

## LUNG TRANSPLANTATION

## Phenotype of airway epithelial cells suggests epithelial to mesenchymal cell transition in clinically stable lung transplant recipients

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**Background:** Obliterative bronchiolitis in chronic rejection of lung allografts is characterised by airway epithelial damage and fibrosis. The process whereby normal epithelium is lost and replaced by fibroblastic scar tissue is poorly understood, but recent findings suggest that epithelial cells can become fibroblasts through epithelial-mesenchymal transition (EMT). It is hypothesised that EMT occurs in lung allografts and plays a potential role in airway remodelling.

**Methods:** Sixteen stable lung transplant recipients underwent bronchoscopy with bronchoalveolar lavage (BAL), endobronchial biopsies, and bronchial brushings. Biopsy sections were stained for the fibroblast marker S100A4. Brushings were cultured on collagen, stained with anti-S100A4, and examined for further EMT markers including matrix metalloproteinase (MMP) zymographic activity and epithelial invasion through collagen coated filters.

**Results:** A median 15% (0-48%) of the biopsy epithelium stained for S100A4 in stable lung transplant recipients and MMP-7 co-localisation was observed. In non-stimulated epithelial cultures from lung allografts, S100A4 staining was identified with MMP-2 and MMP-9 production and zymographic activity. MMP total protein and activity was increased following stimulation with transforming growth factor (TGF)- $\beta_1$ . Non-stimulated transplant epithelial cells were invasive and penetration of collagen coated filters increased following TGF- $\beta_1$  stimulation.

**Conclusions:** This study provides evidence of EMT markers in lung allografts of patients without loss of lung function. The EMT process may represent a final common pathway following injury in more common diseases characterised by airway remodelling.

Although human lung transplantation is now a well accepted therapeutic option for selected patients with advanced cardiopulmonary disease, long term survival is limited by progressive deterioration in lung function due to airway remodelling. There has been no substantial improvement in the reported incidence over the last 10 years, despite improvements in immunosuppression, surgical techniques, and patient management.<sup>1,2</sup> There is an urgent need to understand the pathogenesis of airway remodelling in order to identify new targets for therapeutic development.

Obliterative bronchiolitis (OB) has been defined pathologically as the airway response to chronic allograft rejection and is manifest physiologically as the bronchiolitis obliterans syndrome (BOS). It is an essentially irreversible process, leading directly to patient morbidity and mortality.<sup>1,2</sup> Its pathophysiology is poorly understood, but it is thought to represent an overall response to epithelial injury as a result of immunological and non-immunological mechanisms.<sup>3</sup> Increased staining of the airway epithelium for class II major histocompatibility complex antigens (HLA-DR) has been shown in allografts during rejection<sup>4</sup> and, more recently, anti-HLA antibodies have been implicated in BOS.<sup>5</sup> There is increasing recognition that clinically silent injuries to allograft airway epithelium can lead to airway damage and BOS.<sup>3,6-10</sup>

International registry data indicate that acute rejection and lymphocytic bronchiolitis, a specific form of airway inflammation,<sup>11</sup> are risk factors for BOS. The key pathological findings in chronic lung allograft failure are remodelling and fibrotic obstruction of small airways (OB).<sup>1,2</sup> In apparently clinically stable lung allografts without airflow limitation,

studies have consistently shown high interleukin (IL)-8 levels, neutrophils, natural killer (NK) cells, and CD8 positive lymphocytes trafficking to airway epithelium.<sup>8,12-14</sup> Stable lung allografts also have evidence of subclinical infection, with bacterial *N*-acylhomoserine lactone quorum sensing signalling molecules detectable in airway lavage samples,<sup>6</sup> together with evidence of small airway remodelling as demonstrated by basement membrane thickening.<sup>9,10</sup> Precisely how these changes relate to the development of OB is not understood. In particular, the origin of the activated fibroblasts responsible for airway fibrosis is unknown and represents a key factor in understanding of the basic mechanisms.

Airway fibroblasts potentially originate through a number of pathways including in situ proliferation of resident fibroblasts, recruitment of circulating progenitor cells, and transition of airway smooth muscle.<sup>15</sup> Recent evidence also suggests the existence of epithelial-mesenchymal transition (EMT) as a factor in the fibrotic response.<sup>16,17</sup> EMT involves the acquisition of key proteins and an early marker in the EMT proteome is expression of the human homologue of fibroblast specific protein 1, S100A4 (Swiss Prot P26447, 11.7 kD). S100A4 is a Ca<sup>2+</sup> binding 101 amino acid polypeptide and has been shown in the oncology literature to be a metastasis related protein. Nuclear S100A4 expression

