Tensin1 expression and function in chronic obstructive pulmonary disease

Panayiota Stylianou1, Katherine Clark2, Bibek Gooptu1,3, Dawn Smallwood4, Christopher E. Brightling1, Yassine Amrani1, Katy M. Roach1 & Peter Bradding1*

Chronic obstructive pulmonary disease (COPD) constitutes a major cause of morbidity and mortality. Genome wide association studies have shown significant associations between airflow obstruction or COPD with a non-synonymous SNP in the TNS1 gene, which encodes tensin1. However, the expression, cellular distribution and function of tensin1 in human airway tissue and cells are unknown. We therefore examined these characteristics in tissue and cells from controls and people with COPD or asthma. Airway tissue was immunostained for tensin1. Tensin1 expression in cultured human airway smooth muscle cells (HASMCs) was evaluated using qRT-PCR, western blotting and immunofluorescent staining. siRNAs were used to downregulate tensin1 expression. Tensin1 expression was increased in the airway smooth muscle and lamina propria in COPD tissue, but not asthma, when compared to controls. Tensin1 was expressed in HASMCs and upregulated by TGFβ1. TGFβ1 and fibronectin increased the localisation of tensin1 to fibrillar adhesions. Tensin1 and α-smooth muscle actin (α-SMA) were strongly co-localised, and tensin1 depletion in HASMCs attenuated both α-SMA expression and contraction of collagen gels. In summary, tensin1 expression is increased in COPD airways, and may promote airway obstruction by enhancing the expression of contractile proteins and their localisation to stress fibres in HASMCs.

Chronic obstructive pulmonary disease (COPD) constitutes a major cause of morbidity and mortality with more than 200 million people affected worldwide1,2. It is characterised by irreversible airway narrowing which limits airflow, resulting in breathlessness and in severe cases, respiratory failure1. The most common cause is tobacco smoking3. COPD is characterised by airway remodeling that involves thickening of the airway wall and the airway smooth muscle (ASM) layer, hypersecretion of mucous and metaplasia of epithelial cells4,5. Airway wall thickness results from an increase in each of the airway wall compartments, including the epithelium, ASM and lamina propria6. Under normal conditions ASM has a critical role in regulating the tone of the airways and distribution of airflow by maintaining a balance between contractile and dilatory processes7. In COPD, changes occur in airway smooth muscle, notably in the small airways8,9, and ASM cells mediate deposition of collagens, fibronectin and laminin in extracellular matrix10,11.

Recent evidence suggests that gene-environment interactions constitute a major factor for the development of COPD12. Genome wide association studies (GWAS) have shown a significant association between a non-synonymous single nucleotide polymorphism (SNP) in the coding region of the tensin1 gene (TNS1) and COPD13, as well as airflow obstruction14 and early childhood wheeze15. Tensin1 is a 220kDa cytoplasmic phosphoprotein, which localises to integrin-mediated focal and fibrillar adhesions16. These adhesions provide a bi-directional link between the extracellular matrix and the cytoskeleton17. A phosphotyrosine domain in the C-terminal region of tensin1 facilitates an interaction with the α5β1 integrin, which itself binds to the extracellular matrix protein fibronectin. Furthermore, an actin-binding domain is located on the N-terminal of tensin1 enabling interaction with the actin filaments, and in particular the F-actin protein18,19. The interactions of tensin1 with these intracellular and extracellular molecules contribute to its involvement in cell signaling and function. However, the pattern of expression and (patho)physiological role of tensin1 in human airways is not known.

1Department of Respiratory Sciences, University of Leicester, UK, Institute of Lung Health and NIHR Leicester BRC-Respiratory, Leicester, UK. 2Department of Molecular and Cell Biology, University of Leicester, Leicester, UK. 3Leicester Institute of Structural and Chemical Biology, University of Leicester, Leicester, UK. *Faculty of Health and Life Sciences, De Montfort University, Leicester, UK. *email: pb46@le.ac.uk
We have therefore characterised the localisation of tensin1 and its cellular function in the context of COPD. We have further investigated whether the TNS1 SNP (2q35, rs257114 C > T, resulting in R1197W amino acid substitution) could play a pathogenic role in COPD.

Results

Clinical characteristics. The clinical characteristics of the controls, COPD and asthma subjects used for immunohistochemistry and cell culture studies are summarised in Tables 1–3.

Tensin1 immunoreactivity is increased in both the airway smooth muscle and lamina propria in patients with COPD but not asthma. To identify the expression and cellular localisation of tensin1 in human airways in vivo, tissue was immunostained for tensin1. Immunostaining was performed on non-COPD control (n = 11) and COPD (n = 13) airway tissue from lung resections, and healthy (n = 9) and asthmatic (n = 10) bronchial biopsies. Positive tensin1 staining was observed in the apical epithelium, ASM bundles and lamina propria (Fig. 1A,B). Quantification of tensin1 immunostaining was performed using a thresholding technique as previously described20. A significant increase in tensin1 immunostaining was detected in both the lamina propria and ASM in COPD compared to non-COPD control tissue (Fig. 1C). There was no significant difference between COPD and control airway epithelium (Fig. 1C).

Since smoking is the most common causative factor for COPD and the COPD group had a greater smoking history than non-COPD controls, we examined the correlation of tensin1 and tobacco smoking. The extent of tensin1 immunostaining was not affected by the smoking status of non-COPD control individuals (Fig. 1C), and there was no correlation between tensin1 immunostaining and smoking in the COPD subjects in any compartment (p > 0.05 for all comparisons). The COPD patients were also a little older than the control group (Table 1) but tensin1 immunostaining was not correlated with age in any tissue compartment in either non-COPD controls or COPD subjects (p > 0.05 for all comparisons).

Tensin1 immunostaining was not increased in any airway compartment in asthma (Fig. 1C). Tensin1 immunostaining correlated positively with the area of the smooth muscle bundles in COPD subjects (R = 0.6286, p = 0.0286) (Fig. 1D). Isotype control staining was negative. Specificity of the antibody for tensin1 was confirmed by immunoprecipitation (Figure S1A in the Supplementary Information).

