Oncogene (2004) 23, 8920–8930

www.nature.com/onc

Fibronectin and type IV collagen activate ERα AF-1 by c-Src pathway: effect on breast cancer cell motility

Diego Sisci[1,3], Saveria Aquila[1,3], Emilia Middea[1], Mariaelena Gentile[2], Marcello Maggiolini[1], Fabrizia Mastroianni[1], Daniela Montanaro[1] and Sebastiano Andò*[,2]

[1]*Dipartimento Farmaco-Biologico, Università della Calabria, Arcavacata di Rende, Italy;* [2]*Dipartimento di Biologia Cellulare, Centro Sanitario, Università della Calabria, Arcavacata di Rende, Cosenza 87030, Italy*

The expression of estrogen receptor alpha (ERα) is generally associated with a less invasive and aggressive phenotype in breast carcinoma. In an attempt to understand the role of ERα in regulating breast cancer cells invasiveness, we have demonstrated that cell adhesion on fibronectin (Fn) and type IV Collagen (Col) induces ERα-mediated transcription and reduces cell migration in MCF-7 and in MDA-MB-231 cell lines expressing ERα. Analysis of deleted mutants of ERα indicates that the transcriptional activation function (AF)-1 is required for ERα-mediated transcription as well as for the inhibition of cell migration induced by cell adhesion on extracellular matrix (ECM) proteins. In addition, the nuclear localization signal region and some serine residues in the AF-1 of the ERα are both required for the regulation of cell invasiveness as we have observed in HeLa cells. It is worth noting that c-Src activation is coincident with adhesion of cells to ECM proteins and that the inhibition of c-Src activity by PP2 or the expression of a dominant-negative c-Src abolishes ERα-mediated transcription and partially reverts the inhibition of cell invasiveness in ERα-positive cancer cells. These findings address the integrated role of ECM proteins and ERα in influencing breast cancer cell motility through a mechanism that involves c-Src and seems not to be related to a specific cell type.
Oncogene (2004) **23,** 8920–8930. doi:10.1038/sj.onc.1208098
Published online 27 September 2004

Keywords: estrogen receptor alpha; extracellular matrix; fibronectin; type IV collagen; c-Src

Introduction

Several clinical studies have demonstrated that estrogen receptor alpha (ERα)-positive tumors have lower metastatic potential than ERα-negative tumors (Fraker *et al*., 1984; Osborne *et al*., 1985; McGuire, 1986; Price *et al*., 1990). Many reports correlate ERα expression to lower matrigel invasiveness and to a reduced metastatic potential of breast cancer cell lines (Liotta *et al*., 1991; Thompson *et al*., 1992; Rochefort *et al*., 1998); however, the molecular mechanisms that define this process are still unclear. The interaction of cells with the extracellular matrix (ECM) influences many aspects of cell behavior, including growth, morphology, migratory properties and differentiation (Hynes, 1990; Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999). The adhesion to ECM is mediated by integrin receptors that are reported to control growth factor signaling pathways. Specifically, cells adherent to ECM show an enhanced activation of the p42 and p44 forms of the mitogen-activated protein kinase (MAPK) (Miyamoto *et al*., 1996; Lin *et al*., 1997; Renshaw *et al*., 1997; Aplin and Juliano, 1999). In addition, binding of the integrin receptor with ECM proteins causes a direct transient activation of MAP kinase in the absence of growth factors (Chen *et al*., 1994; Schlaepfer *et al*., 1994; Zhu and Assoian, 1995).

The transcriptional activation function (AF)-1 of the N-terminal ERα is a target of various protein kinases such as MAPK, PI3-k, Akt and c-Src, which are activated by growth factor pathways, either in the presence or in the absence of 17β-estradiol (E_2) (Power *et al*., 1991; Chalbos *et al*., 1993; Aronica *et al*., 1994; Kato *et al*., 1994; Couse *et al*., 1995; Ignar-Trowbridge *et al*., 1995; Bunone *et al*., 1996; Weigel, 1996; Joel *et al*., 1998). c-Src is also one of the first protein kinases activated by cell adhesion to ECM (Guan, 1997; Schlaepfer *et al*., 1997; Schaller *et al*., 1999) and it has been shown to play a significant role in several phases of outside–in signaling in many cell types (Kaplan *et al*., 1995; Lowell *et al*., 1996; Suen *et al*., 1999). The overexpression and activation of Src family kinases have been identified in a range of human cancers (Irby and Yeatman, 2000) and these have been indicated to contribute not only to the growth and survival of breast cancer cells but also to increase their metastatic potential (Summy and Gallick, 2003).

In this study, we have tested the hypothesis that cell adhesion on ECM modulates ERα transcriptional activity, producing a reduction of cell migration. Our results show that estrogen receptor activation function 1 (AF-1)/ERα is an effector of the adhesion protein signals. Cell adhesion on Fibronectin (Fn) and type IV collagen (Col) induces the transcriptional activation of

*Correspondence: S Andò; E-mail: sebastiano.ando@unical.it
[3]These authors contributed equally to this work
Received 27 January 2004; revised 21 May 2004; accepted 8 July 2004; published online 27 September 2004

ERα and the reduction of cell migration in MCF-7 cells, as well as in MDA-MB-231 and in HeLa cells both engineered to express ERα. In addition, we have found that c-Src is essential for ERα activation in response to cell adhesion. Focusing on the relationship between c-Src activity and ERα expression in breast cancer cells, we have shown that the transactivation of ERα upon cell adhesion on ECM proteins counteracts the action of c-Src on cell motility and invasiveness in cancerous cells.

Results

Cell adhesion on Fn or Col induces ERα translocation into the nuclear compartment

First, we determined whether cell adhesion on either Fn or Col may influence ERα expression. MCF-7 cells, serum-starved for 24 h, were plated onto Fn-, Col- and P-Lys- (negative control) coated dishes and incubated for 30 min, 1 h, 4 h and 8 h. Equal amounts of cytosolic and total (nuclear + cytosolic) protein lysates were resolved by SDS–PAGE and analysed by Western blot for ERα detection. Figure 1b shows a significant decrease of ERα content in the cytosol 30 min to 4 h after cell adhesion on Fn and Col with respect to the control reported in Figure 1a. No substantial changes in total ERα content were observed in our time course study (Figure 1b). These results support the conclusion that cell adhesion on either Fn or Col induced a translocation of ERα into the nuclear compartment. The observed compartmentalization of ERα was confirmed by immunocytochemistry using MCF-7 cells maintained in a serum-free medium for 96 h, then detached, plated onto Fn-, Col- and P-Lys-coated slides and incubated for 2 h (Figure 1c). No signal was detected in the control cells (P-Lys); this may be due to the binding of ERα with chaperon proteins that possibly mask the epitope for the ERα antibody.

Fn and Col induce estrogen-responsive element (ERE) transcription

Since Fn and Col were able to translocate ERα into the nuclear compartment, we evaluated their ability to induce ERα-mediated transcription. MCF-7 cells were transfected with the ERE responsive reporter plasmid (XETL) and the pRL-Tk plasmid expressing *Renilla luciferase*, as an internal control. The cells were then serum starved for 24 h and exposed to E_2, Fn, Col or P-Lys before luciferase assays. Figure 2a shows a significant increase in ERE-mediated transcription induced by both Fn and Col in the absence of ER ligand. In contrast, P-Lys failed to induce ERα-mediated transcription, showing that the observed effect is specifically due to the integrin substrates.

The same results were obtained in MDA-MB-231 cells transiently expressing ERα and the reporter gene, suggesting that the Fn/Col-induced transcriptional activation is specifically mediated by ERα (Figure 2c). A slight but significant increase in luciferase expression was observed in wild-type MDA-MB-231 treated with both Fn and Col (Figure 2b), probably due to the expression of ERβ (Lazennec *et al*., 2001), as we detected in the present study (data not shown). The antiestrogen ICI reversed this upregulatory effect induced by both ECM proteins in all the experimental conditions and in both cell lines.

In addition, no substantial differences were observed in luciferase expression when transfected MCF-7 and MDA-MB-231 were plated onto both Fn- and Col-coated dishes (data not shown).

ERE-mediated transcription induced by Fn and Col upregulates pS2 and cathepsin D mRNA

Owing to their ability to induce ERE-mediated transcription, Fn and Col were assessed for their capacity to regulate the expression of endogenous ERα-specific target genes as pS2 (Figure 3a) and cathepsin D (Figure 3b).

