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Kanji Mori, Frédéric Blanchard, Céline Charrier, Séverine Battaglia, Kosei Ando, Laurence Duplomb, Leonard D. Shultz, Françoise Rédini, Dominique Heymann

Conditioned media from osteosarcoma cells up-regulated MC3T3-E1 osteoblastic cell proliferation *via* JAKs and PI3-K/Akt signal crosstalk

Kanji MORI [1, 2, 5 *], Frederic BLANCHARD [1, 2], Celine CHARRIER [1, 2], Severine BATTAGLIA[1, 2], Kosei ANDO [3], Laurence DUPLOMB[1, 2, 5], Leonard D. SHULTZ [4], Francoise REDINI [1, 2], Dominique HEYMANN [1, 2, 5*]

1. INSERM, ERI 7, Nantes, F-44035 France
2. Université de Nantes, Nantes atlantique universités, Laboratoire de Physiopathologie de la Résorption Osseuse et Thérapie des Tumeurs Osseuses Primitives, EA3822, Nantes, F-44035 France
3. Department of Orthopaedic Surgery, Shiga University of Medical Science. Tsukinowa-cho, Seta, Otsu, Shiga, 520-2192, Japan.
4. The Jackson Laboratory, Bar Harbor, ME, USA
5. CHU de Nantes, pôle de Biologie, Nantes, France

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Correspondence and reprint request to:
Dr. MORI K. or Dr. HEYMANN D. INSERM, ERI 7 ; Université de Nantes, Nantes Atlantiques Universités EA 3822;Physiopathologie de la Résorption Osseuse et Thérapie des Tumeurs Osseuses Primitives. Faculté de Médecine, 1 rue Gaston Veil, 44035, Nantes cedex 1, France
Tel: +33 2 40 41 28 45, Fax: +33 2 40 41 28 60
e-mail: kanchi@belle.shiga-med.ac.jp or dominique.heymann@univ-nantes.fr

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The present address of Kanji MORI: Department of Orthopaedic Surgery, Shiga University of Medical Science. Tsukinowa-cho, Seta, Otsu, Shiga, 520-2192, Japan.

Summary

The maintenance of bone mass requires a strict balance between bone formation by osteoblasts and bone resorption by osteoclasts. In tumoral bone environment, tumor cells frequently disturb this balance by interaction with bone cells to create a favorable site for tumor growth, and to promote pathological bone changes. Thus, elucidation of the mechanisms underlying interactions between tumor cells and bone cells might eventually lead to a more rational strategy for therapeutic intervention. In this study, the effects of mouse osteosarcoma cells on mouse pre-osteoblastic cells were determined by assessement of cell viability, osteoblastic differentiation and signal transduction pathways. MOS-J/POS-1 conditioned media (CMs) significantly induced MC3T3-E1 cell proliferation in a dose-dependent manner and reduced both alkaline phosphatase activity as well as mineralized nodule formation. Piceatannol, AG490, LY294002 and rapamycin significantly abrogated this up-regulated cell proliferation; however UO126 and STAT3 inhibitor peptide did not affect this up-regulated cell proliferation. MOS-J/POS-1 CMs activated ERK 1/2, STAT3 and Akt signal transduction pathways; however MOS-J/POS-1 CMs-induced pro-proliferating signal was transmitted *via* Akt not *via* the ERK 1/2 and STAT3 pathway. Furthermore, Western blots clearly revealed novel signal crosstalk between JAKs and PI3-K/Akt in osteoblastic cells. The specific

factor(s) involved in MOS-J/POS-1 CMs-induced MC3T3-E1 cell proliferation *via* JAKs/PI3-K/Akt/mTOR pathway remain(s) to be determined. Determination of the specific factor(s) that participate in pro-proliferating JAKs and PI3-K/Akt signal crosstalk will offers new insight to understand osteosarcoma, as well as other bone-related diseases.

Introduction

Osteosarcoma is the most common form of primary malignant bone tumor typically affecting children and young adults [1]. Approximately 1000 new cases are seen per year in North America and a similar number occurs in Europe [2]. In spite of the dramatic improvement of the prognosis with aggressive chemotherapy in non-metastatic osteosarcoma [2], patients with metastasis and/or chemotherapy-resistant osteosarcoma have poor outcomes [3]. Therefore, alternative treatment that improves survival of the patients with osteosarcoma is critically needed. Better understanding of disease pathogenesis is essential to devise new therapeutic arms; however the role of osteosarcoma growth and cellular biology in bone microenvironment are not fully understood.

Bone remodeling (bone formation and bone resorption) is tightly regulated soluble, membranous and matrix factors. Pathological bone remodeling can be considered as a consequence of imbalance between osteoblast and osteoclast activity. In osteolytic bone metastases, this hypothesis has been well addressed. Thus, the establishment of the interaction between bone cells and tumor cells is considered as a persuasive explanation of tumor growth in bone [4]. These findings suggest that bone environment established by interaction of bone cells with tumor cells plays a pivotal role

in bone tumor development. In this context, osteosarcoma development might also create a favorable environment for tumor growth by complex interaction with bone cells and ultimately determine its osteogenic profile. Thus, the elucidation of the mechanisms implicated in disturbed bone remodeling by osteosarcoma cells could eventually offer new insight to understanding not only osteosarcoma, but also bone cellular biology.

In the present study, we investigated the effects of mouse osteosarcoma cell lines, MOS-J and POS-1 on mouse pre-osteoblastic cells to reveal the significant role of osteosarcoma cells in bone environment.

Materials and methods

Cell culture

MOS-J [5] and POS-1 [6, 7] cell lines were derived from spontaneous mouse osteosarcoma. MOS-J was obtained from The Jackson laboratory (ME, USA) and POS-1 was kindly provided by Dr. A. Kamijo (Kanagawa Cancer Center, Kanagawa, Japan). MOS-J and POS-1 cells express respectively osteogenic and undifferentiated phenotype. MC3T3-E1, a mouse calvaria-derived pre-osteoblast cell line was obtained from the RIKEN Cell Bank (Tsukuba, Japan). These cell lines were cultured in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS, Hyclone, Brebières, France), at 37°C in a humidified atmosphere containing 5% CO_2.

The medium was changed twice a week and adherent cells were harvested using 0.05% trypsin-0.02% EDTA solution (Lonza).

Preparation of osteosarcoma conditioned medium

MOS-J and POS-1 conditioned media (MOS-J CM, POS-1 CM) were prepared as previously described with slight modifications [8]. MOS-J or POS-1 cells (10^4 cells/cm^2) were grown in a 25cm^2 flask until 70-80% confluence in RPMI 1640 supplemented with 10% FBS. Then, cells were washed three times with phosphate buffered saline (PBS, Lonza) and the medium was changed into serum-free RPMI 1640. After 24 h, CMs were collected and centrifuged at 1000 rpm for 10 min and stored at -80°C until use. The same volume of serum-free RPMI 1640 in the same conditions without cells was used as a control CM (CT CM).

