ARTICLE
Journal of Cellular Biochemistry 114:1135–1144 (2013)

Journal of Cellular Biochemistry

Gefitinib Inhibits the Cross-Talk Between Mesenchymal Stem Cells and Prostate Cancer Cells Leading to Tumor Cell Proliferation and Inhibition of Docetaxel Activity

Cinzia Borghese,[1] Lara Cattaruzza,[1] Eliana Pivetta,[1] Nicola Normanno,[2] Antonella De Luca,[2] Mario Mazzucato,[3] Marta Celegato,[1] Alfonso Colombatti,[1,4,5] and Donatella Aldinucci[1]*

[1] *Experimental Oncology 2, C.R.O. National Cancer Institute-IRCCS, Aviano, Italy*
[2] *Cell Biology and Biotherapy Unit, INT-Fondazione Pascale, Naples, Italy*
[3] *Stem Cells Collection and Processing Unit, C.R.O. National Cancer Institute-IRCCS, Aviano, Italy*
[4] *Department of Medical and Biomedical Science, University of Udine, Udine, Italy*
[5] *MATI (Microgravity Ageing Training Immobility) Excellence Center, University of Udine, Udine, Italy*

ABSTRACT

Increasing evidence suggests that bone marrow derived mesenchymal stem cells (BM-MSCs) are recruited into the stroma of developing tumors where they contribute to progression by enhancing tumor growth and metastasis, or by inducing anticancer-drug resistance. Prostate cancer cells secrete ligands of epidermal growth factor receptor (EGFR) and EGFR signaling could play an important role in the cross-talk between mesenchymal stem cells and prostate cancer cells. In this study, we showed that treatment of human primary MSCs with conditioned medium (CM) derived from the bone metastatic PC3 carcinoma cells (PC3-CM) resulted in: a significant activation of EGFR; increased proliferation; increased osteoblastic but decreased adipocitic differentiation; inhibition of senescence induced by serum starvation; increased CCL5 secretion. These activities were significantly inhibited in the presence of the EGFR tyrosine kinase inhibitor gefitinib. PC3-CM directly inhibited osteoclastogenesis as well as the ability of osteoblasts to induce osteoclast differentiation. The increased MSCs migration by PC3-CM and PC3 cells was partially mediated by CCL5. MSC-CM increased the formation of colonies by PC3 cells and inhibited the anti-proliferative activity of Docetaxel. Activation of EGFR expressed on MSCs by PC3-CM enhanced their capability to increase PC3 cells proliferation and to inhibit Docetaxel activity. These findings, by showing that the tumor-promoting interactions between PC3 cells and MSCs are mediated, at least in part, by EGFR, suggest a novel application of the EGFR-tyrosine kinase inhibitors in the treatment of prostate cancer. J. Cell. Biochem. 114: 1135–1144, 2013.

KEY WORDS: PROSTATE CANCER; MESENCHYMAL STEM CELLS; EGFR; GEFITINIB; TUMOR MICROENVIRONMENT; DOCETAXEL; CCL5

Prostate cancer is no longer viewed as a disease of abnormally proliferating epithelial cells but rather as a disease of complex interactions between prostate cancer epithelial cells (epithelial compartment) and the surrounding tissues (stromal compartment) in which they reside [Jin et al., 2011]. Multiple signaling pathways provide cross-talk between the epithelial and the stromal compartments to enhance tumor growth, including androgen receptor signaling, tyrosine kinase receptor signaling, and immune

Abbreviation used: AM, adipogenic medium; BrdU, bromodeoxyuridine; BM, bone marrow; CCL5/Rantes, regulated on activation normal T-cell expressed and secreted; CM, conditioned medium; EGF, epidermal growth factor; ELISA, enzyme-linked immunoadsorbent assay; IgG, immunoglobulins; IL, interleukin; mAb, monoclonal antibody; MSC, mesenchymal stem cells; OBs, osteoblasts; OCs, osteoclasts; OM, osteogenic medium; PCa, prostate cancer; R, receptor; RANKL, receptor activator of nuclear factor kappa B ligand; M-CSF, macrophage colony-stimulating factor; SF, serum free; TGF-alpha, Transforming growth factor-alpha; TRAcP, tartrate-resistant acid phosphatase.

No potential conflicts of interest to disclose.

Grant sponsor: Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.); Grant sponsor: Ministero della Salute, Ricerca Finalizzata FSN, I.R.C.C.S., Rome, Italy.

*Correspondence to: Donatella Aldinucci, Experimental Oncology 2, Centro di Riferimento Oncologico, Via F. Gallini 2, Aviano I-33081, Italy. E-mail: daldinucci@cro.it

Manuscript Received: 2 March 2012; Manuscript Accepted: 5 November 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 28 November 2012

DOI 10.1002/jcb.24456 • © 2012 Wiley Periodicals, Inc.

surveillance [Karlou et al., 2010]. To disrupt this "two-compartments" cross-talk, drugs that target tumor stromal elements in addition to the neoplastic epithelial cell were developed [Guise, 2010; Karlou et al., 2010; Dayyani et al., 2011; Jin et al., 2011].

Mesenchymal stem cells (MSCs) constitute a self-renewing population of multipotent cells present in bone marrow (BM) and in many other adult tissues. MSCs represent a reservoir of reparative cells that migrate toward sites of active tumorigenesis, integrate into the specialized tumor niche and contribute to the development of cancer-associated-fibroblasts (CAF) [Bergfeld and DeClerck, 2010; Kuhn and Tuan, 2010; Cirri and Chiarugi, 2011]. Within the BM microenvironment, MSCs favor the formation of BM metastasis by inducing tumor cell proliferation and protect cancer cells against chemotherapeutic agents [Guise, 2010; McMillin et al., 2010; Salem and Thiemermann, 2010; Suva et al., 2011]. Therefore, the elucidation of the cellular and molecular mechanisms involved in the interactions between prostate cancer (PCa) cells and MSCs can provide new targets and new therapeutic strategies for therapies to control PCa progression [Karlou et al., 2010]. Because of their tumor-tropic migratory responses, MSCs are emerging as promising anti-cancer agents for delivering effective targeted therapy to tumors [Shah, 2011]. Using an orthotopic C4-2B xenograft model system studies by Placencio et al. [2010] revealed that MSCs incorporated into regrowing prostate, could contribute to PCa progression by enhancing Wnt signaling and suggested their use as a vehicle for transporting genetic information with potential therapeutic effects on castration-resistant prostate cancer.

The EGFR belongs to the human epidermal receptor (HER) axis, a dynamic and interconnected family of receptors forming complexes which activate downstream signaling events critical for cell growth and proliferation [Normanno et al., 2006]. The EGFR is expressed in almost all types of non-transformed cells, including MSCs and their progeny, and is frequently over-expressed in carcinoma cells [Normanno et al., 2006]. Several EGFR ligands are secreted by human primary PCa and by prostate cancer cell lines, including PC3 cells [Gullick, 2009]. In PCa cells, suppression of EGFR signaling by the tyrosine kinase inhibitor gefitinib reduces the incidence of metastasis in nude mice [Angelucci et al., 2006]. In addition, we previously demonstrated that factors released under the control of EGFR signaling modulate bone remodeling through the induction of RANKL in bone marrow stromal cells [Normanno et al., 2005; Normanno and Gullick, 2006]. EGFR signaling also regulates the ability of MSCs to release growth factors that promote neo-angiogenesis and tumor cell migration [De Luca et al., 2011].

The purpose of our study was to determine if the inhibition of EGFR signaling could affect the interaction between MSCs and PCa cells, and to assess whether this phenomenon might represent a novel target for therapeutic intervention in PCa.

