JCB Report

Extracellular matrix mineralization is regulated locally; different roles of two gla-containing proteins

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Extracellular matrix mineralization (ECMM) is a physiologic process in the skeleton and in teeth and a pathologic one in other organs. The molecular mechanisms controlling ECMM are poorly understood. Inactivation of *Matrix gla protein* (*Mgp*) revealed that MGP is an inhibitor of ECMM. The fact that MGP is present in the general circulation raises the question of whether ECMM is regulated locally and/or systemically. Here, we show that restoration of *Mgp* expression in arteries rescues the arterial mineralization phenotype of *Mgp*−/− mice, whereas its expression in osteoblasts prevents bone mineralization. In contrast, raising the serum level of MGP does not affect mineralization of any ECM. In vivo mutagenesis experiments show that the anti-ECMM function of MGP requires four amino acids which are γ-carboxylated (gla residues). Surprisingly, another gla protein specific to bone and teeth (osteocalcin) does not display the anti-ECMM function of MGP. These results indicate that ECMM is regulated locally in animals and uncover a striking disparity of function between proteins sharing identical structural motifs.

Introduction

ECM mineralization (ECMM) is a physiologic process in bone, teeth, and hypertrophic cartilage, whereas in other locations it must be inhibited. To date, few proteins acting as inhibitors of ECMM have been identified through mouse and human genetic studies. They include: Ank, a transmembrane protein controlling extracellular export of pyrophosphate, a small molecule that itself inhibits ECMM; NPPS, an ectoenzyme also generating pyrophosphate extracellularly; matrix gla protein (MGP), a mineral-binding protein of the ECM; and fetuin, a circulating protein that accumulates in bone ECM (Jahnen-Dechent et al., 1997; Luo et al., 1997; Okawa et al., 1998; Nakamura et al., 1999; Hagmann, 2000; Ho et al., 2000; Nurnberg et al., 2001; Schafer et al., 2003). Understanding at the molecular level how each of these proteins inhibits ECMM is a prerequisite to better understanding how ectopic ECMM develops, such as that observed in atherosclerosis or in osteoarthritis. Elucidation of the mechanisms behind protein inhibition of ECMM may lead eventually to the identification of novel therapeutic strategies for the treatment of these diseases.

With the long-term goal of understanding how ECMM is prevented in some tissues, whereas favored in others, our laboratory has embarked on a detailed study of the functions and mechanisms of action of proteins containing gla (or γ-carboxylated glutamic acid) residues (Pudota et al., 2000; Bandyopadhyay et al., 2002). This posttranslational modification confers to proteins a high affinity for hydroxyapatite crystals, the major mineral crystal present in mineralized ECMs (Romberg et al., 1986; Roy and Nishimoto, 2002; Hoang et al., 2003). We focused our work on two gla residue-containing proteins, namely MGP and bone gla protein (BGP or osteocalcin), the latter being a protein long thought to be involved in bone ECMM (Price et al., 1976, 1983; Celeste et al., 1986). *Mgp* is expressed in vascular smooth muscle cells (VSMCs) and in chondrocytes but not in osteoblasts, whereas *Osteocalcin* is expressed in osteoblasts and odontoblasts only (Ducy and Karsenty, 1995; Luo et al., 1995). In addition, both MGP and osteocalcin are circulating proteins (Lian et al., 1987; Ismail et al., 1988; Price et al., 2003). Consistent with the pattern of *Mgp* expression, MGP-deficient mice develop abnormal ECMM in their arteries and growth plate cartilage establishing that MGP is an inhibitor of ECMM in the vicinity of the cells expressing it (Luo et al., 1997). In contrast, osteocalcin-deficient mice did not have any detectable defect of bone ECMM indicating that osteocalcin is not required for bone mineralization (Ducy et al., 1996). This latter experiment did not address however, whether osteocalcin, like MGP, could inhibit ECMM.

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Key words: MGP; osteocalcin; ECM; mineralization; local regulation

Abbreviations used in this paper: ECMM, ECM mineralization; MGP, matrix gla protein; VSMC, vascular smooth muscle cell; WT, wild-type.