**Abbreviations:** BAL, bronchoalveolar lavage; BOS, bronchiolitis obliterans syndrome; EBB, endobronchial biopsy; EMT, epithelial-mesenchymal transition; IL, interleukin; MMP, matrix metalloproteinase; NK, natural killer; OB, obliterative bronchiolitis; PBEC, primary bronchial epithelial cell; TGF- $\beta_1$ , transforming growth factor  $\beta_1$

is associated with aggressive metastatic disease in a variety of tumours,<sup>18–20</sup> and there is a correlation between S100A4 expression and poor prognosis in several epithelial cancer types.<sup>18</sup> Further markers of epithelial damage and EMT are the matrix metalloproteinases MMP-7,<sup>21</sup> MMP-2, and MMP-9 (92 kDa gelatinase).<sup>17</sup> Collagen type IV, which makes up the true reticular basement membrane of airway epithelia, is a common substrate for these MMPs, and these enzymes can therefore disrupt the basement membrane of differentiated epithelia, promoting translocation, damage, inflammation, and further EMT.<sup>17</sup> Our group has shown that chronically activated intraepithelial CD103/CD8 T cells elicit ongoing production of transforming growth factor (TGF)- $\beta$  which drives EMT in chronic renal allograft nephropathy.<sup>23</sup> Levels of TGF- $\beta$  are increased in lung allografts,<sup>24</sup> and it is considered a key initiator of EMT in a number of organs and pathologies.<sup>17</sup>

We therefore hypothesise that donor airway epithelial cells—as well as being a recognised pathological target in lung transplantation—may be a significant source of effector fibroblasts through EMT.

## METHODS

### Patients

Sixteen allograft recipients who had undergone lung transplantation at least 1 month earlier were recruited when undergoing routine surveillance transbronchial biopsy (TBB) and bronchoalveolar lavage (BAL). All patients had stable lung function (FEV<sub>1</sub>, FEF<sub>25–75</sub>), no evidence of infection or acute rejection and, in particular, no evidence of BOS. Full patient data are given in table 1. Four volunteer controls were recruited to provide normal endobronchial biopsy specimens.

For further evaluation of non-transplanted airways, explanted tissue blocks from 30 lungs were collected. These were from donor lungs assessed for, but not used in, the Freeman lung transplantation program.

The study was approved by the local research ethics committee and written consent was obtained from all participants. Separate applications were obtained for lung transplant patients and normal control volunteers.

### Bronchoscopy, BAL, and TBB

Subjects were premedicated with intravenous midazolam. 4% lignocaine was applied topically to the nose, pharynx and larynx and below the cords in 1 ml aliquots, as required, up to a maximum dose of 8 mg/kg body weight.

### Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage used a standardised 3×60 ml procedure.<sup>8</sup> Samples were routinely processed for clinical microbiological assessment and differential cell counts performed on Giemsa stained cytocentrifuged preparations.

### Transbronchial biopsy (TBB) processing

Five to seven TBB specimens were taken at each bronchoscopy, fixed in 10% formalin, embedded in paraffin, and stained with haematoxylin and eosin. These TBB specimens were not used for research purposes but rather fulfilled a clinical requirement to assess acute or chronic rejection according to a pulmonary pathologist. TBB specimens were not taken from normal controls.

### Endobronchial biopsy (EBB) processing

Six EBB specimens were taken from around the segmental subcarinae of the right lower lobe of each patient using alligator forceps (Olympus code no. FB 15C) and immediately taken to the laboratory for further processing by an investigator. They were placed in OCT embedding medium (Sakura Finetek) and snap frozen on a liquid N<sub>2</sub> isopentane slurry. Coded blocks were cut by a blinded experienced individual (7  $\mu$ m sections, Thermo Shandon Cryotome E). These samples were used for assessment of markers of EMT in vivo.

### EBB staining and quantification of S100A4

A modified immunoperoxidase staining method was used using a polyclonal antibody (S100A4, A5114; Dako 1:200) or rabbit immunoglobulin as a negative control and an indirect reporter system kit (Envision; Dako). Positive staining was developed with diaminobenzadine chromogen and the slides were counterstained with Mayer's haematoxylin.