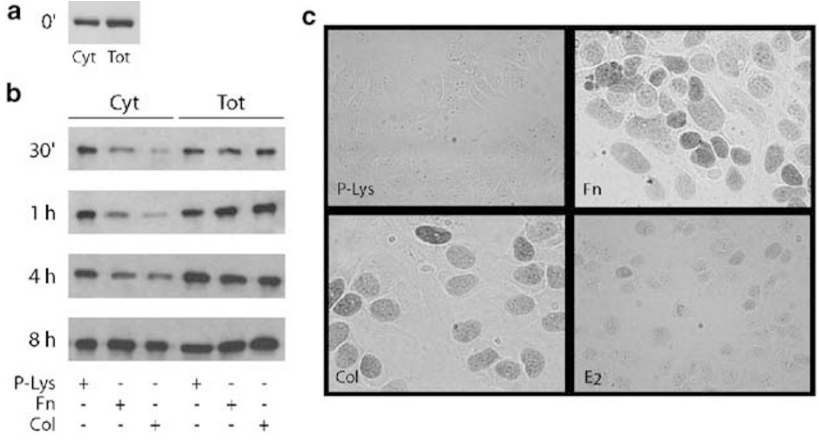

Figure 1 Cell adhesion on Fn or Col induces ERα translocation into the nuclear compartment. (**a**) MCF-7 cells serum-starved for 24 h were detached and an aliquot was lysed and used as control (time 0); (**b**) a further aliquot was plated in PRF-SFM on P-Lys- (2 μg/cm²), Fn- (30 μg/ml) or Col- (30 μg/ml) coated dishes. After 30 min, 1 h, 4 h and 8 h of incubation, cytosolic (Cyt) and total (cytosolic and nuclear) (Tot) protein lysates were subjected to Western blotting with an anti-ERα monoclonal antibody. These results are representative of five independent experiments. (**c**) MCF-7 cells serum starved for 96 h were detached and plated in PRF-SFM on P-Lys- (2 μg/cm²), Fn- (30 μg/ml) and Col- (30 μg/ml) coated slides or plated on culture-treated slide and incubated with E_2 (10 nM). After 2 h, cells were fixed, probed with anti-ERα antibody and stained as described in Materials and methods

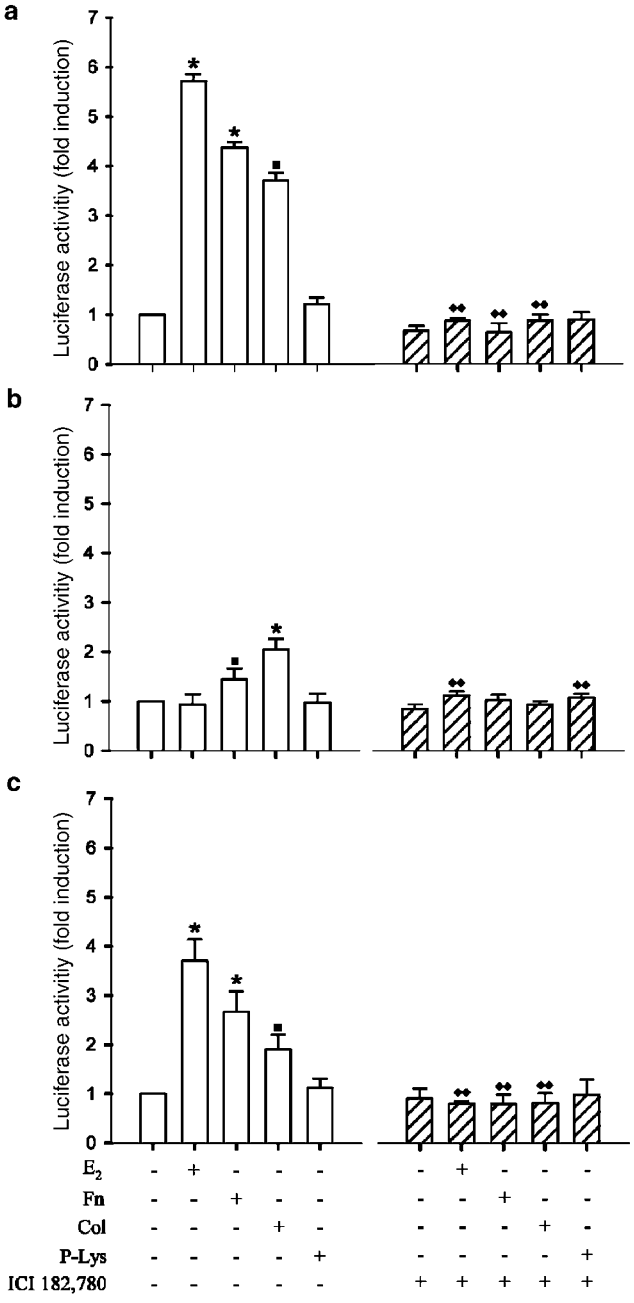

Figure 2 Fn and Col activate ERE-mediated transcription. MCF-7 (**a**) and MDA-MB-231 (**b**) cells were transfected with 0.3 μg/well of pSG5 and 0.6 μg/well of XETL by calcium phosphate method. MDA-MB-231 were also cotransfected with 0.3 μg/well HeG0 and 0.6 μg/well of XETL (**c**). Cells were incubated in the transfection cocktail for 6 h, serum starved for an additional 24 h and then incubated for 16 h in PRF-SFM (untreated) or in PRF-SFM containing either 10 nM estradiol (E_2), 30 μg/ml Fn, 30 μg/ml Col or 15 μg/ml P-Lys. The same treatments were also carried out in the presence of 100 nM ICI 182,780. Firefly luciferase activity was internally normalized to *R. luciferase* and expressed as fold of increase with respect to the PRF-SFM (untreated) sample. Results represent the mean $\pm$ s.d. of at least five independent experiments. $^{\star}P<0.001$ *vs* PRF-SFM; $^{\blacksquare}P<0.05$ *vs* PRF-SFM; $^{\blacklozenge\blacklozenge}P<0.001$ *vs* the homologues samples without ICI 182,780

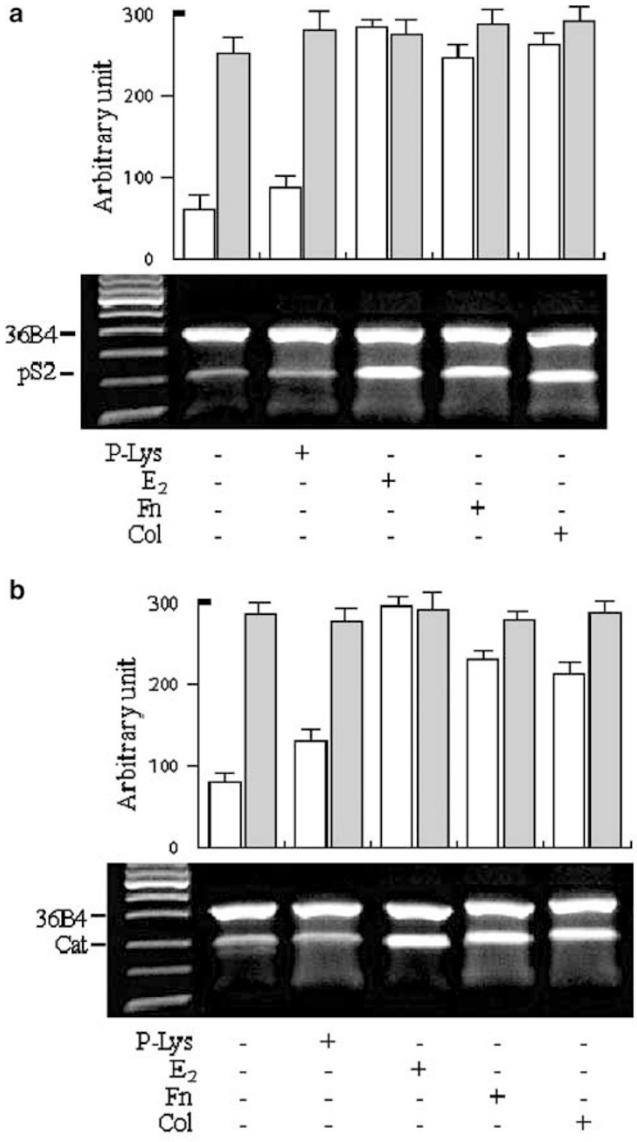

Figure 3 Adhesion of breast cancer cells on Fn and Col upregulates pS2 and cathepsin D mRNA levels. Semiquantitative RT–PCR of pS2 mRNA (**a**) and cathepsin D (**b**). Serum-starved MCF-7 cells were detached and plated, in PRF-SFM, on dishes previously coated with 2 μg/cm^2 P-Lys, 30 μg/ml Fn (Fn), 30 μg/ml or plated on uncoated dishes and treated with 10 nM estradiol (E_2). The 36B4 mRNA levels were determined in the same amplification tube as control. The quantitative representation of three independent experiments expressing the optical density of pS2 (□) and 36B4 (▩) RT–PCR products (**a**) and of cathepsin D (□) and 36B4 (▩) RT–PCR products (**b**) is reported in the histograms

The results show the ability of Fn and Col, but not of P-Lys, to upregulate both pS2 and cathepsin D mRNAs.

