Regulation of A Disintegrin And Metalloprotease-33 Expression by Transforming Growth Factor- β

Youwen Yang^{1*}, James Wicks^{1*}, Hans Michael Haitchi¹, Robert M. Powell¹, Wiparat Manuyakorn¹, Peter H. Howarth¹, Stephen T. Holgate¹, and Donna E. Davies¹

¹Clinical and Experimental Sciences and the Southampton National Institute for Health Research Respiratory Biomedical Research Unit, University of Southampton Faculty of Medicine, Sir Henry Wellcome Laboratories, Southampton General Hospital, Southampton, United Kingdom

The asthma susceptibility gene, *a disintegrin and metalloprotease-33* (*ADAM33*), is selectively expressed in mesenchymal cells, and the activity of soluble *ADAM33* has been linked to angiogenesis and airway remodeling. Transforming growth factor (TGF)- β is a profibrogenic growth factor, the expression of which is increased in asthma, and recent studies show that it enhances shedding of soluble *ADAM33*. In this study, we hypothesized that TGF- β also affects *ADAM33* expression in bronchial fibroblasts in asthma. Primary fibroblasts were grown from bronchial biopsies from donors with and those without asthma, and treated with TGF- β_2 to induce myofibroblast differentiation. *ADAM33* expression was assessed using quantitative RT-PCR and Western blotting. To examine the mechanisms whereby TGF- β_2 affected *ADAM33* expression, quantitative methylation-sensitive PCR, chromatin immunoprecipitation, and nuclear accessibility assays were conducted on the *ADAM33* promoter. We found that TGF- β_2 caused a time- and concentration-dependent reduction in *ADAM33* mRNA expression in normal and asthmatic fibroblasts, affecting levels of splice variants similarly. TGF- β_2 also induced *ADAM33* protein turnover and appearance of a cell-associated C-terminal fragment. TGF- β_2 down-regulated *ADAM33* mRNA expression by causing chromatin condensation around the *ADAM33* promoter with deacetylation of histone H3, demethylation of H3 on lysine-4, and hypermethylation of H3 on lysine-9. However, the methylation status of the *ADAM33* promoter did not change. Together, these data suggest that TGF- β_2 suppresses expression of *ADAM33* mRNA in normal or asthmatic fibroblasts. This occurs by altering chromatin structure, rather than by gene silencing through DNA methylation as in epithelial cells. This may provide a mechanism for fine regulation of levels of *ADAM33* expression in fibroblasts, and may self-limit TGF- β_2 -induced ectodomain shedding of *ADAM33*.

Keywords: *a disintegrin and metalloprotease-33*; myofibroblast; transforming growth factor- β ; histone modification

Asthma is a disease caused by interactions between genetic and environmental factors. It is characterized by variable airflow obstruction and bronchial hyperresponsiveness (BHR) due to airway inflammation and remodeling. In chronic severe asthma,

CLINICAL RELEVANCE

The asthma susceptibility gene, *a disintegrin and metalloprotease-33* (*ADAM33*), has been implicated in airway remodeling as a soluble form of *ADAM33* (s*ADAM33*) which is produced as a consequence of transforming growth factor (TGF)- β -induced ectodomain shedding, is proangiogenic. We now demonstrate that the activity of TGF- β towards *ADAM33* is self-limited, as it also down-regulates *ADAM33* mRNA expression via chromatin modification. Although this may enable fine-tuning of *ADAM33* levels in fibroblasts, the high levels of s*ADAM33* found in bronchoalveolar lavage fluid of subjects with asthma suggest that other factors may override this normal feedback mechanism in asthma.

airway inflammation and structural changes both become more intense and are paralleled by an increase in BHR that is only partially or nonresponsive to treatment with corticosteroids (1).

A disintegrin and metalloprotease-33 (*ADAM33*) is a susceptibility gene that has been strongly linked to asthma and BHR (2). There have been a number of case-control and family-based association studies focused on *ADAM33*, with the majority, including two meta-analyses, confirming the original finding across a wide range of populations (3–5). Furthermore, by applying a candidate gene approach to a genome-wide association study data set, the strongest evidence for true association between a previous candidate single-nucleotide polymorphism and asthma was found for rs528557 in *ADAM33* (6). Polymorphic variation in *ADAM33* also predicts impaired lung function in young children, suggesting that it may contribute to the early-life origins of asthma (7). A similar association of *ADAM33* with impaired lung function and accelerated decline over time has also been reported in asthma (8), the general population (9), and also in chronic obstructive pulmonary disease (10, 11).

ADAM33 belongs to a family of 40 *ADAM* proteins expressed in many cell types, and plays diverse roles in cell surface remodeling, ectodomain shedding of growth factors and receptors, and mediating cell-cell and cell-matrix interaction (12). *ADAM33* mRNA is selectively expressed in mesenchymal cells, including bronchial fibroblasts, myofibroblasts, and smooth muscle (2), where its transcripts undergo alternative splicing to generate several protein isoforms (13). Alternatively spliced mRNA variants of *ADAM33* have been identified in adult bronchial biopsies and human embryonic lungs (13), although no disease-related differences in splice variant expression have yet been detected (13, 14). Alternatively spliced variants have also been detected in bronchial fibroblasts (15) and in smooth muscle cells, where IFN- γ was found to down-regulate expression of both the α and β isoforms (16). We have found that *ADAM33* is not expressed in epithelial cells, due to methylation

(Received in original form January 27, 2011 and in final form December 21, 2011)

*These authors contributed equally to this work.

This work was supported by the Medical Research Council (MRC) UK, the Wellcome Trust, the Rayne Foundation, the Asthma Allergy and Inflammation Research Charity, and the Roger Brooke Charitable Trust. S.T.H. is supported by a MRC Clinical Professorship and W.M. was funded by a studentship from the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. H.M.H. is supported by an MRC Clinician Scientist Fellowship.

