Aromatase inhibitor specific metastasis is driven by the steroid receptor coactivator SRC-1

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Aromatase inhibitor specific metastasis is driven by the steroid receptor coactivator SRC-1

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Abbreviations

4-OHT	4-hydroxy-tamoxifen
AD	Activation domain
AF-1 and AF-2	Activation function domains
AI	Aromatase inhibitor
AIB1	Amplified-in-breast cancer 1
Andro	Androstenedione
AP1	Activating protein
AR	Androgen receptor
ARNO	Arimidex-Novaldex
Aro	Aromatase overexpressing MCF7 cells
ATAC	Arimidex, Tamoxifen alone or in combination
Bcl-2	B-cell lymphoma 2
BCA	Bichinchonic Assay
BIG	Breast International Group
bp	Base pairs
BRCA	Breast cancer susceptibility gene
BSA	Bovine serum albumin
CARM1	Coactivator-associated arginine methyltransferase 1
CBP	CREB-binding protein
cDNA	Complementary DNA
CDS-FCS	Charcoal dextran-stripped fetal calf serum
ChIP	Chromatin Immunoprecipitation
CK	Cytokeratin
Co-IP	Co-Immunoprecipitation
CYP	cytochrome protein
DAB	3,3-diaminobenzidine tetrahydrochloride
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DCIS	Ductal carcinoma *in situ*
dH_2O	Distilled water
DFS	Disease-free survival
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
E1	Estrone
E2	17β-estradiol

EBS	Ets binding site
ECL	Enhanced chemiluminescent reagent
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
Erb	Erythroblastic Leukemia Viral Oncogene
ERE	Estrogen response elements
ERK	Extracellular signal-regulated kinase
Ets	E26 transformation-specific sequence
G-418	Geneticin sulphate
GF	Growth factor
GPR30	G-protein-coupled receptor-30
GTP	Guanosine triphosphate
HAT	Histone acetyltransferase
HER2	Human epidermal growth factor receptor 2
HMT	Histone methyltransferase
HRP	Horseradish peroxidase
ICCG	International Collaborative Cancer Group
IF	Immunofluorescence
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin
IMS	Industrial methylated spirits
ITA	Italian Tamoxifen Anastrozole trial
kD	Kilodalton
LBD	Ligand binding domain
LetR	Letrozole resistant Aro cells
LH	Luteinising hormone
LHRH	Luteinising hormone releasing hormone
LiCl	Lithium Chloride
LTED	Long-term estrogen deprivation
Lys	Lysine
MAPK	Mitogen-activated protein kinase
MCF-7_{Ca}	Aromatase overexpressing MCF7 cells
MEM	Minimal essential medium
$MgCl_2$	Magnesium chloride
MMP	Matrix metalloproteinase

mTOR	Mammalian target of rapamycin
NCoA	Nuclear coactivator
NCoR	Nuclear co-repressor
NR	Nuclear receptor
NSABP	National Surgical Adjuvant Breast and Bowel Project
OS	Overall survival
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol-3 kinase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative PCR
RID	Receptor interaction domain
RTK	Receptor tyrosine kinases
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
SERM	Selective estrogen receptor modulator
SRC	Steroid receptor coactivators
STI	Signal transduction inhibitors
SUMO	Small ubuiqitin-like modifier
TAE	Tris acetate-EDTA
TAM	Tamoxifen
Taq	Taq polymerase (*Thermophilus aquaticus*)
TBS	Tris buffered saline
TBS-T	Tris buffered saline plus 1% Tween
TEAM	Tamoxifen Exemestane Adjuvant Multinational study
TF	Transcription factor
TKI	Tyrosine kinase inhibitors
TMA	Tissue microarray
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume

Summary

Introduction

Breast cancer is the second most common cancer in women worldwide. Approximately 80% of breast cancer patients are postmenopausal women and about two thirds of those are diagnosed with hormone receptor positive breast cancer. Therefore, endocrine therapy to block ER activity and signaling is the most successful and most commonly used therapy. Aromatase Inhibitors (AIs) are currently one of the most promising treatments for estrogen-receptor positive breast cancer in postmenopausal women. Even though many women initially respond to the treatment, approximately 40% will acquire resistance and relapse within 5 year. The mechanisms involved in the development of resistance to AIs however are poorly understood as long-term follow up is only now becoming available.

It is though that the development of resistance and resulting tumour recurrence is due, at least in part, to cellular plasticity leading to a shift in the phenotype of the tumour cell from steroid dependence to steroid independence / growth factor dependence. Consequently, the resistant cancer cells may utilize steroid receptor-independent mechanisms to drive tumour progression.

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 tumour recurrence. 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 tumour progression and the development of metastasis in tamoxifen treated patients.

Hypothesis

The hypothesis of this thesis is to investigate if the steroid receptor coactivator SRC-1 plays an important role in advancing the metastatic phenotype in Aromatase Inhibitor resistance. It will be investigated if such a role is dependent on or independent of estrogen receptor signaling.

Results

The development of AI resistance in cell lines gave rise to a phenotype displaying an increase in motility and invasiveness along with a loss of organisation. Both the resistant cell model and AI resistant tumour samples expressed high levels of the steroid receptor coactivator SRC-1. We found that SRC-1 interacts with the transcription factor Ets to regulate Myc and MMP9 expression and that SRC-1 was required for the aggressive AI resistant phenotype. In patients treated with a first-line AI (n=89), we found that hormone receptor switching between the primary tumour and the resistant metastasis was a common feature of disease recurrence. A significant coassociation between SRC-1 and Ets2 in the nucleus of the recurrent tissue compared with the matched primary tumour was also observed ($p=0.0004$, $n=3$). We also observed an increase in Myc and MMP9 protein expression in the recurrent tissue in comparison to the matched primary tumour.

Conclusion

SRC-1 plays a key functional role in the mediation of an AI resistant aggressive phenotype by utilising Ets to regulate Myc and MMP9. Targeting downstream proteins of the SRC-1 signaling pathway may offer clinical potential to treat tumour recurrence.

Chapter 1

General Introduction

1.1 Breast Cancer

Breast cancer is the second most common cancer in women worldwide after skin cancer (Parkin DM *et al.*, 2002). Though treatment has dramatically improved over the last years, it still is the leading cause of cancer-related death for women. In a recent survey, Irish women ranked fourth highest in European countries for both incidence and mortality of breast cancer (National Cancer Registry Ireland, 2011; Figure 1.1).

Approximately 80% of breast cancers occur in postmenopausal women and out of these about two thirds are diagnosed with hormone receptor positive breast cancer (Fabian CJ and Kimler BF, 2005). Breast cancer development and progression are highly influenced by both Estrogen receptor (ER) and growth factor receptor signaling. In the past decades multiple drugs have been developed to inhibit tumour growth and invasion. However, endocrine therapy to block ER activity and signaling is still the most successful and most commonly used therapy in the treatment of ER positive breast cancer.

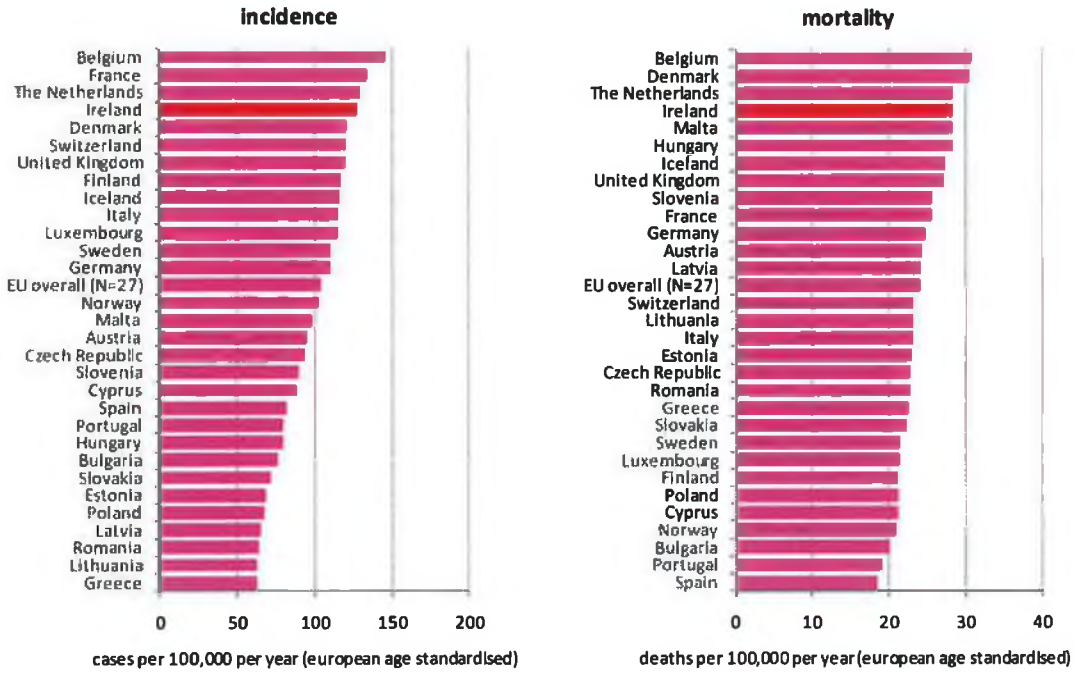


Figure 1.1: Estimated breast cancer incidence and mortality in Europe 2008. *Adapted from NCRI Annual Report (National Cancer Registry Ireland, 2011).*

1.1.1 Breast Cancer incidence and mortality in Ireland

Except for non-melanoma skin cancer, breast cancer is the most common malignancy in Irish women (Figure 1.2). An annual average of 2692 breast cancer cases (20 of which were diagnosed in men) were registered during the three year period 2007-2009 (National Cancer Registry Ireland, 2011), representing an increase of 13% from the annual average over the previous three year period 2005-2007 (National Cancer Registry Ireland, 2009). It is likely that this increase is linked to the development of advanced and organised screening methods in recent years.

Interestingly, the five-year relative survival of breast cancer patients has improved by over 10% between the 1994-1997 and 2003-2007 periods (Figure 1.3), probably because, due to an improvement in detection techniques, earlier diagnosis as well as better treatment is possible.

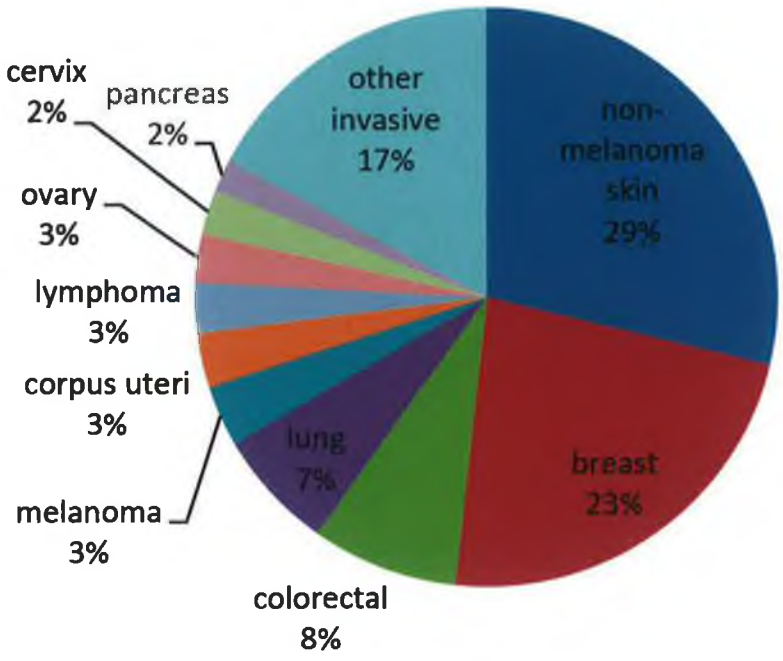


Figure 1.2: Relative frequency of the main invasive cancers diagnosed between 2007 and 2009 in Ireland. *Adapted from the NCRI Annual Report (National Cancer Registry Ireland, 2011).*

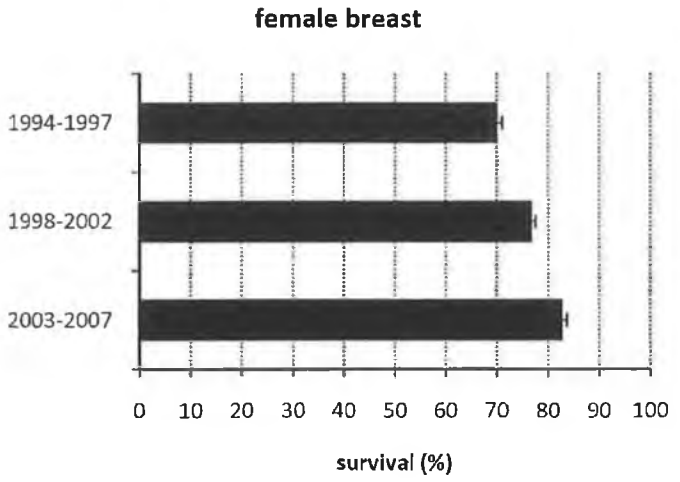


Figure 1.3: Five-year relative survival for cancers diagnosed in Ireland during the periods 1994-1997, 1998-2002 and 2003-2007. *Adapted from the NCRI Annual Report (National Cancer Registry Ireland, 2011).*

1.1.2 Risk factors

Identifying risk factors for breast cancer is an important diagnostic tool for at least two reasons: patients thought to be at risk can be monitored more closely for earlier diagnosis and a better prognosis; also, understanding the pathophysiology of the risk factor's association with breast cancer development may lead to novel ways of prevention and/or the development of more effective therapies (Gradishar and Morrow, 1996). Several risk factors have been uncovered over the last years, the most strongly associated ones being gender and age (Table 1.1).

Breast cancer is fairly rare in men accounting for less than 1% of all breast cancers cases (Fentiman IS *et al.*, 2006; NCRI, 2011). In women, breast cancer incidence increases dramatically with age. This might be due to an increase in endogenous estrogen over time. The risk of breast cancer increases with cumulative number of ovarian cycles and is decreased by 15% for each year of delay in age of menarche but increases by 3% for each year of delay in age at menopause (Colditz GA *et al.*, 2006).

Table 1.1: Factors that increase the risk for breast cancer in women. *Adapted from Breast Cancer Fact & Figures 2011-2012 (American Cancer Society, 2011).*

Relative Risk	Factor
>4.0	• Age (65+ vs. <65 years, although risk increases across all ages until age 80) • Biopsy-confirmed atypical hyperplasia • Certain inherited genetic mutations for breast cancer (BRCA1 and/or BRCA2) • Mammographically dense breasts • Personal history of breast cancer
2.1-4.0	• High endogenous estrogen or testosterone levels • High bone density (postmenopausal) • High-dose radiation to chest • Two first-degree relatives with breast cancer
1.1-2.0	• Alcohol consumption • Ashkenazi Jewish heritage • Early menarche (<12 years) • Height (tall) • High socioeconomic status • Late age at first full-term pregnancy (>30 years) • Late menopause (>55 years) • Never breastfed a child • No full-term pregnancies • Obesity (postmenopausal)/adult weight gain • One first-degree relative with breast cancer • Personal history of endometrium, ovary, or colon cancer • Recent and long-term use of menopausal hormone therapy containing estrogen and progestin • Recent oral contraceptive use

Pregnancy increases the short-term risk of breast cancer probably due to heightened free estrogen levels during the first trimester. However, pregnancy seems to have a long-term beneficial effect due to high levels of prolactin and a decrease in sex-hormone binding globulin. Also, lactation has proved to lower breast cancer risk due to the suppression of ovulatory function caused by nursing (Collaborative Group on Hormonal Factors in Breast Cancer, 2002).

The administration of exogenous hormones has also been shown to associate with breast cancer risk. In current and recent users of oral contraceptives the risk was found to increase by 15-25% compared to never-users (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). Interestingly though, this risk reduces to that of never-users 10 years after stopping oral contraceptive use (La Vecchia C *et al.*, 2004).

Postmenopausal women on recent and long-term hormone replacement therapy (HRT) containing estrogen and progestin are also at higher risk of developing breast cancer (Colditz GA *et al.*, 1995).

Family history is associated with a 2-3-fold higher risk and this risk increases with the number of affected first degree relatives (Collaborative Group on Hormonal Factors in Breast Cancer, 2001). In this group of women low-penetrance genes associated with hormonal metabolism and regulation, DNA damage and repair are thought to be involved in the development of breast cancer. There is also evidence that polymorphisms in genes that are involved in the biosynthesis of estradiol, especially the CYP19 gene, can increase the risk for breast cancer (Haiman CA *et al.*, 2000). Mutations of several high-penetrance genes, such as BRCA1, BRCA2 and p53, increase the cumulative lifetime risk of breast cancer in carriers of these genes by over 50%. However, they are rare in most populations and explain only a small fraction of total cases (2-5%) (WHO International Agency for Research on Cancer, 2008).

Poor lifestyle choices such as lack of physical activity, high alcohol consumption and bad nutrition are also believed to increase the risk for breast cancer (McTiernan A, 2003). Postmenopausal obesity is linked to high breast cancer risk, which may be due to the increase in estrogen exposure due to high hormone levels in the adipose tissue (Siiteri PK, 1987) (Table 1.1).

1.1.3 Hormone receptor status in breast cancer

Hormone receptor (HR) status is a crucial parameter in determining the prognosis and treatment of breast cancer. HR-positive (HR+) breast cancer can be defined as estrogen receptor-positive (ER+), progesterone receptor-positive (PR+) or both (Rugo HS, 2008). A patient that is classed HR+ has hormone-sensitive breast cancer and thus responds to endocrine therapies that block or interfere with the function of estrogen or progesterone. The first evidence of effective endocrine therapy in the treatment of breast cancer was the response of metastatic disease to ovarian suppression (Rugo HS, 2008). Only about one third of patients responded to the therapy, suggesting that better identification of responsive breast cancers and more specific therapies were needed

(Jensen EV and Jorndan VC, 2003). The discovery that a receptor for estrogen was found in estrogen target tissue but not in nontarget tissue raised the question of whether these concepts translated to the clinic to predict hormone responsiveness of the tumour. Jensen *et al.* reasoned that if the ER was necessary for estrogen-induced proliferation, the detection of ER in a tumour specimen might be highly useful in determining the prognosis and treatment. In 1971, Jensen *et al.* reported that ER-rich breast cancers were more likely to respond to endocrine therapy than ER-poor breast cancers (Jensen EV *et al.*, 1971). Today, hormone receptor status is analysed by immunohistochemical (IHC) staining, either on paraffin-embedded or frozen tumour tissue (Allred DC *et al.*, 1990; Harvey JM *et al.*, 1999). Several scoring systems are available to assess the degree of hormone positivity but the best validated one to date is the Allred score (Rugo HS, 2008). Still, due to the simplicity of interpretation, the greater part of laboratories report results as positive or negative and central retesting has shown that a small percentage of results from IHC may in fact be false negatives (Layfield LJ *et al.*, 2003). A definite connection between the relative degree of hormone receptor positivity and response to endocrine therapy has yet to be drawn, even though it has become evident that minimally HR+ tumours have a lower response rate (Allred DC *et al.*, 1998).

1.1.4 Molecular classification of breast cancer

The advent of microarray-based gene expression profiling had a significant effect on our understanding of breast cancer. It is no longer perceived as one disease with altering histological and clinical behaviour but rather as a heterogeneous group of diseases consisting of molecular very distinct entities. This heterogeneity cannot be explained by clinical parameters (tumour size, histological grade, etc.) or biomarkers (ER, PR and HER2 status) alone. The classification by these traditional parameters has been joined by rankings based on gene expression (Eroles P *et al.*, 2011). The seminal class-discovery studies undertaken by Perou *et al.* (2000) and Sorlie *et al.* (2001) revealed that ER+ and ER- breast cancers are distinct diseases in molecular terms. Cluster analysis of intrinsic genes initially revealed the existence of four subtypes – luminal, HER2-enriched, basal-

like and normal breast-like – but lately the luminal subtype has been divided further into luminal A and luminal B. Overall, up to seven intrinsic subtypes have been characterised so far (Figure 1.4) and it is likely that the number of subtypes is going to increase over the next years.

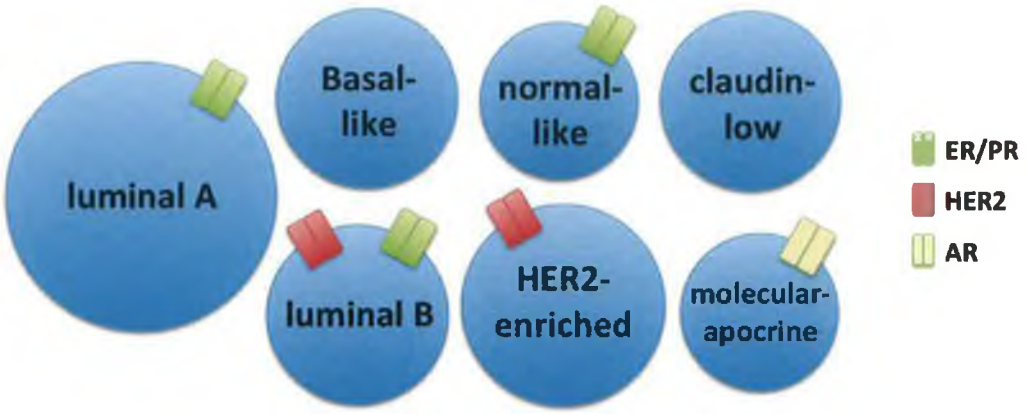


Figure 1.4: Molecular subtypes of breast cancer. *Simplified model of the seven subtypes depicts receptor expression (ER = estrogen receptor, PR = progesterone receptor, HER2 = human epidermal growth factor receptor, AR = androgen receptor) as well as the occurence of the subtypes in breast cancer patients (size of the circles).*

1.1.4.1 Luminal A

The most common subtype is luminal A and makes up 50-60% of all breast cancer. It is characterised by the expression of genes in the luminal epithelial cells lining mammary ducts as well as a low expression of genes that are involved in cell proliferation (Perou CM *et al.*, 2000; Sorlie T *et al.*, 2001). All lobular carcinomas *in situ* as well as most infiltrating lobular carcinomas belong to this class due to their molecular profile. The luminal A immunohistochemistry profile is characterised by the expression of ER, PR, Bcl-2 and cytokeratin CK8/18, an absence of HER2, a low rate of proliferation measured by Ki67 and a low histological grade (Eroles P *et al.*, 2011). Patients diagnosed with this subtype of cancer have a good prognosis; the relapse rate of 27.8% is significantly lower than for any other subtype (Kennecke H *et al.*, 2010) and survival from the time of relapse is also longer with a median of 2.2 years. In luminal A breast cancer, metastases

predominantly occur in the bone (47%), less frequently in the liver (17.7%) and rarely in the lung (8.5%) (Sihto H *et al.*, 2011). The treatment of this subtype is based on selective estrogen receptor modulators, pure selective regulators of ER in premenopausal women as well as aromatase inhibitors in postmenopausal women (Guarneri V and Conte P, 2009).

1.1.4.2 Luminal B

10 to 20% of all breast cancers are classed luminal B. They exhibit a more aggressive phenotype, higher histological grade and proliferative index than luminal A. The pattern of distant metastases also differs: whereas the bone is still the most common site of recurrence (34.9%), this subtype has a much higher recurrence rate in the lung (16.3%) (Sihto H *et al.*, 2011). Patients with luminal B breast cancer have worse prognosis and the survival from time of relapse is lower (1.6 years) (Kennecke H *et al.*, 2010). Like luminal A, luminal B expresses ER, but there are several biological differences between the two subtypes: luminal B breast cancers exhibit an increase in proliferative genes and often express EGFR and HER2. Various different gene expression microarray platforms classify luminal B tumours ER+ with poor prognosis (Loi S *et al.*, 2007). Immunohistochemically, there have been attempts to differentiate luminal A and B using Ki67 protein expression as a potential marker (Cheang MC *et al.*, 2009). In comparison to the ER+/HER2- Ki67 low profile in luminal A breast cancers, luminal B subtypes exhibit an ER+/HER2- Ki67 high or ER+/HER2+ profile. However, a minority of up to 6% of luminal B tumours are clinically ER-/HER2-. The treatment of this subtype is currently challenging as the exact mechanisms that lead to their survival, proliferation and metastasation have yet to be revealed. Still, a number of inhibitors of the PI3K pathway are currently being tested with a particular focus on the treatment of the luminal B subtype (Eroles P *et al.*, 2011).

1.1.4.3 Basal-like

The basal-like subtype represents 10-20% of all breast cancers and expresses genes usually present in normal breast myoepithelial cells, explaining the name of this subtype.

They also express genes characteristic of luminal epithelium but at levels much lower than those of luminal breast cancers (Eroles P *et al.*, 2011). Metastatic relapse is very aggressive with a majority of metastasis in visceral organs such as lung (20.8%), brain (9.5%) and non-regional lymph nodes (11.9%) (Sihto H *et al.*, 2011).

The most prominent feature of this type of breast cancer is the absence of the three key receptors: ER, PR and HER2. Therefore, this subtype is more commonly known as triple negative in clinical practice, even though these two are not equivalent terms. A discordance of 30% between those two groups has been observed (Kreike B *et al.*, 2007). Eventually, five markers have been identified to classifiy this subtype: ER, PR, HER2, EGFR and CK5/6 (Nielsen TO *et al.*, 2004).

1.1.4.4 HER2-enriched

15-20% of all breast cancers fall into the HER2-positive or –enriched subtype. This subtype is characterised by a high expression HER2 and other genes associated with the HER2 pathway and/or the HER2 amplicon located on the 17q12 chromosome. It also exhibits an over-expression of genes involved in cellular proliferation (Eroles P *et al.*, 2011). Tumours of this subtype are highly proliferative, 75% have a high histological grade and more than 40% have p53 mutations. The IHC profile does not absolutely correspond with the intrinsic subtype, as 70% of tumours that have been classed HER2+ by microarray do not overexpress the protein by IHC. Equally, not all tumours with an amplification or overexpression of HER2 are included in the cluster of HER2+ by microarray analysis (Parker JS *et al.*, 2009). As mentioned before, a fraction of tumours that are clinically ER+/HER2+ are classified molecularly as luminal B.

HER2 enriched breast cancer metastasis occur in the liver (27.1%) more frequently than in any other molecular subtype, yet, they also give rise to bone (29.2%) and lung (22.9%) metastasis quite frequently (Sihto H *et al.*, 2011).

Patients diagnosed with the HER2-enriched subtype have poor prognosis, even though anti-HER2 treatment has dramatically improved over the last decade (Piccart-Gebhart MJ *et al.*, 2005; Slamon DJ *et al.*, 2001).

1.1.4.5 Normal breast-like

Only about 6-10% of all breast tumours fall into this molecular subtype. This subtype of breast cancer is rather poorly characterised and consistently cluster together with samples of fibroadenomas and normal breast, hence the name (Fan C *et al.*, 2006). These tumours are mostly small and tend to have good prognosis and are more common amongst postmenopausal women (Calza S *et al.*, 2006). Its clinical significance however has yet to be determined (Reis-Filho JS *et al.*, 2006). Some researchers question whether these tumours are a distinct subtype or if this subtype might rather be an artefact of expression profiling due to a disproportionally high content of stromal cells in the sample (Weigelt B *et al.*, 2010).

1.1.4.6 Claudin-low

Only 12-14% of breast carcinomas are claudin-low, making it a relatively rare subset of tumours. This subtype was the last one to be identified in 2007 and is characterised by a low expression of genes involved in tight junctions and intercellular adhesion, namely claudin-3, -4 and -7 as well as E-cadherin. This subtype is similar to basal-like tumours in that it exhibits low expression of HER2 and luminal gene cluster. However, this group differs from the basal-like subtype in that it overexpresses a set of 40 genes related to immune response (Prat A *et al.*, 2010).

Even though the claudin-low subtype exhibits a low expression of genes involved in cell proliferation, patients diagnosed with this specific subtype have a poor prognosis and are insufficiently responsive to neoadjuvant chemotherapy (Prat A *et al.*, 2011). Claudin-low breast cancer cells overexpress a subset of genes linked to mesenchymal differentiation and EMT, which is associated with acquisition of a cancer stem cell phenotype (Hennessy BT *et al.*, 2009).

As with basal-like tumours, claudin-low tumours are triple negative by IHC, however, the concordance is not 100% and about 20% of tumours are positive for hormone receptors (Prat A *et al.*, 2011).

1.1.4.7 Molecular apocrine

8-12% of breast cancers are classed molecular apocrine (Farmer P *et al.*, 2005). This subtype of tumours is ER- but AR+ and expresses genes that are normally expressed in ER+ luminal tumours such as XBP-1, SCUBE2, SPDEF and FOXA1 (Doane AS *et al.*, 2006). For example, it has previously been shown that in these tumours AR driven transcription of genes that influence cell proliferation is mediated by FOXA1 in an ER-independent manner (Robinson JL *et al.*, 2011).

1.1.5 History of breast cancer management

Breast cancer is one of the oldest types of cancers to be documented. From ancient Egypt until today, breast cancer management has evolved through the ages and continues to be an ongoing research area.

The earliest description of breast cancer can be found in the so-called "Edwin Smith Surgical Papyrus" from ancient Egypt and dates back to around 1600 B.C. (Cooper WA, 1941). The first known record of a mastectomy goes back to Roman times (de Moulin D, 1983) and was performed in more or less the same way until the end of the 18th century. However, with the discovery of nitrous oxide as an anesthetic inhalant by Horace Wells in 1846, the microscopic histopathological observation established by Rudolf Virchow in 1855 and eventually the introduction of carbolic acid spray as an antiseptic by Joseph Lister in 1867, a new era in surgery began (Ekmektzoglou KA *et al.*, 2009). It was American surgeon William Halsted who performed the first properly documented mastectomy of the 19th century. Based on Virchow's theory that cancer is a local disease and that the lymph nodes are the natural barrier against cancer spread, Halsted proposed that cancer cells move to distant organs through adjacent tissues (Ekmektzoglou KA *et al.*, 2009). It was his belief that by surgically removing the tumour and its what he called "spreading tentacles", women could be cured from the disease. Even though this theory turned out to be incorrect, it became the cornerstone of the surgical treatment of cancer. In 1889, Halsted performed his first radical mastectomy, which involved removing the affected breast, the nearby lymph nodes and the two chest wall muscles to ensure elimination of all cancer cells from the body (Halsted WS, 1894).

This technique became the mainstay of breast cancer surgery for almost one hundred years. However, the development of distant metastasis in patients that had undergone Halsted's radical surgical procedure suggested that breast cancer was a systemic disease (Fisher B *et al.*, 1972). This finding led to the introduction of simple mastectomy followed by regional adjuvant radiotherapy, which had been shown to result in similar 10-year survival rates in breast cancer patients with stage I disease when compared to women that had undergone radical mastectomy (McWhirter R, 1955; Atkins H *et al.*, 1972). Cytotoxic chemotherapy was also introduced into the clinic as a form of systemic adjuvant therapy and promising results were published in 1976 (Bonadonna G *et al.*, 1976).

The first use of estrogen suppression as a therapy for breast cancer was more than a century ago when oophorectomy was shown to have antitumour effects in premenopausal women with breast cancer (Beatson GT, 1896). Beatson had unconsciously studied the effect of estrogen on breast cancer, as at the time estrogen had not yet been discovered. Half a century later, adrenalectomy and hypophysectomy was shown to have antitumour effects in postmenopausal women (Dao TL *et al.*, 1955; Fracchia AA *et al.*, 1971). Those surgeries were decided to be the therapy of choice because at the time it was believed that the adrenal gland was the site of estrogen synthesis in postmenopausal women. Modern research however has shown that the adrenal gland produces estrogen precursors called androgens, which are peripherally converted into estrogens through aromatisation (Lonning PE *et al.*, 2010). Even though these surgeries did show success, the fact that only about one third of patients responded to any form of ablative surgery meant that two third of women were undergoing surgery and extensive medical care without any favourable outcome.

The discovery of the estrogen receptor and the representation of the estradiol synthesis by Toft and Gorski in 1966 as well as the discovery of intracellular ER in breast cancers by Elwood Jensen a year later linked hormone signaling through the ER to its effect on breast cancer cells for the first time (Toft D and Gorski J, 1966; Jensen EV *et al.*, 1967). This discovery could distinguish between patients that would or would not benefit from ablative therapy and paved the way for the development of novel endocrine therapies.

1.2 Endocrine therapy

Sustained exposure to endogenous or exogenous estrogen is a well-established cause of breast cancer (Colditz GA *et al.*, 1998). Randomised trials have demonstrated that adjuvant endocrine therapy can effectively reduce the risk of recurrence in estrogen sensitive tumours (Nadji M *et al.*, 1998). Since approximately 75% of all breast tumours are estrogen receptor-positive (ER+) (Possinger K, 2004; Fabian CJ and Kimler BF, 2005) and 55% are progesterone receptor-positive (PR+) (Nadji M *et al.*, 2005), adjuvant hormonal therapy that interferes with steroid action and signaling has become increasingly important in the management of hormone receptor positive breast cancer treatment besides cytotoxic chemotherapy and targeted therapy (Osborne CK, 1998; EBCTCG, 2005).

Endocrine therapy involves the manipulation of the endocrine system through exogenous administration of specific hormones, particularly steroid hormones, or drugs that inhibit either the production or the activity of such hormones. It has a relatively low morbidity, and there is evidence that antihormonal treatments have had a significant effect in reducing mortality in breast cancer patients. Despite this, resistance to endocrine therapy, either primary de novo or acquired during treatment, occurs in the majority of patients, and is a major obstacle to optimal clinical management (Larionov AA and Miller WR, 2009).

Since the endocrine system changes during a woman's life, a variety of drugs have been developed to affect different sites of hormonal synthesis. However, the most familiar example of endocrine therapy in oncology is the use of the selective estrogen-response modulator (SERM) tamoxifen for the treatment of breast cancer. A novel class of hormonal agents called aromatase inhibitors (AIs) display a different mechanism of action and side effects than tamoxifen and have gained an increasing importance in the treatment of ER+ breast cancer in postmenopausal women (Dellapasqua S and Colleoni M, 2010).

1.2.1 Selective Estrogen-Receptor Modulators (SERMs)

SERMs are synthetic, non-steroidal molecules and are an important class of hormonal therapy agents that act as competitive antagonists of the estrogen receptor in breast tissue (Jordan VC, 1976). This class of drugs is used primarily for the treatment and chemoprevention of breast cancer. By competitively binding to the ER, they trigger a change in the biological activity of the receptor to prevent cell proliferation (Oseni T *et al.*, 2008). SERMs act as antagonists in breast tissue but also as agonists in other organs such as bone, liver and the cardiovascular system. SERMs have also been shown to display mixed properties in the uterus. Recruitment of coactivators is a prerequisite for agonist properties of SERMs in certain tissues, which is determined by the availability of those proteins as well as conformational changes in the ER induced by SERM binding (Shanle EK and Xu W, 2010). The exact mechanisms that determine tissue selectivity are unclear and it is therefore difficult to predict the tissue specific effects of SERMs (Katzenellenbogen BS and Katzenellenbogen JA, 2002).

1.2.1.1 Tamoxifen

One of the most frequently utilised SERMs is tamoxifen (TAM) and it has been used as first-line treatment of pre-menopausal women with estrogen receptor-positive breast cancer for nearly four decades (Jordan VC, 2008). Response to the drug in ER- breast cancers is quite rare, as tamoxifen acts as a competitive anti-estrogen to inhibit estrogen signaling. Tamoxifen is a pro-drug that is converted to antiestrogenic metabolites that are more potent than tamoxifen itself (Desta Z *et al.*, 2004). *In vitro* and *in vivo* studies have revealed that tamoxifen undergoes oxidation by the cytochrome P450 system, mainly CYP3A and CYP2D6, resulting in a range of primary and secondary metabolites that display a significant increase in activity (Poon GK *et al.*, 1993; White IN, 2003). Two of the most studied metabolites of tamoxifen are the primary metabolite 4-hydroxy-TAM (4-OHT) and the secondary metabolite 4-hydroxy-*N*-desmethyl-TAM (endoxifen) (Jordan VC *et al.*, 1977). 4-OHT has been shown to possess a high affinity for ERs and to exhibit a 30- to 100-fold increase in potency than tamoxifen in suppressing estrogen-dependent cell proliferation (Coezy E *et al.*, 1982; Robertson DW *et al.*, 1982). Endoxifen

has been demonstrated to be similarly potent as 4-OHT with regards to ER binding affinity, suppression of estrogen-dependent growth and gene expression (Stearns V *et al.*, 2003; Johnson MD *et al.*, 2004).

Tamoxifen competes with estrogen for the estrogen receptor. Binding to the receptor causes dimerisation, conformational changes in the ER/SERM complex and binding of the complex to the ERE (Figure 1.5). The conformational change in the AF2 domain of the ER caused by tamoxifen leads to different downstream effects (Brzozowski AM, 1997). It is thought that tamoxifen might block gene transcription through the AF2 domain, whereas AF1-mediated gene transcription might still occur, offering an explanation for the partial agonist/antagonist action of tamoxifen (Tora L, 1989).

Five years of adjuvant therapy with TAM has shown to halve the rate of disease recurrence (47% disease reduction) as well as contra-lateral breast cancer (47% reduction) and reduces breast cancer deaths per year by one third (26% mortality reduction) (Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 1998 and 2005). A few protective properties have been shown such as protection against osteopenia or hypercholesterolaemia; however, serious side effects include endometrial cancer and thromoembolic events (McDonald CC *et al.*, 1995; Sismondi P, 1994).

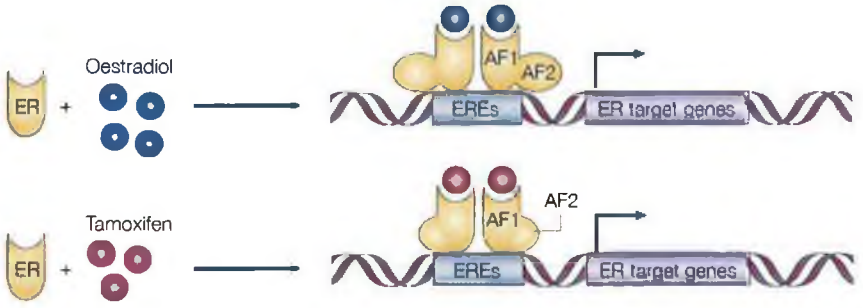


Figure 1.5: Molecular effects of tamoxifen on estrogen receptor. Both estradiol and tamoxifen bind to the ER, which leads to dimerisation and a conformational change in the AF2 domain and binding to the EREs. The conformational change with tamoxifen is different from that with estradiol and leads to persistent but less efficient transcription of most estrogen-dependent genes. *Adapted from Nature Reviews (Johnston SR and Dowsett M, 2003).*

1.2.2 Aromatase Inhibitors (AIs)

Whereas tamoxifen competitively binds to the estrogen receptor to inhibit ER signaling, AIs bind to the p450 subunit of the aromatase enzyme to prevent the conversion of androgenal precursors into estrogen. This interaction causes a severe decrease in estrogen levels inside the cell, subsequently inhibiting cell proliferation by inducing cell cycle arrest in the G_0-G_1 phase which is coupled with increased apoptosis in hormone-sensitive breast cancer cells (Thiantanawat A *et al.*, 2003).

Aromatase is a member of the cytochrome P450 family and is a product of the CYP19A1 gene, which is located on chromosome 15 (Chen SA *et al.*, 1988). It is the only vertebrate enzyme that can aromatise a six-membered ring and is therefore the only source of estrogen in the body (Amarneh B *et al.*, 1993). Breast cancer cells express the aromatase enzyme and produce higher levels of estrogen than non-cancerous cells, making it a great target in the treatment of breast cancer (Harada N, 1997). Aromatisation is the final step of steroid biosynthesis and is rate-limiting for estrogen synthesis (Figure 1.6).

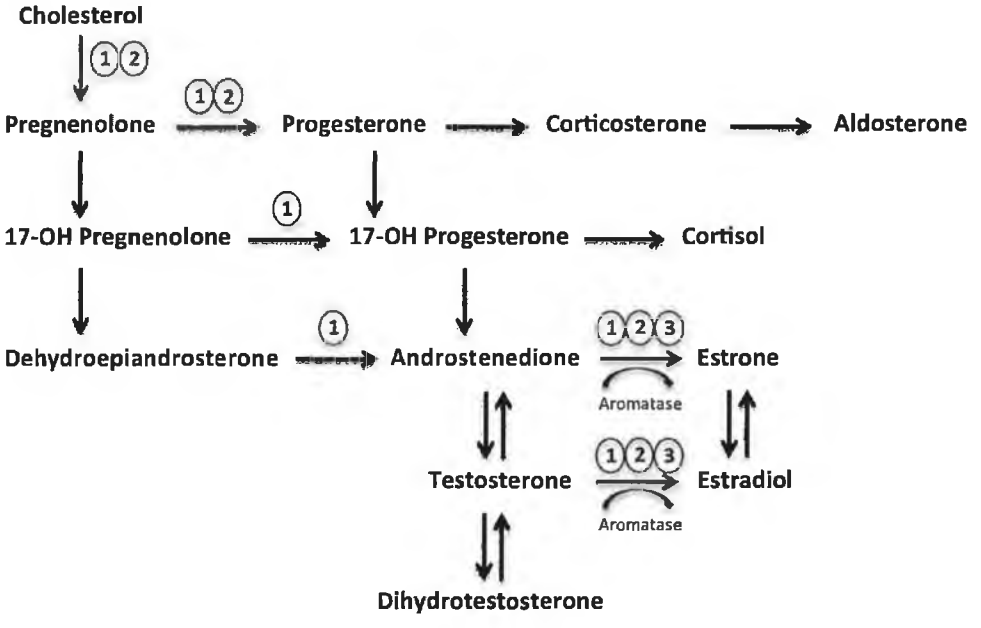


Figure 1.6: Metabolic pathways differentially targeted by AIs. (1) 1st generation AIs reduce aldosterone and cortisol in addition to estrone and estradiol. (2) 2nd generation AIs reduce aldosterone and cortisol in addition to estrone and estradiol. (3) 3rd generation AIs and inactivators block only conversion of androstenedione and testosterone to estrone and estradiol. *Derived from Fabian CJ, 2007.*

Therefore, synthesis of precursors is not affected by inhibition of the enzyme. These unique features of the aromatisation reaction provided the opportunity to develop inhibitors selectively for $P450_{arom}$ (Brodie A *et al.*, 2009).

Aromatase inhibitors can only be used in postmenopausal breast cancer patients, as they possess a limiting ability to decrease circulating estrogen. This is due to the fact that in premenopausal women ovarian aromatase is responsible for the synthesis of the majority of circulating estrogen in the body and is exquisitely sensitive to changes in luteinising hormone (LH) (Fabian CJ, 2007). The exquisite sensitivity of the ovarian aromatase promoter to gonadotrophins, which radically increase after AI administration, renders AIs useless in the treatment of premenopausal women. However, AIs have been shown to successfully decrease estrogen synthesis in premenopausal women when given in combination with a gonadotrophin inhibitor (Winer EP, 2005).

In postmenopausal women, estrogen production in the ovaries has ceased, but other organs, such as adipose tissue, brain, blood vessels, skin, bone, endometrium and breast tissue can still synthesise estrogen by converting androgens produced by the adrenal glands through the aromatase enzyme (Chumsri S *et al.*, 2011). The aromatase gene promoter in breast tissue for example is less sensitive to changes in LH than the gene promoter in the ovaries. However, the gene promoter in breast tissue is more sensitive to an increase in inflammatory cytokines. Since circulating inflammatory cytokines increase with age and breast tissue inflammatory cytokines increase with breast cancer progression, aromatase activity in breast tissue is highly increased in postmenopausal breast cancer patients (Simpson ER and Davis SR, 2001).

1.2.2.1 Evolution of Aromatase Inhibitors

The first generation of AIs included potent but non-selective inhibitors of adrenal steroid synthesis. One of them, aminoglutethimide (Figure 1.7 c and d left), lacked selectivity for aromatase and inhibited cortisol, aldosterone, thyroid hormone as well as aromatase biosynthesis (Figure 1.6) (Santen RJ *et al.*, 1977). Even though this drug exhibited response rates between 20% and 40% in metastatic breast cancer (Santen RJ *et al.*,

1978), its toxicity and the need for chronic corticosteroid substitution led to the development of a new class of aromatase inhibitors.