Human airway smooth muscle cells express tensin1 mRNA, which is up-regulated by TGFβ1. As tensin1 was most strongly increased in the ASM in COPD subjects when compared to controls, its role in cultured HASMCs was studied further. We studied expression at the mRNA and protein level in HASMCs derived from healthy, asthmatic and COPD airway tissue collected at bronchoscopy, basally and following stimulation with TGFβ1 or fibronectin.

Tensin1 mRNA was expressed at high levels in HASMCs, with no differences detected between patients with COPD or asthma and healthy controls (Fig. 2A). Tensin1 mRNA expression by HASMCs was significantly upregulated by TGFβ1 stimulation and to a similar extent in both healthy (n = 3) and COPD (n = 3) subjects (Fig. 2B). Overall, in vitro, tensin1 protein expression was similar between HASMCs derived from healthy and COPD tissue (Fig. 2C) under basal conditions. As tensin1 facilitates integrin binding to fibronectin, we examined whether culturing HASMCs on fibronectin altered tensin1 distribution, but there was no significant change (Fig. 2D). However, stimulating HASMCs cultured on plastic or fibronectin with TGFβ1 significantly increased the length of fibrillar adhesions defined by tensin1 immunostaining in HASMCs derived from both healthy and COPD donors (Fig. 2D). In contrast to the transcriptomic data, and findings in tissue ex vivo, these stimuli did not significantly increase overall tensin1 protein levels in the HASMCs, assessed by immunostaining and greyscale analysis (Supplementary Fig. S1B).

siRNA transfection achieved around 92% silencing of tensin1 mRNA after 48 hours, as quantified by the 2−ΔΔCt method (n = 3) (Supplementary Fig. S1C). Silencing of tensin1 did not have any effect on the survival or proliferation of HASMCs in culture (n = 4 healthy and n = 4 COPD) assessed by the MTS assay (Supplementary Fig. S2A). Tensin1 silencing was associated with a small but significant reduction in TGFβ1 secretion in COPD-derived HASMCs, but this was not evident in supernatants when HASMCs derived from healthy subjects were used (Fig. 2E).

Taken together, these data indicate that the apparent increase in cellular tensin1 protein signal in COPD ASM in vivo may be driven by interactions with the abnormal matrix in disease rather than inherent changes within the cells in the disease state. TGFβ1 and fibronectin stimulation does not appear to account for this, but they do alter the cellular distribution of the protein to associate more with fibrillar adhesions of greater length in HASMCs from both healthy and COPD tissue.

Tensin1 stimulates αSMA expression and interacts with it in HASMCs, and mediates contraction. We next assessed whether the increased length of fibrillar adhesions following TGFβ1 stimulation related to increased interactions with, and/or increased expression of, αSMA in HASMCs derived from healthy controls (n = 4) and COPD individuals (n = 3). Colocalisation and close physical association of tensin1 and αSMA was demonstrated by co-immunoprecipitation (co-IP, Fig. 3A) and confocal microscopy (Fig. 3B). In all conditions, strong co-localisation of tensin1 and αSMA immunofluorescence was observed (Fig. 3B). Quantification of the degree of colocalisation by overlap analysis (Mander’s overlap coefficient = 0.8 and Pearson’s correlation = 0.6) revealed strong spatial colocalisation between the two proteins (Fig. 3C).

Tensin1 silencing significantly reduced αSMA expression at the mRNA (Fig. 4A) and protein levels (Figs. 4B,C, S2B) in both non-stimulated and TGFβ1-stimulated HASMCs derived from healthy and COPD lung tissue. There were no apparent differences relating to the health/disease status of the original lung tissue.
To assess the mechanistic significance of tensin1-αSMA interactions, collagen gel contraction assays were performed to study HASMC contraction. A representative example of these experiments can be seen in Fig. S3 (Supplementary Information). These studies indicated that tensin1 depletion greatly reduced HASMC contraction, with no difference between HASMCs derived from healthy (n = 4) and COPD (n = 4) subjects (Fig. 5A). Collagen gel contraction increased significantly following stimulation with the contractile agonist bradykinin in controls (untreated cells, transfection reagent alone, siCON control), but not in cells subjected to tensin1 knockdown (Figs. 5B and S4 [Supplementary Figure]). Taken together these data indicate that tensin1 interactions with αSMA

### Table 1. Clinical characteristics of non-COPD controls and COPD subjects used for immunohistochemistry.

|                      | Lung resections | Statistical analysis |
|----------------------|-----------------|----------------------|
|                      | non-COPD Lung   | COPD Lung             |
|                      | resections (n = 11) | resections (n = 13)   |
| **Age (years)**      | 66.9 ± 8.6      | 74.3 ± 9.4            | p = 0.0284 |
| **Gender (M/F)**     | 8/3             | 9/4                  | NS        |
| **FEV1 (% predicted)** | 83.3 ± 9.5      | 68.2 ± 10.9           | p = 0.0004 |
| **FEV1/FVC ratio (%)** | 79.8 ± 6.7      | 60.0 ± 8.8            | p < 0.0001 |
| **Smoking (pack years)** | 15.5 ± 15.3    | 34.7 ± 16.6           | p = 0.0024 |
| **Inhaled corticosteroid dose (µg)** | 0              | 0                    | NS        |
| **Severity**         | NA              | GOLD 1 (2) & GOLD 2 (11) | NA        |

Table 2. Clinical characteristics of healthy controls and asthma subjects used for immunohistochemistry.

