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Loss of integrin αvβ6-mediated TGF-β activation causes Mmp12-dependent emphysema

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Integrins are heterodimeric cell-surface proteins that regulate cell growth, migration and survival. We have shown previously that the epithelial-restricted integrin αvβ6 has another critical function; that is, it binds and activates latent transforming growth factor-β (TGF-β)[1,2]. Through a global analysis of pulmonary gene expression in the lungs of mice lacking this integrin (*Itgb6* null mice) we have identified a marked induction of macrophage metalloelastase (Mmp12)—a metalloproteinase that preferentially degrades elastin and has been implicated in the chronic lung disease emphysema[3]. Here we report that *Itgb6*-null mice develop age-related emphysema that is completely abrogated either by transgenic expression of versions of the β6 integrin subunit that support TGF-β activation, or by the loss of Mmp12. Furthermore, we show that the effects of *Itgb6* deletion are overcome by simultaneous transgenic expression of active TGF-β1. We have uncovered a pathway in which the loss of integrin-mediated activation of latent TGF-β causes age-dependent pulmonary emphysema through alterations of macrophage Mmp12 expression. Furthermore, we show that a functional alteration in the TGF-β activation pathway affects susceptibility to this disease.

Pulmonary emphysema, which is characterized by simplification of alveolar architecture, loss of lung elasticity, and enlargement of alveolar airspaces, is a worldwide health problem largely attributable to exposure to tobacco smoke. Extracellular proteases, which regulate extracellular matrix homeostasis, have been implicated in tobacco-smoke-induced pulmonary emphysema. Nevertheless, the regulation of these proteases *in vivo* is poorly understood and a regulatory role of lung epithelial cells in this process has yet to be described.

Mice that do not express the β6 subunit of the αvβ6 integrin (*Itgb6*$^-$) spontaneously develop increased expression of the extracellular macrophage metalloproteinase Mmp12 (macrophage metalloelastase) in their lungs by eight weeks of age. In fact, on the basis of whole-organ gene expression profiling using the

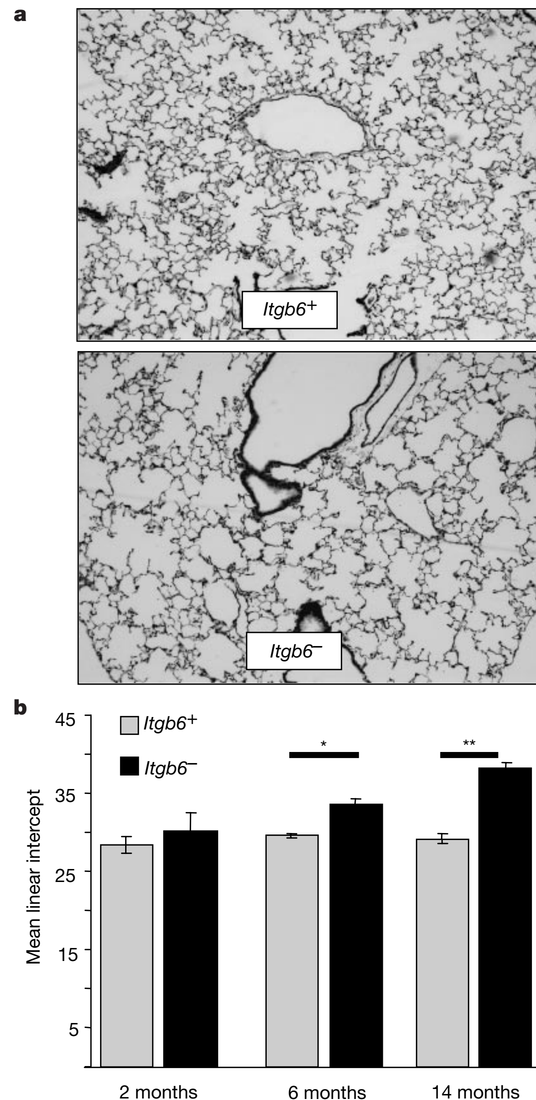

Figure 1 Spontaneous, progressive pulmonary emphysema in *Itgb6*$^-$ mice. **a**, Representative histological sections (×10 objective) of lungs from *Itgb6*$^+$ and *Itgb6*$^-$ mice at 14 months of age show enlarged alveoli indicative of emphysema in *Itgb6*$^-$ mice. **b**, Mean linear intercepts (μm ± s.e.m.) of alveolar septae measured in the lungs of five *Itgb6*$^+$ (wild type) and five *Itgb6*$^-$ mice at 2, 6 and 14 months of age. Asterisk, $P = 0.0006$; double asterisk, $P = 0.0002$.

