Endothelial-Like Progenitor Cells Engineered to Produce Prostacyclin Rescue Monocrotaline-Induced Pulmonary Arterial Hypertension and Provide Right Ventricle Benefits

Running title: Zhou et al.; Prostacyclin-producing ELPCs rescue PAH

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Abstract:

**Background**—Intravenous prostacyclin is approved for treating pulmonary arterial hypertension (PAH), but it has a short half-life and must be delivered systemically via an indwelling intravenous catheter. We hypothesize that localized jugular vein delivery of prostacyclin-producing cells may provide sustained therapeutic effects without the limitations of systemic delivery.

**Methods and Results**—We generated a vector expressing a human cyclooxygenase isoform 1 (COX1) and prostacyclin synthase (PGIS) fusion protein that produces prostacyclin from arachidonic acid. Endothelial-like progenitor cells (ELPCs) were transfected with the COX1-PGIS plasmid and labeled with lentivirus expressing nuclear-localized red fluorescent protein (nuRFP). The engineered ELPCs (expressing COX1-PGIS and nuRFP) were tested in rats with monocrotaline (MCT)-induced PAH. In PAH prevention studies, treatment with engineered ELPCs or control ELPCs (expressing nuRFP alone) attenuated MCT-induced right ventricular systolic pressure (RVSP) increase, right ventricular (RV) hypertrophy, and pulmonary vessel wall thickening. Engineered ELPCs were more effective than control ELPCs in all variables evaluated. In PAH reversal studies, engineered ELPCs or control ELPCs increased the survival rate of rats with established PAH and decreased RV hypertrophy. Engineered ELPCs provided a survival benefit 2 weeks earlier than did control ELPCs. Microarray-based gene ontology analysis of the right ventricle revealed that a number of MCT-altered genes and neurotransmitter pathways (dopamine, serotonin, and gamma-aminobutyric acid [GABA]) were restored after ELPC-based prostacyclin gene therapy.

**Conclusions**—COX1-PGIS–expressing ELPCs reversed MCT-induced PAH. A single jugular vein injection offered survival benefits for at least 4 weeks and may provide a promising option for PAH patients.

**Key words**: prostacyclin, gene therapy, right ventricle, hypertrophy, microarray, neurotransmitter pathways, bone marrow-derived cells
Pulmonary arterial hypertension (PAH) is a progressive disorder characterized by abnormally high blood pressure in the pulmonary artery, right ventricular (RV) overload, and, eventually, right heart failure leading to death. The estimated median survival of patients diagnosed with idiopathic PAH is less than 3 years. The only treatment option that has been shown to increase the survival rate is the continuous systemic administration of prostacyclin; the median survival rate increases from 2.8 years in untreated patients to 5 years in treated patients. However, systemic prostacyclin therapy has serious limitations, including a short half-life and the need for a permanent intravenous catheter. Other treatment options are needed. One alternative being studied is cell-based gene therapy using bone marrow-derived endothelial-like progenitor cells (ELPCs) that express endothelial nitric oxide synthase (eNOS). However, unlike synthetic prostacyclin and its analogs, eNOS treatment is not an established therapeutic approach and has not been included in the PAH treatment algorithm used by clinicians. In the present study, we hypothesize that prostacyclin-producing ELPCs, delivered via the jugular vein, may provide sustained therapeutic effects without the limitations associated with systemic delivery of prostacyclin. Because prostacyclin is produced from arachidonic acid in a serial fashion by the enzymes cyclooxygenase (COX) and prostacyclin synthase (PGIS), we have generated a non-viral vector expressing a human COX isoform 1-PGIS fusion protein. The fusion enzyme is anchored across the membrane of the endoplasmic reticulum via a 10-amino acid linker so that it increases prostacyclin production more efficiently than the 2 enzymes expressed separately.

Autologous bone marrow-derived cell therapy has been shown to improve left ventricular function, infarct size, and variables of remodeling. However, the mechanism(s) underlying the benefits of cell therapy is not well understood, but paracrine factors produced by the transplanted cells appear to contribute to the improvement in heart function. This understanding comes from
studies of the treatment of a compromised left ventricle (as occurs in ischemic heart disease, chronic heart failure, and dilated cardiomyopathy); however, when bone marrow-derived cells are used to treat a compromised right ventricle (as in patients with PAH), the mechanism(s) may be different.

In this study, we sought to examine the benefits associated with ELPC-based prostacyclin gene therapy in the treatment of PAH. First, we generated engineered ELPCs that expressed both the COX1-PGIS fusion protein and nuclear-localized red fluorescent protein (nuRFP). Then, using a monocrotaline (MCT)-induced rat model of PAH, we examined the ability of these engineered ELPCs to prevent or reverse PAH (ie, cell treatments administered before or after development of PAH, respectively). Finally, the potential mechanism(s) underlying RV benefit from bone marrow-derived ELPC-based prostacyclin gene therapy was explored by using genome gene expression analysis.

Methods

We isolated bone marrow mononuclear cells (BMMNCs) from syngeneic Fisher 344 rats and labeled the cells using lentivirus expressing nuRFP for cell tracking. Lentivirus encoding nuRFP was generated in a manner similar to that described previously, except that nuRFP expression was driven by a constitutive cytomegalovirus (CMV) promoter. Engineered BMMNCs that expressed both nuRFP and the COX1-PGIS fusion protein were generated by transfecting BMMNCs with a COX1-PGIS plasmid (CP) containing a G418 resistance gene for selection (Figure 1A) and a lentivirus encoding nuRFP (Figure 1B). After nuRFP flow-sorting and G418 selection, the cells underwent endothelial induction to generate engineered ELPCs (ie, ELPCs-nuRFP/CP, Figure 1C). The ELPCs expressing nuRFP alone (ELPCs-nuRFP) were used as a
control. Engineered ELPCs were characterized by the presence of an endothelial phenotype, COX1-PGIS fusion protein expression, and prostacyclin production. All animal studies were performed in accordance with the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at Houston, Texas.

Monocrotaline (60 mg/kg; Sigma-Aldrich Corporation, St. Louis, MO) was intraperitoneally injected into 6-week-old male Fisher 344 rats. Either 3 days (prevention study) or 21 days (reversal study) after MCT injection, we infused 1.5x10^6 engineered ELPCs (ELPCs-nuRFP/CP) or control ELPCs (ELPCs-nuRFP) via the jugular vein. Analysis was performed at either 25 days (prevention study) or at 4 weeks (reversal study) after cell transplantation. Lung cell retention was calculated as previously described.5 We analyzed the tissue distribution of the COX1-PGIS transgene by using polymerase chain reaction (PCR) and used immunofluorescence to analyze COX1-PGIS fusion protein expression in the transplanted engineered ELPCs, pulmonary vessel wall thickness, cell proliferation, and apoptosis. Methods for measuring right ventricular systolic pressure (RVSP) and assessing RV hypertrophy were described previously.5 Right ventricle genome gene expression was assessed by using Affymetrix Rat Genome 230 2.0 arrays (Affymetrix, Santa Clara, CA). Data analysis was performed as described previously.10 In the reversal study, survival rates were compared by using the log-rank test in the SPSS 11.0 software (IBM Corporation, Somers, NY). Data were expressed as the mean ± standard error of the mean (SEM). The Kolmogorov-Smirnov test was used to verify normal distribution of the variables. Statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS 11.0 software. A P < 0.05 was considered statistically significant. See Online Data Supplement for details.
Results

Engineered ELPCs Exhibited an Endothelial Phenotype, Expressed the COX1-PGIS Protein, and Produced Prostacyclin

Flow cytometry analysis showed that control ELPCs and engineered ELPCs expressed high levels of the endothelial markers KDR (63.9% and 67.7%), Tie2 (74% and 77.4%), and CD31 (43.7% and 52.9%), but very low levels of the macrophage marker CD14 (3.88% and 3.54%) and the leukocyte marker CD45 (0.58% and 0.27%) (Figure 1D). Both control ELPCs and engineered ELPCs expressed low levels of the hematopoietic precursor marker CD34 (1.28% and 2.07%) after endothelial induction (Figure 1D). Lentivirus labeling and COX1-PGIS gene transfection did not alter the endothelial induction potential of BMMNCs (Figure 1D). Moreover, control ELPCs and engineered ELPCs were able to internalize acetylated low-density lipoprotein (AcLDL) and expressed the Tie2 receptor, both of which are characteristic of endothelial cells (Figure 2A). The engineered cells expressed COX1-PGIS fusion protein in the cytoplasm and the perinuclear region, which differs from the predominantly cytoplasmic expression patterns of endogenous PGIS and COX1 (Figure 2B). Production of prostacyclin by the engineered ELPCs was confirmed by showing that the level of 6-keto prostaglandin F₁α (PGF₁α), the stable metabolite of prostacyclin, was higher in the engineered ELPCs than in the control ELPCs expressing nuRFP alone (mean fold change over expression in the nontransfected ELPCs, 2.20±0.10 vs 0.98±0.05, P < 0.01, Figure 2C). The results were normalized to the 6-keto PGF₁α level in the nontransfected ELPC group, which was set as 1.0.