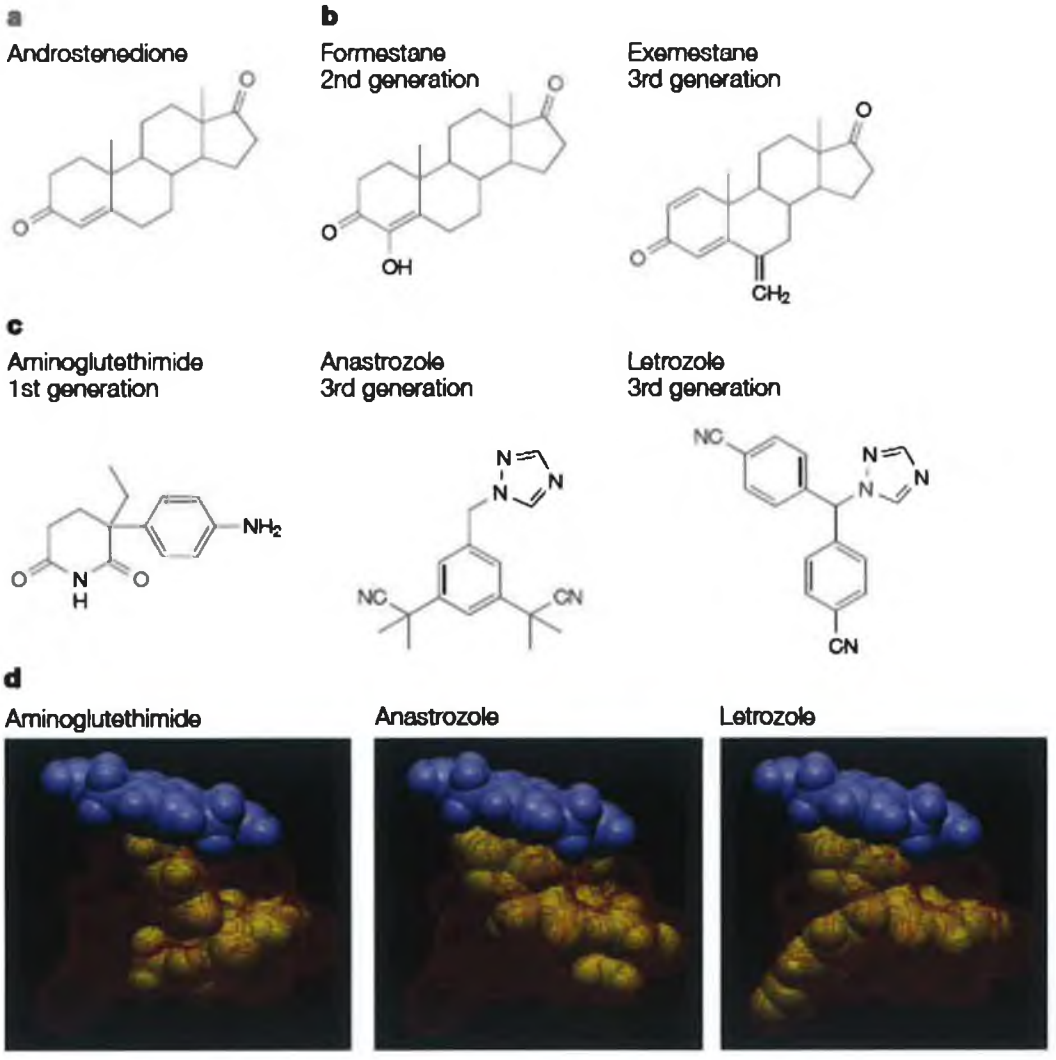


Figure 1.7: Molecular structures of AIs in clinical use. **a)** Androstenedione (natural substrate). **b)** Steroidal drugs (substrate analogues). **c)** Non-steroidal drugs (bind to haem group of aromatase as illustrated in d). **d)** Computer models depicting the two third-generation AIs and aminoglutethimide interacting with the active site of aromatase. Aminoglutethimide is a poorer fit and less space filling than anastrozole and particularly letrozole (Red = substrate-binding pocket; blue = haem prosthetic group; yellow = inhibitor). *Adapted from Nature Reviews Cancer (Johnston SR, 2003).*

The second generation AIs encompassed the non-steroidal inhibitor fadrozole and the steroidal inhibitor formestane (Figure 1.7 b). Even though fadrozole was superior to aminoglutethimide in regards to potency, selectivity and safety, its selectivity was not optimal (Figure 1.6). Additionally, results from clinical trials indicated that it was not more successful than tamoxifen (Santen RJ *et al.*, 1991; Falkson CI *et al.*, 1996; Thurlimann B *et al.*, 1996). Formestane on the other hand proved to be quite successful in metastatic disease and as neoadjuvant therapy, yet its use was limited due to the need of parenteral application (Dowsett M *et al.*, 1994).

The third generation AIs gave rise to a class of very potent and selective inhibitors of aromatase activity. Compared with the first generation non-selective inhibitor aminogluthetimide this generation of AIs has been shown to be 100- to 10,000-fold more potent as well as monospecific for the aromatase enzyme (Santen RJ *et al.*, 2009). The third generation AIs can be split up into irreversible steroidal AIs such as formestane and exemestane and reversible non-steroidal AIs such as anastrozole and letrozole (Figure 1.7 c and d middle and right) (Campos SM, 2004).

Both groups of third generation AIs have demonstrated good clinical efficacy without cross-resistance as well as acceptable short-term toxicity profiles in post-menopausal women with advanced disease (Brueggemeier RW, 2004). The earliest use of third generation AIs in the management of advanced breast cancer was reported in the 1990s (Reddy P, 1998) and initial results of their use in an adjuvant setting were published in 2002 (Baum M *et al.*, 2002).

1.2.2.2 Steroidal vs. non-steroidal Aromatase Inhibitors

All AIs are similar in that they prevent estrogen biosynthesis by inhibiting aromatase activity, however, there are distinct differences between them. The two classes of AIs, namely steroidal and non-steroidal, differ with regards to their mechanism of binding to aromatase.

Steroidal AIs bind to the substrate-binding site of the aromatase enzyme due to their similar structure to the original aromatase substrate, androstenedione. After binding, the steroidal AI is converted to a reactive intermediate that covalently binds to

aromatase causing irreversible inaction. As such, they have become known as aromatase inactivators or suicide inhibitors as the enzyme is inactivated by its own function (Brodie AM *et al.*, 1981).

Non-steroidal AIs bind to the haem moiety of the enzyme and prevent binding of androgens by saturating the binding-site. The inhibition with non-steroidal AIs is competitive and thus reversible (Chen SA *et al.*, 1988). Examples for this type of AIs are fadrozole, vorozole, rogletimide, letrozole and anastrozole. Even though formestane, fadrozole, vorozole and rogletimide exhibited some clinical activity in tamoxifen resistant breast cancer they are no longer in clinical use because they did not prove to be more effective than tamoxifen. Additionally, they had undesirable side effects, caused suppression of aldosterone or required intramuscular injection (Chumsri S *et al.*, 2011). These drugs were replaced by the latest generation of AIs that offer fewer side effects and better oral bioavailability (Smith IE and Dowsett M, 2003).

The steroidal AI exemestane is structurally related to androstenedione. It has been shown that its androgenic structure may give rise to hormonal effects apart from estrogen depletion, which makes it distinct from the non-steroidal AIs letrozole and anastrozole (Miller WR *et al.*, 2008). The main metabolite of exemestane can bind to the androgen receptor (AR) with high affinity and it has been shown that, following the approved daily doses, the circulating levels of this metabolite are 15% that of unchanged exemestane (Ariazi EA *et al.*, 2007; Traina TA *et al.*, 2008). Still, it needs to be evaluated if steroidal AIs like exemestane can exert an androgenic effect in breast cancer that is clinically relevant.

Steroidal, irreversible AIs like exemestane are thought to cause longer aromatase inhibition than non-steroidal, reversible AIs since estrogen synthesis can only resume after *de novo* synthesis of the enzyme. However, *in vivo* studies demonstrated that *de novo* synthesis ocurrs rather quickly in the span of one to two days (Dowsett M *et al.*, 1987).

Recent studies revealed that differences in binding between steroidal and non-steroidal AIs lead to conflicting effects on aromatase. For example, the steroidal AI exemestane destabilises aromatase in MCF7aro, a breast cancer cell line that overexpresses the

enzyme, leading to a faster degradation of the aromatase protein by proteosomic enzyme than in the absence of the AI (Wang X and Chen S, 2006). In contrast, non-steroidal AIs have been shown to increase aromatase protein levels, probably due to stabilisation of the enzyme or the induction of transcription of aromatase mRNA, which may result in an increase in aromatase activity (Chen S *et al.*, 1999; Miller WR and Dixon JM, 2001; Soudon J, 2000). Therefore, it is thought that prolonged treatment with non-steroidal AIs may result in an increase in aromatase protein levels and subsequent estrogen biosynthesis, which could contribute to the development of AI resistance (Miller WR *et al.*, 2008).

The AIs approved by the FDA for postmenopausal women with ER+ breast cancer and currently in clinical use in both the adjuvant and metastatic setting are the steroidal AI exemestane (Aromasin®) as well as the non-steroidal AIs anastrozole (Arimidex®) and letrozole (Femara®) (Janicke F, 2004).

1.2.2.3 Anti-estrogenic efficacy of Aromatase Inhibitors

Even though exemestane, anastrozole and letrozole are currently all in clinical use, it is worth noting that some of them are more efficient than others with regards to blocking aromatase activity. Studies have shown that letrozole is the most potent in suppressing aromatisation. Bernardi *et al.* compared the anti-estrogenic activities of anastrozole (1mg), letrozole (2.5mg) and exemestane (25mg) in postmenopausal women with no previous AI therapy. The results of this study revealed the most substantial effect on estrogen production by letrozole, that is 84% suppression of plasma estrone (E1) and 47% suppression of plasma estradiol (E2), when compared with the other two aromatase inhibitors. Anastrozole displayed a similar level of estrone suppression (75%) but was not as efficient in suppressing estradiol (E2) production (26%). Exemestane proved to be the least potent with 25% of estrone and 21% of estradiol suppression (Bernardi *et al.*, 2002) (Figure. 1.8).

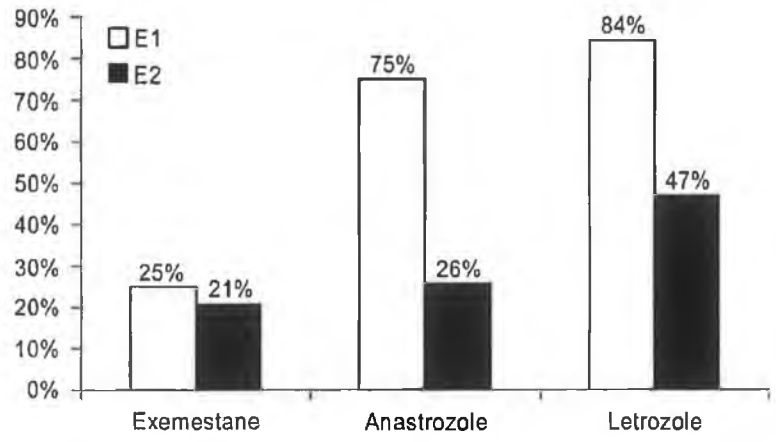


Figure 1.8: Anti-estrogenic activities of AIs. Percent suppression from baseline of plasma estrone (E1) and estradiol (E2) after 1 month of AI therapy. *Adapted from The Breast (Monnier A, 2006).*

1.2.2.4 Letrozole

Letrozole was developed in 1986 by Ciba-Geigy (now Novartis), a company that was also involved in the development of several other AIs in the 1980s. When tested in an *in vivo* assay it appeared to have a profound effect on the rat uterus at doses significantly lower than doses found to have an effect with other AIs. This finding suggested that this molecule was substantially more potent than any other AIs previously tested by the company (Bhatnagar AS, 2007). It was subjected to dose-finding Phase I studies in healthy postmenopausal women (Iveson TJ *et al.*, 1993a), postmenopausal patients with advanced breast cancer (Iveson TJ *et al.*, 1993b) and healthy male volunteers (Trunet PF *et al.*, 1993). Two Phase III trials followed which tested two different doses of letrozole against each other and revealed that 2.5 mg of letrozole was significantly more potent than the 0.5 mg dose (Dombernowsky P *et al.*, 1998; Buzdar A *et al.*, 2001). It was approved for the treatment of advanced breast cancer in Europe in 1996 and one year later in the United States (Dellapasqua S, 2010).

The Femara PO25 study, a Phase III trial, eventually revealed that letrozole proved to be superior to tamoxifen as first-line therapy for postmenopausal women with advanced breast cancer (Mouridsen H *et al.*, 2001).

1.2.3 Tamoxifen vs Letrozole

Even though tamoxifen is known as the gold standard of endocrine therapy, development of resistance is still quite common (Clarke R *et al.*, 2001). It is not effective for more than five years and the risk of recurrence remains even after that period (Brewester AM *et al.*, 2008). More than two third of deaths occur after completed therapy and low rates of compliance have been reported with no more than one third of patients adhering to five years of treatment (Barron TI *et al.*, 2007).

Aromatase inhibitors have been introduced as a prospective alternative. They have been shown to cause less gynaecological and menopausal symptoms than tamoxifen, but have been observed to cause fractures and hypercholesterolaemia more frequently (Monnier A, 2006).

1.2.3.1 Comparison of tamoxifen vs. letrozole in preclinical studies

Angela Brodie's group compared the efficacy of letrozole vs tamoxifen in xenograft models that were treated with either endocrine therapy over a period of 56 weeks (Figure 1.9) (Jelovac D *et al.*, 2005). They inoculated ovariectomized mice with MCF-7Ca cells (MCF-7 cells overexpressing aromatase) and divided them into three groups (n = 20 per group) to receive vehicle, letrozole (10 μg/d), or tamoxifen (100 μg/d) as soon as the tumours had reached a measurable size (300 mm^3). All groups received androstenedione supplement (100 μg/d) throughout the duration of the experiment. Tumor volumes were measured weekly and expressed as percentage change relative to the initial tumor volume (Fig. 1.9). As previously reported (Long BJ *et al.*, 2004), both treatments were effective in controlling tumor growth compared with vehicle, yet, letrozole was more effective and delayed tumor progression twice as long as tamoxifen. Tumors treated with letrozole initially shrunk to half their size (4 weeks). After 18 weeks, however, they had regained their starting size and thereafter were clearly unresponsive to the drug (Fig. 1.9).

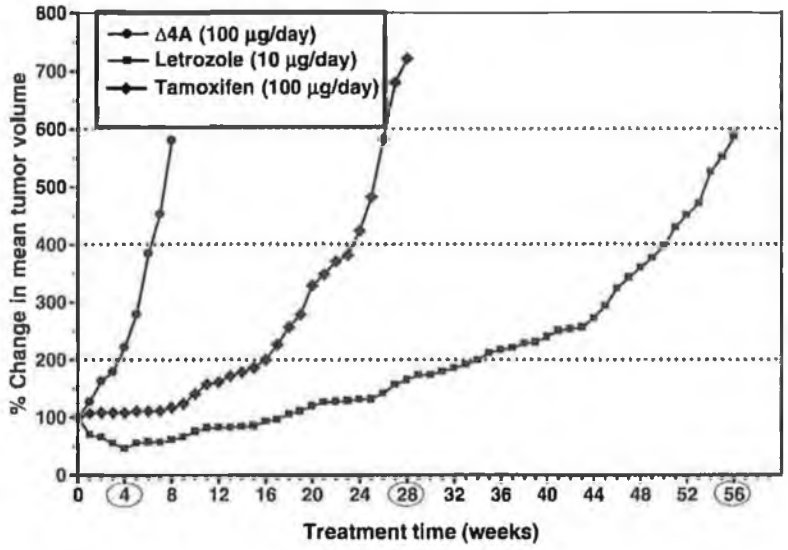


Figure 1.9: Effect of letrozole and tamoxifen on the growth of MCF-7Ca xenografts. Animals were inoculated with MCF-7Ca cells and were supplemented with androstenedione for the duration of the experiment. They were assigned to three groups (n=20) and injected daily with vehicle (control), tamoxifen (100 µg/d) or letrozole (10 µg/d) as soon as tumours reached measurable size (300 mm^3). Tumour volumes were measured weekly and were expressed as the percent change relative to the initial volume. Two mice per group were sacrificed and tumors were collected for analysis at 4, 28, and 56 weeks as indicated. *Adapted from Cancer Research (Jelovac D et al., 2005).*

1.2.3.2 Comparison of tamoxifen vs. letrozole in clinical studies

The first clinical study evaluating an aromatase inhibitor as adjuvant therapy for breast cancer was performed at the Royal Marsden Hospital in London three decades ago. The study compared the first generation AI aminoglutethimide to placebo (Coombes RC *et al.*, 1982). The trial revealed that aminoglutethimide improved short-term, but had no sustainable effect on overall survival (Jones AL *et al.*, 1992).

To date, several trials have been conducted to evaluate the efficacy of the third generation AIs anastrozole, letrozole and exemestane (Figure 1.10). In general, there are three different treatment strategies under investigation: early adjuvant therapy (replacement of tamoxifen as adjuvant therapy for five years), early sequential adjuvant therapy (sequencing of tamoxifen before or after an aromatase inhibitor during the first

five years) and extended adjuvant therapy (aromatase inhibitor therapy following five years of tamoxifen treatment) (Mouridsen HT and Robert NJ, 2005).

1.2.3.2.1 Early adjuvant trials

The first early adjuvant trial comparing an AI with tamoxifen was the so-called **ATAC** (Anastrozole, Tamoxifen, Alone or in Combination) trial. It was initially designed as a three-arm study to assess the efficacy and safety of anastrozole, tamoxifen or a combination of both drugs during five years of adjuvant therapy (Baum M *et al.*, 2002; Howell A *et al.*, 2005; Forbes JF *et al.*, 2008; Cuzick J *et al.*, 2010). After five years of treatment, there was a significant improvement in disease free survival (DFS) of 2.5% (p=0.005) in the group of patients treated with the anastrozole alone when compared with five years of tamoxifen (Howell A *et al.*, 2005), however, no significant improvement in overall survival has been observed beyond 100 months of median follow-up.

The **BIG 1-98** (Breast International Group) collaborative group study represents a phase III, double-blind trial that initially randomised 1828 patients to either letrozole or tamoxifen for five years (Mouridsen H *et al.*, 2009). It was later modified to a four-arm study and randomised 6182 patients to letrozole for five years, tamoxifen for five years, letrozole for two years followed by tamoxifen for three years or tamoxifen for two years followed by letrozole for three years (Coates AS *et al.*, 2007). 26 months follow-up data from the BIG 1-98 study revealed that letrozole significantly increased the DFS rate (p=0.003), decreasing recurrent cancer from 13.6% to 10.4%. Five years of letrozole monotherapy has shown a significant benefit over tamoxifen, especially in the reduction of distant metastasis (Mouridsen H *et al.*, 2009). Interestingly, long-term follow up has revealed an overall survival benefit for patients on letrozole in comparison to patients that received tamoxifen monotherapy (Lao Romera J *et al.*, 2011).

TEAM is a multinational, phase III trial. Approximately 4400 postmenopausal, HR+ patients with early breast cancer were randomised after concluding primary treatment with surgery, chemotherapy and radiation therapy to either tamoxifen or exemestane as adjuvant monotherapy for five years (van de Velde CJ *et al.*, 2011).

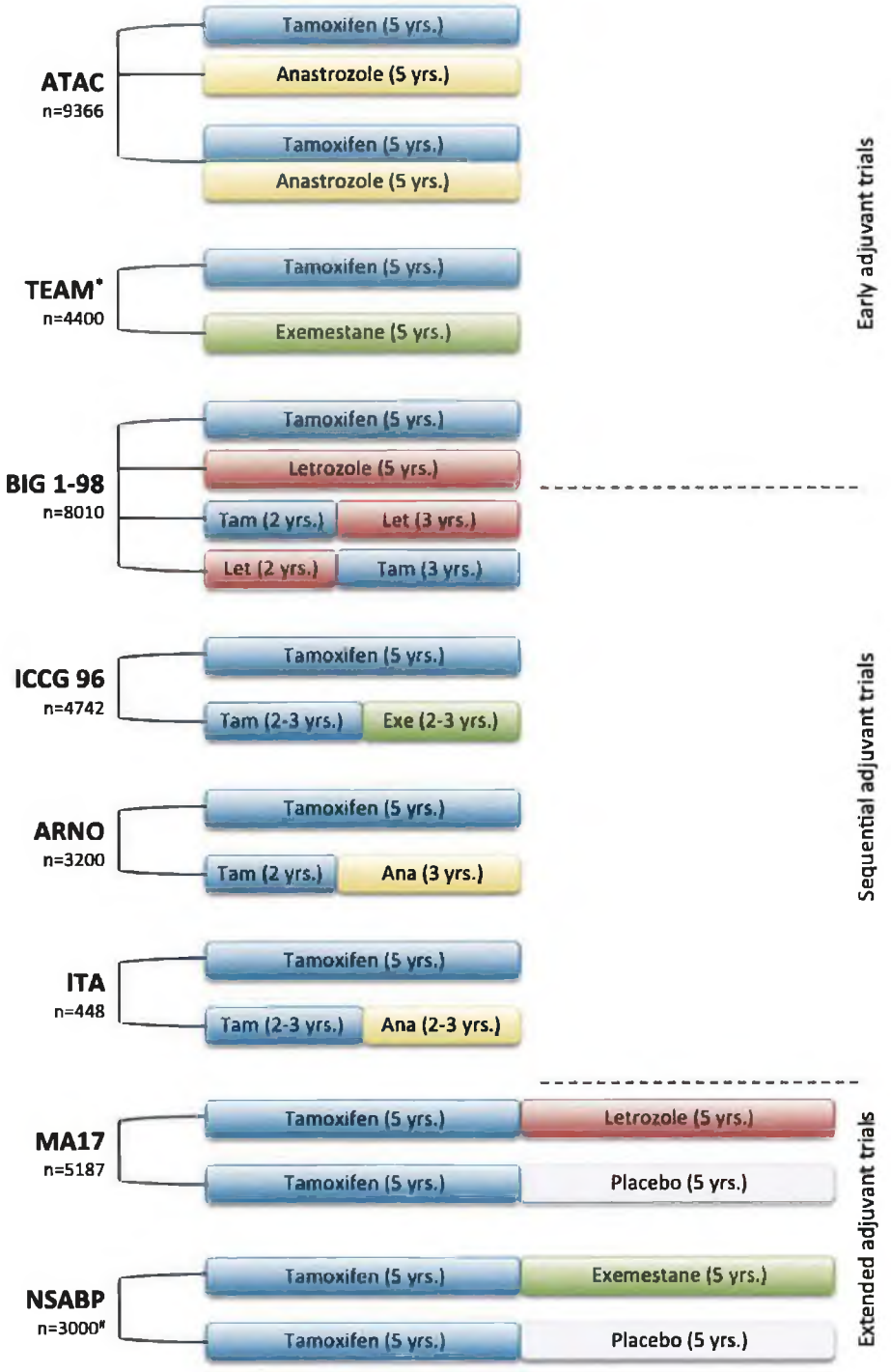


Figure 1.10: Design of various clinical trials. Tam = tamoxifen, Ana = anastrozole, Let = letrozole, Exe = exemestane. * TEAM trial design was amended later on; patients in the tamoxifen monotherapy arm were switched to exemestane after 2-3 years of tamoxifen treatment. # Planned study size, but placebo arm was closed prematurely due to reults from the MA.17 study.

Due to recent findings of the ICCG 96 trial (see below) that suggested that switching from tamoxifen to exemestane after two to three years improves disease free survival (DFS) compared with remaining on tamoxifen, the TEAM study has been amended accordingly (Mouridsen HT and Robert NJ, 2005).

A multicenter, randomized trial performed by the **International Letrozole Breast Cancer Group** was designed exactly like the monotherapy arms of the BIG 1-98 study. It confirmed that Letrozole was more efficient than tamoxifen as a first-line treatment for women with advanced metastatic breast cancer. Results from 907 patients randomized into two groups (letrozole n=453, tamoxifen n=454) revealed that patients on letrozole experienced significantly longer time to progression (9.4 months) when compared with those on tamoxifen (6 months, p<0.0001). Letrozole continued to have a significant advantage over tamoxifen at a median of 32 months. Patients treated with the AI attained a significantly greater overall objective response rate (ORR) of 32% than those treated with the SERM (21%, p=0.0002) as well as a higher rate of clinical benefit (50%) than with tamoxifen (38%, p=0.0004) (Figure 1.11) (Mouridsen H *et al.*, 2003).

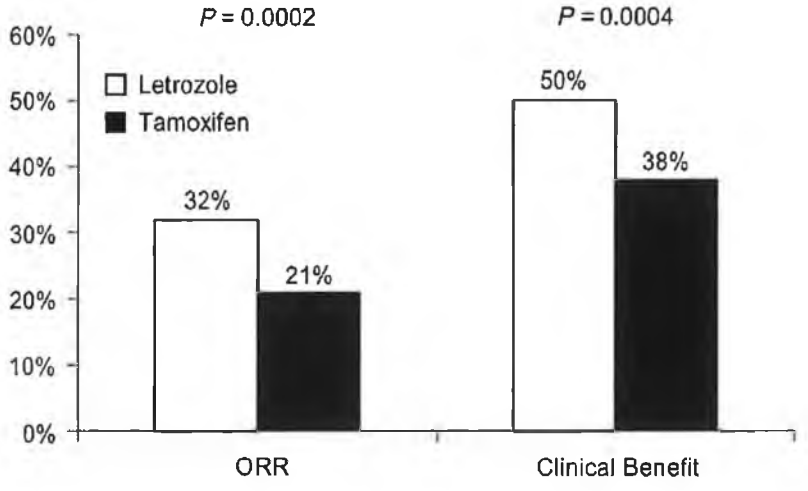


Figure 1.11: Efficacy of letrozole and tamoxifen treatment in patients with advanced metastatic breast cancer. Results at median 32-month follow-up. *Adapted from The Breast (Monnier A et al., 2006).*

1.2.3.2.2 Early sequential adjuvant trials

In the early sequential adjuvant therapy arms of the four-arm **BIG 1-98** trial patients received either two years of letrozole followed by three years of tamoxifen or two years of tamoxifen followed by three years of letrozole as mentioned earlier. When letrozole was compared to each one of the sequential arms it emerged that sequential treatment with letrozole followed by tamoxifen or tamoxifen followed by letrozole was not superior to five years of letrozole monotherapy with regard to improving disease outcome (Regan MM *et al.*, 2011).

The **ICCG 96** / IES (International Collaborative Cancer Group / Intergroup Exemestane Study) is a large, double-blind trial designed to compare two to three years of exemestane with two to three years of tamoxifen in patients that have already received tamoxifen for two to three years. Postmenopausal ER+ patients with early breast cancer who were disease-free after initial tamoxifen treatment were randomised to continue on tamoxifen therapy or to switch to exemestane, with an overall treatment of five years of adjuvant therapy (Coombes RC *et al.*, 2004).

The **ARNO** trial is designed to compare five years of tamoxifen versus sequential tamoxifen for two years followed by three years of anastrozole treatment. This trial began in 1996 and was carried out by two study groups: the Austrian Breast Cancer Study Group and the German Adjuvant Breast Cancer Group (Goss PE, 2001). Their trial design differed slightly in that the Austrian group randomised the patients upfront whereas the German group randomised the patients during the first two years after surgery. Postmenopausal patients with hormone-sensitive breast cancer and no prior adjuvant chemotherapy were recruited for this trial. The trial demonstrated that switching to anastrozole resulted in a 39% relative improvement in DFS (p=0.049) and 52% improvement in overall survival (p=0.045) at a median follow-up of 30 months when compared to continuing on tamoxifen treatment (Kaufmann M *et al.*, 2006).

The **ITA** is an Italian open-label trial that was designed to compare the standard five-year tamoxifen treatment with two to three years of tamoxifen followed by two to three years of anastrozole for a total of five years (Boccardo F *et al.*, 2006). Postmenopausal ER+ and node-positive patients were enrolled, out of which 45% had prior adjuvant

chemotherapy. This study however was limited to the apparent small size (n=448), the open label design and in particular the changes in inclusion criteria. The criteria had specified that the patients in the sequential arm of the trial would receive tamoxifen for a maximum of two to three years, but the swith to anastrozole actually took place at highly inconsistent time points with a median of 28 months (range from 20 up to 40 months).

1.2.3.2.3 Extended adjuvant therapy

Due to the appreciable late recurrence rates in women with ER+ breast cancer following five years of first-line tamoxifen therapy, the **MA.17** trial was designed to evaluate additional five years of letrozole administration after completing adjuvant tamoxifen would improve DFS in those patients. Postmenopausal HR+ breast cancer patients were randomized to receive either five years of letrozole or placebo after five years of tamoxifen treatment (Goss PE *et al.*, 2003). However, the trial was discontinued after a planned interim analysis showed significantly better four-year disease free survival estimates with letrozole. The results of this trial led to the approval of letrozole as extended adjuvant therapy in the treatment of early breast cancer in patients who received adjuvant tamoxifen treatment in the United States as well as several European countries (Mouridsen HT and Robert NJ, 2005).

The **NSABP B-33** study randomised patients to either exemestane or placebo for an additional five years after completed five-year tamoxifen therapy. It was planned to recruit a total of 3000 patients but the placebo arm was closed in October 2003 due to the results of the MA.17 study (Mamounas EP *et al.*, 2008).

1.2.3.3 Side effects

Due to their different mechanisms of action, AIs and tamoxifen have very different side effects, even though in general, AIs have proven to cause fewer. One of the most severe unwanted side effects associated with tamoxifen therapy is the partial agonist activity of the drug in the endometrium. It has been shown to increase the risk for endometrial cancer by 2 – 5 fold (Smith LL *et al.*, 2000). Cardiovascular disease is the second most

common cause of death in women with breast cancer (Yancik R *et al.*, 2001). Due to their menopausal status, age, higher rates of hypertension as well as administered therapies, postmenopausal breast cancer patients are at a higher risk for myocardial infarction and stroke (Nilsson G *et al.*, 2005). Results from the BIG 1-98 trial revealed that patients treated with letrozole experienced less thromboembolic events, such as deep venous thrombosis and pulmonary embolism, than those treated with tamoxifen but displayed significantly higher rates of serious or fatal cardiac events (Bundred NJ, 2005; Coates AS *et al.*, 2007). Other side effects of tamoxifen that AIs rarely cause are hot flushes and gynaecologic complications (Swaby R *et al.*, 2007). In contrast to tamoxifen, which has an agonist effect on bone density, continous estrogen depletion caused by AI treatment can result in acceleration of bone loss in postmenopausal women (Bundred NJ, 2009). Recent studies have succefully used a combination of AIs with either bisphosphonates or vitamin D to prevent bone mineral loss (Gnant MF *et al.*, 2007; Geisler J *et al.*, 2006). Another downside to AI therapy, which is not as such a side effect, is the significantly higher cost of AI therapy (Figure 1.12).

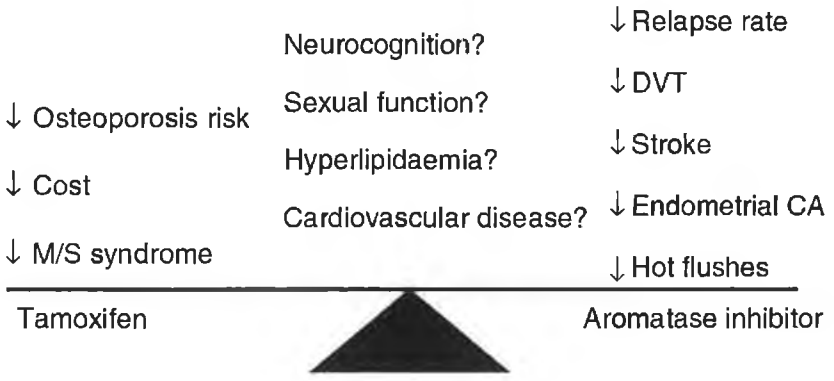


Figure 1.12: Comparison of side effects of Tamoxifen vs AI from the ATAC trial
(CA = cancer, DVT = deep venous thrombosis, M/S = musculoskeletal). *Adapted from British Journal of Cancer (Wong ZW and Ellis MJ, 2004).*

1.3 Disease progression in breast cancer

1.3.1 Metastasis

Early breast cancer is characterized as an invasive cancer that has not spread beyond the breast or the axillary lymph nodes and is therefore potentially curable, as the tumour as well as any nodal matastases can be surgically removed (National Institute for Health and Clinical Excellence, 2006). Despite efforts such as improvements in diagnosis, surgical techniques and local and systemic adjuvant therapies, most deaths from breast cancer are due to micro-metastases that remain undetected or the progressive growth of metastases that are resistant to therapy. The organ microenvironment can modify the response of metastatic tumour cells to therapy and alter the efficacy of anticancer agents (Fidler IJ, 2001). However, the main obstacle in treating metastasis is the biological heterogeneity of primary tumours and metastases: by the time of diagnosis, cancers contain a variety of genetically unstable cell populations with diverse growth rates, cell-surface properties, antigenicities, marker enzymes, sensitivity to cytotoxic drugs, abilities to invade and produce metastasis etc. (Fidler IJ, 1990).

Metastatic disease increases the mortality rate in breast cancer patients by 70%. Even though metastasis usually correlates with later stages of the disease it is thought that the metastatic process may begin early on in breast cancer development (Marsden CG *et al.*, 2012). Metastases can remain undetectable for many months and years but eventually lead to recurrence at the primary site and/or dissemination to distant sites (Allan AL *et al.*, 2006; Kim MY *et al.*, 2009). For this to happen the primary tumour needs to undergo a complex multistep process. The outcome of the metastatic process depends on both intrinsic properties of the tumour cells and their interactions with host factors (Langley RR, 2007).

After the initial transformation neoplastic cell proliferation must be progressive. At this point the expanding tumour mass receives nutrients via simple diffusion. When the tumour exceeds a size of approximately 1-2 mm in diameter it has to undergo extensive neo-angiogenesis to allow supply with fresh nutrients and oxygen and removal of

metabolic waste from the hypoxic center of the tumour mass (Folkman J, 1986). Angiogenesis is regulated by a balance of positive and negative signaling events that are mediated by GFs and their receptors as well as cell adhesion to the ECM (Cheresh DA and Stupack DG, 2008). Certain proangiogenic factors that play a key role in establishing a neocapillary network from the surrounding vasculature are synthesised and secreted by the tumour cells to aid in this process (Langley RR *et al.*, 2007). The next step in the metastatic process is the detachment of carcinoma cells from the epithelium and subsequent invasion of the underlying stroma. This process resembles the well-characterised epithelial-to-mesenchymal transition (EMT) observed in embryogenesis, both on a cellular and a molecular level (Kalluri R and Weinberg RA, 2009). The loss of intracellular adhesion molecules, such as E-cadherin, and cytokeratins as well as an increase in N-cadherin and integrins is crucial for EMT and leads to dramatic changes in the physical and mechanical properties of a cell (Figure 1.13). Reduction in intercellular adhesion as well as a morphological change from cuboidal epithelial to mesenchymal is a hallmark of the transition (Polyak K and Weinberg RA, 2009). These changes eventually result in detachment of cancer cells from the primary tumour and the acquisition of a motile phenotype (Thiery JP and Sleeman JP, 2006).

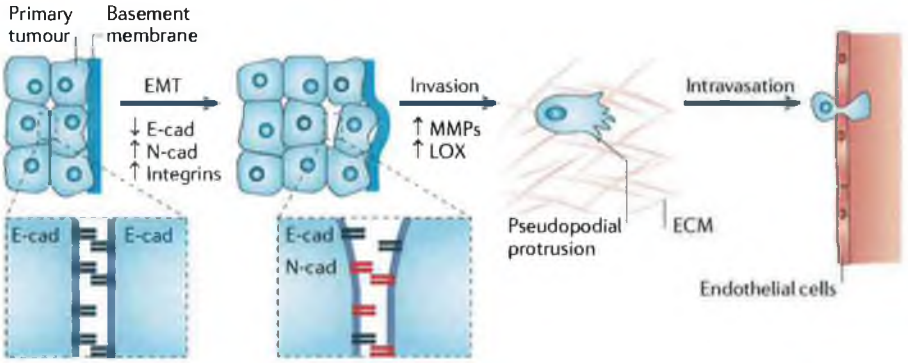


Figure 1.13: The physics of invasion and intravasation. EMT is associated with a loss of adhesion through downregulation of E-cadherin (E-cad) and a change in morphology. Invasion by tumour cells of the surrounding tissue and subsequent motion is dictated by the physicochemical properties of the ECM. Tumour cells enter the vascular system by intravasation. *Adapted from Nature Reviews Cancer (Wirtz D et al., 2011).*

Cancer cells need to express certain secreted and/or membrane-bound matrix metalloproteinases (MMPs) to digest the laminin- and collagen-IV-rich basement membrane (Hotary K *et al.*, 2006). After leaving the tumour microenvironment, the cells encounter the architecturally complex extracellular marix (ECM) that is rich in collagen-I and fibronectin (Wirtz D *et al.*, 2011). The matrix surrounding a mammary tumour is often stiffer than in normal tissue. This is thought to be due to augmented collagen deposition (Levental KR *et al.*, 2009) and crosslinking of the collagen fibres by tumour-associated fibroblasts (De Wever O *et al.*, 2008), the latter enhances integrin signalling and bundling of individual fibres (Provenzano PP *et al.*, 2009). These changes increase proliferation and invasion in a positive feedback loop (Levental KR *et al.*, 2009). However, the exact molecular and physical mechanisms that drive motile cancer cells away from their primary tumour and into the stromal space, especially at a subcellular level, have yet to be elucidated (Wirtz D *et al.*, 2011). Invasion of the surrounding tissue by tumour cells and subsequent migration is dictated by the physiochemical properties of the ECM.

During intravasation, tumour cells have to undergo dramatic shape changes that are driven by cytoskeletal remodelling to penetrate endothelial cell-cell juntions. Once the cancer cells have managed to migrate through the ECM they can enter the vascular system by squeezing between endothelial cells of veins or capillaries to desseminate to distant organs in the body. Cancer cells also frequently invade thin-walled lymphatics, as they offer low resistance to penetration by cancer cells and therefore provide a common pathway for entry into the circulation (Fidler IJ, 2003). Once inside the vasculature, cancer cells often form so called clusters or emboli. Tumour cells are thought to have a better chance of survival within those clusters compared to individual cells in circulation (Liotta LA *et al.*, 1976). A process called cell-induced platelet cell aggregation facilitates vascular embolisation as well as the formation of metastatic foci (Jurasz P *et al.*, 2004). Another way for cancer cells to survive inside the vasculature is the formation of so-called tumour nests that are enveloped with endothelial cells. It is thought to be a means of non-invasive metastasis and can be observed in patients with inflammatory breast cancer (Yui S *et al.*, 2005). A mouse model study revealed that those nests are

enveloped with endothelial cells derived from the sinusoidal vasculature of highly angiogenic breast tumours and grow inside blood vessels in the secondary organ such as the lung (Sugino T *et al.*, 2002).

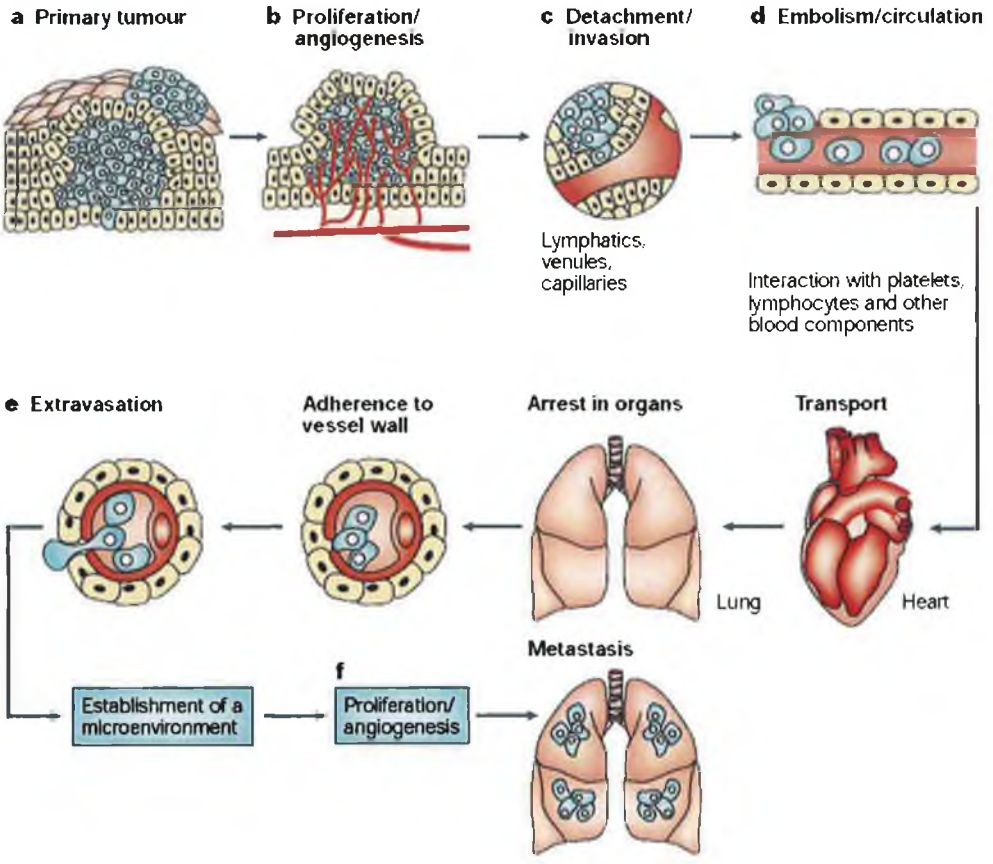


Figure 1.14: The metastatic process. Each step is rate-limiting in that failure of a tumour cell to complete a step terminates the process. **a)** Cellular transformation and tumour proliferation. Tumour is initially supplied with nutrients by simple diffusion. **b)** Vascularisation occurs if a tumour exceeds 1-2 mm in diameter. Angiogenic factors secreted by the tumour establish a capillary network from surrounding tissue. **c)** Detachment of carcinoma cells from the epithelium and subsequent invasion of the underlying stroma. Intravasation of capillaries, venules or, most commonly, lymphatics. **d)** Embolisation of tumour cells. Cells that haven't been destroyed remain in circulation and become trapped in capillary beds of distant organs where they adhere to vessel walls. **e)** Extravasation and establishment of microenvironment. **f)** Proliferation and angiogenesis inside the organ parenchyma completes the metastatic process. *Adapted from Nature Reviews Cancer (Fidler IJ, 2003).*

Primary tumours have been found to metastasise to various distant sites in the body; however, there is a higher probability of metastasis at certain sites. For example, breast cancer tends to metastasise to bone marrow and the lungs, whereas prostate cancer predominantly metastasises to bone marrow and the liver (Wirtz D *et al.*, 2011). Two hypotheses have been postulated over the last years to explain the patterns of metastasis. The "seed and soil" hypothesis states that a primary tumour cell will metastasise to a site with a favourable microenvironment (Paget S, 1889). The mechanical hypothesis on the other hand states that the occurrence of a metastasis is based on the pattern of blood flow. Both mechanisms are thought to have complementary roles in influencing the location of a metastatic site (Weiss L, 2000).

Eventually, the circulating tumour cells arrest in the capillaries of the secondary organ. Two main mechanisms, namely physical occlusion and adhesion, are thought to be involved in this process. Arrest can simply be caused by the large size of the tumour in relation to the capillary lumen (Naumov GN *et al.*, 1999). Attachment of the cancer cells to vessel walls by adhesion is a specific and highly regulated process involved in organ-selective metastatic formation. This non-random arrest of tumours may be determined by the expression of specific adhesion molecules and their corresponding ligands in the lining of the capillaries inside the secondary organs as well as on the tumour cell surface respectively (Gassmann P *et al.*, 2004). Attachment is usually followed by the recruitment of leukocytes, which are thought to be the first cells to extravasate, followed by the cancer cells (Wood S Jr, 1958). In some cases, cancer cells proliferate intravascularly, which can cause physical disruption of the endothelium due to an increase in tumour mass, allowing tumour cells to enter the surrounding tissue (Wong CW *et al.*, 2002). However, studies in breast tumour cells have shown the development of cell protrusions and deformation of the nucleus while crossing the endothelium, suggesting that extravasation is an active process (Tsuji K *et al.*, 2006). It is thought that the same mechanisms required for cell motility also play a role in extravasation (Sahai E, 2007). Most of the primary tumour cells that have arrived at the secondary site will undergo apoptosis within 24 hours (Chambers AF *et al.*, 2002; Fidler IJ, 1970). A study conducted by Kim and colleagues revealed that non-metastatic cells that were lodged in

the capillaries at the secondary site were more prone to apoptosis than metastatic cells, suggesting that increased survival at this stage in the metastatic process correlated with overall metastatic capability (Kim JW *et al.*, 2004). Still, not all tumour cells that survive at the secondary site will start to proliferate, which can be due to the fact that not all cancer cells have the same replicative potential. It has been established that a subset of solid tumour cells may have properties similar to stem cells (Bjerkvig R *et al.*, 2005). Only those stem cell-like cells are thought to be able to form a macroscopic metastasis and interact with the tumour microenvironment to enable vascularisation of the secondary tumour. It has frequently been observed that disseminated cells can remain dormant for a prolonged period of time prior to resuming proliferation, however, the molecular basis is not well understood (Naumov GN *et al.*, 2002).

