Matrix GLA Protein, an Inhibitory Morphogen in Pulmonary Vascular Development

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Deficiency of matrix GLA protein (MGP), an inhibitor of bone morphogenetic protein (BMP)-2/4, is known to cause arterial calcification and peripheral pulmonary artery stenosis. Yet the vascular role of MGP remains poorly understood. To further investigate MGP, we created a new MGP transgenic mouse model with high expression of the transgene in the lungs. The excess MGP led to a disruption of the pulmonary pattern of BMP-4, and resulted in significant morphological defects in the pulmonary artery tree. Specifically, the vascular branching pattern lacked characteristic side branching, whereas control lungs had extensive side branching accounting for as much as 40% of the vascular endothelium. The vascular changes could be explained by a dramatic reduction of phosphorylated SMAD1/5/8 in the alveolar epithelium, and in epithelial expression of the activin-like kinase receptor 1 and vascular endothelial growth factor, both critical in vascular formation. Abnormalities were also found in the terminal airways and in lung cell differentiation; high levels of surfactant protein-B were distributed in an abnormal pattern suggesting lost coordination between vasculature and airways. Ex vivo, lung cells from MGP transgenic mice showed higher proliferation, in particular surfactant protein-B-expressing cells, and conditioned medium from these cells poorly supported in vitro angiogenesis compared with normal lung cells. The vascular branching defect can be mechanistically explained by a computational model based on activator/inhibitor reaction-diffusion dynamics, where BMP-4 and MGP are considered as an activating and inhibitory morphogen, respectively, suggesting that morphogen interactions are important for vascular branching.

Matrix GLA protein (MGP) is a known inhibitor of vascular calcification. Lack of MGP causes arterial calcification in mice (1) and the human Keutel syndrome, which is due to mutations in the MGP gene (2). The arterial calcification in mice is associated with profound changes in cell differentiation as arterial smooth muscle cells are replaced by chondrocyte-like cells undergoing progressive mineralization (1). MGP deficiency also causes peripheral pulmonary artery stenosis and other morphological defects, including nasal hypoplasia and abnormal cartilage and bone calcification (2, 3). Furthermore, alterations in MGP expression has been reported in various tumors including a loss of MGP during progression of lung and colorectal carcinomas (4, 5), which may be related to tumor vascularization given that differential MGP expression has been observed in endothelial cells during angiogenesis (6).

Previous work by our group and others has suggested that MGP can act as an inhibitory morphogen, acting antiangiotically to bone morphogenetic proteins (BMP)-2 and -4 (7–9). Our previous studies also showed close links between BMP-4, MGP, the activin-like kinase receptor 1 (ALK1), a transforming growth factor (TGF)-β receptor essential in vascular formation, and vascular endothelial growth factor (VEGF) (9). BMP-4 stimulated expression of the ALK1 receptor in vascular endothelial cells, which when activated, enhanced expression of MGP and VEGF. Stimulation of the BMP receptors as well as ALK1 is known to activate the SMAD signaling system by phosphorylating SMAD1/5/8, whereas stimulation of the TGF-β receptor ALK5 causes phosphorylation of SMAD2/3 (10).

However, the role of MGP in vascular biology is far from clear. Here, we report that a newly generated human MGP transgenic mouse suffers severe morphological defects in the pulmonary artery tree, which can be explained by considering BMP-4 and MGP as an activating and inhibitory vascular morphogen, respectively, in the developing lung vasculature. Specifically, we show that in the transgenic mouse, the branching pattern in the developing lung vasculature lack characteristic side branching, whereas the control mouse has extensive side branching. We found that excess MGP disrupted the distribution of BMP-4 in the lung tissue, and caused a significant reduction in phosphorylation of SMAD1/5/8, and in epithelial expression of ALK1 and VEGF. The abnormal vascular development was associated with abnormal morphology and cell differentiation in the terminal airways.
Matrix GLA Protein, a Vascular Inhibitor

Recent work has explored the role of morphogens in the creation of branching pattern formation (11). We present evidence, based on a computational model of the process, that MGP acts in lung vascular development as an inhibitory morphogen, acting antagonistically to BMP-4 in an activator/inhibitor reaction-diffusion dynamic system. It provides a mechanistic explanation for the specific lack of side branching in the MGP transgenic mouse, and strengthens the claim that morphogen interactions and dynamics play an important role in the morphogenesis of branched structures.

