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Mesenchymal Stem Cell-Derived Interleukin-6 and Vascular Endothelial Growth Factor Promote Breast Cancer Cell Migration

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ABSTRACT

Several different cytokines and growth factors secreted by mesenchymal stem cells (MSCs) have been hypothesized to play a role in breast cancer progression. By using a small panel of breast cancer cell lines (MCF-7, T47D, and SK-Br-3 cells), we analyzed the role of interleukin-6 (IL-6) and vascular endothelial growth factor A (VEGF) in the cross-talk between MSCs and breast cancer cells. We performed migration assays in which breast cancer cells were allowed to migrate in response to conditioned medium from MSCs (MSCs-CM), in absence or in presence of the anti-VEGF antibody bevacizumab or an anti-IL-6 antibody, alone or in combination. We found that anti-VEGF and anti-IL-6 antibodies inhibited the migration of breast cancer cells and that the combination had an higher inhibitory effect. We next evaluated the effects of recombinant VEGF and IL-6 proteins on breast cancer cell growth and migration. IL-6 and VEGF had not significant effects on the proliferation of breast carcinoma cells. In contrast, both VEGF and IL-6 significantly increased the ability to migrate of MCF-7, T47D and SK-Br-3 cells, with the combination showing a greater effect as compared with treatment with a single protein. The combination of VEGF and IL-6 produced in breast cancer cells a more significant and more persistent activation of MAPK, AKT, and p38MAPK intracellular signaling pathways. These results suggest that MSC-secreted IL-6 and VEGF may act as paracrine factors to sustain breast cancer cell migration. J. Cell. Biochem. 113: 3363–3370, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: MESENCHYMAL STEM CELLS; IL-6; VEGF; BREAST CANCER; MIGRATION

The dynamic interaction between cancer cells and the tumor microenvironment is increasingly considered as an important regulator of malignant progression. In this regard, tumor cells secrete chemokines, cytokines, and growth factors that are able to recruit and activate a variety of non-transformed stromal cells. In turn, stromal cells provide signals that promote the ability of tumor cells to invade and metastasize [De Luca et al., 2008; Joyce and Pollard, 2009; El-Haibi and Karnoub, 2010].

Mesenchymal stem cells (MSCs) are non-transformed stromal cells that reside predominantly in the bone-marrow and display multi-lineage features, particularly osteogenesis, chondrogenesis, and adipogenesis [Deans and Moseley, 2000; Kuhn and Tuan, 2010]. MSCs are able to selectively home to sites of tissue damage as well as to tumors. Indeed, actively growing tumors recruit MSCs in their surrounding environment where they promote tumor growth at the primary site and facilitate metastatic dissemination to distant organs [Hall et al., 2007; Joyce and Pollard, 2009]. The interaction between MSCs and tumor cells might also occur within the bone marrow microenvironment where MSCs can contribute to form niches for dormant micrometastases that may later seed distant metastases [Psaila and Lyden, 2009].

Several studies demonstrated that different factors secreted by MSCs play an important role in tumor progression. Karnoub et al. [2007] showed that MSCs are recruited to developing tumors where they increase breast cancer cells motility, invasion, and metastatic potential through the secretion of the chemokine (C-C motif) ligand 5 (CCL5 also known as RANTES). MSC-derived monocyte chemotactic protein-1 (MCP-1/CCL2) and interleukin 17B (IL-17B) have been also demonstrated to promote breast cancer cell migration [Molloy et al., 2009; Goldstein et al., 2010].

Angiogenesis is a key event in tumor progression. MSCs secrete a number of angiogenic growth factors including vascular endothelial

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growth factor A (VEGF) and interleukin-6 (IL-6) [Beckermann et al., 2008; De Luca et al., 2011]. Intriguingly, these factors have been also shown to promote breast cancer cell migration and/or invasion. Expression of vascular endothelial growth factor receptor 1(VEGFR-1) has been demonstrated to occur in a number of breast cancer cell lines in which VEGF stimulated proliferation and survival [Wu et al., 2006]. In addition, VEGF was able to stimulate the invasion of breast cancer cells through the activation of MAPK and PI3K/AKT signaling [Price et al., 2001]. Finally, breast cancer cells transfected with VEGF showed an increased expression of Snail, an E-cadherin transcriptional repressor that is involved in the epithelial–mesenchymal transition (EMT) and that is associated with increased invasiveness of breast cancer cells [Wanami et al., 2008].

The pleiotropic cytokine IL-6 also plays an important role in cancer progression [Ara and Declerck, 2010]. Through the interaction with an high-affinity receptor complex consisting of the IL-6-binding receptor protein (IL-6R) and the signal transducer protein gp130 (gp130), IL-6 activates several intracellular signaling pathways, including the Jak/STAT3, the Ras/Raf/MEK/MAPK, and the PI3K/Akt pathways. IL-6 was found to stimulate breast cancer cell growth and migration, by acting through autocrine or paracrine mechanisms [Badache and Hynes, 2001; Sasser et al., 2007]. In addition, exposure to IL-6 induced an EMT phenotype and promoted the invasiveness of breast cancer cells [Sullivan et al., 2009]. It has also been recently suggested that IL-6 might facilitate the generation of breast cancer cells with stem cell-like properties [Xie et al., 2012].

