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Supplemental Information

Long Noncoding RNA-Maternally Expressed Gene 3 Contributes to Hypoxic Pulmonary Hypertension

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Supplemental Figure S1

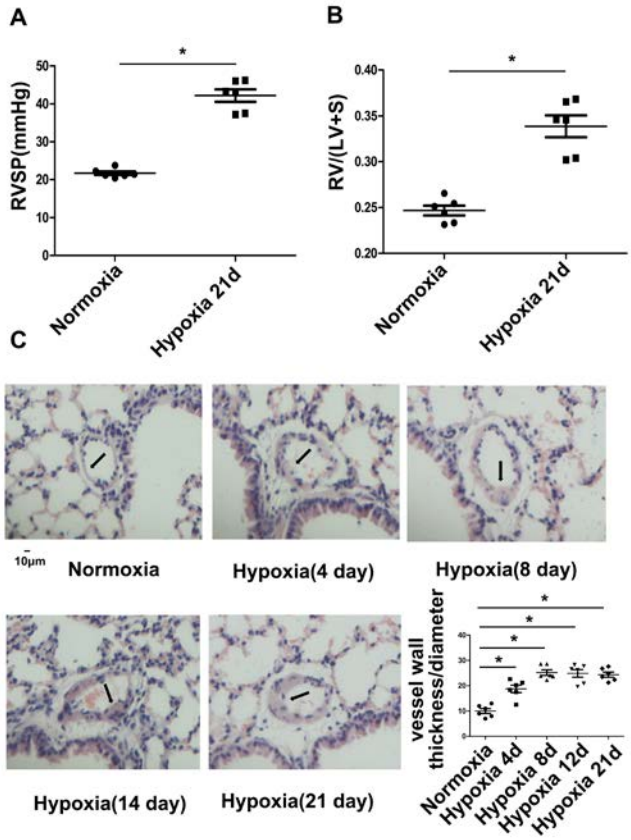

Figure S1. Chronic Hypoxia-Induced PAH mice model. (A) The right ventricular systolic pressure (RVSP) and (B) the right ventricular to left ventricular + spatial weight ratio, two indirect indicators of pulmonary arterial systolic pressure were significantly higher in the 21days hypoxic group than in normal controls. (C) H&E staining demonstrated that the morphology of pulmonary vascular remodeling, as the vessel wall to vessel diameter ratio was significantly higher in hypoxia treated animals groups. $n = 4$ independent experiments. *$P < 0.05$.

Supplemental Figure S2

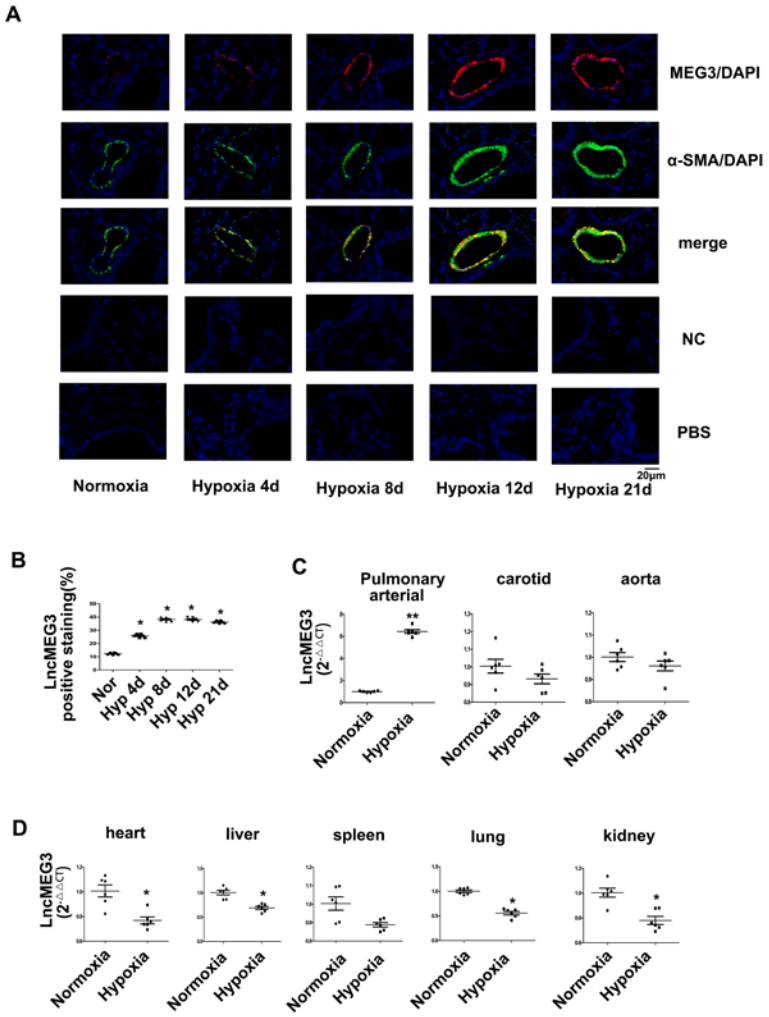

Figure S2. Effects of hypoxia on long noncoding RNA (lncRNA) – Maternally Expressed Gene 3 (MEG3) expression. (A) Mouse was exposed to hypoxia (10% FiO_2) for indicated times, lung section was used to detected lncRNA-MEG3 expression by fluorescence in situ hybridization (FISH). FISH probes targeting to αSMA mRNA were used as positive control, probes target to SUC2 mRNA and PBS were used as negative control. (B) Bar graph showed that hypoxia time-dependently increased the lncRNA-MEG3 expression in mouse pulmonary arteries (PAs) (n = 3). (C) LncRNA-MEG3 expression was detected by real-time PCR in isolated pulmonary arterials, carotid artery and aorta, and (D) in heart, liver, spleen, lung, kidney. (n = 4). **P*<0.05, ***P*<0.01.

