Coactivator-mediated estrogen response in human squamous cell carcinoma lines

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Abstract

Steroid hormones such as 17β-estradiol (E2) are critical to diverse cellular processes including tumorigenesis. A number of cofactors such as nuclear receptor corepressor (NCoR), CREB-binding protein (CBP), and steroid receptor coactivator 1 (SRC-1) interact with estrogen receptors (ERs) to regulate transcriptional repression or activation of target genes. Estrogen signaling in non-reproductive tract tissues such as skin is less well characterized and the effectiveness of anti-estrogen therapy for cancer arising from these tissues is unknown. We show that tamoxifen (TAM) treatment inhibited cell cycle progression and proliferation of human cancer lines derived from stratified squamous epithelium squamous cell carcinoma (SCC). E2 had no effect on proliferation of these lines despite low levels of ERα expression. The E2 treatment promoted displacement of the NCoR from ERα and recruitment of CBP to the receptor. SRC-1 expression was not detected in these SCC lines; however, transient transfection of SRC-1, CBP, or both coactivators enhanced transactivation of an estrogen responsive promoter in cancer cells treated with E2 or TAM. In stable clones expressing SRC-1, the coactivator was recruited to ERα along with CBP in E2 but not in TAM-treated cells. SRC-1 expression restored the E2-mediated proliferative response to human SCC lines. This increased proliferation correlated with increased extracellular signal regulated kinase 1 (ERK1) expression. SRC-1 and CBP were recruited to the proximal ERK1 promoter region in E2 but not in TAM-treated cells. We concluded that SRC-1 was a key molecular determinant of estrogen-mediated proliferation in human SCC lines.

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Introduction

Steroid hormones such as 17β-estradiol (E2) are critical to diverse cellular processes including development, reproduction, and tumorigenesis (Kim *et al.* 2001). The E2 functions through its receptors ERα and ERβ (Couse & Korach 1999). ERs belong to the superfamily of nuclear hormone receptors that are ligand-dependent transcription factors (Mangelsdorf *et al.* 1995). The ERα binds to estrogen response elements in the promoters of responsive genes. A number of cofactors interact with ERα to regulate transcriptional repression or activation. Among the repressive cofactors is nuclear receptor corepressor (NCoR), which interacts with ERα in the presence of the anti-estrogen 4-hydroxytamoxifen (TAM; Huang *et al.* 2002). Chimeric NCoR–ERα proteins have been shown to silence basal transcription of ERα responsive genes (Chien *et al.* 1999). Dissociation of corepressors from ERα correlates with estrogen-dependent responses (Carroll *et al.* 2003). Loss of corepressor interaction with ERα leads to recruitment of coactivators, which serve to recruit other cofactors, acetylate nucleosomal histones, and bind basal transcriptional machinery (Ratajczak 2001). Histone acetylation results in more open chromatin structure and increased transcriptional activity (Kornberg & Lorch 1999). Interactions between activated ERα bound to DNA and coactivators such as steroid receptor coactivator 1 (SRC-1) and CREB-binding protein (CBP) regulate the transcription of estrogen target genes (Robyr *et al.* 2000).

SRC-1 functions primarily as coactivator for nuclear receptors and binds directly to liganded ERα through helical LXXLL motifs (Needham *et al.* 2000). The SRC-1 contributes to transcriptional activation by interacting with other coactivators such as CBP (Smith *et al.* 1996, Sheppard *et al.* 2001). Coactivators such as SRC-1 and CBP possess histone acetyltransferase activity, which can disrupt nucleosomal structure leading to transcriptional activation (Spencer *et al.* 1997). Disruption of the *SRC-1* gene in mice leads to partial hormone resistance in target organs such as uterus, prostate, testis, and mammary gland (Xu *et al.* 1998).

The estrogen response in non-reproductive tract tissues such as skin and other stratified squamous epithelia is less well characterized. Estrogen has clinically important functions in epidermis, hair follicles, and secretory glands (for review see Thornton 2002). The E2 treatment increased proliferation and

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thickness of epidermis in wild-type but not ERα in null mutant mice (Moverare *et al.* 2002); E2 enhanced proliferation of human keratinocytes *in vitro* by inducing cyclin D2 expression (Kanda & Watanabe 2004). In human clinical trials, E2 treatment increased epidermal thickness and reduced the prevalence of histologic features associated with aging (Fuchs *et al.* 2003). E2 increased expression of type I collagen, tropoelastin, fibrillin-1, and elastic fibers in aged skin *in vivo* (Son *et al.* 2005). These studies indicate that ERα signaling can increase keratinocyte proliferation and extracellular matrix production in human skin cells *in vivo* and *in vitro*.

Conversely, treatment with antiestrogens, such as TAM, inhibits proliferation of estrogen-responsive cancer cells. Proliferation of breast and ovarian cancer cell lines was inhibited by TAM (Lindner & Borden 1997, Cariou *et al.* 2000). High doses of antiestrogens have also been shown to inhibit proliferation and induce apoptosis in an ERα negative ovarian carcinoma cell line (Ercoli *et al.* 1998). However, the mechanism by which TAM inhibits proliferation of cancer cells from non-reproductive tissues such as stratified squamous epithelium is not well characterized. Furthermore, ERα expression is reportedly low in stratified squamous epithelia from different anatomic sites (Ojanotko-Harri *et al.* 1992). Coactivator expression and interaction with ERα in regulating cancer cell proliferation from this tissue are largely unknown. We demonstrate that TAM but not E2 regulates cell cycle progression and proliferation of human squamous cell carcinoma (SCC) lines. We show that the SRC-1 coactivator protein is a key molecular determinant of this differential response to ERα signaling.

Materials and Methods

Cell culture and stable transfection

The human SCC lines used in this study were purchased from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle medium without phenol red, 10% charcoal-stripped fetal bovine serum, 40 μg/ml gentamicin at 37 °C in a humidified atmosphere of 5% CO_2. SCC4, SCC9, or SCC25 cells were transfected with 5 μg human SRC-1 expression vector (kindly provided by Dr Ronald Evans) or neomycin-resistance plasmid alone using Lipofectamine reagent according to the manufacturer's recommendations (Invitrogen). Cells were selected in 400 μg/ml G418 for 14 days. Resistant clones were picked for expansion and characterization. The human breast cancer cell lines MCF7 (ERα positive) and MDA-MB-231 (ERα negative) were used as well characterized controls for ERα expression.

