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Bone Marrow Endothelial Progenitor Cells Are the Cellular Mediators of Pulmonary Hypertension in the Murine Monocrotaline Injury Model

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Key Words. Monocrotaline • Pulmonary hypertension • Endothelial progenitor cells • Extracellular vesicles • Mesenchymal stem cells

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ABSTRACT

The role of bone marrow (BM) cells in modulating pulmonary hypertensive responses is not well understood. Determine if BM-derived endothelial progenitor cells (EPCs) induce pulmonary hypertension (PH) and if this is attenuated by mesenchymal stem cell (MSC)-derived extracellular vesicles (EVs). Three BM populations were studied: (a) BM from vehicle and monocrotaline (MCT)-treated mice (PH induction), (b) BM from vehicle-, MCT-treated mice that received MSC-EV infusion after vehicle, MCT treatment (PH reversal, in vivo), (c) BM from vehicle-, MCT-treated mice cultured with MSC-EVs (PH reversal, in vitro). BM was separated into EPCs (sca-1+/c-kit+/VEGFR2+) and non-EPCs (sca-1-/c-kit-/VEGFR2-) and transplanted into healthy mice. Right ventricular (RV) hypertrophy was assessed by RV-to-left ventricle+septum (RV/LV+S) ratio and pulmonary vascular remodeling by blood vessel wall thickness-to-diameter (WT/D) ratio. EPCs but not non-EPCs from mice with MCT-induced PH (MCT-PH) increased RV/LV+S, WT/D ratios in healthy mice (PH induction). EPCs from MCT-PH mice treated with MSC-EVs did not increase RV/LV+S, WT/D ratios in healthy mice (PH reversal, in vivo). Similarly, EPCs from MCT-PH mice treated with MSC-EVs pre-transplantation did not increase RV/LV+S, WT/D ratios in healthy mice (PH reversal, in vitro). MSC-EV infusion reversed increases in BM-EPCs and increased lung tissue expression of EPC genes and their receptors/ligands in MCT-PH mice. These findings suggest that the pulmonary hypertensive effects of BM are mediated by EPCs and those MSC-EVs attenuate these effects. These findings provide new insights into the pathogenesis of PH and offer a potential target for development of novel PH therapies. STEM CELLS TRANSLATIONAL MEDICINE *2017;6:1595–1606*

SIGNIFICANCE STATEMENT

This work is significant as it demonstrates that there is an important connection between the bone marrow and pulmonary vasculature in the pathogenesis of pulmonary hypertension. Our data demonstrate that in monocrotaline model of pulmonary hypertension in mice, pulmonary vascular endothelial cells release extracellular vesicles that induce changes in bone marrow-derived endothelial progenitor cells, converting them into pathologic progenitors that traffic to the lung and induce pulmonary vascular remodeling. The pathologic properties of endothelial progenitor cells can be reversed by exposure to mesenchymal stem cell-derived extracellular vesicles, both in vitro and in vivo, and thus their ability to induce pulmonary hypertension in healthy mice.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a disease of unknown etiology that is characterized by pulmonary vascular remodeling causing a progressive elevation of pulmonary vascular resistance and subsequent right ventricular (RV) failure and death. A combination of increased pulmonary vascular tone, pulmonary vascular endothelial cell proliferation, pulmonary vascular smooth muscle cell transformation to fibromyocytes, extracellular matrix protein deposition and perivascular inflammation contribute to the vascular remodeling that is seen in PAH [1].

Mechanisms responsible for the pulmonary vascular remodeling in PAH are not fully understood, but recent findings suggest an intriguing connection between the bone marrow (BM) and the remodeled pulmonary vasculature. In particular, increased levels of circulating BM-derived

endothelial progenitor cells (EPCs) can be found in patients with PAH compared with normal controls [2, 3]. This may be due to the release of hypoxia-inducible factors (erythropoietin, HGF, SCF, SDF-1, VEGF) from the remodeled pulmonary vasculature into the circulation which, in turn, impacts BM differentiation and mobilization [3]. BM-derived EPCs can be found in remodeled blood vessels of patients with PAH, but whether these cells are reparative or pathogenic is uncertain [4]. Animal studies have demonstrated that BM-derived EPCs isolated from patients with PAH induce a pulmonary hypertension (PH) phenotype in mice [5] whereas infusion of EPCs isolated from healthy animals into rodent models of PH ameliorate the PH phenotype [6, 7]. Additionally, the impact of infused EPCs may be attributable to paracrine effects [8] and may be independent of long-term engraftment into the pulmonary vasculature [9]. In contrast, BM-derived mesenchymal stem cells (MSC) have been found to attenuate several pulmonary disease models in rodents including silica-induced pulmonary fibrosis [10], idiopathic pneumonia syndrome [11] and asthma [12]. Infused MSCs may do so independent of significant tissue engraftment, suggesting that some MSC-based paracrine factor may also be responsible for these beneficial effects.

Extracellular vesicles (EVs) are a diverse group of cell-derived vesicles which include exosomes and microvesicles. They are released from different cell types, including pulmonary vascular endothelial cells and MSCs, and have been shown to enter other cells and alter gene and protein expression [13–15]. Patients with PAH have higher circulating EV levels which correlate directly with pulmonary vascular resistance [16], functional impairment [17] and mortality [18]. Although initially considered a biomarker of disease severity, EVs from animals with PH can directly induce endothelial cell dysfunction [19]. Our group has shown that EVs isolated from the lungs and plasma of mice with monocrotaline-induced PH (MCT-PH) induces RV hypertrophy and pulmonary vascular remodeling when injected into normal mice [20]. BM-derived EPCs may be central in this process as lineage-depleted BM cells cultured with lung and plasma-derived EVs of MCT-injured mice have up regulated EPC gene expression and induce RV hypertrophy and pulmonary vascular remodeling when transplanted into healthy mice [20]. These findings suggest that MCT injury releases pathogenic EVs which influence BM differentiation, leading to the production of BM-derived progenitors that home to the lung and contribute to pulmonary vascular remodeling.

At the same time, other investigators have shown that MSCs and the EVs they secrete have reparative properties in a variety of diseases and conditions. For example, Monsel et al. have reported that microvesicles derived from human MSCs improved survival in a murine model of *Escherichia coli*-induced pneumonia, in part by enhancing bacterial phagocytosis by monocytes [21]. In addition, The Kourembanas lab has reported that conditioned media of cultured MSCs, which contains EVs as well as other secreted factors, can reverse hyperoxia-induced bronchopulmonary dysplasia and its associated pulmonary hypertension in newborn mice [22]. The same group has also reported that MSC-derived exosomes inhibit hypoxia-induced PH in mice and do so by suppression of hyperproliferative pathways, including STAT3-mediated signaling induced by hypoxia [23].

In present studies, we examined the role of BM-derived EPCs in the pathogenesis of PH and in MSC-mediated reversal of PH. We hypothesized that EVs released from the lungs induce EPC differentiation and release from the BM and homing to the lung where they contribute to pulmonary vascular remodeling. In addition, EVs released from BM-derived MSCs act to counter these effects.

Materials and Methods

Experimental Animals

All mouse studies were approved by the Institutional Animal Care and Use Committee at Rhode Island Hospital (CMTT# 0080-13). Six- to eight-week-old male C57BL/6 mice (Jackson Laboratories) were used for all studies (535 total mice were used).

Isolation and Transplantation of Whole Bone Marrow Cells from MCT-Injured or Vehicle-Injected Mice

The goal of these experiments was to determine if BM cells isolated from mice with MCT-PH were capable of inducing pulmonary hypertensive changes after transplantation into healthy mice (Supporting Information Fig. 1, Study Protocol). In these experiments, mice received weekly subcutaneous injections of MCT (600 mg/kg, Sigma) or PBS (vehicle) for 4 weeks. One week after the last series of injections, mice were sacrificed for BM cell harvest or for analysis as described below. Whole bone marrow (WBM) cells were isolated by flushing tibiae, femurs, and iliac crests with PBS supplemented with 5% fetal bovine serum (FBS). Cells were strained through a 40 μm cell strainer then centrifuged (300*g*, 10 minutes) and resuspended in PBS/5% FBS. Cells were counted and viability was determined using trypan blue staining. 2×10^6 WBM cells isolated individually from each mouse were then infused via tail vein into lethally-irradiated mice (950 centigray, Gammacell 40 Exactor Irradiator, MDS Nordion) so each individual transplant recipient received WBM from only one WBM donor mouse. Mice were sacrificed 4 weeks later for analysis.

Lung Histology

Right and left lungs were embedded separately in paraffin and hematoxylin and eosin staining was performed on five micron sections from each paraffin block. The first 10 blood vessels (on end, diameters 20–50 μm), were analyzed. The pulmonary vessel wall thickness-to-blood vessel wall diameter (WT/D) ratio was determined by measuring the thickness of the vessel wall (internal lamina to adventitia) and dividing by the diameter of the blood vessel (adventitia to adventitia).

