Treprostinil inhibits the recruitment of bone marrow derived circulating fibrocytes in chronic hypoxic pulmonary hypertension

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Abstract:
A unique subpopulation of peripheral blood mononuclear cells that exhibits a parallel expression of haematopoietic and mesenchymal markers has been described as ‘Circulating Fibrocytes’ (CF). These cells were demonstrated to obtain a fibroblastic phenotype in tissues or cell culture and contribute to pulmonary fibrotic disorders and tissue remodelling processes.

The objective of our study was to characterise the recruitment of CF in vivo in the model of chronic hypoxic pulmonary hypertension in mice and to analyse the therapeutic effect of the stable prostacyclin analogue treprostinil with respect to this cell population.

To track CF in vivo, we transplanted wild type mice with bone marrow from ubiquitously eGFP expressing mice and subjected them to chronic hypoxia. We observed significantly increased recruitment of CF to the remodelled pulmonary resistance arteries in response to hypoxia. Treatment with treprostinil significantly reduced the recruitment of these cells as compared to normoxic mice. Treprostinil also reduced right ventricular systolic pressure, slightly reduced the vascular remodelling but failed to reverse the right ventricular hypertrophy.

In summary, we show that CF contribute to hypoxic pulmonary vascular remodelling and may be specifically targeted by a prostacyclin analogue. Further investigations of cellular and paracrine mechanisms are warranted to decipher their role in pulmonary hypertension.

Key words- Circulating fibrocytes, prostacyclin, pulmonary hypertension, pulmonary vascular disease.
Introduction:

Pulmonary hypertension is a chronic and potentially fatal disease resulting from various causes [1]. Lung diseases like chronic obstructive or interstitial pulmonary disease may lead to chronic intrapulmonary hypoxia and subsequent pulmonary hypertension. It is characterised by increased pulmonary vascular resistance, elevation of pulmonary artery pressure, subsequent right ventricular dysfunction and ventricular hypertrophy. The prominent histological features are hypertrophy and hyperplasia of all vascular layers of the arteries and arterioles leading to vascular obliteration [2, 3]. Vascular remodelling in pulmonary hypertension is thought to be orchestrated by an interplay between various cell types (adventitial fibroblast, smooth muscle cells, endothelial cells or progenitor cells) either of resident origin or extra pulmonary origin, and their homing, differentiation and release of various mediators [4, 5]. Prostacyclin and its stable analogues iloprost and treprostinil are approved therapies for PAH patients [1, 6]. They act predominantly as vasodilators but display also antiproliferative, anti-inflammatory, and anti-aggregatory effects [7, 8].

Bone marrow-derived cells have been shown to participate in angiogenesis, vascular repair and tissue remodelling of various organs [9-11]. The circulating progenitor cells were demonstrated to assume a mesenchymal or fibroblastic phenotype at the site of tissue injury in animal models of pathological conditions. In this regard, recent reports suggested that a subpopulation of leukocytes contributes to various fibroproliferative pathologies like pulmonary fibrosis, airway remodelling in asthma and organ fibrosis in scleroderma [12-17]. These circulating progenitor cells from peripheral blood (less than 1%), designated ‘circulating fibrocytes’ by Buccala et al., exhibit a parallel expression of haematopoietic and mesenchymal antigens [CD45+, CD34+, Collagen I and III, vimentin] [18]. A study by Hashimoto et al., using a chimeric mouse model with GFP expressing bone marrow cells, has shown the significant contribution of bone marrow derived GFP+/CollagenI+ fibroblasts to
bleomycin induced lung fibrosis [19]. The contribution of circulating fibrocytes to organ fibrosis and extracellular matrix deposition in experimental pulmonary fibrosis was suggested by correlation of CD45+/collagenI+/CXCR4+ circulating fibrocyte recruitment with increased collagen deposition [14]. Massive cellular recruitment to especially the adventitial layer of pulmonary arteries was shown in the setting of hypoxia induced pulmonary hypertension in a rat and a calf model of pulmonary hypertension and was linked to increased numbers of fibroblasts in the adventitia [20]. Bone marrow derived smooth muscle cell recruitment to pulmonary tissues has been reported in chronic hypoxic mice [21].

Despite the growing understanding of the pathobiology of pulmonary hypertension, it is still an open question if apart from local residing cells, cells from extra-pulmonary origin have a potential impact on the process of vascular remodelling, which are the mechanisms that lead to the recruitment of these cells to the site of injury and how differentiation of recruited cells is regulated and mediates vascular remodelling in the progression of disease. Additionally, the impact of the currently approved therapies for pulmonary arterial hypertension such as prostacyclin and its analogues has not been studied with regard to these extra-pulmonary originating cells. Therefore, we aimed to investigate whether these circulating progenitors do contribute to pulmonary vascular remodelling in a murine model of pulmonary hypertension and how prostacyclin analogues modulate circulating fibrocyte adhesion, differentiation, in vivo recruitment and vascular remodelling.

To address this, we first identified and characterised circulating fibrocytes isolated from peripheral blood in cell culture as well as in vivo using a panel of mesenchymal and haematopoietic markers. We further investigated the recruitment of circulating fibrocytes to pulmonary resistance arteries in response to chronic hypoxia in GFP-bone marrow chimeric
mice and evaluated the influence of continuous intravenous infusion of the prostanoid analogue treprostinil.
Material and methods

Immunohistochemistry on paraffin embedded lung tissue of PAH patients

Use of patient material was approved by the local IRB and patients’ informed consent was obtained before tissue or blood donation. Lungs were fixed overnight in 4% paraformaldehyde (PFA) at 4°C, dehydrated over a graded series of alcohol, and paraffin embedded. Sections of 4 to 10 µm were cut on a microtome (Leica Germany). Antigen retrieval was performed by incubation with trypsin solution (Digest All 2, Zymed, Berlin, Germany) for 10 minutes at 37°C or microwave heating for 8 min using 1mM EDTA. Antibody staining was performed following standard procedures. All incubations and washes were done with buffer containing 3 % bovine serum albumin (BSA) and 0.2 % Triton X-100 in 1X phosphate buffer saline (PBS), pH 7.4. Unspecific binding sites were blocked for 45-60 minutes with goat serum and buffer (1:1 (v/v) ratio). The sections were then incubated with primary and secondary antibodies for 60 min followed by nuclear staining for 5-10 min with TO®-PRO-3 iodide (Molecular probes). The sections were examined with a Leica TCS confocal microscope (Leica, Germany).

