Global characterization of the SRC-1 transcriptome identifies ADAM22 as an ER-independent mediator of endocrine resistant breast cancer

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ADAM22 is an SRC-1 target and mediates cancer metastasis

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Abstract

The development of breast cancer resistance to endocrine therapy results from an increase in cellular plasticity that permits the emergence of a hormone independent tumor. The steroid coactivator protein SRC-1, through interactions with developmental proteins and other non-steroidal transcription factors, drives this tumor adaptability. In this discovery study we identified ADAM22, a non-protease member of the ADAM family of disintegrins, as a direct ER-independent target of SRC-1. We confirmed SRC-1 as a regulator of ADAM22 by molecular, cellular and in vivo studies. ADAM22 functioned in cellular migration and differentiation and its levels were increased endocrine resistant tumors compared to endocrine sensitive tumors in a mouse xenograft models of human breast cancer. Clinically ADAM22 was found to serve as an independent predictor of poor disease-free survival. Taken together, our findings suggest that SRC-1 switches steroid-responsive tumors to a steroid resistant state in which the SRC-1 target gene ADAM22 has a critical role, suggesting this molecule as a prognostic and therapeutic drug target that could help improve the treatment of endocrine-resistant breast cancer.
Introduction

Endocrine therapies such as tamoxifen are the treatment of choice for ER-positive tumours and, although most patients initially respond to treatment, many eventually relapse. At a molecular and cellular level increased tumour plasticity occurs in endocrine resistant breast cancer in comparison to endocrine sensitive tumours (1). Alterations in steroid receptor profile observed in clinical studies between primary and metastatic breast cancer, in particular with loss of progesterone receptor status, supports the phenomenon of tumour adaptability in endocrine resistant patients (2). These alterations are marked by increased signalling through growth factor networks and are driven, at least in part, through crosstalk between steroid and developmental pathways (3).

SRC-1, is central to the development of the endocrine resistant phenotype and is an independent predictor of disease free survival in tamoxifen-treated patients (4). As a coactivator protein, SRC-1 does not directly interact with the DNA, but rather partners with other transcription factors to regulate gene expression. Relative to endocrine sensitive tumours, increased SRC-1–estrogen receptor alpha (ER) interactions are observed in patients who are resistant to treatment (4). As the tumour progresses however, increasing evidence suggests that SRC-1 engages in transcriptional interactions independently of ER.

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 (5). These transcription factor interactions may represent one of the consequences of increased growth factor pathway signalling described in endocrine resistance. Work from this group and others has reported functional interactions between SRC-1 and the Ets family of transcription factors, Ets-2 and PEA3, and that this relationship is important in tumour progression and the development of metastasis (6-9). This occurs in part through SRC-1 mediated TWIST suppressing luminal markers such as E-cadherin and beta-catenin during epithelial-mesenchymal transition (7).

Using discovery tools we investigated the signalling network central to SRC-1 mediated endocrine resistance. We identified a new ER-independent target of SRC-1, ADAM22. This disintegrin plays a role in endocrine related tumour metastasis and opens up new possibilities as a therapeutic target.
Materials and Methods

Cell culture and treatments. Endocrine-sensitive MCF-7, metastatic MDA-MB-231 and endocrine insensitive SKBR3 were obtained from American Type Culture Collection (ATCC) and endocrine-resistant LY2 cells were a kind gift from R. Clarke, Georgetown, DC (10) Cells were grown as previously described (3). Letrozole resistant cells (LetR) were created by long-term treatment of over-expressing aromatase MCF7 cells to letrozole (Novartis) as previously described (3). Cells were maintained in steroid depleted medium for 72 h before treatment with hormones [estadiol (E2) $10^{-8}$ mol/L, 4-hydroxytamoxifen (4-OHT) $10^{-7}$ mol/L; Sigma Aldrich] over varying time periods. Primary cell cultures derived from patient tumours were cultured for 72h in prior to experiments as described (3). All cell lines were tested (Source Biosciences, life Sciences, Nottingham, UK) for authenticity in accordance with ATCC guidelines.

