Matrix GLA protein modulates branching morphogenesis in fetal rat lung

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Gilbert, Kirk A., and Stephen R. Rannels. Matrix GLA protein modulates branching morphogenesis in fetal rat lung. Am J Physiol Lung Cell Mol Physiol 286: L1179–L1187, 2004; 10.1152/ajplung.00188.2003.—The regulation of matrix γ-carboxyglutamic acid protein (MGP) expression during the process of lung branching morphogenesis and development was investigated. MGP mRNA expression was determined over an embryonic and postnatal time course and shown to be developmentally regulated. Immunohistochemical analysis revealed increased staining for MGP in peripheral mesenchyme surrounding distal epithelial tubules. Fetal lung explants were used as an in vitro growth model to examine expression and regulation of MGP during branching morphogenesis. MGP mRNA expression over the culture interval mimicked the in vivo time course. Explants cultured in the presence of antibodies against MGP showed gross dilation and reduced terminal lung bud counts, accompanied by changes in MGP, sonic hedgehog, and patched mRNA expression. Similarly, antifibronectin antibody treatment resulted in explant dilation and reduced MGP expression, providing evidence for an interaction with MGP and fibronectin. Conversely, intraluminal microinjection of anti-MGP antibodies had no effect either on explant growth or MGP expression, supporting the hypothesis that MGP exerts its effects through the mesenchyme. Taken together, the results suggest that MGP plays a role in lung growth and development, likely via temporally and spatially specific interactions with other branching morphogenesis-related proteins to influence growth processes.

MAMMALIAN LUNG BRANCHING MORPHOGENESIS is a complex growth process involving temporal and spatial expression of numerous gene products from diverse gene families. From the initial formation of the rudimentary lung bud, a continual complex of its function and lend support to its potential regulatory role in cellular processes of growth and differentiation.

Although present in most vertebrate tissues examined, the physiological function of MGP is not completely understood. Recently, it has been demonstrated that mice lacking MGP develop extensive vascular and cartilaginous calcification to such an extreme that null mutant animals die within 2 mo postpartum due to rupture of the thoracic or abdominal aorta (27). The extensive arterial calcification, osteopenia, and growth plate inhibition present in these MGP animals suggest that one of MGP’s primary roles may be as an inhibitor of tissue calcification. Indeed, virally driven MGP overexpression in cultured embryonic chick chondrocytes results in blocked mineralization of these cells (44). Similarly, in vivo overexpression of MGP in developing chick limb bud causes inhibition of cartilage mineralization, delayed chondrocyte maturation, and inhibition of endochondral bone ossification (44).

Given the increasing evidence that MGP is an important regulator of growth and differentiation and that MGP is highly expressed in the lung, we sought to further define the role of MGP in the process of lung development. This study provides...
a detailed description of lung MGP expression over a time course of pre- and postnatal periods of lung growth and development. In addition, fetal lung explants were used as a model of lung branching morphogenesis to specifically address MGP’s involvement in this process.

**METHODS**

Isolation of fetal lung explants. Timed pregnant adult Wistar rats were purchased (Charles River Laboratories) and housed according to institutional policies. Pregnant rats were euthanized on 13–14 d of gestation, and embryonic (E) 13–14 d fetal rat pups were aseptically dissected from the uterine decidua into ice-cold, sterile PBS. With the aid of a dissecting stereomicroscope, lung bud rudiments were dissected free from the embryos and placed on cell culture inserts (0.4 µm size; Falcon) in six-well tissue culture plates (Falcon). Lung explants were cultured in BGJb medium (Fitzon-Jackson Modification; Life Technologies) supplemented with 5% carbon-stripped fetal calf serum. Typical culture conditions consisted of three to five explants per well. The medium was changed at 24-h intervals, and lung explants were photographed on a daily basis over the culture interval. At specific times in the culture interval, explants were removed from the inserts and flash-frozen in liquid nitrogen, with subsequent storage at −80°C until RNA isolation was performed. For experiments involving immunohistochemistry, explants were carefully removed from the inserts and fixed for 1 h at 4°C in 4% paraformaldehyde, followed by dehydration through an alcohol series and storage at −20°C until further use.

