Pulmonary Hypertension

Monocrotaline-Induced Pulmonary Hypertension Involves Downregulation of Antiaging Protein Klotho and eNOS Activity

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See Editorial Commentary, pp 1106–1107

Abstract—The objective of this study is to investigate whether stem cell delivery of secreted Klotho (SKL), an aging-suppressor protein, attenuates monocrotaline-induced pulmonary vascular dysfunction and remodeling. Overexpression of SKL in mesenchymal stem cells (MSCs) was achieved by transfecting MSCs with lentiviral vectors expressing SKL-green fluorescent protein (GFP). Four groups of rats were treated with monocrotaline, whereas an additional group was given saline (control). Three days later, 4 monocrotaline-treated groups received intravenous delivery of nontransfected MSCs, MSC-GFP, MSC-SKL-GFP, and PBS, respectively. Ex vivo vascular relaxing responses to acetylcholine were diminished in small pulmonary arteries (PAs) in monocrotaline-treated rats, indicating pulmonary vascular endothelial dysfunction. Interestingly, delivery of MSCs overexpressing SKL (MSC-SKL-GFP) abolished monocrotaline-induced pulmonary vascular endothelial dysfunction and PA remodeling. Monocrotaline significantly increased right ventricular systolic blood pressure, which was attenuated significantly by MSC-SKL-GFP, indicating improved PA hypertension. MSC-SKL-GFP attenuated right ventricular hypertrophy. Nontransfected MSCs slightly, but not significantly, improved PA hypertension and pulmonary vascular endothelial dysfunction. MSC-SKL-GFP attenuated monocrotaline-induced inflammation, as evidenced by decreased macrophage infiltration around PAs. MSC-SKL-GFP increased SKL levels, which rescued the downregulation of SIRT1 (Sirtuin 1) expression and endothelial NO synthase (eNOS) phosphorylation in the lungs of monocrotaline-treated rats. In cultured endothelial cells, SKL abolished monocrotaline-induced downregulation of eNOS activity and NO levels and enhanced cell viability. Therefore, stem cell delivery of SKL is an effective therapeutic strategy for pulmonary vascular endothelial dysfunction and PA remodeling. SKL attenuates monocrotaline-induced PA remodeling and PA smooth muscle cell proliferation, likely by reducing inflammation and restoring SIRT1 levels and eNOS activity. (Hypertension. 2016;68:1255–1263. DOI: 10.1161/HYPERTENSIONAHA.116.08184.) • Online Data Supplement

Key Words: familial primary pulmonary hypertension • genetic therapy • Klotho protein • mesenchymal stromal cells • SIRT1 protein, human

Pulmonary arterial hypertension (PAH) is a progressive disease involving an increase in pulmonary vascular resistance and pulmonary arterial (PA) pressure, which leads to right ventricular (RV) dysfunction and failure and ultimately death. Although fairly uncommon (15 cases per million), PAH is associated with high mortality (5-year survival rates ranging from 34% to 58%). Treatment is expensive and is often based on vasodilators, which relieve the symptoms but do not cure the disease. PAH pathogenesis is complex and involves multiple factors (metabolism, the immune system/inflammation, and RV function) that contribute to disease progression. Monocrotaline-induced PAH in rats involves initial endothelial dysfunction followed by rapid medial remodeling in small PAs, which leads to increased PA pressure, RV dysfunction, and death in animals at 4 to 6 weeks after insult. The monocrotaline model is associated with endothelial toxicity, endothelial dysfunction, increased endothelial permeability in small PAs, significant inflammation in lungs, overproliferation of PA-smooth muscle cells (SMCs), and PA medial remodeling. Several types of progenitor cells, such as endothelial progenitor cells, induced pluripotent stem cells, and mesenchymal stem cells (MSCs), have been investigated for the treatment of PAH. Some cell-based therapies are at various stages of preclinical or clinical trials. MSCs were reported to have anti-inflammatory and immune-modulating properties and were shown to prevent or repair endothelial injury. Thus, MSC therapy is an attractive strategy for PAH treatment because inflammation and endothelial injury play an important role in the pathogenesis of PAH. It was previously reported that MSCs improve PAH, lung pathology, RV dysfunction, PA
remodeling and impaired PA responses to vasodilators in monocrotaline models of PAH in rats. We chose to use MSCs in this study because they also have other characteristics that facilitate their application, such as their expansion potential, ease of collection, and decreased susceptibility to genetic mutations during in vitro passaging. MSCs engineered with the desired therapeutic genes may expand and have enhanced therapeutic potential. Genetically modified MSCs overexpressing genes of interest, eg, eNOS (endothelial NO synthase) or prostacyclin synthase, have also been used in the treatment of monocrotaline-induced PAH in rats. Nevertheless, MSC-based therapy has been less effective than expected.