Cell viability assays

-XTT test

The proliferation of mouse pre-osteoblast MC3T3-E1 cells was determined as previously reported with some modifications [8]. MC3T3-E1 cells were seeded into a 96-multiwell plate (2.8 x 10^3 cells/well) and cultured for 72 h in CT CM or MOS-J/POS-1 CMs supplemented with 5% FBS. The media were changed every 48 h and cell proliferation was determined by a sodium 3′

[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) based method using Cell Proliferation Kit II (Roche Applied Science, Mannheim, Germany) following the supplier's recommendations.

Take into account of the result of signal transduction analyses in this study as well as the common knowledge that MEK/ERK, Janus kinases (JAKs), signal transducer and activator of transduction (STAT), phosphatidylinositol 3-kinase (PI3-K) and mammalian target of rapamycin (mTOR) pathways were frequently implicated in cell proliferation; the following inhibitors were tested: MEK/ERK inhibitor (UO126, 10 μM, Calbiochem, CA, USA), a JAK1 inhibitor (piceatannol, 10 μM, Calbiochem), a JAK2 inhibitor (AG490, 20 μM, Calbiochem), a JAK3 inhibitor (JAK3 Inhibitor I, 10 μM, Calbiochem), a STAT3 inhibitor (STAT3 inhibitor peptide, 50 μM, Calbiochem), a PI3-K inhibitor (LY294002, 20 μM, Calbiochem) and a mTOR inhibitor (rapamycin, 10 nM, Calbiochem). These inhibitors were tested to identify which signal transduction pathway plays a crucial role in MOS-J/POS-1 CMs-induced MC3T3-E1 cell proliferation. These inhibitors were added 1 h before of the treatment by osteosarcoma CMs except for AG490 that was added 16 h before. All inhibitors were maintained until the end of culture period at the indicated concentrations. In addition, several neutralizing antibodies were also tested to detect the specific factor(s) that play a pivotal role in MOS-J/POS-1

CMs-induced MC3T3-E1 cell proliferation in the same experimental conditions except for the concentrations of CMs (50% vol/vol were used). Anti-mouse interleukin (IL)-6 receptor antibody (anti-IL-6R, 10 µg/ml, R&D systems, MN, USA), anti-mouse IL-11 receptor antibody (anti-IL-11R, 100 µg/ml, R&D systems), anti-mouse gp130 antibody (anti-gp130, 20 µg/ml, R&D systems), anti-mouse insulin-like growth factor (IGF)-I antibody (anti-IGF-I, 20 µg/ml, R&D systems) and anti-mouse IGF-II antibody (anti-IGF-II, 20 µg/ml, R&D systems) were assessed. Anti-goat IgG (10, 20, 100 µg/ml, R&D systems) was used as a control. These antibodies were pre-incubated for 1 h at 37°C with MC3T3-E1 cells or MOS-J/POS-1 CMs and maintained during the culture period at the indicated concentrations. Recombinant human epidermal growth factor (rhEGF, 100 ng/ml, R&D systems), human growth hormone (rhGH, 100 ng/ml, Lilly France, France), human IL-6 (rhIL-6, 100 ng/ml, R&D systems) alone or association with human soluble IL-6R (rhIL-6Rs:rhIL-6 = 500 ng/ml:100 ng/ml, R&D systems), human IL-11 (rhIL-11, 100 ng/ml, R&D systems), human leukemia inhibitory factor (rhLIF, 100 ng/ml, R&D systems) and human IGF-I (rhIGF-I, 100 ng/ml, R&D systems) were employed and MC3T3-E1 cell proliferation was determined in the presence or the absence of these factors.

- Trypan blue exclusion

Trypan blue exclusion was used to quantify all viability. MC3T3-E1 cells were seeded into a 24-multiwell plate (5 x 10^3 cells/well) and cultured in CT CM or MOS-J/POS-1 CMs supplemented with 5% FBS, then the viable and dead cell numbers were determined at day 1, 4 and 7. Furthermore, under the same experimental conditions, MC3T3-E1 cells were cultured in the presence or the absence of suramin, a pan-growth factor blocker (50-200 µg/ml, Sigma-Aldrich, St. Louis, MO, USA). MC3T3-E1 cell proliferation was measured by direct cell counting using the trypan blue exclusion method, as suramin may interfere with cell proliferation assays based on mitochondrial enzyme activity [9].

Signal transduction analyses by Western Blot

Signal transduction analyses were performed as previously described with slight modifications [8]. Briefly, MC3T3-E1 cells were seeded (10^4 cells/cm^2) into a 6-multiwell plate in RPMI 1640 supplemented with 10% FBS. At 70-80% of confluence, MC3T3-E1 cells were washed three times with serum-free RPMI 1640 and serum starved for 24 h, and then the cells were incubated for 30 min with CT CM. After that MC3T3-E1 cells were incubated for 2, 5, 10, 15 and 30 min in the presence or the absence of MOS-J/POS-1 CMs. In the experiments designed to determine the responsible signal transduction pathway for MOS-J/POS-1 CMs-induced cell proliferation, several signal

transduction inhibitors were used as described above. After pre-treatment with these inhibitors, MC3T3-E1 cells were then cultured in the presence or the absence of MOS-J/POS-1 CMs for 15 min. In addition, to elucidate the signal transduction pathways activated by IL-6 family cytokines (rhIL-6 alone, rhIL-6+rhIL-6Rs, rhIL-11 and rhLIF), MC3T3-E1 cells were similarly treated for 15 min in the presence of these cytokines at the same concentrations as described above. After the treatments, the cells were lysed in ice-cold buffer (NaCl 150 mM, Tris 50 mM, Nonidet P-40 1%, sodium deoxycholate 0.25%, NaF 1 mM, $NaVO_4$ 1 mM, leupeptine 10 mg/ml, aprotinin 10 mg/ml, PMSF 0.5 mM) and same amounts of proteins were subjected to the study. The induction of the phosphorylated forms of ERK 1/2 (Thr202/Tyr204), p38 (Thr180/Tyr182), STAT1 (Tyr701), STAT3 (Tyr705) (Cell Signaling Technologies, MA, USA), STAT5 (Tyr694, ZYMED Laboratories, CA, USA), Akt (Ser473) and mTOR (Ser2448, Cell Signaling Technologies) were determined as well as total forms of ERK 1/2 (Cell Signaling Technologies), p38 (R&D systems), STAT1, STAT3, STAT5 (BD Biosciences, CA, USA), Akt and actin (Cell Signaling Technologies) according to the manufacture's instructions, and revealed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, USA).

