

## MG-63 Osteoblast-like Cells Enhance the Osteoprotegerin Expression of PC-3 Prostate Cancer Cells

HARALAMPOS KATOPODIS<sup>1</sup>, ANASTASSIOS PHILIPPOU<sup>1</sup>, ROXANE TENTA<sup>3</sup>, CHARLES DOILLON<sup>4</sup>, KATERINA K. PAPACHRONI<sup>2</sup>, ATHANASIOS G. PAPAVASSILIOU<sup>2</sup> and MICHAEL KOUTSILIERIS<sup>1</sup>

*Departments of <sup>1</sup>Experimental Physiology and <sup>2</sup>Biological Chemistry,  
Medical School, University of Athens, Goudi, Athens 115 27;*

*<sup>3</sup>Department of Nutrition Science - Dietetics, Harokopio University, Athens, Greece;*

*<sup>4</sup>Molecular Endocrinology Laboratory, Research Center,  
Laval University Hospital, CHUL-CHUQ, Quebec, Canada*

**Abstract.** *Background:* Osteoprotegerin (OPG) expression participates in the pathophysiology of osteoblastic metastasis in prostate cancer. *Materials and Methods:* We investigated whether the expression of OPG of PC-3 prostate cancer cells grown in 3-D collagen gels is stimulated by co-culture with MG-63 osteoblast-like cells. The expression of Runx2 (*Cbfa1*) and OPG were assessed by reverse transcription-polymerase chain reaction and Western blot analysis. *Results:* OPG and Runx2 were expressed in both PC-3 and MG-63 cells. OPG expression was remarkably enhanced in PC-3 cells grown in co-culture with MG-63 cells in a time-dependent manner. Runx2 expression of PC-3 cells was not altered by their co-culture with MG-63 cells. OPG expression of PC-3 cells was altered neither by insulin-like growth factor I (IGF-1), transforming growth factor  $\beta$ 1 (TGF $\beta$ 1), interleukin 6 (IL-6) nor by dexamethasone and zoledronic acid exogenously added to PC-3 cells. *Conclusion:* The enhancement of the OPG expression in PC-3 cells by MG-63 cells is not mediated by IGF-1, IL-6 and TGF $\beta$ 1.

Advanced prostate cancer spreads almost always to the bones being responsible for the high morbidity and mortality of prostate cancer patients (1-3). Interestingly, metastasis of prostate cancer in bone produces osteoblastic lesions, characterized by an increased synthesis of woven bone (1-

5). However, the initial phase of bone metastasis requires active bone resorption (1-3).

Osteoblasts express the receptor activator of nuclear factor kappa B (NF- $\kappa$ B) ligand (RANKL) while its receptor RANK is expressed on circulating progenitor osteoclasts (6). The binding of RANKL to RANK stimulates osteoclastogenesis and marks the initiation site of bone remodeling (6-8). Moreover, osteoblasts secrete osteoprotegerin (OPG), which acts as a decoy receptor for RANKL, thus inhibiting osteoclastogenesis (9, 10). Furthermore, there is strong evidence that OPG, RANKL and RANK are involved in the pathophysiology of osteoblastic metastasis caused by prostate cancer (11-14).

Three-dimensional (3-D) type I collagen cell culture systems have been used for studying the biology of endothelial cells, endometrial cells, hepatocytes, osteoblasts, fibroblasts and various cancer cell lines *in vitro* inducing complex processes, such as angiogenesis, wound healing, functional and architectural integrity of various tissues under physiological and pathophysiological conditions (15-18). In particular, the 3-D collagen system has been proven useful *in vitro* models for analyzing the cell cell interactions between osteoblast-like cells and cancer cells in the context of bone metastasis, including that from prostate cancer (19-23). Moreover, several studies have suggested that a mechanism which involves an epithelial to mesenchymal transition of gene expression, facilitating tumour invasion and metastasis of tumours, includes the OPG/RANK/RANKL system (24-26).

