Reversing the Malignant Proliferation and Doxorubicin Resistance in Human Triple Negative Breast Cancer Cell Line MDA-MB-231 by CRISPR/Cas9-Mediated Deletion of the *KISS1R* gene

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A research component submitted to the Auckland University of Technology in parital fulfilment of the requirements for the degree Master of Science (Research)

January 2025

School of Science

Abstract:

Triple negative breast cancer (TNBC) is a treatment resistant strain of breast cancer (Aysola et al., 2012). Women with TNBC do not benefit from endocrine therapy or Human Epithelial Growth Factor Receptor 2 (HER2) targeted therapies as the relevant drug targets are absent, thus the overall survival (OS) rates of patients diagnosed with TNBC are poor (Aysola et al., 2012). Fayaz and colleagues (2019) suggest the 10-year OS rate of patients with non-metastatic TNBC is 66%, 16% lower than the average breast cancer OS rate, and the 10-year OS rate of patients with advanced TNBC is 0%. These survival rates are likely the result of the lack of effective systemic treatments.

Chemotherapy is one of the only effective systemic treatments available for the treatment of TNBC, with neoadjuvant chemotherapy (NAC) producing a pathological complete response (pCR) in approximately 40-50% of patients with TNBC (van der Ende et al., 2023). Unfortunately, studies show that if timely clearance is not achieved, long-term use often results in the acquisition of broad-spectrum drug resistance.

The use of genetic medicine to overcome chemotherapeutic drug resistance has been posited as a solution. By modifying genes that govern traits such as angiogenesis, immune evasion, replication or the expression of drug efflux channels, all of which contribute to multidrug resistance (MDR), it is possible to improve clinical outcomes in TNBC.

A known modulator of neoplastic behaviours in TNBC cells is the KISS1 receptor (KISS1R), a galanin-like G protein coupled receptor associated with a family of signal peptides, called kisspeptins. The KISS1/KISS1R mechanism is believed to influence several pathways, which regulate cell proliferation and invasiveness. A notable body of research associates KISS1R overexpression with increased proliferation in oestrogen receptor alpha (ERα) negative breast cancer cell lines (Blake et al. 2017).

qRT PCR analysis of KISS1R overexpressing cell lines suggests overexpression of KISS1R directly correlates with Breast Cancer Resistance Protein (BCRP) expression levels. BCRP, also known as MXR/ABCG2, is a key contributor to the acquisition of multidrug resistance in TNBC (Mao and Unadkat, 2014). Conversely, analysis of cell proliferation rates in MDA-MB-231 breast cancer cell cultures treated with KISS1R siRNA showed reduced proliferation, invasiveness and cell viability in screened cell lines, further evincing KISS1Rs impact on TNBCs. This suggests KISS1R is an ideal target for therapeutic intervention.

The CRISPR-Cas9 gene editing system can be used to delete or remove a gene of interest in an in vivo cell model. We hypothesized this system can be used to halt KISS1R expression in an in vitro cell model, resulting in a reversal of multidrug resistance, and a reduction in proliferation and invasiveness in MDA-MB-231 cancer cell cultures.

Two different MDA-MB-231 cell cultures were transformed by the delivery of CRISPR Cas9 ribonucleoprotein complexes, using the Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent. The efficiency of the KISS1R knockout was assessed using a T7 endonuclease cleavage assay and a Western Blot Analysis. An MTT assay was performed to compare the differences of Doxorubicin sensitivity between KISS1R knockout, and the wildtype MDA-MB-231 cells and sequence analysis was performed. The results demonstrate the feasibility of the CRISPR-Cas9 system in the targeted deletion of the KISS1R gene. The genomic cleavage detection assay indicated a cleavage fraction of 30.18 ± 2.2% (95% CI) and the cleavage efficiency of 54.94% in cultures transformed using the CRISPR Cas9 RNP complexed with sgRNA KISS1R C3. TIDE sequence trace decomposition analysis suggests the transformation efficiency was lower, at approximately 31.2%. The MTT assay results support the results of prior studies that KISS1R inhibition also affects cell viability, cancer cell migration and invasiveness, with cell viability reduced by a factor of 3.25. MTT results also suggest a reversion in chemosensitivity in KISS1R knockout cells, with a 6-fold reduction in IC50 in the KISS1R knockout cell line D1-3 when compared to wild type MDA-MB-231 with wild type IC50 = 54 nM (95% CI; 33.83 to 86.11) and D1-3

IC50 = 9 nM (95% CI; 4.420 to 18.14) This supports the targeting of the KISS1 receptor for therapeutic purposes in TNBC as well as validates the utility of the CRISPRCas9 ribonucleoprotein in inducing KO mutations for therapeutic purposes.

Keywords: KISS1R, Triple Negative Breast Cancer, Doxorubicin, CRISPR Cas9, Chemosensitivity, Multidrug Resistance.

Acknowledgements

First, I'd like to thank everyone involved in this project, both directly and indirectly. Dr Yan Li, many thanks for the opportunity to expand my skillset to include mammalian cell culture and the use of CRISPR-Cas9 technologies and CRISPR-Cas9 ribonucleoprotein transfection, as well as the use of lipofection technologies. Your guidance and training were invaluable during this process as was your patience. I'd also like to thank him for his support in bringing one of my projects to life and I look forward to working on some exciting projects this year.

I'd also like to thank Tim and Leo for their help in the lab, the amount of just raw material in the lab is a mission to navigate in and of itself having someone that knows their way around and knows all the tips and tricks for getting the best results in any particular experiment is a godsend.

Lastly, I'd like to thank my family, my Mum, Dad and siblings for their support during difficult times and my partner MJ for the support and unwavering belief, I was glad I got to spend 5 wonderful years with you, I love you and you will be missed.

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor used artificial intelligence tools or generative artificial intelligence tools (unless it is clearly stated, and referenced, along with the purpose of use), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signature:

Wayman Puna

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Abbreviations

°C Degrees celsius

µl Microlitre

3DCRT 3-Dimensional conformal radiotherapy

5-FU Fluorouracil

Å³ Cubic angstrom

AAV Adeno-associated virus

ABCB1 ATP binding cassette B1

ABCG2 ATP binding cassette G2

AC Doxorubicin and Cyclophosphamide polychemotherapy regimen

ACT Doxorubicin, Cyclophosphamide and Docetaxel polychemotherapy regimen

ADCC Antibody-dependent cellular cytotoxicity

ADCP Antibody-dependent cell mediated phagocytosis

AHR Aryl hydrocarbon receptor

AI Aromatase inhibitors

AJCC American joint committee on cancer

ALL Acute lymphoblastic leukemia

AR Androgen receptor

ARH Arcuate nucleus of the hypothalamus

ATCC American Type Culture Collection

ATM Ataxia telangiectasia mutated gene

ATP Adenosine triphosphate

BCRA1/2 Breast cancer resistance gene ½

BCRP Breast cancer resistance protein

BSA Bovine serum albumin

CAM Chorioallantotic membrane

CAR-T Chimeric antigen receptor T cell

CD28 Cluster of differentiation 28

CDC Compliment dependent cytotoxicity

CDK4/6 Cyclin dependent kinase 4/6

CEM Contrast-enhanced mammography

CESM Contrast-enhanced spectral mammography

CHEK2 Checkpoint kinase 2

CM Culture medium

CMF Carboplatin, Methotrexate and Fluorouracil polychemotherapy regimen

CNB Core needle biopsy

COX-2 Cyclooxygenase 2

CRISPR Clustered regularly interspaced short palindromic repeats

CSC Cancer stem cells

CT Computed tomography

CT2 Carnitine transporter 2

DAG Diacylglycerol

DNA Deoxyribose nucleic acid

DCIS Ductal carcinoma in situ

DDT Dithiothreitol

DFS Disease free survival

DOX Doxorubicin

DSB Double strand break

E2K Ubiquitin conjugating enzyme E2K

EBCTCG Early breast cancer trials collaborative group

EC50 Half maximal effective concentration

ECM Extracellular matrix

EGF Epidermal growth factor

EGFR Epidermal growth factor receptor

EMT Epithelial to mesenchymal transition

ER Oestrogen receptor

ERα Oestrogen receptor alpha

ERBB2 Erythroblastic oncogene B2

ERK Extracellular signal-regulated kinase

FBS Fetal bovine serum

FDG Fludeoxyglucose F18

Fe-Dox Iron Doxorubicin complex

FNA Fine needle aspiration

FSH Follicular stimulating hormone

G1 phase Gap 1 phase

Gaq11 G q protein alpha subunit

GnRH Gonadotropin releasing hormone

G Protein Guanosine nucleotide binding proteins

GPCR G protein coupled receptor

g Grams

gRNA Guide RNA

HDR Homologous directed repair

HER2 Human epidermal growth factor receptor

HIF-1 Hypoxia inducible factor 1

HPG Axis Hypothalamic pituitary gland gonadal axis

HR Hormone receptor

HR Hazard ratio

HSVtk Tyrosine kinase of the herpes simplex virus

IDC Invasive ductal carcinoma

IgG1 Immunoglobulin G1

IL2γ Interleukin 2 receptor γ

ILC Invasive lobular carcinoma

IMRT Intensity modulated radiation therapy

IP3 Inositol 1,4,5 triphosphate

IQGAP IQ motif containing GTPase activating protein

IRT Intraoperative radiation therapy

IV Intravenous

JAK/STAT Pathway Janus kinase signal transducer and activator of transcription pathway

JUNB JunB proto-oncogene

KD Dissociation constant

KISS1 KISS1 gene

KISS1R Kisspeptin 1 receptor

KP Kisspeptin

KRAS Kirsten rat sarcoma virus

L Liter

LCIS Lobular carcinoma in situ

LH Luteinizing hormone

LNB Lymph node biopsy

m^2 Square meter

mAb Monoclonal antibody

MAPK Mitogen activated protein kinase

MDR Multidrug resistance

MDRP Multidrug resistance protein

mg Milligram

MHz Megahertz

ml Milliliter

MMP Matrix metalloprotease

MMR Mismatch repair

mPgR Membrane progesterone receptor

mPR Progesterone receptor

MRI Magnetic resonance imaging

mRNA Messenger RNA

MRP Multi-drug resistance protein

mTOR Mammalian target of rapamycin

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

NADPH Nicotinamide adenine dinucleotide phosphate

NFR2 Nuclear factor erythroid 2-related factor 2

NHEJ Non-homologous end joining

NOS3 Nitric oxide synthase 3

NQO1 NADPH dehydrogenase quinone 1

OS rates Overall survival rates

p-4E-BP1 Phosphorylated eukaryotic translation factor 4E binding protein

p63GEF p63 Guanine exchange factor

PALB2 Partner and localizer of BRCA2

PAM Proto-spacer adjacent motif

PARP Poly ADP ribose polymerase

PAX2 Paired box gene 2

PBI Partial breast irradiation

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PD-1 Programmed cell death protein 1

PD-L1/2 Programmed cell death ligand 1/2

PEM Positron emission mammography

PET Positron emission tomography

PFS Progression free survival

PI3K Phosphoinositide 3-kinase

PI3K-PKB Phosphoinositide 3-kinase-protein kinase B

PKC Protein kinase C

PLC Phospholipase C

pmol Picomole

PP2A Protein phosphatase 2a

PVDF Polyvinylidene fluoride

PyMT Polyoma virus middle T

Ras-GTP Rat sacrovirus guanosine triphosphate

RhoA Ras homolog gene family member A

RISM Radiation induced secondary malignancies

RISC RNA induced silencing complex

RNA Ribonucleic acid

RNP Ribonucleoprotein

ROI Region of interest

ROS Reactive oxidation species

rpm Rotations per minute

RR Risk ratio

RUNX2 Runt-related transcription factor 2

S phase synthesis phase

SD Standard deviation

SDS PAGE Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

sgRNA Small guide RNA

SHP-2 Src homology region 2 domain containing phosphatase

siRNA Small interfering RNA

SNAIL/SLUG Superfamily of C2H2 type zinc finger transcription factors

SNP Single nucleotide polymorphism

SSB Single stranded breaks

T7E1 T7 Endonuclease 1

TALENS Transcription activator-like effector nucleases

TCR T cell receptor

TIMP-1 Tissue inhibitor of metalloprotease 1

TNBC Triple Negative Breast Cancer

TP53 Transformation related protein 53

TP2 Topoisomerase 2

TROP2 Trophoblast cell surface antigen 2

VAB Vacuum assisted breast biopsy

VEGF Vascular endothelial growth factor

VEGFR Vascular endothelial growth factor receptor

WBI Whole breast irradiation

Wnt Wingless related integration site

WT Wild type

XDH Xanthine dehydrogenase

ZFNs Zinc finger nucleases

1. Chapter One: Introduction

In 2020, an estimated 10.0 million cancer associated deaths, and some 19.3 million new patients were diagnosed with some form of cancer (Deo et al., 2022). Projections concerning the global cancer burden predict case numbers will reach 28.4 million in 2040, with burden increase predominantly affecting developing nations (64% to 95%). This is likely due to changes in population demographics, which includes an increase in population, a shift in the age profile of the developing nations, urbanization, changing diets and differences in the availability of care (Globocan, 2012). The current and predicted impact has prompted research to better ascertain a more detailed understanding of the pathology of and underlying mechanisms of various cancers, which can in turn allow for the development of more effective strategies to manage, treat or eliminate various cancers.

Breast cancer is one of the more prevalent types of cancer and poses a significant burden to women, with breast cancer affecting one in nine women during their lifetime. The current roster of treatments serves us well in most situations and has significantly reduced the mortality rates of early stage and non-metastatic cancer, with 5-year survival rates of 96% stage I non-metastatic breast cancer. However, the efficacy of treatment for metastatic breast cancer is quite poor (Du and Li, 2023). This is in no small part due to the tendency for breast cancer to develop multidrug resistance and stem cell like traits, including increased invasiveness, immune evasion and higher rates of cell proliferation. The poor clinical outcome is particularly common in a subtype of breast cancer known as triple-negative breast cancer (TNBC), which is defined by the absence of Oestrogen Receptor Alpha (ERα), the progesterone receptor (mPR) and *HER2neu*. To address these shortcomings and thus improve survival metrics, an increase in the number of systemic treatments for late-stage TNBC is essential.

The use of gene editing in the treatment of different cancers has been suggested as a means of improving clinical outcomes. Gene editing technology utilizes nucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENS), and clustered regularly interspaced palindromic repeats associated RNA guided Cas9 (CRISPR Cas9) (Maeder & Gersbach, 2016) to generate double

strand breaks (DSB) in targeted regions of the host genome. These DSBs can be used to facilitate the induction of indel mutations or the insertion of an exogenous fragment of DNA. The type of mutation that occurs at the DSB is dictated by the repair pathway elicited in each system (Suleiman et al., 2021).

These modifications can alter the expression of or eliminate one or more genes linked to resistance or malignancy in cancer or facilitate the insertion of a gene which can be used to improve the clearance of cancer cells or partially restore normal function. An example of genes that are directly associated with development of chemotherapeutic resistance is the ABC transporter family of drug efflux channels (Fletcher er al. 2010). Directly targeting these genes or the genes that govern their expression have demonstrated a partial or complete reversal of drug resistance in vitro (Yin et al. 2021). There are various other sources of chemotherapeutic resistance, and targeting these genes has demonstrated promising results in several in vitro experiments.

Targeted application of gene editing to facilitate the removal of traits that contribute to the chemotherapeutic resistance of TNBC should improve the efficacy of current treatments and allow them to retain more of their anticancer effects over time, ideally impacting cancer survival rates.

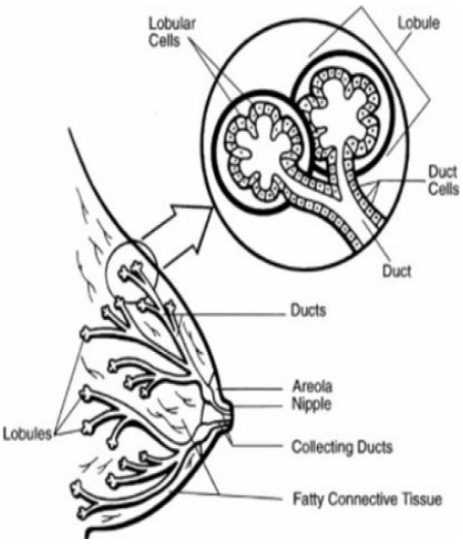
1.1 Breast Cancer

1.1.1 What is Breast Cancer?

Breast cancer is a broad descriptor of cancers that occur in breast tissue. Worldwide, breast cancer is the second most frequently diagnosed cancer and primarily affects women (Arnold et al., 2022). The economic burden of breast cancer is estimated to be the highest among all cancers, accounting for approximately 7.7% of the total medical costs of all cancers (Chen et al., 2023).

Breast cancer is the result of the transformation of non-malignant breast cells to malignant, through a variety of mechanisms including hereditary loss or gain of function mutations, changes in gene expression levels or changes in the microenvironment (Hulka & Stark, 1995). These dysfunctions lead to morphological and behavioural changes in the cell, such as dedifferentiation, increased angiogenesis, loss of apoptotic signalling, a loss of cell-cell adhesion, immune evasion etc. which results in the highly dysregulated growth of cancerous cells. The cancerous mass imposes as significant metabolic and physical burden on the patient, that if left untreated, can result in death.

Invasive ductal carcinoma (IDC) and invasive lobular carcinomas (ILC) are two of the most prevalent types of breast cancer and are detected in roughly 50% - 75% and 5% -15% of breast cancer patients respectively (Arps et al., 2013). The remainder of patients likely have either a mixed ductal/lobular carcinoma or one of several rarer histologies, which includes micropapillary carcinoma or tubular carcinoma (Makki, 2015).



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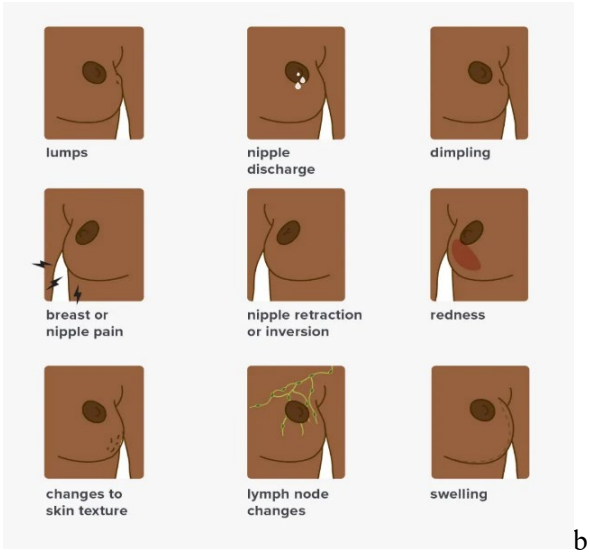

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Figure 1.1 (a) Caricatures of a sagittal cross section of the breast and generalized structure of the lobules and (b) visible signs of breast cancer (Sharma et al., 2024; Waks & Winer, 2019).

The tumour, node, metastasis (TNM) system of classification developed by the American Joint Committee on Cancer (AJCC), classifies different cancers by various metrics such as the site of the original tumour, size of the tumour, various cellular characteristics and metastatic state (Benson et al., 2003). Identifying the stage at which a patient's cancer has progressed can be used to determine the efficacy of certain treatment regimen and predict the patient's odds of survival. Stage 0 cancers are non-invasive, meaning the tumour is and remains restricted to the lactiferous ducts or lobules of the breast and are unlikely to undergo metastasis. This class of lesion is termed benign hyperplasia (Sharma et al., 2024). Ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) are two types of benign hyperplasia, and while DCIS and LCIS exhibit a number of traits indicative of malignancy, such an pleomorphism, and increase in the number of mitotic cells, and central luminal necrosis, they remain confined to the primary tissue site and have not breached the basement membrane. (Shaaban, 2021; van de Vijver, 2005). As the cancer progresses and begins to infiltrate proximal tissues, it is classed as invasive and invasive cancers pose an increased risk to the patient, as the cancer is likely to metastasize. The most common invasive breast cancers are invasive lobular carcinomas (ILC) and invasive ductal

carcinomas (IDC) (Arps et al., 2013). These cancers can usually be detected in the lymph nodes. The proliferation of the cancer through the lymphatic system and the size of the primary tumour generally define which stage the patients breast cancer has reached, with Stage I breast cancers having little to no presence in the lymph nodes and a primary tumour size of < 2cm, Stage II breast cancer having spread to 1-3 lymph nodes and a primary tumour diameter of < 5cm, and Stage III cancers having traces of cancer cells in > 4 lymph nodes and a tumour diameter of > 5 cm. Stage IV breast cancer is the point at which breast cancer has metastasized and is detected in the peripheral tissues, including organs, such as the brain, lungs and the liver. Stage IV breast cancer is often deemed inoperable, and the OS rate is poor (Sharma et al., 2024).

Cancers can be further divided into phenotypic subtypes. This is usually determined histologically or more specifically based on the presence of certain cell surface receptors. These include the vascular endothelial growth factor receptor (VEGFR), ERα, the mPgR and HER2 (Parise et al., 2009; Waks & Winer, 2019).

The first major subtype of breast cancer cell types are hormone receptor positive (HR+) cancer cells. These are tumours that screen positive for either the oestrogen or progesterone hormone receptors, or both. Approximately 80% of all breast cancer tumours are HR+ (Badowska-Kozakiewicz et al., 2017). These tumours can also be HER2+ or HER2-, with HER2+ having elevated levels of the HER2 receptor and HER2- having lower levels or effectively zero HER2 expression. The biological implications of HER2+ and HER2 overexpressing breast cancer include an increased rate of proliferation, increased likelihood of lymph node metastases and a reduced rate of cell apoptosis (Hou et al., 2019) whereas HER2 low and HER2- likely has a lower rate of proliferation and have improved rates of clearance, with HR+/HER2-: 95.1% and HR+/HER2+: 91.5%, however HER2- are resistant to several targeted therapies, which limits treatment options.

The other major category of breast cancer cell types are the hormone receptor negative (HR-) breast cancers, which lack the oestrogen and progesterone cell surface receptors. Of these cancer types, there are

two subcategories defined by the presence of the HER2 receptor, HR - / HER2 + breast cancer cells and HR - / HER2 - breast cancer, the latter group is also known as triple negative breast cancer (TNBC).

Stage I HR + breast cancers, and HER2 + receptor positive stage 1 breast cancers are relatively mild forms of breast cancer, with survival rates of 99% and 94% respectively (Waks & Winer, 2019). TNBCs make up the smallest proportion of breast cancers, however they have a much lower survival rate, with stage 4 TNBC having a 5-year survival rate of 12% and a 10-year survival rate of 0% (Ahmad, 2019).

Early-stage TNBC is usually treated via surgical resection of the primary mass and surrounding tissue and adjuvant radiotherapy. Chemotherapy, a form of systemic treatment, is often used in the treatment of cancer at every stage of cancer development to reduce the size of a tumour so it can be surgically resected, to reduce the likelihood of recurrence or to treat malignant cancers that may have reached the peripheral tissues and organs, a behaviour typical of stage IV breast cancer. The most widely used TNBC chemotherapeutic regimens use anthracycline/taxane-based regimens and often include the anthracycline Doxorubicin. Doxorubicin is considered the most effective chemotherapeutic agent in the treatment of TNBC and can be used alone or in combination with other chemotherapeutic agents such as paclitaxel and Cyclophosphamide as a polychemotherapy. OS rates can be further improved by use of polychemotherapy concomitantly with targeted therapies and immunotherapy, in a practice called combination therapy.

Unfortunately, the efficacy of Doxorubicin-based treatment over time is often reduced, in no small part due to the acquisition of multiple traits including modifications that result in the increase in the rate of cell proliferation, malignancy and MDR. This notably impacts clinical outcomes in advanced TNBC.

Recent research has linked the long-term use of Doxorubicin with changes in the tumour cell secretome, resulting in the efflux of anticancer therapeutics out of the cell. This has profound effects on their efficacy. Doxorubicin for example, intercalates with DNA and acts to inhibit the function of topoisomerase 2 (TP2). The therapeutic targets of Doxorubicin are mostly localized to the nucleus.

Therefore, any action that results in a reduction in the concentration of chemotherapeutic agents from the site of action in the cell will likely reduce the agent's efficacy. It is also likely that other mechanisms facilitate chemotherapeutic resistance, including the induction of the epithelial mesenchymal transition however, genetic and epigenetic modifications that alter the rate of efflux of small molecule compounds seems to have the most notable effect on chemotherapeutic resistance.

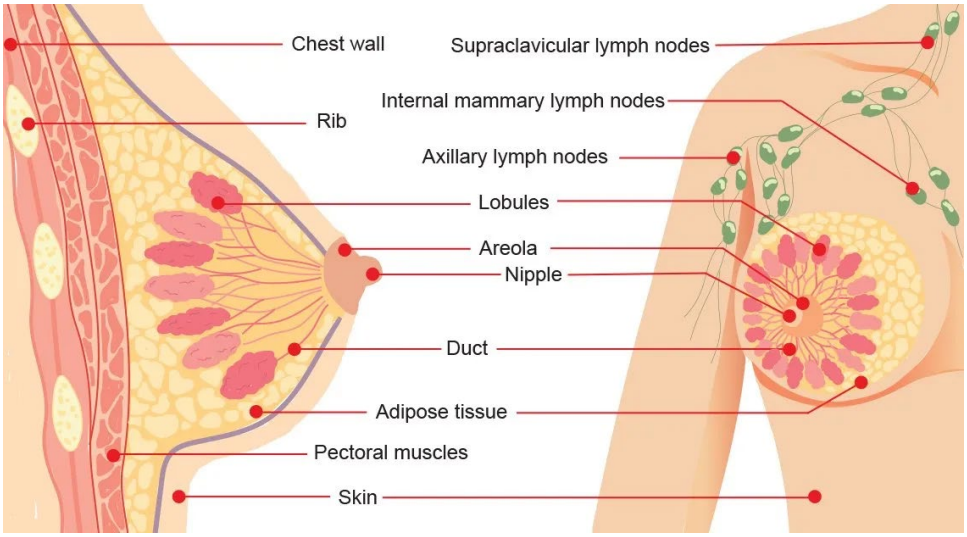


Figure 1.2 Diagrams of the anatomy of the breast and enlarged sagittal cross-sections. The breasts consist of milk producing glands that are connected to the nipple via ducts and are surrounded by adipose tissue. These sit atop the pectoralis muscles and the rib cage and are proximal to the lymph nodes (Geddes, 2007)

1.1.2 How Breast Cancer Affects New Zealand and the Rest of the World

Breast cancers are considered the largest single source of expenditure in global cancer spending, accounting for 7.7 % of all cancer related spending, and is the leading cause of cancer related deaths among women (Chen et al., 2023). 2.3 million women were diagnosed with breast cancer in 2020, and contributed to over 685 000 deaths (Arnold et al., 2022). From the beginning of 2015 to the end of 2020, 7.8 million patients were diagnosed with breast cancer. Predicted changes in population demographics,

including population growth and aging, suggest breast cancer morbidity and mortality rates are likely to increase, with some models predicting an excess of 3 million new cases and 1 million deaths annually by 2040 (Arnold et al., 2022). Much of that growth is expected to occur in developing nations, regions which are unlikely to have adequate infrastructure and the necessary capital to meet these growing needs, likely exacerbating social and economic inequities between developing and developed nations.

Breast cancer is the most common type of cancer in New Zealand and is linked to over 600 deaths per year. Between the years 2016 – 2019, the number of diagnoses has increased, from 3331 to 3478 cases (Meharry et al., 2021). Of note is the disparity between races, with Māori and Pacific women having a higher excess mortality rate of 1.76 and 1.97 respectively, when compared with non-Māori, non-Pacific Island women (Meharry et al., 2021). This is likely linked to issues with care, as these at-risk groups are also more likely to be diagnosed later and less likely to receive timely treatment.

TNBC, as mentioned prior, is a rarer but more aggressive type of breast cancer, has a poorer mean 5-year OS rate than other breast cancer types and poorer overall outcomes. A study by Hsu et al. (2022) suggests the 5-year OS rate of TNBC is 81.28% compared to is 86.50% for non-TNBC, and the prognosis is notably worse if the cancer is metastatic, with metastatic TNBC having a 5-year survival rate of 10.81% compared with metastatic non-TNBC, at 33.46%.

Despite the predicted increase in breast cancer associated mortalities, analyses suggest that, while the global incidence of breast cancer is increasing, the mortality has not increased at a comparable rate. (Ahmad, 2019). For example, women diagnosed between 2010 and 2015, had an expected five-year mortality risk of 4.9% (95% CI; 4.8% to 5.0%) or less compared with woman diagnosed between 1993 and 1999, who had a mortality risk of 14.4% (95% CI; 14.2% to 14.6%) (Taylor et al., 2023).

This reduction in the growth rate of breast cancer associated mortality is likely the result of improved diagnostics and an increase in the number of treatments. These treatments include the development of more novel therapies such as trastuzumab and pertuzumab, anti-HER2 antibody therapies, which have

been reported to increase survival rates, with anti-HER2 antibody therapies increasing the 10-year OS rate from 75.2% to 84% and the 10-year disease-free survival (DFS) rate from 62.2% to 73.7% in non-metastatic TNBC breast cancer (Nahta and Esteva, 2007; Perez et al., 2014). However, survival for Stage IV metastatic TNBC breast cancer remains extremely low.

1.1.3 Methods for the Imaging and Diagnosing of Breast Cancer

The diagnosis and treatment of breast cancer is complex and dynamic. An accurate diagnosis depends on several factors, the most important of which is the physiology of cancer and its stage of progression. Multiple analyses are often performed to arrive at a diagnosis and to determine the appropriate treatment regimen for breast cancer, the initial stages of diagnosis are relatively rudimentary and involve an analysis of the patient's medical and family history and a clinical breast exam. An analysis of the patient's lineage can help inform the practitioner if any signs of cancer are a cause for concern, as a first-degree relative such as a sister or mother being diagnosed with breast cancer increases the probability of breast cancer in the patient by a factor of 1.9x (95% CI, 1.7-2.0) (Pharoah et al., 1997). An analysis of a patients' medical history allows for the identification of additional risk factors that would suggest that any aberrations detected during a planned clinical breast exam are a cause for concern. These factors include gender, age, the age of menopausal onset, diet, smoking habits, BMI as well as diabetes status, and level of exposure to ionizing radiation (Momenimovahed & Salehiniya, 2019). A patient's medical history may also contain information from any prior genetic screening, and the presence of certain mutations are considered strong indicators of an elevated risk of cancer and can influence the patient's suitability for certain treatments.

The assessment of the patient's medical and family history (Pharoah et al., 1997) usually accompanies a clinical breast examination, which involves a visual assessment and palpation of the breast area and surrounding lymph nodes. The goal is to detect the presence of desmoplasia, or any changes in the

morphology of the breast that would signal a cancerous lesion is present (Walker, 2001). Reliable indicators include changes in skin texture and colour, changes in breast volume, dimpling, pain, changes to the lymph nodes, changes to the morphology of the nipple, including the retraction of and inversion of the nipple and swelling. Research shows that clinical examination of the breast and surrounding area is a reliable initial indicator of the presence of cancer and preliminary assessment from a general practitioner (GP) having a negative predictive value of 94% (Donnelly, 2010).

If an aberrant growth is detected, verification is usually obtained first from any of several image-based technologies such as ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) (Basurto-Hurtado et al., 2022). While imaging of the breast is not in and of itself diagnostic, they provide useful information that may indicate some kind of intervention may be required. These imaging techniques will be discussed in the following sections.

1.1.3.1 Mammograms

A mammogram is the primary imaging modality used in the diagnosis of breast cancer. A mammogram involves the administration of a beam of x rays to the breast, and as the x rays pass through the breast, the deflection and scattering of the x ray beams is captured and used to produce an image of the internal structures of the breast. To reduce the amount of ionizing radiation the patient is exposed to, both breasts are pressed between two plates to modify the dimensions of the breast to reduce any potential scattering of the beams, which allows for a reliable scan to be produced with reduced radiation exposure. The average sensitivity of a mammogram is approximately 85% (Chen et al., 2021). Mammography allows for the detection of abnormalities in breast tissue which may suggest the possibility of breast cancer, such as a lump. This is highly useful for women who have no readily apparent physical signs of breast cancer (Autier and Boniol, 2018).

Mammography alone cannot prove that an abnormal region of the breast is cancer, but it raises a medically significant suspicion of cancer. If there is a high likelihood the aberrant structure is cancerous, a biopsy of the tissue of interest is standard protocol. Breast tissue is removed by needle or via surgical biopsy and examined using various histological and immunohybridization methods to determine if the growth is cancerous. Mammography also has reduced sensitivity in young women under 50 (87.5%) (Kerlikowske et al., 1996) and women with dense breast tissue (62–68%) (Freer., 2015).

1.1.3.2 Ultrasound

Ultrasound imaging involves the use of ultrasonic sound waves (2 – 18 MHz) to produce images of the internal structures of the breast. An image is formed using the soundwaves that are reflected and/or scattered, and that information is compiled into a series of 2D images which can be used to identify and determine the location of any potential abnormalities in the breast (Wells, 2006). More recent technology allows for the ultrasonic scan to produce a 3D scan of the breast, providing more useful information for biopsy or surgery (Sehgal et al., 2006). Ultrasonic imaging is considered the safest form of the imaging technologies used to screen the breast, as it is noninvasive and does not use radiation. It is considered effective in the assessment of high-density breast tissue but is considered the least sensitive of the imaging techniques used, with an average sensitivity of 40 – 75 % (Lee et al., 2019). In addition to the lower sensitivity than either mammography or MRI, it also has difficulty detecting the presence of calcified masses and has an elevated rate of false positives.

1.1.3.3 Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging (MRI) is a medical imaging modality that employs the use of radiowaves, strong magnetic fields and magnetic field gradients, to produce images of the internal structures and the physiological processes of the body (Katti et al., 2011). One of the biggest advantages of MRI is its safety. MRI can be used to produce images of the internal structures of the breast without the use of ionizing radiation, which distinguishes it from other imaging methods, such as mammography. An MRI also has a higher degree of sensitivity than mammography or ultrasonography, with sensitivity reaching 100% (Nguyen et al., 2022). MRI is also extremely useful in the assessment of the success of neoadjuvant chemotherapy treatments as it can be used to assess small changes in the morphology of tumours due to its high resolution.

1.1.3.4 Radionuclide Imaging

Radionuclide imaging is an imaging modality that involves the IV administration of small doses of radioactive substances which have an affinity for cells with an elevated metabolism, a trait common in most cancer cells. A specialized detection system is then used to locate the tracer in the regions of interest, including the breast and the lymph nodes. Radionucleotide imaging is useful in locating cancer cells that have metastasized and spread to the peripheral tissues and organs if the radionucleotide selected has appropriate selectivity. However, radionucleotide imaging generally exposes the entire body to radiation, which is a notable drawback (Regulla & Eder, 2005).

1.1.3.5 Positron Emission Tomography (PET) Scans

Positron emission tomography (PET) is an imaging modality that involves the IV administration of a solution containing a radioactive tracer (Pisano & Parham, 2000) such as fludeoxyglucose F18 (FDG) a radioactive glucose analog, which is known to accumulate in breast cancer cells, due to their elevated metabolism, allowing for easy detection. PET scans are useful in detecting and measuring the spread of cancer to the peripheral tissues of the body and organs. Variations in the use of tracers can produce more refined images.

1.1.3.6 Positron Emission Mammography (PEM) Scans

Positron emission mammography (PEM) uses a radioactive tracer to improve the resolution of a mammogram (Abreu et al., 2006). PEM is superior to conventional mammography as it can detect small clusters of cancer cells within the breast so more useful information is derived from a PEM than a mammogram (Thompson et al., 1995). Unfortunately, as it relies on the use of radionucleotides, PEM exposes the whole body to radiation and thus has limited utility (Regulla & Eder, 2005).

1.1.3.7 Computerized Tomography (CT) Scans

Computerized tomography is commonly used imaging modality that uses an X ray source attached to a motorized gantry to direct a beam through the body of the patient. As the patient passes through the gantry, the scattering of the X rays as it passes through the body produces a pattern and that is recorded and compiled to produce a 3D image of the patient's breast and internal structures. CT scans have a relatively high specificity, sensitivity, accuracy, at 99.3%, 84.21% and 98.68% respectively (Desperito et al., 2022).

1.1.3.8 More Recent Imaging Methods

Unfortunately, PEM, PET and CT scans, like most other radionucleotide imaging technologies, expose the whole body to radiation and as such have limited applications throughout the patient's lifetime (Regulla & Eder, 2005). Newer types of imaging modalities are now being developed for breast imaging and are currently undergoing assessment for their suitability in diagnosing breast cancer and include abbreviated MRI (fast breast MRI) which involves the administration of intravenous gadolinium to produce higher resolution images while requiring fewer images to be taken (Geach et al., 2021), Scintimammography, which is a type of radionuclide labelling that involves the use of intravenous tecnetium-99m sestamibi to produce more useful images (Liberman et al., 2003). Contrast-enhanced mammography (CEM) (Gelardi et al., 2022) and contrast-enhanced spectral mammography (CESM) use an intravenous iodine solution to produce a greater contrast in x ray images. CESM also uses multiple X ray frequencies during the scanning process to produce a more refined image (Phillips et al., 2017). Both CEM and CESM of useful in producing more detailed images in denser regions of the breast than a standard mammogram. CESM is currently undergoing testing to determine if it is as reliable as an MRI, meaning it could be used to improve the quality of screening without the addition of significant cost, ideally improving clinical outcomes.

