Targets of anti-endothelial cell antibodies in pulmonary hypertension and scleroderma

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**Supplemental data**

**Methods**

**Endothelial cell (EC) culture**

Human umbilical vein EC (HUVECs) were isolated by digestion of freshly obtained umbilical cords and cultured as previously described [1 , 2]. Pulmonary (p) and dermal (d) human microvascular EC (HMVEC) were purchased from PromoCell (Heidelberg, Germany). They were cultured with microvascular EC growth medium containing 2% fetal calf serum, 5ng/ml human recombinant epidermal growth factor, 10ng/ml human recombinant basic fibroblast growth factor, 20ng/ml insulin growth factor, 0.5 ng/ml human recombinant vascular endothelial growth factor. 1µg/ml ascorbic acid, 0.2µg/ml hydrocortisone. After the second passage for HUVEC and the fourth passage for HMVEC, cells were either seeded onto multiple-chamber glass slides (EZslide, Merck-Millipore, Billerica, MA, USA) for indirect immunofluorescence or prepared for protein extract for 1-D immunoblot or 2-D immunoblot. Protein quantification involved the Bradford method [3].

**Indirect immunofluorescence**

EC were cultured until 60% confluence in multiple-chamber slides then fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), washed with PBS, incubated with sera from patients or healthy controls at a 1:100 dilution, tested individually for 1 h at room temperature and finally washed twice with PBS. A goat anti-human IgG secondary antibody conjugated with FITC (Invitrogen, Carlsbad, CA, USA) was then added for 1 h at room temperature. Slides were mounted with mounting medium containing 4',6'-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) and cells visualized under a Zeiss Axio Observer.Z1 microscope equipped for fluorescence (Carl Zeiss, Oberkochen, Germany). All slides from each single experiment were visualized at the same time of exposure.

**1-D immunoblot**

Protein extracts were prepared in 125 mM Tris/HCl pH 6.8 containing 4% SDS, 1.45 M 2-mercaptoethanol, 1 µg/ml aprotinin, 1 µg/ml pepstatin and 1 µg/ml leupeptin on ice and sonicated 4 x 30 secs. Equal amounts of loading buffer with solubilized proteins (140µl per gel) were subjected to 10% polyacrylamide gel electrophoresis (SDS PAGE), transferred onto nitrocellulose membranes, incubated for 4 h at room temperature with the sera using a Cassette Miniblot System (Immunetics Inc., Cambridge, MA, USA). Mouse polyclonal anti-vinculin, (Abnova, Taipei City, Taiwan), rabbit anti-lamin A (Abcam, Cambridge, UK) and rabbit anti-beta tubulin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) antibodies were tested in 1D-immunoblots during 90 min at room temperature at

dilution of 1:200 for reactivity with HUVEC and HMVEC-d and p proteins extracts. The membranes were then extensively washed and incubated with an anti-Fcγ chain-specific secondary goat anti-human IgG antibodies coupled to alkaline phosphatase (Dako, Glostrup, Denmark). Immunoreactivities were revealed with NBT/BCIP. Quantification of immunoreactivities was performed by densitometry in reflective mode (Epson Perfection 1200S densitometer, Seiko Epson Corporation, Nagano-ken, Japan).

**2-D electrophoresis (2-DE)**

In-gel digestion involved use of trypsin as described [4] with minor modifications and involved for all steps a Freedom EVO 100 digester/spotter robot (Tecan, Männedorf, Switzerland). Spots were first de-stained twice with a mixture of 100 mM ammonium bicarbonate (ABC) and 50% acetonitrile (ACN) for 45 min at 22°C and then dried with use of 100% ACN for 15 min. Protein spots were then treated with 25 mM ABC containing 10 mM DTT for 1 h at 60°C and then alkylated by use of 55 mM iodoacetamide in 25 mM ABC for 30 min in the dark at 22°C. Gel pieces were washed twice with 25 mM ABC and then shrunk twice with 100% ACN for 15 min and dried with 100% ACN for 10 min. Bands were completely dehydrated after 1 h at 60°C. Gel pieces were incubated with 13 µl Sequencing Grade Modified Trypsin (Promega, WI, USA; 12.5 µg/ml in 40 mM ABC–10% ACN, pH 8.0) overnight at 40°C. After digestion, peptides were washed with 30 µl of 25 mM ABC, shrunk with 100% ACN and extracted twice with a mixture of 50% ACN–5% formic acid (FA). Extracts were then dried by