Based on these data, we have used a 3-D collagen co-culture system to study the expression of osteoblast-related genes in PC-3 prostate cancer cells co-cultured with MG-63 osteoblast-like osteosarcoma cells. Therefore, we analysed the expression of OPG and Runx2 (*Cbfa1*), the latter being an osteoblast-specific transcription factor, in PC-3 prostate cancer cells co-cultured with MG-63 osteoblast-like cells.

*Correspondence to:* Dr. Michael Koutsilieris, MD, Ph.D, Department of Experimental Physiology, Medical School, University of Athens, 75 Micras Asias, Goudi, Athens, 115 27 Greece. Tel: +30 2107462597, Fax: +30 2107462571, e-mail: mkoutsil@med.uoa.gr

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## Materials and Methods

**Cell culture.** PC-3 cells, an androgen-insensitive, p53-negative and Kirsten-Ras (K-Ras) mutated human prostate cancer cell line and the human MG-63 osteoblast-like osteosarcoma cell line were obtained from the American Type Cell Culture (ATCC, Bethesda, MD, USA). Cells were grown in 75 cm<sup>3</sup> culture flasks at 37°C in 5% CO<sub>2</sub> using Dulbecco's modified Eagle's medium (DMEM; Cambrex, Walkerville, MD, USA) containing 10% fetal bovine serum (FBS; Cambrex), 100 µU/ml, 100 mg/ml penicillin/streptomycin (Cambrex), 1% L-glutamine (Cambrex) and 1% Hepes (Cambrex). The PC-3 and the MG-63 cells were grown to confluence and then detached by trypsin-EDTA solution and collected by centrifugation.

**Preparation of the 3-D collagen gel co-culture system.** Collagens were prepared using native type I collagen extracted from rat-tail tendons. Three-dimensional gels of native type I collagen were prepared in 24-well plates (6 each time) by rapidly mixing cells (at a final density of 10<sup>6</sup> cells/ml of gel) with type I collagen in a mixture of fivefold concentrated minimum essential medium (MEM)-sodium hydroxide (0.1 M), and sodium bicarbonate (0.26 M) as previously reported (23-24). Co-cultures of PC-3 and MG-63 cells were created by putting gels containing PC-3 cells (6 gels, 10<sup>6</sup> cells/gel) in flasks containing MG-63 cells (about 6×10<sup>6</sup> cells; final ratio 1:1) and *vice versa* as well as collagen gels containing PC-3 and MG-63 in empty flasks (19-23). The cell culture conditions in the co-culture system were the same as described above.

**Cell treatments.** In addition, we have assessed the effects of transforming growth factor β1 (TGFβ1), insulin-like growth factor I (IGF-1) and interleukin 6 (IL-6) on runx2 and opg expression in PC-3 cells. The selected doses [TGFβ1 (50 ng/ml; 48 h), IL-6 (50 ng/ml; 48 h), IGF-1 (50 ng/ml; 48 h)], which has been previously proven to be maximally effective in a PC-3 cell proliferation assay (27-29), were assessed for induction of runx2 and opg expression in PC-3 cells. In addition, in our study we assessed the effects of zoledronic acid (50 µM; 48 h) and of dexamethasone (100 nM; 48h), therapeutic agents used in the treatment of advanced prostate cancer, on the expression of runx2 and opg in PC-3 cells (27).

**RNA isolation and RT-PCR.** Trizol Reagent (Invitrogen Corp., Carlsbad, CA, USA), SuperScript II RNase H- Reverse Transcriptase (Invitrogen), 100 mM dNTP Set (Invitrogen), Random Primers (Invitrogen), Marker 100 bp DNA Ladder (Invitrogen), Taq DNA Polymerase recombinant (Invitrogen), Hot Start Taq DNA Polymerase kit (Qiagen, Hilden, Germany) were used for RNA isolation and RT-PCR. Total RNA from MG-63 and PC-3 cells was extracted using the Trizol Reagent. First-strand cDNA was synthesized from 2 µg of RNA mixed with 0.5 mM dNTPs, 0.015 µg/µl Random Hexamer Primer and made up to 12 µl with diethylpyrocarbonate (DEPC)-treated ddH<sub>2</sub>O. The reaction mixture was then treated to 65°C for 5 min and quick-chilled on iced water. RT-buffer containing 200 U/µl of Superscript II Reverse Transcriptase was then added and the reactants were then incubated at 42°C for 50 min and 70°C for 20 min. The PCR mix for the amplification of the genes was carried out in 25 µl consisting of 5 U/µl Taq DNA Polymerase recombinant, 10XPCR Buffer, 10 mM of each dNTP, 50 mM MgCl<sub>2</sub>, 2 µl 18S Primer:Competimer