**Lung RNA isolation.** Total RNA was isolated from fetal lung explants based on the method of Chomczynski and Sacchi (12). Briefly, lung explants were homogenized in 0.75 ml of Tri-Reagent (Molecular Research), with manual disruption of the tissue by using conical-shaped pestles designed to fit 1.5-ml microcentrifuge tubes. Samples were homogenized on ice according to the Tri-Reagent protocol. RNA was quantified by spectrophotometry and stored at −80°C for subsequent use in RT-PCR; DNase digestion of total RNA samples was performed before spectrophotometric quantitation.

**RT-PCR analysis.** Total RNA (0.1–1 µg) was reverse-transcribed for 1 h at 42°C by using poly(dT) primers, 500 µM deoxynucleotides, 10 mM dithiothreitol, 1× RT buffer, and an RNase H– reverse transcriptase (Superscript H–; Invitrogen) in a total volume of 20 µl. After RT, samples were variably diluted for subsequent PCR amplification; dilutions were empirically based on relative abundance of the respective gene product in fetal lung. MGP mRNA expression was quantified by competitive PCR, as described previously (17). Both endogenous MGP and an MGP cDNA mimic amplified with equal efficiency by using the same set of primers that included forward primer 5′-CGGAGAAATGCAACACCTT-3′ and reverse primer 5′-GCAAGCAACTACCTTG-3′. PCR reactions included 200 fg of MGP mimic, 5 ng of reverse-transcribed total RNA containing 200 µM each of deoxynucleotide, 5 µl 1× polymerase buffer, 250 µM each of forward and reverse primers, and one unit of Pfu polymerase (Clontech Laboratories) in a total volume of 50 µl. Products were amplified in a programmable thermocycler under the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C (20 s), annealing at 50°C (30 s), extension at 72°C (1 min), with a final extension at 72°C for 8 min. For analysis, amplified products were run on 1.5% agarose gels with ethidium bromide staining. Gels were scanned on a Fluorimagager imaging system (Molecular Dynamics) and quantified with the ImageQuant software package. An accurate measure of experimentally induced changes in MGP mRNA expression for any given series of samples could be determined by calculating the ratio of MGP to mimic.

Semiquantitative PCR was used to measure sonic hedgehog (Shh), patched (Ptc), bone morphogenetic protein (BMP)-4, and fibroblast growth factor (FGF)-10 mRNA expression in cultured explants, with the rat homologue to prokaryotic elongation factor Tu (EFTu) or GAPDH used to normalize expression of these genes. Gene-specific primers included the following: Shh forward primer 5′-GAAAAA-CACTGGAGAGGACC-3′ from mouse sequence base pairs 28–47; Shh reverse primer 5′-CCACGAATCTCTGTTTGC-3′ from base pairs 335–316 to give a 309-base pair product for Shh amplification; Ptc forward primer 5′-TCGGTATGGGACAGATTTCC-3′ from base pairs 750–731 to give a 486-base pair product for BMP-4 amplification; FGF-10 forward primer 5′-ACGTACAGGATGTTTGG-3′ from mouse sequence base pairs 136–155; Ptc reverse primer 5′-AGTG-CTGATCCAGGAGGTTG-3′ from base pairs 505–486 to give a 371-base pair product for FGF amplification; BMP-4 forward primer 5′-CTCTTCGAGCTTCTCAGC-3′ from mouse sequence base pairs 266–285; BMP-4 reverse primer 5′-CTCGGAGTGTCTCCAGATG-3′ from base pairs 750–731 to give a 486-base pair product for BMP-4 amplification; FGF-10 forward primer 5′-GGCAAACTACATTTGCTGCC-3′ from base pairs 556–537 to give a 554-base pair product for FGF-10 amplification; GAPDH forward primer 5′-ACACAGTCCATGCACACATC-3′; GAPDH reverse primer 5′-CAGAAGGGGAGGATTGAGGG-3′; EFTu forward primer 5′-ACTTGGTTGTTGAAACAGC-3′; and EFTu reverse primer 5′-GAACAGGATCAGTCCATCT-3′.

