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Bone-Metastatic Prostate Carcinoma Favors Mesenchymal Stem Cell Differentiation Toward Osteoblasts and Reduces their Osteoclastogenic Potential

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ABSTRACT

Bone homeostasis is achieved by the balance between osteoclast-dependent bone resorption and osteoblastic events involving differentiation of adult mesenchymal stem cells (MSCs). Prostate carcinoma (PC) cells display the propensity to metastasize to bone marrow where they disrupt bone homeostasis as a result of mixed osteolytic and osteoblastic lesions. The PC-dependent activation of osteoclasts represents the initial step of tumor engraftment into bone, followed by an accelerated osteoblastic activity and exaggerated bone formation. However, the interactions between PC cells and MSCs and their participation in the disease progression remain as yet unclear. In this study, we show that bone metastatic PC-3 carcinoma cells release factors that increase the expression by human (h)MSCs of several known pro-osteoblastic commitment factors, such as α5/β1 integrins, fibronectin, and osteoprotegerin. As a consequence, as shown in an osteogenesis assay, hMSCs treated with conditioned medium ($C^{ed}M$) derived from PC-3 cells have an enhanced potential to differentiate into osteoblasts, as compared to hMSCs treated with control medium or with $C^{ed}M$ from non-metastatic 22RV1 cells. We demonstrate that FGF-9, one of the factors produced by PC-3 cells, is involved in this process. Furthermore, we show that PC-3 $C^{ed}M$ decreases the pro-osteoclastic activity of hMSCs. Altogether, these findings allow us to propose clues to understand the mechanisms by which PC favors bone synthesis by regulating MSC outcome and properties. J. Cell. Biochem. 112: 3234–3245, 2011. © 2011 Wiley Periodicals, Inc.

KEY WORDS: FGF-9; MESENCHYMAL STEM CELLS; OSTEOGENESIS; OSTEOCLASTOGENESIS; PROSTATE CARCINOMA

Prostate cancer (PC) is the most commonly diagnosed cancer and the second leading cause of cancer death in western men after middle age. PC cells possess high affinity for bone marrow, as 90% of patients with advanced disease will develop metastases to bone [Mundy, 2002]. These skeletal metastases are life-threatening, resulting in clinical complications such as pathological fractures, hypercalcemia, nerve compression syndromes, and severe pain [Roodman, 2004]. At this stage of the disease, hormonal treatment remains temporary effective, but no curative treatment is available [So et al., 2005]. The implantation of PC cells into bone depends on their ability to interact with the bone microenvironment and ultimately leads to the disruption of the normal bone remodeling process, resulting in the formation of mixed osteoblastic and osteolytic lesions [Keller et al., 2001].

Within the bone marrow, two major types of stem cells are present; the hematopoietic stem cells (HSCs) that give rise to all the blood cell types including myeloid and lymphoid lineages, and the mesenchymal stem cells (MSCs) that can differentiate into

Abbreviations used: $C^{ed}M$, conditioned medium; MSCs, mesenchymal stem cells; PC, prostate carcinoma.

Conflict of interest: None

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mesenchymal lineages including bone, cartilage, adipose, muscle, and tendon [Pittenger et al., 1999]. Normal bone remodeling is a balance between bone resorption by osteoclasts that are derived from mononuclear precursors in the myeloid lineage of HSCs, and new bone formation by osteoblasts that are derived from MSCs. It is now recognized that PC cells can alter bone homeostasis either directly, affecting osteoblast and osteoclast functions, or indirectly by altering the molecular composition of the matrix. The PC-dependent activation of osteoclasts is required for the initial step of tumor bone engrafment [Lee et al., 2003]. On the other hand, metastatic PC cells can stimulate new bone formation through the production of various factors that either exert direct stimulatory effect on osteogenic differentiation and activity, such as bone morphogenetic proteins (BMPs) and endotheline-1 (ET-1), or inhibit osteoblast apoptosis, such as the peptide related to parathyroid hormone (PTHrP) (reviewed in [Goltzman et al., 1992; Koeneman et al., 1999]). Other secreted proteases, including the prostate-specific antigen (PSA) and the urokinase-type plasminogen activator (uPA), can activate latent osteogenic proteins that are present in the bone matrix such as the transforming growth factor (TGF)-β. At present however, little is known about the interactions between PC cells and bone marrow MSCs, and how they might participate in the disease progression by orientating bone metastasis toward an osteoblastic or an osteolytic phenotype. In the present study, we aimed at characterizing how bone metastatic PC cells modulate the gene expression profile of human (h)MSCs and to what extent this may influence their differentiation capacity toward the osteoblastic lineage. The results of this study allow us to propose mechanisms explaining the in vivo pro-osteoblastic effets of PC metastasis that characterize this pathology.

MATERIALS AND METHODS

CELLS AND CELL CULTURE CONDITIONS

Human (h)-MSCs were derived from fresh human bone marrow specimens collected from healthy donors after informed consent was obtained. The cells were isolated as previously described [Djouad et al., 2007] and were characterized by their phenotype and their differentiation potential (data not shown). hMSCs were shown by fluorescence activated cell sorting (FACS) analysis to lack expression of typical hematopoietic cell surface markers, including CD14, CD34, and CD45, and to be positive for CD44, CD73, CD90, and CD105. hMSCs were maintained as monolayer cultures in α-MEM medium supplemented with 10% fetal calf serum (FCS) (Perbio Science), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and bFGF (1 ng/ml) (R&D Systems) at 37°C in 5% CO_2. MSCs between passage 3 and 6 were used for our experiments. Between 2 and 5 different hMSCs samples were used for each of our experiments. Recombinant human (rh) FGF9 and neutralizing anti-FGF9 mAb (clone 36912) were purchased to R&D Systems.

The human non metastatic 22RV1 and bone metastatic PC-3 prostate carcinoma (PC) cell lines, obtained from ATCC, were maintained as monolayer cultures in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium (Sigma Aldrich, l'Isle d'Abeau, France), supplemented with 10% FCS, 2 mM L-Glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C in 5% CO_2.

Human osteoclasts were generated from purified peripheral blood monocytes (PBMC) from healthy donors obtained after informed consent. PBMC were isolated from a buffy coat by centrifugation over ficol hypaque. They were then incubated on gelatin-coated culture dishes for 30 min at 37°C. Non-adherent cells were removed by repeated washing with PBS, supplemented with 10% FCS. After addition of 10 mM EDTA in PBS to the culture dishes, followed by an incubation for 10 min at 37°C, the adherent cells were collected, washed once with culture medium, and analyzed for the expression of CD3, CD20, and CD14 by FACS. Purity of the cells was >90%.

PREPARATION OF PC-3 AND 22RV1 CONDITIONED MEDIUM

Conditioned medium ($C^{ed}M$) from PC-3 or 22RV1 cells was obtained by culturing sub-confluent cells for 24 h in α-MEM medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C in 5% CO_2. Then, the $C^{ed}M$ was removed, centrifuged at 300 g for 10 min and filtered (0.2 μm). $C^{ed}M$ was added to subconfluent primary hMSCs for the time indicated. For osteogenic differentiation experiments, PC-conditioned osteogenic induction (OI) medium consisted of PC-$C^{ed}M$ supplemented with 0.1 μM dexamethasone and 50 μM ascorbic acid-2 phosphate just prior incubation with hMSC cultures. For mineralization experiments, 0.1 μM dexamethasone, 50 μM ascorbic acid-2 phosphate, and NH_2PO_4 (3 mM) were added to the PC-$C^{ed}M$ just prior addition to hMSC culture.

