Matrix GLA Protein Modulates Differentiation Induced by Bone Morphogenetic Protein-2 in C3H10T1/2 Cells

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Matrix GLA protein (MGP) is ubiquitously expressed with high accumulation in bone and cartilage, where it was found to associate with bone morphogenetic proteins (BMP) during protein purification. To test whether MGP affects BMP-induced differentiation, three sets of experiments were performed. First, pluripotent C3H10T1/2 cells transfected with human MGP (hMGP) or antisense to hMGP (AS-hMGP) were treated with BMP-2. In cells overexpressing hMGP, osteogenic and chondrogenic differentiation was inhibited indicating decreased BMP-2 activity. Conversely, in cells overexpressing AS-hMGP, BMP-2 activity was enhanced. Second, cells were prepared from homozygous and heterozygous MGP-deficient mice. When treated with BMP-2, these cells underwent chondrogenic and osteogenic differentiation, respectively, whereas controls did not. Third, FLAG-tagged hMGP with the same biological effect as native hMGP inhibited BMP-induced differentiation, when exogenously added to culture media. Together, these results suggest that MGP modulates BMP activity. To test whether hMGP fragments would retain the effect of full-length hMGP, three subdomains were overexpressed in C3H10T1/2 cells. In cells expressing the mid-region, alone (amino acids (aa) 35–54) or in combination with the N terminus (aa 1–54) but not the C terminus (aa 35–84), osteogenic differentiation was enhanced and occurred even without added BMP-2. Thus, two subdomains had the opposite effect of full-length hMGP, possibly due to different expression levels or domain characteristics.

Matrix GLA protein (MGP) is a small ubiquitous matrix protein containing carboxyglutamic acid (GLA) (calculated mass of mature protein 10.4 kDa), initially isolated from bone and characterized by Price et al. (1). Results from other investigators suggest that MGP affects differentiation in developing cartilage and bone. Luo et al. (2) observed that MGP is expressed in early and late stages of chondrogenic differentiation but not in the intermediate stage. Yagami et al. (3) found that MGP had an effect on mineralization in chondrocytes that was dependent on cell stage; it affected mineralization in hypertrophic chondrocytes but not in proliferative chondrocytes. They also found that overexpression of MGP in developing limb buds delayed chondrocyte maturation and blocked endochondral ossification. In MGP null (MGP−/−) mice (4), the epiphyseal growth plates of bones showed inappropriate calcification in the layer of proliferating chondrocytes that failed to differentiate into hypertrophic chondrocytes. In addition, the mice unexpectedly developed severe vascular calcification, resulting from a replacement of the aortic medial layer by chondrocyte-like cells, producing a typical cartilage matrix that progressively calcified.

When Urist and colleagues (5) first discovered bone morphogenetic protein (BMP), they observed a tight association with MGP in vitro during protein purification requiring strong denaturants to break. Although complex formation was not shown in vivo or in situ, it was suggested that MGP may sequester BMP in bone tissue and as such regulate its activity (5). Since BMP mediates its biological response through a cell surface receptor, MGP could potentially act extracellularly to form an inactive complex with BMP. It has not been shown whether MGP affects BMP function by forming such a biologically inactive complex whose formation blocks BMP action on cells. In this paper, we test the hypothesis that the mechanism for the effect of MGP on cell differentiation is through modulation of BMP activity.

A useful model for assaying BMP-2 functional activity in mesenchymal differentiation is the "undifferentiated" mesenchymal cell line, C3H10T1/2. These multipotent mouse embryonic cells, also known as vascular precursor cells (6), differentiate along adipogenic, osteogenic, and chondrogenic lineages when stimulated with BMP-2 (7, 8). Once stimulated, colonies of lineage form within the same culture and time frame.

To test the hypothesis that MGP modulates BMP-2-induced mesenchymal differentiation, we overexpressed human MGP (hMGP) and antisense to hMGP (AS-hMGP), and we assayed differentiation along each lineage in response to recombinant human BMP-2 (rhBMP-2). We also compared responses to rhBMP-2 in cells isolated from aortas of homozygous and heterozygous MGP-deficient mice to those isolated from aortas of wild-type mice. FLAG-tagged hMGP with the same biological effect as native hMGP was used to localize the MGP effect to the extracellular space. In addition, the effect of overexpression of selected hMGP subdomains was compared with that of full-length hMGP.
These results support the concept that MGP modulates BMP activity in mesenchymal differentiation and that the physical interaction observed during protein purification from bone may have physiological significance.

