

## ORIGINAL PAPER

# Heterogeneous response of nasal and lung fibroblasts to transforming growth factor- $\beta_1$

M. Nonaka, R. Pawankar, A. Fukumoto and T. Yagi

Department of Otorhinolaryngology, Nippon Medical School, Tokyo, Japan

## Clinical and Experimental Allergy

### Summary

**Background** Nasal polyposis is characterized by marked oedema, sparse extracellular matrix (ECM) and proliferating blood vessels. Pulmonary fibrosis is characterized by inflammatory cells accumulation, considerable ECM deposition and vascular abnormalities. Although lung fibrosis is not only and necessarily an inflammatory disorder, we hypothesized that the difference between nasal polyposis and pulmonary fibrosis may, in part, be due to the heterogeneity between nasal and lung fibroblasts. Fibroblasts participate in the inflammatory response by releasing ECM proteins and cytokines. TGF- $\beta$  is thought to participate in chronic inflammation and fibrosis. Myofibroblasts are the activated form of fibroblasts. A phenotypic hallmark of myofibroblasts is the expression of smooth muscle  $\alpha$ -actin (SMA).

**Objective** We examined whether there is any heterogeneity between nasal and lung fibroblasts upon stimulation with TGF- $\beta_1$  with regard to the synthesis of SMA, pro-collagen type I and vascular endothelial growth factor (VEGF) as well as translocation of Smad proteins.

**Methods** Fibroblasts lines were established from human biopsy tissue. The expression of SMA, pro-collagen type I, VEGF mRNA was evaluated by reverse transcriptase RT-PCR. The amount of pro-collagen type I and VEGF was measured by ELISA. By immunocytochemistry, we analysed the expression of SMA and Smad2, 3, 4 in cultured fibroblasts.

**Results** TGF- $\beta_1$  induced SMA and pro-collagen type I synthesis in lung, but not in nasal fibroblasts. By contrast, TGF- $\beta_1$  induced VEGF synthesis in both lung and nasal fibroblasts. After stimulation with TGF- $\beta_1$ , Smad2, 3, 4 were translocated from the cytoplasm to the nucleus in lung fibroblasts, whereas only Smad3 was translocated in nasal fibroblasts.

**Conclusion** These results establish the heterogeneous responsiveness of fibroblast populations in the airways to TGF- $\beta_1$  and that such a heterogeneity may contribute, at least in part, to the different pathological outcomes of inflammation in the upper and lower airways.

**Keywords** fibroblasts, pro-collagen type I, Smad, smooth muscle  $\alpha$ -actin, TGF- $\beta_1$ , VEGF

*Submitted 12 July 2006; revised 4 January 2008; accepted 15 January 2008*

### Correspondence:

Manabu Nonaka, Department of Otorhinolaryngology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan.  
E-mail: nonaka@nms.ac.jp

### Introduction

Nasal polyposis is characterized by marked oedema, sparse extracellular matrix (ECM) and proliferating blood vessels [1]. Pulmonary fibrosis is characterized by inflammatory cell accumulation, considerable ECM deposition and vascular abnormalities [2]. Although lung fibrosis is not only and necessarily an inflammatory disorder, it is well known that fibroblasts are the main source of ECM proteins such as collagens, thus contributing to the fibrotic process. Tissue-specific heterogeneity in fibroblasts function or disease-specific heterogeneity with respect to proliferative features and cytokines expression

has already been described [3–5]. However, the differences within the same tissue appear to be less dramatic than those between tissues [3–5]. TGF- $\beta$  is a 25 kDa homodimeric or heterodimeric protein that can mediate a broad spectrum of biological activities [6]. TGF- $\beta$  was initially recognized by its ability to transform normal rat fibroblasts into myofibroblasts *in vitro* [7]. It has been subsequently shown that this factor induces fibroblast smooth muscle  $\alpha$ -actin (SMA) expression, and enhances fibroblast pro-collagen type I production [8, 9]. The study on the intracellular signal transduction system of TGF- $\beta$  has progressed and an important role of the Smad family was revealed [10]. Recently, the importance of Smad proteins

in SMA expression in fibroblasts has been clarified [11]. There is no doubt that TGF- $\beta$  has an important role in the development of chronic inflammation and fibrosis. In addition, it has recently been shown that fibroblasts and other tissue structural cells can also produce a variety of cytokines such as vascular endothelial growth factor (VEGF), suggesting a role for these cells as a secondary source of mediators in the amplification of the inflammatory response. VEGF is a new family of secreted growth factors capable of directly stimulating endothelial cells proliferation [12]. Members of this family have been referred to as angiogenic factor, VEGF, and vascular permeability factor [13], but molecular cloning has shown that all derive from the same gene [12, 13]. It was recently reported that VEGF is expressed in inflamed tissue in the airways, where it is thought to be produced by structural cells such as fibroblasts and epithelial cells, and activated macrophages upon stimulation by TGF- $\beta$  [14–17]. VEGF production by fibroblasts may play an important role in the vascular changes, one of the features of airway remodelling. Therefore, the present study was designed to compare the effects of TGF- $\beta$  on primary fibroblast lines derived from nasal tissue (upper airway) and lung tissue (lower airway).

## Materials and methods

### Cells sources and culture

Primary fibroblasts lines were established from human biopsied tissue and characterized as previously described [4, 18]. Nasal polyp fibroblasts lines were derived from seven patients with chronic rhinosinusitis with nasal polyps (four males and three females aged  $59.4 \pm 11.9$  years (mean  $\pm$  SEM)) who underwent polypectomy. Normal nasal fibroblasts lines were established from the uncinat process of six patients (three males and three females aged  $50.2 \pm 18.3$  years (mean  $\pm$  SEM)) who underwent surgery for a blowout fracture. Nasal fibroblast lines were used between the fourth and ninth passages. Normal lung fibroblasts lines were derived from normal areas of surgically excised lung specimens from six patients (two males and four females aged  $60.3 \pm 12.3$  years (mean  $\pm$  SEM)) undergoing resective surgery for lung cancer. Fibrotic lung fibroblasts lines were established from histologically proven fibrotic lung tissue of six patients (four males and two females aged  $62.3 \pm 10.8$  years (mean  $\pm$  SEM)) with active interstitial lung disease and lung cancer. Lung fibroblast lines were used between the fourth and 10th passages. All specimens were taken from patients who had not received any inhaled or systemic steroids, chemotherapy or irradiation. All subjects had given written informed consent, and the study protocol was approved by the Ethical Committee of Nippon Medical School Hospital.

