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Airway epithelium-derived transforming growth factor- β is a regulator of fibroblast proliferation in both fibrotic and normal subjectsK. E. Hostettler^{*†‡}, M. Roth^{*†}, J. K. Burgess^{†‡}, M. M. Gencay^{*}, F. Gambazzi[§], J. L. Black^{†‡}, M. Tamm^{*} and P. Borger^{*†}

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Clinical and
Experimental
Allergy

Summary

Background In the healthy lung, airway epithelial cells (AEC) regulate fibroblast proliferation through release of soluble factors, such as prostaglandins and proteins. Fibroproliferative diseases and airway remodelling may result from an inadequate generation of suppressive factors by AEC or the inability of fibroblasts to respond to them appropriately.

Objective The aim of this study was to study the effect of primary human AEC on the proliferation of fibroblasts obtained from healthy and fibrotic lungs in an interactive cell culture model.

Results Conditioned medium (CM) from 14 out of 16 AEC lines significantly inhibited proliferation of normal human lung fibroblasts by $51.2 \pm 6.0\%$. The proliferation of fibroblasts derived from patients with lung fibrosis was equally inhibited by CM of AEC. The inhibitory effect of AEC-CM was completely reversed when fibroblasts were pre-incubated with $2.5 \mu\text{M}$ indomethacin. Furthermore, primary human AEC, but not fibroblasts, secrete TGF- β , and the inhibitory effect of the AEC-CM was blocked by neutralizing anti-TGF- β antibodies.

Conclusion These results demonstrate that AEC actively inhibit the proliferation of both normal and fibrotic fibroblasts via TGF- β , which induces the prostaglandin E_2 synthesis in fibroblasts. The data indicate that proliferative lung diseases may be treated using the epithelial cell as the target of medication.

Keywords airway epithelial cell, airway remodelling, cell–cell interaction, epithelial–mesenchymal trophic unit, fibrosis, lung fibroblast, molecular mechanism

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Introduction

Remodelling and fibrosis are hallmarks of pulmonary disorders, including interstitial lung diseases or asthma. Increased fibroblast proliferation and deposition of extracellular matrix proteins cause a distortion of the lung architecture or airway wall thickening [1, 2].

The airway epithelium is in a key position to regulate the activity of the underlying cell layers and their response to external stimuli, thus forming the epithelial–mesenchymal trophic unit (EMTU) [2]. The importance of epithelial–mesenchymal interaction is emphasized in the current pathophysiological concepts of asthma [2, 3], idiopathic pulmonary fibrosis [4], and other extra-pulmonary diseases [5]. Idiopathic pulmonary fibrosis has

been suggested to be an ‘epithelial–fibroblastic disease’, in which the disturbed epithelium–fibroblast cross-talk was proposed to be the crucial event in the pathogenesis [6].

In the healthy lung, airway epithelial cells (AEC) are a source of factors that suppress fibroblast proliferation, and fibrosis may result from an inadequate generation of such suppressive factors by AEC or from an inadequate response of fibroblasts to these factors [7–10]. Prostaglandin E_2 (PGE $_2$) is a potent inhibitor of fibroblast proliferation [11] and collagen synthesis [12, 13], and it is protective in bleomycin-induced pulmonary fibrosis [14]. Furthermore, fibroblasts cultured from patients with idiopathic pulmonary fibrosis as well as the respiratory epithelium from patients with aspirin-sensitive asthma have a diminished capacity to synthesize PGE $_2$ [15, 16].

In primary human lung fibroblasts, PGE₂ synthesis is up-regulated by TGF- β [17], a growth factor involved in the regulation of pivotal cell functions, including proliferation, differentiation, apoptosis, and migration [18]. TGF- β is associated with tissue fibrosis and structural remodelling, as has been demonstrated both *in vitro* and *in vivo* in human and animal studies [19–24]. Conversely, TGF- β inhibits the proliferation of many cell types [17, 18, 25, 26], emphasizing the complexity of this cytokine. Previous studies suggested that PGE₂ and/or the cyclin-dependent kinase inhibitor p27^{kip1} are involved in the mediation of TGF- β 's anti-proliferative effect [17, 27, 28]. In mammalian cells, three isoforms of TGF- β (TGF- β ₁, TGF- β ₂, and TGF- β ₃) are expressed, and importantly, AEC have been demonstrated to secrete TGF- β spontaneously [29–31].

As interstitial fibrosis and airway remodelling are generally refractory to standard therapy with anti-inflammatory agents, it is necessary to look for new pathophysiological concepts and therapeutic approaches [2, 6]. Here, we evaluated the role of the AEC as a regulator of fibroblast proliferation using primary human cells of healthy and fibrotic lung tissue.

Materials and methods

Cell culture, antibodies, and reagents

Material has been purchased from as follows: Invitrogen (Heidelberg, Vic., Australia): Dulbecco's modified Eagle's medium, penicillin, streptomycin, amphotericin B; JRH (Melbourne, Vic., Australia): fetal bovine serum (FBS), phosphate-buffered saline; Sigma-Aldrich (St Louis, MO, USA): trypsin, indomethacin, anti-pan cytokeratin antibody; Affiland (Ans-Liege, Belgium): fibronectin; Cambrex (Clonetics, Walkersville, MD, USA): bronchial epithelial growth medium (BEGM), BEGM-supplements; R&D Systems (Minneapolis, MN, USA): pan-specific TGF- β antibody; Santa Cruz Biotechnology (Santa Cruz, CA, USA): primary antibodies p-p21^{waf1/cip1}, p-p27^{kip1}.