vacuum centrifuge (Eppendorf, Hamburg, Germany). Finally, peptides were desalted with use of $C_{18}$-ZipTips (Millipore) and 2 elutions, first with 50% ACN–5% FA and then 80% ACN–5% FA. Pooled elutions were allowed to dry at room temperature.

**Protein identification by mass spectrometry**

For mass spectrometry (MS) and MS/MS analysis, peptides were redissolved in 4 µL CHCA (5 mg/mL in 50% (v/v) ACN-0.1% (v/v) TFA). One microliter and a half of each sample was spotted directly onto a MALDI plate (Applied Biosystems, Foster City, CA). Droplets were allowed to dry at room temperature. The sample analysis involved a MALDI-TOF-TOF 4800 mass spectrometer (Applied Biosystems). Spectra acquisition and processing involved use of the 4000 series explorer software (Applied Biosystems) version 3.5.28193 in positive reflectron mode at fixed laser fluency with low mass gate and delayed extraction. External plate calibration was by four calibration points spotted onto the four corners of the plate with a mixture of five external standards (PepMix 1, LaserBio Labs, Sophia Antipolis, France). Peptide masses were acquired by steps of 50 spectra for 900 to 4000 Da. MS spectra were summed from 1000 laser shots by an Nd-YAG laser operating at 355 nm and 200 Hz. After filtering tryptic-, keratin- and matrix-contaminant peaks, up to 15 parent ions were selected for subsequent MS/MS fragmentation according to mass range, signal intensity, S/N, and absence of neighboring masses in the MS spectrum. MS/MS spectra were acquired in 1-kV positive mode, and 1000 shots were summed in increments of

50. Database searching involved MASCOT 2.2 (MatrixScience, London, UK) [5] *via* GPS explorer software (Applied Biosystems) version 3.6 combining MS and MS/MS interrogations on human proteins from Swiss-Prot databank release 54.5, 17 253 entries (www.expasy.org). The search parameters were as follows: carbamidomethylation as a variable modification for cysteins and oxidation as a variable modification for methionines. Up to one missed tryptic cleavage was permitted and mass accuracy tolerance of 30 ppm for precursors and 0.3 Da for fragments were used for all tryptic mass searches. Positive identification was based on a MASCOT score above the significance level (*i.e.*< 5%). In the case of peptides match to multiple members of a protein family, the reported protein is the one with the highest number of peptide matches.

**References**

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**Supplemental table 1.** Clinical and immunological characteristics of patients with idiopathic pulmonary arterial hypertension and patients with systemic sclerosis with or without pulmonary arterial hypertension.