Taken together, these findings suggest that several MSC-derived growth factors and cytokines can promote different mechanisms that are involved in breast cancer progression, including angiogenesis and migration. In particular, the above summarized data indicate that VEGF and IL-6 might cooperate in sustaining breast cancer cell migration. Therefore, we analyzed the effects of these MSCs-secreted proteins alone or in combination on the ability to migrate of a panel of breast cancer cell lines.

MATERIALS AND METHODS

CELL LINES AND MATERIALS

MCF-7, SK-Br-3, and T47D human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF-7 and SK-Br-3 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium with GlutaMAX supplemented with 10% fetal bovine serum (FBS; all from Invitrogen, Milan, Italy). T47D cells were routinely maintained in RPMI 1640 medium with GlutaMAX, 10% FBS and 0.2 units/ml bovine insulin. Bone marrow-derived human MSCs were purchased from Lonza (Verviers, Belgium). These cells were positive for CD29, CD44, CD105, CD166, and negative for markers of the hematopoietic lineage, such as CD14, CD34, CD45, by flow cytometric analysis. Cells were thawed and cultured in MSCGM bullet kit (Lonza) in a humidified atmosphere at 37°C and 5% CO_2, according to the manufacturer's instructions.

Recombinant human IL-6 was purchased from PeproTech (London, UK). Recombinant human VEGF was from BD Biosciences (Bedford, MA). The anti-human IL-6 monoclonal antibody (MAB206, clone 6708) was obtained from R&D Systems (Minneapolis, MN). Bevacizumab was from Roche (Milan, Italy).

PREPARATION OF CONDITIONED MEDIA AND IMMUNOASSAYS

Conditioned media were collected from MSCs cultured for 48 h in serum-free medium, sterile filtered and stored in aliquots at −80°C until required.

The levels of VEGF and IL-6 were assessed in the MSCs conditioned medium using the Bio-Plex Human Cytokine Singleplex Assays for VEGF and IL-6 (Bio-Rad Life Science, Milan, Italy), according to the manufacturer's protocols.

The levels of secreted proteins measured in conditioned medium were referred to picograms per 10^5 cells as determined on harvesting day, 48 h after seeding.

RT-PCR

Total RNA from breast cancer cell lines was extracted using TRI Reagent solution (Ambion/Applied Biosystems, Milan, Italy) and treated with RNase-free DNase I (Promega Italia, Milan, Italy) to remove potential genomic DNA contamination. cDNA synthesis was performed with Superscript II-Reverse Transcriptase (Invitrogen, Milan, Italy), using 2 μg aliquots of total cellular RNA and random hexamers as primers. PCR amplification to detect VEGFR-1, IL-6 receptor, and the gp130 subunit mRNAs expression, was performed using the Gene Amp 9700 System (Applied Biosystems). The primer sequences will be provided upon request.

ANCHORAGE-DEPENDENT GROWTH ASSAY

MCF-7 and SK-Br-3 cells were seeded in 12-well cell culture plates at a concentration of 40×10^3 and 30×10^3 cells/well, respectively, in serum containing medium. After 24 h, cells were treated with recombinant IL-6 (25 ng/ml) or VEGF (100 ng/ml), alone or in combination, and counted at the indicated time points with an automated Z1 Coulter Counter (Beckman Coulter, Milan, Italy).

FLOW CYTOMETRY ANALYSIS

Cells were detached by incubation with 0.02% EDTA in PBS, centrifuged at 4°C and washed in PBS containing 0.5% BSA and 0.1% sodium azide. The cells were incubated for 30 min at 4°C with the following antibodies: anti-human VEGFR1 (Flt-1)-phycoerythrin (PE) or anti-human IL-6-R-PE or anti-human gp130-PE (CD130) or anti-IgG1-PE antibodies (all from R&D Systems). After a washing with PBS/0.1% sodium azide, the cells were re-suspended in PBS and analyzed with a FacsAria II (Becton Dickinson, Mountain View, CA).

WESTERN BLOT ANALYSIS

Breast cancer cells were plated in 60-mm culture dishes in serum-containing medium. Then, cells were washed twice with PBS and cultured in serum-free medium. After 24 h, cells were treated with recombinant IL-6 (25 ng/ml) or recombinant VEGF (100 ng/ml) alone or in combination for the indicated times.

Whole protein extracts were prepared and analyzed using the chemiluminescence ECL Western blotting kit (GE Healthcare, Milan, Italy). The following antibodies were used: (i) anti-phospho-p44/p42 MAPK (Thr202/Tyr204) antibody (Cell Signaling Technology,

Beverly, MA); (ii) anti-phospho-AKT (Ser473) antibody (Cell Signaling); (iii) anti-phospho-STAT3 (Tyr705, clone 3E2) antibody (Cell Signaling); (iv) anti-phospho-p38-MAPK (Thr180/Tyr182, clone D3F9) antibody (Cell Signaling); (v) anti-α-tubulin (clone DM1A) antibody (Sigma–Aldrich, Milan, Italy).