Correspondence and requests for reprints should be addressed to Donna Davies, Ph.D., Mailpoint 810, Southampton General Hospital, Southampton, SO16 6YD, UK. E-mail: donnad@soton.ac.uk

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 46, Iss. 5, pp 633–640, May 2012

Published 2012 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2011-0030OC on January 6, 2012

Internet address: www.atsjournals.org

and silencing of the *ADAM33* promoter (14); however, ADAM33 protein has been localized to epithelial cells by immunohistochemistry (17–19). This might be because a soluble 55-kD protein (sADAM33) is present in the airways, and may bind to epithelial cells; high levels of sADAM33 have been reported in bronchoalveolar lavage fluid of subjects with asthma, and correlated both with disease severity and reduced lung function (20).

The selective expression of *ADAM33* in mesenchymal cells and its genetic association with BHR and reduced lung function have led to the proposal that it is involved in airway remodeling (3). In support of this, we have recently reported that a secreted form of ADAM33 that includes the metalloprotease domain promotes angiogenesis (21). In the same study, we also reported that sADAM33 can be released from the cell surface by ectodomain shedding in response to transforming growth factor (TGF)- β (21). This led us to postulate that shedding of ADAM33 liberated it from its regulatory transmembrane and cytoplasmic domains, allowing uncontrolled access to new substrates, resulting in a TGF- β -regulated, disease-related gain of function.

TGF- β is a key profibrogenic cytokine, the expression of which is increased in asthma (22). Its activity has been linked to airway remodeling via increased numbers of myofibroblasts and structural changes, including increased deposition of extracellular matrix proteins and proteoglycans. It is a potent inducer of myofibroblast differentiation, and also promotes the expression of smooth muscle-related mRNA transcripts, such as α -smooth muscle actin (α -SMA), heavy chain myosin, calponin 1, γ -actin, and desmin in bronchial fibroblasts, especially those derived from asthmatic airways (23). Because TGF- β can promote ADAM33 ectodomain shedding (21), we postulated that TGF- β also affects *ADAM33* mRNA expression in asthma. Thus, in the present study, we investigated the influence of TGF- β on *ADAM33* expression during differentiation of normal and asthmatic myofibroblasts, and then identified the mechanisms employed in its regulation.

MATERIALS AND METHODS

Bronchoscopy and Cell Culture

Bronchial biopsies were obtained by fiberoptic bronchoscopy in accordance with standard guidelines (24) after ethical approval and informed consent. The normal subjects (4:2 [male:female]; mean age, 21 [range, 20–21] yr) had an FEV₁ of 100.7 ($\pm$ 9.3)% predicted, whereas the subjects with asthma (4:3 [male:female]; mean age, 26 [range, 20–36] yr) had a prebronchodilator FEV₁ of 76.4 ($\pm$ 7.8)% predicted. All subjects with asthma were using inhaled β_2 -adrenoceptor agonists as required, and four were also using inhaled corticosteroids. Detailed descriptions of the growth of primary bronchial fibroblast (PBF) cultures and treatments of cell lines are described in the online supplement.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Paisley, UK). Quantitative RT-PCR (RT-qPCR) assays and sequence verification were undertaken as described in the online supplement.

Western Blotting

Western blot analysis was performed using an affinity-purified rabbit anti-ADAM33 antibody (RP3; TriplePoint Biological, Forest Grove, OR) raised against the C-terminal residues of the ADAM33 cytoplasmic domain. Control blots were performed using antibody that had been preadsorbed with the immunizing peptide (2 μ g/ml; TriplePoint Biological) and/or an isotype-matched control antibody.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed by following the Upstate Biotechnology CHIP assay kit protocol (no. 17–

295; Upstate Biotech, Milton Keynes, UK). Chromatin was immunoprecipitated using antibodies against acetyl-histone H3 (H3Ac), histone H3 dimethyl lysine 4 (H3K4 diMe), and histone H3 dimethyl lysine 9 (H3K9 diMe), as recommended by the manufacturer, and using rabbit IgG as a negative control. DNA purified from nonimmunoprecipitated (input, for normalization) and immunoprecipitated samples was subjected to qPCR for the *ADAM33* promoter. Data were normalized by PCR for the glyceraldehyde 3-phosphate dehydrogenase promoter and a region on chromosome 16 centromere (CEN16).

Methylation-Sensitive PCR

Genomic DNA was isolated from untreated or TGF- β_2 -treated PBFs and cell lines (Wizard Genomic Purification Kit; Promega, Southampton, UK) and digested overnight at 37°C with *Hpa*II and *Pst*I (NEB, Herts, UK). The samples were amplified by real-time PCR using the primer pairs as in the ChIP assay. These primers amplified a sequence in the *ADAM33* promoter containing an *Hpa*II recognition site; the other primer pair located on CEN16 amplified a sequence that does not contain an *Hpa*II site, and served as an internal control to normalize the assays.

Nuclear Accessibility

The chromatin accessibility assay was a modification of a previous method (14), as described in the online supplement. qPCRs were performed on the purified DNA in a triplex format. The primers and probe for detecting the *ADAM33* promoter were the same as in the ChIP assay. As *Msp*I and *Hpa*II are isoschizomers, the internal control assay used to normalize the data employed the same primer and probe sets used for analysis of methylation.

Statistical Analysis

Normally distributed data were analyzed using ANOVA or Student's *t* test; non-normally distributed data were analyzed using Kruskal Wallis nonparametric ANOVA, followed by Wilcoxon rank sum test for within-group comparisons or Mann-Whitney U test for between-group comparisons. Where appropriate, corrections were made for multiple testing. A *P* value less than 0.05 was considered significant.