Engineered ELPCs Resided Primarily in the Lungs after Cell Transplantation via Jugular Vein Infusion and Produced Prostacyclin In Vivo

Twenty-four hours after cell infusion, 84.0±6.1% of the engineered ELPCs were retained in the
lungs. Cell retention in the lungs remained high throughout the study: 79.9±2.7% at 72 hours after infusion, 70.4±4.6% at 1 week, 62.4±5.7% at 2 weeks, 57.6±6.0% at 3 weeks, and 48.3±1.9% at 25 days (Figure 3A). Our PCR analysis showed that the COX1-PGIS transgene was localized mainly in the lungs 25 days after cell transplantation. Very faint signals were detected in the liver and kidney (Figure 3B). Nuclear red fluorescent signals indicated that most of the transplanted cells localized in the lung parenchyma at 25 days after cell transplantation (Figure 3C, higher magnification shown in Supplemental Figure S1). Some transplanted cells integrated into the pulmonary vessel wall (Figure 3D), but intimal integration was rarely observed. Seven days after the engineered ELPCs were transplanted, in vivo COX1-PGIS fusion protein expression was detected in the engineered ELPCs by using an antibody that specifically recognizes human PGIS (Figure 3E), and a significant increase in prostacyclin production (determined by 6-keto PGF1α, the stable metabolite of prostacyclin) was found in the blood (Figure 3F). Control ELPC-treated and engineered ELPC-treated groups showed no significant increases in blood levels of alanine aminotransferase (ALT), blood urea nitrogen (BUN), or creatinine (CRE), as compared with the vehicle-treated group (Supplemental Figure S2), indicating that ELPC-based prostacyclin gene therapy did not impair hepatocellular or renal function.

Engineered ELPCs Attenuated the RVSP Increase and RV Hypertrophy Induced by MCT

We measured RVSP at different times after MCT injection (Supplemental Figure S3) and found that RVSP was normal at 3 days after injection, but significantly increased at days 7 through 14, reaching a plateau thereafter. We used these data to establish time points for cell infusions in the prevention and reversal studies (3 and 21 days, respectively).

In the prevention study, MCT induced a significant (97%) increase in RVSP as compared
with that in normal, untreated controls ($P < 0.01$, Figure 4A). This increase in RVSP was significantly attenuated by treatment with either control ELPCs (56% vs 97%, $P < 0.01$) or ELPCs engineered to express the COX1-PGIS transgene (28% vs 97%, $P < 0.01$), but engineered ELPCs were significantly more effective than control ELPCs (28% vs 56%, $P < 0.01$; Figure 4A).

Monocrotaline induced a significant (35%) increase in the weight ratio of the right ventricle to left ventricle plus interventricular septum (RV/[LV + IVS]) as compared with that in normal, untreated controls ($P < 0.01$, Figure 4B), indicating the development of RV hypertrophy in rats with MCT-induced PAH. The increase in RV/(LV + IVS) weight ratio was attenuated by treatment with either control ELPCs (21% vs 35%, $P < 0.01$) or ELPCs engineered to express the COX1-PGIS transgene (11% vs 35%, $P < 0.01$; Figure 4B), but engineered ELPCs were significantly more effective than control ELPCs (11% vs 21%, $P < 0.05$; Figure 4B).

Supplemental Table S1 presents the mean RVSP values and RV/(LV + IVS) weight ratios in the prevention study.

Engineered ELPCs are Comparable to the Combined Use of Control ELPCs and the Synthetic Prostacyclin Analog Treprostinil in Preventing RVSP Increase and RV Hypertrophy

Treprostinil (TP; United Therapeutics Corporation, Silver Spring, MD) is a synthetic prostacyclin analog commonly used for the treatment of PAH. We compared the efficacy of individual therapies (control ELPCs, engineered ELPCs, and TP) and combined therapies in preventing MCT-induced PAH. For these experiments, both control and engineered ELPCs were administered as a single dose ($1.5 \times 10^6$ cells) at 3 days after MCT injection. To determine the appropriate TP dosing regimen to be used with cell therapy, we first compared 3 dosing regimens
of TP administered subcutaneously: 1) 0.05 mg/kg body weight, twice daily; 2) 0.15 mg/kg body weight, twice daily; and 3) 0.5 mg/kg body weight, once daily. Both twice-daily regimens were highly effective at preventing the MCT-induced increase in RVSP (34.8±1.7 mmHg and 30.6±1.6 mmHg vs 51.9±2.2 mmHg, \( P < 0.01 \); Supplemental Figure S4). The once-daily regimen was less effective (43.5±3.9 mmHg vs 51.9±2.2 mmHg, \( P < 0.05 \); Supplemental Figure S4) and was chosen as a “sub-optimal” dosing scheme for TP use with cell therapy. This strategy allowed us to detect improvements with the combined therapies above the basal treatment effects.

We found that the RVSP increase (51.9±2.2 mmHg) was significantly attenuated by the once-daily subcutaneous TP treatment (43.5±3.9 mmHg, \( P < 0.05 \)), the control ELPC treatment (41.0±1.5 mmHg, \( P < 0.01 \)), and the engineered ELPC treatment (33.7±1.6 mmHg, \( P < 0.01 \)) (Supplemental Table S1). Right ventricular hypertrophy (0.36±0.01) was also significantly prevented by the once-daily TP treatment (0.29±0.01, \( P < 0.01 \)), the control ELPC treatment (0.32±0.01, \( P < 0.01 \)), and the engineered ELPC treatment (0.30±0.01, \( P < 0.01 \)) (Supplemental Table S1). The efficacy of once-daily TP in preventing an RVSP increase was comparable to that of control ELPCs (43.5±3.9 mmHg vs 41.0±1.5 mmHg, \( P = 0.394 \)), but less than that of engineered ELPCs (43.5±3.9 mmHg vs 33.7±1.6 mmHg, \( P < 0.01 \)) (Supplemental Table S1). The efficacy of the engineered ELPC treatment was similar to the combination of control ELPCs plus once-daily TP in preventing RVSP increase (33.7±1.6 mmHg vs 29.1±2.7 mmHg, \( P = 0.086 \)) and RV hypertrophy (0.30±0.01 vs 0.28±0.01, \( P = 0.143 \)) (Supplemental Table S1). In contrast, the combined therapy of engineered ELPCs plus once-daily TP showed an increased efficacy over that of the engineered ELPCs alone in preventing an RVSP increase (23.3±1.5 mmHg vs 33.7±1.6 mmHg, \( P < 0.01 \)) and RV hypertrophy (0.24±0.01 vs 0.30±0.1, \( P < 0.01 \))
(Supplemental Table S1).

**Engineered ELPCs Reduced MCT-induced Pulmonary Vessel Wall Thickening**

Monocrotaline treatment significantly increased pulmonary vessel wall thickness, as compared with that in normal, untreated controls (Figure 5A). The calculation of pulmonary vessel thickness index (VTI) reflected both intimal thickening and medial smooth muscle hypertrophy (Figure 5B). The MCT-induced increase in VTI was significantly reduced by treatment with either control ELPCs (86% vs 108%, \( P < 0.01 \); Figure 5C) or ELPCs engineered to express the COX1-PGIS transgene (67% vs 108%, \( P < 0.01 \); Figure 5C), but engineered ELPCs were significantly more effective than control ELPCs (67% vs 86%, \( P < 0.01 \); Figure 5C).

**Supplemental Table S2** shows the mean pulmonary VTI for each group.

**Engineered ELPCs Reduced MCT-induced Cell Proliferation in the Intimal and Medial Smooth Muscle Layer and Enhanced Adventitial Cell Apoptosis in the Pulmonary Vessel Walls**

We used Ki-67 immunostaining to analyze cell proliferation in the pulmonary vessel walls (Figure 6A). Monocrotaline treatment significantly increased cell proliferation in the intimal and medial smooth muscle layers of the pulmonary vessel wall as compared with that in normal controls (Ki-67+ cells per pulmonary vessel section, 0.67±0.05 vs 0.07±0.01, \( P < 0.01 \); Figure 6B). The increase in cell proliferation was significantly reduced by treatment with either control ELPCs (0.22±0.01 vs 0.67±0.05, \( P < 0.01 \); Figure 6B) or with ELPCs engineered to express the COX1-PGIS transgene (0.10±0.01 vs 0.67±0.05, \( P < 0.01 \); Figure 6B), but engineered ELPCs were significantly more effective than control ELPCs (0.10±0.01 vs 0.22±0.01, \( P < 0.05 \); Figure 6B).

Normally, apoptosis is rarely detected in pulmonary vessel walls. Four weeks after MCT
injection, cell apoptosis significantly increased in the pulmonary vessel walls, primarily occurring in the adventitial layer (TUNEL+ cells per pulmonary vessel section, 0.52±0.03 vs. 0, P < 0.01) (Supplemental Figure S5). Control ELPCs or engineered ELPCs significantly enhanced adventitial cell apoptosis 25 days after cell transplantation as compared with the vehicle control (control ELPCs, 0.90±0.05 vs. 0.52±0.03, P < 0.01; engineered ELPCs, 0.91±0.04 vs. 0.52±0.03, P < 0.01) (Supplemental Figure S5), although the overall levels were still low.

