PTHrP-induced MCP-1 production by human bone marrow endothelial cells and osteoblasts promotes osteoclast differentiation and prostate cancer cell proliferation and invasion *in vitro*

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Prostate cancer (PCa) preferentially metastasizes to bone resulting in osteoblastic lesions with underlying osteolytic activities. The mechanisms through which PCa cells promote osteolytic activities and subsequent osteoblastic bone formation remain poorly understood. Parathyroid hormone-related protein (PTHrP), produced by bone cells and PCa, binds to receptors on osteoblasts and stimulates bone formation and resorption. We have previously reported that MCP-1 acts as a paracrine and autocrine factor for PCa progression. However, the role of PTHrP in regulating MCP-1 expression in bone microenvironment, specifically by human bone marrow endothelial cells (HBME) and osteoblasts (hFOB), as well as by PCa cells, has not been studied. Accordingly, we first determined the effect of PTHrP on MCP-1 expression by bone cells and PCa cells. PTHrP induced both MCP-1 protein and mRNA expression by HBME and hFOB cells, but not by PCa LNCaP and PC3 cells. To further determine the mechanisms of PTHrP-induced MCP-1 transcription, analysis of the MCP-1 promoter was performed. MCP-1 promoter activity was induced by PTHrP. Both C/EBPβ and NF-κB binding elements are required for PTHrP-induced MCP-1 transcription. Finally, when a constitutively-active PTH receptor construct was transfected into HBME and hFOB cells, MCP-1 production was increased. The conditioned media collected from these cells induced osteoclast differentiation and PC3 proliferation and invasion *in vitro*. These inductions were partially inhibited by MCP-1 neutralizing antibody. We conclude that PTHrP-induced MCP-1 production by HBME and hFOB cells promotes osteoclast differentiation *in vitro* and such induction may play a critical role in PCa development in the bone microenvironment.
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Bone is the most common site of prostate cancer (PCa) metastasis with skeletal metastases occurring in up to 90% of patients dying from PCa.[1,2] Histological studies have revealed that bone metastatic lesions are a heterogeneous mixture of osteoblastic and osteolytic lesions, although they are most often radiographically characterized as predominately osteoblastic responses.[3–6] Many reports have showed that osteoblastic metastases form on trabecular bone at the sites of previous osteoclastic resorption, and that such resorption may be required for subsequent osteoblastic bone formation.[2,6] Thus, bone destruction appears to be an early stage in the development of PCa bone metastasis. However, the mechanisms through which PCa cells promote bone resorption and subsequent osteoblastic bone formation remain poorly understood.

PCa metastasis develops in the skeleton through crosstalk between tumor cells and bone microenvironment. The bone microenvironment, which consists of the extracellular matrix, stromal cells, osteoblasts, osteoclasts, endothelial cells, adipocytes and hematopoietic cell lineages, produces multiple factors which can enhance the growth of PCa cells and promote tumor development in bone. These factors include chemotactic factors, adhesion factors, growth factors and other inflammatory mediators. Paget proposed the ''seed and soil hypothesis'' in 1889 to account for the predilection of metastasis to certain organs [as reviewed in Ref. 7].

Chemokines are classified, based upon the relative position of cysteine residues near the NH2-terminus, into 4 major families: CC, CXC, C and CX3C. In addition to their roles in chemotaxis, one important role for chemokines in tumor biology that has been suggested is that they may regulate the metastatic behavior of tumor cells. For example, the CXC chemokine, stromal-derived factor (SDF-1/CXCL12) and its receptor CXCR4, are responsible for hematopoietic stem cell homing to the marrow and has also been implicated in the pathogenesis of PCa bone metastasis.[8,9] Tumor cells that frequently metastasize to bone (myeloma, breast cancer, PCa and neuroblastoma) all express CXCR4.[10–15] However, blocking CXCR4 only partially inhibits bone metastasis.[11,16] These results suggest that other factors produced by tumors and/or cells in the bone microenvironment can attract and stimulate the tumor growth.

MCP-1, a member of the CC chemokine superfamily, plays a critical role in the recruitment and activation of monocytes during acute inflammation and angiogenesis. *In vitro* studies have shown that for most normal cell types there is little or no MCP-1 expression and exogenous stimulation is required for the induction of MCP-1.[17] However, a variety of cancer cells that home to bone, including PCa, breast cancer and myeloma cells, express MCP-1 and its receptor CCR2.[18–20] We have recently demonstrated that MCP-1 is chemotactic for PCa cells.[18] In addition, MCP-1 is produced by bone cells such as human bone marrow endothelial (HBME) cells,[21] osteoblasts[22,23] and osteoclasts.[24–27] Both receptor activator of NF-κB ligand (RANKL) and Tumor necrosis factor-α (TNF-α) modulate MCP-1 expression in osteoclasts.[24–27] Thus, MCP-1 appears to play a role in tumor progression in bone.

Parathyroid hormone-related protein (PTHrP), a 141-amino acid protein that has limited homology to PTH, binds the same receptor as PTH with similar biological activity.[28,29] It acts as an autocrine and paracrine factor on osteoblasts and modulates bone formation and resorption.[28,30] Furthermore, PTHrP is highly expressed on metastatic bone lesions[31] compared to a moderate expression on localized PCa tissues[32,33] and cell lines.[34] PTHrP has been shown to enhance bone metastases in animal models of PCa[35] and breast cancer.[36–39] However, how PTHrP promotes the tumor-associated osteolysis is not clear. In this study, we investigated the role of PTHrP in MCP-1 production in the bone micro-

environment, specifically by bone HBME cells and osteoblasts, as well as by PCa cells.

Material and methods

Antibodies and reagents

Rabbit polyclonal antibodies against human PTH receptor (PTHR) and GAPDH, mouse monoclonal antibodies against human C/EBPβ and NFκB (p65), C/EBPβ siRNA, NFκB (p65) siRNA and control siRNA (scrambled siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Murine MCP-1 neutralizing antibody, isotype IgG control antibody, recombinant human MCP-1 (rhMCP-1), recombinant murine M-CSF (M-CSF), and recombinant murine RANKL (rmRANKL), were purchased from R & D Systems (Minneapolis, MN). Human PTHrP(1–34) were obtained from Bachem (Torrance, CA). All other chemical reagents were purchased from Sigma (St. Louis, MO).

Cell culture

HBME, hFOB (SV 40 large T antigen transfected and immortalized human osteoblasts), human PCa cell lines LNCaP and PC3 were obtained from American Type Tissue Collection (ATCC, Rockville, MD). HBME cells were cultured in DMEM media. Human FOB cells were grown in media with 50% DMEM and 50% Ham's F12 (Invitrogen) at 34°C. At this temperature, the cells exhibit rapid cell division since this cell line was established by transfection of a temperature sensitive expression vector pUCSVtsA58. LNCaP and PC3 cells were cultured in RPMI 1640 media (Invitrogen, Carlsbad, CA). All the media were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. HBME, LNCaP and PC3 cells were maintained in 100-mm tissue culture dishes in a 37°C incubator equilibrated with 5% CO_2 in humidified air.

