

## Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Svegliati Baroni S, Santillo M, Bevilacqua F, et al. Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. *N Engl J Med* 2006;354:2667-76.

## SUPPLEMENTARY APPENDIX

### Cell Lines

Mouse embryo fibroblasts derived from PDGF receptor knock-out embryos that do not express  $\alpha$  and  $\beta$  chains PDGFR subunits (F<sup>-/-</sup> cells) and F<sup>-/-</sup> cells infected with PDGFR  $\alpha$  and  $\beta$  subunits (F  $\alpha$ ; F  $\beta$ ; F  $\alpha\beta$ ) have been described elsewhere (18)

### Fibroblast Cultures

Human skin fibroblasts were obtained from punch biopsies taken from the forearms of normal volunteers after approval by the Institutional Ethics Committee (Università Politecnica delle Marche, Ancona) and oral and written, informed consent. Fibroblasts between the fourth and the sixth passage were used for all experiments.

### Purification of IgG

IgG fractions were purified by affinity chromatography on protein A/G-sepharose column (Pierce, Rockford, IL). from serum of normal subjects, of scleroderma and systemic lupus erythematosus patients, and from patients with rheumatoid arthritis and primary Raynaud's phenomenon **and idiopathic pulmonary fibrosis**. Protein concentrations were estimated by

**Supplementary Figure 1 Purified antibodies to PDGFR from SSe scleroderma patients stimulate collagen and  $\alpha$ -SMA expression**

**Panel A.**  $\alpha$ -SMA protein induction in normal human fibroblasts stimulated by 1 ml of supernatant derived from clones 4G11 and 1F5, purified from EBV-infected SSe scleroderma B lymphocytes cultures, for 30 minutes in the presence and in the absence of AG 1296 (2  $\mu$ M for 1 hour before treatment). The concentration of immunoglobulin (M type) was approximately 1-2  $\mu$ g/ml. The experiment shown is a representative of 2 independent experiments. Densitometric analysis of 2 independent experiments is shown in the lower panel. Data represents the mean value  $\pm$  SE of 2 independent experiments. C Ig indicates medium from one clone which did not induce ROS. **Panel B.** Type I collagen  $\alpha$ 1 and  $\alpha$ 2 chain gene mRNAs in normal human fibroblasts stimulated by 1 ml (approximately 1-2  $\mu$ g/ml) of supernatant derived from clones 4G11 and 1F5, purified from EBV-infected SSe scleroderma B lymphocyte cultures, for 15 minutes in the presence and in the absence of AG 1296 (2  $\mu$ M for 1 hour before treatment). Northern blot analysis for  $\alpha$ 1(I) and  $\alpha$ 2(I) collagen mRNAs (upper panel). Representative results from 1 of 2 experiments are shown. Real-time quantitative RT-PCR of  $\alpha$ 1(I) and  $\alpha$ 2(I) collagen gene transcripts is shown in the lower panel. C Ig indicates medium from one clone which did not induce ROS. FCS indicates foetal calf serum.

**Supplementary Figure 2. Time course of ROS induction by scleroderma IgG and PDGF.**

Isolated IgG (200  $\mu\text{g/ml}$ ) from a single patient or PDGF (1  $\mu\text{g/ml}$ ) were incubated for 15 min with  $F_{\alpha}$  cells and the ROS measured at different periods as described in Methods. Data represents the mean value  $\pm$  SE of 3 independent experiments.

## **Cell Lysis and Immunoblotting**

Cell culture plates were lysed with 0.3 ml of cold RIPA buffer (1x PBS, 1 percent Nonidet P-40, 0.5 percent sodium deoxycholate, 0.1 percent SDS, 2 mM sodium orthovanadate, 2  $\mu$ g/ml aprotinin, 1mM PMSF) and processed for immunoblotting as described (8).

## **Immunoprecipitation Assays**

PDGF receptor was immunoprecipitated from cultured cells with 200  $\mu$ g of IgG. Immunocomplexes were isolated, subjected to electrophoresis, and immunoblotted with anti PDGFR  $\alpha$  and  $\beta$  subunits antibodies and with anti EGF receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and revealed by chemiluminescence (Amersham, Sweden).

Ras proteins were immunoprecipitated from cultured fibroblasts with monoclonal anti pan Ras antibody (Santa Cruz) following recommended procedures from the manufacturer. Immunocomplexes were isolated, subjected to electrophoresis, and immunoblotted with anti Ha-Ras antibodies and revealed by chemiluminescence (Amersham, Sweden).