1.3.2 Resistance to endocrine therapy

The development of resistance is the major factor limiting endocrine therapy and is most often observed during the treatment of advanced disease. Breast tumours have been shown to start growing 1-3 years into the treatment despite continued tamoxifen administration. Interestingly, it has been shown that resistant tumours become growth dependent on tamoxifen and can be stimulated by the drug in a dose-dependent manner (Gottardis and Jordan, 1988). This mean that the drug has no beneficial effect any more, but furthermore, it actually begins to have a negative effect on the tumour. Therefore, understanding resistance and how it develops is crucial.

1.3.2.1 Absence of ER

The primary and most obvious mechanism of resistance to endocrine treatment is the lack of ERα. Also, a recent study has shown that women that are carrying inactive alleles of cytochrome P450 2D6 (*CYP2D6*), which is needed to convert tamoxifen into its active metabolite endoxifen, may be less responsive to the treatment (Hoskins JM, 2009). These types of mechanisms are coined *de novo* or initial resistance. Acquired resistance on the other hand seems to be due to a variety of mechanisms following prolonged exposure to endocrine therapy. Yet, the most obvious mechanism seems to involve a

simple selection process due to the heterogeneity of the disease. Each breast tumour consists of both estrogen responsive as well as independent cells. During treatment, the number of ER+ cells reduces, while the ER- cells become the prevailing type of cancer cells within the tumour, resulting in a tumour that will not respond to endocrine therapy any more, even if clinically, these tumours still appear to be ER+ (Chen S *et al.*, 2006).

Early studies suggested that the loss of ERα expression or mutations are a potential cause of resistance, but the loss of ERα expression as well as mutations in the receptor occur only in a minority of resistant patients (Gutierrez MC *et al.*, 2005; Herynk MH *et al.*, 2004).

1.3.2.2 Ineffective or compromised inhibition of aromatase

Unsucessful AI therapy is not necessarily a result of *de novo* resistance but more likely due to inefficient or compromised treatment. A plethora of reasons, such as lack of drug potency, poor or adverse pharmacokinetics, compensatory endocrine loops or mutant aromatase molecules that cannot be blocked by the AI might be responsible for the development of resistance (Miller WR, 2010). As mentioned before, early generation AIs were not very specific and did not completely block estrogen biosynthesis, potentially resulting in residual estrogen maintaining proliferation of hormone-dependent tumours (Figure 1.15) (Miller WR, 2006; Lonning PE, 1996). In fact, studies have shown that apparently resistant tumours successfully responded to treatment with more potent AIs (Miller WR *et al.*, 2008; Thurlimann B *et al.*, 1997).

It has also been suggested that some breast cancer patients exhibit adverse pharmacokinetics to AIs (Ingle JN, 2008). The 1st generation AI aminoglutethimide has been shown to catalyse its own metabolism by activating liver cytochrome p450 enzymes (Santen RJ and Misbin RI, 1981). Drug interactions between AIs and tamoxifen are also quite frequent. Results from the ATAC study revealed that administration of anastrozole or letrozole in combination with tamoxifen substantially decreased the plasma levels of letrozole by 30 – 40% and of anastrozole by 20 – 30%, even though estrogen suppression did not seem to be affected by this, at least in the case of anastrozole (Dowsett M *et al.*, 1999a, 1999b, 2001).

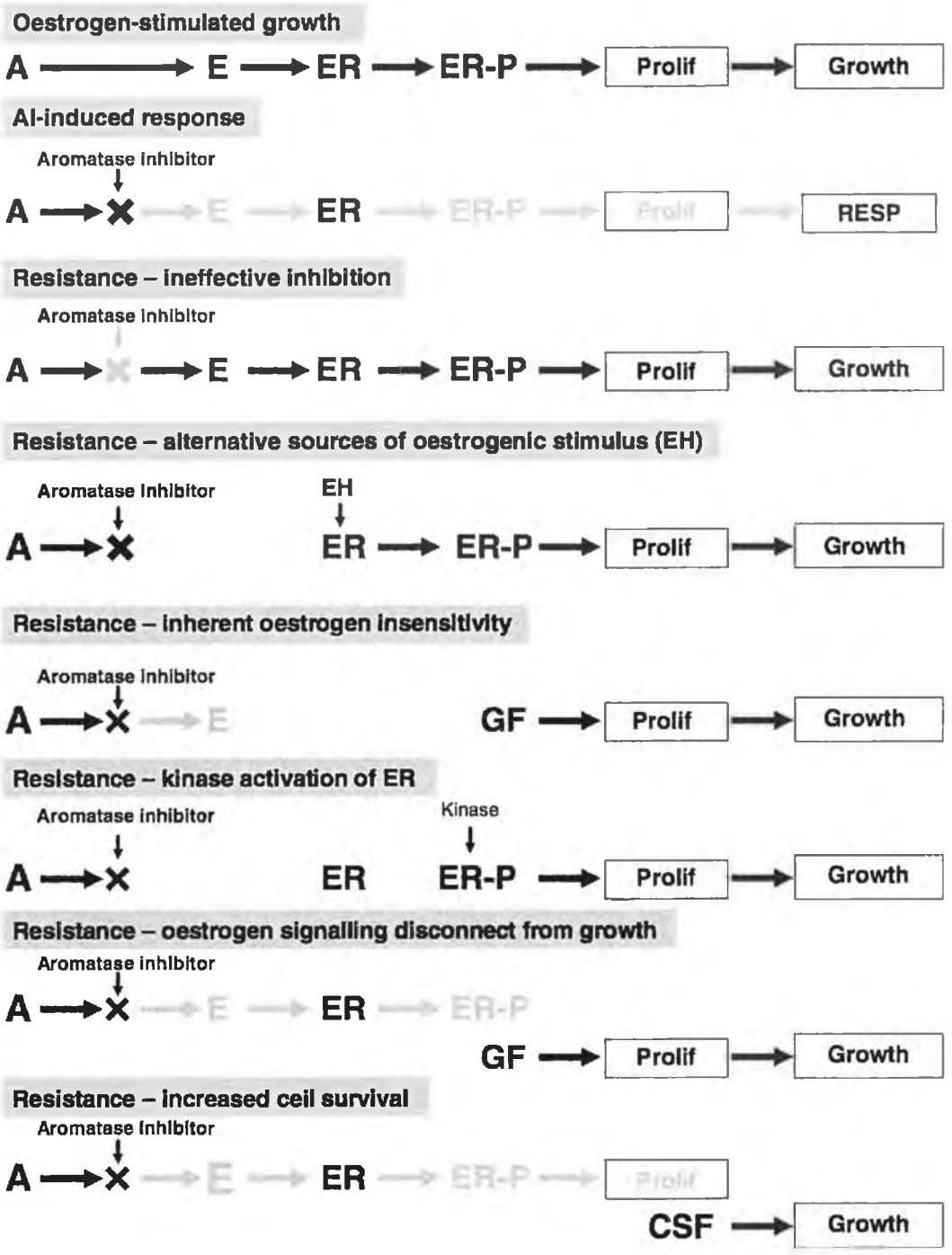


Figure 1.15: Mechanism of estrogen-stimulated proliferation, AI-induced response and mechanisms of AI resistance. A = androgen precursor, CSF = Cell survival factor, E = estrogen, EH = estrogenic hormone, ER = estrogen receptor, ER-P = phosphorylated ER, GF = growth factor, Prolif = proliferation, Resp = response. *Adapted from Expert Opinion Pharmacother. (Miller WR, 2010).*

Another possible reason for ineffective aromatase inhibition by AIs might be the expression of UDP-glucurosyltransferases, a family of enzymes that are involved in the deactivation and clearance of AIs (Lazarus P and Sun D, 2010).

High levels of aromatase enzyme might also prevent effective inhibition by the AIs, which might be especially the case in premenopausal women, who express high levels of aromatase enzyme in the ovaries. Compensatory feedback loops also cause a problem as they increase levels of gonadotrophins, which stimulate androgen synthesis and aromatase activity in the ovaries (Goss PE and Strasser-Weippl K, 2004). Therefore, AIs can only be given to premenopausal women when administered in combination with a luteneizing hormone releasing hormone (LHRH) agonist to inhibit the increase of gonadotrophins (Miller WR, 2004). Recent findings have suggested that two common functional polymorphisms in *CYP19,* the gene encoding aromatase, are linked to differences in response to AIs (Wang L *et al.*, 2010).

1.3.2.3 Alternative sources of estrogenic hormones

The third generation AIs have been praised for their specificity in only blocking aromatisation of androstenedione and testosterone into estrone and estradiol (Figure 1.15). However, this also means that synthesis of other steroids that can potentially interact with the ER to some extent, such as adrenal androgens, is not affected by these AIs. Also, AIs only block endogenous synthesis of estrogens and have no effect on exogenous estrogens, for example synthetic or phytoestrogens. Still, as AIs tend to be equivalent or even superior to other endocrine therapies such as tamoxifen, it is very unlikely that these alternative sources of estrogenic hormones are causing AI resistance (Miller WR, 2010).

1.3.2.4 Altered ER activity

Due to their mode of action, AIs are not used in the treatment of ER- tumours (Miller WR *et al.*, 2002). Surprisingly though, many AI resistant tumours are ER+, yet the tumour does not regress upon estrogen deprivation by AIs (Johnston SR and Dowsett M, 2003). A possible explanation for this phenomenon is that, in those breast cancer patients, ER is

The deregulation of these pathways is a result of genetic or epigenetic modifications, including amplification of HER2 and methylation of PTEN, a tumour suppressor that inhibits PI3K. However, deregulations of these pathways may also reflect aberrations in upstream regulators such as the activation of Akt in association with the loss of PTEN expression or overexpression of HER2 (Arpino G *et al.*, 2008). Loss of PTEN has also been shown to result in the activation IGF1R and HER3 (Miller TW *et al.*, 2009). However, it has not yet been fully elucidated how these events mediate resistance to tamoxifen.

Overexpression of HER2 is one of the best-characterised mechanisms of endocrine resistance (Arpino G *et al.*, 2008). Recent studies suggest that the loss of transcriptional repressors and amplification of *HER2* are responsible for increased expression of this receptor.

One of the reasons that cross talk between ER and growth factor pathways may lead to endocrine resistance involves the phosphorylation of co-activator proteins (Osborne CK *et al.*, 2003). An association between expression of the p160 proteins SRC-1 and AIB-1 and HER2 in a cohort or patients with breast tumour has previously been described (Fleming FJ *et al.*, 2004).

1.4 Hormone receptor signaling

Nuclear hormone receptors (NRs) are ligand-inducible, DNA-binding transcription factors (TFs) that can regulate gene expression by recruiting coregulators to gene promoters (Evans RM, 1988). Members of this superfamily respond to endocrine, paracrine and possibly autocrine signals and thus modulate diverse aspects of development, differentiation, homeostasis and behaviour in vertebrates (Leng X *et al.*, Hormones and Cancer, 1996). They can be divided into 3 families or classes:

The steroid receptor family (class I) includes all the classic steroid horomone receptors such as progesterone receptor (PR), estrogen receptor (ER), glucocorticoid receptor (GR), androgen receptor (AR) & mineralocorticoid receptor. In the absence of ligand, these receptors are complexed with heat shock proteins (HSPs) and are not able to bind DNA. Hormone binding to the receptor causes the HSPs to dissociate, resulting in activation and dimerisation of receptor proteins (Leng X *et al.*, 1996). The homodimer is now able to bind to the hormone response element (HRE) on the DNA and either stimulate or repress transcription of target genes.

The thyroid/retinoid family (class II) includes thyroid receptor (TR), vitamin D receptor (VDR), retinoic acid receptor (RAR) & peroxisome proliferator-activated receptor (PPAR) and its members are located in the nucleus where they are consistently associated with the chromatin. These receptors are able to bind to HREs as homo- or heterodimers in the absence of ligand, but hormone binding radically changes the activity of this class of nuclear receptors (Leng X *et al.*, 1996).

The orphan receptor family (class III) is a set of proteins, which was identified as members of the nuclear receptor family by comparative sequence analysis; their cognate ligands however remain unknown. This class of receptors can bind to DNA as monomers and may potentially be constitutively active or regulated through other mechanisms such as posttranslational modification (Huang P *et al.*, 2010). It is not clear though if these receptors can also form homodimers or heterodimerise with other NRs (Leng X *et al.*, 1996).

1.4.1 Estrogen Receptor

Estrogens, such as 17ß-estradiol (E2), are cyclically secreted in adult women from puberty until menopause and have been shown to influence physiology and behaviour of females. They are known to be key promoters of cell proliferation, both in normal and neoplastic breast epithelium, and mediate their cellular effects via binding to the estrogen receptor (ER) (Jensen EV *et al.*, 2010). Two ERs, ERα and ERβ, have been identified to date and are encoded by separate genes: ESR1 and ESR2. ERα was identified in the late 1950s and purified a couple of years later (Jensen EV and Jordan VC, 2003). Physiologically, ERs play an important role in the reproductive system, bone metabolism as well as the maintenance of the cardiovascular and central nervous system (Chen GG *et al.*, 2008). They function as estrogen-driven transcription factors and are involved in the stimulation of target genes that are implicated in the regulation of cell cycle progression and growth of breast epithelium (Singh RR and Kumar R, 2005).

1.4.1.1 Structure and functional features

All nuclear receptors are part of the zinc finger family of transcription factors and share common structures with domains of distinct functions, suggesting that they are all evolutionary linked and may be derived from the same ancestral receptor (Leng X *et al.*, 1996). Intermolecular sequence analysis and mutational dissection of NRs led to the definition of six functional regions (A-F) (Figure 1.16): The A/B domain at the N-terminus contains the autonomous transactivation function AF-1. The highly conserved C region harbours the DNA binding domain (DBD), which mediates the binding of the receptor to the DNA following ligand activation (Webster NJ *et al.*, 1988). The conserved E region contains a second transcriptional activation function (AF-2) in the C-terminal ligand-binding domain (LBD). The LBD mediates interaction between the receptor and its ligand. Both AF domains are able to recruit a range of co-regulatory proteins to the DNA bound receptor, the only difference is that AF-1 is regulated by phosphorylation and its activity is hormone-independent whereas AF-2 is hormone-dependent. They usually act synergistically but some gene promoters can be independently transactivated by either of the AFs (Osborne CK *et al.*, 2001). The two remaining regions, D and F, are of variable

size and are not conserved: D can be considered as a linker peptide between the DBD and the LBD, whereas F is a C-terminal extension region of the LBD (Ruff M *et al.*, 2000).

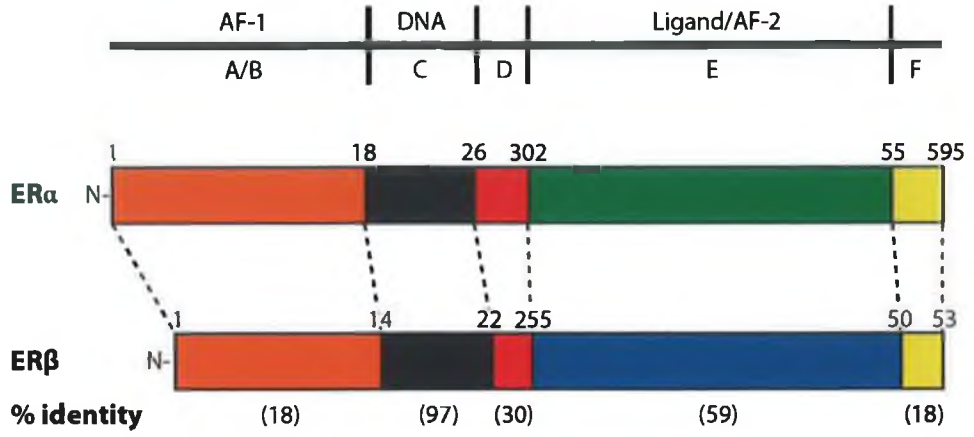


Figure 1.16: Structural organization of NRs consists of six functional regions (A-F). Shown are the relationships of their amino acid sequences, including the level of conservation (in parentheses). *Adapted from Annual Review of Physiology (Huang P et al., 2010).*

1.4.1.2 ER in Breast Cancer

The discovery of the estrogen receptor (ER) as a mediator of estrogen's biological effects revolutionized the understanding of breast cancer biology and led to the classification of breast cancer into two groups: ER positive (ER+) and ER negative (ER-). Each of these groups is understood to have their own distinct biological and clinical features.

The lack of ER is associated with a more aggressive type of breast cancer. Patients who are ER+ tend to be older and seem to have a better overall chance of survival compared to ER- patients (Osborne CK *et al.*, 1980). The presence of ER is also an excellent predictor of response to endocrine therapy with two third of ER+ tumours responding to the anti estrogen tamoxifen.

1.4.2 Estrogen mediated signaling pathways

Estrogen binding to its receptor results in a diverse range of physiological and pathological cell functions (Ascenzi P *et al.*, 2006; Pearce ST and Jordan VC, 2004). The

mechanism of its action, however, can generally be categorised into two pathways: genomic and non-genomic (Figure 1.17).

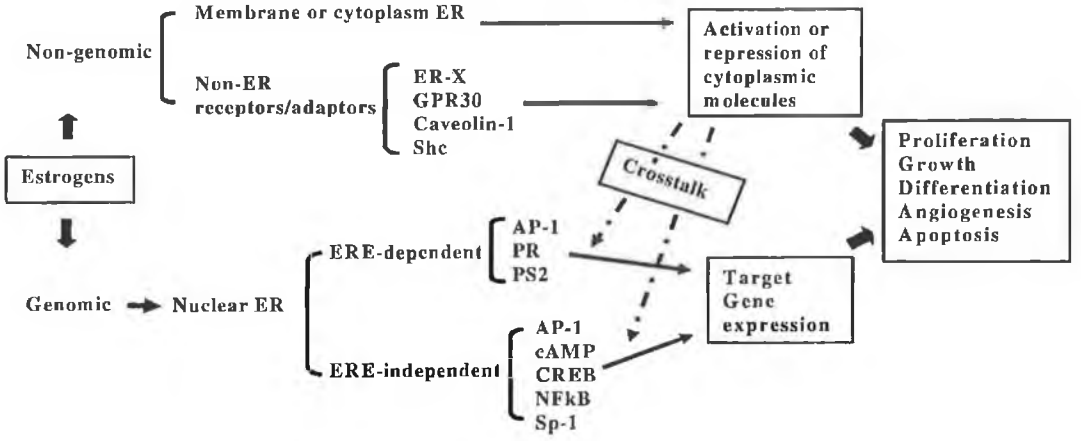


Figure 1.17: Estrogen-mediated genomic and non-genomic pathways. *Adapted from Medicinal Research Reviews (Chen GG et al., 2008).*

1.4.2.1 Genomic pathway

This pathway is commonly known as the classical pathway as it describes the mechanism by which estrogen exerts its function via ERα and ERβ (Figure 1.17). It involves estrogen-dependent formation of nuclear ER homo- or heterodimers, followed by binding of the ligand-receptor complex to the estrogen response element (ERE) sequence in the promoter region of estrogen responsive genes, resulting in recruitment of coregulatory proteins to the promoter. This leads to an increase or decrease in mRNA levels, depending on the type of coregulatory protein recruited (co-activator or co-repressor respectively) followed by the production of a protein and eventually a physiological effect (Chen GG *et al.*, 2008). This process usually happens over the course of hours.

There is growing evidence that both ERs can regulate transcription of certain genes with the aid of other DNA-bound transcription factors independent of ERE (Ascenzi P *et al.*, 2006; Pearce ST and Jordan VC, 2004). This observation may explain why one third of estrogen-stimulated genes lack functional EREs (Pietras RJ and Márquez-Garbán DC,

2007). Examples for non-ERE DNA-bound transcription factors that interact with ERs are AP-1, Fox and NF-κB (Cvoro A *et al.*, 2006; Carroll JS and Brown M, 2006).

1.4.2.2 Non-genomic pathway

In contrast to the genomic pathway, estrogen acts either via ER located in or adjacent to the plasma membrane or via other non-ER plasma membrane-associated estrogen-binding proteins (Figure 1.17) (Pietras RJ and Márquez-Garbán DC, 2007; Levin ER, 2005). Estrogen action in the non-genomic pathway results in cellular responses like increased levels of calcium and the activation of intracellular kinase cascades such as mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K) as well as protein kinase A (PKA) and C (PKC). So called adaptor proteins like caveolin-1 or Shc may target the ER to the plasma membrane.

AP-1 response elements may be regulated indirectly via interactions between the ERs and c-fos and c-jun, two known AP-1 transcription factors. AP-1 dependent transcription is also thought to be directly activated by binding of estradiol to ERs in the cytoplasm that can form complexes with transcription factors such as SRC-1, p300 or pol II (Cascio S *et al.*, 2007; Chen GG *et al.*, 2008). These TFs can regulate genes that are implicated in cellular processes like proliferation, differentiation, cell motility and apoptosis. Events in this pathway occur in a span of a few minutes, meaning it is too fast to be mediated by biosynthesis of RNA or proteins (Chen GG *et al.*, 2008).

1.4.2.3 G-protein-coupled receptor-30

Recent studies have found a range of new estrogen receptors. One of them, the G-protein-coupled receptor-30 (GPR30), has been shown to play a role in carcinogenesis and metastasis (Prossnitz ER *et al.*, 2008; Filardo EJ *et al.*, 2008). Studies conducted in the SKBR3 breast cancer cell line showed that estrogen signalling via the GPR30 can activate MAPK and ERK1/2 and transactivate the epidermal growth factor receptor (EGFR) independent of the estrogen receptor (Filardo EJ *et al.*, 2008; Thomas P *et al.*, 2005). Interestingly though, GPR30 does not play a role in estrogen mediated MAPK activation in MCF-7 breast cancer cells, suggesting that the GPR30-mediated estrogen

signaling is exclusive to some cell lines (Pedram A *et al.*, 2006). This signalling mechanism is similar to the non-genomic signalling mechanism and has therefore been established as an alternative pathway.

1.4.2.4 Non-genomic pathway crosstalk to genomic pathway

It has also been suggested that crosstalk can occur between the genomic and non-genomic pathways (Levin ER, 2005; Pietras RJ and Márquez-Garbán DC, 2007). It has been shown that the non-genomic activation of signalling pathways in the cytoplasm may regulate gene expression independent of an ERE. For example, the non-genomic patheway can activate MAPK to phosphorylate and recruit certain coactivators such as SRC-1, which in turn enhances nuclear ER transcriptional activity (Chen GG *et al.*, 2008).

1.4.3 Coactivators

Initially it was thought that NRs facilitated general transcription factors (GTFs) and RNA polymerase II to assemble at the promoter site to initiate mRNA synthesis (Xu J wu omalley 2009). In the early 1970s, scientists started to look for nuclear non-histone helper proteins that were thought to aid the binding to DNA and the transcriptional function of NRs (Spelsberg TC *et al.*, 1971). It was found that the activation of an overexpressed NR could indirectly inhibit the transcriptional activity of another NR, suggesting that steroid receptors may compete for factors that mediate their enhancer function (Meyer ME *et al.*, 1989). Moreover, it was found that *in vitro* transcription systems consisting of purified NRs and GTFs were inefficient indicating that additional transcription activators were needed for efficient hormone-stimulated transcriptional activation (Klein-Hitpass L *et al.*, 1990).

The first nuclear receptor coactivator (NCoA1) was cloned in 1995 and was aptly named steroid receptor coactivator-1 (SRC-1). It interacted with hormone receptors in a steroid-dependent manner and strongly increased the transcriptional activities of steroid receptors (Onate SA *et al.*, 1995). Two homologous proteins were characterised as steroid receptor coactivators shortly after: SRC-2 or NCoA2, which is also known as TIF2 or GRIP1 (Voegel JJ *et al.*, 1996; Hong H *et al.*, 1996) and SRC-3 or NCoA3, which is

frequently called AIB1 but is also known as p/CIP, RAC3, ACTR or TRAM-1 (Anzick SL, 1997; Torchia J *et al.*, 1997; Li H *et al.*, 1997; Chen H *et al.*, 1997; Takeshita A *et al.*, 1997). These three proteins comprise the p160 family of steroid receptor coactivators, named so because they are all approximately 160kD in size (Darimont BD *et al.*, 1998). To date, over 300 coactivators have been identified and are associated with a range of diseases (Lanz RB *et al.*, 2008; Xu J *et al.*, 2009). They are defined by their inability to bind to DNA, clearly differentiating them from classic transcription factors (Johnson AB, 2011).

Coactivators were initially described as molecules that bridge NRs to the general transcription machinery. Even though this is their primary function, coactivators can also modify chromatin within promoter and enhancer regions or enable secondary coactivators, so called co-coactivators, that modify the chromatin in a manner that supports binding of enhancer regulatory proteins and GTFs, for example via histone acetylation and specific sites of histone methylation (Figure 1.18). Coactivators are a diverse group of proteins and lack a uniform structure such as that found in the super family of nuclear receptors (Johnson AB, 2011).

Figure 1.18: SRC-mediated coactivation of NRs. SRC proteins are recruited to hormone bound NRs and bind via their three LXXLL motifs. SRCs can recruit multiple secondary coactivator complexes by binding to their three activation domains (ADs). Three examples are shown: histone acetyltransferase, p300/CBP; histone methyltransferases, PRMT1 and CARM1; and chromatin remodeling complex, SWI/SNF. These secondary coactivators modify the chromatin and bridge the NR complex with the general transcription machinery to elicit transcriptional activation. SRCs (steroid receptor coactivators); bHLH/PAS (basic helix-loop-helix/Per-Arnt-Sim); S/T (serine/ threonine-rich region); NR (nuclear receptor); Ac (acetylation); Me (methylation); HRE (hormone response element); L (LXXLL motifs). *Adapted from Molecular and Cellular Endocrinology (Johnson AB, 2011).*

In 1997 however, Heery and Torchia identified a motif within several coactivators including the p160 family of SRCs (Heery DM *et al.*, 1997, Torchia J *et al.*, 1997). It was named LXXLL (L = leucine, X = any amino acid) motif or NR box and binds to the hydrophobic pocket created within helix 12 of the LBD upon ligand binding. Heery *et al.* showed that mutation of these residues prevents binding of SRC-1 to the LBD in ERα *in vitro* and SRC-1-mediated activation of the receptor *in vivo* (Heery DM *et al.*, 1997). Nevertheless, a recent analysis of nuclear receptor coregulator motifs revealed that of 303 coregulators only 149 had at least one LXXLL motif meaning while it is a common motif amongst coregulators it is not collectively shared (Lanz RB *et al.*, 2008).

1.4.3.1 p160 family of steroid receptor coactivators

The most extensively studied family of coactivators is the p160 family of steroid receptor coactivators. The founding member of the p160 family, SRC-1, was identified in a yeast two-hybrid screen as a protein that interacts with the progesterone receptor LBD (Onate SA *et al.*, 1995). SRC-2 was found shortly after as a protein that interacted with a variety of NRs (Hong H *et al.*, 1997; Voegel JJ *et al.*, 1996). The third and last member of the family, SRC-3, was cloned by numerous labs and therefore has many different names, but due to the fact that it was found to be amplified in breast cancer it received the fitting name amplified in breast cancer 1, AIB-1 (Anzick SL, 1997). The p160 proteins are all approximately the same size (160 kD) and share 50-55% sequence similarity with a range of structural domains conserved (Kim JH and Stallcup MR, 2008).

1.4.3.2 Structure and function of steroid receptor coactivators

SRCs contain three structural domains. The basic helix-loop-helix-Per/ARNT/Sim (bHLH-PAS) domain at the N-terminus is the most conserved one (75% similarity and 60% identity) and is required for protein-protein interactions (Kim JH and Stallcup MR, 2008). It can interact with various transcription factors and co-coactivators as shown in Figure 1.19 to potentiate transcription (Kim JH *et al.*, 2003; Belandia B *et al.*, 2000). The bHLH-PAS contains nuclear import and export signals as SRCs localise in the nucleus as well as the cytoplasm (Amazit L *et al.*, 2003). The domain is also essential for proteasome-

dependent turnover of the coactivators (Li C *et al.*, 2007). The central region of the protein contains three LXXLL motifs and counts for direct interaction with liganded NRs (Heery DM *et al.*, 1997). The C-terminus is made up of two transcriptional activation domains, namely AD1 and AD2. The AD1 domain helps in the recruitment of CBP and p300 histone acetyltransferase (HAT) to the chromatin context by direct binding which is essential for SRC-mediated transcriptional activation. AD2 can interact with histone methyltransferases (HMTs) such as coactivator-associated arginine methyltransferase 1 (CARM1) or protein arginine methyltransferases (PRMT1) (Yao TP *et al.*, 1996; Koh SS *et al.*, 2001). Both SRC-1 and SRC-3 contain weak c-terminal HAT activity, but the importance of this activity and its cellular substrates has not been completely identified to date (Spencer TE *et al.*, 1997; Chen H *et al.*, 1997).

These structural and functional features provide the SRCs with a suitable base for the recruitment of additional coregulators or GTFs, resulting in chromatin remodeling, assembly of GTFs and recruitment of RNA Polymerase II (Xu J *et al.*, 2009).

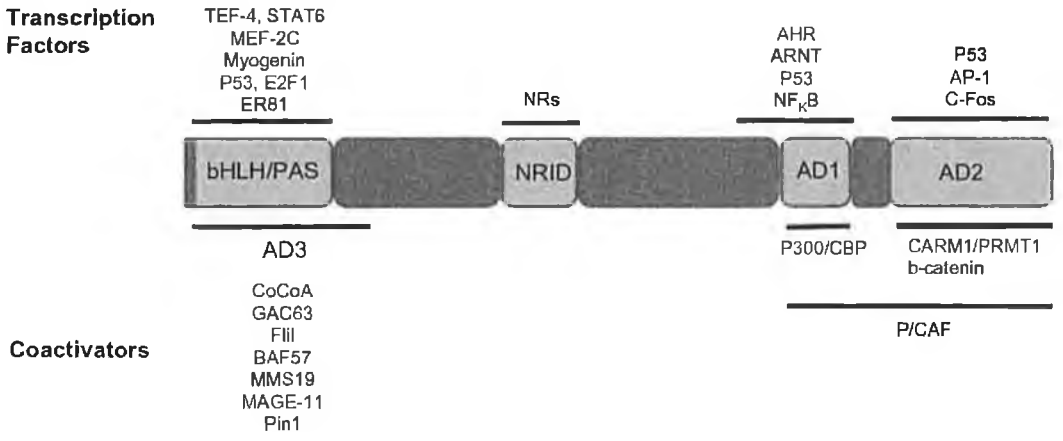


Figure 1.19: SRC-interacting proteins. SRCs coactivate nuclear receptors (NRs), as well as numerous transcription factors. Once tethered to chromatin via these interactions, SRCs recruit a number of secondary coactivators that interact with its activation domains (ADs). This is a representative list of just some of SRC's interacting proteins, of which SRC-interacting domains have been mapped. *Adapted from Molecular and Cellular Endocrinology (Johnson AB, 2011).*

Not only do SRCs serve as coactivators for NRs but they can also serve as coactivators for other TFs such as NF-κB, STATs, p53 and C-Fos as well as secondary coactivators (frequently called co-coactivators) such as p300, CARM1 or CoCoA (Figure 1.19). Another role of SRCs is to promote gene transcription by interacting with kinases, phosphatases, ubiquitin/SUMO ligases, HATs and HMTs.

1.4.3.3 Posttranslational modifications of SRCs

The levels of SRCs inside a cell are not necessarily the sole determinants of their physiological as well as pathological functions. Several studies have suggested that signaling pathways stimulated by either hormones, GFs or cytokines can induce post-translational modifications of steroid receptor coactivators, such as phosphorylation, acetylation, methylation, ubiquiylation and sumoylation (Figure 1.20). These modifications play important roles in determining protein stability, TF interaction specificity and transcriptional activity of SRCs. Deregultated posttranslational modification of SRCs has considerable implications In cancer (Xu J *et al.*, 2009).

Phosphorylation is a temporally controlled, tightly regulated and well-characterised event and leads to conformational changes at particular coactivator sites, resulting in the creation of surface binding sites for other proteins (Li S and Shang Y, 2007).

Phosphorylation changes the coactivator's affinity for particular NRs, resulting in a change in NR-dependent gene expression (Lopez GN *et al.*, 2001; Giamas G *et al.*, 2009). A range of phosphorylation sites has been identified in the p160 coactivator family. SRC-1 contains seven phosphorylation sites, namely Ser-372, Ser-395, Ser-517, Ser-569, Ser-1033, Thr-1179 and Ser-1185, all of which hold a consensus sequence for proline-directed protein kinases (Li S and Shang Y, 2007). For example, phosphorylation of SRC-1 on Thr1179 and Ser1185 can be induced by stimulation with EGF, interleukin 6 (IL-6) and cyclic AMP and it has been demonstrated that phosphorylated SRC-1 has higher coactivator function in ligand-dependent as well as ligand-independent NR pathways (Rowan BG *et al.*, 2000; Ueda T *et al.*, 2002). MAPK-mediated phosphorylation on the same sites has been shown to increase the affinity of SRC-1 for AR in prostate cancer, suggesting its contribution to prostate cancer recurrence (Ueda T *et al.*, 2002; Gregory

CW *et al.*, 2004). The oncogenic kinase Src has been seen to mediate SRC-1 phosphorylation in endometrial cancer cells, causing a significant increase in the agonist activity of the SERM tamoxifen, indicating that the coactivator may have a role in tamoxifen-induced endometrial proliferation and the development of endometrial cancer that is associated with the use of the drug (Shang Y and Brown M, 2002; Shah YM and Rowan BG, 2005).

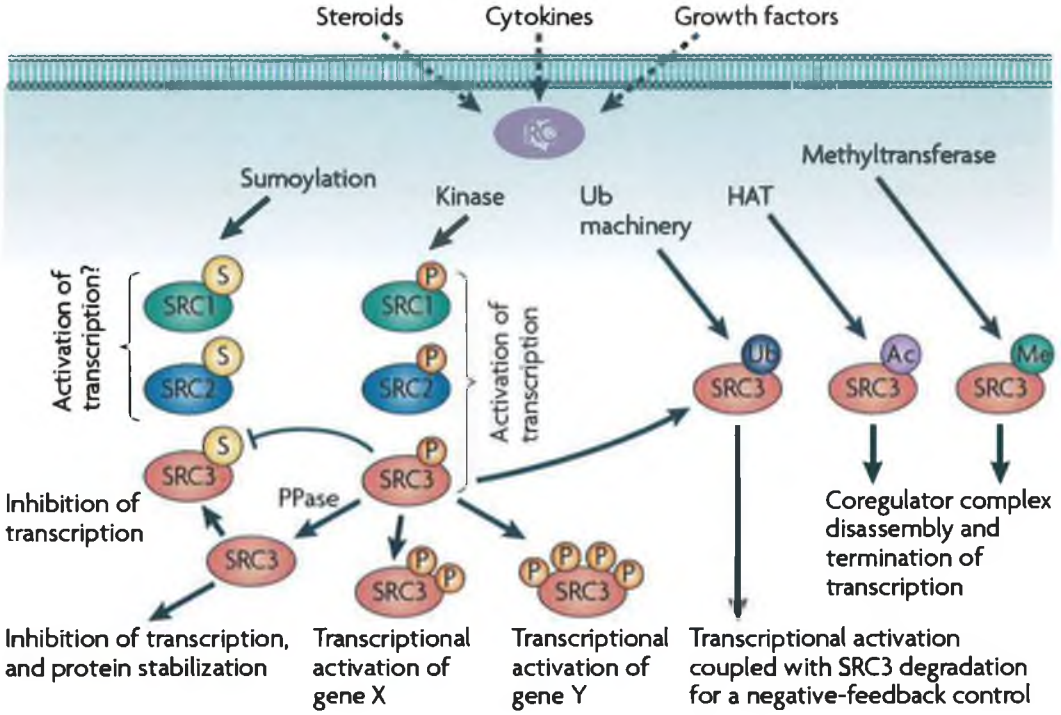


Figure 1.20: Posttranslational modifications of SRCs. Phosphorylation results in activation of SRCs. Phosphorylation of SRC-3 determines its selectivity for different TFs, promotes sequential ubiquitination from mono- (activation) to poly-ubiquitination (degradation), and controls the duration of transcriptional activation by SRC-3. Conversely, de-phosphorylation by PPase promotes its sumoylation, stabilizes it and inhibits its activity. *Adapted from Nat. Rev. Cancer (Xu J et al., 2009).*

SRCs are regulated by subcellular localisation and intracellular trafficking and phosphorylation can change the availability of a certain coactivator in a subcellular compartment by regulating its trafficking. EGF-induced phosphorylation of SRC-3 for example can redistribute the coactivator from the cytoplasm to the nucleus (Wu RC *et*

al., 2002). Estrogen-induced phosphorylation of SRC-3 on the other hand leads to an interaction between the coactivator and ERα, indicating that activation of SRC-3 may have a role in non-genomic estrogen receptor signaling (Zheng FF *et al.*, 2005).

Ubiquitination, also refered to as ubiquitylation, is involved in the fine-tuning of a wide range of substrates and entails further complexity and a broader range of biochemical effects than phosphorylation (Kodadek T el al, 2006; Dennis AP and O'Malley BW, 2005). There are two forms of ubiquitination that can result in two very different outcomes: the addition of a single ubiquitin is a reversible process and regulates the activity and transportation of various cellular proteins (Gill G, 2004; Staub O and Rotin D, 2006); addition of a polyubiquitin chain on the other hand is a irreversible process and results in selective degradation of proteins via the 26S ubiquitin-proteasome pathway (Glickman MH and Ciechanover A, 2002; Pickart CM 2004). It was found that 26S proteasome-degradation regulates the function of NRs and the turnover of activated NRs and SRCs (Shao W *et al.*, 2004; Yan F *et al.*, 2003; Lonard DM *et al.*, 2000). *In vitro* experiments revealed that the p160 coactivators were expressed at a higher level and exhibited an increase in transcriptional activity in the presence of a proteasome inhibitor (Yan F *et al.*, 2003; Lonard DM *et al.*, 2000; Baumann CT *et al.*, 2001).

Sumoylation is another mechanism that can modify SRCs. The enzymatic machinery that adds and removes the small ubuiqtin-like modifier (SUMO) is mechanistically very similar to the ubiquitination machinery (Kotaja N *et al.*, 2002; Chauchereau A *et al.*, 2003; Wu H *et al.*, 2006). However, sumoylation differs from ubiquitination in that it does not promote degradation of its substrates but is involved in modifying their function and directing their localisation in the cell (Hay RT, 2005; Johnson ES, 2004; Melchior F *et al.*, 2003; Schwartz DC *et al.*, 2003). All three members of the p160 coactivators share two common sumoylation sites in the NID: SRC-1 at Lys-732, 774, SRC-2 at Lys-731, 788 and SRC-3 at Lys-723, 786. As an example, sumoylation of SRC-1 at Lys-732 and Lys-774 has been shown to result in an increase in interaction with PR and a prolonged nuclear retention (Chauchereau A *et al.*, 2003).

Acetylation causes the addition of a relatively small acetyl group (Clayton AL *et al.*, 2006; Eberharter A and Becker PB, 2002; Freiman RN and Tjian R, 2003). It was initially thought

that acetylation of histones aids chromatin remodelling and transcriptional activity, however recent studies have revealed other HAT substrates besides histones, such as hormone receptors and coactivators representing a dynamic regulatory mechanism in hormone signaling (Chen H *et al.*, 1999; Fu M *et al.*, 2000; Grunstein M, 1997; Vidali G *et al.*, 1968; Wang C *et al.*, 2001).

Methylation, a process that is known to play an important role in histone modification and transcriptional enhancement (Bannister AJ *et al.*, 2002), has recently been found to repress transcription through modifications of coactivators such as CBP/p300 (Lee YH *et al.*, 2005) but also SRCs (Feng Q *et al.*, 2006; Naeem H *et al.*, 2006). All three members of the p160 proteins have been identified as substrates for CARM-dependent methylation, but only SRC-3 is relatively well-studied (Li S and Shang Y, 2007). SRC-3 contains a methylation site at Arg-1171, which is located in a CARM1-binding site and it has been shown that estrogen induces CARM1-mediated SRC-3 methylation at that site resulting in the termination of transcription by disassembling the SRC-3 coactivator complexes and increasing SRC-3 degradation (Feng Q *et al.*, 2006; Naeem H *et al.*, 2006).

It needs to be mentioned that different combinations of post-translational modifications are very common. They can determine the coactivator potency and selectivity of the SRCs and allow them to incorporate a multitude of upstream signals into the delicately orchestrated regulation of gene expression. Since post-translational modifications influence cellular concentrations as well as activities of the SRCs, altering or inhibiting those modifications may be important in controlling overexpressed SRCs in cancer (Xu J, 2009).

1.4.3.4 In vivo functions of SRC family members

The current understanding of the diverse *in vivo* functions of the p160 family members has mainly been gathered from knockout mouse model experiments. The results of these studies suggest that each member has both specific and redundant physiological functions in embryonic and adult mice (Xu J *et al.*, 2009). Although *Src1*$^{-/-}$ mice showed no major defects in development and growth, it came to light that SRC-1 plays an important *in vivo* role in organ physiology. In mammary gland development SRC-1 has

been shown to be required for normal mammary duct elongation during puberty and alveolar development during pregnancy (Walsh CA *et al.*, 2012). Experiments conducted in SRC-1 null mice have established that ductal density as well as alveoli number is reduced in those animals, yet they are still able to produce milk (Xu J et al., 1998).

The additive severity observed in double-knockout mice points towards the existence of cooperative physiological functions among SRC family members. Most *Src1*$^{-/-}$;*Src2*$^{-/-}$ mice do not survive and *Src1*$^{+/-}$;*Src2*$^{-/-}$ mice are all infertile (Mark M *et al.*, 2004). SRC-1 and SRC-3 cooperatively regulate viability, metabolism and energy balance (Xu J *et al.*, 2009). Most *Src1*$^{-/-}$;*Src3*$^{-/-}$ mice die before birth and mice that do survive exhibit compromised regulation of select peroxisome proliferator-activated receptor-γ (PPARγ) target genes that are involved in adipogenesis and mitochondrial uncoupling. These mice are leaner and resistant to obesity due to a high basal metabolic rate and increased physical activity caused by high leptin levels, a defect in adaptive thermogenesis and a developmental arrest in interscapular brown fat (Wang Z *et al.*, 2006). In normal human breast, protein levels of the three SRCs in epithelial cells are variable but they are generally almost undetectable (Fleming FJ *et al.*, 2004b; Hudelist G *et al.*, 2003; List HJ *et al.*, 2001).

1.4.3.5 SRC expression and function in cancer

Coactivators are associated with a wide range of human diseases, including metabolic syndrome and several types of cancers (Lanz RB *et al.*, 2008). All three p160 SRCs have been found to be overexpressed in several types of human cancer, particularly in steroid hormone-dependent breast and prostate cancer. *SRC3* on human chromosome 20q21 is frequently amplified in cancer while amplification of either *SRC1* or *SRC2* is fairly rare (Xu J *et al.*, 2009). This thesis will mainly focus on the role of SRC-1 in breast cancer.

Many studies have looked into the mechanisms through which SRCs promote carcinogenesis and suggest that SRCs have important and distinct roles in promoting cancer initiation, progression and metastasis through alteration of multiple signaling pathways (Xu J *et al.*, 2009) (Figure 1.21). The precise mechanisms that are involved in the overexpression of SRCs in human cancers, however, are still unclear.

1.4.3.5.1 SRC-1

Several studies established that SRC-1 is significantly elevated in up to 30% of breast tumours and an increase in SRC-1 positively correlates with HER2 expression, lymph node metastasis, disease recurrence and poor disease-free survival (DFS) (Fleming FJ *et al.*, 2004a; Fleming FJ *et al.*, 2004b; Myers E *et al.*, 2004; Hudelist G *et al.* 2003).