Fn and Col induce ERE-mediated transcription through AF-1 activation

Subsequently, we questioned which functional domain of ERα was affected by the adhesion protein signals. To exclude the influence of specific factors present in breast cancer cells, we transiently cotransfected HeLa cells with

the reporter plasmid XETL and with either HeG0 (data not shown) or HE15 (Figure 4a) or HE19 (Figure 4b) coding for the carboxyl-terminal and amino-terminal truncated receptor, respectively. The treatment with either E_2 or Fn and Col leads to an increase of luciferase activity in HeLa cells expressing ERα corresponding to that observed in MCF-7 (data not shown). Both ECM proteins induced ERE-mediated transcription only in HeLa cells transiently expressing the AF-1/DBD domains of ERα (Figure 4a), while the treatment with E_2 gave a significant increase of ERE-mediated transcription when the AF-2/DBD domains of ERα were expressed (Figure 4b). ICI abolished the upregulatory effects induced by Fn, Col and E_2, while 4-OH tamoxifen (4OH-Tam) treatment was able to negatively interfere only with E_2-induced activation in cells expressing either ERα (data not shown) or the AF-2/DBD domains of ERα. In summary, these data demonstrate that either Fn or Col is able to activate ERα by targeting the AF-1 domain.

Fn and Col activate ERα through c-Src

On the basis of previous findings demonstrating that c-Src is activated by Fn (Schlaepfer *et al*., 1997) and that ERα is activated in its AF-1 domain by signals transduced from c-Src (Feng *et al*., 2001), we questioned whether c-Src might be involved in ERα activation induced by cell adhesion/treatment to Fn and Col. We investigated the role of c-Src in MCF-7 cells transfected with pcDNA3 as control vector, c-Src(+) and c-Src(−) (dominant negative of c-Src) (Figure 5a). The overexpression of c-Src potentiates ERα transactivation both in basal condition as well as upon E_2, Fn and Col exposure. The expression of the dominant-negative c-Src as well as the treatment with PP2-abrogated ERE-mediated transcription induced by both Fn and Col, however, only attenuated the response to E_2. Altogether, these data suggest that c-Src is required for ERα activation by signals derived from cell adhesion to ECM. Under the same experimental conditions, we evaluated the autophosphorylation of c-Src and the phosphorylation of the exogenous substrate enolase (Figure 5b) after 5 min of exposure. The indicated time was chosen after a time course study (here not reported) performed at 0, 5, 10 and 20 min displaying the maximal enzymatic activity at 5 min. The results provide evidence that both E_2, Fn and Col activate c-Src, while either the expression of a dominant-negative c-Src or the presence of PP2 strongly reduces both the autophosphorylation of c-Src and the phosphorylation of enolase.

E_2, Fn and Col reduce cell motility

Since previous studies have shown that ERα expression leads to a lower matrigel invasiveness and to a reduced metastatic potential in breast cancer cells (Rochefort *et al*., 1998; Platet *et al*., 2000), we investigated whether

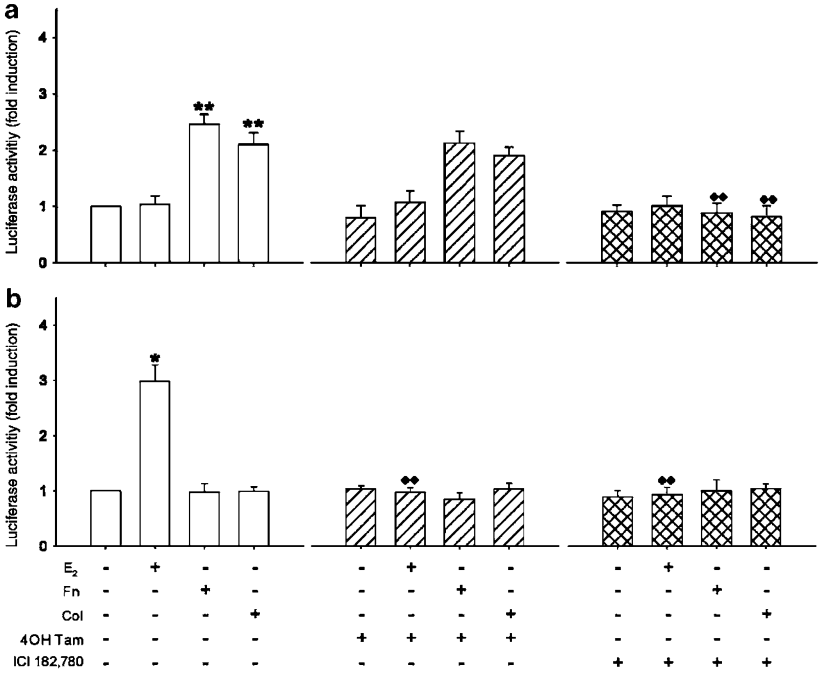

Figure 4 Fn and Col induce ligand-independent transcriptional activation of the ERα. HeLa cells were transfected with 0.3 μg/well HE15 and 0.6 μg/well XETL (**a**) or with 0.3 μg/well HE19 and 0.6 μg/well XETL (**b**). At 6 h after transfection cocktail addition, cells were shifted in PRF-SFM for 24 h and then incubated for 16 h in PRF-SFM containing either 15 μg/ml P-Lys, 10 nM estradiol (E_2), 30 μg/ml Fn or 30 μg/ml Col. The same treatments were also carried out in the presence of 100 nM 4-OH Tam and 100 nM ICI 182,780. Firefly luciferase activity was internally normalized to *R. luciferase* and expressed as fold of increase with respect to the PRF-SFM (untreated) samples. Results represent the mean ± s.d. of five independent experiments. $^{**}P<0.001$ and $^{*}P<0.01$ *vs* PRF-SFM (untreated); $^{\blacklozenge\blacklozenge}P<0.005$ *vs* the homologues samples without 4-OH Tam and ICI 182,780

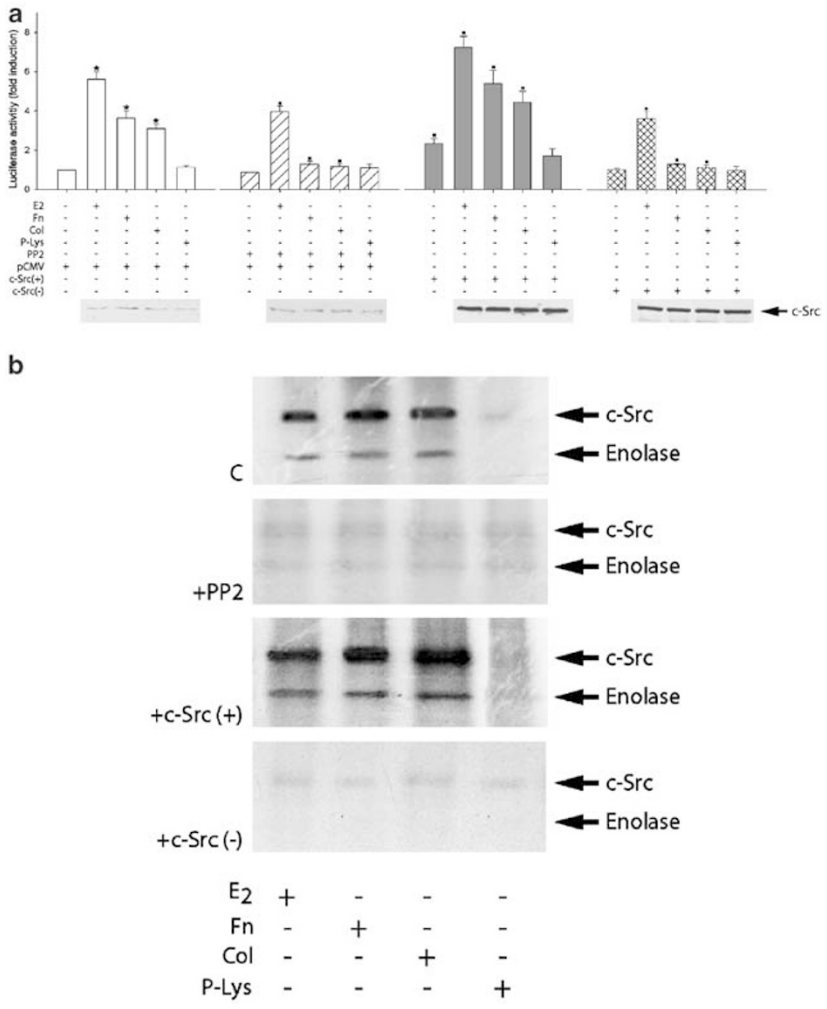

Figure 5 Fn and Col activate unliganded-ERα through c-Src. (**a**) MCF-7 cells were cotransfected with calcium phosphate precipitation method using 0.5 μg/well of XETL and 0.5 μg/well of pCMV empty vector or 0.5 μg/well of XETL plasmid with 0.5 μg/well of c-Src(+) or 0.5 μg/well of c-Src(−). At 6 h after transfection, cells were serum starved for 24 h and then incubated for 16 h in PRF-SFM (untreated) or PRF-SFM containing either 10 nM estradiol (E_2), 30 μg/ml Fn, 30 μg/ml Col or 15 μg/ml P-Lys. The same treatments were also carried out in the presence of 3 μM of PP2. Firefly luciferase activity was internally normalized to *R. luciferase* and expressed as fold of increase with respect to the PRF-SFM (untreated) sample. Results represent the mean ± s.d. of five independent experiments. The inset pictures present the expression of c-Src and were assessed by Western blotting as described in Materials and methods using 30 μg of total cell lysates. (**b**) MCF-7 cells, transfected and treated as before for 5 min, were lysed and immunoprecipitated with an anti c-Src antibody/protein A/G complex and assayed for c-Src-kinase activity using acid-treated enolase as described in Materials and methods. These results are representative of three independent experiments. ★$P<0.001$ *vs* the PRF-SFM (untreated); ■$P<0.01$ *vs* the homologues samples without PP2 and cSrc(−); ◆$P<0.05$ *vs* the homologues samples without PP2 and cSrc(−)

