Supplementary Information

Supplementary Methods:

Reagents

The MK571 was from Alexis Biochemicals. Recombinant human PDGF-BB was from R&D Systems. Staurosporine was purchased by Sigma Aldrich (S5921). All media and sera for culture of human PASMC and human PAEC were respectively from PromoCell (PromoCell GmbH) and Lonza ; antibiotics were from Invitrogen. Sildenafil citrate was provided by Pitié-Salpétrière hospital pharmacy.

Animals and experimental design

After mice were euthanized, a thoracotomy was performed and after exsanguination, the left lung was fixed for histology in 10% neutral buffered formalin, the right lung was snap-frozen in liquid nitrogen. Haematoxylin-Eosin (H&E) staining was performed on 6 µm sections according to common procedures. To assess the type of remodeling of muscular pulmonary arteries, microscopic images were analyzed using a computerized morphometric system (Leica). For each mouse, medial thickness of 50 intraracinar arteries in 4 different sections was measured and reported to their cross sectional diameters. Arteries were categorized according to their external diameter: category I included arteries with an external diameter between 15 to 30 µm ; category II with an external diameter between 31

µm and 50 µm, category III between 51 µm and 75 µm and category IV between 76 µm and 100µm. Analysis was done in a blinded fashion.

Patients with pulmonary arterial hypertension and controls

Preoperative echocardiography was performed in the controls to rule out PH. The lung specimen from the controls were collected at a distance from tumor foci.

Immunofluorescence staining

For immunofluorescence staining, lung sections were hydrated in PBS, incubated for 1 hour in a protein-blocking solution then overnight with anti-MRP4 antibody (M4-I80, Abcam, 1:50), anti-alpha-smooth muscle actin FITC conjugate (clone1A4, Sigma, 1:250), CD68 (ab53444, Abcam), Ki67 (ab15580, Abcam). Antibodies were washed off and incubated with Alexa 546-conjugated secondary antibodies (Invitrogen Corp). After several washings, the sections were mounted with Dako fluorescent mounting media (DakoCytomation).

Immunohistochemistry was performed on 5 micrometer-thick sections of frozen lung tissue. After routine preparation and fixation with acetone, slides were processed with rabbit anti–MRP4 anibody (M4-I80, Abcam). A secondary biotinylated antibody (Goat-anti-rabbit RTU Microm, France, ref: F/TR-060-BN) and eventual streptavidine amplification (Streptavidin Peroxidase RTU, Microm, France, ref: F/TS-060-HR) was used for primary antibody detection, AEC (Diagomics, France, ref: ZUC054-200) was the chromogen.

RNA interference and cellular experiments in PASMCs and PAECs

Silencing RNA against human MRP4 and MRP1 was specifically designed by using the academic Web-based siRNA design program SiSearch. The siRNA sequence was designed to target several MRP4 splicing variants (NM_005845, BC041560, AY081219, AF541977, AY133680, AY133679, AY133678). The sense sequence is 5'-CAGUGUUCUUACACUUCCUTT-3' and the antisense sequence 5'-AGGAAGUGUAAGAACACUGTT-3'. As for MRP1, the sense sequence is 5'-CCUCCCACACUGAAUGGCA-3' and the antisense sequence 5'-UGCCAUUCAGUGUGGGAGG-3. A non silencing siRNA with no homology with mammalian genes (All Stars Negative Control, Qiagen) was used in parallel (scrambled siRNA). Cells were transfected with siRNA (50nM) in Foetal Calf Serum-free medium for 6 h, then the medium was replaced with Foetal Calf Serum-containing medium for a further 66 h. Transfection was performed using Lipofectamine 2000 (Invitrogen) or electroporation using Amaxa® Nucleofector technology according to the manufacturer's instructions.