Primary cell culture

Normal and diseased lung tissue was obtained from resected human lung (Human Ethics Committee, Sydney University, South West Sydney Area Health Service; Ethics Committee, Faculty of Medicine, University Hospital Basel). Cultures of normal primary human lung fibroblasts were established as reported previously [11], and fibroblast cultures from diseased, fibrotic lungs were established similarly from peripheral lung tissue. Fibroblasts stained negative for α muscle actin [32], and all experiments were performed using fibroblasts at passages 3–6. Cultures of primary human AEC were set up from the same lung specimens used for fibroblast cultures. Small airways were dissected from surrounding tissue; the

epithelium was separated, cut into small pieces, and placed in six-well plates that were pre-coated with bovine fibronectin (1 μ g/ μ L). Complete culture medium consisted of BEGM supplemented with 4 μ L/mL bovine pituitary extract, 5 mg/mL insulin, 0.5 mg/mL hydrocortisone, 1 μ L/mL gentamycin/amphotericin B, 0.1 μ L/mL retinoic acid, 10 mg/mL transferrin, 1 μ L/mL triiodothyronine, 0.5 mg/mL epinephrine, and 0.5 μ g/mL human epidermal growth factor. Complete BEGM was replaced every fourth day. Before cells reached confluence (80–90%), they were detached by 0.25% trypsin in 1 mM Na₂EDTA and passaged. Epithelial cells stained positive for a pan-cytokeratin antibody, confirming the epithelial phenotype, and were used for all experiments at passages 1–3.

To obtain conditioned medium (CM) from AEC (AEC-CM), fresh BEGM was added to 90% confluent cell layers and incubated for 24 h (37 °C, 5% CO₂). The AEC-CM was collected, centrifuged (2000 g, 5 min) to remove any cell debris, split into aliquots, and stored at –20 °C. CM derived from the one AEC line was pooled.

[³H]-Thymidine incorporation into DNA

Cell proliferation assays were performed as described previously [11]. Proliferation was measured by [³H]-thymidine incorporation. Fibroblasts were seeded into 96-well plates (10⁴ cells/well), grown until 60–70% confluence, and serum-deprived for 24 h. Fibroblasts were incubated for 24 h with AEC-CM, or with complete BEGM (control). Fibroblasts incubated with 10% FBS were used as a control. Indomethacin (2.5 μ M) was used to block PGE₂ synthesis and was added to fibroblast cultures 30 min before the addition of AEC-CM. AEC-CM was pre-incubated with a pan-specific anti-TGF- β antibody (1 μ g/mL) for 30 min before its addition to the fibroblasts.

Prostaglandin E₂ analysis

PGE₂ levels were quantified in the AEC-CM using a commercial ELISA kit as instructed (Cayman Chemical, Ann Arbor, MI, USA).

Protein extractions and Western blot analysis

Total protein extracts were prepared from 80% confluent fibroblast cultures [33, 34]. To determine phosphorylated (p-) p21^{waf1/cip1} and p-p27^{kip1} expression, equal protein amounts (5 μ g) were size-fractionated in a 10% SDS-polyacrylamide gel, transferred onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) (25 mA/gel, 100 V, 120 min, 4 °C), and stained with Ponceau's red. Membranes were blocked for 1 h in 10 mM Tris, 150 mM NaCl, 0.05% Tween 20, and 5% FBS, followed by incubation with a primary antibody [rabbit-anti-human (p-p27^{kip1}) or goat-anti-human (p-p21^{waf1/cip1}), 1 : 500 in

blocking buffer (4 °C overnight)]. Membranes were washed (3 × 5 min) with blocking buffer and incubated with a secondary horseradish peroxidase-conjugated goat-anti-rabbit (DakoCytomation, Glostrup, Denmark) or donkey-anti-goat antibody (1:3000, 1 h room temperature). Protein detection was performed by chemiluminescence (Pierce, Rockford, IL, USA).

Filter experiments

To determine the characteristics of the growth-inhibitory activity, AEC-CM was size fractionated through centrifugal filters with different pore sizes (100, 50, and 30 kDa) (Millipore, Bedford, MA, USA) as recommended by the manufacturer.

One-dimensional gel electrophoresis

Equal amounts (10 µL) of AEC-CM were size fractionated on a 12% polyacrylamide gel. Protein bands were fixed, stained, and destained using a commercially available silver staining kit (Invitrogen).

Transforming growth factor- β analysis

Active TGF- β_1 was measured in the AEC-CM using a commercial ELISA kit (R&D Systems).

Statistical analysis

ANOVA was used for repeated measures, Student's paired *t*-test for paired data, or Pearson's correlation test for correlation calculations (StatView 5.0, SAS Institute, Cary, NY, USA). A *P*-value ≤ 0.05 was considered to be significant. Data are shown as mean $\pm$ standard error of the mean.

Results

Fibroblast proliferation is inhibited by epithelium-derived conditioned medium

Normal AEC-CM obtained from 16 different primary AEC lines were tested for their effect on fibroblast proliferation on 13 normal fibroblast cell lines. The anti-proliferative effect of each AEC line was tested on at least three different fibroblast cell lines. Compared with complete BEGM (control), the overall inhibitory effect of AEC-CM on fibroblast proliferation was $51.2 \pm 6.0\%$ ($n = 16$, Student's paired *t*-test, $P < 0.0001$; Fig. 1a). AEC-CM from 14 out of 16 AEC lines caused a significant inhibition of fibroblast proliferation ($n = 16$, Student's paired *t*-test, $P \leq 0.05$; Fig. 1b). Despite a high fibroblast cell line-specific variability in base line proliferation, the inhibitory effect was reproducible in all 13 tested cell lines.

