The p160 family coactivators regulate breast cancer cell proliferation and invasion through autocrine/paracrine activity of SDF-1α/CXCL12

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Estrogen receptors (ERs) regulate the transcription of genes involved in breast cancer cell proliferation, invasion and metastasis. In addition to ligand concentration, phosphorylation and coactivator/corepressor levels control ER-dependent transcription. In this study, we used MCF-7 breast cancer sublines with variable levels of the steroid receptor coactivator 1 (SRC-1) to investigate the importance of coactivator levels in basal and estrogen-inducible expression of SDF-1α/CXCL12, cathepsin D and cMyc. Basal expression of SDF-1α and cMyc but not of cathepsin D was substantially lower in a MCF-7 subline lacking SRC-1 ((MCF-7/p2) compared with MCF-7 sublines expressing SRC-1 (MCF-7/p1 and LCC2). Although estrogen efficiently induced SDF-1α in MCF-7/p1 cells, very little induction of this gene was observed in MCF-7/p2 cells. The absence of SRC-1 had no effect on estrogen-inducible expression cMyc and cathepsin D suggesting that coactivator levels determine the expression of only a subset of estrogen-regulated genes. Introduction of SRC-1, SRC-2/TIF-2 or SRC-3/AIB1 increased basal expression of SDF-1α in MCF-7/p2 cells. Consistent with the role of SDF-1α in mediating estrogen-induced proliferation, estrogen failed to increase proliferation of MCF-7/p2 cells. In matrigel invasion assays, conditioned media from MCF-7/p1 but not MCF-7/p2 cells increased invasion of cancer cells expressing metastasis-associated genes and CXCR4, the receptor for SDF-1α. These results suggest that coactivators control SDF-1α expression, which mediates estrogen-induced proliferation and invasion through autocrine and paracrine mechanisms, respectively. These results also provide a molecular explanation for recent observations linking co-overexpression of coactivators and her2/neu with poor prognosis: coactivators increase SDF-1α expression whereas her2/neu stabilize CXCR4 protein.

Introduction

Breast cancer is the most common cancer of women in the western world. In many cases, breast cancer proliferation is estrogen dependent, with estrogens serving as ligands for estrogen receptors (ERs) (1,2). ER is a member of the nuclear receptor (NR) superfamily and regulates transcription of specific target genes in response to ligand binding and phosphorylation. Two ERs, ERα and ERβ, associate with estrogen and bind to the estrogen response element (ERE) of target genes as homodimers or heterodimers (3,4). ERα interacts with a large group of proteins referred to as NR coregulators, including coactivators and corepressors (5–7). Coactivators, such as steroid receptor coactivator 1 (SRC-1) and CREB-binding protein (CBP)/p300, interact with ERα in an estrogen-dependent manner to increase transactivation, in part owing to intrinsic histone acetyltransferase activity. One of the best characterized groups of NR coactivators is the p160 family, which includes SRC-1, SRC-2/TIF-2/GRIP-1 and SRC-3/ACTR/AIB1 (7,8). When bound to estradiol (E2), ERα engages signature motifs (LXXLL) in the center of the p160 molecule (8,9). ERα-bound p160 coactivators facilitate the recruitment of additional proteins in a dynamic manner leading to histone acetylation and activation of transcription (10,11). Binding of tamoxifen, an antiestrogen, to ERα leads to an enhanced interaction with corepressors instead of coactivators (12,13). Such antiestrogen–ERα–corepressor complexes bind ERE but fail to activate target genes.

Recent studies suggest that aberrant expression of coactivator molecules is sufficient for breast cancer initiation. For example, overexpression of AIB1 in transgenic mice leads to the development of malignant mammary tumors (14). Patients with tumors overexpressing her2/neu and AIB1 undergoing tamoxifen therapy show a reduced disease-free survival (15). Similar association between her2/neu and SRC-1 overexpressions and reduced disease-free survival was recently reported (16). The downstream target(s) of coactivators that may contribute to disease progression is under intense investigation. To address this issue, we utilized the ERα-positive sublines of MCF-7 cells expressing variable levels of SRC-1. There are at least six sublines of MCF-7 cells, which show differences in sensitivity to estrogen, antiestrogens and tumor necrosis factor or ERα expression levels (17,18). We have isolated a subline of MCF-7 lacking SRC-1 (MCF-7/p2) and characterized it for basal and estrogen-inducible expression of estrogen-inducible genes stromal cell-derived factor alpha (SDF-1α), cMyc, pS2 and cathepsin D. SDF-1α is of particular interest because it mediates estrogen-induced proliferation of breast and ovarian, but not uterine epithelial cells (19). In addition, along with its receptor CXCR4, SDF-1α controls metastasis of breast cancer

(20). Moreover, her2/neu stabilizes CXCR4 protein, and CXCR4 is an integral part of her2/neu-mediated breast cancer metastasis (21,22). We observed that basal and estrogen-inducible expression of SDF-1α was substantially lower in MCF-7/p2 cells compared with SRC-1 expressing MCF-7 cells. Overexpression of SRC-1, TIF-2 or AIB1 restored SDF-1α expression in MCF-7/p2 cells suggesting that the overall levels of p160 family coactivators determine SDF-1α expression in breast cancer cells.

Materials and methods

Cell types and cell culture

MCF-7/p1 and its tamoxifen-resistant variant LCC2 (LCC2-1 and LCC2-2 correspond to LCC2 cells of different passage numbers from Clarke's lab) are described previously (17). MCF-7/p2 cells were a clonal variant of MCF-7/p1 cells isolated in H.N.'s lab by growing cells in Eagle minimum essential media (EMEM) supplemented with 10% fetal bovine serum (FBS) instead of phenol red free improved minimal essential medium (IMEM)/5% charcoal–dextran-treated FBS (CCS) used in Clarke's lab. After several passages in this media, the clonal variant with reduced growth in phenol red free EMEM plus 5% CCS emerged. ZR-75-1 were maintained in RPMI media with 10% FBS. LMD231 cells, which were used for invasion assay, are a lung metastatic variant of MDA-MB-231 cells and described previously (23). For experiments described in this study, all cell lines were grown in phenol red free EMEM plus 5% CCS for at least 2 days prior to experiments in the same media.

DNA transfections and chloramphenicol acetyl transferase (CAT) assay

All transfections were performed by the calcium phosphate method. Cells were cotransfected with 5 μg of ERE-TATA-CAT reporter gene and 2 μg of β-galactosidase expression vector, which was used to measure the transfection efficiency. Reporter activity was measured as previously described (24). Transient overexpression of coactivators in MCF-7/p2 cells was performed using lipofectamine reagent (Invitrogen Corporation, Carlsbad, CA).

