Statin ameliorates hypoxia-induced pulmonary hypertension associated with down-regulated stromal cell-derived factor-1

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Aims Mobilization of stem cells/progenitors is regulated by the interaction between stromal cell-derived factor-1 (SDF-1) and its ligand, CXC chemokine receptor 4 (CXCR4). Statins have been suggested to ameliorate pulmonary arterial hypertension (PAH); however, the mechanisms involved, especially their effects on progenitors, are largely unknown. Therefore, we examined whether pravastatin ameliorates hypoxia-induced PAH in mice, and if so, which type of progenitors and what mechanism(s) are involved.

Methods and results Chronic hypoxia (10% O2 for 5 weeks) increased the plasma levels of SDF-1 and mobilization of CXCR4+/vascular endothelial growth factor receptor (VEGFR)2+/c-kit+/cells from bone marrow (BM) to pulmonary artery adventitia in Balb/c mice in vivo, both of which were significantly suppressed by simultaneous oral treatment with pravastatin (2 mg/kg/day). Furthermore, in vitro experiments demonstrated that hypoxia enhances differentiation of VEGFR2+/c-kit + cells into α-smooth muscle actin + cells. Importantly, pravastatin ameliorated hypoxia-induced PAH associated with a decrease in the number of BM-derived progenitors accumulating in the pulmonary artery adventitia. The expression of intercellular adhesion molecule-1 (ICAM-1) and its ligand, CD18 (β2-integrin), were enhanced by hypoxia and were again suppressed by pravastatin.

Conclusions These results suggest that pravastatin ameliorates hypoxia-induced PAH through suppression of SDF-1/CXCR4 and ICAM-1/CD18 pathways with a resultant reduction in the mobilization and homing of BM-derived progenitor cells.

1. Introduction
Recent reports demonstrated that circulating endothelial progenitor cells (EPCs) promote endothelial repair, which is enhanced by 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins).1–3 Moreover, the number of circulating EPCs correlates with endothelial function and the degree of coronary artery disease in humans.4 Accumulating evidence suggests that circulating EPCs are mobilized to the site of ischaemia by several humoral factors, such as vascular endothelial growth factor (VEGF), stromal cell-derived factor-1 (SDF-1), CD18, and intercellular adhesion molecule-1 (ICAM-1), contributing to the neovascularization.1,5,6 Recently, it has also been reported that CXC chemokine receptor 4 (CXCR4), the receptor for SDF-1, plays an important role in the mobilization and recruitment of bone marrow (BM)-derived cells.7 We have recently reported that EPCs are mobilized under hypoxic conditions and are incorporated into the pulmonary endothelium in pulmonary arterial hypertension (PAH) in mice.8 Recent studies also demonstrated the therapeutic effects of EPC transplantation in animal models of PAH.9,10 However, it remains to be examined whether EPCs also exert beneficial effects in patients with PAH, which is characterized by plexiform lesion composed of actively proliferating endothelial cells.11

It has been demonstrated that statins could prevent the development of PAH in animal models.12–14 Although statins could mobilize EPCs,15,16 long-term statin treatment has been reported to reduce the number of circulating EPCs in patients with coronary artery disease.17 Therefore,
Statins might have biphasic effects on mobilization of EPCs, as well as other circulating progenitor cells, which have also been reported to exist and differentiate into α-smooth muscle actin (SMA)⁺ cells in humans, and may participate in the progression of atherosclerosis and lung fibrosis. Recent reports also showed the existence of circulating progenitors that exhibit fibroblast-like properties, migrate into the pulmonary vasculature, and promote adventitial remodelling as myofibroblasts (α-SMA-expressing fibroblasts) in hypoxia-induced PAH. Indeed, the BM-derived progenitors could differentiate into α-SMA⁺ cells in hypoxia-induced PAH in mice. However, it largely remains unknown whether statins affect mobilization and recruitment of those progenitors.

