

17 β -Estradiol Promotes Breast Cancer Cell Proliferation-Inducing Stromal Cell-Derived Factor-1-Mediated Epidermal Growth Factor Receptor Transactivation: Reversal by Gefitinib Pretreatment

Alessandra Pattarozzi, Monica Gatti, Federica Barbieri, Roberto Würth, Carola Porcile, Gianluigi Lunardi, Alessandra Ratto, Roberto Favoni, Adriana Bajetto, Angelo Ferrari, and Tullio Florio

Section of Pharmacology, Department of Oncology, Biology and Genetics University of Genova, Genova, Italy (A.P., M.G., F.B., R.W., C.P., A.B., T.F.); National Cancer Institute (IST), Genova, Italy (G.L., R.F.); and Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle D'Aosta, National Reference Center of Veterinary and Comparative Oncology, Genova, Italy (A.R., A.F.)

Received July 18, 2007; accepted October 23, 2007

ABSTRACT

The coordinated activity of estrogens and epidermal growth factor receptor (EGFR) family agonists represents the main determinant of breast cancer cell proliferation. Stromal cell-derived factor-1 (SDF-1) enhances extracellular signal-regulated kinases 1 and 2 (ERK1/2) activity via the transactivation of EGFR and 17 β -estradiol (E2) induces SDF-1 production to exert autocrine proliferative effects. On this basis, we evaluated whether the inhibition of the tyrosine kinase (TK) activity of EGFR may control different mitogenic stimuli in breast tumors using the EGFR-TK inhibitor gefitinib to antagonize the proliferation induced by E2 in T47D human breast cancer cells. EGF, E2, and SDF-1 induced a dose-dependent T47D cell proliferation, that being nonadditive suggested the activation of common intracellular pathways. Gefitinib treatment inhibited not only the EGF-dependent proliferation and ERK1/2 activation but also the effects of SDF-1 and E2, suggesting that these activities were mediated by EGFR transactivation. Indeed, both

SDF-1 and E2 caused EGFR tyrosine phosphorylation. The molecular link between E2 and SDF-1 proliferative effects was identified because 1,1'-(1,4-phenylenebis(methylene))-bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride (AMD3100), a CXCR4 antagonist, inhibited SDF-1- and E2-dependent proliferation and EGFR and ERK1/2 phosphorylation. EGFR transactivation was dependent on c-Src activation. E2 treatment caused a powerful SDF-1 release from T47D cells. Finally, in SKBR3, E2-resistant cells, EGFR was constitutively activated, and AMD3100 reduced EGFR phosphorylation and cell proliferation, whereas HER2-neu was transactivated by SDF-1 in SKBR3 but not in T47D cells. In conclusion, we show that activation of CXCR4 transduces proliferative signals from the E2 receptor to EGFR, whose inhibition is able to revert breast cancer cell proliferation induced by multiple receptor activation.

Breast cancer is the most frequent cause of cancer-related deaths in women. Whether or not breast cancers are estro-

gen-dependent represents a critical factor for patients' prognosis and feasibility of antiestrogenic therapy. Two thirds of breast carcinomas express estrogen receptor- α (ER α) and most of them are responsive to antiestrogens or aromatase inhibitors (Nilsson et al., 2001). However, many initially responsive, ER α -positive breast carcinomas frequently acquire resistance to endocrine therapy. The overexpression or

This work was supported by a research grant from Astra-Zeneca (Milano, Italy) and Italian Association for Cancer Research.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.107.039974.

ABBREVIATIONS: ER, estrogen receptor; E2, 17 β -estradiol; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; GPCR, G protein-coupled receptors; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide; SDF-1, stromal cell derived factor-1; PP1, 4-amino-5-methylphenyl-7-(t-buthyl)pyrazolo 3,4-[d]pyrimidine; TK, tyrosine kinase; AMD3100, 1,1'-(1,4-phenylenebis(methylene))bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride; MEK, mitogen-activated protein kinase kinase; DMEM, Dulbecco's modified Eagle's medium; dn, dominant negative; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular signal-regulated kinases 1 and 2; PD98059, 2'-amino-3'-methoxyflavone; ICI 128,436, 3-(4-bromo-2-fluorobenzyl)-3,4-dihydro-4-oxo-1-phthalazineacetic acid; AZD0530, 4-(6-chloro-2,3-methylenedioxylanilino)-7-(2-(4-methylpiperazin-1-yl)ethoxy)-5-tetrahydropyran-4-yloxyquinazoline.

hyperactivation of epidermal growth factor receptor (EGFR) represents one of the main determinants of the antiestrogen resistance (Nicholson et al., 1994), mainly through ERK1/2 activation (Gee et al., 2001). Altered EGFR activity is involved in the initiation, development, and progression of different tumors, with its hyperactivation generally considered to be a negative prognostic feature (Nicholson et al., 2001). The activation of ERK1/2 via EGFR plays a significant role in breast cancer tumorigenesis, with EGFR (in particular HER2-neu) overexpression often responsible for increased duration and magnitude of ERK1/2 activity. ERK1/2, besides its direct role in cell proliferation, also increases ER activity via the phosphorylation of serine¹¹⁸ (Yue et al., 2002). In turn, ER potentiates ERK1/2 activity (Improta-Brears et al., 1999) in a self-propagating loop. Moreover, elevated ERK1/2 activity was associated with a highly invasive and metastatic behavior of breast cancer (Sivaraman et al., 1997). Thus, deregulation of ERK1/2 via EGFR accounts for most of the intracellular mechanisms associated with antiestrogen resistance in breast cancer.

Besides growth factor receptors, G protein-coupled receptors (GPCRs) activate ERK1/2 via the regulation of small G proteins, the direct activation of Raf or MEK (Della Rocca et al., 1999), or the transactivation of EGFR through the Src-dependent phosphorylation of its intracellular domain. EGFR phosphorylation allows the assembly of the Shc/Grb2/SoS complex and the activation of the mitogen-activated protein kinase cascade (Luttrell et al., 1999).

Stromal cell-derived factor-1 (SDF-1, or CXCL12), upon binding to CXCR4, activates ERK1/2 via a Src-dependent transactivation of EGFR (Cabioglu et al., 2005; Porcile et al., 2005). SDF-1 is an autocrine/paracrine chemokine acting as a proliferative and chemotactic factor for B and T lymphocytes and endothelial cells, stimulating cell migration through the reorganization of the cytoskeleton. SDF-1 and CXCR4 are essential components of organogenesis, hematopoiesis, immune response (Tachibana et al., 1998), and the development and functioning of the central nervous system (Bonavia et al., 2003). Besides its physiological functions, SDF-1 is also a powerful mitogen for normal (Bonavia et al., 2003) and cancer cells (Barbero et al., 2003; Balkwill, 2004) via the activation of ERK1/2. SDF-1 and CXCR4 are overexpressed in ovarian and breast cancer cells, affecting tumor cell migration and metastasization (Müller et al., 2001; Kang et al., 2005). CXCR4 overexpression was recently recognized as a requirement for HER2-mediated metastasis (Li et al., 2004) and breast cancer cell proliferation. Silencing of CXCR4 causes a significant reduction of breast cancer cell proliferation in vivo and in vitro (Smith et al., 2004; Lapteva et al., 2005), suggesting a possible autocrine/paracrine growth factor role of SDF-1 (Luker and Luker, 2006). It is noteworthy that it was proposed that 17 β -estradiol (E2)-induced proliferation of T47D and MCF-7 breast cancer cell lines is mediated by an increased synthesis and release of SDF-1, identified as a novel estrogen-responsive gene that, in an autocrine/paracrine fashion, controls ERK1/2 activity (Hall and Korach, 2003).

In the perspective of an integration between E2 and EGF signaling (Silva and Shupnik, 2007), the inhibition of EGFR tyrosine kinase (TK) activity and downstream signaling cascade provides the theoretical basis for the use of specific TK inhibitors for the treatment of breast cancer. Gefitinib

(ZD1839, Iressa) is a nonpeptide anilino-quinazoline compound that selectively inhibits the EGFR-TK activity with an IC₅₀ value of 0.02 μ M (Herbst et al., 2004). In tumor cell lines, gefitinib abolishes the activity of HER2-neu, the heterodimerization partner of EGFR, resulting in the inhibition of downstream signaling (Sewell et al., 2002). Gefitinib inhibits the growth of tumor cell lines that express EGFR and induces complete regression of well-established tumor xenografts (Ciardiello et al., 2000; Heimberger et al., 2002).

Here we evaluated the effects of gefitinib on E2-induced proliferation of T47D human breast cancer cells. In particular, we investigated the role of SDF-1/CXCR4 as a convergence point between E2 and EGFR intracellular pathways. We demonstrate that E2-induced breast cancer cell proliferation is dependent on the autocrine activation of CXCR4 followed by a c-Src-dependent transactivation of EGFR. As a consequence, the inhibition of the EGFR-TK by gefitinib reduces SDF-1- and E2-mediated cell proliferation and ERK1/2 activation. Conversely, in the E2-insensitive SKBR3 cells, EGFR and HER2-neu transactivation is potentiated by a constitutive CXCR4 activity.

Materials and Methods

Antibodies and Reagents. Antibodies against phospho-ERK1/2, ERK1/2, phospho-HER2-neu/ErbB2 (Tyr1221/1222), HER2-neu/ErbB2, phospho-Akt (Ser473), Akt, and phospho-Src (Tyr416) were purchased from Cell Signaling Technology (Danvers, MA); anti-phospho-EGFR (Tyr1173) and anti-EGFR were from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-phospho-Src (Tyr529) and anti-Src pan antibodies were purchased from BioSource International (Nivelles, Belgium). SDF-1 and EGF were obtained from PeproTech (London, UK).

E2 (stock solution 1 mM in ethanol), AMD3100 (stock solution 10 mM in distilled water), PD98059, and wortmannin (stock solution 10 mM in dimethyl sulfoxide) were purchased from Sigma-Aldrich (Milano, Italy). c-Src inhibitor 4-amino-5-methylphenyl-7-(*t*-buthyl)-pyrazolo 3,4-[d]pyrimidine (PP1) (stock solution 1 mM in DMSO) was from BIOMOL Research Laboratories (Plymouth, PA), and gefitinib (ZD1839; Iressa, stock solution 1 mM in DMSO) was kindly provided by Astra Zeneca (Milano, Italy). When appropriate, the same dilution of the different vehicles was added in the respective control samples.