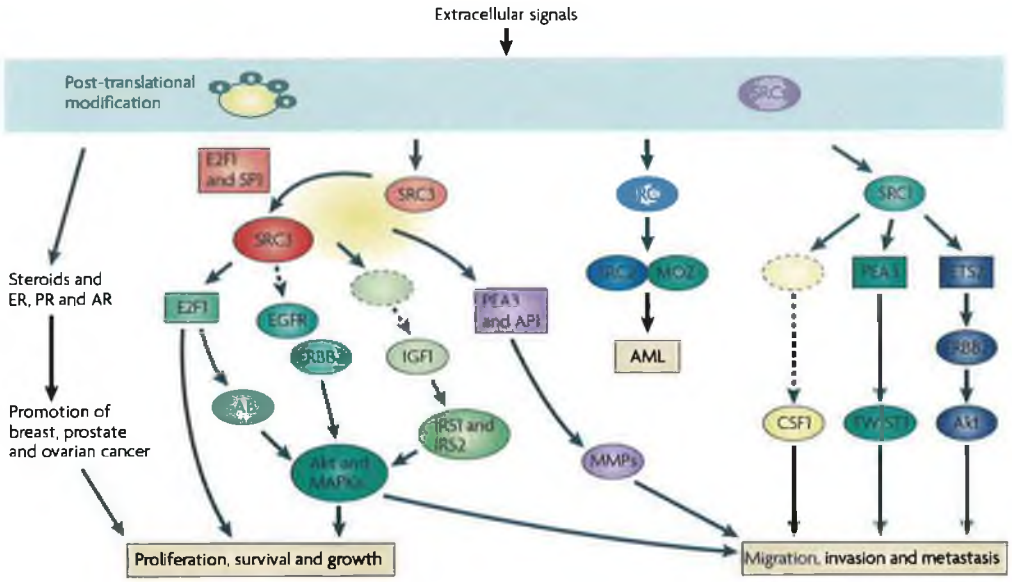


Figure 1.21: SRCs promote carcinogenesis through multiple pathways. Extracellular signals and their pathways cause posttranslational modifications that regulate the cellular concentrations, activities and specificities of SRCs. In general, SRCs enhance steroid receptor functions and enable hormonal promotion of breast, prostate and ovarian cancers. Specifically, SRC-1 enhances Ets2-mediated HER2 expression and PEA3-mediated Twist expression and upregulates CSF-1 expression to promote breast tumour cell migration, invasion and metastasis. SRC-3 upregulates its own expression by serving as a coactivator for E2F1 and SP1. The overexpressed SRC-3 enhances PEA3 and AP-1-mediated MMP expression to promote breast and prostate tumour cell metastasis. SRC-3 also enhances E2F1-mediated cell cycle progression and GAB2 expression that activates Akt. In addition, SRC-3 upregulates IGF-1, IRS-1 and IRS-2 to promote the IGF-1 signaling pathway and to activate EGFR and ERBB2 to enhance Akt and MAPK activities, resulting in hyperactivation of Akt and MAPK which contribute to cancer cell proliferation, growth, survival, invasion and metastasis. *Adapted from Nat. Rev. Cancer (Xu J et al., 2009).*

A clinical study conducted in our lab also revealed elevated SRC-1 to be an independent predictor of breast cancer recurrence following endocrine treatment with tamoxifen (Redmond A *et al.*, 2009).

In vitro, SRC-1 is believed to affect cancer cell proliferation and invasiveness through several different pathways. SRC-1 overexpression in MCF-7 cells potentiates estrogen-induced cell proliferation along with an Increase in estrogen-responsive genes, indicating that SRC-1 plays an important role in ER-mediated proliferation (Tai H *et al.*, 2000). Knockdown of SRC-1 in MCF-7 cells reduced proliferation as well as estrogen-dependent DNA synthesis and pS2 gene expression (Cavarretta IT *et al.*, 2002).

A mouse mammary tumour virus (MMTV) model of breast cancer revealed that loss of SRC-1 does not affect tumour initiation or proliferation, but significantly inhibits tumour metastasis to the lung (Wang S *et al.*, 2009). This is due to the loss of colony stimulating factor 1 (CSF-1), a protein that is involved in the recruitment of tumour-associated macrophages, and loss of PEA3-induced expression of the epithelial-mesenchymal transition (EMT)-promoting gene TWIST (Johnson AB and O'Malley BW, 2012). A study carried out in our own lab revealed that SRC-1 and PEA3 significantly associated with tumour recurrence in a cohort of 70 HER2-positive primary breast tumour patients (Fleming FJ *et al.*, 2004b). An *in vitro* experiment was carried out in our lab to assess associations and interaction between Ets and coregulatory proteins in breast cancer cell lines. It was found that the growth factors EGF and bFGF increase the association of Ets factors with their DNA binding elements as well as SRC-1 resulting in an increase in transcription (Myers E *et al.*, 2005). These findings offer a potential pathway for SRC-1-mediated activation of target genes independent of estrogen signaling and thus making it a great prognostic indicator.

1.4.3.5.2 SRC-2

To date, there has only been a few reports suggesting a potential role for SRC-2 in oncogenesis and a lot of them are contradicting each other. Whereas one group reported no significant change in SRC-2 protein levels between normal and breast cancer tissue (Hudelist G *et al.*, 2003) another revealed a significant correlation between

all members of the p160 family and cyclin D1 expression in ER+ breast tumours (Girault I *et al.*, 2003). SRC-2 has also been implicated in other cancers such as meningiomas, prostate tumours and acute myeloid leukemia (AML) (Gregory CW *et al.*, 2001; Troke PJ *et al.*, 2006). These initial results propose a need for more research into the role of SRC-2 in breast carcinogenesis.

1.4.3.5.3 SRC-3

NCOA3, the gene encoding SRC-3, is amplified in approximately 5-10% of breast cancer cases, while its mRNA is overexpressed in about 30-60% (Anzick SL *et al.*, 1997). It is associated with larger tumour size, higher tumour grade and worse DFS (Xu J *et al.*, 2009). Studies have revealed that SRC-3 overexpression is associated with resistance to tamoxifen in HER2-positive tumours (Kirkegaard T *et al.*, 2007), making it a useful prognostic and diagnostic marker for breast cancer. It has also been shown that SRC-3 is involved in tumour initiation, as overexpression of the coactivator in mice resulted in increased tumorigenesis (Torres-Arzayus MI *et al.*, 2004) and loss of SRC-3 disrupted tumorigenesis in experiments conducted in a tumour mouse model (Kuang SQ *et al.*, 2004). Also, results from SRC-3 knockout mice revealed a role for SRC-3 in the promotion of tumour metastasis to the lung by coactivating PEA3 and subsequently upregulating the expression of the matrix metalloproteinases MMP2 and MMP9 (Qin L *et al.*, 2008).

1.4.4 Ets family of transcription factors

A variety of signaling cascades that transduce extracellular signals from ligand-activated cell surface receptors to the nucleus coordinate cellular responses to environmental stimuli. Even though most pathways were thought to be linear, it has become clear that there is a dynamic interplay between signaling pathways that result in cell-type specific responses necessary for proliferation, differentiation and survival. An example for a group of nuclear effectors of these signaling pathways is the Ets family of transcription factors (Yordy JS *et al.*, 2000). The Ets family consists of a large number (25 human and 26 murine) of evolutionary conserved transcription factors. They control specific genes that play key roles in physiological as well as tumorigenic processes, such as

proliferation, apoptosis, differentiation, lymphoid cell development, angiogenesis and invasion (Sementchenko VI and Watson DK, 2000). Studies in this field have focused on individual Ets family members, however, more recent findings suggest that a variety of Ets factors come together in a coordinated program that affects tumour progression towards metastasis (Hsu T *et al.*, 2004).

Discovery of the avian leukemia virus oncogene v-ets in 1983 uncovered a large family of coserved genes (Leprince D *et al.*, 1983). The Ets proteins were finally identified as transcription factors that share a conserved DNA binding domain (ETS domain) that recognises unique DNA sequences containing GGAA/T, so called Ets binding sites (EBS). Some Ets proteins contain a HLH domain for protein-protein interactions called Pointed (PNT) domain. They can act as positive or negative regulators of genes that are involved in a number of biological processes such as proliferation, differentiation, angiogenesis and tissue remodeling. Over 200 Ets genes have been identified to date and the number of genes regulated via EBS is constantly growing (Sementchenko VI and Watson DK, 2000).

1.4.4.1 Modulation of Ets function

It has been shown that protein-protein interactions of Ets factors with other proteins, including other sequence specific TFs, result in transcriptional regulation and define target gene specificity (Li R *et al.*, 2000a). Binding of an Ets factor in the vincinity of other TFs can even result in higher affinity interaction and synergistic repression or activation of specific target genes. Recent studies revealed that Ets-associated proteins (EAPs) can modulate Ets activities through different mechanisms like DNA binding, subnuclear sequestering or inhibiting synergistic interaction with co-factors (Li R *et al.*, 2000b; Pei H *et al.*, 2003).

Since many Ets proteins are effector molecules of a variety of signalling pathways it has been well established that their functions are post-translationally modified by phosphorylation-mediated effects on DNA binding as well as protein-protein interactions, transcriptional activation and subcellular localisation (Yordy JS and Muise-

Helmericks RC, 2000). The mitogen-activated protein kinases (MAPK) Erk, JNK and p38 belong to the Ets modulators that have been best characterised so far. For example, phosphorylation of a MAPK site adjacent to the PNT domain can positively regulate transcriptional activity of Ets1 and Ets2 and it can also affect the sub-cellular localisation of those Ets proteins. Integrin signalling has also been shown to regulate Ets activity via cell adhesion (Aplin AE *et al.*, 2001).

1.4.4.2 Ets factors in cancer

Abnormalities in signalling pathways are often observed during carcinogenesis and some of the final downstream effectors of these pathways belong to the Ets family of transcription factors (Sharrocks AD, 2001). Ets1 and Ets2 are phosphorylated via the ras/Raf/Mek/Erk signalling pathway and play important roles in the regulation of genes such as *c-myc* and *cyclin D1* that are involved in proliferation and cell cycle control (Bassuk AG and Leiden JM, 1997; Albanese C *et al.*, 1995). Ets proteins are also involved in the transcriptional regulation of anti-apoptotic genes such as *bcl-2* and *bcl-XL* as well as growth factor receptor genes such as *HER2* and *VEGFR*, all of which are frequently amplified in human tumours (Oikawa T *et al.*, 2004) (Figure 1.22). Ets transcription factors are also known to regulate the expression of growth factor receptor genes. It has been shown that Ets-binding sites are located in the promoter of the HER2 gene, the expression of which is regulated by several Ets TFs, including Ets1 and PEA3 (Scott GK *et al.*, 2000). *HER2* is one of several genes that are amplified in 20 – 30% of human breast cancers, generally resulting in a more aggressive phenotype with poor prognosis (Hogdall EV *et al.*, 2003). Ets1 and Ets2 also play a vital role in coordinating endothelial cell activities during angiogenesis (Wei G *et al.*, 2009), which is a critical process in embryogenesis but is also highly implicated in cancer progression (Folkman J, 2006; Zetter BR, 1998). In addition to the malignant transformation of cells, several Ets TFs are also involved in progression of tumours by initiating invasion and metastasis-related genes. A recent study on isolated aortic endothelial cells *in vitro* revealed a role for both Ets1 and Ets2 in directly regulating *Mmp9,* a gene coding for an extracellular protease that is involved in invasion and endothelial cell migration (Iwasaka C *et al.*, 1996; and

Wei G *et al.*, 2009). Work from our group and others has previously shown that interactions with Ets TFs such as Ets2 and PEA3 and the nuclear coactivator SRC-1 regulate the transcription of genes that are involved in tumour progression and metastasis, like *MMP9* and *myc* (Myers E *et al.*, 2004; Al-azawi D *et al.*, 2008; Qin L *et al.*, 2009).

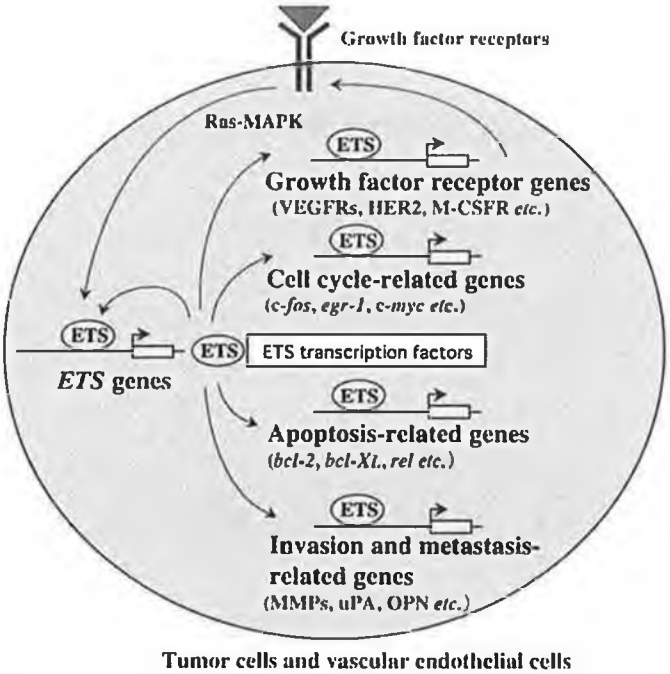


Figure 1.22: Ets target genes. Ets transcription factors are important for the regulation of expression of a variety of genes that are involved in GF signalling, cell cycle control, apoptosis, angiogenesis, invasion and metastasis. *Adapted from Cancer Science (Oikawa T, 2004).*

1.5 Hypothesis

Our group has previously shown that SRC-1 is increased in almost one third of breast carcinomas and that this increase positively correlates with poor disease-free survival and recurrence. SRC-1 was found to be a predictor for breast cancer recurrence in tamoxifen treated patients. The majority of this previous data was collected prior to the introduction of AIs to the breast cancer clinic and a role for SRC-1 in AI resistance has not yet been established.

Here we propose that SRC-1 plays a role independent of ER in advancing the metastatic phenotype in Aromatase Inhibitor resistance (Figure 1.23).

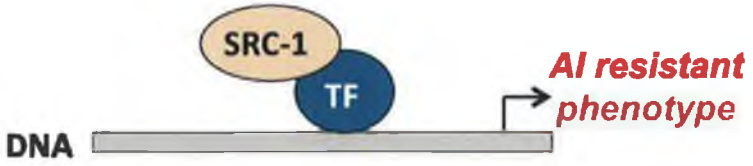


Figure 1.23: Proposed model of AI resistance. *SRC-1 may coactivate transcription factors to induce transcription of genes that are implicated in cancer progression and metastasis, resulting in an AI resistant phenotype.*

1.6 Aims

The objective of this thesis is to further establish the role of SRC-1 and its target genes in the development and most importantly recurrence of breast cancer. Furthermore, the aim of this work is to show, for the first time, that SRC-1 has a key role in the development of resistance to Aromatase Inhibitors (AIs).

The first portion of this work will deal with the characterisation of an AI resistant breast cancer model.

The second part of this work is to establish the molecular and functional role of SRC-1 in mediating an AI resistant phenotype by elucidating the signaling mechanisms and SRC-1 target genes involved in this process.

The last part of this study is to determine the clinical significance of SRC-1 signaling in the development and metastasis of AI resistant breast cancer.

The combination of molecular *in vitro* studies and clinical *in vivo* data offers a translational approach to finding novel signalling networks that can be targeted in the treatment of AI resistant breast cancer.

Chapter 2

Materials and Methods

2.1 Breast cancer cell line culture

2.1.1 Cell culture environment

Cell culture was performed in a sterile environment using a laminar airflow cabinet. All cell lines were maintained in a humid 5% (v/v) CO_2 atmosphere at 37°C, with the exception of MDA-MB231 cells, which were maintained in the absence of CO_2.

2.1.2 Recovering cells from cryo-storage

Cryovials containing cells were removed from storage in liquid nitrogen and warmed to 37°C as quickly as possible. Cells were transferred into a T25 tissue culture flask containing 5ml of culturing medium and transfer into the incubator to slowly bring them to 37°C. Medium was taken off after 5h and fresh medium was added to the cells.

2.1.3 Routine cell culture

Breast cancer cells were cultured in T75 flasks (Sarstedt, Germany). Cells were split at approximately 80% confluency by washing them twice with 5ml phosphatase buffered saline (PBS; Oxoid Limited, Basingstoke, Hampshire, England) and incubating them with 2ml of 0.05% trypsin/0.02% EDTA solution (Sigma Aldrich, Germany) for 5 minutes at 37°C. Trypsin was subsequently quenched by adding 8ml of normal cell culture media to the flask. The cell suspension was transferred into a 15ml conical tube (Greiner Bio-One, Germany) and centrifuged at 1200rpm for 4 minutes. The cell pellet was resuspended in the required volume of normal cell culture medium and divided into new tissue culture flasks with a 1:2 split used for the MCF7 derived cell lines and 1:4 for MDA-MB-231 for routine cell culture. Recipes for additives are listed in the Appendix I.

2.1.4 Cell Counting

To estimate the number of cells for those experiments that required a specific number of cells (transient transfections and functional cell assays), cells were counted using a haemocytometer. 20µl of cell suspension was mixed with 20µl of Trypan blue (Sigma

Aldrich) and 10µl of the suspension was pipetted onto the haemocytometer. The number of cells in the two grids on the left and on the right were averaged and multiplied by 10^4 to obtain the number of cells per ml of cell suspension. Counting was performed in duplicate and an average was calculated. The required volume of cell suspension was calculated and seeded into the appropriate cell culture vessel.

2.1.5 MCF7 cell line

MCF7 cells were acquired from the American Type Culture Collection (ATCC). MCF7 cells are positive for ER and PR and are HER2 negative representing the luminal A breast cancer cell phenotype. The cells were sub-cultured in Minimum Essential Media (MEM) supplemented with 10% fetal calf serum (FCS), 1% Pen/Strep (solution stabilised with 10,000 units penicillin and 10mg streptomycin/ml) and 1% of 200mM L-glutamine (all reagents by Sigma Aldrich).

2.1.6 Aro cell line

To investigate the molecular mechanisms involved in the development of resistance to Aromatase Inhibitors (AI) an estrogen-receptor positive (ER+) breast cancer cell model expressing high levels of aromatase was needed. A cell model overexpressing human aromatase had previously been established in the lab. In short, ER+ MCF-7 breast cancer cells were transfected with a pcDNA-DEST47 vector containing the full-length human aromatase gene *CYP19* to mimic the estrogen production in postmenopausal women. The vector contains a neomycin resistance cassette that makes the cells resistant to the drug Geneticin (G418; Invitrogen). Upon transfection the cells were subcultured in minimum essential media (MEM; Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), penicillin, streptomycin, L-glutamine and the selective agent G418 (200µg/ml), allowing only cells that have been successfully transfected with the vector containing the neomycin resistance gene to grow. These aromatase overexpressing breast cancer cells are from here on referred to as Aro.

2.1.7 LetR cell line

To create a cell line that is resistant to Aromatase Inhibitors, Aro cells were exposed to the AI Letrozole (Novartis) for a minimum of 12 weeks. The cells were continuously cultured in phenol red free MEM containing 10% charcoal dextran stripped fetal calf serum (CDS-FCS) to ensure low levels of steroid hormones, androstenedione ($25x10^{-9}$ M, Sigma Aldrich), letrozole (10^{-6} M) and G418 (200µg/ml). The AI resistant cell model was designated LetR.

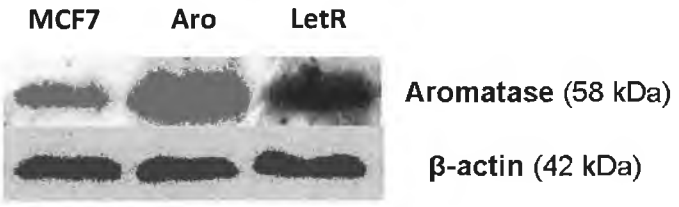


Figure 2.1: Aro and LetR cells stably overexpress aromatase protein. *Protein lysate was dissolved by SDS-PAGE and immunoblotted for Aromatase. β-Actin was used as a loading control. Images are a representative of three separate experiments (n=3). (O'Hara J et al, 2012 – manuscript submitted).*

2.1.8 MDA-MB-231 cell line

MDA-MB-231 cells are ER, PR and HER2 negative basal like cancer cells that possess a highly invasive phenotype. Cells were obtained from ATCC and were cultured in Leibovitz L-15 media (Invitrogen, Carlsbad, California, USA) supplemented with 10% FCS. Tissue culture flasks were closed with air tight caps as these cells need to be grown in the absence of CO_2 at 37°C.

2.1.9 SKBR3 cell line

The SKBR3 breast cancer cell line exhibits c-erbB2 amplification and as a result overexpresses HER2. The cell line was obtained from ATCC and was cultured in RPMI 1640 medium (Sigma Aldrich) supplemented with 10% FCS and 1% Pen/Strep (solution stabilised, with 10,000 units penicillin and 10mg streptomycin/ml).

2.1.10 Cell treatments

Cell culture treatments were performed when the cells were 70% confluent. Prior to endocrine treatments, cell culture medium was decanted, cells were washed in PBS and steroid depleted for 72h in phenol red free MEM containing 10% CDS-FCS (CDS-PRF-MEM) to prevent stimulation by steroidal hormones present in FCS and phenol red. Prior to treatment with growth factors, cells were additionally serum starved for 24h. Medium was decanted and the cells were incubated under the treatment conditions outlined in table 2.1.

Table 2.1: Cell treatment conditions

Treatment	Final Concentration	Treatment time
Letrozole (Novartis)	10^{-6}M in CDS-PRF-MEM	4 hours (RNA) 8 hours (protein) 24 hours (migration assays)
Androstenedione	10^{-7}M in CDS-PRF-MEM	4 hours (RNA) 8 hours (protein) 24 hours (migration assays)
Estrogen	10^{-8}M in CDS-PRF-MEM	4 hours (RNA) 8 hours (protein) 24 hours (migration assays)
EGF	1ng/µl In serum free MEM	4 hours (RNA) 8 hours (protein)
Ethanol (vehicle)	0.01% in CDS-PRF-MEM / serum free MEM	4 hours (RNA) 8 hours (protein) 24 hours (migration assays)

2.2 Functional Cell Assays

2.2.1 Cell Motility Assay

Cell motility is a key activity in a number of biological as well as pathological processes such as wound healing, embryonic development, inflammation, angiogenesis as well as cancer cell invasion and metastasis. Cell movement occurs via a concerted interaction of cell adhesion molecules, the cytoskeleton and a vast network of signalling molecules.

To assess individual movement per cell the Cellomics® cell motility kit (Pierce, Il, USA) was employed.

The wells of a 96-well plate were coated with collagen and incubated for 1h at room temperature (RT). Wells were washed twice with 200µl of 1x Wash Buffer (PBS) and air-dryed for 30 minutes in the laminar flow hood. 75µl of fluorescent beads were added to each well and incubated for 1h at 37°C in the incubator. In the meantime, cells were trypsinised, counted and a cell supension of 5000 cells/ml was prepared. Wells were subsequently washed thrice with 200µl of 1x Wash Buffer and 100µl of the cell suspension was seeded into each well. The plate was placed into the incubator and left for 22h at 37°C. 200µl of a formaldehyde solution was added to each well and cells were fixed for 1h. The formaldehyde was aspirated and 100µl of 1x Permeabilisation Buffer (Kit) was pipetted into each well and left at RT for 15 min. Wells were carefully washed and stained for 30 min with Rhodamine Phalloidin. Wells were washed four times with 1x Wash Buffer and images were taken on an inverted microscope. Track areas were measured using Olympus cell^F imaging software and compared with a Student t-test.

Figure 2.2: Schematic of the cell motility assay. *Gray layer depicts collagen coating. Blue layer is a layer of fluorescent beads, which is covered with cells stained for the cytoskeletal protein F-actin with rhodamine-phalloidin (red).*

2.2.2 Cell Invasion Assay

In order to disseminate to distant sites in the body cancer cells must cross so-called basement membranes. The 'chemoinvasion assay', using a reconstituted basement membrane, matrigel, on top of a microporous polycarbonate membrane was developed 25 years ago as a tool for invasion and metastasis research (Albini A *et al.*, 1986).

Invasion chambers were used to study the invasive properties of breast cancer cells *in vitro.* The highly invasive MDA cells were used as a positive control in this assay. The invasion chamber inserts (Falcon, BD Biosciences, USA) consist of a membrane with a pore size of 8µm are coated with a thin layer of Matrigel® basement membrane matrix (BD Biosciences, MA, USA) to inhibit non-invasive cells from migrating. Only invasive cells are able to detach and break down the matrix and migrate into the pores.

Prior to the experiment, a Matrigel® solution at a concentration of 1mg/ml was prepared on ice and 100µl were quickly layered on top of the membrane inside the insert. The inserts were incubated at 37°C for 2h to allow the matrigel to polymerise. Inserts were then rehydrated with serum free medium (MEM for Aro and LetR cells, Leibovitz's L-15 medium for MDA-MB231 cells) for one hour in the incubator. In the meantime, a cell suspension containing cells was prepared in serum free media. 500 µl of chemoattractant, in this case normal culturing medium supplemented with an additional 10% FCS (20% final concentration), was added to the wells of the 24 well plate. Serum free medium was also added into one well as a control (see figure 2.3). The inserts were then placed into the wells containing the chemoattractant and 500 µl of the relevant cell suspension was added to the insert. The 24 well plate was returned into the incubator and was incubated for 22 hours at 37°C. The non-adherent cells were then removed with a moist swab and the cells stuck inside the pores and on the lower surface of the membrane were fixed with 100% methanol for 10 minutes, followed by staining with crystal violet for another 10 minutes. The membrane was thoroughly washed in tap water, carefully removed from the insert and mounted on a microscope slide. Using an inverted light microscope, the number of invading cells was counted on four separate field views in triplicate and expressed in percentage of invading cells.

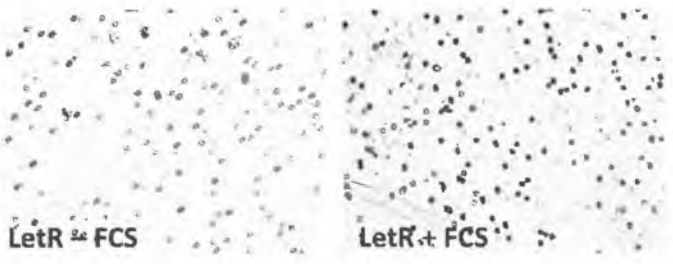


Figure 2.3: Cell invasion in response to chemoattractant. *LetR cells in serum free medium were seeded into an insert and placed into a well containing either serum free medium (control) or MEM containing 20% FCS (chemoattractant). Cells only invaded the membrane in response to the chemoattractant.*

2.2.3 3D Assay

3D assays are a means to assess the level of differentiation and polarisation of a cell. Cells are grown in a three-dimensional basement membrane matrix where differentiated cells are able to form organised and polarised acini structures with a hollow lumen. The less differentiated the cells, the less able they are to form polarised acini.

Growth factor reduced Matrigel matrix was thawed on ice. Eight well chamber slides were prepared by adding the matrigel into the chambers. $5x10^4$ cells in 400µl of their respective medium (as above) and 2% Matrigel (BD Biosciences) were seeded into each well and cultured for 14 days at 37°C and 5% CO_2. 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 and 1% bovine serum albumin for 1h. Cells were stained with Phalloidin 594 (Molecular Probes) for 20 minutes and DAPI for 5 minutes. Slides were mounted (Dako) and examined by confocal microscopy.

2.3 Transfections

2.3.1 Overexpression

Overexpression studies were performed using Lipofectamine® 2000 (Invitrogen). This transfection reagent contains specially designed cationic lipids that facilitate DNA and siRNA delivery into cells (Liu D *et al.*, 2003). The overexpressions were transient and performed using vectors for the genes of interest that had already been validated in the lab. The plasmid backbones were pcDNA3.1 (Invitrogen) for SRC-1 and pCGN (Addgene) for Ets2 (table 2.2).

Table 2.2: DNA vectors used in transient transfections. *(EV = empty vector)*

Vector	Stock Conc.	Amount per 6 well
pCGN EV	0.8759 µg/µl	4µg
pCGN Ets2	0.9195 µg/µl	4µg
pcDNA 3.1 EV	0.5576 µg/µl	4µg
pcDNA3.1 SRC1	2.186 µg/µl	4µg

24h prior to transfection, $5x10^5$ cells were seeded into 6-well plates in their normal growth medium without antibiotics. Plasmid DNA was mixed with OPTI-MEM (Invitrogen) in one eppendorf tube and Lipofectamine 2000 (Invitrogen) reagent was mixed with OPTI-MEM in another tube (table 2.3).

Table 2.3: Components of the transfection mix.

	6 well/T25
Cell plating volume	2.5ml
Number of cells plated	$5x10^5$
Lipofectamine 2000	10µl
OPTI-MEM to	500µl
Plasmid DNA	4µg
OPTI-MEM to	500µl
Final transfection volume	1ml

The solutions were incubated for 5 minutes, then mixed together and left for another 20 minutes. Cells were washed with PBS and overlayed with the transfection mix. 5 hours later the transfection mix was taken off and cells were incubated in normal growth media for the time as listed in table 2.4.

Table 2.4: Transfection times for assays performed using overexpression vectors.

Assay	Time to assay following transfection
RNA for overexpression validation	24 hours
Protein extraction	24 hours
Migration assays	72 hours

2.3.2 RNA interference

The Nobel Prize-winning discovery by Andrew Fire and Craig C. Mello in 1998, that short double stranded RNA molecules can cause suppression of gene activity in a sequence specific manner, marked a new era in molecular research (Fire A *et al.*, 1998). RNA interference (RNAi) enables researchers to experimentally assess the functional role of any gene in a cell. During RNAi, the double stranded RNA is processed to 21-25bp fragments of dsRNA with dinucleotide 3' overhangs, coined short interfering (si)RNA. One strand of the siRNA, called guide strand, is assembled into an RNA induced silencing complex (RISC) that cleaves the target mRNA. Target specificity to RISC is provided by the siRNA through base pairing of the guide strand with the target mRNA.

A transient approach of RNAi was used in this thesis to assess the functional role of *Src1* and *ERα*. Prevalidated siRNAs for these target genes were used and are listed in table 2.5.

Table 2.5: siRNA used in transient transfections

Gene of interest	siRNA	Source	Stock RNA Concentration	Final RNA concentration
SRC-1	AM16706	Ambion	50μM	40nM
ERα	4392421	Ambion	50μM	20nM
Control	AM4635	Ambion	50μM	40nM

The transfection agent Lipofectamine 2000 was used to aid the transport of siRNA into the cells. Cells were plated in antibiotics free normal growth medium 24 hours prior to transfection. Seeding density and reagents used are listed in table 2.6.

Table 2.6: Reagents used for transient transfections with SRC-1 / ERα siRNA

	6 well/T25
Cell plating volume	2.5ml
Number of cells plated	$5x10^5$
Lipofectamine 2000	5µl
OPTI-MEM to	500µl
siRNA stock (table 2.5)	4µl / 2µl
OPTI-MEM to	500µl
Final transfection volume	1ml

OPTI-MEM reduced serum medium (Invitrogen) and the transfetion agent Lipofectamine 2000 (Invitrogen) were brought to room temperature. Lipofectamine 2000 was diluted in OPTI-MEM in one eppendorf tube and the required amount of siRNA was diluted in another tube containing OPTI-MEM (see table 2.6 for specific amounts) and incubated for 5 minutes at room temperature. The contents of the two tubes was then mixed and left for another 20 minutes at room temperature. In the meantime, cells were washed with PBS and the nucleic acid/Lipofectamine transfection complex was transferred onto the cells. The plate was gently tilted back and forth to evenly distribute the solution over the cells and then returned to the incubator. 5 hours later the transfection mix was taken off and normal growth medium was added and cells were incubated for the time listed in table 2.7, depending on the assay performed.

Table 2.7: Transfection times for assays using siRNA

Assay	**Time to assay following transfection**
RNA for knockdown validation	24 hours
Protein extraction	48 hours
Cell invasion assays	72 hours
3D assay	72 hours
Migration assays	72 hours

2.4 Protein Biochemistry

2.4.1 Protein extraction

Cells were trypsInised as described in 2.1.3 and pelleted at 3000rpm for 5 minutes at 4°C. Radioimmunoprecipitation (RIPA) lysis buffer supplemented with 10% protease inhibitor cocktail (P8340, Sigma Aldrich) was added to the cell pellet, vortexed for 30 seconds and incubated on ice for 15 minutes. Cells were then centrifuged at 13,000rpm for 15min at 4°C and the supernatant was transferred into a fresh pre-chilled eppendorf tube and stored at -80°C until further use.

2.4.2 Protein Quantification

A bicinchoninic acid (BCA) assay (Pierce, Il, USA) was performed according to the manufacturer's protocol to assess the protein concentration in the lysate samples. Protein standards with a range of albumin concentrations between 0 and 1.4mg/ml as well as 1:20 dilutions of the samples were prepared. 25µl of each standard and sample was pipetted into a 96-well plate in duplicate. BCA reagent was prepared by mixing 49 parts of reagent A (Bicinchoninic acid and tartrate in alkaline carbonate buffer) with 1 part of reagent B (4% copper sulfate pentahydrate solution). 200µl of this solution was added to each well and incubated at 37°C for 30 min. Absorbance was read at 560nm on a spectrophotometer (Perkin Elmer, MA, USA). Simple linear regression was used to determine the equation of the standard curve. This equation was then used to determine the protein content in the samples.

2.4.3 Co-Immunoprecipitation

Co-immunoprecipitation or Co-IP is a technique used to assess protein-protein interactions. An antibody against a specific epitope on a protein is added to cell lysate to form an immune complex with that particular target. Protein A beads are added to the solution to capture the antibody-protein complex, proteins not bound by the

immobilised protein A are washed away. The bound immunocomplex is then eluted from the beads, run on SDS-PAGE and analysed by Western Blot.

Protein A beads (Santa Cruz, CA, USA) were preblocked in bovine serum albumin (BSA). Cell lysate was precleared by incubating it with 1µg rabbit IgG and 20µl of protein A beads at 4°C for 30 minutes to reduce non-specific binding. Beads were removed by centrifugation at 1,000g and 4°C for 5 minutes and the clear lysate as incubated with 5µg of immunoprecipitating antibody on a rotor at 4°C for 3 hours. After antibody incubation, 20µl of the preblocked beads was added to the lysate and Incubated on the rotor at 4°C for another hour, during which the protein complex will bind to the antibodies on the beads. The beads were collected by centrifugation at 1,000g at 4°C for 5 minutes and the supernatant was discarded. The beads were washed four times in 1ml of RIPA lysis buffer containing 1% protease inhibitor cocktail and centrifuged at 1,000g at 4°C for 5 minutes after each wash. After the final wash, the supernatant was discarded and the beads were resuspended in 20µl of 2x Laemli sample buffer. Samples were boiled for 10 minutes at 95°C to release the protein complex from the beads. Following this step, the samples were ready for gel electrophoresis (2.4.4) or stored at -20°C for further use.

2.4.4 Western Blotting

Western blotting is a technique that is used to detect proteins in tissue or cell lysate. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins based on their size. The gel is transferred to a membrane by applying an electrical current. This membrane can then be stained with a specific antibody against an epitope in the protein.

To allow access of the antibody to the epitope of interest the protein has to be denatured using an anionic denaturing loading buffer. To do so, the protein lysate was mixed with one fifth of 6x loading buffer, briefly vortexed and boiled at 95°C for 8 minutes. This loading buffer contains sodium dodecyl sulfate (SDS), which causes proteins to become negatively charged by their attachment to SDS anions. SDS denatures proteins by "wrapping around" the polypeptide backbone. In so doing, SDS

confers a negative charge to the polypeptide in proportion to its length. In denaturing SDS-PAGE separations, therefore, migration is determined by molecular weight.

Polyacrylamide gels are used to separate proteins in a sample. They are made up of acrylamide and bisacrylamide (Sigma Aldrich) and a cross-linking agent called N,N,N,N-tetramethyletylenediamine (TEMED; Sigma Aldrich). The more acrylamide is added to the mix, the smaller the pore size, allowing seperation of molecules relative to their size. Ammonium persulfate (APS; Sigma Aldrich) and TEMED are added to initiate polymerisation of the gel.

Gels were prepared and run on the ATTO electrophoresis system (ATTO, Tokyo, Japan). The resolving gel was poured between the glass plates, overlayed with Isopropanol and allowed to polymerise for 30 minutes at room temperature. The stacking gel (5%) was then poured on top of the resolving gel, a 1.5mm, 10 well comb was inserted and the gel was allowed to dry for an additional 30 minutes at room temperature. See table 2.8 for the composition of gels of different percentage.

Table 2.8: Gel preparation for SDS-PAGE. *Volumes (ml) are for preparation of 10ml of resolving gel and 2ml of stacking gel for preparation of one gel on an ATTO 1.5mm plate. *1M Tris (pH 6.8) was used for the stacking gel.*

	6% Resolving	8% Resolving	10% Resolving	5% Stacking
H_20	5.3	4.6	4.0	1.4
30% acrylamide mix	2.0	2.7	3.3	0.33
1.5M Tris (pH 8.8)*	2.5	2.5	2.5	0.25
10% SDS	0.1	0.1	0.1	0.02
10% ammonium persulphate	0.1	0.1	0.1	0.02
TEMED	0.008	0.006	0.004	0.002

Gels were placed into an ATTO electrophoresis unit filled with 500 ml of 1x running buffer (Appendix I). Lysate samples were loaded into the wells alongside a molecular weight marker (BioRad) for estimation of molecular weight. A constant voltage of 130V was applied and gels were run for 3 – 3.5 hours, depending on the protein of interest.

Proteins were then transferred onto a nitrocellulose membrane for visualisation. A nitrocellulose membrane and ten sheets of Whatman paper were submerged in ice-cold 1x transfer buffer (Appendix I) for 5 minutes. The gel was removed from the glass plates and a sandwich of 5 sheets of Whatman paper, nitrocellulose membrane, gel and 5 sheets of Whatman paper was assembled and placed between the anode and the cathode of a semi-dry transfer rig (ATTO, Tokyo, Japan). A constant current of 250mA was applied to the transfer rig for varying times according to the molecular weight of the protein of interest as recorded in table 2.9.

Table 2.9: Antibody dillutions and conditions for western blotting.

Protein	Molecular Weight	Gel %	Transfer	Primary Antibody		Secondary Antibody	
				Antibody	Conc	Antibody	Conc
ß-Actin	42kD	n/a	60 min	Mouse monoclonal	1:8000	Anti-mouse	1:10000
ERα	68kD	10%	60 min	Rabbit polyclonal	1:500	Anti-rabbit	1:1000
PR	116kD	8%	2 hours	Rabbit polyclonal	1:200	Anti-rabbit	1:4000
HER2	165kD	6%	2 hours	Rabbit polyclonal	1:1000	Anti-rabbit	1:2000
SRC-1	160kD	6%	2 hours	Rabbit polyclonal	1:100	Anti-rabbit	1:2000
c-MYC	67kD	10%	60 min	Rabbit polyclonal	1:200	Anti-rabbit	1:2000
Ets2	56kD	10%	60 min	Rabbit polyclonal	1:250	Anti-rabbit	1:1000
p-Ets2	56kD	10%	60 min	Rabbit polyclonal	1:1000	Anti-rabbit	1:4000

The nitrocellulose membrane was removed from the transfer rig and blocked in 5% (w/v) non-fat dry milk (Marvel, UK) in tris-buffered saline (TBS) containing 0.1% Tween (Sigma Aldrich) (TBS-T) on a rocker for 1 hour at room temperature. The primary antibody was diluted in 5 ml of 5% non-fat dry milk in TBS-T and incubated on a rocker at 4°C over night. Specific dillutions are recorded in table 2.9.

Following primary antibody incubation, the nitrocellulose membrane was washed in TBS-T buffer for 5 minutes three times. Horseradish peroxidase (HRP)-conjugated

secondary antibody was reconstituted to the concentration specified in table 2.9 in 5% non-fat cry milk in TBS-T, added to the membrane and incubated for 1h at RT. Following secondary antibody incubation, the membrane was washed in TBS-T for 5 minutes three times and developed by enhanced chemiluminescence (ECL; Pierce, Il, USA). ECL substrate is used to detect HRP activity. A luminol and a peroxide solution are added in a ratio of 1:1 and the working solution is layered on top of the membrane and incubated for 1 minute. The blot was quickly placed into an X-ray cassette and exposed to X-ray film (Fuji, Tokyo, Japan) in the dark room. The film was then processed by immersion in developing solution for 2 minutes, followed by fixer solution (both Kodak, USA) for 2 minutes and a quick rinse in water. The film was left to air dry for 10 minutes and bands were visualised.

2.4.5 Zymography

Zymography is an electrophoretic technique where protein samples are run out on a polyacrylamide gel that contains a certain substrate to assess the enzyme activity of a specific protein.

Aro and LetR cells were seeded at approximately 50% confluency and cultured in the respective normal culturing media for 24 hours. MMP9 Is a secreted metallo-proteinase, therefore media was taken off the cells after 2 days and the secreted protein was concentrated using Amicon Ultra4 filters (50 K pore size; Millipore). Filter units were centrifuged at 4,000g for 20 minutes. The media above the filter was transferred into a fresh eppendorf tube and protein concentration was assessed as previously described (2.4.2). 20µg of protein was mixed with 2x non-reducing loading dye (Appendix I). Samples must not be heated, as it will irreversibly inactivate MMPs in the sample.

To assess the enzyme activity of the gelatinase MMP9, samples were run on a precast Novex® 10% Zymogram gel containing gelatin (Invitrogen). The gel was placed into an XCell SureLock electrophoresis chamber (Invitrogen) filled with 1x Novex® Tris-Glycine SDS Running Buffer (supplied with the zymography gel, Invitrogen) and run at 125V for approximately 1h until the running front reaches the bottom of the gel. After electrophoresis, the enzyme was renatured by incubating the gel for 30 minutes in 1x

Novex® Zymogram Renaturing Buffer (Invitrogen) at room temperature with gentle agitation. The Renaturing buffer was decanted and 1x Novex® Developing Buffer (Invitrogen) was added to the gel for 30 minutes at room temperature with gentle agitation. The buffer was decanted and the gel was incubated in fresh developing buffer for at least 4 hours at 37°C. The gel was stained with Coomassie Brilliant Blue for an hour, followed by Incubation with Destain Buffer until bands became visible. MMP9 bands were identified by size (Pro-MMP9: 92 kD, active MMP9: 82 kD).

2.5 Nucleic acid biochemistry

2.5.1 mRNA purification

mRNA was extracted from cells and purified using an RNeasy Kit (Qiagen, Hilden, Germany), consisting of pre-mixed buffers and columns. RLT lysis buffer was supplemented with 1% mercaptoethanol (Sigma Aldrich) and 350µl of the solution was added to the cells and homogenised by passing the lysate 5 times through a 24-gauge needle fitted to a 1ml sterile syringe. One volume of 70% ethanol (EtOH) was added to the lysate and mixed. The sample was transferred to the supplied RNeasy spin column and centrifuged at 8,000 rpm for 15 sec. Flow-through was decanted from the collection tube and the columns were washed, once with 700µl of wash buffer RW1 and twice with 500µl of RPE buffer, followed by a 2 minute spin to dry the membrane inside the column. The spin column was placed in a clean eppendorf tube and RNA was eluted from the membrane by adding 30µl of RNAse-free water and centrifuging at 10,000 rpm for 1 minutes. A NanoDrop spectrophotometer (Thermo Scientific, DE, USA) was used to determine the RNA concentration of the sample. RNA was stored at -80°C until further use.

2.5.2 Reverse transcription

Reverse transcription is a technique used to transcribe single stranded mRNA into complementary DNA (cDNA). 1 µg of RNA was made up to a total volume of 8 µl with DEPC-treated water and mixed with 1 µl of random hexamers (50 ng/µl) and 1 µl of dNTPs (10 mM). The reaction mix was incubated at 65°C for 5 minutes and quickly placed on ice. 10 µl of a cDNA synthesis mastermix (components added in the order as indicated in table 2.10) was added to each RNA/primer mix and incubated for 10 minutes at 25°C, followed by 50 minutes at 50°C. The reaction was terminated by incubating the samples for 5 minutes at 85°C and the samples were quickly transferred to ice and stored at -20°C freezer until further use.

Table 2.10: cDNA synthesis mastermix

Reagent	Volume (µl)
10x RT buffer	2
25 mM $MgCl_2$	4
0.1 M DTT	2
RNAse OUT (40 U/µl)	1
Superscript III (200 U/µl)	1

2.5.3 Polymerase chain reaction

Polymerase chain reaction (PCR) is a technique used to amplify a large amount of copies of DNA from a relatively small amount of starting DNA material. During a PCR reaction, a heat stable DNA polymerase assembles a new DNA strand from template DNA using specific DNA primers. The three main steps of a PCR are denaturation, annealing and extension, which are each repeated for a specific number of cycles.

During **initial denaturation**, template DNA is denatured, while the Taq DNA polymerase gets activated.

DNA denaturation is then performed for 45 seconds again at the beginning of each cycle to ensure DNA denaturation. DNA is melted during each denaturation step by disrupting the hydrogen bonds between complementary bases, resulting in single stranded DNA.

The denaturation step is followed by primer **annealing**, during which the temperature is lowered to allow the primer to anneal to the DNA. Temperature and duration of this step depends on the specific primers used.

During **extension**, the DNA polymerase synthesises a new complimentary DNA strand by adding dNTPs in antisense direction. This cycle is repeated up to 35 times, depending on the amount of template DNA in the sample.

A final **elongation** step after the last cycle is to ensure that any remaining single stranded DNA is fully extended.

PCR reactions were made up to 25µl (see table 2.11 for reagents and 2.12 for primers) in 200µl domed PCR tubes (StarLab, Germany) and PCR was carried out on a thermocycler (Biosciences, Dublin, Ireland) (see table 2.13 for thermocycler settings).