OSTEOGENIC DIFFERENTIATION AND MINERALIZATION ASSAY

Osteogenic differentiation of primary hMSCs was induced by plating cells at low density (2,500 cells per cm^2), allowing them to grow to confluence before performing a 21 day-culture in OI medium. In co-culture experiments, 5×10^3 PC-3 or 22RV1 cells were added to hMSC cultures 4 days after induction of osteogenic differentiation and maintained for a supplementary 16 days. Alternatively, hMSCs were cultured in PC-3- or 22RV1-conditioned OI medium. Culture medium was used as a negative control. Osteogenic differentiation was assessed by measuring the expression of the osteoblastic markers such as osteocalcin (OC), bone phosphatase alkaline (ALP), and cbfa1. For evaluation of ALP protein expression, cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, and then incubated for 30 min with a solution of 2% Fast violet B salt (Sigma) and 4% Naphtol AS-MX phosphate alkaline solution (Sigma). ALP activity in the culture supernatant was assessed by measuring the p-nitrophenol formed from the enzymatic hydrolysis of p-nitrophenylphosphate (Sigma), used as the substrate by spectrometry at 405 nm. Alizarin Red S staining was performed as described before [Fritz et al., 2008] to assess ECM mineralization 21 days after OI.

RNA PREPARATION AND RT-PCR

Total RNA from hMSCs, PC-3, and 22RV1 cells was extracted using the Rneasy Mini Kit according to the supplier's recommendations

(Qiagen S. A., Courtaboeuf, France). Total RNA (100 ng) was reverse transcribed using Multiscribe reverse transcriptase (Applied Biosystems, Courtaboeuf, France). Quantitative (q)-PCR was conducted using the Assays-on-Demands primers specific for human β2-microglobulin (β2-M), RSP9, OC, ALP, cbfa1, the integrin α2, α5, αv, β1, and β3, osteoprotegerin (OPG), and the TaqMan PCR master mix (Applied Biosystems), under the following conditions: 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 s at 95°C and 1 min at 60°C. Fibronectin primers were a kind gift from Dr Ellen Van Obberghen (Centre Antoine Lacassagne, CNRS UMR 6543, Nice). Measurement and analysis of gene expression were performed using ABI Prism 7000 Sequence Detection System Software (Applied Biosystems). Values are expressed as the relative mRNA level of specific gene expression using the formulae $2^{-(\Delta Ct)}$ by normalization with endogenous β2-M reference genes or as fold change using the formulae $2^{-(\Delta\Delta Ct)}$. Semiquantitative RT-PCR was conducted using the gene-specific primers for FGF-9 and Gapdh, as previously described [11] and PCR products were resolved on 2% agarose gels.

TaqMan low-density arrays (TLDA, microfluidic cards; Applied Biosytems) pre-loaded with predesigned fluorogenic TaqMan probes and primers were used in a two-step process [Jorgensen et al., 2004]. First-strand cDNA was synthetized from 3 μg total RNA using the high-capacity cDNA archive kit (Applied Biosystems). Quantitative PCR reactions were then carried out using the microfluidic cards and the ABI PRISM 7900HT Sequence Detection System (Applied Biosystem) as previously described [Djouad et al., 2007]. Values are expressed as the relative mRNA level of specific gene expression - using the formulae $2^{-(\Delta Ct)}$ by normalization with endogenous β2-M reference genes or as fold change using the formulae $2^{-(\Delta\Delta Ct)}$.

FLUORESCENCE-ACTIVATED CELL SORTING (FACS) ANALYSIS

hMSCs were plated in tissue-culture plates and allowed to grow overnight. After a wash with PBS, hMSCs were cultured for another 24 h in control culture medium or PC-3-C^{ed}M. Cells were then harvested by treatment with 0.05% trypsin and 0.53 nM EDTA. After a wash with PBS, isolated hMSCs were suspended in PBS containing 0.1% BSA and 0.01% sodium azide and incubated with a primary mouse monoclonal antibody (mAbs) for 30 min at room temperature (RT). The mAbs used were specific for human CD14, CD34, CD44, CD45, CD73, CD90 and CD105, and for CD49e (integrin α5), CD51 (integrin αv), CD29 (integrin β1), and CD61 (integrin β3) or isotypic controls (R&D Systems, Lille, France). Flow cytometry was performed on a FACSscan (BD Pharmigen, Le Pont de Claix, France) and data were analyzed with the Cellquest software (BD). Data were expressed as the number of cells negative or positive for the marker at the indicated fluorescence intensity.

INDIRECT IMMUNOFLUORESCENCE

For immunocytochemical analysis, hMSCs were grown on glass coverslips (2×10^5 per 3.5-cm plate) for 6 h, then treated with culture medium or PC-3- C^{ed}M for 18 h. Briefly, after fixation in 4% formaldehyde, cells were permeabilized with 0.3% triton in PBS and incubated in blocking buffer (PBS-1% BSA). Immunodetection of proteins was then performed using primary mAbs specific for fibronectin (1:1,000; Sigma F6142), laminin (1:500; Sigma L9393), vitronectin (VN) (1:100; Abcam) for 1 h at 37°C and revealed with Alexa 436/548-conjugated secondary antibody (1:1,000; Molecular Probes, USA), for 30 min at 37°C. DNA was labelled with Hoescht.

OSTEOCLASTOGENIC ASSAY

Enriched $CD3^+CD14^+CD20^+$ monocytes were seeded into 24 well plates (50,000 cells per well) or onto dentine slices (5 mm in diameter, IDS, France) in 24 well plates (200,000 cells per well). The cells were incubated in either complete α-MEM medium (containing 10% FCS, 50 IU/ml penicillin, 50 mg/ml Streptomycin and 2 mM L-glutamine) or C^{ed}M from hMSCs that were pre-treated or not with supernatant of PC-3 cells during 24 h. Control cells were incubated with complete α-MEM medium containing either 20 ng/ml of RANK-L and 20 ng/ml of CSF (both from Preprotech, France) or 5 ng/ml of RANK-L and 20 ng/ml of CSF, respectively.

After 7 days incubation, the average number or percentage of cells with three nuclei or more were counted. After 9 days incubation, the dentine slices were fixed in 4% formaldehyde, washed with PBS, and stained for tartrate-resistant acid phosphatase (TRAP) using the 387A-1KT kit according to the manufacturer's instructions (Sigma). At day 9, cells having settled onto the dentine slices were stained with toluidine blue (solution of 4% glutaraldehyde in 0.2 M Na-cacodylate, 1% toluidine blue in 0.5% tetraborate solution). The percentage of eroded dentine surface (area covered by resorption lacunae) was calculated using an ordinary light microscope equipped with an ocular containing 10 crosses.

OPG AND RANKL ELISA

Supernatant from hMSCs or PC3-C^{ed}M-treated MSCs were then recovered for analysis of OPG and RANKL secretion. Supernatants were not concentrated before analysis. Soluble OPG was quantified using the Millipore Immunoassays & Multiplex kits (Human Panel 1A, Cat. No.HBN1A-51K). Soluble RANKL was analyzed by ELISA (Biomedica).

STATISTICAL ANALYSIS

Experiments were performed independently in triplicate. Statistical analysis was done using unpaired Student's *t*-test. Differences were considered statistically significant at $P < 0.05$. ($^{*}P < 0.05$; $^{**}P < 0.01$, and $^{***}P < 0.001$).

RESULTS

SOLUBLE FACTORS PRODUCED BY PC-3 CELLS INDUCE CHANGES IN GENE EXPRESSION PROFILES OF hMSCs

We first investigated whether bone metastatic PC-3 cells could alter bone microenvironment by modulating the gene expression profile of hMSCs. In particular, we chose to screen for genes implied in development and morphogenesis, cell-matrix interaction, and matrix remodeling. For this purpose, we analyzed the transcriptional profiles of hMSCs from three different patients, following 24 h of incubation with control medium or PC-3-C^{ed}M. Using the TLDA microfluidic cards, we were able to quantify simultaneously the expression of 86 mRNAs. A significant change in the mRNA expression levels was observed between hMSCs cultured with PC-3 C^{ed}M or in medium. Indeed, 16 genes exhibit significant fold

changes in their expression profile in samples cultured in PC-3-$C^{ed}M$. These genes were adhesion molecules, chemokines, matrix proteases, and osteogenic factors. Among the adhesion molecules, we detected the expression of the integrins (ITG) α1, α4, α5, α6, αv, β1, and β7 (Table I). The expression of the subunits α4, α6, and β1 was up-regulated more than two-fold in the three tested hMSC samples, and that of the α5 and αv subunits was up-regulated more than eight-fold in two of the three tested hMSCs samples. Moreover, we present evidence for the up-regulation of the osteogenic factors osteoprotegerin (OPG) in all hMSC samples and transforming growth factor (TGF)-β1 in two out of the three tested samples.