Before reverse transcriptase RT-PCR, fibroblasts ( $2 \times 10^6$  cells) were plated in 100 mm culture dishes (Corning, NY, USA). Before pro-collagen type I and VEGF assays, fibroblasts ( $1 \times 10^5$  cells) were plated in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA). Plated fibroblasts were allowed to grow to subconfluence in regular growth medium (RGM; Dulbecco's modified Eagle medium containing L-glutamine, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 2.5  $\mu$ g/mL amphotericin B) containing 10% foetal bovine serum (FBS). Twenty-four hours before the experiments, the FBS concentration was reduced to 0.4%. This concentration of FBS was used for the rest of the experiments. At specified time points, cells were harvested to extract RNA, and the supernatant was collected and centrifuged for the pro-collagen type I and VEGF protein assays.

### *Analysis of vascular endothelial growth factor, smooth muscle $\alpha$ -actin and pro-collagen type I messenger ribonucleic acid expression by reverse transcription polymerase chain reaction*

Specific sense (forward) and antisense (reverse) primers for SMA, pro-collagen type I and VEGF were constructed according to the published sequences. Sequence of the PCR primers for SMA were sense 5' CCACCCTGCTCAG GAGGCAC 3' and antisense 5' CATAGTGGTGCCCCCTGA TAG 3' (product size 611 bp) (NM001613); for pro-collagen were sense 5' GTCTTCCTGGCCCCCTCTGGTG 3' and antisense 5' GCGGGACCAGCAGGACCAGTC 3' (product size 303 bp) (NM\_000088); for VEGF were sense 5' CACATAG GAGAGATGAGCTTC 3' and antisense 5' CCGCCTCGGCTT GTACAT 3' (product size 228 bp) (m32977). At specified time points, cells were collected to extract RNA. Total RNA was extracted from fibroblasts cultured with or without TGF- $\beta_1$  at various doses, and reverse transcribed as previously described [19]. SMA, pro-collagen type I and VEGF gene segments from the resultant cDNA samples were PCR amplified in the presence of specific sense and antisense primers (1  $\mu$ M each), 200  $\mu$ M dNTP, 0.5 U/ $\mu$ L Ampi Taq (Cetus Corp., Emeryville, CA, USA), 1 U Perfect Match (Stratagene, La Jolla, CA, USA) and PCR buffer (2.5  $\mu$ M MgCl<sub>2</sub>, 50  $\mu$ M KCl, 10  $\mu$ M Tris-HCl, pH 8.3, 0.001% gelatin) in a final reaction volume of 20  $\mu$ L. PCR was performed for 30 cycles, each cycle including denaturation (94 °C, 1 min), annealing (60 °C, 2 min) and extension (72 °C, 3 min) and a final incubation (72 °C, 10 min) after the last cycle [20].  $\beta$ -Actin cDNA was amplified as the internal control [20] and cDNA was substituted with distilled water (DW) as the negative control. PCR products were electrophoresed onto 2% agarose gels containing ethidium bromide, and blotted onto nylon membranes; the DNA fragments were detected using an alkaline phosphatase-labelled anti-digoxigenin antibody and visualized using the chemiluminiscent substrate AMPPD,

as previously described [20]. PCR products were semi-quantified relative to  $\beta$ -actin, the housekeeping gene, by densitometry using the Masterscan, Scanalytics (Fairfax, VA, USA).

#### *Pro-collagen type I and vascular endothelial growth factor assay*

The levels of pro-collagen type I and VEGF in the cultured supernatants were measured by enzyme immuno assay (EIA) (Takara Biomedicals, Tokyo, Japan) and ELISA (R&D Systems, Minneapolis, MN, USA), respectively. The sensitivity of these commercially available kits for pro-collagen type I and VEGF was 10 ng/mL and 5.0 pg/mL, respectively. The amounts of pro-collagen type I and VEGF are expressed in nanograms per  $1 \times 10^6$  cells recovered at the end of the culture period.

#### *Effect of transforming growth factor $\beta_1$ on smooth muscle $\alpha$ -actin expression in cultured airway fibroblasts*

We designed the following experiment to look at the long-term effect of TGF- $\beta_1$  on the phenotypic change of fibroblasts. Fibroblasts were plated in four-well Lab-Tek chamber slides (Nunc Inc., IL, USA) in RGM containing 10% FBS, at a density of 4500 cells/cm<sup>2</sup>. This was referred to as day 2. Twenty-four hours later (day 1), the FBS concentration was reduced to 0.4% in order to decrease the proliferating rate of fibroblasts, this concentration of FBS was used for the rest of the experiments. Twenty-four hours later (day 0), the medium was replaced by medium containing TGF- $\beta_1$  (Genzyme Co., Cambridge, MA, USA) at 8, 40, 200, 1000 pg/mL or diluent only (0 pg/mL TGF- $\beta_1$ ). At the end of day 6, the medium was discarded, cells were rinsed with PBS and the slides were fixed in PLP for 10 min and then washed in a solution of 15% sucrose in phosphate buffer (0.1 M, pH 7.4) for 10 min. After air-drying, the slides were stored at  $-70^\circ\text{C}$  until immunocytochemistry was carried out.

#### *Immunocytochemistry for smooth muscle $\alpha$ -actin*

Immunocytochemistry for SMA was performed as follows. To enhance permeability and block non-specific reactions, the slides were rinsed in Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 0.01 M HEPES buffer and 0.01% saponin (Sigma-Aldrich Co., St Louis, MO, USA) (DPBS [H+S]) for 10 min at room temperature (RT). To block non-specific binding, slides were pre-incubated in DPBS (H+S) containing 75% heat-inactivated human AB sera for 1 h and then in the same buffer with 25% heat-inactivated normal rabbit serum (Sigma) for 30 min at RT. Mouse monoclonal anti-human SMA (Sigma) was diluted in DPBS (H+S), containing 1% BSA at a concentration of 2  $\mu\text{g/mL}$ . Briefly,

after rinsing with DPBS (H+S), the slides were incubated overnight at  $4^\circ\text{C}$  with the monoclonal antibody and washed in the same buffer. Labelling of the monoclonal antibody was detected by the alkaline phosphate anti-alkaline phosphatase method (APAAP Kit, Dako, Carpinteria, CA, USA) following the manufacturer's instructions. For counterstaining, Mayer's haematoxylin solution (Sigma) was used, and specific controls were included in each staining run. Mouse Ig G2a (Zymed Laboratories Inc., San Francisco, CA, USA) was used as the negative control.

#### *Morphometric assessment of smooth muscle $\alpha$ -actin immunoreactivity*

The specimens were counted blindly by one person who was familiar with immunocytochemistry. Those cells that did not show any staining at all were considered negative, whereas those that showed even a minimal positive immunoreactivity were considered positive. Ten randomly selected fields on each slide were examined under a light microscope with an eyepiece graticule (0.25 mm<sup>2</sup>) and the number of positive cells in each area as well as the total number of cells in the same area was counted at  $\times 200$  magnification. The percentage of positive cells was calculated out of the total number of cells in each area and the average of 10 such areas was expressed as the percentage positive SMA cells (results are shown in Fig. 2). The within-observer coefficient for repeated counts was  $<5\%$ .

