ORIGINAL RESEARCH

Airway Smooth Muscle Hyperproliferation Is Regulated by MicroRNA-221 in Severe Asthma

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Abstract

Increased airway smooth muscle (ASM) mass is a feature of asthmatic airways, and could result from augmented proliferation. We determined whether proliferation and IL-6 release are abnormal in ASM cells (ASMCs) from patients with severe asthma, and whether these features could be mediated by microRNA-221 and microRNA-222, through modulation of the cyclin-dependent kinase inhibitors, $p21^{WAF1}$ and $p27^{kip1}$. ASMCs cultured from bronchial biopsies of healthy subjects and patients with nonsevere or severe asthma were studied. Proliferation was measured by the incorporation of bromodeoxyuridine and IL-6 by ELISA. FCS and transforming growth factor (TGF)-β caused greater proliferation and IL-6 release in patients with severe compared with nonsevere asthma and normal subjects. FCS + TGF-β inhibited $p21^{WAF1}$ and $p27^{kip1}$ expression, and increased microRNA-221 (miR-221) expression in ASMCs from individuals with severe asthma. miR-221, and not miR-222, mimics the increased proliferation and IL-6 release induced by FCS + TGF in healthy ASM, whereas in patients with severe asthma, the inhibition of miR-221, but not miR-222, inhibited proliferation and IL-6 release. miR-221 inhibition led to the increased expression of FCS + TGF-β–induced $p21^{WAF1}$ and $p27^{kip1}$. Dexamethasone suppressed proliferation in healthy subjects, but not in subjects with asthma. IL-6 was less suppressible by dexamethasone in patients with nonsevere and severe asthma, compared with healthy subjects. miR-221 did not influence the effects of dexamethasone. ASM from patients with severe asthma shows greater proliferation and IL-6 release than in patients with nonsevere asthma, but both groups show corticosteroid insensitivity. miR-221 regulates $p21^{WAF1}$ and $p27^{kip1}$ expression levels. Furthermore, miR-221 regulates the hyperproliferation and IL-6 release of ASMCs from patients with severe asthma, but does not regulate corticosteroid insensitivity.

Keywords: microRNA; ASM; proliferation; IL-6; steroid insensitivity

Clinical Relevance

This research shows that microRNA-221 regulates the hyperproliferation of airway smooth muscle cells and the release of IL-6 from patients with severe asthma, but does not regulate corticosteroid insensitivity. Blocking the effects of this micro-RNA may exert beneficial effects in severe asthma.

Asthma is characterized by chronic airflow obstruction and chronic airway inflammation and remodeling (1). Airway smooth muscle (ASM) is likely to be an important contributor to chronic airflow obstruction because of its greater prominence through its hyperplastic and hypertrophic qualities, leading to increased thickening and narrowing of the airways. ASM cells (ASMCs) may also participate in the chronic inflammatory response in the airways by expressing cytokines, growth factors, and proteases (2) that could induce the phenotypic changes of ASM (3). Transforming growth factor (TGF)-β is an important growth factor that may promote aberrant ASM function by increasing ASMC proliferation and size, and also by inducing ASM-mediated proinflammatory effects. TGF-β also induces the release of IL-6, which is involved in ASM-induced

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mast-cell activation and the release of eotaxin and vascular endothelial growth factor (4,5).

Asthma can present with varying levels of severity, and a particular group labeled as "severely asthmatic" is characterized by the persistence of symptoms, despite therapy with corticosteroids (6). Examinations of bronchial airways from this group with severe asthma show a greater amount of ASM mass and subepithelial fibrosis, compared with nonsevere asthma (7, 8). *Ex vivo*, ASMCs from patients with mild-to-moderate asthma proliferate faster in response to FCS than does ASM from normal subjects (9). ASMCs from patients with severe asthma demonstrate increased cell growth and proliferation (10), and an increase in proliferating cell nuclear antigen, a marker of proliferation, has been reported in ASMCs from patients with severe asthma (11). We previously demonstrated corticosteroid insensitivity in blood monocytes and alveolar macrophages from severe asthma compared with nonsevere asthma (12, 13). Furthermore, the enhanced proliferation of ASMCs from patients with mild asthma is resistant to dexamethasone (14).

MicroRNAs (miRNAs) comprise small, noncoding RNAs that negatively regulate gene expression at the translational level (15). miR-25 regulates inflammatory cytokine expression, extracellular matrix turnover, and contractile proteins via a posttranslational mechanism (16). However, no miRNAs have been linked to regulating the proliferation of ASMCs. miR-221 and miR-222 are potential regulators of ASM growth, because some cancer cell lines require the high activity of these two miRNAs to maintain low levels of the cyclin-dependent kinase inhibitor $p27^{kip1}$, and to promote continuous proliferation (17). Furthermore, miR-221 and miR-222 are regulators of vascular smooth muscle cell proliferation through $p27^{kip1}$ (18), and the targeting of miR-221 and miR-222 inhibits cell proliferation in alveolar epithelial cells (19). These miRNAs mediate not only vascular smooth muscle proliferation, but also hyperplasia (18, 20).

We hypothesized that increased ASM proliferation in asthma is mediated by the TGF-β–induced miRNA deregulation of miR-221 and miR-222. We examined the effects of combined FCS and TGF-β in the presence of dexamethasone upon ASM proliferation and IL-6 release from patients with nonsevere asthma and severe asthma, and in healthy individuals. We then examined the effects of modulating the expression levels of miR-221 and miR-222 in these cells on proliferation and IL-6 release, on the expression of $p21^{WAF1}$ and $p27^{kip1}$, and on the actions of dexamethasone. miR-221, and not miR-222, accounted for the excessive induced proliferation of ASMCs from patients with severe asthma, but not for the corticosteroid insensitivity.

Materials and Methods

Patient Recruitment

Patients with nonsevere and severe asthma, as defined by American Thoracic Society Workshop on Refractory Asthma (21), were recruited. Current smokers and former smokers with a greater than 5 pack-year history were excluded. Nonasthmatic normal subjects with no previous history of asthma and a provocative concentration producing a 20% fall in forced expiratory volume in one second (PC_{20}) greater than 16 mg/ml were also recruited. The subjects' characteristics are shown in Table 1. Each subject underwent a fiberoptic bronchoscopic study under sedation, with midazolam and topical anesthesia to the airways with lidocaine. Airway biopsies were taken from the segmental and subsegmental airways of the right lower lobe. This study was approved by the local Ethics Committee, and all subjects gave informed consent.

ASMC Culture and Stimulation

ASM cells were cultured as previously described (22). ASMCs were plated onto 96-well plates for the measurement of DNA synthesis and cytokine release, and six-well plates for RNA and protein extraction. Confluent cells were growth-arrested by FCS deprivation for 24 hours in Dulbecco's Modified Eagle's Medium supplemented with sodium pyruvate (1 mM), L-glutamine (2 mM), nonessential amino acids (1:100), penicillin (100 U/ml)/streptomycin (100 μg/ml), amphotericin B (1.5 μg/ml), and BSA (0.1%). At confluence, ASMC cultures exhibited a typical "hill-and-valley" appearance. Immunofluorescence techniques revealed that greater than 80% of the cells displayed the characteristics of smooth muscle cells in culture. Human

Table 1: Characteristics of Subjects

	Non-Asthma	Nonsevere Asthma	Severe Asthma
n	9	9	9
Age (yr)	36.4 ± 12.7	42.4 ± 16.2	40.9 ± 11
Sex (M/F)	7/2	6/4	3/6
Duration of asthma (yr)	N/A	22.2 ± 16.8	25.6 ± 13.2
Inhaled corticosteroid dose (μg BDP equivalent)	0	580 ± 576.9	1688.9 ± 176.4
Atopy (*n*)*	0	8	7
Receiving oral corticosteroids (*n*)	0	0	3
FEV_1 (L)	4.02 ± 0.48	2.81 ± 0.71	2.7 ± 0.82
FEV_1 (% predicted)	104.23 ± 7.28	84.68 ± 12.31	84.48 ± 18.34
FEV_1/FVC (%)	78.89 ± 5.98	69.87 ± 9.27	73.98 ± 9.68
β-agonist reversibility (%)†	N/A	12.3 ± 11.9	19.5 ± 14.6
PC_{20} (mg/ml)	> 16	0.69 ± 0.64	0.20 ± 0.39

Definition of abbreviations: BDP, beclomethasone dipropionate; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; N/A, not available; PC_{20}, provocative concentration of methacholine causing a 20% fall in FEV_1.
*Defined as positive skin prick tests to one or more common aeroallergens.
†Measured as percent increase in FEV_1 after 400 μg salbutamol. Data shown as mean ± SEM.

airway smooth muscle cells at Passages 3–4 from nine different donors were used.

Cells were pretreated with dexamethasone (10^{-10}–10^{-6} M) before being stimulated in triplicate ± 2.5% FCS and ± TGF-β (1 ng/ml) at the indicated times. The supernatants were removed, and IL-6 levels were determined by DuoSet ELISA (R&D Systems, Abingdon, UK). IL-6 levels were normalized back to cell numbers by dividing with the fold increase detected by the bromodeoxyuridine (BrdU) assay. Cell proliferation was measured with a Cell Proliferation ELISA BrdU kit (Roche Diagnostics, Burgess Hill, UK), an assay producing results comparable to actual cell numbers as confirmed by FACS analysis (23).

miRNA and mRNA Expression

The human (hsa)–miR-221 and hsa–miR-222 and $p21^{WAF1}$ and $p27^{kip1}$ expression levels were measured as previously described (24–26). A full description is available in the online supplement.

Western Blotting

Proteins were measured as previously described (25). The p21 Waf1/Cip1 (12D1) rabbit monoclonal antibody and p27 Kip1 (D69C12) XP rabbit monoclonal were obtained from New England Biolabs (Herts, UK). All primary antibodies were used at 1:200 or 1:400, and were incubated overnight. Labeling of the first antibody was detected using relevant secondary antibodies conjugated to horseradish peroxidase (Dako, Ltd., Stockport, UK) and ECL reagents (GE Healthcare, Hertforshire, UK).

Transfection with miR-221 and miR-222 Mimics and Inhibitors

ASM cells were transfected as previously described (26). miR-221 and miR-222 mimics and controls were obtained from Ambion/Applied Biosystems, Ltd. (Paisley, UK), and locked nucleic acid–based miR-221 and miR-222 inhibitors and controls were obtained from Exiqon, Ltd (Vedbaek, Denmark). Transfected cells were plated into 96-well or 6-well plates, and left to adhere overnight before being serum-starved for 6 hours before stimulation with 2.5% FCS and 1 ng/ml TGF-β for the indicated times.

Data Analysis

Data were analyzed using GraphPad Prism, version 5.03 (GraphPad Software, San Diego, CA). Data were not normally distributed (as assessed by the Kolmogorov-Smirnov test), and therefore groups were compared using the Dunn nonparametric test. All data are expressed as means ± SEMs. Significance was defined as a P value of less than 0.05.

