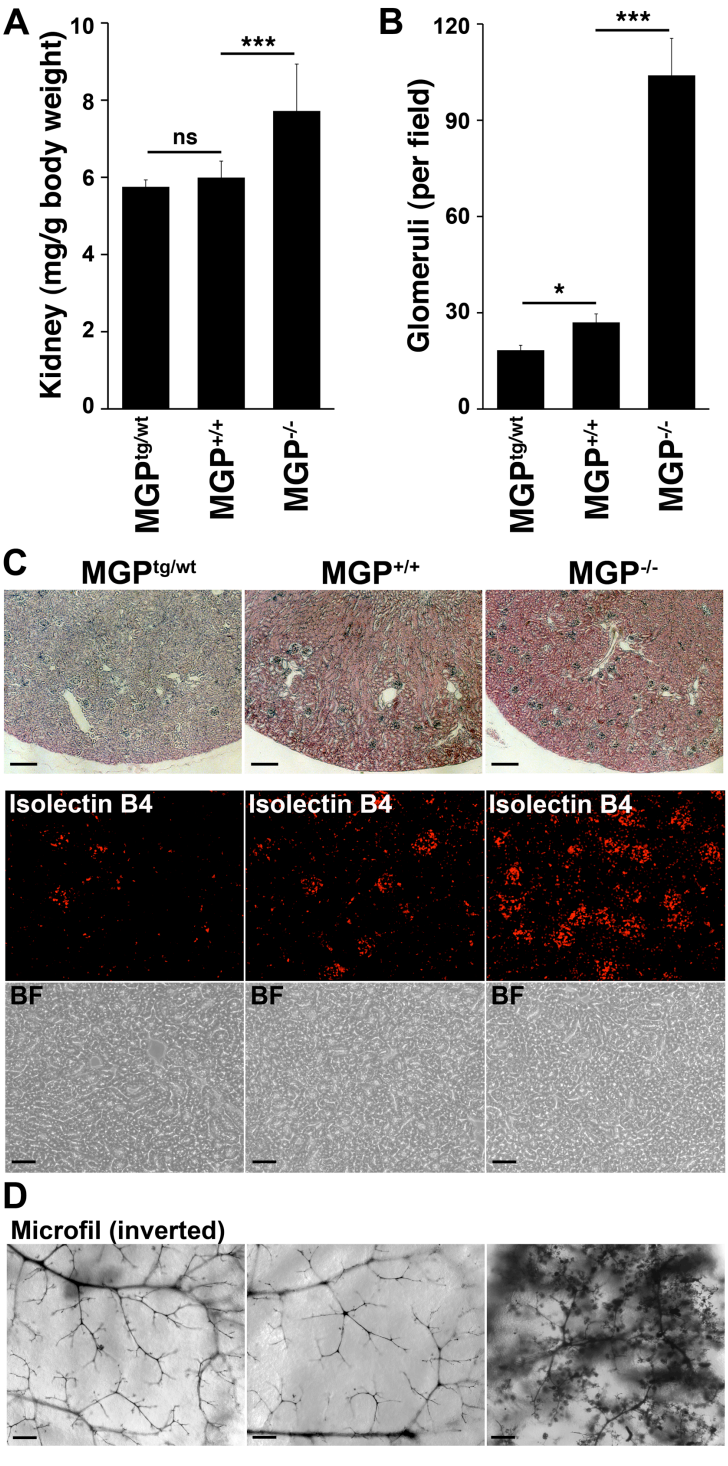
ONLINE SUPPLEMENT

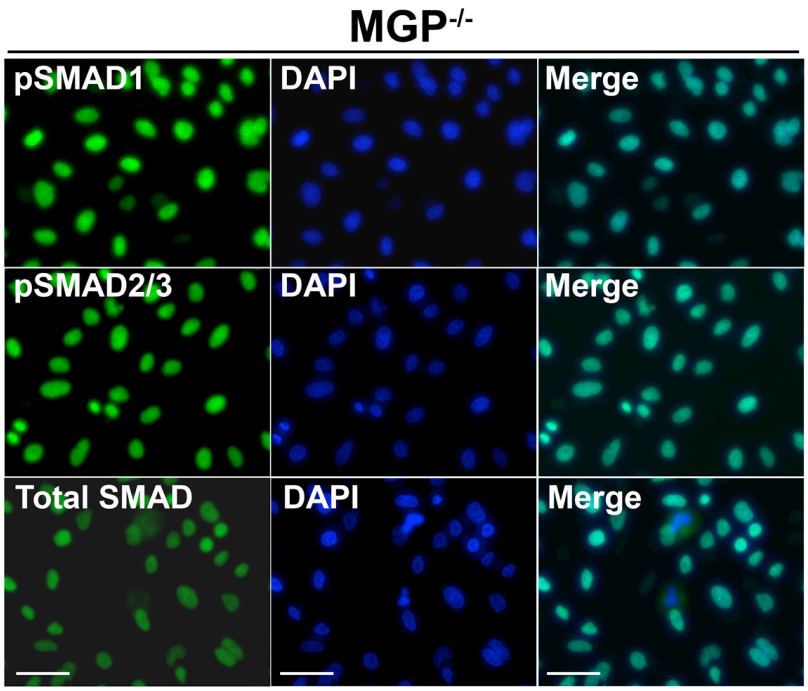
Supplemental Data 1 - Increased number of glomeruli in MGP$^{-/-}$ mice.

The kidneys were heavier in the MGP$^{-/-}$ mice when normalized to total body weight as compared to wild type and MGP$^{tg/wt}$ mice (Supplemental Figure 1A). We also compared the number of glomeruli of the three mice using H&E staining and immunofluorescence with isolectin B4 as a marker of capillary ECs. The results showed significantly more glomeruli per section area in the MGP$^{-/-}$ mice than in wild type and MGP$^{tg/wt}$ mice (Supplemental Figure 1B,C). This was also demonstrated by the vascular casts when viewed by light microscopy (Supplemental Figure 1D).



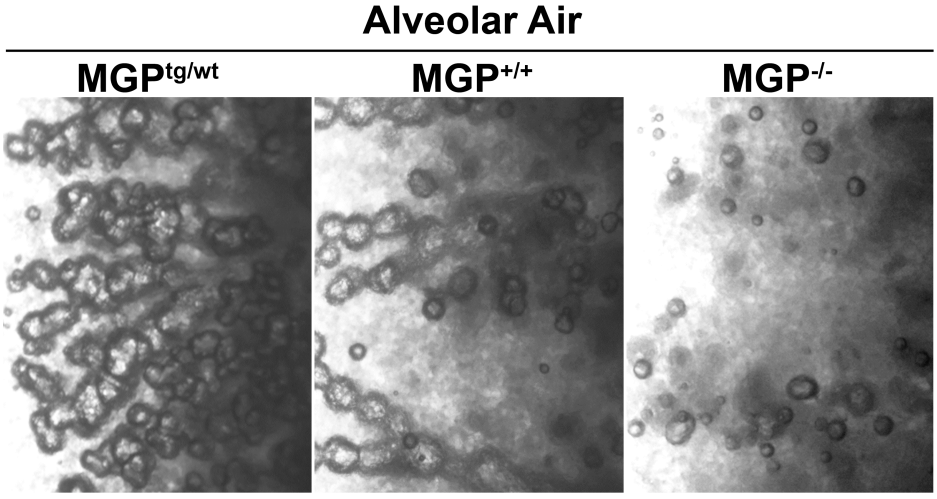
Supplemental Figure 1
Increased number of glomeruli in MGP$^{-/-}$ kidneys.
(A) The weight of kidneys from MGP$^{tg/wt}$, MGP$^{+/+}$ and MGP$^{-/-}$ mice, shown per body weight (n=6). **(B)** Number of glomeruli in kidneys from MGP$^{tg/wt}$, MGP$^{+/+}$ and MGP$^{-/-}$ mice, shown per microscopic field (n=6). **(C)** Kidney sections from MGP$^{tg/wt}$, MGP$^{+/+}$ and MGP$^{-/-}$ mice stained with H&E (top) and isolectin B4 (red immunofluorescence) (bottom). (D) Vascular casts as viewed by light microscopy; black and white images were inverted to improve visualization. Asterisks indicate statistically significant differences compared to wild type (MGP$^{+/+}$). * <0.05, *** <0.001, *Tukey's test*. Scale bars: 200 microns (C, top); 50 microns (C, bottom); 100 microns (D).

