The Development and Characterisation of Everolimus Resistant Breast Cancer Cells

A Thesis Submitted for the Degree of Doctor of Philosophy

By

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Declaration

I declare that the research presented for this thesis is my own work, except where otherwise stated and referenced and has not been submitted for any other degree.

Stephen Hare

Acknowledgements

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Abstract

The mTOR inhibitor and rapalogue everolimus was approved use in 2012, in HR+, HER-2-, post-menopausal patients, who had previously failed aromatase inhibitor treatment. mTOR pathway activation has been associated with resistance to breast cancer therapies, but how cells may become resistant to mTOR therapies themselves in breast cancer is currently not well explored, due to the relative recentness of everolimus approval. Drug resistance across all areas of cancer research is a major clinical issue, often leading to the spread of a patient's cancer. This project set out to create *in vitro* breast cancer models that were resistant to everolimus, and thus explore any changes that had developed in these models, help determine the mechanisms behind resistance and discover drugs/drug combinations that could overcome resistance. Cell lines T47D and MDA-MB-361 were subsequently developed into everolimus resistant lines (EveR) over the course of 4-6 months using an on/off exposure routine.

The exact mechanism behind the everolimus resistance was not fully determined but EveR cells did show multiple intriguing characteristics. An increase in dormancy and stem-cell like phenotype was noted, as revealed by a decrease in cell cycle progression and an increase in increase ALDH activity. mTORC2 components and signalling was up-regulated although siRNA down-regulation of PKCα did not decrease everolimus resistance, suggesting other mTORC2 targets may be involved. The rapalogue 'receptor', FKBP12, was up-regulated which was accompanied by an increased growth inhibition by the rapalogue, temsirolimus, possible due to temsirolimus lower binding affinity for FKBP12 compared to everolimus. No resistance to the dual mTOR/PI3K inhibitor BEZ-235 was observed, in line with similar published work. The combination of vitamin D/calcitriol and everolimus had no added effect compared to everolimus alone, in parental cells, but the addition of 1μM calcitriol did drastically lower EveR cell resistance to everolimus. Future work focusing on the exact nature of calcitriol's interaction with the mTOR pathway is required to advance calcitriols role as a breast cancer therapeutic. Research with everolimus resistant breast cancer patients has not yet been published on, but the work presented here aims to help guide such studies, when they are carried out in the future.

Conference Presentations and Publications of this Work

The research for this thesis was presented at a range of conferences both internally and externally, before submission.

A poster presentation of this work won 3rd place prize at the 2016 College of Health and Life Sciences PhD Research Conference.

On oral presentation of this work won 1st prize at the 2017 College of Health and Life Sciences PhD Research Conference.

Information from this thesis was used for my entry into the 2017 3 minute thesis competition, where I was selected from the college heats to present at the University final.

Major findings of this thesis were presented at the EACR25 Conference in Amsterdam June 2018, with funds obtained through travel prizes from the British Association of Cancer Research and The College of Health and Life Sciences (Brunel). The conference was organised and hosted by the European Association of Cancer Research.

Major sections from the introduction of this thesis were used to write a review article that was published in the American Journal of Cancer Research, in 2017. Full reference:
Hare, S.H. and Harvey, A.J. (2017) 'mTOR function and therapeutic targeting in breast cancer', *American journal of cancer research,* 7(3), pp. 383-404.

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Abbreviations

361 – MDA-MB-361
4E-BP – eukaryotic translation initiation factor 4E-binding protein
Ab - antibody
ABC – ATP binding cassette
ALDH – Aldehyde dehydrogenase
ALL- acute lymphoblastic lymphoma
AR – androgen receptor
B – BSA
BCRP- breast cancer resistance protein
BLAST – basic local alignment search tool
BOLERO – breast cancer trials of oral everolimus
bp – base pair
BSA – bovine serum albumin
Cal - calcitriol
cDNA – complementary deoxyribonucleic acid
CSCs – cancer stem cells
CST – Cell signalling technologies
CT – cycle threshold
DMEM – Dulbecco's modified Eagle's medium
DMF – dimethylformamide
DMSO – dimethyl sulfoxide
DNA - deoxyribonucleic acid
DST – disseminating tumour cell
ECL - Enhanced chemiluminescence
EGFR - epidermal growth factor receptor
eIF4E - translation initiation factor 4E
EMT – epithelial to mesenchymal transition
Enz - enzalutamide
ER – oestrogen receptor
EU – European Union
Eve - everolimus
EveR – everolimus resistant

FBS – foetal bovine serum

FKBP12 - FK506 binding protein 12 kDa

FLCN - folliculin

GI60 – growth inhibition 60%

HCG – hydroxychloroquine

HDAC - histone deacetylase

HER – human epidermal growth factor receptor

HIF1α - hypoxia inducible factor 1 alpha

HR – hormone receptor

hrs - hours

IDC – invasive ductal carcinoma

IGF-1 – insulin-like growth factor 1

IGF-1R - insulin-like growth factor 1 receptor

ILC – Invasive lobular carcinoma

IRS – insulin receptor substrate

KD – kinase domain

kDa - kilodalton

LT – long term

M – milk

mAb – monoclonal antibody

MAM - mitochondria-associated endoplasmic reticulum

MAPK – mitogen activated protein kinase

MDR – multidrug resistance

MET – mesenchymal to epithelial transition

min – minute

MIQE – minimum information for the publication of qPCR experiments

MRP – multidrug resistance associated protein

mTOR - mechanistic target of rapamycin

mTORC – mechanistic target of rapamycin complex

NCBI – National Center for Biotechnology Information

NT – non-targeting

OSCC - oral squamous cell carcinoma

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PFS - progression free survival

PGP- P-glyvoprotein

PH - pleckstrin homology

PI – propidium iodide

PI3K – phosphoinositide 3-kinase

PIKK - phosphoinositide 3-kinase related kinase

PNET - pancreatic neuroendocrine tumour

poly-HEMA - poly(2-hydroxyethyl methacrylate)

PR – progesterone receptor

PRAS40 - proline-rich Akt substrate of 40 kDa

Q – glutamine

QoL – quality of life

qPCR – quantitative polymerase chain reaction

QPS – glutamine penicillin streptomycin

REDD - regulated in DNA damage and development 1

Rheb - ras-homolog enriched in brain

RNA - ribonucleic acid

RPM – revolutions per minute

rpS6 - ribosomal protein S6

RQ – relative quantity

RT – reverse transcription

s - second

S6K – ribosomal protein S6 kinase

SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

sec - second

siRNA – small interfering ribonucleic acid

SRB – sulforhodamine B

TBS-T – tris buffered saline – tween20

TCA – trichloroacetic acid

TGFβR1 - TGF-beta type-I receptors

TGM2 - transglutaminase 2

Tr – treatment/treatment cycle

TSC – tuberous sclerosis complex

UK – United Kingdom

VDR – vitamin D receptor

VHL - Von Hippel-Lindau

XAV – XAV-939

Chapter 1: Introduction

1.1 - Breast Cancer: Background Information and Statistics

Breast cancer is one of the most common cancers seen clinically, with over 50,000 women diagnosed each year in the UK; this means breast cancer makes up around 15% of the 350,000+ people diagnosed with cancer each year. Whilst around 1 in 2 people will not survive beyond 10 years with any form of cancer in the UK, breast cancer has a much better than average survival rate with around 83% surviving 5 years and 78% surviving 10 years, after diagnosis. Data shows that around 1 in 8 women will be diagnosed with a breast cancer (Cancer Research UK, 2017a; Cancer Research UK, 2017b; Breast Cancer Now, 2017). Whilst there is some variation in incidence rates in developed nations, trends are roughly the same between them. In the U.S, again around 1 in 8 women will develop the disease within their lifetime, which equates to around 230,000 new cases each year (DeSantis et al, 2013). It is worth noting that despite the fact that most breast cancers diagnosed are in women, a very small percentage of men can also develop breast cancer, with an incidence of around 400 new cases each year in the U.K (Cancer Research UK, 2017a; Ruddy and Winer, 2013).

In terms of physiology, breast cancer has number of histological subtypes. Invasive ductal carcinoma (IDC) is the most common of these accounting for between 55-75% of all breast cancers diagnosed, with invasive lobular carcinoma (ILC) being the next most common, with around 10% of cases (Thor and Osunkoya, 2009). Metastasis is one of the most important prognostic factors, and women with distant metastasis from breast cancer have a much worse outcome; median survival at this point is estimated at 2 to 2.5 years (Nicolini et al, 2006). Breast cancer often first spreads locally to sites such as the axillary (armpit) lymph nodes, before metastasising to sites such as the brain or bones (Kozłowski et al, 2015; Thor and Osunkoya, 2009). Any cancer with distant metastasis is classified (TNM system) as stage IV (Yalcin, 2013).

Risk factors for breast cancer are wide and varied. They have been well studied, reviewed and meta-analysed by Barnard and colleagues (2015) and Dumalaon-Canaria and colleagues (2014), but will be summarised here. Whilst obvious factors such as BRCA1/2 mutations can dramatically increase risk for individuals with these alleles, they only account for around 2-9% of all breast cancer cases. Many factors have shown strong links to increasing breast cancer risk, with age arguably being the biggest risk factor found thus far. Others include high breast density, a family history of breast cancer, older age at menopause and exposure to certain toxic substances such as cadmium. Meta-analysis of parity and its effect on breast cancer risk suggests it may lower the risks in developing some types of breast cancer (luminal A) but increase the risk of developing others (triple negative). Increased duration of breast feeding and physical exercise (in post-menopausal women) has been shown to decrease risk of breast cancer. Whilst data suggests that higher fat diets increase breast cancer risks, linking association either positively or negatively with any one diet has proven inconsistent across studies, although increased alcohol consumption has been shown to significantly increase risk (Albuquerque et al, 2014; Barnard et al, 2015; Dumalaon-Canaria et al, 2014).

1.2 - Molecular Subtypes of Breast Cancer

Cancers are by their very nature highly heterogeneous, especially between patients. Thus for the sake of patient care, it is vital that rigorous and robust classification systems are in place to ensure patients are given the correct treatments from the point of diagnosis. Whilst there are many histological types of breast cancer, most will end being classified as either ductal or lobular (75-85%) making this system a rather ‘blunt’ tool and less useful for tailoring patient treatment and thus improving prognosis (Viale, 2012).

Within the last two decades, a more robust system has since been developed to subtype a patient’s cancer using its molecular profile. These intrinsic molecular subtypes are usually broken down into 4 key groups (although others can be distinguished); Luminal A and B groups, HER-2+/HER-2 over expressing and basal-like (see table 1.1 for a summary). The efficacy and usefulness of the system is such that it has proven perhaps the most widely used in the field today. Seminal work in

developing this system came from groups in Norway and America who published several key papers exploring this idea (Perou et al, 2000; Sørlie et al, 2001). Several methods can be used to identify sub-type using gene signature, but perhaps the most well-known and one of the most robust is the PAM 50. This determined a 50 gene panel that was readily able to distinguish the 4 main subtypes, as well as helping to give treatment and prognostic information, such as stating a risk of recurrence score, using this gene signature (Dai et al, 2015; Prat et al, 2015). This information can also help give information of potential survival/mortality, as shown in figure 1.1.

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Subtype	Description	% of BC	Relative Prognosis
Luminal A	ER and/or PR+. Gene expression more similar to luminal cells of the breast. Low proliferative markers and low grade. Best prognosis	50%	Good
Luminal B	ER and/or PR+. Gene expression more similar to luminal cells of the breast. Higher expression of proliferative markers than luminal A	15-20%	Worse than Luminal A
HER-2+	HER-2+ and expression of genes associated with HER-2. Higher grade and worse prognosis	15-20%	Poor
Basal-like	Usually triple negative (ER-/PR-/HER-2-). Poor grade and poorest prognosis. Gene expression similar to basal-epithelium and high expression of proliferative markers such as *KRT5* and *EGFR*	10-20%	Poorest among subtypes

Table 1.1: Table to summarise the 4 primary intrinsic molecular subtypes of breast cancer. Information from: Dai et al (2015), Ho-Yen et al (2012), Perou et al (2000), Prat et al (2015), Sahlberg et al (2013), Sørlie et al (2001) and Yersal and Barutca, (2014).

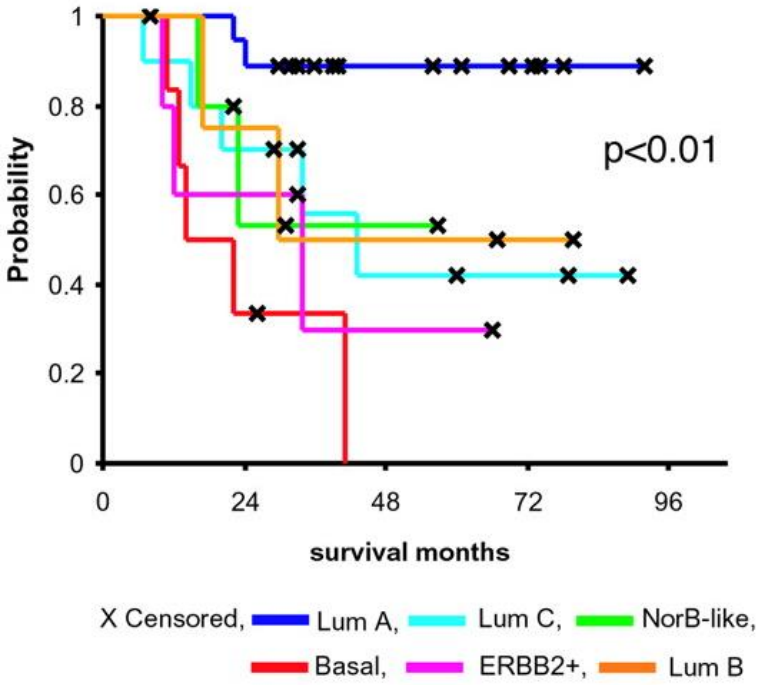


Figure 1.1: Graph showing how breast cancer subtype can greatly effect potential survival, when studying luminal type A, B and C, HER-2/ERB2+, basal- and normal-like subtypes (Sørlie et al (2001).

Luminal breast cancers are generally divided into the two groups of A and B but they both share some key commonality. Both have low HER-2 (HER-2-/negative) expression and expression of oestrogen receptor (ER) and/or progesterone receptor (PR) (hormone receptor positive/HR+). Luminal A comprises the bulk of breast cancer cases (around 50%) and is also accompanied by the best prognosis of the subtypes. Luminal A cancers are characterised by low expression of proliferative markers and usually have a low histological grade (Yersal and Barutca, 2014). Related to the hormone receptor status, luminal A cancers have increased expression of genes that pertain to ER activation including *LIV1* and *CCND1* and express luminal cytokeratins (*KRT8* and *18*) (Carey, 2010). Luminal A tumours also frequently expresses mutant versions of *TP53* (although this gene is also a significant biomarker in other subtypes*)*, *MAP3K1* and *PIK3CA* (Ciriello et al, 2013). Interestingly, studies of multiple data sets, suggests that luminal A cancers generally express PR more than luminal B cancers (Prat et al, 2013); on top of this it is generally observed in breast cancer that an expression of either ER and/or PR increases survival rates, although prognosis has been steadily improving among

patient sub-groups lacking either or both receptors, in recent decades (Chen et al, 2014a; Grann et al, 2005; Wang et al, 2014).

The luminal B subtype has a worse prognosis, compared to luminal A (although not as poor as HER-2 or basal-like subtypes), and in line with this tumours tend to have a higher grade (Dai et al, 2015). Luminal B cancers generally possess a higher expression of proliferative genes such as *MKI67*, *CCNB1* and *AURKA* and appear to have large numbers of chromosomal aberrations and copy number alterations, relative to the other subtypes. Examples of commonly expressed oncogenes in this subtype include *SIX1* and *ZNF703*, with the latter involved in the Wnt and notch pathways (Ades et al, 2014; Prat et al, 2015).

The next subtype, HER-2+, is relatively self-explanatory, with cancers here generally expressing increased levels of the ErbB family receptor HER-2 (ErbB2). Genes in the HER-2 amplicon such as *GRB7*, *STARD3* and *PSMD3* are also commonly up-regulated in the HER-2 positive subtype and have been shown to contribute to growth and survival of the cancer (Perou et al, 2000; Sahlberg et al, 2013). The tumours amount to around 15-20% of cases, with HER-2 expression generally pertaining to a poor histological grade and prognosis (Yersal and Barutca, 2014).

Basal-like breast cancers are often known as triple negative, which refers to a lack of expression of both ER and PR and a low expression of HER-2. Whilst most basal-like cases are triple negative, the two terms are not necessarily synonymous, with a genetic/microarray definition of basal-like tumours being wider than just the expression of these key receptors, of which the receptors are usually defined by immunohistochemistry (Prat et al, 2013; Yersal and Barutca, 2014). Basal-like cancers (again making up around 10-20% of breast cancers) usually possess a poor histological grade and have generally the poorest survival and prognosis amongst the subtypes defined here (Ho-Yen et al, 2012; Rattani and Swift-Scanlan, 2014). This subtype generally expresses genes that are more similar to basal epithelium, rather than luminal cells, like luminal A and B subtypes. In terms of genetic markers for basal-like cancers, these include proliferation markers like *MKI67, TOP2A* and *PCNA* as well keratins 5 and 17 (*KRT5* and *17*); a high expression of *EGFR*, *FOXC1* and p-cadherin (*CDH3*) are also common (Ho-Yen et al, 2012; Prat et al, 2015).

On top of this many further sub-groups of breast cancer have now been identified, that have built upon the original classification of 4 subtypes, and which may one day help further enhance individualised therapy for patients. These include but are not limited to interferon-enriched (Hu et al, 2006), claudin-low (Prat et al, 2010; Sabatier et al, 2014) and several further distinctions and sub-groupings of triple negative breast cancers (Lehmann et al, 2011).

1.3 - Using Intrinsic Molecular Subtyping to Guide Breast Cancer Therapy

Breast cancer, in most forms, is generally considered a highly treatable disease with multiple options for therapy, these varying for each type of breast cancer and stage. However there is still a great deal of standardisation across the board, in terms of treatment offered to most people with breast cancer. Patients will generally receive some form of surgery, with radiotherapy an option afterwards (Board, 2017). Standard chemotherapy use (non-targeted) is commonly used, especially in patients with metastatic disease. Many drugs are used in this setting, but can include 5-fluorouracil, cyclophosphamide, docetaxel, doxorubicin hydrochloride, gemcitabine, methotrexate and paclitaxel (NIH National Cancer Institute, 2017).

These standard chemotherapies are relatively blunt tools, and are non-specific, meaning they can often have a great burden on the patient in terms of side effects. It is at this stage that the intrinsic molecular subtypes can be used for great effect in guiding patient therapy, directing which of the more specific and targeted drugs a patient could benefit from (usually in conjunction with standard chemotherapy, radiotherapy and surgery).

Since one of the main defining features of luminal breast cancers is the presence of hormone receptors, mainly ER, this is a primary target for selective therapy against this subtype (Guarneri and Conte, 2009). Selective estrogen receptor modulators or SERMs are one of the most widely used classes of drugs to target ERs, either acting as agonists or antagonists to specific ER isoforms (Mirkin and Pickar, 2015). Tamoxifen (which blocks ER activation in the breast) is the first among these to be used in breast cancer, being shown to not only reduce the risk of women developing

breast cancer by around 49%, when administered to higher risk groups across a 5 year period (Fisher et al, 1998), but also giving it in an adjuvant setting across a 5 or 10 year period is extremely useful in reducing the risk of recurrence (Davies et al, 2013). Other SERMs have been developed since that have relatively similar effects, whilst lowering their off target activation of different ER isoforms, to reduce possible increased risks of other cancers and conditions (Chlebowski et al 2015). For example, raloxifene is one such SERM, having similar effects in reducing breast cancer incidence in varying groups (Barrett-Connor et al, 2006; Cauley et al, 2001), whilst also posing a reduced risk of developing endometrial cancer when compared to tamoxifen treatment (Freedmam et al, 2011).

Fulvestrant is often called a selective estrogen receptor down-regulator (SERD) and acts with a similar mechanism to SERMs but also helps to down-regulate, as the name suggests, ER expression. It is often used in women whose previous endocrine treatment (e.g. with anastrazole or a SERM such as tamoxifen) has failed to stop the advance of the cancer (Ciruelos et al, 2014; Howell et al, 2002; Howell et al, 2004).

SERM/SERDs are of course not the only way of targeting oestrogen within breast cancer therapy. In post-menopausal women, a major source of oestrogens, will be from the conversion of androgens to oestrogens, by the enzyme aromatase (CYP19) (Baum et al, 2014). Anastrazole is in the class of non-steroidal aromatase inhibitors, and has been widely shown in its efficacy and use as an adjuvant drug for post-menopausal women with hormone responsive breast cancers. Anastrazole has shown good use, even more so than compared to tamoxifen (but can also be used in conjunction it), in this group of patients, with clinical trials showing an increase in survival rates and decrease in recurrence compared to tamoxifen use alone (Baum et al, 2003; Dowsett et al, 2010; Dunbier et al, 2011). Steroidal based aromatase inhibitors are also sometimes used, such as the drug exemestane, with data suggesting it is as least as effective as anastrazole (Goss et al, 2013). Exemestane is now also approved, in conjunction with the mTOR inhibitor everolimus, for use in patients whose previous treatment with anastrazole (or other aromatase inhibitors) has failed (European Medicines Agency, 2016a; Geisler, 2011; Wazir et al, 2014).

Despite the decreased prognosis and average survival rates (compared to luminal types) that HER-2+ breast cancers are associated with, multiple HER-2 targeting therapy agents are approved for use to combat this subtype of the disease, including both monoclonal antibodies (mAb) and small molecular inhibitors. Trastuzumab is a mAb used clinically in breast cancer since 2005, targeting extracellular domains to block HER-2 activity as well as disrupting HER-2/HER-3 dimers (Ahmed et al, 2015; Guarneri and Conte, 2009). Trastuzumab has proved its effectiveness as an adjuvant therapy when combined with standard chemotherapy, with clinical trials in HER-2+ patients, showing significant and large increases in disease free survival, compared to standard chemotherapy regimens alone (Joensuu et al, 2006; Romond et al, 2005).

Due to the depth of research into more advanced breast cancer therapies over the past 30 years, trastuzumab is by no means the only option, even in terms of mAbs, for treating HER-2+ breast cancers. Pertuzumab is another mAb used in this setting, although having a different mechanism of action to trastuzumab, in that it binds to HER-2 inhibiting its dimerization with HER-3. This differing mechanism to trastuzumab has to lead to pertuzumab often being used in conjunction with the former as a ‘two pronged attack’ against HER family receptors. Pertuzumab has shown effectiveness when used as a neoadjuvant therapy (Ahmed et al, 2015; Jhaveri and Esteva, 2014) and adjuvantly in conjunction with docetaxel and trastuzumab (Swain et al, 2015)

In terms of small molecule inhibitors of HER-2, lapatinib is the most prevalent and most widely used in breast cancer. Lapatinib is a tyrosine kinase inhibitor, acting by binding to the intracellular domains and inhibit kinase function, thus limiting downstream response (Medina et al, 2008). Much like pertuzumab, most studies into its effectiveness in a clinical setting have been done in combination with trastuzumab, again due to the varying mechanism of action, with it used adjuvantly (Blackwell et al, 2012) and neoadjuvantly (Hicks et al, 2015)

Due to the fact that triple negative breast cancer by its very definition lacks the expression of ER, PR or HER-2 (overexpression), it also lacks the primary targets for therapy currently approved in breast cancer, in terms of targeted drugs. Because of

this, triple-negative patients have a far more limited range of therapies available, which partially factors in to the worsening of prognosis in this subtype (along with factors like greater aggressiveness). Thus standard chemotherapy regimens are the primary drug therapy sets used in these patients. Despite a poorer prognosis, triple negative cases can still respond well to chemotherapy, both adjuvant and neoadjuvant. Some courses have even shown to help improve surgery success rates, although if these treatments fail patients have a very poor outlook due to the lack of more diverse treatments from that point (Golshan et al, 2015; Guarneri and Conte, 2009; Liedtke et al, 2008; Prat et al, 2015).

This is not to say that there is no use for more targeted drugs in triple negative patients, but these of course have had to look beyond the ER/PR/HER-2 boundary. Bevacizumab is a mAb approved for use in metastatic breast cancers, in combination with chemotherapy agents like paclitaxel, with it targeting VEGF; thus helping to stop the growth of cancers via inhibition of new blood vessel formation (European Medicines Agency, 2017). It has shown effectiveness in multiple drug trials looking at the use of this drug with chemotherapy versus chemotherapy alone, predominantly showing increased pathologic complete response rates and increased progression free survival (Minckwitz et al, 2014; Sikov et al, 2014).

With the gulf in treatment between the subtypes now being vastly apparent, much research effort has now turned to finding similarly good specific targets for triple negative cancers, which is in turn related to increasing efforts to further subtype these patients, and thus find new targets. A range of strategies are being explored including PARP inhibition, targeting p53, notch and Wnt signalling inhibition, targeting androgen receptors and even the exploration of inhibiting mTOR signalling using everolimus or temsirolimus in triple negative cancers (Jamdade et al, 2015; Mayer et al,2015; Palma et al, 2015).

1.4 - The mTOR Pathway: Discovery of Rapamycin and an Overview of mTOR Signalling

The story of rapamycin is an interesting case of serendipitous discovery that led to the uncovering of a brand new signalling pathway. In 1975 a Canadian research

group published a paper describing a novel compound isolated from the bacterium *Streptomyces hygroscopicus*. This microorganism was observed in soil samples from Easter Island and was found to produce a compound that strongly inhibited the growth of fungal species such as *Candida albicans*. They named this compound rapamycin, with the name derived from the native term for Easter Island, Rapa Nui (Vezina et al, 1975). Further studies on rapamycin, by the same group, revealed it to be a potent anti-fungal agent *in vitro* and *in vivo* (Baker et al, 1978), although their testing failed to find a clear mechanism of action (Singh et al, 1979).

Over the next decade, through the 1980s and early 1990s, rapamycin was revealed to also be a strong immunosuppressant, blocking T-cell activation (Dumont et al, 1990), as well as having key action as an anti-cancer drug, including the inhibition of breast cancer cell growth (Eng et al, 1984; Seto, 2012). It wasn't until the early 1990s that the mechanism of rapamycin action began to be elucidated. In yeast, the products of two genes, *TOR1* and *TOR2* (target of rapamycin), were found to participate in rapamycin action (Heitman et al, 1991), with studies on yeast strains carrying TOR mutants, showing that rapamycin in a complex with its intracellular receptor FKBP12, binds to TOR to act (Cafferkey et al, 1993; Kunz et al, 1993).

Only a few years later the mammalian homologue of TOR1 and 2 was discovered by several research groups (Brown et al, 1994; Sabatini et al, 1994; Sabers et al 1995), of which only one protein was found in mammals compared to the two in yeast. This protein was eventually named the mammalian target of rapamycin (mTOR) although today mTOR is generally referred to as the mechanistic target of rapamycin. Since then, the mTOR pathway has been slowly built upon in terms of our understanding of its complexity, but also of its significance in a number of biological fields.

Overall, mTOR signalling causes an increase in protein mass, cellular growth and inhibits autophagy, with it generally acting as a cellular sensor to nutrients and growth factors, as well as being an important effecter of PI3K signalling (Zarogoulidis et al, 2014). mTOR is a serine/threonine kinase which acts in one of two protein complexes; mTORC1 or mTORC2. Key proteins in mTORC1 include mTOR, mLST8 (also known as GβL), raptor, PRAS40, DEPTOR and the Tel2-Tti1 sub-complex. mTORC2 shares the common subunits of mTOR, mLST8, DEPTOR and the Tel2-

Tti1 sub-complex, as well as possessing the unique mTORC2 subunits of rictor, mSIN1, protor-1 and 2 (also known as PRR5 and PRR5L respectively) (Saran et al, 2015; Xu et al, 2014).

A variety of factors can lead to the activation of mTORC1, such as varying amino acid levels, but the most well understood mechanism of activation is via the inactivation of the tuberous sclerosis complex (TSC). Akt, which is activated in response to growth signals such as insulin, phosphorylates TSC2, which stops its role as a GTPase activating protein (GAP) for rheb; a small ras-like GTPase. Now bound to GTP, rheb activates mTORC1. While less well defined, growth factor signalling can also lead to the activation of mTORC2 (Huang and Fingar, 2014). Downstream of mTORC1, protein synthesis is one of the most important outcomes of its signalling, with the complex phosphorylating translation initiation factor 4E (eIF4E) and S6K1/2, to increase translation. mTORC1 also regulates lipid synthesis, ATP production and inhibits autophagy. mTORC2's role is less well understood across most aspects of mTOR signalling, but it is known to phosphorylate Akt, which is of course an important regulator of cellular growth and survival (Manning and Cantley, 2007). Among other things, mTORC2 also phosphorylates proteins that control cytoskeletal re-organisation, such as PKC-α (Laplante and Sabatini, 2012). A broad schematic overview of mTOR signalling, can be seen in figure 1.2.

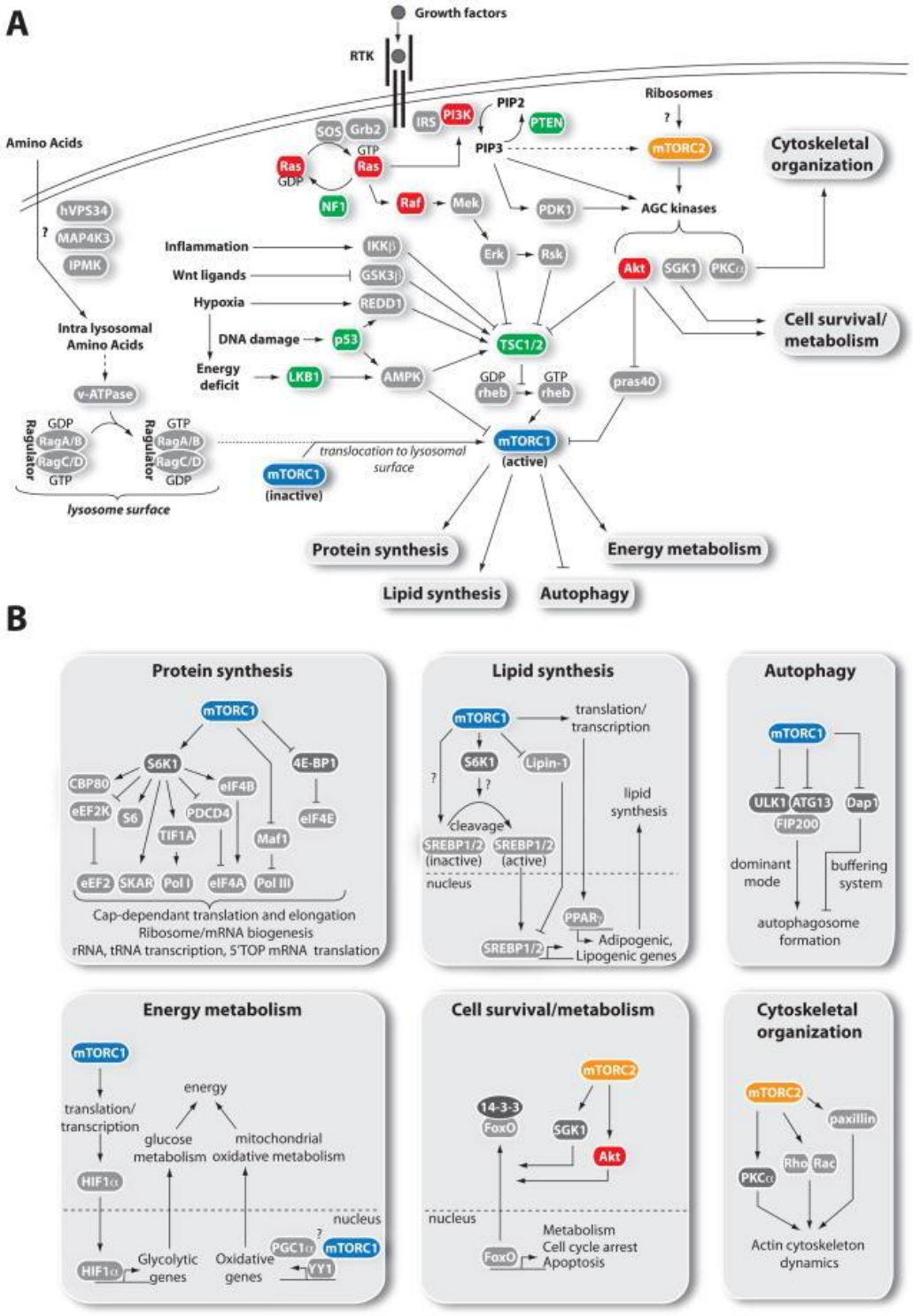


*Figure 1.2: Schematic overview of mTOR signalling (*Laplante and Sabatini, 2012)

1.5 - The mTOR Pathway: mTORC1 Components

mTOR is a 289 kDa/2549 amino acid serine/threonine kinase, belonging to the phosphoinositide 3-kinase related kinase (PIKK) family and is expressed in most mammalian cells (Brown et al, 1994; Lakhlili et al, 2015). Residues 1-1375 of mTOR are not as well defined as the rest of the protein, but predictive modelling techniques and information from related kinases suggest this N-terminal half of the protein consists mostly of HEAT repeats (Knutson, 2010). The remaining structure of the protein is well defined, by crystal structure, as shown in figure 1.3. The FAT domain is made of 28 α-helices, with several wrapping round the kinase domain (KD). The kinase domain is actually formed of two separate sections of the protein, labelled the N-lobe and C-lobe. The FRB domain is inserted within the C-lobe, but forms a distinct section from the kinase domain; this section binds the complex of rapamycin-FKBP12. The FATC domain lies near the c-terminal of the protein and is critical for kinase domain structure and function. ATP binds within a cleft in the kinase domain, with mTOR then catalysing the transfer of the γ-phosphate of ATP onto the substrate (Sauer et al, 2013; Yang et al, 2013).