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Affymetrix mu6500 microarray, expression of Mmp12 is increased in the lungs of *Itgb6*$^{-}$ mice to at least 18-fold above the level of expression in wild-type mice[3] (Supplementary Fig. 1). Using real-time quantitative polymerase chain reaction (PCR) analysis, we found that alveolar macrophages from the lungs of *Itgb6*$^{-}$ mice expressed 200-fold more Mmp12 messenger RNA than alveolar macrophages from wild-type mice (ratio of Mmp12 mRNA abundance in *Itgb6*$^{-}$ compared with *Itgb6*$^{+}$ mice $= 228 \pm 20$; $P = 0.0008$). Casein zymography confirmed increased levels of Mmp12 protein in the bronchoalveolar lavage fluid of *Itgb6*$^{-}$ mice by eight weeks of age (data not shown).

Mmp12 is an extracellular matrix-degrading metalloproteinase that is expressed only by tissue macrophages and placental trophoblasts[4–6]. Mice lacking Mmp12 (*Mmp12*$^{-}$) do not develop alveolar enlargement—the definitive characteristic of pulmonary emphysema—after exposure to cigarette smoke, suggesting that Mmp12 is important in the development of this disease[7]. Our observation that *Mmp12* gene expression is markedly increased in the lungs of *Itgb6*$^{-}$ mice also raised the possibility that loss of the αvβ6 integrin could result in the development of emphysema over time. To test this hypothesis, we examined alveolar size in lungs of *Itgb6*$^{-}$ and wild-type mice by measuring the mean linear intercept of alveolar septae at 2, 6 and 14 months of age. We found that *Itgb6*$^{-}$ mice, despite having normal alveolar size at 2 months of age, spontaneously developed progressive alveolar enlargement, or pulmonary emphysema, over time, whereas wild-type mice did not (Fig. 1).

The αvβ6 integrin has two distinct functions *in vivo* that depend on specific regions of the β6 subunit: enhancement of epithelial cell proliferation, which depends on the carboxy-terminal 11 amino acids[8], and the activation of latent TGF-β, which is independent of these residues[1]. Emphysema in *Itgb6*-deleted mice might conceivably be due to a loss of either of these functions. To determine which of the functions of the β6 integrin subunit is critical to prevent the development of spontaneous emphysema, we studied alveolar size, Mmp12 expression levels and alveolar macrophage morphology in