RESULTS

Expression of *ADAM33* Splice Variant mRNA in Asthmatic and Nonasthmatic Fibroblasts

Using primers in the 3' untranslated region that is common to all *ADAM33* transcripts, there was no significant difference between fibroblasts derived from nonasthmatic or asthmatic bronchial biopsies (Figure 1A, inset). We also measured levels of three splice variants of *ADAM33*: the β isoform, the soluble form, and all forms containing the metalloprotease domain. These assays were validated and shown to be of equal efficiency (15). The expression of each variant was compared with the total expression levels of *ADAM33* mRNA, determined using the assay targeting the 3' untranslated region of *ADAM33*. The β form was detected at around 20% of total *ADAM33* transcripts. The putative soluble form was detected at less than 1%, whereas the metalloprotease-containing transcripts were detected at less than 5% of total *ADAM33* mRNA transcript levels. No differences in expression levels of splice variants were detected between healthy control and asthmatic fibroblasts (Figure 1A).

ADAM33 Splice Variant mRNA Expression Is Decreased during Myofibroblast Differentiation

In fibroblasts cultured in serum-free medium for 48 hours, there was an approximately twofold increase in *ADAM33* mRNA

Figure 1. A disintegrin and metalloprotease-33 (ADAM33) mRNA splice variant expression relative to the geometric mean of *ubiquitin C* and *phospholipase A2* in primary bronchial fibroblast (PBFs) from normal subjects ($n = 6$, open boxes) or subjects with asthma ($n = 7$, shaded boxes) (A). Inset shows expression of ADAM33 transcripts measured using primers and probe in the 3' untranslated region (UTR). Time course (B) and concentration response (C) showing suppression of ADAM33 3' UTR mRNA transcripts by transforming growth factor (TGF)- β_2 . Data are displayed as box and whisker plots showing the median and interquartile range with 95% confidence intervals. In (B), the data for normal and asthmatic PBFs are combined ($n = 13$) for ease of viewing, as there were no differences in the responses between the groups. ** $P < 0.01$ versus untreated controls ($n = 13$); * $P < 0.05$ versus untreated controls ($n = 6$ or 7). MP, metalloprotease.

expression relative to time zero (i.e., in cells that had previously been exposed to serum-containing medium) (Figure 1B). To mimic the effect of epithelial-mesenchymal signaling in the epithelial-mesenchymal trophic unit (25), cells were treated with TGF- β_2 , which is the major isoform of TGF- β expressed by airway epithelial cells (26). This caused a significant time- and concentration-dependent down-regulation of ADAM33 mRNA expression in both normal and asthmatic fibroblasts when compared with untreated (serum-starved) conditions (Figures 1B and 1C; *see also* Figures E1 and E2 in the online supplement). Maximal inhibition of mRNA expression was observed after treatment with 10 ng/ml TGF- β_2 for 24 hours or longer. Under the same conditions, α -SMA mRNA expression increased 20- to 100-fold in response to TGF- β_2 (data not shown), consistent with myofibroblast differentiation. The regulation of alternatively spliced variants encoding the β isoform, the metalloprotease domain, or the putative secreted isoform of ADAM33 were also analyzed, and these were shown to be down-regulated in an identical manner to total levels of ADAM33 mRNA (Figures E1 and E2). ADAM33 mRNA expression was also decreased in HFF1 and WI38 fibroblast cell lines treated with TGF- β_2 (data not shown), suggesting that the effect of TGF- β_2 on ADAM33 is not restricted to bronchial fibroblasts.

ADAM33 Protein Is Broken Down during Myofibroblast Differentiation

To determine whether the effect of TGF- β_2 on ADAM33 mRNA was matched at the protein level, primary airway fibroblasts were cultured in the absence or presence of 10 ng/ml TGF- β_2 for 48 hours, and ADAM33 protein detected by immunofluorescent staining or Western blotting. As expected, exposure to TGF- β_2 for 48 hours caused myofibroblast differentiation, as evidenced by the marked increase in immunostaining for filamentous α -SMA (Figures 2A and 2B). ADAM33 immunostaining in untreated fibroblast cultures appeared diffuse, and was mainly located within the cell (Figure 2C). After 48 hours of TGF- β_2 treatment, ADAM33 staining was reduced to levels approaching the threshold of detection (Figure 2D), and was not different from control cells stained with isotype control Igs (Figure 2E). Western blot analysis of ADAM33 protein expression showed a number of ADAM33 isoforms to be present (Figure 3A), as previously reported (15). The specific nature of the antibody interaction with these bands was shown by isotype-matched control Igs, and by preadsorbing the anti-ADAM33 antibody with the immunizing peptide (15). Upon treatment with TGF- β_2 , bands at 35 kD and 19 kD were shown to accumulate in Western blots of lysates of PBFs probed with an antibody to the C-terminal tail of ADAM33, suggesting either accumulation of cell-associated breakdown products derived from larger ADAM33 proteins or induction of a novel splice variant (Figure 3A). Similar results were obtained using fibroblasts from either normal subjects or subjects with asthma.

As we had failed to identify any evidence of induction of the putative secreted splice variant of ADAM33 by TGF- β_2 , we postulated that the fragments were degradation products of ADAM33 resulting from TGF- β_2 -mediated ectodomain shedding of ADAM33. To examine this, ADAM33 transfected human embryonic kidney (HEK293) cells (21), which do not express any endogenous ADAM33 (14), were treated in the absence or presence of TGF- β_2 for 48 hours and then analyzed by Western blotting. As found with the fibroblasts, TGF- β_2 treatment resulted in appearance of a 19-kD band, which was not detectable in control, nontransfected HEK293 cells (Figure 3B). Further confirmation that the peptide represented a breakdown product

Figure 2. Immunofluorescent staining of PBFs cultured for 48 hours in serum-free medium in the absence (A and C) or presence (B and D) of 10 ng/ml TGF- β_2 . Antibodies recognized α -smooth muscle actin (A and B) or ADAM33 (C and D). The inset (E) shows control staining in the absence of primary antibody. Nuclei were counterstained with TO-PRO-3 iodide (purple-blue). Scale bar, 40 μ m.

of ADAM33 was suggested by the observation that its accumulation was abolished when fibroblasts were pretreated with a protease inhibitor cocktail (Figure 3C) or with MG132, a proteasome inhibitor (data not shown).