ChIP-sequencing and Microarray analysis. To identify SRC-1 target genes ChIP-seq was performed in endocrine resistant LY2 cells that were treated with vehicle or tamoxifen (4-OHT) for 45min and immunoprecipitated with anti-SRC-1 (Santa Cruz, sc-8993) antibody. To estimate the background an input control, omitting immunoprecipitation was performed. Sequencing (35 base read length) was carried out by Illumina using the Illumina Genome Analyzer system as described previously, with technical replicates (3). ChIPseq results are based on the analysis of a single sample, without biological replicates. To identify functionally relevant SRC-1 target genes, mRNA was hybridised to whole genome expression arrays. LY2 cells were transfected with siSRC-1 or scrambled siRNA. 24h post transfection, total RNA was extracted using RNeasy Kit (Qiagen) as per manufacturer’s instructions. Efficiency of knockdown was confirmed by qPCR. Microarray studies were performed on Affymetrix HGU133 Plus 2.0 arrays by Almac Diagnostics (Craigavon, BT63 5QD, Northern Ireland). DNAase treatment, amplification, fragmentation, labelling, hybridisation and array scanning were performed by Almac as per manufacturer’s instructions. Experiments were repeated in triplicate.

Bioinformatics analysis. Sequence reads were aligned to the human genome (hg19) using Bowtie (11) allowing for 2 mismatches and discarding reads with greater than a single mapping. ChIP peaks were identified using MACS with a cutoff of $p<1e-05$ (12). Peaks were filtered with a false discovery rate (FDR) of either $\leq 5\%$ or $\leq 1\%$. Analysis of microarray data was performed using the BioConductor (version 2.4) suite of R packages (13). Briefly, data were preprocessed using gcrma (14) and an evident batch effect was removed with the ComBat approach (15). Differential expression was assessed using linear
modelling and variance shrinkage through the empirical Bayes framework implemented in the limma package (16). After correction for multiple hypothesis testing (17), probesets with an adjusted p-value <0.05 were selected as differentially expressed. To identify genes containing an SRC-1 peak in the promoter and also displaying differential expression in response to SRC-1 status, promoters were defined as 5kbp of sequence upstream of RefSeq genes and found those which contained a SRC-1 peak. We selected the probe sets which were downregulated in the siSRC1 samples, both in the presence and absence of 4-OHT. Affymetrix probe set ID's were mapped to RefSeq genes and the intersection of these sets was determined.

**siRNA and plasmids.** Predesigned small interfering RNA directed against SRC-1 (Ambion AM16706), AIB1 (Ambion AM), MYB (Ambion AM)ADAM22 (Ambion 4390824), ER alpha (Ambion, 4392421) and a non-targeting siRNA (Ambion, AM4635) were used to knock down gene expression. The pcDNA3.1 plasmid containing full length SRC-1 was used for over-expression studies, empty pcDNA3.1 was used as a control plasmid.

**Knockout mouse studies.** Knockout (KO) and wild type (WT) mammary tumour cell lines were developed from primary tumours in SRC-1⁻/⁻/PyMT and WT/PyMT mice as described in (7).

**qPCR.** mRNA levels of ADAM22 and SRC-1 were measured by qPCR. RNA was extracted from 3 wild type cell lines (WT1, WT2 and WT3) derived from mammary tumours of WT/PyMT mice and three independent knockout cell lines KO1, KO2 and KO3 derived from mammary tumours of SRC-1⁻/⁻/PyMT mice. cDNA was prepared from 1µg of RNA and qPCR was carried out using matched universal Taqman probes and gene specific primers (Roche). Results are expressed as mean ± SD, n=3.