#### *Smad translocation in response to transforming growth factor $\beta_1$*

Fibroblasts were plated in four-well Lab-Tek chamber slides in RGM containing 10% FBS at a density of 9000 cells/cm<sup>2</sup>. Twenty-four hours later, the FBS concentration was reduced to 0.4%. Twenty-four hours later, the medium was replaced by medium containing 1 ng/mL TGF- $\beta_1$  or diluent only (0 pg/mL TGF- $\beta_1$ ). After incubation for 1 h, the slides were fixed in PLP for 10 min and then washed in a solution of 15% sucrose in phosphate buffer (0.1 M, pH 7.4) for 10 min. After air-drying, the slides were stored at  $-70^\circ\text{C}$  until the immunocytochemistry was carried out. Immunolocalization experiments were repeated three times.

#### *Immunocytochemistry for Smads*

Immunocytochemistry for Smads was performed as follows. The streptavidin-biotin system (Nichirei Inc., Tokyo, Japan) was used. Slides were rinsed twice in 0.1% TRIS buffer solution supplemented with 0.01% saponin (TRIS+) for 5 min at RT. To block endogenous peroxidase, slides were pre-incubated for 15 min in 3% H<sub>2</sub>O<sub>2</sub>.

Subsequently, they were incubated with goat polyclonal anti-human Smad2 (sc-6200) at a 1 : 100 dilution, Smad3 (sc-6202) at a 1 : 100 dilution or Smad4 (sc-1909) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a 1 : 100 dilution for 4 h. The slides were then washed in TRIS+ and incubated with biotinylated rabbit anti-goat antibody for 10 min at RT. After each incubation period, the slides were washed twice of 5 in TRIS+, for 5 min each time. Then, the slides were incubated with peroxidase-conjugated streptavidin for 5 min at RT followed by incubation in the chromogen solution 3-amino-9-ethyl-carbazide (DAKO). Specific controls were included in each run. Goat Ig G (Vector Laboratories Inc., Burlingame, CA, USA) was used as the negative control.

To verify the specificity of immunocytochemistry for Smads, absorption test was also performed as follows. Before immunocytochemistry for Smads, goat polyclonal anti-human Smad2 (sc-6200) at a 1 : 100 dilution, goat polyclonal anti-human Smad3 (sc-6202) at a 1 : 100 dilution and goat polyclonal anti-human Smad4 (sc-1909) at a 1 : 100 dilution were incubated with the blocking peptide (sc-6200P, 1 µg/mL) for sc-6200, the blocking peptide (sc-6202P, 1 µg/mL) for sc-6202 and the blocking peptide (sc-1909P, 1 µg/mL) for sc-1909, respectively, for 1 h. Then, immunocytochemistry for Smads was carried out to investigate whether the immunoreactivity of goat polyclonal anti-human Smad2 (sc-6200), goat polyclonal anti-human Smad3 (sc-6202) and goat polyclonal anti-human Smad4 (sc-1909) was abolished.

#### Statistical analysis

For statistical analysis, the Mann-Whitney *U*-test was used for intergroup comparisons, and Wilcoxon's signed rank test was used for paired data. A *P*-value of <0.05 was considered as statistically significant.

## Results

### *Effect of transforming growth factor $\beta_1$ on smooth muscle $\alpha$ -actin expression in fibroblasts*

**Reverse transcription polymerase chain reaction.** Unstimulated nasal and lung fibroblasts expressed a weak but specific message for SMA. Expression of SMA mRNA was enhanced in lung fibroblasts upon incubation with TGF- $\beta_1$  in a dose-dependant manner, but not in nasal fibroblasts (Fig. 1).

**Immunocytochemistry.** The morphology of cultured lung fibroblasts was more spindle shaped as compared to that of nasal fibroblasts in the absence of TGF- $\beta_1$  stimulation (Fig. 2A (e, j)). The percentage positive cells for SMA in the absence of TGF- $\beta_1$  tended to be higher in fibroblasts derived from inflamed tissues (nasal polyp and fibrotic lung) as compared with those from normal tissues (normal nasal mucosa and normal lung), but did not reach statistical significance (Fig. 2B). In lung fibroblasts, but not in nasal fibroblasts, TGF- $\beta_1$  increased the number of myofibroblasts (SMA-positive fibroblasts) in a dose-dependant manner (Fig. 2). The up-regulation of SMA in lung fibroblasts was similar whether they were inflamed or not (Fig. 2B).

### *Effect of transforming growth factor $\beta_1$ on pro-collagen type 1 synthesis by fibroblasts*

**Reverse transcription polymerase chain reaction.** Unstimulated nasal and lung fibroblasts expressed a weak message for pro-collagen type I. TGF- $\beta_1$  induced pro-collagen type I mRNA in lung fibroblasts, but not in nasal fibroblasts, in a dose-dependant manner (Fig. 3).

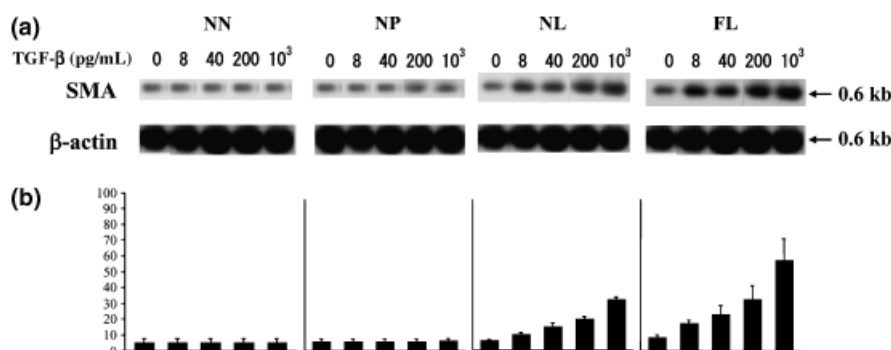


Fig. 1. (a) The representative results of SMA mRNA expression in normal nasal fibroblasts (NN), nasal polyp fibroblasts (NP), normal lung fibroblasts (NL) and fibrotic lung fibroblasts (FL), stimulated with different doses of TGF- $\beta_1$  for 24 h. (b) Densitometric analysis of SMA mRNA. The vertical bar represents percentage expression of SMA mRNA vs.  $\beta$ -actin mRNA. All values are mean  $\pm$  SEM of three experiments. SMA, smooth muscle  $\alpha$ -actin.