Supplemental Figure S3

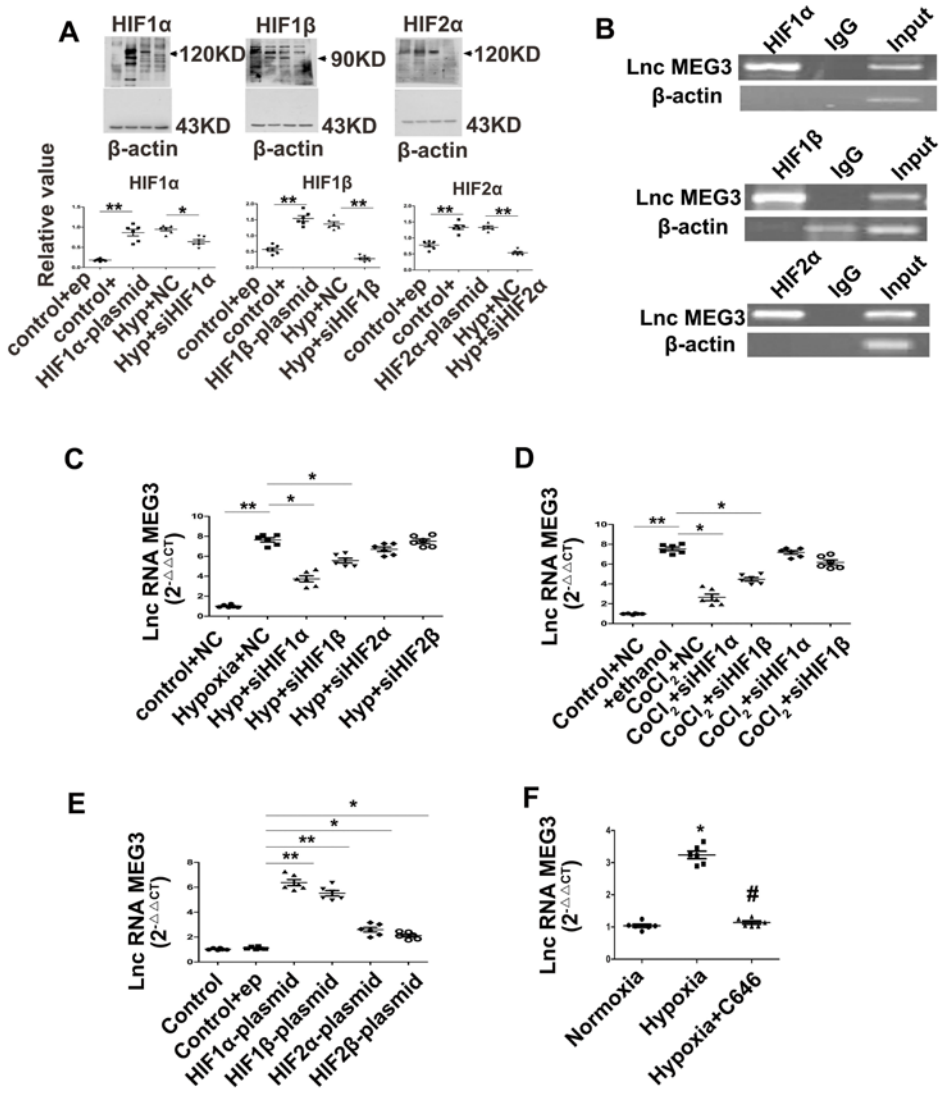

Figure S3. Hypoxia regulated lncRNA-MEG3 though HIF1. (A) Represent image of knockdown and overexpression of HIF1α, HIF1β and HIF2α. (B) CHIP experiment found HIF1α HIF1β, and HIF2α were able to binding to the promoter region of MEG3. (C) LncRNA-MEG3 expression was detected by real-time PCR in mPASMCs was transfected with siRNA targeting HIF1α, HIF1β, HIF2α and HIF2β, then exposured to hypoxia or cobalt chloride ($CoCl_2$, 100μM) (D) for 24 h. (E) mPASMCs was transfected with plasmids overexpressing HIF1α, HIF2α, HIF1β and HIF2β. (F) Effects of histone acetyltransferase inhibitor C646 on lncRNA-MEG3 expression under hypoxia condition. n = 6 independent experiments. **P* < 0.05, ***P* < 0.01.

Supplemental Figure S4

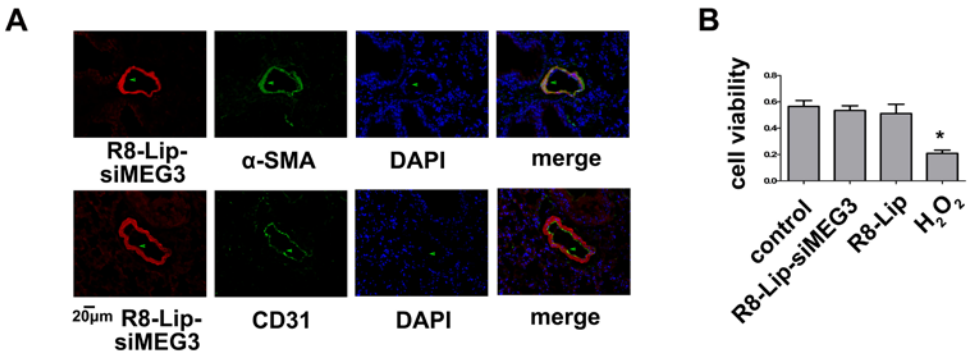

Figure S4. Lung specific knockdown of lncNRA-MEG3 prevented hypoxia-induced PASMCs proliferation. (A) Colocalization of labeled siMEG3 (red) with immunostaining of αSMA (up panel, green) but not CD31 (lower panel, green) indicated that siRNA was delivered to the smooth muscle layer by the R8-liposome delivery system. (B) MTT data showed that R8-modified liposomes were not cytotoxic towards PASMCs after 24 h. n = 6 independent experiments. **P* < 0.05, ***P* < 0.01.