Table 2.11: PCR reaction reagents

Reagent	Volume (µl)
10x PCR buffer	2.5
50mM $MgCl_2$	0.75
10mM dNTP mix	0.5
Primer Mix (Fwd & Rev)	1
Taq DNA polymerase	0.1
Template DNA	1
DNAse-free dH_2O	Up to 25

Table 2.12: Primers for PCR

Gene	Forward primer sequence 5' → 3'	Reverse primer sequence 5' → 3'
SRC-1	CATGGTCAGGCAAAAACCTT	GCTTGCCGATTTTGGTGTAT
Ets2	GCAAGGCTGTGATGAGTCAA	CCTCTGCAGATTCACGTTCA
Myc	TTCGGGTAGTGGAAAACCAG	CAGCAGCTCGAATTTCTTCC
MMP9	CGCAGACATCGTCATCCAGT	GGATTGGCCTTGGAAGATGA
Actin	TCACCCACACTGTGCCCATCTA	CAGCGGAACCGCTCATTGCCA

Table 2.13: Thermocycler settings for DNA amplification. **except MMP9 = 35 cycles and Actin = 20 cycles.*

Step	Temperature (°C)	Time
1. Initial Denaturation	94	3 minutes
2. Denaturation	94	45 seconds
3. Annealing	60	30 seconds
4. Extension	72	1.5 minutes
Repeat Step 2 – 4 x 28* times		
5. Elongation	72	10 minutes
6. Hold	4	Forever

2.5.4 Preparing and running standard agarose DNA gels

PCR products were analysed by agrose gel electrophoresis for size separation. Agarose DNA gels were prepared by dissolving 1.5% (w/v) agarose powder (Promega, Germany)

with heating in 1x tris acetate EDTA (TAE) buffer (Appendix I). After cooling, the intercalating agent SybrSafe (Invitrogen, USA) was added to the solution at a dilution of 1:10,000, transferred into a gel casting tray containing a sample comb and left to set for 30 minutes at room temperature. The comb was then removed and the gel was overlayed with 1x TAE buffer. Samples were mixed with clear loading dye and pipetted into the wells of the gel. The gel was run at 100V for 30 minutes. Bands were visualised on an ultraviolet transilluminator and the image was recorded using LAS3000 imaging software (Fuji, Japan).

2.5.5 Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation or ChIP is a technique used to assess protein-DNA interactions at a particular location within the genome. In short, protein-DNA complexes are fixed by crosslinking with formaldehyde, cells are lysed and chromatin is sheared into shorter fragments of approximately 500bp. Complexes are immunoprecipitated with an antibody against the protein of interest. DNA is then purified from the isolated chromatin and specific genomic regions are amplified by PCR and analysed on a light cycler.

Aro and LetR cells were plated at equal confluence in T75 flasks and cultured in 10ml of phenol red free CDS-FCS MEM for 72 hours prior to treatments. Cells were treated according to table 2.1 for 45 minutes and cross-linked for 10 minutes with formaldehyde at a final concentration of 1% (Appendix I) immediately afterwards. Fixation was quenched by adding glycine solution at a final concentration of 125mM (Appendix I) to the media and incubated for a further 5 minutes. Flasks were washed once with 3ml ice-cold PBS and another 3ml of ice-cold PBS supplemented with Protease Inhibitor cocktail (P8340; Sigma Aldrich) was added to the flask and cells were removed from the flask with a cell scraper (Sarstedt). Cells were transferred into a 15ml falcon tube and centrifuged at 2,000rpm for 5 minutes at 4°C to pellet the cells. The supernatant was discarded and the pellets were stored at -80°C until cell lysis and sonication at a later time.

Prior to sonication, cells were lysed in 600µl SDS lysis buffer (Appendix I) supplemented with 1% protease inhibitor cocktail and placed on ice for 10 minutes. Cross-linked DNA was sheared by sonicating each sample 6 times for 10-second pulses with a 1-minute interval on ice between pulses with a Sonifier 250 sonicator (Branson, USA) at power output 4 – 5. Following sonication, the samples were centrifuged for 10 minutes at 13,000rpm at 4°C after which the lysates were transferred into fresh tubes. DNA was quantified using a Nanodrop 2000c spectrophotometer (Thermo Scientific) to determine an estimation of chromatin mass in the sample. An equal mass of starting material was calculated for each sample and diluted up to 2ml in ChIP dilution Buffer (Appendix I) containing 1% protease inhibitor cocktail. A 40µl aliquot of each sample was transferred into a fresh eppendorf tube to be used as an input control. The DNA in the aliquot was precipitated in 100% ethanol at -80°C for 1 hour, centrifuged at 13,000rpm for 20 minutes at 4°C. The pellet was air dryed and DNA was isolated by a chelex-based procedure as explained below.

The diluted cell lysate was pre-cleared with 75µl Salmon Sperm DNA/Protein A Agarose-50% Slurry (Millipore, USA) for 30 minutes at 4°C on a rotor to reduce non-specific background. The samples were centrifuged at 1,000rpm for 1 minute at 4°C and the supernatant was transferred into a fresh 15ml falcon tube. 6µg of immunoprecipitating antibody was added to each sample (see table 2.14) and incubated on a rotor at 4°C over night. The negative control was incubated with the non-immune IgG fraction from the species in which the respective antibody was raised.

Table 2.14: Immunoprecipitating antibodies used in ChIP experiments

Antigen of interest	Immunoprecipitating antibody	Mass of antibody	Volume of antibody	Non-immune IgG	Mass of non-immune IgG
SRC-1	Rabbit polyclonal	6µg	30µl	Rabbit	6µg
Ets2	Rabbit polyclonal	6µg	30µl	Rabbit	6µg
Histone H4	Rabbit polyclonal	7µg	7µl	Mouse	5µg

After the incubation, 60µl of Salmon Sperm DNA/Protein A Agarose-50% Slurry (Millipore) was added to the sample and incubated for another 60 minutes on the rotor at 4°C to bind the antibody/histone complex. The agarose was pelleted by centrifuging the tubes at 1,000rpm at 4°C for 1 minute. The supernatant was discarded and the agarose was washed on a rotor for 5 minutes each with 1ml of the following buffers: low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer, TE buffer. After the last wash, the pellet was resuspended in 1ml TE buffer, transferred into a fresh eppendorf tube and incubated on a rotor for 5 minutes at room temperature. The tubes were centrifuged at 1,000rpm at 4°C for 1 minute and supernatant was discarded. Cross-linking was reversed and DNA isolated by incubating the agarose beads with 100µl of 10% Chelex 100 slurry (BioRad) at 95°C for 10 minutes. Samples were chilled on ice before adding 2µl of proteinase K solution (10µg/µl; Fluka, Sigma Aldrich) to each sample and incubating them at 55°C for 30 minutes in a thermomixer (Eppendorf, Germany). The proteinase K was inactivated by boiling the samples at 95°C for 10 minutes. Samples were centrifuged for 1 minute at 13,000rpm and 4°C and the supernatant was transferred into a fresh eppendorf tube. 120µl of DNAse/RNAse-free water (Qiagen) was added to the beads, vortexed for 10 seconds, centrifuged, and the supernatant was added to the previous supernatant of the respective sample. The DNA concentration in the samples was assessed using the Nanodrop 2000c spectrophotometer (Thermo Scientific). Samples were either subjected to a PCR (15µl input DNA and 30µl sample DNA; 2.5.3) and prepared to run on a gel (2.5.4) or subjected to semi-quantitative PCR (2.5.6).

2.5.6 Semi-quantitative real time PCR

Semi-quantitative real time PCR, or qPCR, allows detection and quantification of specific DNA sequences in a sample. This technique differs from conventional PCR in that it measures the amount of amplified DNA template throughout the reaction rather than assessing the amount of the template at the end. By focusing on measuring during the exponentional phase of the reaction it allows for accurate quantification of the samples. The PCR products are detected by using fluorescent dyes that bind to dsDNA.

In this case, the fluorescent dye used was SYBR Green (Qiagen). Experiments were carried out on the LightCycler 2.0 (Roche Diagnostics, Switzerland). 80ng starting material was used in the PCR reactions. Reactions were prepared in standard 20µl LightCycler capillaries (see table 2.15 for reagents).

Table 2.15: qPCR reaction reagents

Reagent	Amount
SYBR Green Master Mix (Qiagen)	10µl
1.25µM Primer Mix (table 2.5.3.b)	1µl
Template cDNA	80ng
DNAse-free dH_2O	Up to 20µl

A standard curve was generated for each gene of interest using serial dillutions of input cDNA (neat – 1:10,000). Capillaries were then loaded onto the LightCycler and run under the conditions listed in table 2.16.

Table 2.16: Run conditions for qPCR reactions

qPCR Product	Denature		Anneal		Extend		No. of Cycles
	Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)	
Myc mRNA	94	15	60	20	72	20	50
MMP9 mRNA	94	15	60	20	72	20	50
β-Actin mRNA	94	15	60	20	72	20	42

Endogenous reference gene was β-Actin. Analysis is based on relative quantification. The target gene of interest as well as β-Actin was amplified from the same sample and the normalised value was determined to allow comparison of gene expression between the samples.

2.6 Translational Research

Ethical approval for studies on human samples was obtained from the Medical Ethics Committee, Beaumont Hospital, Dublin 9. Biological samples were only used from patients who provided informed consent for the use of their tissue. Detailed information about the patient cohort and the construction of the TMA can be found in chapter 5.

2.6.1 Immunohistochemistry

Immunohistochemistry (IHC) is a technique used to assess the presence and cellular localisation of a specific protein in the context of intact tissue.

IHC was performed on formalin fixed paraffin embedded tissue in form of previously prepared full face tumour sections or tissue micro arrays (TMAs) that consisted of 0.8 mm diameter cores of patient tumour samples. Slides were baked at 65°C for 6 hours to fix the tissue onto the slides. Tissue was deparaffinised by immersion in xylene for 3 minutes followed by sequential passage for 3 minutes each through decreasing concentrations of industrial methylated spirits (IMS; 100% - 100% - 70%). Slides were washed once in tap water and twice in PBS for 5 minutes. Endogenous peroxidase activity was depleted by treatment with 3% hydrogen peroxide (H_2O_2; Sigma Aldrich, Germany) twice for 10 minutes in the dark, followed by a 5-minute PBS wash. Heat-mediated antigen retrieval was conducted by submerging the slides in a container filled with 10 mM sodium citrate buffer (pH 6.0) and heating the closed container in a microwave for 7 minutes at maximum power, followed by 20 minutes cooling at room temperature. Slides were washed twice in PBS-T and non-specific binding of secondary antibody was minimised by pre-incubating slides with 3% of serum from the species in which the secondary antibody was raised, in this case goat serum, which was supplied with the Vectastain ABC kit (Vectorlabs), made up in PBS. Primary antibody concentrations were determined according to the manufacturer's instructions and optimisation in the lab. Primary antibody was diluted in PBS to the required concentration as listed in table 2.17 and incubated for 90 minutes at room temperature.

Table 2.17: Antibodies and conditions used for immunohistochemistry

Target Protein	Blocking Solution	1° antibody	1° antibody concentration	2° antibody
SRC-1	Goat serum	Rabbit polyclonal anti SRC-1 (sc-8995)	1 in 100 (2.0 µg/ml)	Anti rabbit IgG
MMP9	Goat serum	mouse monoclonal anti MMP9 (sc-21733)	1 in 100 (2.0 µg/ml)	Anti mouse IgG
Myc	Goat serum	Rabbit polyclonal anti Myc (sc-517)	1 in 100 (2.0 µg/ml)	Anti rabbit IgG

Slides were washed thrice in PBS for 5 minutes and incubated with the appropriate biotinylated secondary antibody at a dilution of 0.5% in 1.5% block in PBS for 30 minutes. The signal was then amplified by incubating the slides for an additional 30 minutes with an Avidin Biotin Complex (ABC) supplied with the kit. Slides were washed thrice with PBS for 5 minutes and product was developed using 3,3'-Diaminobenzidine tetrahydrochloride (DAB) (Sigma Aldrich) for 2 minutes until colour developed. Sections were counterstained with haematoxylin (Sigma Aldrich) for 3 minutes, after which slides were washed in running tap water for 5 minutes. Tissue was dehydrated again by passing though increasing concentrations of IMS (70% - 100% - 100%) for 3 minutes each, followed by immersion in xylene twice for 3 minutes. Tissue was air dryed and mounted with DPX (Sigma Aldrich) and a cover slip. Slides were left to dry and stored at room temperature.

Slides were scored under an inverted microscope (Olympus) by two observers using the Allred scoring system. The scoring system consists of two scores: a proportion score to represent the area of tissue stained (none = 0, <1% = 1, >1<10% = 2, >10%<33% = 3, >33%<66% = 4, >66% = 5) and an intensity score that represents the average intensity of the positive tumour cells (none = 0, weak = 1, medium = 2, strong = 3). The scores are then combined to reach a total score between 0 and 8. A total score of greater than 3 was called positive.

2.6.2 Colocalisation studies

In fluorescence microscopy, colocalisation refers to the observation of the spatial overlap between signals of two or more different fluorophores and is often used to visualise the biological interaction between proteins inside the cell (Kreft M *et al.*, 2004, Adler J *et al.*, 2008). The ability to demonstrate correlation between proteins was greatly enhanced by the introduction of the Pearson's correlation coefficient R(r), which aids to characterise the degree of overlap between images (Adler J and Parmryd I, 2010). It can have a value anywhere between -1 and 1; the larger r, the stronger the association between the variables. For example, an r of -1 or 1 means that the two variables are perfectly correlated whereas an r of 0 implies that there is no correlation between the variables.

To assess the colocalisation between SRC-1 and ER and SRC-1 and p-Ets2 respectively, paraffin-embedded full-face tumour sections were pre-treated as described in 2.6.1 in detail. In short, sections were deparaffinised, followed by endogenous peroxidase activity depletion and heat-mediated antigen retrieval. Non-specific binding of secondary antibody was minimised by pre-incubating slides for 90 minutes with 10% of serum from the species in which the secondary antibody was raised, in this case goat serum. Slides were washed in PBS for 5 minutes. The first primary antibody was diluted in 10% human serum in PBS to the required concentration as listed in table 2.18 and incubated for 90 minutes at room temperature.

Table 2.18: Antibodies and conditions used for colocalisation studies

Target Protein	Blocking Solution	1° antibody	1° antibody concentration	2° antibody
SRC-1	Goat serum	Rabbit polyclonal anti SRC-1 (sc-8995)	1 in 100 (2.0 µg/ml)	Anti rabbit 488
ER	Goat serum	Mouse monoclonal anti ER (NCL-ER-6F11)	1 in 100 (2.0 µg/ml)	Anti mouse 568
SRC-1	Goat serum	Mouse monoclonal anti SRC-1 (05-522)	1 in 100 (10µg/ml)	Anti mouse 488
p-Ets2	Goat serum	Rabbit polyclonal anti p-Ets2 (441105G)	1 in 50 (2.0µg/ml)	Anti rabbit 594

Slides were washed thrice in PBS for 5 minutes and incubated for 60 minutes with the appropriate fluorescent secondary antibody at a dilution of 1 in 200 in PBS. Slides were washed thrice in PBS for 5 minutes and blocked in 3% of goat serum for 90 minutes. Slides were washed for 5 minutes in PBS and incubated with the second primary antibody for 90 minutes. Slides were washed thrice in PBS for 5 minutes and incubated for 60 minutes with the appropriate fluorescent secondary antibody at a dilution of 1 in 200 in PBS. Slides were washed in PBS and incubated with DAPI (1:15,000; Invitrogen) for 5 minutes to counterstain the cell nucleus. Slides were washed in water for 5 minutes and mounted with a fluorescein mounting media (DAKO). Staining was viewed under a Zeiss Confocal microscope and the Pearson's coefficient for nuclear colocalisation between SRC-1 and p-Ets2 was assessed using the WCIF colocalisation plug-in for the ImageJ image analysis software.

2.7 Immunofluorescence

Immunofluorescence is a technique that uses fluorophore-labeled antibodies to visualise a certain protein within a cell. To assess the cellular localisation of SRC-1 upon treatment, Aro and LetR cells were grown on collagen-coated coverslips. A collagen solution with the concentration of 50 µg/ml was prepared in 0.115% Acetic Acid (17.4 N) / PBS. The coverslips were washed in 70% IMS and then dried for 20 mins prior to coating them with the collagen solution. Coverslips were incubated at 37°C for 30 minutes and then washed with PBS for 5 minutes. Cells were plated at a density of 2.5 x 10^4 cells / well and steroid depleted for 72 hours. Cells were then treated with the androstenedione, estrogen, letrozole and a combination of androstenedione and letrozole at the concentrations listed in table 2.1 for 45 minutes. Cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature and permeabilised with 0.5% Triton in PBS for a further 10 minutes at 4°C. Cells were washed thrice in PBS for 5 minutes. Cells were blocked with 10% goat serum in PBS for 1h at room temperature. Cover slips were washed for 5 minutes in PBS. 100µl of SRC-1 antibody (1:50) was added to each coverslip and covered with parafilm to prevent evaporation of the solution. After 1h of incubation, coverslips were washed thrice in PBS for 5 minutes. Secondary Antibody was added for 45 minutes, followed by three 5-minute PBS washes. Cytoskeleton was stained with Phalloidin 594 (Molecular Probes) at a dilution of 1:200 for 20 minutes and the nucleus was stainined using DAPI at a dilution of 1:15,000 for 5 minutes, followed by a rinse in tap water. Coverslips were fixed on a slide with mounting media (DAKO).

2.8 Statistical Analysis

Anonymous patient databases were maintained in Microsoft Excel (Microsoft, USA). Multivariate analysis was performed using STATA 10 data analysis software (Stata Corp., Texas, USA). Fisher's exact test was used for comparison of categorical data. Student's t test was used for continuous variables. A p value smaller than 0.05 was considered significant.

Chapter 3

Characterisation of an AI resistant cell model

3.1 Introduction

To understand the mechanisms of aromatase inhibitor resistance and identify novel approaches to develop better therapies to overcome resistance, designing cellular models for endocrine resistant breast cancer appeared to be essential.

Until the 1990s, the most common method for screening aromatase inhibitors was an *in vitro* enzyme assay using aromatase expressing human placental microsomes. However, this assay represented a very artifical environment, as NADPH-cytochrome P450 reductase and a NADPH-regenerating system needed to be added to the assay. Another method established by Hausler in 1989 used luteinising hormone (LH)-treated adult female hamster ovarian tissue, but, since this method used animal tissue it was prone to issues related to tissue heterogeneity as well as species-related differences. Another downside of this assay was the large number of animals needed to harvest enough tissue for the assay (Hausler A *et al.*, 1989).

The obvious choice was to use cultured human breast cancer cells. Since AIs are used in the treatment of ER+ breast cancer a cell line expressing ERa was needed. One ER+ breast cancer cell line that has proven to be the model of choice is the MCF7 cell line as it responds to estrogen stimulation as well as anti-estrogen suppression. Yet, MCF7 cells appear to express the aromatase enzyme at very low levels, probably because in the body, aromatisation happens in several other organs besides the breast (Zhou D *et al.*, 1990). Therefore, the first step in creating an AI resistant cell line was to generate an aromatase overexpressing cell line. In 1990, Shiuan Chen's group successfully constructed an expression plasmid containing the full-length aromatase cDNA CYP19 and transfected it into the MCF7 cells. An enzyme assay could be carried out with an extremely short incubation period of 30 minutes due to high levels of aromatase stably overexpressed in those cells (Zhou D *et al.*, 1990). A reliable cell model that mimicks the estrogen production in postmenopausal women was generated and was from now on called MCF-7aro or MCF-7_{Ca} (Yue W *et al.*, 1994).

Two other ER+ breast cancer cell lines that are frequently used to study ER-mediated response are T47D and ZR75-1 and aromatase expressing versions of these cell lines, T47Daro and ZR75-1aro, have recently been generated and used to study AI resistance (Wong C and Chen S, 2012).

Two fairly different approaches were taken to generate a cell model that could be employed to investigate mechanisms involved in AI resistance:
One approach was to grow breast cancer cells in complete absence of estrogen. Resistance to aromatase inhibitors is thought to be, in part, a result of estrogen hypersensitivity or estrogen-independent activation of ER due to a constant lack of estrogen caused by the AIs. To address this question, several groups created long-term estrogen deprived (LTED) cells by continuously growing MCF-7 cells in steroid depleted media (Martin LA *et al.*, 2005; Nicholson RI, 2004; Sabnis GJ *et al.*, 2005). LTED cells as well as UMB-1Ca cells (aromatase overexpressing LTED cells) (Sabnis GJ *et al.*, 2007) are characterised by an increased activity of GF pathways, HER2 and IGF-1R in particular, but also an increase in ER expression. These finding suggested that, in this setting, GF receptors may crosstalk with ER, resulting in activation and phosphorylation of the receptor in a ligand-independent manner (Chen S *et al.*, 2006). Recent studies have shown that LTED cells are a model for late stage acquired resistance as those cells show no response to AIs or tamoxifen any more (Chen S, 2011).

To generate a physiologically more relevant cell model, aromatase overexpressing breast cancer cells were exposed to aromatase inhibitors until they became resistant to the drug. MCF7-aro cells were exposed to testosterone and the three AIs exemestane, anastrozole and Letrozole for a prolonged time (>8 months) (Chen S *et al.*, 2006). Angela Brodie's group generated a similar model at the same time by culturing early passage MCF-7Ca cells in steroid-depleted medium containing the aromatase substrate androstenedione and the AI letrozole. Cells began to proliferate after 8 weeks of treatment and gradually lost sensitivity to letrozole until they were completely

insensitive to the AI at about 52 weeks of treatment with the drug. These cells were therefore designated long term letrozole cultured or LTLC (Belosay A *et al.*, 2006). The group also generated a long-term letrozole treated (LTLT) cell model by inoculating mice with the MCF-7Ca cells and treating those animals for 56 weeks with the AI until tumours were growing despite administration of the drug. The AI resistant tumours were excised and cells were isolated (Jelovac D *et al.*, 2005).

It was decided to generate a cell model very similar to the LTLC model. A construct containing the full-length aromatase gene was designed and transfected into ER+ MCF7 breast cancer cells. To generate an AI resistant cell model, cells stably overexpressing the aromatase enzyme, designated Aro, were cultured in steroid free medium supplemented with androstenedione and the AI letrozole for a minimum of 12 weeks prior to experiments. To check if the cells were resistant to the AI, a proliferation assay was performed in which Aro and LetR cells were treated with letrozole. Proliferation was inhibited by letrozole in the normally cultured Aro cells but not in the Aro cells subjected to the AI for 12 weeks, suggesting that they have become insensitive to the drug (O'Hara J *et al.*, 2012). These cells were designated LetR (Letrozole Resistant).

Based on previous findings by other labs that endocrine resistance is often characterised by a switch from endocrine- to growth factor signaling, the AI resistant model was expected to be less responsive to steroids and more responsive to growth factors. To investigate this a proliferation assay was performed where cells had been treated with either estrogen or EGF after 48 hours of steroid depletion followed by 24 hours of serum starvation. As expected, the LetR cell model showed loss of response to the steroid and an increased response to the growth factor in comparison to the AI sensitive, steroid dependent Aro cells (Figure 3.1) (McBryan J *et al.*, 2012), suggesting that a shift from steroid- to growth factor responsiveness might be involved in the transition from AI sensitivity to AI resistance *in vitro.*

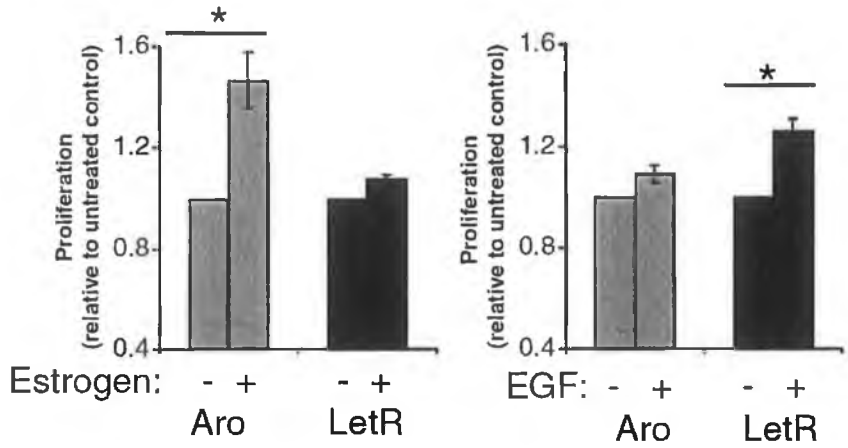


Figure 3.1: LetR cells show reduced response to the steroid Estrogen and increased response to the epidermal growth factor EGF. The AI-resistant cells (LetR, black bars) show reduced proliferative response to steroids and increased growth factor response when compared with the AI-sensitive cells (Aro, grey bars). Results are mean SEM (n = 3) (* $p < 0.01$).

3.2 Aims

The aim of this chapter was to characterize the letrozole resistant LetR cell line in comparison to the AI sensitive Aro cell line in regards to the following:

- Hormone receptor expression
- Migratory capacity
- 3D organization and polarization
- Invasiveness

3.3 Results

3.3.1 Expression of hormone receptors in LetR cells

In ER positive breast cancer patients, development of resistance to endocrine therapy often comes with a loss of ER and PR and a gain of HER2 receptor status. To characterise the AI sensitive and resistant cell models, total protein was examined for hormone receptor status by western blot analysis.

A strong increase in HER2 expression was observed in the AI resistant LetR cell model compared to the AI sensitive Aro cells (Figure 3.2). This observation is consistent with both clinical findings and the increased responsiveness of LetR cells to growth factors (Figure 3.1). However, the LetR cells also displayed a slight increase in ER and PR protein levels (Figure 3.2), despite the finding that these cells are less responsive to steroids than the sensitive Aro cells (Figure 3.1).

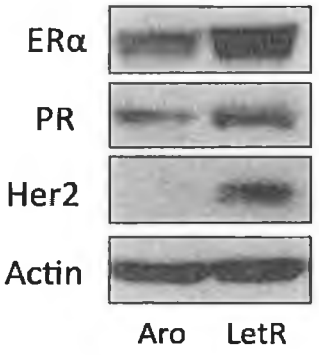


Figure 3.2: LetR cells show a slight increase in ER and PR and a dramatic increase in HER2 protein expression. *Aro and LetR cells were lysed and run on a 6% polyacrylamide gel for HER2 and a 10% gel for ER and PR. Gels were transferred and probed with antibodies against the respective receptor. Western blot analysis showed a slight increase in ER and PR protein expression and a severe increase in HER2 protein expression in LetR cells when compared with Aro cells.*

3.3.2 LetR cells exhibit a decrease in differentiation

The development of resistance to endocrine treatment results in metastasis, a process that is preceeded by endothelial to mesenchymal transition (EMT). It is believed that EMT promotes cancer cell progression and invasion into the surrounding microenvironment. EMT is a process by which cancer cells undergo molecular changes that cause dysfunctional cell-cell adhesion and junctions and reorganisation of the cytoskeleton, resulting in the loss of apical polarity as well as the acquisition of a more spindle-like shape (Creighton CJ *et al.*, 2010).

To investigate if the letrozole resistant cell model LetR possesses a less polarised and organised phenotype, sensitive and resistant cells were subjected to a 3D assay. A 3D assay is used to assess if cells can form organized and polarised acini or mammospheres in a three dimensional matrix. Cells were grown in matrigel for 16 days and stained for Phalloidin and DAPI. It was observed that Aro cells were able to form highly organized and polarized acini structures with a hollow lumen, comparable to the highly differentiated, non tumourigenic MCF10A cell line. The LetR cells on the other hand displayed a lack of organization as well as the lack of a hollow lumen, appearing more similar to the poorly differentiated, metastatic SKBR3 cells (Figure 3.3).

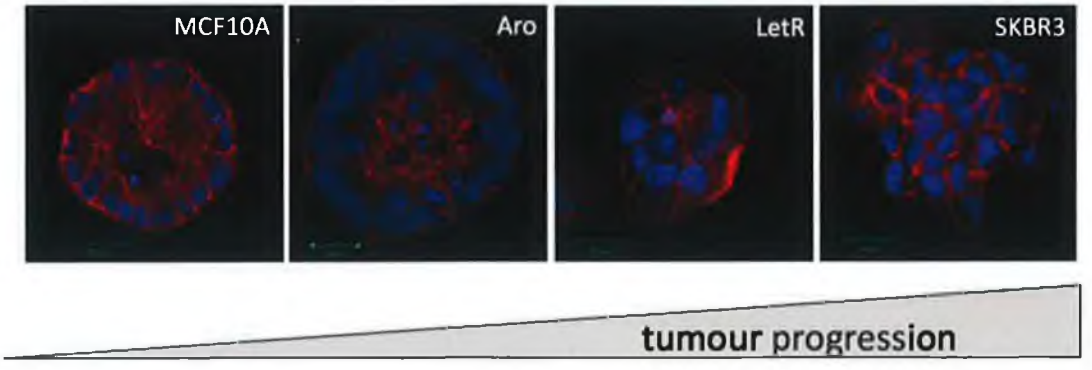


Figure 3.3: LetR cells display a loss of organisation and polarisation. *Aro cells form 3D organized structures with hollow lumen similar to the highly polarized MCF10A cells. LetR cells fail to hollow out a lumen and remain disorganized, more comparable to SKBR3 cells. Cells are stained with DAPI (blue) and phalloidin (red) and images are representative of three separate experiments. (Scale bars 20μm).*

3.3.3 LetR cells show an increase in MMP9 mRNA and express the active, proteolytic form of MMP9

For a cell to be able to invade the tumour microenvironment and metastasise to distant organs in the body it has to be able to degrade its surrounding basement membrane. MMP9 is a member of the family of gelatinases that can degrade gelatine as well as collagen type IV, a major component of the basement membrane (Stellas D *et al.*, 2010). MMP9 has frequently been implicated in cancer cell progression and invasion (Hyuga S *et al.*, 1994; Beliveau A *et al.*, 2010). Owing to the decrease in differentiation in LetR cells, it was decided to examine MMP9 expression in these cells.

RNA was extracted from Aro and LetR cells and was subjected to a PCR using MMP9 primers. At transcript level an increase in MMP9 mRNA was observed in the LetR cells when compared to the highly differentiated Aro cells (Figure 3.4, left).

To assess active levels of the MMP9 enzyme, conditioned medium from both cell lines was collected and analysed by zymography. Zymography is an electrophoretic technique to measure enzymatic activity of a certain protein. Samples are run on a so-called zymogram; a gel that contains a particular protein that serves as a substrate. Since MMP9 is a gelatinase, the conditioned media was run on a gel containing gelatine. The gel is then subjected to a developing buffer at 37°C for digestion followed by staining with Coomassie Blue. The areas of digestion appear as clear bands on a blue background. These bands indicate the size of the enzymatic protein that has degraded the substrate.

In contrast to Aro cells, LetR cells express the active, proteolytic form of MMP9, indicating that cells that become resistant to the AI letrozole are able to degrade ECM components to invade their surrounding tissue and disseminate to distant sites of the body (Figure 3.4, right).

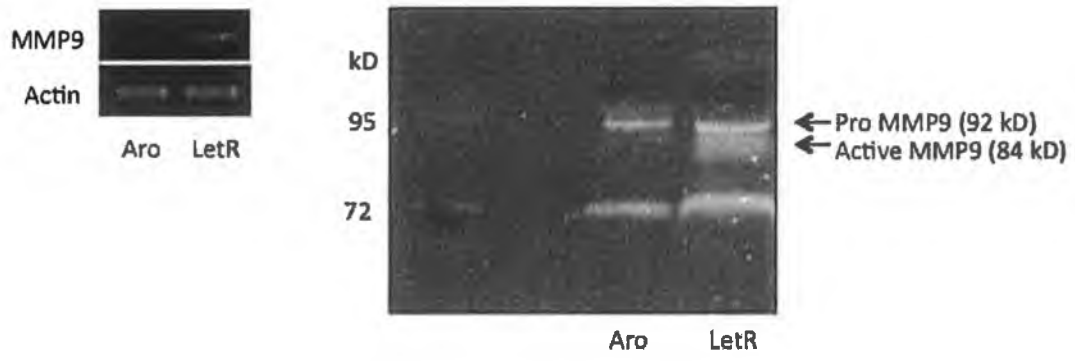


Figure 3.4: LetR cells express high levels of MMP9 mRNA and express the active form of the proteolytic enzyme. *mRNA was extracted from Aro and LetR cells and PCR was performed using MMP9 primers. Conditioned media from Aro and LetR cells was subjected to a zymography gel, stained according to protocol and bands were visualised on a light box.*

3.3.4 LetR cells display higher migratory capacity than Aro cells

The LetR cells appeared to be less organized and polarized in 3D than the AI sensitive Aro cells. Therefore, it was decided to investigate if they displayed an increase in motility in comparison to the sensitive Aro cells. Migratory capacity is an important prerequisite for a cell's ability to metastasise.

Cells were seeded into a collagen coated 96-well plate onto a lawn of fluorescent beads. As they move across the well they push away and phagocytose the beads, clearing a track behind them. The track area is proportional to the cells' ability to migrate. After an incubation period of 22h cells were fixed, stained with rhodamin phalloidin and visualised under a microscope. Track area was measured in μm^2.

The LetR cells were significantly more motile than Aro cells displaying more than 6 times the migratory capacity during the 22-hour analysis period (4,945 μm^2 per Aro cell compared to 30,572 μm^2 per LetR cell) (Figure 3.5). In relation to other cell lines, LetR cells displayed a similar migratory capacity to the highly invasive MDA-MB231 cells (35,579 μm^2 per cell).

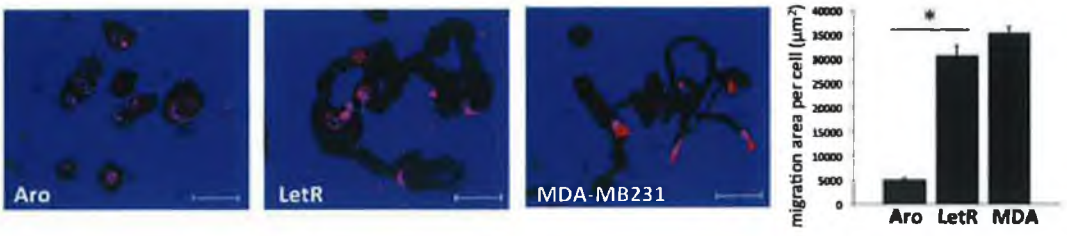


Figure 3.5: LetR cells are more motile than Aro cells. *Cells are seeded onto a lawn of microscopic fluorescent beads (blue), fixed after 22h and stained for Rhodamine Phalloidin (red). Graph shows migration area per cell in μm^2 + SEM (n=3). MDA-MB231 cells were used as a positive control (Scale bar 200µm, * p=0.0001).*

3.3.5 LetR cells are more motile that Aro cells independent of treatment conditions

Since LetR cells are cultured under slightly different conditions than Aro cells, a cell motility assay was performed using various treatments. LetR cells were steroid depleted for 72 hours and treated with Androstenedione, Letrozole and a combination of the two for 8 hours before subjecting them to a migration assay. Cells were treated for another 22 hours, then fixed and stained as described before. Images of the tracks were taken on an inverted microscope and migration area in μm^2 per cell was measured using iCell software.

A slight increase in migration was seen between the different treatments, with the biggest increase being between the vehicle sample and the Androstenedione + Letrozole treated cells (Figure 3.6). However, this increase was not significant (p=0.2375). As observed under basal conditions, the LetR cells treated with vehicle were significantly more motile than the Aro cells (p=0.0001).

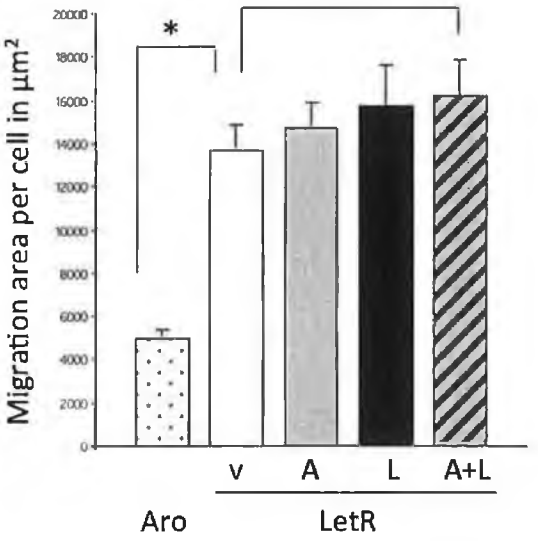


Figure 3.6: Effect of steroid treatment on migration. *Cells were treated with Vehicle (V), Androstenedione (A), Letrozole (L), or a combination (A+L). Aro cells were added for comparison. Histogram shows the mean migratory area per cell (μm^2) + SEM (n=3). (* p=0.0001)*

3.3.6 LetR cells are capable of invading an artificial matrix membrane

To further investigate the cells' potential to metastasise, sensitive and resistant cells were subjected to an invasion assay. Invasion assays have been developed to mimic the process of a cancer cell crossing a basement membrane to disseminate to distant organs in the body. The assay is set up by applying a layer of reconstituted basement membrane, matrigel, on top of a microporous membrane. The membrane is attached to an insert that can be placed into a well filled with a chemoattractant of choice. The idea is that the cells move towards the chemoattractant and by doing so, breaking down the basement membrane to migrate through the pores into the well. The membrane is stained, invading cells are counted and an average percentage of invading cells can be calculated.

Aro cells failed to cross the basement membrane and migrate into the pores (5.3% invasion) (Figure 3.7). The resistant LetR cells, however, displayed a dramatic increase in invasion (87.8%), comparable to the highly invasive MDA-MB231 cells (80.6%), and appeared to be significantly more invasive than the non-invasive Aro cell line ($p<0.0001$).

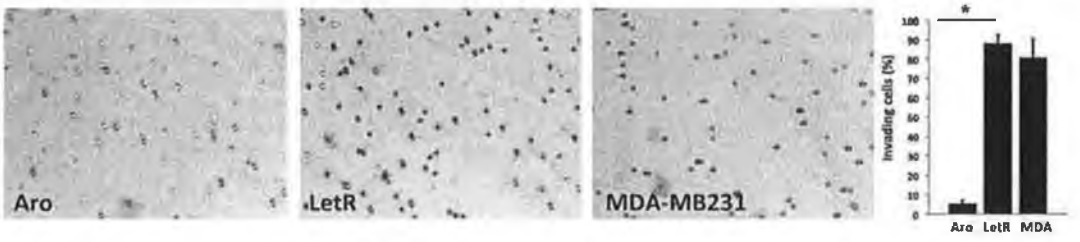


Figure 3.7: LetR cells are highly invasive. *Aro, LetR and the highly invasive MDA-MB231 cells were seeded into invasion chambers. 24h later the artificial membranes were removed from the chambers, fixed and stained with crystal violet. Invading cells were visualized under a microscope and 5 different fields were counted. Graph shows average percentage of invading cells + SEM (n=3; * p<0.0001).*

3.4 Discussion

In the patient, development of resistance to endocrine therapy is frequently accompanied by a loss in hormone receptor status and an increase in growth factor receptor status commonly known as a phenomenon called receptor switching. Even though a dramatic increase in HER2 expression was found, no decrease was found in hormone receptor protein expression in the AI resistant cell model; in fact, the resistant cells exhibited a slightly higher level of hormone receptor protein. Still, other groups studying cell models of endocrine resistance have reported similar findings (Belosay A *et al.*, 2006). For example, the Brodie LTED cell model UMB-1Ca exhibited an increase in ER expression, which was thought to be due to ligand independent activation of the receptor by crosstalk with GF signalling pathways (Brodie A *et al.*, 2005). By contrast the LTLT-Ca cells, which were isolated from MCF-7Ca tumours of mice treated with the AI Letrozole for 56 weeks, display a decrease in hormone receptor expression (Jelovac D *et al.*, 2005). To date, the LetR cells generated in our lab have not yet been used to generate tumours in mice; however, we successfully generated a xenograft model by inoculating tamoxifen resistant LY2 cells. Preliminary findings show a loss of ER and PR as well as a gain in HER2 in cells isolated from LY2 tumours. These results in combination with the proliferation data shown in Figure 3.1 suggest that LetR cells have lost steroid responsiveness but signals from the tumour microenvironment may be required for complete loss of expression of these receptors. In fact, a recent paper reported that macrophages elicit loss of ER and that crosstalk with the tumour microenvironment offers an alternative mechanism that can lead to endocrine resistance (Stossi F *et al.*, 2011).

Breast cancer progression and subsequent metastasis is a multi-step process that involves a range of events such as epithelial to mesenchymal transition (EMT), expression of matrix degrading proteins and an increase in motility and invasion.

In healthy mammary tissue, differentiated epithelium is composed of two epithelial cell linings that form an inner apicobasal-polarised luminal layer and a basal layer of myoepithelial cells, which is surrounded by a basement membrane (Dairkee SH *et al.*, 1985). The void in the middle of those acinar structures is generated through apoptosis and is referred to as a hollow lumen. The integrity of these polarised, highly organised acinar structures, so called mammospheres or acini, is highly influenced by cell-cell and cell-stromal interactions and is necessary for the maintenance of epithelial cell function (Grobstein C, 1967; Bissell M *et al.*, 1982).

One of the first characteristics of metastasis is a process known as EMT during which cancer cells lose polarity and epithelial differentiation (Krause *et al.*, 2008). Another prominent feature of EMT is the loss of E-cadherin, a protein that is responsible for stable cell-cell adhesion between epithelial cells. *In vitro*, this process can be replicated by growing cells in 3D cultures using reconstituted basement membrane as matrix to allow the formation of mammary gland structures resembling the ones found *in vivo* (Swamydas M *et al.*, 2010). MCF10A cells are often used as a positive control as they are a perfect example of well-differentiated cells that form polarised acini with a hollow lumen. The less differentiated a cell, the less polarised it usually is. Acini formed by less differentiated cells also lack a hollow lumen, which is a result of anti-apoptotic signaling inside those cells. As expected, the AI sensistive Aro cells, which are derived from the well-differentiated, non-motile MCF7 breast cancer cells, behaved very similar to MCF10A cells when grown in 3D cultures. The AI resistant LetR cells however, displayed a clear lack of organisation and polarisation and the absence of a hollow lumen, suggesting that those cells were undergoing EMT.

A common result of dedifferentiation and loss of polarisation is the invasion of the surrounding tumour microenvironment. Extracellular matrix (ECM) degradation, mediated by matrixmetalloproteinases (MMPs), is an essential step that needs to occur prior to invasion. An MMP that is highly implicated in human cancer is MMP9, also known as gelatinase B. This MMP degrades collagen IV, a major component of the

basement membrane (Opdenakker G *et al.*, 2001; Timpl R and Dziadek M, 1986). Crossing the basement membrane is a crucial step in invasion and metastasis. MMP9 has previously been shown to be regulated by the steroid receptor coactivator AIB-1 to promote breast cancer metastasis to the lung in a mouse mammary tumour model (Qin L etl al, 2008). Another recent study has shown that MMP9 is involved in osteolysis to promote breast cancer metastasis to the bone (Nannuru KC *et al.*, 2010). LetR cells expressed high levels of the active form of MMP9, whereas Aro cells only expressed the inactive precursor, pro-MMP9. The LetR cells did indeed exhibit a dramatic increase in invasion over the AI sensitive Aro cells. The LetR cells also displayed a severe increase in motility, which allows the cell to move to distant sites in the body.

Taken together, these findings suggest that Aro cells that have become resistant to the AI letrozole are driven towards a more aggressive phenotype and may obtain metastatic properties.

Chapter 4

Functional role of SRC-1 in AI resistance

4.1 Introduction

SRC-1 is known to be involved aggressive cancer behaviour as described earlier. Findings from our lab have proposed a role for SRC-1 in the development of resistance to tamoxifen. The aim of this chapter is to establish a potential role for SRC-1 in conferring the aggressive AI resistant phenotype described in chapter 3.

Initially portrayed as nuclear receptor coactivator proteins, SRC-1 and SRC-3 (AIB1), two members of the p160 family of steroid receptor coactivators, have been shown to interact with transcription factors downstream of growth factor (GF) pathways. These interactions may represent one of the consequences of GF pathway cross-talk described in the development of resistance to endocrine therapies and tumour recurrence (Myers E *et al.*, 2004; Osborne CK *et al.*, 2003; Redmond AM *et al.*, 2009). Functional interactions between SRC-1 and transcription factors other than ER, like the MAP-kinase dependent TFs Ets2 and PEA3, have previously been reported and these interactions have been shown to regulate target genes that are implicated in tumour progression and metastasis, such as Myc and MMP-9 (Myers E *et al.*, 2004; Al-azawi D *et al.*, 2008; Qin L *et al.*, 2009).

