Metastatic progression with resistance to aromatase inhibitors is driven by the steroid receptor coactivator SRC-1

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Abstract

Aromatase Inhibitors (AIs) are a standard of care treatment for post-menopausal, estrogen receptor-positive breast cancers. Although tumor recurrence on AI therapy occurs, the mechanisms underlying acquired resistance to AIs remain unknown. In this study we examined a cohort of endocrine-treated breast cancer patients and employed a cell line model of resistance to the AI letrozole. In patients treated with a first-line AI, hormone receptor switching between primary and resistant tumors was a common feature of disease recurrence. Resistant cells exhibited a switch from steroid-responsive growth to growth factor-responsive and endocrine-independent growth, which was accompanied by the development of a more migratory and disorganized phenotype. Both the resistant cells and tumors from AI-resistant patients showed high expression of the steroid receptor coactivator SRC-1. Direct interactions between SRC-1 and the transcription factor Ets2 regulated Myc and MMP9. SRC-1 was required for the aggressive and motile phenotype of AI-resistant cells. Interestingly, SRC-1 expression in primary and/or recurrent tumors was associated with a reduction in disease-free survival in treated patients. Moreover, there was a significant association between SRC-1 and Ets2 in the recurrent tissue compared with the matched primary tumor. Together, our findings elucidate a mechanism of AI-specific metastatic progression in which interactions between SRC-1 and Ets2 promote de-differentiation and migration in hormone-dependent breast cancer.
Introduction

Endocrine therapies, including estrogen receptor (ER) modulators and aromatase inhibitors (AIs), are first line treatment for ER positive breast cancer. The development of third-generation AIs has brought about a major change in the therapeutic approach to patients with hormone-sensitive breast cancer. A meta-analysis of trials comparing AI and tamoxifen for the adjuvant treatment of women with early breast cancer concluded that AIs should be the treatment of choice in postmenopausal women (1,2). AIs however do not remove all of the estrogen ligand - data from molecular and *in vivo* studies suggest that this can result in adaptive hypersensitivity of the intact ER via increased signaling through growth factor pathways (3). The significance of this hypersensitivity and resultant resistance to the AI therapy will only become evident as long-term follow-up becomes available.

The development of resistance to endocrine therapy, and resulting tumor recurrence, is due at least in part to cellular plasticity leading to a shift in the phenotype of the tumor cell from steroid dependence to steroid independence/growth factor dependence. Consequently, the resistant cancer cells may also utilize steroid receptor-independent mechanisms to drive tumor progression. Alterations in steroid receptor profile observed in clinical studies between primary and metastatic breast cancer, in particular with loss of progesterone receptor (PR) status, supports the phenomenon of tumor adaptability in endocrine resistant patients (4). Furthermore conversion from serum Her2 negative to positive has been reported as an independent risk factor for decreased survival in both tamoxifen and AI treated patients (5).

Aberrant expression of the p160 steroid receptor coactivators SRC-1 and SRC-3 (AIB1) in patients has been associated with resistance to endocrine therapies and the development of tumor recurrence (6-8). Although initially described as a nuclear receptor coactivator protein, SRC-1 has been shown to interact with transcription factors running downstream of an activated MAP kinase pathway. These transcription factor interactions may represent one of the consequences of growth factor pathway cross-talk described in endocrine resistance. Functional interactions between SRC-1 and the Ets family of transcription factors, Ets2 and PEA3 have previously been reported, and this relationship has been shown to be important in tumor progression and the development of metastasis (6,9,10).

In this study negative PR status predicted early disease recurrence on AI treatment and loss of steroid receptor status between matched primary and metastatic tumors was observed. In a cell model of AI resistance, developed using the AI letrozole, we found elevated cell
migration and loss of differentiation compared with the parental endocrine sensitive cells. We provide evidence that SRC-1 can drive this aggressive phenotype by partnering with Ets2 to regulate expression of Myc and MMP9. Furthermore, elevated SRC-1 expression and functional transcriptional interactions were observed in AI-specific metastatic tumors. Taken together these data suggest a role for SRC-1 in the steroid independent adaptation of breast cancer to AI therapy and subsequent disease recurrence.

