

## Phosphate stimulates Matrix Gla Protein expression in chondrocytes through the ERK signaling pathway

M. Julien<sup>1</sup>, D. Magne<sup>2</sup>, M. Masson<sup>1</sup>, M. Rolli-Derkinderen<sup>3</sup>, O. Chassande<sup>4</sup>, C. Cario-Toumaniantz<sup>3</sup>, Y. Chere<sup>5</sup>, P. Weiss<sup>1</sup>, J. Guicheux<sup>1\*</sup>

<sup>1</sup>INSERM UMRS 791, Laboratory of Osteoarticular and Dental Tissue Engineering; University of Nantes, 1 Place Alexis Ricordeau, 44042 Nantes cedex 1, France.

<sup>2</sup>INSERM-ERI 002, LR2B; ULCO, Boulogne-sur-mer, France.

<sup>3</sup>INSERM U533; University of Nantes, France.

<sup>4</sup>INSERM U443; Biomaterials and tissue repair, University of Bordeaux II, Bordeaux, France.

<sup>5</sup>INRA/ENVN UMR 703, National veterinary school, Department of anatomopathology, Nantes, France.

\* Correspondence to

Dr J. Guicheux, INSERM U791, Laboratory of Osteoarticular and Dental Tissue Engineering; University of Nantes, 1 Place Alexis Ricordeau, 44042 Nantes cedex 1, France. Phone +33 240412919. Fax +33240083712. Email: [Jerome.guicheux@nantes.inserm.fr](mailto:Jerome.guicheux@nantes.inserm.fr)

### Short title

Phosphate stimulates MGP expression via ERK

## **Abstract**

Whereas increasing evidences suggest that inorganic phosphate (Pi) may act as a signaling molecule in mineralization-competent cells, its mechanisms of action remain largely unknown. The aims of the present work were to determine whether Pi regulates expression of matrix Gla protein (MGP), a mineralization inhibitor, in growth plate chondrocytes and to identify the involved signaling pathways. Chondrogenic ATDC5 cells and primary growth plate chondrocytes were used. Messenger RNA analysis was performed by RT-PCR and real-time quantitative PCR. Activation and role of mitogen-activated protein kinases (MAPK) were respectively determined by Western blotting and the use of specific inhibitors. Immunohistological detection of extracellular signal-regulated kinase 1 and 2 (ERK1/2) was performed in rib organ cultures from newborn mice. Results indicated that Pi markedly stimulated expression of MGP in ATDC5 cells and primary growth plate chondrocytes. Investigation of the involved intracellular signaling pathways revealed that Pi activated ERK1/2. The activation of ERK1/2 appeared cell-specific. Indeed, although Pi stimulated ERK1/2 in MC3T3-E1 osteoblasts and ST2 stromal cells, ERK1/2 phosphorylation could not be detected in L929 fibroblasts or C2C12 myogenic cells. Accordingly, immunohistological detection of ERK1/2 phosphorylation in rib growth plates revealed a marked signal in chondrocytes. Finally, a specific ERK1/2 inhibitor, UO126, blocked Pi-stimulated MGP expression in ATDC5 cells, indicating that ERK1/2 mediates, at least in part, the effects of Pi. These data demonstrate for the first time that Pi regulates MGP expression in growth plate chondrocytes, thereby suggesting a key role for Pi and ERK1/2 in the regulation of bone formation.

**248 words**

Key words: phosphate, growth plate chondrocytes, matrix Gla protein, ERK1/2

## Introduction

Longitudinal bone growth occurs by endochondral ossification. In this process, a growth plate cartilage template is replaced by bone in a temporally and spatially coordinated manner (1). Chondrogenesis is initiated with condensation of mesenchymal precursor cells, which is followed by their differentiation into chondrocytes. In a second step, growth plate chondrocytes enter a phase of proliferation before they differentiate into hypertrophic chondrocytes. These chondrocytes mineralize their extracellular matrix (ECM) and undergo apoptosis; they are finally replaced by osteoblasts that produce a bony matrix (2). Many extracellular factors, including growth factors and hormones, are involved in the regulation of chondrogenesis, chondrocyte proliferation and differentiation (3). Among these numerous factors, extracellular inorganic phosphate (Pi) has been proposed as a regulator of growth plate chondrocyte differentiation, apoptosis, and ECM mineralization (4-6). Phosphate homeostasis in mammals is mainly regulated by three organs: kidney, intestine, and bone. Deregulation of the proteins or hormones that control phosphatemia can conduct to hypophosphatemic rickets and osteomalacia characterized by defective cartilage and bone formation (7, 8). Conversely, hyperphosphatemia which is associated with chronic renal disease may induce vascular calcification (9). Interestingly, vascular calcification is likely due to Pi-induced transdifferentiation of vascular smooth muscle cells (VSMC) toward bone-like cells (9). Although the mechanisms by which phosphate induces vascular calcification are not yet fully elucidated, it has been proposed that it could be related to the ability of phosphate to downregulate matrix Gla protein (MGP) expression (10).

