ADAM22 as a predictive marker for endocrine resistant metastatic breast cancer and an LGI1 mimetic as a companion therapeutic

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Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree Doctor of Philosophy is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Abbreviations

µl – Microliter
µm – Micrometer
µM – Micromolar
4-OHT – 4-Hydroxytamoxifen
ADAM - A disintegrin and metalloproteinase
AF – Activation function domain
AI – Aromatase inhibitor
AIB1 – Amplified in breast cancer 1
ATLAS - Adjuvant Tamoxifen: Longer Against Shorter
ATTC – American type culture collection
ATTOM - Adjuvant Tamoxifen Treatment Offers More
BBB – Blood brain barrier
BCA – Bicinchoninic Acid assay
BDNF – Brain derived neurotrophic factor
BLAST - Basic Local Alignment Search Tool
bp – Base pair
cAMP – Cyclic adenosine monophosphate
CBP – CREB-binding protein
CCND1 - Cyclin D1
CDK - Cyclin-dependent kinase
cDNA – complementary DNA
CDS – Charcoal dextran stripped
CHIPseq – Chromatin Immunoprecipitation with sequencing
cm - Centimetre

CREB – cAMP response element binding protein

CRISPR – Clustered regularly interspersed palindromic repeats

CSC – Cancer stem cell

CSF-1 – Colony stimulating factor 1

CTCs – Circulating tumour cells

ctDNA – Circulating tumour DNA

DCIS – Ductal carcinoma in situ

DMSO – Dimethyl sulphoxide

DNA – Deoxyribonucleic acid

DSB – Double stranded break

E2 – Oestrogen

EBCTCG - Early Breast Cancer Trialists Collaborative Group

ECL – Enhance chemiluminescence

EDTA - Ethylenediaminetetraacetic acid

EGF – Epidermal growth factor

EGFR - Epidermal growth factor receptor

EMT – Epithelial to mesenchymal transition

EOX – End of experiment

ER – Oestrogen receptor

ERE – Oestrogen response element

ESR1 – Oestrogen receptor alpha gene

ESR2 – Oestrogen receptor beta gene

FACS – Fluorescence activated cell sorting
FAK – Focal adhesion kinase
FANTOM – Functional Annotation of Mammalian Genomes
FCS – Foetal calf serum
FDA - Food and drug administration
FFPE – Formalin-fixed paraffin-embedded
FGF1R - Fibroblast growth factor receptor 1
gDNA – Genomic DNA
GFP – Green fluorescent protein
GOBO – Gene expression-based outcome for breast cancer online
GTEx - Genotype-Tissue Expression
HDR – Homology directed repair
HER2 – Human epidermal growth factor receptor 2
HIF1-α - Hypoxia inducible factor 1 alpha
HK2 - Hexokinase-2
HPA – Human Protein Atlas
HRP – Horse radish peroxidase
IDC – Invasive ductal carcinoma
IGF-1R - Insulin like growth factor-1 receptor
IgG – Immunoglobulin
IHC – Immunohistochemistry
ILC – Invasive lobular carcinoma
IMS - Industrial methylated spirits
InsR - Insulin receptor
IP – Intraperitoneal
ITGA5 – Integrin alpha 5
KEGG – Kyoto encyclopaedia of genes and genomes
KO – Knockout
LG - L-Glutamine
LG11 - Leucine rich glioma inactivated 1
LY2luc – LY2 luciferase tagged
M – Molar
mAb – Monoclonal antibody
MCT4 – Monocarboxylic acid transporter 4
MEME – Minimum essential media eagle
MeOH – Methanol
MFE – Mammosphere forming efficiency
ml – Millilitre
MMP9 – Matrix metalloproteinase 9
MMTV PyMT - Mouse mammary tumour virus polyoma middle T antigen
MOI – Multiplicity of infections
mTOR - Mammalian target of rapamycin
n.s – Not significant
NGF – Nerve growth factor
NHEJ – Non-homologous end joining
nM – Nanomolar
NT – Neurotrophin
O/N – Overnight
pERK1/2 – phosphorylated ERK1/2
P/S – Penicillin-streptomycin
PAGE – Polyacrylamide gel electrophoresis
PALOMA - Palbociclib: Ongoing Trials in the Management of Breast Cancer
PBS – Phosphate buffer saline
PCR – Polymerase chain reaction
PDK1 – Phosphoinositide-dependent kinase-1
PI3K-AKT - Phosphatidylinositol 3-kinase – protein kinase B
PR- Progesterone receptor
PRF – Phenol red free
PSA – Prostate specific antigen
RANK - Receptor activator of nuclear factor kappa-beta
RANKL - Receptor activator of nuclear factor kappa-beta ligand
rcf – Relative centrifugal force
RFP – Red fluorescent protein
RIPA – Radioimmunoprecipitation assay
RISC – RNA induced silencing complex
RNA – Ribonucleic acid
RNAi – RNA interference
RNAseq – RNA sequencing
RPKM – Reads per kilobase gene model and Million mapped reads
RPPA – Reverse phase protein array
RT – Room temperature
RTK - Receptor tyrosine kinase
RT-PCR – Real time PCR
SDS – Sodium dodecyl sulphate
SERD – Selective oestrogen receptor downregulator
SERM – Selective oestrogen receptor modulator
sgRNA – Single guide RNA
siRNA – Short interfering RNA
soFEA - Study of Faslodex Versus Exemestane With or Without Arimidex
SRC-1 – Steroid receptor co-activator 1
TBST – Tris buffer saline 0.001% Tween
TMA – Tissue microarray
TNM – Tumour node metastasis
TPM – Transcripts per million
Trk – Neurotrophin tyrosine kinase receptor
TU – Transducing Units
UV – Ultraviolet
VEGFA - Vascular endothelial growth factor A
VEGFR2 – Vascular endothelial growth factor receptor 2
XIAP – X-linked inhibitor of apoptosis protein
Summary

Approximately 70% of breast cancer patients are classified as oestrogen receptor positive. While initial prognosis is favourable, the risk of recurrence remains long after diagnosis. Furthermore, the ability to treat recurrent tumours becomes harder over time as resistance to endocrine therapy develops. This resistant phenotype is caused in part by overexpression of the nuclear receptor co-activator SRC-1. Moreover, SRC-1 has been shown to promote metastatic development in several breast cancer models.

Previously, our lab identified the neuronal protein ADAM22 as an SRC-1 target gene involved in endocrine resistant breast cancer metastases. Here, this metastatic role was further characterised through knockdown, knockout and overexpression studies along with a high throughput proteomic study. This study demonstrates the potential of ADAM22 as a biomarker for predicting metastatic development in endocrine resistant patients. Finally, an ADAM22 targeting peptide mimetic was shown here to reverse ADAM22 mediated metastatic characteristics both in vitro and in vivo.
1 Introduction
1.1 Breast cancer: incidence and epidemiology

Breast cancer is the most commonly diagnosed tumour found in women in Ireland, with about 2500 new cases per year [1]. Some of the risk factors associated with breast cancer include age, obesity, smoking, drinking alcohol, diet, late age of menarche, nulliparity, excess endogenous and exogenous hormones (particularly estradiols) and familial history (inherited mutations); while childbearing, breastfeeding and a healthy lifestyle appear to be protective against breast cancer [2]. Despite an annual increase in incidence, mortality rates are on the decline in the European Union [3]. This can largely be attributed to early detection and improvements in therapeutic options [4].

1.2 Classification of breast cancer:

Breast cancer is a heterogeneous disease and as such, much work has gone into subdividing the disease into several distinct clinical, histological and molecular classifications, which aid prognosis and effective treatment decisions.

Breast cancer can be initially classified based on the location of the cancerous cells and on whether they have infiltrated surrounding tissue. The most common sites for breast cancer are the ducts and lobules of the breast, with ductal carcinoma in situ (DCIS) accounting for approximately 90% of all non-invasive breast cancers; while invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) account for approximately 95% of all invasive breast cancers [5].

Staging of the tumour is based on the Tumour Node Metastasis (TNM) system, which classifies the severity of the disease, allowing clinicians to determine the progression and stage of the disease depending on the size or extent of the primary tumour; whether the tumour is present in the nodes and whether the tumour has metastasised [6].

The most important histological classification in breast cancer is receptor status. Both the oestrogen receptor alpha (ERα) and progesterone receptor (PR) were implicated as important prognostic biomarkers in breast cancer in the 1970s [7, 8]. In 1987, a prognostic role for the human epidermal growth factor receptor 2 (HER2) was described [9]. These three receptors form the basis of typing used still to this
day as breast cancer can be broadly categorised as hormone receptor positive (ER+ and/or PR+), HER2 positive (HER2+), or triple negative (ER- PR- and HER2-).

Gene expression profiling in the early 2000s by Perou et al led to the first true molecular classification of breast cancers into five distinct subtypes [10, 11]. While seven distinct subtypes are recognised today: luminal A, luminal B, HER2-enriched, basal-like, claudin-low, triple negative and normal-like [12, 13]; up to ten molecularly distinct subtypes have now been described, further adding to the complex nature of breast cancer as a highly heterogeneous disease [14]. This intrinsic subtype model has significantly improved our understanding of breast cancer [15].

Luminal A tumours account for approximately 50-60% of breast cancer and have the most favourable prognosis of all the subtypes. They are histologically defined by high levels of ER with or without PR, coupled with low expression of proliferative markers [16]. Luminal B tumours account for 10-20% of breast cancers and are defined by low to moderate ER/PR expression, high expression of the proliferative marker ki67 and can either be HER2 positive or negative [16]. Clinically, it can be hard to distinguish between the two luminal subtypes on ki67 positivity alone, highlighting the need for standardisation of scoring techniques and routine use of multi-gene assays to determine subtype [4]. Endocrine based therapies are the standard treatment for luminal type tumours; however, luminal B tumours can be more challenging to treat and tend to respond better to neoadjuvant chemotherapy [4, 16].

HER2-enriched tumours account for approximately 15-20% of breast cancers and are defined by HER2 amplification or overexpression and ER/PR negativity [16]. Several distinct HER2 subgroups have now been described [16]. While traditionally HER2 positive breast cancer was associated with poor prognosis, the development of anti-HER2 therapies has greatly improved survival rates [17].

Basal like tumours account for approximately 10-20% of breast cancers, are defined by lack of ER, PR and HER2 and carry the worst prognosis of all subtypes [16]. Similarly, the normal-like and claudin-low subtypes can be classified as triple negative; with the normal-like subtype expressing a gene signature close to adipose tissue and the claudin-low subtype overexpressing genes associated with an epithelial to mesenchymal (EMT) cancer stem cell (CSC) phenotype [16].
Classifying tumours into the above subtypes has allowed researchers and clinicians alike to improve treatment strategies for breast cancer patients [4, 18, 19]. However, despite improving survival rates for breast cancer patients, particularly those on endocrine and anti-HER2 therapies, a significant proportion of patients go on to recur [20]. Figure 1.1 shows survival rates for luminal A, luminal B, HER2-enriched and Basal subtypes.

![Figure 1.1: Kaplan meier survival estimates for breast cancer subtypes.](image)

Figure 1.1: Kaplan meier survival estimates for breast cancer subtypes. Survival probability from a large cohort of breast cancer patients (n=934). Luminal A type patients have the best prognosis; however, a significant proportion will develop recurrent breast cancer. [20]

### 1.3 Gene expression platforms

Current research is focused on developing new diagnostic / prognostic tests which can help identify those patients likely to have a recurrence and predict therapeutic response. The use of gene expression platforms, such as Mammaprint, Prosigna (PAM50) and Oncotype DX, has allowed researchers to further classify breast cancer subtypes based on multi gene signatures [14]; to better understand the signalling pathways which drive progression of the disease; to predict early and late recurrence; and to identify patients likely to benefit from chemotherapy [16]. While
the benefit of these assays have been validated in prospective clinical trials [4], they have yet to become routinely implemented in the clinic [21].

1.4 The development of metastases

The majority of cancer patients die after developing overt untreatable metastases [22]. While advances in our understanding of breast cancer has led to improvements in prognostic tools and tailored therapeutics, a significant number of patients will go on to develop metastases [23]. Recent reports, analysing patient outcome between 2005 and 2015, have shown that, while a modest survival gain was observed for patients with regional disease, for patients with distant metastases no improvement has been observed in 5 year survival rates [24]. Indeed, a recent analysis of metastatic breast cancer patients over a 30 year period has shown there has been no significant improvement in survival [25]. This may be complicated by the fact that heavy treatment with targeted therapies leads to increased mutation rates at distant sites [4]. Recent studies have also reported intrinsic subtype switching in metastatic tissue when compared to the matched primary tissue [26, 27]. This complicates treatment strategies somewhat, and so, subtyping of metastatic tumours in the clinic is now advised where possible [28]. The risk of recurrence is subtype dependent. While luminal type breast cancer patients initially have the best prognosis, the risk of recurrence in these hormone receptor positive patients remains beyond 20 years from diagnosis [29].

1.5 Oestrogen signalling:

Oestrogens (E2) are necessary for the development of the normal breast and play important roles in cell differentiation and proliferation in many tissues throughout the body [30]. Oestrogens primarily act through their binding to and activation of the classical oestrogen receptors ERα and ERβ, encoded by \( ESR1 \) and \( ESR2 \) respectively [30]. These nuclear hormone receptors act as transcription factors to regulate oestrogen and growth factor mediated gene expression [30]. The hormone dependent activation of the ER receptors is carried out in the activation function 2 (AF2) domain found within the ligand binding domain of the receptor [30]. Binding of E2 to ER causes dimerisation, consequent phosphorylation and nuclear translocation [30, 31]. Here it binds with oestrogen response elements (ERE) in genes, leading to the formation of a transcriptional complex involving the steroid
receptor co-activator 1 (SRC-1), amplified in breast cancer 1 (AIB1), cAMP response element binding protein (CREB)-binding protein (CBP), the histone acetyltransferase p300 and other chromatin modellers to regulate gene expression [30, 31]. This process is known as classical genomic ER signalling. Non-genomic ER signalling involves the E2 mediated activation of membrane associated ERs and the consequent activation of downstream signal transduction pathways [32]. Alternatively, ER can be activated in a ligand-independent manner downstream of receptor tyrosine kinases [33]. This non-classical genomic ER signalling occurs after the ER gets phosphorylated by the effectors of these growth factor pathways [34, 35]. Figure 1.2 illustrates the different types of ER signalling.

Figure 1.2: The major oestrogen receptor signalling pathways: (a) Oestrogen acts upon ER causing nuclear translocation, recruitment of co-regulators and chromatin modifiers to act upon oestrogen response genes (ERE); (b) ligand-independent signalling involves the activation of ER via receptor tyrosine kinases (RTKs) and consequent target gene expression, (c) Oestrogen can regulate non-oestrogen response genes via crosstalk with other signalling pathways. Image taken from [35]
1.6 Oestrogen receptor positive breast cancer and endocrine therapy

In the normal breast oestrogen receptors are expressed at relatively low levels, however, approximately 70% of breast cancers are classified as ER positive, with oestrogen as the driving force behind tumorigenesis and tumour survival. In all luminal patients, endocrine therapy is the standard treatment [4]. Adjuvant or neoadjuvant chemotherapy is only given on the basis of grade, proliferation and risk of recurrence [4].

1.6.1 Selective oestrogen receptor modulators (SERMs)

SERMs are a class of drugs which show both agonistic and antagonistic effects on the oestrogen receptor in a tissue dependent fashion [36]. SERMs have anti-oestrogenic effects in the breast and are the standard treatment option for premenopausal ER+ breast cancer patients. The most common SERM used in the clinic is tamoxifen, which was approved in 1977 [36]. In the Early Breast Cancer Trialists Collaborative Group (EBCTCG) tamoxifen trials, tamoxifen treatment was shown to reduce the risk of recurrence and improve overall survival rates in ER+ patients [37, 38]. While in both the Adjuvant Tamoxifen: Longer Against Shorter (ATLAS) and the Adjuvant Tamoxifen Treatment Offers More (ATTOM) trials, continued adjuvant tamoxifen treatment for 10 years was shown to reduce both recurrence risk and reduce overall mortality in ER+ patients [39, 40]. Other clinically used SERMs include raloxifene [36].

1.6.2 Aromatase inhibitors (AI)

In postmenopausal patients, the primary source of oestrogen comes from the aromatisation of androstenedione [30]. As such, aromatase inhibitors act by blocking this oestrogen source through inhibition of the aromatase enzyme [41]. Three third generation AIs are currently in use in the clinic: the non-steroidal AIs letrozole and anastrozole; and the steroidal exemestane [41]. AIs are currently the preferred therapy for both postmenopausal ER+ breast cancer patients and patients with advanced metastatic hormone receptor positive disease; and have been shown to significantly reduce the risk of recurrence and improve survival rates [41-43].
1.6.3 Selective oestrogen receptor downregulators (SERDs)

A third class of endocrine therapy, known as selective oestrogen receptor downregulators (SERDS), such as fulvestrant are also in use. Mechanistically, fulvestrant acts by blocking both the AF1 and AF2 domains of ER, resulting in a complete block of oestrogen signalling [44]. Furthermore, the binding of fulvestrant to ER results in its rapid degradation [45]. Recent studies suggest fulvestrant may be of particular benefit to patients with \( ESR1 \) mutations [46]. As such, SERDs are generally used for the treatment of advanced ER positive breast cancer [47].

1.7 Mechanisms of endocrine resistance

Despite the largely successful treatment of breast cancer with endocrine based therapies, close to one third of patients will relapse [38]. The development of endocrine resistant breast cancer is largely caused by changes on a molecular and cellular level leading to tumour adaptability. This acquired resistance to endocrine therapy is best seen in recurrent patients as response rates significantly drop for each line of therapy a patient receives [48]. Furthermore, while loss of ER\( \alpha \) does occur in endocrine resistant breast cancer, the frequency rates do not suggest it is a key resistance mechanism, implying ER\( \alpha \) still plays a key role in both the survival and proliferation of these tumour cells [49]. This suggests that in the face of endocrine therapy, resistant cells can overcome the cytostatic properties of anti-oestrogens, activating ER\( \alpha \) through other means. As such researchers have focused their attention on identifying novel biological signatures to help predict therapeutic response and to discover new druggable targets to overcome this resistance [35].

1.7.1 \( ESR1 \) mutations

Recent analyses of endocrine resistant metastatic tissue have identified several mutational hotspots within the \( ESR1 \) gene, which lead to the constitutive hormone independent activation of the ER\( \alpha \) ligand binding domain, and consequent resistance to anti-oestrogen therapy [50-52]. Interestingly, the frequency of \( ESR1 \) mutations was higher in ER\( + \) metastatic tissue over primary tissue; while the frequency was also higher in patients who had more regimens of endocrine therapy [53]. The frequency of activating \( ESR1 \) mutations is thus in part driven by continued endocrine therapy, leading to enhanced non-classical genomic oestrogen-independent ER
mediated proliferation. In a prospective-retrospective analyses of patient plasma samples from the SoFEA (Study of Faslodex Versus Exemestane With or Without Arimidex) trial and the PALOMA3 (Palbociclib Combined With Fulvestrant in Hormone Receptor-Positive HER2-Negative Metastatic Breast Cancer After Endocrine Failure) trial, frequency rates of ESR1 mutations were 39% and 25% respectively; with a clear increase in frequency observed in patients with prior AI treatment [54]. Interestingly, the results of this study also suggest some benefit to patients taking fulvestrant over exemestane after failing non-steroidal AI therapy; while the combination of fulvestrant and palbociclib was also beneficial [54]. More recently, both fulvestrant and other SERDs have shown efficacy against ESR1 mutated tumours [55].

1.7.2 Growth factor signalling

In the absence of oestrogen, ERα can be activated through growth factor pathway crosstalk, as seen in non-classical genomic ER signalling. As such, a lot of research has focused on the relevance of growth factor signalling in the development of endocrine resistance. The overexpression of receptor tyrosine kinase receptors is frequently observed in endocrine resistant breast cancer [35]. Increased expression of epidermal growth factor receptor (EGFR) and its downstream signalling effectors are associated with resistance [56]. The fibroblast growth factor receptor 1 (FGF1R) is overexpressed in 10% of breast cancers, with this overexpression also leading to enhanced ligand independent signalling and consequent endocrine resistance [57]. Similarly the insulin receptor (InsR) and insulin like growth factor-1 receptor (IGF-1R) are also frequently overexpressed in breast cancers, leading to enhanced activation of the phosphatidylinositol 3-kinase (PI3K-AKT) pathway and consequent reduced sensitivity to anti-oestrogens [58]. While HER2 positivity is often present in the primary tumour, the level of HER2 overexpression is also strongly associated with resistance to therapy [59]. Interestingly, ERα has been shown to regulate the expression of growth factor receptors, suggesting a feedback loop mechanism employed by resistant cell lines [60-62]. Each of these overexpressed tyrosine kinase receptors mediates a signalling cascade capable of activating ERα independently of oestrogen. Furthermore, activating mutations are commonly found within components of these signalling cascades, primarily in members of the
PI3K/AKT/mTOR (mammalian target of rapamycin) pathway [48, 49, 63]. As such, a new generation of therapies, targeting the PI3K/AKT/mTOR pathway have been developed, which have shown some benefit in the clinic today. The most promising of which are mTOR inhibitors such as everolimus [47].

Preclinical studies showed that mTOR activation was responsible for oestrogen induced proliferation of breast cancer cells and that dual treatment with letrozole and everolimus synergistically inhibited both growth and survival [64]. In the TAMRAD and BOLERO-2 trials, for patients who have failed non-steroidal aromatase inhibitors and developed metastatic breast cancer, combining tamoxifen with everolimus or exemestane with everolimus has been shown to improve both time to progression and overall survival [65, 66]. The clinical impact of these studies demonstrates the potential of inhibiting growth factor pathways in the resistant setting.

1.7.3 Overexpression of cyclin D1

Proliferation is a tightly controlled process in cells. At the heart of this process is the protein cyclin D1, encoded by the gene *CCND1*. Cyclin D1 is responsible for mediating the cellular response to pro-proliferative signals, through its ability to interact with and activate cyclin-dependent kinase 4 (CDK4) and CDK6, which in turn promote progression through the cell cycle and consequent cell division [67]. Approximately 15% of all cancers overexpress cyclin D1, which in turn leads to hyperactive CDK4/6 [47]. Interestingly, in ER+ breast cancer, the frequency of this *CCND1* amplification is 29% for luminal A patients and 58% for luminal B patients [63]. Cyclin D1 has also been shown to co-activate ligand independent ER transcriptional activity, suggesting a positive feedback mechanism [68].

In preclinical studies, CDK4/6 inhibitors were shown to inhibit the proliferation of ER+ cells; while a synergistic effect was observed with tamoxifen in endocrine resistant cell lines [69]. The most promising results in clinical trials have been seen with the CDK4/6 inhibitor palbociclib [47]. In the PALOMA-1 and PALOMA-2 trials, endocrine sensitive patients were treated with letrozole plus palbociclib versus letrozole alone, and showed significant improvement in progression free survival [70, 71]. While in the PALOMA-3 trial, similar improvements in progression free survival were observed for endocrine resistant patients treated with fulvestrant plus palbociclib [72].
1.7.4 ER co-regulatory proteins

The resistance mechanisms outlined above converge to activate ER in a ligand independent fashion, leading to ER mediated gene expression. Interestingly, this ER induced transcriptional activity is dependent on co-regulatory proteins, which aid in the process of target gene expression [73]. Unsurprisingly, many of the co-regulatory proteins involved in the ERα transcriptional complex have been implicated in the development of endocrine resistance [35]. Response to therapy is regulated at least in part by the relative expression levels of these co-activators and co-repressors [74].

The p160 family of co-activators, including steroid receptor co-activator 1 (SRC-1), SRC-2 and amplified in breast cancer 1 (AIB1), are key regulators of nuclear receptor and transcription factor activity. P160 interactions with multiple protein complexes allow them to regulate a host of cellular functions in healthy tissue, while deregulation of their expression often leads to disease [75]. In the breast cancer setting, this family of proteins are key to the ligand-dependent transcriptional activities of ERα, although they have been shown to function as activators for other non-steroidal transcription factors as well, promoting expression of non-estrogen response genes [76, 77]. For example, under normal estrogenic conditions, SRC-1 acts predominantly with ERα; however, in anti-estrogenic conditions, such as after tamoxifen treatment, SRC-1 is sequestered to the promoter of ERBB2, promoting HER2 expression, which is strongly associated with the development of endocrine resistance [78]. Interestingly, studies have shown that tamoxifen treatment alone is sufficient to drive increased expression of SRC-1 and AIB1 [79, 80]. Furthermore, this increase in co-activator expression is enhanced in resistant cell lines compared to sensitive parental cell lines [81]. Moreover, in patient analyses, both SRC-1 and AIB1 expression strongly associate with endocrine therapy resistance and poor disease free survival [82-87].