MATERIALS AND METHODS

Vector Construction—Full-length human MGP (hMGP) cDNA subcloned into the EcoRI site of the pBSSK(-) plasmid (Stratagene, La Jolla, CA) was obtained from the American Tissue Culture Collection (Manassas, VA) (9). The hMGP sequence was excised and subcloned into pcDNA3.1(-) Zeo (Invitrogen, Carlsbad, CA) in sense or antisense orientation using the HindIII and XbaI sites for sense orientation, and the NotI and ApaI sites for antisense orientation. A schematic overview of the vectors used in this study is provided in Fig. 1.

To construct the N-MGP vector (containing the N-terminal and the central region of hMGP, aa 1–54, Fig. 1), the correct fragment was generated by PCR of hMGP cDNA using primers ERIFL-5 and MGPX-3 (Table I) and then subcloned into the EcoRI and XbaI sites in the pcDNA3.1(-) plasmid after digestion of the restriction enzyme sites in the primers. To construct the mid-MGP vector (containing the central region of hMGP alone, aa 35–54, Fig. 1), the correct fragment was generated by PCR of hMGP cDNA using primers MGPH-5 and XBAFL-3 (Table I), and the control vector (containing only the leader sequence, Fig. 1), the hMGP-leader fragment was excised from the hMGP cDNA using EcoRI and a HindIII site located just downstream of the leader sequence. Fragments containing the C-MGP and the mid-MGP regions were generated by PCR of hMGP cDNA using primers MGPB-5' and XBAFL-3', and MGPB-5' and MPBX-3' (Table I), respectively. The fragments were subsequently ligated into the pcDNA3.1(-) vector downstream of and in frame with the leader sequence, after digestion of the restriction enzyme sites in the PCR primers. For the control vector, the EcoRI-HindIII fragment was ligated into pcDNA3.1(-) digested with EcoRI and EcoRV. This vector contains the leader sequence alone followed by a stop codon 1 base downstream of the HindIII site.

To construct the FLAG-tagged hMGP vector, a fragment containing full-length hMGP in which the stop codon had been replaced by an XhoI site, was generated by PCR of hMGP cDNA using primers ERIFL-5' and mgpFLAG-3' (Table I). The FLAG tag was placed in the C terminus of the resulting protein by subcloning the fragment into the EcoRI and XhoI sites in the pCMV-Tag4 plasmid (Stratagene), after digestion of the restriction enzyme sites in the primers.

To construct the human BMP-2 vector, the correct fragment containing the complete coding sequence was generated by PCR of human BMP-2 cDNA using primers bmp2ERT-5' and bmp2XBA-3' (Table I), and then subcloned into the EcoRI and XhoI sites in the pcDNA3.1(-) plasmid, after digestion of the restriction enzyme sites in the primers. All constructs were confirmed by restriction enzyme analysis and DNA sequencing of the complete coding sequences.

Cell Culture—C3H10T1/2 mouse cells were cultured in α-minimal Eagle's medium with Earle's salt (Irvine Scientific, Santa Ana, CA) and HEK293 cells in Dulbecco's modified Eagle's medium (Irvine Scientific), both supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), penicillin (100 units/ml), streptomycin (100 units/ml), sodium pyruvate (1 mmol/liter), and L-glutamine (2 mM). To prepare aortic cells from MGP-deficient and control mice, whole aortas were obtained and cleaned of visible surrounding fat and other tissue. Aortic cells from homozygous MGP−/− and normal mice were prepared by enzymatic digestion with cold trypsin as described previously (10). Because of cartilaginous metaplasia, homozygous MGP−/− aortas required use of enzymatic digestion adapted to cartilage as described previously (10) for release of cells. After digestion, the cells were plated in gelatin-coated 12-well plates and grown in F-12 medium (Irvine Scientific) supplemented with 20% heat-inactivated FBS, penicillin (100 units/ml), streptomycin (100 units/ml), sodium pyruvate (1 mmol/liter), and L-glutamine (2 mM). The MGP−/− cells did not tolerate subsequent trypsinization and replating and thus were treated with rhBMP-2 as they reached ~75% confluency. Cells from normal mice and MGP+/− mice were trypsinized, plated, and treated with rhBMP-2 as they reached ~75% confluency. Treatment with rhBMP-2 was continued for a total of 14–16 days. The cells were then taken to histochemical staining, RT-PCR, or immunoblotting.