MGP is mainly expressed in VSMC and chondrocytes (11). MGP-deficient mice develop severe calcification of arteries and cartilage, attributing a role for MGP as a calcification inhibitor (12). Overexpression of MGP in chick limb bud has also shown that MGP could inhibit both cartilage mineralization and endochondral ossification (13). An *in vitro* study in ATDC5 mouse chondrogenic cell line has finally shown that MGP was expressed in late hypertrophic cells, and controlled both apoptosis and mineralization (14), therefore confirming a role for MGP in regulating mineralization by chondrocytes. Since Pi has been suggested to be a regulator of this late differentiation stage of growth plate chondrocytes (6), we therefore speculate that Pi might modulate MGP expression in growth plate chondrocytes. Despite the large body of evidence indicating that Pi is a specific signal for differentiation of chondrocytes (6), osteoblasts (15), and VSMC (9), the intracellular signaling pathways

activated by Pi are poorly investigated. Only one recent study indicates that Pi modulates osteopontin gene expression in osteoblastic cells through a well defined member of the mitogen-activated protein kinases (MAPK) (16). MAPK are members of the family of serine/threonine kinases. All of the MAPK pathways consist in cascades of phosphorylation in which MAPK-kinase-kinases (MKKK) first activate downstream MAPK kinases (MKK) which then phosphorylate MAPK. Targets of MAPK include cytoplasmic proteins and transcription factors (17). Three major MAPK-dependent signaling cascades have been identified in mammalian cells: extracellular signal regulated kinases (ERK1/2), p38 kinases, and c-Jun-N-terminal kinases (JNK1/2). The role of MAPK signaling pathways in regulating chondrocyte proliferation and differentiation has been widely investigated (18-20). Surprisingly and despite growing evidences indicating a role for MAPK and Pi in chondrocyte differentiation, the effect of Pi on signaling pathways in growth plate chondrocytes has not yet been investigated.

Viewing the above mentioned data and to better understand the molecular mechanisms induced by Pi in chondrocytes, we sought to investigate the effects of Pi on MGP expression and MAPK activation in ATDC5 cells and primary mouse chondrocytes. Here, we demonstrate for the first time that Pi stimulates expression of MGP at least through the ERK1/2 signaling pathway in growth plate chondrocytes.

## Materials and Methods

### Materials

Cell culture plastic ware was purchased from Corning-Costar (Corning BV Life Sciences, Schiphol-Rijk, Netherlands). Fetal calf serum (FCS) was obtained from D. Dutscher (Brumath, France). A 1:1 mixture of DMEM and Ham's F12 medium (DMEM/F12) was provided by ICN Biochemicals (Orsay, France).  $\alpha$ -MEM, MEM, DMEM, L-glutamine, penicillin and streptomycin (P/S), trypsin/EDTA, TRIzol reagent, DNase, dNTPs, TaqDNA polymerase, NuPAGE™ 4-12% Bis-Tris gel, and PVDF Invitrolon membrane were obtained from Invitrogen Corporation (Paisley, UK). Anisomycin, dimethylsulfoxide (DMSO), bovine insulin, transferrin, sodium selenite, amphotericin B, gentamicin, protease, collagenase, ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid tetrasodium salt (EGTA), dithiothreitol,  $\beta$ -glycerophosphate, sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ), phenylmethanesulfonyl fluoride (PMSF), sodium fluoride (NaF),  $\beta$ -mercaptoethanol, sodium dodecyl sulphate (SDS), and Bovine Serum Albumin (BSA) were purchased from Sigma-Aldrich Corporation (St Quentin Fallavier, France). UO126 was purchased from CalBiochem (Merck Eurolab, Germany). Avian myeloblastosis virus-reverse transcriptase (AMV-RT), random hexaprimers, and recombinant ribonuclease inhibitor (RNasin) were purchased from Promega (Charbonnières, France). SyBr Green detection was obtained from Molecular Probes Inc. (The Netherlands) and Titanium *Taq* DNA polymerase from Clontech.

Protein content was determined using the Pierce Coomassie Plus assay (Pierce, Rockford, IL, USA). The anti-phospho-ERK1/2, phospho-JNK1/2, phospho-p38, ERK1/2, p38, JNK1/2 and anti-rabbit IgG HRP-linked antibodies were purchased from Cell Signaling Inc. (Beverly, MA). Western blotting Detection System was obtained from Amersham Biosciences. Goat serum X0907 was obtained from Dako. Immunoreactivity was detected with a streptavidin-biotin-peroxidase technique (P0397, Dako) and 3, 3'-diaminobenzidine was used as a chromogen (K3465, Dako).

### Cells and culture conditions

ATDC5 cells (21) were used with low passages and were routinely grown in maintenance medium consisting of DMEM/F12 (1:1) containing 5% FCS, 1% P/S, and 1% L-glutamine. Cells were subcultured once a week using trypsin/EDTA and maintained at 37°C in a

humidified atmosphere with 5% CO<sub>2</sub> in air. To induce chondrogenesis and nodule formation, ATDC5 cells (1.5 x 10<sup>4</sup>/cm<sup>2</sup>) were seeded in a differentiation medium consisting of maintenance medium supplemented with 10µg/ml of bovine insulin (I), 10µg/ml human transferrin (T), and 3 x 10<sup>-8</sup>M sodium selenite (S) and cultured for 21 days. Medium was replaced every two days. To reduce the non-specific effects of agonists present in culture medium, cells were incubated in ITS-free MEM with 0.5% FCS for 24h before stimulation with inorganic phosphate (Pi). Pi was used as a mixture of NaH<sub>2</sub>PO<sub>4</sub> and Na<sub>2</sub>HPO<sub>4</sub> (pH 7.2). When UO126 was added, an equivalent amount of DMSO was used as control.