**Table 1** Demographic details of lung allograft recipients

| Subject  | S100A4 (% epithelia)* | Age at transplant (years) | Sex | Pretransplant diagnosis | Operation | Time after operation (months) | BOS stage† | TBBX‡   |
|----------|-----------------------|---------------------------|-----|-------------------------|-----------|-------------------------------|------------|---------|
| 1        | 7                     | 39                        | M   | CF                      | BLT       | 6                             | 0          | a0b0    |
| 2        | 13                    | 55                        | F   | CFA                     | SLT       | 1                             | 0          | a0b1    |
| 3        | 27                    | 25                        | F   | CF                      | BLT       | 3                             | 0          | a1/2bx  |
| 4        | 42                    | 31                        | F   | CF                      | BLT       | 12                            | 0          | a1b1    |
| 5        | 0                     | 37                        | M   | Emphysema               | BLT       | 6                             | 0          | a1b1    |
| 6        | 8                     | 32                        | M   | CF                      | BLT       | 6                             | 0          | a1/2 b1 |
| 7        | 32                    | 49                        | M   | Emphysema               | BLT       | 3                             | 0          | a1bx    |
| 8        | 13                    | 28                        | M   | CF                      | BLT       | 3                             | 0          | a1bx    |
| 9        | 16                    | 59                        | F   | Emphysema               | SLT       | 12                            | 0          | a0b0    |
| 10       | 48                    | 40                        | M   | Fibrosis (RA)           | SLT       | 12                            | 0          | a0bx    |
| Cultures |                       |                           |     |                         |           |                               |            |         |
| 1        | –                     | 39                        | M   | CF                      | BLT       | 6                             | 0          | a0b0    |
| 9        | –                     | 59                        | F   | Emphysema               | SLT       | 12                            | 0          | a0b0    |
| 11       | –                     | 28                        | F   | CF                      | BLT       | 6                             | 0          | a0bx    |
| 12       | –                     | 51                        | F   | Emphysema               | BLT       | 6                             | 0          | a0b0    |
| 13       | –                     | 47                        | F   | Emphysema               | SLT       | 12                            | 0          | a1b0    |
| 14       | –                     | 49                        | F   | Emphysema               | SLT       | 12                            | 0          | a1bx    |
| 15       | –                     | 22                        | F   | CF                      | BLT       | 7                             | 0          | axbx    |
| 16       | –                     | 23                        | M   | CF                      | BLT       | 1                             | 1          | a0b0    |

CF, cystic fibrosis; CFA, cryptogenic fibrosing alveolitis; RA, rheumatoid arthritis; SLT, single lung transplantation; BLT, double lung transplantation; BOS, bronchiolitis obliterans syndrome; TBBX, transbronchial biopsy.

\*S100A4 (% epithelia), % of biopsy epithelium stained for S100A4.

†BOS stage: 0=no BOS, 1=FEV<sub>1,0</sub> 60–80% best after transplantation.

‡TBBX, transbronchial biopsy assessed by pathologist for rejection: a=acute rejection, <a1 non-significant, b=airway assessment, axbx=no material for assessment.

Bronchial epithelial staining for S100A4 was quantified in at least five non-overlapping  $\times 40$  objective high power fields of the biopsy sections as previously reported.<sup>8</sup> An interactive computer based image analysis system was used (Image Pro Plus 4.0, USA) with results expressed as the percentage of epithelium staining positively for S100A4.

In three subjects with positive staining for S100A4, staining of separate sequential sections with intact epithelium was possible. These sections were stained with a monoclonal antibody for MMP-7 (Oncogene Research Products) as a further marker of EMT and Ki67 as a marker of epithelial proliferation. Staining was developed using an indirect reporter system (Envision; Dako) and diaminobenzidine chromogen, and the slides were counterstained with Mayer's haematoxylin.

### Processing and staining of non-transplant resected lung blocks

Three to five airway blocks were dissected out from each of 30 donor lungs evaluated for, but not used in, the Freeman lung transplant program, which were then archived. Blocks were taken with the aim of providing intermediate/large airways of 1–5 mm diameter with intact columnar airway epithelia. These blocks were fixed in 10% formalin, embedded in paraffin, and stained with haematoxylin and eosin for assessment of possible pathological changes by a consultant histopathologist. If sections were assessed as being free from fibrosis and significant inflammatory changes, they were subjected to high temperature antigen retrieval in a citrate buffer and subsequently stained for S100A4 using an immunoperoxidase method.