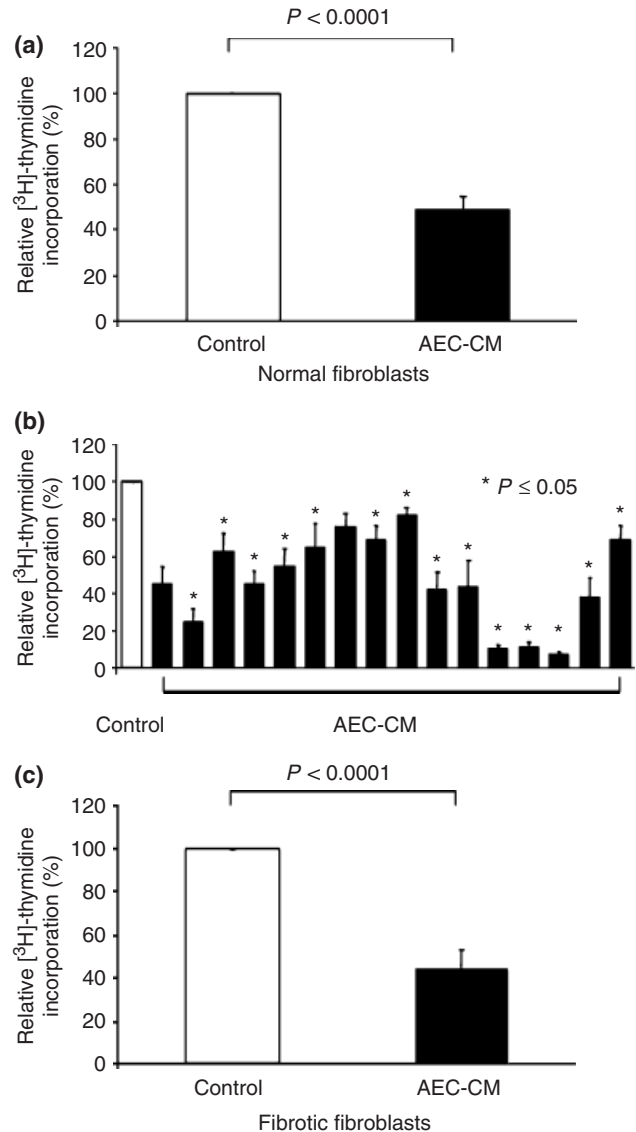


Fig. 1. Conditioned medium (CM) derived from primary airway epithelial cells (AEC) inhibits proliferation of primary human lung fibroblasts. (a) Overall effect of AEC-CM (black bar) on fibroblast proliferation compared with bronchial epithelial growth medium (BEGM) plus additives (complete BEGM) (open bar). Normal primary human lung fibroblasts obtained from 13 different subjects were stimulated with AEC-CM derived from 16 different normal subjects. Values are presented as mean $\pm$ standard error of the mean (SEM) of independent experiments. (b) Effect of AEC-CM derived from 16 different donors (black bars) on fibroblast proliferation compared with complete BEGM (open bar). Normal primary human lung fibroblasts were stimulated with AEC-CM derived from 16 different normal subjects. Values are presented as mean $\pm$ SEM of independent experiments performed in at least three different fibroblast cell lines. (c) Effect of AEC-CM (black bar) on proliferation of fibroblasts derived from fibrotic lungs compared with that of complete BEGM (open bar). Primary human lung fibroblasts obtained from seven different subjects with fibroproliferative lung disorders were incubated with AEC-CM. Values are presented as mean $\pm$ SEM of independent experiments performed in seven different fibroblast cell lines.

Next, we assessed whether the proliferation of fibroblasts derived from seven patients with fibroproliferative lung disease was equally suppressed by AEC-CM. The baseline proliferation rate of fibrotic fibroblasts in the presence of normal growth medium did not differ significantly from that of normal fibroblasts (data not shown). As depicted in Fig. 1c, proliferation of fibroblasts obtained from fibrotic lungs was significantly inhibited by the AEC-CM ($55.9 \pm 9.0\%$, $n = 7$, Student's paired t -test, $P < 0.0001$), and thus the effect was not significantly different from that of normal fibroblasts (ANOVA, $P = 0.58$).

The inhibitory effect of airway epithelial cell-conditioned medium is mediated by fibroblast-derived prostaglandin E_2 and correlates with the phosphorylation of p27^{kip1}

Absolute PGE₂ levels (pg/mL) of AEC-CM were plotted against the inhibitory effect of AEC-CM, but no correlation was found between the two variables ($n = 19$, Pearson's correlation test, $P = 0.6$; Fig. 2a). This was in accordance with our observation that inhibition of cyclooxygenases (COX) in AEC by 2.5 μ M indomethacin did not alter the anti-proliferative effect of AEC-CM (data not shown).

Next, we assessed PGE₂ levels in AEC-CM after a 24-h incubation with fibroblasts and plotted the data against its anti-proliferative capacity. Figure 2b shows a significant correlation between the logarithm of the PGE₂ levels in fibroblast-exposed AEC-CM and proliferation rates ($n = 18$, Pearson's correlation test, $R = 0.75$, $P < 0.001$. PGE₂ data have been transformed into logarithm for statistical calculations, but for visualization in Fig. 2b absolute PGE₂ values were chosen). Furthermore, pretreatment of fibroblasts with indomethacin (2.5 μ M) completely reversed the inhibitory effect of AEC-CM ($n = 3$, ANOVA, $P = 0.001$; Fig. 2c), confirming that PGE₂ originated from fibroblasts. Our data show that the PGE₂ level in the AEC-CM increased significantly during the 24-h incubation with fibroblasts ($n = 3$, ANOVA, $P \leq 0.05$; Fig. 2d), which was prevented by 2.5 μ M indomethacin ($n = 3$, ANOVA, $P \leq 0.05$; Fig. 2d).

Because COX inhibits cell proliferation via a mechanism that involves cyclin-dependent kinase inhibitors [35], we assessed the effect of AEC-CM on the expression and phosphorylation of p27^{kip1} and p21^{waf1/cip1}, two cyclin-dependent kinase inhibitors [36]. Resting, non-cycling fibroblasts expressed a significant level of phosphorylated (P) p27^{kip1} (Fig. 3, upper panel), and the addition of AEC-CM up-regulated the phosphorylation of p27^{kip1}, whereas control medium diminished p27^{kip1} phosphorylation. In contrast, p21^{waf1/cip1} was only weakly phosphorylated in resting fibroblasts and was not affected by AEC-CM (Fig. 3, lower panel). Together, these data suggest that the anti-proliferative activity of AEC-CM on fibroblasts is mediated by an epithelium-derived factor that induced

PGE₂ synthesis in fibroblasts and the phosphorylation of p27^{kip1}.

Airway epithelial cell-derived factor(s) < 50 kDa mediate the anti-proliferative effect

To further characterize AEC-derived soluble factor(s) that induce PGE₂ production by fibroblasts, AEC-CM was subjected to size-exclusion filtration, and the filtrates were used to determine their anti-proliferative capacity on fibroblasts. As shown in Fig. 4a, filtrates containing proteins > 50 kDa had no significant effect on fibroblast proliferation, whereas filtrates containing proteins < 50 kDa exerted an anti-proliferative effect ($n = 3$, ANOVA, $P < 0.0001$; Fig. 4a). Fractions of filtered complete BEGM were used as negative controls and showed no anti-proliferative activity (data not shown).

