Supplemental Data

Title: Calpain mediates pulmonary vascular remodeling in rodent models of pulmonary hypertension and its inhibition attenuates pathologic features of disease

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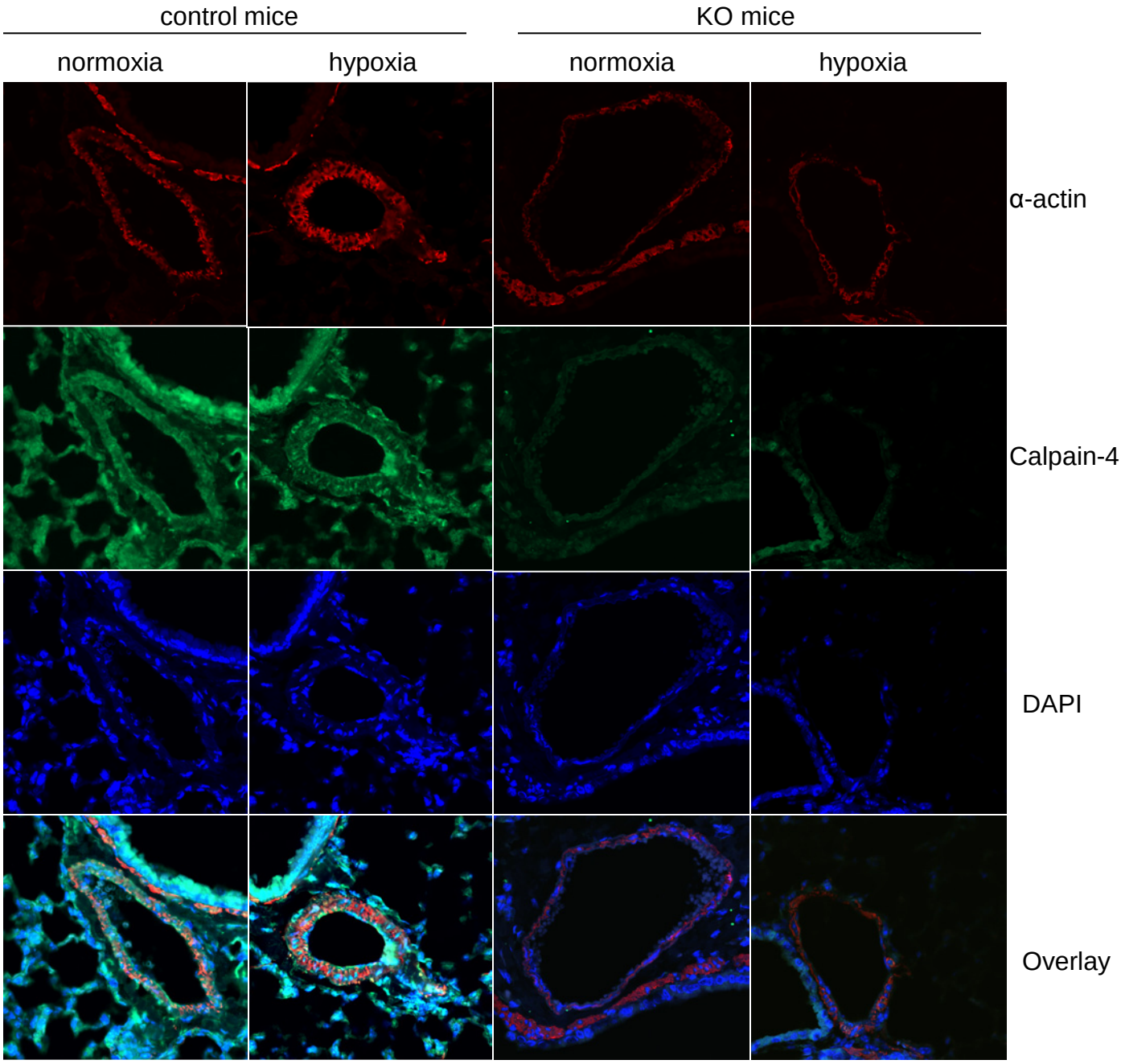
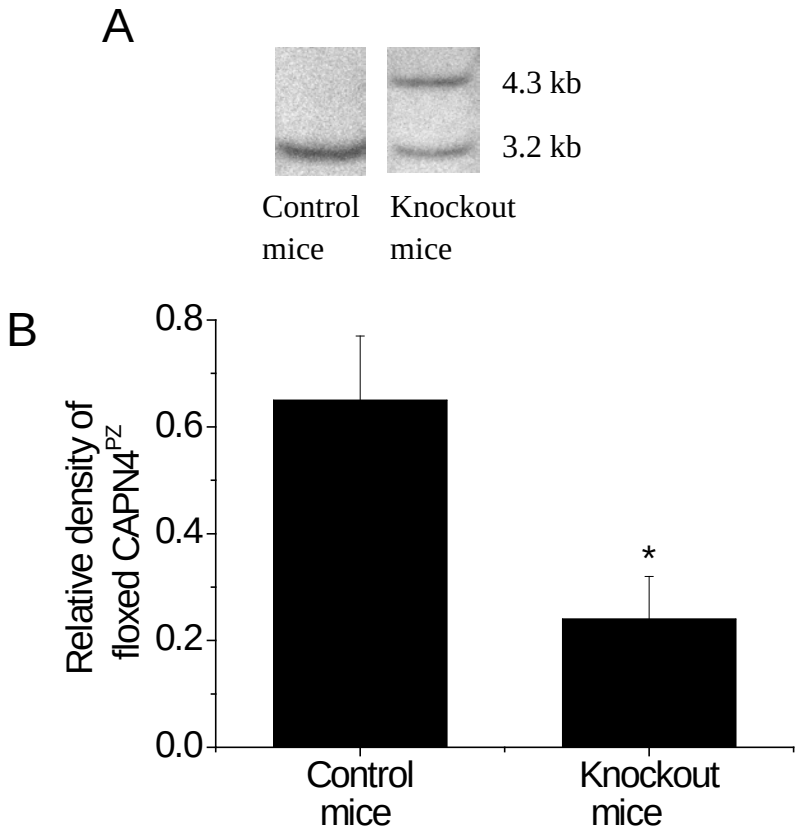
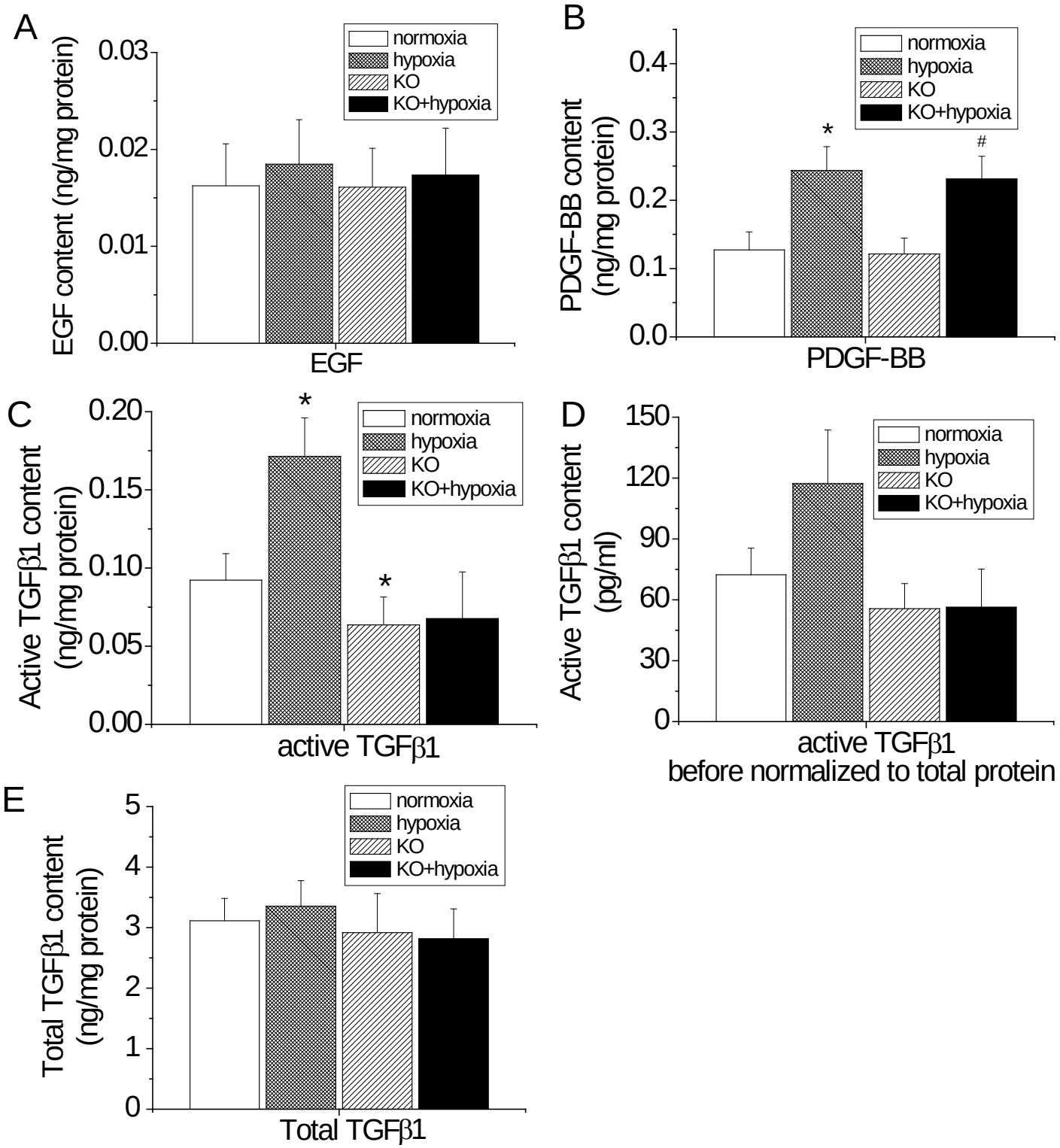


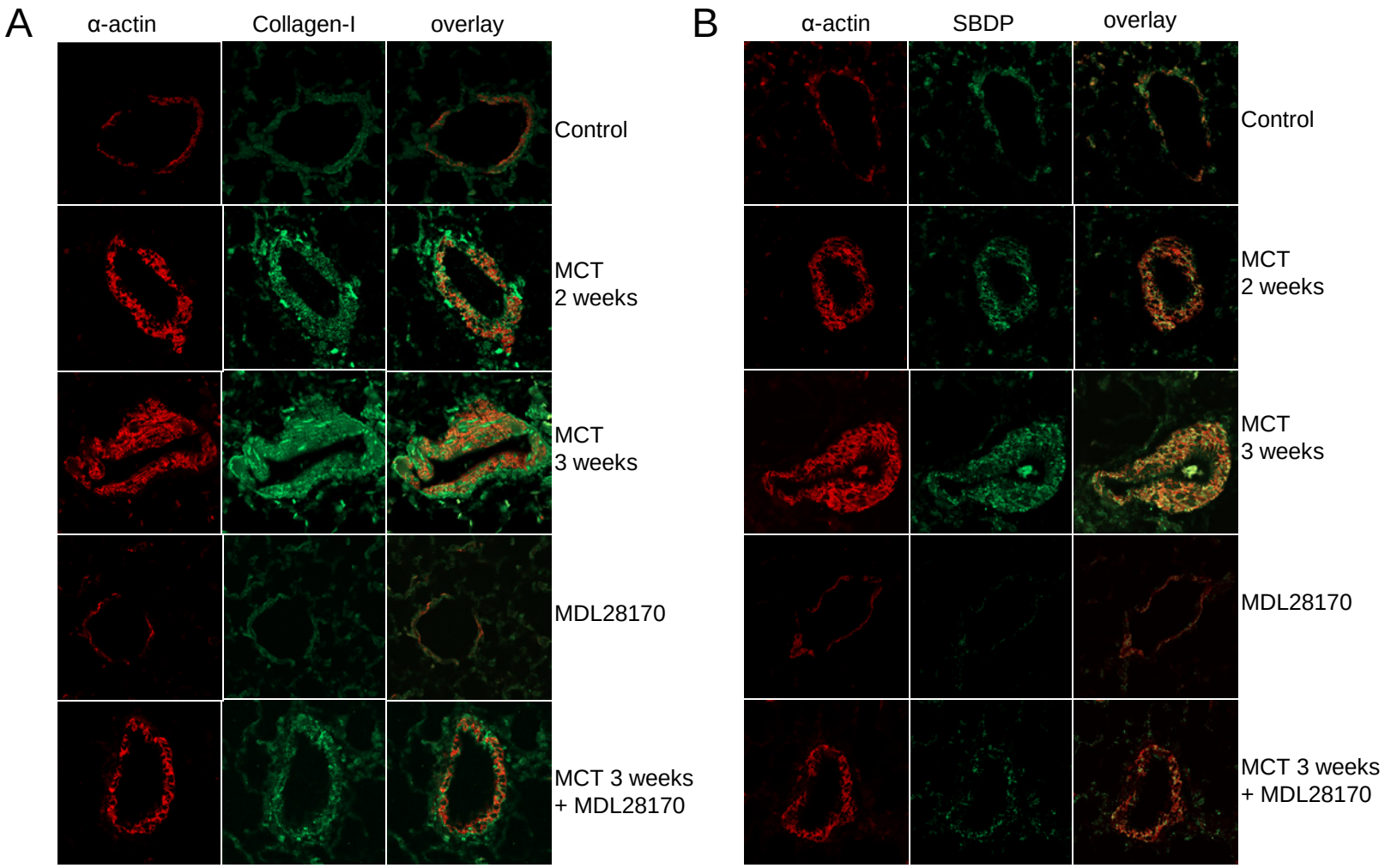
Supplemental Figure 1. Induction of calpain-4 knockout in the smooth muscle of pulmonary arterioles of mice with hypoxic pulmonary hypertension. 