**Universidade de Aveiro**
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**Departamento de Ciências Médicas**

**Catarina Costa Ribeiro Ruivo**

# ESTUDO DA REGULAÇÃO DO RECETOR DE ESTROGÉNIO NA RESISTÊNCIA À TERAPIA HORMONAL

# STUDY OF ESTROGEN RECEPTOR REGULATION IN RESISTANCE TO HORMONAL THERAPY

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular realizada sob a orientação científica da Doutora Luisa Alejandra Helguero Shepherd, Professora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro.

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**O júri**

Presidente

Prof. Doutora Odete Abreu Beirão Da Cruz e Silva
Professora Associada com Agregação da Universidade de Aveiro

Prof. Doutora Luisa Alejandra Helguero Shepherd
Professora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro

Doutora Joana Vieira da Silva
Investigadora de Pós-Doutoramento do Instituto de Investigação e Inovação em Saúde e do Instituto de Biologia Molecular e Celular

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**palavras-chave**

Cancro da mama, recetor de estrogénio α, co-reguladores da transcrição, SETD7 metiltransferase, (R)-PFI-2, proliferação, terapia hormonal, MCF-7, T-47D

**resumo**

O cancro da mama permanece o cancro mais predominante na mulher e estima-se que a mortalidade aumente 43% até 2030 em todo o mundo. Aproximadamente 70% dos tumores mamários são positivos para o recetor de estrogénio (ER), tornando possível o tratamento com terapia hormonal. O cancro da mama é uma doença heterogénea com múltiplos fatores associados à sua origem e comportamento invasivo, e apesar dos avanços científicos, a resistência à terapia continua a ser um assunto na ordem do dia. Este projeto procurou investigar como é que a atividade do ER pode mudar na presença de diversos fatores e a relação com a resistência à terapia endócrina. Primeiramente testámos por imunocitoquímica uma combinação de anticorpos que permitiu avaliar a interação do ER com dois co-ativadores, PPARγ e PGC-1β. Os testes realizados sugerem a existência de co-localização nas células MCF-7 resistentes ao tratamento com tamoxifeno, o que poderá resultar numa ativação ligando-independente do ER e resistência endócrina. Depois fomos investigar o efeito do inibidor da metiltransferase SETD7, (R)-PFI-2 só ou em combinação com 17β-estradiol ($E_2$), no ciclo celular e na proliferação, em células MCF-7 e T-47D. Observamos que o tratamento com (R)-PFI-2 pareceu induzir paragem do ciclo em G0/G1 em ambas as linhas celulares. No entanto, a combinação (R)-PFI-2 + $E_2$ não inibiu o aumento do ciclo celular provocado pelo $E_2$. Nas mesmas células quisemos também ver o efeito do inibidor na expressão de ER e um dos seus genes alvo, o recetor de progesterona (PR). Os nossos resultados indicam que não houve estimulação da expressão de PR. Estes resultados vão de encontro ao descrito na literatura, que indica que a atividade de SETD7 é necessária para o recrutamento eficiente de ER para os promotores dos genes alvo e consequentemente para ativar a transcrição. Portanto, não encontramos uma correlação entre os efeitos da inibição da SETD7 na expressão de PR e no ciclo celular estimulado por $E_2$. Além disso fomos observar o efeito dos antagonistas 4-OH-Tamoxifeno e ICI 182 780 quando combinados com (R)-PFI-2. Nas células MCF-7 a proliferação foi inibida na presença destes compostos, mas quando juntamos (R)-PFI-2 não houve um efeito aditivo inibitório. Por outro lado, nas células T-47D não observamos efeito dos antagonistas, o que indica resistência à terapia, mas quando combinados com (R)-PFI-2, este foi capaz de reduzir a resistência endócrina demonstrada por estas células. Mais estudos são necessários de forma a confirmar estes resultados e o papel da SETD7 no cancro da mama. No entanto, propormos que a inibição de SETD7 em células resistentes aos antagonistas estudados pode ser uma estratégia para diminuir a resistência.

**keywords**

Breast cancer, estrogen receptor α, transcriptional coregulators, SETD7 methyltransferase, (R)-PFI-2, proliferation, hormone therapy, MCF-7, T-47D

**abstract**

Breast cancer remains the most prevalent cancer in women and its mortality is estimated to increase 43% until 2030 worldwide. Approximately 70% of breast cancers are positive for the estrogen receptor (ER), making it possible to treat them with hormone therapy. Breast cancer is a heterogeneous disease with multiple factors associated with its origin and invasive behavior, and despite scientific advances, resistance to therapy remains a major issue. This project sought to investigate how ER activity can change in the presence of several factors and the relationship with resistance to endocrine therapy. Firstly, we tested for immunocytochemistry a combination of antibodies that allowed us to evaluate the interaction of ER with two co-activators, PPARγ and PGC-1β. The performed tests suggest co-localization in MCF-7 cells resistant to tamoxifen treatment, which may result in a ligand-independent activation of ER and endocrine resistance. We then investigated the effect of the methyltransferase inhibitor SETD7, (R)-PFI-2 alone or in combination with 17β-estradiol ($E_2$) on the cell cycle and proliferation in MCF-7 and T-47D cells. We observed that treatment with (R)-PFI-2 appeared to induce a G0/G1 cell cycle arrest in both cell lines. However, the (R)-PFI-2 + $E_2$ combination did not inhibit $E_2$ cell cycle enhancement. In the same cells, we also wanted to see the effect of the inhibitor on ER expression and one of its target genes, the progesterone receptor (PR). Our results indicate that there was no increase in the expression of PR. These results are in agreement with the literature, which indicates that the activity of SETD7 is necessary for the efficient recruitment of ER to the promoters of the target genes and consequently to activate the transcription. Therefore, we did not find a correlation between the effects of the SETD7 inhibitor on ER-mediated expression of PR and $E_2$-stimulated cell cycle. In addition, we investigated the effect of 4-OH-Tamoxifen and ICI 182 780 antagonists when combined with (R)-PFI-2. In MCF-7 cells proliferation was inhibited in the presence of these compounds, but when (R)-PFI-2 was joined there was no inhibitory additive effect. On the other hand, in T-47D cells no effect of the antagonists was observed, indicating resistance to therapy, but when combined with (R)-PFI-2, the antagonists were able to reduce the endocrine resistance demonstrated by these cells. Further studies are needed to confirm these findings and the role of SETD7 in breast cancer. However, we propose that the inhibition of SETD7 in cells resistant to the antagonists studied could be a strategy to decrease resistance.

# INDEX

## I. List of abbreviations

**AF1 -** activation function 1

**AF2 -** activation function 2

**AI -** aromatase inhibitor

**AIB1 -** amplified in breast cancer 1

**AP-1 -** activator protein 1

**ATF -** activating transcription factor

**BC -** breast cancer

**BSA -** bovine serum albumin

**CARM1 -** coactivator-associated arginine methyltransferase-1

**CBP/p300 -** CREB-binding protein/p300

**CDKs -** cyclin-dependent kinases

**CoReg -** coregulators

**DBD -** DNA-binding domain

**DCC -** dextran coated charcoal

**DNA -** deoxyribonucleic acid

**ECM -** extracellular matrix

**EGF -** epidermal growth factor

**EGFR -** epidermal growth factor receptor

**ER –** estrogen receptor

**ER+ -** estrogen receptor-positive

**ERα -** estrogen receptor α

**ERβ -** estrogen receptor β

**ERE -** estrogen response elements

**ERK - e**xtracellular-signal regulated kinase

**ERR -** estrogen-related receptor

**$E_2$ -** 17β-estradiol

**E2F1 -** E2F transcription factor 1

**FBS -** fetal bovine serum

**FOxO3 -** forkhead box O3

**GRIP1 -** glucocorticoid receptor interacting protein 1

**GPR30 -** G protein-coupled receptor

**H –** hinge domain

**HDAC -** histone deacetylase protein complexes

**HER2 -** human epidermal growth factor receptor 2

**Hsp70 -** heat shock protein 70

**Hsp90 -** heat shock protein 90

**ICI 182 780 -** fulvestrant

**IGF-1 -** insulin-like growth factor 1

**IGFR -** insulin-like growth factor I receptor

**K302 -** lysine 302

**LBD -** ligand binding domain

**LTED -** long-term $E_2$ deprivation

**MAPK** - mitogen activated protein kinase

**mRNA -** messenger RNA

**NR -** nuclear receptor

**NCOR1 -** nuclear receptor corepressor 1

**P -** phosphorylation

**PA -** palmitoylation

**PARP1 -** poly-ADP-ribose polymerase 1

**PEST -** penicillin-Streptomycin

**PGC-1α -** proliferator-activated receptor gamma coactivator-1-alpha

**PI -** propidium iodide

**PI3K** - phosphatidylinositol-4,5-bisphosphate 3-kinase

**PKA -** protein kinase A

**PKC -** protein kinase C

**PLA -** proximity ligation assay

**PPARγ -** peroxisome proliferator activated receptor gamma

**PR -** progesterone receptor

**pRb -** retinoblastoma protein

**PTM -** post-translational modifications

**R -** restriction point

**RE -** response elements

**RTK -** tyrosine kinase receptor

**SDS-PAGE -** sodium dodecyl sulfate polyacrylamide gel electrophoresis

**SERMs -** selective estrogen receptor modulators

**SERDs -** selective estrogen receptor downregulators

**SET -** Su(var)3-9, Enhancer-of-zeste and Trithorax

**SETD7 -** SET domain containing lysine methyltransferase 7

**shRNAs -** short hairpin RNA

**SNPs -** single nucleotide polymorphisms

**Sp-1 - s**pecificity protein-1

**STAT3 -** signal transducer and activator of transcription 3

**TGF-β -** transforming growth factor-β

**TF -** transcription factor

**YY1 -** Yin Yang 1

**4-hydroxy-N-desmethyl tamoxifen -** endoxifen

**4-OH-TAM -** tamoxifen

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# Chapter I

# Introduction

# 1. Breast Cancer

Breast cancer (BC) is the most common cancer in women and it is estimated that there were 561,334 deaths worldwide in 2015, being the majority due to metastatic breast cancer. Mortality is projected to rise by 43% by the year 2030 [1]. Breast cancer is a heterogeneous disease, including distinct molecular and/or histological subtypes, with different clinical outcomes. The initiation of breast cancer is due to accumulation of transforming (genetic and epigenetic) events. The progression of BC involves several pathological stages, which begins with ductal hyperproliferation, followed by in situ carcinoma, invasive carcinoma, and finally metastatic disease (Fig. 1) [2].

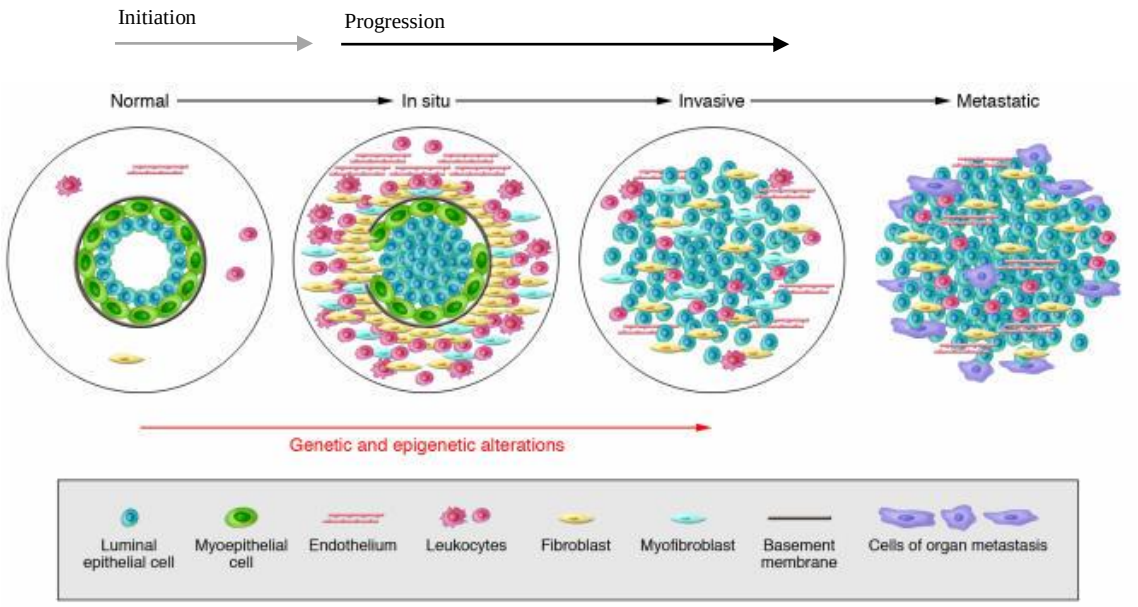


*Figure 1 - Schematic view of the carcinogenic process. Normal mammary ducts are composed of basement membrane, luminal epithelial and myoepithelial cells. Genetic and epigenetic alterations initiate the cell transformation process leading to de-regulated growth and loss of differentiation. Degradation of the basement membrane causes phenotypic changes of myoepithelial cells and decrease of their numbers, while the number of fibroblasts, myofibroblasts, lymphocytes and endothelial cells increases. These changes leads the invasion of tumor cells into adjacent tissues, eventually leading to metastases. Adapted from* [2].

*In vivo* and *in vitro* studies have demonstrated that cells of the microenvironment (myoepithelial, endothelial, fibroblasts, myofibroblasts, leukocytes and other cell types) together with extracellular matrix (ECM) molecules are important for carcinogenesis, modulating the growth and survival of cells, as well as their invasive behavior [2].

The diversity and heterogeneity found in a tumor can be explained by two main theories: 1) Cancer stem cell hypotheses or 2) The clonal evolution hypothesis. Although both hypothesis agree that tumors originate from only one cell that has acquired multiple mutations and proliferative potential, there are some differences, for example: the first

hypothesis identifies the stem cells as the target cells of the tumor-initiating transforming event, while in the second they do not define a particular cell type. Regarding tumor resistance, the first hypothesis claims that cancer stem cells are inherently drug resistant, while in the second hypothesis they are selected through Darwinian evolution [3].

Traditional therapies for breast cancer include surgery, chemotherapy, radiotherapy, and hormone therapy. Despite all scientific and technological advances, around 40% of patients suffer from recurrence of the disease [4].

### 1.1. Classification of breast carcinomas

The majority of breast tumors originate in epithelial cells, about 15% originate in basal/myoepithelial cells, and can be considered tumors with high proliferative rates and worse prognosis than those of epithelial origin [5]. It is possible to classify tumors based on the histological type, tumor grade, lymph node status and the presence of predictive markers estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 (HER2). Using microarray technology, several studies have shown that breast tumors can be classified into five major distinct molecular subtypes: luminal A, luminal B, HER2-enriched, basal-like, claudin-low and normal-like [6][7]. These molecular subtypes correlate with the three groups histologically defined (Table 1).

Table 1–Correlation of breast cancer molecular subtypes with histological subtypes.

| | *Histological Subtype* | | |
|---|---|---|---|
| *Molecular subtypes* | ER/PR | HER2 | Ki-67* |
| *Luminal A* | + | - | low |
| *Luminal B* | + | + | high |
| *HER 2* | - | + | high |
| *Basal-like* | - | - | high |
| *Claudin-low* | - | - | low |
| *Normal-like* | + | - | low |

* Ki67: proliferation marker.

### 1.2. Hormone receptors in breast cancer

ER-positive (ER+) breast cancer is the most prevalent type of breast cancer diagnosed in women (70% of total cases), partly because ER is the most common type of hormone receptor expressed by breast epithelial cells [8]. ER belongs to the superfamily of nuclear receptors (NR), which also includes receptors for other steroid and non-steroidal hormones. There are two ER isoforms – α and β – that regulate expression of different sets of gene. In BC, ERα (from now on ER) is the primary ligand-inducible transcription factor. ERβ either enhances or counteracts ER activity, and several reports suggest that ERβ inhibits breast cancer cell proliferation and functions as a tumor repressor [9]. Nevertheless, recent studies suggest that BC express very low levels of ERβ [10]. For this reason, this receptor will not be the focus of our work.

In addition to ER, PR also plays an important role in BC, being present in over two-thirds of ER+ BC [11]. PR also belongs to the NR family and has two isoforms (A and B) [12]. PR expression is directly activated by ER. Thus, in ER+ BC, PR expression indicates a functional ER, and is therefore a valuable molecular predictor of response to ER antagonist therapy [11]. ER and PR function as hormone-activated transcription factors since they modulate the transcription of target genes that are critical in distinct cellular processes, regulating cancer growth and progression, such as cell division, survival and death [13]. PR and ER control each other´s chromatin binding and transcriptional activity [14][13] which increases the diversity of gene expression programs they regulate.

## 2. Estrogens receptors as therapeutic target

### 2.1. Structure of ER

ER is encoded by ESR1 gene, which is located on chromosome 6q25.1. It is structurally formed by five main domains (Fig. 2). One of these domains is the ligand-binding domain (LBD), which is the region of the protein where the hormone binds. After binding, the ER translocates to the nucleus where the DNA-binding domain (DBD) usually binds to the estrogen response element (ERE) in target genes [15]. ER is directed towards the nucleus through nuclear localization signal located in the hinge domain (H) [16]. The LBD and DBD contain surfaces that allow ER homo or heterodimerization [17]. Also, there are two activation functions (AF1 and AF2) located in the A/B and E domains respectively. AFs consist of conserved amino acid sequences that are responsible for regulating

transcriptional activity by protein-protein interactions with transcription coregulators [18] which are influenced by different post-translational modifications [15]. Depending on the ER phosphorylation status, AF1 may be activated independently of the ligand, by mitogen-activated protein kinase (MAPK) signaling, in particular in the serine 118 residue [18]. AF2 is a hormone-dependent transcriptional activation function.