the activation of ERα by both ECM proteins was correlated with the motility of breast cancer cells. MCF-7, MDA-MB-231 and HeLa cells were used to evaluate cell invasion on Transwell chambers previously coated with Fn, Col or P-Lys and incubated overnight in PRF-SFM or in PRF-SFM containing E_2. In agreement with previous studies (Rochefort *et al*., 1998), E_2 treatment produced a marked reduction of cell migration in MCF-7 (Figure 6a) as well as in both MDA-MB-231 (data not shown) and HeLa cells expressing ERα (Figure 6b). Similar inhibition was observed after cell adhesion to either Fn or Col. To further clarify the contribution of the two ERα/AF domains in regulating cell motility, HeLa cells were transfected with either the AF-1- or AF-2-deleted constructs and plated onto transwells coated with both ECM proteins. Cells expressing the AF-1/DBD domains (Figure 6c) showed a strong reduction of cell invasion induced by both ECM proteins and reversed by ICI (data not shown). In contrast, only E_2 treatment reduced cell migration when the AF-2/DBD domains of the ERα were expressed (Figure 6d).

Fn and Col reduce cell motility through c-Src in breast cancer cells expressing ERα

Having established the role of c-Src in mediating ERα activation induced by cell adhesion to both Fn and Col, we examined its involvement in the regulation of breast

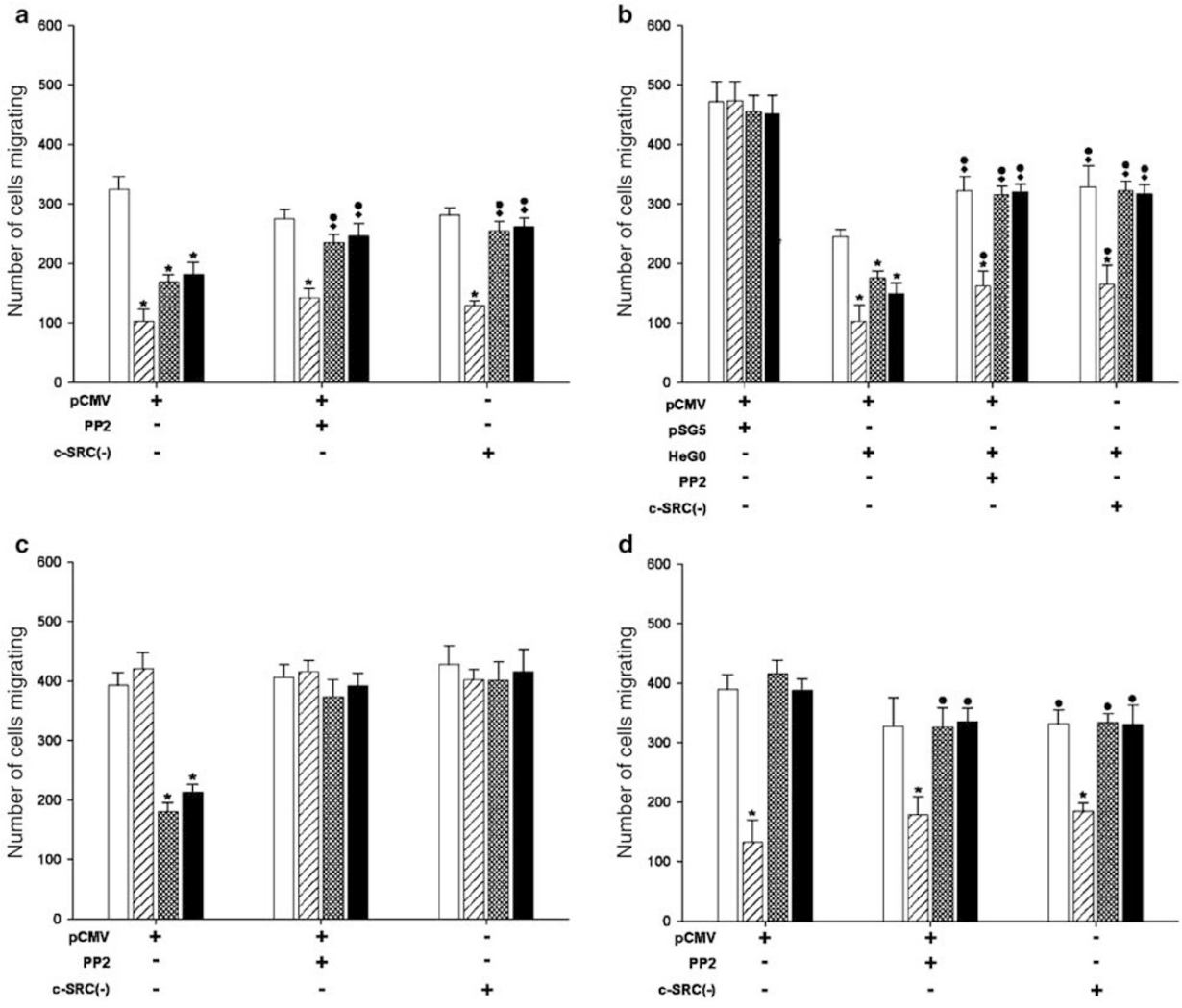

Figure 6 Cell adhesion on Fn and Col activate AF-1/ERα through c-Src reducing cell invasion. MCF-7 cells transiently transfected with 1 μg/well of c-Src(−) or pCMV (**a**) and HeLa cells transfected with 0.5 μg/well of the following plasmids; (**b**) pCMV and pSG5, pCMV and HeG0 or HeG0 and c-Src(−); (**c**) pCMV and pSG5, pCMV and HE15 or HE15 and c-Src(−); (**d**) pCMV and pSG5, pCMV and HE19 or HE19 and c-Src(−), after 6 h cells were serum starved for 24 h, detached, plated and allowed to migrate for 16 h on membranes coated either with 2 μg/cm^2 P-Lys (□) or 30 μg/ml Fn (⊠) or 30 μg/ml Col (■) or treated with 10 nM estradiol (▨). The effects induced by P-Lys, Fn, Col and E_2 on cell motility were also evaluated upon exposure to 3 μM PP2. Cells were then fixed, stained and the cells that migrated to the lower surface of the membranes were counted. The results represent the mean ± s.d. of four independent experiments. *$P<0.001$ *vs* P-Lys; •$P<0.001$ *vs* the homologues samples in vectors; ◆$P<0.05$ *vs* vectors/P-Lys

cancer cell motility. MCF-7, HeLa cells and HeLa expressing ectopic ERα as well as ERα deleted in the AF-1 or AF-2 domain were plated onto either Fn- or Col-coated membranes and incubated in the presence of PP2 or cotransfected with c-Src(−).

In MCF-7 (Figure 6a), the presence of either c-Src inhibitor or dominant-negative c-Src partially reverses the decrease in cell invasion caused by cell adhesion on both ECM proteins and, to a lesser extent, by E_2. In agreement with previous reports (reviewed in Summy and Gallick, 2003), the inhibition of c-Src elicits a slight decrease in both cell motility and invasiveness of about 20% ($P<0.05$) in wild-type HeLa cells (data not shown), while the ectopic expression of ERα induces a strong reduction in cell motility (Figure 6b), which appears emphasized by the adhesion on both ECM proteins, and is partially reversed by the inhibition of c-Src. Under the same circumstances, the dramatic reduction induced by E_2 still persists. Finally, the inhibition of c-Src with PP2 or the dominant-negative c-Src partially reverts the decrease on cell invasion induced by both ECM proteins in HeLa cells expressing the AF-1/DBD domains (Figure 6c). These data clearly show that c-Src is required for ligand-independent ERα-mediated reduction of breast cancer cell invasion.