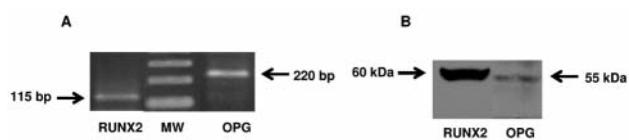


Figure 1. Representative RT-PCR and Western Blot analysis of Runx2 and OPG in PC-3 prostate cancer cells. A, Runx2 and OPG mRNA expression in PC-3 cells. B, Runx2 and OPG expression at protein level in PC-3 cells. Marker 100 bp DNA ladder (Invitrogen) and PageRuler Prestained Protein Ladder (Fermentas) were used for the approximation of molecular weight of PCR products and blotted proteins, respectively (see Material and Methods section).

mixture and 1 µl cDNA. The Primer: Competimer ratios were 1:9 for OPG and 1:9 for Runx2. After amplification, PCR samples were run in 1.8% agarose gels and the products were visualized by ethidium bromide staining and photographed by a Kodak DC290 camera. The quantifications of PCR products were performed with Kodak EDAS 290 program (27).

**Protein isolation and Western blot.** Tris-HCl (Sigma, St. Louis, MO, USA), EDTA (Sigma), dichlorodiphenyltrichloroethane (DTT) (Sigma), NaCl (Sigma), leupeptin (Sigma), pepstatin (Sigma), aprotinin (Sigma), phenylmethanesulphonylfluoride (PMSF) (Sigma), NaF (Sigma), Na<sub>3</sub>VO<sub>4</sub> (Sigma), nitrocellulose membrane (BIO-RAD Laboratories, Hercules, CA, USA) were used for protein isolation and Western blot analysis. After the co-cultivation, the cells were detached using trypsin-EDTA and then collected by centrifugation. The pellet was then lysed in a lysis buffer consisted of 20 mM Tris-HCl, pH-7.4, 1% Triton<sup>R</sup> X-100, 1 mM EDTA, 5 mM DTT and 150 mM NaCl, supplemented with protease inhibitors (10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml aprotinin, 1 mM PMSF, 1 mM NaF and 1 mM Na<sub>3</sub>VO<sub>4</sub>). This solution was left on ice for 20 min and re-centrifuged. The protein content was determined using the Bradford protein assay. A total of 10-20 µg of total protein was resolved by 10% SDS-PAGE for the detection of OPG and Runx2 proteins. The gels were transferred onto nitrocellulose transfer membranes and blocked with 5% non-fat dry milk for 1 hour at room temperature. The membranes were incubated overnight at 4°C, with OPG antibody (R&D Systems, Minneapolis, MN, USA) at a 1:500 dilution or Runx2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:1000 dilution, in TBS-Tween containing 1% non-fat dry milk. The blots were then washed and incubated with the appropriate secondary antibodies for 1 hour at room temperature (Santa Cruz Biotechnology; 1:2000 dilution). Expression of GAPDH (Santa Cruz Biotechnology) as reference protein was also analysed in the same protein extracts. The bands were visualized by exposure to X-ray film after incubation of the blots with SuperSignal ECL substrate (Pierce Biotechnology, Rockford, IL, USA). Quantification was performed using Kodak imaging software (EDAS Kodak software) (27).

**Statistical analysis.** All experiments were performed in triplicate. Values are means±standard deviation (SD). Statistical analysis was performed by Student's *t*-test. The level of statistical significance was set at *p*<0.05.