### Primary bronchial epithelial cell cultures (PBECS) from endobronchial brushings

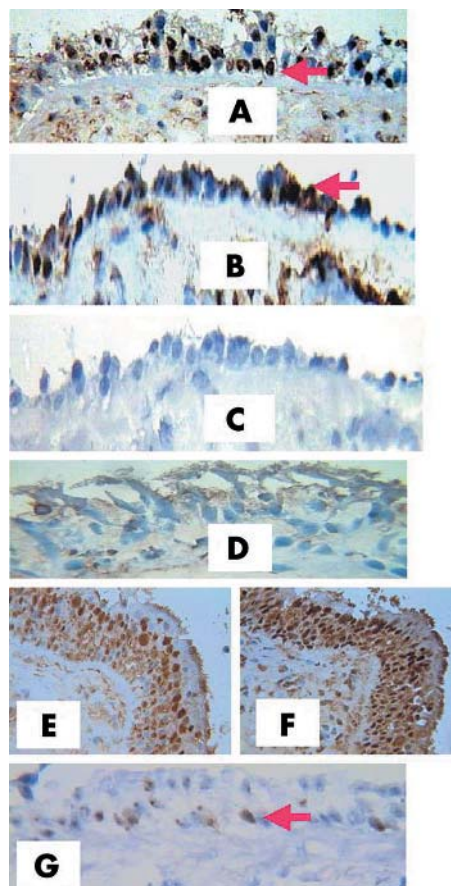
Airway brushings from six allograft recipients were performed using a previously described method.<sup>25</sup> The primary bronchial epithelial cells (PBECS) isolated from the allograft were cultured in 25 cm<sup>2</sup> collagen coated (Vitrogen 100, Nutacon) flasks to confluence in Basal Epithelial Growth Medium (Clonetics, Cambrex Bioproducts) with 1% penicillin/streptomycin.  $5 \times 10^4$  cells were transferred to 8-chamber slides (Lab-Tek, Nunc) and grown to confluence for experimentation, staining, and confocal microscopy or into chambers for invasion studies.

### Immunocytochemistry of non-stimulated PBECS

Eight chamber slides from four allograft recipients were fixed in 4% phosphate buffered paraformaldehyde and permeabilised with 1% Triton X-100 (Sigma). The cells were then treated with 5% normal lamb serum in phosphate buffered saline (PBS, Sigma) for 1 hour to minimise non-specific antibody binding, then incubated with primary antibodies specific for S100A4 (1:25, Polyclonal, DAKO) and cytokeratin (1:50, monoclonal, DAKO) in normal lamb serum for 2 hours at room temperature.<sup>23</sup> After washing with PBS, the slides were treated with appropriate FITC conjugated secondary reagents. Final incubation was carried out for 1 hour at room temperature in the dark before mounting in fluorescence mounting medium (DakoCytomation). Rabbit IgG was the negative control and DAPI was included as a nuclear counterstain. The slides were examined using confocal microscopy.<sup>23</sup>

### Measurement of MMP-2 and MMP-9 protein from PBECS

PBECS from six allograft recipients were grown to confluence and serum starved for 24 hours before the addition of 0.05, 0.5, 5 or 50 ng/ml TGF- $\beta_1$  (Biosource) for 72 hours with cell medium as control. Supernatants were assayed for MMP-2



**Figure 1A–G** Epithelial-mesenchymal transition in vivo. Representative S100A4 positive staining in airway biopsy specimens from lung allografts (B and C) together with a negative staining control (A). S100A4 staining (arrowed) was sometimes observed in areas of the epithelium where morphology was consistent with injury, with only residual basal epithelium remaining on the section (B, same subject as control section). (D) Biopsy specimen from a normal volunteer was borderline positive for epithelial S100A4 due to staining in intraepithelial immune cells but not in epithelial cells. In some subjects sections sequential to S100A4 staining (E) with intact airway epithelium were available and stained positively for the collagenase MMP-7 (F), a further marker of EMT.<sup>21–22</sup> This was in the context of a frankly hyperplastic and proliferative epithelium expressing Ki67 in one subject (G).

and MMP-9 total protein using a commercially available ELISA (R&D Ltd) used in accordance with the manufacturer's instructions.

### Zymographic analysis of MMP-2 and MMP-9 from PBECS

PBECS from six allograft recipients were grown to confluence and serum starved for 24 hours before the addition of 10 ng/ml TGF- $\beta_1$  (Biosource) for 72 hours or cell medium as control. Supernatants were run on SDS-PAGE gelatin zymograms in the presence or absence of a specific MMP inhibitor (2 mM 1,10-phenanthroline (1,10-PA)) and analysed by densitometry.

### Invasion assays

Spontaneous and TGF- $\beta_1$  stimulated invasion of PBECS up to 72 hours was evaluated by assessing penetration of collagen (Matrigel) coated millipore filters contained in commercially available invasion chambers. These were used according to the manufacturer's protocol (8  $\mu$ m pores; BioCoat Invasion Chamber, Becton Dickinson). Briefly, 50 000 cells in 200  $\mu$ l

cell medium, with or without 10 ng/ml TGF- $\beta_1$  (Biosource), were loaded in the upper chamber of the invasion chambers. The lower chamber contained 800  $\mu$ l of basal epithelial growth. Invasive cells were determined following Romanovsky staining and scored at bright field microscopy by a blinded observer. Assays were performed in triplicate using cells derived from four separate PBEC primary cell lines.