MIGRATION ASSAYS

MCF-7, SK-Br-3, and T47D cells (110×10^3 cells/insert) were seeded in serum-free medium in the upper wells and allowed to migrate for 20 h through a fibronectin-coated Boyden chamber using the QCM-FN Haptotaxis Cell Migration Assay-Fibronectin, Colorimetric (Chemicon/Millipore, Milan, Italy). Serum containing media or conditioned media from MSCs in presence or in absence of bevacizumab (50 μg/ml) and/or a neutralizing anti-IL-6 antibody (400 ng/ml); or serum-free medium added with recombinant human VEGF (100 ng/ml) or IL-6 (25 ng/ml) alone or in combination, were used as chemoattractant in the lower Boyden chambers.

STATISTICAL ANALYSIS

Significance was determined using two-tailed Student's *t*-test. *P*-values <0.05 were considered statistically significant.

RESULTS

MSCs-SECRETED IL-6 AND VEGF INCREASE BREAST CANCER CELL MIGRATION

We analyzed the levels of VEGF and IL-6 in the conditioned medium (CM) from MSCs by using the XMAP Bio-Plex Cytokine array system. The analysis revealed that MSCs secrete significant amounts of both VEGF and IL-6 (Table I). In order to investigate whether VEGF and IL-6 produced by MSCs might promote breast cancer cell migration, we performed migration assays in which a panel of breast cancer cell lines representative of different breast cancer subtypes (the ER-positive MCF-7 and T47D cell lines and the ER-negative, ErbB-2 overexpressing SK-Br-3 cell line) were allowed to migrate through a fibronectin-coated membrane in response to MSC-CM, in absence or in presence of the anti-VEGF antibody bevacizumab and/or an anti-IL-6 antibody. We observed that CM from MSCs was able to induce the migration of MCF-7 breast cancer cells at a similar extent of serum that we used as positive control (Fig. 1A). Treatment with bevacizumab or with the anti-IL-6 antibody significantly inhibited the migration of MCF-7 cells induced by MSC-CM. The combination of bevacizumab and the anti-IL-6 antibody produced a greater inhibition of the migration of MCF-7 cells as compared with treatment with a single agent ($P < 0.01$; Fig. 1A). Similar results were obtained in SK-Br-3 and T47D cells; addition of anti-VEGF and anti-IL-6 antibodies to MSC-CM resulted in a significant reduction in its ability to induce migration of these breast cancer cell lines (Fig. 1B,C). Interestingly, simultaneous treatment with bevacizumab and anti-IL-6 antibody also inhibited the basal levels of migration of T47D cells, suggesting that these factors are involved in the spontaneous, MSC-independent migration of these cells (Fig. 1B).

TABLE I. Quantification of VEGF and IL-6 secretion by MSCs

Growth factor	pg/48 h/10^5 cells (mean ± SEM)
IL-6	776.46 ± 86.32
VEGF	678.09 ± 3.38

Values were referred to 1×10^5 cells as determined on the harvesting day, 48 h after seeding. The data presented above were obtained from three independent measurements.

EXPRESSION OF VEGFR-1 AND IL-6R/GP130 IN BREAST CANCER CELLS

We hypothesized that VEGF and IL-6 secreted by MSCs might act on breast cancer cells as paracrine factors. For this purpose, we analyzed the expression of VEGFR-1 and IL-6R/gp130 receptors in the MCF-7, T47D, and SK-Br-3 cell lines by RT-PCR (Fig. 2A). In agreement with previous studies, we found expression of the VEGFR-1 transcript in all the analyzed cell lines (Fig. 2A) [Price et al., 2001; Wu et al., 2006]. In addition, IL-6R and gp130 mRNAs were also detected in MCF-7, T47D, and SK-Br-3 cells (Fig. 2A).

We next assessed the expression of VEGFR-1, IL-6R, and gp130 proteins by flow cytometry analysis. In agreement with RT-PCR data, expression of VEGFR-1, IL-6R, and gp130 was detected in all the breast cancer cell lines. Representative results are shown in Figure 2B.

RECOMBINANT IL-6 AND VEGF AFFECT THE MIGRATION OF BREAST CANCER CELLS

In order to further address the role of VEGF and IL-6 in the pathogenesis and progression of breast cancer, we first evaluated the effects of these proteins, alone or in combination, on the growth of MCF-7 and SK-Br-3 cells. We found that treatment of breast cancer cells with recombinant VEGF or IL-6 or the combination of the two proteins had not significant effects on their proliferation (Fig. 3). We next investigated the effects of the recombinant proteins on breast cancer cell migration, by using assays in which breast cancer cell lines were allowed to migrate in response to IL-6 or VEGF, alone or in combination (Fig. 4). Both recombinant VEGF and IL-6 produced a significant increase in the ability to migrate of MCF-7, T47D, and SK-Br-3 cells. VEGF showed a slightly higher ability to induce migration of MCF-7 and SK-Br-3 cells as compared with IL-6, whereas the two growth factors induced migration of the T47D breast cancer cell line at a similar extent. More importantly, the combination of the two growth factors produced a greater effect on the migration of breast cancer cells, as compared with treatment with a single agent ($P < 0.01$; Fig. 4).