MATERIALS AND METHODS

Generation and Identification of Mice Transgenic for Human MGP—Transgenic mice were generated using bacterial artificial chromosomes (BAC) as previously described (12). The study had been approved by the Institutional Review Board of the University of California, Los Angeles. The BAC clone containing the human mgp gene (Invitrogen), was digested by BsrFI, and the DNA fragment containing the mgp gene was purified using a QIAEX II kit (Qiagen). The purified fragment contained 18.5-kb upstream and 10.5-kb downstream DNA sequences of the mgp gene. The DNA was injected into the pronuclei of C57BL/6 mouse eggs at a concentration of 3 ng/μl. Noon of the day of injection was defined as 0.5 days post coitus. The genotype was determined by PCR analysis of genomic DNA, extracted from the mouse tails. Two primer pairs specific for the human mgp gene (GenBank™ accession number NM_000900) were used. The first primer pair spanned the 5’-junction of exon I (5’-CCAGACACGATCCTGTGTA-3’) and 5’-GAACAGTGAGAGAGCTG-3’) and resulted in a 159-bp product. The second primer pair spanned the 3’-junction of exon IV (5’-GCTCTAAGCGCCCGTACG-3’) and 5’-GCTGTACAGGGGGATACAA-3’) and resulted in a 201-bp product. PCR thermocycling parameters for the genomic DNA were 30 cycles of 95°C for 30 s, 68°C for 30 s, and 72°C for 1 min. The PCR products were analyzed on agarose gels.

RNA Analysis—Total RNA was isolated from tissues or cultured cells using the RNEasy kit (Qiagen). Real time PCR assays were performed as previously described (9). The following primers and probes were used: human MGP (hMGP) forward, 5’-GGGAGACCTGTGACTAGA-3’, hMGP reverse (5’-CGGATTTAGGAGACATTGTGATCCA-3’; hMGP TaqMan probe, FAM-TTGCGAACGCTACGCCATGGTTT-TAMRA; mouse MGP forward, 5’-TTGCGAACGCTACGCCATGGTTT-TAMRA; mouse MGP reverse, 5’-GGTTGCTCTTGGACACGCACAT-3’; TaqMan probe, FAM-CCCACGTCAAGAGCACAACAT-TAMRA; mouse vascular endothelial growth factor (mVEGF) forward, 5’-TGAAGCTTGGAGTTCGTG-3’, mVEGF reverse, 5’-AGGTCTGTACGTCGATG-3’; and TaqMan probe, FAM-CCCACGTCAAGAGCACAACAT-TAMRA. The primer and probes for mouse BMP-2 and BMP-4 were obtained from Applied Biosystems.

Visualization of Lung Vasculature—Corrosion casting of the pulmonary arteries was performed at 1 month of age as previously described (13) using a pressure of 15 mm Hg through the right ventricle. Tissue was removed by maceration in 20% KOH for 2 days. The casts were cleaned in formic acid and distilled water. Lungs were photographed at E15.5, and to optimize visualization of the vasculature, the images were sharpened in NIH Image J (imagejdocu.tudor.lu) using a 3-point moving average algorithm, with weights (−1,1,−1) in both X and Y directions.

Immunohistochemistry—The lung tissues were collected from MGP transgenic and wild type embryos on embryonic day (E) 18.5. Tissues were fixed in 4% paraformaldehyde and immunohistochemistry was performed as previously described (14). Specific antibodies to MGP were obtained from American Diagnostica Inc. Antibodies to platelet endothelial cell adhesion molecule 1 (PECAM-1; CD31, a marker for the endothelial cell population (15)), VEGF, ALK1, BMP-4, P-SMAD2/3, and total SMAD were from Santa Cruz Biotechnology. Antibodies to surfactant protein (SP) B were from Upstate, and antibodies to P-SMAD1/5/8 were from Cell Signaling. Permeabilization of the tissue with 0.1 mg/ml of Proteinase K for 15 min was performed prior to immunohistochemistry using the ALK1 antibody. ABC reagents (Vector Laboratories) were used to detect antibody binding. Eosin was used for counterstaining. Cy3 donkey anti-goat IgG (Jackson ImmunoResearch) was used for immunofluorescence to visualize binding of anti-BMP-4 antibodies. Controls with no primary antibody showed no fluorescence labeling. Images were acquired using a Nikon Labphot-2 microscope, a Nikon FX-35DX camera, and SPOT imaging software, version 4.0.1.