To further identify the AEC-derived anti-proliferative factor(s), different filter fractions of AEC-CM were analysed by one-dimensional gel electrophoresis. We found specific protein bands between 10 and 20 kDa (Fig. 4b, lanes 4 and 5), which were not present in the filter fraction with molecules > 50 kDa (Fig. 4b, lane 3) and in the negative control (Fig. 4b, lane 2).

The Inhibitory effect of airway epithelial cell-conditioned medium is mediated by transforming growth factor- β

TGF- β is a factor produced by AEC and falls into the molecular size of the inhibitory protein fraction described above. Therefore, we determined levels of active TGF- β_1 – the most abundant isoform in the human lung [37] – in AEC-CM. Significantly higher levels of active TGF- β_1 (78.3 ± 15.3 pg/mL, $n = 11$; Fig. 5a) were detected in the AEC-CM compared with control medium (2.2 ± 0.2 pg/mL, Student's paired t -test, $P < 0.0001$; Fig. 5a). The TGF- β_1 level of AEC-CM was significantly reduced after 24-h incubation with fibroblasts ($n = 3$, ANOVA, $P < 0.0001$; Fig. 5b), indicating that fibroblasts did not secrete further TGF- β_1 , but rather consume/bind/internalize it.

Finally, a pan-specific neutralizing anti-TGF- β antibody (1 μ g/mL) significantly inhibited the anti-proliferative effect of the AEC-CM ($n = 7$, ANOVA, $P < 0.05$; Fig. 5c), confirming that TGF- β is the anti-proliferative factor released by AEC. Equivalent doses of an iso-antibody showed no effect (data not shown).

Discussion

Interactions between epithelial and mesenchymal cells of the EMTU have been assumed to play an important role in the pathogenesis of disorders characterized by fibroproliferation and tissue remodelling [2, 6]. In this model with primary human cells, we demonstrate the significance of AEC in exerting an anti-proliferative effect on lung

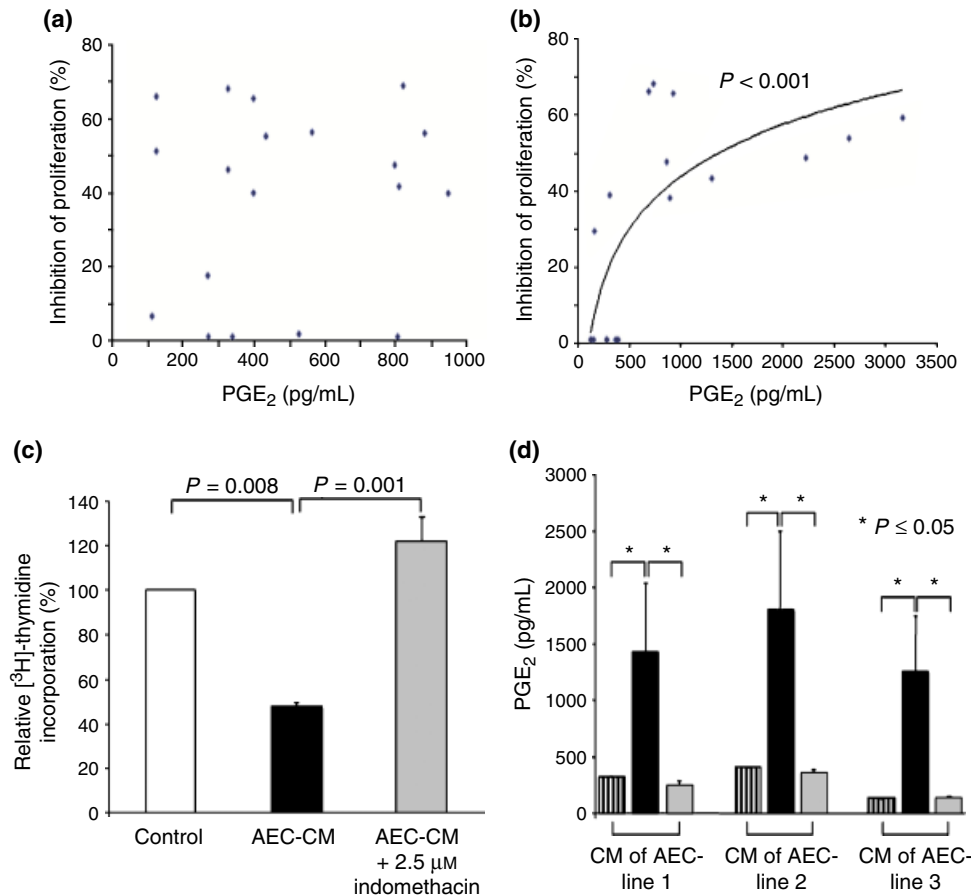


Fig. 2. Fibroblast-derived prostaglandin E₂ (PGE₂) mediates the anti-proliferative effect of airway epithelial cell-conditioned medium (AEC-CM). (a) Correlation between PGE₂ content in AEC-CM and the inhibitory effect of AEC-CM on fibroblast proliferation. Each value represents PGE₂ content in the AEC-CM and the corresponding inhibitory effect of the AEC-CM on fibroblast proliferation ($n = 19$, Pearson's correlation test, $P = 0.6$). (b) Correlation between PGE₂ content in AEC-CM after 24-h incubation with fibroblasts and the inhibitory effect of the AEC-CM on fibroblast proliferation ($n = 18$, Pearson's correlation test, $R = 0.75$, $P < 0.001$). PGE₂ data have been transformed into logarithm for statistical calculations, but for visualization absolute PGE₂ values were chosen). (c) Effect of AEC-CM on fibroblast proliferation in the presence (grey bar) or absence (black bar) of 2.5 μM indomethacin compared with that of complete bronchial epithelial growth medium (open bar). Normal primary human lung fibroblasts were stimulated with AEC-CM, +/- indomethacin. Values are presented as mean $\pm$ standard error of the mean (SEM) of independent experiments performed in three different fibroblast cell lines. (d) PGE₂ levels in the AEC-CM of three different AEC lines before adding to the fibroblasts (striped bar) and after 24-h incubation with fibroblasts, with (grey bar) and without (black bar) 2.5 μM indomethacin. Values are presented as mean $\pm$ SEM of independent experiments performed in three different fibroblast cell lines.

fibroblasts via the release of TGF- β , which stimulates fibroblasts to produce PGE₂. Until now, data on the proliferative properties of fibroblasts obtained from patients with lung fibrosis were scant and contradictory [38, 39]. We demonstrate that fibroblast cultures derived from normal and fibrotic lung tissue are equally sensitive to the anti-proliferative effect of primary human AEC and that it is the activity of the AEC that determines the fibroblasts' proliferative capacity. Our novel data suggest a crucial role of the airway epithelium in directing the pathogenesis of fibroproliferative lung disorders in humans.

