Table 8 Relation of IPF preliminary core set instruments to aspects of OMERACT filter in IPF

IPF	Dyspnoea		Cough		HRQoL		Lung physiology		Lung imaging	Survival
Instruments	D-12	MRC	UCSD-SBQ	LCM	SGRQ	SF-36	FVC	DLCO	HRCT—overall extent of disease	All-cause mortality
Truth										
Face validity	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Content validity	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Construct validity	Y	Y	Y	NT	Y	Y	Y	Y	Y	Y
Criterion validity	NT	NT	NT	NT	NT	NT	No	No	Y	Y
Discrimination										
Discriminatory	NT	NT	Y	NT	NT	NT	±	±	Y	No
Reliable	NT	NT	NT	NT	Y	Y	Y	N	Y	Y
Reproducible	NT	NT	NT	NT	Y	NT	Y	±	Y	N/A
Sensitive to change	NT	NT	Y	NT	Y	Y	Y	Y	Yes but relatively slow	N/A
Feasibility										
Cost effective	Y	Y	Y	Y	Y	Y	Y	Y	Y	No*
Interpretability	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Readily available	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Safe for patients	Y	Y	Y	Y	Y	Y	Y	Y	±	Y
Patient-derived content†	Y	No	No	No	No	No	N/A	N/A	N/A	N/A

*Not cost effective as a primary efficacy endpoint but highly cost effective as a secondary endpoint to detect treatment toxicity—see text for discussion on 'survival'.
†US Food and Drug Administration advocates patient-reported instruments be developed by qualitative data supplied by patients.[18 19]
±, ambiguous; D-12, Dyspnea-12; DLCO, diffusion capacity of lung for carbon monoxide; FVC, forced vital capacity; HRCT, high-resolution CT; IPF, idiopathic pulmonary fibrosis; LCM, Leicester Cough Monitor; MRC, Medical Research Council Dyspnea Scale; N/A, not applicable; NT, not yet tested; OMERACT, Outcome Measures in Rheumatology; SGRQ, St George's Respiratory Questionnaire; SF-36, Short Form 36; UCSD, University of San Diego Shortness of Breath Questionnaire; Y, yes.

recently developed PROMs, such as the King's Brief ILD Health Assessment Questionnaire (K-BILD).[30]

The extent of ground-glass opacities, honeycombing and/or reticulations on high-resolution CT (HRCT) scan each merited careful consideration as outcome measures. However, taken separately each was felt to incompletely capture disease progression in either CTD-ILD or IPF. The overall extent of ILD on HRCT was accepted to provisionally describe the most appropriate and feasible composite of radiological abnormalities to monitor for disease progression.[31 32] No specific assessment tool at this time was able to be confidently identified as it is not yet clear whether subjective or automated objective assessment is the more accurate approach. Though serial HRCT raises concern for patient safety, validation studies of less radio-intense methods of HRCT serial assessment[33] are underway.

Progression-free survival in IPF was agreed to have merit,[34] however the group was undecided as to the practicality of this endpoint in the context of a trial limited to 1 year's duration. Mortality was minimal or absent in two recent RCTs of SSc-ILD.[35 36] There are cogent arguments for and against survival as the primary outcome in studies of IPF.[34 37] Regardless of this unresolved debate, mortality was recognised as an essential endpoint in all treatment trials as it provides a harm signal,[34 37] with all-cause mortality identified as a valid measure of survival in CTD-ILD and IPF. The utility of other measures of progression-free survival in RCTs requires further investigation of candidate instruments before recommending their use in RCTs.

While the domain of *Cough* did not survive the Delphi process, it was important to patient participants. Additionally, there is a correlation between cough and IPF progression[38] and with ILD severity in SSc.[39] In SSc-ILD, cough adversely impacted HRQoL and improved with treatment.[39] The LCM was selected as an interim measure as it was deemed more able to capture frequency, quality and intensity, and impact on HRQoL. It was also most feasible to administer.[40]

Primary and secondary endpoint status of the proposed measures were considered, intensely discussed and even voted upon during the NGT. However, at this preliminary stage and given the lack of full validation of the core measures, the consensus was to pursue further data. A more careful approach to endpoint status declarations entails ad hoc and prospective performance analyses of these measures.

Though we recommend these proposed measures for all future research ventures, continued use of measures outside this core set, for clinical practice and research purposes, is fully expected with further research into their performance anticipated and necessary. Rather, this endeavour defines the currently available, best validated and feasible instruments while providing a much needed prioritised research agenda focus to the research community.

This project applied rigorous multi-investigational processes that captured the perspectives of the international ILD expert community and the life experience of patients with ILD to identify a set of domains and measures. Participation remained robust through all tiers of the consensus process.

The importance of patient participation is supported by the incorporation of *HRQoL*, *Participation* and *Fatigue* in the RA core set for RCTs. From a practical perspective, qualitative data collection involved only English-speaking patients from North America, and results may be affected by cultural, environmental and resource-related effects requiring further investigations to follow up our reported findings. Nevertheless, the engagement of patients as partners in the iterative process was important in identifying and re-capturing areas of potentially meaningful measures of disease activity.

CONCLUSIONS

It is critical that valid and clinically useful instruments be developed and validated to assess the likelihood of treatment response in these disorders. Identification of consensus

preliminary domains and instruments to measure them was attained and is a major advance anticipated to facilitate multicentre RCTs in the field. However, none of the provisional endpoints were ultimately felt to be either ideal or fully validated. Feasible endpoints like FVC are not perfect; more rigorous endpoints like mortality, particularly in the setting of CTD-ILD, lack feasibility. Thus, selecting the best non-ideal endpoints from a larger group of non-ideal endpoints still leaves us with much work which includes further validation of existing and development of new instruments.

Author affiliations
[1]Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA
[2]University of Toronto, Toronto, Canada
[3]German Rheumatism Research Centre, Berlin, Germany
[4]Charité Universitaetsmedizin, Berlin, Germany
[5]University of Michigan, Ann Arbor, Michigan, USA
[6]Brigham and Womens Hospital, Boston, Massachusetts, USA
[7]University Hospital Zurich, Zurich, Switzerland
[8]University of Manitoba, Manitoba, Canada
[9]University of Pittsburgh, Pittsburgh, Pennsylvania, USA
[10]Royal Free Hospital, London, UK
[11]National Jewish Health, Denver, Colorado, USA
[12]Medical University of Bialystok, Bialystok, Poland
[13]Patient Research Partner, Office of Public Health, New Orleans, Louisiana, USA
[14]University of Pennsylvania, Philadelphia, Pennsylvania, USA
[15]University of Dresden, Dresden, Germany
[16]University of Crete, Heraklion, Greece
[17]University of Cincinnati, Cincinnati, Ohio, USA
[18]Massachusetts General Hospital, Boston, Massachusetts, USA
[19]Boston University School of Medicine, Boston, Massachusetts, USA
[20]Johns Hopkins University, Baltimore, Maryland, USA
[21]University of California-San Francisco, San Francisco, California, USA
[22]Claude Bernard University, Lyon, France
[23]Cleveland Clinic, Cleveland, Ohio, USA
[24]Asan Medical Center University of Ulsan, Ulsan, South Korea
[25]National Institute of Environmental Health Sciences, National Institutes of Health, Bethesda, Maryland, USA
[26]University of Adelaide, Adelaide, Australia
[27]University Hospital of Modena, Modena, Italy
[28]Mayo Clinic College of Medicine, Rochester, Minnesota, USA
[29]Patient Research Partner, Maryland, USA
[30]Royal Brompton Hospital and National Heart and Lung Institute, London, UK
[31]Stanford University, Palo Alto, California, USA
[32]Scleroderma Research Consultants, LLC, Avon, Connecticut, USA

Acknowledgements Acknowledgements of thanks for essential and gracious assistance: Kourtnie Augustin, Louisiana State University Health Sciences Center – New Orleans, USA; Reed Barrios, Patient Expert, New Orleans, USA; Bennett deBoisblanc, Louisiana State University Health Sciences Center – New Orleans, USA; Kerri Connolly, Scleroderma Foundation, Danvers, MA, USA; Luis R Espinoza, Louisiana State University Health Sciences Center – New Orleans, USA; Daniel E and Elaine Furst, University of California – Los Angeles, CA, USA; Robert Hedlund, Patient Expert, Virginia, USA; Matthew R Lammi, Louisiana State University Health Sciences Center – New Orleans, USA; Steve Nathan, Innova, Fairfax, Virginia, USA; Karen Nichols, Patient Expert, Virginia, USA; Frank Smart, Louisiana State University Health Sciences Center – New Orleans; Virginia Steen, Georgetown University, Washington DC, USA; Valorie Thompson, DINORA; Pieter van den Assum, Patient Expert, Virginia, USA. Ms LeSage and Ms Sarver are co-investigators and have contributed their expertise as patients to key decision-making in the design, implementation as well as analysis and interpretation of data and thus listed as authors. Research assistance with analysis and coding of patient perspective manuscripts provided by: Sophia L Cenac and Harmanjot K Grewal from LSU – New Orleans, USA; Angela M Christensen and Sancia Ferguson from Tulane University and Maithy Tran from University of Toronto.

