[Frontiers in Bioscience E3, 1249-1258, June 1, 2011]

# Novel molecular mechanisms by inorganic phosphate in osteosarcoma U2OS cells

**Silvio Naviglio, Davide Di Gesto, Vittoria Borrelli, Mafalda Forni, Fausto Illiano, Raffaella D'Auria, Annunziata Sorrentino, Emilio Chiosi, Gennaro Illiano, Annamaria Spina**

*Department of Biochemistry and Biophysics, Second University of Naples, Medical School, Via L. De Crecchio 7, 80138 Naples, Italy*

## TABLE OF CONTENTS



## 1. ABSTRACT

Osteosarcoma is the most common malignant primary bone tumor in children and adolescents and is characterized by a high metastatic potential. Its clinical outcome remains discouraging despite aggressive treatments. Thus, novel therapeutic approaches are needed. Recent results indicate that inorganic phosphate (Pi) is capable of affecting specific signal transduction pathways and of acting as an active regulator of cell behaviour. Previously, we found that Pi inhibits proliferation of human osteosarcoma U2OS cells via an adenylate cyclase/cAMP mediated mechanism. Here, we report that upon Pi treatment, U2OS cells become extremely hard to dislodge with trypsin. The lack of sensitivity to the trypsin action was paralleled by relevant changes in integrin subunits expression and accompanied by an increase of cell adhesion in cell-matrix adhesion assays. Interestingly, exposure of U2OS cells to Pi results also in a strong activation and protein level up-regulation of Rap1 small GTPase and in an early increase followed by a sustained inhibition of Erk1/2 phosphorylation. Importantly, the Pi-induced increase of cell adhesion was enforced by a cAMP analogue which specifically activated Epac/Rap1 and insensitive to PKA and MEK1/2 inhibitors. Our results enforce the evidences of inorganic phosphate as a signalling molecule, identify beta3 integrin, Rap1, ERK1/2 as proteins whose expression and function are relevantly affected by Pi in osteosarcoma U2OS cells The clinical significance and potential therapeutic applications by our data will be discussed.

## 2. INTRODUCTION

Osteosarcoma (OS) is the most common primary malignant tumor of bone, occurring most frequently in children and adolescents. Surgery, radiotherapy and high-dose chemotherapy are mainly effective in patients with localized disease and have improved the prognosis. However, despite aggressive treatment, more than one third of patients develop recurrent high-grade osteosarcomas, with metastatic disease typically affecting the lung or, less frequently, liver and bone itself, so that the 5-year survival rates are still not more than 60%. Moreover, clinically evident metastatic disease is present in 10-20% of patients at diagnosis (1). Thus, there is a pressing need for the development of alternative approaches to the treatment of osteosarcoma and a novel strategy that would efficiently inhibit its metastasis is highly desirable.

Metastasis formation is a complex, multistage process, involving the coordination of several signaling pathways that allow changes in cell morphology, in adhesion and migration capabilities between the cells and the extracellular matrix (ECM) and changes in cell–cell interaction (2,3).

Integrins are the primary receptors for cellular adhesion to ECM molecules. Integrins are a family of transmembrane adhesion glycoproteins comprising of 19alpha and 8beta subunits that interact noncovalently to form up to 24 different heterodimeric receptors. The combination of different integrin subunits on the cell

surface allows cells to recognize and respond to a variety of different ECM proteins including collagen, fibronectin, laminin, and vitronectin (4). Integrins act as crucial transducers of bidirectional cell signaling, regulating cell survival, differentiation, proliferation, migration and tissue remodeling, and have been heavily implicated in tumor development and metastasis (5). Ras-associated protein-1, Rap1, a Ras family member of the small GTPases, has emerged as a key mediator in integrin-mediated cell adhesion (6, 7). Epac 1 and 2, the exchange proteins directly activated by cAMP, are guanine nucleotide exchange factors (GEFs) and activators of Rap1 (8).

Inorganic phosphate (Pi) is an essential nutrient to living organisms. It plays a key role in diverse physiological functions, including osteoblast differentiation and skeletal mineralization (9-11). Serum Pi level is maintained within a narrow range through a complex interplay between intestinal absorption, exchange with intracellular and bone storage pools, and renal tubular reabsorption and depends mainly on the activity of Na/Pi cotransporters. Pi is abundant in the diet, and intestinal absorption of Pi is efficient and minimally regulated. The kidney is a major regulator of Pi homeostasis and can increase or decrease its Pi reabsorptive capacity to accommodate Pi need (12). Adequate control of Pi homeostasis is crucial, as a moderate increase in serum Pi concentration and polymorphisms in genes involved in Pi metabolism may result in bone impairment and influence the ageing process and lifespan (12-14). As the amount of Pi in the human diet, and in particular the western diet, continues to increase (12,15), it will be important to fully understand the influence of Pi on cell function and the possible relationship to tumorigenesis (16, 17).

Relevantly, Pi is emerging as an important signalling molecule capable of affecting multiple cellular functions by modulating signal transduction pathways, gene expression and protein abundance in many cell types (10, 18-24). However, little research has been directed at determining the consequences and the underlying mechanisms of elevated Pi on behaviour of osteosarcoma cells.

Previously, we have shown that Pi inhibits proliferation of human osteosarcoma U2OS cells via an adenylate cyclase/cAMP-mediated mechanism (25).

Here we investigate the effects of Pi on integrin-mediated cell adhesion and the possible role of cAMP/Epac/Rap1 axis and ERK1/2 signalling pathway in U2OS cells.

## 3. MATERIALS & METHODS

### 3.1. Materials

All cell culture materials were from Gibco–Life Technologies (Gaithersburg, MD). cAMP analogue 8-pCPT-2'-O-Me-cAMP, Protein Kinase A inhibitor KT5720, MEK inhibitor PD98059 were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Anti-tubulin antibodies were obtained from Oncogene-Calbiochem (La Jolla, CA). Anti-p-ERK antibodies were obtained from Cell Signaling Technology (Danvers, MA). All other antibodies were obtained from Santa Cruz Biotechnology (San Diego, CA).

### 3.2. Cell culture and treatment

Human osteosarcoma U2OS and Saos cell lines were obtained from the American Type Culture Collection (Rockville, MD). U2OS and Saos cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS) and cultured at 37° C in a 5% CO2 humidified atmosphere. Unless noted, all experiments were done in the above medium which contains 1 mM of Pi, and concentrations listed in the figures are final Pi medium concentrations. Added Pi was in the form of $NaPO_4$, pH 7.4, from Sigma (17, 25). Typically, subconfluent cells were split ($5x10^5$/10cm plate). After 24 hours, the cells were washed with PBS and incubated with fresh medium (time 0), supplemented or not with Pi, and grown for the times indicated.

