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).

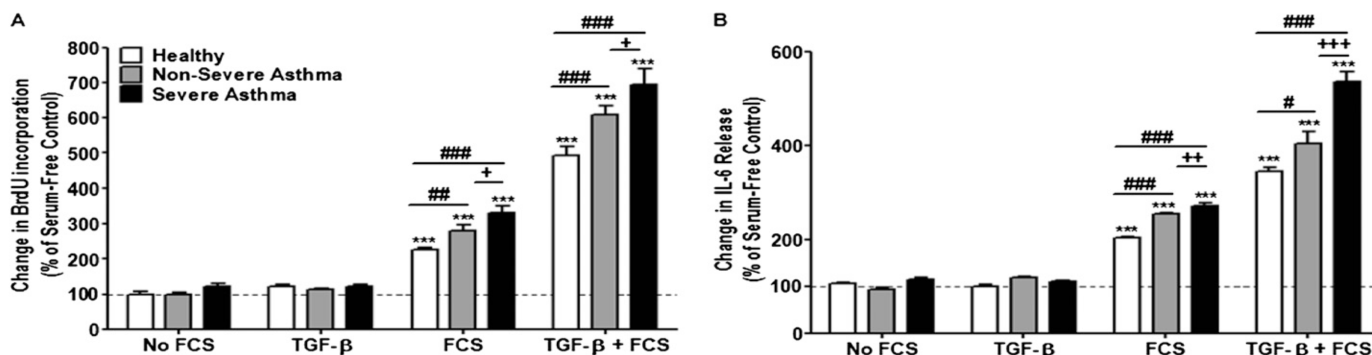

Figure 1. Effects of FCS and transforming growth factor–β (TGF-β) on airway smooth muscle (ASM) proliferation (*A*) and IL-6 release (*B*) from the ASM cells of healthy subjects, patients with nonsevere asthma, and patients with severe asthma at 8 days. *Bars/points* represent the means ± SEMs from nine 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.