Contributors LAS: PI, methodologist and project leader of all three components, author of manuscript. SM: Co-PI of patient perspective, NGT panel member (content, data collection, analysis), manuscript reviewer (MR). DH: Steering Committee for Delphi, Joint Analysis Decision, Interpretation of Delphi Data (SC1), methodologist, designer of Delphi database, statistician to all three components, MR. DK: SC1, NGT panel member, MR. PFD: SC1, NGT panel member, MR. OD: SC1, NGT panel member, methodology, MR. KRF: SC1, NGT panel member, MR. SF: Steering Committee for Patient Perspective, Qualitative analyses, Coding, Interpretation (SC2), methodology, analysis, MR. CVO: SC1, NGT panel member, MR. CPD: SC1, NGT panel member, MR. AF: SC2, NGT panel member, MR. OMK-B: SC1, database design, NGT panel member, MR. DL: SC2, patient research partner (assist with design, analysis, interpretation), analysis, NGT panel member, MR. PAM: SC1, NGT panel member, MR. KP: SC1, MR. DP: SC1, database construction, MR. JS: SC2, NGT panel member, MR. KA: NGT panel member, MR. RPB: NGT panel member, MR. FVC: SC2, patient data collection, analysis. RBC: literature review, database content collection and assembly, MR. LC-S: SC2, patient data collection, analysis, MR. HRC: content, analysis, interpretation, MR. VC: content, analysis, interpretation, MR. SD: SC2, patient data collection, analysis, MR. KBH: NGT panel member, MR. LH: SC2, patient data collection, analysis, MR. AAS: SC2, patient data collection, analysis, MR. DSK: NGT panel member, MR. DAL: NGT panel member, MR. FWM: NGT panel member, MR. SMP: NGT panel member, MR. LR: NGT panel member, MR. JHR: NGT panel member, MR. NS: literature review, database content collection and assembly, MR. CS: SC2, patient research partner, analysis NGT panel member, MR. AUW: SC1, NGT panel member, MR. VS: SC1, NGT panel member, MR. ELM: SC1, NGT panel member, MR. KKB: SC1, NGT panel member, MR. JRS: SC1, NGT panel member, MR.

Collaborators Delphi Co-Authors: *indicates Disease Committee Member. Rohit Aggarwal*, University of Pittsburgh School of Medicine, USA; Gillian Ainslie, University of Cape Town & Groote Schuur Hospital, South Africa; Firas Alkassab, University of North Carolina-Chapel Hill, Charlotte, USA; Yannick Allanore*, Université Paris Descartes, France; Marina E Anderson, University of Liverpool, UK; Andrew P Andonopoulos, University of Patras School of Medicine, Greece; Danielle Antin-Ozerkis, Yale University School of Medicine, USA; Ana Arrobas, Centro Hospitalar de Coimbra, Portugal; Dana P Ascherman*, University of Miami Miller School of Medicine, USA; Shervin Assassi, University of Texas Health Science Center at Houston, USA; Murray Baron, Jewish General Hospital, McGill University, USA; Joan M Bathon*, Columbia University College of Physicians & Surgeons, USA; Juergen Behr, Ludwig-Maximilians University, Munich, Germany; Lorenzo Beretta, Referral Center for Systemic Autoimmune Diseases-Milan, Italy; Clifton O Bingham III, Johns Hopkins University, USA; Matthew Binnie, St. Michael's Hospital, Toronto, Canada; Surinder S Birring, King's College Hospital, UK; Francesco Boin, Johns Hopkins University; USA; Tim Bongartz*, Mayo Clinic College of Medicine, USA; Arnaud Bourdin, Département de Pneumologie et Addictologie INSERM U1046 - Université Montpellier; Demosthenes Bouros, Democritus University of Thrace, Greece; Richard Brasington, Washington University, St Louis, USA; Paul Bresser, Onze Lieve Vrouwe Gasthuis, Netherlands; Maya H Buch, University of Leeds, UK; P Sherwood Burge, Birmingham Hearlands Hospital, UK; Loreto Carmona, Universidad Camilo José Cela and Institute for Musculoskeletal Health, Spain; Patricia E Carreira, Hospital Universitario, Spain; Carlos RR Carvalho, University of Sao Paulo Medical School, Brasil; Luis J Catoggio, Hospital Italiano de Buenos Aires, Argentina; Kevin M Chan, University of Michigan Health Systems, USA; Jeffrey Chapman, Cleveland Clinic, USA; Soumya Chatterjee, Cleveland Clinic, USA; Felix Chua*, St. George's Hospital NHS Trust, UK; Lorinda Chung, Stanford University School of Medicine, USA; Matthew Conron, St Vincent's Hospital, Australia; Tamera Corte, University of Sidney, Australia; Gregory Cosgrove, National Jewish Health, USA; Ulrich Costabel, University of Duisburg-Essen, Germany; Gerard Cox, McMaster University, Canada; Bruno Crestani, Centre de Compétences Maladies Rares Pulmonaires, Paris, France; Leslie J Crofford, University of Kentucky College of Medicine, USA; Mary E Csuka, Medical College of Wisconsin, USA; Pablo Curbelo, Universidad de la República, Montevideo, Uruguay; László Czirják, University of Pécs, Hungary; Zoe Daniil, University of Thessaly, Larissa, Greece; Christine L D'Arsigny, Queen's University, Canada; Gerald S Davis, College of Medicine University of Vermont, USA; Joao A de Andrade, University of Alabama at Birmingham, USA; Paul De Vuyst, Hopital Erasme, Université Libre de Bruxelles, Belgium; Owen J Dempsey, Aberdeen Royal Infirmary Foresterhill, Scotland, UK; Chris T Derk, University of Pennsylvania, USA; Jörg Distler, University of Erlangen-Nuremberg, Germany; William G Dixon*, University of Manchester, UK; Gregory Downey, National Jewish Health, USA; Mittie K Doyle, Alexion Pharmaceuticals Inc, Cambridge, USA; Marjolein Drent, Maastricht University, Maastricht, Netherlands; Lakshmi Durairaj, Carver College of Medicine, University of Iowa, USA; Paul Emery, University of Leeds, UK; Luis R Espinoza, Louisiana State University Health Sciences Center, New Orleans, USA; Dominique Farge, St. Louis Hospital, Paris, France; Maryam Fathi, Karolinska Institutet, Sweden; Charlene D Fell, University of Calgary, Canada; Barri J Fessler, University of Alabama at Birmingham, USA; John E Fitzgerald, University of Texas Southwestern Medical Center, USA; Ivan Foeldvari, University of Hamburg, Germany; George A Fox, Memorial University of Newfoundland, Canada; Tracy M Frech, University of Utah, USA; Sara Freitas, Coimbra Hospital and University Center, Portugal; Daniel E Furst*, University of California Los Angeles, USA; Armando Gabrielli, Università Politecnica delle Marche, Ancona, Italy; Rosario García-Vicuña, Hospital Universitario de la Princesa. IISP; Ognian B Georgiev, University Hospital Alexandrovska, Sofia, Bulgaria; Anthony Gerbino, Virginia Mason Medical Center, USA; Adrian Gillisen, General Hospital Kassel, Germany; Dafna D Gladman, University of Toronto, Canada; Marilyn Glassberg, University of Miami Miller School of Medicine, USA; Bernadette R Gochuico, National Human Genome Research Institute, National Institutes of Health, USA; Athena Gogali, University Hospital of Ioannina, Greece; Nicole S Goh*, Alfred Hospitals, Melbourne, Australia; Avram Goldberg, Hofstra North Shore LIJ School of Medicine, USA; Hilary J Goldberg, Brigham and Women's Hospital, Harvard Medical School, USA; Mark F Gourley*, National Institutes of Health, USA; Leroy Griffing, Mayo Clinic College of Medicine, USA; Jan C Grutters, University Medical Center Utrecht, Netherlands;