1.1.3.9 Tissue Biopsy

While radiolabelling and imaging techniques inform us of the size, location and stage of progression, including nodal status, an analysis of biopsied tissue is requisite for the positive identification of a cancerous lesion and identification of the tumour subtype.

A biopsy involves the removal of a portion of tissue from a region of tissue believed to be cancerous, for analysis using chemical, histological immunohistochemical methods. There are various methods by which the tissue can be biopsied, including fine needle aspiration (FNA), vacuum assisted breast biopsy (VAB), surgical biopsy, stereotactic biopsy, core needle biopsy (CNB), mammotome biopsy, lymph node biopsy (LNB), liquid biopsy and others (Niikura et al., 2013; Versaggi and De Leucio, 2023).

FNA involves the use of a thin hypodermic needle, usually 21-27 gauge, to aspirate a portion of the suspected cancerous tissue. CNB is similar, however the needle is larger diameter, and more tissue is collected. This is the preferred method of breast tissue biopsy and can be used to definitively identify cancerous tissue (Niikura et al., 2013; Versaggi and De Leucio, 2023). A surgical biopsy involves the resection of the tumour and surrounding tissue. Surgical biopsies are both highly invasive and costly and thus are generally avoided (Kasraeian et al., 2010). A lymph node biopsy can involve either the surgical resection of one or more lymph nodes or employ the use of core needle biopsy techniques. A sentinel node biopsy (SNB) is the biopsy of one or more axillary lymph nodes with the aim of detecting and predicting the likelihood of more distally located cancer cells when radiolabelling and radioimaging suggest no evidence of lymph node involvement (Kim et al., 2005). LNBs can have adverse side effects, including an increased risk of lymphedema (Cabanas, 1992). Other biopsy methods, such as stereotactic biopsy, mammotome biopsy and most VAB use live imaging techniques such as mammography and/or ultrasonography and/or MRI either alone or in tandem, to guide the insertion of a core needle, sometimes through multiple regions of interest to extract a tissue sample (Guo et al., 2018). Liquid biopsies are a more recent technique and involve the detection of altered biomarker levels in the blood or other bodily fluid samples to verify the presence of cancer (Alimirzaie et al., 2019).

Once a sample of the tissue of interest is acquired, the tissue undergoes histopathological and immunohistochemical analysis. Histopathological analysis uses haemotoxylin and eosin ('H + E') chemical staining protocol to determine the cancers nuclear and histological grade (Elston & Ellis, 1993), with both being a measurement of the extent to which cancer cell tissue has differentiated from normal breast tissue and includes the observation of increased rates of mitosis. Histochemical analysis can also be used to identify DNA ploidy, (Joensuu et al., 1991), S phase fraction (Gazic et al., 2008) vascular invasion and levels of necrosis (Schlor et al., 1993). These can be used to partially assess the probability of long-term survival. Immunohistochemical analysis is often used to determine the mPgR (Paterson et al., 1990), ERα and HER2 status of a breast cancer lesion (Williams et al., 2009). The results of the analysis allow for the verification of the status of a lesion as cancerous or benign and for the sorting of samples into biologically and clinically significant groups. This allows for the curation of an appropriate treatment regimen for the patient and the delivery of a prognosis.

1.1.3.10 Genetic Screening

Useful stratagems employed in the diagnosis and treatment of breast cancer now include the genomic screening of the patient for breast cancer associated genes, which is reasonable as 5-10% of all breast cancers are hereditary (Mehrgou & Akouchekian, 2016). Some genes most commonly associated with breast include Breast Cancer Gene 1 (*BRCA1*) and Breast Cancer Gene 2 (*BRCA2*). Genetic screening can significantly impact treatment, as an individual with either or both *BRCA1* and *BRCA2* is likely to have poor clinical outcomes without aggressive intervention (Low et al., 2017). If patients test positive for more moderate-risk mutations, like *PALB2*, *CHEK2* or *ATM* mutations then metastasis is likely and surgical intervention is recommended (Low et al., 2017). The presence of other more generalist cancer related genes can also affect treatment, for example, individuals with a mutation have a higher risk of

secondary malignancies if they were to undergo radiotherapy and thus radiotherapy is not recommended for this group.

Further expanding on the utility of genetic screening, pharmacogenetics is a subdiscipline of genetic analysis which selects therapies for a patient that best matches the patient's and the tumour's genetic profile (el Hassouni et al., 2019), modifying treatment regimens for individuals predisposed to elevated risks from certain therapeutic agents (Lee et al., 2005) and identifying those that will likely benefit from others (el Hassouni et al., 2019) with the goal of producing the most effective treatment regimen for the individual. Such practices will likely be widely adopted when the practice becomes more economically viable.

1.2 Treatment of Breast Cancers

While treatment schedules differ between patients due to a number of factors, the protocol for the treatment of early-stage and locally advanced breast cancer is uniform. Surgical resection of the lesion and a portion of the surrounding tissues is recommended as the first line of treatment of LCIS, DCIS and early-stage invasive breast cancer. If the primary tumour is < 5cm in diameter, it is likely key structures in the breast can be conserved, and thus a lumpectomy is recommended. A lumpectomy involves the removal of the tumour and a portion of healthy tissue that surrounds it, called the margin (Hazard et al., 2008; Mogal et al., 2016). If the cancer is considered invasive, adjuvant radiotherapy is recommended. If the size of the tumour > 5cm in diameter, the removal of the entire breast, called a mastectomy, is performed (Mogal et al., 2016). There are several types of mastectomies, most of which have the aim of preserving non-cancerous tissue for use in cosmetic treatments to restore the appearance of the breast. If the patient has inherited a deleterious mutant *BRCA1* or *BRCA2* gene, a double mastectomy is recommended. Adjuvant radiotherapy or chemotherapy is recommended following mastectomy and often

performed with other systemic treatments to reduce the total amount of residual cancer cells, thus reducing the recurrence rate.

1.2.1 Radiotherapy

Radiotherapy is the use of high-energy particle beams such as X rays; to inflict as much genetic and physical damage to cancer cells it triggers mitotic catastrophe. Radiotherapy is a common procedure in the treatment of many different cancers (Yang & Ho, 2013). The molecular mechanisms that produce the anticancer effects associated with radiotherapy are relatively straightforward, a cancerous lesion is exposed to X ray or gamma radiation, which has high penetrance and causes DNA damage via direct ionization, causing double stranded breaks (DSB) or indirect ionization, where the radiolysis of water produces free radicals that damage DNA and trigger apoptosis (Wang et al., 2018). A standard radiotherapy treatment schedule would involve administration of radiotherapy once a day, 5 days a week, for a number of weeks. Radiotherapy is very effective at reducing the risk of recurrence in the early stages of breast cancer, with the rate of recurrence being less than 5% in 10 years after treatment and 6% - 7% in 20 years for Stage 1 and 2 DCIS (Punglia et al., 2012; Narod and Rackovitch, 2014).

Radiation therapy can be given prior to surgical resection of the primary tumour to reduce the tumour size until it is considered operable. This is called neoadjuvant radiotherapy. Radiotherapy can also be administered after surgical resection of the primary tumour. This is termed adjuvant radiotherapy and is recommended if the patient has a large tumour, or the tumour has infiltrated surrounding tissue. Adjuvant radiotherapy reduces the likelihood of local recurrence by an excess of 65% (Early Breast Cancer Trials Collaborative Group, 1995). If the patient's axillary lymph nodes are screened and cancer cells are detected in one or more lymph nodes, radiotherapy may also be administered to the surrounding lymph nodes, inducing the cervical lymph nodes.

There are two major classes of radiotherapy, external beam radiotherapy, and intraoperative radiotherapy. These classes are distinguished from each other via the location of the radiation source and whether radiation is generated from an external source and directed toward the lesion, called external beam radiotherapy, or the source of radiation is inserted into the lesion, as is the case with intraoperative radiotherapy. The two major classes of external beam irradiation are whole breast irradiation (WBI) and partial breast irradiation (PBI) (Hickey & Lehman, 2021). Whole breast irradiation involves the irradiation of the entire breast while partial breast irradiation (PBI) targets radiation directly to the region of the tumour. This reduces the level of radiation exposure required to achieve a therapeutic effect, reducing the overall risk associated with this procedure (Hickey & Lehman, 2021).

Intensity-modulated radiation therapy (IMRT) is a modality of radiation therapy that administers high intensity x ray bursts in rapid succession that are focused on multiple small volumes throughout the lesion surrounding tissue, administering a medically effective dose of radiation to the targeted region while also reducing mean radiation exposure (Teh et al., 1999). This is achieved through precise control of several parameters of the x ray beam, including the duration, intensity and cross-sectional area of the beam.

3D Conformal Radiotherapy (3DCRT) uses CT scans to produce 3D images of the lesion and computational analysis is used to determine the optimal dose distribution. This allows for the highly precise administration of radiotherapy, allowing treatment to avoid internal structures while still achieving the recommended dose (Mehta et al., 2010).

Proton therapy is a relatively new form of radiation therapy that is believed to further reduce the effects of radiation exposure by limiting exposure to a highly controlled beam (Kammerer et al., 2018). Alternative radiation therapies include intra-operative radiation therapy (IRT), which involves administration of radiation using a surgical probe and brachytherapy is the administration of radiation therapy by placing ribbons, seeds or capsules containing radioactive material in the region of the tumour, leading to the formation of reactive oxidation species (ROS), increased DNA damage and synthetic lethality.

Radiation therapy causes a number of side effects, including fatigue, skin discoloration, swelling, pain and blistering in the affected areas of the skin. In a small number of cases, radiation therapy can result in pneumonitis (Sourati et al., 2017). The adverse effects of radiation therapy are dependent on a number of factors including the frequency and duration of exposure as well as the size of the area treated, but the effects lessen with time. The long-term use of radiotherapy can lead to radiation induced secondary malignancies (RISM) (Overgaard & Grantzau, 2015).

1.2.2 Systemic Therapies.

Effective treatment plans for many cancers often include the administration of chemical or biological agents that inhibit the proliferation of and/or eliminate cancer cells. These are classed as systemic therapies due to their ability to act on cancers throughout the body, including the peripheral tissues and organs (Palumbo et al., 2013). Categories of systemic therapies include chemotherapeutic agents and targeted therapeutics such as hormone therapy and immunotherapy. These are usually administered as part of a comprehensive treatment plan that may also include radiation therapy and or surgery. The use of various treatments and drug combinations can be used to treat breast cancer with differing physiologies and at different stages of disease progression.

1.2.2.1 Endocrine Therapy

Endocrine therapy is a class of targeted therapy used in the treatment of tumours which test positive for progesterone (mPgR+) or oestrogen (ERα+) receptors in immunohistochemical analyses (Waks & Winer, 2019). Breast cancers often exploit mechanisms that aid in their proliferation and increase their resistance

to therapeutics. Conventionally, the ERα and mPgR receptors activate the Rat Sarcomavirus guanosine triphosphatase (Ras-GTP) (Simanshu et al., 2017) and Phosphoinositide 3-kinase-protein kinase B (PI3K-PKB/Akt) (Hemmings & Restuccia, 2012) signalling pathways, and when an appropriate ligand is bound, it triggers a cascade of reactions that increase the expression of the erythroblastic oncogene B2 (ERBB2), androgen receptors (AR), the aryl hydrocarbon receptor (AHR), Paired box gene 2 (PAX2) and Runt-related transcription factor 2 (RUNX2), which in turn promotes a series of behaviours that increase cell stemness and heterogeneity (Kunc et al., 2021). As the endocrine receptors are overexpressed in HR+ breast cancers, the signals promoting cell stemness are elevated, resulting in increased proliferation and malignancy.

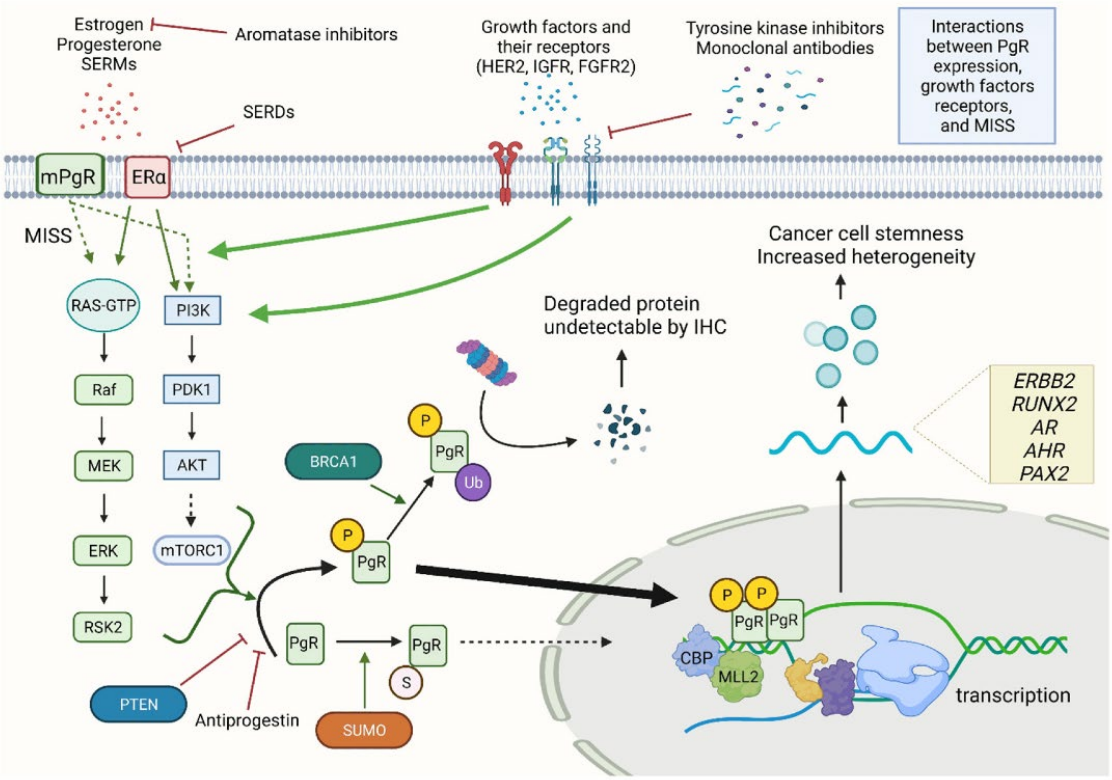


Figure 1.3. Diagram of medically relevant receptors in breast cancer therapy, the systemic treatments associated with each receptor and pathways associated with these receptors (Kunc et al., 2021).

The use of receptor antagonists such as aromatase inhibitors (AI) and selective oestrogen receptor degraders (SERDs) bind to or degrade ERα or mPgR, which prevents signal transduction, thus reducing stemness (Sainsbury, 2013). These receptor antagonists are used as an effective tool in the prevention of cancer recurrence and death when used by themselves or as an ongoing treatment following chemotherapy.

Endocrine therapy can be used as neoadjuvant therapy, with the intent of reducing the size of HR + breast cancers and thus reduce the risk associated with surgical intervention and the risk of recurrence (ROR) (Sainsbury, 2013). Endocrine therapy is either given 3 to 6 months prior to surgery and continued for several months after or given as a monotherapy following surgery to reduce the ROR. The continuation of hormone therapy post-surgical resection is called adjuvant hormonal therapy and can be continued for 5 – 10 years. Adjuvant hormonal therapy has been shown to reduce the likelihood of cancer recurrence by 50% (Rastelli, 2018).

Examples of endocrine therapy include Tamoxifen. Tamoxifen is an ERα receptor antagonist, meaning it binds to and interferes with the default oestrogen binding and signal transduction. This reduces the overall rate of effective signal transduction and inhibits pro-metastatic behaviours associated with ERα overexpressing cancers. This lowers the risk of recurrence, the risk of cancer progression, and the risk of distal recurrence. Aromatase inhibitors (AIs) such as exemestane (Aromasin) or anastrozole (Arimidex) decrease the amount of systemic oestrogen present in the blood by inhibiting aromatase. Aromatase catalyzes the conversion of a subset of androgens into oestrogen. Abemaciclib (Verzenio), a CDK4/6 inhibitor, is often used in combination with hormone therapies such as tamoxifen or an aromatase inhibitor. CDK4/6 is linked to increased proliferation due to the effect it has on E2K levels, which control the transition from G1 to S phase.

1.2.2.2 Chemotherapy

Chemotherapy is an important therapeutic stratagem in the treatment of many cancers, including breast cancer, and is particularly effective in increasing the probability of curative resection. It is also the primary curative agent used in the treatment of TNBC and metastatic breast cancers. The overall goal of chemotherapy is to eradicate tumour cells while minimizing damaging to non-cancerous tissue (Kwok et al., 2017). This is usually because chemotherapeutic agents have an affinity for and interfere with biological targets that are overexpressed in cells with one or more cancer hallmarks. This means any apoptotic effects will occur at an elevated rate in cells with these hallmarks. However, this does mean it can have a detrimental effect on noncancerous cells, which is why there is there are a maximum cumulative dose for most chemotherapeutic agents. In cases of curative resection of non-metastatic breast cancer, chemotherapeutic agents can be used as an both a neoadjuvant and adjuvant therapy, to reduce the tumour size, which allows for surgical resection, as well as improve the rate of disease-free survival in early stage breast cancer, with various regimens such as the AC (Doxorubicin and Cyclophosphamide) having a relapse free survival rate of 87% in non-nodal breast cancer patients (Fisher et al., 2001).

Anthracyclines and taxanes are the most effective and thus the most regularly used chemotherapeutic agents in the treatment of various breast cancers. Anthracycline monotherapies have consistently demonstrated a medically significant response in 20–80% of patients with non-metastatic breast cancer (Orlando et al., 2024). Anthracyclines also are reported to reduce breast cancer mortality in late-stage breast cancer by 20-30% (Sakshi & Anampa, 2018). Like radiotherapy, chemotherapy can be used as a neoadjuvant therapy to reduce the size of a large tumour, which improves surgical resection success rates, or be used as an adjuvant therapy to reduce the ROR.

A standard chemotherapy regimen is comprised of one or a combination of chemotherapeutic agents given in a number of cycles over a set period, usually defined by the presence of certain cancer hallmarks and the current stage of cancer progression. The dosage and regimen depend on various factors, including the patient's BMI, age, their prior treatment history, however it is most likely the treatment schedule will

mirror the results of existing clinical trials (Shien & Iwata, 2020). Chemotherapeutic agents are administered every 1, 2 or 3 weeks. Common therapeutic agents used include Capecitabine, Docetaxel, Doxorubicin, Fluorouracil (5-FU), Epirubicin, Paclitaxel, Carboplatin and Methotrexate. Research has demonstrated the advantages of polychemotherapeutic regimens, the use of two or more chemotherapeutic agents, when compared to monotherapies, the use of single agents, in the treatment of breast cancer (Mamounas, 2019). In an analysis of the efficacy of polychemotherapy regimens compared with single agent chemotherapy regimens, polychemotherapy regimens were associated with a higher overall tumour response rate compared to sequential or single agent chemotherapy regimens, with a relative response rate of 1.16 (Dear et al., 2013).

The following monotherapies or polychemotherapy regimens are recommended as neoadjuvant or adjuvant chemotherapy for early-stage and locally advanced breast cancer:

Treatment Regimen	Chemotherapeutic Agents
AC	Doxorubicin, Cyclophosphamide
EC	Epirubicin, Cyclophosphamide
AC or EC followed by T (paclitaxel or Docetaxel)	Doxorubicin or Epirubicin, Cyclophosphamide, Paclitaxel or Docetaxel
AC or EC followed by T (paclitaxel or Docetaxel) and carboplatin	Doxorubicin or Epirubicin, Cyclophosphamide, Paclitaxel or Docetaxel, Carboplatin
CAF	Cyclophosphamide, Doxorubicin, 5-FU
CEF	Cyclophosphamide, Epirubicin, 5-FU
CMF	Cyclophosphamide, Methotrexate, 5-FU
TAC	Docetaxel, Doxorubicin, Cyclophosphamide
TC	Docetaxel, Cyclophosphamide
Capecitabine (Xeloda)	Capecitabine (Xeloda)

Table 1.1 Various anthracycline and taxane based chemotherapy regimens used in the treatment of breast cancer.

While the overall goal of cancer chemotherapy is to eradicate tumour cells without damaging normal host tissue (Kwok et al., 2017), it is usually difficult to predict what a patient's response will be to any therapeutic agent. The side effects of chemotherapy vary significantly between patients and different

treatment regimens and can include fatigue, white blood cell depletion, febrile neutropenia, nausea and vomiting, pain, hair loss, diarrhoea, loss of appetite, constipation, weight gain and cognitive dysfunction (Carr et al., 2008). Many of these side effects can be managed with treatment. More adverse effects include cancer treatment related mucosal injury, including oral and gastrointestinal mucositis and secondary neoplasms.

In the treatment of last stage metastatic TNBC, chemotherapy remains one of a small number of treatments, although more recent developments suggest at least one targeted antibody therapy and some immunotherapy may be useful in improving OS rates.

1.2.2.3 Targeted Therapy

Targeted therapy is a category of antineoplastic therapies that act on features unique to a cancer and that often facilitate proliferation and invasiveness. This form of treatment is particularly useful as specific features that are common to a particular cancer type are targeted, therefore the therapeutic action is mostly limited to target tissue, minimizing damage to healthy tissue (Tsimberidou, 2015). As a result, these treatments have significantly fewer side effects than chemotherapy or radiotherapy. Drugs of this treatment category require a more detailed understanding of the physiology of the cancer being treated. For example, Trastuzumab and Pertuzumab are recombinant IgG1 monoclonal antibody (mAb) therapies which act on tumours that test positive for the overexpression of HER2. Trastuzumab and Pertuzumab (Capelan et al., 2013), bind to different regions of the extracellular domain of HER2, stereochemically occluding the HER2 binding site (Boekhout et al., 2011) significantly reducing HER2 signal transduction. This reduces Ras and P13K signalling, reducing cancer stemness and heterogeneity (Kunc et al., 2021). Additionally, the binding of antibodies to the exterior of cancer cells can induce apoptosis via a number of mechanisms such as antibody-dependent cellular toxicity (ADCC) complement-dependent cytotoxicity

(CDC) and antibody-dependent cell-mediated phagocytosis (ADCP) (He et al., 2004). Pertuzumab / trastuzumab / hyaluronidase-zzfx (Phesgo) (Perkey, 2021) and Ado-trastuzumab emtansine (Kadcyla) (Thungappa et al. 2021) are also recombinant IgG1 monoclonal antibody-based therapies developed for the treatment of HER2 overexpressing cancer such as HER2+ breast cancers. Targeted therapies are often used in combination with other therapies, such as chemotherapy to improve OS rates in the treatment of Stage I-III HER2+ breast cancer.

Olaparib is a targeted therapy that inhibits poly ADP ribose polymerase (PARP), which targets cancer cells by preventing the repair of single stranded breaks, resulting in the accumulation of genetic damage, ultimately leading to synthetic lethality (Mandapati & Lukong, 2022). Olaparib is used in the treatment of patients with early-stage, HER2-, ΔBRCA1/ΔBRCA2 breast cancer (Goulooze et al., 2015). Ribociclib and Palbociclib are newly developed CDK4/6 inhibitors that are being screened for their utility in treating advanced ERα positive, HER2- breast cancers (Purohit et al. 2024). A more succinct summary of systemic treatment is described in the following table

Breast cancer subtype/classification			Adjuvant systemic therapy		
Phenotypic subtype		Intrinsic subtype	Endocrine therapy	Anti-HER2 therapy	Chemotherapy
Hormone receptors	HER2 overexpression				
+	-	Luminal A or B	Yes	No	Yes (if high risk)
+	+	Luminal B or HER2 enriched	Yes	Yes	Yes
-	-	Basal	No	No	Yes
-	+	HER2 enriched	No	Yes	Yes

Table 1.2. Summary of the subtypes of breast cancer and suitable systemic treatment regimens

Treatment Regimen	Drugs
AC-TH	Doxorubicin, Cyclophosphamide, Paclitaxel or Docetaxel, Trastuzumab
AC-THP	Doxorubicin, Cyclophosphamide, Paclitaxel or Docetaxel, Trastuzumab, Pertuzumab
TCH	Paclitaxel or Docetaxel, Carboplatin, Trastuzumab
TCHP	Paclitaxel or Docetaxel, Carboplatin, Trastuzumab, Pertuzumab

TH	Paclitaxel, Trastuzumab

Table 1.3. Common combination therapy regimens for Stage 1-2 HER2-positive breast cancer containing either one or two targeted antibody therapies and combination chemotherapy (Slamon et al. 2001).

Sacituzumab govitecan-hziy is a novel recombinant IgG1 antibody therapy approved for use in the treatment of TNBC (Syed, 2020). Sacituzumab govitecan-hziy is an antibody drug conjugate that binds to the TROP2 receptor, and has multiple mechanisms of action, including the inhibition of the activity of TROP2 via binding and occlusion, CDC, ADCC, ADCP and the release of the cytotoxic agent SN-38, which is a potent topoisomerase inhibitor and acts to increase the rate of DNA damage and induce apoptosis in cells overexpressing TROP2. Sacituzumab govitecan-hziy has demonstrated notable efficacy in clinical trials. In patients with unresectable locally advanced TNBC and advanced TNBC that have received two different forms of systemic treatment prior to treatment with Sacituzumab govitecan-hziy, the progression free survival (PFS) was shown to have tripled over the course of a year (7% to 21%) (Rugo et al. 2023).

1.2.2.4 Immunotherapy

Immunotherapy is a category of targeted therapy that involves the use of medical agents, usually recombinant monoclonal antibodies, but also recombinant cytokines, soluble cytokine receptors or small molecule drugs to alter immune mechanisms to improve the immune system's ability to detect, form an immune response to and clear cancerous cells (Schuster et al., 2006). A recent immunotherapeutic target of note is the PD-1/PD-L1 mechanism, which is an immune checkpoint, where upon PD-1 binding to PD-L1 or PD-L2 triggers the autophosphorylation of the immunoreceptor tyrosine-based inhibitory and switch motifs. This in turn activates the Src homology region 2 domain-containing phosphatase (SHP-2), which reverses the phosphorylation of T cell receptor (TCR) and cluster of differentiation 28 (CD28)

signalling, and as a consequence, T cell function and immune response are both suppressed (Jiang et al., 2019). In non-cancerous tissue, this mechanism mediates the induction and maintenance of self-tolerance in healthy cells. In cancerous tissue, this mechanism is often coopted by cancer cells to promote immune evasion.

A targeted antibody therapy was developed to prevent the activation of the PD-1/PD-L1 mechanism, Pembrolizumab. Pembrolizumab is an immune checkpoint inhibitor, binding to the extracellular domain of PD-1 and preventing the binding of PD-L1 and PD-L2 (Khoja et al., 2015), which allows the host T cells to generate an immune response.

Pembrolizumab is often used as part of many combination therapies to treat early to late-stage breast cancer as combination therapy that includes immunotherapy has been shown to have a synergistic effect in multiple cancers, improving efficacy over either treatment alone. In the treatment of TNBC, the median overall survival of individuals that received the combination therapy using pembrolizumab and chemotherapy was 23.0 months, those that received a placebo and chemotherapy survived only 16.1 months, however they found this effect was reduced if the expression level of PD-L1 was low (Cortes et al., 2022). Some of the regimens that utilize pembrolizumab are described in the following table.

Treatment Regimen	Drugs
TC/pembrolizumab-AC/pembrolizumab	Paclitaxel, Carboplatin, Pembrolizumab, Doxorubicin, Cyclophosphamide
TC/pembrolizumab-EC/pembrolizumab	Paclitaxel, Carboplatin, Pembrolizumab, Epirubicin, Cyclophosphamide

Table 1.4 Common combination therapy regimens for the treatment of TNBC using immunotherapeutic agent pembrolizumab (Cortes et al., 2022)

1.3 Treatment of TNBC.

As mentioned earlier, there are few effective treatments available for TNBC, due to the lack of druggable targets (ERα -, mPgR-, HER2-). Surgical resection, radiotherapy and some systemic therapies are the primary modalities for the treatment of TNBC. While all of these can treat breast cancer effectively on their own, due to the shortcomings of each modality, a curated multimodal approach is recommended to improve clinical outcomes.

At Stage 0, a mastectomy or a lumpectomy and adjuvant radiotherapy are recommended. If the endocrine receptor status of the tumor is known, endocrine therapy may be used as both a neoadjuvant and adjuvant treatment (Waks & Winer, 2019). Adjuvant radiotherapy is not recommended due to the elevated risk of secondary neoplasms associated with radiotherapy dose.

1.3.1 Stages I-III of TNBC.

If the TNBC tumour is an appropriate size < 1cm in diameter, it is considered a stage I breast cancer, and a mastectomy of the lesion and surrounding tissue and a lymph node biopsy is recommended. If the tumour or if the lymph nodes are found to have cancer, adjuvant radiotherapy may follow surgery. Adjuvant chemotherapy may reduce the ROR and is recommended if the tumour demonstrates some degree of invasiveness. A lumpectomy with adjuvant radiotherapy is also a viable treatment regimen, and if the cancer is expected to be lymph node positive, either a sentinel or axillary lymph node biopsy is recommended.

If a primary tumour is between 2-5cm in diameter or any axillary lymph nodes have tested positive for breast cancer, the tumour is classed Stage II. Recommendations for the treatment of stage II TNBC include a total mastectomy with adjuvant chemotherapy, or a lumpectomy with adjuvant radiotherapy and chemotherapy (Trayes & Cokenakes, 2021).

Nodal diagnostics and treatment are recommended and will require either a lymph node biopsy or a sentinel node biopsy and radiotherapy to supraclavicular and mammary nodes to achieve clearance. Stage II TNBCs can be treated using immunotherapies such as pembrolizumab and a Doxorubicin-based polychemotherapy regimen, which can improve ROR (Jiang et al. 2019). If the patient has undergone genetic screening and tests positive for a pathogenic variant of *BRCA1* or *BRCA2*, the use of Olaparib for up to 12 months following tumour excision is recommended.

If TNBC cells are detected in more distal tissue, including >3 lymph nodes, the cancer is classed as a Stage III TNBC, which requires more aggressive and complex treatment to produce any therapeutic benefit for the patient. A complete mastectomy and an adjuvant therapy such as radiotherapy or chemotherapy is recommended for stage III A TNBC (Trayes & Cokenakes, 2021). For treating stage III B TNBC, neoadjuvant combination therapy, such as Pembrolizumab and a standard chemotherapy regimen such as ACT (Shien & Iwata, 2020 Cortes et al., 2022) may be required to reduce tumour size before it can be safely removed. For Stage III B cancers with a larger primary tumour, a lumpectomy and adjuvant chemotherapy or radiotherapy may be employed to reduce the size of the tumour, followed by a total mastectomy and adjuvant therapy to remove the remaining cancer and reduce the likelihood of recurrence. Stage III C breast cancers are particularly advanced and will likely require axillary node lymphadenectomy and adjuvant radiotherapy to the internal mammary and supraclavicular lymph nodes to address proliferation throughout the upper lymphatic system in addition to one or several lumpectomies with adjuvant therapy and/or a mastectomy with adjuvant therapy.

Treatment is likely to continue after surgical resection of the primary tumour with some form of systemic treatment to improve the likelihood of complete clearance.

If cancer is still detected following neoadjuvant chemotherapy and surgical resection, capecitabine, a fluorouracil precursor, may be given for 18 to 24 weeks following surgery to improve the likelihood of clearance. This regimen has notably improved 5-year OS rates among patients with early-stage TNBC. Additionally, Olaparib may be taken for one year if the patient has a *BRCA1* or *BRCA2* mutation to help reduce the ROR (Goulooze et al., 2015).

1.3.2 Stage IV (Metastatic) Triple-Negative Breast Cancer

Systemic therapies may be employed as a form of palliative care, to improve 2 year and 5-year OS statistics in patients with Stage IV metastatic breast cancer, however the 10-year OS rate of Stage IV metastatic TNBC is effectively 0%. Polychemotherapy is usually the primary treatment modality for Stage IV metastatic breast cancer and is generally used in combination therapy regimens. These combination therapies include immunotherapies such as pembrolizumab as the overexpression of PD-L1 is common in TNBCs, occurring in approximately 1 out of 5 TNBCs (Jiang et al., 2019) and pembrolizumab this has demonstrated the ability to interfere with the PD-1/PD-L1 immunomodulatory mechanism that facilitates immune evasion. Recent clinical trials recommended regimens include the substitution of Pembrolizumab with Atezolizumab. Atezolizumab is an anti PD-L1 mAb that has both immunomodulatory functions and directly acts on the cancer cell. Atezolizumab monotherapy produces a median OS of 17.6 months (95% CI, 10.2 months to not estimable) in patients with metastatic TNBC although its effect is variable and depends on the level of PD-L1 expression in immune cells, with patients with > 1% PD-L1 expression having no notable response to Atezolizumab monotherapy (Emens et al. 2019). The addition of Sacituzumab govitecan-hziy is recommended upon the failure of two other treatment regimens. Sacituzumab govitecan-hziy is an antibody-drug conjugate that targets cells overexpressing the TROP2 receptor, a common trait in TNBC (Stepan et al., 2016). Recently the approval

of Sacituzumab govitecan-hziy has notably improved OS rates, with the median OS rate of metastatic TNBC patients receiving a Sacituzumab govitecan-hziy monothearpy increasing to 12.1 months (95% CI, 10.7 to 14.0) compared to 6.7 months (95% CI, 5.8 to 7.7) with only chemotherapy (Bardia et al., 2021).

At this stage of disease progression, surgical resection (Ruiterkamp et al. 2009) and radiotherapy may not be curative but could potentially improve clinical outcomes and provide relief from the disease burden and should be considered. For patients with TNBC that have developed MDR to the standard polychemotherapeutic regimen of anthracyclines and taxanes, platinum chemotherapeutics such as cisplatin and the use of targeted therapeutics such as PARP inhibitors have demonstrated moderate efficacy (Zhu et al. 2022). For advanced TNBC in which cancer cells have been screened and test positive for the PD-L1 receptor, the first treatment is likely to be a combination therapy containing the immunotherapeutic agent pembrolizumab (Shien & Iwata, 2020). A regimen of carboplatin and paclitaxel, followed by Cyclophosphamide and Doxorubicin while receiving pembrolizumab is a commonly used regimen. Recommended treatment regimens are listed on Table 1.4. Recent clinical trials suggest Atezolizumab, an anti PD-L1 antibody therapy may also be useful in the treatment of TNBCs which overexpress PD-L1 (Jiang et al., 2019).

For advanced TNBC that has shown little or no response for two or more treatments, Sacituzumab govitecan-hziy (Trodelvy) a recently developed antibody-drug conjugate, may be used (Syed, 2020). Surgery and radiation may also be considered, if it is likely to improve survival or have palliative effects.

To treat recurrent TNBC that cannot be removed via surgical resection, and test positive for PD-L1 overexpression, one of the scheduled combination therapies containing pembrolizumab and chemotherapy is recommended. Examples of appropriate regimens are described in Table 1.4. If possible, adjuvant Atezolizumab is recommended to improve clinical outcomes. If the cancer recurs in more distal regions of the body, the regimen may be modified to include the antibody-drug conjugate Sacituzumab govitecan-hziy (Trodelvy).

If the cancer is a recurrent metastatic TNBC, it is likely that more aggressive treatments are needed, as the 5-year survival rate is poor (10.81%) (Hsu et al., 2022). Research recommends a regimen of systemic treatments, including biologics, chemotherapeutic agents and, in certain circumstances, radiotherapy and surgical resection. Recommendations for treatment are similar to the treatment of metastatic TNBC described in Table 1.4; however it is assumed at this stage the cancer is more severe, and the attainment of a curative state is unlikely. As mentioned prior, the treatment regimen may be modified to include atezolizumab till failure is reached, and the antibody-drug conjugate Sacituzumab govitecan-hziy (Trodelvy) when two or more treatments have failed.

While treatments have improved, the low number of systemic treatments available to treat metastatic and non-metastatic TNBC is an issue, and that is reflected in the reduced OS rate for patients with TNBC. To further improve outcomes, newer targets and strategies for improving outcomes are currently being developed.

1.4 Genetic Medicine

Initially developed to target hereditary diseases, genetic medicine, which is defined as the use of RNA or DNA based technologies to diagnose or treat a patient through the sequencing of or direct modification of the host genome (Porteus, 2015) or by altering host gene expression through the coopting of cellular mechanisms such as the RNA induced silencing complex (DICER/RISC) (Agrawal et al., 2003). Genetic medicine has made it possible to treat diseases associated with both changes to the host DNA sequence, and to diseases involved in the abnormal expression of genes. (O'Connor & Crystal, 2006). In 2017, the U.S. Food and Drug Administration approved the CAR-T therapy Kymriah (Tisagenlecleucel), for use in treatment of acute lymphoblastic leukaemia (ALL) in patients under 25 years.