**Lung protein isolation and Western blot analysis.** Proteins (20–25 µg) were separated by SDS-PAGE on 15% SDS-polyacrylamide gels, followed by transfer to nitrocellulose membranes with the use of a Transblot apparatus (Bio-Rad Laboratories). Immunodetection of transferred proteins was performed by standard Western blot procedure. Briefly, nonspecific binding sites were blocked with 5% nonfat dry milk and 0.05% Tween-20 in PBS) for 1 h. Membranes were incubated in primary antibody (1:2,000 dilution in blotto) for 2 h at room temperature and then washed extensively. A subsequent incubation in a solution containing the biotinylated secondary antibody (Vector Laboratories) was performed for 1 h, followed by another round of extensive washing. Immunodetection was visualized by using enhanced chemiluminescence detection reagents (ECL; Amersham) and short exposure to X-ray film.

**Immunohistochemistry.** For experiments aimed at in situ localization of specific proteins, lungs were fixed and paraffin embedded for histological processing into 4-µm tissue sections. Sections were mounted on glass slides, deparaffinized, and rehydrated. Antigen retrieval was accomplished by microwave heating in 10 mM citric acid (pH 6.0). Sections were then incubated in blocking solution containing 10% goat serum in PBS Tween-20. Primary antibody incubation was performed overnight at 4°C, followed by a series of washes in Tris-buffered saline. Detection of primary antibody was performed by using a Vectastain avidin-biotin complex kit (Vector Laboratories). Vector VIP was the substrate chromogen with methyl green used as a counterstain. Parallel sections were stained with hematoxylin-eosin to visualize general morphology.

**Antibody treatment of fetal lung explants.** Two synthetic peptides corresponding to specific rat MGP protein sequences were used to generate anti-MGP antibodies. The first sequence was a 12-residue peptide located in the propeptide-like region of rat MGP and contains the sequence NH2-P T N R N A N T F I-COOH. This region binds the vitamin K-dependent γ-carboxylase required for conversion of glutamic acid residues to γ-carboxylated GLA residues. The antibodies generated with this peptide have been termed the “midpeptide antibody.” The second peptide sequence was a 12-residue peptide located at the extreme C-terminal portion of rat MGP and contains the sequence NH2-Y N R Y F R R G A K-COOH. The antibodies generated with this peptide were termed the “C-terminal antibody.” Affinity-purified antibodies isolated from rabbits challenged with these peptide immunogens were routinely used in explant cultures at a 1:100 to 1:200 dilution of 1 mg/ml antibody, for a final concentration of 5–10 ng/ml. Overnight incubation of anti-MGP antibodies with the respective peptideused for generating the antibodies before their use in explant culture or immunohistochemistry was performed to successfully block anti-MGP antibody effects and/or immunostaining.
The fibronectin antibody was a mouse monoclonal antibody (EP5; sc-8422, Santa Cruz Biotechnology; stock concentration 200 μg/ml) raised against a recombinant protein corresponding to fibronectin of human origin; dilution of this antibody for use in explant culture experiments was 1:100, resulting in a final concentration of 2 ng/µl. Microinjection of antibodies into the lung lumen. A microinjector system (Narishige, MO-22) was mounted to a stereoscope for injection of nanoliter amounts of undiluted MGP antibodies or PBS (control) into the lumen of lung explants positioned on cell culture inserts. Micropipettes were drawn by using a Narishige PC-10 device, and injection pressures were generated with a Femtojet (Eppendorf) system. Injection volumes were precalibrated, and both control and experimental solutions were initially colorized with Coomassie blue to visualize fluid distribution. Subsequent experiments were performed without the addition of Coomassie blue. Beginning on day 2 of culture, ~50–100 nl of antibody were injected directly into the trachea and distal luminal spaces, resulting in an even distribution of solution throughout the lumen of the whole lung or individual lobes. It was estimated that the local exposure of antibody was as much as four times that used for external, mesenchymal application. After microinjections, cell culture inserts were replaced, and explants were incubated at 37°C for the remainder of the culture interval.