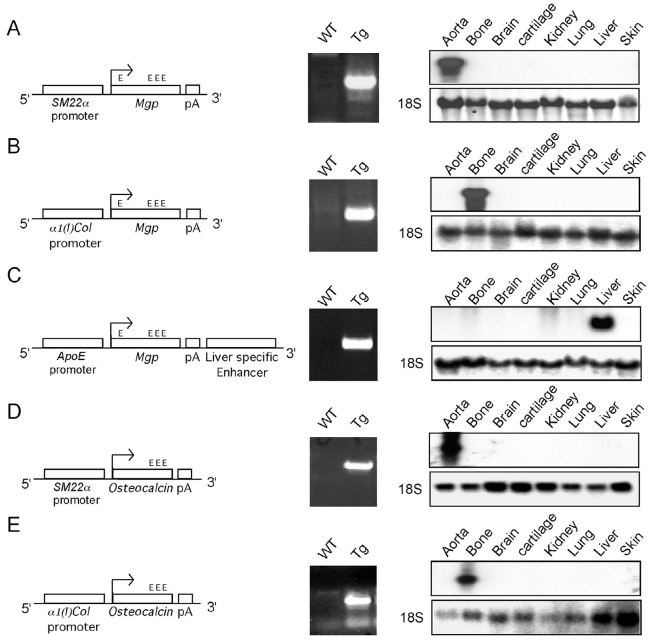

Figure 1. **Transgene expression analysis.** Schematic representation of the transgene constructs (left), transgene detection by PCR (middle), and Northern blot analysis showing tissue-specific expression (right) of *SM22α-Mgp* (A), *α1(I)Col-Mgp* (B), *ApoE-Mgp* (C), *SM22α-Osteocalcin* (D), and *α1(I)Col-Osteocalcin* (E) transgenes. The letter E on transgenes represents γ-carboxylated glutamic acid residues.

The striking differences between MGP and osteocalcin functions already revealed by gene deletion experiments (Ducy et al., 1996; Luo et al., 1997), together with the fact that these proteins are circulating systemically raised a series of questions: first, do these proteins act only after local secretion and/or do they act systemically by reaching various tissues through the circulation? This is an important question as mice deficient in fetuin, a circulating protein, develop ectopic ECMM when fed a high calcium and high phosphorus diet (Schafer et al., 2003). Second, can we identify in vivo the residues in MGP critical for its anti-ECMM function? Lastly, because loss of function experiments failed to uncover a function for osteocalcin during ECMM, could gain of function experiments help to provide definitive information on whether osteocalcin is involved in ECMM?

To address these questions, we used MGP-deficient mice and other transgenics to assess the vascular ECMM by gla-containing proteins, and to assess the influence of these proteins on bone mineralization. Our results are consistent with the hypothesis whereby inhibitors of ECMM act locally and not systemically. They also demonstrate that osteocalcin does not carry out the anti-ECMM function of MGP in vivo.

Results and discussion

Generation of transgenic mice

To study the roles of MGP and osteocalcin during ECMM, we generated several mouse models expressing, in a cell-specific manner, wild-type (WT) or mutated protein*s* (Fig. 1, A–E). We used a *SM22α* promoter fragment to drive their expression specifically in VSMCs, whereas liver-specific expression was achieved by using the promoter of the *apolipoprotein E (ApoE)* gene and its liver-specific enhancer (Simonet et al., 1993; Solway et al., 1995). To achieve osteoblast-specific expression, we used the 2.3-kb promoter fragment of the *α1(I) collagen* gene (Rossert et al., 1995). In each case, we obtained at least two different lines expressing the transgene of interest and we verified cell- or tissue-specific expression for each transgene (Fig. 1, A–E). *SM22α-Mgp, ApoE-Mgp, SM22α-Osteocalcin*, and *α1(I)Col-Osteocalcin* transgenics had no metabolic or histological abnormalities. In particular, they had normally mineralized bone and no sign of ectopic ECMM (Table I and not depicted). These various transgenic mice were then used for subsequent experiments presented below.

