Exosomes Mediate the Cytoprotective Action of Mesenchymal Stromal Cells on Hypoxia-Induced Pulmonary Hypertension

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Background—Hypoxia induces an inflammatory response in the lung manifested by alternative activation of macrophages with elevation of proinflammatory mediators that are critical for the later development of hypoxic pulmonary hypertension. Mesenchymal stromal cell transplantation inhibits lung inflammation, vascular remodeling, and right heart failure and reverses hypoxic pulmonary hypertension in experimental models of disease. In this study, we aimed to investigate the paracrine mechanisms by which mesenchymal stromal cells are protective in hypoxic pulmonary hypertension.

Methods and Results—We fractionated mouse mesenchymal stromal cell–conditioned media to identify the biologically active component affecting in vivo hypoxic signaling and determined that exosomes, secreted membrane microvesicles, suppressed the hypoxic pulmonary influx of macrophages and the induction of proinflammatory and proproliferative mediators, including monocyte chemoattractant protein-1 and hypoxia-inducible mitogenic factor, in the murine model of hypoxic pulmonary hypertension. Intravenous delivery of mesenchymal stromal cell–derived exosomes (MEX) inhibited vascular remodeling and hypoxic pulmonary hypertension, whereas MEX-depleted media or fibroblast-derived exosomes had no effect. MEX suppressed the hypoxic activation of signal transducer and activator of transcription 3 (STAT3) and the upregulation of the miR-17 superfamily of microRNA clusters, whereas it increased lung levels of miR-204, a key microRNA, the expression of which is decreased in human pulmonary hypertension. MEX produced by human umbilical cord mesenchymal stromal cells inhibited STAT3 signaling in isolated human pulmonary artery endothelial cells, demonstrating a direct effect of MEX on hypoxic vascular cells.

Conclusion—This study indicates that MEX exert a pleiotropic protective effect on the lung and inhibit pulmonary hypertension through suppression of hyperproliferative pathways, including STAT3-mediated signaling induced by hypoxia. (Circulation. 2012;126:2601-2611.)

Key Words: hypoxia ■ hypertension, pulmonary ■ inflammation ■ signal transduction

The response of the lungs to low levels of environmental oxygen is multifactorial. Diverse signaling pathways, activated or impaired by alveolar hypoxia, converge on endothelial and vascular smooth muscle cells to perturb pulmonary vascular homeostasis. Chronic hypoxia results in pulmonary vascular remodeling, a key pathological feature of pulmonary hypertension (PH). Inflammation plays a prominent detrimental role in most types of human PH and in animal models of the disease such as the monocrotaline-induced PH and hypoxia-induced PH (HPH) in rodents. The early component of hypoxia-induced lung inflammation, peaking during the first 2 to 3 days of hypoxic exposure,1 is characterized by alternative activation of alveolar macrophages and appears to be causal to the subsequent vascular remodeling and the development of HPH.2

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Despite the significant progress in our understanding of the pathophysiology of PH and the treatment of its symptoms,
there is no cure for this disease, and no single therapy has been proven effective. Given the complex pathways involved in the pathogenesis of PH, therapies aimed at more than one pathway and perhaps more than one cellular target may prove to be more efficacious. Stem cell–based therapeutic approaches hold such promise because they may simultaneously target multiple signaling pathways and have long-lasting effects. The therapeutic potential of mesenchymal stromal cells (MSCs; also referred to as mesenchymal stem cells or multipotent stromal cells) derived from the bone marrow, adipose, and other tissues has been recognized in several animal models of lung disease. In models of proinflammatory lung diseases such as bleomycin- or endotoxin-induced lung injuries and the neonatal murine model of bronchopulmonary dysplasia, MSC delivery ameliorated lung injury, decreased lung inflammation and fibrosis, and increased survival. MSC delivery was reported to inhibit PH induced by monocrotaline in the rat and HPH in the mouse. However, although a robust protection against lung injury on MSC treatment was observed in most of the above-mentioned animal models, only a small fraction of donor cells were retained in the recipient lung. This observation suggested that engraftment and direct tissue repair were not the sole mechanisms of MSC therapeutic function, and paracrine mechanisms were contemplated. In support of this, we observed that injections with culture media conditioned by MSCs can efficiently inhibit parenchymal injury, vascular remodeling, and right ventricular hypertrophy, completely supplanting MSC treatment on the neonatal murine model of bronchopulmonary dysplasia. In vitro experiments demonstrated antiproliferative properties of the MSC secretome on pulmonary vascular smooth muscle cells, again suggesting that MSC paracrine factors can play a major role in preventing lung injury and vascular remodeling. Concordant with our observations, paracrine and immunomodulatory paradigms have recently been proposed to account for enhanced MSC therapeutic function in the context of a number of disease models.

We have previously performed proteomic analysis of MSC-conditioned media (MSC-CM), which, in addition to immunomodulatory factors, revealed the presence of a number of proteins, including CD63, CD81, moesin, lactadherin (MFGE8), heat shock protein 90, and heat shock protein 70, reported to be associated with secreted vesicles known as exosomes. Secreted membrane microvesicles, especially the better-defined subclass represented by exosomes, have been recognized as important mediators of cell-to-cell communication and as participants in immunomodulatory mechanisms. Exosomes are small heterogeneous microvesicles, 30 to 100 nm in diameter, that are stored within multivesicular bodies and released into the environment on fusion of the multivesicular bodies with the plasma membrane. Exosomes and microvesicles have been isolated and characterized from various cell types, including dendritic cells, macrophages, tumor cells, and embryonic stem cells, and information is rapidly accumulating on their diverse biological function and their cell type–specific molecular composition. The physiological relevance of MSC-derived exosomes (MEX) has not yet been evaluated in lung diseases, even though their cellular origin and the recently recognized paracrine function of MSCs may imply a promising therapeutic potential for secreted microvesicles in lung injury.

To address the above questions, we fractionated MSC-CM by size-exclusion chromatography to identify the biologically active component protecting against hypoxia-induced lung inflammation and HPH. Using the murine model of HPH, we demonstrate here that MEX are the critical vectors of MSC action: MEX delivery in vivo suppressed HPH and vascular remodeling. Moreover, proliferative pathways were also blocked by MEX treatment, as evidenced by the suppression of signal transducer and activator of transcription-3 (STAT3) phosphorylation, resulting in increased lung levels of miR-204, a microRNA enriched in distal pulmonary arterioles that is downregulated in both human PH and experimental models of disease. We found that hypoxia upregulates members of the miR-17 family of microRNA clusters in lung tissue, microRNAs shown to be under the regulatory control of STAT3, and show that MEX treatment efficiently suppresses this proliferative signal. Combined, our findings point to MEX as the key effectors of MSC paracrine function with the potential to serve as vehicles of lung-targeted therapy.

Methods

Animal Model and Hypoxic Exposure

The HPH mouse model has been well established and used by our group extensively in previously published work. The hypoxic exposure and treatment protocols used in this study are described in the online-only Data Supplement. All animal experiments were approved by the Boston Children’s Hospital Animal Care and Use Committee.