TGF- β_2 -Induced Suppression of ADAM33 Expression during Myofibroblast Differentiation Does Not Involve DNA Methylation

As DNA cytosine-guanine oligodeoxynucleotide island (CpG) methylation of the ADAM33 promoter silences its expression in epithelial cells (14), we next investigated if the ADAM33 promoter became methylated in response to TGF- β_2 treatment. After treatment with TGF- β_2 , DNA was extracted from the cells and digested with *Hpa*II, which is a methylation-sensitive restriction endonuclease that cuts only unmethylated DNA sequences on CCGG while leaving methylated DNA intact. Because specificity protein 1 (SP1) binding sites are strongly associated with epigenetic modification at the promoters of some genes (27, 28), methylation was analyzed by real-time PCR targeting a potential SP1 binding site, GGCCCCGG (predicted by <http://www.bimas.cit.nih.gov/molbio/signal/>) on the ADAM33 promoter (Figure E3). Two pairs of primers were used: one spanning the CCGG site overlapped the SP1 binding site within the ADAM33 core promoter that would not be amplified if it had been digested by *Hpa*II; and another that did not contain an *Hpa*II recognition site (the latter was used to normalize the data and for comparison with nondigested DNA) (Figure 4A). Using this analysis, we found that the ADAM33 promoter was hypomethylated in primary fibroblasts or fibroblast cell lines, but hypermethylated in H292 epithelial cells (Figure 4B and inset). However, the depression of ADAM33 mRNA expression observed during TGF- β_2 -induced myofibroblast differentiation did not involve any changes in ADAM33 promoter methylation (Figure 4B). These data suggest that other

epigenetic mechanisms regulate ADAM33 mRNA expression during the myofibroblast differentiation.

Reduction of ADAM33 Expression during Myofibroblast Differentiation Is Associated with Histone Modification

Post-translational modification of histones plays a key role in regulation of gene expression. In particular, increased histone H3 acetylation (H3Ac) and methylation on lysine 4 (H3K4) are frequently located in actively transcribed regions (29). In contrast, methylation of histone H3 lysine 9 (H3K9) has been shown to correlate with transcriptional repression (29). To determine whether histone acetylation and methylation influenced ADAM33 mRNA expression, ChIP assays were performed using antibodies directed against H3Ac, H3K4 diMe, and H3K9 diMe. After immunoprecipitation, the enriched fractions of genomic DNA were isolated and analyzed by qPCR for the presence of the ADAM33 promoter sequence (Figure E3), using the constitutively active glyceraldehyde 3-phosphate dehydrogenase promoter and a silent region on CEN16 as internal controls for normalization (30). Initial experiments compared the pattern of histone modification in H292 epithelial cells and WI38 and HFF fibroblast cell lines. The fibroblasts showed hyperacetylation of histone H3, hypermethylation of H3K4 diMe, and hypomethylation of H3K9 diMe, consistent with an active promoter supporting ADAM33 gene expression. In contrast, in H292 cells, H3Ac hypoacetylation, H3K4 diMe hypomethylation, and H3K9 diMe hypermethylation was consistent with an inactivated promoter and ADAM33 silencing (Figure E4). After demonstrating that histone modifications on the ADAM33 promoter regulated gene expression, we further investigated whether the histone modifications on the ADAM33 promoter in primary fibroblasts changed in response to TGF- β_2 exposure. Bronchial fibroblasts were incubated in serum-free medium or treated with TGF- β_2 (10 ng/ml) for 4, 8, 24, and 48 hours, and the chromatin immunoprecipitated with antibodies against AcH3, H3K4 diMe, and H3K9 diMe. PCR analysis revealed that TGF- β_2 significantly reduced enrichment of the H3Ac and H3K4 diMe immunoprecipitates with the ADAM33 promoter, but increased enrichment in the H3K9 diMe fraction (Figures 5A–5C). These changes were evident at 4–8 hours, and were maximal by 24 hours. This time course for histone

Figure 3. Western blots of lysates from asthmatic PBFs (A and C) or human embryonic kidney (HEK) 293 cells (B) transfected with full-length ADAM33 (ADAM33) or parental HEK293 cells. Cells were treated for 48 hours in the absence or presence of 10 ng/ml TGF- β_2 and protease inhibitor (PI) cocktail, as indicated. Blots were stained with an ADAM33 antibody directed to the cytoplasmic domain. Black arrowheads indicate specific ADAM33 bands. The white arrowheads show nonspecific bands that could not be blocked with the immunizing peptide.

Figure 4. The effect of TGF- β ₂ on *ADAM33* promoter methylation by methylation-sensitive PCR. (A) The principle of the assay using *HpaII*-digested genomic DNA; (B) its application to DNA from PBFs derived from two subjects (Fibroblasts 1 and 2) treated for 48 hours in the absence or presence of 10 ng/ml TGF- β ₂, as indicated. *Inset* shows a comparison of *ADAM33* promoter methylation in H292 epithelial cells and WI38 and HFF fibroblast cell lines, where data are means ($\pm$ SD) ($n = 3$). $^{**}P < 0.001$ versus uncut DNA.

modification closely followed the time course for suppression of *ADAM33* mRNA expression by TGF- β ₂ (Figure 1B). The specificity of the ChIP assays was confirmed using rabbit IgG (Figure E5). These data suggest that TGF- β ₂ induces local alteration in post-translational modifications of histones to modulate gene expression (Figure 5D).

The *ADAM33* Promoter Becomes Condensed during TGF- β ₂-Induced Myofibroblast Differentiation

To examine further the chromatin structure within the *ADAM33* promoter, chromatin nuclease accessibility assays were performed using *MspI*, a restriction endonuclease that is able to cut open chromatin, but not closed chromatin (31). Thus, accessible regions will be digested, destroying the PCR template and yielding quantitatively less PCR product in the real-time PCR reaction. In control experiments, differences in chromatin accessibility were demonstrated using epithelial and fibroblast cell lines (Figure E6). When this method was applied to PBFs, TGF- β ₂ significantly reduced chromatin accessibility at the *ADAM33* gene promoter (Figure 6), consistent with its ability to suppress *ADAM33* mRNA expression.

