# NOD2 deficiency exacerbates hypoxia-induced pulmonary hypertension and enhances pulmonary vascular smooth muscle cell proliferation

## SUPPLEMENTARY MATERIALS

## MATERIAL AND METHODS

### Bone marrow derived macrophages (BMDMs) isolation and culture

Bone marrow was flushed from femurs and tibias of C57BL/6 mice using 25-gauge needles with 10% heat-inactivated fetal bovine serum (FBS) containing PBS. Red blood cells were lysed in RBC lysis buffer (0.15 mole/L NH4Cl, 10 mmole/L KHCO3, and 0.1 mmole/L EDTA, pH 7.4). Isolated cells were incubated in RPMI 1640 Medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS (Life Technologies, Grand Island, NY), penicillin (100 u/mL), and streptomycin (100 μg/mL), 1% glutamine, and recombinant granulocyte macrophage-colony stimulating factor (GM-CSF, 40 ng/mL). Cells were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. After 5–6 days, adherent cells were washed, counted, and replated in RPMI (without GM-CSF) at a density of $5 \times 10^6$ cells/well (6-well plate, Corning, Inc., Corning, NY). To induce NOD2 expression, cells were treated with LPS (100 ng/mL, Sigma-Aldrich Co. LLC., St Louis, MO), IFN-γ (10 ng/mL, PeproTech, London, UK) for 12 hours.

### Cell migration assay

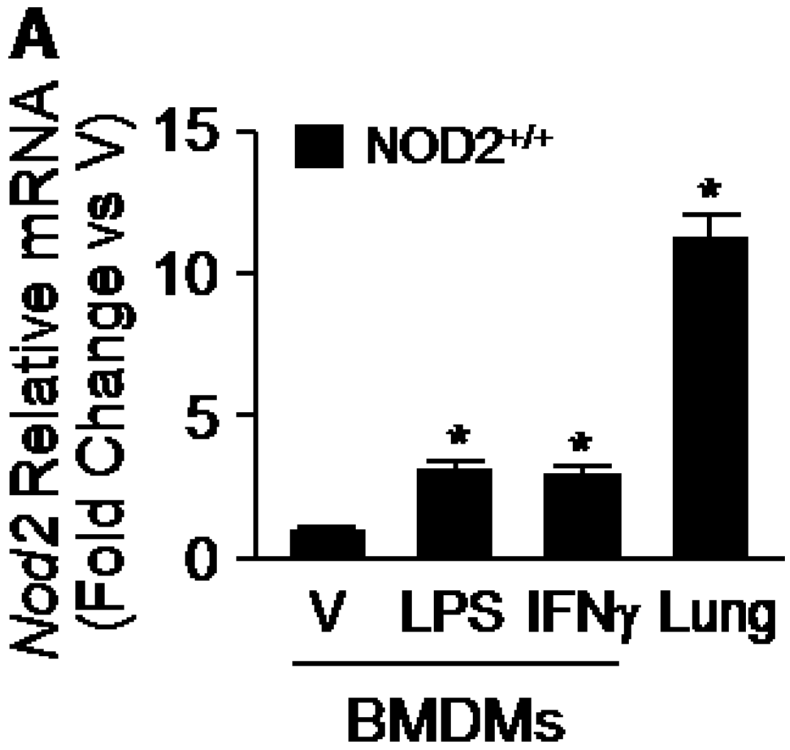
Cell migration was assessed using gelatin-coated 24-well transwell chambers (Corning, Inc., Corning, NY). To assess migration, the cells were placed in the upper chamber of transwell plates and the bottom chambers were filled with 10% FBS medium. The cells were exposed to hypoxia (1% $O_2$). Transwell membranes were scraped and washed out to remove non-migrated cells. The cells that migrated to the lower sides of the filter membranes were fixed and stained with Crystal Violet kit (Sigma-Aldrich Co. LLC., St Louis, MO). Experiments were performed at least 3 times in duplicate.