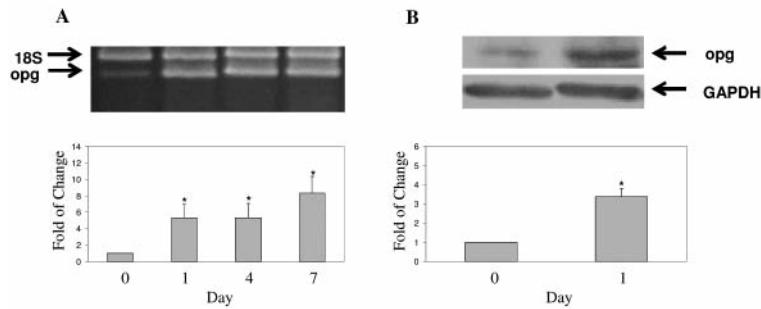


Figure 2. The effect of the co-culture of MG-63 osteoblast-like cells on OPG expression of PC-3 prostate cancer cells using our 3D-collagen gel system. A, Representative image of OPG expression and 18S, using relative quantitative RT-PCR. B, Representative image of OPG expression and GAPDH, using Western blot analysis. Data are represented as fold of change in mRNA and protein expression, respectively, [mean±SE ( $n=3$ )]. Statistical analysis was performed by Student's *t*-test; \* $p<0.05$ .

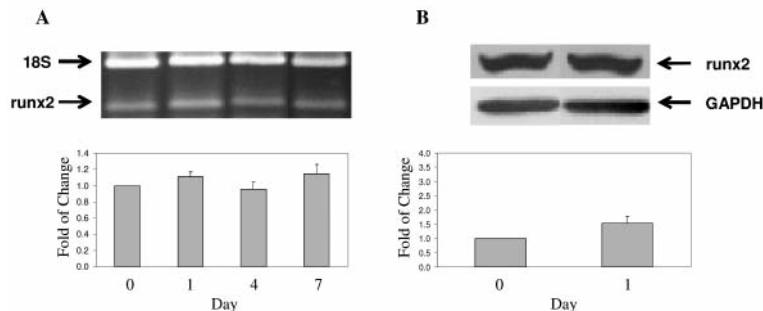


Figure 3. The effect of the co-culture of MG-63 osteoblast-like cells on Runx2 expression of PC-3 prostate cancer cells using our 3D-collagen gel system. There was no significant change in either mRNA or protein expression. A, Representative image of OPG mRNA expression and house keeping gene 18S, using relative quantitative RT-PCR. B, Similar results were obtained by Western blot analysis. Data are represented as fold of change in mRNA and protein expression, respectively, [mean±SE ( $n=3$ )]. Statistical analysis was performed by Student's *t*-test;  $p$ -values did not reach significance ( $p>0.05$ ).

## Results

**Expression of OPG and Runx2 in PC-3 and MG-63 cells.** OPG and Runx2 were expressed in the PC-3 prostate cancer cell line both at the mRNA (Figure 1A) and at the protein level (Figure 1B). These data suggest that PC-3 (human bone metastasis-derived prostate cancer cells) expressed genes, which have been strongly related with osteoblast/mesenchymal cell phenotype. OPG and Runx2 expression was also detected in MG-63 cells, as expected (data not shown).

The expression of OPG was significantly increased in 3-D collagen gels, containing PC-3 cells, co-cultured in flasks with MG-63 osteoblast-like cells at both mRNA (Figure 2A) and at protein level (Figure 2B). This was a time-dependent effect. However, we detected no significant changes in Runx2 expression in PC-3 cells co-cultured with MG-63 cells, neither at the mRNA (Figure 3A) nor at the protein level (Figure 3B). These data suggest that the enhancement of OPG expression was gene specific in this co-culture experiment.

In addition, IGF-1, TGF $\beta$ 1 and IL-6 (50 ng/ml final concentration) did not significantly alter OPG (Figure 4A) or Runx2 expression (Figure 4B) in PC-3 cells. Nor did, zoledronic acid (50  $\mu$ M) or dexamethasone (100 nM) affect the baseline OPG (Figure 5A) and Runx2 expression (Figure 5B) of PC-3 cells. These data suggested that enhancement of OPG expression in PC-3 cells induced by MG-63 cells was not mediated by IGF-1, TGF $\beta$ 1 or IL-6 production of MG-63 cells.