4.1.1 Myc

c-Myc (Myc) is a transcription factor and the most extensively studied member of the bHLH proteins, a superfamily of transcriptional regulators that are involved in critical cellular processes (Ledent V *et al.*, 2002). It was first discovered in Burkitt's lymphoma, where cancer cells show chromosomal translocation of the gene on chromosome 8 (Dalla-Favera R *et al.*, 1982). It was identified as a v-Myc avian myelocytomatosis viral oncogene homolog by restriction endonuclease mapping of the gene (Vennstrom B *et al.*, 1982). Myc regulates up to 15% of human genes and controls cellular processes such as cell proliferation, metabolism, differentiation and apoptosis, making it the most influential transcription factor (Dang CV *et al.*, 2006).

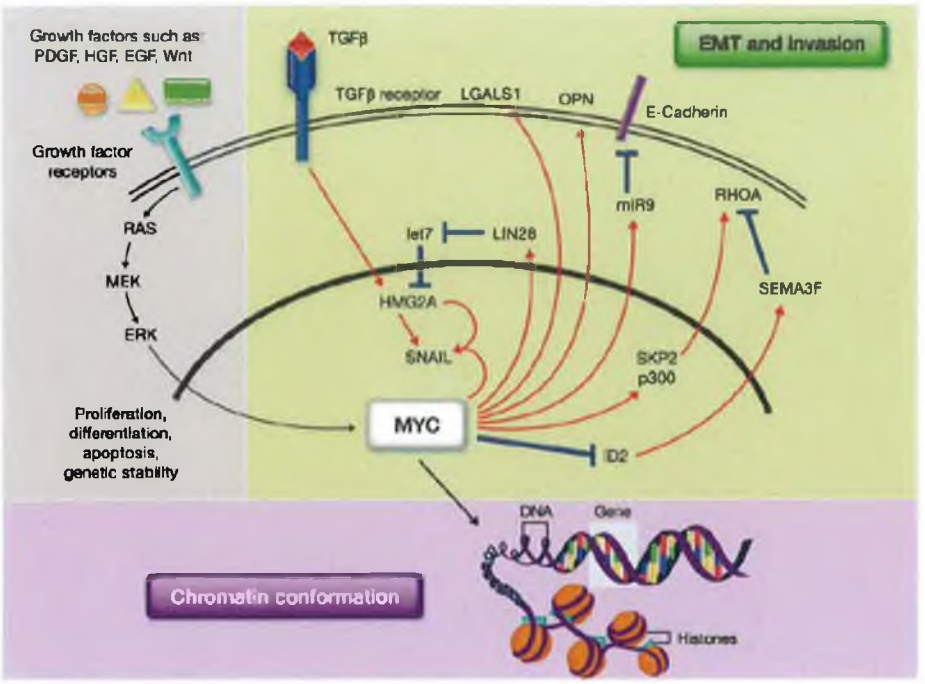


Figure 4.1: Regulation and functional role of Myc. Myc is regulated by a variety of signal transduction pathways to induce proliferation, differentiation, survival and genetic stability (gray box). Recent findings have elucidated mechanisms by which Myc can directly regulate genes involved in cell migration, invasion and EMT (green box). It is also believed that the effect of Myc on chromatin conformation may set a "metastasis enabling" epigenomic landscape (purple box). EGF, epidermal growth factor; ERK, extracellular signal regulated kinase; HGF, hepatocyte growth factor; MEK, MAP/ERK kinase; PDGF, platelet derived growth factor. *Adapted from Cancer Research Reviews (Wolfer A and Ramaswamy S, 2011).*

In cancer cells, the Myc oncoprotein promotes proliferation, cell survival, inhibition of differentiation, genetic instability and angiogenesis, all of which are thought to indirectly contribute to metastasis (Figure 4.1) (Grandori C *et al.*, 2000; Ma L *et al.*, 2010; Rapp UR *et al.*, 2009). Over the recent years, Myc has been established as one of the most important somatically mutated oncogenes and is frequently deregulated and overexpressed in a variety of human cancers where it hijacks the cell's diverse intra- and extracellular mechanisms that promote normal cell proliferation (Eilers M and Eisenman RN, 2008; Meyer N and Penn LZ, 2008). Since Myc was found to be amplified and overexpressed in human breast cancer in 1986, a number of studies have investigated

the status of Myc in breast cancer (Escot C *et al.*, 1986). It was found that amplification of Myc consistently correlated with tumour progression and poor outcome (Chen Y and Olopade OI, 2008). Myc is a downstream effector of a variety of signaling pathways, including the Wnt, Notch, Ras/Raf/MAPK and TGFβ pathways (Figure 4.1) (Chen Y, 2008), all of which are critical in breast cancer and may be potential drug targets (Xu J *et al.*, 2010). Furthermore, Myc has been shown to be regulated by *cis* regulatory elements as well as other transcription factors (Wierstra I and Alves J, 2008). It has previously been established that the transcription factor Ets2 can regulate Myc through E2F binding motifs in its promoter region (Roussel MF *et al.*, 1994; Carbone GM *et al.*, 2004). Additionally, our group has revealed that SRC-1 can utilise Ets2 to regulate the oncogene Myc in tamoxifen resistant breast cancer cells (Al-azawi D *et al.*, 2008). A recent study has also elucidated a direct role for Myc in controlling invasion and motility and subsequently metastasis by regulating expression of specific downstream programs such as EMT (Smith AP *et al.*, 2009). Taken together, these studies suggest a role for Myc in the development of resistance to endocrine therapies (McNeil *et al.*, 2006).

4.1.2 MMP9

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that play a key role in remodelling of the ECM (Vu T and Werb Z, 2000) and have therefore been associated with cancer invasion and metastasis (Fingleton B, 2006). In fact, MMPs are overexpressed in almost every human cancer and their expression is often an indicator for poor survival. They can increase cancer cell proliferation, motility, invasion, angiogenesis and metastasis by cleaving a range of substrates such as structural components of the ECM, growth-factor-binding proteins, GF precursors, RTKs and cell-adhesion molecules (Egeblad M and Werb Z, 2002). MMPs are synthesised as inactive precursors, which are referred to as pro-MMPs or zymogens. Activation of MMPs frequently takes place outside the cell and requires cleavage of the propeptide prodomain by other activated MMPs or serine proteases (Sternlicht MD and Werb Z, 2001). MMPs were originally divided into groups depending on their specificity for ECM components but with the growing list of MMP substrates a sequential numbering system

has been put into place. MMP9 is a member of the gelatinases, a group of secreted MMPs that degrades denatured collagens (gelatin) and type IV collagen, a major component of the basement membrane (Egeblad M and Werb Z, 2002). MMP9 has been found to be expressed in the cytoplasm of malignant as well as stromal cells and positive expression in the stroma was related to HER2 overexpression as well as shorter recurrence-free survival (RFS; p = 0.0389) and breast cancer-related survival (BCRS; p = 0.0081) in ER+ breast cancer (Pellikainen, JM *et al.*, 2004). *In vivo* studies have revealed that intravenously injected cancer cells were less able to metastasise to the lungs of MMP-9 deficient mice when compared to wild-type animals indicating that MMP9 plays a key role in tumour invasion and metastasis (Itoh T *et al.*, 1999).

A range of Ets transcription factors have been shown to play an important role in the activation of MMPs that are involved in invasion and metastasis. It has been revealed that *Mmp9* contains an Ets binding site in its promoter region (Figure 4.2) (Oikawa T, 2004).

Ets transcription factors Ets1 and Ets2 play important roles in embryonic angiogenesis and studies on isolated murine aortic endothelial cells revealed a function for Ets1 and Ets2 in directly regulating *Mmp9* as well as antiapoptotic genes (Wei G *et al.*, 2009). In cancer, MMP9 has been identified as an Ets1-responsive protease to promote cancer cell progression, particularly in highly invasive breast cancer cells such as MDA-MB231 (Dittmer J, 2003, 2004). Recently, Ets1 has also been found to upregulate MMP9 expression in prostate cancer cells, resulting in a chemoresistant and highly invasive phenotype (Kato T *et al.*, 2012).

Figure 4.2: Ets binding site in the MMP9 gene promoter. A Ras responsive element (RRE) consisting of an Ets and AP1 binding site is located in the promoter of the MMP9 gene, a matrix metalloproteinase that is involved in invasion and metastasis. *Adapted from Cancer Science (Oikawa T, 2004).*

4.3 Aims

The aims of this chapter were to:

- Examine the expression and regulation of SRC-1 in the letrozole resistant cell line
- Assess the functional contribution of SRC-1 to the LetR aggressive phenotype
- Elucidate the molecular mechanism of SRC-1 action in LetR cells

4.3 Results

4.3.1 SRC-1 expression is increased in LetR cells

It has previously been reported that the steroid receptor coactivator SRC-1 is aberrantly expressed in tamoxifen resistant cells (Al-azawi D *et al.*, 2008). In the patient, SRC-1 positive tumours significantly associate with increased occurrence of distant metastases and poor outcome (Redmond AM *et al.*, 2009).

To see if SRC-1 was increased in the LetR cell model, RNA as well as protein was extracted from the parental MCF7 as well as the Aro and LetR cells.

An increase in SRC-1 mRNA as well as protein levels was observed in the AI resistant LetR cells in comparison with the AI sensitive Aro and MCF7 cells (Figure 4.3), indicating a potentially similar role for the steroid receptor coactivator in the development of resistance to letrozole.

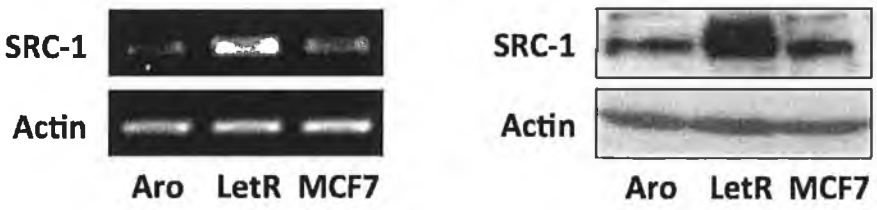


Figure 4.3: LetR cells express higher levels of SRC-1 mRNA and protein. Left: *RNA was extracted from MCF7, Aro and LetR cells. cDNA was generated and subjected to PCR using primers for SRC-1;* ***Right:*** *Protein was extracted from the cells and run on a 6% gel. Western Blot was performed using an antibody against SRC-1. Actin was used as a loading control.*

4.3.2 SRC-1 has a functional impact on motility in LetR cells

To assess if the significant increase in SRC-1 protein levels is essential for the migratory phenotype of LetR cells, the expression of the protein was knocked down using siRNA. LetR cells were transfected with a non-targeting siRNA and siRNA against SRC-1 for 72h and subsequently subjected to a motility assay.

LetR cells transfected with a non-targeting siRNA displayed a high level of motility (21,605 μm^2), which was lost upon transfection with a siRNA directed against SRC-1 (7,256 μm^2). The loss of migratory capacity in those cells was highly significant (p=0.0007), comparable to the non-motile phenotype of the AI sensitive Aro cell line (4,900 μm^2) (Figure 4.4). Thus, SRC-1 appears to be essential for the migratory phenotype of LetR cells.

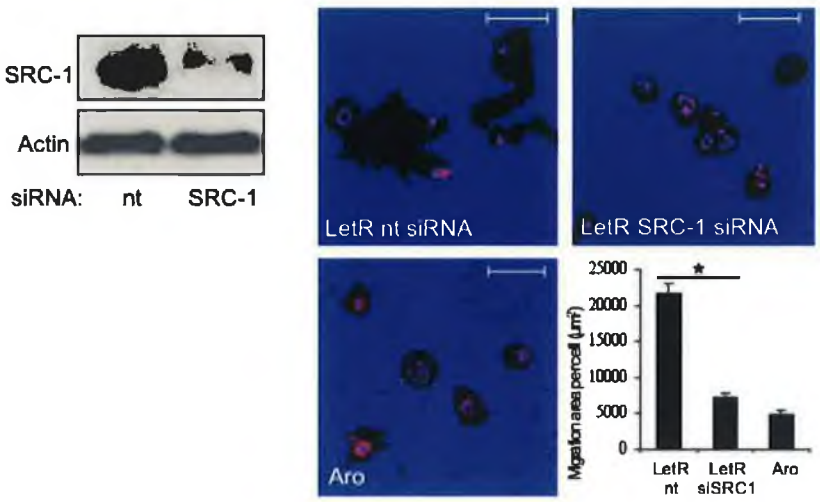


Figure 4.4: SRC-1 knockdown decreases motility in LetR cells. *Histogram shows mean migratory area per cell (μm^2) ± SEM and was significantly (*) less for SRC-1 knockdown than for non-targeting (nt) control (p=0.0007). Aro cells are shown for comparison. (Scale bars 200µm). Western blot confirms SRC-1 protein knockdown (n=3). Images show cells stained with rhodamine-phalloidin.*

4.3.3 Motility in LetR cells is not dependent on ERα

SRC-1 is an established nuclear receptor coactivator. However, nuclear coactivators have been shown to act independent of ERα. To investigate if SRC-1 has a role independent of ERα in AI resistance, LetR cells were transfected with siRNA against ERα and subjected to a motility assay.

LetR cells transfected with a non-targeting siRNA displayed a high level of migratory capacity (22,076 μm^2), whereas knockdown of ERα only caused a slight decrease in motility (13,017 μm^2). SRC-1 knockdown had a significantly greater impact on migration when compared with ERα (6,067 μm^2; p=0.0377) (Figure 4.5).

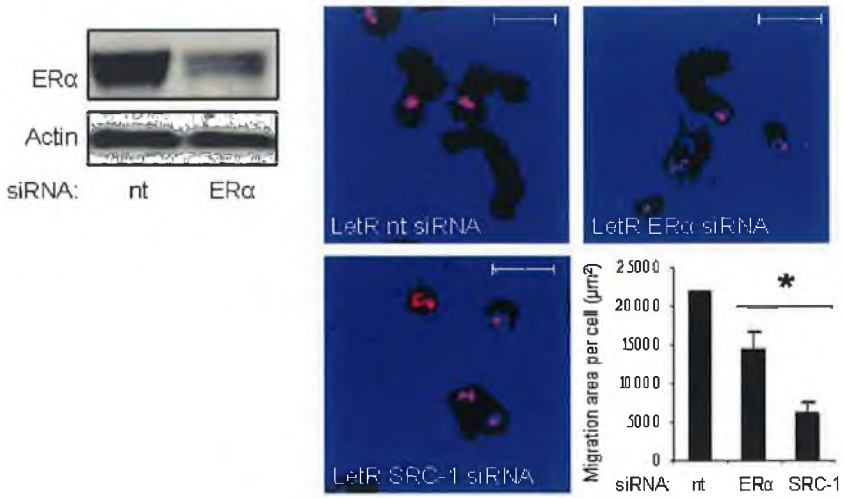


Figure 4.5: Functional migratory role of SRC-1 in AI resistance is not dependent on ERα. *Western blot confirms successful ERα knockdown with siRNA. Histogram shows only a marginal decrease in the mean migratory area per cell in LetR cells following ERalpha knockdown. These cells migrate significantly more than LetR cells with SRC-1 knockdown (shown for comparison, p=0.0377). Images show cells stained with rhodamine-phalloidin.*

4.3.4 SRC-1 overexpression has no effect on migratory capacity in Aro cells

Since LetR cells display an increase in SRC-1 and motility, we wanted to investigate if SRC-1 overexpression in non-motile Aro cells would increase their capacity to migrate. LetR cells were maintained in antibiotics free media for 24h and transfected with SRC-1 for 72 hours. Cells were then subjected to a migration assay as described before.

Aro cells transfected with the SRC-1 plasmid did not display a significant increase in motility ($p = 0.3947$). Their migration area per cell (6,167 μm^2) changed minimally in comparison to that of Aro cells transfected with the empty vector (5,384 μm^2) and therefore, the LetR cells presented a significantly higher ability to migrate than the SRC-1 expressing Aro cells (30,572 μm^2; $p < 0.0001$) (Figure 4.6). Thus, although SRC-1 is essential for the migratory phenotype of LetR cells, temporary overexpression of SRC-1 alone is not sufficient to induce this phenotype in Aro cells.

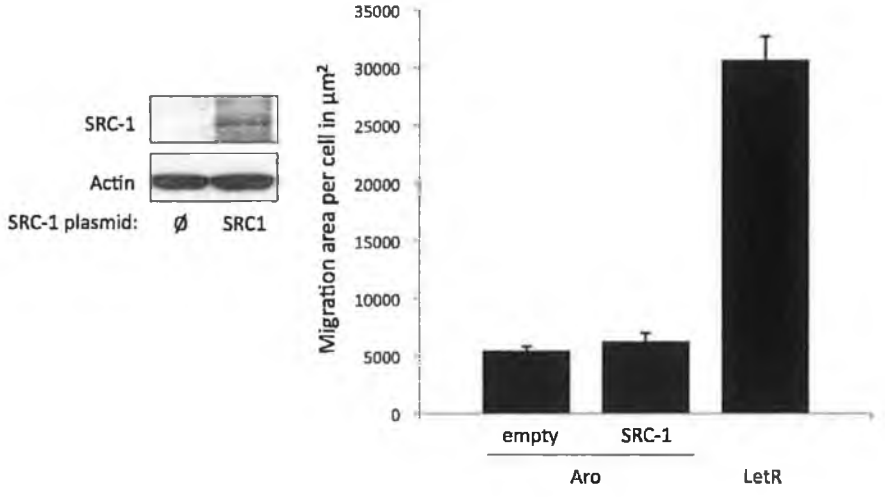


Figure 4.6: SRC-1 overexpression does not increase motility in Aro cells. *Aro cells were transfected with a pcDNA3.1 empty vector and SRC-1 overexpression vector. Western Blot analysis illustrates successful overexpression of SRC-1. Histogram shows the mean migratory area per cell (μm^2) ± SEM (n=3). LetR cells were added for comparison.*

4.3.5 SRC-1 has a functional impact on 3D organisation in LetR cells

LetR cells display a severe loss of 3D organisation and polarisation. Since those cells express a high level of SRC-1 we wanted to examine if knockdown of this protein would restore the cells ability to form acini structures.

Cells were transfected with SRC-1 siRNA and subjected to a 3D assay as before.

LetR cells transfected with a non-targeting siRNA displayed a severe loss of polarisation and formed less organised acini. In contrast, LetR cells transfected with siRNA targeted against SRC-1 regained the ability to form organised 3D acini structures and hollow out a lumen, comparable to the differentiated AI sensitive cell line (Figure 4.7).

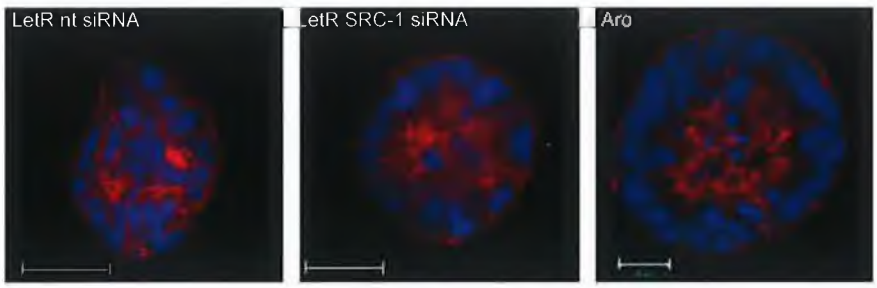


Figure 4.7: SRC-1 knockdown increases ability to form polarized, organized 3D acini in LetR cells. *LetR cells transfected with siRNA against SRC-1 form 3D organized structures with hollow lumen similar to the polarized Aro cells. LetR cells transfected with non-targeting siRNA fail to hollow out a lumen and remain disorganized. Cells are stained with DAPI (blue) and phalloidin (red) and images are representative of three separate experiments (Scale bars 20μm).*

4.3.6 SRC-1 has a functional impact on invasiveness of LetR cells

LetR cells display invasive potential as shown in chapter 3. To investigate if invasion is regulated by SRC-1 in those cells, a knockdown was performed using siRNA against the coactivator. LetR cells were subsequently subjected to an invasion assay as described before. Four individual areas of the membrane were imaged and invading cells were counted. The level of invasiveness was calculated as a percentage of cells blocking the pores of the membrane.

LetR cells transfected with a non-targeting siRNA displayed a high level of invasiveness (84%) as expected from previously described findings. The LetR cells transfected with siRNA against SRC-1, however, failed to invade the matrigel-covered membrane as they were not able to migrate into the pores (11.2% invasion), similar to the non-invasive Aro cell line (5.3%), revealing a significant role for SRC-1 in regulating invasion of letrozole resistant cells (p=0.0001) (Figure 4.8).

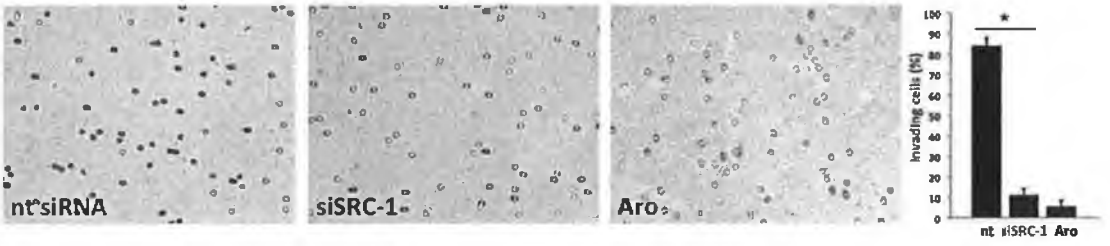


Figure 4.8: SRC-1 knockdown in LetR cells inhibits invasivion. *LetR cells transfected with siRNA against SRC-1 are able to invade matrigel and migrate into pores of the membrane inside an invasion chamber. LetR cells transfected with non-targeting siRNA fail to invade the matrigel layer and cannot migrate into the pores. Aro cells were added for comparison. Cells are stained with Crystal Violet. Invasive potential is expressed in % of cells invading the membrane.*

4.3.7 Ets2 protein expression is increased in letrozole resistant cells

Since SRC-1 seems to act independent of ERα in LetR cells we wanted to identify the transcription factor SRC-1 interacts with to confer the AI resistant phenotype. Our lab has previously reported that, in Tamoxifen resistance, SRC-1 can recruit the MAP-Kinase dependent transcription factor Ets2 to regulate the expression of pro-proliferative genes (Al-azawi D *et al.*, 2008).

The first step was to assess if Ets2 expression was increased in LetR cells in comparison to AI sensitive cells. RNA was extracted from MCF-7, Aro and LetR cells and subjected to PCR to look for changes of Ets2 on a transcriptional level. Protein extracted from Aro, LetR and MCF-7 cells was resolved by SDS-PAGE and immunoblotted for overall and phosphorylated Ets2.

No change in Ets2 mRNA levels was observed. However, Ets2 protein levels were elevated with a dramatic increase in the phosphorylated form of the transcription factor (Figure 4.9). These findings reflect the expression pattern of SRC-1 and support a role for SRC-1 and Ets2 in the development of resistance to AIs.

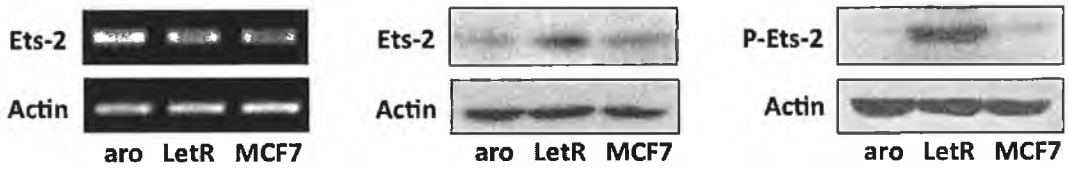


Figure 4.9: Ets2 and P-Ets2 protein levels, but not Ets2 mRNA levels, are increased in LetR cells. *RNA was extracted from MCF7, Aro and LetR cells. cDNA was generated and PCR was performed using primers for Ets2. Protein was extracted from from MCF7, Aro and LetR cells. Western Blot was performed using an antibody against Ets2 and the phosphorylated form of the protein (P-Ets2). Actin was used as a loading control. PCR and Western blot images are representative (n=3).*

4.3.8 Ets2 binds to SRC-1 in the presence of Androstenedione

To show that SRC-1 binds to the MAP Kinase dependent transcription factor Ets2, Co-immunoprecipitation (Co-IP) was performed. Aro cells were treated with androstenedione for 0', 45', 2h, 3h and 4h. Cell lysate was immunoprecipitated with an antibody against Ets2 immobilised on protein A beads, protein was eluted from the beads and run on SDS-PAGE. The gel was transferred onto a membrane, which was immunoblotted for SRC-1 and Ets2. The results of the Co-IP showed that the steroid receptor coactivator SRC-1 and the transcription factor Ets2 can interact after 45 minutes of Androstenedione treatment (Figure 4.10).

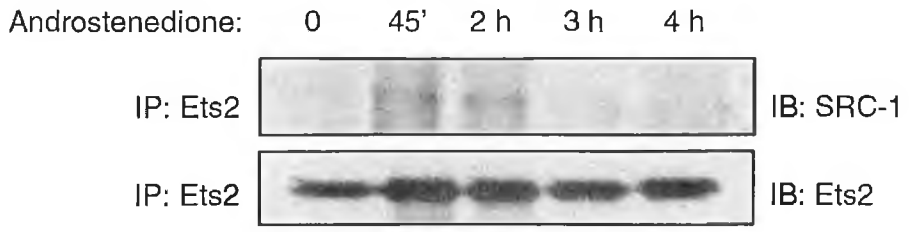


Figure 4.10: SRC-1 and Ets2 co-immunoprecipitate with strongest interaction after 45 minutes steroid treatment. *Aro cells were treated with androstenedione for 0-4 hours. Protein was immunoprecipitated (IP) with an anti-Ets2 antibody and immunoblotted (IB) for SRC-1 and Ets2.*

4.3.9 Myc expression is increased in LetR cells

Myc has previously been identified as an SRC-1 and Ets2 target in tamoxifen resistance by ChIP and luciferase (Al-azawi D *et al.*, 2008). Additional ChIPseq analysis performed in tamoxifen resistant LY2 cells confirmed these findings, revealing a strong SRC-1 binding peak located within the proximal promoter region of the oncogene (Figure 4.11) (McBryan J *et al.*, 2012).

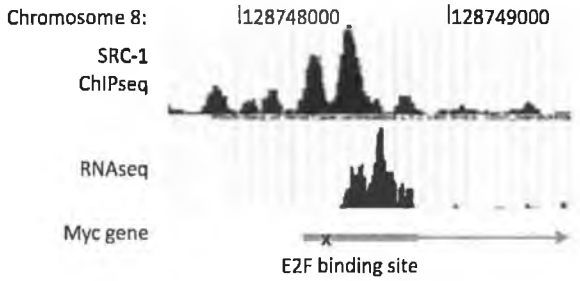


Figure 4.11: Location of SRC-1 binding peak within the proximal promoter region of Myc gene as detected by ChIP sequencing analysis in endocrine resistant LY2 cells. *RNA sequencing confirms expression of Myc mRNA in these cells. X marks the location of an E2F-binding site within the Myc promoter.*

To see, if Myc is involved in the development of resistance to aromatase inhibitors, RNA was extracted from MCF-7, Aro and LetR cells and subjected to PCR to assess changes in Myc at a transcriptional level. Protein was extracted from MCF-7, Aro and LetR cells and analysed for Myc expression by Western Blot analysis.

An increase in Myc mRNA levels was found in the AI resistant cells LetR when compared to the steroid dependent Aro and MCF7 cells (Figure 4.12), indicating a potentially similar role for the steroid receptor coactivator in the development of resistance to letrozole. A substantial increase was also observed in Myc protein levels in the LetR cells. These findings together with the observation that SRC-1 interacts with Ets2 suggest a role for these proteins in regulating the expression of the oncogene Myc in AI resistance.

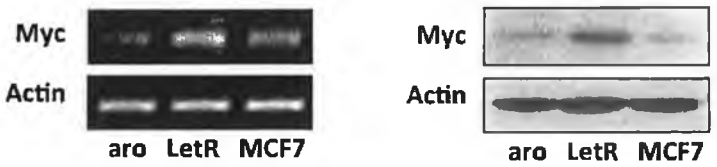


Figure 4.12: LetR cells express higher levels of Myc mRNA and protein. *RNA was extracted from MCF7, Aro and LetR cells. cDNA was generated and PCR was performed using primers for Myc. Protein was extracted from MCF7, Aro and LetR cells and resolved on a 10% gel. Western Blot analysis was performed using an antibody against Myc. Actin was used as a loading control. PCR and Western blot images are representative (n=3).*

4.3.10 SRC-1, Ets2 and Myc protein expression is insensitive to letrozole in LetR cells

To investigate molecular changes induced by steroid treatment, cells were steroid depleted for 72h and treated with estrogen, androstenedione, letrozole and a combination of androstenedione and letrozole for 8h and protein expression was analysed (Figure 4.13).

We found that SRC-1 and Myc protein expression increased upon steroidal treatment but decreased upon treatment with letrozole in Aro cells. However, expression of both proteins was insensitive to Letrozole in the resistant cells. In fact, protein expression of both SRC-1 and Myc seemed to be slightly increased upon AI treatment to the same extent, supporting our theory that SRC-1 is regulating Myc. Ets2 expression was not regulated by steroid treatments or letrozole in either cell line.

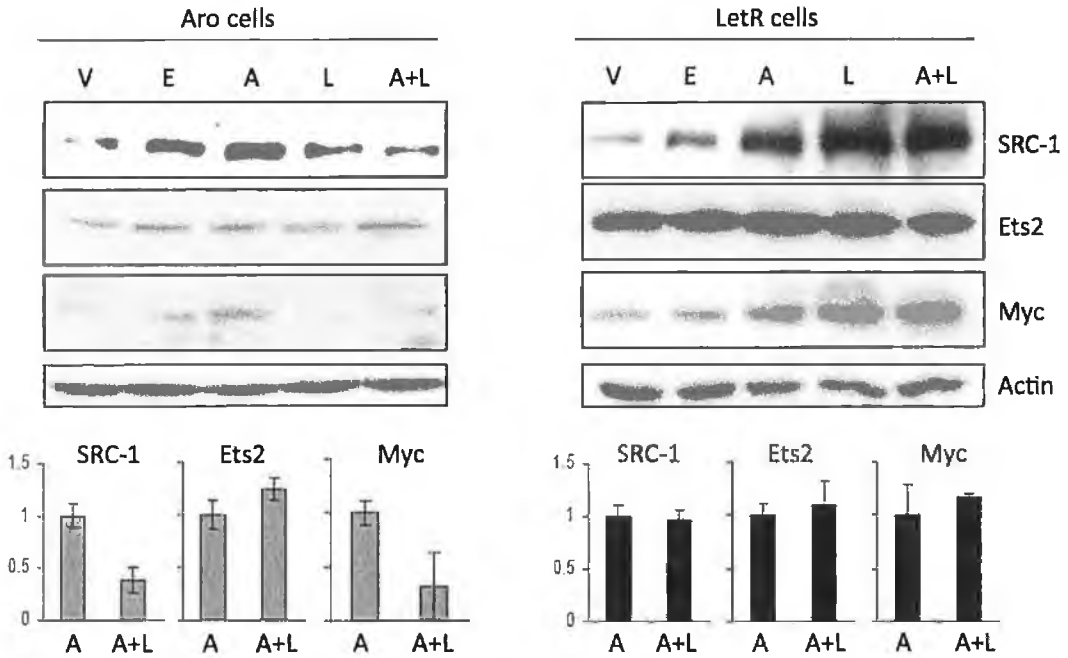


Figure 4.13: Response of SRC-1, Ets2 and Myc to steroid treatments *SRC-1 and Myc protein expression is sensitive to letrozole treatment in Aro cells but insensitive to letrozole in LetR cells. Ets2 expression is not regulated by steroid treatments in either cell line. Cells were treated with Vehicle (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.*

4.3.11 Localisation of SRC-1 upon steroid treatment

As a transcriptional coactivator, SRC-1 is active when it is located in the nucleus and interacting with transcription factors to regulate expression of target genes.

To investigate if SRC-1 is located in different cell compartments upon treatment with the androgen androstenedione and the AI letrozole, cells were grown on cover slips, steroid depleted for 72h and treated for 40 minutes. The cells were then fixed and stained for SRC-1, Phalloidin and DAPI by Immunofluorescence (Figure 4.14).

In the sensitive Aro cells we found that SRC-1 staining intensity was increased and SRC-1 localisation became more nuclear upon treatment with androstenedione. This effect was reversed in Aro cells upon treatment with the AI. In the resistant cell line however SRC-1 levels were already high, particularly in the nucleus, in the steroid depleted vehicle sample and didn't change upon treatment, neither with androstenedione nor the AI letrozole.

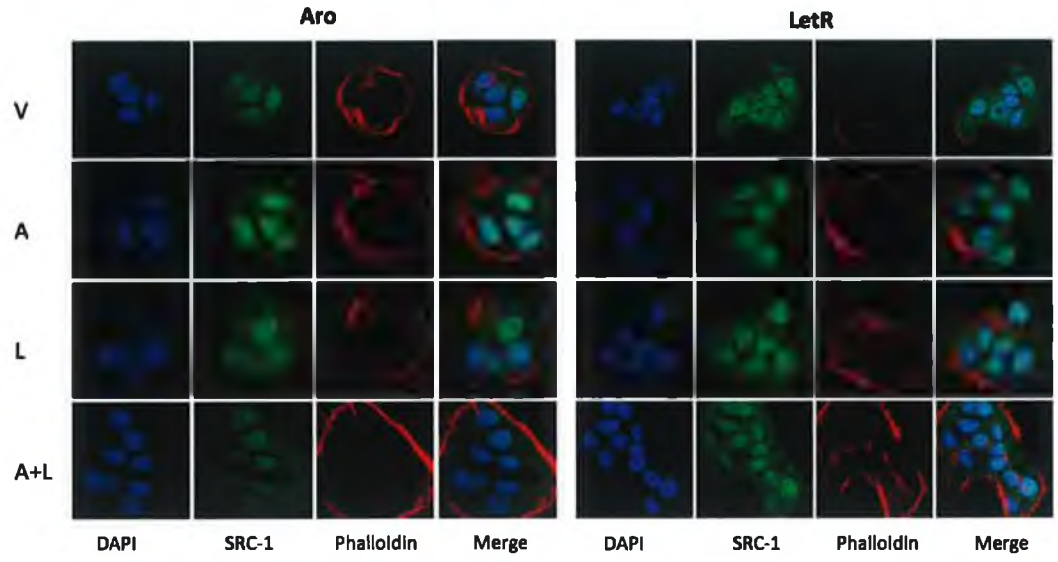


Figure 4.14: Localisation of SRC-1 upon steroid treatments. *Confocal images of SRC-1 localisation in Aro and LetR cells in the presence and absence of androstenedione and letrozole alone and in combination. Nuclear localisation of SRC-1 increased in Aro cells in response to androstenedione and was reduced when letrozole was added. By contrast, nuclear intensity of SRC-1 was strong in LetR cells independent of treatments. Images are taken at 40X magnification with a confocal fluorescent microscope and image exposures were kept constant for ease of comparison.*

4.3.12 SRC-1 and Ets2 are recruited to the promoters of Myc and MMP9

To investigate if SRC-1 and Ets2 recruit to the promoter of the target genes Myc and MMP9, Chromatin Immunoprecipitation (ChIP) was performed at 45 minutes of treatment as previously established.

It was found that SRC-1 as well as Ets2 were recruited to the promoters of both Myc and MMP9 in response to estrogen and androstenedione. This recruitment was sensitive to letrozole in the Aro cells but not the AI resistant LetR cell line, reflecting the expression pattern of the target proteins (Figure 4.15).

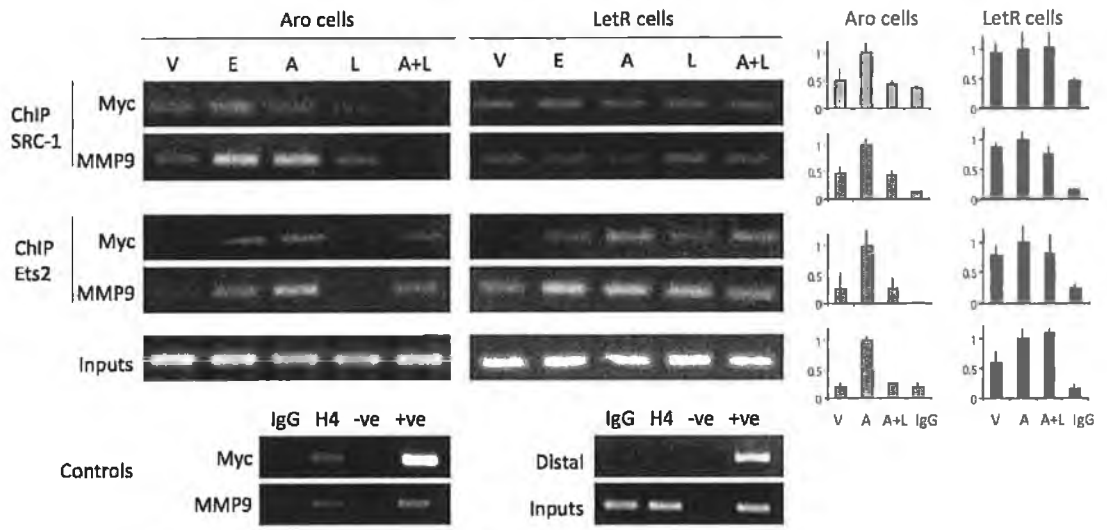


Figure 4.15: SRC-1 and Ets2 are recruited to the Myc and MMP9 promoters. *ChIP analysis in Aro and LetR cells. Cells were treated with vehicle (V), estrogen (E), androstenedione (A), letrozole (L), or a combination (A+L). Recruitment to both promoters was letrozole sensitive in Aro cells and letrozole 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.*

4.3.13 SRC-1 and Ets2 regulate expression of target genes Myc and MMP9

To see if SRC-1 and Ets2 have an effect on expression of those proteins we conducted transient overexpression studies in the AI sensitive Aro cells.

SRC-1 overexpression resulted in an increase of Myc and MMP9 at the transcript level. Myc protein expression was increased upon SRC-1 overexpression, but no change was observed in protein levels of the active, secreted form of MMP9, which was assessed by zymography (Figure 4.16 A).

An increase of Myc and MMP9 mRNA was also observed when Ets2 was overexpressed. Ets2 overexpression resulted in an increase in Myc protein levels, but no change in expression levels of the active, secreted form of MMP9 (Figure 4.16 B).

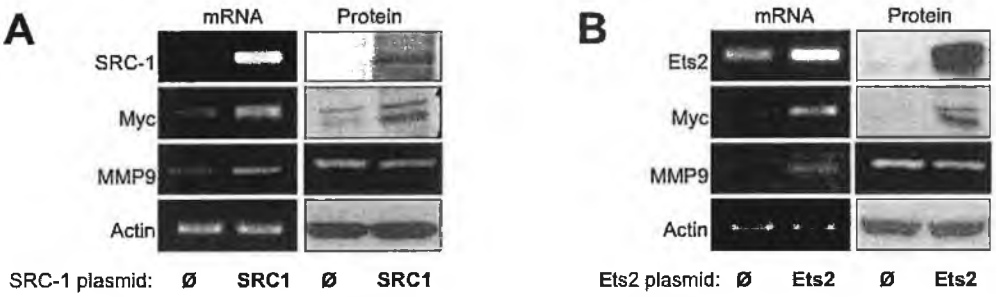


Figure 4.16: Myc and MMP9 are regulated by SRC-1 and Ets2. A, *Overexpression of SRC-1 resulted in increased transcript levels of both Myc and MMP9 (RT-PCR analysis) in Aro cells. Increased Myc expression was also seen at the protein level (western blot) but no change in secreted levels of MMP9 protein was observed (zymography).* ***B,*** *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 was observed (zymography). Images are representative (n=3).*

4.3.14 Ets2 induced Myc and MMP9 expression is dependent on SRC-1

To further assess if SRC-1 and Ets2 are acting in combination or independently to regulate Myc and MMP9 expression, LetR cells were simultaneously transfected with an Ets2 overexpression vector and siRNA directed against SRC-1. As previously shown in the Aro cells, overexpression of Ets2 caused an increase in Myc and MMP9 mRNA levels. However, this increase was abrogated when the cells were concomitantly transfected with siRNA against SRC-1, suggesting that the steroid receptor coactivator is required for the Ets2-mediated upregulation of Myc and MMP9 (Figure 4.17).

These findings reveal that SRC-1 coactivates Ets2 to regulate Myc and MMP9 expression in the aggressive AI resistant phenotype.

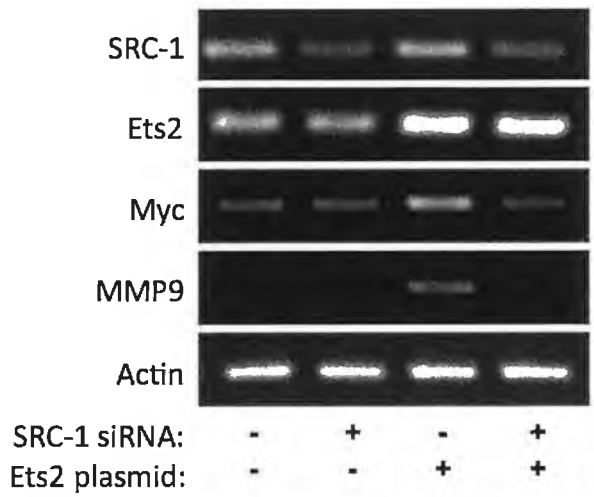


Figure 4.17: Ets2 overexpression increases Myc and MMP9 in an SRC-1-dependent manner. *LetR cells were transfected with empty vector or Ets2 and non-targeting and siRNA against SRC-1. Levels of Myc and MMP9 mRNA were increased when Ets2 was overexpressed in those cells, but this increase was inhibited upon concomitant transfection with SRC-1 siRNA. Images are representative (n=3).*

4.4 Discussion

Steroid receptor coactivators, especially members of the p160 family, have been implicated in a variety of human diseases such as metabolic syndrome and various cancers (Lanz RB *et al.*, 2008; Yan J *et al.*, 2008; York B and O'Malley BW, 2010). SRC-1 has been shown to be overexpressed particularly in breast cancer. It has been reported to be overexpressed in up to 30% of samples and has been shown to positively correlate with breast carcinogenesis (Xu J *et al.*, 2009). SRC-1 expression associates with poor disease-free survival and correlates positively with HER2 expression and resistance to tamoxifen (Fleming FJ *et al.*, 2004a,b; Myers E *et al.*, 2004). It has also been reported that SRC-1 mRNA levels are elevated in patients during neo-adjuvant treatment with AIs (Flageng MH *et al.*, 2009). Here we show that the steroid receptor coactivator SRC-1 is significantly increased in the aromatase inhibitor resistant cell model LetR, both at transcript and protein level.

To investigate the functional role of SRC-1 on migratory capacity and metastatic potential, SRC-1 expression was abrogated by RNA interference. The experiment revealed a dramatic decrease in motility and invasion in response to SRC-1 silencing, suggesting that the coactivator plays a key role in regulating these cellular processes in AI resistant breast cancer cells. Our results are consistent with results from xenograft studies performed in Jianming Xu's and David Crowe's group. They revealed that, while disruption of the SRC-1 gene did not affect tumour initiation or growth, it significantly suppressed breast cancer metastasis to the lung in mice, supporting these findings (Wang S *et al.*, 2009; Qin L *et al.*, 2009; Han JS and Crowe DL, 2010).

On the other hand, overexpression of SRC-1 in hormone sensitive Aro cells did not result in an increase in motility. This might be due to the fact that SRC-1 cannot bind to DNA directly. To promote cell motility SRC-1 needs to interact with other transcription factors to induce genes that are implicated in migration (Edwards DP, 2000; O'Malley BW, 2007). Unless these TFs are endogenously expressed in abundance or artificially overexpressed, an increase in SRC-1 might not be sufficient to affect the cells ability to migrate. Another possible explanation might be that although SRC-1 is overexpressed, it

may require activation, potentially by GF signalling, which is not active in Aro cells, in order to affect migration.

It is also possible that temporary overexpression of SRC-1 is not sufficient to induce cellular changes required for increased migration. After all, LetR cells only begin to exhibit the highly motile, SRC-1 overexpressing phenotype following months of drug exposure.

Silencing SRC-1 resulted in regained ability to form organised and polarised acini structures in 3D culture as well as the loss of invasiveness and motility in the LetR cell model. As discussed in chapter 3, EMT is an important event during cancer progression. Data from Jianming Xu's group revealed that SRC-1 can serve as a coactivator for PEA3 to increase Twist1 expression in breast cancer. Twist1 is an EMT-promoting gene that enhances invasion and metastasis by recruiting the NuRD protein complex to inhibit E-Cadherin expression (Qin L et at, 2009; Fu J *et al.*, 2011). Another paper from the same group has recently reported that SRC-1 is involved in the upregulation of Integrin α_5 which results in the promotion of cell adhesion and migration (Qin L *et al.*, 2011). Therefore, knocking down SRC-1 may result in normal E-Cadherin expression and subsequent restoration of cell-cell adhesion, serving as an explanation for the findings presented in this chapter.

