Effect of Cell Density and Growth Factors on Matrix GLA Protein Expression by Normal Rat Kidney Cells

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The present studies demonstrate that the expression of the vitamin K-dependent matrix Gla protein (MGP) is critically dependent on cell density in culture. Subculture of confluent NRK cells to 1/30 of the confluent cell density causes a 50- to 100-fold decline in MGP expression per cell within two days. MGP expression subsequently increases with increasing cell density and eventually attains a level of expression per cell at five days post-confluence that is over 2,000-fold greater than was seen in the cells two days after the 1 to 30 subculture. These reversible, density-dependent changes in MGP expression are far larger than have been previously reported for other secreted proteins and suggest that the as yet unknown function of MGP requires its expression at high cell density but not at low. We have also observed that human epidermal growth factor (EGF) causes a 20-fold reduction in MGP expression in post-confluent, non-dividing cultures and suggest that the suppression of MGP function at high density may be a prelude to cell migration or division in response to appropriate signals. **J. Cell. Physiol. 171:125–134, 1997.** © 1997 Wiley-Liss, Inc.

Matrix Gla protein (MGP) is a 10 kDa secreted protein which contains five residues of the vitamin K-dependent Ca^{2+} binding amino acid, γ-carboxyglutamic acid (Gla) (Price et al., 1983; Price and Williamson, 1985). MGP is unique among the presently known vitamin K-dependent proteins in that it is synthesized by an exceptionally broad array of tissues and cells, while the vitamin K-dependent proteins involved in blood coagulation are synthesized primarily in the liver, and the bone Gla protein is synthesized only in bone and dentin. MGP mRNA has been detected in all vertebrate tissues tested (Fraser and Price, 1988; Hale et al., 1988), and MGP is secreted into culture medium by osteoblasts, chondrocytes, cardiac myocytes, vascular endothelial cells, breast cells, fibroblasts, pneumocytes, and kidney cells (Fraser and Price, 1988; Hale et al., 1988; Chen et al., 1990; Cancela and Price, 1992; Rannels et al., 1993). In spite of the broad tissue distribution of MGP synthesis, only three tissues have been shown to accumulate significant levels of MGP in an extracellular matrix, bone, cartilage, and calcified cartilage (Price et al., 1983; Hale et al., 1988; Rice et al., 1994).

Although the function of MGP is presently unknown, there is evidence to suggest that the protein could regulate an aspect of cell growth and differentiation. MGP gene transcription is strongly induced by retinoic acid in all normal human cell types tested (Cancela and Price, 1992), and the human MGP gene promoter contains a perfect direct repeat, which is nearly identical to the retinoic acid response element in the human retinoic acid receptor β gene promoter (Cancela et al., 1990). These observations suggest that MGP could mediate some of the known actions of retinoic acid on cell growth and differentiation (Cancela and Price, 1992). Following the original isolation and sequencing of MGP from bone (Price et al., 1983; Price and Williamson, 1985), MGP has been independently discovered by differential cDNA screening as a gene that is overexpressed by breast cancer cells (Chen et al., 1990), by prostate epithelial cells undergoing apoptosis (Briehl and Miesfeld, 1991), and by vascular smooth muscle cells undergoing dedifferentiation in cell culture (Shanahan et al., 1993). While each of these three independent discoveries of MGP is based on increased MGP production in cells undergoing fundamentally different transitions, the fact that MGP is one of the few genes overexpressed in each instance does suggest that MGP expression is driven by transitions in cell growth and differentiation and that the protein may in fact play a role in these processes.

MGP is the target of several post-translational modifications in addition to γ-carboxylation, which may regulate aspects of its activity. Specific proteolytic cleavage at a conserved dibasic site in the C-terminal region

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has been observed for MGP isolated from human, bovine, and shark tissues (Price et al., 1994); and conserved phosphorylation of three serines in the N-terminal region has been observed for MGP from shark, lamb, rat, cow, and human tissues. The recognition motif for serine phosphorylation in MGP, Ser-X-Glu/Ser(P), has also been found in most secreted phosphoproteins and is the expected specificity of the secretory pathway protein kinase (Meggio et al., 1988; Price et al., 1994). Proteins with multiple phosphorylated residues can be regulated in principle, with considerable sensitivity by changes in the extent of phosphorylation (Price et al., 1994), and there is recent evidence to suggest that such changes in phosphorylation and activity can be produced by regulated changes in the secretory pathway protein kinase (Akhoundi et al., 1994) and by extracellular phosphatases (Ek-Rylander et al., 1994).

The initial objective of the present investigations was to characterize the effect of cell density on MGP expression in cultured mammalian cells. These studies revealed that subculture of growth-arrested, confluent cells to very low density caused a rapid resumption of cell growth and reduced MGP expression by over 100-fold. This result suggests that MGP expression may be incompatible with rapid cell division in culture. To further test the possible inverse relationship between MGP expression and cell growth, additional experiments were carried out to assess the possibility that growth factors might down-regulate MGP expression as an aspect of their growth promoting activity.

METHODS

Materials

^{125}I (4×10^{18} cpm/mol), [α-^{32}P] dCTP (>3,000 Ci/mmol), and [α-^{32}P]UTP (800 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Vitamin C, human platelet-derived growth factor (PDGF), human epidermal growth factor (EGF), human transforming growth factor β1 (TGFβ1), and human basic fibroblast growth factor (bFGF) were purchased from Gibco (Grand Island, NY). RNase-free DNase I, RNase A, and proteinase-K were from Boeheringer Mannheim (Indianapolis, IN). Normal rat kidney (NRK) 52E cells were purchased from the American Type Culture Collection (Rockville, MD). Matrigel, human fibronectin, and rat tail collagen Type I were purchased from Collaborative Research Incorporated (Bedford, MA).

Cell culture

NRK cells were cultured in Coon's F12 medium supplemented with 10% fetal calf serum, 50 μg/ml vitamin C, 1 μg/ml vitamin K_1, and antibiotics (Fraser and Price, 1988). For the time course experiment shown in Figures 1 and 2, NRK cells were subcultured into 100 mm plates at a 1 : 30 ratio (Seeding density = 0.25×10^4 cells/cm^2) one day after they had attained visual

