Persistent MCF-7 spheroids exhibit heterogenous phenotypic characteristics and responses to clinically relevant concentrations of Tamoxifen

James Meterissian

Faculty of Medicine

Division of Experimental Medicine

McGill University, Montreal

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ABSTRACT

Breast cancer is the most frequently diagnosed cancer among women worldwide, accounting for over 500,000 deaths annually. Approximately 70% of cases are luminal breast cancers, characterized primarily by estrogen receptor expression. ER has been shown to drive tumorigenesis through the upregulation of proteins such as cyclin D1 and various growth factors, contributing to cell survival, tissue proliferation, and angiogenesis. Tamoxifen, a selective estrogen receptor modulator, is the most widely used form of adjuvant endocrine therapy for ER-positive breast cancer, having been shown to reduce recurrence by 50% and annual mortality by approximately 30%. Despite its successful use in clinical settings, 20-40% of patients will experience relapse or metastatic progression due to intrinsic or acquired resistance to Tamoxifen. Over the past two decades, extensive investigation into the biological mechanisms underlying Tamoxifen resistance have been performed, though more research is required to overcome this important hurdle. Additionally, the optimal concentration range for Tamoxifen, and its metabolites' pharmacokinetic properties remain to be established in humans.

To address these gaps in knowledge, my thesis project aims to characterize and strengthen our understanding of phenotypic profiles associated with eventual Tamoxifen resistance in order to target them ahead of time to provide more effective anti-cancer treatment. This was achieved via immunofluorescent staining and microscopy, single cell cloning, and flow cytometric analysis. The relationship between hormone receptor expression and proliferation following treatment with increasing concentrations of Tamoxifen was studied in semi-embedded MCF-7 spheroids. This line of experimentation indicates the presence of multiple sub-populations of cells that continue to persist, regardless of their hormone-receptor or proliferative state. The single cell cloning data

suggests that hormone receptor expression may be transient, cycling on and off throughout the course of treatment. Lastly, flow cytometry showed that the lower concentration of Tamoxifen could be just as effective as the higher concentration in the induction of G1-phase cell cycle arrest. Results from this thesis further highlight the importance of studying cellular heterogeneity in the context of tumor therapeutic response and therapeutic failure and may advocate for the use of low-dose Tamoxifen with similar anti-cancer benefits.

RÉSUMÉ

Le cancer du sein est le cancer le plus fréquemment diagnostiqué chez les femmes à travers le monde, avec plus de 500 000 décès par an. Environ 70 % des cas sont des cancers du sein luminaux, caractérisés principalement par l'expression du récepteur d'œstrogène. Il a été démontré que le récepteur d'œstrogène favorise la tumorigenèse en favorisant la transcription et traduction de protéines telles que la cycline D1 et divers facteurs de croissance, contribuant ainsi à la survie des cellules, à la prolifération des tissus et à l'angiogenèse. Le Tamoxifène, un modulateur sélectif des récepteurs œstrogéniques, est la forme la plus répandue de thérapie endocrinienne adjuvante pour le cancer du sein ER-positif. Il a été démontré qu'il réduit les récidives de 50 % et la mortalité annuelle d'environ 30 %. Malgré son utilisation courant en milieu clinique, 20 à 40 % des patients connaissent une rechute ou une progression métastatique en raison d'une résistance intrinsèque ou acquise au Tamoxifène. Au cours des deux dernières décennies, des recherches approfondies ont été menées sur les mécanismes biologiques qui sous-tendent la résistance au Tamoxifène, mais des recherches supplémentaires sont nécessaires pour surmonter cet obstacle important. En outre, la concentration optimale du Tamoxifène et les propriétés pharmacocinétiques de ses métabolites n'ont pas encore été établies chez l'Homme. Pour combler ces lacunes, mon projet de thèse vise à caractériser et à renforcer notre compréhension des profils phénotypiques associés à une éventuelle résistance au Tamoxifène afin de les cibler à l'avance et d'offrir un traitement anticancéreux plus efficace. Cet objectif a été atteint grâce à la coloration et microscopie immunofluorescente, au clonage de cellules uniques et à l'analyse par cytométrie de flux. La relation entre l'expression des récepteurs hormonaux et la prolifération suivant des traitements de Tamoxifène, à concentrations croissantes a été étudiée dans des sphéroïdes MCF-7 semi-encastrés. Cette ligne d'expérimentation indique la présence de multiples sous-populations de cellules qui continuent à persister,

indépendamment de l'expression de récepteurs hormonaux ou de leur état prolifératif. Les données de clonage de cellules uniques suggèrent que l'expression des récepteurs hormonaux peut être transitoire, passant d'un état actif à inactif au cours du traitement. Enfin, la cytométrie en flux a montré que la plus faible concentration de Tamoxifène pouvait être tout aussi efficace que la plus forte concentration dans l'induction de l'arrêt dans la phase G1 du cycle cellulaire. Les résultats de cette thèse soulignent l'importance de l'étude de l'hétérogénéité cellulaire dans le contexte de la réponse et de l'échec thérapeutique des tumeurs, et peuvent plaider en faveur de l'utilisation d'une faible dose de Tamoxifène avec des bénéfices anticancéreux similaires.

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Finally, I dedicate this work to my parents, family, and friends, who's endless love, devotion, and encouragement have shaped me into the young man that I am today.

CONTRIBUTIONS

Technical assistance

Dr. Maia Al-Masri assisted with optimizing BrdU staining protocol (Section 3.3.1 and 3.3.1.1).

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Experimental Design and analysis

The author conducted all experiments presented in this thesis.

Experimental design and analysis were performed by the author.

The author wrote this entire thesis.

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LIST OF ABBREVIATIONS

[]: concentration

2D: two-dimensional

3D: three-dimensional

4-OHT: 4-hydroxytamoxifen

AF: activation function

AI: aromatase inhibitor

anti-ERα: anti-estrogen receptor alpha antibody

anti-PRβ: anti-progesterone receptor beta antibody

AREG: amphiregulin

ASCO: American society of clinical oncology

BME: basement membrane extract

BrdU: 5-bromo-2'-deoxyuridine

CAP: College of American Pathologists

Ccnd1: cyclin delta 1

CDK: cyclin-dependent kinase

CDKI: cycling-dependent kinase inhibitors

CoA: co-activator

CoR: co-repressor

CTGF: connective tissue growth factor

Cultrex: Cultrex Pathway Reduced Growth Factor

CYP: hepatic cytochrome P450

DES: diethylstilbestrol

DBD: DNA binding domain

DCIS: ductal carcinoma in situ

DMSO: dimethyl sulfoxide

DTTP: deoxythymidine triphosphate

E_2: estradiol

EBCTCG: Early Breast Cancer Trialist's Collaborative Group

ECM: extracellular matrix

EdU: 5-ethynyl-2'-deoxyuridine

EGF: epithelial growth factor

EGFR: epidermal growth factor receptor

ER *(ESR1/2)*: estrogen receptor *(gene name)*

ERE: estrogen response element

FFPE: formalin-fixed, paraffin-embedded

FGF: fibroblast growth factor

FSH: follicle stimulating hormone

GH: growth hormone

GHRH: growth hormone-releasing hormone

GHRL growth hormone receptor

GPCR: G-protein coupled receptor

GPER-1/GPR30: G protein-coupled estrogen receptor-1

GS: goat serum

GnRH: gonadotropic-releasing hormone

H-#: Helix-#

HER-2: human epidermal growth factor receptor 2

Hoechst 33258: pentahydrate (bis-Benzimide)-10

IDC: invasive ductal carcinoma

IGF-1: insulin-like growth factor 1

IGF-1R: IGF receptor 1

ILC: invasive lobular carcinoma

LBD: ligand binding domain

LCIS: lobular carcinoma in situ

LH: luteinizing hormone

MAPK: mitogen-activated protein kinase

MISS: membrane-initiated steroid signaling

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoliμM

NGS: Nottingham grading system

NISS: nuclear-initiated steroid signaling

NTD: amino terminal domain

P_4: progesterone

PAQR: ADIPOQ receptors

PBS: phosphate buffered saline

PDX: patient-derived xenograft

PFA: paraformadehyde

PI3K: phosphatidylinositol-3'kinase

PI: propidium iodide

PR: progesterone receptor

PRE: progesterone response element

RNase A: ribonuclease alpha

ROR: risk of recurrence

Rb: retinoblastoma protein

SCC: single cell cloning

SERD: selective estrogen down regulators

SERM: selective estrogen receptor modulator

SRC3: nuclear receptor coactivator 3

STAT: signal transducer and activator of transcription family of proteins

TAM: tamoxifen

TEB: terminal end bud

TNFSF11/RANKL: tumor necrosis factor ligand superfamily, member 11

TNM: tumor, node, metastasis

TRAC-1/NCoR: thyroid hormone and retinoic acid receptor co-repressor 1/nuclear receptor co-repressor

Transducin b like protein 1 (TBL1)

WHO: World Health Organization

1. INTRODUCTION

1.1 CANCER OVERVIEW

The World Health Organization defines cancer as the generic term used to describe a group of diseases characterized by abnormal cells that divide without control [1]. This uncontrolled division is acquired through a process known as malignant transformation, during which cancer cells undergo a series of genetic modifications that provide them with the capacity to bypass the highly regulated cellular growth and division cycle, invade surrounding healthy tissue, induce sustained angiogenesis, and evade apoptosis. Carcinogenesis, stimulated both by genetic predispositions and environmental causes, has the potential to occur in every cell, tissue, and organ of the body, though it is most likely to be epithelial in origin [2].

1.1.1 BREAST CANCER

According to the GLOBOCAN 2020 estimates, breast cancer is one of the most common cancers and the 2nd cause of cancer-related deaths worldwide among women. The World Health Organization (WHO), states that breast cancer is the most frequently diagnosed cancer in women worldwide, with over two million new cases in 2020, accounting for approximately 685,000 deaths. The five-year survival rate is estimated at 88%. However, this number is highly contingent upon the afflicted individual's geography (i.e., whether they reside in a country with a developed health care system), socio-economic background, breast cancer classification and tumour stage, grade, and molecular subtype [3].

1.1.2 Breast cancer classification

Breast cancers are generally classified based on their geographic location (e.g., duct, lobule, nipple, etc.) and their capacity to spread to surrounding tissues, known as invasiveness.

1.1.2.1 Non-invasive (in situ) breast cancer

In situ breast cancer cells are non-invasive, meaning they have not (yet) spread beyond the area from which they initially developed.

Ductal carcinoma *in situ* (DCIS) is the most common form of non-invasive breast cancer, and accounts for approximately 20% of all new breast cancer diagnoses. It is characterized by an accumulation of neoplastic cells confined to the mammary ducts, responsible for carrying milk from the lobules to the nipple [4].

Lobular carcinoma *in situ* (LCIS) is a non-invasive lesion that arises in the terminal duct lobules of the breast. LCIS is technically no longer considered a form of cancer and is more accurately described as a non-obligate precursor lesion [5].

1.1.2.2 Invasive breast cancer

Approximately 80% of all breast cancers are invasive in nature, meaning they have breached the basement membrane and infiltrated the surrounding fatty and connective tissues (stroma) of the breast. Following this, malignant cells may metastasize to other parts of the body through the bloodstream or lymph nodes [6].

Invasive ductal carcinoma (IDC) (which prior to infiltration, is classified as DCIS) is the most diagnosed and heavily researched type of breast cancer [7]. The second most common type, accounting for 5-10% of diagnoses, begins as LCIS and may progress towards the Invasive lobular

carcinoma (ILC) stage [8]. Other less common types of invasive breast cancer include inflammatory breast cancer, mutinous (or colloid) carcinomas, tubular carcinomas, and Paget's disease of the nipple [7].

1.1.3 Stage, grade and molecular subtypes

Breast cancer, like other cancers, is heterogeneous in nature, exhibiting wide variability with regards to its histological and molecular characterization. The advent of modern medicine and the many advancements in both clinical and basic research have culminated in a sophisticated clinical classification system based on breast cancer molecular subtype (e.g., luminal, basal-like and HER-2 enriched), grade (NGS grading system), and stage (TMS staging system) [7].

There also exist multi-gene prognostic tests used to provide better insight with regards to treatment selection and response, as well as risk of relapse. For example, the Prosigna™ breast cancer Prognostic Gene Signature Assay, (the successor to the 50-gene PCR-based PAM50 test), utilizes the Nanostring nCounter Dx Analysis System to provide an accurate measurement of mRNA expression levels of up to 800 genes in formalin-fixed, paraffin-embedded (FFPE) breast tumor tissue samples. This assay informs the healthcare team on crucial patient-specific information, including their Risk Group (low, intermediate, or high), Risk of Recurrence (ROR) score, and Intrinsic Subtype (Luminal A, Luminal B, HER-2-enriched, or basal-like) [9].

1.1.3.1 Common Molecular subtypes within ductal carcinomas

Luminal breast cancers are estrogen receptor-positive, comprising approximately 70% of all cases of breast cancer [10]. This subtype displays upregulated luminal-associated proteins and mRNA, as well as hormone-driven proliferative events [11]. This subtype is the least aggressive

of all subtypes and responds relatively well to endocrine therapies. They can be further subdivided into Luminal A and Luminal B, with differing clinical outcomes.

Luminal A tumors are characterized as estrogen receptor (ER) and progesterone receptor (PR) positive (+) if at least 20% of cells express ER and PR (≥20%). They are also Human epidermal growth factor receptor 2 (HER-2) negative (-) and positive for Ki67 (a nuclear protein associated with cellular proliferation) in over 14% of cells (<14%). Clinically, Luminal A are slow-growing tumors with the best prognosis. This subtype often responds favorably to hormone therapy (e.g., selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs)) [12].

Luminal B tumors are ER-positive, HER2-positive/negative and have at least one of the following characteristics: Ki67 ≥20% and/or PR-negative or ≤20%. This subtype also has a high expression of proliferation-associated genes (e.g., MKI67 and AURKA). Consequently, they tend to grow faster than Luminal A, and do not respond as well to targeted hormone therapy [13]. However, this subtype does benefit from chemotherapy to a greater extent compared to Luminal A [14].

The HER-2 enriched subtype accounts for 15-25% of breast cancers [15]. This group is characterised by the high expression of HER-2-associated proliferative genes at both the RNA and protein level. They tend to express low to intermediate levels of luminal genes (e.g., ESR1 and PGR) and basal genes [16]. The HER-2 enriched cancers are more aggressive and faster growing than their luminal counterparts, leading to generally worse prognoses. However, the introduction of monoclonal antibody therapies such as Trastuzumab and tyrosine kinase inhibitors, namely Lapatinib and Neratinib, in addition to targeted chemotherapy have greatly increased recurrence-free survival rates in recent years [17].

Basal-like tumors represent about 15% of diagnoses and are characterized by a high expression of basal markers such as cytokeratin 5, 6 and 17, and epidermal growth factor receptor (EGFR) [18]. Most tumors that fall within this category are known as triple negative, as they do not express ER, PR or HER-2. Thus, it is important to note that not all basal-like breast cancers are triple-negative, and vice versa. Most basal-like/triple negative tumors are considered high grade, characterized by accelerated growth and metastasis. Patients diagnosed with these subtypes are primarily treated with chemotherapy because, for the most part, cannot benefit from endocrine or monoclonal antibody therapies [19].

1.1.3.2 GRADE

The Nottingham Grading System (NGS) is a three-tiered grading system used to assess the degree to which invasive carcinomas resemble normal breast epithelial cells. It is based on the assessment of three morphological features: degree of tubule formation, nuclear pleomorphism and mitotic count. For example, grade 1 tumors are well differentiated, and demonstrate a mild degree of pleomorphism and low mitotic count. At the other extreme, grade 3 tumors are poorly differentiated, have significant cellular pleomorphism, and are highly proliferative [20].

1.1.3.3 STAGE

The tumor, node, metastasis (TNM) staging system is an internationally recognized system used to determine the extent of disease progression and provide insight on prognosis and treatment options [21]. It classifies tumors based on the primary tumor type (invasive or *in situ*), their size (T), the state if lymph node involvement (N) and the presence or absence of distant metastases (M). The overall anatomic stage is determined based on the combination of T, N, and M

parameters. In short, it is comprised of stages 0 through IV (with increasing severity and worse prognosis), where ductal carcinoma *in situ* (DCIS) is considered stage 0 and metastasis as IV [22].

1.2 Mammary gland development and maturation

The mammary gland is an exocrine gland that has evolved from apocrine-like glands associated with hair follicles and primitive sweat glands [23]. They are unique to mammals and are arranged in organs such as breasts in primates (e.g., humans and chimpanzees) and udders in ruminants (e.g., cows and goats) with the primary function of feeding young offspring [24].

1.2.1 Embryological origins

Human breast development is initiated during embryonic life, arising from the ventral epidermal ridges (milk lines) in a five-week embryo [25]. More specifically, these bilateral ridges disappear during normal fetal development, except for a pair of thickenings formed by epithelial buds that surround condensed mesenchymal tissue. Columns of epithelial cells grow bilaterally downward, branch, and transform into ducts that will result in lobules. These lobules form a lobe, eventually becoming the breast [26].

1.2.2 Birth and Pre-pubertal years

At birth, the breast is made up of 10-12 primitive ductal structures lying just below the nipple and areola, that can be easily distinguished by their unique appearance and histologic organization. During these pre-pubertal years, the elementary ducts undergo slow and progressive growth and branching [27].

1.2.3 PUBERTY

Puberty is the first step in rapid glandular maturation. In females, puberty begins in between the ages of 10-12 years old, initiated and regulated through the release of hypothalamic gonadotropin-releasing hormone (GnRH) [28]. This stage of mammary gland development is primarily regulated by growth hormone (GH), insulin-like growth factor 1 (IGF-1) and estrogen [29].

During puberty, the hypothalamus secretes growth hormone-releasing hormone (GHRH), stimulating the release of GH from the pituitary gland. GH binds to growth hormone receptors (GHR) on mammary stromal cells, triggering the release of IGF1. In parallel, circulating GnRH promotes the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary gland, driving the secretion of estradiol (E_2) (as well as progesterone (P_4)) in the ovaries and the commencement of the menstrual cycle [30]. Both E_2 and IGF1 induce rapid cellular proliferation and ductal elongation via the stimulation of terminal end buds (TEBs), club-shaped structures responsible for the formation of mature cells and the ductal tree. Additionally, E_2 triggers branching of mammary ducts via epidermal and fibroblast growth factors (EGF and FGF respectively). More specifically, E_2 binds to ERα, prompting the release of amphiregulin (AREG, a member of the EGF family), which binds to EFG receptors (EGFRs) on stromal fibroblasts [31]. EGFR then induces the expression of FGF to stimulate cell proliferation and branching through FGF receptor 2 (FGFR2), found on mammary epithelium [32].

1.2.4 Pregnancy and Lactation

The mammary gland undergoes important structural changes in preparation for pregnancy and lactation. These changes are primarily under the control of P_4 and prolactin (synthesized in the anterior pituitary gland). The binding of P_4 to its receptor PR is thought to upregulate tumor necrosis factor ligand superfamily member 11 (TNFSF11), also known as RANKL. RANKL stimulates proliferation and lateral side branching through the upregulation of target genes such as Cyclin delta 1 (Ccnd1) via binding to its receptor, RANK, on the surface of neighboring cells [33]. P_4 also synergizes with prolactin to promote alveologenesis, initiating the differentiation of proliferating mammary stem cells into alveoli, capable of synthesizing and secreting milk during the process of lactation [34].

1.2.5 Infant Weaning: First Involution

Weaning of the infant induces mammary gland involution and remodelling to a pre-pregnancy state. The first stage of involution is reversible and is mediated by the signal transducer and activator of transcription (STAT) family of proteins [35]. This stage is marked by apoptosis, an increase of shed cells in the lumen, and alveolar cell detachment. The second stage of involution is irreversible and is characterized by extracellular matrix (ECM) breakdown and a second wave of apoptosis, leading to the replacement of secretory epithelial units by adipocytes. This stage is regulated by serine proteases and matrix metalloproteases [36].

1.2.6 Menopause: Second Involution

During menopause, lobular involution occurs, provoking the replacement of glandular epithelium and interlobular connective tissue with fat. At this stage, the number of functional acini

and ducts are significantly reduced. The reduction in mammary epithelial tissue alongside the increase in adipose tissue is thought to have a protective effect from epithelial-derived carcinogenesis [37].

1.3 Steroid molecules and their receptors: Signaling mechanisms and link to breast cancer

Steroid hormones are all derived from cholesterol and are synthesized in the mitochondria and smooth endoplasmic reticulum of cells in the adrenal cortex, gonads, and placenta. Their lipophilic nature makes them insoluble in blood plasma and other fluids, rendering them reliant on transport proteins for transport and distribution within the body. Steroid hormones mediate their effects through the binding of intracellular nuclear receptors (and membrane receptors, to a lesser extent), forming a complex that will ultimately result in gene transcription [38]. Estrogen and Progesterone and their receptors are instrumental in the regulation of biological processes, such as cholesterol mobilization, maintenance of bone density, and sexual organ development [39].

1.3.1 Estrogen receptor

The mammalian estrogen receptor (ER) is a family of ligand-dependant transcription factors responsible for the regulation of numerous physiological processes, such as the development of the female reproductive system, maintenance of bone mass and the differentiation of axons and dendrites in the central and peripheral nervous systems [40]. ER is divided into two subtypes, ERα and ERβ, discovered and subsequently characterized in the 1960s and 1990s, respectively [41]. These subtypes are encoded by two distinct genes and are expressed either together or separately, at varying levels, depending on the tissue [42]. ERα is encoded by *ESR1* and resides on chromosome 6 [43]. ERβ is encoded by *ESR2* and resides on chromosome 14 [44].

ERα is present in the mammary glands, adipose tissue, and in both female and male reproductive tracts, whereas ERβ exerts its effects in the liver, bladder, colon, and multiple organs of the immune system. Both subtypes are expressed in the cardiovascular and central nervous systems [41].

Estrogen Receptors, depicted in Figure 1, have six distinct functional domains: A, B, C, D, E and F [45]. Both isoforms contain a hypervariable A/F domain, subdivided into activation function (AF) -1 (A/B domain), with a transcriptional activation function and ligand-independent transactivation, as well as AF-2 (E/F domain), which facilitates transcriptional activation of the receptor, located in the COOH terminus of the ligand binding domain (LBD, E domain) [46]. The DNA binding domain (DBD, C domain) facilitates ER binding to DNA and contains two zinc finger structures that aid in receptor dimerization and binding to specific DNA sequences [47]. It also mediates the interaction with heat shock proteins and induces conformational changes in the receptor that determine gene activation or repression [48]. The hinge domain (D domain) contains the nuclear localization signal and links the DBD to the LBD. The LBD is a globular region that contains a hormone-binding site and dimerization interface [49].

