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 FiO_2 regulated by an OxyCycler controller (BioSpherix, Redfield, NY). Hypoxic exposures were performed at 8.5±0.5 % O_2 and ventilation was adjusted to insure that CO_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 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[3, 4] in compliance with published MSC criteria[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[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 α-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 *xg* for 10 mins, and at 13,000 x*g* for 30 mins. The clarified CM were subsequently filtered through a 0.2 μm 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 *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^{o}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_2O_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^{o}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 inVivo$_2$ 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-Light^{PLUS} (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 & 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 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^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec, 60^{o}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

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Quantities of exosomes from cultures of mMSCs and MLFs

	No. of Cells ($x10^6$) per dish	Total Secreted Protein (µg)	Total Exosomal Protein (µg)	Exosomal Fraction as % of Total Secreted Protein	No. of Cells ($x\ 10^3$) per 0.1 µg Exosomes
mMSCs	3.5	200	4.2	2.1	83
MLFs	1.7	35	0.8	2.2	220

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

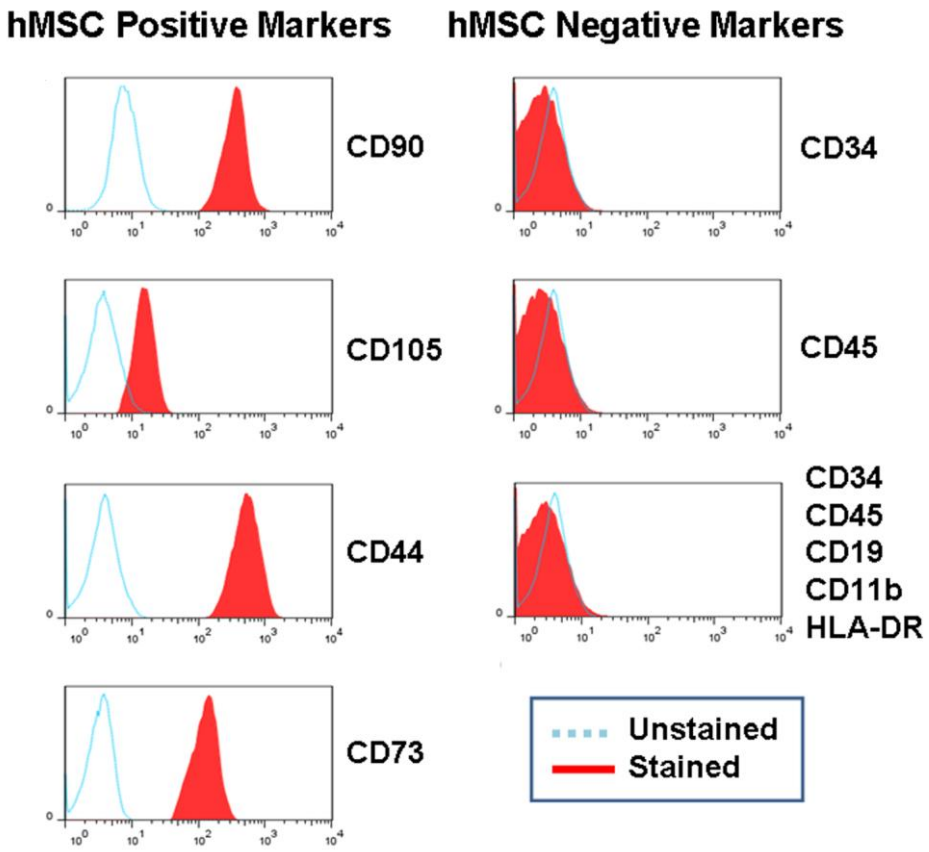
Dose-dependent inhibition of SMC proliferation by MEX. Cultured rat PASMCs were serum-deprived for 48 hours followed by treatment with mMEX (16 to 125 ng/ml) in the presence of 5% FBS and their proliferation rate was quantified relative to the treatment with FBS alone. Data are expressed as mean values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