Relationship between ERα and c-Src in mediating cell motility

It was previously demonstrated that elevated expression and/or activity of c-Src drastically increases motility, invasiveness and the metastatic potential of cancerous

cells (Summy and Gallick, 2003). To further clarify the role of ERα and c-Src in regulating cell motility, we transfected MDA-MB-231 with both HeG0 and Y527F, a constitutively active c-Src (Figure 7). The different levels of c-Src ectopically expressed in MDA-MB-231 cells does not induce substantial changes in cell motility, while the concomitant expression of ERα is sufficient to reduce cell motility. Namely, when ERα and active c-Src were expressed in a 4 : 1 ratio, a stronger reduction of cell motility was observed with respect to that obtained in cells expressing ERα alone. However, when MDA-MB-231 were transfected with ERα/c-Src using a 1 : 4 ratio, the reduction was partially reversed. These results indicate an opposite role for ERα and c-Src activity in regulating cell motility.

Nuclear localization of ERα is required to inhibit cell motility

Next, we questioned whether the nuclear localization of ERα is required for the reduction of cell invasion. With this aim, we tested cell invasion in HeLa cells transfected with either ERα or ERα in which serine 104, 106 and 118 were replaced by alanine residues (ER/Ser/A) and with the ERα lacking the NLS domain (E241G) in the presence of E_2, Fn and Col. In HeLa cells expressing the wild-type ERα E_2, Fn and Col induced a functional

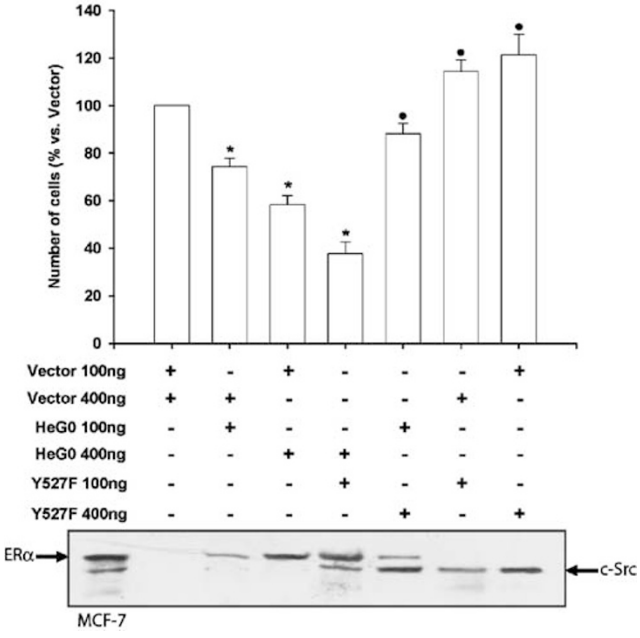

Figure 7 Relationship between ERα and c-Src in the regulation of cell motility. MDA-MB-231 cells were transfected, by calcium phosphate DNA co-precipitation method, using HeG0 and Y527F in a 4 : 1 or 1 : 4 ratio. The same plasmids were transfected either with pcDNA3 or pSG5 as vector of Y527F and HeG0, respectively. At 6 h after transfection, cells were serum starved for 24 h and then detached, counted and plated onto porous membranes in DMEM/F12 + 5% CS for 16 h. Cells were then fixed and stained, and cells which migrated to the lower surface of the filters were counted. The inset picture presents the expression of both ERα and c-Src in MDA-MB-231 cells after transfection. ERα and c-Src expression was assessed by Western blot as described in Materials and methods using 20 μg of total cell lysates. A measure of 20 μg MCF-7 cell lysates was used as positive control. The results represent the mean ± s.d. of three independent experiments. $^{*}P<0.01$ *vs* vectors, $^{\bullet}P<0.05$ *vs* vectors

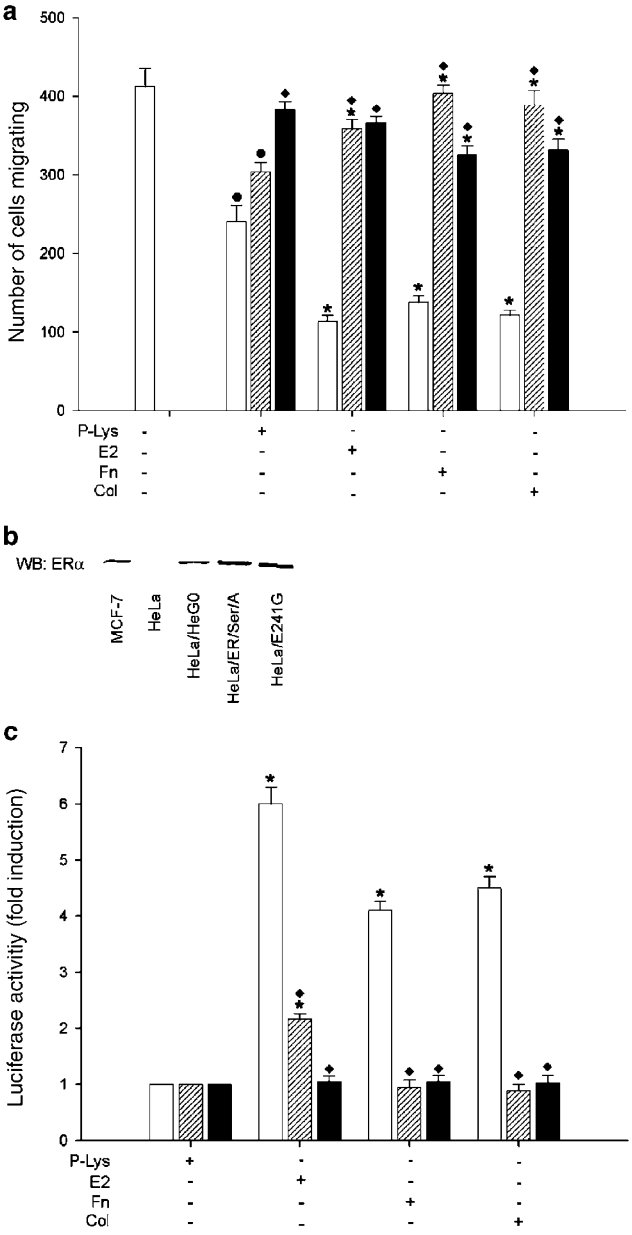

Figure 8 NLS and the AF-1 domain of ERα are required for ligand-dependent and -independent regulation of cell migration. HeLa cells were transfected with either HeG0 (□) or ER/Ser/A (▨) or E241G (■) by the calcium phosphate DNA co-precipitation method. After 6 h, cells were serum starved for 24 h and then detached and plated onto porous membranes coated either with 2 μg/cm² P-Lys, 30 μg/ml Fn (Fn), 30 μg/ml Col (Col) or treated with 10 nM estradiol (E_2) and incubated for 16 h, allowing cells to migrate. Cells were then fixed, stained and cells which migrated to the lower surface of the filters were counted (**a**). The inset picture presents the expression of ERα by Western blot after transfection (**b**). In the same experimental conditions, HeLa cells were co-transfected with either 0.3 μg/well HeG0, ER/Ser/A, E241G plus 0.6 μg/well of XETL (**c**). Firefly luciferase activity was internally normalized to *R. luciferase* and expressed as fold of increase with respect to the P-Lys samples. The results represent the mean ± s.d. of four independent experiments. $^{\bullet}P<0.01$ *vs* C0; $^{*}P<0.05$ *vs* the respective C; $^{\blacklozenge}P<0.01$ *vs* the respective treatment in HeG0

transactivation of ERα (Figure 8a) together with a dramatic reduction of cell invasiveness (Figure 8c). In the presence of ER/Ser/A, only E_2 induced a slight functional transactivation of ERα (Figure 8a), while none of the treatments produced substantial effects on cell invasiveness (Figure 8c). In HeLa cells expressing ERα deleted in the NLS region, the functional transactivation of ERα was abolished (Figure 8a), while just a slight decrease of cell invasiveness was observed after the cells adhesion on membranes coated with Fn and Col (Figure 8c).

Discussion

Several previous studies have indicated an important role for E_2 in decreasing '*in vitro*' invasiveness and motility of ERα-positive breast and ovarian cancer cells (Thompson *et al*., 1992; Hayashido *et al*., 1998; Rochefort *et al*., 1998). We found that cell adhesion on Fn and Col induces ERα translocation from the cytoplasm into the nucleus increasing the expression of estrogen-responsive genes, such as PS2 and cathepsin D, together with a downregulation of cell motility and invasion. Using ERα mutants lacking the AF-1 or the AF-2 region, we have provided evidence that both Fn and Col activate ERα in a ligand-independent manner as efficiently as the AF-2/ERα-mediated transcription induced by E_2.