Klotho is an antiaging gene that causes extensive premature aging phenotypes and shortens life span when disrupted and slows the aging process and extends life span when overexpressed. Klotho gene mutation causes lung inflammation and emphysema, whereas unmutated Klotho has been shown to reduce inflammation and oxidative stress in kidneys. Secreted Klotho (SKL) was reported to protect against endothelial dysfunction and endothelial cell (EC) apoptosis and attenuate vascular remodeling associated with systemic hypertension. MSCs entail secretory function. In this study, we expressed Klotho gene mutation causes lung inflammation and emphysema, whereas unmutated Klotho has been shown to reduce inflammation and oxidative stress in kidneys. Secreted Klotho (SKL) was reported to protect against endothelial dysfunction and endothelial cell (EC) apoptosis and attenuate vascular remodeling associated with systemic hypertension. MSCs entail secretory function. In this study, we engineered mouse MSCs to overexpress SKL and intravenously injected the modified MSCs into monocrotaline-treated rats. To our knowledge, this is the first study to investigate whether MSC delivery of SKL attenuates monocrotaline-induced PAH.

Materials and Methods
A detailed method section is available in the Methods and Data in the online-only Data Supplement.

Generation of MSCs Overexpressing SKL
MSCs overexpressing SKL were generated by transducing MSCs with lentiviral-based transfer of SKL gene (MSC-SKL-green fluorescent protein [GFP]) or GFP gene (MSC-GFP; see Methods in the online-only Data Supplement).

Animal Studies
Briefly, 5 groups of rats (6 rats per group) were used for the study: saline, monocrotaline, monocrotaline+MSC, monocrotaline+MSC-GFP, and monocrotaline+MSC-SKL-GFP. Four groups were given monocrotaline daily (60 mg/kg) via subcutaneous injections, whereas the last group was given saline and served as a control. After 3 days of monocrotaline injections, 3 monocrotaline-treated groups were administered MSCs, MSCs carrying enhanced green fluorescent protein (eGFP) or GFP gene (MSC-GFP; see Methods in the online-only Data Supplement).

Immunohistochemical Analysis of the Lungs
The immunohistochemistry was performed as described recently. A detailed protocol is provided in the Methods in the online-only Data Supplement.

Western Blot for SKL, SIRT1, eNOS, and p-eNOS Expression in Serum and Lung Lysates
Western blotting was performed as described previously. A detailed protocol is provided in the Methods in the online-only Data Supplement.

Cell Culture Experiments
A detailed protocol is provided in the Methods in the online-only Data Supplement.

Statistical Analysis
All data are presented as mean±SEM unless otherwise specified. Data were analyzed using 1-way ANOVA and Student t test. The Newman–Keuls procedure was used to assess the significance of differences between groups, and P<0.05 was considered statistically significant.

Results

Transfection of MSCs With the Mouse SKL Gene
A lentiviral vector construct, pLenti-SRCMVi-mouse-secreted Klotho (mSKL)-GFP (Figure 1A), was constructed in which the expression of mSKL was driven by the CMV (cytomegalovirus) major immediate-early promoter, with extra enhancers from the SV40 (simian virus 40) early promoter and RSV (rous sarcoma virus) long terminal repeat promoter and an intron from the backbone of the pAAV-MCS (Catalog #240071; Agilent Technologies, Santa Clara, CA) plasmid for enhancing expression of the mSKL gene. Besides mSKL, it also expresses an internal ribosome-entering site–mediated eGFP for easy detection of the transduction of target cells. A lentiviral vector construct, pLenti-SRCMVi-GFP, was also constructed as a control construct (Figure 1B). MSCs were in a healthy condition (Figure 1C). After transfection of 293T cells, robust expression of eGFP was observed as an indication of efficient production of infectious lentiviral vectors, and the target MSCs were transduced with ≥100% efficiency after infection of suspended cells at a multiplicity of infection of 10 to 20 (Figure 1D). Western blot analysis of SKL protein expression in MSC lysates and serum-free conditioned medium from the transduced MSCs and untransfected MSCs showed that mSKL was expressed in these cells and was also secreted into the medium (Figure 1E). The full-length Klotho protein (130 kDa) was not detectable in cell lysates and medium. Overexpression of mSKL increased SKL protein levels in cell lysates and medium (Figure 1E). Because of attachment with Flag-tag and His-tag in the C-terminal, the size of the transgenic SKL protein is ∼3 kDa larger than the endogenous SKL protein. The slightly increased size of the secreted SKL protein in the medium is likely attributed to the post-translational modifications (eg, glycosylation).