The members of the p160 family of steroid receptor coactivators were originally thought to only be involved in the progression of hormone-dependent cancers, such as ER+ breast cancer, mainly by acting as transcriptional activators of nuclear steroid receptors. Clinical data however suggested that dysregulation of steroid receptor coactivators in hormone-independent cancers correlated with pathological factors and clinical prognosis (Lee K *et al.*, 2011). Our group has just recently shown that SRC-1 affects motility and 3D organisation in tamoxifen resistant breast cancer cells in an estrogen-independent manner (McCartan D *et al.*, 2012). In line with these findings, the data shown here demonstrates that motility becomes ER-independent when breast cancer

cells develop resistance to aromatase inhibitors. Overall, these findings suggest another role for SRCs in cancer besides just being a nuclear receptor coactivator.

It has frequently been suggested that SRC-1 may bind transcription factors other than nuclear steroid receptors to induce transcription of target genes. SRC-1 has previously been shown to interact with a range of TFs such as Ets2 to regulate Myc (Al-azawi D *et al.*, 2008), MYB to regulate ADAM22 (McCartan D *et al.*, 2012) and HOXC11 to regulate S100β (McIlroy M *et al.*, 2010) in the tamoxifen resistant breast cancer cell line LY2. Here we chose to examine the interaction between SRC-1 and Ets2 in AI resistance. The TF Ets2 has been shown to be highly expressed in invasive breast tumour cell lines (Watabe T *et al.*, 1998). Additionally, Ets2 expression associated with reduced disease free survival in a cohort of endocrine treated breast cancer patients with locally advanced disease (Al-azawi D *et al.*, 2008). Findings from our group have demonstrated that SRC-1 complexes with the transcription factor Ets2 in endocrine resistant breast cancer (Myers E *et al.*, 2005) and that Ets2-mediated expression of the oncogene Myc was reliant on SRC-1 in those cells (Al-azawi D *et al.*, 2008). The results presented in this thesis confirm a similar role for this signalling mechanism in the development of resistance to the aromatase inhibitor letrozole. Here we show that Ets2 and SRC-1 overexpression in Aro cells has a direct effect on regulating Myc and MMP9, yet, overexpression of Ets2 and concomitant knockdown of SRC-1 in LetR cells revealed that regulation of these target genes is dependent on SRC-1, suggesting a key role for SRC-1 in the development of AI resistance.

Furthermore, it has been shown that SRC-1, Ets2 and Myc protein expression as well as SRC-1 localisation in LetR cells is insensitive to steroidal and aromatase inhibitor treatment, again supporting our finding that this signaling mechanism is independent of ER in AI resistance. Although not examined here, EGF stimulation of SKBR3 cells has previously been shown to stimulate recruitment of Ets2 to the Myc promoter and perhaps this is a possible mechanism by which growth factors are stimulating growth in

LetR cells (Al-azawi D *et al.*, 2008). In the LetR cells, SRC-1 and Ets2 recruitment to the promoters of Myc and MMP9 was unaffected by letrozole treatment when compared to the AI sensitive Aro cells, indicating that AI treatment has no longer an effect on the regulation of target genes that are involved in proliferation and metastasis.

The results in this chapter successfully illustrate a functional role for SRC-1 in the development of resistance to aromatase inhibitors.

Chapter 5

Clinical significance of SRC-1 in AI resistance

5.1 Introduction

In vitro cell models are valuable tools to investigate molecular mechanisms that are involved in oncogenesis and the development of resistance to aromatase inhibitors; yet, these cell lines are simply models and are not absolutely true representations of the disease itself (Chen S, 2012). Most AI resistant cell lines are derived from epithelial breast cancer cells that have been stably transfected to overexpress the aromatase enzyme. However, in breast tumours, the stromal cells surrounding the epithelial cells also express aromatase mRNA at high levels (Bulun SE *et al.*, 1993; Harada N, 1997), suggesting that paracrine effects could be overlooked in the *in vitro* setting. Therefore, to fully determine the importance of observations made in the AI resistant cell model, they must be compared to *in vivo* results, either from animal models or ideally from clinical samples. However, the use of AIs as initial adjuvant treatment of breast cancer in post-menopausal women has only been approved a little over five years ago and therefore obtaining clinical samples, both from the primary and resistant tumour, proves to be rather difficult, as recurrences are only emerging now.

Aromatase inhibitors were initially developed to serve as an alternative to tamoxifen in patients that exhibited cancer progression following antiestrogenic therapy. AIs were approved by the FDA as second-line metastatic therapy in 1997 and as first-line metastatic treatment of locally advanced or metastatic disease in 2001 (Figure 5.1).

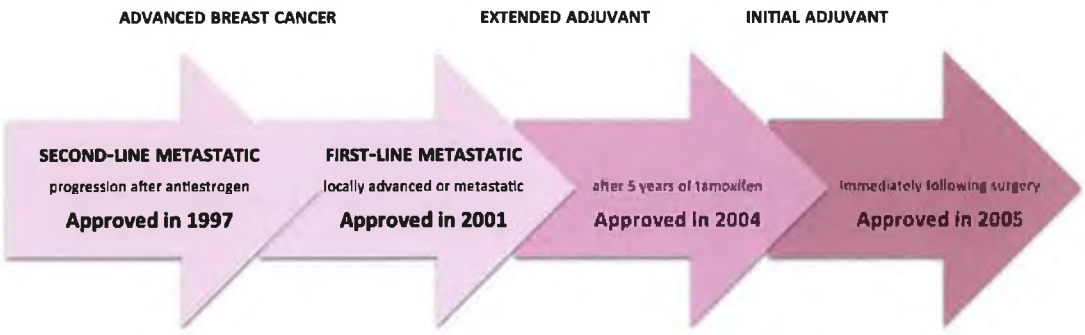


Figure 5.1: Timeline depicting approval of the AI letrozole (Femara®) for various breast cancer stages. *Information derived from Femara.com.*

When results from comparative studies revealed that the non-steroidal AIs letrozole and anastrozole were in fact superior to tamoxifen as first-line treatment in postmenopausal women, they were approved as initial adjuvant therapy in 2005 (Goss PE *et al.*, 2003; Aydiner A, 2008).

5.1.1 Patient information

In order to study mechanisms of AI resistance in the *in vivo* setting, clinical samples were gathered from a cohort of breast cancer patients. Following ethical approval, 159 endocrine-treated breast cancer patients that were diagnosed between 2004 and 2008 were included in this study. Core biopsy was used for diagnosis and to determine ER, PR and HER2 status by immunohistochemical staining. FISH was also used for HER2 calling where necessary and hormone receptor status was then used to determine the subsequent treatment strategy. Depending on the size, tumour stage and grade, patients underwent breast conserving surgery or mastectomy. Following surgery, the majority of patients received chemotherapy and radiation therapy prior to commencing adjuvant hormonal therapy. 75 patients received adjuvant tamoxifen therapy and 84 patients received adjuvant aromatase inhibitor therapy. Those patients who were HER2 positive also received herceptin.

Disease free survival was defined as the time of the initial surgery to the time of disease recurrence with a main follow up time of 56 months. Recurrent tumours that were biopsied or removed by surgery were also examined for hormone receptor status. However, not all recurrences were removed by surgery, depending on patient circumstances.

5.2 Aims

The aims of this chapter were to:

- Examine the expression of SRC-1 in clinical samples
- Assess the association between SRC-1 expression and clinicopathologic variables
- Analyse the expression of the SRC-1 signaling pathway in clinical samples

5.3 Results

5.3.1 SRC-1 associates with poor disease free survival in endocrine treated patients

A tissue microarray was constructed from primary tumours of 159 endocrine treated patients with a minimum of 4 cores per patient represented on the TMA. The TMA was then stained for SRC-1 by immunohistochemistry and scored. Strong positive staining for SRC-1 was detected in the cytoplasm as well as the nucleus of breast tumour epithelial cells (Figure 5.2 A). Kaplan-Meier survival estimates revealed that, in the overall endocrine treated patient cohort, expression of SRC-1 significantly associated with a reduced period of disease-free survival (p=0.0255) (Figure 5.2 B). When looked at the two treatments separately, SRC-1 positive primary tumours also significantly associated with reduced disease free survival in the breast cancer patients treated with tamoxifen (p=0.0326). However, this significant reduction in survival was not observed in the AI treated patient cohort (p=0.6894) (Figure 5.2 C).

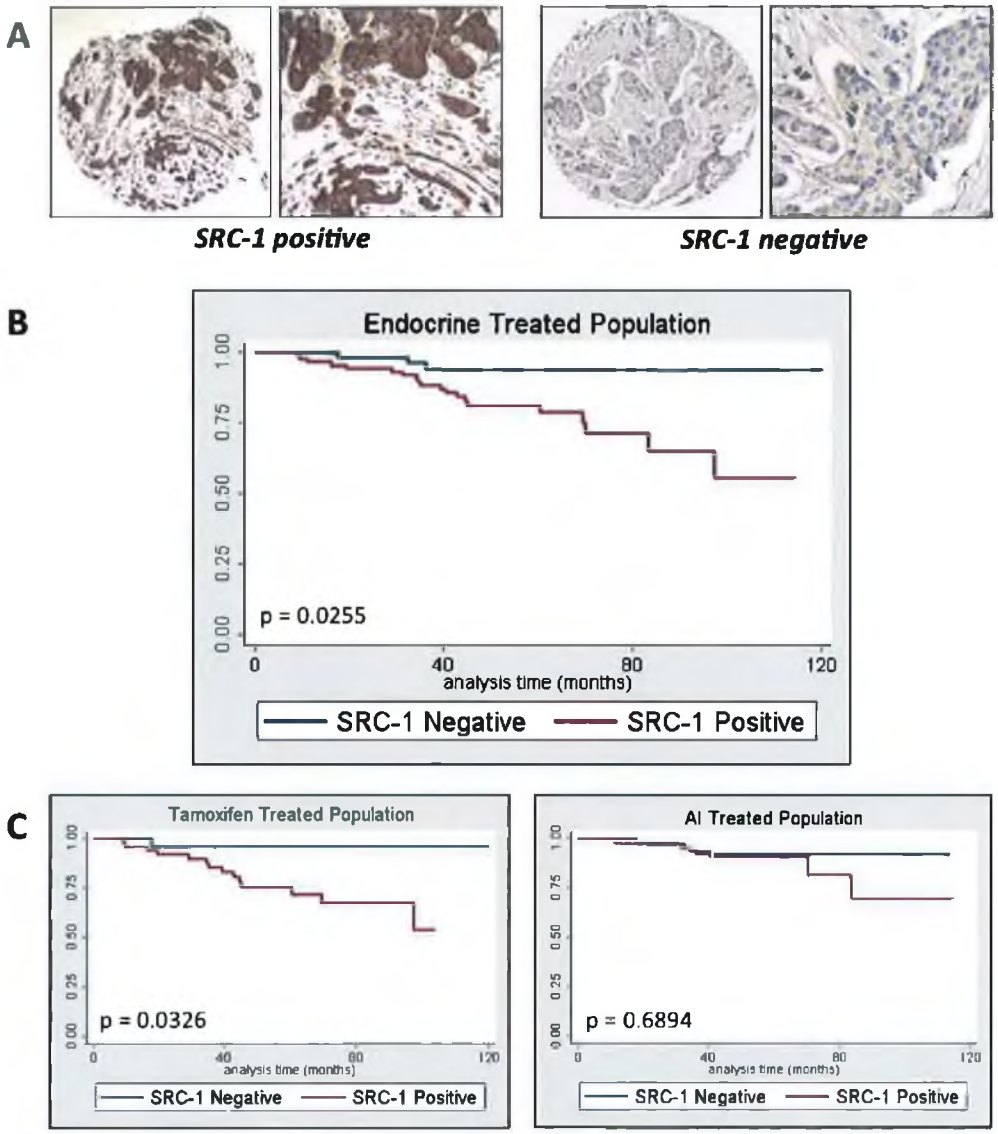


Figure 5.2: A, *Immunohistochemical staining of SRC-1 in tissue microarray cores, counterstained with haematoxylin. Examples of SRC-1 positive and negative primary tumours are shown.* ***B,C,*** *Kaplan Meier estimates of disease free survival in the overall endocrine (n=159), tamoxifen (n=75) and AI (n=84) treated populations. SRC-1 positive primary tumours (red line) significantly associated with reduced disease free survival in the overall endocrine (p=0.0255) and tamoxifen treated population (p=0.0326) but not significantly in the AI treated population (p=0.6894).*

5.3.2 SRC-1 associates with recurrence and tumour stage in endocrine treated patients

This group has previously observed a significant association between SRC-1 and recurrence in a cohort of endocrine treated patients (Al-azawi D *et al.*, 2008; Redmond AM *et al.*, 2009). It was investigated if SRC-1 associated with clinicopathologic variables in the endocrine treated patient population. Fisher's exact test was used to assess PR and HER2 status, recurrence, nodal status as well as tumour grade and stage.

No significant association between SRC-1 and PR status, HER2 status, nodal status or tumour grade, neither in the overall endocrine nor the AI and tamoxifen treated cohorts was observed (Table 5.1). We did, however, see significant associations between SRC-1 and recurrence (p=0.009) as well as tumour stage (p=0.003) in the overall endocrine treated patients. Interestingly, SRC-1 only significantly associated with recurrence in the tamoxifen treated (p=0.015) and not the AI treated population (p=0.494), but significantly associated with tumour stage in the AI treated patients (p=0.001) but not the tamoxifen treated patients (p=0.513).

Table 5.1: *Associations of SRC-1 with clinicopathologic variables using Fisher's exact test in endocrine, AI and Tamoxifen treated patient populations.*

Parameter	Endocrine treated population			AI (n=84)		Tamoxifen (n=75)	
	n=141	SRC-1 %	p value	SRC-1 %	p value	SRC-1 %	p value
PR status							
+ve	106	61.3		57.4		62.5	
-ve	35	62.9	1.000	43.5	0.328	78.9	0.263
Her2 status							
+ve	26	65.4		46.7		70.6	
-ve	115	60.9	0.834	55.1	0.659	65.5	1.000
Recurrence							
+ve	23	87.0		66.7		93.3	
-ve	118	56.8	**0.009**	52.0	0.494	60.0	**0.015**
Node							
+ve	71	67.6		79.5		70.7	
-ve	68	55.9	0.167	50.0	0.658	61.8	0.466
Tumour grade							
I	21	57.1		50.0		66.7	
II	79	63.3		56.5		65.1	
III	41	61.0	0.861	50.0	0.818	70.0	0.943
Tumour stage							
I	56	53.6		41.9		62.1	
II	64	57.8		50.0		63.9	
III	18	94.4		100.0		85.7	
IV	3	100.0	**0.003**	-	**0.001**	100.0	0.513

5.3.3 SRC-1 in primary and/or metastasis predicts poor outcome in AI treated patients

The LetR cells exhibited a dramatic increase in SRC-1 protein expression in comparison to the AI sensitive Aro cells. To investigate if this change in SRC-1 expression can also be observed in patient samples, SRC-1 negative primary and SRC-1 positive resistant tumour samples were obtained. Out of the patients that relapsed (n=9), only three didn't express SRC-1 in the primary breast tumour. The matched primary breast tumour and resistant metastasis were stained for SRC-1 by immunohistochemistry.

SRC-1 protein was very weakly expressed in the cytoplasm of cells in the primary breast tumour. An increase in SRC-1 protein expression, especially in the nucleus, was observed in the AI resistant metastasis (Figure 5.3 A).

SRC-1 protein expression in the primary tumour didn't prove to be a significant indicator for poor disease free survival or recurrence in AI resistant patients. However, SRC-1 expression in either the primary or the AI resistant metastasis significantly associated with poor disease free survival in patients treated with an AI (p=0.0106) (Figure 5.3 B).

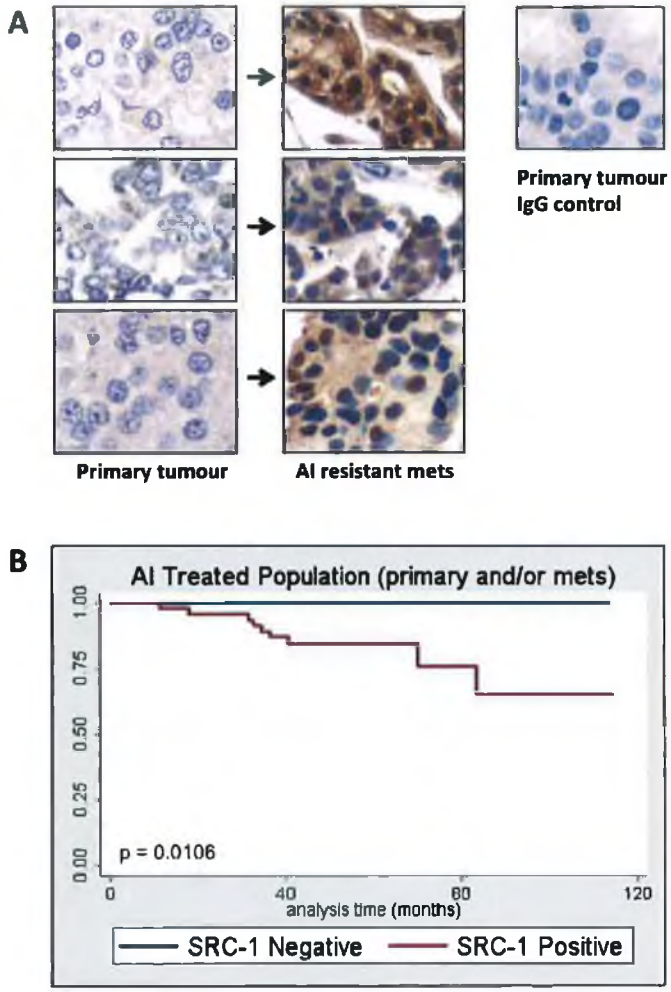


Figure 5.3: SRC-1 positivity in primary and/or mets associates with reduced disease free survival in patients on AI therapy. ***A,*** *SRC-1 protein expression was increased and more nuclear in the AI resistant tumours in comparison to the matched primary tumours. Images shown above are matched primary breast tumours and AI resistant metastases (n=3). IgG was used as a negative control.* ***B,*** *Kaplan Meier estimates of disease free survival in an AI treated patient cohort (n=84) according to SRC-1 staining in the primary and/or resistant tumour. SRC-1 significantly associated with poor disease free survival (p=0.0106).*

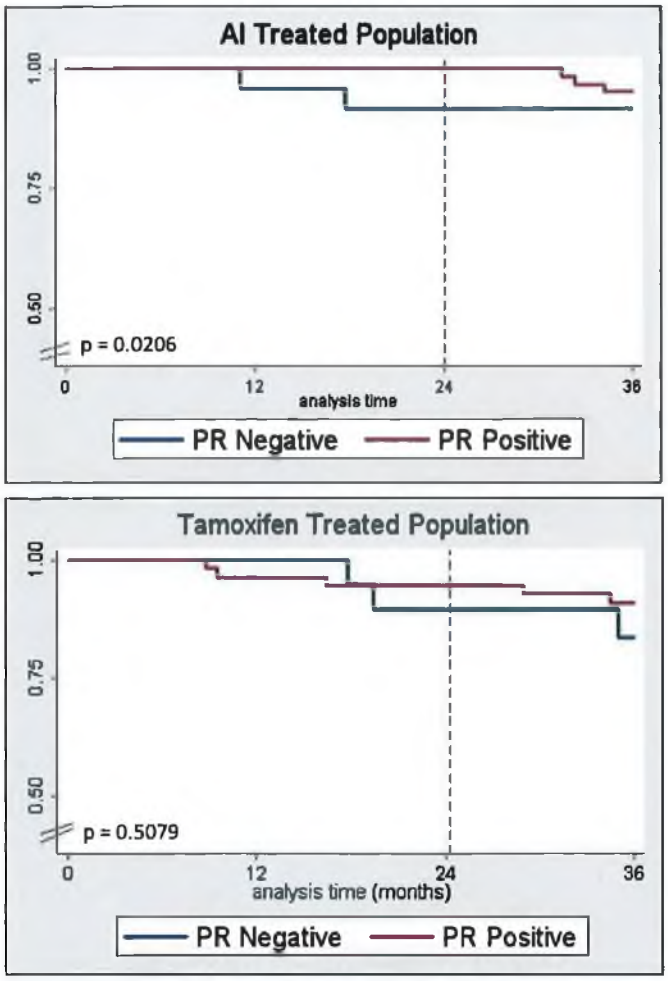
5.3.4 AI resistance is characterised by hormone receptor switching

The development of resistance to endocrine therapy is thought to be marked by a switch from hormone to growth factor signalling which may result in a steroid independent tumour (Sabnis G and Brodie A, 2010).

To investigate this, hormone receptor status in an endocrine treated patient population was assessed. We found significant association between the lack of PR status in the primary tumour and a reduction in early response to AI treatments (p=0.02). However, this association was not significant in the tamoxifen treated patients (0.5079) (Figure 5.4 A).

Matched primary and resistant metastasis of six AI treated breast cancer patients were analysed for hormone receptor status. A trend towards loss of ER and PR and occasional gain of HER2 was frequently seen in those patients. Two of the six patients showed the classical pattern of hormone switching: the primary breast tumour was staged ER+/PR+/HER2- whereas the resistant metastasis had switched to ER-/PR-/HER2+. Two patients had ER+/PR-/HER2- primary tumours with the resistant metastasis only displaying a loss of ER with no gain of HER2. One patient had an ER+/PR-/HER2+ tumour and the subsequent metastasis showed a loss of ER. Only one of these patients was positive for all three receptors in the primary tumour and the resistant metastasis surprisingly has lost HER2 expression (Figure 5.4 B).

A



B

AI treated Patient	Time to recur (months)	Primary ER	Primary PR	Primary Her2	Resistant ER	Resistant PR	Resistant Her2
1	12	+	-	-	-	-	-
2	18	+	-	+	-	-	+
3	34	+	+	-	-	-	+
4	36	+	+	-	-	-	+
5	70	+	-	-	-	-	-
6	79	+	+	+	+	+	-

Figure 5.4: Development of resistance to AI treatment is accompanied by hormone receptor switching. ***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 tumours for 6 AI treated patients. Changes in receptor status are highlighted in pink.*

5.3.5 Differences in SRC-1/ERα expression pattern in patients with same HR status

Breast cancer is a heterogenous disease meaning that most tumours are made up of a group of molecular subtypes. Although a tumour may be characterised ER+ and SRC-1+, it does not necessarily mean that ER and SRC-1 are expressed in the same cells of that tumour. To investigate this further, a number of ER/PR/SRC-1+ primary tumours (n=6) were co-immunostained to examine the localisation of SRC-1 and ER in these tumours (2 examples are shown in figure 5.5). Tumour X is an example of a tumour with strong nuclear and cytoplasmic colocalisation of both proteins. By contrast, tumour Y is an example of a tumour, which is also positive for both proteins but where the two proteins do not colocalise to the same cells. Staining was mostly nuclear in this type of tumour. This implies, that SRC-1, if functional in those cells, is functioning independently of ERα. The patients whose primary tumours were stained had not relapsed on AI therapy and therefore no conclusion can be drawn about the significance of this staining in relation to disease progression.

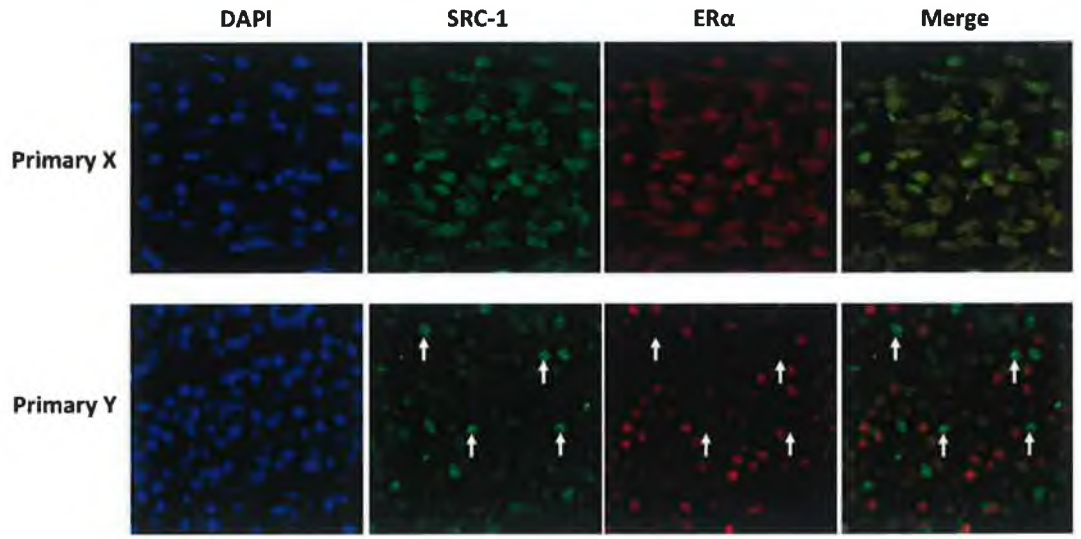


Figure 5.5: Immunofluorescent analysis of SRC-1 (green) and ERα (red) in primary breast tumour samples, counterstained with DAPI. *Primary breast cancer tissue from patients with same hormone receptor status (ER+ PR+ HER2-) show differences in SRC-1 and ERα protein expression.* ***Primary X*** *displays cytosolic and nuclear expression of ERα and SRC-1. Merged images show colocalisation in every cell.* ***Primary Y*** *displays more nuclear expression of both proteins with occasional loss of ERα in some cells (visible in merged image, indicated by arrows).*

5.3.6 SRC-1 colocalises with Ets2 in the nucleus of AI resistant metastases

Data from our cell lines as well as results shown in 5.5 strongly suggest that SRC-1 acts independent of ERα. To investigate if SRC-1 interacts with the transcription factor Ets2 in the AI resistant phenotype matched primary breast tumour and AI resistant metastasis tissue samples were stained for SRC-1 and phospho-Ets2 by immunofluorescence.

SRC-1 seemed to be solely located in the cytoplasm and more perinuclear in the primary breast tumour. Phospho-Ets2 was found in the nucleus and in the cytoplasm, however, SRC-1 and phospho-Ets2 did not colocalise in the primary tissue cells. Also, the overall SRC-1 and phospho-Ets2 staining was quite weak, reflecting the protein expression in the aromatase expressing AI sensitive cell model.

The AI resistant metastasis tissue sample on the other hand displayed high expression levels of both proteins, particularly in the nucleus (Figure 5.6; white arrow). The extent of nuclear coassociation was analysed by Pearson's correlation and it was found that SRC-1 and phospho-Ets2 significantly colocalise in the nucleus (p=0.0004).

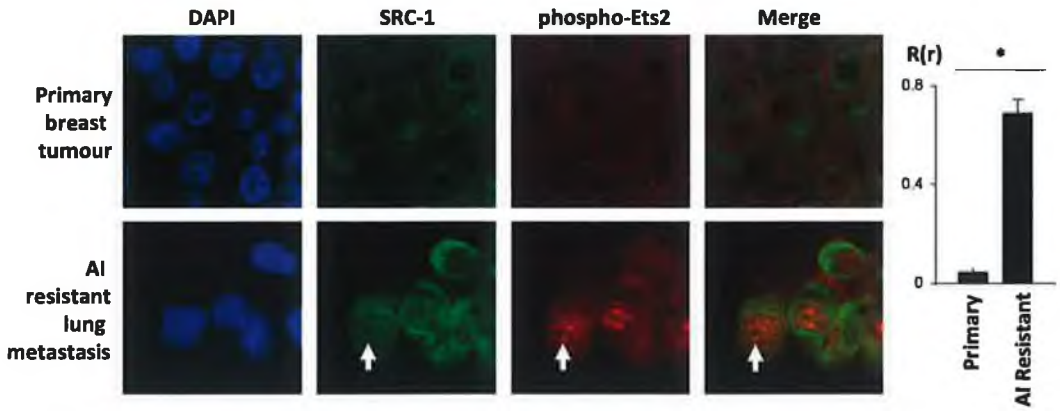


Figure 5.6: Immunofluorescent staining of SRC-1 (green) and phospho-Ets2 (red) in matched primary breast tumour and AI resistant metastasis (n=3), counterstained with DAPI. *Expression of both proteins was stronger and more nuclear in the metastasis samples. Images shown are representative (n=3). Merged image shows that SRC-1 and phospho-Ets2 colocalise in the metastatic cells (indicated by white arrows). Magnitude of coassociation was measured by Pearson's correlation, R(r), and is significantly higher in the metastatic tissue than in the primary tumour sample (p=0.0004).*

5.3.7 AI resistant metastases express higher levels of Myc and MMP9 protein

In the AI resistant cell model LetR it was found that SRC-1 and Ets2 can regulate Myc and MMP9 expression. To confirm the *in vitro* findings in the patient setting, matched primary and resistant metastasis tissue samples were immunohistochemically stained for Myc and MMP9.

Myc was found to be weakly expressed in the cytoplasm of the primary tumour cells (Figure 5.7). In comparison to the primary tumour Myc was highly expressed in the cytoplasm and occasionally in the nucleus of the resistant metastasis cells. When stained for MMP9 it was found that the protein was weakly expressed in the cytoplasm in the metastasis with no expression in the primary tumour sample.

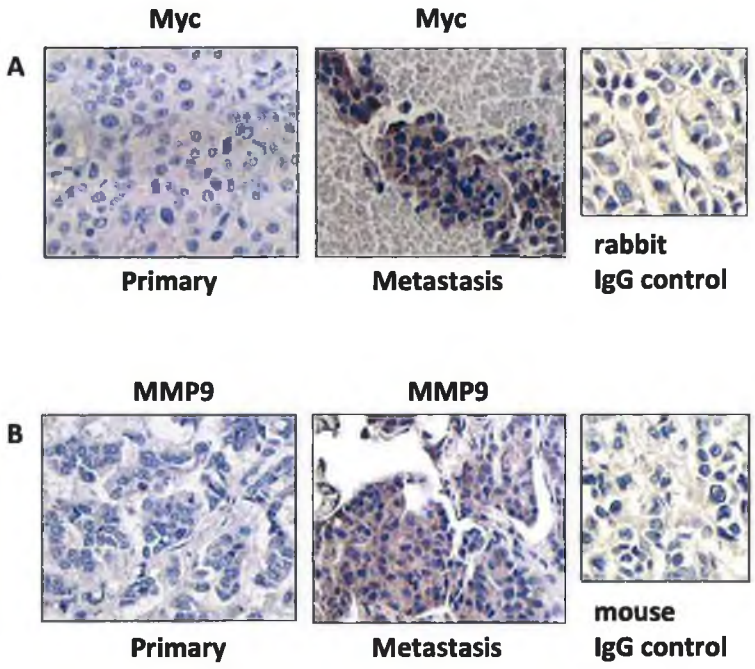


Figure 5.7: Immunohistochemical analysis of Myc and MMP9 in matched primary breast tumour and metastatic tissue samples (n=3), counterstained with haematoxylin. Images shown are representative (n=3). ***A,*** *Myc was strongly expressed in the AI resistant metastatic tissue* ***B,*** *MMP9 was absent in the primary tumour and weakly expressed in the AI resistant metastasis.*

5.4 Discussion

Based on previous results in tamoxifen resistance and observations made in our letrozole resistant cell line we were interested to see if the role of SRC-1 in the development of AI resistance can be translated into the patient.

Previous studies revealed that SRC-1 expression in the primary tumour is an independent predictor of poor disease free survival in tamoxifen treated patients (Myers E *et al.*, 2004; Redmond A *et al.*, 2009). These findings were confirmed in the overall patient cohort and the tamoxifen treated patients included in this study. However, SRC-1 did not significantly associate with poor disease free survival in the AI treated patient group. Additionally, results from the Fisher's exact test also revealed that SRC-1 only associated with recurrence in the overall endocrine and the tamoxifen treated patient group, which was not observed in the AI treated patients. The small size of the AI treated patient population could be one possible explanation for why SRC-1 did not associate with recurrence in this particular patient population. Therefore, a larger cohort would need to be examined in the future to see if significance can be reached.

Interestingly though, SRC-1 associated with tumour stage in the AI treated patients, but not in the tamoxifen treated group. Tumour stage is a variable that describes the severity of a patient's cancer based on the extent of the primary tumour (stage I – III) and whether or not the cancer has metastasised to distant sites in the body (stage IV). SRC-1 has been shown to play a crucial role in migration and invasion, both in this thesis and by other groups (Wang S *et al.*, 2009; Qin L *et al.*, 2009; Han JS and Crowe DL, 2010), which explains why expression of the coactivator strongly associated with higher tumour stage. In fact, analysis of initially SRC-1 negative primary breast tumours and their matched AI resistant metastasis showed a severe increase in SRC-1, suggesting that SRC-1 plays a role in driving the cell toward a more aggressive phenotype.

Two thirds of the patients that suffered a tumour recurrence displayed SRC-1 expression in the primary tumour. Those patients who were negative for SRC-1 in the primary,

however, exhibited an SRC-1+ tumour recurrence, suggesting that SRC-1 may potentially play a role in driving the metastatic phenotype in AI resistance.

Breast cancer progression and the development of endocrine resistance are marked by a process that is known as hormone receptor switching (Sabnis G *et al.*, 2010). It describes the shift from steroid-dependent to steroid-independent/growth factor dependent tumour status. It is well understood that only ER+ breast cancer responds to endocrine therapies, due to their method of action. It is also thought that only ER+/PR+ tumours would respond well to endocrine therapies. PR expression is regulated by ER activity. Therefore, it is believed that ER+/PR- tumours would be less responsive to endocrine therapy because lack of PR expression mirrors a non-functional ER pathway (McGuire WL *et al.*, 1977). Supporting this hypothesis, it has been shown that lack of PR expression is associated with shorter time to treatment failure in patients treated with AIs (Anderson H *et al.*, 2011). In line with this, the results from this study showed that a decrease in PR expression is associated with increased early disease recurrence in patients receiving adjuvant AI therapy.

Additionally, the AI treated patients included in this study occasionally exhibited an increase in HER2 status, suggesting that tumour proliferation was driven by the growth factor pathway independent of ER signalling. In breast cancer patients taking tamoxifen over an extended time period, HER2 has been shown to be co-expressed with Ets-2 and SRC-1 (Myers E *et al.*, 2005) and to associate with a decrease in disease free survival (Lipton *et al.*, 2005). In line with these findings, the increase in growth factor receptor expression as found in the AI treated breast cancer patients included in this study may result in increased proliferation and escape from hormonal therapy.

Even though approximately 75% of postmenopausal breast cancer patients are ER+ as indicated by immunohistochemical staining, not all ER+ patients respond equally well to endocrine treatment. This is caused by a selection process due to the heterogeneity of the disease. Each tumour is made up of ER+ and ER- cells and during treatment the number of ER+ tumour cells shrinks whereas the ER- cells become predominant,

resulting in a tumour that does not respond to endocrine treatment any more. Because of the heterogeneity of the disease, we were interested to investigate the expression pattern of SRC-1 and ER in primary ER+/SRC-1+ tumours. Their status was based on immunohistochemical staining which was also used to determine the treatment strategy for the patient. However, our results revealed inter- as well as intra-tumoural differences in the expression pattern of the two proteins. Whereas SRC-1 was coexpressed with ER in every cell of one tumour, suggesting that it was still functioning as an original nuclear receptor coactivator, it was mostly expressed in the nucleus of cells that were lacking in ER in the other tumour, suggesting that it was acting independently of ER. These findings support the proposal that better diagnostic tools as well as personalised treatment options are desperately needed in the management of breast cancer.

To confirm that SRC-1 was in fact interacting with other TFs to confer AI resistance we assessed co-expression of SRC-1 and Ets2 in matched primary and AI resistant tumours. It was found that SRC-1 colocalises with the TF Ets2 in the nucleus of AI resistant metastases, supporting the *in vitro* data from our LetR cell line. This is the first time this interaction has been reported in AI resistant patients. An increase was also found in the target genes Myc and MMP9 in the AI resistant metastases, suggesting that SRC-1 is coactivating Ets2 in those AI resistant tumours.

These findings strongly support our *in vitro* data. It appears that during the development of resistance to AIs, tumour cells lose their responsiveness to estrogen as well as endocrine treatment and develop an aggressive AI resistant phenotype that is driven by GF signaling. The coactivator SRC-1 appears to be involved in the development of a more aggressive resistant phenotype as previously observed in the AI resistant cell model LetR and we hypothesise that SRC-1 coactivation of Ets2 to regulate MMP9 and Myc is GF-dependent. However, it needs to be elucidated which specific pathways are involved in this process.

Chapter 6

General Discussion

Even though detection and treatment of breast cancer have dramatically improved over the last decades, there is still a high incidence of mortality as a result of recurrence and failure of therapies (Kamangar F *et al.*, 2006). One of the main obstacles in reaching an effective diagnosis and determining the right treatment strategy is tumour heterogeneity (Marusyk A and Polyak K, 2010). Different patients may have different types of breast cancer, known as inter-tumour heterogeneity, and gene expression profiling as well as biomarkers have helped a great deal to classify these subtypes and find appropriate individualised treatments for patients. Intra-tumour heterogeneity on the other hand refers to differences inside the same tumour and can have different causes as well as clinical outcomes. We have seen that global calling of ER does not necessarily group identical tumours. The results from the immunofluorescent staining of ER+ primary tumours cleary revealed major inter-tumour as well as intra-tumour differences in the expression pattern of ER. This observation can have a detrimental impact on the clinical management of breast cancer, as tumour staging by classical tools such as immunohistochemistry is only based on a particular area of a tumour and may not represent the heterogeneity of the whole tumour, resulting in unpredictable response to treatment. Additionally, tumour cells are plastic and may undergo additional genetic and epigenetic changes during tumour progression, leading to an increase in heterogeneity. We have observed that a loss of HR and gain in SRC-1 expression marks the development of resistance to AIs, suggesting that it is essential to monitor tumours over time rather than assess a tumour profile at the point of diagnosis alone. Thus, inter- and intra-tumour heterogeneity as well as tumour evolution should be taken in consideration when assessing current therapies as well as designing novel, more effective anti-cancer drugs (Almendro V and Fuster G, 2011).

We employed the letrozole resistant LetR cell model to establish the role of SRC-1 in AI resistance. A variety of cell models of AI resistance have been generated over the last decades as discussed in chapter three. Initially, a majority of studies had been performed in long-term estrogen deprived (LTED) cells. However, even though these cells are subjected to estrogen depletion, which occurs during AI treatment, this setting

was not optimal to investigate AI resistance, as these cells have never been exposed to the drug. Although they may also be resistant to AIs, it is clear from our work that the cells are different and therefore both cell lines offer independent, useful models of resistance. Additionally, it has been suggested that the resistance mechanisms of the three AIs anastrozole, letrozole and exemestane might be slightly different (Chen S, 2011). Our patients usually received either anastrozole or letrozole, which both belong to the class of non-steroidal AIs. However, close reflection of our results from the AI resistant cell model and patients suggests that SRC-1 pathways discovered in the LetR cells are relevant to multiple AI resistant mechanisms.

It has recently become evident that SRC-1 plays an important role in the development of metastasis (Wang S *et al.*, 2009; Qin L *et al.*, 2011). Here, we have established a clear role for SRC-1 in progression and metastasis. We have observed that SRC-1 is highly expressed in the LetR cell line and that it plays a key role in driving motility, invasion and de-differentiation. Additionally, we have shown that SRC-1 is highly expressed in AI resistant metastases; therefore, monitoring SRC-1 expression may be useful to screen for disease progression and detect disease advancement before metastases appear. However, since the detection of SRC-1 tissue levels is not feasible it is necessary to determine SRC-1 targets that can easily be detected in blood samples from patients. Our group has previously shown that serum levels of the SRC-1 target s100β are highly elevated in patients resistant to tamoxifen, and therefore it would be interesting to see if this finding could be tranferred to AIs (McIlroy M *et al.*, 2010).

Taken together, these findings strongly suggest that SRC-1 plays a pivotal role in the development of AI resistance and could therefore serve as potential novel drug target. As mentioned before, acquired resistance to endocrine therapy has been shown to be a result of crosstalk between ER and HER2 or between signaling pathways downstream of these receptors, namely PI3K/AKT/mTOR (Prat A and Baselga J, 2008). Our lab has previously reported associations between HER2 and SRC-1 overexpression and poor DFS in endocrine resistant breast cancer (Fleming FJ *et al.*, 2004) and several studies have

shown that, in HER2+ patients, the concentration of coactivators can determine active AKT levels (Osborne CK *et al.*, 2003, Torres-Arzayus MI *et al.*, 2004; Zhou G *et al.*, 2003).

Therefore, it is thought that AIs in combination with therapies that target signaling pathways that are involved in crosstalk may work better than alone. A number of trials are currently trying to establish if AIs in combination with drugs such as signal transduction inhibitors, tyrosine kinase inhibitor, multikinase inhibitors or mTOR antagonists, may offer a solution to overcoming resistance or delaying its development (Bedard PL *et al.*, 2008). For example, it has been demonstrated that treatment with the AI anastrozole in combination with the monoclonal anti-HER2 antibody trastuzamab led to an improvement in disease free survival in HR+ metastatic breast cancer patients overexpressing HER2 (Kaufman B *et al.*, 2009). Since trastuzamab may not inhibit proliferation of cells that express low levels of HER2, another trial was launched to evaluate the benefit of the anti-HER2 tyrosine kinase inhibitor lapatinib in combination with the AI letrozole. Published data from this trial revealed an improvement in DFS in patients that were treated with the combination therapy (Johnston S *et al.*, 2009).

The use of Src TKIs has also been suggested to be a logical strategy for the management of metastatic disease. Src is a non-receptor tyrosine kinase that is involved in breast cancer cell proliferation, invasion and metastasis, suggesting that it plays a role in the development of endocrine resistance. Additionally, it is involved in the regulation of osteoclast-mediated bone turnover, which can compromise bone strength, leading to osteoporosis, one of the major side effects associated with AI therapy. Results published by Hiscox *et al.* demonstrated that combination therapy with Src inhibitors such as dasatinib and AIs inhibited proliferation and metastasis of both endocrine responsive and resistant breast cancer cells lines more effectively than either therapy alone (Hiscox S *et al.*, 2010). Src inhibition was also able to suppress osteoclast formation, suggesting that the combination therapy may not only represent a novel approach to overcome acquired resistance but may also offer an alternative strategy to delay bone pathology.

This lab is currently investigating the effect of AIs in combination with dasatinib in the development of resistance.

Another potential approach to prevent the development of AI resistance is the use of AIs in combination with the ER-degrading antiestrogen fulvestrant. A xenograft model was employed to assess the efficacy of this therapy *in vivo*. Animals were treated with letrozole, fulvestrant or a combination of the two. Whereas the size of tumours treated with the letrozole alone and fulvestrant alone had doubled after 10 and 21 weeks respectively, the tumours of animals treated with the combination therapy regressed over 29 weeks of treatment by 45%, making it more effective in suppressing growth than either letrozole of fulvestrant alone (Jelovac D *et al.*, 2005). Even though we have seen a loss of ER activity over time in our AI resistant cell model, this evidence suggests benefit from fulvestrant, potentially in preventing AI resistance from developing rather than treating AI resistant metastasis. Another potential benefit of this combination therapy could be seen in AI resistant cells that express a hyperactive ER. Loss of ER can inhibit interactions of coactivators such as AIB-1 with the receptor to drive AI resistance, as previously observed in our lab (O'Hara J *et al.*, 2012).

However, the success of these combination therapies may still be limited, as the cancer cells might eventually begin to compensate for the downregulation of the particular pathways by upregulating other pathways, resulting in resistance to those novel therapies. Downregulation of SRC-1 itself offers a great potential as illustrated by the results from functional cell assays demonstrated in this thesis. Therefore, the development of small molecule inhibitors (SMIs) against SRCs to circumvent resistance would be an interesting approach in designing new breast cancer therapies, as targeting oncogenic coactivators could lead to simultaneous downregulation of a range of target genes that are involved in the progression and metastasis of cancer cells (Figure 6.1) (O'Malley BW and Kumar R, 2009).