It has been well documented that human ERα is phosphorylated by c-Src *in vitro* (Arnold *et al*., 1995) and that breast tumors, exhibiting an enhanced c-Src activity, frequently express a progesterone receptor. These findings raise the possibility that Src family kinases may contribute to the hormone dependence of tumor cell growth (Lehrer *et al*., 1989). In the same scenario, c-Src is able to transactivate the AF-1 domain of unliganded ERα partly through the ERK1/ERK2 signaling cascade and partly through the JNK signaling cascade (Feng *et al*., 2001).

Cell adhesion to ECM proteins induces the recruitment and activation of the FAK/c-Src kinase complex, which may be a crucial step in integrin-mediated signal transduction processes (Chen *et al*., 1994; Schlaepfer *et al*., 1994; Morino *et al*., 1995; Schlaepfer and Hunter, 1996). These findings well suit our data demonstrating that c-Src is activated by cell adhesion on both Fn and Col, leading to the functional transactivation of ERα with a concomitant reduction of cell invasion. Both ERα transactivation and the reduction of cell invasion, caused by both ECM proteins, were reversed by either PP2 or the expression of a dominant-negative c-Src. However, E_2 is still able to activate ERα with a reduction in cell invasion. These data strengthen previous findings demonstrating the existence of two distinct mechanisms through which ERα inhibits breast cancer cell motility according to its unliganded or liganded status (Platet *et al*., 2000). The ability of both ECM proteins to transactivate ERα via c-Src is not linked to specific factors present in breast cancer cells since it was reproduced in HeLa cells engineered to express ERα, ERβ (data not shown) and ERα deleted in the AF-2 domain. The expression of either ERα or AF-1/ERα is sufficient to reduce cell invasion that results enhanced by cell interactions with both Fn and Col. These data, taken together, address how A/B region of ERα is crucial for both ligand-independent transcriptional activation and the reduction of cell invasion induced by the two ECM proteins, thus confirming that the AF-2 domain may be dispensable for hormone-independent inhibition of cell invasiveness and motility (Platet *et al*., 2000).

C-Src is an effector of multiple protein tyrosine kinase signals, particularly active during the process of tumorigenesis (Summy and Gallick, 2003). For instance, the enhanced expression of c-Src in cells expressing elevated levels of EGF-R resulted in increased DNA synthesis, soft agar growth and tumor formation in nude mice (Maa *et al*., 1995). The sustained activation of c-Src, observed in mammary epithelial cells overexpressing Erb-B2 (Sheffield, 1998) and in TGF-α-induced mammary tumor cell line (Amundadottir and Leder, 1998), enhances the anchorage-independent growth of these cells. Activated c-Src in mammary tumors has been well studied in transgenic mice. Mice expressing viral polyoma middle T antigen under the control of the MMTV promoter produce highly metastatic mammary tumors with elevated c-Src kinase activity (Guy *et al*., 1994). Besides, mice overexpressing the *neu* oncogene also develop mammary tumors with 6–8-fold higher c-Src kinase activity than the adjacent normal tissue (Muthuswamy *et al*., 1994). Thus, it appears from the above reported findings that c-Src lies at the hub of a very complex network of signaling pathways, which integrate a variety of intracellular and extracellular events. Part of this network links the adhesion proteins to ERα, leading to the reduction of cell motility. The latter effect appears to be mediated at the nuclear level since it is abrogated in the presence of ERα deleted in the NLS. This strongly suggests that ERα may induce the expression of genes able to enhance cell adhesion and negatively control cell migration.

Importantly, it has been demonstrated that ERα activation upregulates fibulin-1, an Fn-binding ECM protein (Hayashido *et al*, 1998), while it decreases metalloproteinase 9 (Crowe and Brown, 1999) and type IV collagenase (Abbas Abidi *et al*., 1997), producing a decrease of cell migration. In other words, we have defined an autoregulatory loop linking ECM–integrin–FAK–c-Src signals to ERα. The output of this loop is dependent on the delicate balance between c-Src and ERα. However, this balance is distorted during tumorigenesis, resulting in a sustained marked activation of c-Src. When a continued activation on c-Src occurs, the balance of the autoregulatory loop between integrins–FAK–c-Src kinase and ERα may be over-ridden and the effect of constitutive active c-Src in enhancing cell migration and invasiveness becomes dominant. Indeed, we observed that the reduced cell migration induced by ectopic expression of ERα in MDA-MB-231 cells is reversed when c-Src content substantially exceeds that of ERα.

On the basis of our findings, it is reasonable to assume that a coordinate role between ECM proteins and ERα exists in controlling cell motility and cell metastatic potential in ERα-positive breast cancer cells. Even though several substrates of the FAK/c-Src complex have been recently identified (Cary *et al*., 1998; Klemke *et al*., 1998; Petit *et al*., 2000), the mechanisms whereby c-Src pathways regulate cell migration remain, however, to be fully elucidated.

Materials and methods

Cell lines and cell culture conditions

Two human breast cancer epithelial cell lines, MCF-7 (ER+) and MDA-MB-231 (ER−) and a *cervicae* carcinoma cell line HeLa (ER−) were used. MCF-7 and MDA-MB-231 cells were maintained in a monolayer culture in Dulbecco's modified Eagle's/Ham's F-12 medium (1 : 1) (DMEM/F12; Eurobio, F) supplemented with 5% calf serum (CS; Eurobio, F), 100 UI/ml penicillin (Eurobio, F), 100 μg/ml streptomycin (Eurobio, F) and 0.2 mM L-glutamine (Eurobio, F). HeLa cells were maintained in monolayer cultures in minimal essential medium (MEM) with Earle's salts (Eurobio, F) supplemented with 10% fetal bovine serum (Eurobio, F), 100 UI/ml penicillin, 100 μg/ml streptomycin and 0.2 mM L-glutamine. Cells were passed weekly using trypsin–EDTA (Eurobio, F) and media were changed every 2 days.

In all experiments, steroids and growth factors were withdrawn from cells, and were grown in phenol red-free DMEM (PRF-SFM; Eurobio, F) containing 0.5% BSA and 2 mM L-glutamine for 24 h.

Western blotting

To establish the influence of both ECM proteins on ERα protein expression, MCF-7 cells, serum starved for 24 h, were detached with versene (Eurobio, F), resuspended in PRF-SFM and plated for 30 min, 1 h, 4 h and 8 h on culture-coated wells with either 30 μg/ml Fn (Sigma, USA) in PBS or 30 μg/ml Col (Sigma, USA) in 10 mM acetic acid or 2 μg/cm^2 poly-L-lysine (P-Lys; Sigma, USA) in PBS. At the end of each incubation time, cells were washed with ice-cold PBS and lysed for 1 min at 4°C in Triton lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton X-100, 0.2 mM Na_3VO_4, 1% PMSF, 1% Aprotinin) for the cytosolic lysates and in SDS lysis buffer (62.5 mM Tris-HCl (pH 6.8), 50 mM dithiothreitol, 2% SDS, 10% glycerol, 0.2 mM Na_3VO_4, 1% phenylmethyl-sulfonylfluoride) for the total (cytosolic and nuclear) lysates. Cell lysates were cleared by centrifugation (14 000 r.p.m. for 10 min at 4°C) and the protein content was determined by the Bradford method. Cellular lysates (20 μg of protein/lane) were resolved by SDS–PAGE, then transferred to nitrocellulose membranes and probed with an ERα monoclonal antibody (F-10 clone; Santa Cruz Biotechnology, USA). The antigen–antibody complexes were detected by incubation of the membranes with peroxidase-coupled anti-mouse IgG and developed using the ECL Plus Western Blotting detection system (Amersham Pharmacia Biotech, UK).

Immunocytochemical staining

MCF-7 cells, serum starved for 96 h, were detached with versene, resuspended in PRF-SFM and plated either on 30 μg/ml Fn-, 30 μg/ml Col- or 2 μg/cm^2 P-Lys-coated wells, as described previously, or treated with 10 nM of E_2. After 2 h of incubation, the cells were fixed with paraformaldehyde (2% PFA for 30 min). Endogenous peroxidase activity was inhibited by hydrogen peroxide (3% in absolute methanol for 30 min) and nonspecific sites were blocked by normal horse serum (10% for 30 min). ERα immunostaining was then performed incubating the primary antibody (F-10 clone) overnight at 4°C, while a biotinylated horse-anti-mouse IgG was utilized at room temperature for 1 h as a secondary antibody. Avidin–biotin–horseradish peroxidase complex (ABC/HRP) was applied for 30 min and the chromogen 3-3′-diaminobenzidine tetrachloride dihydrate was then used as detection system for 5 min. TBS-T (0.05 M Tris-HCl plus 0.15 M NaCl (pH 7.6) containing 0.05% Triton X-100) served as a washing buffer. The primary antibody was replaced by normal mouse serum at the same concentration in control experiments on MCF-7-cultured cells (not shown).