Cell proliferation and BrdU incorporation analysis

Triplicate cultures of 5×10^4 parental SCC lines, SRC-1, or vector control clones were plated into six-well plates and treated with 10–1000 nM E2 or 4-hydroxytamoxifen for up to 6 days. The control cultures were treated with 0·1% ethanol vehicle for the same time period and they were trypsinized and counted at 2-day intervals using a hemacytometer. For bromodeoxyuridine (BrdU) incorporation analysis, cells were treated with ligands or vehicle for 1 day followed by 1-h incubation in 10 μM BrdU. After washing in PBS, cells were fixed in 70% ethanol, 50 mM glycine (pH 2) for 30 min at −20 °C. After extensive washing in PBS, cells were incubated with mouse anti-BrdU primary antibody at 37 °C for 30 min (Roche Molecular Biochemicals). After washing in PBS, cells were incubated with anti-mouse IgG secondary antibody conjugated to fluorescein at 37 °C for 30 min. Following extensive washing in PBS, BrdU-positive cells were visualized by fluorescence microscopy. The number of positive cells was expressed as a percentage of total cells counted in ten randomly selected high power fields.

Reverse transcription-PCR

RNA was extracted from SCC and breast cancer cell lines using a commercially available kit (Qiagen) and reverse transcribed using SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). cDNA was amplified using ERα specific primers (5′-CCACCAACCAGTGCACCATT-3′ and 5′-GGTCTTTTCGTATCCCACCTTTC-3′) in 20 mM Tris–HCl (pH 8·3), 1·5 mM $MgCl_2$, 63 mM KCl, 0·05% Tween 20, 1 mM EGTA, 50 μM of each dNTP, and 2·5 U Taq DNA polymerase (Roche Molecular Biochemicals). Amplification with β-actin cDNA using primers 5′-ACAGGAAGTCCCTTGCCATC-3′ and 5′-ACTGGTCTCAAGTCAGTGTACAGG-3′ as the internal control was carried out by real-time PCR (iCycler, BioRad) using cycle parameters 94 °C for 25 s, 55 °C for 1 min, and 72 °C for 1 min.

Immunoprecipitation and western blot

Cultures of ligand or vehicle-treated SCC lines and clones were lysed in 50 mM HEPES (pH 7·5), 150 mM NaCl, 1 mM EDTA, 2·5 mM EGTA, 1 mM dithiothreitol, 1% Nonidet P-40, 10% glycerol, and protease inhibitors for 30 min at 4 °C. Lysates were centrifuged at 10 000 ***g*** for 10 min and anti-human primary antibody to ERα or preimmune IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was incubated with the supernatants for 1 h at 4 °C. Antigen–antibody complexes were precipitated by incubation with protein A/G agarose (Santa Cruz Biotechnology) for 1 h at 4 °C. Immunoprecipitated protein complexes were washed thrice with 1 ml lysis buffer and separated by SDS-PAGE as described below. The blots were incubated with anti-SRC-1, CBP, and NCoR antibodies to determine interaction with ERα in cellular lysates and also stripped and incubated with anti-ERα antibody to determine the amount of immunoprecipitated protein in each lane. For western blots, 75 μg total cellular protein was separated by

SDS-PAGE on 10% resolving gels under denaturing and reducing conditions. Separated proteins were electroblotted to PVDF membranes according to the manufacturer's recommendations (Roche Molecular Biochemicals) and the blots were incubated with antibodies to human ERα, SRC-1, p21, p27, extracellular signal regulated kinase 1 (ERK1), cyclin A, cyclin B, cyclin D1, cyclin E, cdk1, cdk2, cdk6, c-myc, and β-actin (Santa Cruz Biotechnology) for 16 h at 4 °C. After washing in Tris-buffered saline containing 0·1% Tween 20 (TBST, pH 7·4), blots were incubated for 30 min at room temperature with anti-IgG secondary antibody conjugated to horseradish peroxidase. Following extensive washing in TBST, bands were visualized by the enhanced chemiluminescence method (Roche Molecular Biochemicals) and quantitated using laser densitometry. Statistical analysis was performed by *t*-test.

Transient transfection and reporter gene analysis

Triplicate cultures of 50% confluent SCC25 cells were transiently transfected with 5 μg of the estrogen-responsive ERE-luc (estrogen response element fused to luciferase cDNA) or ERK1 promoter/reporter vectors (Chu *et al.* 2005) along with 2 μg SRC-1, CBP, NCoR, or blank expression plasmids using Lipofectamine according to the manufacturer's recommendations (Invitrogen). One microgram β-galactosidase expression plasmid was used to normalize for transfection efficiency. Cultures were treated with 100 nM E2, TAM, or vehicle for 24 h. Cells were harvested and the reporter gene activity determined using a commercially available kit (Tropix, Bedford, MA, USA). Luciferase activity was normalized to β-galactosidase levels for each sample.

Chromatin immunoprecipitation

SCC25 clones were treated with 100 nM E2, TAM, or vehicle for up to 4 h. After washing in PBS, cells were fixed in 1% formaldehyde for 10 min at room temperature. The cells were washed in PBS and lysed in immunoprecipitation buffer containing protease inhibitors for 30 min at 4 °C, sheared and centrifuged at 10 000 ***g*** for 10 min and the supernatants were cleared with 2 μg sheared salmon sperm DNA, 20 μl preimmune serum, and 20 μl protein A/G sepharose beads for 2 h at 4 °C. Aliquots of the supernatant were used as input DNA for normalization and amplified with β-actin PCR primers (5′-ACAGGAAGTCCCTTGCCATC-3′ and 5′-ACTGGTCTCAAGTCAGTGTACAGG-3′). Immunoprecipitation using anti-SRC-1 or anti-CBP antibodies (Santa Cruz Biotechnology) was performed overnight at 4 °C. Preimmune IgG was used as the negative control antibody. The immunoprecipitates were washed extensively in immunoprecipitation buffer, resuspended in 10 mM Tris–HCl, 1 mM EDTA (TE, pH 8) and incubated at 65 °C for 6 h to reverse crosslinks. The supernatants were extracted with phenol/chloroform and ethanol precipitated. Following washing in 70% ethanol, pellets were dried and suspended in 50 μl TE. For PCR, 1 μl template was amplified in buffer containing 10 mM Tris–HCl (pH 8·3), 50 mM KCl, 2·5 mM $MgCl_2$, 200 nM each dNTP, and 100 ng each primer (5′-CCACCACATAGAGAGCCTTTGG-3′ and 5′-CACTCCTGCCGCCTCCCC-3′) flanking the −390 to −10 region of the ERK1 promoter. The optimized cycle parameters were one cycle at 94 °C for 3 min followed by 25 cycles of 94 °C for 25 s, 55 °C for 60 s, 72 °C for 60 s, and one final cycle at 72 °C for 10 min. Amplified products were separated by agarose gel electrophoresis.