Supplemental Data 2 – Phosphorylated SMADs localized to nuclei in isolated MGP$^{-/-}$ lung cells.



Supplemental Figure 2
Phosphorylated (p)SMADs localization in nuclei of MGP$^{-/-}$ lung cells.
Isolated MGP$^{-/-}$ lung cells were stained for pSMAD1, pSMAD2/3 and total SMADs (green immunofluorescence), and nuclei were visualized by DAPI (blue immunofluorescence).
Scale bars: 10 microns.

Supplemental Data 3 – Alveolar air in the terminal airways of MGP$^{tg/wt}$, wild type and MGP$^{-/-}$ lungs.



Supplemental Figure 3
Terminal airways in MGP$^{tg/wt}$, wild type and MGP$^{-/-}$ lungs.
Terminal airways in MGP$^{tg/wt}$, wild type and MGP$^{-/-}$ at one month of age. The inflated lungs were studied under a light microscope - Original magnification of 10X.

Supplemental Data 4 - BMP-regulation of VEGF expression in type II epithelial lung cells.

To determine if the BMP regulatory pathway regulated VEGF expression in type II epithelial cells from $MGP^{-/-}$, $MGP^{tg/wt}$ and wild type mice, we first pretreated the cells with BMP-4 (40 ng/ml) or control (vehicle only) for 12 hours to induce ALK1 expression. Soluble ALK1 fragments, which bind BMP-9, prevented ALK1 activation during the BMP-4 treatment. The BMP-4 and the ALK1 fragments were then removed, and the cells were treated for another 12 hours with either (i) BMP-9 (10 ng/ml) to stimulate ALK1 and induce ALK5, (ii) TGF-ß1 (1 ng/ml) to activate ALK5 and induce VEGF expression, or (iii) BMP-9 and TGF-ß1 to induce ALK5 expression and activate ALK5 and induce VEGF expression. We subsequently determined the levels of ALK2, ALK1, ALK5 and secreted VEGF by immunoblotting; ß-actin was used as loading control.