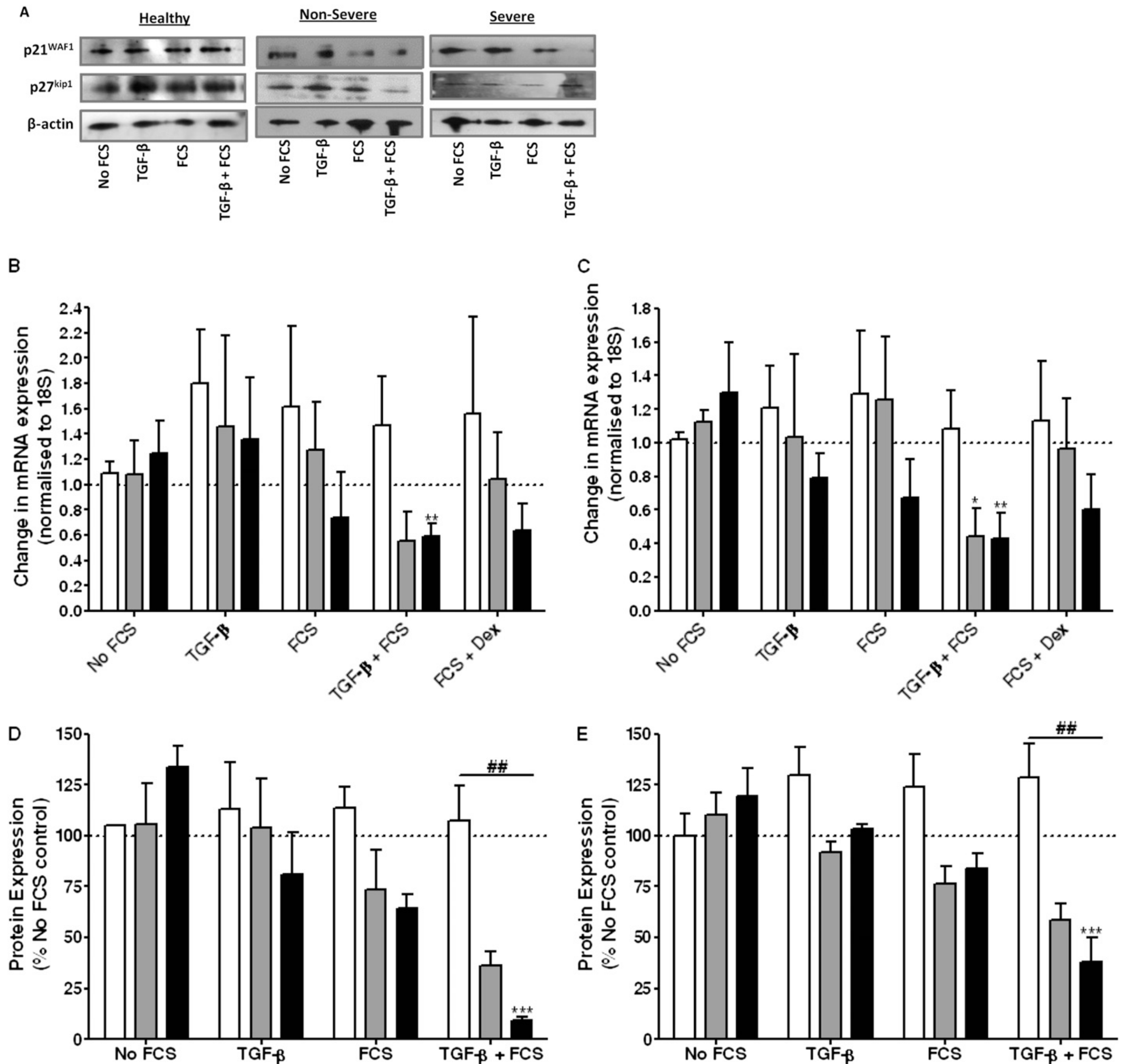

Figure 2. Effects of FCS and TGF-β on the cyclin-dependent kinase inhibitors p21^{WAF1} and p27^{kip1} mRNA (*B* and *C*) and protein expression (*A*, *D*, and *E*) at 24 hours. (*A*) Examples of Western blots of protein expression from extracts of ASM cells exposed to control (No FCS), TGF-β, FCS, and [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

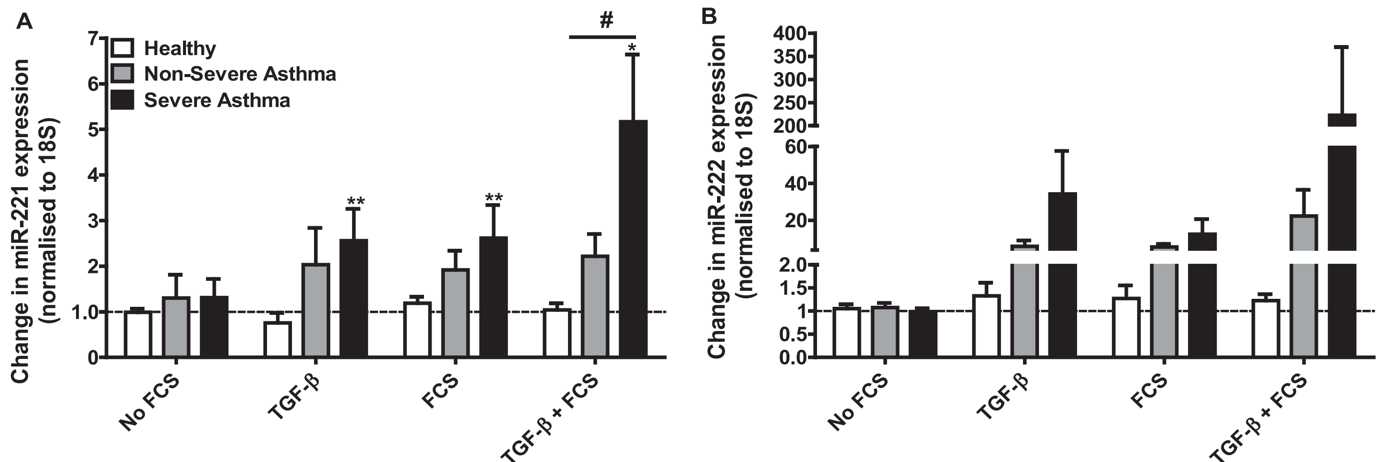

Figure 3. Effects of FCS and TGF-β on microRNA-221 (miR-221) and miR-222 expression between different subject groups at 24 hours. Mean miR-221 and miR-222 expressions, as measured by TaqMan RT-PCR, are shown in *A* and *B*, respectively. *Bars* represent the means ± SEMs of nine ASMC donors in each group.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

