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

Running title: Lee et al.; Exosomes as paracrine vectors of MSC action

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Abstract:

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

Methods and Results—We fractionated mouse MSC-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 pro-inflammatory and pro-proliferative mediators, including monocyte chemoattractant protein-1 and hypoxia-inducible mitogenic factor, in the murine model of HPH. Intravenous delivery of MSC-derived exosomes (MEX) inhibited vascular remodeling and HPH, 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 whose expression is decreased in human PH. MEX produced by human umbilical cord MSCs inhibited STAT3 signaling in isolated human pulmonary artery endothelial cells demonstrating a direct effect of MEX on hypoxic vascular cells.

Conclusions—This study indicates that MEX exert a pleiotropic protective effect on the lung and inhibit PH through suppression of hyperproliferative pathways, including STAT-3 mediated signaling induced by hypoxia.

Key words: hypertension, pulmonary; inflammation; hypoxia; signal transduction
The lung’s response 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 also in animal models of the disease, such as the monocrotaline- and hypoxia-induced PH (HPH) in rodents. The early component of hypoxia-induced lung inflammation, peaking during the first two to three 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\(^2\).

Despite the significant progress in our understanding of the pathophysiology of PH as well as 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 a promise as 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\(^3\). In models of pro-inflammatory lung diseases, such as bleomycin or endotoxin-induced lung injuries\(^4,6\) as well as the neonatal murine model of bronchopulmonary dysplasia (BPD)\(^7,8\), 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\(^9\) and HPH in the mouse\(^10\). However, although a
robust protection against lung injury upon MSC treatment was observed in most of the above animal models, only a small fraction of donor cells were retained in the recipient lung. This observation suggested that engraftment and direct tissue repair was not the sole mechanism 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 (RVH), completely supplanting MSC treatment on the neonatal murine model of BPD\textsuperscript{7,11}. In vitro experiments demonstrated anti-proliferative properties of the MSC secretome on pulmonary vascular smooth muscle cells\textsuperscript{10}, 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 been recently proposed to account for enhanced MSC therapeutic function in the context of a number of disease models\textsuperscript{12}.

We have previously performed proteomic analysis of MSC-conditioned medium\textsuperscript{7}, which in addition to immunomodulatory factors, revealed the presence of a number of proteins including CD63, CD81, moesin, lactadherin (MFGE8), heat-shock protein 90 (hsp90), and hsp70, reported to be associated with secreted vesicles known as exosomes\textsuperscript{13,14}. Secreted membrane microvesicles, especially the better-defined subclass represented by exosomes\textsuperscript{15}, have been recognized as important mediators of cell-to-cell communication and as participants in immunomodulatory mechanisms\textsuperscript{16}. Exosomes are small heterogeneous microvesicles, 30–100 nm in diameter, that are stored within multivesicular bodies (MVB) and released into the environment upon fusion of the MVB with the plasma membrane. Exosomes and microvesicles have been isolated and characterized from various cell types including dendritic cells\textsuperscript{17}, macrophages\textsuperscript{18}, tumor cells\textsuperscript{19}, and embryonic stem cells\textsuperscript{20} and information is rapidly
accumulating on their diverse biological function and their cell type-specific molecular composition. The physiologic 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-conditioned media (CM) through 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, pro-proliferative pathways were also blocked by MEX treatment, as evidenced by the suppression of signal transducer and activator of transcription (STAT3) phosphorylation resulting in increased lung levels of miR-204, a microRNA enriched in distal pulmonary arterioles that is down-regulated in both human PH and in experimental models of disease\(^1\). 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 pro-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\(^1,2,22\). The hypoxic exposure and treatment protocols used in this
study are described in the Supplemental Material. 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 (hUC-MSCs) followed by immunoselection (Supplemental Figure 1) and collection of conditioned media (CM) is outlined in the Supplemental Material.

Concentrated conditioned media 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 mM sodium phosphate (pH 7.4) and 300 mM NaCl using an ÄKTA purifier liquid chromatography system (GE Healthcare, Piscataway, NJ). 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 this standard’s retention volume were pooled and further analyzed.

For the isolation of exosomes from hUC-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 cut-off (Millipore). Exosomes in CM were precipitated with 1/3 volume of polyethylene glycol (PEG) buffer (33.4% PEG 4000, 50 mM HEPES (pH 7.4), 1 M NaCl) overnight at 4°C followed by centrifugation at 12,000 xg for 5 min and resuspension in PBS (pH 7.4). Exosomes in PEG-precipitated fraction were further purified by S200 size-exclusion chromatography. Seventy-five μl sample was applied on a S200 column (Clontech, Mountain View, CA) preequilibrated with PBS by spinning at 700 xg for 5 min and the exosomal fraction was subsequently eluted in the flow-through by centrifugation at 700 xg for 5 min.