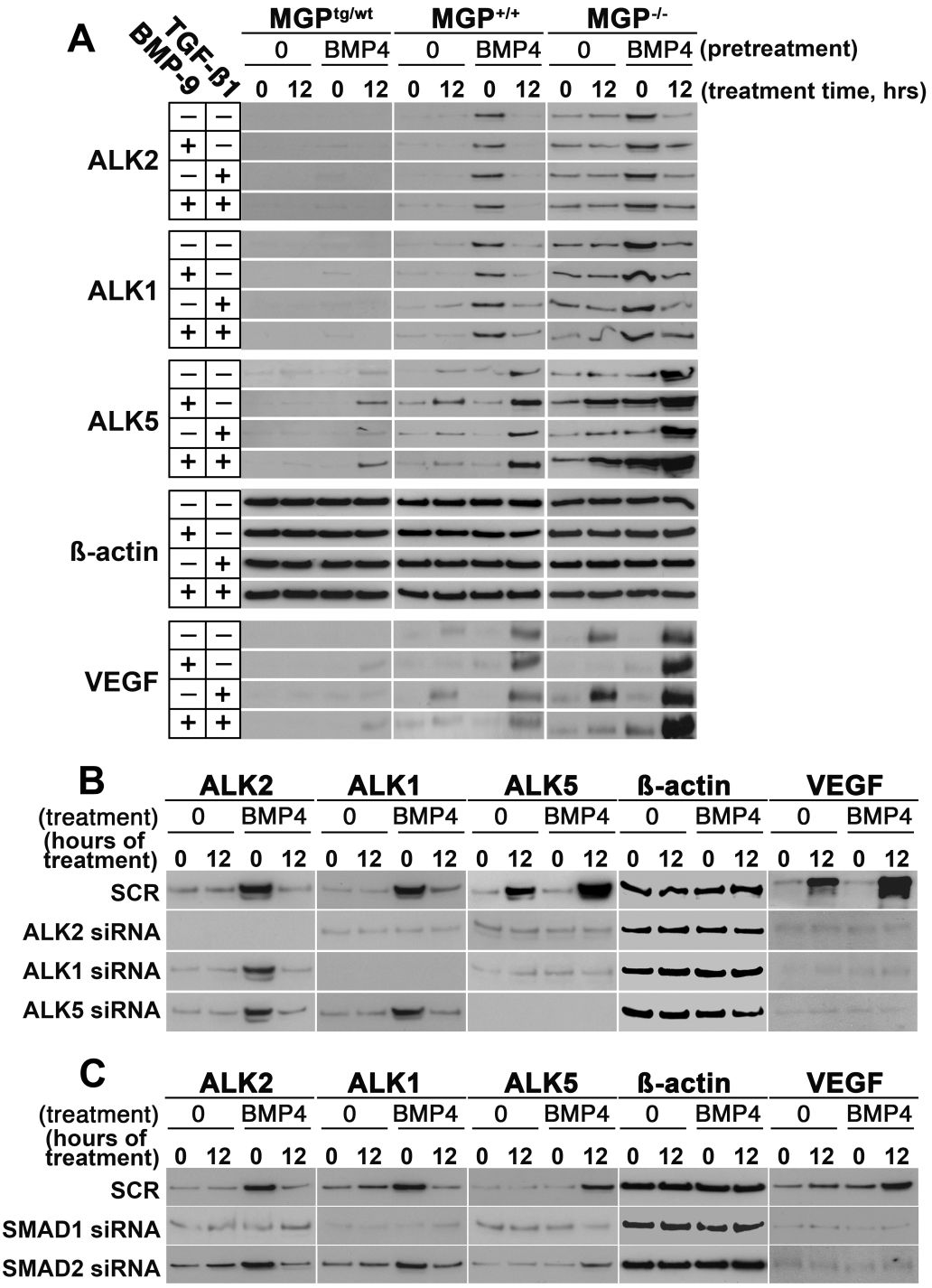
In control-treated cells with control pretreatment, ALK2 and ALK1 were increased in $MGP^{-/-}$ cells, but barely detectable in the $MGP^{tg/wt}$ cells as compared to wild type cells (Supplemental Figure 2A). Pretreatment with BMP-4 increased ALK2 and ALK1 levels, which returned to baseline after 12 hours in all cells, although not well visualized in the $MGP^{tg/wt}$ cells. BMP-9, TGF-ß1 or the combination of the two was added after 12 hours of pretreatment with control or BMP-4. These did not affect expression of ALK1 and ALK2, which supports previous findings that BMP-4 is more important for regulation of ALK1 and ALK2 (1, 2).

In control-treated cells with control pretreatment, expression of ALK5 was increased in $MGP^{-/-}$ cells, but was barely detectable in the $MGP^{tg/wt}$ cells as compared to wild type cells. The ALK5 levels were less affected by pretreatment with BMP-4, but increased 12 hours after treatment with BMP-9 with or without TGF-ß1 in $MGP^{tg/wt}$ and wild type cells (Supplemental Figure 2A). ALK5 was high after all treatments in $MGP^{-/-}$ cells, likely due to high sensitivity to BMP-9 present in the culture medium (3). Overall, the results support that ALK1/BMP-9 signaling induces ALK5 as previously reported for endothelial cells (2).

VEGF levels increased similarly to ALK5, and were highest after treatment with TGF-ß1 or combined treatment with BMP-9 and TGF-ß1 (Supplemental Figure 2A). An important exception was that treatment with BMP-9 inhibited VEGF secretion unless the cells were pretreated with BMP-4 (Supplemental Figure IIa, bottom panels). This is consistent with our previous studies, which showed that BMP-9/ALK1 signaling suppressed VEGF but induced ALK5. ALK5 and TGF-ß1 subsequently allowed VEGF induction (2). Because BMP-4 induces ALK1 (4), the pretreatment with BMP-4 appears to overcome the inhibitory effect. Altogether, our results suggest that BMP-4 induces VEGF through ALK2, ALK1 and ALK5 in type II epithelial lung cells similarly to endothelial cells (1, 2, 4).