Rescue of arterial but not of cartilage phenotype in Mgp−/−; SM22α-Mgp mice

SM22α-Mgp mice had no phenotypic abnormalities and serum PTH, phosphate and calcium levels were normal. To test if this transgene could rescue the arterial phenotype of *Mgp−/−* mice, we intercrossed *SM22α-Mgp* mice with *Mgp+/−* mice to obtain *Mgp−/−; SM22α-Mgp* mice. WT, *Mgp−/− and Mgp−/−; SM22α-Mgp* mice were analyzed at 4 wk old, the age at which most *Mgp−/−* mice die due to vascular rupture and hemorrhaging, by Alizarin red staining of skeletal preparations and histological analyses (Luo et al., 1997). Alizarin red staining of skeletal preparations failed to detect any abnormal mineralization of the aorta or of any arteries in *Mgp−/−; SM22α-Mgp* mice at that age or at 6 mo old (Fig. 2 A and not depicted). Accordingly, histological examination using von Kossa staining for mineral deposits failed to detect any ECMM in the arteries of *Mgp−/−; SM22α-Mgp* mice at 4 or 24 wk old (Fig. 2 B and not depicted). In contrast, mineralization of cartilage, an avascular tissue, was not prevented in *Mgp−/−; SM22α-Mgp* mice (Fig. 2 D). Thus, reintroducing MGP in VSMCs could rescue only the arterial phenotype of MGP-deficient mice.

Table I. **Serum phosphate (Pi), calcium (Ca), and parathyroid hormone (PTH) concentrations in the transgenic mice**

Transgenic mice	[Pi] mg/dL (σ_M)	$[Ca]_{total}$ mg/dL (σ_M)	[PTH] pg/mL (σ_M)
SM22α-Mgp	7.91 (0.38)	9.58 (0.23)	34.76 (1.38)
ApoE-Mgp	7.05 (0.29)	10.20 (0.20)	29.67 (5.24)
α1(I)Col-Mgp	7.39 (0.06)	9.91 (0.08)	38.11 (7.56)
SM22α-Osteocalcin	7.35 (0.25)	10.00 (0.09)	25.95 (0.54)
α1(I)Col-Osteocalcin	8.01 (0.19)	10.37 (0.15)	31.28 (4.47)
WT	7.89 (0.92)	10.37 (0.06)	29.02 (5.30)

SEM (σ_M) values are presented in the parentheses.

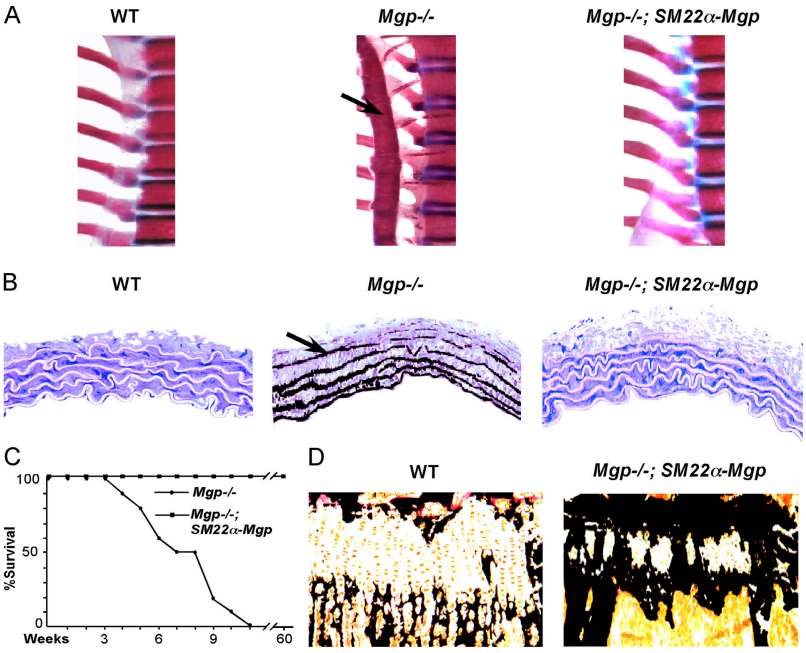

Figure 2. **VSMC-specific expression of *Mgp* prevents arterial mineralization of *Mgp*−/− mice.** (A) Skeletal preparation of the thoracic aorta from a 4-wk-old *Mgp*−/−; *SM22*α-*Mgp* mouse shows the absence of mineral-specific Alizarin red staining, which is present in *Mgp*−/− aorta (arrow). (B) von Kossa staining of a 4-wk-old *Mgp*−/−; *SM22*α-*Mgp* aorta section shows complete lack of mineral deposition, whereas an *Mgp*−/− aorta section shows mineral depositions (arrow) along the elastic lamina. (C) *Mgp*−/−; *SM22*α-*Mgp* mice have a normal life span. (D) VSMC-specific expression of *Mgp* failed to prevent the progressive mineralization of the growth plate cartilage in these mice.