Animals

C57BL/6N mice were obtained from Charles River Laboratories and C57BL/6TgN [eGFP] transgenic mice were obtained from Jackson Labs [22]. Animals were housed under controlled temperature and lighting [12/12-hour light/dark cycle], fed with commercial animal feed and water ad libitum. All experiments were performed according to the institutional guidelines that comply with national and international regulations.

Enhanced GFP chimeric mice [23]

C57BL/6N wild type mice, 8-10 weeks of age, were lethally irradiated with 11 Gy from a Cesium source and received $2 \times 10^6$ bone marrow cells from eGFP positive mice by tail
vein injection. Bone marrow was harvested by flushing tibias and femurs of transgenic mice which expressed the reporter gene enhanced green fluorescent protein (eGFP) under the control of a chicken beta-actin promoter and cytomegalovirus enhancer. These transgenic mice ubiquitously express green fluorescence and were bred in the identical C57BL/6N background as the cell recipients. Success of bone marrow transplantation was monitored by flow cytometry of peripheral blood (FACSan, Becton Dickinson). Four weeks following the transplantation more than 80% of blood cells expressed eGFP.

**Mouse model of hypoxia induced pulmonary hypertension**

Bone marrow transplanted (BMT) mice were divided into five different groups with each group consisting of 6-10 mice. One group of mice was exposed to hypoxia (10% inspired oxygen fraction) in a normobaric chamber as described previously [24, 25] whereas the second group (control BMT) animals were placed in a normoxic chamber with a normal oxygen environment (21% inspired O₂ fraction) for 28 days respectively. Sham group mice received saline treatment whereas two other groups of mice received treprostinil infusions of different dose levels (14 ng/kg/min and 70 ng/kg/min) and exposed to hypoxia for 4 week. For comparison, human infusion rates in PAH therapy vary from 10 to 60 ng/kg/min [26-29].

**Surgical procedure for osmotic minipump implantation in the mice**

Osmotic minipumps (Model number 2004; infusion rate 0.25 µl/min; Alzet, CA, USA) were filled with either treprostinil (Remodulin®) or sterile 0.9% injectable saline (Braun, Melsungen, Germany) and equilibrated at 37°C in phosphate buffered saline for 48 hours before implantation. The mice were anaesthetised with 30-60 mg/kg ketamine (Pfizer, Germany) and 5-10 mg/kg, xylazine (Bayer, Germany) and a 2 cm² area distal to the head on the left ventral and dorsal side were shaved. The minipump was implanted subcutaneously on the back and the cannula connected to the pump was tunnelled subcutaneously to be inserted
in the external jugular vein and was kept in position with a silk suture. Incisions were closed with surgical ligatures and animals had free access to food and water. The animals received postoperative analgesia with buprenorphin injections twice daily for 3 days.

**Hemodynamic measurements and ventricular mass**

The animals were anaesthetized by intraperitoneal injection of ketamine and xylazine as described above and placed on a heating pad to maintain the body temperature in the physiological range. The trachea was cannulated and attached to a mouse respirator (SAR830A/P, IITC, CA, USA) with 10 ml/kg body weight tidal volume and 100/min breath frequency. Inspiratory oxygen (FIO₂) was set to 0.5, and a positive end-expiratory pressure of 1.0 cm H₂O was applied. Systemic blood pressure was recorded from the left carotid artery by a cannula attached to a fluid filled pressure transducer (Braun, Germany). The right jugular vein was used to measure the right ventricular pressure by inserting a custom made silicon catheter through the jugular vein into the right ventricle. Before measurements were taken all instruments used were calibrated.

After systemic and right ventricular pressure measurement, the lung and heart were flushed with saline followed by perfusion through the pulmonary artery and instillation of the trachea with 1% buffered paraformaldehyde (1% PFA) [Sigma-Aldrich, Germany] with pressures of 20 cm and 10 cm H₂O, respectively. Thus lung and heart were isolated, the right ventricle (RV) was dissected from the left ventricle + septum (LV+S), and these dissected samples were dried and weighed to obtain the right to left ventricle plus septum ratio (RV/LV+S).

**Immunohistochemistry of paraffin embedded mouse lung tissue**

The mice lungs were harvested as described in the section hemodynamic measurement. Antibody staining was performed following standard procedures. Lungs were post-fixed
overnight in 1% PFA at 4°C, dehydrated over a graded series of alcohol, and paraffin embedded. Sections of 4 to 10 µm were cut on a microtome (Leica Germany). Antigen retrieval was performed by incubation with trypsin solution (Digest All 2, Zymed, Berlin, Germany) for 10 minutes at 37°C or microwave heating for 8 min using 1mM EDTA. All incubations and washes were done with histobuffer containing 3 % BSA and 0.2 % Triton X-100 in 1× PBS, pH 7.4. Unspecific binding sites were blocked over 45-60 minutes with goat serum and histobuffer (1:1 (v/v) ratio). The sections were then incubated with primary and secondary antibodies for 60 min followed by nuclear staining. The sections were examined with a Leica TCS confocal microscope (Leica, Germany).