Results

Effect of FCS and TGF-β on ASM Proliferation and IL-6 Release

At 8 days, TGF-β (1 ng/ml) alone exerted no effect upon ASM proliferation in any of the patient groups. FCS (2.5%) increased DNA synthesis in cells from subjects without asthma by approximately 1.2-fold ($P < 0.001$), with a greater increase in cells from patients with nonsevere asthma (~ 1.8-fold; $P < 0.001$), and an even greater increase in those from severe asthma (~ 2.1-fold; $P < 0.001$). Patients with severe asthma were significantly different from patients with nonsevere asthma ($P < 0.001$). When ASMCs were stimulated with both TGF-β and FCS together, this increase in DNA synthesis was increased by approximately twofold in all groups ($P < 0.001$), when compared with FCS alone (Figure 1A). Interestingly, this increase in DNA synthesis was greater in the cells from patients with severe asthma (~ 7-fold; $P < 0.001$), compared with cells from subjects without asthma (~ 5-fold; $P < 0.001$). A similar pattern was observed for the increase in IL-6 release (Figure 1B).

Effects of FCS and TGF-β on $p21^{WAF1}$ and $p27^{kip1}$ mRNA and Protein Expression

The basal levels of $p21^{WAF1}$ and $p27^{kip1}$ mRNA and protein were not increased in patients with both nonsevere and severe asthma, compared with healthy subjects (Figure 2). Stimulation with either FCS (2.5%) or TGF-β (1 ng/ml) alone did not increase $p21^{WAF1}$ or $p27^{kip1}$ mRNA and protein in the ASMCs from any of the cohorts (Figure 2). Combined FCS and TGF-β inhibited the expression of $p27^{kip1}$ mRNA in the ASMCs from patients with nonsevere asthma ($P < 0.05$) (Figure 2C), although no effect upon protein expression was observed (Figure 2E). In patients with severe asthma, FCS and TGF-β caused a significant decrease in $p21^{WAF1}$ and $p27^{kip1}$ mRNA ($P < 0.01$) (Figures 2B and 2C) and in protein levels ($P < 0.001$) (Figures 2D and 2E).

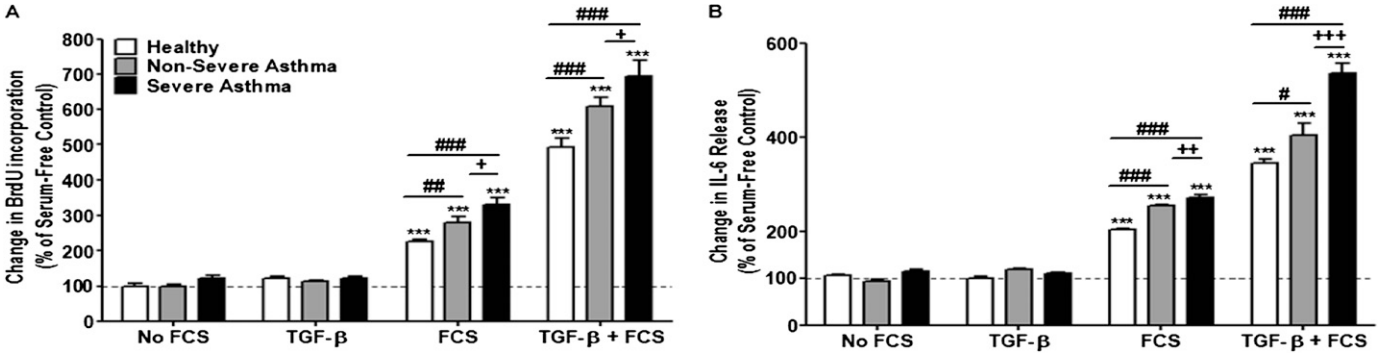

Figure 1. Effects of FCS and transforming growth factor–β (TGF-β) on airway smooth muscle (ASM) proliferation (*A*) and IL-6 release (*B*) from the ASM cells of healthy subjects, patients with nonsevere asthma, and patients with severe asthma at 8 days. *Bars/points* represent the means ± SEMs from nine ASM donors in each group. $^{\#}P < 0.05$. $^{+}P < 0.05$. $^{\#\#}P < 0.01$. $^{++}P < 0.01$. $^{***}P < 0.001$. $^{+++}P < 0.001$. $^{\#\#\#}P < 0.001$. *Asterisks* indicate comparison with no FCS control. *Pound signs* indicate comparison with healthy ASM cells. *Plus signs* indicate comparison with nonsevere ASM cells.

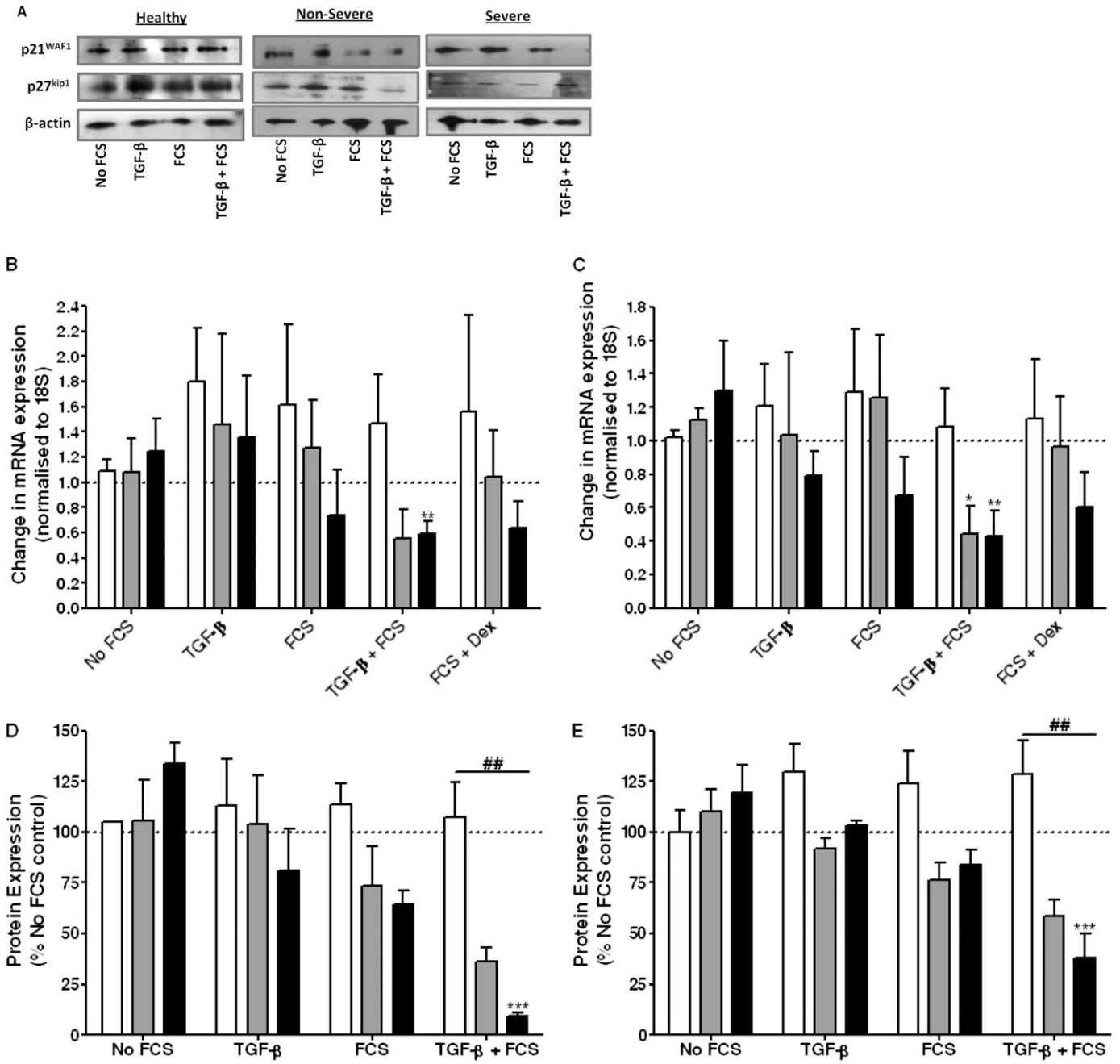

Figure 2. Effects of FCS and TGF-β on the cyclin-dependent kinase inhibitors p21^{WAF1} and p27^{kip1} mRNA (*B* and *C*) and protein expression (*A*, *D*, and *E*) at 24 hours. (*A*) Examples of Western blots of protein expression from extracts of ASM cells exposed to control (No FCS), TGF-β, FCS, and [FCS+TGF-β] from one healthy subject, one subject with nonsevere asthma, and one subject with severe asthma for p21^{WAF1}, p27^{Kip1}, and β-actin. Each gel was probed with the three relevant antibodies separately, and each figure represents a composite gel of the four experimental conditions in each subject category. Protein quantification was normalized to control actin. (*F* and *G*) Correlated data of protein expression versus bromodeoxyuridine (BrdU) incorporation after stimulation with FCS and TGF-β. *Bars* represent the means ± SEMs of nine ASMC donors in each group. *Open bars*, healthy; *shaded bars*, nonsevere asthma; *solid bars*, severe asthma. *Dotted lines* represent 100%. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{\#\#}P < 0.01$. $^{***}P < 0.001$.

Effects of FCS and TGF-β on miR-221 and miR-222 Expression

Combined FCS and TGF-β induced the expression of miR-221, as measured by TaqMan RT-PCR in the ASMCs from patients with severe asthma ($P < 0.05$) (Figure 3A). Under these treatment conditions, a nonsignificant increase in miR-222 expression was found, likely attributable to patient variability (Figure 3B).

Effects of Manipulation of miR-221 and miR-222 on Proliferation and IL-6 Release

To clarify the role of miR-221 and miR-222, we examined the actions of their respective

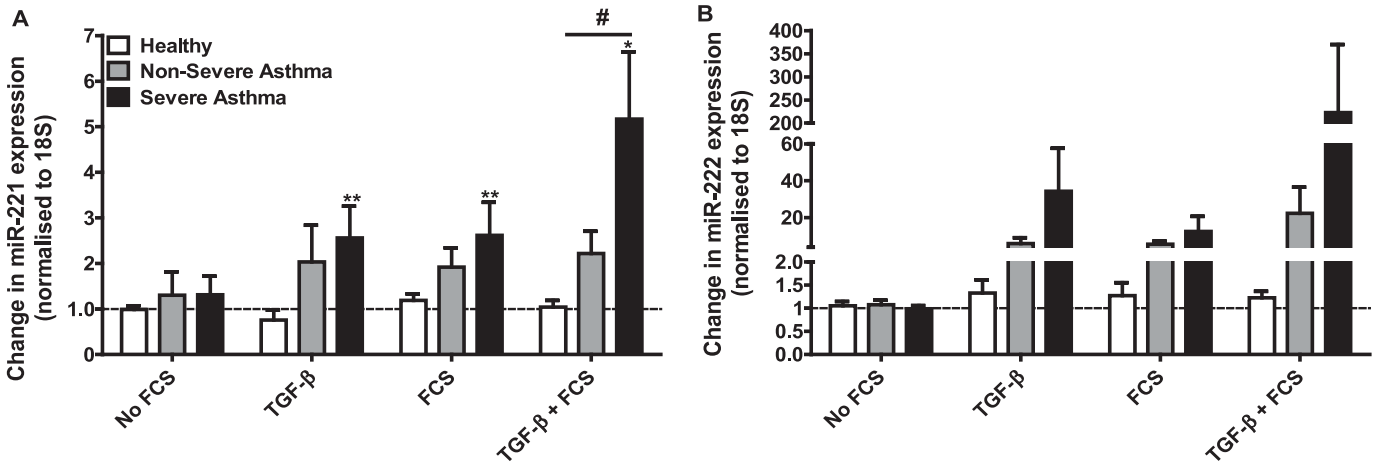

Figure 3. Effects of FCS and TGF-β on microRNA-221 (miR-221) and miR-222 expression between different subject groups at 24 hours. Mean miR-221 and miR-222 expressions, as measured by TaqMan RT-PCR, are shown in *A* and *B*, respectively. *Bars* represent the means ± SEMs of nine ASMC donors in each group. $^{*}P < 0.05$. $^{\#}P < 0.05$. $^{**}P < 0.01$.

inhibitors and mimics on combined FCS-induced and TGF-β–induced DNA synthesis and IL-6 release. Transfection using Amaxa electroporation (Lonza, Slough, UK) showed that miR-221 mimics (100 nM) increased DNA synthesis by approximately 650% ($P < 0.05$), and increased IL-6 release by approximately 450% ($P < 0.001$), on top of the increase already observed with the combination of FCS and TGF-β in healthy ASMCs (Figures 4A and 4B), comparable to the increase observed in patients with severe asthma (Figures 1A and 1B). Conversely, the miR-221 inhibitors (100 nM) decreased both DNA synthesis (∼ 150%; $P < 0.001$) and IL-6 release (∼ 290%; $P < 0.001$), compared with ASMCs from patients with severe asthma stimulated with FCS and TGF-β (Figures 5A and 5B), comparable to the increase observed in healthy individuals (Figures 1A and 1B).