Low-passage MC3T3-E1 cells (22) were cultured for 8-10 days (10,000 cells/cm<sup>2</sup>) in  $\alpha$ -MEM containing 10% FCS, 1% P/S, and 1% L-glutamine. Cells were cultured at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in air and medium was replaced every two days. Pi was added 24h after incubation in MEM containing low-serum (0.5%). The fibroblastic L929 cell line (23), the myogenic C2C12 cell line (24), and the stromal ST2 cell line (25) were grown in DMEM supplemented with 10% FCS, 1% L-glutamine, and 1% P/S. Cells were cultured until confluence at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in air and medium was replaced every two days. Pi was added 24h after incubation in medium containing low-serum (0.5%).

Primary chondrocytes were prepared from ventral rib cages of one to three days old mice as previously described (26). Briefly, chondrocytes were isolated from rib cages by digestion of cartilages with bacterial collagenase after complete elimination of soft tissues by preliminary digestions with pronase and bacterial collagenase. Cells were then plated at a density of 50 000 cells/cm<sup>2</sup> and cultured in DMEM containing 10% FCS, 1% P/S, and 1% L-glutamine for 10 days at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in air. Medium was replaced every two days. These cells were previously demonstrated to express the major chondrocytic markers including type II collagen and type X collagen (26, 27). Pi was added 24h after incubation in medium containing low-serum (0.5%).

### **RNA isolation**

Cells were seeded in 25cm<sup>2</sup> flasks for RNA isolation. Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. Briefly, lysis of the cells in TRIzol was followed by centrifugation at 10,000g for 15min, at 4°C in the presence of chloroform. The upper aqueous phase was collected, and the RNA was precipitated by addition of isopropanol and centrifugation at 7,500g for 5min, at 4°C. RNA pellets were washed with cold 75% ethanol, dried, reconstituted in sterile water, and quantified by spectrometry.

### **Reverse transcription and polymerase chain reaction analysis (RT-PCR)**

After DNase I digestion, RNA samples (2.5µg) were reverse transcribed using AMV-RT and random hexamer primers in a total volume of 30µl. Template cDNAs (2.5µl) were then amplified in a 50µl PCR mix containing 20mM Tris-HCl (pH8.4), 50mM KCl, 1µM of the respective primers, 200µM dNTP, and 2.5U of *Taq*DNA polymerase. The magnesium chloride concentration was 1.5mM. The absence of DNA contamination in RNA preparations was tested by including RNA samples that had not been reverse-transcribed. The housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as internal control. Amplifications were carried out in an Eppendorf master cycler under the following conditions: denaturation for 3min at 94°C followed by cycles (22 cycles for GAPDH and 28 cycles for MGP) of 20s denaturation at 94°C, 20s annealing at 60°C, and 15s elongation at 72°C. The sequences of primers for mouse MGP cDNA were 5'-TCAACAGGAGAAATGCCAACAC-3' (forward) and 5'-CGGTTGTAGGCAGCGTTGT-3' (reverse) generating a 178-bp fragment. The sequences of primers for mouse GAPDH cDNA were 5'-GAAGGTCGGTGTGAACGGAT-3' (forward) and 5'-CGTTGAATTTGCCGTGAGTG-3' (reverse) generating a 161-bp fragment. PCR primers were synthesized by MWG Biotech (Ebersberg, Germany). PCR products were electrophoresed on 2% agarose gels and visualized by U.V. transilluminator.

### **Real-time quantitative PCR**

Real-time quantitative PCR was performed in the iCycleriQ Detection System (Bio-Rad Laboratories) using SyBr Green detection and Titanium *Taq* DNA polymerase, according to the manufacturer's recommendations. The following temperature profile was used: 40 cycles of 15s at 95°C and 1min at 60°C. Cycle numbers obtained at the log-linear phase of the reaction were plotted against a standard curve prepared with serially diluted cDNA samples. Expression of target gene was normalized to GAPDH levels. The sequences of primers for mouse MGP cDNA and for mouse GAPDH cDNA were identical to those used for RT-PCR. The delta Ct ( $\delta$  Ct) (cycle threshold) method was used to calculate relative expression levels, as previously described (28). Results are reported as fold changes in gene expression relative to control conditions (untreated cultures).

### **Western blotting**

After treatment, cells were rapidly frozen in liquid nitrogen before lysis at 4°C and conserved at -80°C until use. Cells were lysed by addition of a buffer containing 20mM Tris-HCl pH 7.5, 100mM potassium chloride, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol, 20mM β-glycerophosphate, 2mM Na<sub>3</sub>VO<sub>4</sub>, 1mM PMSF, and 1mM NaF. The insoluble material was pelleted at 12,000g for 10min at 4°C. The protein concentration of cell lysates was determined with a Pierce Coomassie-Plus-protein assay. 20μg of total protein were resolved by SDS-PAGE. After electrophoresis, proteins were transferred to PVDF (polyvinylidene difluoride) membrane according to the manufacturer's protocol. Membranes for ERK1/2, phospho-ERK1/2, p38 and phospho-p38 were blocked and probed in 5% non-fat dry milk in PBS/Tween20 (1/1000). For JNK1/2 and phospho-JNK1/2, membranes were blocked in 5% non-fat dry milk in PBS/Tween20 and probed in 5% BSA in PBS/Tween20 (1/1000). Primary antibodies were detected using anti-rabbit HorseRadish Peroxidase (HRP)-conjugated secondary antibody diluted in 5% non-fat dry milk in PBS/Tween20 (1/2000). The blots were visualized by Enhanced ChemiLuminescence (ECL) development using a Western blotting Detection System. For reprobing membranes were incubated in a stripping buffer (100mM β-mercaptoethanol, 2% SDS (w/v), and 62.5mM Tris-HCl, pH6.7) for 30min at 60°C, and then reblocked.