1.4.1 RNA therapeutics

RNA therapeutics are a more recent class of RNA based cancer therapies that have shown promise in early-stage clinical trials. RNA therapeutics involve the use of exogenous RNA to coopt cellular defence mechanisms to alter the expression levels of various proteins in the cell, replace nonfunctional proteins with a functional wild type (WT) or to introduce novel proteins. RNA therapeutics are reversible and involve no change to the hosts genome and are thus considered safer than using gene editing technologies. One example includes the use of siRNA induced knockdown in the work of Zhou et al (2017). Their work demonstrated that the use of Notch1 siRNA to inhibit the expression of the Notch1 protein in MDA-MB-231 and MCF-7 breast cancer cell lines reversed chemoresistance. In the work of Divita et al. (2022), p53 mRNA and a tumour selective nanocarrier were used to rescue the tumour suppressor functions of p53 in ovarian cancer in vitro and in vivo, which in turn restored chemosensitivity to PARP inhibitors.

1.4.2 Genetic Modification and Gene Editing

Directly modifying or editing the host genome is another strategy proposed to treat various cancers. By the generation of deletion, insertion or substitution events in either the hosts genome, or the genomes of targeted tissues such as tumour cells, it is possible to halt or modify the expression of certain genes or to introduce entirely novel genes to improve treatment outcomes.

There are a broad range of technologies that have historically been used to alter the host genome. This technology includes zinc finger nucleases (ZNFs) and transcription activator-like effector nucleases

(TALENS). More recent developments include the use of the ultra-precise variants of CRISPR Cas9 that allow for highly accurate modifications to cell tissue.

The goal of gene editing and gene modification in cancer is to utilize one of the aforementioned mechanisms to alter genes in cancer cells to produce alterations in the genome of cancer cells that result in a loss, gain of or modification of traits that will likely improve treatment outcomes.

These technologies utilize nucleases to generate breaks in the host genome, either single stranded breaks (SSB) or double strand breaks (DSB) which are usually followed by cell mediated repair that is specific to each system (Suleiman et al., 2021). Often these double stranded break repairs will incorporate errors, ideally a frameshift mutation, and these errors can result in a loss of function in the targeted gene or facilitate the insertion of an exogenous DNA fragment.

A recent example of genetic modification being used in the treatment of cancer is the use of CAR-T cell technology. CAR-T cell technology is the modification of host T cells to produce a surface antibody that binds to one or more surface proteins that are overexpressed in a patient's cancer. The modified T cell selectively binds to cancer cells characteristically overexpressing this protein and an immune response is initiated (Jiang et al. 2019). There are limitations to this technology, including how cost prohibitive a single CAR-T treatment is, and its low efficacy in solid tumours, which make up approximately 90% of all cancers. Additionally, if the patient's cancer is heterogeneous, CAR-T treatment alone will likely be insufficient to resolve a patient's cancer and if there are no known surface markers that differentiate the targeted cancer from the host patients' cells, it is unlikely that CAR-T would be of any use in that particular instance (Liu et al., 2019). There are also risks associated with CAR-T cell therapy, including CAR-T cell therapy related toxicities, which includes neurotoxicity, cytokine release syndrome as well as coagulopathy, disseminated intravascular coagulation and secondary neoplasms.

While genetic medicine shows promise, there are still some major challenges that impede their success. CAR-T therapy for example, has many issues, that include high cost of production, problems with

scalability, niche applications and inability to target solid tumours effectively. These issues mean that CAR-T is currently unsuitable for the treatment of most cancers, including TNBC (Liu et al., 2019). A more reasonable approach would be to improve the success and safety of more reliable, economical and broader acting treatments, such as chemotherapy by reversing drug resistance.

1.5 Doxorubicin

Doxorubicin is one of the most effective chemotherapeutic agents used in the treatment of solid tumours of diverse origins, such as lymphocytic leukaemia, lymphoma, and bladder and breast cancer (Micallef & Baron, 2020). Doxorubicin is a member of the anthracycline group of chemotherapeutic agents and is comprised of the sugar Daunosamine bound to aglycone Adriamycinone (Martins-Teixeira & Carvalho, 2020). Doxorubicin is the primary chemotherapeutic agent used in the treatment of breast cancer, and is given intravenously, often with other therapeutic agents, including other chemotherapeutics, targeted antibody therapies and immunotherapies. A response rate of 33.25 % has been established when Doxorubicin is administered at doses of ≥ 60 to 75 mg/m2 on a 3-week cycle (Toma et al. 1992).

Doxorubicin is the current standard for the chemotherapeutic treatment of breast cancer due to the efficacy of Doxorubicin when compared with other chemotherapeutic agents (Barrett-Lee et al., 2009). A clinical study comparing the efficacy of Doxorubicin to paclitaxel monotherapies in the treatment of TNBC found the response rate for Doxorubicin was 41% compared to 25% for Paclitaxel (Paridaens et al., 2016).

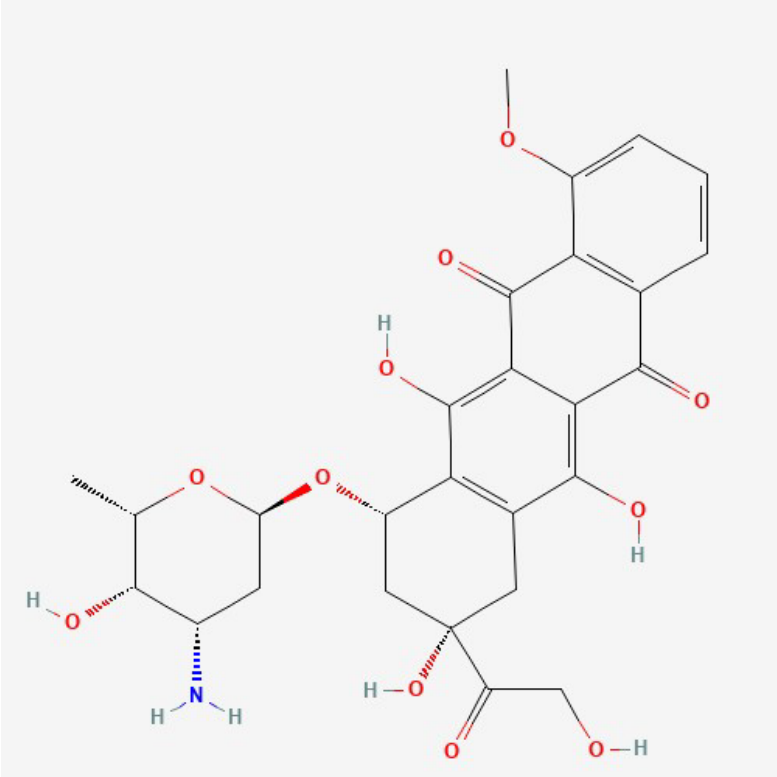


Figure 1.4 The molecular structure of Doxorubicin (Arcamone et al., 1972)

While highly efficacious, long-term administration of Doxorubicin leads to active resistance, contributing to poor clinical outcomes in TNBC breast cancer patients (Chen et al., 2023).

1.5.1 Clinical Use

Anthracycline-based chemotherapy is the current standard of treatment for TNBC in patients under 75 years of age. Early clinical trials using Doxorubicin and other anthracyclines in the treatment of breast cancer produced a notable reduction in the rates of recurrence and improved survival when compared with standard chemotherapy regimens at the time, which lacked anthracycline class chemotherapeutic agents (Early Breast Cancer Trialists' Collaborative Group, 1995). As a direct result of these trials, the use

of adjuvant anthracyclines became the dominant form of treatment for individuals with breast cancer, with more than 70% of women under 70 years with node-negative breast cancer receiving an anthracycline-based chemotherapy regimen as treatment by the year 2000 (Vuger et al., 2022). As a monotherapy, the acceptable dosage range is Doxorubicin is 40 to 75 mg/m², however in the treatment of solid tumours, a higher dose, in the range of 60 to 75 mg/m² IV is recommended on a 21-day or 29-day cycle. The maximum lifetime cumulative dose of Doxorubicin is 550 mg/m² (Gabani et al., 2021) before the probability of therapy related myelodysplastic syndrome is significantly elevated (Morton et al., 2019).

To further improve the efficacy of chemotherapy in the treatment of breast cancer, and to reduce the likelihood of acquired chemotherapeutic resistance, Doxorubicin is now more commonly used as part of a polychemotherapeutic regimen, alongside other agents such as Cyclophosphamide or Docetaxel. Multiple clinical trials were performed that focused on the efficacy of Doxorubicin and Doxorubicin-based polychemotherapies in the treatment of both metastatic and non-metastatic node positive breast cancer. In an analysis of 18 successive Doxorubicin containing protocols on patients with metastatic breast cancer, Doxorubicin-containing chemotherapy was demonstrated to have a RR of 65% and a CRR of 16.6%. PFS and OS were 11.5 months and 21.3 months for participants that exhibited a partial response and 22.4 months and 41.8 months for participants that demonstrated a complete response, respectively (Rahman et al., 1999).

A common breast cancer polychemotherapy regimen is the AC regimen, which includes the intravenous administration of 60 mg/m² of Doxorubicin on a 21-day cycle, usually with Cyclophosphamide, although this is not the only regimen and there are many variations that can include 2 or more other chemotherapeutic agents. Patients receive at least four cycles; however, this can be increased to treat larger, more aggressive and less responsive cancers.

ACT is another common polychemotherapeutic treatment regimen for metastatic breast cancer. This regimen includes the intravenous administration of Docetaxel, Cyclophosphamide and Doxorubicin in 6

to 8 21-day cycles, with the concentration of Doxorubicin, Cyclophosphamide and Docetaxel being 60 mg/m^2, 600 mg/m^2 and 100 mg/m^2 respectively. This protocol is derived from the National Surgical Adjuvant Breast and Bowel Project Protocol B-27 clinical trial, which involved the addition of Docetaxel (T) to a standard AC regimen. As the use of AC chemotherapeutic treatment was explored, the addition of Docetaxel was found to improve disease clearance and reduce the incidence of local recurrences (P = .0034) (Early Breast Cancer Trialists' Collaborative Group, 1995). Neoadjuvant Docetaxel was also shown to significantly improve the likelihood of achieving a disease-free state in participants who demonstrated a partial response to treatment with AC therapy alone, with a hazard ratio (HR) = 0.71 and a p value of .007. Furthermore, the complete response rate was doubled by addition of preoperative Docetaxel, with a HR of 0.33 and a p value of < .0001. Preoperative and postoperative Docetaxel after preoperative Doxorubicin and Cyclophosphamide improved disease-free survival (DFS) rates and the incidence of local recurrences (Bear et al., 2006).

1.5.2 Mechanisms of Action

Most chemotherapeutic agents are cytotoxic, and their mechanisms of action usually involve triggering one or several DNA or cellular damage response mechanisms that lead to cell death. This may involve the creation of DNA lesions, the inhibition of DNA or RNA synthesis, or the initiation of an immune response (Alcindor & Beauger, 2011). The trafficking of Doxorubicin into the cancer cell is believed to be the result of passive diffusion, however, this is likely limited by the biochemical makeup of the membranes. Recent research suggests Doxorubicin uptake may be partially facilitated by the *SLC22A16* gene, which encodes the carnitine transporter, CT2 (Okabe et al., 2005). The inhibition of topoisomerase II A is the primary mechanism by which Doxorubicin triggers synthetic lethality in cancer cells. Topoisomerase II A is involved in the cellular replication process and creates double stranded breaks in

the DNA that relieve torsion during the replication process. Doxorubicin is believed to interfere with the activity of topoisomerase II A, arresting its activity and causing occlusions which prevent DNA repair mechanisms from acting on and repairing double-stranded breaks (DSB) generated by topoisomerase II A (Okabe et al., 2005). This sustained DSB can trigger apoptosis. Furthermore, Doxorubicin is also known to cause free radical oxidative damage, as the quinone structure of Doxorubicin gives rise to a semiquinone free radical via the addition of an electron via interaction with intracellular enzymes NQO1, NOS3 and XDH. The interconversion of Doxorubicin to Doxorubicin semiquinone produces ROS that have a number of cellular effects. ROS are believed to contribute to the oxidation of the phospholipid bilayer, which results in membrane damage and the byproducts of this process are known to trigger a number of intracellular mechanisms that promote apoptosis. Furthermore, when supplemented with iron, the two components form 4 Fe-Dox complexes, which is known to further increase the production of ROS. The accumulation of genetic damage resulting from oxidative reduction, in turn, further promotes apoptosis. Doxorubicin is also a known intercalator of DNA and prefers an intercalation site near GC and likely forms a hydrogen bond with the local guanine. This bond is reportedly stabilized by local formaldehyde produced from free radical oxidation and then forms a covalent bond with complexed DNA. They have found this intercalation of Doxorubicin results in the activation of DNA repair mechanisms. Intercalation, however, does not appear to be the primary mechanism of action as the rate of intercalation is low per unit of Doxorubicin administered, with 4.4+/-1.0 adducts per 10^7 bp.

There are also several other mechanisms via which Doxorubicin acts, including the excessive production of ceramide, which has been strongly associated with improved clinical outcomes and histone eviction with studies demonstrating its effects on the efficacy of DNA repair mechanisms.

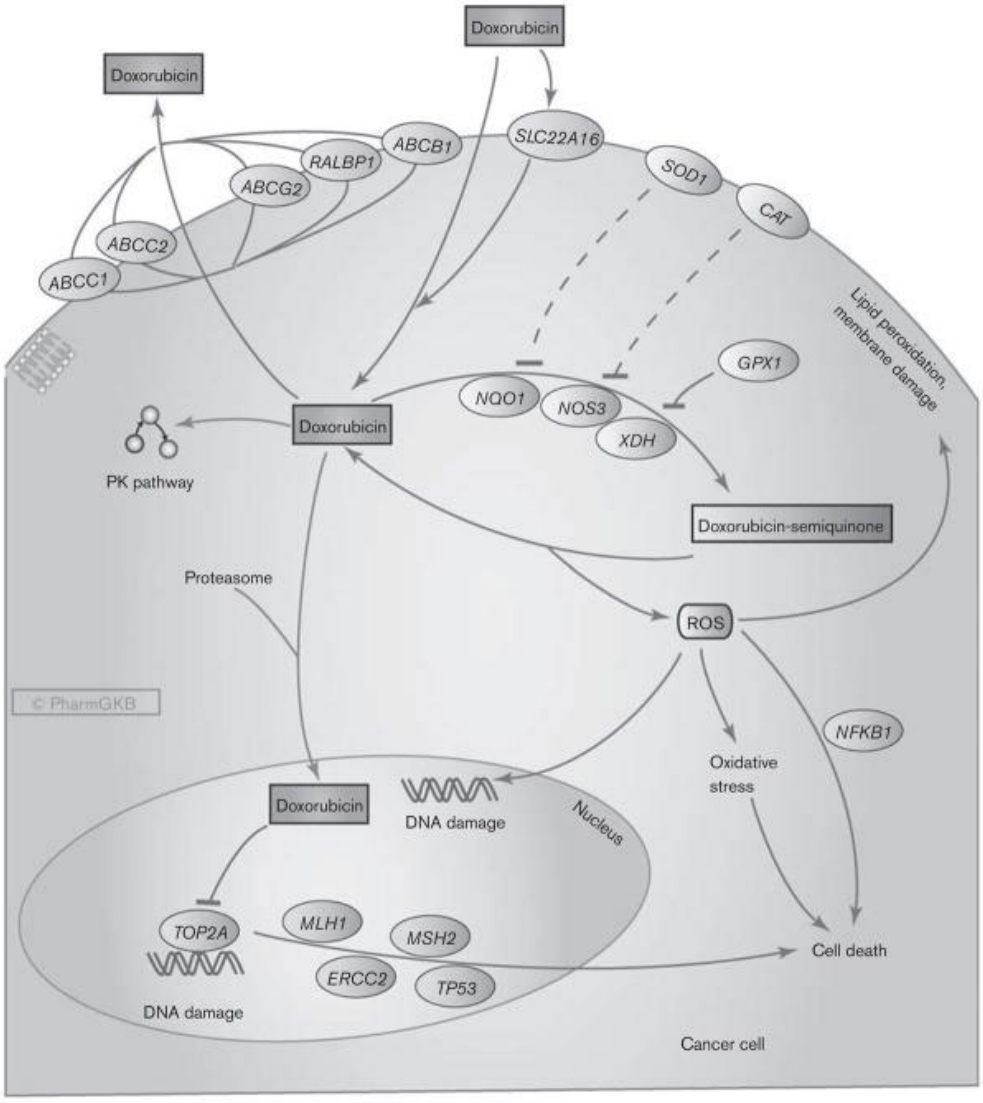


Figure 1.5 A chart describing the mechanisms through which Doxorubicin enters the cell and has a therapeutic effect. Doxorubicin either undergoes catalytic modification and induces oxidative stress or enters the nucleus and interacts with DNA and Topoisomerase 2 A (TOP2A), causing DNA damage and triggering apoptosis in cancer cells. The chart also visualizes the role the ABC transporter family plays in the development of resistance, via the efflux of Doxorubicin outside the cell. (Thorn et al. 2011).

Most chemotherapeutic agents interact with metabolically active and rapidly dividing cells at a higher rate than surrounding noncancerous tissue. For example, Doxorubicin interacts with Topoisomerase II A, an enzyme involved in the uncoiling of dsDNA during replication and mitotic cell division by transiently cleaving one or both strands of DNA (Nitiss, 2009). The efficacy of Doxorubicin relies on its ability to

interact with the target during periods of the target cell's life cycle where there are increased rates of replication and division. As the rate of cellular replication is elevated in cancer, the cytotoxic effect of Doxorubicin is overrepresented in cancer cells. However, this does not mean that the effects of chemotherapeutic agents, additionally, for tissue types that have reduced rates of replacement such as nervous tissue, the damage is cumulative, hence why most chemotherapeutic agents have a maximum lifetime cumulative dose.

1.5.3 Pharmacokinetics

Identifying the pharmacokinetic parameters of any therapeutic agent is important for achieving maximal therapeutic benefit while minimizing any adverse effects. Doxorubicin has undergone extensive clinical evaluation to determine key features like effective dosage, appropriate administrative protocol, etc. to allow for the safe and effective administration of Doxorubicin hydrochloride in the treatment of breast cancer. The Early Breast Cancer Trialists Collaborative Group (EBCTCG) metanalysis of adjuvant Doxorubicin containing regimens collated the results of 19 clinical trials that included 7523 patients and found a reduction in the hazard ratio (HR) reduction of 9%, implying a general improvement in patient survival rates, when compared with a Carboplatin, Methotrexate and flourouracil (CMF) treatment regimen. Larger and more recent trials are consistent with these results (EBCTCG, 2012). General recommendations of the EBCTCG suggest Doxorubicin be intravenously administered at 60 mg/m^2 - 75 mg/m^2, on a 21-day treatment cycle, in combination with Cyclophosphamide, and administered by IV infusion over 2 to 6 hours (Pfizer New Zealand Limited, 1990).

When first assessing Doxorubicin is reached at the end of infusion and is dependent on the time of peak delivery during a treatment regimen (Freyer et al., 2000).

The elimination half-life of plasma Doxorubicin was determined to be approximately 5 minutes, and the rate of plasma clearance is 324 mL/min/m^2 to 809 mL/min/m^2 (Ritzmo, 2009). Clearance is the result of metabolism and biliary excretion, with renal excretion contributing little to overall clearance. The terminal half-life is between 20 to 48 hours, suggesting a significant amount of retention in the organs and peripheral tissues.

Research has found that while 50–85% of IV Doxorubicin can bind to plasma protein, excess accumulation in the blood plasma is not typical as Doxorubicin is rapidly distributed throughout the peripheral tissues and organs, and as a result, the plasma concentration of Doxorubicin rapidly declines.

The volume of distribution of Doxorubicin is in the range of 20 to 30 L/kg (Pfizer New Zealand Limited, 1990). The metabolic breakdown of Doxorubicin usually occurs in kidney, liver and red blood cells, usually via the two-electron reduction pathway, which involves the reduction of Doxorubicin by cytoplasmic NADPH-dependent aldo-keto reductases to Doxorubicinol, a 13-hydoxy1 metabolite, aglycones, 7- deoxydoxorubicinone and Doxorubicinone (Novotna et al., 2008).

It is expected that 50% of IV Doxorubicin hydrochloride passes via biliary secretion within 7 days, with cumulative faecal excretion of Doxorubicin reaching an estimated 25 to 45%. 5% – 10% of the is excreted in the urine (Freyer et al., 2000). In praxis, Doxorubicin is administered as part of a polychemotherapy, with drugs such as Cyclophosphamide, Docetaxel or 5-FU, as of these treatments have greater therapeutic benefit when used concomitantly than when used alone (Early Breast Cancer Trialists' Collaborative Group, 2012). Any adverse effects resulting from the use of Doxorubicin and other chemotherapeutic agents concomitantly is considered of greater benefit to the patient than alternatives, and the risk of cardiotoxicity, neurotoxicity and other adverse effects are a calculated risk, which is why treatment should not cumulatively exceed 550 mg/m^2 over course of the patient's lifetime (Pfizer New Zealand Limited, 1990).

1.5.4 Doxorubicin Toxicity

While it is possible to achieve clearance using chemotherapy alone, and Doxorubicin is one of the more effective chemotherapeutic agents used in the treatment of breast cancer, there are dose limiting side effects that severely restrict therapeutic potential. Cardiotoxicity is a noted side effect and, in some instances, can be lethal (van Dalen et al., 2016). Cardiotoxicity often presents as myopericarditis, arrythmia or ventricular dysfunction. Arrhythmias such as supraventricular tachycardia, premature atrial and ventricular contractions and sinus tachycardia, occur in approximately 26% of patients who receive Doxorubicin-based chemotherapy. Ventricular dysfunction is another side effect of note but is rare and usually reversible. Chronic cardiac toxicity is rare, occurring in only 1.7% of patients and is potentially lethal. Irreversible cardiomyopathy can occur within a few months of treatment cessation but is extremely rare. There are currently no reliable predictors for which symptoms are likely to arise in any patient or the intensity of symptoms a patient may experience.

Dose limiting side effects also include myelosuppression, which can predispose a patient to infection, sepsis and mucositis. Mucositis is very common and occurs in roughly 40% of patients receiving Doxorubicin-based treatment for their breast cancer.

Doxorubicin can also negatively impact multiple subcellular structures, with Doxorubicin-based chemotherapy regimens being linked with increased reactive oxidation species production and mitochondrial damage in cardiomyocytes. Doxorubicin is also known to increase permeability in the cardiac endothelial cell barrier by affecting tight junctions. Doxorubicin also impacts skeletal muscle microcirculation and smooth muscle cells.

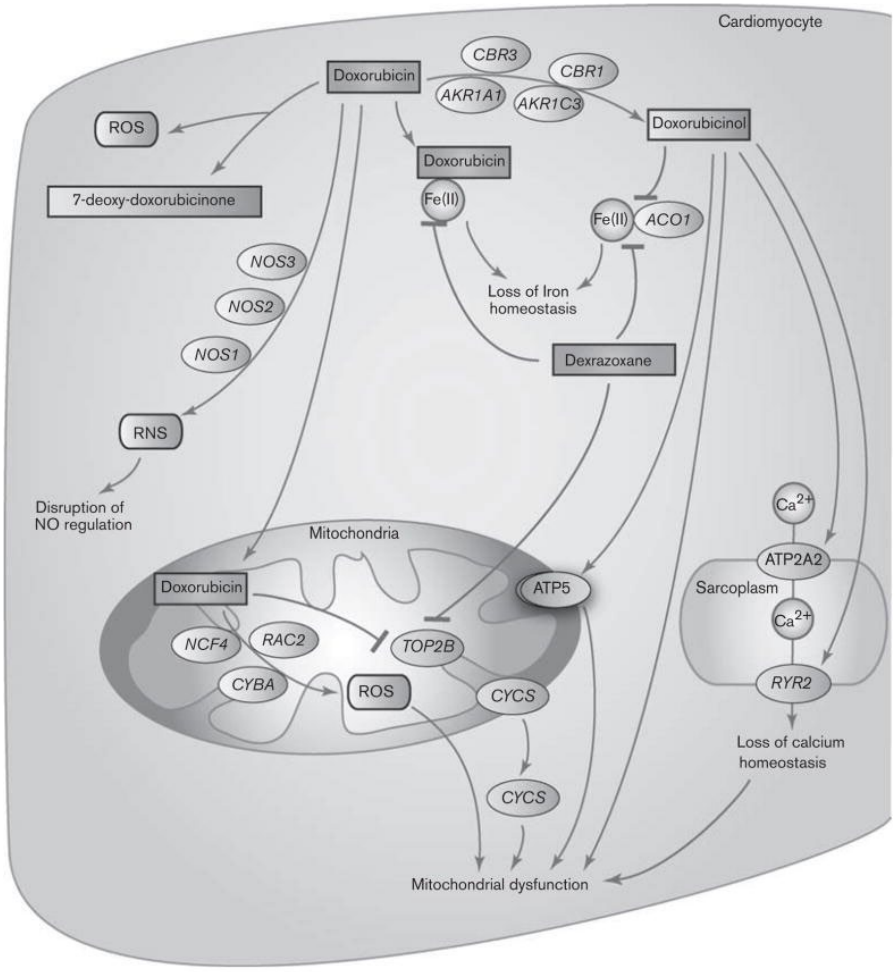


Figure 1.6. Image describing the primary mechanisms by which Doxorubicin induces synthetic lethality in cancer cells. Methods include loss of calcium homeostasis, the induction of mitochondrial dysfunction, loss of iron homeostasis, and disruption of NO regulation (Thorn et al, 2011).

The functionality of Doxorubicin and other anthracyclines can be improved through the use of additives. Small molecule compounds and some biologics have been used to improve therapeutic effect of Doxorubicin, reduce cardiotoxicity and even reverse cardiomyopathy. Quercetin demonstrated synergistic effects in invitro when administered with Doxorubicin, improving the anti-tumour effect of Doxorubicin and potentially reducing the dose required to achieve a therapeutic effect (Zhang et al., 2021). qPCR analysis suggests this is likely due to the downregulation of ABC transporters such as Breast Cancer Resistance Protein (BCRP) and ATP binding cassette B1 (ABCB1). Additionally, quercetin has

antioxidative properties, which has been linked to reduced cardiotoxicity and hepatotoxicity in in vitro assays (Zhang et al., 2021).

Comprehensive meta-analyses were used to determine the optimal dose regimen of Doxorubicin and other anthracycline based treatments for minimizing cardiotoxicity while retaining therapeutic effect in humans (Elvira et al. 2016). A metanalysis of 11 different studies which included 5820 predominantly adult participants with solid tumours, showed a notable reduction in the occurrence of clinical heart failure with an infusion duration of six or more hours (risk ratio (RR) 0.27; 95% CI 0.09 to 0.81) Conversely, the effect of dosage in the range of 0-110 mg/ml was found to have no statistically significant effect (Elvira et al., 2016).

An analysis of Doxorubicin induced cardiomyopathy in mice revealed that Doxorubicin induces subcellular changes in cardiomyocytes, including the degradation of myofibrils and aberrant vacuole formation in the cytosol. An increase in the density of extracellular matrix proteins was also detected, which suggests disorganization of the extracellular spaces in cardiac tissue and structural analysis indicated endothelial cell degradation in cardiac vasculature (Podyacheva et al., 2021). The results of this study align with the conclusions of Elvira and colleagues (2016) metanalysis which suggested that mice treated with shorter interval times and excessive doses had an increased severity in symptoms. Their results show all mice treated with Doxorubicin show subcellular changes linked with cardiotoxicity and found the severity of the subcellular changes were related to peak dosage (Podyacheva et al., 2021) From this we can infer patients will more than likely still suffer from some form of cardiotoxicity from the use of Doxorubicin in the treatment of neoplasms, however, the severity of symptoms are likely the result of the treatment schedule and a reduced peak dosage is recommended.

Other potential side effects of Doxorubicin-based therapy regimens include hematologic toxicity secondary oral neoplasms, tumour lysis syndrome and myelosuppression, however, hematologic toxicity and myelosuppression are common side effects of multiple chemotherapeutic agents (Carr et al., 2008), Patients are likely to experience nausea and vomiting, which are treated with antiemetic therapy and

corticosteroids. It is strongly advised that Doxorubicin-based treatment regimens be terminated if the cumulative lifelong dosage reaches 550 mg/m^2 due to the elevated risk of cardiotoxicity (Pfizer New Zealand Limited, 1990).

1.5.5 Limitations

While Doxorubicin is an important therapeutic agent used in treatment of node positive and malignant breast cancers as well as several other malignancies, there are limitations associated with its use. While high doses of Doxorubicin can eliminate TNBC, these high doses produce the adverse effects mentioned in Section 1.5.4. In addition to these, sustained cell loss in the tumour region and surrounding area will likely alter the tumour microenvironment and promote sub-standard drug accumulation, which necessitates an increase in dosage to maintain therapeutic effects (Li et al., 2015). However, any increase in dosage will likely exacerbate its toxic effects (Carvalho et al., 2009). Conversely, low doses of Doxorubicin are known to elicit a poor response and can lead to eventual drug resistance.

MDR severely limits the therapeutic potential of many chemotherapeutic agents. MDR occurs for several reasons, primarily through enhanced efflux, but also through other mechanisms, including an increase in the metabolism of the therapeutic agent, intracellular sequestration, and increased DNA repair (Rivankar, 2014). These mechanisms of resistance lead to a decrease in intracellular concentrations of the active form of Doxorubicin inside the cell, a reduction in the frequency and intensity of effective therapeutic activity, such as the formation of DNA abducts in neoplastic cells, and thus a reduction in its anticancer effects (Misset et al., 1999).

1.6 Chemotherapeutic Resistance

Improving the long-term efficacy of chemotherapeutic agents will require addressing chemotherapeutic resistance. MDR is argued to be the result of a selective process, where the elevated rate of mutation common in most cancers and dramatic shifts in epigenetic expression (Fath et al. 2022) that occur due to stochastic changes in the local environment is acted on by the use one or more therapeutic agents (Bukowski et al., 2020). This leads to the accumulation of traits that promote cell persistence, survival and replication, reducing the efficacy of various treatments over time (Álvarez-Arenas et al., 2019; Bukowski et al., 2020). The genes that contribute to these aberrant behaviours are known as multidrug resistance (MDR) genes. MDR genes are numerous and modifications to any of these genes can promote a number of traits, such as dysregulated proliferation, modification of and detoxification of xenobiotics, the excretion of metabolites, the loss or modification of druggable targets, alterations in pathways associated with cell division and development. The acquisition of multiple MDR genes allows cancer growth to outpace the effects of therapeutic intervention (Álvarez-Arenas et al, 2019). This becomes a problem, because as mentioned in section 1.6, Doxorubicin has finite utility due to dose limiting effects such as cytotoxicity myelosuppression and secondary neoplasms

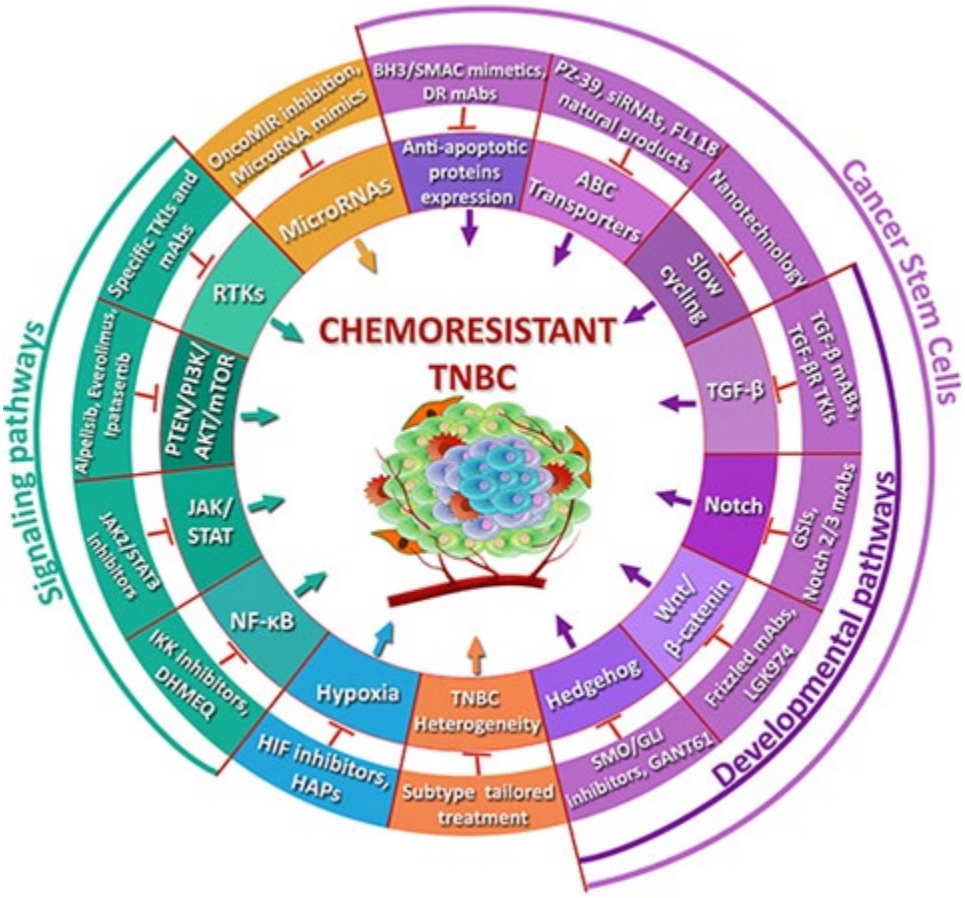


Figure 1.7 Chart describing the differing sources of chemoresistance in TNBC, the treatments that have indicated their ability to act on these sources of chemoresistance in at least in vitro screens, with cancer stem cell behaviour and their associated inhibitors indicated in purple, hypoxia related traits and their associated inhibitors in blue, various signalling pathways and their inhibitors indicated in green and miRNAs and their inhibitors in amber (Nedeljković & Damjanović, 2019).

Multiple studies have implicated numerous genes in the acquisition of drug resistance (Christowitz et al., 2019). The dysregulation of the MAPK, mTOR, WNT, NOTCH, JAK/STAT, PI3K, Hippo etc. pathways has been linked to various cancers. This makes sense as these genes and their associated pathways are known to regulate numerous cell behaviours, including the expression and recruitment of membrane transporters, cellular metabolism, the metabolism of therapeutics, apoptosis, and the activation of transcription factors (Nedeljković & Damjanović, 2019).

The alteration of one or more of these genes during treatment, in a manner that provides a selective advantage is likely to result in the accumulation of treatment resistant cells. This phenomenon is common in the disease pathology of multiple cancers. In various in vitro studies and clinical trials, the role of multiple different signalling and developmental pathways has been deemed responsible for the acquisition of stem cell traits, including the maintenance of growth, cell proliferation and survival in multiple cancer cell lines including leukemia, colorectal cancer, ovarian cancer and glioblastoma (You et al., 2023). Other notable examples of MDR genes include TNF, JUNB and Clusterin. TNF overexpression in TNBC has been linked with increased intratumoural invasiveness and neovasculogenesis in animal models (Narasimhan et al., 2022). JUNB has been associated with the epithelial mesenchymal transition (EMT) and Clusterin (CLU) has been linked to resistance to apoptosis.

A group of MDR genes of particular note is the ABC transporter family, which have been detected in multiple strains of drug-resistant cancers, including colon, ovarian, and breast cancers (Duvivier et al., 2023). The ABC transporter family has a noteworthy effect on the development of chemotherapeutic resistance in multiple cancers as it facilitates the trafficking of xenobiotics as well as other small molecules (Duvivier et al., 2023). The overexpression of ABC transporters promote resistance by removing several substrates from the intracellular environment, and this often includes chemotherapeutic agents (Fletcher et al., 2010). The reduction in the intracellular accumulation of various chemotherapeutic agents alters the pharmacokinetics of that agent, reducing the frequency of synthetic lethality. This necessitates an increase in the dose required to achieve a therapeutic effect. ABC transporters that are commonly overexpressed in TNBC include ABCC1/MRP1, P-gp and BCRP/MXR/ABCG2.