|                      | Bronchial biopsies | Statistical analysis |
|----------------------|---------------------|----------------------|
|                      | Healthy Biopsies    | Asthmatic Biopsies   |
|                      | (n = 9)             | (n = 10)             |
| **Age (years)**      | 31.6 ± 15.9         | 37.7 ± 12.4          | NS        |
| **Gender (M/F)**     | 5/4                 | 4/6                  | NS        |
| **FEV1 (% predicted)** | 102.2 ± 11.6       | 79.4 ± 13.2          | p = 0.0117 |
| **FEV1/FVC ratio (%)** | 81.4 ± 7.4         | 66.9 ± 8.4           | p = 0.0180 |
| **Smoking (pack years)** | 0.4 ± 1.2         | 0.9 ± 2.3            | NS        |
| **PC20 methacholine (mg/ml), geometric mean (95% CI)** | >16                       | 6.8 (2.9–10.5)       | p = 0.0006 |
| **Reversibility to β-agonist (%)** | 1.6 ± 4.9 | 19.7 ± 25.1       | p = 0.0465 |
| **Serum IgE (KU/L), geometric mean (95% CI)** | 43.8 (16.2–71.4) | 172.1 (97.5–246.6) | p = 0.0054 |
| **Inhaled corticosteroid dose (µg)** | 0                        | 192.9 ± 148.4       | p = 0.0008 |
| **Severity**         | NA                  | Mild (3), Moderate (2) | NA        |
| **Age of onset**     | NA                  | 20.3 ± 14.3          | NA        |

Table 3. Clinical characteristics of healthy controls, COPD and asthmatic subjects used for cell culture studies.

|                      | Healthy smooth muscle cells | Asthma | Statistical analysis |
|----------------------|-----------------------------|--------|----------------------|
| **Age (years)**      | 42.5 ± 4.7                  | 69.7 ± 1.5 | 44.5 ± 5.2          | p = 0.0001 |
| **Gender (M/F)**     | 5/7                          | 7/3     | 6/3                  | NS        |
| **FEV1 (% predicted)** | 95.6 ± 3.2                  | 54.4 ± 5.4 | 96.5 ± 4.8          | p < 0.0001 |
| **FEV1/FVC ratio (%)** | 80.2 ± 1.5                 | 53.7 ± 3.1 | 79.1 ± 2.4          | p < 0.0001 |
| **Pack Years (y)**   | 1.7 ± 0.9                   | 41.7 ± 4.5 | 6.1 ± 5.4           | p < 0.0001 |
| **PC20 methacholine (mg/ml), geometric mean (95% CI)** | 9.3 (3.9–14.6) | ND | 4.3 (1.3–7.3) | p = 0.0463 |
| **Reversibility to β-agonist (%)** | NA | ND | –1.3 ± 2.4 | NA |
| **Serum IgE (KU/L), geometric mean (95% CI)** | 4.9 (4.2–5.5) | ND | 498 (396.3–599.6) | p < 0.0001 |
| **Inhaled corticosteroid dose (µg)** | 0                           | 500     | 633.3 ± 190.5       | p = 0.0012 |
| **Severity**         | NA                           | GOLD 1 – GOLD 4 | GINA 1 – GINA 5 | NA |
| **Age of Onset**     | NA                           | 57 ± 2.3 | 31.5 ± 3.6          | p = 0.0005 |
Figure 1. Tensin1 immunostaining in non-disease controls and COPD lung resection and healthy and asthmatic bronchial biopsies. (A) Examples of tensin1 and isotype control immunostaining in COPD (n = 13) and non-COPD lung resections (n = 11) (EP: epithelium; ASM: airway smooth muscle; LP: lamina propria). (B) Examples of tensin1 and isotype control immunostaining in bronchial biopsies from patients with asthma (n = 10) and healthy controls (n = 9) (EP: epithelium; ASM: airway smooth muscle; LP: lamina propria, RBM: reticular basement membrane). (C) The extent of tensin1 immunostaining in airway epithelium, airway smooth muscle and lamina propria analysed using threshold measurements. Increased tensin1 immunostaining was observed in the airway smooth muscle (**p = 0.007) and lamina propria (*p = 0.012) in COPD subjects when compared to non-COPD controls (Mann-Whitney unpaired non-parametric test). Tensin1 immunostaining in lung resections was not affected by the smoking status of non-COPD control individuals. (D) Tensin1 immunostaining in COPD subjects was positively correlated with the smooth muscle bundle area, calculated by Pearson’s correlation (R = 0.6286, p = 0.0286).
Figure 2. Tensin1 mRNA and protein expression in HASMCs in health and disease and the effect of TGF\(\beta\)1 and fibronectin. (A) Quantitative Reverse Transcription - PCR (qRT-PCR) was used to quantify tensin1 mRNA expression in HASMCs isolated from healthy donors and patients with COPD or asthma using the 2^(-\(\Delta\Delta C_t\)) method (n = 7 of each, Mean ± SEM). (B) Tensin1 mRNA expression in human airway smooth muscle cells (HASMCs) was increased following TGF\(\beta\)1-dependent stimulation in both COPD (n = 3) (*p = 0.0418) and healthy (n = 3) (**)p = 0.0042) donors (Tukey's multiple comparison test as part of one-way ANOVA, one-way ANOVA test: **p = 0.0019). (C) HASMC tensin1 immunofluorescent staining was measured by grey scale intensity in n = 6 healthy and n = 6 COPD subjects. No difference was observed in the grey scale intensity of tensin1 immunostaining in the two phenotypes (Mean ± SEM). (D) Cells were stimulated with TGF\(\beta\)1 and/or fibronectin. Stimulation with TGF\(\beta\)1 and fibronectin did not significantly increase the grey scale intensity of tensin1. However, the length of fibrillar adhesions was significantly increased in HASMCs stimulated with TGF\(\beta\)1 alone (*p = 0.0386) and TGF\(\beta\)1 + fibronectin (***p = 0.0002) (Dunnett's multiple comparison test as
part of one-way ANOVA, one-way ANOVA test: ***p = 0.0004). Data shown is pooled COPD and healthy donors which did not differ (n = 3 of each, Mean ± SEM). (E) Cells were transfected with siRNA directed against tensin1 and supernatants were assessed for TGFβ31 expression using ELISA in both COPD (n = 4) and healthy donors (n = 4) (Mean ± SEM). Tensin1 depleted HASMC supernatants derived from COPD subjects had a small but significant reduction of TGFβ31 secretion when compared to controls (*p = 0.0477) (paired t-test).

increase oSMA expression, are increased in association with increased fibrillar adhesion length in HASMCs following TGFβ31 stimulation, and that these responses enhance contraction, irrespective of disease status.

