

Selective dysfunction of p53 for mitochondrial biogenesis induces cellular proliferation in bronchial smooth muscle from asthmatic patients

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Background: Increase of bronchial smooth muscle (BSM) mass is a crucial feature of asthma remodeling. The mechanisms of such an increased BSM mass are complex but involve enhanced mitochondrial biogenesis, leading to increased proliferation of BSM cells in asthmatic patients. The major tumor suppressor protein p53 is a key cell regulator involved in cell proliferation and has also been implicated in mitochondrial biogenesis.

However, the role of p53 in BSM cell proliferation and mitochondrial biogenesis has not been investigated thus far.

Objective: We sought to evaluate the role of p53 in proliferation of BSM cells in asthmatic patients and mitochondrial biogenesis.

Methods: The expression of p53 was assessed both *in vitro* by using flow cytometry and Western blotting and *ex vivo* by using RT-PCR after laser microdissection. The role of p53 was assessed with small hairpin RNA lentivirus in both asthmatic patients and control subjects with BSM cell proliferation by using 5-bromo-2'-deoxyuridine and cell counting and in the expression of p21, BCL2-associated X protein, mitochondrial transcription factor A (TFAM), and peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α).

Results: Twenty-nine patients with moderate-to-severe asthma and 26 control subjects were enrolled in the study. p53 expression was increased in BSM from asthmatic patients both *ex vivo* and *in vitro*, with a decreased interaction with mouse double minute 2 homolog (Mdm2) and an increased phosphorylation of serine 20. p53 did not inhibit the transcription of both TFAM and PGC-1 α in BSM cells from asthmatic patients. As a consequence, p53 is unable to slow the increased mitochondrial biogenesis and hence the subsequent increased proliferation of BSM cells in asthmatic patients. **Conclusion:** this study suggests that p53 might act as a new potential therapeutic target against BSM remodeling in asthmatic patients. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Asthma, remodeling, lentivirus, peroxisome proliferator-activated receptor γ coactivator 1 α , mitochondrial transcription factor A

Asthma is a common respiratory disease characterized by bronchial hyperresponsiveness, inflammation, and remodeling.¹ Bronchial remodeling corresponds to an increased thickening of the bronchial wall caused by various structural alterations, including abnormal epithelium, subepithelial membrane thickening, alteration in extracellular matrix deposition, mucus gland hypertrophy, and increased bronchial smooth muscle (BSM) mass.¹ This latter feature is crucial because it correlates with the decrease in lung function.^{2,3} In this connection bronchial thermoplasty, an invasive technique that is supposed to decrease BSM mass, is associated with a decrease in exacerbation rate.⁴ Together, these findings point out the main role of BSM remodeling in asthma physiopathology.

The mechanisms of this asthma-induced increase in BSM mass are complex and remain controversial. An increased proliferation of BSM cells in asthmatic patients has been proposed and largely documented both *in vitro* and *in vivo*,^{3,5-7} the mechanism of which involves an increased mitochondrial biogenesis and mitochondrial mass through the increase in peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) and mitochondrial transcription factor A (TFAM) expression.⁷ The role of abnormal calcium entry in BSM cells in asthmatic patients has also been suggested *in vitro*.⁷ We very recently demonstrated the beneficial long-term effect of the Ca²⁺ channel blocker gallopamil on BSM remodeling in patients with severe asthma.⁸ Compared with placebo, gallopamil added to conventional anti-inflammatory treatments significantly reduced normalized BSM thickness *in vivo*.⁸ Nevertheless, the complex mechanisms of such an increased mitochondrial biogenesis are still not fully understood.

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Abbreviations used

Bax: BCL2-associated X protein
 BrdU: 5-Bromo-2'-deoxyuridine
 BSM: Bronchial smooth muscle
 DMEM: Dulbecco modified Eagle medium
 FITC: Fluorescein isothiocyanate
 GFP: Green fluorescent protein
 Mdm2: Mouse double minute 2 homolog
 PGC-1 α : peroxisome proliferator-activated receptor γ coactivator 1 α
 shRNA: Small hairpin RNA
 α -SMA: α -Smooth muscle actin
 TFAM: Mitochondrial transcription factor A
 TOM 20: Translocase of outer mitochondrial membranes 20

The major tumor suppressor protein p53 is a key cell regulator involved in cell proliferation.^{9,10} Indeed, p53 has been shown to be involved not only in cancer cell proliferation^{11,12} but also in noncancer cells, such as mesenchymal progenitor cells¹³ or aortic vascular smooth muscle.¹⁴ p53 interacts with a number of cofactors to regulate cell homeostasis. For example, p53 can act on the cell-cycle inhibitor p21 (WAF1/CIP1)¹⁵ or BCL2-associated X protein (Bax)¹⁶ to modulate cell proliferation or apoptosis, respectively. p53 has also been implicated in mitochondrial biogenesis.¹⁷ For example, in skeletal muscle p53 has been shown to be an activator of TFAM,¹⁸ whereas in cardiomyocytes p53 acts as a repressor of PGC-1 α .¹⁹ Consequently, p53 might be a key player in BSM cell proliferation and mitochondrial biogenesis, but this issue has not been investigated thus far. Thus we hypothesized that p53 can modulate BSM cell mitochondrial biogenesis and hence proliferation and that this modulation is disrupted in cells from asthmatic patients.

METHODS**Study populations**

A total of 29 patients with moderate-to-severe persistent asthma and 26 nonasthmatic subjects were prospectively recruited from Centre Hospitalier Universitaire de Bordeaux, France, according to Global Initiative for Asthma criteria.²⁰ All subjects provided written informed consent to participate in the study after the nature of the procedure had been fully explained. The study received approval from the local ethics committee. Bronchial specimens from all subjects were obtained by means of either fiberoptic bronchoscopy or lobectomy, as previously described.²¹

Optical microscopy

Bronchial specimens were embedded in paraffin. Assessable BSM was identified by a pathologist in a blinded fashion by using both morphologic characteristics and α -smooth muscle actin (α -SMA) staining, as previously described.⁷ The total area of the BSM layer was calculated manually in a blind fashion by using ScanView software (Soft Imaging System, Berlin, Germany) at a magnification of $\times 200$. This BSM area was normalized by the whole area of the corresponding tissues and presented as a percentage of the whole area.

Electronic microscopy

Bronchial specimens were also embedded in EPON for electron microscopy (Tecnai 12; Philips, FEI, Hillsboro, Ore), as previously described.²² Ten 60-nm-thick ultrathin serial sections per specimen were cut and examined by a pathologist to locate whole nucleated BSM cells. The number of mitochondrial sections normalized by the whole-cell area was assessed manually from 10 to 25 measurements per section in a blind fashion by using ScanView and ImageJ (National Institutes of Health, Bethesda, Md) at a magnification of $\times 6000$.

Laser microdissection

Laser dissection was performed with a PALM MicroBeam microdissection system, version 4.0–1206, equipped with a P.A.L.M. RoboSoftware (P.A.L.M. Microlaser Technologies, Munich, Germany), as described previously.⁶ Briefly, frozen sections (30 μ m) of bronchial biopsy specimens were obtained and mounted on PEN-membrane 1-mm glass slides. mRNAs were extracted, and p53 expression levels were assessed by using RT-PCR.

Primary cell culture and proliferation

Primary cultures of BSM cells were established from bronchial specimens, as previously described.^{23,24} All experiments were performed on cells with a confirmed BSM phenotype (ie, >90% of cultured cells were positive for both anti- α -SMA and anti-calponin antibody) between passages 2 and 5 to avoid the potential differentiation of BSM cells into myofibroblasts or fibroblasts. Cell proliferation was evaluated by using both 5-bromo-2'-deoxyuridine (BrdU) incorporation and manual cell counting.

Lentivirus experiments

We used dedicated lentivirus producing small hairpin RNA (shRNA) directed against p53 or TFAM, as previously described.^{21,24} The lentivirus produced a green fluorescent protein (GFP) used to follow its expression in infected cells. Moreover, we also used an empty vector, which did not produce any shRNA, as a control. Epithelial supernatants were obtained from confluent HBE160- cells in Dulbecco modified Eagle medium (DMEM) supplemented in 10% FBS.

RT-PCR

After reverse transcription, PCR was performed on a Rotor-Gene 2000 (Corbett Research, Mortlake, Australia)⁷ by using appropriate primers designed to target p53, TFAM, PGC-1 α , p21, and Bax. The mRNA expression levels of these genes of interest were normalized to those of endogenous references by using geometric averaging of 3 internal housekeeping genes (ie, YWHAZ, HPRT-1, and PO), as described previously^{6,7,23–25} and according to geNorm software.²⁶

Protein expression

BSM expression of porin, translocase of outer mitochondrial membranes 20 (TOM20), p53, and mouse double minute 2 homolog (Mdm2) protein was determined by means of immunoblotting on cell protein extracts, flow cytometry, or both. Coimmunoprecipitation was performed with Dynabeads (Life Technologies, Grand Island, NY) associated with p53 antibody and precipitated proteins revealed by means of Western blotting with p53 and Mdm2 antibodies.

p53 gene sequencing

p53 gene sequencing was performed on DNA extracted from BSM cells with the 3100 Genetic Analyzer (Applied Biosystems Hitachi, Carlsbad, Calif). The primer details used for p53 gene sequencing are presented in Table E1 in this article's Online Repository at www.jacionline.org.

p53 transcription factor binding assay

p53 binding was assessed on nuclear protein extract from BSM cells by using the p53 transcription factor binding assay, according to the manufacturer's protocol (Cayman, Ann Arbor, Mich).

Statistical analysis

Statistical analysis was performed with Prism 6 software (GraphPad Software, La Jolla, Calif). Values are presented as means $\pm$ SEMs. Statistical significance was analyzed by using paired or unpaired *t* tests, Mann-Whitney *U* tests, the Fisher exact test for comparison of proportions, ANOVA, and Turkey tests.

TABLE I. Clinical and functional characteristics of subjects

Characteristics	Control subjects	Asthmatic patients	P value
No. of patients	26	29	
Age (y)	56.65 ± 2.12	52.55 ± 2.19	.2
Range	32-75	29-72	
Body mass index (kg/m ²)	24.44 ± 0.90	28.01 ± 1.64	.8
Sex ratio (M/F)	17/9	6/23	.0011*
Smoking status			
Nonsmoker (no.)	6	29	
Former smoker (no.)	13	0	
Current smoker (no.)	7	0	
Treatments			
LABA (no.)	0	26	
ICS (no.)	0	29	
OCS (no.)	0	6	
Moderate/severe asthma (no.)	—	14/15	
FEV ₁ (% predicted)	89.82 ± 3.45	79.51 ± 2.92	.02
FVC (% predicted)	77.65 ± 0.93	72.53 ± 1.54	.01

Values are means ± SEMs, where shown. *P* values were calculated with use of a 2-sided independent *t* test for variables with a parametric distribution and the Mann-Whitney *U* test for comparison of nonparametric variables.

FVC, Forced vital capacity; ICS, inhaled corticosteroid; LABA, long-acting β_2 -agonist; OCS, oral corticosteroid.

*Sex ratios were compared by using the Fisher exact test.

A complete description of all methods is available in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS

Study populations

Subjects' and patients' characteristics are presented in [Table I](#). There were more female subjects in the asthmatic group. All asthmatic patients used inhaled corticosteroids continuously, and their FEV₁ was significantly lower than that of control subjects ([Table I](#)). However, control subjects and asthmatic patients were similar in terms of age and body mass index.