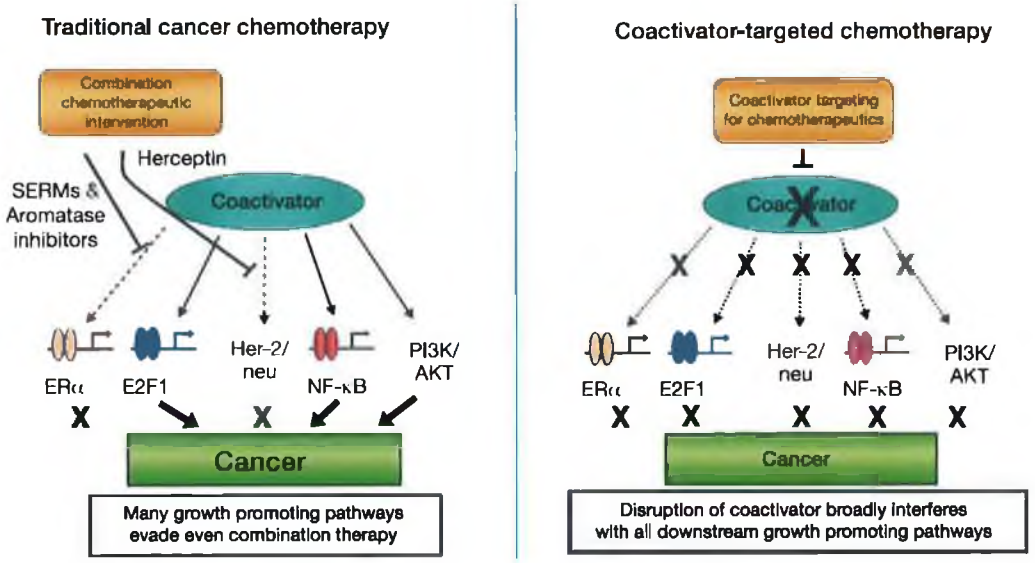


Figure 6.1: Targeting coactivators for anti-cancer therapy. **Left panel:** Coactivator regulates expression of a variety of target genes (ER-responsive; HER2-responsive; NF-κB-responsive; PI3K/AKT-responsive), which are all required for proliferation and metastasis of cancer cells, Targeting the individual pathways with SERMs/AIs (ER) or herceptin (HER2) only inhibits one or two pathways (in case of combination). Tumour growth is slowed, while other pathways are upregulated to compensate, potentially causing resistance to treatment. **Right panel:** Targeting and inhibiting the function of the coactivator leads to simultaneous suppression of all pathways, blocking compensatory upregulation of alternate pathways, potentially decreasing onset of drug resistance. *Adapted from Cancer Research Reviews (O'Malley BW and Kumar R, 2009).*

However, this was initially thought to be impossible as SRCs are large proteins that lack high affinity binding sites as well as other features that exist in regular so-called "druggable" targets. Still, SMIs against other regulatory proteins have lately been successfully designed (Wells JA and McClendon CL 2007).

Bert O'Malley's group has recently identified 2,2'-bis-(Formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene) (gossypol) as an SMI against SRC-1 and SRC-3 (Wang Y *et al.*, 2011). Gossypol, a natural polyphenol found in cotton seeds, was initially considered as a male infertility drug. After it was declared unsuitable due to the risk of permanent infertility (Hadley MA and Burgos MH, 1986) it was proposed as a potential cancer drug based on its anticancer properties (Gilbert NE *et al.*, 1995; Wolter KG *et al.*,

2006; Zhang M *et al.*, 2003). The published data from O'Malley's lab revealed that gossypol selectively reduced the cellular protein concentration of SRC-1 and SRC-3 in MCF7 breast cancer cells, without changing expression of other proteins, in particular other coactivators (Wang Y *et al.*, 2011). These findings suggest that gossypol may have the potential to overcome acquired endocrine resistance.

In summary, we have established that SRC-1 has an essential role in AI resistance, which opens possibilities to expand the results gained from studies on SRC-1 in tamoxifen resistance to AI resistance. In the future, it would be interesting to analyse the serum levels of SRC-1 targets such as S100β in blood samples of AI resistant patients to investigate if conclusions gained from studies in tamoxifen resistant patients can be transferred to AI resistance. It is also necessary to perform a global ChIPseq of SRC-1 in AI resistant cells, because, although SRC-1 evidently has some similar roles in AI and tamoxifen resistance, there are also clear differences, probably due to the different molecular mechanisms of the drugs. Broadening our understanding of these differences and the SRC-1 signaling mechanism in general will help design more effective, targeted therapies for the treatment of AI resistant breast cancer.

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Appendix I

Table 7.1: Primary Antibodies used

Antigen	Primary antibody	Product Code	Concentration	Supplier
SRC-1*⁺†	Rabbit polyclonal	sc-8995	200µg/ml	Santa Cruz
SRC-1⁺	Mouse monoclonal	05-522	1mg/ml	Millipore
c-MYC*†	Rabbit polyclonal	sc-517	200µg/ml	Santa Cruz
Ets2*	Rabbit polyclonal	sc-351	200µg/ml	Santa Cruz
pEts2⁺	Rabbit polyclonal	44-1105G	100µg/ml	Invitrogen
ERα*	Rabbit polyclonal	sc-543	200µg/ml	Santa Cruz
ERα⁺	Mouse monoclonal	NCL-ER-6F11	3.9mg/ml	Novocastra
MMP9†	mouse monoclonal	sc-21733	200µg/ml	Santa Cruz
β-Actin*	Mouse monoclonal	A1978	Not specified	Sigma Aldrich

* Used for western blotting. ⁺ Used for immunofluorescence. † Used for immunohistochemistry.

Table 7.2: Secondary Antibodies used

Species	Product Code	Suppliers
Anti mouse IgG	A3682	Sigma Aldrich
Anti rabbit IgG	A0545	Sigma Aldrich
Anti rabbit 488	A11008	Invitrogen
Anti mouse 568	A11004	Invitrogen
Anti mouse 488	A11001	Invitrogen
Anti rabbit 594	A11012	Invitrogen

Table 7.3: Cell culture reagents

Product	Code	Supplier
Minimum Essential Media (MEM)	M4526	Sigma Aldrich
Leibovitz's L15 media	11415	Gibco (Invitrogen)
Fetal Bovine Serum	F7524	Sigma Aldrich
Penicillin/Streptomycin	P4333	Sigma Aldrich
Tryspin EDTA Solution	T4174	Sigma Aldrich
L-Glutamine	G7513	Sigma Aldrich
Charcoal Dextran	C6241	Sigma Aldrich
Trypan Blue	T8154	Sigma Aldrich
OPTIMEM	11058	Gibco (Invitrogen)
Phenol red free MEM	M3024	Sigma Aldrich

Table 7.4: Buffers used in Western Blotting

RIPA Lysis Buffer	Running Buffer	Semi dry Transfer Buffer
150mM sodium chloride	1.92M Glycine	390mM Glycine
1.0% NP-40	250mM Trizma base	480mM Trizma base
0.5% sodium deoxycholate	1% SDS	0.37% SDS
0.1% SDS (sodium dodecyl sulphate)	dH_2O to 1L	20% methanol by volume
50 mM Tris, pH 8.0		dH_2O to 1L

Table 7.5: Solutions for Zymography *(all other buffers are supplied with the Novex® Zymogram Kit)*

Coomassie Blue Stain	Destain Buffer
0.05% Coomassie Brilliant Blue R250	
2.5% Acetic Acid	10% Acetic Acid
50% Ethanol	20% Methanol
Fill up with dH_2O	Fill up with dH_2O

Table 7.6: Buffers used in nucleic acid biochemistry

10x TAE Buffer (1L)
48.4g Tris
11.4ml glacial acetic acid
20ml 0.5M EDTA
dH_2O to 1L
pH 8.0

Buffers used for Chromatin Immunoprecipitation (ChIP)

Table 7.7: Buffers used in ChIP cell fixation

Formaldehyde Solution (20ml)	Glycine Solution (20ml)	PBS Ipegal (100ml)
6ml 37% formaldehyde (11%)	3.75g Glycine (2.5M)	100ml PBS
0.4ml 5M NaCl (0.1M)		500µl NP-40 (0.5%)
40µl 0.5M EDTA (pH 8.0) (1mM)		
1ml 1M HEPES (pH 7.9) (50mM)		
dH_2O to 20ml	dH_2O to 20ml	

Table 7.8: Buffers used in chromatin preparation during ChIP

SDS Lysis Buffer	ChIP Dilution Buffer
1% SDS	0.01% SDS
10mM EDTA	1.1% Triton X- 100
50mM Tris Hcl	1.2mM EDTA
	16.7mM Tris-HCl
	167mM NaCl
pH: 8.10	pH: 8.10

Table 7.9: Wash buffers used in ChIP

LiCl Immune Complex Wash Buffer	Low Salt Immune Complex Wash Buffer	High Salt Immune Complex Wash Buffer	TE Buffer
0.25M LiCl	0.1% SDS	0.1% SDS	10mM Tris-HCl
1% NP40	1% Triton X-100	1% Triton X-100	1mM EDTA
1% deoxycholic acid	150mM NaCl	500mM NaCl	
1mM EDTA	2mM EDTA	2mM EDTA	
10mM Tris Hcl	20mM Tris-HCl	20mM Tris-HCl	
pH: 8.10	pH: 8.10	pH: 8.10	pH: 8.00

Appendix II

Publications, awards and presentations obtained during the course of this research

Publication

"Metastatic progression with resistance to aromatase inhibitors is driven by the steroid receptor coactivator SRC-1." McBryan J, Theissen SM, Byrne C, Hughes E, Cocchiglia S, Sande S, O'Hara J, Tibbits P, Hill AD, Young LS. *Cancer Res January 15, 2012; 72:548-559; Published OnlineFirst November 22, 2011 (Appendix III).*

Awards

- Scholar-in-Training Award, San Antonio Breast Cancer Symposium, awarded by AACR supported by Susan G. Komen for the Cure, December 2011.
- Ireland-Northern Ireland Cancer Consortium Scholarship to attend the NIH-NCI Summer Curriculum in Molecular Cancer Prevention in Bethesda, MD, August 2011.

Invited oral presentations

- Invited seminar talk: "Aromatase inhibitor specific metastasis is driven by the steroid receptor coactivator SRC-1." Theissen SM, McBryan J, Byrne C, Hughes E, Cocchiglia S, Hill AD, Young LS. *Women's Cancer Research Center, Pittsburgh, August 16, 2011.*
- Proffered paper talk: "New signalling networks in aromatase inhibitor resistant breast cancer". Theissen SM, Byrne C, Sande S, Young LS. *Irish Association for Cancer Research Annual Conference, Galway, Ireland, March 3 – 5, 2010.*

Poster presentations

- "Aromatase inhibitor specific metastasis is driven by the steroid receptor coactivator SRC-1." Theissen SM, McBryan J, Byrne C, Hughes E, Cocchiglia S, Hill AD, Young LS. *San Antonio Breast Cancer Symposium, December 6 – 10, 2011.*

- "Aromatase inhibitor specific metastasis is driven by the steroid receptor coactivator SRC-1." Theissen SM, McBryan J, Byrne C, Hughes E, Cocchiglia S, Hill AD, Young LS. *Young Life Scientists Ireland Symposium, Dublin, November 12, 2011.*
- "New signalling networks in aromatase inhibitor resistant breast cancer." Theissen SM, Byrne C, Sande S, Young LS. *RCSI Research Day, April 7, 2010.*

Appendix III – Publication

Tumor and Stem Cell Biology

Cancer Research

Metastatic Progression with Resistance to Aromatase Inhibitors Is Driven by the Steroid Receptor Coactivator SRC-1

Jean McBryan, Sarah M. Theissen, Christopher Byrne, Eamon Hughes, Sinead Cocchiglia, Stephen Sande, Jane O'Hara, Paul Tibbitts, Arnold D.K. Hill, and Leonie S. Young

Abstract

Aromatase inhibitors (AI) are a standard-of-care treatment for postmenopausal, 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 used 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 dedifferentiation and migration in hormone-dependent breast cancer. *Cancer Res; 72(2); 1–12. ©2011 AACR.*

Introduction

Endocrine therapies, including estrogen receptor (ER) modulators and aromatase inhibitors (AI), 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 AIs 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 use 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, support 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 mitogen-activated protein kinase (MAPK) pathway. These transcription factor interactions may represent one of the consequences of growth factor pathway cross-talk described in endocrine resistance. Functional interactions

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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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 University, Washington, DC) were grown as previously described (11). MCF10A cells (ATCC) were cultured in DMEM/F12 with 15 mmol/L hepes buffer, 5% horse serum, 10 μg/mL insulin, 20 ng/mL EGF, 100 ng/mL cholera-toxin, and 0.5 μg/mL hydrocortisone. AI-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 200 μg/mL Geneticin (G418, Gibco Invitrogen). Letrozole-resistant (LetR) cells were generated by long-term (>3 months) culture of Aro cells with letrozole (10^{-6} mol/L; Novartis) and androstenedione (25×10^{-9} mol/L; Sigma Aldrich) in MEM supplemented with 10% charcoal-dextran-stripped FCS, 1% L-Glutamine, 1% Pen/Strep, and 200 μg/mL G418. All cells were maintained in steroid-depleted medium 72 hours prior to treatment with estradiol (10^{-8} mol/L; Sigma Aldrich), androstenedione (10^{-7} mol/L), or letrozole (10^{-6} mol/L). SiRNA (Ambion) directed against SRC-1 (AM16706) and ERα (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 3-dimensional (3D) cultures, cells were seeded 72 hours after transfection. All other experiments were carried out 24 hours after 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 1×10^4 cells/mL. Mean track areas (minimum of 90 cell tracks per condition) were analyzed with Olympus cell 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 0.5×10^4 cells per well. The cells were serum starved for a further 24 hours before being treated with vehicle (acetic acid; 0.01%), androstenedione (100 nmol/L), or 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, 5×10^4 cells in 400 μL of their respective medium (as above) and 2% Matrigel (BD Biosciences) were seeded onto the growth factor reduced matrigel matrix in 8 well-chamber slides (BD Biosciences) and cultured for 14 days at 37°C/5% CO_2. Cells were fixed in 4% paraformaldehyde and permeabilized with 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 4′,6-diamidino-2-phenylindole (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 were collected 24 hours later. Protein was concentrated with Amicon Ultra4 filters (50 K pore size, Millipore). Twenty micrograms of protein was 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 (92 kDa) and active (82 kDa) 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 carried out 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 ERα (1:500, sc-8002, Santa Cruz) or β-actin (1:7,500; Sigma-Aldrich).

Chromatin immunoprecipitation assay and PCR

Aro and LetR cells were treated with vehicle, estrogen, androstenedione, letrozole or androstenedione and letrozole for 45 minutes, and chromatin immunoprecipitation (ChIP) analysis was carried out 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 4°C 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 immunoglobulin G (IgG) as a negative control or 7 μL antiacetylated H4 (Millipore) as a positive control. Reverse cross-linking and DNA recovery were carried out with Chelex 100 (Bio-Rad). Real-time PCR was carried out in duplicate by SYBR Green PCR (Qiagen) using a Lightcycler (Roche), and primers are listed in Supplementary Table S1. Semiquantitative reverse-transcriptase PCR (RT-PCR) was carried out 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) and treatment with radiotherapy, chemotherapy, tamoxifen, or AIs. Detailed follow-up data (median, 56 months) were 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 carried out using Fisher exact test for categorical variables and Wilcoxon test for continuous variables.

Immunofluorescent microscopy and quantitative colocalization

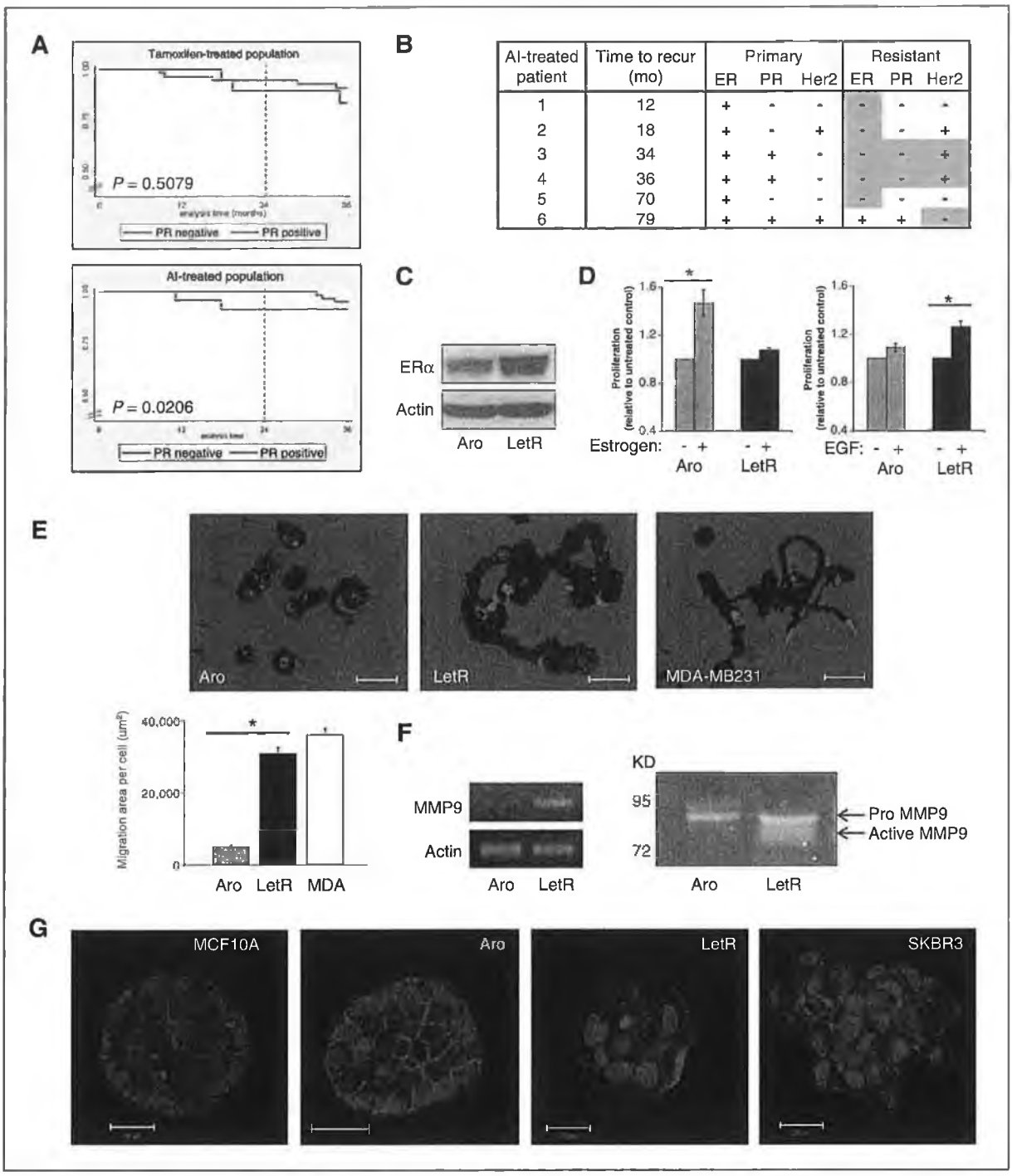
Cell lines, grown on collagen coated coverslips, were fixed and permeabilized as per the 3D assay. Cells were blocked with 10% goat serum for 1 hour, incubated with rabbit anti-human SRC-1 antibody (Santa Cruz) followed by goat anti-rabbit 488 (Molecular Probes), phalloidin for 20 minutes, and DAPI for 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 ×40 objective lens (1.40 NA). Quantitative colocalization analyses (minimum 9 images per sample) were carried out with Zeiss 510 META Software using the Pearson correlation coefficient, R(r) (17).

Results

AI resistance is characterized 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 Fig. 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 were 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 with sensitive cells (Fig. 1D).

To further characterize the AI-resistant phenotype, migratory assays and 3D culture assays were carried out. 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 Fig. S1B). Consistent with the increased motility of LetR cells, increased levels of the matrix metalloproteinase MMP9 were also detected in these resistant cells compared with sensitive cells (both mRNA and levels of the secreted active MMP9 protein; Fig. 1F). In 3D culture assays, sensitive cells were capable of organizing 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 with the disorganized endocrine insensitive SKBR3 cells (Fig. 1G and Supplementary Fig. S1C). The increased migration and decreased polarization 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.



AI-treated patient	Time to recur (mo)	Primary ER	Primary PR	Primary Her2	Resistant ER	Resistant PR	Resistant Her2
1	12	+	-	-	-	-	-
2	18	+	-	+	-	-	+
3	34	+	+	-	-	-	+
4	36	+	+	-	-	-	+
5	70	+	-	-	-	-	-
6	79	+	+	+	+	+	-

Figure 1. AI resistance is characterized 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 ERα in LetR cells compared with Aro cells. D, AI-resistant cell model (LetR cells, black bars) shows reduced proliferative response to steroids and increased growth factor response compared with sensitive cells (Aro cells, gray bars). Results are mean ± SEM ($n = 3$). *, $P < 0.01$. E, LetR cells are more motile than Aro cells ($P < 0.0001$). Histogram shows the mean migratory area per cell (μm^2) ± SEM ($n = 3$). The metastatic MDA-MB231 cells are shown for comparison. (Scale bars, 200 μm). F, higher levels of both MMP9 mRNA by RT-PCR and active MMP9 by gelatin zymography in LetR cells compared with 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 with SKBR3 cells. Cells are stained with DAPI (blue) and phalloidin (red), and images are representative of 3 separate experiments. (Scale bars, 20 μm).

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, conducted 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

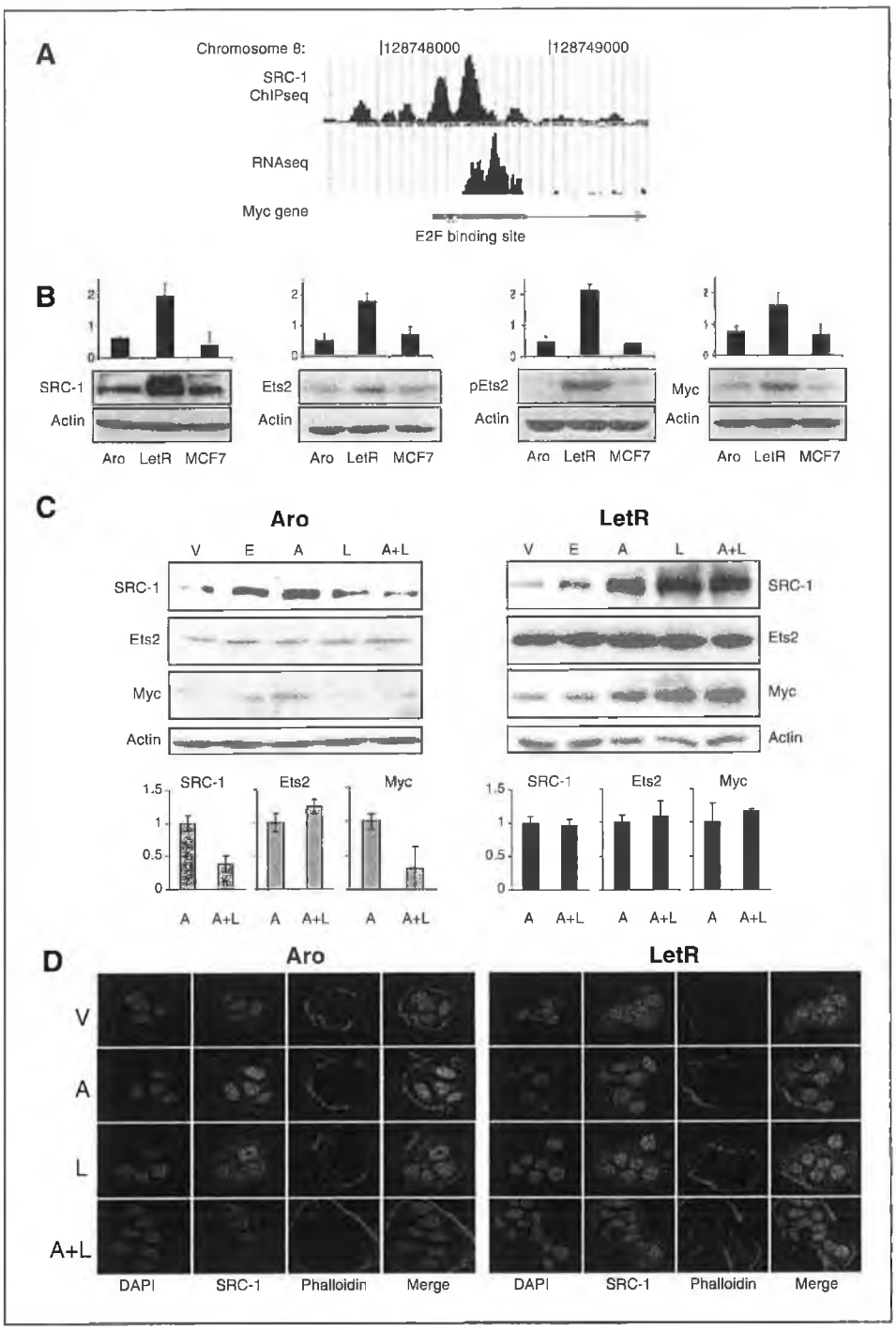


Figure 2. Response of SRC-1, Ets2, and Myc to steroid treatments. A, location of SRC-1–binding peak within the proximal promoter region of Myc gene as detected by ChIP sequencing analysis in endocrine-resistant LY2 cells. RNA sequencing confirms expression of Myc mRNA in these cells. X marks the location 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 localization in Aro and LetR cells in the presence and absence of androstenedione and letrozole alone and in combination. Nuclear localization 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 ×40 magnification with a confocal fluorescent microscope.

between Aro and LetR cells. SRC-1, Ets2, phospho-Ets2, and Myc expression was higher in resistant cells compared with 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). 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). Consistent with these observations, immunofluorescent staining identified increased nuclear localization of SRC-1 in Aro cells treated with androstenedione compared with all other treated Aro cells. Strong nuclear localization of SRC-1 was observed in all LetR cells independent of treatments (Fig. 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 seems to have become dysregulated in the LetR model of AI resistance.

Coimmunoprecipitation analysis revealed that SRC-1 and Ets2 can interact after 45 minutes of steroid treatment (Fig. 3A). ChIP studies were therefore carried out 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 ChIP sequencing 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 and D). 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 posttranslational 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 with that of the AI-sensitive Aro cells (Fig. 4A). Furthermore, the LetR cells with SRC-1 knockdown were capable of forming more organized 3D acini in a manner comparable with 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, ERα knockdown had a minimal effect on migration (Fig. 4C). The significantly greater impact of SRC-1 on migration in comparison with ERα ($P = 0.0377$, Fig. 4C) supports a steroid-independent mechanism for SRC-1 in driving AI-mediated metastasis.

SRC-1 is significantly associated 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 patients who received endocrine therapy, 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 was significantly associated with poor disease-free survival in the total endocrine-treated population ($P = 0.0255$, Fig. 5A and Supplementary Fig. 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 was 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 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.

Among 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–

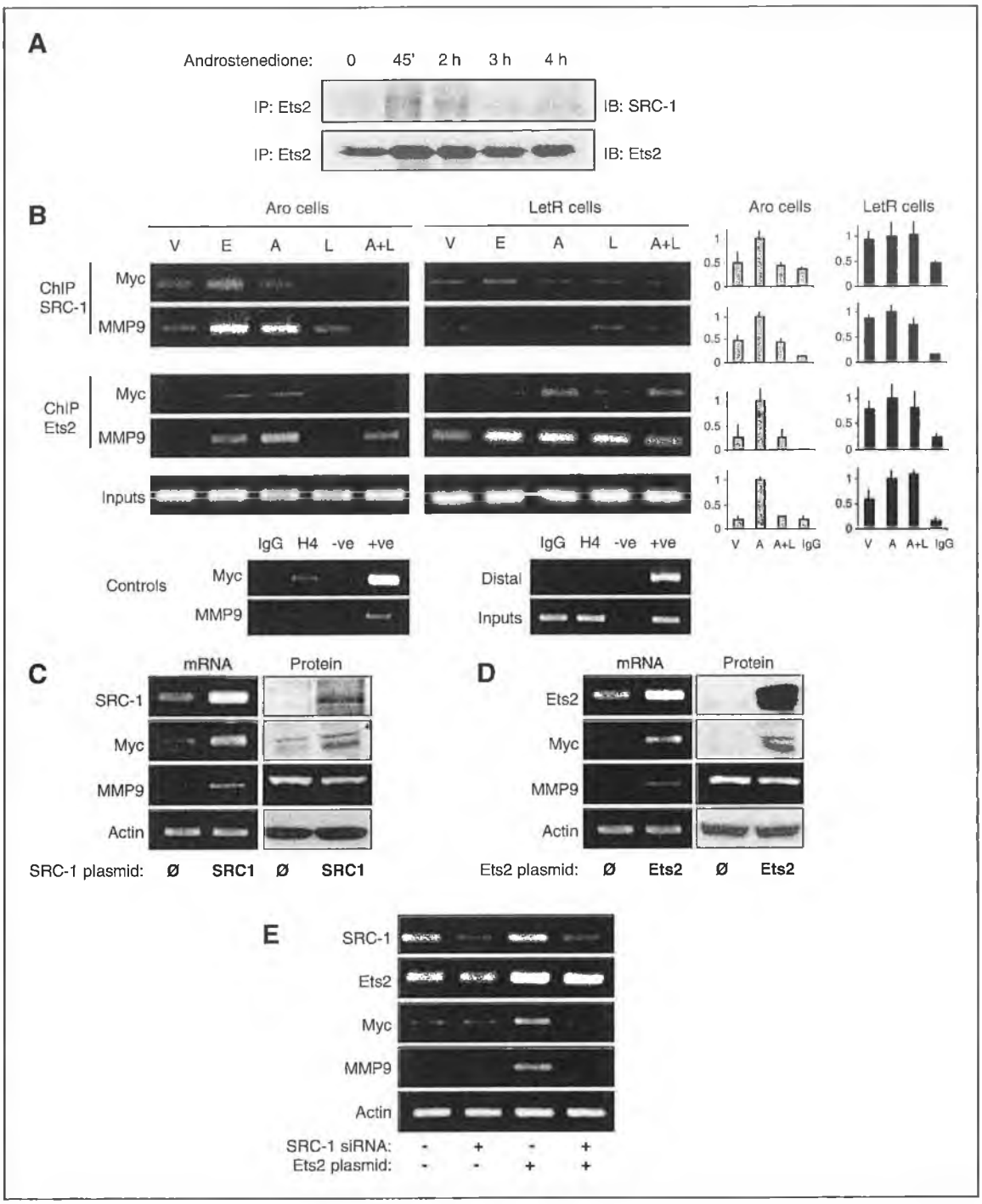


Figure 3. SRC-1 and Ets2 regulate expression of target genes Myc and MMP9. A, SRC-1 and Ets2 coimmunoprecipitate with strongest interaction after 45-minute steroid treatment. Aro cells were treated with androstenedione for 0 to 4 hours. Protein was immunoprecipitated (IP) with an anti-Ets2 antibody and 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 Fig. 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 was 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 was 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.

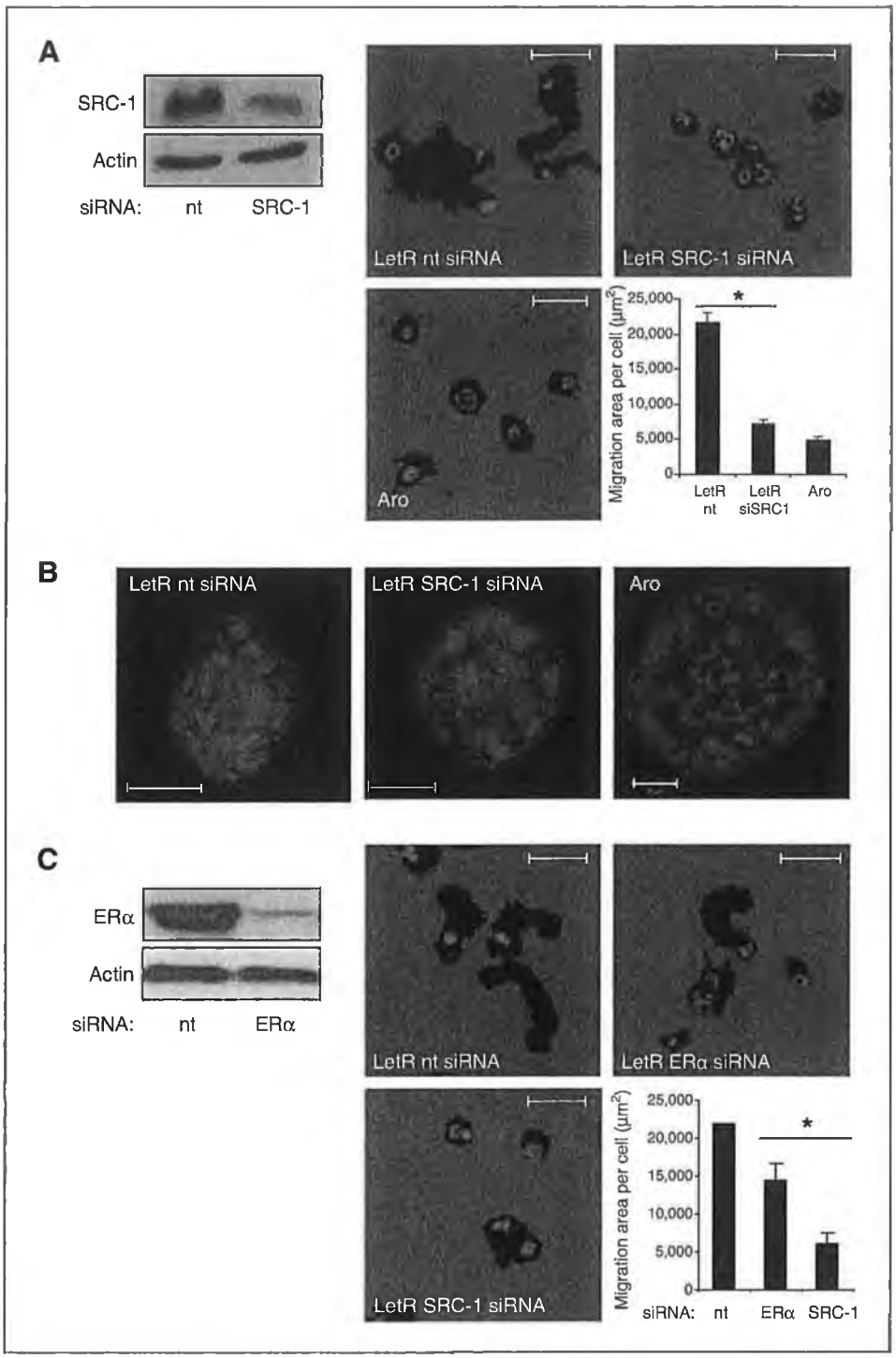


Figure 4. SRC-1 has a functional role in migration and inhibits acini formation in LetR cells. A, SRC-1 knockdown in LetR cells results in decreased migration. Histogram shows the mean migratory area per cell (μm^2) ± SEM and was significantly less for SRC-1 knockdown than for nontargeting (nt) control ($P = 0.0007$). Aro cells are shown for comparison. (Scale bars, 200 μm). 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 3 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 ERα. Western blot confirms successful ERα knockdown with siRNA. Histogram shows only a marginal decrease in the mean migratory area per cell in LetR cells following ERα knockdown. These cells migrate significantly more than LetR cells with SRC-1 knockdown ($P = 0.0377$).

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 carried out in the 3 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). In addition, both proteins significantly colocalized, within the nuclei of the resistant tumor cells in comparison with the matched primary tumor (Pearson 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

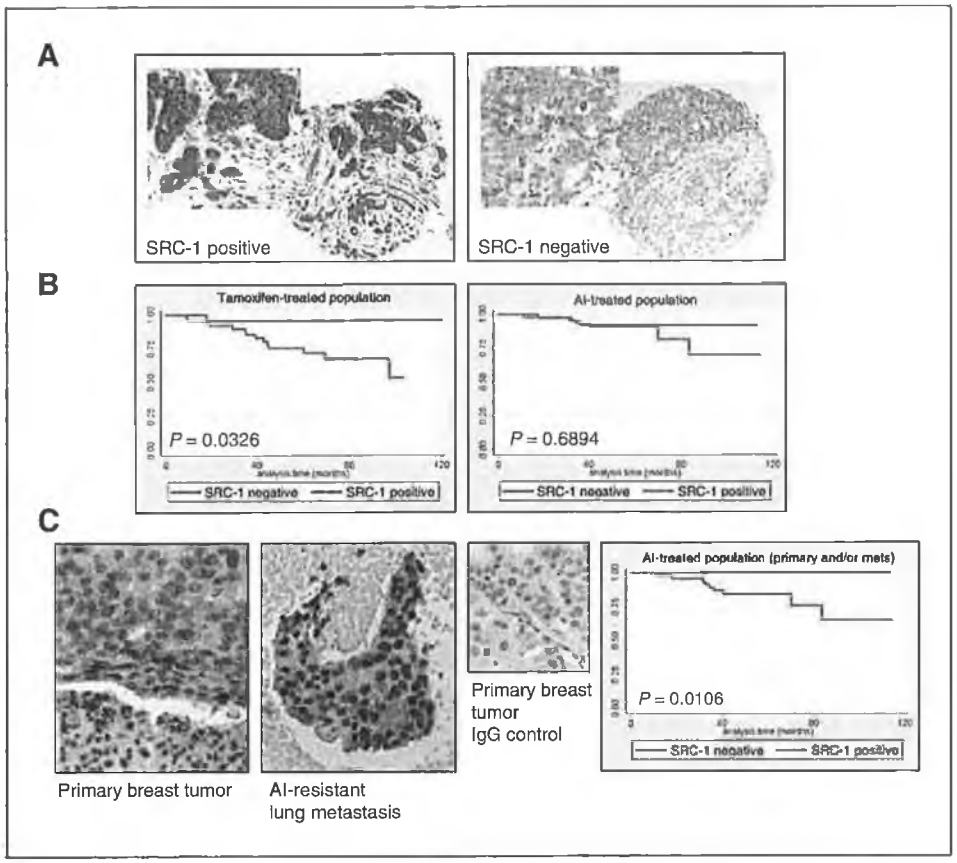


Figure 5. SRC-1 significantly associates with disease recurrence in AI-treated patients. A, immunohistochemical staining of SRC-1 in tissue microarray cores, counterstained with hematoxylin. Examples of SRC-1–positive and–negative primary tumors are shown. B, Kaplan–Meier estimates of disease-free survival in the tamoxifen- ($n = 75$) and AI- ($n = 84$) treated populations. SRC-1–positive primary tumors (red line) were 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 1 AI-treated patient. IgG was used as a negative control. Kaplan–Meier estimates represent disease-free survival in the AI-treated population ($n = 84$) according to SRC-1 staining in the primary or resistant metastatic tumor. SRC-1 is significantly associated with reduced disease-free survival ($P = 0.0106$). *(continued on following page)*

primary tumors (representative images shown in Fig. 5E). These clinical data confirm 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 AI 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 have revealed that PR expression is associated 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 with the matched primary tumor in AI-treated patients. In the LetR cell line model, a slight increase in ERα expression was observed, consistent with the model of long-term letrozole treatment (22). However, we also observed an enhanced proliferative response to EGF in cells resistant to AIs, in comparison with 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 overexpressing 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, LetR cells displayed a reduced proliferative response to steroid treatment and an increased sensitivity to growth factors in

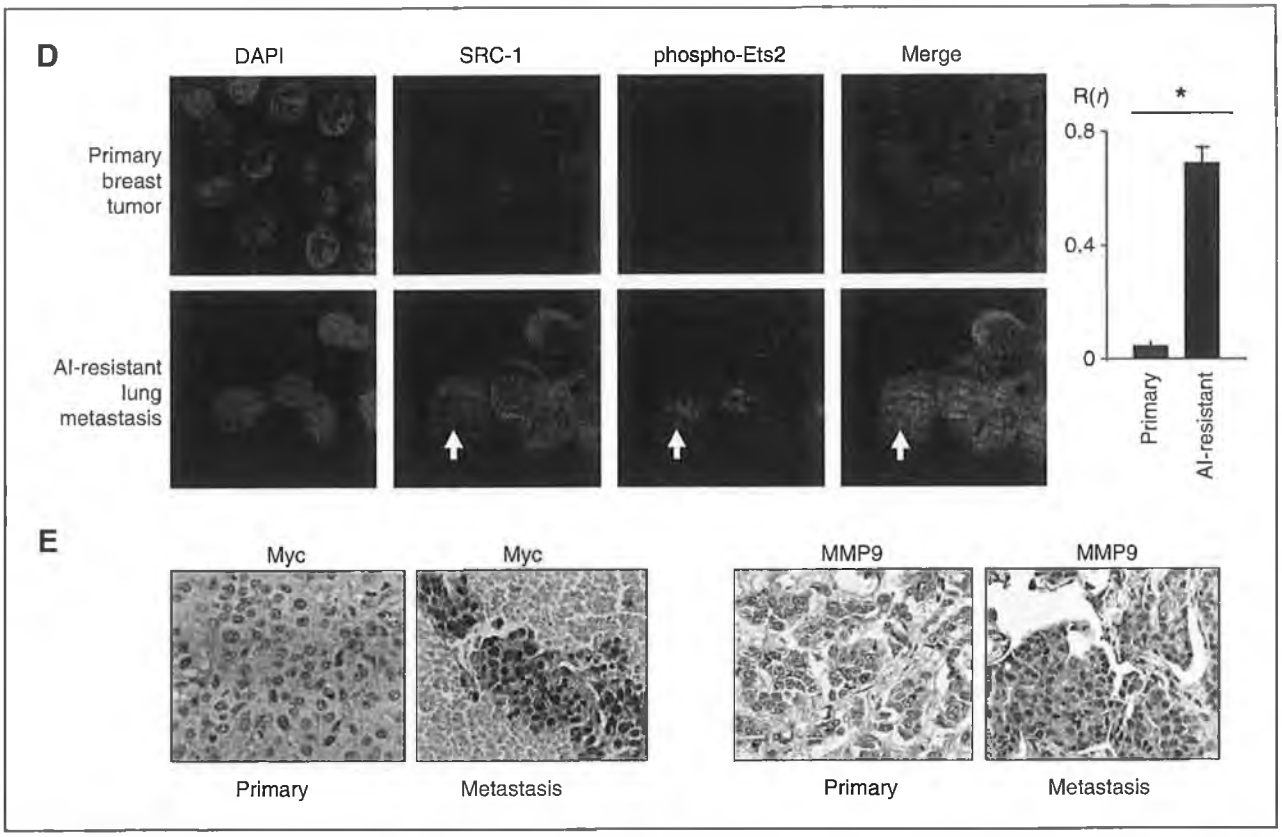


Figure 5. (*Continued*) D, immunofluorescent analysis of SRC-1 (green) and phospho-Ets2 (red) expression in matched primary breast and AI-resistant tumor samples ($n = 3$), counterstained with DAPI (blue). Expression of both proteins was stronger and more nuclear in the resistant samples. Representative images are shown ($n = 3$). Merged image shows that both proteins colocalize in the nucleus of these metastatic cells (white arrows). The extent of coassociation was measured by Pearson 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 hematoxylin. Representative images are shown. Myc was strongly expressed in resistant tumors. MMP9 was weakly expressed in resistant tumors and absent from primary tumors.

comparison with the parental endocrine sensitive cells. Similar elevations in growth factor signaling activity have 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 ERα-dependent pathways are suppressed by letrozole, anastrozole, and exemestane, and ERα can then be activated through different cross-talk 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 Belosay and colleagues (22).

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 MAPK pathway. This group and others have reported functional interactions between SRC-1 and the Ets family of transcription factors, Ets2 and PEA3, and shown 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 β-catenin during epithelial–mesenchymal transition (10). Here, we show that SRC-1 can use Ets2 to regulate the production of Myc and MMP9. The production of both the oncogene

Table 1. Associations of SRC-1 with clinicopathologic variables using Fisher exact test in endocrine, AI-, and tamoxifen-treated patient populations

Parameter	Endocrine-treated population			AI ($N = 84$)		Tamoxifen ($N = 75$)	
	$N = 141$	SRC-1%	*P*	SRC-1%	*P*	SRC-1%	*P*
PR status							
positive	106	61.3		57.4		62.5	
negative	35	62.9	1.000	43.5	0.328	78.9	0.263
Her2 status							
positive	26	65.4		46.7		70.6	
negative	115	60.9	0.834	55.1	0.659	65.5	1.000
Recurrence							
positive	23	87.0		66.7		93.3	
negative	118	56.8	0.009	52.0	0.494	60.0	0.015
Nodal status							
positive	71	67.6		79.5		70.7	
negative	68	55.9	0.167	50.0	0.658	61.8	0.466
Tumor grade							
I	21	57.1		50.0		66.7	
II	79	63.3		56.5		65.1	
III	41	61.0	0.861	50.0	0.818	70.0	0.943
Tumor stage							
I	56	53.6		41.9		62.1	
II	64	57.8		50.0		63.9	
III	18	94.4		100.0		85.7	
IV	3	100.0	0.003	—	0.001	100.0	0.513

NOTE: The percentage of SRC-1% patients within each parameter is listed. Parameters include recurrence (positive) or no recurrence (negative); node positive (1 or more nodes positive) or negative (no positive nodes).

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 ERα 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 with 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 an SRC-1–positive tumor recurrence. This switch from SRC-1 negative to positive in the recurrent tissue echoes the loss of steroid receptor status that 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 promigratory 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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