ELISA

HBME, hFOB, LNCaP and PC3 cells were plated in their maintenance media into 6-well-plates at 3×10^5/well in triplicates. Cells were grown for 12 hr, and then the media was changed to serum-free media. In one experiment, the cells were treated with either vehicle, or 10^{-8} M PTHrP or 20 ng/ml TNFα for 6 hr or 24 hr. In another experiment, the HBME and hFOB cells were treated with 10^{-8} M PTHrP for 6 hr, then exposed to either vehicle, or cycloheximide (10 ug/ml) for 0, 1 or 2 hr. The supernatant was collected and MCP-1 level was measured by ELISA. Quantikine Human MCP-1 ELISA kits were purchased from R & D Systems. ELISAs were performed according to the manufacturer's instructions.

Quantification of PTHR and MCP-1 mRNA expression by real-time RT-PCR

Total RNA was extracted from nontreated HBME, hFOB, LNCaP and PC3 cells and the cells treated as earlier. Total RNA was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD), then subjected to real-time RT-PCR in an iCycler iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA) using the iScript one-step RT-PCR kit with SYBR Green (Bio-Rad). Melting curve analysis was performed to confirm the purity of the PCR products. Duplicate samples were run for each primer set. The relative expression of PTHR to GAPDH and MCP-1 to GAPDH (as housekeeping gene control) was calculated. Primers for PTHR consisted of sense 5′-ACC AAT GAG ACT CGT GAA CGG-3′, and antisense 5′-AAG GAC AGG AAC AGG TGC ATG-3′. MCP-1 primers were: sense 5′-TCG CGA GCT ATA GAA GAA TCA-3′ and antisense 5′-TGT TCA AGT CTT CGG AGT TTG-3. GAPDH primers were: sense 5′-CCA TGG AGA AGG CTG GGG-3′ and antisense 5′-CAA AGT TGT CAT GGA TGA CC-3′.

Reverse transcription-PCR

Total RNA from HBME, hFOB, LNCaP and PC3 cells was extracted. RT-PCR was performed with 1μg of total RNA using the Access RT-PCR system (Promega Corp., Madsion, WI) in a thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, CA) under the following conditions: first strand cDNA was synthesized at 48°C for 45 min; then denatured at 94°C for 2 min for the first cycle and at 30 sec for additional 35 cycles; annealing was performed at 55°C for 30 sec and extension at 72°C for 60 sec. Final extension was at 72°C for 7 min. The PCR products were subjected to electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. RT-PCR primers for PTH receptor consisted of sense 5′-ACC AAT GAG ACT CGT GAA CGG-3′, and antisense 5′-AAG GAC AGG AAC AGG TGC ATG-3′, resulting in a PCR product of 167 bp. GAPDH primers were: sense 5′-CCA TGG AGA AGG CTG GGG-3′ and antisense 5′-CAA AGT TGT CAT GGA TGA CC-3′, resulting in a PCR product of 194 bp.

Western blot analysis

Cell lysates from HBME, hFOB, LNCaP, PC3 cells and HBME and hFOB cells transfected with PTHR (H223R) were prepared using standard procedures. All the samples were measured for total protein content using a BCA assay (Pierce, Rockford, IL) to ensure equal loading. Loading buffer was added to 40 μg protein and samples were boiled prior to being resolved on 12% SDS-PAGE gels and then transferred onto PVDF membranes (Bio-Rad). The blots were blocked using blocking reagents overnight at 4°C with shaking and then incubated for 2 hr with primary antibody for PTHR (diluted 1:200 in blocking solution). The blots were washed and incubated for 1 hr with goat antirabbit IgG-HRP (1:3,000). After washing, bands were detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and exposed to light-sensitive film. As control for equal loading of the proteins, immunoblots for GAPDH (Santa Cruz, CA) were performed on the stripped membranes. PTHR was normalized to GAPDH, and the relative change was calculated using the mean value of changes from 3 replicates. Cell lysates from HBME cells transfected with C/EBPβ siRNA, NFkB p65 siRNA or control siRNA were used to determine C/EBPβ and NFkB p65 protein expression using the same protocol.

Effects of actinomycin D on MCP-1 expression in HBME cells

HBME cells were plated into 6-well-plates at 3×10^5/well. HBME Cells were treated with either vehicle or 10^{-8} M PTHrP for 6 hr and then exposed to either the vehicle or actinomycin D (1 μg/ml) for 0, 1 or 2 hr. Total RNA was collected and MCP-1 mRNA expression was determined by real-time RT-PCR.