In the present study, we thus examined whether pravastatin ameliorates hypoxia-induced PAH in mice, and if so, which type of progenitors and what mechanism(s) are involved.

2. Methods
All procedures were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 8523, revised 1985). The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the article as written.

2.1 Animal preparation
In the present study, we used 16-week-old wild-type (WT, n=56) mice of Balb/c background. ROSA26 (LacZ) mice that express β-galactosidase (β-gal) activity in all tissues were purchased from Jackson Laboratory (Bar Harbor, ME, USA).

2.2 Hypoxia-induced pulmonary arterial hypertension model in mice and statin treatment
The present study is a preventive study in nature. Four weeks after the BM transplantation, β-gal-BM chimeric mice were randomized to receive either pravastatin (2 mg/kg/day) or vehicle by daily gavage, and then exposed to hypoxia (10% O₂) for 7 days. To confirm the differentiation capacity, isolated peripheral blood mononuclear cells (PBMCs) from hypoxic mice (10% O₂ for 7 days) were cultured on collagen I-coated chamber slides (BioCoat; Becton Dickinson, San Jose, USA) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in hypoxic conditions (2% O₂, 5% CO₂, and 93% nitrogen, 33°C, for in vitro experiment). For immunohistological staining, monoclonal antibody to fluorescein isothiocyanate (FITC)-labelled α-SMA (1:400, Sigma, St Louis, USA) was used for primary antibody.

2.3 Ex vivo culture of mononuclear cells
To confirm the differentiation capacity, isolated peripheral blood mononuclear cells (PBMCs) from hypoxic mice (10% O₂ for 7 days) were cultured on collagen I-coated chamber slides (BioCoat; Becton Dickinson, San Jose, USA) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in hypoxic conditions (2% O₂, 5% CO₂, and 93% nitrogen, 33°C, for in vitro experiment). For immunohistological staining, monoclonal antibody to fluorescein isothiocyanate (FITC)-labelled α-SMA (1:400, Sigma, St Louis, USA) was used for primary antibody.

2.4 Fluorescence-activated cell sorter analysis
Fluorescence-activated cell sorter (FACS) analysis was performed as described previously. To quantify the number of VEGFR2⁺/c-kit⁺ cells, we used phycoerythrin-labelled anti-mouse VEGFR2, FITC-labelled anti-mouse c-kit (ebioscience), and biotinylated anti-mouse lineage antibodies (Mac-1, Gr-1, B220, CD4, CD8, and Ter119) and APC-labelled streptavidin (BD Pharmingen). Quantitative analysis was performed by FACS analysis (FACSCalibur; Becton Dickinson).

2.5 Cell sorting by fluorescence-activated cell sorter
BM cells were obtained from hypoxic mice, and BM mononuclear cells were isolated by density gradient centrifugation with HypoPrep Animal 1.077 (Axis-Shield). Lin⁻/VEGFR2⁺/c-kit⁺ cells were selected with a cell sorting system (FACSARia; Becton Dickinson Immucytometry Systems).

2.6 Bone marrow transplantation
BM transplantation was performed as described previously. The chimeric rate was >95% by FACS analysis.

2.7 Immunofluorescence staining
Immunofluorescence staining was performed on 4% paraformaldehyde-fixed frozen sections as previously described. The primary antibodies used were FITC-labelled anti-β-gal (1:400; Abcam Ltd, Cambridge, UK), anti-mouse ICAM-1 (1:400; BD Pharmingen), anti-mouse CD18 (1:400; BD Pharmingen), Cy3-labelled anti-α-SMA (1:400; Sigma), anti-VEGFR2 (1:200; Santa Cruz), anti-CD31, biotinylated anti-c-kit, and biotinylated anti-CXCR4 (1:400; BD Pharmingen, San Diego). As the secondary antibodies, Cy3- or Cy5-labelled antibodies were used (Jackson Immunoresearch Laboratories). Vectashield mounting medium with DAPI (vector) was used to counterstain nuclei. Slides were viewed with a confocal fluorescence microscope (Fluoview FY1000, Olympus, Tokyo, Japan). As a negative control, species- and isotype-matched IgG were used in place of the primary antibody.