### 3.3. Cell adhesion assay

U2OS cells were treated or not for 24 hours with Pi, and with or without 8-pCPT-2'-O-Me-cAMP, KT5720, PD98059 compounds. Before the adhesion assay, cells were washed four times in PBS, detached from monolayers by brief exposure to a solution of 0.25% trypsin. Cells were washed twice, centrifuged at 1,500 rpm for 5 min and resuspended in the culturing medium at 37°C for 1.5–2 hours with gentle rotation in suspension to allow recovery of cell surface markers (6). Cells were centrifuged, resuspended in the same medium with or without stimuli, counted, and plated on 96 multiwell dishes ($10x10^3$ cells per well) coated with type I collagen and cultured for 30 min. Then, non-adherent cells were removed by PBS washes and adherent cells were fixed with 1% formaldehyde. After fixation, attached cells were air dried at room temperature and then 100 µl of 0.1% crystal violet in 0.2 M boric acid, pH 9, was added to each well and the microtiter plate was shaken at 600 rpm on a plate mixer for 20 min. (The staining solution was prepared fresh from a stock solution of 5% crystal violet in 20% methanol.) Excess stain was removed by three washes with water. Stained cells were again air-dried before the crystal violet was solubilized by adding 100 µl of 10% acetic acid to each well and then shaking the plate at 600 rpm for 20 min. Absorbance at 590 nm was measured with a dual-wavelength microtiter plate reader (THERMOmax, Molecular Devices, Menlo Park, CA) and that reading was corrected for light scattering by subtraction of the absorbance at 450 nm (26). Each condition was run in replicates of 6 wells and all experiments were repeated at least three times with comparable results. Specific adhesion (%) was determined (counts in cells bound/counts in total input × 100) and plotted either directly or relative to the basal adhesion of control cells. Error bars represent average deviation among experiments, and where representative experiments are depicted error bars represent average SD within each experiment.

### 3.4. Cell proliferation assay

Viable cells were determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described (27). Briefly, cells

were seeded in 96 multiwell plates at the density of $5x10^3$ cells/well. Cells were treated with Pi for up to 72 hours (see the figure legends). Before harvesting, 100 µl of MTT solution (5 mg/ml) was added to each well and incubated at 37 °C for 3 hours, then the formazan product was solubilized by the addition of 100 µl 0.04 N HCl isopropanol. The optical density of each sample was determined by measuring the absorbance at 570 nm versus 650 nm using an enzyme-linked immunosorbent assay reader (Molecular Device). Cell proliferation assays were performed at least three times (in replicates of 6 wells for each data point in each experiment). Data are presented as means ± standard deviation for a representative experiment.

### 3.5. Rap1 activation assay and phosphorylation of ERK and CREB

Rap1 activation assays were performed as described previously (6). Briefly, cells were treated or not with Pi as indicated, and lysed in 750 µl lysis buffer (10% glycerol, 1% Nonidet P-40, 50 mM Tris-Cl, pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 1 µM leupeptin, 0.1 µM aprotinin, 5 mM NaF, 1 mM $NaVO_3$). Lysates were clarified by centrifugation, and 500 µl of lysate (1.5-2 µg/µl) was incubated with GST-tagged RBD of RalGDS precoupled to glutathione beads to specifically pull down the GTP-bound forms of Rap1. Samples were incubated for 1 hour at 4°C while tumbling. Beads were washed four times in lysis buffer, and remaining fluid was removed with an insulin syringe. Proteins were eluted with Laemmli sample buffer and analyzed by SDS-PAGE and Western blotting using Rap1 antibodies (Santa Cruz Biotechnology, Inc.). To 100 µl of clarified lysate 25 µl 5× Laemmli sample buffer was added, and phosphorylation of ERKs was analyzed by Western blotting using the phospho-specific antibody against $p42/44^{MAPK}$. Phosphorylation of CREB was analyzed by Western blotting using a phospho-specific antibody directed against phosphorylated $Ser^{133}$.

### 3.6. Preparation of cell lysates

Cell extracts were prepared as described previously (27). Briefly, 3-5 volumes of RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 10µg/ml aprotinin, leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added to recovered cells. After incubation on ice for 1 hour, samples were centrifuged at 18.000 g in an Eppendorf microcentrifuge for 15 min at 4°C and the supernatant (SDS total extract) was recovered. Some aliquots were taken for protein quantification according to Bradford method (28); others were diluted in 4x Laemmli buffer, boiled and stored as samples for immunoblotting analysis.

### 3.7. Immunodetection of proteins

Typically, we employed 20-40 µg of total extracts for immunoblotting. Proteins from cell preparations were separated by SDS-PAGE and transferred onto nitrocellulose sheets (Schleicher & Schuell, Dassel, Germany) by a Mini Trans-Blot apparatus BioRad (Hercules, CA). II goat anti-rabbit or anti-mouse antibodies, conjugated with horseradish peroxidase (BioRad), were used as a detection system (ECL) according to the manufacturer's instructions Amersham Biosciences (UK).

### 3.8. Statistical analysis

Most of experiments were performed at least three times with replicate samples, except where otherwise indicated. Data are plotted as mean ± SD (standard deviation). The means were compared using analysis of variance (ANOVA) plus Bonferroni's t-test. P values of less than 0.05 were considered significant. National Institutes of Health Image J 1.42Q (NIH, Bethesda, MD) software were used for densitometric analysis.

## 4. RESULTS

We have been using the human osteosarcoma U2OS cell line in our studies for many years (25, 29-31). The human osteosarcoma U2OS cells grow as adherent cells and are detached from monolayers by brief exposure to a solution of trypsin. However, when we started to perform experiments aimed to studying the consequences of elevated inorganic phosphate (Pi) on behaviour of osteosarcoma cells, we immediately noted that U2OS cells cultured in presence of supplementation of Pi became extremely hard to dislodge with trypsin, whereas other cell lines, including human osteosarcoma Saos cells, did not. Throughout our studies, we have used a spectrum of final concentration to cover the physiologic range in humans and according to most of published studies on Pi-triggered effects.