### Statistical analysis

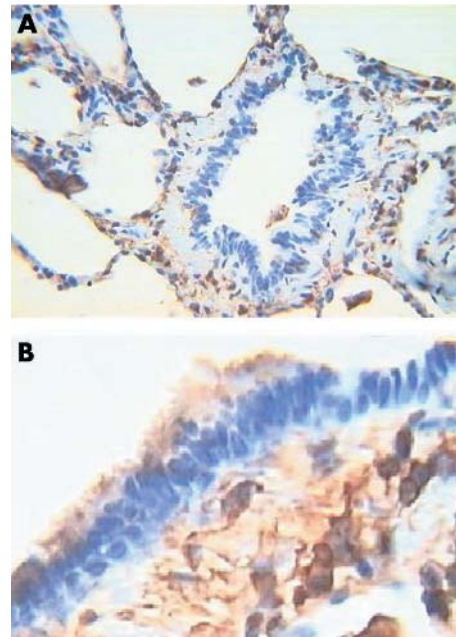
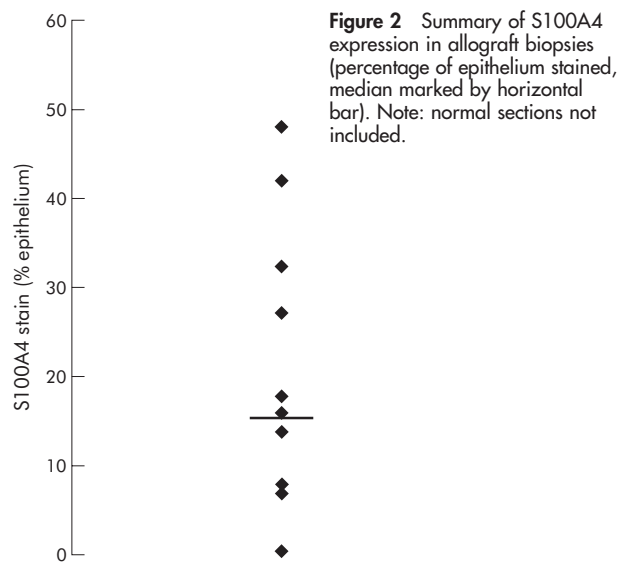
Non-parametric methods were used for non-normally distributed data and parametric methods for normal data. Medians and ranges are quoted for descriptive, non-parametric data and means for normally distributed data. Non-parametric comparisons were made using the Mann-Whitney test for unpaired data and the unpaired *t* test was used for normally distributed data. *p* values of <0.05 were considered significant.

## RESULTS

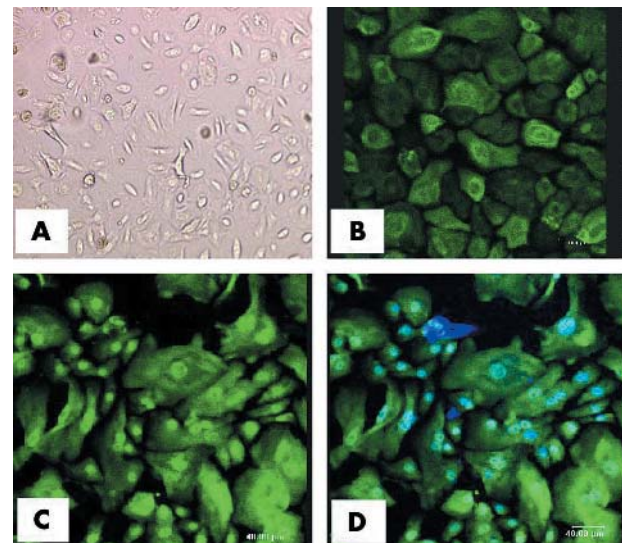
### Expression of markers of EMT and epithelial activation in vivo

Ten allograft biopsy specimens gave evaluable epithelial S100A4 staining. Seven of the 10 biopsy specimens were positive for S100A4 (>10% of the epithelium stained, evidence of nuclear and cytoplasmic staining; fig 1A and B). S100A4 staining was observed in areas of the epithelium where morphology was consistent with injury, with only residual basal epithelium remaining (fig 1B). Figure 1C is the rabbit immunoglobulin control for the section in fig 1B.

The median percentage of epithelium stained positive for S100A4 in allograft biopsies was 15% (range 0–48%, fig 2). One biopsy specimen from four normal volunteer controls gave evaluable epithelial S100A4 staining. Minimal staining was seen due to staining within intraepithelial immune cells and not epithelial cells themselves (fig 1D). In three subjects with positive staining for S100A4 evaluation of separate sequential sections with a monoclonal antibody for MMP-7 was possible. This revealed epithelial expression of the collagenase MMP-7 (fig 1E and F), a further marker of EMT,<sup>21, 22</sup> and this was in the context of a frankly hyperplastic and proliferative epithelium expressing Ki67 in one subject (fig 1G).