MSCs Overexpressing SKL Abolished Monocrotaline-Induced Pulmonary Vascular Dysfunction
To measure PA responses to vasodilators in our experimental model of PAH, we isolated small intralobar PA rings (third order) from the lungs at 3 weeks after monocrotaline injection and mounted them on a wire myograph (DMT) for measuring PA relaxation responses to the endothelium–dependent vasodilator ACh or the endothelium–independent vasodilator SNP. The third-order branches are regarded as resistance PAs. Compared with PAs from the saline group, PAs from the monocrotaline group had markedly decreased relaxation in response to ACh (Figure 2A),
indicating that monocrotaline impairs pulmonary vascular endothelial function. PAs from the monocrotaline+MSC and monocrotaline+MSC-GFP groups had slightly, but not significantly, better relaxation responses to ACh than PAs from the monocrotaline group. It is noteworthy that the PAs from the monocrotaline+MSC-SKL-GFP group showed a significantly greater relaxation in response to ACh, which was comparable with that of the saline group (Figure 2A).

This result suggests that implantation of MSCs overexpressing SKL rescues monocrotaline-induced pulmonary vascular endothelial dysfunction.

There was no significant difference in PA relaxation responses to the vasodilator SNP among all groups (Figure 2B), suggesting that endothelium-independent PA relaxation function is not affected significantly by administration of monocrotaline or implantation of MSCs overexpressing SKL.

MSCs Overexpressing SKL Attenuated Monocrotaline-Induced PA Remodeling

Human PAH is characterized by significant medial hyperplasia/hypertrophy in small PAs. We analyzed PA sections using hematoxylin and eosin staining, and medial hyperplasia was quantified by measuring the medial thickness and lumen area in the small third-order PAs (diameter, 50–80 µm). Monocrotaline significantly increased medial thickness and decreased lumen area (Figure 3A–3C), indicating medial hypertrophy and occlusion of small PAs. Monocrotaline-induced PA remodeling was abolished by MSC-SKL-GFPs. The monocrotaline-induced medial thickening or hypertrophy was likely because of increased proliferation of PA-SMCs because monocrotaline significantly increased expression of medial α-smooth muscle actin (Figure 3D and 3E). α-Smooth muscle actin is a marker of SMCs, and monocrotaline-induced SMC overproliferation was eliminated by MSC-SKL-GFPs. Thus, implantation of MSCs overexpressing SKL effectively rescued monocrotaline-induced PA-SMC proliferation and PA remodeling.

MSCs Overexpressing SKL Attenuated Monocrotaline-Induced PAH and RV Hypertrophy

RV systolic pressure was significantly higher in rats from the monocrotaline group than from the saline group (31.88±1.39 versus 17.27±4.86 mm Hg; Figure 4A), indicating PAH. Treatment with control MSCs slightly, but not significantly, attenuated the RV systolic pressure increase. By contrast, treatment with MSC-SKL-GFP (23.35±3.26 mm Hg) significantly attenuated the monocrotaline-induced increase in RV systolic pressure but not to the control level (Figure 4A). The ratio of RV to LV+S weights was increased significantly in the monocrotaline group compared with the saline group, indicating RV hypertrophy. Treatment with MSCs, especially
MSC-SKL-GFP, decreased RV hypertrophy in monocrotaline rats (Figure 4B). RV wall thickness, measured in hematoxylin and eosin–stained heart sections, was also used as an indicator of RV hypertrophy. Monocrotaline significantly increased RV wall hypertrophy, and treatment with MSCs slightly, but significantly, attenuated RV hypertrophy (Figure 4C). By contrast, treatment with MSC-SKL-GFP more effectively attenuated RV hypertrophy than did untransduced MSCs (Figure 4C). Thus, MSCs overexpressing SKL improved monocrotaline-induced PAH and RV hypertrophy.

MSCs Engrafted Into the Lung Parenchyma and Expressed SKL

Eighteen days after intravenous delivery of MSCs (via the right jugular vein), eGFP-positive MSCs were found in clusters or as single cells in the lung parenchyma in Optimal Cutting Temperature compound–embedded lung sections from the monocrotaline+MSC-SKL-GFP and monocrotaline+MSC-GFP groups (Figure 5A). This result suggests that MSCs homed to the lung. eGFP-Positive cells were not seen in the saline, monocrotaline, or monocrotaline+MSC groups. Unexpectedly, the transgenic MSCs did not differentiate into epithelial cells, ECs, or SMCs.

Western blot analysis showed that SKL expression was significantly decreased by monocrotaline (Figure 5B). SKL levels in the lung were significantly higher in the monocrotaline+MSC-SKL-GFP group (Figure S3). These results suggest that the MSCs secreted SKL to the lungs in a paracrine fashion and that only a minor amount of the secreted SKL entered the circulation. Thus, MSC-SKL-GFP effectively rescued the downregulation of SKL levels in the lungs in monocrotaline-treated rats.