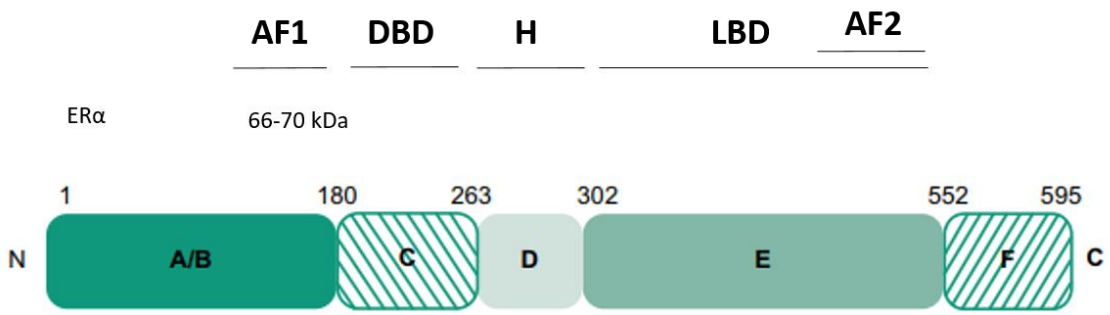


*Figure 2 – ER Structural Organization. A-F domains contain the Activation function one (AF1), DNA binding domain (DBD), hinge domain (H), Ligand binding domain (LBD) and Activation function two (AF2). Human ESR1 gene encodes a protein of 595 amino acid that has a molecular mass of 66-70 kDa. Adapted from* [18].

### 2.2. ER mechanisms of action

In order to improve BC diagnosis, prognosis and to invest in new therapeutic targets, it is of extreme importance to understand the various signaling pathways that result in ER activation and how they can contribute to BC progression. ER may be activated through different mechanisms, including ligand-dependent activation (genomic and non-genomic pathways) and ligand-independent activation (genomic pathway). These concepts will be discussed in detail in the following section (Fig. 3) [19].

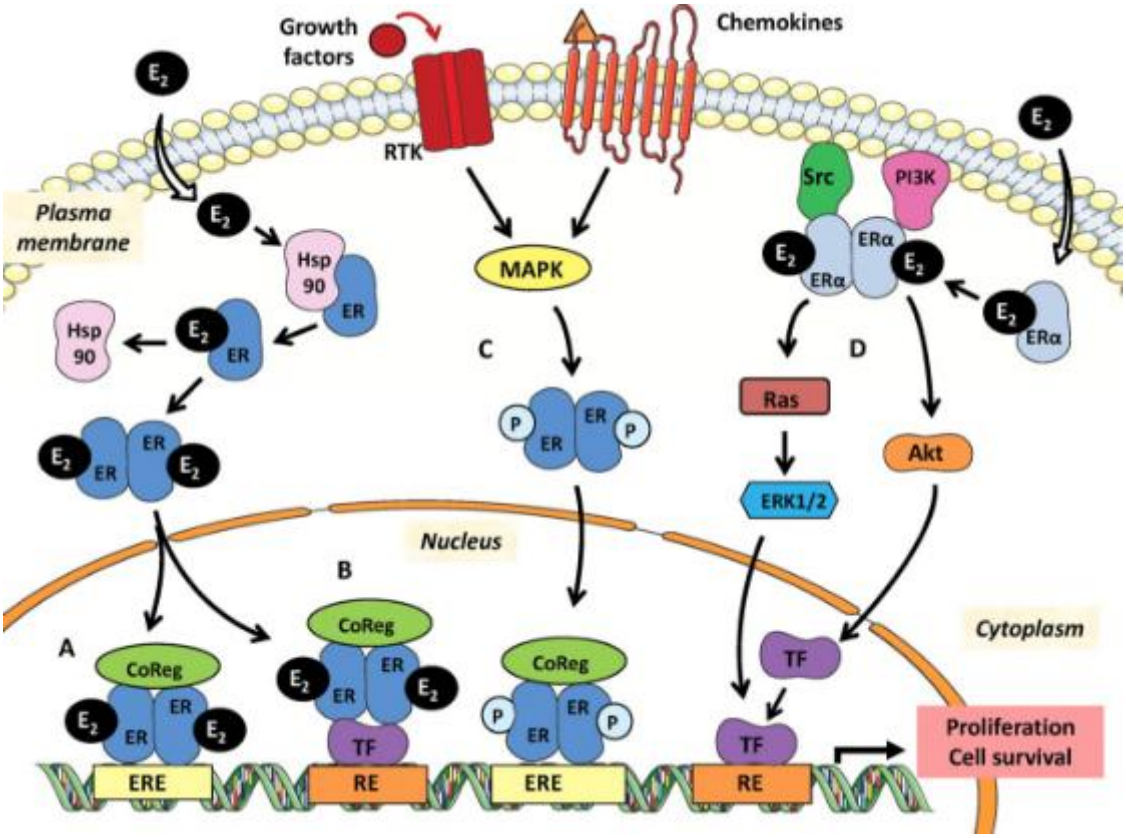


*Figure 3 - Estrogen Receptor Signaling Pathways. Four pathways are shown. Pathway A: Ligand-dependent genomic activation of ER. Pathway B: Non-classical genomic pathway (ERE-Independent Genomic Actions of ER). Pathway C: Ligand-independent genomic activation of ER. Pathway D: The non-genomic pathway (Estrogen receptor-mediated membrane signaling). ER – Estrogen Receptor; RE - Response elements to other transcription factors; ERE- Estrogen Response Elements; P - phosphorylation; RTK - tyrosine kinase receptor; CoReg - coregulators; TF-transcription factor; Hsp90-heat shock protein 90. Adapted from* [19].

### 2.2.1. Ligand-dependent genomic activation

The classical pathway (Fig. 3, pathway A) is the most well characterized pathway and relies on direct interaction of ER with the DNA. Once ER binds its hormone (agonist) it becomes activated, there is a conformational change and estrogen-induced receptor dissociation from the inhibitory chaperones Hsp70 and Hsp90. Next, ER dimerizes with itself and in the nucleus, the ER homodimer binds to the EREs and recruits transcriptional coregulators to activate transcription. Many coregulators have nuclear recognition motif boxes (NR boxes), which bind to the LBD of ER. These coregulators can act either as connectors between ER and chromatin or as RNA processing enzymes. One example of coactivators is SRC family that recruits the essential coactivator CBP/p300 to allow ER transcriptional activity [3]. Other important coactivators are peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) [20] and coactivator-associated arginine

methyltransferase-1 (CARM1) [21]. In addition to coactivators, ER can also recruits corepressors. That interact with ER in order to reduce the transcription of ER target genes. This mechanism occurs because corepressors compete with coactivators and inhibit ER dimerization or DNA binding. Corepressors also act by modifying chromatin by recruiting histone deacetylase protein complexes (HDAC) and interact with ER mainly when it is bound to antagonists [3]. Example of a well-known ER corepressor is N-CoR that interact with the inactive ER [22]. It should be noted that in the same way that the coactivators present will recruit more coactivators, the same happens with corepressors recruitment [23]. Another mechanism of genomic action is by indirect DNA binding, where ER functions as a coactivator to other transcription factors (TFs) (Fig. 3, pathway B). For example, ER can activate the expression of an immensity of genes by protein-protein interactions with activator protein 1 (AP-1) and specificity protein-1 (Sp-1) [24]. AP-1 is a collective term referring to dimeric transcription factors composed of Jun, Fos or activating transcription factor (ATF) subunits that bind to AP-1-binding site in the DNA [25]. In this way, ER can regulate estrogen-responsive genes lacking EREs, for example the insulin-like growth factor 1 (IGF-1) and Cyclin D1 genes [23].

### 2.2.2. Ligand-independent genomic activation

ER can be activated in the absence of the ligand (Fig. 3 pathway C). This pathway include activation of growth factor receptors such as epidermal growth factor receptor (EGFR), insulin-like growth factor I receptor (IGFR); factors that regulate cellular phosphorylation levels such as Protein kinase A (PKA), Protein kinase C (PKC), MAPK and transforming growth factor-β (TGF-β). These, in turn, activate phosphorylation in threonine/serine residues in the AF-1 and AF-2 domains of the ER. Accordingly, increased phosphorylation at the receptor causes its dimerization, recruitment of coactivators and activation at EREs [26].

Phosphorylation of corepressors and coactivators by the same pathways enhances ER ligand-independent activation, for example, the EGF pathway phosphorylation of serine 736 in glucocorticoid receptor interacting protein 1 (GRIP1) enhances its coactivator function on ER [27].

### 2.2.3. Non-genomic activation

This type of signaling (Fig. 3, pathway D) is characterized by: a) fast cellular responses within a few minutes b) which are independent of protein synthesis c) involving many times mobilization of second messenger such as $Ca^{2+}$, cAMP, phosphotidylinositol, and nitric oxide (NO) and d) activation of protein kinase cascades [3]. In this type of signaling, $E_2$ binds to ER resulting in the activation of several protein kinase cascades that in turn lead to indirect changes in gene expression due to the phosphorylation of transcription factors [28]. It is often referred to extranuclear estrogen action because ER location may be in the mitochondria, endoplasmic reticulum or plasma membrane [29]. Immunohistochemical studies suggest that the plasma membrane-associated ER is the same protein as the nuclear-localized receptor [30].

Membrane adapter molecules are important, since the estrogen receptor will require the interaction of these proteins to initiate non-genomic actions. Moreover, the post-translational modification palmitoylation (PA) allows interaction with the membrane protein caveolin-1 and activation of mitogenic signaling pathways such as the activation of MAPK/ERK1/2 and PI3K/AKT pathways, leading to cyclin D1 expression and DNA replication [31]. In addition to membrane ER, the G protein-coupled receptor (GPR30) was identified as a membrane-associated estrogen-binding protein that requires EGFR. Signaling through GPR30 follows EGFR activation of Src, resulting in stimulation of the MAPK and PI3K pathways. This activation induces the expression of transcription factors, leading to the expression of target genes whose promoters do not contain EREs [32].

## 2.3. ER post-translational modifications

ER can suffer several post-translational modifications (PTM) which regulate ER localization, interaction with coregulatory proteins and with the DNA. Phosphorylations are the most studied PTMs. ER has many phosphorylation sites in response to estrogen or other factors. One well-studied example is serine 118. In response to EGF and IGF-1 growth factors, ERK 1/2 become activated and induce phosphorylation in serine 118, enhancing transcription. Other phosphorylations that activates transcription occur in serines 104, 106, 167 and 305. Dimerization of the receptor is induced by serine 236 phosphorylation and regulation of estradiol binding to ER by tyrosine 537 phosphorylation [33]. Acetylations are other PTM, and may have repressor or activator effect in ER

transcriptional activity [33]. More recently, the protein methyltransferase SETD7 has been shown to monomethylate ER in the hinge region, in lysine 302 (K302), *in vivo* and *in vitro* [34]. This modification results in increased ER stability and enhanced ER transcriptional activation. The effect of SETD7 inhibition on ER signaling is one of the focuses of our work and will be discussed later.

### 2.4. Endocrine therapy for breast cancer treatment

Endocrine therapies have been used to treat BC for more than a century and their primary goal is to reduce the risk of local or distant recurrence of hormone-dependent cancers and thus increase the patient's long-term survival [35]. In general, we can classify endocrine agents into two categories according to their function: ER antagonists and Aromatase Inhibitors (AIs) [35].

#### 2.4.1. ER antagonists

An antagonist is a compound capable of neutralizing a cellular function or process, thereby attenuating agonist actions [36]. ER antagonists can be divided into two sub-groups: selective estrogen receptor modulators (SERMs) and selective estrogen receptor down regulators (SERDs) [37]. SERMs are able to compete with estrogens for binding to ER, and thereby alter the coregulators with which it associates, modulating its activity [37]. The most studied of this category is tamoxifen. It is metabolized in the liver to its higher affinity metabolite 4-hydroxytamoxifen (4OH-TAM). Tamoxifen has both ER agonistic and antagonistic properties depending on the tissue where it is acting. In the breast, 4-OH-TAM has an antagonistic effect [38]. Tamoxifen was developed in 1970 and has been for 48 years, the standard adjuvant treatment for ER+ BC [8]. When 4-OH-TAM is bound to ER, the AF2 domain is not functional, while the AF1 domain remains active. The inactivity of AF2 results in an reduction of transcription of the estrogen-responsive gene and of coactivator binding [39][40]. Tamoxifen causes a dose-dependent decrease in the percentage of cells in S phase, and an increase in the number of cells in G0/G1phase, retarding cell proliferation in cells that depend on ER activation proliferation genes (i.e. cyclin D1) [41][35]. SERDs also compete with estrogens for ER binding and function by inducing ER degradation, thus they are considered pure antiestrogens [37]. Fulvestrant (ICI 182 780) is an example. It has high affinity for the ER (similar to that of estradiol and 50

times greater than that of 4-OH-TAM) and has no weak agonist effect. Sold as Faslodex®, is the only SERD that is currently used in the clinics [35]. Upon ICI 182 780 binding, the AF1 and AF2 domains are both inactivated because ER dimerization is inhibited [35]. Thus, the translocation of ER to the nucleus is reduced and degradation is accelerated, resulting in inhibition of gene expression [42].

#### 2.4.2. Aromatase inhibitors

The CYP19 gene encodes the aromatase enzyme (also called estrogen synthetase), which catalyzes the final stage of estrogen production in humans [35][43]. In BC, the deprivation of this hormone is beneficial in a wide spectrum of patients and one way to achieve this is to inhibit its biosynthesis with AIs. This results in lack of ligand-dependent ER activation and reduced proliferation [44].

## 3. Molecular mechanisms of resistance to endocrine therapy

Even when a cancer can be drastically reduced in size by a specific treatment, a resurgence of the drug-resistant disease can occur months or years later. This evidences that the initial treatment failed to destroy small fractions of cells in the original tumor cell population, which had intrinsic or acquired resistance due to genetic instability. Resistance may be a consequence of lack of primary response (*de novo*/intrinsic resistance), or development of resistance a time after the initiation of treatment (acquired resistance). It is known that about 50% of patients with advanced disease can't respond to first-line treatment with 4-OH-TAM (*de novo* endocrine resistance) [45], and they tend to gain resistance within 2 to 3 years after initiation of endocrine therapy [46]. The mechanisms underlying endocrine resistance, described in detail below, may be fitted into three main categories: (1) modifications of estrogen receptor status; (2) alterations in intracellular microenvironment; (3) anomalies in the metabolism of endocrine agents [47].

### 3.1. Modifications of estrogen receptor status

#### 3.1.1. Loss of ER expression

The lack of ER expression is the main mechanism of resistance to hormone therapy. One of the causes is aberrant methylation of the CpG islands, located in the regulatory regions 5 of the ESR1 gene. Another cause is the increase in histone deacetylation, compacting the nucleosome and limiting transcription of ESR1. In order to

circumvent this problem, histone de-acetylation can be inhibited, and a DNA methyltransferase-1 inhibitor can be used. In fact, in the ER-negative breast cancer cells MDA-MB-435 the restoration of 4-OH-TAM sensitivity, by this method, was demonstrated *in vivo* and *in vitro* [48].

#### 3.1.2. ESR1 mutations

The frequency of ESR1 mutations varies from 11 to 39% [49]. In 4-OH-TAM-resistant MCF-7 cells a mutation has been described that causes tyrosine substitution by aspartate at amino-acid 351 in the ER ligand-binding domain. Another substitution, arginine in place of lysine 303, was detected in 20 of 59 hyperplastic breast lesions, increasing the sensitivity of ER to estrogen and consequently promoting tumor growth [47]. Also, a study demonstrated that MCF-7 cells that had the mutated ESR1 leading to lysine 303 substitution by arginine causes resistance to tamoxifen and and anastrozole (AI) and confers hypersensitivity to estrogen [50]. In addition, mutations of tyrosine 537 to serine are related to changes in the selectivity of ER to hormone binding and the kinetics of this process [51].

### 3.2. ER activation

#### 3.2.1. Altered expression of coregulators

The effects of hormone therapy can be reduced by over-expression of coactivators and/or down-regulation of corepressors. AIB1 (SRC-3) is an ER coactivator and when over-expressed in cells, it reduces the antagonist activity of tamoxifen-bound ERs. Signaling through the HER-2 receptor pathway activates AIB1 by phosphorylation. When tumors over-expressing AIB1 and HER-2 combined with administration of 4-OH-TAM, patients had worse prognosis. Those with low AIB1 levels had a more favorable prognosis [52]. *In vitro* studies have established that when NCOR1 mRNA expression levels are low, patients receiving adjuvant 4-OH-TAM, experience poor prognosis because 4-OH-TAM antagonism needs high levels of NCOR1 function [53].

#### 3.2.2. Increased growth factor signalling

EGFR/HER2 pathways activate ERK1/2 and AKT pathways, which phosphorylate the ER. During endocrine therapy resistance, amphiregulin (EGFR ligand) maintains cell

proliferation [47]. A study claims that ER can interact with the AP-1 transcription factor complex and this may allow 4-OH-TAM to exert an agonist response on AP-1-regulated genes. These data suggest that 4-OH-TAM-stimulated tumor growth may be mediated by increased transcription of AP-1 [54]. Also, BT474 breast cancer cells, over-expressing HER2 demonstrate resistance to 4-OH-TAM [47]. It is known that there is a crosstalk between the ER and HER2 pathways, triggering MAPK signaling. In this context, the 4-OH-TAM-ER complex recruits coactivators instead of corepressors resulting in increased expression of estrogen-regulated genes. Treatment with an EGFR inhibitor (gefitinib) blocked signaling, re-established the corepressor complexes with the 4-OH-TAM-bound ER, restoring its anti-tumor activity in these cells [55].