Transfections were performed using Superfect™ (Qiagen, Chatsworth, CA), after optimization of the ratio of DNA to transfection agent as per manufacturer's instructions. Stable, mixed mass transfectants of C3H10T1/2 cells were selected and maintained by adding Zeocin™ (Invitrogen) at a concentration of 500 ng/ml to all culture media.

Recombinant human BMP-2 was graciously supplied by Genetics Institute or generated in our laboratory from HEK293 cells transiently transfected with the human BMP-2 vector. The concentration of rhBMP-2 in undiluted conditioned media was estimated by comparison with purified rhBMP-2 from Genetics Institute using Western blotting.

Differentiation Assay, C3H10T1/2 Cells—Two thousand C3H10T1/2 cells were plated in 60-mm Petri dishes, or 150 cells were plated per well in 12-well plates. BMP-2 was added to the cultures the day after plating at a concentration of 500 ng/ml. Because of the sparse plating, the initial medium with rhBMP-2 was left on the cells for 7 days;
Matrix GLA Protein Modulates BMP-2

Table I
Primer sequences for vector construction and RT-PCR

| Target gene      | Primer sequence                          | Position (Ref.) | Annealing Temperature | Cycle No.* |
|------------------|------------------------------------------|-----------------|-----------------------|------------|
| PPAR-γ2          | 5'-AGCTTATTTGCGTGAACCTGTCTG-3'           | 34-54 (32)       | 58                     | 38         |
|                  | 5'-ATAGGCTGGATGATGGTGTTTGCCT-3'          | 384-364         |                        |            |
| Osteocalcin      | 5'-CCTCGTTCTCTGCTACCTACAG-3'             | 91-112 (14)     | 62                     | 32         |
|                  | 5'-GGAATCTTTCTAATTAGACGTCACCATAC-3'      | 450-429         |                        |            |
| Collagen IX      | 5'-TTTTGAAAACCTCAGTGGATGGTTAGTCTA-3'     | 591-663 (33)    | 58                     | 38         |
| GAPDH            | 5'-ACTCCGACCCTGCTCAGCAGAG-3'             | 1011-990        |                        |            |
| Mouse MGP        | 5'-CTTCCTGTTGATCCTGATGAGCA-3'            | 275-297 (38)    | 58                     | 22         |
| Human MGP        | 5'-CCTAGCGCTAGTCAATTTTCTGCTG-3'          | 82-103 (30)     | 55                     | 32         |

* Cycle numbers refer to the number of cycles used for RT-PCR for the respective primer pair.

RESULTS

Transfection of Sense and Antisense Human MGP in C3H10T1/2 Mesenchymal Cells—To determine the effect of MGP on BMP-2-directed differentiation, we used low density cultures of the multipotent mouse mesenchymal cell line, C3H10T1/2 cells, which commit to various mesenchymal lineages when stimulated with exogenous BMP-2 (7, 8), even after brief, transient exposures (7). Without stimulation, there are low levels of spontaneous differentiation at base line (7). We used these cells as a bioassay to study effects of MGP on BMP-2 response by varying MGP expression.

MGP expression is highly dependent on cell density in cultured rat kidney (NRK) cells (13); the expression at low density substrate dissolved in 20 ml of Sigma 221 Alkaline Buffer Solution diluted 1:2 with distilled water per well and incubating for 60 min at 37°C. Absorbance at 405 nm was read using a microplate reader (Molecular Devices, Sunnyvale, CA), and activity was expressed as units per mg of cellular protein.

Histochemical Staining—Oil Red O, von Kossa, Alizarin Red, and Alcian Blue histochemical stains were performed using standard methods. Alkaline phosphatase stain was performed as described previously (11).