Increased expression of p53 in BSM from asthmatic patients

Using optical microscopy, we first confirmed that the BSM area was increased in biopsy specimens from asthmatic patients compared with that in control subjects ([Fig 1, A](#)). This increase in BSM mass *ex vivo* was associated with an increase in BSM cell proliferation *in vitro* by using both BrdU incorporation ([Fig 1, B](#)) and cell counting (see [Fig E1](#) in this article's Online Repository at www.jacionline.org). We also assessed the BSM mitochondrial mass both *ex vivo* by using electronic microscopy and *in vitro* by using Western blotting and flow cytometry. The number of mitochondrial sections ([Fig 1, C](#), and see [Fig E2](#) in this article's Online Repository at www.jacionline.org) and both the porin ([Fig 1, D](#)) and TOM20 contents (see [Fig E3](#) in this article's Online Repository at www.jacionline.org) were all increased in BSM cells from asthmatic patients compared with those in control subjects. We then assessed the expression of p53 *ex vivo* by using laser-microdissected BSM and RT-PCR and *in vitro* by using Western blotting and flow cytometry. Both *ex vivo* ([Fig 1, E](#)) and *in vitro* ([Fig 1, F](#), and see [Fig E4](#) in this article's Online Repository at www.jacionline.org) levels of p53 were increased in BSM from asthmatic patients compared with those in control subjects.

To evaluate the potential involvement of Mdm2 in this increased level of p53 within BSM cells from asthmatic patients, we first assessed its total protein expression. Protein levels of Mdm2 were similar in BSM cells from asthmatic patients and control subjects by using both Western blotting ([Fig 2, A](#)) and flow cytometry ([Fig 2, B](#)). Because p53 degradation through Mdm2 involved Mdm2/p53 protein interaction, we coimmunoprecipitated p53 and assessed Mdm2 interaction of both proteins by using Western blotting. Interestingly, we found that Mdm2/p53 interaction was decreased in BSM cells from asthmatic patients compared with that in control subjects ([Fig 2, C](#)). To investigate the mechanism of such a decreased interaction, we assessed the level of serine 20 phosphorylation of p53 (p53Pser20), which inhibits p53 and Mdm2 interaction.²⁷ We found that the p53Pser20/total p53 ratio was increased in BSM cells from asthmatic patients compared with that in control subjects ([Fig 2, D](#)).

Role of p53 in BSM cell proliferation

We then assessed the role of p53 in proliferation of BSM cells from both asthmatic patients and control subjects. For this purpose, a dedicated lentivirus producing a specific shRNA targeting p53 mRNA to specifically knock down its expression was designed. More than 80% of BSM cells were stably transduced by such a lentivirus or the dedicated control lentivirus. There was no difference in transduction efficiency between BSM cells from asthmatic patients and those from control subjects for either p53shRNA or the control lentivirus (see [Fig E5](#) in this article's Online Repository at www.jacionline.org). Thus we assessed the effect of this dedicated lentivirus on p53 mRNA expression and found that it specifically downregulates p53 mRNA in BSM cells from both asthmatic patients and control subjects compared with that in nontransduced cells and BSM cells transduced by the control lentivirus ([Fig 3, A](#)). Similarly, p53 protein expression was also drastically reduced in BSM cells from both asthmatic patients and control subjects compared with that in nontransduced cells and BSM cells transduced by the control lentivirus ([Fig 3, B](#)).

We then evaluated the effect of this lentivirus on BSM cell proliferation. Using cell counting, we found a marked increase in the proliferation of control BSM cells transduced with the lentivirus producing shRNA against p53 compared with that of both nontransduced cells and BSM cells transduced by the control lentivirus ([Fig 3, C](#)). This latter result suggests that p53 is able to limit cell proliferation in the control BSM. Interestingly, however, the same p53shRNA lentivirus did not alter BSM cell proliferation in asthmatic patients ([Fig 3, C](#)), suggesting that p53 might not be completely functional in these cells.

However, because BSM cell proliferation in asthmatic patients is already increased in nontransduced BSM cells compared with that in BSM cells from control subjects ([Fig 1, B](#)), one might suggest that the proliferation of BSM cells from asthmatic patients is already at the maximal possible rate. To explore this possibility, we stimulated BSM cells with epithelial supernatant instead of 10% FBS because we very recently demonstrated that BSM cell proliferation in asthmatic patients was also increased with epithelial supernatant.⁶ Using BrdU incorporation, we found that such stimulation further increased the proliferation of BSM cells in asthmatic patients, thus ruling out the abovementioned hypothesis. However, lentivirus producing shRNA against

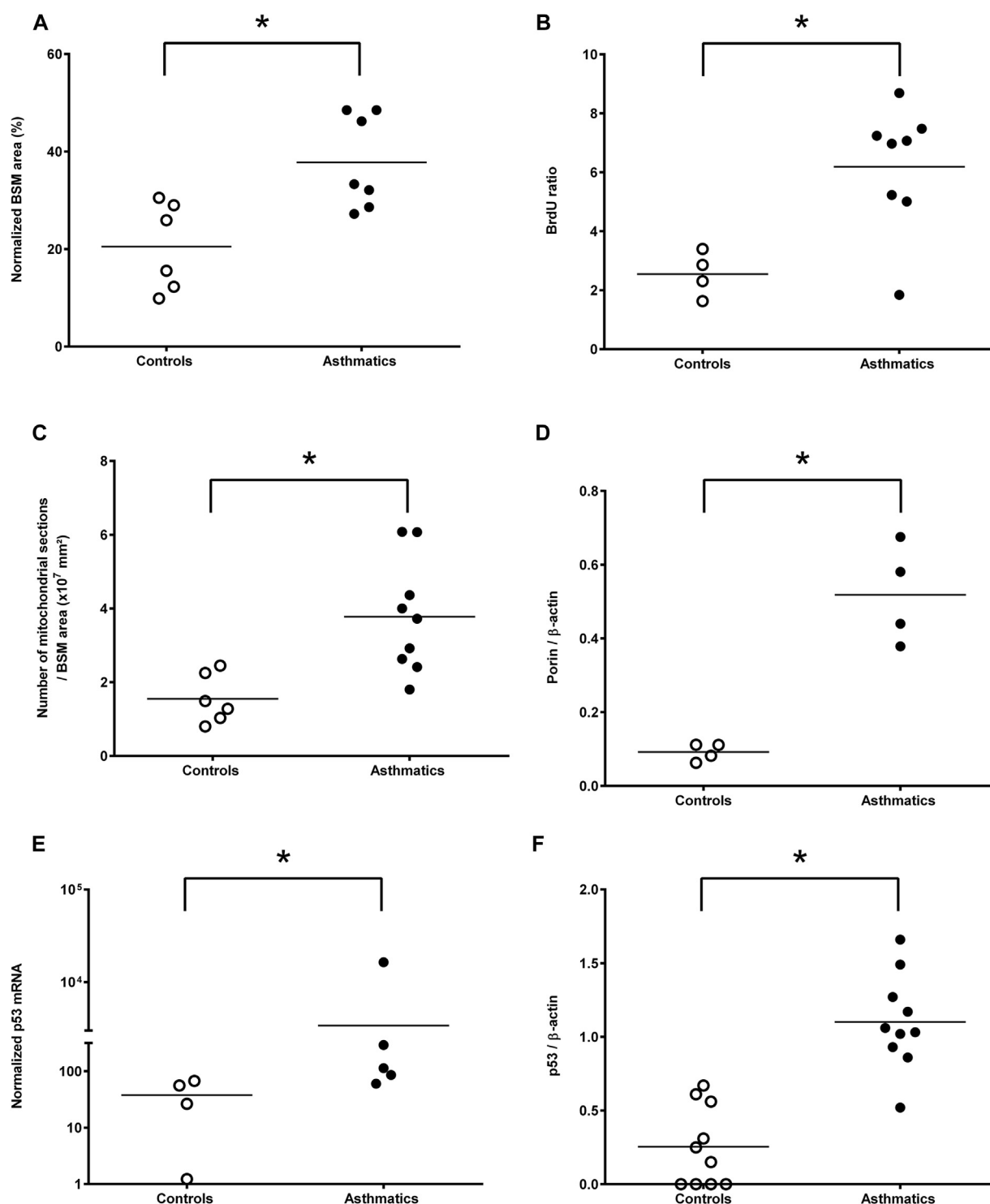


FIG 1. Bronchial specimens and BSM were obtained from control subjects (*open circles*) and asthmatic patients (*solid circles*). **A**, Normalized BSM area was assessed on biopsy specimens and is presented as a percentage of the whole biopsy area (control subjects, $n = 6$; asthmatic patients, $n = 7$). **B**, BSM cell proliferation was assessed by using BrdU incorporation in DMEM supplemented with 10% FBS (control subjects, $n = 4$; asthmatic patients, $n = 8$). **C**, Number of mitochondria per BSM cell was assessed from electronic microscopic images (control subjects, $n = 6$; asthmatic patients, $n = 9$). **D**, Quantification of porin content was assessed from Western blots and normalized by β -actin content (control subjects, $n = 4$; asthmatic patients, $n = 4$). **E** and **F**, Expression of p53 in BSM was assessed *in vivo* by using RT-PCR after laser microdissection (control subjects, $n = 4$; asthmatic patients, $n = 5$; Fig 1, *E*) and *in vitro* by means of Western blotting of BSM cell lysates (control subjects, $n = 10$; asthmatic patients, $n = 10$; Fig 1, *F*). Individual data are shown, and lines represent means. * $P < .05$, Student t tests.

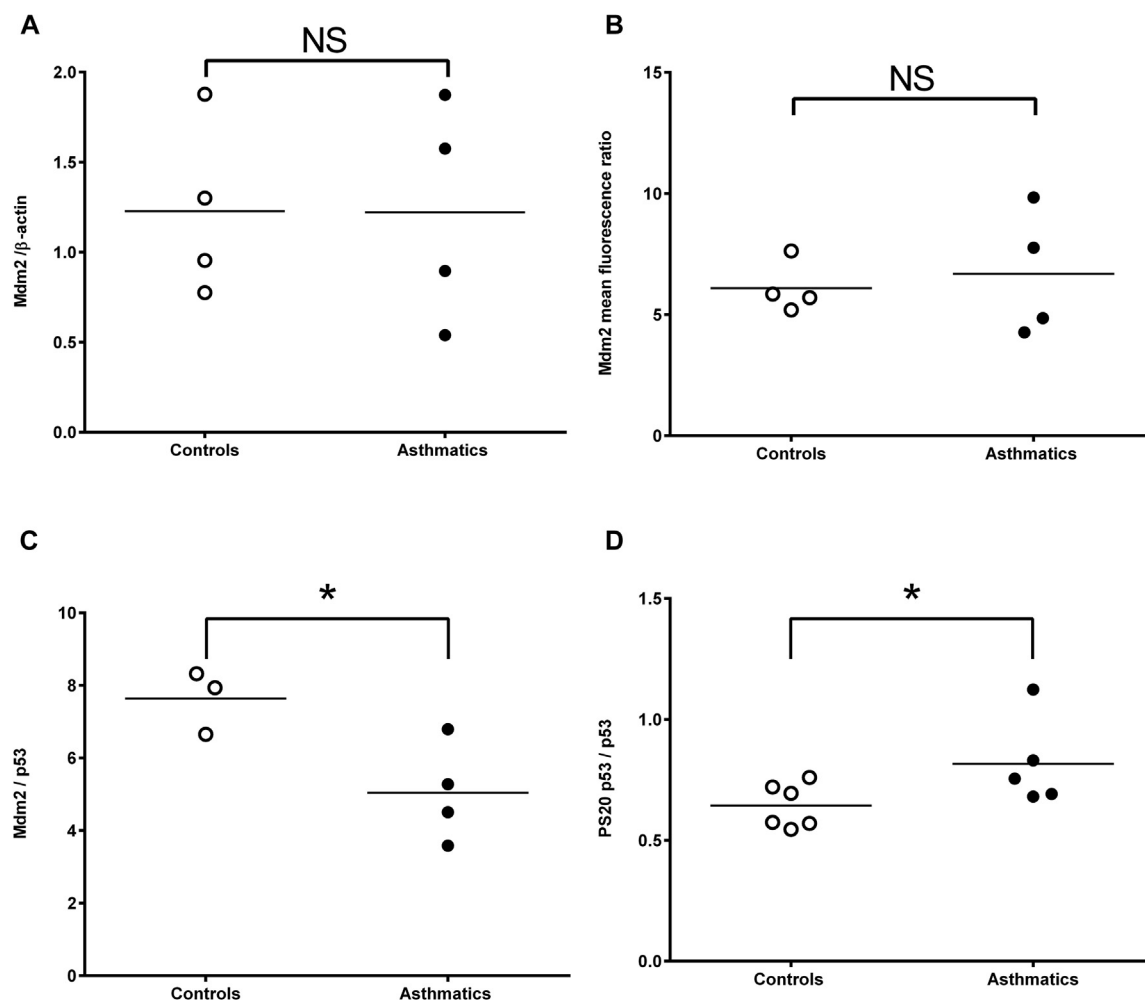


FIG 2. BSM cells were obtained from control subjects (*open circles*) and asthmatic patients (*solid circles*). **A**, Quantification of whole Mdm2 content was assessed from Western blots and normalized by β -actin content (control subjects, $n = 4$; asthmatic patients, $n = 4$). **B**, Mean fluorescence intensity of Mdm2 antibody was assessed by using flow cytometry and normalized by irrelevant antibody (control subjects, $n = 4$; asthmatic patients, $n = 4$). **C**, After coimmunoprecipitation of p53, quantification of Mdm2 was assessed from Western blots and normalized by p53 (control subjects, $n = 3$; asthmatic patients, $n = 4$). **D**, Quantification of phospho-ser20-p53 content was assessed from Western blots and normalized by p53 content (control subjects, $n = 6$; asthmatic patients, $n = 5$). Individual data are shown, and *lines* represent means. * $P < .05$, Student t tests. NS, Nonsignificant.