EFFECTS OF VEGF AND IL-6 ON THE ACTIVATION OF DOWNSTREAM SIGNALING PATHWAYS

We evaluated the effects of VEGF and IL-6 on the activation of p42/p44 MAPK, AKT, STAT3, and p38MAPK signaling proteins in MCF-7, T47D, and SK-Br-3 cells. Exogenous VEGF markedly increased MAPK phosphorylation with a peak after 5 min of treatment in all the cell lines, and AKT with a peak after 30 min (T47D and SK-Br-3 cells) or after 1 h of treatment (MCF-7 cells). Following treatment with VEGF, an increase in the phosphorylation of p38MAPK was also observed, with a peak at 5 min in T47D and SK-Br-3 cells, and at 1 h in MCF-7 cells. Treatment with recombinant VEGF did not affect the activation of STAT3 in these cell lines. IL-6 induced an increase of the phosphorylation of MAPK

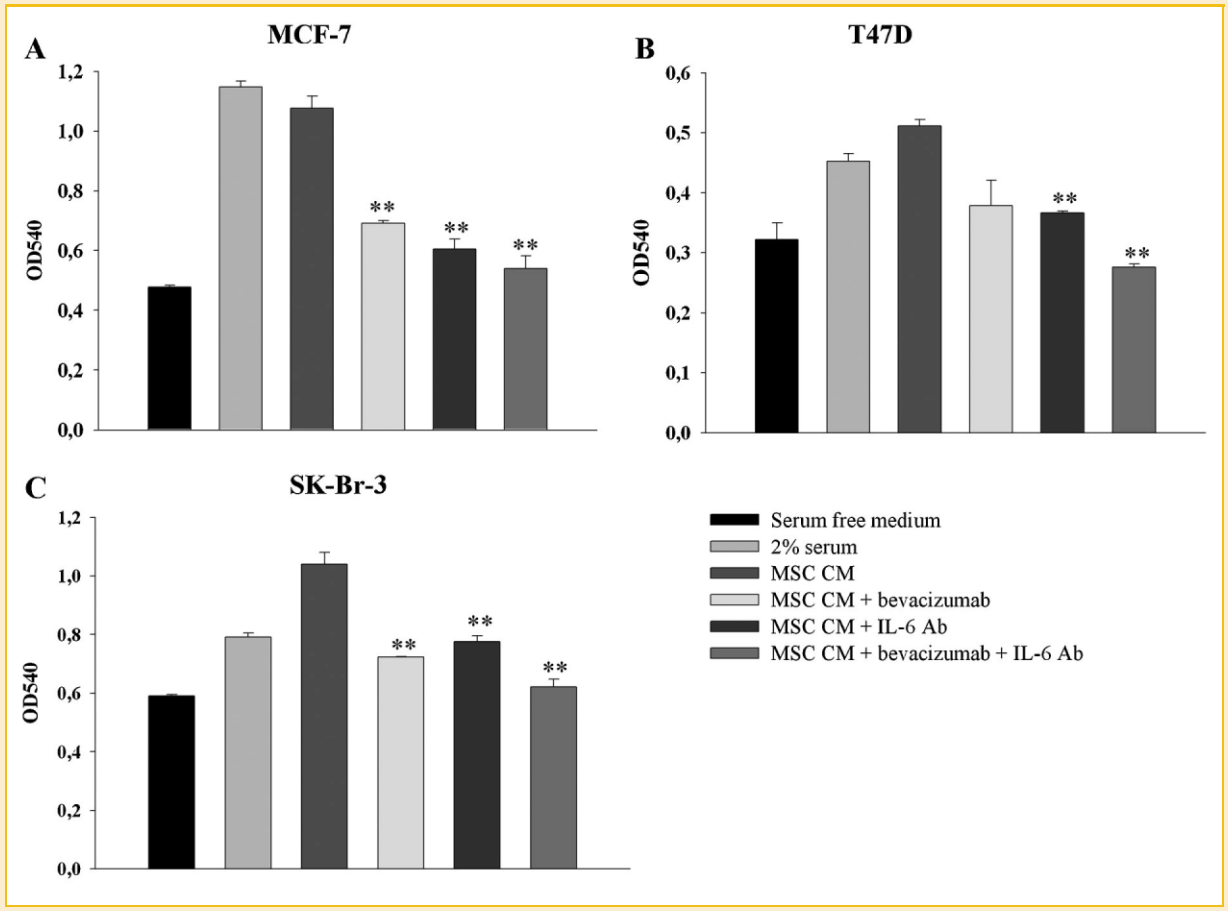

Fig. 1. Effects of MSC-conditioned medium (CM) in presence or in absence of anti-VEGF (bevacizumab) and/or anti-IL-6 antibodies. MCF-7 (A), T47D (B), and SK-Br-3 (C) cells were allowed to migrate for 20 h through a fibronectin-coated membrane in response to: serum-free medium; culture medium supplemented with 2% serum; MSC-CM; or MSC-CM in presence of bevacizumab and anti-IL-6 antibody alone or in combination. $^{**}P < 0.01$ compared with MSCs-CM, as determined by two-tailed Student's *t*-test.

and STAT-3 in all the analyzed cell lines, with peaks at 5 and 30 min, respectively. AKT phosphorylation increased after treatment with IL-6 with a peak at 30 min. IL-6 treatment also produced an increase in the activation of p38MAPK, with a peak at 5 min in SK-Br-3 cells and at 30 min in the MCF-7 and T47D cell lines. Treatment with the combination of VEGF and IL-6 resulted in a more significant and/or more persistent activation of MAPK, AKT, and p38MAPK signaling pathways in MCF-7, T47D, and SK-Br-3 cells (Fig. 5).