DISCUSSION

Based on the roles of TGF- β and *ADAM33* in airway remodeling in chronic asthma, our data provide a novel insight into the regulation of *ADAM33* mRNA and protein by TGF- β ₂ in bronchial fibroblasts during their differentiation into myofibroblasts. We showed that *ADAM33* mRNA expression was depressed and *ADAM33* protein broken down into smaller forms, as the cells differentiated into myofibroblasts in response to TGF- β ₂ treatment. This was accompanied by epigenetic changes around the *ADAM33* promoter involving histone modifications and chromatin accessibility,

Figure 5. Effect of TGF- β ₂ on chromatin remodeling around the *ADAM33* promoter. PBFs were treated in the absence (*open bars*) or presence (*solid bars*) of 10 ng/ml TGF- β ₂ for the times indicated, and chromatin immunoprecipitation (ChIP) performed using antibodies to detect histone modifications, as indicated. (A–C) The relative enrichment compared to $t=0$ (*shaded bar*) of the *ADAM33* promoter sequence in the ChIP using antibodies for acetyl-histone H3 (H3Ac) (A), histone H3 dimethyl lysine 4 (H3K4 diMe) (B), and histone H3 dimethyl lysine 9 (H3K9 diMe) (C) during the time course, as determined by quantitative RT-PCR (RT-qPCR). Data are means ($\pm$ SD) ($n = 3$); $^{**}P < 0.01$ and $^{***}P < 0.001$ versus untreated controls. (D) A model for cell type-specific regulation of the *ADAM33* promoter by DNA methylation (*oblong box*) or histone H3 modifications (*triangle, diamond, and circle*). In the scheme, *filled symbols* indicate hypermethylation or hyperacetylation and *open symbols* indicate hypomethylation or hypoacetylation.

but not DNA methylation. Thus, in common with many regulatory mechanisms, TGF- β can exert opposing effects on a single molecule: it stimulates *ADAM33* ectodomain shedding (21), but it inhibits *ADAM33* gene expression. This type of dual regulatory

Figure 6. Analysis of the effect of TGF- β_2 on ADAM33 promoter accessibility by *MspI* restriction endonuclease-sensitive PCR. PBFs cultures treated in the absence or presence of 10 ng/ml TGF- β_2 for 48 hours before nuclear extracts were prepared and digested with *MspI*. Samples were processed for analysis of the ADAM33 promoter by PCR and normalized with undigested DNA relative to chromosome 16 centromere. Data are means ($\pm$ SD) ($n = 3$); * $P < 0.05$ versus untreated controls.

mechanism is often used to control the dynamic behavior of a system, with a view to maintaining tissue homeostasis (32).

Although real-time PCR analysis of *ADAM33* mRNA expression showed no difference in transcript levels between asthmatic and healthy control cells, this finding was unsurprising, as few of the asthma-associated single-nucleotide polymorphisms are located in the promoter region of *ADAM33* (2, 4). Furthermore, the fact that none of the splice variants were differentially regulated in disease versus healthy cells is consistent with our previous studies of bronchial biopsies from normal subjects and subjects with asthma, where no difference in splice variant expression was detected (13, 14). Surprisingly, we found that TGF- β_2 treatment led to down-regulation of *ADAM33* mRNA in a time- and concentration-dependent manner. Although the relative changes in transcript levels from time 0 are small ($\sim$ two- to fourfold), the difference in *ADAM33* mRNA expression at the later time points in the untreated versus TGF- β_2 -treated cells is significantly larger ($\sim$ 10-fold). The tendency for *ADAM33* expression to increase during culture in serum-free medium suggests that some factors present in serum exert a suppressive effect on *ADAM33* mRNA. The fact that serum is rich in TGF- β (33) may explain the ability of serum to cause a reduction in *ADAM33* mRNA levels.

In our studies, we elected to test the effect of TGF- β_2 on *ADAM33* expression, as TGF- β_2 expression is increased in severe eosinophilic asthma (34), and it is the major isoform expressed by damaged epithelial cells *in vitro* (35), suggesting a role in epithelial-mesenchymal signaling during tissue repair. The down-regulation in *ADAM33* mRNA expression by TGF- β_2 coincided with a down-regulation in ADAM33 protein in cells which had differentiated into myofibroblasts. This was detectable by immunofluorescent staining of the cells with an antibody to the cytoplasmic domain of ADAM33. This also revealed that the majority of the ADAM33 was localized intracellularly. Studies on the trafficking and maturation of ADAM9, -15, and -17 have also shown that the majority of these proteins reside in the Golgi apparatus before regulated trafficking to the

cell surface (36–38). This may prove to be the case for ADAM33, suggesting that further studies on the cellular distribution and trafficking of ADAM33 are required.

Western blot analysis has previously shown a number of ADAM33 protein isoforms to be present in primary airway fibroblasts, and these were presumed to be alternatively spliced variants (15). The present study now suggests that the isoforms at 35 and 19 kD may also represent products of ADAM33 degradation. The 19-kD protein was shown to accumulate not only in TGF- β_2 -treated primary airway fibroblasts, but also in HEK293 cells stably transfected with a full-length ADAM33 cDNA clone (21), which is intron-free and so cannot give rise to alternatively spliced variants. Therefore, the only explanation for the existence of the 19-kD form of ADAM33 in HEK293 cells is as a result of degradation of the full-length protein. Consistent with this interpretation, pretreatment of the cells with a protease inhibitor cocktail or a proteasome inhibitor before TGF- β_2 treatment led to suppression of ADAM33 degradation, supporting a protease-dependent event. Eukaryotic cells contain two major systems for protein degradation: the lysosome containing multiple acid proteases and other hydrolases, which degrade membrane-associated proteins or extracellular proteins; and the proteasome, which degrades cytosolic substrates that are marked by covalent linkage to multiple molecules of ubiquitin (39). As ADAM33 can occur as multiple isoforms, some of which may be membrane anchored, further work will be required to determine how it is targeted for degradation, and if this involves multiple mechanisms. It is also noteworthy that a 25-kD ADAM33 protein isoform was detected in human embryonic lung tissue by Western blotting (13). Because TGF- β plays a key role in branching morphogenesis (40), further studies would be required to determine whether mesenchyme-derived ADAM33 is cleaved in response to endogenous TGF- β , and whether the shed ADAM33 ectodomain plays any role during airway development.