Expression of the R1197 and R1197W tensin1 variants in healthy, asthmatic and COPD HASMCs. Having characterised tensin1 expression in COPD airway tissue and TGFβ31-stimulated HASMCs, and confirmed its interaction with oSMA was a key mediator of HASMC contraction, we then assessed the pathogenic potential of the R1197W mutation. RFLP analysis was used to genotype subjects for the presence of the polymorphism as its presence results in disruption of the formation of a restriction enzyme site (Fig. 6A). pEGFP constructs containing the full length of TNS1 cDNA with the C or T alleles were used as controls to validate the method (Fig. 6B). In Fig. 6C, the PCR-RFLP detection assay can be seen demonstrating the presence of the C or T alleles in HASMCs from healthy or COPD subjects. RFLP analysis revealed the predominant presence of the C/C homozygous genotype (which generates R1197) in healthy controls (11 of the 14 tested), and the heterozygote C/T or homozygous T/T (R1197W) genotype in COPD donors (12 of the 12 tested) (p < 0.0001) (Table 4). This indicates a strong link between the R1197W amino acid change and COPD, consistent with the GWAS signal (p = 1.11 × 10−12) [14]. In patients with asthma ranging from mild to severe, 8 of 9 samples tested were the heterozygote C/T or homozygous T/T genotype, similar to COPD (Table 4). Taken together, these data suggest the mutation is a risk factor for obstructive airway disease as a whole, but the lack of increased tensin1 protein expression in asthmatic airways suggests different handling of tensin1 in these two conditions despite a frequently shared genotype.

qRT-PCR demonstrated similar tensin1 mRNA expression levels between the different TNS1 genotypes in HASMCs (Fig. 6D).

HASMCs were transfected with pEGFP constructs containing full length TNS1 cDNA with either the C or T allele and both constructs revealed similar transfection efficiency. Overexpression of tensin1 using pEGFP constructs containing the full length of TNS1 cDNA with the C or T alleles also did not have any effect on survival or proliferation (Supplementary Fig. S5B). Since the TNS1 SNP does not appear to affect transcription or translation of the TNS1 gene product tensin1, we further hypothesised that the mutation's effects were likely to be mediated by differential turnover. Tensins are known to be highly susceptible to proteolysis23. Consistent with this, the mutation changes the predicted proteolytic sensitivities of tensin1 as assessed by 'PeptideCutter' prediction tool (Fig. 6D). Moreover, western blot analysis of HASMC lysates derived from healthy and COPD patient tissue provides some support for this hypothesis with a differential cleavage profile observed between the two (Fig. 6E). Much of the tensin1 protein from COPD-derived HASMCs appeared to be proteolytically modified, whilst that from HASMCs derived from healthy tissue appeared to be present solely in its full-length, 220kDa form (Fig. 6E).

Discussion

Modern genetic approaches such as GWAS can provide powerful insight in understanding disease by identifying novel pathogenic mutations and highlighting important pathways that may mediate the pathological response. This study follows on from the GWAS findings that a non-synonymous SNP in the coding region of TNS1 is associated with COPD risk. We therefore assessed the roles and interactions of tensin1 in healthy, asthmatic and COPD airways. We have demonstrated for the first time that tensin1 is expressed in the apical airway epithelium, ASM bundles and airway lamina propria in both non-COPD control and COPD lung resections, and healthy and asthmatic endobronchial biopsies. A significant increase in tensin1 immunostaining was detected in the ASM and lamina propria in COPD subjects when compared to non-COPD controls, but no differences were evident between asthma and health. Tensin1 mRNA and protein expression were present in cultured HASMCs. Of note, tensin1 co-localised and interacted physically with oSMA in actin stress fibres, and influenced oSMA expression, as tensin1 silencing led to downregulation of oSMA protein. As a result, tensin1 appears to make a critical contribution to the contractile properties of HASMCs.

Tensin1 protein expression was upregulated in the ASM bundles in COPD compared to non-COPD controls, but not in cultured HASMCs, suggesting that the in vivo findings are regulated by local factors that are lost in cell culture. TGFβ31 is a candidate molecule regulating this in vivo as it is upregulated in COPD airways24, and we found that tensin1 mRNA expression was markedly upregulated by TGFβ31 in HASMCs in vitro. This is in keeping with the effects of TGFβ31 on tensin1 expression in parenchymal lung fibroblasts obtained from patients with idiopathic pulmonary fibrosis25. However, TGFβ31 did not have a detectable effect on total tensin1 expression in HASMCs, but instead altered its intracellular distribution promoting the formation of long tensin1-containing fibrillar adhesions. To account for the increased overall levels of tensin1 protein evident in COPD airways relative to asthma and healthy tissue biopsies, factors in addition to TGFβ31 are likely to contribute. These factors would be less critical in asthmatic than COPD patients. Candidate factors would include other pro-inflammatory mediators present in COPD, the airway response to cigarette smoke and associated epigenetic changes, or the response to impaired airway stretch present in COPD. These various hypotheses will need testing in future work, but may explain why tensin1 expression was not increased in asthmatic airways.

Tensins are known to play a key role in remodeling the extracellular matrix through their interactions with integrins26,27, and so the high expression in patients with COPD may be particularly relevant to the fixed airflow...
obstruction and small airway fibrosis present in COPD. This would be in keeping with the pro-fibrotic role of tensin1 identified in patients with idiopathic pulmonary fibrosis. In addition, an actin-binding domain is located on the N-terminal of tensin1 enabling interaction with actin filaments of the cytoskeleton, specifically within fibrillar adhesions that cause cellular stiffening in IPF. The co-localisation of tensin1 with αSMA within stress fibres, and the marked downregulation of αSMA following tensin1 silencing, suggest feedback between tensin1 gene transcription and/or protein expression and factors regulating αSMA gene regulation. The fact that there were differences in the demographics of the groups providing HASMCs for culture, but no difference in the way their HASMCs behave in culture, indicates that the role of tensin1 in ASM contraction is a fundamental role irrespective of disease status and patient characteristics. Our data do not permit relative quantitative comparisons of the direct contribution tensin1 makes to the contractile properties of HASMCs versus indirect effects through regulation of αSMA expression. However they indicate tensin1 is associated with αSMA transcription and, potentially, retention of the protein product within the cell.