Transfection and MCP-1 promoter activity measurement

Reporter vectors pGL3-MCP1 (MCP1-3.6kb/Luc), MCP1-C/EBPMAB/Luc, which has mutated C/EBP binding sites, and MCP1-NFκBMAB/Luc, which has mutated NF-κB binding sites, were constructed and used as described previously.[40] HBME or hFOB cells were grown to 80% confluence in T75 flasks, trypsinized, washed in PBS, and replated at 3×10^4 cells/well in 24-well culture plate in maintaining media. Twelve hours after plating, cells were cotransfected with pGL3-MCP1, MCP1-C/EBPMAB/Luc, MCP1-NFκBMAB/Luc, PGL-3 basic (empty vector) and promoter-linked Renilla luciferase vector, pRL-TK (Promega Corp) as a normalization reference for transfection efficiency using FuGENE 6 Transfection Reagent (Roche Diagnostic Corp., Indianapolis, IN). Twenty-four hours after the transient transfection, medium was changed to DMEM with 0.5% charcoal-stripped FBS, and cells were then treated with either vehicle or 10^{-8} M PTHrP, 20 ng/ml TNFα for 24 hr. The cells were lysed with lysis buffer (Promega Corp) and the luciferase activity was quantified using a microplate luminometer (Turner Biosystems,
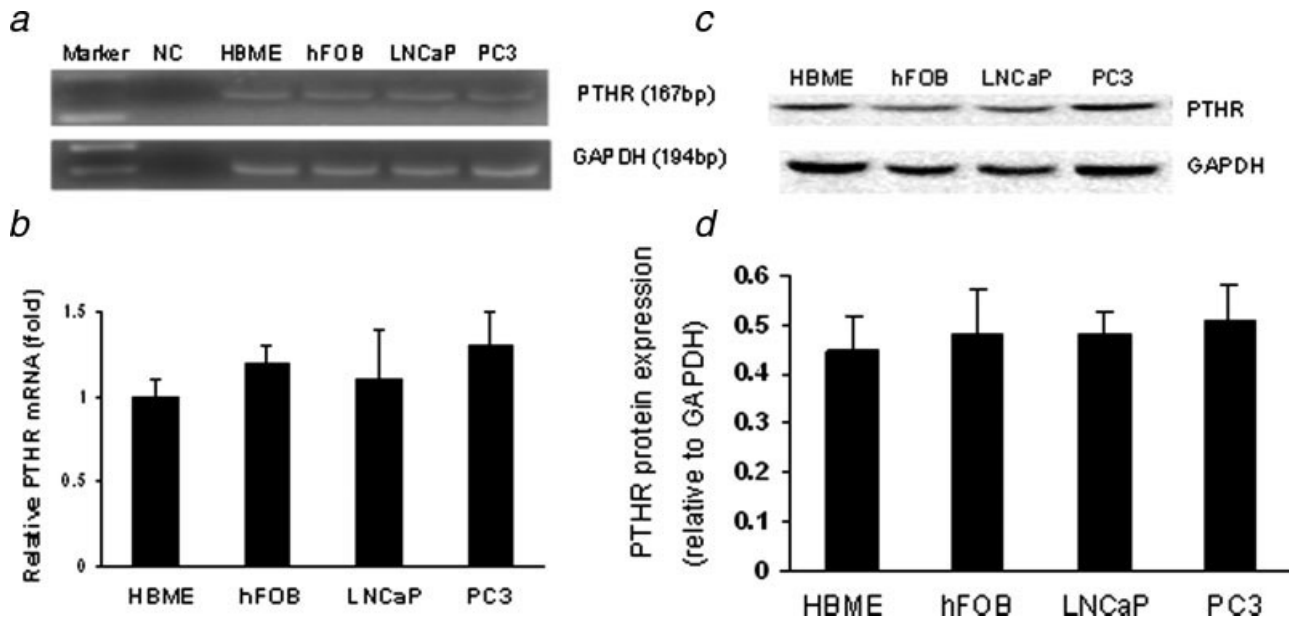
FIGURE 1 – PTH receptors are expressed on HBME, hFOB, LNCaP and PC3 cells. Total RNA and cell lysates were extracted from HBME, hFOB, LNCaP and PC3 cells. (*a*) PTHR mRNA expression was determined by RT-PCR. The PCR products were subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide. (*b*) Relative PTHR mRNA expression was determined by Real-time RT-PCR. Data is representative of 3 separate experiments and is presented as mean ± SE from triplicates. (*c*) PTHR protein expression was determined by Western blot. (*d*) Quantitive data for Western blot. The data is representative of 2 separate experiments and is presented as mean ± SE from replicates.

Sunnyvale, CA) using the protocol from Dual-Luciferase Reporter assay system (Promega Corp).

siRNA knock down experiment

The designed C/EBPβ siRNA, NFκB P65 siRNA, or scrambled siRNA were transfected into HBME cells using the transfection reagents from Santa Cruz. In brief, HBME cells were cultured in 6-well plates (3×10^5/well) with antibiotic-free cell growth medium 1 day prior to transfection. For each transfection, 1 µg of siRNA duplex was diluted in siRNA transfection medium to a final volume of 100 µl. Six microliter siRNA transfection reagent was diluted into 100 µl siRNA transfection medium and added directly into the siRNA duplex solution. After 30 min incubation at room temperature, 0.8 ml transfection medium was added to each tube containing the siRNA and transfection reagent mixture, then overlayed the mixture onto the cells which had been washed by siRNA transfection medium. Cells were incubated for 6 hr at 37°C and cell growth media containing 2× serum and antibiotics were then added. The cells were incubated for an additional 24 hr and the media was replaced with cell growth media with charcoal-stripped FBS plus 10^{-8} M PTHrP, and continued to culture for 24 hr. Cell lysates were collected for Western blot and the cell culture supernatants were collected for MCP-1 ELISA measurement.

PTH receptor transfection into HBME and hFOB cells and obtaining CM

Transient transfection was performed in 35-mm tissue culture dishes (HBME and hFOB cells at 2×10^5) using a constitutively active PTH receptor (H223R) or empty vector constructs (kindly provided by Dr. E. Schipani, Department of Medicine, Massachusetts General Hospital and Harvard Medical School). Briefly, HBME or hFOB cells were co-transfected, using FuGENE 6 (Roche Diagnostic Corp.) reagents, with either PTH receptor (H223R) or empty vector plus β-gal expression vector as an internal control for normalization of transfection efficiency. Twenty-four hours after transfection, medium was changed to DMEM with 0.5% charcoal-stripped FBS with either vehicle or H89

(10 µM), a protein kinase A inhibitor. After 48 hr, the CM was collected and MCP-1 production was measured by ELISA. The CM was used in osteoclast formation assays as described later. To normalize for differences in cell density because of proliferation during the culture period, cells from each plate were collected and the total DNA content/plate was determined (spectrophotometric absorbance, 260 nm). The CM was then normalized for DNA content between samples by adding DMEM.

Osteoclast formation assay

Primary mouse bone marrow cells were obtained by flushing femora from 4–6 weeks old C57BL/6 mice. The animal protocol was approved by the Institutional Animal Care and Use Committee, University of Pittsburgh. Briefly, the bone marrow cells were incubated in α-MEM with 20% FBS overnight at 37°C in 100-mm tissue culture plates to separate nonadherent and adherent cells. Osteoclast formation assays were performed by culturing 1×10^5/well nonadherent cells in 96-well plates in 0.1 ml of α-MEM with 20% FBS for 7 days. The cells were incubated with rmM-CSF (10 ng/ml) and/or rmRANKL (50 ng/ml) and 10% CM collected from the HBME or hFOB PTH receptor-transfected cells in the presence of various doses of antimouse MCP-1 neutralizing antibody or isotype control antibody. In each case, half of the media was changed at day 4. After 7 days of culture, the cells were fixed with 2% formaldehyde and stained with K-ASSAY TRAP staining kit (Kamiya Biomedical, Seattle, WA). Using an inverted microscope, the positive staining cells that contained 3 or more nuclei were counted as osteoclast-like cells. Analysis of all osteoclast formation experiments included data from 3 independent experiments.