Altering the endogenous levels of miR-222 exerted no effect in either healthy ASMCs or those from patients with severe asthma. Inhibiting either miRNA in healthy cells, or overexpressing the miRNAs in the cells of patients with severe asthma, exerted no effect (Figure E1 in the online supplement).

To confirm efficient transfection, the levels of miR-221 in cells electroporated with miR-221 mimics were measured by TaqMan RT-PCR, and showed efficient transfection (Figure E2A). Under the same conditions, we also demonstrated the complete abolition of miR-221 expression in the presence of the miR-221 inhibitor (Figure E2B). To provide additional evidence of transfection, we undertook parallel studies that examined the effects of a small, interfering RNA (100 nM) targeted to IL-6. As demonstrated previously (26), we showed a reduction in IL-6 release induced by FCS and TGF-β stimulation in ASMCs from healthy subjects ($P < 0.001$) and in those with severe asthma ($P < 0.01$) (Figures E2E and E2F).

Effects of miR-221 and miR-222 on $p21^{WAF1}$ and $p27^{kip1}$

We next determined whether miR-221 and miR-222 could regulate $p21^{WAF1}$ and $p27^{kip1}$ expression. The miR-221 mimics (100 nM) decreased the [FCS + TGF-β]–induced expression of $p21^{WAF1}$ mRNA by approximately 190% ($P < 0.01$), and $p27^{kip1}$ mRNA by approximately 150% ($P < 0.05$), in healthy ASMCs (Figures 4C and 4D), comparable to the decrease observed in patients with severe asthma (Figures 2A and 2B). Conversely, the miR-221 inhibitors (100 nM) increased the [FCS + TGF-β]–induced expression of both $p21^{WAF1}$ mRNA (∼ 130%; $P < 0.05$) and $p27^{kip1}$ (∼ 145%; $P < 0.01$) in ASMCs from patients with severe asthma (Figures 5C and 5D), comparable to the increase observed in healthy individuals (Figures 2A and 2B). Altering the endogenous levels of miR-222 exerted no effect on $p21^{WAF1}$ or $p27^{kip1}$ expression in either the healthy ASMCs or those from patients with severe asthma (Figures 4 and 5).

The miR-221 mimics (100 nM) decreased the [FCS + TGF-β]–induced expression of $p21^{WAF1}$ protein by approximately 70% ($P < 0.01$), and of $p27^{kip1}$ protein by approximately 73% ($P < 0.01$), in healthy ASMCs (Figures 6A–6C). Conversely, the miR-221 inhibitors (100 nM) increased the [FCS + TGF-β]–induced expression of both $p21^{WAF1}$ protein (∼ 80%; $P < 0.001$) and $p27^{kip1}$ (∼ 50%; $P < 0.01$) in ASMCs from patients with severe asthma (Figures 6A, 6D, and 6E). Further examples of these blots can be viewed in the online supplement. In contrast, altering the endogenous levels of miR-222 exerted no effect in either the ASMCs from healthy subjects or in those from patients with severe asthma on $p21^{WAF1}$ or $p27^{kip1}$ expression. Inhibiting either miRNA in the healthy cells, or overexpressing the miRNAs in the cells from patients with severe asthma, exerted no effect (Figure E3).

Effects of Dexamethasone on Asthmatic ASM

When ASMCs from healthy individuals were treated with dexamethasone (10^{-8}–10^{-6} M) for 2 hours before stimulation with both FCS (2.5%) and TGF-β (1 ng/ml) for 8 days, a significant, concentration-dependent decrease in DNA synthesis to approximately 50% ($P < 0.001$) was observed. ASMCs isolated from patients with either nonsevere asthma or severe asthma did not respond to dexamethasone (Figure 7A). The inhibition of IL-6 release from ASMCs from healthy individuals was almost complete at 10^{-9}–10^{-6} M, but was only observed at

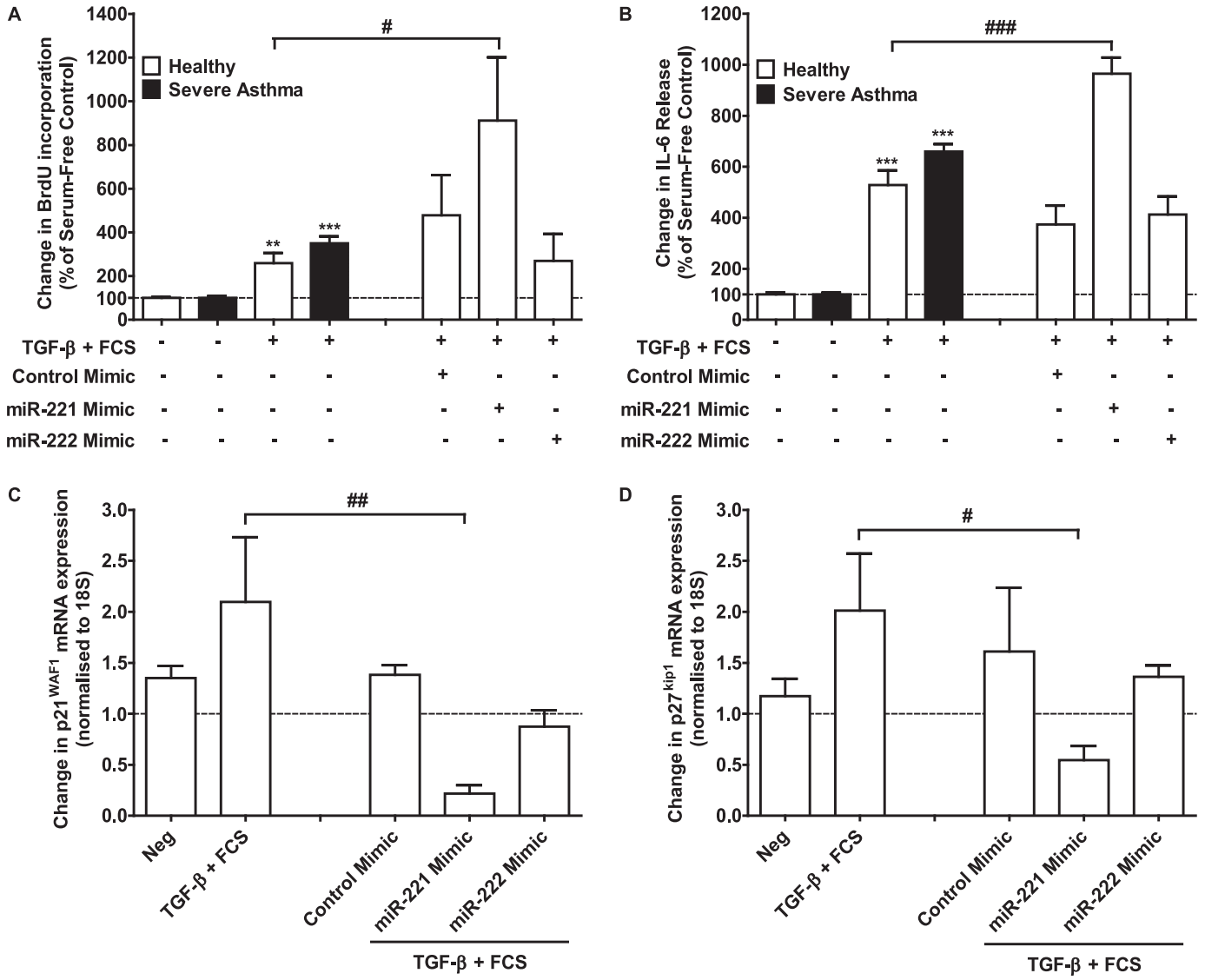

Figure 4. Effects of the overexpression of miR-221 and miR-222 in ASM cells from healthy subjects. DNA synthesis (*A*), IL-6 release (*B*), and $p21^{WAF1}$ (*C*) and $p27^{kip1}$ (*D*) mRNA expression were measured by BrdU ELISA, a DuoSet ELISA Assay (R&D Systems, Abingdon, UK), or TaqMan RT-PCR, respectively. DNA synthesis and IL-6 release were measured at 8 days, and mRNA expression was measured at 24 hours. *Bars* represent the means ± SEMs of nine ASMC healthy donors. "Neg" refers to no FCS. $^{\#}P < 0.05$. $^{**}P < 0.01$. $^{\#\#}P < 0.01$. $^{***}P < 0.001$. $^{\#\#\#}P < 0.001$.

10^{-7}–10^{-6} M in those ASMCs from patients with nonsevere and severe asthma ($P < 0.05$) (Figure 7B). Thus, relative corticosteroid insensitivity was demonstrated in ASMCs from patients with both severe and nonsevere asthma. Interestingly, dexamethasone (10^{-7} M) exerted no effect on either $p21^{WAF1}$ or $p27^{kip1}$ mRNA expression (Figure 7C) or on the expression of miR-221 or miR-222 (Figure 7D), compared with FCS and TGF-β alone. Furthermore, overexpressing or inhibiting these miRNAs exerted no effect upon the inhibitory action of dexamethasone in either the ASMCs from healthy subjects or those with severe asthma (Figure E4).