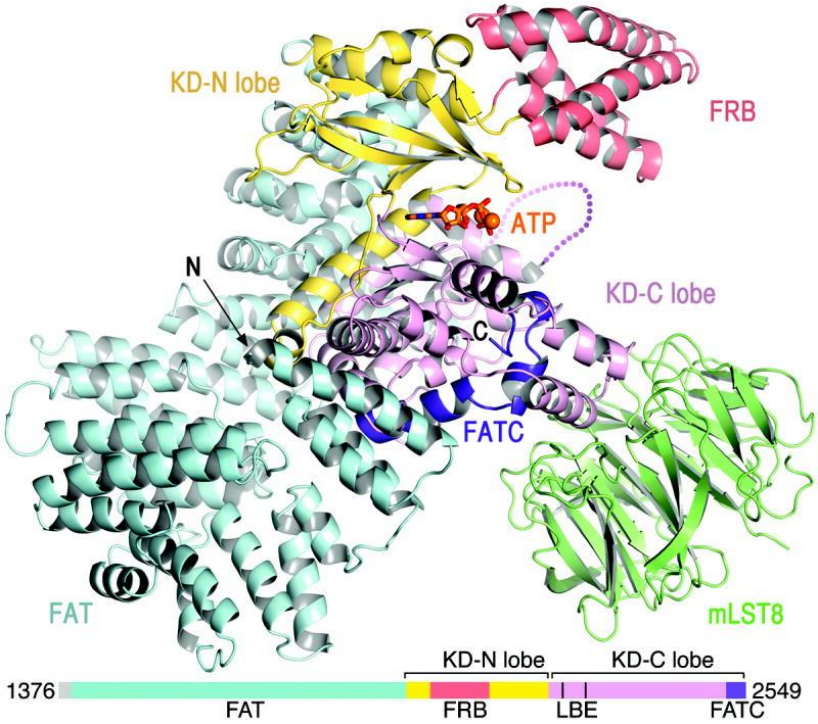


Figure 1.3: Structure of the mTOR$^{\Delta N}$-mLST8 complex. Residues 1-1375 of mTOR are not shown (Yang et al, 2013).

As shown by the crystal structure, mLST8 binds to mTOR at the kinase domain C-lobe. Experiments suggest that mLST8 is needed for mTOR kinase function as well as helping to stabilise the interaction between mTOR and raptor, in mTORC1 (Kim et al, 2003). The work by Yang and colleagues (2013) shown above, suggests that mLST8 may function in activating mTOR catalytic function, via its interactions with the kinase domain.

Extremely important to mTORC1 function is raptor, a 149 kDa protein that is usually found in a complex with mTOR, binding to the mTOR HEAT repeats. Raptor acts as a scaffold for mTORC1, not having catalytic activity itself, but is required for activation of mTORC1 and mTOR kinase activity towards most mTOR substrates. Raptor appears to interact with mTOR on multiple sites, not only helping to activate mTOR but also to stabilise the mTOR-raptor complex and inhibit mTOR catalytic function in low nutrient conditions (Hara et al, 2002; Kim et al, 2002; Kwak et al, 2016).

Raptor is not the only scaffolding component needed for mTORC1 formation and stability, with the sub-complex of Tel2 and Tti1 also contributing to this role. Tel2 also binds to mTOR on its HEAT repeats with both Tel2 and Tti1 helping to stabilise, not only mTOR, but other PIKK family members. The Tel2-Tti1 complex interacts with mTOR in both mTORC1 and 2 to stabilise the complexes, with the knockdown of either Tel2 or Tti1 causing the breakdown of both mTORC1 and 2 (Kaizuka et al, 2010; Takai et al, 2007). On top of this, research has shown that heat shock protein 90 (Hsp90), is needed as a chaperone for the Tel2-Tti1 complex, in relation to its function with PIKKs such as mTOR (Izumi et al, 2012; Takai et al, 2010).

The mTOR complexes also contain sub-units that act as inhibitors of mTOR function. Unique to mTORC1 is the proline-rich Akt substrate of 40 kDa (PRAS40), which binds to the complex via raptor. PRAS40 is believed to have an inhibitory effect on mTORC1 function, with most studies showing increased mTORC1/mTOR activity in the absence of PRAS40, although some research suggests this may be more tissue specific (possibly activating mTORC1 in select tissues and cell lines such as HEK-293/kidney cells) (Thedieck et al, 2007; Wiza et al, 2012); PRAS40's inhibitory effect

is speculated to be due to the inhibition of substrate binding (Wang et al, 2007). PRAS40 is part of the mTORC1 complex, but is also a phosphorylation target of mTOR (on Ser183) (Oshiro et al, 2007) as well as the upstream activator Akt (on Thr246) (Kovacina et al, 2003). This phosphorylation increases PRAS40 binding to 14-3-3 proteins, disassociating it from mTORC1, relieving its inhibitory effect on the complex (Oshiro et al, 2007).

DEPTOR is the last major component of mTORC1, but is also found in mTORC2. DEPTOR is also an inhibitor of mTOR function, binding to mTOR's FAT domain via its PDZ domain (Peterson et al, 2009), with research showing an increase in phosphorylation of mTOR targets after DEPTOR knock down (Kazi et al, 2011). DEPTOR regulation is via its degradation, with mTOR signalling triggering the phosphorylation of DEPTOR, leading to its ubiquitination by the E3 ligase, $SCF^{\beta TRCP}$ (Gao et al, 2011; Zhao et al, 2011).

1.6 - The mTOR Pathway: mTORC2 Components

mTORC2 is less studied than mTORC1, but many years of research have begun to elucidate more components and functions of the second complex. Whilst mTORC2 has a very different set of functions to mTORC1, it does contain many of the same subunits in a similar role; these include mTOR, mLST8, DEPTOR and Tel2-Tti1. A defining component of mTORC2 is rictor, which forms the basis of this second complex, along with mTOR and mLST8 (also binding in the HEAT repeats of mTOR). Like mLST8, rictor is needed for proper function of mTORC2 catalytic activity. Rictor also acts as a scaffold for many proteins in the complex, as will be discussed below (Jacinto et al, 2004; Laplante and Sabatini, 2012; Sarbassov et al, 2004). Research by Martin and colleagues (2008) suggests that rictor may act as a point of binding for Hsp70, with this study also implicating Hsp70 as a key regulator of mTORC2 function. Currently this is the only research substantiating the connection between Hsp70 and mTORC2 but future work in a wider variety of cell lines may further shed light on this area and establish Hsp70 as a major mTORC2 component.

mTORC2 has a second scaffold protein, called mSIN1, which binds to the complex via rictor. Like rictor, mSIN1 is suggested to be needed for mTORC2 formation, with it also stabilising mTOR-rictor interaction. mTORC2 targets such as Akt also show markedly decreased phosphorylation in the absence of mSIN1, highlighting its role in regulating kinase activity of the complex (Jacinto et al, 2006; Yang et al, 2006). mSIN1 activity in mTORC2 is believed to be regulated by phosphorylation although the effect of this is debatable. Yang and colleagues (2015) show that phosphorylation of Thr86 has a positive effect on mSIN1 activity; however, other studies have suggested that this same phosphorylation, is in fact a negative regulator of mSIN1 and therefore mTORC2 activity (Liu et al, 2013; Liu et al, 2014a).

Protor 1 and 2 are the last major components of mTORC2. Protor-1 and 2 bind to rictor within the complex, but, unlike rictor or mSIN1, they are not needed to stabilise parts of the complex (Pearce et al, 2007; Thedieck et al, 2007; Woo et al, 2007). Protor-1 appears to play a role in regulating mTORC2 activity towards one of its substrates, SGK1, with a markedly decreased phosphorylation of this target in protor-1 absence (Pearce et al, 2011; Woo et al, 2007). Like protor-1, protor-2 also appears to modulate mTORC2 in a substrate specific manner; with work by Gan et al (2012) showing protor-2 may suppress mTORC2 phosphorylation of PKC.

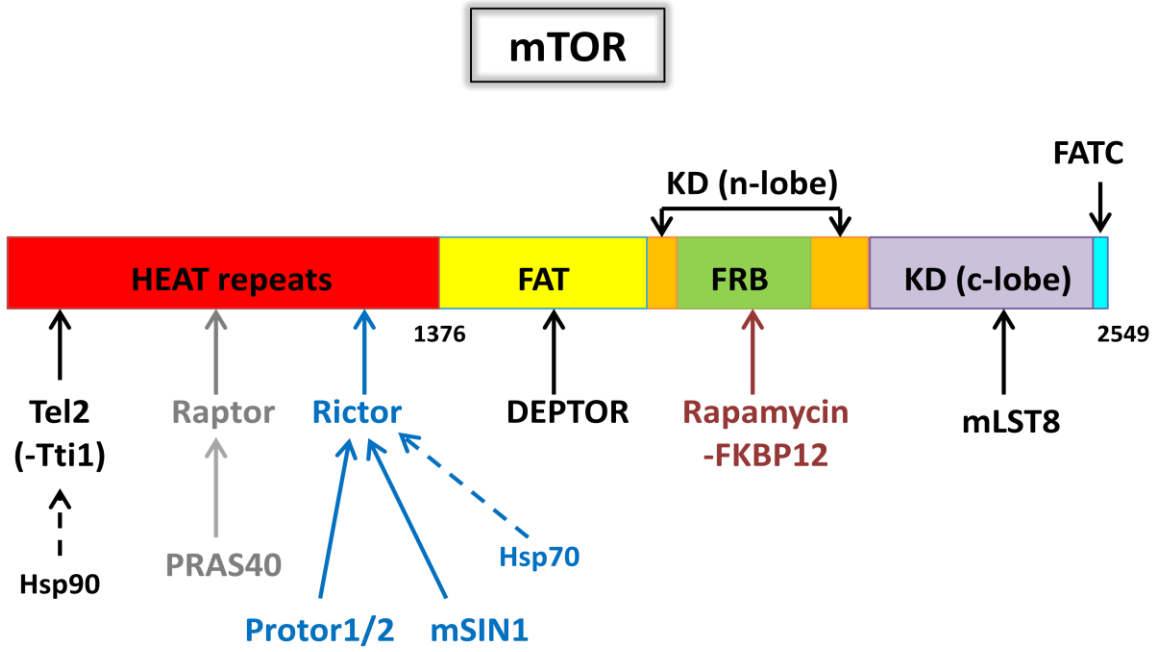


Figure 1.4: Basic structure of the 2549 residue protein, mTOR. The components of mTORC1 and 2 are marked as to which mTOR domain, or complex protein, they bind to. Components of both complexes are marked in black, specific mTORC1 components in grey and specific mTORC2 components in blue. Information from Izumi et al (2012); Knutson, (2010); Laplante and Sabatini, (2012); Martin et al, (2008); Sauer et al, (2013); Yang et al, (2013) Takai et al, (2010).

1.7 - The mTOR Pathway: Upstream of mTORC1

mTORC1 has a variety of upstream pathways which control its activation, including growth factor signalling, amino acid levels, cellular energy levels and stress (reviewed in Sengupta et al, 2010). The tubular sclerosis complex (TSC) is a convergence point for many of these upstream factors and is a key regulator of mTORC1 activity. The complex consists of TSC1 (also known as Hamartin), TSC2 (also known as Tuberin) and TBC1D7 (Dibble et al, 2012). The TSC is effectively an inhibitor of mTORC1, via the protein Rheb (ras-homolog enriched in brain). Rheb functions to bind to and activate mTORC1 (the exact mechanism of Rheb action is not precisely understood), when Rheb is bound to GTP. TSC (with TSC2 being the catalytic part of TSC) acts as a GTPase activating protein (GAP) for Rheb, causing it to convert bound GTP to GDP and therefore no longer activate mTORC1 (Inoki et al,

2003a; Tee et al, 2003). The GEF for Rheb has remained elusive, although work by Maeurer and colleagues (2009) suggests this may be the E3 ubiquitin ligase PAM. Lysosomal localisation is important for mTORC1 activation (as will be discussed below) with recent research suggesting that the phosphorylation of TSC actually causes TSC to dissociate from the lysosome, away from mTORC1 and Rheb, thus helping to activate mTORC1 (Zheng et al, 2014).

The PI3K pathway is a key upstream regulator of mTORC1, via the TSC. Growth factors such as insulin-like growth factor 1 (IGF-1) and insulin activate phosphoinositide 3-kinase (PI3K), which in turn generates PIP_3 from membrane bound PIP_2. This recruits downstream effectors such as PDK1 and Akt (also known as protein kinase B) via their pleckstrin homology (PH) domains. Akt can then be activated by PDK1 via phosphorylation on Thr308 and Ser473 (Dibble and Cantley, 2015). Akt is a critical regulator of TSC, with active Akt phosphorylating TSC2 at multiple sites, to weaken its interaction with TSC1 and destabilise the TSC2 protein. This results in activation of mTORC1 as TSC2 can no longer act as GAP for Rheb (Inoki et al, 2002; Potter et al, 2002). Akt also regulates mTORC1 activity by phosphorylating PRAS40, causing it to bind to 14-3-3 proteins, thus relieving its inhibitory effect on the complex (Wiza et al 2012).

Interestingly mTOR itself is also phosphorylated at multiple sites, including a level of auto-phosphorylation at Ser2481 (Soliman et al, 2010), with some of this phosphorylation induced by growth factor signalling. Research suggests many of these phosphorylated sites (such as Ser2448) increase mTOR activity and may be needed for normal mTORC1 function (Acosta-Jaquez et al, 2009; Ekim et al, 2011; Huang and Fingar, 2014, Rosner et al, 2010). Intriguingly, work by Copp and colleagues (2009), suggested that Ser2481 phosphorylation of mTOR may act as a good biomarker for intact mTORC2 complexes; their work showed that mTORC2 had predominantly Ser2481 phosphorylation, whilst mTORC1 had predominantly Ser2448 phosphorylation.

The Ras-Erk MAPK pathway can also lead to downstream activation of mTORC1. Like PI3K, signalling via this pathway can be activated by various growth factors such as IGF-1 and insulin. As is the case with virtually all membrane receptors, more

than one downstream pathway is activated, as evidenced by these growth factors activating multiple signalling cascades (Mendoza et al 2011). Once Erk is activated, it can directly phosphorylate and inactive TSC2 on Ser664 (Ma et al, 2005; Saini et al 2013). However, Erk can also activate p90 ribosomal S6 kinase 1 (RSK1), which can phosphorylate TSC2 at Ser1798 and again inactivate it (Roux et al, 2004).

Amino acids are critical regulators of mTORC1 function, with increased levels of amino acids leading to mTORC1 activation, with growth factors unable to activate mTORC1 without sufficient levels of amino acids (Huang and Fingar, 2014). Research by Hara and colleagues (1998) has shown how different amino acid concentrations precisely effect mTORC1 activation. The Rag GTPases are central to this regulation, acting as dimers of either RagA or B dimerised with either Rag C or D. In its active state, this complex has RagA/B GTP bound whilst RagC/D is GDP bound (Bar-Peled and Sabatini, 2014; Duan et al, 2015). In this state the complex binds raptor, to localise mTORC1 to the lysosome, bringing it into contact with Rheb (which is also localised to the lysosome), to activate mTORC1 (Huang and Fingar, 2014; Sancak et al, 2008). Localisation at the lysosome is critical to mTORC1 activation and the ragulator complex is needed to localise the Rag GTPases to the lysosomal surface; the ragulator complex consists of the proteins p18, p14, MP1, HBXIP and C7orf59 (Bar-Peled et al, 2012; Sancak et al 2010).

The activation state of Rag proteins are regulated by proteins such as the GATOR1 and 2 complexes and the folliculin (FLCN)/FNIP1/2 complex (Bar-Peled et al, 2013; Tsun et al 2013). How the cell exactly translates amino acid levels to mTORC1 activation is not well understood, but many proteins are now being revealed to have roles in this amino acid sensing. The molecular pump v-ATPase is required for activation of mTORC1, with it directly interacting with the ragulator complex and in turn amino acids directly regulating this interaction (Zoncu et al, 2011). Of interest is work by Pena-Llopis and colleagues (2011) which showed that mTORC1 may be involved in a positive feedback loop, with mTORC1 activation increasing v-ATPase expression. It is probable that the full extent of the amino acid sensing ‘machinery’ (in relation to mTORC1) is yet to elucidated, but as research continues new proteins are being shown to have roles in this capacity, such as MAP4K3 (Findlay et al,

2007), SLC38A9 (Jung et al, 2015; Rebsamen et al, 2015) and PAT1 (SLC36A1) (Ogmundsdottir et al, 2012).

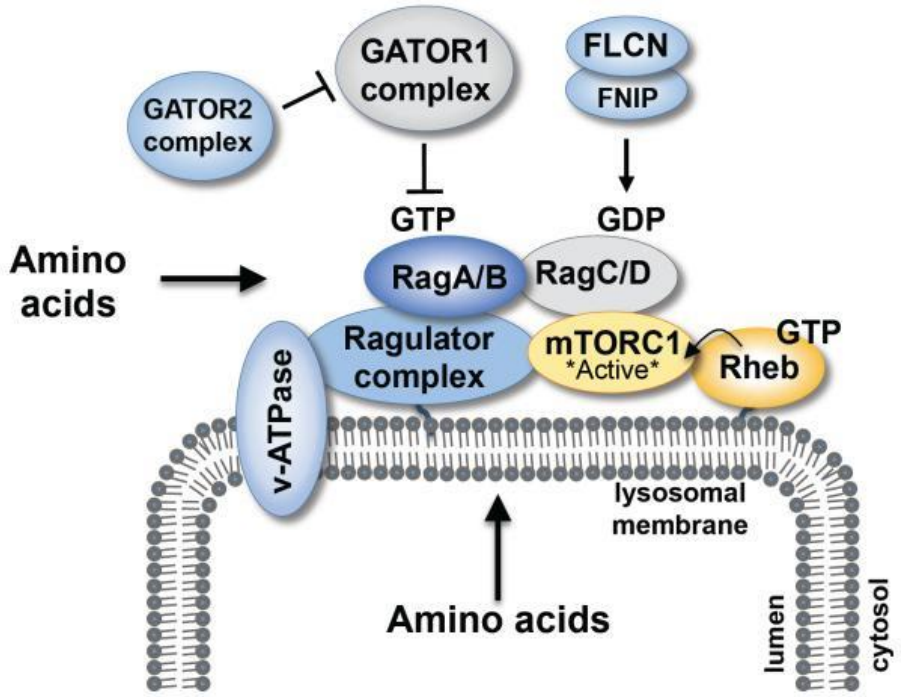


Figure 1.5: The basic 'machinery' needed for amino acid related activation of mTORC1. The complex of either RagA or B with Rag C or D helps tether mTORC1 to the lysosome, where its activator Rheb resides. For the Rag GTPases to do this RagA/B must be GTP bound whilst RagC/D must be GDP bound. The GATOR1 complex of proteins acts as a GAP for Rag A/B, thus inactivating them, although the activity of GATOR1 is inhibited by the GATOR2 complex. Folliculin (FLCN) in complex with FNIP1 and 2 act as a GAP for RagC/D, thus activating them. The ragulator complex of proteins, keeps the Rag GTPases bound to the lysosome. Amino acid sensing proteins such as v-ATPase help transmit amino acid levels directly to mTORC1 activation (Bar-Peled et al; 2013 Huang and Fingar, 2014; Tsun et al, 2013. Figure from Huang and Fingar, 2014).

Cellular energy levels also regulate mTORC1 activity, with low energy generally inhibiting mTORC1, and reducing protein synthesis. This is mainly via cellular levels of AMP increasing, when ATP is low, which in turn activates AMPK. Activated AMPK phosphorylates raptor, causing it to bind to 14-3-3 proteins, sequestering it away from mTORC1 (Gwinn et al, 2008). Activated AMPK also phosphorylates TSC2 on Thr1227 and Ser1345 to activate (rather than inactivate, as is the case when Akt

phosphorylates TSC2 on Ser924 and Thr1518) the TSC to further decrease mTORC1 signalling (Inoki et al, 2002; Inoki et al, 2003b). Since downstream mTORC1 activates protein synthesis (as will be discussed below) it is important the cell only activates mTORC1 signalling when it has the required resources, such as ATP/energy and amino acids. Lower cellular oxygen levels and other cellular stresses also reduce the activity of mTORC1. For example stress such as hypoxia can induce regulated in DNA damage and development 1 (REDD1), which inhibits mTORC1 function (Sofer et al, 2005).

1.8 - The mTOR Pathway: Upstream of mTORC2

Although knowledge of mTORC2 signalling is by and large less defined than mTORC1, research is beginning to fill in gaps in our knowledge. It has been known for a while that like mTORC1, mTORC2 is activated by growth factors such as insulin and IGF-1 (Cybulski and Hall, 2009), although only mTORC2 complexes containing mSIN1 isoforms 1 and 2 (not 5) are activated by insulin (Frias et al, 2006). Recent research has shown that mSIN1 is a critical mediator for growth factors to activate mTORC2, with PI3K signalling linking the two. Membrane bound PIP3, produced by PI3K signalling, binds mSIN1 via its PH domain, relieving its interactions with mTORC2, thus activating it (Liu et al, 2015a; Yuan and Guan, 2015). This research seems at contrast to research by Jacinto and colleagues (2006) and Yang and colleagues (2006) which shows that mSIN1 is needed for mTORC2 activity. These seemingly conflicting reports highlights our relatively poor understanding on the precise mechanism of mTORC2 action and activation.

Active PI3K signalling promotes mTORC2 binding to ribosomes (active mTORC2 bound to the ribosomes); possibly as a mechanism to limit its activation only in growing cells with a high enough ribosome content (Zinzalla et al, 2011). Remarkably, whilst the TSC inhibits mTORC1 function, research suggests that in at least some cell lines (including the breast cancer cell line MCF7) the complex is needed for proper mTORC2 activation, as well as physically interacting with mTORC2, independent of its function with Rheb (Huang et al, 2008).

1.9 - The mTOR Pathway: Downstream of mTORC1

The molecular and cellular effects of mTORC1 activation are well characterised, with a number of processes regulated from this point. The translation of proteins/protein synthesis is critically regulated by mTORC1. mTORC1 phosphorylates S6 kinases including S6K1 and 2, with S6K1 having isoforms including p70-S6K1 and p85-S6K1. (p70-) S6K1 is first phosphorylated on multiple sites which subsequently allows mTORC1 to phosphorylate it on Thr389. PDK1 then phosphorylates S6K1 on Thr229 to fully activate the kinase (Ma and Blenis, 2009). S6K1/2 then phosphorylates ribosomal protein S6 (rpS6), part of the 40s ribosomal unit, with rpS6 needed for protein synthesis. S6K1 also phosphorylates multiple other proteins involved in translation and ones that form the translation machinery, including eIF4B (Tavares et al, 2015). S6K1 activation is also believed to promote transcription via its interactions with transcription factors such as ERα, as well as S6K1 regulating ribosomal gene transcription (Hannan et al, 2003; Tavares et al, 2015). Unsurprisingly negative, feedback loops exist along the mTORC1 axis involving S6K1, with the active protein both repressing the expression of insulin receptor substrate 1 (IRS-1) and phosphorylating it on inhibitory serine residues (Magnuson et al, 2012). mTORC1 also serves to feedback onto mTORC2, with S6K directly phosphorylating rictor, which may serve to control activation of Akt (Dibble et al, 2009).

mTORC1 also controls translation via phosphorylation of the eIF4E- binding proteins (4E-BPs). When un-phosphorylated, the 4E-BPs bind to eIF4E and stop its subsequent binding to eIF4G; a pre-requisite for the formation of the pre-translation machinery (Ma and Blenis, 2009). mTOR phosphorylates 4E-BP1 on sites including Thr37, 46 and 70 and Ser65, with other phosphorylation sites also existing on 4E-BP1. This phosphorylation then stops the inhibitory action of the 4E-BPs on eIF4E to allow the latter, which is bound to the m^7 G-cap structure at the 5' end of mRNA, to bind subsequent factors and initiate cap-dependent translation (Gingras et al, 2001; Hay and Sonenberg, 2004).

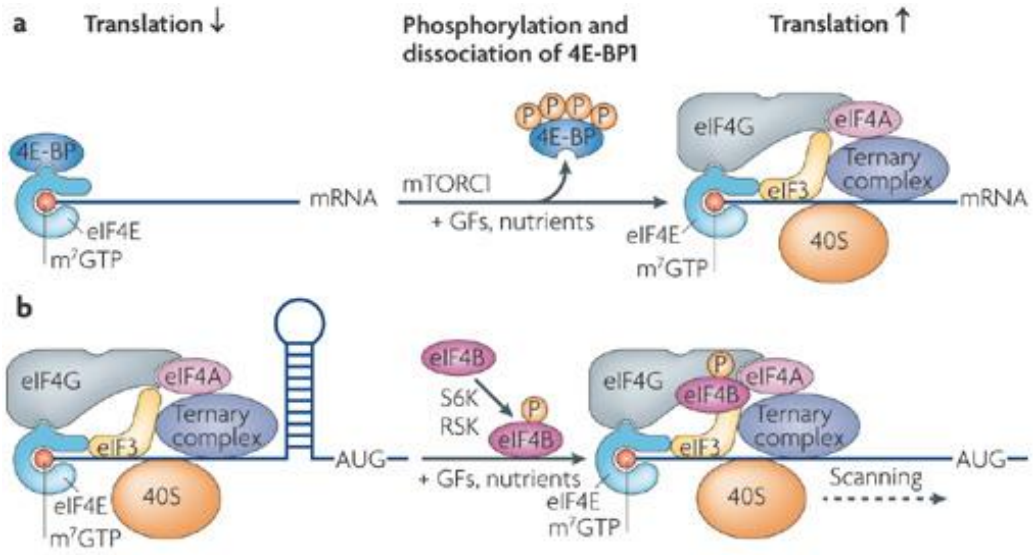


Figure 1.6: (a) When in a hypo-phosphorylated state, 4E-BPs such as 4E-BP1 bind the translation initiation factor eIF4E, which itself binds to the m^7 G-cap structure at the 5' end of mRNA. With 4E-BPs bound, eIF4G cannot bind to eIF4E. When mTORC1 is activated by growth factors (e.g. insulin) and an adequate supply of amino acids, it phosphorylates 4E-BPs, causing them to dissociate from eIF4E. eIF4G can now bind and the pre-translation complex/machinery form at this point, including the binding of further factors such as eIF4A. eIF3 and the ternary complex of proteins subsequently bind, as well as the 40s ribosomal subunit , to allow the complex to begin scanning the mRNA and begin cap-dependent translation. (b) mRNA often forms hair-pins and loops within itself and the helicase activity of eIF4A is needed to unwind these and allow translation to continue. Its co-factor eIF4B helps stimulate this process. When mTORC1 is activated, in the previously mentioned conditions, it phosphorylates S6K1, which then phosphorylates eIF4B to allow its recruitment to the pre-translation complex (Ma and Blenis, 2009; Wilson and Cate, 2012. Figure from Ma and Blenis, 2009).

Autophagy is an important cellular process, by which materials (such as damaged organelles) are delivered to the lysosome, degraded and often recycled, with mTORC1 having critical regulation over this, as well as protein synthesis (Mizushima and Komatsu, 2011). Autophagy is generally not needed when the cell is healthy and has plenty of nutrients, thus nutrients activating mTORC1 causes an overall inactivation of autophagy. mTORC1 phosphorylates the kinases ULK1/2 (ULK1 on

Ser758) as well as ATG13. These proteins usually form a complex with FIP200 to initiate autophagy and the phosphorylation of ULK reduces its kinase activity, leading to a reduction in autophagy (Dunlop and Tee, 2014; Jung et al, 2009; Kim and Guan, 2015).

The ULK complex also cross-talks with another set of proteins, known as the beclin1 or VSP34 complex, which is also involved in initiating autophagy. mTORC1 can phosphorylate a member of this complex called AMBRA1, to reduce ubiquitination of ULK1 by the VSP34 complex protein, TRAF6. Rather than destroy the protein, this ubiquitination actually increases its activity (Nazio et al, 2013). As mentioned previously, AMPK reduces mTOR signalling via its inhibition of mTOR, which in turn reduces its inhibitory effect on autophagy. AMPK also phosphorylates ULK1 on a number of sites to actually stimulate the ULK1 activity. Thus AMPK increases autophagy in cellular stress, in opposition to the mTOR pathway (Egan et al, 2011).

Aside from these functions, mTORC1 is also partially involved in regulating other important cellular processes related to metabolism. Research has shown mTORC1 helps regulate lipid metabolism, including synthesis, storage as well as adipocyte function (Ricoult and Manning, 2013). mTORC1 exerts control over the activity of SREBPs, which along with protein synthesis, can contribute to the way in which mTORC1 activity increases cell mass (Porstmann et al, 2008). Active S6K1 phosphorylates CAD-S1859, which stimulates de novo synthesis of pyrimidines (Robitaille et al, 2013). Active mTORC1 also stimulates metabolism via control of hypoxia inducible factor (HIF1α), which in turn can activate pathways such as glycolysis (Dodd et al, 2015; Duvel et al, 2010; Hudson et al, 2002).

1.10 - The mTOR Pathway: Downstream of mTORC2

As has been demonstrated already, mTORC2 signalling is not as well as characterised as mTORC1 signalling, with mTORC2 importance not wholly understood. However, mTORC2 does regulate the activity of several proteins belonging to the AGC kinase family. mTORC2, can in one sense, be thought of as ‘upstream’ of mTORC1 as it is one of the many regulators of the AGC kinase, Akt. Akt has many downstream effectors of its own, increasing proliferation, cellular

growth (e.g. its role in mTORC1 activation via TSC2), cell survival, angiogenesis and metabolic processes (Manning and Cantley, 2007). mTORC2 directly phosphorylates Akt on Ser473, which is required for its maximal activation (Sarbassov et al, 2005). However mTORC2 is not the only activator of Akt, with research showing that Akt substrates such as FoxO1 had activation impaired by mTORC2 depletion, but others such as GSK3β were not affected (Guertin et al 2006; Jacinto et al, 2006).

mTORC2 also phosphorylates the AGC kinase SGK1, to help control its activity. SGK1 has multiple roles, downstream of mTORC2 control. These include the regulation of proliferation and apoptosis via FoxO3a (Mori et al, 2014), ion channels such as Na^+ (Lang and Pearce, 2016) and regulating differentiation in cell types such as TH1 and TH2 immune cells (Heikamp et al, 2014). mTORC2 can effect cellular shape, structure and morphology, specifically by altering the actin cytoskeleton, with part of this control, at least, down to mTORC2 regulation of PKC, another member of the AGC kinases (Angliker and Ruegg, 2013; Jacinto et al, 2004; Sarbassov et al, 2004).

As well as associating with ribosomes (Zinzalla et al, 2011), mTORC2 also associates with the endoplasmic reticulum endoplasmic reticulum sub-compartment called the mitochondria-associated endoplasmic reticulum (MAM). This sub compartment is a key part of calcium and lipid transfer (and thus assists the regulation of apoptosis) in this region of the cell, with mTORC2 deficiency directly leading to a disruption of these functions and MAM integrity (Betz et al, 2013).

1.11 - mTOR Signalling in Cancer

Looking at the multitude of cellular events mTOR complexes help regulate, it is of no surprise that the activation of mTOR signalling is associated with cancer and is perceived as being oncogenic. The activation of mTOR complexes will give tumours a vast growth advantage, with an increased amount of protein synthesis, as well increased inhibition of autophagy. Thus whilst growing at an increased rate, these cells are also less likely to die. Research has generally shown that activated mTOR signalling leads to an increase in tumour progression and often a decrease in patient survival (Chiang and Abraham, 2007; Xu et al, 2014).