Increased receptor tyrosine kinases (RTKs) signaling (particularly HER2 signaling) is associated with acquired endocrine resistance. The acquired resistance to 4-OH-TAM was demonstrated in a study where there was an over-expressed HER2 or amplification of the gene, and where strong correlations between ER phosphorylation and MAPK activation were observed [56]. Data suggest that in tumors under these conditions, the most effective endocrine treatment is with aromatase inhibitors, compared to 4-OH-TAM. In fact, patients treated with a combination of anti-HER2 and AI endocrine therapy achieved better outcomes than patients treated with only AI [57]. This is expected since with estrogen reduction in circulation, the ER-mediated signaling pathways in the nucleus and in the membrane is shut off (inhibiting AF-2 function) and RTK signaling inhibition inhibits AF-1 function.

### 3.2.3. Estrogen hypersensitivity

The hypersensitivity to estrogen is the ability of a tumor to continue to grow even in the presence of very low levels of estrogen. In order to study this mechanism in ER-positive MCF-7 cells, a medium that did not contain estrogen (LTED - long-term $E_2$ deprivation) was developed. What happened in these cells was that ER levels increased (4 to 10-fold) [38]. This can be explained by the non-genomic pathway of the ER, where estradiol binds to the receptor present on the cell membrane, leading to MAPK activation [62]. However, this mechanism was blocked by the action of ICI 182 780, indicating that ER is a key component in the activation of MAPK signaling in these cells [38]. Nevertheless, MAPK are probably not the only signaling pathway involved in the growth

of these cells, since MAPK inhibitors do not completely block the effect of estrogen hypersensitivity [63].

### 3.3. Anomalies in the metabolism of endocrine agents

Tamoxifen is metabolized in more active forms by various cytochrome P450 enzymes (CYP2D6, CYP3A4, CYP3A5, CYP2C9 and CYP2C19). The two most active forms are: 4-hydroxy-N-desmethyl tamoxifen (also known as endoxifen) and 4-hydroxytamoxifen (4-OH-TAM). Each metabolite has its own specific binding affinity for ER, with 4-OH-TAM having a 100-fold greater affinity for the estrogen receptor and 30 to 100-fold greater potency to inhibit cell proliferation when compared to the parent drug [47]. Studies suggest that specific single nucleotide polymorphisms (SNPs) of CYP2D6 may be the enzyme's zero activity ratio, resulting in a poor clinical outcome for patients treated with 4-OH-TAM [64]. It is observed that in the presence of the two functional alleles of CYP2D6 there is a better clinical outcome than only a functional or reduced function allele [65].

## 4. Cell cycle

Neoplasias are the result of low rate of cell death and high rate of proliferation. This rate of cell proliferation occurs due to the cell cycle, a synchronized cycle of DNA replication and cell growth [66]. The cell cycle consists of the following phases: Phase G0, quiescent or “standby” state, which can be reversible; Phase G1, where the cell size increases and it prepares to replicate its DNA; Phase S, where the replication of the genetic material occurs; Phase G2, when the cell continues to grow and prepares for cell division; and phase M (mitosis), where the cell stops growing and divides into two daughter cells. The transition past the restriction (R) checkpoint of the cycle is physiologically irreversible [67]. A cell that has recently been generated by cell division must decide whether to start a new cycle of growth and division or retreat to the non-growth state (G0). This decision is greatly influenced by mitogenic factors and inhibitory factors stimulating the cell [66].

### 4.1. Cyclin-dependent kinases and cell cycle quality control

The most important cell cycle regulators are cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases, which become active only when binding to cyclins. When active, they phosphorylate other proteins, inducing downstream processes [67]. At each stage of the cell cycle, there is accumulation of different cyclins (Fig. 4). The complexes of cyclin D with CDK4 occurs during the G1 phase, when cyclin D expression is increased, whereas CDK2 complexes with cyclins E and A are mostly needed during the S phase and CDK1-cyclin B complex is needed to for mitosis to proceed [67]. Proteins such as $p21^{Cip1}$, $p27^{Kip1}$, p53 and retinoblastoma protein (pRb) regulates CDK and cyclin expression and activation; they constitute negative regulators of the cell cycle [68] which can become activated in the checkpoints that monitor each step of the cell cycle progression. For example, in G1 phase it is necessary to verify whether DNA has no errors and if there are enough cell resources for replication. One of these checkpoints is p53, which in anomalies stops the cell cycle in G1, triggering the production of inhibitory proteins of CDK. These bind to the CDK-cyclin complexes and block their activity, gaining time for DNA repair or activate the programmed cell death [67]. Another important regulator is the tumor suppressor pRb. The unphosphorylated state of this protein suppresses the advance in G1 by binding to transcription factors called E2Fs, blocking their transactivation domain, which are used to activate transcription of cyclin E to allow cell cycle progression. pRb recruits other proteins that repress E2F1 transcription, for example histone deacetylases. The mutated pRb in the tumors is not capable of binding and negatively regulating E2F1, resulting in excessive cell growth [69]. Moreover, cells respond to mitogenic and inhibitory extracellular signals only during a specific period of time window. The end of this window is called the restriction point (R), which indicates the point in time in which the cell must commit to advance the cell cycle to mitosis, stay in G1, or leave the cell cycle [67]. Mitogenic signals such as growth factors and estrogens increase cyclin D expression, which contributes to formation of the cyclin D/CDK4/6 complexes which in turn phosphorylate pRb and allow E2F1 activation of its target genes.

Due to activation of oncogenes and inactivation of tumor suppressor genes, many types of cancer cells have one or more of their checkpoints changed or disabled. In this way, tumor cells can accumulate more rapidly the mutant genes and altered karyotypes that drive neoplastic growth and disease progression [66]. Endocrine therapy in the cell cycle

induces its arrest in the G1/S phase - a characteristic that is absent in cells resistant to therapy. One of the cyclins that is expressed in cell cycle is the cyclin D1, which is associated with the activation of CDK4 and CDK6 and progression to the S phase of the cell cycle. Studies relate the induction of cyclin D1 and cyclin E2 to 4-OH-TAM resistance [58][59]. Aberrant expression of other cell cycle regulators such as c-MYC, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (kinase inhibitor protein family) have also been associated with, endocrine resistance [60][61].

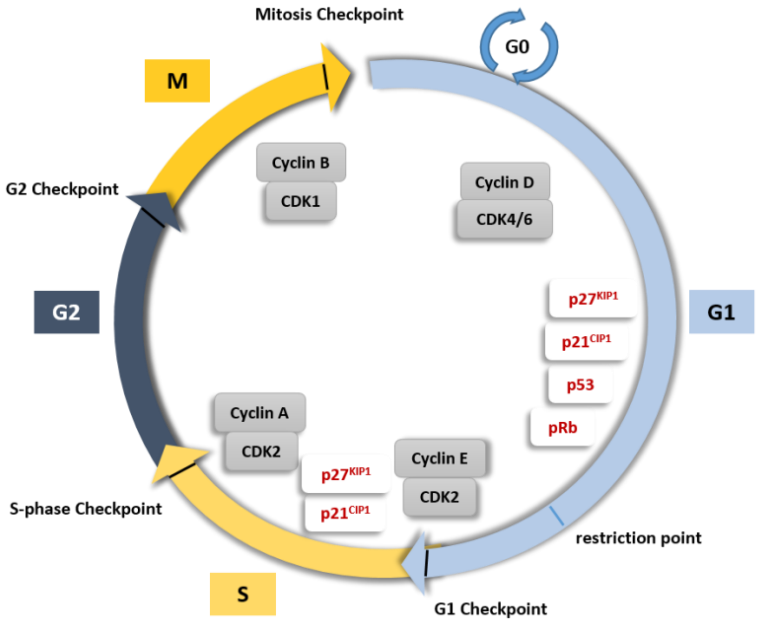


*Figure 4 - Illustration of the cell cycle and the control of its regulation. Cell cycle regulation is controlled by negative regulators such as p53 and pRb and the oscillation of CDKs-cyclin complexes and their inhibition by CDK inhibitory proteins (Cip/Kip).*

## 5. Regulation of cell cycle and ER signaling by SETD7

SETD7 (also known as SET7, SET9, SET7/9) is a SET domain containing lysine methyltransferase 7 that monomethylates a diverse number of proteins including transcription factors, chromatin remodelers and histones (such as lysine 4 methylation in histone 3) [70]. SETD7 regulates the transfer of a methyl group to a lysine residue of its various substrates. Because it is an enzyme with the ability to regulate transcription, either by monomethylating histone H3 in lysine 4, or by targeting a series of transcription factors, the change in its normal activity is associated with various diseases, including cancer. This makes this molecule a potential target in the treatment of this disease [71].

Associated to breast cancer, SETD7 has contradictory effects. A 2017 study has shown that SETD7 activity has negative implications, as it increases cell proliferation and induces anti-apoptotic effects [72]. However, other studies indicated that down-regulation of SETD7 leads to reduced ER recruitment to its target genes, which implies that in this case, SETD7 has positive implications for BC since it blocks gene transcription mediated by ER [34]. Also, SETD7 knockdown enhanced the cell proliferation, migration, and invasion of BC cell lines [73] which suggests that SETD7 activity is necessary to maintain normal proliferation and prevent invasion.

SETD7 substrates are important for maintenance of cell homeostasis, contributing to proper biological function, such as cell cycle regulation, DNA damage response, RNA Polymerase II dependent gene transcription, chromatin modulation, cellular differentiation and regulation of developmental pathways [70]. Methylation of transcription factors plays an important role in gene expression regulation since it alters their affinity for DNA promoter sequences and transcriptional coregulators [70].

SETD7 targets a group of proteins that regulate cell cycle progression (Fig. 5). SETD7 methylates E2F1 at lysine 185 which contributes to E2F1 ubiquitination and proteasomal degradation and decreased transcription of its target gene Cyclin E [74]. However, SETD7 activation inhibits p53 activity and consequently can enhance E2F1 activity and Cyclin E transcription. SETD7 also can methylate pRB in lysine 873, which increases its stability and consequently leads to cell cycle arrest. pRb methylation by SETD7 in lysine 810 prevents its phosphorylation, which contributes to stabilize the pRb/E2F1 complex and reduce Cyclin E transcription. p53, is also methylated by SETD7 in lysine 37, which promotes cell cycle arrest in conditions of stress. In addition, SETD7 methylates forkhead box O3 (FOxO3) in lysine 271. Since FOxO3 is a transcription factor that promotes DNA damage repair, SETD7 methylation of FOxO3 activates pro-apoptotic genes and stops the cell cycle [75]. In MCF-7 BC cells, FOxO3 interacts with ER in order to inhibit cell proliferation and transcription [76]. Also, poly-ADP-ribose polymerase 1 (PARP1) is an enzymatic substrate of SETD7 in K508, which increases its activity. PARP1 play an important role in chromatin replication, transcriptional regulation, cell death and cell cycle arrest [70].

In the context of cell cycle, ER and PR activation leads to stimulation of Cyclin D1 gene (CCND1) in a genomic ligand-dependent manner [77][78]. As mentioned above, ER is a

monomethylated in lysine 302 by SETD7 [34][79]. Using shRNAs to reduce SETD7 levels it was shown that it contributes to ER recruitment to PR and PS2 gene promoters and enhances ER transcriptional activity. In the same study, the authors reported that this methylation affect ER turnover within the cell, because the region K302 constitutes a calmodulin-binding motif and this interaction prevents ubiquitination and subsequent ER degradation. This data indicates that there is efficacy in ER-regulated gene transcription in the presence of methylation by SETD7 [34], increasing receptor stability and increasing recruitment of ER to its target genes [80]. Therefore, given that ER activates the cyclin D1 gene expression in a ligand-dependent genomic manner, then SETD7 would contribute to promote the cell cycle transition, although this has not been tested. Moreover, the ER transcriptional coactivator PGC-1α is methylated in lysine 779 by SETD7. After methylation, there is transcription of PGC-1α target genes, including ER and estrogen-related receptors (ERRs) [81][20] supporting the idea that SETD7 stimulates ER-mediated gene expression. On the contrary, SETD7 dimethylates signal transducer and activator of transcription 3 (STAT3) in lysine 140. STAT3 can activate anti-apoptotic, proliferative (cyclin D1) and inflammatory factors. STAT3 is active in BC, contributing to its progression [82]. However, when dimethylated, its transcriptional function is inhibited [83].

In summary, SETD7 could contribute to cancer cell proliferation and ER signaling, by directly activating ER activity (when methylates ER) or indirectly by modifying histone H3 lysine 4 methylation levels present in ER target gene promoters as well as in ER transcriptional coregulators (Fig. 5). In this scenario, SETD7 could promote cell proliferation. On the contrary, SETD7 could block cell cycle transition by regulating E2F1, pRB and p53. While SETD7 could be therapeutically exploited in some instances, there is still much to be learned about how its inhibition can affect specific BC cell types.


*Figure 5 - Main effects of SETD7-mediated methylation on cell cycle. SETD7 methylates the proteins PARP1, p53, STAT3, pRb and FoxO3, which in turn has consequences on cell cycle arrest. In the ER and E2F1 proteins, methylation has cell cycle progression effects. Adapted from* [70].

# Chapter II

# Aims of the Study

BC is a disease which in women is responsible for the highest number of cancer-related deaths. Most BCs express ER, indicating a better prognosis, since endocrine therapy may be used. However, despite scientific advances in recent years, many cases may respond to endocrine therapy in the first instance, but about 50% of these later become resistant. The acquired resistance may be the result of the unregulated interaction of ER with coregulators and also of the effects of post-transcriptional modifications on ER. Therefore, the general objective of this dissertation was to characterize how ER activity could be changed by different factors and its relation to endocrine resistance. For this purpose, we focused on three specific objectives, which are based on ongoing work in the lab:

i) Test antibodies that will allow us to study if ER interacts differently with transcriptional coactivators in human MCF-7 BC cell lines which are sensitive or resistant to 4-OH-TAM. Specifically, the antibodies for the coactivators tested were PPARγ and PGC-1β using immunofluorescence;
ii) Verify if SETD7 inhibition influences the ER and PR protein levels using Western Blot;
iii) Analyze the effect of SETD7 inhibition on cell proliferation in the absence or presence of $E_2$, 4-OH-TAM or ICI 182 780, using Crystal Violet and flow cytometry.

# Chapter III

# Material and Methods

## 1. Cell lines

In this study, two adherent cell lines derived from human BC were selected: MCF-7 and T-47D. These cell lines were chosen because they are representative of different relevant features of Luminal A subtype.

### 1.1. MCF-7 cell line

MCF-7 (ATCC® HTB-22™) is a human breast cancer cell line which was established from a pleural effusion at the Michigan Cancer Foundation in 1973 and is the most commonly used xenograft model of breast cancer [84]. It belongs to the luminal A molecular subtype and proves to be a suitable model for BC investigations worldwide [85]. Despite its origin from the metastases of an advanced tumor, the cell line is non invasive and represents a model of early-stage disease due to the presence of functional ER and estrogen dependence for growth both *in vitro* and *in vivo*. This model has been particularly valuable in preclinical testing of antiestrogen therapies (e.g. 4-OH-TAM, AI) and identification of mechanisms of resistance to such drugs [84]. MCF-7 cell line resistant to 4-OH-TAM (MCF-7R) was kindly provided from Dr. Julia Gee at Cardiff University.

### 1.2. T-47D cell line

T-47D (ATCC HTB-133) was isolated by I. Keydar from a pleural effusion obtained from a 54 year old female patient with an infiltrating ductal carcinoma of the breast. Due to their high expression of PR, T-47D cells are commonly used to study progesterone signaling in breast cancer and therapeutic efficacy of antiprogestins [84]. Neverthless, this cell line also express ERα and is inhibited by antiestrogens.

## 2. Cell culture

Cells were grown in RPMI-1640 phenol-red medium supplemented with 5% fetal bovine serum (FBS), 1% Penicillin-Streptomycin (PEST) and 2% L-Glutamine (all from Life Technologies). MCF-7R were mantained with addition of 500 nM 4-OH-TAM (Sigma). Cells were kept at 37°C in humidified 5% $CO^2$ atmosphere. Hormone treatments were carried out in the same medium but with 2% dextran coated charcoal (DCC)-treated FBS.

### 2.1. Agonists, antagonists and inhibitors

To study endocrine resistance in breast cancer, we used several hormones: 4-OH-TAM, ICI 182780 (Sigma), $E_2$ (Sigma) as well as the SETD7 inhibitor (R)-PFI-2 (kind gift from Dr. Peter J. Brown, University of Toronto). In the course of this experimental work, we used $E_2$ at the concentration of 10 nM. We used 4-OH-TAM at concentration of 500 nM and ICI 182 780 at the concentration of 250 nM, both dissolved in ethanol. The same volume of absolute ethanol was used for the no treatment control. (R)-PFI-2 is a potent and selective inhibitor of SETD7 activity by binding to it in a cellular environment [99]. The inhibitor was used at two concentrations: 1 nM and 8 nM, diluted in dimethyl sulfoxide (DMSO) (Sigma). This is one of the solvents most used in the laboratory and presents low toxicity. The treatments without (R)-PFI-2 were compensated with equivalent volume of DMSO.