Systemic presence of MGP does not rescue the phenotype of MGP−/− mice

Because MGP is found in the general circulation where it forms a complex with small mineral nuclei and other circulating proteins (Price et al., 2003), the question arises as to its mode of action. Is MGP acting locally in the vicinity of the cells synthesizing it and/or is it using blood transport as a means to inhibit ECMM in locations where it is not synthesized? To address this question, we used the promoter and liver-specific enhancer of *ApoE* to achieve ectopic expression of *Mgp* in liver and thereby in blood. Systemic presence of MGP in these mice was confirmed by dot blot experiments using a polyclonal serum raised against a COOH-terminal MGP peptide. Using this assay, *ApoE-Mgp* transgenics showed a 6–10-fold increase of serum MGP in comparison to their WT littermates (Fig. 3 A). To demonstrate that MGP originating from the transgene has the ability to inhibit mineralization we cultured WT osteoblasts in the presence of either WT or *ApoE-Mgp* mouse serum. Whereas osteoblasts cultured in the presence of WT serum formed nodules that mineralized, those cultured in the presence of *ApoE-Mgp* serum showed a marked reduction of mineralization (Fig. 3 B). This was not due to an absence of osteoblastic nodules because alkaline phosphatase staining showed a comparable number of nodules in both culture conditions (Fig. 3 B). Thus, MGP originating from this transgene has conserved its antimineralization function.

ApoE-Mgp mice had no phenotypic abnormalities and had normal serum PTH, calcium, and phosphate levels (Table I and not depicted). They were intercrossed with *Mgp*+/− mice to eventually generate *Mgp*−/−; *ApoE-Mgp* mice. At 4 wk old, Alizarin red staining of skeletal preparations revealed that *Mgp*−/−; *ApoE-Mgp* mice developed mineralization of aorta similar to that observed in *Mgp*−/− mice (Fig. 3 C). Likewise, histological analysis showed the presence of ECMM in *Mgp*−/−; *ApoE-Mgp* arteries (Fig. 3 D). These results provided the first evidence that, MGP acts locally not systemically to prevent ECMM.

To extend these observations, we asked whether bone mineralization could be affected by either local or systemic expression of *Mgp*. To that end, we studied bone mineralization at 4 wk old in WT, *ApoE-Mgp*, and in α*1(I)Col-Mgp* mice that express *Mgp* only in osteoblasts (Fig. 1 B). Skeletal preparations of 10-d-old skulls showed no mineralization defect in *ApoE-Mgp* mice, whereas a severe decrease in intramembranous bone mineralization was observed in α*1(I)Col-Mgp* mice (Fig. 3 E). Likewise, histological analysis of vertebrae of *ApoE-Mgp* mice failed to show any increase in unmineralized bone, whereas histological analysis of vertebrae of α*1(I)Col-Mgp* mice showed a marked, i.e., 8–12-fold, increase of unmineralized bone tissue (Fig. 3 F). When together, the analyses of *Mgp*−/−; *SM22*α-*Mgp*, *ApoE-Mgp*, and α*1(I)Col-Mgp* mice establish that, in animals fed a normal diet, MGP inhibits ECMM locally and not systemically.

MGP and osteocalcin do not share an antimineralization function despite structural similarities

The osteoidosis, i.e., increase in unmineralized bone ECM, observed in the α*1(I)Col-Mgp* mice provided us with an in vivo model to test the function of the gla residues within MGP and other proteins. In this context, we generated transgenic mice that produced two distinct mutated forms of MGP in osteoblasts. In MGP mutant1 (MGPm1), three of the four glutamic acid residues present in the mouse protein were replaced by aspartic acid residues. In MGP mutant2 (MGPm2) all four glutamic acid residues undergoing γ-carboxylation were replaced by aspartic acid residues. Histological analysis performed in 4-wk-old mice failed to detect any evidence of osteoidosis in the α*1(I)Col-Mgpm2* mice, whereas osteoidosis was considerably milder in α*1(I)Col-Mgpm1* mice than in α*1(I)Col-Mgp* (Fig. 4 C). These results establish that the gla residues are required for MGP antimineralization function.

