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Effect of fasting mimicking conditions on hormone therapy efficacy and resistance acquisition in breast cancer

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List of Abbreviations

a.U.	Arbitrary Unit
aa	amino acid
Ab	Antibody
Abs	Absorption
Akt	Protein kinase B (PKB)
BC	Breast Cancer
BCA	bicinchoninic acid
CCND1	Cyclin D1
CDK	Cyclin dependant kinase
CNRQ	Calibrated normalized relative quantity
CTR	Control
CV	Cristal Violet
DDN2A	Cyclin A2
E2	Estradiol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen Receptor
ERK	extra cellular signal-regulated kinase (also MAPK)
ESR1	ER-alpha gene
ET	Endocrine therapy
FMD	Fasting mimicking diet
FOXM1	Forkhead box protein 1
Ful	Fulvestrant
HER 2	Human epidermal growth factor (EGF) receptor
HRP	Horse-redish peroxidase
HT	Hormone therapy
i.e.	It est (latin: that means)
IGF	Insulin growth factor
IGFR	Insulin growth factor receptor
IHC	Immuno histochemical
Ins	Insulin
IR	Insulin receptor
kDa	kilo Dalton
mTOR	mammalian Target Of Rapamycin

n.s	Not significant
NSG	NOD SCID gamma (Il2 RG null)
Pal	Palbociclib
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI3K	Phosphatidol-inositol-3phosphate kinase
PR	Progesteron Receptor
PTEN	Phophatase and tensin homolog
Rb	Retinoblastoma
RTK	Receptor tyrosin kinase
SASP	Senescence Associated Secretory Phenotype
s.c	sub cutaneously
SDS	Sodium dodecyl sulfate
SE	Standard error
SEM	Standard error mean
StDEV	Standard deviation
STS	Short-term-starvation
Tam	Tamoxifen
TNBC	Triple Negative Breast Cancer
TFF 1	Trefoil factor 1
Vin	Vinculin
WB	Western Blot

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1 ABSTRACT

Breast cancer (BC) is the most common cancer diagnosed among women worldwide; 60-80% of all BC cases belong to the ER^+BC subtype. Development of resistance is almost inevitable and is the major driver of ER^+BC-related death. Well-tolerated treatments that allow concomitant and broad targeting of the heterogeneous cancer cell population are needed in order to abolish resistance acquisition from the beginning. Fasting and fasting mimicking diets (FMD's) have shown great potential to protect the healthy cells of the body during chemotherapeutic treatment, while augmenting treatment efficacy, mainly through lowered levels of blood glucose and circulating Insulin-like growth factor-I (IGF-I) availability. In ER^+BC the interconnection of the ER signalling and the IGF1R signalling builds up a strong network difficult to target with specifically acting drugs. This work provides evidence that cycles of FMD have the potential to postpone development of resistance to the Selective Estrogen Receptor Degrader (SERD) Fulvestrant and more importantly circumvent development of resistance to the combined treatment of Fulvestrant plus the selective CDK4/6 inhibitor Palbociclib *in vivo*. FMD is capable of supressing resistance if given at the onset of the treatment but also if given at later stages, when insensitivity to these drugs has been acquired. Concomitant and early targeting of ER^+BC exhibits a killing effect *in vitro* and *in vivo*, potentially on resistance driving subpopulations. *In vitro* data hint at induction of senescence in the surviving cancer cell population. The combination of Fulvestrant and FMD resulted in resistance acquisition through the Retinoblastoma (Rb) pathway via up-regulation of CDK6. Combined Fulvestrant and Palbocilib treatment instead forces ER^+BC cells to rely on the Insulin- and IGF-I receptor signalling, which FMD can efficiently blunt. Due to its beneficial effects, FMD cycles represent a feasible treatment addition to these pharmaceuticals even over extended periods.

2 INTRODUCTION

2.1 Breast cancer

Breast cancer (BC) is one of the most diagnosed cancer types worldwide and remains a major challenge, mainly due to the intra-tumoral heterogeneity [1,2]. Coexistence of differentially programmed cancer cells, complicates specific targeting in the long term and allows development of resistance by switching growth-dependency [3,4].

2.1.1 Subtypes

Traditional classification of breast cancer patients is mainly based on the histological evaluation of the composition of three distinct biomarkers within the tumor sample and remains the basis for subsequent therapy selection. All three biomarkers are cell surface receptors that promote cell growth and differentiation, hence, if overexpressed, uncontrolled cell division promotes the formation of malignant tissue. They are namely the oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). HER2 impaired breast cancer is characterized by a predominant overexpression of the Her2 receptor and/or other genes of the Her2 pathway in the majority of the cancer cell population. ERs and PRs are absent or overexpressed in only a minor subpopulation. Estrogen receptor-positive breast cancer (ER^+BC) is characterized by a strong overexpression of the ER in the majority of the cancer cell subpopulations. This phenotype is usually, but not always accompanied by

increased levels of PR. Depending on concomitant overexpression or absence of the Her2 receptor, ER^{+}BC can be further classified into Luminal A subtype (ER-positive, PR-positive or -negative, HER2-negative) and Luminal B subtype (ER-positive, PR-positive or -negative, HER2-positive). Triple negative or basal-like breast cancer is characterized by a very low level or complete absence of all three previously named receptors (ER-negative, PR-negative, HER2-negative) in the majority of the cancer cells.

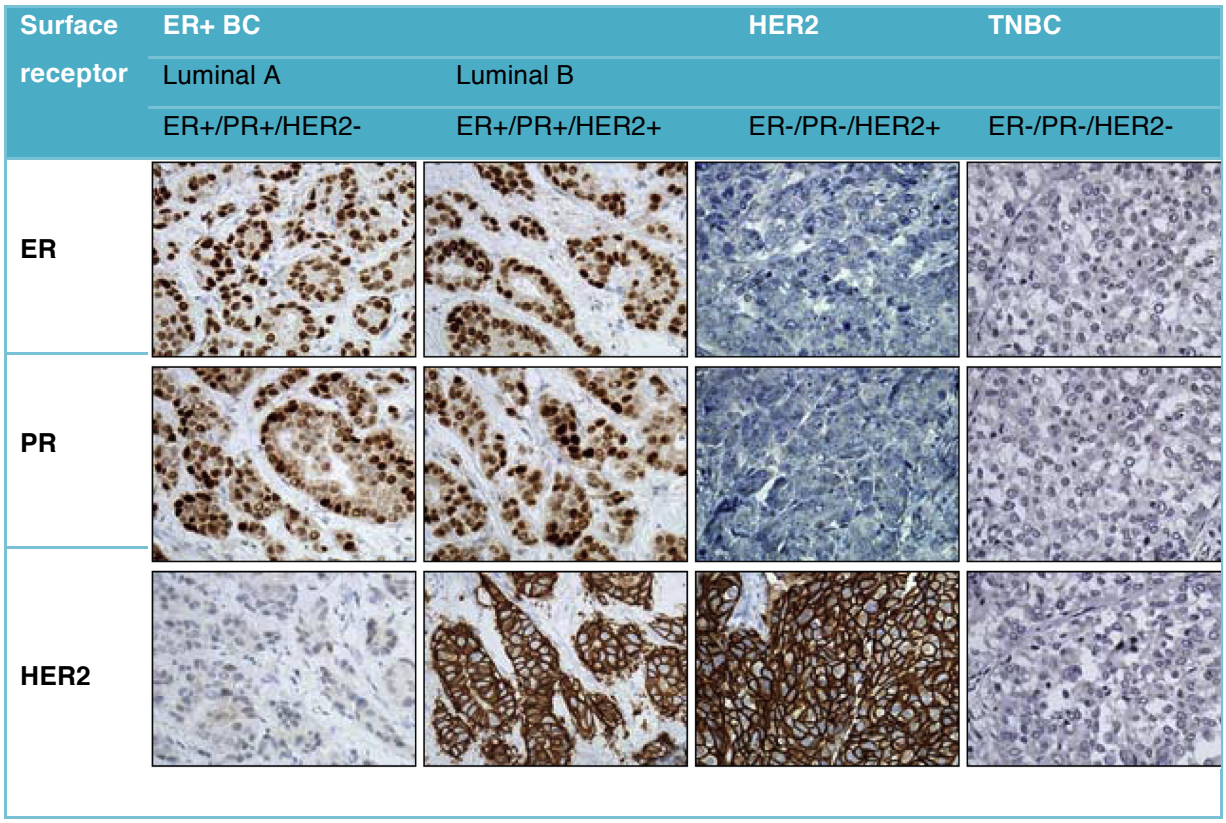

Figure 2-1 Traditional sub classification of breast cancer.

Traditional sub classification of BC is based on histological evaluation of the three main biomarkers: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Figure adapted from Ashley G.Rivenbark et al., The American Journal of Pathology, 2013.

As mentioned above, this archaic classification of breast tumors is the principal basis for treatment choice although it has been refined greatly during the last

decades. Optimized and innovative methods, especially high throughput technologies (i.e. microarrays) provided a deeper insight into the molecular players and can lead to a more detailed characterization of each subtype and further sub-classification [5]. Perou et al. [6] was the first group to develop a molecular classification of breast cancer which has since been validated by independent groups. In this manner, in addition to the expression of the Her2 receptor, Luminal B subtype could be classified with a higher expression of proliferation genes such as MKI67, Cyclin B1, and the epidermal growth factor receptor (EGFR) compared to Luminal A. These markers are linked to its poor prognosis in comparison to the Luminal A subtype. Sub-classifications of TNBC went through several stages depending on the type of analysis [6-8] until Burstein and his group [9] described four stable molecularly derived TNBC subtypes: 1) luminal androgen receptor (AR; LAR), 2) mesenchymal (MES), 3) basal-like immunosuppressed (BLIS), and 4) basal-like immune-activated (BLIA) subtype. Building up on this knowledge, The American Society of Clinical Oncology (ASCO) guidelines of 2016 and 2017, indorsed five gene expression assays, which showed evidence of clinical utility in order to help clinicians sub-classify breast cancer and choose the most effective treatment [10]. Although discrepancies were observed when the results of these assays are compared in a particular patient, the overall benefit is indisputable. Unfortunately, lack of full standardization, as well as associated costs render clinical application of gene expression profiling difficult.

2.1.2 Breast cancer screening and diagnosis

Breast cancer screening should start with manual self-observation and visual scanning at home as early as 30 years of age. Typically, at an age of 40-50 years women should visit a doctor annually for mammographic breast cancer screening as suggested by the American breast cancer society (www.cancer.org; www.breastcancercare.org.uk). However, classical screening and diagnosis of breast cancer does not follow a uniquely set procedure world-wide but rather depends on each country's guidelines and on the possibilities to follow them which rely on the different healthcare environments. Even following the same guidelines, the outcome is greatly influenced by the training and skills of medical professionals that harvest tissue samples, execute the laboratory work, and/or analyse the results, and also the technical equipment itself. According to the *European guidelines for quality assurance in breast cancer screening and diagnosis* [11] the standard operating protocol comprises of clinical examinations regarding general health status (physical examinations, blood tests, liver and renal function, palpation, history, etc.) combined with specific screenings based on imaging techniques (mammography, breast ultrasound, breast magnetic resonance imaging-MRI). When non-invasive examinations are ambiguous, a core needle biopsy of the tumor is performed with needle aspiration or core needle biopsy of suspicious lymph tissue. Final pathological diagnosis and classification are not straightforward and cut–off points often depend on the specific laboratory, which is why analysis of all tissues should follow the World Health Organization (WHO) classification [12] and the tumor-node-metastases (TNM) staging system. These guidelines include immunohistochemical (IHC) analysis of ER, PR, and Her2

status, as well as the proliferation marker Ki67. If elevated or unclear Her2 levels are measured, *in situ* hybridization methods can help evaluate Her2 gene amplification status. Staging assessment occurs depending on all previously collected results and in case metastasis may have occurred, upon applying different methodologies such as chest computed tomography (CT), abdominal ultrasound and/or a bone scan [13]. High throughput assays, explained previously, are costly and therefore not yet included in standardized diagnosis, in some cases laboratories apply, if available, first-generation genomic tests (such as MammaPrint® or Oncotype DX®), especially for luminal cases. Therefore, therapy selection is made based on traditional classification (**Figure 2-1**). The outlined obstacles in making a proper diagnosis should underscore that treatments are based on a very simplified classification system and though major improvements in the development of new drugs have been made during the last decades, breast cancer remains a big challenge due to unavoidable development of resistance to current treatments.

2.1.3 Subtype specific treatments

Breast cancer medication is chosen based on individual circumstances. Not only the evaluation of the present subtype is pivotal. Determining stage and size of the cancer as well as patient's age and health status play a key role when a therapy is selected. In many cases, surgery is part of the treatment and depending on the size of the tumor, either breast conserving surgery or mastectomy (removal of the entire breast) is chosen, with or without excision of nearby lymph nodes. Pharmaceutical treatments can be applied before surgery (neo-adjuvant), after

surgery (adjuvant), or both. As described by the American Cancer Society, pharmaceutical approaches in breast cancer comprise three main strategies and are guided by the traditional classification system: chemotherapeutic treatment, targeted therapy or hormone therapy (HT) also referred to as endocrine therapy (ET). Chemotherapeutic treatments are mainly applied in cases with TNBC cases. The lack of overexpression of specific growth promoting receptor or any other known “achillis heel” requires the general targeting of fast proliferating cells. Chemotherapeutics can be are effective, but are poorly selective, as they interfere with the process of DNA synthesis, DNA repair, or dynamics of the cytoskeleton, to supress cell division and/or promote cell death. For this reason, healthy cells that are constantly dividing in our body (cells of the immune system, intestinal tract, and lung epithelia) are also targeted, which is why chemotherapy is accompanied by deleterious side effects. Her2 impaired breast cancer can be specifically targeted due to the development of the anti-Her2 inhibitory antibody (Trastuzumab) [14] in the late 1990’s, usually without the addition of chemotherapy [15]. Interruption of the main growth-promoting signal in this subtype is still the most efficient way of controlling this cancer subtype. For the ER^+BC subgroup that makes up the majority of all diagnosed breast cancer cases [16,17], the mainstay is endocrine or hormone therapy (ET or HT). ET comprises various classes of pharmaceuticals that target ER signalling, mainly as an adjuvant, but also in a neo-adjuvant setting. ET treatment success in ER^+BC has been significant, nevertheless an unmet challenge remains in the development of a variable but persistent onset of acquired resistance in these types of cancers.

In the work described here, I focus on the challenge of suppressing the development of resistance to endocrine therapy in ER$^+$BC. Therefore the following part will discuss this breast cancer subtype, including the molecular basis of the ER, its signalling, classical treatment approaches and an alternative treatment to circumvent resistance acquisition.

2.2 Estrogen Receptor positive Breast Cancer (ER$^+$BC)

2.2.1 Characteristics, Incidence and Outcome

ER$^+$BC or hormone receptor positive (HR$^+$) breast cancer is characterized by predominant overexpression of the ER. It should be noted that in this context, literature refers to the ERα isoform if not otherwise indicated (and so it is in this work) as it is well accepted that specifically ERα fuels ER$^+$BC progression, at least in the initial stage [18-21]. The second isoform, ERβ, was discovered later and even though some studies connected it to breast and other cancer types, it does not represent the driving force of cancer progression, instead it is associated with better outcome in ER$^+$BC. The biology of ERα is discussed in more detail bellow. Classification of ER$^+$BC as explained before, is usually based on IHC staining of tissue samples. Thresholds for a positive staining of the ER vary between countries and range from ≥1% to ≥20% of positive cells [22,23], while also the intensity of the staining is taken into account. Samples analysed via IHC, that have positive staining for ER in only 1-9% of all cells, are considered low level ER, while patients that pass the 10% threshold are considered good responders to ET [22,24,25]. Fortunately, the majority of ER$^+$BC patients fall into the high level ER class. Sub-classification into Luminal A or Luminal B is mainly useful for validation of

survival expectancies and frequency of controls as Luminal B breast cancer is linked to higher aggressiveness and subsequently worse outcome [26,27]. However, of all diagnosed breast cancers cases, the great majority falls into the ER^+ group (60-80%) [22,28-30]. The ER^+ group has generally better prognosis [31,32] and mortality rates have declined to a greater extent at least in the United states [31-33], compared to the other main BC subtypes (Her2 impaired and TNBC). Endocrine therapy, which will be discussed in detail below, for decades has been the most successful treatment of BC and remains the first choice as an adjuvant or neo-adjuvant. Chemotherapy is chosen only in rare cases were advanced age and/or stage of the disease, which is often connected to *de novo* resistance, might hinder its success [34]. Overall survival rates are reduced due to acquired resistance that occurs according to some sources in at least half of all patients [21,35]. Insensitivity to the applied treatment is usually discovered when the formation of a metastatic disease occurs, a state where median survival in the ER^+BC is limited to only a few years [36]. Therefore, circumventing resistance is the main obstacle in order to increase overall breast cancer survival rates and thus fighting the main cause of death in women with cancer.

2.2.2 The Estrogen receptors

ERs exist in two isoforms, ERα and ERβ, which belong to the nuclear hormone receptor family of ligand-activated transcription factors. Though both ER receptors are considered isoforms, each receptor is product of independent genes, located on different chromosomes, yet sharing roughly 50% overall amino acid sequence identity. They own similar structural organization and are similar to all other

nuclear receptors divided into six functional regions. Both can be bound and activated by estrogens, the primary females sex hormones, because of high sequence conservation in the ligand-binding domain (LBD). A high degree of conservation in the DNA-binding domain (DBD) is responsible for binding of mostly the same consensus sequences. Nevertheless, the differences in sequence as well as only partially overlapping tissue specific expression patterns of the receptors themselves but also of their co-activators and co-repressors, expounds the distinct physiological function [37], hence their divers role in ER^+ tumor progression.