Having investigated the expression and function of tensin1 in COPD, we next examined the association and functional effects of the COPD-associated SNP in the TNS1 gene, identified in GWAS. The polymorphism is a non synonymous SNP (rs2571445) in the TNS1 gene and the base pair change encoded by this SNP leads to the amino acid substitution R1197W. The SNP is present in approximately 40% of the population, and its presence is associated with reduced lung function in GWAS. It was therefore remarkable, that in spite of the relatively small number of subjects studied here, there was very strong segregation between homozygotes for C/C (which generates R1197) who were largely healthy, and patients with COPD or asthma who were predominantly heterozygous C/T or occasionally homozygous T/T (R1197W). These data lend support to the GWAS findings. They suggest that R1197 in tensin1 is potentially protective against the development of both COPD and asthma, and/or that R1197W is a factor promoting the development of COPD and asthma. The exact role that R1197W plays in COPD susceptibility remains unclear as we were unable to find any differences in gene expression or survival between cells expressing R1197W and those homozygous for R1197.
Tensin1 protein expression was increased in the ASM bundles in COPD patients, but not in asthma, indicating that its increased expression is associated with marked fixed airflow obstruction. These data suggest that tissue factors regulating tensin1 turnover may be important. The R1197W mutation is predicted to alter the protease sensitivity of the protein, and *ex vivo* samples supported differential tensin1 cleavage profiles in HASMCs derived from healthy and COPD tissue. We therefore hypothesize that the R1197W mutation and COPD-specific cofactors together modulate tensin1 degradation and thereby affect αSMA expression, and ASM mass (Fig. 6F, schema). This would rationalise the different observations in tissue from asthmatic patients.

In conclusion, we have demonstrated immunoreactivity for tensin1 in the ASM, lamina propria and airway epithelium in human lung tissue. Tensin1 expression was increased in the ASM and lamina propria in COPD donors when compared to non-disease controls, but was not increased in asthma. Tensin1 was enriched in fibrillar adhesions in HASMCs upon stimulation with TGFβ1 and fibronectin. This is likely of great importance as it allows tensin1 to interact with other proteins, leading to activation of signaling pathways and remodeling of the extracellular matrix. Furthermore tensin1 plays a critical role in regulating HASMC αSMA expression and contraction suggesting it may play an important role in both COPD and asthma pathophysiology.
Methods

Subjects. COPD and control tissue for immunohistochemistry. To study COPD airway tissue by immunohistochemistry, we used 2nd-5th generation airway tissue collected at the time of lung resection for lung cancer. The tissue used was well demarcated from the tumour and not affected by the tumour process. The COPD group were all ex or current smokers with spirometric evidence of airflow obstruction and met Global Obstructive Lung Disease (GOLD) criteria for COPD. Non-COPD control tissue was used from patients who did not have evidence of COPD (see Table 1).

Asthma and control tissue for immunohistochemistry. People with asthma and healthy controls underwent fibreoptic bronchoscopy as described previously. Subjects with a history of asthma had a < 10 pack year smoking history and met a diagnosis for asthma as described previously (see Table 2).

Asthma and COPD tissue for primary human airway smooth muscle culture. For the study of primary human ASM cells (HASMCs) in culture, healthy control subjects and subjects with COPD or asthma underwent fibreoptic bronchoscopy as described previously (see Table 3), and HASMCs cultured as described below.

All research participants gave written informed consent, and the collection of tissue was approved by the National Research Ethics Service (reference numbers: 07/MRE08/42, 04/Q2502/74, 08/H0406/189). All methods were performed in accordance with the relevant guidelines and regulations.
Figure 6. The distribution of C and T alleles generating amino acid 1997 of tensin1 and schematic illustrating potential role of tensin1 in disease. (A) A diagram illustrating the base substitution at the site of the variant nucleotide base, the Eag1 restriction site formation in the presence of C versus T, and the different tensin1 genotypes evident on digested PCR products. (B) pEGFP-C1 constructs with the tensin1 variants were used as controls to confirm validity of the RFLP technique. (C) An agarose gel image illustrating the different tensin1 phenotypes in HAMSCs. Lane 1 = ladder. Lane 3 = Heterozygous, Lane 5, 7 and 9 = homozygous (-CC-) (encodes R1197 tensin1) and Lane 11 = homozygous (-TT-) (encodes R1197W tensin1). (D) Tensin1 mRNA expression was assessed between the different genotypes that segregated with health and disease. (E) A western blotting analysis illustrating differential cleavage of tensin1 in COPD subjects when compared to healthy controls (left). 'PeptideCutter' prediction tool showing differential cleavage at the site of mutation (right). (F) A schematic illustrating our hypothesis that the R1197W mutation and COPD-specific co-factors together modulate tensin1 degradation and thereby affect αSMA expression, and ASM mass.
indirectly labelled with FITC.

α-smooth muscle actin (SMA) (FITC directly conjugated) and myosin using the Dako EnVision FLEX (Olympus). The thresholding technique was used to quantify tensin1 immunostaining based on the hue saturation and intensity (HSI) value as described previously. Tensin1 antibodies were validated further using immunoprecipitation and siRNA downregulation (see below).

Images of complete tissue sections were collected and tensin1 immunostaining was quantified by a blinded observer in the airway epithelium, lamina propria and airway smooth muscle bundle area measurements.

**Quantitative real time PCR.** HASMC RNAs were isolated using the RNeasy Plus Kit (Qiagen, Manchester, UK) according to manufacturer’s instructions. Detection of TNS1 and αSMA mRNA was performed using the Fast SYBR Green Master Mix, alongside primers targeting the internal normalised gene β-actin. PCR products were run on an agarose gel to confirm the product amplified was the correct size, and were also sequenced. TNS1 and αSMA mRNA expression was quantified using the ΔΔCT method. Full experimental details can be found in the Supplementary Information.