Supplemental Figure S5

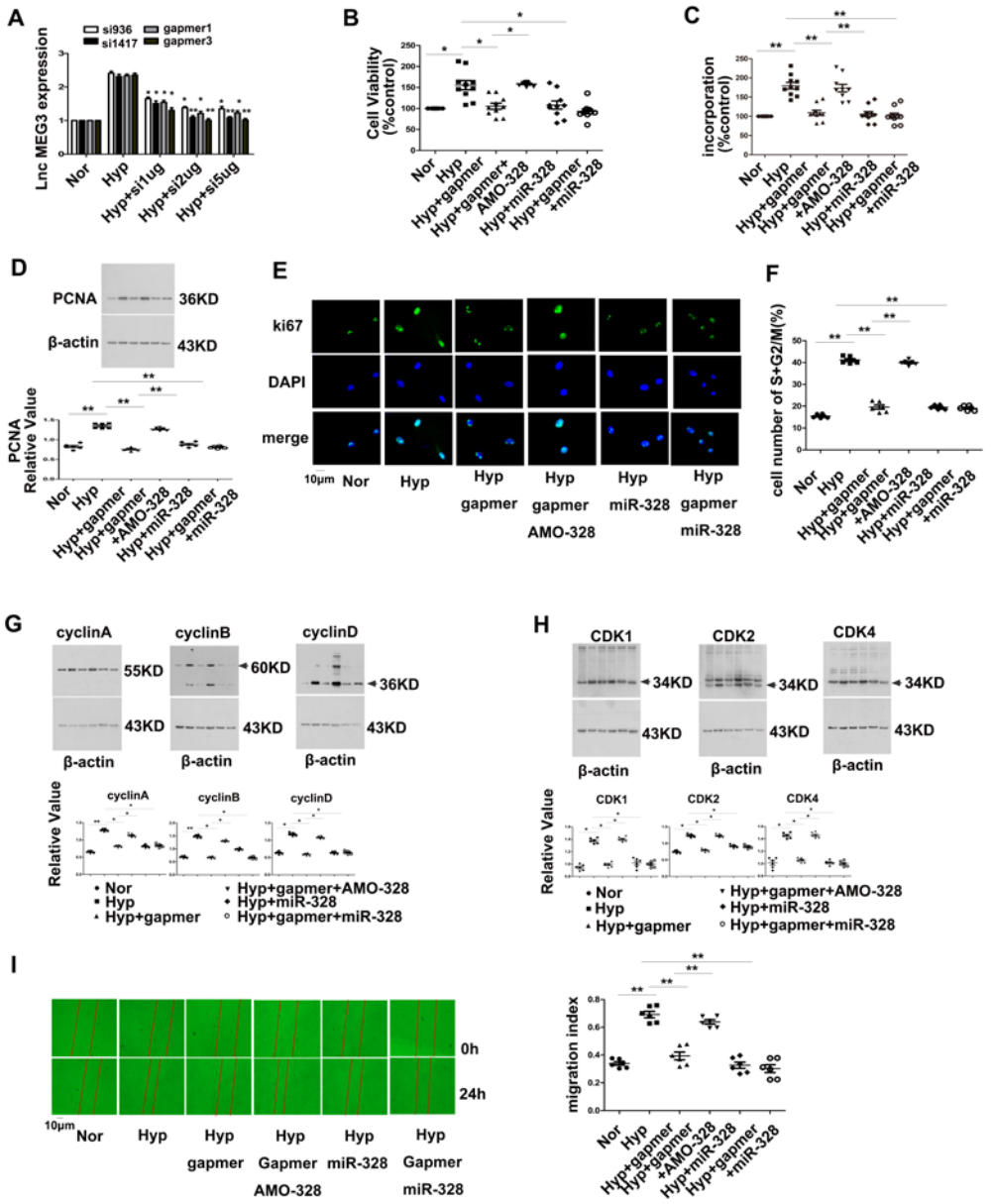

Figure S5. Knockdown of lncRNA-MEG3 by gapmer reversed hypoxia-induced PASMCs proliferation, cell cycle progression and cellular migration. (A) The dose-dependent manners of two small interference RNA (si936 & si1417) and two different gapmeRs were designed and transfected to knockdown the expression of lncRNA-MEG3. The gapmers of lncRNA-MEG3 significantly abolished the increasing PASMCs cell viability induced by hypoxia determined by MTT (**B**), The BrdU incorporation (**C**), and PCNA expression **(D)** determined by immunoblotting and Ki67 staining (**E**) by immunocytochemistry. (**F**) Cell cycle progression detected by flow cytometery, (**G**) Cyclinsand (**H**) CDKs expression, and (**I**)cellular migration showed the gapmer of lncRNA-MEG3 on hypoxia-evoked cell cycle progression and cellular migration. PCNA represents proliferating cell nuclear antigen. CDK represents cyclin-dependent kinases. *n* = 6 independent experiments. *P<0.05, **P<0.01.