5 days after regimen of tamoxifen administration, control mice and ER-Cre$^{+/-}$Capn4$^{flox/flox}$ mutant mice were exposed to room air (normoxia) or 10% oxygen (hypoxia) for 3 weeks. (A): Lung slides from ER-Cre$^{+/-}$Capn4$^{flox/flox}$ mutant and control mice exposed to normoxia or hypoxia were double-stained for α-actin (red) and calpain-4 (green) and then counter stained with DAPI. Images are representative of 6 independent experiments. Magnification x400.



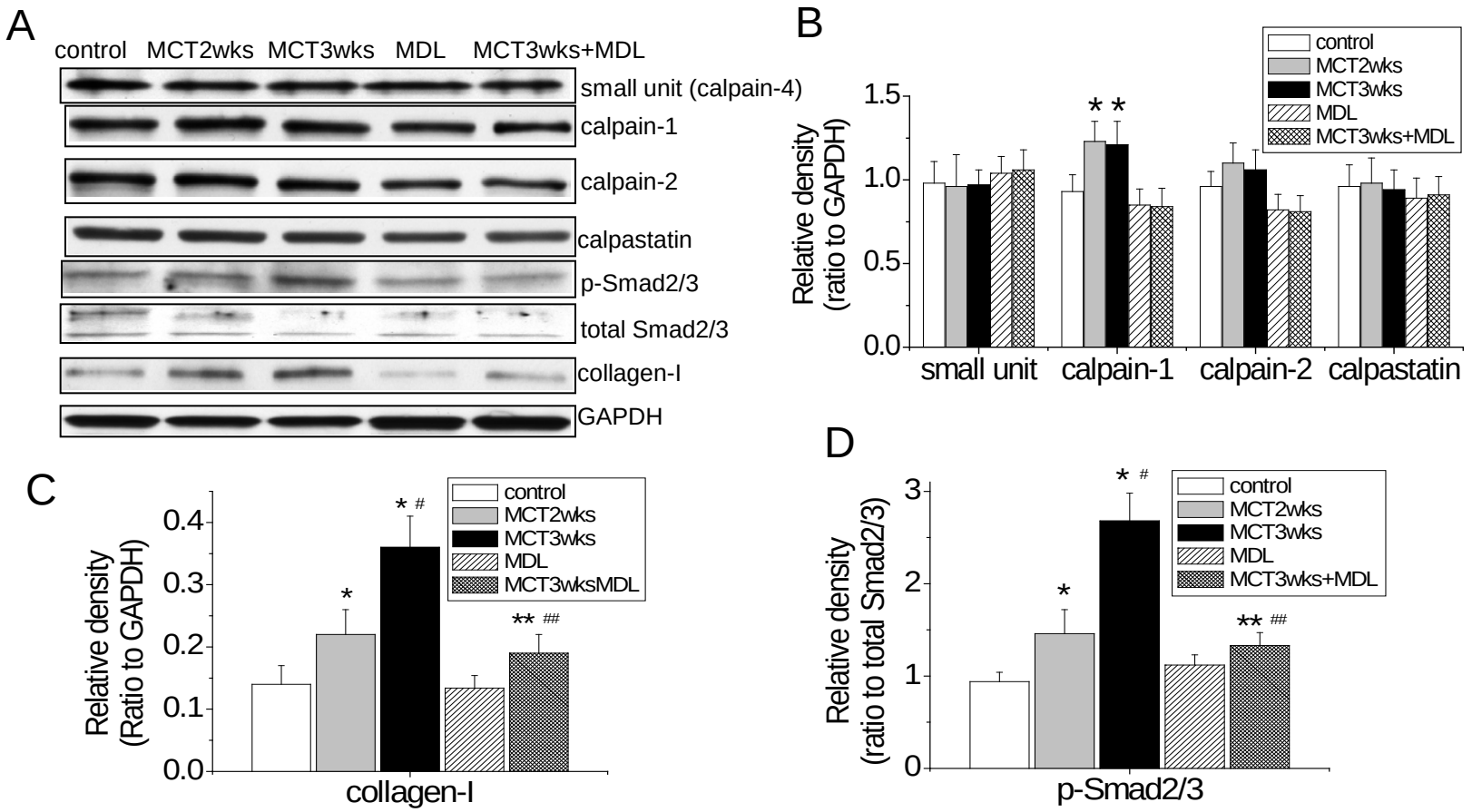
Supplemental Figure 2. Southern blot analysis of lung tissues after tamoxifen induction. To assess the efficiency of Cre-mediated deletion of the loxP-flanked Calpain-4 