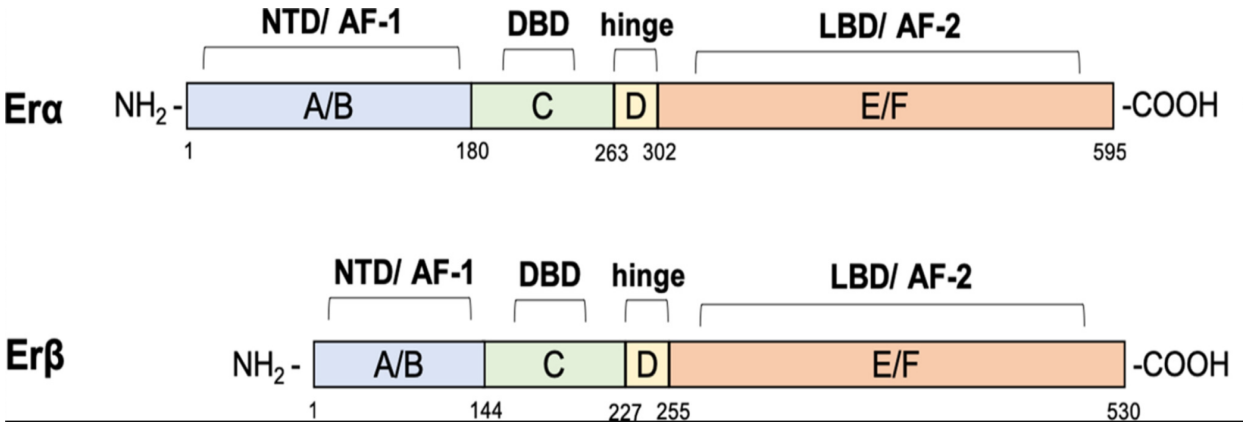

Figure 1: Structural organization of estrogen receptors. Functional domains A/B, C, D, E/F are depicted. Domains A and F are hypervariable and can be subdivided into AF-1 (A/B) responsible for ligand-dependent and independent activation and AF-2 (E/F), which aids in transcriptional activation of the receptor. C is the DNA binding domain, and D is the hinge domain, containing the nuclear localization signal. Used with permission from [50].

1.3.1.1 Mechanism of estrogen signalling

17β-estradiol (E_2) is the most prevalent and highly selective source of endogenous estrogen in the body [51]. Its physiological functions are mediated through its association with the estrogen receptor and the subsequent formation of the E_2-ER complex, acting as a cytoplasmic and nuclear signal that generates a multitude of cellular processes, most notably cell proliferation [52]. Estrogen signaling can be divided into two mechanistically distinct pathways: the genomic and the non-genomic pathway, depicted in Figure 2:

a) Genomic/Classical Pathway: A free hydrophobic steroid molecule, such as E_2, will passively diffuse through the plasma membrane and bind to cytoplasmic ER with high affinity. This binding results in a conformational change in both tertiary and quaternary structures of the receptor, leading to homodimerization and an active ligand-receptor (E_2-ER) complex [53]. The complex is now capable of translocating into the nucleus where it can trigger nuclear-initiated steroid signaling (NISS) via binding of sequences of DNA called estrogen response elements

(ERE; present in the promoter regions of specific target genes) [54]. The activated transcriptional complex can recruit (depending on the position of ER helices, such as Helix 12) neighboring coregulator proteins [55]. Coregulators can either have an excitatory (coactivators-CoA), or an inhibitory (corepressors-CoR) effect on the ER transcription complex, influencing ER target gene expression through post-translational modification of transcription factors and the binding affinity of RNA polymerase II in estrogen-responsive cells, often resulting in increased cellular proliferation [56, 52].

b) Nongenomic (extranuclear)/Non-Classical Pathway: Estrogen molecules incapable of traversing the lipid bilayer (often due to being coupled to high molecular weight substances) bind to membrane bound estrogen receptors [57]. Once bound, membrane-initiated steroid signaling (MISS) commences via the recruitment of membrane and/or cytosolic signaling elements such as Ca^{2+} and nitric oxide (acting as secondary messengers), IGF receptor1 (IGF-1R), EGFR (with receptor tyrosine kinase activity), G protein coupled receptors (GPCRs), and various protein kinases, such as phosphatidylinositol-3'kinase (PI3K), mitogen-activated protein kinase (MAPK) family members, and protein kinase A and C [58]. The vast and highly variable extranuclear pathway, like its genomic counterpart, has the capacity to influence breast tissue growth through control of apoptosis, protein synthesis and cellular proliferation.

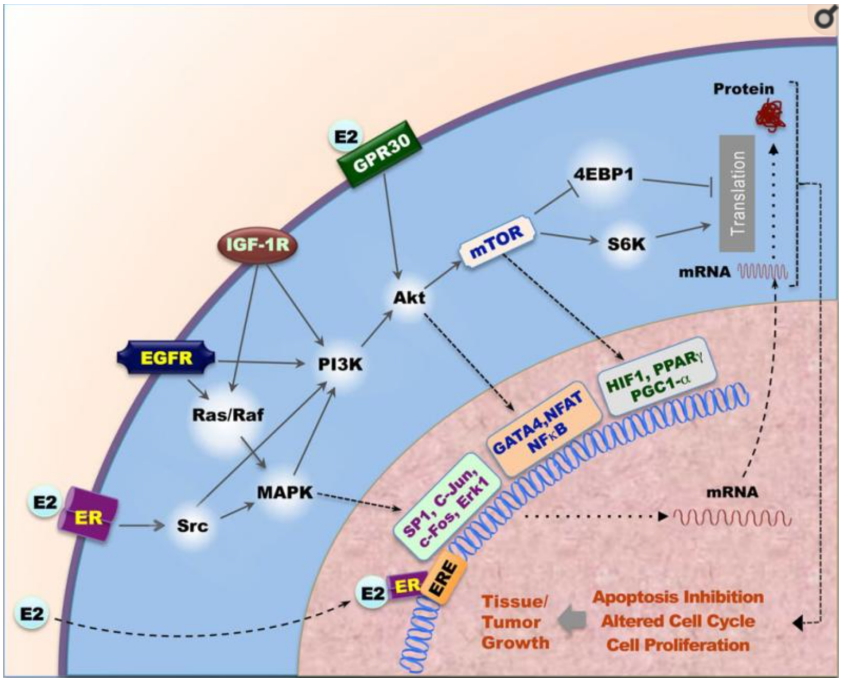

Figure 2: Overview of Estrogen Signaling Pathways: Genomic and non-genomic. The genomic pathway involves ligand diffusion through the membrane, and the translocation of the E_2-ER complex into the nucleus, where it binds to EREs to trigger NISS. The non-genomic pathway involves membrane-bound receptors forming a complex with a ligand incapable of traversing the lipid bilayer. Once the complex has formed, MISS occurs following recruitment of downstream signaling elements. Used with permission from [54].

1.3.2 Progesterone Receptor

The mammalian progesterone receptor (PR), also a member of the steroid receptor family of ligand-dependant transcription factors, is a key player in many biological processes, such as the thickening of the endometrium following ovulation, and the maintenance of metabolic homeostasis via the regulation of insulin and leptin secretion, as well as glucose and lipid metabolism [59].

PR has two predominant isoforms, PRα and PRβ, discovered in 1970 by O'Malley et al. [60]. These isoforms are transcribed from the same gene, PGR (located on chromosome q22-q23 of chromosome 11), differing in their molecular weight [61]. The PRβ isoform is considered a full-length receptor, as it contains an additional 164 amino acids at the N-terminal region called the B-upstream segment (conferring AF-3 activity), which is absent in PRα [62].

The PGR gene can be activated by E_2 due to ERE half-sites found in proximity to its SP1 binding sites [63]. Additionally, PR has long been used as a marker of ER activity because transcription of PR is partly driven by ERα-mediated events [64]. However, more recent, albeit inconsistent data has emerged demonstrating that PR may act as an ER binding partner and modifier of downstream ER gene targets [65].

Both receptors, illustrated below in Figure 3, share identical C-terminal LBD, central DNA-binding domains, and amino-terminal domain (NTD), save for the 164 amino acids mentioned prior. PR has two main transcriptional AFs, that allow for interactions with coregulatory proteins such as SRC-1/2/3 and p300-CPB [66]: AF-1 in the NTD and AF-2 in the LBD [67]. Depending on the target gene, AF-1 and AF-2 can function independently or may operate in synergy via intra-molecular interactions. This additional cooperative activation function is known as AF-3 and can only occur in PRβ via its B-upstream segment [68].

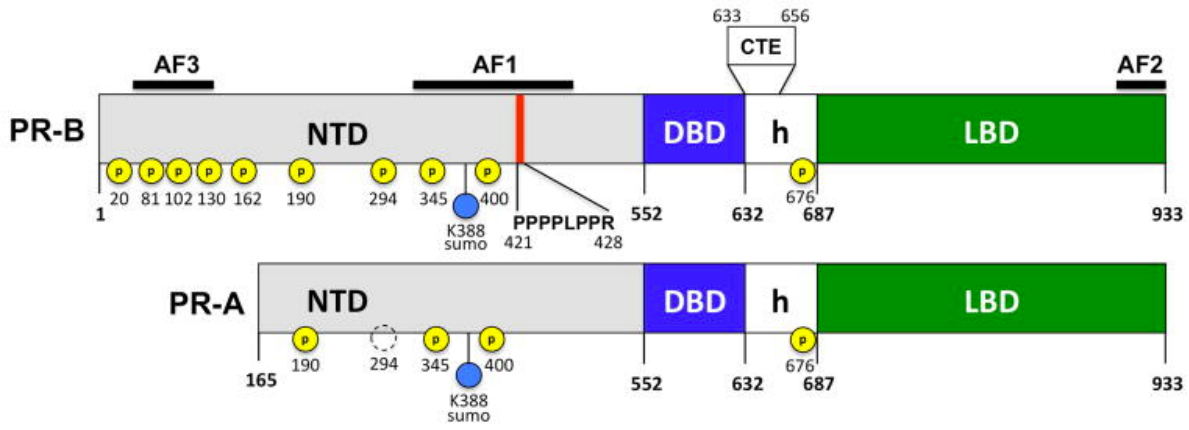

Figure 3: Structural organization of progesterone receptors. PRβ and PRα share identical C-terminal ligand- binding domains (LBD) and central DNA-binding domains (DBD). The amino-terminal domain (NTD) of PRβ contains an additional 164 amino acids, called the B-upstream segment, conferring AF-3 activity. Used with permission from [69].

1.3.2.1. MECHANISM OF PROGESTERONE SIGNALLING

Progesterone (P_4) is the most prevalent and high affinity endogenous ligand for the progesterone receptor (PR) [70]. Its physiological functions are mediated through its association with the progesterone receptor and the formation of the P_4-PR complex, acting as a cytoplasmic and nuclear signal that generates a multitude of cellular processes involved in mammary gland development and menstrual cycle regulation [71]. Progesterone signaling, like estrogen signaling, is divided into two mechanistically distinct pathways: the genomic (classical) and the non-genomic (non-classical) pathway, outlined in Figure 4.

a) Classical signaling: Similar to ERs, classical PRs are part of the nuclear receptor family of transcription factors. In the absence of a ligand, PRs are inactive due to the binding of multiple chaperone protein complexes [72]. When a hydrophobic P_4 molecule diffuses through the plasma membrane, it binds to its receptor with high affinity, forming a P_4-PR complex. This process provokes conformational changes in the receptor, leading to chaperone remodelling and receptor dimerization, followed by translocation into the nucleus where it binds to progesterone response elements (PREs). The newly activated transcriptional complex can now interact with coregulatory proteins that will regulate PR-target gene expression [73].

b) Non-Classical signaling: Recent human studies have demonstrated the ability for P_4 to participate in rapid progestin-activated signaling through cell-surface receptors [74]. In this instance, P_4 binds to membrane-bound PRs with high affinity, rather than diffusing through the lipid bilayer. More specifically, these surface receptors are a class 7 transmembrane domain proteins, called progestin and ADIPOQ receptors (PAQRs), which are similar in organization to G protein-coupled receptors [75]. The P_4-PAQR binding eventually results

in various signal transduction cascades involving intracellular CA^{2+}, MAPKs and ERk1/2 [76].

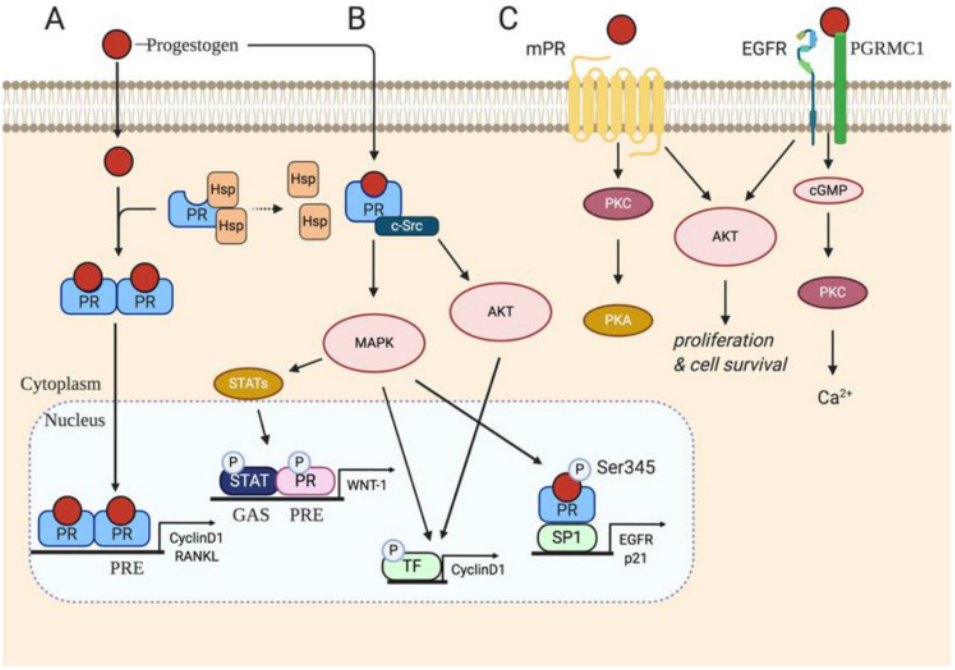

Figure 4: Overview of Progesterone Signaling Pathways: Genomic and non-genomic. The genomic pathway involves ligand diffusion through the membrane, and the translocation of the P_4-PR complex into the nucleus, where it binds to PREs (Progesterone Response Elements) to trigger NISS. The non-genomic pathway involves membrane-bound receptors forming a complex with a ligand incapable of traversing the lipid bilayer. Once the complex has formed, MISS occurs following recruitment of downstream signaling elements. Used with permission from [73].

In normal healthy adult breast tissue, PR isotypes PRα and PRβ are expressed equally. However, in breast cancer, PRβ regulates the expression of more genes than PRα and is the primary mediator of P_4-induced proliferation [77]. Additionally, PRβ predominantly mediates the extranuclear signaling actions of PR, due to PRα's inability to efficiently mediate rapid activation of protein kinase signaling pathways [78]. Lastly, in breast cancer cell lines, PRα has proven to be necessary for appropriate responsiveness to P_4 [79].

1.4 Steroid Hormones and breast cancer initiation

It has been well established that the steroid hormone estradiol plays a key role in both the initiation and progression of breast cancer. Its effects are mediated through the formation of the E_2-ERα/β complex. Interestingly, recent evidence suggests that ERβ expression has antiproliferative (tumor suppressive) properties in breast tissue [80].

The problem, however, lies in ERα's equally important role in breast cancer initiation and progression. Dysfunctional estrogen receptor signaling joins BRCA1/BRACA2, PTEN and TP53 mutations, exposure to HRT or carcinogens, and obesity as noteworthy risk factors for neoplastic disease [81].

Researchers have recently highlighted 2 major mechanisms responsible for estrogen-induced carcinogenesis and subsequent progression. The first mechanism involves the deregulation of the cyclin D1/CDK4/6 pathway. Specifically, estrogen plays a key role in the transition from G1 to S-phase of the cell cycle through the activation and binding of cyclin D1 to dimerized CDK4/6, increasing rates of cellular division, and opportunities for further genetic damage [82]. This is accomplished through the downstream induction of growth factors that directly affect cellular proliferation through the activation of specific nuclear transcription factors, such as AP-1 [83].

The second mechanism highlights the relationship between estrogen metabolism and genotoxicity. [84] Recent *in vitro* studies suggest that the metabolism of estrogens can lead to the formation of DNA depurinating adducts such as catechol estrogens, quinones, free radicals, and reactive oxygen species, all of which contribute to the accumulation of mutations and the induction of neoplastic transformation in breast tissue [85].

Recent mouse model data suggest that endogenous P_4 promotes pre-neoplastic progression via paracrine stimulation of normal breast epithelium and/or mammary stem cell pools [86]. It has also been shown that a switch from paracrine to autocrine regulation of P_4-induced proliferation is a feature of early breast cancer progression [39]. However, it is important to note that currently, there is no conclusive evidence indicating that P_4 has the capacity to initiate tumors through activation of oncogenes or via inducing proteins and enzymes involved in nucleic acid synthesis [65].

1.5 ER AND BREAST CANCER THERAPY

The 19th century saw the beginning of modern, systematic treatment of breast cancer. Observations by the German physician Rudolf Virchow helped solidify the notion that cancer was cellular in nature (dismissing previous humoral theories from physicians of past) and that dissemination via blood and lymphatic vessels is at the core of metastasis [87]. The improvements in surgical and aseptic technique alongside the development of anesthesia allowed pioneers such as American surgeon William Halsted to develop the radical Halsted mastectomy, which involved the removal of the breast, underlying pectoral muscle, lymphatic vessels, and axillary lymph nodes [88]. In 1896, British surgeon Thomas Beatson observed that advanced breast tumours in animal models regressed significantly following oophorectomy. These findings would be corroborated approximately 100 years later, when Bocchinfuso et al. demonstrated that mammary glands of ERα deficient mice (αERKO mutants) did not undergo ductal elongation, nor did they form terminal end buds [89].

Beatson's observations launched the investigation of the relationship between sexual hormones and the onset and progression of breast cancer [90]. The second half of the 20th century

saw the rapid development of a multimodal breast cancer treatment approach involving surgery, radiotherapy, chemotherapy, and endocrine therapy [87].

The discovery of the estrogen receptor in 1967 by Dr. Elwood Jensen and colleagues highlighted the importance of this protein as a diagnostic tool and kickstarted the rapid development of oestrogen-modulating drugs, known as Selective Estrogen-Receptor Modulator (modifiers), or SERMs [91].

SERMs are a relatively new class of therapeutic agents that demonstrate tissue-selective effects. For example, they display an agonistic effect in bone and liver tissue, antagonistic effect in breast and neural tissue, and a mixed effect in uterine tissue [92]. The first SERM to be approved in the United States was Clomiphene, used for the treatment of infertility in women who suffered from ovulatory dysfunction. It would require another ten years before Tamoxifen would be approved for the treatment of metastatic breast cancer in both women and men. Other SERMs, such as Toremifene and Raloxifene, would be approved in subsequent years, catering more specifically to postmenopausal women [93].

Our understanding of the molecular mechanism of action of SERMs in breast cancer is well documented due largely to numerous laboratory and clinical studies on Tamoxifen and Raloxifene, dating back to the 1980s. To this day, of all the currently approved SERMs, Tamoxifen remains the standard of care for hormone therapy of breast tumors [94].

Endocrine therapy is the gold standard for treatment of ER-positive breast cancer. Currently, there exist three classes of endocrine therapy that are at the physician's disposal: SERMS, such as TAM, selective estrogen receptor down regulators (SERDs), such as Fulvestrant, and aromatase inhibitors (AIs) such as Lentrozole and Anastrozole. Since its approval in the 1970s, TAM remains to this day the most wildly used and first line form of adjuvant therapy for

ER-positive breast cancer. TAM has proven to reduce breast cancer recurrence by 50% and annual mortality rate by 31% [95].

1.6 TAMOXIFEN

1.6.1 PHARMACOLOGY AND METABOLISM

Tamoxifen (TAM) is a non-steroidal SERM, marketed as a single trans isomer of p-β-dimethylaminoethoxy-1,2-diphenylbut-1-ene [96]. It is primarily used in the prevention and treatment of estrogen-receptor positive breast cancer.

TAM is a pro-drug, meaning its active metabolites (N-desmethyltamoxifen, 4-hydroxy-N-desmethyltamoxifen, and 4-hydroxytamoxifen) must first be generated in the liver via the hepatic cytochrome P450 (CYP) system. These metabolites have high affinity for the estrogen receptor, competing with circulating estrogens, inhibiting tumour growth [97].

Metabolism occurs through 4-hydroxylation and N-demethylation pathways, resulting in the potent secondary metabolite, 4-hydroxy-N-desmethyltamoxifen, known as Endoxifen [98]. The 4-hydroxylation pathway catalyzed primarily by CYP2D6, results in 4-hydroxytamoxifen (4-OHT), a metabolite that has been shown to be 30 to 100 times more potent than TAM itself. However, this pathway only accounts for 7% of TAM metabolism [99, 100]. On the other hand, the N-demethylation pathway, catalyzed by CYP3A4 and CYP3A5, contributes to approximately 92% of TAM metabolism [101, 99]. It results in N-desmethyltamoxifen, which is further oxidized to many metabolites, the most important being Endoxifen [102, 98].

With regards to antiestrogenic activity, 4-OHT and Endoxifen exhibit similar potencies of effect. However, plasma concentrations of Endoxifen are commonly 10-times higher than that of 4-OHT in patients receiving Tamoxifen therapy [103]. Lastly, of the three most common

metabolites, Endoxifen has the unique capacity to target ERα for proteasomal degradation, thus decreasing its protein levels [104].

1.6.2 Mechanism of action of Tamoxifen

All ERα ligands bind to the C-terminal of the ligand binding domain (E domain). The LBD recognises a variety of molecules (differing in terms of molecular weight, three-dimensional structure, as well as agonist/antagonist properties), and undergoes specific conformational changes depending on the ligand in question [105]. When an agonist molecule (depicted in Figure 5a), such as estradiol, accesses and binds the LBD, the carboxyl-terminal α-helix of Helix 12 (H12) folds and presses against H3, H5/6 and H11, partially obscuring the ligand binding pocket like a lid, effectively trapping the ligand [106]. This conformational shift allows the AF-2 region of the LBD to be more effectively recognized by transcriptional coactivators that will subsequently mediate transcriptional activation of agonist-dependent target genes, resulting in cellular proliferation and differentiation [55].