Human pulmonary smooth muscle cells were cultured in 96-well tissue culture plates for 3 days in Smooth Muscle Cell Basal Medium 2 supplemented with 5% FCS. Medium containing 0.1% S was used for the growth-arrest control. Cells were incubated with siRNA for 72 h in medium containing PDGF. Cell proliferation was measured 3 days after adding siRNA. BrdU was added for the last 16 hours. The plates were washed, and a colorimetric BrdU cell proliferation assay was performed according to the manufacturer's instructions (Roche).

Migration of hPASMC was assessed using a micro Boyden Chamber QCM™ 24-Well Colorimetric Cell Migration Assay (ECD508, Chemicon international) and used according to the manufacturer's instructions. Cells were transfected for 72 h with either scrambled or MRP4 siRNAs, serum-starved and spread to the upper chamber (150000/300µL). Different

concentrations of serum were added to the lower chamber (0.1% or 5% FCS). The Transwell chambers were then incubated in a humidified incubator with 5% CO_2 for 12 hours. After incubation, the inserts were incubated with cell stain solution for 20 min then rinsed with water and swabbed with a cotton swab to remove the non-migrated cells. Subsequently, migrated cells were extracted and detected by colorimetric assay on a microplate reader at 560 nm.

Apoptosis was assessed by detecting the DNA fragmentation with the TUNEL assay. Isolated hPASMC were transfected for 72 h with either scrambled or MRP4 siRNAs, then fixed by paraformaldehyde in 1% and permeabilized using a cold mix of ethanol and acetic acid. Apoptosis was assessed by using the ApopTag® Red *In Situ* Apoptosis Detection Kit (S7165, Chemicon international) according to the manufacturer's instructions. For positive control, cells were treated with staurosporine (1µM).

Cyclic GMP and AMP were measured in culture supernatants and lysates of hPASMC transfected by electroporation using Amaxa® Nucleofector technology for 72 h with either scrambled, MRP4 or MRP1 siRNAs by specific competitive enzyme immunoassay as recommended by the manufacturer (R&D systems).

Quantitative real-time PCR and Western blotting

Total RNA was prepared with RNeasy Mini kits (Invitrogen) and the quality of the sample was checked by using RNA nano Labchip® and 2100 Bioanalyzer (Agilent Technology). 200 ng was reverse-transcribed with a standard protocol. One-tenth of the resulting cDNA was amplified by 35 cycles of 30 s at 94°C, 30 s at Tm (60°C for MRP4, MRP1, PDE5 and RPL32) and 30 s at

72℃, followed by a final amplification step at 72℃ for 10 min, using 1 unit of BIOTAQ DNA Polymerase (Bioline) and 200 pmol each of the following primers: human MRP4 sense primer, 5'-TGGTGCAGAAGGGGACTTAC -3' and antisense primer, 5'-GCTCTCCAGAGCACCATCTT-3' ; human MRP1, the sense sequence is 5'-CACATGAAGAGCAAAGACAA-3' and the antisense sequence 5'-CAGCACCTTCAGCTCCTCCT-3' and human RPL32 sense primer, 5'- GCCCAAGATCGTCAAAAAGA-3' and antisense primer, 5'-GTCAATGCCTCTGGGTTT-3'. Gene-specific primers were used to amplify mRNA by qPCR on an Mx4000 apparatus (Stratagene) using the Qiagen SYBR Green master mix. The specificity of each primer set was monitored by analysing the dissociation curve. The sample volume was 25 µl, with 1X (final concentration) SYBR Green PCR master mix, 400 nM gene-specific primers, and 5 µl of template.