Characterization of mouse osteosarcoma cell lines

- RNA extraction and semi-quantitative RT-PCR analysis

Total RNA was extracted from the mouse osteosarcoma cell lines using TRIzol reagent (Invitrogen, Eragny, France) following manufacturer's recommendations. First, total RNA (5 µg) was reverse transcribed (RT) into cDNA using 400 U MMLV-RT (Invitrogen) and 0.5 µg random primers (Promega, WI, USA). Two microliters of the RT products were subjected to PCR using 1.25 U of Ampli *Taq* Gold (Applied Biosystems, CA, USA) and upstream and downstream primers (20 pmol each) to determine the expression of IL-6 [5'-ccggagaggagacttcacag-3' (sense), 5'-ggaaattggggtaggaagga-3' (anti-sense)], IL-11 [5'-tgtggggacatgaactgtgt-3' (sense), 5'-tactcgaagccttgtcagca-3' (anti-sense)], IGF-I [5'-tggatgctcttcagttcgtg-3' (sense), 5'-gtcttgggcatgtcagtgtg-3' (anti-sense)], IGF-II [5'-ggaagtcgatgttggtgctt-3' (sense), 5'-cgtttggcctctctgaactc-3' (anti-sense)], LIF [5'-acggcaacctcatgaaccaga-3' (sense), 5'-ctagaaggcctggaccaccac-3' (anti-sense)], oncostatin M (OSM) [5'-cctgaacacacctgacctga-3' (sense), 5'-cgatggtatccccagagaaa-3' (anti-sense)] and 18S [5'-tcaagaacgaaagtcggaggtc-3' (sense), 5'-ttattgctcaatctcgggtggct-3' (anti-sense)]. The thermal cycle profile was as follows: denaturation for 30 sec at 95°C, annealing for 30 sec at 60°C (IL-11, IGF-I, IGF-II) and 62°C (IL-6, LIF, OSM, 18S) respectively, and extension for 60 sec at 72°C. After the number of PCR cycles was increased, a plot was done for each sample and the cycle

values corresponding to the linear part of the amplification curve were determined and used to quantify the messages versus the 18S signal determined in the same way. The cycle number used was 30 cycles for IL-6, LIF, 35 cycles for IL-11, OSM, IGF-I, IGF-II and 20 cycles for 18S, respectively. The PCR products were electrophoresed on 1% agarose gel and stained by GelRed (Biotium, CA, USA). The mRNA levels of each gene were standardized to 18S levels.

Osteoblastic differentiation assay

- Alkaline phosphatase (ALP) activity

Alkaline phosphatase (ALP) activity was determined in of mouse calvaria osteoblasts and MC3T3-E1 cells as previously reported with slight modifications [8]. Briefly, primary cultured mouse calvaria osteoblasts isolated as previously described [10] or MC3T3-E1 cells were seeded into a 24-multiwell plate (5 x 10^3 cells/well) in CT CM or MOS-J/POS-1 CMs supplemented with 5% FBS in the presence of 50 µg/ml ascorbic acid (Sigma-Aldrich). Culture medium was changed every 48 h for 7 days. At the end of the culture period, the cells were lyzed in ice-cold buffer [NaCl 150 mM, Tris 50 mM, Nonidet P-40 1%, sodium deoxycholate 0.25%, NaF 1 mM, leupeptine 10 mg/ml, aprotinin 10 mg/ml, PMSF 0.5 mM]. ALP activity was determined by *U*/µg protein using Enzyline PAL optimisé (bioMérieux, Marcy-l'Etoile, France) according to the supplier's

instructions.

- Mineralization assay

Alizarin red-S staining was used to detect the *in vitro* formation of mineralized nodule as previously reported with slight modifications [8]. In brief, mouse calvaria osteoblasts were seeded (10^4 cells/cm^2) into a 24-multiwell plate in CT CM or MOS-J/POS-1 CMs supplemented with 10% FBS containing 10^{-8} M dexamethasone (Sigma-Aldrich), 50 µg/ml ascorbic acid and 10 mM Na-β-glycerophosphate (Sigma-Aldrich). The culture medium was changed every 48 h for 2.5 weeks. Using similar experimental conditions, MC3T3-E1 cells were maintained for 4.5 weeks. Adherent cells were washed with PBS, and then fixed by ice-cold 70% ethanol for 1 h. The fixed cells were washed with distilled water and incubated with alizarin red-S (Merck KGaA, Darmstadt, Germany, 40 mM, pH 7.4) for 10 min at room temperature. After extensive washing, the mineralized nodules were counted by light microscope in three random fields and were averaged for each condition.

Statistical analyses

Represented data are the results of three independent studies at least. Mann-Whitney's *U* test was used to assess differences in experimental groups. *P* values less than 0.05 were considered statistically significant.

Results

-Soluble factor(s) produced by MOS-J/POS-1 cells stimulated MC3T3-E1 cell proliferation

To determine whether osteosarcoma cells produce soluble factor(s) modulating bone cell survival and proliferation, osteoblast proliferation was analyzed in the presence of CMs from osteosarcoma cells. MOS-J/POS-1 CMs induced a significant up-regulation of MC3T3-E1 cell proliferation in a dose-dependent manner (Figure 1A: MOS-J, B: POS-1). MOS-J CM induced around 20 % higher MC3T3-E1 cell proliferation compared to that of POS-1 CM (Figure 1A-D). Furthermore, soluble factor(s) secreted from MOS-J and POS-1 acted as survival factor(s) of MC3T3-E1 cells by increasing alive cell percentage (Figure 1E) and decreasing cell death (Figure 1F).

To identify the factor(s) associated with the up-regulation of pre-osteoblast proliferation, the effects of several signal transduction inhibitors and cytokines/growth factors were assessed in the MC3T3-E1 proliferation assay. Suramin, a pan-growth factor blocker, significantly and dose-dependently abrogated the up-regulated MC3T3-E1 cell proliferation induced by MOS-J CM (Figure 2A) and POS-1 CM (Figure 2B). This up-regulation was also abolished in the presence of piceatannol, AG490, LY294002 or

rapamycin (Figure 2C), whereas both UO126 and STAT3 inhibitor peptide had no significant effect (Figure 2D).

-MOS-J/POS-1 CMs activate ERK 1/2, STAT3, Akt and mTOR signal transduction pathways in MC3T3-E1 pre-osteoblast cell

When MC3T3-E1 cells were incubated in the presence of MOS-J/POS-1 CMs, an overt activation (phosphorylation) of ERK 1/2, STAT3 and Akt were observed after around 2 and 5 min and reached a plateau after 10 min for ERK1/2, 15 min for STAT3 and Akt, respectively (Figure 3A). Clear activation of mTOR by MOS-J/POS-1 CMs was also confirmed after 30 min incubation (Figure 3B) and no activation of p38, STAT1 and STAT5. LY294002 was evidenced. Furthermore, piceatannol and AG490 completely abolished Akt phosphorylation (Figure 3C); while JAK3 inhibitor I did not affect Akt phosphorylation in MC3T3-E1 cells (Figure 3C). Similar to JAK3 inhibitor I, UO126 did not affect the MOS-J/POS-1 CMs-induced Akt status (data not shown).

