RESEARCH COMMUNICATION

# Monocyte Chemoattractant Protein-1 Modulates Invasion and Apoptosis of PC-3M Prostate Cancer Cells Via Regulating Expression of VEGF, MMP9 and Caspase-3

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## Abstract

**Prostate cancer is a form of malignancy that is most likely to develop in older males, but because of the propensity to metastasize to parts of the body, particularly the bones, can have a deleterious impact on quality of life. Recently monocyte chemoattractant protein-1 (MCP-1) has been shown to play important role in prostate cancer progression and metastasi. In this study we aimed to investigate the mechanisms underlying its functional roles. In vitro transwell invasion assays with PC-3M prostate cancer cells demonstrated MCP-1 promotion of invasion, while annexin V-FITC and TUNEL confirmed inhibition of apoptosis. Treatment MCP-1 further led to significant upregulation of VEGF and MMP-9 and downregulation of Caspase-3 at both mRNA and protein levels compared with untreated control (P<0.05), while siRNA mediated knockdown reversed these changes. Taken together, our results indicate important roles of MCP-1 in prostate cancer progression and metastasis and our finding of regulation of VEGF, MMP-9 and Caspase-3 expression open up new possibilities for targeted therapy.**

**Keywords:** MCP-1 - VEGF - MMP-9 - prostate cancer - invasion - metastasis

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## Introduction

Prostate cancer is a form of malignancy that most likely develops in older males. It is the second leading cause of cancer death in American men. Because prostate cancer may metastasize to other parts of the body, particularly the bones, prostate cancer has deleterious impact on the survival and quality of living for patients. In fact about 80% patients who die of prostate cancer develop bone metastasis (Loberg et al., 2005; Taichman et al., 2007). Therefore, investigation of molecular mechanisms underlying the metastasis of prostate cancer to bone will be important for the prevention and therapy of prostate cancer metastasis.

Monocyte chemoattractant protein-1 (MCP-1)/CCL2, is an inducible CC-chemokine that binds its G protein coupled receptor (GPCR), CCR2 and plays a vital role in the recruitment and activation of monocytes during inflammation (Rossi and Zlotnik, 2000).

More recent studies have demonstrated that MCP-1 plays crucial role in the growth, invasion, and metastasis of prostate cancer (Loberg et al., 2006; 2007b; Lu et al., 2006). Therefore, in this study we employed a highly metastatic prostate cancer cell line PC-3M as an in vitro model to characterize the effect of MCP-1 on the cellular behaviors of PC-3M and the underling mechanism. Our results suggest that MCP-1 regulates the expression of VEGF, MMP9 and Caspase-3 to modulate the apoptosis and invasion of prostate cancer cells.

## Materials and Methods

*pFIVSi-MCP-1 construct*

A construct was synthesized by Sangon Biotech (Shanghai, China) MCP-1-siRNA: 5'-AAAGTCACCTGCTGTTATAACTTCACCAA-3' (sense strand) and cloned into pFIVSi-H1/U6 vector (SBI, Mountain View, CA, USA) to make pFIVSi-MCP-1 construct. Random scramble siRNA was cloned into pFIVSi-H1/U6 vector to make pFIVSi-scramble as negative control.

*Cell culture and transfection*

PC-3M prostate cancer cell line was purchased from Institute of Urology, Peking University and grown in IMDM supplemented with penicillin (50 U/ml), streptomycin (50 lg/ml) and 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37ºC in 5% CO2. The cells were transfected with pFIVSi-MCP-1 or pFIVSi-scramble constructs using Lipofectime

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2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. For the following experiments the PC-3M cells were divided into 4 groups. Control group: untransfected and untreated; MCP-1 group: treated with 50 ng/ml MCP-1 (Sigma, Saint Louis, Missouri, USA); Si-scramble group: transfected with pFIVSi-scramble construct; and Si-MCP-1 group: transfected with pFIVSi-MCP-1 construct.