TGF- β exerts its biological activity through transcription regulation of diverse genes via SMAD transcription factors (41), which directly regulate gene transcription in conjunction with transcription factors, chromatin-remodeling complexes, and histone-modifying enzymes (42). It is interesting that TGF- β_2 suppressed *ADAM33* gene expression by altering the post-translational modifications of histone H3 at the *ADAM33* promoter, but it did not completely silence the gene. Because there are no SMAD binding sites on the *ADAM33* promoter, and we observed a relatively slow suppression of *ADAM33* mRNA by TGF- β_2 , it is likely that the effect was linked to induction of expression of new genes by TGF- β_2 . Recently, several histone methyltransferases have been identified, including SUV39H1, SUV39H2, and EZH2, which methylate H3K9, as well as LSD1, which is a chromatin-modifying enzyme that specifically removes methyl groups from mono- and dimethylated H3K4 (43–48). Because we have found that TGF- β_2 can increase *SUV39H2* and *EZH2* mRNA expression levels in fibroblasts (Y.Y., unpublished data), this might be the mechanism by which TGF- β_2 modulates the methylation status of histone H3 at the *ADAM33* promoter. It is also noteworthy that there are several putative SP1 binding sites on the *ADAM33* promoter. This transcription factor is ubiquitously expressed and can act as both a negative and positive regulator of gene expression, depending on physiologic and pathological stimuli (49, 50). For example, SP1-dependent gene regulation can involve competition between the transcriptional repressor, histone deacetylase 1, and the transactivating factor, E2F1 (50). It would be of interest to assess the association of SP1 with the *ADAM33* promoter, and whether TGF- β affects its interaction with histone-modifying enzymes.

In our previous studies, we have shown that *ADAM33* mRNA expression is silenced in epithelial cells by methylation of the *ADAM33* promoter (14). Although TGF- β_2 reduced *ADAM33* mRNA expression in fibroblasts by changing the histone modifications at the *ADAM33* promoter, it did not affect DNA methylation. This suggests that factors are recruited to the locus that are not dependent on DNA methylation, but can lead to a moderately closed chromatin configuration that can reduce the *ADAM33* gene expression (Figure 5D). This may facilitate reactivation of transcriptional activity in response to changing conditions, as observed during culture in serum-free medium. Although no DNA methylation was detectable in our experiments, a recent study has demonstrated that a sequential loss of acetylation at H3K9, loss of methylation at H3K4, and gain of methylation at H3K9 was accompanied a gradual gain of DNA methylation following deoxynucleotide terminal transferase silencing in thymus cells (51). Thus, we cannot exclude the possibility that, over a longer period, the *ADAM33* promoter may become methylated. However, the CpG dinucleotide that we studied is embraced within a putative SP1 binding site, which may protect the CpG island from *de novo* methylation (52).

Overall, the data presented in the current study indicate that TGF- β_2 suppresses *ADAM33* mRNA expression by affecting post-translational histone modifications at the promoter of *ADAM33*. TGF- β_2 also causes breakdown of ADAM33 protein, depleting the myofibroblast of any functional cell-associated ADAM33 ectodomain. This may represent a normal homeostatic mechanism that would limit continuous and excessive stimulation of angiogenesis by sADAM33. However, we have recently found that in an A/J (*bhr1⁺/Adam33⁺*) mouse model, maternal allergy affects *Adam33* expression in postnatal lungs of offspring, causing suppression of *Adam33* mRNA expression, but enhancing accumulation of smaller isoforms of Adam33 protein detected by an antibody to the Adam33 ectodomain (53). Because the T helper type 2 cytokine, IL-13, can induce TGF- β_2 production by epithelial cells (54), these results suggest that an allergic milieu may have a dual effect on *Adam33*, one involving its transcriptional down-regulation by histone modification, and the other involving post-translational processing of the full-length molecule, leading to ectodomain shedding of Adam33. Because TGF- β_2 -mediated shedding of the ADAM33 ectodomain may provide a disease-related gain of function in asthma, further studies will be required to determine how an allergic predisposition overrides this normal feedback mechanism, leading to accumulation of high levels of sADAM33 in bronchoalveolar lavage fluid of subjects with asthma. Some of this work has been presented in abstract form (55).

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

Acknowledgments: The authors thank Prof. Gillian Murphy, University of Cambridge, for provision of human embryonic kidney 293 cells stably expressing a disintegrin and metalloprotease-33 and the nurses in the Wellcome Trust Clinical Research Facility for assistance with sample collection.