Morphometry of pulmonary artery remodelling

The degree of muscularisation due to hypoxia induced pulmonary hypertension in BMT mice was assessed by double staining of 3 µm lung sections with alpha-smooth muscle actin (clone 1A4; 1:400; Sigma, Germany) and von Willebrand factor antibodies (dilution 1:900, Dako, Hamburg, Germany), as previously described [25]. The nuclei were visualised by methylgreen counter stain and the sections evaluated by light microscopy. The degree of muscularisation was measured with a morphometric software as described before [25]. At 40X magnification, 80-100 small vessels, which were either surrounded by alveolar ducts or alveoli, were counted. The blood vessels were classified as to the degree of muscularisation into non-muscularised (NM), partially muscularised (PM) and completely muscularised (CM). The percent of blood vessels in each category was determined by dividing the number of vessels in that category by the total number of blood vessels counted in the same experimental group.

Vessel wall thickness measurement

To measure the vessel wall thickness, the outer (Ro) and inner (Ri) radius was calculated using volume densities of the vessel lumen and the vessel wall using the stereological point
counting grids based on the Cavalieri principle in the newCAST software (Visiopharm, Denmark). The point counting grid was used for volume density analysis. The relative wall thickness of each artery (VWT) was calculated by the formula $VWT = (Ro – Ri)/Ro$ [30]. Three sections of each mouse lung from 5-6 animals per group were analysed.

**Quantitative PCR for chemokine expression from formalin fixed paraffin embedded (FFPE) mouse lung tissue**

For total RNA isolation, four 10 µm thick sections were cut from formalin fixed paraffin embedded (FFPE) tissue block. The “RecoverAll™ Total Nucleic Acid Isolation Optimized for FFPE Samples” (Ambion Inc., Austin, TX) was used for the RNA isolation. The RNA was isolated as per manufacturer’s instruction. Briefly, the protocol follows following steps-deparaffinisation with 100% xylene followed by washing with 100% ethanol. Then tissue was digested with Proteinase K and RNA was isolated. The isolated RNA was incubated with DNase and finally purified. For reverse transcription (RT) analysis, extracted RNA (500 ng) was converted to cDNA using the following components: 2 µl of 10X RT buffer II, 4 µl of MgCl$_2$ (25 mM), 2 µl of deoxynucleotide mixture (5mM), 0.5 µl of Rnase inhibitor, 1 µl of Random hexamer and 1 µl of Maloney murine leukemia virus (MuLV) (All components were from Applied Biosystem). The volume of the mixture made up to 20 µl. The mixture is then incubated at 43°C for 75 min followed by inactivation of reverse transcriptase at 95°C. Quantitative PCR was performed by the CFX96™ C1000Thermal cycler (BioRad). 18S ribosomal RNA, a ubiquitously as well as consistently expressed gene was used as reference. The reactions (final volume: 25 µl) were set up with the SYBR™Green PCR Core Reagents (Applied Biosystems) according to the manufacturer's protocol using 2 µl of cDNA. Cycling conditions were 95°C for 6 min, followed by 45 cycles of 95°C for 20 s, 59°C for 30 s and 73°C for 30 s. Due to the non-selective dsDNA binding of the SYBR™Green I dye, melting curve analysis and gel electrophoresis were performed to confirm the exclusive amplification
of the expected PCR product. The primers used for the real time PCR are listed in the table below.

Table I. Primers for quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4</td>
<td>5’GCTTCCGGGATGAAAACGTC3’</td>
<td>5’ACCAATCCATTGCCGACTATG3’</td>
<td>94</td>
</tr>
<tr>
<td>CCR7</td>
<td>5’CCATGACGGATACCTACCTGC3’</td>
<td>5’GATCCAGGACCTGCTTC GCT 3’</td>
<td>98</td>
</tr>
</tbody>
</table>

**Statistical analysis**

The results are expressed as mean ± SEM if not mentioned differently. The data were analysed by one way analysis of variance (ANOVA) with Bonferroni’s multiple comparison post hoc test using GraphPad Prism statistical software. P values < 0.05 were considered to be significant. Error bars depict S.E.M.
Results

Hypoxia induced pulmonary hypertension in eGFP bone marrow chimeric mice and treprostinil infusion improved hemodynamic

The success of bone marrow transplantation was assessed by flow cytometry of peripheral blood cells after 4 week of reconstitution. At that time already 80-90 % of the peripheral blood cells expressed GFP.

The GFP chimeric mice kept in hypoxia alone, in hypoxia with continuous saline or treprostinil infusion, or in normoxia were analysed for hemodynamic parameters as described in the materials and methods section [24, 25]. The hypoxic mice showed a remarkable increase in right ventricular systolic pressure (36.57 ± 0.705 mmHg) as compared to mice kept in normoxic chambers (25.2 ± 0.43 mmHg) as shown in Figure 1A and Table II.

The sham group mice received continuous saline infusion through subcutaneously implanted minipumps via the jugular vein in the same way as treprostinil infused animals and were kept under hypoxia. The sham group mice showed an increase in RVSP (30.3 ± 0.8 mmHg) as compared to normoxic control animals without implanted pumps. The treprostinil infusion animals at both dose levels (14 and 70 ng/kg/min) showed a significant decrease in RVSP compared to the hypoxic and sham group animals (Figure 1A and Table II).

Right ventricular hypertrophy could not be reversed with treprostinil treatment in hypoxia induced pulmonary hypertension animals

The right ventricular hypertrophy was assessed by the dry weight ratio of right ventricle (RV) to left ventricle with septum (LV+S). The ratio of RV/ (LV+S) exhibited significant hypertrophy of the right ventricle of the hypoxic chimeric mice in comparison to normoxic chimeric mice (Figure 1B and Table II). Sham group animals showed significant increase in right ventricular hypertrophy as compared to normoxic animals. However the treprostinil treatment failed to reverse the ratio significantly as shown in Figure 1B and Table II.
The haematocrit values and hemodynamic measurements from mice kept under hypoxia, normoxia and with treatment are summarised in Table II.

