Online Data Supplement

Methods

Animals

Fra-2 tg mice (n=12) and wt littermates (n=6) were analyzed. Fra-2 tg mice were backcrossed from a mixed (C57BL/6×CBA) genetic background on a pure C57/Bl6 background for at least six generations (1). A subgroup of Fra-2 tg mice (n=6) was treated with nilotinib 2x37.5 mg/d by oral gavage from 8 weeks of age. The doses of nilotinib used herein result in pharmacologically relevant serum levels in mice (2). Fra-2 tg mice in the control group (n=6) received the vehicle 1-methyl-2-pyrrolidone/PEG300 in equivalent volumes of 2x100 µl/d by oral gavage. Mice were sacrificed at 16 weeks. The animal protocol was approved by the local animal care and use committee.

Histology

Lung sections of Fra-2 tg and wt mice were fixed in 4% formalin in a state of distension and embedded in paraffin for subsequent analysis as described previously (3). Lung samples had been collected from the periphery to ensure the systematic analysis of lobular and prelobular vessels. Five µm thick sections were stained with hematoxylin and eosin (HE). Masson's trichrome staining was used for extracellular matrix characterization according to standard protocols.

Immunohistochemistry

Incubation with the primary antibodies was performed at 4°C overnight, and the following antibodies were used: polyclonal rabbit anti-vWF (abcam, Cambridge, UK), monoclonal mouse anti-SM22α (abcam), monoclonal mouse anti-α-SMA

(Sigma-Aldrich, Buchs, Switzerland), monoclonal rat anti-PCNA (Imgenex, San Diego, CA), monoclonal mouse anti-CD3 (abcam), monoclonal rat anti-F4/80 (AbD Serotec, Düsseldorf, Germany), polyclonal rabbit anti-PDGF-BB (abcam), polyclonal rabbit anti-p-PDGFRβ (abcam). Slides were incubated for 30 min at room temperature with the following secondary antibodies: horseradish peroxidase (HRP)-labeled secondary goat anti-rabbit and goat anti-mouse antibodies, alkaline phosphatase (AP)-conjugated secondary goat anti-mouse antibodies (Jackson ImmunoResearch, Soham, UK). Staining was visualized with peroxidase or AP substrate kits (all from Vector, Burlingame, USA). Irrelevant isotype-matched antibodies for monoclonal antibodies or IgGs for polyclonal antibodies at the same concentrations were used as controls.

Analysis of histological and immunohistochemical stainings

The analyses of all histological and immunohistochemical stainings were performed by two blinded independent examiners. Slides were analyzed twice by each examiner. In case of disagreement of >10%, the respective slides were re-assessed. Pictures from 5 randomly selected HPF were taken at x100, x200 or x400 with a digital camera on an Imager1 microscope (Carl-Zeiss AG, Feldbach, Switzerland), using AxioVision software Release 4.6. The analyses included 12 Fra-2 tg mice and 6 wt littermates.

Pulmonary histopathology

The histological analysis included the assessment of vascular remodeling and ILD. Microscopic criteria for the assessment of interstitial lung changes were: pattern and distribution of inflammation, deposition of extracellular matrix, and architectural changes according to established definitions (4). We also assessed the lung

specimens of Fra-2 tg mice for the presence of NSIP and usual interstitial pneumonitis (UIP) (4).

Vascular changes were evaluated according to the consensus on the assessment of vasculopathies in PH established at the Third World Symposium on Pulmonary Hypertension (5). With respect to vasculopathy we analyzed the presence of constrictive lesions (medial hypertrophy, intimal and adventitial fibrosis), complex lesions (plexiform, dilation lesions, arteritis, postthrombotic lesions), pulmonary occlusive venopathy (POV, formerly PVOD), and microvasculopathy (PM). Intimal thickening was defined as either concentric (mainly due to expansion of fibroblasts, myofibroblasts, smooth muscle cells) or eccentric/concentric nonlaminar (predominantly composed of fibroblasts and connective tissue matrix). As thrombotic changes, occlusion of small lobular arteries with signs of re-canalization, so-called colander-like lesions, were considered. Plexiform lesions were defined as arterial lesions consisting of a plexus of slit-like channels enclosed within or in continuity with a dilated segment of the affected artery with predominant endothelial cell proliferation and dilation.

Vascular remodeling

Muscular blood vessels were identified by positive staining for α-SMA and SM22α. Whereas α-SMA is also expressed by myofibroblasts, the expression of SM22α is restricted to vascular smooth muscle cells. The vessel wall thickness of pulmonary arteries as a marker for vascular remodeling was assessed based on established methods by measuring the thickness of vWF+/α-SMA and SM22α-positive vessel walls. An average vessel wall diameter >10% of total vessel diameter was considered pathological (6, 7). The $median_{(Q1,Q3)}$ thickness of walls of α-SMA-

positive/SM22α-positive vessels was evaluated by 5 measurements/vessel and was then calculated using GraphPad Prism software.

As an additional method of measuring vascular remodeling, the degree of luminal occlusion of pulmonary arteries (α-SMA and SM22α-positive) was examined by manually counting the numbers of occluded lumina/HPF (8). The percentage of obliterated vessels was then calculated using GraphPad Prism software.