PCa cell proliferation

Cell proliferation was measured using a CellTiter 96 AQeous Nonradioactive cell proliferation assay (Promega, Madison, WI). Briefly, PC3 cells were plated in 96-well plates at a density of 2,000 cells/well, in 200 µl of RPMI 1640 plus 0.5% FBS. CM (10%) collected from PTHR (H223R) with either MCP-1 neutralizing
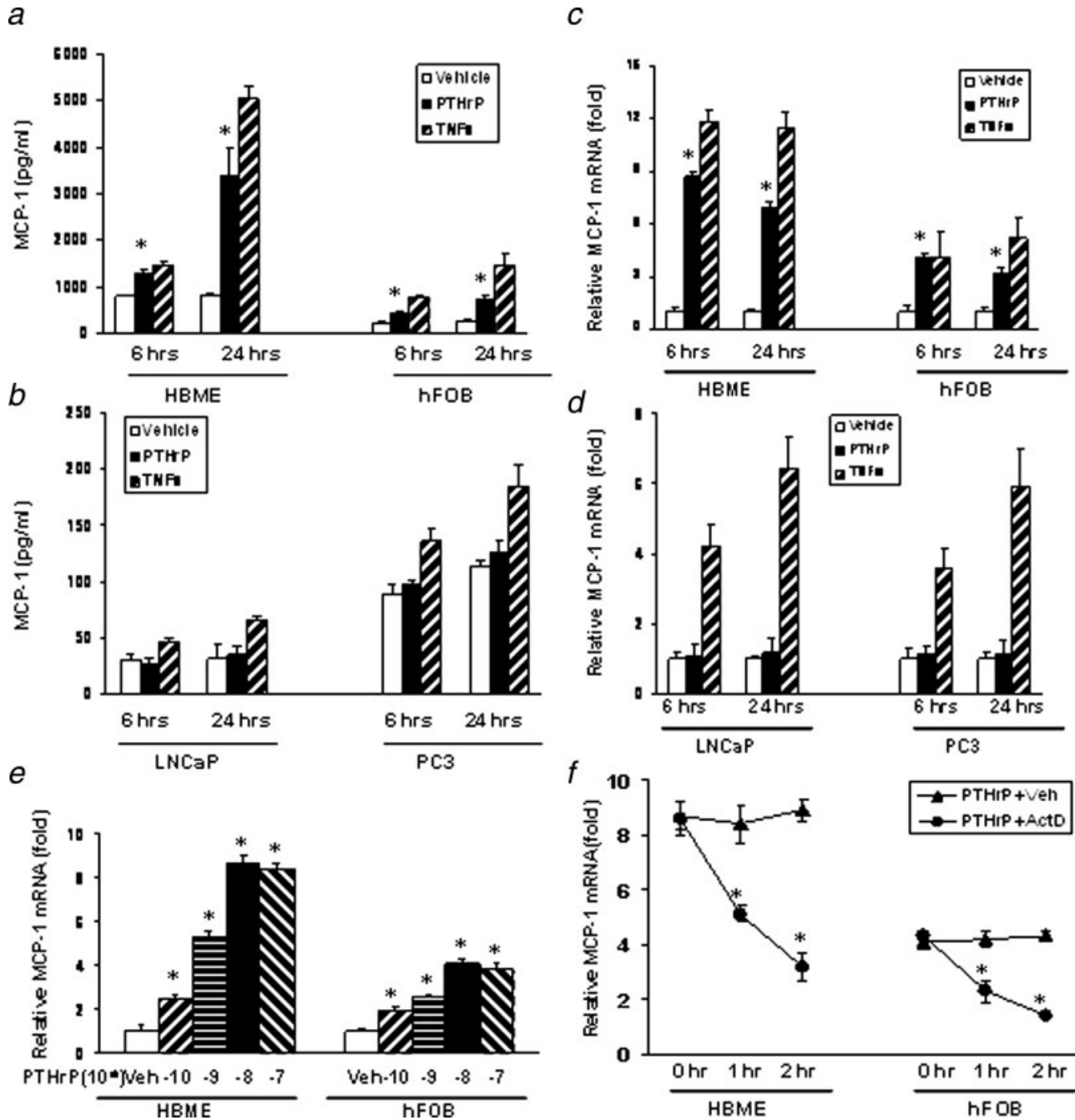
FIGURE 2 – PTHrP induced MCP-1 expression by HBME and hFOB cells, but not by PCa cells. (*a,c,e,f*) HBME, hFOB and (*b,d*) LNCaP, PC3. Cells (3×10^5/well) were plated in a 6-well plate in cell culture media plus 10% FBS for 12 hr, and then the media was changed to serum-free DMEM media. For (*a,b,c,d*), the cells were treated with either vehicle, or 10^{-8} M PTHrP or 20 ng/ml TNFα (as a positive control) for 6 hr or 24 hr. For (*e*), the cells were treated with indicated concentration of PTHrP for 6 hr. For (*f*), the cells were treated with 10^{-8} M PTHrP for 6 hr, and then exposed to either vehicle or actinomycin D (1 ug/ml) for 0, 1 or 2 hr. (*a,b*). The supernatants were collected and MCP-1 levels were measured by ELISA. Data are presented as mean ± SD from triplicates. Data is a representative of 2 separate experiments. *$p < 0.001$ compared to the vehicle-treated cells. (*c,d,e,f*). Total RNA was extracted from the cells, and then subjected to real-time RT-PCR using appropriate primers. GAPDH was served as an internal control. MCP-1 mRNA expression was calculated relative to the expression of GAPDH. Data is representative of 3 separate experiments and is presented as mean ± SE from triplicates. *$p < 0.001$ compared to the vehicle-treated cells.

antibody (1μg/ml) or control IgG (1 μg/ml) was added into the cultures. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 24, 48, 72 hr, then 20 μl of combined MTS/PMS solution was added. After incubation for 2 hr at 37°C, the absorbance of each well at 490 nm was recorded by using an ELISA plate reader. Data represent the average absorbance of 4 wells.