Discussion

We have made several important observations regarding the behavior of ASMCs from patients with asthma, in particular of those from patients with severe asthma. First, we showed that the combination of FCS and TGF-β increased both ASMC proliferation and IL-6 release in the asthmatic cohorts to a greater degree than those from healthy subjects, with ASMC counts from subjects with severe asthma being the highest. This became significant on Day 8. We determined the role of miR-221 and miR-222 in the abnormalities observed in ASMCs from patients with severe asthma. miR-221, but not miR-222, regulates the enhanced proliferation and IL-6 release seen in the ASMCs of patients with severe asthma. Because miR-221 regulates the levels of $p21^{WAF1}$and $p27^{kip1}$, miR-221 may act through these cyclin-dependent kinase

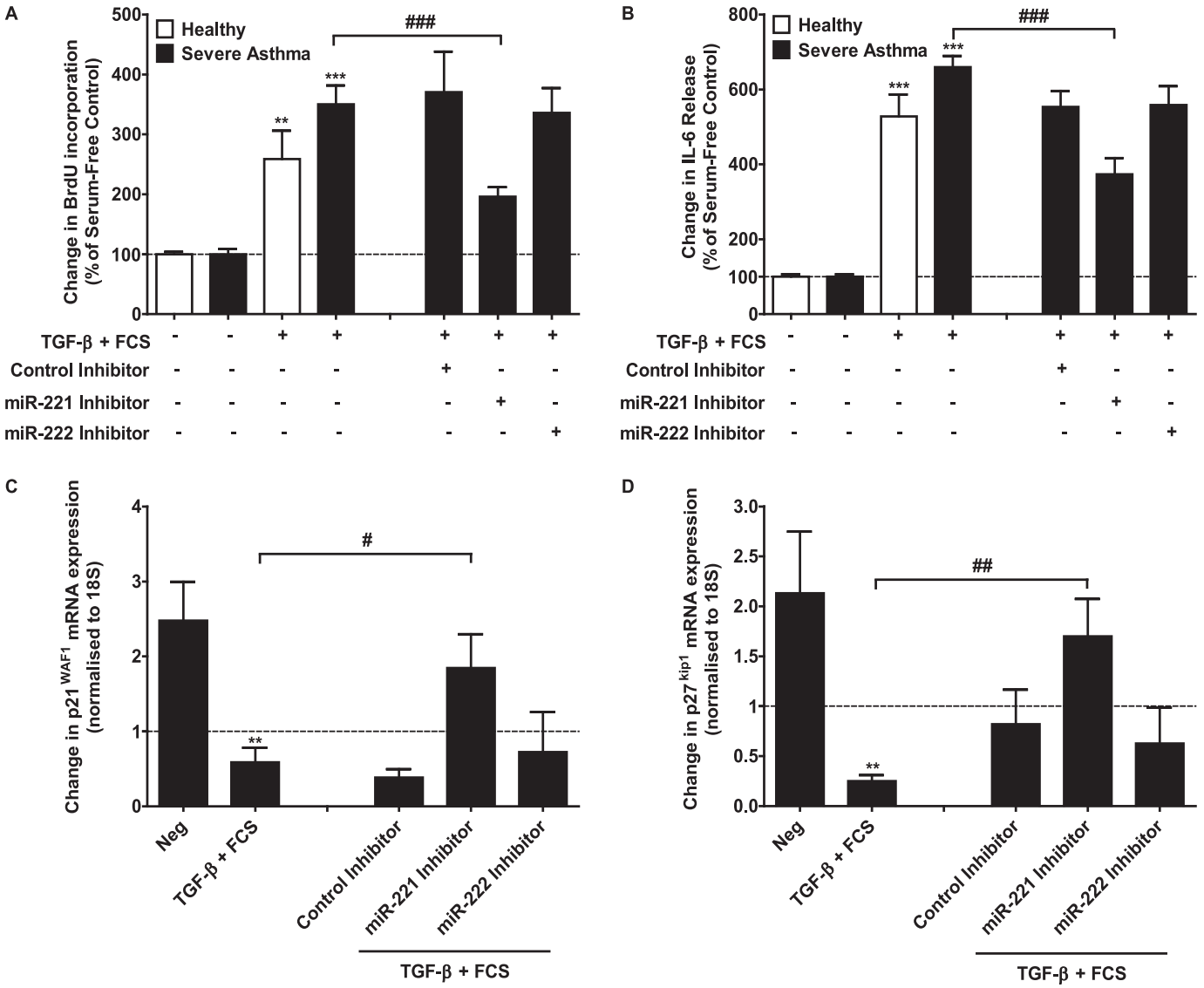

Figure 5. Effects of inhibition of miR-221 and miR-222 in ASM cells from subjects with severe asthma. DNA synthesis (*A*), IL-6 release (*B*), and p21^{WAF1} mRNA (*C*) and p27^{kip1} mRNA (*D*) expression were subsequently measured by BrdU ELISA, a DuoSet ELISA Assay, or TaqMan RT-PCR, respectively. DNA synthesis and IL-6 release were measured at 8 days, and mRNA expression was measured at 24 hours. *Bars* represent the means ± SEMs of nine ASM donors with severe asthma. "Neg" refers to no FCS. $^{\#}P < 0.05$. $^{**}P < 0.01$. $^{\#\#}P < 0.01$. $^{***}P < 0.001$. $^{\#\#\#}P < 0.001$.

inhibitors. Moreover, a difference was evident in the effects of dexamethasone on both the proliferative response and IL-6 release between groups, with ASMCs from both the severe and nonsevere groups being less sensitive to the suppressive effects of dexamethasone, compared with the cells from control subjects without asthma. This was most striking, with a complete lack of suppressive effect by dexamethasone at the highest concentration of 10^{-6} M on proliferation. Neither miRNAs interfered with the effect of dexamethasone on ASMCs, indicating that they were unlikely to be involved in the corticosteroid insensitivity demonstrated in ASMCs from the subjects with asthma.

We have confirmed previous observations on the hyperproliferation of ASMCs cultured from patients with asthma compared with nonasthmatic ASMCs (9). In addition, we now show that the combination of FCS and TGF-β, which is the optimal combination to induce ASM proliferation (27), causes the greatest degree of proliferation in ASMCs from severe asthma compared with nonsevere asthma. We confirmed that the BrdU incorporation method we used reflected ASM proliferation by comparing it to total cell counts using flow cytometry. The increases in ASMC counts seen with FCS and TGF-β in ASMCs from a nonasthmatic and from a severely asthmatic subject were similar to those measured by BrDU incorporation (data not shown), confirming the similar results we previously reported (23). ASMCs from patients with asthma released greater amounts of IL-6 in response to FCS and TGF-β, with the greatest response in the patients with severe asthma. This is also an extension of previous reports indicating the

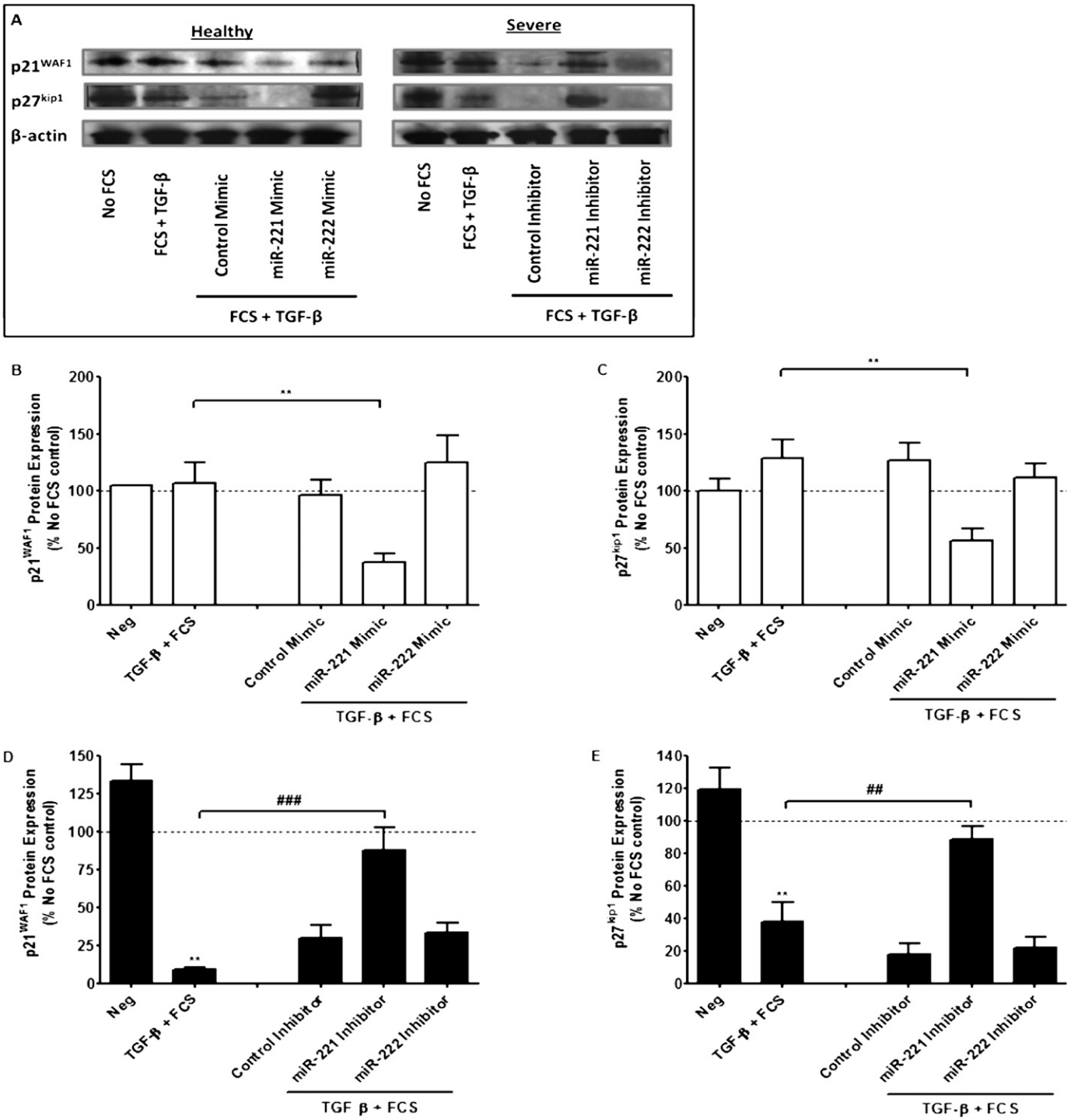

Figure 6. Effects of overexpression or inhibition of miR-221 and miR-222 on $p21^{WAF1}$ and $p27^{kip1}$ protein in ASM cells at 24 hours. Representative results from a subjects without asthma and from a subject with severe asthma are shown in *A*. Mean protein expression, quantified by densitometry, normalized against β-actin expression, and expressed as the percent change versus untreated control subjects, is shown in *B–E*. *Bars* represent the means ± SEMs of nine ASM donors in each group. "Neg" refers to no FCS. $^{**}P < 0.01$. $^{\#\#}P < 0.01$. $^{\#\#\#}P < 0.001$.

increased release of cytokines such as connective tissue growth factor (28), chemokine (C-X-C motif) ligand (CXCL) 10 (29), CXCL8 (30), and C-C motif chemokine 11 (31) from asthmatic ASMCs. The potential contribution of the ASMC as a hyperproliferative and proinflammatory cell in severe asthma to the asthmatic process is likely to occur through the generation of greater contractile response, with greater contributions to the degree of airway inflammation (32).