In some experiments, exosomes were isolated by ultracentrifugation at 100,000 xg 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 Bradford assay (Bio-Rad, Hercules, CA). Expression of exosomal markers between the two preparations was similar, as shown in Supplemental Figure 2.

Statistical Analysis

All values are expressed as mean ± standard deviation (SD). All comparisons between experimental and control groups were performed by One-way ANOVA (analysis of variance) with 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 to indicate statistically significant differences. Student’s t-test was used to compare two groups.

See Supplemental Material for a detailed description of further experimental methods.

Results

Factors secreted by MSCs can prevent hypoxia-induced pulmonary inflammation.

To determine if 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% O2) 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\(^2\),\(^{23}\) and levels of hypoxia-induced mitogenic factor (HIMF), a pleiotropic factor with
pro-inflammatory, 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 BPD, the protective effects of MSC treatment in HPH involve mainly paracrine mechanisms.

The anti-inflammatory activity in MSC-CM is associated with exosomes

In order to identify the biologically-active component of MSC-CM, we fractionated concentrated conditioned media 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 (Figure 2A, Fraction I). Negative staining electron microscopic analysis revealed Fraction I to contain heterogeneous microvesicles that were absent in fractions corresponding to the retention volume of moieties of smaller size (Figure 2B, Fraction II). Fraction I was highly enriched in microvesicles 30-100 nm in diameter exhibiting biconcave morphology, a distinct morphological feature of exosomes (Fig 2C, arrows). Exosomes were present in both MSC- and MLF-CM and, in this report, MSC exosome preparations are termed MEX, whereas MLF exosome preparations are termed FEX. Both MEX and FEX contain diverse mature microRNAs (see below) and also Dicer (Figure 2D), a component of the cytoplasmic microRNA maturation complex. However, 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 pro-
inflammatory 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. This inflammatory response is transient in the murine model, peaking within 2-3 days of hypoxic exposure and subsiding by day 7 (Figure 3A, left panel). 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 µg/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 towards later times (Figure 3A, middle panel). The observed temporal profile of pulmonary macrophage influx was paralleled by the temporal profile of induction of the pro-inflammatory markers, MCP-1, interleukin-6 (IL-6), Galectin-3, and HIMF, in cell-free BALF, which also shifted to a later time (4 to 7 days, Figure 3B). In contrast, a treatment consisting of two sequential injections of MEX, one prior to exposure to hypoxia and a second injection at day 4, just prior to the delayed inflammatory peak (Figure 3A, middle panel), efficiently suppressed pulmonary influx of macrophages over the entire period of inflammatory responses to early hypoxia (Figure 3A, right panel). However the peak of pro-inflammatory 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 pulmonary hypertension, right ventricular hypertrophy, and lung vascular remodeling**
The physiologic consequences of partial or complete abrogation of the early inflammatory response to hypoxia are seen in Figure 3C and 3D. A single low dose of MEX did not protect against the elevation of RVSP or the development of RVH after three weeks of hypoxic exposure, whereas the double injection regimen significantly improved both variables. These results mirror the physiologic response we had observed using pulses of heme oxygenase-1 (HO-1) overexpression to completely or partially suppress the early hypoxic lung inflammation and importantly, also completely abrogated the elevation of pro-inflammatory marker FIZZ-1/HIMF in the lung for the entire period of early hypoxic responses. An equivalent dose of FEX had no effect (Figure 5B). Next, to examine the efficacy of higher dose of MEX on vascular
remodeling and HPH, 10 μg of MEX were injected and mice were exposed to chronic hypoxia for three weeks. Compared to vehicle-injected (PBS) controls, the MEX-treated group had significantly (p < 0.001, One-way ANOVA) decreased RVSP (Figure 6A) and did not develop RVH in response to chronic hypoxia (Figure 6B). MEX treatment prevented pulmonary vascular remodeling, as assessed by α-SMA 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 pro-proliferative pathways in the vascular wall thereby increasing medial wall thickness and altering vascular cell phenotype.