### **Growth plate organ culture**

Skeletal preparations were performed as following. One-to-three-days old mice were killed, skinned, and eviscerated. Ribs were collected in ice-cold PBS solution containing antibiotics. Each rib was incubated in a well containing 2ml FCS-free DMEM supplemented with antibiotics. Explants were then treated with 10mM Pi for 15min at 37°C, in a humidified atmosphere with 5% CO<sub>2</sub> in air. After the incubation period, explants were immediately immersed in 4% phosphate-buffered formalin.

### **Immunohistochemistry**

Ribs were fixed in 4% phosphate-buffered formalin for 24h and embedded in paraffin. Longitudinal sections (5μm) were collected on Polylysine-coated slides. Sections were deparaffined in xylene and dehydrated in graded ethanol. Sections were incubated in 3% H<sub>2</sub>O<sub>2</sub> for 10min to inhibit endogenous peroxidase followed by three rinsing with PBS. The sections were then blocked in goat serum diluted 1:5 in PBS to block nonspecific binding sites, for 1h at room temperature. A rabbit polyclonal primary antibody directed against mouse phospho-

ERK1/2 or mouse ERK1/2 was then diluted in blocking solution (1:100) and sections were incubated overnight at 4°C. After a triple wash in PBS, the secondary antibody diluted in blocking solution (1:300; goat anti-rabbit antibody conjugated with biotin) was incubated for 30min at room temperature. Immunoreactivity was detected with a Dako streptavidin-biotin-peroxidase kit using the manufacturer's instructions. Sections were counterstained for 30s in haematoxylin, dehydrated, and mounted with permanent mounting fluid. Control experiments were performed by omission of the primary antibody. Cells exhibiting a positive immunostaining were brown stained.

### **Statistical analysis**

Each experiment was repeated at least three times with similar results. Results are expressed as mean  $\pm$  SEM of triplicate determinations. Comparative studies of means were performed by using one-way ANOVA followed by post-hoc test (Fisher's projected least significant difference) with a statistical significance at  $p < 0.05$ .

## Results

### Effect of Pi on MGP expression

To determine whether Pi has a role in regulating expression of MGP in growth plate chondrocytes, we first tested the effect of Pi on day 21 in ATDC5 cells, at a stage when MGP is expressed (14). As illustrated in figure 1A, a 24h treatment with 10mM Pi stimulated the steady-state level of mRNA encoding MGP. To quantify the rate of stimulation of MGP expression in response to Pi, we performed real-time RT-PCR. Our data indicated that Pi induced a significant 6-fold increase in MGP expression as compared to control (Figure 1B). To confirm the stimulatory effect of Pi in a non-transformed chondrocyte model, we used primary growth plate chondrocytes primary chondrocytes isolated from ribs of newborn mice. A 24h treatment with Pi stimulated the steady-state level of mRNA encoding MGP (figure 1C) and the analysis by real-time RT-PCR indicated a significant 3-fold increase in MGP expression as compared to control (figure 1D).

### Activation of MAPKs by Pi in ATDC5 cells

With respect to a previous report in osteoblasts (16), we sought to determine whether Pi could modulate the activation of the three major members of the MAPK signaling pathway (ERK1/2, p38 and JNK1/2) in growth plate chondrocytes. As indicated in figure 2A, Pi was found to trigger a marked increase in the phosphorylation of ERK1/2 in ATDC5 cells. Analysis of the phosphorylation of the other MAPK revealed no detectable phosphorylation of either p38 or JNK1/2 in response to Pi (Figure 2A). To ensure that the absence of detection of p38 and JNK1/2 phosphorylation was not due to a technical problem, MC3T3-E1 cells were treated with anisomycin and used as a positive control (29). In these conditions, a phosphorylation of p38 and JNK1/2 was observed in MC3T3-E1 (Figure 2A). The phosphorylation of the various MAPK was not associated with changes in the respective basal level of MAPK suggesting that their phosphorylation results from the stimulation of regulatory upstream kinases.

To determine whether Pi can affect the phosphorylation of ERK1/2 in a time-dependent manner, we then performed a time-course experiment. Our results indicated that Pi induced the phosphorylation of ERK1/2 as early as 5 minutes with a maximum at 15 minutes. The ERK1/2 phosphorylation returned to basal level after 1h (Figure 2B). The time dependent phosphorylation of ERK1/2 was never associated with changes in the basal level of ERK1/2.

### **Effect of Pi in primary chondrocytes and growth plate organ cultures**

To further address the influence of Pi on MAPK, we then questioned whether Pi may affect ERK1/2, p38 and JNK1/2 phosphorylation in cultured primary chondrocytes. As illustrated in figure 3, Pi enhanced the phosphorylation of ERK1/2 in primary chondrocytes. In contrast, Pi failed to affect the phosphorylation level of p38 and JNK1/2 MAPK (Figure 3). As previously mentioned, anisomycin (5 $\mu$ g/ml) treatment of MC3T3-E1 cells was used as a positive control for p38 and JNK1/2 phosphorylation. As expected, anisomycin was found to induce a marked phosphorylation of both kinases.

To confirm our cell culture data, we finally sought to determine the effects of Pi on the phosphorylation of ERK1/2 in rib growth plate organ cultures. In this model, we could easily distinguish the various areas of the growth plate from the proliferative zone (PZ figure 4A) and hypertrophic zone (HZ figure 4A) to the zone of ossification (ZO figure 4A). Whereas a barely detectable immunostaining was observed in the untreated ribs (figure 4B and C), Pi treatment was found to induce a marked phosphorylation of ERK1/2 as evidenced by the large number of proliferative (Figure 4B) and hypertrophic (figure 4C) chondrocytes exhibiting a brown staining. In contrast, a positive staining for total ERK1/2 was detected both in untreated and Pi-treated ribs in proliferative and hypertrophic chondrocytes (Figure 4B and 4C).