Ragnar Gunnarsson, Oslo University Hospital, Norway; Eric Hachulla, Claude Huriez Hospital, University of Lille, France; Frances C Hall, University of Cambridge, UK; Sergio Harari, U.O. di Pneumologia Ospedale San Giuseppe MultiMedica, Milan, Italy; Ariane L Herrick, University of Manchester, UK; Erica L Herzog, Yale University School of Medicine, USA; Roger Hesselstrand, Lund University, Sweden; Nikhil Hirani, University of Edinburgh, UK; Ulla Hodgson, Helsinki University Hospital, Finland; Helen M Hollingsworth, Pulmonary Center, Boston University School of Medicine, USA; Robert J Homer, Yale University School of Medicine Department of Pathology, USA; Rachel K Hoyles, Oxford Centre for Respiratory Medicine, UK; Vivien M Hsu, University of Medicine and Dentistry of New Jersey, USA; Richard B Hubbard, University of Nottingham, UK; Nicolas Hunzelmann, Deptartment of Dermatology, University of Cologne, Germany; Maria Eloisa Isasi, Hospital Maciel. Montevideo. Uruguay; Elida Susana Isasi, Hospital Maciel. Montevideo. Uruguay; Soren Jacobsen, Rigshospitalet, Copenhagen University Hospital, Denmark; Sergio A Jimenez, Jefferson University, USA; Sindhu R Johnson, University of Toronto, Toronto, Canada; Christine H Jones, University of Vermont College of Medicine, Fletcher Allen Health Care, USA; Bashar Kahaleh, University of Toledo Medical Center, USA; Ronaldo A Kairalla, Heart Institute (InCor), University of São Paulo Medical School, Brazil; Meena Kalluri, University of Alberta, Canada; Sanjay Kalra, Mayo Clinic, USA; Robert J Kaner, Cornell University School of Medicine, USA; Brent W Kinder, University of Cincinnati College of Medicine; USA Goksel Kiter (Kiter G), Pamukkale University, Turkey; Ross C Klingsberg, Tulane University School of Medicine, USA; Maria Kokosi, Sismanoglio General Hospital; Martin RJ Kolb, Mcmaster University, Canada; Joanna Kur-Zalewska, Military Institute of Medicine, Warsaw, Poland; Masataka Kuwana*, Keio University School of Medicine, Japan; Fiona R Lake, University of Western Australia; Edward V Lally, Brown University, USA; Joseph A Lasky, Tulane University School of Medicine, USA; Ileda M Laurindo, University of Sao Paulo, Brasil; Lawrence Able, Sanjay Gandhi Postgraduate Institute of Medical Sciences, India; Peter Lee, University of Toronto, Canada; Colm T Leonard, University Hospital of South Manchester NHS Foundation Trust, UK; Dale C Lien, University of Alberta, Canada; Andrew H Limper, Mayo Clinic College of Medicine, USA; Stamatis-Nick C Liossis, University of Patras Medical School. Greece; Kristine M Lohr, University of Kentucky College of Medicine, USA; James E Loyd, Vanderbilt University, USA; Ingrid E Lundberg*, Karolinska Institutet, Sweden; Yolanda N Mageto, University of Vermont, USA; Toby M Maher, Royal Brompton Hospital, UK; Tafazzul H Mahmud, Shaikh Zayed Medical complex, Lahore, Pakistan; Helene Manganas, CHUM (Notre-Dame Hospital), Canada; Isabelle Marie, Rouen University, France; Theodore K Marras, University Health Network, Mount Sinai hospital and the University of Toronto; José Antônio Baddini Martinez, Faculdade de Medicina de Ribeirao Preto, Brasil; Fernando J Martinez, University of Michigan, USA; Alessandro Mathieu, Università degli Studi di Cagliari, Italy; Marco Matucci-Cerinic*, University of Florence, Italy; Maureen D Mayes*, University of Texas Health Science Center at Houston, USA; Kevin M McKown, University of Wisconsin School of Medicine and Public Health, USA; Thomas A Medsger, Jr., University of Pittsburgh School of Medicine, USA; Richard T Meehan, National Jewish Health, USA; Mendes Ana Cristina, Serviço de Pneumologia 1, CHLN- Hospital de Santa Maria; Keith C Meyer, University of Wisconsin School of Medicine and Public Health, USA; Ann B Millar, University of Bristol, UK; Nesrin Moğulkoç, Ege University, Izmir, Turkey; Jerry A Molitor, University of Minnesota, USA; António Morais, Pneumology Department, Centro Hospitalar São João; Portugal Luc Mouthon, Hôpital Cochin, Paris, France; Veronika Müller, Semmelweis University, Hungary; Joachim Müller-Quernheim, University of Freiburg, Germany; Oleg Nadashkevich, Lviv National Medical University, Ukraine; Roland Nador, University of Alberta, Canada; Peter Nash, University of Queensland, Australia; Steven D Nathan, Inova Fairfax Hospital, USA; Carmen Navarro, Instituto Nacional de Enfermedades Respiratorias, Mexico; Sofia Neves, Centro Hospitalar Vila Nova de Gaia/ Espinho, Portugal; Imre Noth, University of Chicago, USA; Hilario Nunes, Avicenne Hospital, Paris, France; Amy L. Olson, National Jewish Health, USA; Christian F Opitz, DRK Kliniken Berlin-Köpenick, Berlin, Germany; Maria Padilla, Mount Sinai School of Medicine, New York, USA; Dimitrios Pappas, Columbia University College of Physicians & Surgeons, USA; Helen Parfrey, University of Cambridge and Papworth Hospital, UK; José M Pego-Reigosa, Meixoeiro Hospital, Spain; Carlos AC Pereira, Federal University of São Paulo, Brasil; Rafael Perez, University of Kentucky College of Medicine, USA; Janet E Pope*, Western University, Canada; Joanna C. Porter, University College London, UK; Elisabeth A Renzoni, Interstitial Lung Disease Unit, Royal Brompton Hospital, UK; Gabriela Riemekasten, Charité Universitätsmedizin Berlin, Germany; David J Riley, University of Medicine and Dentistry of New Jersey, USA; Maureen Rischmueller, The Queen Elizabeth Hospital and University of Adelaide, Australia; Tatiana S Rodriguez-Reyna, Instituto Nacional de Ciencias Médicas y Nutrición, Mexico; Rojas-Serrano, Instituto Nacional de Enfermedades Respiratorias, Mexico; Jesse Romam, University of Louisville Health Sciences Center, USA; Glenn D Rosen, Stanford University School of Medicine, USA; Milton Rossman, University of Pennsylvania, USA; Naomi Rothfield, University of Connecticut Health Center, USA; Steven A Sahn, Medical University of South Carolina, USA; Alessandro Sanduzzi, Università degli Studi di Napoli "Federico II", Italy; Mary Beth Scholand (Scholand MB for Medline, University of Utah, USA; Moises Selman, Instituto Nacional de Enfermedades Respiratorias, Mexico; Jean-Luc Senécal, University of Montreal, Canada; Philip Seo, Johns Hopkins University, USA; Richard M Silver*, Medical University of South Carolina, USA; Joshua J Solomon, National Jewish Health, USA; Virginia Steen*, Georgetown University, USA; Wendy Stevens, St. Vincent's Hospital, Melbourne, Australia; Charlie Strange, Medical University of South Carolina, USA; Robert Sussman, Pulmonary and Allergy Associates, Summit, NJ, USA; Evelyn D Sutton, Dalhousie University, Canada; Nadera J Sweiss, University of Illinois, Chicago, USA; Göran Tornling, Karolinska Institutet, Sweden; George E Tzelepis, University of Athens Medical School, Greece; Alvaro Undurraga, Thorax National Institute, Santiago, Chile; Allessandra Vacca, University and A.O.U. of Cagliari, Italy; Carlo Vancheri, University of Catania, Italy; Janos Varga, National Koranyi Institute of TB and Pulmonology, Hungary; Douglas J Veale, University College Dublin, Ireland; Suncica Volkov, University of Illinois, Chicago, USA; Ulrich A Walker, Basel University Deptartment of Rheumatology, Felix Platter-Spital, Switzerland; Mark Wencel, Via Christi Clinic, University of Kansas Medical Center – Wichita, USA; Lewis J Wesselius, Mayo Clinic Arizona, USA; Melissa Wickremasinghe, St. Mary's Hospital, London, UK; Pearce Wilcox, University of British Columbia, Canada; Margaret L Wilsher, Auckland District Health Board and University of Auckland, New Zealand; Frank A Wollheim, Lund University, Sweden; Wim A Wuyts, University Hospitals Leuven, Netherlands; Gordon Yung, University of California - San Diego, USA; Pietro Zanon, Ospedale di Circolo di Busto Arsizio, Italy; Christopher J Zappala, Royal Brisbane & Women's Hospital, Brisbane QLD Australia