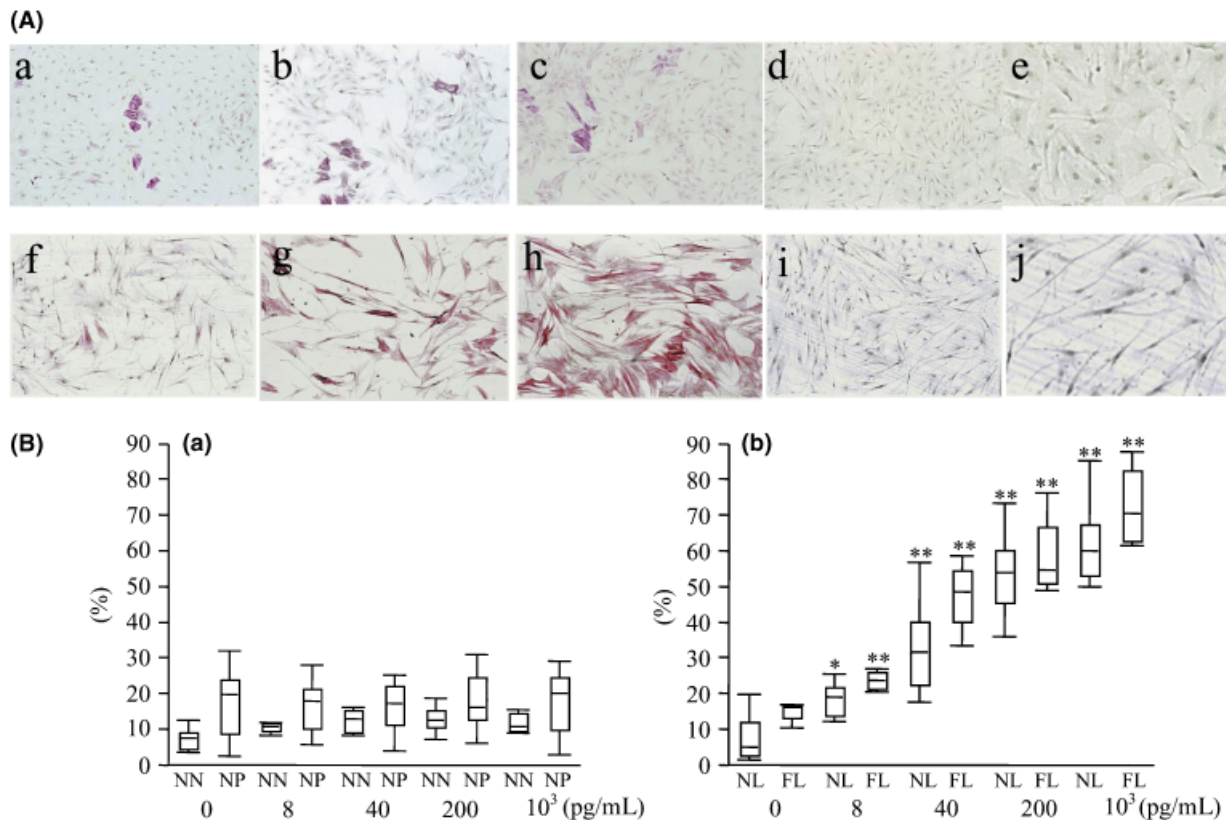


Fig. 2. (A) Representative immunocytochemical staining of SMA in normal nasal fibroblasts (a-e) and in normal lung fibroblasts (f-j) stimulated with (a, f) 0, (b, g) 40, (c, h) 1000 pg/mL TGF- $\beta_1$  for 6 days. (d, e, i, j) Non-immune mouse IgG1 revealed no specific staining [(d, i)  $\times 400$ , (e, j)  $\times 1000$ ]. (B) Induction of SMA in (a) nasal fibroblasts [NN, normal nasal fibroblasts ( $n = 6$ ); NP, nasal polyp fibroblasts ( $n = 7$ )] and in (b) lung fibroblasts [NL, normal lung fibroblasts ( $n = 6$ ); FL, fibrotic lung fibroblasts ( $n = 6$ )] stimulated with different doses of TGF- $\beta_1$  for 6 days. Box plots represent the median values with 25% and 75% interquartiles; the error bars represent the 10th and 90th percentiles. \* $P < 0.05$ , and \*\* $P < 0.01$ , vs. 0 pg/mL TGF- $\beta_1$ . SMA, smooth muscle  $\alpha$ -actin.

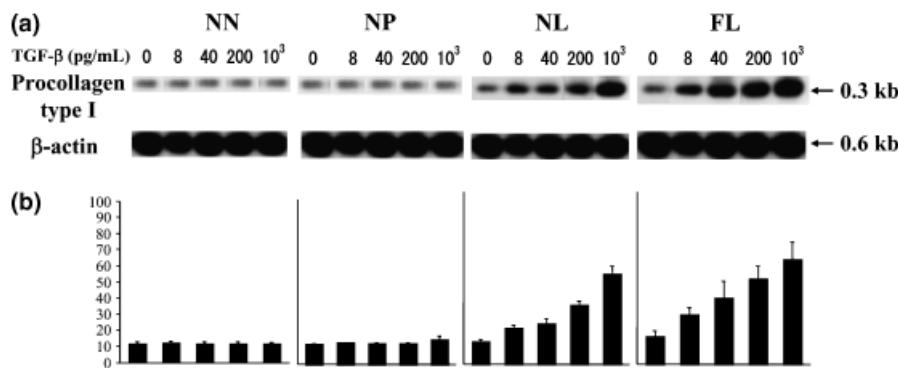


Fig. 3. (a) The representative results of procollagen type I mRNA expression in nasal fibroblasts (NN), nasal polyp fibroblasts (NP), normal lung fibroblasts (NL) and fibrotic lung fibroblasts (FL), stimulated with different doses of TGF- $\beta_1$  for 24 h. (b) Densitometric analysis of procollagen type I mRNA. The vertical bar represents percentage expression of procollagen type I mRNA vs.  $\beta$ -actin mRNA. All values are mean  $\pm$  SEM of three experiments.

**Enzyme-linked immunosorbent assay.** To determine whether airway fibroblasts can synthesize and release pro-collagen type I upon stimulation with TGF- $\beta_1$ , supernatants from cultured nasal and lung fibroblasts were assayed for pro-collagen type I content. In agreement with

the enhanced mRNA expression, TGF- $\beta_1$ -stimulated lung fibroblasts produced significantly greater amounts of pro-collagen type I as compared to the control. This production of pro-collagen type I upon stimulation with TGF- $\beta_1$  occurred in a dose- and time-dependent