Results

To determine how estrogen receptor α (ERα) signaling regulates proliferation of cancer cell lines derived from stratified squamous epithelium, we treated human SCC lines with E2 or TAM for up to 6 days. All SCC lines expressed low levels of ERα as shown by immunoprecipitation (three representative lines shown in Fig. 1A). ERα mRNA levels were 75–90% lower than those observed in the ERα positive human breast cancer cell line MCF7 (Fig. 1B; $P<0·0001$). The ERα expression in the ERα-negative breast cancer cell line MDA-MB-231 is shown for comparison. E2 treatment at concentrations up to 1000 nM had no effect on the proliferation of human SCC lines in these assays. We next tested the effects of TAM treatment at concentrations from 10 to 1000 nM; maximal growth inhibition was achieved using the 100 nM concentration. TAM, 100 nM, inhibited proliferation of SCC lines by 30–40% ($P<0·01$) compared with vehicle-treated control cells, while 50 nM TAM reduced growth by 15–20% (representative lines shown in Fig. 1C and D; $P<0·05$). The E2 effectively blocked the growth inhibitory effects of TAM when the two ligands were combined in culture, indicating that these effects were mediated through ERα. To determine how TAM regulated cell cycle progression of SCC lines, we performed BrdU incorporation analysis and analyzed cell cycle regulatory protein expression. As shown in Fig. 2A, TAM reduced the percentage of BrdU-positive cells in the SCC4, SCC9, and SCC25 lines from 13 to 7%, 14 to 7%, and 16 to 9% respectively ($P<0·02$). E2 had no effect on BrdU incorporation in treated cell lines compared with control cultures. TAM treatment induced expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ by sixfold in SCC4 and SCC9 cells (Fig. 2B). Expression of the G1/S phase cell cycle regulatory protein cyclin E was reduced by sevenfold in TAM-treated cells. Expression of the G1 cyclin-dependent kinase cdk6 also was inhibited by fivefold in TAM-treated SCC4 and SCC9 cells. We also examined expression of the estrogen target genes cyclin D1, c-myc, and $p21^{WAF1/Cip1}$, but these protein levels were too low to be detected by western blot. These results indicate that, while TAM inhibited G1/S phase cell cycle progression of SCC lines, E2 had no effect on the proliferation of these cells.

We hypothesized that the lack of proliferative response to E2 and inhibitory effects of TAM may be due to differential

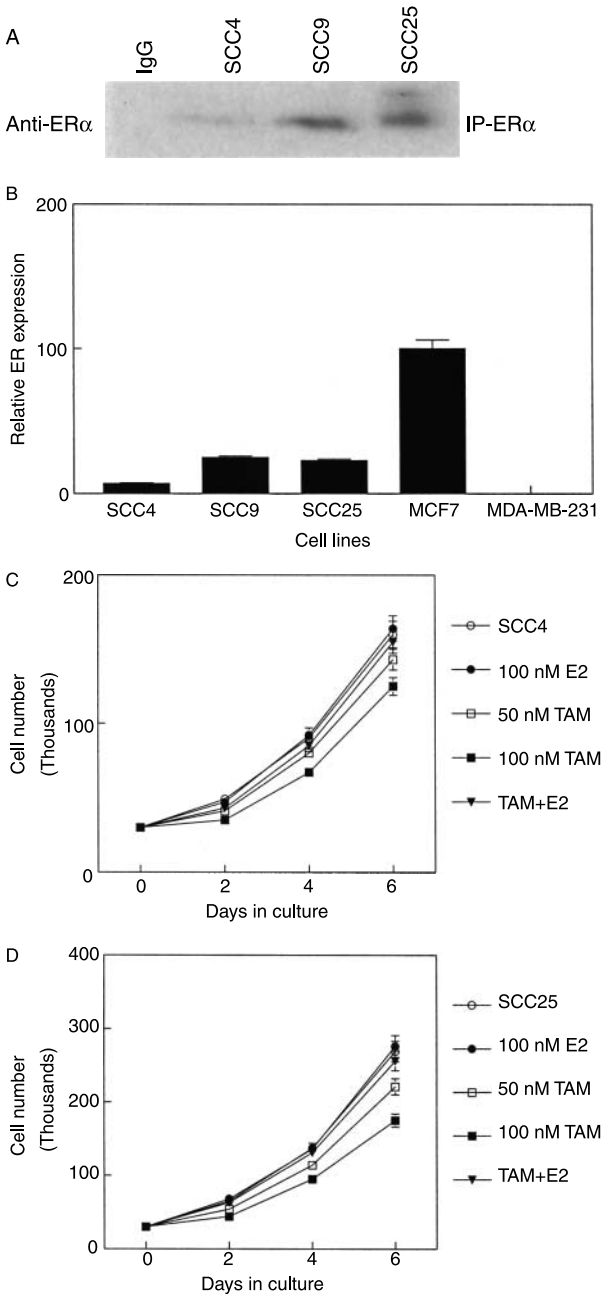


Figure 1 Differential proliferative response of human squamous cell carcinoma lines to E2 and tamoxifen. (A) Human SCC lines express low levels of estrogen receptor α. ERα was immunoprecipitated from SCC lines using anti-ERα antibody (IP ERα). Relative ERα expression was detected by incubating blots with anti-ERα antibody. Preimmune IgG was used as the negative control. These experiments were performed thrice with similar results. Representative blots are shown. (B) Comparison of relative ERα expression between human SCC and breast cancer cell lines (MCF7, MDA-MB-231) by quantitative RT-PCR. (C and D) SCC4 and SCC25 cells were treated with 100 nM E2, 50 nM or 100 nM tamoxifen (TAM), or 50 nM each E2 and TAM (E2 + TAM) for 6 days. At 2-day intervals, triplicate cultures were counted using a hemacytometer. These experiments were performed thrice with similar results. Error bars represent S.E.M.

recruitment of coactivator proteins to ERα. To test this hypothesis, we immunoprecipitated ERα from three E2 and TAM-treated human SCC lines to examine interaction with coactivator proteins. Representative results from SCC25 cells are shown in Fig. 3. The E2 treatment dissociated the NCoR protein NCoR from ERα (Fig. 3A). In contrast, complex formation between ERα and NCoR was increased threefold by TAM treatment compared with vehicle-treated control cultures. These results indicate that NCoR interaction with ERα was largely intact in human SCC lines. Similarly, the coactivator protein CBP was recruited to ERα in E2-treated