p53 remained unable to increase the proliferation of BSM cells from asthmatic patients, whereas it still increased the proliferation of BSM cells from control subjects (Fig 3, D). Thus these results suggest that p53 in BSM cell proliferation is either nonfunctional or that its role is modified in BSM cells from asthmatic patients.

Because increased cell proliferation is accompanied by upregulation of mitochondrial biogenesis in BSM cells from asthmatic patients (Fig 1, A-D), we used the same lentivirus producing shRNA against p53 to assess the role of p53 on mitochondrial biogenesis. p53 in mitochondrial biogenesis acts through inhibition of the PGC-1 α promoter.²⁸ As expected, knocking down p53 in control BSM cells increased the mRNA expression of PGC-1 α (Fig 4, A, and see Fig E6, A, in this article's Online Repository at www.jacionline.org). Surprisingly, a similar knock down of p53 expression in BSM cells from asthmatic patients was

unable to act on PGC-1 α mRNA expression levels (Fig 4, A, and see Fig E6, A). Thus these results strongly suggest that the transrepression of mitochondrial biogenesis by p53 through PGC-1 α is selectively lost in BSM from asthmatic patients. This hypothesis is supported by the fact that p53 knock down in control BSM cells was able to increase the downstream and final processing element of the mitochondrial biogenesis cascade (ie, TFAM), whereas, as for PGC-1 α , p53 knock down had no effect on TFAM in BSM cells from asthmatic patients (Fig 4, B, and see Fig E6, B). By contrast, p53 still acted on p21 (Fig 4, C, and see Fig E6, C) and Bax (Fig 4, D, and see Fig E6, D) expression in BSM cells from both asthmatic patients and control subjects.

To explore the discrepancy between the effect of asthmatic p53 on mitochondrial biogenesis factors (ie, PGC-1 α and TFAM) and p21 or Bax, we addressed the question of the potential role of a somatic mutation of p53 in BSM cells from asthmatic patients

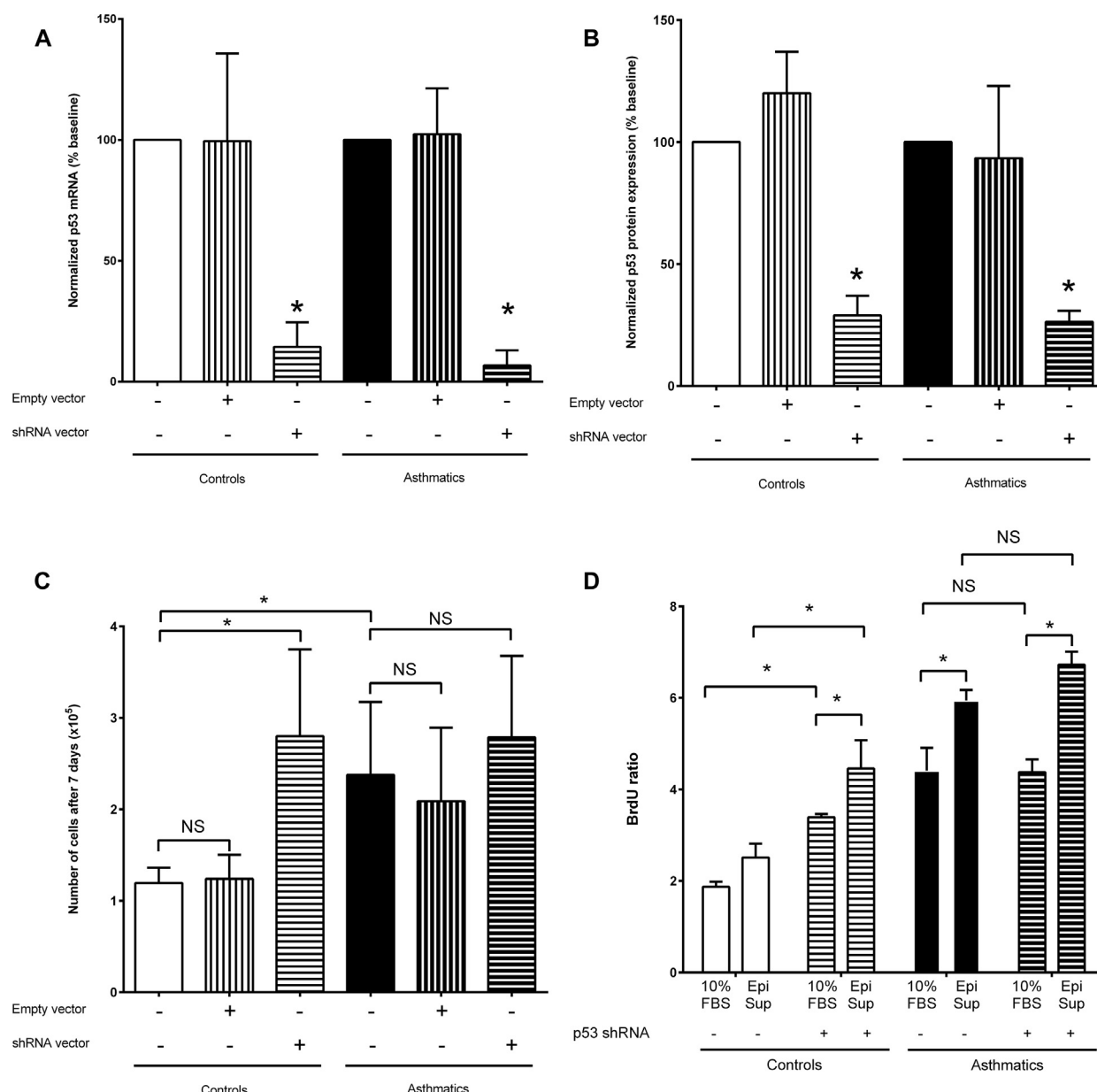


FIG 3. BSM cells were obtained from control subjects (white bars) and asthmatic patients (black bars). **A** and **B**, Expression of p53 in BSM cells was assessed by using quantitative RT-PCR (control subjects, $n = 7$; asthmatic patients, $n = 10$; Fig 3, **A**) and Western blotting (control subjects, $n = 5$; asthmatic patients, $n = 5$; Fig 3, **B**) and expressed as a percentage from nontransduced BSM cells (baseline). **C** and **D**, BSM cell proliferation was assessed by using cell counting (control subjects, $n = 5$; asthmatic patients, $n = 7$; Fig 3, **C**) or BrdU incorporation (control subjects, $n = 4$; asthmatic patients, $n = 5$; Fig 3, **D**) in DMEM supplemented with 10% FBS or bronchial epithelial cell supernatant (Epi Sup). BSM cells were not transduced (empty bars), transduced with an empty lentivirus (vertical hatched bars), or transduced with a lentivirus producing p53 shRNA (horizontal hatched bars). Data are means $\pm$ SEMs. * $P < .05$, ANOVA with the Turkey test. NS, Nonsignificant.

compared with those from control subjects. Using sequencing of p53 exons of 7 asthmatic patients and 6 control subjects, we were unable to find any specific mutation of p53 in BSM cells from asthmatic patients, especially in the hot spots of mutation recorded for p53 by the International Agency for Research on Cancer (data not shown). This latter result indicates that posttranslational mechanisms might have a role in modification of p53 function in BSM cells from asthmatic patients. Moreover,

using a p53 binding assay, we tested the efficacy of nuclear p53 from BSM cells from both asthmatic patients and control subjects to bind to p53 response element. Here, again, we were unable to find any difference between BSM proteins from both asthmatic patients and control subjects (see Fig E7 in this article's Online Repository at www.jacionline.org).

Finally, we used another lentivirus producing shRNA against TFAM to confirm the crucial role of the mitochondrial biogenesis

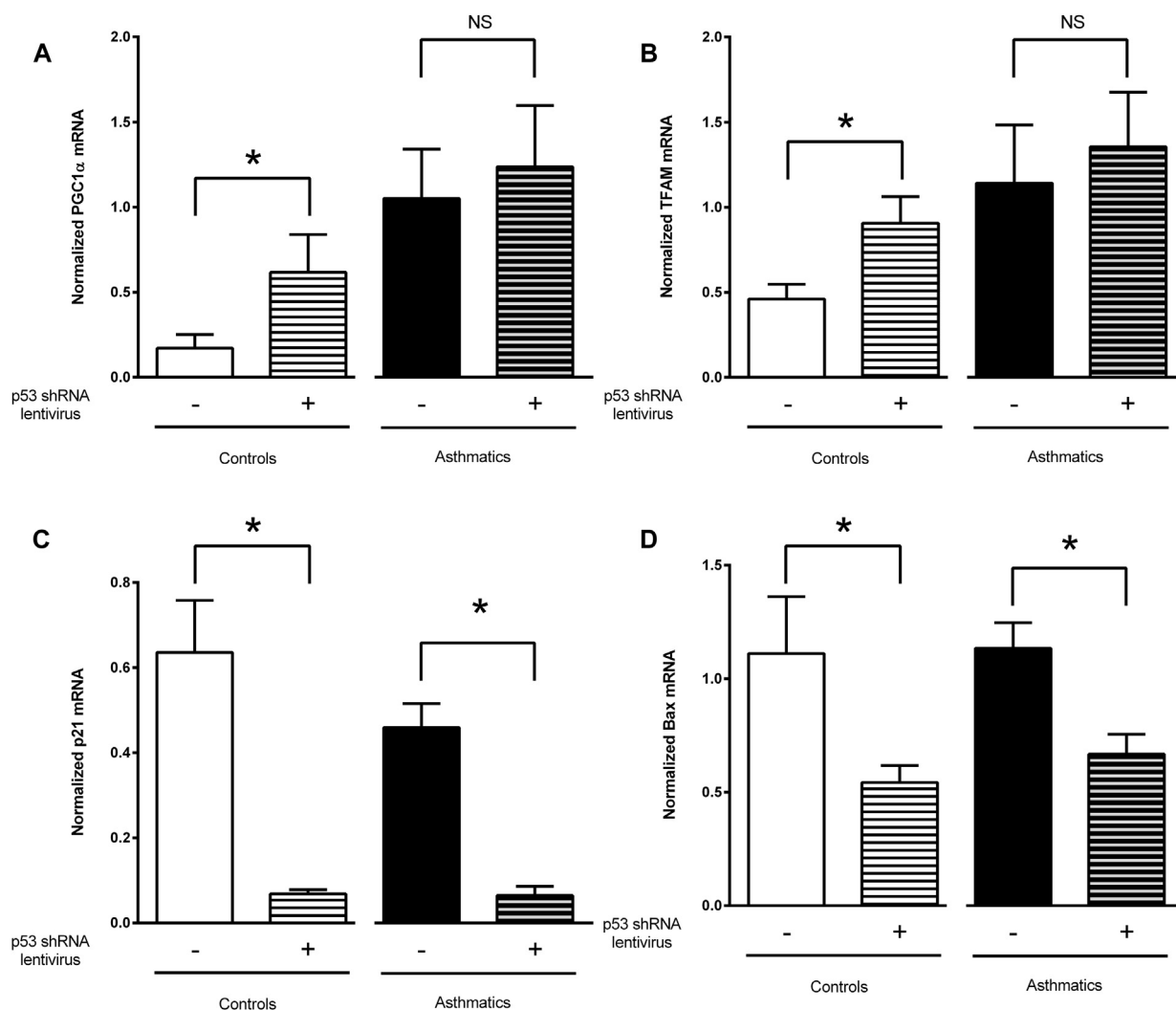


FIG 4. Expression of PGC-1 α (A), TFAM (B), p21 (C), and Bax (D) was assessed by using quantitative RT-PCR. BSM cells were obtained from control subjects (white bars, $n = 7$) and asthmatic patients (black bars, $n = 10$). BSM cells were either not transduced (empty bars) or transduced with a lentivirus producing p53 shRNA (horizontal hatched bars). Data are means $\pm$ SEMs. * $P < .05$, Student paired t tests. NS, Nonsignificant.