**Immunoblotting and co-immunoprecipitation**
Immunoblotting for MYB, SRC-1 and ADAM22 was carried out using rabbit anti-MYB (sc-517 Santa Cruz), anti-SRC-1 (sc-8993 Santa Cruz) and mouse anti-ADAM22 (H00053616-B01 MaxPab) respectively. Protein was immunoprecipitated with rabbit anti-SRC-1 (sc-8995; Santa Cruz, CA) and subsequently blotted with anti-MYB.
**Chromatin immunoprecipitation studies.** ChIP was carried out to confirm SRC-1, MYB and ER alpha recruitment to the ADAM22 promoter in either endocrine resistant LY2 or endocrine sensitive MCF7 cells as previously described (3). PCR was subsequently carried out with primers corresponding to the ADAM22 promoter (forward GGACCTCACAGTCACGAGGT, reverse TCAGTGCTGCATTGTGCTTC).

**3D cultures.** MCF7, LY2 and LetR cells were transfected with either siADAM22 or scrambled non-targeting siRNA. 24h post transfection cells were harvested, 6X10^3 cells from each cell line were mixed in 400μl of medium and 2% Matrigel (BD Biosciences) and subsequently seeded onto 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 permeabilised with phosphate buffered saline (PBS) containing 0.5% Triton X-100 for 10 min at 4°C. Cells were blocked in 10% goat serum (DAKO), 1% bovine serum albumin. Cells were stained with Phalloidin 594 (Molecular Probes) for 20 min at RT and DAPI for 5 min at RT. Slides were mounted (DAKO) and examined by confocal microscopy.

**Migration assay.** Migration assays were carried out as previously described (7).

**Xenograft studies.** Following ethical approval, 8 week old female Balb-C SCID mice (Harlan) were implanted with 17-B Estradiol pellets from Innovative Research of America (0.25mg/pellet, 60 day release). One week later 5X10^6 MCF-7 or LY-2 cells mixed with 50% matrigel (BD biosciences) were implanted by intradermal injection on the rear dorsam. When the tumours reached 100mm^3, the mice were randomly assigned to either treatment (tamoxifen 5mg/pellet 60 day release) or control group (placebo pellet). Tumours were measured weekly until they had quadrupled in size at which point the animals were culled and tumours were stored in RNA later for subsequent Western blot analysis. Statistical analysis was performed by calculating the growth rate of each individual tumour and treatment condition, and analysing them using an unpaired student t-test.

**Tissue microarray / Statistics**

Patient breast tumour samples were collected the tissue microarray (TMA) constructed and data recorded as previously described (18). TMA was immunostained using mouse anti ADAM22. Associations of ADAM22 with clinicopathologic variables and SRC-1 were examined using Fisher’s exact test. Kaplan Meier graphs were used as estimate of disease-free survival. Statistical analyses were
carried out using Minitab software (Pennsylvania, USA) and P values <0.05 were considered significant. Multivariate analysis was performed using STATA 10 data analysis software (Stata Corp. LP, Texas, USA) and was carried out using Cox's proportional hazard model, using the Breslow method for ties. Survival times between groups were compared using the Wilcoxon test adjusted for censored values.

**Accession codes.** All ChiP-seq and expression array data are available from the Gene Expression Omnibus (GEO) database under series entry codes GSE28987 and GSE28645 respectively.

**Results**

**Characterization of SRC-1 target genes in endocrine resistant breast cancer.** To address how SRC-1 mediates tumour adaptability and disease progression, we mapped SRC-1 transcriptional effects in endocrine resistant breast cancer. To this end we combined ChIP-seq/expression array analysis, molecular, cellular and translational studies to define new SRC-1 targets central to the resistant phenotype. SRC-1-ChIP sequencing was performed in endocrine resistant (LY2) cells. Treatment with 4-hydroxytamoxifen (4-OHT) significantly increased the number of ChIP-enriched intervals identified (Fig. 1A). 41% of peaks in the 4-OHT treated sample were close to the transcriptional start site (TSS), in either the promoter, first exon or upstream of the promoter (Fig. 1B). Furthermore a significant SRC-1 ChIP enrichment was observed at the TSS in comparison to the transcriptional termination site (Fig. 1C). As a defined ER coactivator an overlap between ER binding sites and those identified for SRC-1 would be expected. In support of this, 4-OHT induced SRC-1 recruitment to the classic ER target genes, pS2, XBP1 and GREB1 in the endocrine resistant cells (Supplementary Fig. S1A). Recently, examining SRC-3 ChIP DNA, Lanz *et al* reported 28% of SRC-3 peaks (FDR <1) in estrogen treated MCF7 cells have their sequence centres within 1kb of ER binding sites (19). Here, using a modified version of the p53 scan (20), we found 43% of high confidence SRC-1 peaks (FDR<1) in 4-OHT-treated LY2 cells, contained an ERE binding motif within the peak (Fig. 1D). Though this analysis is restricted to ERE motifs rather than total ER binding, it raises the possibility that, in endocrine resistance, SRC-1 can interact with transcription factors to drive transcription, independently of ER.