While clinically the development of drugs which target these endocrine resistant mechanisms has led to an improvement in progression free survival and indeed overall survival, particularly for patients with advanced oestrogen receptor positive breast cancer; ultimately the risk of recurrence never disappears. It is essential that novel biomarkers are discovered which will inform clinicians about therapy response and metastatic progression; while identifying the principal effectors of metastatic
progression may offer novel therapeutic targets, which ultimately will reduce recurrence risk.

1.8 p160 family in metastatic development

While both AIB1 and SRC-1 have been shown to play distinct roles in the development of endocrine resistance, a growing body of evidence has implicated both proteins in the development of breast cancer metastases [77].

Early studies examining the role of AIB1 in breast cancer showed that the nuclear receptor coactivator mediated both the initiation of tumorigenesis and development of metastases through activation of the IGF1R pathway [88]. Follow up studies examining the specific role AIB1 plays in metastatic development, showed that AIB1 ablation led to a significant reduction in lung metastases [89]. Mechanistically, AIB1 was shown to promote both invasion and de-differentiation, through upregulation of mesenchymal markers and through upregulation of matrix metalloproteinases [89].

MMTV-polyoma middle T antigen (PyMT) mice form spontaneous mammary tumours and extensive lung metastases [90]. SRC-1 knockout mice were crossbred with PyMT mice to examine whether SRC-1, like AIB1, played a role in tumorigenesis and metastases [91]. While SRC-1 ablation had no effect on tumorigenesis, SRC-1 knockout mice did form significantly fewer lung metastases [91]. Similar results were observed using the MMTV-neu oncogene model of metastases, where SRC-1 knockout led to both delayed tumorigenesis and an 80% reduction in metastatic development [92]. SRC-1 appears to drive this metastatic phenotype through several different mechanisms. It has been shown to upregulate the Twist transcription factor, which mediates EMT [93]. SRC-1 also directly regulates expression of integrin α5, which enhances migration [94]. Furthermore, SRC-1 is capable of driving the activation of tumour associated macrophages through expression of colony stimulating factor-1 (CSF-1) [91, 95]. Lastly, SRC-1 has also been shown to promote angiogenesis through its ability to upregulate HIF1-α (hypoxia inducible factor alpha) and VEGFA (vascular endothelial growth factor A) [96]. The adaptability of SRC-1 as a co-activator to a plethora of different transcription factors endows it with the ability to drive these multiple phenotypes.

As resistance to endocrine therapy develops, the risk of developing metastases increases and this can partly be explained through overexpression of the p160 family
of proteins, which leads to increased growth factor signalling [87, 89, 97, 98]. This was highlighted in a study where SRC-1 was shown to mediate the metastatic characteristics of AI resistant cells, through increased growth factor signalling and the upregulation of Myc and MMP9 (matrix metalloproteinase 9) [99]. In order to fully understand the mechanisms through which SRC-1 could drive metastases in the endocrine resistant setting, the SRC-1 global transcriptome was characterised in the tamoxifen resistant LY2 cell line [100]. Interestingly, this led to the identification of ADAM22 (a disintegrin and metalloproteinase 22) as a novel SRC-1 mediated gene, involved in metastases [100]. ADAM22 was found to be induced by tamoxifen treatment in an ER independent manner, and was shown to promote both migration and de-differentiation of resistant cell lines [100]. Furthermore, ADAM22 expression was significantly associated with poor disease free survival and recurrence [100]. Treatment with the leucine rich glioma inactivated 1 (LGI1) protein, an ADAM22 ligand, inhibited migration in these cells [100].

1.9 ADAM proteins

The ADAM family is a large group of transmembrane and secreted proteins which are recognisable by their conserved multi-domain structure [101]. In general, the typical ADAM protein structure consists of a pro-domain, a metalloproteinase domain, a disintegrin domain, a cysteine rich domain, an epidermal growth factor (EGF) like domain, a transmembrane domain, and a cytoplasmic tail (figure 1.3) [102].

![Figure 1.3: Typical ADAM protein structure.](image)

Each ADAM protein consists of: a prodomain, a metalloproteinase domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a transmembrane domain and a cytoplasmic tail. Image adapted from [103].
The best described roles for ADAMs comes from those with an active metalloproteinase domain, which acts as a sheddase, releasing extracellular proteins, such as growth factors and cytokines, to promote signalling and cellular response [104]. The disintegrin domain, so called after the snake venom disintegrin, which inhibits platelet aggregation, can bind integrin proteins and is thought to be involved cell-cell interactions and adhesion [101]. While no distinct role has been described for the cysteine-rich and EGF-like domains, they are thought to be involved in substrate specificity and adhesion [102]. Finally, the c-terminal domain of ADAM proteins has been shown to play roles in regulating both sheddase activity and intracellular signalling [106, 107].

Numerous roles in cancers have been described for ADAM proteins. The most common mechanism employed by ADAMs in tumorigenesis involves their ability to release growth factors using their sheddase activity [102]. The most prominent ADAMs involved in this process are ADAM10 and ADAM17, which promote the release of EGFR/HER family ligands, leading to increased proliferation and survival, [108]. ADAM17 has been studied extensively for its role in breast cancer progression and the development of resistance and metastases [109].

While all ADAM proteins contain a metalloproteinase domain, at least 7 of the ADAMs are considered proteolytically inactive [102]. Among those are the three closely related ADAM11, ADAM22 and ADAM23 proteins, whose endogenous expression is found exclusively in the central nervous system [110, 111], where they play functional roles in neuronal development and maintenance [112-117].

While roles in tumorigenesis have been described for each of these ADAM proteins, ADAM23 is by far the most characterised. There is a large body of evidence which shows that the ADAM23 gene gets hyper-methylated during the progression of breast [118], lung [119], head and neck [120], gastric [121] and colorectal cancers [122]. ADAM23 downregulation in the breast was shown to enhance the activity of integrin αvβ3, thereby promoting metastases [123]. Furthermore, the degree of ADAM23 methylation was significantly associated with poor disease free survival [123]. This was also observed in lung cancer, where ADAM23 downregulation led to increased integrin αvβ3 activity and consequent promotion of colony formation,
adhesion, migration and of a cancer stem cell phenotype [124]. Interestingly, ADAM23 intratumoral heterogeneity has been implicated in breast cancer metastases; suggesting that the progression of disease does not rely purely on ADAM23 downregulation, but on the crosstalk between ADAM23 positive and negative cells within the tumour [125]. Furthermore, the authors suggest that the expression of ADAM22 within ADAM23 negative cells may support their proliferation [125].
1.10 Hypothesis

As tumours acquire resistance to endocrine therapy, the p160 nuclear co-activator SRC-1 gets overexpressed, leading to highly adaptable tumours with enhanced growth factor signalling and increased ER independence. Furthermore, this enhanced SRC-1 activity promotes the development of metastases. One of the mechanisms through which SRC-1 achieves this, is through the upregulation of the neuronal transmembrane protein ADAM22, which through its multiple domains, can mediate a pro-metastatic phenotype. Furthermore, ADAM22 is a transmembrane protein, making it a desirable target.

1.11 Aims

1) To elucidate the functional and mechanistic roles played by ADAM22 in endocrine resistant metastatic development.

2) To investigate ADAM22 as a potential biomarker, ADAM22 expression will be examined in patient tissue and in online datasets.

3) To examine the potential of an LGI1 peptide mimetic as a novel companion therapeutic
2 Materials and Methods
2.1 Cell Culture:

All cell lines were adherent and cultured in T75 cm$^2$ flasks (Sarstedt, Germany) @ 37 °C in a humidified 5% (v/v) CO$_2$ atmosphere.

2.1.1 Growing and harvesting cell lines

At 80% confluency, cell lines were passaged. Firstly, growth media was removed, before washing cells twice with sterile phosphate buffer saline (PBS). Cells were detached from culture flasks by incubation with 2 ml of 0.05 % Trypsin/ 0.02 % EDTA for 5 minutes @ 37 °C. Cells were then resuspended in 8 ml of cell line specific media and transferred to a 15 ml falcon tube. Cells were pelleted by centrifugation @ 201 relative centrifugal force (rcf) for 3 minutes. Cells were resuspended in fresh media and seeded at an appropriate density back into T75 cm$^2$ flasks for further incubation.

2.1.2 Counting and seeding cells

For defined seeding density experiments, an aliquot of cell suspension (10 - 40 µl) was mixed in a 1:1 ratio with trypan blue exclusion dye (Sigma Aldrich). 10 µl was then pipetted onto a haemocytometer with a 0.1 mm sample depth. Cells were counted manually under a light microscope, by averaging the number of cells in four separate grids on the haemocytometer. The average cell count was multiplied by 2x10$^4$ to obtain a cell count per ml of suspension. Each count was carried out in duplicate to obtain an average cell density per ml prior to seeding into the appropriate cell culture flask.

2.1.3 Storage and recovery of cells

For cell storage, cells were harvested and pelleted as per section 2.1.1 and any remaining supernatant was removed. Cell pellets were resuspended in cell line specific growth media supplemented with 10% foetal calf serum (FCS) (Sigma Aldrich) and 5% dimethyl sulphoxide (DMSO) (Sigma Aldrich) and transferred to a cryovial (Sarstedt, Germany). Vials were frozen at a rate of 1 °C per minute at -80 °C prior to long term storage in liquid nitrogen. Cells were recovered by rapid thawing of vials and the contents were quickly transferred to a 15 ml centrifuge tube containing 7 ml of media. Cells were then pelleted at 201 rcf and supernatant was removed.
Cell pellets were resuspended in 10 ml of normal growth media and seeded into T75 cm$^2$ flasks. After 24 hours incubation at 37 °C, media was changed to remove any excess DMSO.

2.1.4 Treatments

Prior to endocrine treatments, cells were steroid depleted in phenol red free media supplemented with 3% charcoal dextran stripped (CDS) FCS; 1% L-Glutamine (LG) (Sigma Aldrich) and 1% penicillin-streptomycin (P/S) (Sigma Aldrich) for 72 hours. For signalling experiments, cells were serum starved in phenol red free media supplemented with 1% LG and 1% P/S for the final 24 hours. LGI1 mimetic studies required daily treatment directly into growth media. Drug treatments and concentrations used in cell line experiments are shown in table 2.1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stock conc.</th>
<th>Treatment conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-hydroxytamoxifen (4-OHT)</td>
<td>$10^{-3}$ M</td>
<td>$10^{-7}$ M</td>
</tr>
<tr>
<td>LGI1 mimetic</td>
<td>$10^{-6}$ M</td>
<td>5 - 20 nM</td>
</tr>
<tr>
<td>Vehicle (LGI1 mimetic)</td>
<td>0.005 % methanol</td>
<td>0.0001 % methanol</td>
</tr>
</tbody>
</table>
2.2 Breast Cancer Cell lines:

A series of breast cancer cell lines were used to represent different breast cancer subtypes for comparative studies. These included the endocrine sensitive MCF7 cell line; the endocrine resistant LY2 cell line; the Letrozole resistant LetR cell line; the tamoxifen resistant cell line TamR; the ADAM22 deficient LY2 ADAM22 CRISPR/Cas9 knockout and the ADAM22 overexpressing LY2 lentiADAM22.

2.2.1 MCF7

The MCF7 cell line was obtained from the American Type Culture Collection (ATTC) and represents an endocrine sensitive Luminal A subtype. Cells were maintained in minimum essential media eagle (MEME) (Sigma Aldrich, Germany) supplemented with 10% FCS, 1% LG and 1% P/S.

2.2.2 LY2

The LY2 cell line was created through long term exposure of the MCF7 cell line to the anti-Estrogen LY 117018 [126] and was a gift from Professor Robert Clarke. The cell line represents a tamoxifen resistant luminal B subtype of breast cancer. Cells were maintained in phenol red free (PRF) MEM supplemented with 10 % charcoal dextran stripped (CDS) FCS (Sigma Aldrich), 1% LG, 1% PS and 10^{-8} M 4-OHT (Sigma Aldrich).

2.2.3 LY2 ADAM22 CRISPR Knockout (Clone H)

The LY2 ADAM22 CRISPR knockout cell line was created through electroporation of LY2 cells with a pool of three commercially available ADAM22 CRISPR/Cas9 knockout plasmids (Santa Cruz # sc-405581) and corresponding ADAM22 Homology Directed Repair (HDR) plasmids (Santa Cruz # sc-405581-HDR). Development of the cell line will be discussed in section 2.42.4. Cells were maintained in PRF-MEM supplemented with 10% CDS-FCS, 1% LG, 1% P/S, 4 µg/µl puromycin dihydrochloride (Santa Cruz # sc-108071) and 10^{-8} M 4-OHT.

2.2.4 LY2 lentiADAM22 overexpression cell line

The LY2 ADAM22 lentiviral overexpression cell line was created through transduction of LY2 cells with lentiviral particles containing full length ADAM22
transcript variant 1 (OriGene # RC219272L2V) as discussed in section 2.5. Cells were maintained in PRF-MEM supplemented with 10% CDS-FCS, 1% LG, 1% P/S and 10^{-8} \text{M} 4-OHT.

2.2.5 LetR

The LetR cell line is derivative of the MCF7 cells, resistant to the aromatase inhibitor letrozole. Cells were developed through long term letrozole exposure of MCF7 cells which stably express the full length aromatase gene. Cells were cultured in PRF-MEM supplemented with 10 % CDS-FCS, 1 % LG, 1% P/S, 10^{-9} \text{M} androstenedione (Sigma Aldrich), 10^{-6} \text{M} letrozole (Novartis) and 200 \mu\text{g/ml} G418.

2.2.6 TamR

The TamR cell line was created from the MCF7 cell line through exposure to 10^{-7} \text{M} 4-OHT for 6 months. Cells were maintained in MEME supplemented with 10 % FCS, 1 % LG, 1% P/S and 10^{-6} \text{M} 4-OHT.

2.2.7 LY2 luciferase

In vivo experiments were carried out using the LY2 luciferase tagged cell line (LY2\text{luc}), which stably expresses the luciferase gene along with a blastocidin resistance gene and a GFP tag. Upon reaction with luciferin, cells emit a bioluminescent signal, which allows them to be tracked in vivo using an IVIS system. Cells were maintained in PRF-MEM supplemented with 10 % CDS-FCS, 1 % LG, 1 % P/S, 2.5 \mu\text{g/\mu l} blastocidin and 10^{-8} \text{M} 4-OHT.
2.3 RNA interference:

RNA interference (RNAi) is a technique which silences gene expression in a targeted fashion. Short double stranded RNA specific to the gene of interest is introduced into the cell where it is processed into single stranded short interfering RNA (siRNA). The siRNA, through its interaction with the RNA induced silencing complex (RISC), selectively degrades the messenger RNA of the gene of interest. RNAi is an invaluable technique for studying the function of genes.

2.3.1 Transfection of ADAM22 siRNA

Cells were seeded at a density of $1.5 \times 10^5$ cells per well of a 6 well plate in antibiotic free growth media and incubated at 37°C for 24 hours prior to transfection. Cells were at approximately 40% confluence at time of transfection. Pre-validated siRNA against ADAM22 (Ambion; #4390824) and non-targeting negative control siRNA (Ambion; #4390644) were both used at final concentrations of 10 nM. Lipofectamine 2000 reagent (Life technologies) was used to facilitate entry of siRNA into the target cells using OptiMEM reduced serum media (Life technologies). 5 hours post incubation in the siRNA/ lipofectamine 2000/ Opti-MEM complex, the media was replaced with normal growth media for the duration of the experiment. For functional assays and protein/RNA extraction, cells were trypsinised 48 hours post-transfection.
2.4 CRISPR/Cas9 Knockout (KO)

The recent discovery and development of the CRISPR/Cas9 technique has allowed researchers to selectively knock out genes. CRISPR, or Clustered Regularly Interspersed Palindromic Repeats, were first identified as far back as 1987 as small palindromic DNA repeats separated by spacers in the genomes of microbes [127]. By 2005, several groups hypothesised a potential role for these CRISPR sequences in microbial adaptive immunity against pathogens [128]. These CRISPR sequences acted as guides to selectively target pathogen DNA, where the Cas9 enzyme would act as a molecular scissors to selectively cut and destroy invading pathogens. By 2013, three milestone papers had been published identifying the potential of the CRISPR/Cas9 technique for reprogramming cells [129-131]. Since then, the field of CRISPR/Cas9 genome editing has grown exponentially, offering a cheap, efficient and selective tool for molecular biologists.

The discovery and development of the CRISPR/Cas9 gene editing system has become an invaluable tool for molecular biologists to specifically target genes of interest. The CRISPR/Cas9 nuclease system specifically recognises short sequences of nucleotides (20 nucleotides) upstream of a PAM sequence (ngg) using a complementary single guide RNA strand (sgRNA), where a double stranded break is introduced by the catalytic Cas9 nuclease. A schematic of the CRISPR/Cas9 system is shown in figure 2.1. Endogenous DNA repair mechanisms will quickly ligate the double stranded break using the error prone non-homologous end joining (NHEJ), which regularly leads to genomic insertions and deletions. Alternatively, gene replacement can occur using homology directed repair (HDR).
Pre-designed selective CRISPR/Cas9 plasmids are readily available and utilise the sgRNA sequence specific to the target gene to deliver the Cas9 enzyme, where a double stranded break is then introduced and the target gene is knocked out. Successful transfection or electroporation efficiency of CRISPR/Cas9 knockout plasmids can be examined by fluorescence microscopy using a green fluorescent protein (GFP) tag. Using a target specific homology-directed repair (HDR) plasmid, which contains homologous DNA regions both upstream and downstream of the Cas9 induced double stranded break, homology directed repair can occur with the introduction of a new selection gene (such as the puromycin resistance gene) as well as a tag for clonal selection (Red Fluorescent Protein – RFP). This disruption of the reading frame of the gene leads to efficient knockout.

2.4.1 ADAM22 CRISPR knockout (KO) and homology directed repair (HDR) plasmids

Commercially available ADAM22 CRISPR/Cas9 KO plasmids (sc-405581; appendix figure 7.1) and the ADAM22 HDR plasmids (sc-405581-HDR; appendix figure 7.2) were purchased from Santa Cruz. Both the KO and HDR plasmids each contained a pool of three ADAM22 specific plasmids, each targeting a different region of the ADAM22 gene for Cas9 disruption with corresponding ADAM22 homologous
templates for successful repair of the gene. Guide sequences and predicted cut sites within the ADAM22 gene are shown in figure 2.2.

Guide 1: TGAACACTTACTGAAGTCCG (Exon 5) (CGGACTTCAAGTGTGTCCA)
ADAM22 (88108250 to 88108269) Homo sapiens chromosome 7, GRCh38.p7 Primary Assembly
mRNA variant 1: NUCLEOTIDE position 787-796
CTCCTTTGGAGACTGTAAAGCAACGTTACAGGTG
CC || TGAAGTCATCCACAAAGTCTTTCAATAAAAATGACAAAC
A AAAAAAGTTATATATATATATATACCTTAC 5’

Guide 2: GAGATGAATGACGTTCACA (Exon 3) (TTGAACGTTCATCATACT)
ADAM22 (87978376 to 87978395) Homo sapiens chromosome 7, GRCh38.p7 Primary Assembly
mRNA variant 1: NUCLEOTIDE position 609-629
TCAACTGAGTACAACTGTGCCAGGTCCAACACGG
A AAC|| TTTGCAAGTAAAGTACAGCAGCATCACGCACGATTAGTCA
TTCACTCAACTTACGAGACGGAA AT

Guide 3: AGGGAGAGCCTTTGACTACC (Exon 5)
ADAM22 (88108273 to 88108292) Homo sapiens chromosome 7, GRCh38.p7 Primary Assembly
mRNA variant 1: nucleotide position 716-736
5’ TTGTGACGGAAGACCTTACATTTTATTTCTGTTCAGG
GAGGAGAGCACTGTTACT || ACCAGGGCCATATCCGGGAA
ACCCCTGACCTAGTTGCATTTGT

5’ direction N G G = PAM sequence CRISPR guide RNA || = predicted cut site

Figure 2.2 Blast results of ADAM22 CRISPR guide RNA cut sites in ADAM22 gene.

2.4.2 Electroporation of ADAM22 CRISPR/Cas9 KO and ADAM22 HDR plasmids

LY2 cells were grown to confluency prior to harvesting. As per the recommended Amaxa® cell line nucleofector® kit v protocol (Lonza), a cell suspension of 2X10^6 cells were centrifuged at 90 rcf for 10 minutes and the supernatant was aspirated. The pellet was then resuspended in 100 µl of nucleofector V solution (Lonza) and 2 µg each of the ADAM22 CRISPR/Cas9 KO plasmid (sc-405581) and the ADAM22 HDR plasmid (sc-405581-HDR) were added. The plasmid cell suspension was gently mixed by pipette and transferred into a sterile nucleofector cuvette. Both the CRISPR KO and HDR plasmids were electroporated into the LY2 cells using preset high transfection efficiency programme on a Lonza Nucleofector 2b device. The cells
were quickly transferred into pre-warmed growth media in a 6 well plate and incubated for 72 hours at 37 °C. At this time point, transfection efficiency was determined via GFP and RFP co-expression by fluorescent microscopy. LY2 ADAM22 CRISPR KO cells were cultured for 6 passages prior to puromycin selection for 5 days at a concentration of 8 μg/ml.

2.4.3 Clonal selection of LY2 ADAM22 CRISPR KO cells by fluorescently activated cell sorting (FACS)

As mammalian cells contain two copies of each gene, there were two possible subpopulations within the LY2 ADAM22 CRISPR KO cell line: mono-allelic CRISPR knockout or bi-allelic CRISPR knockout. Mono-allelic knockout cells would still carry a functional ADAM22 gene, whereas bi-allelic knockout would ensure non-functional ADAM22. In order to ensure ADAM22 knockout, the LY2 ADAM2 CRISPR KO cells were FAC sorted as single cells and expanded. All FACS experiments were carried out with Dr Sara Charmsaz.

For FACS experiments, parental LY2 cells were used as a gating control for size and single cellularity, as well as a negative control for RFP expression. LY2 and LY2 CRISPR/Cas9 KO cells were harvested and pelleted by centrifugation and supernatant was removed. Pellets were resuspended in 1 ml of sterile filtered FACS buffer (5 % FCS in PBS) and passed through a 70 μm cell strainer into a sterile FACS tube to obtain a single cell suspension. Cells were loaded into a FACS Aria II and gated by size, single cellularity and finally RFP expression. Single cells were seeded into 96 well plates containing 200 μl of PRF-MEM supplemented with 20% CDS-FCS, 1% LG and 2% P/S. Cells were cultured until observable colonies had formed prior expansion into larger culture flasks. Clone H was used for all future studies.

2.4.4 Genotyping ADAM22 CRISPR/Cas9 clones (Discovery of Clone H)

In order to determine whether successfully expanded ADAM22 knockout clones contained mono-allelic or bi-allelic disruption, primers were designed to flank each of the three sgRNA cut sites within the ADAM22 gene. Parental LY2 cells were expected to contain one band for each set of primers; while two bands were expected for mono-allelic ADAM22 knockout; and one band of larger product size for
bi-allelic ADAM22 knockout (see figure 2.3). Primers were designed using OligoPerfect (ThermoScientific) online primer design tool. As the ADAM22 guide 1 and guide 3 cut sites were in close proximity, one set of primers were sufficient to cover both cut sites. ADAM22 CRISPR gDNA primer information is displayed in table 2.2.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>GC content</th>
<th>Length</th>
<th>Tm ° C</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>gDNA 2 Fwd: ATTGGTGAATGCTTGGCTTC</td>
<td>45 %</td>
<td>20 bp</td>
<td>60.8</td>
<td>709 bp</td>
<td></td>
</tr>
<tr>
<td>Rev: CACCACCACATCAAAGCATC</td>
<td>45 %</td>
<td>20 bp</td>
<td>59.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gDNA 1&amp;3 Fwd: TGTGGAGGTGCATTGTGAGT</td>
<td>50 %</td>
<td>20 bp</td>
<td>60.16</td>
<td>370 bp</td>
<td></td>
</tr>
<tr>
<td>Rev: CGACCATTGTGCAAACCTAAT</td>
<td>45 %</td>
<td>20 bp</td>
<td>59.82</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total genomic DNA was extracted from parental LY2 and LY2 ADAM22 CRISPR KO Clone H using a DNeasy blood and tissue kit (QIAGEN) as per section 2.10.1. DNA concentration was determined using a NanoDrop spectrophotometer 2000c (ThermoFisher Scientific). Prior to polymerase chain reaction (PCR), DNA was diluted 1 in 10 to a concentration of approximately 20-30 ng/μl. PCR amplification was carried out using a Phusion High Fidelity PCR kit (New England Biology) as per section 2.10.2.