**Treprostinil infusion slightly reduced the vascular remodelling in response to chronic hypoxia exposure**

Chronic hypoxia induced remodelling of pulmonary resistance arteries was analysed in immunohistological sections by double staining with αSMA and von Willebrand factor. The degree of muscularisation was assessed quantitatively as described in the materials and methods section [24, 25]. The normoxic control mice exhibited mainly non-muscularised and partially muscularised blood vessels of 20-70 µM diameters and less than 10% fully muscularised blood vessels.

Chronic hypoxia induced in GFP-BMT mice a dramatic increase in muscularisation with concomitant decrease of non-muscularised vessels and increase of partially and fully muscularised blood vessel as shown in Figure 2. The pathophysiological and histological features of hypoxia induced pulmonary hypertension in GFP-BMT mice were similar to the non-irradiated, non-bone marrow transplanted wild type mice subjected to similar conditions as reported earlier [24, 25].

The vascular remodelling was slightly prevented by treprostinil at two different infusion rates in vivo, as shown by a reduction of muscularisation with increase in non-muscularised (NM) blood vessels as compared to the sham infused group, but not significantly reduced compared to hypoxic animals without minipump implantation (Figure 2).

The vessel wall thickness was significantly increased by hypoxia. The treprostinil treatment slightly reduced the vessel wall thickness as compared to sham and hypoxic animals but not statistically significant (Figure 3).
**In vivo characterisation and identification of circulating fibrocytes**

Lung tissue sections were prepared from ten idiopathic PAH patient lungs. Immunohistological analysis for collagen-1 and CD45 double-positive cells showed the regular but rare presence of circulating fibrocytes in pulmonary resistance arteries, as shown in the Figure 4A and in Table III. Two patients had higher numbers of fibrocytes as compared to the rest of the patients (Table III). The number of collagen-1 positive cells was higher, but their origin (resident or recruited) is unclear since there are reports suggesting the loss of hematopoietic markers during differentiations of the fibrocytes [14, 15, 31].

Bone marrow derived GFP$^+$ cells significantly accumulated in the perivascular area and arterial wall of pulmonary arteries in response to hypoxia. The characterisation of circulating GFP$^+$ cells recruited to the perivascular area of pulmonary vessels revealed the co-expression of CD45 and collagen1 as well as vimentin as shown in the Figures 5A-C. Few host derived GFP negative/CD45 positive cells could also be found in the perivascular area and represented cells which had been recruited before bone marrow exchange.

**Recruitment profile of circulating fibrocytes to the pulmonary arterial wall during development of chronic hypoxic pulmonary hypertension**

We quantified the recruitment of bone marrow derived GFP$^+$, Collagen1$^+$/GFP$^+$, Collagen 1$^-$ /GFP$^+$, CD45$^+$/GFP$^+$ and CD45$^-$/GFP$^+$ cells to the pulmonary arteries by counting the cells of 15-20 resistance vessels from randomly taken sections out of five different sections of each mouse lung and six animals (n=6) were taken per group for quantification.

The Collagen1$^+$/GFP$^+$, Collagen 1$^-$/GFP$^+$, CD45$^+$/GFP$^+$ CD45$^-$/GFP$^+$ and GFP$^+$ cells significantly accumulated in the pulmonary resistance arteries in response to chronic hypoxia in comparison to normoxic animals. Continuous treprostinil infusion at a rate of 70 ng/kg/min significantly inhibited the recruitment of Collagen1$^+$/GFP$^+$ CF to the pulmonary arterial wall.
as compared to the sham infusion group. The recruitment of CD45+/GFP+ was significantly reduced with treprostinil treatment at both dose levels (Figure 6A-F).

Chemokine expression in the lung of the mice subjected to chronic hypoxia and treprostinil treatment by quantitative PCR

Circulating fibrocytes are known to express various chemokine receptors like CXCR4, CCR2 and CCR7 [12, 14, 32]. The mRNA expression levels showed up regulation of CXCR4 and CCR7 in mice exposed to hypoxia (hypoxia and sham group animals) as compared to normoxic mice as shown in the Figure 7. The continuous infusion of treprostinil at the dose level of 14 ng/kg/min did not show the down regulation of the chemokine expression as compared to sham group mice. However there is significant down regulation of the CXCR4 and CCR7 expression at 70 ng/kg/min dose of treprostinil treatment as compared to sham group mice as shown in Figure 7.
**Discussion**

Experimental pulmonary hypertension in the setting of hypoxia in mice has been shown to elevate pulmonary arterial pressure due to vascular remodelling [24, 25]. Prominent features of all types of pulmonary hypertension are intimal and medial hypertrophy where the contribution of diverse type of cells (endothelial cells, smooth muscle cells, fibroblast etc.) and their mediators has been proposed in this vascular remodelling process. Several recent reports showed the recruitment of circulating fibrocytes in various fibrotic pathological conditions such as airway remodelling in asthma, parenchymal remodelling in pulmonary fibrosis and vascular remodelling in pulmonary hypertension [14, 15, 20, 21].

In our present study, to track the circulating fibrocytes *in vivo*, we generated GFP bone marrow chimeric mice and subsequently subjected these mice to hypoxia induced pulmonary hypertension. The *in vivo* characterisation of donor derived circulating fibrocytes (bone marrow derived GFP positive cells) showed the expression of CD45 along with fibroblast markers collagen I and vimentin. In addition, we could show the presence of CF (expressing the hematopoietic marker CD45 and the mesenchymal markers collagen1 and vimentin) in the remodelled pulmonary arteries of PAH patients. Our present data provides strong evidence for active recruitment and accumulation of circulating fibrocytes and other bone marrow derived cells in the pulmonary vessel wall in response to hypoxia. Furthermore, CF recruitment was significantly inhibited by continuous treprostinil infusion.