We verified the importance of the BMP regulatory pathway for VEGF induction using small interfering RNA (siRNA) in $MGP^{-/-}$ type II epithelial cells. We transfected scrambled (SCR) control siRNA or siRNA for ALK2, ALK1, and ALK5 into the $MGP^{-/-}$ type II epithelial cells. We treated the cells with BMP-4 or control for 12 hours, but no additional treatment with BMP-9 or TGF-ß1 was used because of high baseline activity. The results revealed that (i) ALK2 depletion prevented induction of ALK1, ALK5 and VEGF, (ii) ALK1 depletion prevented induction of ALK5 and VEGF, and (iii) ALK5 depletion prevented induction of VEGF (Supplemental Figure 2B). Thus, depletion of ALK2, ALK1 and ALK5, respectively, prevented gene induction of “downstream” factors. The results were consistent with those in Supplemental Figure 2A.

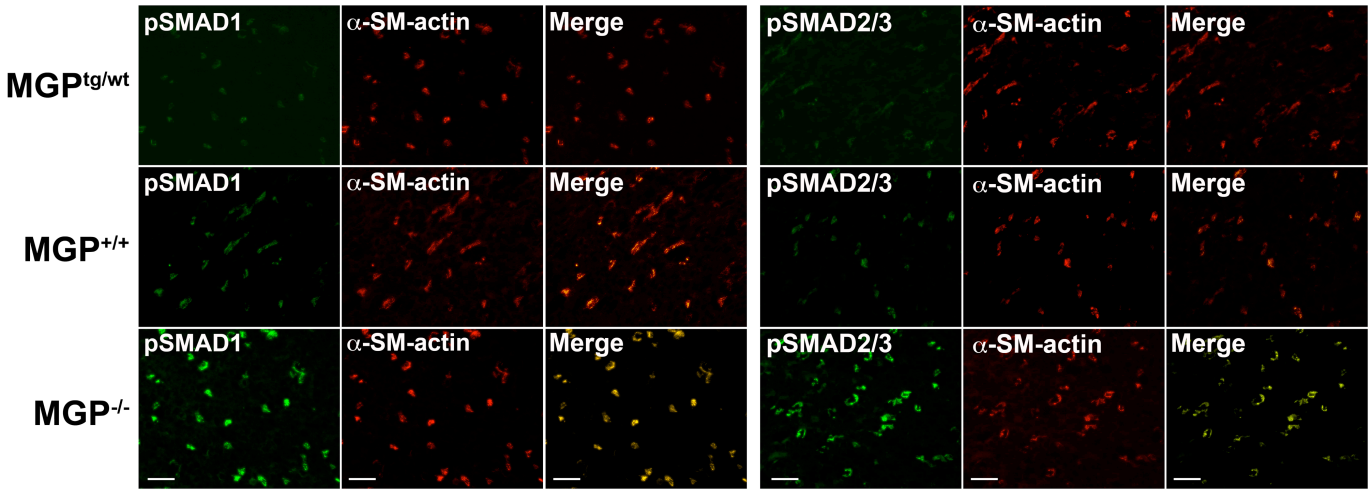
We further confirmed the importance of SMAD1 and SMAD2 in mediating induction of ALK2, ALK1, ALK5 and VEGF using siRNA in $MGP^{-/-}$ type II epithelial cells. After transfection with siRNA, the cells were again treated with BMP-4 or control. The results showed that induction of all of ALK2, ALK1, ALK5 and VEGF was abolished by SMAD1 siRNA (Supplemental Figure 2C), and that induction of VEGF was also abolished by SMAD2 siRNA (Supplemental Figure 2C), consistent with previous findings that VEGF induction is a two-step process involving ALK1 and ALK5 signaling.



Supplemental Figure 4
BMP-regulation of VEGF expression in type II epithelial lung cells.
(A) Expression ALK2, ALK1, ALK5 and secreted VEGF in type II epithelial cells after 12 hours of pretreatment with control vehicle (0) or BMP-4 (40 ng/ml) followed by treatment with control vehicle, BMP-9 (10 ng/ml), TGF-ß1 (1 ng/ml) or both, as determined by immunoblotting. ß-Actin was used as loading control.
(B) Expression ALK2, ALK1, ALK5 and secreted VEGF in type II epithelial cells from $MGP^{-/-}$ mice transfected with scrambled (SCR) siRNA or siRNA to ALK2, ALK1 or ALK5, as determined by immunoblotting. The cells were treated for 12 hours with control vehicle (0) or BMP-4 (40 ng/ml), starting 24 hours after siRNA transfection. ß-Actin was used as loading control.