**MEX inhibit STAT3 activation by hypoxia**

Early hypoxia resulted in activation of STAT3 in the mouse lung through phosphorylation at Tyr-705 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\(^{25, 26}\). Importantly, persistent *ex vivo* STAT3 activation, has been linked to the hyperproliferative and apoptosis-resistant phenotype observed in pulmonary artery endothelial cells (PAECs)\(^{27}\) and pulmonary artery smooth muscle cells (PASMCs)\(^{28}\) from patients with idiopathic pulmonary arterial hypertension (IPAH). Therefore, to determine whether MEX regulate STAT3 activation on lung vascular cells, we exposed primary human PAECs (hPAECs) to hypoxia and assessed pY-STAT3 levels. As depicted in Figure 7B, exposure of hPAECs to hypoxia results in robust activation of STAT3
by Tyr-705 phosphorylation. Treatment with mouse MEX or MEX derived from MSCs isolated from human umbilical cord stroma\textsuperscript{29} completely abrogated this response. In contrast, neither mouse FEX, human FEX, nor the fraction of human umbilical cord MSC-CM depleted of microvesicles (hUC-ExD-CM) had any 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. In concordance, we found that MEX dose-dependently inhibited PASMC proliferation rate in response to serum-derived mitogens (Supplemental Figure 3), confirming that MEX have direct effects on lung vascular cells.

**Differential miRNA content in MEX vs. 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 vs 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 two types of exosomes, let7b pre-miRNA was significantly enriched in MEX vs FEX (>10 fold) (Supplemental Figure 4). 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 anti-proliferative miR-204 in the lung.**

STAT3 (activated by either vascular endothelial growth factor or IL-6) has been reported to
directly regulate the transcription of the miR-17~92 cluster of microRNAs in PAECs, resulting in decreased levels of bone morphogenetic protein receptor-2 (BMPR2), a target of miR-17\(^{32}\). Therefore, we assessed the effect of hypoxia and MEX treatment on the miR-17~92 cluster of microRNAs and its conserved paralog clusters, miR-106b~25 and miR-106a~363. These microRNA clusters have been postulated to be pro-proliferative and to target an array of genes involved in the G1/S phase transition\(^{33}\). We found that select microRNAs representing all three clusters of the miR-17 superfamily were upregulated by hypoxia in the lung, and 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 HIF1\(\alpha\) in cardiac myocytes\(^{34}\), miR-214, which shares the same host gene with miR-199\(^{35}\), or miR-210, a hypoxamir under direct hypoxia-inducible factor-1 (HIF1\(\alpha\) regulation\(^{36}\), 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 (Fig 8A).

Importantly, we observed that MEX treatment, but not FEX, resulted in the increase of 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\(^{21}\). The proliferative and anti-apoptotic phenotype of PASMCs isolated from patients with IPAH 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\(^{21}\). 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 pro-proliferative 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 physiologic 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\textsuperscript{15,16}. Heat-shock protein 72 from tumor-derived exosomes mediates immunosuppressive function to myeloid-derived suppressive cells through activation of STAT3\textsuperscript{19}. 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\textsuperscript{37}.

Supporting our observations here, microvesicles released from MSCs have been recently reported to improve recovery in animal models of experimentally-induced renal failure\textsuperscript{38} and myocardial ischemia/reperfusion injury\textsuperscript{39}, however, the underlying mechanisms mediating these protective effects were not characterized.

Our results show that treatment with MSC-derived exosomes prevents the activation of 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\textsuperscript{40,41} as well as in human PH\textsuperscript{42}.

Egashira \textit{et al.} demonstrated that administration of exogenous recombinant MCP-1 resulted in prominent medial wall thickening of pulmonary arterioles and MCP-1 receptor blockade prevented monocyte recruitment as well as the subsequent vascular remodeling\textsuperscript{41}. HIMF/FIZZ1, a marker of alternative activated macrophages\textsuperscript{43}, is also induced by hypoxia in the respiratory epithelium and plays a critical role in the development of HPH in a murine model and in scleroderma-associated PH\textsuperscript{24,44,45}. The knockdown of HIMF/FIZZ1 partially blocked increases in pulmonary artery pressure, RVH, 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\textsuperscript{40}. We demonstrate here that MEX administration inhibited the hypoxic induction of both MCP-1 and HIMF/FIZZ1 in the lung and this was associated with prevention of HPH.