PC-3 INCREASES THE EXPRESSION OF MATRIX PROTEINS AND INTEGRIN RECEPTORS IN hMSCs

Our next step aimed at validating the previous results for some candidates that were of particular interest due to their known role in bone morphogenesis and cell–matrix interaction. Following a 24 h incubation of hMSCs with PC-3-$C^{ed}M$, we confirmed by RT-qPCR that the mRNA levels of the integrin subunits α5 and αv were increased by a four- and three-fold factor, respectively, whereas β1 mRNA levels were only slightly increased (Fig. 1A). By comparison, we also tested the expression of two other well expressed integrins, the α2 and β3 subunits, which are both over-expressed by a two- to three-fold factor after PC-3-$C^{ed}M$ treatment. A two-fold induction of OPG mRNA in hMSCs was also induced by PC-3-$C^{ed}M$. This increase in expression of integrin transcripts was confirmed at the protein level for cell surface expression of integrins α5 (30%), αv (50%), and β1 (25%), as detected by FACS analysis (Fig. 1B). Because the heterodimeric integrin receptors α5β1 and αvβ3 are specific receptors for the major bone matrix proteins fibronectin (FN) and VN, respectively, we investigated whether PC-3 cells could concomitantly modulate the expression of these proteins by hMSCs. Immunocytochemical analysis revealed that the expression of FN by hMSCs was highly increased following stimulation with PC-3-$C^{ed}M$, favoring the formation of a cellular network (Fig. 1C). On the contrary, staining for vitronectin or laminin, which is another major ECM component in the bone marrow, revealed no difference of expression. The modulation of FN expression by hMSCs following PC-3-$C^{ed}M$ stimulation was further assessed at the mRNA and protein levels. A significant increase of FN mRNA expression level was confirmed by RT-qPCR (Fig. 1D).

PC-3 CELLS FAVOR COMMITMENT OF hMSCs TOWARD OSTEOBLASTS

Cell–ECM interactions are crucial regulators of cell differentiation and both matrix proteins and their corresponding integrin receptors have been shown to regulate osteogenesis and PC cells are known to produce osteoblastic factors. We thus speculated that PC-3 cells could modulate the commitment of hMSCs toward the osteoblastic lineage.

We tested hMSCs for their osteogenic differentiation capacity when cultured alone or co-cultured with PC-3 cells or PC-3-$C^{ed}M$ in OI conditions during 21 days. Osteoblastic differentiation was first assessed by monitoring ALP activity. Culture of hMSCs with PC-3 cells or PC-3-$C^{ed}M$ in OI conditions both significantly increased ALP expression (Fig. 2A) and activity (Fig. 2B) as compared to hMSCs cultured alone or in OI medium. This result was confirmed with hMSC isolated from five different patients (data not shown). The increased amount of ALP was attributable to hMSCs since no significant induction of ALP expression or activity by PC-3 cells in OI conditions could be detected (Fig. 2A and B). To confirm that hMSCs cultured in presence of PC-3-$C^{ed}M$ were fully differentiated osteoblasts, we assessed mineral deposition by quantification of Alizarin Red S staining and quantified the mRNA levels of the specific osteoblastic genes cbfa1, OC, and ALP. In this condition, mineralization was significantly (Fig. 2C) as well as the mRNA expression levels of the osteoblast markers cbfa1, OC, and ALP (Fig. 2D). Altogether, our findings indicate that bone metastatic PC-3 cells favor the commitment of hMSCs toward the osteoblastic lineage and that this effect is mediated by secreted factors.

NON-METASTATIC PC CELLS FAIL TO INDUCE OSTEOBLASTIC DIFFERENTIATION OF hMSCs

We next asked whether the observed pro-osteogenic effect of PC-3 cells was specific of PC cells that have the propensity to form bone metastasis as compared to non bone-metastatic PC cell lines. We used the osteogenic assay to compare the osteoblastic differentiation capacity of hMSCs cultured alone or co-cultured with bone

TABLE I. PC-3 Cells-Induced Changes in Gene Expression Profile of hMSCs

		MSC1			MSC2			MSC3		
Gene name	Accession no.	Control ($2^{-\Delta CT}$)	PC-3 $C^{ed}M$ ($2^{-\Delta CT}$)	Fold ($2^{-\Delta\Delta CT}$)	Control ($2^{-\Delta CT}$)	PC-3 $C^{ed}M$ ($2^{-\Delta CT}$)	Fold ($2^{-\Delta\Delta CT}$)	Control ($2^{-\Delta CT}$)	PC-3 $C^{ed}M$ ($2^{-\Delta CT}$)	Fold ($2^{-\Delta\Delta CT}$)
ITGα1	Hs00235030_m1	2.68	4.66	**1.74**	13.06	8.52	**0.65**	0.70	52.50	**75.29**
ITGα4	Hs00168433_m1	11.53	25.20	**2.19**	112.59	1861.36	**16.53**	5.82	670.03	**115.08**
ITGα5	Hs00233746_m1	2336.36	4346.32	**1.86**	164.83	1395.35	**8.47**	935.80	38827.58	**41.49**
ITGα6	Hs00173952_m1	47.48	111.40	**2.35**	13.68	71.44	**5.22**	2.79	548.18	**196.59**
ITGαV	HS00233808_m1	385.96	425.77	**1.10**	282.74	2627.04	**9.29**	68.61	7135.33	**103.99**
ITGβ1	Hs00559595_m1	8601.79	46571.03	**5.41**	26128.31	66912.28	**2.56**	6717.95	400025.18	**59.55**
ITGβ7	Hs00168469_m1	0.93	nd	**nd**	1.83	71.05	**38.87**	nd	24.50	**nd**
OPG	Hs00171068_m1	466.24	935.07	**2.01**	61.04	1878.49	**30.78**	22.88	879.94	**38.45**
TGF-P1	Hs00171257_m1	3115.19	3326.82	**1.07**	2007.10	57744.63	**28.77**	3088.38	51499.41	**16.68**

Human primary MSCs (n = 3) were grown during 24 h in control medium (Control) or in conditioned medium from PC-3 prostate cancer cells (PC-3-$C^{ed}M$). The mRNA expression levels for selected genes are indicated. Values are expressed as the relative mRNA level of specific genes normalized to the mRNA level of β2M as housekeeping gene and obtained using the formulae $2^{-(\Delta Ct)}$or as fold change and expressed as $2^{-(\Delta\Delta Ct)}$.