Immunoblotting—For immunoblotting, 30 μg per sample of whole cell extract protein was electrophoresed through 3–8% NuPAGE™ Tris acetate gels (NOVEX, San Diego, CA) or 4–12% NuPAGE™ Bis-Tris (MOPS) gels for collagen IX and peroxisome proliferator-activated receptor γ (PPAR-γ), respectively. For detection of FLAG-tagged hMGP and BMP-2 in conditioned media, 30 μl of media were electrophoresed through 10% NuPAGE™ Bis-Tris (MES) gels. Proteins were transferred to nitrocellulose filter using NuPAGE™ Transfer buffer (NOVEX). The blots were incubated with specific antibodies to either collagen IX (monoclonal antibody MAB3304 (12), Chemicon International, Temecula, CA) at a concentration of 2.5 μg/ml, PPAR-γ protein (polyclonal antibody H-100, Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1 μg/ml, the FLAG tag (polyclonal antibody F7425, Sigma) at a concentration of 1 μg/ml, or BMP-2 (polyclonal antibody H-51, Santa Cruz Biotechnology) at a concentration of 1 μg/ml. Specific antibody binding was visualized with the appropriate horseradish peroxidase-conjugated anti-IgG secondary antibody (Santa Cruz Biotechnology).

Immunoradiometric Assay—Mouse osteocalcin was quantitated in undiluted conditioned culture medium after 4 days of incubation according to manufacturer’s instructions using an immunoradiometric assay (Immunoassays, San Clemente, CA), and the results were expressed as ng/ml. There was no cross-reactivity with bovine osteocalcin derived from FBS.

RESULTS
was barely detectable but increased significantly with culture density. We observed the same phenomenon in C3H10T1/2 cells, with barely detectable MGP expression 1 day after low density plating, increasing at least 12–13-fold after 3 weeks in culture.

To test the hypothesis that MGP affects cell differentiation through altered responses to BMP-2, we modulated the MGP expression at low cell density (i.e., minimal expression of endogenous MGP) conditions, using stable cell lines overexpressing human MGP (hMGP) or antisense to MGP (AS-hMGP). We used the human MGP sequence that can be distinguished from mouse MGP using RT-PCR.

One day after low density plating, cells transfected with hMGP had an estimated 3-fold increase in MGP signal (including both sense mouse and human MGP, Fig. 2). Also at low density, cells transfected with AS-hMGP had an estimated 3-fold increase in MGP signal (including both sense mouse and antisense human MGP, Fig. 2) compared with nontransfected control cells. Addition of BMP-2 in all experiments was performed at this low density state.

Effect of MGP Overexpression on BMP-induced Differentiation—To assess whether altered levels of MGP expression modulate BMP-2-induced differentiation, we established a differentiation assay in C3H10T1/2 cells following the methods of Wang et al. (7). Treatment with rhBMP-2 or control vehicle was started at low density and continued for 21 days to allow distinct colonies of differentiated cells to form. We used rhBMP-2 at a concentration of 500 ng/ml based on preliminary experiments showing that this concentration induced differentiation in nontransfected C3H10T1/2 cells detectable by both specific cell markers and histochemical stains used to identify osteogenic, chondrogenic, and adipogenic cell differentiation. RT-PCRs for osteocalcin (14) in combination with von Kossa and Alizarin Red stains were used to identify osteogenic differentiation, RT-PCR for collagen IX (15), and Alcian Blue stain to identify chondrogenic differentiation, RT-PCR for PPAR-γ (16), and Oil Red O stain to identify adipogenic differentiation.

The response to BMP-2 differed with the transfected construct. Nontransfected cells at low density underwent differentiation into a mix of all three cell lineages with cells of each lineage grouped in colonies as expected. No difference was seen between nontransfected cells and control cells transfected with plasmid containing only the MGP-leader sequence. However, in cultures of cells overexpressing hMGP, BMP-2-induced differentiation was inhibited. Conversely, in cultures overexpressing AS-hMGP, osteogenesis and chondrogenesis were enhanced.

These effects were first evident in phenotypic changes. Cells overexpressing hMGP maintained a noninduced phenotype despite BMP-2 treatment (Fig. 3, middle panels). In contrast, even without BMP-2 treatment, AS-hMGP-expressing cells spontaneously underwent these phenotypic changes (Fig. 3, bottom panels).

In hMGP-overexpressing cells, cell-specific markers, osteocalcin and collagen IX, were inhibited based on semi-quantitative RT-PCR (Fig. 4). In fact, both osteocalcin and collagen IX expression increased about 2-fold, and collagen IX expression increased 4-fold compared with non- and control-transfected cells.