MATERIALS AND METHODS

CELL LINES AND CULTURE CONDITIONS

The human PCa-derived PC3 and DU145 cell lines were purchased from an authenticated source (German collection of Micro organisms and cell cultures, Braunschweig, Germany). The cells were maintained in RPMI medium (Cambrex Bio Science, Milano, Italy) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Italy), 0.2 mg/ml penicillin/streptomycin and 0.1% (w/v) L-glutamine (Cambrex) at 37°C in a 5% CO_2 fully humidified atmosphere. Cell lines used in the study were further authenticated for their origin by BMR Genomics (Padova, Italy). BM derived human MSCs were purchased from Lonza (Lonza, Verviers, Belgium) and maintained in MSGM bullet kit (Lonza). These cells are positive for CD29, CD44, CD105, and CD166 and negative for markers of the hematopoietic lineage, such as CD14, CD34, and CD45.

PREPARATION OF CONDITIONED MEDIA AND ELISA ASSAY

PC3 cells were incubated in complete medium. After 5 days, conditioned medium (CM) was collected, filtered and stored in aliquots at −20°C. MSC layers were cultured in medium alone, with PC3-CM (10%, v/v), PC3-CM (10%, v/v)/gefitinib (ZD1839, Iressa, AstraZeneca, Macclesfield, UK), or gefitinib (5 μM) alone. After 10 days supernatants were collected, filtered, aliquoted and stored at −20°C. IL-6 and CCL5/Rantes, EGF and TGF-alpha were assessed with the Quantikine Human Immunoassay kits (R&D, Minneapolis, MN), following the manufacturer's instructions.

EGFR PHOSPHORYLATION

After a 12 h pre-incubation in serum free (SF) medium, MSCs were cultured for 10 min with 50 ng/ml of recombinant EGF (PeproTech, London, UK) or PC3-CM (20%, v/v) in the absence or in the presence of 5 μM gefitinib. The concentration of phosphorylated EGFR was measured by using the Active EGFR EIA kit (Takara, Biomedicals, Tokio, Japan) following the manufacturer's instructions and the concentration was expressed as the amount of phosphorylated EGFR (fmol/ml)/3×10^4 cells.

PROLIFERATION AND COLONY ASSAYS

MSCs (2.5×10^3) were cultured in complete medium in 96-well flat-bottomed plates and allowed to attach for 24 h. Cells were then exposed to increasing amount of PC3-CM that was replaced twice. After a total of 9 days of culture, cell proliferation was evaluated by BrdU assay (Roche Diagnostics, Boehringer-Mannheim, Germany), according to manufacturer's instructions. A similar experiment was performed with OB-differentiated MSCs. MSCs (2.5×10^3) were cultured in complete medium in 96-well flat-bottomed plates and allowed to differentiate into OBs for 14 days. OBs were then cultured in the presence of PC3-CM as described above. In another series of experiments, MSCs and OBs-differentiated MSCs (2.5×10^3) were exposed to increasing concentrations of gefitinib (0.6–20 μM) in the presence of a fixed amount of PC3-CM (20%, v/v). To evaluate PC3 cells capacity for growth at clonal density, 1.0×10^2 cells were cultured in 24-well plates in the presence of MSC-CM (10%, v/v) that was replaced every 5 days. After 10 days, plates were observed under phase-contrast microscopy and aggregates with ≥40 cells were scored as colonies. Senescence studies were performed incubating MSCs in RPMI medium without FBS (serum free medium) for 9 days in the presence or in the absence of PC3-CM (20%, v/v), replaced every 3 days, and gefitinib (2 μM). Senescent cells, positive for β-galactosidase activity, were evaluated with the Senescent cells staining kit (Sigma–Aldrich, Milan, Italy) as described [Itahana et al., 2007]. The percentage of senescent cells (number of blue cells/total

number of cells counted) was calculated from three randomly selected visual fields.

DOCETAXEL ACTIVITY

PC3 cells (2.5×10^3) were cultured in complete medium in the presence or in the absence of Docetaxel (Aventis Pharma, Dagenham, UK; 5 nM). After 24 h cells were washed and cultured in the presence or in the absence of increasing amounts of MSC-CM. In another series of experiment PC3 cells were cultured in the presence of increasing amount of Docetaxel (0.6–10 nM). After 24 h cells were washed and cultured in the presence or in the absence of complete medium (10%, v/v) derived from MSCs cultured in the presence or in the absence of PC3-CM and/or gefitinib. After 72 h, proliferation was evaluated by bromodeoxyuridine (BrdU) incorporation.

MSC DIFFERENTIATION

MSCs (1×10^4) were cultured in complete medium in 24-well plates and allowed to attach. At about 80% cell confluence, the culture medium was replaced by osteogenic medium (OM; 50 nM ascorbic acid, 10 mM β-glycerol phosphate, and 100 nM dexamethasone; Sigma–Aldrich).

Osteogenic differentiation was assessed by alkaline phosphatase staining (Sigma–Aldrich). Adipocyte differentiation, obtained with adipogenic medium (AM; 10 ng/ml insulin, 1 μM dexamethasone, 60 nM indomethacin, and 0.5 mM isobutylmethyl-xanthyne; Sigma–Aldrich), was revealed with the Oil Red-O staining (Sigma–Aldrich). For the period of osteogenic or adipogenic differentiation, MSCs were cultured in the presence or in the absence of PC3-CM (10%) and/or gefitinib (5 μM).

OSTEOCLASTOGENIC ASSAY

OCs were obtained as previously described [Pivetta et al., 2011]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat preparations obtained from healthy donors. Buffy coats were diluted in PBS (1:3) and overlaid on Ficoll-Paque (Amersham, GE Healthcare, Milan, Italy), and centrifuged at 800*g* for 20 min. The interface layers were collected and washed in PBS. PBMCs were resuspended in appropriate volume of RPMI. Cells were seeded at optimal density, incubated for 2 h at 37°C and then washed three times with PBS to remove non-adherent cells. Cultures were grown in RPMI supplemented with 10% heated-inactivated FCS, penicillin (100 IU/ml), streptomycin (100 μg/ml), human M-CSF (30 ng/ml), and human RANKL (40 ng/ml; differentiating agents), and maintained at 37°C in a humidified atmosphere of 5% CO_2 (control cells). The cells were fed every 3 days with fresh medium and differentiating factors. In addition, cells in complete medium plus differentiating factors were cultured with PC3-CM (5%, v/v) or OBs-CM (5%, v/v). After 14 days of culture cells were used for TRAcP staining. To obtain OBs, MSCs were cultured for 14 days in osteogenic medium. OBs-CM were obtained by culturing OBs in the presence or in the absence of PC3-CM (10%, v/v) for 72 h. Then OBs were cultured for additional 24 h in SF medium. For these experiments PC3-CM was obtained by culturing cells for 36 h in complete medium.

TRAcP STAINING

To quantify the formation of tartrate-resistant acid phosphatase-positive (TRAcP) multinucleated cells, cell cultures were stained for TRAcP using Leukocyte Acid Phosphatase Kit (Sigma Diagnostics), according to manufacturer's instruction. Cells positive for TRAcP and having more than three nuclei were considered as TRAcP-positive multinucleated OCs.

CELL MOTILITY ASSAY

Migration was assessed by FATIMA assay as previously described [Spessotto et al., 2009]. Briefly, cells (MSCs: 50,000 cells/insert), tagged with the lipophylic dye Fast DiI (Molecular Probes), were seeded in 150 μl SF medium in the upper side of collagen type I-coated Boyden chamber inserts. Migration was then monitored at different time intervals for 20 h, using a computer-interfaced GeniusPlus microplate reader. FATIMA software determined the percentage of transmigrated cells out of the total amount introduced into the system. Complete medium from PC3 or DU145 supernatants (50%, v/v), PC3 or DU145 cell layers, were used as chemoattractants. Each experiment was performed at least three times, in duplicate. Control experiments were performed in the presence of a neutralizing anti-human-CCL5 monoclonal antibody (mAb) (R&D Systems) or isotype control mAb (mouse IgG1) added to the supernatants at a concentration of 4 μg/ml.