Measurement of RV Hypertrophy

RV free walls were dissected off the interventricular septae and weighed. Wet weight measurements were normalized to body weight (mg/g) and expressed as the RV free wall-to-septum+left ventricle ratio (RV/LV+S).

Lung-Derived Extracellular Vesicle Isolation and Characterization

Lungs from MCT-injured and vehicle-injected mice were filled with dispase (Sigma) and incubated for 45 minutes on ice. Lungs were then mechanically dissociated with forceps into a single cell suspension, filtered through a 40 μm cell strainer and washed with PBS by centrifugation (300*g* for 10 minutes, 4°C). Lung cells were cultured (1×10^6 cells/ml) in Bronchial Epithelial Growth Media (BEGM, Lonza), supplemented with 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 52 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 pg/ml

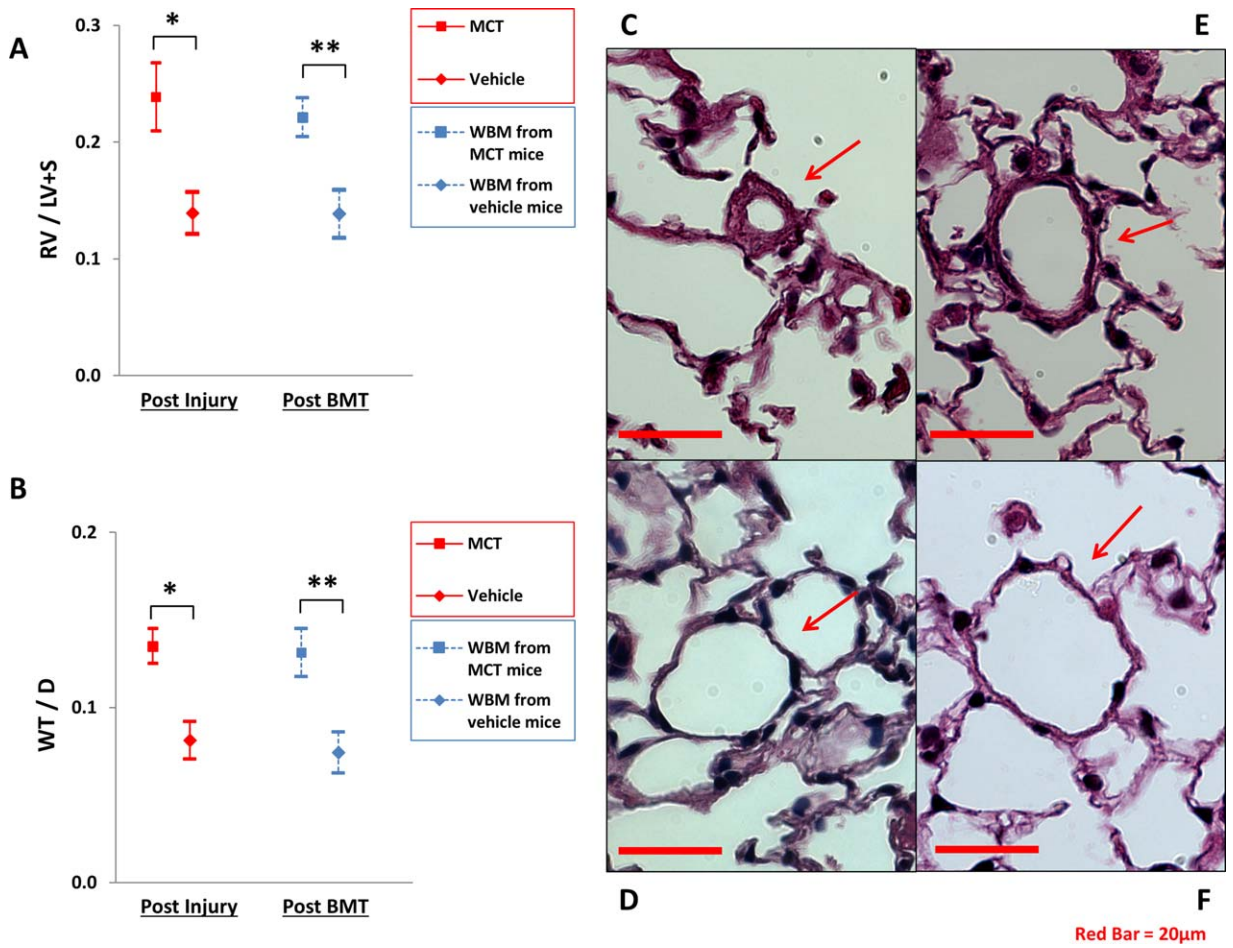

Figure 1. Mice transplanted with bone marrow cells from mice with MCT-induced pulmonary hypertension develop RV hypertrophy and pulmonary vascular remodeling. **(A)**: RV/LV+S ratio, **(B)**: WT/D ratio of MCT-injured (red boxes) and vehicle-injected (red diamonds) mice and ratios 4 weeks after transplantation of bone marrow cells from MCT-injured (blue boxes) or vehicle-injected (blue diamonds) mice. $n = 5$ mice/cohort; *, $p < .05$ versus vehicle-injected mice; **, $p < .05$ versus transplant recipients of bone marrow from vehicle-treated mice. Data presented as means with 95% confidence intervals. Hematoxylin and eosin stained lung sections from **(C)**: MCT-injured mice, **(D)**: vehicle-injected mice, **(E)**: mice transplanted with WBM from MCT-injured mice, and **(F)**: mice transplanted with WBM from vehicle-injected mice. (Red arrow pointing to a pulmonary blood vessel, Red bar = 20 µm). Abbreviations: MCT, monocrotaline; RV, right ventricular; RV/LV+S, RV-to-left ventricle+septum; WBM, whole bone marrow cells; WT/D, wall thickness-to-diameter.

human recombinant epidermal growth factor, 6.5 ng/ml triiodothyronine and exosome-depleted FBS at 37°C/5% CO_2 for 2 days. Prior to use, FBS was ultracentrifuged at 10,000*g* for 1 hour. Pelleted material was discarded and the supernatant was ultracentrifuged at 100,000*g* for 1 hour. The supernatant, containing FBS depleted of exosomes, was then used. Cells were then removed by centrifugation (300*g*, 10 minutes) and the supernatant was ultracentrifuged (100,000*g*, 1 hour). Pelleted material, containing EVs (primarily exosomes and microvesicles), were then quantified using the BCA Protein Assay Kit (Pierce), as per manufacturer's instructions.

Lung-Derived Extracellular Vesicles (LDEVs) isolated by these methods have been fully characterized as described in recent publications by our group [20, 24]. Proteomic analysis of our LDEVs demonstrated the presence of proteins characteristic of both exosomes (including various tetraspanins, such as CD63) and microvesicles (including CD40 ligand) [20]. In addition, western blot analyses of LDEV have confirmed the presence of CD63 [24]. NanoSight NS5000 and transmission electron microscopy analyses revealed EVs in the size range of exosomes and microvesicles [24].

Since lung cells used in these studies are isolated from a single cell suspension of a whole lung digest, the specific population(s) of lung cells from which LDEVs are derived is quite heterogeneous and includes a variety of pulmonary epithelial, endothelial and interstitial cells and hematopoietic cells. Based on RT-PCR [14] and proteomic [20] analyses of EVs isolated from lung cells cultured by these methods, we have shown LDEVs reflect the heterogeneity of their cells of origin. LDEVs are enriched with type I (aquaporin-5) and type II (surfactants A-D) pneumocyte-specific mRNAs [14], as well endothelial-, myeloid-, lymphoid-, erythroid-, and platelet-specific proteins [20].