Supplemental Figure 4

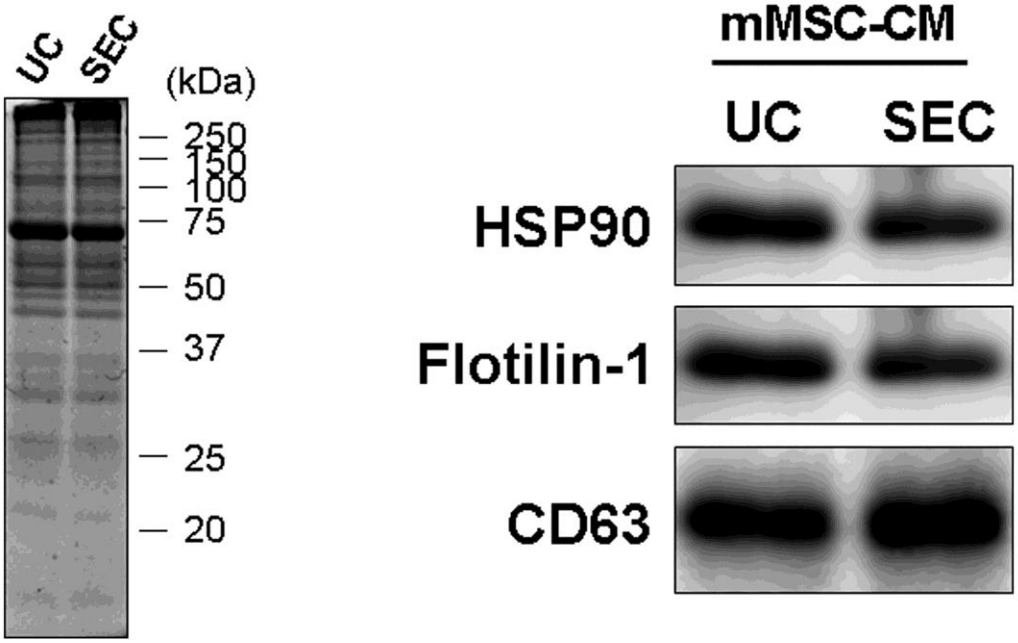
MicroRNA content in mouse MEX compared with FEX. RNAs extracted from equivalent amount of MEX and FEX were subjected to RT-qPCR analysis. Levels of the indicated microRNAs relative to let7a in MEX and FEX are presented on the left panel and comparisons between the level of pre-let7b and let7b relative 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

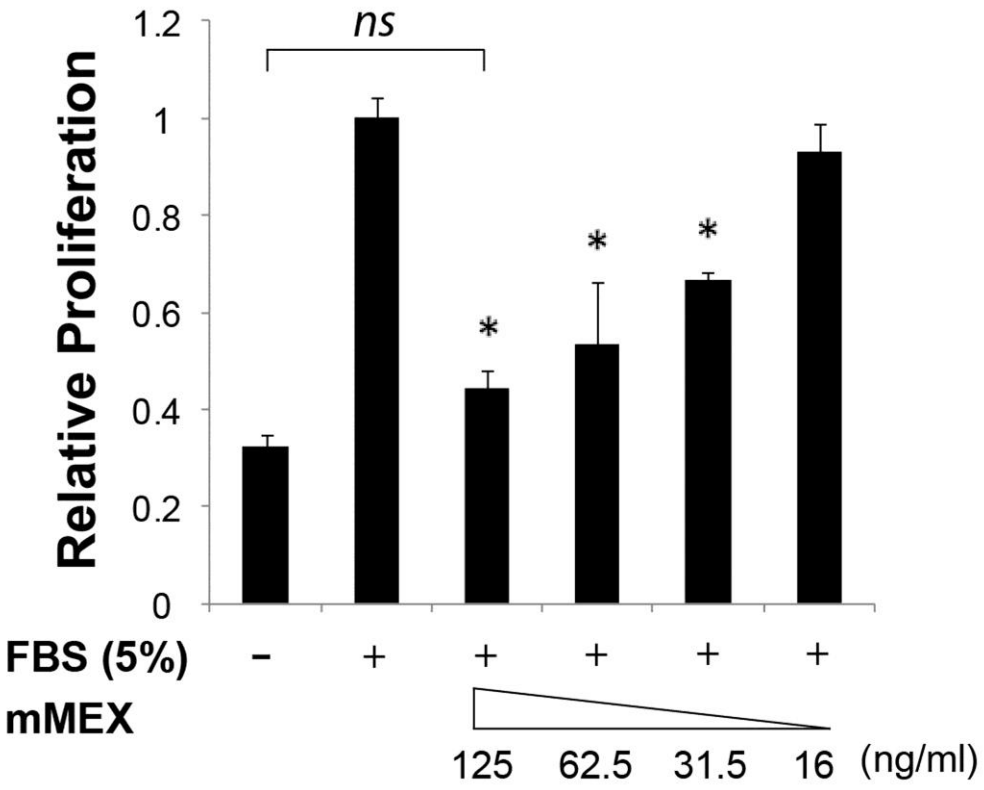
Schema of a hypothesis synthesizing the results of this study with our previous work and published literature. Hypoxia shifts the Th1/Th2 balance of immunomodulators in the lung, resulting in alternative activated alveolar macrophages (AA-AMΦ) and, in the early phase, induces the expression of IL-6, 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.