The tumour microenvironment (TME) also plays a notable role in the acquisition of MDR. The TME is the margin of interstitial tissue that is proximal to cancer cells. The tumour microenvironment itself is made up of the extracellular matrix (ECM) and the non-cancerous cells that surround the cancer cells. Of the non-cancerous cells, these often include fibroblasts, leukocytes, adipocytes, myoepithelial and endothelial cells. The ECM comprises the extracellular macromolecules and minerals in the defined

region of tissue and provides structural and biochemical support to surrounding cells. (Insua-Rodríguez & Thordur Oskarsson, 2016) There is also a fibrous stroma that is common in breast cancer, which is stiffer than most other cancers and gives the breast cancer tumour the rigidity and stiffness that allows for detection via palpation. Excessive activation of fibrogenic signals dysregulates growth factor signalling and the production of ECM, which has a notable effect of the cell microenvironment, increasing the recruitment of myofibroblast-like cells and resulting in ECM stiffening, and malignant transformation of the parenchyma, promoting treatment resistance, resistance to cell death and replicative immortality (Nassar et al., 2019). As such the environment that surrounds a tumour can influence the cancer's rate of proliferation and its resistance to chemotherapy. The chemical nature of the microenvironment is also a significant contributor to the growth of many cancers including breast cancer. For example, a hypoxic microenvironment is a common feature in the TNBC microenvironment as the high metabolic rate of TNBC depletes local oxygen levels. This depletion of oxygen has been linked to a resistance to apoptosis, induction of the glycolytic shift, the recruitment of tumour-associated macrophages and angiogenesis (Gilkes & Semenza, 2013) due to induction of pathways governed by members of the family of hypoxia inducible factors (HIFs). HIFs are a family of proteins that trigger a series of cellular defence mechanisms in response to hypoxia, including angiogenesis, reduced apoptosis and the triggering of the glycolytic shift in response to both acute and sustained hypoxia, with the purpose of improved survival and functionality of normal tissue that has been deprived of oxygen (Gilkes & Semenza, 2013). It is logical then to assume the reduction in available oxygen due to increased cellular metabolism turn activates several HIFs and triggers associated behaviours. This is supported by expression analysis of HIFs in TNBCs. Immunoassays performed by Liu et al. (2022) link overexpression of HIFs to a poor response to chemotherapeutic agents and poorer long term survival statistics in patients.

1.6.1. MDR Genes in TNBC

Multiple pathways are exploited by cancer in the development of MDR and malignancy in TNBC (Luqmani, 2005). Common modifications that contribute to treatment resistance in breast cancer include the loss of key receptors such as the loss of mPgR, ER α and HER2, as is typical in TNBCs (Zattarin et al., 2020). The loss of these druggable targets in TNBC cells have adverse consequences on treatability and clinical outcomes, as endocrine therapy and drugs targeting HER2 demonstrate low efficacy in clinical evaluations of breast cancers absent these targets.

Other well-known MDR genes include the Notch, HIF2a, SRC, STAT and MYC. The Notch developmental pathway usually contributes to multiple processes during embryonic development, including somitogenesis, epidermal differentiation and angiogenesis. Trials targeting the Notch signalling pathway employed anti-notch antibodies and gamma secretase inhibitors (GSI). When tested in women with breast cancer, 11 of the 24 patients screened achieved partial remission and 9 achieved a stable disease state. Other experiments targeting MDR genes in TNBC Src, Stat3 and Myc expression in MDA_MB_435S breast cancer cell line, also demonstrated significant inhibition of cell proliferation by a factor of 3.5 for both Src+Stat3 and Src+Stat3+Myc groups (Bjorge et al., 2011).

The inhibition of HIF expression has also demonstrated significant effects on the malignancy in several different cancers, with HIF 2a inhibition reducing the expression of MMP-2 and resulting in a reduced invasive potency (Li et al., 2016).

While we have an understanding of some of the MDR genes associated with resistance in TNBC, recent metanalysis suggests 110 genes are directly linked with chemotherapeutic resistance in cancer (Baxter et al. 2018) and 70 mutations have been directly linked to chemoresistance in TNBC. Other studies support the conclusion that the acquisition of resistance is a highly complicated process involving numerous genes. Experiments performed by Ciocan-Cartita and colleagues (2020) suggest the number of genes associated with the acquisition of multidrug resistance in TNBC cells may be much higher than suggested by Baxter et al (2018). Their qPCR analysis of Doxorubicin resistant strains of MDA_MB 231 detected changes in the expression level of approximately 311 different genes, with 196 different genes

upregulated and 115 downregulated. 15 mutations were identified as having a causal relationship with the acquisition of drug resistance. These included EGR1, VEGFA, ABCC3, IL-6, IL24, CLU, SNAI1, ABCC6, TNF, TNFSF10, FASN, EPHX1, CXCL1, TP53I11 and JUNB.

1.6.2 ABC Transporters and their Role in Drug Resistance

While Baxter et al. (2018) describe the polygenetic nature of MDR in TNBC, it is likely that certain MDR genes may have a more direct and notable effect on MDR. Nedeljković and colleagues (2019) highlight the significance of ABC transporters in the development of chemoresistance in TNBC. ABC transporters are ubiquitous membrane proteins that traffic diverse substrates across cellular membranes using the energy generated from ATP hydrolysis (Hollenstein et al., 2007) but primarily facilitate nutrient transport and waste removal functions in the blood brain barrier (Girardin, 2006). Members of the ABC transporter family all have similar structures, with domains called walker A and walker B domains, and a C motif which is proximal to the walker B domain (Davidson et al., 2008).

The efflux of anticancer therapeutics by one or more ABC transporters is considered one of the primary mechanisms by which cancers develop multidrug resistance (Fletcher et al., 2016; Mirakhorli et al., 2012) Alterations in the expression levels of ABC transporters are believed to be the underlying mechanism for decreased intracellular accumulation of drugs and drug metabolites, with increased expression of ABC transporters resulting in an increased rate of substrate efflux (Girardin, 2006; Mizuno et al., 2003). There is significant clinical evidence to support this theory, with hallmark overexpression of ABC transporters in multiple cancer cell lines cultured under selection pressure, and at least 12 ABC transporters from four ABC subfamilies were linked to drug resistance of TNBC cells maintained in culture (Huang et al., 2006; Szakács et al., 2006).

Other experiments exploring the role of the ABC transporters in chemoresistance have causally linked overexpression of one or more ABC transporters to resistance to multiple antineoplastic drugs and/or their conjugated metabolites in many cancers, antineoplastic drugs that include various alkylators, antimetabolites, microtubule stabilizers, arsenical and antimonial oxyanions and peptide-based agents (Deeley et al., 2006).

BCRP/ABCG2 is often overexpressed in multiple chemotherapy resistant cell lines (Noma et al., 2008) and has been directly associated with chemotherapeutic resistance in multiple breast cancer cell lines. BCRP was first identified in a northern analysis of S1-M1-80 and MCF-7 AdVp3000 under active selection of several chemotherapeutic agents. S1-M1-80 cells demonstrated elevated levels of expression of a novel gene, which was later identified as BCRP. BCRP expression increased 10-12-fold in MCF-7 AdVp3000 cells under selection. BCRP/ABCG2/MXR has been associated with the efflux of mitoxantrone, epipodophyllotoxins, camptothecins, anthracyclines, bisantrene, Methotrexate, imatinib, and flavopiridol (Fletcher et al., 2010).

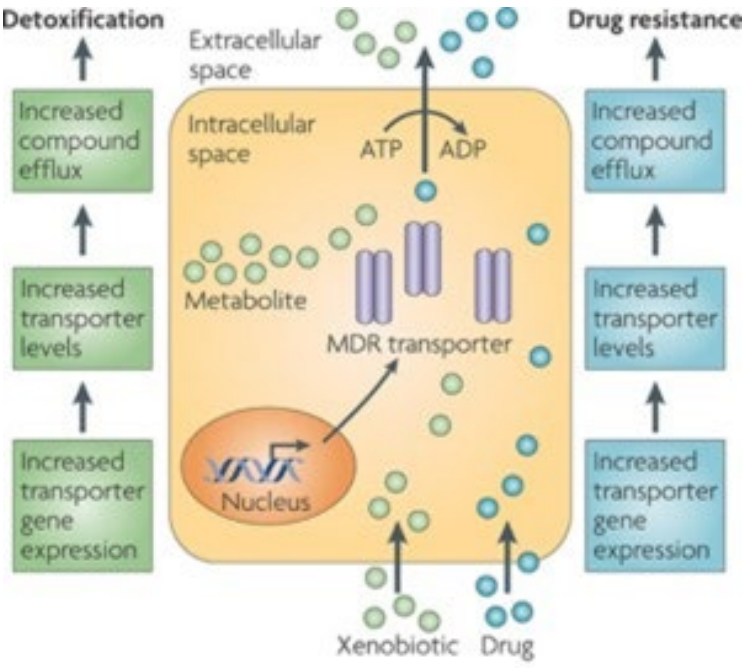


Figure 1.8. Diagram of anticancer and endogenous compounds being exported out of the cell via relevant drug efflux channels

and how that contributes to multidrug resistance (Fletcher et al. 2010).

Immunohistochemical analyses demonstrated significantly elevated levels of ABCG2 expression in breast cancer patients following neoadjuvant chemotherapy (Liu et al. 2015). They also found expression of the ABCG2 transporter was markedly higher in patients whose cancers were resistant to multiple different treatments. The identification of Doxorubicin as a substrate of the ABCG2 transporter was made by Ween and colleagues (2015) in their examination of the role of ABC transporters in the progression of ovarian cancers and the development of chemoresistance. Research by Yang et al. (2014) also found that chemical inhibition of ABCG2 using tivozanib resulted in a reversion of chemotherapeutic resistance in vitro. Experiments modifying the expression levels of MDR1 and BCRP in PLC/PRF/5 and Huh7 cells using pharmacological inhibitors or siRNA (Yin et al. 2021) demonstrated an increase in the intracellular concentration of Doxorubicin upon the inhibition and downregulation of BCRP and PLC.

1.6.3 Chemical Inhibition of Multidrug Resistance Associated Genes

Targeting genes which enhance malignancy or are associated with resistance to treatment, is a promising strategy for the prevention and treatment of metastatic disease. One or several interventions may be sufficient to reverse chemotherapeutic resistance in cancer and allow for effective clearance. Nedeljković and colleagues (2019) describe 6 major classes of genes that contribute to TNBC resistance. These include genes associated with developmental processes, genes that promote stem cell like behaviour, signalling pathways, HIFs, genes contributing to heterogeneity, and microRNAs.

The medical efficacy of targeting multidrug resistance genes has been demonstrated in various tissue assays. Adjuvant chemotherapy and small molecule drugs targeting drug efflux channels have been

demonstrated to influence the viability of several cancers associated with the overexpression of ABC transporters. These in vitro screens act as useful indicators of targeting ABC transporters for inhibition or destruction is a viable therapeutic strategy.

There had been evidence to suggest that the compound curcumin has antitumour properties, including promoting the reversal of chemoresistance in TNBC. Skukla and colleagues (2008) treated Doxorubicin resistant breast cancer cells with curcumin and noted an increase in the intracellular accumulation of Doxorubicin following treatment. They also noted a reduction in the expression levels ABCG2 following treatment, suggesting a likely mechanism of action (Shukla et al. 2008). Another study involving the administration of quinacrine and curcumin increased breast cancer stem cell death by inhibiting BCRP and modulating DNA damage repair pathways (Nayak et al., 2020). The evidence clearly suggests the inhibition or downregulation of ABC transporters associated with the transport of chemotherapeutics is a viable method for cancer cell clearance and improved long term survival rates in patients. Additionally, Xue & Liang (2012) suggest that not all of these drug efflux channels must be targeted to achieve a reversal in chemotherapeutic resistance.

Bocodepsin is another therapeutic compound that has shown a synergistic effect when used with Doxorubicin, reversing chemoresistance in TNBC cancer cell lines. Bocodepsin is a histone deacetylase (HDAC) inhibitor and has been shown to reduce resistance to apoptosis, a key contributor to multidrug resistance (Smoots et al. 2024). These experiments demonstrate the effect that targeting cell stemness can have to further improve clinical outcomes associated with conventional treatment.

Other small molecule drugs have been used to target cancer associated genes include metformin. Metformin has been shown to reduce cell proliferation and induce cell cycle arrest in multiple breast cancer cell tissues, via several mechanisms, including the suppression of phosphorylated-eukaryotic translation initiation factor 4E-binding protein 1 (p-4E-BP1) the reduction of cyclin D1 levels, and the inhibition of cyclooxygenase 2 (COX-2) expression. Thus, metformin likely has potential value as a

synergistic therapy in breast cancer due to its ability to target both the mTOR and COX-2 signalling pathways.

Targeting one or more cancer associated pathways with a small molecule therapeutic has demonstrated antineoplastic effects in vitro, unfortunately many chemical agents used in these experiments have a number of issues in vivo, including toxicity, issues with solubility, or an impractical therapeutic dosage.

1.7 The KISS/KISS1R Pathway: KISS1 and KISS1R Discovery

Emerging research has identified that the KISS1/KISS1R pathway is involved in proliferation and tumour resistance in TNBC and could be a promising therapeutic target.

KISS1 encodes a 145 amino acid long peptide chain that is catalytically cleaved in blood serum into smaller signal peptides, called kisspeptins, the most notable of which is metastin (Gottsch et al., 2008). KISS1 was originally identified by Lee et al. (2005). Lee and colleagues compared non-metastatic C8161.1 and metastatic C8161 cells in a series of subtractive hybridization experiments and identified a cDNA sequence which encodes a 164 AA long peptide, which they named KISS1. mRNA expression analysis detected *KISS1* mRNA in multiple tissues including the brain, ovaries, testis, placenta, as well as skeletal muscle and breasts. The KISS1 peptide is cleaved into smaller peptides of varying lengths, KP-10, KP-13, KP-14, and KP-54 called kisspeptins (KP). The most active of these is KP-10 (peptide sequence: YNWNSFGLRF) which exhibits a dissociation constant (KD) of 1.0 ± 0.1 nM towards KISS1R and has a higher potency than other kisspeptins with an EC50 of 4.13 ± 0.02 nM (Curtis et al., 2009).

1.7.1 KISS1R and KISS1R Signalling

KISS1R is a G protein coupled receptor (GPCR), a known effector of the Ga q/11 signalling pathway and is the target receptor of the kisspeptin family of ligands. Structurally KISS1R resembles other G protein coupled receptors, with 7 transmembrane domains and several extracellular and cytoplasmic domains allowing for the binding of a signal peptide in the extracellular domain and the transmission of a signal to the intracellular environment (Ke et al., 2018).

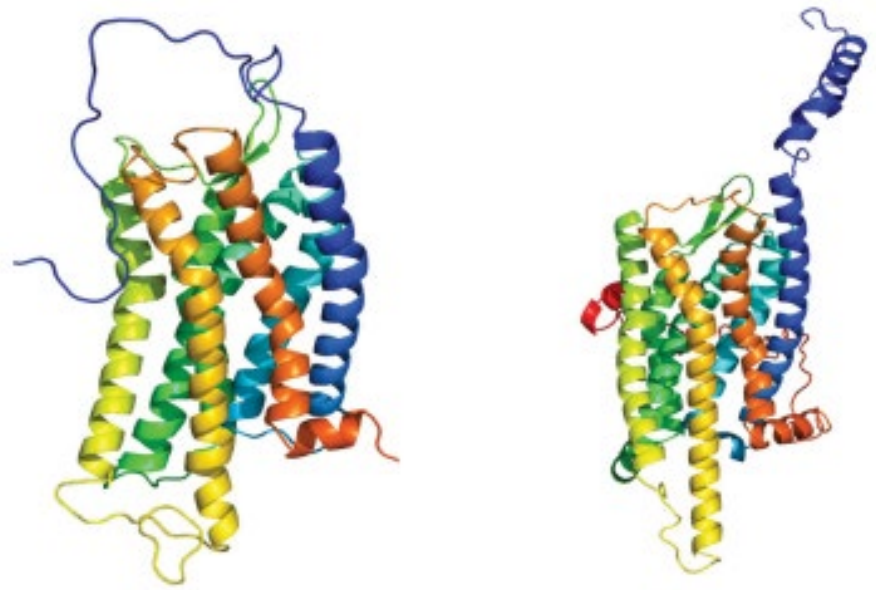


Figure 1.9. A prediction of the 3D structure of the KISS1R receptor using ERRAT overall quality factor and averaged 3D-1D score (Nagarajan & Madhavan, 2016).

Kisspeptins, such as KP-10, once bound to KISS1R, trigger phenomena that drastically affect cell behaviour and morphology. Upon initiation of signal transduction, the KISS1R G protein coupled protein, Gaq11, is released and activates phospholipase C (PLC) and the extracellular signal regulated kinase 1/2 (ERK1/2) (Cvetković et al., 2013). The activation of PLC increases inositol 1,4,5-trisphosphate (IP3) concentration, which in turn increases intracellular calcium levels in a dose dependent manner. The accumulation of diacylglycerol has also been documented and both the increase in calcium and diacylglycerol (DAG) stimulate protein kinase C (PKC). KISS1R has also been linked with β-arrestin-1

and β-arrestin-2 and this association was found to act as a signalling mechanism which contributes to the regulation of ERK 1/2, in a G protein independent manner, with β-arrestin-1 and β-arrestin-2 acting as positive and negative regulators of ERK 1/2 respectively.

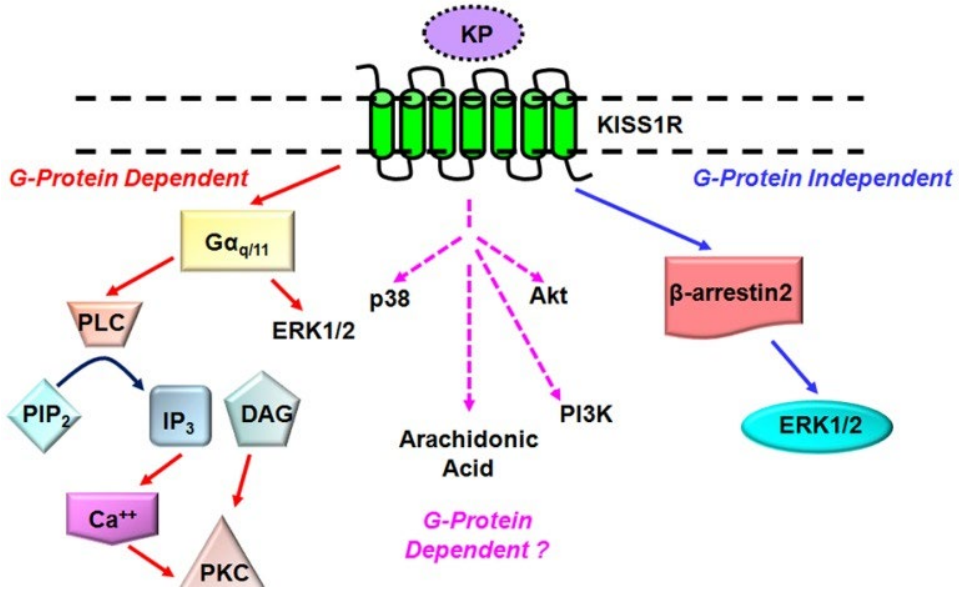


Figure 1.10. Diagram of the signalling pathways associated with KISS1R. KISS1R is a GPCR that when activated, stimulates PLC and ERK1/2, triggers calcium release and upregulates protein kinase C. KISS1R also acts through G protein independent pathways to mediate ERK1/2 activity. KISS1R on multiple different effector proteins, such as PI3K and p38 although the mechanism is not clearly understood (Cvetković et al., 2013).

1.7.2 Roles of KISS1R

KISS1R signalling plays a notable role in the initiation of puberty. This is supported by mutational diseases of the KISS1/KISS1R pathway and their effects on growth, the development of secondary sexual characteristics, and adrenal maturation (Gianetti & Seminara, 2008). An example of this in humans is recessive idiopathic hypogonadotropic hypogonadism, a disease in humans which manifests as delayed growth and puberty and is caused by the ΔL148S mutation of KISS1R (Seminara et al. 2003). Notable

insight on the role of KISS1R is provided by murine models, with mutations of the KISS1 or KISS1R arresting pubertal development. KISS1 mutant murine models were used in kisspeptin dose response experiments to further elucidate the mechanistic role of the KISS1/KISS1R pathway in mammals. Kisspeptin administration in murine models triggered the release of gonadotropin-releasing hormone (GnRH) and premature administration of kisspeptins triggered the early onset of puberty, called precocious puberty (Funes et al., 2003; Seminara et al., 2003). Therefore, the KISS1/KISS1R pathway plays a pivotal role in pubertal maturation via its regulation of GnRH, and GnRHs effect on the Hypothalamic Pituitary Gonadal (HPG) axis.

Kisspeptins are released from the infundibular nucleus in a pulsatile fashion, which triggers the release of GnRH into the hypophyseal portal vein (Han et al., 2005). GnRH signalling affects the anterior pituitary gland, promoting the synthesis and release of luteinizing hormone (LH) and follicular stimulating hormone (FSH), key regulators in the initiation of puberty and the development of secondary sexual characteristics in both males and females. (Abreu & Kaiser, 2016) Further experimentation using KISS1 null mutant murine models support the role of the KISS1/KISS1R pathway in the modulation of the HPG axis as serum levels of LH and FSH were abnormally low in KISS1 null mutants and the administration of kisspeptin 54 partially restored LH and FSH serum levels. Furthermore, the administration of KISS1R antagonists in rat models have consistently resulted in a delay in the onset of puberty (Funes et al., 2003; Seminara et al., 2003). Thus, the relationship between the KISS1/KISS1R pathway, the production of GnRH, the HPG axis and the onset of puberty are quite robust.

Fluctuations in KISS1 and KISS1R levels in human placenta are also indicative of their functions during pregnancy. Analysis of placental levels of KISS1 and KISS1R expression levels found a 900-fold increase in kisspeptin and KISS1R expression during the first trimester, a notable reduction in concentrations of both kisspeptin and KISS1R in the second trimester and 7000-fold increases in kisspeptin and KISS1R the third trimester, coinciding with changes in the invasiveness of the trophoblast (Hu et al., 2018; Reynolds et al., 2009). In vitro studies suggest an inhibitory relationship between

kisspeptins and the trophoblast, with kp-10 signalling appearing to inhibit the migration of trophoblastic cells and lower MMP levels, which are critical for the placental invasion of the uterine walls (Gomes & Sones, 2021).

1.7.3 KISS1/KISS1R Signalling in Cancer

The KISS1/KISS1R pathway's role in cancer is currently an active area of research. KISS1 was first identified as a metastasis suppressor gene by Lee et al., (1996) while attempting to identify genes associated with the suppression of malignancy in melanoma with artificial polyploidy replicate C6. KISS1 analogues were then shown to reduce cancer proliferation in multiple different cancer cell lines, including oesophageal, thyroid, pancreatic and ovarian cancers (Ly et al., 2020). Further analysis of KISS1R found expression levels of KISS1 are lower in malignant cancer cell lines compared with normal tissue as initial experiments involving melanoma found levels of KISS1 expression levels were reduced in metastatic melanoma when compared with non-metastatic cancer cells (Guzman et al. 2018)

Additional research also found when KISS1 was overexpressed, there was a noticeable decline in the motility of cancer cells, and metastasis was suppressed in a dose-dependent manner. This dose response relationship was persistent across multiple cancer cell lines (Stathaki et al., 2019). Overexpression of KISS1 in pancreatic cancer cells led to a reduction in migration of pancreatic cancer cells, however it had no impact on the rate cellular proliferation. qPCR analysis of gastric cancer also found levels of *KISS1* and *KISS1R* mRNA in distal metastasis were much lower than those of the primary gastric tumour, further reinforcing the idea of a dose response relationship.

KISS1R expression has been linked to MMP-9 activity in gastric and is a positive regulator of tissue inhibitor of metalloprotease (TIMP)-1 expression (Matjila et al., 2013).

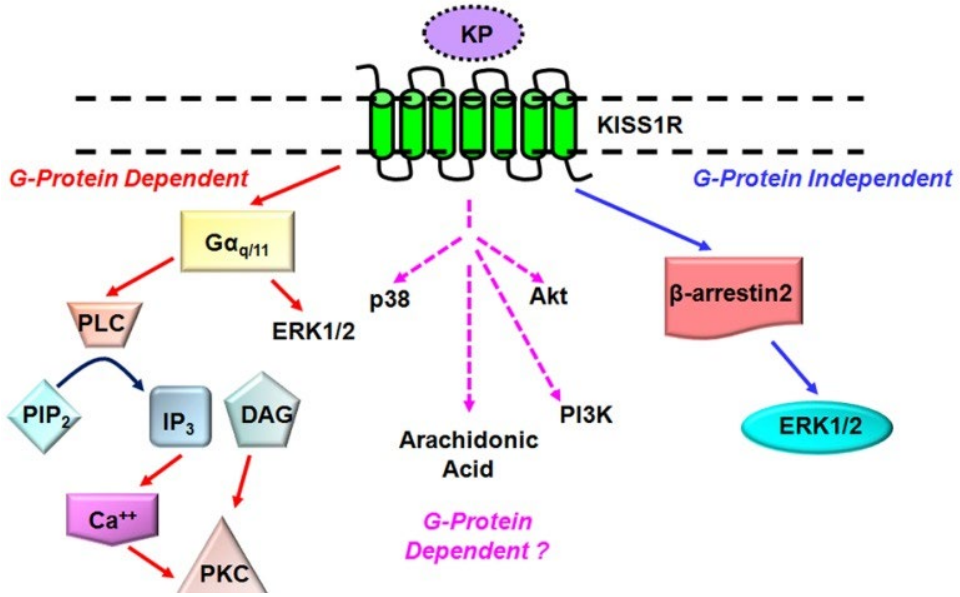


Figure 1.11 Collation of the validated and suggested signalling pathways that define KISS1R activity in ERα – Breast Cancer. KISS1R is activated when a KISS1 peptide or a synthetic analogue is bound. This directly activates ERK1/2 and phospholipase C (PLC) and increases the intracellular concentration of calcium and diacylglycerol. KISS1R also activates ERK1/2 in a β-arrestin-dependent manner and has been linked to other mechanisms that govern cell behaviour, such as p38 and phosphatidylinositol-3-kinase (PI3K), however the mechanism by which they are linked is unclear (Cvetković et al., 2013).

1.7.4 KISS1/KISS1R Signalling in Breast Cancer and its Effect on Tumour Progression

However, the activity of the KISS1 and KISS1R is extremely context dependent, and its activity differs based on various factors that include tissue type, as well as disease state. Martin and colleagues, (2005) performed qRT PCR analysis of *KISS1* and *KISS1R* mRNA levels in TNBC cancer cells and results contradicted prior research and found *KISS1* mRNA expression levels were elevated in breast cancer tissue when compared with relevant controls. Analysis of lymph node biopsies of patients with TNBC also found elevated levels of *KISS1* mRNA levels in comparison with control samples from the primary

tumour. Further examination of TNBC tissue found overexpression of KISS1 and KISS1R in TNBC cell line MDA-MB-231 was linked to increased invasiveness and reduced cellular adhesion in a dose dependent manner. Similar behaviour was identified in a number of cancers including advanced ovarian, bladder and colorectal cancers as well as hepatocellular carcinomas and metastatic osteosarcomas (Makri et al., 2008; Guzman et al., 2019).

Marot et al. (2007) identified a link between the oestrogen status of breast cancer cells and KISS1R expression. Marot and colleagues compared ERα+ and ERα- cancer cell cultures and found ERα- breast tumours expressed seven times the level of kisspeptins of control ERα+ breast tumours, which suggests ERα negatively regulates the expression of KISS1. Analysis of tissue samples from post-menopausal women with ERα+ breast tumours identified dose response relationship with tamoxifen, an ERα antagonist. ERα+ patients taking tamoxifen were found to have higher *KISS1* and *KISS1R* mRNA levels, supporting the postulate ERα negatively regulates KISS1 and KISS1R expression levels. Additional in vitro experiments with ERα+ T47D and MCF7 cell lines revealed an increase in KISS1 and KISS1R expression following treatment with tamoxifen. From a clinical perspective, it is likely then that KISS1 and KISS1R expression levels are markers associated with increased malignancy.

Experiments further elucidating the role ERα plays in the regulation of KISS1R expression in cancer include the treatment of ERα positive MCF7 and T47D cell lines with kisspeptin 10. Treatment with kisspeptin-10 failed to induce the invasive phenotype associated with KISS1R induction supporting the hypothesis that ERα inhibits pro malignant KISS1R signalling. ERα reversion studies in TNBC cell line MDA-MB-231 show elevated *KISS1* mRNA and a subsequent reduction in *KISS1* mRNA when ERα expression was restored. Cho and colleagues (2011) employed heterozygous KISS1R murine models and the MMTV-PyMT transgene cassette which uses the mouse mammary tumour virus promoter (MMTV) to modulate the expression of the polyoma virus middle T antigen (PyMT) to determine the effect KISS1R has on the transformation of mammary epithelium into multifocal adenocarcinoma. Their results suggest reduced KISS1R expression hampers breast tumour formation, cell proliferation, and metastasis

when compared to the homozygotic PyMT-KISS1R+/+ control. qPCR expression analysis of tissue samples from PyMT-KISS1R+/− and PyMT-KISS1R+/+ also revealed the role of the KISS1/KISS1R pathway in the activation of RhoA GTPase, as well as its role in the upregulation of VEGF and MMP-9, which are associated with angiogenesis and other proliferative behaviours.

In several experiments, KISS1R was found to promote stem cell like behaviours, such as detachment from the basement membrane, increased migration and invasiveness (Guzman et al., 2019). MMP-9 was found to play a role in the triggering of metastasis and breast cancer invasion. Expression analysis also found a link between KISS1R expression, SNAIL/SLUG and N-cadherin expression, with KISS1R overexpression increasing the expression levels of both these SNAIL/SLUG and N cadherin expression and contributing to cancer cell stemness (Côme et al., 2006; Nieman et al., 1999). Kisspeptin-10 dose response analysis also linked the KISS1R to the increased expression of EFGR via a B-arrestin mediated pathway in ERα - breast cancer cells. More recently, it has been suggested the actin cytoskeletal binding protein, IQGAP1, likely mediates signal transduction between KISS1R and EGFR (Cvetković et al., 2013).

KISS1R overexpression has also been linked to the induction of the epithelial to mesenchymal transition (EMT) in ERα - breast cancer cells (Guzman et al., 2019). Induction of the EMT leads to the acquisition of multiple cancer stem cell (CSC) traits including the disruption of cell-cell junctions, cytoskeleton remodelling, loss of adhesion, suppression of apoptosis, metastases and extravasation.

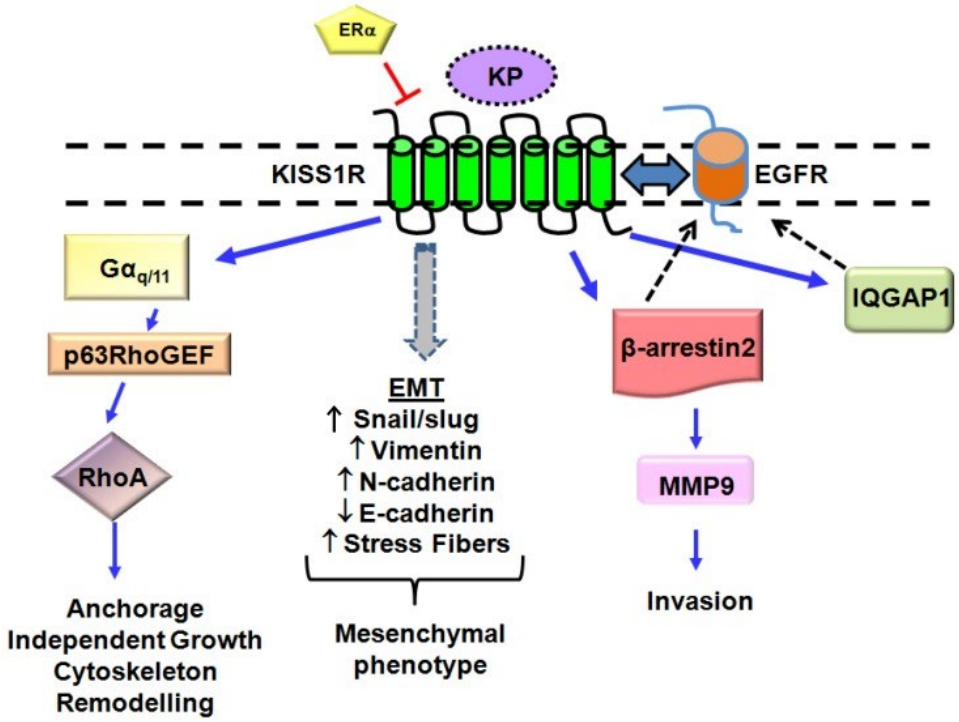


Figure 1.12 The image above describes the proposed KP/KISS1R signalling pathways in ERa- breast cancer cells. If ERα is lost, it is likely the loss of negative feedback regulation will result in increased KISS1R recruitment and thus increased transactivation of EGFR via β-arrestin 2 and IQGAP1 associated mechanisms. KISS1R activation also triggers EMT, reducing E-cadherin expression and promoting the transition to the mesenchymal phenotype, which has a number of notable intracellular effects, including cytoskeleton remodelling and an increase in mesenchymal markers such as E-cadherin and Vimentin. Overexpression of either KISS1 or KISS1R in the pathway can also activate RhoA. The net effect is an increase in the rate of proliferation, invasiveness and a dediffentiation which promotes the growth and spread of the tumour (Cvetković et al., 2013).

1.7.5 Further Developments in Kisspeptin/KISS1R MDR Dynamics: Breast Cancer Resistance Protein (BCRP).

The *ABCG2* multi-drug resistance (MDR) gene encodes the breast cancer resistance protein (BCRP), which is a broad-acting efflux transporter (Robey et al., 2008). BCRP actively extrudes small molecules with amphipathic charge from the cell. BCRP, consisting of 6 transmembrane helices and a nucleotide binding domain, must homodimerize to function as an efflux transporter at the plasma membrane. It

expels various substrates from the cell cytosol, including urate, folate, steroid hormones and anticancer agents such as Doxorubicin (Polgar et al., 2007).

The role of BCRP has been well established as individuals with variation in the ABCG2 transporter gene have a predisposition for gout. One SNP (Gln141Lys or Q141K) can reduce the protein's ability to remove urate by 50%. Another variant (Gln126Ter or Q126X), results in premature termination and thus produces no functional ABCG2 protein (Fohner et al. 2018). The resulting reduction in or loss of ABCG2 functionality reduces the removal of urate from the blood, resulting in the excess accumulation of urate can in the body's joints, forming uric acid crystals and triggering an inflammatory response associated with gout (Chang et al., 2022). BCRP, and various other ABC transporters are known to incur MDR, due to their ability to remove a broad range of small molecule drugs out of the cancer cells. KISS1R overexpression in TNBC tumours are also linked to AXL and BCRP expression, however the precise mechanism of this pathway is unknown (Suleiman et al., 2021). However, it can be safely inferred that the Kisspeptin/KISS1R pathway promotes TNBC chemoresistance via the upregulation of BCRP to the cancer cell membrane. Earlier work also found overexpression of BCRP in chemo resistant ERα - MCF-7 type breast cancer cell lines, supporting the link between KISS1R and BCRP.

BCRP is also marker of pluripotent hematopoietic and tissue stem cells, suggesting it could also have roles in inducing stem cell like behaviours, further enhancing CSC like behaviour by creating a positive feedback loop (Nakanishi & Ross, 2012). Overexpression of ABCG2 has also been linked to the upregulation of cytoprotective factors involved in the oxidative stress response and expression of ABCG2 has been linked with the maintenance of an undifferentiated stem cell state (Evseenko et al., 2007).

1.7.6 Implications of Targeting KISS1R in Treatment.

The aforementioned research suggests KISS1R may be a promising therapeutic target for the treatment of TNBC as it is linked to and controls the expression of multiple traits via the recruitment of multiple pathways, including the transactivation of EGFR via B-arrestin and IQGAP1 associated pathways, initiation of the EMT via upregulation of SNAIL/SLUG, N cadherin, stress fibres and vimentin, and downregulation of E cadherin, and Rho GTPase activation (Cvetković et al., 2013). This leads to chemotherapeutic resistance and increased CSC like behaviour in TNBC. As mentioned in prior sections, analysis of TNBC, (HER2–, ERα–, mPgR–) generally note elevated levels of KISS1 and KISS1R, which are in turn associated with tumour progression, metastasis, and survival (Makri et al., 2008; Guzman et al., 2019) Overexpression of KISS1 and KISS1R have also been associated with the overexpression of BCRP/ABCG2, a member of the ABC transporter family and a notable contributor to drug resistance in multiple cancer types, including TNBC. BCRP can transport uncharged compounds via cotransport with glutathione and thus acts to modulate the pharmacokinetics of many drugs (Jedlitschky et al., 2006). BCRP has also been linked with cell stemness and the maintenance of the undifferentiated stem cell state. Experiments targeting KISS1R expression siRNA screening of ERα- cancer cell lines suggest a reduction in malignancy in TNBC cell lines as well as a reduction in invasiveness.

Standard immunohistochemical screening of biopsied breast cancer tissue could easily identify individuals that would likely benefit from KISS1R targeted therapy, due to the negative regulation of KISS1R by ERα. The loss of ERα is likely to trigger KISS1R overexpression, therefore ERα status may be all that is required to identify breast cancer patients who may benefit from a combination polychemotherapy such as TAC, and KISS1R targeted treatment. To further support the targeting of KISS1R and to support its clinical research, an alternative approach (e.g. CRISPR/Cas9 system) may be required as siRNA-based studies likely have several limitations including as off-target effects.