STATISTICS

All values are given as means ± SD of not less than three measurements (unless otherwise stated). Statistical comparisons were drawn using Student's *t*-test. Differences were considered significant where $P < 0.05$.

RESULTS

PC3 SUPERNATANT INDUCED EGFR PHOSPHORYLATION IN MSCs

It has been previously demonstrated that PCa cells, including PC3 cells, secrete EGFR ligands, such as TGF-alpha and EGF [Torring et al., 2000; Krampera et al., 2005; DeHaan et al., 2009]. We confirmed that PC3 cells secrete high levels of TGF-alpha (approximately 32.26 pg/mg protein) but lower amounts of EGF, as evaluated by ELISA assay (data not shown). To determine whether PC3-supernatants were able to induce EGFR phosporylation in MSCs, we treated MSCs with PC3-CM (20%, v/v) or with EGF (50 ng/ml; positive control), in the presence or in the absence of gefitinib. MSCs treatment with either EGF or PC3-CM determined a significant increase in the levels of phosphorylated-EGFR that was completely abolished by simultaneous treatment with gefitinib (Fig. 1). Under these experimental conditions, treatment with gefitinib alone was unable to significantly affect EGFR phosphorylation basal levels.

GEFITINIB INHIBITED MSC PROLIFERATION INDUCED BY PC3-CM IN VITRO

PC3 conditioned medium promotes proliferation and osteogenic differentiation of human BM-MSCs [Fritz et al., 2011; Tang et al., 2011]. We confirmed that PC3-CM increased MSC proliferation, in a dose dependent manner (Fig. 2A). To demonstrate that this was due

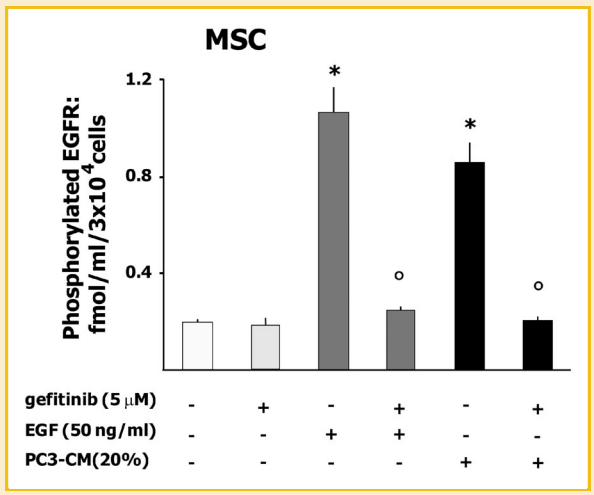

Fig. 1. Effect of PC3-CM on MSCs expression of phosphorylated EGFR. MSCs were cultured with EGF (positive control) or PC3-CM in the absence or in the presence of gefitinib. EGFR phosphorylation levels, determined by a specific ELISA assay, were expressed as the amount of phosphorylated EGFR (fmol/ml)/ 3×10^4 cells. Results represent the mean ± SD of three different experiments in triplicate. $^{*}P < 0.05$, EGF or PC3-CM treated cells versus untreated cells, $^{\circ}P < 0.05$, EGF or PC3-CM treated cells versus EGF or PC3-CM and gefitinib treated cells.

to EGFR activation, we cultured MSCs in a fixed amount of PC3-CM (20%, v/v) in the presence or in the absence of increasing concentrations of gefitinib. PC3-CM increased MSC proliferation that was inhibited in a dose-dependent manner by simultaneous treatment with gefitinib (Fig. 2B).

β-Galactosidase activity at pH 6 is a marker of cell senescence and cultivation in SF medium induced senescence in MSCs (Fig. 2C). Treatment with PC3-CM decreased the mean number of senescent cells from 81 ± 5% (SF medium) to 42 ± 3% (+PC3-CM) and this effect was reverted by simultaneous treatment with gefitinib (79%; Fig. 2C). Gefitinib used alone was fully ineffective on this phenomenon.

These data demonstrate that EGFR activation by PC3-CM increases proliferation and reduces senescence by serum starvation in MSCs.

GEFITINIB INHIBITED OB PROLIFERATION INDUCED BY PC3-CM IN VITRO

Then we evaluated the activity of PC3-CM on MSCs differentiation in the presence or in the absence of gefitinib. Treatment of MSCs with PC3-CM, in the presence of OM or AM, increased the cell number that was strongly reduced by the simultaneous presence of gefitinib (Fig. 3A). The effect of PC3-CM was also evaluated on differentiated OBs, obtained by MSCs cultured for 14 days in OM (the extent of differentiation was quantified by ALP staining). PC3-CM increased OBs proliferation, in a dose-dependent manner (Fig. 3A) and PC3-CM (20%, v/v) activity was inhibited in a dose-dependent manner by simultaneous treatment with gefitinib (Fig. 3C), demonstrating that the increased proliferation was due, at least in part to EGFR activation. Consistent with the finding by Fritz

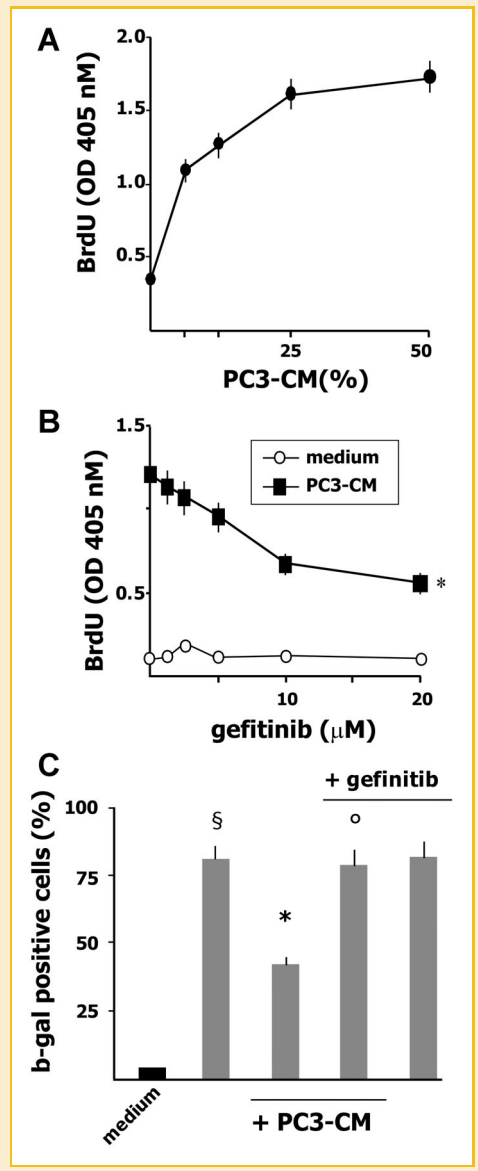

Fig. 2. Effect of gefitinib and PC3-CM on MSC proliferation and senescence. A: MSCs were cultured in the presence of increasing amounts of PC3-CM that was replaced twice B: or in the presence of a fixed amount of PC3-CM (20%, v/v) in the presence or in the absence of increasing amounts of gefitinib. After 9 days, proliferation was evaluated by BrdU assay. Results represent the mean ± SD of three different experiments in triplicate. Data are expressed as percentage of absorbance unit of the respective control. $^{*}P < 0.05$ PC3-CM treated cells versus PC3-CM treated plus gefitinib. C: MSCs were cultured in control medium (plus FBS; black histograms) or in SF medium (gray histograms) for 9 days in the presence or in the absence of PC3-CM (20%, v/v), and gefitinib (2 μM), that was replaced twice. The percentage of senescent β-gal-positive cells (number of blue cells/total number of cells counted) was calculated from three randomly selected fields. Results represent the mean ± SD of three different experiments. $^{\S}P < 0.05$ SF cultured cells versus control medium $^{*}P < 0.05$ PC3-CM treated cells versus untreated $^{\circ}P < 0.05$, PC3-CM treated plus gefitinib versus PC3-CM treated cells.