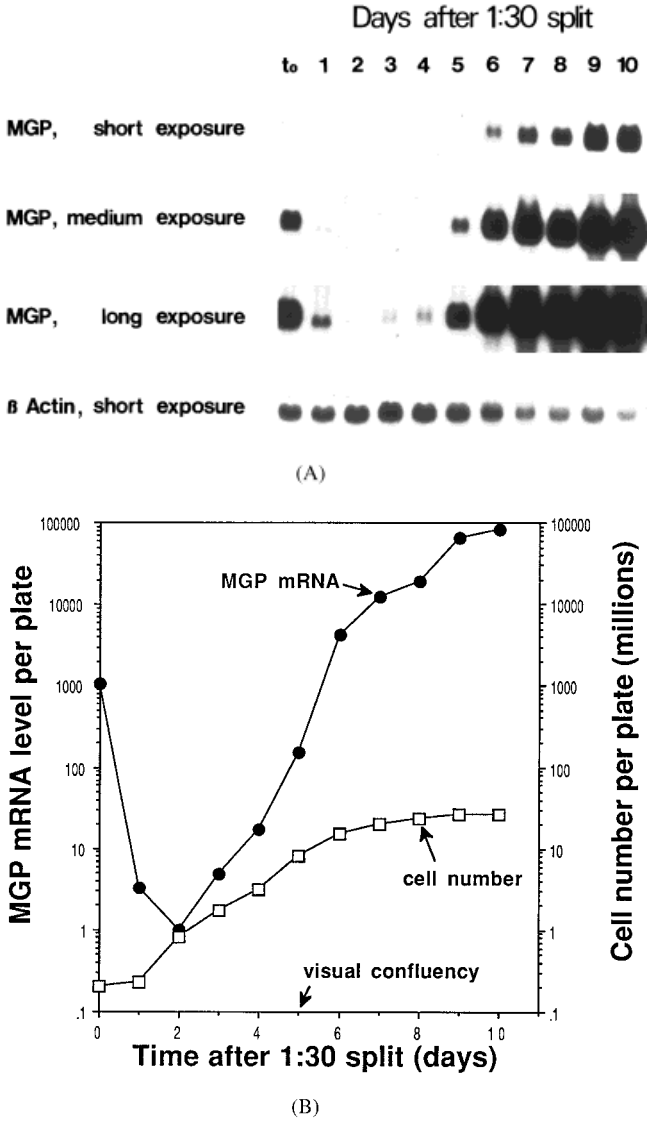

Fig. 1. Effect of increasing cell density on the level of MGP mRNA in NRK Cells. Confluent NRK cells were subcultured at a 1:30 ratio and maintained with daily medium changes for ten days. Total RNA was isolated from cells at the time of subculture (t = 0) and at daily intervals thereafter, and 30 μg of each RNA was analyzed by Northern blot using ^{32}P-labeled rat MGP and β-actin cDNAs as hybridization probes. **A:** Autoradiograms of the Northern blot at different exposure times. **B:** The level of ^{32}P-MGP cDNA hybridization to RNA isolates from the NRK cells at different levels of confluency. The level of ^{32}P-MGP cDNA hybridization were determined by scintillation counting after autoradiography of the blot. Uniform 0.7 × 1.0 cm sections of the filter were chosen by alignment with the autoradiograph so as to include all of a given mRNA band; three equal sized control sections were randomly selected from the remainder of the filter. Specific binding for each band was obtained by subtracting the average counts of the control sections from the total counts of each mRNA band. The specific binding determined on these 30 μg samples of RNA was then multiplied by the total RNA recovered from the plate in order to obtain a measure of the MGP mRNA level in the culture. The resulting values were divided by the day two number so that the changes in MGP mRNA level could be assessed directly in the graph.

Abbreviations

MGP	matrix Gla protein
Gla	γ-carboxyglutamic acid
PBS	phosphate-buffered saline

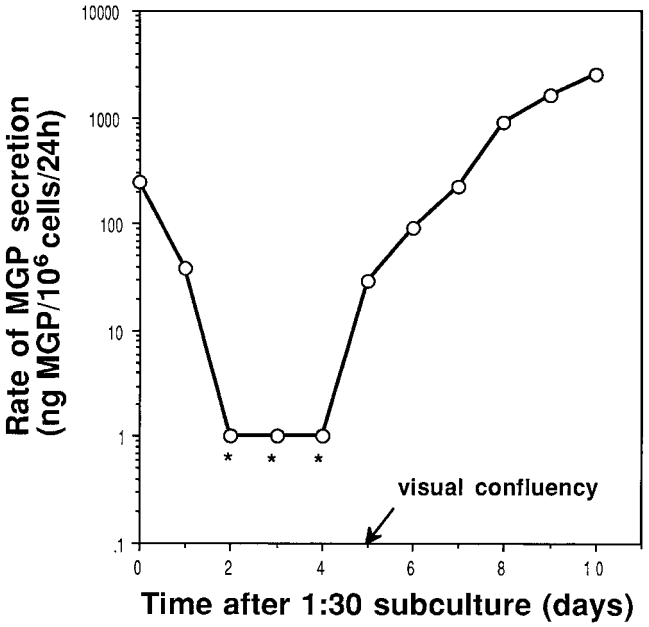

Fig. 2. Effect of increasing cell density on the level of MGP in the conditioned medium of NRK cells. Conditioned medium was obtained daily following a 1:30 subculture of confluent NRK cells in the experiment described in Figure 1 and was analyzed for the level of MGP by radioimmunoassay (see Materials and Methods). Each point is the average MGP level in conditioned medium samples obtained from each of five experimental plates and is expressed as ng of MGP antigen secreted into the conditioned medium per million cells per 24 h. (Asterisks denote the following meaning: below the detection limit of 1 ng/ml.)

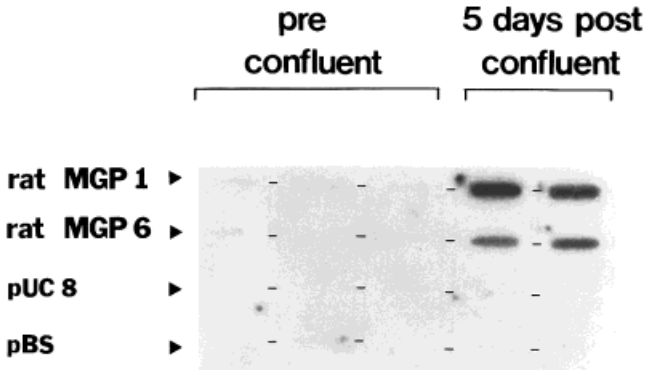

Fig. 3. Effect of cell density on the rate of MGP gene transcription in NRK cells. Nuclei were harvested from preconfluent and five-day postconfluent NRK cultures, and nascent transcripts were elongated in vitro in the presence of [α-^{32}P] UTP (see Methods). After isolation, run-off transcripts were hybridized to the indicated DNAs that had been previously blotted onto the nitrocellulose filters. Rat MGP 1 and Rat MGP 6: Rat MGP cDNAs (see Price et al., 1987, for details); pUC 8 = vector for rat MGP 1; pBS = vector for rat MGP 6. The filters were exposed for four days at −70°C with intensifying screens.

confluence. Culture medium was subsequently exchanged for fresh medium every 24 h throughout the experiment. Conditioned medium was obtained daily from each of five dishes, and aliquots of 0.2 ml or less were analyzed for MGP in triplicate by radioimmunoassay (Otawara and Price, 1986). Each point in Figure 3 is therefore the average of the MGP levels determined individually for the five culture dishes. Total RNA was isolated (Chomczynski and Sacchi, 1987) for Northern blot analysis from a subset of the cells at the time of subculture (t = 0, Fig. 1) and from a suitable number of culture dishes at subsequent 24 h intervals. To determine the intracellular level of MGP, cultures were lysed in 6 M guanidine HCl, and aliquots of 5 µl or less were analyzed for MGP by radioimmunoassay (Otawara and Price, 1986).