*Cell invasion assay*

Cell invasion assay was performed with 24 well transwell chamber (8 $\mu$m, Costa, Lowell, MA, USA) according to the manufacturer's instruction. Briefly, different groups of PC-3M cells ($5\times10^4$) were plated in the upper chamber and IMDM was added in the lower chamber. Four hours latter, cells in the upper chamber were incubated with IMDM supplemented with 1% heat-inactivated fetal bovine serum and the medium in the lower chamber was changed to IMDM supplemented with 10% heat-inactivated fetal bovine serum. The cells were incubated at 37 ºC in 5% $CO_2$ for 24 h and the cells in the lower chamber were stained with 0.5% crystal violet for 5 min. After washing with PBS, the stained cells were counted under microscope (Olympus IX51 at 200×) in five different fields. The assay was performed in triplicate and three times independently.

*Flow cytometry analysis of apoptosis*

Apoptotic cells were quantified using fluorescent Annexin V binding as described previously (van Engeland et al., 1998). Briefly, different groups of PC-3M cells were collected by digestion with EDTA-free trypsin(Invitrogen, Carlsbad, CA, USA). The cell pellet was washed with PBS twice and about $1\text{-}5\times10^5$ cells were resuspended in 400 µlAnnexin V binding buffer (BD-Biosciences, San Jose, CA, USA). The cells were stained with 5µl Annexin V FITC (BD-Biosciences) for 15 min at 4 ºC in the dark. The reaction was stopped by the addition of 10µl PI and incubation for 5 min at 4 ºC in the dark. The samples were subjected to flow cytometry analysis within 1 h. The cell suspension was also analyzed under confocal microscopy (Olympus FV1000). The assay was performed in triplicate and three times independently.

*TUNEL analysis of apoptosis*

Apoptotic cells were detected by TUNEL staining and subsequent confocal microscopy. Different groups of PC-3M cells were collected and seeded on glass slides pretreated with aqueous 0.01% poly-L-lysine. Cells were fixed by covering with a puddle of 1% formaldehyde in PBS for 15 min. Next the slides were incubated with TdT buffer (Promega, Madison, WI, USA) in a humidified chamber for 1 h at 37ºC in the dark. The reaction was stopped by the addition of 2×SSC (Promega, Madison, WI, USA). Next the slides were incubated with 1ug/ml PI for 15 min at RT in the dark. The slides were washed with PBS three times and analyzed under confocal microscopy. The assay was performed in triplicate and three times independently.

*RT-PCR*

Total RNA was extracted from different groups of PC-3M cells using TRIzol reagent (Invertrogen, USA) following the manufacturer's protocol. cDNA was produced by reverse transcription using RT kit (Promega, Madsion, WI, USA) following the manufacturer's protocol. PCR amplification of mcp-1 , VEGF, MMP-9, Caspase-3, and GAPDH was done with Taq Master Mix (Promega, Madison, WI, USA) with cDNA synthesized from PC-3M cells. The primers used were as follows: MCP-1 tcgcgagctatagaagaatca (forward) and tgttcaagtcttcggagtttg (reverse); VEGF atcacgaagtggtgaagttc (forward) and aggatggcttgaagatgtac (reverse); MMP-9 aagctggactcggtcttt (forward) and cacctggttcaactcact (reverse); Caspase-3 gtggaattgatgcgtgatgt (forward) and taaccaggtgctgtggagta (reverse); GAPDH, gggtgatgctggtgctgagtatgt (forward) and aagaatgggagttgctgttgaagtc (reverse). Amplification conditions were as follows: 5 min at 95ºC (one cycle) and 30 sec at 94ºC; 30 sec at the annealing temperature (55ºC for MCP-1 and GAPDH, 54ºC for VEGF, 52ºC for MMP-9 and 50ºC for Caspase-3); and 30 sec at 72ºC (35 cycles) and 72ºC for 5 min (one cycle).

*Western b lot*

Different groups of PC-3M cells were collected after 72 h culture and total protein was isolated from the cells and quantitated by BSA method. 50ug protein was loaded onto a 10% SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA, USA). Next, the membrane was incubated with specific antibody for VEGF, MMP-9, Caspase-3 orβ-actin (Cell Signaling, Danvers, MA, USA) at 4ºC o/n. The membrane was washed with TBST for 5 min 3 times, then incubated with secondary antibody (Cell Signaling, Danvers, MA, USA) for 1 h at room temperature. The membrane was developed using ECL kit (Pierce, Rockford, IL, USA) and exposed to X-ray film. Bands on X-ray films were quantified with Image.plus5.1 software.