1.8 The CRISPR-Cas9 System

Genome editing is now currently dominated by CRISPR (clustered regularly interspaced short palindromic repeats) - Cas9 due to its efficiency, versatility and precision (Bhaya et al., 2011; Cong et al., 2013; Shi et al., 2020). CRISPR-Cas9 has greatly simplified genome editing and is now used in a number of applications, which include the development of therapeutics and the study of disease (ThermoFisher, n.d.) CRISPR-Cas9 occurs in many species of bacteria and archaebacteria, where it generally serves as a bacterial defence system against a number of foreign bodies, including viruses, that have previously entered the cell (van der Oost et al., 2009). Upon degradation of the foreign DNA introduced to the cell by the foreign body, the bacterial host incorporates some of the residual short sequences from the foreign body into a region of its own genome. This region is distinguished by clustered regularly interspaced short palindromic repeats (CRISPR) (Levy et al., 2015) separated by short bands of exogenous DNA (Nishimasu et al., 2018). The process of incorporating foreign DNA into the bacterial host genome is called adaptation (Cong et al., 2013). When these sequences are transcribed, they are usually transcribed as one large primary fragment and processed into crRNA's, this is called the expression phase. The last stage, called interference, is where the crRNA fragments interact with the Cas9 protein complex and are used as a guide to help identify and cleave incoming foreign genetic material that share a high degree of sequence homology with the newly incorporated sequence(s). (Levy et al., 2015).

CRISPR-Cas9 can be used to cause highly targeted damage to the host genome, inducing double stranded breaks in the host genome which can trigger DNA repair mechanisms such as non-homologous end joining (NHEJ) and homology directed repair (HDR). Both pathways utilize the same signalling cascade to elicit cellular responses to DSBs and initiate repair (Yang et al., 2020). The NHEJ repair system is triggered by Ku heterodimers which detect and bind to sequence microhomologies at the ends of dsDNA, like those which occur at the site of DSBs. Ku heterodimers act as a molecular scaffold for guiding the

repair proteins that chemically ligate DSBs back together. Sequence mismatches are acted upon by various enzymes, including various phosphodiesterase's and polymerases to complete the repair (Baliou et al., 2018; Hsu et al., 2014). NHEJ repair is a highly error prone process and has a tendency to incorporate deletions and insertions at the site of repair. This tendency for erroneous repair is exploited in CRISPR Cas9 experiments with the intent of disrupting gene expression. NHEJ mechanisms are constitutively active and thus it is quite convenient to exploit NHEJ in transformation experiments, when compared with HDR, as HDR utilizes homologous recombinant proteins that are expressed during the G2 phase of the cell cycle (Hsu et al., 2014). CRISPR Cas9 produced, NHEJ mediated mutations can have a variety of effects, and while an SNP can occur, the exact location and nature of the mutation can vary, meaning the number of useful mutations is variable and thus the therapeutic effect is likely lower than the total transformation efficiency may infer.

HDR is considered more useful for the incorporation of novel genetic material into the host genome (Baliou et al., 2018; Drost & Clevers, 2016). Upon CRISPR Cas9 mediated cleavage of a target sequence of DNA, Rad51 binds to DSB during the initial phase of HDR and recruits accessory proteins that direct genomic recombination (Hsu et al., 2014). After this, the donor sequence is then incorporated into the genome (Baliou et al., 2018; Drost & Clevers, 2016). There are criteria that are unique to HDR that need to be met in order to facilitate the insertion of a large fragment of DNA. HDR pathways require a donor strand of DNA to match the flanking sequences on both sides of the DSB, these matching regions are known as homology arms. Strategies that manipulate the choice of repair and favor HDR are increasing in popularity as it allows for a more useful edit. (Yang et al., 2020). HDR, while having a relatively low efficiency, can produce more precise modifications, such as introducing stop codons, or entirely new genetic sequences, into host DNA, which means, while the efficiency is poorer, the reliability of the edit is greater, suggesting HDR mediated transformation may have greater therapeutic benefit than NHEJ mediated methods of transformation, if its reliance on transfection at the G2 phase is addressed.

While HDR methods show promise, NHEJ is still the preferred repair pathway in eukaryotes due to the constitutive activity of NHEJ repair mechanisms (Yang et al., 2020).

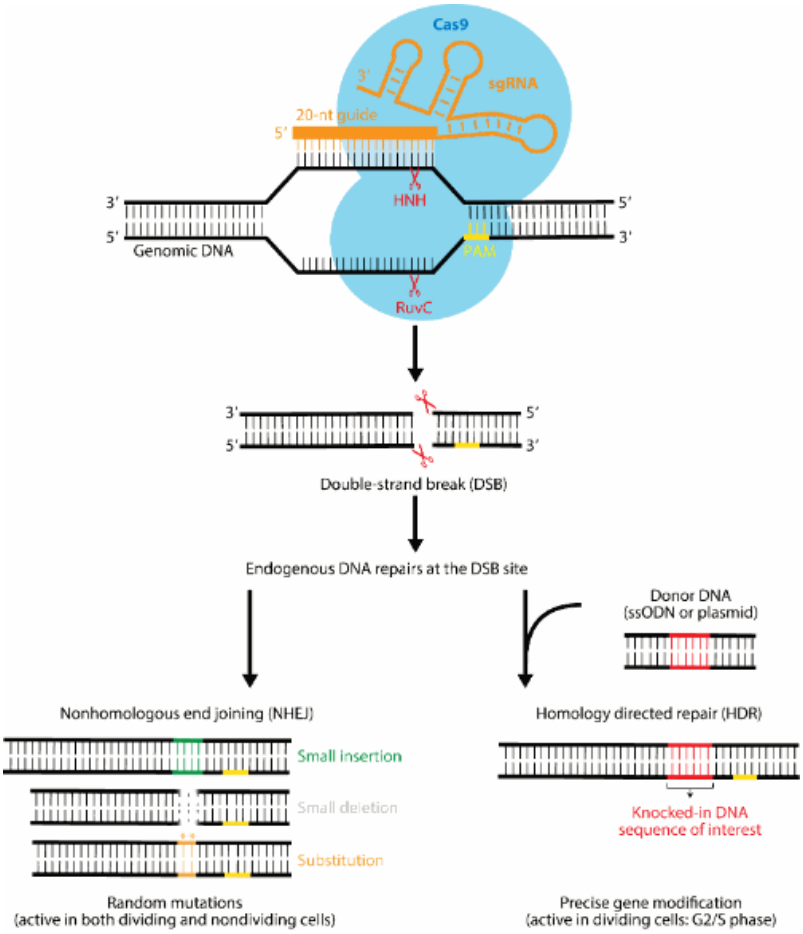


Figure 1.13 The mechanism of the CRISPR-Cas9 system (Chen et al., 2019)

To induce the requisite DSB in a targeted region of DNA requires the Cas9 enzyme and a single-strand guide RNA (sgRNA). The Cas9 protein is responsible for the catalytic cleavage of the host genome and the sgRNA is the template that facilitates site specific binding of the Cas9 protein. The Cas9 nuclease is usually isolated and purified from Streptococcus pyogenes (Saber et al., 2020) and the sgRNA is synthesized using industrial processes. Certain conditions are required for the CRISPR Cas9 system to function, including a target sequence which is proximal to a 2-6 bp long motif called a protospacer adjacent motif (PAM) (Kurien & Scofield, 2006). While this requirement is a notable limitation of the

CRISPR Cas9 system, CRISPR Cas9 mediated transformation allows for greater versatility and precision when compared with other current gene editing techniques, such as TALENS and zinc finger nucleases.

The precision of the CRISPR-Cas9 system has been used to reverse disease-associated genetic mutations in several clinical trials (Acharya et al., 2020). One such technique is the CRISPR-Cas9 ribonucleoprotein (RNP) mediated transformation, which is currently being used as the primary therapeutic mechanism of action in several clinical trials targeting various cancers, including gastrointestinal and pancreatic cancer (Kang et al., 2017). CRISPR Cas9 RNP involves the delivery of a Cas 9 protein complexed with a sgRNA to the host genome where it enzymatically induces a DSB in a targeted region of the host genome, ideally incorporating a deleterious mutation and rendering the targeted gene nonfunctional (Cong et al., 2013; Shi et al., 2020).

A number of systems exist to facilitate the delivery of the necessary systems into the host cell. These include viral encapsidation and delivery, electroporation, lipid nanoparticle mediated transfection, and chemical methods (ThermoFisher, n.d.). Viral vectors are one of the more common methods of delivery, with modified viral vectors acting as delivery vehicles for genome editing systems, including adeno-associated viruses and lentiviruses (Schmidt & Grimm, 2015). Viruses are natural vectors for the transfer of proteins and exogenous genetic material into targeted cells (Kay et al., 2001) and are the primary modality employed in CRISPR genome editing (Xu et al., 2019).

AAV are some of the more commonly used viral vectors for the delivery and expression of CRISPR components into target cells for gene editing (Lau and Suh, 2017). Nonviral vectors are another method of transfection, these include polypeptide and lipid-based vectors (Yin et al., 2014) While it is possible to be very precise regarding the delivery of CRISPR-Cas9 using viral vector-based mediums, there are a number of issues associated with their use, including limitations in cargo size (Li et al., 2015). Lipofection is a lipid-based mechanism for CRISPR transfection that is currently considered the gold standard of vector-mediated transformations. Lipofectamine reagents are widely used in the safe delivery of foreign DNA or RNA into cells (Cardarelli et al., 2016). Lipofection can accommodate a much larger

payload than most AAV and lentiviruses, meaning a Cas9 protein and sgRNA can be efficiently packed into liposomes, which readily pass through the cell membrane (Biagioni et al., 2018; Yip, 2020).

1.8.1 Uses of the CRISPR Cas9 System in Cancer

The CRISPR-Cas9 mechanism has multiple uses in the field of cancer research. CRISPR Cas9 has been used to both identify and target genes associated with MDR (Liu et al., 2019; Manvati & Dhar, 2020) and has demonstrated therapeutic effects in multiple cancers, including lung, breast, liver, colorectal and prostate cancers (Hazafa et al., 2020). One such example includes targeting the expression of adenomatous polyposis coli (APC) and Kirsten rat sarcoma virus (KRAS) mutations via the use of the CRISPR Cas9 RNP in the treatment of colorectal cancer. Treatment showed significant inhibitory effects on xenografted colorectal cancer in murine models (Wan et al. 2021). Unfortunately, there are still numerous issues associated with CRISPR Cas9 derived therapies, including editing efficiency, which is partially the result of suboptimal delivery systems, off-target effects, a decline in the fitness of transgenic cells and the eliciting of an immunogenic response among other adverse side effects (Cheng et al., 2020).

1.8.2 Lipofection

A particularly useful genome editing technique is the combining of the Cas9 protein and sgRNA, into a structure called a ribonucleoprotein, that can be introduced into cancer cells via a lipid-based delivery system called lipofection. By encapsulating the RNP complex into liposomes, and modifying the surface

charge, the system is able to pass through the cell membrane and into the cell (Yip, 2020). Liposomes can be imposed with positive charges, facilitating their entry into the cell.

This method of transfection is beneficial as cells undergo genetic editing without having to synthesize the sgRNA and the Cas9. The process produces a very rapid and robust knockout with the Cas9 clearing 24 hours after transfection. The rapid clearance of Cas9 is useful as it reduces the probability of any off-target effects (Biagioni et al., 2018; Kim et al., 2014; Zuris et al., 2015). RNPs also significantly reduced the rate of off target effects and cell death when compared to plasmid-based modalities of transformation, with insertional mutation being impossible via this method (Biagioni et al., 2018).

1.8.3 Challenges

Despite the therapeutic potential of CRISPR-Cas9, there are several technical issues associated with the technology that prevents its widespread adoption (Kang et al., 2017), such as off target effects, the viability of edited cells, their fitness, editing efficiency, and the lack of adequate delivery methods (M. Chen et al., 2019)

Although new variants of CRISPR Cas 9 are being developed that have improved selectivity or better facilitate the insertion of a gene/DNA fragment of interest via HDR, the probability of off target effects of CRISPR-Cas9 in human cancer cell lines are still quite high (Xiao et al., 2014). Sequence homology and sequence similarity within the host genome still remains a problem, particularly in organisms that have a larger genome, as a larger genome means a higher probability there may be repetitive or highly similar DNA sequences, which may induce off target DSBs, giving rise to mutations (Kang et al., 2017).While targeting specificity is governed by the 20 nucleotide sgRNA and PAM, off target effects can still occur in regions of the genome with up to 5 bp mismatches in the non-PAM region of the sgRNA resulting in

an aberrant DSB (Zhang et al., 2015). Off targets are a cause for concern in CRISPR because off target mutations have the potential to cause genomic instability and disrupt the function of normal genes. The mutations it can cause are often large deletions and genomic rearrangements that have the potential to cause lethal mutations or a loss of function in genes (Naeem et al., 2020; Zhang et al., 2015).

The delivery of Cas9 and the synthesis of guide RNA are the two largest hurdles that limit genomic editing efficiency and ease of use (Liang et al., 2015). For the system to be successful the delivery method needs to be effective at cell targeting, facilitate the rapid elimination of the CRISPR components after transfection and produce minimal cytotoxicity (Mout et al., 2017). The CRISPR Cas9 RNP is relatively large and most transformation modalities have limitations with respect to the size of the payload (Ghosh et al., 2019; Hazafa et al., 2020). For example, AAV's have a loading capacity of approximately 4.7Kb and an internal capsid volume of 2.48 × 106 $Å^3$ (Kay, 2011; Swiech et al., 2015). AAVs also require a long incubation period that can increase the probability of off target effects (Chen et al., 2020). Non-viral delivery methods are recommended due to the lack of size limitations and rapid action (Chen et al., 2020). However, non-viral vectors are unable to penetrate cells as efficiently as viral systems and without significant modification are notably less selective (Jiang & Doudna, 2017; Li et al., 2015) The heterogeneity of cancer is significant, with variation in expression profiles existing between different patients, between different stages of progression and even between different tumour sites within the same patient. This variation poses significant challenges for gene editing therapies in cancer (Chen et al., 2019). The fitness of edited cells is also affected by gene editing, and without a selective advantage, it is likely any therapeutic effects will be temporary unless the transformation efficiency is radically improved (Cox et al., 2015).

The DSB repair mechanism elicited may also affect efficiency. As mentioned previously, NHEJ and HDR differ in efficiency between cell types and cell states. NHEJ repair mechanisms are ubiquitous, but HDR relies on the expression of repair mechanisms associated with the S/G2 phase of mitosis. As a result of this NHEJ has a greater utility and higher efficiency for creating indels to knockout genes, and HDR is

better at performing more precise gene modifications and integrating exogenous DNA into the host genome, but it has considerable disadvantages (Chen et al., 2019; Yin et al., 2016) such as extremely low transformation efficiency (Hsu et al., 2014). Additionally, some cancer cell lines lack HDR mechanisms and can only repair DSB using the NHEJ pathway, and without the compensatory reintroduction of the appropriate HDR repair mechanisms, HDR is not possible (Li et al., 2021)

1.9 Aims

The identification of novel therapeutic targets and the development of novel treatments are essential to the improvement of treatment outcomes. Sacituzumab govetican hziy is a recently developed antibody drug conjugate that acts on the TROP2 receptor, a novel target that is overexpressed in 93% of TNBC (Aslan et al., 2021). Sacituzumab govetican hziy improved OS rates when compared to chemotherapy, increasing mean survival by 3.2 months in Phase 3 clinical trials while reducing symptoms (Rugo et al., 2023).

KISS1R has been suggested as a potential target for the treatment of TNBC due to its overexpression in TNBC and the relationship between KISS1R overexpression and increased proliferation, metastasis and chemotherapeutic resistance.

This thesis aims to support the argument that therapeutic benefit can be achieved by targeting TNBC cells overexpressing KISS1R. Experiments will involve deletion of KISS1R by using the lipofectamine-mediated delivery of KISS1R guide-RNA/Cas9 protein ribonucleoprotein complexes in MDA-MB-231 cell lines only, and to evaluate the effect that KISS1R KO has on the rate of cell proliferation in MDA-MB-231 human breast cancer cells only.

This thesis will also contribute to the growing body of work that supports the use of lipofection and CRISPR Cas9 RNP mediated transformation for use in genetic medicine, due to its notable benefits,

including improved rates of transformation in vitro, reduced off target effects and reduced cellular toxicity.

2. Chapter Two: Methods

2.1 Chemicals and Reagents

Chemicals	Suppliers	Catalogue No.
Agarose RA™	VWR Life Science, US	N605-500G
Anti-Kiss1 receptor antibody	Abcam, NZ	ab137483
Anti-Rabbit IgG (H+L) – HRP antibody produced in goat	Abcam, NZ	ab205718
Bovine serum albumin (BSA) (powder)	Sigma-Aldrich, NZ	A7030
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich, NZ	4693159001
DC Protein Assay Reagents Package	BIO-RAD, NZ	5000116
DEPC-treated water	Bioline, USA	BIO-38030
DMSO (dimethyl sulfoxide) (99.7%)	ThermoFisher Scientific, NZ	FSBBP231-1
Dithiothreitol (DTT)	BIO-RAD, NZ	1610610
Doxorubicin Hydrochloride	Abcam, NZ	Ab120629
Ethanol absolute	ThermoFisher Scientific, NZ	AJA214-20L
Fetal Bovine Serum (FBS)	Medi'Ray, NZ	MG-FBS0820-500ML
GeneArt™ Genomic Cleavage Detection Kit	Life Technologies, NZ	A24372
Greiner high and medium binding 96 black well plate	Sigma-Aldrich, NZ	M5061
L-Glutamine	Life Technologies, NZ	20530081
Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent	ThermoFisher Scientific, NZ	CMAX00003
Lipofectamine™ RNAiMAX reagent	Invitrogen, NZ	13778
Laemmli Sample Buffer (4X)	BIO-RAD, NZ	1610747
Methanol	Sigma-Aldrich, NZ	34860
Milli-Q water	AUT WS212 Lab	-
Mini-PROTEAN® TGX™ Precast Gel (10%)	BIO-RAD, NZ	4561034
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)	Sigma-Aldrich, NZ	M2128-1G
Opti-MEM™ I Reduced Serum Medium	ThermoFisher Scientific, NZ	31985070
Phosphate buffer saline (PBS) pH7.4 (10X)	ThermoFisher Scientific, NZ	70011044
Precision Plus Protein™ Kaleidoscope™ Standards	BIO-RAD, NZ	1610375
Primers	Integrated DNA Technologies / ThermoFisher Scientific, NZ	-
PVDF (polyvinylidene fluoride) membrane	Life Technologies, NZ	22860
Recovery™ Cell Culture Freezing Medium	Life Technologies, NZ	12648010
RPMI-1640 Medium, no glutamine	Life Technologies, NZ	21870092
sgRNA (*KISS1R*) set1	ThermoFisher Scientific, NZ	A35534
sgRNA (*KISS1R*) set2	ThermoFisher Scientific, NZ	A35534
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	ThermoFisher Scientific, NZ	34578

SYBR™ Gold Nucleic Acid Gel Stain (10000X)	ThermoFisher Scientific, NZ	S11494
TaKaRa PCR Amplification Kit	TaKaRa Bio USA, Inc.	R011
TrackIt™ 100 bp DNA Ladder (100 bp-2,000 bp)	Life Technologies, NZ	10488058
Trans-Blot® Turbo™ 5X Transfer buffer	BIO-RAD, NZ	-
Trypan Blue Solution, 0.4%	ThermoFisher Scientific, NZ	15250061
TrueCut Cas9 Protein V2	ThermoFisher Scientific, NZ	A36499
TrypLE™ express	ThermoFisher Scientific, NZ	12604021
Tris-HCl Buffer (0.5 M, pH6.8)	BIO-RAD, NZ	1610799
Triton™ X-100	Sigma-Aldrich, NZ	T8787-250ML
Tween 20 Solution (10%)	BIO-RAD, NZ	1610781

Table 2.1 Chemicals and reagents used in this study and their sources.

2.2 sgRNA Design

Optimal sgRNA design was produced using Invitrogen TrueDesign Genome Editor (Thermofisher, n.d.) and the Chop Chop Online Tool (Labun et al., 2016). These online tools have automated the majority of the sgRNA design process, locating PAM sequences in a gene of interest and comparing the complementary recognition sequences with the rest of the host species genome to determine the likelihood of any off-target effects and provide a predictive score of the transfection efficiency based on the generated sgRNA sequence and its uniqueness. For the Invitrogen TrueDesign Genome Editor, the following inputs were used to generate a list of candidate sgRNA

Species	*Homo sapiens*
Gene Symbol	KISS1R
Knockout Strategy	Frameshift Indels
Predesigned or Custom	Custom
Region	Coding (Exons)

To generate the list of candidates using the Chop Chop Online Platform, the following inputs were used

Target Gene	KISS1R

In	*Homo sapiens*
Using	CRISPR Cas9
For	Knockout

The Invitrogen TrueDesign Genome Editor (Thermofisher, n.d.) and the Chop Chop Online Tool (Labun et al., 2016) autogenerated lists were compared and the two sgRNA designs with the highest predicted efficiency and the lowest number of off target effects were selected for use in these experiments.

2.3 Cell Lines and Cell Culture

The cell lines (MDA-MB-231, ATCC® HTB-26TM, wild type) used in this study were purchased from American Type Culture Collection (ATCC (Cryosite Ltd, NSW, AU)) and stored in a physical containment lab at AUT University.

Cells were thawed from stock by partially immersing the vial of cryopreserved MDA-MB-231 for two minutes in a water bath that was heated to 37°C. The exterior of the vial was sterilized with alcohol once appropriately thawed and the contents were added to 6 ml of complete media (CM) in a T25 flask. The cells were then kept at 37°C in a humidified CO_2 incubator (Forma™ Series II 3110 Water-Jacketed CO_2 Incubator; ThermoFisher Scientific, Inc) to allow the cells to grow on the surface of the flask till the appropriate confluency was reached.

2.4 Transfection

The generation of a KISS1R KO cell line was achieved using reagents and protocol from ThermoFisher. Some slight modifications to conventional protocol were made to improve transformation efficiency and the accuracy of detection assays. Any changes were noted and are contained in the following protocol.

2.4.1 Seeding Cells

Once propagating cells reached 70-80% confluency, the CM was decanted, and the cell monolayer was washed with 4 ml of PBS solution delivered by automatic pipette. Once the PBS solution was decanted, an aliquot of 2ml of TrypLE Express Enzyme was added and the flask. The T25 flask was placed in the CO_2 incubator (Forma™ Series II 3110 Water-Jacketed CO2 Incubator; Thermo Fisher Scientific, Inc) at 37° C for 3-5 minutes. Trypsinization was terminated by the addition of an aliquot 5 ml of culture medium to the T25 flask containing the detached cells. The solution was then transferred to a 15 ml centrifuge tube where cells were to be pulled out of solution by centrifugation for 3-5 minutes at 200 g. The resultant supernatant was decanted and disposed of, and the pellet of cells was retained. 1 ml of warm CM was added to the tube containing the pelleted cells and the concentrated cell suspension was agitated via pipette till the pellet was dissolved and the solution appeared homogenous.10 µl of the cell suspension was removed using a pipette, placed on parafilm, mixed 10 µl with 0.4% Trypan Blue and left to incubate for 10 minutes. A coverslip was placed over the hemocytometer and 10 µl of this mixture was then placed proximal to the juncture of the coverslip and the hemocytometer and capillary action forced the movement of solution between the coverslip and the hemocytometer. Solution was gradually added till the grid was appropriately covered. The cells in each square were counted using a microscope set to 10X objective and the mean number of cells per square was calculated. The viable cell concentrations were then calculated using the equation:

Viable cell concentration (number of cells/mL) = Mean viable cell/square × Dilution Factor × 10^4

MDA-MB-231 cells (100,000 cells per/well) were then placed in a 12 well plate for transfection.

2.4.2 Transfection

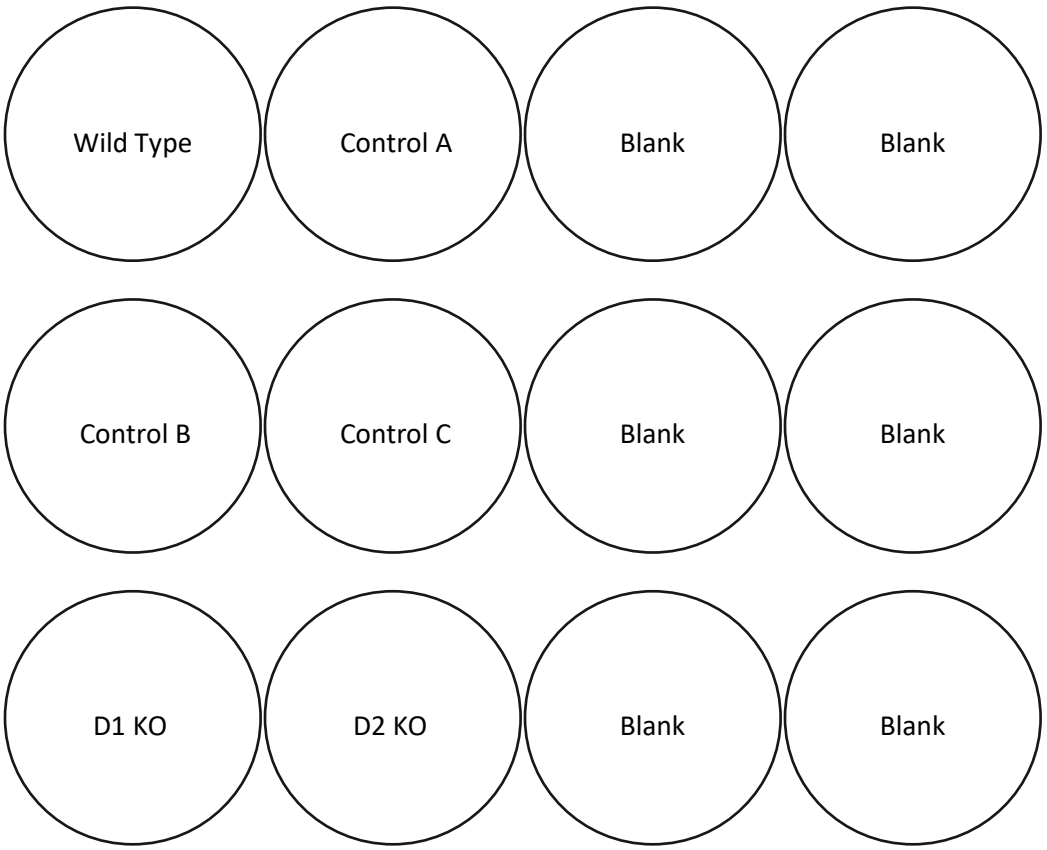


Figure 2.1 The layout of the transfection experiment on a 12 well plate

Five microcentrifuge tubes were prepared as follows:

Firstly, 3 control groups, A, B and C were formulated

No sgRNA (A): 50µl of Opti-MEM medium was added, followed by 0.5µl of TrueCut Cas9 Protein V2 and 5µl of Lipofectamine Cas9 Plus. Lastly a mixture of 3µl of Lipofectamine CRISPRMAX and 50µl Opti-MEM was added to the solution. This mixture was left to rest at room temperature for 15 minutes before being added to the appropriate well.

No Cas9 Protein (B): 50µl of Opti-MEM medium was added, followed by 3µl Invitrogen TrueGuide sgRNA and 5µl of Lipofectamine Cas9 plus. Lastly a mixture of 3µl Lipofectamine CRISPRMAX and 50µL Opti-MEM was added to the solution. This mixture was left to rest at room temperature for 15 minutes before being added to the appropriate well.

No sgRNA or Cas9 Protein (C): 50µl of Opti-MEM was added, followed by Lipofectamine Cas9 plus. Lastly a mixture of 3µl of Lipofectamine CRISPRMAX and 50µl of Opti-MEM to the solution. This mixture was left to rest at room temperature for 15 minutes before being added to the appropriate well.

KISS1R KO (D1): 5µl of Opti-MEM was added, followed 0.5µl of TrueCutTM Cas9 protein, 3µl Invitrogen TrueGuideTM sgRNA sample 1 and 5µl Lipofectamine Cas9 plus. Lastly a mixture of 3µl Lipofectamine CRISPRMAX and 50µl of Opti-MEM was added to the solution. This mixture is left to rest at room temperature for 15 minutes before being added to the appropriate well.

KISS1R KO (D2): 5µl of Opti-MEM was added, followed 0.5µl of TrueCutTM Cas9 protein, 3µl Invitrogen TrueGuide sgRNA 2 and 5µl Lipofectamine Cas9 plus. Lastly a mixture of 3µl Lipofectamine CRISPRMAX and 50µl of Opti-MEM was added to the solution. This mixture is left to rest at room temperature for 15 minutes before being added to the appropriate well.

The plates were briefly agitated then incubated at 37°C for 48 hours in a CO_2 incubator. After the incubation period the CM was removed, and the cells were washed with 500µl of PBS solution and inthe CM was replaced with fresh CM.

Solution 1:

Invitrogen TrueGuideTM sgRNA 1: 45pmol

TrueCutTM Cas9 protein V2: 2500ng

Lipofectamine Cas9 PLUS: 3µl

Opti-MEM medium (no FBS): 50µl for KO reagent

Solution 2:

Lipofectamine CRISPRMAX: 3µl

Opti-MEM medium (no FBS) 50µl

Solution 1:

Invitrogen TrueGuideTM sgRNA 2: 45pmol

TrueCutTM Cas9 protein V2: 2500ng

Lipofectamine Cas9 PLUS: 3µl

Opti-MEM medium (no FBS): 50µl for KO reagent

Solution 2:

Lipofectamine CRISPRMAX: 3µl

Opti-MEM medium (no FBS) 50µl

2.5 Genomic Cleavage Detection Assay

In this study the protocol and reagents used to verify the gene editing efficiency of this experiment were provided by the Invitrogen GeneArt® Genomic Detection Kit. This kit was used as per the manufacturer's instructions.

2.5.1 Harvest Cells

Once transformed cells had reached 70% confluency, cells were harvested, propagated and harvested again, according to the protocol described in 2.3.1. The resultant cells were split into two different groups, one group containing cells designated for use in the analysis of transformation efficiency, and the other group to be propagated for further testing or cryopreserved. The cell lines designated for use in the analysis of transformation efficiency using the Invitrogen GeneArt® Genomic Detection Kit were then separated via centrifugation at 200g for 5 minutes at 4°C and the supernatant was decanted and disposed of.

2.5.2 Cell Lysis and DNA Extraction

50µl of the cell lysis buffer and 2µl of protein degrader from the Invitrogen GeneArt® Genomic Detection Kit were combined in a microcentrifuge tube and this solution was used to resuspend the cell

pellet. The cell suspension was then transferred to a PCR tube and the following program was run in a thermal cycler.

Temp	Time
68°C	15 min
95°C	10 min
4°C	Hold

2.5.3 PCR Amplification

The cell lysate was vortexed, and the components listed below were added to each PCR tube as per instructions provided in the Invitrogen GeneArt® Genomic Detection Kit.

Component	Sample	Control
Cell lysate	2µl	-
10µM F/R primer mix	1µl	-
Control template & primers		1µl
AmpliTaq Gold 360 Master Mix	25µl	25µl
Water	22µl	24µl
Total	50µl	50µl

After the following components were added a PCR reaction was run following the protocol described in the Thermofisher Amplitaq Gold 360 Master Mix Protocol Guide. A portion of the PCR product was aliquoted for sequencing by the University of Otago's DNA Sequencing Service.

Phase	Temperature	Time	Cycle Number
Enzyme activation	95°C	10 min	1

Denaturing	95°C	30 sec	40
Annealing	57°C	30 sec	40
Extension	72°C	30 sec	40
Final extension	72°C	7 min	1
Hold	4°C	Hold	1

2.5.4 Verifying the PCR Product

10µl of PCR product was loaded in a 2% agarose gel with a DNA ladder. The gel was then run for 0.5 hours at 50V then 1.5 hours at 100V. After this the gel was stained via incubation in 50mls of running buffer with 2µl SYBR Gold added. The gel was then imaged, and the running buffer was disposed of.

2.5.5 Cleavage Assay

The purpose of this step is to denature and anneal PCR fragments so DNA strands with and without indels anneal at random and form heterogenous DNA duplexes with mismatches. The T7E1 Detection Enzyme cuts DNA in regions where a DNA mismatch is detected. This produces 2 DNA fragments instead of one, that can be easily differentiated using gel electrophoresis.

2µl of PCR product was combined with 1µl of 10X Detection Reaction Buffer in a 96 well plate and the volume of each well was then brought to 9µl with PCR grade water. Once combined, the 96 well plate was then sealed with an adhesive membrane and briefly spun in a Plate Centrifuge to ensure there was no trapped air. The plate was then sealed with a polymer film and placed in the RT PCR Machine and the following program was run. The modified protocol for the generation of the heterogenous DNA duplexes is as follows

Steps	Temperature, Time
1	95°C, 10 mins
2	95-85°C, -2°C/s
3	85°C, 1 min
4	85-75°C, -0.3°C/s
5	75°C, 1 min
6	75-65°C, -0.3 °C/s
7	65 °C, 1 min
8	65-55 °C, -0.3 °C/s
9	55 °C, 1 min
10	55-45 °C, -0.3 °C/s
11	45 °C, 1 min
12	45-35 °C, -0.3 °C/s
13	35 °C, 1 min
14	35-25 °C, -0.3 °C/s
15	25 °C, 1 min
16	25-4 °C, -0.3 °C/s
17	4 °C, hold

Once the program was finished, enzyme digestion was initiated. This was done by removing the adhesive membrane and adding 1µl of detection enzyme to each test sample and 1 µl of water to all the control samples and these were then incubated at 42°C for 30 minutes in the RT PCR machine. After 30 minutes, the mixture was vortexed briefly and spun down before being immediately loaded to the 2% agarose gel.

2.5.6 Gel Analysis

50 ml of agarose gel solution was prepared in a 250 ml flask and approximately 1g of agar (2%). The flask was heated in the microwave until the agar was fully dissolved. The solution was then poured into a gel mould with a comb insert. Care was taken to avoid producing any defects in the gel. If any defects were detected, the gel was discarded and another gel was made.

Once the gel had set, 10µl of each of the samples mixed with 2µl loading dye and 8 µl of water were added to the wells, totaling 20 mls. A DNA ladder was included to aid in the size differentiation of detected fragments.

The gel was run for 30 minutes at 50V then 1.5 hours at 100V. The gels were then soaked in 50 ml of running buffer containing 2 µl of SYBR Gold Stain for 30 minutes. The gels were then imaged in the gel doc and the images produced were analyzed using ImageJ

2.6 Isolation of Single Clones

Once the cell lines designated for further propagation reached adequate confluency (70%) the medium was removed, and the cells were washed and trypsinized using protocol outlined in Section 2.3.1. These were then spun down in the centrifuge at 200 g for 5 minutes, the supernatant was decanted and 1ml of CM was added to resuspend the pellet. 10µl of the cell suspension was removed and placed on parafilm

and was then mixed with 10µl of 0.4% Trypan Blue. This mixture was added to one half of a haemocytometer until the grid is covered. The number of cells in each counting square on the haemocytometer were counted with microscope set to a 10X objective and the average number of cells per square was calculated. The desired average seeding density per well was 0.8 to reduce the risk of there being more than 1 cell per well. To achieve this, 100 µl of an 8 cells/ml solution was aliquoted to each well of the 96 well plate. One well in the corner of the plate was seeded with 1000 wild type cells, this was done to provide a focal point for the microscope when searching for individual clones.