2.2.3 Estrogen Receptor alpha

ERα was initially described by Elwood Jensen in the late 1950s. Since then the receptor has been subject of extensive research, especially after the discovery of the mechanism of its activation by estrogen [38] and the beneficial effect of interrupting its signalling in breast cancer patients with high expression of the ER [39,40]. ERα is mainly expressed in the female reproductive organs (ovaries, uterus, vagina, endometrium), the mammary glands, but also in the central nervous system, smooth muscle cells and to a lesser extend in the kidney, liver, and adipose tissues [41] (proteinatlas.org). Within the cell the ER is mainly present in the nucleus, but also in the plasma membrane and the cytosol. Upon ligand activation, it can act directly as a transcription factor (TF) of genes involved in cell growth, proliferation, and differentiation, as well as apoptosis. The precise transcriptional activity is dependent on the constellation of recruited co-activators and co-repressors [42]. In the 1990s, a ligand-independent growth promoting action of the

ER via crosstalk with other TF's or growth promoting pathways has been discovered. All mechanisms will be explained more detailed below. Full-length ERα has a molecular weight of 66kDa, a length of 595 amino acids (aa) and consists of six regions [37]: A/B region, C region, D region and E/F region. The **A/B region** contains an amino-terminal transactivation domain 1 (AF1) with a ligand-independent function and a co-regulatory domain that recruits co-activators and co-repressors. The **C region** contains a highly conserved zinc-finger DNA binding domain (DBD) that is responsible for the binding to specific estrogen responsive elements (ERE) in the proximal promotor region or at distal regulatory elements of estrogen-responsive genes [43]. The subsequent **D region** holds a hinge domain and contains part of the ligand-dependent activating domain, as well as the nuclear localization signal. Finally, the carboxy-terminal **E/F region** contains activation function domain 2 (AF2) and the ligand-binding domain. Several transcript variants of ERα have been found [44,45], and their presence *in vivo* has been confirmed [46]. The truncated ERα-36 variant has been studied most extensively and has been associated with endocrine treatment resistance upon Tamoxifen treatment [47] and the agonistic effect from Tamoxifen treatment that is associated with an elevated risk of endometrial cancer [48]. A precise biological function of each variant in health and disease remains elusive and investigations are hindered due to a lack of high-quality antibodies.

2.2.4 Activation of ERs by estrogen

17β-estradiol (E2) is the most potent and prevalent estrogen produced in the body, being the predominant ligand of both ER isoforms. Two metabolites of E2, estrone

and estriol, exert much weaker agonistic effects, even though they are high-affinity ligands [49]. In pre-menopausal women, E2 is mainly produced by the ovaries and to a lesser extent by tissues like the heart, liver, skin, and brain, being essential for development and differentiation of reproductive organs. In post-menopausal women instead, when ovarian function decreases, the main source derives from extra glandular production in the adipose tissue. Expression of aromatase, the enzyme that catalyzes the last step in E2 synthesis increases with age [50] leads therefore at higher age to higher levels of circulating estrogens. Elevated levels of endogenous and exogenous estrogens have been linked to the development of BC [51,52], which increases with higher age and body mass index (BMI). Like all steroid hormones, E2 can diffuse across the plasma membrane and inside the cell can act through ER-dependent or -independent mechanisms [53] that both have been connected to development of BC [54]. In this study, only the ER mediated effects of estrogen have been considered.

2.2.5 Estrogen receptor signalling in cancer

ERs, present in the nucleus, plasma membrane and the cytosol, are known to promote cell growth and proliferation while repressing apoptosis. Their effects can be transmitted in two ways: either in a ligand-dependent way, direct or indirect (named genomic action), or in a ligand-independent way, referred to as non-genomic action. First we look at the different mechanisms of action, then at the outcome in terms of activated and repressed genes. Ligand-dependent activation can occur in two ways. In each case, binding of estrogen to the LBD leads to conformational changes in the ER monomer that allows homo- (or hetero-)

dimerization and translocation into the nucleus. There the ER can act directly as a transcription factor (TF) (**Figure 2-2**). It was long assumed that consensus sequences, known as estrogen-responsive-elements (ERE) are the only binding sites through which ERs exert its direct genomic function. It has been demonstrated, however, that receptors can also bind imperfect EREs or ERE-half-sites, which have been shown to contribute to 30% of the expression of ER-regulated genes [55] and these ER binding sites are now known to not only exist in proximal promotor regions (like initially believed) but the majority are in enhancer regions at distant sites [43,56]. Activation of ERs is accompanied by recruitment of distinct co-activators and co-repressors. Additional co-regulators help to form multi-protein complexes, which then dictate, based on their configuration the resulting genomic actions (enhancing or repressing function). Exact assemblage of these multi-protein complexes is regulated spatially and temporarily and deregulations among these cofactors contribute to abnormal signalling. Alternatively, ERs can execute their genomic action indirectly through stimulation of secondary TFs that do not depend on ER binding sites and lead to transcription of different target genes than the ones controlled by direct genomic action. Investigations that aim at identifying the complete repertoire of genes controlled by ERs in this bivalent genomic way have been continuing for decades and remains on going, yielding a complex and ever-growing network of molecular players and regulatory layers. Like mentioned before, the ER can exert its function also in a ligand-independent way, which represents an extreme form of cross talk between different signalling pathways (**Figure 2-2**). Studies investigating cross talk with other pathways involve the ER-alpha isoform. It can result from direct or indirect

interaction of the ER with growth factors like epidermal growth factor (EGF) [57], insulin or insulin-like growth factor 1 (IGF-I) [58,59], as well as with their associated receptor tyrosine kinases (RTK) [60,61]. Additionally, pro-growth signalling molecules downstream of these RTKs like phosphoinositide 3-kinase (PI3K), protein kinase B (PKB/Akt), mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase (p70S6K) or mitogen-activated protein kinases (MAPK's) are also involved in the transcriptional activation of the ER. All these pro-growth pathway components can phosphorylate the ER mostly at serine residues in the A/B region where the ligand independent AF1 domain lies [62]. The ER can in turn trigger pro-growth pathways by phosphorylating and activating some of these proteins like PI3K or Akt [63,64]. Proliferation is triggered here in a non-genomic way [65]. The impact of this interconnection is not surprising, considering that the PI3K-Akt-mTOR axis is among the most frequently miss-regulated pathways in cancer [66]. Due to the sheer number of possibilities of regulation that are currently known, it is not surprising that many genes cannot be traced back to one activation pathway. Still, the discovery of ER regulated genes helps to better understand the action of ERs and to investigate how to interrupt its signalling in malignant conditions. It should be noted that recently the regulation of non-coding RNAs have been added to the repertoire of how ERs can influence cell homeostasis [67,68] but they are not taken into consideration during this work. Briefly, estrogen regulated expression culminates in the expression of genes that support proliferation, including genes that drive cell cycle progression [69] and DNA synthesis and repair. The ERs influences various levels of the cell cycle, such as G_0/G_1 to S-phase progression by activating Cyclin D1 (CCND1) [70,71] or S-phase and G_2-phase progression by

stimulating expression of Cyclin A1 (CCNA1) [72]. Expression of other cell cycle regulators like the TF Forkhead box protein M1 (FOXM1), indirect effectors like Retinoblastoma binding protein 1 (Rb-BP1) [72], or the general mitogen IGF- I [73] are influenced by the ER as well. Apart of cell cycle key players, genes that support cellular homeostasis during the process of cell division, like chaperones or genes that play a role in protein synthesis like eukaryotic translation initiation factor 5A (EIF5A) [74] are among ER targets. Trefoil factor 1 (TFF1/pS2) has been one of the first target genes discovered [75] and remains an example where ER activity in ER overexpressing breast cancer correlates with its target gene [76], but the exact role remains unclear. TFs that transmit their function through the indirect genomic action of the ER also act on pro-growth promoting pathways driving cell proliferation and/or blocking apoptosis [77-79]. Fostering proliferation in this vast acting mode, genes counteracting cell cycle progression or promoting apoptotic events, are as expected rather supressed. It now seems natural that overexpression of the ER leads to malignant transformation and that interrupting its signalling is the most successful strategy to control tumor progression. Indeed, 520 genes are differentially expressed between ER^+ and ER^- primary breast carcinomas [80].

Summarizing, the signalling network around the ER is extremely complex due to presence of two ERs, splice variants, ligand-dependent and ligand-independent activation, that themselves depend on tissue specific temporal regulation via distinct co-regulators.

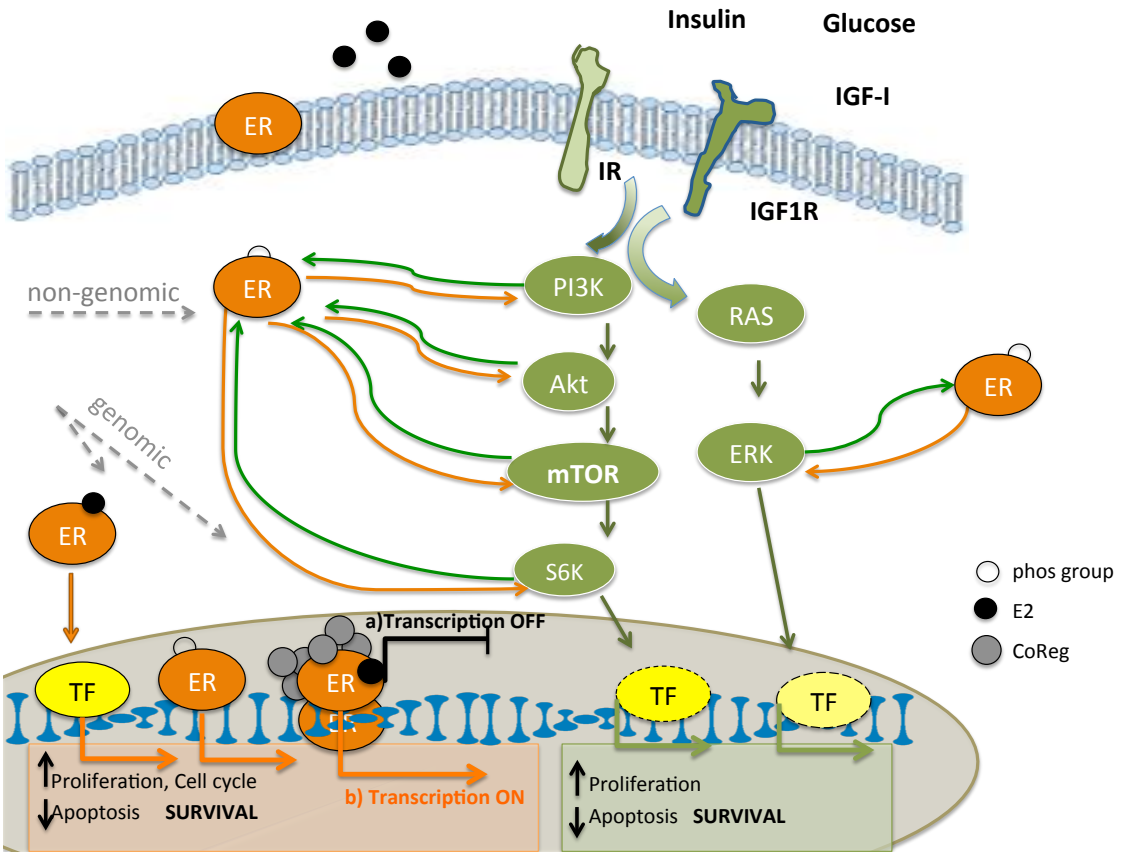

Figure 2-2 Illustration of the ER signalling and its cross talk with other pro-growth pathways

ERs located in the plasma membrane, cytosol or nucleus can trigger transcriptional programs via non-genomic and genomic signalling, while the later one occurs through direct or indirect action. Direct genomic action follows binding of estrogen, which leads to receptor dimerization and translocation into the nucleus. Upon recruitment of Co-activators or Co-repressors (in Figure 2 summerized as Co-regulators) the dimer binds to DNA sequences called estrogen-response elements (ERE) or ERE-half sites and their configuration decides about the following transcriptional action (activation or repression). Activation through estrogen can also lead indirectly to genomic signalling where the ER activates in the cytosol or the nucleus other TFs that than bind DNA sequences different from ERE full- or half-sites and activate transcriptional programs aiming again at cell survival, proliferation and counteracting apoptosis. Non-genomic action relies on interaction with RTKs like the IGF1R or their downstream signalling molecules like PI3K, Akt, mTOR, p70S6K or ERK. The ER can phosphorylate these proteins and thereby trigger pro-growth action in an indirect way. Phosphorylation of the ER by signalling components of the IR/IGF1R pathway can in turn trigger its direct or indirect genomic action.

2.2.6 Endocrine therapy strategies

ET is the mainstay for ER^+BC targeting and the majority of patients with 10% or more positive IHC staining for the ER (considered as high ER levels), therefore greatly benefit from this therapy, while few ER-patients (having less than 10% positive ER staining, making them difficult to target) have less benefits to ET [25]. Therapeutic strategies aim either to block estrogen synthesis with Aromatase-inhibitors, or to target the estrogen receptor itself [81,82]. In this study, I focus on the latter mechanism. Selective Estrogen Receptor Modulators (SERMs) and Selective Estrogen Receptor Degraders (SERDs) impact the ER in two different ways. SERMs change the ER action in a tissue specific way, as binding to the ER induces conformational changes different than those induced by estradiol, subsequently changing the transcriptional program in a tissue specific way but not eliminating the receptor itself. Proliferation of breast cancer cells, therefore, is impaired while estrogenic effect is achieved in other tissues than the breast, which can be both, advantageous and disadvantageous. For the last 40 years, Tamoxifen remains the top treatment currently used in ER^+BC. The therapeutic effects of Tamoxifen are significant, however, so are the associated long-term side-effects, including a correlation with thromboembolic events and a 2-4fold increased risk of developing endometrial cancer. Besides these and other side-effects recurrence within the first 5 years could be reduced but was still inevitable [83], which encouraged the search for novel ER blockers. SERDs lead to the degradation of the receptor, thereby inhibiting estrogenic activity, as well as non-genomic growth promotion. Notably, it has been shown that expression of most genes activated by E2 were entirely abolished upon Fulvestrant treatment *in vitro*

while this was not the case using Tamoxifen [72]. Fulvestrant is the most commonly used selective estrogen receptor degrader, and has been used as the second line of treatment for ER^{+}BC since 2002 [84], and has been approved in 2017 for mono-therapy and in pre-menopausal women An important characteristic of Fulvestrant, and a major reason it being a mainstay therapy, is that it doesn't show cross reactivity with Tamoxifen. Hence, instead of applying immediately cytotoxic chemotherapy, once resistant has developed to Tamoxifen, addition of Fulvestrant or other ET drugs can be used sequentially, with the aim of prolonging the effective duration of well-tolerated treatment [85]. Importantly, the degradation of the ER and subsequent blunting of its signalling including its non-genomic action, should impede crosstalk activity with other signalling pathways. Unfortunately, Fulvestrant treatment also will eventually lead to resistance, the main obstacle, which has led to investigations of new combinatorial treatment approaches [86-88]. Generally all classes of endocrine therapeutics are used in a sequential way, targeting the ER signalling at different points, which is advantageous but apparently not sufficient to avoid resistance acquisition. A novel drug, not used in endocrine therapeutics, yet is showing promising results is Palbociclib (PD-0332991; tradename Ibrance®) a highly selective and reversible cyclin-dependent kinase (CDK) 4/6 inhibitor and by blocking the CDK4/6 complex, its downstream effector protein Retinoblastoma (Rb) remains hypo-phosphorylated. In this hypo-phosphorylated stage, the Rb protein, also called the gate-keeper of the cell cycle, can halt progression from G_1-to S-phase. Addiction to continuous cell cycle progression separates the molecular identity of cancer cells from the healthy counterparts, hence Palbociclib has limited side effects on healthy cells.

Nonetheless, it does not belong to the traditional ET drugs, *in vitro* studies have shown a preferential effect on ER^+BC cell lines [89], which is not surprising given the fact that the upstream regulator of CDK4/6 is Cyclin D1, a downstream target of ER and often amplified in ER^+BC. *In vivo* testing on breast cancer, and also colon, lung and glioblastoma models, have shown beneficial effects of Palbocilcib in various cancers [90]. Finally, clinical trials revealed that addition of Palbociclib to Letrozole (PALOMA2) or Fulvestrant (PALOMA3) treatment augmented progression free survival with low short-term toxicities [91-94]. Due to these striking results for Palbociclib, the FDA accelerated approval in 2015 for the use of it in combination with Letrozole for the treatment of ER^+, $Her2^-$ advanced stage BC. One year later regular approval has been granted for the combination with Fulvestrant for HR^+, $Her2^-$ metastatic BC therapy, meaning only in cases of disease progression following endocrine therapy. Data that support beneficial effects of combined use of these novel drugs at early stage breast cancer are urgently needed.

2.2.7 Resistance acquisition

Endocrine therapy has had great success and has been extensively improved during the last decades due to the development of more potent agents. Presently, drugs of all categories (Aromatase-inhibitors, SERM's and SERD's), whether used alone or sequentially showed great success but will almost inevitably result in resistance within 10 years, which creates the main cause of BC deaths. Further, their combined treatment is only approved for use during advanced stage ER^+BC, mainly due to side-effect burden. Once the tumor becomes insensitive to

previously effective treatments, chemotherapy remains the only way to prolong survival for these individuals. Intra-tumoral heterogeneity of the initial cancer persists even upon resistance acquisition. The Her2 receptor for example can be present in subpopulations of the naïve tumor, as well as in later stages, and being cell surface receptors that stimulate proliferation, Her2 can contribute to the progression especially upon development of insensitivity to former drugs like Tamoxifen [95,96]. The Her2 signalling pathways is not the only one that has been connected to development of resistance, there exists a series of possibilities to escape from previously efficient treatments. Expression of the truncated ERα isoforms ER-α36 has been positively associated with resistance acquisition upon Tamoxifen treatment [97,98]. Mutations in ERα, occurring mostly in the LBD, are mainly found in metastatic breast cancer [99] and can lead to ineffectiveness of drugs that bind to the LBD, while action through the AF1 site is still possible. With Tamoxifen and AI's usually being the initial therapeutic strategy, mutations in the ERα gene (*ESR1*) has been mainly connected to Tamoxifen or AI resistance. Nonetheless, once the ER triggers proliferation independent of its functional LBD, Fulvestrant will also not be effective. CDK6 overexpression has been linked to resistance acquisition specific to Fulvestrant treatment *in vitro* and *in vivo* [100]. Overexpressed CDK6 can compensate for the lowered level of Cyclin D1 (downstream target of the ER) upon Fulvestrant treatment. In this way CDK4/6 complex is able to inactivate an important gate-keeper and tumor suppressor, the Rb protein, to promote cell cycle progression. For this reason, combination of Fulvestrant plus Palbociclib (a specific CDK4/6 inhibitor) prolongs patient's survival. Aberrant Cyclin D1 expression is another candidate to promote tumor

progression upon blunted ER signalling. Importantly, has been highly associated with metastatic ER^+BC [101]. Acting upstream of CDK4/6, Cyclin D1 activity should be blocked with Palbociclib, too, but there is evidence, that CDK4/6 inhibitor induced cytostatic effects can be circumvented by Cyclin D1, via non-canonical action through CDK2 [102]. Considering that Cyclin D1 is not only a direct target of the ER, but that its expression is also stimulated by the growth factor IGF-I, its potential in treatment is significant. Furthermore, there is involvement of the insulin-like growth factor 1 receptor (IGF-IR) signalling or the human epidermal receptor (HER) family signalling in resistance acquisition. Both IGF-1 and Her signalling have been found as compensatory mechanisms that can drive proliferation, promoting cancer cell survival [103-106]. In particular, IGF1R signalling has been found to trigger ER expression, as well as Cyclin D1 and CDK4 expression *in vivo* [107], thereby building a bridge between the ER and the Rb pathway, while still having its own growth supportive effects.