Materials and Methods

Cell lines, treatments and transfections

Breast cancer cells MCF-7, MDA-MB231, SKBR3 [American Type Culture Collection (ATCC)] and LY2 (kind gift from R. Clarke, Georgetown) were grown as previously described (11). MCF10A cells (ATCC) were cultured in DMEM/F12 with 15 mM hepes buffer, 5% horse serum, 10 μg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin and 0.5 μg/ml hydrocortisone. Aromatase inhibitor-sensitive (Aro) cells were generated by stably transfecting MCF-7 cells with the aromatase gene, CYP19 (pcDNA DEST47 destination vector). Aro cells were cultured in MEM supplemented with 10% FCS, 1% L-Glutamine, 1% Pen/Strep and 200μg/ml Geneticin (G418, Gibco® Invitrogen). Letrozole-resistant (LetR) cells were generated by long-term (>3 months) culture of Aro cells with letrozole (10⁻⁶ M, Novartis) and androstenedione (25x10⁻⁹ M, Sigma Aldrich) in MEM supplemented with 10% charcoal-dextran-striped FCS, 1% L-Glutamine, 1% Pen/Strep and 200μg/ml G418. All cells were maintained in steroid depleted medium 72 hours prior to treatment with estradiol (10⁻⁸ M, Sigma Aldrich), androstenedione (10⁻⁷ M) or letrozole (10⁻⁶ M). SiRNA (Ambion) directed against SRC-1 (AM16706) and ERalpha (4392421) were used to knock down gene expression. The pcDNA3.1 and pCGN plasmids containing full length SRC-1 and Ets2 respectively were used for overexpression studies. Empty plasmids were used as a negative control. Plasmids were constructed as previously described (11). Transfections were carried out using Lipofectamine 2000 (Invitrogen) as per manufacturer’s instructions. For the motility assay and the 3D-cultures, cells were seeded 72 hours after transfection. All other experiments were performed 24 hours post transfection.

Cell motility, cell proliferation and 3D culture assays
Cellomics Cell Motility Kit (Thermo Scientific, #K0800011) was used to assess individual cell movement after 22 hours as per manufacturer’s instructions using cells seeded at 1x10^4 cells/ml. Mean track areas (minimum of 90 cell tracks per condition) were analysed with Olympus cellF imaging software and compared with a Student t-test.

For proliferation, Aro and LetR cells were steroid-depleted for 72 hours and seeded into 6-well plates at a density of 0.5x10^4 cells per well. The cells were serum starved for a further 24 hours before treating them with vehicle (acetic acid; 0.01%), androstenedione (100nM) or epidermal growth factor (EGF; 1 ng/ml) for 72 hours. Cells were stained with crystal violet solution (Cruinn), dissolved in 33% glacial acetic acid and the absorbance measured at 620 nm using a plate reader (Greiner).

For 3D assays, 5x10^4 cells in 400μl of their respective medium (as above) and 2% Matrigel (BD Biosciences) were seeded onto the growth factor reduced matrigel matrix in eight well chamber slides (BD Biosciences) and cultured for 14 days at 37°C/5% CO2. Cells were fixed in 4% paraformaldehyde and permeabilized with phosphate buffered saline (PBS) containing 0.5% Triton X-100 for 10 minutes at 4°C. Cells were blocked in 10% goat serum, 1% bovine serum albumin. Cells were stained with Phalloidin 594 (Molecular Probes) for 20 minutes and DAPI for 5 minutes. Alternatively, cells were stained with rat anti-human B1-integrin antibody (552828, BD Transduction Laboratories) followed by goat anti-rat 633 secondary antibody (Alexa-Fluor) and DAPI. Slides were mounted (Dako) and examined by confocal microscopy.

**Zymography**

Aro and LetR cells were seeded in a 6-well plate and media was collected 24 hours later. Protein was concentrated with Amicon Ultra4 filters (50K pore size, Millipore). 20μg of protein were loaded onto a 10% Gelatin Zymogram Gel (Invitrogen) and run according to the manufacturer’s instructions. The gel was stained with Coomassie brilliant blue and destained until bands were visible. Pro (92kDa) and active (82kDa) MMP9 bands were identified by size (12,13).

**Next Generation Sequencing**

SRC-1 ChIP sequencing (vehicle and tamoxifen treated LY2 cells) and RNA sequencing (tamoxifen treated LY2 cells) were carried out using the Illumina Genome Analyzer System as previously described (11).
Coimmunoprecipitation and western blotting

Protein was immunoprecipitated with mouse anti-SRC-1 and blotted for SRC-1 and Ets2. Western blotting was performed as previously described (14). Primary antibodies used were rabbit anti-human Ets2 (1:250, sc-351, Santa Cruz), rabbit anti-human SRC-1 (1:100, sc-8995, Santa Cruz), rabbit anti-human Myc (1:200, sc-788, Santa Cruz), mouse anti-human ERalpha (1:500, sc-8002, Santa Cruz) or β-Actin (1:7500, Sigma-Aldrich).