As the JAK/STAT signaling pathway was activated in MC3T3-E1 cells by MOS-J/POS-1 CMs, we analyzed the mRNA expression, of IL-6 family cytokines wich ar known to activate JAKs/STATs pathway, by osteosarcoma cell. Both MOS-J and POS-1 cells expressed IL-6, IL-11 and LIF transcripts, but not OSM. Therefore, STAT3 and Akt activation by these IL-6 family cytokines expressed by MOS-J/POS-1 cells were

studied. These cytokines did not induce Akt phosphorylation, whereas STAT3 was clearly activated. As previous reported [11], IL-6 can remarkably activate STAT3 in association with its soluble receptor; however STAT3 phosphorylation was slightly induced by IL-6 alone. IL-6 family cytokines-induced STAT3 phosphorylation was significantly abrogated in the presence of anti-gp130. Furthermore, neither all recombinant cytokines/growth factors tested (rhEGF, rhGH, rhIL-6, rhIL-6+rhIL-6Rs, rhIL-11, rhLIF, rhIGF-I) nor neutralizing antibodies (anti-IL-6R, anti-IL-11R, anti-gp130, anti-IGF-I, anti-IGF-II) did not allow to identify the factor produced by MOS-J/POS-1 cells and associated with MC3T3-E1 cell proliferation (data not shown).

-MOS-J/POS-1 cell effects on osteoblastic differentiation

ALP activity of MC3T3-E1 cells was significantly reduced by MOS-J/POS-1 CMs treatment compared to the CT CM [CT CM vs MOS-J/POS-1 CMs (mean ± SD, *U*/µg protein): 0.44 ± 0.064 vs 0.20 ± 0.043/ 0.23 ± 0.078, p=0.02]; however there was no significant difference between MOS-J CM and POS-1 CM (p=0.77). In addition, MOS-J/POS-1 CMs significantly reduced mineralized nodule formed by MC3T3-E1 cells compared to CT CM [CT CM vs MOS-J/POS-1 CMs (mean ± SD): 41 ± 14 vs 11 ± 5/ 5 ± 4, p=0.0039] (Figure 4). MOS-J CM induced clearly bigger and greater number of mineralized nodules compared to those of POS-1 CM (MOS-J CM vs POS-1 CM: 11 ± 5

vs 5 ± 4, p=0.045) (Figure 4). The induction of small mineralized nodules was scarcely observed in the presence of POS-1 CM. The congruent data were observed in the culture of mouse calvaria osteoblasts (data not shown).

Discussion

In the present study, we first evidenced that the conditionned media of MOS-J/POS-1 osteosarcoma cells modulated MC3T3-E1 cell viability and exerted a dose-dependent stimulatory effect on cell proliferation. As suramin, a pan-growth factor blocker, dose-dependently inhibited the effect of MOS-J/POS-1 CMs on MC3T3-E1 cell proliferation, we considered that MOS-J/POS-1 cells produced more than one growth factor which drastically up-regulated MC3T3-E1 cell proliferation.

Interestingly, the present results demonstrated that JAKs/PI3-K/Akt/mTOR pathway, not ERK 1/2 and STAT3, played a crucial role in the effects of MOS-J/POS-1 CMs. Moreover, clear signal crosstalk between JAK1, 2 and PI3-K/Akt cascades was revealed and PI3-K/Akt was downstream target of JAKs (Figure 5). To the best of our knowledge, this is the first report of JAKs and PI3-K/Akt signal crosstalk in osteoblastic cells. Such signal crosstalk has been reported as an optimal anti-apoptotic and pro-proliferating signal in other tumor cell types, including multiple myeloma cells [12-14], hepatoma cells [15] and basal cell carcinoma cells [16]. In agreement with these

reports, this signal also played an essential role in dramatically up-regulated MC3T3-E1 pre-osteoblast cell proliferation.

Upstream signaling between cytokine/growth factor receptors and PI3-K is not fully determined. JAKs are implicated in cytokines/growth hormones signaling and are crucial components of diverse signal transduction pathways that govern cellular survival, proliferation, differentiation and apoptosis [17]. While the STATs are well known and the most investigated downstream target of JAKs, other targets of JAKs have been identified [17]. Indeed, emerging evidence has revealed that JAK kinase function is required for optimal activation of the Src-kinase cascade, the Ras-MAP kinase pathway and the PI3-K/Akt pathway [17]. In JAKs-induced PI3-K activation, several studies have reported the involvement of the p85 subunit of PI3-K [18], insulin-receptor substrate (IRS) proteins (IRS-1, IRS-2 and IRS-3) [19] and gp130 subunit [20]. Taken together, these studies indicate that the cellular signal transduction machinery is programmed to respond to changes in stimuli by integrating diverse signaling pathways and thus to generate an orchestrated response. In these previous reports, IL-6 family cytokines functioned as an activator of JAKs and PI3-K crosstalk [12-16, 20]. We have therefore carefully examined IL-6 family cytokines. Thus, IL-6, IL-11, LIF, OSM but also EGF, GH, IGF-I and IGF-II have been excluded and did not appear responsible for the effects

of CMs observed on osteoblastic cells and the specific factor(s) remain to be determined. It is likely that these cytokine/growth hormone cocktails can uniquely support pro-proliferating effects on pre-osteoblast cells *via* JAKs and PI3-K signal crosstalk by their synergic effect. It has been reported that the rate of bone formation and resorption is largely determined by the numbers of bone-forming (osteoblast) and bone-resorbing (osteoclast) cells responsible for the regeneration of the adult skeleton [21]. Thus, the determination of the specific factor(s) and optimal pre-osteoblast cell pro-proliferating signals undoubtedly will lead to unprecedented therapeutic approach for bone-loss diseases such as osteoporosis. The JAKs and PI3-K signal crosstalk is a good candidate of such signals.

MOS-J/POS-1 CMs significantly reduced mineralized nodule formations in pre-osteoblastic cells; however MOS-J CM could induce bigger and greater number of mineralized nodules compared to those induced by POS-1 CM. MOS-J model mice [5] demonstrated clearly more osteogenic tumors compared to the POS-1 model [7]. Thus, our findings can explain, at least in part, the increased osteogenic profile of MOS-J model.

In conclusion, the present study reveals clear interaction of osteosarcoma cells with bone cells. The pre-osteoblast cell proliferation induced by osteosarcoma cells may

play, at least in part, a role into the osteoblastic profile of osteosarcoma patients. Furthermore, novel JAKs and PI3-K/Akt signal crosstalk that induces pre-osteoblast cell proliferation, one of the principal results obtained from the present study, will offer novel insight into the osteosarcoma biology as well as therapeutic capability for all bone-loss diseases such as osteoporosis.

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Figure legends

Figure 1. MOS-J/POS-1 conditioned media (CMs) significantly up-regulated MC3T3-E1 cell proliferation.

The mouse pre-osteoblast MC3T3-E1 cells were cultured for 72 h with control CM (CT CM) or MOS-J/POS-1 CMs supplemented with 5% FBS as described in the Materials and Methods section. Cell proliferation was determined using a XTT based method. CM from both MOS-J (**A**) and POS-1 (**B**) cells drastically up-regulated MC3T3-E1 cell proliferation in a dose-dependent fashion. Manual viable cell counting with trypan blue exclusion revealed that MOS-J/POS-1 CMs induced MC3T3-E1 cell proliferation (**C**) and reduced cell death (**D**) resulted in an increase of alive cell percentage (**E**) and a decrease of dead cell percentage (**F**).