The activation of mTOR signalling in cancer cells is associated with resistance to multiple drug therapies, especially in breast cancer where this effect is well studied. Resistance to the SERM, tamoxifen, is a common issue (Droog et al, 2013). Whilst there are multiple mechanisms behind this resistance, mTOR appears to have a major role, with the mTOR pathway phosphorylating ERα at Ser118, making it hyper sensitive to activation and less likely to bind tamoxifen (Viedma-Rodriguez et al, 2014). Research has shown that in the long term, breast cancer cells may use the PI3K/Akt/mTOR axis to escape dependency from ER signalling and thus increase their resistance to tamoxifen (Miller et al, 2010). Inhibiting the mTOR pathway has been shown to also help re-sensitise cells to anti-cancerous effects of tamoxifen (deGraffenried et al, 2004).

mTOR signalling has also been linked with resistance to HER-2 therapies in breast cancer, such as with the drug trastuzumab (Margariti et al, 2011), and lapatinib (Brady et al, 2015). Since the mTOR pathway is a key downstream effecter of the ErbB family receptors like HER-2, cells appear to often find other ways to activate mTOR signalling after these receptors have been inhibited. This can include mutations in the PI3K pathway and the use of other growth factor receptors like IGF-1R (in which HER-2-IGF-1R dimers can form), which can all contribute to drug resistance (Gagliato et al, 2016; Thery et al, 2014). Since mTOR does lie downstream of HER-2, it is of no surprise that *in vivo* studies have shown an increased effect when rapamycin is used with trastuzumab (Miller et al, 2009) with some clinical trial data also supporting this theory (discussed later in relation to mTOR inhibitors).

mTOR signalling is also involved in drug resistance/sensitivity in other cancer types. This includes mTOR activation in GI stromal tumours possibly causing resistance to imatinib (Li et al, 2015) and rapamycin increases sensitivity to cetuximab in hepatoma cells (Chen et al, 2014b).

In terms of how the mTOR pathway is altered in cancer, it is found that the majority of alterations and mutations lie upstream of mTOR itself and lead to an increased activation of mTOR signalling overall. Common in many cancers, are alterations to

PI3Ks, which are key activators of mTOR via Akt and TSC1/2 and have been shown to cause over activation of mTOR signalling (Moschetta et al, 2014). The *PIK3CA* gene (which encodes a subunit of PI3K) is often amplified in ovarian cancer and activating mutations have been found to be common in breast, colorectal, endometrial and gastric cancers and are usually centred in kinase domains (His1047) and helical domains (Glu542, Glu545) (McCubrey et al, 2012). PI3K pathway mutations have also been shown to be common in metastatic melanoma (Shull et al 2012). Other common mutations upstream of mTOR occur in *AKT*, with altered or mutated *AKT* detected in breast, pancreatic, prostate and gastric cancers (Strimpakos et al, 2009). Whilst Akt regulates a multitude of cellular events, its control over mTOR signalling is important and its alteration can lead to increased mTOR signalling.

PTEN is a negative regulator of PI3K signalling and is a commonly mutated tumour suppressor gene; its loss of function leads to increased mTOR activation. Whilst this loss of function is seen in sporadic cancers such as those from breast and prostate (Strimpakos et al, 2009), its mutation in a familial sense is the cause behind approximately 70% of Cowden Syndrome cases. Patients exhibit multiple hamartomas (a form of benign tumour) but also have increased risk of developing sporadic cancers of the breast, thyroid and kidneys (Chiarini et al, 2015). The negative regulators of mTOR activation, *TSC1* and *TSC2* are involved in the familial disease for which they are named. Most patients show loss of function of one of these genes, leading to benign growths/hamartomas (Curatolo et al, 2008).

Mutations and alterations of core mTOR components (involved in either of the two mTOR complexes) are by and large a lot rarer than upstream mutations, but have still been noted in cancers, within the last few years. With the availability of more powerful sequencing technology combined with large online databases containing sequencing data, many research groups have been able to identify mutations in mTOR itself (Grabiner et al, 2014; Hardt et al, 2011; Sato et al, 2010). These pieces of research have shown that mutations have occurred in a variety of cancer types and whilst these alterations occur along the length of mTOR (figure 1.7), a high frequency have been found in domains such as the FAT and FATC domains. Since the latter forms part of the kinase domain, it is no surprise that many of the mutations

identified in this research resulted in either increased mTORC1 or 2 activity. Some mutations in *MTOR* also showed decreased binding to the inhibitor DEPTOR, possibly due to mutations in the FAT domain (Grabiner et al, 2014).

Research using tumour samples has also detected mutations in mTOR, with work by Shull et al (2012) showing *MTOR* mutations in metastatic melanoma and work by Kwiatkowski et al (2016) showing mutations in renal carcinoma. Interestingly, the latter piece suggested that these mutations also correlated with rapalogue responsiveness, showing how mutations may in fact one day be used as biomarkers. In relation to its role in causing drug resistance, mTOR components can be differentially regulated in drug resistant cells, with ovarian cancer cells resistant to paclitaxel showing altered expression of many key mTOR proteins (Foster et al, 2010).

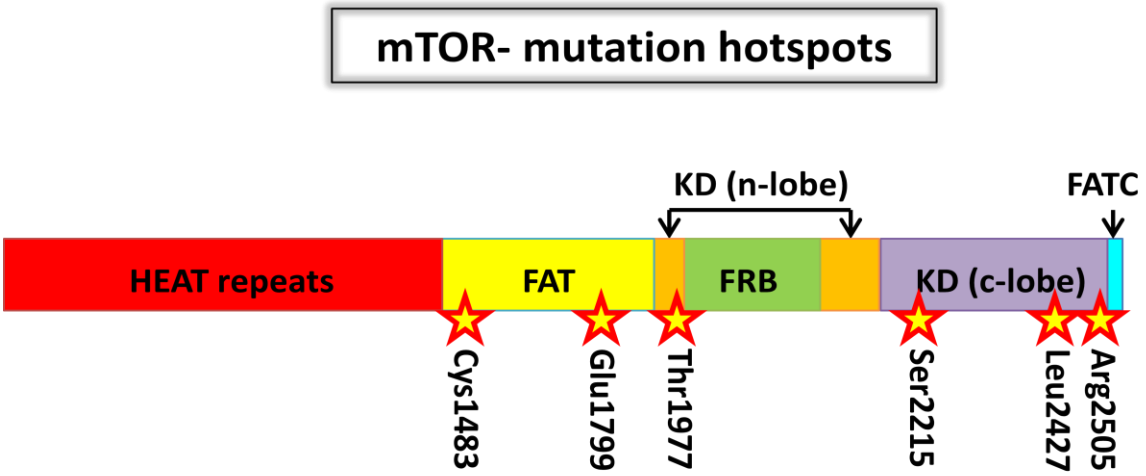


Figure 1.7: Common mutation hotspots along mTOR. Information from Grabiner et al, (2014).

A change in expression of mTOR proteins has been also been noted although it appears that the up or down regulation of specific components may be highly tissue dependent. Squamos cell carcinomas have shown up-regulation of phospho-mTOR (p-mTOR) (Ferrandiz-Pulido et al, 2013), whilst endometrial cancers showed down-regulation of p-mTOR (Kourea et al, 2015). mLST8 expression also appears to correlate with activity of mTOR pathways (Kakumoto et al, 2015). mTORC2 specific

proteins have also been shown to sometimes be up-regulated in cancers, including mSin1 (Moraitis et al, 2014) and rictor (Cheng et al, 2015).

Despite DEPTOR being an mTOR inhibitor, higher DEPTOR expression appears to correlate with a poorer prognosis in some esophageal carcinomas (Liu et al, 2015b) as well as being over expressed in multiple myeloma cells (Peterson et al, 2009). This seems to be in contrast to DEPTOR's role as an inhibitor and with the work by Grabiner et al (2014) showing decreased DEPTOR binding in many cancers. The work by Peterson et al (2009) also showed how DEPTOR over-expression might in fact stop negative feedback on PI3K, causing an activation of PI3K-mTOR signalling, so this may help explain this effect. Again this may reflect the seemingly high tissue dependent nature of mTOR signalling (with different tissues utilizing and using certain components more than others) but also of a poorer understanding of DEPTOR's exact role within carcinogenesis and cells in general.

Considering that DEPTOR's role in mTOR signalling was only discovered relatively recently (Peterson et al, 2009), it is possible that there are still mTOR complex components that have not yet been discovered. If this is the case, it may also explain why there is seemingly conflicting data relating to the role some of these proteins, if there are as yet undiscovered interactions. Research by Luo and colleagues (2015a) found that rapamycin can inhibit mSin1phosphorylation independently of mTORC1 or 2 (raptor and rictor are not required), but the mechanism of inhibition does involve mTOR and mLST8. This again suggests that there may be further mTOR complexes yet to be discovered, that explain the observed effect.

1.12 - mTOR Signalling in Breast Cancer

mTOR involvement in breast cancer has already been mentioned, but there are many alterations that are common features in breast cancer specifically. As is the case with most cancers, mTOR activation in breast cancer is caused by upstream activation. A key biomarker in breast cancer is the relative expression of HER-2. Since HER family receptors can activate PI3K-mTOR signalling, HER-2 expression is important in the over-activation of mTOR signalling in breast cancer. *HER2* is amplified in upwards of 15-20% of all breast cancers, which can result in a nearly

100 fold increase of protein expression. Its status as a key biomarker comes from that fact that HER-2 expression correlates with a much poorer prognosis and a generally more aggressive cancer (Iqbal and Iqbal, 2014; Wu et al, 2015). A truncated form of HER-2 which lacks its extracellular domain, called p95HER-2, has also been observed in breast cancer patients. Work by Molina and colleagues (2002) have showed that in a study of over 300 tumour samples, 20.9% expressed p95HER-2 if node negative but 29.1-36.7% expressed if node positive.

Like its family member, EGFR expression is also commonly altered in breast cancer patients, with EGFR over-expression also correlating (but not high gene copy number) with a poorer prognosis (Lee et al, 2015). EGFR appears to be relatively commonly expressed, with 17.1% of a study of 706 invasive ductal breast carcinomas, having expression of EGFR (Hwangbo et al, 2013). Interestingly expression of EGFR appears to correlate well with HER-2 over-expression, suggesting a therapeutic benefit to inhibiting both types of receptor (Kolesta et al, 2010). HER-3 (EGFR3) and HER-4 (EGFR-4) have also been shown to be up-regulated in breast cancers, although prognosis may actually be improved in HER-4 expression, whilst HER-3 expression again correlated for a worse patient outcome (Witton et al, 2003).

The relative activity/expression of mTOR and its related components also has valid prognostic significance in breast cancer, revealing the importance of this pathway in this cancer type. Research generally suggests that increased mTOR expression correlates for a worse prognosis in breast cancer (Lo et al, 2012; Wazir et al, 2013) with work by Walsh and Colleagues (2012) showing that p-mTOR was more common in triple negative breast cancers. Despite the fact that mTORC2 signalling can increase oncogenic signals via Akt and mTOR signalling, research has suggested that rictor expression, which is required for mTORC2 signalling, is actually lower in breast tumours compared to normal breast tissue (Wazir et al, 2013). This could suggest that mTORC1 signalling is more oncogenic than mTORC2 signalling or that rictor is required in very specific amounts for mTORC2 signalling; with too much or too little ultimately inhibiting the mTORC2 arm.

1.13 - mTOR Therapies: Rapalogues Overview

Since its identification, over four decades ago, rapamycin has been studied as a therapy for a wide variety of diseases. With it being the first mTOR inhibitor to be discovered, work on rapamycin led to a new field devoted to elucidating compounds that inhibited the mTOR pathway. Currently, the most widely used set of compounds are rapamycin and its analogues that are more commonly known as 'rapalogues'. Rapamycin (structure shown in figure 1.8), also known as sirolimus, is a macrocyclic lactone, isolated from the bacterium *Streptomyces hygroscopicus* initially noted for its strong anti-fungal effect (Vezina et al, 1975). It was later found to have strong immunosuppressive effects, blocking T-cell activation (Dumont et al, 1990) and in 1999 was approved for use as an immunosuppressant drug in the USA (Zhou et al, 2010); it is used in procedures such as kidney transplantation, to reduce rejection, risk of infections and also to lower the incidence of post-surgery cancer (Yanik et al, 2015).

Due to its inhibitory effect on mTOR, and thus cellular growth, rapamycin was explored as an anti-cancer agent. It was shown to inhibit cellular proliferation and/or be effective in several types of cancer including pancreatic (Xu et al, 2015), colon (Eng et al, 1984), rhabdomycosarcoma (Dilling et al, 1994) and breast (Zhou et al, 2010). However, rapamycin has on the whole not been taken forward for cancer therapy due to its poor pharmacokinetic properties, including its low solubility (Bjornsti et al, 2004).

Rapalogues have since been developed to tackle these issues, opening up new avenues for treatment, not only cancers, but for a variety of other conditions as well. These include everolimus (RAD-001), temsirolimus (CCI-779), ridaforolimus (deforolimus, AB23573) and zotarolimus (ABT-578). Details of these rapalogues can be found in table 1.2.

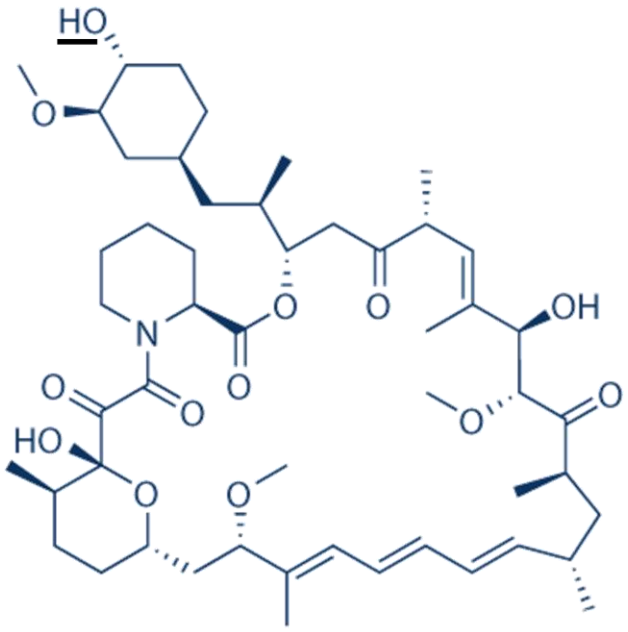


Figure 1.8: Structure of rapamycin (sirolimus). Rapalogues vary from rapamycin mostly on one small side group. This occurs as an O-substitution at carbon-40 on rapamycin, underlined on the primary structure (Ballou and Lin, 2008; Selleckchem, 2016).

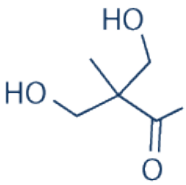
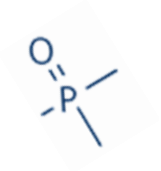
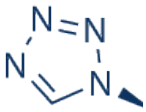
Rapalogue	Side Chain (O-substitution on rapamycin at carbon 40)	Description	Clinical uses	References
Everolimus (RAD-001)		2-hydroxyl-ethyl side chain. Increased solubility over rapamycin, with bioavailability around 15% higher. Administered orally	Approved for use in breast cancer, renal cell carcinoma and neuroendocrine tumours of the pancreas, lungs or gut. Used for kidney transplants and tested in lung and heart transplants	Bjornsti et al, 2004; Carmellini et al, 2015; European Medicines Agency, 2016a; Granata et al, 2016; Kirchner et al, 2004; Kobashigawa et al, 2013; Moes et al, 2015;Parada et al, 2011
Temsirolimus (CCI-779)		Dihydroxymethyl-propanoic acid side group. Increased solubility over rapamycin. Administered orally or IV	Approved for use in renal cell carcinoma and mantle cell lymphoma. Tested at Phase II and III levels in breast cancer	Ballou and Lin, 2008; Chan et al, 2005; Dudkin et al, 2001; European Medicines Agency, 2016b; Wolff et al, 2013
Ridaforolimus (Deforolimus, AP23673)		Phosphine-oxide side group. More soluble than rapamycin in water and organic solvents	Phase II and III testing against sarcomas	Mita et al, 2008; Vignot et al, 2005
Zotarolimus (ABT-578)		Tetrazole side ring replacing hydroxyl group	Inhibits growth of coronary smooth muscle cells and is used to treat stenosis in drug eluting stents	Burke et al, 2006; Chen et al, 2007; Raungaard et al, 2015; Zhou et al, 2010

Table 1.2: Details, including clinical uses of the rapalogues everolimus (RAD-001), temsirolimus (CCI-779), ridaforolimus (deforolimus, AB23573) and zotarolimus (ABT-578).

1.14 - mTOR Therapies: Rapalogue Mechanism of Action

Rapalogues all inhibit mTOR, using the same mechanism of action, which involves the intracellular receptor and immunophilin, FK506 binding protein 12 kDa (FKBP12). FKBP12 binds FK506, and mediates immunosuppressive actions via its alteration of the phosphatase calcineurin, with FKBP12 able to regulate cellular levels of Ca^{2+} (Cameron et al, 1995; Lee et al, 2014). FKBP12 inhibits several important membrane receptors such as TGF-beta type-I receptors (TGFβR1) (Chen et al, 1997; Wang and Donahoe, 2004) and epidermal growth factor receptors (EGFR) (Mathea et al, 2011). FKBP12 also mediates many intracellular proteins, including regulating the trafficking of H-Ras (Ahearn et al, 2011).

FKBP12 was shown early on to bind rapamycin, and mediate its action through its binding to mTOR, causing an inhibition of cell cycle progression (Brown et al, 1994). The FKBP12-rapamycin complex binds to mTOR at the FRB domain, acting through allosteric inhibition and conformational changes in mTOR and mTORC1 (Chen et al, 1995; Choi et al, 1996; Yang et al, 2013). Research suggests that these changes then lead to a decrease in the interaction between mTOR and raptor (Oshiro et al, 2004) which would in theory inhibit the phosphorylation and activation of the major mTORC1 downstream targets including S6K and 4E-BP1. Whilst this is true, more in depth research about events post-rapalogue treatment has revealed a differentiation in the amount of inhibition actually seen on each mTORC1 substrate, with the levels of inhibition of 4E-BP1 phosphorylation compared to S6K varying greatly over time and cell types (Choo et al, 2008; Choo et al, 2009). Interestingly, the level of auto-phosphorylation on mTOR in mTORC1 (but not mTORC2) on Ser2481 is also greatly reduced upon rapamycin treatment (Soliman et al, 2010). Of course treatment of rapalogues will have wider positive molecular effects upon a cell, beyond its effect on translation; for example, in ovarian cancer cells, everolimus treatment has been shown to also reduce the expression of myc (Mabuchi et al, 2007).

Rapalogues were long thought to only inhibit only mTORC1 complexes and their downstream effectors, with evidence at the time backing this theory up (Jacinto et al, 2004). However more in depth study of rapamycin's effect on mTORC2 has revealed that prolonged treatment does in fact inhibit mTORC2 as well as mTORC1, with

rapamycin treatment directly effecting the assembly of mTORC2 components, including rictor. Therefore rather than binding directly to mTORC2, like it does mTORC1, the FKBP12-rapamycin complex binds mTOR and then over time stops the formation of new mTORC2 complexes (Rosner and Hengstschlager, 2008; Sarbassov et al, 2006; Schreiber et al, 2015).

At a cellular level, rapalogues show many effects useful for the treatment of cancer. Due to the inhibition of protein translation, growth of cells can be severely affected, limiting progression through the cell cycle, usually at the G_1 phase, and ultimately inhibiting tumour growth (Brown et al, 1994; Easton and Houghton, 2006). Rapalogues have shown this growth inhibitory effect in a wide variety of cells, with rapamycin inhibiting the growth of cancer cells including prostate (Van der Poel et al, 2003), small cell lung (Seufferlein and Rozengurt, 1996) and rhabdomycosarcoma (Dilling et al, 1994). Acting through similar mechanisms, everolimus has been shown to inhibit the growth of cancer cells including breast (Martin et al, 2012), acute lymphoblastic leukaemia (ALL) (Saunders et al, 2013) and oral squamous cell carcinoma (OSCC) (Naruse et al, 2015).

Rapalogues are also able to induce autophagy in certain cancer types, including breast cancer (Lui et al, 2016) and malignant gliomas (Takeuchi et al, 2005) as well as having an apoptotic effect on human dendritic cells (Woltman et al, 2003). Whilst this increase in autophagy is not surprising due to mTORC1 control over autophagy initiation and ULK1/2 phosphorylation (Dunlop and Tee, 2014), it is not widely noted in cancer types, where cell cycle arrest and growth inhibition appear to be the primary cellular means by which rapalogues act. Everolimus, like rapamycin, can also cause an increase in apoptosis within breast cancer and rhabdomycosarcoma cell cultures (Hosoi et al, 1999; Hurvitz et al, 2015b; Khairi et al, 2014).

However, inhibiting mTOR signalling in this manner has its drawbacks in terms of the desired molecular effect, highlighting possible issues when applying rapalogues in a clinical setting. Usually, negative feedbacks loops exist to perturb over-active mTOR signalling, with S6K inhibiting IRS-1 to reduce mTOR activation via insulin/IGF-1 signalling (Magnuson et al, 2012; Saran et al, 2015). Thus, in rapalogue treatment, cells may actually be more sensitive to PI3K-mTOR activation via growth factors

such as insulin (Huang and Manning, 2009). The inhibition of mTORC1 (on a short term scale) seems to also favour the formation of mTORC2 complexes, shifting mTOR signalling burden from one arm to the other (De et al, 2013). In line with this, and the fact that mTORC2 leads to increased Akt phosphorylation at Ser473 (Sarbassov et al, 2004), rapalogue treatment appears to lead to increased Akt activation. This not only further increases upstream signals activating the mTOR pathway but also increases the activation of various survival pathways associated with Akt activation (Sun et al, 2005). Everolimus and other rapalogues, have been shown to abolish the negative feedback on IRS-1/insulin signalling, up-regulating and further activating growth factor signalling via PI3K and Akt in both cancer cell cultures and patient samples (O'Reilly et al, 2006; Shi et al, 2005). Patients with metastatic cancer have also shown up-regulation of other signalling pathways including MAPK signalling, when treated with everolimus (Carracedo et al, 2008).

1.15 - mTOR Therapies: Clinical Applications of Everolimus and Rapalogues In Breast Cancer

Many rapalogues have now made their way into clinical use, or are being explored for therapeutic use, including in the field of cancer treatment. Whilst not approved for use in breast cancer, rapamycin has shown some small efficacy in the treatment of this disease when used as a combination therapy. Phase II trial data in HER-2 positive patients suggested adding rapamycin may benefit trastuzumab treatment (Acevedo-Gadea et al, 2015) whilst adding resveratrol to rapamycin treatment may stop Akt feedback activation in breast cancer cells (Alayev et al, 2015).

Temsirolimus has been approved for use in renal cell carcinomas since 2007 in the EU (European Medicines Agency, 2016b) and is mainly used as a first line treatment for patients with poor-risk disease. Phase III trial data has shown it improves median survival among this group (Zanardi et al, 2015); however temsirolimus trials in breast cancer have produced inconclusive and mild results at best. One phase II study found no objective response in the observed cohort, although the study size was small at only 31 patients (Fleming et al, 2012) and a separate phase II trial, using a larger cohort, showed a very modest response, with only 9.2% patients showing partial response to the drug (Chan et al, 2005). Phase III trials of this drug combined

with the aromatase inhibitor letrozole, in the HORIZON trials in post-menopausal women, again showed disappointing results and a lack of improved patient survival (Wolff et al, 2013). Similarly, phase II trials with temsirolimus in ovarian cancer have had poor results (Emons et al, 2016). Interestingly, Rangwala and colleagues (2014) showed that combining a rapalogue like temsirolimus with the autophagy inhibitor hydroxychloroquine (HCQ), was well tolerated and showed anti-tumour activity in melanoma patients, suggesting this may be a valuable area of exploration for breast cancer combination therapy in the future.

Although not currently approved for clinical use in cancer treatment, ridaforolimus has been explored in a number of trials for various cancer types including breast cancer. A phase II trial with ridaforolimus combined with trastuzumab, in HER-2 positive-trastuzumab refractory metastatic breast cancer patients, showed good anti-tumour activity. The rate of response was similar to that with patients treated with first line trastuzumab, suggesting that a rapalogue like ridaforolimus may help overcome resistance to trastuzumab (Seiler et al, 2015). Phase II trials of ridaforolimus in endometrial cancer, refractory haematological cancers and soft and bone sarcomas has also shown some promising results in terms of anti-tumour activity, giving cause for possible further investigation (Mita et al, 2013; Oza et al, 2015; Rizzieri et al, 2008).

In breast cancer, everolimus has shown many productive results, across a variety of clinical trials. As such, in 2012 everolimus (marketed as Afinitor) was approved for use in combination with the steroidal aromatase inhibitor exemestane in breast cancer patients who are hormone receptor positive (e.g. ER+), HER-2 negative (non-over-expressing), with advanced cancer, who are post-menopausal and whose prior treatment with a non-steroidal aromatase inhibitor (such as letrozole or anastrazole) had failed (European Medicines Agency, 2016a; Geisler, 2011; Wazir et al, 2014). Key evidence for the use of everolimus in this subset of cancer patients came from the phase III BOLERO-2 (breast cancer trials of oral everolimus) clinical trial. This trial looked at the effect of combining everolimus with exemestane, in this subset of patients, where their cancer was refractory to non-steroidal aromatase inhibitors; all had received prior treatment with either letrozole or anastrazole. The patient set included those who had already been treated with one set of chemotherapy and/or

hormonal therapy, and excluded patients who had already been treated with exemestane or other mTOR inhibitors. Patients treated with the combination of everolimus plus exemestane had a statistically significant increase in progression free survival (PFS), compared to exemestane and placebo treated patients; there was a PFS average of 2.8-4.1 months in the placebo arm compared 6.9-10.6 months in the everolimus arm of the trial. The range of PFS comes from a difference in the analysis of radiographic images, which was used to determine PFS. In terms of toxicity, the combination treatment was also well tolerated, according to quality of life (QoL) end-points and ECOG status (Baselga et al, 2012; Beaver and Park, 2012; Dorris and Jones, 2014). These results are positive compared to the rather flat results of the HORIZON trial; both used a rapalogue in conjunction with an aromatase inhibitor, however it is possible that the use of a steroidal aromatase inhibitor (exemestane) enhanced the effects of the rapalogue in a greater way compared to its non-steroidal counter-part (letrozole).

Since mTOR activation can often confer for resistance to trastuzumab (Margariti et al, 2011) it seems a viable option to use a rapalogue to increase patient sensitivity to this therapy once again. Phase II trial data seemed to validate this thinking, with results showing that patients on a regime of trastuzumab and paclitaxel (who had progressed whilst on trastuzumab treatment and were HER-2 positive) were showing increased PFS times and response rates to the therapy, with the weekly addition of everolimus (Hurvitz et al, 2013). However results from the phase III BOLERO-1 trial in a similar area were not as positive. The trial included patients with HER-2 positive (over-expressing) tumours with advanced disease who had not received chemotherapy (including trastuzumab) within the last 12 months. This time the addition of everolimus to trastuzumab and paclitaxel did not improve outcomes in a significant way although some small advantage to this treatment was noted in women who were HR/ER negative (Hurvitz et al 2015a).

The BOLERO-3 phase III trial also studied women with advanced HER-2 positive cancers who were trastuzumab resistant and had previously received taxane treatment. The addition of everolimus to a regimen of trastuzumab and vinorelbine increased PFS significantly, albeit by a small amount, compared to the addition of placebo, from a median of 5.78 months to 7 months. Again the sub-group of patients

who were HR negative showed an increased efficacy of everolimus (Andre et al, 2014). The data from BOLERO-1 and 3 suggests that in HER-2 positive patients, HR/ER status may be key to everolimus efficacy. Since mTOR signalling can directly alter ER signalling (Viedma-Rodriguez et al, 2014) and is a direct target of growth factor signalling like that of HER family receptors (Huang and Fingar, 2014), it is perhaps no surprise that these multiple pathways connect in relation to therapy efficacy.

Considering the importance of SERMs, everolimus has also been explored in conjunction with tamoxifen. Phase II clinical trial data for this drug combination in post-menopausal advanced breast cancer patients who were HER-2 negative, HR positive, aromatase inhibitor resistant, have been positive. Results suggested a significant increase in time to progression and overall clinical benefit (Bachelot et al, 2012). A small phase II study in triple-negative breast cancer patients has shown that the combination of carboplatin and everolimus may have clinical benefit in this set of breast cancers (Singh et al, 2014). However the addition of everolimus to a regime of paclitaxel and bevacizumab was shown not to significantly increase efficacy of this drug regime, in a phase II study (Yardley et al, 2015).

1.16 - mTOR Therapies: Resistance to Rapalogues

Whilst it is clear that the rapalogues have wide potential in the clinic, as in the case of everolimus use in breast cancer, they also are associated with key issues that may ultimately limit their application and range in terms of therapeutic use. Resistance to rapalogues (and a lack of efficacy to treatment) has been noted in many settings and can been caused by a host of factors. The inhibition of mTOR with rapalogues can alter feedback pathways that exists within PI3K-mTOR signalling as well as activate Akt signalling by shifting the burden of signalling towards mTORC2 (Sarbassov et al, 2004; Sun et al, 2005). This has been shown to reduce the anti-cancer effects that rapalogues have (Carew et al, 2011) and inhibition of Akt directly helps to increase sensitivity of breast and colon cancer cells to rapalogue treatment, partially via increased inhibition of PRAS40 phosphorylation, increasing its inhibitory effect on mTORC1 (Mi et al, 2015). This same feedback effect on Akt has been noted in lung cancer cells with rapamycin treatment, with

PI3K inhibition again re-sensitising the cells to rapamycin treatment (Sun et al, 2005). Since mTOR inhibition can activate apoptosis, a lack of functional apoptotic pathways can reduce their effectiveness as well (Carew et al, 2011). Unsurprisingly, breast cancer cells with a higher reliance/activation of mTORC1 signalling, as shown by over-expression of phosphorylated S6K, show increased inhibition by rapalogues (Noh et al, 2004).

Many other signalling pathways and processes can effect and induce rapalogue resistance. For example MCF-7 breast cancer cells that have developed tamoxifen resistance have also shown intrinsic resistance to everolimus (Jordan et al, 2014). Research suggests that expression of markers of epithelial-mesenchymal transition (EMT) such as snail, increase resistance to rapamycin, whilst expression of pre-EMT markers like E-cadherin in breast cancer cells, can act as a biomarker for rapamycin sensitivity, *in vitro* (Holder et al, 2015). This gives some rationale for testing Wnt inhibitors in conjunction with mTOR inhibitors, since Wnt signalling is a key pathway in EMT (Guo et al, 2014; Kotiyal and Bhattacharya, 2014). Whilst evidence is currently limited for the use of Wnt inhibitors in this context, research with the Wnt pathway inhibitor XAV-939 in breast cancer cells, has shown that it can down-regulate Wnt signalling (Bao et al, 2012), and thus maybe a good candidate for testing in combination with a Wnt inhibitor.

In work with breast cells (including the MCF-7 cell line) that were induced to be everolimus resistant, myc was suggested to play a role in the resistance process, with an up-regulation of myc seen in the resistant lines but also after everolimus treatment; depletion of myc helped re-sensitise the line to everolimus once more (Bihani et al, 2015). Interestingly, vitamin D may offer a way to use this information to help improve breast cancer therapeutics in this area. Research suggests that not only is vitamin D anti-cancerous in a variety of types including breast cancer (Welsh, 2012), but that it can also down-regulate myc, as well as other proliferative markers (LaPorta and Welsh, 2014; Moukayed and Grant, 2013; Shao et al, 2012). This give rise to the possibility that vitamin D could be tested in rapalogue resistant cells, to help over-come resistance, especially if myc has a role in the mechanism behind resistance.

MCF-7 cells treated with rapamycin also show an up-regulation of transglutaminase 2 (TGM2), seemingly also as a compensatory mechanism, with TGM2 inhibition re-sensitising cells to rapamycin treatment (Cao and Huang, 2016). Work with breast, colorectal and renal cancer cells also implicates Met to be involved a mechanism of rapalogue resistance, with increased Met activation conferring resistance (Raimondo et al, 2016). Mutations could also induce rapalogue resistance, with breast cell lines with mutations in the mTOR FRB domain (induced after long term rapamycin treatment) showing insensitivity to rapamycin, even over a period of weeks. Cells with this type of mutation are however still sensitive to ATP-competitive inhibitors of mTOR (Hassan et al, 2014).

Away from breast cancer, other types of malignancies have shown resistance to rapalogues. In pancreatic neuroendocrine tumours, resistance to everolimus and rapalogues can relate to the activation of alternate signalling and growth factor pathways including the MAPK-Erk axis, and like with breast cancer, the activation of the PI3K-Akt pathway. Evidence also suggests that CXCR4-CXCL12-CXCR7 chemokine axis inhibition can increase sensitivity of renal and neuroendocrine pancreatic tumours to everolimus. This suggests that inhibition of this pathway in a combination therapy with everolimus may offer a new targeted approach (Capozzi et al, 2015; Wei et al, 2012). In studies with everolimus-induced resistance via long-term treatment, in pancreatic neuroendocrine cells, PI3K-Akt inhibition again overcame resistance, thus further emphasising the issue of the activation of the PI3K-Akt axis after rapalogue treatment (Vandamme et al, 2016). Similar mechanism of resistance have been observed in renal cell carcinoma (Kornakiewicz et al, 2013; Santoni et al, 2014), highlighting the importance of the activation of these alternative pathways in a broader sense across all cancers treated with rapalogues. Hypoxic conditions could also affect rapalogue activity, with renal cancer cells lacking Von Hippel-Lindau (*VHL*) gene showing increased response to temsirolimus (Thomas et al, 2005). Work by Wagle and colleagues (2014) in a thyroid cancer patient treated with everolimus showed that a particular patient had developed resistance due to mutations in both *TSC2* and *MTOR*. This could be particularly important as this shows that these sorts these mutations can occur in a clinical setting and not just in studies where resistance has been induced *in vitro*.