**Engineered ELPCs Provided Survival Benefit for Rats with Established PAH Along With a Decrease in RV Hypertrophy**

The reversal studies were designed to mimic the treatment for PAH patients in that cell treatments were delivered 21 days after MCT injections, when PAH had fully developed in the rats. An increase in survival rate was detected as early as 2 weeks after cell transplantation in rats treated with engineered ELPCs (P = 0.023, Figure 7A), which was 2 weeks earlier than when benefits were seen in control ELPC-treated rats (P = 0.012, Figure 7B). The survival rate in both cell-treated groups at 4 weeks after cell transplantation was significantly higher than that in the vehicle-treated group (73.3% for control ELPC-treated group and 66.7% for engineered ELPC-treated group vs 30% for vehicle-treated group, P < 0.02, Figures 7A and 7B). The survival benefit was accompanied by a decrease in the RV/(LV + IVS) weight ratio (control ELPCs vs vehicle: 182% vs 242%, P < 0.01; engineered ELPCs vs vehicle: 159% vs 242%, P < 0.01; Figure 7C). However, there was no significant decrease in RVSP in either cell-treated group as compared with the vehicle-treated group (Figure 7D). The mean RVSP values and RV/(LV + IVS) weight ratios (reversal study) are presented in Supplemental Table S3.

**Genome Gene Expression Array Revealed Potential Molecular Basis of Right Ventricle**
Benefits from ELPC-based Prostacyclin Gene Therapy

Our strategy for gene-expression screening was based on the assumption that the target genes and pathways would be altered (upregulated or downregulated) by MCT exposure and that these changes in expression levels would be counteracted by treatment with control or engineered ELPCs. Because both control ELPC and engineered ELPC treatment prevented or reversed RV hypertrophy by 25 days (prevention study, Figure 4B) or 28 days (reversal study, Figure 7C) after cell transplantation and decreased the mortality rates as early as 2 weeks after cell transplantation (Figures 7A and 7B), we assumed that the mechanism(s) underlying these improvements occurred earlier. Therefore, we assessed gene expression levels at 2 weeks post-MCT injection (ie, 11 days after cell transplantation). At that time, the global gene expression pattern in the right ventricle was extensively altered, with or without cell transplantation (Figure 8A). Of the genes that were upregulated by MCT exposure (relative to the normal group), 31 were downregulated by treatment with either control or engineered ELPCs (relative to the vehicle-treated group) (Figure 8B, cluster b), whereas 42 were specifically downregulated by treatment with engineered ELPCs (relative to the vehicle-treated group) (Figure 8B, clusters a and c). Of the genes that were downregulated by MCT exposure (relative to the normal group), 43 were upregulated by treatment with either control or engineered ELPCs (relative to vehicle-treated group) (Figure 8C, clusters d and f), whereas 39 were specifically upregulated by treatment with the engineered ELPCs (relative to vehicle-treated group) (Figure 8C, cluster e).

Likewise, gene ontology (GO) analysis revealed that the pathways that were altered by MCT exposure could be restored by treatment with either control or engineered ELPCs (Supplemental Table S4). Of the MCT-upregulated pathways (relative to the normal group), mitotic sister chromatid segregation was restored by treatment with either control or engineered
ELPCs (relative to vehicle-treated group), whereas steroid delta-isomerase activity, dopamine transporter activity, negative regulation of adenylate cyclase activity, and sodium channel regulator activity were restored only by treatment with engineered ELPCs (relative to the vehicle-treated group) (Supplemental Table S4). Of the MCT-downregulated pathways (relative to the normal group), protein-arginine deiminase activity, gamma-aminobutyric acid (GABA)-β receptor activity, serotonin receptor signaling pathway, and melatonin receptor activity were restored by treatment with both control and engineered ELPCs (relative to vehicle-treated group), whereas cyclin-dependent protein kinase activity, pyruvate kinase activity, dopamine receptor activity, and the serotonin receptor adenylate cyclase activating pathway were restored only by treatment with engineered ELPCs (relative to vehicle-treated group) (Supplemental Table S4). For both the gene expression and gene ontology analyses, the changes that occurred after treatment with the engineered ELPCs, but not with the control ELPCs, were assumed to be due to prostacyclin, whereas those that occurred after treatment with either the control or the engineered ELPCs were assumed to be due to the bone marrow-derived ELPCs.

Discussion

In the present study, we have shown for the first time that ELPCs engineered to express a COX1-PGIS fusion protein can prevent and reverse PAH. Treatment with COX1-PGIS–expressing ELPCs combined the therapeutic effects of prostacyclin and ELPCs. A single dose of 1.5x10⁶ engineered ELPCs delivered via the jugular vein offered sustained therapeutic effects for at least 25 days in our model of PAH prevention and for 4 weeks in our model of PAH reversal. Moreover, in this model of right heart failure, treatment with control or engineered ELPCs significantly improved the survival rate. Additionally, we show for the first time that the
potential mechanism(s) responsible for these therapeutic effects may be the restoration of a number of MCT-altered genes (Adcyap1r1, Clla4, Tead1, Ncoa2, Tln2, Stk11, Fgfr2, Gpr50, etc.) and neurotransmitter pathways (dopamine, serotonin, and GABA) in the right ventricle. Several different gene and cell-based therapies have been proposed for treating patients with PAH, and cell therapy using endothelial progenitor cells has been shown to provide benefits in animal models.\textsuperscript{5,11,12} Therapeutic approaches have included the use of the PGIS gene alone\textsuperscript{13,14} or ELPCs transfected with the eNOS gene.\textsuperscript{5} The latter approach was effective in rats with MCT-induced PAH,\textsuperscript{5} producing results similar to those described here for our COX1-PGIS–expressing ELPCs. The use of eNOS-transfected ELPCs is being studied in a clinical trial (ClinicalTrials.gov Identifier: NCT00469027). We believe that our COX1-PGIS–expressing ELPCs could offer a complementary alternative to the eNOS-expressing ELPCs, as the 2 approaches address 2 distinct, fundamental biochemical pathways that are defective in PAH patients. Drugs designed to target the nitric oxide pathway, such as the PDE-5 inhibitor, sildenafil, are approved for treating PAH patients, as are prostacyclin-replacement drugs, such as epoprostenol. These drugs are used independently or in combination, and individual patients respond differently to each drug.\textsuperscript{15} Thus, providing options offers possible benefits to more patients.

In the present study, we improved the preparation of engineered ELPCs as compared to the approach used in the eNOS gene therapy study in 2 ways.\textsuperscript{5} First, we used a lentivirus encoding nuRFP to genetically label the cells; this approach allows for long-term tracking because the signal does not fade upon cell division. In addition, the use of nuRFP helps to gate fluorescent background noise, thus distinguishing transplanted ELPCs (RFP signal in nuclei) from other cells that have phagocytized degraded transplanted ELPCs (amorphous RFP signal in
cytoplasm). This strategy helps improve the accuracy and specificity of in vivo cell tracking. Second, we used G418 selection to ensure our treatment was enriched with COX1-PGIS–expressing ELPCs for cell transplantation. Our results showed that 48% of the transplanted cells remained in the lungs 25 days after cell transplantation (Figure 3A); in the eNOS gene therapy study, Zhao et al., using a transient cell-labeling technique, reported that < 1% of the transplanted cells remained. However, we cannot exclude the possibility that lentivirus infection and fluorescent cell sorting resulted in the selection of a more proliferative cell population and that the transplanted cells might have proliferated in the lungs.

Although use of the PGIS gene alone has been effective in animal models of PAH,6 our COX1-PGIS fusion gene is significantly more efficient at producing prostacyclin than is the PGIS gene alone.6 Adenovirus-mediated COX1 and PGIS gene transfer have reduced cerebral infarct volume in a rat cerebral ischemia-reperfusion model.16 However, the therapeutic effects of ELPC-based COX1-PGIS gene therapy for PAH have not been reported. In an MCT-induced model of PAH, we showed that engineered ELPCs were more effective than control ELPCs in attenuating MCT-induced increases in RVSP (Figure 4A), RV hypertrophy (Figure 4B), pulmonary vessel wall thickening (Figure 5), and cell proliferation in the pulmonary vessel wall (Figure 6). Furthermore, in rats with established PAH, engineered ELPCs provided an earlier survival benefit than did control ELPCs (Figures 7A and 7B); this benefit may be related to a significant increase in prostacyclin production by the engineered ELPCs (Figure 3F) during the first 2 weeks after cell transplantation. Because the COX1-PGIS transgene is not integrated into the chromosome of the engineered ELPCs, it will be lost gradually in the absence of G418 selection in vivo. Thus, although the engineered ELPCs may remain in the lungs, they may not continue to produce prostacyclin, or they may produce only low levels; therefore, the survival
benefit would be due to the ELPCs themselves. Future studies of multiple doses of the engineered ELPCs may show even better survival rates of rats with PAH. Moreover, the survival benefit was associated with a decrease in RV hypertrophy (Figure 7C) rather than reduced RVSP (Figure 7D). This finding supports the clinical observations that RVSP does not strongly correlate with survival in PAH patients, whereas RV mass and function are strong predictors of survival.17,18 Our measurements were taken 4 weeks after cell injection, so the lack of effect on RVSP could be an artifact of the time at which the measurement was taken. Because RVSP levels may depend more on having continuously high levels of prostacyclin than does RV hypertrophy, we may have seen a difference in RVSP had we been able to measure it at 1 or 2 weeks, since prostacyclin levels were lower at 4 weeks.