## Discussion

More than 90% of patients with advanced prostate cancer finally develop skeletal lesions (1-4). The pathogenesis of bone metastasis involves specific host-tissue recognition of circulating prostate cancer cells, enabling tumour cells to migrate and to invade the bone matrix and, finally, to establish local cell-cell interactions with host tissue, leading to osteoblastic metastasis (1-5). Notably, osteoblastic metastasis represents the stronghold of prostate cancer progression towards the androgen ablation-refractory stage

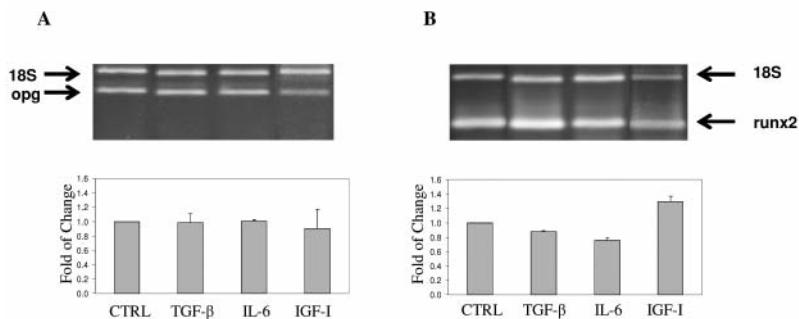


Figure 4. Representative analysis of the effects of TGF $\beta$ , IL-6 and IGF-I (50 ng/ml; 48 h exposure) on OPG (A) and Runx2 (B) mRNA expression of PC-3 prostate cancer cells. There were no significant changes in mRNA expression of either genes. Data are also represented as fold of change in mRNA expression, [mean $\pm$ SE (n=3)]. Statistical analysis was performed by Student's t-test; p-values did not reach significance ( $p>0.05$ ).

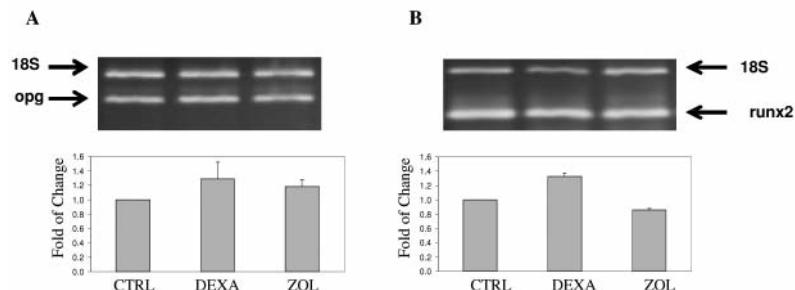


Figure 5. Representative analysis of the effects of dexamethasone (100 nM, 48 h) and zoledronic acid (5  $\mu$ M, 48 h) on OPG and Runx2 mRNA expression in PC-3 prostate cancer cells. There was no significant change in mRNA expression of either genes. A, OPG mRNA expression in PC-3 cells; B, Runx2 mRNA expression in PC-3 cells. Data are also represented as fold of change in mRNA expression [mean $\pm$ SE (n=3)]. Statistical analysis was performed by Student's t-test; p-values did not reach significance ( $p>0.05$ ).

(28, 29). However, the predominantly bone-specific nature of the refractoriness to androgen ablation therapy, implies that specifically local environmental cues, and not only genetic factors related to clonal evolution of the tumour, might be responsible for rescuing prostate cancer cells from apoptosis induced by androgen deprivation (30-33). It might be that host tissues such as bone that are rich in IGF-1, TGF $\beta$ 1 and IL-6 are sanctuaries for prostate cancer cells, and this possibility might also account for the change of prostate cancer cells from an androgen-dependent to an androgen-independent phenotype while they still express active androgen receptors (5, 27-31). Therefore, cell-cell interactions, involving IGF-1, IL-6 and TGF $\beta$ 1, between prostate cancer cells and host tissue (osteoblasts) determine the host tissue reaction (blastic reaction) and tumour cell survival in bone metastasis microenvironment.