gene segment in ER-Cre$^{+/-}$ Capn4$^{flox/+}$ mice, Southern blot analysis was done with genomic DNA from lung tissues of control and knockout mice as described previously by us (Genesis. 2006;44(6):297-303). After PstI digestion, fragments corresponding to floxed CAPN4^{PZ} allele, and excised CAPN4^{P} genes were predicted at sizes of 3.2, and 4.3 kb, respectively. (A): Tamoxifen treatment caused the appearance of a 4.3-kb band and the weakening of the 3.2-kb band in the lungs of ER-Cre$^{+/-}$Capn4$^{flox/+}$ mice. (B) is a bar graph depicting the changes in floxed CAPN4^{PZ} allele. Results are expressed as mean $\pm$ SE; n=6. * $P < 0.05$ vs. control mice.



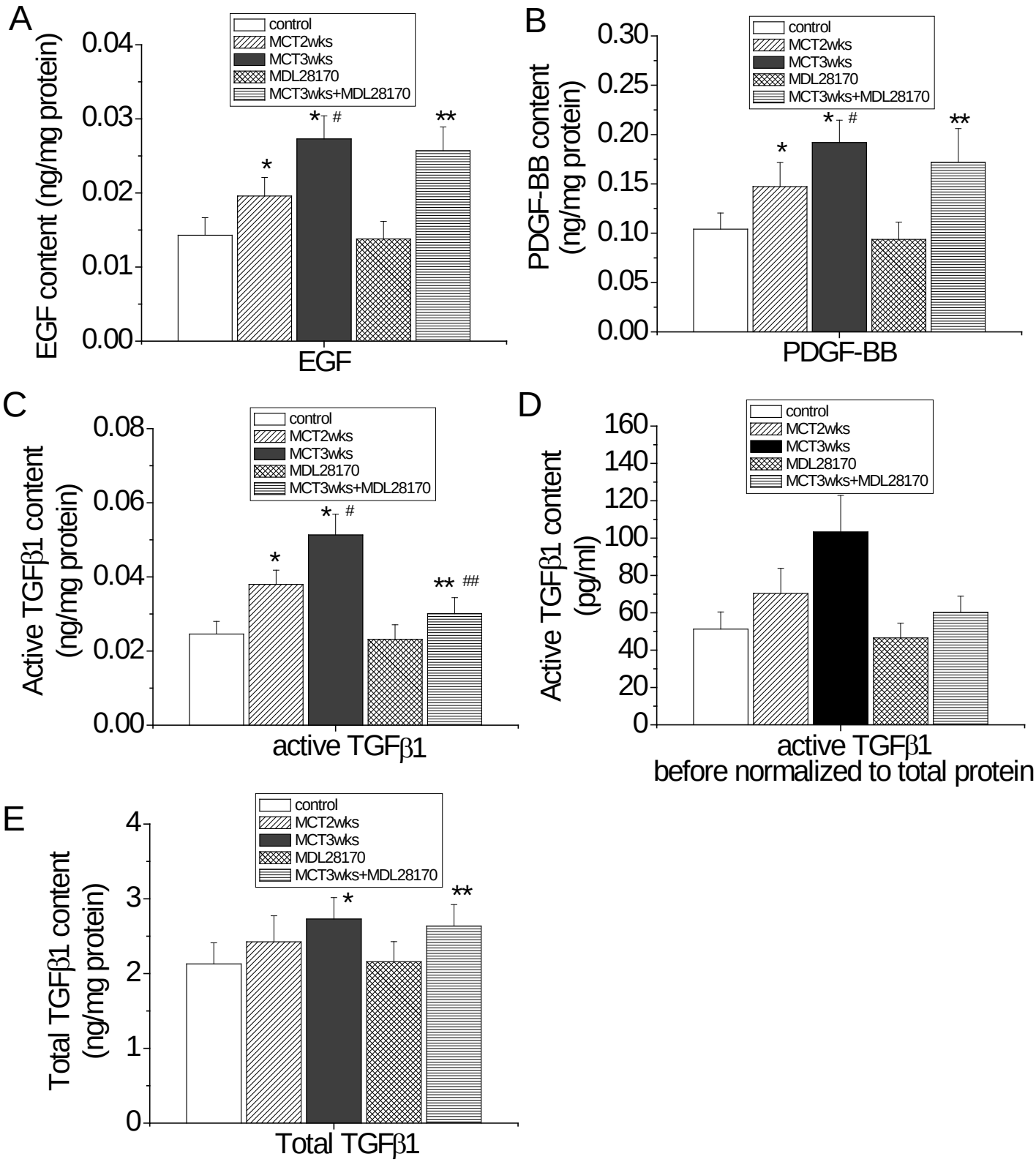
Supplemental Figure 3. Alterations in the contents of EGF, PDGF-BB, active TGFβ1 and total TGFβ1 in the lungs of hypoxia-induced pulmonary hypertension. The contents of EGF (A), PDGF (B), active TGFβ1 (C and D), and total TGFβ1 (E) in the homogenates of lungs of ER-Cre+/-Capn4flox/flox mutant and control mice exposed to normoxia or hypoxia for 3 weeks were assayed using ELISA. Results are expressed as mean ± SE; n=8 experiments. * $P < 0.05$ vs. control; #$P<0.05$ vs. KO (knock-out mice) only.