To test the effect of extracellular matrix on MGP expression, 4×10^5 cells were plated on 100 mm dishes that were either not coated or coated by procedures that achieve protein densities of 2.5 µg/cm^2 human fibronectin, 150 µg/cm^2 Matrigel, or 5 µg/cm^2 rat tail collagen Type I. After three days of culture with daily medium changes, cells were harvested and counted, and RNA was prepared as described above.

To test the effect of medium conditioned by confluent NRK cells on the expression of MGP by sparse cultures of NRK cells, two-day postconfluent cells (t = 0) were subcultured at a 1 : 30 ratio. The resulting daughter plates were treated with 10 ml of fresh medium or 10 ml of 60% conditioned medium obtained from three day postconfluent NRK cultures and containing 1.2 µg MGP antigen per ml. Cells were harvested at the time of subculture and after 72 h culture under each condition, and total RNA was isolated and analyzed by Northern blot. The relative densities of the MGP mRNA bands were determined by densitometer scan.

Northern analysis

Total RNA was fractionated on 1.4% agarose/formaldehyde gels in a 3-[N-morpholino] propanesulphonic acid buffer and transferred to a nylon membrane (0.45 µm; Nytran, Schleicher, and Schuell, Keene, NH) as previously described (Selden, 1993). Relative mRNA levels were determined using rat MGP (Price et al., 1987) and rat β-actin (Nudel et al., 1982) cDNA clones. The cDNAs were separated from the cloning vectors by gel purification, followed by electroelution (Elutrap, Schleicher and Schuell) and labeled with [α-^{32}P] dCTP using a Random Primed DNA Labeling Kit (Boehringer Mannheim) to a specific activity of at least 1×10^8 cpm/µg. Uniformity of the RNA loads and successful RNA transfer to the membrane were verified by staining the gels with ethidium bromide prior to transfer. RNA was visualized both in the gel and in the membrane by ultraviolet illumination (300 nm). Hybridization to RNA immobilized on Nytran was typically performed for 17 h at 42°C with $1–5 \times 10^6$ cpm DNA probe per ml of 50% formamide solution containing 5× SSPE [1 × SSPE is 180 mM NaCl, 10 mM $NaPO_4$ (pH 7.4), and 1 mM EDTA], 5 × Denhardt's, and 100 µg/ml salmon sperm DNA after prehybridization at 42°C in the same solution without the cDNA probe. Filters were washed in 0.1–0.5 × SSPE containing 0.2% sodium dodecyl sulfate for 10 min at room temperature, followed by 1 h at 55°C. Autoradiography was performed at −70°C with Kodak XAR-5 film (Eastman Kodak, Rochester, NY) and DuPont Cronex Lightning Plus screens (DuPont, Wilmington, DE). Probes were removed from the membranes by washing at 65°C for 30–60 min with 50–60% formamide in 6 × SSPE. After autoradiography, the relative intensities of the MGP and β-actin mRNA

bands in the autoradiographs were determined using an LKB Ultrascan XL laser densitometer (Rockville, MD). Unless otherwise stated in the figure legend, small variations in the RNA loads were corrected by dividing the relative intensities obtained for MGP by those obtained for β-actin in the same lane.

Run-off transcription assay

Preparation of nuclei. To obtain nuclei from sparse cells, confluent NRK cells were subcultured at a 1 : 30 ratio and harvested 48 h later. To obtain nuclei from postconfluent cells, NRK cells were maintained for five days after confluence with daily medium changes and then harvested. Nuclei were isolated by dounce homogenization after lysis of the cells in NP-40 buffer, as described (Selden, 1993). Nuclei were counted using an haemocytometer, resuspended at a concentration of 2.5×10^8 nuclei per ml in glycerol storage buffer (50 mM Tris-Cl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA), aliquoted into prechilled eppitubes (200 µl per tube), frozen in liquid nitrogen, and stored at −70°C.

Binding of DNA to nitrocellulose membrane. Plasmids containing either no cDNA insert or rat MGP cDNAs were linearized with the appropriate restriction enzymes, denatured by treatment with 0.1 volume of 1 M NaOH for 30 min at room temperature, neutralized using nine volumes of 6 × SSC (20 × SSC is 3 M NaCl, 0.3 M Na_3 citrate, pH 7.0) and placed on ice. DNA was then spotted onto a nitrocellulose membrane (Schleicher and Schuell, 0.45 µm, BAS-85) using a slot blot apparatus (5 µg DNA per slot), air dried overnight at room temperature, and baked for 2 h at 80°C under vacuum. Membranes were stored in sealed plastic bags at 4°C until used. The identity of the DNA bound to the nitrocellulose filters was confirmed by hybridizing one set of filters to rat MGP cDNA.

Labeling of run-off transcripts. Nuclear run-off transcripts were labeled as described (Selden, 1993) using 4×10^7 nuclei and 200 µCi of (α-^{32}P) UTP per reaction. The labeling was performed at 30°C for 30 min. The samples were then sequentially treated with RNase-free DNase I (90 units per sample, 5 min at 30°C) and proteinase K (200 µg per sample, 30 min at 42°C) and extracted with one volume of phenol/chloroform/isoamyl alcohol (25:24:1). The labeled RNA was precipitated with 10% TCA/60 mM sodium pyrophosphate for 30 min on ice using yeast tRNA as a carrier and recovered by filtration into Whatman GF/A glass filters. After DNase I treatment (150 units, 30 min, 37°C), labeled RNA was eluted from the filters at 65°C using an SDS/Tris/EDTA buffer, treated with proteinase K (90 µg, 30 min, 37°C), phenol-extracted, and precipitated overnight at −20°C as described (Selden, 1993). Labeled RNA transcripts were recovered by centrifugation at 10,000 g for 30 min at 4°C, resuspended in 1 ml of TES buffer (10 mM TES [N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid], pH 7.4; 10 mM EDTA; 0.2% SDS) and counted after spotting 5 µl of each sample in duplicate onto Whatman GF/A glass fiber filters. Samples were diluted where needed with TES buffer to achieve 10×10^6 cpm of ^{32}P-labeled RNA per ml.

Hybridization with run-off transcripts. RNA transcripts were diluted 1:1 with TES-buffer containing 0.6 M NaCl and hybridized to DNA immobilized on strips of nitrocellulose as described above using a total volume of 2 ml RNA solution per condition. Hybridization was performed for two days at 65°C in 5 ml plastic scintillation vials. Filters were then individually washed twice in 25 ml of 2 × SSC for 1 h at 65°C, treated with RNase A (80 µg, 30 min, 37°C), washed once more with 25 ml of 2 × SSC for 1 h at 37°C, blotted dry on Whatman 3 MM paper, and analyzed by autoradiography and densitometry. The hybridizations were performed in triplicate for the sparse cells and duplicate for the five-day confluent cells.