PCR products were run on a 1% agarose gel containing SYBR Safe (Thermo Scientific) for 1 hour at 100 volts as per section 2.10.3. Gels were imaged under UV light to detect PCR products, using a 100 base pair molecular weight ladder to estimate product size.

Figure 2.3 Schematic of potential outcomes for CRISPR induced mutations
2.4.5 Sequencing ADAM22 CRISPR/Cas9 clone H

In order to validate insertions and deletions in clone H, PCR products were purified and prepared for sequencing as per section 2.10.5 using a Big Dye Terminator v.1.1 Cycle Sequencing Kit (Thermo Fischer). Sequencing was carried out at the Queens University Belfast Genomics Core Technology Unit.

2.4.6 Analysis of sequencing results

Analysis and alignment of parental and knockout clone sequencing data was carried out using Ape software and confirmed using nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5 ADAM22 lentiviral overexpression

In order to establish an ADAM22 overexpressing cell line, lentiviral particles, containing recombinant ADAM22 variant 1 with a GFP c-terminal fusion tag, were purchased from OriGene / AMSBIO (#RC219272L2V; appendix figure 7.3). Variant 1 is the predominant ADAM22 variant expressed in the brain, with a fully intact extracellular component and cytoplasmic tail [133]. Lentiviral particles are easily incorporated into host cell genomes allowing for ectopic overexpression of target proteins.

2.5.1 Lentiviral transduction

All lentiviral work was carried out in a BSL2 tissue culture hood and an incubator dedicated to lentiviral work. Cells were seeded at a density of 5 X 10^4 per well of a 24 well plate and cultured overnight (O/N) at 37 °C. The following day, the media was replaced with normal growth media supplemented with 8 μg/ml of polybrene (Sigma). Ranges of multiplicity of infections (MOI) from 1 to 8 were tested per cell line to obtain optimal transduction efficiency. MOI was calculated as follows:

Total number of cells per well x Desired MOI = Total transducing units needed (TU)

Total TU needed / (TU/ml) = Total ml of lentiviral particles to add to each well

lentiADAM22 viral particles: 4 x 10^7 TU/ml

e.g 25000 (cells) X 8 (MOI) = 200000 (Total TU)

200000 / 40000000 = 0.005ml = 5 μl
Transduced cells were cultured for 72 hours and transduction efficiency was determined via GFP expression and fluorescence microscopy. Cells transduced with an MOI of 8 had the highest expression of GFP and were pooled together and seeded into a T25 cm\(^2\) flask for bulking.

### 2.5.2 Purification of LY2 lentiADAM22 overexpressing cells

In order to obtain a pure population of LY2 lentiADAM22 overexpressing cells, cells were bulked in a T175 cm\(^2\) flask and harvested. Cells were pelleted and resuspended in sterile filtered FACS buffer. A single cell suspension was then created by passing cells through a 70 µM cell strainer into a sterile FACS tube. Cells were kept on ice prior to sorting. Parental LY2 cells were used as a control for size and single cellularity and as a negative control for GFP expression. A high GFP expressing population of LY2 lentiADAM22 cells were purified and sorted into a collection tube containing FACS buffer. Cells were centrifuged for 3 minutes at 201 rcf and supernatant was removed. Cells were resuspended in PRF-MEM supplemented with 10 % CDS-FCS, 1% LG, 1% P/S and 10\(^{-8}\)M 4-OHT and seeded into T75 cm\(^2\) flasks.

### 2.5.3 Validation of ADAM22 overexpression

ADAM22 overexpression was validated by western blot (as per section 2.7.3) and real time PCR (as per section 2.9.4).
2.6 Functional Assays:

2.6.1 Cell Motility Assay

Cell motility is a key characteristic of invasive cancer and metastasis. As such, the ability to measure the motility of cancer cells in vitro is essential for studying metastatic potential. The Cellomics® cell motility kit (Pierce, Il, USA) was thus used to investigate the role of ADAM22 in cell motility in the LetR cell line.

The wells of a 96 well plate were coated with 100 μl collagen (BioData Corporation, PA, USA) in 0.02M acetic acid and incubated at room temperature (RT) for 1 hour. Plates were washed twice with sterile PBS and 75 μl of blue fluorescent microspheres were added to each well. The plate was then incubated in the dark at 37°C for 1 hour. LetR cells were harvested, counted and a cell suspension of $10^4$ cells/ml was prepared. The wells were then aspirated and the plate was washed 4 times with PBS. 50 μl of cell suspension was then added to each well (500 cells/well). 50 μl of drug treatments were then added at double concentration with appropriate negative controls. Cells were then incubated for 22 hours at 37°C. 200 μl of 5% formaldehyde in PBS was added to each well for 1 hour at RT. The fixation solution was aspirated and 100 μl of permeabilisation buffer was added to each well and incubated for 15 minutes. The permeabilisation buffer was removed and 100 μl of staining solution (55 μl Rhodamine Phalloidin in 11 ml PBS) was added for 30 minutes. The staining solution was aspirated and the plate was washed 3x with 200 μl PBS. 200 μl of PBS was added and the plate was sealed and stored in tinfoil at 4°C for analysis. Cells were imaged using an inverted microscope under DAPI and FITC filters before cell motility was measured using Cell Sens software. Approximately 10 images were taken per well (~ 5 cells per image). Motility/migration was calculated by subtracting the average cell size from the average area cleared.

2.6.2 Anchorage Independent Growth Assay

Anchorage independent growth is a phenotypic characteristic of metastatic cancer cells, resulting from their ability to overcome a form of programmed cell death called anoikis [134].

300 mg of low melt agarose (Promega) was dissolved in 50 ml of cell line specific media at 65°C to make a 0.6% agarose solution. 2 ml of 0.6% agarose media was
added to each well of a 6 well plate and left at RT for 30 minutes to solidify as a base layer. The remaining agarose was kept at 37°C in the incubator until further use. Cells were trypsinised, counted and a suspension of 4x10^5 cells/2 ml of pre-warmed cell line specific media were made containing any drug treatments required. A 0.3% agarose containing the cell suspension and treatments was added to the solidified 0.6% base layer. Each cell line and treatment was carried out in duplicate (i.e. 2x10^5 cells/2ml). The middle layer was left to solidify at RT for 30 minutes before 300μl of media with any treatments was carefully added to each well. Media was replenished every 2-3 days. Plates were incubated for 14 days at 37 °C to allow colonies to form. Cells were then stained with 400 μl of p-iodinitroetrazolium chloride (1 mg/ml) per well. Plates were then incubated for a further 24 hours before being wrapped in tin foil and left @ 4°C until stain was clearly visible. Colonies of >50 μm in size were counted from 8 different points in each well, using a graticule for measurement. An average of the total number of colonies observed between each duplicate was used for analysis. Average colony size was measured from approximately 50 colonies per sample.

2.6.3 Mammosphere formation assay

The mammosphere formation assay is an invaluable tool for assessing in vitro cancer stem cell activity and self-renewal [135]. Cancer stem cell activity plays a key role in the development of intrinsic resistance to therapy and consequent recurrence [136].

Mammosphere media was made in advance of each experiment as follows: DMEM/F12 (Gibco) was supplemented with B27 (1x), recombinant epidermal growth factor (rEGF, 100 ng/ml) and 4-OHT (10^-8 M). Cells were trypsinised and pelleted as per section 2.1.1. Supernatant was removed and cell pellets were resuspended in ice cold PBS. In order to obtain a single cell suspension, cells were passed through a 25G needle three times. 2 ml of pre-warmed mammosphere media was added to each well of low adherence 6 well plates. Cells were counted and seeded at a final density of 5000 cells per well in duplicate. Cells were then incubated at 37 °C in a humidified 5% (v/v) CO₂ atmosphere without disturbing for 5 days. For LGI1 efficacy studies, either vehicle (0.005% MeOH) or LGI1 mimetic (20nM) was added directly to
each well daily. Total number of mammospheres > 50 µm were counted per well and the mammosphere forming efficiency (MFE) was calculated, as follows.

**Average number of mammospheres/ number of cells seeded X 100**

In order to passage mammospheres to examine 2nd generation mammosphere formation, all cells were collected from wells after counting 1st generation mammospheres. Each well was washed with ice cold PBS and this was added to the corresponding collection tube. Cells were then centrifuged at 115 rcf for 5 minutes. Supernatant was removed and cells were resuspended in 200 µl of trypsin/EDTA. Cells were incubated at 37 °C for 3 minutes before the reaction was neutralised in 600 µl of growth media containing serum. A single cell suspension was achieved by passing the cells through a 25 G needle three times. Cells were counted and seeded at 5000 cells per well of low adherence 6 well plates. Cells were cultured for 5 days. Total number of mammospheres > 50 µm were counted per well and the mammosphere forming efficiency (MFE) was calculated.
2.7 Protein Biochemistry:

2.7.1 Protein Extraction

2.7.1.1 Total cell lysate extraction
Cells were harvested by trypsinisation and centrifugation at 201 rcf. The supernatant was discarded and the cell pellets were resuspended in ~40-100 μl of radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with a protease inhibitor cocktail (Sigma) and phosphatase inhibitors (PhosStop; Roche) to prevent protein degradation. To extract the protein, the resuspended pellets were vortexed for 10 seconds and incubated on ice for 10 minutes three times followed by centrifugation at 20817 rcf for 20 minutes. The supernatant was then transferred to a labelled 1.5 ml tube and stored at -20 °C.

2.7.1.2 Non-denaturing extraction
For interaction studies, a non-denaturing extraction buffer was used (50mM TrisHCl (pH 7.5), 150mM NaCl and 1 % NP-40). Cells were scraped in growth media, collected and centrifuged at 300 rcf for 5 minutes. Supernatant was discarded and pellets were resuspended in 5ml ice cold PBS. Following centrifugation at 300 rcf for 5 minutes, the supernatant was removed and pellets were resuspended in NP-40 lysis buffer. Protein was extracted as per section 2.7.1.1. The supernatant was then transferred to a labelled 1.5 ml tube and stored at -20 °C.

2.7.1.3 Membrane and cytosol extraction
In order to assess membrane specific expression, protein was extracted using the Mem-PER™ Plus membrane extraction kit (Thermo Fisher Scientific). Cells were cultured in 15 cm² culture flasks (Corning). At 75 - 80 % confluency (5 X 10⁶ cells), cells were scraped in growth media using a cell scraper and collected into a 50 ml tube. Cell suspension was centrifuged at 300 rcf for 5 minutes, supernatant was carefully removed and cell pellets were resuspended in 3 ml of Cell Wash Solution (from kit). Cell suspension was centrifuged at 300 rcf for 5 minutes and supernatant was removed. Pellets were resuspended in 1.5 ml of Cell Wash Solution and transferred to a 2ml centrifuge tube. Cell suspension was centrifuged at 300 rcf for 5 minute and supernatant was removed. Cell pellets were resuspended in 750 μl of Permeabilisation Buffer (from kit) and vortexed briefly. Cells were incubated on ice
for 10 minutes with intermittent vortexing every 2 minutes. Permeabilised cells were centrifuged at 16000 rcf for 15 minutes at 4 °C. Supernatant containing cytosolic proteins were carefully collected into labelled tubes. Remaining cell pellets were resuspended in 500 µl of Solubilisation Buffer (from kit) by pipette. Solubilised cells were incubated on ice for 30 minutes with intermittent vortexing every 2 minutes. Solubilised cells were centrifuged at 16000 rcf for 15 minutes at 4 °C. Supernatant containing membrane and membrane associated proteins were collected into new labelled tubes. All protein lysates were stored at -80 °C.

### 2.7.2 Protein Quantification

Protein was quantified by the Bicinchoninic Acid (BCA) assay (Pierce). A range of albumin protein standards were first prepared from 0-1400 μg/ml. Samples were then diluted 10-fold to a total volume of 80 μl. 25 μl of standards/samples were pipetted per well in triplicate onto a 96 well plate (Greiner Bio-One). The BCA working reagent was then prepared in a 49:1 ratio of reagent A: reagent B. 200 μl of the working reagent was added to each well and the plate was left in the incubator @ 37 °C for 30 minutes wrapped in tin foil. The absorbance was then measured @ 560 nm. Protein concentrations were calculated from the standard curve of the albumin standards.

### 2.7.3 Western Blotting

The western blot technique allows for the separation of proteins according to size via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins are then transferred onto a nitrocellulose membrane. Specific antibodies can then be used to detect the protein of interest.

Gels were prepared manually in ATTO gel cast plates (Atto Corporation, Tokyo, Japan) as per Table 2.3. After preparing the resolving gel, a thin layer of isopropanol was added until it had set. The isopropanol was then removed, the stacking gel was added and a 1.5mm comb was inserted to mould the wells. The gel was then placed in an ATTO electrophoresis tank and filled with 500 ml of 1X running buffer. All samples were prepared at equal concentrations and mixed with laemmli buffer. The samples were then heated at 95 °C for 5 minutes before loading on the gel. A molecular weight marker (Spectra Multicolour Broad Range Protein Ladder;
ThermoScientific) was used as a reference for protein size. The gel was run for 2 hours and 15 minutes at 130V. The gel was then transferred by the semi-dry method. Briefly, 10 sheets of filter paper and a sheet of nitrocellulose were pre-soaked in semi-dry transfer buffer and the gel was orientated between them as seen in *figure 2.4*. The transfer was run at 250mA for 1 hour.

<table>
<thead>
<tr>
<th>Table 2.3: Preparation of resolving and stacking gels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resolving gel</strong></td>
</tr>
<tr>
<td><strong>12%</strong></td>
</tr>
<tr>
<td>H2O</td>
</tr>
<tr>
<td>30% acrylamide</td>
</tr>
<tr>
<td>1.5M Tris (pH 8.8)</td>
</tr>
<tr>
<td>1M Tris (pH6.8)</td>
</tr>
<tr>
<td>10% SDS</td>
</tr>
<tr>
<td>10% APS</td>
</tr>
<tr>
<td>TEMED</td>
</tr>
</tbody>
</table>

*Figure 2.4 Orientation of filter paper, gel and nitrocellulose for semi dry transfer*

Membranes were incubated in ponceau stain (SigmaAldrich) to visualise transfer of proteins. The membrane was then blocked in 5% bovine serum albumin (BSA) in Tris buffer saline 0.001% Tween (TBST) for 1 hour at RT. The membrane was then incubated O/N with target primary antibody (see *table 2.4*) in 5% BSA TBST. The membranes were then washed three times with TBST for 10 minutes before incubation in a horseradish peroxide conjugated species specific secondary antibody for 1 hour at RT. The membranes were washed again in TBST three times. The membrane was covered with enhanced chemiluminescence (ECL) substrate (Pierce) for 1 minute before visualisation on an Amersham Imager 600 (GE Healthcare Life Sciences)
### Table 2.4: Antibody list for western blot analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Dilution</th>
<th>Species</th>
<th>2° dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM22</td>
<td>Santa Cruz (#sc-373931)</td>
<td>1:500</td>
<td>Mouse</td>
<td>1:3000</td>
</tr>
<tr>
<td>ADAM22</td>
<td>R&amp;D systems (#MAB49081)</td>
<td>1:1000</td>
<td>Mouse</td>
<td>1:3000</td>
</tr>
<tr>
<td>phospho-P42/P44 MAPK</td>
<td>Cell signalling (#4377)</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>1:3000</td>
</tr>
<tr>
<td>(ERK1/2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P42/44 MAPK (ERK1/2)</td>
<td>Cell Signalling (#9102)</td>
<td>1:1000</td>
<td>Mouse</td>
<td>1:3000</td>
</tr>
<tr>
<td>AKT</td>
<td>Cell Signalling (#2920)</td>
<td>1:1000</td>
<td>Mouse</td>
<td>1:3000</td>
</tr>
<tr>
<td>FAK</td>
<td>Cell Signalling (#13009)</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>1:3000</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Cell Signalling (#2479)</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>1:3000</td>
</tr>
<tr>
<td>β-ACTIN</td>
<td>Sigma Aldrich (#A2228)</td>
<td>1:5000</td>
<td>Mouse</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

### 2.7.4 LGI1 mimetic / ADAM22 interaction studies

#### 2.7.4.1 Biotin-linked LGI1 mimetic pull down of ADAM22

In order to determine LGI1 mimetic interaction with ADAM22, Dynabeads™ magnetic technology was used to capture interacting proteins from a biotin-tagged LGI1 mimetic (JPT Technologies, Germany) (see appendix figure 7.5 for datasheet).

LY2 lentiADAM22 cells were seeded into 15 cm² tissue culture dishes (Corning) and grown to approximately 80 % confluency. Cells were scraped in growth media, collected into 50 ml collection tubes and centrifuged at 300 rcf for 5 minutes. Supernatant was removed and resuspended in 5ml of ice cold PBS. Cells were centrifuged at 300 rcf for 5 minutes. PBS was removed and cells were resuspended in a NP-40 lysis buffer as per section 2.7.1.2 in order to maintain native protein structure. Lysates were quantified as per section 2.7.2. Lysates were then precleared using 10 μl of Dynabeads™ M-280 Streptavidin for 1 hour at 4 °C with rotation. Dynabeads™ were removed using a Dynabeads™ magnetic rack. 500 μg of protein lysate was then mixed gently by rotation with either 50 μg of biotinylated LGI1 or an equivalent volume of PBS (as a no bait control) at 4 °C O/N. The following day 50 µl of Dynabeads™ M-280 Streptavidin beads were aliquoted into two 2 ml tubes and washed three times with ice cold PBS (pH 7.4). After the final wash, PBS was removed and the pre-incubated protein lysates were added and incubated O/N at 4 °C on a roller. The following day, protein bound beads were isolated using a magnet rack and input lysates were collected into labelled tubes and stored for later analysis. The beads were washed three times in NP-40 lysis buffer, followed by three times with ice cold PBS supplemented with 0.5 M NaCl. After the
final wash, Dynabeads™ were isolated and all supernatant was completely removed. Dynabeads™ were resuspended in 20 µl of 1x laemmli buffer and heated at 95 °C for 5 minutes. Bait and prey proteins were dissociated from the Dynabeads™ by vortex for 15 seconds. Using a magnet rack, beads were isolated and supernatant was collected into labelled tubes for western blot analysis.

2.7.4.2 ADAM22 LGI1 mimetic in silico interaction
All in silico work was carried out by Nicola Cosgrove. Briefly, the ADAM22 and LGI1 mimetic predicted 3D structures were obtained from the I-TASSER server [137]. Molecular docking studies were then carried out using the CABS-dock server, using a high quality prediction of < 3 Ångströms [138].

2.7.5 Immunohistochemistry (IHC)

Immunohistochemistry is an invaluable technique for the detection of endogenous proteins in their native tissue structures. A standardised IHC protocol was optimised and adapted to suit each antibody tested (see table 2.5).

Following ethical approval ADAM22 expression was examined in clinical samples, using tissue microarrays (TMA) consisting of 0.6 mm diameter tumour cores from breast cancer patients from St Vincent’s Hospital Dublin (n=647) and Beaumont Hospital Dublin (n=508). Extensive follow up data for each patient was collected in order to determine patterns in disease free survival. Matched primary and metastatic tissues were taken from patients from the above cohorts with approval. Post mortem healthy tissue sections were obtained from Beaumont Hospital.

All staining was carried out using the Dako En-Vision and HRP Kit (Mouse). 5 µm sections of paraffin embedded tissue or tissue microarrays were baked at 65 °C for 6 hours followed by de-paraffinisation and rehydration as follows: xylene (2x 3 minutes); 100% industrial methylated spirits (IMS) (2x 3 minutes); 70% IMS (3 minutes); and finally PBS (5 minutes). Heat induced antigen retrieval, optimised for each antibody used, was carried as shown in table 2.5.
### Table 2.5: IHC primary antibody conditions

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company (cat#)</th>
<th>Species</th>
<th>Concentration</th>
<th>Antigen retrieval</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM22</td>
<td>Santa Cruz (sc-373931)</td>
<td>Mouse</td>
<td>1 μg/ml</td>
<td>EDTA (pH 9) 20 min</td>
<td>O/N 4 °C</td>
</tr>
<tr>
<td>ADAM22</td>
<td>R&amp;D systems (MAB49081)</td>
<td>Mouse</td>
<td>1 μg/ml</td>
<td>EDTA (pH 9) 20 min</td>
<td>1 hr RT</td>
</tr>
<tr>
<td>IgG1</td>
<td>Dako (XO931)</td>
<td>Mouse</td>
<td>1 μg/ml</td>
<td>EDTA (pH 9) 20 min</td>
<td>O/N 4 °C</td>
</tr>
</tbody>
</table>

Samples were allowed to cool to RT for 30 minutes, before washing with TBST for 5 minutes. A waterproof boundary was marked around tissue samples prior to a 5 minute incubation with peroxidise blocking solution (Dako En-Vision Kit). Tissues were washed in TBST for 5 minutes before incubation with primary antibody. Excess primary antibody was washed off with three 5 minute TBST washes before incubation with the HRP labelled secondary antibody (Dako) for 30 minutes at RT. Tissues were washed three times with TBST before development with a DAB substrate (Dako) for a minimum of 2 minutes. Tissues were washed in deionised H_{2}O for 5 minutes before counterstaining with Mayers haemotoxilin for 5 minutes. Tissues were then washed in deionised H_{2}O for 5 minutes. The tissue slides were dehydrated again as follows: 3 minutes in 70% IMS; 2x 3 minutes in 100% IMS; and 2x 3 minutes in 100% Xylene. The slides were mounted with coverslips using DPX mountant and left to dry before analysis. IHC images were captured using an inverted Olympus IX51 light microscope and CellSens software.

TMA scoring was carried out by two independent researchers using the histoscore method:

\[
\text{Histoscore} = \text{staining intensity (0-3) x percentage of cells (0-100)}
\]

### 2.8 Reverse Phase Protein Array

Reverse Phase Protein Array (RPPA) is a high throughput antibody based array which offers the possibility of detecting multiple signal transduction proteins in both phosphorylated and un-phosphorylated forms from a minimal amount of source material [139]. As such, it has broad applications in the mechanistic modelling of disease [139]. RPPA studies were carried out at the RCSI RPPA core facility in Beaumont Hospital Dublin.
Two independent RPPA studies were carried out for this project, utilising both transient ADAM22 siRNA knockdown and CRISPR/Cas9 ADAM22 knockout to measure and elucidate ADAM22 dependent response to tamoxifen treatment in the LY2 cell line.

### 2.8.1 Transient knockdown study
LY2 cells were seeded at a density of $1.5 \times 10^5$ cells / well in antibiotic and steroid free media and incubated O/N at 37˚C. Cells were then transfected with either 10nM non-targeting (NT) siRNA or 10 nM ADAM22 siRNA (as per section 2.3.1) and cultured for 48 hours in steroid free PRF-MEM supplemented with 10% CDS-FCS and 1 % LG. 48 hours post transfection, cells were either left untreated or were treated with $10^{-7}$ M 4-OHT (in PRF-MEM as above) for 15 minutes. Cells were harvested as per sample preparation protocol (section 2.8.3). Three replicates were used for this study. Expression levels of 66 proteins were examined.

### 2.8.2 ADAM22 CRISPR knockout study
LY2 and LY2 ADAM22 knockout Clone H cells were seeded in PRF-MEM (3 % CDS-FCS, 1 % LG and 1% P/S) and cultured for 72 hours. Cells were then serum starved for the final 24 hours in PRF-MEM containing 0 % CDS-FCS. Cells were treated with $10^{-7}$M 4-OHT in serum free media for 15 minutes prior to lysis. Cells were harvested as per sample preparation protocol (section 2.8.3). Four replicates were used for this study. Expression levels of 89 proteins were examined.