Methyl thiazolyl tetrazolium (MTT) and trypan blue exclusion assay

MCF-7 cells were plated at 2000 cells per well in 96-well tissue culture plates in phenol red free EMEM plus 5% CCS medium. E2 was added 2 days after culturing and replaced every 2 days. On indicated days, the number of living cells was determined using the celltiter non-radioactive cell proliferation kit (Promega, Madison, WI) according to the instruction of the manufacturer. In each experiment, cells in eight wells were treated with the same drug. Each datapoint is the average of results obtained from three independent experiments. For trypan blue exclusion assay, 5×10^4 cells were plated in 60 mm plates and cells were counted manually after staining with trypan blue at the indicated time points. Media were changed after 3 days of plating.

siRNA transfection

Cells were seeded with phenol red free plus 5% CCS for 48 h in 60 mm plates, then transfected with 25 nM of double-stranded siRNA using TransIT-TKO Transfection Reagent (Mirus, Madison, WI) according to the manufacturer's protocol. The siRNA against ERα and AIB1 were purchased from Dharmacon RNA Technology (Lafayette, CO). The siRNA against SRC-1 was purchased from Qiagen-Xeragon (Valencia, CA).

RNA preparation and northern blot analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was electrophoresed on an agarose-formaldehyde gel and subjected to northern blot analysis as previously described (24).

RT–PCR and quantitative real-time RT–PCR

RT–PCR was carried out using the proSTAR kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Primers for SRC-1 were as follows: forward, 5′-GGTGAGGCCAACTTTGCTCCA-3′; reverse, 5′-TTCAGTCAG-TAGCTGCTGACG-3′. Primers for AIB1 were: forward, 5′-AGTATGTCGC-CAAACCAAGG-3′; reverse, 5′-AAGTCCCCACACCTTCACTG-3′. Primers for CXCR4 were: forward, 5′-CGGCTGTAGAGCGAGTGTTG-3′; reverse, 5′-CCCCACTTCTTCAGAGTAGTTATCAGA-3′; probe, 6FAMCAT GGA-ACCGATCAGTGMGBNFQ. Quantitative real-time RT–PCR was performed using the Applied Biosystems Mix according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

Western blot analysis

Cell lysates prepared in radioimmunoassay buffer were subjected to western blot as described previously (24). Antibodies against SRC-1 and AIB1 were purchased from (Affinity Bioreagents, Golden, CO). ERα and TIF-2 antibodies were purchased from Chemicon (Chemicon International, Temecula, CA) and Transduction Laboratories (BD, Biosciences, San Diego, CA), respectively.

ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed using a human SDF-1α Quantikine kit (R&D, Minneapolis, MN) according to the manufacturer's protocol.

Invasion assay

Cell invasion assay was done with invasion assay kit (Chemicon International, Temecula, CA) in accordance with the manufacturer's protocol. LMD231 cells (1×10^6/ml) were seeded onto 12-well cell culture chamber using inserts with 8 μM pore size polycarbonate membrane over a thin layer of extracellular matrix. Recombinant human SDF-1α (PeproTech INC, Rocky Hill, NJ) or conditioned media (EMEM phenol red free plus 5% CCS) from MCF-7/p1 and MCF-7/p2 cells were added to the lower chamber. After incubation for 24 h, cells on the lower surface of the membrane were stained and counted under microscope in at least five different fields.

Statistical analysis

Data were analyzed using GraphPad software (Graphpad.com). Analysis of variance was used to determine P-values among mean measurements (two-tailed test). A P-value < 0.05 was deemed significant.

Results

Variable proliferation rate and ERE reporter gene activity in sublines of MCF-7 cells