Adipogenic differentiation appeared to be only partially inhibited in cells expressing hMGP based on only partial inhibition of PPAR-γ. This may be due to the base-line expression of BMP-4 which induces adipogenic differentiation in these cells (17).

To confirm the RT-PCR results, we performed immunoradiometric quantitation of mouse osteocalcin in cell media (Fig. 5, upper panel) and immunoblotting for PPAR-γ protein and collagen IX (Fig. 5, lower panel). These results were consistent with the findings of the RT-PCR. The antibody for PPAR-γ protein recognizes both PPAR-γ1, which is widely expressed in a variety of cells (16), and PPAR-γ2. PPAR-γ2 contains 28 extra amino acids at the N terminus (16) (~3.5 kDa) and thus corresponds to the upper band.
Nonquantitative histochemical stainings (Fig. 6) further confirmed an inhibition of osteogenic and chondrogenic differentiation in hMGP-overexpressing cells. As expected, Oil Red O staining showed occasional adipocytes (Fig. 6, left middle panel). Positive staining for all lineages was seen in nontransfected control cells and in AS-hMGP-overexpressing cells.

Thus, in cells overexpressing hMGP, BMP-2 activity was reduced, and in cells overexpressing AS-hMGP, BMP-2 activity was increased.

Effect of BMP-2 on MGP-deficient Mouse Cells—To test our results ex vivo, mixed aortic cells from homozygous MGP−/− and heterozygous MGP+/− mice as well as wild-type controls were harvested and cultured. The cells were treated with rhBMP-2 at a concentration of 500 ng/ml for 14–16 days.

When MGP−/− cells were treated with BMP-2, chondrogenic differentiation was induced based on strong induction of alkaline phosphatase in colonies of rounded cells (Fig. 7A) and formation of distinct colonies of chondrocytes producing acidic mucopolysaccharides (18) (Fig. 7A). In MGP+/− or control +/+ cells, BMP-2 failed to induce chondrogenic differentiation based on histochemical staining, as well as RT-PCR and immunoblotting for collagen IX (data not shown). However, in MGP+/− cells, BMP-2 did induce osteogenic differentiation based on mineral formation and increased osteocalcin expression (Fig. 7, B and C). Although a low level of osteocalcin expression was found at base line in wild-type cells, this showed a decreasing trend with BMP-2. The differential effect may be the result of a changed ratio of MGP relative to BMP-2 resulting in relatively unopposed BMP activity.

These results differ from those in C3H10T1/2 cells, in that osteogenic and chondrogenic differentiation in mouse cells appeared to be confined to heterozygous or homozygous cells, respectively. It is possible that the C3H10T1/2 cells are less differentiated than the isolated vascular cells, retaining a higher degree of pluripotentiality. Equally possible is that cell isolation procedures and/or culture conditions select for certain types of cells resulting in the observed differences.

Effect of MGP-FLG on BMP-induced Differentiation—In the experiments described above, mRNA levels of MGP were modulated by overexpression of hMGP or AS-hMGP or by using cells from MGP-deficient mice. Even though mRNA levels most commonly reflect protein levels, this may not always be the case. To determine directly the effect of MGP protein on BMP-induced differentiation, we tagged the C-terminal end of hMGP for easy detection in conditioned media (Fig. 8A, inset), and we compared the response to BMP-2 of C3H10T1/2 cells expressing hMGP-FLAG with those expressing nontagged hMGP. No significant difference was detected, here demonstrated by comparing BMP-2-induced alkaline phosphatase activity, an early marker for osteoblastic and chondrocytic differentiation (Fig. 8A). These results indicate that MGP protein is secreted by the transfected cells and that the experimentally tagged protein retains the function of the original MGP.

Nontransfected C3H10T1/2 cells were then treated with conditioned media containing BMP-2 alone or in combination with ~3-fold molar excess hMGP-FLAG. Conditioned media containing BMP-2 (Fig. 8B, inset) or hGMP-FLAG was prepared from HEK293 cells transfected with expression vectors of BMP-2 or hGMP-FLAG. Conditioned media containing both proteins was obtained by cotransfecting the hMGP-FLAG and the BMP-2 vector at a 3:1 molar ratio or by combining conditioned media from singly transfected cells at a comparable ratio.