### 2.8.3 Sample preparation protocol
Media was removed and the plates were kept on ice (Note: Plates kept on ice for duration of lysis and extraction process). Each well was washed twice with ice cold PBS and 150 μl of RPPA lysis buffer (1% Triton X-100, 50mM HEPES, pH 7.4, 150mM NaCl, 1.5mM MgCl₂, 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM Na₃VO₄, 10% glycerol, containing freshly added protease and phosphatase inhibitors from Roche Applied Science Cat. # 05056489001 and 04906837001, respectively) was added. Plates were incubated on ice for 20 minutes with occasional shaking. Cells were then scraped and collected into 1.5 ml collection tubes, centrifuged at 20817 rcf for 10 minutes and the supernatant was collected in
fresh labelled tubes. Protein concentration was quantified as per section 2.7.2 and adjusted to 1 μg/μl in RPPA lysis buffer and 4X SDS sample buffer (40 % Glycerol, 8 % SDS, 0.25 M Tris-HCL, pH 6.8. Bond-Breaker TCEP Solution (Pierce - cat #77720) was added at 1/10 of the volume.

Cell lysates were then two-fold serial diluted for 4 dilutions (undiluted to 1:8 dilution). Lysates were then spotted on nitrocellulose-coated FAST slides (Whatman, Schleicher & Schuell BioScience, Inc., Keene, NH) by a Genetix QArray2 spotter. Samples were probed by the CSA amplification approach and visualised by DAB colorimetric reaction. Slides were then scanned on a flatbed scanner and spots were identified and their density was quantified by MicroVigene. All the data points were normalised for protein loading and transformed to a linear value before final analysis. (see appendix table 7.1 for list of RPPA validated antibodies)

2.8.4 RPPA pipe analysis

RPPA analysis was carried out using the online RPPApipe analysis tool (http://www.cogsys.cs.uni-tuebingen.de/software/RPPApipe) [140]. The RPPA dataset was first formatted according to the online protocol, to include gene symbols and modifications and saved as a text document. After uploading to the RPPApipe server, biological replicates were assigned to defined sample groups (e.g LY2 vs Clone H). These sample groups were then mean-centred and processed using a linear model (LIMMA) statistical method to determine differentially expressed genes between the two subgroups, which were displayed as fold differences between the two groups with associated p-values.

2.8.5 KEGG functional annotation

In order to examine ADAM22 associated pathways, upregulated proteins in the parental LY2 cell line were assigned unique UniProt accession numbers and uploaded to the DAVID functional annotation tool (https://david.ncifcrf.gov/summary.jsp) [141].
2.9 Gene Expression Analysis

2.9.1 RNA Extraction

The RNeasy Mini Kit (Qiagen) was used to extract total RNA from cell pellets. Cells were trypsinised and harvested as per section 2.1.1 and stored in -80 °C until ready for use. To extract RNA 350 µl of RLT buffer containing 1% β-mercaptoethanol was added to each cell pellet and vortexed. An equal volume of 70% ethanol was added and mixed well by pipette. The total lysate (700 µl) was transferred to an RNeasy spin column in a 2 ml collection tube. Samples were centrifuged at RT for 15 seconds @ 10621 rcf and the flow through was discarded. 350 µl of RW1 buffer was then added to wash the spin column and the samples were centrifuged again for 15 seconds @ 10621 rcf and the flow through was discarded. 140 µl of RDD buffer was added to 20 µl aliquots of DNase and mixed by inversion. 80 µl of the DNase incubation mix was then added to each spin column and left to incubate at RT for 15 minutes. 350 µl of RW1 buffer was used to wash the spin column through centrifugation and the flow through was discarded. 500 µl of RPE buffer was added to the column and was centrifuged for 2 minutes @ 10621 rcf. The spin column was then transferred to a new collection tube and centrifuged @ 20817 rcf for 1 minute. The spin column was transferred to a new 1.5 ml collection tube and 30 µl of RNase-free water was added. A final spin @ 10621 rcf for 1 minute was performed and the flow through containing RNA was stored @ -80 °C.

2.9.2 Nucleic acid quantification

Nucleic acid quantification was carried out using a NanoDrop 2000c instrument and NanoDrop 2000 software. Before use, the NanoDrop arm was lifted and the sample pedestal was cleaned using Whatman lens cleaning tissue (SigmaAldrich). The arm was replaced and a routine wavelength check was carried out. A blanking sample (2 µl of elution buffer for DNA/RNA) was then carefully pipetted onto the pedestal and set as the “Blank”. DNA or RNA samples were then measured by pipetting 2 µl of sample directly onto the sample and clicking measure. Concentrations are given as ng/µl, while 260/280 and 260/230 ratios are given to assess RNA/DNA quality and contamination with solvents respectively.
2.9.3 complementary DNA (cDNA) synthesis

For cDNA synthesis, 1000 ng of RNA per sample was used. First, RNA was primed using 1 µl of random hexamers and 1 µl of dNTP mix from a superscript III First Strand Synthesis Kit (Life Technologies) to a total volume of 10 µl in nuclease free H20 in labelled PCR tubes. This was incubated @ 65˚C for 5 minutes in a thermal cycler. For cDNA synthesis, the appropriate amount of superscript III mastermix was prepared. 10 µl was added to each sample (as per table 2.6). Samples were loaded into the thermal cycler and a thermal cycle programme was run as per manufacturer’s instructions (table 2.7).

Table 2.6: Superscript III first strand synthesis mastermix

<table>
<thead>
<tr>
<th>Mastermix</th>
<th>1X</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RT Buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>50mM MgCl2</td>
<td>2 µl</td>
</tr>
<tr>
<td>0.1M DTT</td>
<td>2 µl</td>
</tr>
<tr>
<td>RNase OUT</td>
<td>1 µl</td>
</tr>
<tr>
<td>Superscript III</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>RNase free H20</td>
<td>2.5 µl</td>
</tr>
</tbody>
</table>

Table 2.7: Thermal cycle conditions cDNA synthesis

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 ° C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>50 ° C</td>
<td>50 minutes</td>
</tr>
<tr>
<td>85 ° C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>4 ° C</td>
<td>∞</td>
</tr>
</tbody>
</table>

2.9.4 Real Time PCR (RT-PCR)

Target gene expression was quantified using a Roche LightCycler Real-time PCR machine with SYBR green reagents (QIAGEN). cDNA and predesigned forward and reverse primers for ADAM22 and ACTIN were thawed and diluted 1 in 10 (see table 2.8) . All cDNA, primers and reagents were kept on ice. A SYBR green mastermix was made up as per table 2.9. A separate mastermix was prepared for each gene target (e.g. 1 for ADAM22 and 1 for ACTIN).
Table 2.8: RT-PCR primer information

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Product size</th>
<th>Tm (°C)</th>
<th>GC content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM22</td>
<td>Fw: AGAGGAAGGAAAACCGCTTC</td>
<td>261</td>
<td>58</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Rev: TGCTCCATGTTCATAATGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTIN</td>
<td>Fw: CGGCATCGTCACCAACTG</td>
<td>72</td>
<td>59</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Rev: GCCACACGCAGCTCATTG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.9: SYBR green mastermix

<table>
<thead>
<tr>
<th></th>
<th>Mastermix</th>
<th>1 X</th>
<th>SYBR green</th>
<th>10 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H2O</td>
<td>6 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward Primer</td>
<td>1 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse Primer</td>
<td>1 µl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each LightCycler capillary (Roche) was carefully loaded with 18 µl of appropriate mastermix and 2 µl of cDNA. Three technical replicates were performed for each sample. The capillaries were capped and centrifuged at low speed briefly before loading into the LightCycler machine. The thermal cycling conditions used are shown in table 2.10. Relative gene expression was calculated using the comparative delta Ct method [142].

Table 2.10: LightCycler conditions

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95 °C</td>
<td>15 minutes</td>
<td>1</td>
</tr>
<tr>
<td>94 °C</td>
<td>15 seconds</td>
<td></td>
</tr>
<tr>
<td>61 °C</td>
<td>20 seconds</td>
<td>40</td>
</tr>
<tr>
<td>72 °C</td>
<td>20 seconds</td>
<td></td>
</tr>
<tr>
<td>65 °C</td>
<td>15 seconds</td>
<td>1</td>
</tr>
<tr>
<td>40 °C</td>
<td>30 seconds</td>
<td>1</td>
</tr>
</tbody>
</table>
2.9.5 ADAM22 gene expression studies

2.9.5.1 ADAM22 expression in matched primary and metastatic tissue

RNA extraction was carried out in the lab by Dr Damir Varešlija with samples sequenced at BGI, China.

In order to interrogate the role ADAM22 plays in metastatic development, ADAM22 gene expression was examined in an RNA sequencing (RNAseq) dataset consisting of: 7 primary tumours with no metastases; 3 matched primary and liver metastases; 6 matched primary and brain metastases (+1 unmatched brain metastases); 4 matched primary and bone metastases (+1 unmatched bone metastases); and 3 matched primary and local metastases.

Eligible breast cancer patient cases had formalin-fixed paraffin-embedded (FFPE) tissue from primary and recurrent tumours. This study was reviewed and approved by Institutional Review Boards from Royal College of Surgeons in Ireland (13/09 CTRIAL-IE Protocol 09-07).

ADAM22 expression was also examined in 21 matched primary breast and brain metastases [26]. FFPE sections underwent dual DNA/RNA extraction using Qiagen’s AllPrep kit according to manufacturer’s instructions. Library preparation was performed using 100 ng of RNA and Illumina’s TruSeq RNA Access Library Preparation protocol. Indexed, pooled libraries were then sequenced on a High Output flow cell with an Illumina NextSeq 500 (paired-end reads, 2 X 75 bp). A target of 25-50 million reads per sample was used to plan indexing and sequencing runs. An additional 15 matched samples were processed using Illumina’s TruSeq Ribo-Zero library preparation protocol. Indexed, pooled libraries were then sequenced with an Illumina HiSeq 2000 (paired-end reads, 2 X 90 bp). A target of 30-50 million reads per sample was used to plan indexing and sequencing runs.

FASTQ files were quantified using k-mer based lightweight-alignment (Salmon v0.7.2, quasi-mapping mode, 31-kmer index established from GRCh38 Ensembl v82 transcript annotations, seqBias and gcBias corrections) [143]. Read counts and percentage alignment were calculated. Transcript abundance estimates were collapsed to gene-level values using tximport [144]. Log2 transformed TMM-normalized CPM (log2normCPM) values were implemented for subsequent analyses [145, 146].
2.9.5.2 ADAM22 gene expression in breast cancer patients

ADAM22 gene expression was examined using two online gene expression analysis tools: GOBO (Gene Expression-Based Outcome for Breast Cancer Online) ([http://co.bmc.lu.se/gobo/gsa.pl](http://co.bmc.lu.se/gobo/gsa.pl)) [147] and BreastMark mRNA survival analysis tool ([http://glados.ucd.ie/BreastMark/mRNA_analysis.html](http://glados.ucd.ie/BreastMark/mRNA_analysis.html)) [148].

2.9.5.3 ADAM22 healthy tissue gene expression

ADAM22 healthy tissue expression was examined using Human Protein Atlas ([www.proteinatlas.org](http://www.proteinatlas.org)) [149]. Images used were obtained directly from the human protein atlas website. ADAM22 expression was examined in three separate datasets including the human protein atlas (HPA) RNAseq dataset, which consists of 172 patient tissue samples from 37 tissues; the Genotype-Tissue Expression (GTEx) project RNAseq dataset [150]; and the Functional Annotation of Mammalian Genomes 5 (FANTOM5) project RNAseq dataset [151].

2.10 DNA extraction, amplification and sequencing

2.10.1 DNA extraction

Total genomic DNA was extracted from cell pellets using the DNeasy blood and tissue kit (QIAGEN). Cell pellets were resuspended in 200 µl of PBS. 20 µl of proteinase K was then added, followed by 200 µl of Buffer AL (without ethanol). Samples were mixed thoroughly by vortexing and incubated at 56 °C for 10 minutes. 200 µl of ethanol was then added and vortexed. Samples were pipetted into a DNeasy mini spin column in a 2 ml collection tube and centrifuged at 6000 rcf for 1 minute. Flow through and collection tubes were discarded. DNeasy mini spin columns were placed in new 2 ml collection tubes and 500 µl of Buffer AW1 was added. Columns were centrifuged at 6000 rcf for 1 minute. Flow through and collection tubes were discarded. 500 µl of Buffer AW2 was added and columns were centrifuged at 20000 rcf for 3 minutes. Flow through and collection tubes were discarded. Columns were placed in sterile 1.5 ml eppendorf tubes. 200 µl of Buffer AE was added directly to the DNeasy membrane and left to incubate at RT for 1 minute. Columns were centrifuged at 6000 rcf for 1 minute. Elutes were labelled and stored at 4 °C until quantification using NanoDrop (see section 2.9.2)
2.10.2 Polymerase chain reaction (PCR)

For amplification of PCR products, the Phusion High Fidelity PCR Kit (New England Biology) was used. All kit components were thawed on ice prior to use. Forward and reverse primers were diluted in advance to 10 µM. Genomic DNA was quantified using NanoDrop and diluted to approximately 20 – 30 ng/µl. A mastermix for each set of primers was prepared as per table 2.11. Each reaction was mixed gently and centrifuged briefly before cycling in an Eppendorf Mastercycler EP Gradient S. Conditions for the PCR amplification are shown in table 2.12.

2.10.3 Gel electrophoresis

In order to analyse results of each PCR reaction, PCR products were separated by agarose gel electrophoresis and imaged under UV light. A 1% agarose gel was prepared by dissolving 1 gram of agarose (Sigma) in 100 ml of TAE (Tris Base, Acetic acid, EDTA) in a microwave for 2 minutes. Once dissolved, 20 µl of SYBR Safe DNA gel stain was added and mixed by gentle swirling. The gel was cast using a gel tray with a 16 tooth comb insert. Once set the gel was orientated with the comb at the cathode end of the rig. TAE buffer was added to the electrophoresis rig until the gel was completely covered by at least 5 mm of buffer. The comb was gently removed. PCR products were prepared for loading by adding 6 x Orange G loading dye. A 100 bp or a 1 kb ladder (NEB) was added as a molecular ruler for product sizes. Samples were loaded into each well and the gel was run at 100 V for approximately 1 hour. Gels were then visualised under UV light using a G:BOX imaging system.

<table>
<thead>
<tr>
<th>Table 2.11: Phusion High Fidelity PCR Mastermix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component</td>
</tr>
<tr>
<td>Nuclease free H₂O</td>
</tr>
<tr>
<td>5x Phusion High Fidelity Buffer</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
</tr>
<tr>
<td>10 µM Forward primer</td>
</tr>
<tr>
<td>10 µM Reverse primer</td>
</tr>
<tr>
<td>Template DNA</td>
</tr>
<tr>
<td>Phusion DNA Polymerase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2.12: Thermal cycling conditions for Phusion high fidelity PCR reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
</tr>
<tr>
<td>98 °C</td>
</tr>
<tr>
<td>98 °C</td>
</tr>
</tbody>
</table>
2.10.4 Purification of PCR product

PCR product purification was carried out using the QIAquick PCR purification kit (QIAGEN). 5 volumes of buffer PB was added to each PCR reaction product (e.g. 50 µl of PB to 10 µl of product). Samples were added to labelled QIAquick columns and centrifuged for 1 minute at 17900 rcf. Flow through was discarded. 750 µl of buffer PE was then added to each column and centrifuged for 1 minute at 17900 rcf. Flow through was discarded. Columns were dried by centrifugation for 1 minute. Columns were then placed in sterile 1.5ml eppendorf tubes. 30 µl of elution buffer was added and incubated for 1 minute at RT. Columns were centrifuged at 17900 rcf to collect eluate. Samples were stored at -20 °C.

2.10.5 Sequencing

Sequencing was carried out on PCR products using the Big Dye Terminator v1.1 Cycle Sequencing Kit. Following purification, PCR products were quantified and diluted in DEPC treated H₂O to a concentration of approximately 10 ng/µl. Components of the Big Dye Terminator v1.1 Cycle Sequencing Kit were thawed on ice and a reaction master mix was made for each primer used in the initial PCR reaction (i.e. 1x for forward primer, 1x reverse primer). The sequencing reaction master mix is listed in table 2.13. Sequencing PCR reactions were carried out on a Eppendorf Mastercycler EP Gradient S as per table 2.14.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 °C</td>
<td>1 minutes</td>
<td>1x</td>
</tr>
<tr>
<td>96 °C</td>
<td>10 seconds</td>
<td>1x</td>
</tr>
</tbody>
</table>

Table 2.13: Big Dye Terminator Cycle Sequencing Master Mix

<table>
<thead>
<tr>
<th>Component</th>
<th>1x volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Big Dye Reaction Buffer 5x</td>
<td>2</td>
</tr>
<tr>
<td>DEPC H₂O</td>
<td>4.8</td>
</tr>
<tr>
<td>Sequencing Primer</td>
<td>2</td>
</tr>
<tr>
<td>Big Dye Terminator v1.1 Cycle</td>
<td>0.2</td>
</tr>
<tr>
<td>PCR product</td>
<td>1</td>
</tr>
<tr>
<td>TOTAL</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.14: Big Dye Thermal Cycle conditions
2.11 *In vivo* studies

*In vivo* studies were carried out with Sinéad Cocchiglia and Dr Damir Varešlija. The efficacy of the LGI1 mimetic at inhibiting early metastatic colonisation events was examined in NOD SCID female mice (NOD.CB17-Prkdc<scid>/NcrCrl) by tracking the spread of LY2luc cells. Mice were purchased from Charles River UK at 21-28 days old and LY2luc cells were injected at approximately 42-49 days old.

2.11.1 Oestrogen and tamoxifen pellet implantation

As LY2luc cells are ER+ the mice were supplemented with 17β-estradiol. The steroid, in pellet form, slowly releases 17β-estradiol (~1 mg/pellet; made in house) and was implanted with a slow release tamoxifen pellet (5 mg/pellet; Innovative Research of America) subcutaneously. Prior to pellet implantation, mice were administered buprenorphine (0.1 mg/kg) as analgesic by subcutaneous injection and anaesthesia was induced and maintained with 2-3 % isoflurane. Hair from the surgical area was shaved and sterilised. Steroid pellets were implanted behind the ear above the shoulder. A small incision was created with scissors and the pellets were then inserted using a trochar. Wounds were sealed with surgical glue (SurgiBond).

2.11.2 Orthotopic injection of LY2luc cells

LY2luc cells were collected as per section 2.1.1, resuspended in sterile PBS (100 µl) and mixed in a 1:1 ratio with Matrigel™ matrix (BD Biosciences). 8x10⁵ cells were implanted by orthotopic injection (200 µl total volume) into the 4th inguinal mammary fat pad under anaesthesia.

2.11.3 IVIS imaging

In order to track tumour growth and metastatic spread, mice were imaged once per week using the IVIS system (Xenogen, PerkinElmer). XenoLight D-Luciferin (15 mg/ml in PBS; PerkinElmer) was prepared fresh, sterile filtered and injected subcutaneously into the scruff so that each animal received 10 µl/g of Luciferin.
Anaesthesia was induced 15 minutes later with 3% isoflurane exposure and the animal was placed carefully into the IVIS imaging platform (37 °C). Bioluminescence was recorded and images were analysed with LivingImage software (PerkinElmer).

2.11.4 Caliper measurements

Once the tumours were palpable, growth was measured twice weekly by caliper measurements. Mice were scruffed and tumour width and length were measured. Tumour volume was calculated using the formula: volume = (width$^2$ X length)/2 as previously described [152].

2.11.5 Drug treatment

The LGI1 mimetic was formulated in advance by carefully dissolving the peptide in sterile filtered 5% MeOH in ddH$_2$O to make a $10^{-3}$M stock (see appendix figure 7.5 for datasheet). Vehicle treatments were prepared in advance and consisted of (5% MeOH ddH$_2$O). Once tumours were palpable, mice were randomised to either a vehicle group (40 µl/day) or LGI1 mimetic group (100 µg/mouse/day). Mice were scruffed and treatment was administered daily by intraperitoneal (IP) injection for a total of 30 days.

2.11.6 Animal welfare

Animal score sheets were filled out twice a week to assess any adverse effects from treatment or tumour growth. (See appendix figures 7.6 & 7.7 for welfare score sheets)

2.11.7 End of experiment (EOX)

Animals were sacrificed after 30 days of drug treatment. 5 minutes before sacrifice, mice were injected with luciferin as per section 2.11.3 2.11.3 to assess metastatic spread ex vivo. Mice were exposed to 5% isoflurane, followed by euthanasia by cervical dislocation. Primary tumours and organs were harvested from each animal. Each organ was imaged using the IVIS imager to detect metastatic spread. Primary tumours were photographed with a scale ruler.
2.11.8 Bioluminescent scoring of organs

Images of harvested organs were analysed using LivingImage software (PerkinElmer). For each organ, regions of interest (metastatic signals) were automatically detected and quantified. Signals were recorded as total flux (photons per second). Where no signal was detected, organs were scored as zero. Statistical analysis was carried out using Prism software (GraphPad).

2.11.9 Tissue preservation

For future studies, all metastatic organ tissue and primary tumours were fixed in 10% formalin for 24 hours (with an extra 24 hours in formic acid for bone tissue), followed by 70% ethanol storage. Tissues were then processed through a series of dehydration, clearing and paraffin infiltration steps using a Leica TP1020. Processed samples were then embedded in paraffin blocks for future analyses.
3 ADAM22 function and mechanism in breast cancer
3.1 Introduction

Metastases are responsible for approximately 90% of cancer related deaths [153]. In ER positive breast cancer, metastatic progression is driven by acquired resistance to endocrine therapy; and so, through targeting these resistance pathways, patient survival rates have greatly improved [47]. Despite this, there is an unmet need in the clinic for biomarkers which predict the development of metastases.

In order for a tumour to metastasise, tumour cells must undergo a series of steps, in what is known as the metastatic cascade (figure 3.1).

Figure 3.1: Schematic representation of the metastatic cascade. In order to form a metastatic lesion, cells in the primary tumour must acquire a migratory phenotype which enables their invasion into the adjacent stroma; through the production of matrix metalloproteinases and interaction with tumour associated macrophages, invading cancer cells intravasate into the circulatory system; while here, circulating tumour cells (CTCs) enhance pro-survival pathways to overcome anoikis; cells must then extravasate and colonise the new tissue to form micrometastases; finally, micrometastases form macroscopic metastases through enhanced proliferative signals. Image taken from “rebuilding cancer metastasis in the mouse”[154]

The first step of the metastatic cascade is dissemination of the tumour, which involves the breakdown of the basement membrane. In healthy tissues, the basement membrane helps maintain normal tissue architecture, separating epithelial cells from surrounding connective tissue. This process of dissemination can occur in
three distinct mechanisms: as a collective group of cells (collective transition); via EMT; and through “amoeboid invasion” [155]. The key factors involved in local invasion and tumour dissemination are: upregulation of transcription factors which drive the EMT phenotype, including Slug, Snail, Twist and ZEB1; the production of matrix metalloproteases (MMPs), a family of secreted proteases, which can modify the extracellular environment, breaking down physical barriers to migration, while simultaneously releasing pro-invasive growth factors; and finally, modification of the tumour integrin protein repertoire, which enhances de-differentiation, movement and attachment to the extracellular matrix [155]. Tumour intravasation occurs via upregulation of the vascular endothelial growth factor (VEGF) associated pathway, which stimulate neo-angiogenesis; upregulation of proteases such as MMPs which break down vascular tissue to allow entry into the circulatory system; and activation of tumour associated macrophages, which release chemotactic and chemokinetic factors which promote intravasation [156]. In order to survive and colonise distant sites, circulating tumour cells (CTCs) themselves must have two distinct attributes: firstly, the ability to avoid apoptosis via anoikis resistance while in the circulatory system [157]; and secondly, to have stem cell like properties or tumour initiating potential at the secondary site [158].

The nuclear receptor co-activator SRC-1 has been shown by our lab and others to play a distinct role in several steps of the metastatic cascade [77]. Ablation of SRC-1 expression in the mouse mammary tumour virus-polyoma middle T (PyMT) breast cancer mouse model led to inhibition of both intravasation and metastatic development [91]. Mechanistically, the disruption of SRC-1 expression was shown to cause this phenotype in part through inhibition of AKT signalling and suppression of colony stimulating factor 1 (CSF-1), which aids in macrophage recruitment [91]. The molecular mechanisms through which SRC-1 plays its role in metastatic development was further emphasised through the establishment of cell lines from both the wild type PyMT mice and SRC-1 knockout PyMT mice. The SRC-1 knockout cells were shown to be less migratory and invasive than their SRC-1 positive counterparts; while markers for EMT, such as N-Cadherin and Vimentin were shown to be downregulated in the knockout cells via interactions between SRC-1 and the metastatic transcription factor Twist [93]. The initiation of the EMT phenotype is often accompanied by increased integrin expression and focal
adhesion rearrangement, which leads to changes in the migratory and adhesive properties of the tumour cells [159]. SRC-1 expression was later shown to be strongly associated with the expression of integrin alpha 5 (ITGA5) in both cell line models and breast cancer patients; while functionally, SRC-1 expression associated with increased focal adhesion assembly and adhesion of breast cancer cells, implicating it further in the early stages of metastatic development [94]. Interestingly, forced overexpression of SRC-1 was also shown to enhance metastatic development in mice models of disease through upregulation of CSF-1 [95]. Furthermore, SRC-1 was shown to promote both migration and de-differentiation of letrozole resistant cells through the promotion of MMP-9 in an ER-independent fashion [99]. The adaptable nature of SRC-1 as a co-activator allows it to act in a promiscuous fashion, thereby promoting the characteristics required for metastatic development [77, 160].