In vitro *PCa cell invasion assay*

The invasiveness of PC3 cells was evaluated in 24-well matrigel invasion chamber (BD Biosciences, Bedford, MA), as previously described.[18] Briefly, to assess the ability of the PC3 cells to penetrate the matrigel, PC3 cells (2.5×10^4/well) were placed in 0.5 ml of RPMI medium containing 5% FBS in the upper compartment of
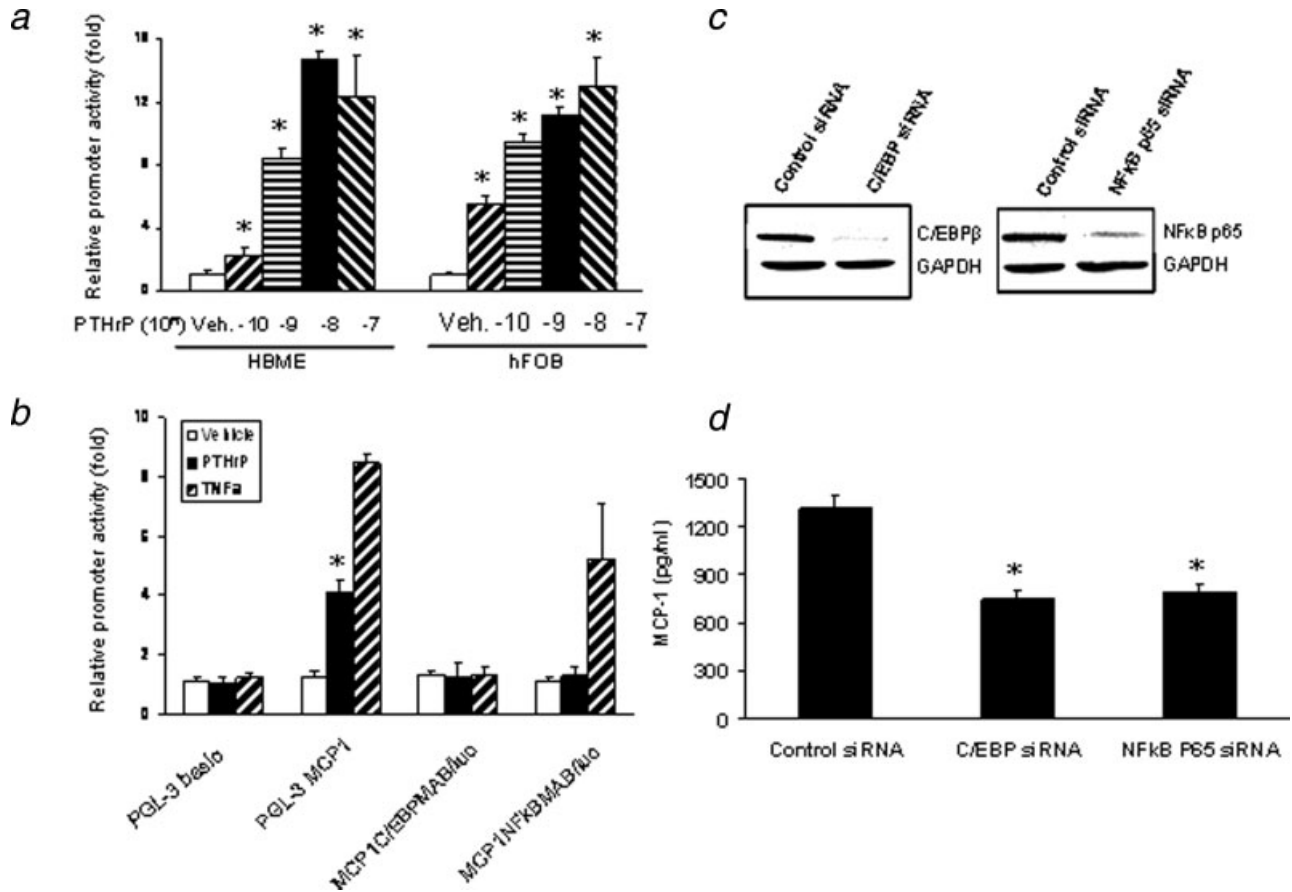
FIGURE 3 – PTHrP induced MCP-1 promoter activity is dependent on the NFκB and C/EBPβ binding elements. (*a*) HBME and hFOB cells (3×10^4/well) were plated in a 24-well plate and transiently cotransfected with PGL3-MCP-1 and pRL-TK plasmids. Twenty-four hours after the transient transfection, the cells were treated with either vehicle, or increasing doses of PTHrP. (*b*) HBME cells were transiently cotransfected with luciferase expression plasmids (PGL3-MCP-1, MCP-1-NFκBMAB, MCP-1-C/EBPMAB or PGL3-Basic) and pRL-TK plasmid. Twenty-four hours after the transient transfection, the cells were treated with either vehicle, or 10^{-8} M PTHrP, 20 ng/ml TNFα for 24 hr. Luciferase activities were measured by dual-luciferase reporter assay system (Promega). Results are expressed as mean ± S.E. of relative luciferase activities. Results are from 3 independent experiments, and each observation was performed in duplicate. *$p < 0.001$ compared to the vehicle-treated cells. (*c,d*) The designed C/EBPβ siRNA, NFκB P65 siRNA, or scrambled siRNA were transfected into HBME cells using the transfection reagents. The cells were treated with 10^{-8} M PTHrP for 24 hr. Cell lysates were collected for Western blot and the cell culture supernatants were collected for MCP-1 ELISA measurement. Data are presented as mean ± SD from triplicates. Data is a representative of 2 separate experiments. *$p < 0.001$ compared to the control siRNA-transfected cells.

wells that were precoated with the reconstituted matrix and 0.75 ml of RPMI medium containing 10% FBS in the lower compartment. In the lower compartments, CM (10%) collected from PTHR (H223R) with either MCP-1 neutralizing antibody (1 μg/ml) or control IgG (1 μg/ml) was added. The transwell chambers were incubated for 24 hr at 37°C in 95% air and 5% CO_2. Cell penetration through the membrane was detected by staining the cells on the porous membrane with a Diff-Quik stain kit (Dade Behring, Newark, DE) and quantified by counting the numbers of cells that penetrated the membrane in 5 microscopic fields (at 200× magnification) per filter. Invasive ability was defined as the proportion of cells that penetrated the matrix-coated membrane divided by the number of cells that migrated through the uncoated membrane (baseline migration). The results are reported from triplicate assays.

Statistical analysis

Statistical significance was determined for multivariate comparisons using ANOVA and Fisher's probable least significant difference for post hoc analysis. Student's *t* test was used for bivariate analyses. Statistical significance was determined as $p < 0.05$. Statistical calculations were performed using Statview software (Abacus Concepts, Berkeley, CA).

Results

PTH receptor expression on HBME, hFOB, LNCaP and PC3 cells

PTHrP functions through binding to its receptors on the target cells. Therefore, we determined if the HBME, hFOB, LNCaP and PC3 cells express the PTH receptor and, thereby, could respond to PTHrP. We found that HBME, hFOB, LNCaP and PC3 cells express both PTH receptor mRNA and protein as identified by RT-PCR and western blot, repectively, (Figs. 1*a* and 1*c*) indicating that these cells are capable of responding to PTHrP. There are no significant differences on PTHR expression levels among these cells (Figs. 1*b* and 1*d*).

Effect of PTHrP on MCP-1 production by the both bone cells and PCa cells

To determine whether PTHrP influences MCP-1 expression by bone cells such as HBME and hFOB, as well as by PCa epithelial cells, HBME, hFOB, LNCaP and PC3 cells were treated with either vehicle, or 10^{-8} M PTHrP, or 20 ng/ml TNFα (as a positive control) for 6 hr or 24 hr. The conditioned media were collected and MCP-1 levels were measured by ELISA. PTHrP significantly induced MCP-1 production by HBME cells, particularly at 24 hr