DISCUSSION

The formation of distant metastases represents a "point of no return" for patients with solid tumors: with few exceptions, a metastatic solid tumor cannot be eradicated with the available therapeutics, and the disease will inexorably progress determining the patient death. Therefore, novel therapeutic strategies that can prevent the formation of metastasis in solid neoplasms are definitely required. In this respect, the search of novel therapeutic approaches for solid tumors has long been based on the identification of molecular alteration of cancer cells that are involved in regulating their proliferation, survival, and ability to form distant metastases. However, it is widely demonstrated that non-malignant cells of the tumor microenvironment strongly influence cancer cell growth and metastatic dissemination to distant sites [Joyce and Pollard, 2009]. In particular, stromal cells provide the soil for the development of tumors and release factors that increase the metastastic potential of cancer cells. In this scenario, recent findings strongly suggest that MSCs play a fundamental role in the progression of breast carcinoma [El-Haibi and Karnoub, 2010].

MSCs are recruited by developing breast carcinomas, and secrete several factors that promote cancer cell proliferation, migration, and invasion such as CCL5/RANTES and IL-17B [Karnoub et al., 2007; Goldstein et al., 2010]. In addition, MSCs produce a number of angiogenic growth factors that facilitate the growth of primary and metastatic tumors by inducing the formation of new vessels [De Luca et al., 2011]. In this study, we demonstrated that VEGF and IL-6 produced by MSCs are also able to sustain the migration of breast cancer cells.

Interestingly, we observed that antibodies directed against VEGF and IL-6 strongly cooperate in inhibiting the migration of breast cancer cells induced by MSC-CM. Analogously, we found that the combination of recombinant VEGF and IL-6 produced a more significant increase in the migration of breast cancer cells as