Recently, it was found that osteoblasts cells express a member of the tumor necrosis factor (TNF)-ligand family, RANKL, which is a membrane associated factor. RANKL interacts with OPG, a soluble decoy receptor for RANKL, preventing the establishment of osteolytic lesions in bone,

although it did not prevent subcutaneous tumour growth, *in vivo* (34). Herein we documented that the presence of MG-63 osteoblast-like cells in our 3-D collagen culture system remarkably stimulated the expression of OPG in PC-3 prostate cancer cells. This enhancement of OPG expression was gene specific because Runx2 expression was not altered by this experimental procedure. Runx2 (Cbfa1) is a member of the runt homology domain family of transcription factors, essential for osteoblast differentiation and bone formation (35). It has been shown that Runx2 regulates the expression of all the major genes expressed by osteoblasts, related to mesenchymal/osteoblast phenotype. Runx2 expression is up-regulated when osteoblasts are treated *in vitro* with conditioned medium obtained from prostate cancer cells (36).

However, in our study Runx2 expression was neither enhanced by the co-culture of PC-3 cells with MG-63 cells nor by IGF-1, IL-6 and TGF $\beta$ 1. Furthermore, the mediator(s) of such an enhancement of OPG expression of PC-3 cells induced by MG-63 cells was apparently not related to osteoblast-derived IGF-1, IL-6 and TGF $\beta$ 1. Moreover,

zoledronic acid and dexamethasone, two pharmaceutical agents used in advanced prostate cancer treatment, did not modify OPG expression of PC-3 cells. Our data are in accordance with previous studies which have shown that OPG overexpression decreases osteolytic lesions although it does not affect the proliferation of cancer cells (34-36).

Therefore, the enhanced secretion of OPG by prostate cancer cells after their establishment in bone can influence the local cell-cell interactions, reducing osteoclastogenesis locally and possibly provides the basis for the development of the blastic reaction in bone metastasis from prostate cancer (9-11). Notably, OPG also facilitates the survival of prostate cancer cells *in vitro* and this anti-apoptotic property is related to its ability to bind to and inhibit the TNF-related apoptosis-inducing ligand (TRAIL) death-activating receptors (9, 34). These findings suggest that bone marrow stromal cells express and secrete OPG in their microenvironment and that of prostate cancer cells, thus increasing the survival capacity of the latter. This OPG overproduction along with other bone-related survival factors, such as IGF-1, IL-6 and TGF $\beta$ 1, could mediate the development of androgen ablation refractoriness and chemotherapy resistance of advanced prostate cancer (27-29).

In addition, the expression of Runx2 and OPG genes by prostate cancer cells, as documented in our study, supports the mesenchymal transition of prostate cancer cells during the metastatic process, as previously suggested (13, 14). Interestingly, bone microenvironment-related factors, such as IGF-1, TGF $\beta$ 1 and IL-6, as well drugs used in the treatment of prostate cancer patients with bone involvement, such as zoledronic acid and dexamethasone, showed no effect on the expression of Runx2 and OPG in PC-3 cells. These data suggest that the epithelial to mesenchymal transition of prostate cancer cells during the process of invasion and metastasis is possibly not dependent on such growth factors. In conclusion, enhancement of OPG expression of PC-3 cells when co-cultured with osteoblast-like cells suggests that OPG may be a key player in the development of blastic reaction in the bone metastasis microenvironment.