References

- Juniper EF, Kline PA, Vanzieleghem MA, Ramsdale EH, O'Byrne PM, Hargreave FE. Effect of long-term treatment with an inhaled corticosteroid (budesonide) on airway hyperresponsiveness and clinical asthma in nonsteroid-dependent asthmatics. *Am Rev Respir Dis* 1990; 142:832–836.
- Van Eerdewegh P, Little RD, Dupuis J, Del Mastro RG, Falls K, Simon J, Torrey D, Pandit S, McKenny J, Braunschweiger K, *et al.* Association of the *ADAM-33* gene with asthma and bronchial hyperresponsiveness. *Nature* 2002;418:426–430.
- Holgate ST, Yang Y, Haitchi HM, Powell RM, Holloway JW, Yoshisue H, Pang YY, Cakebread J, Davies DE. The genetics of asthma: ADAM33 as an example of a susceptibility gene. *Proc Am Thorac Soc* 2006;3:440–443.
- Blakey J, Halapi E, Bjornsdottir US, Wheatley A, Kristinsson S, Upmanyu R, Stefansson K, Hakonarson H, Hall IP. The contribution of ADAM 33 polymorphisms to the population risk of asthma. *Thorax* 2005;60:274–276.
- Li X, Zhang Y, Zhang J, Xiao Y, Huang J, Tian C, He C, Deng Y, Yang Y, Fan H. Asthma susceptible genes in Chinese population: a meta-analysis. *Respir Res* 2010;11:129.
- Michel S, Liang L, Depner M, Klopp N, Ruether A, Kumar A, Schedel M, Vogelberg C, von Mutius E, von Berg A, *et al.* Unifying candidate gene and GWAS approaches in asthma. *PLoS One* 2010;5:e13894.
- Simpson A, Maniatis N, Jury F, Cakebread JA, Lowe LA, Holgate ST, Woodcock A, Ollier WE, Collins A, Custovic A, *et al.* Polymorphisms in a disintegrin and metalloprotease 33 (ADAM33) predict impaired early-life lung function. *Am J Respir Crit Care Med* 2005;172:55–60.
- Jongepier H, Boezen HM, Dijkstra A, Howard TD, Vonk JM, Koppelman GH, Zheng SL, Meyers DA, Bleecker ER, Postma DS. Polymorphisms of the *ADAM33* gene are associated with accelerated lung function decline in asthma. *Clin Exp Allergy* 2004;34:757–760.
- van Diemen CC, Postma DS, Vonk JM, Bruinenberg M, Schouten JP, Boezen HMA, Disintegrin A. Metalloprotease 33 polymorphisms and lung function decline in the general population. *Am J Respir Crit Care Med* 2005;172:329–333.
- Gosman MM, Boezen HM, van Diemen CC, Snoeck-Stroband JB, Lapperre TS, Hiemstra PS, Ten Hacken NH, Stolk J, Postma DS. A disintegrin and metalloproteinase 33 and chronic obstructive pulmonary disease pathophysiology. *Thorax* 2007;62:242–247.
- Xiao J, Han J, Wang X, Hua D, Su D, Bao Y, Lv F. Association of *ADAM33* gene with susceptibility to COPD in Tibetan population of China. *Mol Biol Rep* 2011;38:4941–4945.
- van Goor H, Melenhorst WB, Turner AJ, Holgate ST. Adamalysins in biology and disease. *J Pathol* 2009;219:277–286.
- Haitchi HM, Powell RM, Shaw TJ, Howarth PH, Wilson SJ, Wilson DI, Holgate ST, Davies DE. ADAM33 expression in asthmatic airways and human embryonic lungs. *Am J Respir Crit Care Med* 2005;171:958–965.
- Yang Y, Haitchi HM, Cakebread J, Sammut D, Harvey A, Powell RM, Holloway JW, Howarth P, Holgate ST, Davies DE. Epigenetic mechanisms silence a disintegrin and metalloprotease 33 expression in bronchial epithelial cells. *J Allergy Clin Immunol* 2008;121:1393–1399.
- Powell RM, Wicks J, Holloway JW, Holgate ST, Davies DE. The splicing and fate of ADAM33 transcripts in primary human airways fibroblasts. *Am J Respir Cell Mol Biol* 2004;31:13–21.
- Ito I, Laporte JD, Fiset PO, Asai K, Yamauchi Y, Martin JG, Hamid Q. Downregulation of a disintegrin and metalloproteinase 33 by IFN- γ in human airway smooth muscle cells. *J Allergy Clin Immunol* 2007;119:89–97.
- Dijkstra A, Postma DS, Noordhoek JA, Lodewijk ME, Kauffman HF, Ten Hacken NH, Timens W. Expression of ADAMs (“a disintegrin and metalloprotease”) in the human lung. *Virchows Arch* 2009;454:441–449.
- Foley SC, Mogas AK, Olivenstein R, Fiset PO, Chakir J, Bourbeau J, Ernst P, Lemiere C, Martin JG, Hamid Q. Increased expression of ADAM33 and ADAM8 with disease progression in asthma. *J Allergy Clin Immunol* 2007;119:863–871.
- Erbek SS, Erinanc H, Erbek S, Topal O, Kiyici H. Expression of a disintegrin and metalloproteinase 33 protein in nasal polyposis: an immunohistochemical study. *Am J Rhinol Allergy* 2010;24:79–82.
- Lee JY, Park SW, Chang HK, Kim HY, Rhim T, Lee JH, Jang AS, Koh ES, Park CS. A disintegrin and metalloproteinase 33 protein in patients with asthma: relevance to airflow limitation. *Am J Respir Crit Care Med* 2006;173:729–735.
- Puxeddu I, Pang YY, Harvey A, Haitchi HM, Nicholas B, Yoshisue H, Ribatti D, Clough G, Powell RM, Murphy G, *et al.* The soluble form of a disintegrin and metalloprotease 33 promotes angiogenesis: implications for airway remodeling in asthma. *J Allergy Clin Immunol* 2008;121:1400–1406.
- Redington AE, Madden J, Frew AJ, Djukanovic R, Roche WR, Holgate ST, Howarth PH. Transforming growth factor- β 1 in asthma: measurement in bronchoalveolar lavage fluid. *Am J Respir Crit Care Med* 1997;156:642–647.