MSCs Overexpressing SKL Attenuated Monocrotaline-Induced Inflammation in PAs and Abolished Downregulation of SIRT1 Expression and eNOS Activity in the Lungs

Immunohistochemical analysis revealed extensive infiltration of macrophages (CD68+) around small PAs and in the lungs of monocrotaline-treated rats (Figure 6A and 6B), indicating inflammation. Treatment with MSC-SKL-GFP significantly attenuated monocrotaline-induced macrophage infiltration. By contrast, MSCs or MSC-GFP did not affect macrophage infiltration significantly (Figure 6A and 6B). Thus, implantation of MSCs overexpressing SKL effectively attenuated inflammation in PAs because of monocrotaline insult. These results suggest that SKL enhances the anti-inflammatory capacity of MSCs, which contributes to its beneficial effect on pulmonary vascular dysfunction and PA remodeling.

Western blot analysis indicated that the expression level of SIRT1 (Sirtuin 1), an important deacetylase, was diminished in the lungs of all monocrotaline-treated groups (Figure 6C). Interestingly, MSC-SKL-GFP effectively restored SIRT1 expression to the control level (Figure 6C). The results suggest for the first time that MSCs overexpressing SKL regulate SIRT1 expression in the lungs of monocrotaline-treated rats. Western blot analysis showed that eNOS phosphorylation (p-eNOS) was significantly decreased in the lungs of monocrotaline-treated rats, suggesting that monocrotaline suppresses eNOS activity (Figure S4A). Interestingly,
MSC-SKL-GFP also rescued monocrotaline-induced down-regulation of eNOS activity in lungs (Figure S4A).

### SKL Prevented Monocrotaline-Induced Impairment in EC Viability and Downregulation of NO Bioavailability in HUVECs

To further explore whether SKL affects EC function, we treated HUVECs (human umbilical vein endothelial cells) with different concentrations of monocrotaline for a period of 48 hours. The MTT assay showed a dose-dependent decrease in HUVEC viability in response to monocrotaline treatment (Figure S4B and S4C). Interestingly, the addition of SKL (0.5 μg/mL) to culture medium rescued monocrotaline-induced impairment in cell viability (Figure S4D). Monocrotaline pyrrole, a metabolic product of monocrotaline, diminished NO production, as measured by the DAF-2DA assay.

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**Figure 3.** Mesenchymal stem cells (MSCs) overexpressing secreted Klotho (SKL) attenuated monocrotaline (MCT)-induced pulmonary arterial remodeling. **A**, Representative hematoxylin and eosin staining images of small pulmonary artery (PA) cross sections (60–80 μmol/L) in rat lungs. **B**, Quantitative analysis of medial thickness. **C**, Lumen area in rat small PA cross sections. **D**, α-Smooth muscle actin (α-SMA) staining using immunohistochemical in small PA cross sections. **E**, Quantification of α-SMA staining. Scale bar, 20 μm. *P<0.05 vs saline; #P<0.05 vs MCT. n=6. Data=means±SEM. GFP indicates green fluorescent protein.

**Figure 4.** Mesenchymal stem cells (MSCs) overexpressing secreted Klotho (SKL) attenuated monocrotaline (MCT)-induced pulmonary arterial hypertension and right ventricular (RV) hypertrophy. **A**, Right ventricular systolic blood pressure (RVSP). **B**, The RV/left ventricle (LV)+S weight ratio. **C**, Quantification of RV wall thickness. *P<0.05 vs saline; #P<0.05 vs MCT. n=6. Data=means±SEM. GFP indicates green fluorescent protein; and S, saline.
(4,5-diaminofluorescein diacetate) assay, at 4 days after treatment (Figure S4E). Interestingly, the addition of SKL abolished monocrotaline pyrrole–induced downregulation of NO production (Figure S4E). L-NAME (l-N\(^{G}\)-nitroarginine methyl ester) decreased NO levels (Figure S4E) and prevented an SKL-induced increase in NO production in monocrotaline pyrrole–treated HUVECs (not shown), suggesting that SKL stimulates NO production via activation of eNOS.

**Discussion**

This study demonstrates for the first time that intravenous delivery of MSCs overexpressing SKL effectively prevented...
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monocrotaline-induced pulmonary vascular endothelial dysfunction and PA remodeling. MSC-SKLS also attenuated the elevation of RV pressure and RV hypertrophy in monocrotaline-treated rats. This finding is significant because it provides a new and effective therapeutic approach for PAH, a devastating disease with no cure. By contrast, untransduced MSCs did not significantly improve pulmonary vascular endothelial dysfunction or PA remodeling. Thus, overexpression of SKL enhances the therapeutic potential of MSCs. The beneficial effects of MSCs overexpressing SKL may be achieved through increasing SKL levels in the lungs.