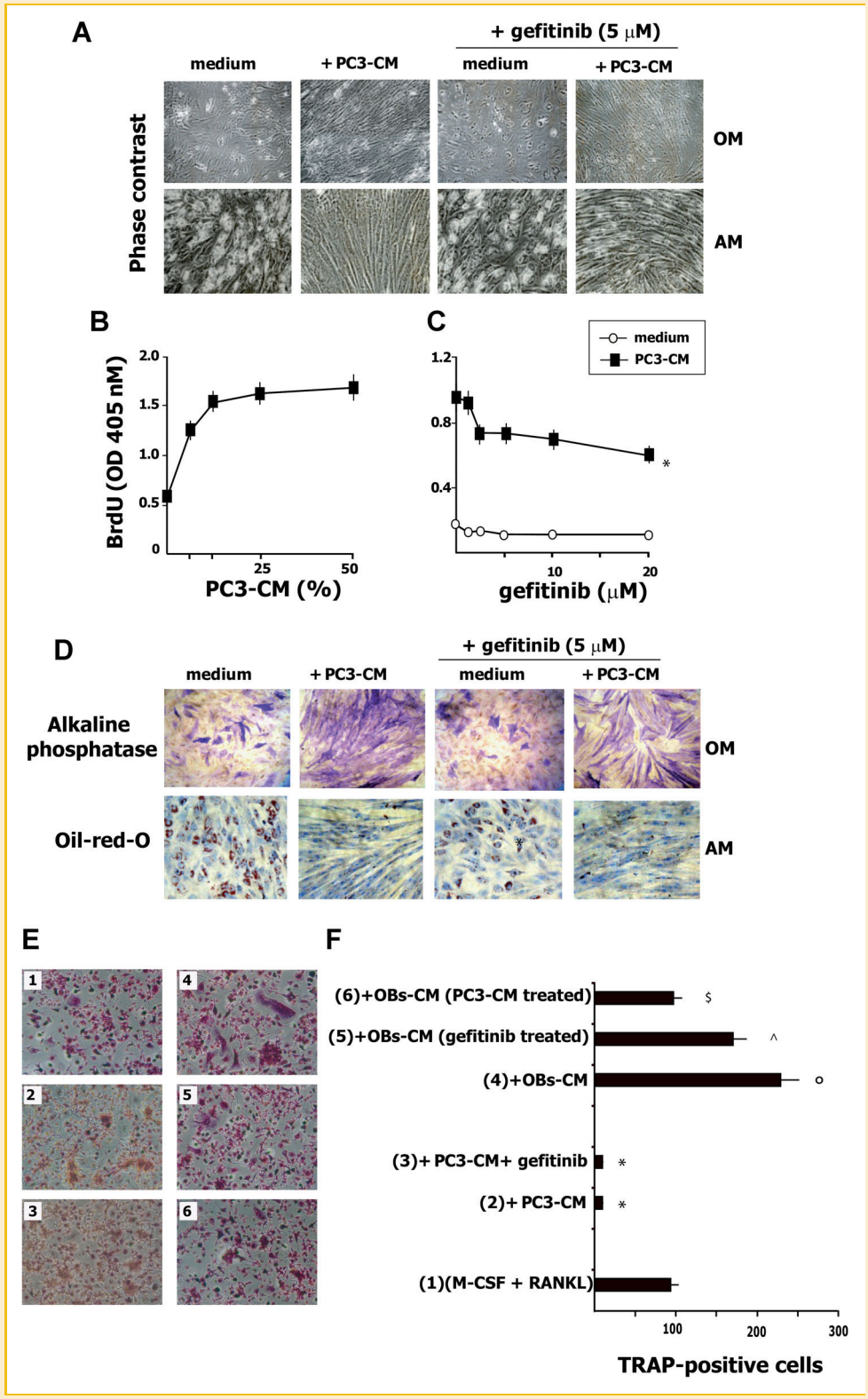

Fig. 3. Effect of gefitinib and PC3-CM on MSC differentiation, OB proliferation and OC differentiation. A: MSCs were cultured with osteogenic (upper panel) or adipogenic (lower panel) medium alone or in the presence of PC3-CM (10%, v/v), PC3-CM plus gefitinib (5 μM), or gefitinib alone. Phase contrast microphotographs showing cell morphology (original magnification, 100×). B: OBs were cultured in the presence of increased amounts of PC3-CM that was replaced twice. C: or in the presence of a fixed amount of PC3-CM (20%, v/v) in the presence or in the absence of increasing amounts of gefitinib. After 9 days, proliferation was evaluated by BrdU assay. Results represent the mean ± SD of three different experiments in triplicate. Data are expressed as percentage of absorbance unit of the respective control. $^{*}P < 0.05$ PC3-CM treated cells versus PC3-CM treated plus gefitinib. D: Osteogenic differentiation was evaluated by ALP staining (upper panel) and adipogenic differentiation with Oil-Red-O staining (lower panel; original magnification, 100×). E: Effect of PC3-CM on OC differentiation. Pre-OC were incubated for 14 days with RANKL+ M-CSF in the absence (1) or in the presence of supernatants from PC3-CM (5%, v/v) (2) in the absence or in the presence (3) of gefitinib (4 μM) or with OBs-CM (5%, v/v) (4) and OBs-CM from cells pretreated with gefitinib (5), or PC3-CM (6). Cells were stained for TRAcP (original magnification 10×). F: Number of multinucleated TRAP-positive cells. $^{*}P < 0.05$ PC3-CM treated (with or without gefitinib) versus untreated cells $^{\circ}P < 0.05$, OBs-CM treated versus untreated cells, $^{\wedge}$gefitinib treated OBs-CM versus OBs-CM treated cells $^{\S}P < 0.05$, PC3-CM treated OBs-CM versus OBs-CM treated cells.

et al. [2011], PC3-CM increased osteoblastic differentiation, as demonstrated by ALP staining (Fig. 3D) and ALP enzymatic activity (data not shown); this effect was strongly reduced by simultaneous treatment with gefitinib (Fig. 3D, upper panel). On the contrary, adipocytic differentiation (Fig. 3D, lower panel) was inhibited by PC3-CM and this effect was not reverted by simultaneous treatment with gefitinib.

PC3-CM INHIBITED OSTEOCLASTOGENESIS

We then evaluated the possibility of direct effect of PC3-CM on OC differentiation and on the ability of OBs to induce OC differentiation. PC3-CM remarkably inhibited osteoclastogenesis induced by RANKL/M-CSF (Fig. 3E,F). This phenomenon was not affected by gefitinib treatment. CM from OBs significantly induced OC differentiation, as expected (Fig. 3E,F). However, when OBs were treated with gefitinib a significant reduction in the ability of OBs to sustain osteoclastogenesis was detected (Fig. 3E,F). Exposure of OBs to PC3-CM produced a further reduction in the OBs-induced OCs differentiation (Fig. 3E,F). Taken together, these experiments suggest that: (1) PC3 cells are able to secrete factors that directly inhibit OC differentiation; (2) the ability of OBs to induce OC differentiation is in part related to activation of the EGFR; and (3) PC3 cells secrete factors that reduce the ability of OBs to induce OC differentiation.