(Fig. 2a). It also, less potently, induced MCP-1 production by hFOB cells. However, PTHrP was not observed to induce either MCP-1 protein production (Fig. 2b) or MCP-1 mRNA production (Fig. 2d) by the 2 PCa cell lines, LNCaP and PC3. After 24-hr PTHrP treatment, the MCP-1 level in the CM from PTHrP-treated HBME and hFOB cells increased 4.1-fold and 2.8-fold, respectively, as compared to the CM from vehicle-treated cells (Fig. 2a). To determine whether this PTHrP-induced MCP-1 protein induction is due to increased MCP-1 mRNA transcription, total RNA from these cells was collected and MCP-1 mRNA expression was quantified by real-time RT-PCR. Consistent with the observed PTHrP induction of MCP-1 protein production, PTHrP also significantly induced MCP-1 mRNA expression by HBME cells (8.7-fold at 6 hr and 6.9-fold at 24 hr) and hFOB cells (4.1-fold at 6 hr and 3.1-fold at 24 hr) (Fig. 2c). Time course analyses for PTHrP (10^{-8} M) induction of MCP-1 mRNA and protein showed that while the peak steady-state MCP-1 mRNA is at 2 hr (Fig. 2c and data not shown), the peak protein accumulation is at 24 hr in both HBME and hFOB cells (Fig. 2a). PTHrP induces MCP-1 mRNA expression in a dose-dependent manner (Fig. 2e). To determine whether the PTHrP-induced MCP-1 mRNA expression may require active mRNA syntheses, we tested the PTHrP-induced (6 hr treatment) and the steady state of MCP-1 mRNA expression in HBME cells after treatment with actinomycin D, an inhibitor of RNA synthesis. PTHrP-induced MCP-1 mRNA expression was significantly diminished within 1–2 hr by the addition of actinomycin D to the cell cultures (Fig. 2f). The cells treated with actinomycin D for 1–2 hr did not change the steady state of MCP-1 mRNA expression in HBME cells (not shown). Consistent with the mRNA findings, we also observed that cycloheximide (10 μg/ml), a protein synthesis inhibitor, diminishes the PTHrP-induced, but not the steady state of MCP-1 protein expression within 1–2 hr treatment determined by ELISA (not shown).

Effect of PTHrP on MCP-1 transcription in bone cells

To further determine the mechanisms of PTHrP-induced MCP-1 transcription in HBME and hFOB cells, a construct containing the full length MCP-1 promoter driving luciferase was transiently transfected into HBME and hFOB cells. PTHrP dose-dependently increased the MCP-1 promoter-driven luciferase activity in both cells (Fig. 3a). To identify the cis-elements important for the MCP-1 promoter response to PTHrP treatment, we used 2 mutant constructs to evaluate the contribution of C/EBP and NF-κB binding sites: MCP1-C/EBPMAB/Luc, which has mutated C/EBP binding sites; and MCP1-NFκBMAB/Luc, which has mutated NF-κB binding sites. PTHrP failed to induce both MCP1-C/EBP-MAB/Luc and MCP1-NFκBMAB/Luc, suggesting that both elements are required for PTHrP-induced MCP-1 transcription in HBME cells (Fig. 3b). In contrast, TNFα induced MCP1-NFκBMAB/Luc promoter activity but not MCP1-C/EBPMAB/Luc promoter activity, indicating that TNFα and PTHrP may induce MCP-1 through different transcription factors. In addition, we observed similar results for MCP-1 transcriptional regulation by PTHrP in hFOB cells (data not shown). To further provide evidence for the role of transcriptional factors C/EBP and NF-κB in PTHrP-induced MCP-1 production, using HBME cells as an example, either C/EBP or NF-κB siRNA were transfected into HBME cells followed by PTHrP treatment. Both C/EBP and NF-κB siRNA significantly diminished MCP-1 production (Figs. 3c and 3d). When either C/EBP and NF-κB siRNA were transfected into LNCaP or PC3 cellls followed by PTHrP treatment, the MCP-1 production by these cells were not altered indicating that the transcriptional factors such as C/EBP and NF-κB may not be induced by PTHrP in this condition (data not shown).

Effect of PTHrP-induced MCP-1 production on osteoclast differentiation in vitro

Using HBME and hFOB cells as examples, we determined the function of this PTHrP-induced MCP-1 production in bone cells. First, a constitutively-active PTH receptor construct (H223R) was
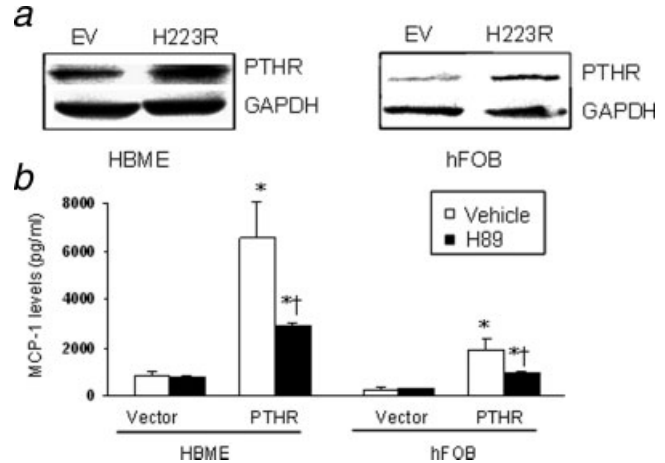

FIGURE 4 – MCP-1 levels in CM from HBME and hFOB cells expressing a constitutively-active PTH receptor. HBME and hFOB cells were transiently cotransfected with either a constitutively-active PTH receptor (H223R) construct or empty vector plus β-gal expression vector as an internal control for normalization of transfection efficiency. (a) PTHR protein expression was determined by Western blot. EV represents empty vector. (b) CM was collected as described in Materials and methods. MCP-1 levels were measured by ELISA. Data are presented as mean ± SD from triplicates. *$p < 0.001$ compared to vector-transfected CM. †$p < 0.001$ compared to the vehicle-treated CM.

transiently transfected into both HBME and hFOB cells (Fig. 4a). Twenty-four hours after transfection, medium was changed to DMEM with 0.5% charcoal-stripped FBS with vehicle or H89, a PKA inhibitor. After 48 hr, we collected the CM and measured MCP-1 levels in the CM by ELISA. MCP-1 protein production by both HBME and hFOB cells was significantly increased by transfection with PTHR (H223R) compared to the vector (Fig. 4b). As PTHR/cAMP-PKA is one of the major cascades in PTHR function, H89 was incorporated into this study. As expected, H89 significantly diminished MCP-1 induction by both cells (Fig. 4b). The CM from the HBME was then utilized for an in vitro osteoclast differentiation assay using murine bone marrow nonadherent cells. The CM (10%) induced osteoclast differentiation in vitro and this induction was dose-dependently inhibited by adding MCP-1 neutralizing antibody, whereas the isotype control antibody did not inhibit (Figs. 5a and 5b). The highest concentration (1 μg/ml) of neutralizing antibody only partially inhibited the CM-induced osteoclast differentiation. We observed similar results using hFOB cells (data not shown).

Effect of CM collected from PTHR (H223R) HBME cells on PC3 cell proliferation and invasion

We have previously reported that MCP-1 induces PCa cell proliferation and invasion in vitro.[18] To further determine whether the CM collected from PTHR (H223R)-transfected HBME cells may, through MCP-1, induce PCa cell proliferation and invasion, PC3 cells were treated with either neutralizing antibody for MCP-1 or control IgG in the presence of CM (10%) collected from PTHR (H223R) HBME cells. The neutralizing MCP-1 antibody significantly diminished both PC3 cell proliferation (Fig. 6a) and invasion (Fig. 6b) induced by the CM.