Total cell lysates were prepared with a standard protocol (Upstate Biotechnology). Cells were lysed and the proteins were extracted by Promokine Mammalian Whole Cell Protein Extraction Kit (Promocell) according to the manufacturer's instructions. Lung tissue samples were homogenized in lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% triton and phosphatase and protease inhibitors cocktail. The proteins concentration was determined using the Bradford protein assay (bio-Rad). Proteins (50 μg) were separated by SDS 12% PAGE, blotted on Hybond-C membranes (Amersham Biosciences) and incubated with various antibodies. The antibodies were: anti-MRP4 (M4I-80, Abcam, 1:200), anti-MRP1 (MRPr1, Abcam, 1:100), anti-PDE5 (#2395, Cell Signaling, 1:1000), anti-GAPDH (ab9485, Abcam, 2:2500), anti-pVASP-Ser157 (#3111, Cell Signaling, 1:1000, anti-pVASP-Ser239 (#3114, Cell Signaling, 1:1000) and anti-VASP (#3112, Cell Signaling, 1:1000), anti-CD45

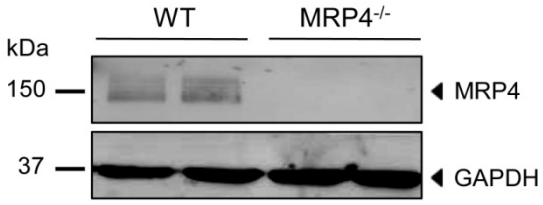
(ab10558, Abcam, 1:500). The PDE3A antibody (1:1000) was kindly provided by Dr Chen Yan (University of Rochester Medical Center, NY) and the PDE4A was from Fabgennis (PDE4-151AP, 1:1000). The GAPDH antibody served as the internal control. Immunoreactive proteins were visualized by using an ECL® (enhanced chemiluminescence) detection system (Amersham Biosciences). Optical density was measured with ImageJ software (National Institutes of Health, Bethesda, MD).

Proliferation index

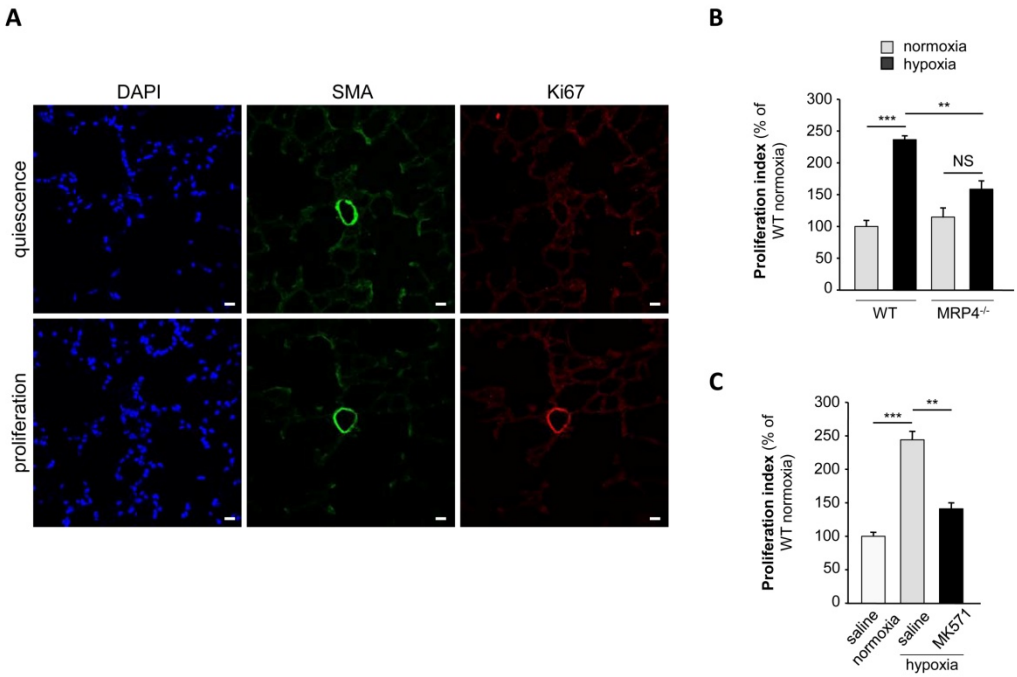
To quantify the proliferation index in KO mice or MK571-treated mice after hypoxia exposure, immunofluorescence were performed on lung section using Ki67 (a proliferation marker) and Smooth Muscle Actin antibodies. Proliferation index was calculated as a ratio of Number of Ki67 positive arterioles / Total of arterioles. Calculation was performed on 5 mice in each groups.