## 3. Evaluation of cell viability by crystal violet assay

For the implementation of this protocol (adapted from [86]), the cells were seed in a 96-well plate (1500 cells/mL). In 3 of the 96 wells, we did not add cells, only medium. These wells served as control for nonspecific binding of the crystal violet dye to the plate. We incubated cells 24h at 37.°C to enable adhesion to the well surface. On day 2, we aspirated the medium and added 200 µL/well of fresh medium supplemented with the drugs appropriate for the experimental goals of each experiment. Then, we incubated cells for 48h. At day 4, we took 100 µL of each well and added 100 µL of new treatments. Incubated 24 hours. Next, we fixed the cells: washed cells twice with PBS (Gibco, Thermo Fisher Scientific) (200 µL/well), added 100 µL of 4% formalin (Sigma) and left at room temperature for half an hour. Then, washed again with 200 µl/well of PBS. The next step was to aspirate the PBS and wash the cells twice with tap water (200 µl/well). We inverted the plate on paper and pressed to remove any remaining liquid. Then added 50 µL of 0,5% crystal violet (Sigma) solution in 80% distilled $H_2O$ and 20% methanol (VWR) to each well and incubated for 20 minutes at room temperature on a bench rocker. The plate was washed four times with tap water and left to dry. The next day, we added 200 µl/well of methanol and incubated 20 minutes on the bench rocker. Finally, we measured the optical density of each well at 570 nm ($OD_{570}$) with a plate reader. For final accounts, we

subtracted the average $OD_{570}$ of the wells without cells from the $OD_{570}$ of each well on the plate.

## 4. Cell treatment for ER and PR analysis

T-47D and MCF-7 were grown in 10 $cm^3$ petri plates to reach a confluency of >70%. The following treatments were applied: cells were treated with 10 nM $E_2$; (R)-PFI-2 in the concentration of 1 nM or 8 nM and $E_2$ combined with (R)-PFI-2 for 2, 6 and 24 hours. Thereafter cells were collected for total protein extracts. The control treatment included the same volume of EtOH and DMSO as the treatments.

## 5. Preparation of protein extracts

To obtain whole cell lysates, T-47D and MCF-7 cells were washed in ice-cold PBS and then centrifuged at 1200 RPM, 10 minutes, 4.ºC. After centrifugation the supernatant was discarded. Then, cells were harvested in lysis buffer [1% NP40, 50 mmol/L Tris-HCl (pH 7.5), 140 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L $Na_3VO_4$] (all from Sigma), freshly supplemented with protease and phosphatase inhibitor cocktails (Roche) and 1 mM DTT (Sigma). The cell suspension was incubated on ice for 20 minutes, vortexing slightly every 5 minutes to homogenize the suspension. The insoluble cell debris was then removed by centrifugation at 12,000 RPM at 4. ºC for 10 min. The supernatant was used as total protein extract. Supernatant was transferred to a new microtube and stored at -80. ºC for further use.

## 6. Protein quantification

Determination of protein concentration was performed by colorimetric method "RC DC Protein Assay" from BioRad. This assay allows the quantification of protein in the presence of reducing agents and detergents. A calibration curve was carry out using bovine serum albumin (BSA) (Sigma) standards with different concentrations (0, 0.3, 0.5, 075, 1, 1.25 and 1.5 mg/mL). After adding the reagents to the samples, the plate was incubated at room temperature for 15 minutes. The optical density values were determined at 750 nm in a spectrophotometer (Tecan). Protein concentration was determined by extrapolation of standard curve values using linear regression.

## 7. SDS-PAGE and western blotting

Forty μg of total proteins were solubilized in 6x SDS loading buffer (87% glycerol, 495 mM SDS, 861 mM DTT and 1,3 mM Bromophenol Blue) (all from Sigma). The sample was resolved in a denaturing 7.5% SDS-PAGE. As marker, we used the Protein Marker MultiColour (GRISP). Proteins were separated in the gel, running at 200 V for approximately 50 min and then transferred to a nitrocellulose blotting membrane (GE Healthcare) by a semi-dry transfer (Trans-Blot Turbo, BioRad). The transfer sandwich was assembled with transfer buffer (70% $dH_2O$, 20% methanol, 191 mM glycine, 25 mM Tris, 3 mM SDS) (all from Sigma). The transfer occurred at 25 V and 1.0 A for 30 minutes. Once finished, the membrane was removed carefully and soaked in TBS (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween, pH 7.5) (all from Sigma). The efficacy of the transfer is evaluated by staining with 0,5% (w/v) Ponceau S (Sigma) in 1% acetic acid. The membrane was then washed with water and TBST and later blocked in 5% non-fat milk in TBST overnight, 4. ºC. The morning after, membranes were washed with TBST. The primary antibodies used were anti-ERα (HC-20, Santa Cruz Biotechnology, dilution 1:200 in TBST) or PR (C-19, Santa Cruz Biotechnology, dilution 1:200 in TBST). The membrane was incubated at room temperature (RT) in agitation, 2h. Subsequently the membrane was washed 3x with TBST at RT for 10 min. Then the secondary antibody (anti-rabbit HRP, Sigma, dilution 1:5000) was applied in 5% non-fat milk in TBST at RT in agitation for at least 1h. Finally, after washed twice 10 min with TBST and once 10 min with TBS the enzymatic reaction was performed using WesternBright ECL HRP substrate solution (Advansta) and visualized with Molecular Imager ChemiDoc (Bio-Rad). The membrane was subjected to a *stripping* process in a specific buffer (200 mM glycine, 0,1% SDS, 1% Tween) (all from Sigma) and re-blotted with total ERK 1/2 primary antibody (BD Biosciences, dilution 1:500) and anti-mouse HRP secondary antibody (Sigma, dilution 1:5000). This step was used as loading control. Densitometric values for each band intensity were determined using Image Lab version 6.0 (BioRad software).

## 8. Cell cycle analysis – flow cytometry

Cells used in this experiment were T-47D and MCF-7 and they were seeded on a 3cm diameter plate in a concentration of $2x10^5$ cells/mL. When the cells reached the desired confluence (70-80%), we applied medium with 1% DCC (RPMI + 1% gentamicin + 1%

glutamine + 1% DCC) so that the cells grew with few nutrients, decrease their proliferation and became synchronized. After 24h, we applied the treatments with the cells being the majority at G0/G1. Cells were incubated for 24h with treatments. Then, the growth medium was collected and the cells were washed with PBS before the addition of 0,5 mL pre-warmed trypsin (Life Technologies) for 2 minutes at 37.ºC. After the complete detachment, the cells were placed in an eppendorf with 1 mL of RPMI supplemented with 10% of FBS to stop trypsin reaction. Then the eppendorf tubes with the cells were centrifuged for 10 minutes at 2500 rpm. Next, the supernatant was discarded and cells washed once more with 1 mL PBS. Then centrifuged again under the same conditions as before. We removed the entire volume of supernatant and added 0.3 mL of ice cold PBS and suspended to obtain a single cell suspension. Finally, we vortexed at low speed, while adding 0.7 mL of ice cold absolute ethanol in order to fix the cells. The cells were stored at 4.ºC until further analysis. At the time of analysis, we spinned the cells at 2500 rpm, 10 minutes at 4.°C. Then we discarded the ethanol and added 1 mL of 0,5% triton (Sigma) in PBS to permeabilize the cells. Afterward, we centrifuged again and discarded the supernatant. Then 0.1 mg/ml RNAse A (Thermo Fisher Scientific) was added and incubated half an hour at 37.°C to eliminate RNA from samples. Finally, we stained the cells with 2 μL of propidium iodide (PI) (Sigma, 1μg/mL) and incubated for 30 minutes at room temperature. The PI is excited at 535 nm and emits at 617 nm so we used the FL3 channel to analyse the cells. Then, 10.000 cells were analysed in a flow cytometer (BD Accuri). Cell cycle analysis was then conducted based on the histogram outputs with the BD Accuri C6 Software.

## 9. Indirect immunocytochemistry assay

Immunofluorescence is a technique used for the labeling of proteins with fluorescent dyes. This method can be divided in three steps: cell culture, fixation and cell staining. In this experiment, we used MCF-7S and MCF-7R cells. At the beginning, $5x10^3$ cells/mL were seeded in a 24-well plate in coverslips and grew until reaching the confluence 70%. Then, cells were washed twice with 1x PBS and the treatments are applied in the plate according to the following scheme (Fig. 6).

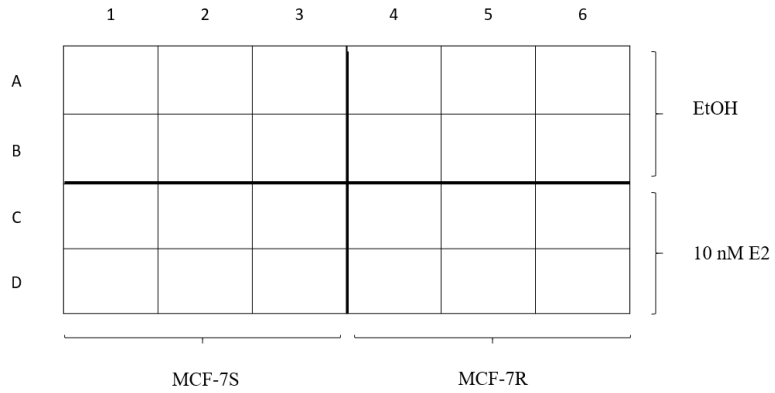


*Figure 6 - Treatments applied to the 24-well plate. Half of the plate with MCF-7 cells sensitive to tamoxifen (MCF-7S) and another half MCF-7 cells resistant to tamoxifen (MCF-7R). Each had the same amount of EtOH treatments and 10 nM $E_2$.*

After applying the treatments, we waited 2h and washed the plate twice with sterile PBS. The fixation step was with 4% paraformaldehyde in PBS for about 20 min at room temperature. After fixation, cells were permeabilized with 0.5% Triton X-100 in PBS. This step is needed to the fully access of the antibody to the antigen. Then washed 3 times with PBS + 0,05% Tween 20, incubating 5 minutes at RT between washes. The cell staining step involves the blocking step, the incubation with the primary antibody and the incubation with secondary antibody labeled with a fluorochrome. Cells were blocked for 1h-1.5h at RT through 250 µL of blocking solution (0,05% Tween and 10% FBS in PBS). Incubations with the sets of primary antibodies were performed overnight at 4.ºC with specific antibody dilutions (in PBS) (Table 2).

Table 2 - Primary and secondary antibodies and respective dilutions used for Immunofluorescence analysis.

| Gene Target | Primary Antibodies | Conditions | Secondary Antibodies |
|---|---|---|---|
| **ERα** | HC-20, rabbit polyclonal, Santa Cruz Biotechnology | 1:300 | FITC |
| **PPARG** | A3409A,mouse monoclonal, Abcam | 1:100 | Alexa 568 |
| **ERα** | ID5, mouse monoclonal, Abcam | 1:50 | Alexa 568 |
| **PPARGC1B** | EPR12370, rabbit monoclonal, Abcam | 1:250 | FITC |

Then, the cells were washed in PBS + 0.05% Tween 20 3 times for 5 minutes each. The cells were then incubated with the appropriate secondary antibodies (dilutions: 1:400 with 0,05% Tween and 10% FBS in PBS) for 1h at room temperature, protected from the light.

After the incubation, the cells were washed with PBS + 0.05% Tween 20 three times 10 minutes each. Next, cells were stained with 100 µl DAPI (Sigma, 1:1000 in PBS), 10 min, RT and washed with PBS, 10 min, RT. Lastly, the coverslips were mounted onto glass slides in anti-fading medium (Invitrogen) and seen under the fluorescence microscope (Axio Imager Microscope, Zeiss).

## 10. Statistical analysis

All data was analysed with the GraphPad software. In crystal violet assay differences between control and treatments were analysed using one-way ANOVA and considered significant if p value <0,05. Flow cytometry assay was analyzed using unpaired t-test with Bonferroni correction for multiple testing and differences were considered significant if p value <0,01 or p<0,008.

# Chapter IV

# Results

## 1. ER co-localization with transcriptional coregulators

It is described that when the hormones bind to ER, its dimerization occurs and thereafter, ER dimmers bind to sequences in the promoters of target genes. What allows the expression of these genes to occur is the interaction with coregulatory proteins that will inhibit (corepressors) or stimulate (coactivators) transcription through chromatin remodeling [87]. The aim of these experiments was to verify whether there was co-localization between ER and two proteins which, in a previous proteomics analysis carried out in a mouse tumor model, we found differentially bound to ER according to the tumor response to 4-OH-TAM. These proteins were the transcriptional coregulators PPARγ and PGC-1β which had previously been found by others in the lab interacting with ER in 4-OH-TAM resistant mouse mammary tumors using mass spectrometry [88]. For this purpose, we used the MCF-7S cell line which is sensitive and MCF-7R cell line which is resistant to 4-OH-TAM. In addition, we tested different antibody combinations in order to choose the most adequate ones for future confocal analysis. Cells were treated with 10 nM $E_2$ and fixed after 2 hours. Then, co-localization was evaluated by immunofluorescence.

The first pair of antibodies tested was to detect ER together with PGC-1β (Fig. 7). PGC-1β is a coactivator of nuclear receptors, which is involved in the regulation of cellular metabolism and homeostasis [87]. PGC-1β has high specificity for ER and potently increases its activity. Its location is mainly in the nucleus [89]. In this experiment, the localization of PGC-1β and ER appears to be both cytoplasmic and mostly nuclear. In MCF-7S cells co-localization was detected only after stimulation with $E_2$. This is in agreement with the literature, since when the ER interacts with its ligand, there is activation of the ER, recruitment of coactivators and consequently, the complex travels towards the nucleus where the transcription is processed [23]. On the other hand, we noticed a greater expression of PGC-1β in MCF-7R cells and ER co-localized with PGC-1β in the nucleus even in the absence of $E_2$, which suggests that in the 4-OH-TAM resistance cell line, PGC-1β activates ER in a ligand-independent way. However, in order to verify that there is a direct protein-protein interaction between ER and PGC-1β it would be necessary to use confocal microscopy and the proximity ligation assay (PLA).

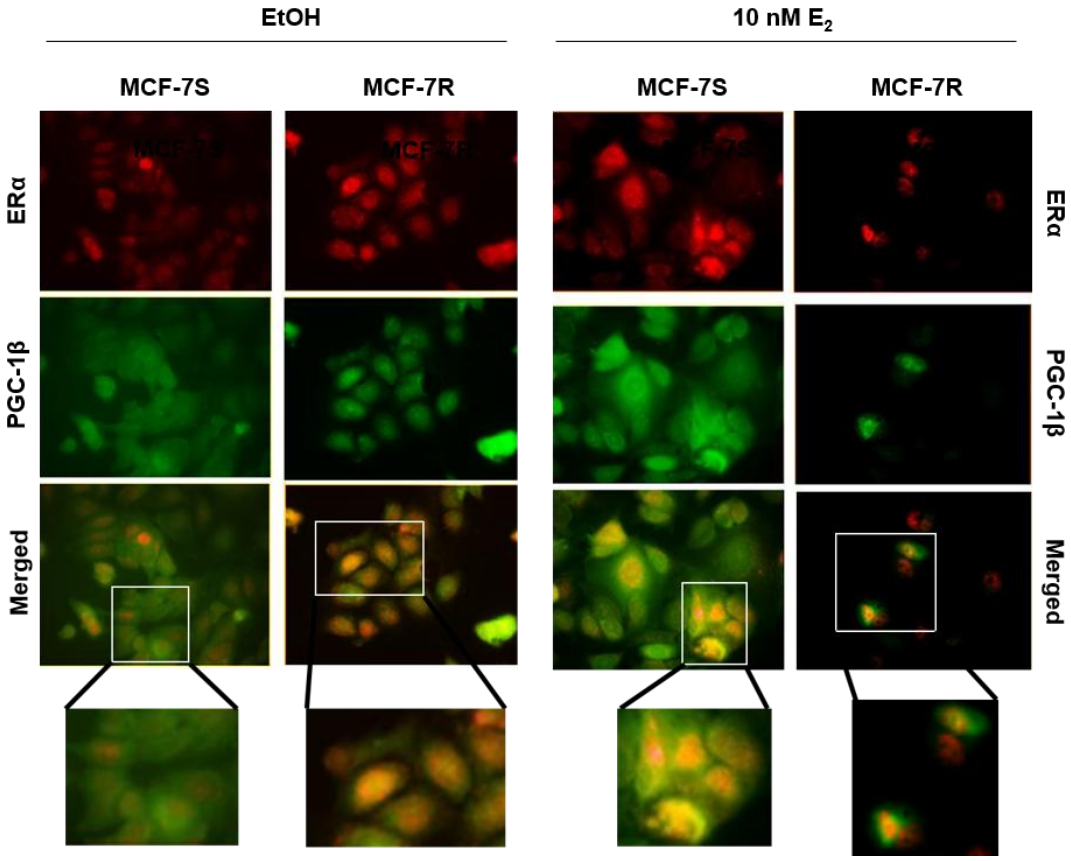


*Figure 7 - Immunofluorescence analysis of ER (1D5) and PGC-1β (EPR12370). The MCF-7S and MCF-7R cells were treated 2h with the same volumes of EtOH or 10 nM $E_2$. There is higher nuclear ER and PGC-1β coactivator in the MCF-7R cells than in the MCF-7S cells. Experiment representative of one. ~ 300 cells were analysed. Magnification: 63x.*

Also, we investigated the interaction of ER with PPARγ (Fig. 8). PPARγ is a ligand-activated transcription factor that belongs to the family of nuclear receptors [90].