GEFITINIB INHIBITED CCL5 SECRETION BY MSCs

The cytokine IL-6 and the chemokine CCL5 are two molecules involved in prostate cancer progression, chemo-resistance, androgen independence, and bone remodeling [Lu et al., 2004; Vaday et al., 2006; Levina et al., 2008; Colombatti et al., 2009; Morrissey et al., 2010; Santer et al., 2010]. Treatment of MSCs with PC3-CM (10%, v/v) significantly increased the secretion of CCL5 (3.0-fold compared to untreated cells; Fig. 4) and IL-6 (1.4-fold; Fig. 4). Addition of gefitinib decreased the basal levels of secreted CCL5 (to 65% respect to untreated cells) but not of IL-6 (Fig. 4). The simultaneous treatment with PC3-CM and gefitinib reduced the secretion of CCL5 by approximately 30% but was ineffective on IL-6 (Fig. 4). Our results suggest that activation of EGFR by PC3-CM regulates only the secretion of CCL5.

CCL5 SECRETED BY PC3 CELLS INDUCED SPECIFIC CHEMOTACTIC RESPONSE BY MSCs

Both PC3-CM and PC3 cells increased MSC migration in a similar manner after 5 h (approximately 2.70-fold compared to control medium) or after 20 h of incubation (3.0-fold increase; Fig. 5A). Similar results were obtained when complete medium or a confluent layer of DU145 PCa cell line were used as chemoattractants (data not shown).

Since PC3 cells secreted significant amounts of CCL5 (153.84 pg/mg of protein) we evaluated if CCL5 secreted by PC3 cells was able to sustain the migration of MSCs [Ponte et al., 2007]. For this purpose, a migration assay in which MSCs were induced to migrate in response to PC3-CM in the presence of neutralizing anti-CCL5 antibodies was performed [Aldinucci et al., 2008; Gallo et al., 2011]. The ability of PC3-CM to induce MSC migration was significantly inhibited by addition of an anti-CCL5 antibody (Fig. 5B). Anti-CCL5 Ab used alone only slightly decreased MSC migration. Isotype control antibodies never affected MSC migration. Our data demonstrate that MSC migration is due, at least in part, to CCL5 secreted by PC3 cells.

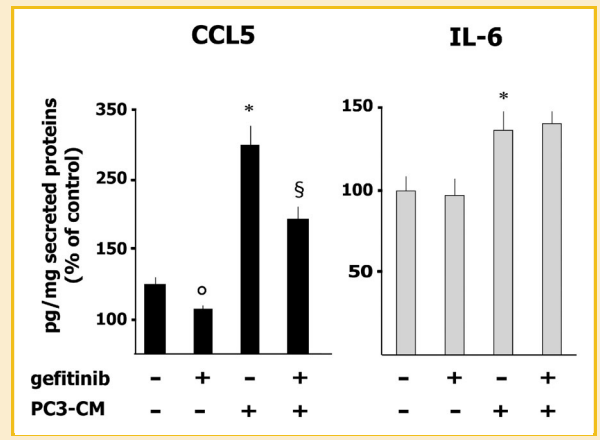

Fig. 4. Effect of gefitinib and PC3-CM on MSC secretion of CCL5 and IL-6. MSCs were cultured in the presence of PC3-CM (10%, v/v), or PC3-CM plus gefitinib (5 μM), or gefitinib alone. After 10 days, supernatants were analyzed for IL-6 and CCL5 concentration using specific ELISA assays. All samples were run in duplicate; supernatants from three different experiments were evaluated. $^{*}P < 0.05$ PC3-CM treated cells versus untreated. $^{\circ}P < 0.05$, gefitinib treated cells versus untreated, $^{\S}P < 0.05$, PC3-CM plus gefitinib treated cells versus PC3-CM treated.

MSC-CM INCREASED PROLIFERATION AND INHIBITED DRUG ACTIVITY ON PC3 CELLS

MSCs in the tumor microenvironment favor tumor cell growth, offering also protection from chemotherapeutic agents [Meads et al.,

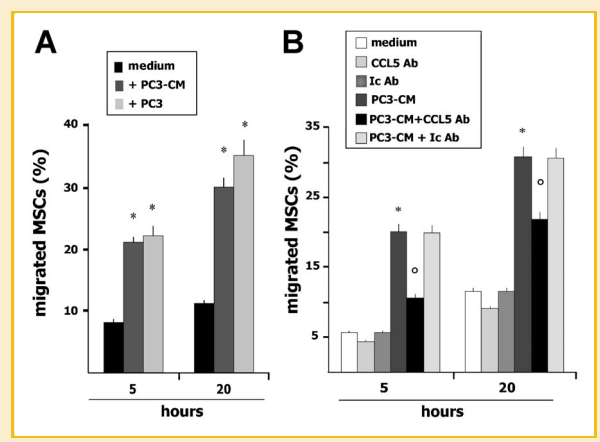

Fig. 5. Effect of PC3-CM on MSCs migration. A: Migration of MSCs through a collagen type I-coated Boyden chamber in response to PC3-CM or PC3 cells $^{*}P < 0.05$, PC3-CM or PC-3 cells layers versus medium alone. B: Inhibition of PC3-CM induced migration of MSCs following treatment with neutralizing anti-CCL5 antibodies (CCL5 Ab) or isotype control (IC) antibodies. $^{*}P < 0.05$ PC3-CM versus medium alone. $^{\circ}P < 0.05$, PC3-CM versus PC3-CM plus anti-CCL5.

2009; Roodhart et al., 2011]. We investigated whether the PC3/MSC cross-talk mediated by the EGF ligand/receptor system could affect PC3 cells proliferation and inhibit Docetaxel activity. For this purpose, we used supernatants derived from: MSCs cultured alone, or in presence of PC3-CM and/or gefitinib. First, we evaluated the effects of MSC-CM on PC3 cells colony formation. As shown in Figure 6A, supernatants from MSCs cultured alone or with gefitinib increased PC3 cells colony number by 3.4-fold. Supernatants from MSCs treated with PC3-CM showed the highest ability to improve PC3 cells clonogenic growth, suggesting that factors secreted by PC3 cells mediate in MSCs the secretion of molecules that promote PCa cell growth. Interestingly, this phenomenon was significantly reduced by treatment of MSCs with PC3-CM in presence of gefitinib. Then, we assessed the activity of Docetaxel on PC3 cell growth [Chin et al., 2010] in the presence or in the absence of MSC-CM. The MSC-CM decreased in a dose-dependent manner the activity of Docetaxel, used at a concentration capable to reduce PC3 cell proliferation to about 50% of control (5 nM; Fig. 6B). Finally, Docetaxel activity was evaluated in the presence of a fixed amount of supernatants from MSCs cultured alone or with gefitinib and/or PC3-CM (Fig. 6C). Docetaxel inhibited in a dose dependent manner PC3 cell proliferation (Fig. 6C). Supernatants from MSCs cultured alone (black circle) or with gefitinib (white circle) significantly decreased the antiproliferative activity of Docetaxel. Complete medium from MSCs pre-treated with PC3-CM (black square) was the most active and almost totally reduced Docetaxel activity. Supernatants from MSCs treated simultaneously with PC3-CM and gefitinib (white square) resulted as active as medium derived from MSCs cultured alone (black circle).

Our results demonstrate that PC3-CM activated the EGFR on MSCs and increased their capability to secrete molecules involved in PC3 cell proliferation and in the inhibition of Docetaxel activity.

