Supplemental Materials

Title: PGC-1α-mediated angiogenesis prevents pulmonary hypertension in mice
Brief title: PGC-1α-mediated angiogenesis in pulmonary hypertension

Takayuki Fujiwara1,2,7, Norifumi Takeda, MD1,#, Hironori Hara1,3, Satoshi Ishii1, Genri Numata1,3, Hiroyuki Tokiwa1, Manami Katoh1, Sonoko Maemura1, Takaaki Suzuki1, Hiroshi Takiguchi1, Tomonobu Yanase1, Yoshiaki Kubota6, Seitaro Nomura1,4, Masaru Hatano1, Kazutaka Ueda1, Mutsuo Harada1,5, Haruhiro Toko1, Eiki Takimoto1, Hiroshi Akazawa1, Hiroyuki Morita1, Satoshi Nishimura7 and Issei Komuro1,#

1Department of Cardiovascular Medicine, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
2Department of Computational Diagnostic Radiology and Preventive Medicine,
3Department of Advanced Translational Research and Medicine in Management of Pulmonary Hypertension
4Department of Therapeutic Strategy for Heart Failure,
5Department of Advanced Clinical Science and Therapeutics, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
6Department of Anatomy, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
7Center for Molecular Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan

Address for correspondence:
Issei Komuro
Department of Cardiovascular Medicine
The University of Tokyo Hospital
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
Phone: +81-3-3815-5411
Fax: +81-3-5800-2087
Email: komuro_tky2000@yahoo.co.jp

Norifumi Takeda
Department of Cardiovascular Medicine
The University of Tokyo Hospital
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
Phone: +81-3-3815-5411
Fax: +81-3-5800-9182
Email: norifutakeda@gmail.com
### Supplemental Table S1
List of primer sequences used for qRT-PCR analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s</td>
<td>CGGCTACCACATCCAAAGGAA</td>
<td>AGCTGGAATTACCGCGGC</td>
</tr>
<tr>
<td>Vegfa</td>
<td>ATCTTCAGGGCTTCTGCTG</td>
<td>GCATTACATCTGTGTGCT</td>
</tr>
<tr>
<td>Ppargc1a</td>
<td>CCGAGAATTCATGGAGCAAT</td>
<td>TTTCTGTGGTTTTGGTGTA</td>
</tr>
<tr>
<td>Cdkn1a</td>
<td>CACAGCTCAGTGGACTGGAA</td>
<td>ACCCTAGACCCACAAATGCAG</td>
</tr>
<tr>
<td>Tnf</td>
<td>TAGCCAGGAGGAGAACAGA</td>
<td>TTTTCTGGAGGGAGTGTGG</td>
</tr>
<tr>
<td>Il6</td>
<td>GATGATGCACTTGCAAGAAAACA</td>
<td>GGCTACTCCAGAAGACCAGAGGAA</td>
</tr>
</tbody>
</table>
Supplemental figure 1. Feature matching and similarity score against 3D reconstructed SMC image of normoxia in multiple PH models
All set of comparison images used to calculate the similarity score (Figure 1H) are shown. Image feature points from two reconstructed images depicting SMC remodeling were automatically extracted by AKAZE feature detector and descriptor, and the similarity score was calculated by mean distance between the matched feature points. Comparison images between normoxia (Nx) and hypoxia (Hx 3w, A) and SU5416/hypoxia mice (SuHx 3w, B).
Supplemental Figure 2

<table>
<thead>
<tr>
<th></th>
<th>Feature matching</th>
<th>Similarity score</th>
<th></th>
<th>Feature matching</th>
<th>Similarity score</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Hx 3w</td>
<td></td>
<td>B</td>
<td>SuHx 3w</td>
<td></td>
</tr>
<tr>
<td>Nx</td>
<td></td>
<td>129.69</td>
<td>Nx</td>
<td></td>
<td>119.76</td>
</tr>
<tr>
<td>Hx</td>
<td></td>
<td>125.19</td>
<td>SuHx</td>
<td></td>
<td>117.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>127.54</td>
<td></td>
<td></td>
<td>118.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>129.98</td>
<td></td>
<td></td>
<td>119.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>122.56</td>
<td></td>
<td></td>
<td>127.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>124.11</td>
<td></td>
<td></td>
<td>119.3</td>
</tr>
</tbody>
</table>

Supplemental figure 2. Feature matching and similarity score against 3D reconstructed EC lineage-tracing image of normoxia in multiple PH models