Chromatin Immunoprecipitation (ChIP) assay and PCR

Aro and LetR cells were treated with vehicle, estrogen, androstenedione, letrozole or androstenedione and letrozole for 45 minutes and ChIP analysis was performed as previously described (14). Cell lysates were quantified after shearing using a Nanodrop (Thermo Scientific) to ensure equal starting material in each sample. The following antibodies were incubated overnight at 4ºC with rotation: 6 μg rabbit anti-human Ets2 (sc-351, Santa Cruz), 6 μg rabbit anti-human SRC-1 (sc-8995, Santa Cruz), 6 μg rabbit IgG as a negative control or 7 μl anti-acetylated H4 (Millipore) as a positive control. Reverse crosslinking and DNA recovery were performed with Chelex 100 (Bio-Rad). Real-time PCR was performed in duplicate by SYBR Green PCR (Qiagen) using a Lightcycler (Roche) and primers listed in Supplementary Table S1. Semi-quantitative RT-PCR was performed using primers listed in Supplementary Table S1.

Patient information and construction of tissue microarray

Patient breast tumor samples were collected and data recorded as previously described (15). Data included pathologic characteristics (tumor stage, grade, lymph node status, ER status, recurrence) as well as treatment with radiotherapy, chemotherapy, tamoxifen or aromatase inhibitors. Detailed follow-up data (median 56 months) was collected on the patients to determine disease-free survival. Tissue microarray (TMA) construction was conducted as previously described (15).

Immunohistochemistry

Breast tissue and TMA sections were deparaffinized and incubated with rabbit anti-human SRC-1 (2 μg/mL; Santa Cruz); rabbit anti-human Myc (2 μg/mL; Santa Cruz), mouse anti-human MMP9 (2 μg/mL; Santa Cruz) or control IgG for 1 hour at room temperature. The slides
were then incubated with the corresponding biotin-labeled secondary (0.5% in PBS; Vector Laboratories) for 30 minutes, followed by peroxidase-labeled avidin biotin complex (Vector Laboratories) for 30 minutes. Sections were developed in 3,3-diaminobenzidine tetrahydrochloride for 2 minutes and counterstained with hematoxylin for 3 minutes, then passed through increasing concentrations of Industrial Methylated Spirits (70% and 100%) and then xylene. The immunostained TMA slides were scored using the Allred scoring system (16). Independent observers, without knowledge of prognostic factors, scored slides. Univariate statistical analysis was performed using Fisher’s exact test for categorical variables and Wilcoxon’s test for continuous variables.

**Immunofluorescent microscopy and quantitative colocalisation**

Cell lines, grown on collagen coated coverslips, were fixed and permeabilized as per the 3D assay. Cells were blocked with 10% goat serum for 1 hour, incubated with rabbit anti-human SRC-1 antibody (Santa Cruz) followed by goat anti-rabbit 488 (Molecular Probes), phalloidin for 20 minutes and DAPI for 5 minutes. Tumor sections were blocked in 10% goat serum for 1 hour, incubated with rabbit anti-human phospho-Ets2 (10 μg/mL in 10% human serum; Invitrogen) for 1.5 hours and then Alexa 594 conjugated goat anti-rabbit antibody (1/200; Molecular Probes) for 1 hour. Sections were blocked again with goat serum for 1 hour, then incubated with mouse anti-human SRC-1 (10 μg/mL in 10% human serum; Upstate) for 1.5 hours, followed by a 1 hour incubation with Alexa 488 conjugated goat anti-mouse antibody (1/200; Molecular Probes). Sections were mounted using fluorescent mounting media (DAKO). Slides were examined under a Zeiss LSM 510 META confocal fluorescent microscope with the 40x objective lens (1.40 NA). Quantitative colocalization analyses (minimum 9 images per sample) were performed with Zeiss 510 META Software using the Pearson’s correlation coefficient, R(r) (17).

**Results**

**AI resistance is characterised by hormone receptor switching and a more motile and disorganized phenotype**

Endocrine resistance is thought to involve, at least in part, a switch from steroid signaling to growth factor signaling, leading to a steroid independent tumor (18). In keeping with this hypothesis, we identified a significant association between lack of PR expression in the
primary tumor and reduced early response specifically to AI treatments (Fig. 1A, p=0.02 at 2 year follow up, Supplementary Figure S1A). In addition, analysis of patients with matched primary and AI resistant tumors highlighted that hormone receptor status regularly switched between primary and subsequent tumors. In particular, a trend for loss of ER and PR expression and occasional gain of Her2 expression was observed (Fig. 1B). Analysis of a cell model system with AI sensitive (Aro) and AI resistant (LetR) cells was also in keeping with this hypothesis of a signaling switch. Resistant cells, although they had slightly elevated expression of ERα (Fig. 1C), showed a reduced proliferative response to estrogen and an increased proliferative response to EGF compared to sensitive cells (Fig. 1D).