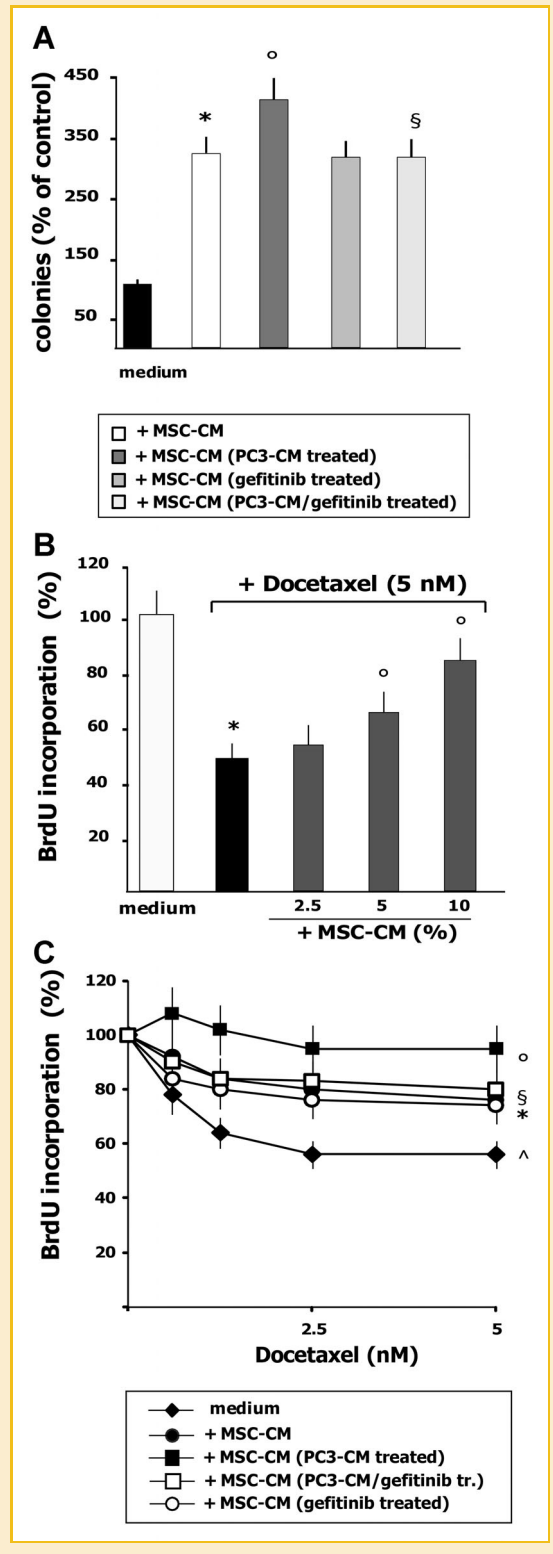

Fig. 6. Effects of MSC-CM on PC3 cells clonogenic growth and Docetaxel activity. A: 1.0×10^2 PC3 cells were cultured in control medium, or in the presence of supernatants derived from MSC-CM, MSCs pre-treated with PC3-CM (10%, v/v), PC3-CM plus gefitinib (5 μM), or gefitinib alone. MSC-CM was replaced every 5 days. After 10 days incubation, plates were observed under phase-contrast microscopy and aggregates with ≥40 cells, were scored as colonies. Results represent the mean ± SD of three replicate wells in triplicate and are expressed as percentage of colony number respect to control (medium alone). $^{*}P < 0.05$ MSC-CM treated cells versus untreated. $^{\circ}P < 0.05$, MSC-CM (PC3-CM treated) treated cells versus MSC-CM treated $^{\S}P < 0.05$, MSC-CM (PC3-CM/gefitinib pre-treated) treated cells versus MSC-CM (PC3-CM pre-treated) treated. B: PC3 cells (2.5×10^3) were cultured in the presence or in the absence of Docetaxel (5 nM) for 24 h. Then cells were washed and cultured in the presence or in the absence of increasing amounts of MSC-CM. After 3 days, proliferation was evaluated by BrdU assay. Results represent the mean ± SD of three different experiments in triplicate. Data are expressed as percentage of absorbance unit of the respective control. $^{*}P < 0.05$ Docetaxel treated cells versus untreated. $^{\circ}P < 0.05$, Docetaxel plus MSC-CM treated cells versus Docetaxel treated. C: PC3 cells were cultured in the presence or in the absence of Docetaxel (0.6–5 nM). After 24 h cells were washed and cultured in the presence or in the absence of MSC-CM (10%, v/v) derived from MSCs pre-treated with PC3-CM (10%, v/v), PC3-CM plus gefitinib (5 μM), or gefitinib alone. After 3 days, proliferation was evaluated by BrdU assay. Results represent the mean ± SD of three different experiments in triplicate. Data are expressed as percentage of absorbance unit of the respective control. $^{\wedge}P < 0.05$ Docetaxel treated cells versus untreated. $^{*}P < 0.05$ MSC-CM, and MSC-CM/gefitinib treated cells versus untreated. $^{\circ}P < 0.05$, MSC-CM (PC3-CM pre-treated) treated cells versus untreated cells $^{\S}P < 0.05$ MSC-CM (PC3-CM/gefitinib pre-treated) treated cells versus MSC-CM (PC3-CM pre-treated) treated.

DISCUSSION

MSCs migrate towards regenerating tissues, primary tumors and metastatic sites where they interact with tumor cells and may sustain progression through the release of growth factors that promote neo-angiogenesis, tumor cell migration and drug resistance [Houthuijzen et al., 2012].

In this study we provided evidence that EGFR signaling is involved in the cross-talk between PCa cells and MSCs. Blockade of EGFR in MSCs was able to affect their ability to sustain PCa cell growth and to inhibit Docetaxel activity on PCa cells.

PC3-CM increases MSC proliferation and osteoblastic differentiation [Tang et al., 2011]. Accordingly, we found that PC3-CM increased both MSCs and OBs proliferation, reduced senescence of serum-starved MSCs and increased osteoblastic differentiation. Using the EGFR tyrosine kinase inhibitor gefitinib we demonstrated that these phenomena were due, at least in part, to EGFR activation by PC3 cells, in line with the observation that PC3 cells secrete TGF-alpha. However, it is likely that different pathways of EGFR transactivation also were involved [Gschwind et al., 2001; Porcile et al., 2005; Singh and Lokeshwar, 2011].

Previous reports demonstrated that an EGFR inhibitor caused a significant reduction in trabecular bone volume [Zhang et al., 2011] and the reduction of experimental metastasis of PC3 cells transplanted in mice [Angelucci et al., 2006]. Gefitinib inhibited the cross-talk between PC3 and MSCs, and our finding suggest that activation of EGFR by PCa cells [De Luca et al., 2008] might favor the abnormal expansion of MSCs and OBs in the tumor microenvironment, leading to the development of osteoblastic metastasis [Ibrahim et al., 2010]. In addition, PC3 cells secreted molecules that directly inhibited osteoclastogenesis and the ability of OBs to induce OC differentiation.

PCa cells/adipocyte interactions result in direct uptake and metabolism of Arachidonic acid [Angelucci et al., 2008] by PCa cells, destruction of adipocytes and subsequent formation of bone metastasis. Our results indicate that the direct effects of PCa cells on MSCs during adipogenic differentiation might be a further mechanism contributing to the absence of differentiated adipocytes in BM stroma invaded by PCa cells. In fact, PC3-CM increased pre-adipocyte proliferation, but at the same time it inhibited adipocyte differentiation. This finding was in line with the observation that PCa and PC3 cells secrete considerable amounts of endothelin (ET-1) [Le et al., 1999; Rosenblatt et al., 2009], which is known to increase MSC proliferation and to inhibit adipogenesis [Bhattacharya and Ullrich, 2006].