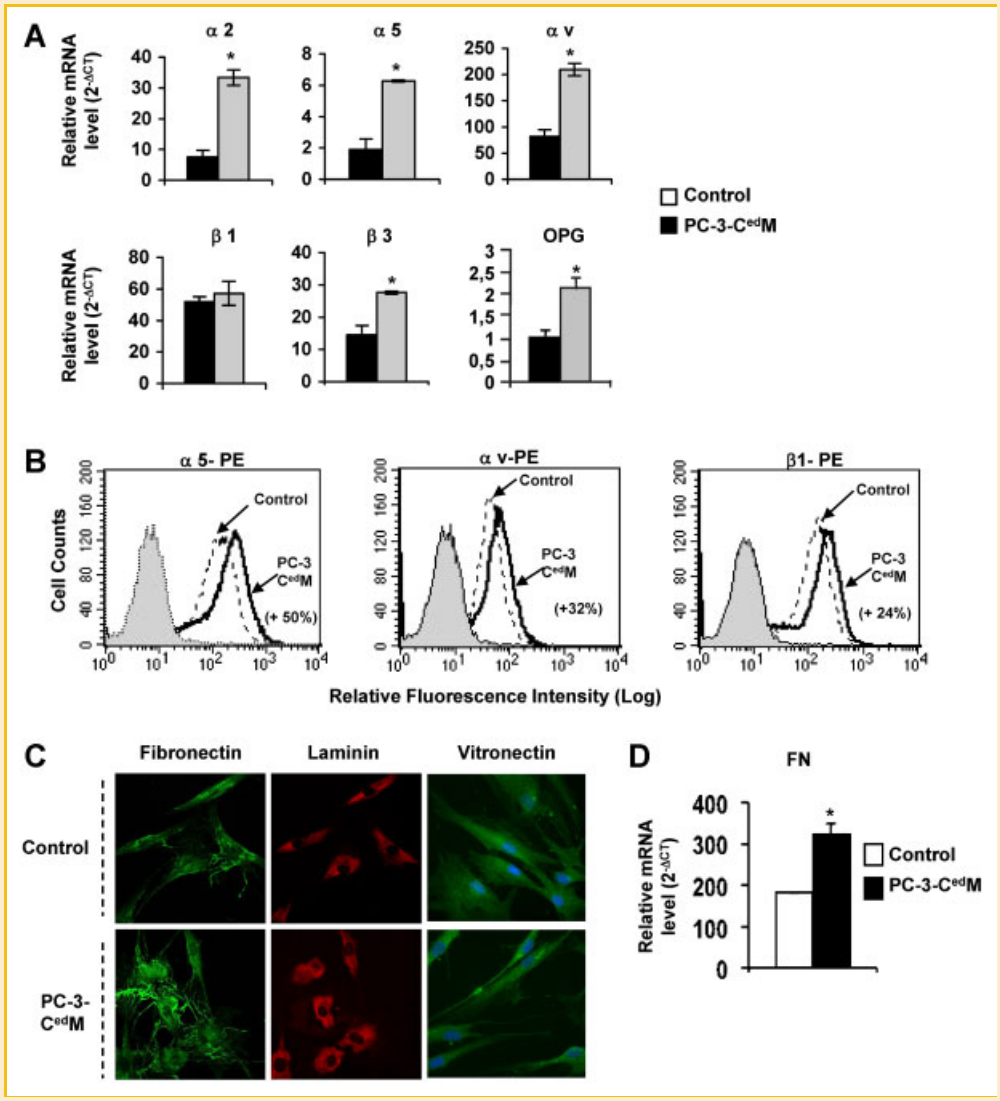

Fig. 1. PC-3-conditioned medium increases integrin and fibronectin expression by hMSCs. Human primary MSCs were cultured in control medium or PC-3-C^{ed}M during 24 h, and then tested for integrin expression and immunocytochemical staining of ECM components. A: Total RNA was extracted from hMSCs ($n = 3$) and analyzed by RT-qPCR for expression of α2-, α5-, αv-, β1-, and β3- integrins and OPG mRNAs, and normalized to β2M as described in methods, using the formulae $2^{-(\Delta CT)}$. B: hMSCs were harvested and analyzed by FACS for the cell surface expression of α5-, αv-, and β1- integrins. Integrin expression at cell surface was quantified by measuring the geometric mean, and the induction of integrins expression by hMSCs following exposure to PC-3 C^{ed}M was expressed as the % of increase of the geometric mean as compared to control condition. C: The level of expression of fibronectin, laminin, and Vitronectin was compared by immunofluorescence in hMSCs 24 h after culture in control medium or PC-3-C^{ed}M. All pictures are 40x magnifications. D: Total RNA was analyzed by RT-qPCR for FN mRNA expression and normalized to β2-microglobulin (β2M) as described in methods, using the formulae $2^{-(\Delta Ct)}$. Data are expressed as mean ± SEM ($n = 3$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

metastatic PC-3 or non-metastatic 22RV1 cells. After 21 days, ALP activity was significantly induced following co-culture with PC-3 cells, whereas it was similar in hMSCs co-cultured with 22RV1 cells or cultured alone (Fig. 3A and B). Furthermore, although the expression levels of Cbfa1, OC, and ALP mRNA were increased in hMSCs cultured in presence of PC-3-C^{ed}M, these transcripts were expressed at similar levels in hMSCs cultured alone or with 22RV1-C^{ed}M (Fig. 3C). All together, these results indicate that the pro-osteoblastic effect of PC cells seems to be specific to those that metastasize to bone. Fibroblast growth factors (FGFs) have important regulatory functions in bone formation and FGF-9 expression was previously shown to increase during progression of PC to a metastatic phenotype. We thus aimed to compare FGF-9 expression in PC-3 and 22RV1 cells. As revealed by RT-PCR, we found that FGF-9 was highly expressed in PC-3 cells whereas it was only barely detectable in 22RV1 cells (Fig. 3D). This result is in concordance with the previous observed correlation between FGF-9 expression and bone metastasis properties [Li et al., 2008].

FGF9 CONTRIBUTES TO PC-3 INDUCED TRANSCRIPTOMIC AND FUNCTIONAL CHANGES IN hMSCs

Because FGF-9 is highly expressed by the bone metastatic PC-3 cells and was demonstrated to exert pro-osteogenic activity in other PC