### 4.1. Inorganic phosphate inhibits the trypsin-induced detachment of U2OS cells

Figure 1A, shows that Pi treatment inhibits trypsinization of U2OS cells. U2OS were cultured in control medium (containing 1 mM Pi) or medium supplemented with Pi to a final concentration of 5 mM for 24, 48, 72 hours. After that, plates were subjected to routine trypsinization to harvest cells, and at indicated times the detached cells were counted. The number of detached cells in each experiment was adjusted by the cell viability assay to correct for antiproliferative effects of Pi. We found that while control U2OS cells are almost fully detached after 3-7 min trypsin incubation, only 65%, 35%, 20% of U2OS treated with Pi for 24, 48, 72 hours, respectively, are removed upon 7 min trypsin incubation. Moreover, leaving Pi-treated cells for a longer time up to 30 minutes with trypsin, only few cells could be further retrieved. In parallel experiments, no obvious change in trypsinization occurs in human osteosarcoma Saos cells in response to Pi treatment (data not shown). In Figure 1B, representative images by phase-contrast microscopy of control and 24 hours Pi treated U2OS cells, upon 10 min trypsin incubation are shown and clearly indicate that Pi treated cells only partially are detached in response to trypsinization Overall, the data described above indicate that Pi treatment inhibits in a time dependent manner the trypsin-induced detachment of U2OS cells.

### 4.2. Inorganic phosphate affects integrin expression and enhances cell adhesion

It is well known that adherent cells attach themselves to surface of cell culture dishes using proteins, produced by the cells, that form tight bridges between cells and plastic surface. To dislodge cells from the dish, the

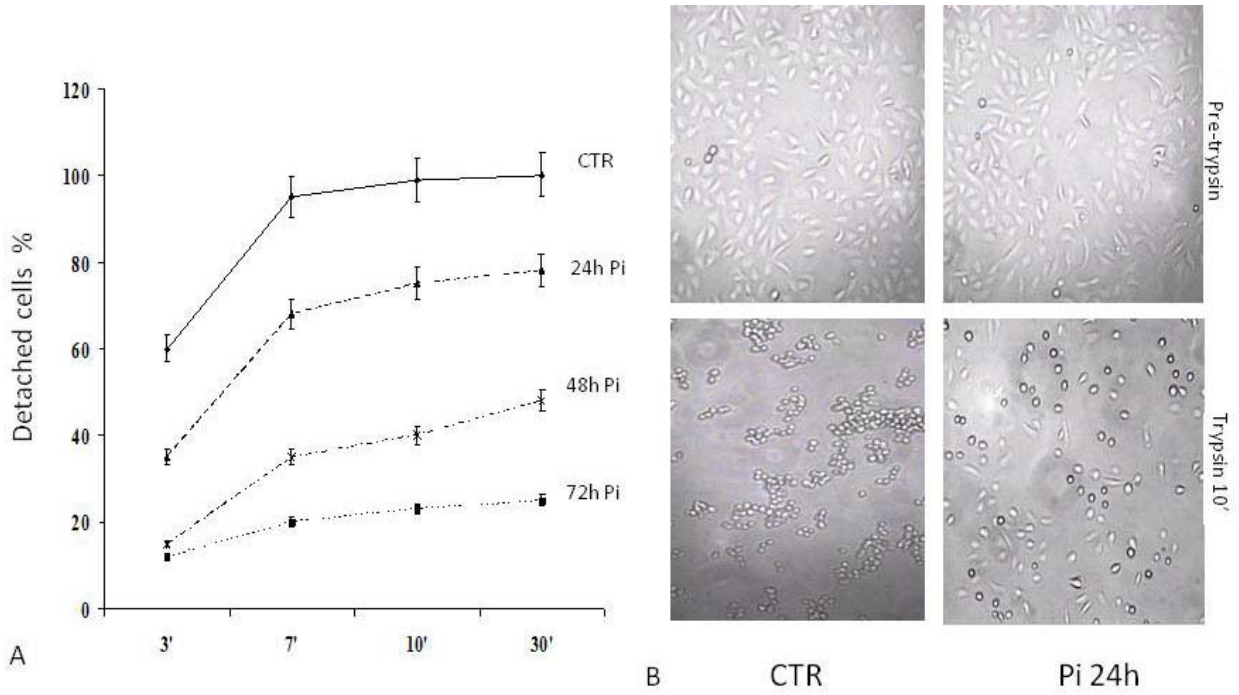


**Figure 1.** Effects of inorganic phosphate on the trypsin-induced detachment of U2OS cells. U2OS cells were cultered in medium supplemented with 5 mM Pi or not (control) for 24, 48, 72 hours. Then, plates were subjected to routine trypsinization and cell number recorded at indicated times. Panel A: Results are expressed as the mean cell counting (% vs control) ± SD (six replicates/treatment). Panel B: Representative images by phase-contrast microscopy of control and 24 hours Pi treated cells, upon 10 min trypsin incubation are shown (10x, Nikon Eclipse TE-300). Images captured by the Arkon-Fluo software (Nikon).

protein bridges must be broken. Trypsin is a proteolityc enzyme that cuts at specific sites the adhesion proteins in cell-cell and cell-matrix interactions. We speculated that the strong reduction of sensitivity to the trypsin action in response to Pi could be dependent on changes of adherence capabilities of U2OS cells. To explore this possibility, we performed conventional cell adhesion assays. U2OS were cultured in control medium (1 mM Pi) or in medium containing 3 mM, 5 mM, 10 mM Pi concentrations for 24 hours. After that, plates were subjected to trypsinization to harvest cells. To note, at this time of 5 mM Pi treatment, a large percentage of U2OS cells could be still recovered (fig.1). Then, the cells were seeded onto multiwell plates coated with type I collagen and the amount of cells that adhered after 30 min was quantified. Figure 2A shows that Pi increases adhesivity of U2OS cells to collagen-coated plates in a dose dependent manner (>30% at 5mM, $P < 0.001$). Moreover, the attachment of U2OS cells to multiwell plates coated with BSA as a control substrate was also promoted upon Pi treatment of about 15% (data not shown). To extend data on the effects of Pi on the adherence capabilities of U2OS cells, we also looked at expression of cell adhesion molecules integrins. To do this, we performed dose-effect and time course experiments. U2OS cells were cultured for 24 hours in presence of increasing Pi concentrations (Figure 2B, right part) and in presence of 5 mM Pi containing medium (a submaximal concentration) for different times up to 72 hours (Figure 2B, left part). Then, cells were collected by scraping, total cell extracts in SDS-containing buffer were prepared, and protein levels of some integrin subunits were monitored by western blotting analysis. Interestingly, we found that beta3 integrin subunit was clearly decreased upon Pi treatment in a dose- and time- dependent manner, with a significant reduction already evident at 24 hours with 3 mM Pi, whereas an increase of beta5 integrin subunit occurs at 24 hours with 5 and 10 mM Pi and more evident after 72 hours; on the other hand, beta1 protein levels appear unchanged. Altogether, the above results indicate that Pi treatment of U2OS affects integrin expression and enhances cell adhesion.