| Patients (N°) | Pool number | PAH etiology | Sex (M/F) | Ethnicity (B/W) | Age (years) | Disease duration of PAH (months) | NYHA | mPAP (mmHg) | Walk test (m) | Form of SSc | Disease duration of SSc (months) | Raynaud's phenomenon | Digital ulcers | Pulmonary fibrosis | Scleroderma renal crisis | Autoantibodies |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 1 | IPAH | F | w | 47 | 11 | 4 | 52 | 75 | N/A | N/A | N | N | N | N/A | N |
| **2** | 1 | IPAH | F | W | 30 | 23 | 2 | 53 | 375 | N/A | N/A | N | N | N | N/A | N |
| **3** | 1 | IPAH | F | W | 49 | 27 | 4 | 73 | 0 | N/A | N/A | N | N | N | N/A | N |
| **4** | 2 | IPAH | F | W | 52 | 27 | 3 | 65 | 290 | N/A | N/A | N | N | N | N/A | N |
| **5** | 2 | IPAH | M | W | 9 | 72 | 2 | 65 | 372 | N/A | N/A | N | N | N | N/A | N |
| **6** | 2 | IPAH | F | W | 49 | 17 | 3 | 90 | 490 | N/A | N/A | N | N | N | N/A | N |
| **7** | 3 | IPAH | F | W | 6 | 241 | 3 | 104 | 185 | N/A | N/A | N | N | N | N/A | N |
| **8** | 3 | IPAH | M | W | 61 | 28 | 2 | 63 | 390 | N/A | N/A | Y | N | N | N/A | N |
| **9** | 3 | IPAH | F | W | 20 | 43 | 2 | 68 | N/A | N/A | N/A | N | N | N | N/A | N |
| **10** | 4 | IPAH | F | W | 22 | 35 | 3 | 79 | 440 | N/A | N/A | N | N | N | N/A | N |
| **11** | 4 | IPAH | F | W | 57 | 26 | 3 | 51 | N/A | N/A | N/A | N | N | N | N/A | N |
| **12** | 4 | IPAH | F | W | 28 | 14 | 2 | 56 | 490 | N/A | N/A | N | N | N | N/A | N |
| **13** | 5 | IPAH | F | W | 47 | 9 | 3 | 64 | 420 | N/A | N/A | N | N | N | N/A | N |
| **14** | 5 | IPAH | F | W | 38 | 8 | 3 | 55 | 385 | N/A | N/A | N | N | N | N/A | N |
| **15** | 5 | IPAH | F | W | 33 | 6 | 4 | 73 | 0 | N/A | N/A | N | N | N | N/A | N |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **16** | 6 | IPAH | F | W | 15 | 41 | 3 | 65 | N/A | N/A | N/A | Y | N | N | N/A | N |
| **17** | 6 | IPAH | F | W | 65 | 2 | 2 | 44 | 510 | N/A | N/A | N | N | N | N/A | N |
| **18** | 6 | IPAH | F | W | 25 | 6 | 4 | 61 | 0 | N/A | N/A | N | N | N | N/A | N |
| **19** | 7 | IPAH | F | W | 29 | 9 | 2 | 73 | 490 | N/A | N/A | N | N | N | N/A | N |
| **20** | 7 | IPAH | M | W | 26 | 12 | 2 | 39 | N/A | N/A | N/A | N | N | N | N/A | N |
| **21** | 7 | IPAH | F | W | 62 | 6 | 3 | 49 | 290 | N/A | N/A | N | N | N | N/A | ANA |
| **22** | 8 | IPAH | M | W | 33 | 4 | 3 | 60 | 550 | N/A | N/A | N | N | N | N/A | N |
| **23** | 8 | IPAH | F | W | 37 | 84 | 3 | 66 | 350 | N/A | N/A | N | N | N | N/A | N |
| **24** | 8 | IPAH | M | W | 55 | 24 | 3 | 76 | 280 | N/A | N/A | N | N | N | N/A | N |
| **25** | 9 | IPAH | F | B | 26 | 19 | 3 | 62 | 310 | N/A | N/A | N | N | N | N/A | N |
| **26** | 9 | IPAH | F | B | 28 | 9 | 2 | 56 | 500 | N/A | N/A | N | N | N | N/A | N |
| **27** | 9 | IPAH | F | B | 32 | 0 | 3 | 51 | 470 | N/A | N/A | N | N | N | N/A | N |
| **28** | 10 | IPAH | M | W | 34 | 29 | 3 | 56 | 370 | N/A | N/A | N | N | N | N/A | N |
| **29** | 10 | IPAH | M | W | 30 | 12 | 3 | 54 | 400 | N/A | N/A | N | N | N | N/A | N |
| **30** | 10 | IPAH | F | W | 26 | 7 | 3 | 60 | 412 | N/A | N/A | N | N | N | N/A | N |
| **31** | 11 | SSc-PAH | F | B | 48 | 12 | 2 | 289 | 90 | LC | 155 | Y | Y | N | N | ACA |
| **32** | 11 | SSc-PAH | F | W | 80 | 2 | 4 | 128 | 50 | LC | 42 | Y | N | N | N | ACA |
| **33** | 11 | SSc-PAH | F | W | 83 | 0 | 2 | 95 | 48 | LC | 32 | Y | N | N | N | ACA |
| **34** | 12 | SSc-PAH | M | W | 78 | 32 | 3 | 290 | 62 | DC | 179 | Y | N | N | N | ATA |
| **35** | 12 | SSc-PAH | F | W | 63 | 23 | 3 | 378 | 60 | DC | 137 | Y | Y | N | N | ATA |
| **36** | 12 | SSc-PAH | M | B | 35 | 40 | 4 | N/A | 55 | DC | 101 | Y | Y | Y | N | ATA |
| **37** | 13 | SSc-PAH | F | W | 38 | 52 | 2 | 324 | 50 | LC | 142 | Y | N | Y | N | ANA |