Klotho was originally identified as an aging-suppressor gene that is primarily expressed in kidney distal tubule epithelial cells and brain choroid plexuses. In this study, we found that SKL is also expressed in the lungs, which was diminished by monocrotaline (Figure 5). Interestingly, MSC-SKLS rescued monocrotaline-induced downregulation of SKL, suggesting that implantation of MSCs overexpressing SKL effectively increased SKL levels in the lung. MSCs have the ability to secrete paracrine factors, leading to the improvement of injured tissue.12,14,35 Although MSCs are known to secrete a variety of regulatory and trophic factors, the complete MSC secretome remains to be determined.36 This study demonstrates that the engineered MSCs exhibit paracrine activity, as the released SKL was limited to the lung (Figure 5), with a minor amount of SKL entering the circulation (Figure S3). Although Klotho deficiency has been implicated in systemic hypertension,21,37,38 this study provides the first evidence that MSCs overexpressing SKL significantly improve pulmonary vascular endothelial dysfunction and PA remodeling. These results also suggest that downregulation of SKL in the lungs contributes to monocrotaline-induced pulmonary vascular endothelial dysfunction, PA remodeling, and PAH.

MSCs are unique in possessing the potential to differentiate into other cell types and home to the injured tissue.12 They can enter the circulation and follow chemotactic gradients to home to sites of injury or inflammation, participating in wound healing and tissue repair via their regenerative and paracrine functions.39-42 We reported recently that SKL regulates adipogenic stem cell proliferation and differentiation.43 Silencing of SKL impairs adipogenic stem cell differential potential, whereas overexpression of SKL enhances adipogenic stem cell viability.33 Nevertheless, whether overexpression of SKL increases MSC therapeutic potential for PAH has never been assessed. In this study, we demonstrated that the engineered MSCs overexpressing SKL were engrafted in the lung parenchyma in monocrotaline-treated rats (Figure 5), suggesting effective homing to the injured lungs. Unexpectedly, these MSCs did not differentiate into any types of lung cells (alveolar epithelial cell type I or II), pulmonary vascular ECs, or SMCs (Figure S2). These results suggest that the beneficial effect of MSCs overexpressing SKL cannot be attributed to their regenerative capacity. Instead, this is likely mediated by their paracrine function, eg, by release of SKL.

Inflammation is a key mediator in monocrotaline-induced PA-SMC proliferation, PA remodeling, and PAH.44 Interestingly, MSCs overexpressing SKL effectively attenuated monocrotaline-induced inflammation, as evidenced by decreased infiltration of macrophages around small PAs (Figure 6). Therefore, the therapeutic effect of MSCs overexpressing SKL may be mediated, at least in part, by their anti-inflammatory effect. We reported recently that haploinsufficiency of Klotho increases the release of chemokines (eg, MCP-1 [monocyte chemotactic protein-1] and cytokines (eg, TNF-α [tumor necrosis factor-α]), leading to macrophage infiltration and inflammation in kidneys.5,7 The current study showed that overexpression of SKL enhances the anti-inflammatory effect of MSCs in PAs and lungs (Figure 6). This finding is supported by a recent report that Klotho may suppress inflammation.45 However, we cannot exclude the possibility that SKL also induces MSCs to release other paracrine factors, such as anti-inflammatory cytokines (eg, interleukin-10). This hypothesis, however, needs to be validated. Recently, SIRT1 has come to the attention of researchers in the field because it may protect against lung inflammation and PA-SMC proliferation.46-48 Resveratrol, a SIRT1 activator, has been shown to prevent or rescue monocrotaline-induced PAH in rats by reducing inflammation and oxidative stress and inhibiting PA-SMC proliferation.46-48 Inhibition of SIRT1 increased SMC proliferation,46 whereas resveratrol prevented SMC proliferation.47,48 Activation of SIRT1 protects ECs and improves endothelial function.49,50 Interestingly, we found that monocrotaline depleted SIRT1 levels in the lungs, which were rescued by MSCs overexpressing SKL (Figure 6). To our knowledge, this is the first study demonstrating that SKL regulates SIRT1 expression in vivo. Thus, it is expected that the enhanced SIRT1 levels contribute to the beneficial effect of MSCs overexpressing SKL on pulmonary vascular endothelial dysfunction and PA remodeling.