Plasmids, transfections and ERE-luciferase assay

The reporter plasmid XETL drives the expression of luciferase by an ERE from the *Xenopus* vitellogenin promoter (Bunone *et al*., 1996). The SV40 promoter-based pSG5 vector encoding ERα (wild-type) pSG5-HeG0 (HeG0, Tora *et al*., 1989). The two deleted constructs of ERα, pSG5-HE15 and pSG5-HE19, code for a carboxyl-terminal truncated receptor (HE15, amino acids 1–281) and for the amino-terminal truncated receptor (HE19, amino acids 179–575), respectively (a gift from D Picard). The pcDNA3-ER-S104/S106/S118/A is an ERα derivative containing Ser 104, 106 and 118 mutated in Ala inserted in a pcDNA3 expression vector (ER/Ser/A; a gift from DA Lannigan). The pCMV-hE241G is a construct of ERα deleted in the nuclear localization signal (NLS) region (amino acids 250–303) and inserted in the pCMV expression plasmid (E241G; a gift from R Song). The empty expression vector, pCMV, and the same vector containing the c-DNA encoding the wild type of c-Src, pCMV-c-Src (c-Src(+)), and the dominant negative of c-Src, pCMV-cSrc-K295R,Y527F (c-Src(−)) were gifts from J Brugge and the active form of c-Src, pcDNA3-cSrc-Y527F, (Y527F) was a gift from DD Boyd. The *R. reniformis* luciferase expression vector used was pRL-Tk (Promega, USA).

To monitor the activation of ERα by ECMs, MCF-7 and MDA-MB-231 cells (5×10^4 density) were plated onto 24-well plates, grown in DMEM/F12 to an approximate confluence of 70–80% and then cotransfected with XETL and pRL-Tk (MCF-7) or XETL, HeG0 and pRL-Tk (MDA-MB-231). All the transfections were carried out using the calcium phosphate DNA co-precipitation method. Cells were transfected in a growing medium and 6 h after transfection were washed twice with PRF-SFM and switched to PRF-SFM for 24 h and then treated for 8 h either with 10 nM E_2, 30 μg/ml Fn, 30 μg/ml Col or 15 μg/ml P-Lys. The same treatments were carried out in the presence of either 100 nM ICI 182,780 (ICI; Zeneca, UK), 100 nM 4-OH-Tam (Sigma, USA) or 3 μM PP2, an inhibitor of c-Src (Calbiochem, USA). To evaluate which ERα functional domain was involved in ERα activation by ECMs, HeLa cells, cultured and treated as before, were cotransfected with pRL-Tk, XETL and HeG0, HE15 or HE19.

Firefly luciferase and *R. reniformis* luciferase activities were determined using the Dual Luciferase reporter assay system (Promega, USA) according to the manufacturer's instructions. Firefly luciferase activity was normalized to *R. reniformis* luciferase activity and expressed as relative luciferase units.

The influence of ERα and c-Src on cell motility was analysed in HeLa cells transfected with HeG0 and Y527F at different

concentrations (ratio 1:4 and 4:1). The same cells were transfected with HeG0 and pcDNA3 or Y527F and pSG5 using the same concentrations mentioned before. To evaluate if ERα acts at the nuclear level in mediating the induced effects of ECM/c-Src on cell motility, MDA-MB-231 cells cultured and treated as before were transfected, as described previously, with ERα mutated in some serine residues (ER/Ser/A, 10 μg/dish), or with ERα deleted in the NLS (E241G, 10 μg/dish).

RT–PCR

MCF-7 cells were incubated for 24 h in PRF-SFM and then detached and plated on coated or uncoated dishes with either 2 μg/cm^2 P-Lys, 30 μg/ml Fn or 30 μg/ml Col as described previously. The cells plated on uncoated dishes were treated both with and without 10 nM E_2. After 24 h, total cellular RNA was extracted using RNeasy (Quiagen, USA) and reverse transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, USA). Briefly, reverse transcription was performed on 1 μg of total RNA in a final volume of 10 μl by incubation at 37°C for 30 min with 200 U of M-MLV reverse transcriptase, 0.4 μg oligo-dT, 0.5 μM deoxynucleotidetriphosphate (dNTP) and 24 U RNAsin, followed by heat denaturation for 5 min at 95°C.

Subsequent PCR analysis was performed on 1 μl of the RT product in a final volume of 25 μl. The following pairs of primers were used to amplify the 210 bp of PS2: 5′-TTCTATCC-TAATACCATCGACG-3′ (PS2 forward) and 5′-TTTGAGTAGTCAAAGTCAGA-GC-3′ (PS2 reverse); the 304 bp of cathepsin D: 5′-AACAACAGGGTGGGCTTC-3′ (Cat forward) and 5′-ATGCACGAAACAGATCTGTGCT-3′ (Cat reverse). The amplification of the 408 bp of ribosomal RNA 36B4 was performed as control using the following primers: 5′-CTCAACA-TCTCCCCCTTCTC-3′ (36B4 forward) and 5′-CAAATCCCATATCCTCGTCC-3′ (36B4 reverse). The PCR mixture consisted of 1.25 U GoTaq DNA Polymerase (Promega, USA), 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl), 2.5 mM $MgCl_2$ and 0.2 mM each dNTP, 0.6 μM of each PS2 primer and 0.2 μM of each 36B4 primer and 0.6 μM of each cathepsin D primer and 0.2 μM of each 36B4 primer. PCR was performed for 20 cycles at 95°C/1 min, 59°C/2 min and 72°C/1 min. A measure of 10 μl of the PCR products were separated on a 1.2% agarose gel.

Immunoprecipitation and kinase activity of c-Src

To assay for c-Src kinase activity MCF-7 cells transfected, as reported in *plasmids and transfection* section, with c-Src(−), c-Src(+) or pcDNA3 were grown in PRF-SFM for 24 h and stimulated with either 15 μg/ml P-Lys, 30 μg/ml Fn, 30 μg/ml Col or E_2 10 nM for 5 min. Another set of cells, transfected with pcDNA3 and treated as before, were incubated with 3 μM PP2. Cells were then lysed with RIPA lysis buffer (500 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100) containing 10 mM PMSF, 1.5 mg/ml aprotinin and 2 mg/ml leupeptin and immunoprecipitated. A Protein G–Agarose and an anti-c-Src antibody complex was prepared to immunopurify the lysates. A measure of 1 μg of mouse monoclonal anti-c-Src antibody (clone 327; Oncogene, USA) and 30 μl of protein G–agarose (Santa Cruz, Biotechnology, USA) were incubated at 4°C for 1 h in 500 μl of PBS with a tube rotator. The complexes were microfuged and washed with 1 ml of lysis buffer for three times. At the end, 500 μg of each cell lysates were added to the protein G–agarose/anti-c-Src antibodies and incubated at 4°C for 2 h rotating. The proteins/complexes were centrifuged and washed three times with the kinase buffer (200 mM PIPES, 100 mM $MnCl_2$). c-Src kinase activity was assayed by a standard *in vitro* kinase assay using acidified enolase as substrate. The incubation was performed in a total volume of 50 μl composed of the immunopurified c-Src protein and the kinase buffer containing 5 mM ATP, 1 μC of [γ^{32}P]ATP and 2.5 μg of acid denatured rabbit muscle enolase (Sigma, USA) as exogenous substrate. Samples were incubated at 30°C for 10 min then reduced with an equal volume of 2 × SDS Laemmli sample buffer (Sigma, USA) and aliquots of them (40 μl) were submitted to SDS–PAGE (acrylamide 11%). The dried gel was exposed to X-omat film (Kodak, USA) for 12 h. All gels were stained with Coomassie blue to ensure that an equal amount of enolase was present in all samples.

Motility assay

To evaluate the role of ERα on cell motility, the following cell lines were used: MCF-7, HeLa and MDA-MB-231 transfected as reported in *plasmids and transfections* section.

Cells maintained in PRF-SFM for 24 h were dispersed with versene (Eurobio, F), washed twice, resuspended in PRF-SFM and counted using a hemocytometer. The 24-well modified Boyden chambers, containing porous (8 μm) polycarbonate membranes (Costar, USA), were coated, on the internal surface, with either 30 μg/ml Fn, 30 μg/ml Col or 2 μg/cm^2 P-Lys by incubation at room temperature (Doerr and Jones, 1996). The lower chambers were loaded with 500 μl of PRF-SFM, while synchronized cells (2×10^4) suspended in 200 μl of PRF-SFM with or without 10 nM E_2 and/or 100 nM ICI were plated into upper chambers. Another set of cells, treated as before, was suspended in 200 μl of PRF-SFM with or without 10 nM E_2 and/or 3 μM PP2. After 16 h of incubation in 5% CO_2 at 37°C, the cells in the upper chamber were removed by a cotton swab, so that only cells that had migrated through the membrane remained. The membranes were then fixed and stained in Coomassie blue solution (0.25 g Coomassie blue, 45 ml water, 45 ml methanol, 10 ml glacial acetic acid) for 5 min, then each well was rinsed three times with distilled water. The migrated cells were determined using an inverted microscope.