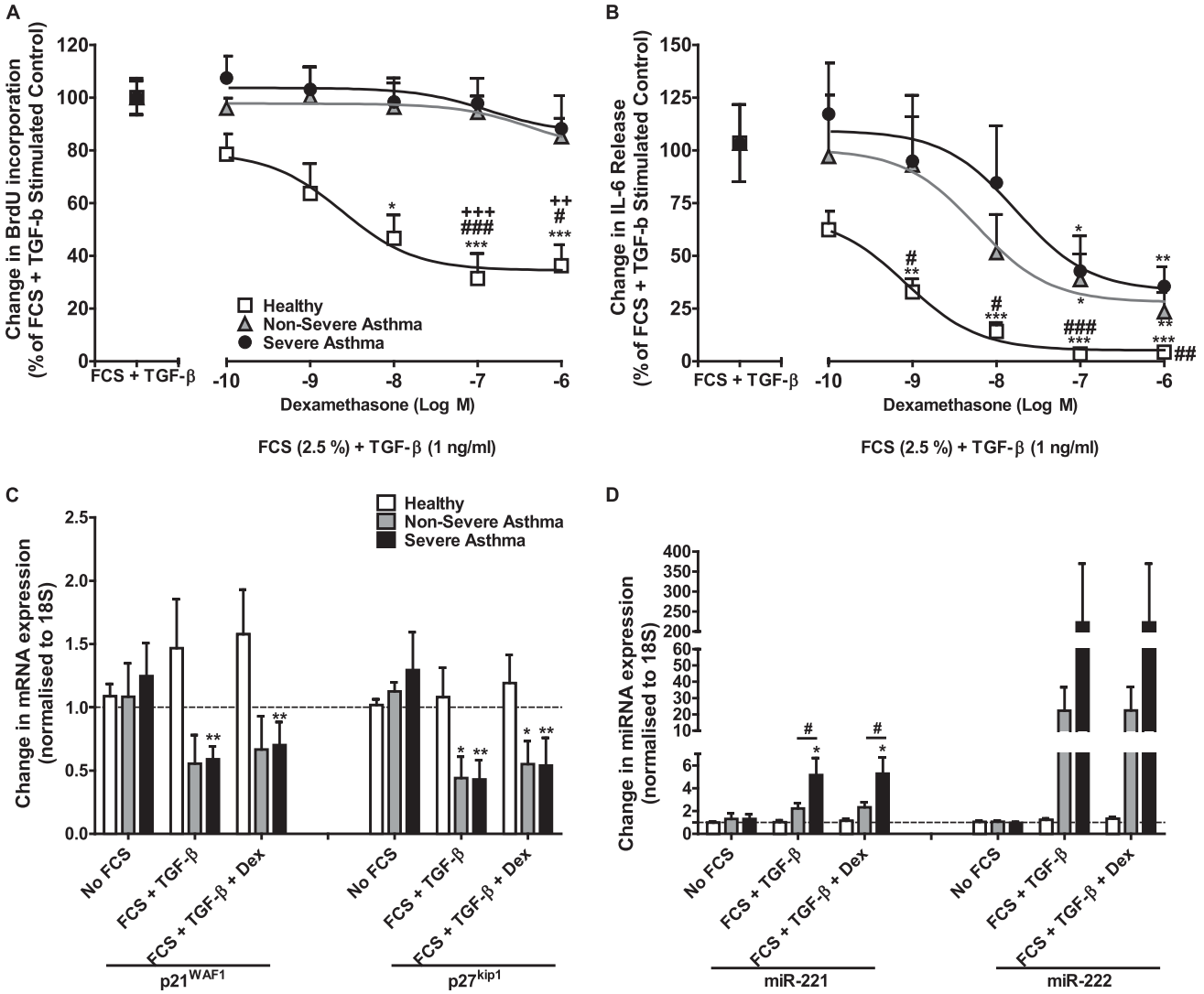

Figure 7. Effects of dexamethasone (Dex) on ASM proliferation, IL-6 release, $p21^{WAF1}$ and $p27^{kip1}$ mRNA, and miR-221 and miR-222 expression in ASM cells from healthy subjects, subjects with nonsevere asthma, and subjects with severe asthma. DNA synthesis (*A*) and IL-6 release (*B*) were measured by BrdU ELISA and DuoSet ELISA assays, respectively. *C* shows changes in $p21^{WAF1}$ and $p27^{kip1}$ mRNA expression, and *D* shows changes in miR-221 and miR-222 expression measured by TaqMan RT-PCR. DNA synthesis and IL-6 release were measured at 8 days, and mRNA and miRNA expression were measured at 24 hours. *Bars/points* represent the means ± SEMs from nine ASM donors in each group. $^{*}P < 0.05$. $^{\#}P < 0.05$. $^{**}P < 0.01$. $^{\#\#}P < 0.01$. $^{++}P < 0.01$. $^{***}P < 0.001$. $^{\#\#\#}P < 0.001$. $^{+++}P < 0.001$.

An important role for miR-221 has been demonstrated in various cancers, hematopoietic cells, endothelial cells, and vascular smooth muscle cells (20, 33–37). The miRNA inhibitors have been used to demonstrate that certain cancer cell lines require a high activity of miR-221 and miR-222 to maintain low $p27^{kip1}$ levels and continuous proliferation (17). Furthermore, miR-221 and miR-222 have been demonstrated to target the tumor suppressor $p27^{kip1}$ directly, and their overexpression may comprise one of the factors contributing to the oncogenesis and progression of prostate carcinoma through $p27^{kip1}$ down-regulation (38). Our results indicate that the miR-221–dependent down-regulation of both $p27^{kip1}$ and $p21^{WAF1}$ is associated with ASMC proliferation. miR-221 is part of a gene cluster that also expresses miR-222, and both miRNAs share an identical seed sequence and are predicted by TargetScan to bind to the p27–3′ untranslated region. However, only miR-221 modulated $p21^{WAF1}$ and $p27^{kip1}$ expression. Depleting both miR-221 and miR-222 in human umbilical vein endothelial cells further modulated the expression of a distinct set of 32 miRNAs, with nine up-regulated and 23 down-regulated miRNAs (39), suggesting that the functional effect seen with the manipulation of one miRNA may be the result of secondary effects on downstream mRNA or protein expression (39).

Both miR-221 and miR-222 were previously demonstrated to be differentially expressed, and they may play slightly different roles in some cancers (40). The reason for the differential expression of miR-221 and miR-222 in severe asthma is likely attributable to the fact that the

expression of these miRNAs is differentially regulated by TGF-β and FCS. The expression of miRNAs can be regulated by different mechanisms, such as transcriptional regulation or the processing of primary miRNA or preliminary miRNA by Drosha or Dicer RNase III enzymes (41–43). miR-221 is the first miRNA gene reported to be regulated by TGF-β in human asthmatic ASM. The expression of miR-221 is under the control of the microphthalmia-associated transcription factor (44), and we speculate that TGF-β may activate miR-221 transcription through the recruitment of microphthalmia-associated transcription factor or other E-box binding proteins under the control of the extracellular regulated kinase, NF-κB, and c-Jun (activator protein 1) signaling pathways (45,46). Determination of the precise mechanism by which TGF-β and FCS induce miR-221 expression in human ASMCs from patients with severe asthma is currently being undertaken.

We have confirmed that asthmatic ASMCs are resistant to the effects of corticosteroids in terms of their proliferative response (14). In the present work, ASMCs from patients with both severe and nonsevere asthma were unresponsive to dexamethasone, with respect to their proliferative response to FCS and TGF-β. Neither the overexpression nor inhibition of miR-221 or miR-222 exerted any influence upon the effect of dexamethasone in either the ASMCs from healthy subjects or from those with severe asthma. We previously performed a similar miRNA expression analysis of 227 miRNAs (including miR-221 and miR-222) in asthmatic airway biopsy samples after inhaled corticosteroid treatment, compared with pretreatment samples (47). Corticosteroids are potent anti-inflammatory agents that inhibit the expression of many proinflammatory cytokines and chemokines through interfering with the binding of transcription factors such as NF-κB and activator protein 1 to the promoter regions of their respective genes (48). Many miRNAs contain NF-κB transcription factor binding sites. However, we previously reported that no alteration in miRNA expression is evident after steroid treatment (47,49). Therefore, the expression of a large percentage of miRNAs, including miR-221 and miR-222, is not altered by corticosteroids. Defining the mechanisms that regulate miR-221 expression may, however, provide a novel approach to suppressing the abnormal inflammatory and proliferative responses seen in ASMCs from patients with severe asthma.

In conclusion, miR-221 is vital in controlling the aberrant proliferation of human ASMCs in severe asthma. This finding may open a new avenue in asthma therapeutics by targeting miRNA-221 for severe asthma, thereby leading to improved airflow obstruction and better control of asthma. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

1. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma: from bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000;161:1720–1745.
2. Chung KF. Should treatments for asthma be aimed at the airway smooth muscle? *Expert Rev Respir Med* 2007;1:209–217.
3. Halayko AJ, Amrani Y. Mechanisms of inflammation-mediated airway smooth muscle plasticity and airways remodeling in asthma. *Respir Physiol Neurobiol* 2003;137:209–222.
4. Michaeloudes C, Chang PJ, Petrou M, Chung KF. Transforming growth factor–β and nuclear factor E2–related factor 2 regulate antioxidant responses in airway smooth muscle cells: role in asthma. *Am J Respir Crit Care Med* 2011;184:894–903.
5. Michaeloudes C, Sukkar MB, Khorasani NM, Bhavsar PK, Chung KF. TGF-β regulates Nox4, MnSOD and catalase expression, and IL-6 release in airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2011;300:L295–L304.
6. Chung KF, Godard P, Adelroth E, Ayres J, Barnes N, Barnes P, Bel E, Burney P, Chanez P, Connett G, *et al*. Difficult/therapy-resistant asthma: the need for an integrated approach to define clinical phenotypes, evaluate risk factors, understand pathophysiology and find novel therapies: ERS Task Force on Difficult/Therapy-Resistant Asthma. *Eur Respir J* 1999;13:1198–1208.
7. Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* 2003;167:1360–1368.
8. Macedo P, Hew M, Torrego A, Jouneau S, Oates T, Durham A, Chung KF. Inflammatory biomarkers in airways of patients with severe asthma compared with non-severe asthma. *Clin Exp Allergy* 2009;39:1668–1676.
9. Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G, Burgess JK, Black JL. Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med* 2001;164:474–477.
10. Trian T, Benard G, Begueret H, Rossignol R, Girodet PO, Ghosh D, Ousova O, Vernejoux JM, Marthan R, Tunon-de-Lara JM, *et al*. Bronchial smooth muscle remodeling involves calcium-dependent enhanced mitochondrial biogenesis in asthma. *J Exp Med* 2007;204:3173–3181.
11. Hassan M, Jo T, Risse PA, Tolloczko B, Lemière C, Olivenstein R, Hamid Q, Martin JG. Airway smooth muscle remodeling is a dynamic process in severe long-standing asthma. *J Allergy Clin Immunol* 2010;125:1037–1045, e3.
12. Bhavsar P, Hew M, Khorasani N, Torrego A, Barnes PJ, Adcock I, Chung KF. Relative corticosteroid insensitivity of alveolar macrophages in severe asthma compared with non-severe asthma. *Thorax* 2008;63:784–790.
13. Hew M, Bhavsar P, Torrego A, Meah S, Khorasani N, Barnes PJ, Adcock I, Chung KF. Relative corticosteroid insensitivity of peripheral blood mononuclear cells in severe asthma. *Am J Respir Crit Care Med* 2006;174:134–141.
14. Roth M, Johnson PR, Borger P, Bihl MP, Rüdiger JJ, King GG, Ge Q, Hostettler K, Burgess JK, Black JL, *et al*. Dysfunctional interaction of C/EBPalpha and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. *N Engl J Med* 2004;351:560–574.
15. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;136:215–233.
16. Kuhn AR, Schlauch K, Lao R, Halayko AJ, Gerthoffer WT, Singer CA. MicroRNA expression in human airway smooth muscle cells: role of miR-25 in regulation of airway smooth muscle phenotype. *Am J Respir Cell Mol Biol* 2010;42:506–513.
17. le Sage C, Nagel R, Egan DA, Schrier M, Mesman E, Mangiola A, Anile C, Maira G, Mercatelli N, Ciafrè SA, *et al*. Regulation of the p27(kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. *EMBO J* 2007;26:3699–3708.
18. Liu X, Cheng Y, Zhang S, Lin Y, Yang J, Zhang C. A necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia. *Circ Res* 2009;104:476–487.
19. Zhang C, Zhang J, Zhang A, Wang Y, Han L, You Y, Pu P, Kang C. PUMA is a novel target of miR-221/222 in human epithelial cancers. *Int J Oncol* 2010;37:1621–1626.
20. Davis BN, Hilyard AC, Nguyen PH, Lagna G, Hata A. Induction of microRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype. *J Biol Chem* 2009;284:3728–3738.