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

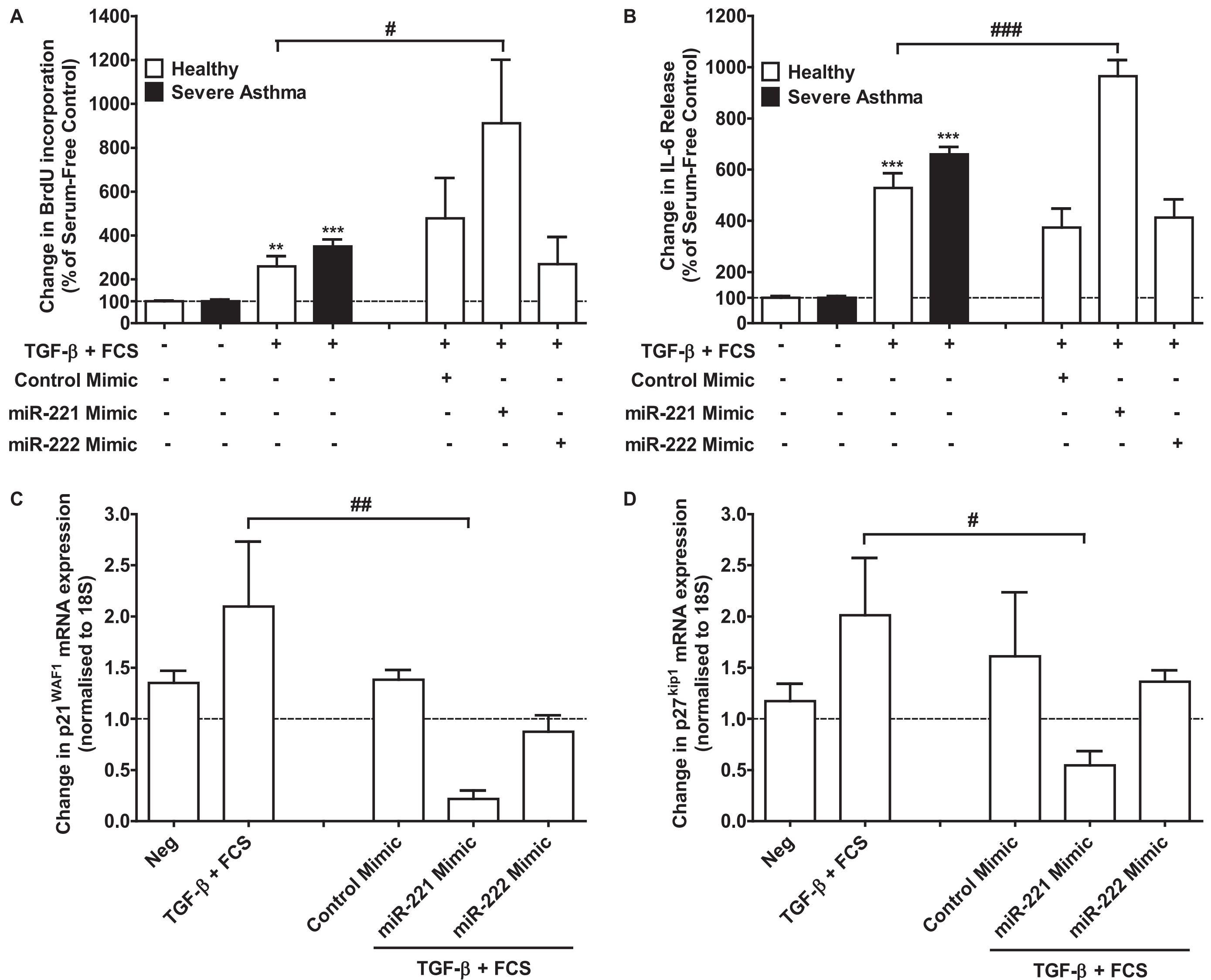

Figure 4. Effects of the overexpression of miR-221 and miR-222 in ASM cells from healthy subjects. DNA synthesis (*A*), IL-6 release (*B*), and p21^{WAF1} (*C*) and p27^{kip1} (*D*) mRNA expression were measured by BrdU ELISA, a DuoSet ELISA Assay (R&D Systems, Abingdon, UK), or TaqMan RT-PCR, respectively. 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