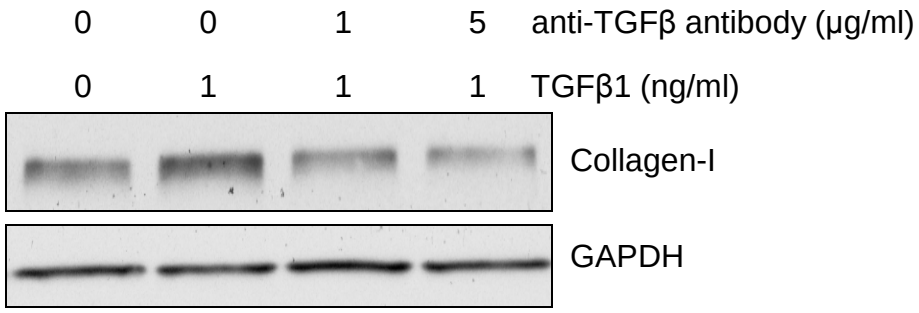
Supplemental Figure 4. Effects of calpain inhibitor MDL28170 on SBDP and collagen-1 in the smooth muscle of pulmonary arterioles of rats with MCT-induced pulmonary hypertension. Male Sprague-Dawley rats with age of 8 weeks were injected subcutaneously without or with MCT (60 mg/kg). After 2 weeks, control rats and MCT-injected rats (MCT 2 weeks) were subjected to determination of pulmonary hypertension and pulmonary vascular remodeling. At same time (the beginning of third week), another groups of control rats (MDL 28170) and MCT-injected rats (MCT 3 weeks+MDL28170) started to receive calpain inhibitor MDL28170 (20 mg/kg, i.p.) once daily. A second group of MCT-injected rats received same volume of vehicle (MCT 3 weeks). Pulmonary hypertension and pulmonary vascular remodeling were accessed one week later (3 weeks after MCT injection). (A): Lung slides were double-stained for α-actin (red) and collagen-I (green). (B): Lung slides were double-stained for α-actin (red) and SBDP (green). Images are representative of 6 independent experiments. Magnification x400.



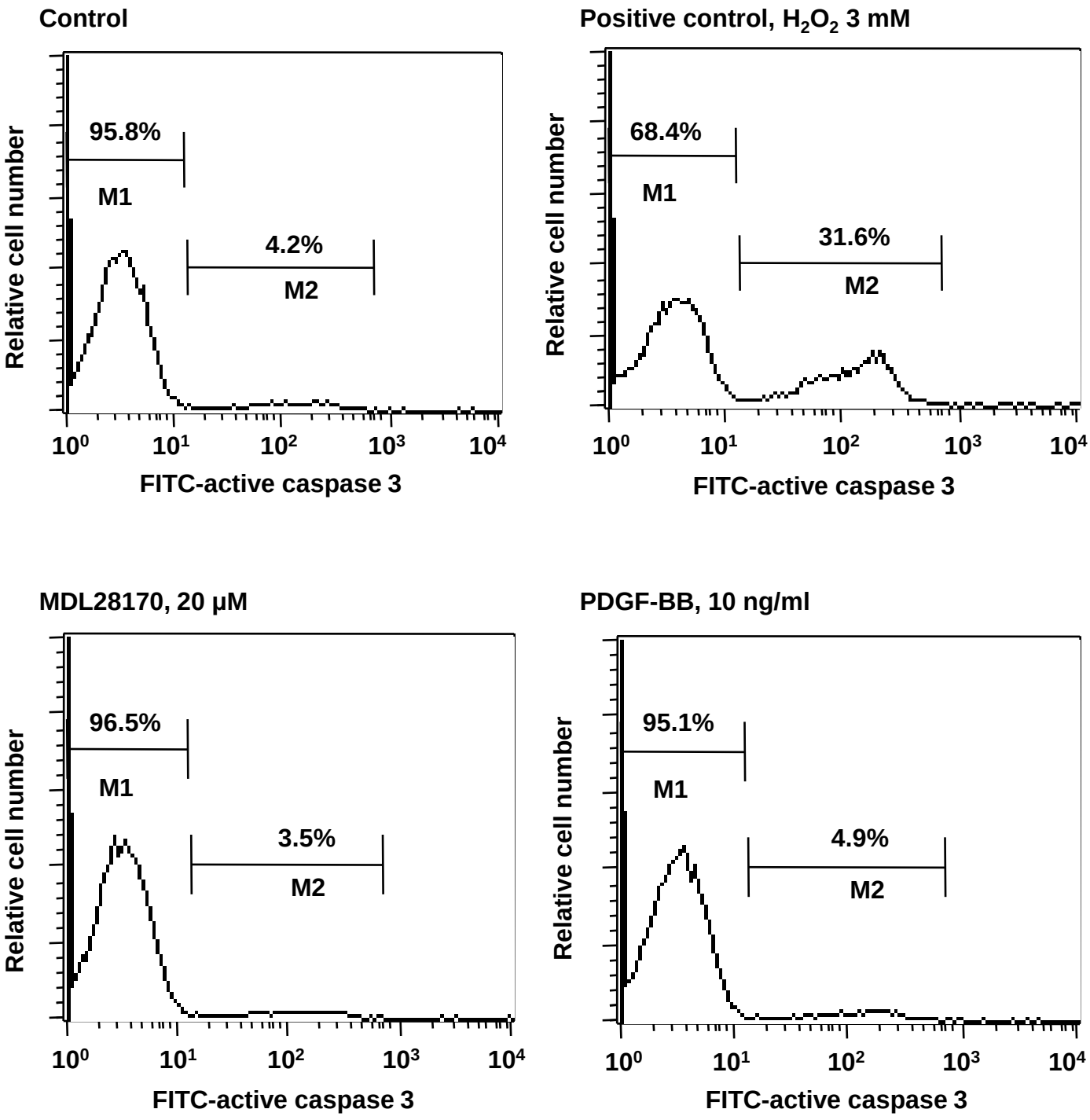