Cells were stimulated with 10 ng/ml transforming growth factor-β1 (TGF-β1) (R&D Systems), cells were grown to confluence and then serum-starved for 24 hours prior to stimulation for a further 24 hours.

**Western blotting.** Tensin1 expression in primary HASMCs was analysed by western blotting as described previously. HASMCs were disrupted in lysis buffer and soluble protein from equivalent number of cells was resolved by 7.5% SDS-page and then transferred to a polyvinylidene (PVDF) membrane. The membrane was blocked using 5% Milk + 0.1% TBS Tween20 and incubated with antibodies to Tensin1 (SAB4200283, 1 μg/ml, Sigma-Aldrich), β-actin (sc-47778, 0.04 μg/ml Santa-Cruz) and αSMA (M0851, 1 μg/ml, Dako). Secondary antibodies conjugated to horseradish peroxidase (HRP) were then applied (goat anti-rabbit HRP [sc-2054, 0.08 μg/ml, Santa-Cruz] or goat anti-mouse HRP [P0447, 0.5 μg/ml, Dako]). Immunolabelled proteins were visualised by chemiluminescence using ECL substrate and the ImageQuant LA S 4000 (GE Healthcare Life Sciences, Little Chalfont, UK). Band intensity was quantified using ImageJ software (National Institutes of Health; http://rsbweb.nih.gov/ij/).

**Immunoprecipitation.** HASMCs were disrupted in lysis buffer and as a pre-clearing step, incubated with Protein A/G beads (sc-2003, Santa-Cruz) for 30 minutes at 4 °C. Separately, Protein A/G beads were incubated with either anti-tensin1 antibody (SAB4200283, 4 μg/ml, Sigma-Aldrich or sc-28542, 2 μg/ml, Santa-Cruz) or isotype control rabbit IgG (X0936, 4 μg/ml, Dako) for 30 minutes at 4 °C. Pre-cleared lysates were then incubated with bead-antibody complexes for 16 hours at 4 °C. Immunoprecipitated complexes were washed three times in lysis buffer and once in PBS, eluted in Laemmli buffer, and denatured for 5 minutes at 95 °C. Western blot analysis was then performed. Immunoprecipitates were probed with two anti-tensin1 antibodies (Sigma-Aldrich [SAB4200283, 1 μg/ml] and Santa-Cruz [sc-28542, 4 μg/ml]) to examine tensin1 antibody specificity.

To examine tensin1-αSMA interaction, Dynabeads Protein G (10003D, Thermo Fisher) were used. Again, HASMCs were disrupted in lysis buffer and as a pre-clearing step, incubated with Protein G Dynabeads for 1 hour at 4 °C. Pre-cleared lysates were then incubated with either anti-tensin1 antibody (SAB4200283, 4 μg/ml, Sigma-Aldrich or sc-28542) or isotype control rabbit IgG (X0936, 4 μg/ml, Dako) for 4 hours.

| Group  | Number | Genotype frequency, no. (%) |
|--------|--------|-----------------------------|
|        | CC     | CT or TT                    |
| Healthy| 14     | 11 (75%) 2 + 1 (25%)        |
| COPD   | 12     | 11 + 1 (100%)               |
| Asthma | 9      | 1 (11.1%) 6 + 2 (88.89%)    |

Table 4. Genotype profile of healthy and diseased subjects for tensin1. HASMCs from 14 healthy controls, 12 COPD subjects and 9 asthmatic subjects were genotyped for the two tensin1 variants. RFLP revealed the presence of the homozygous C/C (R1197) variant only in healthy controls, while COPD donors were predominantly T/C heterozygotes or occasionally T/T homozygotes (p < 0.0001). The distribution in asthma was similar to COPD (p = 0.0075).
at 4 °C. Lysate-antibody complexes were then incubated with Protein G Dynabeads for 16 hours at 4 °C. Immunoprecipitated complexes were washed three times in lysis buffer and once in PBS, eluted in Laemmli buffer at room temperature for 30 minutes. Western blot analysis was then performed. Immunoprecipitates were probed with anti-αSMA (M0851, 1 μg/ml, Dako).

**Immunofluorescence.** HASMCs were seeded into 8-well chamber slides, grown to confluence, and immunostained using mouse monoclonal αSMA (0.7 μg/ml, Dako) and isotype control FITC-conjugated mouse IgG2a (X0933, 10 μg/ml, Dako, Ely, UK), anti-Tensin1 antibody (4.5 μg/ml, Sigma–Aldrich) and isotype control rabbit IgG (4.5 μg/ml, Dako). Secondary antibodies labelled with AlexaFluor594 (A-11012, ThermoFisher) or FITC (FO382, Sigma–Aldrich) were applied and the cells were counterstained with 4′, 6-diamidino-2-phenylindole (DAP) (32670, Sigma–Aldrich). To study the effect of the extracellular matrix protein fibronectin and TGFβ1 on tensin1 expression, slides were coated with human recombinant fibronectin (F0895, Sigma–Aldrich) for 1 hour prior seeding and stimulated with TGFβ1 (10 ng/ml) for 24 hours. When 50% confluent, cells were stained as above and mounted with fluorescent mounting medium. Original images were captured on a confocal immunofluorescence microscope (Leica TCS SP5, UK) and staining was quantified using Cell F imaging software (Olympus UK Ltd.). Matched exposures were used for isotype controls. Quantification of the co-localisation of αSMA and tensin1 was performed using an Image J plugin, JACoP, in which Mander's overlap coefficient and Pearson’s correlation were calculated (National Institutes of Health; http://rsbweb.nih.gov/ij/). Quantification of fibrillar adhesion length was measured by Cell F imaging software (Olympus UK Ltd.).