Because the efficacy of cell-based gene therapy has not been compared with that of prostacyclin or its analogs (the most efficient and last resort for PAH patients), we compared the efficacy of control ELPCs and engineered ELPCs with that of TP. The single intravenous injection of 1.5x10⁶ ELPCs was comparable to a once-a-day subcutaneous injection of TP (0.5 mg/kg body weight) in preventing MCT-induced increases in RVSP (Supplemental Table S1). The efficacy of engineered ELPCs was comparable to that of the combined therapy of control ELPCs and TP in preventing RVSP increase and RV hypertrophy (Supplemental Table S1), again demonstrating that the prostacyclin–producing cells are superior to cell treatment alone. Our results suggest that the use of ELPCs or ELPC-based prostacyclin gene therapy may help to reduce the TP dose needed clinically, thus minimizing the systemic side effects of TP.

The jugular vein is thought to be an ideal route for evenly delivering cells into the pulmonary vasculature. Most of the transplanted cells are trapped by the pulmonary capillary network, and they migrate across the endothelium into the lung parenchyma, where they can
exert their therapeutic effects. However, some cells may pass through the pulmonary capillaries, enter the circulation, and eventually be distributed into different organs where they may produce unnecessary effects. Thus, using PCR, we examined the tissue distribution of the COX1-PGIS transgene as an indicator of the transplanted engineered ELPCs. PCR is more sensitive than histologic analysis in detecting small numbers of cells that are distributed sparsely. Very low levels of COX1-PGIS transgene were detected in the liver and kidney but did not cause hepatocellular injury as determined by ALT levels or renal insufficiency as determined by BUN and CRE levels (Supplemental Figure S2). No transgene DNA was seen in the heart, spleen, brain, or testis at 25 days after cell transplantation (Figure 3B).

To further confirm the efficacy of ELPC-based prostacyclin gene therapy, we analyzed cell proliferation in the pulmonary vessel wall. We found that both control and engineered ELPCs significantly reduced MCT-induced cell proliferation in the vessel wall, but engineered ELPCs were more effective than control ELPCs (Figure 6). This observation supports the previous finding that prostacyclin analogues can inhibit smooth muscle cell proliferation in the pulmonary artery. The decreased thickening of the pulmonary vessel wall may also be related to increased apoptosis. We showed that apoptosis occurred mainly in the adventitial smooth muscle layer of the pulmonary vessel walls, rather than in the intimal or medial layers (Supplemental Figure S5). This finding is interesting because adventitial cells may play an important role in PAH development by differentiating into myofibroblasts or by interacting via crosstalk with medial smooth muscle cells. Moreover, adventitial cells may contribute to increased stiffness of the vessel wall by producing vasoactive molecules, cytokines, and extracellular matrix (including collagen). Our results suggest that ELPCs may attenuate the development of MCT-induced PAH, in part, by enhancing apoptosis of the adventitial cells in the...
pulmonary vessel wall.

The therapeutic mechanism(s) underlying improvements in a compromised right ventricle after treatment with bone marrow-derived cells or prostacyclin is not understood at the genome level. Currently, only 1 study has used the MCT-induced PAH rat model to investigate changes in the whole genome gene expression of the decompensated right ventricle relative to those in the compensated one. However, because the study lacked an effectively treated right ventricle group, the authors were unable to identify potential therapeutic targets in the hypertrophic right ventricle. In the current study, we showed for the first time a genetic restoration signature in the right ventricle that may account for the therapeutic effects of bone marrow-derived ELPC-based prostacyclin gene therapy in PAH. In our model of PAH, several neurotransmitter pathways and gene expression levels were altered, potentially contributing to the RV hypertrophy and dysfunction and the poor survival rates observed; reversing these changes may be the mechanism(s) by which prostacyclin and ELPC therapies improved RV hypertrophy and dysfunction and increased the survival rate in this model of PAH.

Monocrotaline-induced changes in expression of Adcyap1r1, Ctl4, Tead1, Ncoa2, and Tln2 (Figure 8, clusters a, c, and e) could be reversed only with the engineered ELPC treatment (ie, with prostacyclin). Alterations in the expression of these genes have been found to be associated with non-cardiovascular systems as well. Adenylate cyclase activating polypeptide 1 receptor 1 (Adcyap1r1) is a G protein–coupled receptor of pituitary adenylate cyclase–activating polypeptide (PACAP); complete deletion of Adcyap1r1 results in pulmonary hypertension and right heart failure, as well as a deficit in hippocampus-dependent contextual fear conditioning. However, the specific roles of Adcyap1r1 in pulmonary hypertension and RV hypertrophy cannot be determined unless tissue-specific deletion of Adcyap1r1 in the lung or...
right ventricle is investigated. Cytotoxic T-lymphocyte-associated protein 4 (Ctla4) is an inhibitory receptor expressed on activated T cells. Blocking Ctla4 enhances CD8+ cytotoxic T lymphocyte-mediated myocarditis\textsuperscript{25} and cardiac allograft rejection\textsuperscript{26} Although there is no evidence suggesting that the immune response contributes to the development of RV dysfunction, a recent study showed that rats with autoimmune myocarditis benefit from treatment with a prostacyclin agonist\textsuperscript{27} Overexpression of TEA domain family member 1 (Tead1) in the heart has been found to promote age-dependent heart dysfunction\textsuperscript{28} Moreover, a deficiency in nuclear receptor coactivator 2 (Ncoa2), also known as SRC-2, was recently shown to be associated with decreased cardiac functional reserve in response to pressure overload, accentuating its role in maintaining the steady-state transcriptional profile in the adult heart\textsuperscript{29} Talin 2 (Tln2), the major form of talin in adult hearts,\textsuperscript{30} co-localizes with spectrin to cardiac T-tubule membranes\textsuperscript{31} Furthermore, RV failure from acute RV pressure overload has been associated with reduced talin levels and disrupted talin organization\textsuperscript{32} Monocrotaline-induced changes in Stk11 and Fgfr2 expression (Figure 8, clusters b, d, and f) could be reversed by treatment with control or engineered ELPCs (ie, by ELPCs themselves). Deletion of serine/threonine kinase 11 (Stk11), also known as liver kinase B1 (Lkb1), in the heart leads to hypertrophy and dysfunction\textsuperscript{33} whereas Stk11 overexpression destabilizes microtubule assembly in myoblasts\textsuperscript{34} Conditional knockout or over-expression of Stk11 in adult cardiomyocytes may help to elucidate the role of Stk11 in cardiac hypertrophy and dysfunction. Fibroblast growth factor receptor 2 (Fgfr2) is expressed in cardiomyocytes and in the vasculature of normal human adult hearts,\textsuperscript{35} and its ligand, fibroblast growth factor (FGF), has protective effects on ischemic myocardium\textsuperscript{36} Monocrotaline-induced downregulation of melatonin receptor activity (Gpr50), GABA-B
receptor activity (Gabbr2), and the serotonin receptor signaling pathway (Htr1a) was reversed by treatment with either control or engineered ELPCs. Furthermore, prostacyclin enhanced the restoration of the serotonin pathway. G-protein-coupled receptor 50 (Gpr50) is an X-linked orphan receptor that shares homology with melatonin receptors, but its ligand and physiologic function are unknown. In a brain endothelial cell line, Levoye and colleagues37 showed that Gpr50 heterodimerizes with melatonin receptor MT1 and prevents MT1 from binding melatonin and coupling to G proteins. Therefore, Gpr50 was mapped to the GO term of melatonin receptor activity. However, the circadian cycle was found not to be altered in the Gpr50 knockout mice.38 Thus, altered Gpr50 expression may not represent a perturbation of the melatonin receptor activity in the right ventricle. Energy metabolism38 and glucocorticoid receptor signaling39 may be the actual roles of Gpr50 in the right ventricle. Gamma-aminobutyric acid B receptor 2 (Gabbr2) is a B-type receptor for the neurotransmitter gamma-aminobutyric acid (GABA). It is expressed in ventricular cardiomyocytes and may mediate the cardioprotective effects of the GABA B-type receptor agonist, gamma hydroxybutyric acid (GHB).40,41 5-hydroxytryptamine receptor 1A (Htr1a), also known as the 5-HT1A receptor, and 5-hydroxytryptamine receptor 7(Htr7), also known as the 5-HT7 receptor, are 2 receptor subtypes for serotonin (5-hydroxytryptamine, 5-HT). The serotonin system affects pulmonary hypertension through the serotonin transporter (SERT) and receptor subtypes 5-HT1a, 5-HT2a, and 5-HT2b in the pulmonary arteries.42 However, because of the complexity of the serotonin/receptor system (7 classes and 14 subtypes of receptors), cardiovascular responses to serotonin stimulation are different or even opposite, depending on the receptor subtype and its tissue location.43 The main serotonin receptor subtype found in the heart is Htr7,44 whereas Htr1a is mainly expressed in the brain. We showed for the first time that Htr1a is expressed in the right ventricle of the heart, but
the cardiac roles of Htr1a and Htr7 are unknown.