### **Absorption of Autoantibodies with F $\alpha$ Cells**

Scleroderma IgG (200  $\mu$ g/ml) were incubated for 2 hours at 4 °C with PDGFR expressed by F $\alpha$  cells. This mixture was then centrifuged (14,000 g for 15 minutes at 4°C) and the supernatant was again subjected to immunoprecipitation with the extracellular domain of PDGFR  $\alpha$  subunit (R&D Systems, Wiesbaden, Germany), as antigen source, to test whether the PDGFR band had been removed. F $^{-/-}$  cells were used as control cells. Immunoblots were challenged with anti PDGFR  $\alpha$  and  $\beta$  subunits antibodies (Santa Cruz) and revealed by chemiluminescence (Amersham, Sweden).

### **Immunofluorescence**

Fibroblasts, cultured on Lab-Tek chamber glass slides (Nalge-Nunc, IL, USA) and starved for 48 hours before stimulation or addition of inhibitors, were fixed in 4 percent para-formaldehyde, permeabilized by 0.1 percent Triton-X, and stained with a monoclonal antibody against Ha-Ras and then with a tetramethylrhodamine-isothiocyanate (TRITC) conjugated secondary antibody (Molecular Probes). Slides were mounted with Vectarshield (H-100; Vector, Burlingame, CA) and examined using a BioRad (Hemel Hempstead, UK) microradiance confocal laser-scanning microscope equipped with argon and helium/neon lasers. Acquired images were analysed using the Laser Sharp

Processing BioRad software (version 3.2). All images from different slides condition were acquired in double-blinded fashion.

### **Immunofluorescence Flow Cytometry**

Target cells ( $3 \times 10^5$ /sample) were suspended in phosphate-buffered saline with 0.5 percent bovine serum albumin and incubated for 30 minutes at 4°C with 100  $\mu$ l/ml of spent medium obtained from clones derived from EBV-infected B lymphocytes from SSe **scleroderma** patients (or with an anti-PDGF receptor  $\alpha$  mAb). After washing, cells were incubated for 30 minutes at 4°C with F(ab')<sub>2</sub>-RaHIg-FITC (or F(ab')<sub>2</sub>-RaMIg-FITC) and fluorescence was analyzed by a FACS Calibur flow cytometer using CellQuest software (Becton-Dickinson). Background mAb binding was estimated by means of isotype-matched negative control antibody.

### **Immunohistochemical Analysis for $\alpha$ -Smooth-muscle Actin ( $\alpha$ -SMA)**

The cells were stimulated with SSe- **scleroderma** IgG and normal IgG (200  $\mu$ g/ml for 30 minutes), and stained for  $\alpha$ -smooth-muscle actin. In brief, fibroblasts

were fixed in 80 percent acetone for 10 minutes. After several washes in phosphate-buffered saline with 1 percent bovine serum albumin, fibroblasts were permeabilized with 0.1 percent Triton X-100 for 5 minutes. The fibroblasts were then incubated with a mouse polyclonal anti  $\alpha$ -SMA antibody (Sigma) for 2 hours and then with a goat anti mouse Ig antibody coupled to horseradish peroxidase diluted 1:50 for 30 minutes.

The reaction was developed with a buffer containing 0.05 percent 3,3'-diaminobenzidine and 0.01 percent H<sub>2</sub>O<sub>2</sub> (Sigma) in phosphate-buffered saline.  $\alpha$ -SMA positive cells were visualized by a computerized system coupled to an Olympus microscope.

### **RNA Isolation and Northern Analysis**

Total cellular RNA was extracted using the Rneasy Mini kit (Qiagen, Hilden, Germany). Ten micrograms total RNA was then used for Northern blot analysis (7,8).

### **Real Time RT-PCR for Collagen**

Levels of  $\alpha$ 1 (I) and  $\alpha$ 2 (I) collagen RNA transcripts were quantitated by real-time PCR. Normal human fibroblasts were treated with normal and SSe **scleroderma** IgG (200  $\mu$ g/ml for 15 minutes), total RNA from the samples were isolated using RNeasy (QIAGEN) and cDNA sequence derived from NCBI database (NCBI, <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide>; accession

numbers GAPDH: BC013310; COL  $\alpha$ 1(I): BC036531, COL  $\alpha$ 2(I): BC054498) was used to design the forward and reverse primers to the sequence using Beacon Designer software ( PREMIER Biosoft International). The primers were as follows:

GAPDH FW: 5'-CCCTTCATTGACCTCAACTACATG-3';

GAPDH REV: 5'- TGGGATTTCCATTGATGACAAGC-3';