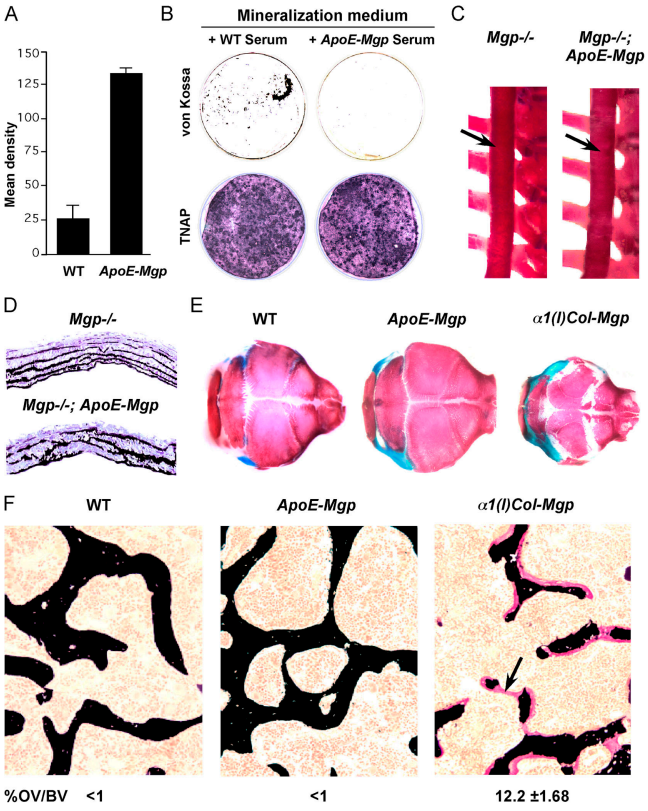

Figure 3. **Increased serum MGP level does not prevent ECMM in vivo.** (A) Dot blot analysis shows a 6–10-fold increase of MGP serum level in *ApoE-Mgp* mice in comparison to their WT littermates. The error bars represent SDs of three independent measurements. (B) *ApoE-Mgp* serum prevents in vitro mineralization of primary osteoblast culture, whereas WT serum does not (top). Note that nodule formation is comparable under both conditions (bottom). Skeletal preparation of the thoracic aorta (C) and von Kossa staining of an aorta section (D) from a 4-wk-old *Mgp−/−; ApoE-Mgp* mouse show mineral deposition as seen in *Mgp−/−* mice. The arrows in C indicate mineralized aortae. (E) Skeletal preparations of 10-d-old *ApoE-Mgp* skulls show normal ECM mineralization, whereas it is severely affected in α*1(I)Col-Mgp* skulls. (F) von Kossa and van Gieson staining show normal bone mineralization in a 4-wk-old *ApoE-Mgp* vertebra and a 12-fold increase of unmineralized bone (arrow) in a 4-wk-old α*1(I)Col-Mgp* vertebra.