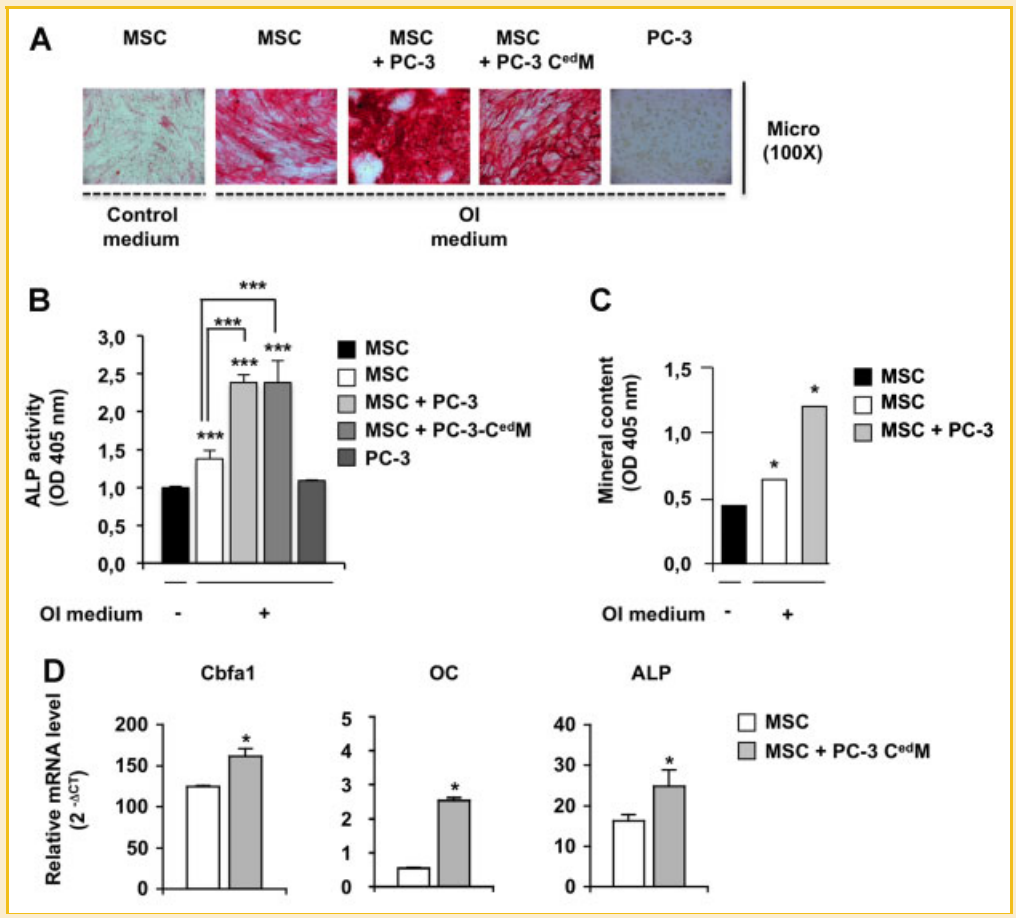

Fig. 2. Tumoral PC-3 cells increase the osteogenic differentiation of hMSCs in vitro. A–D: Primary hMSCs were cultured alone or co-cultured with bone metastatic PC-3 cells or PC-3-C^{ed}M in osteogenic (OI) medium during 21 days and analyzed for their osteogenic differentiation capacity. A: Representative photomicrographs of hMSCs after osteogenic differentiation, evidenced by alkaline phosphatase (ALP) staining, and compared to PC-3 cells ALP staining. B: Quantification of ALP activity in hMSCs and PC-3 supernatants. C: Formation of calcified nodules was quantified after Alizarine S Red staining. D: Primary hMSCs were cultured for 21 days in OI medium or PC-3- OI C^{ed}M and analyzed for their commitment toward osteoblast lineage. Total RNA isolated from 21-day culture cells was analyzed by RT-qPCR for osteoblastic markers: cbfa1, OC and ALP mRNAs, and normalized to β2M as described in methods, using the formulae $2^{-(\Delta Ct)}$. Data are representative of one experiment out of three and expressed as mean ± SEM. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

cell lines [Li et al., 2008], we investigated whether FGF-9 could mediate the effects of PC-3 cells on the expression profile and osteoblastic differentiation of hMSCs. First, we investigated whether a neutralizing anti-FGF-9 antibody (mAb) could inhibit the effect of PC-3-C^{ed}M on the expression levels of Itgα5 and OPG. We showed a dose-dependent decrease of the PC-3-C^{ed}M-induced expression of Itgα5 and OPG in hMSCs by RT-qPCR when the antibody was added (Fig. 4A and B). This antibody failed to modify the PC-3-dependent increase of Itgαv, β1, and β3 mRNA expression levels (data not shown). Moreover, the neutralizing anti-FGF-9 mAb was able to reduce the PC-3-C^{ed}M-induced increase in the protein expression of Itgα5 at the cell surface of hMSCs (Fig. 4C). We next added the recombinant human (rh) FGF-9 protein in the culture medium of hMSCs. Using RT-qPCR, we demonstrated that similar to PC3-C^{ed}M, rhFGF-9 (1 and 10 ng/ml) could up-regulate Itgα5 and OPG mRNAs by hMSCs (Fig. 4A and B). These changes in gene expression were translated in significant changes in cell surface levels of the protein Itgα5, as detected by FACS analysis (Fig. 4C). Altogether, these data indicate that the up-regulation of Itgα5 and OPG transcripts in hMSCs by PC-3-C^{ed}M are dependent on FGF-9 secretion.

We next investigated whether FGF-9 contributes to the pro-osteogenic effect of PC-3, by performing an osteogenic assay in which hMSCs were cultured during 21 days in OI conditions with control medium, PC-3-C^{ed}M, or rhFGF-9, in the presence or absence of the neutralizing anti-FGF-9 mAb. Staining of ALP activity on day 21 revealed that both PC-3-C^{ed}M and rhFGF-9 induced a similar stimulation of osteoblastic differentiation of hMSCs (Fig. 4D). Addition of the neutralizing anti-FGF-9 mAb on hMSCs cultured with PC-3-C^{ed}M abolished the increase in ALP activity, although it did not decrease ALP activity in hMSCs cultured with control OI medium. Moreover, culture of hMSCs with PC-3-C^{ed}M or rhFGF-9 in OI conditions both significantly increased ALP mRNA expression level (Fig. 4E). Addition of the neutralizing anti-FGF-9 mAb on hMSCs cultured with PC-3-C^{ed}M abolished the increase in ALP mRNA level, although it did not decrease ALP mRNA level in hMSCs

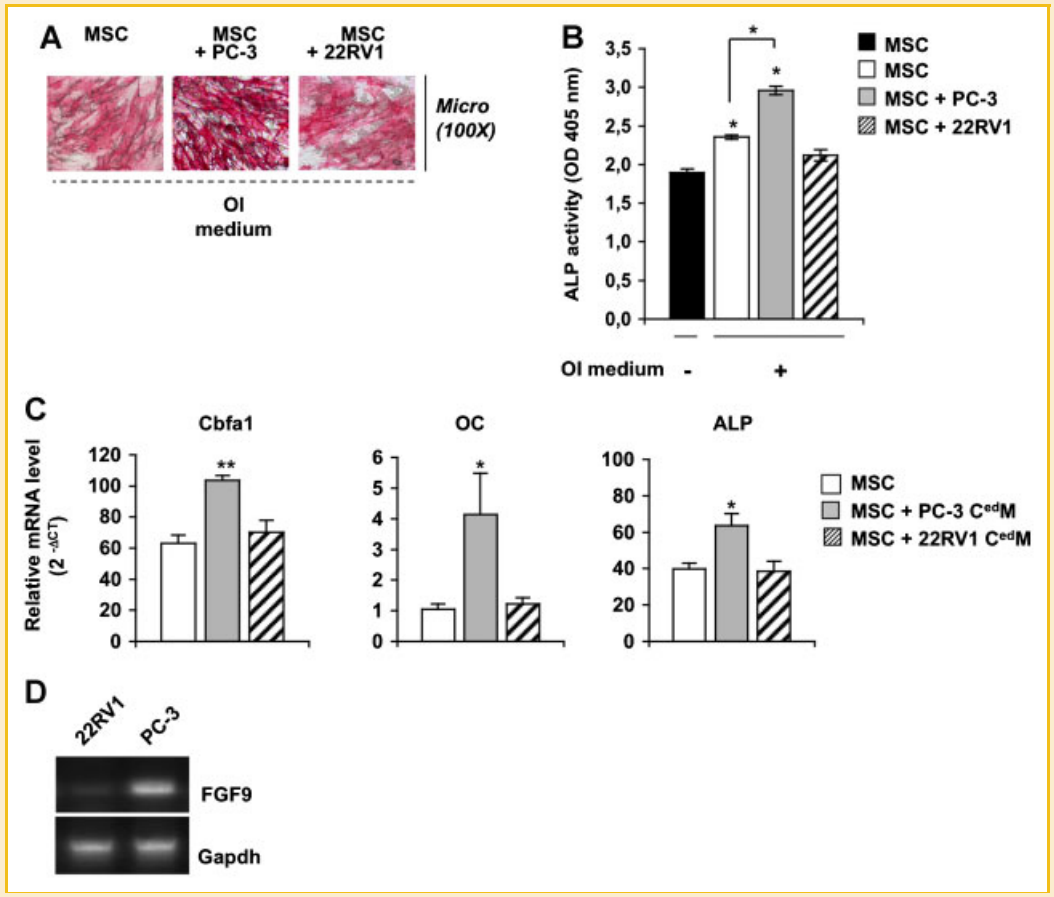

Fig. 3. Osteogenic differentiation of hMSCs is specifically induced by bone metastatic prostate cancer cells. A–B: Primary hMSCs were cultured alone or co-cultured with bone metastatic PC-3 or non-metastatic 22RV1 PC cells in osteogenic (OI) medium during 21 days and analyzed for their osteogenic differentiation capacity. A: Representative microscopic images of hMSC osteoblastic differentiation, evidenced by ALP staining. B: Quantification of ALP activity in hMSCs supernatants. C: Expression of osteoblastic markers by hMSCs cultured in A. Total RNA was analyzed by RT-qPCR and expression levels of cbfa1, OC, and ALP mRNAs were normalized to β2M, using the formulae $2^{-(\Delta Ct)}$. Data are expressed as mean ± SEM ($^*P < 0.05$) and are representatives of one out of three experiments. D: Total mRNA from PC-3 and 22RV1 cells was analyzed by semiquantitative RT-PCR for the expression of FGF-9 and normalized to Gapdh. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

cultured with control OI medium. Altogether, these data indicate that FGF-9 secretion participates to the pro-osteogenic effect of PC-3 on hMSCs.