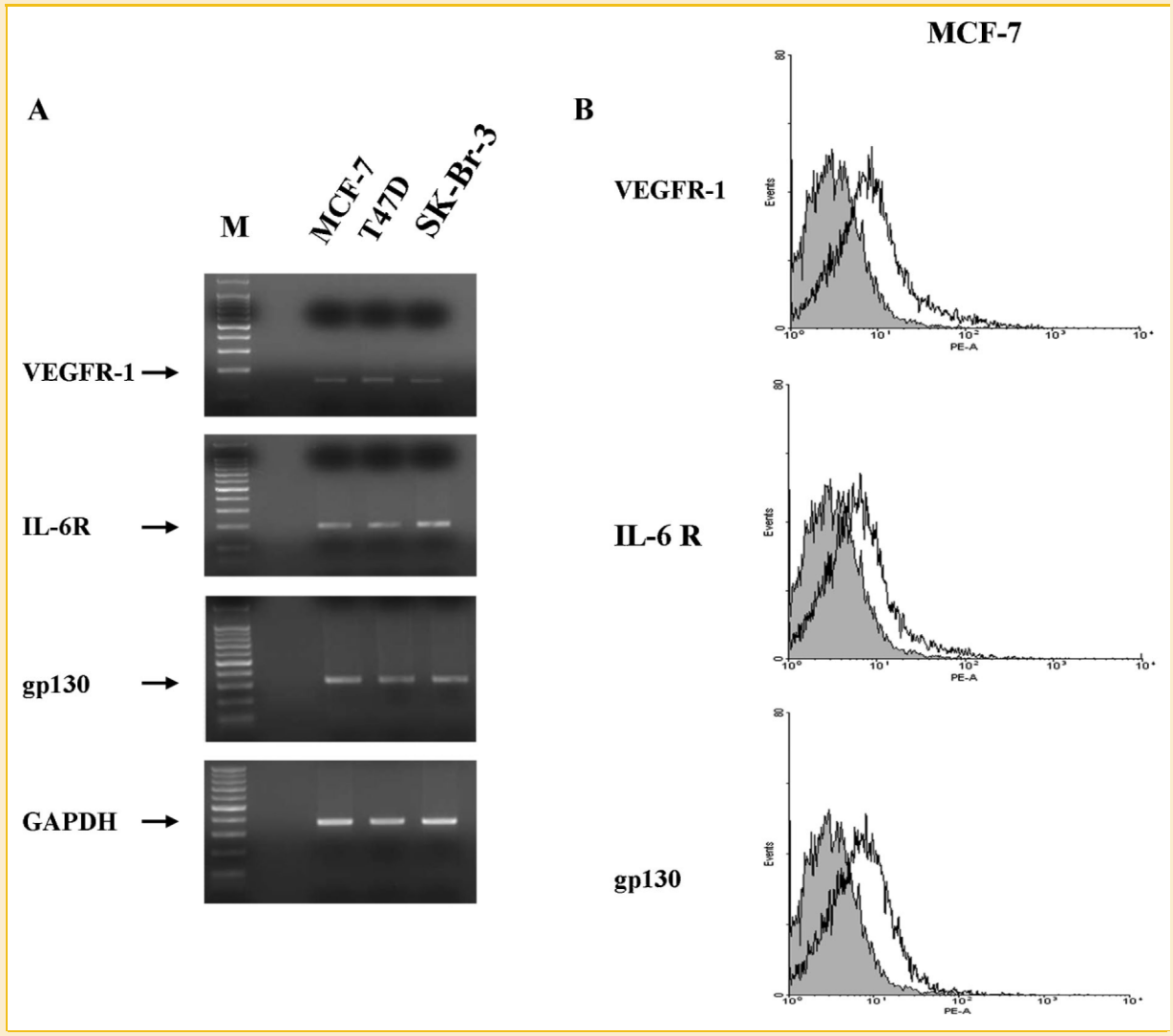

Fig. 2. Expression of VEGFR-1, IL-6R, gp130 mRNAs, and proteins in breast cancer cells. A: VEGFR-1, IL-6R, gp130, and GAPDH transcripts were detected by RT-PCR. M: 100 bp ladder marker. B: Cytofluorimetric analysis of VEGFR-1, IL-6R, gp130 proteins in MCF-7 cells (open peaks). Grey peaks represent the staining with an isotype-matched control IgG1.

compared with treatment with a single factor. In this respect, the role of VEGF and IL-6 in the pathogenesis and progression of breast carcinoma has long been established. As we summarized in the introduction, both these factors have been demonstrated to promote neo-angiogenesis and to stimulate the migration or invasion of breast cancer cells. Several studies have also demonstrated that high levels of expression of VEGF, either measured in the tumor or in serum samples, are associated with poor patients' prognosis [Gasparini, 2001]. Analogously, serum levels of IL-6 have shown to predict a worse outcome in breast cancer patients [Knupfer and Preiss, 2007]. Intriguingly, these two factors are functionally correlated, since IL-6 has been shown to induce VEGF expression. Whether serum levels of VEGF and IL-6 might help to identify patients in which these factors are playing a major role in promoting the metastatic cascade remains to be determined and represents an important question for future clinical applications.

In agreement with previous reports, we found that both VEGF and IL-6 were able to induce in breast cancer cells the activation of signaling pathways that have been previously demonstrated to be involved in migration and invasion of cancer cells, such as the MEK/MAPK, the PI3K/AKT, and the STAT3 pathways [Badache and Hynes, 2001; Price et al., 2001; Sasser et al., 2007]. More importantly, the combination of the two growth factors was able to produce an higher and more prolonged activation of these pathways as compared with a single protein, thus providing a mechanistic explanation of their cooperative effects. These findings are in agreement with previous results from our group demonstrating that RANTES and IL-6 cooperate in inducing migration of breast cancer cells and in activating intracellular signaling pathways associated with tumor cell migration (MAPK, AKT, and STAT3 proteins) [Gallo et al., 2012].

In contrast with previous reports, we found that both VEGF and IL-6 had no significant effects on the proliferation of breast

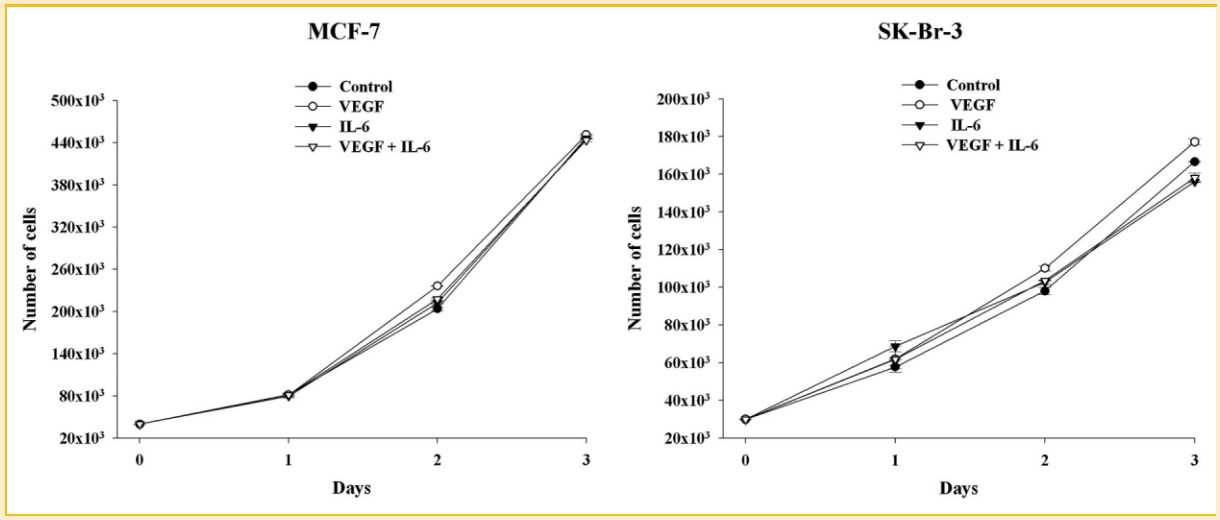

Fig. 3. Effects of recombinant IL-6 and VEGF alone or in combination on the proliferation of MCF-7 and SK-Br-3 breast cancer cells. Cells were cultured in the presence of the recombinant IL-6 (25 ng/ml) or VEGF (100 ng/ml) alone or in combination, and counted at the indicated time points.