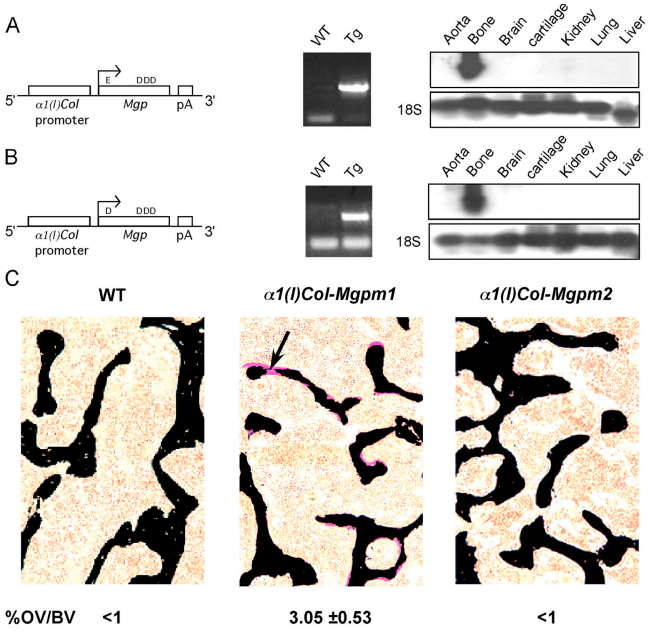

Figure 4. **Gla residues are required for MGP function.** Schematic representation of the transgene constructs (left), transgene detection by PCR (middle), and Northern blot analysis showing tissue-specific expression of the transgenes (right) in α*1(I)Col-Mgpm1* (A) and α*1(I)Col-Mgpm2* (B) transgenics. Letters E and D on transgenes represent γ-carboxylated glutamic acid residues and mutated aspartic acid residues, respectively. (C) von Kossa and van Gieson staining of vertebra sections show a threefold increase of unmineralized bone (arrow) in a 4-wk-old α*1(I)Col-Mgpm1* mouse, whereas bone mineralization is largely unaffected in an α*1(I)Col-Mgm2* mouse.

The demonstration of the functional importance of gla residues in MGP leads to the question of whether other gla-containing proteins are also inhibitors of ECMM. Osteocalcin is the most abundant gla protein synthesized in the skeleton, yet its deletion in mice failed to show impaired ECMM (Ducy et al., 1996). However, because this experiment could not assess whether osteocalcin, like MGP, could act as an inhibitor of ECMM, we subsequently performed here gain of function and ectopic expression experiments.

*SM22*α*-Osteocalcin* mice did not show any metabolic or histological abnormalities (Table I and not depicted) despite a six- to eightfold increase in serum osteocalcin indicating efficient transcription/translation of the transgene (Fig. 5 A). *SM22*α*-Osteocalcin* mice were then intercrossed with *Mgp+/−* mice to obtain *Mgp−/−; SM22*α*-Osteocalcin* mice. Surprisingly, these latter mutant mice did not survive past 2 mo old and possessed the phenotype of *Mgp−/−* mice (unpublished data). Indeed, Alizarin red staining of skeletal preparations and histological analysis showed that at 1 mo old the aorta of *Mgp−/−; SM22*α*-Osteocalcin* was fully mineralized (Fig. 5, B and C). This result indicates that unlike MGP and despite the presence of three gla residues, osteocalcin cannot inhibit ECMM in arteries. Alternatively, it could mean that this function of osteocalcin could not be incurred outside bone, its physiological site of expression. To test this possibility, we generated transgenic mice overexpressing *Osteocalcin* in osteoblasts under the control of the α*1(I) collagen* promoter. Again, unlike what we observed in α*1(I)Col-Mgp* transgenic mice, α*1(I)Col-Osteocalcin* mice had normally mineralized bone. In particular, they had a normal osteoid volume relative to total bone volume (Fig. 5 D). Together, the results of these two experiments indicate that despite the presence of gla residues, osteocalcin is not an inhibitor of ECMM in vivo. Based on the gain of function experiments presented here and on the loss of function experiments described previously it appears that the gla residues do not affect MGP and osteocalcin functions in the same way. Our results raise the hypothesis that, as it is the case for thrombin, only decarboxylated osteocalcin may have a function (Furie and Furie, 1988). Consistent with this hypothesis, it was shown recently that serum level of decarboxylated osteocalcin is a reliable indicator of the severity of osteoporosis in postmenopausal women (Szulc et al., 1996).