**Human airway smooth muscle cell transfection with siRNA.** HASMCs were transfected with siRNA smartpools directed against TNS1 (M-009976-05, Dharmacon) and a non-targeting siRNA control (D-001206-14, Dharmacon). HASMCs were plated in culture media in the absence of antibiotics overnight. Lipofectamine 2000 (11668, Invitrogen) and siRNA mixture was added to cells. The cells were incubated with the complexes for 5 hours. After 5 hours, medium was replaced with antibiotic-free media for 48 hours. Tensin1 siRNA-transfected HASMCs derived from healthy and COPD individuals along with controls (untreated cells, transfection reagent alone, non-targeting siRNA control) were immunostained for both αSMA and tensin1 expression, slides were coated with human recombinant fibronectin (F0895, Sigma–Aldrich) and αSMA (0.7 μg/ml, Dako). Cells were also stimulated with TGFβ1 (10 ng/ml) to examine its role on αSMA expression after silencing tensin1. Full experimental details can be found in the Supplementary information.

**Survival/proliferation assay.** The MTS assay (G3582, Promega) was used to assess survival and proliferation of the cells after tensin1 silencing. 48 hours after transfection, cells were collected and plated into 96-well plates in culture media in the absence of antibiotics overnight. Cells were stimulated with TGFβ1 for 24 hours. Cells were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (32670, Sigma–Aldrich). To study the effect of the extracellular matrix protein fibronectin and TGFβ1 on tensin1 expression, slides were coated with human recombinant fibronectin (F0895, Sigma–Aldrich) for 1 hour prior seeding and stimulated with TGFβ1 (10 ng/ml) for 24 hours. When 50% confluent, cells were stained as above and mounted with fluorescent mounting medium. Original images were captured on a confocal immunofluorescence microscope (Leica TCS SP5, UK) and staining was quantified using Cell F imaging software (Olympus UK Ltd.). Matched exposures were used for isotype controls. Quantification of the co-localisation of αSMA and tensin1 was performed using an Image J plugin, JACoP, in which Mander's overlap coefficient and Pearson’s correlation were calculated (National Institutes of Health; http://rsbweb.nih.gov/ij/). Quantification of fibrillar adhesion length was measured by Cell F imaging software (Olympus UK Ltd.).

**TGFβ1 ELISA.** Tensin1 knockdown was performed as described above and supernatants were collected. A commercial ELISA was used to measure TGFβ1 in tensin1 knockdown sample supernatants according to the manufacturer’s protocol (DY240, R&D systems). Full experimental details can be found in the Supplementary Information.

**Collagen gel contraction assay.** The MTS assay (G3582, Promega) was used to assess survival and proliferation of the cells after tensin1 silencing. 48 hours after transfection, cells were collected and plated into 96-well plates in culture media in the absence of antibiotics overnight. Cells were stimulated with TGFβ1 for 24 hours. Cells were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (32670, Sigma–Aldrich). To study the effect of the extracellular matrix protein fibronectin and TGFβ1 on tensin1 expression, slides were coated with human recombinant fibronectin (F0895, Sigma–Aldrich) for 1 hour prior seeding and stimulated with TGFβ1 (10 ng/ml) to examine its role on αSMA expression after silencing tensin1. Full experimental details can be found in the Supplementary information.

**SNP genotyping.** PCR-restriction fragment length polymorphism (RFLP) analysis was used to genotype cultured cells for the presence of the polymorphism rs2571445. Full experimental details are provided in the Supplementary Information (can be found on the Supplementary information). The homozygote T/T genotype produced one band (278 bp), the homozygote C/C genotype produced two bands (214 and 64 bp) and the heterozygote C/T genotype displayed all three bands (278, 214 and 64 bp).

**Statistical analysis.** Data distribution was tested for normality using the Kolmogorov-Smirnov test. Data across groups were compared with either the ANOVA or Kruskall Wallis tests where appropriate. Between group comparisons were analysed using Dunnett’s/Tukey’s multiple comparison test, or Students unpaired/paired t test, or Mann Whitney U/Wilcoxon Signed Rank for paired and unpaired parametric and non-parametric data, respectively. Data were analysed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). P < 0.05 was taken as statistically significant.

**Ethical approval and consent to participate.** All subjects gave written informed consent, and the collection of tissue was approved by the National Research Ethics Service. Reference numbers: 07/MRE08/42, 04/Q2502/74 and 08/H0406/189.

**Data availability** All data generated or analysed during this study are included in this published article [and its Supplementary Information Files].
References