MCF-7 cells has served as a standard *in vitro* model system for studying ERα-positive breast cancer, particularly with respect to estrogen-stimulated gene expression, antiestrogen resistance, acquisition of hormone-independent growth properties, and ERα activity (2,17,25–27). We have been using these cells for studying the role of extracellular signal activated kinases in antiestrogen resistance. By culturing cells in different growth media (regular serum versus CCS containing media; EMEM versus IMEM), we isolated a subline of MCF-7 cells (called MCF-7p2 hereafter) that showed lower proliferation rate in CCS containing media compared with parental MCF-7 (MCF-7/p1) and its tamoxifen-resistant counterparts LCC2-1 and LCC2-2 (Figure 1A). Similar results were obtained when proliferation was measured by trypan blue exclusion assay (Figure 1B). All sublines showed similar growth pattern in media containing regular FBS (Figure 1C). To determine whether MCF-7/p2 cells are defective in estrogen (E2)-induced proliferation, we treated all four cell types with different concentrations of E2 and measured proliferation after 4 days of treatment. E2 induced proliferation of all cell types except MCF-7/p2 (Figure 1D). To determine whether the failure of estrogen to induce proliferation of MCF-7/p2 cells correlates with reduced ERα activity, we performed transient transfection assay with ERE-TATA-CAT reporter. As expected, both basal and estrogen-inducible ERE-TATA-CAT activity was lower in MCF-7/p2 cells compared with MCF-7/p1 and LCC2 cells (Figure 1E). Similar results were obtained with ERE-TK-CAT reporter (data not shown). All four cell types expressed similar levels of ERα and estrogen was able to induce degradation of ERα to a similar level in MCF-7/p1 and MCF-7/p2 cells (Figure 1F). Thus, differences in the levels/activity of signaling molecules involved in regulating ERα activity may have contributed to cell type specific variation in ERα activity and estrogen-induced proliferation. However, differences in ERα activity and proliferation did not correlate with the levels of two of the major kinases known to regulate ERα activity (1,24,28). In fact, the basal activity of MAPK, which increases ERα activity by phosphorylating
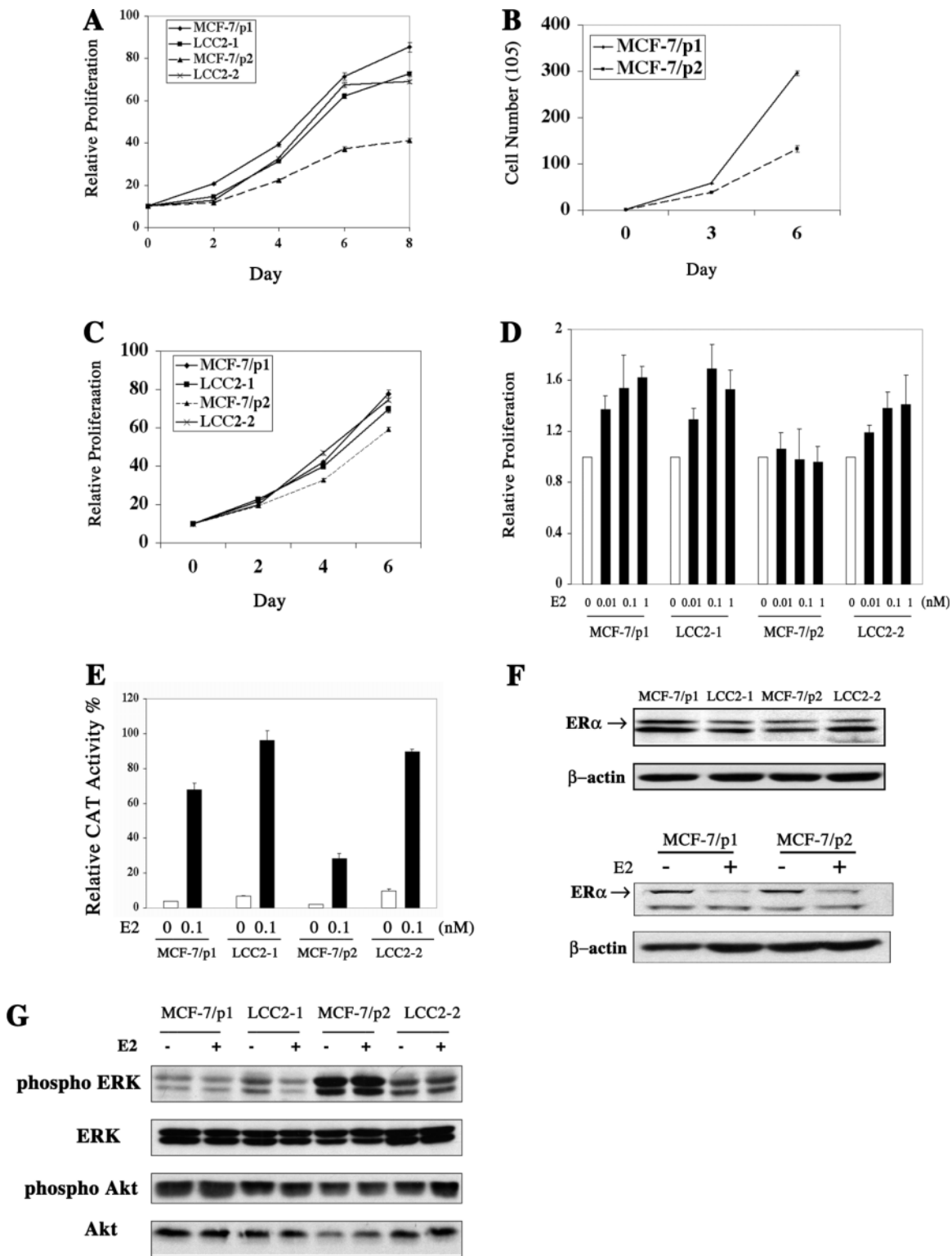
Fig. 1. Variable proliferation rate and ERE reporter gene activity in sublines of MCF-7 cells. (**A**) Proliferation rate of tamoxifen-sensitive MCF-7/p1, MCF-7/p2 cells and tamoxifen-resistant variants LCC2-1 and LCC2-2 cells grown in phenol red free and CCS containing media. Proliferation was measured by MTT assay as described in Materials and methods. $P < 0.0001$, MCF-7/p2 versus other cell types. Mean and standard deviation is given. (**B**) Proliferation rate of MCF-7/p1 and MCF-7/p2 cells as measured by trypan blue assay. (**C**) Proliferation of four cell types in regular media with 10% FBS. Note that all cell types showed similar proliferation rate. (**D**) Sensitivity of MCF-7 parental cells and variants to estrogen (E2) at the indicated concentrations was measured by MTT assay after 4 days of treatment. Mean and standard deviation is given. Estrogen (0.1 nM)-induced increase in proliferation is statistically significant ($P < 0.009$) in MCF-7/p1, LCC2-1 and LCC2-2 but not in MCF-7/p2 cells. (**E**) Cells were transiently transfected with an estrogen response element containing reporter gene (ERE-TATA-CAT, 5 µg) and β-galactosidase expression vector (2 µg). CAT activity in an equal number of β-galactosidase units of untreated or E2-treated cells was measured 24 h after transfection. Difference in estrogen-inducible activation of ERE-TATA-CAT between MCF-7/p1 and MCF-7/p2 cells is statistically significant ($P = 0.002$). (**F**) A representative western blot showing ERα expression level in various cell types. Cells were treated with or without estrogen 0.1 nM for 4 h. The whole cell extracts (50 µg) were subjected to western blot analysis using ERα antibody. (**G**) The cell extracts from cells treated with or without E2 (0.1 nM) for 1 h were subjected to western blot analysis using the indicated antibodies.

S118, was higher in MCF-7/p2 cells compared with MCF-7/p1 cells (Figure 1G). We also did not observe significant differences in levels of activated AKT between cell types, another kinase involved in regulating ERα activity (Figure 1G).

MCF-7/p2 cells lack SRC-1 protein

We next examined whether MCF-7/p1 and MCF-7/p2 cells differ with respect to coactivator and corepressor levels. All four cell types expressed similar levels of coactivators TIF-2, AIB1, and CBP (Figure 2A). Similarly, there was no difference in the levels of corepressors N-CoR and SMRT (Figure 2B). In contrast, SRC-1 protein was detected in MCF-7/p1 and LCC2 cells but not in MCF-7/p2 cells (Figure 2A). SRC-1 transcripts were also lower in MCF-7/p2 cells compared with other cell types (Figure 2B). Specificity of SRC-1 antibody was confirmed by an siRNA against SRC-1. LCC2 cells treated with siRNA against SRC-1 showed lower levels of SRC-1 protein compared with cells treated with control siRNA or AIB1 siRNA (Figure 2C). Thus, MCF-7/p2 cells correspond to naturally occurring ERα-positive SRC-1$^{-/-}$ cells similar to the ERα-negative MCF-7 derivatives, C4 and C4-12 cells (29). Previously, we have made several attempts to generate breast cancer cells stably overexpressing SRC-1 to study its role in ERα-regulated gene expression and antiestrogen sensitivity. There is only one report on generation of SRC-1 overexpressing MCF-7 cells and one on AIB1 overexpressing prostate cancer cells suggesting technical difficulties in generating breast cancer cells overexpressing coactivators (30,31). MCF-7/p2 cells, therefore, provided a model system to study the role of SRC-1 in ERα-regulated gene expression and antiestrogen sensitivity.

Lack of SRC-1 affects basal and estrogen-inducible expression of a subset of ERα-regulated genes