Discussion

Bone is the most frequent site for distant metastasis from PCa. The skeletal lesions form due to the interaction between tumor cells and the bone microenvironment. Chemokines, cytokines, growth factors and other inflammatory mediators produced in the bone microenvironment modulate activity of osteoblasts and osteoclasts. One factor that can be produced by both bone and PCa cells is PTHrP. In the present study, we found that PTHrP induces
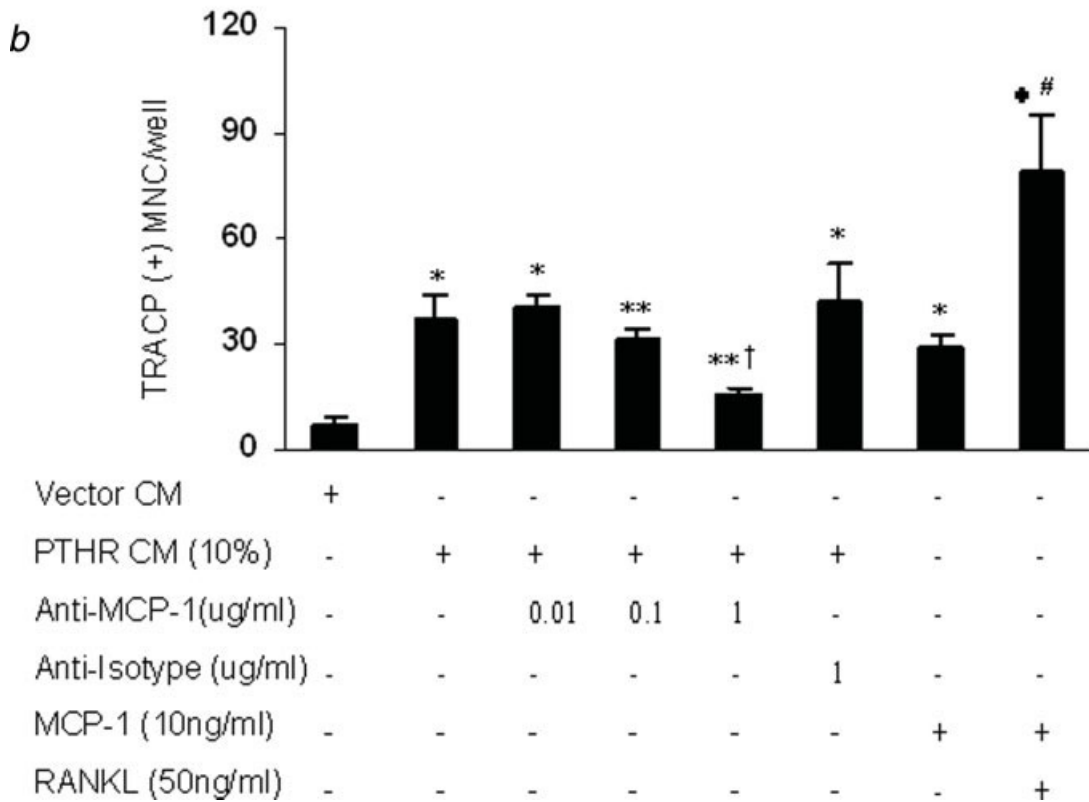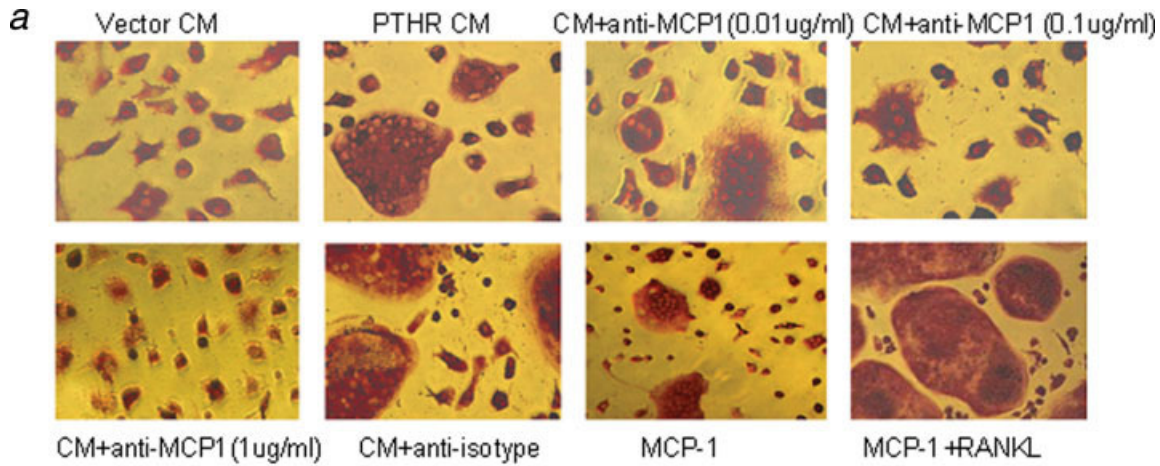
FIGURE 5 – Induction of osteoclast differentiation by PTHrP-induced MCP-1 production *in vitro*. Nonadherent MBMCs were cultured at 1×10^5/well in 96-well plates for 7 days. The cells were incubated with rmM-CSF (10 ng/ml) and/or rmRANKL (50 ng/ml) or 10% CM collected from either vector- or PTHR (H223R)-transfected cells in the presence of various doses (0.01–1 μg/ml) of MCP-1 neutralizing antibody. After 7 days, the cells were fixed and murine osteoclast-like cells were identified by TRAP staining. (*a*) Representative pictures of TRAP staining. (*b*) Number of osteoclast-like multinucleated cells per well were quantified. Samples were evaluated in quadruplicate. Results are reported as mean ± SD. Data were analyzed using one-way ANOVA. *$p < 0.001$ compared to the CM collected from vector-transfected cells; **$p < 0.005$ compared to the CM collected from PTHR (H223R)-transfected cells; †$p < 0.01$ compared to the CM collected from vector-transfected cells; #$p < 0.001$ compared to the MCP-1-treated cells.

MCP-1 expression, at both the mRNA and protein levels, by HBME and hFOB cells (Figs 2*a*, 2*c* and 2*e*). However, although the 2 PCa cell lines, LNCaP and PC3, express the PTH receptor (Fig. 1), PTHrP induces neither MCP-1 protein production nor mRNA expression in these cells (Figs. 2*b* and 2*d*). We also found that PTHrP-induced MCP-1 mRNA expression requires active gene transcription because PTHrP-induced MCP-1 mRNA expression can be significantly inhibited by actinomycin D (Fig. 2*f*). To our knowledge, this is the first report that shows that PTHrP can

induce MCP-1 production by HBME and hFOB cells, and thus play an important role in PCa growth in bone. Of interest, RANKL protein levels were measured by ELISA in the HBME and hFOB cells treated with PTHrP for 24 hr. We observed less than 15 and 11% induction (less potent than MCP-1 induction) in HBME and hFOB cells, respectively (data not shown), suggesting MCP-1 expression is significantly induced by PTHrP in these cells.