Cell Lines and Transfections. The human breast cancer cell lines T47D and SKBR3 were obtained from the Interlab Cell Line Collection (Genova, Italy). Cells were cultured in DMEM containing 10% fetal bovine serum (FBS), penicillin-streptomycin (100 U/ml), and L-glutamine (2 mM). Before all of the experiments, cells were cultured for 48 h in phenol red-free DMEM and estrogen-deprived FBS and starved for further 48 h in DMEM without phenol red alone. All of these reagents were purchased from Euroclone (Milano, Italy).

Transfection of T47D was performed using the Fugene reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Cells were transfected with pUSEamp vector containing the cDNA encoding c-Src wild-type or a Src dominant-negative mutant (K296R/Y528F mutations) (Upstate Biotech, Charlottesville, VA) (Arena et al., 2007). The plasmid encoding MEK dominant-negative mutant (K97R) has been described previously (Florio et al., 1999).

[³H]Thymidine Incorporation Assay. DNA synthesis was measured by means of the [³H]thymidine uptake assay. Cells were plated at 20,000/well in 24-well plates, serum-starved for 48 h, and then treated with SDF-1, E2, or EGF for 24 h; in the last 4 h, cells were pulsed with 1 μ Ci/ml [³H]thymidine (GE Healthcare, Milano, Italy). When indicated, cultures were pretreated with gefitinib (0.1–1 μ M for 10 min), AMD3100 (1–100 μ M for 10 min), PD98059 (10 μ M for 10 min), and wortmannin (1 μ M for 10 min).

Cell Survival Assay. Cell viability was evaluated measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide (MTT). The cleavage of MTT to a purple formazan product by mitochondrial dehydrogenase was quantified spectrophotometrically. In brief, treated and control cells were incubated for 1 h with 0.25 mg/ml MTT in serum-free culture medium at 37°C; after the removal of the medium, formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm.

Human SDF-1 Detection Immunoassay. Quantitative determination of SDF-1 concentration was carried out in cell culture supernatants using the Quantikine kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Quantitative Real Time-PCR. Total RNA was isolated from T47D and SKBR3 cells using the RNeasy isolation system (QIAGEN, Hilden, Germany). First-strand cDNA was synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and random hexamer primers (Promega), according to the manufacturer's instructions. Quantitative real time-PCR was performed using the iQ SYBR Green Supermix (Bio-Rad Laboratories, Milano, Italy) in a Mini Opticon System (Bio-Rad Laboratories).

Standard curves were plotted by using serially diluted cDNA of the SKBR3 and T47D cell lines; for quantification of the results, the CXCR4 and SDF-1 mRNA levels were normalized, in the same reaction, to the L-41 mRNA level and expressed as ratio of the normalized expression of the gene of interest. The following primers were used: human CXCR4, 5'-CCTCCTGCTGACTATTCCCGA-3' and 5'-GGAACACAACCACCCACAAGT-3'; human SDF-1, 5'-GCCCCGTCAGCCTGAGCTACA-3' and 5'-TTCTTCAGCCGGGCTACAATCT-3'; and human L-41, 5'-AGTGGAGGAAGAAGCGAATG-3' and 5'-TTTATGAGCAAGGTGGGTCTC-3'. For each measurement, two independent qPCR analyses were performed.

Western Blotting. Cultures were serum-starved for 48 h and treated as described. Then cells were lysed in a buffer containing 1% Nonidet P-40, 20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM NaF (all from Sigma-Aldrich), and the Complete protease inhibitor cocktail (Roche Diagnostics SpA, Milano, Italy) for 10 min at 4°C. Nuclei were removed by centrifugation (5000 rpm at 4°C) for 10 min, and total protein contents were measured using the Bradford assay (Bio-Rad Laboratories). Proteins (5–10 µg) were diluted in equal volume of 2× reducing sample buffer (2% SDS, 62.5 mM Tris, pH 6.8, 0.01% bromophenol blue, 1.43 mM 2-mercaptoethanol, and 0.1% glycerol), electrophoresed on 10 to 15% SDS-

polyacrylamide gels, transferred on polyvinylidene difluoride membrane (Bio-Rad Laboratories), and probed with specific antibodies. The detection of immunocomplexes was performed using the enhanced chemiluminescence system (GE Healthcare).

Statistical Analysis. Unless otherwise specified, all experiments were performed in quadruplicate and repeated at least three times. Data are expressed as mean ± S.E. values, and statistical significance was assessed by analysis of variance. A *P* value less than or equal to 0.05 was considered statistically significant.

Results

Expression of CXCR4 and SDF1 in T47D and SKBR3

Cell Lines. To study the possible role of the SDF-1/CXCR4 system in the E2- and EGFR-mediated breast carcinoma cell proliferation, we first evaluated the mRNA expression of the chemokine and its receptor in the E2-sensitive T47D and E2-insensitive SKBR3 cell lines. T47D cells were reported previously to express ERα (Htun et al., 1999), EGFR, and moderate levels of HER2-neu (Cabioglu et al., 2005). SKBR3 cells express EGFR and high levels of HER2-neu but not ERα (Cabioglu et al., 2005).

Quantitative real time-PCR experiments showed that T47D cells express both CXCR4 and SDF-1 mRNA, whereas in SKBR3 cells, only CXCR4 mRNA was detected. Mean CXCR4 mRNA levels were 0.02 ± 0.005 and 0.06 ± 0.003 copy/copy L-41 for T47D and SKBR3 cells, respectively. T47D cells also showed high levels of SDF-1 mRNA (1.4 ± 0.02 copy/copy L-41). The different SDF-1 expression levels in the two cell lines were further confirmed evaluating the chemokine content in the cell culture media by ELISA. In normal culture conditions, T47D released 77 ± 5 pg of SDF-1/50,000 cells in 24 h, whereas in the same experimental conditions, the SDF-1 released in the culture medium by SKBR3 cells was lower than the ELISA detection level.

EGF, E2, and SDF-1 Induced Proliferation of Human T47D Breast Cancer Cells. The proliferation pattern of T47D cells in response to EGF, E2, and SDF-1 was assessed using the [³H]thymidine incorporation assay. To avoid estrogen-like activity of the culture medium, before the experiments, breast cancer cells were cultured 2 days in DMEM

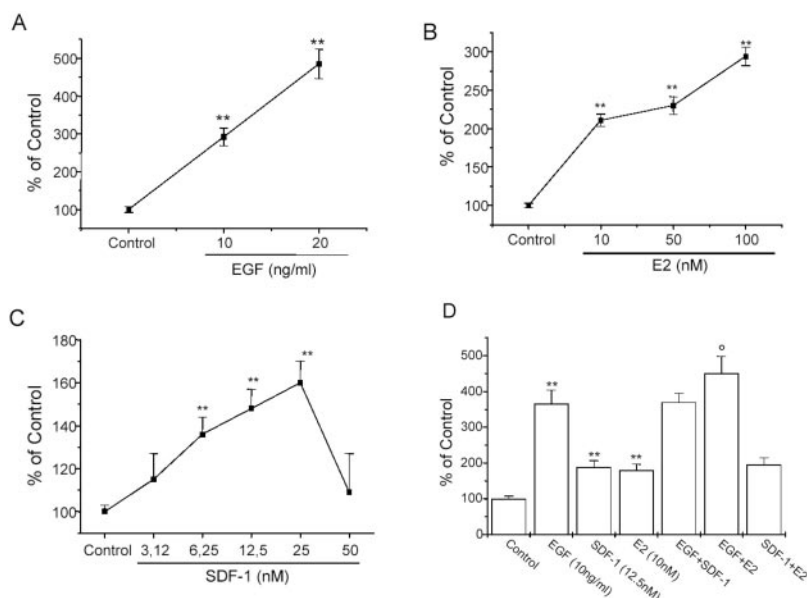


Fig. 1. Activation of DNA synthesis in T47D breast cancer cells in response to EGF, E2, and SDF-1. A to C, serum-starved T47D cells were treated with increasing concentrations of EGF (A), E2 (B), and SDF-1 (C). Data are expressed as a percentage of untreated controls ± S.E. of three replicate determinations (**, *P* < 0.01 versus control value). EGF and E2 significantly stimulate T47D cell proliferation at all of the concentrations tested; SDF-1 induces a significant dose-dependent growth stimulation from 6.25 to 25 nM, whereas for the highest dose tested (50 nM), a lower effect is observed. D, serum-starved T47D cells were cotreated with concentrations of EGF (10 ng/ml), SDF-1 (12.5 nM), and E2 (10 nM) that give submaximal proliferative stimuli. Data are expressed as a percentage of the untreated control. Each bar represents the mean ± S.E. of three replicate determinations (**, *P* < 0.01 versus control, °, *P* < 0.05 versus EGF-stimulated cells). Each molecule induced a significant increase of [³H]thymidine uptake versus the untreated control but only the combination of EGF + E2 showed a slight but statistically significant additive effect.

without phenol red, supplemented with estrogen-deprived FBS and then grown in the same medium without serum for further 48 h (see *Materials and Methods*). EGF treatment (10–20 ng/ml) induced a powerful increase in T47D cell proliferation showing dose-dependence and a maximal stimulation of 4-fold over the basal value (Fig. 1A).

E2 (10–100 nM) also induced T47D cell proliferation in a dose-dependent manner, increasing DNA synthesis up to +200% compared with untreated control cells at the highest concentration tested (Fig. 1B). When T47D cells were treated with SDF-1 (3.12–50 nM), a statistically significant increase of [³H]thymidine incorporation was reached at the concentration of 6.25 nM, whereas a maximal effect was observed at 25 nM (Fig. 1C). Conversely, the highest SDF-1 concentration tested (50 nM) induced a proliferative response lower than that observed with 25 nM SDF-1 (Fig. 1C). A similar effect was observed in other human tumor cell lines (glioblastoma, pituitary adenoma, ovarian carcinoma) and was proposed to be dependent on a down-regulation of CXCR4 (Barbero et al., 2003; Porcile et al., 2005; Florio et al., 2006). These data show that besides E2 and EGF, SDF-1 is also an effective mitogenic factor for T47D cells.

To investigate the possible cross-talk among these receptor systems leading to cell proliferation, we performed [³H]thymidine incorporation experiments in T47D cells treated with the combination of submaximal concentrations of EGF (10 ng/ml), SDF-1 (12.5 nM), and E2 (10 nM). Figure 1D shows that both EGF and E2 did not display additive effect as far as cell proliferation when coadministered with SDF-1, whereas a less than additive potentiation of the EGF-dependent cell proliferation was observed in the presence of E2. These data suggest the possibility that all of these mitogens may act via the activation of the same intracellular pathway.