The ER staining worked as expected. In both treatments, we observed nuclear and cytosolic labeling. However, in this case, nuclear localization did not increase with $E_2$ treatment. Regarding the staining of PPARγ, in both treatment conditions cytosolic location was observed. In the literature, this protein is found mainly in cytosol, especially in tumors [91][92], although it may also have peri-nuclear localization [93]. Accordingly, our results are corroborated by the literature. Therefore, the results showed that there may be a small co-localization in the nucleus (observed in MCF-7R with EtOH), which suggest that PPARγ activates ER in a ligand-independent manner. This interaction needs to be verified in the future using confocal microscopy and other antibodies, as well as PLA.

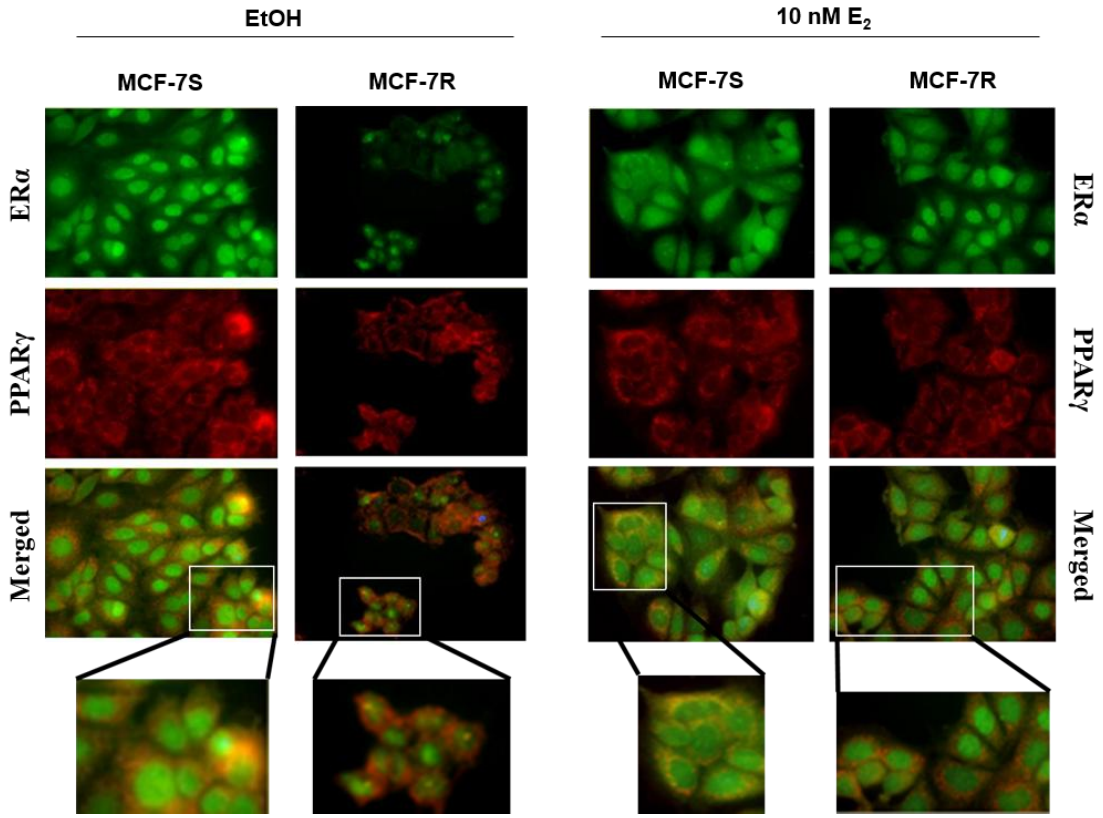


*Figure 8 - Immunofluorescence analysis of ER (HC-20) and A3409A (PPARγ). The MCF-7S and MCF-7R cells were treated 2h with the same volumes of EtOH and $E_2$. Experiment representative of one. ~ 300 cells were analysed. Magnification: 63x (except for MCF-7R cells treated with EtOH which was 20x).*

In summary, two antibodies against ER were tested and showed the expected staining pattern; while with regards to the coregulators, the antibody anti-PGC-1β and possibly anti-PPARγ appear to be specific (Table 3). Moreover, we were able to partially confirm the mass spectrometry results regarding a potential ER-PGC-1β interaction occurring in tamoxifen resistant cells. Full confirmation will require the use of confocal microscopy and PLA.

Table 3 - Main conclusions from the immunofluorescence analysis.

| | Antibody Specificity | Co-localization |
|---|---|---|
| ID5 (ERα) | ✓ | |
| EPR12370 (PGC1β) | ✓ | |
| **ERα- PGC-1β** | | ✓ |
| HC-20 (ERα) | ✓ | |
| A3409A (PPARγ) | ✓ | |
| **ERα - PPARγ** | | ✓ |

## 2. Influence of SETD7 on ER activity

As stated earlier, SETD7 is a protein with paradoxical function, because depending on the target proteins, it could have an oncogenic or tumour suppressive role in breast tumors. Studies indicate that SETD7 methylates the ER in lysine 302 and this methylation is effective in recruiting ER to its target genes and enhancing its transactivation. Therefore, SETD7 could have an important role in ER stabilization [34] and consequently, in development of endocrine resistance.

In order to better understand how SETD7 can regulate ER-mediated effects, we inhibited SETD7 activity with (R)-PFI-2. To do this, we used $E_2$ at the concentration of 10 nM with two concentrations of (R)-PFI-2 (1 nM and 8 nM). The two compounds were tested separately and in combination.

### 2.1. Regulation of cell proliferation by SETD7

In the first part of this project, we wanted to see how SETD7 inhibition regulates cell proliferation. For this purpose, we used the SETD7 inhibitor (R)-PFI-2 on T-47D and MCF-7 cells at concentrations of 1 nM and 8 nM. To measure cell viability we used the crystal violet assay to check whether or not the number of cells changed. In a subsequent step, we performed the flow cytometry assay to analyze the effect of the SETD7 inhibitor on the distribution of the cells throughout the cell cycle.

The crystal violet assay showed that T-47D cells were responsive, since there was a significant increase of cells treated with $E_2$ (Fig. 9). There was no significant effect of 1 or 8 nM (R)-PFI-2. There was cell proliferation in the combination of both treatments, although this effect was not additive or $E_2$ effect inhibited and is possibly due to $E_2$ alone. These data correlates with the flow cytometry assay (Fig. 10 and 11) where we observed a clear tendency towards increase in the S-phase of the cell cycle in cells treated with $E_2$, and similar levels in $E_2$ + (R)-PFI-2. However, in cells treated with (R)-PFI-2 1 nM or 8 nM alone a G0/G1 phase arrest occurred (although this was a tendency and with our number of samples did not reach statistical significance).

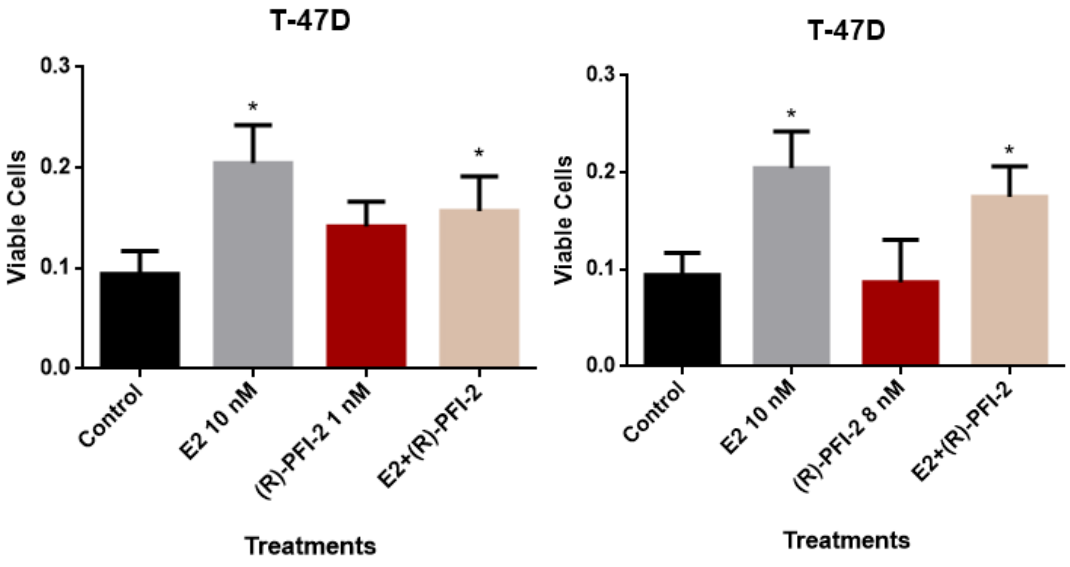


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*Figure 9 - Crystal violet assay on T-47D cells treated with 10 nM $E_2$, 1 nM or 8 nM (R)-PFI-2 and the combination of the two treatments Representative of four experiments. The bars indicate the mean ± SD of quadruplicates. *p<0,05 significant differences relative to the control (one-way ANOVA followed by Dunnett's multiple comparison test).*

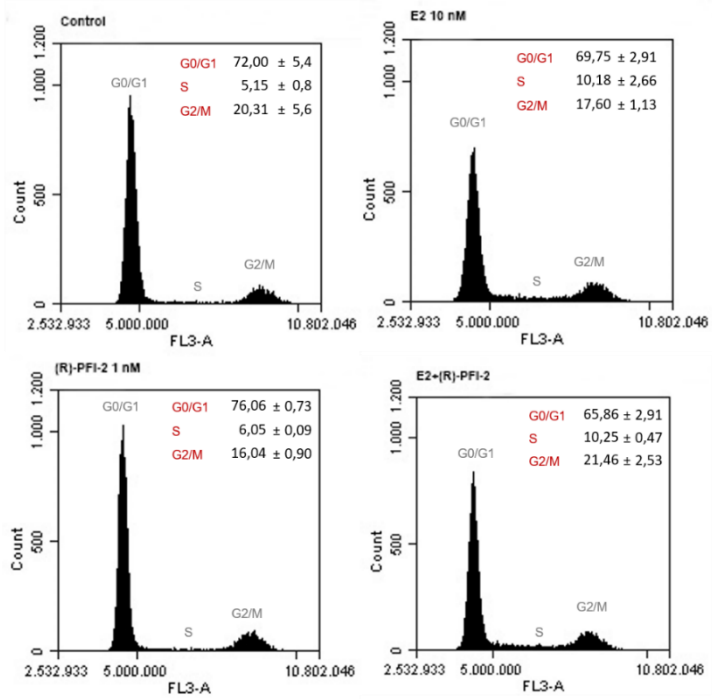


*Figure 10 - Flow cytometry assay with T-47D cells treated with 10 nM $E_2$, 1 nM (R)-PFI-2 and the combination of the two compounds for 24 h. Representative of one experiment carried out in duplicate. Data are represented as means ± SD of technical replicates. No significant differences ($p<0.01$; t-test with Bonferroni correction for multiple comparisons) were found.*

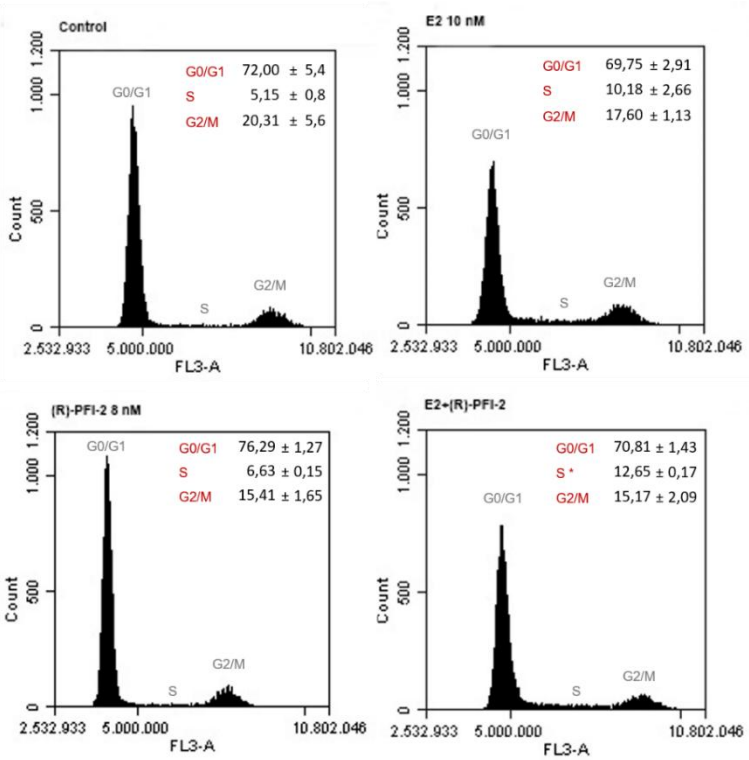


*Figure 11 - Flow cytometry assay with T-47D cells treated with 10 nM $E_2$, 8 nM (R)-PFI-2 and the combination of the two compounds for 24h. Representative of one experiment carried out in duplicate. Data are represented as means ± SD of technical replicates. *$p<0.01$ significant differences relative to (R)-PFI-2 8 nM (unpaired t-test with Bonferroni correction for multiple testing). No significant differences were observed between the treatments and the control group.*

Regarding MCF-7 cells, as expected, 10 nM $E_2$ increased viability, indicating that the cells were responsive (Fig. 12). When treated with 1 nM (R)-PFI-2 we found reduced cell viability, but when treated with the $E_2$ + (R)-PFI-2 combination the effect was the same as with $E_2$ alone, again revealing that cell viability increase was due to $E_2$ alone. These results are consistent with the flow cytometry assay (Fig. 13 and 14) where there seemed to be a cell cycle arrest in the G0/G1 phase with 1 nM (R)-PFI-2 and increase S-phase in cells treated with $E_2$ + (R)-PFI-2. In MCF-7 cells treated with 8 nM (R)-PFI-2 the effect was similar as with 1 nM, in flow cytometry assay. Unfortunately, we cannot compare these results to cell viability.

To sum up, we observed no additive or inhibitor effect of (R)-PFI-2 combined with $E_2$, although (R)-PFI-2 alone inhibits S phase transition. Thus, we can conclude that although SETD7 methylation of ER increases the stability of the protein and its transcriptional activity [34], contrary to the expected, inhibition with (R)-PFI-2 has no effect on ER-mediated proliferation. Moreover, (R)-PFI-2 alone appears to have distinct effects according to the cell lines.

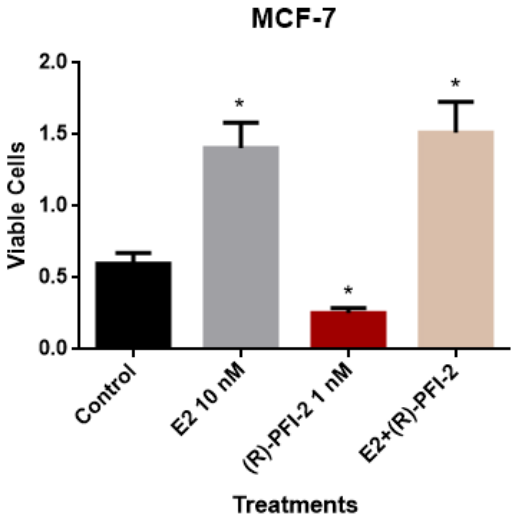


*Figure 12 – Crystal violet assay on MCF-7 cells treated with 10 nM $E_2$, 1 nM (R)-PFI-2 and the combination of the two treatments Representative of three experiments. The bars indicate the mean ± SD of quadruplicates. *p<0,05 significant differences relative to control (one-way ANOVA followed by Dunnett's multiple comparison test).*

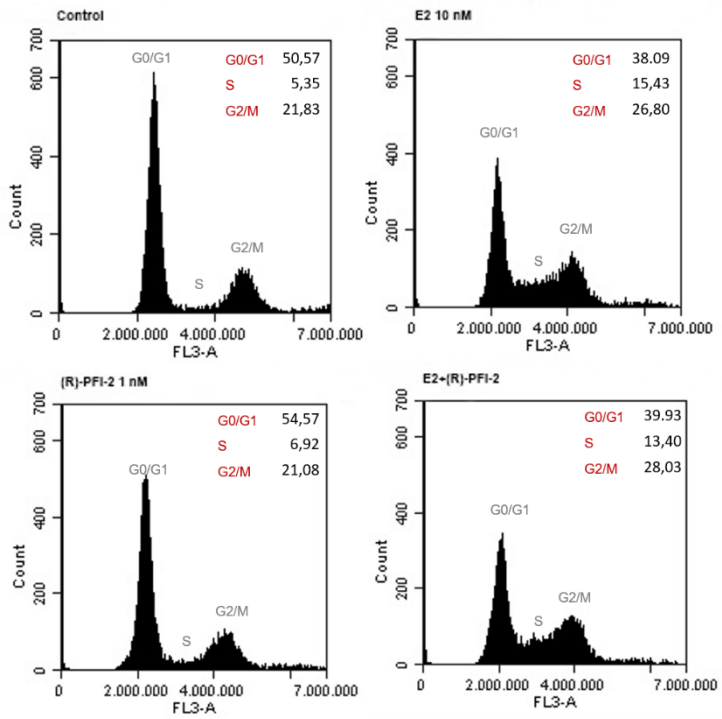


*Figure 13 – Flow cytometry assay with MCF-7 cells treated with 10 nM $E_2$, 1 nM (R)-PFI-2 and the combination of the two compounds. Representative of three experiments (one replicate each).*