To further characterise the AI resistant phenotype, migratory assays and 3D culture assays were performed. The migratory assay identified a significant increase in motility between resistant cells and sensitive cells (approximately 5 fold) (Fig. 1E). As expected, the high motility of the resistant cells was not significantly affected by either steroid or AI treatment (Supplementary Figure S1B). Consistent with the increased motility of LetR cells, increased levels of the matrix metalloproteinase MMP9 were also detected in these resistant cells compared to sensitive cells (both mRNA and levels of the secreted active MMP9 protein)(Fig. 1F). In 3D culture assays, sensitive cells were capable of organising into circular, hollow structures, similar to the highly organized acini of MCF10A cells (Fig. 1G). Resistant cells by contrast, were more disorganized, failed to form round, hollow, polarized spheres and were more comparable to the disorganized endocrine insensitive SKBR3 cells (Fig. 1G and Supplementary Figure S1C). The increased migration and decreased polarisation of AI resistant cells is consistent with a metastatic phenotype. Combined, these results provide evidence of hormone receptor switching as an important feature involved in the development of an aggressive AI resistant phenotype.

**SRC-1 and Ets2 interact to regulate expression of Myc and MMP9 target genes in AI resistance**

The steroid receptor coactivator SRC-1 has previously been shown to play an important role in endocrine resistance and expression of SRC-1 has been associated with reduced disease free survival in a cohort of breast cancer patients with locally advanced disease (9). ChIP sequencing, performed to identify molecular targets of SRC-1 in endocrine resistant cells, identified the oncogene Myc as a potential target gene, with a strong SRC-1 binding peak located within the proximal promoter (Fig. 2A). The transcription factor Ets2 has previously been shown to regulate Myc expression through binding to an E2F binding motif which is also located...
within the Myc proximal promoter (19,20). To investigate possible SRC-1 signaling pathways in AI resistance, basal protein expression was compared between Aro and LetR cells. SRC-1, Ets2, phospho-Ets2 and Myc expression was higher in resistant cells compared to sensitive Aro or parental MCF7 cells (Fig. 2B). To assess regulation of expression of these proteins both Aro and LetR cells were treated with estrogen and androstenedione in the presence or absence of letrozole (Fig. 2C). In Aro cells, expression of SRC-1 and Myc was increased in response to both estrogen and androstenedione. Letrozole inhibited this response to androstenedione, confirming the sensitivity of Aro cells to AIs (Fig. 2C, left hand panel). In LetR cells, expression of SRC-1 and Myc was higher than in Aro cells and increased further in response to androstenedione. This increase was not inhibited by the presence of letrozole (Fig. 2C, right hand panel). Consistent with these observations, immunofluorescent staining identified increased nuclear localisation of SRC-1 in Aro cells treated with androstenedione compared to all other Aro treated cells. Strong nuclear localisation of SRC-1 was observed in all LetR cells independent of treatments (Figure 2D). Ets2 protein expression was not altered by steroid treatments but its expression was constitutively higher in LetR cells than in Aro cells (Fig. 2C). Thus expression of SRC-1 and Myc appears to have become dysregulated in the LetR model of AI resistance.