Transplantation of WBM Cells from Mice with Pulmonary Hypertension Induced by LDEVs from MCT-Injured Mice

The goal of these experiments was to determine if mice that developed PH after infusion of LDEV from MCT-injured mice were capable of transferring this disease phenotype to healthy mice via their BM cells (Supporting Information Fig. 2, Study Protocol). In these experiments, 1 week after the last series of MCT or vehicle

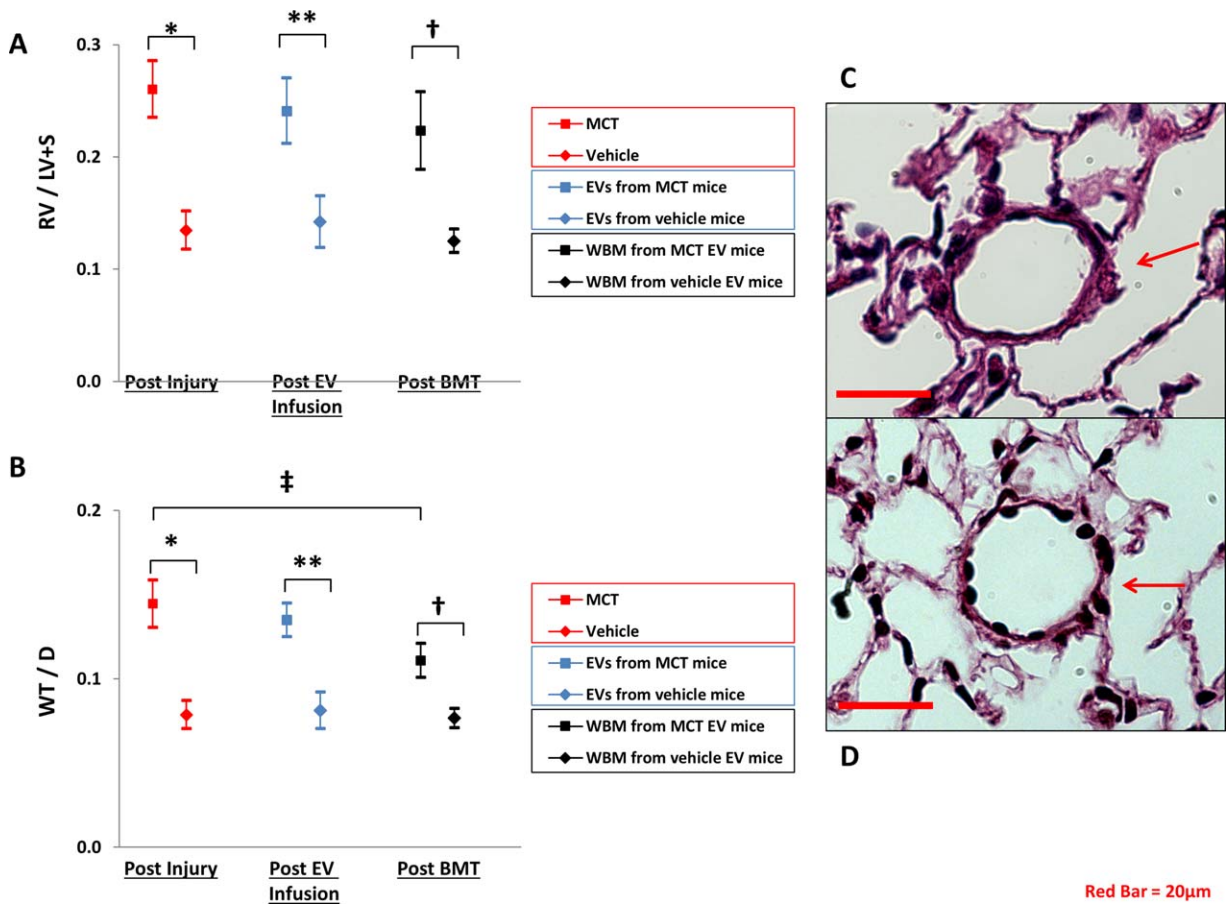

Figure 2. Mice transplanted with bone marrow cells from mice with EV-induced pulmonary hypertension develop RV hypertrophy and pulmonary vascular remodeling. **(A)**: RV/LV+S ratio, **(B)**: WT/D ratio of MCT-injured (red boxes) and vehicle-injected (red diamonds) mice, ratios 4 weeks after infusion of lung EVs isolated from MCT-injured (blue boxes) or vehicle-injected (blue diamonds) mice, ratios 4 weeks after transplantation of WBM isolated from mice infused with EVs from MCT-injured mice (black boxes) or vehicle-injected mice (black diamonds). $n = 5$ mice/cohort; *, $p < .05$ versus vehicle-injected mice; **, $p < .05$ versus mice injected with EVs from vehicle-injected mice; †, $p < .05$ versus mice transplanted with WBM from mice injected with EVs from vehicle-injected mice; ‡, $p < .05$ versus MCT-injured mice. Data presented as means with 95% confidence intervals. Hematoxylin and eosin stained lung sections from **(C)**: mice transplanted with WBM from mice infused with EVs from MCT-injured mice, **(D)**: mice transplanted with WBM from mice infused with EVs from vehicle-injected mice. (Red arrow pointing to a pulmonary blood vessel, Red bar = 20 μm). Abbreviations: EV, extracellular vesicles; MCT, monocrotaline; RV, right ventricular; RV/LV+S, RV-to-left ventricle+septum; WBM, whole bone marrow cells; WT/D, wall thickness-to-diameter.

injections, mice received daily tail vein injections lung-derived EVs, 25 ug/day or an equal volume of PBS, for a total of 3 days. 25 μg of EVs were isolated from approximately 2–4 × 10^6 cultured lungs cells. Four weeks after the last series of injections, mice were sacrificed for BM cell harvest and for analysis. 2 × 10^6 WBM cells isolated individually from each mouse were then infused (tail vein) into lethally-irradiated mice so that cells from one WBM donor was infused into one transplant recipient. Mice were sacrificed 4 weeks later for analysis.

EPC Quantification

Peripheral blood (0.5 ml) was collected from mice and stored in a heparinized tube. Additionally, BM from one femur was flushed with PBS supplemented with 10% exosome-depleted FBS. Red blood cells were lysed using ammonium chloride and cells were labeled with the following anti-mouse antibodies (BD Pharmingen): Fluorescein isothiocyanate (FITC)-conjugated sca-1; Phycoerythrin (PE)-conjugated c-kit; Allophycocyanin (APC)-conjugated VEGFR2. Samples were analyzed using an LSR II flow cytometer (Becton Dickinson) and EPCs were defined as sca-1+/c-kit+/VEGFR2+ cells. EPCs were quantified per 50,000 peripheral blood or BM leukocytes, as defined by the forward and side scatter properties of each sample.

Transplantation of Bone Marrow-Derived EPCs from MCT-PH and Control Mice

The goal of these experiments was to determine if EPCs are the BM cell population responsible for inducing PH after transplantation into healthy mice (Supporting Information Fig. 3, Study Protocol). In these experiments, 1 week after the last series of MCT or vehicle injections, mice were sacrificed and whole BM cells isolated from femurs and tibiae. Red blood cells were lysed using ammonium chloride and cells were labeled with FITC-conjugated sca-1, PE-conjugated and APC-conjugated VEGFR2 antibodies, as described above. Sca-1+/c-kit+/VEGFR2+ cells (EPCs) and Sca-1−/c-kit−/VEGFR2− cells (non-EPC cells) were separated using a 5 laser Becton Dickenson/Cytopeia Influx High Speed Cell Sorter from individual MCT-injured ($n = 5$) and vehicle-injected ($n = 5$) mice and kept separate. Cohorts of lethally-irradiated mice (950 centigray) were then infused, via tail vein, with EPCs from MCT-