Pathology Advisory Team Steve D Groshong, National Jewish Health, USA; Kevin O Leslie, Mayo Clinic College of Medicine, USA; Jeffrey L Myers, University of Michigan, USA; Robert F Padera, Brigham and Women's Hospital, USA

Radiology Advisory Team Sujal R Desai, King's College Hospital, London, UK; Jonathan Goldin, David Geffen School of Medicine, UCLA, USA; Ella A Kazerooni, University of Michigan, Ann Arbor, USA; Jeffrey S Klein, Fletcher Allen Health Care, University of Vermont, USA; David A Lynch, National Jewish Health, Denver, USA

Additional Statistical Support Kevin J Keen, University of Northern British Columbia, Prince George, Canada

Funding Non-profit support: These studies were supported in part by the intramural division of the National Institute of Environmental Health Sciences, National Institutes of Health; and the following non-profit organisations: Brigham and Women's Hospital, Charité Hospital—Berlin, German Rheumatism Research Centre, Ira J Fine Discovery Fund, Jonathan and Lisa Rye Scleroderma Research Foundation, Louisiana State University Health Sciences Center—New Orleans, Mayo Clinic—Rochester, National Jewish Hospital Denver, Louisiana State Office of Public Health—New Orleans, OMERACT (Outcome Measures in Rheumatology), Scleroderma Foundation, Sibley Hospital Foundation, and Sonia Roth AARC Foundation. Commercial Interest Support: Abbott Laboratories Canada, Actelion, Boehringer–Ingelheim Pharmaceuticals, Celgene, Intermune, Sigma Tau, UCB and United Therapeutics.

Competing interests None.

Ethics approval Louisiana State University School of Medicine Institutional Review Board for all components. Patient perspective studies also included approval from Johns Hopkins University, Massachussets General Hospital, University of Manitoba and University of Toronto.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement *We have made all data visible in the online supplement.*

REFERENCES

1 American Thoracic Society/European Respiratory Society international multidisciplinary consensus classification of the idiopathic interstitial pneumonias. *Am J Respir Crit Care Med* 2002;165:277–304.
2 Raghu G, Collard HR, Egan JJ. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *Am J Respir Crit Care Med* 2011;183:788–824.
3 Tyndall AJ, Bannert B, Vonk M, *et al*. Causes and risk factors for death in systemic sclerosis: a study from the EULAR Scleroderma Trials and Research (EUSTAR) database. *Ann Rheum Dis* 2010;69:1809–15.
4 de Lauretis A, Veeraraghavan S, Renzoni E. Review series: aspects of interstitial lung disease: connective tissue disease-associated interstitial lung disease: how does it differ from IPF? How should the clinical approach differ? *Chron Respir Dis* 2011;8:53–82.
5 Olson AL, Swigris JJ, Sprunger DB, *et al*. Rheumatoid arthritis-interstitial lung disease-associated mortality. *Am J Respir Crit Care Med* 2011;183:372–8.
6 Bajwah S, Ross JR, Peacock JL, *et al*. Interventions to improve symptoms and quality of life of patients with fibrotic interstitial lung disease: a systematic review of the literature. *Thorax* 2013;68:867–79.
7 Martinez FJ, Safrin S, Weycker D, *et al*.; IPF Study Group. The clinical course of patients with idiopathic pulmonary fibrosis. *Ann Intern Med* 2005;142:963–7.
8 Collard HR, King TE Jr, Bartelson BB, *et al*. Changes in clinical and physiologic variables predict survival in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2003;168:538–42.
9 Boers M, Brooks P, Strand V, *et al*. The OMERACT filter for outcome measures in rheumatology. *J Rheumatol* 1998;25:2198–9.