Another interesting finding is that MSCs overexpressing SKL almost rescued monocrotaline-induced downregulation of eNOS activity (Figure S4). Consistently, the pulmonary vascular relaxation response to stimulation of eNOS by acetylcholine was increased by MSCs overexpressing SKL in monocrotaline-treated rats (Figure 2), suggesting an enhanced ability of ECs to generate NO. NO not only leads to PA vasodilatation but also inhibits PA-SMC proliferation.34,44 Indeed, MSCs overexpressing SKL significantly improved pulmonary vascular endothelial dysfunction, PA-SMC proliferation, and PA remodeling. It was reported that inhalation of NO improves PAH in patients.44 Implantation of MSCs overexpressing eNOS also attenuates PAH and RV hypertrophy.15 Thus, impaired eNOS activity and NO production may be involved in the pathogenesis of PAH.34,44 We further assessed whether SKL has a direct protective effect on ECs challenged by monocrotaline. Interestingly, SKL significantly improved monocrotaline-induced impairment in EC viability (Figure S4). It is noteworthy that SKL abolished the monocrotaline-induced decrease in NO levels in ECs, suggesting for the first time that SKL rescues the downregulation of eNOS activity. Engineering MSCs for overexpressing therapeutic genes seems to be a promising strategy for the treatment of PAH. In this study, MSCs were genetically engineered to provide a source for the therapeutic protein SKL. Takemiya et al16 genetically modified MSCs to overexpress prostacyclin synthase and found that the transgenic MSCs significantly attenuated PAH-related morbidity in monocrotaline rats, whereas the untransduced MSCs failed to alleviate PAH-associated
pathogenic changes. Implantation of MSCs overexpressing eNOS attenuated monocrotaline-induced PAH. The engineered MSCs overexpressing eNOS provided moderately better treatment outcomes (RV hypertrophy and survival) compared with untransduced MSCs. Some reports indicated that the delivery of MSCs partially decreases PAH and attenuates PA remodeling. In this study, untransduced MSCs slightly, but not significantly, attenuated pulmonary vascular dysfunction, PAH, or PA remodeling (Figures 2–4). Similarly, Takemiya et al reported that untransduced MSCs failed to attenuate monocrotaline-induced PAH and PA remodeling. The variation in the treatment effect of MSCs is likely because of the disparity in the function of the allogeneic MSCs. However, the untransduced MSCs significantly improved monocrotaline-induced hypertrophy (Figure 3), suggesting that MSCs improve the RV impairment, which is independent of PAH. The untransduced MSCs may repair heart damage via their paracrine function.

Perspectives
PAH is a life-threatening disease with high mortality. The 5-year survival rate ranges from 34% to 58%, and the current therapy relieves only the symptoms and does not cure the disease. In this study, we demonstrated that MSCs overexpressing SKL effectively attenuated monocrotaline-induced PA endothelial dysfunction, PAH, PA remodeling, and RV hypertrophy. SKL augments the therapeutic effects of MSCs in PAH by (1) decreasing inflammation and rescuing downregulation of SIRT1 expression and eNOS activity, and (2) improving EC survival and function. This is, to our knowledge, the first report demonstrating the great efficacy of Klotho gene-engineered MSCs for the treatment of PAH.

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Z.S. helped design concept; R.V., Q.A., and C.W. helped perform experiments; and Z.S. and R.V. drafted the manuscript.

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Disclosures
None.

References
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Novelty and Significance

What Is New?

- It is new and interesting that mesenchymal stem cell delivery of aging-suppressor protein secreted Klotho (SKL) abolished monocrotaline-induced pulmonary vascular endothelial dysfunction, pulmonary arterial (PA) hypertension, and right ventricular hypertrophy.

- This study demonstrates, for the first time, that SKL attenuates monocrotaline-induced PA remodeling and PA smooth muscle cell proliferation, likely by reducing inflammation and restoring SIRT1 (Sir2uin1) levels and eNOS (endothelial NO synthase) activity.

What Is Relevant?

- It is significant that a decrease in lung SKL is associated with pulmonary vascular endothelial dysfunction, PA remodeling, and PA hypertension.

This study reveals that stem cell delivery of SKL is an effective therapeutic strategy for pulmonary vascular endothelial dysfunction and PA remodeling.
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Online Supplemental Methods

Construction and packaging of lentiviral vector overexpressing mSKL

To construct a lentiviral vector overexpressing the mouse SKL (mSKL) gene, the SR-CMV promoter from the pSR-CMV-SV-N2A-GFP-polyA (PMID: 20410313) plasmid was PCR amplified with the primers (F) 5’-TTCTAGACGCGTGGCCTGAAATAACCTCT-3’ and (R) 5’-CCTCGAGGATCAGATCGGAATTCCGGCGCCT-3’. The PCR product was digested with Xba I and Xho I and then ligated into the backbone plasmid pHR-cPPT-hB.7-SIN1 digested with the same restriction enzymes. The resulting plasmid was first completely digested with BamH I and then partially digested with Nde I. A fragment containing part of the CMV promoter and the coding sequence of the mSKL gene was released from the pAAV-mSKL plasmid (previously constructed in our lab)² by first completely digesting with Nde I and then partially digesting with Bgl II. Digested DNAs were separated through agarose gel electrophoresis, extracted, and ligated. The desired recombinant plasmids were then identified through colony PCR using primers specific for the mSKL gene. Extra-pure plasmid DNA was extracted using the E.Z.N.A.® Plasmid Mini Kit I (Omega Bio-Tek) and used for packaging of infectious lentiviral vectors through a transient co-transfection method as previously described.³ Passage 3–4 mouse MSCs (Invitrogen) were subsequently transduced at a multiplicity of infection (MOI) of 10–20.