All set of comparison images used to calculate the similarity score (Figure 2C) are shown. Image feature points from two reconstructed images depicting EC lineage-tracing (angiogenesis) were automatically extracted by AKAZE feature detector and descriptor, and the similarity score was calculated by mean distance between the matched feature points. Comparison images between normoxia (Nx) and hypoxia (Hx 3w, A), SU5416/hypoxia mice (SuHx 3w, B).
Supplemental Figure 3. Baicalin treatment did not enhance PGC-1α-mediated angiogenic response, nor attenuate Hx-PH in Ppargc1a<sup>cko</sup> mice

Ppargc1a<sup>cko</sup> mice were treated with baicalin (150 mg/kg/day) or vehicle during 3 weeks of hypoxic conditions. (A) Relative expression of Ppargc1a measured one week after hypoxic exposure (n = 6 per group). (B, C) Western blot analysis of PGC-1α protein in the lung. One representative blot (B) and quantified PGC-1α levels (C) are presented (n = 6 mice per group). (D) Relative expression of Vegfa one week after hypoxic exposure (n = 6 mice per group). (E, F) Pulmonary ECs of Ppargc1α<sup>cko</sup> mice (L1-Cre; Ppargc1α<sup>lofl</sup>) were labeled with tdTomato fluorescence by generating L1-Cre; Rosa26-lsl-ttdTomato; Ppargc1α<sup>lofl</sup> (tdTom; Ppargc1α<sup>cko</sup>). The mice were exposed to Hx with administration of baicalin or vehicle for 3 weeks (Hx 3w). (E) Representative multiphoton images of the lung ECs. Scale bar: 500 μm (and 200 μm for the inset). (F) Quantification of EC volume density (n = 6 mice per group). (G) Representative 2D images showing αSMA-stained arterioles. Scale bar: 50 μm (and 20 μm for the inset). (H) Quantification of SMC thickness (n = 62 vessels from N = 4 mice per group). (I) Representative RVP data. (J) RVSP (n = 6 mice per group). (K) RVH (n = 6 mice per group). Data are representative of three independent experiments. Statistical significance for comparison between means was evaluated using a Student’s t-test.
Supplemental Figure 4. Baicalin treatment increased PGC-1α expression in SuHx mice, but did not enhance angiogenic response or attenuate PH

SuHx mice were treated with baicalin (150 mg/kg/day) or vehicle during 3 weeks of hypoxic conditions. (A) Relative expression of Ppargc1a measured one week after hypoxic exposure (n = 6 per group). (B, C) Western blot analysis of PGC-1α protein in the lung. One representative blot (B) and quantified PGC-1α levels (C) are presented (n = 7 mice per group). (D) Relative expression of Vegfa one week after hypoxic exposure (n = 6 mice per group). (E, F) 3D EC lineage-tracing experiment. (E) Representative 3D EC lineage-tracing images. Scale bar: 500 μm (and 100 μm for the inset). (F) Angiogenesis index (n = 6 mice per group). (G) Representative 2D images showing αSMA-stained arterioles. Scale bar: 50 μm (and 20 μm for the inset). (H) Quantification of SMC thickness (n = 24 vessels from N = 4 mice per group). (I) Representative RVP data. (J) RVSP (n = 8 mice per group). (K) RVH (n = 8 mice per group). Data are representative of three independent experiments. Statistical significance for comparison between means was evaluated using a Student’s t-test. *P < 0.05.
Supplemental video legends

Supplemental video 1. 3D morphological analysis of pulmonary smooth muscle cells in mouse under normoxia
Wild-type mouse was housed in the normoxic chamber for control until the age of 11 weeks. SMCs stained with Cy3-conjugated anti-αSMA antibody were three-dimensionally shown. Nuclei were counterstained with Hoechst 33342.

Supplemental video 2. 3D morphological analysis of pulmonary smooth muscle cells in hypoxia (Hx)-induced pulmonary hypertension mouse model
Wild-type mouse was exposed to hypoxia for 3 weeks from the age of 8 weeks (Hx 3w). SMCs stained with Cy3-conjugated anti-αSMA antibody were three-dimensionally shown. Note the marked SMC elongation into the peripheral lung and/or hyperplasia-like structure. Nuclei were counterstained with Hoechst 33342.

Supplemental video 3. 3D morphological analysis of pulmonary smooth muscle cells in SU5416/hypoxia (SuHx)-induced PH mouse model
Wild-type mouse was subjected to hypoxia and treated with SU5416 for 3 weeks from the age of 8 weeks (SuHx 3w). SMCs stained with Cy3-conjugated anti-αSMA antibody were three-dimensionally shown. Note that SMC elongation into the peripheral lung observed in Hx-PH (Supplemental video 2) was less often in SuHx severe PH model. Nuclei were counterstained with Hoechst 33342.