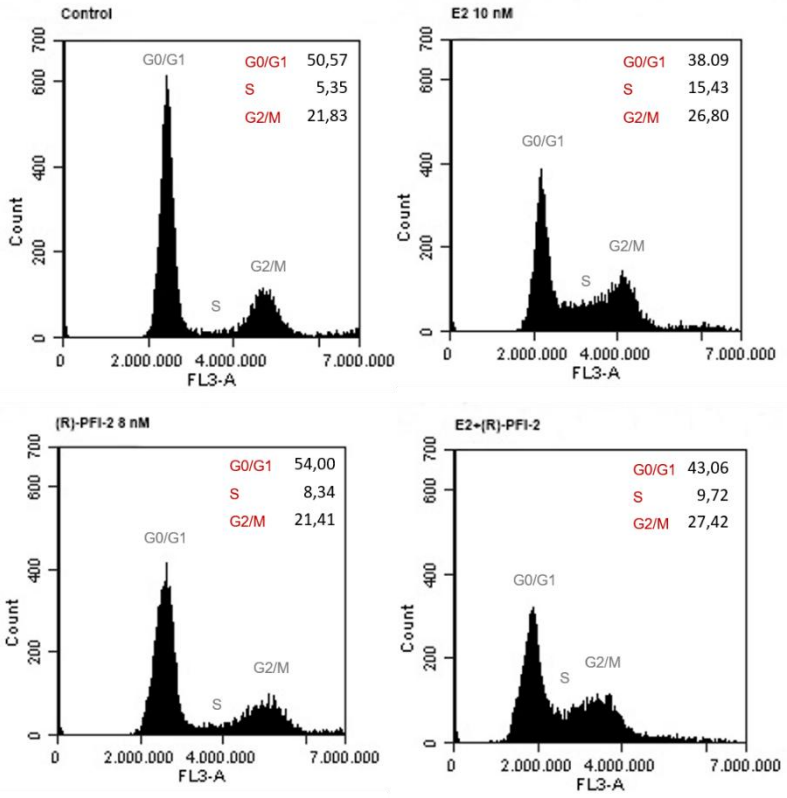


*Figure 14 - Flow cytometry assay with MCF-7 cells treated with 10 nM $E_2$, 8 nM (R)-PFI-2 and the combination of the two compounds. Representative of three experiments (one replicate each).*

### 2.2. Influence of SETD7 on ER and PR protein levels

In order to confirm if treatment with (R)-PFI-2 had any effect on ER and PR protein levels (which are indicative of ER activation and protein stability) we carried out immunoblot analysis after cell treatment with $E_2$ (known to down-regulate ER, and up-regulate PR), (R)-PFI-2 (1 or 8 nM; only 1 nM is shown) and the combination of $E_2$ and (R)-PFI-2. We added the treatments and incubated for 2, 6 and 24 hours so that we could compare what happens over time. In addition, we used the same samples but with the antibody to detect PR, since we know that this is an ER target gene and therefore could be used as indicator of how SETD7 affects ER activity.

In T-47D cells, low amount of ER expression were observed when cells were treated with $E_2$, probably because after gene promoter activation by ER, there is degradation of the receptor by the proteasome [94]. In addition, we see a gradual increase in the expression of the PR up to its maximum peak at 24 hours, indicating that the ER is active and activating the expression of its target gene PR. For cells treated with 1 nM (R)-PFI-2, we again found a decrease in ER compared to the control at 2h and 6h, which was lost after 24h probably because (R)-PFI-2 was no longer having an effect on the cells. With (R)-PFI-2 alone, the PR expression decreased at 6 and 24 hours, indicating that the ER was not active. In cells treated with 1 nM $E_2$ + (R)-PFI-2 we found that ER levels remained down-regulated at all hours relatively equal to cells treated with $E_2$ alone, however we observed that ER was not active since there was no PR expression (Fig. 15 and 16). Similar effects were observed when cells were incubated with 8 nM (R)-PFI-2 (data not shown).

These data may lead us to believe that SETD7 is necessary to have high levels of ER, but when we inhibit SETD7, the ER is not active since there is no expression of PR. These facts are in agreement with the literature that indicates that SETD7 is essential for the stabilization of ER, and the efficient recruitment of ER to its target genes and their transactivation [34] but, contrary to the expected, they do not explain the lack of effect observed in cell viability, which is stimulated through activation of transcription by ER even in the combined treatment $E_2$ + (R)-PFI-2 which had the same effect as $E_2$ alone.

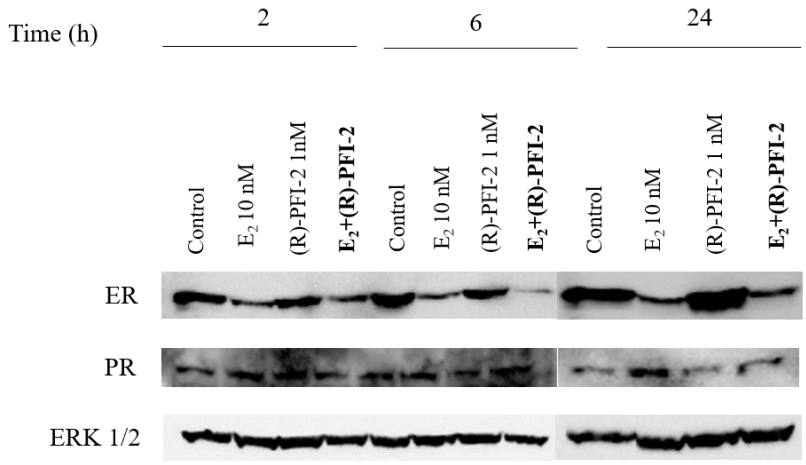


*Figure 15 - Western blot analysis of total ER and PR in T-47D cells treated with 10 nM $E_2$, 1 nM (R)-PFI-2 and the combination of the two compounds. Western blot is representative of 2 experiences with ER and 1 with PR.*

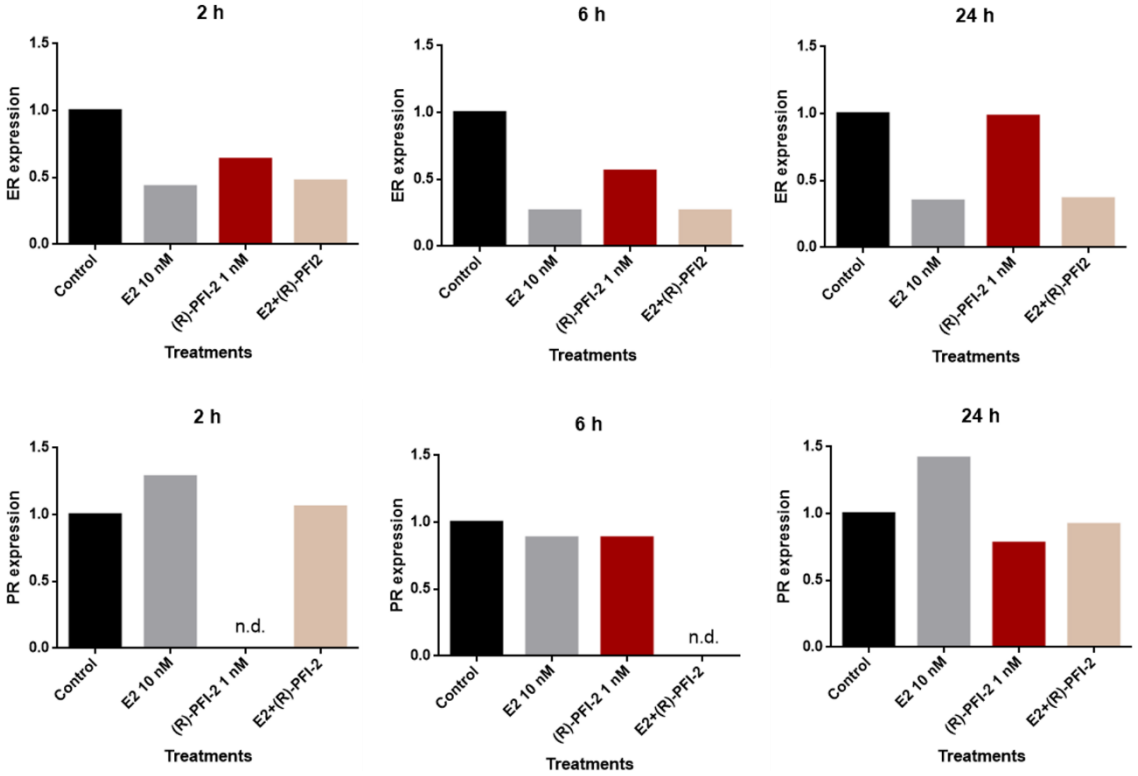


*Figure 16 - Quantification of bands in the Western blot shown in figure 15. ER (top) and PR (bottom) expression levels after 2, 6 and 24 hours of incubation. T-47D cells were treated with 10 nM $E_2$, 1 nM (R)-PFI-2, and the combination of the two compounds. n.d. = not determined due to artifact in the western blot since the WB exhibits higher background in the area of these bands.*

We then investigated the effect of (R)-PFI-2 in ER and PR protein levels in MCF-7 cells (Fig. 17 and 18). We treated the cells with 10 nM $E_2$, 8 nM (R)-PFI-2 and the combination $E_2$+ (R)-PFI-2.

We verified that the ER is active because after $E_2$ treatment there was expression of PR (except at 24 h, which could not be verified, because the WB did not run properly). With (R)-PFI-2, we observed an increase of ER relative to the control at all time points. The expression of the target gene PR was increased only within 2 hours but equaled the control at 6 hours. In the combination of the two compounds, we saw that ER expression levels were similar to $E_2$ and the levels of PR were similar to the control, indicating that there is no expression of target genes and therefore that when SETD7 was inhibited ER was not active. A similar effect was observed with 1 nM (R)-PFI-2 (not shown).

These results partially corroborate those described in the literature, which reports that in MCF-7 cells SETD7 inhibition with shRNAs reduces ER stability, which results in lower protein levels (in our case, protein levels are increased) and reduced PR expression [34].

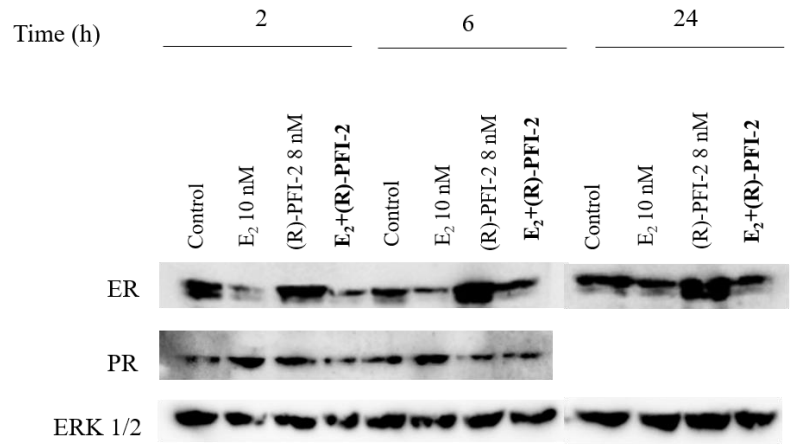


*Figure 17 - Western blot analysis of ER and PR on MCF-7 cells treated with 10 nM $E_2$, 8 nM (R)-PFI-2, and the combination of the two compounds for 24 h. Representative of one experiment.*

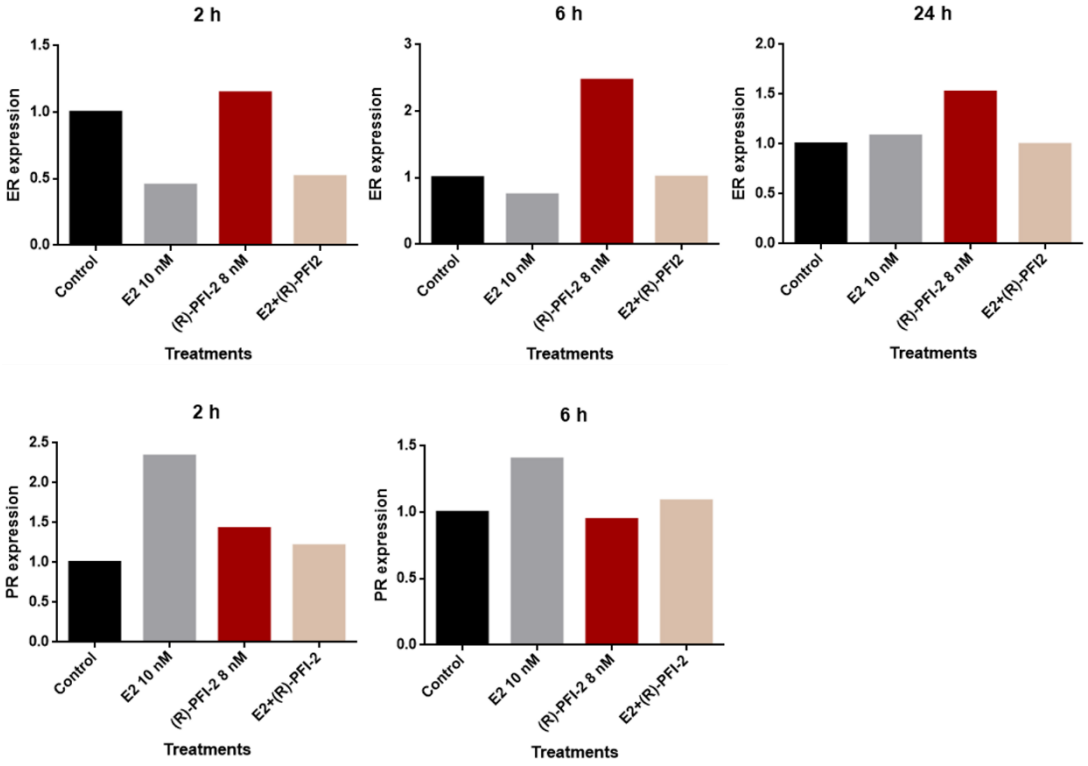


*Figure 18 - Quantification of bands displayed in the western blots shown in Figure 17. ER (top) and PR (bottom) expression levels after 2, 6 and 24 hours of incubation on MCF-7 cells treated with 10 nM $E_2$, 8 nM (R)-PFI-2, and the combination of the two compounds.*

### 2.3. Study of SETD7 inhibition in response to endocrine therapy

Finally, we sought to investigate the role of SETD7 inhibition in the response to endocrine therapy. We know that tamoxifen and ICI 182 780 are two ER antagonist compounds and their binding to the receptor inhibits transcription of proliferation genes [40][42]. Thus, we were interested to see if 1 nM and 8 nM (R)-PFI-2, which we found could inhibit G0/G1 transition, could have an effect when combined with 500 nM Tamoxifen and 250 nM ICI 182 780. For this, we used the crystal violet assay in the two cell lines under investigation, T-47D and MCF-7.

In MCF-7 cells we found increased proliferation with $E_2$ and descreased with 1 nM (R)-PFI-2. By flow cytometry, we see that cell cycle appears to be retarded with (R)-PFI-2 at both concentrations (Fig. 20 and 21). In relation to 4-OH-TAM we clearly saw an inhibition of proliferation, which was maintained when combined to (R)-PFI-2 (Fig. 19A). As we do not see a smaller proliferation with 4-OH-TAM and (R)-PFI-2 we can suggest that (R)-PFI-2 has no additive effect when combined with the antagonist. Similar effects were observed when we combined ICI 182 780 to each of these compounds (Fig. 19B).

Flow cytometry showed that ICI 182 780 acting alone inhibited G1 to S progression (Fig. 20 and 21). However, when combined, we see that there is retardation in the cell cycle, but the percentage remains the same as ICI 182 780 alone, so (R)-PFI-2 doesn't enhance ICI 182 780 inhibition.