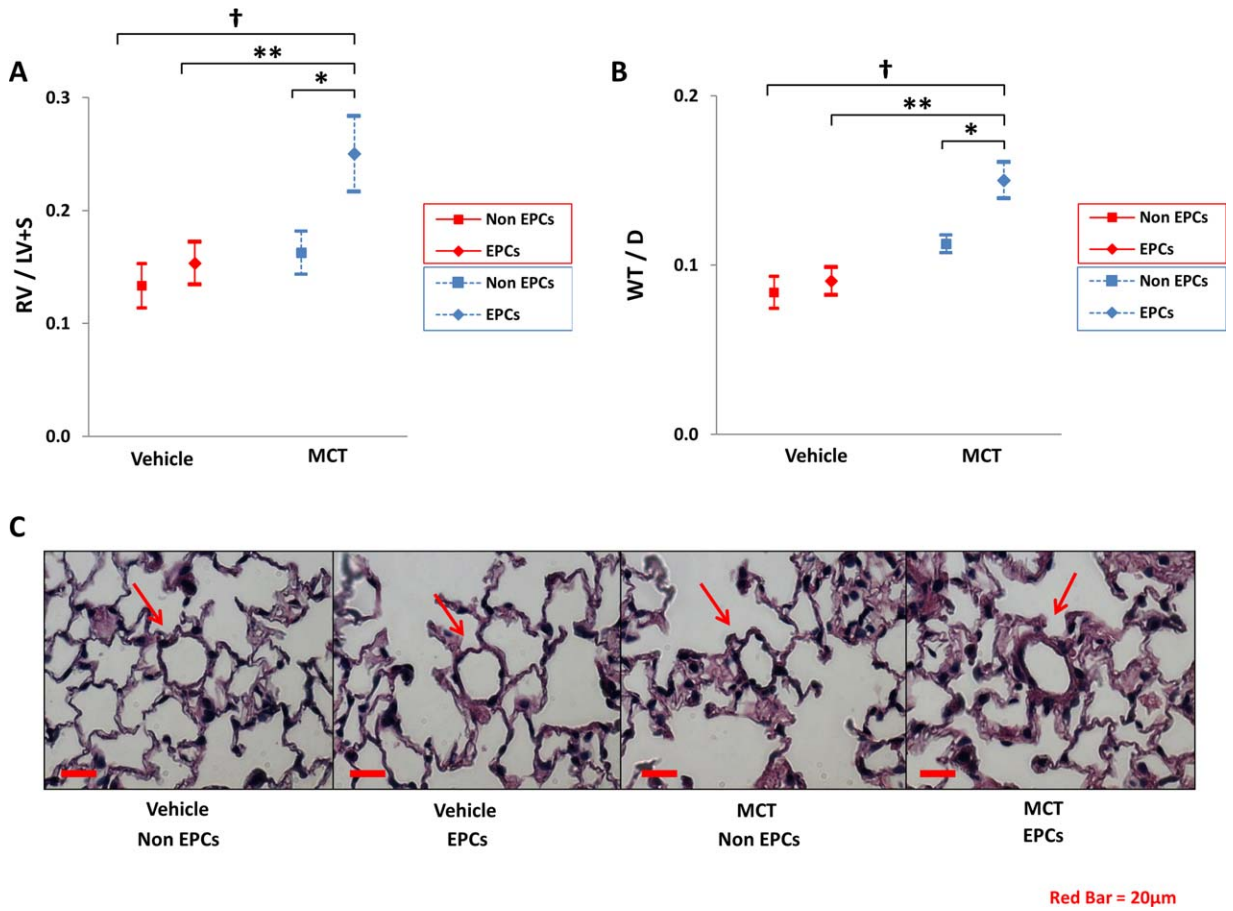

Figure 3. Transplantation of EPCs and non-EPC cells from MCT-injured and vehicle-injected mice. **(A)**: RV/LV+S ratio, **(B)**: WT/D ratio 4 weeks after transplantation of bone marrow-derived EPCs (sca-1+/c-kit+/VEGFR2+ cells, diamonds) and non-EPC cells (sca-1−/c-kit−/VEGFR2−, boxes) isolated from MCT-injured (blue) and vehicle-injected (red) mice. $n = 5$ mice/cohort, *, $p < .05$ versus mice transplanted with non-EPC cells from MCT-injured mice. **, $p < .05$ versus mice transplanted with EPCs from vehicle-injected mice. †, $p < .05$ versus mice transplanted with non-EPC cells from vehicle-injected mice. Data presented as means with 95% confidence intervals. **(C)**: Hematoxylin and eosin staining of lung sections from each cohort. (Red arrow pointing to a pulmonary blood vessel, Red bar = 20 μm). Abbreviations: EPC, endothelial progenitor cells; MCT, monocrotaline; RV/LV+S, right ventricular-to-left ventricle+septum; WT/D, wall thickness-to-diameter.

injured mice (average, 1.8×10^3 cells/mice), EPCs from vehicle-injected mice (average, 1.5×10^3 cells/mice), non-EPCs from MCT-injured mice (average, 6.0×10^5 cells/mice) or non-EPCs from vehicle-injected mice (average, 6.0×10^5 cells/mice). In addition, all mice were transplanted with 3×10^5 unfractionated WBM cells isolated from healthy male C57BL/6 mice to ensure survival after myeloablation. Mice were sacrificed 4 weeks later for analysis.

Isolation and Culture of MSCs

MSCs were isolated and cultured as described by Zhu et al. [25]. Briefly, femurs, tibiae and iliac crests were removed from 10 mice and flushed with α-MEM (Hyclone) supplemented with 2 mM L-glutamine and 10% exosome-depleted FBS to expel hematopoietic marrow cells. Bones were broken into 1–3 mm chips and collagenase-digested for 2 hours at 37°C. Bones were rinsed and placed in a 25 cm^2 plastic culture flask with α-MEM supplemented with 10% FBS and incubated at 37°C for 3 days. On day 3, the culture medium was changed to remove non-adherent cells and tissue debris. On day 5, bone chips and adherent cells (removed with 0.25% trypsin/0.02% EDTA) were reseeded into new flasks and incubated at 37°C. Culture medium was changed every 48 hours. Immunotyping characterization of cells occurred at passage four confirming the presence of CD29+, CD44+ and Sca-1+ but not CD31−, CD45− and CD86− adherent cells. Adipogenic, chondrogenic, and osteogenic differentiation assays were performed at passage five confirming the differentiation capacity of cells into osteoblasts, adipocytes, and chondrocytes.

Isolation and Characterization of MSC-Derived EVs

MSC-derived EVs were isolated from cell-free culture media obtained from MSC culture flasks at passage eight. As MSCs grown using this protocol can be maintained for up to 10 passages, EVs isolated at this time are derived from true MSCs. Cells were removed from culture media by centrifugation and EVs were isolated and quantified as described above. EV preparations were quantified based on their protein content, as determined by using the BCA Protein Assay Kit (Pierce).

MSC-derived EVs isolated using these methods have been fully characterized as described in a recent publication by our group [24]. Western blot analyses of these EVs have confirmed the presence of CD63. NanoSight NS5000 and transmission electron microscopy analyses revealed EVs in the size range of exosomes and microvesicles.

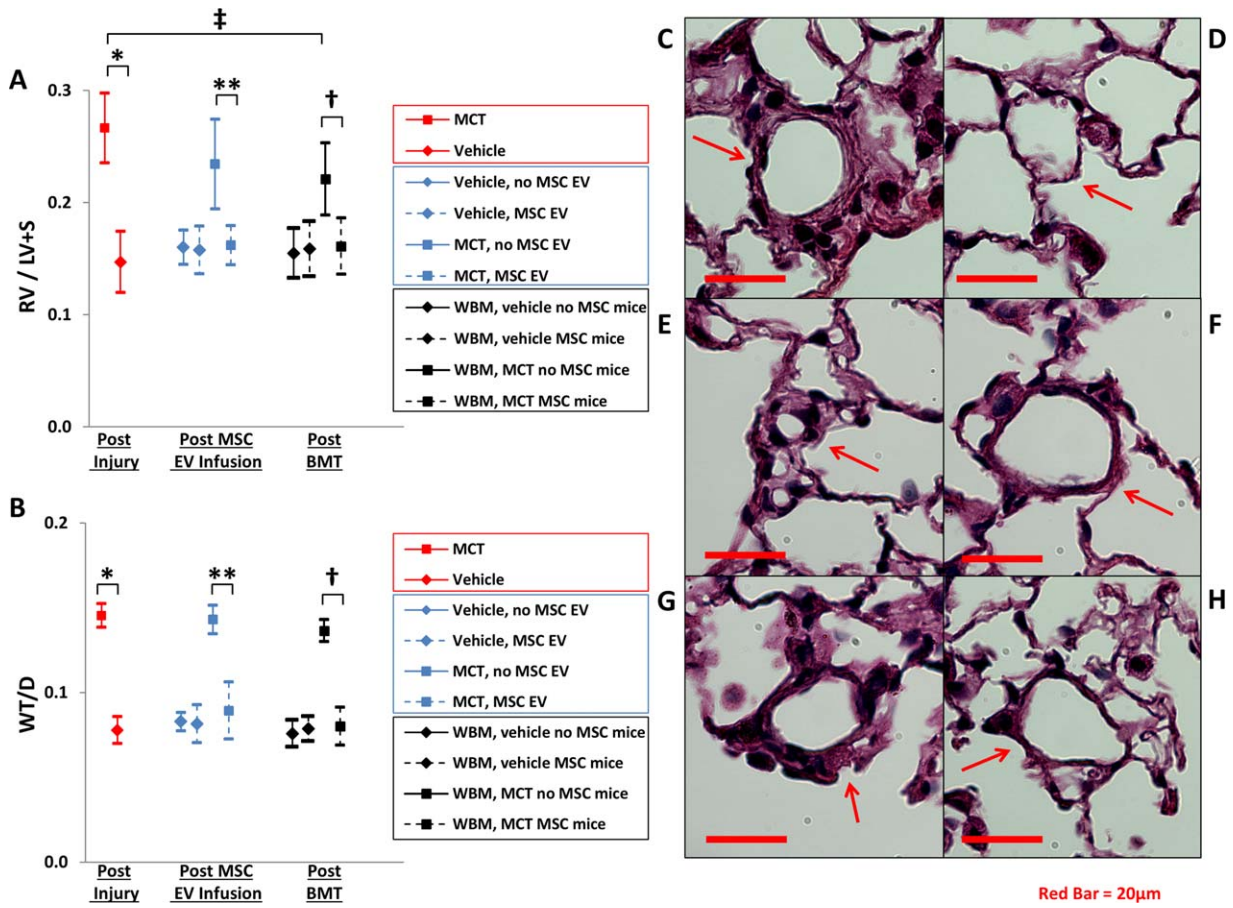

Figure 4. MSC-EV treatment prevents RV hypertrophy and pulmonary vascular remodeling from being transferred via bone marrow transplantation. **(A)**: RV/LV+S ratio, **(B)**: WT/D ratio of MCT-injured (red boxes) or vehicle-injected (red diamonds) mice, MCT-injured or vehicle-injected mice 4 weeks after treatment with MSC-EVs or vehicle (blue boxes, diamonds), recipients 4 weeks after bone marrow transplant using WBM isolated from the MCT-injured and vehicle-injected mice that were treated with MSC-EVs or vehicle (black boxes, diamonds), $n = 5$ mice/cohort; *, $p < .05$ versus vehicle-injected mice; **, $p < .05$ versus MCT-injured mice treated with MSC-EVs; †, $p < .05$ versus mice transplanted with WBM from MCT-injured mice treated with MSC-EVs; ‡, $p < .05$ versus MCT-injured mice. Data presented as means with 95% confidence intervals. Hematoxylin and eosin stained lung sections from **(C)**: MCT-injured mice, **(D)**: vehicle-injected mice, **(E)**: MCT-injured mice treated with vehicle, **(F)**: MCT-injured mice treated with MSC-EVs, **(G)**: mice transplanted with WBM isolated from MCT-injured mice treated with vehicle, **(H)**: mice transplanted with WBM isolated from MCT-injured mice treated with MSC-E versus (Red arrow pointing to a pulmonary blood vessel, Red bar = 20 μm). Abbreviations: MCT, monocrotaline; MSC-EV mesenchymal stem cell-extracellular vesicles; RV, right ventricular; RV/LV+S, RV-to-left ventricle+septum; WBM, whole bone marrow cells; WT/D, wall thickness-to-diameter.