Fibrotic lung diseases are often associated with epithelial injury, i.e. desquamation of the epithelium. Importantly, marked disruption in the integrity of the alveolar

epithelium and slow re-epithelialization of the denuded area are distinct pathologies in idiopathic pulmonary fibrosis and asthma [6, 40]. The observations that fibrotic changes occur in the absence of inflammatory cells and that epithelial cell damage and perturbation of epithelium-fibroblast interaction are sufficient to promote the development of a fibrotic process [41] further strengthen the inhibitory role of the epithelium. The loss of epithelial cells may not only affect the barrier function of the epithelium but may also cause a deficit of anti-proliferative factors, in particular TGF- β , thereby allowing fibroblasts to proliferate less restricted. This may ultimately lead to airway wall remodelling and/or interstitial fibrosis of the lung.

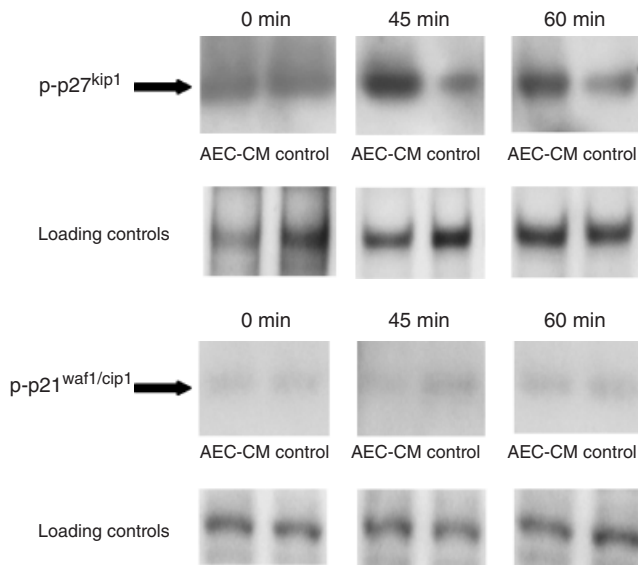


Fig. 3. Expression of phosphorylated p27^{kip1} and phosphorylated p21^{waf1/cip1} in fibroblasts stimulated with airway epithelial cell-conditioned medium (AEC-CM). Representative picture of Western blots showing the expression of phosphorylated p27^{kip1} (upper panel) and phosphorylated p21^{waf1/cip1} (lower panel) in human lung fibroblasts ($n = 3$) after stimulation with AEC-CM or complete bronchial epithelial growth medium (control) as a function of time. Expression of phosphorylated p27^{kip1} and phosphorylated p21^{waf1/cip1} was determined in the total protein fraction. Respective loading controls are shown under the Western blots.

Epithelium-dependent inhibition of fibroblast proliferation in co-culture models is commonly regarded to be mediated by PGE₂ [7, 9, 42]. PGE₂ acts as a negative regulator of fibroblast function, and its protective role against fibroproliferation in the lung has been extensively studied [4, 8–10, 13, 16, 26, 43–46]. Our present data demonstrate that the inhibitory effect of AEC-CM on fibroblast proliferation is mediated through epithelium-derived TGF- β that stimulates fibroblasts to produce PGE₂.

In accordance with previous reports [30, 31, 47, 48], we detected active TGF- β ₁ in AEC-CM obtained from primary human AEC in culture. Furthermore, TGF- β ₁ levels were significantly reduced after 24-h incubation of AEC-CM with fibroblasts. This may indicate that fibroblasts do not secrete significant amounts of TGF- β , but rather actively consume, bind, or internalize TGF- β before synthesis of PGE₂.

The TGF- β specificity of the anti-proliferative response to AEC-CM was demonstrated by a pan-specific anti-TGF- β antibody. To our knowledge, this is the first report to demonstrate in primary human cells the mechanism underlying the EMTU hypothesis in which the injured and repairing airway epithelium initiates the process of remodelling [2]. Complementary to the current understanding of the EMTU, our data suggest that not only an

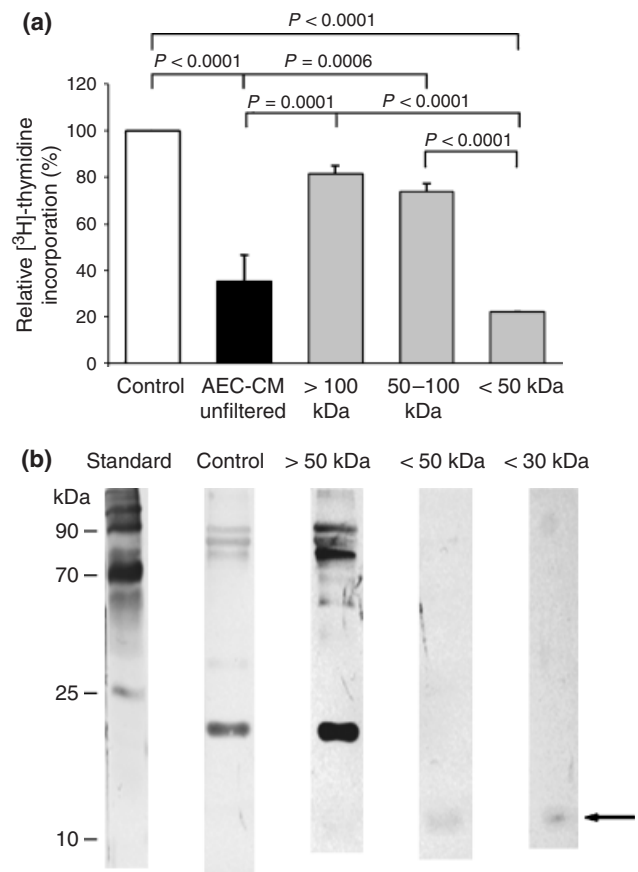


Fig. 4. The inhibitory effect is mediated by an epithelium-derived factor < 50 kDa. (a) Effect of airway epithelial cell-conditioned medium (AEC-CM) on fibroblast proliferation, before (black bar) and after (grey bars) size-fractionation of the AEC-CM compared with complete bronchial epithelial growth medium (BEGM) (open bar). Values are presented as mean $\pm$ standard error of the mean of independent experiments performed in three different fibroblast cell lines. (b) Silver staining of representative one-dimensional gel electrophoresis of AEC-CM after size-fractionation by filtering (lanes 3–5) compared with complete BEGM (lane 2). Lane 1, protein standard; lane 2, unfiltered complete BEGM; lane 3, AEC-CM filter fraction > 50 kDa; lane 4, AEC-CM filter fraction < 50 kDa; lane 5, AEC-CM filter fraction < 30 kDa.