CONDITIONED MEDIUM FROM PC-3 CELLS REDUCES THE PRO-OSTEOCLASTOGENIC ACTIVITY OF hMSCs IN VITRO

Because our data show that PC-3 cells increase the osteoclast-repressing factor OPG in an FGF-9-dependent manner, we next asked whether PC-3 cells could also impact bone remodeling by modulating the ability of hMSCs to regulate osteoclastogenesis. For this purpose, a preparation of human monocytes, used as a source of osteoclast progenitors, were cultured in the presence of M-CSF and RANKL with $C^{ed}M$ from control- or PC-3-treated hMSCs. We then assessed osteoclast formation by counting the multinucleated cells with more than three nuclei and osteoclast activity by counting cells positive for TRAP staining. Following a 7 days culture, a similar percentage of multinucleated cells was measured when monocytes were cultured in control medium or $C^{ed}M$ from hMSCs (47.5 ± 2.6% versus 50.5 ± 2.8% cells, respectively) (Fig. 5A). In contrast, half multinucleated cells were formed in the presence of $C^{ed}M$ from PC-3-primed hMSCs (23.2 ± 1.8%). Consistent with this result, we measured a similar percentage of $TRAP^+$ cells after monocyte cultures with control medium or $C^{ed}M$ from control hMSCs (41.5 ± 1.3 and 40.3 ± 1.3%, respectively), as well as a significantly lower percentage of $TRAP^+$ cells (15.7 ± 0.8%) with $C^{ed}M$ from PC-3-primed hMSCs (Figs. 5B and C).

We then assessed the osteoclastic activity using a pit assay in which monocyte preparations were added to slices of dentine and examined for the formation of aeras of bone resorption (pits). Under control conditions, the formation of pits indicated that the preparation contained active osteoclasts. The eroded surface was similar in control medium or $C^{ed}M$ from control hMSCs (20.7 ± 1.6 and 20.3 ± 1%, respectively) (Fig. 5D and E). On the contrary, culture in the presence of $C^{ed}M$ from PC-3-primed hMSCs resulted in a five-fold decrease in the percentage of eroded surface (3.7 ± 0.2%). Indeed, supernatants from hMSCs primed with PC-3-$C^{ed}M$ were able to suppress osteoclast formation and activity.

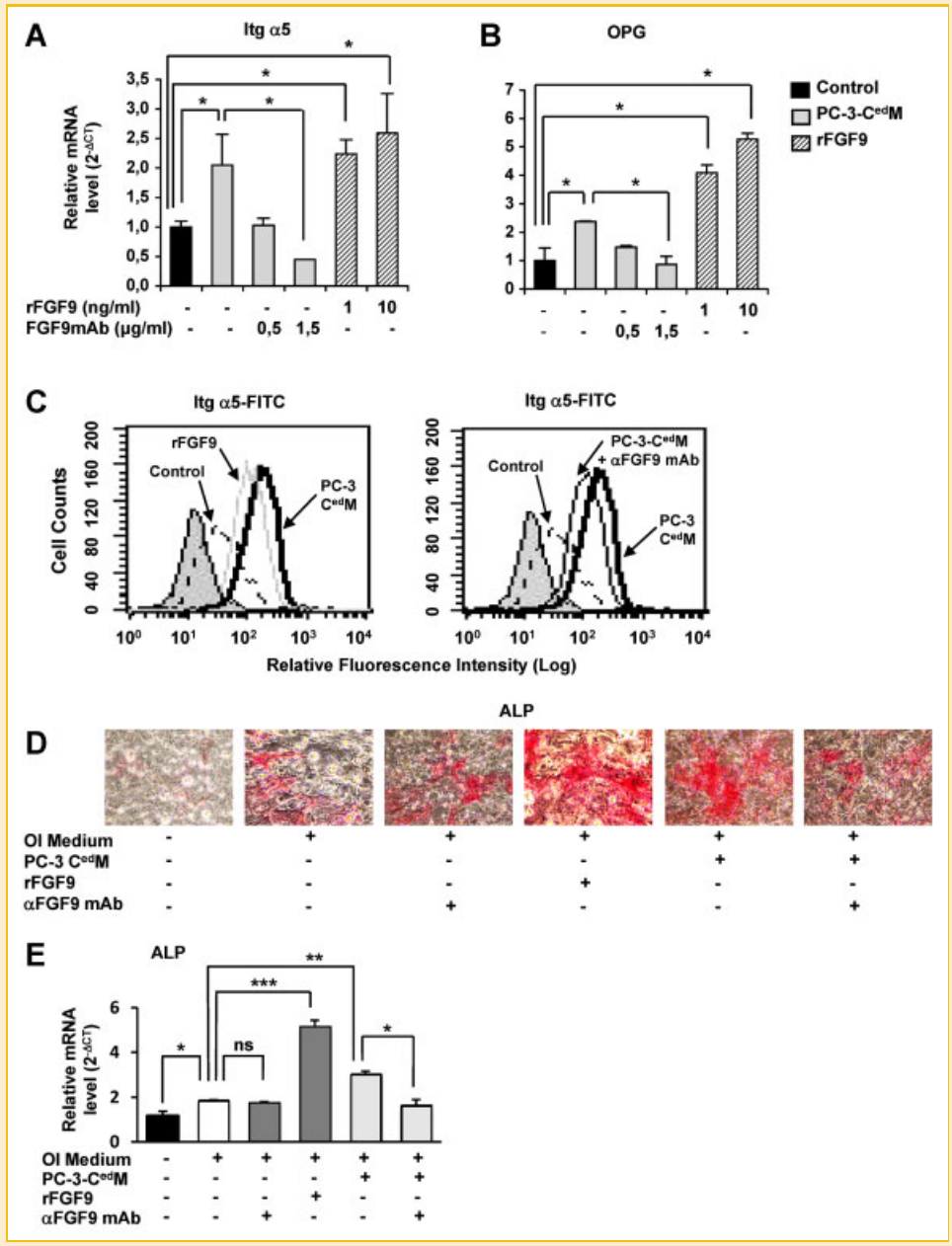

Fig. 4. Soluble FGF-9 produced by PC-3 cells mediates the pro-osteogenic effect of PC-3 cells on hMSCs. A–B: hMSCs were cultured for 24 h with control medium, PC-3-C^{ed}M, 1 or 10 ng/ml rFGF-9. Neutralizing Ab against FGF-9 were added to PC-3 C^{ed}M at the indicated doses. Total RNA was analyzed by RT-qPCR for expression of integrin (Itg) α5 (A) and OPG (B) mRNAs. Data are expressed as mean ± SEM and are representatives of one out of three experiments ($^*P < 0.05$). C: hMSCs were cultured for 24 h with control medium, PC-3-C^{ed}M, rFGF-9 (1 ng/ml) with or without addition of neutralizing Ab against FGF-9 at 1.5 μg/ml, and then analyzed by FACS for cell surface expression of Itg α5. D: Osteoblastic differentiation of hMSCs was assessed by staining of ALP activity after 21 days of culture in OI medium or PC-3-OI C^{ed}M with or without addition of rhFGF-9 or neutralizing Ab against FGF-9 at the doses indicated above. E: Expression of ALP mRNA was analyzed by RT-qPCR and normalized to β2M, using the formulae $2^{-(\Delta Ct)}$. Data are expressed as mean ± SEM ($^*P < 0.05$) and are representatives of one out of three experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

We then determined whether the capacity of hMSCs cultured with PC-3-C^{ed}M to decrease osteoclastogenesis might be involved in the increased OPG expression. We show that secretion of OPG by hMSCs was significantly increased at 12 h (Fig. 5F) and 24 h (data not shown) when they are cultured with PC-3-C^{ed}M, as compared to culture with C^{ed}M from control. In contrast, RANKL secretion by hMSCs was only slightly but not significantly increased following exposure to PC-3-C^{ed}M (Fig. 5G). As a consequence, the OPG:RANKL ratio was increased in hMSCs supernatant after exposure to PC-3-C^{ed}M. Altogether these data demonstrate that exposure of hMSCs to PC-3- C^{ed}M reduces their capacity to stimulate osteoclastogenesis.