10 Rand Organization. Multiple articles and chapters in PDF format by the RAND Organization. http://www.rand.org/international_programs/pardee/pubs/futures_method/delphi.html (accessed 3 Dec 2012).
11 VandeVen AH, Delbecq AL. The effectiveness of nominal, Delphi, and interacting group decision making processes. *Acad Manage J* 1974;17:605–21.
12 Distler O, Behrens F, Huscher D, *et al*. Need for improved outcome measures in pulmonary arterial hypertension related to systemic sclerosis. *Rheumatology (Oxford)* 2006;45:1455–7.
13 Bottomley A, Jones D, Claassens L. Patient-reported outcomes: assessment and current perspectives of the guidelines of the Food and Drug Administration and the reflection paper of the European Medicines Agency. *Eur J Cancer* 2009;45:347–53.
14 Swigris JJ, Stewert AL, Gould MK, *et al*. Patients' perspectives on how idiopathic pulmonary fibrosis affects the quality of their lives. *Health Qual Life Outcomes* 2005;3:61.
15 Pope C, Ziebland S, Mays N. Qualitative research in health care: analysing qualitative data. *Br Med J* 2000;320:114–16.
16 Fink A, Kosecoff J, Chassin M, *et al*. Consensus methods: characteristics and guidelines for use. http://www.rand.org/content/dam/rand/pubs/notes/2007/N3367.pdf (accessed 3 Dec 2012).
17 Beyer C, Distler JH, Allanore Y, *et al*.; EUSTAR Biobanking Group. EUSTAR biobanking: recommendations for the collection, storage and distribution of biospecimens in scleroderma research. *Ann Rheum Dis* 2011;70:1178–82.
18 Manali ED, Lyberopoulos P, Triantafillidou C, *et al*. MRC Chronic Dyspnea Scale: relationships with cardiopulmonary exercise testing and 6-minute walk test in idiopathic pulmonary fibrosis patients: a prospective study. *BMC Pulm Med* 2010;10:32.
19 Nishiyama O, Taniguchi H, Kondoh Y, *et al*. A simple assessment of dyspnoea as a prognostic indicator in idiopathic pulmonary fibrosis. *Eur Respir J* 2010;36:1067–72.
20 Roth MD, Tseng CH, Clements PJ, *et al*.; Scleroderma Lung Study Research Group. Predicting treatment outcomes and responder subsets in scleroderma-related interstitial lung disease. *Arthritis Rheum* 2001;63:2797–808.
21 Swigris JJ, Han M, Vij R, *et al*. The UCSD Shortness of Breath Questionnaire has longitudinal construct validity in idiopathic pulmonary fibrosis. *Respir Med* 2012;106:1447–55.
22 du Bois RM, Weycker D, Albera C, *et al*. Forced vital capacity in patients with idiopathic pulmonary fibrosis: test properties and minimal clinically important difference. *Am J Respir Crit Care Med* 2011;184:1382–9.
23 Kim DK, Jacobson FL, Washko GR, *et al*. Clinical and radiographic correlates of hypoxemia and oxygen therapy in the COPD Gene study. *Respir Med* 2011;105:1211–21.
24 Yorke J, Swigris J, Russell AM, *et al*. Dyspnea-12 is a valid and reliable measure of breathlessness in patients with interstitial lung disease. *Chest* 2011;139:159–64.
25 Swigris JJ, Brown KK, Behr J, *et al*. The SF-36 and SGRQ: validity and first look at minimum important differences in IPF. *Respir Med* 2010;104:296–304.
26 Yorke J, Jones PW, Swigris JJ. Development and validity testing of an IPF-specific version of the St George's Respiratory Questionnaire. *Thorax* 2010;65:921–6.
27 Beretta L, Santaniello A, Lemos A, *et al*. Validity of the Saint George's Respiratory Questionnaire in the evaluation of the health-related quality of life in patients with interstitial lung disease secondary to systemic sclerosis. *Rheumatology (Oxford)* 2007;46:296–301.
28 Swigris JJ, Yorke J, Sprunger DB, *et al*. Assessing dyspnea and its impact on patients with connective tissue disease-related interstitial lung disease. *Respir Med* 2010;104:1350–5.
29 Steen VD, Medsger TA. The value of the Health Assessment Questionnaire and special patient-generated scales to demonstrate change in systemic sclerosis patients over time. *Arthritis Rheum* 1997;40:1984–91.
30 Patel AS, Siegert RJ, Brignall K, *et al*. The development and validation of the King's Brief Interstitial Lung Disease (K-BILD) health status questionnaire. *Thorax* 2012;67:804–10.
31 Wells AU, Desai SR, Rubens MB, *et al*. Idiopathic pulmonary fibrosis: a composite physiologic index derived from disease extent observed by computed tomography. *Am J Respir Crit Care Med* 2003;167:962–9.
32 Goh NS, Desai SR, Veeraraghavan S, *et al*. Interstitial lung disease in systemic sclerosis: a simple staging system. *Am J Respir Crit Care Med* 2008;177:1248–54.
33 Winklehner A, Berger N, Maurer B, *et al*. Screening for interstitial lung disease in systemic sclerosis: the diagnostic accuracy of HRCT image series with high increment and reduced number of slices. *Ann Rheum Dis* 2012;71:549–52.
34 Wells AU, Behr J, Costabel U, *et al*. European IPF Consensus Group. Hot of the breath: mortality as a primary end-point in IPF treatment trials: the best is the enemy of the good. *Thorax* 2012;67:938–40.
35 Tashkin DP, Elashoff R, Clements PJ, *et al*. Cyclophosphamide versus placebo in scleroderma lung disease. *N Engl J Med* 2006;354:2655–66.
36 Seibold JR, Denton C, Furst DE, *et al*. Randomized, prospective, placebo-controlled trial of bosentan in interstial lung disease secondary to systemic sclerosis. *Arthritis Rheum* 2010;62:2101–8.
37 Corte TJ, Goh NS, Glaspole IN, *et al*. Idiopathic pulmonary fibrosis: is all-cause mortality a practical and realistic end-point for clinical trials? *Thorax* 2013;68:491–2.
38 Ryerson CJ, Abbritti M, Ley B, *et al*. Cough predicts prognosis in idiopathic pulmonary fibrosis. *Respirology* 2011;16:969–75.
39 Theodore AC, Tseng CH, Li N, *et al*. Correlation of cough with disease activity and treatment with cyclophosphamide in scleroderma interstitial lung disease: findings from the Scleroderma Lung Study. *Chest* 2012;142:614–21.
40 Key AL, Holt K, Hamilton A, *et al*. Objective cough frequency in idiopathic pulmonary fibrosis. *Cough* 2010;6:4.

ARTHRITIS & RHEUMATOLOGY
Vol. 66, No. 5, May 2014, pp 1300–1305
DOI 10.1002/art.38326

BRIEF REPORT

Impaired In Vivo Neovascularization Capacity of Endothelial Progenitor Cells in Patients With Systemic Sclerosis

Masataka Kuwana and Yuka Okazaki

Objective. **Defective vasculogenesis is thought to play a role in the pathogenesis of systemic sclerosis (SSc). We undertook this study to explore the in vivo functional capacity of CD34+CD133+CD309+ endothelial progenitor cells (EPCs) in SSc patients.**

Methods. **CD133+ cells and EPCs were enumerated by flow cytometry. Immunomagnetically sorted circulating CD133+ cells from 16 patients with SSc and 12 healthy subjects, as well as murine colon carcinoma CT-26 cells, were transplanted beneath the skin of SCID mice. Tumor volume and blood vessel density were measured 10 days later. Human EPC incorporation into the vascular wall was evaluated using tumor sections double stained for mouse CD31 and human CD31.**

Results. **The number of CD133+ cells and EPCs was significantly decreased in SSc patients as compared to healthy controls ($P = 0.001$ and $P = 0.02$, respectively), while the proportion of EPCs in CD133+ cells was similar between the 2 groups. CT-26 cells produced markedly stronger tumor growth and neovessel formation when transplanted with CD133+ cells from healthy subjects than when transplanted with CD133+ cells from SSc patients ($P = 0.001$ and $P = 0.008$, respectively). Tumors from mice that received transplants of CT-26 cells and SSc-derived CD133+ cells formed fewer vessels incorporating human EPC–derived mature endothelial cells than did tumors from mice that received transplants of CT-26 cells and CD133+ cells from healthy control subjects ($P = 0.0002$).**

Conclusion. **We established a system that can be used to evaluate the in vivo neovascularization capacity of freshly isolated EPCs. EPCs contribute to vascularization by incorporating into vessel walls and by differentiating into endothelial cells. These EPC functions are impaired in SSc.**

Systemic sclerosis (SSc) is characterized by widespread vasculopathy and excessive fibrosis of the skin and internal organs. Microvascular abnormalities that begin to appear during the earliest stages of the disease reduce blood flow and cause tissue ischemia, leading to Raynaud's phenomenon and ulcers on the fingers. While SSc vasculopathy has been attributed to increased vascular injury, we recently proposed the theory that defective vasculogenesis inhibits the vascular repair machinery, contributing to the pathogenic process (1). For vasculogenesis to occur, bone marrow–derived circulating endothelial progenitor cells (EPCs) must be recruited to form blood vessels (2). Recent findings indicate that in vascular injury or ischemia, EPCs contribute to vascular healing and remodeling by homing to the injury site and working in concert with existing endothelial cells (ECs); however, whether the numbers of circulating CD34+CD133+CD309+ EPCs are reduced or increased in SSc patients is under debate (3).

While several studies have assessed circulating EPC counts in SSc patients, few have evaluated the functional properties of these EPCs. This is primarily due to the difficulty of preparing sufficient numbers of EPCs for functional analysis. We and others have previously reported that the potential of SSc-derived EPCs to become mature ECs is impaired in in vitro cultures of CD133+ cells or late-outgrowth EPCs (1,4). Although large numbers of late-outgrowth EPCs can be obtained by long-term culture (5), their functional properties may be altered by repeated passages and exposure to high concentrations of proangiogenic factors. Herein we established a system for assaying EPC capacity to promote neovascularization in vivo, and used this system to assess whether EPC functions were impaired in SSc patients.

Supported by an Intractable Diseases Research grant from the Japanese Ministry of Health, Labor, and Welfare.

Masataka Kuwana, MD, PhD, Yuka Okazaki, BS: Keio University School of Medicine, Tokyo, Japan.

Address correspondence to Masataka Kuwana, MD, PhD, Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: kuwanam@z5.keio.jp.