Immunoblotting—Immunoblotting was performed as previously described (9). Equal amounts of protein from whole tissue extracts were used. Blots were incubated with specific antibodies to SP-B (4 μg/ml; Upstate), BMP-4 (4 μg/ml; Santa Cruz Biotechnology), FLAG (2.5 μg/ml; Sigma), PECAM-1 (0.4 μg/ml; Santa Cruz Biotechnology), aquaporin-5 (5 μg/ml; Calbiochem), Clara cell-specific protein (CCSP) (1:200 dilution; Upstate), β-actin (1:5000 dilution; Sigma), and VEGF (0.4 μg/ml; Santa Cruz Biotechnology). Densitometry using NIH Image J was performed to compare the relative protein levels after normalization to β-actin.

Cell Culture—Bovine aortic endothelial cells (BAEC) were purchased from VEC Technologies (Rensselaer, NY) and cultured as previously described (9). A549 cells (American Type Cell Collection) and mice lung cells (prepared as per below) were cultured in Ham’s F12K medium, supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories), penicillin (100 units/ml), streptomycin (100 units/ml), and 1-glutamine (2 mM). Recombinant human BMP-4 (R&D Systems) was added at the time of plating of the cells.
**Matrix GLA Protein, a Vascular Inhibitor**

\[
\frac{\partial H}{\partial t} = cA^2S - \nu H + D_h \nabla^2 H + \rho_h Y \quad \text{(Eq. 2)}
\]

\[
\frac{\partial S}{\partial t} = c_0 - \gamma S - eYS + D_s \nabla^2 S \quad \text{(Eq. 3)}
\]

\[
\frac{\partial Y}{\partial t} = aA - eY + \frac{g^2}{(1 + fg)^2} \quad \text{(Eq. 4)}
\]

Here, the activator \( A \) is produced autocatalytically in a process requiring \( S \) and inhibited by \( H \), is degraded at a rate \( \mu \) and is produced by differentiated cells \( Y \) at a rate \( \rho_h \). It also diffuses, at a rate \( D_h \). The inhibitor \( H \) is produced by activator \( A \) in a process requiring \( S \), degraded at a rate \( \nu \) and produced by differentiated cells at a rate \( \rho_h \). It is this parameter \( \rho_h \), modeling the rate of MGP production by differentiated vascular cells that we will increase to model the effects of the MGP transgenic mouse. \( H \) also diffuses, at a rate \( D_h \). Substrate, \( S \), is supplied at a rate \( \gamma \), degrades at a rate \( \gamma \), is taken up by differentiated cells \( Y \) at a rate \( e \), and diffuses at a rate \( D_s \). Last, \( Y \) is a marker of differentiation: it is increased by the presence of \( A \), decreases at a rate \( e \), and sigmoidally increases from 0 to 1, representing commitment to differentiation.

The model was simulated in a 200 x 200 grid using Matlab code (available on request) that implemented a finite-difference scheme using a forward Euler method for the reaction part and a second-order four-point Laplacian for the diffusion operator. We used no-flux boundary conditions throughout.

**Parameter Values**—The values used are: \( \Delta x = 0.25, \Delta t = 0.0045; D_A = 0.02, D_H = 0.18, D_S = 0.06; c_e = 0.0025, c_i = 0.002; \mu = 0.12, \gamma = 0.02, e = 0.02, d = 0.0033, e = 0.1, f = 10; \rho_A = 0.03, \nu = 0.04; \rho_H = 0.0001 \) or 0.0003.