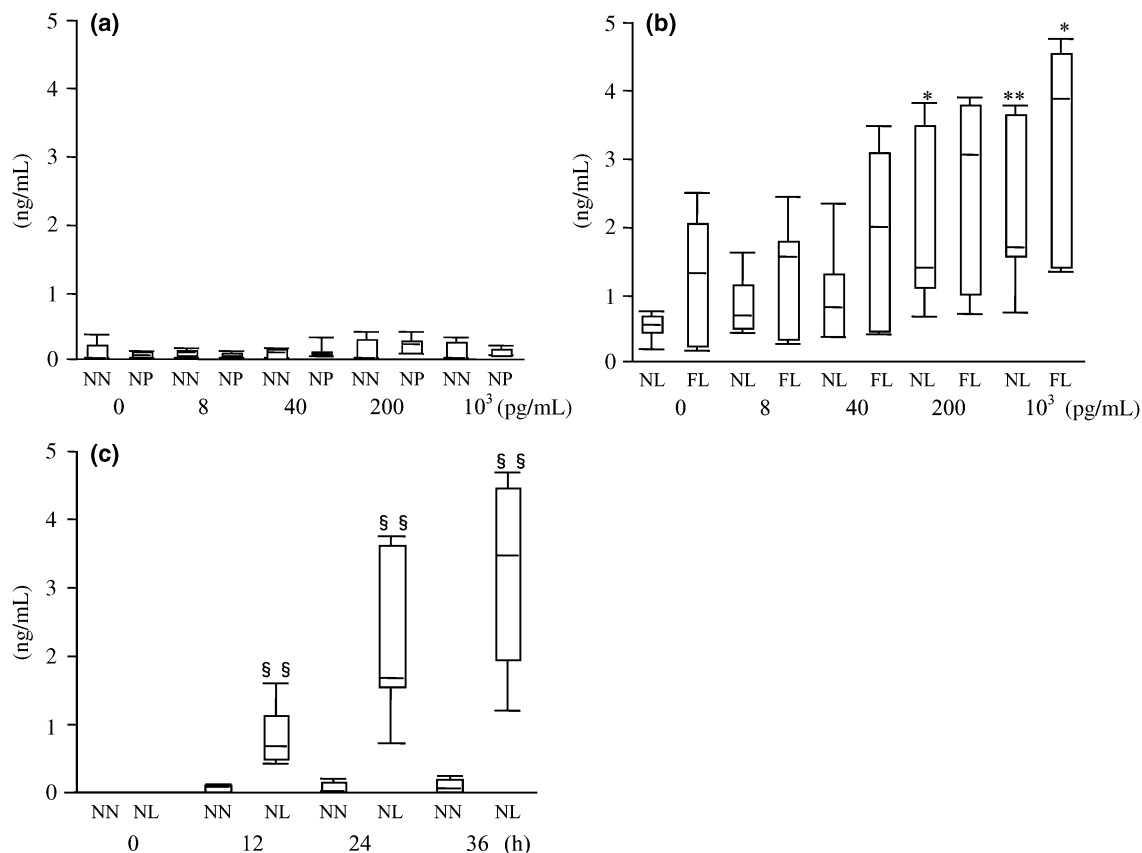


Fig. 4. Induction of procollagen type I release by (a) nasal fibroblasts [NN, normal nasal fibroblasts ( $n = 6$ ); NP, nasal polyp fibroblasts ( $n = 7$ )] and by (b) lung fibroblasts [NL, normal lung fibroblasts ( $n = 6$ ); FL, fibrotic lung fibroblasts ( $n = 6$ )] stimulated with different doses of TGF- $\beta_1$  for 24 h. (c) Kinetics of procollagen type I release by NN ( $n = 6$ ) and NL ( $n = 6$ ) stimulated with 1000 pg/mL TGF- $\beta_1$  for 0, 12, 24, 36 h. The amount of procollagen type I was expressed in nanograms per  $1 \times 10^6$  cells recovered at the end of the culture period. Box plots represent the median values with 25% and 75% interquartiles; the error bars represent the 10th and 90th percentiles. \* $P < 0.05$  and \*\* $P < 0.01$ , vs. 0 pg/mL TGF- $\beta_1$ . §§ $P < 0.01$  compared with normal nasal fibroblasts stimulated with TGF- $\beta_1$  under the same conditions.

manner (Fig. 4). In contrast, TGF- $\beta_1$  failed to significantly increase pro-collagen type I production in nasal fibroblasts (Fig. 4).

#### *Effect of transforming growth factor $\beta_1$ on vascular endothelial growth factor synthesis by fibroblasts*

**Reverse transcription polymerase chain reaction.** Unstimulated nasal and lung fibroblasts expressed a weak but specific message for VEGF. All the nasal and lung fibroblasts used here responded to TGF- $\beta_1$  stimulation resulting in the expression of VEGF mRNA. This induction of VEGF mRNA was enhanced in all fibroblasts in a dose-dependant manner regardless of the site of origin (Fig. 5).

**Enzyme-linked immunosorbent assay.** To determine whether airway fibroblasts can synthesize and release VEGF upon stimulation with TGF- $\beta_1$ , supernatants from the same airway fibroblasts lines used for pro-collagen type I experiments were assayed for VEGF content. In

agreement with the enhanced mRNA expression, TGF- $\beta_1$  stimulated not only lung fibroblasts but also nasal fibroblasts to produce significantly greater amounts of VEGF as compared to control (Fig. 6). The TGF- $\beta_1$ -induced production of VEGF occurred in a dose-dependant manner (Fig. 6).

#### *Effect of transforming growth factor $\beta_1$ on Smad translocation to the nucleus in cultured airway fibroblasts*

Under unstimulated conditions, Smad2, 3, 4 in both nasal fibroblasts and lung fibroblasts were localized in the cytoplasm (Figs 7a, c, e, g, i and k). After 1 h of stimulation with 1 ng/mL TGF- $\beta_1$ , Smad2, 3, 4 translocated to the nucleus in lung fibroblasts (h, j, l) (Fig. 7B), whereas only Smad3, but not Smad2 and Smad4, translocated to the nucleus in nasal fibroblasts (b, d, f) (Fig. 7A). Non-immune goat IgG revealed no specific staining (data not shown). Immunoreactivity for Smad2, 3, 4 was completely abolished in the absorption test (data not shown).

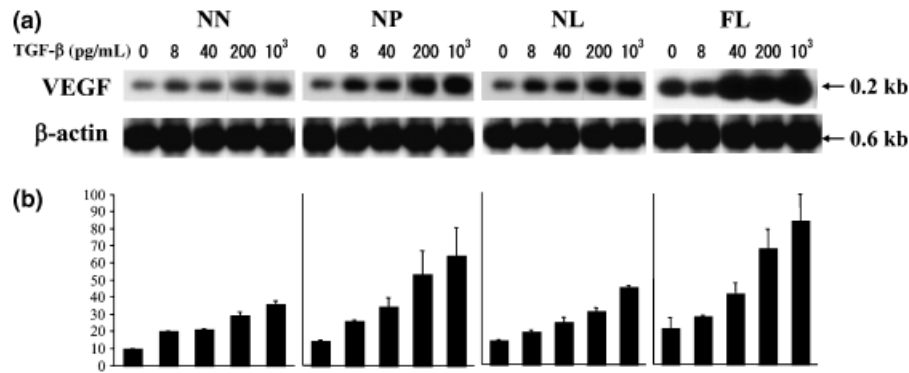


Fig. 5. (a) The representative results of VEGF mRNA expression in normal nasal fibroblasts (NN), nasal polyp fibroblasts (NP), normal lung fibroblasts (NL) and fibrotic lung fibroblasts (FL), stimulated with different doses of TGF- $\beta_1$  for 24 h. (b) Densitometric analysis of VEGF mRNA. The vertical bar represents percentage expression of procollagen type I mRNA vs.  $\beta$ -actin mRNA. All values are mean  $\pm$  SEM of three experiments. VEGF, vascular endothelial growth factor.