(C) Expression ALK2, ALK1, ALK5 and VEGF (secreted) in type II epithelial cells from $MGP^{-/-}$ mice transfected with scrambled (SCR) siRNA or siRNA to SMAD1 or SMAD2, as determined by immunoblotting. The cells were treated for 12 hours with control vehicle (0) or BMP-4 (40 ng/ml), starting 24 hours after siRNA transfection. ß-Actin was used as loading control.

Supplemental Data 5 – Phosphorylated SMADs co-localize with α-smooth muscle cell actin in $MGP^{tg/wt}$, wild type and $MGP^{-/-}$ kidneys.



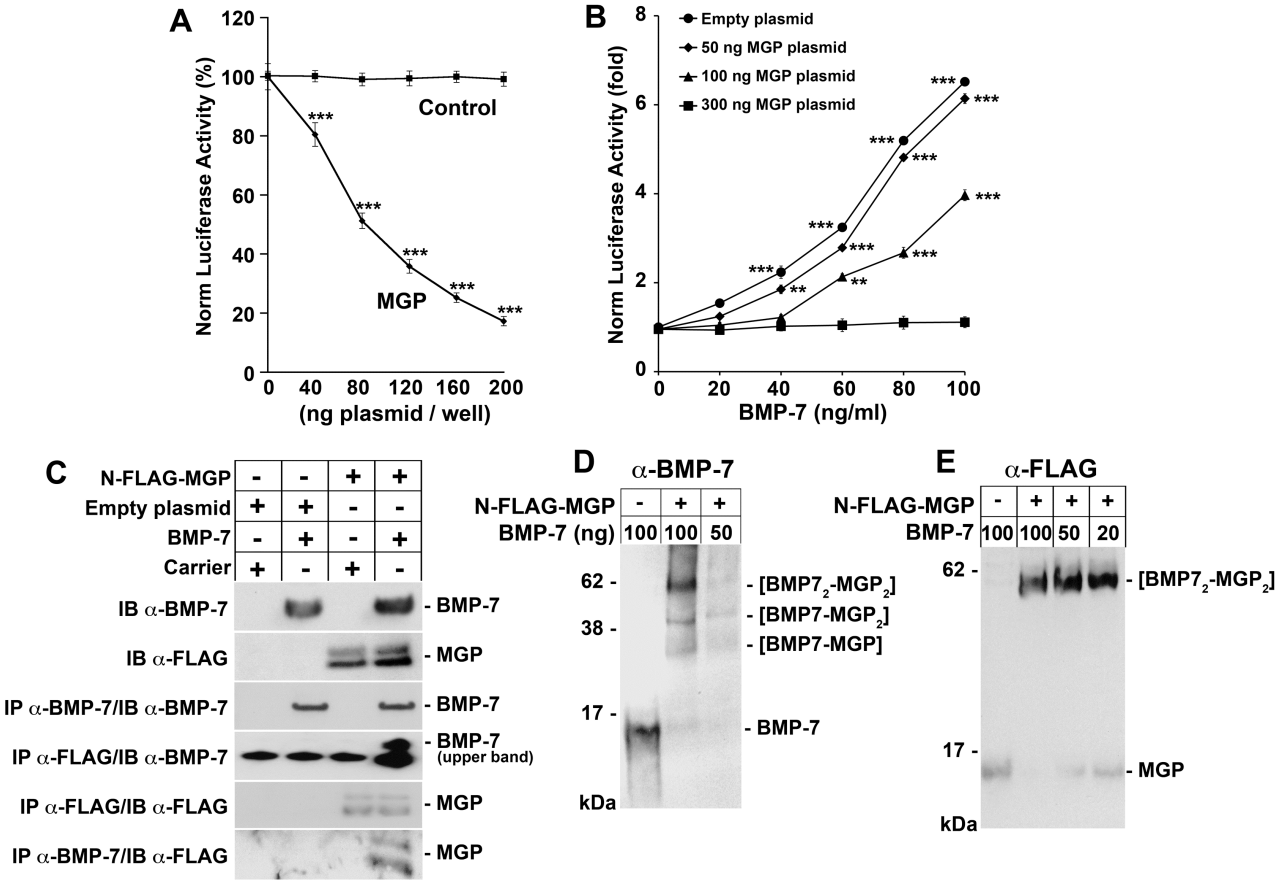
Supplemental Figure 5

Phosphorylated (p)SMADs co-localize with α-smooth muscle cell actin in $MGP^{tg/wt}$, wild type and $MGP^{-/-}$ kidneys.
$MGP^{tg/wt}$, wild type and $MGP^{-/-}$ kidneys were stained for pSMAD1 (left) and pSMAD2/3 (right) (green immunofluorescence), and for α-smooth muscle cell actin (red fluorescence), used as a marker for mesangial cells, and the images were merged.
Scale bars: 10 microns.

Supplemental Data 6 - MGP binds and inhibits BMP-7.

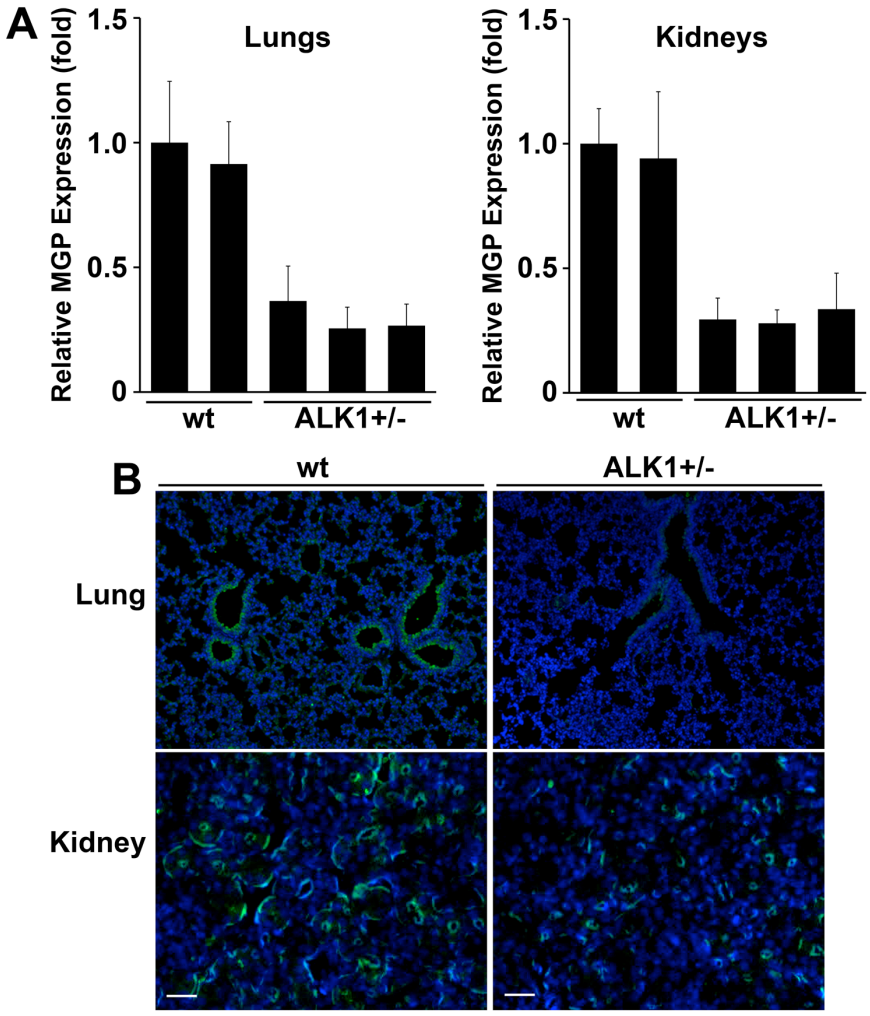
Although BMP-4 and -7 have overlaps in function, MGP has not been shown to inhibit BMP-7. Therefore we tested if MGP interferes with BMP-7 activity and binds BMP-7. Increasing amounts of an MGP expression plasmid (5) were co-transfected with the BRE-luciferase reporter gene (BRE-Luc) into the mesangial cells. The BRE-reporter gene is responsive to a number of BMPs and reflects activation of SMAD1/5/8 (6). The cells were treated with BMP-7 (80 ng/ml) starting on the day after transfection, and luciferase activity was measured after 24 hours of treatment (5). Increasing levels of MGP progressively reduced the BMP-7 activity (Supplemental Figure 6A), suggesting that MGP inhibits BMP-7 activity. We also transfected mesangial cells with an empty plasmid (200 ng) or an MGP expression construct (pN-FLAG-hMGP) (50, 100, or 200 ng), and treated the cells with increasing levels of BMP-7 (0-100 ng/ml). Again, the results showed that increasing levels of MGP progressively reduced the BMP-7 activity (Supplemental Figure 6B).