Considering the heterogeneous nature of a cancer cell population it is likely that alternative routes arise within the same patient. This phenomenon complicates targeting the evolving resistant cancer, with a single and specific acting drug. Furthermore, it has not been proven whether these signalling pathways actually evolve through accumulation of mutational hits that create new subpopulations or whether the different subpopulations, are present from the onset. In this case, concomitant targeting with diverse acting drugs might bring more therapeutic benefit. Specifically supressing ER signalling is the most efficient way in controlling initial tumor progression in ER^+BC patients but progress, regardless, still occurs.

2.3 Cancer, aging, IGF-I signalling and dietary interventions

2.3.1 Cancer and age-related diseases

Cancer is considered an age-related disease as the incidences of most types of cancer increases with age and age itself is a factor considered in all studies of cancer epidemiology [12,108]. Some opinions support the idea that cancer cannot be prevented in older individuals [109] but by lowering or postponing age-related risk factors its incidence could be at least postponed. It is reasonable that extensive effort is and has been made to understand the molecular basis of aging, age-related risk factors and to find interventions that might have the potential to lower these, considering that the worlds population of individuals over 65 years will triple between 2015 and 2050 [110] and the median age of cancer diagnosis is 66 [111].

2.3.2 Dietary interventions slow down aging and cancer incidence

For many decades the beneficial effect of calorie restriction (i.e. reducing calorie intake by 20–50%), starvation, or fasting (both entailing the abstinence from all food, but not water) in short or prolonged intervals (prolonged fasting, PF), have been investigated in all exiting model organisms from yeast to human. Indeed, it has been demonstrated that starvation/fasting slows down the ageing process and extends life expectancy, from yeast to mice [112-114]. Increased life span has a reciprocal effect on incidence of cancer, as well as other age-related diseases like diabetes and neuro-degeneration [114-117]. After years of investigations on the basis of starvation, dietary interventions that are more practical and safer than fasting, once applied to humans, were needed in order to provide a feasible alternative especially for elderly, frail individuals. Therefore, a fasting mimicking diet (FMD),

low in calories, sugars, and protein but high in unsaturated fats, has been developed and investigated for its beneficial effects [118]. This periodic diet has been shown to retain the beneficial effects of prolonged fasting, prolong healthspan, by promoting multisystem regeneration and delayed neoplasm-related death in mice. In humans, cycles of a 5-day FMD are safe, feasible, and have been successful in reducing risk factors for aging and age-related diseases [119].

2.3.3 Molecular mechanism of fasting in health

The underlying molecular mechanism, with which fasting delivers various benefits, is highly complex due to its vast acting profile. The first studies that began to illuminate various areas, took advantage of the simplest eukaryotic model organism, the yeast *S.cerevisiae* and revealed two main nutrient sensing pathways as key players in prolonging replicative life span (i.e. the number of created daughter cells form a single mother cell) as well as chronological life span (i.e. the survival of a population of resting, non dividing cells). One pathway includes the axis between target of rapamycin (TOR) and the serine-threonine kinase Sch9, which is the homologous version of the human protein kinase B (PKB), also known as Akt. This axis is sensitive to amino acid availability in yeast and reduction in their signalling prolonged both types of lifespan [120-125]. The second pathway involves Ras, adenylate cyclase (AC), and protein kinase A (PKA) axis. Down-regulation of Ras signalling allows transcriptional activation of protective enzymes, such as manganese-superoxide dismutase (Mn-SOD) by activation of the homologous master regulators transcription factors Msn2 and Msn4. Although not the only key player, this mechanism seems to be of high

importance as superoxide levels increase with age in the model organism and are reduced in long-lived mutants deficient in Ras-AC-PKA or Tor-Sch9 signalling. [126-128]. The important role of these pathways in mediating an anti-aging effect, has been confirmed in other model organisms such as worms, flies, rodents and, importantly, humans [129]. In more developed organisms, nutrient sensing pathways are more complex. Signalling transducing receptors in worms [130] and flies demonstrated a connection to their homologous versions in rodents: Insulin receptor (IR) and the IGF-I receptor (IGF1R), that are necessary to exert the anti-aging effects demonstrated in lower organism and that are highly conserved in humans. IRs and IGF1Rs initiate a ligand-dependent activation signalling cascade network that leads to activation of a broad spectrum of pathways, including the Ras/MAPK (mitogen-activated protein kinase) pathway, that drive cell cycle progression, inhibition of apoptosis via the PI3K-Akt/PKB axis, stimulation of glucose uptake via Akt mediated expression of Glut4 transporter and stimulation of protein synthesis via the Akt/mTOR (mammalian TOR) axis. Furthermore, it has been demonstrated that mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r) show growth deficiency and can die soon after birth [131]. Overwhelming evidence suggests that IGF1R signalling, known to promote cell growth and proliferation, is a major effector of the positive benefits derived from fasting. In fact, applying fasting mimicking condition to human cell lines, *in vitro* or directly on humans, the benefits are strongly connected to the reduction of blood glucose, circulating insulin and insulin-like growth factor-1 (IGF-1) levels, while ketone bodies and insulin-like growth factor binding protein 1 (IGFBP-1) levels are increased [114,119,132]. A direct

correlation of reduced IGF-I levels and lower cancer incidence becomes clear when looking at individuals with the Laron syndrome (hereditary dwarfism): a mutation in the Growth Hormone Receptor (GHR) interrupts functional Growth Hormone (GH) signalling, thereby lowering secretion of IGF-I by the liver, the main source of circulating IGF-I. These individuals show significantly less development of cancer or cancer-related death compared to the healthy cohort [133]. Importantly, uncontrolled IGF1R signalling has been connected with the onset of many types of cancer, including lung, prostate, and breast cancer [134,135]. Increased IGF1R activity has been related to poor outcome in breast but also other cancers [136].

Periodical cycles of starvation have the potential to support a healthy lifestyle in order to lower the risk or postpone the incidence of cancer development due to reduction of age-related risk factors. Still, the disease cannot be exterminated. Once malignant cells start to grow, they should by definition loose their dependence to external growth signalling [137].

2.3.4 Molecular mechanism of fasting in disease

Currently, cancer treatments generally comprise of the targeting and killing of malignant cells, mostly through chemotherapy, which needs to be limited in dose density and efficacy, due to its severe side effects. There remains a need for more effective and less stressful treatments affording patients a more humane therapy and recovery as relapse almost invariably occurs with a more aggressive pathology. Surprisingly, fasting also has beneficial effects for the healthy tissues of the body. Unspecific targeting of proliferating cells with chemotherapy, these drugs exert their side effects on tissues that have high turnover rates: bone marrow

immune system, intestine, lung. The healthy part of the body can be protected by applying cycles of fasting. Reduction of glucose and IGF1 lower the IR/IGF1R-signalling in normal cells, while cancer cells should be impacted to a lesser extent by nutrient scarcity. Hence, continuous proliferation renders them the main target of chemotherapeutic drugs. Fasting mimicking conditions *in vitro* as well as prolonged fasting (PF) *in vivo*, have been shown to boost efficacy of chemotherapy treatment in many different cancer types [112]. Fasting's protective effect has been demonstrated in mice, where fasting derived glucose reduction induced the expression of cardio-protecting factors through a pathway that involves PKA, AMP-activated protein kinase (AMPK), and early growth response protein 1 (Egr1) [138]. Fasting also slows down tumor progression in several mouse models [117] against the hypothesis that cancer cells do not react on external availability of nutrient [139]. Growth promoting pathways like the PI3K/Akt/S6K and MAPK-kinase pathways, that are downstream of the IGF-IR, have been found to be blunted even in cancers that have nutrient restricting interventions [113,140]. Another way through which these dietary interventions exert their anti-tumor effect involves anti-cancer activity of the immune system. Heme-Oxygenase 1 (HO-1), a key-player in the antioxidant defence, is down-regulated in cancer cells upon cycles of low nutrient uptake *in vivo*, and playing an important role in supporting immune system-mediated tumor cytotoxicity [141]. Playing also an important role in suppression of apoptosis or senescence, it could be hypothesized whether blunting IGF1R signalling might lead to release of pro-apoptotic events in cancer cells, which remains unclear.

2.4 The aim of the project

2.4.1 Why could a fasting mimicking diet abolish resistance acquisition in ER^+BC?

ER^+BC is efficiently controlled with ET until resistance develops. The SERD Fulvestrant, blunts efficiently genomic as well as non-genomic signalling of the ER, while gene expression remains unaffected. Cross talk with other growth-promoting pathways like the IR/IGF1R pathway, have shown to contribute to development of resistance. Our FMD has been demonstrated to improve chemotherapeutic treatment of other cancers while bringing beneficial effects to the healthy part of the body through its vast acting potential. In this work, a first approach aimed at the investigation of the combination of Fulvestrant with our FMD for its potential to overcome the development of resistance. Concomitant targeting of the ER signalling and the IGF1R signalling at the onset of the cancer, should suppress outgrowth of subpopulations. Beside the double targeting of these two interconnected pathways, the proliferation slowing effect of fasting should impede accumulation of mutational hits that could promote the creation of subpopulations capable to escape specific treatments. And concomitant long-term treatment including our diet could even meliorate patient's life quality. Development of endocrine resistance has been connected to up-regulation of Cyclin D1 and particularily to Fulvestrant treatment has been connected to an up-regulated CDK6 pathway, and addition of Palbociclib has demonstrated clinical success while still being well tolerated when combined with Fulvestrant. In case one specific acting

drug combined with FMD is not sufficient to suppress development of resistance, the addition of Palbociclib was expected to lead to the final goal.

Fasting combined with specifically acting ET drugs simultaneously could augment treatment efficacy and feasibility, improving cancer treatment outcomes while promoting a better quality of life for the patient during treatments.

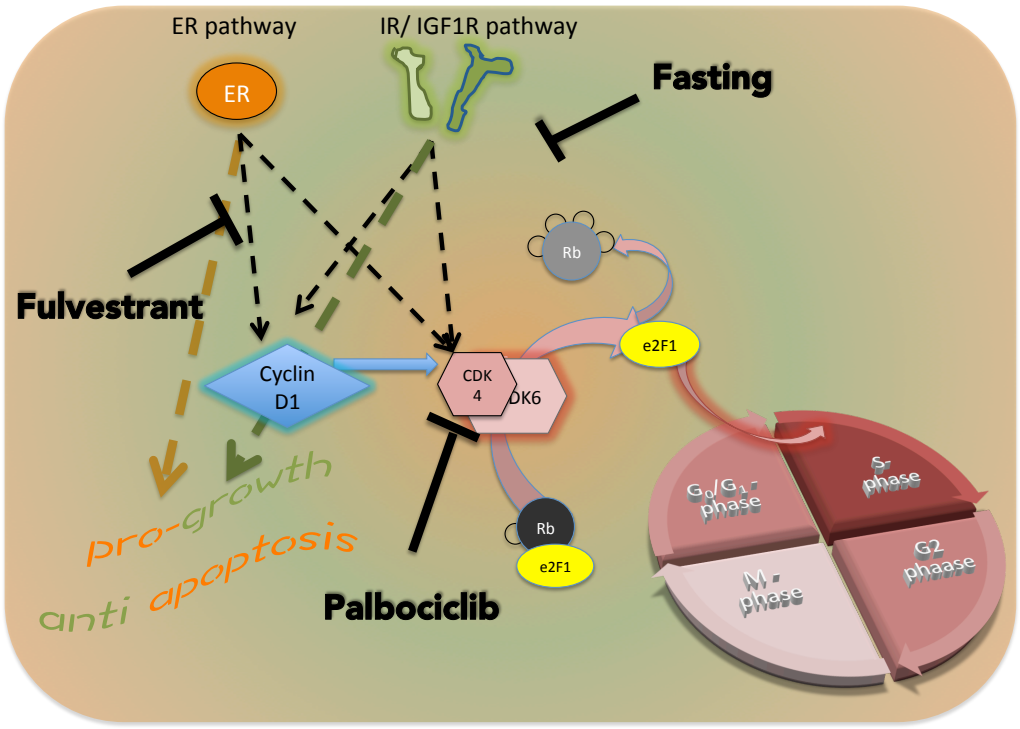

Figure 2-3 Illustration of concomitant targeting of ER$^+$BC

The ER- and the IR/IGF1R signalling trigger distinct and overlapping signalling cascades, creating a solid net to promote proliferation and suppress apoptosis. Concomitant targeting of these two pathways with the specific acting SERD Fulvestrant and the broad acting FMD should blunt uncontrolled proliferation stimuli more effective in ER dependent subpopulations but also target other subpopulations. Up-regulated or amplified Cyclin D1 expression as well as up-regulation of CDK6 can support resistance acquisition in ER$^+$BC, eventually being present already at the onset of tumor formation. Addition of Palbociclib to the concomitant treatment should close the gap and prevent resistance acquisition.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 General materials

Reagent	Supplier	Code
Gram's Cristal violet	VWR	1.09218.0500
DMEM high glucose	Life Technologies	32430-100
DMEM no glucose	Life Technologies	A14430-01
FCS	Sigma	F7524
Glucose solution	Sigma	G8769
Glutamine	Microtec	X-0550
h IGF-I	Peprotec	100-11
h Insulin	Sigma	11376497001
Hepes	Sigma	H4034
NEAA	Biowest	X0557-100
Nitrocellulose Blotting Membran	GE Healthcare	10600001

Muse® count and Viability Kit	Merck Millipore	MCH600103
Paraformaldehyde Solution (4%)	Himedia	TCL-119
PI	Sigma	P4864
pre-casted gradient Acrylamide Gels	Bio Rad	5671094
RNAse A	Invitrogen	12091-021
Trypsin solution	Microtech	L0940
Xylene	Carlo Erba	n.a.
DMEM/F-12	Thermo Fisher	11320-033
Trypsin 10x	Life Technologies	5090-046
Myltenyl MS Colums	Miltenyl Biotec	130-042-201
Hyaluronidase TypeIV	Sigma	H4272
Collagenase TypeI	Sigma	C2674
EpCam (CD326) beads	Miltenyl Biotec	130-061-101
Red Blood Cell Lysis	Sigma	R-7757

Buffer

Table 1 List of general reagents

3.1.2 Drugs

For *in vitro* experiments Fulvestrant (Selleckchem) was dissolved in DMSO. Palbociclib (Selleckchem) was dissolved in H_2O, all aliquots were stored at -20°C for 6 months. For *in vivo* studies Fulvestrant was purchased from Astra Zeneca, and administered as delivered, Palbociclib was purchased from MedChem Express (HY-50767A), dissolved in sterile drinking water under sonication and warming to 37°C and stored at -80°C for maximum of 6 months.

3.1.3 Antibodies Western Blot and IHC

Antibodies used for Western Blot analysis were purchased either from Cell Signaling (Akt #9272; CDK4 #12790; CDK6 #D4S8S; ERα D8H8 #8644 for *in vitro*) IGF1R #9750; IR #3025; S6 #2317; ph(Ser235/236) S6 #4858; mTOR #2983; ph(Ser2448) mTOR #5536;) or from Sigma (Vinculin #V9131) or from Santa Cruz(ERα-F10 #sc8002 for tumor masses). IGF1R #3027 for IHC analysis was purchased from Cell Signaling. Protein Ladders were either Novex® Sharp Pre-stained protein standard (Life Technologies) or Precision Plus Protein™ Dual Color Standard (Bio Rad).

3.1.4 TaqMan® assays for RT-PCR

All TaqMan® assays were purchased from Thermo Fisher Scientific.

Gene	Assay Code
18S	Hs99999901_s1
RPLP0	Hs99999902_m1
TFF1	Hs00907239_m1
PGR	hs00172183_m1
ESR1	hs00174860_m1
CCND1	hs00277039_m1
E2F1	hs00153451_m1
CCNA2	hs00153138_m1
FOXM1	Hs01073586_m1

Table 2 List of TaqMan assays used for RT-PCR

3.1.5 Cell lines and media

MCF7 cells derive from the NCI60 panel and have been maintained in Dulbecco's modified Eagle medium (DMEM) containing 4.5g/L glucose (Thermo Fisher) and supplemented with 10% fetal calf serum (FCS), 5% non-essential aminoacids (NEAA) and 25mM Hepes. Experiments were performed in freshly prepared medium, mimicking physiological levels of glucose and other serum factors of mice fed ad libitum or starved for 72h[132]. That means that DMEM w/o glucose, w/o phenol red, was supplemented always with 5% NEAA 5% glutamine (Gln) and 25mM Hepes. For short-term starvation (STS) conditions medium was additionally

supplemented with 0.5g/L glucose and 1% FCS, for normal (CTR) conditions with 1g/L glucose and 10% FCS.

3.2 METHODS

3.2.1 *In vitro* experiments

For all *in vitro* experiments, similar ratio of cell number and medium volume was kept throughout all experiments in order to guarantee equal drug exposure. Cells were seeded and experiments were only started when small islands of 3 or more cells were present because MCF7 cells, an epithelial cell line, only show continuous growth once islands are formed. Medium was then replaced with freshly prepared CTR or STS medium, after gentle washing with 1x PBS. After 24h 1% FCS was added to STS conditions, followed by drug treatment. If several cycles of STS were applied drug treatment was always carried out 24h after initiation of STS or 12h upon re-feeding. Cells were treated if not otherwise indicated with 0.1μM Fulvestrant and 0.05μM Palbociclib.

3.2.2 Proliferation assay

Proliferation was measured applying Cristal Violet (CV) assay. Cells were seeded in a 96 well plate ($2x10^3$cells/well 50μL medium) and 24 - 48h were given to let cells settle down and form small islands. Once the experiment was started treatment followed the description in 3.2.1. At each time point one plate was taken, cells were washed with 1x PBS, fixed for 10min with 4% PFA, washed with 1x PBS and stained for 1h with 0.1% CV solution. Staining solution was removed and washing was performed with H_2Odd. Plates were kept protected from light until

reagent was solubilized with 10% acetic acid for 30min under agitation and absorption was measured at 595nm with Victor3™ 1420 Multilabel Counter (Perkin Elmer).

3.2.3 Cell death analysis

Cell death was analyzed with the Muse® Count and Viability assay Kit (MerckMillipore). A total of $3x10^4$ cells/well were seeded in 12 well plates (0.8mL of medium). Once the experiment was started treatment followed the description in 3.2.1. At the end of the experiments, cells including supernatant were harvested and treated according to manufactures instructions. Acquisition with the Muse Cell Analyzer allowed elimination of debris and quantification of positively stained cells, which dead or undergoing membrane permeabilization.