Collagen1+/GFP+ circulating fibrocytes were significantly increased in the vessel walls of pulmonary resistance arteries of chronic hypoxic mice. This cell population is highly likely to contribute to the vascular remodelling process in experimental pulmonary hypertension. Collagen1+/GFP+ cells represent cells belonging to the immune system, their role in pulmonary vascular remodelling is unresolved but it is a regular observation that
inflammatory cells contribute to vascular lesions in experimental and human PH, possibly by secretion of fibroproliferative mediators [33, 34]. The data of Stenmark et al. showed strong recruitment of cells of mononuclear origin, predominantly to the adventitial layer in chronic hypoxic calves and rats, with upregulation of various fibroproliferative markers conductive for vascular remodelling processes [20, 34].

We could however not detect bone marrow (BM) derived GFP positive smooth muscle cells in the media of the pulmonary vessels which is in contrast to a previous report [21] but in line with observations of failure of bone marrow to contribute to pulmonary vascular smooth muscle in monocrotaline induced pulmonary hypertension in rats [35]. The observation of differentiation of bone marrow derived precursors into alpha-smooth muscle acting expressing myofibroblasts is also varying amongst different groups. The group of Strieter R., [14] reported myofibroblast generation in experimental lung fibrosis whereas the group of Phan S. H. [19] as well as our group (unpublished data) did not observe this phenomenon in experimental fibrosis. The reason for this is yet unclear; variations in the transplant protocols, cell handling or animal strains may be taken into account. Similar failure of bone marrow derived cells to populate smooth muscle of the lung has been described earlier by us in a model of compensatory lung growth following unilateral pneumonectomy in mice [23].

However, CF accumulation in the perivascular area in response to chronic hypoxia suggests their contribution to the progression of detrimental vascular remodelling apart from other contributing cell types. Recent data demonstrated that sustained hypoxia creates a pulmonary artery specific inflammatory microenvironment with up regulation of various growth factors (VEGF, TGF-β), chemokines and cytokines, adhesion molecules as well as monocyte/fibrocyte growth and differentiation markers in rat model of pulmonary
hypertension all of which may promote recruitment, retention and differentiation of circulating cells of mononuclear origin [34].

A study by Hashimoto et al., using a comparable GFP chimeric mouse model, has shown significant contribution (approximately 27%) of bone marrow derived GFP+/collagenI+ fibroblasts to bleomycin induced lung fibrosis. In addition, they showed that isolated GFP+ lung fibroblast exhibited CXCR4+/CCR7+ chemokine receptor expression [19]. Strieter et al. addressed the role of circulating fibrocytes by the similar marker profile in pulmonary fibrosis as Hashimoto did before and positively correlated the recruitment of CD45+/collagenI+/CXCR4+ circulating fibrocytes to increased collagen deposition in bleomycin induced pulmonary fibrosis. This effect was reversed by treatment with a neutralising antibody against CXCL12 which is the ligand for CXCR4 [14]. Similarly in another study, CCR2 expression of CF has been shown to play an important role in FITC induced pulmonary fibrosis [12]. The data from these studies support the fibroproliferative role of circulating fibrocytes and their chemokine receptors.

The increased expression of chemokines such as SDF-1, fractalkine, RANTES or MCP-1, has been demonstrated in PAH patients as well as in pulmonary hypertension animal models [36-38]. The gene profiling study carried out with laser dissected pulmonary arteries also exhibited the up regulation of various growth factors (vascular growth factor, platelet derived growth factor) and matrix proteins (procollagen1A2, procollagen1A2) [34, 39]. Human circulating fibrocytes express chemokine receptors of the CC family, namely CCR3, CCR5, CCR7 and the CXC family chemokine CXCR4 whereas mouse fibrocytes express CCR2, CCR7 and CXCR4 along with various growth factors, including monocyte/macrophage chemoattractant protein-1 (MCP-1), transforming growth factor α (TGF-α) and TGF-β, vascular endothelial growth factor (VEGF) and platelet derived growth factor A (PDGF-A).
Performing quantitative mRNA expression analysis, we observed a significant increase in the expression of circulating fibrocyte-related chemokine receptors such as CXCR4, CCR7 in response to hypoxia. The recruitment and consecutive secretion of mediators by this cell population may have profound effects on abnormal proliferation of other cells type such as smooth muscle cells or endothelial cells and hence ascribe to the initiation and/or progression of the remodelling process either via autocrine or paracrine loops. Therefore, circulating fibrocytes may partially mediate effects such as exuberant cell proliferation and extracellular matrix deposition in the pulmonary arterial wall that leads to vascular lumen narrowing and increased vascular resistance. Circulating fibrocytes are shown to promote angiogenesis [41], thus may be involved in neovascularisation and possibly serve as conduit for recruitment of new circulating cells from the circulation at the site of injury [41]. The current data on CF recruitment in chronic hypoxia encourage us to further study the cross talk of circulating fibrocytes with other vascular cell types and grow our interest to decipher the molecular mechanism for the vascular remodelling process.