Growth factor treatment

The MGP response to growth factors was first tested in NRK cells, which had just reached confluence. Treatment was initiated by exchanging the cell culture medium with fresh medium containing the desired concentration of EGF, bFGF, PDGF, or TGFβ. The conditioned medium was subsequently exchanged for fresh medium containing the same concentration of the respective growth factor every 12 h. The effect of serum-free culture medium was evaluated in the same experiment by exchanging culture medium with serum-free medium or with serum-free medium plus TGFβ at the start of the experiment and every subsequent 12 h. Conditioned medium levels of MGP were determined by radioimmunoassay in the 0–12, 12–24, 24–36, and 36–48 h conditioned medium from each of the four 35 mm plates in the respective treatment groups. Each MGP level depicted in Figure 4 is the average of the MGP levels determined in the four separate culture dishes. After 48 h treatment, RNA was extracted from the four plates in each treatment group, and 30 µg of this RNA was then analyzed by Northern blot.

In order to determine the time course of the MGP response to EGF in postconfluent, nondividing NRK cells, cells were first cultured until confluent in Coon's F12 medium containing 10% fetal calf serum and then maintained for four days with daily medium changes. EGF treatment was initiated by the replacement of medium with fresh medium containing either 10 ng/ml EGF or no EGF (control). The medium was subsequently exchanged every 6 h for fresh control medium or medium containing 10 ng/ml EGF. RNA was extracted from two 100 mm control and two 100 mm EGF-treated plates at 12, 24, 36, and 48 h. 30 µg of the RNA from each culture plate were then analyzed by Northern blot. The medium levels of MGP shown in Figure 5 were determined by radioimmunoassay in every 6 h conditioned medium from each of three control and three EGF-treated plates.

The dose-dependence of the MGP response to EGF was also measured in postconfluent, nondividing NRK cells. Cells were cultured until confluent and then maintained for four days with daily medium changes. The medium was exchanged for fresh medium containing the desired concentration of EGF at four days postconfluence, and after 24 and 48 h. MGP levels were determined by radioimmunoassay in the 0–24, 24–48, and 48–72 h conditioned medium. The results shown in Figure 6 are the average MGP levels in the conditioned medium from three 35 mm plates at each EGF dosage.

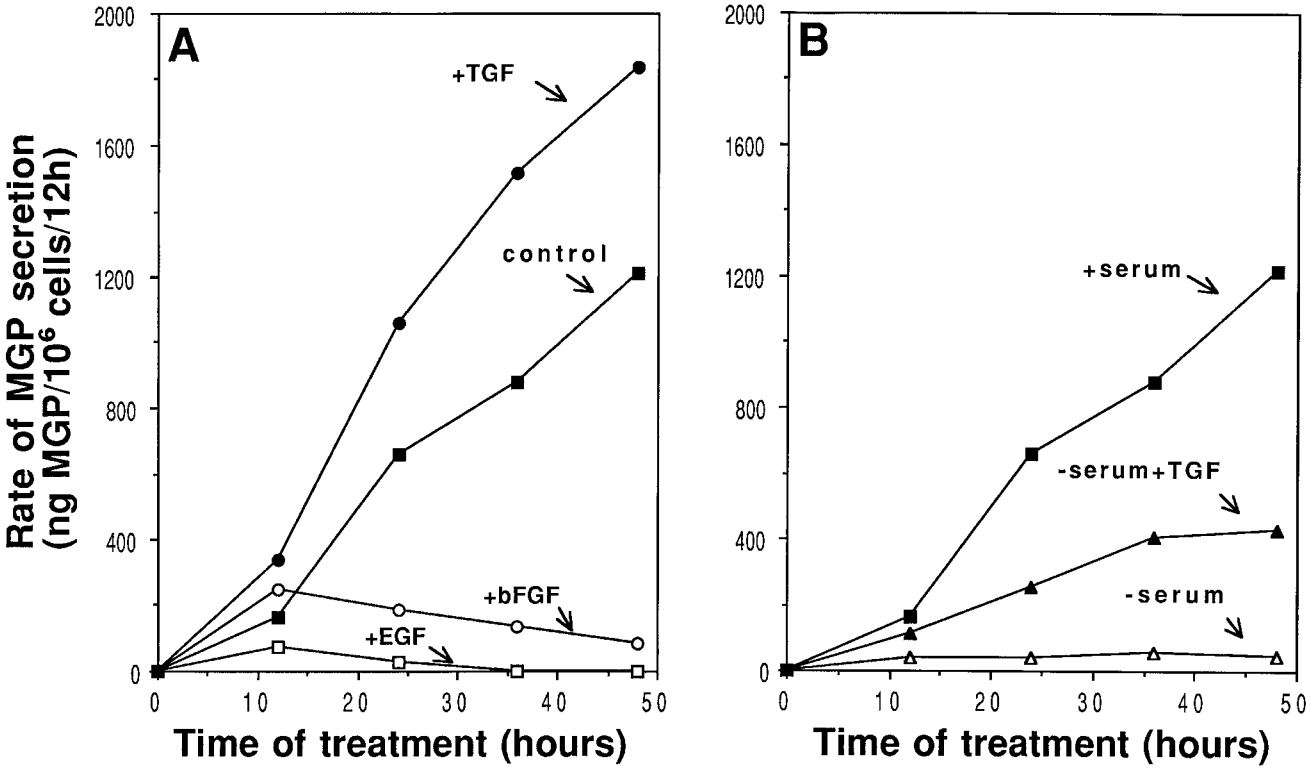

Fig. 4. Effect of EGF, bFGF, and TGFβ on MGP expression in confluent NRK cells. The medium of NRK cells that had just reached confluence was exchanged initially and every subsequent 12 h with the indicated fresh medium, and the levels of MGP in each 12 h conditioned medium were determined by radioimmunoassay. **A:** Rate of MGP secretion by NRK cells cultured for 48 h in medium containing 10% fetal calf serum alone (■), with 5 ng/ml EGF (□), with 5 ng/ml bFGF (○), or with 0.5 ng/ml TGFβ (●). **B:** Rate of MGP secretion by NRK cells cultured for 48 h in medium containing 10% fetal calf serum (■) or in serum-free medium alone (△), or with 0.5 ng/ml TGFβ. **C:** Northern blot analysis of MGP mRNA levels. NRK cells shown in panels (A) and (B) were harvested after 48 h of treatment, and total RNA was prepared and analyzed by Northern blot as described in Methods section.

