INVESTIGATING THE ROLE OF THE MUTANT ESTROGEN RECEPTOR IN MAMMARY GLAND DEVELOPMENT AND TUMORIGENESIS

By

Michaela Jane Moore

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Master of Science

Department of Biochemistry

Goodman Cancer Research Centre

McGill University

Montreal QC, Canada

August 2020

Table of Contents

ABSTRACT

Hormone receptor-positive breast cancer accounts for over 70% of breast cancer diagnoses worldwide. When diagnosed early, the prognosis for patients with estrogen receptor-positive breast cancer is very good and there are several treatment options that have been developed over the last fifty years. However, this disease presents us with a challenge: resistance to endocrine therapy occurs in roughly 25% of cases and recurrence of the tumour or of secondary tumours is common. Mutations in the estrogen receptor are found in endocrine resistant metastatic breast cancer, but rarely in primary tumours. The mechanisms underlying endocrine resistance have not been fully explored and are still overall poorly understood. Because of this, we generated the first immunocompetent mouse model that expresses one of the most common mutations that occurs in human metastatic ER+ breast cancer: $ESR1^{Y537S}$ ($ESR1^{Y541S}$ in mice). We have shown that a full-body knock-in of this point-activated mutant results in a dramatic phenotype, but mammary epithelium-specific expression does not seem to affect the development of the ductal tree in virgin mice or during reproductive development. Ovariectomy of these mice prior to puberty inhibits the development of the mammary gland, however, in the full-body context, a ductal tree appears in 100% of cases. Expression of $ESR1^{Y541S}$ in a PI3K-driven model of breast cancer also does not affect onset or tumour growth overall but may affect metastasis and signalling in later stages or in secondary tumours. This remains to be investigated.

RÉSUMÉ

Les cancers du sein à récepteurs hormonaux positifs représentent 70% des cancers du sein à travers le monde. Un diagnostique précoce pour un cancer du sein positif aux récepteurs d'oestrogènes permet généralement un pronostique favorable puisque plusieurs options de traitements ont étés développées depuis les 50 dernières années. Par contre, cette maladie représente encore un défi pour la recherche puisqu'une résistance à l'hormonothérapie se développe dans 25% des cas et la récurrence de la tumeur au site originel ou secondaire est fréquente. Plusieurs mutations dans le récepteur oestrogen ont été identifiées dans les sites de métastase des femmes atteintes d'un cancer du sein résistant à l'hormonothérapie, mais très rarement dans les tumeurs primaires. Les mécanismes sous-jacents cette résistance n'ont pas été pleinement explorés et demeurent toujours peu compris. Pour cette raison, nous avons généré le premier modèle de souris immunocompétente qui exprime une des mutations les plus communes qui se retrouve dans les métastases des cancers du sein ER+ : ESR1^{Y537S} (ESR1^{Y541S} chez la souris). Nous avons montré que l'expression de cette mutation dans le génome entier d'une souris provoque un phénotype sévère, mais que l'expression spécifique de cette même mutation dans les cellules épithéliales mammaires des souris n'affecte pas le développement des canaux mammaires des femelles vierges ni même durant le développement reproducteur. L'overactomie de ces souris avant la puberté inbibe le développement des glandes mammaires, néamoins, dans le contexte de l'expression dans le génome entier, le développement des glandes mammaires se produit dans 100% des cas. L'expression dans les glandes mammaires de la mutation ESR1^{Y541S} dans un model de cancer de souris exprimant l'antigène T moyen du polyomavirus n'affecte pas non plus le temps de formation des tumeurs ni la croissance de celles-ci, mais pourrait affecter les métastases et les voies de signalisation à un stage plus avancé ou dans les tumeurs secondaires. Ces éléments restent à explorer.

ACKNOWLEDGMENTS

I would like to thank Dr. William Muller for offering me a position in the lab and for giving me an interesting project that lined up with my interest in breast cancer research. Thank you for providing invaluable insight and advice throughout the two years I spent here. Additionally, I would like to thank my Research Advisory Committee, Dr. Vincent Giguère and Dr. Luke McCaffrey for their support and direction during my graduate studies.

Thank you to my boyfriend, Daniel Moehrle, for always supporting me and my decisions. Thank you for being wonderful even when I wasn't, especially when life and lab all got to be a little too much for me to handle. Thank for you always encouraging me and believing I could do whatever I put my mind to, even when I didn't believe in myself.

Thank you to my family for encouraging me throughout my education and for continuing to support me when I moved away to pursue graduate studies.

I would like to thank my Grade 12 Biology teacher, Mr. Darren Yip, for igniting my passion for science and molecular biology.

I would also like to acknowledge the first mentor I ever had, Khalid Al-Zahrani. You taught me how to think like a scientist and how to ask the right questions. You also encouraged me, had tremendous faith in me, and made me feel like I was cut out for the lab and for graduate school, and for that I think I will be forever grateful. You are a wonderful mentor and anyone that has the privilege to work closely with you is very lucky.

I would also like to acknowledge the members of the Muller lab for making the last two years easier than they would have been otherwise. Thank you for the advice when I didn't know what to do next, for the technical help, and emotional support. I would like to thank Virginie for all her help and time she took establishing my cell lines with me, and for teaching me how to do most of the techniques included in this thesis. Thank you for taking on my work when COVID forced us out of the lab. Thank you to Cynthia for her extensive help and advice in the mouse room and for always being willing to listen (and for doing my Cre PCRs when I couldn't make them work for weeks at a time). Thank you to Sherif for always making me think a little bit harder and more critically about things, both science-related and not. And last, but definitely not least, thank you to Ippy for being so open and willing to welcome me into the lab and your life,

for your friendship, your support, and for making me laugh daily. Thank you for always listening and being someone I could lean on (heavily!) for the last two years. Grad school would not have been nearly the same without you.

PREFACE AND CONTRIBUTION OF OTHERS

I hereby state that I carried out the work presented in this thesis unless stated otherwise, analyzed the data, and wrote this thesis. I produced the figures presented. Alexandra Simond characterized the ESR1^{Y541S}/β-actin Cre strain and produced the mice and tissue samples used for the ESR1^{Y541S}/MTB/MIC Western blots. Cynthia Lavoie performed necropsies during the COVID-19 shutdown. Virginie Sanguin-Gendreau performed the invasion assays (data not included). Ipshita Nandi helped establish and maintain the cell lines. Dr. William Muller reviewed and provided feedback on this thesis.

I feel as though it is necessary for me to state here that my research was heavily disrupted by COVID-19. There are instances where there are gaps in my work and times where I will mention what I had been intending to do had I not been prohibited from working for the last 6 months of my degree.

List of figures

List of tables

LIST OF ABBREVIATIONS

ACK	ammonium-chloride-potassium
BPE	Bovine pituitary extract
DBD	DNA binding domain
DCIS	ductal carcinoma *in situ*
ddH_2O	deionized distilled water
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial-to-mesenchymal transition
ERα, ESR1	Estrogen receptor alpha
ERE	Estrogen response element
EtOH	Ethanol
FBS	fetal bovine serum
GEMM	Genetically engineered mouse model
H&E	Hematoxylin and eosin
IDC	Invasive ductal carcinoma
LBD	Ligand binding domain
MMTV	Mouse mammary tumour virus
PyVmT, MT	Polyoma virus middle T antigen
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
SQ	Single quot

1. LITERATURE REVIEW

1.1 Breast cancer

1.1.1 Breast cancer epidemiology

Apart from non-melanoma skin cancers, breast cancer is the most common cancer diagnosed among women. Roughly 1 in 8 women will be affected by the disease in her lifetime, and 1 in 33 dying from breast cancer. According to the Canadian cancer society, breast cancer will represent roughly 25% of all new cancer diagnoses in women in 2020, as well as 13% of all cancer-related deaths. This implies that roughly 27 000 Canadian women will be diagnosed with breast cancer this year, and over 5000 women will die from the disease. The five-year survival rate for breast cancer is relatively high and increasing every year (~88% currently)[1], as research has revealed many key molecular mechanisms behind mammary tumorigenesis and many targets have been identified. Many new detection and diagnosis technologies and breast cancer treatments have been discovered in the last 30 years. However, the decline of the breast cancer death rate is slowing for breast cancer[2], and there is still a need for continuing research and development of new therapies to close the gap that remains. Factors that are responsible for most breast cancer deaths include metastasis, cancer reoccurrence, and development of therapy resistance. Much remains to be discovered to help us effectively treat this disease, as breast cancer is a hugely heterogenous, complex pathology.

1.1.2 Mammary gland development and structure

For us to thoroughly understand breast cancer and all the complexities associated with it, it is essential to first understand the normal state and structure of the human (and in the case of this thesis, the mouse) mammary gland. It functions in mammals to produce and secrete milk for newborn offspring[3]. This provides not only nourishment, but also plays a protective role in the infant's digestive system, a role in passing immunity on to the infant, and promotes bonding between mother and child[4].

The human mammary gland is composed of a glandular tissue surrounded by adipose and connective tissues. The glandular tissue is composed of lobes, which are then further divided into smaller lobules that contain alveoli. A huge variability exists in the size and arrangement of lobes in the human breast. They can be arranged in a radial fashion or intertwined and twisted around each other[3]. The alveoli are the specific tissue where milk is produced during lactation and they form during pregnancy. The glandular tissue is secretory and is filled with a ductal network. During lactation, these ducts (alveoli) produce milk and they transport the milk to the nipple[3].

From birth and puberty to pregnancy, lactation and involution, the mammary gland undergoes extensive physiological remodeling and experiences drastic changes in its gene expression profile[3]. Mammary gland development technically begins embryonically, although the most drastic changes occur during puberty, pregnancy, lactation, and involution. This tissue retains such a plastic phenotype up into adulthood contrasting with most other adult tissues that are relatively static. Puberty is marked by hormonal changes that drive rapid breast growth and development in humans. Ovulation and the establishment of a regular menstrual cycle is associated with the establishment of additional adipose tissue in the breast, and this is what generally contributes to the increase in breast size. Other changes that occur during puberty include the elongation of immature ducts, as well as increased branching into secondary ducts [3]. This ductal development continues throughout a woman's life, with some reports claiming ductal branching is not complete until age 35 or older[3]. Pregnancy is the physiological event that triggers the most significant remodeling of the human breast. Increased circulating levels of hormones (estrogen, progesterone, prolactin) trigger increased ductal growth and branching, as well as formation of alveoli to produce milk after giving birth. During involution, there is rapid regression of the breast tissue as the alveolar cells are cleared from the mammary gland. This results in a non-lactating, mature, mammary gland that can remodel itself many times over as needed.

Not only is the mammary gland a dynamic structure, but it is also a heterogenous tissue composed of many different cell types. Epithelial cells and stromal cells make up the mammary gland. The epithelial ductal cells of the mammary gland are composed of luminal cells (secretory) and myoepithelial basal cells[5], which arise from the differentiation of mammary stem cells[3]. As their name suggests, luminal cells surround a hollow lumen in the ducts. The basal

cells are contractile and form a network around the base of the luminal cells. Their contractility supports milk let down in lactation[5]. These cells are then surrounded by a basement membrane. Other supporting cells make up the surrounding stroma; adipocytes, fibroblasts, immune cells, lymphatic cells, and vascular cells.

1.1.3 Breast cancer development and progression

Normally, the cells that make up our body are tightly regulated by various gene programs that control how they behave, how often and when they divide, as well as how many times they can divide before they undergo apoptosis. Cancer occurs when these processes become disturbed due to genetic alterations, or mutations. Mutations can either be inherited (germline mutations) or they can occur spontaneously in various cell types after birth (somatic mutations) due to DNA damage. Mutations that ultimately result in cancer can cause effects like uncontrolled cell division, evasion of apoptosis, cellular immortality, dysregulated metabolism, and the capability to degrade the extracellular matrix, migrate, and invade surrounding tissues[6]. Other factors, such as epigenetic modifications of DNA (e.g. histone modification, DNA methylation, etc.), can also contribute to the develop of cancers, including breast cancer[7]. Any change that increases the fitness of a cell can lead to the development of cancer, if that cell and its progeny remain unchecked. There are many risk factors associated with the development of breast cancer, such as exposure to environmental chemicals or radiation, as well as a woman's history with certain medications and whether she has had a child (and at what age) or not[8].

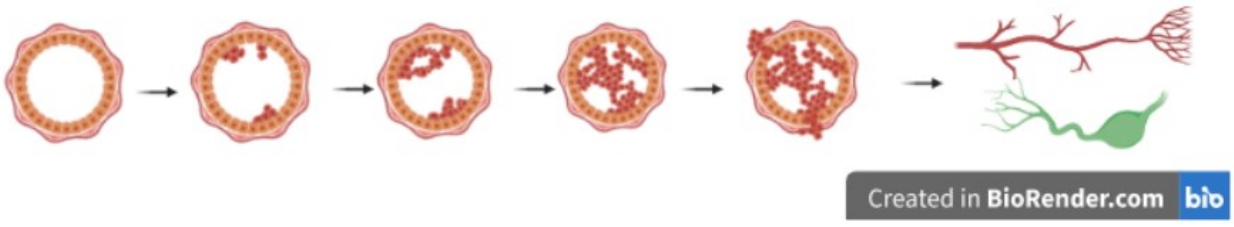

Figure 1. Schematic demonstrating the progression from a healthy duct, to hyperplasia, to ductal carcinoma in situ, to invasive ductal carcinoma, and finally metastasis via the lymphatic and vascular systems.

Breast cancer occurs when the cells of the breast, or more specifically, the mammary gland, are altered in some way. Our current understanding of general breast cancer initiation and progression is that it follows a series of specific steps. Malignancies can originate in either the ductal or lobular cells of the mammary gland, and the resulting disease is referred to either as ductal or lobular breast cancer[8,9]. Initially, a hyperplastic lesion will develop, forming either an atypical ductal or lobular hyperplasia. About 80% of *in situ* breast cancer diagnoses are classified as ductal carcinoma *in situ*[8]. DCIS is characterized by epithelial cells that have become malignant but are confined within the ducts themselves[9]. The basement membrane remains intact and the cells have not invaded through and into the stroma[8,9]. Many cells at this stage also lose their polarity markers that differentiate their apical and basal regions[10]. Cells proliferate to the point where the ductal lumen is lost, however the basement membrane remains intact. Progression from DCIS to invasive ductal carcinoma (IDC) involves changes in gene expression and cellular behaviour, often called the epithelial-to-mesenchymal transition, or EMT[8]. EMT is characterized by cancer cell motility, stemness, and loss of cellular polarity[11]. Epithelial cells lose their characteristics and become more mesenchymal. Progression into IDC is characterized by a loss of the myoepithelial layer as cells infringe on the basement membrane. Malignant breast cells acquire the ability to invade through the basement membrane and into the extracellular matrix (ECM) and stroma[10]. Invasive breast carcinomas can then lead to metastases, where tumour cells colonize distant secondary sites in the body. This requires that the cells acquire the ability to migrate, intravasate into either the lymphatic system or vasculature, extravasate, and adapt to the new secondary site[10]. The most common metastatic sites in breast cancer are the lung, bone, liver, brain, and lymph nodes. Roughly one third of breast cancer patients develop distant metastases[7], and this is a major contributor to breast cancer deaths. The five-year survival rate for women diagnosed with metastatic breast cancer is only 23%. Metastatic breast cancer is also associated with a higher occurrence of the development of resistance to therapy[10,11]. Metastasis was previously believed to be a single-cell process, and this does occur in some cases. However, more recently, it has been shown to also occur as a collective process[12,13], whereby cells break off from the primary tumour but remain attached to each other via cell-to-cell junctions.

Although these sequential steps have been used to describe the progression of breast cancer development, it is also important to remember that all breast cancers are different, and while they

may go through these general steps, they will have differing characteristics. Some breast cancers will progress faster than others, some will metastasize, some will not, some will go preferentially to the lung first, some to the lymph node, etc. Different breast cancers will also exhibit different gene expression patterns and different surface marker profiles. These will be described in section 1.1.4.

1.1.4 Classification of breast cancer into its molecular subtypes

Breast cancers are classified based on morphology, grade, and stage; however, they are also classified more specifically based on their molecular profiles. These different molecular subtypes are associated with different phenotypes, clinical prognoses, and treatment options[9,14]. This classification system is based on the presence or absence of three different protein receptors expressed by the breast tumour cells[9]. These are the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2). Initially, breast cancers were classified into hormone receptor positive (ER^+/PR^+), $HER2^+$, and triple negative ($ER^-/PR^-/HER2^-$) subtypes. However, more recently, Perou et al. demonstrated that breast tumours can be classified more specifically into four different subtypes based on microarray data looking at the expression of thousands of different genes[15]. Currently, the PAM50 system is widely used to look at the transcriptional profile of breast tumours[16]. Based on this system, breast tumours can be classified into one of six molecular subtypes: Luminal A, Luminal B, HER2-enriched, basal-like, normal-like, and claudin-low tumours[16]. Breast tumours tend to cluster into one of these groups based on their transcriptional profiles, which in turn informs how the tumour may behave and what treatments it may respond to. Each subtype has its own associated prognosis in the clinic. However, this thesis will focus on Luminal breast cancers.