Preparation of Exosomes

Isolation of mouse bone marrow–derived MSCs and MSCs from human umbilical cord Wharton’s jelly, immunoselection (Figure I in the online-only Data Supplement), and collection of CM are outlined in the online-only Data Supplement. Concentrated CM were applied on a column of 16/60 HiPrep Sephacryl S-400 HR (GE Healthcare, Piscataway, NJ) that was pre-equilibrated with a buffer containing 20 mmol/L sodium phosphate (pH 7.4) and 300 mmol/L NaCl with an AKTA purifier liquid chromatography system (GE Healthcare). Fractions (1 mL) were collected at a flow rate of 0.5 mL/min. Polystyrene nanospheres of 50-nm diameter (Phosphorex, Fall River, MA) were used as a size reference, and elution fractions corresponding to the retention volume of this standard were pooled and further analyzed.

For the isolation of exosomes from human umbilical cord Wharton’s jelly-MSCs and human dermal fibroblasts, serum-free culture medium conditioned for 24 hours was filtered (0.2 μm) and concentrated by ultrafiltration device with 100-kDa cutoff (Millipore). Exosomes in CM were precipitated with one-third volume of polyethylene glycol buffer (33.4% PEG 4000, 50 mmol/L HEPES [pH 7.4], 1 mol/L NaCl) overnight at 4°C, followed by centrifugation at 12,000 × g for 5 minutes and resuspension in PBS (pH 7.4). Exosomes in polyethylene glycol–precipitated fraction were further purified by S200 size-exclusion chromatography. A 75-μL sample was applied on a S200 column (Clontech, Mountain View, CA) pre-equilibrated with PBS by spinning at 700 g for 5 minutes, and the exosomal fraction was subsequently eluted in the flow-through by centrifugation at 700 g for 5 minutes.

In some experiments, exosomes were isolated by ultracentrifugation at 100,000 × g for 2 hours, and the pellet was subsequently washed with PBS, followed by repeat ultracentrifugation for 2 hours at the same speed. Exosome pellet resuspended in PBS was measured for protein concentration by the Bradford assay (Bio-Rad, Hercules,
Expression of exosomal markers between the 2 preparations was similar, as shown in Figure II in the online-only Data Supplement.

Statistical Analysis
All values are expressed as mean±SD. All comparisons between experimental and control groups were performed by one-way ANOVA with the Tukey-Kramer post test using PRISM 5 statistical software (GraphPad Software, San Diego, CA) unless otherwise indicated. A value of $P<0.05$ was considered statistically significant. Student t test was used to compare 2 groups.

See the online-only Data Supplement for a detailed description of further experimental methods.

Results
Factors Secreted by MSCs Can Prevent Hypoxia-Induced Pulmonary Inflammation
To determine whether hypoxic lung inflammation responds to MSC paracrine signals, we injected mice with concentrated serum-free culture media conditioned by either mouse MSCs (MSC-CM) or by mouse lung fibroblasts (MLF-CM) and exposed the animals to normobaric hypoxia (8.5% O$_2$) for 48 hours. In the control group injected with vehicle (serum-free culture media), hypoxia resulted in pulmonary influx of macrophages, as assessed in bronchoalveolar lavage fluid (BALF), and this response was blocked in animals treated with MSC-CM but not in the group treated with MLF-CM (Figure 1A). We also assessed, in cell-free BALF, levels of monocyte chemoattractant protein-1 (MCP-1), a cytokine transiently upregulated in the lung by early hypoxia, and levels of hypoxia-induced mitogenic factor (HIMF), also known as found in inflammatory zone-1 (FIZZ-1), a pleiotropic factor with proinflammatory, mitogenic, and chemokine-like properties. In animals injected with either vehicle or MLF-CM, BALF levels of both MCP-1 and HIMF were highly increased by hypoxia, and this increase was effectively suppressed by MSC-CM treatment (Figure 1B). These results indicate that, as we have previously reported on the model of hyperoxia-induced bronchopulmonary dysplasia, the protective effects of MSC treatment in HPH involve mainly paracrine mechanisms.

The Antiinflammatory Activity in MSC-CM Is Associated With Exosomes
To identify the biologically active component of MSC-CM, we fractionated concentrated CM through size-exclusion chromatography. Polystyrene nanospheres of 50-nm diameter served as a hydrodynamic radius standard to identify the exosomal fraction, and fractions in a protein peak eluting with a retention volume corresponding to that of the standard were pooled (fraction I, Figure 2A). Negative-staining electron microscopic analysis revealed that fraction I contained heterogeneous microvesicles that were absent in fractions corresponding to the retention volume of moieties of smaller size (fraction II, Figure 2B). Fraction I was highly enriched in microvesicles 30 to 100 nm in diameter exhibiting biconcave morphology, a distinct morphological feature of exosomes (Figure 2C, arrows). Exosomes were present in both MSC-CM and MLF-CM, and in this report, MSC exosome preparations are called MEX, whereas MLF exosome preparations are called FEX. Both MEX and FEX contain diverse mature microRNAs (see below) and Dicer (Figure 2D), a component of the cytoplasmic microRNA maturation complex. However, the relative abundance of each exosomal marker differs, depending on the cellular origin of the microvesicles.

MEX preparations were efficacious in suppressing hypoxic inflammation when injected into animals, whereas the MSC-CM fraction depleted of exosomes (ExD-CM) had no significant effect and FEX had a partial inhibiting effect (Figure 2E). Concordantly, levels of proinflammatory mediators in cell-free BALF of hypoxic animals were suppressed only by MEX but not by FEX or ExD-CM treatment (Figure 2F), indicating that the ability to suppress early hypoxia-induced pulmonary inflammation is associated specifically with exosomes of MSC origin.
Dose-Response Effects of MEX on Lung Inflammation

We have previously reported that suppression of the entire period of the inflammatory response to early hypoxia is required to protect animals from later development of PH.\(^2\) This inflammatory response is transient in the murine model, peaking within 2 to 3 days of hypoxic exposure and subsiding by day 7 (Figure 3A, left). We therefore assessed the effect of MEX treatment on the temporal profile of hypoxic lung inflammation, and we found that a low dose of MEX (0.1 \(\mu\)g per animal, via jugular vein) was able to delay but not to completely suppress the pulmonary influx of macrophages, resulting in a shift of the inflammatory peak toward later times (Figure 3A, middle). The observed temporal profile of pulmonary macrophage influx was paralleled by the temporal profile of induction of the proinflammatory markers MCP-1, interleukin-6, galectin-3, and HIMF in cell-free BALF, which also shifted to a later time (4–7 days; Figure 3B). In contrast, a treatment consisting of 2 sequential injections of MEX, 1 before exposure to hypoxia and a second injection at day 4, just before the delayed inflammatory peak (Figure 3A, middle), efficiently suppressed pulmonary influx of macrophages over the entire period of inflammatory responses to early hypoxia (Figure 3A, right). However, the peak of proinflammatory markers in BALF, although delayed by the first dose, was not affected by the second injection of MEX (Figure 3B).