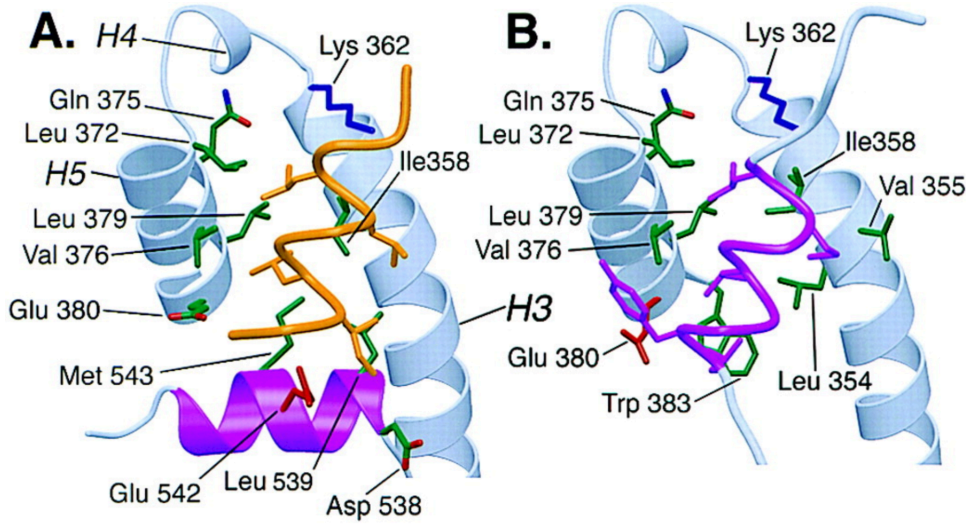

Figure 5: The NR BOX II Peptide/DES-LBD Interface and Helix 12/4-OHT-LBD Interface. This illustrates the differences in ligand-binding domain (LBD) organization when bound to diethylstilbestrol (DES) (mimicking estradiol) (Figure 5A.) versus bound to 4-hydroxytamoxifen (4-OHT) (Figure 5B.) Used with permission from [55].

SERMs such as Tamoxifen demonstrate mostly ER antagonist activity in breast tissue like that of pure anti-estrogens. Similar to estradiol, TAM binds to ERα at the core of the ligand binding domain (see Figure 5b). The difference lies in the conformational response of the estrogen receptor following ligand binding. Specifically, when 4-OHT (the active metabolite of tamoxifen *in vivo*) binds the LBD, Helix-12 repositions itself to bind and obstruct the coactivator-binding groove, effectively blocking AF-2 transcription and preventing nuclear receptor box recognition [55]. This allows TAM to function as an estrogen receptor antagonist of proliferative genes dependant on AF-2 activation. Also, the TAM-ER complex recruits co-repressor proteins such as nuclear receptor co-repressor (NCoR)/ thyroid hormone and retinoic acid receptor co-repressor 1 (TRAC-1) and nuclear receptor co-receptor 2 (SMRT), that function in synergy to deregulate transcription factors via the recruitment of histone deacetylase 3 and transducin b like protein 1 (TBL1) [107].

Briefly put, in estrogen-sensitive breast cancer, Tamoxifen's high affinity for the ERα allows it to compete with free-estrogen for receptor binding, inhibiting estrogenic effects. Additionally, once bound, TAM forms a complex with the ER, preventing the transcription of proliferative genes associated with cellular growth and proliferation and recruits co-repressor proteins that assist in the down regulation of proliferative gene expression.

1.6.3 Tamoxifen resistance and Breast cancer Recurrence

Approximately 20-40% of ER-positive breast cancer tumors are resistant to TAM treatment. This resistance was either present before drug administration or developed throughout the 5 to 10 years of hormone therapy. Consequently, many patients who have received adjuvant TAM therapy will experience relapse or progress towards advanced metastasis within approximately 5-10 years following initial treatment [108].

TAM resistance has been extensively studied over the last two decades, though its precise molecular mechanism remains unclear. This is likely due to an incomplete understanding of the complex array of signal transduction pathways and their interplay with cellular components responsible for proliferation, survival, and apoptosis. Antiestrogen resistance can be divided into two categories:

A. *de novo* resistance, found in cells that are intrinsically non-responsive to TAM therapy from the beginning of treatment. This has been demonstrated in ER-positive cells lines transfected with the HER-2 gene, inducing tumor growth in xenograft mice despite TAM treatment [109]. However, this form of resistance has also been shown in unaltered, parental breast cancer cell lines.

B. acquired resistance, in which ER-positive cells that had initially responded to antiestrogens fail to do so after long-term therapy [110]. Resistant cells may be completely unresponsive to TAM or may exhibit TAM-dependent/stimulated growth while still expressing

ER. This is well documented in xenograft experiments, during which MCF-7 cells were injected into athymic ovariectomized mice treated with TAM. Initially, most of the tumors cease proliferating. However, after approximately a year of treatment, some tumors continued to grow, despite still being on TAM [111].

1.6.4 TAMOXIFEN DELIVERY AND DOSAGE OPTIMIZATION CHALLENGES

Tamoxifen (TAM) is a selective estrogen receptor modulator used to treat Estrogen receptor-positive breast cancer tumors. FDA-approved indications include:

- Treatment of breast cancer in both females and males [112].
- Treatment of female patients with ductal carcinoma *in situ* post-surgery/radiation to reduce the risk of invasive ductal carcinoma [113].
- Adjuvant treatment of breast cancer after patients have successfully completed primary treatment (surgery and radiation therapy) [114].
- Breast cancer risk reduction in patients considered to be at elevated risk (i.e., 1.67% 5-year risk, according to the Gail Model) [115].

TAM is prescribed in tablet form (10mg or 20mg) or as an oral solution (10mg/5 mL) [116]. The American Society of Clinical Oncology (ASCO) proposes a set of guidelines that vary depending on the patient's needs, risk factors and physical status. For adjuvant endocrine therapy of hormone-receptor positive breast cancer, ASCO recommends a dose of 20mg daily following the completion of chemotherapy. The duration of Tamoxifen treatment may last 5 to 10 years, depending on the patient's menopausal status and progression [117]. Patients with DCIS are instructed to take 20mg daily by mouth for a period of five years following tumor excision and X-ray therapy [118].

A wider range of doses are prescribed when tackling metastatic breast cancer. More specifically, the ASCO recommends 20 to 40 mg of TAM daily, though clinical benefits have not been consistently demonstrated for doses above 20 mg daily [116].

Currently, oral TAM administration is still based on the one-dose-fits-all approach, despite on-going interest and experimentation surrounding its therapeutic index, active metabolites, and refining systems of delivery (e.g., localized therapy, such as topical transdermal creams and intraductal injections) [119].

The optimal concentration range for TAM has not yet been established, and the tissue distribution and effects of its metabolites have not been well documented in humans. The biological consequences of varying TAM concentrations at the tissue and cellular level have equally not been well studied either [120]. According to a study by Kisanga et al. (2004), the tamoxifen concentration at 20 mg daily over a period of 2 years ranged from 0.56-6.88 μM in breast cancer tissue [121]. Factors, such as liver and renal function, interactions with other drugs, menopausal status, as well as polymorphisms in TAM-metabolizing enzymes likely contribute to the frequently observed inter-patient variabilities [120].

Pharmacokinetic properties of TAM are also poorly understood. Generally, after having entered the systemic circulation, a drug is distributed to the body's tissues. Unfortunately, distribution is often not uniform due to differences in regional pH, permeability of cell membranes, tissue binding affinity (e.g., contingent upon local lipid content) and blood perfusion [122]. It goes without saying that this constitutes a noteworthy hurdle for both clinicians and researchers, especially when it pertains to a highly variable and rapidly evolving disease such as breast cancer.

1.7 MODELS USED TO STUDY RESISTANCE

1.7.1 *IN VITRO* MODELS

To investigate the biological intricacies of anti-hormone resistant breast cancer cells, populations of ER-positive cell lines such as T47D, BT-474, ZR-75-1 and MCF-7, have been engineered to adapt to various anti-hormone (e.g., TAM-rich) environments [123]. For example, the Lombardi Cancer Center generated TAM resistant MCF-7 cell line via selection by increasing the concentration of TAM up to 1μM as MCF-7 cells become resistance [124]. *In vitro* models have provided researchers with indispensable research tools for studying cellular mechanisms associated with drug resistance and alterations in metabolism and proliferation.

1.7.2 *IN VIVO* MODELS

The transition towards *in vivo* cancer models commenced shortly after the introduction of their *in vitro* counterparts. With regards to drug resistance models, MCF-7 human breast models (e.g., MCF-7/RAL, MCF-7/LCC2, MCF-7/LCC9, and MCF-7: TAM) were transplanted into athymic, ovariectomized mice to investigate acquired resistance to SERMs as early as the 1980s [123].

More recently, patient-derived xenograft (PDX) models are being used to provide a more faithful understanding of the cellular mechanisms driving TAM resistance. The patient-derived tumor tissue is thought to better conserve original tumour heterogenous histology, architecture and vasculature, clinical biomolecular signature, and malignant phenotype compared to the cell line-based in vivo models mentioned previously [125].

1.8 Molecular Mechanisms of Resistance

Examples of mechanisms of resistance, such as those depicted in Figure 6, include loss of ER expression, involving a switch from an initial ER-positive phenotype to ER-negative, likely due to transcriptional repression of *ESR1* via hypermethylation of CpG islands or histone deacetylation [126]. The proliferation of ER-negative cancer stem cells over their arrested ER-positive counterparts may also contribute to the transition from ER status from positive to negative [127]. Impaired ER function due to mutations in *ESR1*, negative feedback regulations of ER protein expression, and abnormal splicing may contribute to the of loss TAM's main molecular target [128]. Additionally, crosstalk between ER and HER-2, along with elevated expression of nuclear receptor coactivator 3 (SRC3) is associated with switching the TAM-ER complex from an antagonistic configuration to an agonistic architecture [129]. There is also evidence that upregulation of certain growth factor receptors such as EGFR, FGFR and IGFIR and alterations in PI3K-PTEN/AKT/mTOR pathway are also implicated in resistance [130].

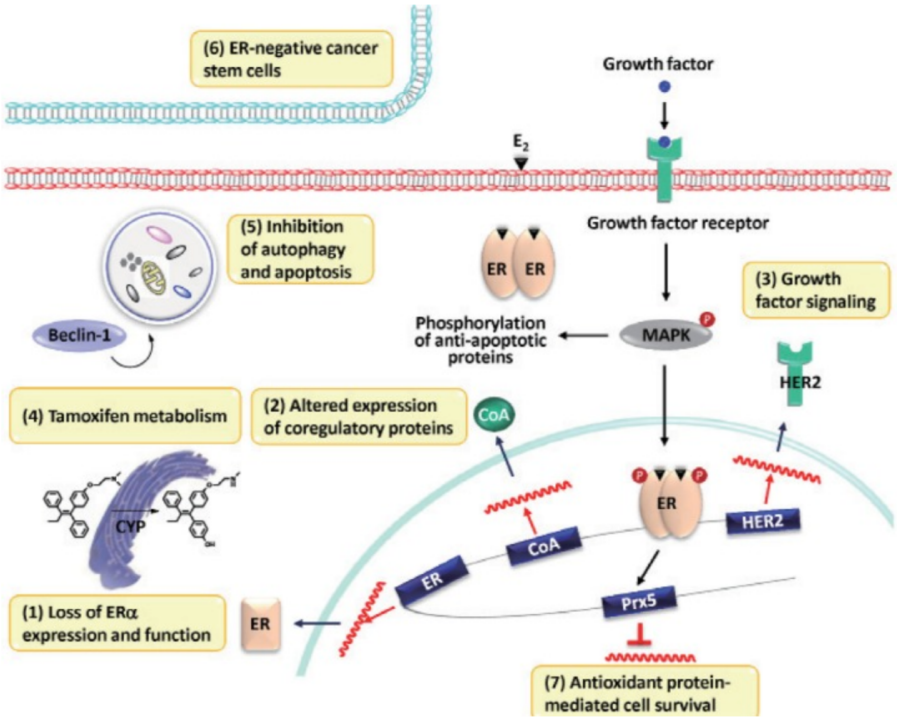

Figure 6: Overview of mechanisms of Tamoxifen resistance in estrogen receptor-positive breast cancer. Examples of proposed mechanisms of resistance include loss of ER expression, impaired ER function, upregulation of growth factors, and altered coregulatory protein expression. Used with permission from [128].

In brief, the body of knowledge pertaining to mechanisms of TAM resistance increases year after year. It is important to note that it is highly unlikely that a single mechanistic pathway or gene is responsible for TAM resistance. Additionally, mechanisms of resistance likely vary within tumors and across patients due to heterogeneity of breast tissue and external environmental factors, respectively.

2. PROJECT OVERVIEW

2.1 PROJECT DESCRIPTION AND RATIONALE

Breast cancer is the most common cancer among women specifically and the second leading cause of death within this group [2]. Of those affected, approximately 70% will have estrogen-receptor positive cancers [3]. Tamoxifen is the oldest and most prescribed form of hormone therapy, having been shown to reduce the risk of developing breast cancer by 50% and to slow the progression of metastatic hormone receptor-positive cancer in both pre- and postmenopausal women [95].

Unfortunately, approximately 1/3 of those diagnosed with ER-positive tumors will experience some form of recurrence despite undergoing extensive and carefully monitored hormone therapy [108]. Current literature suggests that recurrence is due to sub-populations of cancer cells that exhibit intrinsic resistance or have the capacity to acquire resistance to TAM throughout treatment. Molecular mechanisms of TAM resistance have been and continue to be elucidated, both in clinical and laboratory settings. However, there are currently few studies aiming to uncover the cellular profile of cells that ultimately grant the ability to undergo the aforementioned mechanistic changes leading to TAM resistance. This project aims to characterize and strengthen our understanding of the specific phenotypic profiles associated with future resistance in order to target them ahead of time to provide more effective and long-lasting anti-cancer treatment.

2.2 Hypothesis

Breast cancers exhibit cellular heterogeneity that influences acute responses to Tamoxifen at clinically relevant concentrations.

2.3 Project aims

2.3.1 Aim 1

Characterize changes in cell cycle activity in relation to hormone receptor expression (ER and PR) of persistent cells that emerge following acute TAM exposure in MCF-7 semi-embedded spheroid model.

ER-positive MCF-7 breast cancer cells were plated in wells coated with basement membrane extract and incubated with varying concentrations ([]) of Tamoxifen and synthetic nucleoside analogs. Hormone receptor expression and proliferation were assessed using immunofluorescent staining and microscopy. Additionally, cell-cycle analysis was performed using flow cytometry.

2.3.1 Aim 2

Characterize clonal expression of hormone receptor ER and PR.

ER expression is heterogeneous between cells in many ER-positive cancers. It is not well understood if there are different populations of ER-positive and ER-negative cells, or if cells can transiently express ER. MCF-7 cells were sparsely plated in cell culture dishes and incubated with varying TAM concentrations. Cloning rings were used to isolate single cell clones from parental populations. Immunofluorescence was used to visualize and compare hormone receptor status.

3. Experimental Design and Methodology

3.1 Reagents and Materials

3.1.1 Cell lines

MCF-7 cells were obtained from ATCC. MCF-7 cells were cultured in DMEM (Wisent BioProducts, 319-005-CL) supplemented with 10% Fetal Bovine Serum (FBS) (Wisent BioProducts, 080-150). Media was changed every 2-3 days. Cells were cultured in a humidified chamber at 37°C with 5% CO_2.

MCF-7 cell lines are a prototypic *in vitro* model used for the study of human breast cancer and its response to SERM therapy. They are an adherent, epithelial mammary cell line derived from the pleural effusion of a 69-year-old female suffering from metastatic breast cancer [131]. MCF-7 cells serve as an excellent model because they display characteristics similar to normal mammary epithelium: epithelial-like morphology, monolayer-type growth patterns and expression of ERα, as well as androgen, progesterone, and glucocorticoid receptors [132]. It is important to note that MCF-7 cell lines exhibit high variability and plasticity, leading to differences in gene expression profiles, proliferation and apoptotic signaling pathways, and receptor expression [133].

3.1.2 Reagents

3.1.2.1 Tamoxifen

Tamoxifen (≥99%; Sigma-Aldrich, T5648-1G) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D2438-5x10mL) at a concentration of 10mM, aliquoted and stored at -

20°C, courtesy of Dr. Élise Di Lena. TAM-rich media, regardless of concentration, contained 1% DMSO.

3.1.2.2 Synthetic Nucleoside Analogs

5-bromo-2'-deoxyuridine (BrdU) is a synthetic thymidine analog. It is phosphorylated by cells to BrdUTP and incorporated into newly synthesized DNA during S-phase instead of deoxythymidine triphosphate (dTTP) [134]. BrdU (≥99%; Sigma-Aldrich, B5002-1G) was dissolved in DMSO at a concentration of 162.8mM, aliquoted and stored at -20°C, as per the manufacturer's recommendations.

5-ethynyl-2'-deoxyuridine (EdU) is another synthetic thymidine analog that is incorporated into DNA of dividing cells during S-phase. Unlike BrdU, EdU detection can be performed without the use of harsh DNA-denaturing acids or specific antibodies. Instead, it utilizes Click Chemistry, involving covalent coupling of an azide with an alkyne to form a stable triazole ring [135]. Click-iT™ EdU Cell Proliferation Kit (ThermoFisher Scientific, C10337) and its associated reagents were stored at 4°C and -20°C as per the manufacturer's recommendations.

3.1.2.3 Cultrex

Basement membrane extracts (BME) mimic the extracellular matrix (which provides structural support to cells and mediates proliferation and survival dynamics) in a cell culture environment [136]. Cultrex PathClear Reduced Growth Factor (Cultrex) (Cedarlane Labs, 3533-010-02) was aliquoted and stored at -80°C, as per the manufacturer's recommendations.

3.1.2.4 MTS

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoliμM (MTS) assay is a commonly used test to measure cell proliferation, cell viability and cytotoxicity [137]. This assay involves the reduction of MTS tetrazolium by NADP(H)-dependent dehydrogenase in metabolically active mammalian cells to generate formazan dye. This dye can then be quantified by measuring its absorbance at 490-500nM [138]. MTS Assay kit (Abcam, ab197010) was stored at -20°C as per the manufacturer's recommendations.

3.1.2.5 Cloning rings

Cloning cylinders (or cloning rings) are glass/polystyrene disks of varying dimensions that are placed around a single cell (or a group cells) to create a colony of cloned, identical cells [139]. Cloning cylinders (8x8mm; VWR, 10170-802) were autoclaved using standard procedure and stored at room temperature in a sterile container.

3.1.2.6 High-Vacuum Grease

High-Vacuum grease is a non-melting silicone oil used for sealing and lubricating O-rings, chemical processing equipment and control valves. It is a heat stable compound with low volatility and resistant to water, mineral oils, as well as common chemicals and gases [140]. High Vaccuum Grease (DuPont MOLYKOTE, 14-635-5D) was autoclaved under standard procedure and stored at room temperature.

3.1.2.7 Propidium Iodide

Propidium Iodide (PI) is a cell-impermeant fluorescent agent used to stain cells and nucleic acids. PI binds to DNA by intercalating between the bases without sequence preference. It is commonly used to measure DNA content in cell cycle analysis, visualize DNA-containing structures, and assess cell viability [141]. Propidium Iodide Ready Flow™ reagent (PI) (ThermoFisher Scientific, R37169) was stored at room temperature as per the manufacturer's recommendations.

3.1.2.8 Anti-Estrogen Receptor alpha antibody

Anti-Estrogen Receptor alpha antibody (anti-ERα) is a rabbit monoclonal antibody [SP1] to ERα. Anti-ERα (Abcam, ab16660) was aliquoted and stored at -20°C, as per the manufacturer's recommendations.

3.1.2.9 Anti-Progesterone Receptor beta antibody

Anti-Progesterone Receptor beta antibody (anti-PRβ) is a rabbit monoclonal antibody [YR35] to PRβ. Anti-PRβ (Abcam, ab32085) was aliquoted and stored at -20°C, as per the manufacturer's recommendations.

3.1.2.10 Anti-BrdU antibody

Anti-BrdU antibody is a mouse monoclonal antibody to BrdU. Anti-BrdU (DHSB, G3G4) was aliquoted and stored at -20°C, as per the manufacturer's recommendations.

3.1.2.11 Ribonuclease Alpha

Bovine pancreatic ribonuclease alpha (RNase A) is a member of the RNase A protein superfamily. It catalyzes the transphosphorylation and degradation of RNA via the binding of single stranded RNA polynucleotide chains [142]. RNase A (Macherey-Nagel, 740505) was stored at 4°C, as per the manufacturer's recommendations.

3.1.2.12 Molecular Probes Hoechst 33258, Pentahydrate (bis Benzimide)

Pentahydrate (bis-Benzimide)-10 (Hoechst 33258) is an organic compound used as a nuclear counter stain that emits blue fluorescence when bound to double stranded DNA [143]. Hoechst 33258 (ThermoFisher Scientific, H3569) was dissolved as a 10mg/mL solution in water, aliquoted, and stored at 4°C, as per the manufacturer's recommendations.

3.2 Cell plating (Experimentation)

3.2.1 MTS Assay

The aliquot of cultrex was thawed on ice 30 minutes prior to cell culture work. 3µL of cultrex was applied evenly to the bottom of the wells of a 96-well plate. The plate was then incubated for 5 minutes on ice, followed by 15 minutes at room temperature, and finally for 5 minutes in the 37°C humidified CO_2 chamber. During the incubation process, cells were passaged using standard cell passaging technique. They were then plated at a density of 10000 cells in 100 µL of culture media, supplemented with 2% cultrex, per well. The cells were incubated and allowed to grow for 3 days. On the third day, various concentrations of TAM were added to the culture media

Cell culture media was changed every second day throughout the treatment period. Following the treatment, the TAM-rich culture media within each well was replaced with a 100µL solution containing 10µL of MTS and 90µL of PBS. The wells were allowed to incubate for 60 minutes in the 37°C humidified CO_2 chamber. Following incubation, colorimetric analysis was performed using the facility's Varioskan multipurpose plate reader (ThermoFisher Scientific, 5250010) and associated SkanIt software.

3.2.1.1 24-HOUR AND 7-DAY TREATMENTS

The 24-hour treatment and 7-day treatment used the following concentrations of TAM: 0µM, 0.1µM, 0.5µM, 1µM, 5µM, 7.5µM, 10µM, 12.5µM, 15µM, 20µM, 30µM and 100µM.