increased production of pro-proliferative but also a lack of anti-proliferative growth factors, especially TGF- β , induces fibroproliferative processes. Our model suggests that epithelial TGF- β and fibroblast-derived PGE₂ are two key players in the maintenance of the normal lung structure, emphasizing the relevance of the airway epithelium as a protector of lung homeostasis. We acknowledge that our model cannot rule out that *in vivo* fibroblast-derived TGF- β contributes in an autocrine way to the inhibitory effect. However, *in vivo* data demonstrate that the epithelial cells and macrophages are the main source of TGF- β in the lung, whereas fibroblasts produce only low levels of TGF- β [47, 48]. Our interactive cell culture model also does not allow direct cell–cell contact between

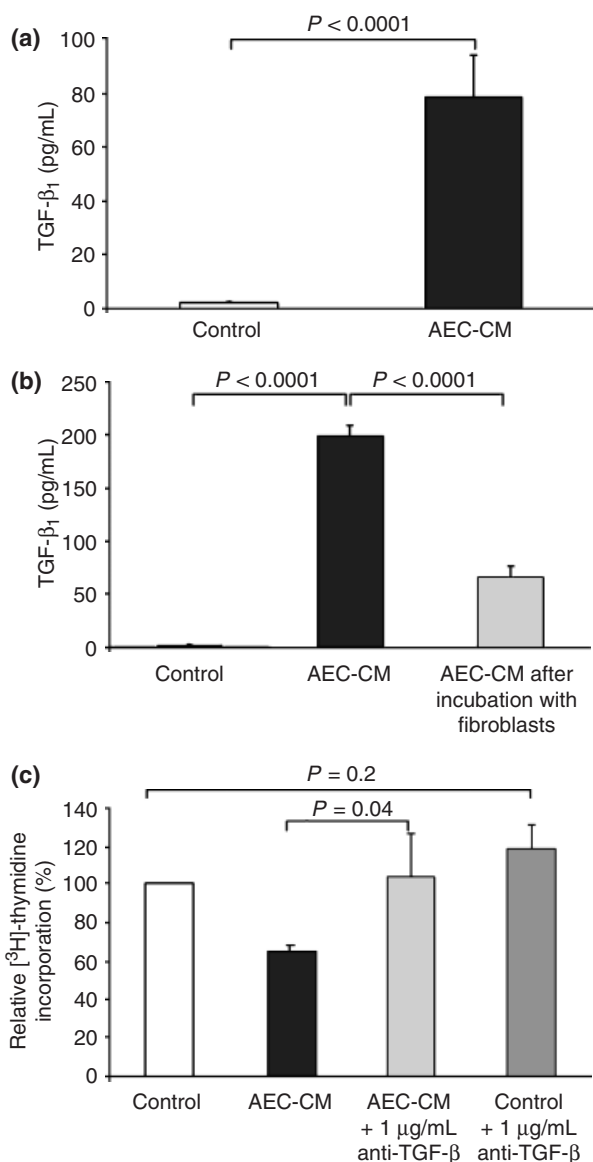


Fig. 5. Anti-TGF- β antibody reverses the anti-proliferative effect of airway epithelial cell-conditioned medium (AEC-CM). (a) TGF- β_1 level in the AEC-CM (black bar) compared with control medium [complete bronchial epithelial growth medium (BEGM)] (open bar). Values are presented as mean $\pm$ standard error of the mean (SEM) of independent experiments performed in AEC-CM of 11 different AEC lines. (b) TGF- β_1 levels in the AEC-CM before adding to the fibroblasts (black bar) and after 24-h incubation with fibroblasts (grey bar) compared with control medium (complete BEGM) (open bar). Values are presented as mean $\pm$ SEM of independent experiments performed in three different fibroblast cell lines. (c) Effect of AEC-CM on fibroblast proliferation in the presence (grey bar) or absence (black bar) of 1 μ g/mL pan-specific anti-TGF- β antibody compared with control (open bar). Primary human lung fibroblasts were stimulated with AEC-CM, $\pm$ anti-TGF- β antibody, or with BEGM, $\pm$ anti-TGF- β antibody. Values are presented as mean $\pm$ SEM of independent experiments performed in seven different fibroblast cell lines.

AEC and fibroblast, but this may not be a major problem, because direct AEC–fibroblast contacts are not frequently observed in the normal adult lung [49].

It is reasonable to assume that latent TGF- β is present in the AEC-CM and only after addition of the AEC-CM onto fibroblasts TGF- β is activated. Because the integrin $\alpha v \beta 6$ is expressed primarily in the epithelium and not in fibroblasts [50, 51], alternative activation mechanisms such as the interaction with thrombospondin 1 [52, 53] or activation by matrix metalloproteinase 2 [54–56] have to be considered in our model.

In conclusion, our data provide a new insight into how AEC control the proliferation of fibroblasts in both normal and fibrotic lung tissue. Our study demonstrates that TGF- β derived from healthy primary AEC induces PGE₂ synthesis by fibroblasts, which in turn inhibits the proliferation of these fibroblasts in an auto-regulatory feedback. This auto-regulation of fibroblast proliferation might represent an important control mechanism in the healthy lung, and its disturbance may lead to enhanced fibroblast proliferation, airway wall remodelling or/and fibrosis. In view of the generally unsatisfactory response of interstitial fibrosis and airway remodelling to current treatments, it might be of great interest to choose the epithelial cell as the target for new therapeutic strategies.