### 4.3. Inorganic phosphate affects multiple signalling pathways and mostly up-regulates the cAMP/Epac/Rap1 axis

To investigate the mechanisms underlying the Pi-induced enhancement of the adherence capabilities of U2OS cells, first we examined the cAMP/Epac/Rap1 function in response to Pi. To this purpose, we cultured U2OS cells in presence of control and 5 mM Pi containing medium for different times, from 15 minutes to 24 hours. Thereafter, cell extracts were prepared and used for Rap1 activation assays and immunoblotting analysis. As shown in Figure 3, we found that exposure of cells to Pi resulted in a significant activation of Rap1 that was detectable at 30 minutes, and maintained for at least 6 hours, To note, a strong increase of Rap1 protein levels after 24 hours of Pi treatment also occurred. To extend data on specificity of this Pi-induced effect, we evaluated also CREB and ERK1/2 phosphorylation and expression. Fig 3 shows also

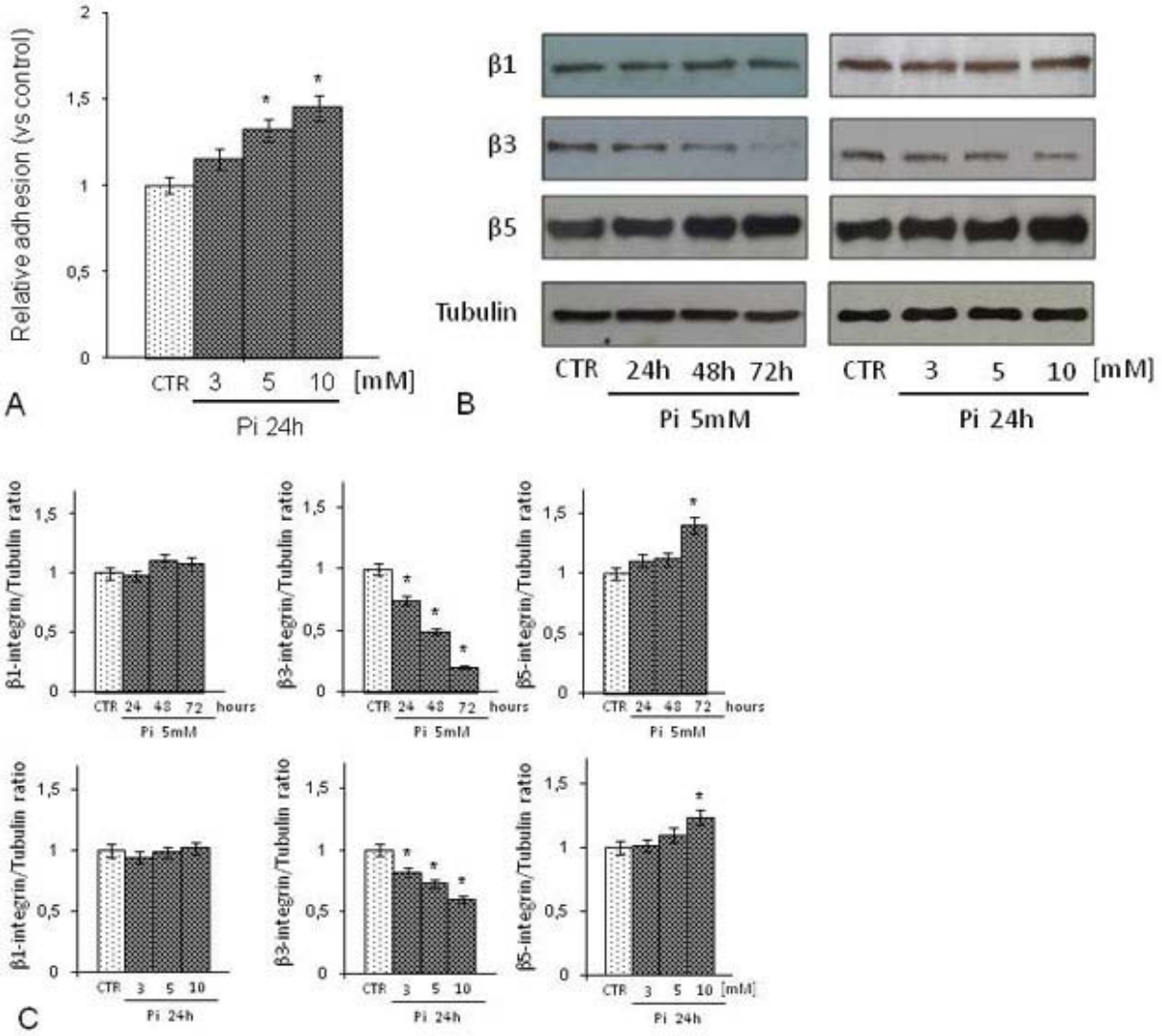


**Figure 2.** Effects of inorganic phosphate on the cell-matrix adhesion and integrin expression. Panel A: Conventional cell-matrix adhesion assay. U2OS were cultered in control medium (1 mM Pi) or in medium containing 3 mM, 5 mM, 10 mM Pi for 24 hours. Each condition was run in replicates of 6 wells and experiment was repeated at least three times with similar results. *, $P<$ 0.01 vs. control cells. Panel B: Western blotting analysis. U2OS were cultered in control medium or in medium containing 3 mM, 5 mM, 10 mM Pi for 24 hours (dose-effects experiment, right part) and in 5 mM Pi containing medium for 24, 48, and 72 hours (time-course experiment, left part). Protein levels of some integrin subunits were monitored by western blotting analysis from 40 μg of SDS-total cell extracts using antibodies against the indicated proteins. The images are representative of three different experiments with similar results. Panel C: Graphs showing the densitometric intensity of beta1 integrin/Tubulin, beta3 integrin/Tubulin, beta5 integrin/Tubulin bands ratio are shown. The intensities of signals were expressed as arbitrary units. *, $P <$ 0.01 vs. control untreated cells.

that Pi treatment results in a rapid and transient increase of Erk1/2 phosphorylation evident at 15 min that declined to basal level at 30 min and followed by a prolonged inhibition up to 24 hours. Moreover, a transient increase of phosphorylation of CREB protein, a major substrate of cAMP-dependent protein kinase A, could be seen only at 15 min. To note, no significant change in the total amount of ERK1/2 and CREB proteins occurs. Overall, the above findings indicate that Pi treatment of U2OS cells affects multiple signalling pathways and mostly up-regulates the cAMP/Epac/Rap1 axis.