We then tested if MGP binds BMP-7 using the same approach that we previously used to establish that MGP binds and inhibits BMP-4 (5). Co-immunoprecipitation using N-terminally FLAG-tagged MGP, BMP-7 and antibodies to FLAG and BMP-7 showed co-precipitation of N-FLAG-MGP and BMP-7 (Supplemental Figure 6C). Crosslinking followed by immunoblotting with antibodies to BMP-7 or FLAG also showed an interaction between BMP-7 and MGP (Supplemental Figure 6D,E), which was similar to that of BMP-4 and MGP (5). A major band corresponding to a $BMP7_2$-MGP_2 complex was detected with both anti-BMP-7 and anti-FLAG antibodies (Supplemental Figure 6D,E). Additional complexes of lower molecular weight that most closely corresponded to BMP-7 bound to one or two MGP proteins were detected with anti-BMP-7 but not anti-FLAG antibodies. Together the results support that MGP is an inhibitor of BMP-7.



Supplemental Figure 6
MGP binds and inhibits BMP-7.
(A) Mesangial cells were cotransfected with a constant amount of the BRE-luc reporter gene and increasing amounts of an expression construct from N-FLAG-MGP. The cells were treated with BMP-7 (80 ng/ml) for 24 hours before luciferase activity was determined and normalized to Renilla. Asterisks indicate statistically significant differences compared with control. *** <0.001, *Tukey's test*.
(B) Mesangial cells were transfected with empty plasmid (200 ng) or MGP expression construct (pN-FLAG-hMGP) (50, 100, or 200 ng). The cells were treated with increasing levels of BMP-7 (0-100 ng/ml) for 24 hours before luciferase activity was determined and normalized to Renilla. Asterisks indicate statistically significant differences compared with control. ** <0.01, *** <0.001, *Tukey's test*.
(C) BMP-7 (100 ng) or carrier was mixed with one ml of culture medium collected from BAEC 24 hours after transfection with expression constructs for N-FLAG-MGP or empty plasmid. The MGP protein level was approximately 40-50 ng/ml. BMP-7 was immunoprecipitated (IP) using specific anti-BMP-7 antibodies, and the immunoprecipitates were analyzed by immunoblotting (IB) with anti-FLAG antibodies. Conversely, N-FLAG-MGP proteins were immunoprecipitated with anti-FLAG antibodies, and the immunoprecipitates were analyzed with anti-BMP-7 antibodies.
(D, E) BMP-7, 50-100 ng (D) or 20-100 ng (E) was added to 80 μl of conditioned medium containing N-FLAG-MGP and chemically crosslinked with DSS. Protein complexes were analyzed by immunoblotting using anti-BMP-7 antibodies (c) or anti-MGP antibodies (d).
All data are from a single experiment but are representative of three repeat experiments.

Supplemental Data 7 - ALK1-deficient mice have decreased expression of MGP in lungs and kidneys.



Supplemental Figure 7
Decreased MGP expression is lungs and kidneys of ALK1$^{+/-}$ mice.
(A, B) Expression of MGP in lungs from wild type and ALK1$^{+/-}$ mice as determined by real-time PCR (A) and immunofluorescence (MGP, green fluorescence) (B).
Scale bars: 100 microns.

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