**RESULTS**

Characterization of the MGP Transgenic Mouse—To elucidate the role of MGP during development and morphogenesis, we generated transgenic mice for MGP on C57BL/6J background. We used a 33-kb BAC clone containing the human mgp (hmgp) gene (Fig. 1A) to allow the human MGP (HMG) promoter to drive expression. No other genes except the MGP gene were included in the BAC clone. We obtained five transgenics, all male, expressing the hmgp transgene, which was verified by real-time PCR with specific primers spanning intron-exon junctions of exons 1 and 4 of HMG (Fig. 1B). Three transgenic lines with high hmgp transgene expression (Fig. 1B) were selected for further studies. Studies of the two mice with low MGP expression were not pursued because one was chimeric and did not generate offspring, and the second had a truncated BAC clone. The distribution of hMGP expression was compared with that of mMGP on E18.5 and at 1 month of age using real time PCR with species-specific primers. In both cases, the highest MGP expression was found in the lungs (Fig. 1C). There was increased perinatal mortality of heterozygous hMGP transgenic mice (Mgp\(^{+\text{n}}\)/") resulting in a decrease of the ratio of Mgp\(^{+\text{n}}\)/" mice to total mice from 0.4 prenatally to 0.25 in adult mice (Fig. 1D). The deaths usually occurred within minutes to a few hours after birth. Furthermore, females had a higher lethality than males, resulting in an increase of the male to female ratio than males, resulting in an increase of the male to female ratio than males, resulting in an increase of the male to female ratio than males, resulting in an increase of the male to female
ratio among MGP<sup>tg/wt</sup> mice from 0.75 prenatally to 1.6 in adult mice (Fig. 1E). The higher lethality of female MGP<sup>tg/wt</sup> mice led to difficulties in obtaining homozygous MGP<sup>tg/tg</sup> mice. Because the phenotype was apparent in MGP<sup>tg/wt</sup> mice, these were used for characterization and the results from the three MGP<sup>tg/tg</sup> mice were incorporated when available (see Fig. 5, B–D).

When determining the cause of the early lethality in MGP<sup>tg/wt</sup> mice, we found that the MGP<sup>tg/wt</sup> mutants were indistinguishable from wild type littermates at birth in regards to body weight and organ weight per body weight (Fig. 1F). The movements of the transgenic pups were normal, suggesting that the peripheral nervous system was normal. Furthermore,
we examined the skeletal development in transgenic and wild type littersates because MGP is expressed in developing bones (1). However, cleared skeletal preparations did not reveal any major skeletal defects and histochemical stains did not reveal significant differences in the growth plates in the MGP transgenic (data not shown). Furthermore, palates and tracheae were without obvious abnormalities (data not shown). Contrary to MGP-deficient aorta, which are calcified at 3 weeks of age (1), histochemical stains did not reveal any abnormalities in the MGP transgenic aorta at 1 month of age (data not shown).

Effect of the MGP Transgene on Airways and Pulmonary Cell Differentiation—MGP has been implicated in lung development, and the MGP transgene was highly expressed in the lungs. We examined the airways at E18.5, but were unable to distinguish significant differences in the airways by serial histological sectioning (Fig. 2A). However, at 1 month of age, the transgenic lungs were larger and buoyant in normal saline, and microscopic examination of the terminal airways revealed terminal air sacs that were more closely spaced and tube-like compared with wild type littersates (Fig. 2B). We hypothesized that the changes in airway morphology might be associated with changes in pulmonary cell differentiation, and analyzed molecular markers for pulmonary cells. Our results showed an increase in SP-B, a marker for type II epithelial cells (17), at E18.5 and 1 month of age, as determined by immunoblotting (E18.5 shown in Fig. 2C, left). Furthermore, immunohistochemical analysis revealed that the distribution of SP-B was significantly altered in transgenic lungs. In normal lungs, SP-B was expressed mostly in the alveolar epithelium, whereas in transgenic lungs, it was spread throughout the epithelium and the mesenchyme (E18.5 shown in Fig. 2C, right) reflecting the abnormal airway morphology. We were unable to detect significant differences for aquaporin-5, a marker for type I epithelial cells (18), or CCSP, a marker for bronchial epithelial cells (19) as determined by immunohistochemistry and immunoblotting (data not shown).