Supplemental Figure S6

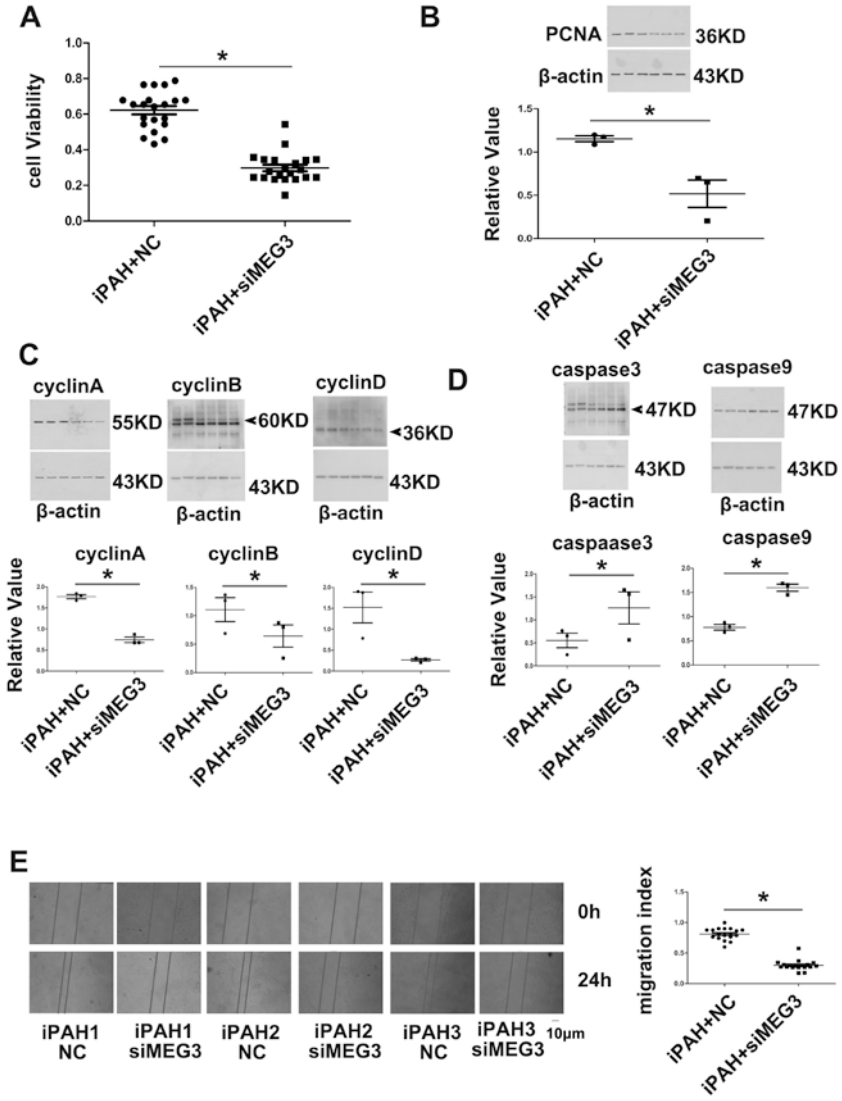

Figure S6. MEG3 reduces iPAH-PASMC viability, proliferation, resistance to apoptosis and migration. (A) Cell viability, (B) PCNA expression, (C) Cyclins expression, and (D) CDK expression detected by western blot, (E) Cell migration experiments. PCNA represents proliferating cell nuclear antigen. CDK represents cyclin-dependent kinases. n = indicated independent experiments. *P<0.05, **P<0.01.

Supplemental Figure S7

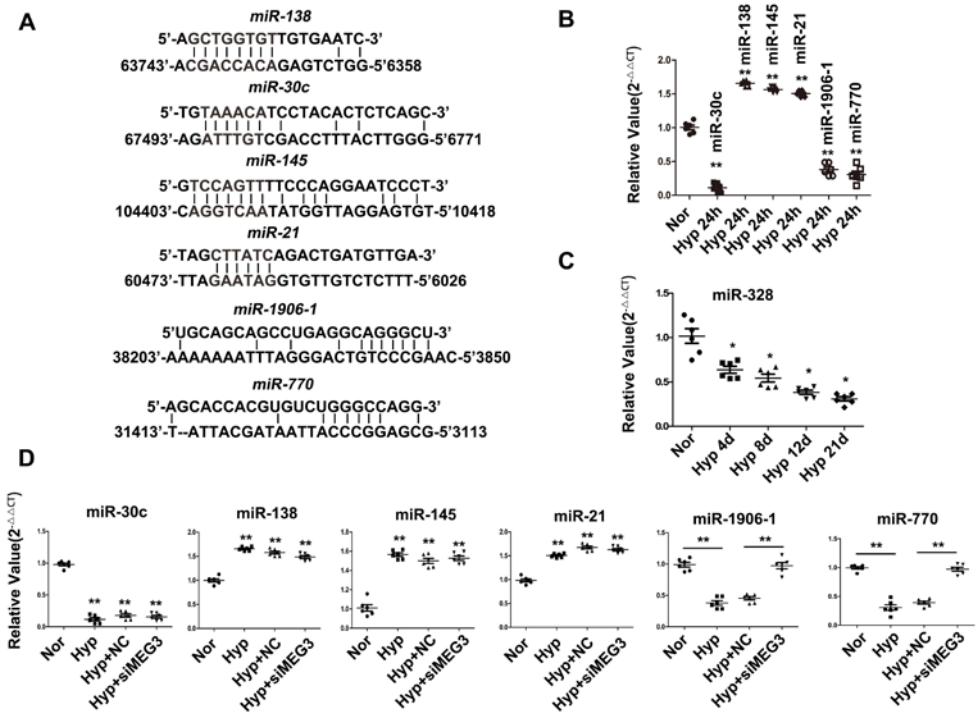

Figure S7. The association of lncRNA-MEG3 with other microRNAs. (A) Prediction of the binding site of lncRNAs-MEG3 with miR-138, miR-30c, miR-145, miR-21, miR-1906-1, and miR-770. (B) Real-time PCR detected the expression of miR-138, miR-30c, miR-145, miR-21, miR-1906-1, and miR-770 in hypoxia treated mPASMCs. (C) The downregulation of miR-30c, and the upregulation of miR-138, miR-145 and miR-21 expression were not affected by lncRNA-MEG3 knockdown, however, miR-1906-1 and miR-770 expression was regulated by lncRNA-MEG3 under hypoxia condition. (D) Real-time PCR revealed that hypoxia inhibited miR-328 expression with a time-dependent manner. n = 6 independent experiments. $^{**}P<0.01$.