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| **38** | 13 | SSc-PAH | F | W | 66 | 70 | 2 | 154 | 64 | DC | 145 | Y | N | Y | N | ANA |
| **39** | 13 | SSc-PAH | F | W | 76 | 3 | 3 | N/A | 44 | LC | 83 | Y | N | N | N | ANA |
| **40** | 14 | SSc-PAH | F | W | 38 | 48 | 2 | 420 | 65 | DC | 204 | Y | N | N | N | ANA |
| **41** | 14 | SSc-PAH | M | W | 65 | 21 | 3 | 142 | 48 | DC | 72 | Y | N | N | N | ANA |
| **42** | 14 | SSc-PAH | F | B | 53 | 6 | 2 | 160 | 70 | DC | 27 | Y | Y | N | N | ANA |
| **43** | 15 | SSc-PAH | F | W | 65 | 71 | 3 | 330 | 35 | LC | 108 | Y | N | N | N | ANA |
| **44** | 15 | SSc-PAH | M | B | 64 | 57 | 2 | 482 | 65 | DC | 312 | Y | Y | N | N | ANA |
| **45** | 16 | SSc-PAH | F | W | 62 | 85 | 2 | 326 | 75 | LC | 195 | Y | N | Y | N | ACA |
| **46** | 16 | SSc-PAH | F | W | 76 | 64 | 2 | 185 | 58 | LC | 202 | Y | N | N | N | ACA |
| **47** | 16 | SSc-PAH | F | W | 86 | 44 | 2 | 224 | 52 | LC | 282 | Y | N | Y | N | ACA |
| **48** | 17 | SSc-PAH | F | W | 76 | 56 | 3 | 148 | 84 | DC | 148 | Y | N | Y | N | ATA |
| **49** | 17 | SSc-PAH | F | B | 53 | 36 | 3 | 212 | 62 | DC | 116 | Y | N | Y | N | ATA |
| **50** | 17 | SSc-PAH | F | W | 66 | 126 | 4 | 92 | 77 | LC | 273 | Y | N | Y | Y | ATA |
| **51** | 18 | SSc | F | W | 66 | N/A | 2 | N/A | N/A | LC | 302 | Y | N | Y | Y | ANA |
| **52** | 18 | SSc | M | W | 64 | N/A | 0 | N/A | N/A | LC | 180 | Y | Y | N | Y | ANA |
| **53** | 18 | SSc | M | W | 65 | N/A | 2 | N/A | N/A | DC | 132 | Y | N | Y | Y | ANA |
| **54** | 19 | SSc | F | W | 64 | N/A | 1 | N/A | N/A | LC | 24 | Y | N | N | N | ACA |
| **55** | 19 | SSc | F | W | 39 | N/A | 1 | N/A | N/A | LC | 33 | Y | N | N | N | ACA |
| **56** | 19 | SSc | F | W | 37 | N/A | 1 | N/A | N/A | LC | 36 | Y | Y | Y | N | ACA |
| **57** | 20 | SSc | F | W | 55 | N/A | 1 | N/A | N/A | DC | 38 | Y | N | N | Y | ANA |
| **58** | 20 | SSc | F | B | 38 | N/A | 2 | N/A | N/A | DC | 35 | Y | Y | Y | N | ANA |
| **59** | 20 | SSc | F | W | 39 | N/A | 1 | N/A | N/A | LC | 91 | Y | Y | N | Y | ANA |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **60** | 21 | SSc | M | W | 40 | N/A | 2 | N/A | N/A | DC | 204 | Y | Y | Y | N | ATA |
| **61** | 21 | SSc | F | W | 51 | N/A | 2 | N/A | N/A | DC | 84 | Y | Y | Y | N | ATA |