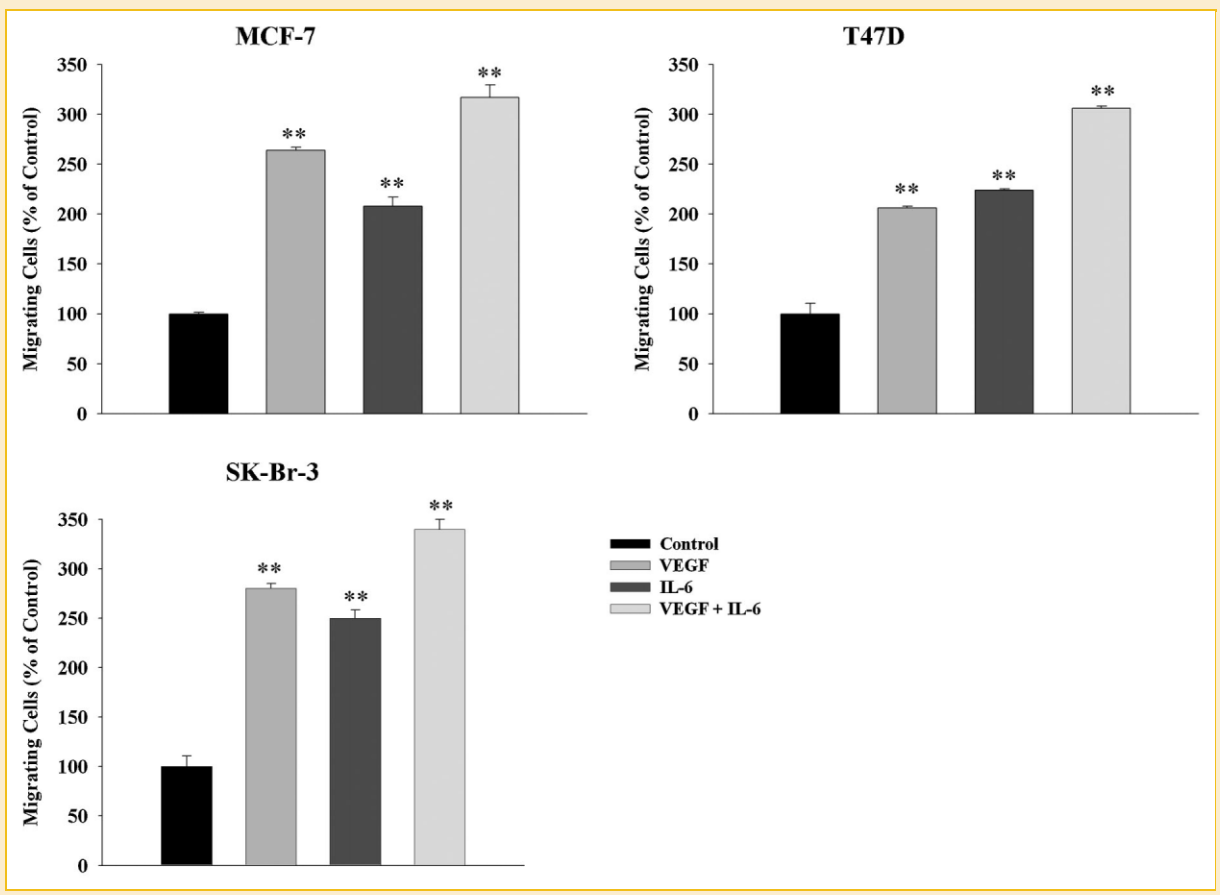

Fig. 4. Effects of recombinant IL-6 (25 ng/ml) and VEGF (100 ng/ml) alone or in combination on the ability to migrate of MCF-7, T47D, and SK-Br-3 breast cancer cells (**$P < 0.01$ compared with untreated MSCs-CM, as determined by two-tailed Student's t-test). The results are expressed as % of control untreated cells. Control OD was 0.320 nm for MCF-7, 0.378 nm for SK-Br-3, and 0.246 nm for T47D cells.