Prostacyclin [PGI2] and its mimetic have been shown to decrease smooth muscle cell proliferation [42]. Apart from this, prostacyclins do inhibit the production of various cytokines, inflammatory mediators and suppress neutrophil adhesion [8, 43]. It has been shown that treprostinil mediates antiproliferative mechanisms of resident lung fibroblast [44]. Another prostacyclin analogue, iloprost was shown to have beneficial effect in asthma [45]. The inhaled iloprost suppressed the cardinal feature of asthma in mouse model by reducing the co stimulatory molecule expression (CD80/CD86) of the dentritic cells (DCs) in effect affecting proinflammatory events. The treatment also altered the cytokine expression and inhibited the migration of the DCs [45]. Circulating fibrocyte also reported to possess the co stimulatory molecules CD80/CD86. The treprostinil treatment may have similar effect as mentioned in this study. Thus, our findings of trepostinil induced reduction of circulating
fibrocytes in parallel to an overall reduction of pulmonary vascular remodelling may be interpreted as i) loss of proliferative signal of CF on resident fibroblasts and smooth muscle cells or ii) as direct antiproliferative effects of treprostinil on resident cells in addition to inhibition of CF recruitment. The true contribution of CF to pulmonary vascular remodelling can thus currently not be delivered, a genetic cell targeting approach to decipher this aspect is ongoing work in this direction.

Treatment of experimental PH can be carried out in ways of “preventive” and “reversing” protocols, i.e. treatment being applied throughout disease development or after onset of significant PH, respectively. As we assumed that hypoxia leads to recruitment of CF as part of the pathogenesis, the hypoxic mice were treated with treprostinil from the beginning of the hypoxia exposure. This was based on a study on hypoxia induced pulmonary hypertension in rats which showed recruitment of monocyte/macrophage lineage cells from day one until 3-4 week of hypoxia exposure [20]. In addition, our in vitro data showed that treprostinil inhibited the adhesion and differentiation of CF from freshly isolated peripheral blood mononuclear cells but it was ineffective on the completely differentiated circulating fibrocytes with respect to adhesion (unpublished data). We thus treated the mice with treprostinil from the beginning to evaluate its influence on the CF during the entire recruitment phase to the hypoxic lung.

The pulmonary hemodynamic changes induced by treprostinil in our experimental PH model closely resembled the clinical effects of this drug. The continuous infusion of treprostinil in hypoxic mice significantly reduced the pulmonary arterial pressure as compared to the hypoxia and sham control group. However, the structural remodelling could not be strongly reversed by treprostinil treatment in the hypoxic chimeric mice with the applied infusion rate of 14 ng/kg/min. This rate of infusion has been shown to exert strong effects on human
pulmonary hemodynamic and is about the starting dose for patients [26-29]. Patients on chronic treprostinil infusion treatment may need increased doses up to 60 ng/kg/min. In an additional set of experiments, we applied a five times higher dose to mice (70 ng/kg/min) with the same outcomes on hemodynamic, vascular remodelling and RV hypertrophy. The systemic pressures of minipump implanted mice (either saline filled (sham) or treprostinil filled minipumps (Hox+T14 and Hox +T70) were less compared to non implanted mice (normoxia and hypoxia). An explanation could be the surgical intervention and catheterisation of the left jugular vein. So whenever the effect of treprostinil had to be assessed, we compared it to sham infused animals rather than to hypoxia group animals alone.

We observed significant changes in cell recruitment and hemodynamic but less changes in vascular remodelling and ventricular hypertrophy due to treprostinil treatment. The underlying mechanisms for hypoxia induced pulmonary hypertension and smooth muscle cell hyperplasia are not yet clear [46]. Direct proliferative effects of hypoxia on vascular smooth muscle cells may account for the observed hypertrophy. The degree of contribution of circulating fibrocytes to smooth muscle cell hypertrophy and hyperplasia has yet to be defined. CF capacity to generate extracellular matrix like collagen 1, fibronectin however suggests a role in structural vessel changes. To our knowledge this is first study showing the non-reversal of structural remodelling in a chimeric murine model with treprostinil treatment.

One recent investigation exhibited similar findings in advanced monocrotaline induced pulmonary hypertension in rat model [47].

The functional connectivity of the parameters hypoxia, pulmonary arterial pressure, right ventricular hypertrophy and pulmonary resistance due to vascular remodelling is not fully understood. Differential effects of various treatments have been observed for hypoxia induced pulmonary hypertension wherein another prostacyclin analogue [iloprost] was as well found
to decrease pulmonary arterial pressure without reversing the right ventricular hypertrophy and sildenafil was found to prevent right ventricular hypertrophy with modest improvement in pulmonary arterial pressures [21]. It is yet unclear if direct effects of hypoxia on myocardial remodelling take place and whether differential impacts of the above mentioned drugs on vasorelaxation, vascular remodelling and myocardial remodelling are operative.

The analysis of lung tissue from idiopathic pulmonary arterial hypertension patients revealed a low frequency of double positive (CD45+/collagen-1+) fibrocytes in pulmonary resistance arteries. This low frequency of fibrocytes in patient tissue could be assigned to i) continuous, long term intravenous prostacyclin therapy, which all patients received before lung transplantation, ii) by the end stage of disease, in which possibly less cell recruitment is ongoing, or iii) by a rather quick transition from the hematopoietic marker expressing fibrocyte to an only mesenchymal markers expressing fibroblast. To resolve the question of cell transition, in vivo cell tracing studies still need to be done.

In summary, we have shown that bone marrow derived circulating fibrocytes are recruited to pulmonary resistance arteries in mice upon chronic hypoxic exposure where they presumably participate in the pathogenesis of PH development. Circulating fibrocyte recruitment can be selectively inhibited by the stable prostacyclin analogue treprostinil. Further investigations with respect to the origin of these cells, the kinetics of recruitment and cell transition and the specific effect on vascular remodelling will be needed to understand the significance of this cell type in the pathogenesis of pulmonary hypertension.
References


Figure 1 Hemodynamic and right ventricular hypertrophy

(A) The right ventricular systolic pressure of mice exposed to hypoxia (4 week) and hypoxia (4 week) + saline (sham) was increased significantly as compared to the mice kept under normoxic condition. The mice kept under hypoxia and treated with treprostinil (Hox +T14 and Hox +T70) showed normalisation of right ventricular pressure. * p < 0.05 considered to be significant vs. normoxia. ** p < 0.05 significant vs. sham and hypoxia. The statistical analysis was done using one way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test (n=6). (B) Right ventricular hypertrophy depicted as RV/ (LV+S) ratio, showed significant increase in hypoxic and sham group animals as compared to normoxic mice. The ratio was not significantly altered by the treprostinil treatment at both dose levels. * p < 0.05 considered to be significant vs. normoxia. # p > 0.05 non significant vs. sham. The statistical analysis was done using one way analysis of variance with Bonferroni’s multiple comparison post hoc test (n=6).