Directly related to the anti-inflammatory action of MEX treatment is the observed prevention of hypoxia-activated pro-proliferative pathways. Examining total lung tissue we found that, in addition to MCP-1, hypoxic levels of IL-6 were also suppressed by MEX administration. IL-6 is a pro-inflammatory cytokine known to activate STAT3\textsuperscript{46} and, in a number of studies, was associated with PH\textsuperscript{32,47}. It is thus possible that STAT3 may be a key mediator of hypoxic, pro-inflammatory signaling leading to PH in the \textit{in vivo} lung. Indeed, phosphorylation of STAT3 at the 705 tyrosine residue is required for STAT3 dimerization and subsequent nuclear translocation\textsuperscript{48,49}, and this was markedly increased in both lung and PAECs in response to hypoxia but significantly suppressed by MEX treatment. Importantly, persistent \textit{ex vivo} STAT3 activation, has been linked to the hyperproliferative and apoptosis-resistant phenotype observed in PAECs\textsuperscript{27} and PASMCs\textsuperscript{28} from patients with IPAH. Mathew \textit{et al.} reported a marked
upregulation of STAT3 phosphorylation in the lungs of rats with monocrotaline-induced PH\cite{50}. STAT3 directly regulates the transcription of the miR-17\textendash{}92 cluster of microRNAs in hPAECs, resulting in decreased levels of BMPR2, a target of miR-17\cite{32} whose downregulation is recognized as a hallmark of PH. We found that hypoxia induced select microRNAs of the miR-17 superfamily in the lung and 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 HIF\cite{36}, 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 IPAH, the suppression of miR-204 (a distal pulmonary artery specific microRNA) correlates with PH severity in human disease and rodent models of PH\cite{21}. 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\cite{21} we consider the fact that MEX treatment increases the basal level of miR-204 as a strong indication that MEX treatment is shifting the balance of the STAT3-miR-204 loop to an anti-proliferative state.

A schema of a hypothesis synthesizing the above results with previous work from our group and the work of others is shown in \textbf{Supplemental Figure 5}. We have previously shown that hypoxia shifts the Th1/Th2 balance of immunomodulators in the lung, resulting in alternative activated alveolar macrophages (AA-AMΦ) and this is inhibited by HO-1 overexpression\cite{2}. Hypoxia also induces the expression of HIMF in the lung epithelium\cite{24} and HIMF mitogenic action on the vasculature requires Th2 cytokines, such as IL-4\cite{44} to result in PH. 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 inflammation, HIMF transcriptional upregulation, and alternative macrophage activation. In addition, MEX treatment may directly suppress STAT3 activation in lung vascular cells and also upregulate miR-204 levels, thus breaking the STAT3-miR-204-STAT3 feed-forward loop and shifting the balance to an anti-proliferative state.

The ability to secrete microparticles that contain not only proteins but RNA or miRNA species which can modulate the expression of multiple genes make 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 physiologic relevance is the differential distribution of 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 are molecules that 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 mRNAs, to be translated into new proteins. The authors identified different miRNAs within exosomes and, in a more recent study, pre- and mature miRNAs, but not larger species, were identified within MSC-derived exosomes. Given the robust, long-lasting anti-inflammatory and
cytoprotective effects of MEX demonstrated for the first time in the present study, it is reasonable to postulate that one or more 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.

In summary, in this study we isolated, identified, and characterized exosomes from mouse and human MSC-CM and demonstrated a robust biologic 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 anti-inflammatory 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 need 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.

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**Figure Legends:**

**Figure 1.** MSC-CM but not MLF-CM suppress hypoxia-induced pulmonary influx of macrophages. (A) Macrophage numbers in the BALF of hypoxic mice and littermate normoxic controls. Animals received either serum-free culture media (vehicle), MSC-CM (5 μg protein) or MLF-CM (5 μg protein) prior to hypoxic exposure for 48 hours. Each dot represents the total number of Kimura stain-positive cells in BALF from individual animals. Horizontal lines represent the group mean and vertical brackets the standard deviation. (B) BALF levels of MCP-1 and HIMF. Cell-free BALF specimens from all animals in each treated group were pooled and
an amount corresponding to one animal equivalent was subjected to western blot analysis. IgA levels were assessed as a control for equal loading. n > 6 for all groups. ¶, p<0.001 vs. untreated normoxic controls; §, p<0.001 vs. PBS-treated hypoxic controls (One-way ANOVA with Tukey-Kramer post-test).