Multiple Administrations of Low Doses of MEX But Not FEX Ameliorate PH, Right Ventricular Hypertrophy, and Lung Vascular Remodeling

The physiological consequences of partial or complete abrogation of the early inflammatory response to hypoxia are shown in Figure 3C and 3D. A single low dose of MEX did not protect against the elevation of right ventricular systolic pressure or the development of right ventricular hypertrophy after 3 weeks of hypoxic exposure, whereas the double-injection regimen significantly improved both variables. These results mirror the physiological response we had observed using pulses of heme oxygenase-1 overexpression to completely or partially suppress the early hypoxic lung inflammation\(^2\) and suggest a dose- and time-sensitive window for antiinflammatory treatments to confer protection from HPH. Importantly, FEX treatment using the double-injection protocol did not have any physiological effect, buttressing the assertion that the function(s) protecting against HPH reside specifically with exosomes produced by MSCs. Furthermore, animals treated with 2 doses of MEX and exposed to 3 weeks of hypoxia did not develop vascular remodeling as determined by \(\alpha\)-smooth muscle actin staining, whereas the same treatment protocol with FEX resulted in medial wall hypertrophy similar to vehicle-treated controls (Figure 4).

A Single High-Dose MEX Treatment Inhibits Hypoxic Inflammation, Vascular Remodeling, and HPH

The incomplete protection from HPH by 2 sequential low doses of MEX could be related to the failure to completely suppress early hypoxic inflammation. To test the efficacy of higher MEX dosages on early hypoxic inflammation and HPH, 10 \(\mu\)g MEX was injected through the tail vein, and mice were exposed to hypoxia for 2 and 7 days. A higher dose of MEX prevented pulmonary influx of macrophages similarly with 2 sequential injections of MEX (Figure 5A) and, importantly, completely abrogated the elevation of the proinflammatory marker FIZZ-1/HIMF in the lung for the entire...
MEX Inhibits STAT3 Activation by Hypoxia

Early hypoxia resulted in activation of STAT3 in the mouse lung through phosphorylation at Tyr705 and without any effect on the total levels of STAT3 protein. This activation was efficiently suppressed by MEX treatment but not FEX (Figure 7A). STAT3 is a transcription factor integral to signaling pathways of many cytokines and growth factors, and its activation plays a critical role in respiratory epithelial inflammatory responses. Importantly, persistent ex vivo STAT3 activation has been linked to the hyperproliferative and apoptosis-resistant phenotype observed in pulmonary artery endothelial cells (PAECs) and pulmonary artery smooth muscle cells from patients with idiopathic pulmonary arterial hypertension. Therefore, to determine whether MEX regulates STAT3 activation on lung vascular cells, we exposed primary human PAECs to hypoxia and assessed pY-STAT3 levels. As depicted in Figure 7B, exposure of human PAECs to hypoxia results in robust activation of STAT3 by Tyr705 phosphorylation. Treatment with mouse MEX or MEX derived from MSCs isolated from human umbilical cord stroma completely abrogated this response. In contrast, mouse FEX, human FEX, and the fraction of human umbilical cord ExD-CM had no effect. Besides demonstrating that suppression of STAT3 activation is a property shared by MEX of both human and mouse origin, these results strongly suggest that direct suppression of hypoxic signaling in pulmonary vascular cells is a primary function underlying the protection conferred by MEX treatment of higher dose of MEX on vascular remodeling and HPH, 10 μg MEX was injected, and mice were exposed to chronic hypoxia for 3 weeks. Compared with vehicle (PBS)–injected controls, the MEX–treated group had significantly (P<0.001, one-way ANOVA) decreased right ventricular systolic pressure (Figure 6A) and did not develop right ventricular hypertrophy in response to chronic hypoxia (Figure 6B). MEX treatment prevented pulmonary vascular remodeling, as assessed by α-smooth muscle actin staining (Figure 6C). Morphometric analyses on small arterioles revealed a significant effect on the medial wall thickness index, with values in the MEX–treated group approximating those of the minimally muscularized normoxic vessels (Figure 6D). Taken together, the above results strongly suggest that the protective mechanism of MEX action is through blocking inflammatory lung responses to early hypoxia, which, when left unchecked, activate proproliferative pathways in the vascular wall, thereby increasing medial wall thickness and altering vascular cell phenotype.

Figure 3. Repeated administration of low-dose (x2) mesenchymal stromal cell exosome preparations (MEX) can suppress pulmonary influx of macrophages and ameliorate pulmonary hypertension. A, Animals were injected with 1 dose of PBS (left; n=14) or 0.1 μg MEX (middle; n=31) through the jugular vein before hypoxic exposure, and alveolar macrophages were measured in bronchoalveolar lavage fluid (BALF) at sacrifice on the days indicated on the x axis. Animals (right; n=15) received 0.1 μg MEX before exposure to hypoxia and 0.1 μg MEX again at day 4 of hypoxia, and alveolar macrophages were measured in BALF at sacrifice on the days indicated on the x axis. Each dot represents an individual animal. Macrophage numbers in BALF from normoxic control animals (NRX; ○) are replicated in all 3 panels for direct comparison. ¶P<0.001 vs normoxic control group (one-way ANOVA with Tukey-Kramer post test). B, Representative Western blot analysis of cytokine levels in BALF in animals treated with a single low-dose MEX. BALF from 6 animals in each group was pooled for immunoblot analysis and repeated at least 3 times. IL-6 indicates interleukin-6. Right ventricular systolic pressure (RVSP; C) and the Fulton Index (D) determined after 3 weeks of hypoxic exposure. The Fulton Index (right ventricle/left ventricle + septum, [RV/(LV+s)]) is a measurement of right ventricular hypertrophy expressed as a weight ratio. MEX (x1); animals were injected once with MEX. MEX(x2) and FEX(x2); animals were injected twice with equivalent amounts of MEX or mouse lung fibroblast exosome preparations (FEX). Black dots represent values for individual animals; horizontal lines, the group mean; and vertical brackets, the standard deviation. ¶P<0.001 vs normoxic control group; §P<0.01 vs normoxic control group; ¶¶P<0.001 vs PBS–injected hypoxic control group (one-way ANOVA with Tukey-Kramer post test). NRX indicates normoxia.