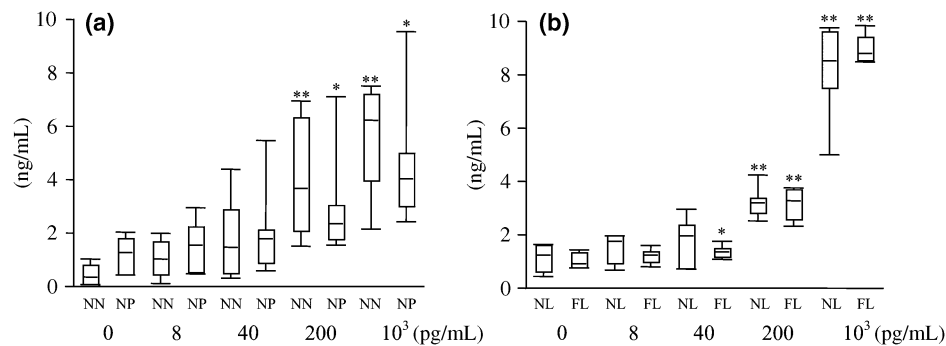


Fig. 6. Induction of VEGF release by (a) nasal fibroblasts (NN, normal nasal fibroblasts ( $n = 6$ ); NP, nasal polyp fibroblasts ( $n = 7$ )) and by (b) lung fibroblasts [(NL, normal lung fibroblasts ( $n = 6$ ); FL, fibrotic lung fibroblasts ( $n = 6$ )] stimulated with different doses of TGF- $\beta_1$  for 24 h. The amount of VEGF was expressed in nanograms per  $1 \times 10^6$  cells recovered at the end of the culture period. Box plots represent the median values with 25% and 75% interquartiles; the error bars represent the 10th and 90th percentiles. \* $P < 0.05$  and \*\* $P < 0.01$ , vs. 0 pg/mL TGF- $\beta_1$ . VEGF, vascular endothelial growth factor.

## Discussion

The differences in responsiveness of nasal and lung fibroblasts to TGF- $\beta$  may lead to different patterns of inflammation between upper and lower airways. TGF- $\beta$  is a family of multi-functional cytokines which includes at least five known isoforms, three of which are expressed by mammalian cells (TGF- $\beta_{1-3}$ ) [21, 22]. These isoforms arise from different genes, and their promoter structures and regulatory elements are different [23]. TGF- $\beta_1$  seems to be the main isoform involved in fibrosis [24, 25]. The present study was therefore designed to investigate the role of TGF- $\beta$  in inflammation of the upper and lower airways by analysing the responsiveness of nasal and lung fibroblasts with TGF- $\beta_1$ . TGF- $\beta_1$  enhanced both SMA expression and pro-collagen type I synthesis in lung fibroblasts, but not in nasal fibroblasts. This is the first report on the differential response of airway fibroblasts to TGF- $\beta_1$  with respect to SMA. Recently, it was clarified that Smad proteins play an important role in intracellular transduction of TGF- $\beta$  [10]. Rachel et al. examined the role of

Smad proteins by transfection of human lung fibroblasts with expression vectors for Smads, and found that transfection with Smad 2 resulted in TGF- $\beta_1$ -independent up-regulation of SMA, and transfection with Smad 4 was also involved in it [11]. In addition, Petridou et al. reported evidence of the translocation of Smad 2 from the cytoplasm to the nucleus in TGF- $\beta$ -induced myofibroblast differentiation [26]. Interestingly, Smad 4 (Dpc 4) knockout mice developed inflammatory polyps in the glandular stomach and duodenum [27]. Thus, the formation of nasal polyps may, at least in part, be related to the characteristics of nasal fibroblasts, and reflect the inability of Smad 4 to translocate from the cytoplasm to the nucleus in nasal fibroblasts. In the present experiment, stimulation by TGF- $\beta_1$  led to the translocation of both Smad2 and 4 from the cytoplasm to the nucleus, in lung but not in nasal fibroblasts. This may explain in part why TGF- $\beta_1$  did not induce SMA expression in nasal fibroblasts, unlike that in lung fibroblasts.

However, nasal polyposis is characterized by marked oedema, coarse ECM and proliferating blood vessels [1].