pathway on BSM cell proliferation. Knocking down TFAM in BSM cells from both asthmatic patients and control subjects dramatically decreased BSM cell proliferation (Fig 5). Moreover, this knock down abolished the difference between BSM cell proliferation in asthmatic patients and control subjects (Fig 5), which supports the hypothesis that mitochondrial biogenesis is the key enhancer of BSM cell proliferation in asthmatic patients.

DISCUSSION

This study indicates that, unlike in BSM from control subjects, p53 does not inhibit the transcription of PGC-1 α in BSM from asthmatic patients (see Fig E8 in this article's Online Repository at www.jacionline.org). As a consequence, p53 is unable to limit the increased mitochondrial biogenesis cascade and the subsequent increase in proliferation of BSM cells in asthmatic patients, despite the fact that its expression appears to be increased as a consequence of a lower interaction with Mdm2. By contrast, in

BSM cells from nonasthmatic subjects, p53 plays an inhibitory role in both mitochondrial biogenesis and proliferation.

Regarding inclusion criteria, for this study, we selected asthmatic patients with both severe and nonsevere disease. There was no difference between these subgroups of asthmatic patients in terms of p53 function and expression (see Fig E9 in this article's Online Repository at www.jacionline.org). Moreover, asthmatic patients and nonasthmatic subjects were similar in terms of age and body mass index, whereas sex ratios appeared different. However, it is unlikely that this difference could explain our results because there was no difference in p53 expression in BSM cells from female and male subjects, and p53 expression remains significantly increased in BSM cells from asthmatic patients compared with those from control subjects among female and male subjects separately (data not shown). Moreover, by using gastric epithelial cells, it has been demonstrated that proliferation induced by *Helicobacter pylori* infection was associated with increased p53 expression but was not sex dependent.²⁹ One could argue that, despite the number of patients included

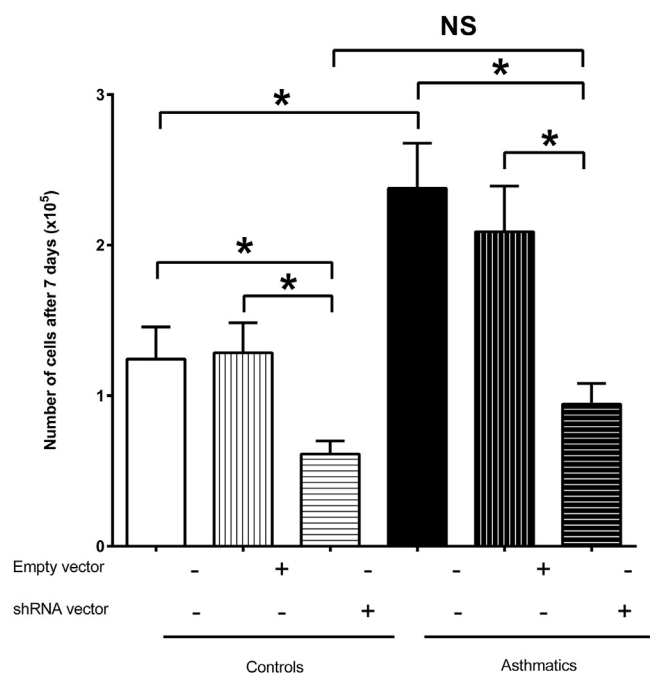


FIG 5. BSM cells were obtained from control subjects (white bars, $n = 7$) and asthmatic patients (black bars, $n = 7$). BSM cell proliferation was assessed by using cell counting in DMEM supplemented with 10% FBS. BSM cells were not transduced (empty bars), transduced with an empty lentivirus (vertical hatched bars), or transduced with a lentivirus producing TFAM shRNA (horizontal hatched bars). Data are means $\pm$ SEMs. * $P < .05$, ANOVA with the Turkey test. NS, Nonsignificant.

in the study (ie, 26 control subjects and 29 asthmatic patients), a subset only of the total population was used for each experiment. It must be taken into account that this study was performed on phenotypically confirmed primary human BSM cells from early passages, and it was thus impossible to perform all experiments on all BSM cells.

The protein p53 has been widely studied in a variety of cell types and in multiple diseases.^{13,30} However, to the best of our knowledge, its role in BSM and, more importantly, in BSM from asthmatic patients whose proliferation is increased has never been studied. For the first time, our results show that p53 limits the proliferation of BSM cells from nonasthmatic subjects. The mechanism of such a limitation is probably complex, but here we demonstrate that p53 activates transcription of p21 and Bax. It has been demonstrated previously that both p21 and Bax limit cell proliferation in various cell types^{31,32} because p21 blocks the cell cycle³³ and Bax induces cell apoptosis.¹⁶ However, in BSM cells from asthmatic patients, despite the evidence that p53 is still active on p21 and Bax, its effect on cell proliferation is lost.

Indeed, in the present study we confirm the crucial role of mitochondrial biogenesis and its final effector, TFAM, in the proliferation of BSM cells. We have observed that TFAM silencing with dedicated lentivirus not only dramatically reduces BSM cell proliferation from both control subjects and asthmatic patients but also abolishes the difference between BSM cell proliferation in control subjects and asthmatic patients. We previously pointed out the crucial role of mitochondrial biogenesis in BSM cells from patients with severe asthma,⁷ which raises the issue of the effect of p53 on this biogenesis. Indeed, we previously demonstrated that BSM cells from asthmatic patients

are characterized by an abnormal calcium entry, which activates various transcription factors, including PGC-1 α and TFAM, which in turn increase mitochondrial biogenesis and cell proliferation.⁷ Moreover, we recently demonstrated in a proof-of-concept clinical trial that targeting such an abnormal calcium entry was able to reduce BSM mass of patients with severe asthma *in vivo*.⁸ The implication of mitochondrial biogenesis in cell proliferation has also been described in other cell types, such as vascular smooth muscle cells and keratinocytes.³⁴⁻³⁶ In the present study we also show that under physiologic conditions, p53 acts as a repressor of the mitochondrial biogenesis pathway in BSM cells from control subjects, whereas in BSM cells from asthmatic patients, this repression is lost, leading to an increase in BSM cell proliferation without any negative feedback. At the molecular level, it has been shown recently that p53 binds to the PGC-1 α promoter to regulate its transcription.^{28,37} In BSM cells PGC-1 α promotes transcription of both nuclear respiratory factor 1 and TFAM, which are implicated in mitochondrial biogenesis.⁷ Depending on the cell type, p53 can be either an activator¹⁷ or a repressor¹⁹ of mitochondrial biogenesis. Therefore one might suggest that the differential effect of p53 on mitochondrial biogenesis in BSM cells from asthmatic patients versus control subjects could be related to a difference in cell phenotype. However, we paid special attention to performing experiments only in cells from early passages (2-5) and checked at each passage that the smooth muscle phenotype of cells from both nonasthmatic and asthmatic subjects was similar. As previously described, we used immunohistochemistry and flow cytometry to assess the presence of both α -SMA and calponin in our cells.²¹

The molecular mechanisms through which some p53 functions are altered in BSM from asthmatic patients deserves additional discussion. In tumors the majority of p53 mutations are found within the DNA binding domain, resulting in an altered conformation and changes in sequence-specific binding to DNA.³⁸ In the present study we show that p53 in asthmatic patients can bind normally to DNA. Nevertheless, p53 in asthmatic patients is unable to regulate PGC-1 α , whereas it remains able to regulate other target genes (ie, p21 and Bax). A similar discrepancy between the presence and absence of p53 regulation on expression of various target genes has already been observed in other cell types. For example, in melanoma metastasis mutations in p53 induce aberrant function on some target genes related to apoptosis and the cell cycle, whereas other target genes remain normally regulated by p53.³⁹ However, we performed sequencing of p53 exons from BSM cells from both asthmatic patients and control subjects and were not able to find any specific mutation of p53. This result indicates that the regulation of p53 function in asthmatic patients can be explained by alternative mechanisms that occur after gene transcription. Indeed, it has been shown that p53 activity is tightly regulated by numerous posttranslational modifications.⁴⁰ The mechanisms by which p53 function is altered in BSM from asthmatic patients thus needs to be addressed specifically in further studies.

Finally, alteration in some p53 functions induces the additional consequence of an increase in p53 expression in BSM cells from asthmatic patients. In patients with some p53-dependent cancers, loss of p53 function is associated with increased p53 expression both *in vitro* and *ex vivo*.⁴¹ In this study we also confirmed the *in vitro* findings *ex vivo* using microdissected BSM obtained from asthmatic and nonasthmatic subjects. Expression of p53 is

regulated by several factors, the most important being Mdm2.^{42,43} In the present study we demonstrated a lower interaction of p53 with Mdm2, which was related to an increased phosphorylation of p53 at serine 20 in BSM cells from asthmatic patients. Such a lower interaction decreased p53 degradation. However, further studies are required to identify the molecular mechanism responsible for this increased p53 phosphorylation.

In conclusion, this study indicates that p53 cannot limit the previously described⁷ increased mitochondrial biogenesis and subsequent increase in proliferation of BSM cells from asthmatic patients. These new findings should be integrated into the complex mechanisms of BSM cell pathologic proliferation in asthmatic patients (see Fig E8). Thus p53 might act as a new potential therapeutic target against BSM remodeling in asthmatic patients that deserves further studies.

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Clinical implications: Because p53 is implicated in the complex mechanism of BSM cell pathologic proliferation in asthmatic patients that leads to BSM remodeling, its role as a new potential therapeutic target against BSM remodeling in asthmatic patients should be examined.