*: $p<0.004$, **: $p<0.0001$ by a Mann-Whitney's *U* test (CT CM vs CMs)

Figure 2. MC3T3-E1 cell proliferation tests with suramin or several signal transduction inhibitors.

Manual cell counting with trypan blue exclusion revealed that suramin, a pan-growth factor blocker, attenuated the effect of MOS-J/POS-1 CMs on MC3T3-E1 cell proliferation from 4 days of culture in a dose-dependent fashion. (**A**) MOS-J CM, (**B**)

POS-1 CM, *: $p<0.004$ by a Mann-Whitney's *U* test (CMs vs others) CT CM: control CM, s50: suramin 50 µg/ml, s100: suramin 100 µg/ml, s200: suramin 200 µg/ml

(**C**) The high proliferation rate of MC3T3-E1 cells induced by MOS-J/POS-1 CMs was reduced to the basal level in the presence of a Janus kinase (JAK) 1 inhibitor (piacetannol, 10 µM), a JAK2 inhibitor (AG490, 20 µM), a phosphatidylinositol 3-kinase (PI3-K) inhibitor (LY294002, 20 µM) or a mammalian target of rapamycin (mTOR) inhibitor (rapamycin, 10 nM). (**D**) Both an ERK 1/2 inhibitor (UO126) and a STAT3 inhibitor (STAT3 inhibitor peptide) had no significant effect. **: $p<0.001$ by a Mann-Whitney's *U* test (vs respective CM)

Figure 3. Results of signal transduction analyses by Western blot.

(A) MC3T3-E1 cells were prepared as described in the Materials and Methods section and then incubated for 30 min with control conditioned media (CT CM). MC3T3-E1 cells were then incubated for 2, 5, 10, 15 and 30 min in the presence or absence of MOS-J (left panel)/POS-1 (right panel) CMs. **(B)** MOS-J/POS-1 CMs significantly activated mTOR after 30 min incubation. **(C)** A phosphatidylinositol 3-kinase (PI3-K) inhibitor (LY294002, 20 µM) completely abrogated MOS-J/POS-1 CMs-induced Akt phosphorylation. A Janus kinase (JAK) 1 inhibitor (piacetannol, 20 µM) and a JAK2

inhibitor (AG490, 20 μM) also completely abrogated MOS-J/POS-1 CMs-induced Akt phosphorylation; however a JAK3 inhibitor (JAK3 inhibitor I, 20 μM) had no effect on Akt phosphorylation.

Figure 4. Representative mineralized nodules induced by CT CM and MOS-J/POS-1 CMs.

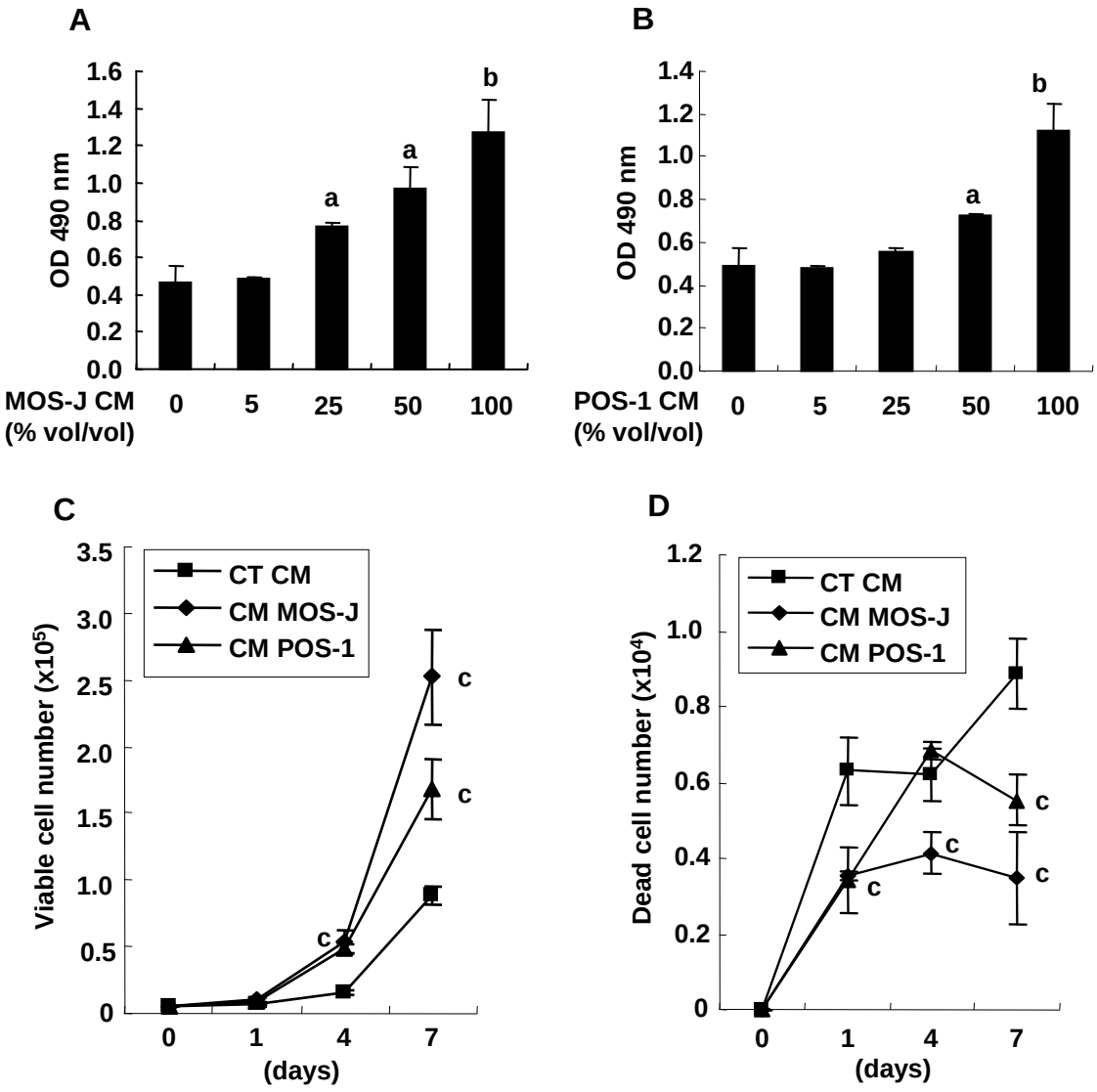
Mineralized nodule induction was detected by alizarin red-S staining as described in Materials and Methods section. MOS-J/POS-1 conditioned media (CMs) significantly reduced mineralized nodule formation compared to control CM (CT CM); however MOS-J CM induced clearly bigger and greater numbers of mineralized nodules compared to those of POS-1 CM. POS-1 CM induced only small mineralized nodules. Original magnification: x100 and x400 (inserts), bar: 25 μm

Figure 5. Schematic representation of signaling pathway involved in pre-osteoblast proliferation induced by MOS-J/POS-1 CMs .