Only treatment with engineered ELPCs (ie, prostacyclin) restored the MCT-induced upregulation of dopamine transporter activity (Slc6a3) and the MCT-induced downregulation of dopamine receptor activity (Drd5) (Figures 8B and 8C, Supplemental Table S4). Solute carrier family 6, member 3 (Slc6a3), also known as the dopamine transporter (DAT), is the primary mediator of dopamine reuptake from nerve endings. Dopamine receptor D5 (Drd5) is expressed in normal human hearts.45 The heart has a complex intrinsic nervous system comprising adrenergic, cholinergic, serotonergic, dopaminergic, and histaminergic nerve fibers.46 Our findings highlight the pivotal roles of neurotransmitter pathways in the development and improvement of RV hypertrophy and dysfunction.

In summary, COX1-PGIS–expressing ELPCs attenuated MCT-induced PAH and increased the survival rate of rats with established PAH. The overall efficacy of COX1-PGIS–expressing ELPCs was better than that of ELPCs alone. ELPC-based prostacyclin gene therapy may help to reduce the dose of prostacyclin or its analogs needed to treat patients with PAH. Moreover, administering the cells via the jugular vein resulted in the cells localizing primarily in the lungs; this delivery approach may minimize the side effects associated with the systemic delivery of prostacyclin. Our single-injection cell delivery approach provided survival benefits for at least 4 weeks and may offer a promising option for PAH patients. Our gene expression array findings may provide novel therapeutic targets for early intervention in the treatment of a compromised right ventricle.

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**Conflict of Interest Disclosures:** None.

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constantly biosynthesize prostacyclin, the vascular protector. *FEBS J.* 2008;275:5820-5829.


**Figure Legends:**

**Figure 1.** Endothelial-like progenitor cells were engineered to express COX1-PGIS fusion protein and nuRFP. A, Schematic of the human COX1-PGIS expressing vector with a G418 resistance gene. B, Schematic of the lentivirus expressing nuRFP for cell labeling. C, Cell preparation procedure. D, Flow cytometry analysis of induced ELPCs. Neo R, G418 resistance gene; DsRed-MST, *Discosoma sp.* red fluorescent protein variant MST; NLS, nuclear localization signal; nuRFP, nuclear-localized red fluorescent protein (ie, DsRed-MST-3xNLS); COX1, cyclooxygenase isoform 1; PGIS, prostacyclin synthase; CP, COX1-PGIS; ELPCs, endothelial-like progenitor cells; BMMNCs, bone marrow mononuclear cells; ELPCs-nuRFP/CP, ELPCs expressing COX1-PGIS and nuRFP (ie, engineered ELPCs); ELPCs-nuRFP, ELPCs expressing nuRFP only.
Figure 2. Engineered ELPCs (ELPCs-nuRFP/CP) exhibited an endothelial phenotype, expressed the COX1-PGIS protein, and produced prostacyclin. A, AcLDL uptake and Tie2 expression in ELPCs-nuRFP/CP. B, COX1-PGIS fusion protein detected by COX1 and PGIS primary antibodies (recognizing both rat and human COX1 or PGIS). COX1-PGIS expression was seen in the cytoplasm and the perinuclear region in ELPCs-nuRFP/CP, but endogenous PGIS and COX1 expression was seen only in the cytoplasm of the ELPCs-nuRFP. C, Prostacyclin production (represented by the prostacyclin metabolite 6-keto prostaglandin F$_{1\alpha}$ [PGF$_{1\alpha}$]) by ELPCs-nuRFP/CP, ELPCs-nuRFP, and ELPCs, as measured in cell culture medium by using ELISA. Results were normalized to the level of 6-keto PGF$_{1\alpha}$ in the ELPCs group (set at 1.0). ELPCs, endothelial-like progenitor cells; nuRFP, nuclear localized red fluorescent protein; CP, COX1-PGIS. Analysis was performed in triplicate. * P < 0.01.

Figure 3. Engineered ELPCs were retained in the lungs after cell transplantation and produced prostacyclin in vivo. Engineered ELPCs ($1.5 \times 10^6$) were infused into the jugular vein of rats 3 days after monocrotaline (MCT) injection. A, Cell retention rate is shown at different time points after cell transplantation (n=3; Hr, hours; D, days; W, weeks). At 25 days after transplantation, (B) the COX1-PGIS transgene was distributed primarily in the lungs, rather than in other organs, as shown by PCR (CP vector, COX1-PGIS plasmid used as a positive control), (C) most of the engineered ELPCs were localized in the lung parenchyma, and (D) the engineered ELPCs occasionally integrated into the lung vessel wall (pulmonary vessel intima shown by vWF staining). E, At 7 days after cell transplantation, expression of the human COX1-PGIS fusion protein was detected in the transplanted engineered ELPCs located in the lungs by using an antibody that specifically recognizes human PGIS. DAPI staining shows nuclei.
localized red fluorescent protein (nuRFP) indicates transplanted engineered ELPCs. F, At 7 days after cell transplantation, an increase in serum prostacyclin production was detected in the engineered ELPC-treated group (as determined by measuring 6-keto PGF1α, by using liquid chromatography–mass spectrometry). The mean of each group is shown beside the dotplot; $P < 0.01$, MCT+vehicle vs MCT+ELPCs-nuRFP/CP. Normal, saline injection (MCT control); Vehicle, Dulbecco’s modified Eagle’s medium (used for cell suspension); ELPCs-nuRFP, ELPCs expressing nuRFP only; ELPCs-nuRFP/CP, engineered ELPCs expressing nuRFP and COX1-PGIS fusion protein.

**Figure 4.** Both engineered ELPCs and ELPCs alone attenuated the monocrotaline (MCT)-induced increase in RVSP and RV hypertrophy. A, RVSP and (B) the RV/(LV + IVS) weight ratio at 4 weeks after MCT injection (ie, 25 days after cell transplantation). RVSP, right ventricular systolic pressure; RV/(LV + IVS), weight ratio of the right ventricle to left ventricle plus interventricular septum; normal, saline injection (MCT control); Vehicle, Dulbecco’s modified Eagle medium (used for cell suspension); ELPCs-nuRFP, ELPCs expressing nuRFP only; ELPCs-nuRFP/CP, engineered ELPCs expressing nuRFP and COX1-PGIS fusion protein. RVSP or RV/(LV + IVS) (% increase above normal), (mean of treated group - mean of normal group) / mean of normal group,*$P < 0.01$; **$P < 0.05$.

**Figure 5.** Engineered ELPCs attenuated monocrotaline (MCT)-induced pulmonary vessel wall thickening. A, Immunofluorescence showing staining for vWF and smooth muscle α-actin in lungs from each treatment group 4 weeks after MCT injection. DAPI staining shows nuclei. B, Pulmonary vessel thickness index (VTI) was calculated as (area of circle 1 – area of circle 2) /
area of circle 1; green represents intimal thickening, and red represents medial smooth muscle hypertrophy. C, Graph comparing the pulmonary VTI values of the different treatment groups (n=3 rats per each group, > 20 vessels analyzed per rat). Normal, saline injection (MCT control); Vehicle, Dulbecco’s modified Eagle medium (used for cell suspension); ELPCs-nuRFP, ELPCs expressing nuRFP only; ELPCs-nuRFP/CP, engineered ELPCs expressing nuRFP and COX1-PGIS fusion protein. Pulmonary VTI (% increase above normal) = (mean of treated group - mean of normal group) / mean of normal group. * P < 0.01.

Figure 6. Both engineered ELPCs and control ELPCs reduced monocrotaline (MCT)-induced cell proliferation in pulmonary vessel walls. A, Immunofluorescence showing staining for Ki-67, vWF, and smooth muscle α-actin in lungs from each treatment group 4 weeks after MCT injection. Arrows indicate the Ki-67+ cells. B, The number of Ki-67+ cells in the intimal and medial smooth muscle layers per pulmonary vessel section for each treatment group. Data are presented as the mean ± standard error of the mean (SEM) (n=3 rats per each group, > 50 vessels analyzed per rat). The mean of each group is shown beside the dotplot. P < 0.01, Normal vs MCT+vehicle; P < 0.01, MCT+vehicle vs MCT+ELPCs-nuRFP; P < 0.01, MCT+vehicle vs MCT+ELPCs-nuRFP/CP; P < 0.05, MCT+ELPCs-nuRFP vs MCT+ELPCs-nuRFP/CP. Normal, saline injection (MCT control); Vehicle, Dulbecco’s modified Eagle medium (used for cell suspension); ELPCs-nuRFP, ELPCs expressing nuRFP only; ELPCs-nuRFP/CP, engineered ELPCs expressing nuRFP and COX1-PGIS fusion protein.

Figure 7. In rats with established PAH, both engineered ELPCs and control ELPCs provided a survival benefit that was accompanied by a decrease in right ventricular hypertrophy. Cells were
transplanted 21 days after monocrotaline (MCT) injection. A and B, Kaplan Meier survival analysis and comparison by the log-rank test (n=15 in each group, except the MCT + vehicle group [n=20]). Numbers of surviving rats at days 21, 35, and 49 after MCT injection are indicated below each graph. A difference in survival rate was detected as early as 2 weeks after cell transplantation in the group treated with engineered ELPCs (B) and at 4 weeks after cell transplantation in the group treated with control ELPCs (A). C, The survival benefit was accompanied by a decrease in RV/(LV+IVS) weight ratio. D, Survival benefit was not accompanied by a decrease in RVSP. Normal, saline injection (MCT control); Vehicle, Dulbecco’s modified Eagle medium (used for cell suspension); ELPCs-nuRFP, ELPCs expressing nuRFP only; ELPCs-nuRFP/CP, engineered ELPCs expressing nuRFP and COX1-PGIS fusion protein; RV/(LV + IVS), weight ratio of the right ventricle to left ventricle plus interventricular septum; RVSP, right ventricular systolic pressure; RVSP or RV/(LV + IVS) (% increase above normal), (mean of treated group - mean of normal group) / mean of normal group. *P < 0.01; **P < 0.05.