Supplemental Figure S8

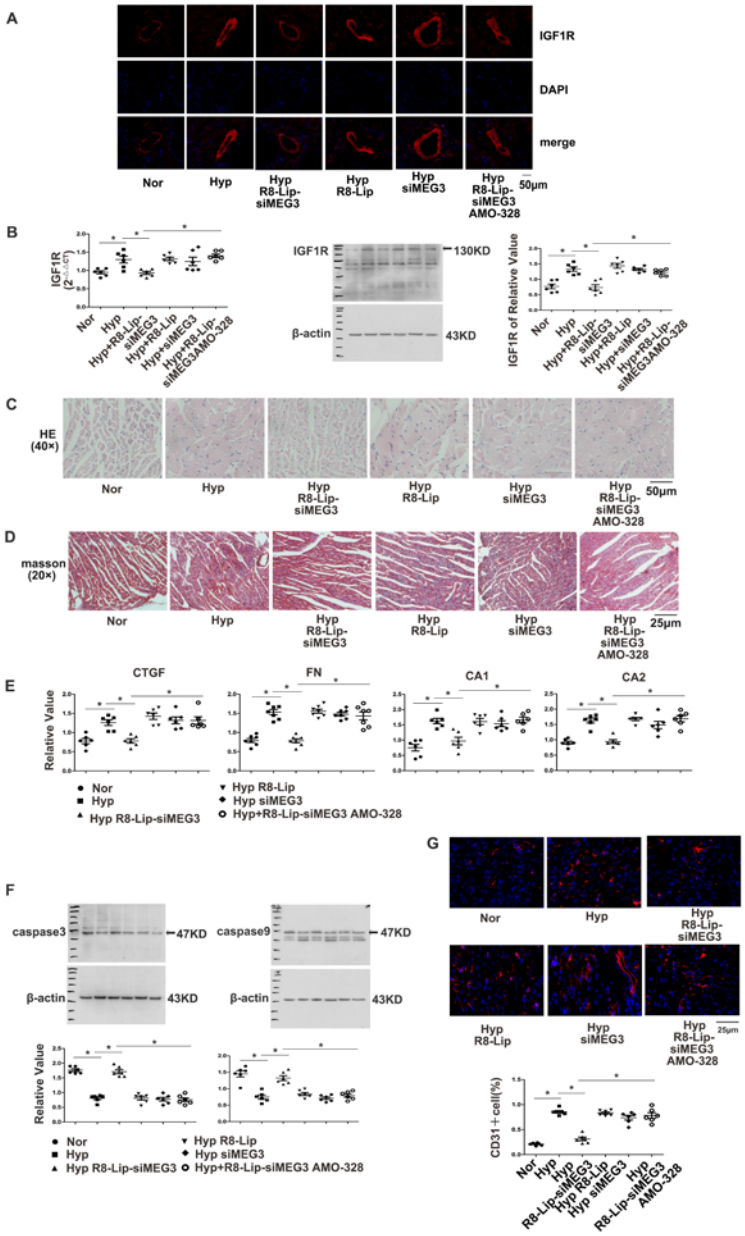

Figure S8. IGF1R expression and right ventricular failure was modulated by lncRNA-MEG3/miR-328-3p in relevant mouse models. (A) Immunofluorescence target to IGF1R in pulmonary artery. (B) IGF1R mRNA expression in right ventricular detected by real-time PCR. (C) IGF1R protein expression in right ventricular detected by western blot. (C) The Cardiomyocytes hypertrophy was detected by H&E staining. (D) The fibrosis of right ventricular was assessed by Masson's trichrome staining, (E) Real-time PCR detected the fibrosis markers, markers, includes connective tissue growth factor, fibronectin, collagens A1 and A2 in right ventricular. (F) The apoptosis of cardiomyocytes was assessed by the expression of caspase 3 and caspase 9. (G) Immunofluorescence target to CD31 was measured in the right ventricular section. The microvessel density was expressed as the proportion of $CD31^+$ cells in the whole section.n = 6 animals, a minimum of 10 resistance pulmonary arteries (<100 μm external diameter) were analyzed per animal. **P<0.01.

Supplemental Figure S9

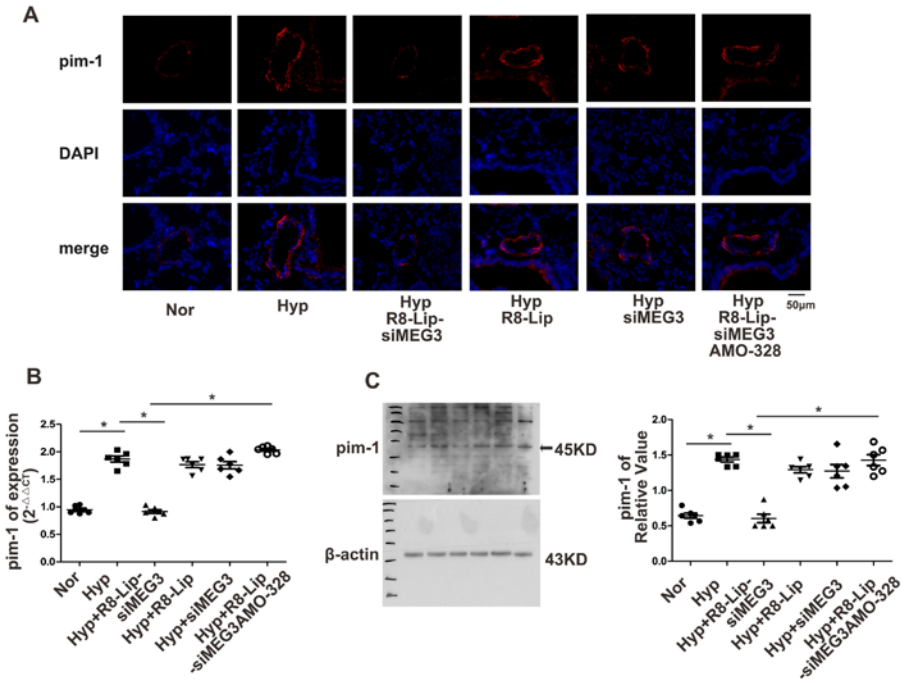

Figure S9. Pim1 expression was modulated by lncRNA-MEG3/miR-328-3p signaling. (A) Pim1 expression was detected by immunofluorescence in lung section. (B) Real-time PCR detected the Pim1 mRNA expression in lung tissue. (C) Western blot examined the Pim1 protein expression in lung tissue. Pim1 expression was increased by in pulmonary artery from HPH mice, pulmonary-specific MEG3 knockdown (R8-Lip-siMEG3) attenuated, whereas siRNA targets to MEG3 without any modification (siMEG3) and R8-Liposome did not prevent the hypoxia-trigged pim1 expression in pulmonary artery. n = 6 independent experiments. *P<0.05.