We carried out microarray experiments on LY2 cells transiently transfected with scrambled siRNA or SRC-1 siRNA, untreated or treated with 4-OHT (Supplementary Fig. S1B) to assess SRC-1-dependent gene expression. Combining the ChIP-seq and expression array datasets, a total of 2,065 genes were significantly downregulated (p<0.05) following SRC-1 knockdown, and harboured a high
confidence SRC-1 peak in the promoter region (Fig. 1E). We analysed this list for novel metastatic oncogenes that represent potential druggable targets. The metalloproteinase ADAM22 was selected for functional validation through a series of molecular, in vivo and translational studies. Interestingly, enrichment of SRC-1 at the ADAM22 promoter was observed in the LY2 cells on treatment with 4-OHT (Fig. 1F). Recruitment of SRC-1 to the ADAM22 promoter was confirmed using standard ChIP analysis (Fig. 1G).

**SRC-1 regulates ADAM22 in endocrine resistant breast cancer.** We hypothesised that ADAM22 may be an effector of SRC-1 mediated endocrine resistant disease progression. Knockdown of SRC-1 in LY2 cells reduced ADAM22 protein expression and conversely, forced expression of SRC-1 in the sensitive MCF7 cells increased ADAM22 expression (Fig. 2A). Knockdown of the p160, AIB1 had minor effects on ADAM22 expression in endocrine resistant cells (Fig. 2A). In primary breast tumours, a significant association between transcript levels of SRC-1 and ADAM22 was observed (Fig. 2B). Furthermore, cells derived from mammary tumours of the SRC-1-/-/PyMT mouse lacked ADAM22 transcript whereas high expression was evident in the wild type PyMT mouse (Fig. 2C). Having established a role for SRC-1 in the regulation of ADAM22 in breast cancer we addressed the question of whether or not this is ER dependent.