*MCP-1 quantification by enzyme-linked immunosorbent assay*

Total protein was isolated from different groups of PC-3M cells and subjected to enzyme-linked immunosorbent assay (ELISA) for MCP-1 according to manufacturer's instructions (R&D Systems, Villejust, France).

*Statistical analysis*

The experiments' data were expressed as mean (X) ± standard deviation (S). Statistical analysis was performed using SPSS17.0 software (SPSS, Inc., Chicago, IL, USA) and $P<0.05$ was considered significant.

## Results

*Evaluation of silencing of MCP-1 in PC-3M cells*

To verify that pFIVSi-MCP-1 vector we constructed could mediate silencing of MCP-1 in PC-3M cells upon transfection, we performed ELISA analysis to examine MCP-1 levels in total protein extracted from different groups of PC-3M cells. The results demonstrated that the concentration of MCP-1 (pg/ug total protein) was 63.5±11.60, 64.9±11.07, 88.1±8.45, and 44.5±11.29 for

control group, Si-scramble group, MCP-1 group and Si-MCP-1 group, respectively (Figuer 1). Compared with control group, the level of MCP-1 was significantly increased in MCP-1 group and decreased in Si-MCP-1 group ($p<0.05$). Thus pFIVSi-MCP-1 vector was effective in silencing MCP-1 in PC-3M cells and could be used for the following experiments.

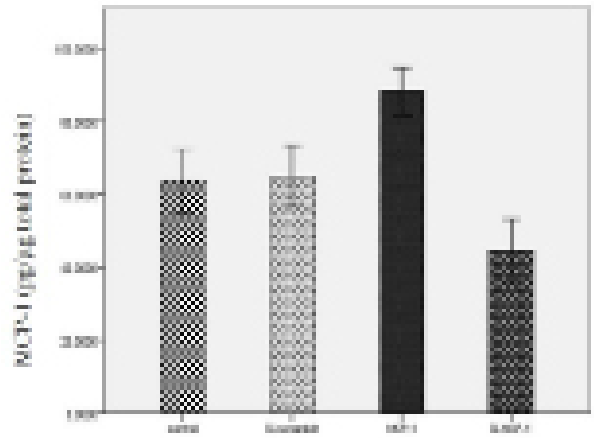

**Figure 1. MCP-1 Levels in PC-3M Prostate Cancer Cells Determined by ELISA.** Total protein was isolated from PC-3M cells in control group, Si-scramble group, MCP-1 group or Si-MCP-1 group and the concentration of MCP-1 (pg/ug total protein) was determined by ELISA. Compared with control group, the level of MCP-1 was significantly increased in MCP-1 group and decreased in Si-MCP-1 group ($p<0.05$)