Despite significant sequence homology among p160 coactivator members, several lines of evidence suggest non-redundant functions to these proteins (7,32). Coactivator specificity may be determined by the nature of response elements involved in ERα-mediated gene expression (32). For example, genes with classical EREs bind to heterodimers of SRC-1:AIB1 or AIB1:TIF-2. In contrast, monomers of SRC-1 or TIF-2 bind to non-ERE containing promoters. To address the effect of lack of SRC-1 on ERα-regulated gene expression, we examined the basal and estrogen-inducible expression of pS2, cathepsin D and cMyc, as well as SDF-1α, which is recently identified as an estrogen-inducible gene, in MCF-7/p1 and MCF-7/p2 cells (19). The pS2 promoter contains ERE element, which functions in cooperation with AP-1 response element (33). Cathepsin D promoter contains classical ERE, whereas cMyc is a non-ERE containing estrogen-inducible gene (34,35). The ERE element in SDF-1α has not been identified. However, based on the available sequence in the database, the SDF-1α promoter contains ERE half sites but not palindromic classical ERE elements. The basal levels of pS2 and cMyc were lower in MCF-7/p2 cells compared with MCF-7/p1 cells, which is consistent with the lower ERα transcriptional activity in MCF-7/p2 cells compared with MCF-7/p1 cells (Figure 3B). Although there was a corresponding decrease in estrogen-inducible expression of pS2 in MCF-7/p2 cells compared with MCF-7/p1 cells, estrogen-inducible cMyc expression was similar in both cell types (Figure 3A). SRC-1 levels do not appear to effect either basal or estrogen-inducible expression of cathepsin D. In fact, estrogen-inducible cathepsin D
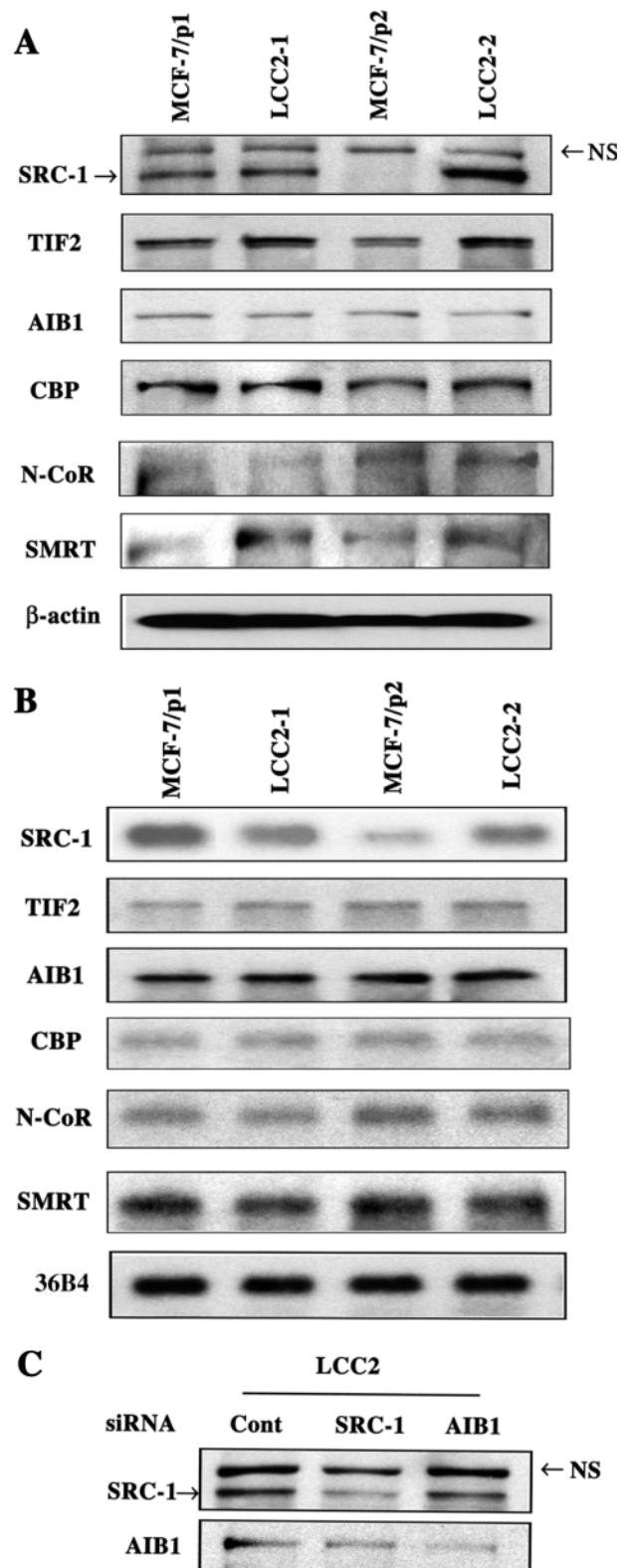
Fig. 2. Expression levels of coactivators and corepressors in MCF-7/p1 and MCF-7/p2, as well as variants LCC2-1 and LCC2-2 cells. (**A**) Whole cell extracts (50 μg protein) were subjected to western blot analysis and probed with the indicated antibodies. NS, non-specific. (**B**) Total RNA (5 μg) was subjected to northern blot analysis with the indicated probes. The integrity of RNA was verified by reprobing the blot with 36B4 ribosomal protein gene. (**C**) LCC2 cells were transfected with the siRNA against SRC-1 or AIB1, and non-specific control siRNA for 72 h using the TransIT-TKO transfection reagent. The cell lysates were subjected to western blot analysis using SRC-1 or AIB1 antibody.
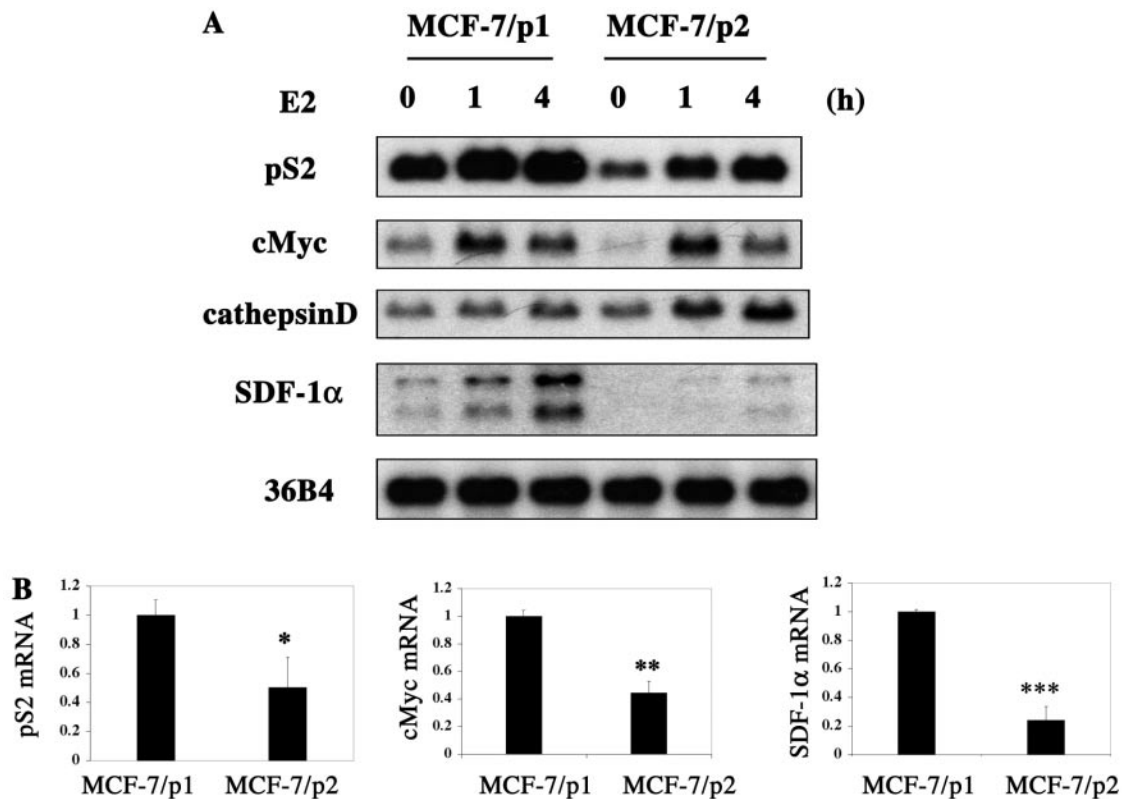
Fig. 3. Basal and estrogen-inducible expression of a subset of ERα-regulated genes in MCF-7/p1 and MCF-7/p2 cells. Total RNA (5 μg) from cells treated with or without E2 0.1 nM at the indicated time point was subjected to northern blot analysis with indicated probes (**A**). Basal expression levels of pS2, cMyc and SDF-1α in MCF-7/p1 and MCF-7/p2 cells from three experiments are shown (**B**). $^{*}P = 0.0272$; $^{**}P = 0.0005$; $^{***}P = 0.0008$.