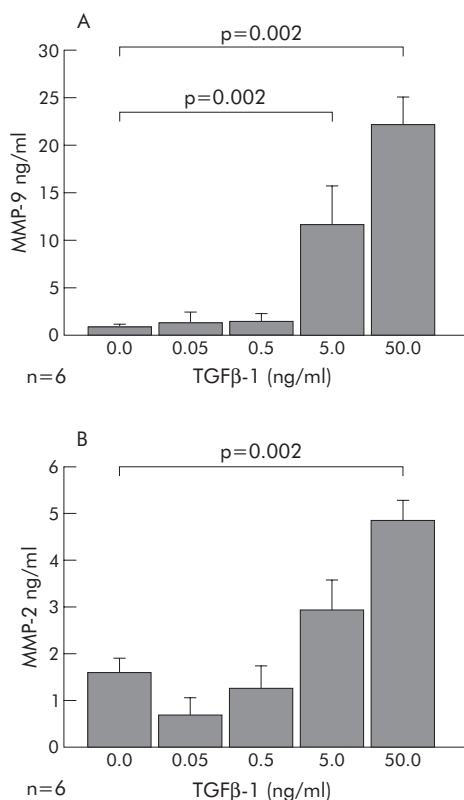
**Figure 3** In non-transplanted explanted donor lung, borderline levels of epithelial layer S100A4 staining were observed but, where present, this was due to staining in intraepithelial immune cells, not epithelial cells.



**Figure 4** Primary bronchial epithelial cell (PBEC) phenotype and S100A4 staining in non-stimulated PBECs. (A) Cultured cells retained the morphological appearance of epithelial cells (Brightfield light microscopy of unstained chamber slide) and (B) expressed cyokeratin (green, FITC staining). (C) S100A4 was expressed in all of the epithelial cultures at baseline (green, FITC staining) with (D) some nuclear co-localisation (blue, DAPI nuclear counterstain).

### Expression of S100A4 in explanted lung

Of the 30 lungs evaluated for, but not used, in the Freeman lung transplant program which were formally assessed for pathological changes, seven were assessed as being free from fibrosis with “normal bronchioles and bronchi”. There was some inflammation reported as “marginating neutrophils” by the examining pathologist. Of these seven samples, airways tissue with preserved columnar epithelium was available in four subjects and these sections were stained for S100A4. In these paraffin embedded sections minimal



**Figure 5** Measurable MMP-2 and MMP-9 protein levels were detected in all cultures. TGF-β<sub>1</sub> stimulated cells produced a reproducible increase in MMP levels.

staining was seen in the airway epithelium and, where present, this was due to staining within intraepithelial immune and inflammatory cells and not the epithelial cells themselves (fig 3A and B).

#### PBEC phenotype and S100A4 staining in non-stimulated PBECs

Cultured cells retained the morphological appearance of epithelial cells (fig 4A) and expressed cytokeratin, a phenotypic marker for epithelia (fig 4B). S100A4 (fig 4C) was expressed in all PBECs at baseline, with some nuclear localisation (fig 4D).

#### MMP-2 and MMP-9 protein production in PBEC supernatants

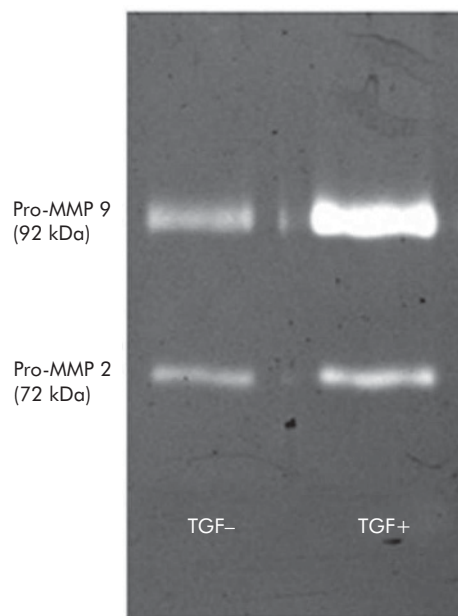
Measurable MMP-2 and MMP-9 protein levels were detected in all cultures. TGF-β<sub>1</sub> stimulated cells produced a reproducible increase in MMP levels (fig 5A).

#### PBEC culture supernatant MMP zymography

Zymography confirmed the presence of MMP activity corresponding to pro-MMP-9 and pro-MMP-2. TGF-β<sub>1</sub> stimulated cells produced a reproducible increase in MMPs in all PBECs confirmed by densitometry: mean MMP-9 170 units at baseline v 708 units after TGF-β<sub>1</sub> (n = 6, p = 0.03, fig 6). MMP activity was abolished by 1,10-PA.