MCT Injury Reversal Studies Using mMSC-EVs

The goal of these experiments was to determine if an infusion of MSC-EVs could reverse pulmonary hypertensive changes in mice with MCT-PH (Supporting Information Fig. 4, Study Protocol). In addition, we wished to determine if BM cells from mice with MCT-PH that had been treated with MSC-EVs no longer transferred a pulmonary hypertensive phenotype when transplanted into healthy mice. In these experiments, mice received weekly subcutaneous injections of MCT (600 mg/kg, Sigma) or PBS (vehicle) for 4 weeks. One week after the last series of injections, mice were sacrificed for BM cell harvest or for analysis as described below. Alternatively, other cohorts of mice received daily tail vein injections of MSC-EVs, 25 μg/day, or an equal volume of PBS, for a total of 3 days. Twenty five micrograms of EVs were isolated from approximately 2–4 $\times$ 10^6 cultured MSCs. Four weeks after the last series of injections, mice were sacrificed for analysis and for BM harvest. 2 $\times$ 10^6 WBM cells were isolated from femurs and tibiae of vehicle-injected or MCT-injured mice that were treated with MSC-EVs or vehicle. WBM cells were infused (tail vein) into cohorts of lethally-irradiated mice so that cells from one WBM donor was infused into one transplant recipient. Mice were sacrificed 4 weeks later for analysis.

RNA Extraction from Paraffin-Embedded Lung Tissue

RNA was extracted from paraffin-embedded lung tissue using the RecoverAll Multi-Sample RNA/DNA Workflow kit (Life Technologies), per manufacturer's instructions. Briefly, lung samples were de-paraffinized with xylene then protease-digested. RNA was then isolated using a series of proprietary isolation additives and wash buffers.

mRNA Analysis

Total RNA extracted from lung tissue was measured for quantity and quality (260/280 ratio) using a Nanodrop ND/1000 spectrophotometer (Thermo Scientific). For each sample, 10 ng of RNA was used to amplify cDNA using the High Capacity cDNA transcription kit (Life Technologies). cDNA amplification reactions were performed on a 9800 Fast Thermal Cycler (Life Technologies) and

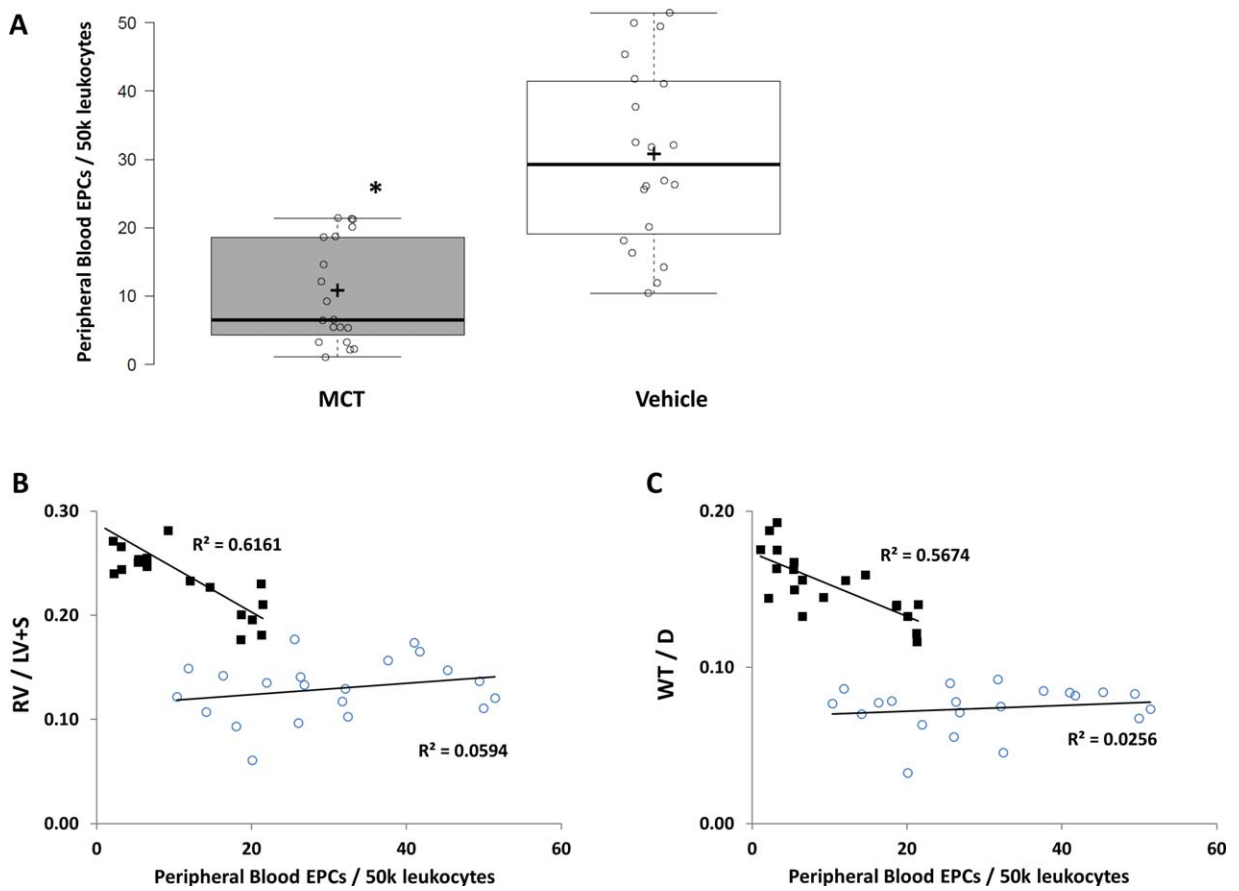

Figure 5. Peripheral blood EPCs in MCT-injured and vehicle-injected mice. **(A)**: Peripheral blood EPCs (sca-1+/c-kit+/VEGFR2+ cells) per 50,000 peripheral blood leukocytes in MCT-injured and (dark grey) and vehicle-injected (white) mice. Box and whisker plots show the median (line), mean (X), 25th and 75th percentiles and 10th and 90th percentiles (error bars). $n = 20$ mice/cohort, *, $p < .01$ versus vehicle-injected mice. Linear regression analysis **(B)**: RV/LV+S, **(C)**: WT/D ratio versus peripheral blood EPCs per 50,000 peripheral blood leukocytes in MCT-injured (black squares) and vehicle-injected mice (white circles). $n = 20$ mice/cohort; $R^2 = 0.609$, 0.026 respectively, RV/LV+S, $p < .05$; $R^2 = 0.636$, 0.048 respectively, WT/D, $p < .05$. Abbreviations: EPC, endothelial progenitor cells; MCT, monocrotaline; RV, right ventricular; RV/LV+S, RV-to-left ventricle+septum; WT/D, wall thickness-to-diameter.

consisted of one cycle (10 minutes, 25°C), one cycle (120 minutes, 37°C), and one cycle (5 minutes, 85°C). All Real Time RT-PCR reactions were performed 7900HT Fast RT PCR System using the following murine primers: β2 microglobulin (housekeeping gene, Mm00437762_m1); Sca-1 (Mm00726565_s1) and its receptor, αVβ3 (Mm00434486_m1); c-kit (Mm00445212_m1) and its ligand stem cell factor (SCF, Mm00442972_m1), VEGFR2 (Mm01222421_m1) and its ligand VEGFA (Mm00437306_m1). Duplicate reactions of the target and housekeeping genes were performed simultaneously for each cDNA template. The PCR reaction consisted of an initial enzyme activation step at (10 minutes, 95°C) followed by 40 cycles (15 seconds, 95°C; 1 minute, 60°C). A cycle threshold value (CT) value was obtained for each sample, and duplicate sample values were averaged. The $2^{-\Delta\Delta CT}$ method was used to calculate relative expression of each target gene [26].