MOS-J/POS-1 conditioned media (CMs) induced novel JAK1, 2 and PI3-K signal crosstalk in osteoblastic cells. The pre-osteoblast proliferating signal induced by MOS-J/POS-1 CMs was transmitted *via* the JAKs/PI3-K/Akt/mTOR pathway. “Soluble

factor(s)": pre-osteoblast cell pro-proliferating factor(s) produced by osteosarcoma cells. Solid line arrow: signaling pathway associated with pre-osteoblast proliferation induced by MOS-J/POS-1 CMs. Dotted line arrow: MOS-J/POS-1 CMs-induced but non-responsible signals for pre-osteoblast proliferation

Figure 1


A
OD 490 nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
MOS-J CM
(% vol/vol)
0
5
25
50
100
B
OD 490 nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
POS-1 CM
(% vol/vol)
0
5
25
50
100
C
Viable cell number (x10⁵)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
CT CM
CM MOS-J
CM POS-1
c
c
c
0
1
4
7
(days)
D
Dead cell number (x10⁴)
1.2
1.0
0.8
0.6
0.4
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0
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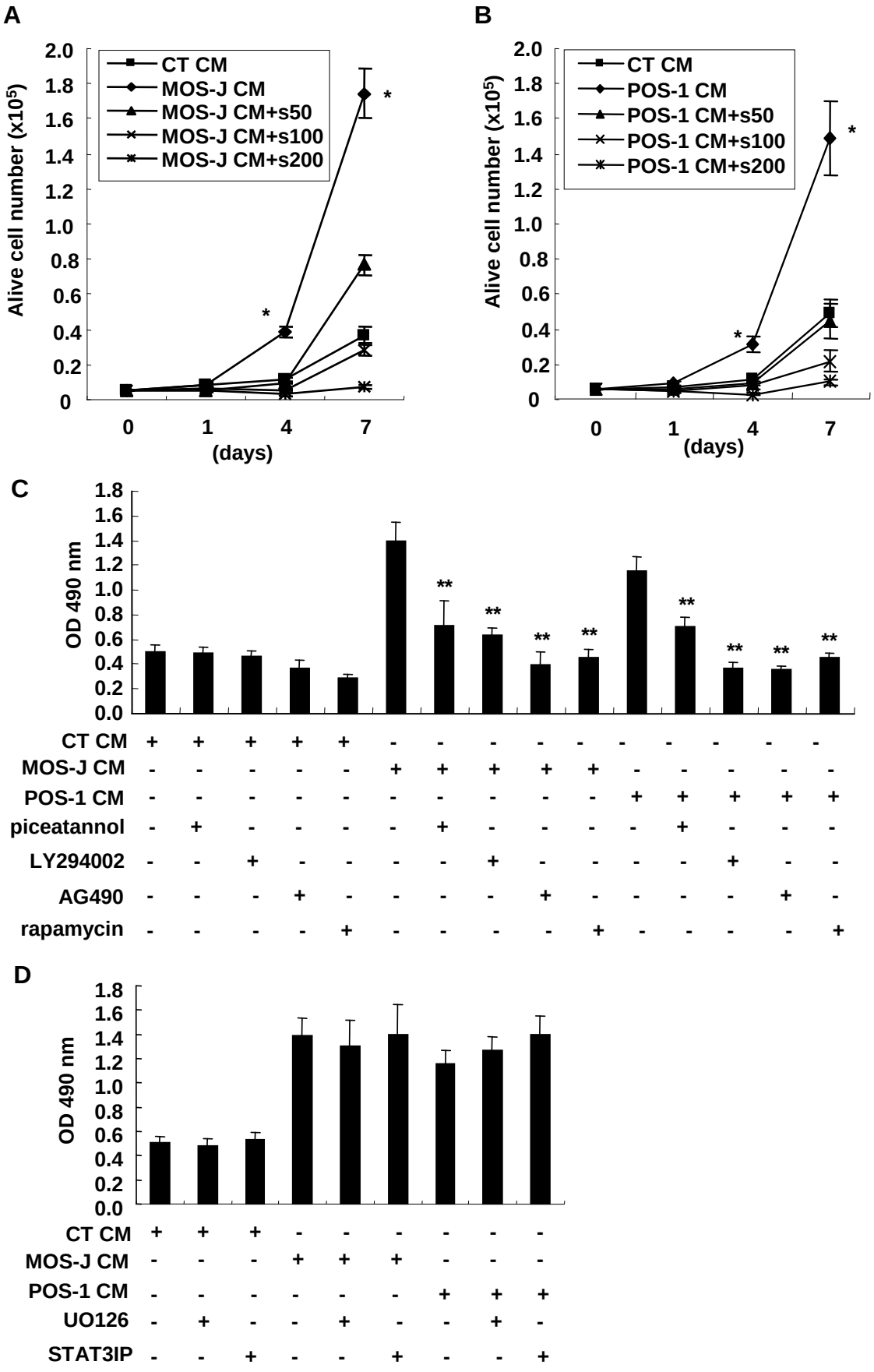