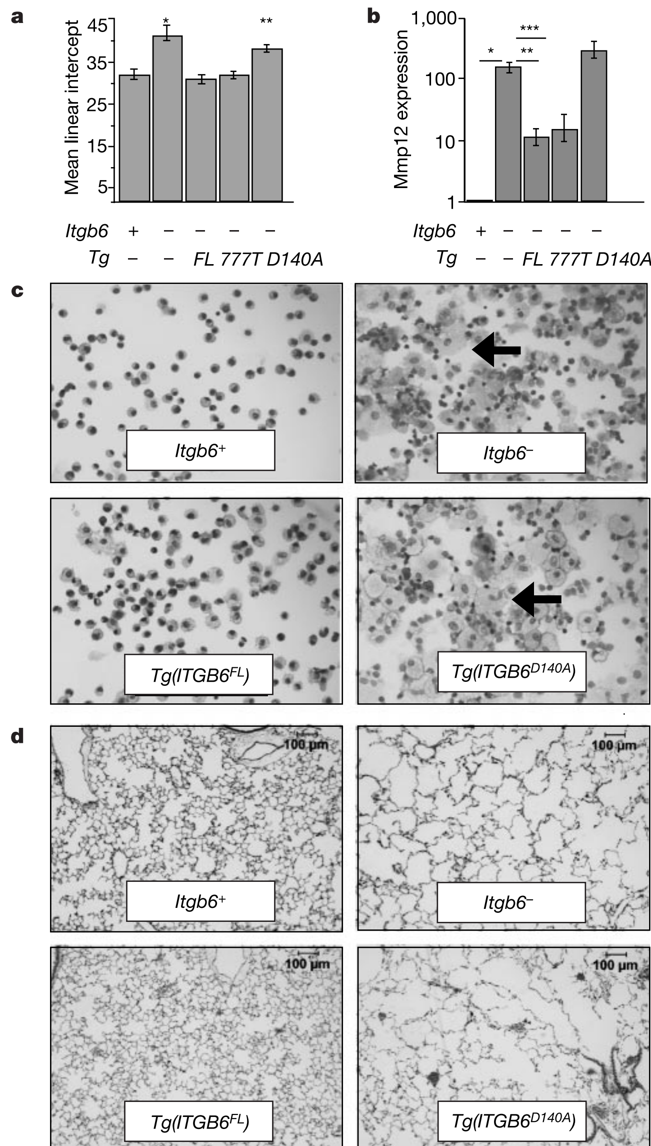

Figure 2 Transgenic expression of human *ITGB6*^{FL} or *ITGB6*^{777T} reduces Mmp12 expression, normalizes macrophage appearance and prevents the development of airspace enlargement in *Itgb6*$^{-}$ mice. **a**, Mean linear intercepts in 9-month-old mice show that *Itgb6*$^{-}$ and *Itgb6*$^{-}$*Tg*(*ITGB6*^{D140A}) mice develop alveolar enlargement whereas *Itgb6*$^{-}$*Tg*(*ITGB6*^{FL}) and *Itgb6*$^{-}$*Tg*(*ITGB6*^{777T}) mice do not. Asterisk, $P = 0.002$; double asterisk, $P = 0.004$ compared with wild-type mice; $n = 5$ per group. **b**, Two-month-old *Itgb6*$^{+}$, *Itgb6*$^{-}$*Tg*(*ITGB6*^{FL}) and *Itgb6*$^{-}$*Tg*(*ITGB6*^{777T}) mice have markedly reduced levels of Mmp12 expression compared with *Itgb6*$^{-}$ and *Itgb6*$^{-}$*Tg*(*ITGB6*^{D140A}) mice. Asterisk, $P = 2.3 \times 10^{-7}$; double asterisk, $P = 0.0001$; triple asterisk, $P = 0.0002$; $n = 4$ per group. **c**, Representative cytospin concentrates from bronchoalveolar lavage fluid ($\times$ 40 objective) from mice of each genotype show abundant, large vacuolated alveolar macrophages from both *Itgb6*$^{-}$ and *Itgb6*$^{-}$*Tg*(*ITGB6*^{D140A}) mice (arrows). Alveolar macrophages from *Itgb6*$^{-}$*Tg*(*ITGB6*^{777T}) mice are nearly normal and indistinguishable from those recovered from *Itgb6*$^{-}$*Tg*(*ITGB6*^{FL}) mice (data not shown). **d**, Representative histological sections ($\times$ 10 objective) of the lungs of *Itgb6*$^{+}$, *Itgb6*$^{-}$, *Itgb6*$^{-}$*Tg*(*ITGB6*^{FL}) and *Itgb6*$^{-}$*Tg*(*ITGB6*^{D140A}) mice.