Co-immunoprecipitation analysis revealed that SRC-1 and Ets2 can interact after 45 minutes of steroid treatment (Fig. 3A). Chromatin immunoprecipitation (ChIP) studies were therefore performed using this time point to investigate the potential recruitment of SRC-1 and Ets2 to the Myc and MMP9 promoters. Though MMP9 was not highlighted by the ChIPsequencing study, owing to the undetectable levels of MMP9 expression in the endocrine resistant LY2 cells used for this analysis, bioinformatic analysis did identify both Ets and E2F binding motifs within the proximal MMP9 promoter. ChIP analysis confirmed that both SRC-1 and Ets2 were recruited to the promoters of Myc and MMP9 target genes in Aro and LetR cells (Fig. 3B). In AI sensitive Aro cells, SRC-1 and Ets2 recruitment to the promoters was driven by steroids and inhibited by letrozole treatment, as confirmed by real-time PCR (Fig. 3B). By contrast, in the resistant LetR cells, SRC-1 and Ets2 were recruited to the target gene promoters independent of steroid treatment, and this recruitment was not inhibited by the presence of letrozole (Fig. 3B). Furthermore, overexpression of either SRC-1 or Ets2 in Aro cells resulted in increased mRNA expression of both Myc and MMP9 target genes (Fig. 3C, 3D). The increased transcript levels of MMP9 did not translate into increased levels of secreted MMP9 protein.
suggesting that mechanisms other than SRC-1 or Ets2 may be important in the post-translational modification and secretion of MMP9. Finally, Ets2 overexpression in LetR cells, resulted in increased Myc and MMP9 transcript expression and concomitant knockdown of SRC-1 using siRNA inhibited the increase (Fig. 3E). Combined, these results suggest that SRC-1 can interact with the transcription factor Ets2 to regulate expression of Myc and MMP9 and that this signaling pathway is dysregulated in AI resistance.

**SRC-1 is required for the motile, disorganized phenotype of AI resistant cells**

To assess the functional role of the SRC-1 driven signaling pathway in AI resistance, SRC-1 was knocked down in LetR cells using siRNA. Reduced SRC-1 expression resulted in a significantly reduced ability of these cells to migrate (p=0.0007), returning the LetR cells to a migratory phenotype comparable to that of the AI sensitive Aro cells (Fig. 4A). Further, the LetR cells with SRC-1 knock down were capable of forming more organized 3D acini in a manner comparable to the AI sensitive Aro cells (Fig. 4B).

Previous reports have indicated that SRC-1, although named as a nuclear receptor coactivator, may interact with other transcription factors such as Ets2, as shown here. In line with these findings ERalpha knockdown had a minimal effect on migration (Fig. 4C). The significantly greater impact of SRC-1 on migration in comparison to ERalpha (p=0.0377, Fig. 4C), supports a steroid independent mechanism for SRC-1 in driving AI mediated metastasis.

**SRC-1 significantly associates with disease recurrence in AI treated patients**

To examine the significance of the SRC-1 signaling pathway in the clinical setting, a tissue microarray was constructed with primary breast tumors from 150 endocrine therapy treated patients, 84 of whom received an AI and 75 of whom received tamoxifen. Median follow up on these patients was 56 months. SRC-1 protein expression significantly associated with poor disease-free survival in the total endocrine treated population (p=0.0255, Fig. 5A and Supplementary Figure S1A) and the tamoxifen treated population (p=0.0326, Fig. 5B) but not in the AI treated population (p=0.6894, Fig. 5B). SRC-1 also associated with recurrence (independent of time to recurrence) in the tamoxifen treated (p=0.015) and total endocrine treated (p=0.009) populations but not in the AI treated (p=0.494) population (Table 1). No associations were observed between SRC-1 expression and PR, Her2 or nodal status. However, a highly significant association was observed between SRC-1 and advanced tumor stage in both
the endocrine treated population (p=0.003) and specifically within the AI treated population (p=0.001, Table 1). This association suggests that although SRC-1 may not be useful as a predictor of response to treatment, it may play an important role in mediating the metastatic phenotype of AI resistance.

Amongst the patients who displayed AI resistance (n=9), only 3 primary tumors were scored as SRC-1 negative. Matched resistant tumor tissue was collected for all 3 of these patients and paired primary and resistant tumors were stained for SRC-1 protein expression. In each case, the resistant tumor tissue was SRC-1 positive (representative images shown in Fig. 5C). This finding is consistent with the proposed role of SRC-1 in mediating the metastatic phenotype of AI resistance. Indeed, expression of SRC-1 in either the primary or resistant tumor of AI treated patients revealed a significant correlation between SRC-1 expression and reduced disease free survival (p=0.0106, Kaplan Meier Fig. 5C). To monitor the potential functional role of SRC-1 expression in these tumor samples, dual immunofluorescent staining for SRC-1 and phospho-Ets2 was performed in the three pairs of matched primary and AI resistant tumors. Expression of not only SRC-1, but also phospho-Ets2 was higher in the resistant tumors (representative images shown in Fig. 5D). Additionally, both proteins significantly colocalized, within the nuclei of the resistant tumor cells in comparison to the matched primary tumor (Pearson’s correlation coefficient, p=0.0004, Fig. 5D). Consistent with these findings, strong Myc expression was observed in each of the AI resistant tumors (representative images shown in Fig. 5E). Weak MMP9 expression was observed in the resistant tumors with no detectable staining in matched primary tumors (representative images shown in Fig. 5E). This clinical data confirms the importance of SRC-1 and its signaling pathway in mediating the aggressive phenotype of AI resistance and metastasis.