Statistical analysis

All data were expressed as the mean ± s.d. (standard deviation) of at least three different experiments. Statistical significances were tested using Student's *t*-test or paired Student's *t*-test where appropriate.

Abbreviations

AF-1, estrogen receptor activation function 1; AF-2, estrogen receptor activation function 2; Col, type IV collagen; ECM, extracellular matrix; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; Fn, fibronectin; ERE, estrogen-responsive element; c-Src(+), c-Src; Src(−), dominant negative of c-Src.

Acknowledgements

We thank Dr Didier Picard for kindly providing XETL, pSG5-HeG0, pSG5-HE15 and pSG5-HE19 plasmids, Dr Joan Brugge for generously providing pCMV-cSrc-K295R, Y527F plasmid, Dr Douglas D Boyd for helpfully providing pcDNA3-c-Src/Y527F plasmid, Dr Deborah A Lannigan for kindly providing pcDNA3/ER-S104/S106/S118/A plasmid and Dr Robert XD Song for helpfully providing hE241G/pCMV plasmid. This work was supported by Grant 'AIRC 2001'.

References

Abbas Abidi SM, Howard EW, Dmytryk JJ and Pento JT. (1997). *Clin. Exp. Metast.*, **15,** 432–439.

Amundadottir LT and Leder P. (1998). *Oncogene*, **16,** 737–746.

Aplin AE and Juliano RL. (1999). *J. Cell Sci.*, **112,** 695–706.

Arnold SF, Vorojeikina DP and Notides AC. (1995). *J. Biol. Chem.*, **270,** 30205–30212.

Aronica SM, Kraus WL, Katzenellenbogen BS and Power RF. (1994). *Proc. Natl. Acad. Sci. USA*, **91,** 8517–8521.

Bunone G, Briand PA, Miksicek RJ and Picard D. (1996). *EMBO J.*, **15,** 2174–2183.

Cary LA, Han DC, Polte TR, Hanks SK and Guan JL. (1998). *J. Cell Biol.*, **140,** 211–221.

Chalbos D, Philips A, Galtier F and Rochefort H. (1993). *Endocrinology*, **133,** 571–576.

Chen Q, Kinch MS, Lin TH, Burridge K and Juliano RL. (1994). *J. Biol. Chem.*, **6,** 26602–26605.

Clark EA and Brugge JS. (1995). *Science*, **268,** 233–239.

Couse JF, Curtis SW, Washburn TF, Eddy EM, Schomberg DW and Korach KS. (1995). *Biochem. Soc. Trans.*, **23,** 929–935.

Crowe DL and Brown TN. (1999). *Neoplasia*, **1,** 368–372.

Doerr ME and Jones JI. (1996). *J. Biol. Chem.*, **271,** 2443–2447.

Feng W, Webb P, Nguyen P, Liu X, Li J, Karin M and Kushner PJ. (2001). *Mol. Endocrinol.*, **15,** 32–45.

Fraker LD, Halter SA and Forbes JT. (1984). *Cancer Res.*, **44,** 5757–5763.

Giancotti F and Ruoslahti E. (1999). *Science*, **285,** 1028–1032.

Guan JL. (1997). *Int. J. Biochem. Cell Biol.*, **29,** 1085–1096.

Guy CT, Muthuswamy SK, Cardiff RD, Soriano P and Muller WJ. (1994). *Genes Dev.*, **8,** 23–32.

Hayashido Y, Lucas A, Rougeot C, Godyna S, Argraves WS and Rochefort H. (1998). *Int. J. Cancer*, **75,** 654–658.

Hynes RO. (1990). *Fibronectins*. Springer: New York.

Ignar-Trowbridge DM, Pimentel M, Teng CT, Korach KS and McLachlan JA. (1995). *Environ. Health Perspect.*, **103,** 35–38.

Irby RB and Yeatman TJ. (2000). *Oncogene*, **19,** 5636–5642.

Joel PB, Traish AM and Lannigan DA. (1998). *J. Biol. Chem.*, **273,** 13317–13323.

Kaplan KB, Swedlow JR, Morgan DO and Varmus HE. (1995). *Genes Dev.*, **9,** 1505–1517.

Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D and Chambon P. (1994). *Science*, **270,** 1491–1494.

Klemke RL, Leng J, Molander R, Brooks PC, Vuori K and Cheresh DA. (1998). *J. Cell Biol.*, **140,** 961–972.

Lazennec G, Bresson D, Lucas A, Chauveau C and Vignon F. (2001). *Endocrinology*, **142,** 4120–4130.

Lehrer S, O'Shaughnessy J, Song HK, Levine E, Savoretti P, Dalton J, Lipsztein R, Kalnicki S and Bloomer WD. (1989). *Mt. Sinai. J. Med.*, **56,** 83–85.

Lin TH, Chen Q, Howe A and Juliano RL. (1997). *J. Biol. Chem.*, **272,** 8849–8852.

Liotta LA, Stracke ML, Aznavoorian SA, Beckner ME and Schiffmann E. (1991). *Semin. Cancer Biol.*, **2,** 111–114.

Lowell CA, Fumagalli L and Berton G. (1996). *J. Cell. Biol.*, **133,** 895–910.

Maa MC, Leu TH, McCarley DJ, Schatzman RC and Parsons SJ. (1995). *Proc. Natl. Acad. Sci. USA*, **92,** 6981–6985.

McGuire WL. (1986). *Cancer Surveys: Hormone Receptors Breast Cancer* Vol. 5. Bulbrook RD (ed). Oxford University Press: Oxford, pp. 527–536.

Miyamoto S, Teramoto H, Gutkind JS and Yamada KM. (1996). *J. Cell Biol.*, **135,** 1633–1642.

Morino N, Mimura T, Hamasaki K, Tobe K, Ueki K, Kikuchi K, Takehara K, Kadowaki T, Yazaki Y and Nojima Y. (1995). *J. Biol. Chem.*, **270,** 269–273.

Muthuswamy SK, Siegel PM, Dankort DL, Webster MA and Muller WJ. (1994). *Mol. Cell. Biol.*, **14,** 735–743.

Osborne CK, Hobbs K and Clark GM. (1985). *Cancer Res.*, **45,** 584–590.

Petit V, Boyer B, Lentz D, Turner CE, Thiery JP and Valles AM. (2000). *J. Cell Biol.*, **148,** 957–970.

Platet N, Cunat S, Chalbos D, Rochefort H and Garcia M. (2000). *Mol. Endocrinol.*, **14,** 999–1009.

Power RF, Mani SK, Codina J, Conneely OM and O' Malley BW. (1991). *Science*, **254,** 1636–1639.

Price JE, Polyzos A, Zhang RD and Daniels LM. (1990). *Cancer Res.*, **50,** 717–721.

Renshaw MW, Ren XD and Schwartz MA. (1997). *EMBO J.*, **16,** 5592–5599.

Rochefort H, Platet N, Hayashido Y, Derocq D, Lucas A, Cunat S and Garcia M. (1998). *J. Steroid Biochem. Mol. Biol.*, **65,** 163–168.

Schaller MD, Hildebrand JD and Parsons JT. (1999). *Mol. Cell. Biol.*, **10,** 3489–3505.

Schlaepfer DD, Broome MA and Hunter T. (1997). *Mol. Cell. Biol.*, **17,** 1702–1713.

Schlaepfer DD, Hanks SK, Hunter T and Van der Geer P. (1994). *Nature*, **372,** 786–791.

Schlaepfer DD and Hunter T. (1996). *Mol. Cell. Biol.*, **16,** 5623–5633.

Sheffield LG. (1998). *Biochem. Biophys. Res. Commun.*, **250,** 27–31.

Suen PW, Ilic D, Caveggion E, Berton G, Damsky CH and Lowell CA. (1999). *J. Cell Sci.*, **112,** 4067–4078.

Summy JM and Gallick GE. (2003). *Cancer Metast. Rev.*, **22,** 337–358.

Thompson EW, Paik S, Brunner N, Sommers CL, Zugmaier G, Clarke R, Shima TB, Torri J, Donahue S, Lippman ME, Martin GR and Dixon RB. (1992). *J. Cell. Physiol.*, **150,** 534–544.

Tora L, Mullick A, Metzger D, Ponglikitmongkol M, Park I and Chambon P. (1989). *EMBO J.*, **8,** 1981–1986.

Weigel N. (1996). *Biochem. J.*, **319,** 657–667.

Zhu X and Assoian RK. (1995). *Mol. Cell. Biol.*, **6,** 273–282.