Supplemental Figure 5. Protein contents of calpain-1, calpain-2, calpain-4, calpastatin, p-Smad2/3, total Smad2/3 and collagen-I in the lungs of rats with MCT-induced pulmonary hypertension. Rats were treated as described in Online Figure 2. Then protein contents of calpain-1, calpain-2, calpain-4, calpastatin, p-Smad2/3, total Smad2/3 and collagen-I in lung homogenates were analyzed using Western blot analysis. (A) is representative immuno-blot from 6 experiments. (B), (C) and (D) are bar graphs depicting the changes in calpain-1, calpain-2, calpain-4, calpastatin, p-Smad2/3, total Smad2/3 and collagen-I quantified by scanning densitometry. Results are expressed as mean ± SE; n=6 experiments. * P < 0.05 vs. control; ** P<0.05 vs. MDL28170 group; # P < 0.05 vs. MCT 2 weeks; ##P<0.05 vs. MCT 3 weeks. MDL=MDL26170.



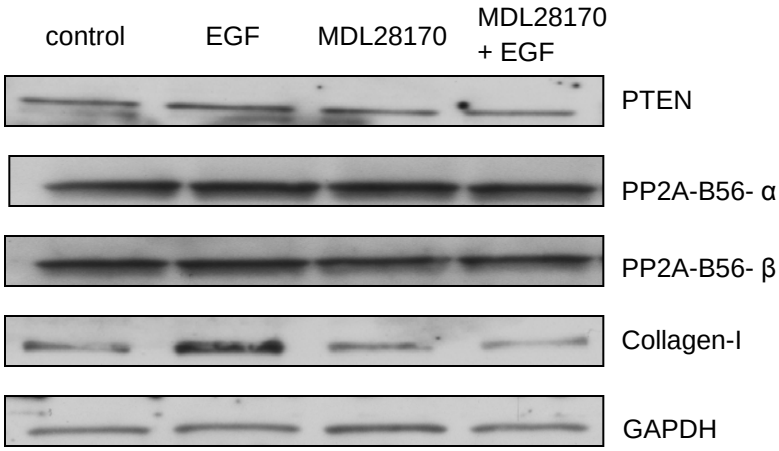
Supplemental Figure 6. Alterations in the contents of EGF, PDGF, active TGFβ1 and total TGFβ1 in the lungs of rats with MCT-induced pulmonary hypertension. Rats were treated as described in Online Figure 2. The contents of EGF (A), PDGF-BB (B), active TGFβ1 (C and D) and