Discussion

The development of resistance to aromatase inhibitor therapy is marked by a shift in cancer cell status from steroid dependent to steroid independent/growth factor dependent. Recent clinical studies of advanced breast cancer observed that PR expression associates with increased time to AI treatment failure (21), suggesting that a move away from steroid signaling may mark the emergence of a more aggressive phenotype. Consistent with this we observed an association between PR negative status in the primary tumor and increased early disease recurrence on first line AI treatment. Furthermore there was a significant loss of steroid
receptor status in metastatic tumor tissue in comparison to the matched primary tumor in AI treated patients. In the LetR cell line model, a slight increase in ERalpha expression was observed, consistent with the Brodie model of long term letrozole treatment (22). However, we also observed an enhanced proliferative response to EGF in cells resistant to AIs, in comparison to the parental sensitive phenotype, indicating increased reliance on growth factor signaling pathways. The ability of AI resistant tumors to alter their receptor status and increase sensitivity to growth factor pathways may be a consequence of increased cellular plasticity leading to the development of a steroid independent phenotype.

Several cell model systems of AI resistance have been described in the literature including the long-term estrogen deprived (LTED) model and an estrogen withdrawal breast cancer cell line over-expressing aromatase (UMB-1Ca). Where the former model displays increased sensitivity to estrogen not observed in the UMB-1Ca cells, both models show increased sensitivity to growth factor signaling (23,24). In this study, letrozole resistant (LetR) cells displayed a reduced proliferative response to steroid treatment and an increased sensitivity to growth factors in comparison to the parental endocrine sensitive cells. Similar elevations in growth factor signaling activity has previously been reported in models of resistance to both letrozole and anastrozole (25). Recently Chen reported that growth factor/signal transduction pathways are upregulated after ERalpha-dependent pathways are suppressed by letrozole, anastrozole and exemestane, and ERalpha can then be activated through different crosstalk mechanisms (26). Increased tumor plasticity occurs in endocrine resistant breast cancer relative to endocrine sensitive tumors (11). This is evident in our model of AI resistance where a decrease in cellular differentiation and a concomitant increase in cell migration were observed. Moreover, alterations in migratory patterns were accompanied by increased activity of the metalloproteinase MMP9. These observations of a more aggressive phenotype are consistent with increased levels of invasion in AI resistant models reported by Brodie et al (24).

Aberrant expression of the p160 steroid receptor coactivators SRC-1 and SRC-3 (AIB1) in patients has been associated with resistance to endocrine therapies and the development of tumor recurrence (6-8,27,28). Unlike other oncogenes, recent studies provide evidence of a specific role for SRC-1 in the development of metastasis (29,30). Of interest, knockdown of SRC-1 can decrease cell proliferation, restore differentiation and decrease migration in tamoxifen resistant breast cancer cells (11). A steroid-independent role for SRC-1 has been established and the coactivator has been shown to interact with transcription factors running downstream of an
activated MAP kinase pathway. Work from this group and others has reported functional interactions between SRC-1 and the Ets family of transcription factors, Ets2 and PEA3, and that this relationship is important in tumor progression and the development of metastasis (9,10,31). In the ER negative PyMT SRC-1 knockout mouse model, SRC-1, though not required for mammary tumor initiation, is essential for the development of metastatic disease. This occurs in part through SRC-1 mediated TWIST suppressing luminal markers such as E-cadherin and beta-catenin during epithelial-mesenchymal transition (10). Here we show that SRC-1 can utilize Ets2 to regulate the production of Myc and MMP9. The production of both the oncogene and metalloproteinase was dysregulated in AI resistance, where treatment of the resistant cells with letrozole failed to prevent recruitment of SRC-1 and Ets2 to the promoters of Myc and MMP9 or inhibit their production. Moreover, at a functional level, knockdown of SRC-1 restored cellular differentiation and reduced cell migration in the AI resistant cells. In line with SRC-1 mediating the metastatic phenotype through steroid independent mechanisms, knockdown of ERalpha had no significant effect on AI resistant cell migration.