Submitted for publication July 23, 2013; accepted in revised form December 17, 2013.

PATIENTS AND METHODS

Patients and controls. This study included 16 female patients who fulfilled the American College of Rheumatology preliminary classification criteria for SSc (6) and were followed up at Keio University Hospital. Patients receiving >10 mg of prednisolone daily, immunosuppressive agents, or statins were excluded. Twelve age- and sex-matched healthy controls were recruited from among hospital staff. None of the subjects had a history of smoking, diabetes, hypertension, or dyslipidemia. The mean ± SD age at initial evaluation was similar among SSc patients and healthy controls (50.7 ± 14.4 years and 50.8 ± 9.5 years, respectively). Half of the SSc patients and controls had already experienced menopause. Among SSc patients, disease duration ranged from 18 months to 175 months from the onset of Raynaud's phenomenon and from 14 months to 120 months from the onset of non–Raynaud's phenomenon symptoms. Nine patients were classified as having diffuse cutaneous SSc. The study protocol was approved by the institutional review board, and written informed consent was obtained from each patient.

Flow cytometry. Circulating counts of CD133+ cells and EPCs were determined by flow cytometry after magnetic-activated cell sorting (MACS) was used to partially enrich CD34+ cells as previously described (3). We used multiparameter flow cytometry to identify EPCs by the expression of CD34, CD133, and CD309, and the viability marker 7-aminoactinomycin D (BD Biosciences). In some instances, phycoerythrin-labeled anti-CD14 monoclonal antibody (mAb) (RMO52; Beckman Coulter) or annexin V (BD Biosciences) were used instead of anti-CD133 mAb. The absolute number of CD133+ cells and EPCs in 1 ml of peripheral blood was calculated using FlowCount fluorospheres (Beckman Coulter) as an internal calibrator.

Mouse model of in vivo tumor neovascularization. The capacity of EPCs to promote neovascularization was examined in a mouse model of in vivo tumor neovascularization as previously described (7), with some modifications. Briefly, to isolate peripheral blood mononuclear cells, heparinized venous blood was subjected to Lymphoprep (Nycomed Pharma) density-gradient centrifugation. CD133+ cells, including CD34+CD133+CD309+ EPCs, were immunomagnetically sorted from peripheral blood mononuclear cells using an anti-CD133 mAb coupled to magnetic beads (Miltenyi Biotech), followed by MACS column separation. Control CD133− cells were also prepared by MACS-based negative selection. Flow cytometric analysis showed that the CD133+ cell fraction was consistently composed of >85% CD34+CD133+ cells, and that the contamination of CD133+ cells in the CD133− cell fraction was <0.01%. For some tests, CD309+ cells were removed from the CD133+ cell fraction using MACS-based negative selection.

SCID mice were obtained from Charles River Japan and were kept under specific pathogen–free conditions according to the guidelines of the Keio University Animal Care and Use Committee. Syngeneic murine colon carcinoma CT-26 cells (2.5×10^5) were transplanted subcutaneously into the backs of the SCID mice, alone or in combination with CD133+ or CD133− cells (2.5×10^3). The subcutaneous tumors were removed 10 days after mice received the transplants. Tumor volume was calculated according to the following formula: 0.5 × longest diameter × (shortest diameter)^2. Tumor size was expressed relative to the size of the tumors that developed in mice that received transplants of CT-26 cells alone. Tumors were fixed with formalin, embedded in paraffin, and stained with hematoxylin and eosin. The number of erythrocyte-bearing blood vessels was counted in 10 independent fields, and the results were expressed as the number per 1 mm^3. Neovessel density was expressed relative to the number of vessels in tumors that developed in mice that received CT-26 cells alone.

For immunohistochemical analysis, frozen tumor sections (10 μm thick) were placed onto slides and were incubated with different combinations of the following antibodies at a 1:1 ratio: rat mAb to mouse-specific CD31, mouse mAb to human-specific CD31 (BD Biosciences), fluorescein isothiocyanate–conjugated mouse mAb to HLA class I (Sigma-Aldrich), and rabbit polyclonal antibodies to human von Willebrand factor (Merck Millipore). Secondary antibodies included species-specific IgG conjugated with AlexaFluor 488, 568, or 647 (Molecular Probes). Nuclei were counterstained with TO-PRO-3 (Molecular Probes). These slides were examined with a confocal laser fluorescence microscope (IX71; Olympus). At least 100 independent blood vessels were observed, and the results were expressed as the proportion of blood vessels that were found to incorporate human ECs expressing CD31. In addition, the proportion of human EPCs that differentiated into CD31+ mature ECs was calculated by examining at least 100 cells expressing HLA class I.

Statistical analysis. All continuous variables were expressed as the mean ± SD. Comparisons between 2 groups were tested for statistical significance using an unpaired *t*-test. The correlation coefficient was determined using a single-regression model.

RESULTS

We obtained counts of circulating CD133+ cells and CD34+CD133+CD309+ EPCs using flow cytometry and partially enriched CD34+ cells. Levels of CD133+ cells were significantly decreased in SSc patients as compared to healthy controls (mean ± SD 222 ± 142 versus 449 ± 193; $P = 0.001$). Levels of EPCs were also significantly decreased in SSc patients as compared to healthy controls (10.8 ± 8.2 versus 21.1 ± 13.0; $P = 0.02$). However, the proportion of EPCs in the CD133+ population was the same in both groups (4.7 ± 1.9% versus 4.8 ± 2.2%). EPCs from both groups lacked expression of monocyte marker CD14 or the early apoptosis marker annexin V.

Murine colon carcinoma CT-26 cells were transplanted, alone or in combination with circulating CD133+ or CD133− cells, into the backs of SCID mice. When cells from healthy controls were used in the assay, tumor growth was markedly stronger when the CT-26 cells were transplanted with CD133+ cells that contained EPCs, while CD133− cells had almost no effect

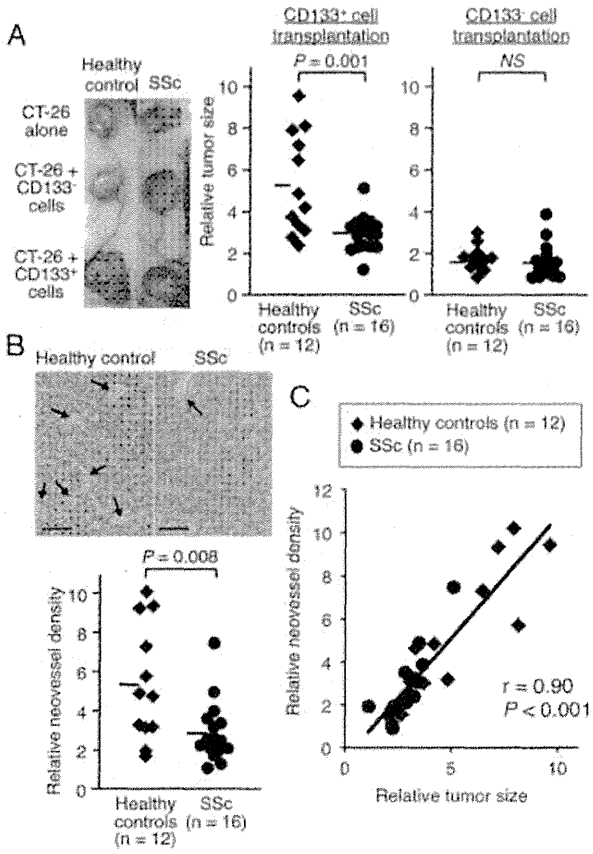

Figure 1. Size and neovessel density of subcutaneous tumors in mice that received transplants of CT-26 cells alone or in combination with CD133+ or CD133− cells from healthy control subjects or systemic sclerosis (SSc) patients. **A,** Representative subcutaneous tumors from mice that received transplants (left) and the relative size of the tumors that developed (right). Each data point represents a single mouse (receiving a transplant from a single patient); horizontal lines show the mean. NS = not significant. **B,** Representative sections of tumors from mice that received transplants of CT-26 cells with CD133+ cells from a healthy control subject or an SSc patient (top). **Arrows** indicate erythrocyte-bearing blood vessels. Hematoxylin and eosin stained; bars = 100 μm. The relative neovessel density of the tumors that developed in mice that received transplants of CT-26 cells with CD133+ cells from healthy control subjects and SSc patients was also determined (bottom). Each data point represents a single mouse (receiving a transplant from a single patient); horizontal lines show the mean. **C,** Positive correlation between tumor size and neovessel density.