Supplemental video 4. 3D lineage-tracing analysis of pulmonary endothelial cells in mouse under normoxia
VE-cadherin-CreER<sup>T2</sup>; Rosa26-lsl-tdTomato mouse was treated with a single dose of tamoxifen at 5 weeks of age, leading to expression of tdTomato fluorescent protein in VE-cadherin-expressing ECs, and housed in the normoxic chamber for control until the age of 11 weeks. tdTomato-labeled cells were three-dimensionally shown.

Supplemental video 5. 3D lineage-tracing analysis of pulmonary endothelial cells in Hx-PH mouse model
VE-cadherin-CreER<sup>T2</sup>; Rosa26-lsl-tdTomato mouse was treated with a single dose of tamoxifen at 5 weeks of age and exposed to hypoxia for 3 weeks from the age of 8 weeks (Hx 3w). tdTomato-labeled cells were three-dimensionally shown. Sprouting and elongation of the tdTomato-labeled pre-existing ECs, indicating angiogenesis, was evident in Hx 3w mild PH model.

Supplemental video 6. 3D lineage-tracing analysis of pulmonary endothelial cells in SuHx-PH mouse model
VE-cadherin-CreER<sup>T2</sup>; Rosa26-lsl-tdTomato mouse was treated with a single dose of tamoxifen at 5 weeks of age, and subjected to hypoxia and treated with SU5416 for 3 weeks from the age of 8 weeks (SuHx 3w). tdTomato-labeled cells were three-dimensionally shown, and tdTomato-labeled neovessel formation from pre-existing ECs observed in Hx-PH (Supplemental video 5) was less often in SuHx 3w severe PH model.

Supplemental video 7. 3D lineage-tracing analysis of pulmonary endothelial cells in Hx-PH mouse model
VE-cadherin-CreER<sup>T2</sup>; Rosa26-lsl-tdTomato mouse was treated with a single dose of tamoxifen at 5 weeks of age and exposed to hypoxia for 3 weeks from the age of 8 weeks (Hx
3w). tdTomato-labeled cells were three-dimensionally shown. tdTomato-labeled neovessel formation from pre-existing ECs was evident in Hx 3w mild PH model.

Supplemental video 8. 3D lineage-tracing analysis of pulmonary endothelial cells in CabHx-PH mouse model

*VE-cadherin-CreERT²; Rosa26-lsl-tdTomato* mouse was treated with a single dose of tamoxifen at 5 weeks of age, and subjected to hypoxia and treated with cabozantinib for 3 weeks from the age of 8 weeks (SuHx 3w). tdTomato-labeled cells were three-dimensionally shown, and tdTomato-labeled neovessel formation from pre-existing ECs was less obvious in CabHx-PH mice compared to Hx-PH mice (Supplemental video 7).

Supplemental video 9. 3D lineage-tracing analysis of pulmonary endothelial cells in Hx-PH mouse model with vehicle

*VE-cadherin-CreERT²; Rosa26-lsl-tdTomato* mouse was treated with a single dose of tamoxifen at 5 weeks of age, and subjected to hypoxia and treated with saline (vehicle) for 3 weeks from the age of 8 weeks. tdTomato-labeled pre-existing ECs and derivatives, indicating angiogenesis, were three-dimensionally shown.

Supplemental video 10. 3D lineage-tracing analysis of pulmonary endothelial cells in Hx-PH mouse treated with baicalin, a flavonoid having a PGC-1α activation effect

*VE-cadherin-CreERT²; Rosa26-lsl-tdTomato* mouse was treated with a single dose of tamoxifen at 5 weeks of age, and subjected to hypoxia and treated with baicalin for 3 weeks from the age of 8 weeks. tdTomato-labeled pre-existing ECs were three-dimensionally shown. Note that sprouting and elongation of the tdTomato-labeled pre-existing ECs, indicating angiogenesis, was enhanced by baicalin treatment, compared to vehicle control (Supplemental video 9).

Supplemental Video 11. 3D lineage-tracing analysis of pulmonary endothelial cells in SuHx-PH mouse model with vehicle

*VE-cadherin-CreERT²; Rosa26-lsl-tdTomato* mouse was treated with a single dose of tamoxifen at 5 weeks of age, and subjected to hypoxia and treated with SU5416 and saline (vehicle) for 3 weeks from the age of 8 weeks. tdTomato-labeled cells were three-dimensionally shown.

Supplemental Video 12. 3D lineage-tracing analysis of pulmonary endothelial cells in SuHx-PH mouse model with baicalin

*VE-cadherin-CreERT²; Rosa26-lsl-tdTomato* mouse was treated with a single dose of tamoxifen at 5 weeks of age, and subjected to hypoxia and treated with SU5416 and baicalin for 3 weeks from the age of 8 weeks. Neovessel formation from pre-existing ECs was not evident as well as SuHx-PH mouse with vehicle (Supplemental Video 11)