Effect of the MGP Transgene on Vascular Growth and Branching—We then hypothesized that the pulmonary vasculature may be abnormal in association with the abnormal airways. Gross dissection of the lungs showed an overall decrease in pulmonary vascularization in MGP transgenic mice compared with wild type littersates, apparent at 1 month of age (Fig. 3A). Visualization of the pulmonary arteries in the transgenics using corrosion casting showed two main vascular trunks and a severe lack of small vessels and side branches when compared with wild type (Fig. 3B). We also examined the effect on endothelial cell differentiation by FACS sorting of disaggregated lung cells and immunohistochemistry using antibodies to PECAM-1, a marker for the endothelial cell population (15). The percentage of PECAM-1 positive lung cells decreased by

**FIGURE 2. Morphological and histological analysis of the lungs in the MGP transgenic mice.** A, lungs of wild type and MGP tg/wt mice on E18.5, H&E staining. Original magnification, ×4 (top), and ×20 (bottom). B, terminal airways in wild type and MGP tg/wt littermates at 1 month of age. The inflated lungs were studied under a light microscope. Original magnification, ×10. C, pulmonary expression of SP-B in MGP transgenic mice (MGP tg/wt) and wild type littersates at E18.5 as determined by immunoblotting and densitometry (n = 3) (left), and immunohistochemistry (right). wt, wild type; tg, transgenic; a, alveolus. Original magnification, ×20. Asterisks indicate statistically significant differences compared with control. **, p < 0.01, Scheffe’s test.
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40%, from 10.2 ± 0.04 to 6.2 ± 0.4% in transgenic lungs (Fig. 3C). The results were confirmed by immunohistochemistry (data not shown), and consistent with a decrease in overall vessel formation.

Pulmonary expression of MGP starts to increase after E14 (20), which coincides with an exponential increase in vascular networks (21). To examine if a branching defect led to the abnormal vasculature in the MGP transgenics, we examined the pulmonary vessels in non-perfused E15.5 and E18.5 embryos. The results revealed a qualitative change in vascular branching, best seen in E15.5 where individual vessels easily could be distinguished. Normal embryos had vascular trees with both two-pronged tip-branching and abundant side branches (Fig. 4, top and bottom right), whereas transgenic vascular trees had retained the tip branching but lacked the side branching (Fig. 4, middle and bottom right), resulting in a sparser network. We determined the number of visible branch points per lower lung field in MGP transgenic and wild type embryos. The results showed that the number of branch points was reduced by 63% compared with wild type (Fig. 4, bottom).

Altered BMP-4 Distribution in MGP Transgenic Lungs—Because MGP is known to inhibit BMP-4 (9) and BMP-4 is a key factor in the regulation of the pulmonary development, we hypothesized that excess MGP affected pulmonary BMP-4 and diminished its activity. To determine expression and distribution of MGP and BMP-4, we examined lungs from E18.5 using real time PCR and immunohistochemical analysis. Total MGP mRNA expression was increased 2.4–6.6-fold (3.8 ± 1.8) (n = 8) in the MGP heterozygous transgenic lungs by real time PCR of whole lung RNA (Fig. 5A). Immunohistochemical analysis of wild type littermates revealed staining for MGP in the mesenchyme and the alveolar epithelium (Fig. 5A). It is consistent with findings in the rat (20) although we detected less bronchiolar staining. In the MGP transgenics, however, MGP staining was enhanced in a wider area surrounding the alveoli and in the mesenchyme, areas where endothelial cell progenitors are found (21) (Fig. 5A). Total BMP-4 expression was unchanged in the transgenic lungs (Fig. 5B), but surprisingly, BMP-4 protein distribution was significantly altered. In normal lung, the high-
The highest BMP-4 concentration was found in the epithelium (Fig. 5B, arrowheads) similar to previous findings (22, 23). In the transgenic lungs, however, BMP-4 was detected mainly in the mesenchyme as shown by immunofluorescence (Fig. 5B), resulting in a relative BMP-4 deficiency in the epithelium. We were unable to detect changes in expression or distribution of BMP-2, a closely related BMP also inhibited by MGP (7) (data not shown).