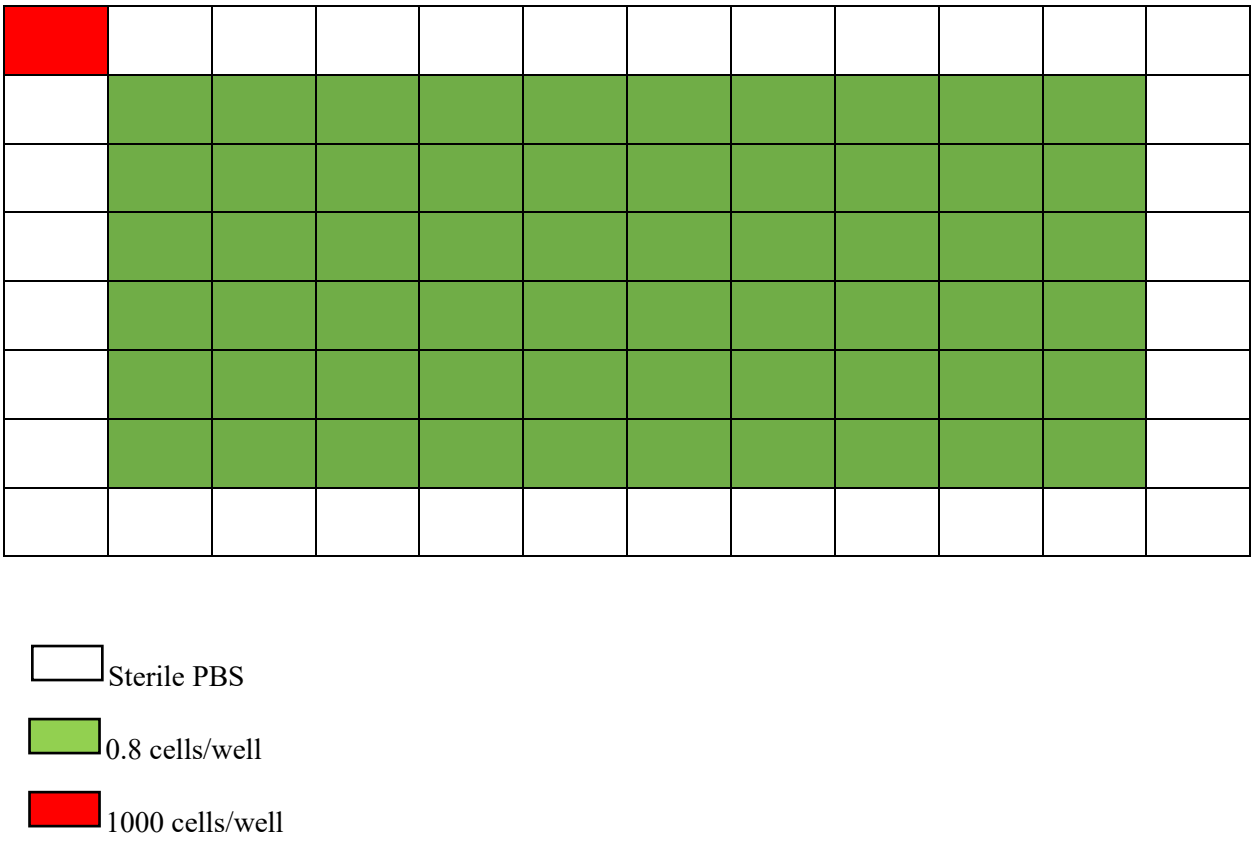


Figure 2.2 96 well plate layout for the isolation and propagation of single cell clones

Once the cells were seeded in the 96 well plate they were observed under a microscope and wells that appeared to contain single cells were marked with a fibre tip pen. The cells were stored in a 37°C 5% CO_2

incubator. The cells were checked every few days to ensure they were growing and to note any signs of colony collapse.

After three weeks of growth in the 96 well plate, viable cell colonies believed to originate from a single cell were moved to 12 well plates. Using a pipette, the complete medium was carefully removed and discarded, the cells were then washed with 100µl PBS and then detached from the surface of the well using 30µl of trypsin. The cells were incubated with TrypLE Express Enzyme for approximately a minute or until cells appeared to have detached from the plate surface as indicated by visualization of wells under a microscope at 10x magnification. Trypsinization was arrested via the addition of 150µl of complete medium and the cell suspension was pipetted into a microcentrifuge tube and spun down to form a pellet at 200g for 2 min. If the cell density was too small for a viable pellet to be produced, 200µl of culture medium was added to each well and then the cell suspension from these wells were transferred to a new well in a 12 well plate, where another 1 ml of CM was added. Wells were labelled according to the sgRNA used (D1, D2 or none), and numbered according to the order in which they were seeded (1 – 70). A total of 70 clones were originally produced across six 12 well plates.

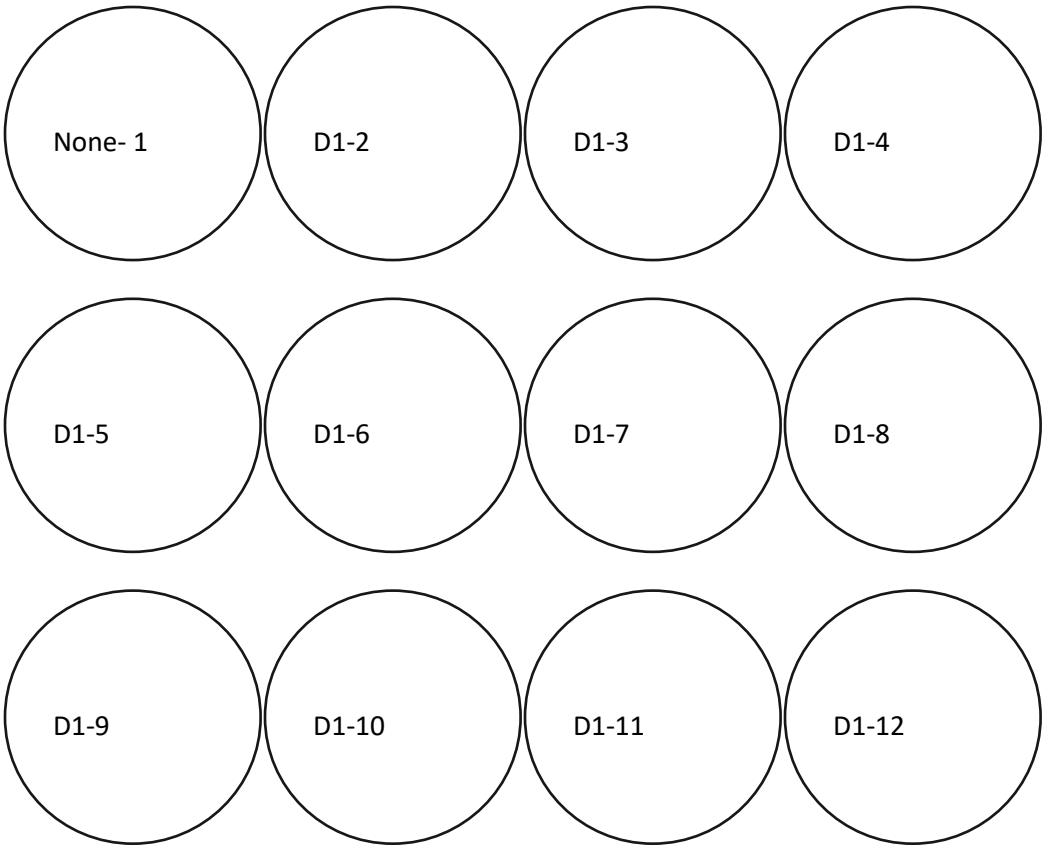


Figure 2.3 Layout of the clones in a 12 well plate, each number indicating the strain from which it was isolated and the order in which it was successfully isolated.

Clones in the 12 well plates were left to grow until they became confluent enough to be transferred to T25 flasks in the 37°C CO_2 incubator. Once they had reached at least 70% confluency the clones were washed with PBS and detached from the surfacing using 0.5 ml of trypsin. After trypsinization, 2ml of complete medium was added again. The cells were not spun down in the centrifuge but instead were directly transferred to the T25 flasks. Each T25 flask had 5 ml of CM added to each flask before the cell suspension was added. The T25 flasks were then returned to the 37°C CO_2 incubator to allow the cells to grow.

2.7 Western Blot Analysis

Western Blot Analysis allows for the identification and quantification of proteins in a tissue sample by separating fragments based on size, charge and structure (Mahmood and Yang 2012). This is done by combining two different techniques to first separate proteins based on size and charge, called Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then the identification of unique structural motifs via antibody selectivity, called immunohybridization. SDS PAGE involves the application of directional charge to a porous gel matrix containing a mixture of charged and denatured peptides, in a salt rich solution. The propagation of the peptides is hindered by the gel medium's density in a way proportional to the size of the peptide, and its associated charge. The propagation of the peptides is hindered by the density of the gel medium in a manner that is proportional to the size of the peptide, and its associated charge. This separation of fragments produces a series of distinct bands in each lane. For precise identification of a protein, trace amounts of peptide are transferred to a polymer membrane and inoculated with a highly specific antibody, for example, an anti-KISS1R antibody, which binds to structural motifs unique to KISS1R. Once the membrane containing the transferred protein is inoculated with the highly selective antibody, it is then washed and inoculated with a generic secondary antibody that is bound to a protein that can produce a signal that can be easily visualized. This practice allows for the easy identification of proteins in a sample and can also indicate relative quantities of protein if a reference is provided. The following protocol will allow us to determine the presence of KISS1R in any of the colonies grown from the isolated single cells.

2.7.1 Propagating Cells for Protein Assay

Wild type and D1 mutant strains were cultivated in T25 flasks using the protocol outlined in Section 2.2 till they reached 70% confluency. These cells were then harvested by first decanting the complete medium (DMEM + 10% FBS) from the flask, washing the cell monolayer with 4 mls of PBS and then treating the cultured monolayer with 2 mls of TrypLE Express Enzyme. Cell monolayers were then incubated for 3-5 minutes at 37°C with the TrypLE. After the incubation period, the trypsinization of the cell monolayer was arrested by dilution with 7 mls of complete medium. The solutions were then transferred to two 15 ml falcon tubes and centrifuged at 200g for 2 minutes. The supernatant was decanted, and the cell pellet was washed twice with 2 mls of PBS and stored in the 4°C fridge.

2.7.2 Reagent Preparation

1 ml of stock dithiothreitol (DTT) containing Laemmli sample buffer [62.5 mM tris-HCl (pH 6.8), 2% LDS, 100 mM dithiothreitol, and 0.01% bromophenol blue] was prepared by combining 100 µls of DTT, 400µl of ddH2O and 500 µl of 4x Laemilli sample buffer in a 1.5 ml Eppindorf tube.

2.7.3 Cell Lysate Preparation

The cell pellet was resuspended in 0.3 mls of DTT containing Laemilli sample buffer and placed on ice. After a 30-minutes on ice, samples were placed in a centrifuge and spun for 15 minutes at 14,000 g, 4°C. The supernatant was transferred to a set of clean tubes and the pellet was discarded.

2.7.4 Gel Electrophoresis

A premade gel cassette was removed from its packaging and rinsed, the comb and tape were removed, and the wells were rinsed three times with running buffer [25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3]. The gel cassette was then loaded into the electrophoresis module, and the module was loaded into the tank. The tank was then filled with running buffer to the 'one gel' mark and the electrophoresis module was filled with running buffer till the wells were covered. 20µl of a prestained molecular weight ladder was loaded into the first well, the second was loaded with a 20µl protein sample from the WT control, and protein samples from single cell colonies were loaded into the remaining wells. The well number and protein samples were recorded. The lid was then placed on the electrophoresis tank, locked into place and the gel was run for 10 mins at 50 V and immediately after, 100 V for one hour.

2.7.5 Protein Transfer

Once the run was complete, the gel cassette was removed from the module and the cassette was pried open with a plastic wedge. A Trans-Blot Turbo Transfer Pack (0.2µm PVDF) was opened, and the gel was placed on the polyvinylidene fluoride (PVDF) membrane and bottom stack. A roller was used to remove any air pockets of air present as they could interfere with the protein transfer. The top stack was then placed on top of the gel and a roller was again used to remove any additional pockets of air and to improve contact as these can affect the efficacy of the transfer and thus the image produced. The 'transfer sandwich' was then placed inside the cassette in the correct orientation with the membrane closest to the

anode and the gel closest to the cathode and the cassette was sealed shut and returned to the BioRad TransBlot Turbo Transfer System. The parameters chosen for the run were as follows

Run	Current	Voltage	Time
Mixed Molecular Weight	1.3A	25V	7 mins

After the run was complete, the membrane was removed and placed in a sealed container for further analysis, and the gel was disposed of.

2.7.6 Protein Quantification

In order to validate both the presence and the quantity of protein in a sample, the Pierce™ Reversible Protein Stain Kit for PVDF Membranes was used on the newly produced membrane. The membrane was first washed with reverse osmosis (RO) purified water and decanted. The membrane was then submerged in approximately 25mls of MemCode™ Sensitizer and shaken on a rotary platform shaker for 2 minutes. The solution was decanted and approximately 25 mls of MemCode™ Stain was added. The membrane was shaken for 1 minute. The solution was decanted, and the membrane was removed from the container and imaged for further analysis.

To remove the staining, the membrane was washed and decanted thrice with MemCode™ Destain. The membrane was then submerged in approximately 25 mls of the Destain/Methanol solution and was shaken on a rotary platform shaker for 5 minutes and then decanted. The membrane was then washed 5 times with ultrapure water, before being washed with the Eraser/Methanol solution on a shaker for 10-20

minutes. Lastly, it was rinsed 5 times with ultrapure water and decanted before being stored in a container in the –4 C fridge for further analysis. The PVDF membrane was then imaged and analyzed.

2.7.7 Antibody Binding

The membrane is submerged in blocking buffer (TBST and 0.5% milk, filtered) for one hour with agitation. The solution was then decanted and washed with TBST for five minutes with agitation. 5 µl of primary antibody solution was added to 10 ml of blocking buffer and vortexed. The membrane was then submerged in solution and incubated overnight in the 4°C fridge. The solution containing the primary antibody was decanted and the membrane was washed with TBST while agitated for 3 minutes. This is repeated three times. After each wash, any excess TBST was removed with a paper towel. A secondary antibody solution was formulated (25 ml TBST, 0.5% milk, filtered, 2.5 µl secondary antibody) and the membrane was submerged in the secondary antibody solution. The tray was then covered in aluminium foil to prevent UV degradation and placed on a shaker for 1 hour. The antibody solution was then decanted, and the membrane washed with TBST for approximately 3 mins. The membrane was washed another two times with RO purified water for three minutes each wash. Pierce ECL Western Blot Substrate Detection Reagents 1 and 2 were mixed in a 1:1 ratio, in quantities of approximately 4 ml each, and the membrane was submerged in this solution for one minute. The membrane was then removed and taken to the ImageQuant LAS 500 Gel Doc for imaging.

2.8 MTT Assay

The MTT assay is a colorimetric assay that measures the conversion of MTT to insoluble formazan, by enzymes of the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase family. This is used as a proxy for cellular metabolic activity and thus cell viability. In cancer research, an MTT can be used to infer the effects of treatments on cancer cell viability, providing some indication of how effective a particular treatment may be in vivo. In this particular set of experiments, MTT is used to determine the effect a KISS1R KO has on the rates of cell proliferation and is also used to detect any changes in sensitivity to Doxorubicin as a result in the loss of the KISS1R receptor.

2.8.1 Reagent Preparation

1ml of filter sterilized PBS was added to one 5mg vial of MTT to produce a 12mM MTT stock solution and mixed by vortex for a minute or until any visible particulate was dissolved. The solution was then sterile filtered to remove any remaining particulate.

2.8.2 Culturing Cells

MDA-MB-231 wild type and D1-3 knock out cells were seeded at 8,000 cells/100µl/well (Figure 2.3). The cells were then incubated for 24 hours 37ºC with 5% CO_2/95% air. After the cells were allowed enough time to appropriately attach to the bottom of the plate, 100µl of Doxorubicin of varying concentrations (see Figure 2.5) was added to each well.

The cells were then stored in an incubator for 72 hours at 37ºC with 5% CO_2/95% air.

PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
PBS	CM	Wild Type 0nM	Wild Type 50nM	Wild Type 140nM	Wild Type 410nM	Wild Type 1.23µM	Wild Type 3.7µM	Wild Type 11.1µM	Wild Type 33.3µM	Wild Type 100µM	PBS
PBS	CM	Wild Type 0nM	Wild Type 50nM	Wild Type 140nM	Wild Type 410nM	Wild Type 1.23µM	Wild Type 3.7µM	Wild Type 11.1µM	Wild Type 33.3µM	Wild Type 100µM	PBS
PBS	CM	Wild Type 0nM	Wild Type 50nM	Wild Type 140nM	Wild Type 410nM	Wild Type 1.23µM	Wild Type 3.7µM	Wild Type 11.1µM	Wild Type 33.3µM	Wild Type 100µM	PBS
PBS	CM	KISS1R KO 0nM	KISS1R KO 50nM	KISS1R KO 140nM	KISS1R KO 410nM	KISS1R KO 1.23µM	KISS1R KO 3.7µM	KISS1R KO 11.1µM	KISS1R KO 33.3µM	KISS1R KO 100µM	PBS
PBS	CM	KISS1R KO 0nM	KISS1R KO 50nM	KISS1R KO 140nM	KISS1R KO 410nM	KISS1R KO 1.23µM	KISS1R KO 3.7µM	KISS1R KO 11.1µM	KISS1R KO 33.3µM	KISS1R KO 100µM	PBS
PBS	CM	KISS1R KO 0nM	KISS1R KO 50nM	KISS1R KO 140nM	KISS1R KO 410nM	KISS1R KO 1.23µM	KISS1R KO 3.7µM	KISS1R KO 11.1µM	KISS1R KO 33.3µM	KISS1R KO 100µM	PBS
PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Figure 2.4 Layout of the MTT assay on a 96-well plate with concentrations of Doxorubicin

2.8.3 Labelling and Detection

After the cultured cells were given sufficient time to inoculate in, approximately 70 hrs, the spent CM was removed from each well and replaced with 100µl of CM and 10µl of MTT stock solution which was formulated according to the protocol outlined in Section 2.7.1

After the three-hour incubation period, approximately 85µl of solution was removed from cells B-G of rows 3-11, leaving 25µl of solution in each well. 150µl of DMSO was then added to these wells and mixed for 30 minutes on an orbital shaker. Once the plate had been mixed for 30 minutes, it was placed in a plate reader and the absorbance was read at 540nm with a reference wavelength of 680nm. Results were analysed using the GraphPad PRISM 10.3.1 software suite.

3. Chapter Three: Results

To investigate the feasibility of inducing an indel mutation in the KISS1R gene in MDA-MB-231 cell lines using CRISPR-Cas9 RNP system, several different sgRNAs were designed using the Invitrogen TrueDesign Genome Editor Online Tool and Chop Chop Online Platform to target *KISS1,* and two sgRNA designs were selected for use in these experiments. The two selected sgRNA were then used to formulate two solutions for the CRISPR-Cas9 RNP mediated KISS1R knockout of two WT MDA-MB-231 cell cultures. These transformed cell lines were then propagated and the genomic cleavage efficiency of this technique was then assessed using a T7 endonuclease assay and Sanger Sequence Analysis. A homozygous knockout clone was isolated and propagated and Western Blotting and an MTT assay using varying doses of Doxorubicin were then used to compare the phenotypic differences between the wild type and the homozygous knockout cell lines produced, including rates of cell viability and levels of resistance to Doxorubicin. Only MDA-MB-231 breast cancer cells were used in these experiments.

3.1 sgRNA Design

Using the Invitrogen TrueDesign Genome Editor, a list of custom sgRNA targeting the gene *KISS1R* using the parameters described in Section 2.2. These were compared with a list of candidate sgRNA produced by the Chop Chop Online Platform, which were also produced using the inputs described in Section 2.2. The two top scoring sgRNA with the least number of off target effects were selected. The number of off target effects for both sgRNA designs were 0.

The selected sgRNA designs are as follows

Name	Target Sequence	PAM	Score	Genomic Location	Strand	Amplicon Size (Cleavage band sizes)
KISS1R_C1	TGTGGCGCCAACGCCTCGGA	CGG	100.00	chr19[917574]	+	446 (296, 150)
KISS1R_C3	AAGTTGGTCACGGTCCGCAT	CGG	100.00	chr19[917738]	-	309 (261, 46)

Table 3.1 sgRNA used in the CRISPR Cas9 mediated knockout of KISS1R, including the location of the target, their target sequence, the associated PAM, their rank score as well as the expected cleavage band sizes.

The selected sgRNA and associated PAM site, supplementary primers produced for use in analysis and the DNA sequence contained within those primers are as follows.

Primers and Amplicon for KISS1R_C1 sgRNA:

GCACGGGCTATAAACGCTCGGCCGCAGCGGCGCCGGCAGAGGAGCCGCCGAGCCCA
GCACAGCTGCCCTCTGGACCCTGCGGACCCCAGCCGAGCCCCTTCCTGAGTTCCACA
GGCGCAGCCCCCGGGCGGTCGGGCGGAGGGGTCCCCGGGGCGGTGCCAGGGCGCA
ATCCTGGAGGGCGGCCGGGAGGAGGAGGTGCGCGCGGCCATGCACACCGTGGCTAC
GTCCGGACCCAACGCGTCCTGGGGGGCACCGGCCAACGCCTCCGGCTGCCCGGGCT
GTGGCGCCAACGCCTCGGACGGCCCAGTCCCTTCGCCGCGGGCCGTGGACGCCTGG
CTCGTGCCGCTCTTCTTCGCGGCGCTGATGCTGCTGGGCCTGGTGGGGAACTCGCTG
GTCATCTACGTCATCTGCCGCCACAAGCCGATGCGGACCGTGACCAACTTCTA

Primers and Amplicon for KISS1R_C3 sgRNA:

GGGAACTCGCTGGTCATCTACGTCATCTGCCGCCACAAGCCGATGCGGACCGTGACC
AACTTCTACATCGGTGAGTGCGGGCGCTGCGCCGCACCTGCTGCCGTCCCGGGGGCT
CCGAGGGCCGAGCGGCCTGGGGCGCCCTCTCGCGACGCATCGGGGCCCTCTCGGAC
CCGGCTCTGTCCCCTGCAGGGGTCCCCCCCAACCTCGAATCTTTTCCCTGTGGTCCCT
GCACCTGAGGCTAGAGGTCGCAAACTCCAGCGCGGGCGGGCCCGGGCGGGCCGGA
GGGTGGGGAGTGTGGCACATGGAAAC

Figure 3.1 Image of the forward and reverse primer (green) bound region and the corresponding KISS1R CRISPRCas9 KISS1R_C1 and KISS1R_C3 sgRNA target sites (yellow) and associated PAM (blue).

3.2 Cell Culture Imaging

Observations of changes in cell morphology and behaviour following treatment with gene editing tools such as CRISPR Cas9 RNP can suggest the existence of relationships between one or more genotypes and a specific set of phenotypic traits. As these experiments explore the relationship between KISS1R expression and its effect on cell viability, metastasis and drug resistance in MDA-MB-231 breast cancer cells, images of WT, D1 and D2 cell lines were collected to better elucidate the effect KISS1R expression has on cell behaviour, cell proliferation and cell viability

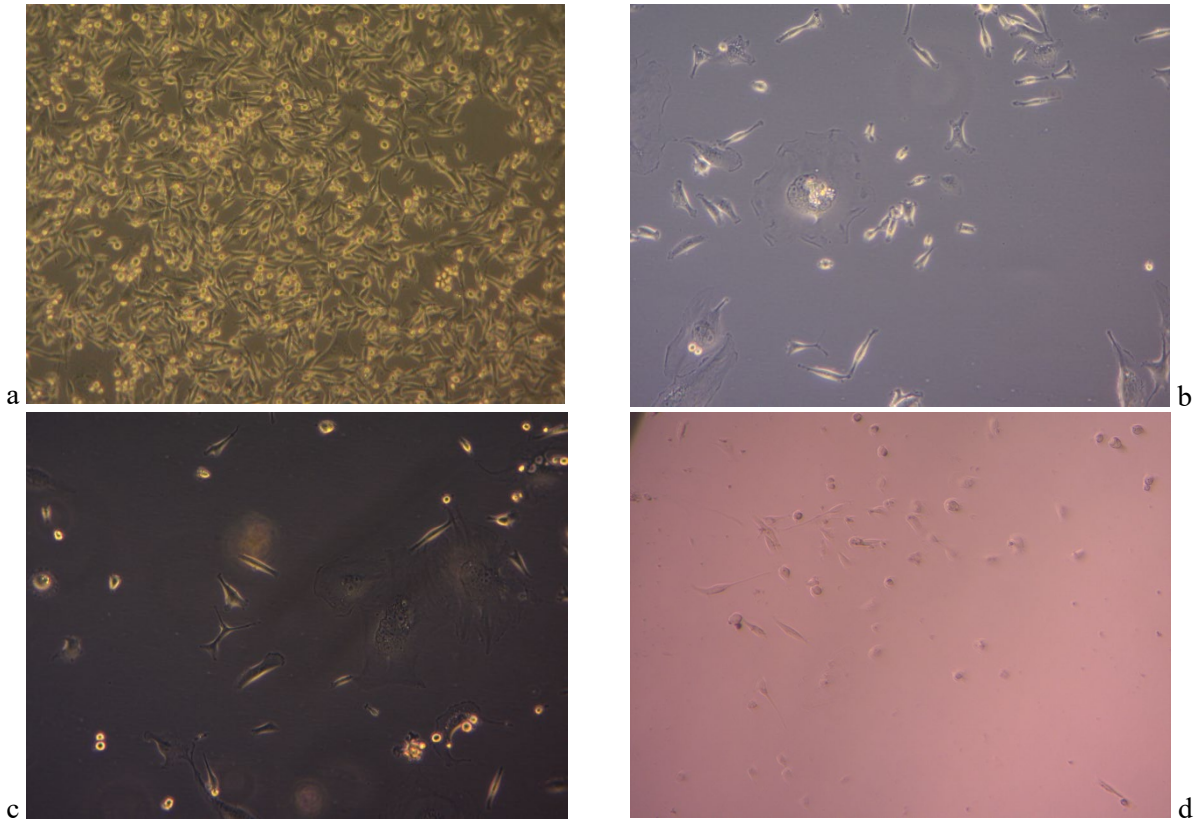


Figure 3.2 (a) An image of high-density culture of MDA-MB-231 wild type population at 72 hours. (b) An image of low-density culture of MDA-MB-231 mixing population knockouts for sgRNA 1 KO after 28 days. (c) An image of low-density culture of MDA-MB-231 mixing population knockouts for sgRNA 2 KO after 28 days (d) Image of expected KISS1R knockout line D1-3 in culture after 14 days of culture.

Figures 3.2b and 3.2c contain D1 and D2 mixed cultures, cultures which contain both WT and KO cells, and Figure 3.2d is an image of the isolated D1-3 culture. The reduction in cell density and the reduced rate of proliferation for both the mixed cultures and the expected homozygous knockout D1-3 cell line suggests the loss of KISS1R likely hampers cell growth and proliferation, however any noted changes in morphology and proliferation suggested by these images are indicative and necessitate further investigation. Figure 3.2a contains images of WT MDA-MB-231 and is used as a comparative control.

3.3 Analyses of Gene Editing Efficiency

The detection of a successful transformation event and the measurement of the transformation efficiency is essential in determining if the approach described in these experiments can have applications in a clinical setting. If the transformation efficiency is poor, this will likely reduce the benefit of the proposed therapy, and if there are any off-target effects, the possibility of complications, such as secondary neoplasms, is elevated. Three key strategies employed in the validation of CRISPR Cas9 mediated gene editing efficiency are sequence analysis, which allows for the direct assessment of the targeted sequence through the use of sequencing technology, enzyme mismatch cleavage, a method of detection that exploits the ability of a particular class of enzymes to detect and cleave mismatches in dsDNA to detect any sequence changes, and Western Blot Analysis, which is used to identify proteins based on size and charge separation and immunohybridization techniques.

3.3.1 Gel Image Analysis

The ThermoFisher GeneArt Genomic Cleavage Detection Kit was used to detect sequence changes in our D1 and D2 mixed lines. The ThermoFisher GeneArt Genomic Cleavage Detection Kit uses the T7 endonuclease 1 (T7E1), which cleaves dsDNA fragments at regions where a sequence mismatch between the two strands is detected by the endonuclease. A heterodimerization reaction of the crude PCR product was performed so as to randomly assort WT and mutant ssDNA into random dsDNA complexes by controlled fluctuations in temperature. The T7 endonuclease then acted to cleave mismatched dsDNA and the ratio of cleaved to uncleaved DNA fragments can be used to infer the efficiency of gene editing protocol. The following images are the results of several repetitions of these experiments.

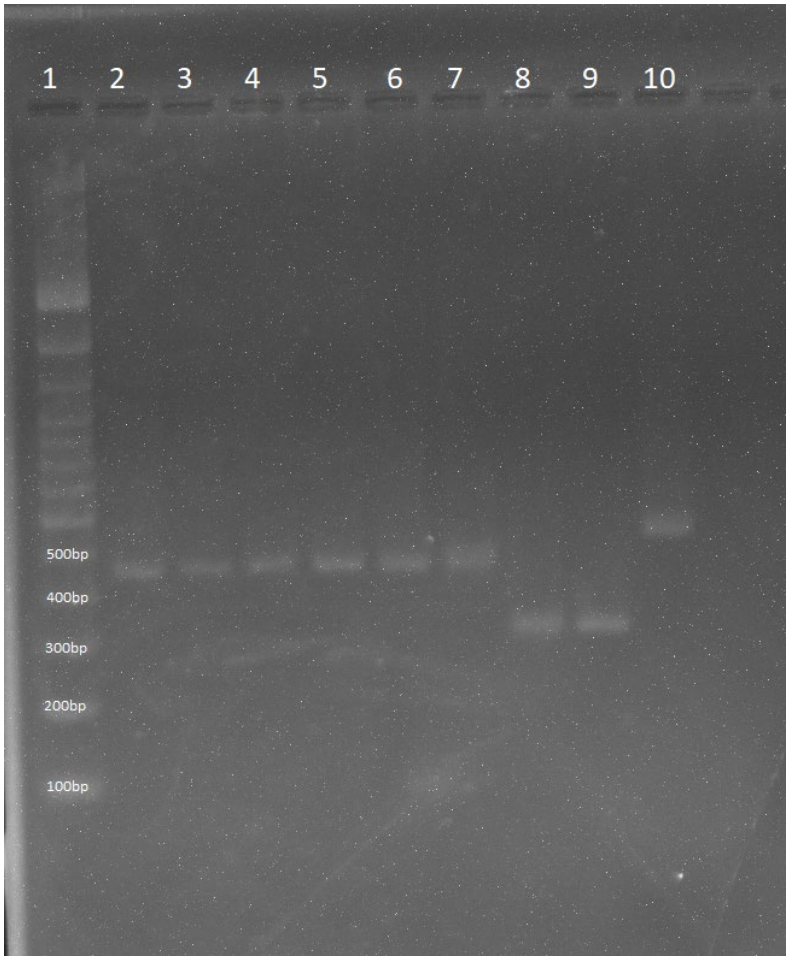
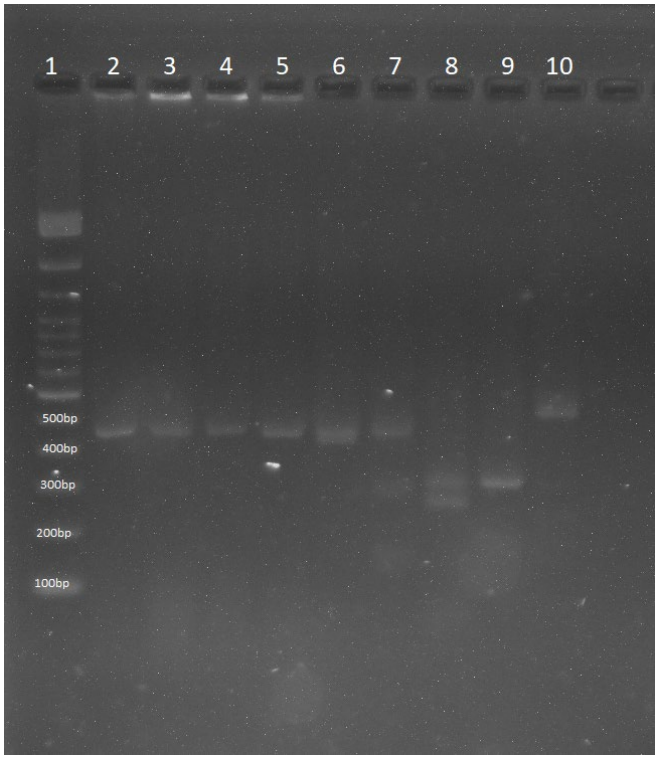
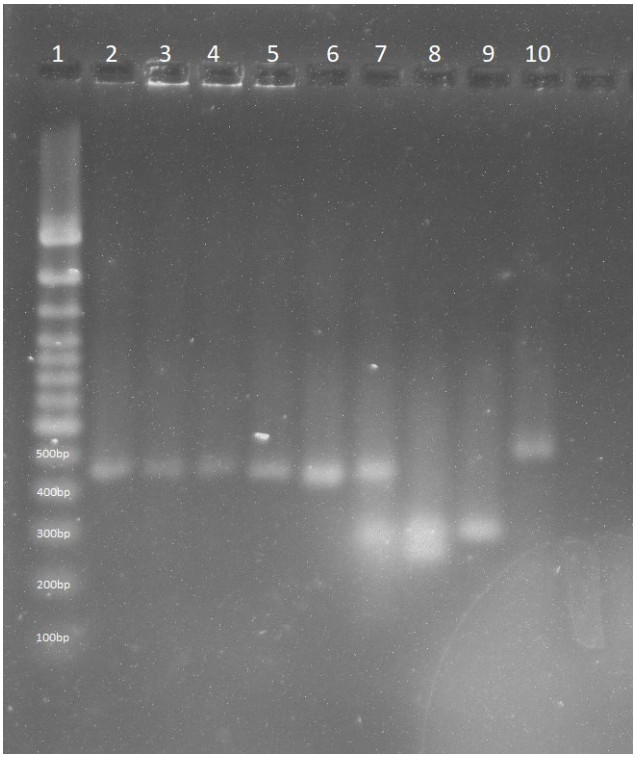


Figure 3.3 The first gel image is a control; untransformed cell lines are screened using identical protocol to the exposure groups. Lanes 2 – 7 are the product of sgRNA 1 forward and reverse primers used on wild type MDA-MB-231. Lanes 8 and 9 are the product of using sgRNA 2 primers on wild type MDA-MB-231. Lane 10 contains the positive control provided by Invitrogen. Lane 1 contains the 100bp DNA Ladder.

The gel in Figure 3.3 is a negative control. DNA was extracted from untransformed MDA-MB-231 cell cultures and underwent PCR using primer sets KISS1R_C3 (Lanes 2-7) and KISS1R_C1 (Lanes 8-9) and treatment using protocol outlined in Section 2.4.5. This GCD assay was performed for two purposes. First to determine if the experimental protocol and components used were performing as expected and there were no artefacts or unusual results. The results of this assay also act as a comparative control, allowing for us to easily visualize what the bands of an untransformed cell line look like, so transformed cell cultures can be more readily identified. The bands produced were all the expected sizes, 446 and 309, suggesting the GCD protocols were correct, adhered to and the reagents were sufficiently functioning.



a



b

Figure 3.4 Images of gels produced in the GCD assay of MDA-MB-231 breast cancer cell lines treated with CRISPR Cas9 ribonucleoprotein targeting KISS1R (a) from left to right, Lane 2 contains the wild type and uses the primer set sgRNA 1, used as a control, Lane 3 contains control 1 (no Cas9), lane 4 contains control 2 (no sgRNA), Lane 5 contains control 3 (no sgRNA or Cas9). Lanes 6 and 7 contain mutant strains D1 and D2 and use the sgRNA 2 set of primers Lanes 8 and 9 contain D1 and D2 but were screened using the sgRNA 1 set of primers. Lane 1 contains a 100bp DNA ladder. Lane 10 is loaded with the positive control provided by Invitrogen. This screen was repeated in (b).

Figures 3.4a and 3.4b are GCD assays of the expected transformant cell cultures D1 and D2, and relevant controls. Figures 3.4a, 3.4b use DNA extracted from the experimental controls including Control A (no sgRNA), Control B (no Cas9) and Control C (no sgRNA nor Cas9) in lanes 3, 4 and 5 respectively, and MDA-MB-231 cell cultures which have been treated with the KISS1R KO (D2) formulation in Lanes 6 and 7, and MDA-MB-231 cell cultures that have been treated with KISS1R KO (D1) formulation in Lanes 8 and 9. Lane 2 contains a sample of WT MDA-MB-231 DNA prepared for use as another control.

The presence of a successful transformation is marked by the production of a parental band and two daughter bands. The daughter bands indicate a sequence mismatch is present and thus a T7 endonuclease mediated cleavage has occurred, likely the result of an insertion or deletion mutation. Figure 3.1 provides predictions of the size of the parental and daughter fragments that we should expect if a successful KISS1R KO (D1) and KISS1R KO (D2) knockout is present. The expected fragment length of the parental bands for KISS1R KO (D1) and KISS1R KO (D2) is 307 bp and 446 bp respectively. The predicted lengths of daughter fragments in an effective KISS1R KO (D1) reaction are 261 bp and 46 bp and the predicted length of daughter fragments in an effective KISS1R KO (D2) reaction are approximately 296 bp and 150 bp.

Lane 2 (WT) 3, 4 and 5 (no sgRNA, no Cas9, no sgRNA nor Cas9) lack daughter bands, therefore we can confidently assume that there are no detectable mutations in these cultures. Lanes 6 and 7 contain the GCD products derived from strains D1 and D2 and use the KISS1R KO (D2) primer set. Lane 6 lacks daughter bands, which is expected, however, Lane 7 contains a parental band and two daughter bands. Lanes 8 and 9 contain GCD assay products derived from D1 and D2 and use the KISS1R KO (D1) primer set. Of these we see that the D1 strain, using the KISS1R KO (D1) primer set produces two daughter bands from one parental band in Lane 8. We can therefore assume D1 and D2 strains contain a successful knockout within the boundaries of their respective primer set.