Figure 2. Vascular remodelling was partially reversed with treprostinil treatement

The percentage of vascular muscularisation categorised as NM (non-muscularised); PM (partially muscularised) and CM (completely muscularised) exhibited significant increase of muscularisation of pulmonary resistance arteries during chronic hypoxia in hypoxic and sham group mice. The treprostinil treatment was partially reversed the muscularisation with increase in non musculualrised blood vessels. *P< 0.05 considered to be significant vs. normoxic group. ** p< 0.05 significant compared to sham group mice. # p > 0.05 non significant vs. sham group. § p > 0.05 non significant vs. hypoxia group mice without minipump implantation. One way of analysis of variance with Bonferroni’s multiple comparison test was performed for statistical analysis (n=6).
Figure 3. Effect of treprostinil treatment on vessel wall thickness

The vessel wall thickness was found to be increased with chronic exposure to hypoxia in hypoxic and sham group animals. The treprostinil treatment at both dose level slightly reduced the vessel wall thickness as compared to sham and hypoxic mice but statistically non significant. * p < 0.05 considered to be significant vs. normoxic group. # p> 0.05 non significant vs. sham group as well as hypoxic mice without minipump implantation. One way of analysis of variance with Bonferroni’s multiple comparison post hoc test was performed (n=6).

Figure 4. Characterisation of circulating fibrocytes in pulmonary arteries of idiopathic PAH patients

The lung tissue was analysed for the presence of circulating fibrocytes using collagen-1 and CD 45 markers along with transmitted light differential interference contrast (DIC) images to illustrate tissue organisation and architecture. (A) Circulating fibrocytes were observed in plexiform lesions (arrows). Collagen-1$^+$ cells (fibroblasts), collagen depositions (arrowheads) and CD45$^+$ (asterisks) cells (leucocytes) were also detected in the tissue. (B) Pulmonary artery with a concentric lesion showing few fibrocytes in the perivascular area (arrows).

Figure 5. In vivo characterisation of circulating fibrocytes in pulmonary arteries of pulmonary hypertensive mice

(A) Murine small pulmonary arteries displayed co-expression of haematopoietic (CD45) and BM derived markers (GFP) in the pulmonary arterial wall (arrow). Host derived CD45 positive cells (green; asterisk) were observed in the arterial wall. (B) The co-expression of collagen1 (mesenchymal marker) and GFP in circulating fibrocytes. (C) Co-expression of vimentin (mesenchymal marker) and GFP by CF recruited in the pulmonary vascular wall (a)
at 1, 5 and 7 o’clock position (dotted square insets). The magnified insets of figure (a) are shown as (b), (c) and (d).

**Figure 6. In vivo recruitment of CF to the perivascular area in response to hypoxia is inhibited by treprostinil infusion.**

(A) The number of recruited Collagen1⁺/GFP⁺ cells increased in response to chronic hypoxia as compared to normoxic mice. The treprostinil treatment at 70 ng/kg/min significantly reduced the Collagen1⁺/GFP⁺ cells. (# p > 0.05 non significant vs. normoxia; ## p > 0.05 non significant vs. sham group; *p < 0.05 significant vs. sham.) (B) Collagen1⁺/GFP⁺ cells significantly accumulated in perivascular area in chronic hypoxia as compared to normoxia and treprostinil treatment remarkably inhibited their accumulation. * p < 0.05 significant vs. normoxia; ** p < 0.05 significant vs. sham. (C) CD45⁺/GFP⁺ cells were accumulated in the perivascular area and their recruitment was inhibited with treprostinil infusion at both dose level. (* p < 0.05 significant vs. normoxia group animals; ** p < 0.05 significant vs. sham. (D) The number of CD45⁻/GFP⁻ Tcells were not significantly inhibited with treprostinil treatment. (# p > 0.05 non significant vs. normoxia and ## p > 0.05 non significant vs. sham). (E) The number of recruited GFP⁺ cells significantly (* p < 0.05) increased in response to chronic hypoxia as compared to normoxic mice. The treatment with treprostinil infusion significantly inhibited the recruitment of CF ** p < 0.05 significant vs. sham group. (F) Lung tissue stained for αSMA and GFP detected no co-expressing cells in the vascular smooth muscle layer in response to chronic hypoxia in GFP chimeric mice. The one way of analysis of variance with Bonferroni’s multiple comparison post hoc test was performed (n=6).

**Figure 7. Chemokine regulation at mRNA level by quantitative PCR**

Upregulation of mRNA of the chemokine receptors CXCR4 and CCR7 in lung tissue of the mice exposed to hypoxic condition (hypoxia and sham group animals) as compared to
normoxic animals. The chemokine expression (CXCR4 and CCR7) did not change with treprostinil treatment at 14ng/Kg/min dose level as compared to sham group animals. However the treatment at 70 ng/kg/min dose significantly down regulated the chemokine expression as compared to sham group mice. The 18S ribosomal RNA was used as internal control for normalisation to calculate ΔCt. Statistical analysis was done with one way of analysis of variance using Bonferroni’s post hoc test (n=5-6). * p < 0.05 significant vs. normoxia; # p>0.05 non significant vs. sham and ** p< 0.05 significant vs. sham group mice.
Figure 1.