Reduction of ALK1 and VEGF Expression in MGP Transgenic Lungs—We previously identified a regulatory pathway that would enable BMP-4 to activate endothelial cells (9). BMP-4 induces expression of ALK1, which is essential in angiogenesis (24). When activated, ALK1 signaling leads to increased expression of VEGF, a well known angiogenic factor. High levels of ALK1 have been reported in the tissue surrounding the bronchi (25) and in pulmonary blood vessels (26). VEGF expression has

FIGURE 5. Expression of MGP, BMP-4, ALK1, and VEGF in wild type and MGP transgenic lungs at E18.5. Pulmonary expression of MGP (A), BMP-4 (B), ALK1 (C), and VEGF (D) in wild type and MGP transgenic lungs at E18.5 as determined by real time PCR (n = 8 in each group for MGP and BMP-4; n = 6 in each group for ALK1 and VEGF) (right panels) and immunohistochemistry or immunofluorescence (middle panels). Negative controls with non-immune rabbit or goat IgG are shown in the left panels. Arrowheads indicate alveolar epithelium. Br, bronchiole; a, alveolus; wt, wild type; tg, transgenic. Original magnification, ×20. Asterisks indicate statistically significant differences compared with control. **, p < 0.01; ***, p < 0.001, Scheffe’s test.
Because ALK1 is involved in TGF-β and the mesenchyme in the normal lungs consistent with pre-staining for P-SMAD2/3 or total SMAD between transgenic (Fig. 5). In contrast, we were unable to detect differences in corresponding to the areas of low BMP-4 and ALK1 (compare 0.20 by real time PCR at E18.5. ALK1 expression was reduced to transgenics compared with wild type littermates, as determined expression of ALK1 and VEGF was significantly reduced in the MGP reduction of both ALK1 and VEGF in the lung tissue. Expression, we determined the level of phosphorylated (P)-SMAD1/5/8 by immunohistochemistry at E18.5. The results showed a clear response to stimulation by BMP-4 and activation of ALK1 (10), been reported in airway epithelium and mesenchyme (15, 27). Our results suggested that BMP-4 as well as ALK1 signaling was significantly reduced in the MGP transgenics compared with wild type littermates, as determined by real time PCR at E18.5. ALK1 expression was reduced to 0.20 ± 0.12 and 0.09 ± 0.08 of normal levels in the heterozygotes and hemizygotes, respectively, whereas VEGF expression was reduced to 0.48 ± 0.12 and 0.30 ± 0.25, respectively (n = 6 in each group) (Fig. 5, C and D). Immunohistochemical analysis showed staining for ALK1 and VEGF in the alveolar epithelium and the mesenchyme in the normal lungs consistent with previous reports. In the MGP transgenic lungs, however, staining for both ALK1 and VEGF was greatly reduced (Fig. 5, C and D). Because ALK1 is involved in TGF-β1 and -β3 signaling (28) and TGF-β1 has been shown to affect VEGF levels in developing lungs (29, 30), we determined expression and distribution of both TGF-β1 and -β3. No significant differences were detected between normal and transgenic lungs as determined by real time PCR and immunostaining (data not shown).

Our results suggested that BMP-4 as well as ALK1 signaling activity might be reduced in the alveolar epithelium in the MGP transgenic lungs. Because SMAD1/5/8 is phosphorylated in response to stimulation by BMP-4 and activation of ALK1 (10), we determined the level of phosphorylated (P)-SMAD1/5/8 by immunohistochemistry at E18.5. The results showed a clear decrease in P-SMAD1/5/8 staining in the transgenic alveolar epithelium compared with controls (Fig. 6, top panels), which corresponded to the areas of low BMP-4 and ALK1 (compare Fig. 5). In contrast, we were unable to detect differences in staining for P-SMAD2/3 or total SMAD between transgenic and normal lungs (Fig. 6, middle and bottom panels). P-SMAD2/3 is phosphorylated in response to TGF-β stimulation of the ALK5 receptor (10). The results were consistent with a reduced BMP-4 and ALK1 signaling activity in the transgenic lung epithelium.