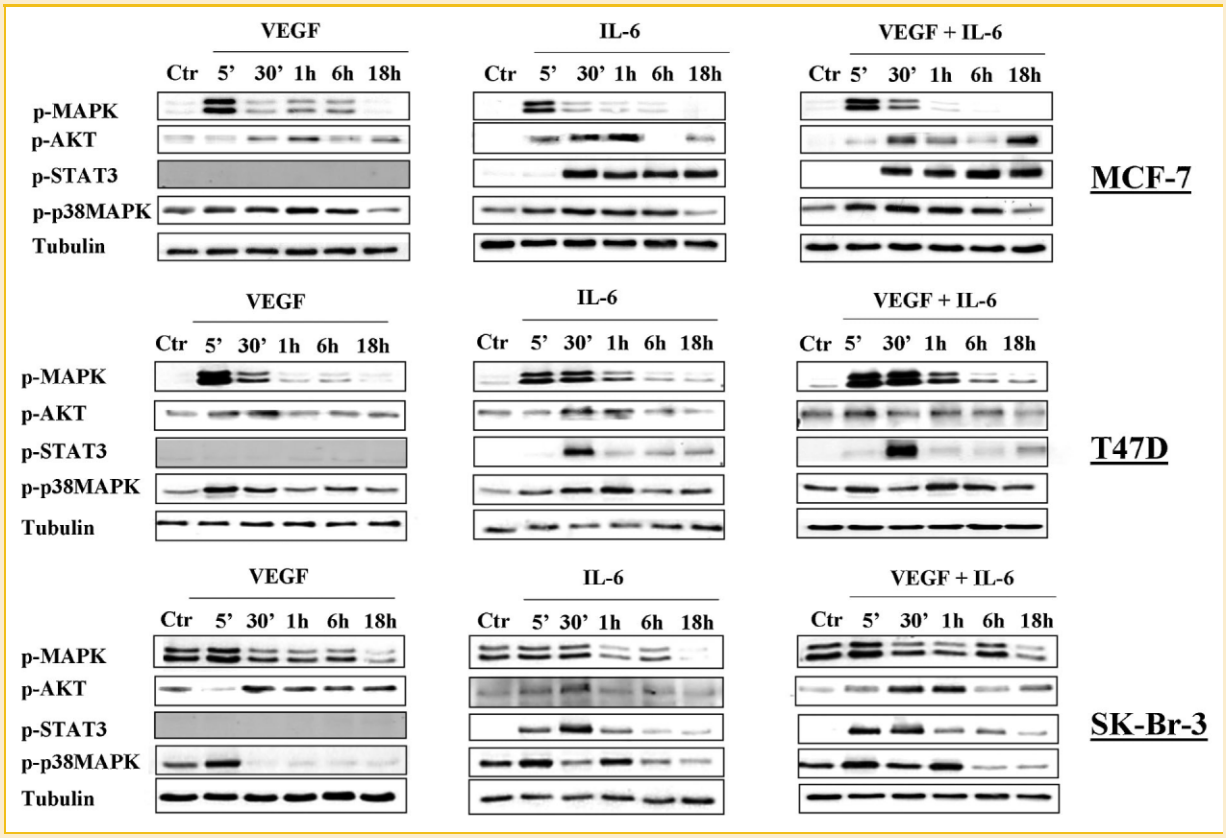

Fig. 5. Western blot analysis of the expression of the activated forms of MAPK, AKT, STAT3, and p38MAPK in MCF-7, T47D, and SK-Br-3 cells untreated or treated with 100 ng/ml VEGF and 25 ng/ml IL-6 alone or in combination. Blots were normalized with an α-tubulin antibody.

cancer cells. These experiments were repeated by using different concentrations of growth factors and cell culture conditions (serum-free vs. serum-containing medium) yielding to the same conclusions (data not shown). Previous studies suggested that IL-6 can either inhibit or stimulate breast cancer cell growth. However, in the studies in which IL-6 was found to promote cell proliferation, a three-dimensional tumor growth assay in which cancer cell were embedded in a basement membrane extract was employed [Sasser et al., 2007; Studebaker et al., 2008]. The membrane extracts are likely to be enriched of a number of different growth factors, and this might have significantly enhanced the proliferative response to exogenous IL-6. On the other hand, the studies that suggested that IL-6 might inhibit breast cancer cell proliferation used growth assays in which cell lines were cultured in medium supplemented with insulin [Danforth and Sgagias, 1993; Badache and Hynes, 2001]. In this regard, it has been demonstrated that IL-6 significantly inhibits the ability of IGF-1 to induce DNA synthesis, thus suggesting that IL-6 can prevent the insulin-induced breast cancer growth rather than directly inhibit breast cancer cell proliferation [Shen et al., 2002].

Taken together, the above-summarized findings strongly support the hypothesis that the metastatic potential of breast cancer cells is determined by the combined activity of a variety of factors secreted by MSCs. At the same time, these results indicate that the interaction between MSCs and breast cancer cells might represent an important therapeutic target to prevent the onset of metastases. In this regard, drugs able to directly inhibit VEGF or IL-6 signaling have been already approved for clinical use. The monoclonal anti-VEGF blocking antibody bevacizumab has been approved for treatment of patients with different carcinoma types including breast cancer. Additional agents directed against VEGF and its receptors have been approved or are currently in clinical development [Morabito et al., 2006; Grothey and Galanis, 2009]. An anti-IL-6 receptor antibody (tocilizumab) is employed for the treatment of rheumatoid arthritis, and antibodies directed against IL-6 are in clinical development in cancer patients [Sansone and Bromberg, 2012]. In addition, a number of signaling inhibitors that are able to block the pathways that we have identified to be activated by IL-6 and VEGF in breast cancer cells are being employed in clinical trials enrolling patients with different tumor types. Therefore, the identification of the mechanisms involved in the formation of tumor metastases might offer the potential for clinical intervention by using different types of agents and their combinations. In this respect, the importance of using combinations of drugs that target different mechanisms in the tumor microenvironment has been recently highlighted [Swartz et al., 2012].

In conclusion, this study has for the first time identified the combined effect of VEGF and IL-6 as an important mediator of

MSC-induced breast cancer cell migration, and has indicated potential therapeutic applications of this finding.

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