3.2.2 SEMI-EMBEDDED SPHEROIDS

The aliquot of cultrex was thawed on ice 30 minutes prior to cell culture work. 10µL of cultrex was applied evenly to the bottom of each well of the 8-well IBIDI plate. The plate was then incubated for 5 minutes on ice, followed by 15 minutes at room temperature, and finally for 5 minutes in the 37°C humidified CO_2 chamber. During the incubation process, cells were passaged using standard cell passaging technique. They were then plated at a density of 10000 cells in 200 µL of culture media, supplemented with 2% cultrex, per well. The cells were incubated and allowed to grow for 3 days. On the third day, various concentrations of TAM were added to the culture media depending on the duration of the treatment period:

3.2.2.1 SHORT-TERM TREATMENTS

Wells were incubated with culture media (supplemented with 2% cultrex) containing 0μM, 1μM, 8μM or 15μM of TAM for a period of 24 hours. Culture media was then removed, replaced with media (supplemented with 2% cultrex) containing 10μM of BrdU, and incubated for a period of 24 hours, before immunostaining and imaging.

3.2.2.2.1 LONG-TERM TREATMENTS A

Wells were incubated with culture media (supplemented with 2% cultrex) containing 0μM, 1μM or 8μM of TAM for a period of 7 days. Culture media containing TAM was then removed, replaced with media (supplemented with 2% cultrex) containing 10μM of BrdU, and incubated for a period of 24 hours before immunostaining and imaging.

3.2.2.2.2 LONG-TERM TREATMENTS B

Wells were incubated with culture media (supplemented with 2% cultrex) containing 0μM, 1μM or 8μM of TAM for a period of 7 days. Following 24 hours of treatment, 10μM EdU was incorporated into the TAM-rich media, for a period of 24 hours, after which it was removed. On the 6th day of TAM treatment, 10μM BrdU was incorporated into the TAM-rich media for a period of 24 hours. Culture media (supplemented with 2% cultrex, TAM, and/or BrdU, and/or EdU) was changed every second day. Immunostaining and imaging were performed subsequently.

3.2.3 SINGLE CELL CLONING (S.C.C.) WITH TAM

A 15 cm cell-culture plate with a confluency of approximately 70% was split using standard cell-passaging protocol. Cells were sparsely re-plated at 1:50 in two 10 cm plates. After

2-3 days of incubation, enough time to form small colonies derived from single cells, cloning rings were placed over the colonies and filled with the appropriate amount of culture media. High-vacuum grease was used to adhere and seal the cloning rings to the bottom of the plate. Following 2 more days of incubation, cells were transferred to IBIDI wells coated with cultrex, and allowed to grow in TAM-rich media (0 µM, 1 µM or 8 µM) for a period of 7 days before immunostaining (for ER and PR) and imaging.

3.2.4 Cell-cycle analysis by flow cytometry

MCF-7 cells were passaged using standard cell passaging technique and plated at a density of 50,000 cells in 1,500uL of media per well. The cells were incubated and allowed to grow for 3 days. On the third day, 0µM, 1µM or 8 µM of TAM-rich media was added to the cultures. Cells were incubated with TAM over a period of 1 week. Culture media was changed every second day. Cells were fixed, then stained with PI in preparation for flow cytometric analysis.

3.3 Immunofluorescent staining

3.3.1 General procedure

(Step 1) Culture media was aspirated, and cells were washed with Phosphate buffered saline (PBS) (Wisent, 311-010-CL) for 3 sequential 5-minute washes. (Step 2) Following the washes, cells were fixed with 4% Paraformaldehyde (PFA) (Electron Microscopy Sciences, 15713-S) for 20 minutes at room temperature. (Step 3) Cells were then blocked with 5% goat serum (GS) (Jackson ImmunoResearch, 005-000-121) and permeabilized with 0.5% Triton X-100 (BioShop Canada, TRX506.100) diluted in PBS for 1 hour at room temperature or overnight at

4°C. (Step 4) Following a single 10-minute wash in PBS, cells were incubated overnight at 4°C with primary antibodies (anti-ER, anti-PR, anti-BrdU) diluted at 1:100 in PBS with 5% GS. (Step 5) The next day, following three 15-minute washes with 0.5% Triton X-100 in PBS, species-specific secondary antibodies, (such as Alexa Fluor 488 (Jackson ImmunoResearch, 711-545-152) diluted at 1:700 in PBS with 5% GS were added to the cells for 1 hour at room temperature. (Step 6) Lastly, Hoechst 33258 diluted at 1:2500 in PBS was added to cells for 10 minutes at room temperature. Samples were stored in PBS at 4°C in a light-proof container until imaging.

3.3.1.1 BrdU-specific procedure

1.0M hydrochloric acid (Sigma-Aldrich, 258148-500ML-GL) diluted in distilled water was added to cells for 45 minutes at room temperature. The treatment with acid unwinds the DNA so that the anti-BrdU (added along with other antibodies in step 4) antibody may access the BrdU-incorporated DNA.

3.3.1.2 EdU-specific procedure

EdU Click-iT™ reaction cocktail was prepared using 1X Click-iT™ EdU reaction buffer additive, 1X Click-iT™ EdU reaction buffer, CuSO4, and AlexaFluor Azide (488nm), all of which were present in the Click-iT™ EdU Cell Proliferation Kit. The cocktail was added to each well and incubated for 30 minutes, protected from light, at room temperature.

3.3.2 PI staining for flow cytometry

Cell culture media was aspirated, and wells were trypsinized and incubated for 5 minutes in the 37°C humidified CO_2 chamber. The solutions were then quenched with media and centrifuged for 5 minutes at 1200 RPM. After discarding the supernatant, the cells were resuspended in cold PBS and underwent a second round of centrifugation for 5 minutes at 1200

RPM. Following this, cells were fixed and permeabilized in 70% ethanol (added dropwise while vortexing) incubated on ice for 30 minutes and centrifuged for a third time. To ensure that only DNA is stained, RNase A was added along with the PI solution for 30 minutes at room temperature. Lastly, the pellet was resuspended in PBS and stored at 4°C.

3.4 MICROSCOPY

A Zeiss LSM700 microscope was used for imaging. A 20x/0.8NA lens was used to image IBIDI wells, with a frame size of 1024 by 1024 pixels, 8-bit date depth, unilateral scan speed of 7, and averaging set to 2. The digital gain for each laser was between 500-700 and the power less than 5%.

3.5 FLOW CYTOMETRY

A BD Fortessa LSR (4 lasers) was used for flow cytometric analysis. A yellow/green (561 nm) laser with a 610/20 detection filter was used. Manual gating was employed to determine quantity of cells in the G0/G1, G2/M and S-phases of the cell cycle.

4. RESULTS

4.1 IN MCF-7 SPHEROIDS, THE APPROXIMATE IC_{50} VALUE OF TAMOXIFEN DECREASES AS DRUG INCUBATION TIME INCREASE.

To represent more accurately what occurs in a clinical setting (i.e., wide range of prescribed doses, unpredictable drug-tissue distribution) I decided to use broad range of clinically relevant TAM concentrations throughout the course of my experiments.

Given the high variability of MCF-7 cell lines, it was important to first establish which concentrations were to be used, rather than relying solely on previously published data. This was achieved by determining the IC_{50}, a quantitative assay used to indicate the concentration of an inhibitory substance needed to inhibit (*in vitro*) a biological process by 50% [144]. To do this, I seeded MCF-7 cells in a 96-well plate coated with cultrex and allowed them to grow for a period of 3 days, after which they were incubated with increasing concentrations of TAM for a period of 24 hours or 7 days. Following the treatment period, an MTS colorimetric assay was performed (see 3.2.1). Figure 7 shows the approximate IC_{50} after 24 hours of exposure of ≈ 33 μM (see Figure 7A), and that after 7 days of exposure of ≈ 14 μM (see Figure 7B).

Based on these values, I decided to use the following TAM concentrations for the upcoming experiments: The short-term experiment (i.e., an acute TAM exposure) (see 3.2.2.1) used 0 μM as a control, 1 μM to mimic what a tumor cell would be exposed to on the lower end, as well 8 and 15 μM to simulate the higher end. The long-term experiments (see 3.2.2.2.1, 3.2.2.2.2, 3.2.4, and 3.2.5) used 0 μM, 1 μM, 8μM as Tamoxifen concentrations. I decided to omit the 15 μM condition due to 100% cell death upon 24hrs of treatment.

4.1.1 SEMI-EMBEDDED SPHEROIDS

Semi-embedded MCF-7 spheroids were used as an *in vitro* model to assess the effects of varying TAM concentrations on MCF-7 proliferation and hormone receptor (ER or PR) expression. The 24-hour TAM exposure (see 3.2.2.1) attempts to model the spheroids' response to an acute drug exposure, whereas the 7-day exposure (see 3.2.2.2.1 and 3.2.2.2.2) models a longer-term exposure, which will identify cells that are less sensitive to an acute dose.

Two-dimensional (2D) cell culture has been the method of choice for researchers for over 100 years [145]. These adherent cultures grow as a monolayer in a flat dish or culture flask, attaching themselves to a plastic surface. 2D cultures are relatively simple and low-cost to maintain, and yield consistent, reproducible results [146]. However, cell-to-cell and cell-to-extracellular environment interactions (which heavily impact gene and protein expression, cell differentiation and proliferation, and responsiveness to drugs and stimuli) are lost. The 2D conditions also lead to altered or inaccurate tissue morphology and the loss of phenotypic diversity [147]. For these reasons, I opted to use a semi-embedded, three-dimensional (3D) model, to more accurately mimic the cellular environment of cells *in vivo*. This was achieved using basement membrane extract (BME) (see 3.1.2.3), containing a mixture of extracellular proteins (such as Laminin I, Collagen I, Collagen IV and Vitronectin) to form a reconstituted basement membrane that promotes and maintains differentiated epithelial phenotypes in vitro [148].

Data from the IC_{50} experiment indicate that cells are heterogeneously sensitive to Tamoxifen. While many cells die, we wondered if the less sensitive residual cells had proliferative capacity.

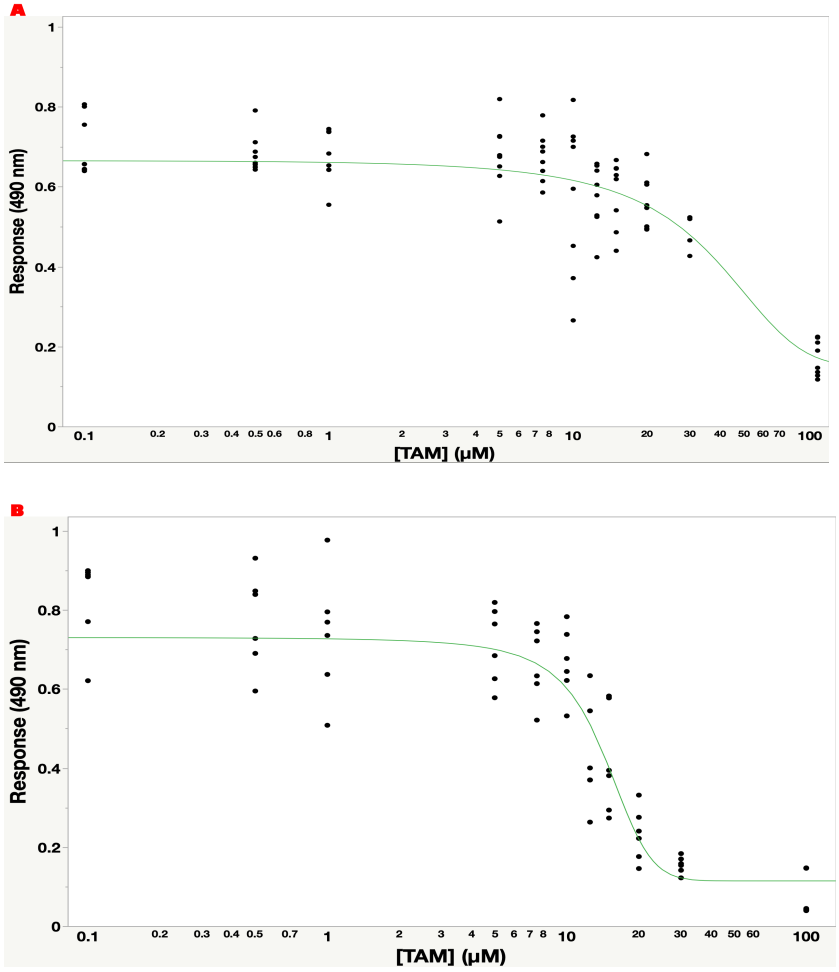

Figure 7: *The approximate IC_{50} value of Tamoxifen decreases as drug incubation time increases*.**A,B** MCF-7 cells were seeded in 96-well plates coated with cultrex and allowed to grow for a period of 3 days, after which they were incubated with increasing concentrations of TAM (0µM, 0.1µM, 0.5µM, 1µM, 5µM, 7.5µM, 10µM, 12.5µM, 15µM, 20µM, 30µM and 100µM) for a period of 24 hours (**A**) or 7 days (**B**). Following treatment, TAM-rich culture media was replaced with 10% MTS solution for 60 minutes. Following incubation, colorimetric analysis was performed on a Varioskan multipurpose plate reader and SkanIt software. The X-axis represents the logarithmic scale of the broad range of Tamoxifen concentrations. In the Y-axis, the reduction of MTS tetrazolium, performed by metabolically active cells, is quantified through measuring its absorbance at 490nm. Each black dote represents a concentration of Tamoxifen. 3 replicates were performed.

4.2 The relationships between hormone receptor expression and BrdU incorporation vary in MCF-7 spheroids when exposed to increasing concentrations of Tamoxifen

4.2.1 The percentage of BrdU-positive cells within a single spheroid decreases as the concentration of Tamoxifen increases

Measuring cellular proliferation is one of many ways to assess cell activity, genotoxicity, and drug efficacy. This is traditionally determined by incubating cells with a single pulse of a nucleoside analog, such as BrdU, that'll incorporate into the DNA during S-phase [134].

MCF-7 cells were seeded in 8-well IBIDI plates coated with cultrex and allowed to grow for 3 days in culture media supplemented with cultrex. Varying concentrations of TAM (0μM, 1μM, 8μM or 15μM for the short-term treatment, see 3.2.2.1; 0μM, 1μM or 8μM for the long-term treatment, see 3.2.2.2.1.) were then added for a period of 24 hours or 7 days. Following TAM treatment, the wells were incubated and labelled with 10 μM of BrdU for a period of 24 hours. Immunostaining and imaging followed subsequently (see 3.3.1 and 3.3.1.1).

The short-term treatment (see 3.2.2.1) indicates that as [TAM] increases, the percentage of BrdU positive cells (%BrdU+) within a single spheroid decreases (see Figure 8A). The long-term treatment A (see 3.2.2.1.1) indicates an inverse relationship between BrdU incorporation and [TAM] (Figure 9A)

4.2.2 The percentage of hormone-positive cells within a spheroid varies as the concentration of Tamoxifen increases

Steroid hormones and their receptors play a crucial role in the development and progression of breast cancer. Furthering our understanding of their relationship in relation to Tamoxifen treatment is crucial to overcoming endocrine therapy resistance.

In the short-term treatment, as [TAM] increases, so does the mean percent of ER-positive cells (%ER+) (see Figure 8B). As [TAM] increases, progesterone receptor expression (%PR+) decreases (Figure 8B). In Long-term experiment A (see 3.2.2.1.1), the proportion of ER-positive cells increase as [TAM] increases. From 1 to 8 μM, there is a statistically significant decrease in percent ER-positive cells (Figure 9B). As [TAM] increases, the percentage of PR-positive cells decreases (Figure 9B).

4.2.3 The percentage of dual-positive (for ER/PR and BrdU) cells within a spheroid varies as the concentration of Tamoxifen increases

Figures 8C and 9C depict the relationship between cells within a single spheroid that are both positive for either ER or PR, and BrdU (i.e., % Dual+) as the concentration of Tamoxifen increases. For the short-term treatment, in the case of the estrogen receptor, as [TAM] increases, the percentage of dual-positive cells decreases. However, from 8 to 15 μM, there is a statistically significant increase (Figure 8C). With regards to the progesterone receptor, as [TAM] increases, the percentage of dual-positive cells decreases (Figure 8C). In the long-term treatment (long-term experiment A; see 3.2.2.1.1), as [TAM] increases, cells that are positive for both ER and BrdU decreases as well (Figure 9C). With regards to PR, as [TAM] increases, the mean percentage of

dual-positive cells does not seem to follow an apparent trend. From 0 to 1 μM, there is an increase, though not statistically significant. At 8μM, the percent dual-positive is significantly lower compared to both the 1 μM and the 0 μM control (Figure 9C).

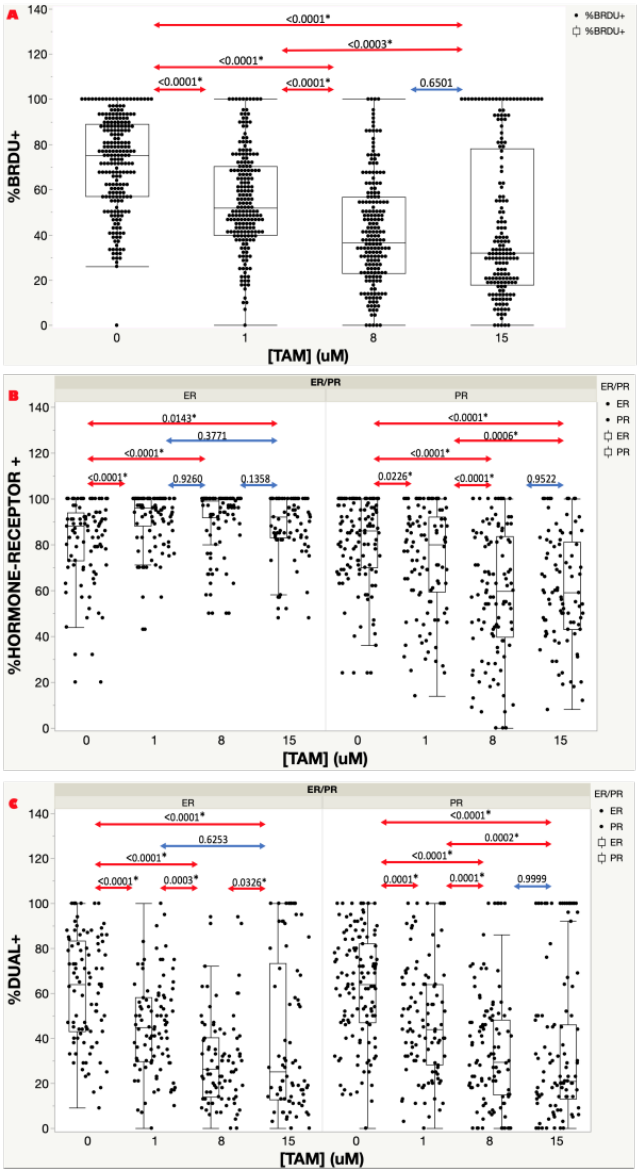

Figure 8: *Following a treatment period of 24 hours, the percentage of BrdU-positive, Hormone-positive, and Dual-positive cells in a spheroid vary as the concentration of Tamoxifen increases*. **A,B,C** MCF-7 cells were plated in IBIDI wells coated with Cultrex, with culture media containing 2% cultrex. Spheroids were allowed to grow for 3 days, after which they were exposed to 0, 1, 8 or 15 µM of TAM for a duration of 24 hours. TAM media was replaced after the treatment period with culture media containing 2% cultrex and 10µM of BrdU, for 24 hours. Immunostaining, fluorescent microscopy, and image analysis followed, in order to determine the percentage of cells within a spheroid that were proliferating (**A;** %BrdU+), expressing hormone receptor (**B**; %Hormone+ (ER+ or PR+)) and proliferating while expressing hormone receptor (**C**; %Dual+), following Tam treatment. Each black dot represents an individual spheroid. 3 replicates were performed. The red lines indicate significant differences between means. The blue lines indicate P-values that are not significant.

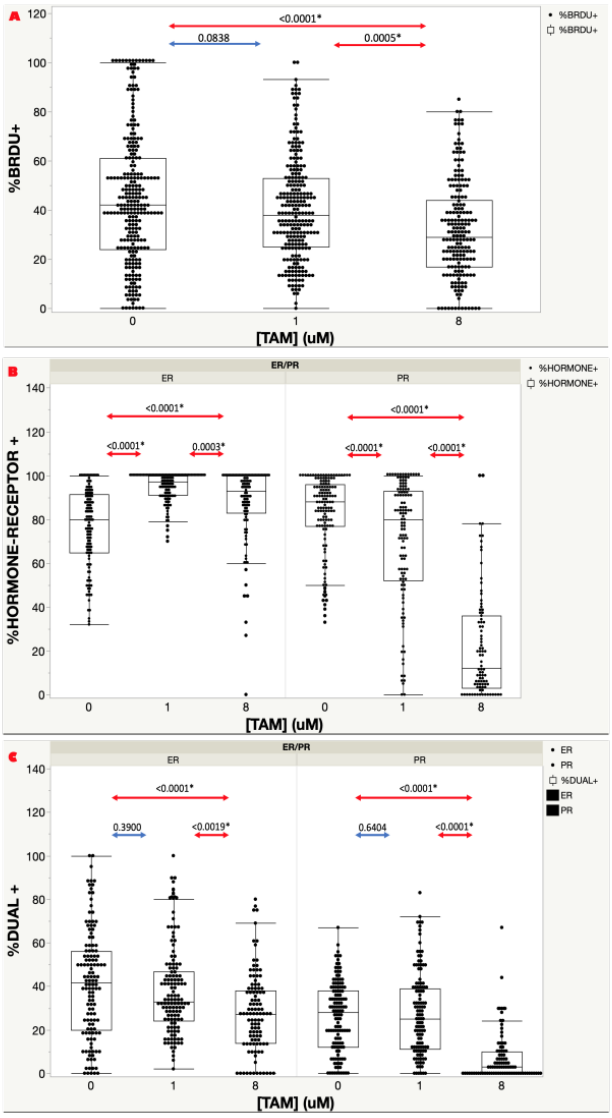

Figure 9: *Following a treatment period of 7 days, the percentage of BrdU-positive, Hormone-positive, and Dual-positive cells in a spheroid vary as the concentration of Tamoxifen increases*. **A,B,C** MCF-7 cells were plated in IBIDI wells coated with cultrex, with culture media containing 2% cultrex. Spheroids were allowed to grow for 3 days, after which they were exposed to 0, 1 or 8 µM of TAM for a duration of 7 days. TAM media was replaced at the end of the 7th day with culture media containing 2% cultrex and 10µM of BrdU, for 24 hours. Immunostaining, fluorescent microscopy, and image analysis followed suit, in order to determine the percentage of cells within a spheroid that were proliferating (**A;** %BrdU+), expressing hormone receptor (**B**; %Hormone+ (ER+ or PR+)) and proliferating while expressing hormone receptor (**C**; %Dual+), following Tam treatment. Each black dot represents an individual spheroid. 3 replicates were performed. The red lines indicate significant differences between means. The blue lines indicate P-values that are not significant.