3.2.4 Cell cycle analysis

MCF7 cells ($0.4x10^6$) have been seeded in 10 cm dishes (10mL of medium) and treatment was started after 24 – 48h according to the description in 3.2.1. At the end of the experiment, cells including supernatant were harvested and total cell number was evaluated. 2 x10^6 cells were washed twice with cold 1x PBS and subsequently resuspended in 250μL 1x PBS, filtered (70μm filters) and fixed with 750μL ice-cold EtOH absolute. After minimum of one hour incubation at 4°C, cells were washed with 1x PBS + 1% BSA and pellet was resuspended in 1mL PI (50μg/mL) + RNAseA (250μg/mL) and incubated over night at 4°C. Resuspension and filtering just before analysis is suggested as MCF7 cells tend to form aggregates. Stained cells were analysed with the fluorescence associated cell

sorter (FACS) Attune NXT (Life Technologies) and data were analyzed using the program FlowJo v10.

3.2.5 High-resolution imaging

MCF7 cells have been seeded at low densitiy ($3.5x10^4$) in a 6 well plate (2mL medium) and were treated after 24h of STS according to 3.2.1 with half the concentration of Fulvestrant (0.05μM) and Palbociclib (0.025μM) for the first 2 cycles (to keep the ratio of cell number and drug exposure similar), than concentration was rised to standard concentration of 0.1μM Fulvestrant and 0.05mM Palbociclib. Cells growth has been followed by acquisition of mosaic pictures in phase contrast of each well with a Olympus IX81 inverted microscope (camera Hamamatsu ORCA-Flash4.0 16bit) An area of 6022 x 6016 microns (18529 x 18512 pixels with a pixel size of 6.5 μm) has been captured, using an 20x objective. Time-points where: pre 1^{st} cycle (Start), post 1^{st} cycle, post 2^{nd} cycle, post 3^{rd} cycle and post 4^{th} cycle. Five regions of interest (ROI) were chosen using an image from an initial time point, coordinates were kept and used for all subsequent analysis in each treatment condition to. With the program Fiji cell count and covered surface was evaluated and area/cell was calculated.

3.2.6 Western Blot analysis

Briefly, the cells were lysed in modified RIPA buffer containing 50 mM HEPES, 0.3% NP-40, 75 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, and EDTA-free protease inhibitor (Thermo Fisher) and phosphatase inhibitors (Roche) on ice. Protein was quantified via Bradford-assay (Pierce) and a total of 30μg of proteins

were separated using pre-casted or home-made Acrylamid gels and transferred to 0.45μM nitrocellulose membranes (for high molecular weight proteins, i.e. over 150kDa) or 0.2μM nitrocellulose membranes (for low molecular weight proteins or gradient gels) (GE Healthcare) over night. The transferred blot was blocked with 5% non-fat dry milk or 5% BSA for detection of phosphorylated proteins, in 1x TBS containing 0.05% Tween20 (TBST) for 1h hours at RT. Membranes were then immune-blotted with primary antibody at 4°C over night. After washing with TBST (3x, 5min) secondary horseradish peroxidase linked antibody was applied for 1h at RT. After washing (3x, 10min) with TBST specific bindings were detected by a chemiluminescence system (Thermo Scientific).

3.2.7 Quantitative real-time (qPCR or RT-PCR) analysis

Total RNA from cells was isolated using the RNeasy mini kit (QIAGEN), RNA from tumors was extracted with QIAzol Lysis Reagent (Qiagen) according to manufacture's instructions. Subsequently, 0.5 µg was reverse transcribed with random hexamers (High Capacity cDNA Archive kit; Applied Biosystems). cDNA was amplified with the TaqMan Gene Expression assay (Applied Biosystems) and a thermocycler (ABI Prism 7900HT; Thermo Fisher Scientific). For any sample, the expression level, normalized to the housekeeping genes encoding RPLPO and S18 was determined by the comparative threshold cycle method as described previously[142].

3.2.8 IHC staining

Tissues were fixed in 4% PFA over night at 4°C and subsequently transferred to 70% EtOH until paraffin embedded with Diapath automatic processor. For IHC analysis paraffin was removed using xylene (undiluted) and the sections were rehydrated by incubation in descending concentration of EtOH (100%, 95%, 80%, each 2x 3min) then rinsed with H_2Odd. Antigen retrieval was carried out using preheated (95°C) target retrieval solution (Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0)) for 45min. Tissue sections were blocked with FBS in 1x PBS for 60min and incubated over night with primary antibody. The antibody binding was detected using a polymer detection kit (GAR-HRP, Microtech) followed by a diaminobenzidine chromogen reaction (Peroxidase substrate kit, DAB, SK-4100; Vector Lab). All sections were counterstained with Mayer's hematoxylin and visualized using an Olympus bright-field microscope (IUpright BX 51). Images were captured at 10x magnification of a representative area of the tumor.

3.2.9 Isolation of primary tumor cells

Tumors are kept in cold 1x PBS between removal from the animal and digestion (maximum1h). Then tumors are transferred into DMEM/F12 medium (w/o FCS; + Hyaluronidase 100U/mL; + Collagenase 300U/mL) and disintegrated with forceps and scalpel. During incubation for 3h at 37°C suspension was pipetted every 30min to support further disintegration. After centrifugation (5min; 80g), SN was decanted and red blood cells were removed from the pellet by resuspending the pellet in 2mL Red blood Lysis Buffer and incubating for 10min at RT. After

centrifugation (5min; 450g) SN was removed and pellet was resuspended in 3mL Trypsin and incubated for 3min at RT. To stop the reaction, 10mL of 1x PBS with 30% FCS and 100μg/mL DNAse was added and pipetted. After centrifugation (5min; 450g) cells were resuspended in 1x PBS, filtered (70μm filters) and counted. Human MCF7 cells were separated from murine tumor microenvironment with magnetic beads for human epithelial cell selection (EpCAM beads from Miltenyl Biotec) according to manufactures instructions. Subsequently cells were plated in DMEM high glucose Medium. After 5 days of recover, when cell have attached to the plate, drug treatment was started to then amplify resistant MCF7-R cells.

3.2.10 *In vivo* studies

Animals derived from in house colonies, kept for a maximum of 3 generations. Breeders (Charles River Laboratories) and experimental animals have been maintained at IFOM-IEO Campus animal facility under specific pathogen-free conditions. $5x10^6$ parental MCF7 cells or $3x10^6$ resistant MCF7 (MCF7-R) cells have been injected subcutaneously near the mammary fat pad into 6 to 8 week old NSG mice, 2 - 3 days after implanting estrogen releasing pellet (90 day release, 0.36mg, Innovative Research of America). Once tumor size (measured with the formula: a*b*c*0.52) reached 50 – 120 mm^3, mice were divided randomly into different groups and FMD cycles together with treatment was started. Fulvestrant (150mg/kg) was administered 1/week subcutaneously at the second day of FMD at the opposite side of tumor injection. Placebo was prepared using corn oil with 10% EtOH absolute and administered at the same time. Palbociclib HCL

(MedChemExpress) was diluted in sterile drinking water (20mg/mL) and dissolved by warming and sonication. Mice were administered via oral gavage 62.5mg/kg of Palbociclib suspension at the 1st, 4th day of the FMD and 2 days upon re-feeding. Mice have been continuously monitored and new rounds were only started if bodyweight was recovered and physical status was stable. Once tumor size reached minimum 400mm^3 or started to ulcerate, animals have been sacrificed and masses were taken for IHC or western blot analysis. Mice showing signs of severe stress, deteriorating health status, or excess tumor load was designated as moribund and euthanized.

3.2.11 Animal housing

Experimental animals have been maintained at IFOM-IEO Campus animal facility under specific pathogen-free conditions. All experiments were performed in accordance with the Italian Laws (D.lgs. 26/2014), which enforce Directive 2010/63/EU (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes). Accordingly, the project has been authorized by the Italian Competent Authority (Ministry of Health) with Authorization # 16/16 PR. Mice are housed and taken care of accordingly to the guidelines set out in Annex III of Directive 2010/63/EU. At the end of experiments, mice will be euthanized by inhalation of CO_2. This method is considered an appropriate method of killing for rodents, as indicated in table 3, Annex IV of Directive 2010/63/EU.

3.2.12 Diet

Mice were fed ad libitum with irradiated TD.7912 rodent chow (Harlan Teklad) containing 15.69 kJ/g of digestible energy (3.92 kJ/g animal-based protein, 9.1 kJ/g carbohydrate, 2.67 kJ/g fat). The FMD is based on a nutritional screen that identified ingredients that allow nourishment during periods of low calorie consumption [143]. The FMD consists of two different components designated as day 1 diet and day 2–4 diet that were fed in this respective order. The day 1 diet consists of a mix of various low-calorie broth powders, a vegetable medley powder, extra virgin olive oil, and essential fatty acids; day 2–4 diet consist of low-calorie broth powders and glycerol. Both formulations were then substituted with hydrogel (Clear H2O) to achieve binding and to allow the supply of the food in the cage feeders. Day 1 diet contains 7.67 kJ/g (provided at ~50% of normal daily intake; 0.46 kJ/g protein, 2.2 kJ/g carbohydrate, 5.00 kJ/g fat); the day 2–4 diet is identical on all feeding days and contains 1.48 kJ/g (provided at ~10% of normal daily intake; 0.01 kJ/g protein/fat, 1.47 kJ/g carbohydrates).

3.2.13 Statistical analysis

To determine statistical significance *in vivo*, Two-way ANOVA (multi-comparison) was applied. For *in vitro* experiments student's t-test (one-tiailed or two-tailed, paired) was used and p-value was indicated as follows (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

4 RESULTS

4.1 Concomitant targeting of ER$^+$BC with Fulvestrant and STS/ FMD

4.1.1 STS augments cytostaticity of Fulvestrant on MCF7 cells *in vitro*

Fulvestrant has been shown to reduce viability in ERα positive cells *in vitro* [144,145] and controls ER$^+$BC progression in patients, until inevitable resistance develops. Also STS decreases viability of several cancer cells *in vitro* and slows tumor progression *in vivo* [117]. Acting on two different pro-growth pathways, that are highly interconnected but can also drive independently cancer cell proliferation, we wondered whether concomitant blockage of these pathways, could increase or at least maintain Fulvestrants effect. To this aim MCF7 cells, the most common model for ER$^+$BC, were grown constantly in CTR medium or underwent cycles of STS (interrupted by 48h growth in CTR medium). Treatment was done with the standard concentration to investigate Fulvestrant resistance *in vitro* [146] and proliferation was followed over 6 days until cells in CTR conditions reached 90% confluence.

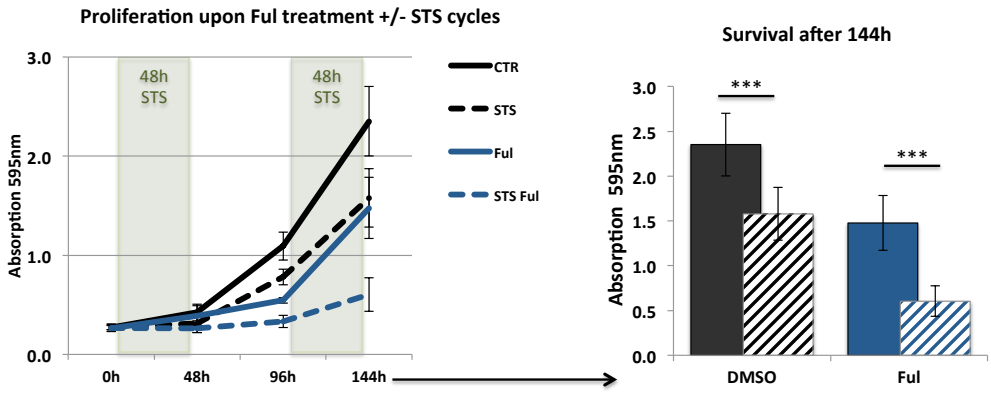

Figure 4-1 STS augments cytostaticity of Fulvestrant on MCF7 cells *in vitro*

MCF7 cells were grown in 96 well plates (six multiplicates) in CTR medium +/- cycles of STS (interrupted by 48h growth in CTR medium) and treated with Fulvestrant [0.01μM]. Fulvestrant was added after the first 24h of STS or 12h upon re-feeding to mimick physiological conditions. At each time point a plate was stained with Cristal Violet. Experiments were stopped once the untreated CTR reached 90% confluence. Data are represented as average ± StDEV, significance was calculated using student's one-tail t-test (*P<0.05, **P<0.01, ***P<0.001).

As expected, STS as well as Fulvestrant slow proliferation of MCF7 cells over a period of 6 days. If combined, this effect is increased as cell proliferation is nearly halted over 96h. During the last 48h treated cells in CTR medium +/- Ful and STS conditions show an increased proliferation rate.

4.1.2 STS augments cytotoxicity of Fulvestrant on MCF7 cells *in vitro*

To see whether reduced viability results from cytotoxicity, we analyzed cell death upon one and three cycles of STS with the automated Muse™ Cell Analyzer. Cycles of STS were like previously, interrupted by 48h in CTR medium (re-feeding).

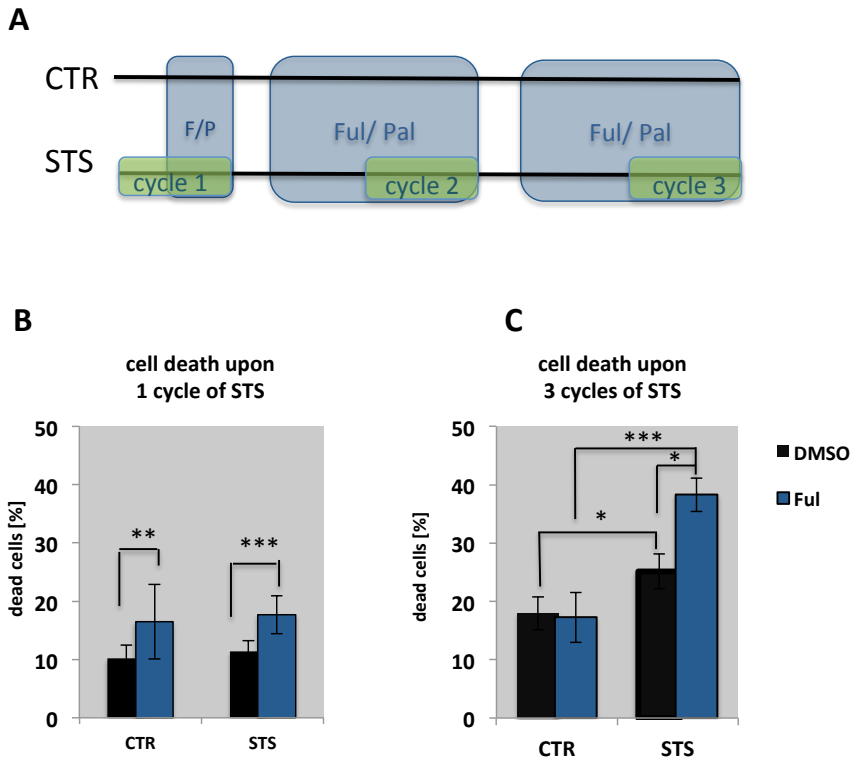

Figure 4-2 STS augments cytotoxicity of Fulvestrant on MCF7 cells *in vitro*

MCF7 cells, were seeded in 12 well plates (triplicates) and grown constantly in CTR medium or underwent cycles of 48h STS (interrupted by 48h growth in CTR medium) and were treated with Fulvestrant [0.1μM]. Fulvestrant was added after the first 24h of STS or 12h upon re-feeding to mimick physiological conditions. **A** Treatment scheme **B** Cell death analysis at the end of one cycle of STS and **C** at the end of the third cycle of STS with the Muse™ Cell Analyzer. Data of 3 independent experiments (A) and two independent experimetns (B) are represented as average ± StDEV, significance was calculated using student's one-tailed t-test (*P<0.05, **P<0.01, ***P<0.001).

STS alone does not show an increase in cell death on MCF7 cells compared to CTR conditions, while Fulvestrant under CTR conditions and more so under STS conditions significantly increases cell death. As we aim at abolishing resistance acquisition, which has been reported to evolve after some weeks of drug exposure, MCF7 cells were then cultured for 8 days under drug exposure and challenged three times with STS. Cells grown in CTR medium did not show

differences in cell death anymore, while STS alone but more so in combination with Fulvestrant, had the potential to increase cell death significantly.

4.1.3 STS plus Fulvestrant are most successful in dampening expression of the ER and IGF1R signalling components

It is proven that Fulvestrant targets specifically the ER leading to its proteasomal degradation, thereby lowering drastically genomic and non-genomic action. As mRNA levels do not seem to correlate with the decrease in protein level in patients [147], there might persist a low but stable amount of ER protein, which is not occupied by Fulvestrant. And ER molecules that are occupied by the drug, which binds to the LBD in the AF2 region, are still free at their AF1 region, that can interact with proteins from other pro-growth pathways, for example mTOR and p70S6K [62]. These proteins execute key events in the IR/IGF1R signalling cascade and can be blunted with STS. Hence STS plus Fulvestrant might be more efficient in reducing both, the ER signalling as well as IGF1R signalling. We therefore looked at the protein level of key players in the IGF1R signalling cascade, the ER and expression of two target genes (Progesteron receptor and trefoil factor 1). Further analyses on other key players have been carried out by a collaborating group and are not shown here.

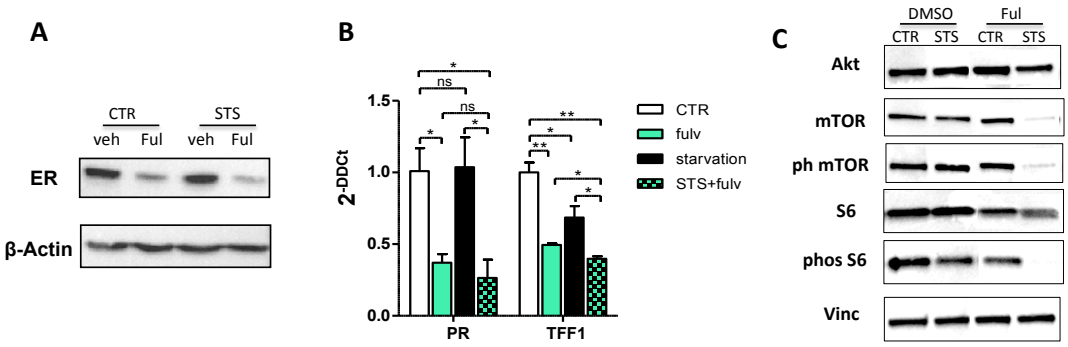

Figure 4-3 STS plus Fulvestrant are most successful in dampening ER and IGF1R signalling MCF7 cells were grown in CTR or STS medium for 48h and treated for the last 24h with 1μM Fulvestrant or vehicle. **A** Cells were harvested and lysed for WB analysis of the full length ER protein, and in **C** IGF1R signalling executors (Akt, mTOR, S6) **B** From cells treated the same way, total RNA was extracted for real-time PCR analysis on ER downstream targets (PR and TFF1). Expression levels were compared to the house-keeping gene β-actin and calculated using the comparative threshold cycle method. Experiments were repeated three independent times (except WB anlaysis of Akt, TOR and S6) and representative WB's are shown. Gene expression is represented as Mean ± SE and significance was calculated using student's t-test (*P<0.05, **P<0.01, ***P<0.001). **A** and **B** was carried out by the collaborative group of Prof. A. Nencioni.