Figure 3A. Repeated administration of low-dose (x2) mesenchymal stromal cell exosome preparations (MEX) can suppress pulmonary influx of macrophages and ameliorate pulmonary hypertension. A, Animals were injected with 1 dose of PBS (left; n=14) or 0.1 μg MEX (middle; n=31) through the jugular vein before hypoxic exposure, and alveolar macrophages were measured in bronchoalveolar lavage fluid (BALF) at sacrifice on the days indicated on the x axis. Animals (right; n=15) received 0.1 μg MEX before exposure to hypoxia and 0.1 μg MEX again at day 4 of hypoxia, and alveolar macrophages were measured in BALF at sacrifice on the days indicated on the x axis. Each dot represents an individual animal. Macrophage numbers in BALF from normoxic control animals (NRX; ○) are replicated in all 3 panels for direct comparison. ¶P<0.001 vs normoxic control group (one-way ANOVA with Tukey-Kramer post test). B, Representative Western blot analysis of cytokine levels in BALF in animals treated with a single low-dose MEX. BALF from 6 animals in each group was pooled for immunoblot analysis and repeated at least 3 times. IL-6 indicates interleukin-6. Right ventricular systolic pressure (RVSP; C) and the Fulton Index (D) determined after 3 weeks of hypoxic exposure. The Fulton Index (right ventricle/left ventricle + septum, [RV/(LV+s)]) is a measurement of right ventricular hypertrophy expressed as a weight ratio. MEX (x1); animals were injected once with MEX. MEX(x2) and FEX(x2); animals were injected twice with equivalent amounts of MEX or mouse lung fibroblast exosome preparations (FEX). Black dots represent values for individual animals; horizontal lines, the group mean; and vertical brackets, the standard deviation. ¶P<0.001 vs normoxic control group; §P<0.01 vs normoxic control group; ¶¶P<0.001 vs PBS–injected hypoxic control group (one-way ANOVA with Tukey-Kramer post test). NRX indicates normoxia.
In concordance, we found that MEX dose-dependently inhibited pulmonary artery smooth muscle cell proliferation rate in response to serum-derived mitogens (Figure III in the online-only Data Supplement), confirming that MEX have direct effects on lung vascular cells.

Differential miRNA Content in MEX Versus FEX

A number of recent studies report the successful horizontal transfer of functional mRNA and miRNA species from exosomes into recipient cells.\textsuperscript{30,31} To evaluate potential differential signals released by MEX versus FEX that could mediate their therapeutic effects in vivo, we quantified relative levels of a number of candidate miRNAs in MEX and FEX preparations. We found that relative to FEX, MEX contain significantly increased levels of miRNA-16 and miRNA-21. Interestingly, although let7b miRNA levels were comparable within these 2 types of exosomes, let7b pre-miRNA was significantly enriched in MEX versus FEX (>10-fold; Figure IV in the online-only Data Supplement). These findings point to distinct and potentially important microRNA-mediated regulatory signals delivered to the lung by MSC-derived exosomes.

MEX Treatment Suppresses the Hypoxic Induction of the miR-17 microRNA Superfamily and Increases Levels of Antiproliferative miR-204 in the Lung

STAT3 (activated by either vascular endothelial growth factor or interleukin-6) has been reported to directly regulate the transcription of the miR-17\textendash;92 cluster of microRNAs in PAECs, resulting in decreased levels of bone morphogenetic protein receptor-2, a target of miR-17.\textsuperscript{32} Therefore, we assessed the effect of hypoxia and MEX treatment on the miR-17\textendash;92 cluster of microRNAs and its conserved paralog clusters, miR-106b\textendash;25 and miR-106a\textendash;363. These microRNA clusters have been postulated to be proproliferative and to target an array of genes involved in the G1/S phase transition.\textsuperscript{33} We found that select microRNAs representing all 3 clusters of the miR-17 superfamily were upregulated by hypoxia in the lung and that this transcriptional activation was efficiently suppressed by MEX treatment (Figure 8A). Interestingly, levels of microRNAs involved in hypoxic signaling networks such as miR-199a-5p (a microRNA reported to stabilize hypoxia-inducible factor-1α in cardiac myocytes), miR-214 (which shares the same host gene with...
miR-199a, or miR-210 (a hypoxami under direct hypoxia-inducible factor-1α regulation) were not affected by MEX treatment (Figure 8B), pointing to targeted effects of MEX on specific hypoxia-regulated signaling pathways. Treatment with an equivalent dose of FEX had no inhibitory effect on the hypoxamirs examined, with only members of the miR106b/25/93 cluster being moderately affected by FEX (Figure 8A).

Importantly, we observed that MEX but not FEX treatment resulted in an increase in lung levels of miR-204 (Figure 8C), a microRNA enriched in distal pulmonary arterioles that is transcriptionally suppressed by STAT3 but also inhibits the activation of STAT3 in a feed-forward regulatory loop. The proliferative and antiapoptotic phenotype of pulmonary artery smooth muscle cells isolated from patients with idiopathic pulmonary arterial hypertension is inversely related to the level of miR-204 and delivery of exogenous miR-204 to the lungs of animals with PH ameliorated established disease. Therefore, we interpret these results as an indication that MEX treatment, by suppressing STAT3 activation at the early stages of hypoxic exposure, prevents the hypoxic induction of the proproliferative miR-17 superfamily in the lung vasculature and blocks the STAT3–miR-204–STAT3 feed-forward loop in distal pulmonary vessels.

Discussion

This report demonstrates that the protective functions of MSCs are mediated by secreted microvesicles. Thus, our work provides an explanation for the paradox of the consistently observed significant physiological effect of MSC treatment despite very low retention of donor cells in the lung. Secreted membrane microvesicles, of which exosomes represent the better characterized subclass, have been recognized as important mediators of intercellular communication, especially in the immune system. It has been proposed that such microvesicles, called exosomes, can act as a vector for the transfer of genetic information (mRNA and microRNAs) or the shuttling of effector proteins to recipient cells. Heat shock protein 72 from tumor-derived exosomes mediates immunosuppressive function to myeloid-derived suppressive cells through activation of STAT3. Through putative transfer of microRNAs and mRNAs, microvesicles secreted from tumor-initiating cells positive for the mesenchymal marker CD105 have been reported to confer angiogenic phenotype to normal endothelial cells. Supporting our observations here, microvesicles released from MSCs have recently been reported to improve recovery in animal models of experimentally induced renal failure and myocardial ischemia/reperfusion injury; however, the underlying mechanisms mediating these protective effects were not characterized.

Our results show that treatment with MSC-derived exosomes prevents the activation of the hypoxic signaling that underlies pulmonary inflammation and the development of PH in the murine model. MCP-1 and HIMF/FIZZ1 are highly upregulated by hypoxia in the lung, and both factors are potent proinflammatory mediators. Moreover, both MCP-1 and HIMF/FIZZ1 have been linked to the development of PH in murine models of disease and in human PH. Egashira et al demonstrated that administration of exogenous recombinant MCP-1 resulted in prominent medial wall thickening of pulmonary arterioles and that MCP-1 receptor blockade prevented monocyte recruitment and the subsequent vascular remodeling. HIMF/FIZZ1, a marker of alternative activated macrophages, is also induced by hypoxia in the respiratory epithelium and plays a critical role in the development of PH in a murine model and in scleroderma-associated PH. The knockdown of HIMF/FIZZ1 partially blocked increases in pulmonary artery pressure, right ventricular hypertrophy, and vascular remodeling caused by chronic hypoxia. The important role of HIMF in the development of PH was further confirmed through intrapulmonary gene transfer of HIMF/FIZZ1, which recapitulated the findings of HPH. We demonstrate here that MEX administration inhib-
ited the hypoxic induction of both MCP-1 and HIMF/FIZZ1 in the lung and that this was associated with the prevention of HPH.