### 4.4. cAMP/Epac/Rap1 up-regulation is relevantly involved in the Pi-induced enhancement of cell adhesion

The involvement of Rap1 in integrin-mediated cell adhesion is largely known (7). To explore the contribution of the Pi-induced cAMP/Epac/Rap1 up-regulation to the enhancement of cell adhesion in response to Pi in U2OS cells, we took advantage of the availability of cAMP analogue that specifically activates Epac/Rap1, but not PKA, cAMP downstream effector (32). We performed a combined treatment of U2OS cells for 24 hours with a not-maximal concentration of Pi (5 mM) and

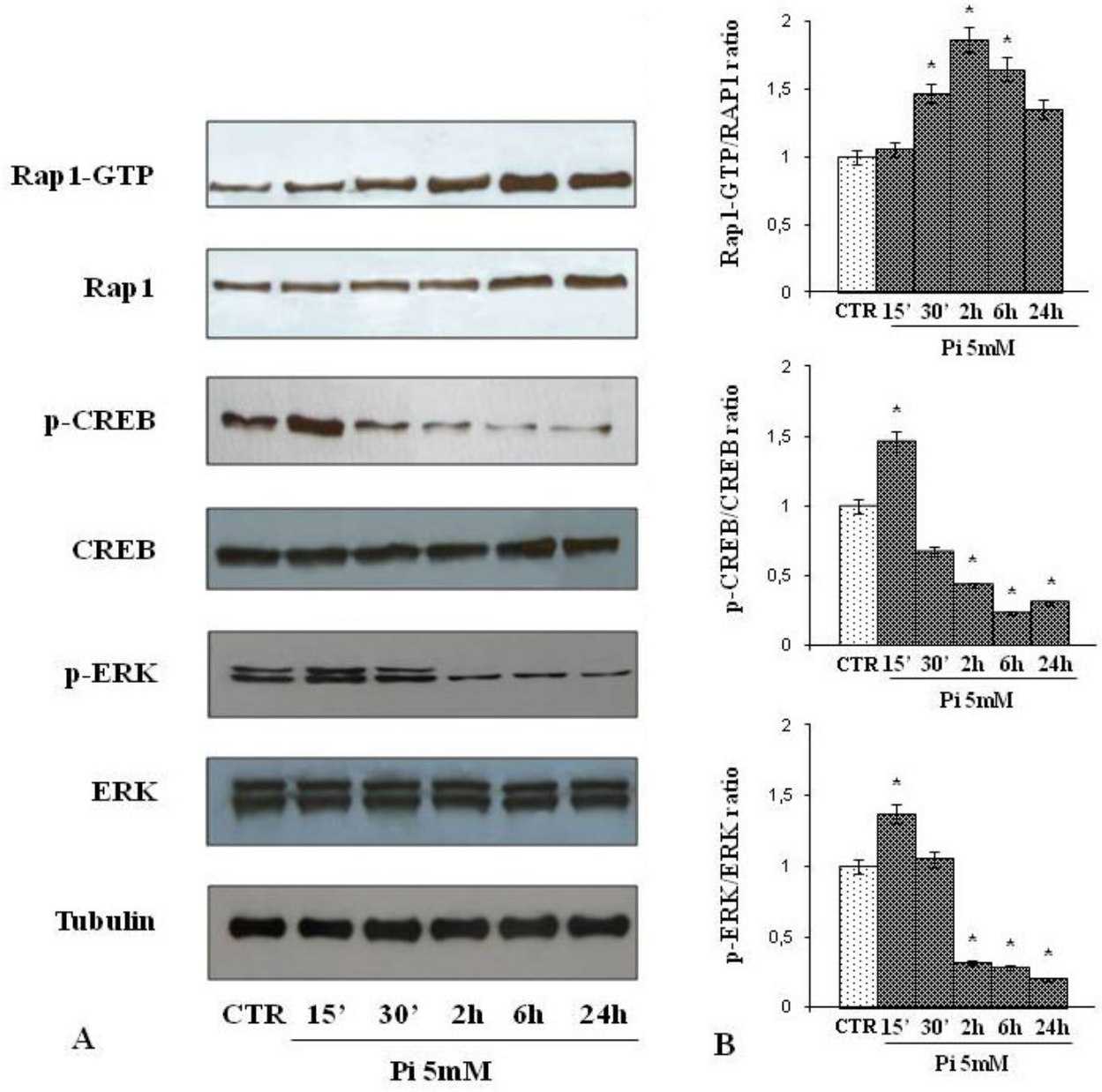


**Figure 3.** Effects of inorganic phosphate on the Rap1 activation and ERK1/2 and CREB phosphorylation. U2OS cells were cultered in control and 5 mM Pi containing medium for the indicated times. Panel A: Cell extracts were prepared and used for Rap1 activation assays and western blotting analysis using antibodies against the indicated proteins. The images are representative of three different experiments with similar results. Panel B: Graphs showing the densitometric intensity of Rap1-GTP/Rap1, pCREB/CREB, pERK/ERK bands ratio are shown. The intensities of signals were expressed as arbitrary units. *, $P <$ 0.01 vs. control untreated cells.

increasing doses of cAMP analogue. After that, plates were subjected to trypsinization to harvest cells and conventional cell adhesion assays were performed. As expected (6), we found that cAMP analogue alone in a dose-dependent manner induced cell adhesion (fig. 4A). Interestingly, figure 4A also shows that the Pi treatment enhanced the cell adhesion induced by cAMP analogue, strongly suggesting that Pi-induced effects on Rap1 function (fig.3) are relevantly linked to increase of cell adhesion in response to Pi. To enforce the significance of these data, we include inhibitors of PKA and MEK1/2 kinases that appeared, at least at early time points, activated by Pi treatment, as suggested by early increase of CREB and ERK1/2 phosphorylation (fig.3). As shown in fig. 4B, cotreatments with KT-5720 and PD98059 inhibitors did not prevent the Pi-induced enhancement of cell adhesion.