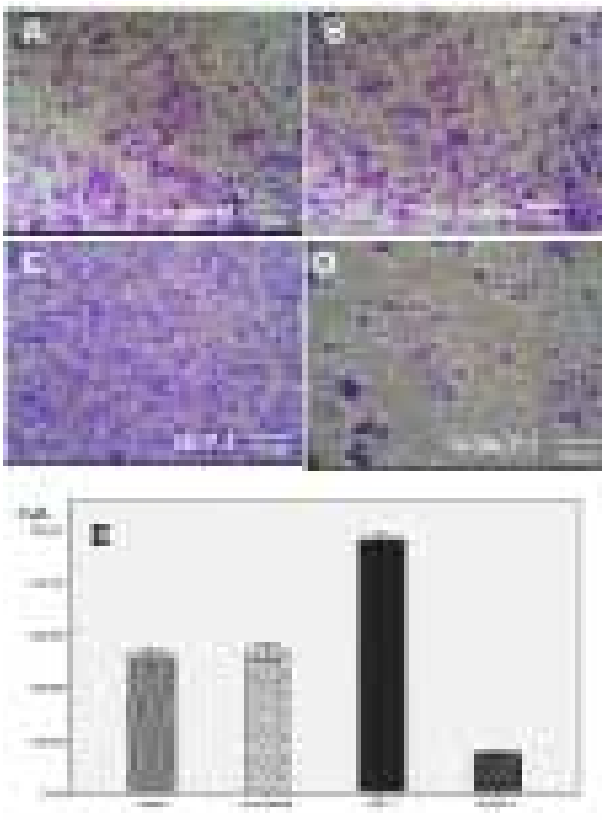

**Figure 2. MCP-1 Promotes the Invasion of PC-3M Prostate Cancer Cells.** PC-3M cells from A. control group, B. Si-scramble group, C. MCP-1 group. D. Si-MCP-1 group migrated into the lower chamber of transwell. E: Quantitive analysis of the number of cells migrated into the lower chamber of transwell. Compared with control group, there was no significant difference for Si-scramble group, however, the number of cells was significant higher in MCP-1 group and significantly lower in Si-MCP-1 group ($p<0.01$). Scale bar: 50 uM

*MCP-1 promotes the invasion of PC-3M cells*

In vitro invasion assay demonstrated that MCP-1 treatment promoted the migration of PC-3M cells into the lower chamber compared with untreated control (Fig.2 A and C). On the other hand, siRNA-mediated knockdown of MCP-1 inhibited the migration of PC-3M cells into the lower chamber compared with scramble siRNA treated cells (Figure 2 B and D). Quantitation of the cells migrated into the lower chamber showed that there were 259.4±45.54, 267.9±48.42, 482.3±55.03, and 77.2±23.93 cells in control group, Si-scramble group, MCP-1 group, and Si-MCP-1 group, respectively (Figure 2 E). Compared with control group, there was no significant difference for Si-scramble group, however, there was significant difference for MCP-1 and Si-MCP-1 group ($p<0.01$). Taken together, these data suggest that MCP-1 promotes the invasion of PC-3M cells.

*MCP-1 inhibits the apoptosis of PC-3M cells*

The apoptosis rate in different groups of PC-3M cells as determined by Annexin V-FITC and propidium iodide (PI) double staining was 5.4±1.12% (control group), 7.0±3.24% (Si-scramble group), 1.0±0.59% (MCP-1 group), and 40.8±3.02% (Si-MCP-1 group) (Figure 3 A). Compared with control group, the apoptosis rate in Si-scramble group was not significantly different ($p>0.05$). Nevertheless, the apoptosis rate was significantly higher in Si-MCP-1 group or significantly lower in MCP-1 group ($p<0.01$). These results suggest that MCP-1 has inhibitory effect on apoptosis of PC-3M cells. To further confirm this, we performed TUNEL assay and found that most cells in MCP-1 group were negative for TUNEL assay, which indicated that very few cells underwent apoptosis. In contrast, most cells in Si-MCP-1 group were positive for TUNEL assay in that their nucleus were condensed and exhibited green florescence (Figure 3 B). Quantitative analysis of TUNEL assay showed that there was no significant difference in the apoptosis rate between control group and Si-scramble group ($p>0.05$). But compared with control group, the apoptosis rate was significantly higher in Si-MCP-1 group or significantly lower in MCP-1 group ($p<0.01$) (Figure 3 C). Taken together, these data suggest that MCP-1 inhibits the apoptosis of PC-3M cells.

*MCP-1 regulates the expression of VEGF, MMP-9 and Caspase-3*

To characterize the molecular mechanisms by which MCP-1 modulate the invasion and apoptosis of PC-3M cells, we investigated the effects of MCP-1 on the expression of VEGF, MMP-9 and Caspase-3 since VEGF and MMP-9 are involved in tumor invasion and metastasis while Caspase-3 is an important regulator of apoptosis. By RT-PCR we found that the mRNA levels of VEGF and MMP-9 were significantly increased in MCP-1 group but significantly decreased in Si-MCP-1 group ($p<0.05$). In contrast, the mRNA level of Caspase-3 was significantly decreased in MCP-1 group but significantly increased in Si-MCP-1 group ($p<0.05$) (Fig. 4 A and B). As control we