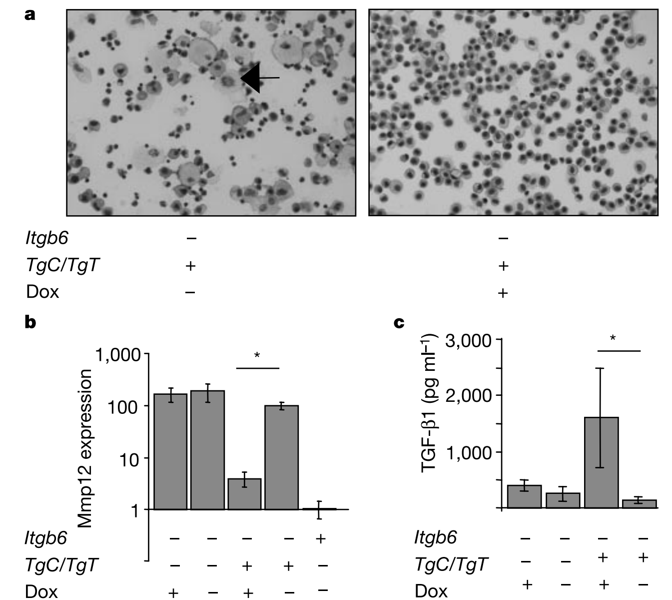

Figure 3 Transgenic expression of *Tgfb1Cys-Ser*^{223,225} normalizes alveolar macrophage appearance and reduces Mmp12 expression in Itgb6 mice. **a**, Representative cytospin concentrates of bronchoalveolar lavage fluid ($\times$ 40 objective) from *Itgb6*$^{-}$ double-transgenic mice show abundant, large vacuolated alveolar macrophages (arrow) from mice without transgene induction (Dox −) and normal-appearing macrophages from mice with transgene induction (Dox +). *TgC/TgT*, *Tg*(*CCSP-rtTA*)*Tg*(*tetO-Tgfb1Cys-Ser*^{223,225}). Alveolar macrophages from water- and doxycycline-treated *Itgb6*$^{-}$*TgC*$^{-}$/*TgT*$^{-}$ mice and water-treated *Itgb6*$^{-}$*TgC*$^{+}$/*TgT*$^{+}$ mice are identical in appearance (data not shown). **b**, Measurement of Mmp12 transcript abundance in bronchoalveolar lavage fluid cells from 6-week-old mice shows that Mmp12 expression in *Itgb6*$^{-}$*TgC*$^{+}$/*TgT*$^{+}$ mice is reduced nearly 45-fold by 21 days of doxycycline-induced gene expression. Asterisk, $P = 4 \times 10^{-6}$; $n = 3$ per group. **c**, Total TGF-β1 in bronchoalveolar lavage fluid in *Itgb6*$^{-}$*TgC*$^{+}$/*TgT*$^{+}$ mice is increased after 14 days of doxycycline treatment. Asterisk, $P = 0.03$; $n = 3$–5 per group.

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Itgb6^{-} mice that expressed transgenic versions of either full-length human β6 (*Itgb6*$^{-}$*Tg*(*ITGB6*^{FL})), a truncated form of the human β6 integrin subunit lacking the C-terminal 11 amino acids (*Itgb6*$^{-}$*Itgb6*$^{-}$*Tg*(*ITGB6*^{777T})), or a ligand-binding-incompetent form of β6 (*Itgb6*$^{-}$*Tg*(*ITGB6*^{D140A})) in distal bronchiolar and type II lung epithelial cells[9,10]. We confirmed lung-specific expression of these proteins *in vivo* by western blotting (data not shown) and have shown previously that each of these mutants is well expressed on the surface of epithelial cells *in vitro*[9,10]. Transgenic introduction of the full-length human β6 integrin subunit (*Tg*(*ITGB6*^{FL})) into *Itgb6*$^{-}$ mice eliminated the development of spontaneous emphysema, substantially reduced the induction of Mmp12, and prevented the development of vacuolated alveolar macrophages (Fig. 2). Transgenic introduction of the β6 truncation mutant (*Tg*(*ITGB6*^{777T}))—a mutant that cannot support enhanced cell growth in three-dimensional culture or *in vivo*[8] but that can bind the latency-associated protein (LAP) and activate TGF-β[1]—also eliminated development of spontaneous emphysema, substantially reduced the induction of Mmp12, and prevented the development of vacuolated alveolar macrophages in *Itgb6*$^{-}$ mice. However, transgenic introduction of a ligand-binding-incompetent mutant of the β6 protein, which can neither bind nor activate TGF-β as a result of an aspartic acid to alanine mutation at position 140 (D140A) in the metal-ion-dependent adhesion site (MIDAS) domain of β6 (*Tg*(*ITGB6*^{D140A})), did not prevent the development of spontaneous emphysema, the induction of Mmp12 expression, or the development of vacuolated alveolar macrophages in *Itgb6*$^{-}$ mice.

These results indicate that although expression of the β6 integrin subunit by epithelial cells in the lung is sufficient to prevent the development of spontaneous emphysema in ageing mice, the prevention of spontaneous emphysema in *Itgb6*-deleted mice seems to be dependent on the ability of the transgenic β6 integrin to bind and activate latent TGF-β. These findings further suggested that the alveolar macrophage phenotype in *Itgb6*-deleted mice was due, in part or in whole to a chronic deficiency of active TGF-β which, in turn, was caused by a defect in the activation of latent TGF-β by the αvβ6 integrin. This possibility is further strengthened by *in vitro* studies showing Smad-3-dependent regulation of Mmp12 expression by TGF-β in peripheral blood-derived macrophages[11,12].

Therefore, we investigated whether expression of active TGF-β itself in the lungs of *Itgb6*-deleted mice was sufficient to bypass the activation defect imposed by Itgb6 deficiency and thereby prevent the development of the early hallmarks of the *Itgb6*-deleted pulmonary phenotype (that is, increased Mmp12 expression and the accumulation of large vacuolated alveolar macrophages). To do this, we first generated mice that expressed constitutively active TGF-β1 under positive tetracycline-regulated transcriptional control (*Itgb6*$^{+}$*Tg*(*CCSP-rtTA*)*Tg*(*tetO-Tgfb1Cys-Ser*^{223,225})). We found that these mice, when bred to be double allelic for both transgenes, had detectable levels of TGF-β1 in bronchoalveolar lavage fluid after two weeks of doxycycline administration, whereas transgene-negative mice, or isogenic mice receiving water, did not (data not shown). We then bred copies of both of these transgenes into syngeneic *Itgb6*$^{-}$ mice. We found that double-transgenic *Itgb6*$^{-}$ mice, when given doxycycline to cause expression of constitutively active TGF-β1 in their lungs, had alveolar macrophages that appeared normal and had substantially reduced Mmp12 expression compared with untreated, double-transgenic *Itgb6*$^{-}$ mice (Fig. 3). Furthermore, expression of active TGF-β1 eliminated the lymphocyte and neutrophil accumulation in the alveoli of *Itgb6*$^{-}$ mice (data not shown). These findings show that expression of active TGF-β1 in the lungs of *Itgb6*$^{-}$ mice corrects the principal abnormalities caused by deletion of the integrin gene—presumably by bypassing the associated defect in latent TGF-β activation.