expression was greater in MCF-7/p2 cells compared with MCF-7/p1 cells. The most striking observation was seen with SDF-1α. Basal SDF-1α mRNA was detectable in MCF-7/p1 cells and increased by estrogen, though with slower kinetics than induction of cMyc transcription. In contrast, basal SDF-1α expression was extremely low in MCF-7/p2 cells, which barely increased after 4 h of estrogen treatment. Note that estrogen at 10 nM, a concentration required for inducing SDF-1α in the MCF-7/BUS subline (36), did not significantly increase SDF-1α expression in MCF-7/p2 cells (data not shown). Taken together, these results suggest that coactivator levels determine both basal and estrogen-inducible expression of select genes.

We next determined whether cell type specific differences in ERα-regulated gene expression have an impact on the sensitivity of cells to antiestrogens. Contrary to our expectation, MTT assay and trypan blue exclusion assays showed similar sensitivity of MCF-7/p1 and MCF-7/p2 cells to tamoxifen and ICI 182 780 (data not shown). This implies that the SRC-1 has a minimal role in determining the sensitivity of MCF-7 breast cancer cells to antiestrogens.

Basal and E2-inducible SDF-1α expression in MCF-7/p1 cells is dependent on ERα and p160 family coactivators

To confirm that basal SDF-1α expression in MCF-7/p1 cells is dependent on ERα and coactivators, we measured SDF-1α expression in the presence of either tamoxifen or ICI 182 780. Both tamoxifen and ICI 182 780 reduced basal SDF-1α expression (Figure 4A). To further ensure the role of ERα in basal SDF-1α expression, we treated MCF-7/p1 cells with either control siRNA or siRNA against ERα and measured

expression levels of SDF-1α mRNA. As shown in Figure 4B, the basal SDF-1α expression was substantially lower in cells treated with siRNA against ERα when compared with cells treated with control siRNA. The siRNA against ERα had similar effect on basal SDF-1α expression in LCC2 cells (data not shown). These results suggest that ERα is required for basal SDF-1α expression.

To determine whether basal SDF-1α expression is dependent on overall coactivator levels or requires specific expression of SRC-1, we used siRNA specifically to reduce SRC-1 or AIB1 levels in MCF-7/p1 cells. Interestingly, basal SDF-1α expression was lower in cells treated with siRNA against either SRC-1 or AIB1 compared with control cells (Figure 4C). Overall effects of siRNA against SRC-1 on pS2 and SDF-1α expression in MCF-7/p1 and LCC2 cells from multiple experiments is shown in Figure 4D. These results suggest that overall coactivator levels but not SRC-1 *per se* determine basal SDF-1α expression.

To determine whether SDF-1α expression in other breast cancer cell lines is regulated by estrogen and p160 coactivators, we first investigated the effect of estrogen on SDF-1α expression in ZR-75-1 cells by RT–PCR (Figure 4E). Estrogen increased SDF-1α expression in these cells. Similar results were obtained in T47-D cells (data not shown). The siRNA against SRC-1 or AIB1 reduced basal SDF-1α expression in ZR-75-1 cells (Figure 4F). Thus, the effect of p160 activators on SDF-1α expression is not specific to MCF-7 cells.