Bone Marrow Transplant Studies Using Marrow Cells Isolated from MCT-Injured Mice Cultured with MSC-EVs

The goal of these experiments was to determine if MSC-EVs were capable of preventing BM-derived EPCs isolated from MCT-injured mice from inducing PH when transplanted in healthy animals (Supporting Information Fig. 5, Study Protocol). In these experiments, 2×10^6 WBM cells were isolated from femurs and tibiae of individual vehicle-injected or MCT-injured mice and plated into individual six-well plastic culture plates (USA Scientific) in 5 ml of DMEM-glutamax at 2.1×10^5 cells/cm^2 (one cell culture well containing cells from one mouse). Twenty five micrograms MSC-EVs in 100 μl PBS or an equal volume of vehicle were added to culture wells and cultures were maintained at 37°C for 48 hours. Cells were then washed, labeled with FITC-conjugated sca-1, PE-conjugated c-kit and APC-conjugated VEGFR2 antibodies and separated into EPCs and non-EPC cells by flow cytometry. Cohorts of lethally-irradiated mice were infused with all EPCs or non-EPC cells isolated from individual culture wells (one mouse transplanted with all EPCs and one mouse transplanted with all non-EPC cells from an individual culture well, see supplemental online Table 1 for # cells transplanted and # mice transplanted in each cohort). Some culture wells failed to yield any EPCs but an ample number of non-EPC cells. When this occurred, one mouse was transplanted with non-EPC cells from that culture well but no mouse was transplanted with EPCs. In addition, all mice were transplanted with 3×10^5 unfractionated WBM cells isolated from healthy male C57BL/6 mice to ensure survival after myeloablation. Four weeks after transplantation, mice were sacrificed for analysis.

Statistical Analysis

Analyses were run using SAS Software 9.4 (SAS Inc.) with the GLIMMIX Q3 procedure. Analyses were accomplished using generalized estimating equations assuming a normal distribution with Sandwich estimation. Multiple comparisons were examined using Tukey corrections. Significance was established at the 0.05 level, and all interval estimates were calculated for 95% confidence. Data are presented as mean values with 95% confidence intervals.

RESULTS

Bone Marrow Cells from Pulmonary Hypertensive Mice Induce PH When Transplanted into Healthy Mice

To determine if BM cells isolated from mice with MCT-PH are capable of inducing pulmonary hypertensive changes in healthy mice, cohorts of healthy irradiated mice were transplanted with WBM cells isolated from mice with established MCT-PH and from vehicle-injected mice (supplemental online Fig. 1, study protocol). Four weeks after transplantation, mice transplanted with WBM cells isolated from MCT-PH mice had significantly increased RV/LV+S and WT/D ratios compared to control mice or mice transplanted with WBM cells from control mice ($p < .05$) (Fig. 1). There was no difference in RV/LV+S or WT/D between mice with MCT-PH and mice transplanted with WBM from mice with MCT-PH.

In an earlier study, we found that injection of EVs isolated from the blood or lungs of mice with MCT-PH induced RV hypertrophy and pulmonary vascular remodeling when injected into healthy mice. To determine if mice that developed PH in this manner are capable of transferring this disease phenotype to healthy mice via their BM cells, cohorts of healthy mice were infused with EVs isolated from the lungs of mice with MCT-PH or from the lungs of mice injected with vehicle. Four weeks after EV infusion, mice were sacrificed for analysis and their BM cells were harvested. WBM from mice with MCT lung EV-induced PH and mice infused with control lung EVs was transplanted into cohorts of lethally-irradiated mice. Four weeks after transplantation, mice were sacrificed for analysis (supplemental online Fig. 2, study protocol). RV/LV+S and WT/D ratios were significantly higher in mice transplanted with WBM harvested from mice with MCT lung EV-induced PH compared with mice transplanted with WBM harvested from mice infused with control lung EVs ($p < .05$) (Fig. 2). RV/LV+S ratios in mice transplanted with WBM cells from mice treated with MCT lung EVs were similar to MCT-PH mice. However, WT/D ratios were significantly lower in mice transplanted with WBM cells from mice treated with MCT lung EVs compared with MCT-PH mice ($p < .05$).

Mice Transplanted with EPCs from MCT-Injured Mice Develop RV Hypertrophy

To determine if EPCs are the BM cell population that is responsible for inducing PH in healthy mice, additional experiments were done in which WBM cells were separated into EPCs and non-EPC fractions prior to transplantation. Cohorts of lethally-irradiated mice were transplanted with EPCs (sca-1+/c-kit+/VEGFR2+) and non-EPC cells (sca-1−/c-kit−/VEGFR2−) isolated from the BM of MCT-PH and control mice (Supporting Information Fig. 3, study protocol). Mice transplanted with BM-derived EPCs from MCT-injured mice had significantly elevated RV/LV+S and WT/D ratios compared with mice transplanted with non-EPCs from MCT-PH mice or EPCs and non-EPCs from control mice ($p < .05$) (Fig. 3). RV/LV+S and WT/D ratios in mice transplanted with non-EPC cells from MCT-injured mice were not significantly different compared with mice transplanted with EPCs and non-EPC cells from vehicle mice.

MSC-EV Treatment Prevents RV Hypertrophy and Pulmonary Vascular Remodeling from Being Transferred via Bone Marrow Transplantation

In previous studies, we found that infusion of MSC-EVs reverses MCT-PH whereas infusion of EVs isolated from lung tissue and a non-MSC BM cell population (lineage-depleted BM cells) fails to reverse MCT-PH [24]. In order to determine if MSC-EV infusion also prevents WBM cells from MCT-PH from inducing PH we performed additional studies. Cohorts of MCT-PH and control mice received tail vein injections of MSC-derived EVs, 25 μg once daily for 3 days, or an equal volume of PBS (vehicle), once daily for 3 days, beginning 1 week after the last injection of MCT or vehicle alone (control mice). Four weeks later, mice were sacrificed for analysis and for BM harvest. Cohorts of lethally-irradiated mice were then transplanted with WBM cells isolated from these mice. Four weeks after transplantation, mice were sacrificed for analysis (supplemental online Fig. 4, study protocol). MCT-injured mice that were then infused with MSC-EVs had significantly lower RV/LV+S and WT/D ratios compared with MCT-injured mice that were infused with vehicle ($p < .05$) (Fig. 4). Mice that were transplanted with WBM cells harvested from MCT-injured mice treated with vehicle had RV/LV+S and WT/D ratios that were significantly elevated compared with those of all other transplant recipient cohorts ($p < .05$). However, mice that were transplanted with WBM cells harvested from MCT-injured mice treated with MSC-EVs had RV/LV+S and WT/D ratios that were similar to those of control mice.

MSC-EV Treatment Reverses EPC Level Alterations in MCT-Injured Mice

To determine if MCT injury influences the quantity of resident and circulating BM-derived EPCs, BM and peripheral blood was isolated from MCT-injured and vehicle-injected mice and EPCs were quantified. Peripheral blood EPC (sca-1+/c-kit+, VEGFR2+ cells) levels were significantly lower in MCT-injured mice compared to vehicle-injected mice ($p < .05$) (Fig. 5A). Linear regression analysis revealed a strong negative correlation between RV/LV+S (Fig. 5B), WT/D ratios (Fig. 5C) and peripheral blood EPC levels in MCT-injured mice, but not in vehicle-injected mice indicating that increases in RV mass were associated with lower levels of circulating EPCs. However, BM EPCs levels were significantly higher in MCT-injured mice compared to vehicle-injected mice ($p < .05$) (Fig. 6A). There was a strong positive correlation between RV/LV+S (Fig. 6B), WT/D (Fig. 6C) ratios and BM EPC levels in MCT-injured mice indicating that increases in RV mass were associated with higher levels of BM EPCs.

Peripheral blood EPC levels were significantly higher in MCT-injured mice treated with MSC-EVs compared to MCT-injured mice treated with vehicle ($p < .01$) but similar to vehicle-injected mice treated with vehicle (Supporting Information Fig. 6A). In addition, BM EPCs levels were significantly lower in MCT-injured mice treated with MSC-EVs compared to MCT-injured mice treated with vehicle ($p < .01$) but similar to vehicle-injected mice treated with vehicle (Supporting Information Fig. 6B).