RESULTS

Effect of cell density on MGP expression by normal rat kidney cells

To evaluate the effect of cell density on MGP expression, confluent NRK cells were seeded onto 100 mm plates at 1/30 of the original density, and the culture medium was subsequently changed daily for ten days. Some cultures were harvested daily for determination of cell number and of intracellular MGP antigen and MGP mRNA levels, and conditioned medium was collected daily for analysis of levels of MGP secretion. As can be seen in Figure 1, the level of MGP mRNA per culture plate fell dramatically over the first two days following subculture. The level of MGP mRNA per culture subsequently increased by 8×10^4 between days 2 and 10 of culture. Since the number of NRK cells per culture increased by only 33-fold over this period (Fig. 1), most of the increase in the level of MGP mRNA per culture is due to an increase in the expression of MGP mRNA per cell. The calculated increase in MGP mRNA level per cell between day 2 and day 10 is 2,400-fold. This increase is specific for MGP mRNA since the levels of β-actin mRNA were found to slightly decrease during the same period of time (see Fig. 1A), while ethidium bromide staining revealed that all lanes were loaded with equivalent amounts of total RNA (results not shown). The rate of MGP secretion per 10^6 cells also

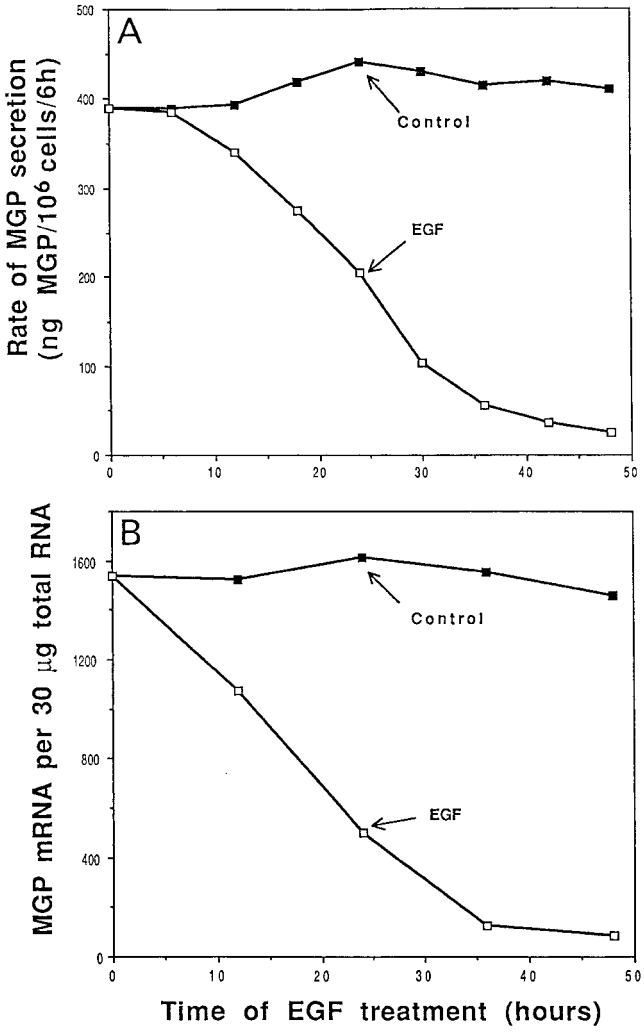

Fig. 5. Time course of the EGF effect on MGP expression in four-day postconfluent NRK cells. NRK cells were maintained at confluence for four days with daily medium changes in order to achieve a non-dividing state with high MGP expression (Figs. 1 and 2). Medium was exchanged with fresh serum containing medium alone or with 10 ng/ml EGF at the start of treatment and every subsequent 6 h. Medium levels of MGP were determined by radioimmunoassay in each 6 h conditioned medium. **A:** Rate of MGP secretion by NRK cells cultured for 48 h in the presence of 10% fetal calf serum (■) or 10% fetal calf serum and 10 ng/ml EGF (□). **B:** Northern blot analysis of MGP mRNA levels. Additional plates of NRK cells treated as in panel (A) were harvested after 12 h of treatment and every subsequent 12 h for a total of 48 h. Total RNA was prepared and analyzed by Northern blot, and the resulting autoradiograph was quantified by densitometer scanning as described in the Methods section.

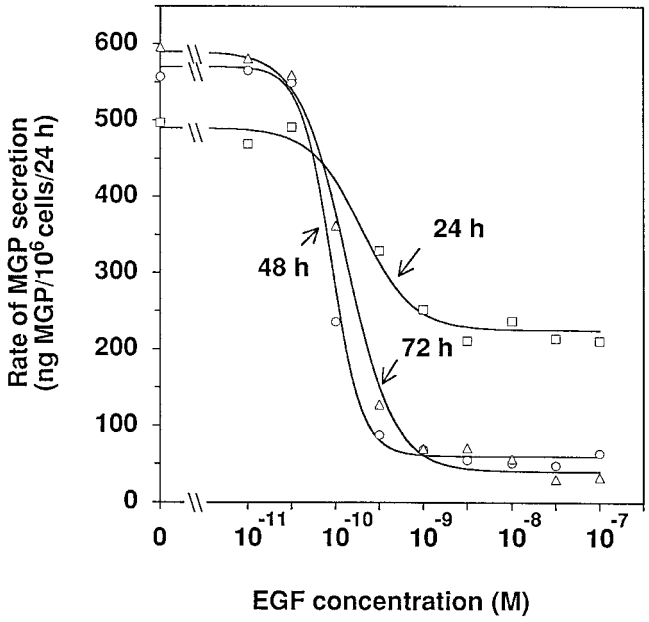

Fig. 6. Dose-dependence of the EGF effect on MGP expression in four-day postconfluent NRK cells. The medium of four-day postconfluent NRK cells was exchanged for fresh serum-containing medium with the indicated concentration of EGF initially and every subsequent 24 h. MGP levels were determined by radioimmunoassay in the 0–24 h (□), 24–48 h (○), and 48–72 h (△) conditioned medium samples.

increased with increasing cell density (Fig. 2). The 80-fold increase in the rate of MGP secretion per 10^6 cells between day 5 and day 10 (Fig. 2) is comparable to the 130-fold increase in MGP mRNA levels per cell over this period. Intracellular levels of MGP rose from 1.7 to 22 ng MGP per 10^6 cells between day 6, the first day at which intracellular levels could be detected, and day 10 (data not shown). This intracellular MGP level is only about 1% of the amount secreted by NRK cells in 24 h and probably represents MGP antigen within the secretory pathway of the cell.

This time course experiment has been repeated several times with identical results. We have also investigated in greater detail the decline in MGP mRNA, which occurs over the first two days following the 1 : 30 subculture. There is no discernable lag in the fall in MGP mRNA levels following subculture, and the decay in MGP mRNA levels is first order in MGP mRNA concentration with a half time of 6 h (data not shown). If cells are maintained at the 1 : 30 subculture density by replating every 48 h, MGP mRNA levels continue to decline until they become undetectable by Northern blot. To assess the possible effect of subculture on MGP expression, confluent NRK cells were trypsinized and replated at the same density. The level of MGP mRNA measured 24 h after subculture was essentially identical to that measured before subculture, and we therefore conclude that subculture itself does not inhibit MGP expression. A similar effect of cell density on MGP expression was also seen when confluent NRK cells were replated to different cell densities rather than grown to these densities following a 1 : 30 subculture. Two days after subculture, the MGP mRNA band on a Northern blot was very intense at the 10^7 cells per 100 mm plate initial subculture density, was barely detectable at the 2×10^6 cells per plate initial density, and was undetectable at the 5×10^5 cells per plate initial density.