To identify the proliferating cells responsible for vascular remodeling, we used PCNA as proliferation marker and performed double stainings with the respective cell markers for vascular smooth muscle cells (α-SMA and SM22α), and myofibroblasts (α-SMA). For the semiquantitative analysis of PCNA-positive nuclei, pictures of 3 randomly chosen HPF/slide at x200 magnification were taken with a digital camera using AxioVision software. Positive nuclei (brown staining) within vessel walls were counted manually and independently confirmed by automated counting by image analysis software (Image J, NIH).

To assess potential differences in the expression of PDGF-BB and phosphorylated Platelet-derived growth factor β (p-PDGFRβ) in the pulmonary vessels, positive vascular cells were quantified by automated analysis of staining intensity using image analysis software (Image J, NIH). Results of computer-assisted imaging were presented 0=no staining, 1=weak staining, 2=moderate staining, and 3=intensive staining. The $median_{(Q1,Q3)}$ of staining intensity was then calculated using Graph Pad Prism software.

For a more detailed analysis of inflammatory infiltrates, stains for T-cells (CD3+) and murine macrophages (F4/80) as well as double stainings with those markers and PDGF-BB/p-PDGFRβ were performed.

To assess potential differences in the expression of PDGF-BB and phosphorylated Platelet-derived growth factor β (p-PDGFRβ) in the pulmonary

vessels, positive vascular cells were evaluated by automated analysis of staining intensity using image analysis software (Image J, NIH). A density threshold was set to quantify the positive staining by using the respective negative controls. The threshold was selected to exclude unspecific background staining. The same thresholds and system settings were used for all slides. The number of pixels falling within the threshold, indicating the quantity of staining positivity, was recorded for each field. Results of computer-assisted imaging were presented 0=no staining, 1=weak staining, 2=moderate staining, and 3=intensive staining. The $median_{(Q1,Q3)}$ of staining intensity was then calculated using Graph Pad Prism software.

Statistical analysis

The Kolmogorov-Smirnov test was used to test for normal distribution. Nonparametric nonrelated data were analyzed with the Mann-Whitney-U test, and data were expressed as $median_{(Q1,\ Q3)}$. P-values less than 0.05 were considered statistically significant. Power calculation was performed using STATA 10.0 (StataCorp, College Station, Texas). For vessel wall thickness of pulmonary arteries as the primary endpoint of the interventional study, with a mean ± standard deviation of 49±8 in vehicle-treated Fra-2 tg mice, of 24±8 for nilotinib treated Fra-2 tg, with alpha set at 0.05 and a given sample size of 6 animals per group, power was calculated at 81% (ß = 19%).

Supplementary results

Pulmonary pathology of Fra-2 tg mice resembles changes in SSc-PH

Interstitial lung disease

Compared to wt mice (suppl. Fig.1A), Fra-2 tg mice developed a severe pneumonitis. The alveolar septal infiltrates showed a diffuse (suppl. Fig.1B) to patchy (Fig.1C) pattern, mainly consisting of macrophages (F40/80+) and to some extent of lymphocytes (CD3+) (suppl. Fig.1D).

Additionally, interstitial lung fibrosis with accumulation of extracellular matrix proteins occurred as analyzed by Masson's trichrome staining (suppl. Figs.1E-G) resulting in an expansion of alveolar septae (suppl. Fig.1E) and in a loss of alveoli with concomitant lung emphysema. There was a uniform expansion of peribronchiolar interstitium due to deposition of extracellular matrix proteins (suppl. Fig.1F). Rarely, fibroblastic foci were observed (suppl. Fig.1G). Thus, the phenotype of ILD in Fra-2 tg mice resembled features of human NSIP, which is known to be the main histological pattern in SSc-ILD (9). In contrast to a previous study (10), honeycombing, severe scarring or other pulmonary changes resembling human UIP were not observed which might be due to the different genetic background of the Fra-2 tg mice.

Supplementary figure legends

Suppl. Fig. 1 Pulmonary pathology of Fra-2 tg mice: interstitial lung disease

(A) Pulmonary histology of wt mice (HE staining). Fra-2 tg mice developed a severe pneumonitis with alveolar septal infiltrates in a diffuse (B, arrows) to patchy (C, arrows) pattern (HE staining). Mononuclear infiltrates mainly consisted of macrophages (F40/80, dark green, arrows) and to some extent of lymphocytes (CD3 positive, purple, short arrows) (D). Additionally, interstitial lung fibrosis and

emphysema with accumulation of extracellular matrix proteins and loss of alveoli, expansion of alveolar septae (E, arrows) and accumulation of peribronchiolar connective tissue (F, arrows) occurred (Masson's trichrome staining, green, arrows), accompanied by inflammatory infiltrates in the interstitium (E-G) occured. Rarely, cellular aggregates reminding of fibroblastic foci could be observed (G, arrow). Pictures are representative examples of 6 wt and 6 Fra-2 tg mice. Data are expressed as median and interquartile range. * indicates p-values <0.05

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