Gefitinib Treatment Inhibited T47D Mitogen-Dependent Cell Proliferation and EGFR and ERK1/2 Activation. Then we evaluated whether E2, SDF-1, and EGF may induce their proliferative effects via the activation of a common intracellular signaling. Indeed, E2 was reported to induce the release of SDF-1 (Hall and Korach, 2003) that may exert its proliferative activity via the transactivation of EGFR (Cabioglu et al., 2005; Porcile et al., 2005). Thus, if this was the case, we should be able to block all these proliferative stimuli by inhibiting the EGFR kinase activity. To verify this hypothesis, we pretreated T47D cells with gefitinib, an inhibitor of the EGFR-TK activity, and measured cell proliferation, EGFR phosphorylation, and ERK1/2 activation in response to EGF, SDF-1, and E2. To demonstrate the specificity of the gefitinib activity and to exclude nonspecific toxic effects, we first evaluated the direct cytotoxicity in vitro of increasing doses of gefitinib (0.01–10 μ M) on T47D cells in the absence of EGF treatment using the MTT assay. A significant (50%) cytotoxicity occurred only at the concentration of 10 μ M, whereas at lower concentrations (0.01–1 μ M), gefitinib per se did not affect cell viability (data not shown). Thus, these concentrations were considered suitable for the following experiments to specifically interfere with EGF intracellular signaling.

We performed [³H]thymidine uptake dose-response experiments to study the effects of gefitinib on T47D cell proliferation induced by EGF (10 ng/ml). Figure 2A shows that gefitinib significantly reduced EGF-dependent T47D cell growth already at the concentration of 0.01 μ M, whereas at

0.1 μ M and 1 μ M, the inhibition was more complete, reducing, for the highest concentration tested, DNA synthesis below the basal level. To confirm the specificity of the effects of gefitinib in the cell proliferation mediated by EGF, we investigated its effects on EGFR and ERK1/2 activation induced by EGF. T47D cells were serum-starved for 48 h, pretreated with increasing concentrations of gefitinib (0.01–1 μ M) for 10 min, and then treated with EGF (10 ng/ml) for 5 min. T47D cell lysates were immunoprobed with anti phospho-EGFR and anti-phospho-ERK1/2 antibodies. In these experimental conditions, EGF treatment caused a marked EGFR and ERK1/2 phosphorylation that was completely reverted by gefitinib in a dose-dependent manner (Fig. 2B).

To analyze whether SDF-1-dependent T47D cell proliferation may involve EGFR transactivation, we evaluated the possible EGFR phosphorylation after treatment with this chemokine. In Fig. 3, we show that SDF-1 treatment increased EGFR tyrosine phosphorylation that was detectable after 10 min of treatment and lasted up to 2 h (Fig. 3A, left). The cotreatment with SDF-1 and EGF resulted in a slight increase in EGFR phosphorylation compared with the individual treatments (Fig. 3A, right). The role of EGFR transactivation in SDF-1 proliferation was then directly demonstrated showing that gefitinib, at concentrations devoid of any aspecific toxic effect (1 μ M and lower), reduced the proliferation induced by the chemokine (Fig. 3B). The proliferative effects of SDF-1 were dependent on ERK1/2 activation because it was completely abolished in the presence of the MEK inhibitor PD98059 or transfecting the cells with a dominant-negative mutant of MEK (mutation K97R, MEKdn)

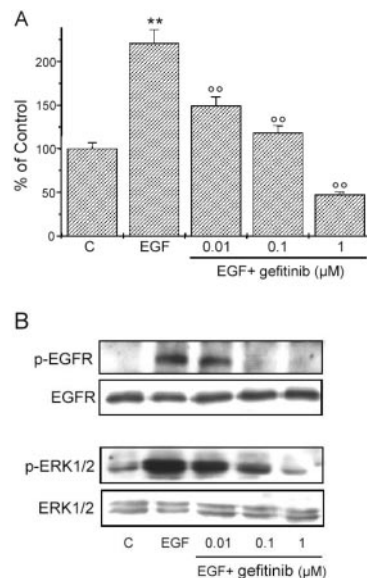


Fig. 2. Effects of gefitinib on the EGF-induced DNA synthesis and EGFR/ERK1/2 activation. A, [³H]thymidine incorporation assay on serum-starved T47D cells treated with EGF (10 ng/ml) in the absence or presence of increasing concentrations of gefitinib. Data are expressed as a percentage of the untreated control (C). Each bar represents the mean \pm S.E. of three replicate determinations. Gefitinib inhibits EGF-induced cell proliferation in a dose-dependent manner (**, $P < 0.01$ versus control cells; °°, $P < 0.01$ versus EGF-stimulated cells). B, cells were treated with EGF (10 ng/ml) for 5 min in the absence or presence of increasing concentrations of gefitinib; cell lysates were analyzed by Western blotting. Blots were probed with anti-phospho-EGFR and anti-phospho-ERK1/2 antibodies. Anti-EGFR and anti-ERK1/2 antibodies were used to reprobe blots to ensure the equal loading of proteins. Gefitinib dose-dependently inhibits the phosphorylation of EGFR and ERK1/2 induced by EGF treatment.

(Fig. 3C). It is noteworthy that ERK1/2 activation induced by SDF-1 was blocked not only by the overexpression of MEKdn, as expected, but also by gefitinib pretreatment (Fig. 3, D and E). Thus, these experiments indicate that the mitogenic activity of SDF-1 is mainly dependent on EGFR transactivation and that it is possible to shut down SDF-1-dependent T47D cell growth, blocking the kinase activity of this receptor.

Likewise, E2 treatment caused EGFR phosphorylation starting after 10 min of treatment, reaching a maximum after 1 h, and then lasting up to 2 h (Fig. 4A). In [³H]thymidine uptake experiments, we show that gefitinib significantly reduced E2-dependent proliferation, although, in the presence of high E2 concentrations (100 nM), the inhibition was not complete (−42% for the gefitinib concentration of 0.1 μM and −62% for 1 μM) (Fig. 4B). E2-dependent proliferation was mediated by ERK1/2 activation, because it was completely abolished in the presence of PD98059 and in cells transfected with MEKdn (Fig. 4C). Furthermore, E2-dependent ERK1/2 activation was also completely abridged in cells transfected with MEKdn (Fig. 4D). In agreement with the involvement of EGFR transactivation in E2-regulated proliferation, the pretreatment with gefitinib significantly reduced E2-induced ERK1/2 activation (Fig. 4E). These data confirm that the E2-dependent proliferation of T47D cells is, in a large part, mediated by the activation of EGFR.

E2 Induced T47D Cell Proliferation Inducing SDF-1 Secretion and Autocrine/Paracrine Activation of CXCR4. In T47D cells, both E2 and SDF-1 exert their proliferative effects, at least in part, through EGFR transactivation. It was reported previously that E2 induces breast cancer cell proliferation through the stimulation of SDF-1 secretion (Hall and Korach, 2003). Thus, we evaluated whether, in T47D cells, a similar mechanism could take part in the proliferative effects of E2. To this aim, we used the selective CXCR4 antagonist AMD3100 (Rosenkilde et al., 2004) to evaluate the effect of CXCR4 inhibition on cell proliferation induced by SDF-1, E2, and EGF. As expected, all SDF-1 mitogenic effects were inhibited by the pretreatment with AMD3100 in a dose-dependent manner (Fig. 5AI). Likewise, ERK1/2 activation induced by SDF-1 was completely inhibited in the presence of AMD3100 (Fig. 5AII).

The pretreatment with AMD3100 also caused a significant inhibition of the E2-dependent proliferation. In particular, the highest AMD3100 concentration tested (100 μM) that per se did not modify DNA synthesis, reduced by 60% E2-dependent cell proliferation (Fig. 5BI). Moreover, we show that in the presence of AMD3100, E2-dependent stimulation of ERK1/2 phosphorylation was significantly reduced (Fig. 5BII). These results clearly demonstrate the involvement of CXCR4 activation in E2-promoted T47D cell proliferation.