Effect of cell density on MGP gene transcription

As could be anticipated from the magnitude of the density-dependent increase in MGP mRNA per cell,

there is a strong density-dependent increase in the rate of synthesis of MGP gene transcripts. The magnitude of the increase in MGP mRNA between days 2 and 10 following the 1 : 30 subculture, about 2,400-fold (Fig. 1B), is far too large to be explained by message stabilization alone and must primarily reflect increased MGP gene transcription. To illustrate this, we first calculate the magnitude of MGP mRNA increase, which could maximally occur if there were no MGP mRNA degradation between days 2 and 10. Although we do not know the exact rate of MGP mRNA synthesis at day 2, we do know that it can be no greater than the rate needed to exactly balance the first-order degradation rate of MGP mRNA; that is, 50% every 6 h (data not shown). Therefore, at two days, 0.5 units of new MGP mRNA must be produced every 6 h to balance the MGP mRNA degradation rate of 50% every 6 h and so give the observed value of 1 unit (see Fig. 1B). If there is no increase in transcription and all degradation of MGP mRNA ceases at day 2, the final MGP mRNA level 8 days later would be $8 \times 24/6 \times 0.5 = 16$-fold greater. This is far lower than the observed 2,400-fold increase in MGP mRNA, so most of the increase in MGP mRNA level must be explained by increased MGP gene transcription. As seen in Figure 3, nuclei from five-day postconfluent NRK cells have a far larger number of nascent MGP transcripts than nuclei from preconfluent NRK cells. Although the strength of the MGP hybridization signal in the preconfluent cells was too near background to permit accurate quantification by densitometer scanning, we estimate that the nascent transcripts in preconfluent cells are less than 1% of those in the five-day postconfluent cells. Increased MGP gene transcription must therefore account for most of the 2,400-fold increase in MGP mRNA levels between the preconfluent and postconfluent cells (Fig. 1).

Nature of the cell density signal that stimulates MGP expression

Several experiments were carried out in order to determine the nature of the signals that stimulate MGP expression at high cell density. To test the effect of extracellular matrix on MGP expression, confluent NRK cells were disaggregated with trypsin and seeded at 1:10 of the original density onto plates coated with matrigel, type I collagen, or fibronectin. When MGP mRNA levels were examined by Northern blot three days later, no difference was noted in the MGP mRNA levels between uncoated control plates and plates coated with the extracellular matrix constituents; in each case, the MGP mRNA level was about 10% of that in the confluent NRK cells at the time of subculture (data not shown).

In order to examine the effect of NRK-conditioned medium on MGP mRNA levels, confluent NRK cells were seeded at 1/30 of the original density and cultured in fresh medium (control cells) or in a mixture of 40% fresh medium and 60% medium conditioned by 24 h of culture with three-day postconfluent NRK cells. Since the MGP antigen levels in this conditioned medium were high, 1.2 μg/ml, there should be, in principle, a high level of putative factors that stimulate MGP expression if the cell density dependence of MGP expression is indeed a reflection of the action of such conditioned medium factors. However, MGP mRNA levels examined after three days of culture in conditioned medium were only 4.4-fold higher than in control cells (data not shown), which is far less than the 70-fold increase that would be needed to raise MGP mRNA levels to the point that they equal those found in the high-density NRK cells that produced the conditioned medium used in these experiments. This experiment indicates that the enhanced MGP expression at high cell density cannot be explained solely by the action of conditioned medium factors.

Effect of growth factors on the expression of MGP by NRK cells

The observation that MGP expression increases by over 2,000-fold as sparse, rapidly growing NRK cell cultures progress to densely confluent, contact-inhibited cultures suggests that MGP could be important to cellular functions that are found only at high cell density. In addition, the near absence of MGP production in the sparse, rapidly dividing cultures suggests that high MGP expression is either incompatible with, or unnecessary for, exponential growth at low cell density. If low MGP expression is indeed a phenotypic feature of the rapid growth state, then it could be argued that growth factors should act on confluent NRK cell cultures to produce the same growth-potentiated state with an associated low level of MGP production as is found at low cell density.

Several growth factors were tested to evaluate their ability to modulate MGP expression in NRK cultures that had just reached confluence. As seen in Figure 4A, EGF and bFGF dramatically reduced the level of MGP in conditioned medium. In contrast, TGFβ stimulated MGP expression (Fig. 4A), and PDGF had no significant effect (data not shown). Conditioned medium levels of MGP were undetectable in cells treated from confluence with serum-free medium (Fig. 4B). Although addition of TGFβ to serum-free medium strongly induced MGP expression, TGFβ alone was unable to restore MGP expression to the level seen in serum-containing medium. The MGP mRNA response to each of these treatments (Fig. 4C) paralleled the changes seen in medium MGP levels.

The effect of EGF on MGP expression was also evaluated in NRK cell cultures that had been confluent for five days. These cultures produce MGP at the highest rate (Fig. 2) and are no longer increasing in cell number (Fig. 1B). As seen in Figure 5A, EGF strongly suppressed MGP secretion into media by the second 6 h assay increment and must therefore achieve its maximal effect on MGP expression by 6 h of treatment. After two days treatment with this dosage of EGF, MGP secretion per 6 h increment was only 6% of that in control cultures. EGF produced a parallel decrease in the level of MGP mRNA (Fig. 5B). Since there is no apparent lag in the decline in MGP mRNA following EGF treatment, it is likely that EGF depresses MGP gene transcription within a short time after the cells have been exposed to the growth factor.

To determine the dose-dependence of the EGF effect on MGP expression, five-day-postconfluent NRK cells were treated for one, two, or three days with different doses of EGF. As seen in Figure 6, the half maximal effect of EGF on MGP expression is found at approximately 0.1 nM EGF. This dose dependence is in good

agreement with that which would be expected based on the 0.11 nM dissociation constant for EGF binding to the high affinity EGF receptor in NRK cells (Masuda et al., 1992).

DISCUSSION

The present studies demonstrate that MGP expression is critically dependent on cell density in culture. MGP is secreted at a very high rate by postconfluent, nondividing NRK cells. A single 100 mm culture dish of five day postconfluent NRK cells will, for example, secrete into culture medium 0.1 mg of MGP every day; and MGP is the most abundant single protein that can be seen in the SDS-PAGE of this conditioned medium. In contrast to the high MGP expression at high cell density, subculture of NRK cells to 1 : 30 of the confluent density rapidly reduces the level of MGP mRNA to nearly undetectable levels by 48 h. Since NRK cells grow rapidly, there are already sufficient cells by 48 h so that the steep decline in MGP mRNA levels is reversed (Fig. 1B). Since there is no discernible lag in the fall in MGP mRNA levels following subculture from high density to low, this apparent cessation in MGP gene transcription must occur within a short time following subculture. The increase in MGP expression per cell from the lowest level in subconfluent cells at two days following the 1 : 30 subculture to the highest level in post confluent cells eight days later is over 2,000-fold. Much of this increase occurs after the cells have attained visual confluence (Fig. 1). These density-dependent changes in MGP expression are reversible and are recapitulated with each subculture during the routine maintenance of the NRK cell line.