Directly related to the antiinflammatory action of MEX treatment is the observed prevention of hypoxia-activated proliferative pathways. Examining total lung tissue, we found that, in addition to MCP-1, hypoxic levels of interleukin-6 were suppressed by MEX administration. Interleukin-6 is a proinflammatory cytokine known to activate STAT3 and, in a number of studies, was associated...

Figure 7. Mesenchymal stromal cell exosome preparations (MEX) of either mouse (m) or human (h) origin suppress the hypoxic phosphorylation (activation) of signal transducer and activator of transcription 3 (STAT3). A, Total protein extracts from lungs of individual animals treated with PBS, MEX (10 μg), or mouse lung fibroblast exosome preparations (FEX; 10 μg). Hypoxia exposure for 2 days resulted in activation of STAT3 through phosphorylation at Tyr705 (pY-STAT3), and this was prevented by treatment with mMEX. Right, Quantification of STAT3 phosphorylation. #P<0.01 vs PBS-treated hypoxic group (1-way ANOVA with Tukey-Kramer post test). B, Primary cultures of human pulmonary artery endothelial cells exposed to hypoxia (1% O2, 6 hours) exhibit robust activation of STAT3 that is efficiently suppressed by mMEX and hMEX. Note that the microvesicle-depleted fraction of media conditioned by human umbilical cord MSCs (hUC-ExD-CM) and human dermal fibroblast or mouse lung fibroblast exosome preparations (hFEX, mFEX) had no effect on STAT3 phosphorylation. Data are representative of at least 3 independent experiments. NRX indicates normoxic group; HPX, hypoxic group.

Figure 8. Mesenchymal stromal cell exosome preparation (MEX) treatment suppresses the hypoxic induction of the miR-17 microRNA superfamily and increases levels of antiproliferative miR-204 in the lung. Mice were treated with PBS, MEX (10 μg), or mouse lung fibroblast exosome exosome preparations (FEX; 10 μg) and then exposed to hypoxia for 7 days. Another untreated group remained in normoxia as control (NRX). MicroRNA levels in total mouse lung were assessed by quantitative polymerase chain reaction, and fold changes in the hypoxic groups (HPX) are presented relative to the mean of the normoxic group. A, Select miRs representing the miR-17–92, miR-106a–3p clusters. B, Select miRs reported to be involved in hypoxic signaling. C, Upregulation of basal levels of the pulmonary arteriole-specific miR-204 on MEX treatment. Dots represent expression levels in individual animals. ¶P<0.001 vs normoxia; †P<0.01 vs normoxia; ‡P<0.05 vs normoxia (one-way ANOVA with Tukey-Kramer post test).
with PH. It is thus possible that STAT3 may be a key mediator of hypoxic, proinflammatory signaling leading to PH in the in vivo lung. Indeed, phosphorylation of STAT3 at the Tyr705 residue is required for STAT3 dimerization and subsequent nuclear translocation, and this was markedly increased in both lung and PAECs in response to hypoxia but significantly suppressed by MEX treatment. Importantly, persistent ex vivo STAT3 activation has been linked to the hyperproliferative and apoptosis-resistant phenotype observed in PAECs and pulmonary artery smooth muscle cells from patients with idiopathic pulmonary arterial hypertension. Mathew et al reported a marked upregulation of STAT3 phosphorylation in the lungs of rats with monocrotaline-induced PH. STAT3 directly regulates the transcription of the miR-17–92 cluster of microRNAs in human PAECs, resulting in decreased levels of bone morphogenetic protein receptor-2, a target of miR-17, the down-regulation of which is recognized as a hallmark of PH. We found that hypoxia induced select microRNAs of the miR-17 superfamily in the lung and that MEX effectively suppressed this induction, whereas MEX did not suppress other microRNAs involved in hypoxic signaling networks, including the hypoxamir miR-210, which is induced by hypoxia-inducible factor, pointing to selective, STAT3-targeted effects of MEX action in the lung rather than global suppression of all hypoxamirs.

In addition to STAT3 being a central determinant of the hyperproliferative vascular cell phenotype in patients with idiopathic pulmonary arterial hypertension, the suppression of miR-204 (a distal pulmonary artery–specific microRNA) correlates with PH severity in human disease and rodent models of PH. In this study, miR-204 was suppressed by STAT3, and miR-204 was shown to inhibit the activation of STAT3 in a self-regulatory loop. Although during the acute hypoxic phase we did not observe the decrease in miR-204 levels observed under chronic hypoxia in the mouse, we consider the fact that MEX treatment increases the basal level of miR-204 a strong indication that MEX treatment is shifting the balance of the STAT3–miR-204 loop to an antiproliferative state.

A schema of a hypothesis synthesizing the above results with previous work from our group and the work of others is shown in Figure V in the online-only Data Supplement. We have previously shown that hypoxia shifts the Th1/Th2 balance of immunomodulators in the lung, resulting in alternative activated alveolar macrophages, and that this is inhibited by hemeoxygenase-1 overexpression. Hypoxia also induces the expression of HIMF in the lung epithelium, and HIMF mitogenic action on the vasculature requires Th2 cytokines such as interleukin-4 to result in PH. Consequences of the shift toward proliferation include the hypoxic activation of STAT3 signaling and the upregulation of the miR-17 family of microRNAs. Treatment with MEX interferes with an early hypoxic signal in the lung, suppressing inflammation, HIMF transcriptional upregulation, and alternative macrophage activation. It addition, MEX treatment may directly suppress STAT3 activation in lung vascular cells and upregulate miR-204 levels, thus breaking the STAT3–miR-204–STAT3 feed-forward loop and shifting the balance to an antiproliferative state.

The ability to secrete microparticles that contain not only proteins but also RNA or miRNA species that can modulate the expression of multiple genes makes these packaging vesicles an attractive and quite plausible means for MSCs to regulate multiple pathways and produce a robust therapeutic effect in vivo. Indeed, exosomes are lipid vesicles of endocytic origin released by many cell types, including vascular cells, dendritic cells, and mast cells, that function to mediate intercellular communication through the exchange of protein and RNA moieties. Of possible physiological relevance is the differential distribution of the tetraspanins CD63 (abundant in mouse MEX) and CD81 (abundant in mouse FEX). Although this differential distribution is not apparent between human MEX and human FEX (results not shown), these molecules may play a role in target-cell specificity of exosomes and could participate in signaling pathways. Full molecular characterization of exosomal preparations produced by mouse bone marrow MSCs and human umbilical cord stroma MSCs is the focus of ongoing work. Exosomes isolated from a mast cell line or from primary bone marrow–derived mast cells were reported to contain mRNAs and microRNAs that were transferable to other mast cells and, in the case of miRNAs, to be translated into new proteins. The authors identified different miRNAs within exosomes, and in a more recent study, premature and mature miRNAs, but not larger species, were identified within MSC-derived exosomes. Given the robust, long-lasting, antiinflammatory and cytoprotective effects of MEX demonstrated for the first time in the present study, it is reasonable to postulate that ≥1 miRNA species that are unique to or highly enriched within MEX serve as master regulator(s) of several genes and pathways underlying the development of PH.