REFERENCES

- Bara I, Ozier A, Tunon de Lara JM, Marthan R, Berger P. Pathophysiology of bronchial smooth muscle remodeling in asthma. *Eur Respir J* 2010;36:1174-84.
- Kaminska M, Foley S, Maghni K, Storness-Bliss C, Coxson H, Ghezzi H, et al. Airway remodeling in subjects with severe asthma with or without chronic persistent airflow obstruction. *J Allergy Clin Immunol* 2009;124:45-51.
- Ramos-Barbon D, Fraga-Iriso R, Brienza NS, Montero-Martinez C, Vereza-Hernando H, Olivenstein R, et al. T Cells localize with proliferating smooth muscle alpha-actin+ cell compartments in asthma. *Am J Respir Crit Care Med* 2010;182:317-24.
- Cox G, Thomson NC, Rubin AS, Niven RM, Corris PA, Siersted HC, et al. Asthma control during the year after bronchial thermoplasty. *N Engl J Med* 2007;356:1327-37.
- Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G, et al. Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med* 2001;164:474-7.
- Trian T, Allard B, Dupin I, Carvalho G, Ousova O, Maurat E, et al. House dust mites induce proliferation of severe asthmatic smooth muscle cells via an epithelium-dependent pathway. *Am J Respir Crit Care Med* 2015;191:538-46.
- Trian T, Benard G, Begueret H, Rossignol R, Girodet PO, Ghosh D, et al. Bronchial smooth muscle remodeling involves calcium-dependent enhanced mitochondrial biogenesis in asthma. *J Exp Med* 2007;204:3173-81.
- Girodet PO, Dournes G, Thumeret M, Begueret H, Dos Santos P, Ozier A, et al. Calcium channel blocker reduces airway remodeling in severe asthma: a proof-of-concept study. *Am J Respir Crit Care Med* 2015;191:876-83.
- Jiang W, Wang XW, Unger T, Forgues M, Kim JW, Hussain SP, et al. Cooperation of tumor-derived HBx mutants and p53-249(ser) mutant in regulating cell proliferation, anchorage-independent growth and aneuploidy in a telomerase-immortalized normal human hepatocyte-derived cell line. *Int J Cancer* 2010;127:1011-20.
- Brosh R, Rotter V. Transcriptional control of the proliferation cluster by the tumor suppressor p53. *Mol Biosyst* 2010;6:17-29.
- Zhang EB, Yin DD, Sun M, Kong R, Liu XH, You LH, et al. P53-regulated long non-coding RNA TUG1 affects cell proliferation in human non-small cell lung cancer, partly through epigenetically regulating HOXB7 expression. *Cell Death Dis* 2014;5:e1243.
- Montano X. Repression of SHP-1 expression by p53 leads to trkA tyrosine phosphorylation and suppression of breast cancer cell proliferation. *Oncogene* 2009;28:3787-800.
- Peng X, Yang L, Chang H, Dai G, Wang F, Duan X, et al. Wnt/beta-catenin signaling regulates the proliferation and differentiation of mesenchymal progenitor cells through the p53 pathway. *PLoS One* 2014;9:e97283.
- Chan KC, Wang CJ, Ho HH, Chen HM, Huang CN. Simvastatin inhibits cell cycle progression in glucose-stimulated proliferation of aortic vascular smooth muscle cells by up-regulating cyclin dependent kinase inhibitors and p53. *Pharmacol Res* 2008;58:247-56.
- Hsieh JK, Kletsas D, Clunn G, Hughes AD, Schachter M, Demoliou-Mason C. p53, p21(WAF1/CIP1), and MDM2 involvement in the proliferation and apoptosis in an in vitro model of conditionally immortalized human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2000;20:973-81.
- Fister S, Gunthert AR, Aicher B, Paulini KW, Emons G, Grundker C. GnRH-II antagonists induce apoptosis in human endometrial, ovarian, and breast cancer cells by up-regulating cyclin dependent kinase inhibitors and p53. *Cancer Res* 2009;69:6473-81.
- Saleem A, Adhithy P, Hood DA. Role of p53 in mitochondrial biogenesis and apoptosis in skeletal muscle. *Physiol Genomics* 2009;37:58-66.
- Saleem A, Hood DA. Acute exercise induces tumour suppressor protein p53 translocation to the mitochondria and promotes a p53-Tfam-mitochondrial DNA complex in skeletal muscle. *J Physiol* 2013;591:3625-36.
- Villeneuve C, Guilbeau-Frugier C, Sicard P, Lairet O, Ordener C, Duparc T, et al. p53-PGC-1alpha pathway mediates oxidative mitochondrial damage and cardiomyocyte necrosis induced by monoamine oxidase-A upregulation: role in chronic left ventricular dysfunction in mice. *Antioxid Redox Signal* 2013;18:5-18.
- Global Initiative for Asthma. Global strategy for asthma management and prevention. Available at: <http://www.ginasthma.org>. Accessed February 3, 2015.
- Bara I, Ozier A, Girodet PO, Carvalho G, Cattiaux J, Begueret H, et al. Role of YKL-40 in bronchial smooth muscle remodeling in asthma. *Am J Respir Crit Care Med* 2012;185:715-22.
- Begueret H, Berger P, Vernejoux JM, Dubuisson L, Marthan R, Tunon-de-Lara JM. Inflammation of bronchial smooth muscle in allergic asthma. *Thorax* 2007;62:8-15.
- Trian T, Girodet PO, Ousova O, Marthan R, Tunon-de-Lara JM, Berger P. RNA interference decreases PAR-2 expression and function in human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 2006;34:49-55.
- Allard B, Bara I, Gilbert G, Carvalho G, Trian T, Ozier A, et al. Protease activated receptor-2 expression and function in asthmatic bronchial smooth muscle. *PLoS One* 2014;9:e86945.
- Girodet PO, Ozier A, Trian T, Begueret H, Ousova O, Vernejoux JM, et al. Mast cell adhesion to bronchial smooth muscle in asthma specifically depends on CD51 and CD44 variant 6. *Allergy* 2010;65:1004-12.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3:RESEARCH0034.
- Craig AL, Burch L, Vojtesek B, Mikutowska J, Thompson A, Hupp TR. Novel phosphorylation sites of human tumour suppressor protein p53 at Ser20 and Thr18 that disrupt the binding of mdm2 (mouse double minute 2) protein are modified in human cancers. *Biochem J* 1999;342:133-41.
- Sahin E, Colla S, Liesa M, Moslehi J, Muller FL, Guo M, et al. Telomere dysfunction induces metabolic and mitochondrial compromise. *Nature* 2011;470:359-65.
- Petersson F, Borch K, Franzen LE. Gastric epithelial proliferation and p53 and p21 expression in a general population sample: relations to age, sex, and mucosal changes associated with *H. pylori* infection. *Dig Dis Sci* 2002;47:1558-66.
- Abriss B, Hollweg G, Gressner AM, Weiskirchen R. Adenoviral-mediated transfer of p53 or retinoblastoma protein blocks cell proliferation and induces apoptosis in culture-activated hepatic stellate cells. *J Hepatol* 2003;38:169-78.
- Masuo T, Okamura S, Zhang Y, Mori M. Cyclosporine A inhibits colorectal cancer proliferation probably by regulating expression levels of c-Myc, p21(WAF1/CIP1) and proliferating cell nuclear antigen. *Cancer Lett* 2009;285:66-72.
- Rundle CH, Wang X, Sheng MH, Wergedal JE, Lau KH, Mohan S. Bax deficiency in mice increases cartilage production during fracture repair through a mechanism involving increased chondrocyte proliferation without changes in apoptosis. *Bone* 2008;43:880-8.
- Liang YY, Zheng LS, Wu YZ, Peng LX, Cao Y, Cao X, et al. RASSF6 promotes p21(Cip1/Waf1)-dependent cell cycle arrest and apoptosis through activation of the JNK/SAPK pathway in clear cell renal cell carcinoma. *Cell Cycle* 2014;13:1440-9.
- Lee CH, Wu SB, Hong CH, Liao WT, Wu CY, Chen GS, et al. Aberrant cell proliferation by enhanced mitochondrial biogenesis via mtTFA in arsenical skin cancers. *Am J Pathol* 2011;178:2066-76.

35. Rao J, Li J, Liu Y, Lu P, Sun X, Sugumaran PK, et al. The key role of PGC-1 α in mitochondrial biogenesis and the proliferation of pulmonary artery vascular smooth muscle cells at an early stage of hypoxic exposure. *Mol Cell Biochem* 2012;367:9-18.
36. Battersby BJ, Richter U. Why translation counts for mitochondria—retrograde signalling links mitochondrial protein synthesis to mitochondrial biogenesis and cell proliferation. *J Cell Sci* 2013;126:4331-8.
37. Aquilano K, Baldelli S, Pagliei B, Cannata SM, Rotilio G, Ciriolo MR. p53 orchestrates the PGC-1 α -mediated antioxidant response upon mild redox and metabolic imbalance. *Antioxid Redox Signal* 2013;18:386-99.
38. Toledo F, Wahl GM. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nat Rev Cancer* 2006;6:909-23.
39. Avery-Kiejda KA, Bowden NA, Croft AJ, Scurr LL, Kairupan CF, Ashton KA, et al. P53 in human melanoma fails to regulate target genes associated with apoptosis and the cell cycle and may contribute to proliferation. *BMC Cancer* 2011;11:203.
40. Bode AM, Dong Z. Post-translational modification of p53 in tumorigenesis. *Nat Rev Cancer* 2004;4:793-805.
41. Yazici AC, Karabulut AA, Ozen O, Eksioglu M, Ustun H. Expression of p53 in lesions and unaffected skin of patients with plaque-type and guttate psoriasis: a quantitative comparative study. *J Dermatol* 2007;34:367-74.
42. Boesten LS, Zadelaar SM, De Clercq S, Francoz S, van Nieuwkoop A, Biessen EA, et al. Mdm2, but not Mdm4, protects terminally differentiated smooth muscle cells from p53-mediated caspase-3-independent cell death. *Cell Death Differ* 2006;13:2089-98.
43. Wade M, Li YC, Wahl GM. MDM2, MDMX and p53 in oncogenesis and cancer therapy. *Nat Rev Cancer* 2013;13:83-96.

METHODS

Study populations

A total of 29 patients with asthma (14 with moderate and 15 with severe persistent asthma) and 26 nonasthmatic subjects were prospectively recruited from the "Centre Hospitalier Universitaire" of Bordeaux according to the Global Initiative for Asthma criteria.^{E1} Control subjects were enrolled by using the following inclusion criteria. Subjects aged more than 18 years must have normal FEV₁ values of greater than 80% of predicted value with an FEV₁/forced vital capacity ratio of greater than 0.7. Control subjects underwent a fiberoptic fibroscopy or a thoracotomy because of hemoptysis or an abnormal image on radiography. They had to be asymptomatic without any treatment, and only those in whom fiberoptic investigation and bronchial mucosa were normal were selected as control subjects. Exclusion criteria were any comorbidity, such as asthma or chronic obstructive pulmonary disease, and recent bacterial or viral infection (<3 months). All subjects provided written informed consent to participate in the study after the nature of the procedure had been fully explained. The study followed recommendations outlined in the Helsinki Declaration and received approval from the local ethics committee. Bronchial specimens from all subjects were obtained by means of either fiberoptic bronchoscopy or lobectomy, as previously described.^{E2}

Optic microscopy

Bronchial specimens were fixed in 10% buffered formalin and processed in standard fashion for paraffin embedding, as described previously.^{E3} Three-micrometer-thick sections were cut and stained with hematoxylin-eosin-safran or processed for immunohistochemistry. After deparaffinization and rehydration through graded alcohols, endogenous peroxidase was inhibited with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide (both from Sigma-Aldrich, Saint Quentin-Fallavier, France) for 30 minutes, followed by 2 rinses in PBS (Invitrogen, Cergy Pontoise, France). Nonspecific staining was blocked with 10% BSA (Dako, Trappes, France) for 30 minutes. Sections were incubated for 2 hours at room temperature with mouse anti-human smooth muscle actin (clone 1A4) or the unrelated mouse antibody (both from Sigma-Aldrich). After rinsing in PBS, biotinylated rabbit anti-mouse F(ab')₂ (Dako) was applied to the sections for 2 hours, followed by the streptavidin-biotin horseradish peroxidase complex (Dako) for a further 2-hour period. After rinsing in PBS, 1 mg/mL 3,3'-diaminobenzidine tetrahydrochloride (Dako) plus 0.02% hydrogen peroxide for 6 minutes was used as substrate to develop a peroxide-dependent brown color reaction at room temperature. Finally, the sections were rinsed and counterstained with Mayer hematoxylin. The total area of the BSM layer was assessed manually in a blinded fashion by using Quancoul software at a magnification of $\times 100$, as described previously.^{E3} This BSM area was normalized by the whole area of the corresponding tissues and presented as a percentage of the whole area.