#### Invasion assays

Invasion assays confirmed the ability of morphologically distinct non-stimulated PBECs to penetrate Matrigel coated millipore filters spontaneously after 24 hours (fig 7A, C). This was significantly increased following TGF-β<sub>1</sub> stimulation (figs 7B and 8).



**Figure 6** Primary bronchial epithelial cell (PBEC) gelatin zymography for MMPs. An illustrative example of a PBEC supernatant gelatin zymogram showing Pro-MMP-2 (72 kDa) and Pro-MMP-9 (92 kDa) activity sensitive to an MMP inhibitor (2 mM 1,10-phenanthroline). TGF-β<sub>1</sub> stimulated cells (10 ng/ml, 72 hours) produced a reproducible increase in MMPs in all PBECs confirmed by densitometry. Mean MMP-9 170 units at baseline v 708 units after TGF-β<sub>1</sub> (p = 0.03, n = 6 cell lines).

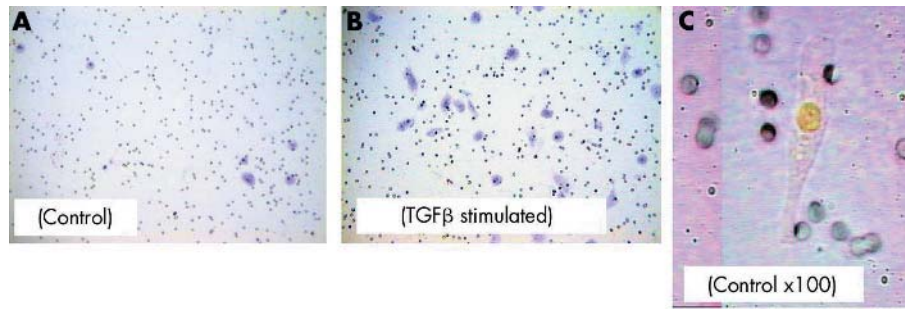
#### DISCUSSION

We have shown phenotypic and functional markers of EMT in both in vivo biopsy specimens and ex vivo primary epithelial cell cultures from lung allograft recipients. These preliminary data support the hypothesis that fibroblasts may originate directly from human airway epithelial cells as a result of EMT, and are the first such evidence in the human airway of which we are aware. Our work follows current focus on EMT, including its role in organ fibrosis which has been comprehensively reviewed.<sup>17</sup>

While axiomatic in developmental biology and other disciplines, EMT has not to date been studied in lung transplantation or inflammatory airways disease. EMT is a widely recognised mechanism in organogenesis, fibroblast formation in injured tissues, and as an initiator of metastasis in epithelial cancer.<sup>16 17 26</sup> During transition the epithelia lose polarity, tight junctions, and the cytokeratins that characterise aggregated and organised epithelial units.<sup>17</sup> Transiting epithelia become motile with upregulation of cytoskeletal machinery and expression of filopodia, while an increasing capacity to secrete MMPs as part of an emergent EMT proteome mediates movement through the basement membrane and tissues.<sup>17</sup>

Expression of the human homologue of fibroblast specific protein 1 (S100A4) has become an important tool for studying EMT. This is because S100A4 is expressed on both fibroblasts and is an early marker of EMT, as shown by direct observation in transgenic mice with epithelium marked with green fluorescent protein.<sup>27</sup>

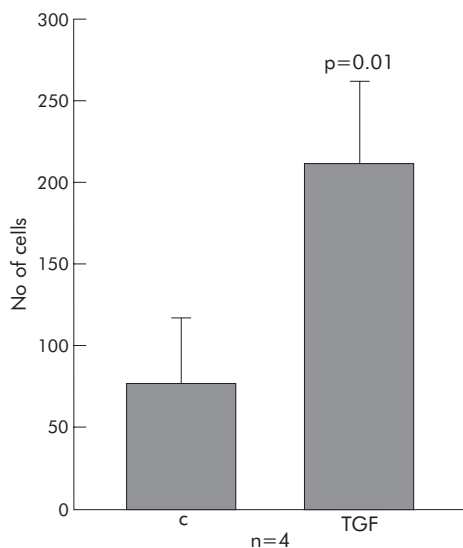
In the present study we showed epithelial expression of S100A4 in large airway biopsy specimens from clinically stable lung allografts without airflow limitation (BOS 0). Expression was both cytoplasmic and nuclear, the potential importance of this being that nuclear expression strongly correlates with functional EMT and metastatic disease in the oncology literature.<sup>18</sup> Image analysis showed that a