4.3 The relationships between hormone receptor expression and incorporation of thymidine analogs EdU and BrdU vary in MCF-7 spheroids when exposed to increasing concentrations of Tamoxifen

In the case of drug efficacy testing, the incorporation of multiple nucleoside analogs at different time points (i.e., dual-pulse or dual-labelling) can aid in better defining cell cycle kinetics and the proliferative nature of cells in culture. I performed an EdU pulse at an early time point (i.e., 24 hours after drug administration), followed by a second pulse with BrdU (the second nucleoside analog) later point (i.e., during the last 24 hours of treatment) in culture.

MCF-7 cells were seeded in 8-well IBIDI plates coated with cultrex and allowed to grow and incubate for 3 days in culture media supplemented with cultrex. Varying concentrations of TAM (0μM, 1μM or 8μM, see Long-term experiment B 3.2.2.2.2.) were then added for a period of 7 days. After 24 hours of exposure to TAM, a 24-hour EdU pulse was performed. On the 6th day of TAM treatment, BrdU was incorporated into the TAM-rich media (i.e., the second consecutive nucleoside pulse) for a period of 24 hours. Following TAM treatment, immunostaining and imaging were performed (see 3.3.1 and 3.3.1.1).

As [TAM] increases, the percentage of EdU-positive cells within a single spheroid (i.e., %EdU+) remains relatively constant. The percentage of BrdU-positive cells decreases as [TAM] increases (see Figure 10A).

Figure 10B depicts the percentage of cells that are actively cycling at both time points, cycling at one of the two time points, or not cycling at either of the time points, representing a state of cellular quiescence.

As [TAM] increases, the percentage of cells within a single spheroid positive for both EdU and BrdU (i.e., Dual positive: Proliferating at both time points; %(EdU/BrdU)+) decreases. Though from 1 and 8 μM, there is a slight, statistically significant increase. The percentage of EdU-positive/BrdU-negative cells remains relatively constant despite the increase in [TAM]. The percentage of EdU-negative/BrdU-positive cells decreases as [TAM] increases. Lastly, the percentage of dual negative cells (i.e., %(EdU/BrdU)-) increases with increasing [TAM] (Figure 10B).

It has been well established that steroid hormones drive breast cancer proliferation in hormone receptor-positive breast cancers. However, a tumor does not require the totality of its cells to be hormone receptor-positive to be classified as such. Figures 10C and 10D venture to more clearly define the relationship between hormone receptor status and proliferation throughout Tamoxifen treatment.

Figure 10C depicts the relationship between cells within a single spheroid that are both positive or negative for either ER or PR, and EdU (i.e., first time point) as the concentration of TAM increases.

As [TAM] increases, the percentage of cells within a single spheroid that are proliferating while expressing ER (i.e., %(H/EdU) +) remains relatively constant. The percentage of ER-positive/EdU-negative (%(H+/EdU-)) cells follows no particular trend: it increases from 0 to 1 μM, then decreases from 1 to 8 μM, both in a statistically significant manner. The percentage of ER-negative/EDU-positive cells (%(H-/EdU+)) decreases as [TAM] increases. Lastly, the percentage of dual negative cells (i.e., (H/EdU)-) increases Tamoxifen concentration increases, despite a statistically insignificant decrease from 0 to 1 μM.

In the case of progesterone receptor, the percentage of dual-positive (%(H/EdU)+)), PR-positive/EdU-negative (%(H+/EdU-)) and PR-negative/EdU-positive (%(H-/EdU+)) decrease with increasing [TAM]. The percentage of dual-negative cells (%(H/EdU)-) is an exception however, where it seems to increase as [TAM] increases. (Figure 10C)

Figure 10D depicts the relationship between cells within a single spheroid that are both positive or negative for either ER or PR, and BrdU (second time point) as the concentration of TAM increases. For both ER and PR:

- as [TAM] increases, the percentage of hormone receptor-positive/BrdU-positive cells (%(H/BrdU)+) decreases.
- The hormone receptor-positive/BrdU-negative (%(H+/BrdU-)) increases from 0 to 1 μM and decreases from 1 to 8 μM in a statistically significant manner.
- The hormone receptor-negative/BrdU-positive (%(H-/BrdU+)) increases with increasing [TAM] (in the case of ER, there is a statistically insignificant decrease from 0 to 1 μM to note).

Lastly, as Tamoxifen increases, the percentage of cells that are negative for both ER and BrdU (i.e., %(H/BrdU)-) does not follow a particular trend: it decreases from 0 to 1 μM, and increases from 1 to 8 μM, both in a statistically significant manner. For progesterone receptor, the percentage of dual negative cells (%(H/BrdU)-) increases as [TAM] increases. (Figure 10D).

Figure 10: *Following a treatment period of 7 days, the relationship between thymidine analog incorporation and hormone receptor expression in MCF-7 spheroids vary as the concentration of Tamoxifen increases*. **A,B,C,D** MCF-7 cells were plated in IBIDI wells coated with cultrex, with culture media containing 2% cultrex. Spheroids were allowed to grow for 3 days, after which they were exposed to 0, 1 or 8 µM of TAM for a duration of 7 days. Following 24 hours of treatment, 10µM EdU was incorporated into the TAM-rich media, for a period of 24 hours, after which it was removed. On the 6th day of TAM treatment, 10µM BrdU was incorporated into the TAM-rich media for a period of 24 hours. Immunostaining, fluorescent microscopy, and image analysis followed suit, in order to determine the percentage of cells within a spheroid that were actively cycling at the first time point (**A**; %EdU+; yellow) or at the second time point (**A**; %BRDU+; green). The red lines indicate significant differences between means. The blue lines indicate P-values that are not significant. The percentage of cells within a spheroid that were actively cycling at both time points (**B**; %(EdU/BrdU)+; blue), at the first time point but not during the second (**B**; %(EdU+/BrdU-); yellow), at the second time point but not the first (**B**; %(EdU-/BrdU+); green) and those not cycling at all (**B**; %(EdU/BrdU)-; grey) was also measured. P-values are show above in a table format. Lastly, the percentage of cells within a spheroid that are double positive, double negative, or single positive for EdU (**C**) or BrdU (**D**) and presence or absence of estrogen or progesterone receptors (H+ or H-) was determined. P-values are show above in a table format. 3 replicates were performed.

4.3 MCF-7 SPHEROIDS DERIVED FROM SINGLE CELL CLONES LIKELY EXHIBIT TRANSIENT ER AND PR EXPRESSION

Single cell cloning (SCC) is a technique used to generate a population of genetically identical cells derived from a single parent cell. It can be used in biological research to study lineage tracing, monoclonal antibody production, and cellular heterogeneity, amongst many other applications [149]. For the purposes of this project, SCC was used to study the expression of ER and PR in individual MCF-7 cells given this cell line's heterogenous and plastic expression of proliferative genes and receptors (see 3.1.1). Variations in ER and PR expression could serve as a valuable insight when predicting a cell line's (or a tumor's) response to hormone therapy, such as Tamoxifen.

MCF-7 cells were sparsely plated as single cells in a 15 cm cell-culture plate. After 2-3 days of incubation, the individual cell colonies were isolated from others using glass cloning rings, sealed with high-vacuum grease. Following 2-3 more days of incubation, the contents of each cloning ring were transferred to IBIDI wells coated with cultrex, so that they may form 3D spheroids. They were then allowed to grow for a period of 7 days in TAM-rich media (0μM, 1μM or 8 μM) before ER/PR immunostaining and fluorescent imaging (see 3.2.3).

As it pertains to estrogen receptor expression, the percentage of ER-positive cells within a MCF-7 spheroid (derived from a single-cell clone) remains very consistent (about 97% ER+) following 7 days of TAM exposure, at concentrations of 0 and 1 μM. This is true, regardless of spheroid size, represented as number of cells. The only statistically significant difference is found at the 8 μM concentration (in the 2-14 cell range), where the mean percent of ER-positive cells is 68% (See Figure 11; ER in blue).

The percentage of PR-positive cells also remain consistent (about 66% PR+) following 7 days of TAM exposure, at concentrations of 0 and 1 µM. This is true, regardless of spheroid size, represented as number of cells. At the 8µM concentration (in the 2-14 cell range), the mean percentage of PR-positive cells is not (statistically) significantly different when compared to the other two concentrations, though its mean is slightly lesser, at approximately 61% PR+ (See Figure 11; PR in red).

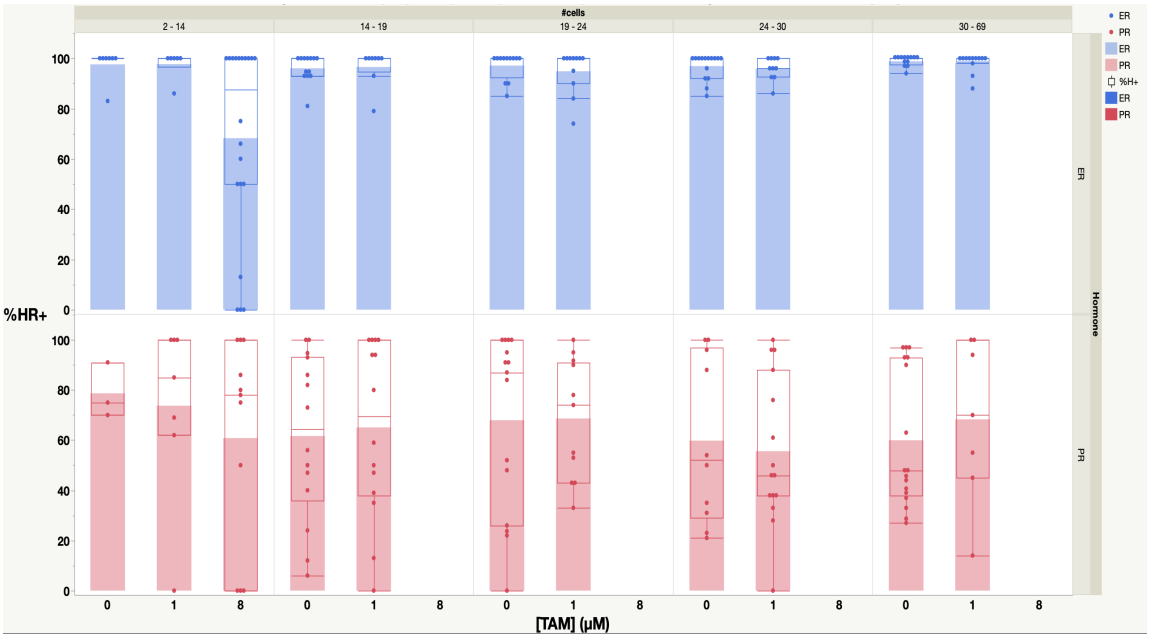

Figure 11: MCF-7 spheroids derived from single cell clones exhibit heterogeneous ER and PR expression. MCF-7 cells were sparsely plated as single cells in a 15 cm cell-culture plate. After 2-3 days of incubation, the individual cell colonies were isolated from others using glass cloning rings, sealed with high-vacuum grease. Following 2-3 more days of incubation, the contents of each cloning ring were transferred to IBIDI wells coated with cultrex, so that they may form 3D spheroids. They were then allowed to grow for a period of 7 days in TAM-rich media (0µM, 1µM or 8 µM) before ER/PR immunostaining and fluorescent imaging. The percentage of cells within a spheroid expressing hormone receptor (ER in blue, PR in red) was determined. Spheroid size is represented as number of cells (#cells). Each dot represents an individual spheroid. 3 replicates were performed.

4.4 TAMOXIFEN INDUCES CELL CYCLE ARREST SPECIFICALLY IN THE G1-PHASE OF THE CELL CYCLE

Cell cycle analysis by flow cytometry is a common technique used to assess the distribution of cells in different phases of the cell cycle. The DNA content of individual cells can be measured using PI, and the proportion of cells in G0/G1, S and G2/M phases can be determined subsequently using a DNA content histogram and manual gating [150].

It has been established that TAM administration can alter the cell cycle progression of ER-positive breast cancer cells through a variety of mechanisms, via the inhibition or altered expression of cyclins (proteins involved in the regulation and progression of the cell cycle) and modulation of Cyclin-Dependent Kinases (CDKs) [151]. However, the dose dependent effects of TAM on cell cycle progression remains unclear and varies greatly depending on the cell in line in question.

MCF-7 cells were seeded in 6-well plates and allowed to grow for 3 days, before being subjected to TAM treatment (0 μM, 1 μM or 8 μM) for a period of 7 of days. They were then stained with PI, as per the protocol found in section 3.3.2.

The data derived from cell cycle analysis by flow cytometry, depicted in Figure 12, indicates that the majority of MCF-7 were found in the G1-phase of the cell cycle, irrespective of the concentration of TAM they were subjected to. The proportion of cells in the G2-phase was consistently higher than those in S-phase at all concentrations as well.

The proportion of cells in G1 saw a statistically significant increase when comparing the control to both the 1 and 8 μM conditions. There were also statistically significant decreases in the proportion of cells in both S and G2 phases of the cell cycle from 0 to 1 μM and 0 to 8 μM of

TAM. There were no statistically significant differences in G1, G2 and S-phase when comparing 1µM to 8µM of TAM.

[TAM] µM	P-Value		
	% G1 phase	% S phase	% G2 phase
0,1	<.0001*	0.0031*	0.0041*
0,8	0.0001*	0.0008*	0.0035*
1,8	0.9687	0.7729	0.9968

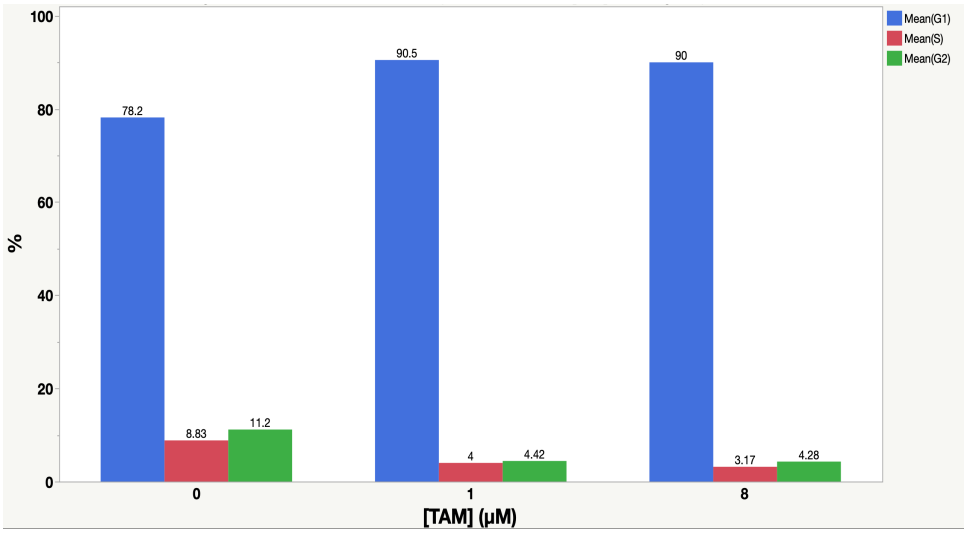

Figure 12: Tamoxifen induces cell cycle arrest specifically in the G1-phase of the cell cycle. MCF-7 cells were seeded in 6-well plates and allowed to grow for 3 days, before being subjected to TAM treatment (0 µM, 1 µM or 8 µM) for a period of 7 of days. They were then stained with PI, followed by cell cycle analysis by flow cytometry. The percentage of cells in G1-phase, (blue), S-phase (red) and G2-phase (green) of the cell cycle was determined. The mean percentage of cells in a particular phase of the cell cycle is labelled above each bar. 3 replicated were performed.

5. DISCUSSION

5.1 THE APPROXIMATE IC_{50} VALUE OF TAMOXIFEN DECREASES AS DRUG INCUBATION TIME INCREASES

Inhibitory concentration curves are dose response curves used in laboratory and pharmaceutical settings to determine the drug concentration needed to decrease the population of viable cells by a specific percentage (in this case 50%) when compared to cells not exposed to the drug in question. The IC_{50} provides an approximate of the drug concentration needed to inhibit a biological process by half [144]. In the context of cancer specifically, using a drug at its IC_{50} concentration translates to inhibiting tumor growth by half. If an IC_{50} is determined to be a lower concentration, this entails that less of the drug is required to be considered effective, which in turn lowers the systemic toxicity when administered to patients, improving outcomes [152].

The cytotoxic effects of TAM in MCF-7 cells have been documented, though it seems to be highly variable based on the literature. For example, Hassan et al. carried out an MTT assay([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliμM bromide, which differs slightly from the MTS compound, in an MCF-7 cell line, and found an IC_{50} of 4.506 ug/mL, equivalent to 12.13 μM after 24 hours of exposure to TAM [153]. On the other hand, Seeger et al. demonstrated that 4-hydroxytamoxifen showed an IC_{50} value of 27μM in MCF-7 cells after 4 days of exposure [154]. These findings seem to contrast with those found throughout my experimentation: A higher IC_{50} was determined following "short-term" exposure to TAM (i.e., ≈ 33 μM; 24 hours of TAM exposure) when compared to the "long-term" exposure (i.e., ≈ 14 μM; 7 days of TAM exposure). This is most likely attributable to the increased duration of time the spheroids were exposed to TAM, allowing more of the cells within the well to metabolize the drug and succumb to its inhibitory effects.

It should be noted however that the IC_{50} value of a drug can be influenced by several factors. For example, drug metabolism and pharmacokinetics can influence its concentration at the target site. Factors such as drug distribution, absorption, metabolism, and elimination can also influence the IC_{50} by altering the drug's bioavailability [155]. The target molecule's (in this instance, ER) biomolecular properties, such as receptor density and expression levels, binding site accessibility and conformational changes may also influence the IC_{50} value. Lastly, the physiological conditions of the cellular environment, such as pH, temperature, and the presence of other molecules can influence the drug's interactions with target molecules, may impact the IC_{50} [155].

Throughout my experimentation, cultrex, a basement membrane extract was used to provide a more "tissue-representative' environment. The presence of proteins such as laminin, collagen and vitronectin may have increased MCF-7 cell survivability, which may explain why my IC50 values are elevated compared to those found in the literature [156].

5.2 Effects of Short and long-term TAM treatment on proliferation of MCF-7 spheroids.

BrdU proliferation assays are a common laboratory technique used to quantify cell proliferation in cancer research. More specifically, this thymidine analog incorporates itself into newly synthesized DNA during S-phase of the cell cycle, allowing for the assessment of the rate of DNA synthesis and cellular proliferation [157].

The data obtained throughout my experimentation following both "short" (24-hour) and long (7-day)-term exposure to TAM suggest that as the concentration of Tamoxifen increases, the percentage of cells incorporating BrdU (i.e., proliferating) decreases (see Figures 8A and 9A). This is also true when looking at the data from the dual labelling experiment (see Long-term treatment

B; 3.2.2.2.2.), during which BrdU labelling occurred while the cells were still receiving the SERM treatment (in contrast to 3.2.2.1. and 3.2.2.2., which were labelled with BrdU following TAM treatment). These results were expected, given the vast swathes of literature (highlighted in section 1.6.2) and clinical evidence indicating TAM's anti-proliferative effects in breast tissue.

EdU, similar to BrdU, is a thymidine analog that is incorporation into dividing cells during S-phase (see 3.1.2.2 for more information regarding the benefits of EdU versus BrdU proliferation assays). Interestingly, EdU can be used in conjunction with BrdU to determine the proliferative state of cells at two different time points throughout the incubation process. For the purposes of my experimentation, using this double labelling procedure allowed me to visualize changes in the proliferative state of individual MCF-7 cells throughout the course of the TAM treatment. The cells were first incubated with EdU, on the second day of TAM treatment (time point #1). BrdU was added second, on the 6th day of TAM treatment (time point #2). The data represented in Figure 10A shows little to no change in EdU incorporation despite the increase in TAM concentration. This seems to contrast with the short-term TAM experiment (see Figure 8A) data, mentioned above. This is most likely attributable to the previously mentioned heterogeneity within MCF-7 cells (even within the same cell line), which may have conferred those used in this specific experiment with inherent resistance to TAM, briefly summarized in Figure 6 (section 1.8).

Figure 10B depicts the percentage of cells within a single spheroid that are actively cycling at both time points (%(EdU/BrdU)+), during one of the two ((%(EdU+/BrdU-) or (%(EdU-/BrdU+)), or not cycling during either (%(EdU/BrdU)-).

The percentage of EdU-positive/BrdU-positive cells should be highest (which it seems to be) at the control (i.e., 0μM of TAM) because there is no inhibition present in the cell culture media. In theory, if TAM is acting solely as an antagonist, then we should see a decrease in dual-

positive cells from 0 to 1 μM (which we do, albeit statistically insignificant), and an even further decrease from 1 to 8 μM. However, the data seems to indicate an increase in EdU-positive/BrdU-positive cells at the highest concentration versus that of the low concentration, suggesting that TAM might be exerting a stimulatory effect at this concentration, within this specific cellular context. Interestingly, recent off-label clinical biomarker-based trials have demonstrated that low-dose tamoxifen (e.g., 10 mg daily, 5 mg daily, 10 mg weekly, etc.) could prove to be equally effective to the standard dosing regimen. Findings such as these suggest a similar anti-cancer benefit, with a diminished risk of TAM-associated thromboembolic events and uterine malignancies [158].

It has been well documented that Tamoxifen, being an ER modulator (i.e., not strictly an agonist or antagonist), can act as an agonist in endometrial epithelium, leading to stimulated cellular proliferation and metabolism [158].

In contrast, mechanistic evidence for its stimulatory effect in mammary epithelial tissue is still lacking, despite having been observed at the clinical level since the late 1970s [159]. Recent studies have pointed towards novel membrane-bound G protein-coupled estrogen receptor-1 (GPER-1 or GPR30) as potential contributors to TAM resistance and TAM-induced proliferation in MCF-7 cells. More specifically, GPER-1 is a GPCR that participates in various biological activities in response to endogenous estrogens [160]. Approximately 60% of breast tumors are GPER-1 positive, which is correlated with over-expression of HER-2, EGFR (HER–1), connective tissue growth factor (CTGF), and positive lymph node status [161]. Interestingly, it has been reported that patients with GPER-1 positive tumors suffered increased tumor size and mass following 4-6 months of Tamoxifen therapy [162]. This could be due to TAM (and its primary

metabolite 4-OHT) acting as GPER-1 agonists, exacerbating its proliferative effects on neighboring epithelial tissue [163].