3.2 Preliminary work

In order to assess the SRC-1 transcriptional network in endocrine resistant breast cancer, its regulated genes were identified through analysis of chromatin immunoprecipitation with sequencing (CHIPseq) studies and an SRC-1 siRNA microarray dataset (figure 3.2.a). ADAM22 was identified and confirmed as an SRC-1 target gene, with potential metastatic roles in the resistant setting. ADAM22 enhanced migration (figure 3.2.b) and de-differentiation (figure 3.2.c) in endocrine resistant LY2 and LetR cell lines, implicating it in the early stages of metastatic disease [100]
Figure 3.2: SRC-1 drives ADAM22 expression to enhance migration and de-differentiation. (a) ADAM22 was identified as a potential metastatic oncogene from SRC-1 CHIP-Seq and siSRC-1 microarray data. (b) ADAM22 siRNA mediated silencing reduces the migratory capacity of the endocrine resistant LY2 and LetR cell lines to a level observed in the endocrine sensitive MCF7 cell line. (c) ADAM22 siRNA silencing in LY2 and LetR cells restores their ability to form well differentiated acini. Adapted from McCartan et al [100].
3.3 Aims

The previous work carried out on SRC-1 has shown that it plays a significant role in metastatic development through its ability to drive different steps along the metastatic cascade [91, 93-96]. In the endocrine resistant breast cancer setting, it appears that SRC-1 drives this phenotype increasingly in an ER independent manner [99, 100]. While ADAM22 is implicated in this phenotype, little is known as to how it contributes to the progression of endocrine resistant breast cancer. As such, the aims of this chapter are:

- To examine the effect of ADAM22 expression in endocrine resistant breast cancer using transient ADAM22 siRNA knockdown, CRISPR/Cas9 ADAM22 knockout and forced ADAM22 lentiviral overexpression.
- To investigate potential ADAM22 dependent mechanistic roles in endocrine resistant models of breast cancer using high throughput reverse phase protein array proteomic analysis.
3.4 Results

3.4.1 ADAM22 expression is specific to endocrine resistant models of breast cancer primed for metastases.

Previous work from our lab has shown that SRC-1 upregulates ADAM22 in the endocrine resistant LY2 cell line after tamoxifen treatment in an ER-independent manner [100]. Furthermore, *in vivo*, ADAM22 expression was only found in tumours which were both endocrine resistant and treated with tamoxifen [100]. To investigate whether ADAM22 expression was specific to the endocrine resistant phenotype, ADAM22 expression was analysed in three MCF7 derived endocrine resistant models of breast cancer: the tamoxifen resistant LY2 and TamR cell lines and the letrozole resistant LetR cell line. ADAM22 expression was detected in the LY2, TamR and LetR cell lines, with no expression detected in the MCF7 parental cell line (*figure 3.3.a*).

Previously, both the endocrine resistant LY2 and LetR cell lines used by our lab were shown to be significantly more migratory than the MCF7 cell line; while both resistant cell lines formed less differentiated acinar structures when cultured as three dimensional spheroids, suggesting a phenotype geared towards metastases [100]. In order to further assess this metastatic phenotype, both MCF7 and LY2 cells were cultured in an anchorage independent state in soft agar for two weeks. The ability to form colonies in soft agar is indicative of anoikis resistance, a key characteristic phenotype of the metastatic cascade [134]. LY2 cells formed significantly more colonies than MCF7 cells (~3 fold higher; n=4; *p*<0.0001) (*figure 3.3.b*).

In order to form metastases at distant sites, CTCs must have tumour initiating properties. The mammosphere formation assay assesses the ability of single cell clones to form mammospheres, thereby gauging the relative stemness of the population [135]. Here, the cancer stem cell like properties of both the MCF7 and LY2 cell lines were examined. LY2 cells had a significantly higher mammosphere forming efficiency (*figure 3.3.c*) than the parental MCF7 cells in both the primary and secondary generations (left, n=4; *p*=0.0015; right, n=4; *p*=0.024).
Figure 3.3: ADAM22 expression is enhanced in endocrine resistant cell lines with a metastatic phenotype. (a) Western blot of basal ADAM22 protein expression in whole cell lysates extracted from the endocrine sensitive MCF7 cell line, tamoxifen resistant LY2 and TamR cell lines and the letrozole resistant LetR cell line. Protein extracted from whole mouse brain was used as a positive control. B-actin was used as a loading control. (n=3) (b) MCF7 and LY2 cells were cultured in soft agar for two weeks to replicate an anchorage independent state. An average number of colonies (> 50 µm) were counted from 8 sections per well in duplicate. (n=4) (c) MCF7 and LY2 cells were grown in low adherence plates for 5 days before the total number of mammospheres were counted (> 50 µm). 1st generation mammosphere forming efficiency (MFE) was measured by dividing the total number of mammospheres by the number of cells seeded per well (n=4). 1st generation mammospheres were collected and cultured for a further 5 days to measure 2nd generation mammosphere forming efficiency (n=4). *p = 0.024; ** p= 0.0015; ***p <0.0001 (paired t-test)
3.4.2 ADAM22 knockdown leads to decreased anoikis resistance

Previously, ADAM22 was shown to play a key role in the migratory and dedifferentiated phenotype of endocrine resistant cells, suggesting it plays a key role in the early seeding events of the metastatic cascade [100]. In order to examine whether ADAM22 also contributes to the anoikis resistant phenotype of the LY2 cell line, ADAM22 expression was transiently silenced using short interfering RNA and cultured in soft agar to examine its role in anchorage independent growth. ADAM22 mRNA expression was significantly reduced (n=3; $p<0.0001$) using an ADAM22 siRNA (figure 3.4.a). ADAM22 knockdown led to a significant decrease in the number of colonies formed (n=3; $p=0.0011$) (figure 3.4.b).

![Figure 3.4: ADAM22 transient knockdown decreases anoikis resistant potential of the LY2 cell line.](image)

LY2 cells were seeded in antibiotic free media 24 hours prior to transfection with 10nM of scrambled negative control siRNA (scr siRNA) or ADAM22 siRNA. 48 hours post transfection, cells were harvested for mRNA quantification by RT-PCR (n=3) or seeded into soft agar and cultured for a further two weeks (n=3). **$p=0.0011$ ***$p<0.0001$ (paired t-test)

3.4.3 ADAM22 CRISPR/Cas9 mediated knockout leads to an inhibition of both anoikis resistance and a reduction in self-renewing capacity.

While transient knockdown of proteins is informative with regard to loss of function studies, the development of the CRISPR/Cas9 tool means a complete knockout of gene expression can be achieved. In order to create a stable ADAM22 CRISPR/Cas9 knockout endocrine resistant cell line, LY2 cells were electroporated
with a pool of three commercially available ADAM22 CRISPR/Cas9 knockout plasmids along with a pool of three corresponding ADAM22 HDR plasmids. Successful electroporation of both sets of plasmids was confirmed by co-expression of a GFP tag on the knockout plasmid and a RFP tag on the HDR plasmid (figure 3.5.a). Cells were passaged six times to establish healthy populations before puromycin selection. After selection, ADAM22 mRNA levels were examined and the functionality of the ADAM22 CRISPR mediated knockout was examined using an anchorage independent growth assay. ADAM22 mRNA was significantly reduced by approximately 90% (n=3; p=0.0004) (figure 3.5.b). This CRISPR mediated ADAM22 knockout also led to a significant reduction in both the number of colonies formed and in the size of the colonies formed (n=4; p<0.05) (figure 3.5.c).

Figure 3.5: Development of an ADAM22 CRISPR/Cas9 knockout cell line. (a) Confirmation of successful co-electroporation of LY2 cells with ADAM22 CRISPR knockout plasmids (GFP) and ADAM22 CRISPR HDR plasmids (RFP). (b) ADAM22 mRNA levels as measured by RT-PCR in parental LY2 and LY2 ADAM22 CRISPR/Cas9 knockout cells. (n=3) (c) Functional effect of ADAM22 CRISPR/Cas9 knockout as shown by a reduction in anchorage independent colonies (left) and colony size (right) (n=4). *p<0.05 ***p<0.0005 (paired t-test)
As residual ADAM22 expression was observed at the transcript level, it was hypothesised that within the population of LY2 ADAM22 CRISPR/Cas9 knockout cells, there were subpopulations of mono-allelic ADAM22 knockout cells with one intact wild type copy of the gene. We hypothesised that bi-allelic ADAM22 knockout cells would be more likely to express higher levels of RFP due to bi-allelic insertion of the RFP gene along with the HDR plasmids. Using fluorescence activated cell sorting (FACS) (with Dr Sara Charmsaz), LY2 ADAM22 CRISPR/Cas9 knockout cells were single cell sorted into 96 well plates based on high RFP expression (figure 3.6.a).

Once colonies were established, ADAM22 expression was analysed by RT-PCR. One clone in particular, Clone H, had significantly decreased levels of ADAM22 mRNA expression (< 4%) when compared to parental LY2 cells (n=3; p<0.0001) (figure 3.6.b). In order to determine whether Clone H was a bi-allelic ADAM22 knockout clone, genomic DNA was extracted for genotyping. Primers specific to the flanking regions of the guide 1&3 and guide 2 cut sites were used to amplify genomic DNA from both parental LY2 and Clone H cells. PCR products were analysed by polyacrylamide gel electrophoresis (figure 3.6.c). A homozygous deletion (ADAM22^del/del) was evident in the Clone H guide 1&3 cut site PCR product (left); while a heterozygous HDR insertion (ADAM22^{WT/HDR}) was evident in the guide 2 cut site (right). In order to determine whether the homozygous deletion in Clone H was enough to disrupt the ADAM22 gene, PCR products were purified and sequenced. Sequencing results were aligned using Ape software and mapped to the ADAM22 gene using BLAST. A resulting homozygous 58 base pair deletion was found in ADAM22 exon 5 directly between the guide 1 and guide 3 RNA CRISPR cut sites (figure 3.6.d).
Figure 3.6: Clonal selection of a bi-allelic ADAM22 knockout cell line. (a) FACS gating strategy to single cell sort ADAM22 CRISPR knockout cells with high RFP expression (right), using LY2 cells as a negative control (left). (b) ADAM22 mRNA levels as measured by RT-PCR in parental LY2 and CRISPR Clone H cells. (n=3; p<0.0001; paired t-test) (c) Genotyping of Clone H by PCR amplification of CRISPR cut sites 1&3 (left) and cut site 2 (right). (d) Confirmation of 58 bp deletion (red) in Clone H between guide site 1 and guide site 3.

The functionality of the bi-allelic ADAM22 knockout in Clone H was then analysed using the anchorage independent growth assay and mammosphere formation assay. Both MCF7 and Clone H cells formed significantly fewer colonies in soft agar than LY2 cells (n=4; p<0.0001) (figure 3.7.a). Similarly, both MCF7 and Clone H cells exhibited a significant reduction in mammosphere forming efficiency compared to LY2 cells (figure 3.7.b) in both the first (left; n=4; p<0.0002) and second (right; n=4; p<0.006) generation mammospheres.
Figure 3.7: ADAM22 knockout inhibits anoikis resistance and cancer stemness
(a) MCF7, LY2 and Clone H cells were seeded in duplicate on soft agar and allowed to grow for two weeks. Colonies were stained with p-iodonitrotrazolium chloride and counted if over 50 µm in size. (n=4) (b) MCF7, LY2 and Clone H cells were seeded into low adherence plates and allowed to form mammospheres. First generation mammospheres were counted on day 5 (n=4) and were then collected, trypsinised and seeded for second generation formation (n=4). **p<0.006 *** p<0.0002 (paired t-test)
3.4.4 ADAM22 lentiviral overexpression enhances metastatic potential

To confirm that ADAM22 expression promotes metastatic development, an ADAM22 overexpressing cell line (LY2lentiA22) was developed. LY2 cells were transduced with lentiviral particles containing a full length cDNA transcript of ADAM22 variant 1 over a 72 hour period. Successful transduction of LY2 cells was assessed by high expression of a GFP tag in the lentiviral particles (figure 3.8.a). FACS was then utilised to purify the population of ADAM22 overexpressing (GFP positive) cells from non-transduced wild type LY2 cells (figure 3.8.b). ADAM22 overexpression was then confirmed in the LY2 lentiA22 cell line using RT-PCR and western blot. ADAM22 mRNA expression was approximately 1000 fold higher in the LY2 lentiA22 cell line versus parental LY2 cells (figure 3.8.c). This significant increase in ADAM22 transcript translated into protein detectable at the predicted molecular weight of 100kDa (figure 3.8.d, top) and was found predominantly in purified membrane extract (figure 3.8.d, bottom). Functionally, forced overexpression of ADAM22 transcript variant 1 in the LY2 lentiA22 cell line led to a significant increase in anchorage independent colony formation (n=3; \( p=0.0007 \)) (figure 3.8.e); while a significant increase in mammosphere formation was also observed (n=4; \( p<0.05 \)) (figure 3.8.f). Consistent with ADAM22 silencing and knockout, these experiments define the key contributions of ADAM22 to the metastatic phenotype and offer key insights into its function.
Figure 3.8: Forced overexpression of ADAM22 leads to a more aggressive metastatic phenotype. (a) GFP expression in LY2 lentiA22 cells 72 hours post transduction with GFP tagged lentiviral particles containing a full length cDNA transcript of ADAM22 variant 1. (b) FACS was used to purify GFP expressing LY2 lentiA22 cells from non-transduced parental LY2 cells. (c) ADAM22 overexpression in purified LY2 lentiA22 cell leads to a 1000 fold increase in ADAM22 mRNA levels (n=2). (d) Representative images of ADAM22 expression in the LY2 lentiA22 cell line. Forced ADAM22 overexpression in LY2 lentiA22 cells leads to detectable overexpressed protein at the predicted molecular mass of 100kDa (top) (n=3); while the protein is predominantly found in the membrane, suggesting correct localisation (n=1). (e) LY2 lentiA22 cells form significantly more colonies than parental LY2 cells in soft agar (n=3). (f) LY2 lentiA22 cells form significantly more 1st (n=3) and 2nd (n=3) generation mammospheres than parental LY2 cells. * p<0.05 ***p<0.007 (paired t-test)
3.4.5 Reverse Phase Protein Array to determine ADAM22 dependent response to tamoxifen (4-OHT)

Having established a strong functional role for ADAM22 in mediating both the early dissemination events as well as providing evidence for its role in the metastatic colonisation process, we set out to explore the mechanism of action through which ADAM22 promotes these characteristics. As a transmembrane multi-domain protein, it was hypothesised that ADAM22 was mediating a more metastatic phenotype in the endocrine resistant cell lines through a complex system of second messengers. Previously, ADAM22 has been shown to bind both to integrins through its disintegrin domain [161], and the 14-3-3 second messenger scaffold proteins through its cytoplasmic tail [162-164]. Furthermore, as ADAM22 lacks a catalytic metalloproteinase domain, it is likely that functionally ADAM22 acts as a membrane receptor for extracellular ligands and as a transducer of second messengers. In order to examine these ADAM22 mediated signalling events, two reverse phase protein array (RPPA) studies were carried out. The benefit of using the RPPA system for mechanism studies is the high throughput proteomic data that is attainable from relatively small amounts of protein, making it an invaluable tool in cancer discovery [165].

The expression levels of 66 different proteins were examined in LY2 cells with or without transient ADAM22 knockdown. Despite variation in the levels of ADAM22 knockdown between the biological replicates (figure 3.9.a) and the consequent variation in the RPPA results, a trend was observed suggesting ADAM22 may be playing an anti-apoptotic role. ADAM22 knockdown led to the upregulation of the pro-apoptotic protein caspase-9 cleaved (p=0.01) (figure 3.9.b). Similarly, the pro-apoptotic proteins caspase-7 cleaved and SMAC/DIABLO were increased after ADAM22 knockdown; however, these results were insignificant (figure 3.9.c). Inversely, the anti-apoptotic proteins HIAP-2 and XIAP were downregulated after ADAM22 knockdown (figure 3.9.d), however, this was also insignificant.
Figure 3.9: RPPA analysis of LY2 cells with transient ADAM22 knockdown suggests a potential anti-apoptotic role for ADAM22 in endocrine resistant breast cancer. (a) ADAM22 protein expression in each replicate of LY2 cells transfected with a scrambled siRNA (scr) or an ADAM22 siRNA. (b) Caspase-9 cleaved normalised protein expression in LY2 scr siRNA cells and LY2 ADAM22 siRNA cells. (c) Caspase-7 cleaved, and SMAC/DIABLO normalised protein expression LY2 scr siRNA cells and LY2 ADAM22 siRNA cells. (d) HIAP-2 and XIAP normalised protein expression LY2 scr siRNA cells and LY2 ADAM22 siRNA cells. **p<0.01 (paired t-test)

Due to the variation in ADAM22 expression in the first RPPA, a second RPPA experiment was carried out comparing response to tamoxifen treatment in the parental LY2 cell line and the ADAM22 CRISPR/Cas9 knockout Clone H.

Four biological replicates were analysed. A total of 89 proteins were analysed. Differential protein expression between the LY2 and Clone H cell lines was analysed
using the online RPPA analysis tool RPPApipe [140]. Significant differences were observed in a total of 21 proteins and are displayed as fold difference in LY2 versus ADAM22 knockout Clone H cells (i.e negative values are indicative of higher expression in the Clone H cell line) (figure 3.10.a). Clone H ADAM22 expression was decreased in each biological replicate used in the study when compared to the LY2 control (figure 3.10.b).

Pathway analysis was then carried out to uncover the potential mechanisms through which ADAM22 mediates its role in breast cancer. Significantly upregulated proteins in the parental LY2 cell line were clustered into functionally annotated groups using the online tool DAVID [141], referencing the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. These ADAM22 associated protein clusters were significantly associated with: proteoglycans in cancer \((p=1e^{-8})\); the PI3K-AKT signalling pathway \((p=4.60e^{-07})\); the insulin signalling pathways \((p=2.10e^{-06})\); the ErbB signalling pathway \((p=1.10e^{-05})\); focal adhesions \((p=0.000015)\); the HIF-1 signalling pathway \((p=1.7e^{-05})\); and the neurotrophin signalling pathway \((p=3.8e^{-05})\) (figure 3.10.c).

In order to validate the role of ADAM22 in these pathways, some of the key proteins were assessed by western blot. In keeping with ADAM22 involvement in the PI3K-AKT pathway, ADAM22 expression was consistently shown to be associated with increased levels of total AKT (figure 3.10.d). Despite an association with the focal adhesion pathway, ADAM22 expression inversely correlated with focal adhesion kinase (FAK) expression (figure 3.10.d); this may be due to the significantly elevated levels of phosphorylated FAK Y925 \((p<0.01)\) observed in ADAM22 positive cells in the RPPA experiment (figure 3.10.a). The HIF1-\(\alpha\) signalling pathway was also significantly associated with ADAM22 expression, implying a potential link with pro-angiogenesis. Accordingly, VEGFR2 expression was also found to be enhanced in ADAM22 positive cells (figure 3.10.e). Finally, 4-OHT treatment was shown to induce enhanced phosphorylation of ERK1/2 (pERK1/2) in ADAM22 positive cells (figure 3.10.f). This is consistent with enhanced ErbB signalling. A spider diagram of ADAM22 mediated pathways with known roles in the metastatic cascade is shown (figure 3.10.g)
Figure 3.10: RPPA analysis suggests ADAM22 enhances growth factor signalling, survival signalling, ECM remodelling, cancer stemness, metabolism, angiogenesis and neurotrophic signalling. (a) Significant differentially expressed proteins between the LY2 and Clone H cell lines displayed as fold differences. (b) ADAM22 protein expression in each biological replicate used for the RPPA study. (c) Top ranking KEGG pathways associated with ADAM22 expression. (d) Representative western blot of ADAM22, FAK and AKT in the LY2 and Clone H cell lines (n=5). (e) Representative western blot of basal VEGFR2 levels in the LY2, Clone H and LentiA22 cell lines (n=3). (f) Representative western blot of 4-OHT (10^{-7} M) induced phosphorylation of ERK1/2 in the LY2, Clone H, and LentiA22 cell lines (n=4). (g) ADAM22 associated pathways with known roles in the development of metastases.
3.5 Discussion

As endocrine resistance is acquired, tumour cells begin to lose their dependency on the oestrogen receptor, leading to increased growth factor signalling and progression on therapy [35]. There is now growing evidence that tumour progression and metastases in this setting is driven in part through overexpression of the nuclear receptor co-activators AIB1 and SRC-1 [100, 166, 167]. Adaptability to treatment is the primary cause of recurrent breast cancer and this can be partly explained through the promiscuous nature of SRC-1 as a co-activator [99, 100, 160]. Modelling of SRC-1 mediated disease both in vitro and in vivo has shown that it plays a significant role in the key stages of metastatic development, through upregulation of EMT markers to enable initial invasion; alteration of the integrin repertoire to allow for invasion of proximal tissue; and stimulation of tumour associated macrophages to promote both MMP production and angiogenic factors to allow for intravasation [93-96].

Previous work from our lab showed SRC-1 mediated this metastatic phenotype via upregulation of the transmembrane protein ADAM22, specifically in the endocrine resistant setting under the pressure of tamoxifen [100]. Here, ADAM22 expression was shown to be specifically expressed in endocrine resistant models of breast cancer. Furthermore, the aggressive phenotypes observed in these cell lines were shown to be alleviated through ADAM22 silencing. Early functional studies using the CRISPR/Cas9 ADAM22 knockout cells showed ADAM22 mediated a pro-clonogenic, pro-proliferative phenotype, by enhancing both the number and size of colonies formed in an anchorage independent state. Interestingly, this reduced size phenotype was specific to the unsorted heterogeneous ADAM22 CRISPR/Cas9 knockout cells. This may be in part be explained by subpopulations of mono-allelic ADAM22 expressing cells, as implied by the residual levels of ADAM22 transcript in these cells. Previous work examining the role of the closely related ADAM23 protein in metastatic development yielded similar findings as a result of ADAM23 intratumoural heterogeneity [125].

One of the issues observed with the Clone H ADAM22 CRISPR/Cas9 knockout cell line was residual ADAM22 protein expression, despite a homozygous 58 base pair deletion in exon 5 of the ADAM22 gene. Previous work from Gödde et al, examining
the coding potential of ADAM22 transcript variants led to the discovery of a second transcription initiation element within the ADAM22 gene, which may explain the presence of residual ADAM22 expression in these Clone H cells [133]. Importantly, the transcripts originating from this secondary transcriptional start site were shown to be functionally inactive [133]. Several controls may have also been included for these ADAM22 knockout studies, such as the use of other knockout clones as additional evidence of ADAM22 involvement in these metastatic phenotypes. Furthermore, the ability to restore ADAM22 expression in these knockout clones would control for potential non-specific global gene effects associated with CRISPR/Cas9 modification, however, restoration of ADAM22 expression was not possible in this case due to a non-homologous end joining mediated deletion.

While a reduction in anchorage independent colonies was observed in all experiments involving reduced ADAM22 expression; importantly, forced overexpression of ADAM22 increased this anchorage independent phenotype. As mentioned previously, a key characteristic of metastatic CTCs is their ability to overcome anoikis or anchorage independent cell death. As such, ADAM22 was shown to mediate this anoikis resistant phenotype in endocrine resistant breast cancer.