Figure 2. (A) Size fractionation of concentrated conditioned media by Sephacryl-400 chromatography. The black trace represents elution of protein (A_{280}) and the gray trace represents the elution profile of 50 nm diameter polystyrene nanospheres (A_{220}). The fractions of conditioned media in the retention volume corresponding to the 50 nm diameter range were pooled and termed Fraction I, and fractions representing moieties of smaller size were pooled as Fraction II. (B) Negative staining electron microscopy at 30,000X magnification revealed heterogeneous microvesicles specifically in Fraction I. (C) Fraction I preparations from either MEX or FEX were enriched in 30-100 nm diameter microvesicles exhibiting typical exosome morphology (arrows). (D) Preparations of exosomes produced by either MSCs or MLFs are associated with typical exosomal markers as well as Dicer. The relative levels of these markers depends on the cellular origin of the microvesicles. Density for each band relative to Alix was measured and relative abundance in MEX compared to FEX for each marker is shown on right. (E) Macrophage numbers in the BALF of animals exposed to hypoxia for 48 hours and treated with either vehicle (PBS), MEX, FEX, or the exosome-depleted fraction of MSC-CM (ExD-CM). Normoxic animals were used as a control. ¶, p<0.001 vs. normoxic control animals; §, p<0.001 vs. PBS-treated hypoxic animals (One-way ANOVA with Tukey-Kramer post-test). (F) Western blot analysis of MCP-1 and HIMF levels in BALF of hypoxic animals treated with isolated exosomes and controls.
Figure 3. Repeated administration of a low MEX dose (x2) can suppress pulmonary influx of macrophages and ameliorate HPH. (A) Animals were injected with one dose of PBS (left panel, n=14) or 0.1 µg MEX (Middle panel, n=31) through the jugular vein prior to hypoxic exposure and alveolar macrophages were measured in BALF at sacrifice on the days indicated on the X axis. Animals (Right panel, n=15) received 0.1 µg MEX prior to 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 (open circles) are replicated in all three 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 of 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. (C) RVSP and (D) Fulton’s Index determined after 3 weeks of hypoxic exposure. The Fulton’s Index (RV/[LV+S]) is a measurement of right ventricular hypertrophy expressed as a weight ratio. RV, right ventricle; LV, left ventricle; S, septum. MEX (x1): Animals were injected once with MEX. MEX(x2), FEX(x2): Animals were injected twice with equivalent amounts of MEX or FEX. Black dots represent values for individual animals, horizontal lines represent 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).

Figure 4. Repeated administration of low MEX dose (x2) inhibits lung vascular remodeling. Representative lung sections are shown from animals exposed to the same conditions as described in the legend of Figure 3 and stained for α-SMA to evaluate medial vessel wall
thickness. (A) 100X magnification and (B) 400X.

**Figure 5.** A single high dose MEX efficiently suppresses the entire period of inflammatory responses to early hypoxia. (A) Macrophage numbers in the BALF of animals injected with a high dose (10 µg) MEX prior to hypoxic exposure for 2 and 7 days. Dots represent values for individual animals. Black dots represent values for MEX-treated group. White and gray dots represent values for normoxic group (NRX) and PBS-treated groups, respectively, which are adopted from Fig 3A. Horizontal lines represent the group mean and vertical brackets the standard deviation. ¶, p<0.001 vs. normoxic control group (One-way ANOVA with Tukey-Kramer post-test). (B) Representative Western blot analysis of FIZZ-1/HIMF levels in BALF of animals treated with a single high dose MEX and FEX (10 µg) prior to hypoxic exposure for 7 days.

**Figure 6.** A single high dose MEX treatment prevents HPH. (A) RVSP measurements and (B) Fulton’s Index of normoxic animals, and animals injected with either PBS or 10 µg MEX prior to a three week hypoxic exposure. Black dots represent values for individual animals, horizontal lines represent the group mean and vertical brackets the standard deviation. ¶, p<0.001 vs. normoxic control group; §, p<0.001 vs. PBS-treated hypoxic control group (One-way ANOVA with Tukey-Kramer post-test). (C) Histology of paraffin-embedded lung sections stained for αSMA at 400X magnification, depicting representative pulmonary arterioles. (D) Assessment of medial wall thickness of small pulmonary arterioles (20-40 µm diameter) in the three experimental groups (sections from four animals per group; n>40 vessels measured per animal). ¶, p<0.001 vs. normoxic control group; §, p<0.001 vs. PBS-treated hypoxic group (One-way ANOVA with Tukey-Kramer post-test).
**Figure 7.** MEX of either mouse or human origin suppress the hypoxic phosphorylation (activation) of STAT3. (A) Total protein extracts from lungs of individual animals treated with 10 μg MEX. Hypoxia exposure for 2 days resulted in activation of STAT3 through phosphorylation at Tyr-705 (pY-STAT3) and this was prevented by treatment with MEX of mouse origin. *Right panel:* Quantitation of STAT3 phosphorylation. #, p<0.01 vs. PBS-treated hypoxic group; ns, statistically non-significant (One-way ANOVA with Tukey-Kramer post-test). (B) Primary cultures of hPAECs exposed to hypoxia (1% O₂, 6 hours) exhibit robust activation of STAT3 that is efficiently suppressed by MEX secreted by MSCs from mouse (mMEX) and human umbilical cord stroma (hMEX). Note that neither the microvesicle-depleted fraction of media conditioned by human umbilical cord MSCs (hUC-ExD-CM), nor hFEX or mFEX, have any effect on STAT3 phosphorylation. Data are representative of at least 3 independent experiments.