The percentage of EdU-positive/BrdU-negative cells should increase as the [TAM] increases. In fact, if TAM is truly strictly inhibitory in this context, then the MCF-7 cells within a spheroid are more likely to be actively cycling early on during Tamoxifen treatment (EdU) than during the later stages, say after 6 days (BrdU). The data in Figure 10B, show that the percentage of cells no longer proliferating towards the latter stages of treatment seems to hover around 50%. Interestingly, this percentage seems to remain relatively stable despite the increase in [TAM]. This seems to suggest that TAM is not having its intended inhibitory affect, seeing as though the proportion of EdU-positive/BrdU-negative cells remained constant.

The percentage of EdU-negative/BrdU-positive cells attempts to provide insight into TAM's potential stimulatory effect in MCF-7 cells. More specifically, if for unknown mechanistic reasons, Tamoxifen was stimulating the proliferation of a subset of MCF-7 cells in culture, then we should see an increase in the percentage of cells that have re-entered the cell cycle at 1 and/or 8 μM TAM conditions. However, this is not reflected in the data, as the %(EdU-/BrdU+) decreases from 0 to 1 μM and remains constant from 1 to 8 μM.

Lastly, the percentage of double negative cells (i.e., %(EdU/BrdU)-) depicts the proportion of cells that were not actively cycling during either of the thymidine analog pulses. In theory, this percentage should increase as the concentration of TAM increases due to the expected inhibition of ER-mediated proliferation. The data from Figure 10B seem to corroborate this theory, though only partially. In fact, there is a significant increase in the percentage of double negative cells when comparing 0-1 μM and 0-8 μM. However, the percentage of dual-negative cells is identical in the 1 and 8 μM conditions, which may suggest that a lower dose of TAM is just as effective as the

more elevated one for the purposes of inducing cellular quiescence and/or slowing proliferation. Lowering the dose is of particular interest to clinicians, who are aiming to lessen the often-debilitating array of side effects associated with Tamoxifen [158].

5.3 Effects of Short and long-term TAM treatment on ER and PR expression in MCF-7 spheroids.

Steroid molecules such as estrogen (estradiol) and progesterone and their respective receptors play a crucial role in several physiological processes, ranging from cholesterol mobilization to the development of sexual organs [38].

Originally it was thought that of the two steroid molecules mentioned above, solely estrogen was responsible for hormone-induced carcinogenesis and breast cancer progression. However, more recent studies suggest that dysfunctions in both ER and PR signaling could induce neoplastic transformation in mammary tissue.

As it pertains to estrogen receptor, the data from both short and long-term BrdU experiments indicate an increase in ER expression as the concentration of TAM increases (see Figures 8B and 9B). These findings are unexpected, given that the literature states that Endoxifen (a metabolite of TAM) has the capacity to target ER for proteasomal degradation, resulting in a reduction in protein levels [104]. A literature review of both in vitro and in vivo studies pertaining to the stimulatory effects of TAM of ER expression in breast tissue was conducted to justify these findings. Unfortunately, there does not appear to be any evidence to corroborate the data collected throughout my experimentation.

The percentage of dual positive cells for ER and BrdU decreases as [TAM] increases (see Figure 8C, 9C and 10D). This is true for both short (24-hour) and long-term (7-day) exposure to

TAM. These results are expected, given that estrogen signaling is the primary driver of proliferation in MCF-7 cells, derived from an estrogen receptor positive tumor. Interestingly, this data also indicates that at all [TAM], there exists a population of ER-positive cells that continue to actively cycle, despite being deprived of their primary source of stimulation. This suggests that this population of cells is relying on other growth factors present in the media to continue proliferating.

The percentage of dual positive cells for ER and EdU remains constant as [TAM] increases (see Figure 10C). This contrasts with the data found in Figure 8C (short-term experiment) due to differences in ER expression in both experiments. In fact, ER expression levels increase throughout the short-term treatment, leading to an increase in the proportion of dual positive cells as Tamoxifen increases. Following this logic, the consistency in ER-positive/EDU-positive cells in long-term experiment B is most likely due to constant ER expression levels.

The percentage of ER-positive/BRDU-negative (i.e., not proliferating; see Figure 10D) increases alongside [TAM]. This is expected, indicating that TAM is exerting its intended effect on this sub-population of cells: the Tamoxifen molecule is competing with circulating estrogen for ER receptor binding, preventing the transcription of proliferative genes associated with cellular growth. The percentage of ER-positive/EDU-negative (i.e., not proliferating; see figure 10C) follows a similar trend.

The percentage of ER-negative/BRDU-positive (i.e., proliferating independent of ER expression; see figure 10D) seems to increase alongside TAM. This sub-population of cells should in theory remain constant, given that TAM molecules have no receptor to bind to. Despite making up a small fraction of the total cells measured, the increase is still statistically significant. This raises concerns regarding the effects of Tamoxifen on the sub-population of cells within a tumor

that are not positive for ERα. Currently, there is no consensus regarding the effectiveness of TAM in ER-positive-low or ER-negative patients. Researchers have recently been looking to more accurately define TAM's ER-independent mechanisms of action: TAM has been shown to stimulate the mitochondrial apoptotic pathway through the upregulation of pro-apoptotic proteins BAX and Caspase-3, disrupt cancer cell metabolism via inhibition of Gln uptake, and induce cell death by directly attacking ETC complex I, leading to the activation of AMPK pathway and inhibition of mTOR signaling [164-166]. Despite these positive findings, results of a meta-analysis conducted by the Early Breast Cancer Trialist's Collaborative Group (EBCTCG) found a 4% increased risk of death from breast cancer in ER-negative patients treated with TAM over a period of 5 years [167]. A more recent review by Merglen et al. (2009) arrived at a similar conclusion [168]. It has also been shown in vitro that the agonist activity of tamoxifen on cell proliferation was exacerbated in cells expressing elevated levels of HER-2 and AIB1 (an ER co-activator), both of which are more frequently expressed in ER-negative disease and associated with TAM resistance [169]. The lack of consensus on this topic raises concerns regarding the guidelines for determining the ER status of tumors, which will ultimately influence the treatment regimen offered to patients. Currently, ASCO and the College of American Pathologists (CAP) guidelines for ER (and PR) testing were updated in 2020 based on a systematic review of medical literature: Tumor samples with 1% to 100% of nuclei positive for ER or PR should be interpreted as positive. More specifically for ER, samples that are 1% to 10% ER positive should be classified as ER-low-positive. Biopsies should be considered ER and PR-negative if less than 1%, or 0% of nuclei are immunoreactive [170]. However, clinical reports show the use of wider, and somewhat arbitrary thresholds values for ER positivity (e.g., >0%, 5%-10%, and >20%) [171]. Recent studies have also presented findings suggesting that the majority of low (1-10%) ER-positive breast cancer

behave more so like hormone receptor negative tumors, calling into question the efficacy of hormone therapy in these particular cases [172].

The percentage of ER-negative/EDU-positive (i.e., proliferating independent of ER; see figure 10C) seems to decrease as the concentration of Tamoxifen increases. These cells also make up a much larger proportion of the total number of cells, likely due to Tamoxifen having less time to exert its likely anti-proliferative effects. This data suggests that this sub-population of cells are showcasing reduced proliferative capacity following an acute (24-hour) exposure to Tamoxifen. Given that these cells do not express ER, it is possible that TAM is inhibiting their growth via an ER-independent mechanism.

Lastly, the percentage of dual negative cells for ER and BRDU/EDU decreases from 0 to 1 μM but increases from 1 to 8 μM of TAM. These dual negative cells represent a sub-population of ER-negative surviving cells that are not actively cycling. This phenotype seems to make up a larger proportion of cells at all concentrations at the second time point (BRDU), compared to the first. This makes sense, as more spheroids are proliferating because TAM has not had as much time to exert its cytostatic/cytotoxic effects. The observed increase is likely attributable to spheroids at the highest concentration having significantly fewer total number cells, thus over-representing the proportion of surviving cells with an ER-negative/EDU, BRDU-negative phenotype. On the basis that Tamoxifen solely inhibits estrogen signaling, it follows that the percentage of dual negative cells remain constant despite increases in TAM, because no receptor is present. Therefore, the observed decrease in the proportion of this phenotype can likely be attributed to ER-independent mechanisms of action, briefly mentioned above.

It has not been addressed in the literature whether TAM has a direct effect on progesterone receptor expression or signaling. However, there is evidence that elevated PR expression levels

predict positive clinical outcomes and the beneficial effect of adjuvant hormonal treatments [173]. In fact, studies have indicated that ER-positive/PR-positive breast cancer have a more favorable breast cancer-specific survival rate than ER-positive/PR-negative subtypes, especially when treated with Tamoxifen [174, 175]

With regards to progesterone receptor, the data from the short and long-term BRDU experiments indicate that progesterone receptor expression decreases as the concentration of Tamoxifen increases (see figures 8B and 9B). These findings are expected given that the PGR gene can be activated by E_2 due to ERE half-sites close to its SP1 binding site, resulting in PR transcription being partially driven by ER-mediated events [61, 62]. However, given that the staining for ER and PR occurred in separate wells, it cannot be stated with certainty that the decrease in PR expression is a product of decreased ER expression following TAM treatment. In fact, as mentioned above, the data from both short and long-term BRDU experiments indicate an increase in ER expression as the concentration of TAM increases, not a decrease. Therefore, future experiments should make use of ER and PR antibodies of different species so that they may be quantified, within the same well so that their relationship can be more accurately understood.

The percentage of dual positive cells for PR and BRDU/EDU decreases as [TAM] increases (see figures 8C, 9C, 10C and 10D). This is true for both short (24-hour; see 3.2.2.1 for BRDU and 3.2.2.2.2 for EDU) and long-term (7-day; see 3.2.2.2.1 and 3.2.2.2.2 for BRDU) exposure to Tamoxifen. Due to a lack of evidence, it is unlikely (though not impossible) that Tamoxifen is directly inhibiting progesterone signaling. If we assume that ER expression was reduced in these wells following TAM treatment, then the decrease in the proportion of PR-positive/Thymidine analog-positive is consistent, given that PR expression is a marker for ER [176]. However, ER and PR levels were not measured simultaneously, and ER expression did not

consistently decrease as [TAM] increased based on my experimentation. However, it is possible that ER activity (which was not measured) decreased with rising Tamoxifen concentrations, which could explain the reduction in PR expression, and the observed PR-positive/BRDU,EDU-positive trends.

The percentage of PR-positive/BRDU-negative cells within a spheroid increases from 0 to 1 μM of TAM but decreases drastically from 1 to 8 μM. The percentage of PR-positive/EDU-negative cells within a spheroid decreases as [TAM] increases. Given that a positive PR status yields more favorable clinical outcomes, it follows that the percentage of cells with this phenotype should increase alongside Tamoxifen. However, this is only true when comparing the control to 1 μM of TAM, after 7 days of treatment (see figure 10C).

The percentage of PR-negative/BRDU,EDU-positive cells within a spheroid increases as [TAM] increases. This phenotype represents a sub-population of cells that proliferate despite not expressing progesterone receptor. The data suggests that at both time points, proliferation of PR-negative cells is enhanced by Tamoxifen. Unfortunately, there is no evidence in the literature to corroborate these findings.

Lastly, the percentage of PR-negative/BRDU,EDU-negative cells within a spheroid increases as [TAM] increases. This phenotype represents a sub-population of PR-negative surviving cells that are not actively proliferating. Given that it is not yet known whether TAM has a direct effect of progesterone signaling, the observed trend is likely attributable to decreased ER activity or ER-independent inhibition due to Tamoxifen.

5.4 MCF-7 SPHEROIDS DERIVED FROM SINGLE CELL CLONES LIKELY EXHIBIT TRANSIENT ER AND PR EXPRESSION

Single cell cloning (S.C.C.) was used to generate a population of genetically identical cells derived from a single parent line to study changes in estrogen and progesterone receptor expression levels in MCF-7 spheroids.

Phenotypic heterogeneity is often observed in *in vitro* cell cultures, even in a controlled environment [177]. This is especially true for MCF-7 cells, which are known to exhibit variability and plasticity in molecular programs driving proliferation and receptor expression [133]. It is well-known that hormone receptor expression is heterogenous between cells in many ER-positive cancers. However, it is not well understood if there are different populations of ER/PR-positive and ER/PR-negative cells, or if hormone receptor expression is transient.

At 0 and 1 μM of Tamoxifen, the mean percentage of ER-positive cells constituting a spheroid remains very consistent, at approximately 97% (see figure 11). The majority of the spheroids were 100% ER-positive, while others had varying degrees of ER expression, with proportionally few cells not expressing ER. At these concentrations, no spheroids were 100% ER-negative. With regards to progesterone receptor, the mean percentage of PR-positive cells within a spheroid is approximately 66%. Additionally, there seems to be a lot more variability in PR-positivity, and the presence of spheroids that are 100% PR-negative and 100% PR-positive, which is not observed when measuring estrogen receptor status. At 8μM concentration, spheroids had a mean ER-positivity of 68%, and a mean PR positivity of 61%. Contrary to the other two concentrations, there exist spheroids that are 100% ER/PR-positive, 100% ER/PR-negative, and others with varying levels of ER/PR-positivity.

Given that a partial ER-negative phenotype was not uncommon in the parental line (see figures 8B and 9B), it is surprising to observe no spheroids (derived from single cell clones) with 100% ER-negative status. Of the 130 spheroids quantified following exposure to 0 and 1 μM of TAM, we would expect 20 spheroids (i.e., 20/130 ≈ 15%) to be 100% ER-negative, because approximately 85% of cells that constitute a spheroid derived from the parental line were ER-positive. Thus, it is plausible to suggest that estrogen receptor expression is transient, cycling on and off throughout treatment duration.

Although not definitive, these findings seem to corroborate the existing evidence in the literature involving a switch from an ER/PR-positive, to an ER/PR-negative phenotype, and vice versa [126]. In the event that ER/PR expression is lost or reduced in tumor cells, Tamoxifen becomes less effective, as its primary target inhibits ER pathway-induced proliferation.

Additionally, the proposed phenomenon of transient ER/PR expression may help to better understand the many unexpected trends observed regarding the relationship between hormone receptor expression and proliferation, discussed above.

In the future, the use of a fluorescent reporter to monitor the expression and activity of ER and PR could provide valuable insight and help to validate the findings of my experimentation.

5.5 Flow cytometry

Cell cycle analysis by flow cytometry is used to assess the distribution of cells in different phases of the cell cycle. The DNA content of individual cells were measured using PI, and the proportion of cells in G0/G1, S and G2/M phases were determined subsequently. In the cell cycle, the transition from one phase to the next is dictated by sequential activation of serine threonine kinases called CDKs [178].

It has been previously shown that Tamoxifen induces cell cycle arrest specifically in the G1-phase of the cell cycle, preventing cells from progressing into S (DNA synthesis) phase [151]. However, little is known regarding the dose-dependent effects of Tamoxifen on the induction of G1-phase arrest.

Through competitive binding of the estrogen receptor, TAM prevents the activation and binding of cyclin D1 to dimerized CDK4/6, that would normally occur had the E_2-ERα complex been formed [82]. Additionally, TAM has been shown to increase the expression of cyclin-dependent kinase inhibitors (CDKIs) such as p21 and p27. These proteins inhibit the activity of cyclin-CDK complexes, which results in halting the cell cycle in the G1-phase, preventing the transition towards S-phase [179]. Lastly, TAM has the capacity to inhibit retinoblastoma protein (Rb) phosphorylation, a key event in G1-phase progression. When Rb is phosphorylated, its inhibitory effect on transcription E2F is withdrawn, allowing E2F to activate genes required for DNA synthesis and entry into S-phase. Thus, the inhibition of Rb phosphorylation by TAM prevents the release of E2F and further progression into the cell cycle [180].

The flow cytometric data acquired throughout my experimentation (see figure 12) seems to confirm the mechanisms outlined above: Following treatment with Tamoxifen for 7 days, the percentage of MCF-7 cells in G1 phase increased when compared to the untreated cells. Naturally, the percentage of cells in S and G2 phases decreased as the concentration of Tamoxifen increased. While TAM is not directly involved in regulating the S to G2 transition, its impact on the cell cycle in the G1-phase indirectly influences subsequent phases of the cell cycle, including S-phase and G2-phase.

Interestingly, none of the observed differences from 1 to 8 μM were statistically significant, which may suggest that under these experimental circumstances, the lower concentration of TAM is just as effective as the higher concentration in the induction of G1-phase cell cycle arrest.

CONCLUSION

The data presented in this thesis suggests the presence of persistent sub-populations of cells that differ with respect to their expression of estrogen and progesterone receptor, and proliferative capacities in response to increasing concentrations of Tamoxifen, which supports my initial hypothesis. Moreover, hormone receptor expression is heterogenous, and may be transient in nature, which could explain the lack of consistent response to Tamoxifen observed in clinical settings. Lastly, my data suggests that lower doses of Tamoxifen may be equally effective in inducing G1-phase cell cycle arrest when compared to more elevated doses.

Tamoxifen has undoubtedly revolutionized the treatment of hormone receptor-positive breast cancer, providing significant benefits to countless patients worldwide. However, the persistent challenge of Tamoxifen resistance and inconsistent efficacy underscores the urgency to delve deeper into the complexities of cellular heterogeneity within breast tumors. A more thorough understanding of the dynamic interplay of diverse cell populations and mechanisms of resistance will hopefully unlock the full potential of Tamoxifen and other personalized therapies, leading to better patient outcomes and longer lasting anti-cancer treatment.

BIBLIOGRAPHY

[1] World Health Organization. (2021, October 22). "Cancer." Retrieved from https://www.who.int/health-topics/cancer#tab=tab_1

[2] Ferlay, J., Ervik, M., Lam, F., Colombet, M., Mery, L., Piñeros, M., ... & Bray, F. (2020). Global cancer observatory: cancer today. International Agency for Research on Cancer. Lyon, France.

[3] Brenner DR, Weir HK, Demers AA, et al . Projected estimates of cancer in Canada in 2020 . Canadian Medical Association Journal (CMAJ) . 2020 : 192:E199–205. https://www.cmaj.ca/content/cmaj/192/9/E199.full.pdf.

[4] Ernster, V. L., Ballard-Barbash, R., Barlow, W. E., Zheng, Y., Weaver, D. L., Cutter, G., Yankaskas, B. C., Rosenberg, R., Carney, P. A., Kerlikowske, K., Taplin, S. H., Urban, N., & Geller, B. M. (2002). Detection of ductal carcinoma in situ in women undergoing screening mammography. Journal of the National Cancer Institute, 94(20), 1546–1554. https://doi.org/10.1093/jnci/94.20.1546

[5] Wen, H. Y., & Brogi, E. (2018). Lobular Carcinoma In Situ. Surgical pathology clinics, 11(1), 123–145. https://doi.org/10.1016/j.path.2017.09.009

[6] Suhail, Y., Cain, M. P., Vanaja, K., Kurywchak, P. A., Levchenko, A., Kalluri, R., & Kshitiz (2019). Systems Biology of Cancer Metastasis. Cell systems, 9(2), 109–127. https://doi.org/10.1016/j.cels.2019.07.003

[7] Watkins E. J. (2019). Overview of breast cancer. JAAPA : official journal of the American Academy of Physician Assistants, 32(10), 13–17. https://doi.org/10.1097/01.JAA.0000580524.95733.3d

[8] Mouabbi, J. A., Hassan, A., Lim, B., Hortobagyi, G. N., Tripathy, D., & Layman, R. M. (2022). Invasive lobular carcinoma: an understudied emergent subtype of breast cancer. Breast cancer research and treatment, 193(2), 253–264. https://doi.org/10.1007/s10549-022-06572-w

[9] Wallden, B., Storhoff, J., Nielsen, T., Dowidar, N., Schaper, C., Ferree, S., ... & Parker, J. S. (2015). Development and verification of the PAM50-based Prosigna breast cancer gene signature assay. BMC medical genomics, 8(1), 1-14.

[10] Howlader, N., Altekruse, S. F., Li, C. I., Chen, V. W., Clarke, C. A., Ries, L. A., & Cronin, K. A. (2014). US incidence of breast cancer subtypes defined by joint hormone receptor and HER2 status. Journal of the National Cancer Institute, 106(5), dju055. https://doi.org/10.1093/jnci/dju055

[11] Yersal, O., & Barutca, S. (2014). Biological subtypes of breast cancer: Prognostic and therapeutic implications. World journal of clinical oncology, 5(3), 412–424. https://doi.org/10.5306/wjco.v5.i3.412

[12] Gao, J. J., & Swain, S. M. (2018). Luminal A Breast cancer and Molecular Assays: A Review. The oncologist, 23(5), 556–565. https://doi.org/10.1634/theoncologist.2017-0535

[13] Li, Z. H., Hu, P. H., Tu, J. H., & Yu, N. S. (2016). LμMinal B breast cancer: patterns of recurrence and clinical outcome. Oncotarget, 7(40), 65024–65033. https://doi.org/10.18632/oncotarget.11344

[14] Łukasiewicz, S., Czeczelewski, M., Forma, A., Baj, J., Sitarz, R., & Stanisławek, A. (2021). Breast cancer-Epidemiology, Risk Factors, Classification, Prognostic Markers, and Current Treatment Strategies-An Updated Review. Cancers, 13(17), 4287. https://doi.org/10.3390/cancers13174287

[15] Krishnamurti U, Silverman JF. HER2 in breast cancer: a review and update. Adv Anat Pathol. 2014 Mar;21(2):100-7. doi: 10.1097/PAP.0000000000000015. PMID: 24508693.

[16] Kolářová I, Vaňásek J, Odrážka K, Dušek L, Šinkorová Z, Hlávka A, Štuk J, Stejskal J, Dvořáková D, Sákra L, Mergancová J, Vilasová Z. Is There a Benefit of HER2-Positive Breast cancer Subtype Determination in Clinical Practice? Klin Onkol. 2019 Winter;32(1):25-30. English. doi: 10.14735/amko2019. PMID: 30764626.

[17] Zhu, X., & Verma, S. (2015). Targeted therapy in her2-positive metastatic breast cancer: a review of the literature. Current oncology (Toronto, Ont.), 22(Suppl 1), S19–S28. https://doi.org/10.3747/co.22.2363

[18] Cleator, S., Heller, W., & Coombes, R. C. (2007). Triple-negative breast cancer: therapeutic options. *The Lancet. Oncology*, *8*(3), 235–244. https://doi.org/10.1016/S1470-2045(07)70074-8

[19] Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. N Engl J Med. 2010 Nov 11;363(20):1938-48. doi: 10.1056/NEJMra1001389. PMID: 21067385.