total TGFβ1 (E) in the homogenates of lungs were assayed using ELISA. Results are expressed as mean ± SE; n=6 experiments. * $P < 0.05$ vs. control; ** $P<0.05$ vs. MDL28170; # $P < 0.05$ vs. MCT 2 weeks; ##$P<0.05$ vs. MCT 3 weeks.



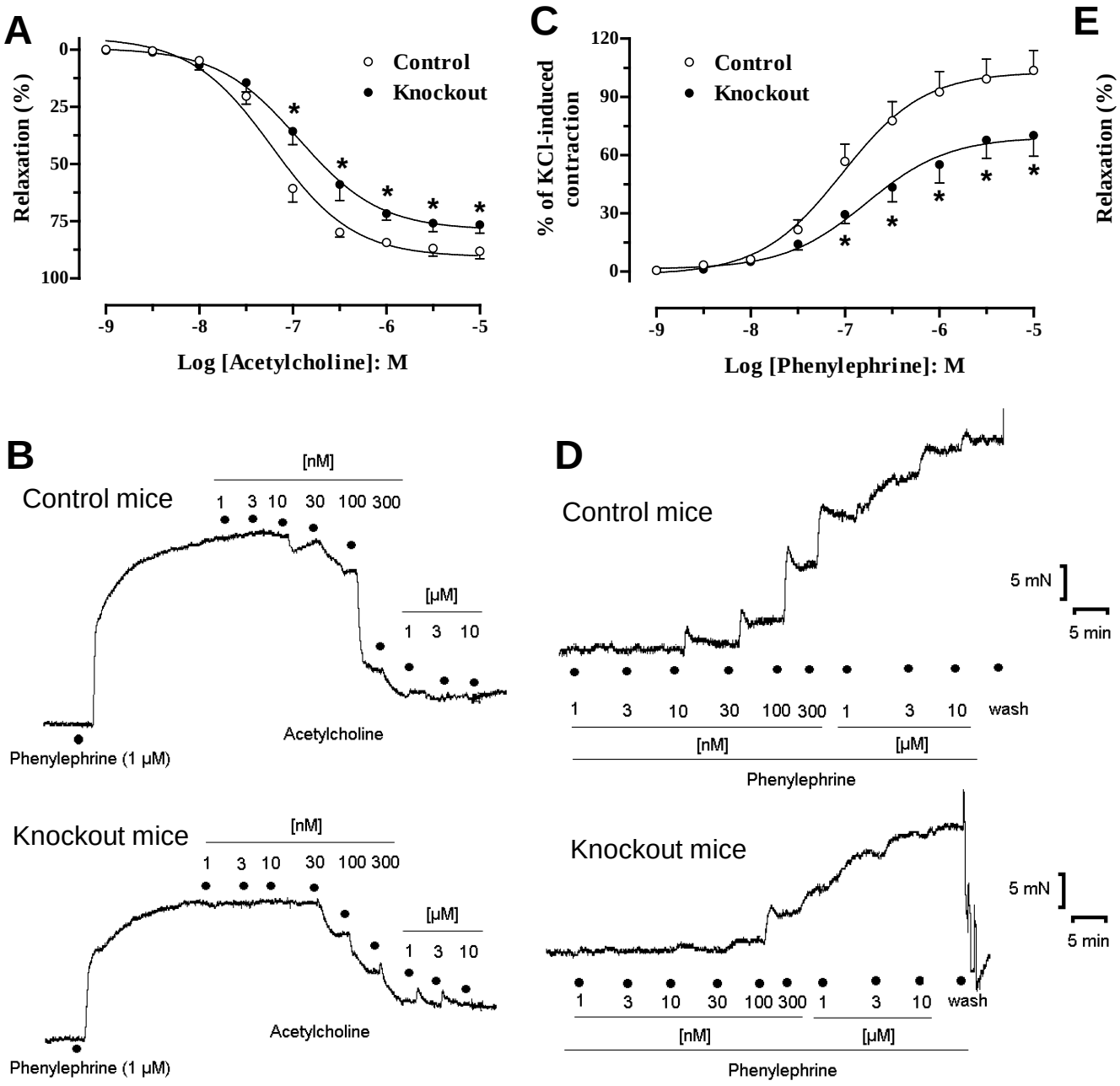
Supplemental Figure 7. Neutralizing effect of anti-TGFβ antibody on collagen synthesis in PASMC. PASMC were incubated without or with active TGFβ1 (1.0 ng/ml) in the absence or presence of anti-TGFβ neutralizing antibody (1.0 μg/ml) for 24 h after which collagen content was measured using Western blot analysis. The images are representative immuno-blots from 3 experiments.



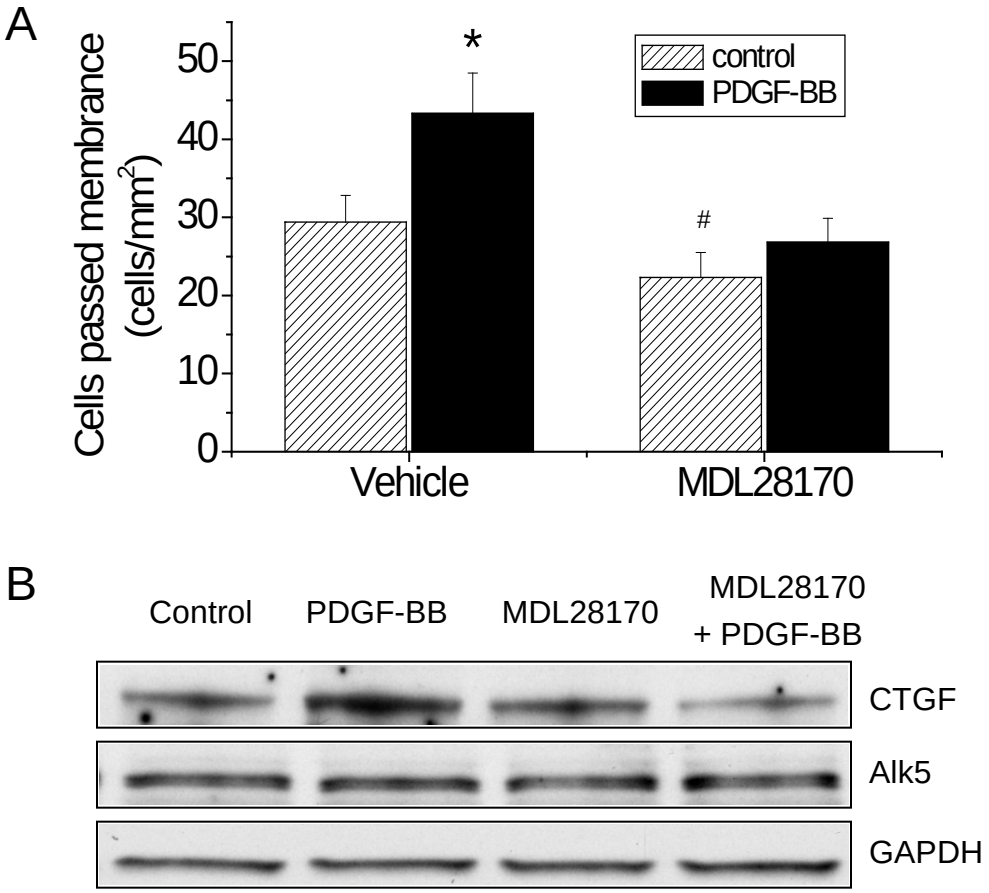
Supplemental Figure 8. PDGF and MDL28170 does not cause apoptosis in PASMC. PASMC were incubated with PDGF-BB (10 ng/ml, 24 h), MDL28170 (20 µM, 24 h), or H_2O_2 (3.0 mM, 24 h, positive control) after which active caspase 3 was assayed using a FITC-active caspase 3 apoptosis kit from BD Biosciences. Data are representative of 3 independent experiments. M1 = mean fluorescence intensity of normal cells. M2 = mean fluorescence intensity of apoptotic cells.