We next investigated whether basal SDF-1α expression in MCF-7/p2 cells can be increased by overexpression of coactivators. Overexpression of SRC-1, TIF-2 or AIB1 increased basal SDF-1α mRNA levels in MCF-7/p2 cells (Figure 4G).
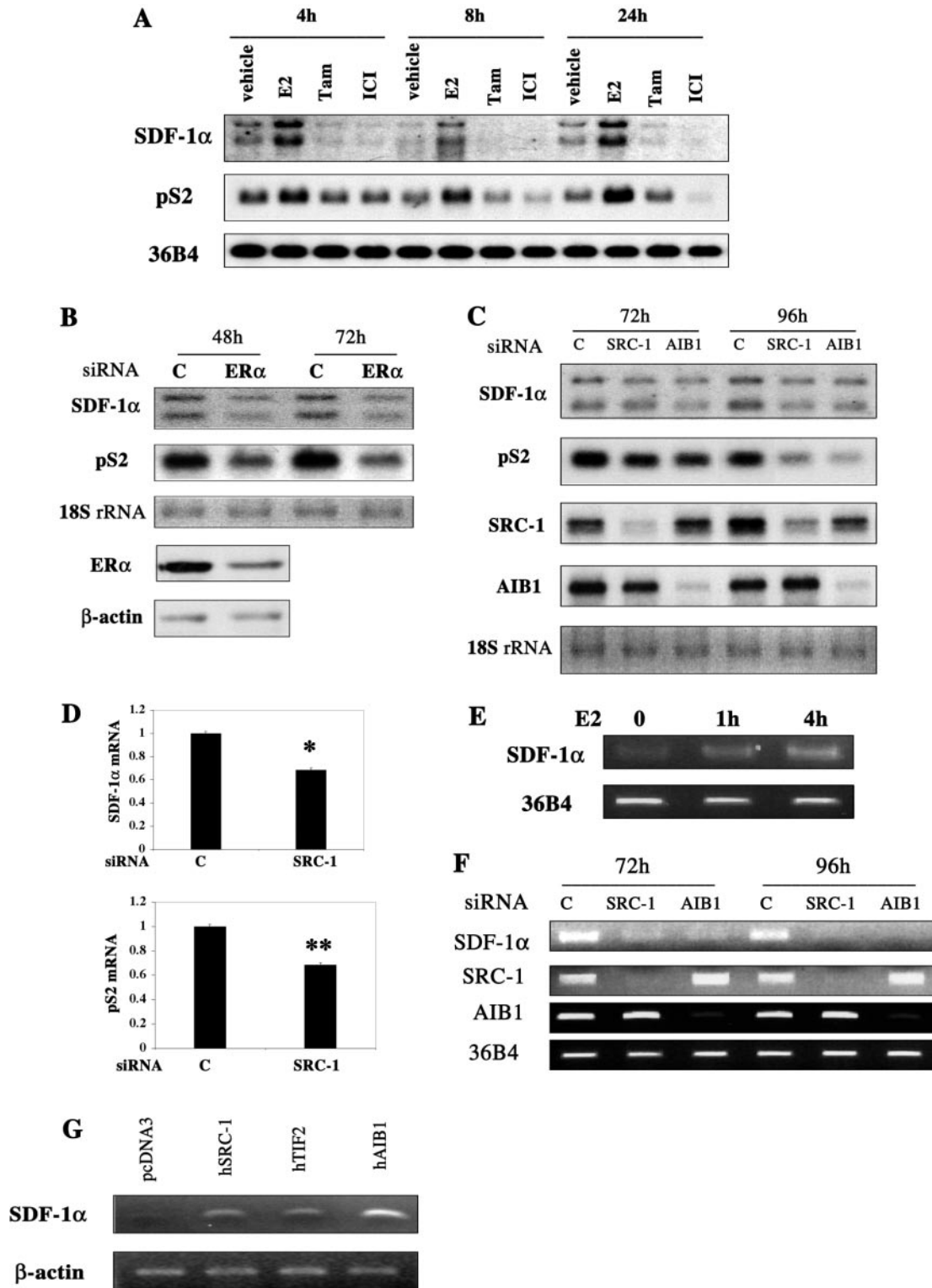
Fig. 4. Basal and E2-inducible SDF-1α expression in MCF-7/p1 cells is dependent on ERα and p160 family coactivators. (**A**) Total RNA (5 μg) from MCF-7/p1 cells treated with vehicle, E2 (0.1 nM), 4-hydroxytamoxifen (Tam, 1 μM) or ICI 182 780 (ICI, 1 μM) for indicated time was subjected to northern blot analysis with SDF-1α and pS2 probes. The integrity of RNA was verified by reprobing the blot with 36B4 ribosomal protein gene. (**B**) MCF-7/p1 cells were transfected with siRNA against ERα or non-specific control siRNA. SDF-1α and pS2 expression was measured after 48 or 72 h of siRNA transfection by northern blot analysis. ERα protein was measured by western blot analysis (bottom two rows). (**C**) Total RNA (5 μg) from MCF-7/p1 cells treated with siRNA against SRC-1 or AIB1 at the indicated time point was subjected to northern blot analysis with SDF-1α or pS2 probe. The same blot was reprobed with SRC-1 or AIB1 to ensure that introduction of siRNA resulted in the degradation of SRC-1 or AIB1 mRNA. (**D**) The effect of siRNA against SRC-1 on the basal expression of SDF-1α and pS2 from three or more experiments is shown. $^*P = 0.0005$; $^{**}P < 0.0001$. (**E**) The effect of estrogen on SDF-1α expression in ZR-75-1 cells was measured by RT–PCR. (**F**) The effect of siRNA against SRC-1 and AIB1 on basal expression of SDF-1α in ZR-75-1 cells. SDF-1α, SRC-1 and AIB1 expression in siRNA-treated cells was determined by RT–PCR. (**G**) All p160 coactivators increase SDF-1α expression. MCF-7/p2 cells were transfected with pcDNA3 vector or SRC-1, TIF-2 and AIB1 expression vectors using Lipofectamine 2000. Total RNA from cells after 72 h of transfection was subjected to RT–PCR using the proSTAR kit (Stratagene).

These results further support the interpretation that overall expression levels of coactivators, but not individual coactivator levels, determine basal SDF-1α expression in breast cancer cells.

SDF-1α stimulates proliferation of ERα positive MCF-7 cells and increases invasion of metastatic MDA-MB-231 cells

To establish a functional role for SDF-1α, we first measured the levels of SDF-1α secreted in MCF-7/p1 and MCF-7/p2 cells by ELISA. SDF-1α could not be detected in the conditioned media from MCF-7/p2 cells, whereas a low amount of SDF-1α was detected in the media of MCF-7/p1 cells. E2 induced robust SDF-1α production in MCF-7/p1 cells at both 8 and 24 h after treatment when compared with MCF-7/p2 cells (Figure 5A).