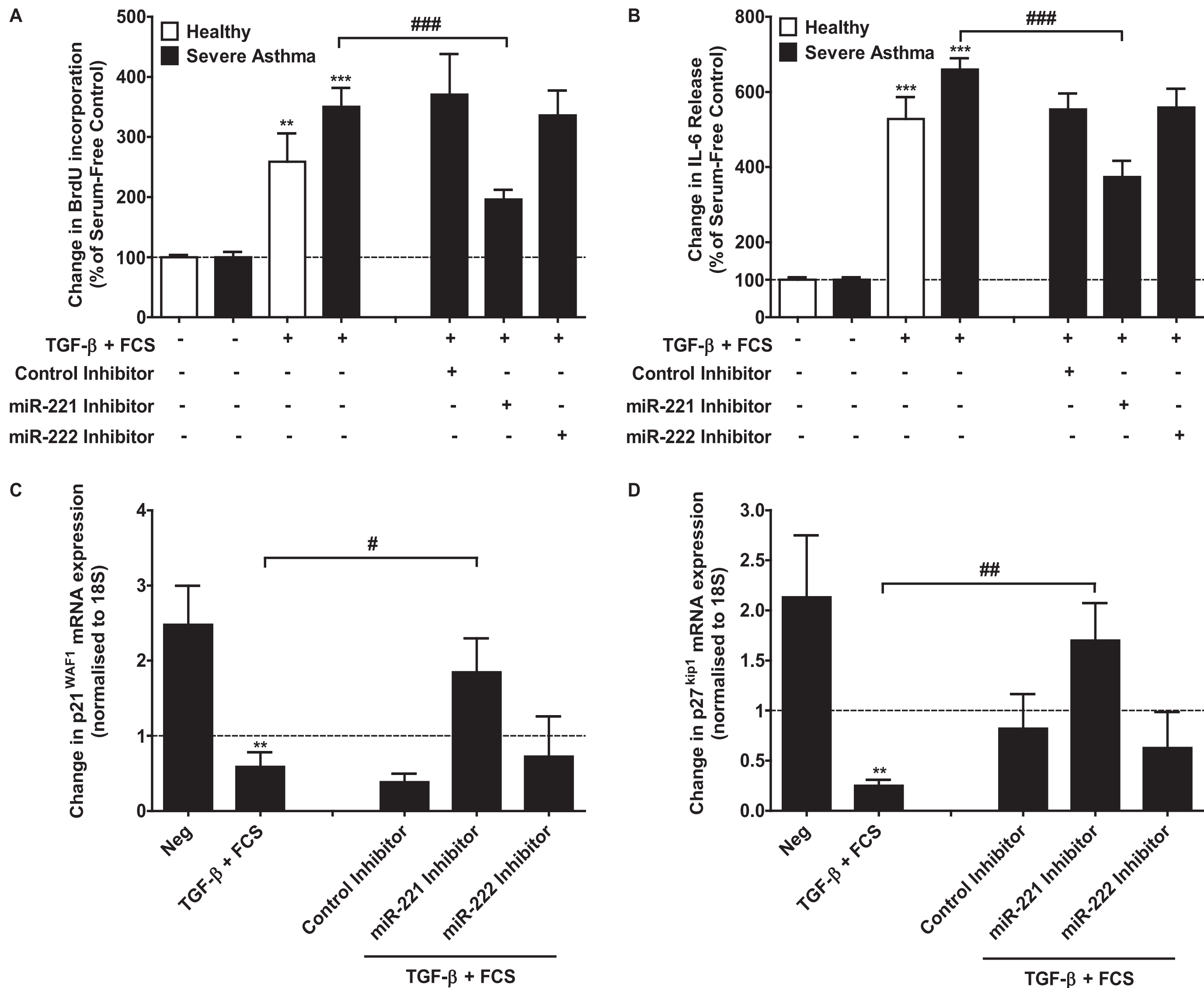

Figure 5. Effects of inhibition of miR-221 and miR-222 in ASM cells from subjects with severe asthma. DNA synthesis (*A*), IL-6 release (*B*), and $p21^{WAF1}$ mRNA (*C*) and $p27^{kip1}$ mRNA (*D*) expression were subsequently measured by BrdU ELISA, a DuoSet ELISA Assay, or TaqMan RT-PCR, respectively. 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