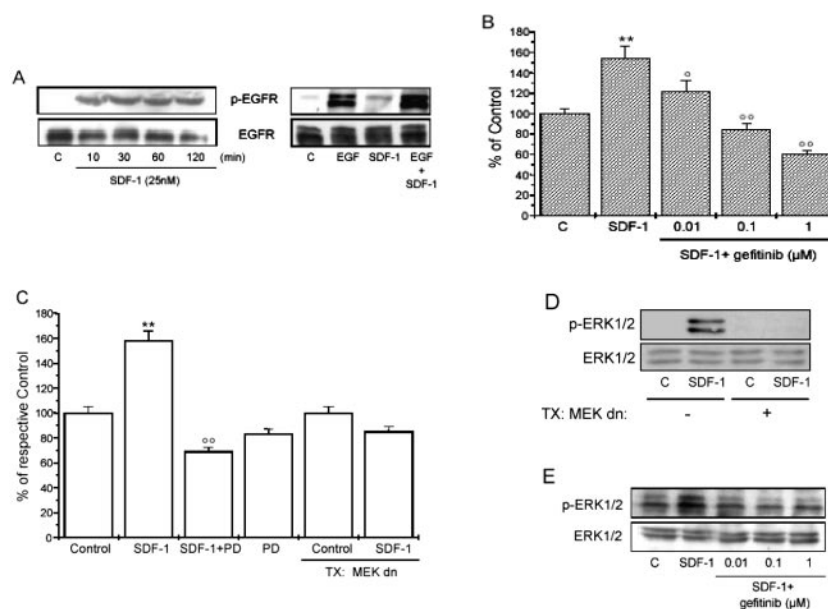


Fig. 3. A, SDF-1 modulation of EGFR phosphorylation in T47D cells. Left, lysates from cells treated with SDF-1 (25 nM) were analyzed by Western blotting. Blots were probed with anti-phospho-EGFR and then reprobed with anti-EGFR antibodies to ensure the equal loading of proteins. EGFR phosphorylation is detected after 10 min of exposure to SDF-1 and lasts up to 120 min. Right, cells treated with EGF (10 ng/ml), SDF-1 (25 nM) alone, or in combination were analyzed by Western blotting using anti-phospho-EGFR and anti-EGFR antibodies. The cotreatment with SDF-1 and EGF causes a slight additive increase in EGFR phosphorylation. B, effects of gefitinib on SDF-1-induced DNA synthesis. [³H]thymidine incorporation assay on serum-starved T47D cells treated with SDF-1 (25 nM) in the absence or presence of increasing concentrations of gefitinib. Data are expressed as a percentage of the untreated control. Each bar represents the mean \pm S.E. of three replicate determinations. Gefitinib significantly inhibits SDF-1-induced cell proliferation in a dose-dependent manner (**, $P < 0.01$ versus control; °, $P < 0.05$ versus stimulated cells; °°, $P < 0.01$ versus stimulated cells). C, effects of MEK inhibition by PD98059 or MEKdn transfection in T47D cells on SDF-1-induced DNA synthesis. [³H]Thymidine incorporation assay on serum-starved T47D cells treated with SDF-1 (25 nM) in the absence or presence of PD98059 (10 μM, PD). Data are expressed as a percentage of untreated controls. Each bar represents the mean \pm S.E. of three replicate determinations. PD98059 significantly inhibits SDF-1-induced cell proliferation in wild-type T47D. SDF-1 does not affect DNA synthesis of MEKdn (dominant-negative mutant of MEK_[K97R])-transfected cells (**, $P < 0.01$ versus control; °°, $P < 0.01$ versus stimulated cells). D, the expression of MEKdn reverses the SDF-1-dependent ERK1/2 phosphorylation Western blot analysis of ERK1/2 phosphorylation after SDF-1 (25 nM) treatment in T47D wt or transfected with MEKdn (dominant-negative mutant of MEK_[K97R]). The expression of MEKdn blocks ERK1/2 activation by SDF-1. E, effects of gefitinib on the SDF-1-induced ERK1/2 activation. Cells were treated with SDF-1 (25 nM) in the absence or presence of increasing concentrations of gefitinib. Cell lysates were analyzed in Western blot experiments using anti-phospho-ERK1/2 antibody and reprobed with anti-ERK1/2 antibody to ensure equal loading of proteins. Gefitinib inhibits ERK1/2 phosphorylation induced by SDF-1 in a dose-dependent manner.

Conversely, EGF-mediated proliferation and ERK1/2 activation were not affected by AMD3100 pretreatment (data not shown), thus confirming that as far as the E2- and SDF-1-dependent proliferation, EGFR activation is downstream of the stimulation of CXCR4.

SDF-1 is functional soon after it is secreted from cells, an event that enables it to signal through its cell surface receptor CXCR4. Thus, we evaluated the possibility that E2 treatment may promote SDF-1 secretion, thus representing an intermediate step between ER α and EGFR activation. In these experiments, T47D cells, plated at 60% confluence in 24-well plates in serum-free medium were treated with E2 (50 nM) for 24 or 48 h, and cell culture supernatants were analyzed for SDF-1 contents by ELISA.

In basal conditions, T47D cells produce and secrete moderate amounts of SDF-1 in a time-dependent manner (Fig. 6A). E2 treatment (50 nM) markedly increased SDF-1 accumulation in the culture medium (3- and 4-fold over basal levels after 24 and 48 h, respectively). E2-induced SDF-1 secretion was inhibited by the pretreatment with the ER antagonist ICI 128,436 (Fig. 6B), confirming the specificity of the E2 effects in the enhancement of SDF-1 secretion. These data show that T47D cells synthesize and release SDF-1 in response to E2 and that this activity may induce an auto-crane-paracrine mechanism of proliferation via CXCR4.

SDF-1 Transactivation of EGFR Was Mediated by a CXCR4 Regulation of Src Activity. Many GPCRs control tyrosine kinase receptor activity via a Src-dependent transactivation. Thus, we verified whether, in T47D cells, CXCR4 modulation of EGFR activity was dependent on the activation of this kinase. We measured Src activation by SDF-1, monitoring the phosphorylation state of the tyrosines 416 and 529 located in the catalytic domain and the carboxyl terminus of the protein, respectively (Arena et al., 2007). Under basal conditions, tyrosine 529 is phosphorylated by the C-terminal Src kinase. Phosphorylated tyrosine 529 acts as a negative regulator, keeping Src inactive through an intramolecular interaction with its SH2 domain. When tyrosine 529 is dephosphorylated, Src conformation changes and allows the autophosphorylation of the tyrosine 416 causing a powerful increase in Src catalytic activity. Using specific antibodies directed against phosphorylated Src Tyr529 and Tyr416, we were able to evaluate in WB experiments the changes in Src phosphorylation at these sites, representing indexes of the kinase activation.

Figure 7, A and B, depicts the time course of the effects of SDF-1 (25 nM) on pSrc^{Y529} and pSrc^{Y416} levels. The chemokine induced the dephosphorylation of the inhibitory tyrosine of Src starting after 60 s of treatment, increasing until 300 s, with a partial recovery toward basal level after 600 s. A

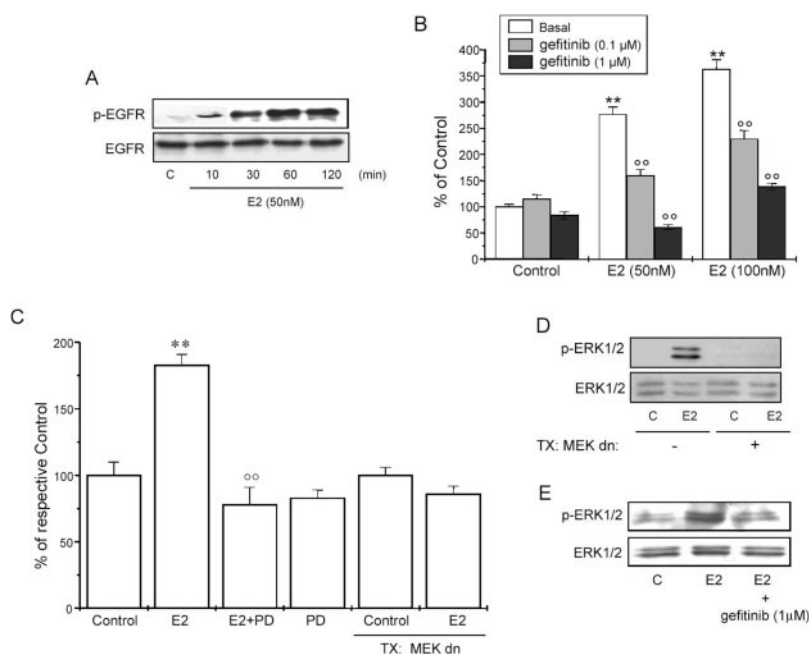


Fig. 4. A, time course of EGFR activation in T47D cells treated with E2. Lysates from cells treated with E2 (50 nM) were analyzed by Western blotting. Blots were probed with anti-phospho-EGFR and reprobed with anti-EGFR antibodies to ensure the equal loading of proteins. E2 treatment induces EGFR phosphorylation starting after 10 min of treatment, reaching a maximum after 60 min, and lasting up to 120 min. B, effects of gefitinib on the E2-induced DNA synthesis. [³H]Thymidine incorporation assay on serum-starved T47D cells treated with E2 in the absence or presence of gefitinib (0.1 or 1 μ M). Data are expressed as a percentage of the untreated controls. Each bar represents the mean \pm S.E. of three replicate determinations. Both concentrations of gefitinib significantly inhibit E2-induced cell proliferation (**, $P < 0.01$ versus control; $^{\circ\circ}$, $P < 0.01$ versus stimulated cells). C, effects of MEK inhibition by PD98059 or MEKdn transfection in T47D cells on E2-induced DNA synthesis. [³H]Thymidine incorporation assay on serum-starved wild-type T47D cells treated with E2 (100 nM) in the absence or presence of PD98059 (10 μ M, PD). Data are expressed as a percentage of the untreated controls. Each bar represents the mean \pm S.E. of three replicate determinations. PD98059 significantly inhibits E2-induced wt T47D cell proliferation, whereas E2 does not increase DNA synthesis in MEKdn (dominant-negative mutant of MEK_[K97R]) transfected cells. (**, $P < 0.01$ versus control; $^{\circ\circ}$, $P < 0.01$ versus stimulated cells). D, the expression of MEKdn reverses the E2-dependent ERK1/2 phosphorylation Western blot analysis of ERK1/2 phosphorylation after E2 (100 nM) treatment in wild-type T47D cells or in cells transfected with MEKdn (dominant-negative mutant of MEK_[K97R]). The expression of MEKdn blocks ERK1/2 activation by E2. E, effects of gefitinib on E2-induced ERK1/2 activation. Cells were treated with E2 (50 nM) in the absence or presence of gefitinib (1 μ M). Cell lysates were analyzed by Western blotting; blots were probed with anti-phospho-ERK1/2 and reprobed with anti-ERK1/2 antibodies to ensure the equal loading of proteins. The phosphorylation of ERK1/2 induced by E2 treatment is inhibited by gefitinib pretreatment.

mirror-like effect was observed on pSrc^{Y416}. To demonstrate the specificity of the SDF-1 effects, we evaluated Src phosphorylation at Tyr529 treating the cells with SDF-1 (25 nM for 5 min) in the presence of the CXCR4 antagonist AMD3100 (1 μ M). As shown in Fig. 7C, AMD3100 inhibits Src^{Y529} dephosphorylation/activation subsequent to SDF-1 treatment, thus confirming the role of CXCR4 in the modulation of Src activity by SDF-1.

To assess the role of Src activation in SDF-1 and E2 proliferative effects, we performed [³H]thymidine incorporation experiments in T47D cells treated with the two mitogens in the presence or absence of the Src inhibitor PP1 (200 nM) (Fig. 8A). The inhibition of Src activation caused a statistically significant reduction of both SDF-1 and E2-induced cell proliferation, thus confirming that the activation of this kinase is also involved in the E2- and SDF-1-induced cell proliferation. Moreover, we analyzed the effects of PP1 on ERK1/2 phosphorylation/activation induced by EGF, SDF-1, and E2. As shown in Fig. 8B, Src activity inhibition by PP1 completely reverted ERK1/2 phosphorylation induced by SDF-1 (I) and E2 (II). Conversely, no effects were observed on EGF-mediated ERK1/2 activation (Fig. 8BI). To further assess the role of Src in E2, SDF-1, and EGF activation of ERK1/2, we used T47D cell transfected with a dominant-negative mutant of Src (Src_[K296R/Y528F] mutations) (Arena et al., 2007). When Src activation was blocked by the overexpression of Src_[K296R/Y528F], the increase in ERK1/2 phosphorylation was not detectable after treatments with either E2 or SDF-1 but only in the presence of EGF (Fig. 8BIII).