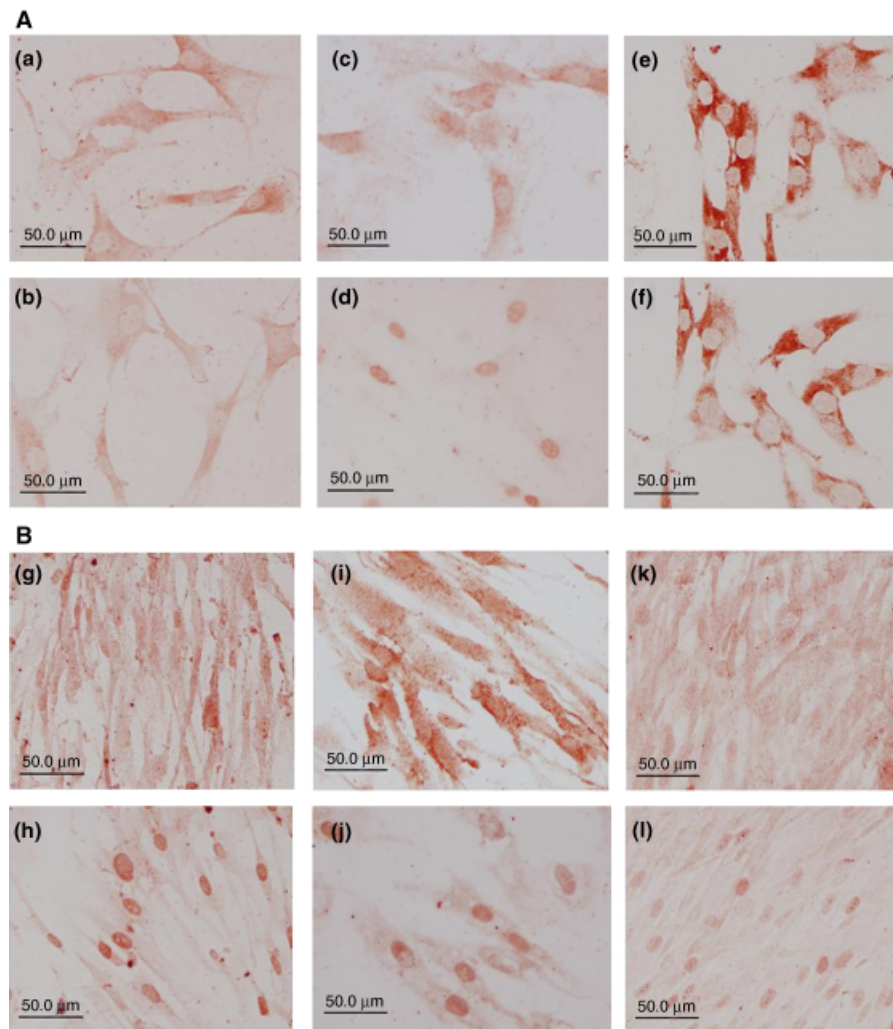


Fig. 7. Smad2, 3, 4 translocation to the nucleus in airway fibroblasts Smad2 (a, b), Smad3 (c, d) and Smad4 (e, f) immunolocalization in normal nasal fibroblasts (A) stimulated with (b, d, f) or without (a, c, e) 1 ng/mL TGF- $\beta_1$  for 1 h. Smad2 (g, h), Smad3 (i, j) and Smad4 (k, l) immunolocalization in normal lung fibroblasts (B) stimulated with (h, j, l) or without (g, i, k) 1 ng/mL TGF- $\beta_1$  for 1 h.

On the other hand, pulmonary fibrosis, which is not only and necessarily an inflammatory disorder, is characterized by inflammatory cell accumulation, excessive ECM deposition such as collagens and vascular abnormalities [2]. The ECM takes up to more than one-third of the dry mass of the lung and the most dominant components are members of the collagen family. The major portion is constituted by interstitial collagens, consisting mainly of types I and III, but also types V and VI collagens. The ratio of abundance between collagen types I and III in the normal lung is around 3–6 : 1 and the ratio is even higher in fibrotic lungs [28]. This difference in the histopathology between upper and lower airway inflammation is unclear. We thought that it may, in part, be due to the heterogeneity between nasal and lung fibroblasts. To clarify the heterogeneous responsiveness of these fibroblasts to TGF- $\beta$ , we established primary fibroblasts lines from nasal and lung tissues for our experiments. We report here that TGF- $\beta_1$

enhanced the expression of SMA and the production of pro-collagen type I only in lung fibroblasts but not in nasal fibroblasts. These differences were clearly seen whether nasal and lung fibroblasts were inflamed or not. Thus, these results indicate that responsiveness to TGF- $\beta$  was site but not disease specific.

Because myofibroblasts were first described in contracting experimental granulation tissue, they have been found in different tissues including the lungs of normal subjects and of those with pathological conditions [29]. It is now believed that these cells play a crucial role in wound healing as well as in human and rat lung fibrosis. Zhang et al. [30] suggested that myofibroblasts may be responsible for most, if not all, of the increased lung collagen gene expression in pulmonary fibrosis in rats. Our present *in vitro* data, showing that SMA-positive lung fibroblasts induced by TGF- $\beta_1$  produce pro-collagen type I were in agreement with their *in vivo* data of bleomycin-

induced lung fibrosis in rats. On the other hand, nasal fibroblasts, which failed to induce SMA and to produce pro-collagen type I by TGF- $\beta_1$ , may be less fibrogenic in nature, at least when stimulated with TGF- $\beta_1$ , as compared with lung fibroblasts. These results may reflect that nasal polyps consist of much less ECM, like collagen type I, than pulmonary fibrosis. Myofibroblasts have been reported to be present in chronic inflammatory processes including pulmonary fibrosis and asthma [30, 31]. Recently, their presence has been highlighted in nasal polyps [32, 33]. Further studies are needed to elucidate the factors important for the induction of myofibroblasts in nasal polyps.

VEGF is a new family of secreted growth factors capable of promoting angiogenesis and vascular permeability [13]. It was recently reported that VEGF is expressed in inflamed tissue in the airways, where it is thought to be produced by structural cells such as fibroblasts and epithelial cells, and by activated macrophages upon stimulation with TGF- $\beta$  [14–17]. The possible role for VEGF in the development of vascular abnormalities in both nasal polyps and pulmonary fibrosis has been evaluated [34, 35]. Thus, we compared synthesis of VEGF from nasal and lung fibroblasts upon stimulation with TGF- $\beta_1$ . TGF- $\beta_1$  induced VEGF in both nasal and lung fibroblasts. This is in contrast to the up-regulation of SMA and synthesis of pro-collagen type I in only lung but not nasal fibroblasts by TGF- $\beta_1$ . Recently, Kobayashi et al. [36] used human lung fibroblasts treated with Smad3 small interfering RNA and suggested that VEGF production induced by TGF- $\beta_1$  depends on Smad3 signalling. Our present data, demonstrating that Smad3 but not Smad2 and Smad4 translocated from the cytoplasm to the nucleus in nasal fibroblasts upon TGF- $\beta_1$  stimulation, whereas all three were translocated in case of lung fibroblasts may explain why TGF- $\beta_1$  only up-regulated VEGF production but not SMA expression and pro-collagen type I production (unlike in lung where all three were up-regulated) in a dose-dependent manner in nasal fibroblasts.

The observation that nasal fibroblasts failed to respond to TGF- $\beta_1$  with respect to SMA expression and pro-collagen type I synthesis suggests a distinct role for these cells in the modulation of the inflammatory response in the upper airway. The nasal mucosa is the first point in the whole respiratory tract that gets in contact with environmental agents and, thus, it is exposed to many potentially injurious agents, including microorganisms. Fibrogenic changes in the nasal mucosa are rather rare, although nasal inflammatory conditions such as sinusitis with nasal polyps and rhinitis are quite prevalent, and the nasal mucosa is often subject to subclinical inflammation without profound symptoms [37]. Interestingly, we also found that nasal fibroblasts responded to TGF- $\beta_1$  with respect to VEGF synthesis. We suggest that nasal fibroblasts may be less susceptible to fibrogenic growth factors in nature as compared with lung fibroblasts, and that these typical characteristics of nasal fibroblasts may at least in part be

responsible for the sparse ECM and vascular changes rather than fibrosis seen in nasal polyps.

## Acknowledgement

We express our gratitude to Ms S. Saito for the technical assistance. This work was supported by Grants-in-Aid from the Ministry of Education, Japan.