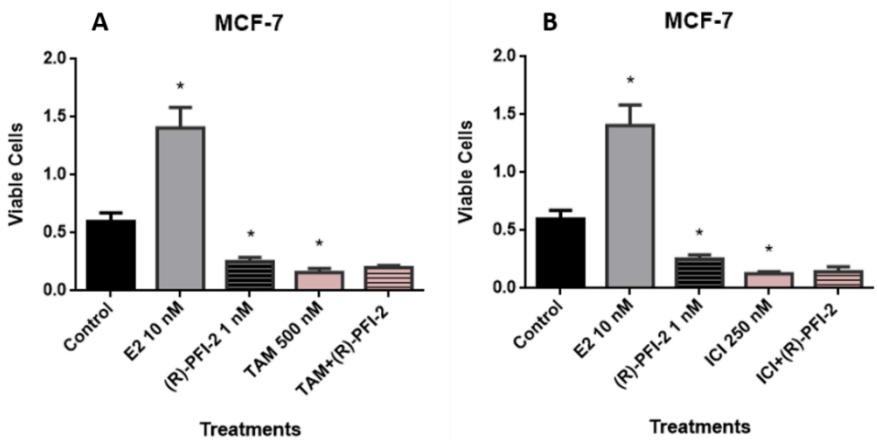


*Figure 19 - Crystal violet assay on MCF-7 cells with 10 nM $E_2$, 500 nM 4-OH-TAM, 250 nM ICI 182 780 and 1 nM (R)-PFI-2. *p<0,05 significant differences relative to control (one-way ANOVA followed by Tukey's multiple comparison test).*

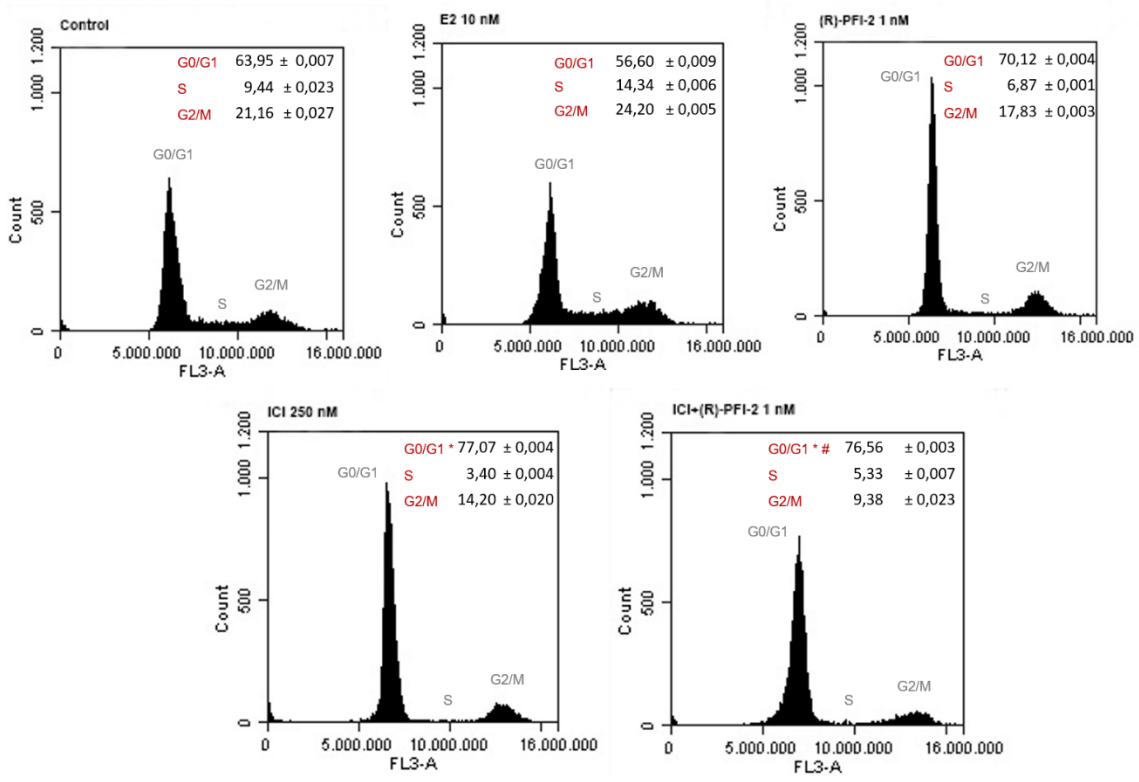


*Figure 20 - Flow cytometry assay with MCF-7 cells treated with 10 nM $E_2$, 1 nM (R)-PFI-2 and 250 nM ICI 182 780. Representative of one experiment carried out in duplicates. *p<0.08 significant differences relative to control. #p<0,08 significant differences relative to (R)-PFI-2 1 nM (unpaired t-test with Bonferroni correction for multiple testing).*

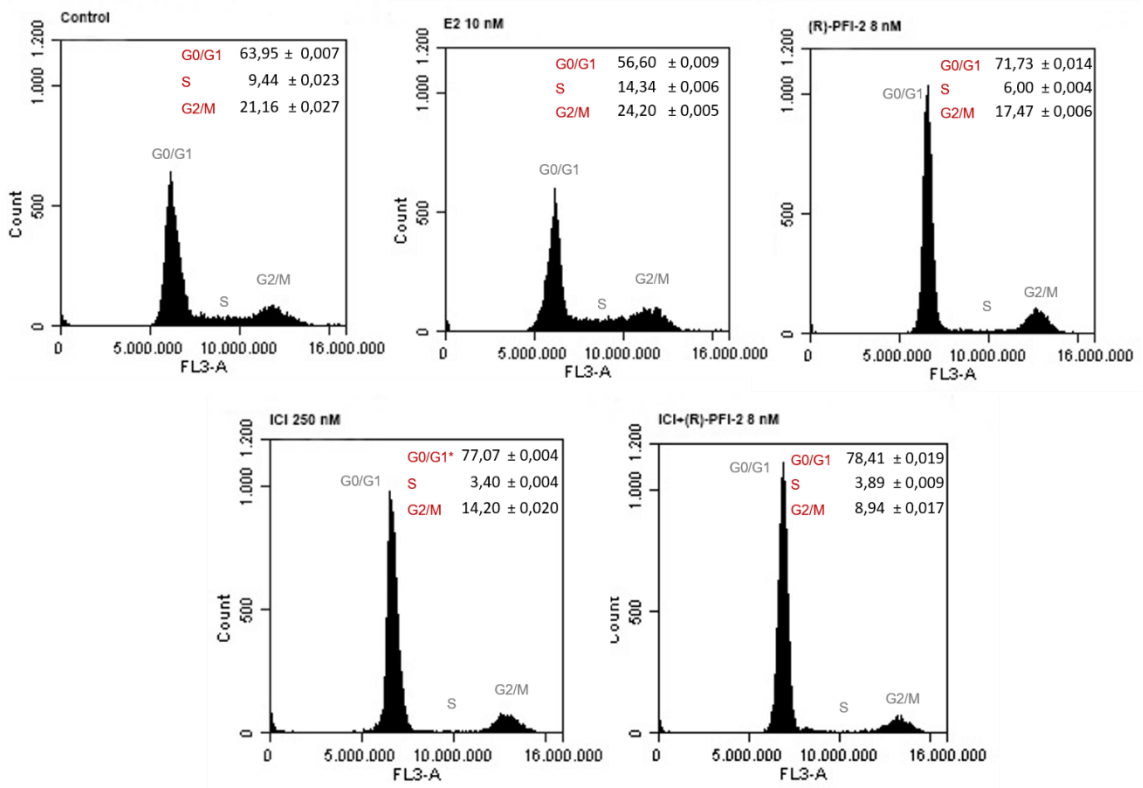


*Figure 21 - Flow cytometry assay with MCF-7 cells treated with 10 nM $E_2$, 8 nM (R)-PFI-2 and 250 nM ICI 182 780. Representative of one experiment carried out in duplicate. *p<0,008 significant differences relative to control (unpaired t-test with Bonferroni correction for multiple testing).*

In the previous experiment (Fig. 19-21) we found that there is no additive response when we combined (R)-PFI-2 to each of the antagonists tested. So, the next step was to investigate if a combination of both (R)-PFI-2 + antagnist could reduced the effect of $E_2$ on cell proliferation, in T-47D cells. Firstly, we saw that the cells were responsive to $E_2$, since proliferation increased (Fig. 22). When (R)-PFI-2 and $E_2$ were combined proliferation was observed. As seen previously, this proliferation is essentially due to the effect of $E_2$. With 4-OH-TAM the cells did not proliferate. $E_2$ + 4-OH-TAM cell levels were similar to $E_2$, which is indicative that these batch of cells had started to become TAM-resistant ($E_2$ effect is not reduced by 4-OH-TAM). With 1 nM (R)-PFI-2 viability levels were similar to 4-OH-TAM or (R)-PFI-2 in combination or alone. However, co-incubation of the cells with $E_2$ and 4-OH-TAM + (R)-PFI-2 reduced the number of cells slightly more than $E_2$ + 4-OH-TAM alone (although did not reach statistical significance). With 8 nM (R)-PFI-2, the effects were also not significant.

With the ICI 182 780 antagonist (Fig. 22B and 23B) we observed a stimulation of viability with $E_2$ + (R)-PFI-2 (1 nM or 8 nM), ICI 182 780 partially reduced $E_2$ effect. In this case, ICI 182 780 in combination with (R)-PFI-2 seemed to be better at reducing the viability stimulated by $E_2$ than ICI 182 780 alone. By flow cytometry, we detected an increase at the G0/G1 phase when the cells were treated with $E_2$ + (R)-PFI-2 1 nM + ICI 182 780 when compared to ICI 182 780 alone. Unfortunately, we do not have a group consisting of $E_2$ + ICI 182 780 alone to compare. With the 8 nM (R)-PFI-2 concentration, no inhibition was observed in the mentioned conditions.

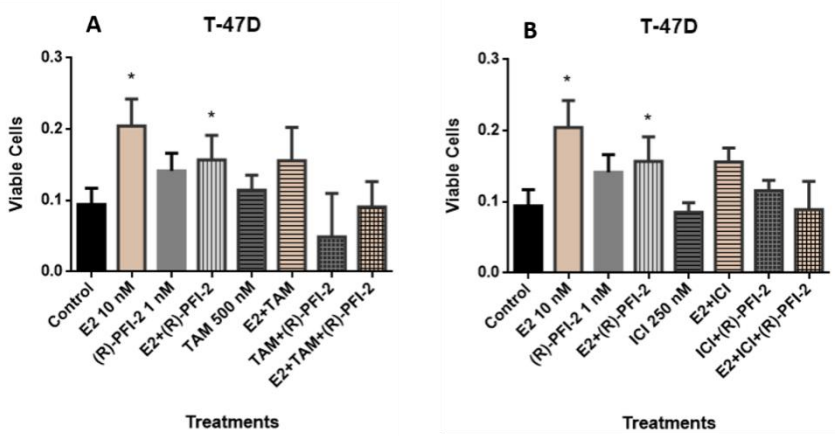


*Figure 22 - Crystal violet assay on T-47D cells treated with 10 nM $E_2$, 500 nM 4-OH-TAM, 250 nM ICI 182 780 and (R)-PFI-2 at concentration of 1 nM. *p<0.05 significant differences relative to control (one-way ANOVA followed by Tukey's multiple comparison test).*

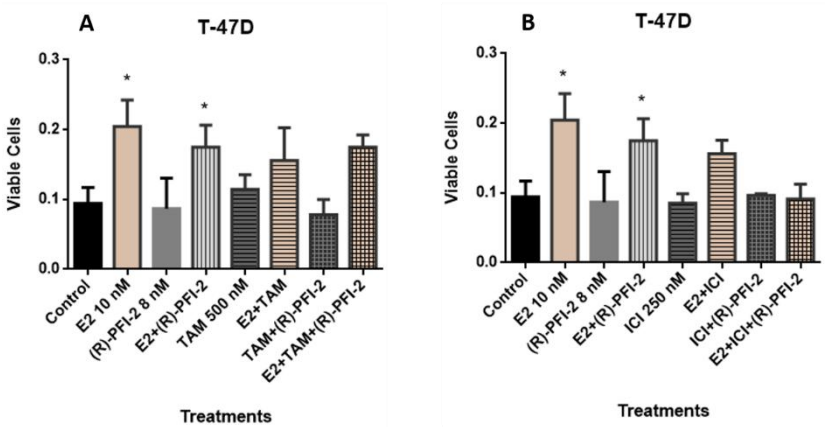


*Figure 23 - Crystal violet assay on T-47D cells treated with 10 nM $E_2$, 500 nM 4-OH-TAM, 250 nM ICI 182 780 and (R)-PFI-2 at concentration of 8 nM. * p<0.05 significant differences relative to control (one-way ANOVA followed by Tukey's multiple comparison test).*

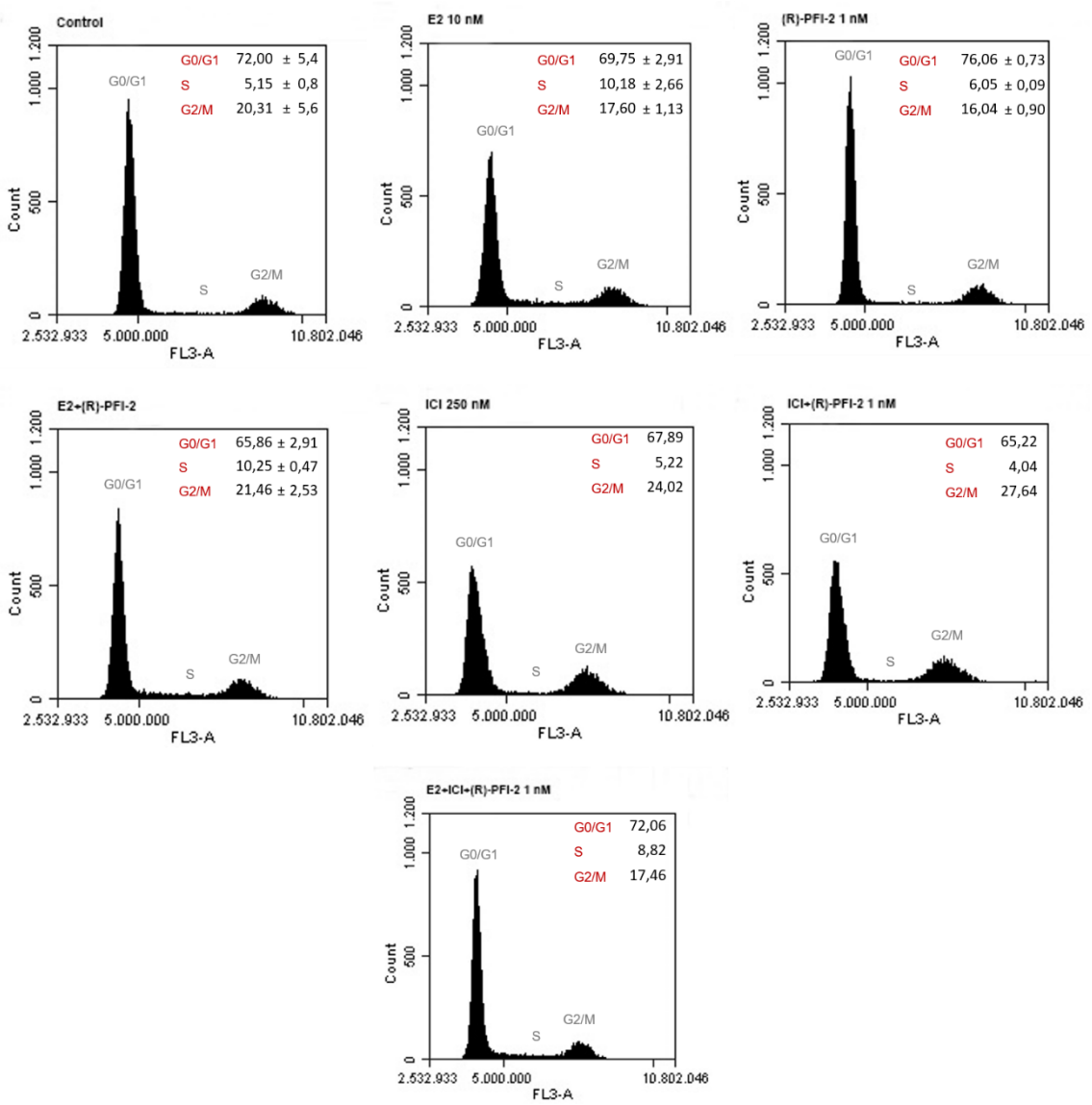


*Figure 24 - Flow cytometry assay with T-47D cells treated with 10 nM $E_2$, 1 nM (R)-PFI-2, the combination of the two compounds and 250 nM ICI 182 780. Representative of one experiment. No significant differences ($p<0.01$) were found.*

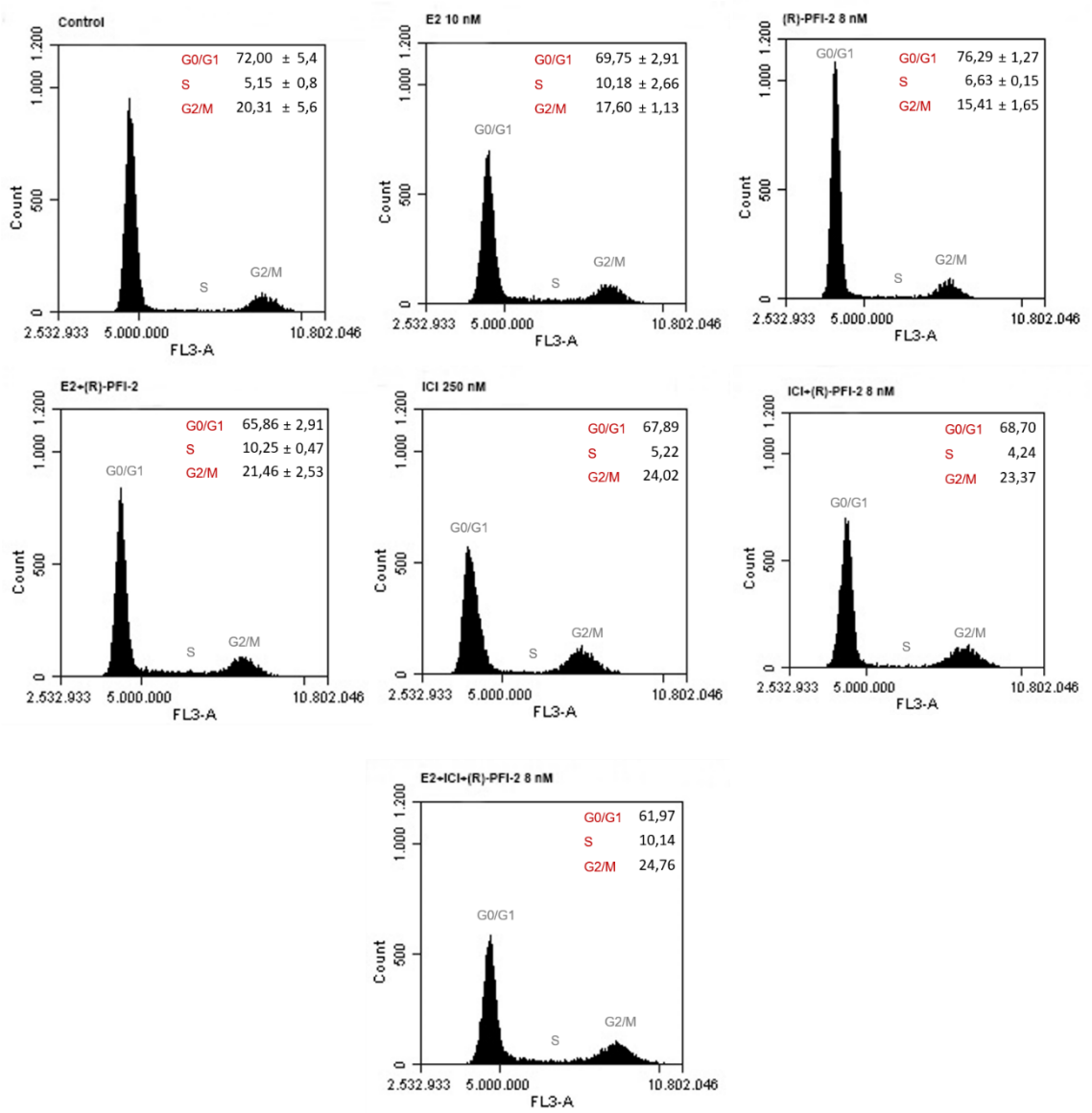


*Figure 25 - Flow cytometry assay with T-47D cells treated with 10 nM $E_2$, 8 nM (R)-PFI-2, the combination of the two compounds and 250 nM ICI 182 780. Representative of one experiment. *p<0,01 significant differences relative to (R)-PFI-2 8 nM (unpaired t-test with Bonferroni correction for multiple testing).*

# Chapter V

# Discussion

This project was part of a larger project already being developed in our laboratory, which seeks to answer questions related to resistance to endocrine therapy specifically in ER-positive BC. To that end, we used T-47D and MCF-7 cells, sensitive and resistant to tamoxifen, the oldest and most-prescribed SERM in the world [95].