Expression of SRC-1 in the primary tumor is an independent predictor of poor response to tamoxifen treatment in breast cancer patients (6,8). Despite this, in patients treated first-line with AI therapies, SRC-1 was not a significant predictor of response to treatment, suggesting that different mechanisms may be important in the initiation of resistance to tamoxifen in comparison to AI therapy. From our patient population that suffered a tumor recurrence on AI treatment, two thirds expressed SRC-1 in the primary tumor. Those patients whose primary tumor was negative for SRC-1 however all had a SRC-1 positive tumor recurrence. This switch from SRC-1 negative to positive in the recurrent tissue echoes the loss of steroid receptor status which can be observed in AI resistant patients. Furthermore, a significant increase in functional associations between the coactivator and Ets2, together with an increase in Myc and MMP9, were also found in the recurrent tumor compared with the matched primary patient tumor tissue. Taken together these data indicate the significance of SRC-1 in advancing the metastatic phenotype in AI resistant patients.

Increased tumor plasticity can enable endocrine sensitive tumors to adapt to therapy through the promotion of growth factor signaling. In this study we provide evidence that SRC-1 can play a significant role in driving AI-related tumor metastasis through the regulation of dedifferentiation and pro-migratory pathways. Understanding the mechanisms of how tumors can
turn off and on key signaling networks in response to AI treatment will enable new strategies to be developed to detect and treat metastatic disease.

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References


Figure 1. AI resistance is characterised by hormone receptor switching and an aggressive phenotype. A, Kaplan Meier estimates of disease free survival in tamoxifen treated (n=77) and AI treated (n=89) patients according to PR expression. PR positive patients treated with an AI did significantly better than PR negative patients during the first 2 years of follow up (p = 0.0206). B, Table showing hormone receptor status of matched primary and resistant tumors for 6 AI treated patients. Changes in receptor status are highlighted in pink. C, Western blot analysis shows slightly increased expression of ERalpha in LetR cells compared to Aro cells. D, AI resistant cell model (LetR cells, black bars) show reduced proliferative response to steroids and increased growth factor response compared to sensitive cells (Aro cells, grey bars). Results are mean ± SEM (n=3). * indicates p<0.01. E, LetR cells are more motile than Aro cells (p<0.0001). Histogram shows the mean migratory area per cell (μm²) ± SEM (n=3). The metastatic MDA-MB231 cells are shown for comparison. (Scale bars 200μm). F, Higher levels of both MMP9 mRNA by RT-PCR and active MMP9 by gelatin zymography in LetR cells compared to Aro cells. G, LetR cells do not form organized acini in 3D culture. Aro cells, similar to the highly polarized MCF10A cells, form 3D organized structures with hollow lumen. LetR cells fail to hollow out a lumen and remain disorganized, more comparable to SKBR3 cells. Cells are stained with DAPI (blue) and phalloidin (red) and images are representative of three separate experiments. (Scale bars 20μm).

Figure 2. Response of SRC-1, Ets2 and Myc to steroid treatments. A, Location of SRC-1 binding peak within the proximal promoter region of Myc gene as detected by ChIP sequencing analysis in endocrine resistant LY2 cells. RNA sequencing confirms expression of Myc mRNA in these cells. X marks the location of an E2F binding site within the Myc promoter. B, Protein levels of SRC-1, Ets2, phospho-Ets2 (pEts2) and Myc are higher in LetR than in Aro and MCF7 cells. Western blot images are representative and densitometry graphs represent relative mean normalized expression (n=3). Error bars represent SEM. C, SRC-1 and Myc protein expression is sensitive to letrozole treatment in Aro cells but insensitive to letrozole in LetR cells. Ets2 expression is not regulated by steroid treatments in either cell line. Cells were treated with Vehicle (V), Estrogen (E), Androstenedione (A), Letrozole (L), or a combination (A+L). Western blot images are representative and densitometry graphs represent relative mean normalized expression (n=3). Error bars represent SEM. D, Confocal images of SRC-1 localisation in Aro and
LetR cells in the presence and absence of androstenedione and letrozole alone and in combination. Nuclear localisation of SRC-1 increases in Aro cells in response to androstenedione and is reduced when letrozole is added. By contrast, nuclear intensity of SRC-1 is strong in LetR cells independent of treatments. Images are taken at 40X magnification with a confocal fluorescent microscope.