Role of Akt Activation in the SDF-1-Induced Proliferation of T47D Cells. It was reported that in several tumor cell lines SDF-1 proliferative effects involve the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway (Barbero et al., 2003). Akt activation by SDF-1 was reported to be independent of ERK1/2 activation

being mainly involved in CXCR4-mediated tumor cell migration (Cabioglu et al., 2005; Peng et al., 2005). To evaluate the role of the PI3K/Akt pathway in the T47D cell proliferation induced by SDF-1, we performed [³H]thymidine uptake experiments in the presence of the PI3K inhibitor wortmannin that completely blocked SDF-1 induced DNA synthesis (Fig. 9A). The involvement of Akt in the intracellular signaling upon CXCR4 activation in T47D cells was directly demonstrated measuring the Akt phosphorylation levels in Western blot experiments (Fig. 9B). Similar results were also obtained after E2 treatment (Fig. 9, A and B), reinforcing the hypothesis that T47D cells are stimulated to proliferation through the sequential activation of ER α and CXCR4.

Constitutive CXCR4/EGFR Signaling Controlled Proliferation in E2-Insensitive SKBR3 Breast Cancer Cell Line. The identification of the molecular link between E2, SDF-1, and EGF in the regulation of breast cancer cell proliferation, led us to evaluate whether, in cells insensitive to E2, the commonly observed constitutive EGFR activation was the resultant of sustained autocrine/paracrine CXCR4 activation. To this aim we used the SKBR3 human breast cancer cell line that was reported to be E2-insensitive but express both EGFR and HER2-neu. These cells express CXCR4 mRNA [as reported previously by Cabioglu et al. (2005) and further verified by qPCR in this study, see above], but do not release SDF-1 (this study).

EGF treatment (10 ng/ml to 1 μ g/ml) of SKBR3 cells did not affect the proliferative activity of these cells (data not shown), whereas gefitinib treatment at concentrations that were noncytotoxic inhibited cell growth in both basal (data not shown) and EGF-treated conditions (Fig. 10A). These results suggest that EGFR may be already maximally activated under basal conditions.

Thus, to confirm this observation, EGFR phosphorylation status was analyzed. As shown in Fig. 10B (left), EGFR was

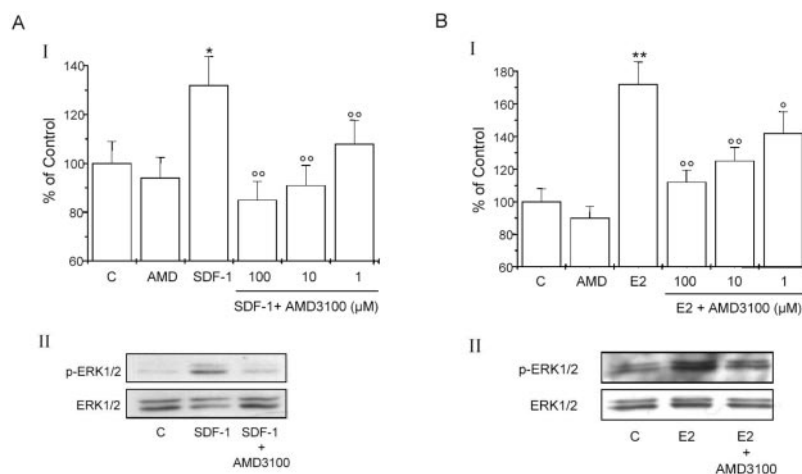


Fig. 5. A, effects of AMD3100 on the DNA synthesis and ERK1/2 activation induced by SDF-1. I, [³H]thymidine incorporation assay on serum-starved T47D cells treated with SDF-1 (25 nM) in the absence or presence of AMD3100 (1–100 μ M). Data are expressed as a percentage of basal value of the untreated controls. Each bar represents the mean \pm S.E. of three replicate determinations. AMD3100 significantly inhibits T47D cell proliferation induced by SDF-1 (*, $P < 0.05$ versus control cells; **, $P < 0.01$ versus SDF-1-treated cells). II, cells were treated with SDF-1 (25 nM) in the absence or presence of AMD3100 (1 μ M). Cell lysates were analyzed by Western blotting using anti-phospho-ERK1/2 antibody and reprobed with anti-ERK1/2 antibody to ensure the equal loading of proteins in different lanes. AMD3100 inhibits ERK1/2 activation induced by SDF-1. B, effects of AMD3100 on DNA synthesis and ERK1/2 activation induced by E2. I, [³H]thymidine incorporation assay on serum-starved T47D cells treated with E2 (50 nM) in the absence or presence of AMD3100 (1–100 μ M). Data are expressed as a percentage of the untreated control. Each bar represents the mean \pm S.E. of three replicate determinations. AMD3100 significantly inhibits T47D cell proliferation induced by E2 (**, $P < 0.01$ versus control cells; *, $P < 0.05$ and **, $P < 0.01$ versus E2-treated cells). II, cells were treated with E2 (50 nM) in the absence or presence of AMD3100 (1 μ M). Cell lysates were analyzed by Western blotting using anti-phospho-ERK1/2 antibody and reprobed with anti-ERK1/2 antibody to ensure the equal loading of proteins in different lanes. AMD3100 inhibits ERK1/2 activation induced by E2.

highly phosphorylated in untreated serum-starved SKBR3 cells. However, after EGF administration, the level of phospho-EGFR markedly increased, and the pretreatment with gefitinib greatly reduced its levels below the basal level.

Several data in literature reported a cross-talk between CXCR4 and HER2-neu/EGFR via receptor transactivation (Cabioglu et al., 2005; Luker and Luker, 2006). We also show that in SKBR3 cells, SDF-1 increased EGFR phosphorylation over the basal level, an effect that was additive to that of EGF (Fig. 10B, right). Therefore, we assayed the effect of the CXCR4 antagonist AMD3100 on cell proliferation.

It is interesting that AMD3100 reduced basal DNA synthesis (-27% , $P > 0.05$; data not shown), thus suggesting that, in SKBR3 cells, basal proliferation, probably dependent on EGFR constitutive activation, is related to the activity of CXCR4.

Moreover, we evaluated the activity of SDF-1 on SKBR3 alone and in combination with gefitinib. SDF-1 treatment induced a weak stimulation of SKBR3 cell proliferation compared with T47D ($+20\%$ versus $+60\%$, Figs. 10C and 1C, respectively) however, in the presence of gefitinib, a highly significant inhibition of DNA synthesis was observed in both basal and SDF-1-treated conditions, indicating the involvement of EGFR in these effects (Fig. 10C). Moreover, SDF-1 dose-response curve in SKBR3 cells was left-shifted compared with T47D cells with the down-regulation of the proliferative response occurring already at the concentration of 25 nM (compared with 50 nM in T47D cells). This different sensibility probably reflects the higher CXCR4 expression level detected in SKBR3 cells by qPCR experiments.

Furthermore, we evaluated the modulation of EGFR phosphorylation in the presence of AMD3100. In agreement with the proliferation data, we found that SDF-1 slightly in-

creased EGFR phosphorylation that, on the other hand, was significantly activated already in untreated cells (Fig. 11A). The inhibition of CXCR4 activity by AMD3100 significantly reduced basal and EGF- or SDF-1-induced EGFR phosphorylation (Fig. 11A, top), clearly implying the occurrence of a constitutive role of CXCR4 activity in EGFR activation. Likewise, AMD3100 slightly reduced ERK1/2 activation after EGF and SDF-1 treatment (Fig. 11A, bottom).

Another important feature of SKBR3 cells is represented by the high levels of expression of HER2-neu compared with the low-level expression in T47D cells (Cabioglu et al., 2005). Thus, we evaluated by comparing side-by-side the effects of SDF-1 on HER2-neu phosphorylation in both T47D and SKBR3 cells in the presence or absence of EGF. In basal conditions, a significant HER2-neu tyrosine phosphorylation was observed in SKBR3 but not in T47D cells. It is interesting that, in agreement with previous data (Cabioglu et al., 2005), EGF treatment increased HER2-neu phosphorylation in both cell lines, but SDF-1 was able to transactivate HER2-neu only in SKBR3 cells, whereas no effects were observed in T47D cells (Fig. 11B). However, differently from the previous study (Cabioglu et al., 2005), no additive effects were detected on HER2-neu phosphorylation by the cotreatment with EGF and SDF-1 in both cell lines (Fig. 11B).

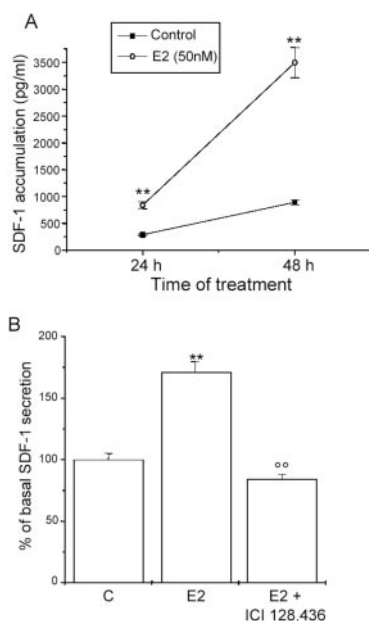


Fig. 6. Effects of E2 on SDF-1 secretion. A, SDF-1 concentrations in the culture medium were assayed in supernatants from serum-starved cells treated or not with E2 (50 nM, 24 or 48 h) by a specific ELISA. T47D cells constitutively released SDF-1 and this secretion is significantly increased after E2 treatment (**, $P < 0.01$ versus respective control values). B, effect of the ER antagonist ICI 128,436 (100 nM) on the E2-induced SDF-1 release from T47D cells (**, $P < 0.01$ versus control cells; **, $P < 0.01$ versus E2-treated cells).