1.1.5 Luminal breast cancers

Luminal breast cancer is the most commonly diagnosed breast cancer, accounting for roughly 50% of invasive cases[9], and 70-80% of all cases[8,15,16]. They arise from the luminal cells of the mammary epithelium[8], in contrast to other types of breast cancer that can arise from the basal cells. Luminal breast cancers have the best prognosis when comparing with HER2+, basal-like, normal-like, and claudin-low tumours. Luminal breast cancers are usually ER-positive and either positive or negative for the progesterone receptor. Breast cancer is defined as ER+ when

anywhere from 1-10% of the tumour cells stain for ER by immunohistochemistry[17]. Luminal A breast cancers are HER2-negative and express low levels of Ki67, while Luminal B breast cancers tend to have variable HER2 expression and Ki67 tends to be more highly expressed. However more recently, Ki67 is less-used as a prognostic indicator as there tends to be subjectivity involved when classifying tumours based on this marker[18]. Luminal B tumours tend to have lower ER expression and lower expression of estrogen receptor-regulated genes, as well as lower (or a lack of) expression of the progesterone receptor. Luminal B breast tumours also tend to be of higher grade and the cells tend to be more highly proliferative[18]. This increased proliferation is driven by higher expression of proliferation-inducing genes, such as the PI3K/Akt axis. They also tend to have lower sensitivity to hormone therapy treatments[18]. This, in part, explains why Luminal B breast cancers have slightly worse prognosis when compared to Luminal A[15,16]. However, it is important to keep in mind that despite being able to cluster breast cancers into different categories, different luminal tumours will still bear different gene expression profiles[18] and will behave differently.

1.1.6 The estrogen receptor: structure and function

A key feature of luminal breast tumours is the expression of the estrogen receptor. There are two isoforms of the estrogen receptor in humans, ERα and ERβ. These isoforms are encoded by different genes and have a homologous domain structure[19]. Several different isoforms exist within ERα itself and ERβ as well, and are expressed differentially depending on the tissue type. However, this thesis will be focusing on ERα as it is more relevant to breast cancer. Apart from the full-length, 66 kDa isoform, alternative splicing events also result in the expression of two major isoforms of ERα, a 46 kDa and 36 kDa isoform[20].

The estrogen receptor is a 595 residue ligand-activated steroid hormone receptor that acts primarily in the nucleus[19]. It exists mainly in the cytoplasm of our cells as an inactive monomer, and also appears in the cellular membrane, likely due to tethering adaptors. Upon estradiol (E_2) stimulation and ligand binding, the receptor undergoes a conformational change and homodimerization occurs. Dimerization of the estrogen receptor has also been shown to be occur independent from ligand binding via phosphorylation of specific residues, such as Ser-118, Ser-

104, and Tyr-537[21]. At this stage, the estrogen receptor dimer can then translocate to the nucleus. Here, the ER dimer acts as a transcription factor and binds to estrogen response elements, or EREs, which are transcriptional promoters. ER binds the ERE with the help of other coactivators, and also interacts with the RNA polymerase complex[19]. Binding of the estrogen receptor to EREs can also occur indirectly through other adaptor proteins. In this case, the estrogen receptor does not actually contact the DNA, but rather its interaction with other proteins that are in contact with DNA provides a favourable environment to promote transcription[18,19]. Some examples of these "tethering" proteins are AP-1 and RARα[19]. Upon binding of ER to EREs (directly or indirectly), these estrogen-responsive genes will then be transcribed and expressed. Some examples of genes regulated by the wild-type estrogen receptor include those coding for ABCA3, cathepsin D, TFF1, and cyclin D1.

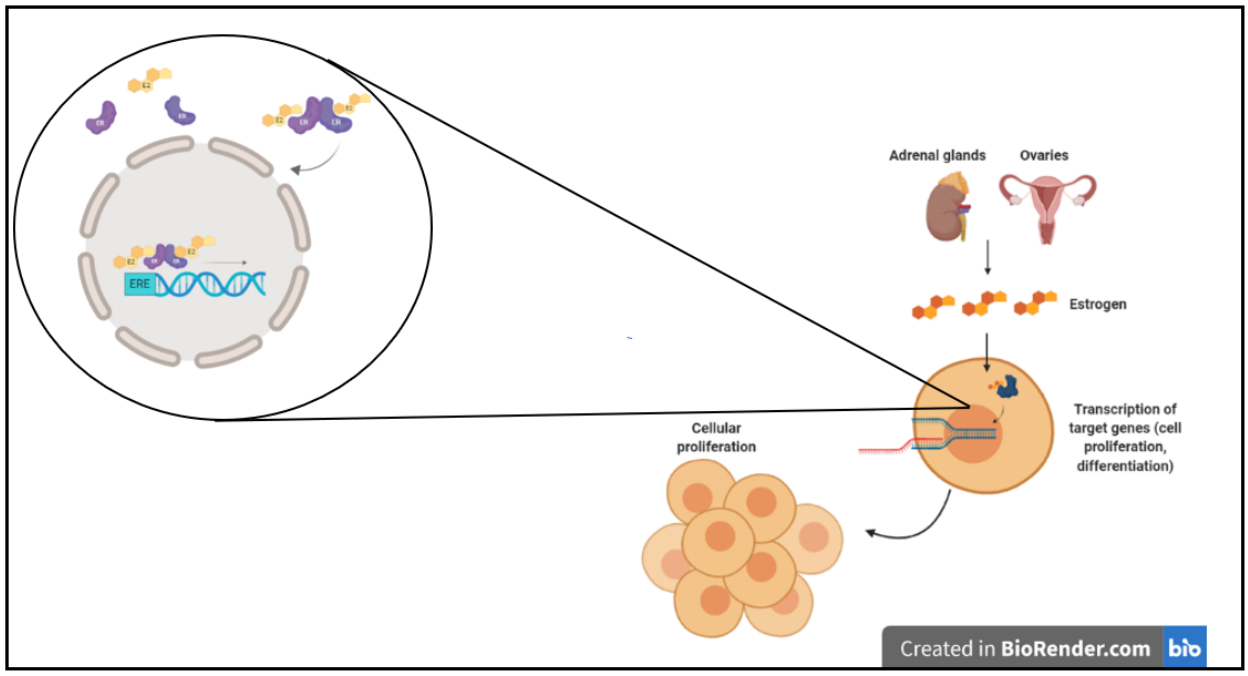

Figure 2. Mechanism of action of the wild-type estrogen receptor. Coactivators and other proteins involved in transcription have been excluded for simplicity.

The estrogen receptor can be described in terms of its domains, which are highly conserved among the nuclear receptor superfamily. The AF-1 domain plays a role in ligand-independent activation[19,21]. The AF-2 domain is responsible for ligand-dependent activation. ER also contains a hinge region, a DNA-binding domain, and a ligand binding domain. See Schematic 1 for a depiction of the domains. The DNA binding domain (DBD) has been shown to be the most highly conserved domain among all nuclear receptors, and they all function similarly[22]. Two alpha helices are arranged perpendicularly, where one directly recognizes the DNA strand by inserting itself into the major groove[22]. The helix that recognizes the ER DNA half-site sequence, also known as a consensus sequence, (5'AGGTCA3') contains specific amino acids on its exposed surface that are responsible for this recognition[22]. The ligand binding domain (LBD) consists of a hydrophobic pocket which attracts lipophilic ligands such as estradiol[22]. The amino acid composition of this pocket determines the specificity of the ligands it attracts. The ER LBD contains twelve alpha helices[22,23]. In the unbound state, helix 12 of the estrogen receptor is positioned far away from the LBD. When ligand binds, this induces a conformational change that brings helix 12 in close proximity to helix 3 of the LBD. This represents the active, E_2-bound conformation of the wild-type estrogen receptor. Helix 12 also plays a role in binding of ER coactivators to the LBD[22]. ER antagonists, like Tamoxifen, function similarly to estrogen, however, they contain a bulky side chain that prevents helix 12 from coming into close enough proximity to the LBD pocket for it to activate the receptor. Thus, Tamoxifen prevents estrogen from binding and simultaneously inhibits the receptor[22].

Schematic 1. Depiction of the conserved domains of ERα and ERβ. Schematic is adapted from Robinson et al and created using BioRender.

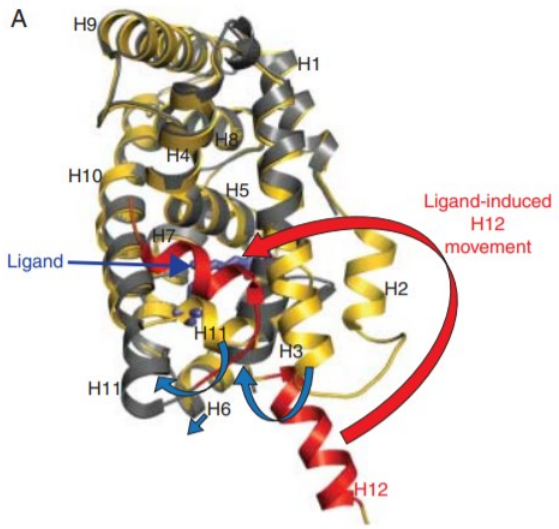

Figure 3. Rastinejad et al. (2013; Fig. 5A) demonstrates how ligand binding in nuclear receptors triggers a conformational change that allows the ligand to be trapped within the hydrophobic pocket of the LBD[22].

1.1.7 Available therapies for estrogen receptor-positive breast cancer

Hormone receptor-positive breast cancer accounts for roughly 70% of breast cancer diagnoses each year. The prognosis for this disease when caught early is very good. Over 90% of patients survive past five years. This is due to the increased understanding we have gained as to how this disease progresses and how it can be targeted. Many therapies have been developed to treat estrogen receptor-positive breast cancer. The first targeted therapy used for estrogen receptor-positive breast cancer was Tamoxifen, a non-steroidal antiestrogen. It was initially synthesized to act as an anti-estrogen to be used for contraceptive purposes. However, it was found that Tamoxifen actually stimulated ovulation, instead of repressing it[24]. It was later re-purposed for use as an anti-cancer drug. Tamoxifen is a non-steroidal selective estrogen receptor modulator (SERM). It competes with estradiol's binding to the estrogen receptor, and effectively prevents it from adopting its activated conformation[25]. This prevents estrogen receptor activation, dimerization, translocation to the nucleus, binding to EREs, and activation of transcription. While Tamoxifen plays an antagonistic role on ER in the mammary gland, it is important to note that it acts as an agonist for ER in some other tissues, such as bone and endometrium[26], and can increase the risk for developing endometrial cancer[24,25]. Previous research has shown that

Tamoxifen-bound ER can still dimerize and enter the nucleus, where transcriptional activity of the AF-2 domain is inhibited but the AF-1 domain is still partially active[26]. This may, in part, explain the different effects of Tamoxifen on different tissues. Tamoxifen was very widely used for the prevention and treatment of ER+ breast cancer and ER+ metastatic breast cancer. Tamoxifen derivatives are more commonly used now since they tend to have fewer negative side effects. In general, these were developed by altering side chains of the Tamoxifen structure. There are many other SERMs that have been developed to treat this disease that are commonly used worldwide. However, it is very common for mammary epithelial cells to adapt over time to chronic SERM exposure and develop resistance to this type of therapy[25,26].

Researchers sought to circumvent the issue of SERMs acting as antiestrogens in some tissues and having agonist activity in others. This led to the development of selective estrogen receptor degraders or down-regulators, also known as SERDs. They are another pharmaceutical development that has positively impacted the treatment and prognosis of patients diagnosed with hormone receptor-positive breast cancer. Fulvestrant is the most commonly prescribed SERD for ER+ breast cancer[26]. Fulvestrant is a steroidal anti-estrogen. It binds the estrogen receptor and inhibits dimerization by sterically interfering at the dimerization interface[27]. The result of this is increased degradation of the estrogen receptor and lower levels of cellular and nuclear ER. Fulvestrant also exhibits an affinity for the estrogen receptor that is 100 times higher than that of Tamoxifen[28]. It does not present with any intrinsic agonistic effects and can also block any agonistic effects that Tamoxifen may have on various tissues[27,28]. Previous research has shown that Fulvestrant may also delay the development of endocrine resistance when used in combination with Tamoxifen[26]. Tamoxifen-resistant cell lines and *in vivo* tumours have been shown to remain sensitive to SERD inhibition[26-28]. Despite this, resistance to SERDs is still a common occurrence.

Another drug class that is used to treat ER+ breast cancer is aromatase inhibitors (AIs). Aromatase inhibitors prevent the production of estrogen by inhibiting the action of the aromatase enzyme complex[29]. The aromatase complex is responsible for a critical step of estradiol synthesis, where it converts androgens into estrogens[30]. They bind, reversibly or irreversibly (depending on the compound), to the aromatase enzyme complex and compete with the endogenous substrate. Therefore, AIs inhibit the production of estrogen. Treatment with AIs

reduces the levels of circulating estrogens to, in most cases, undetectable levels[30]. Aromatase inhibitors are replacing the use of Tamoxifen for the treatment of ER+ breast cancer since they appear to be more efficient at preventing disease progression. AIs come in two flavours: steroidal and non-steroidal[29]. Examples of commonly used aromatase inhibitors include anastrozole and Letrozole[30].

Luteinizing hormone-releasing hormone analogues (LHRHs) prevent the ovaries from producing estrogen. These are most commonly prescribed for premenopausal women, as the inhibition of the ovaries is reversible and they can recover over several months. While breast cancer diagnoses in premenopausal women tend to be associated with worse prognosis and more risk factors (such as a higher rate of triple negative breast cancer), 50% of breast cancer diagnoses in these patients are estrogen receptor-positive[31]. LHRHs function in the pituitary gland by stimulating the release of gonadotropins. These compounds can be used intermittently to treat infertility, or continually in the case of breast cancer treatment[31]. Continual use results in desensitization of the pituitary gland and the downregulation of luteinizing hormone releasing hormone receptors[32,33]. This is then followed by a reduction in the production of luteinizing hormone and follicle stimulating hormone[31,32], which essentially ablates the ovaries' ability to produce estrogen. Lastly, the most extreme and aggressive treatment for ER+ breast cancer is oophorectomy or ovariectomy. This is when the ovaries are surgically removed. This treatment eliminates the body's main source of estrogen. By depriving the body of estrogen, the estrogen-responsive breast tumour's growth is inhibited.

The threshold for diagnosing a breast tumour as ER-positive is quite low: only a minimum of 1% of the tumour cells need to highly express the estrogen receptor for it to be classified as an ER+ tumour. This can give us some insight as to why or how such a large proportion of ER+ breast cancer patients develop resistance to endocrine therapy. To conclude with this in mind, we can say that although ER+ breast cancer is very treatable, resistance to therapy is a major hurdle with many complexities that still need to be addressed in the laboratory and in the clinic.

1.1.8 Resistance to therapy

As previously mentioned, a major challenge in the treatment of hormone receptor-positive breast cancer is the development of drug resistance, which commonly occurs after hormone therapy[34]. Some patients present with *de novo*, or intrinsic resistance, where they do not respond well to endocrine therapy as the first line of attack against their tumour. Many patients acquire resistance after endocrine therapy. They tend to respond well initially, but recurrence or metastases of the cancer are often characterized by mutations in key genes that allow the cancer to continue thriving despite the targeted therapy. Endocrine resistance does not occur only via one mechanism. It is a complex, multifaceted issue to tackle and it needs to be approached from an intersectional point of view. Every patient's cancer is different from another's, patients are diagnosed at different stages and grades of breast cancer, people receive different treatments, and every patient has a unique history that may influence the progression of their disease, initial response to therapy, or their risk of developing resistance. Treatment history plays a critical role in predicting the pathways that become altered in endocrine therapy resistance[35]. Primary courses of treatment impact what kinds of mutations are selected for as well as which molecular pathway(s) may become hyper-activated in breast tumours.

It is estimated that up to 50% of ER+ breast cancers will develop resistance to endocrine therapy and will recur and metastasize. For this subtype, this is what contributes most significantly to patient deaths[36]. Different mechanisms of endocrine resistance have been proposed, such as the existence of breast cancer stem cell populations contributing to intratumoral heterogeneity[36], the activation of key oncogenic pathways like the PI3K/AKT/mTOR axis, and increased HER2 signalling, among many others. The non-classical mechanism of estrogen receptor function, where kinases such as c-Src or HER2 can phosphorylate ER, resulting in activation and modulation of its activity, has been identified as an important factor in endocrine resistance. The resulting differential recruitment of co-regulators and the upregulation of several growth factor receptor axes has been implicated in the development of resistance as well[36].

There are several endocrine resistance mechanisms that can occur in ER+ breast cancer (and other subtypes). Some of these are found in common among patients receiving different courses of treatment; this is referred to as "cross-resistance"[32,36,37]. These processes can be a result of

intrinsic or acquired endocrine resistance. An observation that can be found among roughly 20% of metastatic ER+ breast cancer is the downregulation of ER expression[38,39]. Having lower or non-existent expression of the estrogen receptor makes drugs targeting this protein ineffective for obvious reasons. Amplification of the ER gene can be found in 2% of primary ER+ tumours and 21% of recurrent tumours[40,41], so this could also play a role in resistance. Another example of a cross-resistance mechanism is the reduction of intracellular drug concentration[38]. This can be achieved in multiple ways: the reduction of cellular uptake of pharmaceuticals, increased efflux of pharmaceuticals, or modified drug metabolism phenotypes[38,42]. The cell achieves this by having altered expression of specific influx or efflux proteins or by increased the rate at which the drug is metabolized to an inactive form (another related example would be the inability to convert a pro-drug into its active metabolite, such as is the case with Tamoxifen conversion to endoxifen before it can act on ER)[36]. Other mechanisms of endocrine resistance include the activation of or cooperation of various growth factor pathways with the ER pathway, the activation of various kinases, and modification of, or altered expression of ER co-regulators[37,39]. Although many key molecular players have been identified, endocrine resistance is still a gray area, and much remains to be discovered. A better understanding of the mechanisms behind resistance and the ramifications for disease progression and patient prognosis will help to diagnose patients more effectively and will perhaps allow for a more specific treatment plan.