To determine whether the induction of Mmp12 expression in *Itgb6*$^{-}$ mice is responsible for the alveolar enlargement that we observed, we generated mice deficient in the expression of both β6 and Mmp12 (*Itgb6*$^{-}$ *Mmp12*$^{-}$). By 8 months of age, *Itgb6*$^{-}$ mice had developed significant alveolar enlargement whereas *Itgb6*$^{-}$ *Mmp12*$^{-}$ mice displayed no alveolar enlargement (Fig. 4a, c). These results show that Mmp12 is essential for the development of emphysema in *Itgb6*$^{-}$ mice. Notably, the prevention of spontaneous emphysema in *Itgb6*$^{-}$ *Mmp12*$^{-}$ mice was not due to a

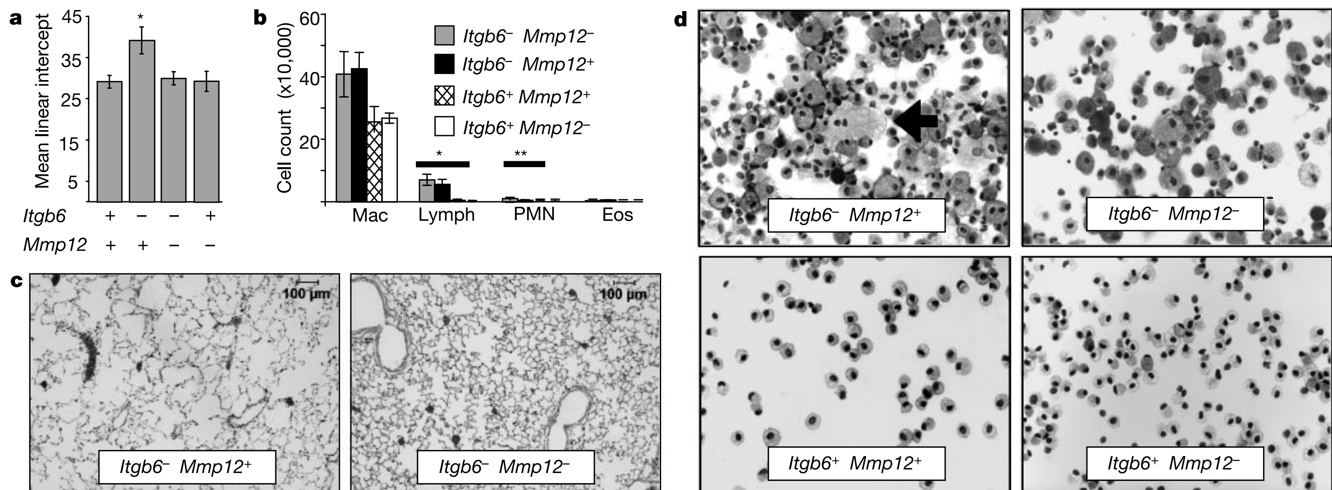

Figure 4 Deletion of Mmp12 prevents spontaneous emphysema in *Itgb6*$^{-}$ mice but does not affect inflammation. **a**, Mean linear intercepts in the lungs of 8-month-old *Itgb6*$^{+}$ *Mmp12*$^{+}$, *Itgb6*$^{-}$ *Mmp12*$^{+}$, *Itgb6*$^{-}$ *Mmp12*$^{-}$ and *Itgb6*$^{+}$ *Mmp12*$^{-}$ mice show that alveolar enlargement in *Itgb6*$^{-}$ mice is prevented by simultaneous deletion of *Mmp12*. Asterisk, $P = 0.009$ for *Itgb6*$^{-}$ *Mmp12*$^{+}$ compared with *Itgb6*$^{-}$ *Mmp12*$^{-}$ mice; $n = 5$ per group. **b**, Comparison of total cell counts in bronchoalveolar lavage fluid from 3-month-old *Itgb6*$^{-}$ *Mmp12*$^{+}$, *Itgb6*$^{-}$ *Mmp12*$^{-}$, *Itgb6*$^{+}$ *Mmp12*$^{+}$ and *Itgb6*$^{+}$ *Mmp12*$^{-}$ mice reveals similar degrees of macrophage, polymorphonuclear leukocyte and lymphocyte accumulation in *Itgb6*$^{-}$ *Mmp12*$^{+}$ and *Itgb6*$^{-}$ *Mmp12*$^{-}$ mice. Mac, alveolar macrophages; lymph, lymphocytes; PMN, polymorphonuclear leukocytes; eos, eosinophils. Asterisk, $P < 0.001$; double asterisk, $P = 0.02$ for *Itgb6*$^{-}$ *Mmp12*$^{+}$ compared with wild-type mice; $n =$ 5–8 per group. **c**, Representative histological sections ($\times$ 10 objective) of the lungs of 8-month-old mice show airspace enlargement in *Itgb6*$^{-}$ *Mmp12*$^{+}$ but not *Itgb6*$^{-}$ *Mmp12*$^{-}$ mice. **d**, Representative cytospin concentrates from bronchoalveolar lavage fluid ($\times$ 40 objective) from mice of each genotype show abundant, large vacuolated alveolar macrophages in both *Itgb6*$^{-}$ *Mmp12*$^{+}$ and *Itgb6*$^{-}$ *Mmp12*$^{-}$ mice (arrow).

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reduction in the degree of lung inflammation, because, by means of bronchoalveolar lavage of the lungs of these double knockout mice, we found that the degree of lung inflammation was similar to that seen in *Itgb6*$^{-}$ mice (Fig. 4b)[10,13]. In addition, *Itgb6*$^{-}$ *Mmp12*$^{-}$ mice still developed patchy juvenile baldness and the associated skin inflammation that we have previously reported in *Itgb6*$^{-}$ mice (data not shown)[13]. Therefore, Mmp12 activity is not required for inflammatory cell recruitment into the lungs or skin of *Itgb6*$^{-}$ mice, and lung inflammation, in the absence of Mmp12, is not sufficient to cause emphysema in *Itgb6*$^{-}$ mice.