[20] Rakha, E. A., Reis-Filho, J. S., Baehner, F., Dabbs, D. J., Decker, T., Eusebi, V., Fox, S. B., Ichihara, S., Jacquemier, J., Lakhani, S. R., Palacios, J., Richardson, A. L., Schnitt, S. J., Schmitt, F. C., Tan, P. H., Tse, G. M., Badve, S., & Ellis, I. O. (2010). Breast cancer prognostic classification in the molecular era: the role of histological grade. Breast cancer research : BREAST CANCERR, 12(4), 207. https://doi.org/10.1186/breast cancerr2607

[21] O'Sullivan, B., Brierley, J., Byrd, D., Bosman, F., Kehoe, S., Kossary, C., Piñeros, M., Van Eycken, E., Weir, H. K., & Gospodarowicz, M. (2017). The TNM classification of malignant tμMours-towards common understanding and reasonable expectations. The Lancet. Oncology, 18(7), 849–851. https://doi.org/10.1016/S1470-2045(17)30438-2

[22] Hortobagyi GN, Edge SB, Giuliano A. New and Important Changes in the TNM Staging System for Breast cancer. Am Soc Clin Oncol EdUc Book. 2018 May 23;38:457-467. doi: 10.1200/EDBK_201313. PMID: 30231399.

[23] Oftedal OT. The mammary gland and its origin during synapsid evolution. J Mammary Gland Biol Neoplasia. 2002 Jul;7(3):225-52. doi: 10.1023/a:1022896515287. PMID: 12751889.

[24] Peaker, M. (2002). The mammary gland in mammalian evolution: a brief commentary on some of the concepts. Journal of mammary gland biology and neoplasia, 7, 347-353.

[25] Howard BA, Gusterson BA. Human breast development. J Mammary Gland Biol Neoplasia. 2000 Apr;5(2):119-37. doi: 10.1023/a:1026487120779. PMID: 11149569.

[26] Balinski, BI. On the prenatal growth of the mammary gland rudiment in the mouse. J Anat. 1950 Jul;84(3):227-35. PMID: 15436328; PMCID: PMC1273299.

[27] Wood CE, Hester JM, Cline JM. Mammary gland development in early pubertal female macaques. Toxicol Pathol. 2007 Oct;35(6):795-805. doi: 10.1080/01926230701584213. PMID: 17943653.

[28] Spaziani M, Tarantino C, Tahani N, Gianfrilli D, Sbardella E, Lenzi A, Radicioni AF. Hypothalamo-Pituitary axis and puberty. Mol Cell Endocrinol. 2021 Jan 15;520:111094. doi: 10.1016/j.mce.2020.111094. Epub 2020 Dec 1. PMID: 33271219.

[29] Macias, H., & Hinck, L. (2012). Mammary gland development. Wiley interdisciplinary reviews. Developmental biology, 1(4), 533–557. https://doi.org/10.1002/wdev.35

[30] Abreu AP, Kaiser UB. Pubertal development and regulation. Lancet Diabetes Endocrinol. 2016 Mar;4(3):254-264. doi: 10.1016/S2213-8587(15)00418-0. Epub 2016 Feb 4. PMID: 26852256; PMCID: PMC5192018.

[31] Paine, I. S., & Lewis, M. T. (2017). The Terminal End Bud: the Little Engine that Could. Journal of mammary gland biology and neoplasia, 22(2), 93–108. https://doi.org/10.1007/s10911-017-9372-0

[32] Unsworth, A., Anderson, R., & Britt, K. (2014). Stromal fibroblasts and the immune microenvironment: partners in mammary gland biology and pathology?. Journal of mammary gland biology and neoplasia, 19(2), 169–182. https://doi.org/10.1007/s10911-014-9326-8

[33] Beleut, M., Rajaram, R. D., Caikovski, M., Ayyanan, A., Germano, D., Choi, Y., Schneider, P., & Brisken, C. (2010). Two distinct mechanisms underlie progesterone-induced proliferation in the mammary gland. Proceedings of the National Academy of Sciences of the United States of America, 107(7), 2989–2994. https://doi.org/10.1073/pnas.0915148107

[34] Goldhar, A. S., Duan, R., Ginsburg, E., & Vonderhaar, B. K. (2011). Progesterone induces expression of the prolactin receptor gene through cooperative action of Sp1 and C/EBP. Molecular and cellular endocrinology, 335(2), 148–157. https://doi.org/10.1016/j.mce.2011.01.004

[35] Haricharan, S., & Li, Y. (2014). STAT signaling in mammary gland differentiation, cell survival and tumorigenesis. Molecular and cellular endocrinology, 382(1), 560–569. https://doi.org/10.1016/j.mce.2013.03.014

[36] Watson, C.J. Key stages in mammary gland development - Involution: apoptosis and tissue remodelling that convert the mammary gland from milk factory to a quiescent organ. Breast cancer Res 8, 203 (2006). https://doi.org/10.1186/breast cancerr1401

[37] Radisky, D. C., & Hartmann, L. C. (2009). Mammary involution and breast cancer risk: transgenic models and clinical studies. *Journal of mammary gland biology and neoplasia*, *14*(2), 181–191. https://doi.org/10.1007/s10911-009-9123-y

[38] Cole, T. J., Short, K. L., & Hooper, S. B. (2019). The science of steroids. *Seminars in fetal & neonatal medicine*, *24*(3), 170–175. https://doi.org/10.1016/j.siny.2019.05.005

[39] Li, Z., Wei, H., Li, S., Wu, P., & Mao, X. (2022). The Role of Progesterone Receptors in Breast cancer. Drug design, development and therapy, 16, 305–314. https://doi.org/10.2147/DDDT.S336643

[40] Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., ... & Gustafsson, J. A. (2007). Estrogen receptors: how do they signal and what are their targets. Physiological reviews, 87(3), 905-931.

[41] Saha, T., Makar, S., Swetha, R., Gutti, G., & Singh, S. K. (2019). Estrogen signaling: An emanating therapeutic target for breast cancer treatment. European journal of medicinal chemistry, 177, 116–143. https://doi.org/10.1016/j.ejmech.2019.05.023

[42] Yaşar, P., Ayaz, G., User, S. D., Güpür, G., & Muyan, M. (2016). Molecular mechanism of estrogen-estrogen receptor signaling. Reproductive medicine and biology, 16(1), 4–20. https://doi.org/10.1002/rmb2.12006

[43] Kos, M., Reid, G., Denger, S., & Gannon, F. (2001). Minireview: genomic organization of the human ER alpha gene promoter region. Molecular endocrinology (Baltimore, Md.), 15(12), 2057–2063. https://doi.org/10.1210/mend.15.12.0731

[44] Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjöld, M., & Gustafsson, J. A. (1997). Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. The Journal of clinical endocrinology and metabolism, 82(12), 4258–4265. https://doi.org/10.1210/jcem.82.12.4470

[45] Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., ... & Gustafsson, J. Å. (2001). Mechanisms of estrogen action. Physiological reviews, 81(4), 1535-1565.

[46] Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., & Gustafsson, J. A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. Proceedings of the National Academy of Sciences, 93(12), 5925-5930.

[47] Schwabe, J. W., Chapman, L., Finch, J. T., & Rhodes, D. (1993). The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell, 75(3), 567-578.

[48] Wurtz, J. M., Bourguet, W., Renaud, J. P., Vivat, V., Chambon, P., Moras, D., & Gronemeyer, H. (1996). A canonical structure for the ligand-binding domain of nuclear receptors. Nature structural biology, 3(1), 87-94.

[49] Montano, M. M., Müller, V., Trobaugh, A., & Katzenellenbogen, B. S. (1995). The carboxy-terminal F domain of the human estrogen receptor: role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. Molecular endocrinology, 9(7), 814-825.

[50] Fuentes, N., & Silveyra, P. (2019). Estrogen receptor signaling mechanisms. Advances in protein chemistry and structural biology, 116, 135–170. https://doi.org/10.1016/bs.apcsb.2019.01.001

[51] Lu, Y., & Liu, W. (2020). Selective Estrogen Receptor Degraders (SERDs): A Promising Strategy for Estrogen Receptor Positive Endocrine-Resistant Breast cancer. Journal of medicinal chemistry, 63(24), 15094–15114. https://doi.org/10.1021/acs.jmedchem.0c00913

[52] Ikeda, K., Horie-Inoue, K., & Inoue, S. (2015). Identification of estrogen-responsive genes based on the DNA binding properties of estrogen receptors using high-throughput sequencing technology. Acta Pharmacologica Sinica, 36(1), 24-31.

[53] KμMar, V., & Chambon, P. (1988). The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell, 55(1), 145-156.

[54] Zhou, Z., Qiao, J. X., Shetty, A., Wu, G., Huang, Y., Davidson, N. E., & Wan, Y. (2014). Regulation of estrogen receptor signaling in breast carcinogenesis and breast cancer therapy. *Cellular and molecular life sciences: CMLS*, *71*(8), 1549. https://doi.org/10.1007/s00018-013-1376-3

[55] Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., & Greene, G. L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell, 95(7), 927-937.

[56] Hall, J. M., & McDonnell, D. P. (2005). Coregulators in nuclear estrogen receptor action. Molecular interventions, 5(6), 343.

[57] Vrtačnik, P., Ostanek, B., Mencej-Bedrač, S., & Marc, J. (2014). The many faces of estrogen signaling. Biochemia medica, 24(3), 329–342. https://doi.org/10.11613/BM.2014.035

[58] Ho, K. J., & Liao, J. K. (2002). Non-nuclear actions of estrogen: new targets for prevention and treatment of cardiovascular disease. Molecular interventions, 2(4), 219–228. https://doi.org/10.1124/mi.2.4.219

[59] Boonyaratanakornkit, V., & Pateetin, P. (2015). The role of ovarian sex steroids in metabolic homeostasis, obesity, and postmenopausal breast cancer: molecular mechanisms and therapeutic implications. BioMed research international, 2015, 140196. https://doi.org/10.1155/2015/140196

[60] O'Malley, B. W., Sherman, M. R., & Toft, D. O. (1970). Progesterone "receptors" in the cytoplasm and nucleus of chick oviduct target tissue. Proceedings of the National Academy of Sciences of the United States of America, 67(2), 501–508. https://doi.org/10.1073/pnas.67.2.501

[61] Graham, J. D., Yeates, C., Balleine, R. L., Harvey, S. S., Milliken, J. S., Bilous, A. M., & Clarke, C. L. (1995). Characterization of progesterone receptor A and B expression in human breast cancer. Cancer research, 55(21), 5063–5068.

[62] Sartorius, C. A., Melville, M. Y., Hovland, A. R., Tung, L., Takimoto, G. S., & Horwitz, K. B. (1994). A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. Molecular endocrinology (Baltimore, Md.), 8(10), 1347–1360. https://doi.org/10.1210/mend.8.10.7854352

[63] Petz, L. N., & Nardulli, A. M. (2000). Sp1 binding sites and an estrogen response element half-site are involved in regulation of the human progesterone receptor A promoter. Molecular endocrinology (Baltimore, Md.), 14(7), 972–985. https://doi.org/10.1210/mend.14.7.0493

[64] Hewitt, S. C., & Korach, K. S. (2000). Progesterone action and responses in the alphaERKO mouse. Steroids, 65(10-11), 551–557. https://doi.org/10.1016/s0039-128x(00)00113-6

[65] Trabert, B., Sherman, M. E., Kannan, N., & Stanczyk, F. Z. (2020). Progesterone and Breast cancer. Endocrine reviews, 41(2), 320–344. https://doi.org/10.1210/endrev/bnz001

[66] McKenna, N. J., Lanz, R. B., & O'Malley, B. W. (1999). Nuclear receptor coregulators: cellular and molecular biology. Endocrine reviews, 20(3), 321–344. https://doi.org/10.1210/edrv.20.3.0366

[67] Takimoto, G. S., Tung, L., Abdel-Hafiz, H., Abel, M. G., Sartorius, C. A., Richer, J. K., Jacobsen, B. M., Bain, D. L., & Horwitz, K. B. (2003). Functional properties of the N-terminal region of progesterone receptors and their mechanistic relationship to structure. The Journal of steroid biochemistry and molecular biology, 85(2-5), 209–219. https://doi.org/10.1016/s0960-0760(03)00197-3

[68] Dong, X., Challis, J. R., & Lye, S. J. (2004). Intramolecular interactions between the AF3 domain and the C-terminus of the human progesterone receptor are mediated through two LXXLL motifs. Journal of molecular endocrinology, 32(3), 843–857. https://doi.org/10.1677/jme.0.0320843

[69] Hill, K. K., Roemer, S. C., Churchill, M. E., & Edwards, D. P. (2012). Structural and functional analysis of domains of the progesterone receptor. Molecular and cellular endocrinology, 348(2), 418–429. https://doi.org/10.1016/j.mce.2011.07.017

[70] Kelder, J., Azevedo, R., Pang, Y., de Vlieg, J., Dong, J., & Thomas, P. (2010). Comparison between steroid binding to membrane progesterone receptor alpha (mPRalpha) and to nuclear progesterone receptor: correlation with physicochemical properties assessed by comparative molecular field analysis and identification of mPRalpha-specific agonists. Steroids, 75(4-5), 314–322. https://doi.org/10.1016/j.steroids.2010.01.010

[71] Conneely, O. M., Jericevic, B. M., & Lydon, J. P. (2003). Progesterone receptors in mammary gland development and tμMorigenesis. Journal of mammary gland biology and neoplasia, 8(2), 205–214. https://doi.org/10.1023/a:1025952924864

[72] Smith D. F. (2000). Chaperones in progesterone receptor complexes. Seminars in cell & developmental biology, 11(1), 45–52. https://doi.org/10.1006/scdb.1999.0350

[73] Pedroza, D. A., Subramani, R., & Lakshmanaswamy, R. (2020). Classical and Non-Classical Progesterone Signaling in Breast cancers. Cancers, 12(9), 2440. https://doi.org/10.3390/cancers12092440

[74] Faivre, E. J., Daniel, A. R., Hillard, C. J., & Lange, C. A. (2008). Progesterone receptor rapid signaling mediates serine 345 phosphorylation and tethering to specificity protein 1 transcription factors. *Molecular Endocrinology*, *22*(4), 823-837.

[75] Valadez-Cosmes, P., Vázquez-Martínez, E. R., Cerbón, M., & Camacho-Arroyo, I. (2016). Membrane progesterone receptors in reproduction and cancer. Molecular and cellular endocrinology, 434, 166–175. https://doi.org/10.1016/j.mce.2016.06.027

[76] Karteris, E., Zervou, S., Pang, Y., Dong, J., Hillhouse, E. W., Randeva, H. S., & Thomas, P. (2006). Progesterone signaling in human myometriμM through two novel membrane G protein-coupled receptors: potential role in functional progesterone withdrawal at term. Molecular endocrinology (Baltimore, Md.), 20(7), 1519–1534. https://doi.org/10.1210/me.2005-0243

[77] Obr, A. E., & Edwards, D. P. (2012). The biology of progesterone receptor in the normal mammary gland and in breast cancer. Molecular and cellular endocrinology, 357(1-2), 4-17.

[78] Boonyaratanakornkit, V., McGowan, E., Sherman, L., Mancini, M. A., Cheskis, B. J., & Edwards, D. P. (2007). The role of extranuclear signaling actions of progesterone receptor in mediating progesterone regulation of gene expression and the cell cycle. Molecular endocrinology (Baltimore, Md.), 21(2), 359–375. https://doi.org/10.1210/me.2006-0337

[79] Miller, M. M., James, R. A., Richer, J. K., Gordon, D. F., Wood, W. M., & Horwitz, K. B. (1997). Progesterone regulated expression of flavin-containing monooxygenase 5 by the B-isoform of progesterone receptors: implications for tamoxifen carcinogenicity. The Journal of clinical endocrinology and metabolism, 82(9), 2956–2961. https://doi.org/10.1210/jcem.82.9.4239

[80] Zhou, Y., & Liu, X. (2020). The role of estrogen receptor beta in breast cancer. Biomarker research, 8, 39. https://doi.org/10.1186/s40364-020-00223-2

[81] Kamińska, M., Ciszewski, T., Łopacka-Szatan, K., Miotła, P., & Starosławska, E. (2015). Breast cancer risk factors. Przeglad menopauzalny = Menopause review, 14(3), 196–202. https://doi.org/10.5114/pm.2015.54346

[82] Foster, J. S., Henley, D. C., Ahamed, S., & Wimalasena, J. (2001). Estrogens and cell-cycle regulation in breast cancer. Trends in Endocrinology & Metabolism, 12(7), 320-327.

[83] Shen, Q., Uray, I. P., Li, Y., Krisko, T. I., Strecker, T. E., Kim, H. T., & Brown, P. H. (2008). The AP-1 transcription factor regulates breast cancer cell growth via cyclins and E2F factors. Oncogene, 27(3), 366–377. https://doi.org/10.1038/sj.onc.1210643

[84] Santen, R. J., Yue, W., & Wang, J. P. (2015). Estrogen metabolites and breast cancer. Steroids, 99(Pt A), 61–66. https://doi.org/10.1016/j.steroids.2014.08.003

[85] Zahid, M., Kohli, E., Saeed, M., Rogan, E., & Cavalieri, E. (2006). The greater reactivity of estradiol-3,4-quinone vs estradiol-2,3-quinone with DNA in the formation of depurinating adducts: implications for tμMor-initiating activity. Chemical research in toxicology, 19(1), 164–172. https://doi.org/10.1021/tx050229y

[86] Conneely, O. M., Lydon, J. P., De Mayo, F., & O'Malley, B. W. (2000). Reproductive functions of the progesterone receptor. Journal of the Society for Gynecologic Investigation, 7(1 Suppl), S25–S32. https://doi.org/10.1016/s1071-5576(99)00062-3

[87] Ades, F., Tryfonidis, K., & Zardavas, D. (2017). The past and future of breast cancer treatment-from the papyrus to individualised treatment approaches. Ecancermedicalscience, 11, 746. https://doi.org/10.3332/ecancer.2017.746

[88] Rankin J. S. (2006). William Stewart Halsted: a lecture by Dr. Peter D. Olch. *Annals of surgery*, *243*(3), 418–425. https://doi.org/10.1097/01.sla.0000201546.94163.00

[89] Bocchinfuso, W. P., & Korach, K. S. (1997). Mammary gland development and tμMorigenesis in estrogen receptor knockout mice. Journal of mammary gland biology and neoplasia, 2(4), 323–334. https://doi.org/10.1023/a:1026339111278

[90] Stockwell S. (1983). Classics in oncology. George Thomas Beatson, M.D. (1848-1933). CA: a cancer journal for clinicians, 33(2), 105–121. https://doi.org/10.3322/canjclin.33.2.105

[91] O'Malley, B. W., & Khan, S. (2013). Elwood V. Jensen (1920-2012): father of the nuclear receptors. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(10), 3707–3708. https://doi.org/10.1073/pnas.1301566110

[92] Lewis, J. S., & Jordan, V. C. (2005). Selective estrogen receptor modulators (SERMs): mechanisms of anticarcinogenesis and drug resistance. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *591*(1-2), 247-263.

[93] Mirkin, S., & Pickar, J. H. (2015). Selective estrogen receptor modulators (SERMs): a review of clinical data. *Maturitas*, *80*(1), 52–57. https://doi.org/10.1016/j.maturitas.2014.10.010

[94] LμMachi, F., Brunello, A., Maruzzo, M., Basso, U., & Basso, S. M. (2013). Treatment of estrogen receptor-positive breast cancer. Current medicinal chemistry, 20(5), 596–604. https://doi.org/10.2174/092986713804999303

[95] Ali, S., Mondal, N., Choudhry, H., Rasool, M., Pushparaj, P. N., Khan, M. A., Mahfooz, M., Sami, G. A., Jarullah, J., Ali, A., & Jamal, M. S. (2016). Current Management Strategies in Breast cancer by Targeting Key Altered Molecular Players. Frontiers in oncology, 6, 45. https://doi.org/10.3389/fonc.2016.00045

[96] Shagufta, & Ahmad, I. (2018). Tamoxifen a pioneering drug: An update on the therapeutic potential of tamoxifen derivatives. European journal of medicinal chemistry, 143, 515–531. https://doi.org/10.1016/j.ejmech.2017.11.056

[97] Brauch, H., Mürdter, T. E., EichelbaμM, M., & Schwab, M. (2009). Pharmacogenomics of tamoxifen therapy. Clinical chemistry, 55(10), 1770–1782. https://doi.org/10.1373/clinchem.2008.121756

[98] Klein, D. J., Thorn, C. F., Desta, Z., Flockhart, D. A., Altman, R. B., & Klein, T. E. (2013). PharmGKB sμMmary: tamoxifen pathway, pharmacokinetics. Pharmacogenetics and genomics, 23(11), 643–647. https://doi.org/10.1097/FPC.0b013e3283656breast cancer1

[99] Desta, Z., Ward, B. A., Soukhova, N. V., & Flockhart, D. A. (2004). Comprehensive evaluation of tamoxifen sequential biotransformation by the humancytochrome P450 system in vitro: prominent roles for CYP3A and CYP2D6. The Journal of pharmacology and experimental therapeutics, 310(3), 1062–1075. https://doi.org/10.1124/jpet.104.065607

[100] Jordan, V. C., Collins, M. M., Rowsby, L., & Prestwich, G. (1977). A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. The Journal of endocrinology, 75(2), 305–316. https://doi.org/10.1677/joe.0.0750305

[101] Stearns, V., Johnson, M. D., Rae, J. M., Morocho, A., Novielli, A., Bhargava, P., Hayes, D. F., Desta, Z., & Flockhart, D. A. (2003). Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. Journal of the National Cancer Institute, 95(23), 1758–1764. https://doi.org/10.1093/jnci/djg108

[102] Kiyotani, K., Mushiroda, T., Nakamura, Y., & Zembutsu, H. (2012). Pharmacogenomics of tamoxifen: roles of drug metabolizing enzymes and transporters. Drug metabolism and pharmacokinetics, 27(1), 122–131. https://doi.org/10.2133/dmpk.dmpk-11-rv-084

[103] Mürdter, T. E., Schroth, W., Bacchus-Gerybadze, L., Winter, S., Heinkele, G., Simon, W., Fasching, P. A., Fehm, T., German Tamoxifen and AI Clinicians Group, EichelbaμM, M., Schwab, M., & Brauch, H. (2011). Activity levels of tamoxifen metabolites at the estrogen receptor and the impact of genetic polymorphisms of phase I and II enzymes on their concentration levels in plasma. Clinical pharmacology and therapeutics, 89(5), 708–717. https://doi.org/10.1038/clpt.2011.27

[104] Wu, X., Hawse, J. R., Subramaniam, M., Goetz, M. P., Ingle, J. N., & Spelsberg, T. C. (2009). The tamoxifen metabolite, endoxifen, is a potent antiestrogen that targets estrogen receptor alpha for degradation in breast cancer cells. Cancer research, 69(5), 1722–1727. https://doi.org/10.1158/0008-5472.CAN-08-3933

[105] Wang, Y., Chirgadze, N. Y., Briggs, S. L., Khan, S., Jensen, E. V., & Burris, T. P. (2006). A second binding site for hydroxytamoxifen within the coactivator-binding groove of estrogen receptor beta. Proceedings of the National Academy of Sciences of the United States of America, 103(26), 9908–9911. https://doi.org/10.1073/pnas.0510596103

[106] Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O., Ohman, L., Greene, G. L., Gustafsson, J. A., & Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. Nature, 389(6652), 753–758. https://doi.org/10.1038/39645

[107] Wong, M. M., Guo, C., & Zhang, J. (2014). Nuclear receptor corepressor complexes in cancer: mechanism, function and regulation. American journal of clinical and experimental urology, 2(3), 169–187.