BM-MSCs are incorporated within prostate epithelia during the process of prostate regrowth suggesting their use as a vehicle for transporting genetic information with potential therapeutic effects on castrate resistant PCa [Placencio et al., 2010]. CCL5 is a chemokine involved in MSCs migration [Ponte et al., 2007] whose expression was demonstrated in PCa cell lines, primary prostate adenocarcinoma cells, and human PCa tissues [Vaday et al., 2006]. We demonstrated here that supernatants from PC3 and DU145 cells increased MSC migration that was partially blocked by neutralizing anti-CCL5 mAbs. These results suggest that CCL5, together with other molecules secreted by PCa cells or by cells of the microenvironment, could be involved in the recruitment of MSCs into the prostate tumor stroma. Our findings imply the possibility to use manipulated MSCs, that have inherent tumor-trophic migratory properties, as vehicles for delivering effective, targeted therapy to isolated tumors and metastatic disease [Shah, 2011].

Increasing evidence indicate that MSCs attracted by tumor cells contribute to a microenvironment that promotes osteolysis, tumor growth, and drug resistance [Bergfeld and DeClerck, 2010]. We found that supernatants from MSCs, in particular when pre-treated with PC3-CM, increased PC3 cells colony growth and decreased the antiproliferative activity of Docetaxel, suggesting that MSCs may contribute to prostate cancer progression by providing additional growth factors and by inhibiting Docetaxel activity. Gefitinib, by inhibiting EGFR activation induced by PC3-CM on MSCs reduced their effects on PC3 cells. These results suggest the possibility that the activation of MSCs by PCa cells could be partially inhibited by EGFR blockade.

Studies by Karnoub et al. [2007] demonstrated that MSCs induce weakly metastatic breast cancer cells to acquire enhanced metastatic abilities through the secretion of CCL5. Is this regard, we found that EGFR activation by PC3-CM increased the secretion by MSCs of CCL5 [Vaday et al., 2006; Colombatti et al., 2009]. Expression of both CCL5 and IL-6 in PCa cells was found to be regulated by prostate specific membrane antigen (PSMA) through the MAPK pathway [Colombatti et al., 2009]. More recently, co-authors of this study have demonstrated that the bis-phosphonate zoledronic acid, a drug that is commonly used for the treatment of bone metastasis in patients affected by different cancer types including PCa, is able to significantly reduce the secretion of CCL5 in MSCs [Gallo et al., 2011] a chemokine involved in PCa cells growth. Since zoledronic acid is currently used for the treatment of metastatic disease in PCa patients, our data suggest that a combined treatment with zoledronic acid and anti-EGFR agents might induce a more significant suppression of PCa cell growth.

CONCLUSION

Our study demonstrated that PCa cells can recruit and activate MSCs and in turn MSCs can enhance tumor cell proliferation and inhibit Docetaxel activity. These effects are mediated, at least in part, by EGFR activation. The inhibition of the cross-talk between MSCs and PCa cells by gefitinib, suggests a novel application of the EGFR-tyrosine kinase inhibitors in the treatment of prostate cancer.

ACKNOWLEDGMENTS

Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.) to Nicola Normanno; and the Ministero della Salute, Ricerca Finalizzata FSN, I.R.C.C.S., Rome, Italy.

REFERENCES

Aldinucci D, Lorenzon D, Cattaruzza L, Pinto A, Gloghini A, Carbone A, Colombatti A. 2008. Expression of CCR5 receptors on Reed-Sternberg cells and Hodgkin lymphoma cell lines: Involvement of CCL5/Rantes in tumor cell growth and microenvironmental interactions. Int J Cancer 122:769–776.

Angelucci A, Gravina GL, Rucci N, Millimaggi D, Festuccia C, Muzi P, Teti A, Vicentini C, Bologna M. 2006. Suppression of EGF-R signaling reduces the incidence of prostate cancer metastasis in nude mice. Endocr Relat Cancer 13:197–210.

Angelucci A, Garofalo S, Speca S, Bovadilla A, Gravina GL, Muzi P, Vicentini C, Bologna M. 2008. Arachidonic acid modulates the crosstalk between prostate carcinoma and bone stromal cells. Endocr Relat Cancer 15:91–100.

Bergfeld SA, DeClerck YA. 2010. Bone marrow-derived mesenchymal stem cells and the tumor microenvironment. Cancer Metastasis Rev 29:249–261.

Bhattacharya I, Ullrich A. 2006. Endothelin-1 inhibits adipogenesis: Role of phosphorylation of Akt and ERK1/2. FEBS Lett 580:5765–5771.

Chin SN, Wang L, Moore M, Sridhar SS. 2010. A review of the patterns of Docetaxel use for hormone-resistant prostate cancer at the Princess Margaret Hospital. Curr Oncol 17:24–29.

Cirri P, Chiarugi P. 2011. Cancer-associated-fibroblasts and tumour cells: A diabolic liaison driving cancer progression. Cancer Metastasis Rev 31:195–208.

Colombatti M, Grasso S, Porzia A, Fracasso G, Scupoli MT, Cingarlini S, Poffe O, Naim HY, Heine M, Tridente G, Mainiero F, Ramarli D. 2009. The prostate specific membrane antigen regulates the expression of IL-6 and CCL5 in prostate tumour cells by activating the MAPK pathways. PLoS ONE 4:e4608.

Dayyani F, Gallick GE, Logothetis CJ, Corn PG. 2011. Novel therapies for metastatic castrate-resistant prostate cancer. J Natl Cancer Inst 103:1665–1675.

De Luca A, Carotenuto A, Rachiglio A, Gallo M, Maiello MR, Aldinucci D, Pinto A, Normanno N. 2008. The role of the EGFR signaling in tumor microenvironment. J Cell Physiol 214:559–567.

De Luca A, Gallo M, Aldinucci D, Ribatti D, Lamura L, D'Alessio A, De FR, Pinto A, Normanno N. 2011. Role of the EGFR ligand/receptor system in the secretion of angiogenic factors in mesenchymal stem cells. J Cell Physiol 226:2131–2138.

DeHaan AM, Wolters NM, Keller ET, Ignatoski KM. 2009. EGFR ligand switch in late stage prostate cancer contributes to changes in cell signaling and bone remodeling. Prostate 69:528–537.

Fritz V, Brondello JM, Gordeladze JO, Reseland JE, Bony C, Yssel H, Noel D, Jorgensen C. 2011. Bone-metastatic prostate carcinoma favors mesenchymal stem cell differentiation toward osteoblasts and reduces their osteoclastogenic potential. J. Cell Biochem 112:3234–3245.

Gallo M, De Luca A, Lamura L, Normanno N. 2011. Zoledronic acid blocks the interaction between mesenchymal stem cells and breast cancer cells: Implications for adjuvant therapy of breast cancer. Ann Oncol 23:597–604.

Gschwind A, Zwick E, Prenzel N, Leserer M, Ullrich A. 2001. Cell communication networks: Epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. Oncogene 20:1594–1600.

Guise T. 2010. Examining the metastatic niche: Targeting the microenvironment. Semin Oncol 37(Suppl 2):S2–S14.

Gullick WJ. 2009. The epidermal growth factor system of ligands and receptors in cancer. Eur J Cancer 45(Suppl 1):205–210.

Houthuijzen JM, Daenen LG, Roodhart JM, Voest EE. 2012. The role of mesenchymal stem cells in anti-cancer drug resistance and tumour progression. Br J Cancer 106:1901–1906.

Ibrahim T, Flamini E, Mercatali L, Sacanna E, Serra P, Amadori D. 2010. Pathogenesis of osteoblastic bone metastases from prostate cancer. Cancer 116:1406–1418.