Electron microscopy

Specimens were fixed in 2.5% glutaraldehyde in cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated, and embedded in EPON, as described previously.^{E4} For each specimen, semithin sections (1 μ m thick) were cut and stained with alkaline toluidine blue. We selected the first semithin section large enough to span from the epithelium to the muscular layer. Five ultrathin serial sections (60 nm thick) were then cut with diamond knives. Four of these latter sections were subsequently selected randomly and placed on grids. Staining was performed with uracile acetate and lead citrate. Grids were then scanned by using transmission electron microscopy (Tecnai 12, Philips) and examined by a pathologist from left to right and from top to bottom to locate every whole nucleated BSM. Each ultrathin section was examined in its entirety. Computerized photographs and measurements were performed in a blind fashion by using Scanview software (Soft Imaging System) at a standard magnification of $\times 4200$ to evaluate the number of mitochondria.

Cell culture

Human BSM cells were derived from bronchial specimens, as described previously.^{E3} Cells were cultured and maintained in DMEM (Invitrogen, Cergy Pontoise, France) containing 10% (vol/vol) FCS (Invitrogen) and 4.5 g/L glucose and supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin B2 (antimycotic/antibiotic solution, all from Invitrogen), 1 mmol/L sodium pyruvate, and 1% (vol/vol) nonessential amino acid mixture (both from Sigma-Aldrich). The medium was changed every 48 to 72 hours. After 6 to 8 weeks, confluent cells were rinsed twice with HBSS and passaged with trypsin-EDTA (both from Invitrogen). An immunofluorescence technique was performed by using both immunocytochemistry and flow cytometry on growth-arrested cells with serum-free DMEM for 48 hours to assess cell purity. For immunocytochemistry, cells were rinsed twice in PBS and fixed with cold methanol for 20 minutes. Nonspecific staining was blocked with PBS containing 3% BSA (Sigma-Aldrich) for 30 minutes. Monoclonal antibodies diluted in PBS with 1% BSA, including anti- α -SMA and anti-calponin (all from Sigma-Aldrich), were incubated for 1 hour. Control slides were treated similarly with an unrelated antibody (mouse IgG, Sigma-Aldrich). After rinsing with PBS containing 0.05% Tween 20 (Sigma-Aldrich), the cells were incubated for 1 hour with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Dako). Slides were mounted with a drop of fluorescent mounting medium (Dako) and observed under a fluorescence microscope (Nikon, Paris, France).

For flow cytometry, cells were harvested, fixed, and permeabilized in 4% paraformaldehyde and 0.1% saponin (Sigma-Aldrich) for 15 minutes on ice. Cells were then labeled with either mouse α -SMA mAb indirectly labeled with a FITC-conjugated secondary antibody or a rabbit monoclonal anti-calponin antibody indirectly labeled with phycoerythrin-conjugated secondary antibody. Fluorescence intensity was compared with appropriate isotype control antibody. Analysis was performed with double-color flow cytometry on a FACSCanto (BD Biosciences, Pont de Claix, France). Depending on the experiments, cells with a confirmed BSM phenotype were seeded on various culture supports (96-, 48-, 24-, and 12-well plates or culture flasks). Early-passage BSM cells were also frozen in liquid nitrogen and used later after thawing. Only cells at passages 2 to 5 with a confirmed BSM phenotype (ie, >90% of cultured cells were positive for both anti- α -SMA and anti-calponin antibody) were used for this study.

Cell proliferation

Cell proliferation was evaluated by using both BrdU incorporation and cell counting, as described previously.^{E3} Regarding BrdU, BSM cells were growth arrested as described above in 96-well plates for 24 hours and stimulated with 10% FCS or human bronchial epithelial cell supernatants obtained after 24 hours of HBE160-culture in DMEM 10% FBS. Control BSM cells remained in serum-deprived DMEM. DNA synthesis was measured with the BrdU kit, according to the manufacturer's instructions (Roche Diagnostics, Meylan, France). Briefly, BSM cells were incubated with 10 μ mol/L BrdU for 24 hours at 37°C. Cells were fixed and denaturated for 30 minutes at room temperature and incubated with the anti-BrdU antibody for 2 hours at room temperature. Absorbance was measured at 370 nm in a microplate reader in triplicate. Results were expressed as a normalized ratio of BrdU incorporation (ie, absorbance for test condition divided by absorbance for control serum-deprived condition).

Regarding cell counting, 1×10^4 BSM cells were seeded in 6-well plates. After 24 hours, growth was arrested for 24 hours, as described above. BSM cells were then challenged with 10% FBS for 10 days, with medium changed every 48 hours. BSM cells were then harvested and counted. Results were expressed as absolute cell numbers.

Lentiviral shRNA against p53 and mitochondrial transcription factor A

Lentivirus production. We used a commercial plasmid expressing p53-targeted shRNA to knockdown p53 gene expression. A proviral plasmid (pLVTH-sip53) containing the p53 shRNA sequence

(ie, 5'-CCGACTCCAGTGGTAATCTACTTCAAGAGAGTAGATTACCAC TGGAGTCTTTTT-3') was purchased from Addgene (Cambridge, Mass). For TFAM shRNA lentivirus, we designed a specific shRNA sequence (ie, 5'-CGCGTCGTTATGATAACAGGTTTCGTCCTTGATATCCGGGACGAAAC CTGTTATCATAATTTTTTCCAAAT-3') that we cloned into the transfer lentiviral plasmid pLVTH containing a GFP reporter gene (Plateforme de vectorologie SFR TransBioMed, Université Bordeaux, France) to obtain the pLVTH-mitochondrial transcription factor A vector. Stbl2 bacteria were transformed with both lentiviral plasmids and grown overnight. DNA was isolated from bacteria by using a Maxi Plasmid DNA Purification Kit (Qiagen, Courtaboeuf, France). Replication-deficient lentiviruses were then produced by transfecting human embryonic kidney cells (293T) with either the pLVTH-sip53 vector containing the p53 shRNA sequence or pLVTH-mitochondrial transcription factor A, the packaging plasmid pVSV-G (Trono, Geneva, Switzerland), and the HIV structure plasmid pCMV (Trono). Three days later, the viral supernatant was collected and filtered to remove cellular debris. Viral titers were estimated by measuring GFP protein with flow cytometry.

Transduction and measure of efficacy. BSM cells (400,000 cells/mL) were transduced with lentivirus in 12-well plates at various viral concentrations, as described previously.^{E5} The efficacy of transduction was assessed by using both flow cytometry and real-time quantitative RT-PCR 7 days later. For flow cytometry, BSM cells were harvested and fixed in 4% paraformaldehyde for 15 minutes on ice. BSM cells were washed with PBS, and GFP fluorescence was evaluated by using the BD FACSCanto (BD Biosciences) flow cytometer. For quantitative RT-PCR, BSM cell total RNA was extracted and reverse transcribed into cDNA by using AMV reverse transcriptase (Sigma-Aldrich), as previously described.^{E6} Briefly, real-time PCR was performed on a Rotor-Gene 2000 (Corbett Research) with specific primers against p53 (NM_000546; sense 5'-AATCTACTGGGACGGAA CAGC-3', antisense 5'-GTTGGGCGAGTCTCGCTTAGT-3'), TFAM (NM_003201; sense 5'-GAAGTCGACTGCGCTCCC-3', antisense 5'-ACTC CGCCCTATAAGCATC-3'), PGC-1 α (NM_013261; sense 5'-GTC ACCACCCAAATCCTTATT-3', antisense 5'-GGCGATCTTGAACGTGAT-3'), p21 (NM_000389; sense 5'-TGATTAGCAGCGGAACAAGG-3', antisense 5'-GAGCCGAGAGAAAACAGTCC-3'), Bax (NM_001291428; sense 5'-AGTGGCAGCTGACATGTTTT-3', antisense 5'-GAGGAAGTCCAATG TCCAGC-3'), and Mdm2 (NM_002392; sense 5'-AGGAACTGGG GAGTCTTGA-3', antisense 5'-GCTTTGGTCTAACCAGGGTC-3'). The efficiency of the PCR reactions was always greater than 90%. The specificity of the amplified PCR products was examined with melting curve analysis and also in 2% agarose gel containing SYBR Green (Invitrogen). The RT-PCR expression of these genes was then presented as an arbitrary unit and normalized to endogenous references (geometric averaging of three internal control genes; ie, YWHAZ, HPRT-1, and PO), as described previously^{E7} and according to geNorm software.^{E6}

Western blotting

Various protein expressions were analyzed by using Western blotting. Immunoblotting was performed on cell protein extracts, as described previously.^{E5} Briefly, cells were lysed with 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% IGEPAL, and 2 mmol/L EDTA supplemented with complete protease inhibitors (Roche, Meylan, France). Lysates were cleared by means of centrifugation at 12,000g at 4°C, and protein concentrations were determined by using the Lowry method (Bio-Rad Laboratories, Marnes-la-Coquette, France). Samples were resolved on 5-20% SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham Biosciences, Fontenay-sous-Bois, France). Immunoblots were then developed by using specific antibody against p53 (BD Biosciences, Pont de Claix, France), specific antibody against porin (Abcam, Paris, France), or specific antibody against Mdm2 (Abcam) after stripping anti-human β -actin (Sigma-Aldrich) for 14 hours at 4°C. For amplification, biotinylated goat secondary anti-mouse antibody (Bio-Rad Laboratories) for 1 hour at room temperature and a streptavidin-biotinylated horseradish peroxidase complex (Dako) were used. Immunoblots were revealed by using Immobilon

enhanced chemiluminescence (Millipore, Molsheim, France). Blot images were acquired with BioCaptMW (Thermo Fisher Scientific, Illkirch, France), and band densities were quantified with ImageJ software.

Flow cytometry

For flow cytometry, cells were harvested, fixed, and permeabilized in 4% paraformaldehyde and 0.1% saponin (Sigma-Aldrich) for 15 minutes on ice. Cells were then labeled with p53 antibody or specific antibody against TOM20 (Santa Cruz, Dallas, Tex, and BD Biosciences) indirectly labeled with an FITC-conjugated secondary antibody. Fluorescence intensity was compared with an appropriate isotype control antibody. Analysis was performed by using double-color flow cytometry on a FACSCanto (BD Biosciences).