Recently, several studies have established that cell density can strongly modulate gene expression in a number of in vitro cell culture systems, with cell density-dependent effects ranging from two- to 20-fold changes in the expression of specific proteins. For instance, in the rat hepatocyte cell line BRL-3A, the levels of insulin-like growth-factor-binding protein 2 mRNA increase 20-fold with increased cell density, while the expression of β tubulin decrease by 4.5-fold (Kutoh et al., 1995). In normal human skin fibroblasts, the increase in cell density from sparse, subconfluent cultures to dense, postconfluent cultures decreases message levels for collagenase and stromelysin by five- to 10-fold (Colige et al., 1992); increases message levels for collagen VI by two-fold (Hatamochi et al., 1989); and does not affect the message levels for collagen I, collagen III, fibronectin, TIMP, laminin, or laminin-binding protein (Hatamochi et al., 1989; Mäkelä et al., 1990; Munaut et al., 1991; Colige et al., 1992). The effect of cell density on MGP expression by NRK cells is substantially greater than that typically seen in other studies. This may be explained in part by the relatively rapid decrease in MGP mRNA levels following subculture and to the deeper split ratio used in the present studies. The induction of MGP expression by increased cell density is not unique to the NRK cell system. Our initial discovery of the density-dependent induction of MGP expression was made, in fact, in human fibroblasts and in the MG-63 human osteosarcoma cell line. NRK cells were chosen for the present investigation in part because a radioimmunoassay was available for the detection of rat MGP but not for human MGP, and so it was possible to monitor the effect of cell density on MGP expression at both the level of message and protein. In these earlier, preliminary experiments, we found an eight-fold increase in MGP mRNA expression per cell with a three-fold increase in the density of normal human fibroblasts, and a 20-fold increase in MGP mRNA level per cell with an 8-fold increase in the density of the MG-63 human osteosarcoma cells. The possibility that MGP is strongly induced by cell density in many cell types is further supported by the previous observations that MGP is expressed by the ROS 17/2 rat osteoblastic cell line only when the cells are in a high-density, nondividing state (Fraser and Price, 1990) and that normal rat osteoblasts and chondrocytes only express high MGP levels after they have been maintained in a state of high cell density for several days (Barone et al., 1991).

The mechanism for the density dependence of MGP expression is presently unclear. Nuclear run off experiments showed that the dramatic increase in MGP expression with increasing cell density is accompanied by a corresponding increase in gene transcription, and it is therefore possible that the entire over-10^3-fold increase in MGP expression with increasing cell density is due to the increased transcription of the MGP gene. There are several candidates for the extracellular signal(s) that could be responsible for the increase in MGP expression with increasing cell density. (1) NRK cells deposit an extracellular matrix with increasing density, and elements of this matrix may interact with cell receptors, such as integrins, to promote MGP expression. Although the direct test of laminin, collagen type I, and fibronectin did not show that these matrix constituents could stimulate MGP expression, the actual matrix produced by NRK cells in culture is likely to be different than can be achieved by coating plastic dishes with these purified molecules; we cannot therefore exclude the possible contribution of cell-matrix interactions to the induction of MGP expression at high density. (2) NRK cells may secrete a factor or factors into culture medium, which can induce MGP gene expression. The concentration of these factors which is attained in the 24 h interval between medium changes would be a function of the number of cells on the plate and could therefore account for the cell density dependence of MGP expression. In the present experiments, we have, however, observed only a rather modest increase in MGP gene expression from the culture of cells in medium conditioned by high-density cultures; and it seems unlikely that such autocrine effects can account for the over-10^3-fold increase in MGP expression with high cell density. (3) Cell–cell contact may induce increased MGP expression. Although we have not tested this possibility directly, NRK cells do divide rapidly following subculture to low density (Fig. 1), and we have observed that the cell progeny tend to distribute near one another so as to create a somewhat non-uniform cell distribution on the plate. Cell contact therefore occurs in some areas within two days of a 1:30 subculture, and the increase in such contacts as the cells continue to divide roughly parallels the subsequent increase in MGP expression.

We have also tested the effect of growth factors on MGP expression in order to evaluate the possibility that the cell density dependence of MGP expression

may be only one aspect of a more general inverse relationship between cell growth and MGP expression. These studies demonstrated that treatment with EGF or bFGF strongly suppresses MGP expression by dividing cultures of NRK cells and therefore support the view that cell growth and MGP expression are inversely related. We also observed, however, that EGF strongly suppressed MGP expression in NRK cells, even when the cells are in a contact-inhibited, non-dividing state. This result indicates that the effect of EGF on MGP expression is not merely a secondary consequence of its ability to increase the rate of NRK cell division.

A possible rationale for the suppression of MGP expression by EGF in non-dividing cells could be that EGF elicits changes in the non-dividing cell at high density that allow the cell to subsequently divide when other signals are present. The down-regulation of MGP expression by EGF could, for example, weaken the cell–cell and cell–matrix contacts that are normally part of the mechanism by which cell division is inhibited at high cell density and thereby enable cells to divide in response to signals other than EGF itself. This hypothesis is supported by the observation that treatment of 30-day post confluent, non-dividing cultures of normal human fibroblasts with EGF for two days causes a five- to ten-fold reduction in collagen I message and a 50- to 2000-fold increase in collagenase message with no associated stimulation of cell division (Colige et al., 1992). It seems probable that decreased collagen expression and increased collagenase expression by these hyperconfluent fibroblasts act in concert to reduce the level of collagen in the extracellular matrix and that reduced collagen levels allow the cell to migrate or divide when other signals are present. Treatment of 30-day postconfluent fibroblasts with EGF also induces stromelysin by ten- to 200-fold (Colige et al., 1992). Since stromelysin can cleave extracellular matrix constituents in addition to collagen, its induction by EGF would be expected to further enable hyperconfluent fibroblasts to divide when other signals are present. These observations suggest that the dramatic down-regulation of MGP expression that occurs in post confluent NRK cells treated with EGF is part of a broader pattern of gene responses to EGF in contact inhibited, post confluent cells that act in concert to enable cells to divide when other signals are present. If the suppression of MGP synthesis in response to EGF, in fact, enables cells to divide in response to other signals, one could further argue that the as yet unknown function of MGP in postconfluent cultures interferes with cell division.

While the function of MGP is not yet known, previous studies have suggested that it could be important for the maintenance of a differentiated state (Briehl and Miesfeld, 1991; Cancela and Price, 1992; Shanahan et al., 1993). In the present studies, we have shown that MGP expression is stimulated by TGFβ, a protein that is known to modulate differentiated function in some cell types (Massagué, 1990), and that MGP expression is highest in post confluent, non-dividing cells. It has been shown previously that maintenance in such a high-density state is necessary for the expression of a differentiated phenotype by cells, such as osteoblasts and chondrocytes in culture (Barone et al., 1991). If MGP does contribute to changes in the phenotypic characteristics of some cells, it is likely that MGP elicits these changes by binding to the cell. Since the mature form of MGP can be modified in vivo by proteolytic cleavages at different sites (Hale et al., 1991) and can be phosphorylated in vivo to different extents (Price et al., 1994), we are currently evaluating the cell binding properties of the different phosphorylation isoforms and proteolytically derived variants of MGP.