21. American Thoracic Society. Proceedings of the ATS Workshop on Refractory Asthma: current understanding, recommendations, and unanswered questions. *Am J Respir Crit Care Med* 2000;162: 2341–2351.
22. Chang PJ, Bhavsar PK, Michaeloudes C, Khorasani N, Chung KF. Corticosteroid insensitivity of chemokine expression in airway smooth muscle of patients with severe asthma. *J Allergy Clin Immunol* 2012;130:877–885.
23. Perry MM, Hui CK, Whiteman M, Wood ME, Adcock I, Kirkham P, Michaeloudes C, Chung KF. Hydrogen sulfide inhibits proliferation and release of IL-8 from human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 2011;45:746–752.
24. Moschos SA, Jones SW, Perry MM, Williams AE, Erjefalt JS, Turner JJ, Barnes PJ, Sproat BS, Gait MJ, Lindsay MA. Lung delivery studies using siRNA conjugated to TAT(48–60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity. *Bioconjug Chem* 2007;18:1450–1459.
25. Perry MM, Moschos SA, Williams AE, Shepherd NJ, Larner-Svensson HM, Lindsay MA. Rapid changes in microRNA-146a expression negatively regulate the IL-1beta–induced inflammatory response in human lung alveolar epithelial cells. *J Immunol* 2008;180: 5689–5698.
26. Larner-Svensson HM, Williams AE, Tsitsiou E, Perry MM, Jiang X, Chung KF, Lindsay MA. Pharmacological studies of the mechanism and function of interleukin-1beta–induced miRNA-146a expression in primary human airway smooth muscle. *Respir Res* 2010;11:68.
27. Xie S, Sukkar MB, Issa R, Khorasani NM, Chung KF. Mechanisms of induction of airway smooth muscle hyperplasia by transforming growth factor–beta. *Am J Physiol Lung Cell Mol Physiol* 2007;293: L245–L253.
28. Burgess JK, Johnson PR, Ge Q, Au WW, Poniris MH, McParland BE, King G, Roth M, Black JL. Expression of connective tissue growth factor in asthmatic airway smooth muscle cells. *Am J Respir Crit Care Med* 2003;167:71–77.
29. Brightling CE, Ammit AJ, Kaur D, Black JL, Wardlaw AJ, Hughes JM, Bradding P. The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle. *Am J Respir Crit Care Med* 2005;171:1103–1108.
30. John AE, Zhu YM, Brightling CE, Pang L, Knox AJ. Human airway smooth muscle cells from asthmatic individuals have CXCL8 hypersecretion due to increased NF-kappa B p65, C/EBP beta, and RNA polymerase II binding to the CXCL8 promoter. *J Immunol* 2009; 183:4682–4692.
31. Chan V, Burgess JK, Ratoff JC, O'Connor BJ, Greenough A, Lee TH, Hirst SJ. Extracellular matrix regulates enhanced eotaxin expression in asthmatic airway smooth muscle cells. *Am J Respir Crit Care Med* 2006;174:379–385.
32. Chung KF. Airway smooth muscle cells: contributing to and regulating airway mucosal inflammation? *Eur Respir J* 2000;15:961–968.
33. Felicetti F, Errico MC, Bottero L, Segnalini P, Stoppacciaro A, Biffoni M, Felli N, Mattia G, Petrini M, Colombo MP, *et al*. The promyelocytic leukemia zinc finger–microRNA-221/-222 pathway controls melanoma progression through multiple oncogenic mechanisms. *Cancer Res* 2008;68:2745–2754.
34. Miller TE, Ghoshal K, Ramaswamy B, Roy S, Datta J, Shapiro CL, Jacob S, Majumder S. MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting $p27^{kip1}$. *J Biol Chem* 2008; 283:29897–29903.
35. Sun T, Yang M, Kantoff PW, Lee GSM. Role of microRNA-221/-222 in cancer development and progression. *Cell Cycle* 2009;8:2315–2316.
36. Chun-Zhi Z, Lei H, An-Ling Z, Yan-Chao F, Xiao Y, Guang-Xiu W, Zhi-Fan J, Pei-Yu P, Qing-Yu Z, Chun-Sheng K. MicroRNA-221 and microRNA-222 regulate gastric carcinoma cell proliferation and radioresistance by targeting PTEN. *BMC Cancer* 2010;10:367.
37. Minami Y, Satoh M, Maesawa C, Takahashi Y, Tabuchi T, Itoh T, Nakamura M. Effect of atorvastatin on microRNA 221/222 expression in endothelial progenitor cells obtained from patients with coronary artery disease. *Eur J Clin Invest* 2009;39:359–367.
38. Galardi S, Mercatelli N, Giorda E, Massalini S, Frajese GV, Ciafrè SA, Farace MG. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting $p27^{kip1}$. *J Biol Chem* 2007;282:23716–23724.
39. Tuccoli A, Poliseno L, Rainaldi G. miRNAs regulate miRNAs: coordinated transcriptional and post-transcriptional regulation. *Cell Cycle* 2006;5:2473–2476.
40. Ladeiro Y, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou S, Zucman-Rossi J. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. *Hepatology* 2008;47:1955–1963.
41. Obernosterer G, Leuschner PJ, Alenius M, Martinez J. Post-transcriptional regulation of microRNA expression. *RNA* 2006;12: 1161–1167.
42. Wulczyn FG, Smirnova L, Rybak A, Brandt C, Kwidzinski E, Ninnemann O, Strehle M, Seiler A, Schumacher S, Nitsch R. Post-transcriptional regulation of the LET-7 microRNA during neural cell specification. *FASEB J* 2007;21:415–426.
43. Davis BN, Hilyard AC, Lagna G, Hata A. Smad proteins control Drosha-mediated microRNA maturation. *Nature* 2008;454:56–61.
44. Ozsolak F, Poling LL, Wang Z, Liu H, Liu XS, Roeder RG, Zhang X, Song JS, Fisher DE. Chromatin structure analyses identify miRNA promoters. *Genes Dev* 2008;22:3172–3183.
45. Galardi S, Mercatelli N, Farace MG, Ciafrè SA. NF-kB and c-Jun induce the expression of the oncogenic miR-221 and miR-222 in prostate carcinoma and glioblastoma cells. *Nucleic Acids Res* 2011;39: 3892–3902.
46. Terasawa K, Ichimura A, Sato F, Shimizu K, Tsujimoto G. Sustained activation of ERK1/2 by NGF induces microRNA-221 and 222 in PC12 cells. *FEBS J* 2009;276:3269–3276.
47. Williams AE, Larner-Svensson H, Perry MM, Campbell GA, Herrick SE, Adcock IM, Erjefalt JS, Chung KF, Lindsay MA. MicroRNA expression profiling in mild asthmatic human airways and effect of corticosteroid therapy. *PLoS ONE* 2009;4:e5889.
48. Ito K, Chung KF, Adcock IM. Update on glucocorticoid action and resistance. *J Allergy Clin Immunol* 2006;117:522–543.
49. Moschos SA, Williams AE, Perry MM, Birrell MA, Belvisi MG, Lindsay MA. Expression profiling in vivo demonstrates rapid changes in lung microRNA levels following lipopolysaccharide-induced inflammation but not in the anti-inflammatory action of glucocorticoids. *BMC Genomics* 2007;8:240.

Online Data Supplement

Airway smooth muscle hyperproliferation is regulated by microRNA-221 in severe asthma

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Materials & Methods

miRNA and mRNA expression

hsa-miR-221 and -222 was carried out on total RNA extracts by two-step TaqMan® reverse transcription polymerase chain reaction protocol (RT-PCR). mRNA expression levels of $p21^{WAF1}$ and $p27^{kip1}$ were determined using semi-quantitative two-step RT-PCR as previously described using Assay on Demand primer/probe sets obtained from Applied Biosystems, UK. The relative quantity of miRNAs from each of the treated plates was compared to the relevant control samples and is therefore described as a normalised fold difference in expression, using the $2^{-\Delta\Delta CT}$ method, which prevents the need for a standard curve for each plate (1). Comparative slopes of the relationship between log[RNA] and Ct (gradient = − 3.3) for both the gene of interest and the endogenous control 18S (Applied Biosystems) indicated that the comparative Ct method (ΔΔCt) could be used for the relative quantification of gene expression.

Results

Effect of manipulation of miR-221 and miR-222 on proliferation, IL-6 release, p21WAF1 and p27kip1 expression

To clarify the role of miR-221 and miR-222, we examined the action of their respective inhibitors and mimics on combined FCS- and TGF-β-induced DNA synthesis and IL-6 release. Transfection using Amaxa electroporation showed that miR-221 mimics (100 nM) increased DNA synthesis by ~650 % ($p < 0.05$), and IL-6 release by ~450 % ($p<0.001$) on top of the increase already observed with combination of FCS and TGF-β in healthy ASM cells, comparable to the increase observed in severe asthmatics. Conversely, miR-221 inhibitors (100 nM) decreased both DNA synthesis (~150 %, $p < 0.001$) and IL-6 release (~290 %,

$p<0.001$), compared to ASM cells from patients with severe asthma stimulated with FCS and TGF-β, comparable to the increase observed in healthy individuals.

Altering endogenous levels of miR-222 had no effect in either the healthy ASM cells or those from severe asthmatics. Inhibiting either miRNA in the healthy cells (**Supplemental Figure 1A&B**), or over-expressing them in the cells from the severe asthmatics had no effect (**Supplemental Figure 1 C&D**).

To confirm efficient transfection, the levels of miR-221 in cells electroporated with miR-221 mimics were measured by TaqMan and showed efficient transfection (**Supplemental Figure 2A**). Under the same condition, we have also demonstrated complete abolition of miR-221 expression in the presence of miR-221 inhibitor (**Supplemental Figure 2B**). To provide additional evidence of transfection, we undertook parallel studies that examined the effect of ansiRNA (100 nM) targeted to IL-6. As demonstrated previously (2), we showed a reduction in IL-6 release induced by FCS and TGF-β stimulation in ASM cells from healthy subjects ($p < 0.001$) and those from severe asthma ($p < 0.01$) (**Supplemental Figure 2C&D**).

Inhibiting either miRNA in the healthy cells, or over-expressing them in the cells from the severe asthmatics had no effect upon expression of p21WAF1 or p27kip1 mRNA expression (**Supplemental Figure 3**).

Effect of manipulation of miR-221 and miR-222 on the action of dexamethasone

Over-expressing these miRNAs in ASM cells from healthy patients (**Supplemental Figure 4A&C**) or inhibiting them in ASM cells from those with severe asthma (**Supplemental Figure 4B&D**) had no effect upon the inhibitory action of dexamethasone.