1.1.9 Mutations in the estrogen receptor: what does it mean?

More recently, mutations in ER have been implicated in endocrine resistance. The estrogen receptor commonly becomes mutated in the metastatic setting: 30-40% of patients present with estrogen receptor mutations after hormone therapy, and the dominant majority occur in the ligand binding domain[41,43,44]. Some literature suggests that next generation sequencing detected ESR1 mutations in 55% of metastatic samples[34]. It is estimated that this occurs in 20-40% of endocrine resistant tumours[41,45]. These mutations tend to be activating mutations that render ER active in the absence of estrogen. One of the most common activating mutations that occurs in humans is a tyrosine to serine substitution at amino acid position 537 (ERαY537S; ERαY541S is the corresponding murine mutation)[34]. This mutation allows for helix 12 in the ligand binding

domain to hydrogen bond with helix 3, effectively mimicking the active conformation of the estrogen receptor[34,44]. A critical characteristic of this mutation (and most other recurrent activating ESR1 mutations that occur in the same hotspot) is that it is primarily found in recurrent and metastatic breast cancers, and not in primary tumours[44-47]. This could be a result of either clonal selection of a rare population of cells that were harboring this mutation in the primary tumour, or the acquisition of new mutations after the selective pressure of hormone therapy.

Many studies have shown that this mutation is commonly detected after hormone treatment[47-49], and it also renders the estrogen receptor resistant to inhibition by both Tamoxifen and Fulvestrant[40,41]. The conformational change that occurs when WT ESR1 is mutated to ESR1(Y538S) allows for the inhibition of antagonist action, as well as reduced affinity for certain endocrine drugs, like Tamoxifen[34]. Martin et al demonstrated that a human breast cancer cell line naturally developed the tyrosine to serine mutation at residue 537 after long-term estrogen deprivation and treatment with Fulvestrant[48]. Over time, the mutation became enriched for *in vitro* and they showed that this mutation altered ESR1's interactome as well as its interactions with the genome. Other *in vitro* studies have shown that, when comparing the Y537S mutation to other common activating mutations, $ESR1^{Y537S}$ had the highest transcriptional activity both in the presence and absence of estradiol. When researchers tested the effect of Fulvestrant and three other SERDs on ER activity, they found that cells expressing $ESR1^{Y537S}$ also required the highest dosage to inhibit ER activity completely (for all drugs tested)[45,47]. Other studies have shown similar results when cells are treated with Tamoxifen[44].

Mutating the estrogen receptor also has ramifications for its interactome and its transcriptome. Point activation of ER allows for the recruitment of its co-activators in the absence of estrogen. The $ESR1^{Y537S}$ interactome is relatively similar to its wild-type counterpart, however recruitment of different co-activators or negative regulators can occur[41,44]. There is evidence showing that $ESR1^{Y537S}$ has increased association with its coactivator AIB-1[44], resulting in increased transcriptional activity. Work by Jeselsohn and colleagues has revealed that there are allele-specific transcriptomes associated with different ESR1 mutations[45]. They show that there are unique differences in transcriptome and genome recruitment between the different mutants they investigated[45]. There is some degree of overlap between mutant and wild-type ER transcriptional

targets, where classic estradiol-responsive genes are expressed ubiquitously (such as GREB1 and MYC). Interestingly, only 18% of the genes transcribed by the Y537S mutant overlapped with the estradiol-stimulated wild-type cells' transcriptome[45]. Other mutants had transcriptional profiles that were more like that of wild-type ER-expressing cells. Therefore, there are significant differences between the transcriptome of wild-type ER and $ESR1^{Y537S}$. Some of the most striking differentially expressed genes (when comparing $ESR1^{Y537S}$ to wild-type) are genes associated with migration and metastasis, as well as upregulation of the AKT1 signalling cascade[45]. Chip-sequencing analysis also revealed that mutant forms of ER had different binding sites in the cellular DNA. $ESR1^{Y537S}$ had the highest number of genome binding sites associated with it, as well as the highest binding intensity when comparing to wildtype and the other mutants tested. Additionally, mutation of specific tyrosine residues in the estrogen receptor can modulate its capacity for phosphorylation by kinases. The conversion of a tyrosine residue to a serine residue has implications for which specific kinases can activate ER or modulate its activity. For example, Kinase A may only be able to phosphorylate tyrosine residues of ER, whereas Kinase B can only phosphorylate serine residues. Downstream effects may change in this respect when mutations occur that ablate or change those residues. This is important to take into consideration when we look at the effect of point mutations. The effects we may see are likely not only due to the activation of the receptor, but also to the differential activation of downstream pathways that may occur.

Mutation of the ligand-binding domain of the estrogen receptor plays a more complex role than previously thought. Mutation of ER does not only render it constitutively active, but also affects co-regulator recruitment and its transcriptional targets. By broadening our understanding of the consequences of these mutations, we hope to exploit any weaknesses we can find and hopefully improve the care of patients presenting with recurrent or metastatic disease. Long term, the aim is to develop a better treatment plan tailored specifically to the patient's tumour and the associated mutations in the estrogen receptor.

1.1.10 The phosphatidylinositol 3' kinase signalling pathway

The phosphatidylinositol 3' kinase (or PI3K) pathway is altered in a large proportion of human cancers. Class I PI3Ks play an important role in normal cells. They transmit signals from receptor tyrosine kinases, like growth factor receptors or HER2, as well as G-protein coupled receptors, to promote cellular proliferation, metabolism, angiogenesis, and survival[50,51]. Polyomavirus middle T antigen (MT) also promotes assembly and activation of the PI3K complex[52,53]. This will become important later in this thesis. PI3K is a heterodimer composed of a catalytic subunit, p110, and a regulatory subunit, p85. Multiple isoforms exist for both subunits. This thesis will focus on p110α, as this is the relevant catalytic isoform for the study. The regulatory subunit acts as an adaptor protein, linking the catalytic p110 subunit to the membrane-bound RTK. Phosphorylation of RTKs allows for p85 to dissociate from p110 and it binds the phosphorylated tyrosine residues. Subsequently, inhibition of p110 by p85 is alleviated and p110 is in its active form[51]. When PI3K is activated, it catalyzes the conversion of phosphatidylinositol biphosphate, PI(4,5)P2, to phosphatidylinositol triphosphate, PI(3.,4,5)P3 via phosphorylation[54]. PIP3 is an important second messenger molecule that causes a cascade of activation of multiple effectors, including activation of the PDK1/Akt/mTOR axis[50]. The tumour suppressor PTEN, antagonizes the action of PI3K by dephosphorylating PI(3,4,5)P3 to PI(4,5)P2, therefore reducing the levels of the messenger molecule responsible for downstream pathway activation[52,55]. The PI3K signalling axis plays an important role in cancer, especially when it becomes deregulated. Unchecked activation of this pathway results in cells that display dysregulated growth and increased survival, leading to tumour progression and potentially to metastasis.

1.1.11 The PI3K signalling pathway and ER+ breast cancer

The PI3K pathway is activated in over 15% of all human cancers, 30% of all breast cancers, and over 70% of ER+ breast cancers[55,56]. The gene encoding the catalytic subunit of PI3K, *PIKC3A,* is the most commonly mutated gene in the PI3K pathway in breast cancer[41], and *PIKC3A* gain-of-function mutations are more highly associated with ER+ breast cancers compared to HR- breast cancers[17]. Activation of PI3K results in activity independent from growth factor/ligand stimulation[57]. Our lab, among many others, has shown that p110α expression is indispensable for

breast cancer progression[52]. More than 80% of mutations that occur in *PIKC3A* are found in the helical or the kinase domain. One of the most common mutations found in PI3K is H1047R, found in exon 20 of the kinase domain. We know that this mutation results in constitutive activation of the receptor through increased membrane recruitment[51], however the mechanism behind this is not fully understood. The loss of PTEN expression also commonly occurs in breast cancer[51], and this synergizes with the activation of PI3K, resulting in increased signalling through the PI3K/AKT/mTOR axis.

Besides the discrepancy in prevalence of *PIK3CA* mutations between different subtypes of breast cancer, there are many emerging studies linking the activation of the PI3K pathway with recurrent and metastatic ER+ breast cancer. Genomic analyses of human ERα-positive breast cancers show that there are recurrent mutations that frequently co-occur along with the activation of ER, including those in the catalytic subunit of phosphatidylinositol 3-kinase[49]. There is also a discrepancy in the reported incidence of these mutations co-occurring, but it ranges from 5-20% depending on the source[42,49]. There is significant cross-talk between PI3K and ER signalling pathways but some studies are contradictory, especially when we compare results from different systems (i.e. cell lines, PDX, etc). Signalling from the PI3K axis has been shown to increase ER activity in the absence of estrogen[58]; this is achieved via phosphorylation of ER by effectors downstream of PI3K. However, there is literature suggesting that reduced levels of ER signalling in ER+ breast cancers is reciprocally associated with PI3K signalling[59]. Similarly, inhibiting PI3K increases estrogen receptor activity and dependence in ER-positive patient-derived xenografts[60]. The estrogen receptor also promotes transcription of genes that are upstream of PI3K activation, such as IGFR and EGFR[58].

Links have been made between endocrine resistance and the activation of PI3K in ER+ breast cancer[34,51], but the significance of this has not been fully explored. Endocrine resistance has also been shown to be associated with reduced levels of PTEN expression[40,58], as well as low estrogen receptor expression levels but high levels of PI3K signalling[58]. Research has shown that endocrine sensitivity can be restored in some cases by targeting downstream effectors of this pathway, such as mTOR or MAPK[40]. Clinical trials have shown that targeting mTOR with everolimus in endocrine resistant breast cancer cases restores sensitivity to Tamoxifen

treatment[61-64]. However, targeting this pathway to treat cancer often results in cellular toxicity because it is an essential pathway in most cell types.

While the evidence for interplay between PI3K and the estrogen receptor is overwhelming, there is some lack of consensus in some areas and it will be important for researchers to carefully dissect the true mechanisms behind this relationship. We have generated a new murine strain expressing activated PI3K ($p110\alpha^{H1047R}$) and activated ER ($ESR1^{Y541S}$). This will enable us to explore the roles that these mutations play in breast cancer progression and metastasis in an *in vivo* context.

1.2.1 Mouse models of breast cancer

Genetically engineered mouse models (GEMMs) have proven to be an invaluable tool in the study of various human diseases, including breast cancer. The mouse mammary gland is structurally like the human mammary gland, and various processes can be closely recapitulated in vivo. Murine mammary glands mimic the development of human mammary glands, from the post-natal immature state through to pregnancy, lactation, and involution. Oncogenic process, such as the initiation of mammary hyperplasia, the progression of breast cancer from DCIS to IDC, and the metastatic cascade can also be studied closely in murine models. Mouse models allow us to control both temporal and spatial expression of various genes, depending on the specific model in question. This provides a critical link from *in vitro* experiments and simpler *in vivo* models to study human disease.

1.2.2 The Cre/LoxP recombination system and the MMTV promoter

The utility of genetically engineered mouse models in studying human disease is amplified by the use of tissue-specific promoters. Germline gene knock-ins or knock-outs provide useful information in specific contexts, such as studying embryonic development or hereditary germline mutations in cancer. However, affecting a gene in a full-body context can have radical ramifications that may not be expected or understood easily, such as when it results in embryonic lethality[65]. Promoters that drive expression of genes of interest specifically to the mammary gland epithelium are useful when studying breast cancer. The Mouse Mammary Tumour Virus

Long Terminal Repeat (MMTV-LTR) promoter is an example of a promoter that, when placed upstream of a transgene, drives expression of the gene of interest specifically to the mammary epithelial cells[65]. This promoter is active throughout mammary development in mice[66]. This allows us to specifically study the effect that a gene has on mammary gland and breast tumour development in our mouse models.

The Cre/LoxP system is another useful tool that scientists can use to control gene expression, both spatially and temporally, in conditional GEMMs. Cre recombinase is usually linked to a tissue-specific promoter, such as MMTV, which drives its expression to the mammary epithelium. The transgene to be studied will be flanked by loxP sites (or the loxP sites will be integrated in the gene in some way to result in a desired outcome). When Cre recombinase is expressed, the loxP sites will be excised by recombination[65] and the flanked gene will be deleted. As you will see in section 1.2.2, loxP sites can also be used within genes to excise specific exons or coding sequences.

1.2.3 Models of estrogen receptor-positive breast cancer

Despite the fact the estrogen receptor-positive breast cancer is the most prevalent subtype that is diagnosed, there is a general lack of clinically relevant *in vivo* models we can use to study it. The majority of studies use *in vitro* approaches, such as stable cell line development as well as their use in immunocompromised mice. However, there are some models that result in ER+ tumours. These models include GEMMs that overexpress ER or other co-factors that are associated with it, such as AIB1[17]. There are also many models that overexpress specific ER-responsive genes, such as cyclin D1, that result in ER+ mammary tumors[17]. Additionally, there are several GEMMs resulting in ER+ tumours that combine either drug or carcinogen treatment with genetic aberrations of genes related to the ER signalling pathway.

1.2.4 The ESR1^{Y541S}/MMTV Cre model

We noticed a general lack of mouse models existing to study recurrent and/or metastatic ER-positive breast cancer. This unfortunately occurs in about 20% of ER-positive breast cancer diagnoses. It is important to note that these recurrent cases of ER-positive breast cancer

contribute the most to patient deaths within this subset of diagnoses (the survival rate for early stage ER-positive breast cancer is very high). It has been shown that deregulated estrogen signalling in mice and in humans results in increased cellular proliferation of the mammary epithelium[17]. Because of this, our laboratory has developed the first genetically engineered mouse model that possesses a Cre-inducible point-activated mutant form of the estrogen receptor, $ESR1^{Y541S}$. This mutation is the corresponding murine mutation to the human $ESR1^{Y537S}$ that was described previously in this thesis. The point mutation change from a tyrosine residue to a serine residue is conserved between mice and humans.

This murine model was generated by engineering the ESR1 gene to contain a mutated form of exon 9, which encodes a portion of the ligand-binding domain of the estrogen receptor. In the absence of Cre, it is out of frame, and therefore not expressed. However, the wild-type exon 9 is expressed in this case. The wild-type exon 9 was engineered to be flanked by loxP sites. When Cre is expressed, it excises the wild-type exon via recombination, and the mutant exon is then placed in frame and can be expressed. See Figure 4. An advantage of this model is that $ESR1^{Y541S}$ expression is driven by the endogenous promoter that normally drives expression of the estrogen receptor. This allows for better understanding of the role that ESR1 plays in mammary gland development and breast cancer, as it is not over-expressed. Overexpression could cause off-target effects to be observed and being expressed at endogenous levels makes this a more physiologically relevant mouse model to work with. We have crossed this strain with a strain that expresses Cre recombinase linked to the MMTV promoter, which drives the expression of Cre, and subsequently the activated form of ESR1, specifically to the mammary gland. We will initially be using this model to investigate the effects that this point-activated form of ER has on mammary gland development, as well as any oncogenic effects it may have.

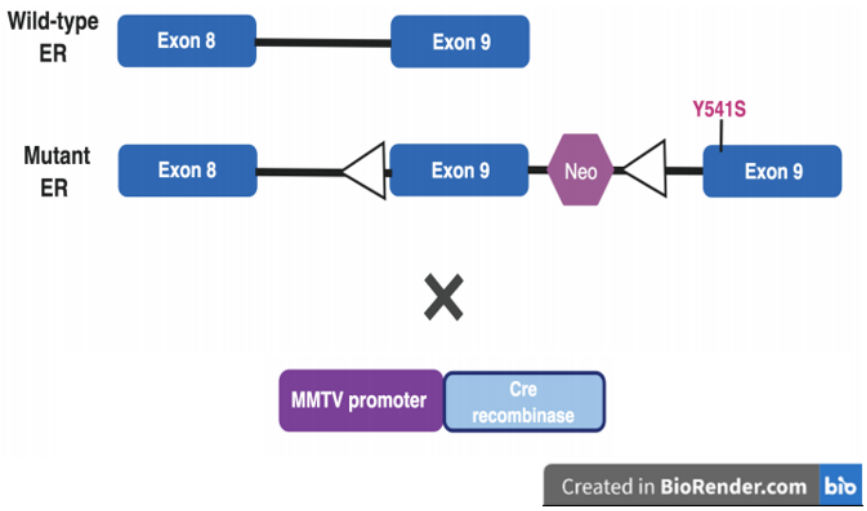

Figure 4. Schematic of the ESR1^{Y541S}/MMTV-Cre GEMM. We crossed the activated ESR1 with the MMTV-Cre strain to generate murine mammary epithelial cells that are heterozygous for the mutant ESR1.

1.2.5 The p110αHR/ESR1^{Y541S}/MMTV-Cre mouse model of ER-positive breast cancer

To expand our investigation into the effects that the point-activated estrogen receptor may have in breast cancer development and progression, we decided to cross our ESR1^{Y541S} mouse strain with a characterized strain where we have already confirmed tumour development occurs. This is a murine strain that expresses a mutant form of PI3K (p110α^{H1047R}), where the point mutation in exon 20 renders it constitutively active. This new strain we generated is the p110αHR/ESR1^{Y541S}/MMTV-Cre strain. See Figure 5. Both p110α^{H1047R} and ESR1^{Y541S} expression is driven by their endogenous promoters, and the MMTV promoter that drives the expression of Cre targets gene expression specifically to the mammary epithelium. The p110α^{H1047R} mouse model has been described and characterized by previous lab members (Manuscript in preparation by Alexandra M. Simond) and by other laboratories[67]. Using this strain, we aim to determine the genetic and molecular mechanisms by which ERα^{Y541S} co-

expressed with activated PI-3K can drive mammary gland tumour progression. We aim to provide important insight into the molecular basis for the emergence of resistance to ERα targeted therapies and to understand how these resistant tumours evolve and further progress. In the future, this work may contribute to defining appropriate therapeutic modalities with single or combined agents to prevent the progression of resistant ER+ breast cancers.