**Figure 8.** MEX treatment suppresses the hypoxic induction of the miR-17 microRNA superfamily and increases levels of anti-proliferative miR-204 in the lung. Mice were treated with either PBS, MEX (10 μg) or FEX (10 μg) and then exposed to hypoxia for 7 days. Another untreated group remained in normoxia as control. MicroRNA levels in total mouse lung were assessed by qPCR and fold changes in the hypoxic groups are presented relative to the mean of the normoxic group. (A) Select miRs representing the miR-17~92, miR-106b~25 and miR-106a~363 clusters. (B) Select miRs reported to be involved in hypoxic signaling. (C) Upregulation of basal levels of the pulmonary arteriole-specific miR-204 upon MEX treatment. Dots represent expression levels in individual animals. NRX : Normoxia; HPX : Hypoxia. ¶, p<0.001 vs. normoxia; ‡, p<0.01 vs. normoxia; †, p
A

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ASSOCIATION

Hypoxia

Normoxia

Vehicle

MLF-CM

MSC-CM

IgA

FIZZ-1/HIMF

Alveolar MΦs (x 10³)

0

200

400

600

1

2

3

4

5

6

7
Figure A shows the relative levels of miR-17, miR-20a, miR-106a, miR-106b, and miR-93 with different symbols indicating different groups.

Figure B displays the relative levels of miR-199a-3p, miR-199a-5p, miR-214, miR-210, and miR-204 with symbols and error bars indicating variability.

Figure C presents the relative levels with different symbols representing NRX, HPX + PBS, HPX + MEX, and HPX + FEX.
Exosomes Mediate the Cytoprotective Action of Mesenchymal Stromal Cells on Hypoxia-Induced Pulmonary Hypertension

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Expanded Methods

Animal model and hypoxic exposure

Eight to 10-week old mice (FVB strain) were housed in large plexiglass chambers and the FiO\textsubscript{2} regulated by an OxyCycler controller (BioSpherix, Redfield, NY). Hypoxic exposures were performed at 8.5±0.5 % O\textsubscript{2} and ventilation was adjusted to insure that CO\textsubscript{2} 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 \(\mu\)g protein in 50 \(\mu\)l) or exosome preparations (0.1 \(\mu\)g protein in 50 \(\mu\)l PBS). A higher dose of exosomes (10 \(\mu\)g protein in 50 \(\mu\)l 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 \(\alpha\)-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 \(\mu\)g protein in 50 \(\mu\)l 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\textsuperscript{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\textsuperscript{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\textsubscript{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\textsuperscript{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 CD79\(\alpha\) 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\(^8\) 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 \( \mu \)m 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 $\alpha$-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 $\alpha$-SMA staining in vessels (20-40 $\mu$m 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 $\alpha$-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 inVivo$^2$ workstation (Ruskin Technology, Bridgend, UK) in the presence or absence of exosomal fraction (1 $\mu$g/ml), or the exosome-depleted fraction of hUC-MSC conditioned media (1 $\mu$g/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 µg 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 β-actin antibody (Sigma) was used.

Proteins in exosome preparations were separated on 12% polyacrylamide gel and then transferred onto 0.45 µm 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 & Sacchi⁹ 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 µl 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 10^3/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.
Supplemental References


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

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<th>Total Exosomal Protein (µg)</th>
<th>Exosomal Fraction as % of Total Secreted Protein</th>
<th>No. of Cells (x 10^6) per 0.1 µg Exosomes</th>
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**Supplemental Table 1**
**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
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.