Figure 3 shows that Fulvestrant alone down-regulates the ER protein and the expression of both downstream targets. STS plus Fulvestrant increased down-regulation of the ER protein and to a little but significant amount expression of *TFF1*. While neither STS nor Fulvestrant alone had obvious effects on lowering total Akt or phosphorylation of mTOR, phosphorylation of S6 is reduced (**Figure 4-3** C). Impressively the combined treatment leads to a lowered level of total Akt, almost complete down-regulation of total and phosphorylated mTOR and completely abolished S6 phosphorylation.

4.1.4 FMD postpones resistance acquisition to Fulvestrant *in vivo*

We wanted to test immediately the *in vivo* potential of our FMD in combination with Fulvestrant. Fasting has been shown to increase the anti-tumor effect of chemotherapy while protecting normal cells *in vitro* and *in vivo* [112,117] and not

impeding the health status of patients undergoing harmful chemotherapy [148]. A fasting mimicking diet (FMD) has been shown to have the same potential [118,138], while being easier applicable in patients. Due to beneficial side effects, we applied both treatments (Fulvestrant and cycles of FMD) concomitantly and over a long period. For our xenograft models, severe immune-compromised mice (NOD SCID gamma) were chosen, being the best option for long-term experiments due to lowest incidence of immune system leakage (i.e. reactivation of parts of the immune defence) and injected them with MCF7 cells. Once masses reached the size of a minimum of 50mm^3, mice were randomly assigned to four groups and challenged either with Placebo or Fulvestrant and constantly fed ad libitum (ad lib) or underwent weekly cycles of FMD with a break every fifth week.

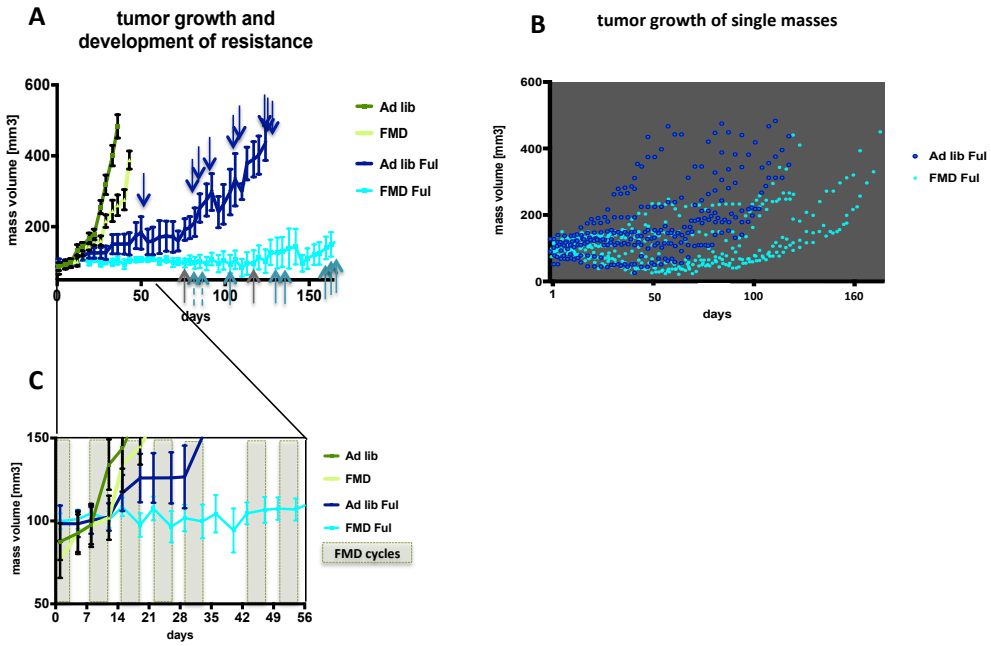

Figure 4-4 FMD postpones resistance acquisition to Fulvestrant *in vivo*

NSG mice had been supplemented with an estrogen-releasing pellet and were injected three days later with 5x10^6 MCF7 cells near the mammary fat pad. After 2 weeks, masses that reached a volume of 50 - 130mm^2 were randomly divided into 4 groups: ad lib (n=9), FMD (n=10), ad lib Ful (n=9) and FMD Ful (n=10). Mice underwent weekly cycles of FMD with a break every fifth week

and were treated subcutaneously at the second day of the diet with 150mg/kg Fulvestrant or Palcebo. Masses that increased in size approximately 4 times were considered resistant and sacrificed. Animals showing not tumor related health problems or ulcerated masses were sacrificed (2-4 mice/group). **A** Tumor growth over time is presented as mean ±SEM. FMD cycles are graphically not represented. Dark and light blue arrows indicate sacrifices of resistant mice (responsible for dropping of the average), grey arrows indicate sacrifices of mice that had tumors still at the initial size. **B** Dot Plot of single masses of the two Fulvestrant treated groups to visualize delayed resistance acquisition of masses that showed initially better response. **C** Tumor shrinkage upon the first 5 cycles of FMD.

The sole application of cycles of FMD delayed tumor growth of our ER^+BC model as it has been reported also for several other tumor models. Treatment with Fulvestrant alone controlled in most cases (8 out of 9) tumor growth over a period of at least 60 days, then masses started to become insensitive to the treatment at different time points. By day 125 every single tumor treated only with Fulvestrant had reached a size four times bigger than in the beginning and mice have been sacrificed. Cycles of FMD could postpone this effect. By day 60 no tumor had developed resistance. Around day 70 three mice had to be sacrificed for welfare reasons (post mortem analysis revealed kidney deformation) and of these three mice, two had tumor masses, which had almost doubled in size. Compared to ad libitum conditions, 5 mice developed resistance after day 125, where all ad libitum fed mice already had become insensitive. However, resistance could not be abolished in a single case. Supporting our *in vitro* data, that showed cell death (**Figure 4-2**), some single masses showed a decrease in size at least at the initial stage, which becomes clear in **Figure 4-4**B, where some light blue dots go down and **Figure 4-4**C were average tumor size declines during every cycle until day 40, indicating potentially occurrence of cell death. Masses that dropped in size initially

developed resistance notably later than other masses in the FMD group, revealing in some cases FMDs potential to postponing tumor growth.

4.1.5 Resistance to FMD plus Fulvestrant *in vivo* might be connected to CDK6 up-regulation

If contemporary targeting of the ER- and IGF1R- signalling still allows for an adaption, a possible alternative could lay downstream of these pathways, for example in the control of the cell cycle machinery, that is often miss-regulated in ER^+BC. In fact, Fulvestrant resistance has been connected to CDK6 up-regulation and efficiency of the combination of Fulvestrant plus Palbociclib, a highly selective CDK4/6 inhibitor, has led to recent approval for treatment of metastatic breast cancer, as the combination further postpones progression free survival. We therefore looked at the protein levels of ER and IGF1R as well as CDK4 and CDK6 in tumor masses of each group before resistance acquisition (day 20) and upon resistance acquisition (endpoint).

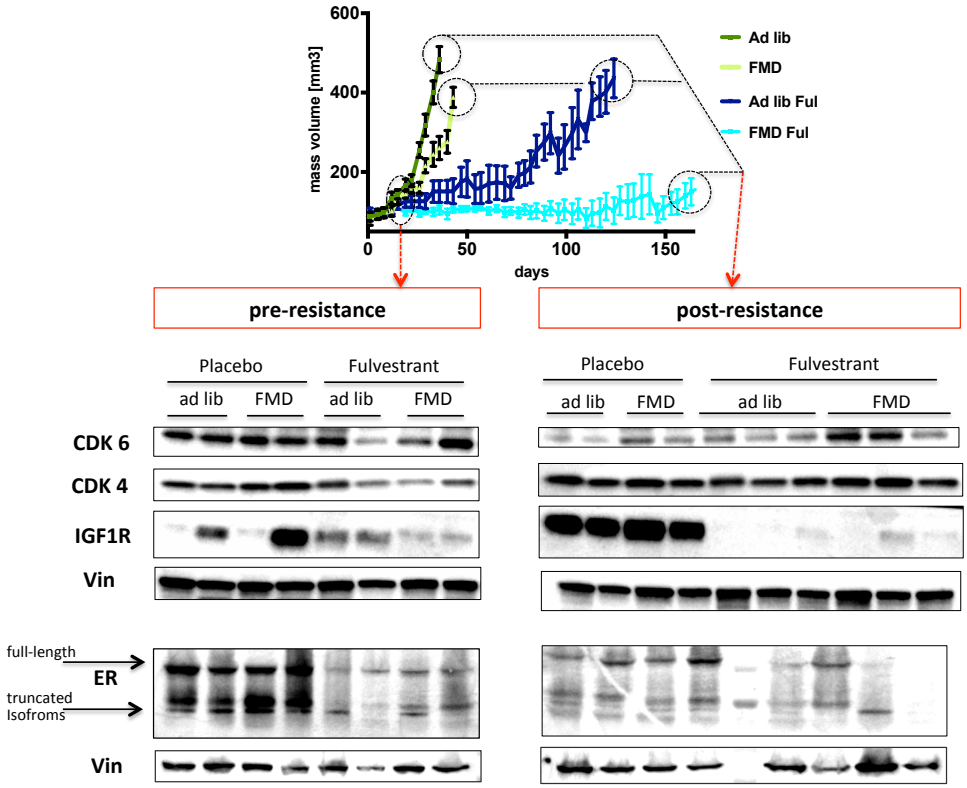

Figure 4-5 Resistance to Fulvestrant treatment *in vivo* shows up-regulation of CDK6

Western blot analysis from flash frozen tumors treated for 20 days (pre-resistance) or until endpoint was reached (post resistance/endpoint). As masses have been collected at different time points, ER protein was only evaluated on two masses resistant to Ful and Ful + FMD, while all other proteins were analyzed on three resistant masses. Expression is compared to the house-keeping gene Vinculin (Vin).

As expected, ER expression (including shorter isoforms) is predominant at the initial stage (day 20) of pharmaceutically untreated tumors (ad lib, FMD), being the driving force or ER^+BC. Fulvestrant leads to its down-regulation already after 20 days, independent of additional dietary interventions. Once untreated mice reach the endpoint, ER expression is less evident compared to the initial stage in all groups. Tumors that have developed resistance to Fulvestrant show still blunted ER expression and in combination with FMD this down-regulation is much more prevalent (considering the overexpressed Vinculin in the second last line) as ER

protein (including shorter isoforms) seems almost lost. In pharmaceutically untreated mice IGF1R expression becomes more dominant over time, while Fulvestrant and FMD plus Fulvestrant treated mice show almost no IGF1R upon resistance acquisition. Along with tumor progression in untreated mice, CDK6 seems to play subordinate role as less expressed at late stage of pharmaceutically untreated mice. CDK4 levels are maintained or slightly up-regulated. Mice treated with Fulvestrant show at an early stage variable CDK4 and CDK6 expression. In each of the two groups (Fulvestrant ad Fulvestrant plus FMD) 1 out of 2 masses shows reduced CDK4 levels, while CDK6 levels are lower only in one mass treated with Fulvestrant under ad lib fed conditions. All three masses resistant to Fulvestrant (endpoint) and constantly fed ad libitum, show low levels of CDK6 but maintained levels of CDK4. Interestingly, in two out of 3 cases where FMD plus Fulvestrant was applied a striking CDK6 up-regulation can be observed, accompanied with a slight increase in CDK4.

As addition of cycles of FMD provided a better outcome in resistance acquisition to Fulvestrant and showed in two out of three cases up-regulation of CDK6 which has been connected to resistance acquisition upon Fulvestrant treatment in patients [100], we decided to test clinically efficient Fulvestrant plus Palbociclib treatment in combination with FMD. In this way the potential resistance driving CDK4/6 complex, could be blocked and close a fundamental gap. Contrarily to the recent approval of subsequent application of Fulvestrant plus Palbociclib on ER^+BC that has developed resistance to previous ET, we want to challenge the cancer concomitantly with all three treatments. Thereby we aimed at the targeting of

different subpopulations in the heterogeneous population to avoid the outgrowth of resistant cells.

4.2 Concomitant targeting of ER$^+$BC with Fulvestrant, STS/ FMD and low dose Palbociclic

4.2.1 STS sensitizes to low dose Palbociclib *in vitro*

As mentioned before, for *in vitro* experiments, Fulvestrant was used in at the standard concentration to investigate resistance acquisition, while Palbociclib had to be tested for a concentration at such a low level where effects were low in CTR medium and hopefully still efficient when combined with STS. Especially because future *in vivo* experiments would not allow medication at the maximum tolerated dose due to combination with other treatments. Being a cell cycle inhibitor, no cell death was expected, which is why a proliferation assay was used to determine the lowest efficient concentration. MCF7 cells were grown in 96 well plates in CTR medium +/- cycles of STS +/- Palbociclib at concentrations ranging from 0.05μM to 1μM. STS cycles were interrupted by growth in CTR medium (re-feeding). Cell cycle arrest, hence slowed proliferation should only be observed above 0.5μM (tested by selleckchem), we hoped to lower the needed concentration applying STS.

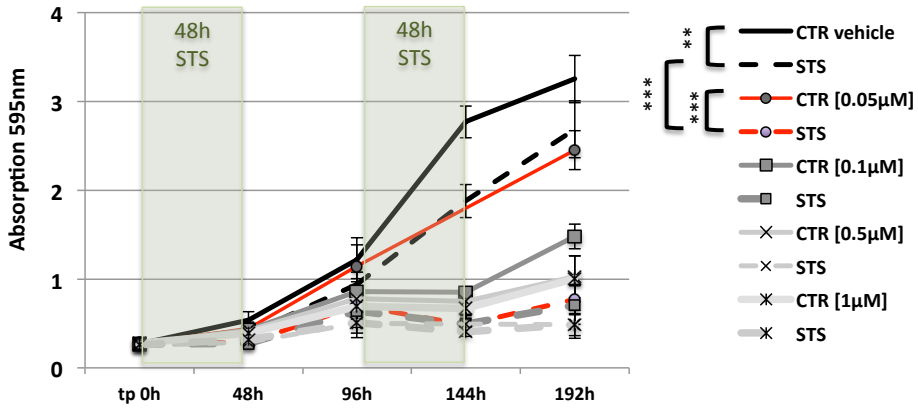

Figure 4-6 STS sensitizes to low dose Palbociclib *in vitro*

MCF7 cells were grown in 96 well plates (six multiplicates) in CTR medium +/- cycles of STS and different concentration of Palbocilib. Repeated STS cycles were interrupted by 48h of re-feeding in CTR medium. Palbociclib was added after the first 24h of STS or 12h upon re-feeding to mimic physiological conditions. At each time point a plate was stained with Cristal Violet. Data are represented as average ± StDEV and significance was calculated using student's one-tail t-test (*P<0.05, **P<0.01, ***P<0.001).

Two cycles of STS alone resulted in a significant growth retarding effect of MCF7 cells over a period of one week. As expected, Palbociclib at a concentration of 0.5μM and 1μM slowed proliferation evidentially in CTR conditions. STS could augment this effect therefore only minimally. A concentration of as low as 0.05μM, blunted the effect of Palbociclib in CTR conditions while under STS conditions growth was still halted to great extent. Cell cycle analysis of MCF7 cells treated with 0.05μM Palbociclib after 1 and after 3 cycles of STS can confirm these observations, revealing a possible arrest in the G_1-pahse of the cell cycle.

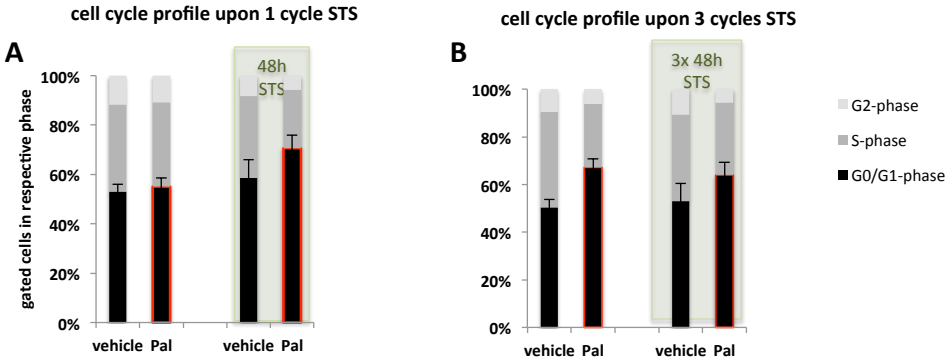

Figure 4-7 Los dose Palbociclib leads to G_1-arrest of MCF7 cells *in vitro*
MCF7 cells were seeded in 10 cm plates and treated with one or three cycles of STS, interrupted by 48h of re-feeding and treated with 0.05μM Palbocilib after 24h of STS or 12h of re-feeding. At the end cells including supernatant were harvested and prepared for cell cycle analysis. Experiments have been repeated twice, data are represented as average ± StDEV.

Low dose Palbociclib leads to an increase of MCF7 cells in G_1-phase if starved for 48h, while treatment in CTR medium does not. G_1-arrest is induced in CTR conditions and slightly decreased in STS conditions when treatment goes on for 10 days.

4.2.2 Low dose Palbociclib augments cytostaticity of Fulvestrant in CTR conditions

Whether addition of low dose Palbociclib could further increase halted proliferation by Fulvestrant plus STS (especially after 96h) was analyzed through a proliferation assay. Like in previous experiments, MCF7 cells were grown in CTR medium or periodically STS medium and were treated +/- Fulvestrant [0.1μM] or Fulvestrant plus Palbociclib [0.1μM + 0.05μM]. Cycles of STS were interrupted by 48h of re-feeding in CTR medium. The experiment was stopped when the cells in untreated CTR medium reached 90% confluence.

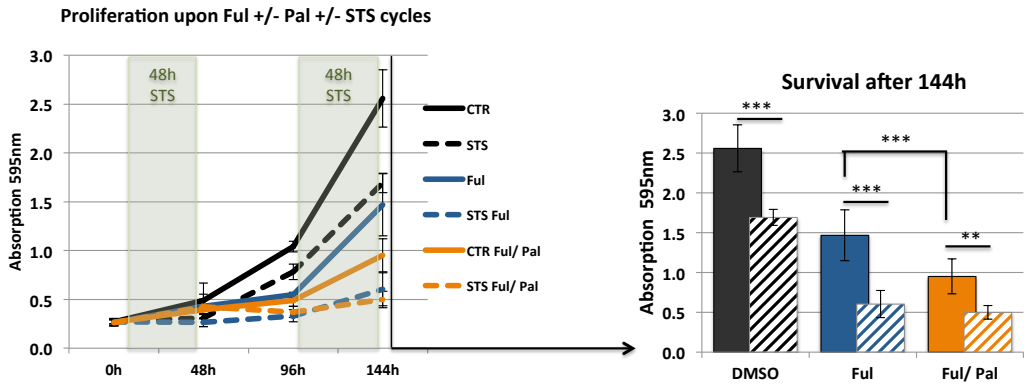

Figure 4-8 Low dose Palbociclib augments cytostaticity of Fulvestrant in CTR conditions
MCF7 cells have been grown in 96 well plates (six multiplicates) in CTR medium +/- cycles of STS (interrupted by 48h growth in CTR medium) and treated with Fulvestrant [0.01μM] and/or Palbociclib [0.05μM]. At each time point a plate was stained with Cristal Violet. Data are represented as average ± StDEV and significance was calculated using student's one-tail t-test (*P<0.05, **P<0.01, ***P<0.001).