**Figure 7** Primary bronchial epithelial cell invasion of Matrigel coated millipore filters. Migration assays ( $n=4$  cultures) confirmed the ability of morphologically distinct epithelial cells to spontaneously penetrate Matrigel coated millipore filter barriers spontaneously at 24 hours (A, C). This was increased with TGF- $\beta_1$  stimulation (10 ng/ml) for 72 hours (B).

significant area of the epithelia available for scoring had S100A4 expression, indicating that the potential for EMT may not be uncommon in the allograft airway. Emergent evidence from the kidney suggests that over a third of disease related fibroblasts might originate from epithelia at the site of injury.<sup>17</sup> Staining of sequential sections with intact epithelium in snap frozen endobronchial biopsies is technically difficult but, where staining was possible, we showed that expression of S100A4 was accompanied by staining for MMP-7. MMP-7 is a collagenase and recognised marker of EMT in murine and human studies.<sup>21–22</sup> Collagen type IV, which makes up the true reticular basement membrane of airway epithelia, is a substrate for MMP-7 and this enzyme can therefore disrupt the basement membrane of differentiated epithelia, promoting translocation, damage, inflammation and further EMT.<sup>17</sup>

Our study included limited normal control data from airway biopsy specimens taken from four volunteers. The small number of these results was mainly due to the low success rate (25%) of sampling intact bronchial epithelium in normal subjects, confirming our previous experience.<sup>8–12–14</sup> This was backed up by evaluating tissue blocks from 30 lungs evaluated for, but not used in, the Freeman lung transplant program which were formally assessed as being free from fibrosis with “normal bronchioles and bronchi”. These data were from an archive and were only available in paraffin



**Figure 8** Summary of migration assay data (number of epithelial cells penetrating through a collagen matrix) with and without TGF- $\beta_1$  stimulation (10 ng/ml).

embedded sections; they cannot be compared quantitatively with data from snap frozen airway biopsies. It was of interest, however, that the minimal positive staining for S100A4 in the airway epithelium in these specimens was due to staining within intraepithelial immune or inflammatory cells rather than the airway epithelium.

The EMT process is a longitudinal series of events and we recognise the limitations of cross sectional studies of biopsy specimens taken at single time points. In complementary work we therefore set up PBECs from airway brushings from allograft recipients which included longitudinal ex vivo stimulation experiments. We felt that use of human tissue for studies was appropriate because much of the previous work on EMT has been in animal models or commercial cell lines.

In our culture system we again showed S100A4 expression, with all cultures showing positivity including nuclear expression denoted by dual label confocal microscopy. In further PBEC culture experiments we showed production of other markers of the EMT proteome<sup>17</sup> including functional potential using gelatin zymography. MMP-2 (72 kDa gelatinase) and MMP-9 (92 kDa gelatinase) are particularly strategic and widely studied in the oncology literature and metastatic disease. The substrate of MMP-2 and MMP-9 (type IV collagen) is a principal component of epithelial basement membrane. The demonstration of MMP-2 and MMP-9 protein and activity in non-stimulated and TGF- $\beta$  stimulated epithelial cell cultures is therefore directly complementary to the S100A4 staining data in ex vivo cultures and in vivo airway biopsies. As well as showing a TGF- $\beta$  inducible increased release of MMP-2 and MMP-9, we confirmed that epithelial cells were functionally invasive and able to penetrate filters with a mixed matrix coating. Again this functional marker of EMT was significantly increased by TGF- $\beta$  stimulation.

Our study is novel but should be considered as preliminary. In particular, both the cell culture and biopsy data in isolation could reasonably be criticised. Loss of cell phenotype in prolonged culture is well described, while airway biopsies are small and difficult to work with. However, our culture experiments were all performed at the point where cells were sub-confluent (around passage 2) on cytokeratin positive cells with epithelial cell morphology. The present findings are also supported by our description of MMP-9 and MMP-7 mRNA and protein expression in lung allograft airway epithelium.<sup>28</sup> Our observation that some S100A4/MMP7 positive epithelia showed frank hyperplasia (fig 1E, F) suggests a mechanism for amplification of this fibrogenic process. Importantly, our work shows—in separate in vivo and ex vivo systems—complementary evidence of early and late markers of EMT in allografts, together with functional assessments indicating that epithelial cells invade collagen

matrices and that this is stimulated by TGF- $\beta$  which is increased in lung allografts.<sup>24</sup>

In common with the relatively more mature literature on MMPs,<sup>29, 30</sup> this initial description of EMT in allografts does not indicate whether EMT is a homeostatic reparative or a deleterious pathological mechanism. This is a key question and, together with others, requires further translational studies of EMT and airway fibrosis.

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