CM MOS-J
CM POS-1
c
c
c
c
0
1
4
7
(days)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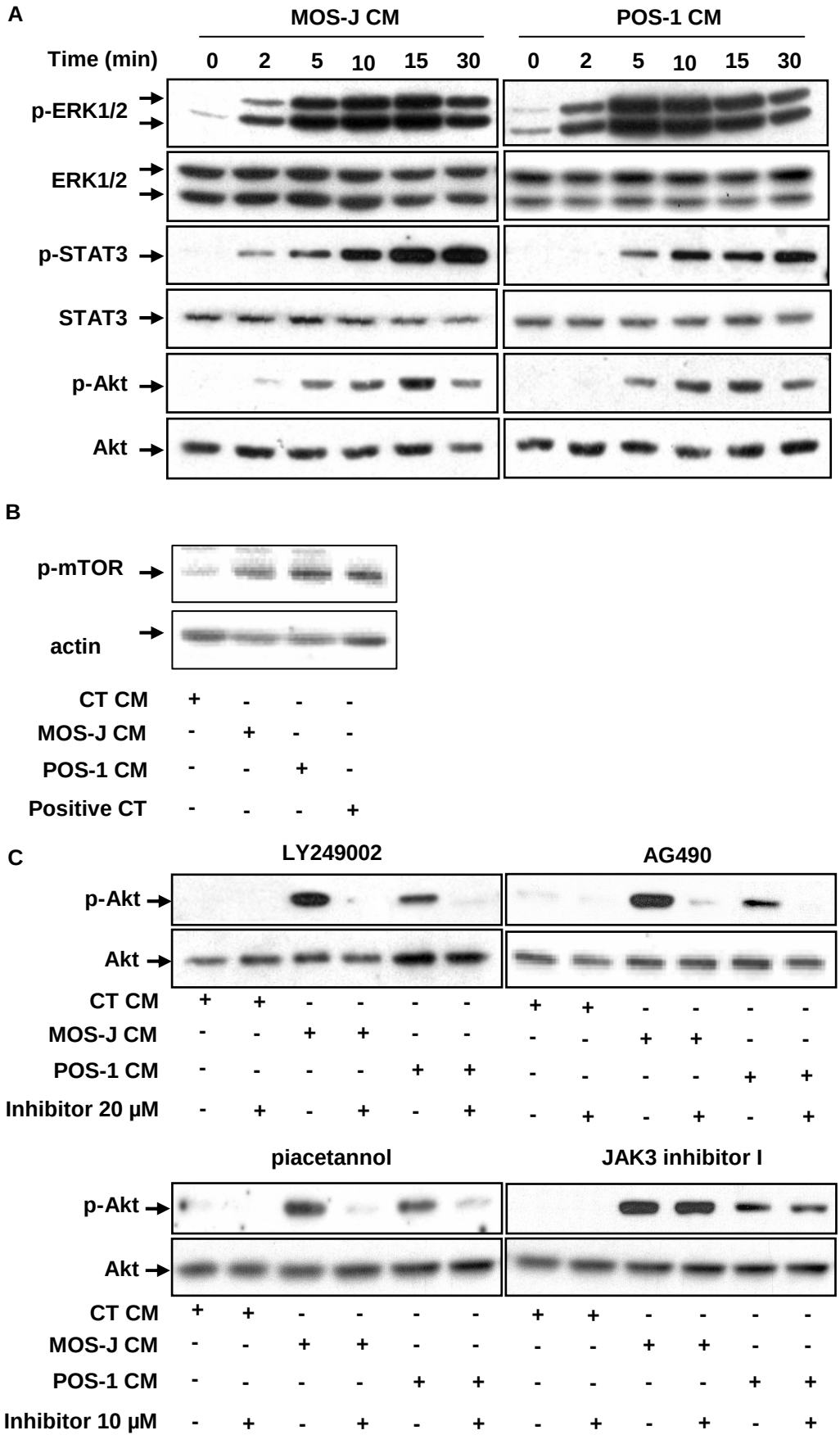
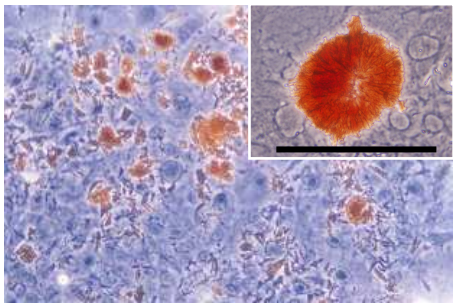
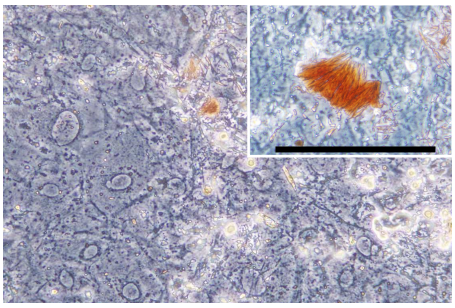
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
CT CM
MOS-J CM
MOS-J CM+s50
MOS-J CM+s100
MOS-J CM+s200
*
*
0
1
4
7
(days)
B
Alive cell number (x10^5)
CT CM
POS-1 CM
POS-1 CM+s50
POS-1 CM+s100
POS-1 CM+s200
*
*
0
1
4
7
(days)
C
OD 490 nm
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
**
**
**
**
**
CT CM + + + + + - - - - - - - - - -
MOS-J CM - - - - - + + + + + - - - - -
POS-1 CM - - - - - - - - - - + + + + +
piceatannol - + - - - - + - - - - + - - -
LY294002 - - + - - - - + - - - - + - -
AG490 - - - + - - - - + - - - - + -
rapamycin - - - - + - - - - + - - - - +
D
OD 490 nm
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CT CM + + + - - - - - -
MOS-J CM - - - + + + - - -
POS-1 CM - - - - - - + + +
UO126 - + - - + - - + -
STAT3IP - - + - - + - - +