Supplemental Figure S10

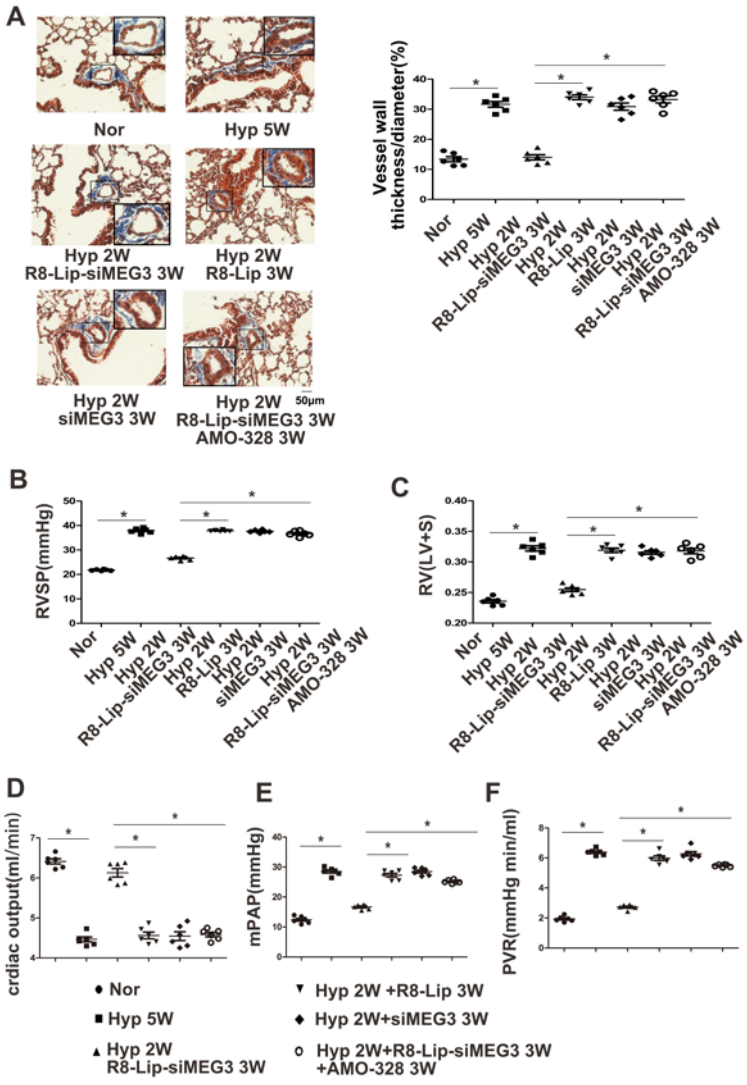

Figure S10. The beneficial effect of R8-Lip-siMEG3 once PH established. (A) Masson trichrome staining, (B) RVSP, (C) RV/LV+S, (D) Cardiac output (CO), (E) mPAP, (F) Pulmonary vascular resistance calculated as mean pulmonary arterial pressure/CO. All these data indicated that lung specific delivery of R8-Lip-siMEG3 was able to reverse the established PH induced by hypoxia. Moreover, miR-328-3p therapy was able to reverse the effects of R8-Lip-siMEG3. n = 6 animals, a minimum of 10 resistance pulmonary arteries (<100 μm external diameter) were analyzed per animal. *P<0.05, **P<0.01.

Supplemental Table S1

Patient	Diagnosis	Origin	Sex	Age	mPAP (mmHg)	（PAWP） (mmHg)	RAP (mmHg)	PVR (WU)	CO (L/min)	LVEF (%)	TAPSE (mm)	NYHA FC#	ERA	PDE5 inhibitor	Epoprostenol
1	iPAH	Surgery	F	48	40	13	11	6.92	3.9	55%	12	II	no	yes	no
2	iPAH	Surgery	M	43	43	11	7	6.95	4.6	60%	11	II	no	yes	no
3	iPAH	Surgery	M	32	47	3	0	9.78	4.5	60%	9	II	yes	yes	no

CO: cardiac output; iPAH: idiopathic pulmonary arterial hypertension; F: female; LVEF: left ventricle ejection fraction; M: male; mmHg: millimetres of mercury; mPAP: mean pulmonary artery pressure; N/A: not available; NYHA FC: New York Heart Association functional class; PAWP: pulmonary artery wedge pressure; PVR: pulmonary vascular resistance; RAP: right atrial pressure; WU: Wood units. ERA: endothelin receptor antagonist; PDE5: phosphodiesterase-5.

Supplemental Methods

Animal Model of Chronic Hypoxia-Induced PH and tissue preparation

Adult male C57BL/6 mice (mean weight of 30 g) were obtained from the Experimental Animal Center of Harbin Medical University, which is completely accredited by policies of the Institutional Animal Care and Use Committee. The animal study was approved by the ethics review board of Harbin Medical University ([2012]-006). The study design was trying to following the guideline by Provencher et.al.[1] Including but not limited to two PH models, invasive hemodynamic assessment, image-base data, randomization, blinded assessment of standardized outcomes et. al. Animals were conditioned at a controlled ambient temperature of 22 ± 2 °C with 50% ± 10% relative humidity and at a 12-h light–dark cycle (lights on at 8:00 am). Standard rat chow and water were provided adlibitum to all mice.

For the chronic hypoxia induced pulmonary hypertension model, Mice were randomized for exposure for the indicated times to normal and hypoxic environments with fractional inspired oxygen (FiO_2) of 0.21 and 0.12, respectively, as previously described.[2] At the end of the indicated hypoxia exposure period, mice were anesthetized with chloral hydrate (40 mg/kg, i.p.). Right ventricular systolic pressure (RVSP) and right ventricular to left ventricular + spatial weight ratio (RV/LV+S), two index of pulmonary artery systolic pressure were measured, the methods were described in the following. In some experiments, cardiac output was measure by left ventricular catheters. Then the chest was opened, following by tissues (heart, spleen, kidney, liver, lungs, aorta and carotid artery) were harvested for the following experiments. The right lung was then cut into pieces that were either fixed with 4% paraformaldehyde PBS solution before being embedded in paraffin blocks for hematoxylin-eosin (H&E) staining or stored at −80°C for protein and mRNA analyses. The external diameter and medial wall thickness were measured using a calibrated micrometer. The percent of vessel wall thickness of each artery was calculated.