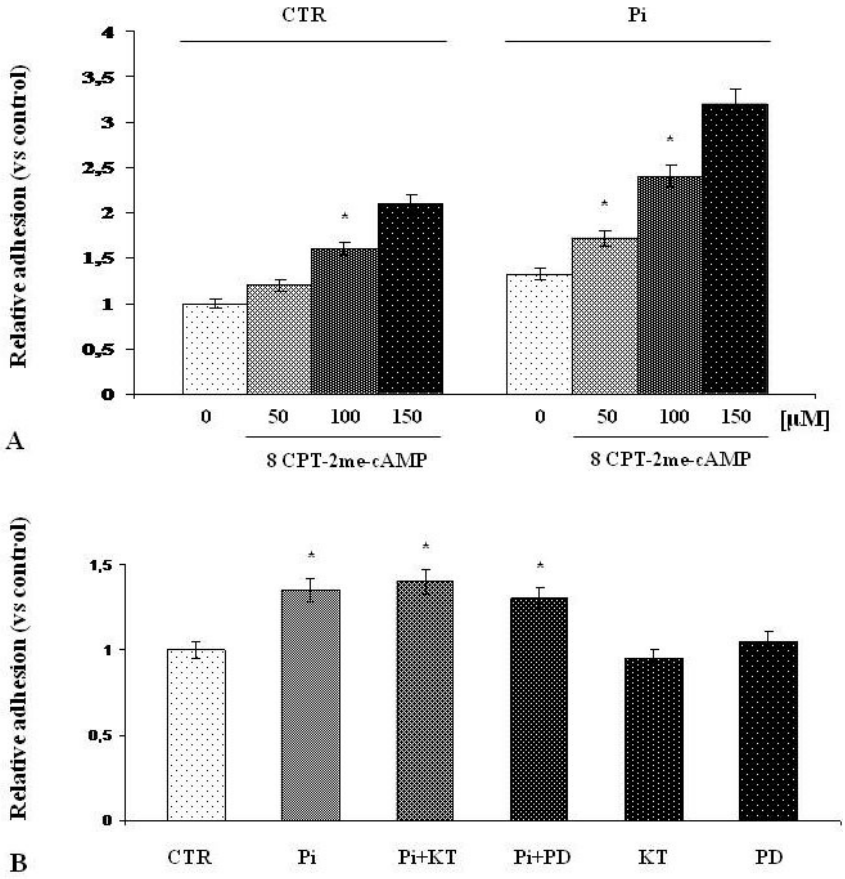


**Figure 4.** Effects of 8-pCPT-Me-cAMP, KT5720, PD98059 compounds on the inorganic phosphate-mediated cell-matrix adhesion. U2OS cells were subjected or not to combined treatments for 24 hours with 5 mM Pi and increasing doses (50 up to 150 µM) of cAMP analogue, 8-pCPT-Me-cAMP (Panel A) or 10 µm PKA inhibitor KT5720 and 10 µm MEK1/2 inhibitor PD98059 (Panel B). Then, conventional cell-matrix adhesion assays were performed. Each condition was run in replicates of 6 wells and experiment was repeated three times with similar results. *, $P < 0.01$ vs. control cells.

Overall, the above results suggest that in U2OS cells cAMP/Epac/Rap1 up-regulation is relevantly involved in the Pi-induced enhancement of cell adhesion.

## 5. DISCUSSION

Diet represents an environmental factor that can be easily manipulated and have profound effects on functional genomics; its potential contribution to cancer is well known (33-35).

Pi is a common dietary component that may directly alter cell behavior in such a manner.

Increasing evidences propose Pi as a novel signalling molecule capable of stimulating specific signal transduction pathways including ERK1/2 and Akt, and to increase cell proliferation in some cell types, such as preosteoblastic MC3T3-E1 cells, human lung cells, epidermal JB6 cells, thereby defining Pi as a novel mitogenic signal for such cells (10, 17-23).

However, little research has been directed at determining the consequences and the underlying mechanisms of elevated Pi on behaviour of osteosarcoma cells.

Previously, we have shown that Pi inhibits proliferation of human osteosarcoma U2OS cells via an adenylate cyclase/cAMP-mediated mechanism (25). Here, we report that treatment of U2OS cells with a spectrum of Pi concentrations physiologically relevant results in a strong reduction of sensitivity to the trypsin action and in an increase of cell adhesion, thus indicating a clear change in adherence capabilities of U2OS cells in response to Pi.

In agreement with our finding, it has been reported that L/B/K ALP, Alkalin Phosphatase, whose main action is to locally increase the Pi levels in extracellular environment, inhibits the aggressiveness and the metastatic ability of U2OS cells, by modulating the expression of genes involved in cell adhesion (36).

Importantly, we demonstrate that the Pi-induced cell-adhesion increase is accompanied by a dramatic decrease of beta3 integrin subunit protein levels. To note, de novo expression or upregulation of beta3 subunit

containing integrins is associated with a metastatic phenotype and increased motility in a variety of cancer cells, such as melanoma, breast and prostate carcinoma, and osteosarcoma (5, 37, 38). Interestingly, we found that in U2OS osteosarcoma cells Pi in a dose- and time-dependent manner profoundly down-regulates the abundance of beta3 integrin subunit protein levels, strongly suggesting that the metastatic potential of U2OS cells would be attenuated in response to Pi.

In agreement with a Pi-induced reduction of metastatic potential in osteosarcoma cells, we also found that Pi treatment of U2OS cells results in a large inhibition of ERK1/2 signalling pathway.

To note, ERK1/2 activation has been involved in the enhancement of motility by chemokine stromal cell-derived factor-1/CXCR4 in human osteosarcoma cells, including U2OS cells (37). Moreover, an inhibition of ERK1/2 phosphorylation mediates the anti-metastatic activity of silibinin in human osteosarcoma cells (39). Furthermore, ERK1/2 signalling pathway has been found activated in an entire osteosarcoma case series by immunohistochemistry and its inhibition by sorafenib inhibitor has been linked to the inhibition of tumor growth, angiogenesis and metastatic potential in preclinical models of osteosarcoma (40).

Very interestingly, here we show that Pi significantly down-regulates ERK1/2 phosphorylation and this well correlates with its antiproliferative and anti-metastatic action in U2OS cells.