Supplementary Figure legends

Supplemental Figure 1: Effect of inhibiting miR-221 and -222 in healthy ASM cells and over-expressing miR-221 and -222 in severe ASM cells on proliferation & IL-6 release. Healthy ASM cells were electroporated in the presence of buffer, control inhibitor, or miR-221/222 inhibitor [100 nM] (Panels A&B). Severe ASM cells were electroporated in the presence of buffer, control mimic, or miR-221/222 [100 nM] (Panels C&D). Cells were then stimulated with FCS (2.5 %) + TGF-β (1 ng/ml) and DNA synthesis (Panels A&C) and IL-6 release (Panels B&D) were subsequently measured by BrdU ELISA and DuoSet ELISA Assay respectively. Bars represent mean ± SEM of 9 ASMC donors. "Neg" refers to no FCS.

Supplemental Figure 2: Confirmation of transfection efficiency. HASM cells were electroporated in the presence of buffer, control mimic/inhibitor, miR-221 mimic/inhibitor, or IL-6 siRNA [100 nM]. Cells were then stimulated with FCS (2.5 %) + TGF-β (1 ng/ml). miR-221 expression was measured by TaqMan RT-PCR (Panels A&B), or IL-6 release (Panels D&D) was measured by DuoSet ELISA Assay. Bars represent mean ± SEM of 9 ASMC donors. ** $p < 0.01$; *** $p < 0.001$.

Supplemental Figure 3: Effect of inhibiting miR-221 and -222 in healthy ASM cells and over-expressing miR-221 and -222 in severe ASM cells on p21WAF1 & p27kip1 mRNA. Healthy ASM cells were electroporated in the presence of buffer, control inhibitor or miR-221/222 inhibitors [100 nM] (Panels A&B). Severe ASM cells were electroporated in the presence of buffer, control mimic or miR-221/222 mimics [100 nM] (Panels C&D). Cells were then stimulated with FCS (2.5 %) + TGF-β (1 ng/ml) and p21WAF1 (Panels A&C) and p27kip1 (Panels B&D) mRNA were subsequently measured by TaqMan RT-PCR. Bars represent mean ± SEM of 9 ASMC donors. "Neg" refers to no FCS. ** $p < 0.01$.

Supplemental Figure 4: Effect of inhibiting miR-221 and -222 in healthy ASM cells and over-expressing miR-221 and -222 in severe ASM cells in the presence of dexamethasone. Healthy ASM cells were electroporated in the presence of buffer, control mimic or miR-221/222 mimics [100 nM] (Panels A&C). Severe ASM cells were electroprated in the presence of buffer, control inhibitor or miR-221/222 inhibitors [100 nM] (Panels B&D). Cells were then treated with dexamethasone (10-7 M) for 2 h before being stimulated with FCS (2.5 %) + TGF-β (1 ng/ml) and DNA synthesis (Panels A&B) and IL-6 release (Panels C&D) were subsequently measured by BrdU ELISA and DuoSet ELISA Assay respectively. Bars represent mean ± SEM of 3 ASMC donors. "Neg" refers to no FCS. # $p < 0.05$; ** $p < 0.01$; ***/### $p < 0.001$.

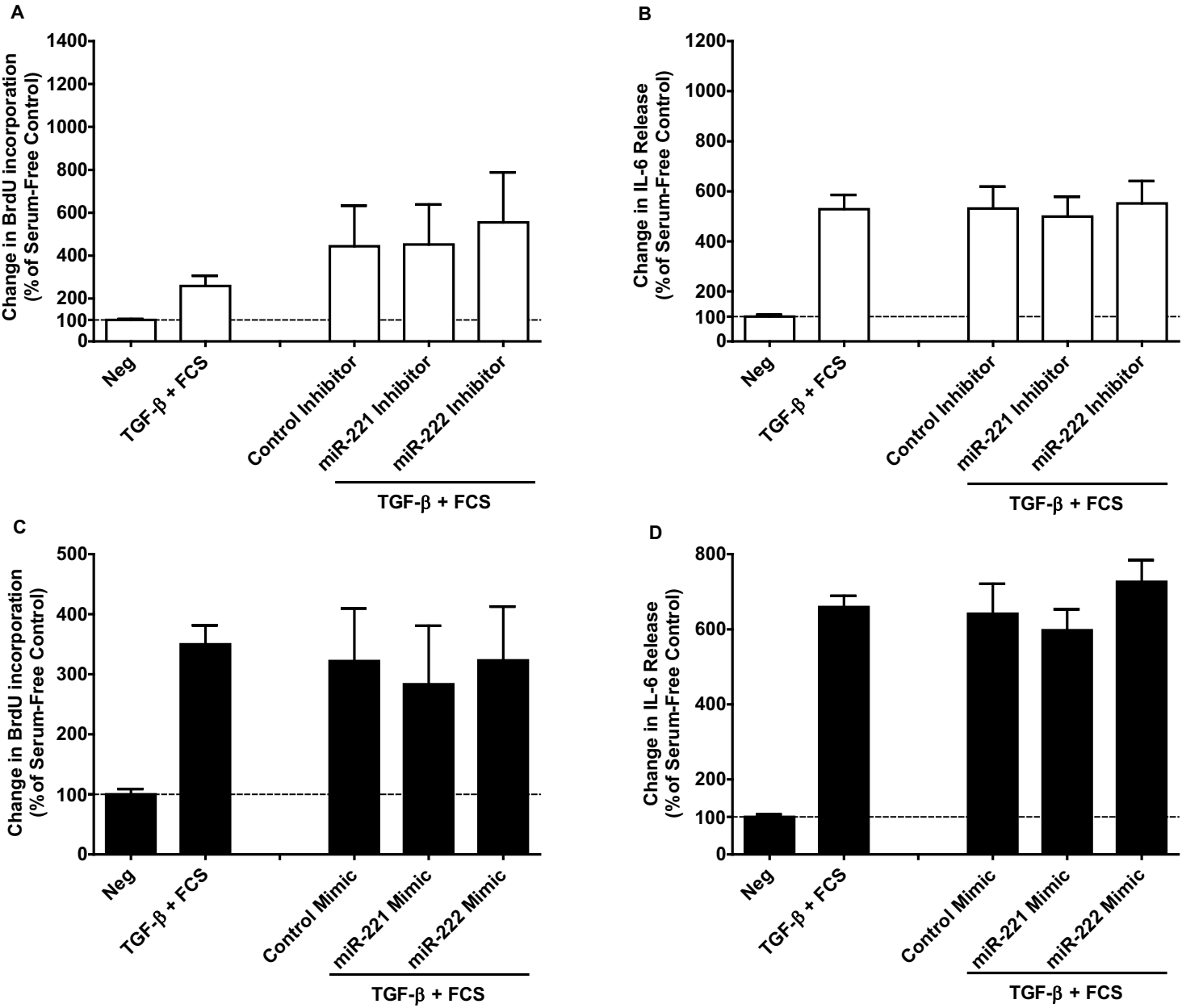
Supplemental Figure 5: Effect of over-expression or inhibition of miR-221 and miR-222 on $p21^{WAF1}$ and $p27^{kip1}$ protein in ASM cells at 24 h. Results from 3 non-asthmatics and 3 severe asthmatic subjects are shown.

Reference List

E1. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408.

E2. Larner-Svensson, H. M., A. E. Williams, E. Tsitsiou, M. M. Perry, X. Jiang, K. F. Chung, and M. A. Lindsay. 2010. Pharmacological studies of the mechanism and function of interleukin-1beta-induced miRNA-146a expression in primary human airway smooth muscle. *Respir. Res* 11: 68.

Supplementary Figure 1



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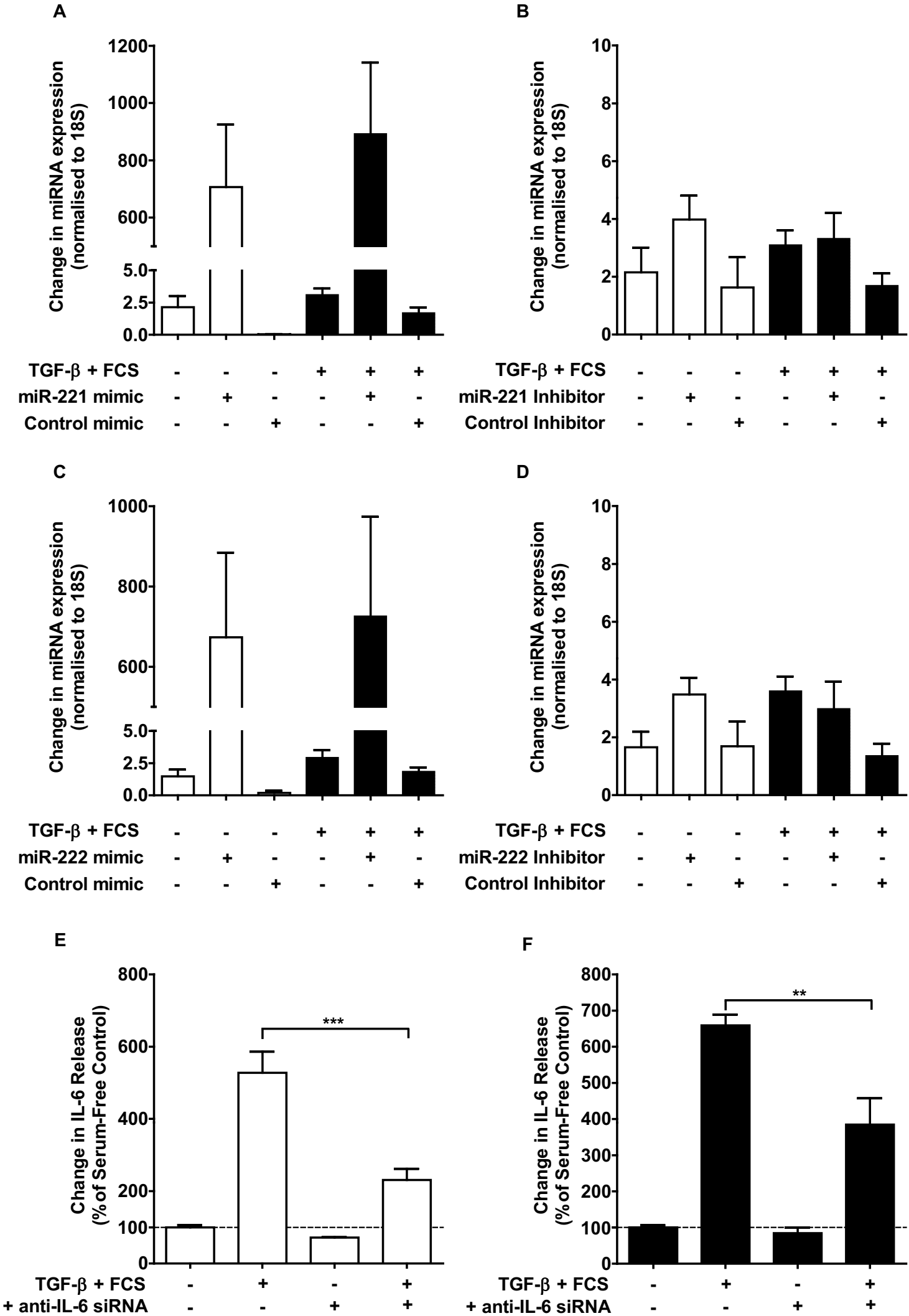