1.17 - mTOR Therapies: Alternatives to Rapalogues

Inhibiting mTOR via FKBP12 is by no means the only way to achieve the overall effect of blocking mTOR activity. In fact there are now multiple well explored avenues to block mTOR signalling, away from the rapalogue mechanism, many of which circumnavigate the issues that arise with rapalogue use. Whilst these still present with their own issues, such as side effects like that of regular chemotherapy (which rapalogues also display), they have shown promising efficacy in the field of cancer treatment and early clinical trial stages and it is very possible that they will make their way into the clinical setting (Gil, 2014).

Rather than allosterically inhibiting mTOR, like the rapalogues do, ATP-competitive inhibitors of mTOR act to block mTORs kinase ability by blocking ATP binding and this aim to block activity of both mTOR complexes. Due to the related sequence nature of mTOR (and other PIKK family proteins) and PI3K, many of the ATP competitive inhibitors also inhibit PI3K as well as mTOR. These inhibitors therefore help reduce signalling across the entire PI3K-Akt-mTOR axis and reduce the problems of feedback activation to PI3K signalling or mTORC2 activation (Garcia-Echeverria, 2010). BEZ235 and PF-04691502 are both dual PI3K-mTOR inhibitor of this class and have been studied for their anti-cancer efficacy in cancers like breast cancer. Both shown an anti-proliferative effect on cancer cells (and some tumours) and inhibition of PI3K-mTOR signalling (Britten et al, 2014; Dey et al, 2016). However, since PI3K signalling controls such a broad range of downstream pathways and processes vital for a cell, inhibiting both PI3K and mTOR may have serious side effects that could limit the clinical application of such inhibitors. For example, in a phase II study of BEZ235 in pancreatic neuroenodrocrine cases that were everolimus resistant, the drug was poorly tolerated, limiting the trial progression (Fazio et al, 2016).

More specific ATP-completive inhibitors, that only target mTOR, and thus block mTORC1 and 2 are becoming more and more favourable in terms of their use and research into them. The drugs MLN0128, CC-223 and ADZ2014 have all shown promising results in terms of their anti-cancerous effects on breast cancer. AZD2014 and MLN0128 both show good anti-proliferative and anti-tumour effect *in vitro* and *in*

vivo in breast cancer, reducing signalling from mTORC1 and mTORC2, with MLN0128 also able to inhibit the growth of breast cancer cells that have acquired resistance to rapamycin (Hassan et al, 2014; Guichard et al, 2015; Wilson-Edell et al, 2014). A phase I study of CC-223 has shown results that are relatively promising, with it being well tolerated, with partial response noted in a breast cancer patient, disease stability in multiple types of cancer as well as good inhibition of mTORC1 and 2 in patients (Bendell et al, 2015). AZD2014 is also currently being explored in a phase II trial, looking to test the use of this drug, against its combination with fulvestrant, in advanced breast cancer patients who are ER+, after failed treatment with an aromatase inhibitor (Cancer Research UK, 2016; NIH, 2017).

Despite the issues of inhibiting PI3K, pan-PI3K inhibitors, such as the buparlisib (BKM120) have shown early promise in tackling breast cancers. Buparlisib widely inhibits PI3Ks but not does not directly inhibit mTOR, with phase I data with buparlisib in combination with either trastuzumab (Saura et al, 2014) or fulvestrant (Ma et al, 2016a) showing the drug to be well tolerated in breast cancer patients as well as some signs of disease management also presenting themselves. PI3K inhibition may also be a viable way of over-coming or avoiding resistance to rapalogues, with buparlisib use in combination with everolimus (or trastuzumab) being shown to reduce the occurrence of resistance to these drugs, whilst also showing good growth inhibition, *in vivo* (Yu et al, 2016).

Inhibiting Akt directly is another alternative therapeutic option to rapalogues that has shown potential at a research stage and early clinical levels. In terms of breast cancer therapeutics, MK-2206, an allosteric inhibitor of Akt is perhaps the promising of the selective Akt inhibitors. Multiple phase I trials have suggested this may hold key therapeutic benefits and has been tested in a similar settings to the BOLERO trails previously mentioned. MK-2206 in combination with paclitaxel and trastuzumab, in HER-2 positive breast cancers (similar to the BOLERO-1 trial), was well tolerated, with 63% of patients showing some clinical response (Chien et al, 2016). Likewise, MK-2206 in combination with anastrazole was also well tolerated and 42% of patients showing clinical benefit. Due to these successes, phase II trials are underway with MK-2206 (Ma et al, 2016b). Preclinical evidence for the efficacy of the ATP competitive inhibitor, AZD5363, is also positive, with breast cancers cells

and xenografts showing some of the best response to this drug of all malignancies tested (Davies et al, 2012).

1.18 - Aims for this Project

mTOR signalling and its role in breast cancer is relatively well understood, however the use of mTOR inhibitors in its treatment is in its infancy, with only a few rapalogues approved for use across all cancer types. Thus there is a gap in our knowledge of the mechanisms of resistance to rapalogues, especially in breast cancer, since everolimus was only approved for use in this disease in 2012. Clinical examples of everolimus resistance in breast cancer have not yet been published on, thus my aim was to help begin close this knowledge gap by developing *in vitro* models of resistance, and hence study these. The knowledge learnt from these models, on resistance mechanisms, characteristics and drug susceptibilities, alongside the small amount of literature currently published on the subject, would then help guide future work when studying patient examples as they arise.

Overall I aimed to do this by:

- Understanding base mTOR signalling and drug responsiveness to everolimus in a range of breast cancer cells
- Developing *in vitro* breast cancer models that are resistant to everolimus
- Testing drugs and drug combinations that can overcome this induced resistance to everolimus
- Studying these models for changes compared to parental control cells and test for alterations that may underline a mechanism for resistance

Chapter 2: Materials and Methods

2.1 - Cell Culture

Breast cancer cell lines T47D, SKBR3, GI101 and MDA-MB-361 were selected for their use in this project due to unpublished data by Harvey et al, showing their responsiveness to calcitriol. T47D, SKBR3 and MDA-MB-361 cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific), supplemented with 10% foetal bovine serum (FBS) (Thermo Fisher Scientific), 2mmol/L glutamine, 100U/mL penicillin and 100µg/mL streptomycin (Thermo Fisher Scientific). GI101 cells were cultured in the same medium, with the addition of 5µg/mL insulin (Thermo Fisher Scientific).

MDA-MB-463 and MDA-MB-231were selected for their use in this project due to their status as 'triple negative' breast cancer cell lines (Holliday and Spiers, 2011). These lines were cultured in identical conditions/medium as the T47D line. MCF10a was also selected to be used, as they are a non-tumourgenic breast cell line. These were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% foetal bovine serum (FBS), 2mmol/L glutamine, 100U/mL penicillin and 100µg/mL streptomycin, 5µg/mL insulin, 10ng/mL epidermal growth factor (EGF) (Sigma) and 0.5µg/mL hydrocortisone (Sigma).

All cells were grown at 37°C in a humidified 5% CO_2 incubator. Cell cultures were split 1-2 times a week and when required. Cells were first washed twice with 1x phosphate buffered saline (PBS) pH 7.4 solution (Severn Biotech). 0.5-2mL TrypLE express (Thermo Fisher Scientific) was then added and incubated at 37°C, for 3-6 minutes, to detach cells from the flask. Cell suspensions were then diluted, with fresh media, at a ratio of 1:2-1:10, depending on the cell line.

2.2 - Development of Everolimus Resistant Cells

T47D cells were seeded into T25 culture flasks at concentrations of 2.5 x 10^5 cells/flask and MDA-MB-361 cells seeded into either a T25 at a concentration of 3.5

x 10^5 cells or a T75 with 1 x 10^6 and left to adhere overnight. Everolimus was then added at the concentration closely matching the GI60 (growth inhibition 60%) for a one week everolimus treatment, with the GI60 obtained using an SRB assay. Cells were then left to grow over the course a week (minimum 3 days, maximum 10 days). A portion of the cells were then used for seeding into a 96-well plate to determine the new GI60, whilst the remaining cells recovered and re-grew post-treatment. The process was then repeated with the new GI60 concentration of everolimus, until a stable GI60 was achieved between 2-3 treatment cycles. Control flasks of both cells (designated as parental) ran in parallel with the treated cells and were treated with 1:1000 (0.1%) DMSO in media. Drug resistant cell lines and the parental controls were used for subsequent experiments for no longer than 15 passages from the point at which the development process was stopped. Drug resistant lines were treated with 100nM everolimus after every 3-4 cell splits, to help maintain the resistance phenotype.

2.3 - Cell Morphology Imaging

Morphology of cells was studied and compared using a FLoid cell imaging station (Thermo Fisher Scientific), with pictures taken at x20 magnification (objective).

2.4 - Drug Treatments

Cells suspensions were counted using a haemocytometer. Cells were then seeded into either 96-well plates (to perform SRB assays), 100µL medium per well or 24-well plates, 500-1000µL medium per well, at concentrations specified in table 2.1. 6 wells for technical replicates were used per concentration of drug, for 96-well plates and 3 for 24-well plates. Plates were incubated overnight to allow the cells to attach to the bottom of the well and then treated with varying concentrations of 5-fluorouracil, cyclophosphamide monohydrate, doxorubicin hydrochloride, (Sigma), everolimus, rapamycin, temsirolimus, BEZ-235, enzalutamide, methotrexate, paclitaxel, XAV-939 and/or calcitriol (Selleckchem). Concentrations tested for these drugs ranged from 0.1-10,000nM, unless stated otherwise. All drugs, apart from BEZ-235, were diluted in DMSO at higher concentrations, before being diluted in medium to add to the plate, with the amount of DMSO added to each well kept at 0.1%, regardless of

concentration of drug. 0.1% DMSO (in media) was used as a control. BEZ-235 was diluted in the same fashion, except using dimethylformamide (DMF) as a vehicle instead, with 0.1% DMF in media as the control. Drug treatments were from 72 hours - 1 week, with media/drug changed half way through the week.

	Cells per well			
	24-well plate		**96-well plate**	
Cell type	**72 hr treatment**	**1 week treatment**	**72 hr treatment**	**1 week treatment**
T47D	$25x10^3$	$10\text{-}15x10^3$	$5x10^3$	$2x10^3$
SKBR3	$6x10^3$	$3x10^3$	$1x10^3$	$0.5x10^3$
GI101	$30x10^3$	$15x10^3$	$5x10^3$	$2.5x10^3$
MDA-MB-361	$30x10^3$	$15\text{-}20x10^3$	$7\text{-}5x10^3$	$3.5\text{-}5x10^3$
T47D-EveR	N/A	$15\text{-}x10^3$	$5x10^3$	$2.5x10^3$
MDA-MB-361-EveR	N/A	$20x10^3$	$9x10^3$	$7x10^3$
T47D-EveR-LT	N/A	N/A	$5x10^3$	$2x10^3$

Table 2.1: Concentration of cells seeded for drug treatments in 96-well plates, used for the SRB assay and concentration of cells seeded for drug treatments in 24-well plates for trypan blue staining.

2.5 - Sulforhodamine B (SRB) Assay

Dose response to drug treatments for 72hr-1 week, was assessed using an SRB assay. This assay was chosen for a number of reasons. The SRB assay has been shown to be an accurate and sensitive method to testing drug sensitivity (Skehan et al, 1990) and testing has shown it is at least as sensitive, if not more so, than the MTT assay, another colour-metric assay for testing drug treated cells.(Keepers et al, 1991). The MTT assay was deemed to not be suitable, as mTOR has been shown to affect mitochondrial function (Morita et al, 2015). Finally, this assay was selected in relation to the development of everolimus resistant cells. The method for this development was adapted from the protocol described by Box and colleagues (2013), in which they also used the SRB assay, so the SRB was used here to be in keeping with their methodology.

Cells were seeded into 96-well plates and each experiment was performed separately, three times, unless specified. At the end of the treatment period, cells were fixed with the addition of 25µL ice-cold 50% (w/v) trichloroacetic acid (TCA) and left for 1hr at 4°C. Wells were then rinsed several times with dH_2O and air dried. To quantify the amount of cellular protein and therefore growth, cells were then stained with 50µL 0.4% (in 1% acetic acid) SRB solution (Sigma) and incubated for 20 minutes at room temperature. Unincorporated dye was then removed with several washes in 1% acetic acid, before being air dried again. To solubilise the protein-bound stain, 100µL 10mM Tris base solution per well was added and the plate incubated for 5 minutes at room temperature, and subsequently mixed by pipetting.

Plates were read using a Thermo Scientific Multiscan EX plate reader, at 405 nM, to account for background, and 570nM, to detect the SRB stain. The final absorbance was gained by subtracting the 405nM absorbance from the 570nM absorbance. Average absorbance for medium only was subtracted from the other average data values, to cancel out non-specific SRB staining.

Data was plotted with drug concentration on the x-axis and relative cell number (RCN) on the y-axis. RCN was used to represent the % absorbance/OD of each sample compared to the control of that experiment. For experiments with one drug the control was the vehicle (DMSO or DMF). For experiments using a combination of everolimus, with the addition of a second drug, the control for that experiment was the second drug as a single agent.

2.6 - Trypan Blue Inclusion Assay

To quantify cell death, trypan blue staining was performed on cells seeded into 24-well plates and either left to grow or treated with drug for 72hr-1 week. To quantify live and dead cells, the media was collected from each well and 200-300µL trypsin added to detach cells. Cell suspensions along with their associated media were centrifuged at 1500 RPM for 5 minutes and the cell pellet then re-suspended in 200µL PBS.

0.4% trypan blue solution (in PBS) was diluted with cell suspension to a final concentration of 0.1%. Cells, both dead and viable, were then counted using a haemocytometer. Dead cells showed up as bright blue compared to viable ones. The experiment was performed a minimum of twice.

2.7 - Wound Healing Assay

Cell migration was studied using a wound healing assay. Cells were seeded into 24-well plates, 3 technical replicates per line; $20x10^4$ cells per well for T47D resistant and parental lines and $30x10^4$ cells for MDA-MB-361 resistant and parental lines. These were allowed to grow to confluency before 5µg/mL mitomycin C (Sigma) for T47D and 2.5µg/mL for MDA-MB-361, diluted in DMSO, was added to the cells for two hours before scratching, to inhibit all cell proliferation. A scratch was then made using a 10µL pipette tip, through the cell monolayer, the media replaced and a picture taken at x20 magnification using a FLoid cell imaging station (Thermo Fisher Scientific). The same area was then photographed 24 and 48 hours later to assess wound closure and thus cell migratory potential.

2.8 - Cell Suspension Assay

To assess cell viability in suspension, cells were seeded into plates coated with poly(2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma). 12-well cell culture plates were prepared with a poly-HEMA coating by adding 300-400µL 10mg/mL poly-HEMA diluted in 95% ethanol, to each well, and air dried in a tissue culture hood, before the process was repeated. Cells were seeded into plates at a concentration of $7.5\ x10^4$ cells and left overnight. Cell suspensions were then collected centrifuged at 1500 RPM for 5 mins and re-suspended in 200µL cold PBS, using vigorous pipetting and the tip to disturb cell clumps. Cells were then stained with trypan blue and assessed as described previously.

2.9 - Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

SDS-PAGE/western blotting was used to analyse protein levels of mTOR associated proteins and breast cancer markers (e.g. ER). Cells were collected by trypsinising, centrifuged and pelleted and 100µL of lysis buffer (table 2.2) per $10x10^5$ cells was used to lyse them. For phospho-protein studies, lysates had 1:100 of phosphatase inhibitor cocktail 2 (Sigma) added. Lysates were aliquoted and stored at -20°C. A volume of lysate equivalent to $1x10^5$ cells was run per sample, on SDS-PAGE gels ranging from 8-15% in size (table 2.3). 5µL of protein ladder (Fisher) was electrophoresed alongside as a molecular weight standard. Gels were run at 250-300v and 40mA for around 1 hour in 1x running buffer, made up with dH_2O (table 2.5). Sample loading was checked using a coomassie stain (table 2.4). Gels were incubated with coomassie stain for 20-30 minutes, followed by a 1 minute wash, then 3-4 washes at 10-20 minutes, with de-stain to visualise protein bands. Bands were studied for intensity and sample loading adjusted to give an even protein load on subsequent gels. Samples were then re-run accordingly.

Protein was transferred to nitro-cellulose membrane by electro-blotting in 1x transfer buffer (100mL 10x transfer buffer, 100mL methanol, 800mL dH_2O) , run at 300v and 400mA for 1-2hr (table 2.5). Membranes were washed in between steps with 1x TBS- 0.1% Tween20 (TBS-T) solution and blocked for a minimum of 1-2hr at room temperature, with 5% milk in TBS-T. Primary antibodies were diluted in either 5% milk or 5% bovine serum albumin (BSA) in TBS-T and membranes incubated with them at 4°C overnight. Secondary antibodies were diluted in 5% milk TBS-T and incubated at room temperature for 1hr with the membrane. All incubations and washes were done on a shaker. Please see table 2.7 for a full antibody list.

Membranes were developed manually with Carestream Kodak autoradiography GBX developer and fixer (Sigma) solutions, using Amersham hyperfilm (GE Healthcare). Enhanced chemiluminescence (ECL) solution (combined ECL A and B, table 2.6) was added across the membrane and left 2-3 minutes before film was exposed to the membrane for varying times depending on the antibodies used. The film was developed for 1-2 minutes, washed in water then fixed for 1 minute, before being

washed in water and left to dry. Western blots were done a minimum of three times to validate results.

Lysis buffer	
Reagent	**Volume (mL)**
1M tris pH 6.8	1
10% SDS	4
Glycerol	2
99% β-mercaptoethanol	0.5
Bromophenol blue	Small amount (<0.05g)
dH_2O	Enough to make up to 10mL total

Table 2.2: Lysis buffer reagents.

	Separating gel (mL per gel)				Stacking gel (mL per gel)
Reagent	**8%**	**10%**	**12%**	**15%**	
Water	2.3	1.9	1.6	1.1	2.1
30% acrylamide	1.3	1.7	2	2.5	0.5
1.5M tris (pH 8.8)	1.3	1.3	1.3	1.3	
1M tris (pH 6.8)					0.38
10% SDS	0.05	0.05	0.05	0.05	0.03
10% ammonium persulfate (APS)	0.05	0.05	0.05	0.05	0.03
TEMED	0.003	0.002	0.002	0.002	0.003

Table 2.3: SDS-PAGE gel reagents.

Coomassie stain solutions		
Reagent	**Coomassie stain**	**Coomassie destain**
dH_2O	300mL	300mL
Methanol	150mL	150mL
Acetic acid	50mL	50mL
Coomassie	0.5g	

Table 2.4: Coomassie solutions.

Western blotting solutions (made up to 1L with dH_2O)			
	Amount (g)		
Reagent	**10x TBS pH 7.6**	**10x Running buffer pH 8**	**10x Transfer buffer pH 8.3**
Tris	60.5	30.3	30.3
Glycine		144.1	144.1
SDS		10	
NaCl	87.6		

Table 2.5: Solutions used for western blotting.

ECL solutions per membrane		
Volume (mL)		
Reagent	**ECL A**	**ECL B**
0.1M tris pH 8.8	2.5	2.5
1.4% coumaric acid	22	
4.4% luminol	55	
Hydrogen peroxide		3

Table 2.6: ECL solutions for developing film after western blotting.

Antibody	Species	Company	Dilution used	Diluted in BSA (B) or Milk (M)
anti-β-actin (ab8226)	Mouse	Abcam	1:1000	B/M
anti-GAPDH (D16H11)	Rabbit	Cell Signalling Technologies (CST)	1:1000	B/M
anti-mTOR (7C10)	Rabbit	CST	1:1000	B
anti-phospho-mTOR Ser2481 (#2974)	Rabbit	CST	1:1000	B
anti-phospho-mTOR Ser2448 (D9C2)	Rabbit	CST	1:1000	B
anti-rictor (D16H9)	Rabbit	CST	1:1000	B
anti-raptor (24C12)	Rabbit	CST	1:1000	B
anti-DEPTOR (D9F5)	Rabbit	CST	1:1000	B
anti-S6 kinase (49D7)	Rabbit	CST	1:1000	B
anti-phospho-S6 kinase Thr389 (108D2)	Rabbit	CST	1:1000	B
anti-Akt (#9272)	Rabbit	CST	1:1000	B
anti-phospho-Akt Ser473 (D9E)	Rabbit	CST	1:2000	B
anti-SGK1 (D27C11)	Rabbit	CST	1:1000	B
anti-phospho-SGK1 Ser422	Rabbit	Abcam	1:1000	B
anti-PKCα (D7E6E)	Rabbit	CST	1:1000/2000	B
anti-phospho-PKCα Ser657 (EPR1901(2))	Rabbit	Abcam	1:1000/2000	B
anti-FKBP12 (ab2918)	Rabbit	Abcam	1:1000	B
anti-vitamin D3 receptor (D2K6W)	Rabbit	CST	1:1000	M
anti-progesterone receptor A/B (D8Q2J)	Rabbit	CST	1:1000	M/B
anti-HER-2 (CB11)	Mouse	CST	1:1000	B
anti-HER-2 (29D8)	Rabbit	CST	1:1000	M
anti-Androgen receptor	Rabbit	CST	1:2000	M
anti-oestrogen receptor α (D8H8)	Rabbit	CST	1:1000	B
anti-mouse Immunoglobulins HRP	Rabbit	CST	1:1000	M
anti-rabbit Immunoglobulins HRP	Goat	CST	1:1000	M
anti-rabbit Immunoglobulins HRP	Swine	CST	1:1000	M

Table 2.7: List of antibodies used for western blotting.

2.10 - RNA Extraction

RNA was extracted from cells for q/PCR experiments, to analyse mRNA levels of mTOR associated genes. Cells were centrifuged down and RNA extracted using a Qiagen RNeasy mini kit, following the manufactures guidelines. Buffer RLT (provided in the kit) was added to re-suspend the cell pellet; either 350µL for $<5x10^6$ cells or 700µL for up to $1x10^7$ cells and then centrifuged for 3 minutes at 13,000 RPM. Cells were then homogenised by passing them several times through a syringe (23g x 1"). 1 volume of 70% ethanol was added to each individual sample, and the sample transferred to an RNeasy spin column (provided in the kit). The column was then centrifuged for 15 sec at 13,000 RPM and the flow through discarded. 700µL of buffer RW1 (provided in the kit) was added to the column, centrifuged again for 15 secs at 13,000 RPM, and the flow through again discarded. 500µL of buffer RPE (provided in the kit) was added to the column and it again centrifuged for 15 secs at 13,000 RPM, and the follow through discarded. 500µL of buffer RPE was again added and the column centrifuged for 2 min at 13,000 RPM, and the follow through discarded. The column was then dried by centrifuging for 1 minute at 13,000 RPM.

The RNA was then eluted by adding 50µL of RNase-free water to the column, and spinning it for 1 min at 13,000 RPM. RNA concentration was then assessed using a Thermo Scientific Nanodrop 2000c spectrophotometer and samples stored at -80°C.

2.11 - Reverse Transcription

Using RNA, extracted as described above, cDNA was made using a reverse transcription (RT) reaction for later use in q/PCR experiments. Heated reactions were performed using a BioRad Tetrad 2 Peltier Thermal Cycler. To 1µg of RNA, 150ng of random primers (Invitrogen) and 1µL of dNTP mix (10mM each of dATP, dCTP, dGTP and dTTP) (Invitrogen), were added and made up to 12µL with dH_2O. This was heated at 65°C for 5 min and then chilled on ice. Reagents were then added from the Supercript II RT (Invitrogen) set. This was 4µL of 5X first-strand buffer and 2µL 0.1M DTT. 1µL of RNaseOUT ribonuclease inhibitor (Invitrogen) was then added and the mixture heated at 25°C for 2 min. Finally 1µL of the Superscript II RT enzyme was added. The samples were then heated to carry out the RT

reaction as follows; 25°C for 10 min, 42°C for 50 min and 70°C for 15 minutes to inactivate the reaction. cDNA was then stored at -20°C.

2.12 - qPCR: geNorm Kit and Analysis for Reference Genes

To determine reference genes for qPCR experiments, a 12 gene geNorm primer kit (Primer Design) was used. geNorm qPCR experiments were carried out with a QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems), using PrecisionPlus SYBR green mastermix (Primer Design). Reactions were carried out in microAmp fast optical 96-well plates (Applied Biosystems), with reactions volumes per sample/well specified in table 2.8. Primers from the kit were re-suspended in 220µL of RNase/DNase free water, as per manufacturer's instructions. cDNA from RT reactions was diluted 1:10 before use in qPCR reactions. Plates were sealed using optical adhesive film (Applied Biosystems) and centrifuged briefly, to ensure all material was at the bottom of the well. Plates were then run for 40 cycles at the conditions shown in table 2.9.

Volumes per reaction/well	
Reagent	**Volume (µL)**
geNorm primer mix	1
PrecisionPlus SYBR green master mix	10
RNase/DNase free H_2O	4
cDNA	5

Table 2.8: Reaction volumes per well for geNorm qPCR experiments.

	Time	**Temperature (°C)**
40x cycles (max)	2min	95
	10s	95
	60s	60

Table 2.9: Cycling conditions for geNorm qPCR experiments. Data collection stage is marked in yellow, at the 60°C for 60s step.

All 7 cell lines (T47D, SKBR3, GI101, MDA-MB-361, MDA-MB-436, MDA-MB-231 and MCF10a) were run through this protocol twice, using two independent sets of cDNA. Analysis of both runs was then done together using qbase+ (Biogazelle). The software was used to determine the most stable of the genes to use (using geNorm M value) and the recommended number of reference genes (using geNorm V value), for all 7 cell lines together. Everolimus resistant and parental cell lines were also run through the geNorm protocol together to similarly determine reference genes for their cumulative qPCR experiments.

2.13 - qPCR: mTOR Genes

mTOR related gene expression was studied using qPCR with reference genes selected using the geNorm kit method described previously. These, again, were run using a QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems), with PrecisionPlus SYBR green mastermix (Primer Design) with reactions carried out in microAmp fast optical 96-well plates (Applied Biosystems) and reactions volumes per sample/well specified in table 2.10. cDNA was again used from RT reactions, after a 1:10 dilution. Plates were run for 40 cycles at the conditions shown in table 2.11.

Volumes per reaction/well	
Reagent	**Volume (µL)**
Forward primer (10µM)	1
Reverse primer (10µM)	1
PrecisionPlus SYBR green master mix	10
RNase/DNase free H_2O	5.5
cDNA	2.5

Table 2.10: Reaction volumes per well for qPCR experiments.

	Time	Temperature (°C)
40x cycles (max)	2min	95
	15s	95
	60s	60

Table 2.11: Cycling conditions for qPCR experiments. Data collection stage is marked in yellow, at the 60°C for 60s step.

As minimum information for the publication of qPCR experiments (MIQE) guidelines (Bustin et al, 2009) state all primer sequences should be published, primers for the reference genes were re-designed and differed from the ones used in the geNorm kit, with re-designed primers developed using Primer-BLAST (NCBI) software and made by Sigma. Sequences for all primers used are shown in table 2.12.

qPCR data was analysed by first calculating the ΔCT; this was done by taking the mean CT value of each gene of interest, and then subtracting the mean CT of reference genes from this. The relative quantity (RQ) of each gene was calculated using the formula $2^{-\Delta CT}$. RQ values were then averaged between experiments and compared between cell lines to determine relative expression of each gene.

Primer name	Sequence	Reference
mTOR-Forward	TGCCAACTACCTTCGGAACC	Rodgers-Broadway et al (2016)
mTOR-Reverse	GCTCGCTTCACCTCAAATTC	Rodgers-Broadway et al (2016)
Raptor-Forward	ACTGATGGAGTCCGAAATGC	Rodgers-Broadway et al (2016)
Raptor-Reverse	TCATCCGATCCTTCATCCTC	Rodgers-Broadway et al (2016)
Rictor-Forward	GGAAGCCTGTTGATGGTGAT	Rodgers-Broadway et al (2016)
Rictor-Reverse	GGCAGCCTGTTTTATGGTGT	Rodgers-Broadway et al (2016)
DEPTOR-Forward	CACCATGTGTGTGATGAGCA	Rodgers-Broadway et al (2016)
DEPTOR-Reverse	TGAAGGTGCGCTCATACTTG	Rodgers-Broadway et al (2016)
FKBP12 (q)-Forward	CAAGCAGGAGGTGATCCGAG	Developed using Primer-BLAST
FKBP12 (q)-Reverse	CATGTGGTGGGATGATGCCT	Developed using Primer-BLAST
TOP1-Forward	CCTTCCCTCTCTCCCATTTC	Developed using Primer-BLAST
TOP1-Reverse	AGCCACGACTGCTTCAAGTT	Developed using Primer-BLAST
YWHAZ-Forward	AGACGGAAGGTGCTGAGAAA	Rodgers-Broadway (2016)
YWHAZ-Reverse	GAAGCATTGGGGATCAAGAA	Rodgers-Broadway (2016)

Table 2.12: Primer sequences for qPCR experiments.

2.14 - Agarose gel electrophoresis

The quality of q/PCR product and the product size was checked by running the post-PCR product on a 1-2% agarose gel. 100mL gels were made with, and run in, 1xTAE (table 2.13), with 5uL per 100mL gel of SYBR safe DNA stain (Invitrogen) to visualise DNA products. 2.5-5uL of post-PCR product was run with 2uL loading buffer (10% glycerol, with a small amount of bromophenol blue diluted in 1xTAE). 5uL of hyperladder II or V (Bioline) was used as a reference.

Reagent	**Volume/weight**
Tris	242g
Acetic acid	57.1mL
0.5M EDTA	100mL
dH_2O	Make up to 1L total

Table 2.13: 50x TAE buffer

Gels were run for around 1hr at 400mA, 100v and then visualised using the UV function on a Bio-Rad Gel Doc EZ Imager.

2.15 - PCR of FKBP12 and mTOR's FRB Domain

Mutations in FKBP12 and mTOR's FRB domain were checked using Sanger sequencing, with the domain first amplified via PCR. Primers were developed using Primer-BLAST (NCBI) software and made by Sigma. Primers were selected that encompassed the entire FKBP12 gene and the entire FRB domain, as well as several dozen nucleotides either side, to produce a 587bp fragment for FKBP12 or a 515bp fragment for the FRB domain. Sequences for these primers are shown in table 2.14. Reactions were carried out on a Labcycler thermocycler (Sensquest), using Phusion High Fidelity DNA Polymerase (Thermo Fisher Scientific), with this polymerase selected due to its accuracy and low error rate. Reactions were carried out in 0.2mL PCR tubes (Fisher), with reaction volumes per sample/tube specified in table 2.15. Annealing temperature for the reaction was calculated using Thermo

Fisher Tm calculator software (ThermoFisher, 2018), with cycling conditions shown in table 2.16. Reactions were checked using agarose gel electrophoresis, as previously detailed.

Primer name	Sequence
FKBP12-Forward	TACTAGGCAGAGCCGTGGAA
FKBP12-Reverse	TCGGAAGCAAAGCTGAGTGA
FRB Dom (mTOR)-Forward	ATGTGTGAGCACAGCAACAC
FRB Dom (mTOR)-Reverse	GGCCTCTGCTTGGATGTGAT

Table 2.14: Primer sequences for PCR experiments to amplify FKBP12 or mTOR's FRB domain.

Volumes per reaction/well	
Reagent	**Volume (µL)**
dH_2O	10.9
Forward primer (10µM)	1
Reverse primer (10µM)	1
dNTPs (10mM)	0.4
Phusion DNA polymerase	0.2
cDNA	2.5

Table 2.15: Reaction volumes per well for PCR experiments to amplify FKBP12 or mTOR's FRB domain.

	Time	Temperature (°C)
	30s	98
30 x cycles	10s	98
	15s	65.1 (FKBP12)/ 63.1 (FRB)
	30s	72
	5mins	72

Table 2.16: Cycling conditions for PCR experiments to amplify FKBP12 or mTOR's FRB domain.

2.16 - PCR Purification

Pure PCR product was required for sequencing, so once samples had the FRB domain or FKBP12 amplified using PCR, the product was then run through a QIAquick PCR purification kit (Qiagen), following the manufactures guidelines. 5 parts buffer PB (provided in the kit) was added to each sample, before being loaded into individual spin columns (provided in the kit) and then centrifuged for 1 minute at 13,000 RPM. The flow through was discarded and 750μL buffer PE (provided in the kit) was added to each column, with the columns again centrifuged for 1 minute at 13,000 RPM. The flow through was again discarded and the columns again centrifuged for 1 minute at 13,000 RPM to dry the column. Purified PCR product was then eluted by adding 50μL of buffer EB (provided in the kit), letting each column stand for 1 minute, then centrifuged for 1 minute at 13,000 RPM.