Alkaline phosphatase was determined using a 96-well assay after 12–14 days of incubation with conditioned media (Fig. 8B). The results show that exogenous addition of hMGP-FLAG to BMP-2-containing media inhibits BMP-2-induced differentiation as measured by alkaline phosphatase. No significant difference was seen in experiments using conditioned media from cotransfected HEK293 cells versus singly transfected cells. This result places the site of action for MGP extracellularly.

Taken together, the three sets of experiments described above suggest that MGP directs mesenchymal differentiation by modulating BMP activity.

Effects of MGP Domains on BMP-induced Differentiation—To test whether fragments of MGP would retain the effect of full-length MGP on BMP-2-induced differentiation, we established stable cell lines overexpressing selected subdomains of hMGP by transfection of C3H10T1/2 cells. Three vectors were transfected, one with the mid-region (usually subjected to γ-carboxylation) alone (mid-MGP, aa 35–54) and the other two with the mid-region combined with either the N terminus (N-MGP, aa 1–54) or the C terminus (C-MGP, aa 35–54) (Fig. 1). Unique RT-PCR products for each vector were detected from cells transfected with the respective vector using
primers recognizing only human MGP (Fig. 9, upper panel). The relative expression by RT-PCR was determined by multiple cycles of RT-PCR using comparable primer pairs and adjustment to GAPDH expression and length of amplified sequence. Expression was compared with that of hMGP-expressing cells (Fig. 9, lower panel). The lowest expression was found in N-MGP cells, which is comparable to that of confluent AS-hMGP. Intermediate expression was found in hMGP and mid-MGP cells, and the highest expression is found in C-MGP cells.

The phenotype of cells expressing N-MGP or the mid-MGP resembled that of cells expressing AS-hMGP (Fig. 4) whereas that of cells expressing C-MGP did not. In N-MGP- and mid-MGP-expressing cells, there was a significant enhancement in osteogenic differentiation as assessed by RT-PCR of osteocalcin (Fig. 10). Even without added BMP-2, osteogenic differentiation occurred in these cells based on an approximate 4-fold increase in osteocalcin expression compared with non- and control-transfected cells (Fig. 10) and positive mineral production (von Kossa staining, data not shown). Addition of BMP-2 had little additional effect on osteocalcin expression in N-MGP cells but induced it about 2-fold in mid-MGP cells (Fig. 10), which may be attributable to the higher relative expression of this construct compared with N-MGP (Fig. 8) or to specific characteristics of this MGP domain. Chondrogenic differentiation was also enhanced about 2-fold in N-MGP cells and 4-fold in mid-MGP cells compared with non- or control-transfected cells as assessed by RT-PCR for collagen IX (Fig. 10). This finding suggests that the mid-MGP fragment is the most efficient in enhancing BMP-2 activity either due to its particular level of expression or domain characteristics.

In cells expressing C-MGP, no enhancement of osteocalcin was seen at base line, and the response to BMP-2 overall was not different from that of control cells (Fig. 10). Induction of adipogenic differentiation was similar in all cells, between 2.5- and 3.5-fold, without clear enhancement or inhibition (data not shown).

Thus, the intact MGP protein appears to be required to inhibit BMP-2 activity, whereas the N-MGP and mid-MGP subdomains have the opposite effect. One possible mechanism is through interference with MGP function.

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**FIG. 5. Effect of varying expression on marker protein levels in response to BMP-2.** Mouse osteocalcin in media as determined by immunoradiometric assay (top), collagen IX (middle), and PPAR-γ protein (bottom) in cell extracts as shown by immunoblotting from C3H10T1/2 cells transfected with hMGP or AS-hMGP after 21 days in culture: treatment with vehicle alone (−) or rhBMP-2 (500 ng/ml) (+), compared with nontransfected (NT) and control-transfected (CT) cells. 30 μg of protein was loaded per lane for immunoblotting. Fold induction was determined by comparison of the signal intensity of treated cells to that of nontreated cells.