(Figure 1A). CT-26 cells formed significantly smaller tumors when transplanted with SSc-derived CD133+ cells versus when they were transplanted with CD133+ cells from healthy control subjects (mean ± SD 2.9 ± 0.9 mm^3 versus 5.3 ± 2.5 mm^3; $P = 0.001$). However, tumor size did not differ among mice that received CD133− cells from either group (1.5 ± 0.8 versus 1.7 ± 0.6).

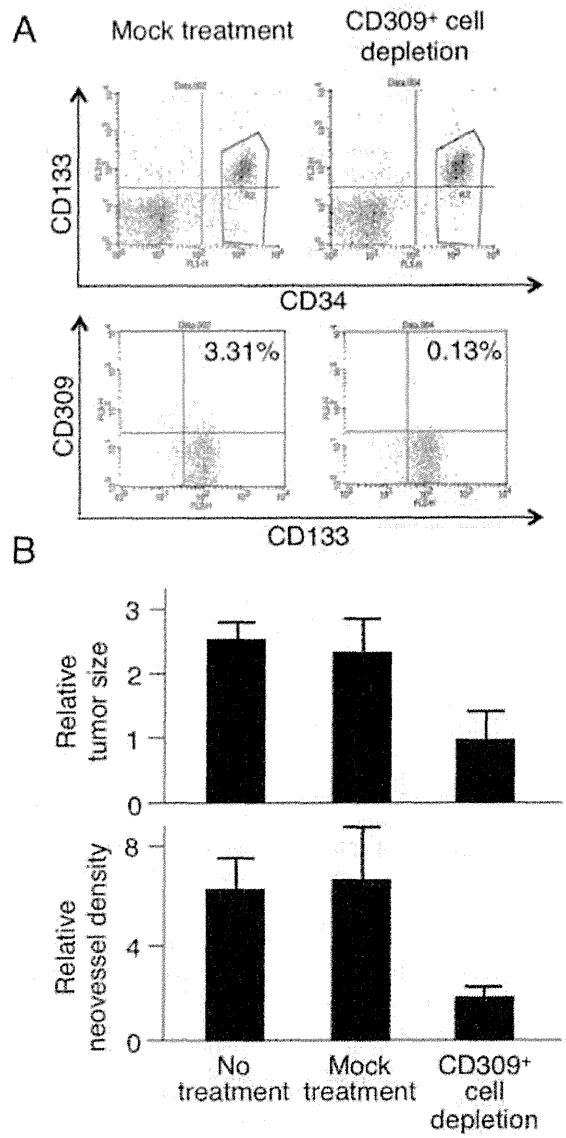

Figure 2. CD133+ cells lose the capacity to promote tumor growth and neovessel formation when depleted of CD309+ cells. **A,** Representative flow cytometric analysis of CD133+ cell fractions from a healthy control subject after mock treatment or CD309+ cell depletion. Top, In the dot plot of CD34 and CD133 cells, the outlined area shows region 2 (R2), which identifies CD34+ cells. Bottom, In the dot plot of CD309 and CD133 cells, region 2 gating for CD133 and CD309 is shown; CD34+ cells positive for CD133 and CD309 were regarded as endothelial progenitor cells (EPCs). The proportion of EPCs in CD133+ cells is indicated in the upper-right quadrants. **B,** Tumor size and neovessel density in mice that received transplants of CT-26 cells with untreated CD133+ cells, mock-treated CD133+ cells, or CD133+ cells depleted of CD309+. Values are the mean ± SD and are representative of independent measurements of samples from 4 healthy control subjects.

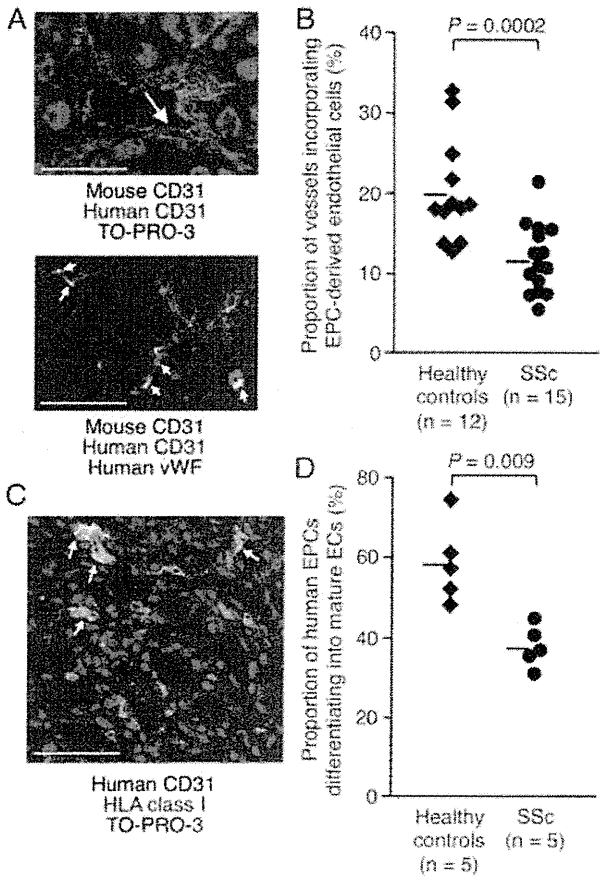

Figure 3. The vasculogenic capacity of human endothelial progenitor cells (EPCs) in mice that received transplants of CT-26 cells with CD133+ cells derived from healthy control subjects or systemic sclerosis (SSc) patients. **A,** Detection of human EPC–derived endothelial cells (ECs) (**arrows**) in a mouse that received a transplant of CD133+ cells from a healthy control subject. Top, A representative tumor vessel shows staining for mouse CD31 (red), human CD31 (green), and nuclei (blue). Bar = 25 μm. Bottom, A representative tumor vessel shows staining for mouse CD31, human CD31, and human von Willebrand factor (vWF) (blue). Bar = 100 μm. **B,** Proportion of tumor vessels that incorporated human EPC–derived ECs in mice that received transplants of CD133+ cells from healthy control subjects and SSc patients. Each data point represents a single mouse (receiving a transplant from a single patient); horizontal lines show the mean. **C,** Detection of human EPC–derived ECs in a mouse that received a transplant of CD133+ cells from an SSc patient. A representative tumor vessel shows staining for human CD31 (red), HLA class I (green), and nuclei (blue). **Arrows** show human EPCs differentiated into mature ECs. Bar = 25 μm. **D,** Proportion of human EPCs that differentiated into mature ECs in mice that received transplants of CD133+ cells from healthy control subjects and SSc patients. Each data point represents a single mouse (receiving a transplant from a single patient); horizontal lines show the mean.

Tumor sections were stained with hematoxylin and eosin and were evaluated for blood vessel formation (Figure 1B). A semiquantitative assessment showed a 5.4-fold increase in neovessel density in tumors that developed when CT-26 cells were transplanted with CD133+ cells from healthy control subjects as compared to the tumors that developed when CT-26 cells were transplanted alone. SSc-derived CD133+ cells had less in vivo capacity to promote neovascularization than did the CD133+ cells from healthy control subjects (mean ± SD 2.8 ± 1.6 versus 5.4 ± 3.1; $P = 0.008$) (Figure 2B). When the effects of cells from all the SSc patients and healthy controls were assessed, there was a positive correlation between neovessel density and tumor size ($r = 0.90$, $P < 0.001$) (Figure 1C), suggesting that neovessel formation is the primary contributor to tumor growth. We failed to find a difference in neovessel density between diffuse cutaneous SSc and limited cutaneous SSc or a correlation between neovessel density and disease duration after onset of Raynaud's phenomenon or non–Raynaud's phenomenon symptoms in SSc patients.