The presence of the correct fragment was again checked using agarose gel electrophoresis, as previously described, and the purity and concentration of each sample checked using a Thermo Scientific Nanodrop 2000c spectrophotometer and samples stored at -20°C.

2.17 - Sanger Sequencing of FKBP12 and mTOR's FRB Domain

Purified PCR product encompassing FKBP12 or mTOR's FRB, was then sent to Genewiz for Sanger sequencing, to analyse for mutations. Ten different sequencing primers, for the FRB domain and 12 for FKBP12, were developed using Primer-BLAST (NCBI) software and made by Sigma. All were selected to have a GC content of 45-55%, a Tm of between 50-60°C and be 18-22bp in length, as per recommendations from Genewiz. Sequences of these primers are shown in figure 2.17. Samples were diluted to 2ng/μL and 10μL per reaction added to 0.2mL PCR tube strips, with 5μL of 5μM primer per tube.

Primer name	Sequence	Location on PCR Product
FRB Seq F-1	GTGTGAGCACAGCAACAC	3-20
FRB Seq F-2	CATGATGGTGAGCGAGGA	36-53
FRB Seq F-3	ATGATGGTGAGCGAGGAG	37-54
FRB Seq F-4	ATCCTCTGGCATGAGATGTG	70-89
FRB Seq F-5	CTCTGGCATGAGATGTGG	73-90
FRB Seq R-1	TCTGCTTGGATGTGATGACT	511-492
FRB Seq R-2	GATGACTTGCAAAGACGGTG	498-479
FRB Seq R-3	AAAGACGGTGCTATGGACTG	488-469
FRB Seq R-4	CTATGGACTGAATGCGAATG	478-459
FRB Seq R-5	GGACTGAATGCGAATGATTG	474-455
FKBP12 Seq F-1	TACTAGGCAGAGCCGTGGA	1-19
FKBP12 Seq F-2	GAGTGCAGGTGGAAACCAT	94-112
FKBP12 Seq F-3	GAGTGCAGGTGGAAACCATC	94-113
FKBP12 Seq F-4	GTGCAGGTGGAAACCATC	96-113
FKBP12 Seq F-5	GCAGGTGGAAACCATCTC	98-115
FKBP12 Seq F-6	GAAACCATCTCCCCAGGA	105-122
FKBP12 Seq R-1	AAAGTGGAGTGGAACATCAGG	534-514
FKBP12 Seq R-2	TGGAACATCAGGAAAAGCTCC	525-505
FKBP12 Seq R-3	TGTGCACATGTCTGGAGG	493-476
FKBP12 Seq R-4	CTCCATGGCAGATCCAAGAA	465-446
FKBP12 Seq R-5	TGGCAGATCCAAGAACAGGG	460-441
FKBP12 Seq R-6	AACAGGGAGCTAAGGGAGGA	447-428

Table 2.17: Primer sequences for Sanger sequencing experiments to analyse FKBP12 or mTOR's FRB domain. Location on PCR product is either 1-515bp for FRB domain or 1-587BP FOR FKBP12.

Sequencing data was then analysed using DNASTAR Lasergene software. Consensus sequences were generated for each sample, from the forward and reverse sequences of that experiment, using the Megalign function, aligning sequences using the Clustal W method. Consensus sequences were then aligned together, to compare EveR cells with the parental lines, and to compare all with published sequences.

2.18 - Cell Cycle Prolife Analysis with Flow Cytometry

The cycling profile of certain cell lines was studied using a propidium iodide staining kit (Abcam) and flow cytometry analysis. Cells were cultured in T25 flasks until 50-60% confluent and which point the cells were harvested. The cells were then pelleted at 500 RPM for 5 minutes, then washed in 1mL of 1x PBS and pelleted again at 500 RPM for 5 minutes. The cell pellet was then fixed by adding 400uL ice cold PBS and then slowly adding 800uL ice cold ethanol (66% final concentration). Cells were stored like this at 4°C for a maximum of 4 weeks.

Once ready to run on the flow cytometer, samples were equilibrated to room temperature and the cells re-suspended before being pelleted again at 500 RPM for 5 minutes. The fixative solution was then removed, the cells washed with 1mL 1x PBS and again centrifuged at 500 RPM for 5 minutes, and the PBS then removed from the cell pellet. The cells were then re-suspended in 200uL 1x propidium iodide + 1x RNase staining solution (provided in the kit) and incubated in the dark for 20-30 minutes. Samples were then placed on ice, covered from the light and run immediately on an ACEA NovoCyte flow cytometer, looking for 12,000 events per sample. Forward scatter and side scatter channels were used to gate out cellular debris and analyse the bulk of the cells in the sample. The cell cycle analysis function was then used to categorise the proportions of cells in each phase of the cell cycle. Each experiment was done a minimum of twice with 3-4 technical replicates of each sample within a single run.

2.19 - ALDH Activity Analysis with ALDEFLUOR™ Kit and Flow Cytometry

Aldehyde dehydrogenase (ALDH) activity was used as a measure of breast cancer ‘stemness’, with data run and analysed using flow cytometry. An ALDEFLUOR kit was purchased from Stemcell Technologies to assess ALDH activity. ALDH processes the ALDEFLUOR reagent to retain a green fluorescent product, which can then be measured. ALDEFLUOR DEAB inhibits ALDH and is used as a negative control to count for background fluorescence. ALDEFLUOR reagent (provided in the kit) was prepared into its active form as per manufacturer’s instructions, and stored in aliquots at -20°C until use.

Cells were cultured in T75 flasks and harvested before use on the day. Cells were counted and then re-suspended in ALDEFLUOR assay buffer (provided in the kit) at a concentration of $5x10^5$ cells per mL.

For each sample, two tubes were prepared, one for the ALDEFLUOR reagent ('test') and one for the ALDEFLUOR DEAB ('control'). 1mL of cell culture was first added to the test tube whilst 5µL ALDEFLUOR DEAB (provided in the kit) was added to the control tube. 5µL of activated ALDEFLUOR reagent was then added to the test tube, mixed and then 0.5mL of this then added to the DEAB containing control tube. This was repeated for all samples, which were then incubated for 45 minutes at 37°C.

All tubes were then centrifuged at 750 RPM for 5 minutes to remove the buffer and ALDEFLUOR reagents. Cells were then re-suspended in 0.5mL ALDEFLUOR assay buffer, put on ice and then immediately measured using the ACEA NovoCyte flow cytometer, looking for 25,000 events per sample. Forward scatter and side scatter channels were used to first gate out cell debris and analyse the bulk of the cells in the sample. Side scatter and FITC was then used to gate for cells having ALDH activity. A polygon gate was then drawn for each control sample to the right (relative to FITC level, plotted on the Y axis) of the main cell bulk. This gate was then copied onto the equivalent test tube and the % of cells in the second gate, in the test tube for that sample, used as a measure of ALDH activity in that sample. All replicates were then averaged to compare the proportion of ALDH activity in each EveR cell line compared to its parental cell line. Each experiment was done three times with two technical replicates per cell line.

2.20 - Antibody Arrays

To give a wider picture of the drug resistant cells vs their parental counter parts, Proteome Profiler antibody arrays, supplied by R&D systems, were used. This included the use of the human phospho-kinase antibody array and the human XL oncology array. By using antibodies dotted onto each array, the expression of many dozens of proteins (and phospho-proteins) could be explored simultaneously. Thus, we could assess the status of multiple important signalling pathways and processes

in the everolimus resistant cells compared to the parental lines. Cells were seeded into T25 flasks, and then lysates taken from them when confluent, as according to the instructions in each kit. Apart from cell culture equipment and reagents, unless otherwise stated all reagents used were provided in the kits.

For the phospho-kinase array: cells were collected and washed in cold PBS with 1:100 of phosphatase inhibitor cocktail 2 (Sigma). Cells were then pelleted at 1500 RPM for 5 minutes, and 1mL of lysis buffer 6 added per $10x10^6$ cells. Solutions were then incubated on a rotating shaker, at 4°C for 30 minutes, before being aliquoted and stored at -80°C. As per manufacture instructions, all reagents from this point (excluding samples, which were thawed on ice) were used at room temperature. 1mL of array buffer 1 was added to each well required of an 8-well plate and array membranes (A and B per sample) added and incubated for 1 hour at room temperature, on a shaker. A maximum of 334uL of lysate was diluted to 2mL in array buffer 1. Array buffer was aspirated from the plate and 1mL of sample per membrane added, with the dish incubated at 4°C overnight, on a shaker.

Membranes A and B for each sample were placed in a plastic dish and washed with 20mL of 1x wash buffer, and the 8-well plate washed with dH_2O. The membranes were then washed 3 times with 1x wash buffer for 10 minutes on a shaker and then added back to the 8 well plate and all buffer was drained from it. For each membrane section A, 20uL of detection antibody cocktail A was added to 1ml of 1x array buffer 2/3, then added across the membrane. For each B membrane, 20uL of detection antibody cocktail B was added to 1ml of 1x array buffer 2/3, then to added across the membrane. The plate was then incubated at room temperature on a shaker for 2hr, then each membrane (A and B separate), removed and the plate was again washed with dH_2O. The membranes were washed 3 times with 1x wash buffer for 10 minutes on a shaker and added back to the plate after all buffer had drained from it. The streptavidin-HRP was then diluted 1:2000 in 1x array buffer 2/3, 1mL added to each membrane in the plate and incubated at room temperature for 30 minutes on a shaker.

Membranes were then washed in plastic dishes with 20mL of 1x wash buffer, then washed again 3 times with 1x wash buffer for 10 minutes on a shaker; the 8-well

plate was also washed with dH_2O. Membranes were then drained and placed on plastic sheets, before 1mL per membrane of chemi reagent mix was added across each membrane, the membranes covered with the plastic sheet and incubated for 1 minute. Excess chemi reagent was then removed by gentle wicking onto blue roll. The arrays were then visualised/ developed in the same fashion as described for developing western blot membranes, using exposures times between 1-12 minutes with Amersham hyperfilm (GE Healthcare) laid on top of the membranes.

Membranes were then analysed using ImageJ. The best exposure time for each target was chosen and then a circle used to analyse the pixel intensity within. Duplicate spots were then averaged, before the negative control value was subtracted to account for background readings. Reference targets on the membranes were used to check if the values needed normalising, when comparing the drug resistant to its parental counterpart. If reference targets differed by a noticeable amount between the two, the data set was normalised according the ratio difference between the reference targets.

For the XL oncology array: cells were collected and washed in cold PBS and then pelleted at 1500 RPM for 5 minutes. 1mL of lysis buffer 17 (R&D Systems) with 10% SIGMAFAST™ protease inhibitor cocktail (Sigma) was added per $10x10^6$ cells, with solutions then incubated on a rotating shaker at 4°C for 30 minutes, before being aliquoted and stored at -80°C. As per manufacture instructions, all reagents from this point (excluding samples) were used at room temperature. 2mL of array buffer 6 was added to each required well of a 4-well plate and membranes added and incubated for 1 hour at room temperature, on a shaker. A maximum of 0.5mL of sample was diluted with 0.5mL of array buffer 4 and the volume adjusted to 1.5mL total using array buffer 6. Array buffer was aspirated from the plate and 1.5mL of sample per membrane added, with the dish incubated at 4°C overnight.

Membranes were then placed in a plastic dish and washed with 20mL of 1x wash buffer, and the 4-well plate washed with dH_2O. The membranes were then washed 3 times with 1x wash buffer for 10 minutes on a shaker and then added back to the 4 well plate and all buffer was drained from it. Per membrane, 30ul of detection antibody cocktail was added to 1.5mL of 1x array buffer 4/6, which was then added

across the membrane. The plate was then incubated at room temperature on a shaker for 1hr, then each membrane removed and the plate was again washed with dH_2O. The membranes were washed 3 times with 1x wash buffer for 10 minutes on a shaker and added back to the plate after all buffer had drained from it. The streptavidin-HRP was then diluted 1:2000 in array buffer 6, 2mL added to each membrane in the plate and incubated at room temperature for 30 minutes on a shaker.

Membranes were then washed in plastic dishes with 20mL of 1x wash buffer, then washed again 3 times with 1x wash buffer for 10 minutes on a shaker; the 4-well plate was also washed with dH_2O. Membranes were then drained and placed on plastic sheets, before 1mL per membrane of chemi reagent mix was added across each membrane, the membranes covered with the plastic sheet and incubated for 1 minute. Excess chemi reagent was then removed by gentle wicking onto blue roll. The arrays were then visualised/ developed in the same fashion as described for developing western blot membranes, using exposures times between 30sec-8 minutes with Amersham hyperfilm (GE Healthcare) laid on top of the membranes.

Membranes were then analysed the same way as described previously for the phospho-kinase arrays.

2.21 - siRNA Transfections: Transfection Optimisation

To test whether specific proteins involved in mTORC2 signalling, namely PKCα and SGK1, are involved in the everolimus resistance mechanism, siRNA based knock-down of the associated genes was used. SMARTpool: ON-TARGETplus siRNA specific for *PRKCA* (PKCα) and *SGK1* along with 5x siRNA buffer, Dharmafect 1 transfection reagent and ON-TARGETplus Non-targeting pool (to be used as a negative control siRNA) were all selected and purchased from Dharamcon (GE Healthcare) for these experiments. All siRNAs used were made up to a stock concentration of 20μM, using 1x siRNA buffer (in RNase free water) and stored at -20°C. All siRNAs (including siGLO) were used at a working concentration of 5μM.

To first optimise the cell and reagent concentration/volumes to be used in the experiments, a series of transfections using siGLO green transfection indicator (Dharamcon), instead of siRNA, were done first. 24-well plates were seeded with T47D-Parental and 361-Parental cells at concentrations of 10, 20 or $30x10^3$ cells per well. This was done in conjunction with adding 0.25, 1, 1.75 or 2.5µL per well of Dharmafect 1, thus to test which concentration of cells worked best with which concentrations of transfection reagent. 2.5uL of 5uM siGLO was used per well, as per manufacturers recommendations.

Cells were seeded into plates and left over night before transfection with the siGLO was done the following day. The siGLO and Dharmafect 1 were diluted with plain RPMI (no glutamine penicillin streptomycin/QPS or FBS), in separate tubes, in volumes specified in table 2.18. Tubes were made separately, mixed gently with pipetting and then left at for 5 minutes. The contents of one siGLO tube were then added to one Dharamfect tube, mixed together and left for another 20 minutes. The combined solution was then made up to its final volume of 500µL per well using RPMI (with glutamine/Q and FBS), using the volumes specified in table 2.18.

Plates from the previous day then had all media removed, and the media containing the Dharamfect and siGLO added to each appropriate well. The FLoid cell imaging station was then used to take pictures of the cells at 0, 24 and 48hr post transfection. The transfection reagent media was left on the plate for 24hrs before being removed, to lower toxicity to cells, and replaced with normal cell culture RPMI. Cells were checked for their relative health and the relative amounts of siGLO that had entered the cells.

For experiments where a subsequent re-transfection with the same siRNA was tested, the media was removed the following day after transfection as per the single transfection, and then then transfection was done again exactly as outlined previously. Media for these plates was then replaced the following day with normal cell culture RPMI.

	Volumes per well (µL) (24-well plate)			
	Dharmafect 1	siGLO (5µM)	Plain RPMI	RPMI (Q+FBS) added after tube 1 and 2 combined
Tube 1		2.5	47.5	
Tube 2	0.25		49.75	400
	1		49	400
	1.75		48.25	400
	2.5		47.5	400

Table 2.18: Volumes per well for siGLO transfections optimisation experiments. Volumes of each reagent were first added to tube 1 and tube 2, with the contents of 1x tube 1 then added to 1x tube 2 (then incubated for 20 minutes). The RPMI (Q+FBS) was then added to each tube to make a final volume of 500µL to be added per well.

2.22 - siRNA Transfections: Testing Knockdown of *PRKCA* and *SGK1*

Once siGLO optimisations were complete, with the volume of Dharmafect to be used and the cell amounts per plate selected, the siRNAs to be used were then tested for their efficiency of knockdown. 12-well plates were set-up with T47D everolimus resistant and parental cell line (to check efficiency of knockdown in a resistant and non-resistant line), with $60x10^3$ cells per well and left over night before transfecting.

A single and double transfection was tested, with both transfections being done exactly as outlined before with the siGLO based experiments. 5µM of either PRKCA, SGK1 or Non-targeting pool (Dharmacon) (as a negative control) siRNA was used in place of the siGLO. Volumes were scaled up from the 24-well plates used previously to match the increased size of the 12-well plates, as shown in table 2.19. Cell lysates were taken at 24, 72hr and 5 days after transfection (or second transfection with the double transfection samples). These were then checked for relative protein content of PKCα and SGK1, using western blotting.

	Volumes per well (µL) (12-well plate)			
	Dharmafect 1	siRNA (5µM)	Plain RPMI	RPMI (Q+FBS) added after tube 1 and 2 combined
Tube 1		5	95	
Tube 2	2		98	800

Table 2.19: Volumes per well for transfections done in a 12-well plate. Volumes of each reagent were first added to tube 1 and tube 2, with the contents of 1x tube 1 then added to 1x tube 2 (then incubated for 20 minutes). The RPMI (Q+FBS) was then added to each tube to make a final volume of 1000µL to be added per well.

2.23 - siRNA Transfections: Knockdown + Everolimus Treatment

Once the efficiency of each siRNA had been checked, the siRNAs were then tested to see if that had an effect on everolimus resistance, using the SRB assay in conjunction with the knockdown of *PRKCA*. In 96 well pates, $2.5x10^3$ T47D-EveR, $2 x10^3$ T47D-Parental, $7 x10^3$ 361-EveR or $5 x10^3$ 361-Parental cells were seeded, and left over night. They were then transfected, again in the same fashion as previously described, with the volumes scaled down for 96-well plates, as shown in table 2.20. PRKCA and non-targeting plates/wells were both transfected using a single transfection. Cells were transfected a day after seeding, with the media changed the following day with normal cell culture media that was laced with everolimus. Plates were then fixed and stained with SRB 5 days from this point.

	Volumes per well (µL) (96-well plate)			
	Dharmafect 1	siRNA (5µM)	Plain RPMI	RPMI (Q+FBS) added after tube 1 and 2 combined
Tube 1		0.5	9.5	
Tube 2	0.2		9.8	80

Table 2.20: Volumes per well for transfections done in a 96-well plate. Volumes of each reagent were first added to tube 1 and tube 2, with the contents of 1x tube 1 then added to 1x tube 2 (then incubated for 20 minutes). The RPMI (Q+FBS) was then added to each tube to make a final volume of 100µL to be added per well.

2.24 - Statistical Analysis

Data was averaged across experimental repeats and equal variance un-paired student T-test used to compare data for statistical significance. T-test was selected due to normally distributed data across treatments. Data was deemed significant when P=<0.05.

Chapter 3: Confirmation of mTOR Signalling in Breast Cancer Cell Lines

3.1 - Introduction

3.1.1 - Breast Cancer Cell Markers in Key Cell Lines

As discussed in sections 1.2-1.3, the presence (or lack off) certain key breast cancer cell markers are greatly important in not only classifying a breast cancer, but also in determining treatment and prognosis; with ER, PR and HER-2 among the most important of these. Checking the status of these markers is important when working with breast cancer cell lines, to help show that each cell is what we believe it to be, and thus validate our work. Shown in table 3.1, are these key markers, and their expression in key cell lines used in this research, according to most widely accepted research.

	Receptor status		
Cell line	**ER**	**PR**	**HER-2**
T47D	+	+	-
SKBR3	-	-	+
GI101	+	?	-
MDA-MB-361	+	-	+
MDA-MB-436	-	-	-
MDA-MB-231	-	-	-
MCF10a	-	-	-

Table 3.1: Table to show the status of breast cancer markers ER, PR and HER-2, for cell lines used in this research. For ER/PR, + = expression of marker, - = no expression of marker. For HER-2, + = over-expression, - = normal expression. GI101 cells are less widely published on, and ER status is only known at a gene level (yellow). Information from Holliday and Speirs (2011), Kao et al (2009), Mackay et al (2009), Morrissey and Raney (1998), Neve et al (2006) and Prat et al (2013).

3.1.2 - Vitamin D and Rapalogue Resistance

Vitamin D has potential as a novel way to overcome rapalogue resistance. This steroid-like molecule has well known roles in bone metabolism but research has also shown its effects in cancer. Vitamin D_3 (common form of vitamin D) is converted to the circulating metabolite 25-hydroxyvitamin D_3 before conversion to the biologically active 1,25-hydroxyvitamin D_3 (calcitriol). Many of its actions, including in breast cancer, are then mediated by its nuclear receptor (VDR) (Matthews et al, 2010).

Vitamin D has tumour suppressive action in a vast range of cancers including skin, prostate, colon and breast (Welsh, 2012). In breast cancer, data indicates a strong link between higher blood levels of vitamin D and decreased incidence and improved prognosis (Rose et al, 2013). Cell and animal studies, have shown that vitamin D often does this by altering expression of genes involved in multiple biological processes relating to tumour development and progression. Cell cycle arrest genes are up-regulated, whilst proliferative genes like c-myc are down-regulated. Metastasis and EMT are also blocked through E-cadherin up-regulation, MMP

inhibition and the blocking of angiogenesis. The oestrogen pathway, which is so important in breast cancer, is also down-regulated. Non-genomic actions of vitamin D have also been reported in breast cancer cells (LaPorta and Welsh, 2014; Moukayed and Grant, 2013; Shao et al, 2012). Research into vitamin D responsive breast cancer cell lines SKBR3, T47D, GI101 and MDA-MB-361 has suggested that vitamin D action is independent of VDR (Harvey, unpublished).

Research in multiple cell types is now suggesting that vitamin D can also regulate the mTOR pathway. mTOR is inhibited in osteoblasts by calcitriol, via direct up-regulation of the mTOR suppressor, DDIT4 (Lisse et al, 2011). Research in T-cells and keratinocytes (relating to immune-mediated psoriasis) has shown Akt activation to be reduced on exposure to vitamin D analogues (Datta-Mitra et al, 2013a; Datta-Mitra et al, 2013b). In breast cancer cells, a reduction in Akt phosphorylation after vitamin D analogue treatment was accompanied by reduced mTOR signalling (O'Kelly et al, 2006). This raises the question as to whether 1,25-hydroxyvitamin D_3/vitamin D can also reduce mTOR signalling in breast cancer cell lines, thereby opening potential new avenues for therapy, by combining it with an mTOR inhibitor.

With rapalogues making their way into the clinic, resistance is very likely to begin occurring and vitamin D may in theory offer a cheap and easy way of once again re-sensitising these cells. The combination of everolimus and 1,25-hydroxyvitamin D_3 has been shown to have potentiation effects in acute myeloid leukaemia (AML) studies, both *in vitro* and *in vivo* (Yang et al, 2010), with the question still remaining as to whether this also occurs in breast cancer cells. As the mTOR pathway is often the cause of resistance to trastuzumab and other therapies that target growth factor signalling, vitamin D and everolimus could be used to overcome resistance to these therapies as well as improve efficacy, if mTOR is regulated by vitamin D in breast cancer cells. Evidence to support this hypothesis comes from Zeichner and colleagues (2015) who showed increased patient survival when vitamin D supplementation was given to patients on trastuzumab.

3.2 - Aims

- The specific aims of this chapter were to:
    - Confirm the receptor status of the breast cancer cells and characterise their cell morphology
    - Characterise the expression of mTOR signalling molecules in breast cancer cell lines to give an indication as to which parts of the pathway may be more active than others.
    - Study the response of breast cancer cell lines to the mTOR inhibitor everolimus and the combination treatment of everolimus with calcitriol (vitamin D).

3.3 - Results

3.3.1 - Characterisation of Cellular Morphology

Breast cancer cell lines T47D, SKBR3, GI101, MDA-MB-361, MDA-MB-436 and MDA-MB-231, as well as the non-tumourgenic breast cell line, MCF10a, were examined to give an overall view of the cells and as a basis for comparison to the everolimus resistant cells that were to be developed. Cells were imaged at x20 magnification (objective) and compared in terms of cell morphology and growth patterns, with representative images shown in figure 3.1. SKBR3, MDA-MB-436 and MDA-MB-231 showed a very distinct morphology, being much less rounded than other cell lines such as the T47D, with multiple cytoplasmic projections, with an almost mesenchymal appearance. This is in contrast to the T47D and GI101 cell types which grow with a relatively more rounded appearance, in ‘crazy paving’ morphology. Unlike the other cell lines, the MDA-MB-361 cells, tended to grow in small colonies. The MCF10a cells, showed elongated, larger cell morphology.

Whilst looking at normal split ratio for each cell line, it was noted that in terms of proliferation, the SKBR3, MDA-MB-436 and MDA-MB-231 cells were by far the fastest growing and the MDA-MB-361 were the slowest growing (of the breast cancer lines), with the T47D and GI101 growing at intermediate rates between them. The MCF10a grew at a slightly slower rate than any of the breast cancer cell lines.

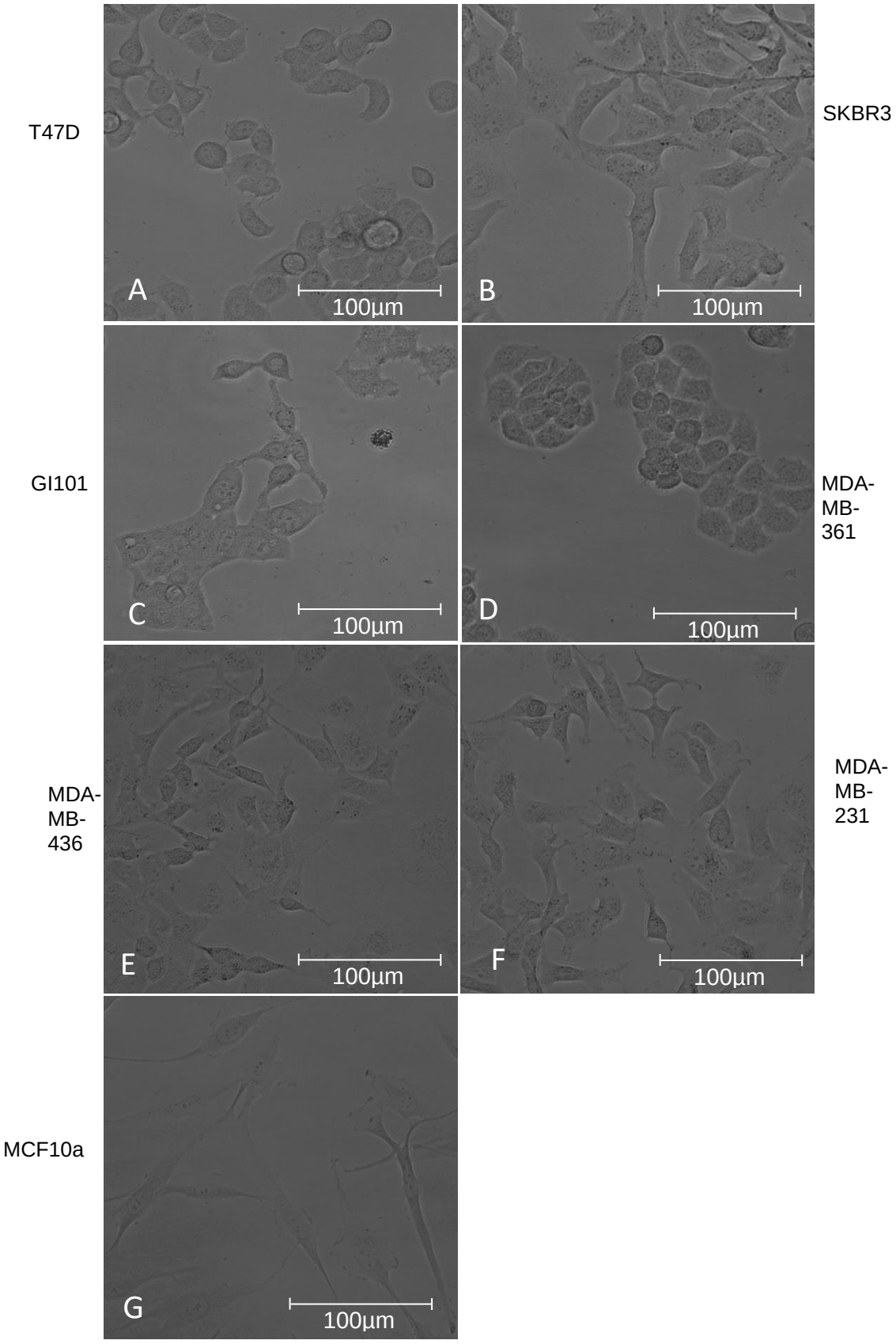


Figure 3.1: Representative images of breast cell lines. *T47D (A), SKBR3 (B), GI101 (C) MDA-MB-361 (D), MDA-MB-436 (E) and MDA-MB-231 (F) and images of non-tumourgenic breast cell line, MCF10a (G) at x20 magnification.*

3.3.2 - Expression of Key Breast Cancer Markers

The status of key breast cancer cell markers, were studied, using western blotting. This included oestrogen receptor α (ERα), progesterone receptor (PR) and HER-2, as shown in figure 3.2. This was to check that the cell lines matched status of these markers, compared to previously published research (Holliday and Speirs, 2011; Kao et al, 2009; Mackay et al, 2009; Morrissey and Raney, 1998; Neve et al, 2006; Prat et al 2013). Cell lines matched previous research in terms of these markers, with only T47D expressing progesterone receptor and T47D, GI101 and MDA-MB-361 expressing ERα. However the SKBR3 did not match their previously stated HER-2 status. Whilst SKBR3 are generally HER-2 over-expressers, like the MDA-MB-361, the SKBR3 showed normal/little expression.

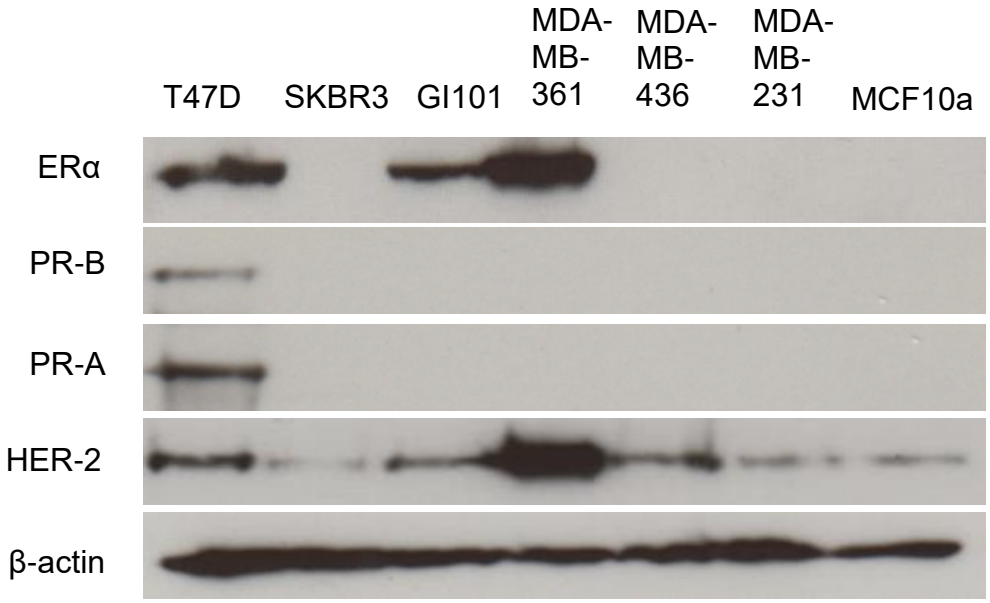


Figure 3.2: Cell lines express varying levels of key cancer biomarkers. *Representative western blots for cell lines T47D, SKBR3, GI101, MDA-MB-361, MDA-MB-436, MDA-MB-231 and MCF10a. n=3 independent experiments. Target proteins were key markers of breast cancer that included oestrogen receptor α (ERα) and progesterone receptor (PR) expression. For HER-2, + = over-expression, - = normal expression. E.g. T47D are – whilst MDA-MB-361 are + in regards to HER-2.*

3.3.3 - Confirming the Expression of mTOR Pathway Components at a Protein Level

At the start of the project there were few papers summarising and comparing the status of mTOR components in a panel of breast cancer cells. Since one of the primary objectives was to develop everolimus resistant cells (and everolimus inhibits mTOR signalling), it was important that cell lines with functional and active mTOR signalling were chosen for this process. Therefore the status of major mTOR components was studied by western blotting. This data is shown in figure 3.3. The data shows that cell lines chosen for this project do express various important mTOR components and thus should respond to various mTOR inhibitors, studied later in this project; however there were some differences between the expression of certain components. Please see section 4.3.2 for more details on cell lines selected and used for development of everolimus resistant models.

The ratio of raptor vs rictor expression was markedly different between cell lines. T47D, MDA-MB-361 and MCF10a showed distinctly lower levels of rictor compared to raptor, unlike SKBR3, GI101, MDA-MB-436 and MDA-MB-231 lines which showed generally equal amounts. The MDA-MB-361 also had much larger levels of total and phospho-S6K (both isoforms) which supports the hypothesis that mTORC1 activation is elevated in comparison to other cell lines, such as GI101 for example.