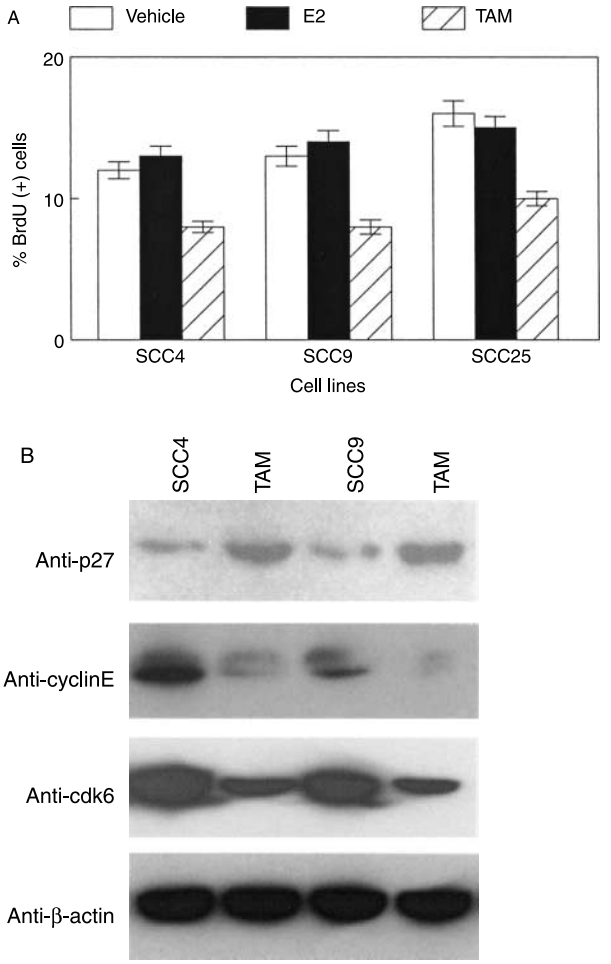


Figure 2 Tamoxifen treatment inhibits G1/S phase progression in human SCC lines. (A) SCC lines were treated with 100 nM E2 or tamoxifen (TAM) for 16 h before being labeled with BrdU as described in Materials and Methods. BrdU-positive cells were identified using immunofluorescence microscopy and reported as the percentage of total cells counted. These experiments were performed thrice with similar results. Error bars indicate S.E.M. (B) Tamoxifen treatment alters expression of G1/S phase cell cycle regulatory proteins. SCC lines were treated with 100 nM tamoxifen (TAM) for 24 h prior to harvesting for western blotting using the antibodies indicated at left. These experiments were performed thrice with similar results. Representative blots are shown.

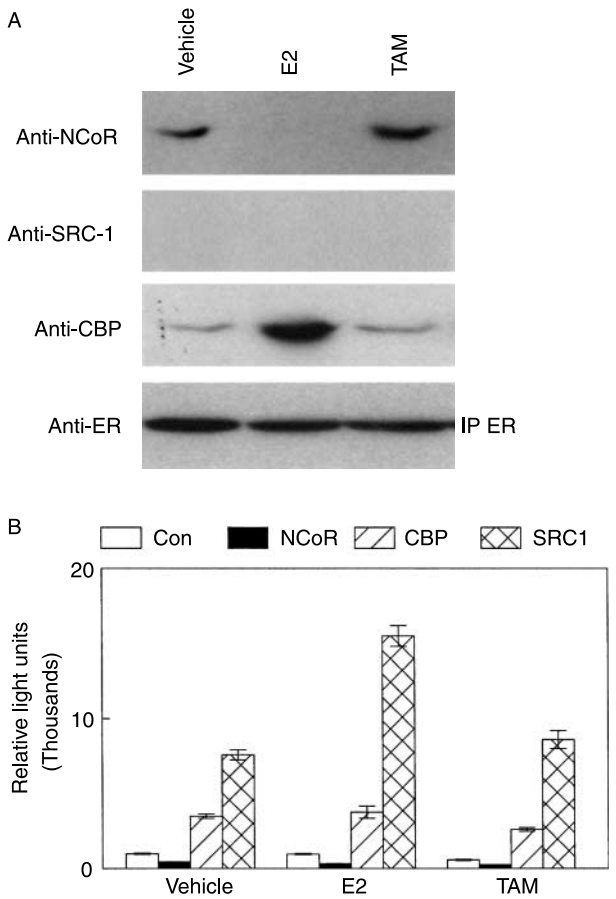
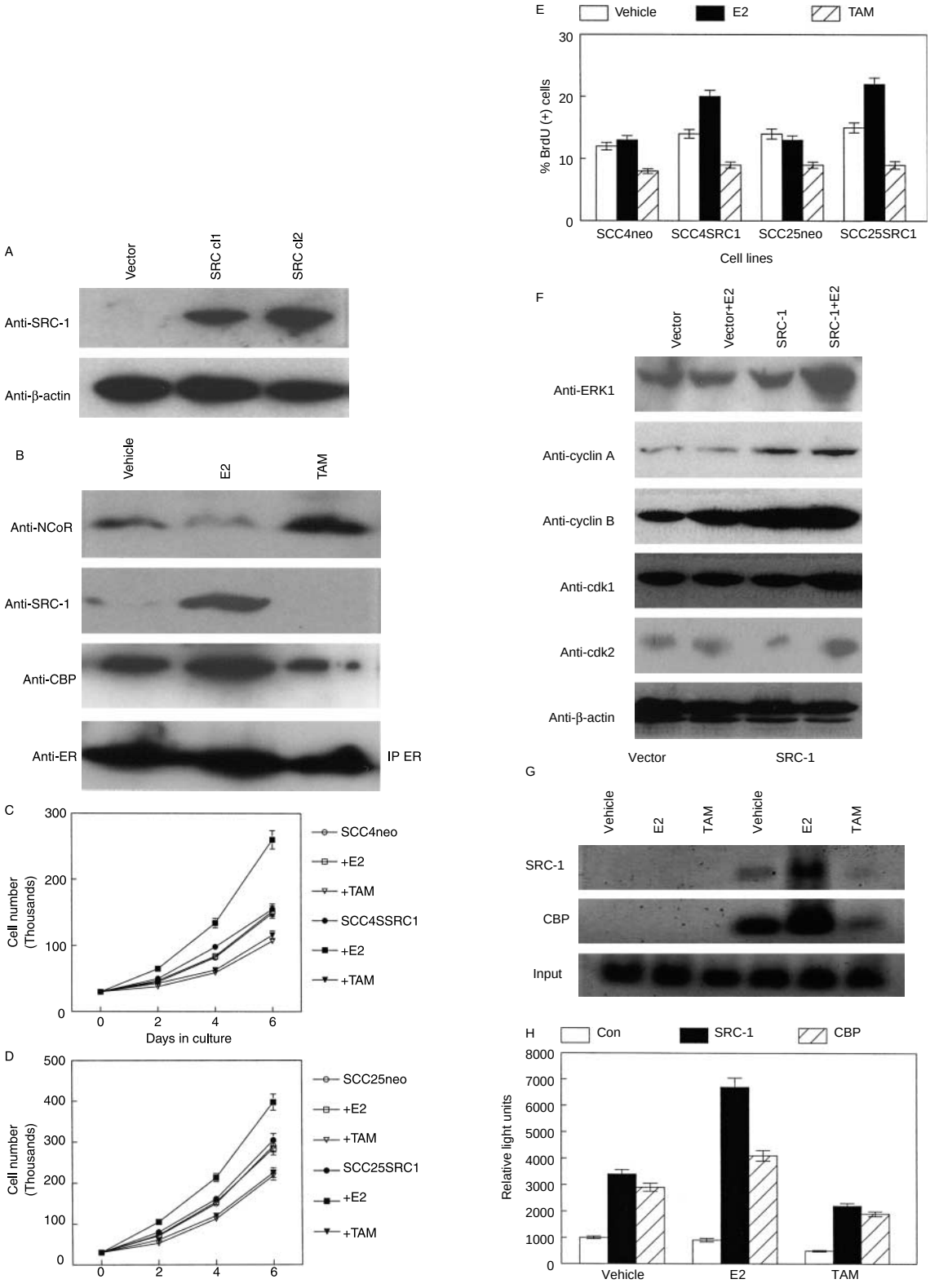