### **Cellular specificity of Pi-induced phosphorylation of ERK1/2**

We have found that ERK1/2 is phosphorylated in response to Pi in growth plate chondrocytes and it has been shown that osteoblasts present also enhanced ERK1/2 phosphorylation in response to Pi treatment (16). To test the cellular specificity of this response, we studied the effect of Pi on different cell types. Our results revealed that Pi enhanced the phosphorylation of ERK1/2 in stromal ST2 cells (Figure 5). In contrast, Pi did not affect the phosphorylation level of ERK1/2 in L929 fibroblasts or myogenic C2C12 cells. In response to Pi, MC3T3-E1 cells showed a stimulation of the phosphorylation of ERK1/2 as compared to untreated cells (Figure5). Basal level of non-phosphorylated ERK1/2 remained constant whatever the conditions.

### **Effect of UO126 on Pi-induced level of MGP expression**

To identify a possible relationship between the ERK1/2 phosphorylation and the stimulation of MGP gene expression induced by Pi in ATDC5 cells, we thought to determine the effects

of UO126, a widely used inhibitor of MKK1/2, the upstream kinases of ERK1/2. As shown in figure 6A and 6B, Pi induced a significant increase in the steady-state level of mRNA encoding MGP as compared to the control. Interestingly, pretreatment with UO126, almost completely blunted Pi-induced level of MGP expression. Treatment of ATDC5 cells with UO126 alone did not significantly affect the endogenous steady-state level of MGP mRNAs. To confirm the inhibitory effect of UO126 on ERK1/2 phosphorylation in response to Pi, we performed a western blot analysis. Our results indicated that UO126 completely blocked the Pi-induced phosphorylation of ERK1/2 without affecting the basal level of ERK1/2 (Figure 6C). Viewed together, these results strongly suggest that Pi-stimulated level of MGP mRNAs is at least in part mediated by ERK1/2 phosphorylation.

## Discussion

The maintenance of phosphate homeostasis is required for normal skeletal development and for preservation of bone integrity. Low serum phosphate levels can result in defective skeletal growth and mineralization, in turn leading to rickets (7). Phosphate homeostasis is also essential to prevent ectopic calcifications. Vascular calcification for instance, is highly correlated with elevated serum phosphate levels in patients with renal failure (30). This calcification has been characterized as an active process in which Pi could induce a transdifferentiation of VSMC in osteochondrogenic cells capable of mineralization.

Despite the clinical impact of Pi homeostasis, the mechanisms through which Pi modulates cell differentiation remain poorly understood. Remarkably, a common effect of Pi on cell behavior is the induction of matrix calcification. Indeed, Pi stimulates mineralization in chondrocyte, osteoblast, and VSMC cultures (31). In VSMC, generation of Pi by beta-glycerophosphate correlates with mineralization and a decrease in matrix Gla protein (MGP) levels (10). MGP has been characterized as an inhibitor of mineralization, as evidenced by the dramatic calcification observed in MGP null mice (12), that is mainly expressed in cartilage and VSMC. In the light of these data, we therefore questioned whether Pi may also modulate MGP expression in chondrocytes.

In this attempt, the effects of Pi on MGP expression were first investigated in a chondrogenic *in vitro* model: the ATDC5 cell line. ATDC5 cells represent a well-characterized culture model for chondrocyte differentiation, since these cells display the mesenchymal condensation stage of proliferating chondrocytes, nodule formation and hypertrophic stages, ending with matrix mineralization (21). Furthermore, an *in vitro* study performed in ATDC5 cells showed that MGP present a biphasic expression pattern during chondrocyte differentiation (14) similar to that in the growth plate *in vivo* (11). Interestingly, we found that, in concentrations inducing mineralization (6), Pi stimulates the steady state level of mRNA encoding MGP both in ATDC5 cells and in primary chondrocytes after a 24 hours treatment. This increase could be related to a transcriptional activation of MGP gene or to an increase of MGP mRNA stability. In addition, whether this increase in MGP expression at the mRNA level is accompanied by an increase at the corresponding protein level remains also to be determined. Unfortunately, a specific antibody against MGP is not yet commercially available.

Since crystal formation occurs in our conditions in the first 8 hours (6), it is possible that the effects of Pi on MGP expression after a 24 hours treatment are mediated by the formation of apatite crystals. Experiments aiming at blocking crystal formation or phosphate transport may help us determine whether these effects are due to phosphate ions or apatitic crystals (32, 33). Nevertheless, the upregulation of MGP mRNA synthesis by Pi may be explained by setting off a feed-back mechanism to control Pi-induced mineralization. Likewise, the overexpression of MGP in late differentiating ATDC5 (14) and chick hypertrophic chondrocytes (13) was demonstrated to reduce matrix mineralization. These data strengthen the hypothesis of a MGP-dependent negative feed-back for controlling mineralization.