Several recent reports have described the development of emphysema in rodent models. These include mice transgenic for interferon-γ (IFN-γ)[14], tumour necrosis factor-α (TNF-α)[15] or interleukin-13 (IL-13)[16], mice deficient in surfactant protein D[17] or tissue inhibitor of metalloproteinase 3 (Timp-3)[18], and rats treated with an inhibitor of vascular endothelial-derived growth factor receptor 2 (Vegfr-2)[19]. In the mouse models involving IL-13, IFN-γ, TNF-α and surfactant protein D, rapidly progressive emphysema follows induction of a multitude of elastinolytic and collagenolytic enzymes. Although our results do not eliminate the possibility that other proteases may interact with Mmp12 in the development of emphysema, the absence of a significant induction of the expression of other proteases in the lungs as measured by microarray analysis, and the lack of emphysema in *Itgb6*$^{-}$ *Mmp12*$^{-}$ mice indicates that Mmp12 is central to this process in *Itgb6*$^{-}$ mice.

We demonstrate a new *in vivo* pathway in which the loss of an epithelial integrin, which is known to cause a local deficiency in active TGF-β1[1,2], results in increased expression of Mmp12 by alveolar macrophages and causes emphysema over time. We also show that the increased expression of Mmp12 can be prevented by expression of constitutively active TGF-β in these integrin-deficient mice. The gradual, age-related emphysema in *Itgb6*$^{-}$ mice, in contrast to the severe and rapidly progressive forms observed in mice that overexpress IL-13, TNF-α and IFN-γ, is more akin to the pace of emphysema commonly observed in humans. Furthermore, the pathological features of *Itgb6*$^{-}$ mice resemble those previously reported in young tobacco smokers[20]. Although cigarette smoke is the major environmental factor responsible for causing emphysema in humans, only a minority of heavy smokers develop emphysema, which suggests that other risk factors are important. Family studies of patients with emphysema have demonstrated clearly the importance of genetic factors in determining an individual's susceptibility to this disease. Although congenital deficiency of α_1-antitrypsin has been shown to increase susceptibility to emphysema, it is a rare condition, and most of the genetic factors affecting susceptibility remain unexplained[21]. The finding that mice lacking the αvβ6 integrin had persistently elevated expression of Mmp12 and developed slowly progressive, age-related emphysema, suggests that the integrity of this pathway may also be important in preventing emphysema in humans. Our results suggest that abnormalities in any of the steps in this pathway of TGF-β activation or signalling (for example, decreased αvβ6 expression or function, decreased expression or function of TGF-β, or defects in the TGF-β signalling pathway in macrophages) may contribute to genetic or acquired susceptibility to this common and debilitating disease. □