23. Wicks J, Haitchi HM, Holgate ST, Davies DE, Powell RM. Enhanced upregulation of smooth muscle related transcripts by TGF beta2 in asthmatic (myo) fibroblasts. *Thorax* 2006;61:313–319.
24. Hurd SZ. Workshop summary and guidelines: investigative use of bronchoscopy, lavage, and bronchial biopsies in asthma and other airway diseases. *J Allergy Clin Immunol* 1991;88:808–814.
25. Holgate ST, Davies DE, Lackie PM, Wilson SJ, Puddicombe SM, Lordan JL. Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J Allergy Clin Immunol* 2000;105:193–204.
26. Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, Davies DE. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J* 2000;14:1362–1374.
27. Green MR, Yoon H, Boss JM. Epigenetic regulation during B cell differentiation controls CIITA promoter accessibility. *J Immunol* 2006;177:3865–3873.
28. Honda H, Pazin MJ, Ji H, Wernyj RP, Morin PJ. Crucial roles of Sp1 and epigenetic modifications in the regulation of the CLDN4 promoter in ovarian cancer cells. *J Biol Chem* 2006;281:21433–21444.
29. Barth TK, Imhof A. Fast signals and slow marks: the dynamics of histone modifications. *Trends Biochem Sci* 2010;35:618–626.
30. Lau JC, Hanel ML, Wevrick R. Tissue-specific and imprinted epigenetic modifications of the human NDN gene. *Nucleic Acids Res* 2004;32:3376–3382.
31. Rao S, Procko E, Shannon MF. Chromatin remodeling, measured by a novel real-time polymerase chain reaction assay, across the proximal promoter region of the IL-2 gene. *J Immunol* 2001;167:4494–4503.
32. Lan RY, Selmi C, Gershwin ME. The regulatory, inflammatory, and T cell programming roles of interleukin-2 (IL-2). *J Autoimmun* 2008;31:7–12.
33. Grainger DJ, Mosedale DE, Metcalfe JC, Weissberg PL, Kemp PR. Active and acid-activatable TGF-beta in human sera, platelets and plasma. *Clin Chim Acta* 1995;235:11–31.
34. Balzar S, Chu HW, Silkoff P, Cundall M, Trudeau JB, Strand M, Wenzel S. Increased TGF-beta2 in severe asthma with eosinophilia. *J Allergy Clin Immunol* 2005;115:110–117.
35. Howat WJ, Holgate ST, Lackie PM. TGF-beta isoform release and activation during in vitro bronchial epithelial wound repair. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L115–L123.
36. Roghani M, Becherer JD, Moss ML, Atherton RE, Erdjument-Bromage H, Arribas J, Blackburn RK, Weskamp G, Tempst P, Blobel CP. Metalloprotease-disintegrin MDC9: intracellular maturation and catalytic activity. *J Biol Chem* 1999;274:3531–3540.
37. Lum L, Reid MS, Blobel CP. Intracellular maturation of the mouse metalloprotease disintegrin MDC15. *J Biol Chem* 1998;273:26236–26247.
38. Soond SM, Everson B, Riches DW, Murphy G. ERK-mediated phosphorylation of Thr735 in TNFalpha-converting enzyme and its potential role in TACE protein trafficking. *J Cell Sci* 2005;118:2371–2380.
39. Lee DH, Goldberg AL. Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* 1998;8:397–403.
40. Warburton D, Bellusci S, De Langhe S, Del Moral PM, Fleury V, Mailleux A, Tefft D, Unbekandt M, Wang K, Shi W. Molecular mechanisms of early lung specification and branching morphogenesis. *Pediatr Res* 2005;57:26R–37R.
41. Derynck R, Zhang Y, Feng XH. Smads: transcriptional activators of TGF-beta responses. *Cell* 1998;95:737–740.
42. Schmierer B, Hill CS. TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol* 2007;8:970–982.
43. Garcia-Cao M, O'Sullivan R, Peters AH, Jenuwein T, Blasco MA. Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases. *Nat Genet* 2004;36:94–99.
44. West AG, Attikum H. Chromatin at the crossroads. Meeting on Signalling to Chromatin Epigenetics. *EMBO Rep* 2006;7:1206–1210.
45. Cloos PA, Christensen J, Agger K, Maiolica A, Rappsilber J, Antal T, Hansen KH, Helin K. The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. *Nature* 2006;442:307–311.
46. Chen Y, Yang Y, Wang F, Wan K, Yamane K, Zhang Y, Lei M. Crystal structure of human histone lysine-specific demethylase 1 (LSD1). *Proc Natl Acad Sci USA* 2006;103:13956–13961.
47. Forneris F, Binda C, Dall'aglio A, Fraaije MW, Battaglioli E, Mattevi A. A highly specific mechanism of histone H3-K4 recognition by histone demethylase LSD1. *J Biol Chem* 2006;281:35289–35295.
48. Cha TL, Zhou BP, Xia W, Wu Y, Yang CC, Chen CT, Ping B, Otte AP, Hung MC. Akt-mediated phosphorylation of EZH2 suppresses methylation of lysine 27 in histone H3. *Science* 2005;310:306–310.
49. Tan NY, Khachigian LM. Sp1 phosphorylation and its regulation of gene transcription. *Mol Cell Biol* 2009;29:2483–2488.
50. Doetzlhofer A, Rotheneder H, Lagner G, Koranda M, Kurtev V, Brosch G, Wintersberger E, Seiser C. Histone deacetylase 1 can repress transcription by binding to Sp1. *Mol Cell Biol* 1999;19:5504–5511.
51. Su RC, Brown KE, Saaber S, Fisher AG, Merckenschlager M, Smale ST. Dynamic assembly of silent chromatin during thymocyte maturation. *Nat Genet* 2004;36:502–506.
52. Siegfried Z, Eden S, Mendelsohn M, Feng X, Tsuberi BZ, Cedar H. DNA methylation represses transcription *in vivo*. *Nat Genet* 1999;22:203–206.
53. Haitchi H-M, Bassett D, Bucchieri F, Gao X, Powell R, Hanley NA, Wilson DI, Holgate ST, Davies DE. Induction of a disintegrin and metalloprotease 33 during embryonic lung development and the influence of IL-13 or maternal allergy. *J Allergy Clin Immunol* 2009;124:590–597.
54. Richter A, Puddicombe SM, Lordan JL, Bucchieri F, Wilson SJ, Djukanovic R, Dent G, Holgate ST, Davies DE. The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. *Am J Respir Cell Mol Biol* 2001;25:385–391.
55. Haitchi HM, Yang Y, Wicks J, Murphy G, Powell RM, Howarth P, Holgate ST, Davies DE. TGF-beta down regulates ADAM33 expression during myofibroblast differentiation via histone modification [abstract]. *Eur Respir J* 2009;28:E3096.