DEPTOR expression also varied greatly between the cell lines. MCF10a then T47D, followed by MDA-MB-361 and GI101 expressed the highest level of DEPTOR protein, with SKBR3 and the triple negative lines (MDA-MB-231/436) showing extremely low levels. In terms of cell breast cancer type, both triple negative lines showed similar expression overall of mTOR components.

Interestingly the greater expression of either raptor or rictor (which form the respective complexes of mTORC1 or 2) did not necessarily correlate with active downstream signalling of either complex, as shown by looking at the p-S6K in comparison with raptor and p-PKCα and p-Akt in comparison with rictor. Whilst cell lines like the T47D and triple negative lines had high raptor levels, they contained less active (phosphorylated) S6K compared to lines such as the MDA-MB-361, which showed the reverse trend. This negative correlation between key complex

component expression (raptor or rictor) and downstream signalling was seen to an even greater extend in regards to rictor and mTORC2 signalling. Rictor seemed to negatively correlate in all 7 cell line with p-Akt levels, as seen in figure 3.4. By ordering the cell lines by rictor expression, DEPTOR as well as p-Akt negatively correlated with rictor levels. Perhaps even more interestingly, we do not see the same negative correlation in regards to rictor and p-PKCα (figure 3.3), which is of course also a downstream target of mTORC2; suggesting perhaps that different mTOR complex amounts may affect downstream targets differentially. The expression of p-mTOR (Ser2481) also negatively correlated with rictor levels.

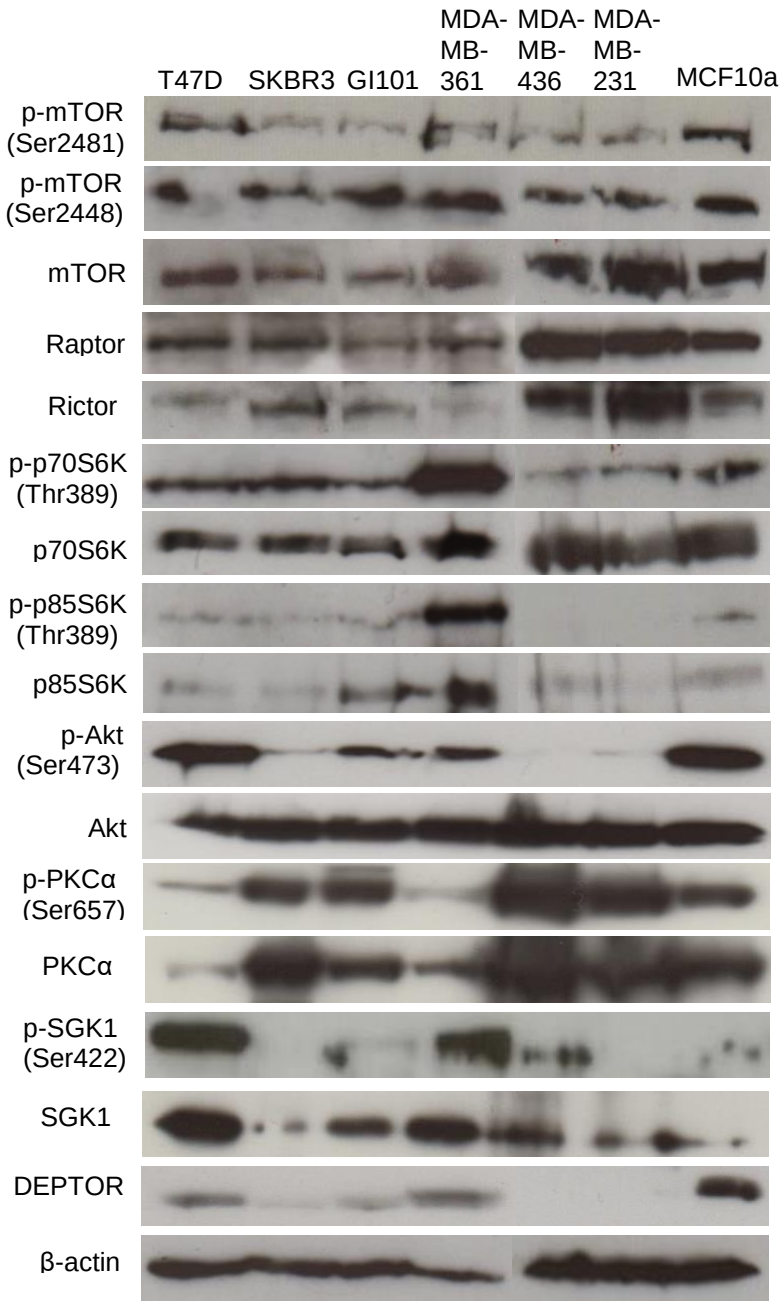


Figure 3.3: All cell lines express mTOR components. *Representative western blots for cell lines T47D, SKBR3, GI101, MDA-MB-361, MDA-MB-436, MDA-MB-231 and MCF10a. n=3 independent experiments. Target proteins included major mTOR signalling pathway components to give an overview of the status of mTOR signalling in these cell lines, with β-actin as a loading control. Raptor and rictor are required for mTORC1 and mTORC2 signalling respectively. Phosphorylated S6K (p-p70/85S6K) at Thr389 shows downstream activation of mTORC1 signalling with phosphorylated PKCα, SGK1 and Akt showing downstream activation of mTORC2.*

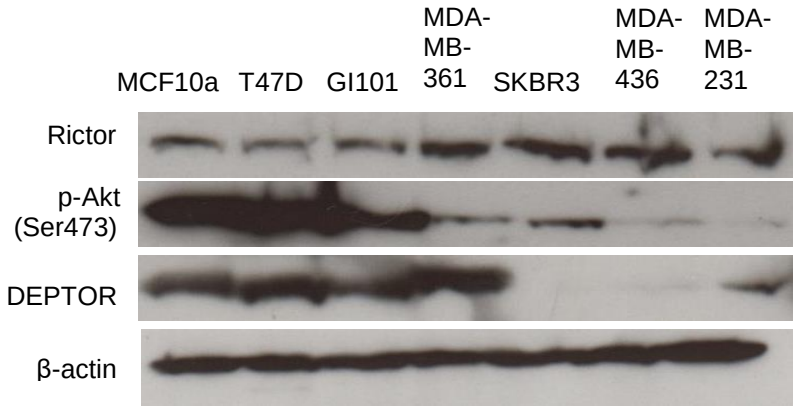


Figure 3.4: Rictor expression negatively correlates with DEPTOR expression and Ser473 Akt phosphorylation*. Western blot comparing rictor expression to p-Akt (Ser473) and DEPTOR expression, with cell lines ordered by rictor expression. n=3 independent experiments. The expression of the mTORC2 critical component rictor, correlates negatively with the levels of the downstream mTORC2 target p-Akt, as well as with the mTOR inhibitor DEPTOR.*

3.3.4 - Confirming the Expression of mTOR Pathway Components at a Gene Level

The status of major mTOR components was also determined at a gene expression level, using qPCR analysis. Before this was done, housekeeping genes were selected using a geNorm primer kit (Primer Design) and qbase+ (Biogazelle) software. All 7 cell lines were assessed to find the most stable genes to use for later mTOR qPCR experiments. Results for this analysis can be seen in figure 3.5. As seen in this figure, qbase software used qPCR results, studying gene expression of 12 different reference genes in all 7 cell lines, to determine a 'M-value' for each gene; with the genes having the lowest M-value being the most stable. The software then calculated a 'V-value' to dictate the ideal number of reference genes to be used in qPCR experiments containing these 7 cell lines. Overall the analysis recommended the use of both *TOP1* and *YWHAZ* as housekeeping genes for these cell lines.

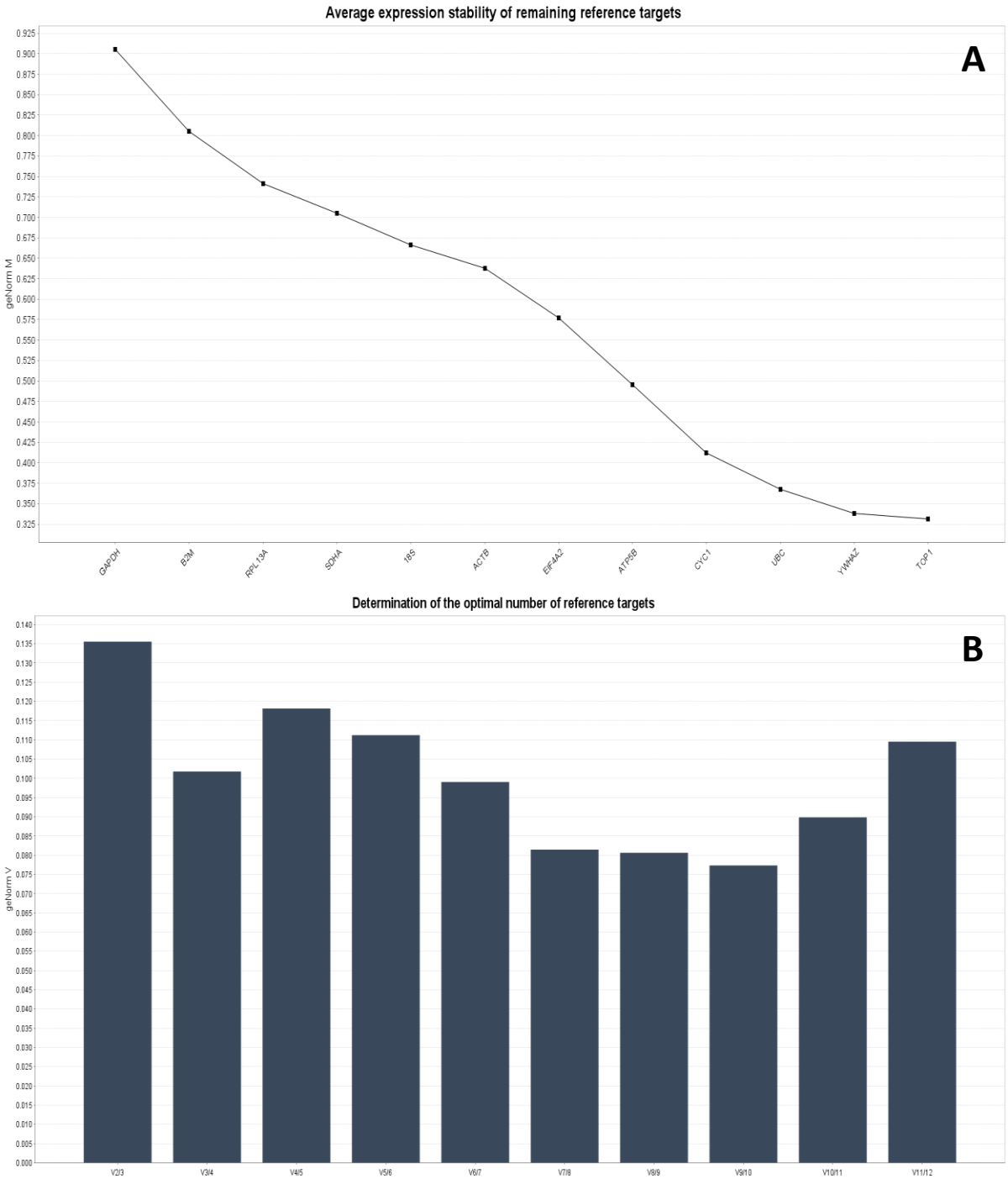


Figure 3.5: TOP1 and YWHAZ are recommended reference genes. *geNorm analysis, using qbase+ (Biogazelle), with the stability of the potential housekeeping genes ranked in graph A, using the 'M' value, with the most stable on the right with the lowest value. Graph B shows the ideal number of reference genes, with any 'V' value lower than 0.15 suitable. The use of 2 genes fit these criteria, with TOP1 and YWHAZ, being selected as they were the highest ranking in terms of stability.*

The expression of *MTOR, RAPTOR, RICTOR* and *DEPTOR* were then studied using the same qPCR method, with the relative quantity (RQ) used to compare expression across all cell lines, shown in figure 3.6. MDA-MB-361 and MCF10a showed the highest expression of *DEPTOR*, whilst the triple negative lines and the SKBR3 had markedly higher *RICTOR* expression than other cell lines. *RAPTOR* and *MTOR* expression showed less obvious trends, or differences between cell lines, although MCF10a did show slightly higher expression of both genes, compared to the other cell lines. Thesis data, along with the western blotting data (figure 3.3), showed that despite being an immortalised non-tumourgenic line, the MCF10a had higher expression of mTOR and raptor at both a protein and a gene level.

In terms of the congruity between this data and the western blotting data (see figure 3.3), there was a good level, with gene expression for *RAPTOR, RICTOR* and *DEPTOR* matching well to the protein expression. Whilst *MTOR* expression was similar for the qPCR data compared to the western data for cell lines T47D, SKBR3, GI101, MDA-MB-361 and MCF10a, this matched less so for the triple negative lines.

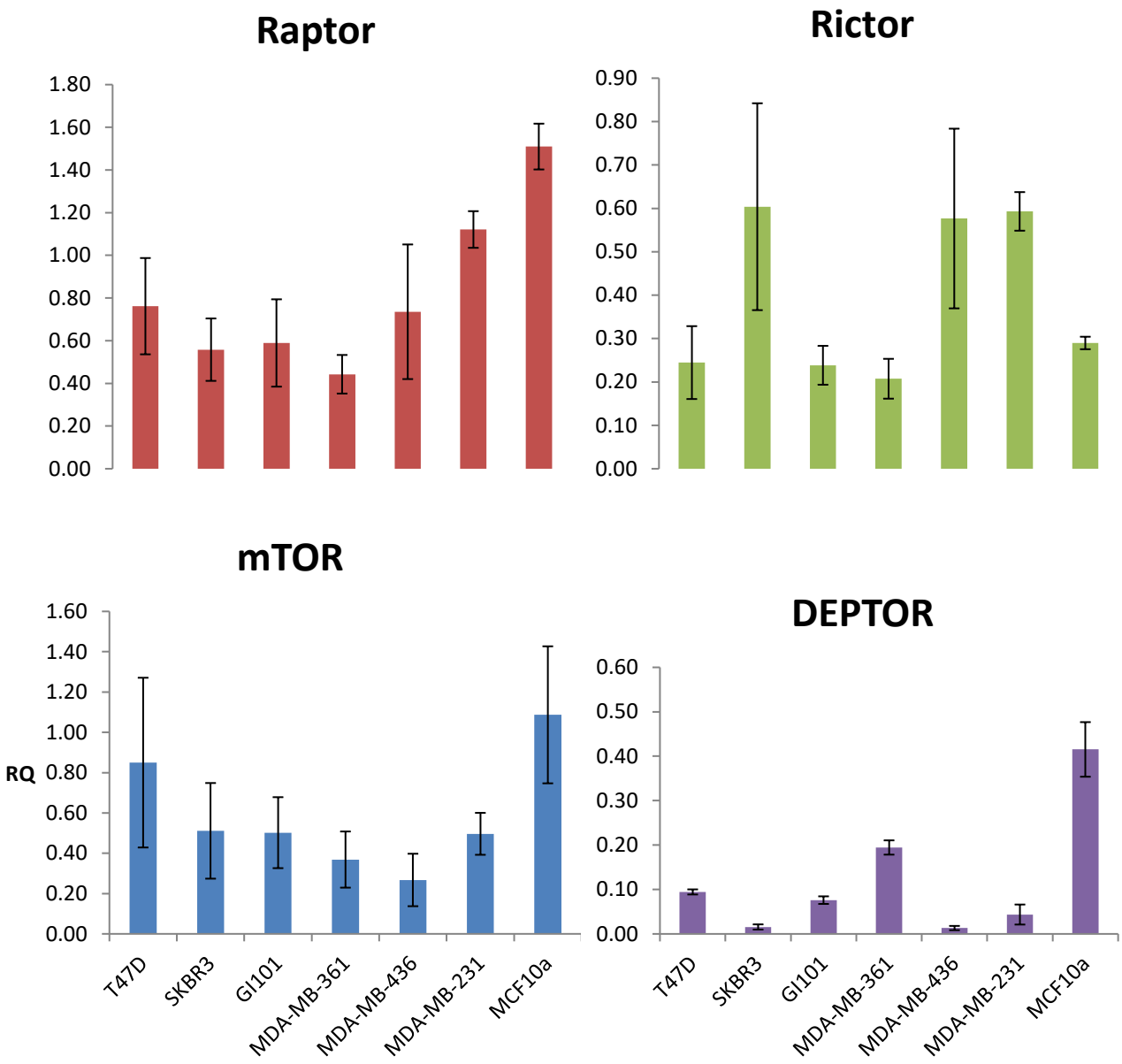


Figure 3.6: Base gene expression for key mTOR components. *qPCR results for individual genes (mean plus or minus standard deviation, n=2 independent experiments) studying base gene expression of MTOR, RAPTOR, RICTOR and DEPTOR, with data displaying RQ (relative quantity).*

To check that primers had amplified the correct sequence, a representative sample of post-PCR product for each cell and for each target was analysed on an agarose gel and studied for the correct DNA product size. Expected product size is shown in table 3.2. All samples showed the expected product size (figure 3.7) confirming that the q-PCR results described previously had been accurate to the aforementioned genes.

A small number of negative controls (water) did show a ct value, suggesting contamination in some samples, hence a secondary reason to check them using an agarose gel. However, all contamination was generally 5-10 ct values away from the sample ct values and were generally higher than 35 (for *DEPTOR*, *RICTOR* and *TOP1*). In line with this, data were still deemed reliable to use. Agarose gels revealed that contamination in the negative control, as seen for *MTOR* and *TOP1* which showed an extra band in this sample, was not of the correct bp size for the corresponding gene. This further suggests that amplification in the samples was genuine and thus reliable.

Gene	**Expected product size**
MTOR	135
RAPTOR	170
RICTOR	117
DEPTOR	202
YWHAZ	127
TOP1	125

Table 3.2: Expected qPCR product sizes.

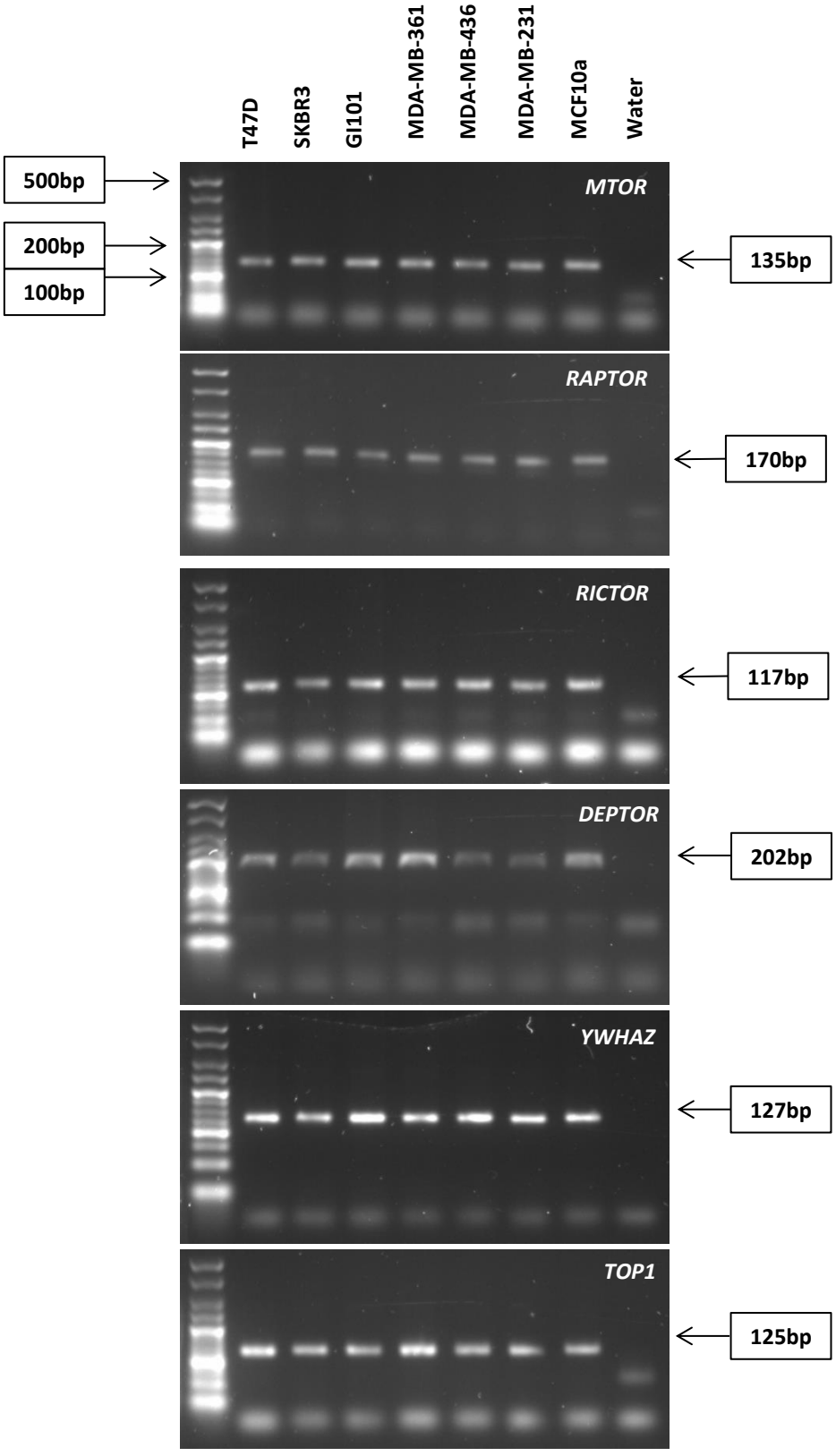


Figure 3.7: qPCR results show expected amplicon size. *Agarose gel images from the post-PCR products of q-PCR experiments detailed previously to check for the presence of expected product size of each primer; all primers showed the expected bp size.*

3.3.5 - Breast Cancer Responsiveness to Everolimus and Calcitriol

Everolimus is an mTOR inhibitor and rapalogue, currently in clinical use for breast cancer. 4 cell lines, from the 7 worked on previously in this chapter, were selected to be tested for their responses to drugs including everolimus. These cell lines were selected based on their use in previously unpublished work by Harvey, with all 4 showing responsiveness to calcitriol in that work. The response of the 4 cell lines to everolimus, was assessed using an SRB assay to stain for total protein as a surrogate for relative cell number (RCN), after a 72 hour treatment, with the results seen in figure 3.8. Whilst physiological concentration of calcitriol (active form of vitamin D) varies depending on the person and the context (i.e. amount of exposure to sunlight), the average individual has plasma concentrations of roughly 10nM (Wacker and Holick, 2013). For this reason, the same concentration range was used as for everolimus, including doses either side. All cell lines responded to the inhibitor with a greatly reduced protein mass and cellular growth, with the T47D showing the greatest inhibition. As can be seen from figure 3.8, response to the drug (after 72hr) generally plateaued after 10nM, which is also close to a physiological dose of everolimus (Kirchner et al, 2004), although a greater increase in growth inhibition was often seen at the much higher concentration of 10,000nM.

Also shown in figure 3.8 is the data for the 72 hour calcitriol treatments. As expected based on previous data, the cell lines responded to calcitriol. The response was less in terms of growth inhibition, compared to everolimus treatments, but the T47D again showed the greatest response with an average of 40% inhibition (60% growth compared to control) at 10,000nM of calcitriol. The response from the SKBR3 cells was more variable, with cells generally not responding to calcitriol.

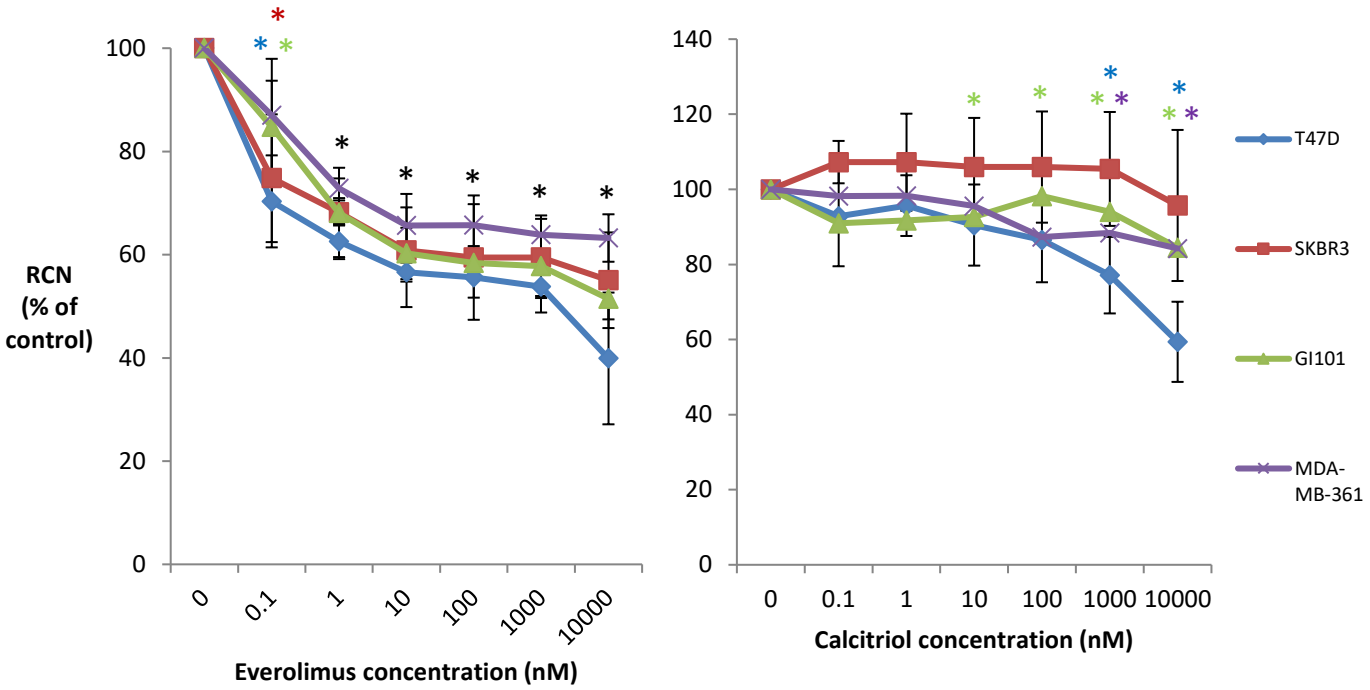


Figure 3.8: Cells show significant growth inhibition by everolimus. *SRB results (mean plus or minus standard deviation, n=3 independent experiments) for 72 hour treatment of either everolimus (left) or calcitriol (right). Relative cell number (RCN) represents the % OD compared to the control. * P<0.05 compared to control (DMSO only), with black * representing all cell lines at that dose.*

3.3.6 - Everolimus and Calcitriol as a Combination Treatment

Since both everolimus and vitamin D can individually inhibit the growth of breast cancer cells, and together show synergistic effects in AML (Yang et al, 2010) the two drugs were tested in combination to see whether this synergy could also be observed in breast cancer cells lines. This combination was tested at the same dose range of everolimus as with previous experiments and one of three calcitriol doses; 0.1nM (low), 10nM (medium) or 1µM (high). The effect of combination treatments was tested using both the SRB assay and cell counting in conjunction with trypan blue staining, which also assessed relative cell death. Figures 3.9-3.10 show the SRB results for the combination treatments after 72 hours. Overall, there was no obvious additional effect of the two drugs in combination and everolimus alone was just as effective as it was in combination with calcitriol. The high calcitriol dose with everolimus in MDA-MB-361 (figure 3.10) was one of the combined data sets to show

some extra affect. However this was a small difference and no data point for the combination treatment for this experiment was significantly lower than the everolimus only. As the MDA-MB-361 did show some small extra affect at this higher calcitriol dose, a 1 week treatment was tested with these cells, across this dose range, to see if the increased treatment time extenuated this affect. As seen in figure 3.11, the 1 week treatment showed no extra affect with the two drugs against everolimus only, which supports the observation that there was no significant difference between treatments at the 72 hour stage in MDA-MB-361 cells.

It should be noted that when comparing data from figure 3.9 to 3.10, the later actually showed an increase (rather than decrease) in RCN in most cell lines, when looking at the 10000nM everolimus concentration. Due to the observing this phenomena across the board for this data set (figure 3.10), it was deemed that was most likely due to a technical error from a particular channel, in our multichannel pipette.