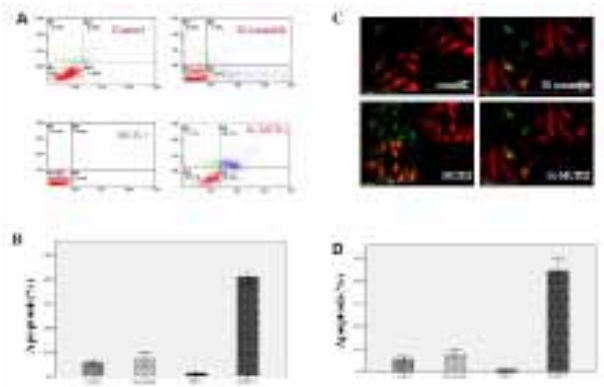

**Figure 3. MCP-1 Inhibits the Apoptosis of PC-3M Prostate Cancer Cells.** A. Quantitive analysis of the apoptotic cells in different groups of PC-3M cells detected by Annexin V staining. Compared with control group, the apoptosis rate was significantly higher in Si-MCP-1 group or significantly lower in MCP-1 group ($p<0.01$). B. The apoptotic PC-3M cells in different groups were detected by TUNEL. C. Quantitive analysis of the apoptotic cells detected by TUNEL staining. Compared with control group, the apoptosis rate was significantly higher in Si-MCP-1 group or significantly lower in MCP-1 group ($p<0.01$). Scale bar: 10 uM

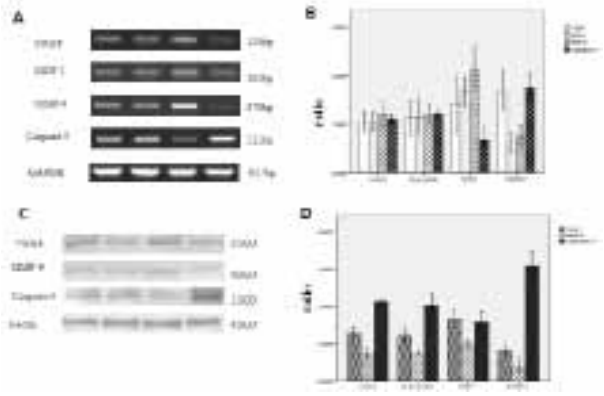

**Figure 4. MCP-1 Upregulates the Expression of VEGF and MMP-9 and Downregulates the Expression of Caspase-3 in PC-3M Prostate Cancer Cells.** A. The mRNA levels of VEGF, MMP-9, MCP-1 and Caspase-3 in PC-3M cells were determined by RT-PCR. B. Quantitive analysis of mRNA levels of VEGF, MMP-9, MCP-1 and Caspase-3 using GAPDH as internal control. The mRNA levels of VEGF, MMP-9 and MCP-1 were significantly increased in MCP-1 group but significantly decreased in Si-MCP-1 group ($p<0.05$). The mRNA level of Caspase-3 was significantly decreased in MCP-1 group but significantly increased in Si-MCP-1 group ($p<0.05$). C. The protein levels of VEGF, MMP-9, and Caspase-3 in PC-3M cells were determined by Western blotting using respective antibody. D. Quantitive analysis of protein levels of VEGF, MMP-9, and Caspase-3 using β-actin as loading control. The protein levels of VEGF and MMP-9 were significantly increased in MCP-1 group but significantly decreased in Si-MCP-1 group ($p<0.05$). The protein level of Caspase-3 was significantly decreased in MCP-1 group but significantly increased in Si-MCP-1 group ($p<0.05$)

observed that the mRNA level of MCP-1 was significantly increased in MCP-1 group but significantly decreased in Si-MCP-1 group ($p<0.05$), consistent with knockdown of MCP-1 in Si-MCP-1 group. Collectively, these results indicate that MCP-1 may upregulate VEGF and MMP-9 and downregulate Caspase-3 to modulate the cellular phenotypes of PC-3M cells.