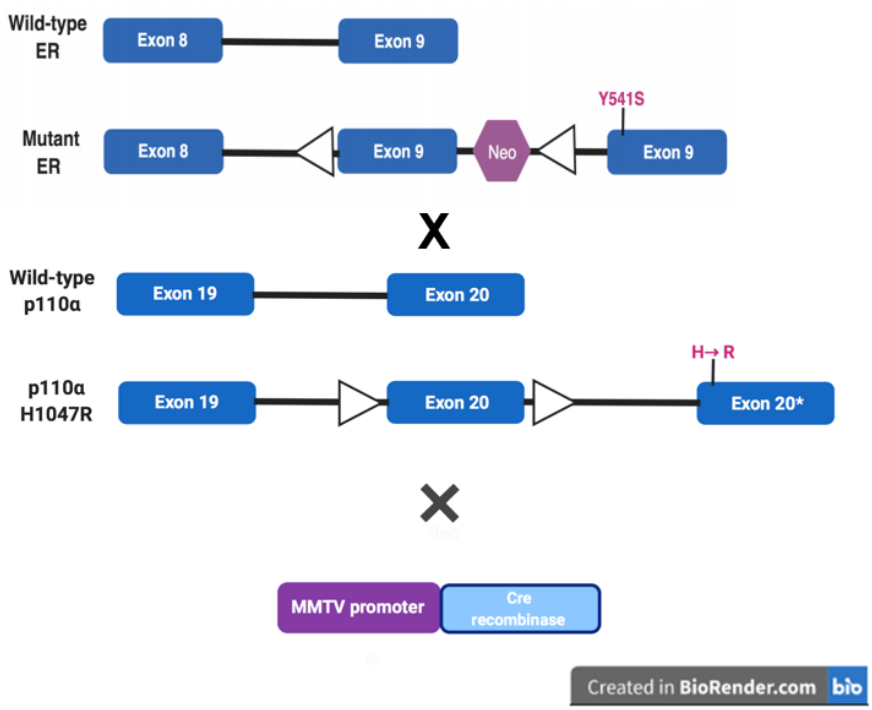

Figure 5. Schematic of the p110aHR/ESR1Y541S/MMTV-Cre GEMM. We crossed our activated ESR1 GEMM with a point-activated form of P13K, and with the MMTV-Cre strain to generate a new strain of mouse where the mammary epithelial cells are heterozygous for both genes of interest.

1.2.6 The polyomavirus middle T antigen

The polyomavirus middle T antigen was first discovered in the 1950s as a protein that could cause tumours in mice[68,69]. While it is not a human oncogene, the PyVmT antigen is a potent viral oncogene that can mediate transformation of many different epithelial and mesenchymal tissue types[70]. The polyomavirus produces three antigens: the large, middle, and small T antigen via alternative splicing events[53]. Only the middle T antigen is required for oncogenic transformation in mice and rats[71,72]. The middle T antigen is a membrane protein that is activated via phosphorylation by tyrosine kinases, such as c-Src family members[53,70,72,73]. Membrane anchorage is critical to its function, as loss of the domain that inserts itself into the cell membrane eliminates the transforming potential of MT[53]. Phosphorylation of specific residues of MT allows it to act as a scaffold for many proteins, such as kinases like PI3K. This allows for the MT antigen to essentially mimic an activated RTK, activating many pathways whose downstream effects include cellular proliferation, survival, and evasion of apoptosis[53]. Middle T has been shown to robustly activate PI3K by recruiting the regulatory subunit, p85[72]. In fact, PI3K expression is required for Middle T-mediated transformation. It has been shown that loss of one allele of p110α delays tumour onset and reduces tumour burden in MMTV-PyVmT mice[53].

Mammary carcinomas resulting from Middle T expression closely recapitulate the stages and progression of human breast carcinoma, starting with hyperplasia, and progressing through adenoma to early carcinoma and late carcinoma[53,74]. Lung metastasis then occurs in the MMTV-PyVmT model with a penetrance of almost 100%[70]. A biomarker profile, like that of human breast cancers, can be seen in these tumours as well. This includes the progressive loss of

expression of ER/PR and β1-integrin, while the overexpression of ErbB2 and cyclin D1 persists throughout progression[74]. This expression profile is associated with Luminal B tumours with poor prognosis[72].

Although PyVmT is not a human oncogene, it mimics the activation of several pathways that are critical for human breast cancer development. By mimicking our PI3K-driven mouse model to some degree, this tool will allow us to study this disease in multiple different ways and in a more efficient manner. It is for these reasons that we have used two MT-driven models (see section 1.2.7 and 2.2.3) in this thesis to further explore and draw parallels with our PI3K-driven mouse model of breast cancer.

1.2.7 The ESR1/MTB/MIC mouse model of ER-positive breast cancer

This section will only briefly describe the ESR1^{Y541S}/MTB/MIC mouse model, as it is it not relevant for the bulk of this thesis. This model was generated in the Muller lab by previous members. See Figure 6 for a schematic of the genetic cross.

Expression of our genes of interest is temporally and spatially controlled in this model by taking advantage of the tetracycline-inducible promoter and tissue-specific promoters. The expression of a reverse tetracycline transactivator transgene (rtTA) is controlled by the MMTV promoter, as described previously in this thesis. Expression of the Polyomavirus Middle T antigen and Cre recombinase is under the control of the TetO promoter. These two genes are linked by an internal ribosome entry site, allowing the expression of both to occur from the same transcript. Upon treatment with doxycycline, an rtTA/doxycycline complex binds TetO to activate transcription. This is referred to as Tet-ON system[72,75]. With doxycycline, PyVmT and Cre will be expressed.

Cre recombinase expression also results in the excision and activation of ESR1^{Y541S}. Each gene is expressed specifically in the mammary gland of mature mice. Mice in this model develop palpable tumours within two weeks after induction with doxycycline, and these tumours progress through the typical stages seen in MT-driven tumours (see section 1.2.6)[72]. Lung metastasis is another phenotype that characterises this model.

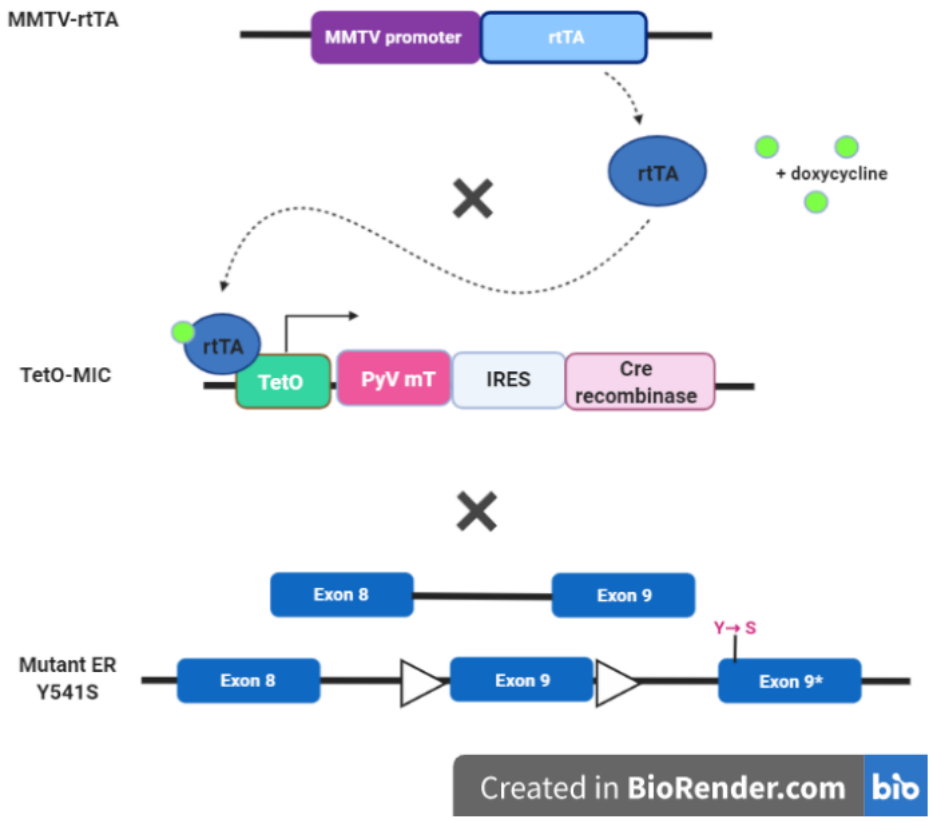

Figure 6. Schematic depicting the ESR1/MTB/MIC GEMM cross. We crossed the novel ESR1^{Y541S} strain with our MTB/MIC strain to generate mice that develop tumours driven by the PyVmT antigen that express the mutant estrogen receptor.

2. RESEARCH FINDINGS AND DISCUSSION

2.1 Characterization of the novel genetically engineered mouse model, ESR1^{Y541S}

Due to the lack of immune-competent mouse models to model the progression of recurrent ER+ breast cancer, our lab (Chen Ling) developed a Cre-inducible mouse model to help us study the effect that this common mutation has on breast cancer progression and metastasis. Termed the ESR1^{Y541S} strain, the point-activated estrogen receptor is driven by its endogenous promoter. We decided it was important to characterize the effect of the activated estrogen receptor in a full-body and a mammary gland specific context before investigating its effect in a breast cancer context. This will enable us to better understand any effects we see in the long-term.

2.1.1 Full-body knock-in characterization

We crossed the ESR1^{Y541S} strain with our β-actin-Cre strain. The β-actin promoter drives Cre expression to every cell of the body. Therefore, every cell should express the point-activated estrogen receptor early in development. Germ-line expression of this protein resulted in dramatic effects that I will summarize briefly in this thesis. Characterization of the ESR1^{Y541S}/β-actin-Cre (full-body) strain was largely performed by Alexandra Simond, another graduate student in the Muller lab. Her data will not be included here as it is currently under review in Genes and Development, but I will touch on her findings where they are relevant to my own. The full-body knock-in demonstrates a striking phenotype in both sexes. Both sexes are runted. Female mice develop ductal ectasia, which is a condition where the ducts of the mammary gland widen and thicken. The ducts also do not exhibit as much lateral branching as wild-type mammary ducts. Additionally, female mice lack a corpus luteum and have a higher density of follicles present in their ovaries. We believe this is because the female mice are unable to ovulate. Progesterone and

the progesterone receptor have been shown to play a role in this, so this data suggests a lack of expression of PR or lack of progesterone production[76]. These mice are also unable to produce pups. This, taken together with the lack of ductal branching, supports my hypothesis that the oestrous cycle is disrupted in these mice, and they are unable to develop a corpus luteum which produces most of the body's progesterone. This, in turn, results in a lack of ovulation and sterility. Female mice also develop uterine and ovarian cysts. They do not survive past twelve weeks of age. As of now, we do not know why exactly, but we hypothesize that it may have to do with ovarian and uterine cysts bursting, which likely results in huge inflammation and sepsis.

2.1.2 Mammary-specific knock-in characterization

The prevalence of ER+ breast cancer and the frequency of our point mutation in recurrent and metastatic breast cancer led us to investigate whether it can play a role in breast cancer initiation. As mentioned previously, this mutation results in the constitutive activation of the estrogen receptor in the absence of estrogen stimulation. As ER activity promotes cellular proliferation and differentiation, we hypothesized that expression of the mutant would result in ductal hyperplasia and tumour development. We wanted to characterize the effect of the activated estrogen receptor on mammary gland development in virgin glands and through various stages of the reproductive cycle. To address this question, we crossed the $ESR1^{Y541S}$ strain with our MMTV-Cre mouse strain to generate a cohort expressing the point-activated mutant specifically in the mammary epithelium. Control mice are referred to as "ESR1" in the figures, as they carry the genetically engineered allele, however they do not express Cre. Therefore, they are effectively wild-type FVB mice. We sacrificed virgin mice at 6 weeks, 8 weeks, and 10 weeks of

age and extracted their mammary glands. A minimum of five mice for each time point, as well as both the control and experimental group, were generated and analyzed. Mammary gland whole-mounts were prepared. For each mammary gland prepared, the presence of the ESR1 allele and the presence/absence of Cre recombinase was confirmed using PCR analysis on tail pieces obtained after sacrifice. Both the ESR1 and Cre alleles were maintained in a heterozygous state in our experimental mice as this is more physiologically relevant (however breeders homozygous for the ESR1 allele were phenotypically normal and fertile). Whole-mounts of these mammary glands are presented in Figure 7A. No differences were observed between our control and experimental mice at the developmental time points chosen.

Since estrogen and the estrogen receptor play a critical role during pregnancy, we decided to also characterize any effect that the $ESR1^{Y541S}$ allele has on ductal development and alveolar expansion during other stages of development in non-virgin mice. We were interested to study whether the activated estrogen receptor has an effect on ductal outgrowth during pregnancy, lactation, and involution. For pregnancy, female mice were checked every morning for the presence or absence of a vaginal plug. The presence of a plug indicates that mating occurred overnight and pregnancy is likely. These mice were sacrificed 10 days after a plug was detected. Pregnancy was confirmed by checking the uterus for pups prior to mammary gland excision. For lactation, mice were sacrificed 24-48 hours after giving birth. For involution, pups were weaned and adult involuting mice were sacrificed 2, 4, or 6 days after weaning. Wholemounts for these developmental stages are presented in Figure 7B. No significant differences were observed during these stages either. This tells us that the expression of $ESR1^{Y541S}$ in the mammary epithelium is not sufficient to induce increased proliferation and hyperplasia. It likely will not contribute to the initiation of mammary tumours in this model.

Something that needs to be considered is the lack of confirmation of mutant ER and Cre expression in these mammary glands. In the future, IHC staining should be used to confirm and quantify the expression of Cre in sections of the mammary glands used for these experiments as controls. Another strategy to confirm that the mutant estrogen receptor is expressed and functional in these tissues would be to perform qPCR using cDNA prepared from RNA from these mammary glands. We could look at targets specifically upregulated by the mutant receptor, such as MMPs or other matrix-degrading proteins, or we could look at expression of putative ER targets to see if they are upregulated in the mutant relative to the control.

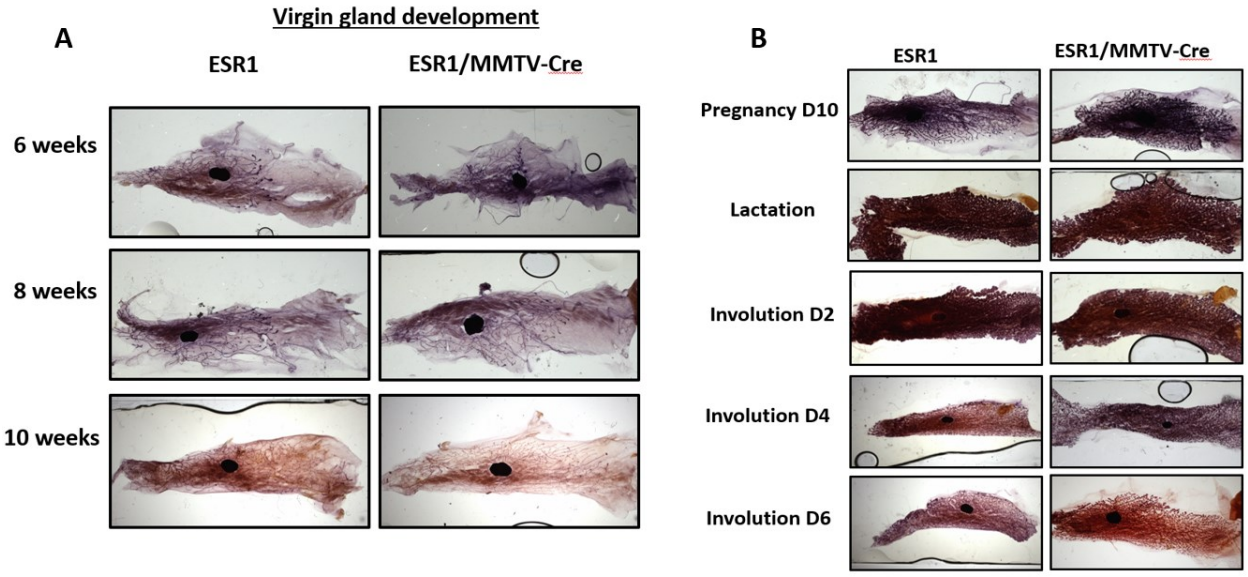

Figure 7. Representative wholemounts of mammary glands from the ESR1^{Y541S}/MMTV-Cre mice and controls. (A) Wholemounts from virgin mice. (B) Wholemounts of mice during different reproductive stages.

2.1.3 Characterizing tumour onset in the mammary-specific knock-in

We generated a cohort of approximately 20 mice to age to characterize tumour onset in this model. Five mice were aged past two years with no tumour development. The remaining cohort

will all reach 365 days old between now and September 2020. Thus far, no tumour development has been observed. See Figure 8.