Laser microdissection

Bronchial biopsy specimens were immediately fixed for 2 hours in 4% paraformaldehyde and stored at -80°C before laser microdissection, as previously described.^{E7} Twenty-micrometer-thick bronchial sections were carried out on frozen tissue at -20°C to prevent mRNA degradation with a CM3050 S microtome (Leica, Wetzlar, Germany). Tissue sections were mounted on PEN-membrane 1-mm glass slides (P.A.L.M. Microlaser Technologies AG Zeiss). Frozen sections were passed through an ethanol bath (100% to 50%) and then stained with 1% cresyl violet (Sigma-Aldrich) and dehydrated in an ethanol bath (50% to 100%). Immediately after dehydration, laser-assisted microdissection of BSM area was performed by using the P.A.L.M. MicroBeam microdissection system, version 4.0-1206, equipped with PALM RoboSoftware (P.A.L.M. Microlaser Technologies AG Zeiss).^{E8} We used a frequency tripled solid-state laser with a wavelength of 355 nm (UV) and a pulse of energy at 100 μJ . After laser microdissection along the BSM circumscribed area, a defined laser pulse catapults the selected specimen out of the object plane into the cap of a collection device located above the tissue section. The presence of captured tissue samples in the cap was checked under low magnification, and then the samples were immediately stored at -80°C until use. Microdissection was performed at $\times 20$ magnification. Laser microdissection and pressure catapulting was performed for no longer than 30 minutes per slide. RNA extraction was performed, as previously described, with both oligo dT and random primers.^{E9} The p53 PCR primers have been adapted to microdissected tissues (sense 5'-CCAGCCAACCTTTTGCATGT-3', antisense CTGAGACAGGTGGATCGC). mRNA expression of p53 was normalized to endogenous references by using a geometric averaging of 3 internal control genes (ie, YWHAZ, HPRT-1, and PO), as described previously^{E7} and according to geNorm software.^{E6}

p53 gene sequencing

DNA was extracted from BSM cells by using a DNA extraction kit (Qiagen), according to the manufacturer's protocol. The sequencing reaction was carried out with the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Courtaboeuf, France), according to the Sanger method. The conditions of this polymerization reaction were as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles consisting of denaturation for 30 seconds at 95°C , hybridization of 10 seconds at 50°C , and finally elongation at 60°C for 4 minutes. The DNA was then precipitated by addition of 125 mmol/L EDTA, 3 mol/L sodium acetate, and 100% ethanol 100% for 15 minutes at room temperature. Centrifugation for 30 minutes at 11,000 rpm was then performed, and the supernatant was completely removed and washed with 70% ethanol. Another centrifugation at 11,000 rpm for 15 minutes was performed. The supernatant was removed, and the pellet was allowed to dry in the dark and then resuspended in formamide (Sigma-Aldrich, Lyon, France). The sequencing reaction products were analyzed with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Hitachi, Carlsbad, Calif). The primer details used for p53 gene sequencing are presented in Table E1.

p53 transcription factor binding assay

p53 binding to double-stranded DNA containing the p53 response element was assessed on nuclear protein extracted from BSM cells by using a p53 transcription factor binding assay (Cayman). Briefly, BSM cells were harvested and centrifuged twice at 300g. Cells were then lysed with hypotonic buffer containing phosphatases inhibitors (Abcam). Nuclear proteins were extracted by using dedicated buffer containing protease and phosphatase inhibitors (Abcam) at 14,000g centrifugation. The total amount of nuclear proteins was assessed by using the Lowry method with the DC Protein Assay Kit (Bio-Rad Laboratories). Then nuclear protein extracts were put in 96-well plates containing the p53 response element. ELISA was performed in duplicate by using specific anti-p53 antibody and horseradish peroxidase-conjugated secondary antibody (Cayman). Results were expressed in OD assessed at 450 nm normalized by the amount of nuclear protein in each well.

REFERENCES

- E1. Crapo RO, Casaburi R, Coates AL, Enright PL, Hankinson JL, Irvin CG, et al. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am J Respir Crit Care Med* 2000;161:309-29.
- E2. Bara I, Ozier A, Girodet PO, Carvalho G, Cattiaux J, Begueret H, et al. Role of YKL-40 in bronchial smooth muscle remodeling in asthma. *Am J Respir Crit Care Med* 2012;185:715-22.
- E3. Trian T, Benard G, Begueret H, Rossignol R, Girodet PO, Ghosh D, et al. Bronchial smooth muscle remodeling involves calcium-dependent enhanced mitochondrial biogenesis in asthma. *J Exp Med* 2007;204:3173-81.
- E4. Begueret H, Berger P, Vernejoux JM, Dubuisson L, Marthan R, Tunon-de-Lara JM. Inflammation of bronchial smooth muscle in allergic asthma. *Thorax* 2007;62:8-15.
- E5. Allard B, Bara I, Gilbert G, Carvalho G, Trian T, Ozier A, et al. Protease activated receptor-2 expression and function in asthmatic bronchial smooth muscle. *PLoS One* 2014;9:e86945.
- E6. Girodet PO, Ozier A, Trian T, Begueret H, Ousova O, Vernejoux JM, et al. Mast cell adhesion to bronchial smooth muscle in asthma specifically depends on CD51 and CD44 variant 6. *Allergy* 2010;65:1004-12.
- E7. Trian T, Allard B, Dupin I, Carvalho G, Ousova O, Maurat E, et al. House dust mites induce proliferation of severe asthmatic smooth muscle cells via an epithelium-dependent pathway. *Am J Respir Crit Care Med* 2015;191:538-46.
- E8. Maitre M, Roullot-Lacarrière V, Piazza PV, Revest JM. Western blot detection of brain phosphoproteins after performing laser microdissection and pressure catapulting (LMPC). *J Neurosci Methods* 2011;198:204-12.
- E9. Trian T, Girodet PO, Ousova O, Marthan R, Tunon-de-Lara JM, Berger P. RNA interference decreases PAR-2 expression and function in human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 2006;34:49-55.

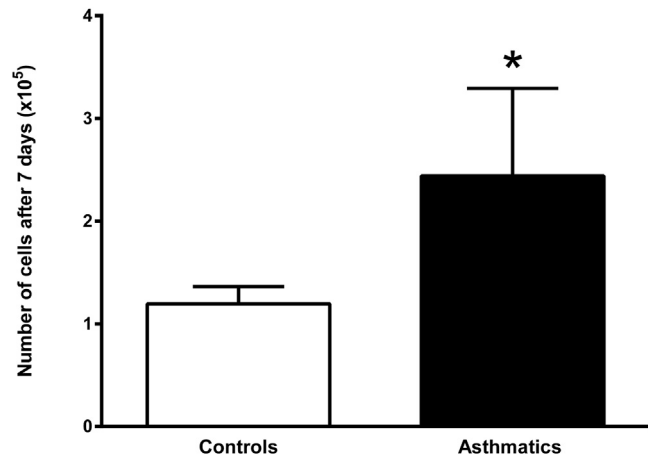


FIG E1. BSM cells were obtained from control subjects (*white bars*, $n = 5$) and asthmatic patients (*black bars*, $n = 6$). BSM cell proliferation was assessed by using cell counting in DMEM supplemented with 10% FBS. Data are means $\pm$ SEMs. $*P < .05$, ANOVA with Turkey tests. NS, Nonsignificant.

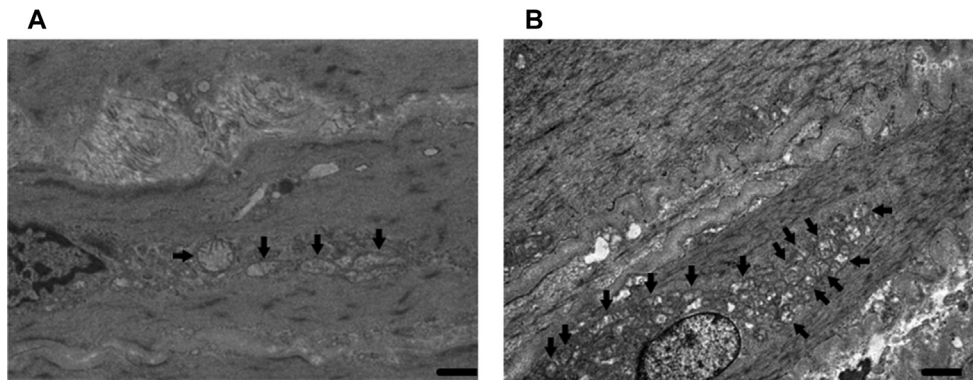


FIG E2. Representative electronic microscopic images from bronchial sections were obtained from a control subject (**A**) and an asthmatic patient (**B**) and observed at $\times 6000$ magnification. Some smooth muscle mitochondria were visualized (*arrows*). Bars = 1 μm .

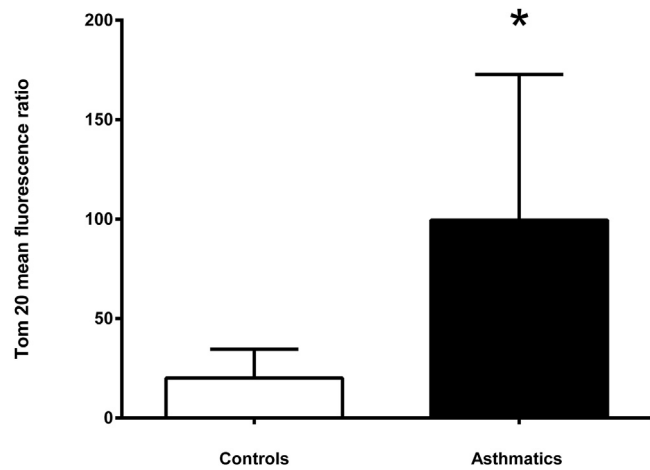


FIG E3. BSM cells were obtained from control subjects (*white bars*, $n = 5$) and asthmatic patients (*black bars*, $n = 10$). Mean fluorescence intensity of TOM20 antibody was assessed by using flow cytometry and normalized by that of irrelevant antibody. Data are means $\pm$ SEMs. $*P < .05$, Student t tests.

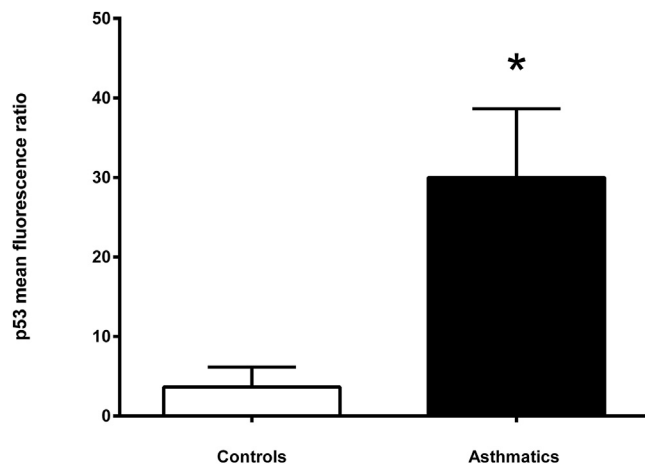


FIG E4. BSM cells were obtained from control subjects (*white bars*, $n = 4$) and asthmatic patients (*black bars*, $n = 4$). Mean fluorescence intensity of p53 antibody was assessed by using flow cytometry and normalized by that of irrelevant antibody. Data are means $\pm$ SEMs. * $P < .05$, Student t tests.

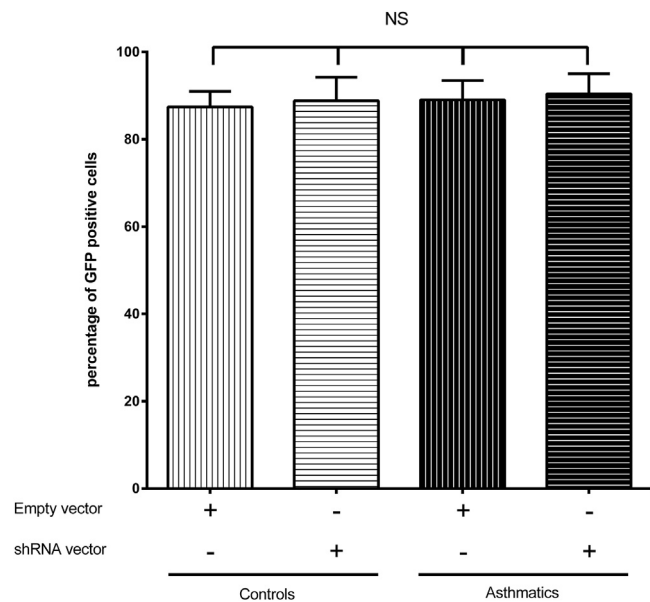


FIG E5. Lentivirus transduction was assessed by means of flow cytometry with lentiviral production of GFP. BSM cells were obtained from control subjects (*white bars*, $n = 7$) and asthmatic patients (*black bars*, $n = 10$). BSM cells were either transduced with an empty lentivirus (*vertical hatched bars*) or transduced with a lentivirus producing p53 shRNA (*horizontal hatched bars*). Data are means $\pm$ SEMs. NS, Nonsignificant. ANOVA with Turkey tests.