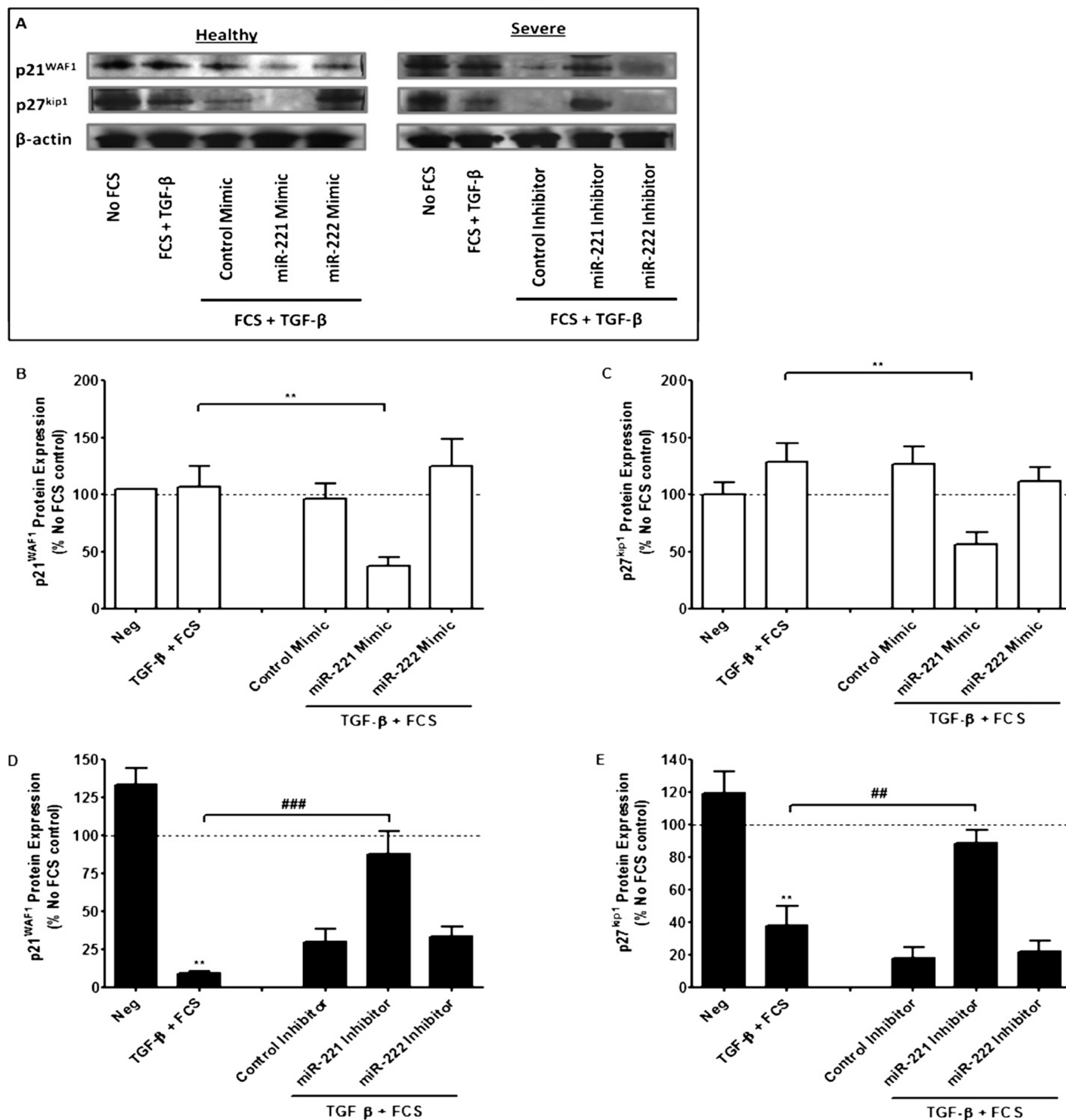

Figure 6. Effects of overexpression or inhibition of miR-221 and miR-222 on $p21^{WAF1}$ and $p27^{kip1}$ protein in ASM cells at 24 hours. Representative results from a subjects without asthma and from a subject with severe asthma are shown in *A*. Mean protein expression, quantified by densitometry, 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).