2.8 X-gal staining
To detect β-gal-positive cells, the lungs were perfusion-fixed with 0.5% glutaraldehyde (pH 7.2) at 4°C and the whole lungs were incubated at 37°C for 18 h in X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) solution as previously described.

2.9 Plasma levels of stromal-derived factor-1
Plasma levels of SDF-1 were evaluated by ELISA with a mouse SDF-1 Quantikine kit (R&D) following the manufacturer’s protocol.

2.10 Statistical analysis
Quantitative results are expressed as means ± SD. Statistical analysis was performed with StatView (StatView 5.0, SAS Institute Inc., Cary, NC, USA). Comparisons of parameters among the three groups were made by one-way ANOVA and those between the two groups under different conditions by two-way ANOVA followed by Bonferroni post hoc test. A value of P < 0.05 was considered to be statistically significant.

3. Results
3.1 Mobilization of vascular endothelial growth factor receptor 2⁺/c-kit⁺ cells in hypoxia-induced pulmonary arterial hypertension in mice
To elucidate the role of circulating VEGFR2⁺/c-kit⁺ cells, we performed FACS analysis on PBMCs from the mice that were
chronically exposed to hypoxia (10% O2) for 5 weeks. Interestingly, the number of cells was significantly increased in hypoxic mice compared with that in normoxic mice at 3 and 5 weeks of hypoxia (Figure 1A). Moreover, cultivation of PBMCs from hypoxic mice revealed a colony formation (Figure 1B). Immunostaining revealed that these cells were positive for α-SMA (Figure 1C). Furthermore, VEGFR2+/c-kit+ cells were accumulated mainly at the adventitia of pulmonary arteries of chronically hypoxic mice (Figure 1D), whereas VEGFR2+/c-kit+ cells were not observed in normoxic condition (Figure 1E). These results suggest a crucial role of circulating VEGFR2+/c-kit+ cells in the pathogenesis of hypoxia-induced PAH.

3.2 Differentiation of vascular endothelial growth factor receptor 2+/c-kit+ cells into α-smooth muscle actin+ cells

To elucidate the role of VEGFR2+/c-kit+ cells, we isolated VEGFR2+/c-kit+ cells from BM of Rosa26 mice by flow cytometry (Figure 2A) and cultured them on primary cultured stromal cell layers (derived from WT mice) under hypoxic conditions (2% O2, 33°C) for 3 weeks (Figure 2B). Interestingly, these cells differentiated into α-SMA+ cells after 3 weeks of culture (Figure 2C).

3.3 Pravastatin reduces the number of bone marrow-derived cells at the pulmonary artery adventitia

To elucidate the effects of pravastatin on the recruitment of the BM-derived cells and the development of pulmonary vascular remodelling, we used chimeric mice with β-gal-labelled BM. These chimeric mice were chronically exposed to hypoxia (10% O2) for 5 weeks with or without simultaneous treatment with pravastatin (2 mg/kg/day). Microscopic observation revealed that the BM-derived (β-gal+) cells migrated and accumulated to the adventitia of pulmonary arteries (Figure 3A) and that pravastatin significantly reduced the number of those cells (Figure 3A and B).

3.4 Pravastatin reduces mobilization of vascular endothelial growth factor receptor 2+/c-kit+ cells and ameliorates pulmonary arterial hypertension

It has been shown that the secretion of SDF-1 is controlled by hypoxia-induced factor-1α (HIF-1α), which have chemotactic power to mobilize the BM-derived progenitors. We therefore assessed the plasma levels of SDF-1 in chronically hypoxic mice with or without pravastatin. Interestingly, plasma levels of SDF-1 were significantly increased in hypoxic mice, which was significantly reduced by pravastatin (Figure 4A). Moreover, immunostaining revealed that hypoxia enhanced the accumulation of CXCR4+ cells in the lung (Figure 4B) and FACS analysis showed the hypoxia-induced increase in the number of circulating CXCR4+/VEGFR2+/c-kit+ cells in peripheral blood (Figure 4C), both of which also were inhibited by pravastatin. Finally, we confirmed that pravastatin ameliorated PH in mice in vivo, as assessed by RVSP (Figure 4D) and RV hypertrophy (Figure 4E).