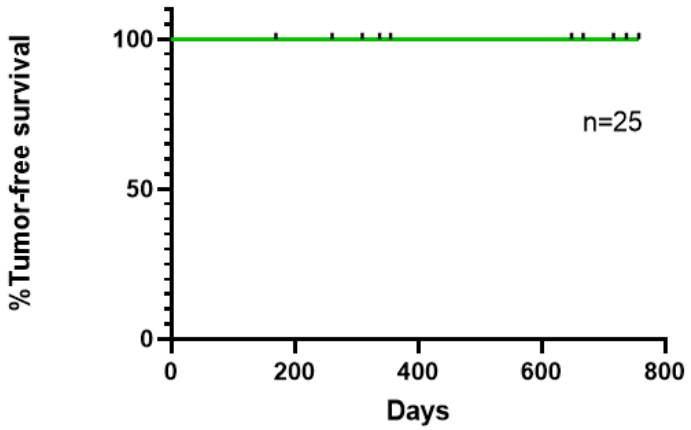

Figure 8. Kaplan-Meier showing no tumour development in the ESR1^{Y541S}/MMTV-Cre GEMM.

Table 1. Aging cohort and associated lack of tumour development.

Age of as August 21, 2020 (days)	Number of mice (ESR1^{Y541S}/MMTV-Cre)	Tumour development (Y/N)
730	6	N
425	3	N
407	5	N
379	1	N
330	3	N
239	2	N
222	2	N
218	1	N
192	1	N

2.1.4 Ovariectomy experiments

Since we did not see any dramatic effects of the ESR1^{Y541S} allele on ductal outgrowth in virgin mice or during various stages of reproduction in the mammary-specific model, we decided to ovariectomize these mice to deprive them of their major source of estrogen. The theory here

being that deprivation of estrogen will prevent any inhibitory signalling that may be occurring from the remaining wild-type estrogen receptor allele. We wanted to see if inhibiting the activity of WT ER, would result in ductal hyperplasia. Ten female control mice, 10 female ESR1^{Y541S}/MMTV-Cre mice and 10 female ESR1^{Y541S}/β-actin-Cre mice were ovariectomized at 4 weeks of age (done by Cynthia Lavoie and Vasilios Papavasiliou). Mice were sacrificed at 10 weeks and whole-mount analysis was performed. Alexandra Simond performed the necropsies for the ESR1^{Y541S}/β-actin-Cre mice. The ESR1^{Y541S}/MMTV-Cre mice did not display any ductal outgrowth at all when comparing to their counterparts that had not been ovariectomized (see Figure 9A). Interestingly, 100% of the ovariectomized ESR1^{Y541S}/β-actin-Cre female mice developed "normal" (normal for the strain) ductal outgrowth at 10 weeks of age. See Figure 9B. This tells us that the expression and activity of the estrogen receptor in the mammary epithelial cells alone is insufficient to drive ductal outgrowth. Since outgrowth occurred in the ESR1^{Y541S}/β-actin-Cre mice, we can say that estrogen/ER signalling in other supporting cell types in the mammary gland is critical for driving this process. This is not unexpected, as it has been shown before that hormonal signalling in mouse stromal cells is essential for epithelial growth in multiple different tissue types, such as the uterus, genital tract, and testicles[77]. In mice, uterine stromal cells express the estrogen receptor before uterine epithelial cells do[77]. Estrogen stimulation prior to epithelial expression of ER results in rapid expansion of uterine epithelial cells. In culture, estrogen stimulation of isolated uterine or vaginal epithelial cells does not result in increased proliferation unless these cells are co-cultured with their steroid receptor-positive stromal cell counterparts[77]. Similar results can be observed in other hormone-sensitive tissues as well. Tissue recombinant studies have been useful in investigating these questions.

Estrogen receptor knock-out mouse models have been critical to demonstrate this principle in the mammary gland. Using tissue recombination from ER knock-out and ER wild-type neonatal mice, it has been shown that ER signalling in stromal cells of the neonatal mammary gland is critical for mammary ductal outgrowth[77-79]. Hormonal regulation of epithelial development and proliferation is mediated not by the epithelial cells themselves, but by hormone receptor-positive stromal cells[77]. When ER knockout mammary fat pad is combined with ER wild-type mammary epithelial cells and transplanted into nude mice, ductal development does not occur[77]. This suggests that estrogen's main target is the estrogen receptor present in the stroma, and activation of this receptor results in some form of communication with epithelial cells that result in their proliferation (whether this is a ligand-receptor system, or another mechanism is unknown). This phenomenon has also been demonstrated in adult mice by injecting either wild-type or ER-knockout epithelial cells into both wild-type and ER-knockout cleared fat pads. ER-knockout epithelial cells isolated from mature mice developed into a full ductal tree when injected into a wild-type fat pad and mice were treated with estradiol[78]. Taken together, these results further reinforce our observations in our mouse models.

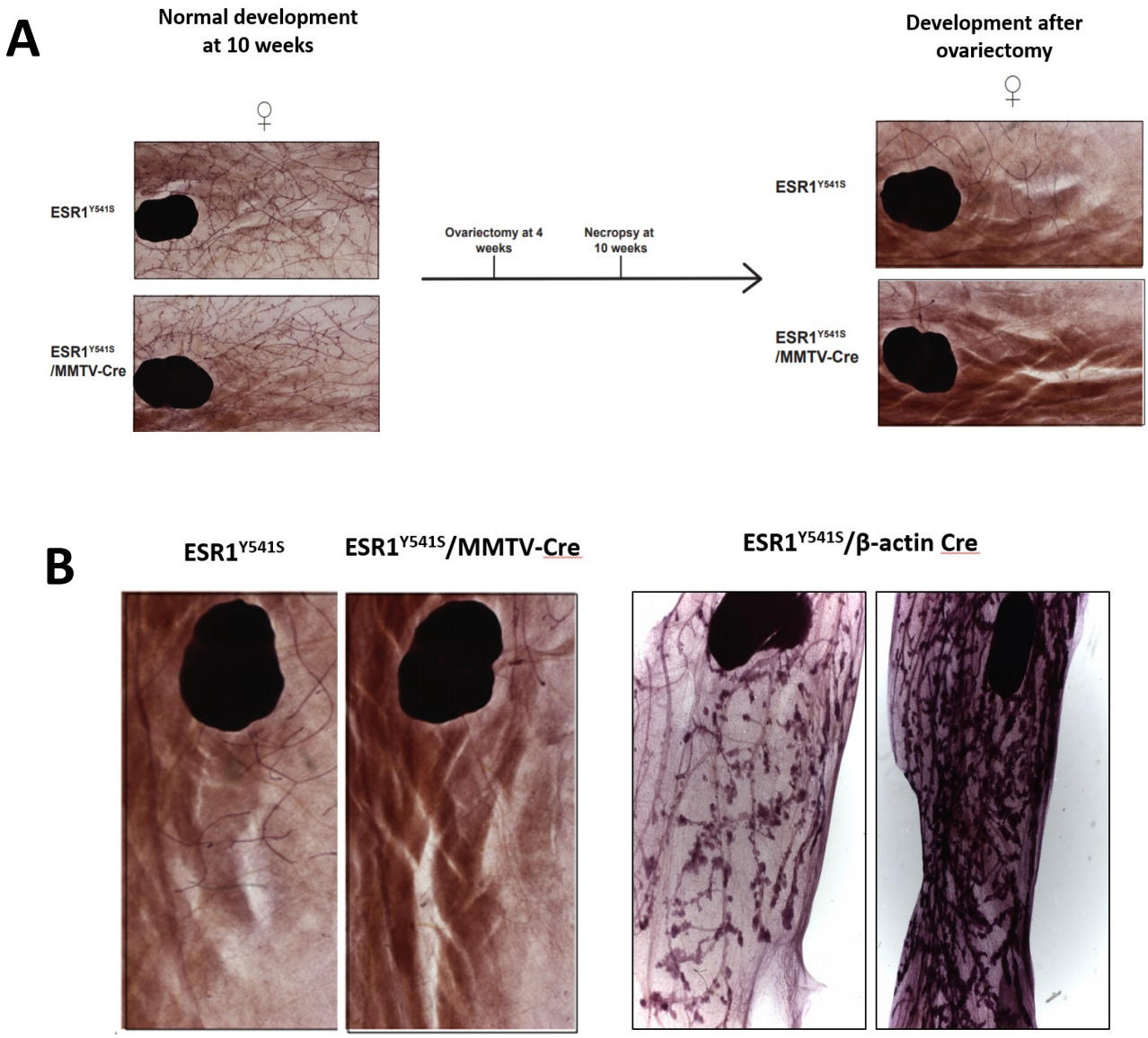

Figure 9. Representative wholemounts of 10 week-old mice post ovariectomy at 4 weeks. (A) Schematic of experiment and results for MMTV-Cre model. (B) Comparison of ductal outgrowth between MMTV-Cre and B-actin-Cre models. Two wholemounts are shown for the B-actin-Cre mice because there are two distinct phenotypes that occurred after ovariectomy.

2.1.5 Characterizing the effect of Tamoxifen on tumour development

Another strategy we used to attempt to induce tumour development in our ESR1^{Y541S}/MMTV-Cre model was to inhibit the wild-type estrogen receptor allele by treating the mice with Tamoxifen. A cohort of 20 control mice carrying the ESR1^{Y541S} allele but not Cre recombinase

as well as 20 ESR1^{Y541S}/MMTV-Cre mice were generated. At weaning, they were housed in the carcinogen room of the animal facility and were fed exclusively with Tamoxifen-containing chow (Envigo, TD.130860). These mice are all currently between 8-10 months of age and thus far, no tumour development has been observed.

2.1.6 Future directions

Although it is less clinically relevant than a heterozygous knock-in, it is possible that we may see a stronger phenotype if we generate mice homozygous for the activated receptor. This would double the dosage of activated ER and might allow us to better understand its effects. On top of this, the mice would not be carrying a wild-type copy of the ESR1 allele, therefore any negative feedback signalling that might be in play would be eliminated. We could also try and generate mice homozygous for Cre recombinase, simply to increase the dosage of Cre expression in the epithelial cells. Cre expression is known to not occur in 100% of cells it is targeted to, and expression can be leaky. By doubling the DNA coding for Cre recombinase, we might be able to induce a higher frequency of excision in these mice.

We will perform further analysis on the wholemounts that have been prepared, such as quantifying total length of the ductal tree and by counting the number of branching points. This could reveal a weak phenotype that we could not observe by using the microscope qualitatively. Histological analyses will reveal if there are any significant differences in cell types/proportion of cell types present in the mammary glands and stroma. We will use immunohistochemistry and Western blot to evaluate levels of various proteins that are implicated in tissue degradation or invasion and metastasis. We will also analyze gene expression in the mammary glands of these mice by qPCR, RNA-SCOPE, or RNA sequencing.

2.2 The role of ESR1^{Y541S} in mouse mammary gland tumorigenesis

ER+ breast cancer makes up roughly 70% of breast cancer diagnoses. Endocrine resistance occurs in over 20% of these cases, and the mechanisms underlying this are poorly understood. Many articles published in recent years describe a link between PI3K signalling and endocrine resistance. Our lab decided to investigate this potential relationship using several different strategies. Multiple unique systems were used to investigate this link between our point-activated ESR1 and the PI3K signalling axis. However, due to time restrictions and the shut down caused by COVID-19, the data presented in the following sections consists of a mix of experiments that are relatively incomplete. Future directions will be largely elaborated on for this section of the thesis.

2.2.1 The role of ESR1^{Y541S} in PI3K-driven mouse model of breast cancer

Due to the lack of understanding of the mechanisms underlying endocrine-resistant ER+ breast cancer and the links that have been made between PI3K and endocrine resistance, we decided to investigate the role of our activated ER in a model of PI3K-driven breast cancer. We generated a new strain of mice that express our point-activated ER (ESR1^{Y541S}), our point-activated PI3K (p110α^{H1047R}), and MMTV-Cre. Both ER and PI3K expression are driven by their endogenous promoters. This model will allow us to examine the effects that ESR1^{Y541S} plays in breast cancer development, progression, and metastasis. We wanted to address two preliminary questions: 1)

Does the expression of ESR1^{Y541S} affect the time of onset in this model? And 2) Does ESR1^{Y541S} expression affect metastasis of these tumours, and if so, how?

The p110α^{H1047R}/MMTV-Cre model has already been characterized by another lab member (Alexandra Simond, manuscript in preparation). Metastasis has not been observed in this strain before, however, they can spontaneously develop primary lung tumours. We hypothesize that co-expressing ESR1^{Y541S} with p110α^{H1047R} will likely not affect onset but will result in more aggressive tumours that display a metastatic phenotype. ESR1^{Y541S} expression may also increase the rate of growth of these tumours, as ER signalling is associated with proliferation and tissue expansion.

To investigate this, we generated cohorts of 20 mice for each of our groups: p110α^{H1047R}/ESR1^{Y541S}/MMTV-Cre, p110α^{H1047R}/MMTV-Cre, and ESR1^{Y541S}/MMTV-Cre. Mice were palpated weekly beginning from 90 days of age. The onset for this model is late and it also has a long latency period. Some mice had palpable tumours for months before they became measurable (measurable here is defined as 5 mm x 5 mm; the smallest accurate caliper measurement we could take). Because of time constraints, we defined tumour endpoint as 12 weeks post-measurability, unless the mouse progressed faster and reached ethical endpoint earlier. As shown in Figure 10, median palpable tumour onset for p110α^{H1047R}/ESR1^{Y541S}/MMTV-Cre was 367 days and 332 days for p110α^{H1047R}/MMTV-Cre, however this difference was not significant. The time difference between palpable tumour and measurable tumour between p110α^{H1047R}/ESR1^{Y541S}/MMTV-Cre and p110α^{H1047R}/MMTV-Cre mice was also not significant. We wanted to compare tumour growth rate in these tumours as there is an additional constitutively active proliferative gene present (ESR1^{Y541S}), however there is no significant difference between the two groups (see Figure 11AB). We thought it seemed

like p110α^{H1047R}/ESR1^{Y541S}/MMTV-Cre mice were developing more tumours in multiple mammary glands when compared to the p110α^{H1047R}/MMTV-Cre controls. However, upon quantification, the number of transformed mammary glands is trending toward significance. As more mice develop tumours, this trend may become statistically significant. In conclusion, the expression of our point-activated estrogen receptor does not seem to affect tumour onset or growth in this model.

The results of this mouse strain that are presented here are very preliminary. Future directions of this project include all the tissue, protein, and RNA analysis. At necropsy, tissues collected included tumour, adjacent mammary gland, lungs, brain, femur bone, liver, and tail piece. These samples have not been prepared for histology due to COVID-19. Histological analysis and IHC of the collected organs will allow us to investigate the metastatic profile of these tumours. It will be especially interesting to further look into the samples corresponding to longer latency periods, as this allows for more opportunity for metastasis to occur. Standard characterization of the tumours' profile will also be performed. This, at the basic level, will include Western blot, as well as IHC staining and evaluating RNA expression of various targets. These will include, but will not be limited to, the standard cancer pathway proteins such as Akt, PTEN, MAPK, and mTOR, as well as target genes of ESR1, such as pS2, Cyclin D1, and c-Myc. Genes involved in metastasis, such as matrix-degrading proteins (which have been shown to be upregulated by ESR1^{Y537S}) will also be investigated. Characterizing the gene expression and protein profile of these tumours will be key in allowing us to pursue this model further.

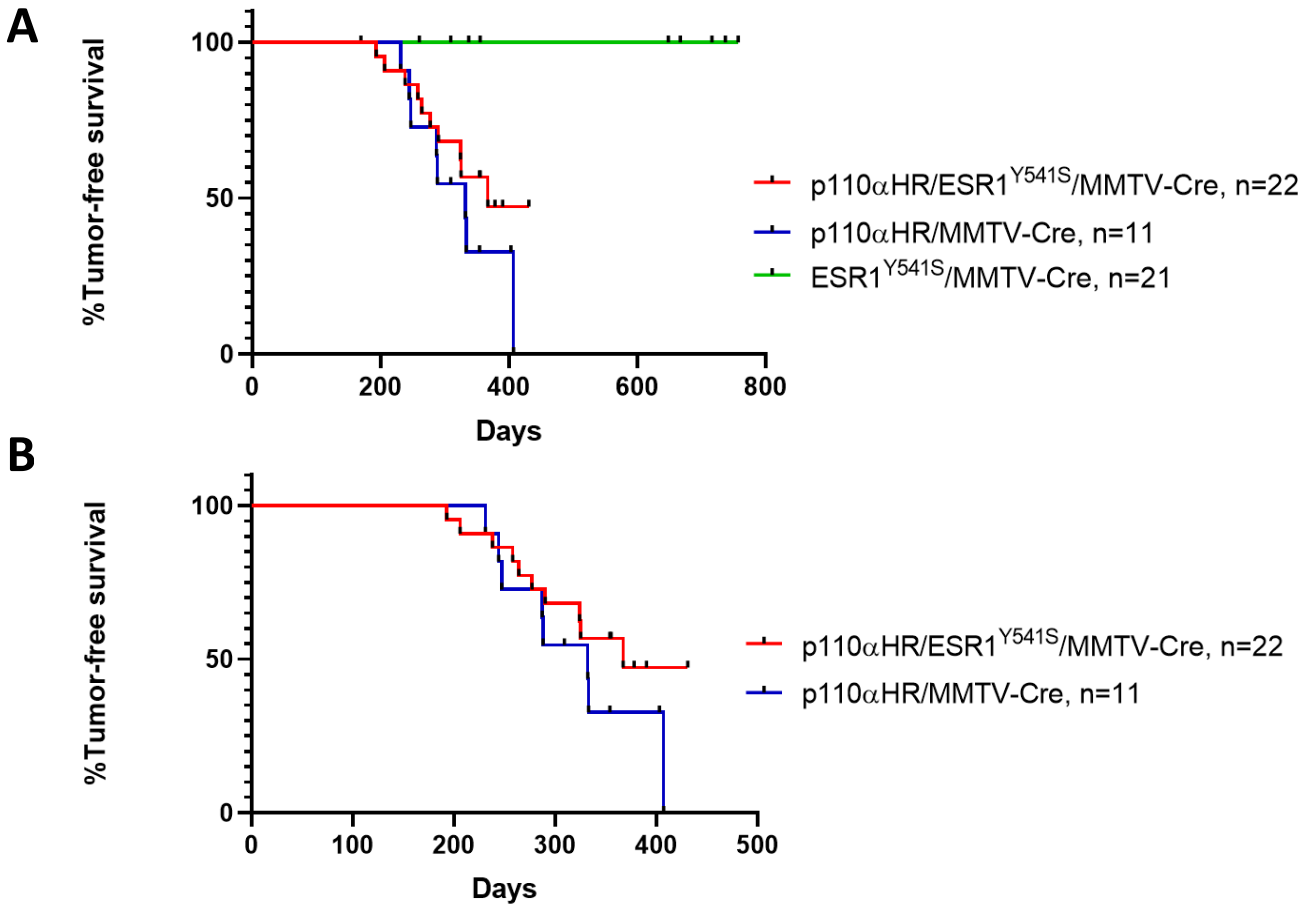

Figure 10. Kaplan-Meier curves of mammary tumour onset in p110α^{H1047R}/ESR1^{Y541S}/MMTV-Cre, p110α^{H1047}/MMTV-Cre, and ESR1^{Y541S}/MMTV-Cre cohorts. (A) Kaplan-Meier comparing experimental cohort with both control groups. (B) Kaplan-Meier curve comparing only p110α^{H1047R}/ESR1^{Y541S}/MMTV-Cre with p110α^{H1047R} /MMTV-Cre for better resolution.