The Rap GTPases are master regulators of integrin activation, cell motility, and the underlying cytoskeletal, adhesion and membrane dynamics (7). The list of Rap effectors is rapidly expanding and contains proteins which are mostly involved in all aspects of cell adhesion and modulation of the actin cytoskeleton. A number of effectors have been implicated in the control of integrins. They may form an "integrin activation complex" consisting of Rap1 and several effectors and perhaps further adapter proteins, required to mediate integrin activation and to induce cell adhesion. Also a number of effectors have been identified that regulate the actin cytoskeleton, in particular the GEFs Vav2 and Tiam1 for Rac proteins and the GAPs Arap3 and RA-RhoGAP for Rho proteins. These effectors apparently determine the balance between Rac and Rho signaling and, as such, regulate the dynamics of the actin cytoskeleton (41).

Remarkably, here we report that exposure of U2OS cells to Pi results in a strong activation and protein level up-regulation of Rap1 and provide evidence that the Epac/Rap1 pathway is involved in Pi-induced increase of U2OS cell adhesion.

Collectively, here we demonstrate that upon supplementation of medium with Pi, U2OS cells became extremely hard to dislodge with trypsin and increase their adherence capabilities. This phenomenom is accompanied by an inhibition of pro-proliferative and pro-metastatic ERK1/2 signalling pathway and a down-regulation of pro-metastatic beta3 integrin subunit expression, and also by an upregulation of Rap1 function.

Notably, most of the effects elicited by Pi in U2OS cells, including Rap1 up-regulation and beta3 down-regulation, could not be seen in Saos osteosarcoma cells, indicating that Pi can produce discrete effects depending on cell type and genetic background.

Due to its high metastatic potential, the clinical outcome for osteosarcoma remains discouraging despite aggressive treatments. New effective therapies are warranted for preventing osteosarcoma metastatis. Dietary Pi is emerging as a novel target for chemoprevention.

Our data enforce the evidences of inorganic phosphate as a signalling molecule, identify beta3 integrin, Rap1, ERK1/2 as proteins whose expression and function are relevantly affected by Pi in human osteosarcoma U2OS cells to ultimately inhibit their aggressiveness and suggest that targeting Pi levels might represent potentially a novel way for therapeutic intervention in osteosarcoma.

## 6. ACKNOWLEDGEMENTS

This work was supported by Italian Minister for Research (PRIN 2005, contract number 2005055874_005)

## 7. REFERENCES

1. AJ Chou, DS Geller, R Gorlick: Therapy for osteosarcoma: where do we go from here? *Paediatr Drugs* 10(5):315-27 (2008)

2. E Behmoaram, K Bijian, TA Bismar, MA Alaoui-Jamali: Early stage cancer cell invasion: signaling, biomarkers and therapeutic targeting. *Front Biosci* 1;13:6314-25 (2008)

3. L Calorini, F Bianchini: Environmental control of invasiveness and metastatic dissemination of tumor cells: role of tumor cell-host cell interactions. *Cell Commun Signal* 7;8(1):24 (2010)

4. FG Giancotti: A structural view of integrin activation and signaling. *Dev Cell* 4(2):149-51 (2003)

5. B Felding-Habermann: Integrin adhesion receptors in tumor metastasis. *Clin Exp Metastasis* 20(3):203-13 (2003)

6. S Rangarajan, JM Enserink, HB Kuiperij, J de Rooij, LS Price, F Schwede, JL Bos: Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor. *J Cell Biol* 160(4):487-93 (2003)

7. B Boettner, L Van Aelst: Control of cell adhesion dynamics by Rap1 signaling. *Curr Opin Cell Biol* 21(5):684-93 (2009)

8. M Gloerich, JL Bos: Epac: defining a new mechanism for cAMP action. *Annu Rev Pharmacol Toxicol* 50:355-75 (2010)

9. CG Bellows, JN Heersche, JE Aubin: Inorganic phosphate added exogenously or released from beta-glycerophosphate initiates mineralization of osteoid nodules in vitro. *Bone Miner* 17 (1):15-29 (1992)

10. GR Jr Beck: Inorganic phosphate as a signaling molecule in osteoblast differentiation. *J Cell Biochem* 90 (2):234-43 (2003)

11. Y Yoshiko, GA Candeliere, N Maeda, JE Aubin: Osteoblast autonomous Pi regulation via Pit1 plays a role in bone mineralization. *Mol Cell Bio*. 27(12):4465-74 (2007)

12. E Takeda, Y Taketani, N Sawada, T Sato, H Yamamoto: The regulation and function of phosphate in the human body. *Biofactors* 21(1-4):345-55 (2004)

13. D Prié, L Beck, P Urena, G Friedlander: Recent findings in phosphate homeostasis. *Curr Opin Nephrol Hypertens* 14(4):318-24 (2005)

14. Y Nishida, Y Taketani, H Yamanaka-Okumura, F Imamura, A Taniguchi, T Sato, E Shuto, K Nashiki, H Arai, H Yamamoto, E Takeda: Acute effect of oral phosphate loading on serum fibroblast growth factor 23 levels in healthy men. *Kidney Int* 70:2141–7 (2006)

15. MS Calvo: Dietary phosphorus, calcium metabolism and bone. *J Nutr* 123:1627–33 (1993)

16. MF McCarty: A moderately low phosphate intake may provide health benefits analogous to those conferred by UV light—a further advantage of vegan diets. *Med Hypotheses* 61:543–60 (2003)

17. CE Camalier, MR Young, G Bobe, CM Perella, NH Colburn, GR Jr Beck: Elevated phosphate activates N-ras and promotes cell transformation and skin tumorigenesis. *Cancer Prev Res* 3(3):359-70 (2010)

18. KA Conrads, M Yi, KA Simpson, DA Lucas, CE Camalier, LR Yu, TD Veenstra, RM Stephens, TP Conrads, GR Jr Beck: A combined proteome and microarray investigation of inorganic phosphate-induced pre-osteoblast cells. *Mol Cell Proteomics* 4(9):1284-96 (2005)

19. VE Kemi, MU Karkkainen, CJ Lamberg-Allardt: High phosphorus intakes acutely and negatively affect Ca and bone metabolism in a dose-dependent manner in healthy young females. *Br J Nutr* 96:545–52 (2006)

20. H Jin, SK Hwang, K Yu, HK Anderson, YS Lee, KH Lee, AC Prats, D Morello, GR Jr Beck, MH Cho: A high inorganic phosphate diet perturbs brain growth, alters Akt-ERK signaling, and results in changes in cap-dependent translation. *Toxicol Sci* 90(1):221-9 (2006)

21. H Jin, SH Chang, CX Xu, JY Shin, YS Chung, SJ Park, YS Lee, GH An, KH Lee, MH Cho: High dietary inorganic phosphate affects lung through altering protein translation, cell cycle, and angiogenesis in developing mice. *Toxicol Sci* 100(1):215-23 (2007)