Figure 1  Vascular endothelial growth factor receptor (VEGFR)2+/c-kit+ cells to the adventitia of pulmonary arteries in hypoxia. (A) Fluorescence-activated cell sorter analysis indicated that hypoxia significantly increased the number of circulating VEGFR2+/c-kit+ cells at 3 and 5 weeks (n = 6 each). Results are expressed as means ± SD. (B) Colonies with a ‘hill and valley’ morphology after cultivation of peripheral blood mononuclear cells for 3 weeks under hypoxic conditions (2% O2). Scale bar: 200 μm. (C) α-SMA immuno-positive cells in the colony. Scale bar: 10 μm. (D,E) VEGFR2+/c-kit+ cells in the adventitia of pulmonary arteries in mice with hypoxic condition (5 weeks) detected by immunostaining (D, arrows), but not in normoxic mice (E). Scale bar: 50 μm.
3.5 Pravastatin reduces accumulation of bone marrow-derived cells by suppressing ICAM-1 expression

It has been implicated that the interaction between ICAM-1 and its ligand, CD18, is crucial for the recruitment of the BM-derived progenitors. We therefore assessed ICAM-1 expression in the hypoxic lung, the number of BM-derived cells, and their CD18 expression. Interestingly, hypoxia enhanced the expression of ICAM-1 in the lung, which was again suppressed by pravastatin (Figure 5A). Moreover, the BM-derived CD18+ cells accumulated into the pulmonary artery adventitia in hypoxic lung, which was again reduced by pravastatin (Figure 5A and B).

4. Discussion

The novel findings of the present study were that: (i) chronic hypoxia significantly mobilized VEGFR2+/c-kit+ cells that accumulated into the pulmonary artery adventitia in vivo and differentiated into α-SMA+ cells in vitro and (ii) pravastatin reduced the plasma levels of SDF-1, mobilization of CXCR4+/VEGFR2+/c-kit+ cells, and the expression of ICAM-1 in the lung, resulting in the reduced accumulation of BM-derived progenitors and amelioration of PAH. The present findings are summarized in Figure 6. To the best of our knowledge, this is the first study that demonstrates the therapeutic effects of a statin for PAH in the cutting edge of circulating progenitors.

4.1 Circulating progenitors and pulmonary arterial hypertension

We have recently demonstrated that circulating progenitors are incorporated into the pulmonary endothelium and that mobilization and homing of progenitors are important in the pulmonary vascular remodelling as they exert beneficial effects in hypoxia-induced PAH in mice. In the present study, circulating mononuclear cells isolated from mice chronically exposed to hypoxia differentiated into α-SMA+ cells in vitro. Consistent with our finding, a recent report demonstrated that circulating c-kit+ progenitor cells are involved in vessel wall thickening in hypoxia-induced PAH in calves. Moreover, it has been shown that BM-derived cells differentiate into α-SMA+ cells in pulmonary arteries in hypoxia-induced PAH in mice. It has also been demonstrated that circulating progenitors are heterogeneous and that haematopoietic stem cell-derived progenitors contribute to neoangiogenesis through adventitial infiltration, without any contributions to pulmonary endothelium in PAH animal models. Therefore, the BM-derived circulating progenitors may play a crucial role in the development of hypoxia-induced pulmonary vascular remodelling.