[108] Pedersen, R. N., Esen, B. Ö., Mellemkjær, L., Christiansen, P., Ejlertsen, B., Lash, T. L., Nørgaard, M., & Cronin-Fenton, D. (2022). The Incidence of Breast cancer Recurrence 10-32 Years After Primary Diagnosis. *Journal of the National Cancer Institute*, *114*(3), 391–399. https://doi.org/10.1093/jnci/djab202

[109] Benz, C. C., Scott, G. K., Sarup, J. C., Johnson, R. M., Tripathy, D., Coronado, E., Shepard, H. M., & Osborne, C. K. (1992). Estrogen-dependent, tamoxifen-resistant tμMorigenic growth of MCF-7 cells transfected with HER2/neu. Breast cancer research and treatment, 24(2), 85–95. https://doi.org/10.1007/BF01961241

[110] Jordan V. C. (2004). Selective estrogen receptor modulation: concept and consequences in cancer. Cancer cell, 5(3), 207–213. https://doi.org/10.1016/s1535-6108(04)00059-5

[111] Gottardis, M. M., & Jordan, V. C. (1988). Development of tamoxifen-stimulated growth of MCF-7 tμMors in athymic mice after long-term antiestrogen administration. Cancer research, 48(18), 5183–5187.

[112] Eggemann, H., Altmann, U., Costa, S. D., & Ignatov, A. (2018). Survival benefit of tamoxifen and aromatase inhibitor in male and female breast cancer. Journal of cancer research and clinical oncology, 144(2), 337–341. https://doi.org/10.1007/s00432-017-2539-7

[113] Staley, H., McCallμM, I., & Bruce, J. (2014). Postoperative Tamoxifen for ductal carcinoma in situ: Cochrane systematic review and meta-analysis. Breast (Edinburgh, Scotland), 23(5), 546–551. https://doi.org/10.1016/j.breast.2014.06.015

[114] Gradishar, W., & Salerno, K. E. (2016). NCCN Guidelines Update: Breast cancer. Journal of the National Comprehensive Cancer Network : JNCCN, 14(5 Suppl), 641–644. https://doi.org/10.6004/jnccn.2016.0181

[115] Sauter E. R. (2018). Breast cancer Prevention: Current Approaches and Future Directions. European journal of breast health, 14(2), 64–71. https://doi.org/10.5152/ejbh.2018.3978

[116] Quirke, V. M. (2017). Tamoxifen from failed contraceptive pill to best-selling breast cancer medicine: a case-study in pharmaceutical innovation. Frontiers in pharmacology, 8, 620.

[117] Burstein, H. J., Temin, S., Anderson, H., Buchholz, T. A., Davidson, N. E., Gelmon, K. E., Giordano, S. H., Hudis, C. A., Rowden, D., Solky, A. J., Stearns, V., Winer, E. P., & Griggs, J. J. (2014). Adjuvant endocrine therapy for women with hormone receptor-positive breast cancer: american society of clinical oncology clinical practice guideline focused update. Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 32(21), 2255–2269. https://doi.org/10.1200/JCO.2013.54.2258

[118] Buttiron Webber, T., Marra, D., Puntoni, M., Giuliano, S., Briata, I. M., Cevasco, I., Clavarezza, M., D'Amico, M., Defferrari, C., Gozza, A., Provinciali, N., Lazzeroni, M., Bonanni, B., & DeCensi, A. (2021). Patient- versus physician-reported outcomes in a low-dose tamoxifen trial in noninvasive breast cancer. The breast journal, 27(11), 817–823. https://doi.org/10.1111/tbj.14296

[119] Lazzeroni, M., Serrano, D., Dunn, B. K., Heckman-Stoddard, B. M., Lee, O., Khan, S., & Decensi, A. (2012). Oral low dose and topical tamoxifen for breast cancer prevention: modern approaches for an old drug. Breast cancer research: BCR, 14(5), 214. https://doi.org/10.1186/bcr3233

[120] Gjerde, J., Gandini, S., Guerrieri-Gonzaga, A., Haugan Moi, L. L., Aristarco, V., Mellgren, G., Decensi, A., & Lien, E. A. (2012). Tissue distribution of 4-hydroxy-N-desmethyltamoxifen and tamoxifen-N-oxide. Breast cancer research and treatment, 134(2), 693–700. https://doi.org/10.1007/s10549-012-2074-9

[121] Kisanga, E. R., Gjerde, J., Guerrieri-Gonzaga, A., Pigatto, F., Pesci-Feltri, A., Robertson, C., Serrano, D., Pelosi, G., Decensi, A., & Lien, E. A. (2004). Tamoxifen and metabolite concentrations in serum and breast cancer tissue during three dose regimens in a randomized preoperative trial. Clinical cancer research : an official journal of the American Association for Cancer Research, 10(7), 2336–2343. https://doi.org/10.1158/1078-0432.ccr-03-0538

[122] Caldwell, J., Gardner, I., & Swales, N. (1995). An introduction to drug disposition: the basic principles of absorption, distribution, metabolism, and excretion. Toxicologic pathology, 23(2), 102–114. https://doi.org/10.1177/019262339502300202

[123] Sweeney, E., McDaniel, R., Maximov, P., Fan, P. & Jordan, V. (2012). Models and mechanisms of acquired antihormone resistance in breast cancer: significant clinical progress despite limitations. Hormone Molecular Biology and Clinical Investigation, 9(2), 143-163. https://doi.org/10.1515/hmbreast canceri-2011-0004

[124] Bronzert, D. A., Greene, G. L., & Lippman, M. E. (1985). Selection and characterization of a breast cancer cell line resistant to the antiestrogen LY 117018. Endocrinology, 117(4), 1409–1417. https://doi.org/10.1210/endo-117-4-1409

[125] Pompili, L., Porru, M., Caruso, C., Biroccio, A., & Leonetti, C. (2016). Patient-derived xenografts: a relevant preclinical model for drug development. Journal of experimental & clinical cancer research: CR, 35(1), 189. https://doi.org/10.1186/s13046-016-0462-4

[126] Sharma, D., BlμM, J., Yang, X., Beaulieu, N., Macleod, A. R., & Davidson, N. E. (2005). Release of methyl CpG binding proteins and histone deacetylase 1 from the Estrogen receptor alpha (ER) promoter upon reactivation in ER-negative human breast cancer cells. Molecular endocrinology (Baltimore, Md.), 19(7), 1740–1751. https://doi.org/10.1210/me.2004-0011

[127] Croker, A. K., Goodale, D., Chu, J., Postenka, C., Hedley, B. D., Hess, D. A., & Allan, A. L. (2009). High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. Journal of cellular and molecular medicine, 13(8B), 2236–2252. https://doi.org/10.1111/j.1582-4934.2008.00455.x

[128] Chang M. (2012). Tamoxifen resistance in breast cancer. *Biomolecules & therapeutics*, *20*(3), 256–267. https://doi.org/10.4062/biomolther.2012.20.3.256

[129] Mc Ilroy, M., Fleming, F. J., Buggy, Y., Hill, A. D., & Young, L. S. (2006). Tamoxifen-induced ER-alpha-SRC-3 interaction in HER2 positive human breast cancer; a possible mechanism for ER isoform specific recurrence. Endocrine-related cancer, 13(4), 1135–1145. https://doi.org/10.1677/erc.1.01222

[130] Ali, S., Rasool, M., Chaoudhry, H., N Pushparaj, P., Jha, P., Hafiz, A., Mahfooz, M., Abdus Sami, G., Azhar Kamal, M., Bashir, S., Ali, A., & Sarwar Jamal, M. (2016). Molecular mechanisms and mode of tamoxifen resistance in breast cancer. *Bioinformation*, *12*(3), 135–139. https://doi.org/10.6026/97320630012135

[131] Soule, H. D., Vazquez, J., Long, A., Albert, S., & Brennan, M. (1973). A human cell line from a pleural effusion derived from a breast carcinoma. Journal of the national cancer institute, 51(5), 1409-1416.

[132] Horwitz, K. B., Costlow, M. E., & McGuire, W. L. (1975). MCF-7; a human breast cancer cell line with estrogen, androgen, progesterone, and glucocorticoid receptors. Steroids, 26(6), 785–795. https://doi.org/10.1016/0039-128x(75)90110-5

[133] Kleensang, A., Vantangoli, M. M., Odwin-DaCosta, S., Andersen, M. E., Boekelheide, K., Bouhifd, M., Fornace, A. J., Jr, Li, H. H., Livi, C. B., Madnick, S., Maertens, A., Rosenberg, M., Yager, J. D., Zhao, L., & Hartung, T. (2016). ErratμM: Genetic variability in a frozen batch of MCF-7 cells invisible in routine authentication affecting cell function. Scientific reports, 6, 33011. https://doi.org/10.1038/srep33011)

[134] Yu, J., Wang, Z., & Wang, Y. (2022). BrdU Incorporation Assay to Analyze the Entry into S Phase. Methods in molecular biology (Clifton, N.J.), 2579, 209–226. https://doi.org/10.1007/978-1-0716-2736-5_16

[135] Bauer, D., Sarrett, S. M., Lewis, J. S., & Zeglis, B. M. (2023). Click chemistry: a transformative technology in nuclear medicine. Nature protocols, 18(6), 1659–1668. https://doi.org/10.1038/s41596-023-00825-8

[136] Kleinman, H. K., & Martin, G. R. (2005). Matrigel: basement membrane matrix with biological activity. Seminars in cancer biology, 15(5), 378–386. https://doi.org/10.1016/j.semcancer.2005.05.004

[137] Wang, Y., Nguyen, D. T., Yang, G., Anesi, J., Chai, Z., Charchar, F., & Golledge, J. (2020). An Improved 3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-TetrazoliμM Proliferation Assay to Overcome the Interference of Hydralazine. *Assay and drug development technologies*, *18*(8), 379–384. https://doi.org/10.1089/adt.2020.1004

[138] Riss, T. L., Moravec, R. A., Niles, A. L., Duellman, S., Benink, H. A., Worzella, T. J., & Minor, L. (2013). Cell Viability Assays. In S. Markossian (Eds.) et. al., Assay Guidance Manual. Eli Lilly & Company and the National Center for Advancing Translational Sciences.

[139] Mathupala, S., & Sloan, A. A. (2009). An agarose-based cloning-ring anchoring method for isolation of viable cell clones. BioTechniques, 46(4), 305–307. https://doi.org/10.2144/000113079

[140] DuPont. (2023, July 21). "MOLYKOTE High Vacuum Grease". Retrieved from https://www.dupont.com/products/molykote-high-vacuum-grease.html

[141] Crowley, L. C., Chojnowski, G., & Waterhouse, N. J. (2016). Measuring the DNA content of cells in apoptosis and at different cell-cycle stages by propidiμM iodide staining and flow cytometry. Cold Spring Harbor Protocols, 2016(10), pdb-prot087247.

[142] Marshall, G. R., Feng, J. A., & Kuster, D. J. (2008). Back to the future: ribonuclease A. Peptide Science, 90(3), 259-277.

[143] Bucevičius, J., Lukinavičius, G., & Gerasimaitė, R. (2018). The use of hoechst dyes for DNA staining and beyond. Chemosensors, 6(2), 18.

[144] Park, A., Joo, M., Kim, K., Son, W. J., Lim, G., Lee, J., Kim, J. H., Lee, D. H., & Nam, S. (2022). A comprehensive evaluation of regression-based drug responsiveness prediction models, using cell viability inhibitory concentrations (IC50 values). Bioinformatics (Oxford, England), 38(10), 2810–2817. https://doi.org/10.1093/bioinformatics/btac177

[145] Ferreira, L. P., Gaspar, V. M., & Mano, J. F. (2018). Design of spherically structured 3D in vitro tumormodels-Advances and prospects. Acta Biomaterialia, 75, 11-34.

[146] Breslin, S., & O'Driscoll, L. (2013). Three-dimensional cell culture: the missing link in drug discovery. Drug discovery today, 18(5-6), 240–249. https://doi.org/10.1016/j.drudis.2012.10.003

[147] Kapałczyńska, M., Kolenda, T., Przybyła, W., Zajączkowska, M., Teresiak, A., Filas, V., Ibbs, M., Bliźniak, R., Łuczewski, Ł., & Lamperska, K. (2018). 2D and 3D cell cultures - a comparison of different types of cancer cell cultures. Archives of medical science : AMS, 14(4), 910–919. https://doi.org/10.5114/aoms.2016.63743

[148] Kleinman, H. K., & Martin, G. R. (2005). Matrigel: basement membrane matrix with biological activity. Seminars in cancer biology, 15(5), 378–386. https://doi.org/10.1016/j.semcancer.2005.05.004

[149] McFarland, D. C. (2000). Preparation of pure cell cultures by cloning. Methods in cell science, 22, 63-66.

[150] Nunez, R. (2001). DNA measurement and cell cycle analysis by flow cytometry. Current issues in molecular biology, 3(3), 67-70.

[151] Osborne, C. K., Boldt, D. H., Clark, G. M., & Trent, J. M. (1983). Effects of tamoxifen on human breast cancer cell cycle kinetics: accumulation of cells in early G1 phase. Cancer Research, 43(8), 3583-3585.

[152] Shapiro, C. L., & Recht, A. (2001). Side effects of adjuvant treatment of breast cancer. New England Journal of Medicine, 344(26), 1997-2008.

[153] Hassan, F., Mohammed, G., El-Hiti, G. A., Alshanon, A., & Yousif, E. (2018). Cytotoxic effects of tamoxifen in breast cancer cells. J. Unexplored Med. Data, 3(3), 1-10.

[154] Seeger, H., Huober, J., Wallwiener, D., & Mueck, A. O. (2004). Inhibition of human breast cancer cell proliferation with estradiol metabolites is as effective as with tamoxifen. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme, 36(5), 277–280. https://doi.org/10.1055/s-2004-814480

[155] Jansson-Löfmark, R., Hjorth, S., & Gabrielsson, J. (2020). Does In Vitro Potency Predict Clinically Efficacious Concentrations?. Clinical pharmacology and therapeutics, 108(2), 298–305. https://doi.org/10.1002/cpt.1846

[156] Berrouet, C., Dorilas, N., Rejniak, K. A., & Tuncer, N. (2020). Comparison of Drug Inhibitory Effects ([Formula: see text]) in Monolayer and Spheroid Cultures. Bulletin of mathematical biology, 82(6), 68. https://doi.org/10.1007/s11538-020-00746-7

[157] Vega-Avila, E., & Pugsley, M. K. (2011, January). An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. In *Proc West Pharmacol Soc* (Vol. 54, No. 10, p. 4).

[158] Mourits, M. J., De Vries, E. G., Willemse, P. H., Ten Hoor, K. A., Hollema, H., & Van der Zee, A. G. (2001). Tamoxifen treatment and gynecologic side effects: a review. Obstetrics & Gynecology, 97(5), 855-866.

[159] McIntosh, I. H., & Thynne, G. S. (1977). TμMour stimulation by anti-oestrogens. Journal of British Surgery, 64(12), 900-901.

[160] Aihara, M., Yamamoto, S., Nishioka, H., Inoue, Y., Hamano, K., Oka, M., & Mizukami, Y. (2012). Optimizing high-resolution melting analysis for the detection of mutations of GPR30/GPER-1 in breast cancer. Gene, 501(2), 118-126.

[161] Filardo, E. J., Graeber, C. T., Quinn, J. A., Resnick, M. B., Giri, D., DeLellis, R. A., ... & Sabo, E. (2006). Distribution of GPR30, a seven membrane–spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumorprogression. Clinical cancer research, 12(21), 6359-6366.

[162] Ignatov, T., Treeck, O., Kalinski, T., Ortmann, O., & Ignatov, A. (2020). GPER-1 expression is associated with a decreased response rate to primary tamoxifen therapy of breast cancer patients. Archives of Gynecology and Obstetrics, 301, 565-571.

[163] Zhou, X., Wang, S., Wang, Z., Feng, X., Liu, P., Lv, X. B., ... & Guan, K. L. (2019). Estrogen regulates Hippo signaling via GPER in breast cancer. The Journal of clinical investigation, 125(5), 2123-2135.

[164] Li, W., Shi, X., Xu, Y., Wan, J., Wei, S., & Zhu, R. (2017). Tamoxifen promotes apoptosis and inhibits invasion in estrogen-positive breast cancer MCF-7 cells. Molecular medicine reports, 16(1), 478–484. https://doi.org/10.3892/mmr.2017.6603

[165] Todorova, V. K., Kaufmann, Y., Luo, S., & Klimberg, V. S. (2011). Tamoxifen and raloxifene suppress the proliferation of estrogen receptor-negative cells through inhibition of glutamine uptake. Cancer chemotherapy and pharmacology, 67(2), 285–291. https://doi.org/10.1007/s00280-010-1316-y

[166] Daurio, N. A., Tuttle, S. W., Worth, A. J., Song, E. Y., Davis, J. M., Snyder, N. W., Blair, I. A., & Koumenis, C. (2016). AMPK Activation and Metabolic Reprogramming by Tamoxifen through Estrogen Receptor-Independent Mechanisms Suggests New Uses for This Therapeutic Modality in Cancer Treatment. Cancer research, 76(11), 3295–3306. https://doi.org/10.1158/0008-5472.CAN-15-2197

[167] Early Breast Cancer Trialists' Collaborative Group. (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. The Lancet, 365(9472), 1687-1717.

[168] Merglen, A., Verkooijen, H. M., Fioretta, G., Neyroud-Caspar, I., Vinh-Hung, V., Vlastos, G., Chappuis, P. O., Castiglione, M., Rapiti, E., & Bouchardy, C. (2009). Hormonal therapy for oestrogen receptor-negative breast cancer is associated with higher disease-specific mortality. Annals of oncology : official journal of the European Society for Medical Oncology, 20(5), 857–861. https://doi.org/10.1093/annonc/mdn688

[169] De Laurentiis, M., Arpino, G., Massarelli, E., Ruggiero, A., Carlomagno, C., Ciardiello, F., ... & De Placido, S. (2005). A meta-analysis on the interaction between HER-2 expression and response to endocrine treatment in advanced breast cancer. Clinical cancer research, 11(13), 4741-4748.

[170] Allison, K. H., Hammond, M. E. H., Dowsett, M., McKernin, S. E., Carey, L. A., Fitzgibbons, P. L., Hayes, D. F., Lakhani, S. R., Chavez-MacGregor, M., Perlmutter, J., Perou, C. M., Regan, M. M., Rimm, D. L., Symmans, W. F., Torlakovic, E. E., Varella, L., Viale, G., Weisberg, T. F., McShane, L. M., & Wolff, A. C. (2020). Estrogen and Progesterone Receptor Testing in Breast cancer: ASCO/CAP Guideline Update. Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 38(12), 1346–1366. https://doi.org/10.1200/JCO.19.02309

[171] Yi, M., Huo, L., Koenig, K. B., Mittendorf, E. A., Meric-Bernstam, F., Kuerer, H. M., Bedrosian, I., Buzdar, A. U., Symmans, W. F., Crow, J. R., Bender, M., Shah, R. R., Hortobagyi, G. N., & Hunt, K. K. (2014). Which threshold for ER positivity? a retrospective study based on 9639 patients. Annals of oncology : official journal of the European Society for Medical Oncology, 25(5), 1004–1011. https://doi.org/10.1093/annonc/mdu053

[172] Prabhu JS, Korlimarla A, Desai K, Alexander A, Raghavan R, Anupama CE, Dendukuri N, Manjunath S, Correa M, Raman N, Kalamdani A, Prasad MSN, Gopinath KS, Srinath BS, Sridhar TS. A Majority of Low (1-10%) ER Positive Breast cancers Behave Like Hormone Receptor Negative TμMors. J Cancer 2014; 5(2):156-165. doi:10.7150/jca.7668. https://www.jcancer.org/v05p0156.htm

[173] Grootes, I., Keeman, R., Blows, F. M., Milne, R. L., Giles, G. G., Swerdlow, A. J., Fasching, P. A., Abubakar, M., Andrulis, I. L., Anton-Culver, H., Beckmann, M. W., Blomqvist, C., Bojesen, S. E., Bolla, M. K., Bonanni, B., Briceno, I., Burwinkel, B., Camp, N. J., Castelao, J. E., Choi, J. Y., … Pharoah, P. D. P. (2022). Incorporating progesterone receptor expression into the PREDICT breast prognostic model. European journal of cancer (Oxford, England : 1990), 173, 178193. https://doi.org/10.1016/j.ejca.2022.06.011

[174] Li Y, Yang D, Yin X, et al. Clinicopathological characteristics and breast cancer-specific survival of patients with single hormone receptor-positive breast cancer. JAMA Network Open. 2020;3(1):e1918160. doi: 10.1001/jamanetworkopen.2019.18160

[175] Arpino G, Weiss H, Lee AV, et al. Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance. J Natl Cancer Inst. 2005;97(17):1254–1261. doi: 10.1093/jnci/dji249

[176] Li, Z., Wei, H., Li, S., Wu, P., & Mao, X. (2022). The Role of Progesterone Receptors in Breast Cancer. Drug design, development and therapy, 16, 305–314. https://doi.org/10.2147/DDDT.S336643

[177] Avery S. V. (2005). Cell individuality: the bistability of competence development. Trends in microbiology, 13(10), 459–462. https://doi.org/10.1016/j.tim.2005.08.006

[178] Lees, E. (1995). Cyclin dependent kinase regulation. Current opinion in cell biology, 7(6), 773-780.

[179] Ichikawa, A., Ando, J., & Suda, K. (2008). G1 arrest and expression of cyclin-dependent kinase inhibitors in tamoxifen-treated MCF-7 human breast cancer cells. Human cell, 21(2), 28–37. https://doi.org/10.1111/j.1749-0774.2008.00048.x

[180] Lee, T. H., Chuang, L. Y., & Hung, W. C. (1999). Tamoxifen induces p21WAF1 and p27KIP1 expression in estrogen receptor-negative lung cancer cells. Oncogene, 18(29), 4269-4274.

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