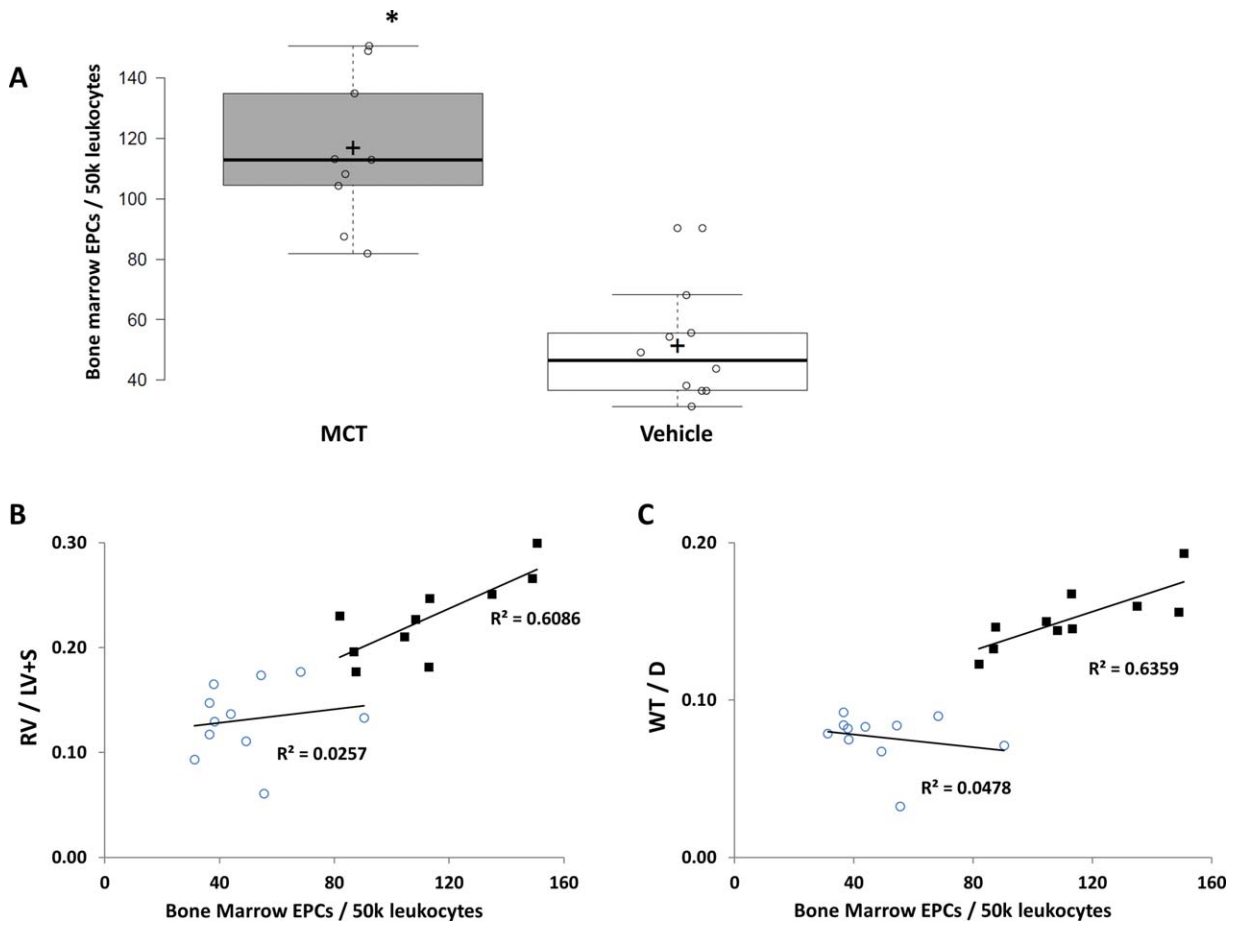

Figure 6. Bone marrow EPCs in MCT-injured and vehicle-injected mice. **(A)**: Bone marrow EPCs (sca-1+/c-kit+/VEGFR2+ cells) per 50,000 bone marrow leukocytes in MCT-injured (dark grey) and vehicle-injected (white) mice. Box and whisker plots show the median (line), mean (X), 25th and 75th percentiles and 10th and 90th percentiles (error bars). $n = 10$ mice/cohort, * $p < .01$ versus vehicle-injected mice. Linear regression analysis **(B)**: RV/LV+S, **(C)**: WT/D ratio versus bone marrow EPCs per 50,000 bone marrow leukocytes in MCT-injured (black squares) and vehicle-injected mice (white circles). $n = 10$ mice/cohort; $R^2 = 0.616$, 0.059 respectively, RV/LV+S, $p < .05$; $R^2 = 0.567$, 0.026 respectively, WT/D, $p < .05$. Abbreviations: EPC, endothelial progenitor cells; MCT, monocrotaline; RV, right ventricular; RV/LV+S, RV-to-left ventricle+septum; WT/D, wall thickness-to-diameter.

MSC-EV Treatment Alters Lung Tissue Gene Expression in MCT-Injured Mice

RT-PCR analysis, focusing on the expression of EPC genes and genes for their ligands or receptors ($\alpha V\beta 3$ for Sca-1, SCF for c-kit; VEGFRA for VEGFR2), was performed on lung tissue isolated from MCT-injured mice treated with MSC-EVs or vehicle. Lung tissue isolated from MCT-injured mice treated with vehicle had increased expression of the EPC genes sca-1, c-kit and VEGFR2 and their ligands or receptors ($\alpha V\beta 3$, SCF, and VEGFRA, respectively) compared with lung tissue isolated from control mice (supplemental online Fig. 7). In MCT-injured mice treated with MSC-EVs, lung tissue expression of all of these genes was significantly lower than expression in lung tissue isolated from MCT-injured mice treated with vehicle ($p < .05$) but no different than expression in lung tissue isolated from control mice.

EPCs From MCT-Injured Mice Cultured with MSC-EVs Do Not Induce RV Hypertrophy or Pulmonary Vascular Remodeling

To determine if MSC-EVs can prevent BM-derived EPCs from MCT-injured mice from inducing PH when transplanted in healthy animals, we performed additional experiments in which the BM-derived EPCs from MCT-PH mice were exposed to MSC-EVs prior to transplantation. WBM cells from MCT-PH and control mice were cultured with or without MSC-EVs for 48 hours before EPCs and non-EPC cells were isolated by flow cytometry and transplanted into lethally-irradiated mice (supplemental online Table 1). Four weeks after transplantation, mice were sacrificed for analysis (supplemental online Fig. 5, study protocol). RV/LV+S and WT/D ratios were significantly higher in mice transplanted with EPCs from MCT-injured mice that were not cultured with MSC-EVs compared to mice transplanted with EPCs from MCT-injured mice that were cultured with MSC-EVs ($p < .05$) (Fig. 7). RV/LV+S and WT/D ratios were not significantly different in mice transplanted with EPCs from MCT-injured mice that were cultured with MSC-EVs compared to mice from all other transplantation cohorts.

Discussion

In the present study, we found that the infusion of only the EPC fraction (Sca-1+/c-kit+/VEGFR2+ cells) of WBM cells isolated from mice with MCT-PH resulted in significant pulmonary