For the SUGEN models, Animals received Su5416 (20 mg/kg subcutaneously; dissolved in DMSO; Sigma-Aldrich, St Louis, MO, USA), immediately followed by hypoxia exposure immediately. After 3 weeks of hypoxia, animals were returned to room air for another 4 weeks.

To access the beneficial effect of R8-Lip-siMEG3 once PH established, mice was exposure to hypoxia for 2 weeks to establish the PH, then R8-Lip-siMEG3 was injected through the tail vein (4 times on day 14, 17, 21 and 28) and housing in hypoxia chamber for another 3 weeks.

To knock down the expression of lncRNA-MEG3 in pulmonary arteries (PAs), siRNA against lncRNA-MEG3 (siMEG3) was loaded with octaarginine (R8) conjugated PEG2000-Lipid (R8-Lip) to form a lung-specific delivery system (R8-Lip-siMEG3) as previously described with some modifications. Briefly, octaarginine (R8) and DSPE-PEG2000-Mal (molar ratio: 1.5:1) were mixed in chloroform/methanol (v/v = 2:1) at room temperature with gentle stirring for 48 h. The mixture was evaporated under vacuum and then re-dissolved in chloroform, after discarding the insoluble material; the supernatant (DSPE-PEG2000-R8) was evaporated again under vacuum. Lipid compositions of the prepared liposomes were as follows: R8 modified liposomes (R8-LIP), SPC/Chol/DSPE-PEG2000/DSPE-PEG2000-R8 (molar ratio 59:33:3:5). A lipid film was produced by rotary evaporation of all lipids in chloroform. The films were left under vacuum for 2 h. Hydration buffer was added to produce a concentration of 10 μmol/3 mL of lipid. The lipid suspensions were sonicated with a probe sonicator at 80 W for 2 min. siMEG3 and lipid ingredients, at a siRNA to lipid molar ratio of 1:40, were dissolved in chloroform to prepare siRNA-loaded liposomes. Free siRNA was removed using a Sephadex-G50 column and the pellets were collected. The bio-distribution of R8-Lip was evaluated with a labeled siRNA (si-MEG3) and fluorescence images were captured. Additionally, αSMA stains or CD31+ stains were used to detect smooth muscle and endothelial compartments with delivery. To efficiently knock down the expression of lncRNA-MEG3, R8-Lip-siMEG3 was repeatedly injected on days 1, 4, 7, and 14 during experiment. R8-Lip and siMEG3 plus Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) were used as controls and repeatedly injected. The injected volume is 100 μL/mice. After injection, mice were returned to normal and hypoxic environments, respectively. No death of animals was observed during the course of the experiments. In some experiments, AMO-328 (miR-328 specific 2-*O*-methyl antisense inhibitory oligoribonucleotide) was loaded into the liposome together with siMEG3. The efficiency of MEG3 knockdown by R8-Lip-siMEG3 was confirmed by using Real-Time PCR and FISH.

Right ventricular systemic pressure and ventricular hypertrophy index measurement

For the hemodynamic measurement, all mice were anesthetized with chloral hydrate (40 mg/kg, i.p.). The right jugular vein was separated; a Mouse Pressure-Volume (PV) Loop Catheters (SPR839, Millar Instrument, Houston, TX) was inserted into the vein and passed into the right ventricle to measure right ventricular systemic pressure (RVSP). The location of the transducer was monitored

by pressure tracing. The pressure signals were exported to a bridge amplifier (PL3508 PowerLab 8/30 Data Acquisition Systems; ADInstruments Pty Ltd., Castle Hill, NSW, Australia) where the signals were amplified and digitized. The data were recorded and later analyzed with the LabChart 8 software.

To evaluate the right ventricular hypertrophy, the right ventricular (RV) was freed from the left ventricular and septum (LV+S), and dried with absorbent paper. The weight ratio of RV/LV+S was calculated.

Echocardiography

In some experiments, right ventricular and pulmonary artery pressure, cardiac output (CO) was assessed by echocardiography (Philips HD11 XE imaging system/S3-1 probe). Total pulmonary vascular resistance (PVR) was calculated as mean pulmonary arterial pressure (mPAP) divided by cardiac output.

Histological and morphometric analyses

Mice tissues were fixed in 4% paraformaldehyde for 24 h, and then dehydrated, cleared, and embedded in paraffin wax. The paraffin-embedded tissues were sliced into 5 μm-thick sections and stained with hematoxylin and eosin (H&E) or Masson trichrome staining, as appropriate. Images were captured with a fluorescence microscope (Nikon) equipped with a digital camera. A minimum of 10 resistance pulmonary arteries (<100 μm external diameter) were analyzed per animal.

Cell Culture

The mice pulmonary artery smooth muscle cells (mPASMCs) and human pulmonary artery smooth muscle cells (hPASMCs) were purchased from the Sciencell Company (Shanghai, China) and were cultured in smooth muscle cell medium (SMCM) (Sciencell, Shanghai, China) in a humidified incubator with 5% CO_2 at 37 °C. Passages 2 - 4 were used for further experiments. The PASMCs of iPAH patients were provided by Professor Jian Wang from State Key Laboratory of Respiratory Diseases, Guangzhou Institute of Respiratory Disease, and The First Affiliated Hospital of Guangzhou Medical University (Guangzhou, China). The clinical information of the iPAH patients was attached in supplemental Table S1. The usage of these human iPAH-PASMCs was followed the recommendation by Bonnet et.al.[3] Passages 12 - 14 of PASMCs of iPAH patients were used for further experiments. Before each experiment, the cells were incubated in serum-free low-glucose

DMEM for 24 h to stop growth. For hypoxic cultivation, the cells were grown in a Tri-Gas incubator (HF100; Heal Force) with 92%N_2 /5% CO_2 /3% O_2 for the indicated times as described previously.[4]