Statistical Analysis

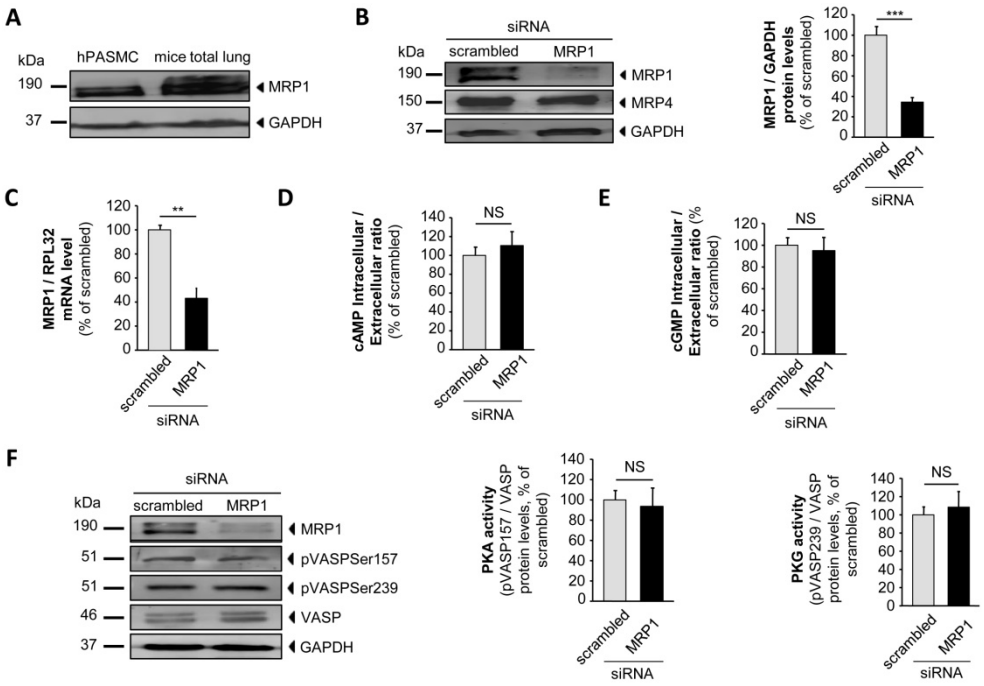
All quantitative data are reported as means ± SEM. Statistical analysis was performed with the Prism software package (GraphPad v3). One-way analysis of variance (ANOVA) was used to compare each parameter. Post hoc t-test comparisons were performed to identify which group differences accounted for significant overall ANOVA results. Differences were considered significant when $P<0.05$.