Quantitation of explant morphology. To assess morphological changes in fetal lung explants, terminal lung buds were counted with the aid of a microscope and statistically compared across culture conditions. Terminal lung buds were defined as acinar structures separated by distinct septae located at the periphery of the growing explants.

Data analysis. Quantitation of morphogenesis was as described above. For some experiments, such as immunostaining or in situ analysis of specific tissue sections or specimens, results were assessed by using a qualitative approach. PCR experiments were repeated at least three times for statistical comparisons. Significant differences in gene expression or morphological measurements resulting from various treatment protocols were determined by using Student’s t-test. Results were considered significant at values of P < 0.05.

RESULTS

MGP expression in developing lung. MGP mRNA levels were determined in rat lung from gestational day 13 (E13) through the perinatal period and in the adult by using a competitive PCR assay, as previously described (17) (Fig. 1). MGP mRNA was found in early lung primordia at E15 and continued to rise during gestation, with a small peak occurring just before birth. Postnatally, levels dropped slightly and were steadily maintained until a sharp increase was observed during the alveolarization interval, beginning in the second postnatal week. MGP mRNA was most highly expressed in adult lung. These results demonstrate that MGP message expression is developmentally regulated, with higher levels of expression occurring during periods of lung development traditionally characterized by low-cell proliferation and increased differentiation.

In addition to overall changes in MGP expression during lung development, site-specific localization of protein was determined. Fetal lungs were prepared as described for immunohistochemical localization of MGP protein by using the C-terminal anti-MGP antibody (Fig. 2). MGP immunostaining was associated predominantly with the mesenchymal cells surrounding epithelial tips of distal buds (Fig. 2, A and C). This staining pattern was compared with that of pro-surfactant protein (SP)-C in adjacent sections, where pro-SP-C was found in the epithelium of early embryonic lungs (Fig. 2, B and D).

Later in gestation, MGP protein became more widely distributed in the lung. By E20, additional staining was observed in the bronchial epithelium, along with continued staining in the mesenchyme (Fig. 2, E and F). As the mesenchyme became more differentiated in early postnatal lung, MGP was localized to airway epithelial cells and vascular walls, as well as light deposition in the surrounding parenchyma (Fig. 2, H and M). In the adult, light distribution throughout the parenchyma and airway cells was still evident, with more intense staining lining the airways (Fig. 2, O and P). This pattern of distribution in adult lung is consistent with other reports (26, 27). No staining was observed in the absence of primary antibody or if anti-MGP antibody was preincubated with a blocking peptide (Fig. 2, G and N).

To further investigate MGP regulation in the developing lung, particularly during early branching morphogenesis, an in vitro explant model was used. Explants remain in the pseudoglandular phase of lung development during culture. This model provides an ideal system in which to examine specific regulators of lung growth and differentiation by using reagent-addition protocols. Increased MGP mRNA expression over the culture interval mimics the early embryonic in vivo time course (17). A stereotypic morphological pattern of explant growth was observed in control cultures, characterized by a high degree of distal epithelial branching (Fig. 3A). Interfering with MGP function by addition of anti-MGP antibodies to the culture medium revealed a completely different explant morphology after 4 days in culture. Compared with control cultures, anti-MGP antibody treatment induced a grossly dilated morphology characterized by elongated epithelial tubules (Fig. 3A). By the end of the culture interval, lung branching, as assessed by terminal lung bud count, was significantly reduced
in anti-MGP antibody-treated explants by using either the midpeptide or C-terminal peptide-derived antibodies (Fig. 3C). Both of these antibodies were specific for MGP, as assessed by Western blotting of purified MGP isolated from rat femurs (Fig. 3B). In contrast to the external application of anti-MGP antibody, explant growth was not altered when antibodies were microinjected directly into the lumen of specific lobes of intact explants (Fig. 4), suggesting that endogenous MGP exerts its effects primarily through the mesenchyme.