| **62** | 21 | SSc | F | W | 57 | N/A | 3 | N/A | N/A | DC | 81 | Y | Y | Y | N | ATA |
| **63** | 22 | SSc | F | W | 79 | N/A | 1 | N/A | N/A | LC | 535 | Y | N | N | N | ACA |
| **64** | 22 | SSc | F | W | 41 | N/A | 2 | N/A | N/A | LC | 72 | Y | N | N | N | ACA |
| **65** | 22 | SSc | F | W | 69 | N/A | 2 | N/A | N/A | LC | 348 | Y | N | N | N | ACA |
| **66** | 23 | SSc | F | B | 37 | N/A | 2 | N/A | N/A | DC | 168 | Y | N | Y | N | ANA |
| **67** | 23 | SSc | F | B | 45 | N/A | 1 | N/A | N/A | DC | 19 | Y | Y | N | Y | ANA |
| **68** | 23 | SSc | M | B | 33 | N/A | 2 | N/A | N/A | DC | 41 | Y | Y | Y | N | ANA |
| **69** | 24 | SSc | F | W | 40 | N/A | 0 | N/A | N/A | DC | 106 | Y | Y | N | Y | ACA |
| **70** | 24 | SSc | F | W | 62 | N/A | 0 | N/A | N/A | LC | 113 | Y | N | N | N | ACA |
| **71** | 24 | SSc | F | W | 59 | N/A | 0 | N/A | N/A | LC | 178 | Y | N | N | N | ACA |
| **72** | 25 | SSc | M | W | 29 | N/A | 3 | N/A | N/A | DC | 58 | Y | N | Y | N | ATA |
| **73** | 25 | SSc | F | W | 30 | N/A | 2 | N/A | N/A | DC | 63 | Y | Y | Y | N | ATA |
| **74** | 25 | SSc | F | W | 59 | N/A | 2 | N/A | N/A | DC | 72 | Y | N | Y | N | ATA |

Legend to supplemental figures

Supplemental figure 1. **Indirect immunofluorescence on unpermeabilised pulmonary human microvacular endothelial cells.** Serum IgG from 2 representative individuals from each group of patients: healthy controls (a, b), patients with systemic sclerosis without pulmonary arterial hypertension (PAH) (c, d), systemic sclerosis-associated PAH (e, f) and idiopathic PAH (g, h). Sera were tested at a 1:200 dilution. Secondary antibody: goat anti-human IgG antibody conjugated with FITC, nuclear staining: DAPI. Magnification x 63.

Supplemental figure 2. **Indirect immunofluorescence on unpermeabilised dermal human microvacular endothelial cells.** Serum IgG from 2 representative individuals from each group of patients: healthy controls (a, b), patients with systemic sclerosis without pulmonary arterial hypertension (PAH) (c, d), systemic sclerosis-associated PAH (e, f) and idiopathic PAH (g, h). Sera were tested at a 1:200 dilution. Secondary antibody: goat anti-human IgG antibody conjugated with FITC, nuclear staining: DAPI. Magnification x 63.