Itahana K, Campisi J, Dimri GP. 2007. Methods to detect biomarkers of cellular senescence: The senescence-associated beta-galactosidase assay. Methods Mol Biol 371:21–31.

Jin JK, Dayyani F, Gallick GE. 2011. Steps in prostate cancer progression that lead to bone metastasis. Int J Cancer 128:2545–2561.

Karlou M, Tzelepi V, Efstathiou E. 2010. Therapeutic targeting of the prostate cancer microenvironment. Nat Rev Urol 7:494–509.

Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA. 2007. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 449:557–563.

Krampera M, Pasini A, Rigo A, Scupoli MT, Tecchio C, Malpeli G, Scarpa A, Dazzi F, Pizzolo G, Vinante F. 2005. HB-EGF/HER-1 signaling in bone marrow mesenchymal stem cells: Inducing cell expansion and reversibly preventing multilineage differentiation. Blood 106:59–66.

Kuhn NZ, Tuan RS. 2010. Regulation of stemness and stem cell niche of mesenchymal stem cells: Implications in tumorigenesis and metastasis. J. Cell Physiol 222:268–277.

Le BG, Aubin P, Soliman H, Ropiquet F, Villette JM, Berthon P, Creminon C, Cussenot O, Fiet J. 1999. Upregulation of endothelin 1 and its precursor by IL-1beta, TNF-alpha, and TGF-beta in the PC3 human prostate cancer cell line. Cytokine 11:157–162.

Levina V, Su Y, Nolen B, Liu X, Gordin Y, Lee M, Lokshin A, Gorelik E. 2008. Chemotherapeutic drugs and human tumor cells cytokine network. Int J Cancer 123:2031–2040.

Lu Y, Zhang J, Dai J, Dehne LA, Mizokami A, Yao Z, Keller ET. 2004. Osteoblasts induce prostate cancer proliferation and PSA expression through interleukin-6-mediated activation of the androgen receptor. Clin Exp Metastasis 21:399–408.

McMillin DW, Delmore J, Weisberg E, Negri JM, Geer DC, Klippel S, Mitsiades N, Schlossman RL, Munshi NC, Kung AL, Griffin JD, Richardson PG, Anderson KC, Mitsiades CS. 2010. Tumor cell-specific bioluminescence platform to identify stroma-induced changes to anticancer drug activity. Nat Med 16:483–489.

Meads MB, Gatenby RA, Dalton WS. 2009. Environment-mediated drug resistance: A major contributor to minimal residual disease. Nat Rev Cancer 9:665–674.

Morrissey C, Lai JS, Brown LG, Wang YC, Roudier MP, Coleman IM, Gulati R, Vakar-Lopez F, True LD, Corey E, Nelson PS, Vessella RL. 2010. The expression of osteoclastogenesis-associated factors and osteoblast response to osteolytic prostate cancer cells. Prostate 70:412–424.

Normanno N, Gullick WJ. 2006. Epidermal growth factor receptor tyrosine kinase inhibitors and bone metastases: Different mechanisms of action for a novel therapeutic application? Endocr Relat Cancer 13:3–6.

Normanno N, De Luca A, Aldinucci D, Maiello MR, Mancino M, D'Antonio A, De Filippi R, Pinto A. 2005. Gefitinib inhibits the ability of human bone marrow stromal cells to induce osteoclast differentiation: Implications for the pathogenesis and treatment of bone metastasis. Endocr Relat Cancer 12:471–482.

Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, Carotenuto A, De FG, Caponigro F, Salomon DS. 2006. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene 366:2–16.

Pivetta E, Scapolan M, Wassermann B, Steffan A, Colombatti A, Spessotto P. 2011. Blood-derived human osteoclast resorption activity is impaired by Hyaluronan-CD44 engagement via a p38-dependent mechanism. J Cell Physiol 226:769–779.

Placencio VR, Li X, Sherrill TP, Fritz G, Bhowmick NA. 2010. Bone marrow derived mesenchymal stem cells incorporate into the prostate during regrowth. PLoS ONE 5:e12920.

Ponte AL, Marais E, Gallay N, Langonne A, Delorme B, Herault O, Charbord P, Domenech J. 2007. The in vitro migration capacity of human bone marrow mesenchymal stem cells: Comparison of chemokine and growth factor chemotactic activities. Stem Cells 25:1737–1745.

Porcile C, Bajetto A, Barbieri F, Barbero S, Bonavia R, Biglieri M, Pirani P, Florio T, Schettini G. 2005. Stromal cell-derived factor-1alpha (SDF-1alpha/CXCL12) stimulates ovarian cancer cell growth through the EGF receptor transactivation. Exp Cell Res 308:241–253.

Roodhart JM, Daenen LG, Stigter EC, Prins HJ, Gerrits J, Houthuijzen JM, Gerritsen MG, Schipper HS, Backer MJ, van AM, Vermaat JS, Moerer P, Ishihara K, Kalkhoven E, Beijnen JH, Derksen PW, Medema RH, Martens AC,

Brenkman AB, Voest EE. 2011. Mesenchymal stem cells induce resistance to chemotherapy through the release of platinum-induced fatty acids. Cancer Cell 20:370–383.

Rosenblatt R, Valdman A, Cheng L, Lopez-Beltran A, Montironi R, Ekman P, Egevad L. 2009. Endothelin-1 expression in prostate cancer and high grade prostatic intraepithelial neoplasia. Anal Quant Cytol Histol 31:137–142.

Salem HK, Thiemermann C. 2010. Mesenchymal stromal cells: Current understanding and clinical status. Stem Cells 28:585–596.

Santer FR, Malinowska K, Culig Z, Cavarretta IT. 2010. Interleukin-6 trans-signalling differentially regulates proliferation, migration, adhesion and maspin expression in human prostate cancer cells. Endocr Relat Cancer 17:241–253.

Shah K. 2011. Mesenchymal stem cells engineered for cancer therapy. Adv Drug Deliv Rev 64:739–748.

Singh RK, Lokeshwar BL. 2011. The IL-8-regulated chemokine receptor CXCR7 stimulates EGFR signaling to promote prostate cancer growth. Cancer Res 71:3268–3277.

Spessotto P, Lacrima K, Nicolosi PA, Pivetta E, Scapolan M, Perris R. 2009. Fluorescence-based assays for in vitro analysis of cell adhesion and migration. Methods Mol Biol 522:221–250.

Suva LJ, Washam C, Nicholas RW, Griffin RJ. 2011. Bone metastasis: Mechanisms and therapeutic opportunities. Nat Rev Endocrinol 7:208–218.

Tang YJ, Sun YH, Xu CL, Wang LH, Gao X, Liu B, Ji JT. 2011. Prostate cancer cell line PC-3 conditioned medium promotes proliferation and osteogenic differentiation of human bone marrow mesenchymal stem cells. Zhonghua Nan Ke Xue 17:229–236.

Torring N, Jorgensen PE, Sorensen BS, Nexo E. 2000. Increased expression of heparin binding EGF (HB-EGF), amphiregulin, TGF alpha and epiregulin in androgen-independent prostate cancer cell lines. Anticancer Res 20:91–95.

Vaday GG, Peehl DM, Kadam PA, Lawrence DM. 2006. Expression of CCL5 (RANTES) and CCR5 in prostate cancer. Prostate 66:124–134.

Zhang X, Tamasi J, Lu X, Zhu J, Chen H, Tian X, Lee TC, Threadgill DW, Kream BE, Kang Y, Partridge NC, Qin L. 2011. Epidermal growth factor receptor plays an anabolic role in bone metabolism in vivo. J Bone Miner Res 26:1022–1034.