To provide more experimental support for this conclusion next we examined the protein levels of VEGF, MMP-9 and Caspase-3 in different groups of PC-3M cells by Western blotting. Our results demonstrated that the protein levels of VEGF and MMP-9 were significantly increased in MCP-1 group but significantly decreased in Si-MCP-1 group ($p<0.05$). In contrast, the protein level of Caspase-3 was significantly decreased in MCP-1 group but significantly increased in Si-MCP-1 group ($p<0.05$) (Figure 4 C and D). These results confirmed that MCP-1 upregulates the expression of VEGF and MMP-9 and downregulates the expression of Caspase-3 in PC-3M cells.

## Discussion

Overwhelming evidence collected from distinct disciplines such as in vitro tissue culture studies, preclinical animal model studies, clinical histopathology, and clinical epidemiology prove that MCP-1 is crucially involved in different phases of cancer development. As a major chemokine MCP-1 can induce the recruitment of macrophages into various human tumors ranging from bladder cancer, cervix cancer, ovary cancer, lung cancer to breast cancer (Conti and Rollins, 2004). In breast cancer, MCP-1 is highly expressed by breast tumor cells at primary tumor sites and there is a significant associations between MCP-1 and advanced disease progression. Significantly, MCP-1 mediates many types of tumor-promoting cross-talks between the tumor cells and the tumor microenvironment and plays important role in metastasis and angiogenesis (Soria and Ben-Baruch, 2008). With regard to prostate cancer, recent studies suggest that MCP-1 could promote prostate cancer proliferation and inhibit cell death (Lu et al., 2006; Roca, Varsos and Pienta, 2008). It has been established that bone metastasis of prostate cancer is closely related with bone absorption of osteoclast (Keller and Brown, 2004; Roodman, 2004; Dougall and Chaisson, 2006; Datta et al., 2008). Notably, MCP-1 promotes prostate cancer growth and bone metastasis through recruitment of macrophages and osteoclast to the tumor site (Mizutani et al., 2009). In addition, MCP-1 promotes prostate cancer growth in the bone (Lu et al., 2009). Taken together, these studies convincingly prove that MCP-1 is implicated in the metastasis of prostate cancer to the bone.

As expected we observed that the mRNA level of MCP-1 was reduced in the MCP-1 siRNA treated PC-3M cells. However, treatment of these cells with MCP-1 protein induces the upregulation of MCP-1 mRNA. This is not surprising since it has been demonstrated that MCP-1 has autocrine and aracrine effects in various cell lines (Loberg et al., 2006; 2007a; Lu et al., 2006)

Angiogenesis is necessary for tumor progression, invasion and metastasis. As the most effective proangiogenetic factor up to now, VEGF is highly expressed in prostate cancer and implicated in prostate cancer initiation and metastasis (El-Gohary et al., 2007; Fukuda et al., 2007). On the other hand, cancer invasion and metastasis involves cellular detachment, mobility through extracelluar matrix and basement membrane

degradation, a series of processes that depend on matrix metalloproteinase (MMP). Interestingly, a most recent study revealed that MMP-9 and MMP-2 play major role in MCP-1 induced human hepatoma cell invasion (Dagouassat et al., 2010).

In the present study, via siRNA mediated knockdown of MCP-1 in PC-3M prostate cancer cells, we observed a significant downregulation of VEGF and MMP-9 at both mRNA and protein levels, and significant decrease of cell migration in in vitro invasion assay. In accordance with this, treatment of PC-3M cells with MCP-1 led to a significant upregulation of VEGF and MMP-9, and significant increase of cell migration. Therefore, our results support the model that MCP-1 induces the expression and secretion of VEGF and MMP-9, thus promoting the invasion and metastasis of prostate cancer.

Furthermore, in this study we found that knockdown of MCP-1 in PC-3M cells led to a significant upregulation of Caspase-3 at both mRNA and protein levels, and significant increase of apoptosis as determined by two independent apoptosis assays. Consistently, treatment of PC-3M cells with MCP-1 led to a significant downregulation of Caspase-3 and significant decrease of apoptosis. Collectively, these data indicate that MCP-1 inhibits apoptosis of prostate cancer cells via downregulating the expression of Caspase-3.