Methods

Itgb6^{-}, *Mmp12*$^{-}$ and transgenic mice

Itgb6^{-} mice were generated as described on a 129Svems genetic background[13]. *Mmp12*$^{-}$ mice were generated as described on a 129Sv genetic background[7]. Transgenic mice expressing a truncated form of the human β6 subunit lacking the last 11 amino acid residues of the cytoplasmic domain (*Tg*(*ITGB6*^{777T})) or an aspartic acid to alanine point mutant (*Tg*(*ITGB6*^{D140A})) under transcriptional control of the tissue-specific surfactant protein C (Spc) promoter were generated using methods as described[10]. The 777T fragment was excised from pCDNAINeo with *Xho*I and *Xba*I, blunt ends were created, and it was cloned into the expression plasmid pUC18SPC3.7 (a gift of J. Whitsett), which had been digested with *Bgl*II and blunt-ended[22,23]. The D140A fragment was subcloned into pMamBlue, digested with *Xho*I and *Xba*I, excised with *Sal*I and cloned into pUC18SPC3.7 that had been digested with *Sal*I. Transgene incorporation in founder lines was confirmed by Southern blot analysis, and tissue-specific protein expression was confirmed using western analysis using the 4B5 rabbit monoclonal anti-human β6 antibody[10]. These transgenic mice were backcrossed five generations onto an *Itgb6*$^{-}$ C57BL/6 background. *Tg*(*CCSP-rtTA*)*Tg*(*tetO-Tgfb1Cys-Ser*^{223,225}) double-transgenic mice were generated by breeding together two independent lines of FVB mice carrying either the *Tg*(*CCSP-rtTA*) transgenic construct (a gift of J. Whitsett)[24] or the *Tg*(*tetO-Tgfb1Cys-Ser*^{223,225}) transgenic construct[25]. Mice carrying copies of both transgenes were verified by PCR genotyping and bred onto an FVB *Itgb6*$^{-}$ genetic background. Doxycycline-inducible expression of TGF-β1 was verified by enzyme-linked immunosorbent assay (Pharmingen).

Quantitative real-time RT–PCR

RNA isolation, treatment, primer design and amplimer detection probe design were carried out as described[26]. All primers and probe sequences are available at http://asthmagenomics.ucsf.edu/pubs/. The mean number of cycles to threshold (C_T) of fluorescence detection was calculated for each sample and the results were normalized to the mean C_T of glyceraldehyde 3-phosphate dehydrogenase (Gapdh) for each sample tested. Results are expressed as a fold increase (or decrease) in complementary DNA abundance compared with control animals. We used the normalized C_T differences (Mmp12 C_T − Gapdh C_T) for all statistical comparisons.

Quantitative morphometry and mean linear intercept

The trachea and lungs were removed together and inflated with 10% buffered neutral formalin (VWR Scientific) to 25 cm water pressure. Parasagitally sectioned tissue was embedded in paraffin, and 5-μm-thick sections were stained with haematoxylin and eosin. Slide images were digitally captured using CAST-grid software version 2.00.1 (Olympus) with a line-counting tool at ×20 magnification with meander sampling beginning at a randomly selected point, sampling all of the tissue in an unbiased fashion. The mean linear intercept (defined as the linear sum of the lengths, in μm, of all lines in all frames counted divided by the number of intercepts (defined as an alveolar septa intersecting with a counting line)) was calculated according to an adaptation of the method of ref. 27. A minimum of 12 fields, 200 intercepts and 300 points for each animal were measured, sampling all lobes.

Bronchoalveolar lavage and cytospin analysis

Animals were terminally anaesthetized with Methoxyfluorane (Metofane), their tracheae cannulated, and lungs washed with five sequential aliquots of 0.8 ml PBS at room temperature. Aliquots were pooled, centrifuged, and the cell pellets re-suspended in red-blood-cell lysis buffer (Sigma). Cells were counted using a haemacytometer and cell concentrates were stained with Diff-Quick (Dade Diagnostics). Cell subsets were counted under ×40 objective magnification (300 cells per slide).

Statistical analysis

All data are reported as mean ± s.e.m. All between-group comparisons of mean linear intercepts were made using the Kruskal–Wallis test, and subsequently the Mann–Whitney *U*-test, using the Bonferroni correction for multiple comparisons. All between-group comparisons of normalized C_T differences, TGF-β protein levels and cell counts were made using analysis of variance followed by Bonferroni-corrected unpaired *t*-tests. Only experimentally relevant comparisons are reported. Comparisons with an α-value of $P < 0.05$ were assigned as significantly different. Statistical analyses were conducted using SYSTAT version 9.0 software (SPSS Science).