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References

- Kim DS, Collard HR, King TE Jr. Classification and natural history of the idiopathic interstitial pneumonias. *Proc Am Thorac Soc* 2006; 3:285–92.
- 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 (Part 1):193–204.
- Knight D. Epithelium–fibroblast interactions in response to airway inflammation. *Immunol Cell Biol* 2001; 79:160–4.
- Selman M, Pardo A. The epithelial/fibroblastic pathway in the pathogenesis of idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2003; 29 (Suppl. 3):S93–7.
- Kinnman N, Housset C. Peribiliary myofibroblasts in biliary type liver fibrosis. *Front Biosci* 2002; 7:d496–503.

- 6 Selman M, King TE, Pardo A American Thoracic Society, European Respiratory Society, American College of Chest Physicians. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* 2001; 134:136–51.
- 7 Portnoy J, Pan T, Dinarello CA *et al*. Alveolar type II cells inhibit fibroblast proliferation: role of IL-1 α . *Am J Physiol Lung Cell Mol Physiol* 2006; 290:L307–16.
- 8 Pan T, Mason RJ, Westcott JY, Shannon JM. Rat alveolar type II cells inhibit lung fibroblast proliferation in vitro. *Am J Respir Cell Mol Biol* 2001; 25:353–61.
- 9 Lama V, Moore BB, Christensen P, Toews GB, Peters-Golden M. Prostaglandin E2 synthesis and suppression of fibroblast proliferation by alveolar epithelial cells is cyclooxygenase-2-dependent. *Am J Respir Cell Mol Biol* 2002; 27:752–8.
- 10 Moore BB, Peters-Golden M, Christensen PJ *et al*. Alveolar epithelial cell inhibition of fibroblast proliferation is regulated by MCP-1/CCR2 and mediated by PGE2. *Am J Physiol Lung Cell Mol Physiol* 2003; 284:L342–9.
- 11 Hostettler KE, Roth M, Burgess JK *et al*. Cyclosporine A mediates fibroproliferation through epithelial cells. *Transplantation* 2004; 77:1886–93.
- 12 Goldstein RH, Polgar P. The effect and interaction of bradykinin and prostaglandins on protein and collagen production by lung fibroblasts. *J Biol Chem* 1982; 257:8630–3.
- 13 Hodges RJ, Jenkins G, Wheeler-Jones C, McNulty RJ. Severity of lung injury in cyclooxygenase-2-deficient mice is dependent on reduced prostaglandin E2 production. *Am J Pathol* 2004; 165:1663–76.
- 14 Moore BB, Coffey MJ, Christensen P *et al*. GM-CSF regulates bleomycin-induced pulmonary fibrosis via a prostaglandin-dependent mechanism. *J Immunol* 2000; 165:4032–9.
- 15 Picado C, Fernandez-Morata JC, Juan M *et al*. Cyclooxygenase-2 mRNA is downexpressed in nasal polyps from aspirin-sensitive asthmatics. *Am J Respir Crit Care Med* 1999; 160:291–6.
- 16 Wilborn J, Crofford LJ, Burdick MD, Kunkel SL, Strieter RM, Peters-Golden M. Cultured lung fibroblasts isolated from patients with idiopathic pulmonary fibrosis have a diminished capacity to synthesize prostaglandin E2 and to express cyclooxygenase-2. *J Clin Invest* 1995; 95:1861–8.
- 17 McNulty RJ, Hernandez-Rodriguez NA, Mutsaers SE, Coker RK, Laurent GJ. Indomethacin suppresses the anti-proliferative effects of transforming growth factor-beta isoforms on fibroblast cell cultures. *Biochem J* 1997; 321 (Part 3):639–43.
- 18 Massague J. TGF-beta signal transduction. *Annu Rev Biochem* 1998; 67:753–91.
- 19 Sagara H, Okada T, Okumura K *et al*. Activation of TGF-beta/Smad2 signaling is associated with airway remodeling in asthma. *J Allergy Clin Immunol* 2002; 110:249–54.
- 20 Chu HW, Balzar S, Seedorf GJ *et al*. Transforming growth factor-beta2 induces bronchial epithelial mucin expression in asthma. *Am J Pathol* 2004; 165:1097–106.
- 21 Balzar S, Chu HW, Silkoff P *et al*. Increased TGF-beta2 in severe asthma with eosinophilia. *J Allergy Clin Immunol* 2005; 115:110–7.
- 22 Anscher MS, Peters WP, Reisenbichler H, Petros WP, Jirtle RL. Transforming growth factor beta as a predictor of liver and lung fibrosis after autologous bone marrow transplantation for advanced breast cancer. *N Engl J Med* 1993; 328:1592–8.
- 23 Westergren-Thorsson G, Hernnas J, Sarnstrand B, Oldberg A, Heinegard D, Malmstrom A. Altered expression of small proteoglycans, collagen, and transforming growth factor-beta 1 in developing bleomycin-induced pulmonary fibrosis in rats. *J Clin Invest* 1993; 92:632–7.
- 24 Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 1997; 100:768–76.
- 25 Piek E, Ju WJ, Heyer J *et al*. Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts. *J Biol Chem* 2001; 276:19945–53.
- 26 Keerthisingam CB, Jenkins RG, Harrison NK *et al*. Cyclooxygenase-2 deficiency results in a loss of the anti-proliferative response to transforming growth factor-beta in human fibrotic lung fibroblasts and promotes bleomycin-induced pulmonary fibrosis in mice. *Am J Pathol* 2001; 158:1411–22.
- 27 Liu W, Wu G, Li W, Lobur D, Wan Y. Cdh1-anaphase-promoting complex targets Skp2 for destruction in transforming growth factor beta-induced growth inhibition. *Mol Cell Biol* 2007; 27:2967–79.
- 28 Reynisdottir I, Polyak K, Iavarone A, Massague J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes Dev* 1995; 9:1831–45.
- 29 Thompson HG, Mih JD, Krasieva TB, Tromberg BJ, George SC. Epithelial-derived TGF-beta2 modulates basal and wound-healing subepithelial matrix homeostasis. *Am J Physiol Lung Cell Mol Physiol* 2006; 291:L1277–85.
- 30 Sacco O, Romberger D, Rizzino A, Beckmann JD, Rennard SI, Spurzem JR. Spontaneous production of transforming growth factor-beta 2 by primary cultures of bronchial epithelial cells. Effects on cell behavior in vitro. *J Clin Invest* 1992; 90:1379–85.
- 31 Kelly MM, Leigh R, Bonniaud P *et al*. Epithelial expression of profibrotic mediators in a model of allergen-induced airway remodeling. *Am J Respir Cell Mol Biol* 2005; 32:99–107.
- 32 Tamm M, Roth M, Malouf M *et al*. Primary fibroblast cell cultures from transbronchial biopsies of lung transplant recipients. *Transplantation* 2001; 71:337–9.
- 33 Borger P, Matsumoto H, Boustany S *et al*. Disease-specific expression and regulation of CCAAT/enhancer-binding proteins in asthma and chronic obstructive pulmonary disease. *J Allergy Clin Immunol* 2007; 119:98–105.
- 34 Roth M, Johnson PR, Borger P *et al*. Dysfunctional interaction of C/EBPalpha and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. *N Engl J Med* 2004; 351:560–74.
- 35 Zahner G, Wolf G, Ayoub M *et al*. Cyclooxygenase-2 overexpression inhibits platelet-derived growth factor-induced mesangial cell proliferation through induction of the tumor suppressor gene p53 and the cyclin-dependent kinase inhibitors p21waf-1/cip-1 and p27kip-1. *J Biol Chem* 2002; 277:9763–71.
- 36 Morgan DO. Principles of CDK regulation. *Nature* 1995; 374:131–4.
- 37 Coker RK, Laurent GJ, Shahzeidi S *et al*. Diverse cellular TGF-beta 1 and TGF-beta 3 gene expression in normal human and murine lung. *Eur Respir J* 1996; 9:2501–7.
- 38 Ramos C, Montano M, Garcia-Alvarez J *et al*. Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression. *Am J Respir Cell Mol Biol* 2001; 24:591–8.