**Figure 8.** Molecular basis of right ventricle (RV) benefit from ELPC-based prostacyclin gene therapy. Gene expression profiles of the RV were obtained 11 days after cell transplantation (ie, 2 weeks after MCT injection). A, Global gene expression. B, Genes that were upregulated by MCT exposure and restored by treatment with either control or engineered ELPCs (a & c, MCT-upregulated genes that were restored only by engineered ELPCs; b, MCT-upregulated genes that were restored by both control and engineered ELPCs). C, Genes that were downregulated by MCT exposure and restored by treatment with control or engineered ELPCs (d & f, MCT-downregulated genes that were restored by both control and engineered ELPCs; e, MCT-
downregulated genes that were restored only by engineered ELPCs). Normal, saline injection; MCT, monocrotaline; Vehicle, Dulbecco’s modified Eagle medium (used for cell suspension); ELPCs-nuRFP, ELPCs expressing nuRFP only; ELPCs-nuRFP/CP, engineered ELPCs expressing nuRFP and COX1-PGIS fusion protein. Each group included biological quadruplicates that were run separately. The heatmap displays an averaged signal (mean intensity) of the 4 samples per group.
Figure 1
Figure 2
Figure 3
Figure 4

A

RVSP (% increase above normal)

- Normal
- MCT+Vehicle
- MCT+ELPCs-nuRFP
- MCT+ELPCs-nuRFP/CP

n=12
n=9
n=22
n=23

* p < 0.05

B

RV/(LV+IVS) (% increase above normal)

- Normal
- MCT+Vehicle
- MCT+ELPCs-nuRFP
- MCT+ELPCs-nuRFP/CP

n=6
n=9
n=22
n=23

* p < 0.05

** p < 0.01
Figure 5
Figure 6
Figure 7

A

Cumulative Survival

MCT+Vehicle
MCT+ELPCs-nuRFP/CP
Normal

Days after MCT injection

Day 21

35

49

15

15

15

B

Cumulative Survival

MCT+Vehicle
MCT+ELPCs-nuRFP
Normal

Days after MCT injection

Day 21

35

49

15

15

15

C

RV/(LV+IVS)

(% increase above normal)

Normal
MCT+Vehicle
MCT+ELPCs-nuRFP
MCT+ELPCs-nuRFP/CP

n=6
n=5
n=4

D

RVSP

(% increase above normal)

Normal
MCT+Vehicle
MCT+ELPCs-nuRFP
MCT+ELPCs-nuRFP/CP

n=12
n=9
n=7
Figure 8

(a & c): Adcyap1r1, Cpa1, Cst11, Ctla4, Cyba, Cyp2c6v1, Cyp2c6v2, Fbxl17, Foxd1, Gjc2, Hira, Hrh3, Hsd3b, Hspb11, Ier5, Lect1, Lnpep, Mab2113, Mip, Prss30, Psg19, Sema6a, Serpinb3, Sftd2, S100a3, Spata5l1, Spdy, Tead1, Tlx2, Tmem151a, Trem2, Trpv5, Zp3

(b): Aspdlh, Baat, Btg4, Camk2b, Cinp, Dsg1b, Fbxo23, Insrr, Lrx4, LOC100362634, LOC100909612, Ucp1, Nkx6-2, Nrtn2, Pex5l, Prm3, Rap2ip, RGD1560871, RGD1566083, Rifip1, Stk11

(d & f): Als2cl, Aqp11, Cdhr4, Dpm1, Drd5, Fam172a, Fgrf2, Gabbr2, Gaint1, Gpr50, Hist1h3f, Hist2h3c, Hist2h3c2, Htr1a, Kdm4d, LOC100192314, LOC364653, Msh5, Odf3, Padi4, Pitx3, Rab17, Rbp3, S100a3, Svop

(e): Ank1, Cdh10, Cep76, Dmrt1a, Elavl3, Fam135a, Fbxo42, Gbx2, Htr7, Kcnc2, LOC100911283, LOC691984, Ncoa2, Nfkbia, Nkx2-2, Nxph1, Pklr, Ppm1n, RGD1308065, RGD1560784, Rnf32, Sptbn1, Tacstd2, Tln2, Tlx2, Tmem130
Endothelial-Like Progenitor Cells Engineered to Produce Prostacyclin Rescue Monocrotaline-Induced Pulmonary Arterial Hypertension and Provide Right Ventricle Benefits
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SUPPLEMENTAL MATERIAL

Methods

Construction of a Lentivirus Encoding nuRFP

A lentivirus encoding nuRFP was generated for in vivo cell tracking. The procedure was similar to that described in our previous publication,¹ except that the nuRFP cDNA linked with an encephalomyocarditis virus-derived internal ribosome entry site (IRES) sequence was cloned into the pLV-CMV-LacZ-IRES-EGFP lentiviral vector (provided by Dr. Douwe E. Atsma, Leiden University Medical Center)² by using PstI and BsrGI restriction sites.

Preparation of Engineered ELPCs Expressing COX1-PGIS Fusion Protein and nuRFP

The endothelial-like progenitor cells (ELPCs) were generated as previously described.³ Briefly, bone marrow mononuclear cells (BMMNCs) were isolated from the bone marrow of 6-week-old male syngeneic Fisher 344 rats (Charles River Laboratories, Wilmington, MA) by Ficoll gradient centrifugation (GE Healthcare, Buckinghamshire, UK). The BMMNCs were then infected with lentivirus encoding nuRFP (multiplicity of infection = 20:1). The BMMNCs expressing nuRFP were sorted by flow cytometry and were transfected with the COX1-PGIS plasmid⁴ (CP) containing a G418 selection marker by using the FuGENE HD transfection reagent (Roche Applied Science, Penzberg, Germany). After G418 selection, BMMNCs that expressed both nuRFP and the CP fusion protein were subjected to 7 to 10 days of endothelial induction in endothelial cell growth medium 2 (EGM-2) (Lonza Group Ltd., Basel, Switzerland) supplemented with 2% fetal bovine serum to generate
engineered ELPCs that express nuRFP and CP (ie, ELPCs-nuRFP/CP). Endothelial-like progenitor cells expressing nuRFP alone (ELPCs-nuRFP) were used as a control.

**Characterization of Engineered ELPCs**

Engineered ELPCs were characterized by the presence of an endothelial phenotype, COX1-PGIS fusion protein expression, and prostacyclin production. The cells were analyzed by flow cytometry to assess the expression of endothelial (KDR, Tie2, CD31), macrophage (CD14), and leukocyte (CD45) lineage markers. Briefly, engineered ELPCs or control ELPCs were detached from cell culture plates by using StemPro Accutase (Invitrogen), washed with Dulbecco's Phosphate-Buffered Saline (DPBS; Invitrogen), and fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 minutes at room temperature. One million cells (engineered ELPCs or control ELPCs) were incubated with rabbit anti-KDR (Abcam, Cambridge, UK), rabbit anti-Tie2 (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-CD14 (Santa Cruz Biotechnology), goat anti-CD31 (Santa Cruz Biotechnology), goat anti-CD34 (Santa Cruz Biotechnology), or FITC-conjugated mouse anti-CD45 (Invitrogen) for 40 minutes at room temperature. For indirect staining (KDR, Tie2, CD14, CD31, and CD34), cells were washed with DPBS and then incubated with FITC-conjugated goat anti-rabbit IgG (Abcam) or FITC-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology), as appropriate, for another 30 minutes at room temperature. Cells incubated with FITC-conjugated isotype controls were used to exclude non-specific staining. Analysis was performed by using a BD LSRII cell analyzer (BD Biosciences, Franklin Lakes, NJ) and FlowJo 7.6.1 software (Tree Star, Inc., Ashland, OR).
The cells were also characterized by their acetylated low-density lipoprotein (AcLDL) uptake ability, which was superimposed with Tie2 receptor immunostaining (2 common markers of endothelial cells). Briefly, engineered ELPCs were incubated with AlexaFluor 488-labeled AcLDL (15 μg/ml; Invitrogen) for 4 hours at 37°C. After fixation in 4% paraformaldehyde, the cells were incubated with Tie2 primary antibody (Santa Cruz Biotechnology), followed by AlexaFluor 350-conjugated secondary antibody (Invitrogen). For the detection of COX1-PGIS fusion protein expression, engineered ELPCs were incubated with COX1 (Santa Cruz Biotechnology) and PGIS primary antibodies (Abcam), followed by AlexaFluor 350- and 488-conjugated secondary antibodies (Invitrogen). Images were collected by using an Olympus FluoView FV1000 confocal laser-scanning microscope (Olympus Corporation, Tokyo, Japan). Because prostacyclin has a short half-life, its stable metabolite, 6-keto prostaglandin F1α (PGF1α), was measured to assess prostacyclin production in cell culture medium after 48 hours by using an enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI).