Animal studies

The experimental protocol was approved by the Institutional Animal Care and Usage Committee of the University of Oklahoma Health Sciences Center. Six- to eight-week-old male Sprague Dawley rats (Harlan Laboratories) were acclimatized for a week at the animal facility. Five groups of rats (six rats per group) were established for the study: Saline, MCT, MCT + MSC, MCT + MSC-GFP and MCT + MSC-SKL-GFP. Four groups were given monocrotaline (MCT) daily (60 mg/kg) via subcutaneous injections, while one group was given saline and served as a control. After 3 days of MCT injections, three MCT-treated groups were administered MSCs, MSCs carrying eGFP (MSC-GFP), and MSCs carrying eGFP-SKL (MSC-SKL-GFP), respectively, via injection into the right jugular vein (3.5 x 10⁶ cells/rat), while one MCT-treated group received no treatment and served as a control. Twenty-one days post MCT injection, RV pressures were measured under anesthesia before the animals were euthanized for tissue collection. The scheme of the experimental protocol is shown in Supplemental Figure 1.

Measurement of right ventricular systolic pressure (RVSP)

Twenty-one days post MCT injection, rats were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg). An incision approximately 3 cm in length
was made, and the trachea was exposed. PE 240 tubing was guided into the trachea and connected to a ventilator (Harvard Apparatus, MA) to control respiration. Animals were placed on a heating pad for maintaining body temperature at 39°C. The chest cavity was opened to expose the heart, and the apex of the right ventricle was localized and held firmly by a pair of blunt forceps. A PA-C40 transmitter (Data Sciences International, MN) was carefully introduced into the right ventricle using a 25-gauge needle. After a stable pressure was obtained and recorded, the rats were euthanized, and PA, heart, and lungs were collected for further analysis as described below.

**Pulmonary vascular responses to vasodilators**

Small intralobar 3rd-order PA rings (2 mm in length) were isolated from euthanized rats and mounted on a wire myograph system (DMT, MI). The PA rings were incubated in physiological saline solution and aerated with 5% CO2 and 95% O2. The vessels were normalized to a resting tension equivalent to 20–29 mmHg, equilibrated for at least 60 minutes, and challenged three times with 60 mM K+ solution before starting experiments for testing relaxation responses to vasodilators. PA rings were pre-constricted with phenylephrine before measuring PA relaxation in response to cumulative doses of acetylcholine (ACh) or sodium nitroprusside (SNP). Data is presented as the percentage relaxation of phenylephrine-induced pre-constriction.

**Determination of RV hypertrophy**

Hearts were weighed before RVs were dissected from left ventricles and septa (LV+S). RV and LV+S weights were measured separately, and RV hypertrophy was assessed by the ratio of RV/LV+S per g body weight. Some of the animals were perfused with saline, and hearts were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, sectioned at a 5-µM thickness, and stained with H&E. The width of the right ventricular wall was measured at the thickest point in each transverse section and used as a measure of RV hypertrophy.

**Immunohistochemical (IHC) analysis of the lungs**

The IHC was performed as described recently.4-6 Lungs from saline-perfused rats were isolated and fixed in 4% PFA or in optimal cutting temperature (OCT) embedding compound (Tissue-Tek, CA). To look at innate eGFP fluorescence in lung tissue, 10-µm-thick cryo-sections of OCT-embedded lungs were thawed at room temperature (RT) for 10 minutes, fixed in 10% neutral buffered formalin (NBF) for 20 minutes, stained with DAPI mounting solution (Santa-Cruz), and observed under a fluorescence microscope (Olympus IX73). For immunofluorescence labeling with epithelial, smooth muscle cells (SMCs) and EC antibodies, NBF-fixed cryo-sections were incubated in 1% SDS for 5 minutes for antigen retrieval and in 3% H2O2 for 15 minutes. After blocking for 1 hour at RT with 5% BSA, the following primary
antibodies were used: EC marker vWF (Abcam, 1:100), SMC marker α-SMA (Abcam, 1:100), alveolar epithelial type I cell marker PDX7 (Santa Cruz), alveolar epithelial type II cell marker LB-180 (BioLegend), GFP antibodies, and Rabbit Alexa Fluor 488 (Life Technologies 1:100), and mouse IgG (Santa Cruz, 1:100). Sections (5-µM thick) from paraffin-embedded lung tissues were used for H&E staining for morphometric analysis of PA medial thickness and lumen area and for IHC staining of CD68 (Abcam, 1:100) and α-SMA (Abcam, 1:500).