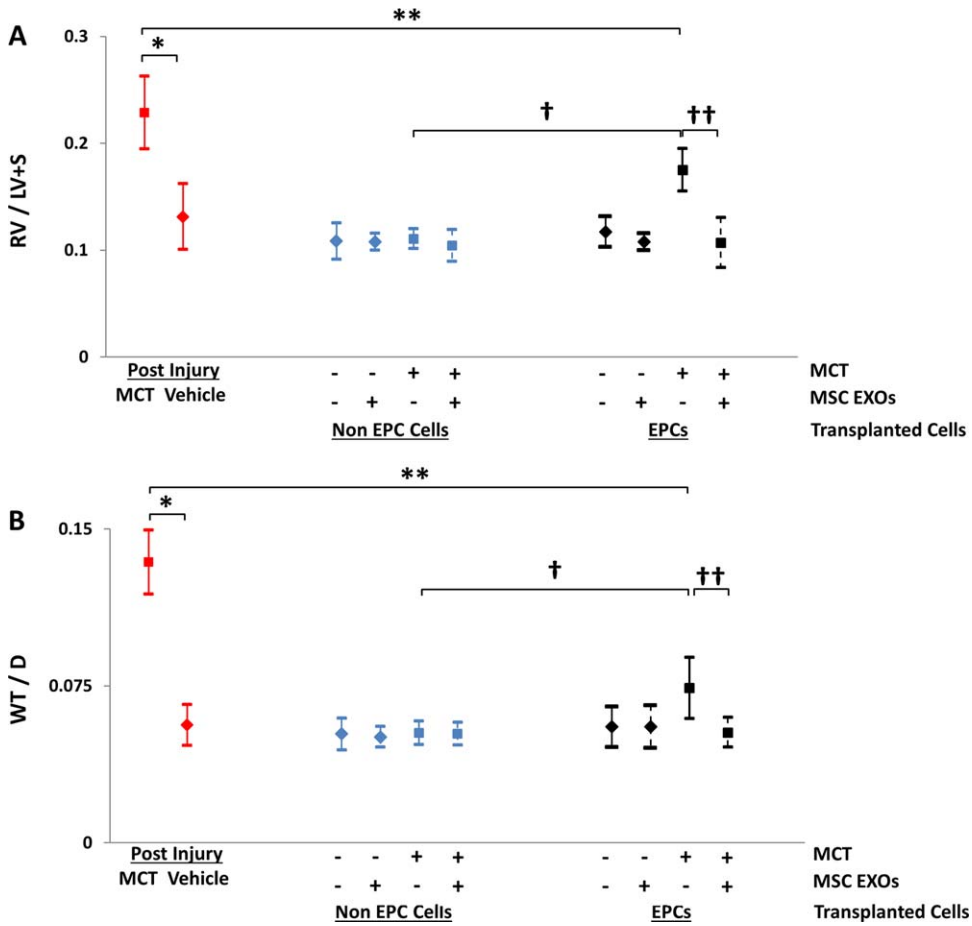

Figure 7. Mice transplanted with EPCs from MCT-injured mice cultured with MSC-EVs prior to transplantation do not develop RV hypertrophy, pulmonary vascular remodeling. **(A)**: RV/LV+S ratio, **(B)**: WT/D ratio of mice injected with MCT-injured (red boxes) or vehicle-injected (red diamonds) mice, mice transplanted with non-EPC cells from vehicle-injected and MCT-injured mice cultured with or without MSC-EVs prior to transplantation (blue boxes, diamonds), mice transplanted with EPCs from vehicle-injected and MCT-injured mice cultured with or without MSC-EVs prior to transplantation (black boxes, diamonds). Analysis performed 4 weeks after transplantation. Data presented as means with 95% confidence intervals. $n = 6$–15 mice/cohort; *, $p < .05$ versus vehicle-injected mice; mice transplanted with EPCs from MCT-injured mice that were not cultured with MSC-EVs versus MCT-injured mice (**, $p < .05$) or versus mice transplanted with non-EPC cells from MCT-injured mice that were not cultured with MSC-EVs († $p < .05$) or versus mice transplanted with EPCs from MCT-injured mice that were cultured with MSC-EVs (††, $p < .05$). Abbreviations: EPC, endothelial progenitor cells; MCT, monocrotaline; MSC-EV mesenchymal stem cell-extracellular vesicles; RV, right ventricular; RV/LV+S, RV-to-left ventricle+septum; WT/D, wall thickness-to-diameter.

hypertensive changes when injected into healthy mice. Interestingly, indices of RV hypertrophy and pulmonary vascular wall thickness in healthy mice given EPCs isolated from the BM of mice with MCT-PH were elevated to the same degree as the mice from which these EPCs were obtained, suggesting that these cells are capable of producing the same severity of disease. In contrast, EPCs isolated from control mice or WBM cells from mice with MCT-PH that lacked the EPC markers Sca-1, c-kit, and VEGFR2 did not induce RV hypertrophy or pulmonary vascular remodeling when infused into healthy mice. These findings suggest that the EPC fraction of WBM in mice with MCT-PH have undergone a transformation to a pathogenic phenotype that is capable of inducing PH when transplanted into otherwise healthy mice.

In addition, the present study and work published by our group suggest that the EPC transformation into a pathogenic phenotype is the result of interactions between cells of the BM and lung-derived EVs. Previously, we had shown that EVs isolated from the lungs or plasma of mice with MCT-PH cause PH when infused into healthy mice [20]. In addition, we demonstrated that BM cells cultured with EVs from mice with MCT-PH had greater EPC gene expression than cells cultured with EVs isolated from control mice [20]. Particularly pertinent to our findings presented were observations that BM cells cultured with EVs from mice with MCT-PH caused PH when injected into healthy mice [20]. These observations suggest that EVs released from pulmonary vasculature of pulmonary hypertensive mice were capable of inducing BM cells to differentiate into EPCs which, in turn, induced a pulmonary hypertensive phenotype. Indeed, further experiments in this study found that healthy mice transplanted with whole BM cells isolated from mice with PH induced by the infusion of lung EVs from mice with MCT-PH develop features of PH. Taken together, these studies suggest that not only can BM cells transfer this disease phenotype, but they do so after interactions with lung-derived EVs released by MCT injury. In addition, these findings suggest that lung-derived EVs from mice with MCT-PH mice act primarily by inducing changes in BM-derived EPCs.

As others have shown the presence of more circulating BM-derived EPCs in humans with PAH [2, 3], we speculated that EPCs might be the BM cell population responsible for transferring PH in our animal model. Although the number of circulating BM-derived EPCs was decreased in mice with MCT-PH, more of these cells could be detected in the BM of mice after MCT injury. Higher expression of EPC genes and genes for their ligands or receptors ($\alpha V\beta 3$ for sca-1, SCF for c-kit; VEGFRA for VEGFR2), was found in the lungs of mice with MCT-PH compared with the lungs of control mice, suggesting the presence of more EPCs in the lungs after MCT injury. These findings may be the result of increased expression of EPC adhesion receptors and ligands in the lung. In spite of the quantitative differences of EPCs between mice with MCT-PH and control mice, an equal number of EPCs from the two cohorts was used in transplantation studies, suggesting that qualitative differences account for the ability of EPCs from MCT-PH mice to induce PH upon transplantation. Taken together, we believe our studies demonstrate that lung-derived EVs induce pulmonary hypertensive changes after MCT injury primarily by influencing BM differentiation into "pathogenic" EPCs and that EPC homing to the lungs may be influenced by an increase in EPC ligand/receptor gene expression in the lungs of these animals. Whether this increase in expression is due to the MCT injury itself or induced by EVs released by the lung in the setting of MCT injury is not known.

The mechanism by which pathogenic EPCs contribute to pulmonary vascular remodeling is not clear. The EPC fraction of WBM cells used in this study may contain a variety of progenitor populations that could give rise to functionally divergent progeny. Yoder and colleagues described endothelial cell colony-forming units (CFU-ECs) as cells that are derived from hematopoietic stem cells and display hematopoietic-restricted and macrophage-specific cellular proteins but are incapable of forming vascular structures in vivo [27]. In contrast, endothelial colony-forming cells express endothelial cell surface proteins and are capable of forming vascular structures in vivo. In addition, many different BM-derived progenitor cell populations may participate in PH-related vascular remodeling, including EPCs, smooth muscle progenitors, fibrocytes and pericytes [28], although their specific role is uncertain. Our work focused on BM progenitors most similar to CFU-ECs as they display hematopoietic markers including c-kit and sca-1; however, the role of the progeny of these progenitors or other progenitor cells, including E-CFUs, in the vasculopathy reported in our current work were not specifically examined and cannot be excluded. Alternatively, EVs released by EPCs or their progeny may be responsible for inducing vasculopathic changes in this injury model. However, our current work did not explore this possibility.

BM-derived MSCs or EVs shed from these cells have been shown to improve a variety of injuries [10–12, 21, 22] but the mechanism responsible for their beneficial effects is not well understood. The findings of the present study suggest that MSC-EVs appear to reverse the BM "defect" that is induced by MCT-injury and by EVs isolated from mice with MCT-PH. We show that mice transplanted with BM cells isolated from those with MCT-PH also develop PH whereas transplant recipients of BM cells isolated from mice who's PH had been successfully reversed by MSC-EVs did not. MSC-EVs appear to reverse MCT-PH specifically by targeting dysfunctional BM-derived EPCs as we have shown that EPCs isolated from MCT-PH mice to induce PH in healthy mice was completely abolished by culturing them with MSC-EVs for 48 hours prior to transplantation. A non-MSC-EV population was not used as a control in EPC cultures, raising the question of whether or not the observed effect is specific to EVs from MSCs. However, work recently published by our group has shown that infusion of MSC-EVs reverses MCT-PH in mice whereas infusion of EVs isolated from lung tissue and lineage-depleted BM cells do not [24]. These data strongly suggest that the reparative effects described in this work are specific to MSC-EVs. Interestingly, MSC-EV infusion in mice with MCT-PH normalized increased lung expression of EPC genes and their receptors/ligands. Based on these data, MSC-EV infusion may also mitigate MCT-induced vasculopathy by attenuating EPC homing to the lung.

Conclusion

We believe that our data demonstrate that in PH, the lung releases EVs that induce change in BM-derived EPCs, converting them into pathologic progenitors, that traffic to the lung and induce pulmonary vascular remodeling. The pathologic properties of BM-derived EPCs can be reversed by exposure to MSC-derived EVs, both in vitro and in vivo, and thus their ability to induce PH in healthy mice.

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Authors Contributions

J.M.A.: Conception and design, financial support, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; M.P.: conception and design, collection and/or assembly of data, manuscript writing; S.W.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; M.S.D.: conception and design, collection and/or assembly of data, data analysis and interpretation; M.D.T., E.P., Y.C. and L.G.: collection and/or assembly of data, data analysis and interpretation; C.E.V. and O.L.: data analysis and interpretation, manuscript writing; J.R.K. and P.J.Q.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

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