Similar to **Figure 4-1**, STS leads to a constantly lowered proliferation of MCF7 cells and Fulvestrant retards proliferation to a similar extend with a slight higher impact. Addition of Palbociclib in CTR medium again increases the effect significantly. Under STS conditions, drugs work significantly more efficient compared to their CTR's but addition of Palbociclib to Fulvestrant and STS does not seem to impact over 144h, which does not exclude an effect over longer periods, as the inhibitor per se seems to affect cell proliferation in CTR conditions.

4.2.3 Immediate cytotoxicity of STS plus Fulvestrant can be further increased with low dose Palbociclib

Nonetheless Palbociclib is expected to act cytostatic, a possible cytotoxic effect of contemporary treatment with Fulvestrant and Palbociclib under STS conditions was analyzed upon 1 and 3 cycles of STS.

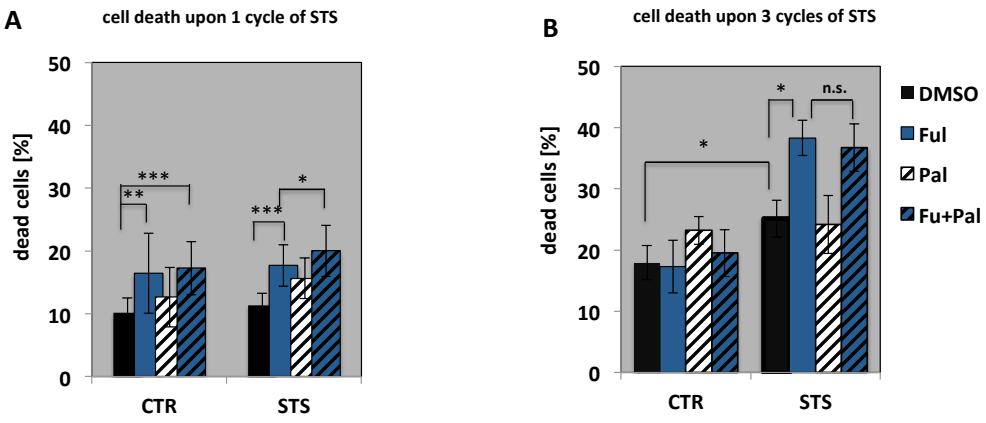

Figure 4-9 Cycles of STS augment cytotoxicity of Fulvestrant on MCF7 cells *in vitro*
MCF7 cells, were seeded in 12 well plates (triplicates) and grown in CTR medium or underwent cycles of 48h STS (interrupted by 48h growth in CTR medium) and were treated with Fulvestrant [0.1μM], Palbociclib [0.05μM] or both drugs (Ful [0.1μM] + Pal [0.05μM]). Drugs were added after the first 24h of STS or 12h upon re-feeding to mimic physiological conditions A Cell death analysis at the end of one cycle of STS and B at the end of the third cycle of STS. Data of 3 independent experiments are represented as average ± StDEV and significance was calculated using student's one-tailed t-test (*P<0.05, **P<0.01, ***P<0.001).

Within the first 48h, addition of Palbociclib to Fulvestrant treatment increased significance of the treatment under CTR conditions, compared to Fulvestrant treatment alone. Under STS conditions the already observed increase in cell death by Fulvestrant (**Figure 4-2**) can be significantly increased by addition of low dose Palbociclib. Upon three cycles of STS (total treatment of 10 days) addition of Palbociclib to Fulvestrant under STS conditions does not further increase this cytotoxicity at that stage.

4.2.4 Greatest down-regulation of both, ER- and Rb-pathway is achieved only with Palbociclib plus Fulvestrant under STS conditions

Addition of low dose Palbociclib to the combination of Fulvestrant plus STS could not significantly augment halted proliferation (**Figure 4-8**) and cell death induction

only upon 48h of treatment (**Figure 4-9**). We aim though at abolishing resistance acquisition, which occurs over long-term. Hence, changes on a molecular basis might be different upon addition of Palbociclib and could help to validate the potential. We showed that combined Fulvestrant plus STS treatment blunts most efficiently the ER and its transcriptional function and only combined, components of the IGF1R signalling pathway (**Figure 2-3**). Literature as well as our *in vivo* data (**Figure 4-5**) support the role of up-regulated CDK4/6, hence up-regulated Rb pathway as a possible escape route. Low dose Palbociclib, could be sufficient in blunting the CDK4/6 – Rb axis, if contemporary treatment with Fulvestrant and STS lead to a supportive down-regulation of Cyclin D1, a target gene of the ER as well as controlled by mitogen stimuli, and direct stimulator of CDK4/6 complex. We checked therefore gene expression down-stream of the ER and the Rb-pathway upon 10 days of treatment with and without cycles of STS.

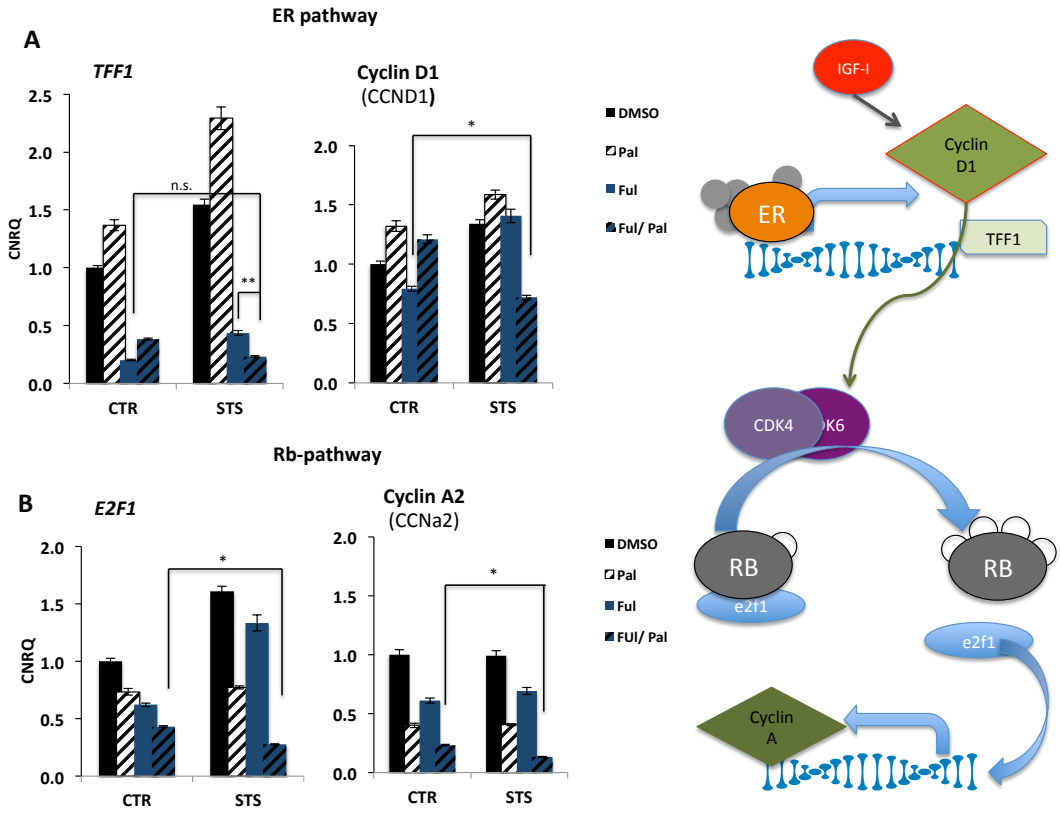

Figure 4-10 Greatest down-regulation of ER and Rb-pathway is achieved with Palbociclib plus Fulvestrant under STS conditions

MCF7 cells were grown in 6 well plates in CTR medium +/- three cycles of STS (48h), interrupted by 48h of re-feeding in CTR medium and treated with vehicle, Palbociclib [0.05μM], Fulvestrant [0.1μM] or the combination of the two. Total RNA was extracted and 0.5μg were reverse transcribed to for real-time PCR analysis on the selected genes. **A** *TFF1* (trefoil factor 1) and *CCND1* (Cyclin D1) as target genes of the ER. **B** *E2F1* (transcription factor e2f1) as an executor of the Rb-pathway; *CCNa2* (Cyclin A2) as target gene of e2f1. Experiments (n=1) was done in technical triplicates, data are presented as mean ±SE, significance is calculated on technical triplicates with student's two tailed t-test (*P<0.05, **P<0.01, ***P<0.001).

Even though STS augments the immediate ER down-regulation especially when cells are treated with Fulvestrant (**Figure 4-3**), 3 cycles of STS seem to induce the opposite effect if looking at the two analyzed target genes (**Figure 4-10**). *TFF1* and *Cyclin D1* expression is increased when cells undergo cycles of STS or under any condition with Palbociclib. Comparing STS plus Fulvestrant to CTR plus

Fulvestrant we see again an up-regulation even if still overall lower expression compared to CTR. Interestingly, addition of low dose Palbociclib can revert this effect by down-regulating particularly under STS conditions both genes, *Cyclin D1* significantly. STS alone and combined with Fulvestrant leads to an up-regulation of *E2F1*, while Palbociclib alone slightly down-regulates the gene, as expected, independent of STS. Expression of a downstream target of E2F1, *Cyclin A* is unchanged upon cycles of STS and slightly down-regulated upon Fulvestrant in CTR and STS conditions. The addition of low dose Palbociclib lead to the greatest down-regulation of both genes especially if combined with STS.

4.2.5 Fulvestrant plus Palbociclib and cycles of STS lead to a great down-regulation of *FOXM1* in MCF7 cells *in vitro*

Based on morphological observation of dishes that were analyzed in previous experiments, a cytosolic expansion of cells treated with Fulvestrant was observable. The ratio between normal sized cells and large cells seemed to increase under STS conditions or if Fulvestrant was combined with Palbociclib and most evident in plates undergoing triple treatment. A large, flat cytosolic area is a recognized marker of senescent cells. If this is the case at least one of two crucial pathways known for their role in senescence induction should be affected: p53/p21 pathway, usually activated by a constant DNA damage response (DDR) or the $p16^{INK4a}$/pRb pathway (where Rb activity is inhibited) usually induced by other stress factors [149]. Recently down-regulation of *FOMX1* induced by blockage of Rb-pathway using Palbociclib, has been connected to senescence [150]. Because in our treatment conditions proliferation was overall halted, and should therefore reduce

possible DNA damage occurring during S-phase, the p53/p21 pathway was not expected to trigger this phenomenon at first sight. MCF7 cells carry a deletion in the *p16*^{INK4a} gene, so we looked directly at the expression levels *FoXM1* in cells that have previously shown down-regulation of ER- and Rb- pathway (**Figure 4-10**).

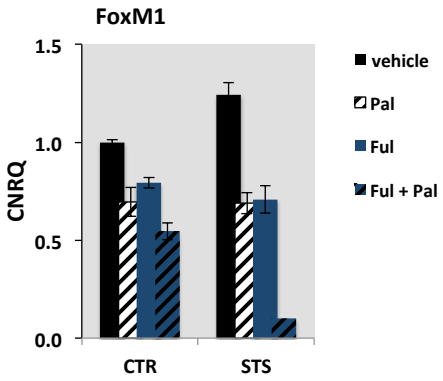

Figure 4-11 Only Fulvestrant plus low dose Palbociclib and cycles of STS greatly down-regulate *FOXM1 in vitro*

MCF7 cells were grown in 6 well plates in CTR medium and underwent three cyles of STS (48h), interrupted by 48h of re-feeding in CTR medium and treated with vehicle, Palbociclib [0.05μM], Fulvestrant [0.1μM] or the combination of the two. Total RNA was extracted and 0.5μg were reverse transcribed to for real-time PCR on the *FOXM1*. Experiment has been done once in technical triplicates, data are represented as mean ±SE.

Low dose Palbociclib, directly acting on blockage of Rb-pathway, down-regulates *FOXM1*, independent of STS. Fulvestrant's effect is similar, while double treatment reveals a better potential in suppression. Interestingly STS, which alone up-regulates *FOXM1* has a striking potential to induce the opposite effect if combined with Palbocicilib and Fulvestrant.

4.2.6 Low dose Palbociclib reveals its potential to augment STS plus Fulvestrant treatment if treated over longer-term *in vitro*

To visualize morphological changes, cell growth was followed with high-resolution mosaic images that always captured the same area of a 6 well plate, over four cycles of STS. Cells treated with vehicle or low dose Palbociclib reached confluence after the third cycle and are compared to cells treated with Fulvestrant or the combination of Fulvestrant plus Palbociclib after four cycles.

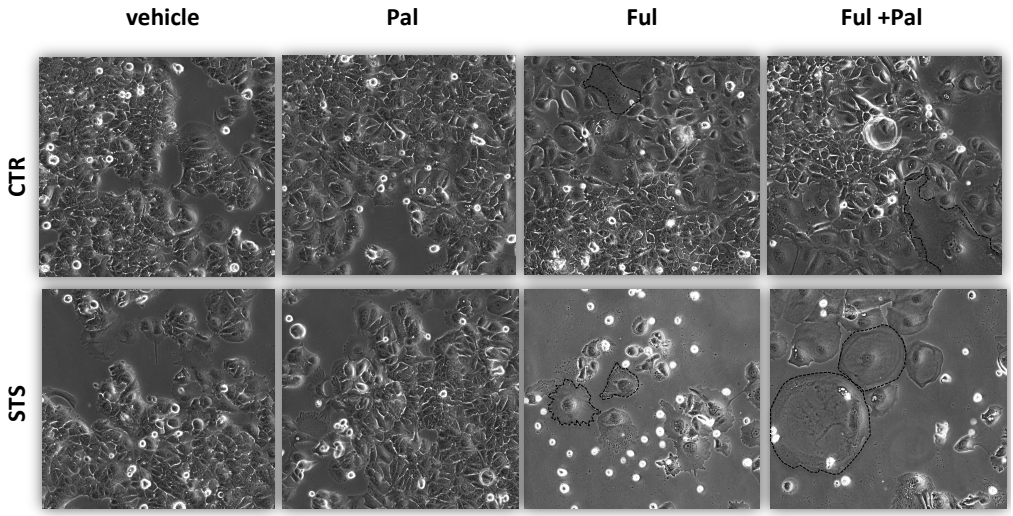

Figure 4-12 Morphological changes in MCF7 cells treated with Fulvestrant and/or Palbocilib and cycles of STS *in vitro*

MCF7 were grown for several weeks in 6 well plates in CTR medium +/- weekly cycles of STS (48h) and were treated with Fulvestrant [0.1μM], Palbocicilib [0.05μM] or both drugs according to the scheme in **Figure 4-2**. After each cycle high resolution mosaic images (20x magnification) of a total area of 6022 x 6016 microns (18529 x 18512 pixels) were captured in phase contrast. Representative areas of 3000px^2 are shown.

MCF7 cells in CTR medium +/- low dose Palbociclib grow to confluence pretty fast, showing a decreasing single cell size over time due to space becoming limited and the typical growth profile of epithelial cells. STS slows growth by reaching a little lower confluence after 3 cycles, without affecting cell morphology. Fulvestrant

treatment with and without addition of Palbociclib induced in parts of the cell population an increase in the cytosolic fraction. These cell population were more evident under STS conditions, but the most in the triple treatment condition, where overall cell number was lower and the ratio between small and large cells more evident. To quantify this increase in cell dimension five regions of interest (ROI's) were chosen blind in one of the first images and coordinates were kept throughout analysis. Cell number and covered surface area was evaluated with the program Fiji and used to calculated the surface covered /cell.

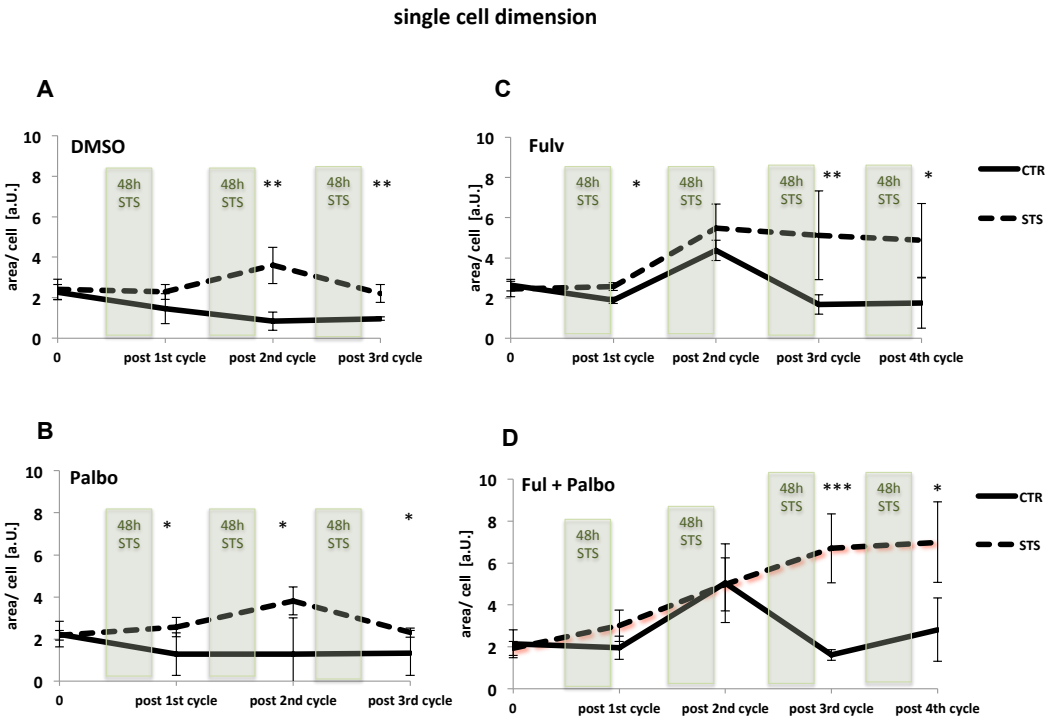

Figure 4-13 Fulvestrant plus Palbociclib and cycles of STS induce a great increase in cell surface area.