In conclusion, our results reveal the important role of MCP-1 in prostate cancer progression and metastasis and our findings of the regulation of VEGF, MMP-9 and Caspase-3 expression by MCP-1 open up new possibility for targeted therapy for prostate cancer.

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## References

Conti I Rollins BJ (2004). CCL2 (monocyte chemoattractant protein-1) and cancer. *Semin Cancer Biol*, **14**, 149-54.

Dagouassat M, Suffee, N, Hlawaty, H, et al (2010). Monocyte chemoattractant protein-1 (MCP-1)/CCL2 secreted by hepatic myofibroblasts promotes migration and invasion of human hepatoma cells. *Int J Cancer*, **126**, 1095-108.

Datta HK, Ng WF, Walker JA, et al (2008). The cell biology of bone metabolism. *J Clin Pathol*, **61**, 577-87.

Dougall WC, Chaisson M (2006). The RANK/RANKL/OPG triad in cancer-induced bone diseases. *Cancer Metastasis Rev*, **25**, 541-9.

El-Gohary YM, Silverman JF, Olson PR, et al (2007). Endoglin (CD105) and vascular endothelial growth factor as prognostic markers in prostatic adenocarcinoma. *Am J Clin Pathol*, **127**, 572.

Fukuda H, Tsuchiya N, Narita S, et al (2007). Clinical implication of vascular endothelial growth factor T-460C polymorphism in the risk and progression of prostate cancer. *Oncol Rep*, **18**, 1155-63.

Keller ET, Brown J (2004). Prostate cancer bone metastases promote both osteolytic and osteoblastic activity. *J Cell Biochem*, **91**, 718-29.

Loberg RD, Day LL, Harwood J, et al (2006). CCL2 is a potent regulator of prostate cancer cell migration and proliferation. *Neoplasia*, **8**, 578-86.

Loberg RD, Logothetis CJ, Keller ET, et al (2005). Pathogenesis and treatment of prostate cancer bone metastases: targeting the lethal phenotype. *J Clin Oncol*, **23**, 8232-41.

Loberg RD, Tantivejkul K, Craig M, et al (2007a). PAR1-mediated RhoA activation facilitates CCL2-induced chemotaxis in PC-3 cells. *J Cell Biochem*, **101**, 1292-300.

Loberg RD, Ying C, Craig M, et al (2007b). Targeting CCL2 with systemic delivery of neutralizing antibodies induces prostate cancer tumor regression in vivo. *Cancer Res*, **67**, 9417-24.

Lu Y, Cai Z, Galson DL, et al (2006). Monocyte chemotactic protein-1 (MCP-1) acts as a paracrine and autocrine factor for prostate cancer growth and invasion. *Prostate*, **66**, 1311-8.

Lu Y, Chen Q, Corey E, et al (2009). Activation of MCP-1/CCR2 axis promotes prostate cancer growth in bone. *Clin Exp Metastasis*, **26**, 161-9.

Mizutani K, Sud S, McGregor NA, et al (2009). The chemokine CCL2 increases prostate tumor growth and bone metastasis through macrophage and osteoclast recruitment. *Neoplasia*, **11**, 1235.

Roca H, Varsos Z, Pienta KJ (2008). CCL2 protects prostate cancer PC3 cells from autophagic death via phosphatidylinositol 3-kinase/AKT-dependent survivin up-regulation. *J Biol Chem*, **283**, 25057-73.

Roodman GD (2004). Mechanisms of bone metastasis. *N Engl J Med*, **350**, 1655-64.

Rossi D, Zlotnik A (2000). The biology of chemokines and their receptors. *Immunology*, **18**, 217.

Soria G, Ben-Baruch A (2008). The inflammatory chemokines CCL2 and CCL5 in breast cancer. *Cancer Lett*, **267**, 271-85.

Taichman RS, Loberg RD, Mehra R, et al (2007). The evolving biology and treatment of prostate cancer. *J Clin Invest*, **117**, 2351-61.

Van Engeland M, Nieland LJ, Ramaekers FC, et al (1998). Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry*, **31**, 1-9.