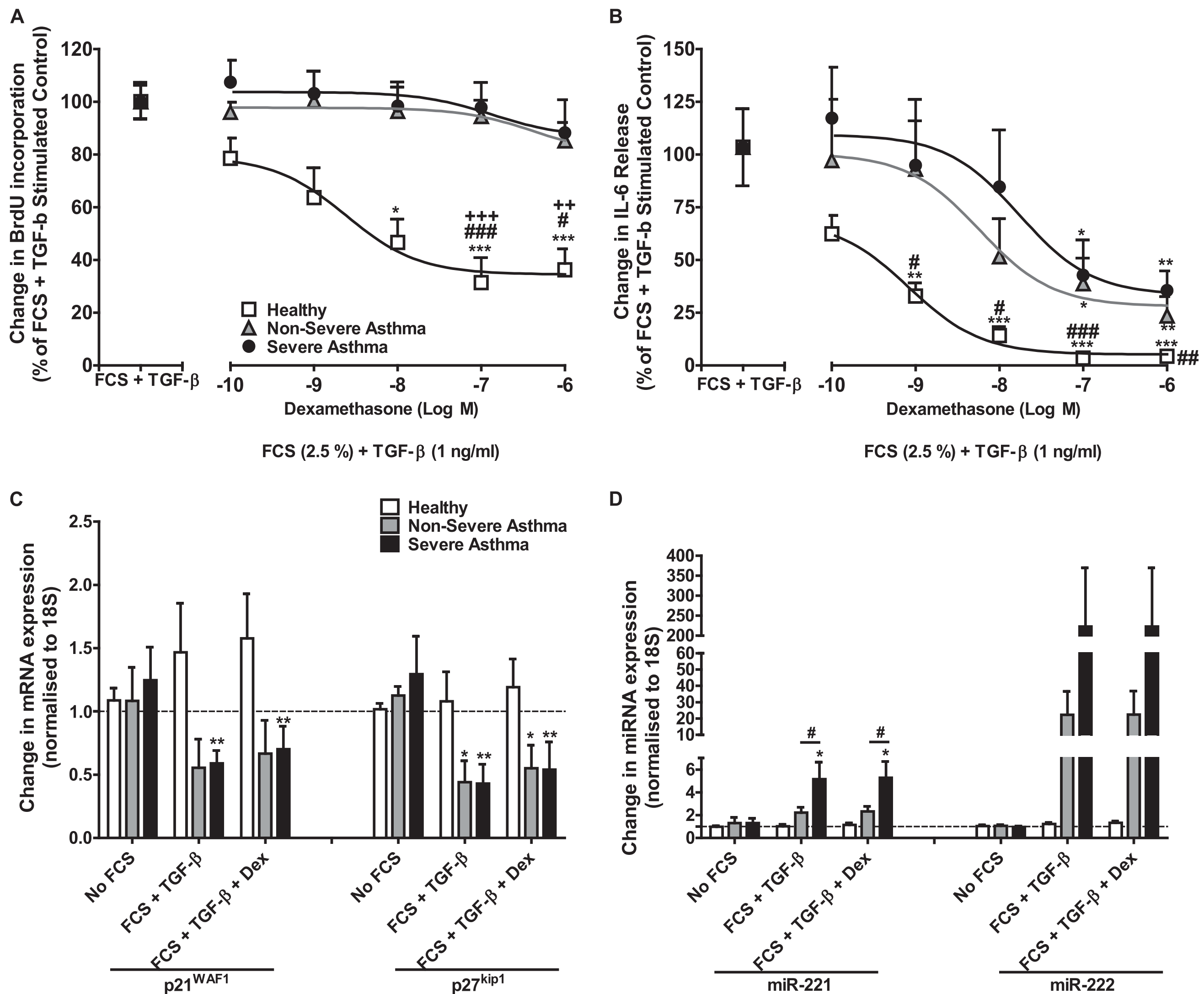

Figure 7. Effects of dexamethasone (Dex) on ASM proliferation, IL-6 release, p21^{WAF1} and p27^{kip1} mRNA, and miR-221 and miR-222 expression in ASM cells from healthy subjects, subjects with nonsevere asthma, and subjects with severe asthma. DNA synthesis (*A*) and IL-6 release (*B*) were measured 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.

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