Furthermore, we have begun to characterize the transcriptional regulation of MCP-1 by PTHrP. PTHrP was found to induce
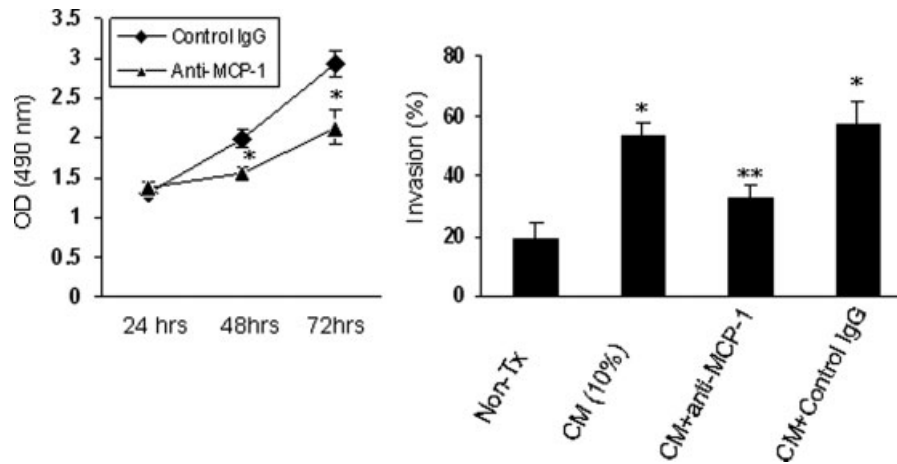
Figure 6 – Effect of CM collected from PTHR (H223R)-transfected HBME cells on the PC3 cell proliferation and invasion. (*a*) PC3 cells were plated in 96-well plates at 2,000 cells/well. CM (10%) collected from PTHR (H223R) with either MCP-1 neutralizing antibody (1 µg/ml) or control IgG (1 µg/ml) was added into the cultures. The cells were cultured for 24, 48, 72 hr. Cell proliferation was measured using a CellTiter 96 AQeous nonradioactive cell proliferation assay. Data represent the average absorbance of 4 wells. *$p < 0.001$ compared to control IgG treated cells. (*b*) PC3 cells were placed in transwell chambers with Matrigel-coated filters in the upper wells and CM (10%) collected from PTHR (H223R) with either MCP-1 neutralizing antibody (1 µg/ml) or control IgG (1 µg/ml) was added into the lower wells. After 24 hr incubation, invasion assays was performed as described in Materials and methods. Data are presented as mean ± SD from triplicates. *$p < 0.001$ compared to the non-treated (Non-Tx) cells. **$p < 0.001$ compared to the CM-treated cells.

MCP-1 promoter activity in HBME and hFOB cells. In accordance with the fact that binding of NF-κB is important for human MCP-1 transcription in human glioblastoma,[41] monocytes,[42] rat kidney tubular epithelial cells[43] and vascular smooth muscle cells,[44] we observed that the NFκB binding elements are required for PTHrP-induced MCP-1 promoter activity in HBME and hFOB cells because mutation of the 2 NFκB binding sites leads to the loss of PTHrP-stimulated promoter activity (Fig. 3). Similarly, the C/EBPβ binding elements were also demonstrated to be necessary for MCP-1 promoter activation stimulated by PTHrP. The latter finding is consistent with reports that the C/EBP elements are essential for induction of MCP-1 gene expression by lipopolysaccharide in a lymphoblastic cell line[45] and by nitric oxide in vascular smooth muscle cells.[46] As Sp1 binding is critical for MCP-1 promoter assembly and activation by TNFα,[47] Sp1 response elements and other *cis*-elements including AP-1 response elements that may be important for the response to PTHrP need to be further characterized. Interestingly, TNFα induced MCP-1 expression in HBME and hFOB cells, but the induction of MCP-1 promoter activity was through the C/EBP elements and not the NF-κB elements. This is consistent with the lack of a requirement for these sites for TNFα induction of the MCP-1 promoter in leiomyosarcoma (SKLMS1) cells, and in contrast to the requirement for these NF-kB elements in order for TNFα to induce the MCP-1 promoter in fibrosarcoma (HT1080) and glioblastoma (A172) cells.[42] Therefore, MCP-1 transcription regulation appears tissue-specific and stimulus-specific.

Finally, the significance of the PTHrP-induced MCP-1 production by HBME and hFOB cells was examined by assessing its role in osteoclast differentiation *in vitro*. To test this, a constitutively-active PTH receptor was first transfected into HBME and hFOB cells (Fig. 4). The CM collected from these cells was tested for its capacity to induce osteoclast differentiation *in vitro* using primary murine nonadherent bone marrow cells. We observed that the CM from cells with the activated PTH receptor induced osteoclast differentiation *in vitro* and that this induction was dose-dependently inhibited by adding MCP-1 neutralizing antibody (Fig. 5). Inter-estingly, the high concentration of neutralizing antibody only partially inhibited the CM-induced osteoclast differentiation suggesting that the activated PTH receptor in HBME stimulated the production of other factors that were secreted into the CM that may also induce osteoclast differentiation.

Although chemokines were first described based upon their capacity to stimulate recruitment and activation of leukocytes, it has become evident that they possess other functions including regulating events in bone marrow. It was reported that MCP-1 is induced by RANKL and it promotes osteoclast fusion and rescues granulocyte macrophage colony-stimulating factor suppression of osteoclast formation.[48] In the current study, the PTHrP-induced MCP-1 production resulted in formation of multinucleated cells in the absence of RANKL suggesting that MCP-1 is sufficient for osteoclast fusion events. This result is consistence with a recent report that MCP-1-induced TRAP positive multinuclear cells represent an arrested stage in osteoclast differentiation.[49]

In this study, we further demonstrated that CM collected from PTHR (H223R)-transfected HBME cells, through MCP-1, induce PC3 cell proliferation and invasion since the MCP-1 neutralizing antibody inhibited the CM-induced PC3 proliferation and invasion (Fig. 6). Together with the recent findings that humanized monoclonal antibody against PTHrP suppresses osteolytic bone metastases of breast cancer cells,[50] we postulate that PTHrP-induced MCP-1 production in the bone microenvironment, specifically by HBME and hFOB cells, may enhance osteoclast differentiation that results in osteolysis. The tumor-associated osteolysis may cause the release of bone matrix-associated factors such as transforming growth factor-β and insulin growth factors, and these factors may increase bone formation through stimulation of osteoblasts and contribute to osteoblastic lesions of PCa bone metastasis. In conclusion, we observed that PTHrP-induced MCP-1 production by HBME and hFOB cells promotes osteoclast differentiation and PC3 proliferation and invasion *in vitro*. This newly characterized function of PTHrP may play a critical role in PCa skeletal metastasis.

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