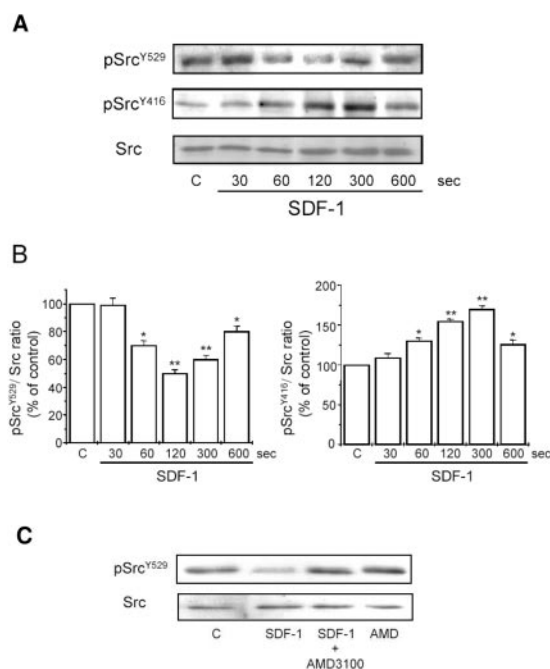


Fig. 7. SDF-1 treatment induces c-Src activation in T47D cells. A, time course of c-Src activation subsequent to SDF-1 treatment in T47D cells. SDF-1 (25 nM) effects were detected using anti pSrc^{Y529} (top) or pSrc^{Y416} (middle) antibody in Western blot. Normalization of protein loading was performed probing the membrane with an antibody directed against the total Src (bottom). B, quantification of SDF-1 effects on c-Src activation measured by densitometric analysis of the blots performed as described in A. Results represent the average of three independent experiments (*, $P < 0.05$ and **, $P < 0.01$ versus control values). C, effects of AMD3100 on SDF-1-induced c-Src activation. Cells were treated with SDF-1 (25 nM, 300 s) in the absence or presence of AMD3100 (1 μ M). Cell lysates were analyzed by Western blotting using anti-pSrc^{Y529} antibody and reprobed with anti-total Src antibody to ensure the equal loading of proteins. The panel reports a blot representative of two independent experiments. AMD3100 inhibits c-Src dephosphorylation/activation induced by SDF-1 treatment.

Discussion

Current antihormonal treatment of estrogen-positive breast cancer commonly leads to incomplete responses and development of resistance, ultimately resulting in disease recurrence. In particular, EGFR overexpression and dysregulated activation, occurring in 50% of breast cancers, has been linked to the acquired resistance to ER antagonists both in vitro and in vivo (Osborne et al., 2005). Thus, the understanding of the underlying molecular pathways, involved in estrogen- and growth factor-mediated signal transduction mechanisms involved in breast tumor cell proliferation, represents a major goal to improve therapies and prevent endocrine resistance.

SDF-1/CXCR4 system was reported to control proliferation, angiogenesis, migration, and metastasis in a variety of cancer cells (Balkwill, 2004), including breast carcinomas (Luker and Luker, 2006). In addition, breast cancer cell lines exhibiting an active autocrine SDF-1/CXCR4 signal pathway display aggressive behavior, increased invasiveness, and faster growth (Kang et al., 2005). E2 regulation of SDF-1 production and the consequent autocrine activation of

CXCR4 in breast carcinoma cells have been described previously (Hall and Korach, 2003), as well as the CXCR4-mediated EGFR and HER2-neu transactivation (Cabioglu et al., 2005; Porcile et al., 2005). Here we evaluated the role of SDF-1 as a convergence factor between the proliferative activity of E2 and EGF in breast cancer cells to establish a role for EGFR TK inhibitors as a tool to control not only EGF-induced proliferation but also E2 and SDF-1 growth effects.

We used the T47D cell line as in vitro model of ER-positive human breast cancer and SKBR3 cells as a model of E2-insensitive and HER2-neu-overexpressing cells. Both cell lines express CXCR4 and EGFR (Cabioglu et al., 2005; Müller et al., 2001, this study). Here we show that T47D cells but not SKBR3 cells release SDF-1 in E2-stimulated conditions. It is interesting that CXCR4 levels were higher in the SKBR3 cells that do not secrete SDF-1, suggesting the possibility of a down-regulation of the receptor in T47D cells. This evidence may reflect possible autocrine CXCR4 activation.

We show that T47D cell proliferation was markedly stimulated by E2, EGF, and SDF-1. In particular, SDF-1 displayed a bell-shape dose-response curve, showing a desensitization of the receptor for high concentrations of the chemokine. This pattern of response was observed in many different kind of tumors, including gliomas, meningiomas, ovarian cancers, and pituitary adenomas (Barbero et al., 2003; Porcile et al., 2005; Florio et al., 2006; Bajetto et al., 2007). Indeed, it was reported that upon SDF-1 binding, β -arrestins are recruited to CXCR4 to promote its endosomal internalization and down-regulation of its intracellular signaling (Cheng et al., 2000).

The main observation we provide in this article is that the three stimuli not only induce proliferation via the same intracellular pathway (i.e., the activation of ERK1/2) but that the sequential activation of E2, SDF-1, and EGF receptors

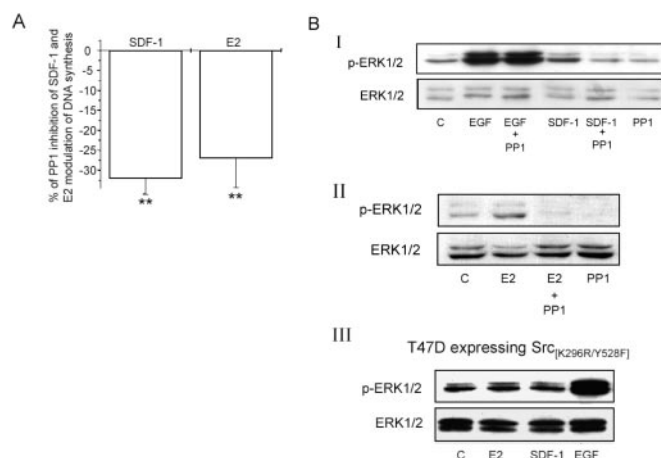


Fig. 8. Role of c-Src in SDF-1- and E2-dependent cell proliferation and ERK1/2 activation. A, effects of the Src inhibitor PP1 on the DNA synthesis induced by SDF-1 and E2. [³H]Thymidine incorporation assay was performed in serum-starved T47D cells treated with SDF-1 (25 nM) and E2 (50 nM) in the absence or presence of PP1 (200 nM). Data are expressed as a percentage of inhibition versus respective treated cells. Each bar represents the mean \pm S.E. of three replicate determinations (**, $P < 0.01$ versus stimulated cells). The inhibition of c-Src activity by PP1 significantly reverts both SDF-1- and E2-induced cell proliferation. B, effects of PP1 on ERK1/2 activation induced by EGF, SDF-1, and E2. I, phosphorylation/activation of ERK1/2 detected by Western blot performed in T47D cells treated with EGF (10 ng/ml) or SDF-1 (25 nM) in the presence or absence of PP1 (200 nM, 10-min pretreatment) using phospho-ERK1/2 antibody. Equal protein loading was demonstrated by reprobing the membrane with ERK1/2 antibody. II, phosphorylation/activation of ERK1/2 detected by Western blot performed in T47D cells treated with E2 (50 nM) in the presence or absence of PP1 (200 nM, 10-min pretreatment) using phospho-ERK1/2 antibody. Equal protein loading was demonstrated by reprobing the membrane with ERK1/2 antibody. The Src inhibitor PP1 completely reverts ERK1/2 phosphorylation induced by SDF-1 and E2, whereas it has no effects on EGF-mediated ERK1/2 activation. III, effects of E2, SDF-1, and EGF in T47D cells expressing Src^[K296R/Y528F]. Western blot showing the effect on ERK1/2 phosphorylation after the treatments with E2 (50 nM), SDF-1 (25 nM), and EGF (10 ng/ml) in T47D cells transfected with the cDNA encoding for the dominant-negative mutant of Src^[K296R/Y528F]. In these cells, an increase in ERK1/2 phosphorylation is detectable only after EGF treatment, indicating that SDF-1- and E2-dependent ERK1/2 phosphorylation requires the activation c-Src. Equal protein loading was demonstrated by reprobing the membrane with an antibody directed against ERK1/2.

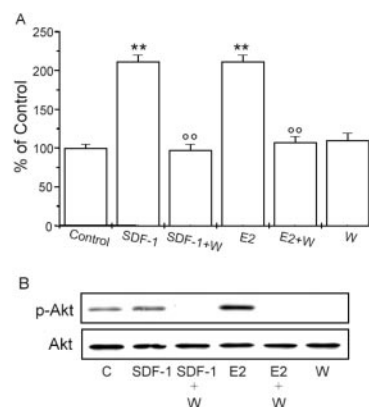


Fig. 9. A, effects of PI3K inhibition by wortmannin pretreatment on SDF-1- and E2-induced DNA synthesis. [³H]Thymidine incorporation assay on serum-starved T47D cells treated with SDF-1 (25 nM) or E2 (50 nM) in the absence or presence of wortmannin (1 μ M). Data are expressed as a percentage of the untreated control. Each bar represents the mean \pm S.E. of three replicate determinations. Wortmannin, although not effective on basal activity, significantly inhibits SDF-1- and E2-induced cell proliferation (**, $P < 0.01$ versus control cells; °°, $P < 0.01$ versus SDF-1-treated cells). B, effects of wortmannin on the SDF-1- and E2-induced Akt phosphorylation/activation. Cells were treated with SDF-1 (25 nM) or E2 (50 nM) for 15 min in the absence or presence of wortmannin (1 μ M, W); cell lysates were analyzed by Western blotting. Blots were probed with anti-phospho-Akt (Ser 473) antibody and reprobed with anti-Akt antibody to ensure the equal loading of proteins. SDF-1 and E2 increase basal Akt activation an effect reverted by pretreatment with wortmannin.

controls T47D cell proliferation. In particular, the blockade of EGFR by gefitinib not only reverted EGF-induced cell proliferation but also the effects of SDF-1 and E2. Likewise, the inhibition of CXCR4, using the selective antagonist AMD3100, affects both the SDF-1- and E2-dependent cell growth without interfering with the EGF proliferative activity. Altogether these experiments suggest a molecular ordering in the activation of mitogenic receptors in T47D cells, in which E2-stimulated cell growth requires the activation of CXCR4 that, in turn, modulates EGFR proliferative activity. Differently from a previous study (Hall and Korach, 2003), in our experiments, not all the proliferative effects of E2 were involved in this pathway, because in the presence of gefitinib and AMD3100, a residual, small proliferative activity was observed. We do not know the reason for such a discrepancy,

but it probably depends on the different cell lines used. Conversely, in our study, the effects of SDF-1 were completely blocked by gefitinib, and SDF-1 and EGF promoted cell proliferation without displaying additive effects, indicating that the proliferative stimuli activated by E2 are mediated by SDF-1/CXCR4 transactivation of EGFR.