Five regions of interest (ROI) were selected randomly in one of the high-resolution mosaic images at the initial time point and coordinates were kept throughout analysis. The ROI's have subsequently been analysis regarding cell number and covered surface area to build a ratio: area/cell for each treatment condition: A vehicle B Palbociclib [0.05μM] C Fulvestrant [0.1μM] D Fulvestrant plus Palbociclib [0.1μM + 0.05μM]

Cells grown in CTR medium decrease in size due to increased confluence and decrease in space. STS has the opposite effect where the cytosolic area increases until the second cycle of STS had passed then cells show a decrease in size ending up almost like cells in CTR medium. The same phenomenon can be observed when cells are treated with low dose Palbociclib. Fulvestrant leads to an increase in cytosolic area over the first two cycles. Then cells in CTR medium show a decrease and end up almost on the same level than CTR cells, while cell under STS conditions maintain on average their larger cytosol. When treatment includes both drugs and cycles of STS, a constant increase in cell dimension can be observed that reached almost 700% of the size of CTR cells after 4 cycles. Considering phase contrast pictures it becomes clear that the amount of cells that stay small and continue to proliferate is lower but not yet eliminated. Parallel to imaging acquisition cells grown in six well plates and treated the same way were used to analyse doubling time. After 23 days, when the first groups reached confluence all cells where harvested and counted to calculated doubling time.

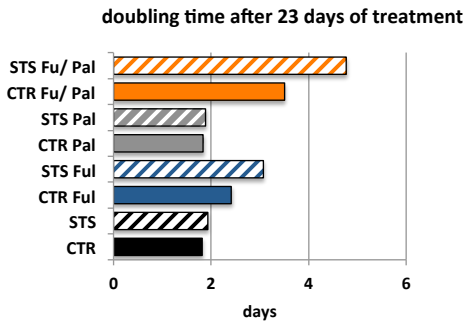

Figure 4-14 Fulvestrant plus Palbociclib and cycles of STS augment doubling time of MCF7 cells *in vitro* the most

MCF7 were grown in CTR medium +/- weekly cycles of STS (48h) and were treated with Fulvestrant [0.1μM], Palbocicilib [0.05μM] or both drugs according to the scheme in **Figure 4-2**. When cells treated with vehicle and Palbociclib reached confluence, all samples were harvested

and counted to calculate doubling time with the formula: ln2*(ln(Xend/Xbeg))*t (x=cell number; t=time). Experiment was done once.

Doubling time was similar among cells in CTR medium or undergoing cycles of STS without treatment or low dose Palbociclib, while Fulvestrant and Fulvestrant plus Palbociclib augmented doubling time. Triple treatment prolonged doubling time the most.

4.2.7 Cycles of FMD show potential to abolish resistance acquisition to combined Palbociclib and Fulvestrant treatment *in vivo*

Even though a triple treatment of low dose Palbociclib, Fulvestrant and cycles of STS did not synergistically blunt proliferation or rise cell death over treatment periods of 10 days, on a molecular level this combination had the most effect in blunting both, the ER- and the Rb- pathway. Before investigating further a possible molecular mechanism, we have been keen to test the combined treatment *in vivo* and repeated the previous experiment (**Figure 4-4**) adding four experimental groups (ad lib Pal, FMD Pal, ad lib Ful+Pal and FMD Ful+Pal). Palbociclib was given alone or in combination with Fulvestrant 3 times per week (before, during and after FMD) at half maximum tolerated dose.

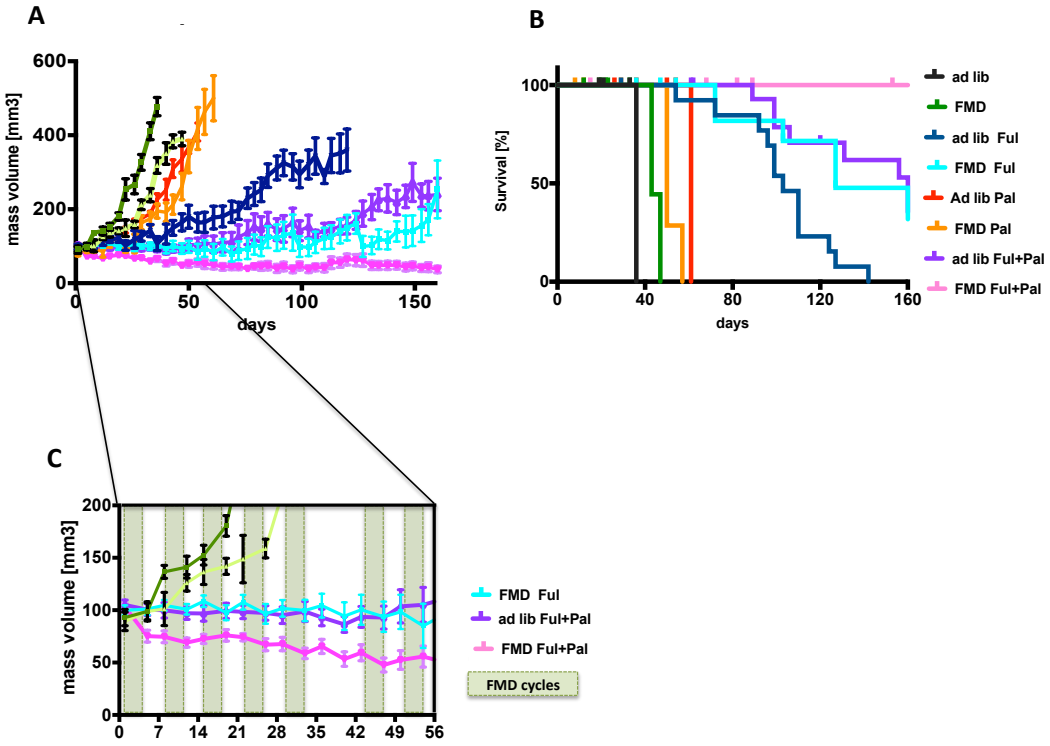

Figure 4-15 Cycles of FMD show potential to abolish resistance acquisition to combined Palbociclib plus Fulvestrant treatment *in vivo*

Experimental procedure like described in **Figure 4-4**: NSG mice have been supplemented with a estrogen-releasing pellet and three days later injected with 5x106 MCF7 cells near the mammary fat pad. After 2 weeks, masses that reached a volume of 50 - 130mm2 were randomly divided into 8 groups: ad lib, FMD, ad lib Ful, FMD Ful, ad lib Pal, FMD Pal, ad lib Ful+Pal, and FMD Ful+Pal. Mice underwent weekly cycles of FMD with a break every fifth week and were treated subcutaneously at the second day of the diet with 150mg/kg Fulvestrant or Palcebo and at the first and forth day of the FMD and 2 days after refeeding with 62.5mg/kg Palbociclib via oral gavage. Masses that increased in size at least and are inicated in Fig 13C A Tumor growth of all mice (incl. the ones from first experiment) over time is presented as mean ±SEM. FMD cycles are graphically not represented. B Survival curve; dropping line indicates death/sacrifice due to resistance acquisition while outreaching symbols indicte death/sacrifice due to health reasons C tumor shrinkage upon the first 5 cycles of FMD in the newly added groups compared to FMD plus Fulvetrant. Total number of animals: ad lib (total n=15), FMD (total n=15), ad lib Ful (total n=16) and FMD Ful (total n=16), ad lib Pal (n=15) FMD Pal (n=10); ad lib Ful + Pal (n=18) and FMD Ful + Pal (n=18)

While cycles of FMD have the potential to postpone overall resistance acquisition to Fulvestrant (**Figure 4-4**), the addition of Palbociclib 3 times a week could

abolish the phenomenon completely over a period of 160 days. Palbociclib was administered at such low dosis, that its sole administration had only minor effect on tumor progression. The combination of Fulvestrant and Palbociclib under ad libitum fed conditions showed a resistance postponement with a similar profile than Fulvestrant and FMD. At the end of the experiment (day 160) in five mice tumor volume has increased 2 to 3 times. Interestingly the triple treatment augmented the overall decrease in tumor size at very early stage (**Figure 4-15**C) to a greater extent as Fulvestrant plus FMD.

These results have been completed recently, which is why analysis on tumor masses from the triple treatment group has not been carried out yet. Another clinically important question arose during the on-going *in vivo* experiment and has been investigated. As only FMD showed potential to induce cell death (represented by a decrease in tumor size) on Fulvestrant or more so Fulvestrant plus Palbociclib treated masses, we wondered whether FMD could also act on masses that have already developed resistance to both drugs under ad libitum condition. Many patients are under therapy at this moment and cannot benefit anymore from synchronous triple treatment from at an early stage.

4.2.8 FMD has the potential to reverse ER$^+$BC resistance to combined Fulvestrant plus Palbociclib treatment *in vivo*

We tested this potential of FMD initially applying two cycles of FMD on just 2 mice that have developed resistance to combined treatment (**Figure 4-15** A) and as tumor size indeed decreased, we isolated cells from a third resistant tumor, amplified them *in vitro* (called MCF7-R) under continuous drug exposure (**Figure**

4-15 B) and re-injected them in NSG mice. Once small masses have formed combined treatment was applied and when tumor size increased, mice were randomly divided into two groups (ad lib and FMD) and cycles of FMD were applied.

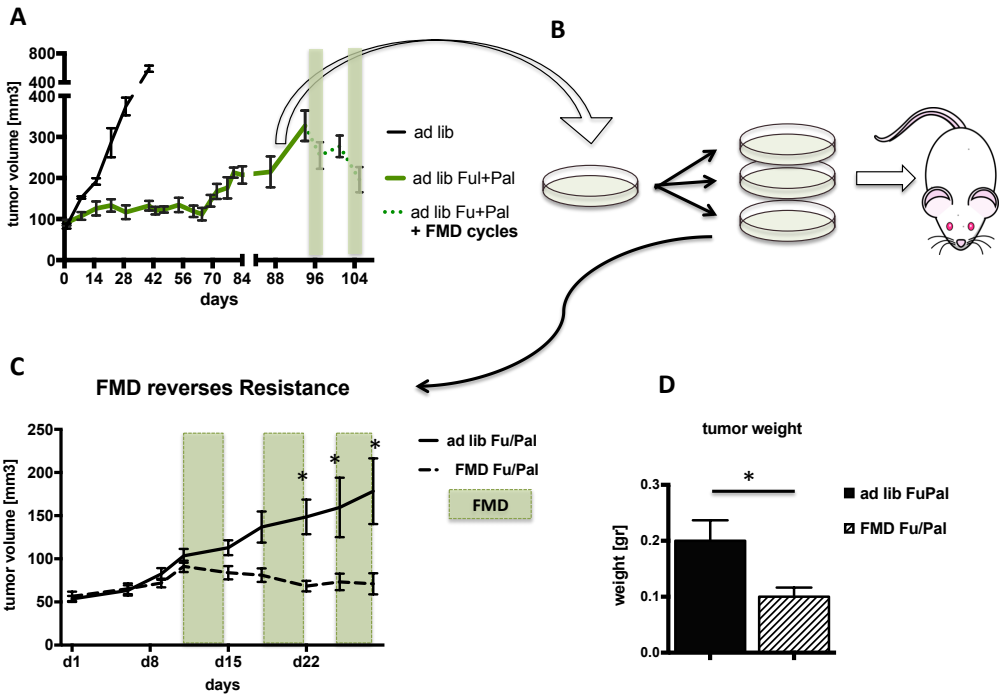

Figure 4-16 FMD reverses ER$^+$BC resistance to combined Fulvestrant plus Palbociclib treatment *in vivo*

A Two NSG mice that developed resistance to combined Fulvestrant plus Palbociclib treatment during the previous experiment, where subjected to two cycles of FMD. A third resistant mass served as donor for resistant cells that have been in **B** amplified in vitro under continuous drug exposure. **C** NSG mice have been supplemented with an estrogen-releasing pellet and three days later injected with 3x10^6 MCF7-R cells near the mammary fat pad on both flanks. When masses reached a volume of 50mm^2 treatment with Fulvestrant [150mg/kg] and Palbociclib [62.5mg/kg] was started like in previous experiments (**Figure 4-15**). Masses that showed continuous growth regardless the treatment were divided into two groups (n=4 each). CTR group was continued fed ad libitum, while the other group started to undergo cycles of FMD. **D** After three cycles mice have been sacrificed and tumors were checked for weight. Data are represented as mean ±SEM; significance was calculated using 2wayANOVA in **C** and unpaired t-test with Welch's correction in 14D (*P<0.05, **P<0.01, ***P<0.001)

With *ex vivo* amplified MCF7-R cells sensitivity to FMD cycles *in vivo* could be repeated on a bigger number of animals. Eight mice that showed immediate resistance to double treatment were divided into two groups and mice that underwent three cycles of FMD showed decreasing tumor masses (**Figure 4-16 C**). From the second cycle on, this difference in size was significant and could be confirmed by tumor weight (**Figure 4-16 D**) when mice were sacrificed. We started to analyze these masses via Western Blot while future experiments aim at depicturing the entire expression landscape through RNA sequencing analysis. Due to the great response to FMD cycles we supposed a re-sensitization of tumors to Insulin/IGF1 signalling especially because ER-signalling as well as the Rb-pathway should be blunted by the pharmaceutics. So we looked at protein levels of ER, IGF1R and IR in four tumor masses.

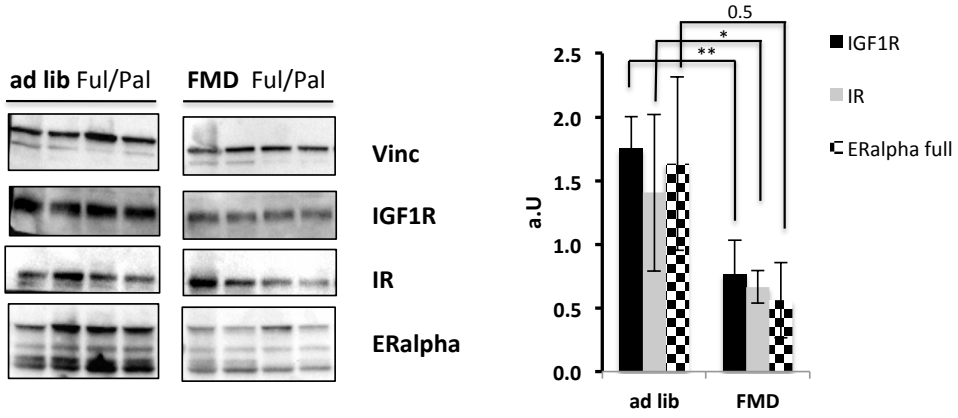

Figure 4-17 Down-regulation of IGF1R and IR in resistant tumors treted with FMD
IGF1R, IR and ER of four tumor masses per group were analyzed on protein level via Western Blot. Relative expression was calculated with Image J. Data are represented as Average ± StDEV, significance was calculated using student's one-tailed t-test (*P<0.05, **P<0.01, ***P<0.001).

Elevated levels of IR, IGF1R and ER (full length and shorter isoforms) in masses resistant to Fulvestrant plus Palbociclib treatment could be down-regulated upon

cycles of FMD. This effect was significant for IR and IGF1R, while close to significance regarding the ER. As Fulvestrant treatment alone almost did not show any expression of IGF1R in the previous experiment (**Figure 4-5**), we tested via IHC analysis, whether disappearance and reactivation of IGF1R correlated.

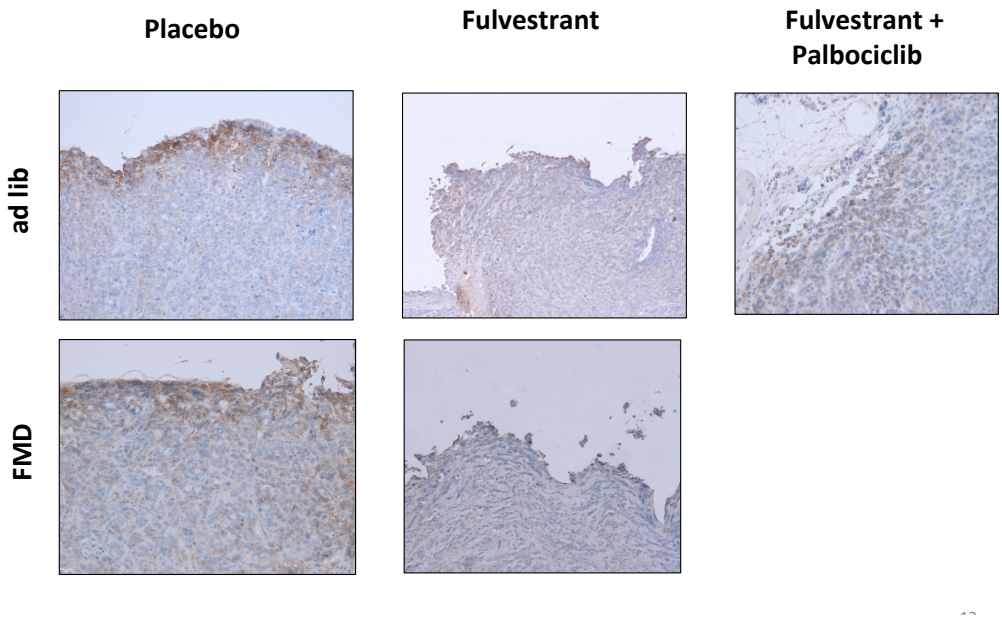

Figure 4-18 Fulvestrant treatment depletes IGF1R that is reactivated upon addition of Palbociclib *in vivo*

Tumor masses (n=2/group) from mice reaching the endpoint have been collected and analyzed immunohistochemically for expression of the IGF1R. Representative pictures (magnification 10x) are shown.

IHC analysis confirmed that IGF1R is not present in Fulvestrant treated tumors independent of FMD, while addition of Palbociclib under ad libitum fed conditions re-activates the receptor. Masses undergoing triple treatment were not collected for IHC analysis, but entirely kept to investigate protein and RNA level. As the reactivation of IR/IGF1R hinted at a dependence on Insulin/IGF-I, we just started to test *in vitro* on the same clone that has been injected, whether STS had a

greater effect on MCF7-R than on parental MCF7 cells and if addition of Insulin and IGF-I could abolish the effect.