Figure 3 Estradiol and tamoxifen differentially recruit nuclear receptor repressors and activators to the estrogen receptor. (A) The SCC lines were treated with 100 nM estradiol (E2), tamoxifen (TAM), or vehicle for 4 h prior to immunoprecipitation using anti-ER antibody (IP ER) as described in Materials and Methods. The blots were incubated with antibodies to the nuclear receptor repressor NCoR or the activators SRC-1 and CBP to determine interaction with ERα. These experiments were performed thrice with similar results. Representative blots are shown. (B) Differential regulation of an estrogen-responsive promoter element by ERα ligands, repressors, and activators. SCC lines were co-transfected with the estrogen-responsive ERE-luc reporter vector along with NCoR, CBP, or SRC-1 expression constructs. Triplicate transfected cultures were treated with 100 nM estradiol (E2), tamoxifen (TAM), or vehicle as described in Materials and Methods. The activity of the luciferase reporter gene was expressed as relative light units normalized to the activity of a control reporter construct. These experiments were performed thrice with similar results. Error bars indicate S.E.M.

cells. This interaction was not observed in TAM-treated cells. Given that SRC-1 can interact with CBP, we expected that SRC-1 would co-immunoprecipitate with ERα. However, we did not detect the presence of SRC-1 protein in the immunoprecipitated complexes from either E2 or TAM-treated cells. This lack of SRC-1 interaction with ERα transcriptional complexes made this coactivator a potential candidate for mediating E2 proliferative responses in human SCC lines. To determine if SRC-1 could enhance transcription from an E2 responsive promoter, we transiently transfected SRC-1, CBP, or NCoR with the ERE-luc reporter vector into E2 or TAM-treated SCC lines. Representative results from SCC25 cells are shown in Fig. 3B. SRC-1 induced the activity of the estrogen-responsive promoter by fourfold in vehicle-treated cells ($P<0{\cdot}01$). This SRC-1-mediated transactivation was increased to sevenfold when transfected cells were treated with E2. However, TAM treatment repressed SRC-1 transactivation by 25% compared with vehicle-treated cells ($P<0{\cdot}05$). CBP overexpression induced ERE-luc activity by threefold, although transactivation by this coactivator was less sensitive to E2 or TAM treatment ($P<0{\cdot}05$). NCoR repressed the activity of the estrogen-responsive promoter by 50% ($P<0{\cdot}02$), which was largely unaffected by E2 or TAM treatment, likely due to overexpression of this corepressor protein. These results indicate that SRC-1 is a key mediator of the transcriptional response to E2 in human SCC lines.

SRC-1 protein expression was not detected in SCC lines by western blot (Fig. 4A). We created stable SRC-1 expressing clones in order to assess the effect of the coactivator on estrogen response in SCC lines. The expression of SRC-1 protein in stable clones is shown in Fig. 4A. To determine if SRC-1 protein could interact with ERα in these clones, we immunoprecipitated ERα from E2 and TAM-treated cultures. E2 treatment recruited SRC-1 and CBP to ERα while displacing NCoR (Fig. 4B). Conversely, NCoR was strongly associated with ERα in TAM-treated cells. The SRC-1 interaction with ERα was undetectable in TAM-treated cells, and receptor association with CBP was reduced by twofold compared with vehicle-treated cultures. These results indicate that SRC-1 can form transcriptional protein complexes with CBP and ERα in SCC lines.

To determine the effect of E2 on proliferation of SRC-1 expressing clones, we treated SCC4 and SCC25 cells expressing SRC-1 protein or the neomycin resistance gene product with E2 for up to 6 days. As shown in Fig. 4C and D, SRC-1 expression produced E2 responsive increases in cellular proliferation *in vitro*. SCC4 clones expressing SRC-1 protein proliferated 60% faster than control clones when treated with E2 ($P<0{\cdot}01$). Additionally, SCC25 cells expressing SRC-1 protein proliferated 25% faster than control clones when treated with E2 ($P<0{\cdot}05$). TAM treatment inhibited proliferation of SRC-1 expressing clones similar to that observed in neomycin resistant cells, likely due to efficient recruitment of NCoR to ERα by TAM and SRC-1 displacement from the receptor in these clones. To determine how E2 regulated cell cycle progression in SRC-1 expressing clones, we examined G1/S phase progression and expression of cell cycle regulatory proteins by western blot. As shown in Fig. 4E, E2 treatment increased BrdU incorporation in SRC-1 expressing clones (13–19% in SCC4 and 12–21% in SCC25; $P<0{\cdot}05$ and $0{\cdot}04$ respectively). E2 treatment had no effect on neomycin resistant clones. S phase progression was inhibited to a similar degree by TAM treatment in both



neomycin resistant and SRC-1 expressing clones. Expression of the mitogen-activated protein kinase ERK1 was induced threefold in SRC-1 expressing clones by E2 treatment (Fig. 4F). Expression of the S and G2 phase cyclins A and B was two- to three-fold higher in SRC-1 expressing clones, but was not affected by E2 treatment. Expression of the S phase cyclin-dependent kinase cdk2 was induced eightfold in SRC-1 expressing clones by E2 treatment, and the G2 phase kinase cdk1 was increased twofold. The E2 treatment had little effect on cell cycle regulatory protein expression in control clones. These results indicate that SRC-1 induces E2-mediated proliferation in human SCC lines.