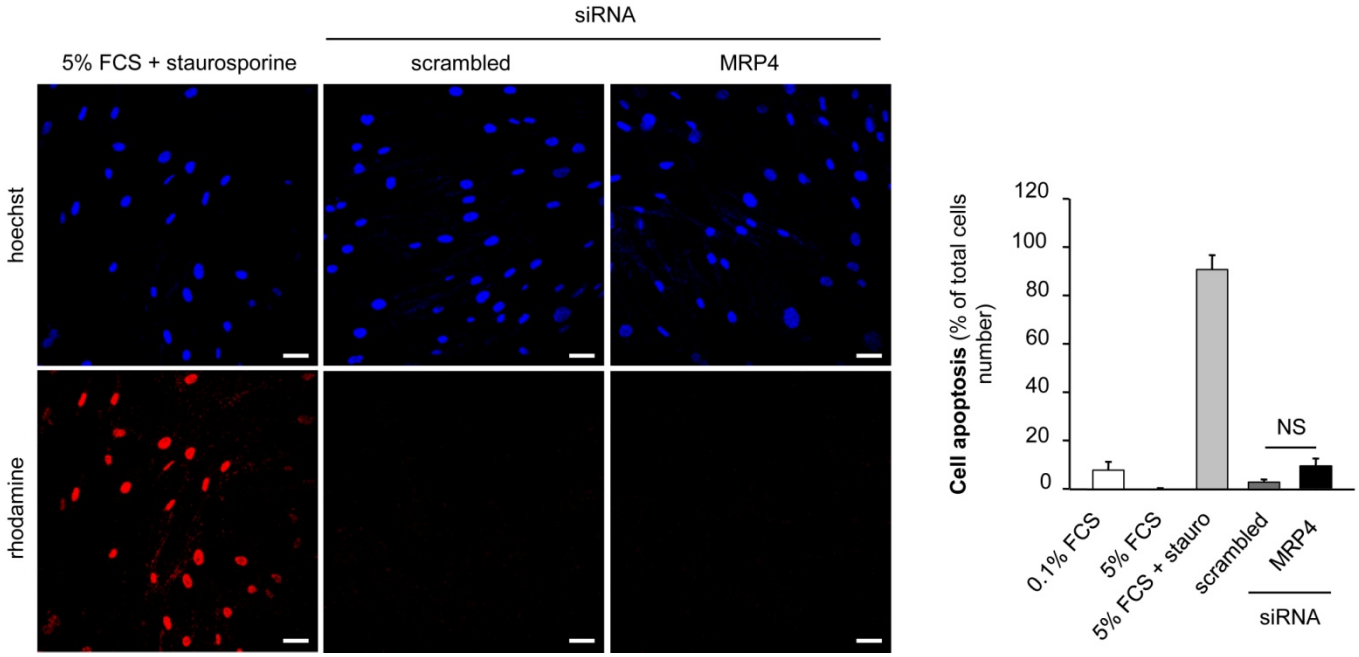
Supplemental Figure 1: MRP4 expression in total lungs from MRP4$^{-/-}$ mice. Western blot analysis from MRP4$^{-/-}$ mice total lung extracts compared to control were performed. GAPDH was used as a loading control.



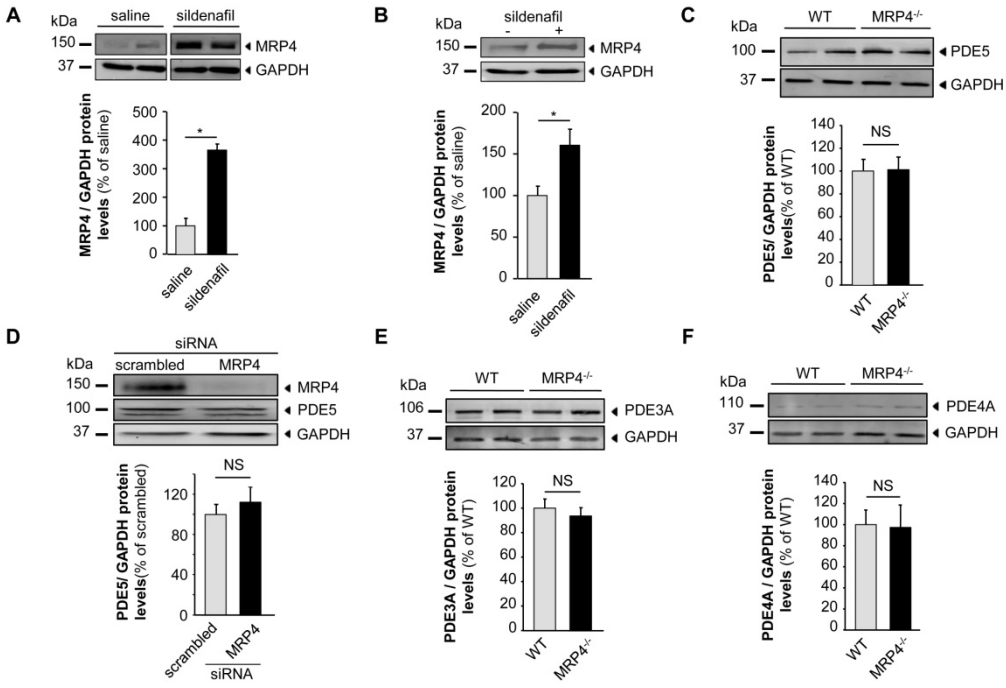
Supplemental Figure 2: Indices of pulmonary smooth muscle cell proliferation in situ. (A) Representative staining of a Ki67-negative (upper line) and a Ki67-positive (lower line) vessel in a lung from a wild-type mouse during hypoxia. **(B&C)** Proliferation index of PASMC in arterioles from MRP4$^{-/-}$ vs WT mice (left) or MK571-treated mice vs vehicle-treated mice (right) after hypoxia or normoxia exposure. Indices were calculated as a ratio of number of Ki67 positive arterioles / total of arterioles. Calculation was performed on 4 different sections for 5 mice in each groups. Scale bar: 15µm.