**FIG. 6. Histochemical staining of C3H10T1/2 cells transfected with hMGP and AS-hMGP after treatment with rhBMP-2 for 21 days.** Nontransfected (NT) control cells are shown in the upper panels, hMGP cells in the middle panels, and AS-hMGP cells in the lower panels. Oil Red O staining (adipogenic differentiation) is shown in the left panels, von Kossa staining (osteogenic differentiation) in the middle panels, and Alcian Blue staining (chondrogenic differentiation) in the right panels. Original magnification × 40.
DISCUSSION

A possible clue to the mechanism of the effect of MGP on differentiation comes from earlier reports that MGP is associated with BMP, during BMP purification from bone (5). To determine whether the observed association has a functional significance, and whether it serves as the mechanism for MGP’s modulation of differentiation, we tested the effect of MGP on BMP activity. The findings presented in this paper suggest that MGP modulates BMP-2-induced differentiation. In C3H10T1/2 cells, MGP overexpression suppresses the effects of BMP-2, whereas overexpression of antisense to MGP enhances these effects. It is not clear how AS-hMGP exerts its effect. The most straightforward explanation is that the 3-fold excess of AS-hMGP mRNA (Fig. 2) reacts with the endogenous MGP mRNA to decrease its translation. Our results show that even when present at only 3-fold excess in the media, hMGP-FLAG inhibits the response to BMP-2, which suggests that small changes in MGP expression and MGP levels may have significant impact. Alternatively, inhibitory peptides may be produced from the reversed hMGP sequence. Inspecting the sequence, translation of two potential peptides (22 and 6 amino acids respectively) is theoretically possible. However, extensive GenBank™ searches did not show any obvious similarity with

![Fig. 7. A, histochemical staining for alkaline phosphatase (top) and cartilage acidic mucopolysaccharides (Alcian Blue) (bottom) demonstrating the effect of rhBMP-2 (500 ng/ml) for 14–16 days on mixed aortic cells isolated from homozygous MGP null mouse (MGP−/−). B, histochemical staining for mineral (Alizarin Red) demonstrating the effect of rhBMP-2 for 14–16 days on aortic cells isolated from wild-type mouse (MGP+/+) (left), and heterozygous MGP null mouse (MGP+/−) (right). Original magnification × 100. C, expression of osteocalcin in MGP+/+ and MGP+/- cells after treatment with vehicle alone (−) or rhBMP-2 (500 ng/ml) (+) for 14–16 days. Fold induction was determined by comparison of the signal intensity for treated cells to that of nontreated cells.

![Fig. 8. A, alkaline phosphatase activity in C3H10T1/2 cells transfected with hMGP and hMGP-FLAG after 12 days in culture with treatment with vehicle alone or rhBMP-2 (500 ng/ml). Nontransfected (NT) and control-transfected (CT) cells are shown for comparison. Inset, Western blot demonstrating secreted hMGp-FLAG in conditioned media. B, alkaline phosphatase activity in nontransfected C3H10T1/2 cells after 12 days in culture with treatment with rhBMP-2 (estimated 500 ng/ml) alone or in combination with ~3-fold molar excess hMGP-FLAG. Control media and hMGP-FLAG alone are shown for comparison. Inset, Western blot demonstrating secreted rhBMP-2 in conditioned media.](image)
known proteins, including MGP and BMP, which could explain the effect of AS-hMGP.

The importance of low density plating of C3H10T1/2 cells to be able to study the differentiation of individual cells or colonies and to avoid confusing results due to too high cell density has been emphasized by Wang et al. (7). Interestingly, there appears to be a lower cell density in cultures that failed to differentiate. Since the cells were plated carefully at identical densities, this difference would be attributed to BMP-2 treatment. Two possibilities are that BMP-2 directly affects cell density or that induction of differentiation by BMP-2 affects cell density. The latter is more likely, based on the results of Wang et al. (7) that induction of differentiation by BMP-2 causes a decrease in contact inhibition resulting in ultimately greater cell density. Thus, the apparent difference in cell density is an expected result, rather than a cause of the differentiation.

All markers of osteogenic differentiation increased in BMP-2-treated AS-hMGP cells except for one, osteocalcin levels measured in the media. This phenomenon of decreased secretion of soluble osteocalcin in BMP-2-stimulated cells undergoing osteogenic differentiation has been described previously (19) and may be attributed to adsorption of osteocalcin by hydroxyapatite mineral.

Although osteogenic and chondrogenic differentiation was affected, the effect of MGP on adipogenic differentiation was variable. Even though C3H10T1/2 cells express minimal amounts of BMP-2 at base line (20), they do express other BMP (20, 21) including significant amounts of BMP-4, a strong adipogenic inducer in these cells (17). Thus, other factors regulating adipogenesis may explain the variable effect on adipogenesis.