## References

- 1 Galasko C: Mechanisms of bone destruction in the development of skeletal metastases. *Nature* 263: 507-508, 1976.
- 2 Galasko C: Mechanisms of lytic and blastic metastatic disease of bone. *Clin Orthop Relat Res* 169: 20-27, 1982.
- 3 Koutsilieris M: Osteoblastic metastasis in advanced prostate cancer. *Anticancer Res* 191: 443-450, 1993.
- 4 Koutsilieris M, Rabbani SA, Bennett HPJ and Goltzman D: Characteristics of prostate-derived growth-factors for cells of the osteoblast phenotype. *J Clin Invest* 80: 941-946, 1987.
- 5 Koutsilieris M, Frenette G, Lazure C, Lehoux JG, Govindan MV and Polychronakos C: Urokinase-type plasminogen activator-A paracrine factor regulating the bioavailability of IGFs in PA-III cell-induced osteoblastic metastases. *Anticancer Res* 13: 481-486, 1993.
- 6 Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Lüthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R and Boyle WJ: Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 89: 309-319, 1997.
- 7 Lacey DL, Timms E, Tan H-L, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J and Boyle WJ: Osteoprotegerin (OPG) ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93: 165-176, 1998.
- 8 Anderson MA, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D and Galibert L: A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* 390: 175-179, 1997.
- 9 Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N and Suda T: Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* 95: 3597-3602, 1998.
- 10 Khosla S: Minireview: The OPG/RANKL/RANK System. *Endocrinology* 142: 5050-5055, 2001.
- 11 Holen I, Croucher PI, Hamdy FC and Eaton CL: Osteoprotegerin (OPG) is a survival factor for human prostate cancer cells. *Cancer Res* 62: 1619-1623, 2002.
- 12 Corey E, Brown LG, Kiefel JA, Quinn JE, Pitts TE, Blair JM and Vessella RL: Osteoprotegerin in prostate cancer bone metastases. *Cancer Res* 65: 1710-1718, 2005.
- 13 Holen I and Shipman CM: Role of osteoprotegerin (OPG) in cancer. *Clin Sci* 110: 279-291, 2006.
- 14 Guise TA, Mohammad KS, Clines G, Stebbins EG, Wong DH, Higgins LS, Vessella R, Corey E, Padalecki S, Suva L and Chirgwin JM: Basic mechanisms responsible for osteolytic and osteoblastic bone metastases. *Clinical Cancer Res* 12: 6213s-6216s, 2006.
- 15 Takeshita K, Ishibashi H, Suzuki M, Yamamoto T, Akaike T and Kodama M: High cell-density culture system of hepatocytes entrapped in a three-dimensional hollow fiber module with collagen gel. *Artif Organs* 19: 191-193, 2005.
- 16 Blackshaw SE, Arkinson S, Cameron C and Davies JA: Promotion of regeneration and axon growth following injury in an invertebrate nervous system by the use of three-dimensional collagen gels. *Proc R Soc Lond B Biol Sci* 264: 657-661, 1997.
- 17 Ushida T, Furukawa K, Toita K and Tateishi T: Three-dimensional seeding of chondrocytes encapsulated in collagen gel into PLLA scaffolds. *Cell Transplant* 11: 489-494, 2002.
- 18 Gagnon E, Cattaruzzi P, Griffith M, Muzakare L, LeFlao K, Faure R, Bélineau R, Hussain SN, Koutsilieris M and Doillon CJ: Human vascular endothelial cells with extended life spans: *in vitro* cell response, protein expression and angiogenesis. *Angiogenesis* 5: 21-33, 2002.
- 19 Koutsilieris M, Sourla A, Pelletier G and Doillon CJ: Three-dimensional type I collagen gel system for the study of osteoblastic metastases produced by metastatic prostate cancer. *J Bone Miner Res* 9: 1823-1832, 1994.