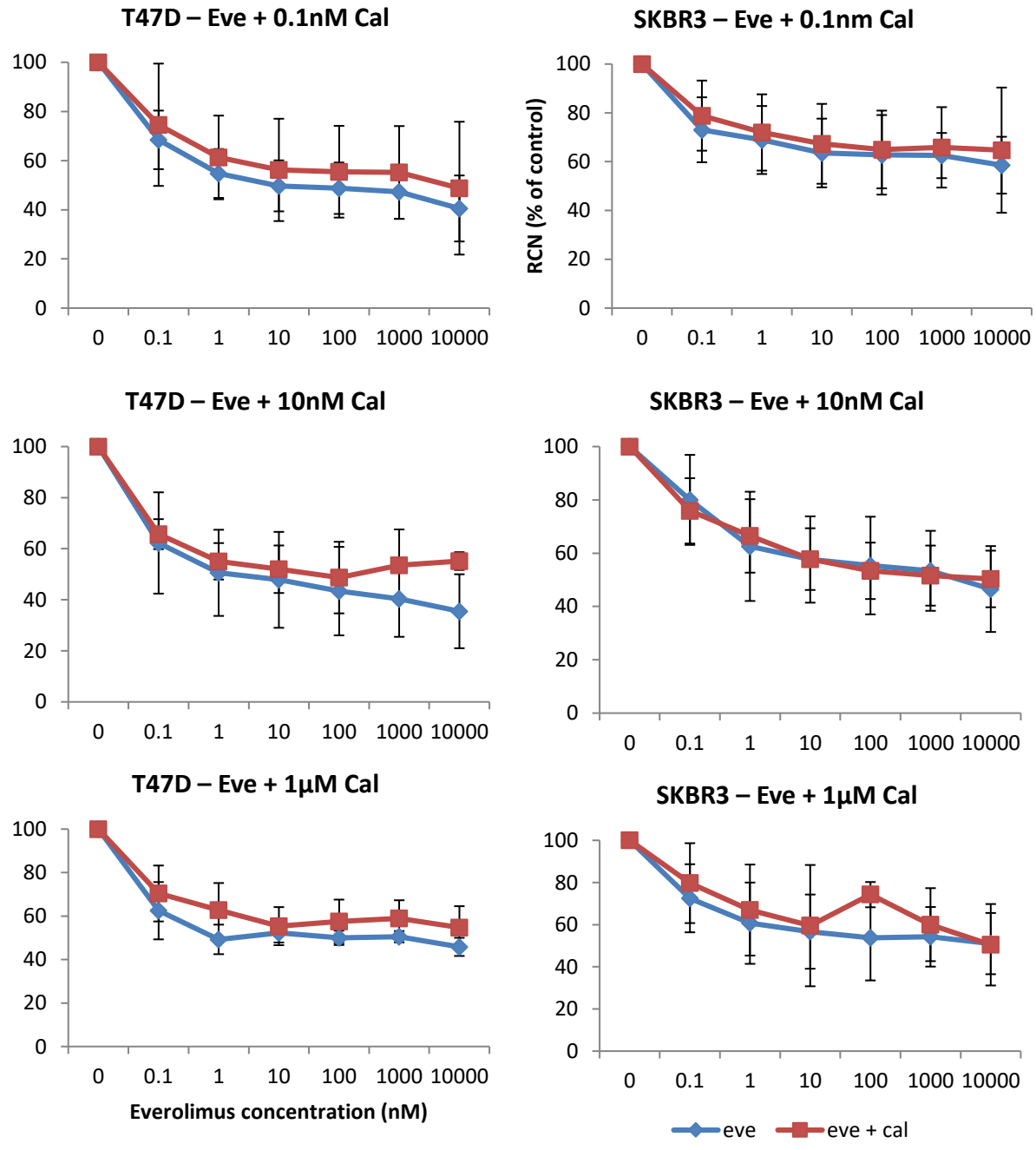


Figure 3.9: Everolimus+0.1nM, 10nM or 1µM calcitriol fails to improve growth inhibition v everolimus only. *SRB result (mean plus or minus standard deviation, n=3 independent experiments) for T47D and SKBR3 cells treated for 72 hours with either everolimus (eve) or everolimus + 0.1nM, 10nM or 1µM calcitriol (eve + cal). Across all dose ranges, the combination of drugs did not inhibit growth more than everolimus alone.*

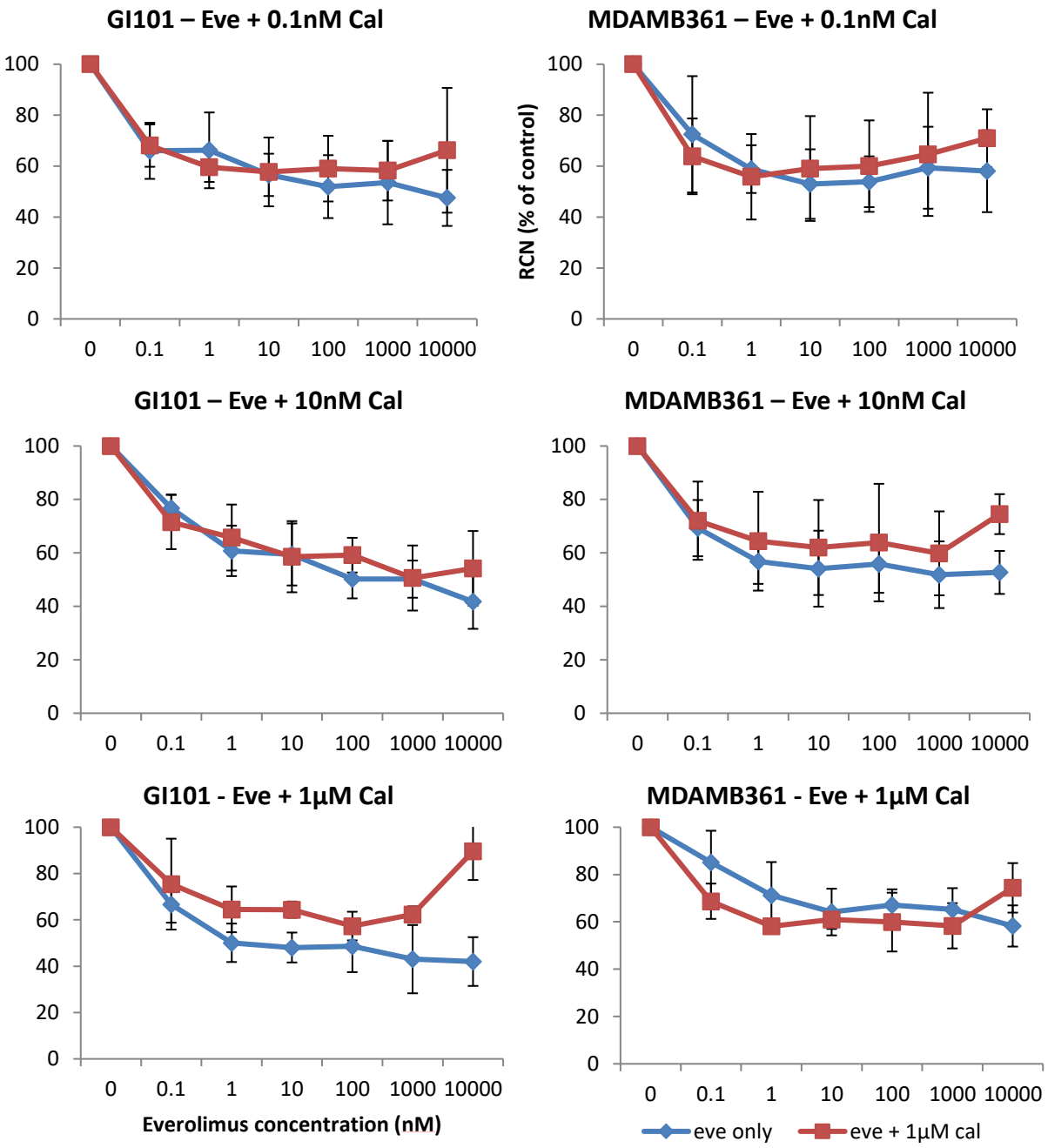


Figure 3.10: Everolimus+0.1nM, 10nM or 1µM calcitriol fails to improve growth inhibition v everolimus only. *SRB result (mean plus or minus standard deviation, n=3 independent experiments) for GI101 and MDA-MB-361 cells treated for 72 hours with either everolimus (eve) or everolimus + 0.1nM, 10nM or 1µM calcitriol (eve + cal). Only the high (1µM) calcitriol with everolimus showed any increased affect over the everolimus only, although no dose inhibited growth significantly more than the everolimus only.*

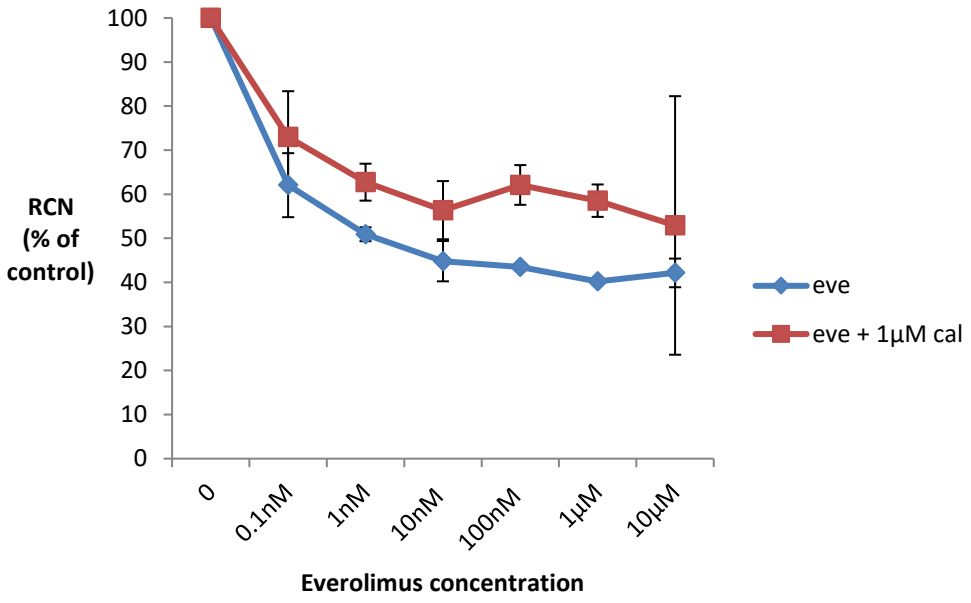


Figure 3.11: Everolimus+calcitriol fails to improve growth inhibition v everolimus only. *SRB result (mean plus or minus standard deviation, n=2 independent experiments) for MDA-MB-361 cells treated for 1 week with either everolimus (eve) or everolimus + 1μM calcitriol (eve + cal). Despite the fact that the equivalent 72 hour treatment showed the combination treatment inhibited growth slightly (but not significantly) less than the everolimus alone, the 1 week treatment, shown here did not show the same trend with the everolimus alone actually inhibiting growth slightly more than the combination.*

The effect of everolimus and calcitriol in combination, on cell viability, was also tested via cell counting after trypan blue staining. Everolimus was tested at doses 0.1nM, 10nM and 1μM and then the same doses in combination with 10nM calcitriol (figures 3.12-3.13). The dose of calcitriol was chosen due to it being an estimated physiological dose (Wacker and Holick, 2013).

Across all dose ranges there was no variation in dead cell number. This included the cells treated with everolimus alone, the calcitriol alone and the cells exposed to both compounds. This suggests that both everolimus and calcitriol as single treatments are having a cytostatic effect on these cells as opposed to a cytotoxic effect. It is also evident that the addition of calcitriol with everolimus has no added effect in terms of cytotoxicity or cell number. Whilst some doses of the combination treatment showed

a slightly lower live cell number than the everolimus only counterpart, this difference was extremely small and not significant. Examples of this can be seen in the MDA-MB-361 data for the 0.1nM everolimus and the GI101 for the 10nM everolimus data points at the 1 week mark in figure 3.13.

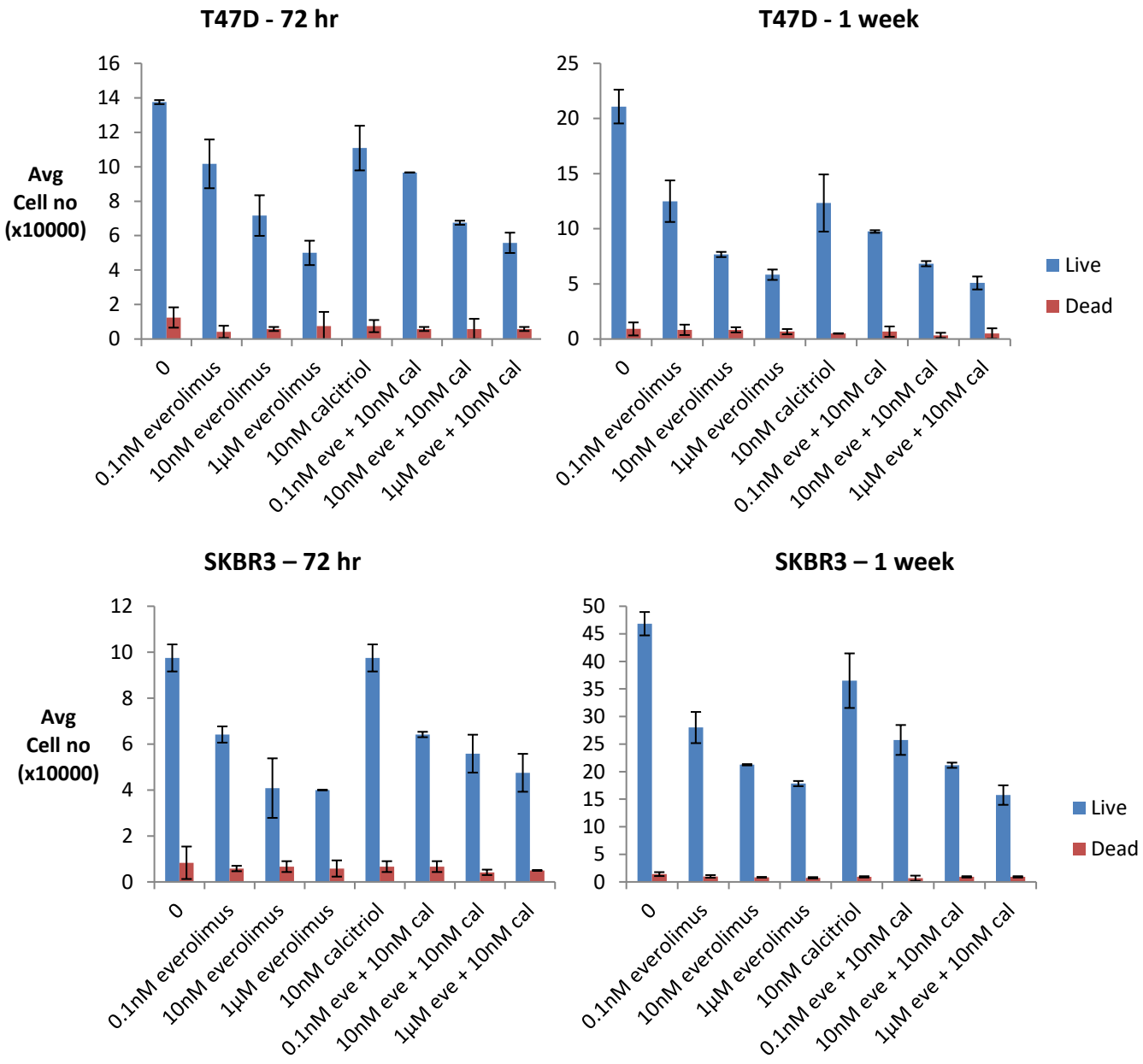


Figure 3.12: Addition of calcitriol to everolimus regimen does not alter cell death rate. *Cell counting and trypan blue staining results (mean plus or minus standard deviation, n=2 independent experiments) for T47D (top row) and SKBR3 (bottom row) treated for either 72 hour (left) or 1 week (right), with either everolimus (eve) or everolimus + 10nM calcitriol (eve + cal). There was no change in cell death across any dose. In terms of live cell numbers, the combination treatment did not inhibit growth significantly more than everolimus only.*

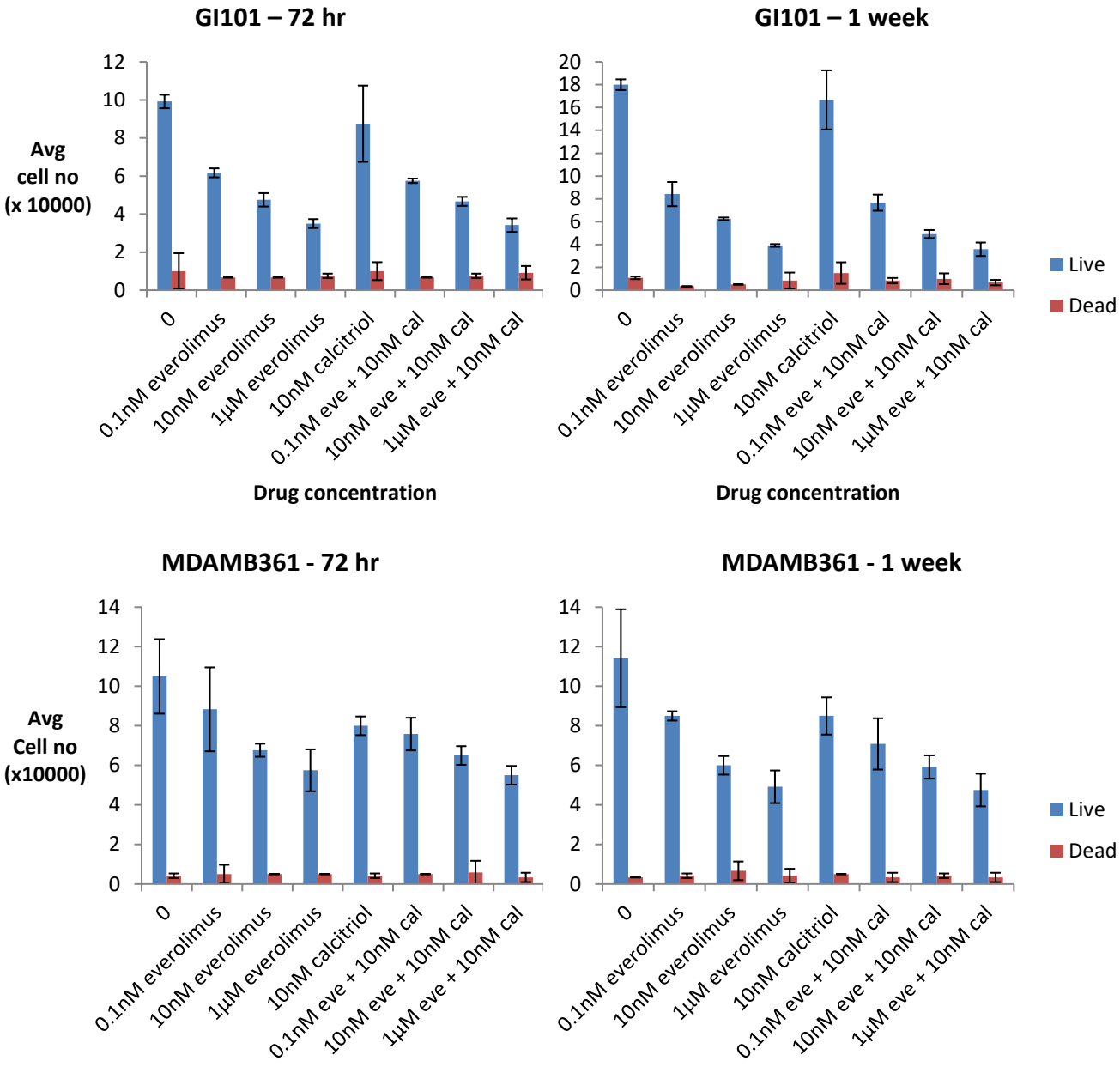


Figure 3.13: Addition of calcitriol to everolimus regimen does not alter cell death rate. *Cell counting and trypan blue staining results (mean plus or minus standard deviation, n=2 independent experiments) for GI101 (top row) and MDA-MB-361 (bottom row) treated for either 72 hour (left) or 1 week (right), with either everolimus (eve) or everolimus + 10nM calcitriol (eve + cal). There was no change in cell death across any dose. In terms of live cell numbers, the combination treatment did not inhibit growth significantly more than everolimus only.*

3.4 - Discussion

This section looked to study various breast cancer cell lines in terms of their expression of key breast cancer cell makers, as well as confirming the expression levels of mTOR components and their downstream targets.

All cell lines were characterised for the breast cancer cell markers of ER, PR and HER-2. All cell lines matched published literature for these markers except the SKBR3 which did not show the expected over-expression of HER-2. This suggests that this particular set/sub-culture of SKBR3 cells that were used for this thesis have mutated to have an altered expression of HER-2. In all likely hood, whilst being sub-cultured, the SKBR3 cells had randomly altered to give this change in HER-2 expression. These sub-cultures (with the differing HER-2 expression from the expected in SKBR3 cells) were then frozen down in liquid nitrogen, and handed to myself for use in this research. This alteration may explain why SKBR3 cells tested here showed very little response to calcitriol (only) treatments whilst unpublished data by Harvey, showed a dramatic response to calcitriol in this cell line, in terms of proliferation. Due to this alteration in their normally observed expression of a key biomarker, SKBR3 cells were not used any further in this piece of work.

Expression of mTOR components was varied across the cell lines studied here. For example, the triple negative cell lines expressed similar amounts of virtually all proteins of the pathway. Interestingly, despite the fact that mTOR activation is generally associated with increased cell growth and an increase in oncogenic potential, the non-tumourgenic and slow growing (compared to tumourgenic breast cancer cell lines) MCF10a cells, actually showed high levels of many mTOR components, including mTOR (and p-mTOR Ser2481), raptor and high levels of phosphorylated downstream mTOR targets including p-S6K and p-Akt. Western blotting data in this thesis also correlated with published literature on the expression of DEPTOR, with work by Zhao and colleagues (2011) showing a high level of T47D DEPTOR expression, but next to no expression in SKBR3 cells.

Western blotting data revealed that high levels of the scaffold proteins in the mTOR complexes (raptor and/or rictor), which are deemed integral for complex function,

may not in fact equate to an increase in downstream signalling. For example, despite having less raptor than other lines, the MDA-MB-361 cells had the highest level of phosphorylated S6K. In regards to the mTORC2 target, phospho-Akt, specific to mTORC2 activation, inversely correlated to rictor expression, even though all lines showed an equal expression of total Akt. The mTORC2 targets SGK1 and PKCα also varied significantly between cell lines, although this appeared to be more closely related to total protein expression (more total protein, equalling more phospho-protein), than a specific difference in phosphorylation status. This suggests that certainly in relation to some mTOR targets (such as Akt), high levels of mTOR complex components is not always linked to signalling and that there may actually be some inhibition of downstream signalling in this circumstance; leading us to theorise that there may be an 'ideal ratio' of complex components to allow for maximal activation, due in part to forms of negative feedback.

This idea is supported by knowledge of negative feedback along the mTOR pathway. Work by Peterson and colleagues (2009) has shown that an increase in the negative mTOR regulator, DEPTOR, may in some cell types actually serve to increase signalling along the PI3K-mTOR axis, by ablating negative feedback. Results shown here seem to suggest that this effect may extend also to mTORC2; thesis data showed that increased DEPTOR positively correlated with an increase in p-Akt, whilst rictor was negatively correlated with it. This work may therefore represent proof of an extension of this principle, with less active complexes of mTORC2 leading to increased active Akt.

As discussed by Copp and colleagues (2009) p-mTOR on Ser2448 may be a good biomarker for mTORC1 complexes, whilst p-mTOR on Ser2481 may be a good biomarker for mTORC2 complexes, hence we studied both here. Whilst there were no noticeable changes or trends in the expression of Ser2448 p-mTOR, Ser2481 p-mTOR was strongest in the cell lines that had the least rictor, but the most p-Akt (T47D, MDA-MB-361 and MCF10a). This further suggests that rictor expression may not equal active complexes and in fact there is some form of feedback that exists (in relation to certain downstream targets) that means higher rictor levels may in fact dampen certain arms of the mTOR path.

Calcitriol was explored in terms of its usefulness as a combination therapy with everolimus. This was studied in breast cancer lines that had previously shown some responsiveness to calcitriol alone, in terms of growth arrest (Harvey, unpublished). Both SRB assays and trypan blue staining showed that the addition of calcitriol to everolimus treatments, had no extra effect on inhibiting the proliferation of these cell lines, and did not alter the rate of cell death. Whilst calcitriol alone did have some inhibitory effects on the proliferation of these cells, there was little/no additive effect with the addition of everolimus. It is still possible that calcitriol may alter mTOR signalling, although this effect was not noted during these drug treatments. These findings are contrary to work by O'Kelly and colleagues (2006), which showed that vitamin D analogues could alter mTOR signalling levels in breast cancer cells and work by Yang and colleagues (2010) which showed a potentiation effect between everolimus and calcitriol in AML. The lack of observed effect in this project is possibly because calcitriol either does not alter mTOR signalling in breast cancer cells, or because it does not alter mTOR signalling in these specific breast cancer cell lines.

Chapter 4: Development of Everolimus Resistant Breast Cancer Cells

4.1 - Introduction

4.1.1 - Everolimus Resistance in Breast Cancer and Other Malignancies

Everolimus is used in a wide variety of clinical settings, in terms of cancer/malignancies, having been approved for use not only in breast cancer but also renal cell carcinoma and neuroendocrine tumours of the gut, lung and pancreas (European Medicines Agency, 2016a). As of the first half of 2018, there is limited research examining everolimus resistance in breast cancer patients (most likely due to its relative recent appearance in the clinic for breast cancer), leaving a current gap in our understanding as to why exactly a breast cancer patient may could develop resistance to this drug. Despite this, clinically observed resistance would still be expected to develop, as it has for other kinase inhibitors.

Some studies looking at everolimus resistance in breast cancer *in vitro* have been published, although currently, no consistent mechanism of resistance runs through these studies. Various known oncogenes have been implicated in resistance to everolimus in breast cancer. In one study looking at breast cancer cells induced to be everolimus resistant, myc up-regulation was seen, with its depletion helping to re-sensitise the cells (Bihani et al, 2015). Other published work has observed that Met up-regulation in similar cells can help induce everolimus resistance, possibly due to its interaction with the FKBP12, which binds rapalogues (Raimondo et al, 2016). EMT marker expression has also been shown to decrease in response to rapalogues (Holder et al, 2015). Taken together, the various signalling pathways implicated pose interesting possibilities for explaining everolimus resistance, but unfortunately form no consistent picture. Possible future research in everolimus resistant patients in breast cancer may help to bridge this gap in knowledge and reveal which pathways have true clinical relevance.

Away from signalling pathways, mutations relating to mTOR signalling have been observed in both in cells and clinically, suggesting these as mechanisms for resistance. Work by Hassan and colleagues (2014) showed that in BT474 breast cancer cells that were rapamycin resistant, a mutation in the FRB domain (S2035F) was present; previous research demonstrated that mutations in this position can drastically lower rapamycin binding (Chen et al, 1995). Clinically, mutations in the FRB domain have also been observed, however this time in a thyoid cancer patient treated with everolimus who had previously responded to the drug. Exome sequencing in this patient revealed a mutation in the FRB domain (F2108L) of mTOR that conferred resistance to rapalogues. This patient also displayed mutations in TSC2, further up-stream in the mTOR pathway, possibly also contributing to resistance to rapalogues (Wagle et al, 2014). Interestingly, both the rapamycin resistant breast cancer cells mentioned previously and cells expressing the F2108L mTOR mutant, were still sensitive to other mTOR inhibitors that were ATP-competitive, such as MLN0128 and torin 1.

Away from breast cancer, everolimus resistance has been observed more widely in the clinic, although precise mechanisms for resistance are still relatively elusive in these settings. In pancreatic neuroendocrine tumours (PNET), response rate to everolimus is relatively low, possibly due to the molecular and histological features of these types of cancer (Capozzi et al, 2015). In the University of Texas MD Anderson Cancer phase II study, response rate averaged at 27% in these patients, whilst in the larger RADIANT-3 phase III study, progression free survival did increase 2.4 fold compared to the placebo arm of the trial. However this study also suggested that everolimus often halted the time to progression, but did not ultimately change the pattern of development, suggesting that the cancer in these patients usually finds a way to become resistant to everolimus (Yao et al, 2013). Similar to the previously mentioned research in breast and thyroid cancer cells, PNET cells that showed resistance to everolimus, were not resistant to ATP-competitive inhibitors of mTOR (Vandamme et al, 2016).

Furthermore, various studies in multiple other cancer types have explored resistance to everolimus in cell lines, often by inducing resistance. Work by Juengel and colleagues (2014) used renal carcinoma cells, and induced everolimus resistance in

them, to establish that histone deacetylase (HDAC)-inhibition could help overcome resistance to everolimus. In research with colon cancer cells, the well-known *BRAF* V600E mutation was able to induce everolimus resistance (He at al, 2016). Away from cancers based on solid tumours, everolimus resistance has also been explored in lymphoma cell lines; here altering autophagy helped overcome resistance to everolimus (Rosich et al, 2012).

4.1.2 - The Androgen Receptor and Breast Cancer

The primary classification of breast cancer involves studying the expression of ER, PR and HER-2, as of course these help direct therapy. However, research in recent years has looked to make a case for possibly expanding this to include the androgen receptor (AR). The AR is already an important biomarker in prostate cancer (Helsen et al, 2014) with evidence now existing that the same may be true for breast cancer.

In breast cancer, expression of AR (clinically) is widely observed, with its expression seemingly varying between subtypes, as well between studies. In a meta-study of breast cancer patients across all subtypes, around 60% had expression of AR, with ER+ cancers having an expression rate of around 75% compared to ER- cancers with a rate of around 30% (Vera-Badillo et al, 2013). Interestingly, in ER+ breast cancers, AR expression seems to correlate positively with outcome/survival (McNamara et al, 2014); however as a counter to this, evidence from 192 breast cancer patients, suggests that a high AR:ER ratio may actually confer for tamoxifen resistance (Cochrane et al, 2014).

In ER- subtypes, AR expression and its relation to patient outcome, is not as well defined, possibly because of both the decreased expression of AR in these cases but also that these patients make up far fewer of the overall patient cohort. In triple-negative breast cancer, expression of AR is at its lowest among the subtypes with some studies reporting as low as 10-20% of this subtype expressing the receptor (McGhan et al, 2014; McNamara et a, 2014), although other studies report far higher values of 30-60%, depending on the assay used for detection (Mrklić et al, 2013). Because of the lack of good biomarkers in triple negative cancers, AR may offer a solution to categorise and give targeted therapy to a group among this subtype,

possibly giving rise to its own subtype of AR+/triple negative breast cancer (Barton et al, 2015). In research by Safarpour and Tavassoli (2014), out of 400 breast cancer cases in their research, 4.5% of cases solely expressed AR of the four receptors, suggesting that a substantial group of patients could benefit from this new classification.

As such, AR inhibitors are being tested clinically and pre-clinically in breast cancer, predominately in triple negative breast cancers, with drugs that that have often shown good efficacy in prostate cancer. Enzalutamide, which binds AR to inhibit the receptor and its translocation to the nucleus, has been shown to have good clinical activity and be well tolerated, in a phase II study involving triple negative cases (Traina et al, 2018), giving hope for its use in phase III trials in the future. In another phase II study, this time using the AR antagonist bicalutamide, women who were AR+ but ER and PR- were treated; with the drug and AR inhibition showing good efficacy and being well tolerated (Gucalp et al, 2013).

There is also preliminary evidence that signalling through the AR may be linked to the PI3K/mTOR axis, with some form of interplay existing between the two. This link has been well explored in prostate cancer, with data showing that either pathway is able to activate the other (Edlind and Hsieh, 2014). As such, the use of PI3K/mTOR inhibition combined with AR inhibition has been shown to have great efficacy in this cancer type (Carver et al, 2011). In triple negative breast cancer, patients with expression of AR have an increased number of *PIK3CA* activating mutations. Also, activation of the AR via dihydrotestosterone was shown to increase PI3K pathway activation (Lehmann et al, 2014) suggesting this link between AR and PI3K/mTOR is also relevant in breast cancer. This interplay may also be important in other breast cancer subtypes. Research by Gordon and colleagues (2017) using cell lines and xenografts of HER-2+ and triple negative breast cancers, showed an increased effect when combining HER-2 or mTOR inhibition with AR inhibition. Like the previously discussed research, this work also showed that each pathway can increase the activation of the other, with everolimus treatment increasing AR levels, whilst enzalutamide treatment increased p-HER-2 levels.

4.2 – Aims

- The main body of work in this chapter aimed to develop everolimus resistant breast cancer cell lines
- This was be done by:
    - Determining the GI60 for everolimus, in the lines that are suitable for the resistance development process
    - Treating cells with everolimus to make them everolimus resistant
    - Determining that resistance has been achieved using the SRB assay, to confirm a stable GI60
- The chapter also aimed to explore drug combinations that could help re-sensitise these cells to everolimus treatment

4.3 - Results

4.3.1 - Determination of GI60

Drug resistant cells were developed using a similar method to that used by Box and colleagues (2013), altered and adapted for use with breast cancer cell lines. This method was chosen partially as it utilised the SRB assay, which I had previously used to assess breast cancer cell responsiveness to everolimus (see section 3.3.5). It was also chosen due to previous collaborations between this group and the Eccles group, who had published that research. This method of developing resistant cells relied on knowing the initial (and continued, during the development process) responsiveness to the drug in question, by using the growth inhibition (GI) % compared to control as a measure; with a GI60 for everolimus (concentration at which 60% growth inhibition was achieved when compared to control cells) selected as the concentration at which the drug would be used in these experiments.

Therefore the GI60 for everolimus needed to be established in potential cell lines. This was determined by treating cell lines with everolimus for 1 week, and plotting a dose-response curve (figure 4.1). SKBR3 cells were not included in this experiment due to concerns over their HER-2 status/expression. Please see section 3.4 for more details. For the T47D cells, the longer treatment did not radically alter the amount of inhibition at each concentration, compared to a 72hr treatment (figure 3.8); however both the GI101 and MDA-MB-361 generally showed higher levels of growth inhibition at the same concentration of everolimus, at 1 week compared to the 72 hour treatment (figure 3.8).

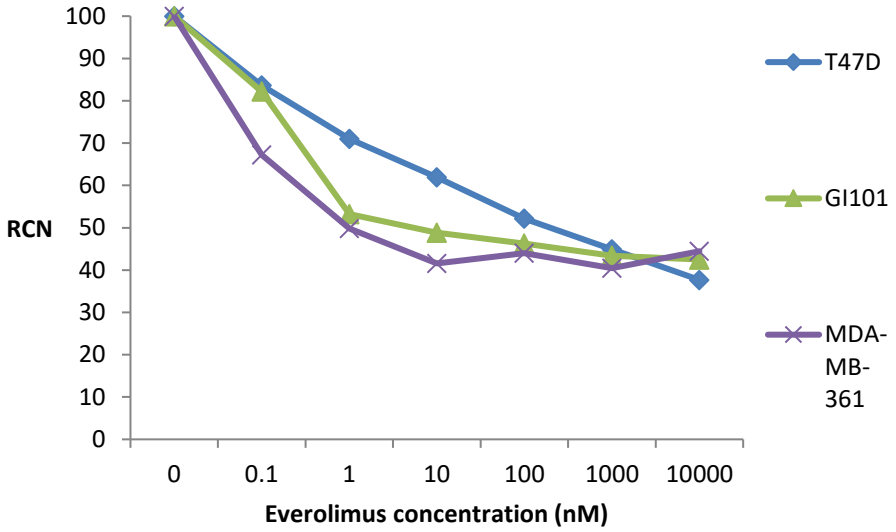


Figure 4.1: Effects of a 1 week everolimus treatment on T47D, GI101 and MDA-MD-361 cells. *SRB results for 1 week treatment of everolimus.*

4.3.2 - Selection of Cell Lines and Summary of Development Process

Selected breast cancer cell lines were then used to construct everolimus resistant cells using a protocol summarised in figure 4.2 (and in the materials and methods), that as previously mentioned, was adapted from that used by Box and colleagues (2013). The long term objective would be to compare the phenotype and behaviours of the resistant cell lines with that of the parental cells.

MDA-MB-361 were selected to go through the development process because they showed some, albeit small (and not significant), extra growth inhibition in the everolimus + calcitriol treatments (figure 3.10). Since there is evidence that vitamin D may alter the mTOR pathway (Datta-Mitra et al, 2013a; Datta-Mitra et al, 2013b; O'Kelly et al, 2006), the MDA-MB-361 in theory provided the best hope for testing whether calcitriol could be used to overcome resistance to everolimus. T47D cells were selected due to their wide use in breast cancer research and their use in various drug resistance models. This includes work by Jordan and colleagues (2014) that used tamoxifen resistant T47D cells alongside their own model of drug resistant MCF-7 cells (which were tamoxifen resistant and intrinsically resistant to everolimus). Both cells are also ER+, and since everolimus is approved for use in

ER+ patients (European Medicines Agency, 2016a), these cells were deemed good candidates for testing everolimus resistance.