### Collagen matrix contraction assay

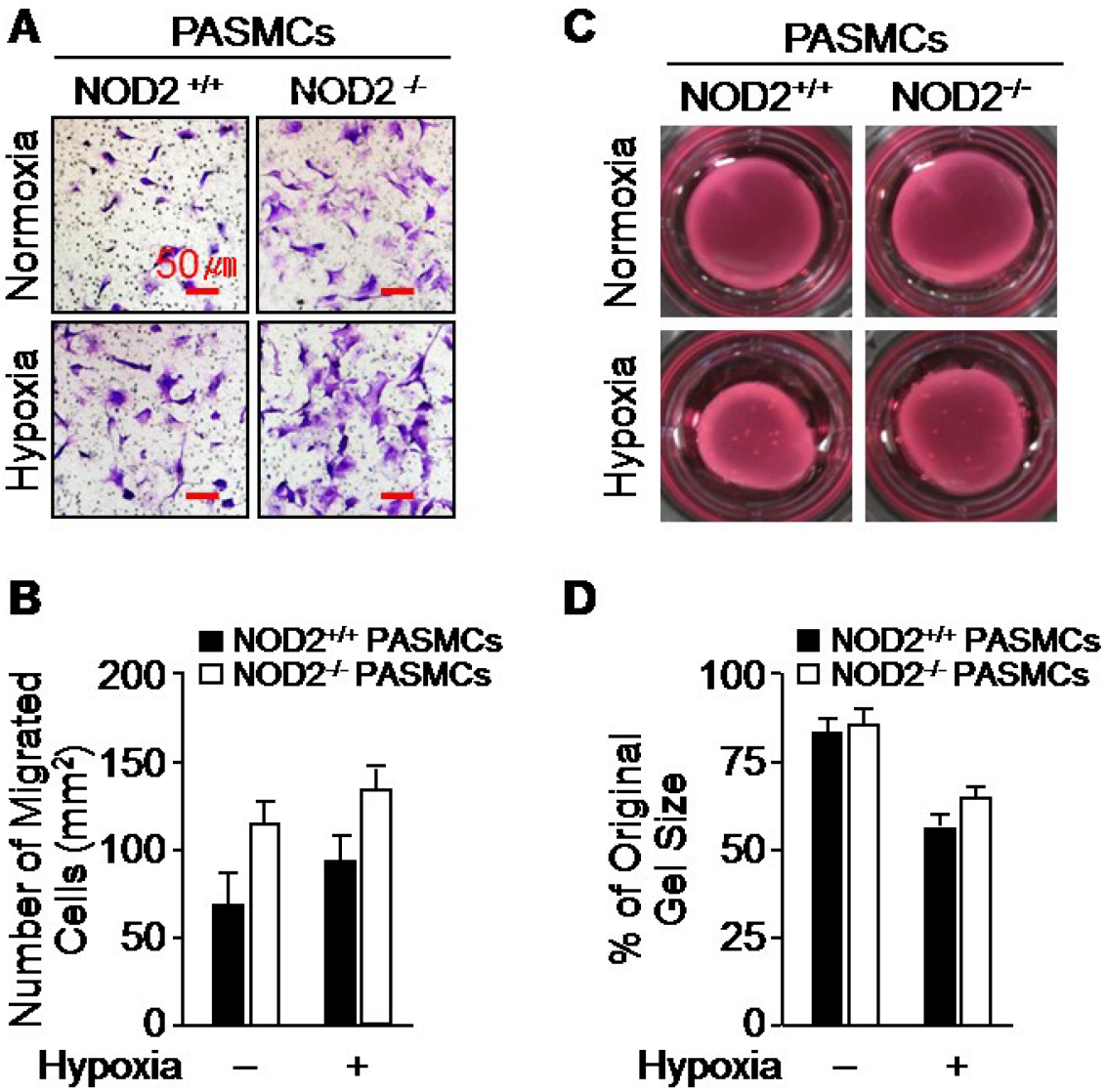
$NOD2^{-/-}$ and $NOD2^{+/+}$ PASMCs were plated on type I collagen gel matrices and exposed to hypoxia or normoxia for 24 hours. Gel size was defined as the sum of the 2 longest gel diameters; gel contraction was expressed as a percentage of the original gel size.

### Statistical analysis

Data represent mean ± SDs. For comparisons between two groups, we used Student's two-tailed unpaired t test. For comparison between more than two groups, and multiple comparisons, we used an ANOVA test. Statistically significant differences were accepted at $p < +0.05$.




**Supplementary Figure 1: Nod2 mRNA is expressed in the lung.** Total RNA was extracted from lungs of NOD2$^{+/+}$ mice under normoxic conditions and from bone marrow derived macrophages (BMDMs) 12 hours after vehicle (V), LPS (100 ng/mL), or IFN-γ (10 ng/mL) administration, as a positive control. Quantitative real-time RT-PCR was performed to assess mRNA levels of Nod2 in NOD2$^{+/+}$ lung and BMDMs. $^{*}p < 0.05$, upregulation of Nod2 mRNA levels vs. vehicle-treated BMDMs. Data are expressed as mean ± SDs.




**Supplementary Figure 2: Absence of NOD2 has no effects on the cell migration and contraction in PASMCs after hypoxia.** (**A**) The migration assay for PASMC was analyzed using 24-Transwell chamber plates in response to normoxic (21% O2) or hypoxic (1% O2) condition. Migrated PASMCs were stained using the Crystal Violet staining kit. (**B**) The number of migrated cells was measured and is represented as a graph. Values are mean ± SDs, $n = 6$. (**C**) Representative photographs of collagen gels from NOD2$^{+/+}$ (left) and NOD2$^{-/-}$ (right) PASMCs under normoxic (top) and hypoxic (bottom) conditions. (**D**) Gel contraction after matrix release was analyzed. Data are presented as the percentage of the original collagen gel size for PASMCs (NOD2$^{+/+}$, black bars; NOD2$^{-/-}$, white bars) exposed to normoxia or hypoxia. Data are expressed as mean ± SDs.