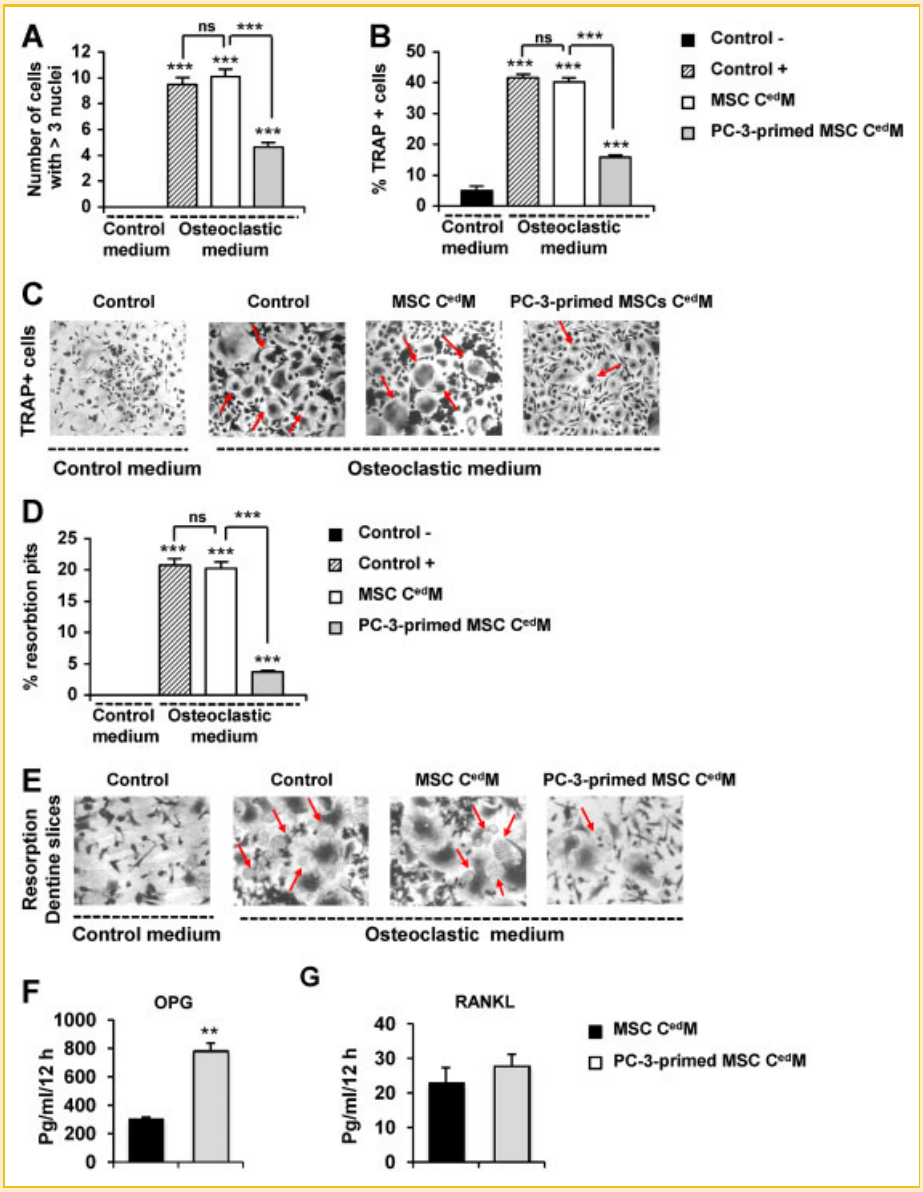

Fig. 5. Pre-treatment of hMSCs with PC-3-C^{ed}M decreases OPG expression by hMSCs and reduces their pro-osteoclastogenic capacity. A–E: Osteoclast differentiation assay using human peripheral blood CD14^{+} monocytes. The formation of mature osteoclastic cells was compared following culture of osteoclast progenitor cells in osteoclastic induction conditions with control medium or with supernatant from hMSCs pre-treated or not with PC-3-C^{ed}M during 24 h. In osteoclastic induction conditions, media contained recombinant human M-CSF (20 ng/ml) and RANKL (5ng/ml). A: Average number of cells containing more than three nuclei on day 7 (±SEM). B: Average % of TRAP positive cells (±SEM) on day 9. C: Photomicrographs of representative dentine slices showing TRAP positive osteoclasts (arrows) (40× magnification). D: The osteoclastic activity was measured on day 9 by counting the average number of pits per slice of dentine (±SEM) from a total of five slices. E: Photomicrographs of representative dentine slices showing pits formed by activated osteoclasts (arrows) (40× magnification). F–G: Secretion of OPG (F) and RANKL (G) were measured in hMSCs supernatants 12 h following priming with control medium or with PC-3-C^{ed}M. Data are expressed as mean ± SEM of ($^{*}P < 0.05$) and are representatives of one out of three experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

DISCUSSION

The findings described in the present study demonstrate that bone metastatic PC-3 cells could modulate bone remodeling by modifying the expression profile and the differentiation capacity of hMSCs. We show herein that bone metastatic PC-3 cells, stimulate the commitment of bone marrow hMSCs toward the osteoblastic lineage. This is consistent with our previous report that PC-3 carcinoma cells increase expression of osteogenic markers by murine primary MSCs or C3H10T1/2 MSC line in vitro [Fritz et al., 2008]. Moreover, we present evidence that PC-3 cells induce the expression of matrix proteins and associated integrin receptors by hMSCs, which may contribute to the formation of a cellular context that is favorable to osteogenesis.

MSC differentiation required specific factors, specific cell–cell contacts and cell–substratum interactions, including adhesion to the extracellular matrix (ECM). The integrin family of adhesion receptors has been shown to mediate interactions between cells and their ECM ligands through specific combinations of α and β subunits, triggering signal transduction that regulates the expression of differentiation genes [Adams and Watt, 1993; Damsky et al., 1992; Huhtala et al., 1995]. Various integrin receptors have been reported to be expressed by osteoblasts, including α2β1, α5β1, and αvβ3 [Hughes et al., 1993; Hultenby et al., 1993; Pistone et al., 1996; Schneider and Burridge, 1994], and prior studies with committed osteoblasts showed that integrin-mediated adhesion to ECM promote osteoblastic differentiation [El-Amin et al., 2002; El-Amin et al., 2003; Schneider et al., 2001] by activating the osteoblast-specific transcription factor Cbfa1 [Franceschi et al., 2003; Xiao et al., 2000] and by regulating BMP-2 activity [Lai and Cheng, 2005]. hMSCs also express several integrin receptors subunits including α1, α2, α3, αv, β1, β3, and β4 (reviewed in [Docheva et al., 2007; Pittenger et al., 1999]). Although ECM proteins and their integrin receptors are known to play a critical role in regulating the commitment of hMSCs toward osteogenesis [Mizuno and Kuboki, 2001; Salasznyk et al., 2004a, b; Weiss and Reddi, 1981], the signaling mechanisms underlying this effect remain unclear.