To determine if transfected SRC-1 formed transcriptional complexes on chromatin, we performed ChIP on the proximal ERK1 promoter in E2 and TAM-treated SCC lines. The ERK1 expression was increased by E2 treatment in SRC-1 expressing clones (Fig. 4F). As shown in Fig. 4G, SRC-1 and CBP bound to the proximal ERK1 promoter in clones expressing SRC-1 but not in control cells. SRC-1 interaction with the proximal ERK1 promoter was increased by fivefold when SRC-1 clones were treated with E2. CBP interaction with the proximal ERK1 promoter increased threefold in E2-treated clones. TAM treatment reduced SRC-1 and CBP binding to the proximal ERK1 promoter. SRC-1 and CBP induced ERK1 promoter activity by threefold in reporter gene assays (Fig. 4H; $P<0{\cdot}04$). E2 treatment increased induction of the ERK1 promoter to sevenfold for SRC-1 ($P<0{\cdot}03$) and fourfold for CBP ($P<0{\cdot}02$). TAM treatment inhibited ERK1 promoter activation by SRC-1 and CBP (twofold induction; $P<0{\cdot}05$). These results indicate that SRC-1 can form E2 responsive transcriptional complexes on the promoters of target genes in human SCC lines.

Discussion

The results of the present study have potentially important clinical ramifications in treatment of cancers from non-reproductive tissues. We showed that TAM inhibited proliferation of human SCC lines by inhibiting G1 to S phase progression. This inhibition correlated with the upregulation of $p27^{Kip1}$ expression and the downregulation of cyclin E and cdk6 protein levels. A similar mechanism of TAM-mediated growth inhibition was shown in human pancreatic cancer cell lines in which the anti-estrogen induced $p21^{WAF1/Cip1}$ expression (Robinson *et al.* 1998). TAM upregulated $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ in human breast cancer cells and inhibited cyclin E-cdk2 kinase activity (Cariou *et al.* 2000). The p27 overexpression induced cell cycle arrest in TAM-treated breast cancer cell lines (Carroll *et al.* 2003). Initiation of cell cycle progression in these cells required dissociation of NCoR from ERα. We have demonstrated that TAM promoted complex formation between NCoR and ERα in agreement with the previous studies (Shang *et al.* 2000). NCoR has been shown to interact with helices 3 and 5 of the receptor ligand-binding domain in a TAM-dependent manner (Yamamoto *et al.* 2001). A tumor-derived ERα mutant containing an amino acid substitution in helix 3 showed reduced interaction with NCoR and high

Figure 4 SRC-1 expression restores proliferative response to estradiol in human SCC lines. (A) SRC-1 expression in human SCC clones. SCC25 cells were stably transfected with a SRC-1 expression construct or blank vector as described in Materials and Methods. SRC-1 expression in stable clones (vector, SRC cl1, SRC cl2) was determined by western blot using anti-SRC-1 antibody. The blots were stripped and incubated with anti-β-actin antibody to determine relative amounts of protein in each lane. These experiments were performed thrice using different cell lines with similar results. Representative blots are shown. (B) Differential recruitment of NCoR and SRC-1 to ERα in response to estradiol and tamoxifen in SRC-1 stable clones. SCC25 clones were treated with vehicle, 100 nM estradiol (E2), or 100 nM tamoxifen (TAM) for 4 h as described in Materials and Methods. ERα was immunoprecipitated from cell lysates (IP ER) using anti-ERα antibody. The immunoprecipitated proteins were blotted and incubated with anti-NCoR, SRC-1, or CBP antibodies to determine complex formation and the blots were also incubated with anti-ERα antibody to determine relative amounts of the immunoprecipitated protein in each lane. These experiments were performed thrice using different clones with similar results. Representative blots are shown. (C and D) SRC-1 expression restores the proliferative response to estradiol in human SCC clones. SCC4 and SCC25 SRC-1 and neomycin resistant (neo) clones were treated with vehicle, 100 nM estradiol (+E2), or 100 nM tamoxifen (+TAM) for 6 days. At 2-day intervals, triplicate cultures were counted using a hemacytometer. These experiments were performed thrice with similar results. Error bars indicate S.E.M. (E) SRC-1 expression increases S phase progression in E2-treated SCC clones. Neomycin resistant (neo) and SRC-1 expressing SCC4 and SCC25 clones were treated with 100 nM estradiol (E2) or tamoxifen (TAM) for 16 h before being labeled with BrdU as described in Materials and Methods. BrdU-positive cells were identified using immunofluorescence microscopy and reported as the percentage of total cells counted. These experiments were performed thrice with similar results. Error bars indicate S.E.M. (F) Estradiol treatment induces cell cycle regulatory protein expression in SRC-1 expressing clones. SRC-1 and vector expressing clones were treated with 100 nM estradiol (+E2) or vehicle for 24 h prior to protein extraction and western blotting using antibodies indicated at left. The blots were stripped and incubated with anti-β-actin antibody to determine relative amounts of protein in each lane. These experiments were performed thrice using different clones with similar results. Representative blots are shown. (G) SRC-1 and CBP are bound to the ERK1 promoter in estradiol-treated SCC clones. Chromatin immunoprecipitation using SRC-1 and CBP antibodies was performed as described in Materials and Methods using lysates from vehicle, estradiol, and tamoxifen-treated SRC-1 and vector expressing clones. Input genomic DNA was amplified prior to immunoprecipitation to determine relative amounts of DNA in each sample. These experiments were performed thrice using lysates from different clones with similar results. Representative gels are shown. (H) SRC-1 and CBP transactivate the ERK1 promoter. The ERK1 promoter was transiently cotransfected with SRC-1 or CBP expression vectors into triplicate cultures of SCC25 cells, which were treated with 100 nM estradiol (E2), tamoxifen (TAM), or vehicle for 24 h prior to harvesting for reporter gene assays. Luciferase activity was measured as relative light units normalized to the activity of a control reporter construct as described in Materials and Methods. These experiments were performed thrice with similar results. Error bars represent S.E.M.