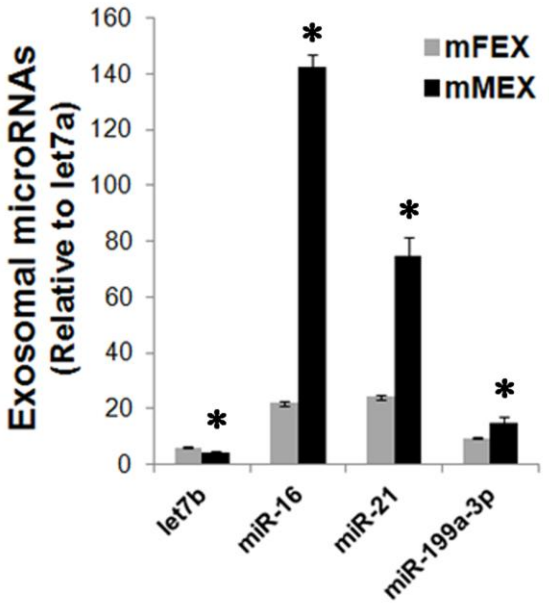
Supplemental Figure 1

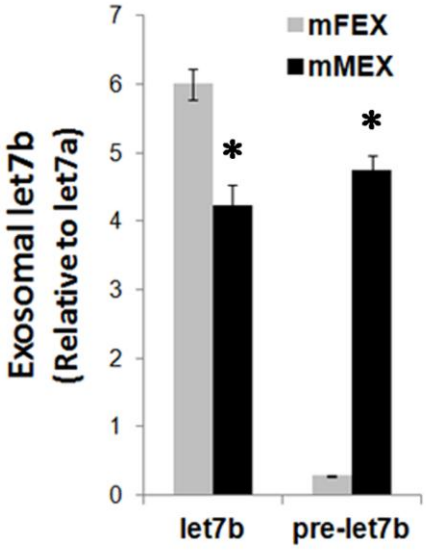


Supplemental Figure 2

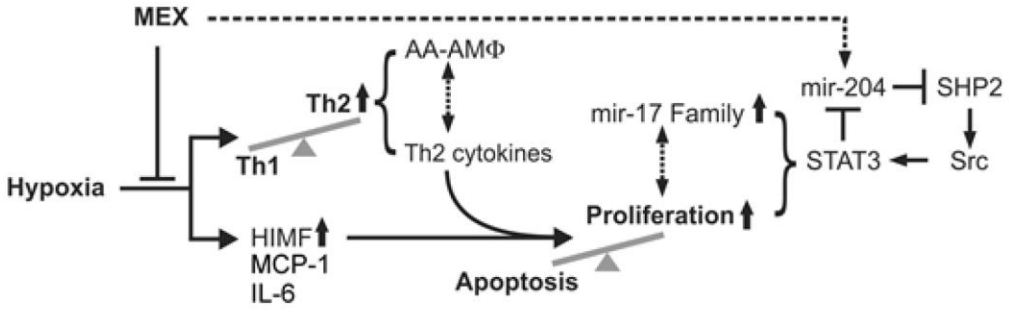


Supplemental Figure 3





Supplemental Figure 4



Supplemental Figure 5