**Western blot for SKL, SIRT1, eNOS, and p-eNOS expression in serum and lung lysates**

Western blotting was performed as we described previously. Lungs were lysed in RIPA buffer containing protease inhibitors. Protein concentrations were measured using a bicinchoninic acid (BCA) assay. Serum samples were diluted 20 fold before adding loading buffer and reducing agents. An equal amount of total protein was loaded for each sample. Primary antibodies were used against SKL (R&D, 1:200), SIRT1 (Abcam, 1:500), eNOS (BD Biosciences, 1:1000), p-eNOS (Millipore, 1:500), and β-actin (Abcam, 1:10000) as loading control. Ponceau-stained membrane was used to quantify total protein as a loading control for serum samples.

**Cell culture experiments**

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza, and passage 3–5 cells were used for viability assays using MTT (Roche). HUVECs were cultured in 2% serum medium EGM-2 (Lonza), seeded at a density of 5000 cells per well in a 96-well plate, and allowed to attach overnight. Various concentrations of MCT were added to see dose-dependent effect on HUVEC viability, and 1 mg/ml MCT and 0.5 µg/ml SKL were added at the same time to see the protective effect of SKL on MCT-induced cell death. Five replicates were assayed for each experimental condition. Two days after adding MCT and SKL, MTT reagent was added, and the viability assay was performed following the manufacturer’s instructions.

The same experimental conditions were used for the DAF-2DA assay for NO production in HUVECs (see below).

**DAF-2DA assay for NO production**

The concentrations used for MCT and SKL were 5 µg/ml and 2 µg/ml, respectively. Both MCT and SKL were added at the same time. After 4 days, cells were washed and incubated in serum-free EBM medium (Lonza) for 1 hour and in 1 µM DAF-2DA for 15 minutes at room temperature. Following this procedure, cells were washed twice with PBS and then fresh EBM medium was added. Mean fluorescence intensity at 515-nm excitation wavelength was measured using a Biotek microplate reader 60 minutes after removing DAF-2DA from the
cells. After measuring fluorescence, images were acquired using an Olympus iX73 inverted fluorescence microscope. To confirm eNOS-specific NO production, 100 µM L-NAME (Sigma-Aldrich) was added along with MCT and SKL for 4 days, followed by incubation with DAF-2DA for 15 minutes.

References

Online Supplemental Data

5. Groups
1. Control (CT)
2. Monocrotaline (MCT)
3. Monocrotaline + MSC (MCT+MSC)
4. Monocrotaline + MSC + GFP (MCT+MSC-GFP)
5. Monocrotaline + MSC + sKlotho (MCT+MSC-SKL-GFP)

Male SD Rats

![Graph showing the experimental design with days of study and groups](image)

**Figure S1. Experimental design.**
Figure S2. SKL-GFP-transfected MSCs do not differentiate into epithelial, endothelial, or smooth muscle cell types in vivo in the lungs of MCT + MSC-SKL-GFP rats.

A) Immunofluorescent staining with endothelial marker vWF (green) and GFP (red) antibodies.

B) Immunofluorescent staining with smooth muscle cell marker α-SMA (red) and GFP (green) antibodies.

C) Immunofluorescent staining with the alveolar epithelial type I cell marker P2X7 (red) and GFP (green) antibody.

D) Immunofluorescent staining with alveolar epithelial type II cell marker LB-180 (red) and GFP (green) antibodies.
Figure S3. Western blot analysis of SKL levels in serum.
Figure S4. SKL prevented MCT-induced downregulation of eNOS activity in the lungs and nitric oxide bioavailability in HUVECs.

A) Western blot analysis of eNOS and p-eNOS in lung lysates. *p<0.05, **p<0.01, ***p<0.001 vs. Saline; #p<0.05 vs. MCT; n=6. Data = means±SEM. B) Photomicrographs of HUVECs under different MCT concentrations. C) MTT assay for HUVEC viability under different MCT concentrations. **p<0.05, **p<0.001 vs. Control. D) MTT assay for HUVEC viability under treatment with MCT and MCT plus SKL. *p<0.05 vs. Control. E) DAF-2DA assay for NO production in HUVECs under treatment with MCT and SKL. **p<0.05 vs. HUVEC. Triplicates were used in each experiment, and each experiment was repeated three times. Data = means±SEM.