Less than 0.1% of CTCs are capable of establishing metastases [22]. Therefore, in order to form metastatic disease, CTCs must have tumour initiating potential or possess a stem cell like phenotype. Mammospheres form from single stem cell clones and are highly associated with drug resistance, tumour aggressiveness and metastatic development [168]. Here, ADAM22 knockout led to a significant reduction in mammosphere forming efficiency, a strong output for measuring cancer cell stemness. Interestingly, ablation of ADAM22 was enough to reduce the mammosphere forming efficiency of the LY2 cell line to levels observed in the endocrine sensitive and non-metastatic MCF7 cell line. Conversely, overexpression of ADAM22 enhanced this cancer stem cell phenotype. While no mechanism for this stem cell like characteristic has been described for ADAM22, ADAM23 downregulation, which in turn leads to integrin αvβ3 activation, has been shown to promote both anchorage independence and a stem cell like phenotype [123, 124]. Interestingly, ADAM22 has also been shown to interact with integrin αvβ3 [161]. While both ADAM22 and ADAM23 proteins share similar tissue distribution in healthy
individuals [169], the inverse appears to be true in cancerous tissue. ADAM23 downregulation is strongly associated with metastatic development [123], while ADAM22 overexpression in endocrine resistant breast cancer at least seems to promote metastases. As integrin αvβ3 has been shown to mediate anchorage independent growth, tumour stemness, angiogenesis, differentiation and metastases [170-172], it is quite possible that ADAM22 may promote these characteristics through interactions with integrin αvβ3.

Using a high throughput reverse phase protein array, the molecular mechanisms through which ADAM22 mediates its role in the endocrine resistant setting were investigated. Due to problems with the efficiency of ADAM22 protein knockdown in the initial RPPA study, no major significant differences were observed; however, a trend was observed suggesting a potential anti-apoptotic role for ADAM22. Evading apoptotic stimuli is a well-established mechanism used by tumours during their progression towards metastases [173]. Furthermore, several studies have established anti-apoptotic roles for other ADAM family proteins [174-178]. For example, ADAM12 was shown to contribute to metastatic breast cancer through its ability to confer apoptotic resistance within the tumour, while inducing apoptosis in surrounding stromal cells [174]. Moreover, work from our lab, has shown that SRC-1 promotes an anti-apoptotic signature, in part through repression of pro-apoptotic genes [160]. Here, ADAM22 was shown functionally to promote anoikis resistance; a form of programmed cell death induced by detachment of cells from the extracellular matrix which can be overcome by upregulation of anti-apoptotic proteins, such as Bcl-2 and XIAP [179, 180].

Interestingly, Bcl-2 expression levels were significantly higher in the CRISPR RPPA study reaffirming a potential role for ADAM22 expression in enhancing an anti-apoptotic role in endocrine resistant cells.

Bypassing external stimuli, tumours cells have also been shown to adapt to anchorage independent environments through hyper activation of pro-survival PI3K-AKT and Ras-ERK pathways, which in turn may upregulate this anti-apoptotic phenotype. KEGG analysis of ADAM22 dependent signalling showed an upregulation in components of the PI3K-AKT pathway, while basal AKT levels were significantly higher in the ADAM22 positive LY2 cell line. Similarly, ADAM22
expression was also associated with enhanced activation of the ERK1/2 pathway in response to tamoxifen treatment. While tamoxifen induced ERK1/2 activation has previously been reported in tamoxifen resistant cell lines [182], work from our lab suggest this elevated ERK1/2 activity is most likely as a result of tamoxifen induced phospho-src expression [166]. This result highlights the increased level of ER independent signalling associated with tamoxifen resistance and further implicates ADAM22 in the progression of ER+ disease.

One of the surprising results from the RPPA study was the significant increase in FAK in the ADAM22 CRISPR knockout cells. Increased FAK expression is usually associated with a more aggressive phenotype [183]. FAK Y925 phosphorylation was significantly higher in the parental LY2 cells despite significantly lower levels of total FAK, suggesting a potential role for ADAM22 in mediating FAK activity. Previous reports have shown FAK Y925 phosphorylation is associated with a pro-angiogenic switch in tumours [184]. Consistent with this, ADAM22 expression was associated with enhanced VEGFR2 expression and the HIF-1α signalling pathway. Interestingly, previous reports have shown both VEGFR2 and integrin β3 can synergistically activate each other to promote migration and angiogenesis [185]. These results suggest a potential role for ADAM22 in promoting neo-angiogenic signalling under hypoxic conditions, possibly through interactions with integrin αvβ3.

The area of cancer cell metabolism and the role it plays in metastatic development is an emerging field. It has long been established that tumours intake significantly higher levels of glucose than surrounding tissue, using the process of aerobic glycolysis to produce lactate in what is known as the Warburg effect [186]. Several recent studies have shown that this glycolytic production of lactate may encourage metastatic development [187, 188]. Here, ADAM22 expression was significantly associated with the expression of hexokinase-2 (HK2), monocarboxylic acid transporter 4 (MCT4) and phosphoinositide-dependent kinase-1 (PDK1), three proteins involved in the glycolysis pathway. HK2 is involved in the first catalytic step of this pathway and has been specifically implicated in the development of metabolic reprogramming of cancer cells under hypoxic conditions and the consequent development of metastases [189-192]. Interestingly, this metabolic reprogramming has been shown to be driven in part by the phosphorylation of src kinase [193]. Similarly, the expression of PDK1 is in part regulated by hypoxia, playing an
essential role in the glycolysis pathway, while also contributing to metastatic development [194, 195]. Additionally, PDK1 expression has been shown to play a significant role in breast cancer stem cell reprogramming [196]. This suggests ADAM22 may mediate the cancer stem cell like phenotype observed here in part through PDK1 activation. Intriguingly, increased ADAM22 expression was previously reported in the subcutaneous tissue of insulin resistant patients [197], lending weight to the possibility that ADAM22 may be associated with dysregulation of metabolism. ADAM22 expression in the endocrine resistant setting was shown here to promote activation of insulin signalling, growth factor signalling and HIF-1 signalling. Taken together, these results suggest ADAM22 promotes enhanced metabolism in endocrine resistant breast cancer, in part through enhanced growth factor and hypoxic signalling cascades, which results in breast cancer stem cell reprogramming and increased invasiveness.

Interestingly, the ADAM22 RPPA signature was significantly associated with the neurotrophin signalling pathway. Considering the roles both play in the development of the central nervous system, this association is somewhat unsurprising [110, 116, 198-201]. There are four human neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5); and three neurotrophin receptors: p75NTR, Trk tyrosine kinase receptors and sortilin [202]. High levels of NGF production has been reported in breast cancer cells, leading to the formation of an autocrine loop which drives their proliferation [203]. Furthermore, autocrine production of neurotrophins has also been shown to promote an anti-apoptotic and pro-invasive phenotype in breast cancer [204, 205]. Interestingly, expression of the neurotrophin receptor TrkB on CTCs was recently shown to promote the development of breast to brain metastases, through its ability to respond to astrocyte derived BDNF released from the brain microenvironment [206]. Moreover, neurotrophin-3 expression in breast cancer cells has also been shown to promote metastatic colonisation of the brain via interactions with the brain metastatic niche [207]. Considering the functional roles both ADAM22 and the neurotrophins play in the development of the nervous system and indeed in breast cancer, it is possible that ADAM22 expression may mediate a similar organotropic phenotype on endocrine resistant cells.
While further studies are necessary to fully elucidate the molecular mechanisms through which ADAM22 functions in endocrine resistant breast, the functional effect ADAM22 expression has on endocrine resistant cells suggests that it plays a key role in early invasive capacity of cells; in their dissemination and survival in the circulatory system; and finally in their ability to colonise distant sites. Furthermore, results from the RPPA studies suggest ADAM22 mediates these roles through its ability to interact with multiple signalling pathways. As a transmembrane protein with potential roles in both intra and extra cellular signalling, ADAM22 is central to the metastatic phenotype observed in endocrine resistant breast cancer.
4 ADAM22 as a biomarker and therapeutic target
4.1 Introduction

In the breast cancer setting, there is a growing need for novel biomarkers to identify patients likely to develop metastatic disease. Despite this need, very few biomarkers make it to FDA approval and consequent use in the clinic [208]. Classic histological typing may aid in risk assessment for approximately 30% of patients, however, for the remaining 70% of patients, new strategies are urgently needed [22]. Despite the prognostic potential of multi-gene assays such as Endopredict, MammaPrint, Oncotype DX and Prosigna in determining risk of recurrence in luminal patients, they are not universally implemented in the clinic [4].

As response to endocrine therapy is directly related to risk of recurrence, the primary focus of translational studies has been on novel methods to accurately predict this response [209]. For example, a highly accurate four gene prognostic signature was recently identified in patients responsive to neoadjuvant AI therapy [210]. Similarly, the proliferative marker ki67 has also shown some promise as a predictive and prognostic biomarker for response to endocrine therapy [211], chemotherapy [212], and palbociclib [213]. While these prognostic tests may be of particular benefit in the earlier stages of disease, recurrence in luminal patients is often a later event. Therefore, the ability to monitor response long term may be of more translational benefit [209].

One particular area of interest is the ability to detect and analyse CTCs [209]. For example, the number of CTCs in patient bloods may be of prognostic value with regard to metastases, progression free survival and overall survival rates [214, 215]. Furthermore, the ability to analyse circulating tumour DNA (ctDNA) has also shown prognostic potential. For example, activating ESR1 mutations which stem from AI resistance are detectable in patient plasma ctDNA and are predictive of poor disease free survival and response to fulvestrant [54, 216]. Numerous studies have also assessed serum protein markers in patient bloods as potential prognostics [217]. For example, elevated levels of the serum marker S100β was recently shown to predict response to endocrine therapy and may also be indicative of sensitivity to tyrosine kinase inhibition [166].

While these examples show extreme promise, a lack of standardisation, equipment and cost are all limiting factors for their implementation in the clinic [209, 217].
Furthermore, while accurately predicting or monitoring response to therapy, these biomarkers also fail to identify markers which mediate metastatic progression.

As shown in chapter 3, ADAM22 mediates these metastatic characteristics in endocrine resistant cell lines, suggesting its potential use as a prognostic biomarker for predicting metastases.

4.2 Preliminary work

Previously, ADAM22 protein expression was examined in a large patient cohort (n=560). Patients expressing ADAM22 had a significantly worse prognosis with regard to disease free survival ($p<0.0001$) (figure 4.1.a) [100]. Furthermore, when compared against other clinicopathological features, such as tumour grade, tumour stage, lymph node status, ER status, treatment history or SRC-1 expression, ADAM22 expression was the strongest independent predictor of poor disease free survival in this patient group (Odds ratio 2.40; $p=0.005$) (figure 4.1.b) [100, 218]. This work emphasises the potential of ADAM22 as a biomarker for endocrine resistant breast cancer.
Figure 4.1: ADAM22 is a strong independent predictor of poor disease free survival. (a) Kaplan Meier survival analysis for ADAM22 expression in a breast cancer patient cohort (n=560; p<0.0001). (b) Odds ratio for disease free survival for clinicopathological variables, SRC-1 and ADAM22 expression. [100]
4.3 Aims

Based on the preliminary clinical studies and the functional roles described in chapter 3, this chapter will examine the role of ADAM22 expression in patient tissue and assess its potential relevance as a biomarker. The three major aims are:

- To assess the clinical relevance of ADAM22 expression in breast cancer
- To assess endogenous ADAM22 expression in normal tissues
- To investigate the levels of ADAM22 expression in metastatic tissue
4.4 Results

4.4.1 Development of an ADAM22 clinical assay

ADAM22 expression was previously shown to significantly predict poor disease free survival in a breast cancer patient cohort using a polyclonal anti-ADAM22 antibody [100]. As polyclonal antibodies can vary in their target specificity and cross reactivity, the use of monoclonal antibodies (mAb) as prognostic tools is preferable [219]. With this in mind, two commercially available ADAM22 monoclonal antibodies from Santa Cruz (sc-37391) and R&D systems (MAB4081) were purchased and tested for potential use in a clinical validation assay. Optimal antibody concentrations, antigen retrieval steps and incubation times were assessed in order to obtain the largest reproducible staining range. Optimal ADAM22 immunostaining, in terms of localisation and range of staining intensity, was observed with the ADAM22 mAb (sc-373931) (figure 4.2.a). ADAM22 immunostaining with the ADAM22 mAb (MAB4081) was poor, with non-specific edge staining observed in all samples and a poor range of staining intensity (figure 4.2.b). As such, the ADAM22 mAb (sc-373931) was chosen for analytical validation.

ADAM22 expression was first assessed in a retrospective breast cancer patient cohort (training set, n=327) using the ADAM22 mAb (sc-373931). Each patient in the training cohort was assigned a histoscore based on the percentage and intensity of ADAM22 expression in each core on the tissue microarray. The training set population was divided into quintiles based on ADAM22 histoscore and each quintile was tested as a threshold for ADAM22 positivity to predict poor disease free survival. Using a cut off histoscore of 270, ADAM22 expression was shown to significantly predict poor disease free survival in the training set cohort (p=0.038) (figure 4.3.a). Having established a clinically significant cut off, ADAM22 expression was then assessed in a second retrospective breast cancer patient cohort (Validation set, n=444), however, this result was insignificant (Figure 4.3.b).

In order to assess the reasons behind the loss of significance observed in the validation set, ADAM22 expression was examined across different patient subtypes. No association was observed between ADAM22 expression and the luminal A, HER2 enriched or triple negative subtypes (data not shown); however, in luminal B
patients, an observable but insignificant trend was seen between ADAM22 positivity and poor disease free survival (n=59; p=0.177) (figure 4.3.c).

![Figure 4.2: Tissue microarray (TMA) staining with ADAM22 monoclonal antibodies.](image)

(a) Representative staining range using the ADAM22 mouse monoclonal antibody (Santa Cruz sc-373931). 5 μm sections were cut from FFPE TMA blocks and deparaffinised. ADAM22 was exposed using EDTA (pH9) antigen retrieval. Tissues were incubated with ADAM22 antibody (1μg/ml) overnight at 4 °C. (b) Representative staining range for the ADAM22 mouse monoclonal antibody (R&D systems MAB4081). 5 μm sections were cut from FFPE TMA blocks and deparaffinised. ADAM22 was exposed using Sodium Citrate (pH6) antigen retrieval. Tissues were incubated with ADAM22 antibody (1μg/ml) for 1 hr at room temperature.
Figure 4.3: Clinical validation with the ADAM22 antibody (Santa Cruz sc-373931).
(a) A training set TMA consisting of breast cancer patients (n=333) was stained with 1μg/ml of ADAM22 mAb (sc-373931) and staining intensity was scored according to the histoscore method. Based on the range of staining, ADAM22 expression was divided into quintiles. The predictive power of each quintile was tested against disease free survival. An ADAM22 histoscore of 270+ significantly predicted poor disease free survival (p=0.0318). (b) Using the training set histoscore cut off of 270, ADAM22 expression was examined in a validation set TMA (n=444) (left). No significance was observed between ADAM22 expression and disease free survival. (c) ADAM22 expression was examined in Luminal B patients only within the validation set.
In order to overcome the limits of patient numbers in our validation cohort and to explore this potential luminal B specific role, ADAM22 expression was assessed in publicly available online gene expression datasets. Using GOBO (Gene Expression-Based Outcome for Breast Cancer Online) [147], which encompasses 1881 breast cancer samples across 11 different gene expression datasets, ADAM22 expression was examined in relation to relapse free survival, in all breast cancer patients and luminal b patients. No correlation was observed between ADAM22 expression and relapse free survival in a total breast cancer patient population (n=914) (figure 4.4.a). However, in luminal B patients, ADAM22 expression significantly associated with poor relapse free survival time (n=130, p=0.00799) (figure 4.4.b). ADAM22 expression was then examined in a second online gene expression analysis tool, BreastMark, which encompasses 4738 samples across 26 different gene sets [148]. ADAM22 expression was found to predict poor disease free survival in all subtypes of breast cancer (n=2652, p=0.025564) (figure 4.4.c) and in luminal B stratified patients alone (n=1013, p=0.040934) (figure 4.4.d).
Figure 4.4: ADAM22 expression predicts relapse free survival in Luminal B patients. ADAM22 expression was examined in all breast cancer subtypes using the GOBO [147] and BreastMark [148] gene set analysis tools. (a) ADAM22 expression has no effect on relapse free survival in all breast cancer subtypes (n=914). (b) ADAM22 expression significantly predicts relapse free survival in luminal B breast cancer patients (n=130, p=0.00799). Sub-stratification was based on [15]. (c) ADAM22 expression significantly predicts poor disease free survival in a large breast cancer patient cohort (n=2652, p=0.025564) (d) ADAM22 expression significantly predicts poor disease free survival in a luminal B stratified cohort (n=1013, p=0.040934)
4.4.2 ADAM22 is predominantly found in normal brain tissue

As ADAM22 expression is implicated functionally in the development of metastases and is associated with poor disease free survival, it could be a promising drug target. One of the desirable characteristics of potentially druggable targets is low endogenous tissue expression [220]. Therefore, ADAM22 expression was examined in normal tissues using online transcriptomic data and in house immunohistochemical staining.

Three online datasets were used to examine endogenous ADAM22 tissue mRNA levels. In the Human Protein Atlas (HPA) RNAseq dataset, ADAM22 expression was predominantly found in the cerebral cortex with a score of approximately 45 transcripts per million (TPM), with low to moderate levels (8-20 TPM) observed in the prostate, adrenal gland, epididymis, cervix, endometrium and seminal vessel (figure 4.5.a). In the Genotype-Tissue Expression (GTEx) RNAseq dataset, ADAM22 expression was highest in the cerebellum with a Reads Per Kilobase gene model and Million mapped reads (RPKM) score of approximately 30, with moderate levels (5-10 RPKM) observed in cerebral cortex, hypothalamus, hippocampus and caudate (figure 4.5.b). Finally, using the FANTOM5 CAGE dataset, ADAM22 expression was highest in the cerebellum with a score of approximately 330 tags per million, with moderate scores (50-150) observed in the hippocampus, brain and caudate (figure 4.5.c). These results indicate endogenous ADAM22 expression is predominantly found in the central nervous tissue.

As the above data is based on transcriptomic results, ADAM22 protein expression was then examined in healthy brain, liver, heart, spleen, lung and kidney tissue obtained from Beaumont Hospital Dublin using the ADAM22 monoclonal antibody (sc-373931). Consistent with the publicly available transcript data, ADAM22 protein expression was exclusively found in normal brain tissue when compared against relevant mouse IgG1 controls (figure 4.6).
Figure 4.5: ADAM22 expression is predominantly found in central nervous tissue. (a) ADAM22 mRNA expression in the HPA RNAseq dataset. (b) ADAM22 mRNA expression in the GTEx RNAseq dataset. (c) ADAM22 mRNA expression in the FANTOM5 CAGE dataset.
Figure 4.6: ADAM22 expression is exclusively found in normal brain tissue. ADAM22 expression was examined in the brain, liver, heart, spleen, lung and kidney of a healthy individual post mortem by IHC. 5 μm sections were cut from FFPE tissue blocks. Tissues were stained overnight with an ADAM22 monoclonal antibody (sc-373931; 1 μg/ml) after EDTA antigen retrieval and visualised using a mouse specific HRP conjugated secondary (Dako) and DAB. A mouse IgG1 antibody (Dako) was used as a negative control. Scale bar = 50 μm (Brain tissue = 20 μm).

4.4.3 ADAM22 expression is maintained in matched primary and metastatic tissue

While ADAM22 expression in primary breast tumour tissue is indicative of poor disease free survival, no studies have examined whether ADAM22 expression is also present in metastatic tissue. As such, ADAM22 expression was examined in matched patient primary and metastatic tissue by immunohistochemistry and using RNAseq data generated within our lab.

ADAM22 protein expression was first examined in matched primary and metastatic ER+ endocrine treated breast cancer tissue. Two sets of tumours with pathologically matched contralateral breast metastases were stained with an ADAM22 polyclonal antibody. Strong ADAM22 expression was maintained in both primary and metastatic tissues tested (figure 4.7). ADAM22 expression was then examined in a larger panel of ER positive matched metastatic tissue using the ADAM22 mAb (sc-373931). ADAM22 protein expression was detected in all primaries and matched metastatic
tissues (*figure 4.8.a*). Clinical characteristics for each patient, including receptor status, treatment history and site of metastases are shown in *figure 4.8.b*.

**Figure 4.7: ADAM22 expression is maintained in matched patient primary and metastatic contralateral breast tissue.** FFPE primary and metastatic tissue were sectioned and stained with 2.5μg/ml of ADAM22 polyclonal antibody (Novus #H00053616-B01) using sodium citrate (pH6) antigen retrieval. Scale bar = 50 μm
Figure 4.8: Confirmation that ADAM22 expression is maintained in matched primary and metastatic tissue. (a) Representative images of primary and matched metastatic tissue stained with the ADAM22 mAb (sc-373931) and imaged at 20x magnification. Scale bar = 50 μm. (b) Patient information for ADAM22 stained primary and matched metastatic tissue, including ER, PR and HER2 status; treatment received; and metastatic site.

In order to further assess the relationship between ADAM22 and metastatic development, ADAM22 mRNA levels were investigated in an RNAseq dataset of breast cancer patient tissue, comprising: 7 primary tissues with no metastases; 3 patient matched primary and liver metastases; 6 patient matched primary and brain metastases (and one unmatched brain metastases); 4 patient matched primary and bone metastases (and one unmatched bone metastases); and 3 patient matched primary and local metastases. ADAM22 transcript expression was first assessed in primary breast tumour tissue from patients who did not recur (n=7) and primary
breast tissue from patients who did recur (n=16). A significant increase in ADAM22 expression was observed in primary breast tumours which went on to metastasise versus primaries which did not recur ($p=0.047$) (figure 4.9.a).

In order to investigate whether ADAM22 expression was associated with a specific metastatic site, transcript levels were then examined in the brain (n=7), bone (n=5), liver (n=5) metastatic tissues and good outcome primary tissue (n=7) and compared against transcript levels seen local metastatic tissue (n=3). Interestingly, ADAM22 expression was significantly higher in metastatic brain tissue ($p=0.0262$) and was elevated in bone metastatic tissue, however this increase was not significant ($p=0.202$) (figure 4.9.b).

As ADAM22 mRNA expression was elevated in brain metastases, this site specific relationship was explored further using an expanded cohort of matched patient primary and brain metastatic tissues (n=21) (figure 4.9.c). ADAM22 mRNA expression was significantly increased in all but one matched brain metastases in a total patient population (left, $p=0.0004$). ADAM22 expression was also significantly increased in brain metastatic tissue from ER positive (centre, $p=0.0117$) and triple negative patients (right, $p=0.0078$).
Figure 4.9: ADAM22 mRNA expression is significantly higher in primary tissue with worse outcomes and is significantly upregulated in brain metastatic tissue. (a) ADAM22 mRNA expression, displayed as counts per million, was examined in primaries with good outcomes (good primary; n=7) and primaries which metastasised (bad primary; n=16; p=0.047) (b) ADAM22 mRNA expression was examined by RNAseq in good primary tissue (n=7) bone (n=4), brain (n=7), liver (n=5) and local (n=3) metastatic tissues. ADAM22 expression in each metastatic site was compared against levels in good primary tissue. *p=0.0262 (c) ADAM22 mRNA expression was examined in matched primary and brain metastatic tissue in all patients (left; n=21; p=0.0004), ER +ve patients (centre; n=9; p=0.0117) and triple negative patients (right; n=8; p=0.0078). (Wilcoxon signed rank test)
4.5 Discussion

The overall aim of this chapter was to examine the potential of ADAM22 as a metastatic biomarker in breast cancer. As previous work from our lab established ADAM22 expression as a predictor of poor disease free survival in breast cancer patients, the first goal of this chapter was to begin the process of developing a highly specific, sensitive and reproducible IHC based ADAM22 assay. IHC based assays are desirable in the clinic for biomarker detection, as they are commonly utilised, are efficient and are relatively cheap to set up [221]. The benefit of using monoclonal antibodies over polyclonal antibodies is well established. Monoclonal antibodies have high reproducibility and low cross reactivity, however, while specificity for a single epitope is high, this can also be seen as a limitation due to epitope masking [221].

Two monoclonal antibodies were investigated in this study, however, only the ADAM22 mAb (sc-373931) showed specific and wide ranging staining of breast cancer tissue. In the training set population, ADAM22 expression predicted poor disease free survival. However, this significance was lost in the validation set. Only partial overlap was observed between the ADAM22 polyclonal and monoclonal antibodies, suggesting recognition of different epitopes on the ADAM22 protein or partial masking of the monoclonal antibody epitope. While it is unclear why there was such a discrepancy between the polyclonal and monoclonal antibodies, it is worth noting that ADAM22 has multiple variants [133]. One of the important considerations in the development of biomarkers is the ability of the antibody used to detect the specific disease causing isoform of the target protein [222]. For example, prostate-specific antigen (PSA), an important biomarker for prostate cancer, is found in several isoforms, resulting in a level of bias in clinical assays which was previously found to be unacceptable for use in the clinic [223]. Future studies should examine the specific role of ADAM22 variants in breast cancer.