In the present study, we focused on the effects of PC cells on the gene expression profile and the differentiation capacity of hMSCs. It is nevertheless important to mention that the cross-talk between bone marrow stromal cells and PC cells during bone metastasis formation also triggers gene expression changes in PC cells, including uPA up-regulation, which tends to favor invasiveness and bone remodeling [Zhang et al., 2009]. In our study, we present evidence that soluble factors from bone metastatic PC up-regulate the expression of the matrix protein FN and its integrin receptor α5β1 in bone marrow hMSCs, conferring significant microenvironment advantage to undergo osteoblast differentiation. Supporting our observation, it has previously been reported that FN plays a role in the early stages of osteogenesis. Indeed, FN synthesis and deposition have been demonstrated in areas of bone tissue, such as the periosteum of rat calvaria [Gronowicz et al., 1991] and the osteoid surrounding implants [Weiss and Reddi, 1980; Weiss and Reddi, 1981], where recruitment and commitment of osteoblast precursor occur. The early stages of osteoblast differentiation are correlated with a higher expression of FN and its accumulation in the matrix [Stein et al., 1990; Winnard et al., 1995]. Moreover, several factors known to regulate osteoblast differentiation, such as PrTH, estrogens, and transforming growth factor beta (TGFβ) also affect osteoblast attachment to FN or FN expression [Breen et al., 1994; Eielson et al., 1994; Gronowicz and McCarthy, 1995]. The interaction between FN and its specific integrin receptor α5β1 was shown to be essential during the early phase of osteoblast differentiation, since disruption of this interaction following treatment with anti-FN or anti-α5 integrin Ab selectively inhibited expression of osteoblast makers ALP and OC as well as nodule formation in fetal calvarial osteoblasts [Moursi et al., 1996; Moursi et al., 1997]. Amplification of α5β1 expression in human osteoblast-like MG-63 cultures was associated with increased mineralization, although anti-α5 and anti-β1 antibodies blocked ALP induction [Dedhar et al., 1989]. Recently, osteoblast differentiation of hMSCs has been shown to require integrin α5 expression and its activation of focal adhesion kinase/ERK1/2-MAPKs and PI3K signaling pathways [Hamidouche et al., 2009]. Another important result is the demonstration that non-metastatic 22RV1 PC cells do not change the osteogenic capacity of hMSCs. This strengthens the fact that pro-osteoblastic activity is associated with the invasiveness of PC cells. It would be therefore interesting to further investigate the link between invasiveness, bone tropism, and pro-osteoblastic capacity of cancer cells by using primary prostate tissues and additional cancer cell lines normally associated with osteolytic vs osteoblastic phenotype.

The important finding of our study is the demonstration that soluble FGF-9 participates to PC-induced effects on hMSCs, in particular the commitment of MSCs toward osteoblasts. Indeed, PC cells are known to produce factors that may orientate bone lesions toward an osteoblastic or an osteolytic phenotype. We have previously shown in an experimental murine model that despite their osteolytic phenotype, PC-3 cells favor osteogenic potential of mouse MSCs in vitro [Fritz et al., 2008]. Our findings indicate that FGF-9 plays an important role in PC-3-induced transcriptional and functional changes in hMSCs, including commitment to osteoblast differentiation. Previous in vitro and in vivo studies have shown that FGFs, including FGF-9 have important regulatory functions in bone formation. Treatment of primary calvarial bone cell populations with FGF-9 has been shown to enhance their intrinsic osteogenic potential by increasing expression of differentiation markers such as OC or Cbfa1, and other osteogenic factors such as BMP-2 and TGF-β1, and to stimulate mineralization [Fakhry et al., 2005; Jackson et al., 2006; Noda and Vogel, 1989]. In another study, FGF-9 treatment of the murine preosteoblastic MC3T3-E1 cells increased Cbfa1 promoter activity [Yu et al., 2007]. Local application of FGF-9 to mouse calvarias led to suture obliteration and fusion in a serum-free organ culture model [Ignelzi et al., 2003]. FGF-9 might participate to intramembranous bone formation and endochondral ossification. Moreover, FGF-9 was able to induced new bone formation in an organ culture assay with new born mouse calvariae [Li et al., 2008]. Since PC-3 cells are known to express several osteogenic factors, such as BMP-2, -3 and -4 [Garofalo et al., 1999], and the BMP receptors Ia, Ib, and II [Hecht et al., 1995], but also IGF-I which has been shown to up-regulate markers of osteoblastic differentiation in hMSCs [Koch et al., 2005], it is likely that FGF-9 might act in cooperation with these other factors to contribute to PC-3 induced osteogenic differentiation of hMSCs.

Important for our study, FGF-9 expression has been found to increase with the severity in clinical stages of PC, suggesting a role in the progression of PC to a metastatic phenotype [Li et al., 2008]. This is supported by the observation that anti-FGF-9 treatment significantly reduced intrafemoral tumor growth and associated new bone formation in an experimental murine model [Li et al., 2008]. In this study, bone metastase-derived cell lines PC-3 and PC-118 were shown to express high levels of FGF-9 protein, which was shown to mediate PC-induced osteogenesis. Interestingly, it was previously demonstrated in prostate tumor that the source of FGF-9 is switched from the stroma to the epithelial cancer cells [Giri et al.,

1999; Kwabi-Addo et al., 2004; Li et al., 2008], and FGF-9 expressed by cancer cells was shown to exhibit paracrine activity through binding to FGF receptor-expressing stromal cells [Jin et al., 2004]. Together with our findings, it supports a role for FGF-9 in the cross-talk between epithelial PC cells and mesenchymal stromal cells and the disruption of bone homeostasis during malignancy.

We and others have previously shown that PC cells increase bone remodeling through a direct stimulation of osteoclast activity [Fritz et al., 2007]. Osteoclast formation is tightly regulated by the ratio between RANKL and OPG, which are respectively osteoclast-inducing and -repressing factors. Expression of both factors by hMSCs is tightly regulated by various local and systemic signals. Herein, we give evidence that bone metastatic PC cells also exert an indirect regulation of osteoclastogenesis by increasing the expression of OPG by hMSCs. In another study, OPG-overexpressing MSCs were shown to prevent PC-3 cells-induced osteolysis in mice, likely du to inhibition of osteoclastogenesis [Chanda et al., 2009]. Moreover, OPG has also been reported to act as a survival factor in prostate cancer cells, by its capacity to conteract TRAIL-induced apoptosis [Holen et al., 2002] and bone marrow stromal cells were previously shown to produce OPG in the context of PC cells interaction [Cross et al., 2007]. Further investigations are needed to determine by which mechanism PC cells modulate OPG expression in hMSCs. However, hMSCs treated with $C^{ed}M$ from PC-3 cells were less potent to stimulate the formation of mature osteoclasts in vitro. Therefore, we propose a model in which PC cells might have a dual effect described in two phases. First, following their metastasis to bone, PC cells directly stimulate osteoclast activity and associated matrix resorption, an essential step for their implantation and survival in this new microenvironment. During the second phase, PC cells acquire pro-osteoblastic properties and act to favor the formation of new bone. Through the secretion of soluble factors, such as FGF-9, PC cells enhance the osteoblastic differentiation of hMSCs and modulate the expression by hMSCs of osteoclastogenic-regulating factors.

CONCLUSION

In conclusion, our study demonstrates that bone metastatic PC-3 cells can modulate bone remodeling by both stimulating the commitment of hMSCs toward the osteoblastic lineage and modulating the expression of osteoclastogenesis-regulating factors by hMSCs. We propose new clues to the understanding of PC bone metastasis process, with the identification of FGF-9 as a factor mediating the interactions between PC cells and hMSCs.

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