- 39 Jordana M, Schulman J, McSharry C *et al.* Heterogeneous proliferative characteristics of human adult lung fibroblast lines and clonally derived fibroblasts from control and fibrotic tissue. *Am Rev Respir Dis* 1988; **137**:579–84.
- 40 Holgate ST, Holloway J, Wilson S *et al.* Understanding the pathophysiology of severe asthma to generate new therapeutic opportunities. *J Allergy Clin Immunol* 2006; **117**:496–506; quiz 507.
- 41 Adamson IY, Young L, Bowden DH. Relationship of alveolar epithelial injury and repair to the induction of pulmonary fibrosis. *Am J Pathol* 1988; **130**:377–83.
- 42 Peters-Golden M. When defenses against fibroproliferation fail: spotlight on an axis of prophylaxis. *Am J Respir Crit Care Med* 2003; **168**:1141–2.
- 43 Lovgren AK, Jania LA, Hartney JM *et al.* COX-2-derived prostacyclin protects against bleomycin-induced pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2006; **291**:L144–56.
- 44 Charbeneau RP, Christensen PJ, Chrisman CJ *et al.* Impaired synthesis of prostaglandin E2 by lung fibroblasts and alveolar epithelial cells from GM-CSF – / – mice: implications for fibroproliferation. *Am J Physiol Lung Cell Mol Physiol* 2003; **284**:L1103–11.
- 45 Ghosh M, Stewart A, Tucker DE, Bonventre JV, Murphy RC, Leslie CC. Role of cytosolic phospholipase A(2) in prostaglandin E(2) production by lung fibroblasts. *Am J Respir Cell Mol Biol* 2004; **30**:91–100.
- 46 Bonner JC, Rice AB, Ingram JL *et al.* Susceptibility of cyclooxygenase-2-deficient mice to pulmonary fibrogenesis. *Am J Pathol* 2002; **161**:459–70.
- 47 Coker RK, Laurent GJ, Jeffery PK, du Bois RM, Black CM, McAnulty RJ. Localisation of transforming growth factor beta1 and beta3 mRNA transcripts in normal and fibrotic human lung. *Thorax* 2001; **56**:549–56.
- 48 Khalil N, Bereznay O, Sporn M, Greenberg AH. Macrophage production of transforming growth factor beta and fibroblast collagen synthesis in chronic pulmonary inflammation. *J Exp Med* 1989; **170**:727–37.
- 49 Adamson IY, Hedgecock C, Bowden DH. Epithelial cell–fibroblast interactions in lung injury and repair. *Am J Pathol* 1990; **137**:385–92.
- 50 Breuss JM, Gallo J, DeLisser HM *et al.* Expression of the beta 6 integrin subunit in development, neoplasia and tissue repair suggests a role in epithelial remodeling. *J Cell Sci* 1995; **108** (Part 6):2241–51.
- 51 Araya J, Cambier S, Morris A, Finkbeiner W, Nishimura SL. Integrin-mediated transforming growth factor-beta activation regulates homeostasis of the pulmonary epithelial–mesenchymal trophic unit. *Am J Pathol* 2006; **169**:405–15.
- 52 Crawford SE, Stellmach V, Murphy-Ullrich JE *et al.* Thrombospondin-1 is a major activator of TGF-beta1 in vivo. *Cell* 1998; **93**:1159–70.
- 53 Schultz-Cherry S, Chen H, Mosher DF *et al.* Regulation of transforming growth factor-beta activation by discrete sequences of thrombospondin 1. *J Biol Chem* 1995; **270**:7304–10.
- 54 Coutts A, Chen G, Stephens N *et al.* Release of biologically active TGF-beta from airway smooth muscle cells induces autocrine synthesis of collagen. *Am J Physiol Lung Cell Mol Physiol* 2001; **280**:L999–1008.
- 55 Karsdal MA, Larsen L, Engsig MT *et al.* Matrix metalloproteinase-dependent activation of latent transforming growth factor-beta controls the conversion of osteoblasts into osteocytes by blocking osteoblast apoptosis. *J Biol Chem* 2002; **277**:44061–7.
- 56 George SJ, Johnson JL, Smith MA, Angelini GD, Jackson CL. Transforming growth factor-beta is activated by plasmin and inhibits smooth muscle cell death in human saphenous vein. *J Vasc Res* 2005; **42**:247–54.