Conclusions

In this study, we isolated, identified, and characterized exosomes from mouse and human MSC-CM and demonstrated a robust biological effect that is unique to MEX versus exosomes derived from other cells such as fibroblasts. Importantly, we demonstrate for the first time that MEX are the major paracrine antiinflammatory and therapeutic mediators of MSC action on the lung, acting, at least in part, through inhibition of hypoxic STAT3 signaling. Further work is required to identify the critical components of MEX, be they protein, lipid, or nucleic acid species. Although the applicability of our findings to a human disease model needs to be verified, the efficacy of this treatment in preventing PH makes these microvesicles an attractive candidate for exploring models of therapeutic interventions in PH and other diseases with no definitive therapy to date.

Acknowledgments

We would like to thank Xianlan Liu for technical expertise, Sarah Gately for assistance in preparing the manuscript, and Dr Georg Hansmann for critical review of the manuscript.

Sources of Funding

This work was supported by National Institutes of Health grants R01 HL055454 and R01 HL085446 (Drs Kourembanas and Mitsialis).
Disclosures

None.

References


CLINICAL PERSPECTIVE

Pulmonary arterial hypertension remains without cure despite significant progress in our understanding of its pathophysiology. Given the complex molecular and cellular pathways underlying the development of pulmonary arterial hypertension, therapies aimed at multiple pathways and cellular targets may prove to be more efficacious. Stem cell–based therapies hold such a promise because they can simultaneously target diverse signaling pathways and have long-lasting effects. Accumulating studies support an important cytoprotective, antiinflammatory role for mesenchymal stem cells with their impact on cellular function.

Exosomes as Paracrine Vectors of MSC Action

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ACRYLIC PERSPECTIVE

Pulmonary arterial hypertension remains without cure despite significant progress in our understanding of its pathophysiology. Given the complex molecular and cellular pathways underlying the development of pulmonary arterial hypertension, therapies aimed at multiple pathways and cellular targets may prove to be more efficacious. Stem cell–based therapies hold such a promise because they can simultaneously target diverse signaling pathways and have long-lasting effects. Accumulating studies support an important cytoprotective, antiinflammatory role for mesenchymal stem cells with their impact on cellular function.

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Exosomes Mediate the Cytoprotective Action of Mesenchymal Stromal Cells on Hypoxia-Induced Pulmonary Hypertension
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_Circulation_. 2012;126:2601-2611; originally published online October 31, 2012;
doi: 10.1161/CIRCULATIONAHA.112.114173

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/126/22/2601

Data Supplement (unedited) at:
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Correction

In the article by Lee et al, “Exosomes Mediate the Cytoprotective Action of Mesenchymal Stromal Cells on Hypoxia-Induced Pulmonary Hypertension,” which was published ahead of print on October 31, 2012, an error occurred.

In the initial publication of the article, Figure 6 was incorrect. The error has been corrected in the current online version of the article.

The Editorial Office regrets the error.
SUPPLEMENTAL MATERIAL

Expanded Methods

Animal model and hypoxic exposure

Eight to 10-week old mice (FVB strain) were housed in large plexiglass chambers and the FiO₂ regulated by an OxyCycler controller (BioSpherix, Redfield, NY). Hypoxic exposures were performed at 8.5±0.5 % O₂ and ventilation was adjusted to insure that CO₂ levels did not exceed 5,000 ppm (average range 1,000-3,000 ppm). Ammonia was removed by ventilation and activated charcoal filtration through an air purifier. Under these conditions, the ammonia concentration is less than 2.5 ppm, the limit of detection of Gastec passive dosimetry tubes (Sigma). Animals were anesthetized with pentobarbital (50 mg/kg, I.P.) and injected through the left jugular vein with concentrated conditioned media (5 μg protein in 50 μl) or exosome preparations (0.1 μg protein in 50 μl PBS). A higher dose of exosomes (10 μg protein in 50 μl PBS) was delivered through the tail vein. Table 1 lists the number of cells used and the exosomal protein recovery from each cell type. Approximately 2% of secreted proteins in the conditioned media of both mMSCs and MLFs are associated with the exosomal fraction and roughly 3-fold more MLFs were required to extract equal amounts of exosomes for the injections. An equal volume of PBS or serum-free α-MEM media was injected in control experimental groups. Mice were allowed to recover for 3 hours before placement in hypoxic chambers. In certain time-course and dose-dependent studies, a second injection of MEX (0.1 μg protein in 50 μl PBS) was performed on the contralateral jugular vein after 4 days of hypoxic exposure.
Isolation of human MSCs from umbilical cord Wharton’s Jelly

Human umbilical cord Wharton’s jelly derived MSCs (hUC-MSCs) were isolated according to published methods\(^1,2\) with minor modifications. Cord was rinsed twice with cold sterile PBS, cut longitudinally, and arteries and vein were removed. The soft gel tissues were scraped out, finely chopped (2-3 mm\(^2\)) and directly placed on 100 mm dishes (15 pieces per dish) with DMEM/F12 (1:1) (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, and penicillin/streptomycin, and incubated for 5 days at 37°C in a humidified atmosphere of 5% CO\(_2\). After removal of tissue and medium, the plates were washed 3 times with PBS, the attached cells were cultured and fresh media replaced 3 times per week. At 70-80% confluence, cells were collected and stained with PE conjugated antibodies for CD34 (Miltenybiotec, Auburn, CA) and CD45 (Miltenybiotec, Auburn, CA). Immunodepletion was performed using the anti-PE-microbeads (Miltenybiotec, Auburn, CA) and MSCS column (Miltenybiotec, Auburn, CA) according to manufacturer’s instructions. The CD34 and CD45 negative populations were further propagated and selected for the expression of MSC markers (CD105, CD90, CD44, and CD73) and the absence of CD11b, CD19, and HLA-DR by using a set of fluorescently-labeled antibodies designed for the characterization of human MSCs (BD Biosciences, San Diego, CA) and a MoFlo flow cytometry (Beckman Coulter).

Cell culture and collection of conditioned media

Bone marrow-derived MSCs, isolated from the femurs and tibiae of 5-7 week old male FVB mice, were selected and their differentiation potential assessed as previously described\(^3\). Briefly, after 3-4 passages, plastic adherent cells were immunoselected using mouse specific antibodies (BD Biosciences Pharmingen, San Diego, CA) and a MoFlo fluorescence-activated cell sorter (FACS) (Dakocytomation, Fort Collins, CO), as
we reported previously\textsuperscript{3,4} in compliance with published MSC criteria\textsuperscript{5}. Cells were negatively selected for CD11b, CD14, CD19, CD31, CD34, CD45, and CD79α antigens, and positively selected for CD73, CD90, CD105, c-kit and Sca-1 antigens. Primary MLF cultures were derived according to standard methods\textsuperscript{6,7}.