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

Total RNA from cultured PASMCs and mouse tissues were isolated using miRNeasy kits (Qiagen, Hilden, Germany) according to the protocol of Xing et al.[5] For measuring microRNAs or lncRNAs, 100 ng total RNA was reverse transcribed using Prime Script® RT reagent Kit (TaKaRa Biotechnology) in a 20 μL reaction. cDNA was used as template for quantitative real-time polymerase chain reaction (PCR) using Fast SYBR Green (Applied Biosystems, Forster City, CA) with lncRNA-MEG3 primer (mouse MEG3, sense 5'-CTTAGCGTGTCTGCCTGTGT-3'; antisense 5'-GGAGGCCCAAT GTGTGTATG-3'; human MEG3, sense 5'-GAGTGTTTCCCTCCCCAAGG-3'; antisense 5'-GCGTGCCTTTGGTGATTCAG-3'); miR-328-3p (sense 5'-GGGGGGCAGGAGGGGC-3'; antisense 5'-AGTGCAGGGTCCGAGGTATT-3'). 18s and U6 mRNA was used for normalization. Analysis of relative gene expression levels was performed using the formula $2^{-\Delta\Delta CT}$ with $\Delta\Delta CT=(CT_{(\text{target gene in hypoxia group})}- CT_{(\text{control gene in hypoxia group})})-(CT_{(\text{target gene in control group})}- CT_{(\text{control in control group})})$.

Oligonucleotides design and transfection in vitro

To silence the expression of lncRNA-MEG3, PASMCs were transfected with the small interfering RNA (siRNA) targets to lncRNA-MEG3 (siMEG3-936, 5'-GCGUCUUCCUGUGCC AUUUTT-3' sense; 5'-AAAUGGCACAGGAAGACGCTT-3' antisense), (siMEG3-1147, 5'-CC UCCUGGAUUAGGCCAAATT-3' sense; 5'-UUUGGCCUAAUCCAGGAGGTT-3' antisense) Non-targeted control siRNA (siNC, 5'-UUCUCCGAACGUGUCACGUTT-3' sense; 5'-ACGUG ACACGUUCGGAGAATT-3' antisense), using X-treme Gene siRNA Transfection Reagent (Roche). In some experiments, lncRNA-MEG3 was knocked down with gapmers (gapmer-1, TCCATTTGCCTCATAA; gapmer-3, CACTCCATCACTCATA), which was designed and synthesized by Exiqon (Vedbaek, Denmark).

Cell viability assay

The mPASMCs were transfected with siNC, siMEG3, respectively and cultivated for another 48 h. After 48 h of the incubation in 37°C, the cells were incubated for 4 h in a medium containing 0.5% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT). The absorbance was read at 540 nm in a spectrophotometer.

BrdU assay

5-Bromo-2-deoxyuridine (BrdU) incorporation was detected with Millipore©'s BrdU Cell Proliferation Assay Kit (Millipore, US) according to the provided protocol. Briefly, the cells were treated as indicated, and BrdU was added into the wells and incubated for 4 h. After being fixed by the fixer solution, the anti-BrdU monoclonal antibody and the second antibody were added and incubated for the indicated time period. TMB Peroxidase Substrate was added and incubated for 30 min at room temperature in the dark. Positive wells were visible by a blue color, the intensity of which was proportional to the amount of BrdU incorporation in the proliferating cells. The plates were read using a spectrophotometer microplate reader set at a single wavelength of 450 nm.

Protein preparation and western blot analysis

Isolated PAs or mPASMCs (25 cm^2 culture flask) were lysed into 400 μl of protein lysis buffer containing protease inhibitors. A total of 20μg samples were heated in SDS-PAGE sample buffer at 95°C for 5 min and then were separated by SDS-PAGE gels. Primary antibodies against PCNA (1:500), CyclinA (1:200), CyclinD (1:400), CyclinB (1:400), CDK1 (1:200), CDK2 (1:500), CDK4 (1:500) were incubated in 5% BSA-TBST. Blots were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:7000) in 5% BSA-TBST for 1 h at room temperature. The immunoreactivity was visualized using enhanced chemiluminescence kit (Beyotime, Guanzhou, China).

Immunofluorescence assay

The sections of lung tissue or the cultured PASMCs were incubated with antibody against Ki67 (1:100), IGF1R (1:100), and Pim 1 (1:150) overnight at 4°C, followed by secondary IgG (Santa Cruz) (1:1000) conjugated with fluorescein isothiocyanate (FITC) or CY3, respectively, for 2 h at 37°C. DAPI (4,6-diamidino-2-phenylindole) was used to label nuclei. Fluorescence images were obtained with a fluorescence microscope.

Cell cycle progression analysis

The proportion of cells in the G0/G1, S and G2/M phases was detected by flow cytometry, as reported previously.[4] Briefly, the cells were pre-treated with hypoxia with or without other indicated agents and then harvested by trypsinization. Cells were then fixed with 70% ethanol. 25μl of PI (propidium iodide) was added, and then the cells were filtered once through 400-mesh sieves and detected by flow cytometry.

Cellular migration assay

A "wound" was created by a pipette in a mPASMC cell monolayer. Images were captured and marked as beginning. The cells were treated as indicated, following hypoxia for 24h, the images were captured, and the images were compared to beginning images to quantify the migration rate of the cells.

Computational prediction of lncRNA target

We used established lncRNA target-prediction algorithms including microran.org and generunner to identify the candidate microRNAs that were potentially targeted by lncRNA-MEG3.

Statistical Analysis

Quantitative data are expressed as the means ± SEM. Data analysis was performed with paired Student's t test (for two means) or one-way analysis of variance followed by Dunnett's test (for >2 means) where appropriate. All the experiments were performed using an unbiased method: all the experiments were blindly performed and analysed. Finally, a biostatistician has performed and approved the statistics used in the present study. Sample sizes (n) were reported in the corresponding figure legend. Significance levels of $P < 0.01$ (**) and 0.05 (*) were considered statistically significant.

Reference for Online Supplement

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