A

![Bar chart showing RVSP (mm Hg) for different groups: Normoxia, Hypoxia, Sham, Hox + T14, Hox + T70.](image)

B

![Bar chart showing RV/(LV+S) for different groups: Normoxia, Hypoxia, Sham, Hox + T14, Hox + T70.](image)
Figure 2.

![Graph showing % vascular muscularisation (20-70 μm vessel diameter)]

- Normoxia
- Hypoxia
- Sham
- Hox+T14
- Hox+T70

Legend:
- NM
- PM
- CM

Significance:
- * p < 0.05
- ** p < 0.01
- § p < 0.005
- # p < 0.001

Figure 3.

![Graph showing vessel thickness (μm)]

- Normoxia
- Hypoxia
- Sham
- Hox+T14
- Hox+T70

Significance:
- * p < 0.05
- # p < 0.001
Table II. Hemodynamic parameters with treprostinil treatment in BMT mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>RVSP (mm of Hg)</th>
<th>SAP (mm of Hg)</th>
<th>Hematocrit (%)</th>
<th>RV/(LV+S)</th>
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</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>25.2 ± 0.43</td>
<td>76 ± 5.1</td>
<td>45.8 ± 0.37</td>
<td>0.24 ± 0.014</td>
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<tr>
<td>Hypoxia</td>
<td>36.6 ± 0.7</td>
<td>76.6 ± 2.9</td>
<td>63.0 ± 1.3</td>
<td>0.34 ± 0.01</td>
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<tr>
<td>Sham</td>
<td>30.29 ±0.8</td>
<td>59.1 ± 3.1</td>
<td>53.2 ± 1.2</td>
<td>0.31 ± 0.02</td>
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<tr>
<td>Hox + T14</td>
<td>25.75 ± 0.5</td>
<td>62.2 ± 3.9</td>
<td>55.0 ± 1.1</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>Hox +T70</td>
<td>24.75 ± 0.22</td>
<td>57.8 ± 2.4</td>
<td>62.4 ± 1.03</td>
<td>0.32 ± 0.01</td>
</tr>
</tbody>
</table>

RVSP = right ventricular systolic pressure; SAP = systemic arterial pressure. Hox= Hypoxia; T14 = Treprostinil (14ng/kg/min); T70 = Treprostinil (70 ng/kg/min). Data Mean ± SEM; n= 6 mice.
Figure 4.

A

B
### Table III Patient Data

<table>
<thead>
<tr>
<th>Etiology</th>
<th>Gender</th>
<th>Age (years)</th>
<th>mPAP (mm Hg)</th>
<th>Specific medication</th>
<th>Lumen</th>
<th>Vessel wall</th>
<th>Perivascular area</th>
<th>Blood vessels analysed</th>
<th>Observations</th>
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<tbody>
<tr>
<td>iPAH</td>
<td>F</td>
<td>45</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>35</td>
<td>Pulmonary arteries severely remodelled. Presence of CD45+ cells. Occurrence of plexiform lesions.</td>
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<tr>
<td>iPAH</td>
<td>M</td>
<td>29</td>
<td>N.D.</td>
<td>Prostacycline, Treprostinil</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>35</td>
<td>Pulmonary arteries severely remodelled. No plexiform lesions.</td>
</tr>
<tr>
<td>iPAH</td>
<td>M</td>
<td>52</td>
<td>60</td>
<td>Prostacyclin</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>35</td>
<td>Pulmonary arteries severely remodelled. Presence of plexiform lesions with collagen depositions, CD45+ cells and few CD45+/collagen1+ fibrocytes.</td>
</tr>
<tr>
<td>iPAH</td>
<td>M</td>
<td>44</td>
<td>83</td>
<td>Flolan, Iloprost</td>
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<td>0</td>
<td>2</td>
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<tr>
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<td>Prostacyclin</td>
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<tr>
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<td>80</td>
<td>Prostacyclin, Bosentan</td>
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<td>4</td>
<td>2</td>
<td>30</td>
<td>Pulmonary arteries severely remodelled. Presence of CD45+ cells. No plexiform lesions.</td>
</tr>
<tr>
<td>iPAH</td>
<td>F</td>
<td>27</td>
<td>82</td>
<td>Bosentan, Prostacyclin</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>40</td>
<td>CD45 positive cells in the lumen of the vessel. No plexiform lesions.</td>
</tr>
<tr>
<td>iPAH</td>
<td>M</td>
<td>45</td>
<td>N.D.</td>
<td>Sildenafil</td>
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<td>1</td>
<td>0</td>
<td>35</td>
<td>Rare occurrence of plexiform lesions with collagen depositions and CD45 cells. Remodelled vessels.</td>
</tr>
</tbody>
</table>

N.D. not determine

mPAP = mean pulmonary arterial pressure; Col1 = collagen-1; F = female; M = male; iPAH = idiopathic pulmonary arterial hypertension
Figure 5.

A  CD45 + GFP

B  GFP + Collagen1

C  Vimentin + GFP
Figure 6.

A

Ratio of Col1+/GFP+ cells/number of blood vessel

B

Ratio of Col1-/GFP+ cells/number of blood vessel
C

Ratio of CD45+/GFP+ cells/number of blood vessel

D

Ratio of CD45+/GFP+ cells/number of blood vessel
E

Ratio of GFP\(^{+}\) cells/number of blood vessel

F

<table>
<thead>
<tr>
<th>Condition</th>
<th>Image 1</th>
<th>Image 2</th>
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<td>Hox + T70</td>
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</tr>
</tbody>
</table>
Figure 7.

![Bar graph showing relative chemokine expression (ΔCt) for CXCR4 and CCR7 under different conditions.](image)