We next sought to investigate whether lower proliferation rate of MCF-7/p2 cells compared with MCF-7p1 cells is a consequence of lower SDF-1α in these cells. SDF-1α has been shown to mediate the mitogenic effects of estrogen in ovarian and breast cancer cells (19). We first ensured that MCF-7/p1 and MCF-p2 cells do not differ with respect to level of CXCR4, the receptor for SDF-1α. A quantitative real-time PCR was used to assess the expression of CXCR4 mRNA. Both MCF-7/p1 and MCF-7/p2 cells express low but comparable levels of CXCR4 mRNA (Figure 5B). We established two stable clones of MCF-7/p2 cells overexpressing SDF-1α; these clones secrete higher levels of SDF-1α compared with cells containing vector alone (Figure 5C). Proliferation of cells stably overexpressing SDF-1α and blank vector control cells was assessed by MTT assay. As shown in
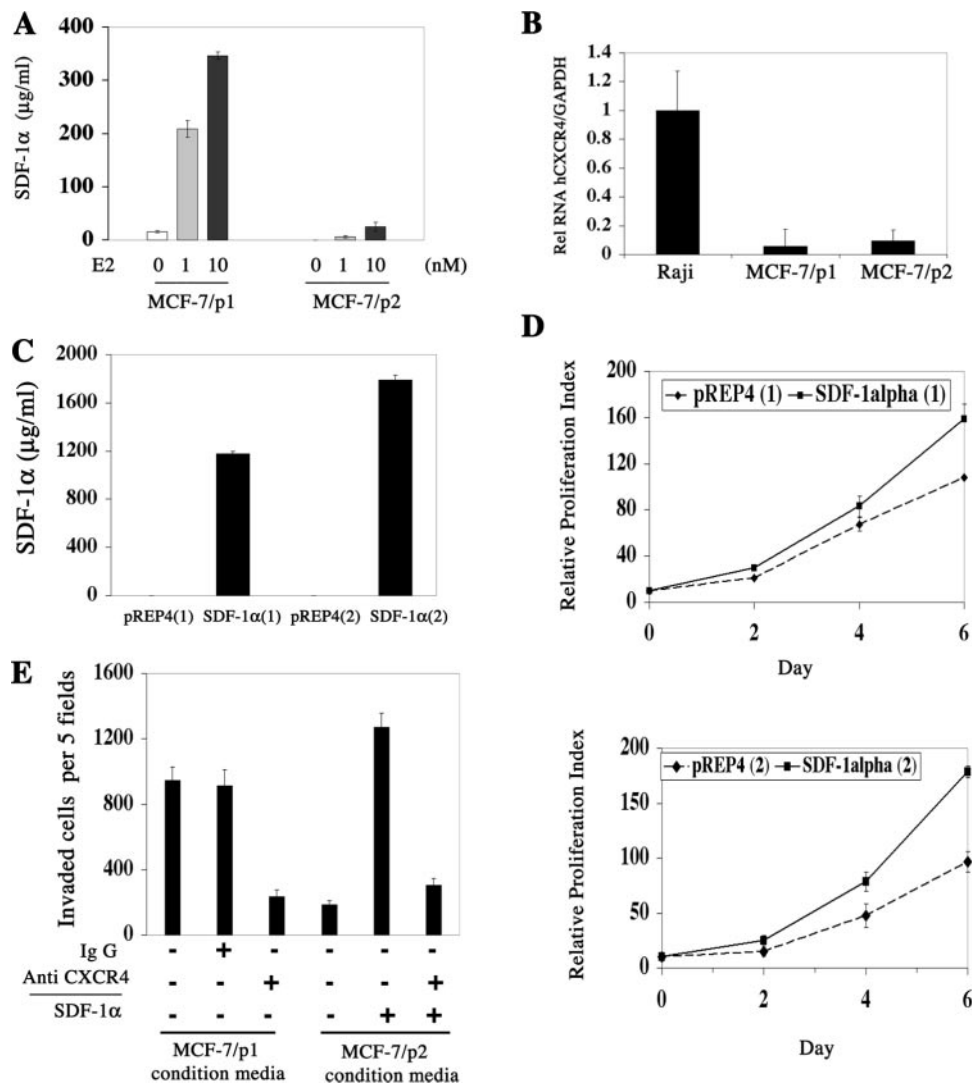
Fig. 5. SDF-1α stimulates proliferation of ERα-positive MCF-7 cells and increases invasion of metastatic MDA-MB-231 cells. (**A**) MCF-7/p1 and MCF-7/p2 cells were cultured in 5% phenol red free CCS containing media for 3 days. Conditioned media from cells treated with or without 1 or 10 nM E2 for 24 h were subjected to ELISA using a human SDF-1α Quantikine kit (R&D) according to the manufacturer's protocol. (**B**) Total RNA (5 μg) from MCF-7/p1, MCF-7/p2 cells and Raji cells, which served as a positive control, was subjected to quantitative real-time RT–PCR as described in Materials and methods. Difference in CXCR4 expression between MCF-7/p1 and MCF-7/p2 cells is not statistically significant ($P > 0.05$). (**C**) The conditioned media from MCF-7 /p2 cells stably overexpressing SDF-1α and blank control vector were subjected to ELISA using a human SDF-1α Quantikine kit (R&D) according to the manufacturer's protocol. (**D**) The proliferation rate of MCF-7/p2 cells stably overexpressing SDF-1α and blank control vector was measured by MTT assay. $P = 0.0006$, control versus SDF-1α overexpressing cells. (**E**) Effects of the conditioned media of MCF-7/p1 and MCF-7/p2 cells on the invasion of LMD 231 cells. Cell invasion assay was carried out as described in Materials and methods. Results are expressed as the mean number of invaded cells per well. $P = 0.008$, invasion with MCF-7/p1 versus MCF-7/p2 condition media. $P = 0.006$, invasion with MCF-7/p1 conditioned media versus same media treated with anti-CXCR4 antibody.

Figure 5D, SDF-1α overexpressing cells grew faster than blank vector cells suggesting that lack of SDF-1α expression is responsible for reduced proliferation of MCF-7/p2 cells compared with MCF-7p1 cells.

To determine the significance of SDF-1α expression in other processes of cancer progression, we compared migration and invasion of MCF-7/p1 and MCF-7p2 cells using *in vitro* chemotaxis and matrigel invasion assays. Neither MCF-7/p1 nor MCF-7/p2 cells showed any migration or invasion in these assays (data not shown). MCF-7 cells do not express urokinase plasminogen activator (uPA), an enzyme required for invasion (37,38). Lower levels of CXCR4 are unlikely to explain the failure of MCF-7/p1 or MCF-7/p2 cells to invade because MCF-7 cells overexpressing interleukin-1 alpha (IL-1α) express higher levels of CXCR4 compared with parental cells but fail to show invasion and metastasis in xenograft models (39). Thus, it appears that SDF-1α:CXCR4 signaling confers a proliferative advantage but not an invasive capacity to MCF-7 cells.

We have recently developed variants of MDA-MB-231 cells with different levels of CXCR4 (23). Cells that have metastasized to lungs (LMD231) express the highest level of CXCR4. MDA-MB-231 cells express several genes involved in invasion including uPA and efficiently invade through matrigel in *in vitro* assays (40). We compared the ability of conditioned media from MCF-7/p1 and MCF-7/p2 cells to enhance invasion of LMD231 through matrigel. Conditioned media from MCF-7/p1 cells but not MCF-7/p2 cells increased invasion of LMD231 cells (Figure 5E). Addition of neutralizing anti-CXCR4 antibody to the MCF-7/p1 cell conditioned media, which blocks SDF-1α:CXCR4 interactions, reduced invasion of LMD231 cells through matrigel. In contrast, adding SDF-1α to the conditioned media from MCF-7/p2 cells dramatically increased invasion of LMD231 cells. Taken together, these results suggest that coactivators play an important role in determining the proliferation capacity of breast cancer cells through SDF-1α. These coactivators also influence SDF-1α:CXCR4 mediated invasion of cancer cells provided they express additional genes involved in invasion.

Discussion

In this study, we have characterized a MCF-7 cell variant lacking SRC-1 for ERα-dependent gene expression, proliferation and invasion. We observed that coactivator levels determine the basal and estrogen-inducible expression of SDF-1α, a secreted protein that controls breast cancer cell proliferation and invasion through autocrine and paracrine mechanisms (19). SDF-1α has been shown to be required for estrogen-induced proliferation of breast and ovarian cancer cells but not uterine epithelial cells (19). Consistent with the significant role of SDF-1α in breast cancer progression, high levels of SDF-1α is present in the breast cancer microenvironment (41).

Coactivator specificity is a subject of intense investigation. SRC-1, TIF-2 and AIB1 knockout animals show distinct phenotypes. SRC-1 knockout animals show partial resistance to several hormones, whereas AIB1 knockout animals show impaired female reproductive functions (42–45). Both TIF-2 and SRC-1 are involved in energy balance (46). At the molecular level, it was shown that coactivator levels determine both inducible and temporal expression pattern of genes induced by nuclear receptors (32). For example, the expression level

of early response genes with non-classical EREs is dependent on overall coactivator levels, although SRC-1 is favored over TIF-2 or AIB1. Elevating levels of any one of the coactivators increases the expression of early response genes. In contrast, the expression of late response genes with classical EREs is dependent primarily on AIB1. In our study, the maximum effect of lack of SRC-1 was on basal expression of three genes; the early response genes *cMyc* and *pS2*, and the late response gene *SDF-1α* (Figure 3). Absence of SRC-1 had no effect on basal expression of cathepsin D, a late response gene with a classical ERE. Note that lack of SRC-1 had no effect on the expression of recently identified estrogen-responsive genes *WISP2* (connective tissue growth factor family), *FLH2* (Four-and-a-half LIM domain 2) and *RRM2* (ribonucleotide reductase M2) (36) (data not shown). With respect to inducible expression, cells lacking SRC-1 showed lower induction of pS2 and SDF-1α but not cathepsin D. In fact, inducible expression of cathepsin D was higher in cells lacking SRC-1. Although our results are not in complete agreement with the results of Zhang *et al*. (32) with respect to coactivator specificity in induction of early and late response genes, they highlight the importance of coactivator levels in regulating basal expression of estrogen-responsive genes.