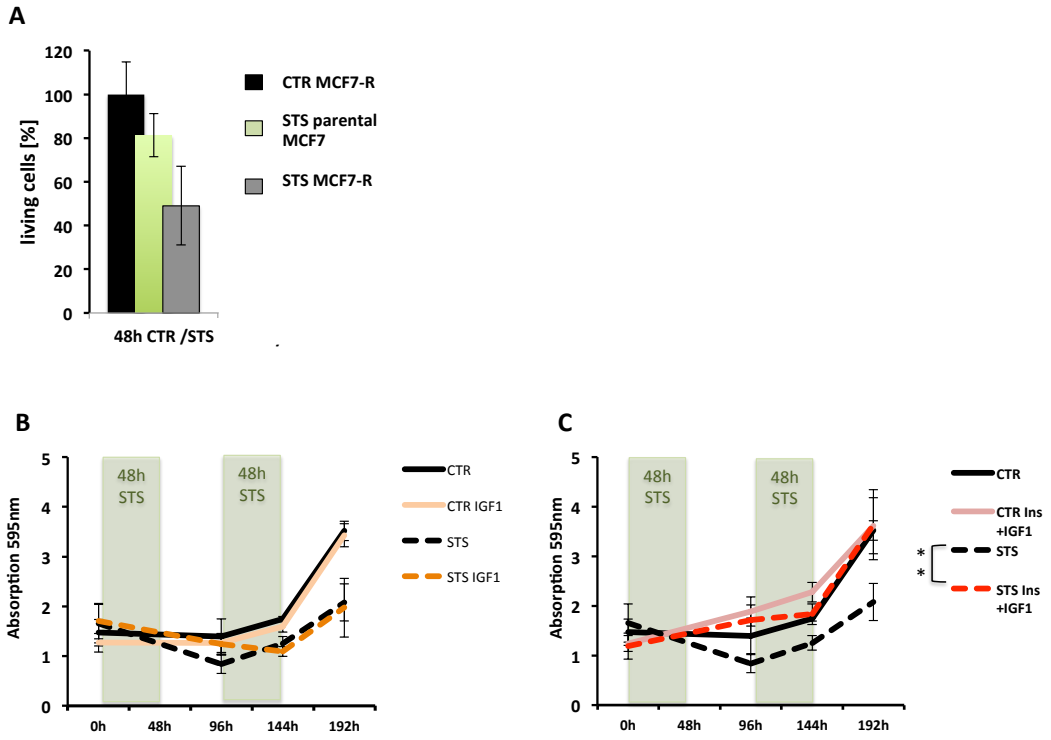

Figure 4-19 IGF-I plus Insulin can abolish STS induced growth retardation of MCF7-R cells
A MCF7-R cells were seeded in 12 well plates (triplicates), and upon attachment in CTR or STS medium, supplemented with Fulvestrant [0.1μm] and Palbociclib [0.05μm]. After 48h cell were harvested and anlayzed with the Muse™ Cell Analyzer. Data are presented as average ±StDEV. Survival is compared to parental MCF 7 cultured in STS medium only. **B** and **C** MCF7-R cells were seeded in 96 well plates (six multiplicates) and grown in CTR medium or underwent cycles of STS (48h) interrupted by 48h of re-feeding. Medium was supplemented constantly with Fulvestrant [0.1μm] and Palbociclib [0.05μm]. Addition of physiological concentrations of Insulin (5ng/mL) and/or IGF-I (5ng/mL) occurred during STS. Experiment was carried out once, data are represented as average ±StDEV, significance is calculated on six technical replicates using student's one-tailed t-test (*P<0.05, **P<0.01, ***P<0.001).

MCF7-R cells show lower survival upon 48h of STS than parental MCF7 cells (**Figure 4-19** A). Analyzing proliferation a decrease of the line implies occurring cell death within the first 96h (**Figure 4-19** B and C), which support *in vivo* observation but remains to be proven on the *in vitro* model. Addition of only IGF-I

could not rescue the reduced proliferation (**Figure 4-19** B), while addition of Insulin and IGF-I at physiological concentrations abolished the effect of STS completely.

5 DISCUSSION

5.1.1 FMD postpones resistance acquisition of ER$^+$BC to Fulvestrant

We first aimed at abolishing resistance acquisition to the innovative and effective SERD Fulvestrant, that is a pure anti-estrogen and has no estrogenic activity like the most commonly used SERM Tamoxifen, and up to date no known side effects.

Our *in vitro* data showed that proliferation can be most efficiently halted (**Figure 4-1**) and cell death mostly increased (**Figure 4-2**) when Fulvestrant is combined with cycles of STS. An evident increase in the proliferation rate of Fulvestrant treated cells after 96h (**Figure 4-1**) could imply an early adaption to *in vitro* treatment. Many studies on MCF7 resistance to Fulvestrant report adaption after several weeks only. It should be noted, that in most of these studies, cells are grown in low serum conditions, which are similar to our STS medium where proliferation seems almost completely halted even after 96h. Cell death analysis of Fulvestrant treatment in CTR conditions led to an increase in cell death after the first 24h of treatment, but did not show elevated cell death compared to the CTR after 10 days. A technical limit can be responsible for the increase of cell death in the untreated CTR condition from 10% after 1 cycle to 20% after 3 cycles as cells in CTR conditions reach high confluence. The high variability in response to Fulvestrant treatment under CTR conditions can be explained with a variable response of a heterogeneous population, which is lowered by adding STS. Importantly the combined treatment was most efficient. Western Blot analysis (**Figure 4-3**) showed a combined effect of STS plus Fulvestrant in down-regulating

the ER protein. Out of two proteins whose expression is controlled by the ER, one confirmed the additional effect of combined treatment. *TFF1* expression was mostly down-regulated upon combined treatment, while *PR* mRNA levels were mostly down-regulated upon Fulvestrant alone. Protein levels of key components of the IGF1R signalling, that have been connected to endocrine resistance [151] were efficiently blunted under combined treatment (**Figure 4-3**), while single treatment only affected phosphorylation status of S6 (phS6). These data are supported by analysis of our collaborators that have seen also a synergistic down-regulation of phosphorylated p70S6K, phAkt, as well as up-regulation of 4E-BP1, a negative effector of the eukaryotic translation initiation factor 4 (data not shown). Based on these observations, concomitant targeting of the ER- and IR/IGF1R signalling seems more efficient in targeting MCF7 cells *in vitro* than Fulvestrant alone. Even though Fulvestrant leads to degradation of the ER, expression of the gene (*ESR1*) is not influenced [147]. Hence, a low but stable amount of the ER protein, which is or is not occupied by Fulvestrant at the LBD in the AF1 region, persists within the time window between translation and degradation. This low amount of ER can still trigger through its AF2 region genomic and non-genomic signalling and thereby promoting proliferation [62] until cells have found a more proficient way to escape this suppression. Downstream targets of the IR/IGF1R signalling pathway, such as Akt, mTOR or p70S6K can in turn still activate remaining ER [62]. Therefore concomitant blunting of both pathways might be capable to reduce cross activity to a minimum. To further prove this hypothesis, we need to look at expression of genes regulated by the IR/IGF1R axis and

phosphorylation status of the ER specifically in the N-terminal A/B domain, where action of the AF1 region is regulated.

Based on these results we wanted to test the combination soon in the context of an organism, as *in vitro* mechanism can easily miss-guide. The subsequent analysis on our xenograft model proved the potential of FMD to postpone resistance acquisition to Fulvestrant and most interestingly showed in some cases an initial tumor size decrease, a phenomenon that is rarely found in published studies. Hence, FMD not only slowed tumor growth (which has been demonstrated in several mouse models) but in our case also showed potential to kill a subpopulation (**Figure 4-4** C), which is supported by *in vitro* data in **Figure 4-2**. Tumors that decreased in size at an early stage, developed resistance to combined treatment as last (**Figure 4-4** B), implying that FMD killed specifically part of the resistance driving cells. Heterogeneity of the tumor might be responsible for resistance development at different time points and the fact that not all tumors show a strong reaction to the combined treatment. Initial analysis on tumor masses showed an increase of the IGF1R in pharmaceutically untreated mice upon tumor progression, thereby underlining the importance of the IGF1R signalling in onset and progression of the disease. ER expression in contrast was down-regulated, a phenomenon that has been observed also in patients diagnosed with ER^+BC and showing invasive status [152]. However resistance to Fulvestrant and FMD plus Fulvestrant treatment was what we focused on and the ER was efficiently down-regulated upon Fulvestrant treatment and almost disappeared when FMD was added (**Figure 4-5**), confirming our *in vitro* data.

IGF1R expression was evidently on the lowest level once masses were resistant to Fulvestrant or FMD plus Fulvestrant pointing towards a different pathway being responsible for tumor progression at that stage. Studies on resistant MCF7 cells and more importantly on patients show that resistance to specifically Fulvestrant treatment (but not Tamoxifen treatment) can arise through CDK6 up-regulation [100]. We recall here again the fact that *in vitro* resistance to Fulvestrant is generated by growth in low serum medium (1% FCS), which is similar to our STS condition that in turn mimics FMD conditions *in vivo*. Data on patient studies are reported disregarding diet. Importantly we could appreciate an up-regulation of CDK6 in two out of three tumors resistant to FMD plus Fulvestrant, accompanied by a slight increase of CDK4. Recently a very selective CDK4/6 inhibitor, named Palbociclib, has been approved for combined treatment with Fulvestrant upon resistance development to previous ET, prolonging progression free survival (PFS). We therefore next tested combined treatment of FMD, Fulvestrant and Palbociclib, targeting contrarily to clinical application the tumor at an early stage.

5.1.2 FMD has potential to abolish resistance acquisition to combined Fulvestrant plus low dose Palbociclib treatment

Applying three treatments synchronously, Palbociclib, which is more harmful to healthy tissues than Fulvestrant or FMD, was administered at the half maximum dose *in vivo*. Due to the long timeline we started the xenograft experiment while trying to look for a possible mechanism *in vitro*. A concentration that had low effect in CTR medium, while maintaining the effect under STS conditions was evaluated and maintained throughout subsequent experiments. Proliferation analysis (**Figure**

4-6) showed a bigger effect of low dose Palbociclib under STS conditions than in CTR medium. Cell cycle analysis (**Figure 4-7**) confirmed the cytostatic potential of Palbociclib in combination with STS within the first 48h, while upon 3 cycles, a slight regression in G_1-arrest implied adaption. Subsequent analysis of the combination of low dose Palbociclib, Fulvestrant and 3 cycles of STS *in vitro* could not prove an increase in halted proliferation or cell death compared to STS plus Fulvestrant (**Figure 4-8** and **Figure 4-9**). To evaluate the potential of the CDK inhibitor better, investigations of long-term treated cells could be helpful. Gene expression analysis was used to evaluate an additive effect at early stage, as molecular changes often occur sooner than cellular effects. To this aim, we started to look at gene expression of key players in the ER and Rb pathways. While TFF1 is assumed, to be regulated only by the ER, *Cyclin D1* expression is additionally influenced by the extracellular matrix (ECM), soluble growth factor stimuli [153,154], and TFs other than the ER. With IGF-I, being among the mitotic stimuli that influence *Cyclin D1* expression, Fulvestrant plus STS was expected to impact more on down-regulating *Cyclin D1* expression than Fulvestrant treatment alone, allowing low dose Palbociclib to blunt under these circumstances the Rb pathway. **Figure 4-10** shows that Fulvestrant plus STS reversed the Fulvestrant induced reduction of *Cyclin D1* mRNA, while addition of Palbociclib to the combined treatment led to the greatest down-regulation. Importantly most efficient dampening of both, the ER- and the Rb pathway was achieved by the combination of Fulvestrant, low dose Palbociclib and cycles of STS. Interestingly, it becomes clear how cells might compensate for single treatments. Fulvestrant treatment in CTR conditions induced a down-regulation of all genes, while the addition of STS

abolished this effect to little extent regarding expression of TFF1 and reversed down-regulation of *Cyclin D1* and *E2F1*. Up-regulated *Cyclin D1* and *E2F1* upon Fulvestrant plus STS show a compensation of ER- and IGF1R signalling blockage with elevated Rb signalling, probably through TF's that act independent of ER and IGF1R on *Cyclin D1* [153,154]. A similar phenomenon is visible upon Palbociclib treatment that down-regulated both genes of the Rb pathway but led to up-regulation of both genes connected to the ER pathway. To further support this hypothesis, we need to analyse gene expression downstream of the IR/IGF1R signalling.

MCF7 cells treated with Fulvestrant, Palbocilib and STS showed a flattened morphology and a great cytosolic expansion that related to a senescent morphology. This increase in cell dimension was observed to a lesser extent in cells treated only with Fulvestrant (+/-STS) or Fulvestrant plus Palbociclib in CTR conditions. Senescence in cancer cells can be induced by activation of at least one of two crucial pathways. The p53/p21 pathway which is usually activated upon irrepairable DNA damage that leads to maintained DNA damage response (DDR) and the $p16^{INK4a}$/pRb pathway (where Rb activity is inhibited) by other stress factors [149]. Having a cell cycle inhibitor among our treatments and with DDR occurring mostly during S-phase, the p53/p21 pathway was not expected to trigger this phenomenon primarily. MCF7 cells carry a deletion in the $p16^{INK4a}$ gene but down-regulation of *FOMX1* as a result of blockage of the Rb pathway using Palbociclib, has been connected to senescence [150] and which is why we looked first at the expression levels *FOXM1*. In the study by Anders et al., Palbociclib was

used as a single treatment and at higher concentrations. In our case, low dose Palbociclib did not greatly impact on *FOXM1* expression while triple treatment was the only condition under which *FOXM1* expression was strikingly down-regulated (**Figure 4-11**). We also tried to visualize morphological changes capturing high-resolution images (**Figure 4-12**) and to measure cell surface area (**Figure 4-13**). When cells grew in CTR or STS conditions and were treated with vehicle or low dose Palbociclib, cell dimension did not increase but decreased over time due to a higher confluence in the plate. Doubling time of cells is equal among untreated and Palbociclib treated groups (**Figure 4-14**). Low dose Palbociclib in combination with STS was here less efficient in halting proliferation than previous experiments (**Figure 4-6**) where cycles of STS were applied every second day and not at a distance of 5 days. Applying cycles only once per week rather represents the *in vivo* applied schedule, where sole low dose Palbociclib did not show marked effects either (**Figure 4-15**). Treatment with Fulvestrant +/- STS and the combination with Palbociclib +/- cycles of STS showed still great effect on slowing proliferation, even when cycles of STS were applied once per week, which can be confirmed in **Figure 4-14**. Initial increase in cytosolic area of cells treated with Fulvestrant or Palbociclib plus Fulvestrant in CTR conditions could not be maintained, implying adaption. Fulvestrant plus FMD induced an increase in cytosolic area, which was only maintained upon the second cycle of STS, while addition of low dose Palbociclib had the greatest effect leading to a constant increase in cell dimension that reached on average 7 times the size of CTR cells after 4 cycles. Measuring doubling time of cells grown parallel to imaging acquisition showed that triple treatment is most efficiently (**Figure 4-14**) but

indicated also that there might still persist some cells that proliferate on a very low level. Long-term experiments are needed to see whether these cells can be completely eliminated and whether all surviving cells enter definite growth arrest.

Summarizing, *in vitro* experiments with addition of low dose Palbociclib to Fulvestrant plus STS revealed that triple treatment is able to most efficiently and significantly down-regulate genes belonging to both, the ER- and the Rb pathway and to increase doubling time to the greatest extent. A senescence-like phenotype could be observed in many cells that survived triple treatment and only these conditions had the capacity to greatly down-regulate *FOXM1*, which has been connected to senescence induction. However, more experiments, especially on long-term treated cells are needed to prove the induction of irreversible cell cycle arrest, hopefully in all cells that survive long-term treatment. We therefore will investigate the involvement of the p53/p21 pathway, senescence-associated beta-galactosidase activity, expression of senescence associated secretory phenotype (SASP) and measure BrdU incorporation. Of highest importance was the *in vivo* potential of the combined treatment. Our Xenograft experiment showed that FMD had the potential to abolish development of resistance to combined Fulvestrant plus Palbociclib treatment over 160 days. Not a single animal showed insensitivity to our treatment at this time point. Contrarily to the former experiment, all tumors decreased in size within the first days of treatment (**Figure 4-15** C) showing a unique response. The experiment was terminated recently and analysis on protein and mRNA of the tumor are planned in order to prove shut down of all three targeted pathways and probable senescence induction *in vivo* in the remaining

cancer cells. Based on the observations, another question with clinical importance arose. Many patients are undergoing therapy at the moment and cannot benefit anymore from concomitant treatment at the moment of diagnosis. FMD showed its potential to kill subpopulations within some tumors treated with Fulvestrant alone and all tumors treated with the combination of Fulvestrant and Palbociclib. We wondered whether FMDs potential to kill the resistance driving cells is maintained until a later stage, when insensitivity arises.

5.1.3 FMD has the potential to reverse ER^+BC resistance to combined Fulvestrant plus Palbociclib treatment

To this aim, two mice that have developed resistance to combined Fulvestrant plus Palbociclib treatment underwent just two cycles of FMD (**Figure 4-16** A) and tumor size decreased. We next used another resistant mass as a donor of resistant cells, amplified them under continuous drug exposure *in vitro* (**Figure 4-16** B) and re-injected them into new mice. Once tumor progression was guaranteed in presence of administration of both drugs, half of the mice underwent cycles of FMD. Impressively a decrease in tumor size to a significant extent after the second cycle of FMD(**Figure 4-16** C and D) could be measured. This great response to our diet that lowers circulating glucose, insulin and IGF-I, hinted at reactivation of the IR/IGF1R signalling. Western Blot analysis on tumor masses showed a high expression of IR and IGF1R that were significantly decreased by cycles of FMD (**Figure 4-17**). The reactivation of IGF1R in Fulvestrant plus Palbociclib resistant mice compared to Fulvestrant resistant mice could be proven via IHC staining of tumors (**Figure 4-18**). Also ER expression seemed to be reactivated in masses

resistant to Fulestrant plus Palbociclib (**Figure 4-17**) compared to previous analysis on tumors only treated with Fulvestrant (**Figure 4-5**). FMD was able to down-regulate the ER even if not significantly. We know that FMD has a broad acting effect and therefore aim at depicturing the entire transcriptome with RNA sequencing analyses that are on going and should help to better understand molecular changes upon FMD. Insulin and IGF-I dependence of resistant cells could be proven on MCF7-R clones *in vitro*. MCF7-R cells show a greater response to STS than parental MCF7 cells (**Figure 4-19** A) and addition of Insulin plus IGF-I could abolish the effect (**Figure 4-19** C).

Summarizing, we can state that FMD has great potential to abolish resistance acquisition to combined Fulvestrant and Palbociclib treatment *in vivo*, eventually through elimination of resistance driving subpopulations within the heterogeneous tumor. As *in vitro* conditions are hard to use to investigate the real underlying mechanism, collected tumors from *in vivo* experiments will be analyzed extensively on protein and RNA level to reveal the mechanism through which FMD exerts its potential. Evidence for adaption to single or combined treatment by alternatively up-regulating ER- or Rb- signalling is shown in **Figure 4-10**. Our *in vivo* data support this phenomenon as IGF1R disappears in tumors treated with Fulvestrant and in some of these masses CDK6 up-regulation can be observed (**Figure 4-5**). If tumors are treated instead with Fulvestrant and Palbociclib without applying FMD, the IGF1R is present again (**Figure 4-18**) and the potential target through which FMD exerts its effect on resistant tumors.

6 References

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