From bioinformatic studies, no ERE was observed in the promoter region of ADAM22. In endocrine resistant LY2 cells, analysing a 290 base pair fragment of the ADAM22 promoter encompassing the SRC-1 peak, no recruitment of ER was observed, in the presence of either estrogen or 4-OHT (Fig. 2D). Moreover, knockdown of ER in LY2 cells did not alter ADAM22 expression (Fig. 2E). Further analysis of the promoter revealed a binding motif for the transcription factor MYB close to the SRC-1 peak (Fig. 2F). MYB expression has been reported in 64% of primary breast cancers and is associated with ER positive tumours. Co-immunoprecipitation established that 4-OHT could drive MYB-SRC-1 interactions in endocrine resistant tumours (Fig. 2G). ChIP analysis indicated that neither SRC-1 nor MYB are recruited to the ADAM22 promoter in the endocrine sensitive MCF7 cells. In endocrine resistant cells however, both SRC-1 and MYB were recruited to the promoter in the presence of estrogen and in particular 4-OHT (Fig. 2H). These findings support the hypothesis that, as disease progression occurs in endocrine resistant breast cancer, cellular adaptability can enable SRC-1 to regulate genes independently of ER.
ADAM22 promotes tumour progression in endocrine resistant breast cancer. ADAM22 has a defined role as a postsynaptic neuro-receptor but recent work examining its structure intimates a possible role for the protein in cell adhesion, spreading and migration (21-23). These processes are critical to the metastatic phenotype so we explored the role of ADAM22 in cell migration in endocrine resistant breast cancer. We profiled a range of endocrine sensitive, insensitive and resistant cells along with metastatic breast cancer cells for expression of SRC-1 and ADAM22 (Supplementary Fig. S2). Low levels of both ADAM22 and SRC-1 were found in the endocrine sensitive and insensitive cells, while stronger expression of both was found in the resistant and metastatic cells. Furthermore, a marked increase in cell migration and de-differentiation was observed in both the 4-OHT resistant LY2 and the letrozole resistant LetR cells (Fig. 3A and B). Though MYB alone did not affect cell migration (Supplementary Fig. S3A), knockdown of ADAM22 with siRNA, significantly reduced cell migration and restored differentiation in the endocrine resistant cells (Supplementary Fig. S3B, Fig. 3A and B) and in the ER negative MDA-MB-231 (Supplementary Fig. S3C). Moreover, ADAM22 knockdown reverses the pro-migratory effects of 4-OHT observed in the endocrine resistant cells (Fig. 3C). Additionally, in xenograft studies, expression of ADAM22 was absent from both the 4-OHT treated and untreated sensitive tumours and from the untreated resistant tumours. Treatment with 4-OHT however, not only increased tumour volume in the resistant tumours, but also induced ADAM22 expression (Fig. 3D and E). The neuronal protein, LGI1 serves as a specific extracellular ligand for ADAM22 (24). We found that treatment with recombinant LGI1 reduced cellular migration in endocrine resistant cells in a similar manner to that observed with knockdown of ADAM22 (Fig. 3F). These data raise the distinct possibility of ADAM22 as a viable drug target for the treatment of endocrine resistant breast cancer.

ADAM22 predicts poor disease free survival in breast cancer patients. Previous molecular, in vivo and clinical studies from this group and others have established SRC-1 as a key mediator of disease recurrence in endocrine treated breast cancer (4,8,9). To determine the significance of the putative SRC-1 target ADAM22 in mediating disease progression we examined its expression levels in a large cohort of breast cancer patients and compared this with classic clinicopathological parameters. ADAM22 was found to localise to the cell membrane of the tumour epithelial cells with strong cytoplasmic staining also observed (Fig. 4A). In this cohort, expression of ADAM22 was found to significantly associate with SRC-1 (p=0.002) and also with disease recurrence (p<0.001) (Table 1). In line with our bioinformatic, molecular and in vivo studies, no association between ADAM22 expression and ER was
observed (Table 1). Kaplan Meier estimates of disease free survival indicated that the absence of ADAM22 is a strong predictor of disease free survival in breast cancer (p<0.0001) (Fig. 4B). Furthermore in multivariate analysis employing standard clinicopathological parameters as well as SRC-1 and ADAM22, both SRC-1 and ADAM22 were found to be significant independent predictors of disease recurrence (odds ratio; 2.18 and 2.4 respectively) (Table 2). These clinical ex vivo data firmly support our molecular observations that SRC-1 and its target ADAM22 can drive tumour progression in endocrine resistant breast cancer (Figure 5).
Discussion

Endocrine tumours can adapt to overcome targeted therapy. There is now substantial evidence that the coactivator SRC-1 is central to this process. Though other members of the p160 family have been investigated in endocrine related cancer, it is only SRC-1 that has been associated with the metastatic phenotype (7). SRC-1, a member of the p160 family of steroid coactivator proteins, is a master transcriptional regulator. SRC-1 was recently identified as the gene with the strongest selective pressure among ethnic populations analysed by the International HapMap Project (25), illustrating the importance of the coactivator in human evolutionary adaptation. In this study we use discovery tools to identify new SRC-1 target genes. Our intention was to uncover mechanisms of endocrine related tumour metastasis and identify biomarkers and drug targets in this class.