In the present study, we were also able to demonstrate the significant mobilization and accumulation of VEGFR2+/c-kit+ cells at the pulmonary artery adventitia in vivo and their differentiation into α-SMA+ cells ex vivo. Recent reports demonstrated that α-SMA+ cells at the pulmonary artery adventitia can be derived from circulating...
progenitors and have a potential to migrate and incorporate into the media and contribute to medial thickening. These reports and our present findings suggest the existence of circulating progenitor cells for \( \alpha \)-SMA\(^+\) cells and their crucial roles in the pathogenesis of pulmonary vascular remodelling in hypoxia-induced PAH.

### 4.2 Statins and pulmonary arterial hypertension

It has been recently demonstrated that statins induce apoptosis of neointimal smooth muscle cells and ameliorate monocrotaline-induced PAH in rats. Statins have also been shown to prevent pulmonary artery muscularization and progression of PAH by protecting expression and activity of endothelial nitric oxide synthase (eNOS) at post-transcriptional level in hypoxia-induced PAH in rats. Although the mechanisms underlying the beneficial effects of statins on PAH are not fully elucidated, these studies suggest that statins may improve endothelial function, at least in part, through NO-dependent mechanisms.

On the other hand, it also has been shown that statins mobilize progenitors and activate their function through up-regulation of eNOS. However, in the present study, the number of circulating progenitors assessed by FACS analysis was significantly reduced by pravastatin in hypoxic mice. Therefore, the effect of statins on mobilization of progenitors under hypoxic conditions may have a complex mechanism (Figure 6).

Recently, it has been shown that SDF-1 plays a crucial role in mobilizing and controlling BM-derived CXCR4\(^+\) cells to the ischaemic tissue. Moreover, it has been shown that the activated perivascular myofibroblasts produce abundant SDF-1, which plays a crucial role in the recruitment of BM-derived cells to the perivascular area. Again, recruited CXCR4\(^+\) cells secret angiogenic cytokines and promote the proliferation and differentiation of resident cells in vascular walls. In the present study, we demonstrated that pravastatin significantly reduced the plasma levels of SDF-1, which could explain, at least in part, for the reduced mobilization of progenitors by statin. Pravastatin also suppressed the expression of ICAM-1 that is important for homing of BM-derived cells to the ischaemic tissue. Taken together, we were able to demonstrate that pravastatin significantly reduces mobilization and homing of

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**Figure 3** Bone marrow (BM)-derived cells in the adventitia of pulmonary arteries of chimeric mice. (A) X-gal staining showed that \( \beta \)-galactosidase (\( \beta \)-gal)\(^+\) BM-derived cells were increased by hypoxia and accumulated to the pulmonary artery adventitia (arrowheads) in hypoxic mice, while the number of BM-derived cells was reduced by pravastatin (arrowheads). (B) The number of \( \beta \)-gal\(^+\) cells was significantly increased in the hypoxic lung, which was significantly suppressed by pravastatin. Results are expressed as means \( \pm \) SD. Normoxia, normoxic mice; hypoxia, mice exposed to 5 weeks of hypoxia (10\% O\(_2\)) with or without pravastatin (2 mg/kg/day). LPF, low-power field (\( \times 200\)).
BM-derived cells, resulting in the inhibition of hypoxia-induced pulmonary artery remodelling and PAH (Figure 6).

### 4.3 Limitations of the study

There are several limitations should be mentioned for the present study.

First, hypoxia-induced PAH model may not fully represent the primary PAH in humans because this model shows considerably high plasma levels of cytokines/chemokines.³⁹ It has been shown that hypoxia increases the expression of SDF-1 through HIF-1-dependent mechanisms.⁴⁰ Consistently, in the present study, plasma level of SDF-1 was significantly increased in WT mice in response to hypoxia. Additionally, pravastatin significantly reduced the plasma levels of SDF-1 and ameliorated hypoxia-induced PAH, although the precise mechanism remains to be elucidated in future studies. On the other hand, blockade of SDF-1 by specific antibody has been shown to reduce neointimal formation in ApoE⁻/⁻ mice by regulating neointimal smooth muscle content.⁴¹ Taken together, it is possible that SDF-1 also plays a crucial role in the development of pulmonary vascular remodelling in PH.