- 20 Sourla A, Doillon C and Koutsilieris M: Three-dimensional type I collagen gel system containing MG-63 osteoblast-like cells as a model for studying local bone reaction caused by metastatic cancer cells. *Anticancer Res* 16: 2773-2780, 1996.
- 21 Janvier R, Sourla A, Koutsilieris M and Doillon CJ: Stromal fibroblasts are required for PC-3 human prostate cancer cells to produce capillary-like formation of endothelial cells in a three-dimensional co-culture system. *Anticancer Res* 17: 1551-1557, 1997.
- 22 Doillon C, Gagnon E, Paradis R and Koutsilieris M: Three-dimensional type I collagen system as a model for studying cancer cell invasion capacity and anticancer drug sensitivity. *Anticancer Res* 24: 2169-2177, 2004.
- 23 Themistocleous GS, Katopodis H, Sourla A, Lembessis P, Doillon CJ, Souacos PN and Koutsilieris M: Three-dimensional type I collagen cell culture systems for the study of bone pathophysiology. *In Vivo* 18: 687-696, 2004.
- 24 Scott LJ, Clarke NW, George NJ, Shanks JH, Testa NG and Lang SH: Interactions of human prostatic epithelial cells with bone marrow endothelium: binding and invasion. *Br J Cancer* 84: 1417-1423, 2001.
- 25 Chen G, Sircar K, Aprikian A, Potti A, Goltzman D and Rabbani SA: Expression of RANKL/RANK/OPG in primary and metastatic human prostate cancer as markers of disease stage and functional regulation. *Cancer* 107: 289-298, 2006.
- 26 Chen HX, Li HZ, Li HJ, Shi BB, Jin W and Cheng XQ: Serum osteoprotegerin as a novel marker of bone metastasis in prostate cancer. *Zhonghua Wai Ke Za Zhi* 45: 412-414, 2007 (Article in Chinese).
- 27 Tenta R, Tiblalexi D, Sotiriou E, Lembessis P, Manoussakis M and Koutsilieris M: Bone microenvironment-related growth factors modulate differentially the anticancer actions of zoledronic acid and doxorubicin on PC-3 prostate cancer cells. *Prostate* 59: 120-131, 2004.
- 28 Reyes-Moreno C, Sourla A, Choki I, Doillon C and Koutsilieris M: Osteoblast-derived survival factors protect PC-3 human prostate cancer cells from adriamycin apoptosis. *Urology* 52: 341-347, 1998.
- 29 Koutsilieris M, Mitsiades C and Sourla A: Insulin-like growth factor I and urokinase-type plasminogen activator bioregulation system as a survival mechanism of prostate cancer cells in osteoblastic metastases: development of anti-survival factor therapy for hormone-refractory prostate cancer. *Mol Med* 6: 251-267, 2000.
- 30 Tolis G, Faure N, Koutsilieris M, Lemay A, Klloze S, Yakabow A and Fazekas AT: Suppression of testicular steroidogenesis by the GNRH agonist analog buserelin (HOE-766) in patients with prostatic-cancer – studies in relation to dose and route of administration. *J Steroid Biochem Mol Biol* 19: 995-998, 1983.
- 31 Mitsiades CS, Mitsiades N and Koutsilieris M: The Akt pathway: Molecular targets for anti-cancer drug development. *Current Cancer Drug Targ* 4: 235-256, 2004.
- 32 Koutsilieris M, Bogdano J, Milathianakis C, Dimopoulos P, Dimopoulos T, Karamanolakis D, Halapas A, Tenta R, Katopodis H, Papageorgiou E, Pitulis N, Pissimisis N, Lembessis P and Sourla A: Combination therapy using LHRH and somatostatin analogues plus dexamethasone in androgen ablation refractory prostate cancer patients with bone involvement: a bench to bedside approach. *Expert Opin Investig Drugs* 15: 795-804, 2006.
- 33 Mitsiades CS and Koutsilieris M: Molecular biology and cellular physiology of refractoriness to androgen ablation therapy in advanced prostate cancer. *Expert Opin Invest Drugs* 10: 1099-1115, 2001.
- 34 Nyambo R, Cross N, Lippitt J, Holen I, Bryden G, Hamdy FC and Eaton CL: Human bone marrow stromal cells protect prostate cancer cells from TRAIL-induced apoptosis. *J Bone Miner Res* 19: 1712-1721, 2004.
- 35 Yang J, Fizazi K, Peleg S, Sikes CR, Raymond AK, Jamal N, Hu M, Olive M, Martinez LA, Wood CG, Logothetis CJ, Karsenty G and Navone NM: Prostate cancer cells induce osteoblast differentiation through a Cbfa1-dependent pathway. *Cancer Res* 61: 5652-5659, 2001.
- 36 Zhang J, Dai J, Qi Y, Lin DL, Smith P, Strayhorn C, Mizokami A, Fu Z, Westman J and Keller ET: Osteoprotegerin inhibits prostate cancer induced osteoclastogenesis and prevents tumor growth in the bone. *J Clin Invest* 107: 1235-1244, 2001.

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