**Figure 3. SRC-1 and Ets2 regulate expression of target genes Myc and MMP9.** A, SRC-1 and Ets2 co-immunoprecipitate with strongest interaction after 45 minutes steroid treatment. Aro cells were treated with androstenedione for 0-4 hours. Protein was immunoprecipitated (IP) with an anti-Ets2 antibody and immunoblotted (IB) for SRC-1 and Ets2. B, SRC-1 and Ets2 are recruited to the Myc and MMP9 promoters. ChIP analysis in Aro and LetR cells with the same treatments as in Figure 2C. Recruitment to both promoters was letrozole sensitive in Aro cells and letrozole insensitive in LetR cells. Graphs show real-time PCR relative quantification of ChIP results. Anti-H4 antibody was used as a positive control and IgG as a negative ChIP control. Genomic DNA (+ve) and water (-ve) were used as PCR controls. A distal promoter region was used to confirm specificity of recruitment to the promoter region. C, Overexpression of SRC-1 resulted in increased transcript levels of both Myc and MMP9 (RT-PCR analysis) in Aro cells. Increased Myc expression was also seen at the protein level (western blot) but no change in secreted levels of MMP9 protein were observed (zymography). D, Overexpression of Ets2 resulted in increased transcript levels of both Myc and MMP9 (RT-PCR analysis) in Aro cells. Increased Myc expression was also seen at the protein level (western blot) but no change in secreted levels of MMP9 protein were observed (zymography). E, Overexpression of Ets2 resulted in increased expression of Myc and MMP9 in an SRC-1 dependent manner. Myc and MMP9 mRNA expression was increased in response to Ets2 overexpression in LetR cells but this increase was inhibited when the cells were concomitantly transfected with SRC-1 siRNA.

**Figure 4. SRC-1 has a functional role in migration and inhibits acini formation in LetR cells.** A, SRC-1 knockdown in LetR cells results in decreased migration. Histogram shows the mean migratory area per cell (μm²) ± SEM and was significantly less for SRC-1 knockdown than for non-targeting (nt) control (p=0.0007). Aro cells are shown for comparison. (Scale bars 200μm). Western blot confirms SRC-1 protein knockdown. B, SRC-1 knockdown in LetR cells results in increased ability to form organized 3D acini. Cells from 3D assay are stained with DAPI (blue) and
phalloidin (red) and results are representative of three separate experiments. Aro cells are shown for comparison. (Scale bars 20 μm). C, Functional migratory role of SRC-1 in AI resistance is not dependent on ERalpha. Western blot confirms successful ERalpha knockdown with siRNA. Histogram shows only a marginal decrease in the mean migratory area per cell in LetR cells following ERalpha knockdown. These cells migrate significantly more than LetR cells with SRC-1 knockdown (p=0.0377).

**Figure 5. SRC-1 significantly associates with disease recurrence in AI treated patients.** A, Immunohistochemical staining of SRC-1 in tissue microarray cores, counterstained with haematoxylin. Examples of SRC-1 positive and negative primary tumors are shown. B, Kaplan Meier estimates of disease free survival in the tamoxifen (n=75) and AI (n=84) treated populations. SRC-1 positive primary tumors (red line) significantly associated with reduced disease free survival in the tamoxifen treated population (p=0.0326) but not significantly in the AI treated population (p=0.6894). C, Expression of SRC-1 was increased and more nuclear in AI resistant metastatic tumors than in matched primary tumors (n=3). Representative images are shown of a matched primary breast tumor and a metastatic lung tumor from one AI treated patient. IgG was used as a negative control. Kaplan Meier estimates of disease free survival in the AI treated population (n=84) according to SRC-1 staining in the primary or resistant metastatic tumor. SRC-1 significantly associated with reduced disease free survival (p=0.0106). D, Immunofluorescent analysis of SRC-1 (green) and phospho-Ets2 (red) expression in matched primary breast and AI resistant tumor samples (n=3), counterstained with DAPI (blue). Expression of both proteins was stronger and more nuclear in the resistant samples. Representative images are shown (n=3). Merged image shows that both proteins co-localize in the nucleus of these metastatic cells (white arrows). The extent of co-association was measured by Pearson’s correlation, R(r), and is significantly higher in the resistant tissue than in the primary breast tissue (p=0.0004, n=3). E, Immunohistochemical analysis of Myc and MMP9 in matched primary breast and AI resistant tumor samples (n=3), counterstained with haematoxylin. Representative images are shown. Myc was strongly expressed in resistant tumors. MMP9 was weakly expressed in resistant tumors and absent from primary tumors.
Table 1: Associations of SRC-1 with clinicopathologic variables using Fisher’s exact test in endocrine, AI and Tamoxifen treated patient populations. The percentage of SRC-1 % patients within each parameter are listed. Parameters include recurrence (+ve) or no recurrence (-ve); Node +ve (1 or more nodes positive) or -ve (no positive nodes).

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Jean McBryan, Sarah M. Theissen, Christopher Byrne, et al.

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