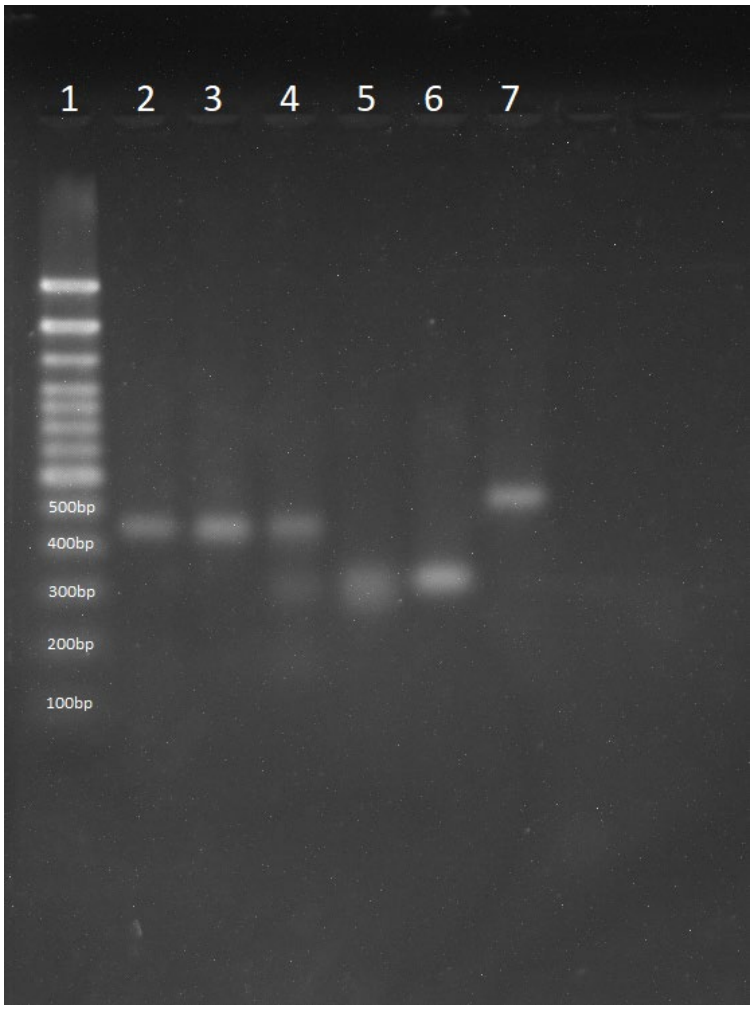


Figure 3.5 Gel image of Genomic Cleavage Detection Assay with only positive transformants and controls. PCR products derived from the WT in lane 2, and the D1 transformed lines and the sgRNA 2 primer set are in Lane 3. Lane 4 is from a D2 transformed line using the sgRNA 2 primer set, Lane 5 contains PCR products from the D1 transformed lines using the sgRNA 1 primer set, and Lane 6 contains the D2 transformed lines and used the sgRNA 1 primer set. Lane 1 contains a 100bp DNA ladder.

Figure 3.5 is a final repetition of the more significant elements of the GCD assay, which was done further validate the results of the previous two GCD assays. The gel image in Figure 3.5 contains GCD assay products derived from WT MDA-MB-231 and the KISS1R KO (D2) primer set in lane 2, PCR product from D1 and D2 transformed MDA-MB-231 cell lines and the sgRNA 2 primer set in Lane 3 and Lane 4 respectively, and Lanes 5 and 6 contain D1 and D2 transformed lines using the sgRNA1 primer set. Lane 6 contains the positive control provided by Thermofisher. Lane 1 contains a 100bp DNA ladder. The results of this assay are consistent with both previous assays, with daughter bands being produced in Lane

4, which contains the GCD assay product of D2 and the sgRNA2 primer set, and daughter bands being produced in Lane 5, which contains the GCD assay product of D1 and the sgRNA1 primer set.

We can conclude a mutant genotype was produced in D1 and D2 cultures as the presence and position of the daughter fragments support the occurrence of a cleavage event at the targeted sites, which is indicative of a sequence mismatch. Lane 7 of the imaged gels in Figures 3.4a, 3.4b produce daughter bands that are very distinct, and the location of the strand is consistent with the size estimations indicated in Figure 3.1. of approximately 296 bp and 150 bp for a positive KISS1R KO (D2) cell line and a predicted parental band of 307 bp and daughter bands of 260 bp and 46 bp in the KISS1R KO (D1) cell line, however GCD assays only imaged one daughter band of approximately 260 bp. It is likely second daughter band was lost or removed during electrophoresis due to its miniscule size. In total, three genomic cleavage detection assays were run, (Figures 3.4a, 3.4b, and 3.5). There was slight variation in the propagation of these fragments as well as variances in their intensity, and thus the assay was repeated. However, these replicate experiments produced nearly identical results.

Image J software was used to compare relative differences in intensities between parental and daughter bands, and these were used to approximate the cleavage fraction

These were calculated using the following formula:

Fraction Cleaved = sum of cleaved band intensities / (sum of the cleaved and parental band intensities)

All relevant cleavage fractions are recorded in percentages and recorded in Table 3.2

Cell Culture	Gel 3.3a	Gel 3.3b	Gel 3.4
D1	28.16	32.12	30.27
D2	18.03	20.19	19.15

Table 3.2 Fraction cleavage values of mixed cell lines D1 and D2 as determined by band intensity analysis using Image J

The mean value for the fraction cleaved for D1 and D2 populations are 30.18 ± 2.2% and 20.01 ± 1.22% (95% CI). The fraction cleaved was then used to calculate cleavage efficiencies using the following formula:

Cleavage Efficiency = 1– [(1–fraction cleaved) ½],

which was determined to be 0.5494 and 0.4472 for the KISS1R KO D1 and KISS1R KO D2 cell lines respectively.

While these experiments can be used to determine whether a transformation event has occurred and infer the efficiency of the techniques used, it does not provide any information on the nature of the transformation induced, thus it was unknown if homozygous knockouts were present in the mixing population.

3.3.2 TIDE Sequence Trace Decomposition Analysis

To more accurately validate the presence of a KO mutation, gauge the transformation efficiency of this particular transformation strategy and determine the nature of the CRISPR Cas9 RNP mediated KISS1R KO, some PCR product produced for use in the transformation efficiency analysis using ThermoFisher GeneArt Genomic Cleavage Detection system was aliquoted and sent for Sanger sequencing. The sequences produced were then aligned using TIDE Sequence Analysis. TIDE allows for the detection of mutations generated in of a mixed population of cells by genome editing tools such as CRISPR/Cas9. TIDE provides information on both the proportion of cells that have undergone at least a partial transformation and what particular transformations had occurred.

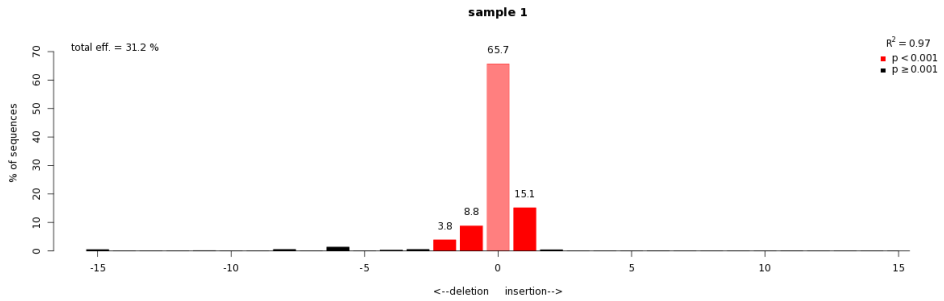


Figure 3.6 Graph describing the frequency of indel mutations at the expected DSB site. The opaque red bar in the centre represents the WT/non transformed cell population, the solid red bars to the left represent deletion mutations, and the bar on the right represents the number of insertion mutations ($p < 0.001$)

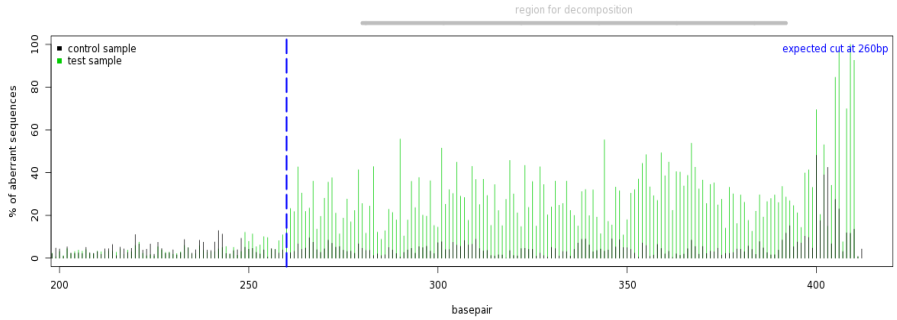


Figure 3.7 Graph illustrating the frequency of sequence mismatches between a WT sample and the D1 mixed cell culture. The blue line indicates the expected site of cleavage, and the green bars are indicative of a sequence mismatch between the WT control and the D1 mutant population.

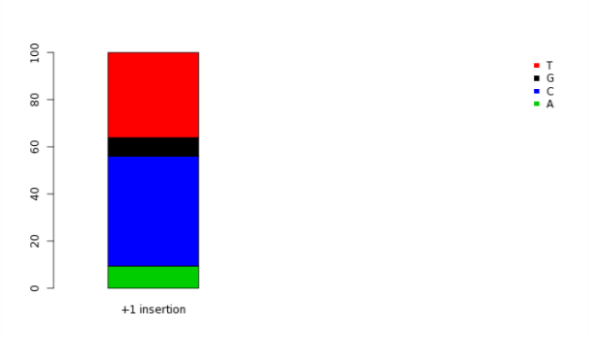


Figure 3.8 Bar graph describing the nature of insertion mutations induced by CRISPR Cas9 RNP transformation using sgRNA1. The colours represent different nucleotide identities, with red representing thiamine, black representing guanine, blue representing cytosine and green representing adenine.

Figure 3.7 details the location of the indel, at roughly 260 bp, which corresponds with the expected cleavage band size. Figure 3.6 provides details on the nature of the indel produced in these experiments. TIDE quantification of indel frequencies of D1 mixed populations suggests the overall transformation efficiency was 31.2 %, with 15.1% of these being insertion mutations, and Figure 3.8 provides an assessment on the nature of the insertion, suggesting the majority of the insertion mutations are cytosine and thiamine insertions.

3.4 Western Blot Analysis

Western Blot analysis was used to determine if any of the isolated strains have any recognizable expression of the KISS1R receptor. Western Blot analysis works by denaturing all proteins present in the cell tissue sample, separating them based on size and charge via gel electrophoresis, and labelling them using the highly selective binding affinity of antibodies. The results managed to identify the strain D1-3 as a strain that did not either express KISS1R or the mutations produced by the CRISPR Cas9 Lipofectamine system resulted in sufficient changes to the KISS1R molecule so as for it to no longer be recognized in the antibody assay.

Figure 3.9 Image of isolated D1-3 KISS1R line and heterozygous or mixed populations.

The Pierce™ Reversible Protein Stain Kit for PVDF Membranes was used on a newly produced PVDF membrane to validate both the quality of the samples being tested and to determine if the quantity of protein in the sample was sufficient for the sample to be trusted. The image suggests there is sufficient protein in the samples taken to suggest that any results from the antibody screening portion of this process are likely representative of the true nature of that cell line.

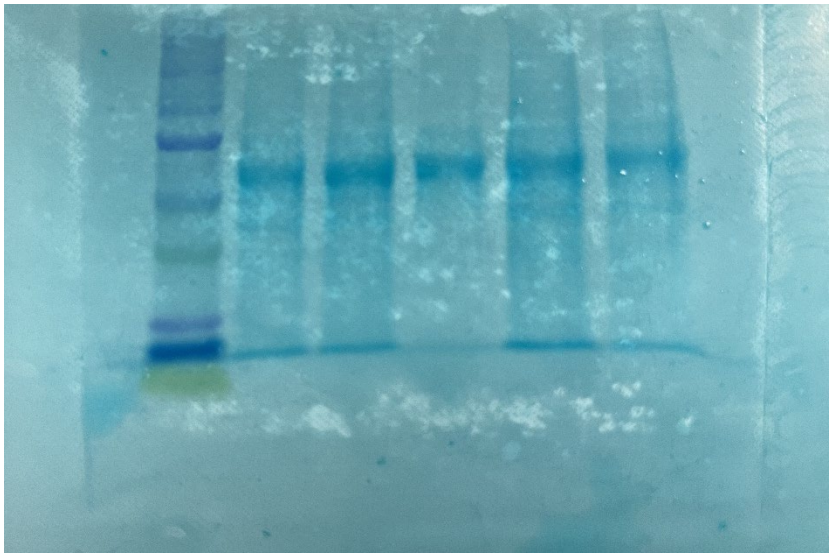


Figure 3.10 Image of PVDF Pierce™ Reversible Protein Stain Kit on PVDF with transferred protein. The first lane contains the prestained molecular weight ladder. The second lane contains wild type MDA-MB-231 protein extract, lane 3 contains the MDA_MB_ 231 KO line D1-2, lane 4 contains the MDA_MB_ 231 KO line D1-3, lane 5 contains the MDA_MB_ 231 KO line D1-4 and lane 6 contains the MDA_MB_ 231 KO line D1-5. Blue bands in lanes 2-6 are indicative of the presence and quantity of protein.

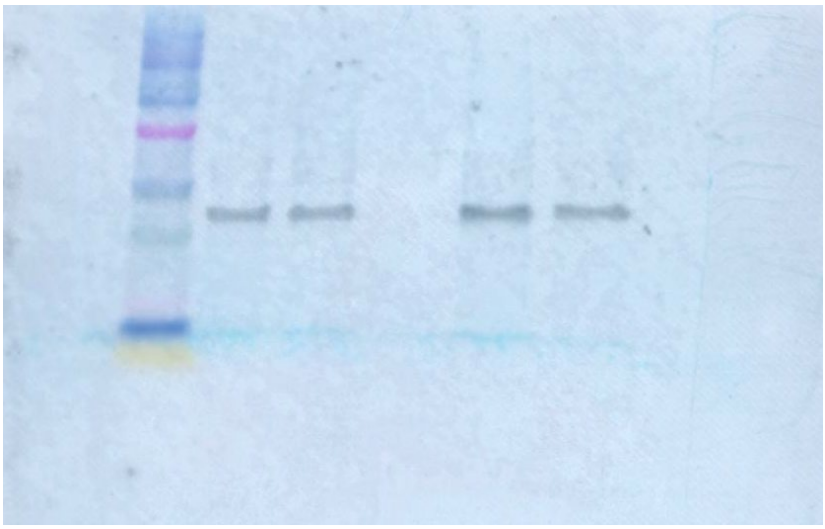


Figure 3.11 Results of the Western Blot. Protein has been transferred to PVDF membrane and stained using an anti GPR54 rabbit antibody as the primary antibody. The first lane contains the prestained molecular weight ladder. The second lane contains wild type MDA-MB-231 protein extract, lane 3 contains the MDA_MB_ 231 KO line D1-2, lane 4 contains the MDA_MB_ 231 KO line D1-3, lane 5 contains the MDA_MB_ 231 KO line D1-4 and lane 6 contains the MDA_MB_ 231 KO line D1-5. Black lines indicate the presence of KISS1R.

The antibody used in the screening of these samples was the anti GPR54 rabbit antibody, the secondary antibody used was the anti-rabbit antibody linked to the horseradish peroxidase enzyme for chemiluminescent detection of the protein of interest. The Western Blot Analysis results in Figure 3.11 show that the WT, D1-2, D1-4 and D1-5 mutants tested positive for the KISS1 receptor, with grey bands in the 50kDa range, however the mutant strain isolate D1-3 screened as a homozygous knock out for the KISS1R protein as the expected 50 kDa band is absent. It can therefore be assumed that the D1-3 cell line was a successful homozygous KO.

3.5 Cytotoxicity and Cell Viability in KISS1R KO MDA-MB-231 Cell Line

An MTT assay was performed according to the protocol outlined in Section 2.7 and the results obtained were as follows.

DOX conc (μM)	0	0.05	0.14	0.41	1.23	3.70	11.1	33.3	100
	1.7508	1.1123	0.4752	0.5003	0.5887	0.4573	0.3397	0.2462	0.2432
	1.9525	1.3760	0.5484	0.5710	0.6591	0.5212	0.3373	0.2527	0.2279
	1.8554	1.4633	0.6438	0.6977	0.6680	0.5376	0.3786	0.5623	0.2493
	0.5686	0.3787	0.3023	0.2879	0.2679	0.2855	0.2982	0.2789	0.2029
	0.5452	0.3715	0.3067	0.3187	0.3072	0.3195	0.3191	0.3136	0.2571
	0.5992	0.3118	0.2617	0.3347	0.2263	0.2417	0.3141	0.2784	0.1898

Table 3.3 Results of a 72-hr MTT analysis of cytotoxic effect of DOX on KISS1R KO mutant strain D1-3 (yellow) and a WT control (green). The numbers indicate detected absorbance values at 540 nm.

This data was analysed using the PRISM software suite and the results are as follows.

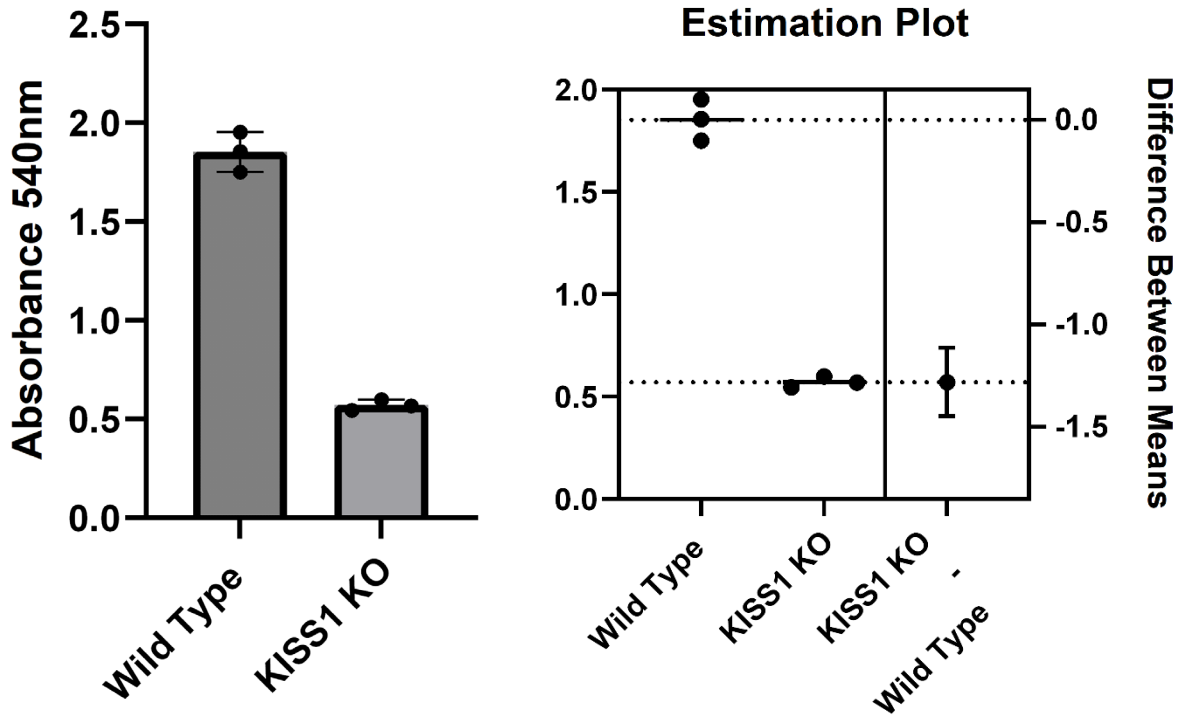


Figure 3.12 The results of an unpaired t-test used to compare the MTT results of an untreated WT MDA-MB-231 and untreated D1-3 KISS1 KO mutant MDA-MB-231. The graph on the left provides the estimated cell viability of both WT and the KISS1R KO strain D1-3. The dot plot describes the difference in means between WT and KISS1R KO MDA-MB-231 breast cancer cell lines ($p = < 0.0001$).

Figure 3.12 contains the results of an unpaired t-test comparing the MTT results of WT MDA-MB-231 and KISS1R KO mutant MDA-MB-231 labelled D1-3, both not treated with Doxorubicin. The graph is a comparison estimated cell viability, and the dot plot was used to represent the difference in means between WT and D1-3 breast cancer cell lines not treated with Doxorubicin. This comparison helps determine the effect a loss of KISS1R has on MDA-MB-231 breast cancer cells. Statistical analysis found the mean absorbance of WT MDA-MB-231 was 1.853 and the mean absorbance of D1-3 cell lines is 0.571, suggesting the loss of KISS1R produces a 3.25-fold reduction in absorbance ($p = < 0.0001$). This implies a significant reduction in cell viability when the KISS1R gene is inactivated, and thus a notable reduction in cancer cell proliferation rates and supports the postulate that KISS1R plays a notable role in the malignancy of TNBC and the CRISPR Cas9 RNP mediated knockout of KISS1R alone can be used to treat TNBC.

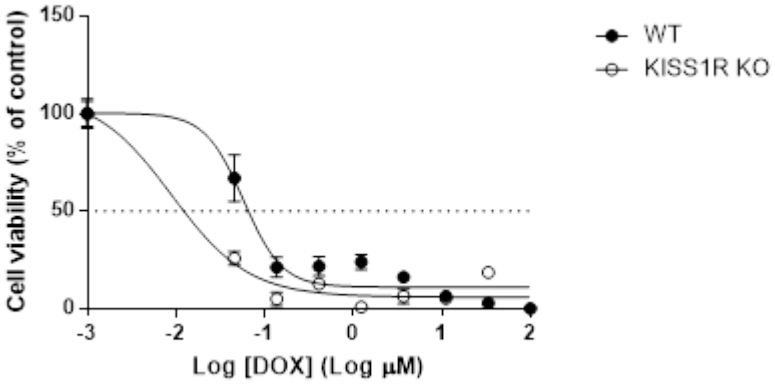


Figure 3.13 Concentration-dependent effect of Doxorubicin on cytotoxicity in wild type and KISS1R knockout MDA-MB-231 cells.

	WT	KISS1R-KO (D1-3)
IC50 (nM)	54	9
95% CI	33.83 to 86.11	4.420 to 18.14

Table 3.4 The IC50 values of the wild type and KISS1R knock out MDA-MB-231 (D1-3) cells from DOX induced toxicity

Furthermore, we compared the chemosensitivity of the KISS1R-/- MDA-MB-231 cell line D1-3 with the WT MDA_MB-231 cell line as shown in Figure 3.13 by exposing both to varying concentrations of Doxorubicin.

The concentration versus cell viability curves fit the sigmoidal model, both cell lines show an increase in cell viability at lower concentrations, and the change in viability is significant. There is a consistent decrease in cell viability as the concentration increases before cell viability begins to plateau.

D1-3 has an IC50 value of 9 nM while the WT cell line has an IC50 of 54 nM. This suggests a 6-fold reduction in IC50, which is indicative of an increase in chemosensitivity. This is a preliminary result; further studies may be required to further clarify the impact of a KISS1R deletion has on Doxorubicin cytotoxicity, because according to the research of Goertzen and colleagues (2016) and our own results, KISS1R inhibition alone already has a notable impact on rates of proliferation and invasiveness. It is likely the mechanisms by which chemosensitization occurs act synergistically with the antiproliferative effects of KISS1R deletion, likely exaggerating the effect KISS1R deletion has on drug efflux dynamics and will require more work to be better understood. Furthermore, while it is likely that the chemosensitization of the D1-3 line is the result of a KISS1R knockout associated reduction in the expression of BCRP, as KISS1R has been linked to reduced BCRP levels and a reduction in BCRP has been linked to the reduction in the IC50 of multiple antineoplastic drugs, analysis of BCRP expression and cellular drug retention in the D1-3 line is required to link KISS1R deletion and reduced BCRP expression in drug transportation dynamics in TNBC.

Of further note, multiple factors can affect the results of an MTT assay. One potential issue is the potential interference of Doxorubicin on MTT measurements, as Doxorubicin is a red pigment, and its residual presence in solution could affect colorimetric assays such as MTT. However, in our 72-hr MTT setup, DOX at 100 μM was not able to generate any significant absorbance values. Thus, the interference from DOX residue would be minimal in our MTT analysis. Other variables likely to affect MTT results include the age and the passage number of the cells. While this method is designed to measure cell viability, in actuality it measures the activity of mitochondrial oxidoreductases (Gutiérrez et al. 2017), which is used as a proxy for cell viability. The MTT is taken up through passive diffusion and is reduced by NADPH oxidoreductases and thus the activity of these oxidoreductases is used to infer cell viability and cell population density (Kuete et al., 2017). However, cell health and viability are believed to decrease as the passage number increases, and it is recommended cells with a high passage number are discarded. However, there are no recommendations for the range of optimal passages (Kwist et al., 2016). The age of and passage number of our cells needed to be taken into consideration, as the MTT was performed at a high passage the supposed decrease in cell health could affect the cells background levels of mitochondrial enzymes potentially altering the results of the assay. Two cell lines were used in this assay, wild type MDA-MB-231 cells and clone D1-3 knockout cells. Both cell lines were of a similar passage number this should reduce any inconsistencies that could have occurred between cell lines.

4. Chapter 4: Discussion

4.1 Introduction

Cancer constitutes a significant societal burden, and the prevalence of cancer is expected to increase due to various population trends such as an increasing and aging population as well as changes in factors linked to increasing urbanization (Torre et al., 2015). Breast Cancer is one of the most common cancers and has been stated by the WHO as the second leading cause of cancer related deaths worldwide, second only to gastrointestinal cancers.

Generally, the first line of treatment for breast cancer is surgical resection and radiotherapy. More recently, treatment of breast cancer has expanded to include chemotherapy and various targeted therapies. However, the likelihood that various treatment regimens would be successful depends on the nature of the cancer, the stage to which the cancer has progressed and whether the cancer cells bare hallmarks that allow them to be treated by existing therapies. Recent approaches involve using chemotherapy in combination with other systemic treatments to improve OS rates.

Triple negative breast cancers are a type of breast cancer that have three loss of function mutations that affect the behaviour of cancer cells, altering cell physiology, increasing invasiveness, metastasis and significantly reducing sensitivity to various treatments. TNBCs have lost two key hormonal receptors, the ERα and mPgR, and the HER2 receptor, and represent over 10% of all breast cancers. Triple negative breast cancers are usually more aggressive, are harder to treat and have a higher rate of recurrence than other breast cancer types. TNBCs can be cured if they are detected early, and the treatment is immediate and aggressive. However, if TNBC has already metastasized and spread to the peripheral tissues and organs, there is no known cure. The use of chemotherapeutic agents as adjuvant and neoadjuvant therapies has been shown to improve outcomes. One of the standard and preferred chemotherapeutic agents used in the treatment of TNBC is Doxorubicin. However, the progression of TNBCs is often linked with resistance to Doxorubicin and Doxorubicin-based chemotherapy regimens.

There are multiple traits that are believed to develop in breast cancer that promote resistance to Doxorubicin or modify the behaviour of the cell so that the dose required to achieve a perceivable therapeutic effect is increased. Previous sections have discussed the role the KISS1/KISS1R pathway has

in the behaviour of invasive TNBC cell lines. It is likely KISS1/KISS1R mechanisms associated with implantation and trophoblast regulation during pregnancy, and pubertal maturation, have been coopted in part by more advanced breast cancers, facilitating the growth of the tumour and spread of the tumour cells from their site(s) of origin. This is supported by multiple analyses of KISS1R expression in ERα- breast cancer cell lines, where KISS1R overexpression has been associated with invadopodia formation, the EMT and increased proliferation (Cvetkovic et al., 2013). Various analysis of KISS1/KISS1R suggests that KISS1/KISS1R overexpression in ERα- breast cancer cell lines trigger several cell responses, including in the transactivation of EFGR via β-arrestin2 and IQGAP1 associated mechanisms, which in turn promotes the expression of MMP 2/9, that facilitates the dissolution of the basement membrane and promote invasiveness (Guzman et al. 2019). β-arrestin and Gαq/11 both also activate ERK which increases the levels of cortactin, cofilin and MT1-MMP, promoting cellular remodelling and invadopodia formation. P63GEF is also activated, which promotes RhoA and further promotes metastasis (Cho et al., 2011). Further research also suggests increased KISS1/KISS1R signalling resulting from KISS1R overexpression promotes the EMT.

The overexpression of drug efflux transporters has also been linked to the KISS1 receptor and MDR in TNBCs. The role of ABC transporters in drug trafficking and the acquisition of drug resistance has been discussed in Section 1.7.2 (Girardin, 2006). The ABC family of transporters extrude structurally diverse small molecule compounds via the consumption of ATP. In cases of MDR in multiple cancer cell lines, a number of ABC transporters are overexpressed on the cell membrane and increase the rate of drug efflux. Research strongly indicated targeting ABC transporters can restore the levels of intracellular accumulation of chemotherapeutic agents in treatment resistant cell lines (e.g Caco-2, HepG2 and PANC1). Recent studies suggest that KISS1R overexpression increases levels of intracellular calcium, which is known to increase the expression of the ABC transporters such as P-gp (Chen et al 2023). KISS1R is also likely to trigger BCRP expression via AXL, however the mechanisms for this are not well understood (Blake et al. 2017).

It is reasonable to assume then that targeting KISS1R expression may have a provide a solution to chemotherapeutic resistance in TNBC. Targeting KISS1R via gene editing techniques such as the CRISPR-Cas9 RNP (Bhaya et al., 2011) would likely provide an efficient means of altering the genome of the host in a highly specific manner (Cong et al., 2013; Shi et al., 2020). The CRISPR Cas9 RNP system is composed of two major components, an sgRNA and a Cas9 nuclease. The Cas9 nuclease uses the guide RNA to locate the appropriate site to cleave the DNA (Saber et al., 2020). Once the Cas9 protein cleaves the DNA, causing a double strand break, one of two repair pathways will be initiated, NHEJ and HDR. To create knockouts, the NHEJ repair pathway is commonly exploited, as NHEJ is considered the most effective DNA repair mechanism exploited in the process of genetic editing (Acharya et al., 2020; Ghosh et al., 2019). NHEJ chemically ligates DSB together, however, the mechanism is highly error prone and results in the creation of indels and disrupting the gene of interest (Baliou et al., 2018; Ghosh et al., 2019; Menon & Povirk, 2016). The CRISPR-Cas9 system has been used to incorporate sequence errors in target genes, inhibiting tumour cell growth in multiple different cancer types, including breast, lung, liver, colorectal and prostate cancer by targeting oncogenes with relative success (Hazafa et al., 2020).

The aim of this study was to determine the effect the deletion of KISS1R has on the viability of the MDA-MB-231 breast cancer cell line and its responsiveness to treatment with Doxorubicin. The KISS1R gene is believed to affect a number of behaviours such as implantation and trophoblast migration in the developing embryo, and this control over proliferation, migration and differentiation is exploited in ERα- breast cancer cells. KISS1R overexpression has been linked to an increase in the rate of proliferation in TNBC cell line and recruitment of the ABC transporter ABCG2/BCRP to the cell membrane. Overexpression of KISS1R in TNBC results in increased proliferation and an increased efflux of Doxorubicin in a number of experiments. CRISPR-Cas9 knockout would likely reverse these behaviours, reducing the rate of proliferation and metastasis as well as improve the efficiency of DOX based therapies.

4.2 Summary of Findings

The results of this project showed that by transfecting MDA-MB-231 cells transformed with the CRISPR-Cas9 ribonucleoprotein with sgRNA sequences targeting the KISS1R gene, we were able to produce a cell line with a KISS1R knockout. We ran multiple assays to determine if the transfection was successful. A genomic cleavage detection assay was undertaken using the T7 endonuclease method and the fraction cleaved was 30.18 ± 2.2% and the cleavage efficiency was 54.94

in the MDA-MB-231 KISS1R KO cell line, D1-3 as determined by analysis with ImageJ. TIDE indel analysis suggests a 31.2% efficient transformation. Clones of the potential knockout cells were isolated and propagated and a Western Blot analysis was performed to verify the deletion of KISS1R from the WT cell line (Fig 3.7). The results showed that one of our clones, the D1-3 clone, either expresses a mutant form of the KISS1R receptor that is not recognized by the anti KISS1R antibody or fails to produce KISS1R entirely. The D1-3 strain was propagated and used in an MTT assay (Table 3.3). D1-3 and wild type cells were treated with varying concentrations of Doxorubicin and compared with each other and respective controls.

The difference in growth rates between WT and D1-3 control groups were used to infer differences in rates of proliferation. MTT analysis noted a 3.25-fold reduction in absorption, suggesting a notable reduction in cell proliferation and cell viability. Furthermore, detected changes in the IC50 (Table 3.4) suggest a decrease in cell viability at a much lower concentration for D1-3 when compared to wild type MDA-MB-231, with an IC50 value of 9 nM in D1-3 cells (KISS1R knockouts) and an IC50 value of 54 nM in wild type MDA-MB-231 cells. These results suggest that by using gene editing techniques to produce a nonfunctional KISS1R, rates of cell proliferation and drug resistance traits in cancer are reduced. In these experiments we have been able to make MDA-MB-231 cancer cells less malignant, less

proliferative and more susceptible to treatment with Doxorubicin, however, further research will need to be done to determine if this effect is not due to a hitherto unknown secondary effect, although this is less likely as, according to BLAST sequence analysis, the selected sgRNA did not have any matching sequences in H. sapiens genome. Sequence analysis further validates the results of the GCD assay, TIDE sequence analysis, and Western blot.

4.3 Gene Editing System for the Improvement of Cancer Treatment

Our results provide evidence that gene editing in combination with chemotherapy acts to limit TNBC cell viability significantly and reverses chemoresistance. The synergistic effects of both treatments will likely provide notable improvements in treatment over Doxorubicin-based chemotherapy alone. We can therefore assume that tumour growth and proliferation would likely be reduced in vivo. These results support the targeting of KISS1R using the CRISPR Cas9 RNP in breast cancer as a means of improving OS rate of TNBC. This is useful as TNBC has fewer treatment options than other types of invasive breast cancer, as TNBC lacks many of the targets that render most breast cancers susceptible to standard therapies such as endocrine therapy and targeted anti-HER2 therapies (Neilsen et al., 2013). Chemotherapy, surgical resection and radiotherapy are usually the only available treatments for non-metastatic and metastatic TNBC, although Sacituzumab govitecan-hziy was approved for use in 2023 by the US FDA and other treatments are also undergoing assessment and show promise.

For most with multidrug resistant TNBC, surgical resection and adjuvant radiotherapy is recommended at the earliest opportunity. For both resectable and non-resectable breast tumours, chemotherapy can be used to improve the survival rates of patients (Szakács et al., 2006). Anthracycline based combination

chemotherapy regimens, such as ACT, a treatment regimen which contains Doxorubicin, Cyclophosphamide and Docetaxel, given at 21-day intervals 6 – 8 times, is a standard regimen for both reducing the size of the tumour and targeting nodal metastasis. Historical studies involving the anthracycline Doxorubicin have shown a response rate of 40% at doses of ≥ 60 to 75 mg/m^2 and given every 3 weeks (Sakshi and Anampa, 2018). Doxorubicin is considered the most effective chemotherapeutic agent currently used to treat TNBC. A major limitation with Doxorubicin, as well as many other chemotherapeutic agents, is the dose limiting toxicity, with adverse effects such as cardiotoxicity and the development of secondary neoplasms resulting from excessive dosage. High doses of Doxorubicin, either as a monotherapy or as part of a regimen, are effective at eliminating breast cancer but cause adverse side effects, while low doses elicit a poor tumour response and result in acquired chemotherapeutic resistance (Li et al., 2015). Prolonged chemotherapy can also incur resistance (Li et al., 2015). By using gene editing we should be able to enhance the efficiency of the treatment by targeting genes, which confer chemoresistance and other anti-cancer traits to TNBC and could lead to actively killing cells at a lower dose or restore chemosensitivity while inhibiting proliferation. If we can lower the dose of chemotherapeutic agents required to achieve clearance, we should be able to reduce the levels of toxicity and improve the outcome of the cancer treatment. Furthermore, if we can revert chemoresistant cancers to a chemosensitive state, it will allow breast cancer patients to receive effective treatment for longer, ideally improving OSR and rates of clearance.