Ex vivo results showed that BMP-2 induced chondrogenic and osteogenic differentiation in MGP−/− and MGP+/− cells, respectively, which differs from the results in the C3H10T1/2 cells. In C3H10T1/2 cells, intermediate levels of BMP-2 have been reported to favor formation of osteoblastic cells, and high levels to favor chondrocytes (7), but not to the exclusion of differentiation along other lineages. It is possible that the procedures used to obtain cells from the respective aortas may select for cells with more limited inducibility compared with C3H10T1/2 cells. Culture conditions and time in culture may be other factors affecting the observed differentiation.

MGP appears to be an important factor in ensuring correct differentiation of vascular smooth muscle cells based on the profound changes seen in MGP-deficient mice where whole media are replaced by chondrocyte-like cells undergoing endochondral ossification (4). Interestingly, BMP-2 is expressed in the embryonic aorta (embryonic day 10.5) when the aorta is still a tube lined with a single layer of cells (22), coinciding with the time of initial media formation (23). In vitro experiments have shown that BMP-2 is able to induce smooth muscle cell differentiation in neural crest-derived stem cells (22). We speculate that the absence of MGP may allow for uncontrolled BMP-2 activity at a stage when smooth muscle cell precursors are easily triggered to undergo endochondral bone formation leading to the phenotype seen in MGP-deficient mice.

By FLAG-tagging hMGP and using HEK293 cells for producing MGP, we facilitated both the detection of the protein in medium and avoided handling of purified hMGP which is poorly soluble in water-based buffers (1). Functionally, hMGP-FLAG retained the function of untagged hMGP when expressed in C3H10T1/2 cells. The same effect was seen when hMGP-FLAG was added to the culture medium that localizes the action of MGP to the extracellular space. A possible in vivo scenario is that MGP binds BMP-2, preventing its interaction with cell surface receptors and retaining BMP during integration with bone matrix. This mechanism would account for the earlier observation of BMP being tightly associated with MGP during purification of bone proteins (5).

The effect of full-length MGP did not seem to be retained by any of the three selected subdomains. Instead, two of the three subdomains had the reverse effect of full-length MGP, may be due to different expression levels or domain characteristics. MGP is mainly attached to the organic matrix if bone (24). Thus, possible mechanisms for the MGP fragments would be to
compared with nontransfected (NT) their widely different primary structures. In the case of trans-
forming growth factor-β, a member of the same superfamily of growth factors as BMP, the matrix protein betaglycan seque-
ters transforming growth factor-β and limits access to signaling receptors (28, 29).

Together, our findings suggest MGP modulates mesenchymal cell differentiation and that the mechanism is, at least in part, modulation of the potent bone differentiation factor, BMP-2.

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Fig. 10. Upper panel, expression of cell-specific differentiation markers in cells transfected with selected domains of hMGP after 21 days of culture: treatment with vehicle alone (−) or rhBMP-2 (500 ng/ml) (+), compared with nontransfected (NT) and control-transfected (CT) cells. Total RNA and cDNA were prepared, and PCR was performed using specific primers for osteocalcin (osteogenic differentiation) and collagen IX (chondrogenic differentiation). GAPDH is shown for comparison. Lower panel, relative signal intensity of the two markers after normal-
ization to GAPDH. The results are expressed as fold increase of expres-
sion in respective vehicle-treated control and are the means of at least three independent determinations.

interfere with the matrix attachment of MGP or its interaction with BMP, in that way enhancing BMP activity. Alternatively, the MGP domains may activate other or additional osteoinduc-
tive factors such as GDF-7 (21), possibly explaining the baseline differen-
tiation observed without BMP-2 treatment in N-
MGP- and mid-MGP-expressing cells.

Further support for the role of MGP in cell differentiation is that MGP expression is strictly limited to specific stages and zones of chondrocyte development (2) and that the MGP null mouse has disorganized ephysseal growth plates (4). In addi-
tion, Yagami et al. (3) showed that overexpression of MGP in developing limb buds delayed chondrocyte maturation and blocked endochondral ossification.

There are precedents for modulation of BMP activity. Embryonic regulators including chordin (25), noggin (26), gremlin, cerberus, and DAN (27) directly bind and inhibit BMP despite their widely different primary structures. In the case of trans-