To identify the intracellular mechanisms responsible for such a signaling cascade, we evaluated the capability of E2 to induce SDF-1 release. SDF-1 acts mainly as an autocrine/paracrine factor not only in breast cancer (Hall and Korach, 2003) but also in other cell types. Indeed, not only SDF-1 and its receptor were often identified in the same tumor cells (Barbero et al., 2003; Bajetto et al., 2007) but recently, it was proposed that stromal fibroblast could also provide SDF-1 to promote breast carcinoma growth and neovascularization (Orimo et al., 2005). Here we show that E2 treatment induced SDF-1 release from breast cancer cells and that this event was mediated via ER activation. Thus, SDF-1 released after E2 treatment may directly interact with CXCR4 expressed by the same or nearby cell. Subsequently, the activated receptor determines the phosphorylation/activation of EGFR.

We directly evaluated the molecular mechanism of such transactivation process, demonstrating a pivotal role for the cytosolic tyrosine kinase c-Src in this effect. Indeed, the blockade of this kinase significantly reverted E2 and SDF-1-dependent cell proliferation and prevented ERK1/2 activation. These data extend on cell proliferation activity, previous reports in which the transactivation of EGFR and HER2-neu via c-Src was responsible for the breast cancer cell migration (Cabioglu et al., 2005). We propose a model in which E2

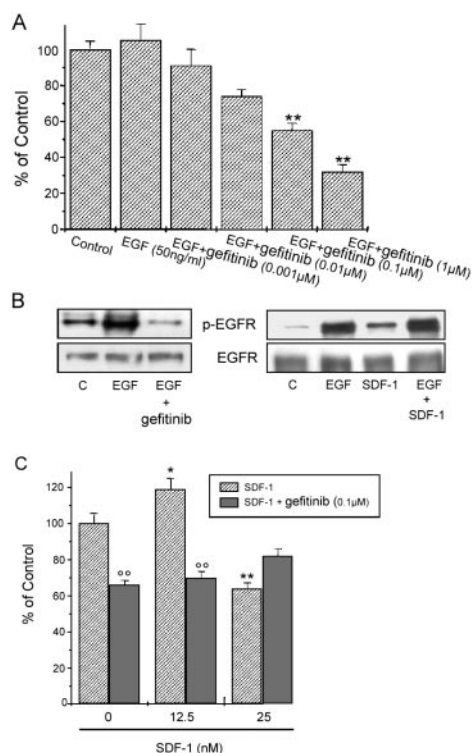


Fig. 10. Role of EGFR inhibition by gefitinib in the control of SKBR3 cell proliferation. A, effects of gefitinib on the EGF-induced DNA synthesis. [3 H]Thymidine incorporation assay on serum-starved SKBR3 cells treated with EGF (50 ng/ml) in the absence or presence of increasing concentrations of gefitinib. Data are expressed as a percentage of the untreated control. Each bar represents the mean \pm S.E. of three replicate determinations. Gefitinib inhibits EGF-induced cell proliferation in a dose-dependent manner (**, $P < 0.01$ versus EGF-treated cells). B, effects of gefitinib on the EGF-induced EGFR activation. Left, cells were treated with EGF (50 ng/ml) for 5 min in the absence or presence of gefitinib (1 μ M), and cell lysates were analyzed by Western blotting. Blots were probed with anti-phospho-EGFR antibody. The anti-EGFR antibody was used to reprobe blots to ensure the equal loading of proteins. EGF increased basal EGFR activation and the pretreatment with gefitinib reduced the phosphorylation of EGFR below the control level. Right, lysates from cells treated with EGF (10 ng/ml), SDF-1 (25 nM) alone, or in combination were analyzed by Western blotting using anti-phospho-EGFR and anti-EGFR antibodies. The cotreatment with SDF-1 and EGF causes a slight additive increase in EGFR phosphorylation. C, effects of gefitinib on the SDF-1-induced DNA synthesis. [3 H]Thymidine incorporation assay on serum-starved SKBR3 cells treated with SDF-1 (10–25 nM) in the absence or presence of gefitinib (0.1 μ M). Data are expressed as a percentage of the untreated controls. Each bar represents the mean \pm S.E. of three replicate determinations. Gefitinib significantly inhibits basal and SDF-1-induced cell proliferation (**, $P < 0.01$ versus control; *, $P < 0.05$ versus control; °, $P < 0.01$ versus stimulated cells).

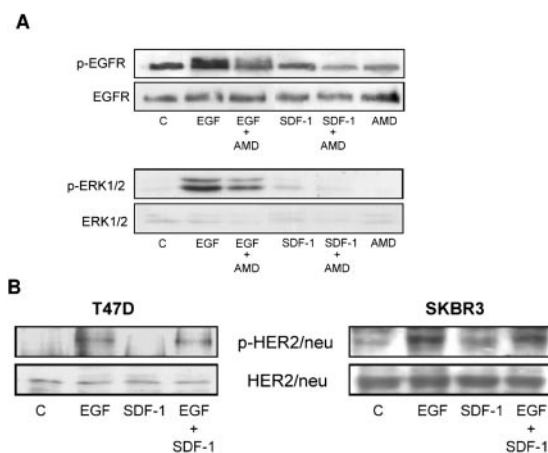


Fig. 11. A, effects of AMD3100 on the EGF- and SDF-1-induced EGFR/ERK1/2 activation. SKBR3 were treated with EGF (10 ng/ml) or SDF-1 (25 nM) in the absence or presence of AMD3100 (1 μ M), and cell lysates were analyzed by Western blotting. Blots were probed with anti-phospho-EGFR (top) and anti-phospho-ERK1/2 (bottom) antibodies. Anti-EGFR and anti-ERK1/2 antibodies were used to reprobe the blots to ensure the equal loading of proteins. The inhibition of CXCR4 activity induced by AMD3100 significantly reduces EGFR and ERK1/2 activation induced by EGF and SDF-1. B, effects of EGF and SDF-1 on HER2-neu activation in T47D and SKBR3 cells. Cells were treated with EGF (10 ng/ml) or SDF-1 (25 nM) and cell lysates analyzed by Western blotting. Blots were probed with anti-phospho-HER2-neu (Tyr1221/1222) antibody. Anti-HER2 antibody was used to reprobe blots to ensure the equal loading of proteins. In T47D cells, expressing moderate HER2-neu levels SDF-1 did not transactivate HER2-neu, whereas in the highly expressing SKBR3 cells, an increased HER2-neu phosphorylation was detected after SDF-1 treatment. The cotreatment with EGF and SDF-1 did not result in an additive HER2-neu activation in both cell lines.

induces cell proliferation via SDF-1/CXCR4-mediated activation of EGFR that in turn activates both Akt and ERK1/2 cascade. In fact, we show that besides a role in cell migration, Akt activation by E2 and SDF-1 was also involved in breast cancer cell proliferation. Because this model directly connects E2 and EGF activity as mediators of breast cancer proliferation, we can speculate that, being the E2 proliferative effects mediated by EGFR, if cancer cells develop an independent EGFR activity, the effects of antiestrogens are likely to be lost inducing tumor resistance. It is interesting that a recent article described that elevated c-Src activity was responsible of proliferation in tamoxifen-resistant breast cancer cells that was inhibited by cotreatment with the c-Src inhibitor AZD0530 and gefitinib (Hiscox et al., 2006). This observation well fit with the model we propose, because CXCR4 may be responsible of the hyperactivation of c-Src and EGFR transactivation. In this perspective, it is particularly relevant to our observation that in the SKBR3 estrogen-resistant cell line, EGFR is constitutively transactivated through a CXCR4-dependent mechanism being inhibited by AMD3100 altogether with cell proliferation.

Because SKBR3 cells are insensitive to E2 and neither express SDF-1 mRNA nor secrete the protein, we hypothesized that this phenotype is dependent on the lack of the ER-mediated regulation of SDF-1 gene, identified as E2-responsive gene (Hall and Korach, 2003; Kishimoto et al., 2005). In addition, these cells display a high expression of HER2-neu that does not bind any specific ligand, probably being a coreceptor and part of a heterodimer complex with EGFR (Citri and Yarden, 2006). HER2-neu is one of the most commonly analyzed proto-oncogenes in human cancer studies because it plays a pivotal role in tumorigenesis and metastasization (Ménard et al., 2003). Patients with HER2-neu overexpressing breast cancers are associated with unfavorable prognosis, shorter relapse time, and low survival rate. HER2-neu and EGFR transactivation may be mediated by several GPCRs, including CXCR4 (Cabioglu et al., 2005). On the other hand, HER2-neu contributes to increase cell surface levels of CXCR4. It has been proposed that CXCR4 and HER2-neu expression are linked, because most CXCR4-positive cells were also HER2-neu-positive (Li et al., 2004). These authors demonstrated that HER2-neu up-regulates CXCR4 expression either enhancing CXCR4 translation, through the activation of the PI3K/Akt/mTor signaling pathway, or inhibiting its degradation, preventing CXCR4 ubiquitination, a process that sorts internalized GPCRs to the degradative pathway (Li et al., 2004).

CXCR4 signaling is mainly dependent on ligand binding, but there is recent evidence that ligand-independent mechanisms can be activated in different tumor types. In glioblastomas, EGFR activation can stimulate CXCR4 phosphorylation to induce its activity (Woerner et al., 2005). The effects of AMD3100 observed in our study in SKBR3 cells may support these findings. In fact, also in the absence of SDF-1, the CXCR4 antagonist significantly reduced cell proliferation, EGFR phosphorylation, and ERK1/2 activation. Thus, we can hypothesize that in these cell lines, a constitutive proliferative loop may occur in which HER2-neu ligand-independent activity may sustain a constitutive CXCR4 activation (as demonstrated by the effects of AMD3100 in untreated cells and, indirectly, by the low responsivity to exogenous SDF-1) that, in turn, may control both EGFR and HER2-neu activa-

tion. It is interesting that, as reported previously (Cabioglu et al., 2005), SDF-1 induces HER2-neu activation mainly in cells overexpressing this receptor (SKBR3 cells) but not when HER2-neu levels are low (T47D cells). This mechanism may sustain some of the E2-independent proliferation of breast tumors. Further studies will be required to support this hypothesis. In conclusion, from our results, we propose that 1) the SDF-1/CXCR4 system represents the link between the proliferative stimuli induced by E2 and EGF, with E2-induced SDF-1 release can control EGFR phosphorylation through a CXCR4-mediated c-Src activation; 2) alterations in CXCR4 activity may bypass the requirement for E2 in estrogen-resistant cells; and 3) the occurrence of such transductional cascade have to be considered when evaluating the pharmacological approaches to breast cancer.