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LITERATURE CITED

Akhoundi, C., Amiot, M., Auberger, P., Le Cam, A., and Rossi, B. (1994) Insulin and interleukin-1 differentially regulate PP63, an acute phase phosphoprotein in hepatoma cell line. J. Biol. Chem., *269:*15925–15930.

Barone, L.M., Owen, T.A., Tassinari, M.S., Bortell, R., Stein, G.S., and Lian, J.B. (1991) Developmental expression and hormonal regulation of the rat matrix Gla protein (MGP) gene in chondrogenesis and osteogenesis. J. Cell. Biochem., *46:*351–365.

Briehl, M.M., and Miesfeld, R.L. (1991) Isolation and characterization of transcripts induced by androgen withdrawal and apoptotic cell death in the rat ventral prostate. Mol. Endocrinol., *5:*1381–1388.

Cancela, M.L., and Price, P.A. (1992) Retinoic acid induces matrix Gla protein gene expression in human cells. Endocrinol., *130:*102–108.

Cancela, L., Hsieh, C.-L., Francke, U., and Price, P.A. (1990) Molecular structure, chromosome assignment and promoter organization of the human matrix Gla protein gene. J. Biol. Chem., *265:*15040–15048.

Chen, L., O'Bryan, J.P., Smith, H.S., and Liu, E. (1990) Overexpression of matrix Gla protein messenger RNA in malignant human breast cells—isolation by differential cDNA hybridization. Oncogene, *5:*1391–1395.

Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid-guanidinium-thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162:*156–159.

Colige, A.C., Lambert, C.A., Nusgens, B.V., and Lapière, C.M. (1992) Effect of cell–cell and cell–matrix interactions on the response of fibroblasts to epidermal growth factor in vitro-expression of collagen Type-1, collagenase, stromelysin and tissue-inhibitor of metalloproteinases. Biochem. J., *285:*215–221.

Ek-Rylander, B., Flores, M., Wendel, M., Heinegård, D., and Andersson, G. (1994) Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase-modulation of osteoclast adhesion in vitro. J. Biol. Chem., *269:*14853–14856.

Fraser, J.D., and Price, P.A. (1988) Lung, heart and kidney express high levels of mRNA for the vitamin K-dependent matrix Gla protein. J. Biol. Chem., *263:*11033–11036.

Fraser, J.D., and Price, P.A. (1990) Induction of matrix Gla protein synthesis during prolonged 1,25-dihydroxyvitamin D_3 treatment of osteosarcoma cells. Calcif. Tiss. Int., *46:*270–279.

Hale, J.E., Fraser, J.D., and Price, P.A. (1988) The identification of matrix Gla protein in cartilage. J. Biol. Chem., *263:*5820–5824.

Hale, J.E., Williamson, M.K., and Price, P.A. (1991) Carboxyl–terminal proteolytic processing of matrix Gla protein. J. Biol. Chem., *266:*21145–21149.

Hatamochi, A., Aumailley, M., Mauch, C., Chu, M.-L., Timpl, R., and Krieg, T. (1989) Regulation of collagen VI expression in fibroblasts. Effects of cell density, cell–matrix interactions and chemical transformation. J. Biol. Chem., *264:*3494–3499.

Kutoh, E., Schwander, J., and Margot, J.B. (1995) Cell density dependent modulation of the rat insulin-like growth factor binding protein 2 and its gene. Eur. J. Biochem., *234:*557–562.

Mäkelä, J.K., Vuorio, T., and Vuorio, E. (1990) Growth dependent modulation of Type-1 collagen production and messenger RNA levels in cultured human skin fibroblasts. Biochim. Biophys. Acta, *1049:*171–176.

Massagué, J. (1990) The transforming growth factor β-family. Ann. Rev. Biol., *64:*597–641.

Masuda, A., Kizaka-Kondoh, S., Miwatani, H., Terada, Y., Nojima, H., and Okayama, H. (1992) Signal transduction cascade shared by epidermal growth factor and platelet-derived growth factor is a major pathway for oncogenic transformation in NRK cells. New Biologist, *4:*489–503.

Meggio, F., Boulton, A.P., Marchiori, F., Borin, G., Lennon, D.P.W., Calderan, A., and Pinna, L.A. (1988) Substrate-specificity determinants for a membrane-bound casein kinase of lactating mammary gland. Eur. J. Biochem., *177:*281–284.

Munaut, C., Noël, A., Sobel, M., and Foidart, J.M. (1991) Expression of laminin by human fibroblasts, HT-1080 fibrosarcoma cells and MCF-7 breast adenocarcinoma cells—lack of regulation by the cell density and extracellular matrix. Cell Biol. Intl. Rep., *15:*499–509.

Nudel, U., Katcoff, D., Zakut, R., Shani, M., Carmon, Y., Finer, M., Czosnek, H., Ginsburg, I., and Yaffe, D. (1982) Isolation and characterization of rat skeletal muscle and cytoplasmic actin genes. Proc. Natl. Acad. Sci. U.S.A., *79:*2763–2767.

Otawara, Y., and Price, P.A. (1986) Developmental appearance of matrix Gla protein during calcification in the rat. J. Biol. Chem., *261:*10828–10832.

Price, P.A., and Williamson, M.K. (1985) Primary structure of bovine matrix Gla protein, a new vitamin K-dependent bone protein. J. Biol. Chem., *260:*14971–14975.

Price, P.A., Urist, M.R., and Otawara, Y. (1983) Matrix Gla protein, a new γ-carboxyglutamic acid-containing protein which is associated with the organic matrix of bone. Biochem. Biophys. Res. Commun., *117:*765–771.

Price, P.A., Fraser, J.D., and Metz-Virca, G. (1987) Molecular cloning of matrix Gla protein: Implications for substrate recognition by the vitamin K-dependent γ-carboxylase. Proc. Natl. Acad. Sci. U.S.A., *84:*8335–8339.

Price, P.A., Rice, J.S., and Williamson, M.K. (1994) Conserved phosphorylation of serines in the Ser-X-Glu/Ser(P) sequences of the vitamin K-dependent matrix Gla protein from shark, lamb, rat, cow, and human. Protein Sci., *3:*822–830.

Rannels, S.R., Cancela, M.L., Wolpert, E.B., and Price, P.A. (1993) Matrix Gla protein mRNA expression in cultured Type II pneumocytes. Amer. J. Physiol., *265:*L270–L278.

Rice, J.S., Williamson, M.K., and Price, P.A. (1994) Isolation and sequence of the vitamin K-dependent matrix Gla protein from the calcified cartilage of the soupfin shark. J. Bone Min. Res., *9:*567–576.

Selden, R.F. (1993) Identification of newly transcribed RNA. In: Current protocols in molecular biology. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds. Greene Publishing Associates and Wiley-Interscience, Brooklyn, New York, Unit 4-10.

Shanahan, C.M., Weissberg, P.L., and Metcalfe, J.C. (1993) Isolation of gene markers of differentiated and proliferating vascular smooth muscle cells. Circul. Res., *73:*193–204.

Shanahan, C.M., Cary, N.R.B., Metcalfe, J.C., and Weissberg, P.L. (1994) High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. J. Clin. Invest., *93:*2393–2402.