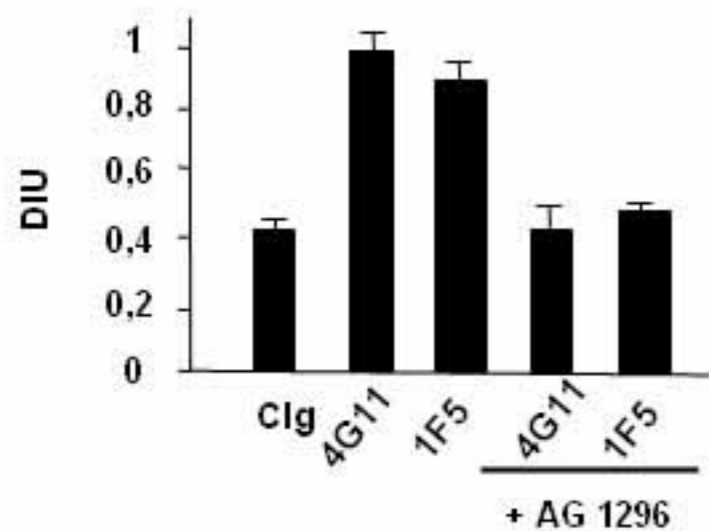
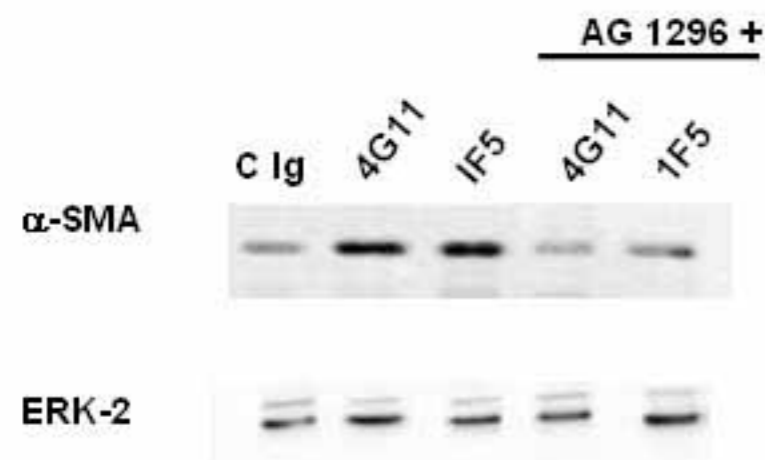
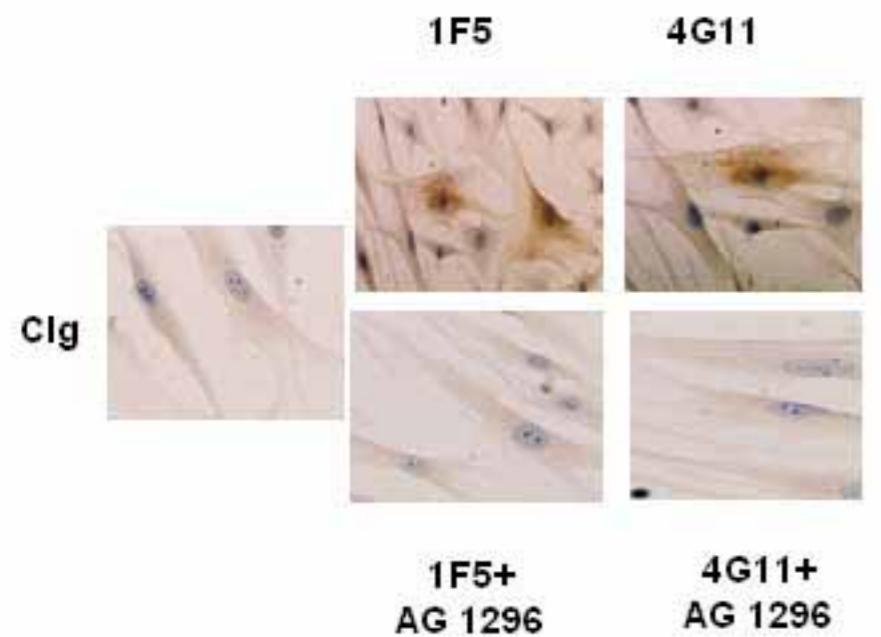
COL  $\alpha$ 1 (I) FW: 5'-AGGGCCAAGACGAAGACATC-3';

COL  $\alpha$ 1 (I) REV: 5'-AGATCACGTCATCGCACAACA-3';

COL  $\alpha$ 2 (I) FW: 5'-AGGTCAAACAGGAGCCCGTGGG-3'

COL  $\alpha$ 2 (I) REV: 5'-GCACCTGGGAAGCCTGGAGGG-3'

cDNA was reverse transcribed from 2  $\mu$ g of total RNA each sample, using M-MLV RT kit (Gibco). Complementary DNAs were mixed with SYBR-Green PCR IQ Super mix (Biorad) and primers, and real-time PCR were performed. GAPDH gene was used as internal control. For each single well amplification reaction, a threshold cycle (CT) was observed in the exponential phase of amplification, and the quantitation of relative expression levels was achieved using standard curves for both the target and endogenous controls. All assays were performed in triplicate.

**A****B**