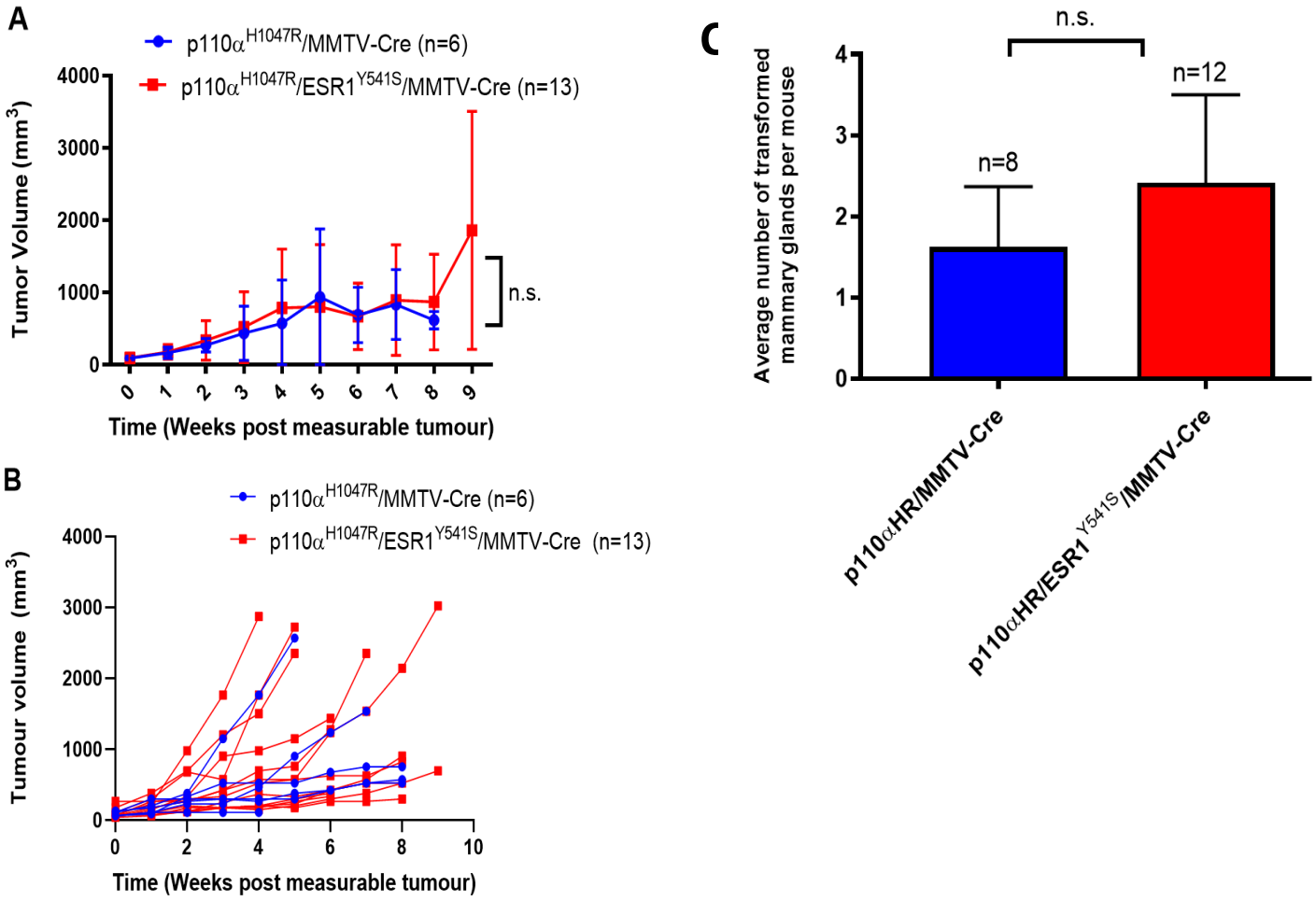

Figure 11. Characterization of tumour growth in the p110aHR/ESR1/MMTV-Cre mouse model. (A) Weekly quantification of average tumour volume from the time tumours were measurable (5 mm x 5 mm). (B) Quantification of growth of individual tumours. (C) Quantification of the number of transformed mammary glands at the defined endpoint.

2.2.2 Characterizing protein levels using the ESR1^{Y541S}/MTB/MIC mouse model

Another student in our lab has been working on the ESR1^{Y541S}/MTB/MIC model of breast cancer, as described in the introduction. This is relevant to the p110α^{H1047R}/ESR1^{Y541S}/MMTV-Cre mouse model, as MT activates similar downstream pathways as PI3K. Frozen tumour pieces were obtained from the student and Western blots were performed to characterize the protein levels in these tumours (Figure 12). No clear differences were observed, apart from phospho-MAPKs possibly being slightly elevated. This may indicate increased activation of this pathway, but further inquiry will be required to make any clear conclusions. Validation will include repetition of Western blots followed by quantification of expression thereafter. We blotted for Chi3L1, or chitinase-3-like protein 1, as peers in the lab have observed a possible correlation between ESR1 and Chi3L1 expression. Colleagues have also identified an estrogen response element upstream of the Chi3L1 gene, which suggests its expression is regulated, in part, by the estrogen receptor. Chi3L1 is a secreted glycoprotein and our lab has implicated Chi3L1 as being involved in the immune microenvironment and the inflammation response of tumours from a different model being studied (unpublished). However, no differences in expression were observed in this model.

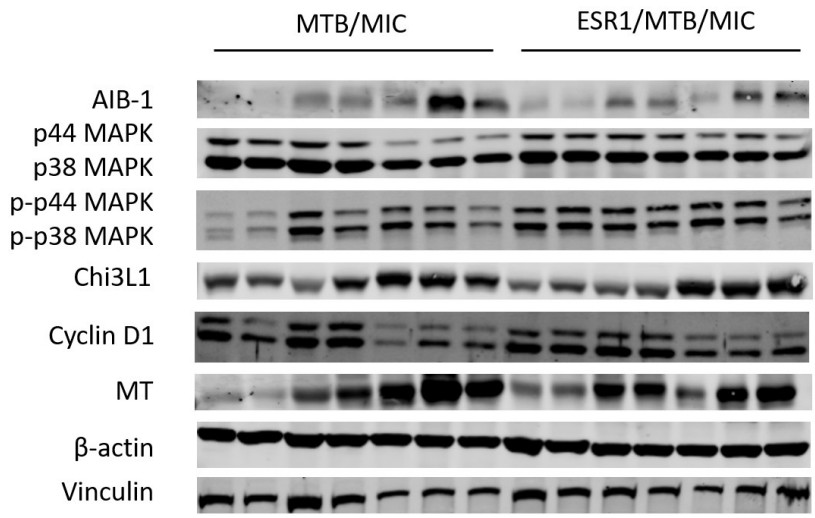

Figure 12. Immunoblots using ESR1Y541S/MTB/MIC and MTB/MIC tumour lysates.

RNA-sequencing analysis of these tumours revealed that cholesterol biosynthesis pathways may be upregulated in these mice. Next steps in this project involve investigating that further, first by blotting for HMG-CoA Reductase, which is the rate-limiting enzyme in the cholesterol biosynthesis pathway. The RNA sequencing also revealed a potential metabolic phenotype, where glycolysis is up-regulated and the TCA cycle is downregulated. However, again, this requires validation and investigation using Seahorse analysis or BIONOVA before any conclusions can be made.

2.2.3 Generation of a new syngeneic ESR1^{Y541S}/MMTV-PyVmT system to characterize the effect of ESR1 activation on breast cancer progression and metastasis

In collaboration with a colleague, Bin Xiao, we developed a new system to investigate the effects that the point-activated estrogen receptor has on mouse mammary gland tumour development and progression. As shown in Figure 13A, we crossed two mouse strains: our ESR1^{Y541S} with our MMTV-PyVmT strain. These mice develop tumours in roughly 90 days that are effectively PyVmT tumours. The cells carry the allele containing the mutant ER exon 9, but since there is no

Cre recombinase expressed, no excision occurs. Therefore, the tumours are MT tumours and express the wild-type estrogen receptor. When tumours reached maximum dimensions of 15 mm x 15 mm, the mice were sacrificed, and the tumours were collected. Cell lines were generated by dissociating the tissue and culturing them in the appropriate medium. We established these cell lines over several months. Virginie Sanguin-Gendreau helped with this process. We will use adenovirus infection with Ad-Cre (and Ad-Lacz as a control) to cause excision of the wild-type ER allele and allow expression of the mutant ER.

This tool is very useful because it is an isogenic system. Comparisons can be done between identical cells, where the only difference is the activation of ESR1. Any differences we see in phenotype, whether it be with *in vitro* assays or *in vivo*, we can attribute to the activation of $ESR1^{Y541S}$. This system will allow us to dissect specific effects that the expression of $ESR1^{Y541S}$ has in PyVmT cells.

Due to COVID-19, we have been unable to generate data with these cell lines. This section of the thesis will consist of future directions. We will begin by optimizing excision of the ESR1 allele. Preliminary tests show that 96 hours is sufficient for excision to occur using both Ad-Cre and Ad-CreGFP (see Figure 14). However, infection with Ad-CreGFP showed signs of cellular toxicity and immense cell death so we will be proceeding only with Ad-Cre. Additionally, it seems as though excision was lost 2 weeks after adenovirus infection. This might indicate that the activation of ESR1 is unfavourable for the cell, which is unexpected, so this will need to be pursued further. We will perform excision PCR analysis at multiple time points to pin-point when exactly excision is lost, and what impact this may have on future experiments. We plan to carry out *in vitro* assays to determine if ESR1 activation causes an effect on various parameters. These will include experiments such as invasion-migration assays, proliferation assays, and soft-

agar colony formation assays. If we find there is a phenotype, we will proceed with *in vivo* experiments using NSG mice. These will include tail vein injections to assess the capacity of the cells to colonize the lung, as well as orthotopic injections. We will assess primary tumour growth and metastasis using this technique. Our hypothesis is that cells infected with Ad-Cre will be more efficient at colonizing the lung, as well as having a higher capacity to metastasize from the mammary fat pad to other sites in the body.

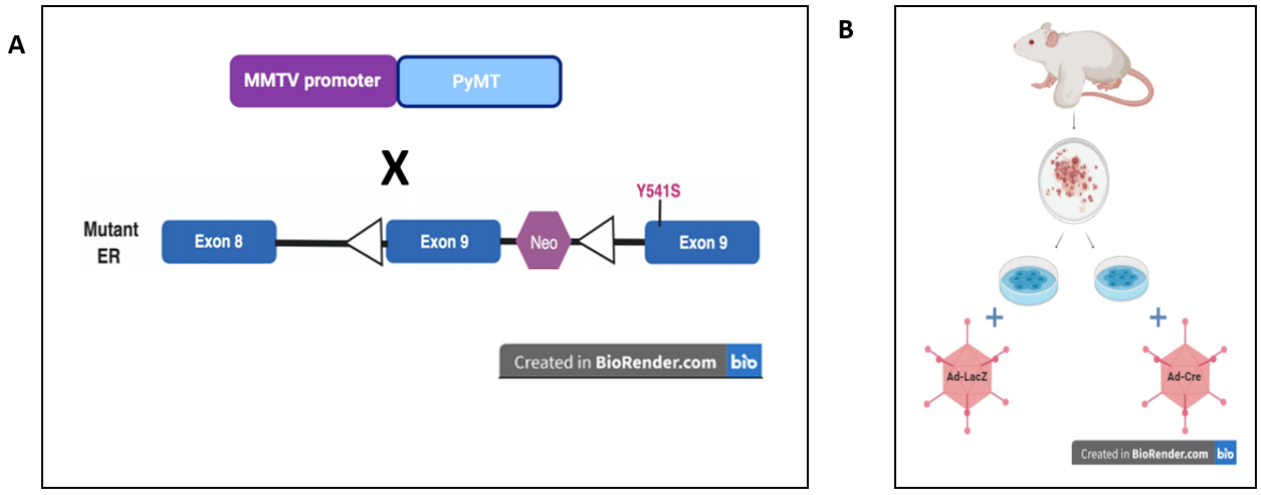

Figure 13. Schematics depicting the syngeneic system developed to study the effects of ESR1(Y541S). (A) Diagram depicting the genetic cross. (B) Schematic showing the experimental plan using this system.

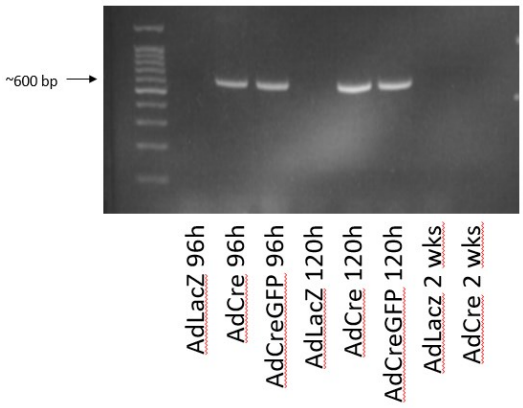

Figure 14. Agarose gel electrophoresis using the product of the excision PCR. Presence of a band indicates excision occurred. Excision was maintained 120 hours post-infection but was lost 2 weeks after infection.

3. MATERIALS AND METHODS

3.1 Animal work

3.1.1 Animal care and husbandry

All the mice were housed in the Animal Facility of the Goodman Cancer Research Centre. All mouse manipulation and experimental protocols were performed in accordance with the standards established by the McGill Facility Animal Care Committee and the Canadian Council on Animal Care. Strains were backcrossed and maintained on an FVB background. The ESR1^{Y541S} mouse strain was developed by Dr. Chen Ling.

3.1.2 Tumour monitoring

Mouse mammary tumour development and growth was monitored weekly by manual palpation and by taking caliper measurements. P110αHR/ESR1^{Y541S}/MMTV Cre mice were sacrificed when they reached endpoint or 12 weeks post-tumour measurability, whichever came first. PyVmT/ESR1^{Y541S} mice were sacrificed when they reached endpoint (defined as a total tumour burden of 6 cm^3 or by an individual tumour reaching 2.5 cm^3). Tumour volume was calculated using the formula: $[(4/3)\pi(\text{length}/2)\ (\text{width}/2)^2]$. Mice for development timepoints were sacrificed at the specified ages.

3.1.3 Necropsy and tissue collection

After cervical dislocation of ESR1^{Y541S}/MMTV Cre mice, mammary glands L4 and R4 were excised for embedding and wholemount, respectively. Glands L&R 2/3 were flash frozen for future protein/DNA/RNA extraction, and stored either at -80°C or in liquid nitrogen. Mammary gland, breast tumour, femur, liver and brain were collected specifically from p110αHR/ESR1^{Y541S}/MMTV Cre mice. Only breast tumour was collected from PyVMT/ESR1^{Y541S} mice for dissociation to generate cell lines. Tissues were fixed in either 4% PFA or 10% neutral buffered formalin, then embedded in paraffin. The fixed and embedded mammary glands were sectioned at 4 uM thickness and stained with H&E. Lungs were sectioned five times at 50 uM intervals in order to detect and score and metastatic lesions.

3.1.4 Mammary gland wholemounts

Animals were sacrificed at specific timepoints or stages of development. Mammary glands were collected and spread on glass slides. Slides were then incubated in 100% acetone for 24 hours up to 1 week. Glands were then incubated in 100% Harris Modified Hematoxylin (Fisher) overnight. Thicker glands (such as for involution and lactation) were stained for roughly 20 hours. Glands were then washed repeatedly with destaining solution (70% ethanol, 1% concentrated HCl) until the ducts were seen in sharp contrast to the fat pad. Glands were then dehydrated in 100% ethanol twice for 30 minutes. They were then incubated in xylenes for 48 hours. Permount mounting media (Fisher SP15-500) was then used to mount the mammary glands with a glass coverslip. Images of the mammary glands were taken at 3.5X and 17X magnification using an AxioZoom microscope which was equipped with a digital camera (Carl Zeiss, Inc.).