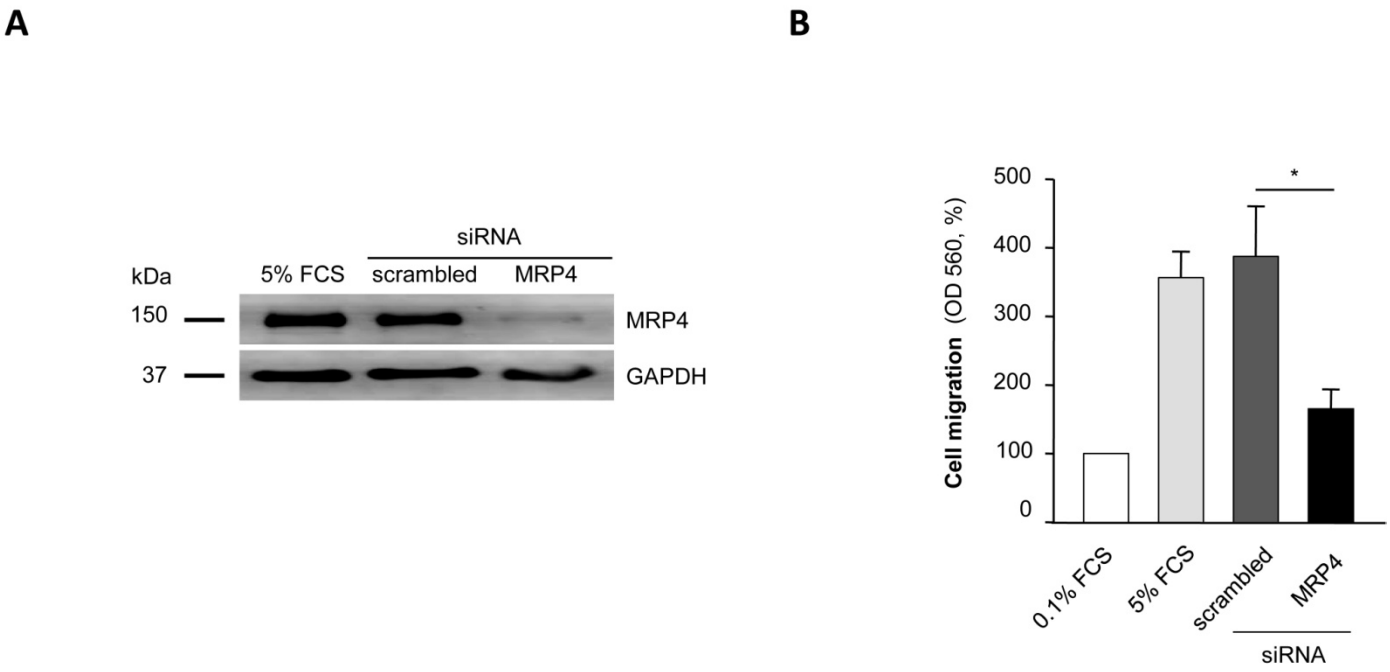
Supplemental Figure 3: **Silencing MRP1 has no effect on PKA and PKG pathways. (A)** Western blot analysis of MRP1 in total lysates from hPASMC and from mice lung, showing physiological expression of MRP1. GAPDH is used as a loading control. **(B)** Quantitative real-time PCR with gene-specific primers of MRP1 in hPASMC transfected for 72h with MRP1 or scrambled siRNAs; relative levels of MRP1 mRNA is normalized to RPL32 mRNA. Three experiments were done (***P*<0.01). **(C)** Western-Blot analysis of MRP1 and MRP4 from total lysates from hPASMC transfected for 72 h with MRP1 or scrambled siRNAs, showing efficient and specific silencing of MRP1. Proteins were incubated with anti-MRP1, anti-MRP4 or GAPDH antibodies. Five experiments were done (****P*<0.001). **(D-E)** Intracellular/extracellular ratios of cAMP and cGMP, measured with a specific competitive enzyme immunoassay, in isolated hPASMCs transfected with scrambled or MRP1 siRNAs for 72 h. Three experiments were done (NS: Non Significant). **(F)** Western-Blot analysis of total lysates from hPASMC transfected with scrambled or MRP1 siRNAs for 72 h. Proteins were incubated with anti-pVASP-Ser157 to assess PKA activity and anti-pVASP-Ser239 to assess PKG activity. Anti-VASP was used for normalization and anti-GAPDH as a loading control. Three experiments were done (NS=Non Significant)