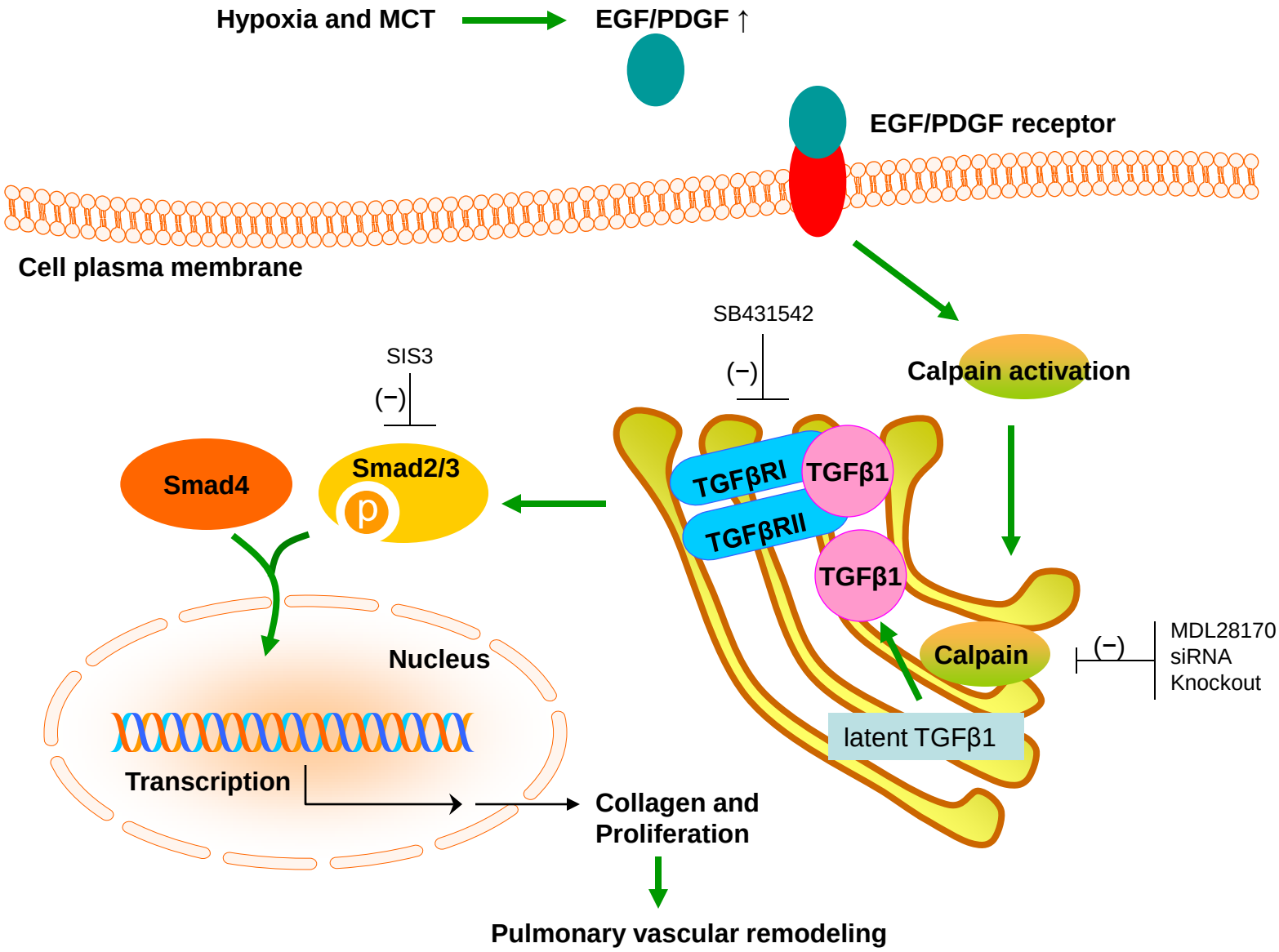
Supplemental Figure 9. EGF does not affect the protein contents of PTEN and PP2A subunits B56-α and B56-β in PASMC. PASMC were incubated with EGF (10 ng/ml) in the presence and absence of MDL28170 (20 μM) for 24 h after which the protein contents of PTEN, PP2A subunits B56-α and B56-β, and intracellular collagen-I were measured using Western blot analysis. The images are representative immuno-blots from 3 experiments.



Supplemental Figure 10. (A) and (B): knockout of calpain-4 decreases endothelium-dependent relaxation. (C) and (D): knockout of calpain-4 decreases smooth muscle contraction induced by phenylephrine. (E): knockout of calpain-4 does not affect NO donor SNP-induced relaxation. Results are expressed as mean ± SE; n=4-5 experiments. $P < 0.05$ *vs.* control mice.



Supplemental Figure 11. Effects of PDGF-BB and MDL28170 on cell migration and protein contents of CTGF and Alk5 in PASMC. (A) Migration of PASMC was measured using Boyden chamber in the presence or absence of PDGF-BB (10 ng/ml) or MDL28170 (20 μM). Results are expressed as mean ± SE; n=3 experiments. * $P<0.05$ vs. control; #$P<0.05$ vs. control in Vehicle group. (B) PASMC were incubated with PDGF-BB (10 ng/ml) in the presence or absence of MDL28170 (20 μM) for 24 h after which the protein contents of CTGF and Alk5 were measured using Western blot analysis. The images are representative immuno-blots from 3 experiments.



Supplemental Figure 12. A schematic pathway illustrating the role of calpain in pulmonary vascular remodeling of pulmonary hypertension. PDGF and EGF released in hypoxia- or MCT-induced pulmonary hypertension cause activation of calpain which translocates to Golgi and induces activation of TGFβ. TGFβ/p-Smad signaling leads to collagen synthesis and smooth muscle proliferation which contributes to pulmonary vascular remodeling and pulmonary hypertension.