A series of experiments demonstrated the dynamic nature of the anti-MGP antibody-induced dilation of fetal lung explants. The presence of antibody throughout the culture interval resulted in marked dilation of distal airways compared with untreated lungs (Fig. 5, A and B). When C-terminal antibody
was externally applied after 1 or 2 days of culture, distal bud
dilation was evident, whereas morphology of medial structures
was comparable to controls (Fig. 5, C and D, respectively).
Conversely, when antibody was withdrawn after 1 or 2 days in
culture, normal terminal branching and bud formation re-
sumed, with the more medial structures remaining dilated (Fig.
5, E and F, respectively). Blocking peptide was also added to
explant cultures following anti-MGP antibody withdrawal to
block any residual antibody that may have remained in the
tissue (Fig. 5, G and H). The resulting morphology was similar
to antibody withdrawal in the absence of blocking peptide, as
seen in Fig. 5, E and F. Peptide alone had no effect on gross
explant morphology and growth (Fig. 5I). Preincubation of
anti-MGP antibody with its immunogenic peptide completely
blocked the antibody-induced dilation (Fig. 5J). Thus addition
or withdrawal of anti-MGP antibody at different times during
the culture interval altered only the most distal morphology.
These results suggest that MGP expression and function are
both site specific and time dependent, requiring cyclic deposi-
tion with each new generation of epithelial branching.

Based on our results demonstrating altered branching with
MGP antibody treatment (Figs. 3 and 5), we examined the
expression of some genes known to play a role in lung
branching morphogenesis in antibody-treated explants (Table
1). Anti-MGP antibody treatment caused a significant down-
regulation of MGP mRNA expression (Table 1 and Fig. 6).
Antibody treatment also significantly downregulated the ex-
pression of Shh and Ptc mRNA, whereas the expression of
Bmp-4 and Fgf-10 was not significantly different from that of
controls (Table 1). Interestingly, antifibronectin antibody treat-
ment of fetal explants caused a similar dilated morphology as
that seen with anti-MGP (Fig. 6), reflected by a 21% decrease
in terminal bud counts (71.75 ± 4.66 in control vs. 56.75 ±
1.38 in fibronectin antibody-treated explants; Fig. 6B). Com-
bined treatment with both anti-MGP and antifibronectin anti-
odies resulted in an even greater reduction in terminal bud
counts. Additionally, antifibronectin antibody also caused a
similar decrease in MGP mRNA expression as that seen with
anti-MGP antibody (Fig. 6C); however, the combination of
antifibronectin and anti-MGP antibodies did not further reduce
MGP mRNA expression. These results suggest that these two
proteins coordinate preferentially to contribute to regulation of
branching morphogenesis in the developing lung.

DISCUSSION

The present results identify MGP as a potential regulatory
factor in the process of lung growth and development. The time
course of expression during the pre- and postnatal periods

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Fig. 3. Terminal lung bud counts are reduced with MGP
antibody treatment. Two different domain-specific antibodies to
MGP were added independently to explant culture medium, and
explants were harvested after 4 days in culture. A: the photomi-
crographs shown are representative of the explant morphology
normally observed under control conditions or in the presence
of peptide-specific anti-MGP antibodies. B: Western blot of
purified MGP isolated from rat femur shows specificity of both
the C-terminal antibody (C-term Ab) and mid-peptide antibody
(Mid-Pep Ab) for MGP. MW, molecular weight. C: 16 explants
each were photographed and used for analysis of terminal bud
counts. Values are means ± SE. Significant reduction in ter-