Despite the great number of studies dealing with the inhibitory effects of MGP on calcification, the mechanisms by which MGP inhibits mineralization remain however unclear. At least two mechanisms could account for the inhibitory function of MGP on calcification. On the one hand, MGP is a member of the mineral-binding Gla protein family (34) which includes a number of coagulation factors and osteocalcin. MGP binds calcium ions and hydroxyapatite via its five  $\gamma$ -carboxylated glutamic acid (Gla) residues. Inhibition of the  $\gamma$ -carboxylation of Gla residues with warfarin in both cell culture and *in vivo*, results in increased matrix mineralization suggesting that the mineral-binding Gla residues are crucial for regulation of matrix mineralization (13, 35). Recently, *in vivo* mutagenesis experiments showed that the Gla residues are required for MGP anti-mineralization function (36). On the other hand, MGP was found to be in tight association with members of TGF- $\beta$  superfamily such as BMPs (37). It was thereafter reported that MGP modulates BMP activity in mesenchymal differentiation (38). More recently, MGP has been demonstrated to exert a dose-dependent inhibitory effect on osteoblastic differentiation through interference with binding of BMP-2 to its receptor (39). Viewed together, these data highlight the possibility that MGP inhibits mineralization at least through two concomitant mechanisms involving its calcium and BMP binding abilities.

To better understand the physiological effect of Pi on cell differentiation and MGP expression, we investigated the cellular mechanisms activated by Pi. We first found that Pi enhances ERK1/2 phosphorylation in a time-dependent manner in ATDC5 cells. We also confirmed that Pi activates ERK1/2 using primary chondrocytes. However, ATDC5 and primary chondrocyte cultures may contain cells at different stages of differentiation: undifferentiated cells around nodules and probably proliferative, pre-hypertrophic and hypertrophic chondrocytes inside nodules. These models are therefore poorly adapted to the

identification of cells in which ERK1/2 is activated in response to Pi. In this context, to clearly localize the cellular populations in which Pi induces ERK1/2 phosphorylation, we embarked on growth plate organ cultures. Our results indicate that Pi enhances ERK1/2 phosphorylation in hypertrophic and proliferative chondrocytes. In addition, the phosphorylation of ERK1/2 induced by Pi does not seem to be restricted to growth plate chondrocytes because Pi also enhances the ERK1/2 phosphorylation in osteoblastic MC3T3-E1 and bone-marrow derived stromal ST2 cells. Since stromal cells have the capacity to differentiate into bone-forming cells (40), one can assume that ST2 cells share a common feature with osteoblasts and chondrocytes that would allow stromal cells to trigger a signal for ERK1/2 activation.

We found that the stimulation of MGP expression by Pi was prevented by specific blockade of ERK1/2 signaling pathway. A residual effect of Pi on MGP expression in the presence of ERK1/2 inhibitor remained however detectable by quantitative real-time PCR suggesting that ERK1/2 is not the exclusive pathway activated by Pi. While Protein Kinase C and proteasome have already been suggested to mediate the effects of Pi on gene expression in osteoblastic cells (16), it remains to be determined whether these pathways could play a role in Pi-induced level of MGP. Finally, the molecular mechanisms by which ERK could mediate the effects of Pi on MGP expression are unknown. Downstream signaling elements from ERK involve transcription factors that regulate gene expression (17). Interestingly, it has been shown that the MGP gene is a specific target of the Fos-related antigen (Fra-1), which is associated with the regulation of bone mass through bone matrix production by osteoblasts and chondrocytes (41). The relationship between Fra-1 and Pi-induced level of MGP expression deserves consideration.

## **Conclusion**

This study demonstrates for the first time that Pi stimulates the expression of MGP in growth plate chondrocytes. In addition, we demonstrated that Pi is able to activate at least one member of the MAP kinase family, ERK1/2. Finally, our data indicated a role for ERK1/2 in the regulation of MGP. These findings provide new insights in the molecular mechanisms induced by inorganic phosphate in growth plate.

## **Acknowledgments**

Authors gratefully acknowledge Lydie Guigand and Jérôme Amiaud from the National Veterinary School of Nantes as well as Françoise Moreau from INSERM U791 for their technical assistance in histological investigation. This work was partially supported by grants from INSERM, French society of rheumatology, and French ministry of research. MJ received a fellowship from INSERM and Région des Pays de Loire.

## Figure legends

### Figure 1. Effect of Pi on MGP mRNA expression in chondrocytes

ATDC5 cells and primary ribs-derived chondrocytes were cultured as described in the Materials and Methods section before treatment with 10mM Pi for 24h. The effect of 10mM Pi on MGP mRNA level was assessed by RT-PCR (**A and C**) and real-time PCR (**B and D**) as described in the Materials and Methods section. Results are reported as fold increase in gene expression. \* $p < 0.0005$  compared with untreated cells. Data are representative of experiments with similar results.

### Figure 2. Effect of Pi on MAPK phosphorylation in ATDC5 cells

ATDC5 cells were cultured as described in the Materials and Methods section before treatment with 10mM Pi for the indicated times. At the end of the incubation period, cells were rapidly frozen in liquid nitrogen before lysis at 4°C. The resulting samples were analyzed by western blotting using specific antibodies against P-ERK1/2, P-p38, and P-JNK1/2 or the corresponding antibodies against ERK1/2, p38, and JNK1/2 as indicated. Confluent MC3T3-E1 cells treated with 5µg/ml anisomycin (Ani) for 30min were used as control for the phosphorylation of p38 and JNK1/2 MAPK; CT: control. (**A**) Analysis of MAPK phosphorylation. (**B**) Time-course experiment.