TAM-induced transcriptional activation. These preclinical studies suggest that TAM may be a useful clinical adjunct in cancers from non-reproductive tissues, perhaps in combination with standard chemotherapy agents (Tavassoli *et al.* 2002).

One of the key results of this study was the dependence on SRC-1 for E2-mediated proliferative response in cancer cells from non-reproductive tissues. Stratified squamous epithelia such as skin are E2 responsive (Kanda & Watanabe 2004). Clinically, decreased estrogen levels associated with menopause correlate with epidermal thinning, and estrogen containing skin creams have been shown to increase epidermal thickness (Fuchs *et al.* 2003, Hall & Phillips 2005). The E2 induces proliferation of neonatal keratinocytes *in vitro*, which express both ERα and ERβ (Verdier-Sevrain *et al.* 2004). ERβ was expressed by many cell types in skin from the human scalp, while ERα was localized to the dermal papilla and sebocytes (Thornton *et al.* 2003). However, a study using ERα null mutant mice indicated that only ERα mediated keratinocyte proliferation *in vivo* (Moverare *et al.* 2002). Little is known about SRC-1 expression in normal epidermal keratinocytes or ERα expression in SCCs, but the lack of E2 response in these cancers suggests that SRC-1 expression may be lost during carcinogenesis. One previous report demonstrated that TAM induced programmed cell death in SCC lines (Hoffmann *et al.* 2002), but only at high doses (up to 10 μM). TAM has been shown to have opposing effects on different tissues such as breast and uterus (Shang & Brown 2002). The estrogenic effect of TAM in the uterus was shown to require high levels of SRC-1 expression. In mouse epidermis, TAM inhibits u.v.-induced DNA damage (Wei *et al.* 1998) and is used in clinical treatment of dendritic cell-mediated allergic dermatitis (Yotsumoto *et al.* 2003). These studies suggest that as a potential chemotherapeutic agent, TAM would be more effective against cancer cells with low levels of coactivator expression.

Our results demonstrated that ERK1 expression is induced by SRC-1. This induction was mediated at the transcriptional level through direct interaction of SRC-1 and CBP with the proximal ERK1 promoter. These interactions were enhanced by E2 treatment and inhibited by cellular exposure to TAM. The proximal ERK1 promoter contains a number of transcription factor binding sites including those for AP-1 (Chu *et al.* 2005). The SRC-1 has been shown to bind directly to fos and jun subunits (Lee *et al.* 1998). Coexpression of CBP/p300 enhanced SRC-1-dependent transactivation, which was corroborated by our results using the ERK1 promoter. SRC-1 also has been shown to interact with serum response factor and enhances transactivation from this responsive element (Kim *et al.* 1998). Coexpression of CBP/p300 also enhanced transactivation from serum response elements. Alternatively, ERα also has been shown to interact directly with and transactivate AP-1 subunits *in vitro* (Cheung *et al.* 2005). These results suggest the existence of multiple mechanisms by which E2 can activate target gene expression through coactivators.

In summary, we show that SRC-1 was a key molecular determinant of estrogen-mediated proliferation in human SCC lines. TAM treatment inhibited cell cycle progression and proliferation of human cancer lines derived from stratified squamous epithelium. SRC-1 expression was not detected in these SCC lines; however, transient transfection of SRC-1, CBP, or both coactivators enhanced transactivation of an estrogen-responsive promoter in cancer cells treated with E2 or TAM. SRC-1 expression restored the E2-mediated proliferative response to human SCC lines. This increased proliferation correlated with increased ERK1 expression. SRC-1 and CBP were recruited to the proximal ERK1 promoter region in E2 but not TAM-treated cells. Future studies will focus on specific interactions of SRC-1 and CBP with transcription factor response elements in the proximal ERK1 promoter and examine the role of ERβ in SCC proliferation.

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References

Cariou S, Donovan JC, Flanagan WM, Milic A, Bhattacharya N & Slingerland JM 2000 Downregulation of $p21^{WAF1/Cip1}$ or $p27^{Kip1}$ abrogates anti-estrogen mediated cell cycle arrest in human breast cancer cells. *PNAS* **97** 9042–9046.

Carroll JS, Lynch DK, Swarbrick A, Renoir JM, Sarcevic B, Daly RJ, Musgrove EA & Sutherland RL 2003 $p27^{Kip1}$ induces quiescence and growth factor insensitivity in tamoxifen treated breast cancer cells. *Cancer Research* **63** 4322–4326.

Cheung E, Acevedo ML, Cole PA & Kraus WL 2005 Altered pharmacology and distinct coactivator usage for estrogen receptor dependent transcription through activating protein 1. *PNAS* **102** 559–564.

Chien PY, Ito M, Park Y, Tagami T, Gehm BD & Jameson JL 1999 A fusion protein of the estrogen receptor (ER) and nuclear receptor corepressor (NCoR) strongly inhibits estrogen dependent responses in breast cancer cells. *Molecular Endocrinology* **13** 2122–2136.

Chu BY, Tran KV, Ku TKS & Crowe DL 2005 Regulation of *ERK1* gene expression by coactivator proteins. *Biochemical Journal* **392** 589–599.

Couse JF & Korach KS 1999 Estrogen receptor null mice: what have we learned and where will they lead us? *Endocrine Reviews* **20** 358–417.

Ercoli A, Scambia G, Fattorossi A, Raspaglio G, Battaglia A, Cicchillitti L, Malorni W, Rainaldi G, Benedetti Panici P & Mancuso S 1998 Comparative study on the induction of cytostasis and apoptosis by ICI 182,780 and tamoxifen in an estrogen receptor negative ovarian cancer cell line. *International Journal of Oncology* **76** 47–54.

Fuchs KO, Solis O, Tapawan R & Paranjpe J 2003 The effects of an estrogen and glycolic acid cream on the facial skin of postmenopausal women: a randomized histologic study. *Cutis* **71** 481–488.

Hall G & Phillips TJ 2005 Estrogen and skin: the effects of estrogen, menopause, and hormone replacement therapy on the skin. *Journal of the American Academy of Dermatology* **53** 555–568.

Hoffmann TK, Bojar H, Eckel J, van Lierop A, Balz V, Friebe-Hoffmann U, Hauser U & Bier H 2002 Effects of tamoxifen on human squamous cell carcinoma lines of the head and neck. *Anti-Cancer Drugs* **13** 521–531.