Fig. 4. Luminal microinjection of MGP antibody does not alter explant growth.
Intact explants were microinjected after 24 h in culture with 50–100 nl of
either anti-MGP antibody (1 mg/ml) or PBS (control) into each luminal space
(arrows indicate which spaces were microinjected). Limiting antibody expo-
sure to the epithelium of intact explants did not significantly alter gross lung
morphology after 3 days in culture. The solutions that were microinjected into
the explants shown above contained 0.1% Coomassie blue to better visualize
fluid distribution. Subsequent experiments were performed in the absence of
the dye with identical results.
Fig. 5. Effects of MGP antibody addition and withdrawal on explant morphology. C-term Ab (final concentration of 10 ng/l) added to the medium surrounding explants for the entire culture interval (B) resulted in dramatically dilated airways and tubules compared with the highly branched control cultures that terminated in numerous distal buds (A). When antibody was withdrawn after 1 day (D1; E) or 2 days (D2; F) in culture, proper branching and bud formation resumed; the extent of recovery was directly dependent on how long the antibody was present. F and H: for example, explants treated with antibody for 2 days had many dilated proximal structures and a rim of normal distal buds. C and D: applying antibody at different times during the culture interval altered only the most distal morphology. G and H: addition of blocking peptide following antibody withdrawal did not further affect morphology, suggesting that free antibody did not remain in the cultures. Indeed, blocking peptide alone had no effect on explant morphology (I), with resulting growth resembling control conditions (A). J: finally, preincubation of anti-MGP antibodies with blocking peptide before the addition to explant culture completely blocked the antibody-induced inhibition of branching. D3, day 0; D0, day 0.

Table 1. Gene expression analysis following anti-MGP antibody treatment of fetal explants

<table>
<thead>
<tr>
<th>Gene</th>
<th>MGP mRNA</th>
<th>Bmp-4 mRNA</th>
<th>Fgf-10 mRNA</th>
<th>Shh mRNA</th>
<th>Ptc mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±5.2</td>
<td>100±9.3</td>
<td>100±6.0</td>
<td>100±10.9</td>
<td>100±7.3</td>
</tr>
<tr>
<td>Anti-MGP</td>
<td>46.4±5.5*</td>
<td>90.6±3.7</td>
<td>92.5±3.7</td>
<td>63.7±7.2†</td>
<td>80.9±8.3‡</td>
</tr>
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Values are means ± SE. Explants were cultured for 4 days in the absence (Control) or presence of anti-matrix γ-carboxyglutamic acid protein (MGP) antibody (Anti-MGP). Control values were averaged across 8 experiments for each given gene product and normalized to 100%. Competitive PCR analysis of MGP mRNA expression following 4 days of culture resulted in a downregulation of MGP expression. Semiquantitative PCR was used to determine message expression of 4 genes involved in branching morphogenesis, bone morphogenetic protein-4 (Bmp-4), fibroblast growth factor-10 (Fgf-10), sonic hedgehog (Shh), and patched (Ptc), and normalized against expression of the control gene elongation factor Tu. *P < 0.0001; †P < 0.01; ‡P < 0.05.

suggests that MGP is developmentally regulated, with peak expression occurring during periods of relatively low cellular proliferation and increased cellular differentiation. The prenatal rise in mRNA expression occurs at a time when the proximal airway epithelium loses the ability to respond to distal mesenchymal signals, suggestive of a terminally differentiated phenotype (36). Similarly, the increase in MGP expression occurring in the second postnatal week coincides with alveolar differentiation and lung maturation. The time course of expression that we observed is similar to that reported in a limited study in the mouse (26), in which MGP mRNA was expressed peripherally at the epithelial-mesenchymal border early in gestation and became more restricted to proximal airways later in development. The high levels observed in the adult are consistent with a proposed role of the lung in secretion of MGP into the vascular system, where MGP is secreted into the vascular system, where MGP is thought to play a role in the maintenance of vascular integrity (32). Because of this, it is possible that high-circulating blood levels of MGP could cross the placenta and rescue an MGP-deficient fetus (27) during development in a manner similar to that observed in transforming growth factor (TGF)-β1−/− mice (23). The lethal phenotype associated with the absence of MGP would then be acquired postnata tally, resulting ultimately in arterial calcification and death (27).