### Figure 3. Effect of Pi on ERK1/2 phosphorylation in primary chondrocytes

Primary ribs-derived chondrocytes were cultured as described in the Materials and Methods section and exposed to 10mM Pi for 15min. At the end of the incubation period, cells were rapidly frozen in liquid nitrogen before lysis at 4°C. The resulting samples were analyzed by western blotting using specific antibodies against P-ERK1/2, P-p38, and P-JNK1/2 or the corresponding antibodies against ERK1/2, p38, and JNK1/2 as indicated. Confluent MC3T3-E1 cells treated with 5µg/ml anisomycin (Ani) for 30min were used as control for the phosphorylation of p38 and JNK1/2 MAPK; CT: control.

### Figure 4. Immunohistochemical localization of phospho-ERK1/2 in the growth plate

Non-decalcified sections of rib growth plates of three-day-old mice were stained with a rabbit antibody against mouse phospho-ERK1/2 or ERK1/2 and detected by a goat anti-rabbit antibody conjugated with biotin. (**A**) Haematoxylin-eosin staining of rib growth plate without

treatment showing the different zones of the growth plate differentiation (magnification, x10); PZ: proliferative zone; HZ: hypertrophic zone; OZ: ossification zone. **(B)** Immunohistochemical staining for phospho-ERK1/2 (P-ERK1/2) or ERK1/2 in PZ. **(C)** Immunohistochemical staining for phospho-ERK1/2 (P-ERK1/2) or ERK1/2 in HZ. CT: control; Pi: 15min treatment with 10mM Pi (magnification, x40). Arrows indicate the presence of immunostained cells (brown staining). Data are representative of experiments with similar results.

### **Figure 5. Cellular specificity of Pi-induced phosphorylation of ERK1/2**

Confluent L929, C2C12, ST2, and MC3T3-E1 cells were cultured as described in the Materials and Methods section and treated with 10mM Pi. At the end of the incubation period, cells were rapidly frozen in liquid nitrogen before lysis at 4°C. The resulting samples were analyzed by western blotting using specific antibodies directed against P-ERK1/2 or ERK1/2.

### **Figure 6. Effect of UO126 on Pi-induced level of MGP expression in ATDC5 cells**

ATDC5 cells were cultured as described in the Materials and Methods section for 21 days before treatment. ATDC5 cells were pretreated with UO126 (30µM) for 1h followed by treatment with 10mM Pi for 24h. The effect of UO126 and 10mM Pi on MGP mRNA level was assessed by RT-PCR **(A)** and real-time PCR **(B)**, as described in the Materials and Methods section. In parallel, cultures of ATDC5 cells were processed for western blot analysis to assess UO126 efficiency as described in the Materials and Methods section **(C)**. Results are reported as fold increase in gene expression.\* $p < 0.05$  compared with untreated cells. Data are representative of experiments with similar results.

1. **Magne D, Julien M, Vinatier C, Merhi-Soussi F, Weiss P, Guicheux J** 2005 Cartilage formation in growth plate and arteries: from physiology to pathology. *Bioessays* 27:708-16
2. **Kronenberg HM** 2003 Developmental regulation of the growth plate. *Nature* 423:332-6
3. **Karsenty G, Wagner EF** 2002 Reaching a genetic and molecular understanding of skeletal development. *Dev Cell* 2:389-406
4. **Wang D, Canaff L, Davidson D, et al.** 2001 Alterations in the sensing and transport of phosphate and calcium by differentiating chondrocytes. *J Biol Chem* 276:33995-4005
5. **Mansfield K, Teixeira CC, Adams CS, Shapiro IM** 2001 Phosphate ions mediate chondrocyte apoptosis through a plasma membrane transporter mechanism. *Bone* 28:1-8
6. **Magne D, Bluteau G, Faucheux C, et al.** 2003 Phosphate is a specific signal for ATDC5 chondrocyte maturation and apoptosis-associated mineralization: possible implication of apoptosis in the regulation of endochondral ossification. *J Bone Miner Res* 18:1430-42
7. **Quarles LD** 2003 FGF23, PHEX, and MEPE regulation of phosphate homeostasis and skeletal mineralization. *Am J Physiol Endocrinol Metab* 285:E1-9
8. **Sabbagh Y, Carpenter TO, Demay MB** 2005 Hypophosphatemia leads to rickets by impairing caspase-mediated apoptosis of hypertrophic chondrocytes. *Proc Natl Acad Sci U S A* 102:9637-42
9. **Jono S, McKee MD, Murry CE, et al.** 2000 Phosphate regulation of vascular smooth muscle cell calcification. *Circ Res* 87:E10-7
10. **Mori K, Shioi A, Jono S, Nishizawa Y, Morii H** 1998 Expression of matrix Gla protein (MGP) in an in vitro model of vascular calcification. *FEBS Lett* 433:19-22
11. **Luo G, D'Souza R, Hogue D, Karsenty G** 1995 The matrix Gla protein gene is a marker of the chondrogenesis cell lineage during mouse development. *J Bone Miner Res* 10:325-34
12. **Luo G, Ducy P, McKee MD, et al.** 1997 Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature* 386:78-81
13. **Yagami K, Suh JY, Enomoto-Iwamoto M, et al.** 1999 Matrix GLA protein is a developmental regulator of chondrocyte mineralization and, when constitutively expressed, blocks endochondral and intramembranous ossification in the limb. *J Cell Biol* 147:1097-108
14. **Newman B, Gigout LI, Sudre L, Grant ME, Wallis GA** 2001 Coordinated expression of matrix Gla protein is required during endochondral ossification for chondrocyte survival. *J Cell Biol* 154:659-66
15. **Beck GR, Jr., Moran E, Knecht N** 2003 Inorganic phosphate regulates multiple genes during osteoblast differentiation, including Nrf2. *Exp Cell Res* 288:288-300
16. **Beck GR, Jr., Knecht N** 2003 Osteopontin regulation by inorganic phosphate is ERK1/2-, protein kinase C-, and proteasome-dependent. *J Biol Chem* 278:41921-9
17. **Pearson G, Robinson F, Beers Gibson T, et al.** 2001 Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 22:153-83
18. **Oh CD, Chang SH, Yoon YM, et al.** 2000 Opposing role of mitogen-activated protein kinase subtypes, erk-1/2 and p38, in the regulation of chondrogenesis of mesenchymes. *J Biol Chem* 275:5613-9