Interestingly, when the validation set was subdivided into different subtypes, the closest association between ADAM22 positivity and poor disease free survival was seen in the luminal B patient group. While the correlation was insignificant, it is worth noting that luminal B patient numbers were limited. When examined in online gene expression datasets, a clear and significant association was observed between
ADAM22 expression and disease free survival, specifically in luminal b patients. Previous reports implicating SRC-1 as a driver of metastases in the SRC-1\(^{+}\);MMTV-PyMT and SRC-1\(^{-}\);MMTV-neu breast cancer models were both ER negative, suggesting SRC-1 drives this phenotype in an ER-independent manner [77]. Similarly, SRC-1 was shown to drive ADAM22 expression in an ER-independent manner in an endocrine resistant luminal B cell line [100]. As the luminal B subtype is in part characterised by low expression of ER regulated genes [224], this suggests that, as resistance to endocrine therapy develops, SRC-1 increasingly acts in an ER-independent manner, upregulating ADAM22 expression to promote metastases.

In order to assess the druggability of ADAM22, its endogenous mRNA and protein expression levels were examined in healthy tissues. As expected, ADAM22 was predominantly found in tissues of the central nervous system. ADAM22 was first discovered as a protein with high expression levels in mouse brain tissue [111]. A functional role in neural development was then suggested as ADAM22 knockout mice suffered from severe ataxia and were found to have peripheral nerve hypomyelination [163, 200]. Similar roles for ADAM22 in neuronal development were later established through interactions with the post synaptic scaffolding proteins LGI1 and PSD-95 [114, 225] and through interactions with LGI4 to regulate Schwann cell differentiation [116]. ADAM22 and the closely related ADAM11 and ADAM23 proteins are unique to the ADAM family of proteins in that their tissue distribution is limited almost exclusively in central nervous tissues [110]. One of the key characteristics of druggable targets is low or specific tissue expression, so as to avoid off target side effects [220]. No studies have examined the potential side effects of targeting ADAM22 in normal tissue; however it is important to note that previous ADAM22 knockout studies in mice led to severe ataxia and hypomyelination [200]. While this suggests ADAM22 inhibition could impair this process in the early stages of development, it is unclear what toxicities could be observed in adult tissues.

Here, the role of ADAM22 in metastatic development was further established by examining ADAM22 expression in matched primary and metastatic tissues. ADAM22 protein expression was maintained in all ER+ matched patient primary and metastatic tissues examined by IHC. This result is consistent with the functional role ADAM22 plays in both early seeding and late colonisation events of the metastatic
cascade. Furthermore, it suggests ADAM22 may be a candidate drug target both in primary tumours to prevent metastases as well as in established metastases.

ADAM22 mRNA expression was examined in high throughput RNAseq data from a cohort of matched primary and metastatic tissues. The first finding was that ADAM22 expression was higher in primary breast tumours which went on to metastasise than in primaries which did not. While this result was insignificant, it is consistent with the role ADAM22 expression plays in metastatic development, and further emphasises its potential as a predictive biomarker for metastases.

Interestingly, ADAM22 expression was significantly higher in brain metastatic tissue than other sites of metastases. To explore this further, in an expanded cohort of matched primary and brain metastatic tissues, a gain in ADAM22 expression was observed in all but one case. It is important to note that all RNAseq data was obtained from primary and metastatic tissue with a high tumour content (>70 %), which suggests that any detectable ADAM22 expression observed was breast specific. This suggests that ADAM22 may be conferring a brain specific organotropism in the endocrine resistant setting.

The concept of site specific metastases is not in any way new. In fact, it was Stephen Paget in 1889, who first suggested the seed and soil hypothesis, while trying to understand “what organs shall suffer a case of disseminated cancer” [226]. This organotropism is evident in the clinic, where different cancers preferentially colonise specific organs. For example, oestrogen receptor positive breast cancer is known to preferentially metastasise to the bone [227]. This is in part explained by osteomimicry, whereby proteins, normally expressed in the bone microenvironment, are upregulated in the breast [228]. The expression of RANK (receptor activator of NF-κB) and RANKL (receptor activator of NF-κB ligand), proteins involved in the bone remodelling pathway, are known mediators in the ER/PR signalling axis in the mammary epithelium, and are specifically implicated in the development of bone metastases [229].

The results here suggest ADAM22 expression primes breast cancer cells to metastasise to the brain. ADAM22 expression may confer a level of neuromimicry in disseminated breast cancer cells, which enables them to adhere to and interact with the brain microenvironment. In a landmark paper examining the genes which
mediate breast to brain metastases, Bos et al showed that ST6GALNAC5, a protein whose expression is normally restricted to the brain, was upregulated in breast cancer and mediated brain metastases through its interaction with the blood brain barrier [230]. This brain specific tropism is also consistent with findings in the previous chapter. ADAM22 expression was shown to promote hexokinase-2 and VEGFR2, two proteins associated with the development of brain metastases [191, 231]. Furthermore, ADAM22 expression was also significantly associated with neurotrophin signalling, a neural development signalling pathway recently implicated in the establishment of breast cancer brain metastases [206, 207].

While traditionally, the development of breast cancer brain metastases is a late event [232], the incidence is on the rise, largely due to the efficacy of systemic therapies at targeting extracranial disease [231, 233]. In total, close to one third of breast cancer patients develop brain metastases, although the incidence rate varies greatly by subtype [231]. For instance, brain metastases are increasingly the first site of relapse in HER2 positive breast cancer [234]. The frequency of brain metastases as their first site of relapse in luminal B patients has been reported as 0%, compared to a frequency 11% of luminal b patients who eventually develop brain metastases [233]. Of note, a reported 40.2 % of luminal A patients have been shown to switch to a luminal B subtype from primary to brain metastases [27]. Similarly, a number of ER + primaries have been reported to switch towards a HER2 subtype in matched brain metastases, highlighting the importance of subtyping brain tumours where possible [26]. It is also worth noting that current targeted therapies, such as Trastuzumab show limited activity in the brain [231]. This outlines not just the need for novel biomarkers, but also the need for novel therapeutic strategies, particularly in the treatment of emerging diseases such as brain metastases.

While further work is needed to establish an ADAM22 assay, the results here suggest ADAM22 has strong prognostic potential as a biomarker for endocrine resistant metastatic breast cancer. High ADAM22 expression predicts poor disease free survival; elevated levels in the primary tissue are associated with metastatic development; and ADAM22 may be promoting colonisation of the brain. Finally, relatively exclusive expression of ADAM22 in the central nervous tissue suggests that ADAM22 targeting would be safe; however, potential adverse neurological effects should be monitored carefully.
5 An LGI1 mimetic as companion therapeutic
5.1 Introduction

Metastatic breast cancer is a largely untreatable disease. Despite improvements in treatment options and standard of care, no significant improvement in survival has been seen in patients with distant recurrences over a thirty year period [25]. The development of endocrine resistance makes it harder to treat overt metastases and so, current treatment strategies for this advanced patient group involves overcoming the more defined modes of resistance.

The most common mechanisms of endocrine resistance in the clinic involve activating mutations in ESR1 [47]; hyper-activation of the PI3K/AKT/mTOR signalling pathway [66]; and overexpression of Cyclin D1, which leads to activation of CDK4 and CDK6 [63]. Understanding these mechanisms of resistance has allowed several new targeted therapies to emerge as a way of overcoming these resistance pathways. Mutations in the ligand binding domain of ESR1 commonly occur in patients pre-treated with aromatase inhibitors, which leads to the ligand independent activation of the oestrogen receptor [51]. For these patients, fulvestrant, which actively degrades ER, may be of benefit [54, 55]. The FIRST and FALCON studies have shown that fulvestrant is the most effective endocrine therapy for treatment naïve patients with advanced breast cancer [235-238]. In the PALOMA-3 trial, fulvestrant showed similar benefits for endocrine resistant patients when used in combination with the CDK inhibitor palbociclib [239]. CDK inhibition has also shown great promise for patients with advanced breast cancer alongside both first and second line endocrine therapy and has received regulatory approval in the US [47].

One of the most significant mechanisms of resistance to therapy is hyperactivation of the PI3K/AKT/mTOR pathway [47]. This has led to the development of the mTOR inhibitors, such as everolimus. In the BOLERO-2 trial, patients with metastatic breast cancer who had recurred on prior AI therapies were treated with exemestane and everolimus or exemestane alone, with the former leading to a significant improvement in progression free survival [66]. Despite the prevalence of PIK3C mutations in advanced breast cancer, clinical trials using pan- and isoform specific inhibitors have yielded only limited success [47]. While these novel therapies have shown great promise, there is still a lack of clinically relevant biomarkers to identify which patients are likely to gain the most benefit from them [47]. Furthermore, while these therapies target the more common endocrine resistant pathways, there is still
a need for novel therapeutics which can target the mediators of the metastatic cascade.

While many preclinical tests for anti-metastatic therapies yield great promise, the success rate in clinical trials has been disappointingly poor. The reasons for this are multi-faceted. Traditional therapies target aberrant pathways within the primary tumour, with the intention of preventing progression. As such, traditionally approved drugs are assessed on the basis of their anti-tumorigenic potential (i.e. their ability to shrink the tumour in the clinic) [24]. Clinical trials also assess the efficacy of treatment using end points such as progression free survival and overall survival [24]. This poses a problem with regard to anti-metastatic therapies, as those in development, may not directly affect the size of the primary tumour, but instead halt metastatic outgrowth; therefore, they may have no direct effect on overall or progression free survival end points [24].

One of the rare success stories in the clinic has been the use of the monoclonal antibody therapy denosumab, which targets RANKL, a key protein involved in the development of bone metastases [240]. In clinical trials, skeletal related events (i.e. events related to the development of bone metastases) were used as clinical end points. Using this output, clinically relevant responses to denosumab were shown in patients with pre-existing bone metastases; while in the adjuvant setting, the development of bone metastases was also significantly delayed [241, 242]. Despite the success of denosumab, other anti-metastatic therapies have not shown as much promise. The anti-VEGF monoclonal antibody bevacizumab, now FDA approved, showed great pre-clinical efficacy as an inhibitor of primary tumour growth and metastases [243, 244]. However, mixed responses have been observed in the clinic [245, 246]. Similarly, the SRC kinase inhibitor dasatinib showed great promise in the pre-clinical setting [247], with overall disappointing results in the clinic, particularly in the metastatic breast cancer setting [248]. Effective anti-metastatic therapies are close, however, a lack of suitable biomarkers and a lack of suitable metastases related clinical trial end points (i.e. such as in the denosumab trials) may hinder this process.
As up to 90 % of cancer related deaths are caused by the development of metastases [249], it is vital that new anti-metastatic therapies are developed with achievable end point results in clinical trials.

Each stage of the metastatic process is potentially targetable [24]. Some of the key characteristics which are desirable in druggable targets include: membrane localisation; involvement in crucial biological pathways; presence of distinct binding sites; structural characterisation; and limited but distinct tissue expression [250]. ADAM22, as a target, is quite attractive in this sense, as functionally, it would seem to play distinct roles in the metastatic cascade. ADAM22 is also a transmembrane protein with multiple extracellular binding domains with tissue expression specific to the brain, suggesting anti ADAM22 therapies would have a relatively low toxicity profile. Furthermore, the efficacy of any targeted therapy against ADAM22 would be significantly aided by the use of ADAM22 as a biomarker.

5.2 Rationale for targeting ADAM22 with LGI1

LGI1 or leucine rich glioma inactivated 1, was first discovered while mapping a chromosomal region which was frequently lost in glioma [251]. LGI1 expression is highly expressed in the brain; however, LGI1 expression is reduced and finally lost during the progression of glioma [251]. It was first shown to be an endogenous ligand of ADAM22 in the brain, forming a complex with the PSD-95 protein [114]. The LGI1 ADAM22 interaction was later confirmed in a separate study [110].

Aside from its ADAM22 related functions, LGI1 has also been described as an active tumour suppressor in several glioma cell lines, where forced LGI1 expression was shown to inhibit proliferation, migration and anchorage independent growth [252]. LGI1 was found to actively inhibit ERK1/2, leading to downregulation of several matrix metalloproteases, thereby inhibiting metastases [253]. In neuroblastoma cells LGI1 expression also impaired cell growth, by increasing apoptotic activity, further enhancing its potential role as a tumour suppressor [254]. Similar LGI1 mediated anti-proliferative and pro-apoptotic effects were also observed in HeLa cells [255]. Both its roles as a tumour suppressor and a potent ligand for ADAM22 provided the rationale to explore its potential as an ADAM22 targeting therapeutic.
5.3 Preliminary work

Previous reports have shown that recombinant LGI1 treatment was sufficient to inhibit migration in endocrine resistant cell lines (*figure 5.1.b*) [100]. Treatment with LGI1 also restored differentiation (*figure 5.1.c*) and inhibited anchorage independent growth (*figure 5.1.d*) [Unpublished work by Dr, Jarlath Bolger]. LGI1 treatment was clearly shown to be an effective strategy for reversing ADAM22 associated metastatic phenotypes in endocrine resistant cells. With this in mind, a small peptide mimetic of LGI1 was designed.

The binding region on LGI1 for ADAM22 had previously been described [114]. Using 3D modelling, a small peptide mimetic of LGI1 was designed (by Dr Kieran Brennan) using the I-Gasser server, based on the unobstructed and charged regions of the LGI1 and ADAM22 proteins. The resulting 22 amino acid peptide (KGDVYICLTRFIGDSKVMKWGG) was modified to contain a serine residue instead of a cysteine residue to prevent disulphide binding and improve water solubility (KGDVYISLTRFIGDSKVMKWGG). This LGI1 peptide mimetic was synthesised by JPT, Berlin to a purity of > 95%.

In vitro efficacy of the LGI1 mimetic was then shown in the endocrine resistant models. Both full length LGI1 and the LGI1 mimetic inhibited the migration of LY2 and LetR cells (*figure 5.1.e*), while the peptide mimetic also restored the ability of LY2 cells to form more differentiated acinar structures (*figure 5.1.f*).
Figure 5.1: LGI1 and its peptide derivative are biologically active in vitro. (a) Schematic of ADAM22 and likely binding site of the LGI1 mimetic (illustration adapted from [103]). (b) MCF7, LY2 and LetR cells were seeded onto collagen and blue fluorescent microsphere coated wells and treated with 5nM of recombinant LGI1 or a vehicle control. 20 hours post seeding, cells were fixed and imaged under blue fluorescent light. Average migration was calculated based on the area cleared by individual cells. [100] (c) MCF7, LY2 and LetR cells were cultured in matrigel for 2 weeks and stained with DAPI (blue) and Phaloidin (red), to examine the effect of LGI1 treatment on their ability to form differentiated acini. Cells were imaged with a confocal microscope. [100] (d) MCF7 and LY2 cells were cultured in soft agar for two weeks and stained with p-iodinotetrazolium chloride. Average number of colonies were counted per well. (e) MCF7, LY2 and LetR cells were seeded onto collagen and blue fluorescent microsphere coated wells and treated with 5 nM of recombinant LGI1, 10 nM of LGI1 mimic or a vehicle control. 20 hours post seeding, cells were fixed and imaged under blue fluorescent light. Average migration was calculated based on the area cleared by individual cells. (f) LY2 cells were cultured in matrigel for 2 weeks and stained with DAPI (blue) and Phaloidin (red), to examine the effect of LGI1 treatment on their ability to form differentiation acini. Cells were imaged with a confocal microscope. [Work carried out by Dr Damian McCartan and Dr Jarlath Bolger (unpublished)]
Preclinical modelling of disease *in vivo* is paramount to drug development, particularly with regard to toxicity and efficacy studies. As such, toxicity was examined (by Dr Jarlath Bolger) in NOD/SCID mice treated with three concentrations of the LGI1 mimetic. In order to assess adverse effects, variations in weight, behaviour and gait were monitored throughout the treatment period. No difference in weight was observed between groups, suggesting treatment studies with the LGI1 mimetic should yield no adverse effects on the welfare of mice *in vivo* (*n*=6, 2 mice per treatment group) (*figure 5.2.a*).

A preliminary efficacy study was also carried out (by Dr Christopher Byrne) to examine whether the LGI1 mimetic had an effect on primary tumour growth. Daily treatment with the LGI1 mimetic inhibited primary tumour growth when compared to vehicle treated mice (*figure 5.2.b*), however, the study lacked sufficient numbers to be deemed significant (*n*=4, 2 mice per group). As ADAM22 expression is implicated in the development of metastases, the potential anti-metastatic effect of LGI1 mimetic treatment was explored (by Dr Damir Vareslija and Sinead Cocchi glia). Although insignificant, this preliminary study shows the LGI1 mimetic may be an effective anti-metastatic therapy (*figure 5.2.c*).
Figure 5.2: Preliminary testing shows the LGI1 mimetic is safe and effective in vivo. (a) NOD SCID mice were given LGI1 mimetic at 1, 10 or 100 µg/mouse/day for 30 days by intraperitoneal (IP) injection. Weight changes were the primary measure for toxicity. (b) 1 x 10^6 LY2luc cells in a PBS/Matrigel mixture were injected into the mammary fat pad of NOD SCID mice and allowed to form primary tumours. Mice were split into two treatment arms: Vehicle treated vs LGI1 mimetic (100 µg/mouse/day) (2 mice per group). Mice were treated daily for 30 days by IP injection. Caliper measurements were taken weekly. (c) Slow release estrogen and 4-OHT pellets were implanted subcutaneously in NOD SCID mice. 1 x 10^6 LY2luc tagged cells in a PBS/Matrigel mixture were injected into the mammary fat pad mice and allowed to form primary tumours. At week 5, primary tumours were surgically removed and mice were split into two daily treatment arms: Vehicle vs LGI1 mimetic (100 µg/mouse/day). Local and distant recurrences were examined using an IVIS imaging system, after injection with luciferin (10 µl / gram of a 15mg/ml stock). At week 15, mice were sacrificed and metastatic development was examined in brain, bone lung and liver by IVIS imaging.
5.4 Aims

Preliminary data shows the LGI1 mimetic is biologically active and can reverse the metastatic phenotypes associated with ADAM22 expression in endocrine resistant breast cancer. Furthermore, preliminary studies showed the LGI1 mimetic to be non-toxic to healthy mice, while having both anti-proliferative and anti-metastatic effect in xenograft mouse models. As such the aims of this study are:

- To show interaction of the LGI1 mimetic with ADAM22
- To further establish in vitro efficacy of the LGI1 mimetic
- To investigate the anti-metastatic potential of the peptide \emph{in vivo}
5.5 Results

5.5.1 LGI1 mimetic interacts with ADAM22

Previous studies have shown that LGI1 is the endogenous ligand to ADAM22 in the brain [110, 114, 201, 256, 257]. One of the key characteristics of peptidomimetics is their ability to bind to and block critical binding “hotspots” on target proteins [258]. By mimicking the conformational structure of endogenous ligands, peptidomimetics offer the benefit of interacting with these binding hotspots, improving membrane permeability, while often avoiding peptidase degradation and immunogenicity [258]. As mentioned earlier, both full length LGI1 and the LGI1 mimetic were shown to be functionally active, inhibiting both migration and de-differentiation of endocrine resistant cell lines. In order to show that this observed functional activity is a result of targeting ADAM22, both in silico (by Nicola Cosgrove) and in vitro interaction studies were carried out.

In order to examine whether the LGI1 mimetic interacts with ADAM22 in silico, predicted 3D models for ADAM22 and the LGI1 mimetic were first generated using the I-TASSER server [137]. Using the CABS-dock server, an online tool which allows for an accurate and highly flexible prediction of peptide-protein interactions without prior knowledge of binding sites, the ADAM22-LGI1 mimetic predicted binding sites were analysed [138]. A stringent threshold distance of less than 3 Ångströms was set for determining most likely ADAM22-LGI1 mimic interactions. As predicted, the LGI1 mimetic (pink) binds to and interacts with the disintegrin domain (yellow) of the ADAM22 protein (grey) (figure 5.3.a). Contact hotspots for this predicted in silico interaction show the LGI1 mimetic is likely to bind to the ADAM22 disintegrin domain between amino acid residues 492 – 498 (figure 5.3.b).

The ADAM22 LGI1 mimetic interaction was then examined in vitro, using a biotinylated form of the peptide and streptavidin Dynabead™ technology, as previously described [259]. Biotinylated LGI1 mimetic (Biotin-LGI1) was able to pull down ADAM22 following incubation with LY2 lentiADAM22 lysate and elution from streptavidin beads (figure 5.3.c).
Figure 5.3: *In silico* and *in vitro* confirmation of the ADAM22 and LGI1 mimetic interaction. (a) CABS-dock predicted binding of the LGI1 mimetic (pink) to the ADAM22 disintegrin domain (yellow). (b) CABS-dock contact map for LGI1 mimetic and ADAM22 protein using a <3 Angstrom threshold. Y-axis contains amino acid residues for the LGI1 mimetic and contact point amino acid number within the ADAM22 disintegrin domain. X-axis contains ADAM22 amino acid sequences of alternative LGI1 mimetic contact points. (c) Western blot confirmation of ADAM22 interaction with a biotinylated LGI1 mimetic (n=3). Total protein from LY2 lentiADAM22 cells was extracted and incubated with either biotinylated LGI1 or a no bait control. Using dynabead M280 streptavidin beads, proteins interacting with biotin-LGI1 were pulled out of the input lysates (input). Interacting proteins (prey) were dissociated from the dynabeads and blotted with an ADAM22 specific antibody (R&D systems – MAB49081).
5.5.2 The LGI1 mimetic inhibits migration, anchorage independent growth and self-renewing capacity of endocrine resistant cells

Functionally, the LGI1 peptide mimetic was previously shown to inhibit migration of both tamoxifen and letrozole resistant cell lines to similar levels observed after ADAM22 siRNA knockdown (figure 5.1.e). Furthermore, treatment of these cell lines with the LGI1 mimetic restored their ability to form well differentiated acinar structures, as previously described after ADAM22 knockdown (figure 5.1.f). Together, these preliminary findings suggest the LGI1 mimetic, through its ability bind to ADAM22, can inhibit ADAM22 mediated phenotypes in endocrine resistant cells.

As described in chapter 3, ADAM22 expression in endocrine resistant cell lines contributes to the metastatic phenotype through its ability to promote anoikis resistance and a cancer stem cell phenotype. Here, the effect of the LGI1 mimetic on these metastatic characteristics was investigated.

The ability of the LGI1 mimetic to inhibit the migration of the highly motile LetR cells was first examined to account for any potential batch-to-batch variation or improper formulation. The LGI1 mimetic significantly inhibited LetR migration at each concentration tested (n=3; \(p<0.0001\)) compared to untreated and vehicle treated controls (figure 5.4.a). As ADAM22 expression confers an anoikis resistant and stem cell like phenotype in these endocrine resistant cell lines, the effect of LGI1 mimetic treatment on these features was examined. Treatment with the LGI1 mimetic (20nM) significantly inhibited LY2 anchorage independent colony formation by approximately two fold (n=3; \(p=0.0022\); figure 5.4.b). Similarly, LGI1 mimetic treatment had an inhibitory effect of both first (n=5; \(p=0.0014\); figure 5.4.c, left) and second generation (n=4; \(p=0.0085\); figure 5.4.c, right) mammosphere forming efficiency when compared against untreated and vehicle controls. No significant difference was seen in mammosphere forming efficiency between untreated and vehicle treated cells in the first generation; while a small but significant increase in mammosphere forming efficiency was observed in the second generation between untreated and vehicle treated groups (\(p=0.0198\)).
Figure 5.4: The LGI1 mimetic inhibits migration, anoikis resistance and mammosphere formation. (a) LetR cells were seeded onto collagen coated and blue fluorescent microsphere coated plates and were cultured in growth media alone, or supplemented with 5, 10 and 20nM of the LGI1 mimetic or a vehicle control. Cells were fixed and imaged to assess individual cell migration. Average migration was calculated from a minimum of 100 cells per treatment. Results representative of n=3 (p=0.0001). (b) The inhibitory effect of LGI1 mimetic treatment on anoikis resistance was examined in LY2 cells using an anchorage independent growth assay. LGI1 mimetic treatment resulted in a significant reduction in the number of colonies formed (n=3; p=0.0022). (c) LY2 mammosphere formation was examined after treatment with the LGI1 mimetic. The LGI1 mimetic significantly inhibited 1st generation (left) MFE (n=5; p=0.0014) and 2nd generation (right) MFE (n=4; p=0.0085).
5.5.3 The LGI1 mimetic inhibits the metastatic potential in vivo

Functionally, the LGI1 mimetic inhibits metastatic characteristics associated with ADAM22 expression in endocrine resistant cells in vitro. Preliminary studies examining the efficacy of the LGI1 mimetic in vivo were extremely promising; both as a tumour growth inhibitor (figure 5.2.b) and as an anti-metastatic therapeutic (figure 5.2.c). Interestingly, in chapter 4, ADAM22 expression was found to be higher in primary tumours which metastasised versus those that did not. Furthermore, ADAM22 expression was found in all matched metastatic tissue. These results suggest ADAM22 is involved in the early seeding events of metastases. In the clinical setting, the dissemination of tumour cells is an early event, however, only a tiny fraction of these cells has the capacity to form distant metastases [22]. Here, the ability of the LGI1 mimetic to inhibit the metastatic potential of these early disseminating cells was examined.