This is reliant on the identification of an appropriate target or set of targets. This series of experiments targeted KISS1R. KISS1R was an ideal target as it has been strongly associated with multiple traits associated with TNBC chemoresistance. In healthy individuals, the KISS1/KISS1R pathway governs several traits that contribute to the increased proliferation and metastasis of TNBC. KISS1R overexpression increases Gaq/11 signalling, activating PLC and p63RhoGEF, which increases intracellular calcium levels, activates PCK and induces cytoskeletal remodelling and invasion. KISS1R also transactivates EGFR, activates IQGAP1, β-arrestin2 and Fibulin 3 through non-G protein coupled

pathways, promoting invasion by the production of various MMPs and inducing invadopodia formation. KISS1R signalling also triggers the EMT transition, which reduces cell adhesion by lowering E-cadherin expression and promotes the transition of epithelial to mesenchymal cells as indicated by the increase in the intracellular concentration of mesenchymal markers and the formation of stress fibers (Guzman, 2019).

KISS1R also influences the expression of a key drug efflux transporter, BCRP, which plays an important role in detoxification via the transport of chemotherapeutic agents by increasing efflux of agents out of the cells (Chen et al., 2016). This is mediated via AXL, however the precise mechanisms of this are unclear. Previous studies had shown that certain cancer cells overexpressing KISS1R have increased mRNA levels of the BCRG, which was linked to the reduced accumulation of chemotherapeutic agents in cells.

We can be confident the D1-3 MDA-MB-231 cell line is a knockout cell line due to several key findings. The results of the genomic cleavage detection assay both detected the presence of an indel and was used to infer the cleavage efficiency of the CRISPR Cas9 mediated RNP transformation system using the sgRNA KISS1R C3. Multiple GCD assays suggest the D1 cell line contains an indel and the induction of a mismatch repair using this method is 54.94 % efficient, which suggests we successfully transfected approximately 54.94% of D1 cells. This is further supported by TIDE analysis of the D1 mixed line, which detected indel frequencies in the target site of approximately 31.2% ($p = 0.001$).

The successful KO of KISS1R in the D1-3 cell line is supported by the results of the Western Blot analysis, as the immunohybridization screening did not detect the KISS1R protein in the D1-3 strain. This is indicated by the absence of any banding in Lane 4 of Figure 3.10. This suggests we have produced a line of cells that are phenotypically distinct from the untransformed lines and does not produce the KISS1 receptor.

The effect of this KISS1R deletion was determined by MTT assay. Absorbance readings produced in the MTT assay were analyzed using GraphPad PRISM 10.3.1. Student t test results suggest the KISS1R deletion that occurred in D1-3 cell lines produced a 3.25-fold reduction in absorbance at 540 nm, absent treatment with DOX. This suggests the loss of KISS1R in D1-3 cell lines directly impacts cell proliferation rates and cell viability. These results align with existing research. Anti-KISS1R siRNA screening of TNBC demonstrated a reduction in invasiveness and cell viability when KISS1R expression levels were reduced in ERα– breast cancer cell lines.

DOX treated WT and D1-3 mutant strains were analyzed to determine the effects of KISS1R deletion on chemosensitivity. Nonlinear regression analysis was performed using GraphPad PRISM 10.3.1 and was used to identify the IC50 for both MDA-MB-231 WT and KISS1R KO strain D1-3. Analysis detected a remarkable 6-fold difference in IC50, with WT IC50 = 54 nM (95% CI; 33.83 to 86.11) and D1-3 IC50 = 9 nM (95% CI; 4.420 to 18.14) respectively.

4.4 CRISPR-Cas9 Efficiency

CRISPR-Cas9 has shown great therapeutic potential, however there are still many technical issues that prevent this technology from being more widely used (Kang et al., 2017), including the effect gene editing has on the fitness of edited cells, poor editing efficiency and the fact that transformation efficiencies less than 100% are likely to result in cancer recurrence without some form of combination therapy. This is in no small part due to inefficient delivery mechanisms, and it is unlikely that the current roster of delivery mechanisms will be able to achieve transformation efficiencies of 100% (Yahya and Alqadhi, 2021) There are also potential off target effects that can occur due to sequence similarity and random error (Chen et al., 2019). Larger genomes are likely to have DNA sequences that are identical or closely resemble a sequence of interest, this can result in non-specific cleavage, giving rise to mutations,

called off-target effects (Kang et al., 2017). Additionally, genetic variation between individuals and an increased rate of mutation common in many cancers is likely to result in DNA sequences that differ from the target sgRNA sequences somewhat, which may lower CRISPR-Cas9 RNP cleavage efficiency, and thus overall transformation efficiency.

The decision on what sgRNA to use was based on the results produced by the Thermofisher TrueDesign Genome Editor and the Chop Chop sgRNA Design Tool. The guide RNAs chosen were the two top scoring options suggested by Thermofisher. To ensure that the guide RNAs selected were suitable for use, a list of sgRNA suggestions were produced by the Chop Chop sgRNA design tool, which includes an estimate of the efficiency of transformation and the probability of off target effects. By comparing the sequences produced by Thermofisher TruDesign Genome Editor with the sequences produced by Chop Chop sgRNA Design Tool, we are able to estimate the off-target effects of the sgRNAs of interest. Chop Chop sgRNA Design Tool results indicated that the sequences we chose had 0 potential off target effects when using either sgRNA1 or sgRNA2. The top scoring Thermofisher designed sgRNAs matched those produced by the Chop Chop sgRNA Design Tool and the sgRNA selected had zero predicted off target effects. The efficiency of the sgRNA was predicted to be 65.84%, however, our analysis of efficiency of knockouts only showed to be 54.94% and the TIDE indel analysis suggests transformation efficiency was lower, at 31.2% ($p=0.001$).

We used more than one guide RNA on two sperate MDA-MB-231 cell cultures to improve the overall impact of the project as we would have not only been able to compare the efficiencies of multiple guide RNA but been able to see if the cell lines transfected with each guide RNA had varying cell viabilities. Unfortunately, we only managed to isolate one homozygous clone from either sgRNA transformation experiment. D2 cells lines proved more difficult to culture, which may indicate off target effects that affect transformant viability that were not detected by the Chop Chop sgRNA Design Tool.

The heterogeneity of cancer is also a source of concern. Sequence variation is significant between tumours in different patients but also between tumours at different stages within one patient (Chen et al.,

2019). This could suggest that although the sgRNA chosen for this project yielded good results in vitro, the diversity of the cancer genome means that the sgRNA may not be as effective in a clinical setting. This is an unfortunate limitation of the gene editing model in cancer therapy. Striking the correct balance between precision of sequence recognition and sufficient tolerance in sequence variation to lift the efficiency of transformation while also minimizing any off-target effects will likely remain a challenge

A genetic screen may have been a useful technique in these experiments, as we could test multiple sgRNA against one cell line and find the most effective sgRNA for that cell line. As discussed earlier the repair pathway chosen plays a significant role in the success of gene editing. For this study we chose to use the NHEJ repair method to produce our knockouts. This method is an error prone repair pathway that often produces indels after double strand breaks (Baliou et al., 2018; Ghosh et al., 2019; Menon & Povirk, 2016). This method of producing indels is active during all stages of the cell cycle, making CRISPR Cas9 RNP mediated editing flexible and efficient (Chen et al., 2019; Yin et al., 2016). We chose this repair pathway due to its efficiency compared to HDR. Strategies that utilize HDR are becoming more popular and can assist in improving the editing precision of the CRISPR-Cas9 system, which should increase the number of therapeutically effective edits per transformation event. However, studies have shown that the efficiency of HDR is low, due to the mechanisms required for HDR only being expressed in the G2 phase of the cell cycle (Hsu et al., 2014). The HDR pathway also requires a donor strand of DNA to contain sequences matching the flanking sequences on each side of the DSB, called homology arms (Baliou et al., 2018; Drost & Clevers, 2016; Ran et al., 2013). Therefore, high sequence homology for both generation of the cleavage event, but also for the binding of the homology arms to the liberated portions of the DNA fragment is required for successful HDR mediated transformation to occur (Yang et al., 2020). Attempts at improving HDR mediated transformation efficiency have produced promising results. Work by Devkota and colleagues (2018) suggests that by inhibiting the NHEJ pathway, HDR can be activated as a compensatory mechanism after the generation of a Cas9 mediated DSB (Devkota, 2018). A 2015 study looked at suppressing the NHEJ pathway so it cannot disrupt the HDR pathway. The key molecules they

looked into suppressing SCR7 which influences DNA ligase IV and KU70 expression, they also investigated the co-expression of adenovirus 4 E1B55K and E4orf6p proteins. Suppressing DNA ligase IV and KU70 showed a 5-fold increase in the efficiency of HDR. When expressed with the Cas9 system E1B55k and E4orf6, the efficiency of HDR mediated transformation increased 8-fold. Another approach to increase HDR is to use siRNA or shRNA to knockdown NHEJ effectors (Devkota, 2018). Robert et al (2015) used siRNA to target DNA-PKCs and DNA Ligase IV, the results showed that knockdown of the DNA ligase and DNA-PKCs improved HDR by approximately 3-fold while reducing NHEJ. With the ever-improving rate of CRISPR-Cas9 for producing knock ins and utilizing the HDR pathway a possible next step for this study would be to use the HDR pathway to insert one or more stop codons into our gene of interest.

There are a number of problems associated with the exploitation of the NHEJ pathway for the generation of an indel, including its ability to produce off target effects. Another notable weakness of this strategy is the changes made to the genome are random and so we cannot be certain of the nature of these without going through the extensive process of gene sequencing. Conversely, while the HDR pathway can produce precise changes to the genome, HDR has a much lower transfection rate than NHEJ. If we have the ability to increase the rate of transfection using the HDR pathway, the insertion of a stop codon in a protein coding region of a target gene can result in the premature termination of transcription, producing an incomplete and likely nonfunctional protein (Kato, 2019). The introduction of stop codons can produce a more reliable knockout/knock down effect than the generation of random indels. If it were feasible to introduce multiple stop codons into the KISS1R gene with higher efficiency, we could have a higher chance of producing a more effective knockout.

4.5 KISS1R, Invasiveness and the EMT

The KISS1/KISS1R is a very important pathway that is involved in a number of functions, including the initiation of puberty and the proliferation of the trophoblast during pregnancy. The KISS1R protein is activated by members of a family of short peptide hormones called kisspeptins, the most active of which is kisspeptin 10. In many cancers, kisspeptin expression acts to suppress malignancy and proliferation, usually by affecting expression of PP2A, which is an antimetastatic gene that suppresses invasive phenotypes. KISS1R also inhibits RhoA, PI3K and ERK, which all have well documented metastatic effects. However, the function of KISS1/KISS1R is tissue dependent. In the case of TNBC, the absence of an ERα receptor results in a notably different response than other tissues, with KISS1R overexpression being linked to increased migration and invasion in TNBC. The mechanism by which this occurs is triggered by the overexpression of KISS1R due to the loss of ERα, which results in excess KISS1R activation in breast cancer cell lines. This results in excess activation of B-arrestin 2, IQGAP1, ERK and p63GEF expression and activation, which promote the expression of MMPs, cortactin, cofilin and RhoA, and initiate invadopodia formation, increase migration and promote metastasis. KISS1R overexpression also triggers the EMT, which is hallmarked by the reduction in E-cadherin and an increase in the intracellular concentration of mesenchymal markers and stress fibers. By targeting KISS1R in TNBC, we expect a halt in the KISS1R signalling in effectively transformed cells, thus producing antiproliterative and antineoplastic effects in TNBC. The effects of KISS1R on cancer cell malignancy is one of the primary reasons why KISS1R was chosen as a target for therapeutic intervention. The reduction of KISS1R signalling in vivo results in the reduction in proliferation and spread of TNBC. This is supported by qRT PCR expression analysis of haploinsufficient murine models. Haploinsufficient mice experienced delays in the PyMT induction of breast cancer when compared to wild type controls. Our experiments support these results. GraphPad PRISM 10.3.1 was used to analyze control populations in our MTT experiments. A student t test analysis was performed on the untreated WT MDA-MB-231 and D1-3 KISS1R KO cell lines. Our KISS1R KO line D1-3 demonstrated a notable reduction in rates of cell proliferation. The mean difference in detected absorbance was roughly 3.25x, with the KISS1R KO line D1-3 having notably lower absorbance when compared with wild type MDA-MB-231. This difference in

absorption between WT and D1-3 cell lines implies a notable reduction in cell viability when KISS1R functionality is lost, aligning with previous research. This detected difference in means between WT and D1-3 control groups is also extremely useful as it suggests the targeting of KISS1R has therapeutic benefits independent of treatment with Doxorubicin.

These results are superior to many other previous tests due to the secondary effects associated with the use of other screening methods, such as siRNA and its tendency to produce off target effects, or experiments utilizing heterozygous KISS1R KO murine models, which provide an indication of the function of KISS1R in various cancers, but fail to appropriately model the effect an effective homozygous KISS1R KO would have in KISS1R overexpressing cancers.

4.6 KISS1R and BCRP-Mediated Active Transport of Doxorubicin

ABC transporters are ubiquitous membrane proteins that utilize ATP to transport various substrates across membranes (Hollenstein et al., 2007). Mutations in ABC transporters have contributed to a number of different disorders (Davidson & Chen, 2004), and several ABC transporters are notably overexpressed in treatment resistant cancer cell lines, including TNBC (Szakács et al., 2006). Due to the overexpression of ABC transporters in cancer cells, the intracellular accumulation of chemotherapeutic agents is reduced, meaning the requisite dose required to achieve cancer cell clearance is increased. This particular phenomenon has been identified in numerous MDR cancer cell lines, and thus the overexpression of ABC transporters has become one of the most widely recognized mechanisms of MDR (Fletcher et al., 2016). In the polarized cells of the colon, small intestine and the blood brain barrier, BCRP functions as an efflux pump, facilitating the unidirectional flow of substrates and contributing to the maintenance of the tissue's barrier function. In many tumour cell lines, BCRP is overexpressed, which contributes significantly to chemotherapeutic resistance. A 2021 study demonstrated the effect that BCRP levels have on

Doxorubicin sensitivity, with siRNA inhibition of BCRP reversing the Doxorubicin resistance phenotype and increasing the total amount of intracellular Doxorubicin accumulation in multiple breast cancer cell lines. Research suggests KISS1R governs the expression of BCRP, a drug efflux channel, via AXL, however the precise mechanisms governing this pathway are not well understood. The results of this study support the hypothesis that KISS1R likely plays a role in the acquisition of multidrug resistance to Doxorubicin (Kim et al., 2013). In Chapter 3, we show that by inducing an indel in the KISS1R gene, we are able to significantly alter the IC50 of Doxorubicin (Figure 3.4). By treating the D1-3 KISS1R KO and MDA-MB-231 breast cancer cell line with Doxorubicin and comparing the cell viability of the two strains, we were able to see a decrease in cell viability occur at much lower concentrations of Doxorubicin for KISS1R KO cell line D1-3 than MDA-MB-231 WT with an IC50 of WT = 54 nM (95% CI; 33.83 to 86.11) and D1-3 = 9 nM (95% CI; 4.420 to 18.14) (Figure 3.5). This suggests that the elevated efflux of Doxorubicin out of the cell was inhibited once KISS1R function was disrupted. This supports prior work that suggests that KISS1R overexpression in ERα – cancer cell lines promote chemoresistance.

4.7 Limitations of this Thesis

One of the major limitations of this study was the number of functional assays undertaken. While this study involved repeats of the genomic cleavage detection assay, MTT assay and Western Blot, it would have been beneficial to have high quality sequences to further validate the KISS1R KO. One round of genetic sequencing was performed, and the quality of the reads provided was not ideal.

The functional assays produced were useful in validating the presence of a deleterious mutation. By performing several genomic cleavage detection assays, we were able verify that some cells had undergone a DSB and incorporated a detectable change in their genetic sequence. This also allowed us to estimate the efficiency at which the cells were transfected, or the percentage of cells that may have a knockout

mutation. As discussed in chapter 3 the cleavage assay does not indicate what proportion of the knockouts induced by CRISPR-Cas9 RNP were heterozygous or homozygous. However, we were able to produce a Western Blot Analysis of an isolated cell line of D1 clones and compared them to wild type MDA-MB-231 cells and of the 70 viable clones that were produced in the dilution cloning process, the first 20 were screened via western blot and the D1-3 clone produced is likely a homozygous KISS1R knockout clone, based on the results of the Western Blot Analysis. An MTT assay was performed on the D1-3 line and allowed us to identify the effect that knocking out the KISS1R protein had on both MDA-MB-231 cell viability and sensitivity of MDA-MB-231 cells to Doxorubicin. Our results support those of a growing body of evidence which suggests KISS1R KO likely reduces MDR and cancer cell proliferation rates. However, all studies and assays have their weaknesses. To address the weaknesses of this study, resequencing of the D1-3 cell line is recommended. Confidence in initial results were low and resequencing may be required to better confirm the D1-3 cell line is an entirely homologous knockout. Additionally, the reduced viability of the D2 knockout line hampered propagation efforts of this particular KISS1R KO line, thus verification of the effects of KISS1R KO in these experiments are limited to the D1-3 line. Further attempts at propagating D2 cell lines, or the generation of cell lines using alternative sgRNAs is recommended.

A limitation of most in vitro studies is their applicability. In vitro studies are carried out in ideal conditions. Cells in this experiment were cultured in an optimum environment and thus the results here are unlikely to reflect how treatment would affect patients participating in clinical trials. Furthermore, transfection of cell tissue culture likely does not reflect in vivo transfection rates. It is likely that transformation efficiency would be notably poorer in human patients as the transfection process would be hindered by different elements in the body, including physical barriers, uneven systemic distribution of the liposomes or immune clearance. Another issue is in vitro studies only use a small sample of cancer cells, cells which are likely genetically very similar. Cancer heterogeneity may hamper transformation efficiency. Sequence variation even within a single tumour may result in very poor transfection efficiency

in comparison to in vitro studies. Thus, it is difficult to predict whether results produced in an in vitro study would have a similar impact in vivo or notably improve clinical outcomes.

Furthermore, recent studies suggest the relationship between KISS1R, proliferation and metastasis in TNBC may not be a simple cause and effect relationship. Recent research has found that the response of malignant breast cancer BT20 cell lines does not mirror the behaviour of most TNBC cancer cell lines and inoculation of BT20 TNBC with KISS10 has no notable effect on the invasiveness in BT20 Azubuike and colleagues (2022) suggest this may be the result of different complements in B-arrestin 1 and 2, however further investigation is needed. Additionally, little information was gleaned from this study that directly suggests the KISS1R/AXL/BCRP mechanism would be affected by KISS1R deletion, and experiments to determine expression levels of BCRP and that monitor drug retention in D1-3 cell lines are recommended.

4.7 Future Research

As animal trials are considered more indicative of potential therapeutic impact than in vitro tissue cultures, to further explore the utility of CRISPR Cas9 RNP in the treatment of TNBC, animal trials are recommended. Animal trials are useful in the identification of any off-target effects, such as neurotoxicity.

Relevant examples of the use of murine models in the assessment of potential cancer treatments include an in vivo study performed by Castillo-Rodriguez et al. (2014), which used the suicide gene therapy HSVtk on subcutaneous xenografts of MDA-MB-231 treated mice to model treatment of TNBC and achieved a 55% reduction in growth of the transformed tissue after a single treatment. Another animal trial that bears some similarity to our own is the functional CRISPR Cas9 mediated knockout of nuclear factor erythroid 2-related factor 2 (NFR2) in lung cancer cells. An NFR2 functional knockout

demonstrated increased sensitivity to various chemotherapeutic agents such as cisplatin and carboplatin (Bialk et al. 2018). Mice were implanted with MDA-MB-231 cancer cells to simulate tumour induction and growth, once a tumour was established mice were treated with various chemotherapeutic agents. They found that the combined treatment of NFR2 CRISPR Cas9 mediated KO slowed tumour growth and extended the survival of mice. Additionally, the therapeutic effects were shown to persist over time, with signs of complete arrest in tumour growth for the duration of the trial. Both of these studies are useful templates for the use of murine models in future research.

The work of Bialk et al. (2018) describes protocol for the implantation of tumour cells subcutaneously into mice ideally inducing TNBC cancer tumours. The mice would then need to be transfected using lipid encapsulated CRISPR-Cas9 RNP as we did with the tissue cultures in this study, in attempt to KO the KISS1R receptor protein in tumour cells within mice. To reduce the probability of off target effects, surface functionalization is a useful strategy whereby incorporating targeting ligands to the surface of the liposome improves selectivity (Zhang et al., 2018). This strategy, accompanied with intertumoural injection will likely sufficiently reduce the probability of off target effects. Once transfection is completed, the mice would be treated with Doxorubicin and tested in various aspects to understand if tumour growth was impacted. By using an animal model, we would be better able to simulate this procedure in humans with similar protocols in place, making the research more clinically relevant.

The results from our study provide evidence that KISS1R overexpression induces a malignant phenotype in MDA-MB-231 cells, and a deletion of the KISS1R gene significantly reduces rates of cell proliferation and malignancy and sensitizes cancer cells to treatment with Doxorubicin. This will likely have a notable impact on the future treatment of TNBCs.

If treatment response is poor in people who differentially express KISS1R and our data suggests KISS1R overexpression can negatively impact OSR, then screening KISS1R expression levels in breast tissue biopsies is recommended (Sukumar et al., 2021).

By utilizing the gene editing techniques developed as part of this research project, KISS1R KO in TNBCs may lower levels of KISS1R in patients, reducing the rate of cancer cell proliferation and the level of BCRP in transformed TNBC cells and lowering the amount of chemotherapeutic agent needed to treat TNBC. Lowering the dose of Doxorubicin required to achieve a therapeutic effect may further reduce toxicity in patients and lower the likelihood of Doxorubicin resistance. KISS1R KO will also hamper the progress of the tumour, further improving the likelihood of achieving a disease-free status in patients. It is likely this therapy may be employed both as a neoadjuvant therapy and as an adjuvant therapy in the treatment of TNBC.

The validation of KISS1Rs role as an MDR gene in TNBC, and other cancers where KISS1R overexpression leads to an increase in malignancy, provides a useful target for future therapies. The development non peptidic KISS1R antagonists for the reversal of drug resistance in TNBC was explored in the work of Scanlon (2022). His research found two of his prototype non peptidic antagonists showed reasonable affinity for the KISS1 receptor and one prototype antagonist demonstrated mild inhibitory effect in the MCF-7 cell line.

The development of a number of antibody-drug conjugates targeting cell surface receptors unique to advanced cancers has shown promise. The development of antibody-drug conjugates such as Sacituzumab govetican, Polatuzumab vedotin and Moxetumomab pasudotox all demonstrate clinical superiority when compared with equivalent targeted antibody therapy or chemotherapy alone. With the identification of KISS1R as a candidate drug target, the design of a KISS1R antibody-drug conjugate should be achievable as the anti KISS1R antibody structure is already known and a range of chemistries exist by which a chemotherapeutic agent could be reversibly linked to an antibody that have already been tested.

Improvements to the design of the prototype gene therapy developed in these experiments could also be explored. If HDR efficiency can be improved, HDR mediated insertion of one or a number of anticancer genes into the site of the KISS1R gene with the intent of further improving ROC, may be a useful strategy. Examples of candidate antineoplastic genes for HDR insertion into the cancer genome include

HSVtk, and cytosine deaminase, which both convert nontoxic precursors into chemotherapeutic agents as part of an anticancer strategy more commonly known as suicide gene therapy. Combining multiple anticancer strategies into one treatment may have potent synergistic effects.

The use of multiple guide RNAs in a single treatment may also be useful. The induction of multiple DSB events may have an increased likelihood of producing an indel and rendering KISS1R nonfunctional. The use of multiple sgRNAs may also improve the probability of a functional KO across multiple cell lines, which may improve clinical efficacy in vivo.

Further improvements include repeating experiments in multiple KISS1R overexpressing cancer cell lines. This may be used to infer this antineoplastic effect is consistent across a more diverse range of cancers and thus increase the number of clinical applications possible through this treatment. Experiments to further improve the utility of the prototype gene therapy developed in this project, the use of anti ERα endocrine therapies have been shown to induce KISS1R overexpression. Endocrine therapy may sensitize non-TNBC cell lines to the effects of a CRISPR Cas9 RNP KO of KISS1R. These dual therapies may have a synergistic effect.

4.7 Overall Conclusions

In summary, this thesis has demonstrated the use of the CRISPR-Cas9 RNP gene editing system can produce KISS1R knockouts in MDA-MB-231 cell lines and that the KISS1R KO has had a notable effect on cancer cell viability, with reduced cell viability in D1-3 clone lines when compared with WT MDA-MB-231. This is consistent with other studies, including KISS1R siRNA experiments on MDA-MB-231 cell lines. The loss of KISS1R also had a significant effect on Doxorubicin resistance, with sensitivity to the chemotherapeutic anthracycline Doxorubicin being increased. The KISS1R KO demonstrated reduced cell viability, reduced invasiveness, reduced migration and reduced proliferation, which aligns with

previous studies targeting KISS1R expression. The mechanisms that influence this behaviour in breast cancer cell lines overexpressing KISS1R are likely the transactivation of EFGR, the activation of P13K and protein kinase c associated pathways, as well as the initiation of the EMT. Recent research also suggests KISS1R KO affects BCRP expression, which is known to facilitate the efflux of anthracyclines such as Doxorubicin from the cell, impacting the long-term efficacy of Doxorubicin-based chemotherapy regimens. BCRP has also been associated with the maintenance of an undifferentiated stem cell like state in cancer. Targeting the KISS1R gene likely affects multiple mechanisms involved in the malignancy and MDR of TNBC, resulting in a reduced malignancy and the restoration of sensitivity to Doxorubicin in MDA-MB-231 cell lines. These hypotheses are supported by our MTT results, with KISS1R KO strain D1-3 having a generally reduced mean cell viability when compared with wild type MDA-MB-231, and the KISS1R KO strain D1-3 having a greater sensitivity to Doxorubicin than wild type MDA-MB-231. It is likely that a KISS1R KO, or therapies targeting the KISS1/KISS1R pathway present themselves as promising avenues for the treatment of TNBC.

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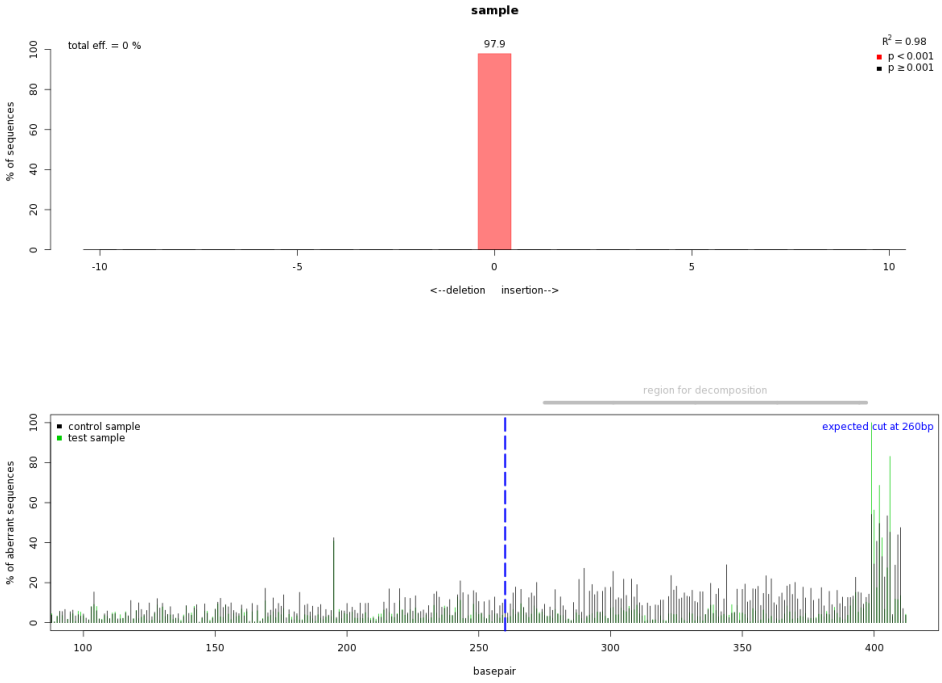
Supplementary Material

TIDE Quantitative Analysis of Indels of WT MDA-MB-231 as a Control



Default settings:

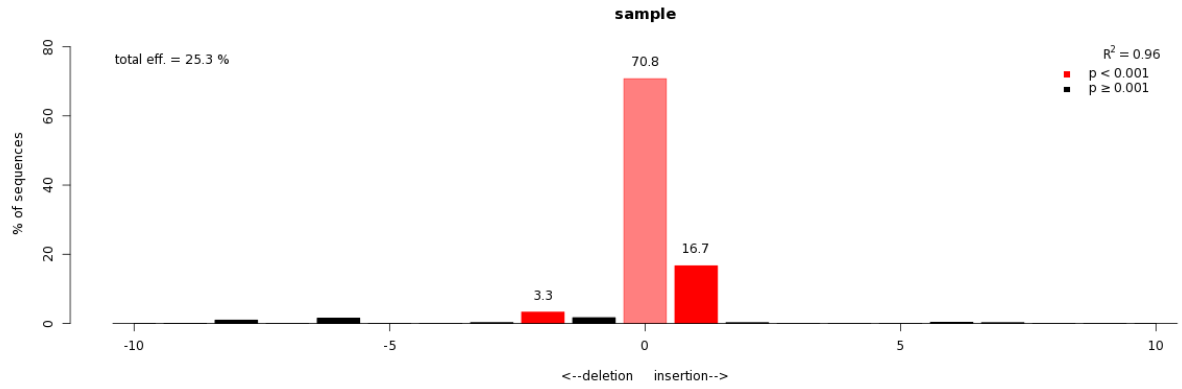
alignment window = 100 - 250

decomposition window = 275 - 397

indel size = 10

p threshold = 0.001

TIDE Quantitative Analysis of Indels of D2 Knock Out Line (Mixed Population)



Default settings:

alignment window = 100 - 250

decomposition window = 275 - 397

indel size = 10

p threshold = 0.001

A control Western Blot of WT MDA-MB-231 using an anti-GCPR 54 (anti-KISS1R) antibody.

DNA Extraction and Sequencing

To further validate the presence of a mutation, D1-3 and WT cells were and lysed and had their DNA extracted using the Qiagen DNeasy Extraction Kit and purified using a QiaQuick DNA Gel Extraction Kit for sequencing using the DNA Sequencing Services provided by Otago University.

DNA Extraction

Cells were cultured according to the protocol in Section 2.3.1 and cell pellets of D1-3 and WT MDA-MB-231 cell lines were immediately treated with Proteinase K and 180µl of ATL Buffer. The cell solutions were quickly vortexed and incubated in a heating block for 3 hours at 55 °C. The samples were removed from the heating block and 200µl of AL buffer was added to each sample and these were returned to the heating block and heated at 70 °C for a further 10 minutes. The samples were removed from the heating block and 200µl of 100% ethanol was then added to the samples. The resulting solution was added to the spin columns provided in the kit. These were centrifuged at 8000 rpm for 60 seconds and the liquid was discarded. 500µl of AW1 buffer was added to each of the columns, and these were centrifuged for 60 seconds at 8000 rpm, and again, the resultant flow through was discarded. 500µl of AW2 buffer was then added to the spin columns, the filter columns were returned to the centrifuge and the columns were centrifuged at 1300 rpm for 3 minutes and the flow through was disposed of. The columns were then placed in a 1.5 ml centrifuge tube and 200 µl of AE buffer was added to each column, and after one minute of incubation at room temperature, the columns were spun down for one minute at 8000 rpm. Another 200µl was added to the column and was left to incubate at room temperature for a minute and once again, the column was spun down at 8000 rpm for one minute. The supernatant was collected and cleaned for use in Sanger Sequencing via gel purification.

Gel DNA Purification and Sequencing Preparation

An 0.8% agarose gel was prepared using the protocol described in Section 2.4.6 and the products of the DNA extraction process were placed in separate wells. The gel was run for 30 mins at 50V then at 100V for 90 minutes using the protocol described in Section 2.4.6. Bands were visualized using the ImageQuant

LAS 500 gel docs UV image setting and were removed using a sterilized scalpel and placed in separate centrifuge tubes. The centrifuge tubes were weighed on a scale that had been zeroed with the weight of each respective centrifuge tube. To dissolve the gel, approximately 1µl of QG buffer per mg of gel was added to each vial. This was then incubated for 10 minutes at 55 ° C and vortexed as needed. The equivalent volume of isopropanol was added to each vial, which was then placed inside a collection column and spun at 1000 rpm for 60 seconds and the flow through was discarded. An additional 500µl of QG buffer was added, and the solution was centrifuged again. The column was then placed in a centrifuge tube and 50µl of EB buffer was added to elute the DNA. The flow through was collected for quantification.

The amount of DNA present in each sample was quantified using fluorometric quantification using a ThermoFisher Qubit dsDNA Kit and a QuBit Flurometer. Qubit™ dsDNA BR Reagent was combined with the Qubit™ dsDNA BR Buffer in a 1:200 ratio to produce a working solution. 10µl of each sample was then added to their own individual Qubit Tube, along with 190µl of working solution, vortexed, then allowed to rest at room temperature for 2 minutes. The fluorescence of each sample was measured.

Once the fluorescence of each sample was measured the concentration of DNA was calculated, and the D1 forward primer was added to one of the vials containing the D1-3 genomic DNA and the D2 reverse primer was added to the other D1-3 sample to produce samples with the recommended concentrations: 3.2 pmol/5µl for primers and 1ng/100bp/5µl for template DNA. Premixed samples were then sent to the DNA Sequencing Service at the University of Otago for analysis.

DNA Sequencing Results

Sanger sequencing was used to determine the genetic sequence of the potential mutant strain D1-3. The sequencing results were aligned with a WT control using the BLAST sequence alignment tool. This was used to validate the presence of a mutation and determine the nature of the mutation.

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3. Query  9     TGGGGGGAGATGACGTASATGACCAGCGAGTTTCC-ACCAKGCCCAGYAGCATCAGCGCC  67
4.              ||| || |||||||||| |||||||||||||| || |||| |||||| ||||||||||||
5. Sbjct  5370  TGGCGGCAGATGACGTAGATGACCAGCGAGTTCCCCACCAGGCCCAGCAGCATCAGCGCC  5311
6.
7. Query  68    GCraasaakaGCGGCACGAGCCAGGCGTCCACGGCCCGCGGCGAAGGGACTGGGCCGTCC  127
8.              || || || ||||||||||||||||||||||||||||||||||||||||||||||||||
9. Sbjct  5310  GCGAAGAAGAGCGGCACGAGCCAGGCGTCCACGGCCCGCGGCGAAGGGACTGGGCCGTCC  5251
10.
11. Query  128   GAGGCGKTGGCGCCACRSCCCGGGCAASGGGAGGCGTTGGGCGGAGCCCCCCRSGACCCT  187
12.             |||||| |||||||||  ||||||||   |||||||||||| ||| |||||||  ||| |
13. Sbjct  5250  GAGGCGTTGGCGCCACAGCCCGGGCAGCCGGAGGCGTTGGCCGGTGCCCCCCAGGACGCG 5191
14.
15. Query  188   TTGGGTCCGGACTTAGCCACGGKGTGCATGGYCGCGCGCACCTCCTCCTCCCGGCCGCCC  247
16.             |||||||||||| ||||||||| |||||||| ||||||||||||||||||||||||||||
17. Sbjct  5190  TTGGGTCCGGACGTAGCCACGGTGTGCATGGCCGCGCGCACCTCCTCCTCCCGGCCGCCC 5131
18.
19. Query  248   TCCASGATTGTGCCCTGGCACCGCCCCGGGGACCCCTCCSCCCGACCGCCCGGGGGCTGC  307
20.             |||| ||||| |||||||||||||||||||||||||| ||||||||||||||||||||
21. Sbjct  5130  TCCAGGATTGCGCCCTGGCACCGCCCCGGGGACCCCTCCGCCCGACCGCCCGGGGGCTGC 5071
22.
23. Query  308   GCCTGTGGAACTCAGGAAGGGGCTCGGCTGGGGTCCGCAGGGTCCAGAGGGCAGSTGTGC  367
24.             |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
25. Sbjct  5070  GCCTGTGGAACTCAGGAAGGGGCTCGGCTGGGGTCCGCAGGGTCCAGAGGGCAGCTGTGC 5011
26.
27. Query  368   TGGGCTCGGCGGCTCCTCTGCCGGCGCCGCTGCGGSCGARCGTTTATASCCCGTGC  423
28.             |||||||||||||||||||||||||||||||| ||| |||||||| |||||||
29. Sbjct  5010  TGGGCTCGGCGGCTCCTCTGCCGGCGCCGCTGCGGCCGAGCGTTTATAGCCCGTGC  4955
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The results of a sequence alignment between a WT MDA_MB_231 (Sbjct) and the sequencing results produced from the Section 2.8 sequencing of the cell line labelled D1-3 (Query). Sequences highlighted in green are primers.

The K2 Reverse read produced a relatively high quality read of a portion of the KISS1R C1 amplicon, however, it does not contain the target sequence as the read terminated prematurely. Furthermore, the quality of the K1 Reverse read of the KISS1R C3 amplicon is poor and not likely useful in the identification of any indels with any degree of reliability.