3.2 DNA Analyses

3.2.1 Tail DNA Extraction

Tail pieces (2-5mm long) were cut from 2-3 week old mice. Tails were digested in 500 uL of tail buffer (10 mM Tris pH 7.4, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) with 10 uL of Proteinase K (20 mg/mL) overnight at 55°C. The next day, 200 uL of 5M NaCl was added to each tube. Tubes were inverted 10 times and centrifuged at 12 000 rpm for 5 minutes. The supernatant was then transferred to a new tube and 1 mL of 100% ethanol was added to this. Tubes were inverted 10 times and were centrifuged at 12 000 rpm for 10 minutes at 4°C. The supernatant was aspirated and tubes were left open to dry at room temperature for 30 minutes. DNA pellets were then resuspended in 150 uL of TE buffer (10 mM Tris, 0.5 mM EDTA, pH 7.8). Resuspended DNA was then stored either at 4°C for long-term storage or at room temperature for short term storage.

3.2.2. Phenol-Chloroform Extraction

Cell pellets were lysed in 500 uL RLT Buffer (Qiagen, Cat #79216) containing 5 uL of β-mercaptoethanol on ice for 30 minutes. One volume of phenol-chloroform (25:24) was added to the lysate. Tubes were shaken and centrifuged at 12 000 rpm for 5 minutes. The aqueous top layer was transferred to a new tube. Phenol-chloroform was repeated. One volume of chloroform was then added to the tubes. Tubes were shaken and centrifuged at 12 000 rpm for 5 minutes. The aqueous top layer was transferred to a new tube and tubes were filled with 100% ethanol. Tubes were inverted and centrifuged at 12 000 rpm for 10 minutes at 4°C. DNA pellets were then washed twice with 100% ethanol, and once with 70% ethanol. Pellets were allowed to dry at room temperature for 1 hour. DNA was then resuspended in 200 uL of distilled water.

3.2.3 Genotyping

Mouse genotyping was carried out via PCR. Genotyping master mixes were prepared using 10X EasyTaq Buffer (Civic Bioscience AP111), 5 mM dNTPs, 10 uM primers, ddH_2O, and 0.5 units

of EasyTaq DNA Polymerase. Addtionally, DMSO was added for genotyping of MMTV Cre. PCR programs and primer sequences are listed in Table 2.

Table 2. Primer sequences and PCR programs for genotyping

Transgene	Primer sequences (5' to 3')	PCR program
Cre	(F): GCTTCTGTCCGTTTGCCG (R): ACTGTGTCCAGACCAGGC	1. 94°C – 2 mins 2. 94°C – 30s 3. 58°C – 45s 4. 72°C – 1 min Repeat 2-4, 29x 5. 72°C – 2 mins 6. 4°C – pause
ESR1	(F): GCCTTTGGAGTTGCTCATCC (R): TTGTAGAGATGCTCCATGCC	
PyVmT	(F): GGAAGCAAGTACTTCACAAGGG (R): GGAAAGTCACTAGGAGCAGG	
p110α HR		
ESR1 excision PCR	(F): TGTCTAGGCTTCAGAGAGCC (R): ATCTCCAGGAGCAGGTCGG Neo6: CACAACAGACAATCGGCTGC	

3.3 Tissue culture

3.3.1 Tumour dissociation and establishment of cell lines

Prior to sacrificing MT/ESR1 mice, dissociation media was prepared for each tumour by dissolving 25 mg of Collagenase B (Roche, 11088831001) and 25 mg of Dispase II ((Roche, 4942078001) in 10 mL DMEM (Wisent) containing 1% penicillin streptomycin (Wisent). This solution was filtered through a 40 uM filter prior to use with tumour material. Healthy tumours were excised from mice (see 4.1.3) and kept on ice in a 60 mm plate containing sterile 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Tumours were then homogenized using a tissue chopper (McIlwain). Tumour slurry was transferred to a 50 mL conical tube (Falcon) containing 10 mL of the pre-prepared dissociation media. Tubes were then incubated, rotating, at 39°C for 1-2 hours. The media-tumour mixture was then transferred to a 15 mL conical tube (Falcon) and spun at 800 rpm for 3 min at 4°C. The supernatant was aspirated, and the pellet was resuspended in 10 mL of ACK lysis buffer (150 mM NH_4Cl, 10

mM $KHCO_3$, 0.2 mM Na_2EDTA). This mixture was allowed to sit at room temperature for 1-2 minutes before being spun down again at 800 rpm for 3 min at 4°C. The supernatant was aspirated, and the pellet was washed with sterile 1X PBS. Tubes were spun down at 800 rpm for 3 min at 4°C. In total, pellets were washed 3X with PBS. Depending on the final size of the pellet, it was either plated in 1-4 100 mm NUNC plates (Thermofisher) in 8 mL of 5% SQ media (see 4.3.2 for SQ media recipe).

To establish primary cell lines, cells were allowed to grow until confluency and passed at decreasing densities until they could withstand being passed 1:4 and could survive a -80°C freeze-thaw cycle. If fibroblasts were detected, cells were cultured in 0-2% SQ media for 1 week to eliminate them.

3.3.2 General tissue culture and culture materials

Primary cells were cultured on Nunc plates in 5% DMEM media plus single-quot supplements (5% fetal bovine serum (Wisent), 5 µg/mL human insulin, 1µg/mL hydrocortisone, 5 ng/mL EGF, 35 µg/mL BPE, 50 µg/mL gentamycin, 1% penicillin streptomycin, 1% amphotericin).

Phoenix and 293T cells were cultured in DMEM containing 10% FBS, 50 µg/mL gentamycin, 1% penicillin streptomycin, 1% amphotericin.

Once cells reached confluency, cells were washed with sterile 1X PBS and treated with trypsin for 5-10 minutes at 37°C to detach cells from plates. The trypsin was neutralized using DMEM with 10% serum and cells were collected in 15 mL Falcon tubes. Tubes were then spun at 800 rpm for 3 minutes at 4°C. The supernatant was aspirated, and the pellet was resuspended in an appropriate volume of media for desired passage density. Cells were then plated on fresh Nunc plates.

All cells were cultured in incubators at 37°C with 5% CO_2.

3.3.3 Viable freezing of tumours and cell lines

To freeze cell lines for future use, cells were treated with trypsin as described above and pellets were resuspended in a solution of freezing media, composed of 90% FBS and 10% DMSO (v/v). The volume of freezing media used depended on the size of the pellet. Cell lines were frozen in

cryo vials (Thermofisher) at -80°C for short term storage, or in liquid nitrogen for long term storage.

To viably freeze tumour pieces for future use, tumours were excised from endpoint mice and placed in sterile 1X PBS on ice. Tumours were then cut into small pieces (~32mm^3) and placed in cryo vials with 1 mL of tumour freezing media. Tumour freezing media is composed of 50% FBS, 40% DMEM, and 10% DMSO (v/v). Tubes were then stored at -80°C for future use in either transplants or dissociations.

3.3.4 *in vitro* proliferation assays

On Day 0, cells were seeded in 96-well optical-bottom plates (Nunc) in quadruplicate in 100 µL of complete media. Transfected 293T cells were plated at 2000 cells per well, MT/ESR1 cells at 5000 cells per well? The plate was placed in an IncuCyte Live Cell Analysis Device (10x objective) (Essen Bioscience). Cellular proliferation was assessed by quantifying cellular confluency over a period of 96 hours. This was done using phase contrast scanning every 6 hours at 37°C and 5% CO_2. Images were analyzed using the IncuCyte ZOOM software.

3.3.5 *in vitro* migration and invasion assays

Boyden chambers (8 µM pores, BD Falcon) were placed in the wells of a 24-well plate (BD Falcon). The upper sides of the membranes were either coated with 50 µL of DMEM (in the case of migration), or with 50 µL of DMEM with 6% Matrigel (for invasion, VWR). The lower chamber contained 1 mL of complete growth media (5% SQ). The plates were then incubated at 37°C for 30 minutes to 1 hour to allow the media to warm and for the Matrigel to polymerize. MT/ESR1 cells were treated with trypsin as described above and counted using trypan blue and an automated cytometer. Cells were then aliquoted and resuspended to form a master-mix containing 300,000 cells/500 µL/well in DMEM containing 1% penicillin streptomycin. Cells were then plated in the upper chamber at a density of 300,000 cells per well in 500 µL. Plates were then incubated for 24 hours at 37°C. The Boyden chamber membranes were then fixed for 20 minutes in 10% neutral buffered formalin. They were then washed 3X in ddH_2O and stained for 20 minutes in crystal violet solution (Sigma). The Boyden chambers were then washed multiple times to remove excess stain. Cells remaining in the top chamber were removed using

cotton swabs. The chambers were then allowed to dry upside-down overnight. Images were taken of each chamber using the AxioZoom microscope at 14.6X magnification.

3.3.6 Adenovirus infection

The following protocol will refer to adenovirus infection done in 60 mm plates. MT/ESR1 cell lines were tested to optimize seeding density prior to infection protocol design. On day 0, cells were trypsinized and counted. Cells were plated according to dish size and cell line used (this varied from 1.0 x 10^6 to 3.0 x 10^6 for a 60 mm dish), in 1 mL of 5% SQ media. After, 1 mL of 0% SQ media was added, for a final FBS concentration of 2.5%. Adenovirus was added to each plate for an MOI of 25. Cells were distributed evenly and plates were placed back in the incubator overnight. Media was changed the following day. Cells were allowed to grow until the optimal gene excision was obtained, which was about 96 hours for this system. Excision PCR was used to confirm excision of the wild-type ESR1 allele.

3.4 Protein analyses

3.4.1 Cell lysis

Cells were washed three times with cold non-sterile 1X PBS. Plates were then scraped and lysed with 500 µL/1 mL of RIPA buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl). RIPA was aliquoted prior to use and protease inhibitors were added as follows: 1 µg/mL aprotinin, 1 µ/mL leupeptin, 1 mM sodium orthovanadate, and 1 mM PMSF. Cell pellets were resuspended in the RIPA solution and lysed for 30 minutes on ice. Cells were then centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was collected and the pellet discarded.

3.4.2 Tumour lysis

Both cell and tumour lysates are prepared similarly. Frozen mammary tumours were crushed to a powder in liquid nitrogen using a mortar and pestle. They were then lysed for 30 minutes on ice or rotating at 4°C using the same RIPA recipe as described in 4.4.1 with the same protease inhibitors. Lysates were centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was collected and the pellet discarded.

3.4.3 Immunoblotting

Bradford assay (BioRad) was used to quantify protein concentration of the cell and tumour lysates. Protein lysates were diluted to equal concentrations (4 μg/μL) using additional RIPA buffer. Additionally, 6X SDS-PAGE loading buffer (375 mM Tris pH 6.8, 10% SDS, 60% glycerol, 0.6 mM DTT, 0.06% bromophenol blue) was added to each lysate to a final concentration of 1X. Lysates were boiled at 95°C for 5 minutes and either used immediately or stored at -20°C for future use. Lysates were run on polyacrylamide gels of various concentrations (6-20%). Gels were then transferred onto PDVF membranes (Immobilon-FL, Millipore). Membranes were blocked for an hour, shaking, at room temperature in blocking solution (5% BSA, 1% casein in TBST). Membranes were then incubated overnight at 4°C in primary antibody solutions (see Table 3). Membranes were then washed 3X for 5 minutes with 1X TBST (137 mM NaCl, 2.7 mM KCl, 19 mM Tris base, 0.1% Tween20). The membranes were then incubated with fluorescently-labelled secondary antibodies for 1 hour at room temperature. Antibodies were diluted in blocking solution. Membranes were then washed again 3X for 5 minutes with TBST, and then scanned using the Odyssey CL-X infrared imaging system. Results were analyzed using Image Studio software (Licor Biosciences). Membranes were stored at 4°C in 1X TBS (137 mM NaCl, 2.7 mM KCl, 19 mM Tris base).

Table 3. Antibodies and dilutions used in Western blotting

Target	Company	Catalog number	Dilution
AIB-1	BD Transduction	611104	1:250
Akt	Cell Signaling	2920	1:1000
p-Akt	Cell Signaling	4506	1:1000
β-actin	Sigma Aldrich	A5441	
Chi3L1	Invitrogen	PA5-81355	
Cyclin D1	Cell Signaling	2978	1:1000
PyVmT	Dilworth Lab	ab750	1:500
p38 MAPK	Cell Signaling	8690	1:1000
p-p38 MAPK	Cell Signaling	4631	1:1000

p44 MAPK	Cell Signaling	9102	1:1000
p-p44 MAPK	Cell Signaling	4370	1:1000
pS2	Novus Biologicals	JM37-86	
Stat3	Cell Signaling	9132	1:1000
p-Stat3	Cell Signaling		1:1000
Tubulin	Cell Signaling	2148	1:1000
Vinculin	Chemicon	MAB3545	1:1000

3.5 RNA analyses

3.5.1 RNA extraction from cells

Cells were washed 3X with cold non-sterile 1X PBS then scraped in 500 µL-1 mL of RLT buffer (Qiagen). Total RNA was then extracted from samples using an RNEasy Midi kit (Qiagen) according to manufacturer's instructions. Total RNA was quantified and quality was verified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.).

3.5.2 RNA extraction from tumours

Flash-frozen mammary tumour samples were crushed in liquid nitrogen with a mortar and pestle. Total RNA was then extracted from using the same RNEasy Midi kit (Qiagen) according to manufacturer's instructions. RNA was quantified using the NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.).

3.5.3 cDNA synthesis and qRT-PCR

cDNA was prepared by reverse transcribing RNA isolated from cells or tumours using the TransGen Biotech kit (AT341). qRT-PCR was performed using a Roche LC480 SYBR Green RT-PCR kit (Roche). Samples were run using a LightCycler (Roche) and each reaction was run in triplicate, minimally. The resulting crossing point values were normalized against GAPDH or

B-actin to generate the relative transcript levels using the formula: 2^(average GAPDH crossing point - average target X crossing point). Data not included in thesis.

3.6 Statistics

All figures and associated statistical analyses were made and performed using Prism software. (Graphpad, San Diego, CA) Unless otherwise indicated, a two-tailed unpaired Student's t-test was used to determine statistical significance.

4. CONCLUSIONS AND FUTURE DIRECTIONS

Mutation of the estrogen receptor in hormone-receptor positive breast cancer is very common after endocrine therapy. This is typically the first line of defense for patients diagnosed with this disease. While it is very effective and prognoses tend to be very good when the disease is caught early, there is still a large proportion of patients who present with recurrent or metastatic breast cancer. In these cases, mutated forms of the estrogen receptor are commonly selected for by the pharmaceuticals used to treat the primary tumour. Mutant, activated forms of the estrogen receptor are favorable for the cell to maintain as they result in constitutive proliferative signalling in the cell. This, combined with decreased apoptosis and many other factors, gives the cell harboring these mutations a survival advantage relative to other cells in the tumour that do not harbor mutant estrogen receptors. The specific mutant we discuss in this thesis, $ESR1^{Y537S}$, regulates a different set of genes compared to the wild-type receptor. While there is overlap, it has been shown that this form of ER is involved in the transcription of many hundreds of new genes. Many of these have been implicated in pathways involving cell cycle progression, matrix degradation, angiogenesis, and cell survival. It is also possible and likely that some of the newly-transcribed genes play important roles in organ colonization and survival. Metastasis requires that the cell is able to enter the blood or lymphatic system, survive long enough to get to the new tissue, exit the circulatory system in question, and establish itself in a new location. Many tumour cells are unable to do this prior to EMT, therefore it is reasonable to assume that these processes are enhanced when the estrogen receptor is mutated and capable of transcribing a new set of genes.

To summarize the findings of this thesis, the expression of our novel point-activated estrogen receptor mutant, $ESR1^{Y541S}$, does not seem to impact mammary gland development in virgin mice or throughout various stages of reproductive development. Additionally, it does not affect the onset of tumorigenesis in the activated PI3K model we used. The tumour growth rate is also unaffected in these mice. However, the bigenic mice may present with a higher number of transformed mammary glands when comparing to the monogenic control, but more replicates are required before stating definitive conclusions.

Future directions will include characterizing the histological make up of these tumours and investigating the metastatic phenotype. It will be interesting to see how expression of our activated ER effects protein levels in these tumours and what the ramifications of this might be. As mentioned in the introduction, our p110α^{H1047R}/MMTV-Cre mice present with non-metastatic tumours. We hypothesize that expression of our mutant ER will result in metastasis to the brain, bone, liver or lungs, as these are the most common sites of breast tumour metastasis. Sections of our bigenic lungs, brain, liver and bone will be prepared and evaluated for metastases. We will also perform protein and RNA analyses of these tumours and mammary glands.

Down the line, it will be interesting to pursue investigating endocrine resistance in this model. The first thing to do would be to confirm that these mice are indeed resistant to endocrine therapy, for example with Tamoxifen treatment. If they are intrinsically resistant to therapy, then treatment should not affect the onset or growth of mammary tumours in this model. This is expected as the mutant form of ER appears after endocrine therapy in the majority of cases. We could try to induce resistance by mimicking a standard treatment protocol for early stage ER+ breast cancer if these mice do not present with intrinsic resistance to hormone therapy. Following this, we could treat with a dual-pronged approach, whereby we treat the mice with a PI3K inhibitor as well as another endocrine therapy. It would be interesting to investigate how treatment affects downstream pathway activation and inhibition, and whether or not other tumour drivers appear and compensate for this inhibition. In the future, this model may prove useful in informing therapeutic strategies for anti-estrogen resistance in recurrent and metastatic breast cancers that express mutant estrogen receptors.