ADAM22 was identified, and through a series of molecular and in vivo studies, confirmed as an SRC-1 target in endocrine resistant breast cancer. ADAMs are multidomain transmembrane glycoproteins that have a diverse role in physiology and disease, with several members being targets for cancer therapy (21). Although most members of the ADAM family are active zinc metalloproteinases, 8 (including ADAM22) of 21 ADAMs lack functional metalloproteinase domains and are implicated in adhesion rather than membrane protein ectodomain shedding (26,27). ADAM22 acts as a receptor on the surface of the postsynaptic neuron to regulate signal transmission, but a function for ADAM22 outside the nervous system has not been described to date (24).

Despite its well documented role as an ER coactivator protein, evidence from ChIP-seq, bioinformatic and molecular studies reported here suggest that SRC-1 can regulate ADAM22 independently of ER. A steroid receptor-independent role for SRC-1 is fast emerging. Interactions between SRC-1 and Ets family proteins have been implicated in tumour progression and the development of breast cancer metastasis (7-9). Furthermore, in the ER negative PyMT SRC-1 knockout mouse model, although SRC-1 is not required for mammary tumour initiation, it is essential for the development of metastatic disease (28). Here, examination of the ADAM22 promoter revealed a binding motif for the transcription factor MYB. MYB is a proto-oncogene, though reports of its actions in breast cancer are limited, a role for MYB in steroid regulation has been suggested in several studies (reviewed in 29). Although MYB alone had no significant effect on cell migration, results of our molecular experiments suggest that SRC-1 can regulate ADAM22, at least in part, through interaction with MYB, specifically in endocrine resistant cells.
LGI1 acts as a specific extracellular ligand for the neuro-receptor ADAM22. It functions as a tumour suppressor of glioblastoma and neuroblastoma and has recently been shown to impair proliferation and survival in HeLa cells (30-32). Both LGI1 and ADAM22 are genetically linked to epilepsy and the ligand/receptor complex has been suggested as a therapeutic target for synaptic disorders (24). Treatment of breast cancer cells resistant to either 4-OHT or letrozole, with recombinant LGI1, reduced cell migration. The anti-migratory action of LGI1 observed here is consistent with the suppression of cell invasion observed in glioma cells (30). LGI1 may function by inhibiting the extracellular disintegrin domain of ADAM22. Furthermore, in a significant cohort of breast cancer patients, ADAM22, along with SRC-1, was found to be an independent predictor of poor disease free survival. Taken together these studies provide strong evidence of ADAM22 as a mediator of metastasis and as a potential drug target for the treatment of endocrine related metastatic disease.

SRC proteins are master regulators (33). SRC-1 through interactions with developmental proteins can increase cellular plasticity and enable tumours to adapt to endocrine therapy (3). Discovery studies described here have uncovered a steroid independent SRC-1 mediated network in endocrine resistant breast cancer, which has led to the identification of a new SRC-1 target, ADAM22. There is currently no effective treatment for patients with endocrine related tumour metastasis. ADAM22 represents a rational new therapeutic target with a robust companion biomarker for the treatment of endocrine resistant tumours.

Accession codes. All ChiP-seq and expression array data are available from the Gene Expression Omnibus (GEO) database under series entry codes GSE28987 and GSE28645 respectively.

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References


Table 1
Associations of ADAM22 in breast cancer patient TMA with clinicopathologic variables and SRC-1 using Fisher’s exact test

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<th>Age</th>
<th>ER+ve</th>
<th>HER2+ve</th>
<th>T-Stage 3/4</th>
<th>Nodal Disease</th>
<th>High Grade</th>
<th>Adjuvant Tx</th>
<th>SRC-1+ve</th>
<th>Disease Recurrence</th>
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<td>22%</td>
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<td>49%</td>
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<td>18%</td>
<td>57%</td>
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<td>20%</td>
<td>61%</td>
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Table 2
Odds ratio for disease free survival for clinicopathologic variables, SRC-1 and ADAM22 in breast cancer patient TMA.

<table>
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<td>0.335</td>
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<td>2.40</td>
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Figure 1
Figure 2
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Figure 5
Global characterization of the SRC-1 transcriptome identifies ADAM22 as an ER-independent mediator of endocrine resistant breast cancer

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