**Rat Pulmonary Arterial Hypertension (PAH) Model and Cell Transplantation**

All animal studies were performed in accordance with the Institutional Animal Care and Use Committee of The University of Texas Health Science Center. Saline or monocrotaline (MCT) (60 mg/kg; Sigma-Aldrich Corporation, St. Louis, MO) was intraperitoneally injected into 6-week-old male Fisher 344 rats (Charles River Laboratories). Three days after MCT injection (prevention study) or 21 days after MCT injection (reversal study), rats were anesthetized with 2% isoflurane (Baxter, Deerfield, IL) in an air-oxygen mixture, and 1.5x10^6 engineered ELPCs (ELPCs-nuRFP/CP) or
control ELPCs (ELPCs-nuRFP), suspended in 0.5 ml of Dulbecco's modified Eagle’s medium (DMEM), were infused through the jugular vein via a 23G needle (BD Biosciences, San Jose, CA). An equal volume (0.5 ml) of DMEM was used as a vehicle control. Analyses were performed at 25 days (prevention study) or at 4 weeks (reversal study) after cell transplantation.

Analysis of Lung Cell Retention Rate, Tissue Distribution of COX1-PGIS Transgene, and COX1-PGIS Fusion Protein Expression In Vivo

In the prevention study, rats were euthanized at various time points (24 hours, 3 days, 1 week, 2 weeks, 3 weeks, and 25 days) after cell transplantation, and the lungs of each animal were harvested. The rate of lung cell retention was calculated as previously described. Briefly, the lungs were cryosectioned into 5-μm thick slices at apical, middle, and basal levels. The number of fluorescent cells in each lung section was counted. The total cell number present in the lungs was mathematically approximated by using Simpson’s rule for the volume of a truncated cone. The rate of lung cell retention was calculated as the total number of fluorescent cells in the lungs divided by the total number of cells transplanted.

Tissue distribution of the COX1-PGIS transgene was analyzed by using polymerase chain reaction (PCR). Briefly, tissues were harvested 25 days after cell transplantation and were lysed with a solution containing 1% sodium lauroyl sarcosine, 250 mmol/L EDTA, and 50 mmol/L Tris-HCl (pH 8.0). Cell lysates were treated with 0.1 mg/ml RNase A for 1 hour at 37°C and 0.4 mg/ml proteinase K overnight at 56°C. The DNA was extracted with phenol/chloroform and dissolved in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA; pH 8.0). Then, PCR analysis was performed by using 0.3 g of
DNA from each tissue and primers specific for COX1 and PGIS. The COX1-PGIS plasmid (40 ng) was used as a positive control. Primer sequences are available upon request.

To analyze the expression of the COX1-PGIS fusion protein in the transplanted engineered ELPCs, lung cryosections (obtained 7 days after cell transplantation) were incubated with an antibody that specifically recognizes human PGIS (Cayman Chemical Company). Then, an AlexaFluor 488-conjugated secondary antibody (Invitrogen) was used for visualization. Images were collected by using an Olympus FluoView FV1000 confocal laser-scanning microscope.

**Measurement of Right Ventricular Systolic Pressure (RVSP) and Evaluation of Right Ventricular (RV) Hypertrophy**

Rats were anesthetized at 25 days (prevention study) or 4 weeks (reversal study) after cell transplantation, and RVSP was measured by using a 3F Millar microtip pressure transducer (Millar Instruments, Houston, TX) positioned in the right ventricle via the right jugular vein. After RVSP measurements were taken, the rats were euthanized, and the hearts and lungs were harvested. To evaluate RV hypertrophy, the weight ratio of the right ventricle to left ventricle plus interventricular septum (RV/[LV + IVS]) was calculated.

**Analysis of Pulmonary Vessel Thickness Index (VTI), Cell Proliferation, and Apoptosis in Pulmonary Vessel Walls**

In the prevention study, cryosectioned lung samples were incubated with Ki-67 (Santa Cruz Biotechnology), von Willebrand factor (vWF; Abcam), and smooth muscle α-actin (Abcam) primary antibodies, followed by incubation with secondary antibodies conjugated to AlexaFluor 350, 488,
594, or 647 (Invitrogen). For analysis of apoptosis, cells were subjected to terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL; Roche Applied Bioscience) with fluorescein before immunostaining. Nuclear staining with DAPI (4’,6-diamidino-2-phenylindole) was used when necessary. Vessels with a diameter less than 200 μm were analyzed. Pulmonary VTI was calculated as the ratio of the encircled area of the intima and medial smooth muscle to the medial smooth muscle outer margin; this reflects intimal thickening and medial smooth muscle hypertrophy. For each pulmonary vessel section, the number of Ki-67–positive cells in the intimal and medial smooth muscle layers and the number of TUNEL-positive cells in the adventitial layer were calculated.

**Right Ventricle Microarray and Gene Ontology Analysis**

Rats were euthanized 2 weeks after MCT injection (prevention study). Right ventricles were dissected from the harvested hearts. Total RNA was isolated by using Trizol reagent (Invitrogen) followed by DNase I (Qiagen) treatment. Samples were compared by using Affymetrix Rat Genome 230 2.0 arrays. Each group included 4 right ventricle samples that were run separately and provided 4 independent gene expression profiles per group. These 4 data sets were averaged and used for comparison. Data analysis was performed by using dChip2010, GenMAPP, and the GeneVenn web application, as indicated previously.¹ Monocrotaline-upregulated or -downregulated genes were defined as genes with an absolute fold change ≥ 2.0 relative to the normal group. Genes upregulated or downregulated by control or engineered ELPCs were defined as genes with an absolute fold change ≥ 2.0 relative to the vehicle (DMEM) treated group. A permutation test was applied for multiple testing correction, and a permute P value was calculated by using a non-parametric bootstrapping
approach. Gene ontology terms with a permute $P < 0.05$ and a nested gene change percentage $\geq 25\%$ were considered to be significantly upregulated or downregulated. Each group included biologic quadruplicates.

**Survival Analysis**

In the reversal study, rats were monitored daily after cell transplantation to determine survival rates. Kaplan-Meier survival curves were plotted and compared at each intermediate time point (2 and 3 weeks after cell transplantation) and at the end point (4 weeks after cell transplantation) by the log-rank test by using SPSS 11.0 software (IBM Corporation, Somers, NY).

**Blood Chemistry Analysis**

In the prevention study, blood samples were collected 25 days after cell transplantation and subjected to chemistry analysis by using an IDEXX VetLab station (IDEXX Laboratories, Westbrook, ME).

**Serum Prostacyclin Measurement after Cell Transplantation**

In the prevention study, blood samples were collected 7 days after cell transplantation. Serum prostacyclin levels were determined by measuring 6-keto PGF$_{1\alpha}$, a stable prostacyclin metabolite, by using liquid chromatography–mass spectrometry (LC-MS). Briefly, serum was applied to a C18 cartridge. After washing with double distilled water (ddH$_2$O), 6-keto PGF$_{1\alpha}$ that was bound to the cartridge was eluted with 100% acetone, blow-dried by nitrogen gas, and dissolved in solvent A (35% acetonitrile with 0.1% acetic acid in ddH$_2$O). The samples were then injected into a Waters Micromass LC-MSMS system. The components in the sample were separated by the reversed-phase high-performance liquid chromatography (RP-HPLC) C18 column and injected into the mass detector.
with an electrospray ionization (ESI) source in a negative mode. A 6-keto PGF$_{1\alpha}$ standard (Cayman Chemical Company, Ann Arbor, MI) was used to calibrate the LC-MS system and to identify and normalize the retention time limits and sensitivities.

**Statistical Analysis**

Statistical analysis was performed by using SPSS 11.0 software. A Kolmogorov-Smirnov test was used to verify the normal distribution of the variables. Results were expressed as the mean ± standard error of the mean (SEM). Statistical significance was evaluated by one-way analysis of variance (ANOVA) for normally distributed variables. A $P < 0.05$ was considered statistically significant.
Supplemental Table S1. Effects of ELPC and Treprostinil Treatments on RVSP and RV Hypertrophy in Rats With MCT-induced PAH

<table>
<thead>
<tr>
<th></th>
<th>RVSP (mmHg)</th>
<th>P value</th>
<th>RV/(LV + IVS) weight ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>26.3±1.5 (n=12)</td>
<td></td>
<td>0.27±0.01 (n=6)</td>
<td></td>
</tr>
<tr>
<td>MCT+Vehicle</td>
<td>51.9±2.2 (n=9)</td>
<td>*</td>
<td>0.36±0.01 (n=9)</td>
<td>*</td>
</tr>
<tr>
<td>MCT+ELPCs-nuRFP</td>
<td>41.0±1.5 (n=22)</td>
<td>*</td>
<td>0.32±0.01 (n=14)</td>
<td>*</td>
</tr>
<tr>
<td>MCT+ELPCs-nuRFP/CP</td>
<td>33.7±1.6 (n=23)</td>
<td>*</td>
<td>0.30±0.01 (n=14)</td>
<td>*</td>
</tr>
<tr>
<td>MCT+TP</td>
<td>43.5±3.9 (n=8)</td>
<td>0.394†</td>
<td>0.29±0.01 (n=8)</td>
<td>0.045†</td>
</tr>
<tr>
<td>MCT+ELPCs-nuRFP+TP</td>
<td>29.1±2.7 (n=10)</td>
<td>0.086‡</td>
<td>0.28±0.01 (n=10)</td>
<td>0.143‡</td>
</tr>
<tr>
<td>MCT+ELPCs-nuRFP/CP+TP</td>
<td>23.3±1.5 (n=10)</td>
<td>&lt;0.01‡</td>
<td>0.24±0.01 (n=10)</td>
<td>&lt;0.01‡</td>
</tr>
</tbody>
</table>