19. **Watanabe H, de Caestecker MP, Yamada Y** 2001 Transcriptional cross-talk between Smad, ERK1/2, and p38 mitogen-activated protein kinase pathways regulates transforming growth factor-beta-induced aggrecan gene expression in chondrogenic ATDC5 cells. *J Biol Chem* 276:14466-73
20. **Stanton LA, Sabari S, Sampaio AV, Underhill TM, Beier F** 2004 p38 MAP kinase signalling is required for hypertrophic chondrocyte differentiation. *Biochem J* 378:53-62
21. **Shukunami C, Ishizeki K, Atsumi T, Ohta Y, Suzuki F, Hiraki Y** 1997 Cellular hypertrophy and calcification of embryonal carcinoma-derived chondrogenic cell line ATDC5 in vitro. *J Bone Miner Res* 12:1174-88
22. **Guicheux J, Lemonnier J, Ghayor C, Suzuki A, Palmer G, Caverzasio J** 2003 Activation of p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation. *J Bone Miner Res* 18:2060-8
23. **Zhang CL, Wu LJ, Tashiro S, Onodera S, Ikejima T** 2004 Oridonin induces a caspase-independent but mitochondria- and MAPK-dependent cell death in the murine fibrosarcoma cell line L929. *Biol Pharm Bull* 27:1527-31
24. **Maroni P, Bendinelli P, Piccoletti R** 2005 Intracellular signal transduction pathways induced by leptin in C2C12 cells. *Cell Biol Int* 29:542-50
25. **Otsuka E, Yamaguchi A, Hirose S, Hagiwara H** 1999 Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid. *Am J Physiol* 277:C132-8
26. **Lefebvre V, Garofalo S, Zhou G, Metsaranta M, Vuorio E, De Crombrughe B** 1994 Characterization of primary cultures of chondrocytes from type II collagen/beta-galactosidase transgenic mice. *Matrix Biol* 14:329-35
27. **Valcourt U, Gouttenoire J, Moustakas A, Herbage D, Mallein-Gerin F** 2002 Functions of transforming growth factor-beta family type I receptors and Smad proteins in the hypertrophic maturation and osteoblastic differentiation of chondrocytes. *J Biol Chem* 277:33545-58
28. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402-8
29. **Cano E, Hazzalin CA, Mahadevan LC** 1994 Anisomycin-activated protein kinases p45 and p55 but not mitogen-activated protein kinases ERK-1 and -2 are implicated in the induction of c-fos and c-jun. *Mol Cell Biol* 14:7352-62
30. **Cozzolino M, Dusso AS, Slatopolsky E** 2001 Role of calcium-phosphate product and bone-associated proteins on vascular calcification in renal failure. *J Am Soc Nephrol* 12:2511-6
31. **Reynolds JL, Joannides AJ, Skepper JN, et al.** 2004 Human vascular smooth muscle cells undergo vesicle-mediated calcification in response to changes in extracellular calcium and phosphate concentrations: a potential mechanism for accelerated vascular calcification in ESRD. *J Am Soc Nephrol* 15:2857-67
32. **Li X, Yang HY, Giachelli CM** 2006 Role of the sodium-dependent phosphate cotransporter, Pit-1, in vascular smooth muscle cell calcification. *Circ Res* 98:905-12
33. **Suzuki A, Ghayor C, Guicheux J, et al.** 2006 Enhanced expression of the inorganic phosphate transporter Pit-1 is involved in BMP-2-induced matrix mineralization in osteoblast-like cells. *J Bone Miner Res* in press
34. **Price PA, Williamson MK** 1985 Primary structure of bovine matrix Gla protein, a new vitamin K-dependent bone protein. *J Biol Chem* 260:14971-5

35. **Price PA, Faus SA, Williamson MK** 1998 Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. *Arterioscler Thromb Vasc Biol* 18:1400-7
36. **Murshed M, Schinke T, McKee MD, Karsenty G** 2004 Extracellular matrix mineralization is regulated locally; different roles of two gla-containing proteins. *J Cell Biol* 165:625-30
37. **Urist MR, Huo YK, Brownell AG, et al.** 1984 Purification of bovine bone morphogenetic protein by hydroxyapatite chromatography. *Proc Natl Acad Sci U S A* 81:371-5
38. **Bostrom K, Tsao D, Shen S, Wang Y, Demer LL** 2001 Matrix GLA protein modulates differentiation induced by bone morphogenetic protein-2 in C3H10T1/2 cells. *J Biol Chem* 276:14044-52
39. **Zebboudj AF, Imura M, Bostrom K** 2002 Matrix GLA protein, a regulatory protein for bone morphogenetic protein-2. *J Biol Chem* 277:4388-94
40. **Prockop DJ** 1997 Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 276:71-4
41. **Eferl R, Hoebertz A, Schilling AF, et al.** 2004 The Fos-related antigen Fra-1 is an activator of bone matrix formation. *Embo J* 23:2789-99