Long term future plans with this project include a new murine model that is in the process of being generated. We aim to generate an ER+ breast cancer model by linking the expression of AIB-1 to the TetO system (described already for the ESR1/MTB/MIC cross). This dox-inducible system will result in AIB-1, a major coactivator for ER expression, being highly expressed specifically in the mammary epithelium. After initial characterization, we will see if this model results in ER+ breast tumours. Later plans include treatment with Tamoxifen to induce endocrine resistance and mutation of ESR1. We also plan to cross this with our activated ER to try and increase expression and allow us to investigate associated phenotypes.

For our isogenic ESR1^{Y541S} /MMTV-PyVmT cell lines, we are currently in the process of optimizing excision PCR conditions and we are performing preliminary invasion-migration assays and qPCR validations. Detailed future directions are provided in the results section. Lastly, we plan to carry out a non-directed CRISPR screen to identify any tumour suppressors that may be implicated in the pathways we are specifically interested in.

References

1 Brenner, D. R. *et al.* Projected estimates of cancer in Canada in 2020. *Canadian Medical Association Journal* **192**, E199-E205, doi:10.1503/cmaj.191292 (2020).

2 Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2020. *CA: A Cancer Journal for Clinicians* **70**, 7-30, doi:10.3322/caac.21590 (2020).

3 Hassiotou, F. & Geddes, D. Anatomy of the human mammary gland: Current status of knowledge. *Clinical anatomy (New York, N.Y.)* **26**, 29-48, doi:10.1002/ca.22165 (2013).

4 Brock, E. G. & Long, L. Breast feeding. *Obstetrics, Gynaecology & Reproductive Medicine* **29**, 136-140, doi:10.1016/j.ogrm.2019.02.003 (2019).

5 Hennighausen, L. & Robinson, G. W. Information networks in the mammary gland. *Nature reviews. Molecular cell biology* **6**, 715-725 (2005).

6 Hanahan, D. & Weinberg, R. A. The Hallmarks of Cancer. *Cell* **100**, 57-70, doi:10.1016/S0092-8674(00)81683-9 (2000).

7 Pasculli, B., Barbano, R. & Parrella, P. Epigenetics of breast cancer: Biology and clinical implication in the era of precision medicine. *Seminars in cancer biology* **51**, 22-35, doi:10.1016/j.semcancer.2018.01.007 (2018).

8 Russo, J. (Springer, Switzerland, 2016).

9 Makki, J. Diversity of breast carcinoma: Histological subtypes and clinical relevance. *Clinical Medicine Insights: Pathology* **8**, 23-31, doi:10.4137/CPath.s31563 (2015).

10 Rejon, C., Al-Masri, M. & McCaffrey, L. Cell Polarity Proteins in Breast Cancer Progression. *Journal of Cellular Biochemistry* **117**, 2215-2223, doi:10.1002/jcb.25553 (2016).

11 Pastushenko, I. *et al.* Identification of the tumour transition states occurring during EMT. *Nature* **556**, 463-468, doi:10.1038/s41586-018-0040-3 (2018).

12 Khalil, A. A. *et al.* Collective invasion in ductal and lobular breast cancer associates with distant metastasis. *Clinical & Experimental Metastasis : Official Journal of the Metastasis Research Society* **34**, 421-429, doi:10.1007/s10585-017-9858-6 (2017).

13 Cheung, K. J., Gabrielson, E., Werb, Z. & Ewald, A. J. Collective invasion in breast cancer requires a conserved basal epithelial program. *Cell* **155**, 1639-1651, doi:10.1016/j.cell.2013.11.029 (2013).

14 Sanpaolo, P., Barbieri, V. & Genovesi, D. Prognostic value of breast cancer subtypes on breast cancer specific survival, distant metastases and local relapse rates in conservatively managed early stage breast cancer: A retrospective clinical study. *European Journal of Surgical Oncology* **37**, 876-882, doi:10.1016/j.ejso.2011.07.001 (2011).

15 Perou, C. M. *et al.* Molecular portraits of human breast tumours. *Nature* **406**, 747-752 (2000).

16 Parker, J. S. *et al.* Supervised risk predictor of breast cancer based on intrinsic subtypes. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **27**, 1160-1167, doi:10.1200/JCO.2008.18.1370 (2009).

17 Dabydeen, S. A. & Furth, P. A. Genetically engineered ERα-positive breast cancer mouse models. *Endocrine-Related Cancer* **21**, R195-R208, doi:10.1530/ERC-13-0512 (2014).

18 Ignatiadis, M. & Sotiriou, C. Luminal breast cancer: from biology to treatment. *Nature reviews. Clinical oncology* **10**, 494-506, doi:10.1038/nrclinonc.2013.124 (2013).

19 Klinge, C. M. Estrogen receptor interaction with estrogen response elements. *Nucleic acids research* **29**, 2905-2919 (2001).

20 Nilsson, S. *et al.* Mechanisms of Estrogen Action. *PHYSIOLOGICAL REVIEWS* **81**, 1535-1566 (2001).
21 Lannigan, D. A. Estrogen receptor phosphorylation. *Steroids* **68**, 1-9, doi:10.1016/S0039-128X(02)00110-1 (2003).
22 Rastinejad, F., Huang, P., Chandra, V. & Khorasanizadeh, S. Understanding nuclear receptor form and function using structural biology. *Journal of molecular endocrinology* **51**, T1-T21, doi:10.1530/JME-13-0173 (2013).
23 McEwan, I. J. & Kumar, R. *Online access with purchase: Springer (t)* (Springer, Cham, 2015).
24 Quirke, V. M. Tamoxifen from Failed Contraceptive Pill to Best-Selling Breast Cancer Medicine: A Case-Study in Pharmaceutical Innovation. *Frontiers in pharmacology* **8**, 620, doi:10.3389/fphar.2017.00620 (2017).
25 Zhang, X. *Cancer drug discovery and development* (Humana Press, Cham, Switzerland, 2019).
26 Johnston, S. R. Endocrinology and hormone therapy in breast cancer: selective oestrogen receptor modulators and downregulators for breast cancer - have they lost their way? *Breast cancer research : BCR* **7**, 119-130 (2005).
27 Parker, M. G. Action of "pure" antiestrogens in inhibiting estrogen receptor action. *Breast Cancer Research and Treatment* **26**, 131-137, doi:10.1007/BF00689686 (1993).
28 Howell, A., Osborne, C. K., Morris, C. & Wakeling, A. E. ICI 182,780 (Faslodex): development of a novel, "pure" antiestrogen. *Cancer* **89**, 817-825 (2000).
29 Burstein, H. J. *Oxford American pocket notes* (Oxford University Press, Oxford ;, 2011).
30 Buzdar, A. U. *Oxford oncology library* (Oxford University Press, Oxford ;, 2007).
31 Tan, S.-H. & Wolff, A. C. Luteinizing Hormone-Releasing Hormone Agonists in Premenopausal Hormone Receptor-Positive Breast Cancer. *Clinical Breast Cancer* **7**, 455-464, doi:10.3816/CBC.2007.n.002 (2007).
32 Osborne, C. K. *Cancer Treatment and Research, 0927-3042 ; 39* (Springer US, Boston, MA, 1988).
33 Tolkach, Y., Joniau, S. & Van Poppel, H. Luteinizing hormone-releasing hormone (LHRH) receptor agonists vs antagonists: a matter of the receptors? *BJU International* **111**, 1021-1030, doi:10.1111/j.1464-410X.2013.11796.x (2013).
34 Reinert, T. s. M. D., Gonçalves, R. M. D. M. P. & Bines, J. M. D. P. Implications of ESR1 Mutations in Hormone Receptor-Positive Breast Cancer. *Current Treatment Options in Oncology* **19**, 1-13, doi:10.1007/s11864-018-0542-0 (2018).
35 Larionov, A. *Resistance to targeted anti-cancer therapeutics, 2196-551X ; volume 8* (Springer, Cham, 2015).
36 Prosperi, J. R. *Resistance to targeted anti-cancer therapeutics, 2196-551X ; volume 16* (Springer, Cham, Switzerland, 2017).
37 Fu, X. *et al.* Abstract 2290: Crosstalk between ER and PI3K signaling pathways in human luminal type breast cancer and its therapeutic implications. *Cancer Research* **71**, 2290, doi:10.1158/1538-7445.AM2011-2290 (2011).
38 Clarke, R., Tyson, J. J. & Dixon, J. M. Endocrine resistance in breast cancer - An overview and update. *Molecular and Cellular Endocrinology* **418**, 220-234, doi:10.1016/j.mce.2015.09.035 (2015).

39 Fu, X., Osborne, C. K. & Schiff, R. Biology and therapeutic potential of PI3K signaling in ER+/HER2-negative breast cancer. *The Breast: Supplement 2* **22**, S12-S18, doi:10.1016/j.breast.2013.08.001 (2013).
40 Fu, X. *et al.* Overcoming endocrine resistance due to reduced PTEN levels in estrogen receptor-positive breast cancer by co-targeting mammalian target of rapamycin, protein kinase B, or mitogen-activated protein kinase kinase. *Breast Cancer Research* **16**, 1-17, doi:10.1186/s13058-014-0430-x (2014).
41 Fu, X., De Angelis, C., Veeraraghavan, J., Osborne, C. K. & Schiff, R. in *Estrogen Receptor and Breast Cancer: Celebrating the 60th Anniversary of the Discovery of ER* (ed Xiaoting Zhang) 265-307 (Springer International Publishing, 2019).
42 Schiff, R., Massarweh, S., Shou, J. & Osborne, C. K. Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. *Clinical cancer research : an official journal of the American Association for Cancer Research* **9**, 447S-454S (2003).
43 Robinson, D. R. *et al.* Activating ESR1 mutations in hormone-resistant metastatic breast cancer. *Nature genetics* **45**, 1446-1451, doi:10.1038/ng.2823 (2013).
44 Toy, W. *et al.* ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nature genetics* **45**, 1439-1445, doi:10.1038/ng.2822 (2013).
45 Jeselsohn, R. *et al.* Allele-Specific Chromatin Recruitment and Therapeutic Vulnerabilities of ESR1 Activating Mutations. *Cancer cell* **33**, 173-186, doi:10.1016/j.ccell.2018.01.004 (2018).
46 Jeselsohn, R., Buchwalter, G., De Angelis, C., Brown, M. & Schiff, R. ESR1 mutations—a mechanism for acquired endocrine resistance in breast cancer. *Nature reviews. Clinical oncology* **12**, 573-583, doi:10.1038/nrclinonc.2015.117 (2015).
47 Toy, W. *et al.* Activating ESR1 Mutations Differentially Affect the Efficacy of ER Antagonists. *Cancer discovery* **7**, 277-287, doi:10.1158/2159-8290.CD-15-1523 (2017).
48 Lesley-Ann, M. *et al.* Discovery of naturally occurring ESR1 mutations in breast cancer cell lines modelling endocrine resistance. *Nature Communications* **8**, 1-15 (2017).
49 Spoerke, J. M. *et al.* Heterogeneity and clinical significance of ESR1 mutations in ER-positive metastatic breast cancer patients receiving fulvestrant. *Nature Communications* **7**, doi:10.1038/ncomms11579 (2016).
50 Michael, R. G., Martin, T. & Klaus, O. *Frontiers Research Topics* (Frontiers Media SA, 2015).
51 Guerrero-Zotano, A., Mayer, I. A. & Arteaga, C. L. PI3K/AKT/mTOR: role in breast cancer progression, drug resistance, and treatment. *Cancer metastasis reviews* **35**, 515-524, doi:10.1007/s10555-016-9637-x (2016).
52 Utermark, T. *et al.* The p110α and p110β isoforms of PI3K play divergent roles in mammary gland development and tumorigenesis. *Genes & development* **26**, 1573-1586, doi:10.1101/gad.191973.112 (2012).
53 Schaffhausen, B. S. & Roberts, T. M. Lessons from polyoma middle T antigen on signaling and transformation: A DNA tumor virus contribution to the war on cancer. *Virology* **384**, 304-316, doi:10.1016/j.virol.2008.09.042 (2009).
54 Hiles, I. D. *et al.* Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell* **70**, 419-429 (1992).
55 Hosford, S. R. *et al.* Combined Inhibition of Both p110α and p110β Isoforms of Phosphatidylinositol 3-Kinase Is Required for Sustained Therapeutic Effect in PTEN-

Deficient, ER + Breast Cancer. *Clinical Cancer Research* **23**, 2795-2805, doi:10.1158/1078-0432.CCR-15-2764 (2017).
56 Todd, W. M., Brent, N. R., Joan, T. G. & Carlos, L. A. Mutations in the phosphatidylinositol 3-kinase pathway: role in tumor progression and therapeutic implications in breast cancer. *Breast Cancer Research* **13**, 224 (2011).
57 Tserga, A., Chatziandreou, I., Michalopoulos, N. V., Patsouris, E. & Saetta, A. A. Mutation of genes of the PI3K/AKT pathway in breast cancer supports their potential importance as biomarker for breast cancer aggressiveness. *Virchows Archiv : an international journal of pathology* **469**, 35-43, doi:10.1007/s00428-016-1938-5 (2016).
58 Ciruelos Gil, E. M. Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive breast cancer. *Cancer Treatment Reviews* **40**, 862-871, doi:10.1016/j.ctrv.2014.03.004 (2014).
59 Creighton, C. J. *et al.* Proteomic and transcriptomic profiling reveals a link between the PI3K pathway and lower estrogen-receptor (ER) levels and activity in ER+ breast cancer. *Breast cancer research : BCR* **12**, R40, doi:10.1186/bcr2594 (2010).
60 Bosch, A. *et al.* PI3K inhibition results in enhanced estrogen receptor function and dependence in hormone receptor–positive breast cancer. *Science Translational Medicine* **7**, 283ra251, doi:10.1126/scitranslmed.aaa4442 (2015).
61 Sanchez, C. G. *et al.* Preclinical modeling of combined phosphatidylinositol-3-kinase inhibition with endocrine therapy for estrogen receptor-positive breast cancer. *Breast Cancer Research* **13** (2011).
62 Bachelot, T. *et al.* Randomized phase II trial of everolimus in combination with tamoxifen in patients with hormone receptor-positive, human epidermal growth factor receptor 2-negative metastatic breast cancer with prior exposure to aromatase inhibitors: a GINECO study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **30**, 2718-2724, doi:10.1200/JCO.2011.39.0708 (2012).
63 deGraffenried, L. A. *et al.* Inhibition of mTOR activity restores tamoxifen response in breast cancer cells with aberrant Akt Activity. *Clinical cancer research : an official journal of the American Association for Cancer Research* **10**, 8059-8067 (2004).
64 Wolff, A. C. *et al.* Randomized phase III placebo-controlled trial of letrozole plus oral temsirolimus as first-line endocrine therapy in postmenopausal women with locally advanced or metastatic breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **31**, 195-202, doi:10.1200/JCO.2011.38.3331 (2013).
65 Greenow, K. R. & Smalley, M. J. Overview of Genetically Engineered Mouse Models of Breast Cancer Used in Translational Biology and Drug Development. *Current Protocols in Pharmacology* **70**, 1-14, doi:10.1002/0471141755.ph1436s70 (2015).
66 John, N. H. & William, J. M. Transgenic mouse models of human breast cancer. *Oncogene*, 6130-6137 (2000).
67 Kinross, K. M. *et al.* An activating Pik3ca mutation coupled with Pten loss is sufficient to initiate ovarian tumorigenesis in mice. *The Journal of clinical investigation* **122**, 553-557, doi:10.1172/JCI59309 (2012).
68 Gross, L. A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)* **83**, 414-421 (1953).

69 Rowe, W. P. The epidemiology of mouse polyoma virus infection. *Bacteriological reviews* **25**, 18-31 (1961).
70 Guy, C. T., Cardiff, R. D. & Muller, W. J. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Molecular and cellular biology* **12**, 954-961 (1992).
71 Treisman, R., Novak, U., Favaloro, J. & Kamen, R. Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein. *Nature* **292**, 595-600 (1981).
72 Rao, T. *Investigating the phosphatidylinositol 3' kinase signalling pathway in transgenic mouse models of breast cancer*, McGill University Libraries, (2015).
73 Schaffhausen, B. S., Dorai, H., Arakere, G. & Benjamin, T. L. Polyoma virus middle T antigen: relationship to cell membranes and apparent lack of ATP-binding activity. *Molecular and cellular biology* **2**, 1187-1198 (1982).
74 Lin, E. Y. *et al.* Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *The American journal of pathology* **163**, 2113-2126 (2003).
75 Trisha, R. *et al.* Inducible and coupled expression of the polyomavirus middle T antigen and Cre recombinase in transgenic mice: an in vivo model for synthetic viability in mammary tumour progression. *Breast Cancer Research* **16**, R11 (2014).
76 Arendt, L. M. & Kuperwasser, C. Form and Function: how Estrogen and Progesterone Regulate the Mammary Epithelial Hierarchy. *Journal of Mammary Gland Biology and Neoplasia* **20**, 9-25, doi:10.1007/s10911-015-9337-0 (2015).
77 Cunha, G. R. *et al.* Elucidation of a Role for Stromal Steroid Hormone Receptors in Mammary Gland Growth and Development Using Tissue Recombinants. *Journal of Mammary Gland Biology and Neoplasia* **2**, 393-402, doi:10.1023/A:1026303630843 (1997).
78 Mueller, S. O. Mammary Gland Development in Adult Mice Requires Epithelial and Stromal Estrogen Receptor *Endocrinology* **143**, 2357-2365, doi:10.1210/en.143.6.2357 (2002).
79 Feng, Y., Manka, D., Wagner, K.-U. & Khan, S. A. Estrogen Receptor-α Expression in the Mammary Epithelium Is Required for Ductal and Alveolar Morphogenesis in Mice. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 14718-14723 (2007).