**BMP-4 Induces Expression of ALK1 in A549 Pulmonary Cells**—The immunostaining showed that ALK1 and VEGF are expressed in the lung epithelium. To determine whether BMP-4 induces ALK1 in pulmonary cells similarly to endothelial cells (9), we used the A549 cells, a pulmonary epithelial tumor cell line responsive to BMP-4 (31). First, we compared baseline levels of secreted BMP-4 and mRNA expression of ALK1, VEGF, and MGP between BAEC and A549 cells. The results showed that BMP-4 was easily detected in the medium of A549 cells but not in BAEC after 24 h of culture as determined by immunoblotting (Fig. 7A). As would be expected if BMP-4 had an autocrine effect on A549 cells, ALK1, VEGF, and MGP were all up-regulated compared with BAEC as determined by real time PCR. ALK1 was increased 6-fold (6.0 ± 0.5), VEGF 4-fold (3.5 ± 0.5), and MGP 10-fold (10.6 ± 1.7) (Fig. 6A). However, addition of BMP-4 (0–100 ng/ml) to A549 cells further increased ALK1 expression in a dose-dependent fashion up to 2.5-fold (2.6 ± 0.3) above baseline (Fig. 7B), demonstrating that pulmonary epithelial cells are able to induce ALK1 expression in response to BMP-4.

**In Vitro Angiogenesis Is Poorly Supported by MGP Transgenic Lung Cells**—The increase in lung size, airways, and SP-B expression suggested that pulmonary cell proliferation was increased in the MGP transgenic lungs. To compare lung cell proliferation in MGP transgenic and wild type littermates, we prepared total lungs cells by enzymatic disaggregation. We determined cell proliferation by [3H]thymidine incorporation, we collected conditioned media from transgenic and wild type lung epithelium.

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To examine the ability of the lung cells to support angiogenesis, we collected conditioned media from transgenic and wild type lung cells after 4 days of culture. VEGF levels were significantly decreased in the medium from the transgenic lung cells compared with wild type cells as determined by immunoblotting (Fig. 8C). We then performed tube formation assays in the presence of the conditioned media. The results showed that the medium from the normal lung cells strongly supported tube formation (Fig. 8D, second panel from left) compared with non-conditioned medium and medium from transgenic lung cells.
and time between MGP and BMP-4 as inhibitor and activator. Using our 4-variable reaction-diffusion model (Fig. 9A), we asked whether an increase in MGP production by differentiated cells, the rate $\rho_P$, could account for the specific loss of side branching. We found this to be true. Using standard parameter values, the result was a branching process that had both side branching and tip branching, as seen in the normal mouse (Fig. 9B, left). But when the value of $\rho_P$ was increased from 0.0001 to 0.0003, a similar fold increase to that of MGP expression in transgenic lungs in vivo, side branching was specifically inhibited (Fig. 9B, right) (see also supplemental data Movie 1).

**DISCUSSION**

Our studies suggest that MGP is an inhibitory vascular morphogen in the pulmonary vascular tree, acting antagonistically to BMP-4. Excess MGP was associated with disrupted lung pattern of BMP-4 in vivo, and decreased levels of P-SMAD1/5/8, ALK1, and VEGF consistent with previous in vitro findings (9). On one hand, proper dynamics of BMP-4 and MGP appears to be critical for formation of vascular side branching, as evidenced by the lack of side branching in the newly generated MGP transgenic mouse. Most likely, this is the underlying mechanism of the increased perinatal lethality in the MGP transgenics. On the other hand, excess MGP deregulates airway formation, lung cell proliferation, and SP-B expression, thus acting as an activator of pulmonary cells.

**BMP and MGP as Activator and Inhibitor in Vascular Cell Development**—Our studies suggest that BMP-4 signaling activates vascular cell differentiation and vascular formation. This is supported by results from other investigators who demonstrated that BMP-4 is required for generation of endothelial cells from embryonic stem cells in vitro (32), and by our previous identification of BMP-4 as a stimulator of expression of ALK1 and VEGF (9). Because outgrowth of blood vessel sprouts has been shown to occur toward a gradient of VEGF (33, 34), it is possible that BMP-4 determines the expression of VEGF, and that VEGF is the main vascular activator and the connection between BMP-4 and branching. Furthermore, Zeng et al. (35) showed that overexpression of VEGF in developing respiratory epithelium results in increased growth of pulmonary blood vessels and disrupted branching morphogenesis.