All our studies were conducted in cells maintained in phenol red free media with 5% CCS. CCS contains ∼10 pM of estrogen (as per product description from Hyclone), which gives a final concentration of 0.5 pM. It is possible that basal expression that we describe, in part, is because of residual ligand, which is sufficient for receptor activation under adequate coactivator levels. In this respect, tamoxifen (which stabilizes ERα but inhibits activity) and ICI 182 780 (which induces ERα degradation) reduced the basal expression of SDF-1α in MCF-7/p1 cells (Figure 4A).

Although SDF-1α induced proliferation of MCF-7 cells, it failed to induce migration and invasion of these cells (Figure 5E), which is consistent with the observations by Hall and Korach (19). There are several reasons why MCF-7 cells failed to invade through matrigel. One is that the level of CXCR4 in these cells is sufficient for activating proliferation signals but not invasion. Estrogen has counter-balancing actions that limit autocrine SDF-1α:CXCR4 signaling pathways. In spite of increasing SDF-1α expression, estrogen reduces CXCR4 as well as the signaling protein B cell linker (BLNK), a scaffolding protein that regulates CXCR4 activity (25). We do not favor this possibility because we have generated MCF-7 cells overexpressing IL-1α. These cells express higher levels of CXCR4 than control cells but fail to show invasion and metastasis in a xenograft model (39). A more probable explanation is that MCF-7 cells do not express genes that are downstream of the SDF-1α:CXCR4-induced invasion process. SDF-1α:CXCR4-induced proliferation is dependent on MAPK and AKT pathways, whereas migration/invasion is dependent on Tec family tyrosine kinase ITK and matrix metalloproteinases 2 and 9 (MMP2 and MMP9) (47–49). Invasion and metastasis requires the coordinated activity of MMPs and the uPA/uPAR system (38). MCF-7 cells show very little MMP activity and do not express uPA because of promoter methylation (37,39). Thus, with respect to invasion and metastasis, paracrine action of ERα-induced SDF-1α may be important. We propose that overexpression of p160 coactivators in ERα-positive cancer cells leads to increased SDF-1α expression, which induces invasion and metastasis of cancer cells expressing higher levels of CXCR4 and other
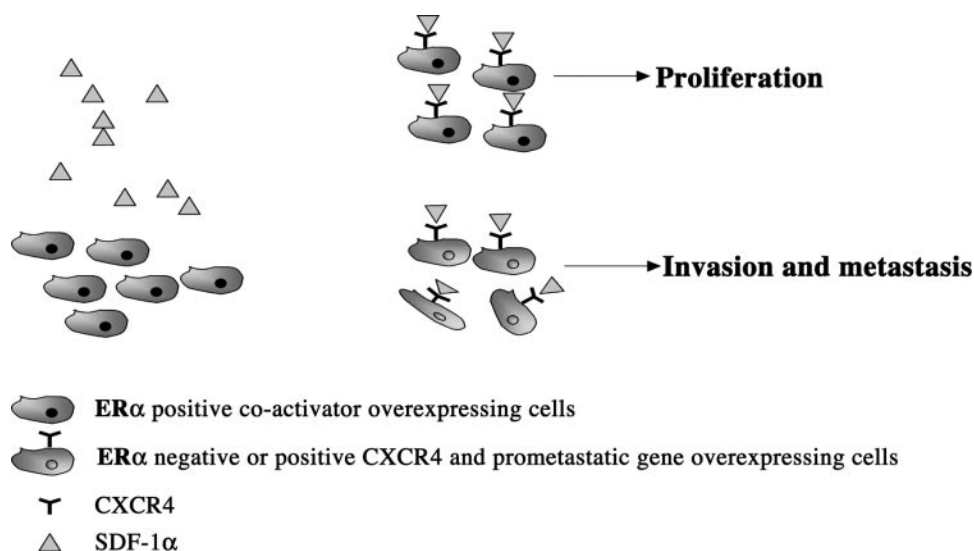
Fig. 6. An integrated model depicting autocrine/paracrine action of SDF-1α induced as a consequence of coactivator overexpression. ER α-positive cells that overexpress coactivators secrete SDF-1α, which increases proliferation in an autocrine manner. SDF-1α in the tumor microenvironment may enhance invasion/metastasis of cancer cells that overexpress CXCR4 as well as other prometastatic genes, such as UPA and MMPs. her2/neu overexpressing cells may potentially benefit from SDF-1α because they usually overexpress prometastatic genes and CXCR4 in these cells is stabilized by her2/neu (21). See online Supplementary material for a colour version of this figure.

prometastatic genes (Figure 6). Tumors are heterogeneous with respect to ERα expression with ∼56% of cytokeratin-positive cells being ERα-positive in one study (50).

Several recent studies suggest that the concentration of coactivators determines active AKT levels, response to selective estrogen receptor modulators (SERMs) and disease free survival, particularly in patients with her2/neu overexpression (14,15,31,51). For example, in endometrial cancer cells, SRC-1 is responsible for the estrogen-like activity of tamoxifen (51). In the MCF-7 cell model system used here, coactivator levels do not appear to determine tamoxifen sensitivity because coactivator levels in MCF-7/p1 and tamoxifen-resistant LCC2 cells were similar, and MCF-7/p1 and MCF-7/p2 cells showed similar sensitivity to tamoxifen. Our attempt to address the possibility that the estrogenic activity of tamoxifen in endometrial cells is through SRC-1:ERα:tamoxifen-induced SDF-1α expression was not successful because the endometrial cell line Ishikawa cells do not express SDF-1α (data not shown). In another study, it was shown that simultaneous overexpression of AIB1 and her2/neu is associated with short disease free survival and poor response to tamoxifen therapy (15). A similar link is also established between co-overexpression of SRC-1 and her2/neu (16). It is possible that the poor prognosis in these patients is because of coactivator-mediated overexpression of SDF-1α and her2/neu-mediated stabilization of CXCR4 (21). SDF-1α:CXCR4 signaling is required for proliferation of cancer cells at sites of metastasis, although it is not required for the establishment of micrometastasis in colon carcinoma cells (52). Thus, it is possible that cancer cells that co-overexpress coactivators and her2/neu are better equipped to proliferate at sites of metastasis. Additional studies with primary tumor samples are required to test this possibility.

Supplementary material

Supplementary material can be found at http://carcin. oxfordjournals.org/.

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