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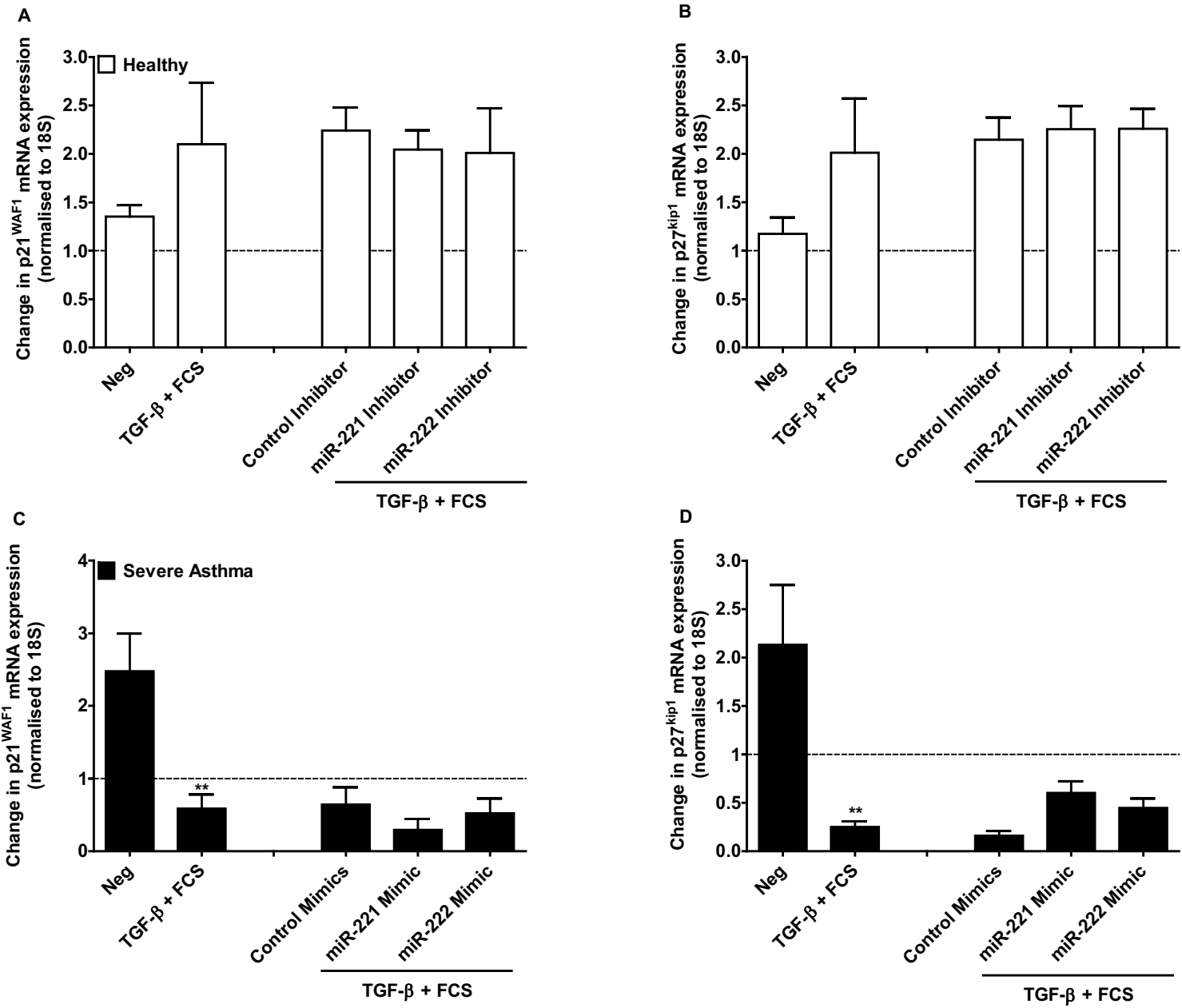

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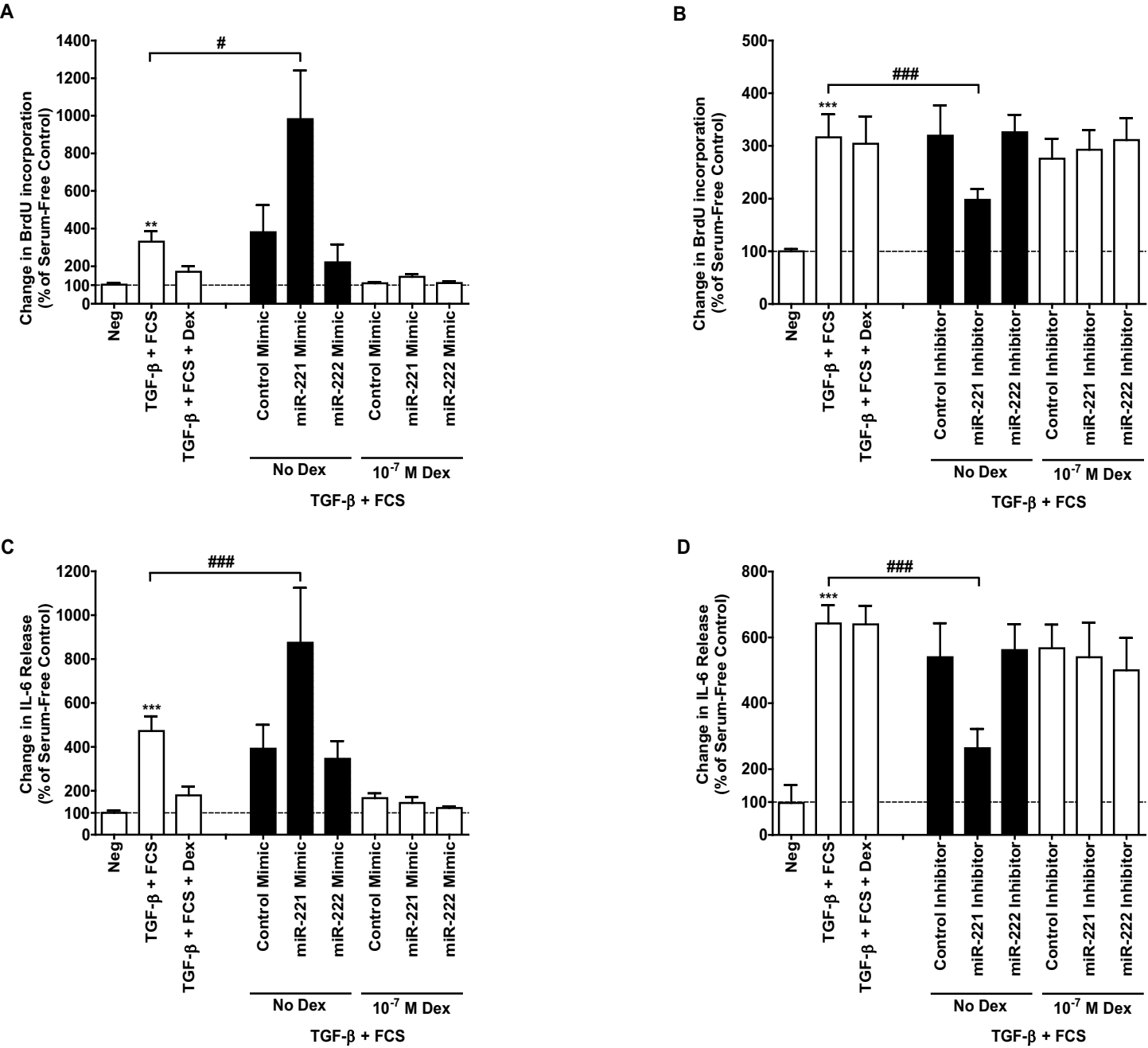
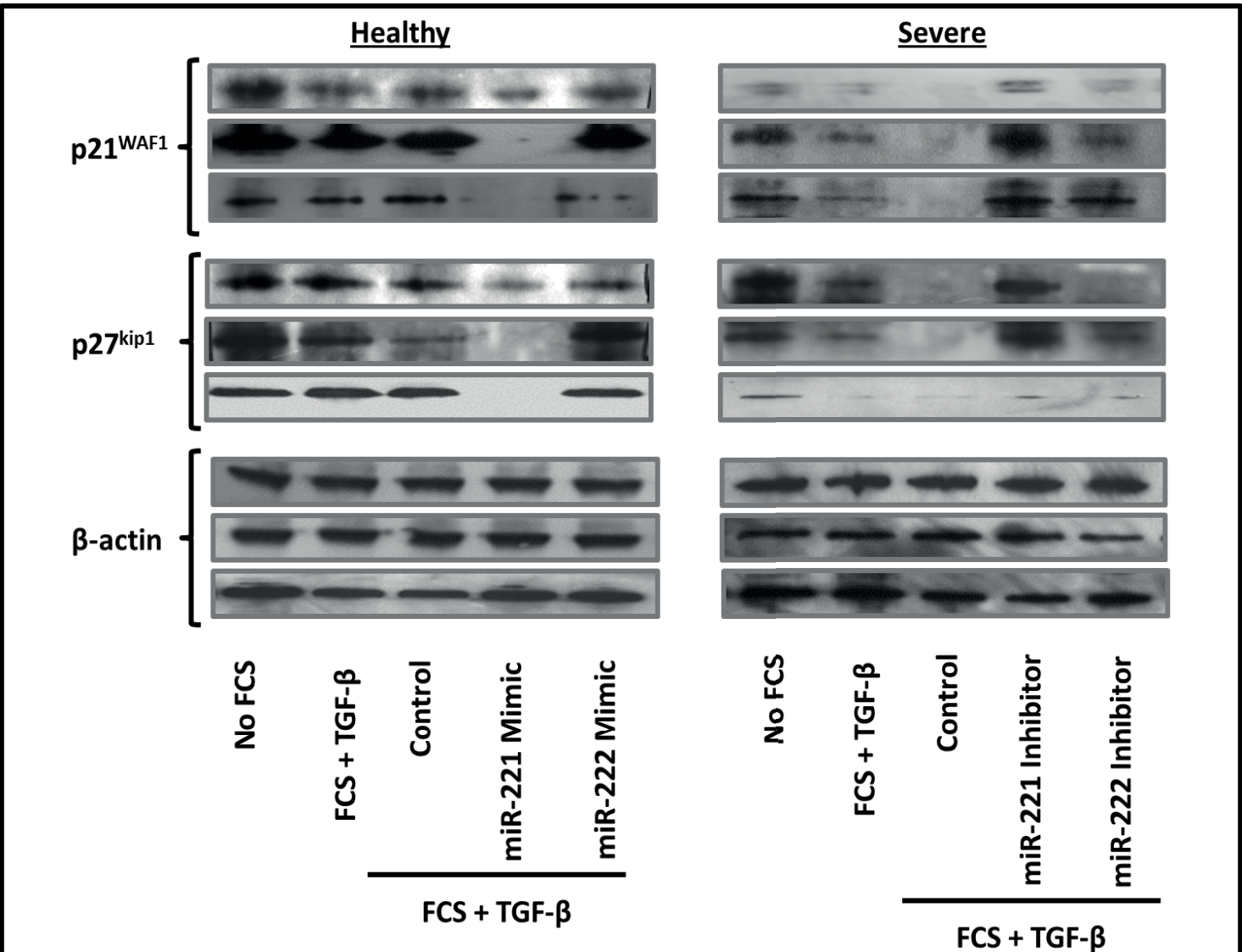
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