To exclude contamination from serum-derived microvesicles, serum used for propagation of cell cultures and the collection of CM was clarified by ultracentrifugation at 100,000 x $g$ for 18 hours. MSCs were cultured in $\alpha$-MEM media supplemented with 10\% (v/v) FBS (Hyclone), 10\% (v/v) Horse Serum (Hyclone), 2 mM L-glutamine (GIBCO), and antibiotics. MLFs were cultured in DMEM (Invitrogen) supplemented with 10\% (v/v) FBS and 2 mM L-glutamine. Cultures at 70\% confluence were washed twice with PBS and incubated with serum-free media supplemented with 2 mM L-glutamine for 24 hours under standard culture conditions. Conditioned media were collected and cells and debris were removed by differential centrifugations at 400 x $g$ for 5 mins, at 2,000 x $g$ for 10 mins, and at 13,000 x $g$ for 30 mins. The clarified CM were subsequently filtered through a 0.2 $\mu$m filter unit and concentrated using an Ultracel-10K (Millipore) centrifugal filter device, to a protein concentration range of 0.1-0.5 mg/ml. Protein levels in the CM were determined by Bradford assay (Bio-Rad, Hercules, CA).

**Bronchoalveolar lavage**

Animals were anesthetized with Avertin (250 mg/Kg \textit{i.p.}) and their trachea cannulated with a blunt-ended 20 gauge Luer Stub Adapter (Becton Dickinson). BALF was collected via sequential administration of PBS supplemented with 5 mM EDTA (0.8 ml, 0.8 ml, 0.8 ml, and 0.9 ml) and approximately 3.0 ml (+/- 0.1 ml) of BALF was recovered
per animal. Cells in BALF were collected by centrifugation at 400 xg for 10 min and leukocytes stained with Kimura solution for counting.

**Right ventricular systolic pressure measurements**

Mice were anesthetized with 60 mg/kg of pentobarbital and remained spontaneously breathing. A small incision was made in the abdominal wall, and the translucent diaphragm exposed. A 23-gauge butterfly needle with tubing attached to a pressure transducer was inserted through the diaphragm into the right ventricle and pressure measurements were recorded with PowerLab (ADInstruments, Colorado Springs, CO) monitoring hardware and software. Animals with heart rates less than 300 beats per minute were considered over-anesthetized and their RVSP measurements were excluded. Mean RVSP over the first ten stable heartbeats was recorded.

**Right ventricular weight measurements**

Hearts and pulmonary vasculature were perfused in situ with cold 1X PBS injection into the right ventricle; hearts were excised and used for Fulton’s Index measurements (ratio of RV weight over left ventricle plus septal weight, RV/[LV+S]). Both ventricles were weighed first, then the right ventricular free wall was dissected and the remaining LV and ventricular septum was weighed.

**Pulmonary histology**

Lungs were inflated by tracheotomy and perfused with 4 % paraformaldehyde, excised, and fixed in 4 % PFA overnight at 4°C followed by paraffin embedding. Sections (two per animal) from 4 individuals in each group (group n > 7) were analyzed for pulmonary histology. For pulmonary vascular morphometry, paraffin-embedded lung sections were stained with hematoxylin and eosin. For immunohistochemical analysis, 5 μm lung
tissue sections were deparaffinized in xylene and rehydrated. Tissue slides were treated with 0.3% H$_2$O$_2$ in methanol to inactivate endogenous peroxidases and blocked with horse serum for 1 hour. After incubating with monoclonal anti-mouse α-SMA antibody (Sigma) at a dilution of 1:125 overnight at 4°C, secondary antibodies and peroxidase staining was applied according to manufacturer's instructions (Vector Laboratories, Burlingame, CA). Vessel wall thickness was assessed by measuring α-SMA staining in vessels (20-40 μm in diameter) within each field (40-50 fields per section) captured at 400X magnification with a microscope digital camera system (Nikon, Tokyo, Japan), and using Metamorph image analysis program (Molecular Devices, Sunnyvale, CA). The medial wall thickness index was calculated by the following formula: Wall thickness (%) = 100 x (area[ext] – area[int]) / area[ext] where area[ext] and area[int] denote the areas bounded by the α-SMA layer.

**In vitro hypoxia**

Human PAECs were purchased from GIBCO and cultured in M200 medium (Invitrogen) supplemented with LSGS (Invitrogen). At 80% confluence, cells were exposed to 1% O$_2$ for 6 hours in an inVivo2 workstation (Ruskin Technology, Bridgend, UK) in the presence or absence of exosomal fraction (1 μg/ml), or the exosome-depleted fraction of hUC-MSC conditioned media (1 μg/ml). Cells were lysed and proteins in whole cell lysates were separated on 8% SDS-polyacrylamide gel electrophoresis followed by western blot analysis using rabbit monoclonal antibody for phospho-STAT3 (Y705) and mouse monoclonal STAT3 antibody (Cell Signaling).
**Electron microscopic analysis**

EM analysis was performed at the Harvard Medical School electron microscope facility. Exosome preparations were adsorbed to a carbon coated grid that had been made hydrophilic by a 30 second exposure to a glow discharge. Excess liquid was removed and the samples were stained with 0.75% uranyl formate for 30 seconds. After removing the excess uranyl formate, the grids were examined in a JEOL 1200EX Transmission electron microscope and images were recorded with an AMT 2k CCD camera.

**Protein extraction and immunoblotting**

BALF (3 ml) was centrifuged at 420 x g for 10 min and cell-free BALF supernatants were used for protein analysis. Equal volumes of BALF specimens from individual animals in the same group were pooled (1 ml) and proteins precipitated overnight by 20% trichloroacetic acid (Sigma). A fraction equivalent to 30% of each protein pellet was dissolved in 1x sodium lauryl sulfate (SDS)-loading buffer was separated on a denaturing 15% polyacrylamide gel. After transfer to 0.2 μm PVDF membranes (Millipore), blots were blocked with 5% skim milk and incubated with 1:1,000 diluted rabbit polyclonal MCP-1, galectin-3, or HIMF/FIZZ1 antibody (Abcam) for overnight at 4°C. To detect mouse Immunoglobulin A, 1:5,000 diluted goat anti-mouse IgA antibody (Abcam) was used. Peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotech) was used in 1:20,000 dilution to visualize immunoreactive bands either by the enhanced chemiluminescence reagent (Pierce) or Lumi-LightPLUS (Roche).

For analysis of proteins from whole lung, frozen lung tissues were homogenized for 5 seconds with Polytron in cold PBS containing 2 mM phenylmethanesulfonyl fluoride
(Sigma) and centrifuged at 3,000 \(xg\) for 3 mins. Tissue pellets were washed twice with cold PBS containing 2 mM PMSF followed by centrifugation at 3,000 \(xg\) for 3 mins and lysed in RIPA buffer containing protease inhibitor (Roche) and phosphatase inhibitor cocktails (Thermo). Forty \(\mu g\) of lung tissue extracts were separated on 10-20% gradient gel (Invitrogen). Antibodies for MCP-1, HIMF, IL-6, STAT3, and phospho-STAT3 (Y705) were used for immunoblotting. For loading control, mouse monoclonal \(\beta\)-actin antibody (Sigma) was used.