22. CX Xu, H Jin, HT Lim, JE Kim, JY Shin, ES Lee, YS Chung, YS Lee, G Jr Beck, KH Lee, MH Cho: High dietary inorganic phosphate enhances cap-dependent protein translation, cell-cycle progression, and angiogenesis in the livers of young mice. *Am J Physiol Gastrointest Liver Physiol* 295(4):G654-63 (2008)

23. H Jin, CX Xu, HT Lim, SJ Park, JY Shin, YS Chung, SC Park, SH Chang, HJ Youn, KH Lee, YS Lee, YC Ha, CH Chae, GR Jr Beck, MH Cho: High dietary inorganic phosphate increases lung tumorigenesis and alters Akt signaling. *Am J Respir Crit Care Med* 179(1):59-68 (2009)

24. CX Xu, H Jin, HT Lim, YC Ha, CH Chae, GH An, KH Lee, MH Cho: Low dietary inorganic phosphate stimulates lung tumorigenesis through altering protein translation and cell cycle in K-ras(LA1) mice. *Nutr Cancer* 62(4):525-32 (2010)

25. S Naviglio, A Spina, E Chiosi, A Fusco, F Illiano, M Pagano, M Romano, G Senatore, A Sorrentino, L Sorvillo, G Illiano Inorganic phosphate inhibits growth of human osteosarcoma U2OS cells via adenylate cyclase/cAMP pathway. *J Cell Biochem* 98(6):1584-96 (2006)

26. RA Clark, JQ An, D Greiling, A Khan, JE Schwarzbauer: Fibroblast migration on fibronectin requires three distinct functional domains. *J Invest Dermatol* 121(4):695-705 (2003)

27. S Naviglio, D Di Gesto, F Illiano, E Chiosi, A Giordano,G Illiano, A Spina: Leptin potentiates antiproliferative action of cAMP elevation via protein kinase A down-regulation in breast cancer cells. *J Cell Physiol* 225(3):801-9 (2010)

28. MM Bradford: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72:248-254 (1976)

29. S Naviglio, C Mattecucci, B Matoskova, T Nagase, N Nomura, PP Di Fiore, GF Draetta: UBPY: a growth-regulated human ubiquitin isopeptidase. *EMBO J* 17(12):3241-50 (1998)

30. S Naviglio, M Pagano, M Romano, A Sorrentino, A Fusco, F Illiano, E Chiosi, A Spina, G Illiano: Adenylate cyclase regulation via proteasome-mediated modulation of Galphas levels. *Cell Signal* 16(11):1229-37 (2004)

31. E Chiosi, A Spina, A Sorrentino, M Romano, L Sorvillo, G Senatore, R D'Auria, A Abbruzzese, M Caraglia, S Naviglio, G Illiano: Change in TNF-alpha receptor expression is a relevant event in doxorubicin-induced H9c2 cardiomyocyte cell death. *J Interferon Cytokine Res* 27(7):589-97 (2007)

32. GG Holz, OG Chepurny, F Schwede: Epac-selective cAMP analogs: new tools with which to evaluate the signal transduction properties of cAMP-regulated guanine

nucleotide exchange factors. *Cell Signal* 20(1):10-20 (2008)

33. R Doll, R Peto: The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst* 66:1191–308 (1981)

34. PM Newberne, MW Conner: Dietary modifiers of cancer. *Prog Clin Biol Res* 259:105–29 (1988)

35. VL Go, DA Wong, Y Wang, RR Butrum, HA Norman, L Wilkerson: Diet and cancer prevention: evidence-based medicine to genomic medicine. *J Nutr* 134:3513–6S (2004)

36. C Zucchini, M Bianchini, L Valvassori, S Perdichizzi, S Benini, MC Manara, R Solmi, P Strippoli, P Picci, P Carinci, K Scotlandi: Identification of candidate genes involved in the reversal of malignant phenotype of osteosarcoma cells transfected with the liver/bone/kidney alkaline phosphatase gene. *Bone* 34(4):672-9 (2004)

37. CY Huang, CY Lee, MY Chen, WH Yang, YH Chen, CH Chang, HC Hsu, YC Fong, CH Tang: Stromal cell-derived factor-1/CXCR4 enhanced motility of human osteosarcoma cells involves MEK1/2, ERK and NF-kappaB-dependent pathways. *J Cell Physiol* 221(1):204-12 (2009)

38. A Karadag, KU Ogbureke, NS Fedarko, LW Fisher: Bone sialoprotein, matrix metalloproteinase 2, and alpha(v)beta3 integrin in osteotropic cancer cell invasion. *J Natl Cancer Inst* 96(12):956-65 (2004)

39. YS Hsieh, SC Chu, SF Yang, PN Chen, YC Liu, LH Lu: Silibinin suppresses human osteosarcoma MG-63 cell invasion by inhibiting the ERK-dependent c-Jun/AP-1 induction of MMP-2. *Carcinogenesis* 28(5):977-87 (2007)

40. Y Pignochino, G Grignani, G Cavalloni, M Motta, M Tapparo, S Bruno, A Bottos, L Gammaitoni, G Migliardi, G Camussi, M Alberghini, B Torchio, S Ferrari, F Bussolino, F Fagioli, P Picci, M Aglietta: Sorafenib blocks tumour growth, angiogenesis and metastatic potential in preclinical models of osteosarcoma through a mechanism potentially involving the inhibition of ERK1/2, MCL-1 and ezrin pathways. *Mol Cancer* 8:1-18 (2009)

41. JH Raaijmakers and JL Bos: Specificity in Ras and Rap Signaling. *J Biol Chem* 284 (17) 10995–10999 (2009)

**Abbreviations**: cAMP, 3'-5'-cyclic adenosine monophosphate, PKA, Protein Kinase A, ERK, extracellular signal-regulated kinases, MAPK, mitogen activated protein kinases; MEK-1, mitogen-activated kinase kinase, Rap1, Ras-associated protein-1, Epac, exchange proteins activated by cAMP, Pi, inorganic phosphate.

**Key Words:** Inorganic Phosphate, cAMP, Rap1, ERK1/2, integrins, Osteosarcoma

**Send correspondence to**: Silvio Naviglio, Department of Biochemistry and Biophysics, Second University of Naples, Medical School, Via L. De Crecchio 7, 80138 Naples, Italy, Tel 39-0815667517, Fax: 39-0815665863, E-mail: silvio.naviglio@unina2.it

http://www.bioscience.org/current/volE3.htm