Antibody-directed interference of MGP function with peptide-derived anti-MGP antibody addition to cultured fetal lung explants resulted in grossly dilated epithelial tubules characterized by significantly reduced branching. We did not observe any signs of antibody-induced toxicity on explant growth, as exemplified by the antibody addition and withdrawal experiments (see Fig. 5). Although dilated, antibody-treated explants grew to a similar size as controls, and preincubation of the antibody with blocking peptide abolished the dilatory effects of anti-MGP. Explant growth with peptide treatment alone resembled controls. Additionally, microinjection of undiluted MGP antibody into the lumen of intact explants (see Fig. 4) did not alter growth over the culture interval.

Expression of specific genes known to regulate the process was altered with MGP antibody treatment, suggesting that mesenchymal MGP may interact with or regulate additional proteins involved in lung growth and development. Evidence for this interaction has been demonstrated in other tissues. Attempts to purify MGP from bone extracts by using CaCl2/urea-based protein purification protocols demonstrated that...
MGP is tightly associated with fibronectin and a member of the BMP protein family, subsequently identified as BMP-2 (39). Indeed, more recent reports have shown that MGP binds strongly with BMP-2 in ligand-binding studies (41) and that BMP-2 co-precipitates with MGP in transfected human embryonic kidney 293 cells (45), suggesting that MGP may regulate or contribute to the functional role of BMP-2 through their physical interaction. BMPs are members of the TGF-β superfamily and function in many diverse cellular processes during development, including cellular differentiation and tissue morphogenesis. Several BMP antagonists, some of which act as BMP binding proteins, limit the activity or availability of BMPs and thus regulate BMP function. In this respect, one of the roles of MGP may be to limit the availability of these proteins by binding and sequestering the protein in specific locations during growth and differentiation.

Although BMP-2 expression has not been reported in lung, a closely related family member, BMP-4, plays an important role in lung branching morphogenesis, where its expression is limited to the epithelial tips of distal lung buds and surrounding mesenchyme during lung development. It is hypothesized that lung BMP-4 functions to inhibit local epithelial proliferation at distal bud tips, forming a suppression zone at the branch point and forcing lateral growth (4). The regulation of BMP-4 expression or function in early lung development is not completely understood. Exogenous addition of BMP-4 to E11.5 mouse lung explant cultures actually enhanced lung branching morphogenesis (5, 37), an effect that was blocked by adenoviral-induced overexpression of the BMP-4 antagonist gremlin (37). Lung branching was not affected in explants, however, when BMP-4 was microinjected into the lung lumen, thereby restricting exposure to the luminal epithelium (5). Targeted epithelial misexpression of Bmpr-4 using the SP-C enhancer/promoter produced normal mouse lungs at E11.5 and E12.5, but, by E15.5, transgenic lungs were significantly smaller and less branched than normal (4). These studies underscore the importance of tightly regulated spatial and temporal expression of BMP-4 for normal lung branching to occur. A recent hypothesis suggests that BMP-4 signaling in regions of high expression (such as at the bud tips) could induce inhibitors of BMP-4 that, in turn, modulate or inhibit local BMP activity, allowing bud induction to occur (13). Thus it is possible that MGP may act as a binding protein to BMP-4, limiting its inhibitory capacity. The reduced branching observed in the presence of anti-MGP antibodies is consistent with this hypothesis.