Supplemental Figure 4: Effect of MRP4 silencing on hPASMC apoptosis. Cells nuclei were stained by Hoechst (blue), and apoptosis positive cells were detected by rhodamine (red) labelling the free 3'OH DNA termini resulted from the DNA fragmentation. Results of hPASMC apoptosis were expressed as a ratio of apoptotic cells (red) and total number of cells (blue). The photographs are representative of each condition. 3 experiments were done (NS = Non Significant).



Supplemental Figure 5: Balance between PDE5 and MRP4. **(A)** Western blot analysis of MRP4 in total lung extract from mice treated with saline or sildenafil in normoxia conditions (the lane were run on the same gel but were not contiguous). GAPDH is used as a loading control. Quantification of MRP4 is shown in the bar graph. Immunoblots are representative of 4 individuals lung for each groups (**P*<0.05). **(B)** Western blot analysis of MRP4 in lysates from isolated hPASMC treated or not by sildenafil (1µM) during 48 hours. Four experiments were done. **(C)** Western-blot analysis of PDE5 in total lung lysates from MRP4$^{-/-}$ and wild-type mice. Immunoblots are representative of lung from 4 individuals for each group (NS = Non Significant). **(D)** Western-blot analysis of MRP4 and PDE5 in lysates from isolated hPASMC transfected with scrambled or MRP4 siRNAs for 72 h. Quantification of PDE5 is shown in the bar graph. Six experiments were done. **(E&F)** Protein expression of PDE3A and PDE4A in MRP4$^{-/-}$ mice lungs. Immunoblots are representative of 4 individuals lung for each groups.



Supplemental Figure 6: Inhibition of PAEC migration by MRP4 silencing. (A) Western-Blot analysis of MRP4 from hPAEC transfected with scrambled or MRP4 siRNAs for 72 h. **(B)** Effect of MRP4 siRNA on hPASMC migration (assessed by colorimetric assay OD at 560nm) compared to scrambled siRNA. Migration was normalized to the value obtained in 0.1% PDGF. 4 experiments were done in triplicate (*P<0.05).