## References

- 1 Cauna N, Hinderer KH, Manzetti GW, Swanson EW. Fine structure of nasal polyps. *Ann Otol* 1972; 81:41–58.
- 2 Crystal RG, Ferrans VJ, Basset F. Biologic basis of pulmonary fibrosis. In: Crystal RG, West JB, Barns PJ, Cherniack NS, Weibel ER, eds. *The lung: scientific foundations*. New York: Raven Press, 1991; 2031–46.
- 3 Harper RA, Grove G. Human skin fibroblasts derived from papillary and reticular dermis: differences in growth potential *in vitro*. *Science* 1979; 204:526–7.
- 4 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 J Respir Dis* 1988; 137:579–84.
- 5 Schmitt-Graff A, Desmouliere A, Gabbiani G. Heterogeneity of myofibroblast phenotypic features: an example of fibroblastic cell plasticity. *Virchows Arch* 1994; 425:3–24.
- 6 Sporn MB, Roberts AB. Transforming growth factor-beta: recent progress and new challenges. *J Cell Biol* 1992; 119:1017–21.
- 7 Moses HL, Branum EL, Proper JA, Robinson RA. Transforming growth factor production by chemically transformed cells. *Cancer Res* 1981; 41:2842–8.
- 8 Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor- $\beta_1$  induces  $\alpha$ -smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 1993; 122:103–11.
- 9 Khalil N, Greenberg AH. The role of TGF- $\beta$  in pulmonary fibrosis. *Ciba Found Symp* 1991; 157:194–207.
- 10 Massague J. TGF- $\beta$  signal transduction. *Annu Rev Biochem* 1998; 67:753–91.
- 11 Rachel AE, Ya CT, Robert S, Aled OP. TGF- $\beta_1$ -mediated fibroblast-myofibroblast terminal differentiation the role of Smad proteins. *Exp Cell Res* 2003; 282:90–100.
- 12 Ferrara N, Houck K, Jakeman L, Leung DW. Molecular and biological properties of the vascular endothelial growth family of proteins. *Endocr Rev* 1992; 13:18–32.
- 13 Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev* 1997; 18:4–25.
- 14 Acarregui MJ, Penisten ST, Goss KL, Ramirez K, Snyder JM. Vascular endothelial growth factor gene expression in human fetal lung *in vitro*. *Am J Respir Cell Mol Biol* 1999; 20:14–23.
- 15 Berse B, Hunt JA, Diegel RJ *et al*. Hypoxia augments cytokine (transforming growth factor-beta (TGF- $\beta$  and IL-1)-induced vascular endothelial growth factor secretion by human synovial fibroblasts. *Clin Exp Immunol* 1999; 115:176–82.
- 16 Coste A, Brugel L, Boussat S *et al*. Inflammatory cells as well as epithelial cells in nasal polyps express vascular endothelial growth factor. *Eur Respir J* 2000; 15:367–72.

- 17 Cosgrove GP, Brown KK, Schiemann WP *et al*. Pigment epithelium-derived factor in idiopathic pulmonary fibrosis: a role in aberrant angiogenesis. *Am J Respir Crit Care Med* 2004; 170:242–51.
- 18 Vancheri C, Ohtoshi T, Cox G *et al*. Neutrophilic differentiation induced by human upper airway fibroblast-derived granulocyte/macrophage colony-stimulating factor (GM-CSF). *Am J Respir Cell Mol Biol* 1991; 4:11–7.
- 19 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987; 162:156–9.
- 20 Pawankar R, Okuda M, Hasegawa S *et al*. Interleukin-13 gene expression in the nasal mucosa of perennial allergic rhinitics. *Am J Respir Crit Care Med* 1995; 152:2059–67.
- 21 Massague J, Cheifetz S, Liao M, Palph DA, Weis FM, Zentella A. Transforming growth factor-beta. *Cancer Surv* 1992; 12:81–103.
- 22 Roberts AB, Sporn MB. Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). *Growth Factors* 1993; 8:1–9.
- 23 Barton DE, Foellmer BE, Du J, Tamm J, Derynck R, Franke U. Chromosomal mapping of genes for transforming growth factor beta 2 and beta 3 in man and mouse: dispersion of TGF-beta gene family. *Oncogene Res* 1988; 3:323–31.
- 24 Coker RK, Laurent GJ, Shahzeidi S *et al*. Transforming growth factors – beta 1, -beta 2, and -beta 3 stimulate fibroblast procollagen production *in vitro* but are differentially expressed during bleomycin-induced lung fibrosis. *Am J Pathol* 1997; 150:981–91.
- 25 Coker RK, Laurent GJ, Jeffery PK, du Bois RM, Black CM, McNulty. Localisation of transforming growth factor  $\beta$ 1 and  $\beta$ 3 mRNA transcripts in normal and fibrotic human lung. *Thorax* 2001; 56:549–56.
- 26 Petridou S, Maltseva O, Spanakis S, Masur SK. TGF- $\beta$  receptor expression and Smad2 localization are cell density dependent in fibroblasts. *Invest Ophthalmol Vis Sci* 2000; 41:89–95.
- 27 Takaku K, Miyoshi H, Matsunaga A, Oshima M, Sasaki N, Taketo MM. Gastric and duodenal polyps in Smad4 (Dpc4) knockout mice. *Cancer Res* 1999; 59:6113–7.
- 28 Adams SL. Collagen gene expression. *Am J Respir Cell Mol Biol* 1989; 1:161–8.
- 29 Leslie KO, Mitchell J, Low R. Lung myofibroblasts. *Cell Motil Cytoskeleton* 1992; 22:92–8.
- 30 Zhang K, Rekhter MD, Gordon D, Phan SH. Myofibroblasts and their role in lung collagen gene expression during pulmonary fibrosis. A combined immunohistochemical and *in situ* hybridization study. *Am J Pathol* 1994; 145:114–25.
- 31 Roche WR. Fibroblasts and extracellular matrix in bronchial asthma. In: Busse WW, Holgate ST, eds. *Asthma and rhinitis*. Boston: Blackwell Scientific Publications, 1995; 554–62.
- 32 Nakayama M, Wenig BM, Heffner DK. Atypical stromal cells in inflammatory nasal polyps: immunohistochemical and ultrastructural analysis in defining histogenesis. *Laryngoscope* 1995; 105:127–34.
- 33 Wang QP, Escudier E, Roudot-Thoraval F, Samad I, Peynegre R. Myofibroblast accumulation induced by transforming growth factor-[beta] is involved in the pathogenesis of nasal polyps. *Laryngoscope* 1997; 107:926–31.
- 34 Wittekindt C, Hess A, Bloch W, Sultan S, Michel O. Immunohistochemical expression of VEGF and VEGF receptors in nasal polyps as compared to normal turbinate mucosa. *Eur Arch Otorhinolaryngol* 2002; 259:294–8.
- 35 Ebina M, Shimizukawa M, Shibata N *et al*. Heterogeneous increase in CD34-positive alveolar capillaries in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2004; 169:1203–8.
- 36 Kobayashi T, Liu X, Wen F *et al*. Smad3 mediates TGF- $\beta$ <sub>1</sub> induction of VEGF production in lung fibroblasts. *Biochem Biophys Res Commun* 2005; 327:393–8.
- 37 Nonaka M, Nonaka R, Jordana M, Dolovich J. GM-CSF, IL-8, IL-1R, TNF-alpha R, and HLA-DR in nasal epithelial cells in allergic rhinitis. *Am J Respir Crit Care Med* 1996; 153:1675–81.