Since CD34+CD133+CD309+ EPCs comprised only 1.9–8.9% of the CD133+ cells, the effect of transplanted CD133+ cells might have been mediated by non-EPCs in this cell fraction. To exclude this possibility, CT-26 cells were cotransplanted with CD133+ cells that had been depleted of CD309+ cells (Figure 2). The depletion of CD309+ cells almost completely cancelled the ability of CD133+ cells to promote tumor growth and neovessel formation, indicating that EPCs contained in the CD133+ fraction were primarily responsible for promoting tumor neovascularization in our assay system.

The examination of tumor sections occasionally showed cells expressing human-specific CD31, but not mouse CD31, in addition to ECs expressing mouse CD31, within the vascular wall (Figure 3A). These cells coexpressed the human-specific mature-EC marker von Willebrand factor, indicating that transplanted human EPCs had been incorporated into the endothelium and had differentiated into mature ECs in vivo. Human ECs were found in ~20% of the blood vessels in tumors from mice that had received CD133+ cells from healthy control subjects, but the proportion was significantly lower in tumors from mice that had received CD133+ cells from SSc patients (mean ± SD 20.1 ± 6.4% versus 11.4 ± 4.3%; $P = 0.0002$) (Figure 3B). In addition, efficiency of endothelial differentiation in transplanted human EPCs (the proportion of human CD31+ cells in HLA class I–positive cells) was significantly lower in mice that had received CD133+ cells from SSc patients as compared with mice that had received CD133+ cells from healthy control subjects ($P = 0.009$) (Figures 3C and D). There was no correlation between the

circulating EPC count and the ability of EPCs to promote tumor growth or neovessel formation, or to differentiate into mature ECs.

DISCUSSION

We have successfully established a system for evaluating the in vivo function of circulating CD14− CD34+CD133+CD309+ EPCs using a murine model of tumor neovascularization. This system allows us to evaluate EPC function with regard to their ability to promote neovascularization either by direct incorporation into the vascular structure and differentiation into mature ECs (vasculogenesis), or by activating mouse ECs through such methods as secreted proangiogenic factors (angiogenesis). The system uses freshly isolated EPCs rather than cells cultured with exogenous proangiogenic factors. A potential limitation of this system is that it uses CD133+ cells instead of CD34+ CD133+CD309+ EPCs. Since EPCs are extremely rare in the circulating cell population (8), it is not feasible to isolate sufficient numbers of these cells for transplantation. However, experiments using CD133+ cells that had been depleted of CD309+ cells clearly demonstrated that while the percentage of EPCs contained in the CD133+ cell population was small, EPCs were the primary contributor to neovascularization.

Using our assay system to evaluate the function of circulating EPCs in vivo, we demonstrated that the neovascularization capacity of circulating EPCs in SSc patients is impaired, partly due to a deficiency in the vasculogenesis ability of these cells. Therefore, the defects in vasculogenesis observed in SSc patients are likely to be mediated through impaired EPC function, irrespective of EPC quantity. Aberrant functional property was also reported in another circulating EPC subset, CD45+CD14+CD34−CD133− monocytic EPCs; these cells exerted enhanced angiogenesis but were impaired in vasculogenesis (9).

Currently, little is known about the mechanisms behind these aberrations in circulating CD34+CD133+ CD309+ EPCs in SSc patients. Late-outgrowth EPCs derived from SSc patients were found to have an aberrant gene expression profile (a proadhesive, proinflammatory, and activated phenotype [10]), although a direct link between circulating CD34+CD133+CD309+ EPCs and late-outgrowth EPCs from long-term culture has not been established (11). Del Papa and colleagues reported a low CD34+CD133+CD309+ EPC count in the bone marrow of patients with SSc and found that the EPCs were defective in their ability to proliferate in long-term culture (despite the presence of multiple proangiogenic factors) (12), suggesting that these EPCs were functionally altered before their release into the circulation. A recent study of the bone marrow of patients with diffuse cutaneous SSc demonstrated markedly reduced microvascular density and increased fibrosis (13), despite a strong proangiogenic push provided by an up-regulation of vascular endothelial growth factor (13–15). This dysregulated microenvironment within the bone marrow may alter the developmental process of EPCs. Alternatively, the stem cells that will develop into EPCs may be intrinsically altered and become hyporesponsive to proangiogenic signals. Further studies are necessary to investigate more fully the mechanisms that create these dysfunctional EPCs and their roles in vasculopathy development in SSc.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kuwana had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Kuwana.

Acquisition of data. Kuwana, Okazaki.

Analysis and interpretation of data. Kuwana, Okazaki.

REFERENCES

1. Kuwana M, Okazaki Y, Yasuoka H, Kawakami Y, Ikeda Y. Defective vasculogenesis in systemic sclerosis. Lancet 2004;364: 603–10.
2. Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. Circ Res 2004;95:343–53.
3. Kuwana M, Okazaki Y. Quantification of circulating endothelial progenitor cells in systemic sclerosis: a direct comparison of protocols. Ann Rheum Dis 2012;71:617–20.
4. Avouac J, Wipff J, Goldman O, Ruiz B, Couraud PO, Chiocchia G, et al. Angiogenesis in systemic sclerosis: impaired expression of vascular endothelial growth factor receptor 1 in endothelial progenitor–derived cells under hypoxic conditions. Arthritis Rheum 2008;58:3550–61.
5. Distler JH, Allanore Y, Avouac J, Giacomelli R, Guiducci S, Moritz F, et al. EULAR Scleroderma Trials and Research group statement and recommendations on endothelial precursor cells. Ann Rheum Dis 2009;68:163–8.
6. Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 1980;23:581–90.
7. Kuwana M, Okazaki Y, Kodama H, Satoh T, Kawakami Y, Ikeda Y. Endothelial differentiation potential of human monocyte-derived multipotential cells. Stem Cells 2006;24:2733–43.
8. Prater DN, Case J, Ingram DA, Yoder MC. Working hypothesis to redefine endothelial progenitor cells. Leukemia 2007;21:1141–9.
9. Yamaguchi Y, Okazaki Y, Seta N, Satoh T, Takahashi K, Ikezawa Z, et al. Enhanced angiogenic potency of monocytic endothelial

progenitor cells in patients with systemic sclerosis. Arthritis Res Ther 2010;12:R205.
10. Avouac J, Cagnard N, Distler JH, Schoindre Y, Ruiz B, Couraud PO, et al. Insights into the pathogenesis of systemic sclerosis based on the gene expression profile of progenitor-derived endothelial cells. Arthritis Rheum 2011;63:3552–62.
11. Richardson MR, Yoder MC. Endothelial progenitor cells: quo vadis? J Mol Cell Cardiol 2011;50:266–72.
12. Del Papa N, Quirici N, Soligo D, Scavullo C, Cortiana M, Borsotti C, et al. Bone marrow endothelial progenitors are defective in systemic sclerosis. Arthritis Rheum 2006;54:2605–15.
13. Carrai V, Miniati I, Guiducci S, Capaccioli G, Alterini R, Saccardi R, et al. Evidence for reduced angiogenesis in bone marrow in SSc: immunohistochemistry and multiparametric computerized imaging analysis. Rheumatology (Oxford) 2012;51:1042–8.
14. Guiducci S, Manetti M, Romano E, Mazzanti B, Ceccarelli C, Dal Pozzo S, et al. Bone marrow-derived mesenchymal stem cells from early diffuse systemic sclerosis exhibit a paracrine machinery and stimulate angiogenesis in vitro. Ann Rheum Dis 2011;70:2011–21.
15. Cipriani P, Marrelli A, Benedetto PD, Liakouli V, Carubbi F, Ruscitti P, et al. Scleroderma mesenchymal stem cells display a different phenotype from healthy controls: implications for regenerative medicine. Angiogenesis 2013;16:595–607.