The estrogen receptor interacts with several coregulatory proteins to activate (coactivators) or inhibit (corepressors) transcription. Given its function, it is important to emphasize the role that these proteins may play in the development of the disease, such as modulating the agonist and antagonist activity of a drug such as tamoxifen [96].

We sought to find suitable antibodies to investigate if PGC-1β and PPARγ interact with ER in MCF-7S and MCF-7R cells. Focusing on the first, it is a coactivator that has high transcriptional activity when bound to the DNA binding domain or when bound to transcription factors, as is the case of the estrogen receptor [97]. Previously we had seen using mass spectrometry that PGC-1β interacted with ER in mouse tumors resistant to tamoxifen [88]. Our results corroborated this finding, since we observed a greater expression of PGC-1β in human breast cancer cells resistant to tamoxifen and found antibody combinations that will allow further studies in mouse and human tumours. Our results are in agreement with the literature that showed that sensitivity to tamoxifen can be restored by silencing the expression of PGC-1β in MCF-7 cells resistant to tamoxifen [98], indicating once again its important role in endocrine resistance. We have also seen that ER co-localizes with PGC-1β in MCF-7R cells, even in the absence of $E_2$, which may indicate that PGC-1β activates ER in a ligand-independent way. In fact, PGC1α, closest homolog of PGC-1β can bind and coactivate members of the nuclear receptor family in a ligand-dependent and ligand-independent manner [97]. We know that when tamoxifen binds to ER it induces changes in the receptor [37]. One study has shown that these changes result in interaction with PGC-1β, which cooperates with another coactivator, SRC-1, activating the agonist effect of tamoxifen [99]. This may explain the fact that PGC-1β is more expressed in tamoxifen-resistant cells.

Peroxisome Proliferator-Activated Receptor-γ is a nuclear receptor activated by different types of lipids, and consequent translocation to the nucleus, where it acts as a transcription factor. It plays an essential role in energy metabolism, where it regulates energy storage [100]. In this experiment, we found PPARγ was localized mainly in the

cytoplasm. These results are in agreement with what is described in the literature. An immunohistochemical study of 170 breast cancer samples, 101 of the samples demonstrated the cytoplasmic expression of PPARγ, contrasting with only 2 samples that showed nuclear localization [91]. More specifically, in another study, it has been described by immunofluorescence that in MCF-7 cells PPARγ has a mostly cytoplasmic and peri-nuclear location "with very limited nucleus staining" [93]. Studies with preadipocytes also indicate cytosolic localization of the protein [92]. In cells in breast cancer, PPARγ transactivation induces apoptosis-related morphological changes. It is described that in these cells, the ER binds to the PPARγ response element in target genes and inhibits their transactivation [90][101]. Our results suggest that ER is interacting with PPARγ, because there is a small co-localization in the nucleus in cells resistant to tamofixen, treated only with EtOH. This may suggest that PPARγ regulates ER activity in a ligand-independent manner, a hypothesis that we plan to test in the future.

The second part of our project was related to SETD7 methyltransferase. Initially, we investigated the effects of its inhibition on cell proliferation. The response was distinct in the two cell lines. In T-47D cells we observed that inhibition with 1 nM (R)-PFI-2 slightly increased proliferation, although not significantly. On the other hand, we found that there was a cell cycle retardation in G0/G1, which may mean that the slight (yet not significant) increase that we verified with the crystal violet assay may be due to the increase in the number of cells that resulted from apoptosis inhibition. When treated with 8 nM (R)-PFI-2 there was no effect on the number of cells, the level being relatively equal to the control. Studies done previously in our laboratory with T-47D cells and using a cell count method indicated a significant increase in cell number in the T-47D cells treated with 1 nM (R)-PFI-2 and no effect with 8 nM (R)-PFI-2 [102]. So, we assume that the lack of significance observed with crystal violet may be due to the sensibility of the method. In MCF-7 cells we observed, as expected, an increase in stimulation with $E_2$ and when combined with (R)-PFI-2, levels were equal to $E_2$ alone. When the cells were treated with 1 nM (R)-PFI-2 alone we saw a significant decrease in the crystal violet assay, which was confirmed by increasing the cell cycle in G0/G1. Similar effects were seen when cells were treated with 8 nM (R)-PFI-2. Therefore, we can conclude that in MCF-7 cells, SETD7 is necessary for cell cycle progression and consequent cell proliferation in the absence of $E_2$ stimulation, but SETD7 activity is dispensable when proliferation is mediated by ER.

In summary, we have seen that in both cell lines when treated with 1 nM R-PFI-2 alone there is a cell cycle arrest in G0/G1. Thus, we may suggest that SETD7 is required for cell cycle progression but that it is not necessary if cell proliferation is stimulated by ER. When cells are stimulated with $E_2$ we know that there is stimulation of transcription of ER target genes, such as cyclin D1 [77] and in the cells treated with $E_2$ + (R)-PFI-2 condition we found proliferation occurring, indicating that (R)-PFI-2 has no effect and that SETD7 is not required to activate cyclin D1 activity. Therefore, we found that in the absence of $E_2$ the effects on proliferation are possibly due to other cell cycle proteins known to be methylated by SETD7, such as E2F1 that increases the transcription of one of its target genes, cyclin E and repression of TP53. We can also specify the methylation of Yin Yang 1 (YY1), which causes an increase in its oncogenic activity [103]. The combination of these methylations must be necessary for a certain level of proliferation to occur. Nevertheless, when ER is activated, it also plays an important role in the progression of the cell cycle, and its activity can be influenced by methylations [34][79]. However, we did not observe any effect on ER stimulation of the cell cycle. These may be because there are other compensating ER activating mechanisms that support Cyclin D1 expression or that SETD7 targets cell cycle proteins up-stream of those regulated by ER. Thus, when cells are stimulated with $E_2$, ER induction of cell cycle genes such as Cyclin D1 would override the effect of SETD7 inhibition on the cell cycle.

Next, we wanted to verify if SETD7 inhibition influenced the ER and PR protein levels. What we observed in the two cell lines treated with (R)-PFI-2 was no increase in PR levels, one of the ER target genes, indicating that ER is not active. These results are in agreement with the literature, where Subramanian *et al*., demonstrated that ER methylation by SETD7 is necessary for protein stabilization and consequent efficient transcription of ER target genes [34]. However, the work did not address the effects on cell viability or $E_2$-regulation of the cell cycle. Our results do not explain the lack of effect observed in cell viability, where the combined treatment $E_2$ + (R)-PFI-2 had the same effect as $E_2$ alone. In the crystal violet assay and flow cytometry, we observed that there was indeed proliferation when the cells were treated with the two compounds. This result can be explained by the fact that ER undergoes several PTMs, and even if its methylation by SETD7 does not occur in cell with (R)-PFI-2, other modifications may occur that eventually lead to protein activation. In fact, ER undergoes PTMs in the hinge region and a

crosstalk may also be occurring with residues close to K302 leading to transcription changes. A brief example is what Subramanian *et al*., describe in the study demonstrating that acetylation of K303 attenuates ER-driven transcription. Another example is phosphorylations occurring in S104-106 or S118, which upregulate transcription [33]. These two examples show the influence that other PTMs may have on regulating the transcription of ER target genes. Inhibition of SETD7 may in fact favor other post-transcriptional modifications, but we still saw that the protein was inactive to induce PR upregulation. Our study was limited in this case since we only studied the PR protein levels. The fact that SETD7 inhibition affects PR expression, but has not effect on the cell cycle, was unexpected. This because, ER and also PR, both stimulate CCDN1 gene expression. Subramanian *et al*. demonstrate in their study that, unlike some known genes that are regulated by estrogen, PR has no ER binding site on the gene promoter. Instead, they found ER recruitment for a putative enhancer 169 kB upstream of PR [34]. Cyclin D1 gene promoter contains canonical ERE and PREs regulatory sequences [14]. In addition, the promoter of the gene contains multiple regulatory elements such as E2F and Sp1 that may be involved in its transcriptional regulation [104]. Another example of how the organization of chromatin in the promoters of the target genes will affect the ER activity was described by Cicatiello *et al*. [77]. In quiescent cells, when CCND1 transcription is at baseline, specific regulatory elements are occupied by a complex between YY1 and histone deacetylase 1 (HDAC1), and repress transcription, but when cells are stimulated by $E_{2,}$ the complex is replaced by AP-1 and Oct-1 which interact in order to occurs gene transcription [77]. Therefore, ER methylation levels could be necessary to recruit the transcriptional machinery and specific chromatin remodelers to the PR gene, but not the CCND1 gene. In addition, other PTMs in ER itself or its interacting proteins may be favored or blocked when methylation is inhibited (Fig. 26).

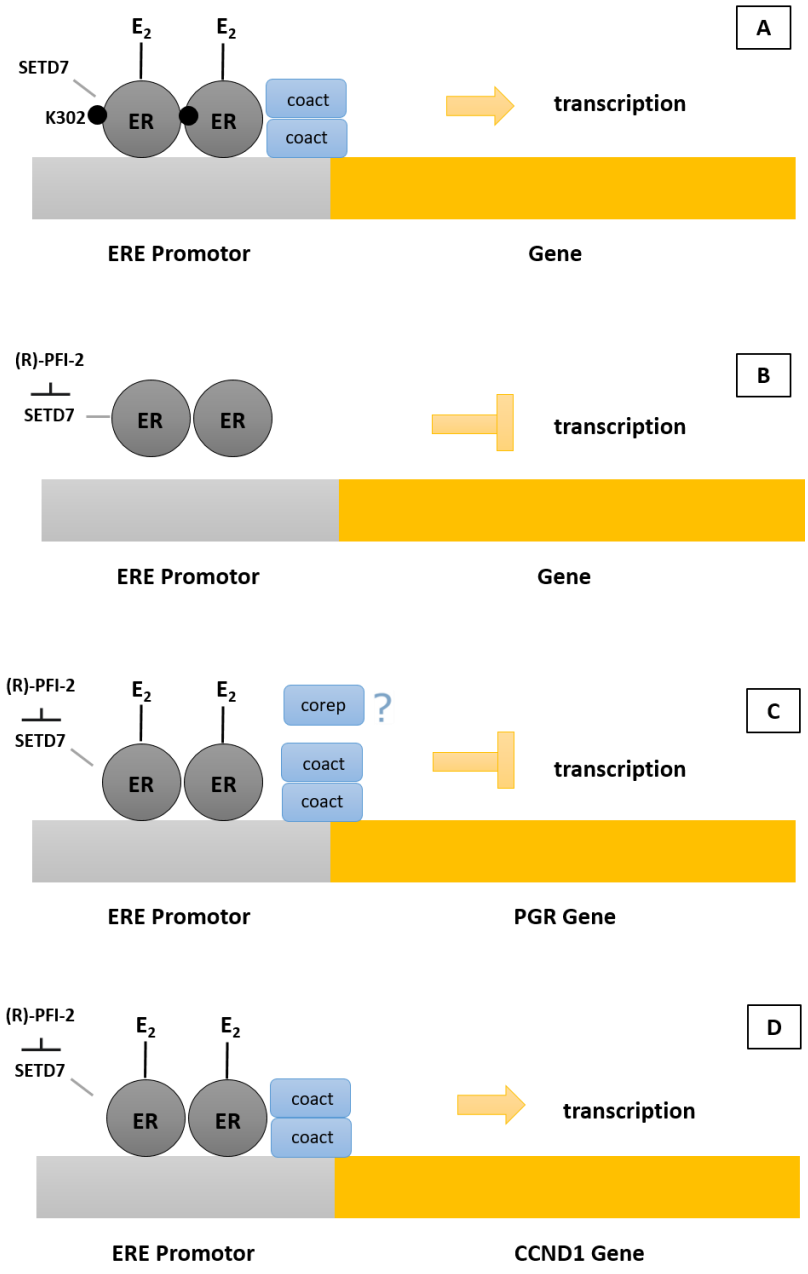


*Figure 26 - Several responses of $E_2$ induction in cells and combination with (R)-PFI-2. A) $E_2$ stimulation in the cell promotes ER dimerization, recruitment to the ERE of the target gene and consequent transcription. B) Without stimulation of $E_2$ and with inhibition of SETD7, no transcription is observed. C) and D) The combined effect of $E_2$ + (R)-PFI-2 may promote different responses at the level of transcription in progesterone receptor (PGR) gene and CCND1 gene, respectively. ERE – Estrogen response elements; corep – corepressors; coact – coactivators. Model based on our results and Subramanian et al. results* [34].

Finally, we investigated the effect of the 4-OH-TAM and ICI 182 780 antagonists when combined with (R)-PFI-2 at the two concentrations. The effect of 4-OH-TAM and ICI182 780 was different in the two cell lines. In MCF-7 there was clearly an inhibition of the number of cells when treated with 4-OH-TAM, which we unfortunately can't confirm

with flow cytometry, but based on the literature, we believe it is a decrease in proliferation. In addition, we found a retardation in the cell cycle when the cells were treated with ICI 182 780. However, we did not see an additive effect of (R)-PFI-2 when combined. The same thing happened with tamoxifen. We propose that the lack of additive effect of (R)-PFI-2 in this cell line was because they are already quite efficient in their inhibition by the antagonists.

In T-47D, antagonists had no effect on the number of cells or on the cell cycle. Interestingly, the literature says that the proliferation of these cells can be inhibited by antiestrogens [106], and we have seen the same in the lab before. Therefore, we suggest that the cells used, acquired resistance to antiestrogens. In fact, variants of this cell line have already been described as resistant to antiestrogens [107]. More studies are needed to confirm this situation. However, we were able to see that combining ER antagonism with 1 nM (R)-PFI-2 could increase the inhibition of $E_2$ effects, which is a promising result given that the cells appeared to be resistant to the antagonists alone.

# Chapter VI

# Concluding Remarks and Future Perspectives

This dissertation aimed to find out how ER activity can be changed by different factors and its relation to endocrine resistance. Our first specific objective was to test antibodies for future studies on the interaction of the estrogen receptor with two coactivators, PPARγ and PGC-1β. We found suitable antibodies and showed that both coactivators are possibly co-localizing with ER. Our next specific objective was to analyze the effect of SETD7 inhibition on cell proliferation in the presence of $E_2$, 4-OH-TAM and ICI 182 780. We observed that in the two cell lines there was a cell cycle retardation when they were treated with both (R)-PFI-2 concentrations, which indicates that SETD7 activity is necessary for cell cycle progression. We also wanted to verify whether inhibition of methyltransferase influenced the levels of ER and one of its targets, PR. In our results we didn't observe expression of the PR in the two cell lines under test, which indicates that the ER is not active. However, we observed increased number of the cells when they were treated with (R)-PFI-2 and $E_2$. We suggest that ER needs to be methylated by SETD7 to recruit coactivators and activate PR transcription, but to activate the transcription of other genes, such as the cyclin D1 gene, protein required for cell cycle progression, will not be necessary this methylation. Still, the lack of methylation by SETD7 can probably be offset by the favoring of other post-translational modifications. In addition, we admit that the transcriptional effect depends on the machinery of each gene, so the cellular responses will be different, which may explain the lack of expression of PR and the increase in the number of cells in the condition mentioned above. Regarding the endocrine response we obtained interesting results since the T-47D cells presented endocrine resistance to 4-OH-TAM and ICI 182 780 antagonists, and yet the addition of (R)-PFI-2 was able to decrease this condition.

In the future, it would be interesting to see if the inhibition of SETD7 leads to a decrease in the methylation of the ER. To support our hypothesis that inhibition of SETD7 inhibits ER activation and consequently PR but not the cyclin D1, we propose to check whether (R)-PFI-2 has any effect on ER binding to the PR and CCND1 gene promoters and their activation and to verify the effects exerted by (R)-PFI-2 on levels and their methylation of proteins involved in the cell cycle, such as retinoblastoma and E2F1, as well as cyclin D1.

# Chapter VII

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