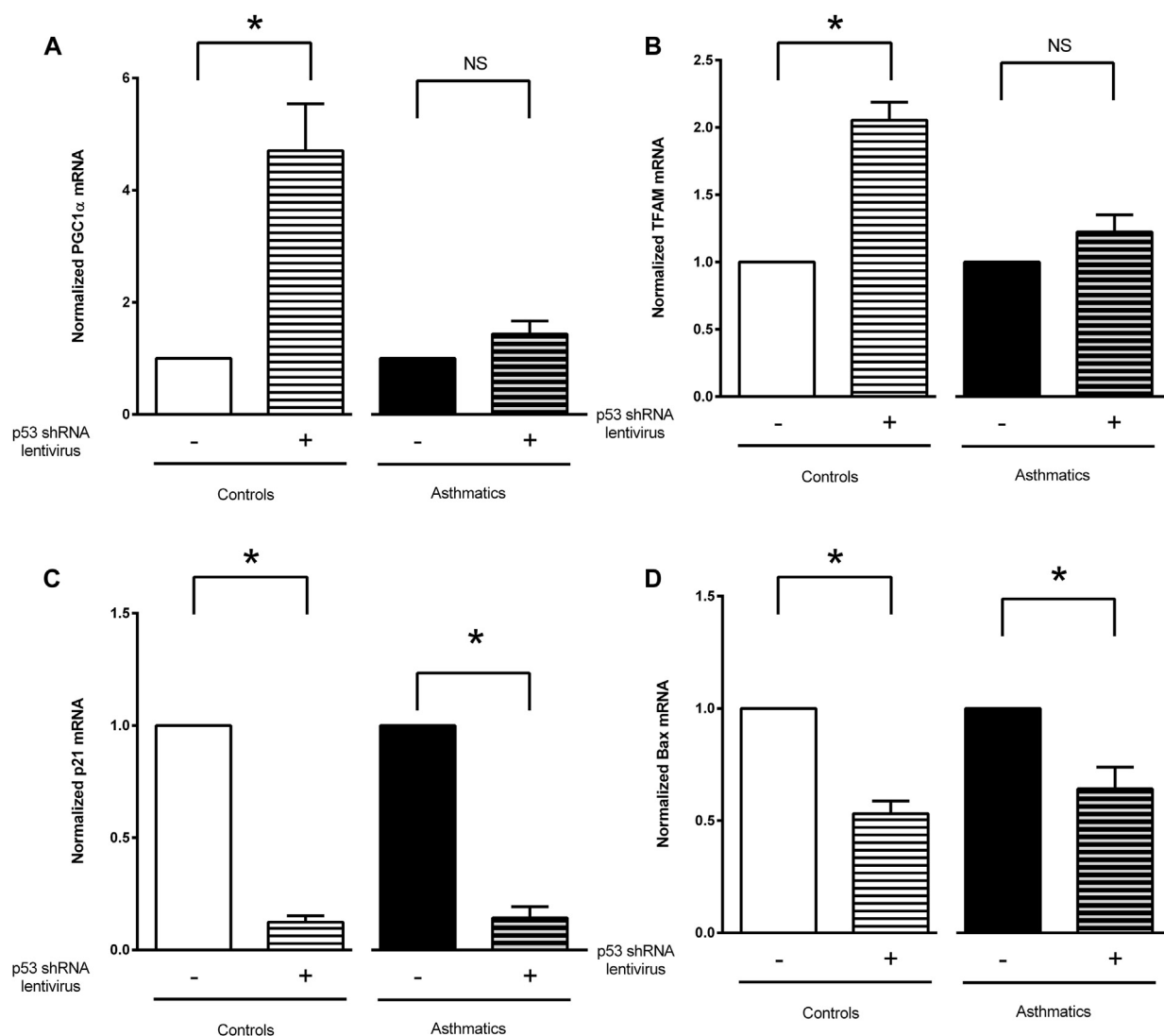


FIG E6. Expression of PGC-1α (A), TFAM (B), p21 (C), and Bax (D) was assessed by using quantitative RT-PCR. BSM cells were obtained from control subjects (white bars, $n = 7$) and asthmatic patients (black bars, $n = 10$). BSM cells were either not transduced (empty bars) or transduced with a lentivirus producing p53 shRNA (horizontal hatched bars). Data are expressed as fold changes compared with control values and presented as means $\pm$ SEMs. * $P < .05$, Student paired t tests. NS, Nonsignificant.

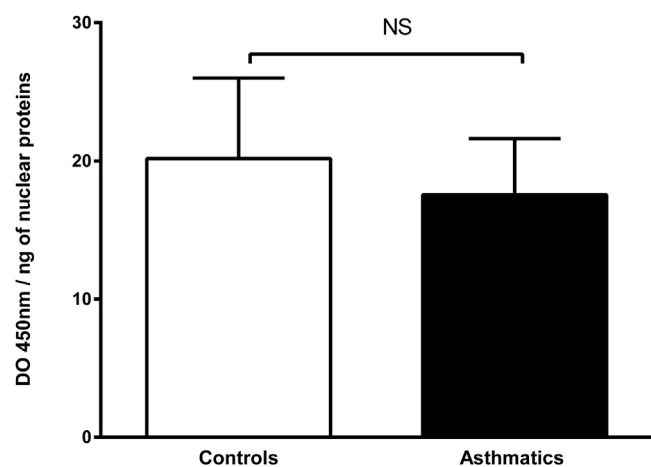


FIG E7. The p53 binding assay was performed on nuclear protein extracted from BSM cells by using the p53 transcription factor binding assay. BSM cells were obtained from control subjects (*white bars*, $n = 5$) and asthmatic patients (*black bars*, $n = 10$). Data are expressed as a ratio between the OD value measured at 450 nm and the quantity of nuclear protein assessed by using the Lowry method in nanograms. Data are presented as means $\pm$ SEMs. *NS*, Nonsignificant by using Student *t* tests.

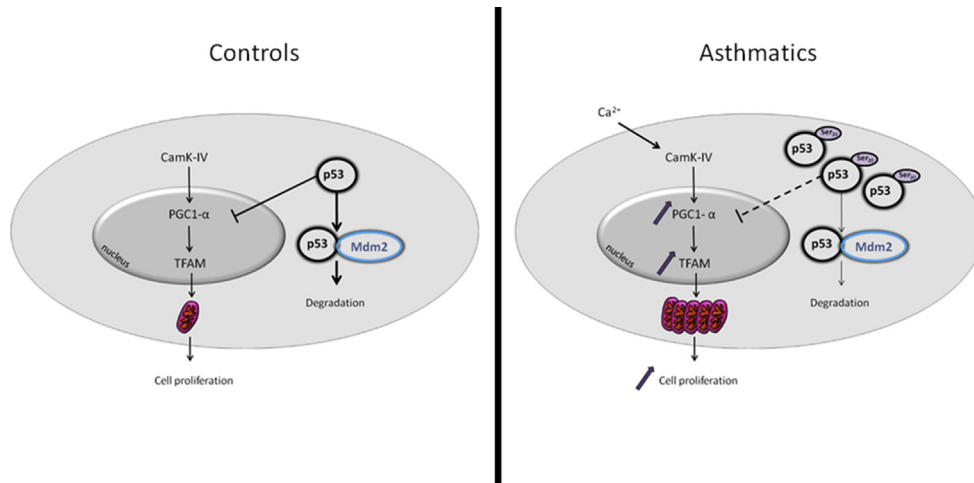


FIG E8. Proposed schematic diagram of the cellular pathway involved in BSM proliferation in asthmatic patients compared with that in control subjects.

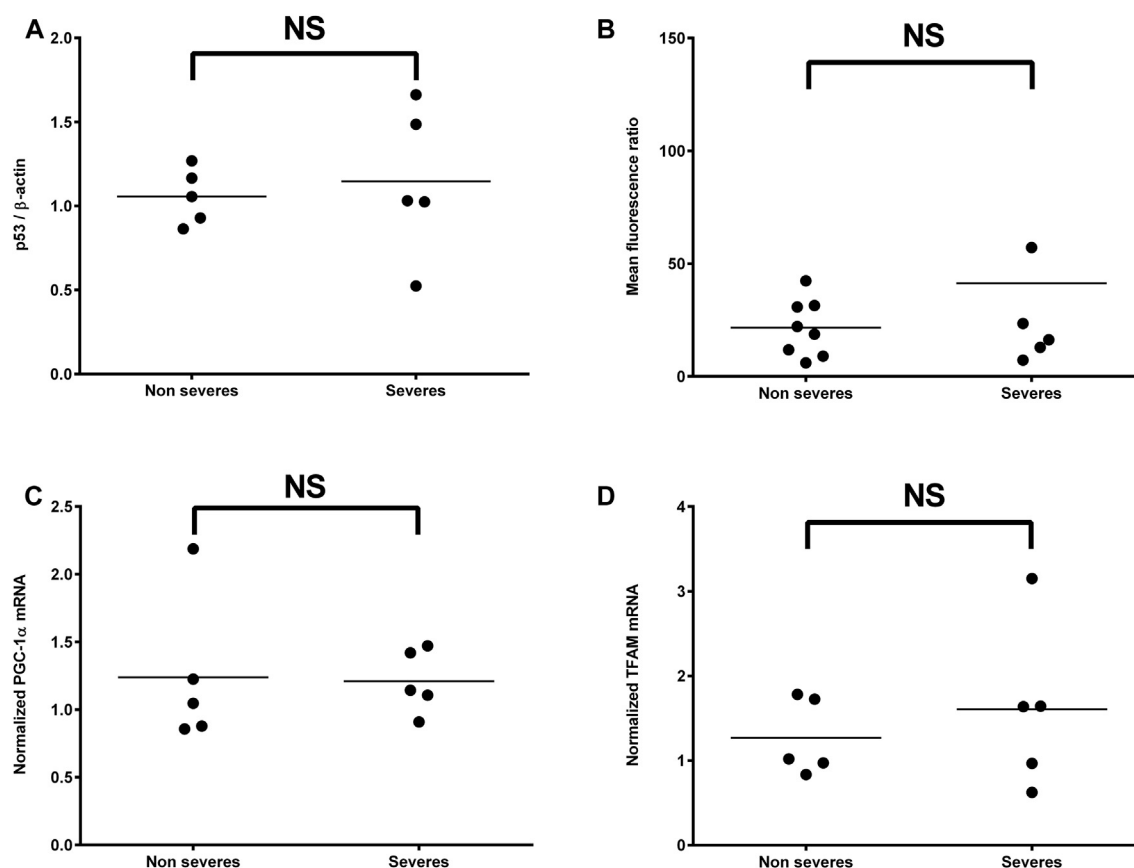


FIG E9. **A** and **B**, Expression of p53 in BSM was assessed *in vitro* by means of Western blotting (patients with moderate asthma, $n = 5$; patients with severe asthma, $n = 5$; Fig E9, **A**) and flow cytometry (patients with moderate asthma, $n = 8$; patients with severe asthma, $n = 6$; Fig E9, **B**). **C** and **D**, Expression of PGC-1 α (Fig E9, **C**) and TFAM (Fig E9, **D**) was assessed by using quantitative RT-PCR. BSM cells were transduced with a lentivirus producing p53 shRNA and obtained from patients with moderate asthma ($n = 5$) and those with severe asthma ($n = 5$). Individual data are shown, and *lines* represent means. *NS*, Nonsignificant by using Student *t* tests.

TABLE E1. *p53* gene sequencing primers

Exon (s)	Primer sense	Primer antisense	Amplicon size (pb)	Annealing temperature (°C)
2-3	TCTCAGACACTGGCATGGTG	GGGGACAGCATCAAATCATC	500	60
4	CGTTCTGGTAAGGACAAGGG	GGAATCCCAAAGTTCCAAAC	484	60
5-6	GTTTGTTTCTTTGCTGCCG	TCATGGGGTTATAGGGAGGTC	585	60
7	CCTCCCCTGCTTGCCAC	GAGGTGGATGGGTAGTAGTATGG	293	60
8-9	TTGGGAGTAGATGGAGCCTG	AAACAGTCAAGAAGAAAACGGC	473	60
10	TCAAACAATTGTAAC TTGAACCATC	GGCAGGATGAGAATGGAATC	269	60
11	GGGAAAAGGGGCACAGAC	GCAAGCAAGGGTTCAAAGAC	244	60