Huang HJ, Norris JD & McDonnell DP 2002 Identification of a negative regulatory surface within estrogen receptor α provides evidence in support of a role for corepressors in regulating cellular responses to agonists and antagonists. *Molecular Endocrinology* **16** 1778–1792.

Kanda N & Watanabe S 2004 17β-Estradiol stimulates the growth of human keratinocytes by inducing cyclin D2 expression. *Journal of Investigative Dermatology* **123** 319–328.

Kim HJ, Kim JH & Lee JW 1998 Steroid receptor coactivator 1 interacts with serum response factor and coactivates serum response element mediated transactivations. *Journal of Biological Chemistry* **273** 28564–28567.

Kim MY, Hsiao SJ & Kraus WLA 2001 A role for coactivators and histone acetylation in estrogen receptor α mediated transcription initiation. *EMBO Journal* **20** 6084–6094.

Kornberg RD & Lorch Y 1999 Chromatin modifying and remodeling complexes. *Current Opinion in Genetics and Development* **9** 148–151.

Lee SK, Kim HJ, Na SY, Kim TS, Choi HS, Im SY & Lee JW 1998 Steroid receptor coactivator 1 coactivates activating protein 1 mediated transactivations through interaction with the c-jun and c-fos subunits. *Journal of Biological Chemistry* **273** 16651–16654.

Lindner DJ & Borden EC 1997 Synergistic antitumor effects of a combination of interferon and tamoxifen on estrogen receptor positive and receptor negative human tumor cell lines *in vivo* and *in vitro*. *Journal of Interferon and Cytokine Research* **17** 681–693.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P *et al*. 1995 The nuclear receptor superfamily: the second decade. *Cell* **83** 835–839.

Moverare S, Lindberg MK, Faergemann J, Gustafsson JA & Ohlsson C 2002 Estrogen receptor α, but not estrogen receptor β, is involved in the regulation of the hair follicle cycling as well as the thickness of epidermis in male mice. *Journal of Experimental Dermatology* **119** 1053–1058.

Needham M, Raines S, McPheat J, Stacey C, Ellston J, Hoare S & Parker M 2000 Differential interaction of steroid hormone receptors with LXXLL motifs in SRC-1a depends on residues flanking the motif. *Journal of Steroid Biochemistry and Molecular Biology* **72** 35–46.

Ojanotko-Harri A, Forssell H, Laine M, Hurttia H, Blauer M & Tuohimaa P 1992 Immunohistochemical detection of androgen receptors in human oral mucosa. *Archives of Oral Biology* **37** 511–514.

Ratajczak T 2001 Protein coregulators that mediate estrogen receptor function. *Reproduction, Fertility and Development* **13** 221–229.

Robinson EK, Grau AM, Evans DB, Smid CM, Chiao PJ, Abbruzzese JL & Grimm EA 1998 Cell cycle regulation of human pancreatic cancer by tamoxifen. *Annals of Surgical Oncology* **5** 342–349.

Robyr D, Wolffe AP & Wahli W 2000 Nuclear hormone receptor coregulators in action: diversity for shared tasks. *Molecular Endocrinology* **14** 329–347.

Shang Y & Brown M 2002 Molecular determinants for the tissue specificity of SERMs. *Science* **295** 2465–2468.

Shang Y, Hu X, DiRenzo J, Lazar MA & Brown M 2000 Cofactor dynamics and sufficiency in estrogen receptor regulated transcription. *Cell* **103** 843–852.

Sheppard HM, Harries JC, Hussain S, Bevan C & Heery DM 2001 Analysis of the steroid receptor coactivator 1 (SRC1)-CREB binding protein interaction interface and its importance for the function of SRC1. *Molecular and Cellular Biology* **21** 39–50.

Smith CL, Onate SA, Tsai MJ & O'Malley BM 1996 CREB binding protein acts synergistically with steroid receptor coactivator 1 to enhance steroid receptor dependent transcription. *PNAS* **93** 8884–8888.

Son ED, Lee JY, Lee S, Kim MS, Lee BG, Chang IS & Chung JH 2005 Topical application of 17β-estradiol increases extracellular matrix protein synthesis by stimulating TGF-β signaling in aged human skin *in vivo*. *Journal of Investigative Dermatology* **124** 1149–1161.

Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, McKenna NJ, Onate SA, Tsai SY, Tsai MJ *et al*. 1997 Steroid receptor coactivator 1 is a histone acetyltransferase. *Nature* **389** 194–198.

Tavassoli M, Soltaninia J, Rudnicka J, Mashanyare D, Johnson N & Gaken J 2002 Tamoxifen inhibits the growth of head and neck cancer cells and sensitizes these cells to cisplatin induced apoptosis: role of TGFβ1. *Carcinogenesis* **23** 1569–1575.

Thornton MJ 2002 The biological actions of estrogens on skin. *Experimental Dermatology* **11** 487–502.

Thornton MJ, Taylor AH, Mulligan K, Al-Azzawi F, Lyon CC, O'Driscoll J & Messenger AG 2003 Oestrogen receptor beta is the predominant oestrogen receptor in human scalp skin. *Experimental Dermatology* **12** 181–190.

Verdier-Sevrain S, Yaar M, Cantatore J, Traish A & Gilchrest BA 2004 Estradiol induces proliferation of keratinocytes via a receptor mediated mechanism. *FASEB Journal* **18** 1252–1254.

Wei H, Cai Q, Tian L & Lebwohl M 1998 Tamoxifen reduces endogenous and UV light induced oxidative damage to DNA, lipid, and protein *in vitro* and *in vivo*. *Carcinogenesis* **19** 1013–1018.

Xu JM, Qiu Y, DeMayo FJ, Tsai SY, Tsai MJ & O'Malley BW 1998 Partial hormone resistance in mice with disruption of the steroid receptor coactivator 1 (*SRC-1*) gene. *Science* **279** 1922–1925.

Yamamoto Y, Wada O, Suzawa M, Yogiashi Y, Yano T, Kato S & Yanagisawa J 2001 The tamoxifen responsive estrogen receptor α mutant D351Y shows reduced tamoxifen dependent interaction with corepressor complexes. *Journal of Biological Chemistry* **276** 42684–42691.

Yotsumoto S, Shimomai K, Hashiguchi T, Uchimiya H, Usuki K, Nishi M, Kanekura T & Kanzaki T 2003 Estrogen dermatitis: a dendritic cell mediated allergic condition. *Dermotology* **207** 265–268.

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