1. Yang, Q. et al. NO and EDHF pathways in pulmonary arteries and veins are impaired in COPD patients. *VascuL PharmacoL* 57, 113–118, https://doi.org/10.1016/j.vph.2012.05.004 (2012).
2. Shakhiev, R. & Crystal, R. G. Innate immunity and chronic obstructive pulmonary disease: a mini-review. *Gerontology* 59, 481–489, https://doi.org/10.1159/000354173 (2013).
3. Zhang, J., He, J., Xia, J., Chen, Z. & Chen, X. Delayed apoptosis by neutrophils from COPD patients is associated with altered Bak, Bel-x1, and Mcl-1 mRNA expression. *Diagn Pathol* 7, 65, https://doi.org/10.1186/1746-1596-7-65 (2012).
4. Decramer, M., Janssens, W. & Miravitlles, M. Chronic obstructive pulmonary disease. *Lancet* 379, 1341–1351, https://doi.org/10.1016/s0140-6736(15)60698-9 (2012).
5. Pauwels, R. A., Buist, A. S., Calverley, P. M., Jenkins, C. R. & Hurd, S. S. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 163, 1256–1276, https://doi.org/10.1164/ajrccm.163.5.2101309 (2001).
6. Hogg, J. C., Macklem, P. T. & Thrulbeck, W. M. Site and nature of airway obstruction in chronic obstructive lung disease. *N Engl J Med* 278, 1335–1360. https://doi.org/10.1056/NEJMoa023158 (2004).
7. James, A. & Carroll, N. Airway smooth muscle in health and disease; methods of measurement and relation to function. *Eur Respir J* 15, 782–789 (2000).
8. Bosken, C. H., Wiggs, B. R., Pare, P. D. & Hogg, J. C. Small airway dimensions in smokers with obstruction to airflow. *Am Rev Respir Dis* 142, 563–570, https://doi.org/10.1164/arccrimj.142.3.563 (1990).
9. Johnson, P. R. Role of human airway smooth muscle in altered extracellular matrix production in asthma. *Clin Exp Pharmacol Physiol* 28, 233–236 (2001).
10. Aoshiba, K. & Nagai, A. Differences in airway remodeling between asthma and chronic obstructive pulmonary disease. *Clin Rev Allergy Immunol* 27, 35–43, https://doi.org/10.1385/crai:27:1:335 (2004).
11. Agusti, A., Sobradillo, P. & Celli, B. Addressing the complexity of chronic obstructive pulmonary disease: from phenotypes and biomarkers to scale-free networks, systems biology, and P4 medicine. *Am J Respir Crit Care Med* 183, 1129–1137, https://doi.org/10.1164/ajrccm.201009-1414PP (2011).
12. Soler Artigas, M. et al. Effect of five genetic variants associated with lung function on the risk of chronic obstructive lung disease, and their joint effects on lung function. *Am J Respir Crit Care Med* 184, 786–795, https://doi.org/10.1164/ajrccm.201102-0192OC (2011).
13. Repapi, E. et al. Genome-wide association study identifies five loci associated with lung function. *Nat Genet* 42, 36–44, https://doi.org/10.1038/ng.501 (2010).
14. Kerkhof, M. et al. Transient early wheeze and lung function in early childhood associated with chronic obstructive pulmonary disease genes. *J Allergy Clin Immunol* 133(68–76), 661–64, https://doi.org/10.1016/j.jaci.2013.06.004 (2014).
15. McCleverty, C. J., Lin, D. C. & Liddington, R. C. Structure of the PTB domain of tensin1 and a model for its recruitment to fibrillar collagen. *Nature* 408, 1233–1239, https://doi.org/10.1038/35097070 (2001).
16. Geiger, B., Bershadsky, A., Pankov, R. & Yamada, K. M. Transmembrane crosstalk between the extracellular matrix–cytoskeleton crosstalk. *Nat Rev Mol Cell Biol* 2, 793–805, https://doi.org/10.1038/35099066 (2001).
17. Zamir, E. et al. Dynamics and segregation of cell–matrix adhesions in cultured fibroblasts. *Nat Cell Biol* 2, 191–196, https://doi.org/10.1038/35088607 (2000).
18. Lo S. H. Tensin, *Int J Biochem Cell Biol* 36, 31–34 (2004).
19. Shikotra, A. et al. Increased expression of immunoreactive thymic stromal lymphopoietin in patients with severe asthma. *J Allergy Clin Immunol* 129(104–111), e101–109, https://doi.org/10.1016/j.jaci.2011.08.031 (2012).
20. Sakota, Y., Ozawa, Y., Yamashita, H., Tanaka, H. & Inagaki, N. Collagen gel contraction assay using human bronchial smooth muscle cells and its application for evaluation of inhibitory effect of formoterol. *Biol Pharm Bull* 37, 1014–1020 (2014).
21. Lewis, R. J., Chachi, L., Newby, C., Amrani, Y. & Bradding, P. Bidirectional TGF-beta signaling mediates the effect of recombinant protein on lung development. *Am J Respir Crit Care Med* 183, 1103–1108, https://doi.org/10.1164/rccm.200409-1220OC (2005).
22. Chuang, J. Z., Lin, D. C. & Lin, S. Molecular cloning, expression, and mapping of the high affinity actin-capping domain of chicken cardiac tropomyosin. *J Biol Chem* 278, 1105–1109 (1995).
23. Takizawa, H. et al. Increased expression of transforming growth factor-beta1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD). *Am J Respir Crit Care Med* 163, 1476–1483, https://doi.org/10.1164/ajrccm.20136.69908135 (2001).
24. Bernau, K. P. et al. TNS1 is Essential for Myofibroblast Differentiation and Extracellular Matrix Formation. *Am J Respir Cell Mol Biol* https://doi.org/10.1165/rcmb.2016-0104OC (2016).
25. Strategy G. From the Global Strategy for the Diagnosis, Management and Prevention of COPD, Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2016. http://goldcopd.org/ (2016).
26. Brightling, C. E. et al. The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle. *Am J Respir Crit Care Med* 171, 1103–1108, https://doi.org/10.1164/rccm.200409-1220OC (2005).
27. Bradding, P. et al. Interleukin 4 is localized to and released by human mast cells. *J Exp Med* 176, 1381–1386 (1992).
28. Lvk, K. J. & Schmittingen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta CT(T)) Method. *Methods* 25, 402–408, https://doi.org/10.1016/meth.2001.1202 (2001).
29. Hollins, F. et al. Human airway smooth muscle promotes human lung mast cell survival, proliferation, and constitutive activation: cooperative roles for CADM1, stem cell factor, and IL-6. *J Immunol* 181, 2772–2780 (2008).
30. Phillips, P. A. (American Pancreatic Association, Pancrepedia: Exocrine Pancreas Knowledge Base, 2011).
31. Woodman, L. et al. Mast cells promote airway smooth muscle cell differentiation via autocrine up-regulation of TGF-beta 1. *J Immunol* 181, 5001–5007 (2008).

Acknowledgements
This work was supported by the National Institute for Health Research Leicester Respiratory Biomedical Research Unit (PhD studentship, PS). BG’s research is supported by the Medical Research Council (UK), and the British Lung Foundation. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. This work has been published as a PhD thesis by PS. “The Functional Relevance of Tensin1 in Chronic Obstructive Pulmonary Disease”, University of Leicester, 2017 (http://hdl.handle.net/2381/40290). We thank Dr. Kees Straatman for support with confocal microscopy.
Author contributions
Conception: P.B. and Y.A. Study design: P.B., B.G., K.C., C.E.B., Y.A. and K.M.R. Data acquisition: P.S. and D.S. Data analysis P.S., B.G., K.C., D.S., Y.A., K.M.R. and P.B. Writing of the manuscript: P.S., B.G., K.C. and P.B. All authors reviewed and approved the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-019-55405-2.

Correspondence and requests for materials should be addressed to P.B.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019