Acknowledgments

We thank Dr. Alessandro Massa for the scientific and technical contribution to the development of this study.

References

- Arena S, Pattarozzi A, Massa A, Esteve JP, Iuliano R, Fusco A, Susini C, and Florio T (2007) An intracellular multi-effector complex mediates somatostatin receptor 1 activation of phospho-tyrosine phosphatase eta. *Mol Endocrinol* **21**:229–246.
- Bajetto A, Barbieri F, Pattarozzi A, Dorcaratto A, Porcile C, Ravetti JL, Zona G, Spaziante R, Schettini G, and Florio T (2007) CXCR4 and SDF1 expression in human meningiomas: a proliferative role in tumoral meningotheial cells in vitro. *Neuro-oncol* **9**:3–11.
- Balkwill F (2004) The significance of cancer cell expression of the chemokine receptor CXCR4. *Semin Cancer Biol* **14**:171–179.
- Barbero S, Bonavia R, Bajetto A, Porcile C, Pirani P, Ravetti JL, Zona GL, Spaziante R, Florio T, and Schettini G (2003) Stromal cell-derived factor 1alpha stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt. *Cancer Res* **63**:1969–1974.
- Bonavia R, Bajetto A, Barbero S, Pirani P, Florio T, and Schettini G (2003) Chemokines and their receptors in the CNS: expression of CXCL12/SDF-1 and CXCR4 and their role in astrocyte proliferation. *Toxicol Lett* **139**:181–189.
- Cabioglu N, Summy J, Miller C, Parikh NU, Sahin AA, Tuzlali S, Pumiglia K, Gallick GE, and Price JE (2005) CXCL12/stromal cell-derived factor-1alpha transactivates HER2-neu in breast cancer cells by a novel pathway involving Src kinase activation. *Cancer Res* **65**:6493–6497.
- Cheng ZJ, Zhao J, Sun Y, Hu W, Wu YL, Cen B, Wu GX, and Pei G (2000) β -Arrestin differentially regulates the chemokine receptor CXCR4-mediated signaling and receptor internalization, and this implicates multiple interaction sites between beta-arrestin and CXCR4. *J Biol Chem* **275**:2479–2485.
- Ciardiello F, Caputo R, Bianco R, Damiano V, Pomato G, De Placido S, Bianco AR, and Tortora G (2000) Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* **6**:2053–2063.
- Citri A and Yarden Y (2006) EGF-ERBB signalling: towards the systems level. *Nat Rev Mol Cell Biol* **7**:505–516.
- Della Rocca GJ, Maudsley S, Daaka Y, Lefkowitz RJ, and Luttrell LM (1999) Pleiotropic coupling of G protein-coupled receptors to the mitogen-activated protein kinase cascade. Role of focal adhesions and receptor tyrosine kinases. *J Biol Chem* **274**:13978–13984.
- Florio T, Casagrande S, Diana F, Bajetto A, Porcile C, Zona G, Thellung S, Arena S, Pattarozzi A, Corsaro A, et al. (2006) Chemokine stromal cell-derived factor 1 α induces proliferation and growth hormone release in GH4C1 rat pituitary adenoma cell line through multiple intracellular signals. *Mol Pharmacol* **69**:539–546.
- Florio T, Yao H, Carey KD, Dillon TJ, and Stork PJ (1999) Somatostatin activation of mitogen-activated protein kinase via somatostatin receptor 1 (SSTR1). *Mol Endocrinol* **13**:24–37.
- Gee JM, Robertson JF, Ellis IO, and Nicholson RI (2001) Phosphorylation of ERK1/2 mitogen-activated protein kinase is associated with poor response to anti-hormonal therapy and decreased patient survival in clinical breast cancer. *Int J Cancer* **95**:247–254.
- Hall JM and Korach KS (2003) Stromal cell-derived factor 1, a novel target of estrogen receptor action, mediates the mitogenic effects of estradiol in ovarian and breast cancer cells. *Mol Endocrinol* **17**:792–803.
- Heimberger AB, Learn CA, Archer GE, McLendon RE, Chewning TA, Tuck FL, Pracyk JB, Friedman AH, Friedman HS, Bigner DD, et al. (2002) Brain tumors in mice are susceptible to blockade of epidermal growth factor receptor (EGFR) with the oral, specific, EGFR-tyrosine kinase inhibitor ZD1839 (Iressa). *Clin Cancer Res* **8**:3496–3502.
- Herbst RS, Fukuoka M, and Baselga J (2004) Gefitinib—a novel targeted approach to treating cancer. *Nat Rev Cancer* **4**:956–965.
- Hiscox S, Morgan L, Green TP, Barrow D, Gee J, and Nicholson RI (2006) Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. *Breast Cancer Res Treat* **97**:263–274.
- Htun H, Holth LT, Walker D, Davie JR, and Hager GL (1999) Direct visualization of

- the human estrogen receptor alpha reveals a role for ligand in the nuclear distribution of the receptor. *Mol Biol Cell* **10**:471–486.
- Improta-Brears T, Whorton AR, Codazzi F, York JD, Meyer T, and McDonnell DP (1999) Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. *Proc Natl Acad Sci U S A* **96**:4686–4691.
- Kang H, Watkins G, Parr C, Douglas-Jones A, Mansel RE, and Jiang WG (2005) Stromal cell derived factor-1: its influence on invasiveness and migration of breast cancer cells in vitro, and its association with prognosis and survival in human breast cancer. *Breast Cancer Res* **7**:R402–R410.
- Kishimoto H, Wang Z, Bhat-Nakshatri P, Chang D, Clarke R, and Nakshatri H (2005) The p160 family coactivators regulate breast cancer cell proliferation and invasion through autocrine/paracrine activity of SDF-1alpha/CXCL12. *Carcinogenesis* **26**:1706–1715.
- Lapteva N, Yang AG, Sanders DE, Strube RW, and Chen SY (2005) CXCR4 knock-down by small interfering RNA abrogates breast tumor growth in vivo. *Cancer Gene Ther* **12**:84–89.
- Li YM, Pan Y, Wei Y, Cheng X, Zhou BP, Tan M, Zhou X, Xia W, Hortobagyi GN, Yu D, et al. (2004) Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. *Cancer Cell* **6**:459–469.
- Luker KE and Luker GD (2006) Functions of CXCL12 and CXCR4 in breast cancer. *Cancer Lett* **238**:30–41.
- Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, et al. (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* **283**:655–661.
- Ménard S, Pupa SM, Campiglio M, and Tagliabue E (2003) Biologic and therapeutic role of HER2 in cancer. *Oncogene* **22**:6570–6578.
- Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, et al. (2001) Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410**:50–56.
- Nicholson RI, Gee JM, and Harper ME (2001) EGFR and cancer prognosis. *Eur J Cancer* **37** (Suppl 4):S9–S15.
- Nicholson RI, McClelland RA, Gee JM, Manning DL, Cannon P, Robertson JF, Ellis IO, and Blamey RW (1994) Epidermal growth factor receptor expression in breast cancer: association with response to endocrine therapy. *Breast Cancer Res Treat* **29**:117–125.
- Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, and Gustafsson JA (2001) Mechanisms of estrogen action. *Physiol Rev* **81**:1535–1565.
- Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, and Weinberg RA (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* **121**:335–348.

- Osborne CK, Shou J, Massarweh S, and Schiff R (2005) Crosstalk between estrogen receptor and growth factor receptor pathways as a cause for endocrine therapy resistance in breast cancer. *Clin Cancer Res* **11**:865S–870S.
- Peng SB, Peek V, Zhai Y, Paul DC, Lou Q, Xia X, Eessalu T, Kohn W, and Tang S (2005) Akt activation, but not extracellular signal-regulated kinase activation, is required for SDF-1alpha/CXCR4-mediated migration of epithelioid carcinoma cells. *Mol Cancer Res* **3**:227–236.
- Porcile C, Bajetto A, Barbieri F, Barbero S, Bonavia R, Biglieri M, Pirani P, Florio T, and 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.
- Rosenkilde MM, Gerlach LO, Jakobsen JS, Skerlj RT, Bridger GJ, and Schwartz TW (2004) Molecular mechanism of AMD3100 antagonism in the CXCR4 receptor: transfer of binding site to the CXCR3 receptor. *J Biol Chem* **279**:3033–3041.
- Sewell JM, Macleod KG, Ritchie A, Smyth JF, and Langdon SP (2002) Targeting the EGF receptor in ovarian cancer with the tyrosine kinase inhibitor ZD 1839 (“Iressa”). *Br J Cancer* **86**:456–462.
- Silva CM and Shupnik MA (2007) Integration of steroid and growth factor pathways in breast cancer: focus on signal transducers and activators of transcription and their potential role in resistance. *Mol Endocrinol* **21**:1499–1512.
- Sivaraman VS, Wang H, Nuovo GJ, and Malbon CC (1997) Hyperexpression of mitogen-activated protein kinase in human breast cancer. *J Clin Invest* **99**:1478–1483.
- Smith MC, Luker KE, Garbow JR, Prior JL, Jackson E, Piwnica-Worms D, and Luker GD (2004) CXCR4 regulates growth of both primary and metastatic breast cancer. *Cancer Res* **64**:8604–8612.
- Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, et al. (1998) The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* **393**:591–594.
- Woerner BM, Warrington NM, Kung AL, Perry A, and Rubin JB (2005) Widespread CXCR4 activation in astrocytomas revealed by phospho-CXCR4-specific antibodies. *Cancer Res* **65**:11392–11399.
- Yue W, Wang JP, Conaway M, Masamura S, Li Y, and Santen RJ (2002) Activation of the MAPK pathway enhances sensitivity of MCF-7 breast cancer cells to the mitogenic effect of estradiol. *Endocrinology* **143**:3221–3229.

Address correspondence to: Dr. Tullio Florio, Sezione Farmacologia, Dipartimento Oncologia, Biologia e Genetica, Università di Genova, Viale Benedetto XV, 2, 16132 Genova, Italy. E-mail: tullio.florio@unige.it