In summary, using genetic and in vivo bioassay models of physiologic (bone) and pathologic (arterial) ECMM, we

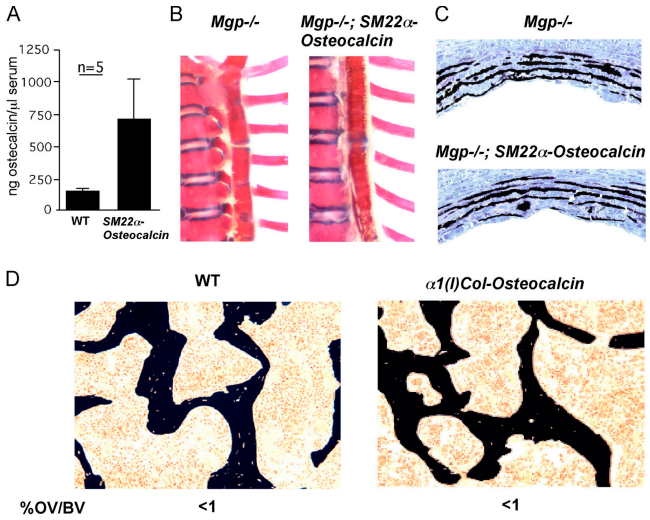

Figure 5. **Osteocalcin does not share antimineralization function of MGP.** (A) Radioimmuno-assay shows a six- to eightfold increase of osteocalcin serum level in *SM22α-Osteocalcin* mice in comparison to their WT littermates. The error bars represent SDs of five independent measurements. Skeletal preparation of the thoracic aorta (B) and von Kossa staining of aorta sections from a 4-wk-old *Mgp−/−; SM22α-Osteocalcin* (C) mouse show mineral deposition as seen in *Mgp−/−* mice. (D) von Kossa and van Gieson staining of a vertebra section show normal bone mineralization in 4-wk-old *α1(I)Col-Osteocalcin* mice.

demonstrate that inhibition of ECMM by proteins is exerted locally and not systemically. They also uncover great functional disparity between proteins sharing identical functional motifs thought to be pivotal in controlling ECMM. Indeed, our mutagenesis experiments establish that the gla residues are required for MGP inhibition of ECMM function, yet gla residue-containing osteocalcin cannot inhibit ECMM. This observation again underscores the importance of in vivo tests for protein function even when their structure might suggest an obvious function. The observation that MGP can inhibit ECMM in arteries and bone suggests that the function of inhibitors of ECMM does not overtly depend on the composition of a given ECM but rather depends on the expression of these inhibitors. The similarity of function of MGP in bone and in preventing arterial ECMM implies also that if one can elucidate the molecular bases for the spatial restriction of ECMM to bone then this information could possibly be used to prevent ectopic mineralization in diseases of arteries and joints.

Materials and methods

DNA constructs

DNA constructs for VSMCs and osteoblast-specific expressions of *Mgp* transgenes were generated using a plasmid (p*SMS4*) containing *Mgp* ORF with a downstream SV40 polyadenylation signal. To generate *pSM22α-Mgp*, a 3-kb *SM22α* promoter fragment was inserted at a XhoI site upstream to the *Mgp* ORF in p*SMS4*. Similarly, to generate p*α1(I)Col-Mgp*, a 2.3-kb *α1(I) collagen* promoter fragment was inserted upstream to the *Mgp* ORF. p*α1(I)Col-Mgpm1* with three gla-encoding codons mutated to Asp encoding codons was generated by PCR mutagenesis. The mutated amplicons were digested by NcoI to replace the NcoI fragment of *Mgp* ORF in p*SMS4* and was followed by the insertion of a 2.3-kb *α1(I) collagen* promoter at an upstream XhoI site. To generate p*α1(I)Col-Mgpm2*, the remaining gla encoding codon in p*α1(I)Col-Mgpm1* construct was mutated to an aspartic acid encoding codon by PCR mutagenesis. The mutated amplicon was digested by ClaI and PpuM1 to replace the ClaI and PpuMI digested fragment of *α1(I)Col-Mgpm1*. p*ApoE-Mgp* was generated by inserting the *Mgp* ORF at a HpaI site of p*Liv7* containing a 3-kb *ApoE* promoter fragment, the first intron, polyadenylation signal, and a liver-specific enhancer (Simonet et al., 1993). To construct p*SM22α-Osteocalcin*, the complete *Osteocalcin* ORF was first subcloned in pBlueScript (Stratagene). The SV40 polyadenylation signal was introduced at a 3′ XbaI site and the 3-kb *SM22α* promoter fragment was inserted at an upstream XhoI site. p*α1(I)Col-Osteocalcin* was constructed by inserting the 2.3-kb *α1(I) collagen* promoter fragment upstream to the *Osteocalcin* ORF and SV40 polyadenylation signal.