Interestingly, MGP expression in a mouse chondrocyte cell line (MC615) is reduced in the presence of either BMP-2 or BMP-4 (40). In osteogenic sarcoma cells, however, MGP expression is unaltered by BMP-4, except in the presence of retinoic acid (RA), where the cytokine further enhanced the RA-induced expression of MGP (50). These studies suggest a cell type-specific regulatory interaction with MGP and BMP-4 that likely is dependent on the presence of additional molecular regulators. In our model system, anti-MGP antibody treatment did not significantly alter overall BMP-4 expression, but site-specific or cell type-specific changes in expression remain to be determined. It is of interest to note that RA has been reported to repress endogenous MGP expression in a cell-type-specific manner through RA receptor binding to a response element located on the MGP promoter (22). Other studies, however, demonstrated elevated MGP expression in the presence of RA (7). It is known that RA, an important morphogen in the control of lung branching morphogenesis (10), exerts its effects on gene expression in a concentration-dependent manner. It is also likely that RA-induced changes in gene expression alter both the spatial and temporal balance of specific regulatory proteins, which, in turn, affect the local growth characteristics of the developing lung. Indeed, BMP-4 expression patterns become much more diffuse, with severely attenuated expression at the epithelial tips in explants cultured in the presence of RA (28). We speculate that MGP and BMP-4 may be integral components in a regulatory complex of proteins in the cleft region and that paracrine factors such as RA may contribute to the regulation of expression at specific sites.

The effects of anti-MGP antibody treatment on expression of Shh and Ptc are likely the result of the antibody-induced

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Fig. 6. Anti-fibronectin antibodies reduce fetal lung branching and MGP mRNA expression. Explants treated for 4 days with antibodies to fibronectin (FN; 1:100 dilution of 200 g/ml stock), MGP (1:100 dilution of 1 mg/ml stock), or in combination were analyzed for changes in morphology and for alterations in MGP mRNA expression. A similar dilated morphology was observed with either antibody treatment (A), resulting in a significant reduction in terminal bud counts compared with controls (B). Combined antibody treatment resulted in a further reduction in terminal branching compared with treatment with only 1 of the antibodies. In addition to altered branching, antibody treatments also reduced MGP mRNA expression, as determined by competitive PCR (C). Terminal bud counts were calculated from 4 explants each and are expressed as means ± SE from 3 separate experiments. #P < 0.03; *P < 0.001. RNA expression data are expressed as BMP-2 (39).
decrease in branching, as these proteins are important mediators of this process. Several members of the Shh signaling pathway are required for normal pattern formation and morphogenesis in a variety of developing tissues, including the lung. Shh expression begins very early in lung development, where its highest levels are found in the distal epithelium of growing lung bud tips (4, 38). Its receptor, Ptc, is expressed at high levels in the distal mesenchyme, adjacent to the Shh-expressing epithelium (2). Several lines of evidence suggest that Shh plays a role in controlling site-specific FGF-10 expression in the developing lung. Overexpression of Shh in transgenic mice causes downregulation of Fgf-10 and Ptc (3), whereas Shh null mutant mice are characterized by abnormal, diffuse mesenchymal expression of Fgf-10 and severely altered lung development (31). Inhibition of Shh and Ptc mRNA expression in the presence of anti-MGP antibody would prevent the normal local downregulation of Fgf-10 by Shh, ultimately resulting in altered cleft formation and attenuated lung branching. Our data suggest that MGP may normally contribute to the induction of Shh, which, in turn, dampens the growth-stimulatory function of FGF-10. This relationship may be reciprocal in nature, as evidenced by the consistent downregulation of MGP mRNA expression observed in the presence of anti-MGP. We speculate that a critical balance of key gene products is required for normal branch formation to occur and that, if an imbalance arises in one component, expression of other regulatory proteins is affected.

TGF-β is another important signaling molecule in lung development that also has a regulatory influence on MGP expression. Zhou et al. (51) demonstrated arrested lung sacculation and delayed epithelial cell differentiation in transgenic mice with a constitutively activated TGF-β1 gene. At day 16 of development, transgenic mouse lungs contained fewer acinar buds than their control nontransgenic littermates. Perturbation of the TGF-β type II receptor or TGF-β1 antisense oligonucleotides further demonstrated the negative influence of TGF-β1 on embryonic murine lung morphogenesis (46). It is likely that TGF-β1 is an important signal for the local induction of MGP, because this growth factor is known to regulate protein-protein interactions are presently under investigation to further define the role of MGP in fetal lung growth and differentiation.

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GRANTS

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