RVSP, right ventricular systolic pressure; RV/(LV + IVS), weight ratio of the right ventricle to left ventricle plus interventricular septum; normal, saline injection (MCT control); Vehicle, Dulbecco's modified Eagle's medium (used for cell suspension); ELPCs-nuRFP, ELPCs expressing nuRFP only; ELPCs-nuRFP/CP, engineered ELPCs expressing nuRFP and COX1-PGIS fusion protein; MCT, monocrotaline; TP, treprostinil. Data are presented as the mean ± standard error of the mean (SEM). Sample sizes (n) are indicated accordingly. *P values are indicated in Figure 4. †Compared with MCT+ELPCs-nuRFP. ‡Compared with MCT+ELPCs-nuRFP/CP.
Supplemental Table S2. Effects of ELPC Treatment on Pulmonary Vessel Thickness in Rats

With MCT-induced PAH

<table>
<thead>
<tr>
<th></th>
<th>Pulmonary VTI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.44±0.03 (n=3)</td>
</tr>
<tr>
<td>MCT+Vehicle</td>
<td>0.92±0.01 (n=6)</td>
</tr>
<tr>
<td>MCT+ELPCs-nuRFP</td>
<td>0.83±0.01 (n=6)</td>
</tr>
<tr>
<td>MCT+ELPCs-nuRFP/CP</td>
<td>0.74±0.02 (n=6)</td>
</tr>
</tbody>
</table>

VTI, vessel thickness index; Normal, saline injection (MCT control); Vehicle, Dulbecco's modified Eagle’s medium (used for cell suspension); ELPCs-nuRFP, ELPCs expressing nuRFP only; ELPCs-nuRFP/CP, engineered ELPCs expressing nuRFP and COX1-PGIS fusion protein. Data are represented as the mean ± standard error of the mean (SEM). Samples sizes (n) are indicated accordingly.
Supplemental Table S3. Effects of ELPC Treatment on RVSP and RV Hypertrophy in Rats

With Established PAH

<table>
<thead>
<tr>
<th></th>
<th>RVSP (mmHg)</th>
<th>RV/(LV + IVS) weight ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>26.3±1.5 (n=12)</td>
<td>0.27±0.01 (n=6)</td>
</tr>
<tr>
<td>MCT+Vehicle</td>
<td>70.5±4.4 (n=4)</td>
<td>0.91±0.06 (n=4)</td>
</tr>
<tr>
<td>MCT+ELPCs-nuRFP</td>
<td>74.8±7.0 (n=9)</td>
<td>0.75±0.02 (n=6)</td>
</tr>
<tr>
<td>MCT+ELPCs-nuRFP/CP</td>
<td>66.3±4.9 (n=7)</td>
<td>0.69±0.06 (n=5)</td>
</tr>
</tbody>
</table>

RVSP, right ventricular systolic pressure; RV/(LV + IVS), weight ratio of the right ventricle to left ventricle plus interventricular septum; Normal, saline injection (MCT control); Vehicle, Dulbecco’s modified Eagle’s medium (used for cell suspension); ELPCs-nuRFP, ELPCs expressing nuRFP only; ELPCs-nuRFP/CP, engineered ELPCs expressing nuRFP and COX1-PGIS fusion protein. Data are represented as the mean ± standard error of the mean (SEM). Samples sizes (n) are indicated accordingly.
<table>
<thead>
<tr>
<th>Pathways restored by both control and engineered ELPCs</th>
<th>Pathways restored by engineered ELPCs only</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MCT-downregulated pathways</strong></td>
<td></td>
</tr>
<tr>
<td>Protein-arginine deiminase activity</td>
<td>Cyclin-dependent protein kinase activity</td>
</tr>
<tr>
<td>Melatonin receptor activity</td>
<td>Pyruvate kinase activity</td>
</tr>
<tr>
<td>Serotonin receptor signaling pathway</td>
<td>Dopamine receptor activity</td>
</tr>
<tr>
<td>Gamma-aminobutyric acid (GABA)-β receptor activity</td>
<td>Serotonin receptor/adenylate cyclase</td>
</tr>
<tr>
<td></td>
<td>activating pathway</td>
</tr>
<tr>
<td><strong>MCT-upregulated pathways</strong></td>
<td></td>
</tr>
<tr>
<td>Mitotic sister chromatid segregation</td>
<td>Steroid delta-isomerase activity</td>
</tr>
<tr>
<td></td>
<td>Dopamine transporter activity</td>
</tr>
<tr>
<td></td>
<td>Sodium channel regulator activity</td>
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<tr>
<td></td>
<td>Negative regulation of adenylate cyclase</td>
</tr>
<tr>
<td></td>
<td>activity</td>
</tr>
</tbody>
</table>

Gene ontology terms with a permute $P < 0.05$ and a nested gene change percentage $\geq 25\%$ were considered to be significantly upregulated or downregulated.
Supplemental Figure S1. Engineered ELPCs resided in the lungs after cell transplantation.

Engineered ELPCs (1.5x10^6) were infused into the jugular vein of rats 3 days after monocrotaline injection. Twenty-five days after cell transplantation, lung cryosections were directly visualized under fluorescent microscopy. DAPI staining shows nuclei. Nuclear-localized red fluorescent protein (nuRFP) indicates transplanted engineered ELPCs.
Supplemental Figure S2

A

![Bar chart A]

B

![Bar chart B]

C

![Bar chart C]

Supplemental Figure S2. Treatment with control ELPCs or engineered ELPCs did not further affect hepatocellular and renal function. Three days after MCT injection, $1.5 \times 10^6$ cells were infused via the jugular vein. Chemical analysis was conducted on blood samples collected 25 days after cell transplantation ($n=6$ per group). ALT, alanine aminotransferase; BUN, blood urea nitrogen; CRE, creatinine; Normal, saline injection as MCT control; Vehicle, DMEM used for cell suspension; ELPCs-nuRFP, ELPCs expressing nuRFP only; ELPCs-nuRFP/CP, engineered ELPCs expressing nuRFP and COX1-PGIS fusion protein. *$P < 0.01$; **$P < 0.05$; $N$, no significant difference.
Supplemental Figure S3. Right ventricular systolic pressure (RVSP) was measured at different time points after monocrotaline (MCT) injection. Monocrotaline (60 mg/kg) was injected intraperitoneally, and RVSP was measured before injection (n=12) and at 3 days (n=12), 7 days (n=5), 14 days (n=6), 21 days (n=5), and 28 days (n=5) after injection. *P < 0.01 as compared with Day 0.
Supplemental Figure S4. The efficacies of 3 different dosing schemes of subcutaneous treprostinil (a synthetic prostacyclin analog) used to prevent MCT-induced RVSP increase were compared. Three days after monocrotaline (MCT) injection, treprostinil (TP) was administered subcutaneously according to 3 different dosing schemes: TP1, 0.05 mg/kg body weight, twice daily; TP2, 0.15 mg/kg body weight, twice daily; and TP3, 0.5 mg/kg body weight, once daily. RVSP was measured 28 days after MCT injection. TP3 was the least effective and was chosen as a “sub-optimal” dosing scheme for TP use in conjunction with cell therapy. Normal, saline injection (MCT control); Vehicle, Dulbecco’s modified Eagle’s medium. *P < 0.01, **P < 0.05.
Supplemental Figure S5. Engineered ELPCs and control ELPCs enhanced apoptosis in the adventitia of pulmonary vessel walls. A, Four weeks after monocrotaline (MCT) injection, lungs were harvested and cryosectioned. The sections were subjected to TUNEL staining (fluorescein-labeled) and
incubated with vWF and smooth muscle α-actin primary antibodies, which were visualized by AlexaFluor 647 and 594, respectively. DAPI staining shows nuclei. TUNEL+ cells (arrows) can primarily be seen in the adventitia. B, The number of TUNEL+ cells in the adventitia per pulmonary vessel section is shown. Data are presented as the mean ± standard error of the mean (SEM) (n=3 for each group). More than 50 vessels per rat were analyzed. Normal, saline injection (MCT control); Vehicle, Dulbecco’s modified Eagle’s medium (used for cell suspension); ELPCs-nuRFP, ELPCs expressing nuRFP only; ELPCs-nuRFP/CP, engineered ELPCs expressing nuRFP and COX1-PGIS fusion protein. *P < 0.01.
References


4. Ruan KH, So SP, Cervantes V, Wu H, Wijaya C, Jentzen RR. An active triple-catalytic hybrid enzyme engineered by linking cyclo-oxygenase isoform-1 to prostacyclin synthase that can constantly biosynthesize prostacyclin, the vascular protector. Febs J. 2008;275:5820-5829