Mice

Generation of *Mgp−/−* mice was described previously (Luo et al., 1997). Transgenic founders were generated by pronuclear injection according to standard techniques. All mice were maintained in a pathogen-free standard animal facility.

Genotyping and expression analysis

Genotypes were determined by PCR using isolated tail DNA. The following sets of primers were used: for *SM22α-Mgp* transgene 5′-AAGGAAGGGTTTCAGGGTCCTG-3′ and 5′-CGGGAAAGATGAGGAAGAAGGG-3′; for *α1(I)Col-Mgp*, *α1(I)Col-Mgpm1*, and *α1(I)Col-Mgpm2* transgenes 5′-CCAGGATGCCTGAAAGATTACTAGC-3′ and 5′-CGGGAAAGATGAGGAAGAAGGG-3′; for *ApoE-Mgp* transgene 5′-TTAGAGGAAATCACAGGGGGAGGC-3′ and 5′-GGATCGCAACAAGCCTGCCTACGATATCAACAGAGATGC-3′; for *SM22α-Osteocalcin* transgene 5′-AAGGAAGGGTTTCAGGGTCCTG-3′ and 5′-GGGGATCTGGGCTGGGGACTGGACTGAGG-3′ and for *α1(I)Col-Osteocalcin* transgene 5′-CCAGGATGCCTGAAAGATTACTAGC-3′ and 5′-GGGGATCTGGGCTGGGGACTGAGG-3′. For analysis of transgene expression, RNA was isolated as previously described and analyzed by Northern blotting (Ausubel et al., 1996). Probes used were SV40 and *ApoE* polyadenylation signals.

Primary osteoblast culture

WT osteoblasts were cultured in Alpha-MEM (Invitrogen) containing 10% WT or *ApoE-Mgp* serum and supplemented by 100 μg/ml ascorbic acid (Sigma-Aldrich). After formation of osteoblastic nodules at 4 d, 5 mM β-glycerophosphate (Sigma-Aldrich) was added to the culture medium and cells were grown for another 4 d. Fresh medium was added in every 48 h. von Kossa staining for mineral and alkaline phosphatase staining for osteoblasts were performed at the end of the culture period.

Skeletal preparation

Thoracic aorta together with vertebrae were dissected, fixed overnight in 100% ethanol, and stained in Alcian blue dye followed by Alizarin red solution as described previously (Luo et al., 1997).

Histology

Vertebrae were fixed overnight in 4% PFA/PBS, embedded in methyl methacrylate, sectioned (7 μm), and stained by von Kossa and van Gieson. Unmineralized bone was measured using Osteomeasure software (Osteometrics Inc.). Aortas were fixed in 1% glutaraldehyde overnight, washed in 0.1 M sodium cacodylate buffer, serially dehydrated in ethanol, and embedded in paraffin. 7-μm sections were stained by von Kossa and counterstained by Toluidine blue. Images were captured with a light microscope (model DMLB; Leica) using a SPOT CCD camera, acquired with SPOT software v2.1 (Diagnostic Instruments), and processed using Adobe Photoshop®.

Serum biochemistry

Serum calcium and phosphate were measured using commercially available kits (Sigma-Aldrich). PTH was measured using an ELISA kit for immunodetection (Immunotopics). Dot blot analysis to detect serum MGP was performed using a polyclonal rabbit serum raised against a COOH-terminal MGP peptide (ERYAMVYGYNAAYNRYFRQRRGAKY). A commercially available anti–rabbit antibody conjugated with HRP was used as a secondary antibody. HRP activity was detected by using standard protocols and signal intensity on an imaging film was measured by NIH software for densitometric analysis. Serum osteocalcin level was measured using an osteocalcin RIA kit (Biomedical Technologies Inc.).

We acknowledge Dr. P. Ducy from Baylor College of Medicine for very useful suggestions.

This paper was supported by National Institutes of Health grant NIH PO1 AR42919, March of Dimes Foundation grant 1-FY99-489, and Canadian Institute of Health Research grant MT11360. M. Murshed is supported by a Postdoctoral Fellowship from American Heart Association (Application ID 0325220Y).

Submitted: 9 February 2004
Accepted: 30 April 2004

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