Second, the role of EPCs in the development of PAH in humans remains to be elucidated. It was demonstrated that the number of circulating endothelial cells was significantly increased in patients with PAH.¹¹ The number of circulating EPCs is regulated not only by their recruitment or mobilization but also by their activity and consumption in the peripheral vasculature.¹⁻⁴ Therefore, when considering a therapy focusing on BM-derived progenitors, it would be important to evaluate their mobilization and homing as well as the character of each progenitor.

Third, it remains to be examined whether statins could ameliorate PH in humans through down-regulation of SDF-1. However, this important issue is beyond the scope of the present study, and we would like to address this issue in future studies.

Fourth, in the present study, we have demonstrated that the number of progenitor cells is decreased by co-treatment with pravastatin in which finding is different from that in atherosclerotic model.⁴² Thus, future studies are needed to determine the role of progenitor cells, especially at the adventitia.

Fifth, the present study is a preventive study in nature. Because oxidative stress contributes to the initiation and the development of hypoxia-induced tissue injury and PAH, statins may be expected to reduce initial oxidative and subsequent events that lead to PAH. In this regard, statins have direct antioxidant effects: inhibit NAD[P]H activity and up-regulate the activity of antioxidant enzymes, such as catalase and paraoxonase, and also reduce circulating markers of oxidation, such as F2-isoprostane and nitrotyrosine. Thus, it remains to be examined in future studies whether statin and pulmonary hypertension
statins exerts beneficial effects in animals with fully developed disease.

4.4 Clinical implications

We have previously demonstrated that statins alter smooth muscle cell accumulation and collagen content in established atheroma in rabbits. It has recently been demonstrated that statins ameliorate congestive heart failure, ischaemia/reperfusion injury, and PAH in humans. In the present study, we were able to demonstrate that pravastatin ameliorates PAH in mice associated with a marked reduction in the number of BM-derived progenitors at the pulmonary artery adventitia. Indeed, it was shown that in vivo depletion of circulating progenitors for α-SMA+ cells resulted in marked attenuation of hypoxia-induced pulmonary vascular remodelling, such as adventitial thickening, perivascular fibrosis, and myofibroblast accumulation. Therefore, modulation of mobilization and homing of BM-derived cells by statins could be a new therapeutic strategy for the treatment of PAH in humans.

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Figure 5 Bone marrow (BM)-derived cells in the adventitia of pulmonary arteries of chimeric mice through the suppression of intercellular adhesion molecule (ICAM)-1/CD18 interaction. (A) Immunostaining revealed that the expression of ICAM-1 (red signals) is enhanced in the lung and CD18+ cells (blue signals) are accumulated to the pulmonary artery adventitia (arrowheads) in hypoxic mice. Pravastatin reduced the number of CD18+/β-galactosidase (β-gal)+ cells that accumulated to the pulmonary artery adventitia (CD18/ICAM-1/β-gal). β-gal is expressed as green signals. (B) The number of CD18+ cells was significantly increased in hypoxic lung, which was significantly suppressed by pravastatin. Results are expressed as means ± SD. Normoxia, normoxic mice; hypoxia, mice exposed to 5 weeks of hypoxia (10% O2) with or without pravastatin (2 mg/kg/day). HPF, high-power field (×400).
Figure 6 Summary of the present study. Stromal cell-derived factor (SDF)-1 mediates the mobilization and chemotaxis of bone marrow (BM)-derived progenitors in response to hypoxia. During the development of hypoxia-induced pulmonary vascular remodelling, pravastatin reduces the plasma levels of SDF-1 and the expression of intercellular adhesion molecule (ICAM)-1 in the lung, resulting in the reduced number of BM-derived progenitors in the adventitia and the amelioration of pulmonary arterial hypertension. Solid line, proven mechanism; dashed line, proposed mechanism; plus, stimulation; minus, inhibition.

Conflict of interest: none declared.

References