LY2 luciferase tagged cells were orthotopically injected into the mammary fat pad of mice treated with both oestrogen and tamoxifen. In this model of the disease, tamoxifen drives both the proliferation of these endocrine resistant tumours and ADAM22 expression [100]. In order to replicate the early seeding events of metastatic endocrine resistant breast cancer and to represent realistic tumour size as seen in the clinic, tumour cells were seeded a lower density than in preliminary studies. A schematic of the experimental design is shown in figure 5.5.a. Once the primary tumours were palpable, mice were treated daily with either the LGI1 mimetic (100 µg/day) or a vehicle control for a total of 30 days by IP injection. Primary tumour growth was measured twice weekly by calliper measurement and weekly by IVIS imaging. The mice were monitored daily for signs of toxicity, with scoresheets assessing changes in weight, mobility, appearance and behaviour filled out twice weekly throughout the treatment period. Primary tumours were significantly smaller than those in preliminary studies (figure 5.5.b; bottom vs top). No significant difference in primary tumour growth was observed between treatment groups using calliper measurements (figure 5.5.b; bottom) or bioluminescent readings (figure 5.5.c), however, one tumour in the LGI1 treated group (bottom right) was unexpectedly larger (approximately 2-fold) than all other tumours (figure 5.5.d). There were no adverse effects associated with either treatment.
In order to examine the effect of the LGI1 mimetic as an inhibitor of early metastatic disease, metastatic bioluminescence was examined in the bone, brain, liver and lung ex vivo by IVIS imaging. Metastatic burden was observably reduced in the LGI1 mimetic treated mice in all organs except the liver (figure 5.6). The level of metastatic spread in the bone and lung were observably diminished in LGI1 mimetic treated mice; while the LGI1 mimetic prevented all metastatic development in the brain. Using Living Image analysis software, each organ was examined for detectable bioluminescence. A total flux score (photons per second) for each organ is represented in figure 5.7. LGI1 mimetic treatment significantly reduced bioluminescent signal in the bone ($p=0.007$; figure 5.7.a) and brain ($p=0.038$; figure 5.7.b). No significant differences in bioluminescence were observed for the liver (figure 5.7.c) or lungs (figure 5.7.d).
Figure 5.5: The LGI1 mimetic does not inhibit early tumour growth in vivo. (a) Schematic of the in vivo experiment to assess the effect of LGI1 mimetic treatment on early metastatic development. Once primary tumours were palpable, vehicle or LGI1 mimetic (100 µg/mouse) was administered by daily IP for a total of 30 days (b) Comparison of tumour growth in the preliminary (top) and early seeding studies (bottom). Mean primary tumour growth was examined by calliper measurements for each mouse twice a week. No significant differences in tumour growth was observed in the early seeding study between LGI1 mimetic (red; n=7) and vehicle treated mice (blue; n=7). (c) Mean primary tumour growth was assessed by bioluminescence weekly. Mice were injected with luciferin (150 µg/g) 15 minutes prior to imaging. Mice were then anaesthetised by isoflurane exposure and imaged in an IVIS imaging system. No significant differences were observed between LGI1 mimetic (red) and vehicle treated mice (blue). (d) Representative photograph of primary tumours at EOX.
Figure 5.6: The LGI1 mimetic inhibits brain and bone metastases, while reducing metastatic burden in the lungs. Metastatic development in major organs was assessed by IVIS imaging at EOX. Bioluminescent images for bone, brain, liver and lung are displayed with vehicle treated shown on the left and LGI1 mimetic treated on the right.
Figure 5.7: LGI1 mimetic significantly inhibits metastatic development to the brain and metastatic burden to the bone. (a) Metastatic burden was significantly reduced in the bone \((p=0.0070)\). (b) Metastatic development in the brain was inhibited in all LGI1 mimetic treated except one mouse \((p=0.0338)\). (c) No significant difference in liver metastases was observed in LGI1 mimetic or vehicle treated groups. (d) Average bioluminescence was reduced in LGI1 mimetic treated mice, however this was not significant. Bioluminescence was recorded for each site of metastases using Living image software. Statistical significance was analysed using a two tailed Mann Whitney non-parametric t test.
5.6 Discussion

In previous chapters, the role ADAM22 plays in the development of endocrine resistant metastatic breast cancer was established and characterised. The evidence suggests that as ER positive tumours progress, their dependency on ER is lost, increasingly through SRC-1 mediated transcriptional control [100]. SRC-1 promotes the initiation of metastases through the upregulation of ADAM22 [100]. Functionally, ADAM22 enhances de-differentiation and migration in the early stages of disease; promotes survival of circulating tumour cells; and finally, through its ability to regulate stem cell properties, promotes colonisation. While ADAM22 expression predicts poor disease free survival in patients, its expression is also elevated both in primary tumours which metastasise as well as in metastatic tissue, suggesting it is involved in both early dissemination events and later colonisation events.

LGI1, the endogenous ligand to ADAM22, was previously described as a potent tumour suppressor in several cancer cell lines [252-255]. Previous work from our lab had shown that recombinant LGI1 could inhibit ADAM22 mediated migration in endocrine resistant cell lines [100], leading to the design of a small LGI1 peptide mimetic as a potential ADAM22 companion therapeutic. Preliminary work from our lab showed the LGI1 mimetic was biologically active in endocrine resistant cells both in vitro and in vivo. Here, the ability of an LGI1 mimetic to inhibit ADAM22 mediated metastatic function and disease was explored further.

While LGI1 and ADAM22 are known to interact, no previous studies examined the ability of the LGI1 mimetic to bind ADAM22. Here, 3D modelling and molecular docking of the LGI1 mimetic with ADAM22 was examined in silico. Previous reports have shown the ADAM22 disintegrin domain is important for its interaction with both LGI1 and LGI4 [114, 116]. Using the CABS-dock method, which has recently been shown to be an invaluable tool for studying the dynamic molecular interactions of peptides and receptors [260], the LGI1 mimetic was predicted to bind specifically to the ADAM22 disintegrin domain. Moreover, the LGI1 mimetic was shown to pull out ADAM22 protein from whole cell lysate. These results suggest that the biological activity of the mimetic is likely to be through its ability to interact with and inhibit ADAM22.
Functionally, LGI1 mimetic treatment was sufficient to inhibit each of the metastatic phenotypes ADAM22 was previously shown to drive. While preliminary work showed its ability to inhibit migration and restore differentiation in endocrine resistant tumours; here, a significant reduction was observed in both anchorage independent colony formation and mammosphere forming efficiency. These results suggest the LGI1 mimetic is capable of inhibiting each of the pathways ADAM22 expression confers on endocrine resistant cells.

Previously, ectopic expression of the LGI1 gene in glioma cell lines was shown to inhibit both their migration and anchorage independent growth [252]. Interestingly, this LGI1 mediated inhibition was shown to be mediated in part through negative regulation of ERK1/2 and consequent downregulation of MMP1 and MMP3 [253] As previously mentioned, anoikis resistance is largely dependent on pro-survival signals, specifically through activation of PI3K-AKT, ERK1/2 and integrin signalling pathways [179-181]. ADAM22 expression in endocrine resistant cell lines was shown to promote enhanced PI3K-AKT and ERK1/2 signalling, suggesting the LGI1 mimetic may reverse these phenotypes in part through inhibition of these pathways. Mechanistically, LGI1 has also been shown to promote pro-apoptotic responses in neuroblastoma and HeLa cell lines, through its ability to downregulate Bcl-2 expression [254]. Interestingly, both RPPA studies suggested ADAM22 was playing an anti-apoptotic role in the endocrine resistant setting, in part through upregulation of XIAP and Bcl-2. Taken together these results suggest the LGI1 mimetic may also inhibit pro-survival stimuli which ADAM22 confers in these cells.

As inferred in chapter 3, the possibility that ADAM22 mediates its metastatic characteristics through its ability to bind integrin αvβ3 is intriguing [161]. Although this link has not been established, the specific region of the ADAM22 protein that was shown to be important for both LGI1 and LGI4 binding [114, 116], also happens to be the homologous region in the ADAM23 protein where integrin αvβ3 binds [261]. Interestingly, the LGI1 mimetic was originally designed using this section of the ADAM22 disintegrin domain as a template. Here the CABS-dock LGI1 mimetic predicted binding site is within the vicinity of this integrin binding region. This suggests that ADAM22 may promote de-differentiation, migration, anoikis resistance and stemness through an interaction with integrin αvβ3 and that the LGI1 mimetic
may be able to inhibit this interaction. Future studies are needed to explore this potential relationship.

Preliminary studies in vivo were extremely promising, despite the lack of numbers to show significance. Both functional and clinical studies in chapter 3 and 4, implicate ADAM22 in the early dissemination of tumour cells, which have the potential to form metastases. Here, the ability of the LGI1 mimetic to inhibit these early colonisation events was investigated. In this early stage model, no significant differences in tumour growth were observed, however, as one of the tumours in the LGI1 mimetic treated group was excessively large, the data may be somewhat skewed. On the other hand, as discussed in the introduction to this chapter, anti-metastatic therapies are often dismissed in the clinical setting, due to their lack of effect on tumour growth [24]. While no effect on primary tumour growth was observed, the LGI1 mimetic significantly inhibited all metastases to the brain, with the exception of a single micrometastatic signal in the mouse with the abnormally large primary tumour. Metastatic burden was observably and significantly reduced in the bone; while an observable but modest reduction was also observed in the lung. These results suggest that the LGI1 mimetic is an effective inhibitor of early metastatic events to the brain and potentially to the bone. Importantly, no mice displayed any signs of adverse effects from the treatment.

The efficacy of the LGI1 mimetic at inhibiting the development of brain metastases is particularly interesting. The result ties in well with the suggestion in chapter 4, that ADAM22 expression, may be conferring a level of brain seeking organotropism in endocrine resistant breast cancer CTCs. ADAM22 expression was found to be highest in brain metastatic tissue versus all other metastatic sites. Furthermore, as discovered in chapter 3, ADAM22 expression was significantly associated with neurotrophin signalling, which has been associated with brain tropic metastases in HER2 positive breast cancer [234]. Interestingly, the full length LGI1 protein shares a high level of homology with the neurotrophin receptor Trk, responsible for this brain seeking signal in HER2 positive breast cancer. While the LGI1 mimetic does not contain this homologous region, it lends weight to the concept that ADAM22, as an LGI1 receptor, activates a neurotrophic pathway, leading to the establishment of brain metastases.
The results of the *in vivo* study suggest the LGI1 mimetic is highly potent inhibitor of this ADAM22 mediated brain colonisation. Future studies need to be carried out to examine whether: (a) the LGI1 mimetic can cross the blood brain barrier (BBB); (b) whether crossing the BBB could lead to adverse effects to endogenous ADAM22/LGI1 functions; (c) if the LGI1 mimetic could be used to treat and inhibit established overt metastases.

As previously mentioned, the incidence of breast cancer brain metastases is on the rise, particularly with the advent of improved systemic therapies [231]. Worryingly, while many patients succumb to extracranial disease, autopsy reports have shown that a significant number of breast cancer patients have overt undiagnosed brain metastases [262]. The incidence of brain metastases in luminal b patients is approximately 11% and tends to be a late event as opposed to site of first relapse [233]. This is significant with regard to the potential utility of ADAM22 as metastatic biomarker with the LGI1 mimetic as a companion therapeutic. While a significant number of patients with advanced metastatic disease are living longer; the development of brain metastases remains highly untreatable [263]. As such, there is a growing need for new anti-metastatic therapies, such as the LGI1 mimetic, which can interrupt brain colonisation. Importantly, a number of anti-metastatic therapies have had little success in the clinic due in part through a lack of suitable biomarkers [24]. This highlights the potential of ADAM22 as a biomarker with the LGI1 mimetic as a companion therapeutic.
6 General Discussion
Endocrine therapy is the mainstay treatment for ER positive breast cancer. Unfortunately, a large proportion of patients will eventually acquire resistance and go on to develop metastatic breast cancer [38]. This is highlighted by the fact that while luminal patients initially have the best prognosis; the risk of recurrence is maintained long after initial diagnosis [38].

Several targetable resistance mechanisms have now been described, including activating ESR1 mutations; hyperactive growth factor signalling; and dysregulation of the cell cycle components [47]. Clinically, there has been some success in targeting these resistance mechanisms. For example, fulvestrant, everolimus and palbociclib have all shown efficacy in advanced breast cancer patients with endocrine resistant metastases [48]. While clearly these therapeutic strategies will aid in future clinical decisions; novel strategies for targeting the root cause of metastases need to be established and translated into viable options for these patients [24].

Recent work from our lab and others has highlighted the importance of the nuclear receptor co-activators in the development of resistance to tamoxifen and aromatase inhibitors [83, 85, 98, 264]. These co-regulatory proteins, in the face of endocrine therapy, become overexpressed [81], which can lead to both ligand independent activation of ER and to the activation of other oncogenic transcription factors [97]. In the endocrine resistant setting, SRC-1 in particular acts increasingly in an ER independent manner [89, 97]. This is particularly relevant for the luminal B subtype of breast cancer which is in part distinguished from the luminal A subtype by its relatively low expression of ER regulated genes and increased proliferative index [265].

While the promiscuous nature of SRC-1 enhances endocrine insensitivity, a significant role in promoting the development of metastases has been described [93-95, 99]. In the endocrine resistant setting, this metastatic phenotype is in part driven by ADAM22, a neuronal development protein [100]. ADAM22 promotes the migration and de-differentiation of tumour cells, hallmarks of EMT, suggesting it plays a role in the early stages of metastatic development [100]. Interestingly, the research here also demonstrates ADAM22 enhances anoikis resistance, thereby promoting the survival of CTCs. Moreover, ADAM22 expression enriches the cancer stem cell population, implying involvement in the colonisation of distant sites. This versatile
role is explained in part through its association with multiple pro-metastatic pathways.

Consistent with these findings, ADAM22 expression was higher in patient primary tissue which went on to metastasise versus primaries with favourable outcomes. Furthermore, ADAM22 expression was also maintained in all matched primary and metastatic tissue, further implicating it in both the early dissemination and later colonisation events associated with metastases.

Although the ADAM22 clinical assay will need to be developed further, the results here suggest ADAM22 expression may be particularly useful as a predictive tool in the luminal B setting. Clinically, luminal B patients have an unfavourable outcome when compared to luminal A patients, particularly in the early stages of disease [224]. Despite clinical acknowledgement that multi-gene assays have prognostic benefit in identifying luminal patients with high risk of recurrence, these techniques are not globally implemented [4]. Instead, clinicians rely on the classical ER, PR and HER2 markers as the cornerstone for prognosis, with ki67 assessed in some institutions for marginal cases [265]. Thus, the concept of a metastases specific protein biomarker, such as ADAM22, which could be implemented in the clinic make it highly desirable particularly in the era of precision medicine.

One of the key findings of this study was the relative increase in ADAM22 expression in brain metastatic tumour cells compared to primary breast tumour cells. Gene expression analyses of brain seeking tumours are beginning to unravel the mechanisms through which they develop. One large transcriptome analysis study recently showed that the brain microenvironment promoted a complete reprogramming of metastatic cancer cells so that they expressed genes associated with neuronal development [266]. Consistent with this, ADAM22 is primarily expressed in the brain with functions predominantly associated with neuronal development. Furthermore, ADAM22 expression was found here to be associated with the neurotrophin signalling pathway. While not explored in this study, both ADAM22 and neurotrophin signalling both play significant and similar roles in the development of the central nervous system. Additionally, a growing body of evidence has implicated members of the neurotrophin signalling cascade in both the
progression of breast cancer [202]; and more recently in the establishment of breast cancer brain metastases [206, 207].

The primary endogenous ADAM22 ligand in the developing brain is LGI1 [257]. A significant number of studies have described LGI1 as a potent tumour suppressor gene [252-255]. Here, a small LGI1 peptide mimetic capable of binding and inhibiting ADAM22 was established. In our endocrine resistant models, treatment with the LGI1 mimetic reversed each of the metastatic phenotypes associated with enhanced ADAM22 expression. This was perhaps most evident in vivo where the LGI1 mimetic was capable of completely inhibiting the development of metastases to the brain; while a significant reduction in metastatic burden in the bone was also observed. Coupled with elevated levels of ADAM22 expression in brain metastatic tissue and the neurotrophic signature, this suggests ADAM22 expression enhances metastatic colonisation of the brain and the LGI1 mimetic can effectively inhibits this process.

Clinically, the incidence of brain metastases is on the rise [233]. This has been largely attributed to the success of treating extracranial disease with targeted therapies [231]. Moreover, there is an unmet need in the clinic for drugs which can specifically target the metastatic process [24]. Kodack et al recently stated that – “Treatment studies that begin prior to colonization translate, clinically, into prevention studies...If preventative measures are to succeed in the clinic, methods to identify predisposed patients are necessary, and this will entail identification of the expression of relevant proteins in primary or systemic metastases of patient tissue” [231]. Using this as a template for success, it could be suggested that ADAM22 is a legitimate candidate biomarker for identifying predisposed patients, while its companion LGI1 mimetic therapeutic could effectively inhibit this ADAM22 mediated metastases.

ADAM22 knockout mice suffer from ataxia and hypomyelination [200]; while mutations in LGI1 are associated with a rare form of epilepsy [114]. Therefore, any adverse effect from ADAM22 targeting would most likely be neurological. Importantly, no toxicity was observed in any of the preliminary in vivo studies or the preclinical in vivo study carried out here. Evidence suggests that endogenous ADAM22 expression is critical in the early development of the nervous system [267].
Future studies will be required to as whether targeting ADAM22 in adult tissues would be more favourable.

The work described here shows ADAM22 is an important player in the development of endocrine resistant metastatic breast cancer. Throughout the metastatic cascade, ADAM22 contributes to the aggressive and adaptable nature of tumour cells, allowing them to invade, disseminate, survive and colonise distant sites. These findings translate into the clinic, where ADAM22 expression is associated with poor disease free survival in luminal B patients. While ADAM22 expression is higher in primary tissues which go on to metastasise, a significant gain in expression was also observed in brain metastases, suggesting ADAM22 primes circulating tumour cells towards brain colonisation. While this form of the disease is on the rise, outcome is unfavourable and treatment options are limited. Here, a peptide mimetic of LGI1 was shown to effectively reverse the metastatic characteristics induced by ADAM22 in endocrine resistant cell lines. Furthermore, in vivo studies suggest the LGI1 mimetic is not toxic and can prevent the establishment of overt brain metastases. This work suggests ADAM22 is a candidate predictive biomarker for metastatic breast cancer with the LGI1 mimetic as an effective companion therapeutic.


71. Finn, R.S., et al., PALOMA-2: Primary results from a phase III trial of palbociclib (P) with letrozole (L) compared with letrozole alone in postmenopausal women with ER/HER2 advanced breast cancer (ABC). Journal of Clinical Oncology, 2016. 34(15).


7 Appendix

ADAM22 CRISPR/Cas9 KO Plasmid (h) is recommended for the disruption of gene expression in human cells.

Figure 7.1: ADAM22 CRISPR/Cas9 Knockout plasmid (Santa Cruz - sc-405581)
ADAM22 HDR Plasmid (h) is recommended for co-transfection with ADAM22 CRISPR/Cas9 KO Plasmid (h): sc-405581 and designed for repair of the site-specific Cas9-induced DNA cleavage within the ADAM22 (human) gene. During repair, the ADAM22 HDR Plasmid (h) incorporates a puromycin resistance gene to enable selection of stable knockout (KO) cells and an RFP gene to visually confirm transfection.

Figure 7.2: ADAM22 CRISPR/Cas9 HDR plasmid (Santa Cruz sc-405581-HDR)
Figure 7.3: ADAM22 lentiviral plasmid datasheet (AMSBIO – RC219272L2V)
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<td>Peptide content [%]</td>
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**Figure 7.4:** LGI1 mimetic analytical data sheet (JPT, Berlin)
### Analytical Data Sheet

**JE.#**  33238_1  
**PO.#**  8565866  
**Customer**  Royal College of Surgeons in Ireland  
  Mr. Ben Doherty  
**Product**  LGI1  
**Sequence**  Biotin-Ttds-KGDVYISLTRFIGDSKVMKWGG-OH  
**Amount**  1.1 mg  
**Batch no.**  110717L-02  
**Molecular weight (net)**  2986.52 g/mol (average)  
**Specification**  >95% (HPLC - 220nm - C18 - linear gradient)

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<th>Results</th>
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<tbody>
<tr>
<td>Appearance</td>
<td>lyophilized material</td>
</tr>
<tr>
<td>MS [m/z]</td>
<td>996.3 [M+3H]3+  ESI</td>
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<td></td>
<td>747.5 [M+4H]4+  ESI</td>
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<td></td>
<td>598.4 [M+5H]5+  ESI</td>
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<tr>
<td>Purity found [%]</td>
<td>96.3 (see raw data enclosed)</td>
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<tr>
<td>AAA calcd.</td>
<td>n.d.</td>
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<tr>
<td>AAA found</td>
<td>n.d.</td>
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<tr>
<td>Peptide content [%]</td>
<td>n.d. (Non-normalized; see raw data enclosed. Amino acids labeled with an asterisk could not be properly determined due to incomplete hydrolysis or decomposition. Such amino acids were excluded from determination of the peptide content.)</td>
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<tr>
<td>Remark</td>
<td>This product is supplied as a trifluoroacetate salt.</td>
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Figure 7.5: Biotin linked LGI1 mimetic analytical datasheet (JPT, Berlin)
### General Health

#### Eating
- 0: drinking and eating well
- 1: change in eating and drinking habit
- 2: inappetance
- 3: not eating/not drinking

#### Locomotion
- 0: walking normally
- 1: limping, (favouring uninjured limb), stiffness,
- 2: swollen limbs
- 3: severely restricted mobility

#### Behaviour
- 0: normal
- 1: minor changes away from littermates
- 2: aggressive/huddled/ina corner/active
- 3: severe distress/self mutilation/SES/unsolicited vocalization/immobile

#### Physical Appearance
- 0: normal
- 1: ruffled fur/lack of grooming
- 2: rough coat/nasal or ocular discharge
- 3: hunched/abnormal posture/very rough coat

#### Weight Loss
- 0: normal
- 1: 5-10%
- 2: 10-19%
- 3: >20%

#### Tumour Volume
\[ V = \frac{\pi}{6} \times \text{largest diameter} \times (\text{smallest diameter})^2 \]

#### Comments
- M1
- M2
- M3
- M4
- M5

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**Caliper Measurements**

0 = normal

3 in one category = euthanize

1-2 = minor changes, monitor daily

6 = significant changes, monitor twice daily

23/09/2000

Tumour volume: 0.02

Caliper: 28mm

Mice: 318 319

Mice: 320 321
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Presentations:

February 2015 – The development of ADAM22 as a predictive marker for endocrine resistant breast cancer and an LGI1 mimetic as a companion therapeutic. Irish Association for Cancer Research annual meeting (oral presentation)

March 2015 - The development of ADAM22 as a predictive marker for endocrine resistant breast cancer and an LGI1 mimetic as a companion therapeutic. RCSI Research Day (poster presentation)

February 2016 – ADAM22 as a biomarker for endocrine resistant breast cancer. Irish Association for Cancer Research annual meeting (poster presentation)

September 2016 – ADAM22 as a predictive marker in endocrine resistant breast cancer. Irish Cancer Society BreastPredict SAB meeting (moderated poster presentation winner)

January 2017 – ADAM22 as a predictive marker for endocrine resistance with a companion therapeutic. Society of Academic and Research Surgery (SARS) annual meeting (oral presentation)

September 2017 – Deciphering ADAM22 mechanisms in endocrine resistant breast cancer. 7th Global Reverse Phase Protein Array Workshop (display poster)