Our results also show that ALK1 is induced in pulmonary cells, which may contribute to the establishment of a VEGF gradient and vascular activation. Lung buds normally contain high expression of both ALK1 and VEGF (25, 36), but the transgenic lungs showed a decrease in ALK1 and VEGF expression in areas with reduced BMP-4 and P-SMAD1/5/8. TGF-β1 overexpression has been shown to decrease VEGF expression and inhibit vascular development in fetal lungs (30). However, we were unable to identify changes in expression of TGF-β1, TGF-β3, or P-SMAD2/3 in the transgenics.

**The Role of MGP and BMP-4 in the Coordination of Vascular and Airways**—BMP-4 is detected very early in the lung mesenchyme, but it is not present in the epithelium until airway branching starts (23). The highest BMP-4 levels are subsequently found in the tips of the distal airways (compare Fig. 9B, lower panels). The precise role of BMP-4 in the developing lung is still unclear (23). It has been proposed that BMP-4 is induced

(Fig. 8D, middle). The tube formation in the medium from the transgenic cells could be rescued by the addition of exogenous VEGF (10 or 50 ng/ml) (Fig. 8D, right panels). These results were consistent with our in vivo findings and suggested that MGP overexpression leads to increased proliferation of SP-B expressing type II epithelial cells with poor ability to express VEGF and to support angiogenesis.

**The Role of MGP in Inhibiting Side Branching: Computational Model**—We tested the hypothesis that elimination of side branching (but not tip branching) in the MGP transgenic mouse was specifically due to the dynamic interactions in space

![BMP dose dependently induces expression of ALK1 in A549 pulmonary cells](image-url)
in the epithelium of the distal buds during branching to limit bud outgrowth (23, 37, 38); overexpression of BMP-4 in the distal epithelium results in lung hypoplasia and a decrease in the number of SP-C-positive type II epithelial cells (39). However, when BMP-4 is administered to intact lung explants in which epithelium and mesenchyme are present, branching is paradoxically enhanced (40). A model has been proposed to explain how BMP-4 can have both positive and negative effects in the lung. It proposes that the mesenchyme influences the ability of the epithelium to respond to BMP-4. Thus, autocrine BMP-4 signaling in the epithelium would inhibit proliferation, whereas paracrine BMP-4 stimulation of the adjacent mesenchyme in the intact lung would induce a mesenchymal signal that enhances epithelial proliferation (23, 40). The increase in airways and SP-B expression in the MGP transgenics suggest that MGP might be such a signal. Our results suggest that correct BMP-4 patterning is essential for correct positioning of SP-B expressing cells. It is possible that continued expression of BMP-4 in the mesenchyme (41) plays a role in maintaining the coordination between vasculature and airways, important for optimization of oxygen uptake. Furthermore, BMP-4 has been shown to promote vascular remodeling in hypoxic pulmonary hypertension (42), possibly by affecting ALK1 and VEGF.

Our results are consistent with those of Gilbert et al. (20) who reported that in fetal lung explants, high MGP increased branching of the saccules and caused dilation of conducting airways. Intraluminal MGP, however, did not have any effect in their experiments, supporting the concept that exact localization of each stimulus is essential for tissue organization. Changes in the airways in our transgenics may also be promoted postnatally by changes in blood oxygen tension due to poor vascularization.

**BMP-4 and MGP in a Vascular Reaction-Diffusion Dynamic System**—Our previous studies showed that activation of ALK1 leads to increased MGP expression (9), allowing for restriction of BMP activity. The identification of BMP-4 and MGP as activator and inhibitor, respectively, suggested that they interact in a pattern formation dynamic that could explain the organization of airways and vasculature. Mathematical models of development using reaction-diffusion partial differential equations involving activator and inhibitor "morphogens" were first proposed by Turing (58), who coined the word. The identity of these morphogens long remained unclear, which limited the applicability of this type of model, but in the last decade, a number of important morphogens have been identified, including: retinoic acid (43), members of the TGF-β superfamily, such as squint and several BMPs (44, 45), growth factors such as fibroblast growth factor and epidermal growth factor (46, 47), and shh/decapentaplegic (Dpp) (48).

Reaction-diffusion models of activators and inhibitors have been proposed for a number of biological processes (8, 49–54).
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