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Free fatty acids regulate insulin secretion from pancreatic β cells through GPR40

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Diabetes, a disease in which carbohydrate and lipid metabolism are regulated improperly by insulin, is a serious worldwide health issue[1,2]. Insulin is secreted from pancreatic β cells in response to elevated plasma glucose, with various factors modifying its secretion[3]. Free fatty acids (FFAs) provide an important energy source as nutrients, and they also act as signalling molecules in various cellular processes, including insulin secretion[4,5]. Although FFAs are thought to promote insulin secretion in an acute phase, this mechanism is not clearly understood[6]. Here we show that a G-protein-coupled receptor, GPR40, which is abundantly expressed in the pancreas, functions as a receptor for long-chain FFAs. Furthermore, we show that long-chain FFAs amplify glucose-stimulated insulin secretion from pancreatic β cells by activating GPR40. Our results indicate that GPR40 agonists and/or antagonists show potential for the development of new anti-diabetic drugs.

GPR40 is an orphan (that is, its ligands are unidentified) G-protein-coupled receptor (GPCR) isolated originally from a human genomic DNA fragment[7]. We isolated complementary DNAs from human, monkey, mouse, rat and hamster (DDBJ/EMBL/GenBank accession numbers AF024687, AB095743, AB095744, AB095745 and AB095746, respectively) and found that their amino acid sequences (300 amino acids) were highly conserved. Analyses for the distribution of GPR40 messenger RNA in rat tissue by quantitative polymerase chain reaction with reverse transcription (RT-PCR)[8] demonstrated its highest expression level in the pancreas (Fig. 1a). In addition, we found that expression of GPR40 mRNA

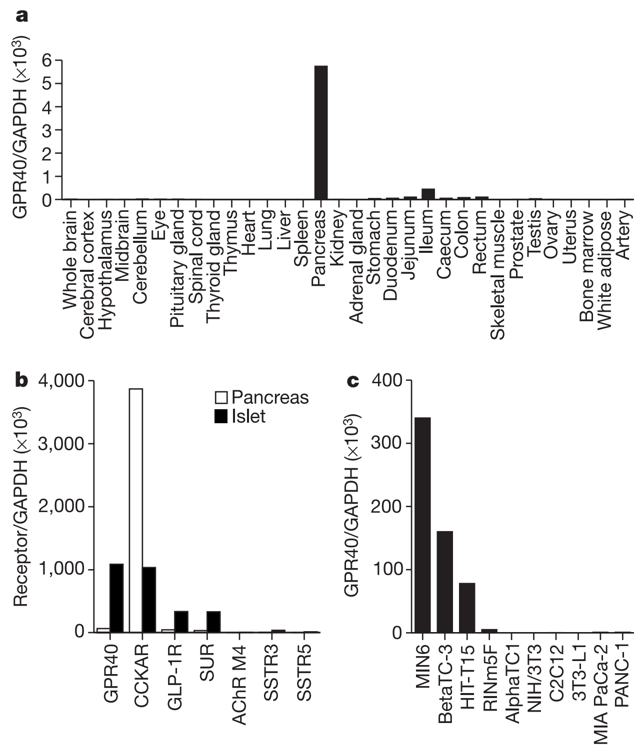

Figure 1 GPR40 mRNA is expressed abundantly in pancreatic β cells. Data represent the ratios of GPR40 and other mRNAs to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. **a**, Distribution of GPR40 mRNA in rat tissues. **b**, Specific expression of GPR40 mRNA in rat pancreatic islets. CCKAR, type A cholecystokinin receptor; SSTR, somatostatin receptor; SUR, sulphonylurea receptor. **c**, Expression of GPR40 mRNA in pancreatic β-cell lines. MIN6, mouse pancreatic β cells; betaTC-3, mouse pancreatic β cells; HIT-T15, Syrian golden hamster pancreatic β cells; RINm5F, rat pancreatic β cells; alphaTC1, mouse pancreatic α cells; NIH/3T3, mouse embryonic cells; C2C12, mouse myoblasts; 3T3-L1, mouse embryonic fibroblasts; MIA PaCa-2, human pancreatic carcinoma cells; PANC-1, human pancreatic carcinoma cells. **d**, Localization of GPR40 mRNA in rat islet cells. IHC, immunohistochemistry; ISH, *in situ* hybridization. Scale bar, 50 μm.

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