Proteins in exosome preparations were separated on 12% polyacrylamide gel and then transferred onto 0.45 \(\mu m\) PVDF membrane (Millipore). Goat polyclonal anti-CD63 antibody (Santa Cruz Biotech), mouse monoclonal Alix and TSG101 antibodies (Santa Cruz Biotech), rabbit polyclonal CD81, CD9, hsp90, and flotillin-1 antibodies (Santa Cruz Biotech), and rabbit polyclonal Dicer (Abcam) antibody were used for immunoblotting.

**Isolation and Quantification of microRNAs**

Total lung RNA was extracted by the method of Chomczynski & Sacchi\(^9\) and 750 ng was used as a template for reverse transcriptase with specific primers for each target microRNA (TaqMan Reverse Transcription Kit, Applied Biosystems, Foster City, CA). Each reverse transcription reaction included also the primer for the small nuclear RNA sno202, which was used as an internal control. 37.5 ng cDNA was used for each 20 \(\mu l\) qPCR reaction with TaqMan universal master mix II with no UNG (Applied Biosystems) in the presence of probes specific for the indicated microRNAs and the internal control (TaqMan microRNA assay, Applied Biosystems). Amplification was performed at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min, on a StepOne Plus platform (Applied Biosystems).
RNAs from isolated exosomes were extracted by Trizol reagent (Invitrogen). Briefly, 30 µg exosomal protein was mixed with 0.5 ml Trizol reagent per manufacturer’s recommendation. 20 µg RNase-free glycogen (Ambion) was applied as a carrier prior to RNA precipitation with isopropyl alcohol and samples were placed at -80 °C for overnight. 150 ng exosomal RNAs were used as a template in reverse transcription reactions with specific primers for target microRNAs (TaqMan Reverse Transcription Kit, Applied Biosystems). To quantify pre-let7b, 300 ng of exosomal RNAs were reverse transcribed by High Capacity RNA-to-cDNA kit (Applied Biosystems) per manufacturer’s recommendations. 7.5 ng of cDNA for each microRNA assay and 11.5 ng of cDNA for pre-let7b (TaqMan gene expression assay, Applied Biosystems) were used for qPCR reaction in the presence of specific probes. Amplification was performed as described above. Let7a was used as an internal control.

**Smooth Muscle Cell Proliferation Assay**

Primary rat PASMCs were inoculated at a concentration of 2 x 10³/well on a 96 well plate in DMEM containing 5% FBS and incubated for 24 hours under standard culture conditions. After serum starvation for 2 days in 0.1% FBS/DMEM, cells were pretreated either with vehicle or varying doses of mMEX (16, 31.5, 62.5, 125 ng/ml) for 30 min then FBS was added at 5% (v/v) to each well. After incubation for 48 hours, cell proliferation reagent WST-1 (Roche), which is cleaved by mitochondrial dehydrogenases in metabolically active cells to form formazan dye, was directly applied to the cells followed by further incubation for 3 hours. Intensity of solubilized dark red formazan was determined at 440 nm using a microplate reader.


### Quantities of exosomes from cultures of mMSCs and MLFs

<table>
<thead>
<tr>
<th></th>
<th>No. of Cells (x10⁴) per dish</th>
<th>Total Secreted Protein (μg)</th>
<th>Total Exosomal Protein (μg)</th>
<th>Exosomal Fraction as % of Total Secreted Protein</th>
<th>No. of Cells (x 10⁴) per 0.1 μg Exosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>mMSCs</td>
<td>3.5</td>
<td>200</td>
<td>4.2</td>
<td>2.1</td>
<td>83</td>
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<tr>
<td>MLFs</td>
<td>1.7</td>
<td>35</td>
<td>0.8</td>
<td>2.2</td>
<td>220</td>
</tr>
</tbody>
</table>
Supplemental Figure Legends

Supplemental Figure 1
Flow cytometric analysis of surface-markers for human MSCs. Human MSCs from umbilical stroma (hUC-MSCs) were cultured in DMEM/F-12(1:1) supplemented with 10% FBS. Human UC-MSCs at passage 5 express CD90, CD105, CD73, CD44, but lack expression of CD19, CD34, CD45, CD11b, and HLA-DR in flow cytometric analysis. Isotype-matched IgG controls are shown with non-shaded dotted curves, and hUC-MSCs curves are shown in red shaded area.

Supplemental Figure 2
Comparison of exosomes from two methods of isolation. Exosomes in the medium conditioned by mMSCs were isolated by ultracentrifugation (UCF) or S200 size-exclusion chromatography (SEC). Two μg of proteins from each preparation were loaded onto 12% SDS-PAGE and total proteins stained by SimplyBlue (Invitrogen) (left panel). Exosomal markers, HSP90, flotillin-1, and CD63 were detected by immunoblotting and are comparable in both preparations (right panel).

Supplemental Figure 3
Dose-dependent inhibition of SMC proliferation by MEX. Cultured rat PASMCs were serum-deprived for 48 hours followed by treatment with mMEX (16 to 125 ng/ml) in the presence of 5% FBS and their proliferation rate was quantified relative to the treatment with FBS alone. Data are expressed as mean values ± SD. *, p < 0.001 vs. FBS-alone (One-way ANOVA with Tukey-Kramer post-test).
**Supplemental Figure 4**

MicroRNA content in mouse MEX compared with FEX. RNAs extracted from equivalent amount of MEX and FEX were subjected to RT-qPCR analysis. Levels of the indicated microRNAs relative to let7a in MEX and FEX are presented on the left panel and comparisons between the level of pre-let7b and let7b relative to let7a in MEX and FEX are shown on the right. Data are presented as mean values ±SD. *, p < 0.001 MEX vs. FEX (Student's t-test).

**Supplemental Figure 5**

Schema of a hypothesis synthesizing the results of this study with our previous work and published literature. Hypoxia shifts the Th1/Th2 balance of immunomodulators in the lung, resulting in alternative activated alveolar macrophages (AA-AMΦ) and, in the early phase, induces the expression of IL-6, MCP-1, and HIMF in the lung epithelium. HIMF mitogenic action on the vasculature requires Th2 cytokines, such as IL-4. Consequences of the shift towards proliferation include the hypoxic activation of STAT3 signaling and the upregulation of the miR-17 family of microRNAs. Treatment with MEX interferes with an early hypoxic signal in the lung, suppressing both inflammation and HIMF transcriptional upregulation. It addition, MEX treatment may directly upregulate miR-204 levels, thus breaking the STAT3-miR-204-STAT3 feed-forward loop, and shifting the balance to an anti-proliferative state.
Supplemental Figure 1
Supplemental Figure 2
Supplemental Figure 3
Supplemental Figure 4
Supplemental Figure 5