A
MOS-J CM
POS-1 CM
Time (min)
0 2 5 10 15 30
0 2 5 10 15 30
p-ERK1/2
ERK1/2
p-STAT3
STAT3
p-Akt
Akt
B
p-mTOR
actin
CT CM + - - -
MOS-J CM - + - -
POS-1 CM - - + -
Positive CT - - - +
C
LY249002
AG490
p-Akt
Akt
CT CM + + - - - - + + - - - -
MOS-J CM - - + + - - - - + + - -
POS-1 CM - - - - + + - - - - + +
Inhibitor 20 μM - + - + - + - + - + - +
piacetannol
JAK3 inhibitor I
p-Akt
Akt
CT CM + + - - - - + + - - - -
MOS-J CM - - + + - - - - + + - -
POS-1 CM - - - - + + - - - - + +
Inhibitor 10 μM - + - + - + - + - + - +

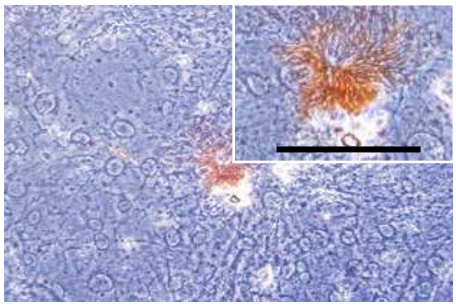
CT CM



MOS-J CM



POS-1 CM



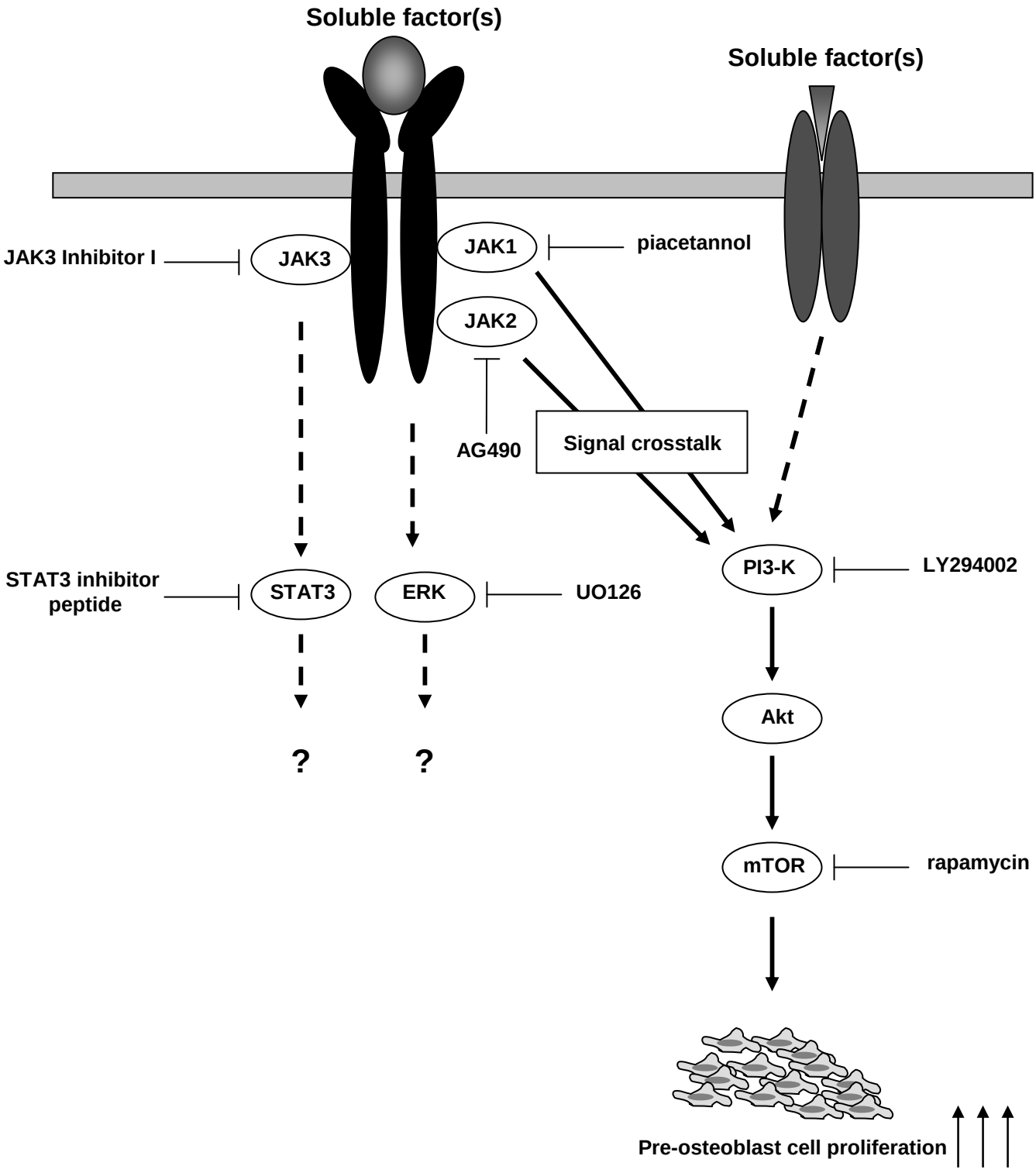
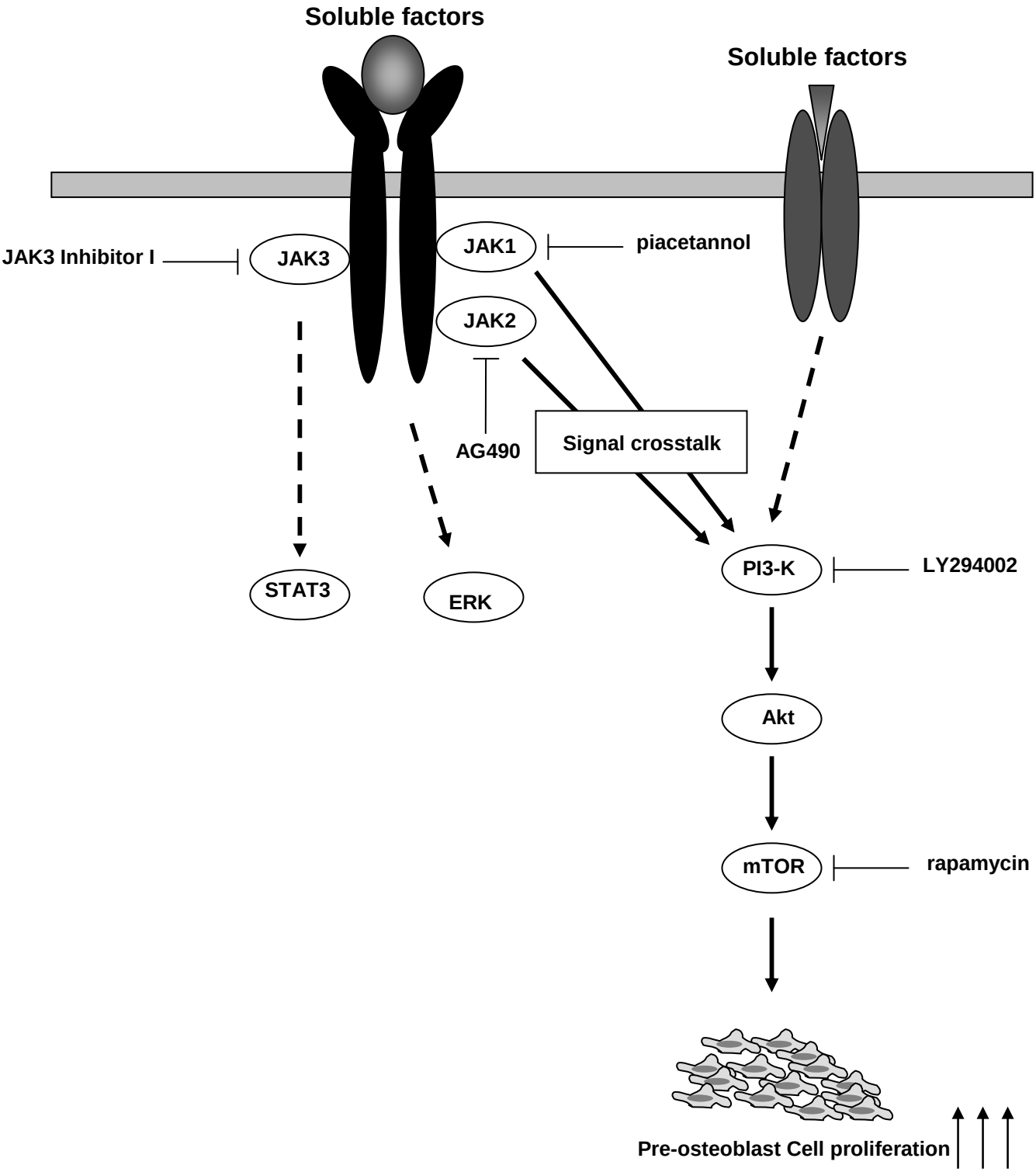

Soluble factor(s)
Soluble factor(s)
JAK3 Inhibitor I
JAK3
JAK1
piacetannol
JAK2
AG490
Signal crosstalk
STAT3 inhibitor peptide
STAT3
ERK
UO126
PI3-K
LY294002
Akt
mTOR
rapamycin
?
?
Pre-osteoblast cell proliferation

Figure 6


Soluble factors
Soluble factors
JAK3 Inhibitor I
JAK3
JAK1
piacetannol
JAK2
AG490
Signal crosstalk
STAT3
ERK
PI3-K
LY294002
Akt
mTOR
rapamycin
Pre-osteoblast Cell proliferation