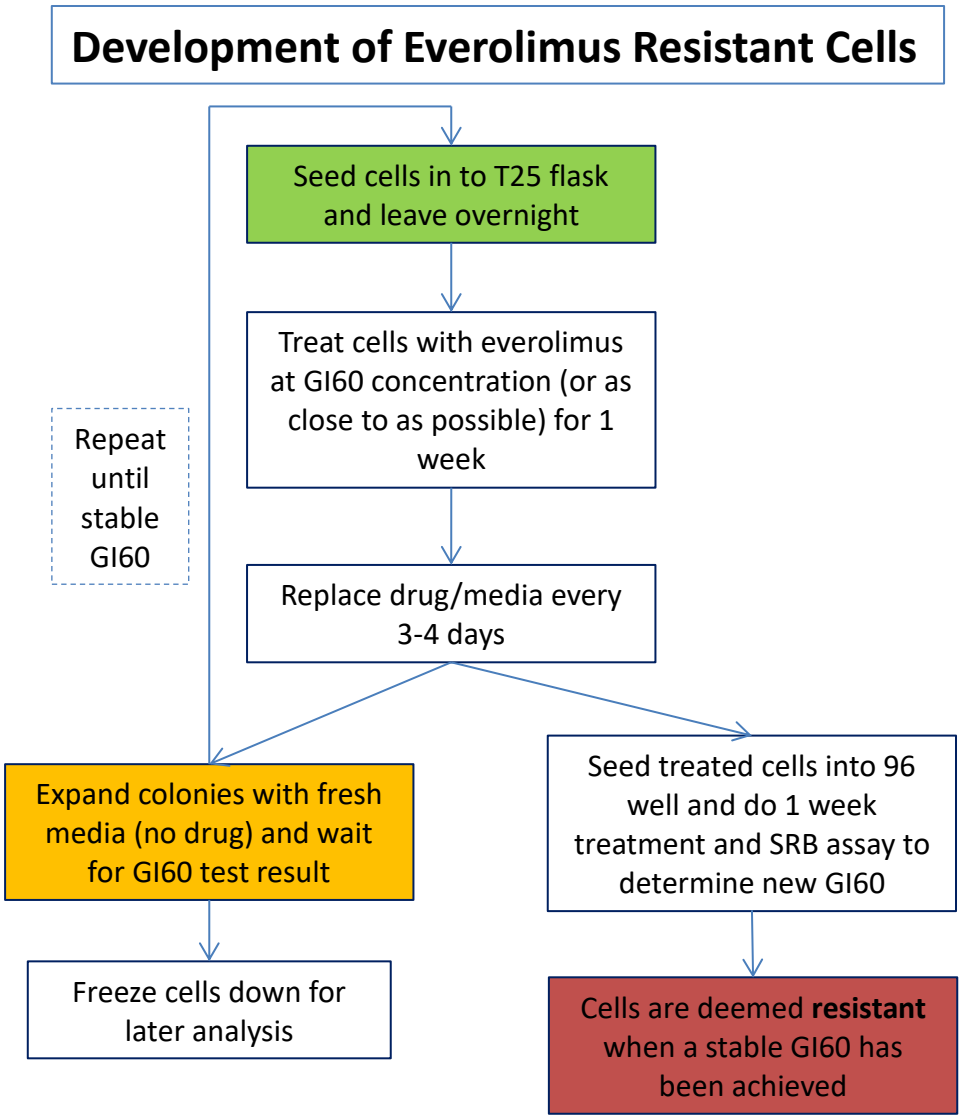


Figure 4.2: Flow chart summary of developing everolimus resistant cells. *Method adapted from work by Box and colleagues (2013). Control flasks of each cell line (parental cells) were in parallel and split as and when needed.*

4.3.3 - Development of Everolimus Resistant T47D Cells

T47D cells went through a total of 10 treatment cycles (as shown in figure 4.2) until it was deemed a stable response and resistance to everolimus was achieved. These

cells were dubbed T47D-EveR, with their control counter parts dubbed T47D-Parental. Parental cells were treated with DMSO during each treatment cycle as a control.

For treatment cycles 1-7, T47D-EveR cells only were tested in the SRB to give an up to date GI60. These first 7 cycles showed very little in terms of any development of a resistant phenotype, with a stable GI60 of around 100nM (figure 4.3). However, SRB testing on cells after treatment 8, showed that they had begun to change in terms of their everolimus responsiveness, with the GI60 now closer to 10,000nM rather than 5000nM (figure 4.3), with the treatment dose of everolimus adjusted to match the new GI60. At the same time it was also noted that the cells appeared to be growing at a slower rate than their parental counter parts, with 2000 cells per well of a 96 well plate, yielding lower relative protein amount; hence, after some testing with new cell numbers for the SRB, 2500 T47D-EveR cells per well was used for treatment cycle 9 testing and for all plates afterwards. This altered cell number gave relative protein amounts (according the SRB assay) closer to that of parental cells which were still seeded at 2000 cells per well.

Since a shift in the cell responsiveness to everolimus had been noted, SRB assays for treatments 9-10 were carried out to include the T47D-Parental cells, to give the most accurate judge of resistance. Treatment 9 (figure 4.4) showed further increases in resistance, and a large gap in RCN of around 30% compared to the parental lines, at most tested everolimus concentrations. This level of resistance was maintained through to treatment 10 (figure 4.4) with everolimus concentrations of 15-20,000nM yielding a GI60, with the everolimus concentration in the treatment again adjusted accordingly to match this. As a high level of resistance compared to the parental lines and a relatively stable GI60 had been achieved, the development of the T47D-EveR cell line was stopped.

GI60 (nM)			
Tr1	100	Tr2	100
Tr5	100	Tr8	5000

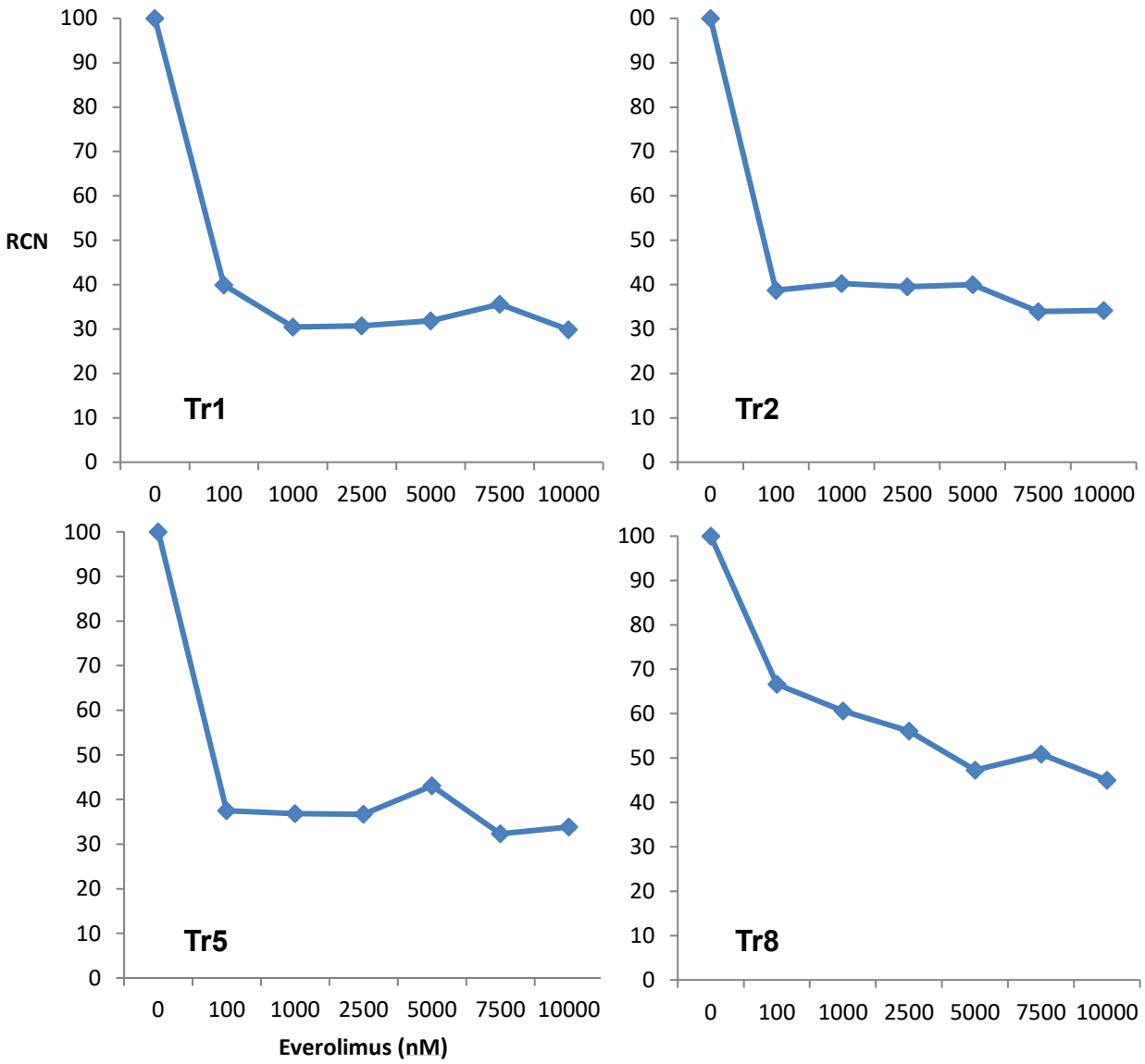


Figure 4.3: GI60 to everolimus, in T47D cells, shifts after 8 treatment cycles. *Selection of SRB results for the development of T47D-EveR cells between treatment cycles 1-8. Treatments (Tr) 1-7 had little effect on the everolimus responsiveness of T47D-EveR cells, but after Tr8, cells begun to show decreased growth inhibition during treatment, with the GI60 shifting from around 1000 to 10000nM.*

		GI60 (nM)		
EveR	Tr9	15000	Tr10	15000
Parental	Tr9	100	Tr10	100

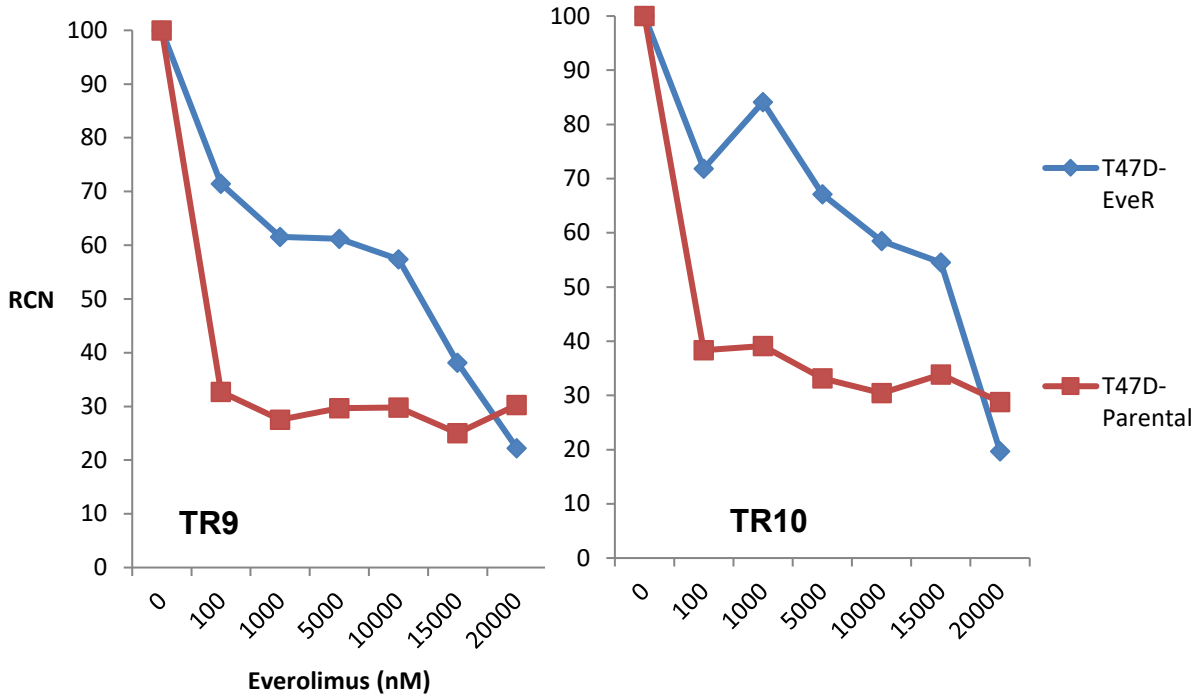


Figure 4.4: T47D cells show resistance to everolimus after 9/10 treatment cycles. *SRB results for the development of T47D-EveR cells, for treatment cycles (Tr) 9-10. T47D-EveR cells showed a relatively stable resistance to everolimus over the course of these treatments, compared to T47D-Parental.*

Cell morphology and growth was studied throughout the development process by taking pictures of the cells, after each treatment cycle. Examples of these images can be seen in figure 4.5. For the first 5-6 treatments T47D-EveR cells showed very little difference, in terms of how they presented down the microscope. However from treatment 7 onwards, the EveR cells appeared to show a change in how they grew, with cells often growing in large colonies. This is in contrast to the T47D-Parental cells which generally do not show this behaviour. This change appears to coincide roughly (within one or two treatment cycles) with the apparent change in growth rate and everolimus responsiveness.

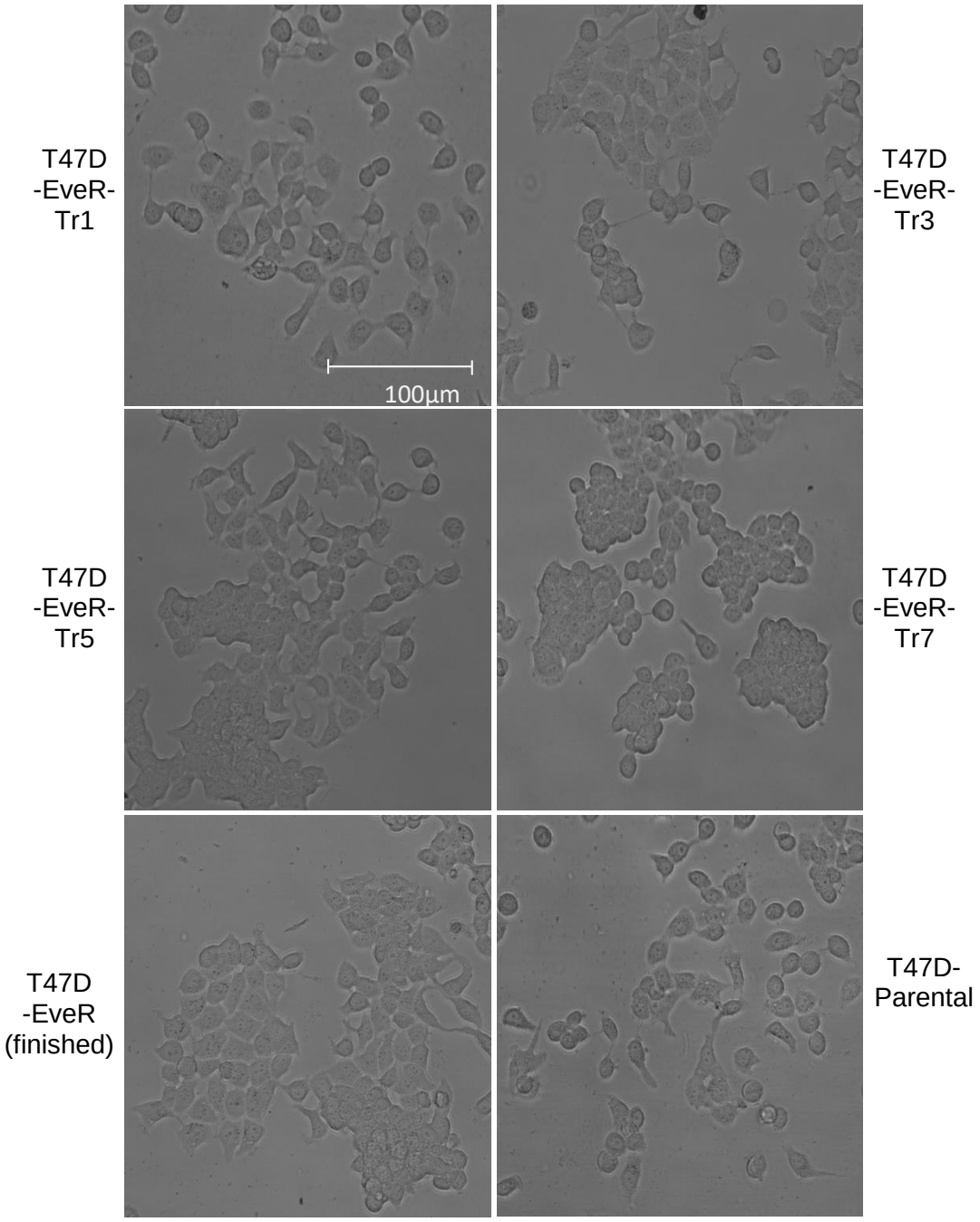


Figure 4.5: T47D-EveR cells show morphological changes. *Representative images of T47D-EveR cells taken throughout the development process after various treatment cycles (Tr) and showing the T47D-EveR and T47D-Parental cells after the process (bottom row). T47D-EveR cells show a gradual shift in morphology, shown by cells growing in a more colonial fashion compared to the parental cells, and themselves at earlier treatment steps. Magnification = x20.*

4.3.4 - Development of Everolimus Resistant MDA-MB-361 Cells

MDA-MB-361 cells went through a total of 14 treatment cycles. A stable resistance was deemed viable at this point, and the resistant line was dubbed MDA-MB-361-EveR (abbreviated as 361-EveR) and the control cells MDA-MB-361-Parental (abbreviated as 361-Parental). Parental cells were treated with DMSO during each treatment cycle as a control.

Similar to the T47D development process, the 361-EveR alone were tested for changes in GI60 to everolimus. Between treatments 2-4 (figure 4.6), some small changes in everolimus responsiveness were noted compared to the initial 1 week treatment, shown in figure 4.1; this was seen with a GI60 shifting from around 10-50nM to 500-1000nM, with the treatment concentration of everolimus adjusted to match the GI60.

Data for treatment cycles 5-7 was difficult to obtain as SRB assays failed, with 361-EveR cells failing to grow properly in the plate. Similar to the T47D-EveR cells, it was noted that cells appeared to be growing slower than the controls. Since the MDA-MB-361 is a slower growing cell line, it was hypothesised that this was why cultures were failing to grow. Experiments with varying cell numbers showed that an increased number of 7000 cells per well, compared to the 5000 cells used for the parental cells, gave similar levels of protein between the EveR and parental line (according the SRB assay), and allowed us once again to accurately gather data from these cells. Treatment cycle 8 (figure 4.7), demonstrates this new cell number. Since shifts in GI60 were noted, cells were now tested with the parental counterparts in the SRB assay, similar to the T47D cells, to accurately determine resistance to everolimus.

Despite observed small changes in responsiveness to everolimus, by the 361-EveR cells, treatment cycles 8-10 (figure 4.7) showed very little difference in terms of everolimus responsiveness between the EveR and parental cells. Treatment cycle 11 showed the first difference between the two lines (figure 4.7), with a difference of about 10% RCN between most everolimus concentrations. This widened to around 20-25% by treatment 13 with no more changes noted at treatment cycle 14. Since

the resistance appeared to have become stable, the development process was then stopped.

GI60 (nM)			
Tr1	50	Tr2	500
Tr3	500	Tr4	1000

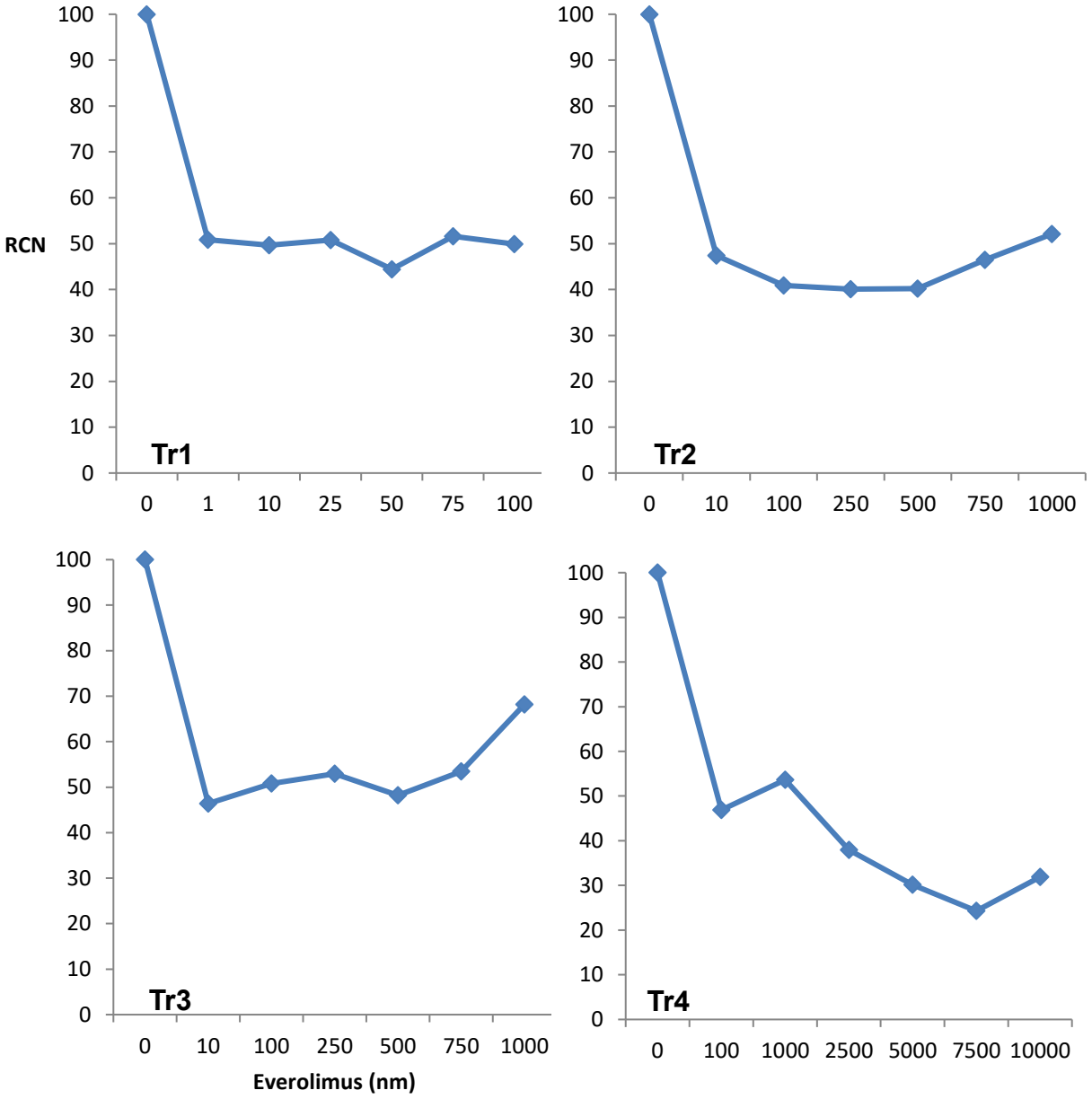


Figure 4.6: GI60 to everolimus, in 361 cells, shifts after 4 treatment cycles. *SRB results for the development of 361-EveR cells between treatment cycles (Tr) 1-4. 361-EveR cells appeared to show small increases in drug resistance during these treatments, with the GI60 shifting from around 10M, to 100-1000nM by Tr4.*

	GI60 (nM)							
EveR	Tr8	1000	Tr9	N/A	Tr11	N/A	Tr14	N/A
Parental	Tr8	1000	Tr9	N/A	Tr11	100	Tr14	10-100

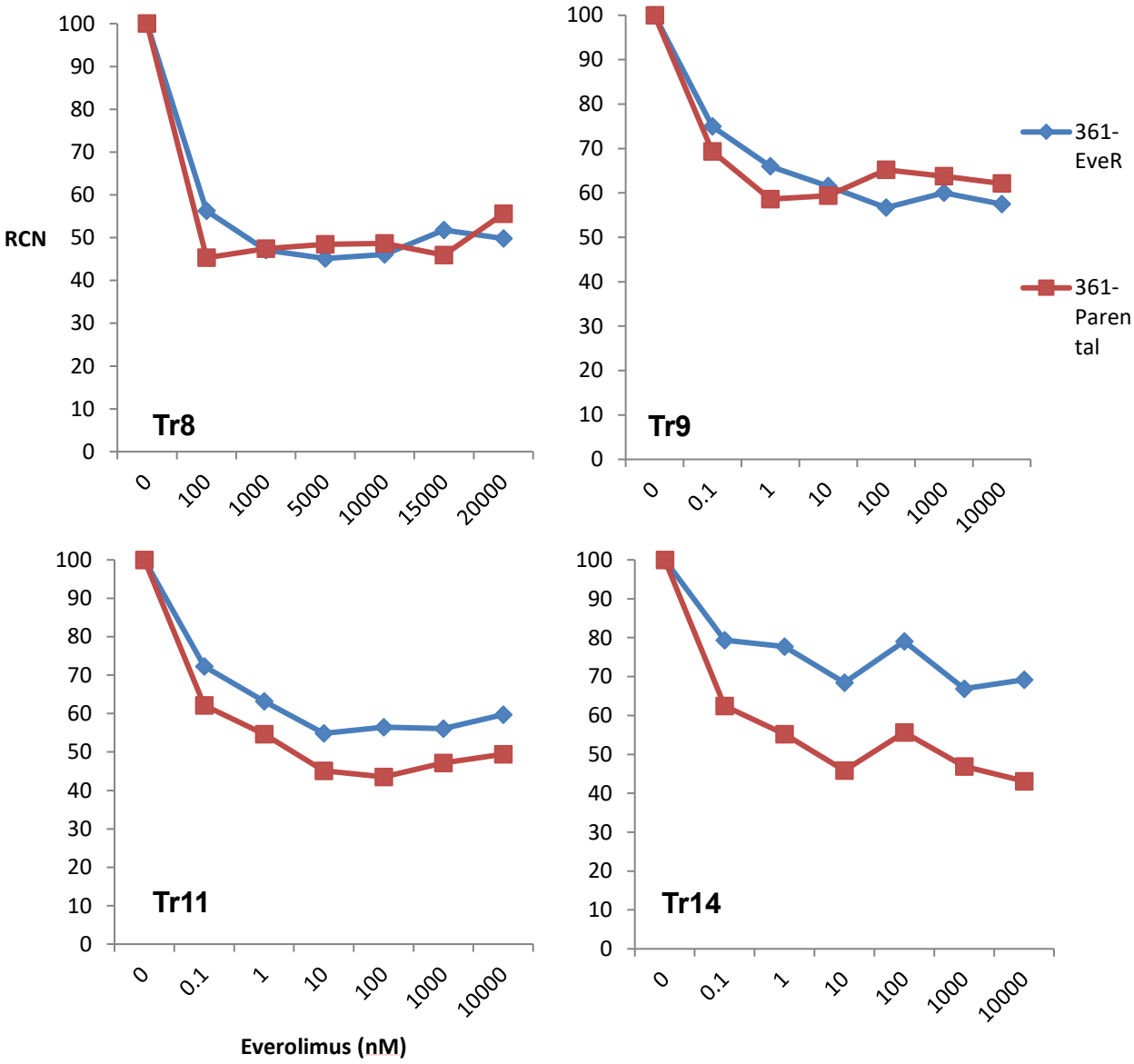


Figure 4.7: 361 cells show resistance to everolimus after 14 treatment cycles. *Selection of SRB results for the development of 361-EveR cells between treatment cycles (Tr) 8-14. 361-EveR cells showed little resistance compared to 361-Parenal cells until Tr11. By Tr14 a stable resistance to everolimus was achieved, compared to the parental cells.*

As with the T47D cells, MDA-MB-361 cell morphology and growth was studied throughout the development process using microscopy, with examples of these images shown in figure 4.8. Overall, the cells showed very little change in cell morphology across the development process and compared to the parental line. As mentioned previously the MDA-MB-361 grew in small colonies and this phenotype persisted in the EveR cells across the process, with next to no observed change, despite the apparent change in growth rate.

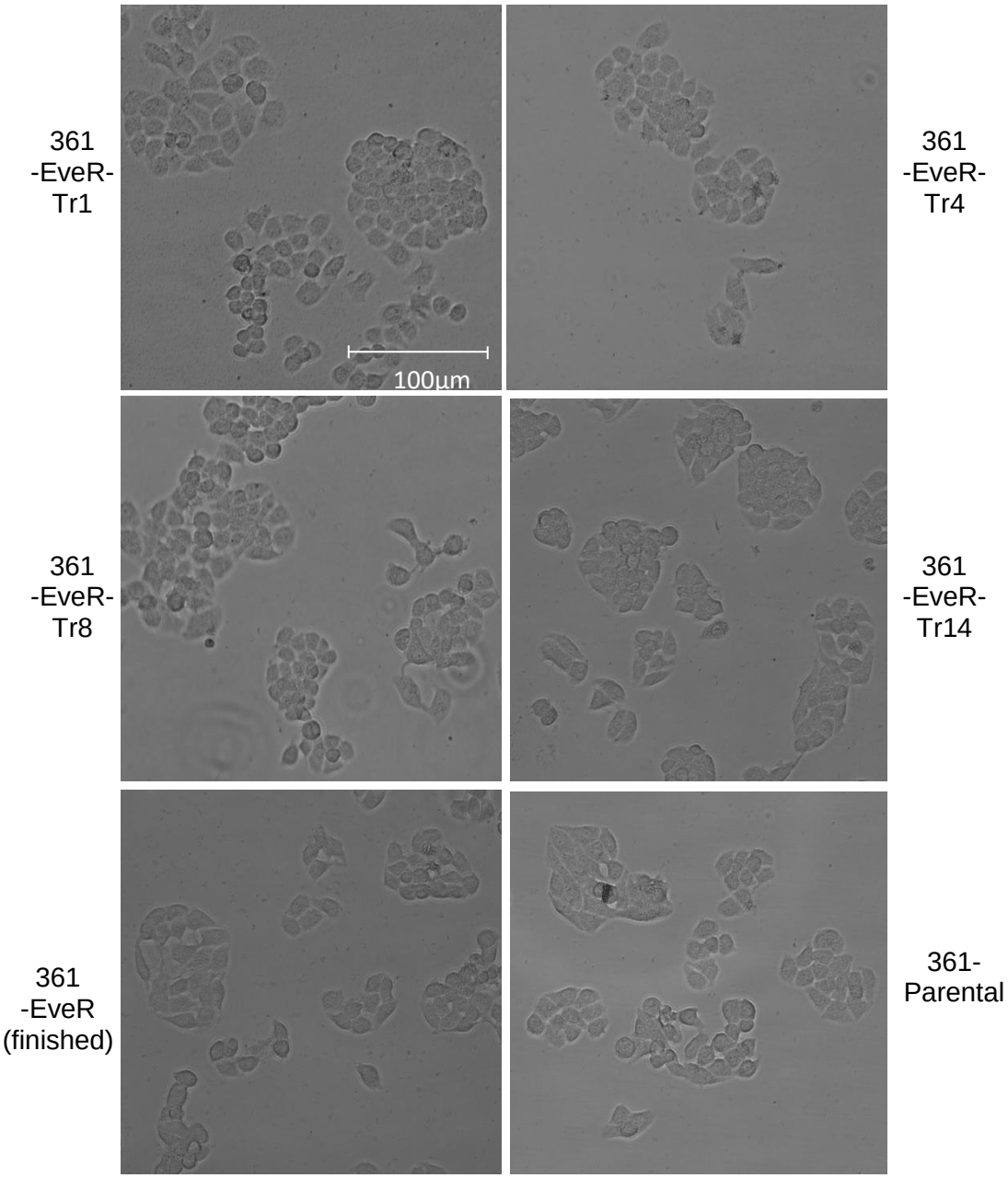


Figure 4.8: 361-EveR cells show no morphological changes. *Example images of MDA-MD-361-EveR (361-EveR) cells taken throughout the development process after various treatment cycles (Tr) and showing the 361-EveR and 361-Parental cells after the process (bottom row). No major morphological changes were noted in the 361-EveR cells throughout the development process, with the 361-EveR cells after the process matching the 361-Parental cells.*

4.3.5 - T47D and MDA-MB-361-EveR Responsiveness to Everolimus at 72 hr and 1 Week

Once the development process was complete both newly developed resistant lines were tested multiple times, against the parental cells, at multiple times points for their everolimus responsiveness (figure 4.9).

T47D-EveR cells showed significant resistance versus the parental T47D cells at almost every concentration, across both time points, excluding the highest and lowest concentrations tested of everolimus at 72hr. This was a difference of around 20-25% greater RCN at 72hr and around 30-35% at one week.

361-EveR cells showed significant resistance after one week treatments with everolimus, similar to the T47D-EveR cells, shown by a growth difference of around 30-35% across most concentrations of everolimus, between the EveR and parental line. Unlike T47D, the EveR cells showed no significant resistance to everolimus at 72hr.

	GI60 (nM)							
	361				T47D			
EveR	72hr	N/A	1 week	N/A	72hr	N/A	1 week	10000
Parental	72hr	N/A	1 week	100	72hr	N/A	1 week	10

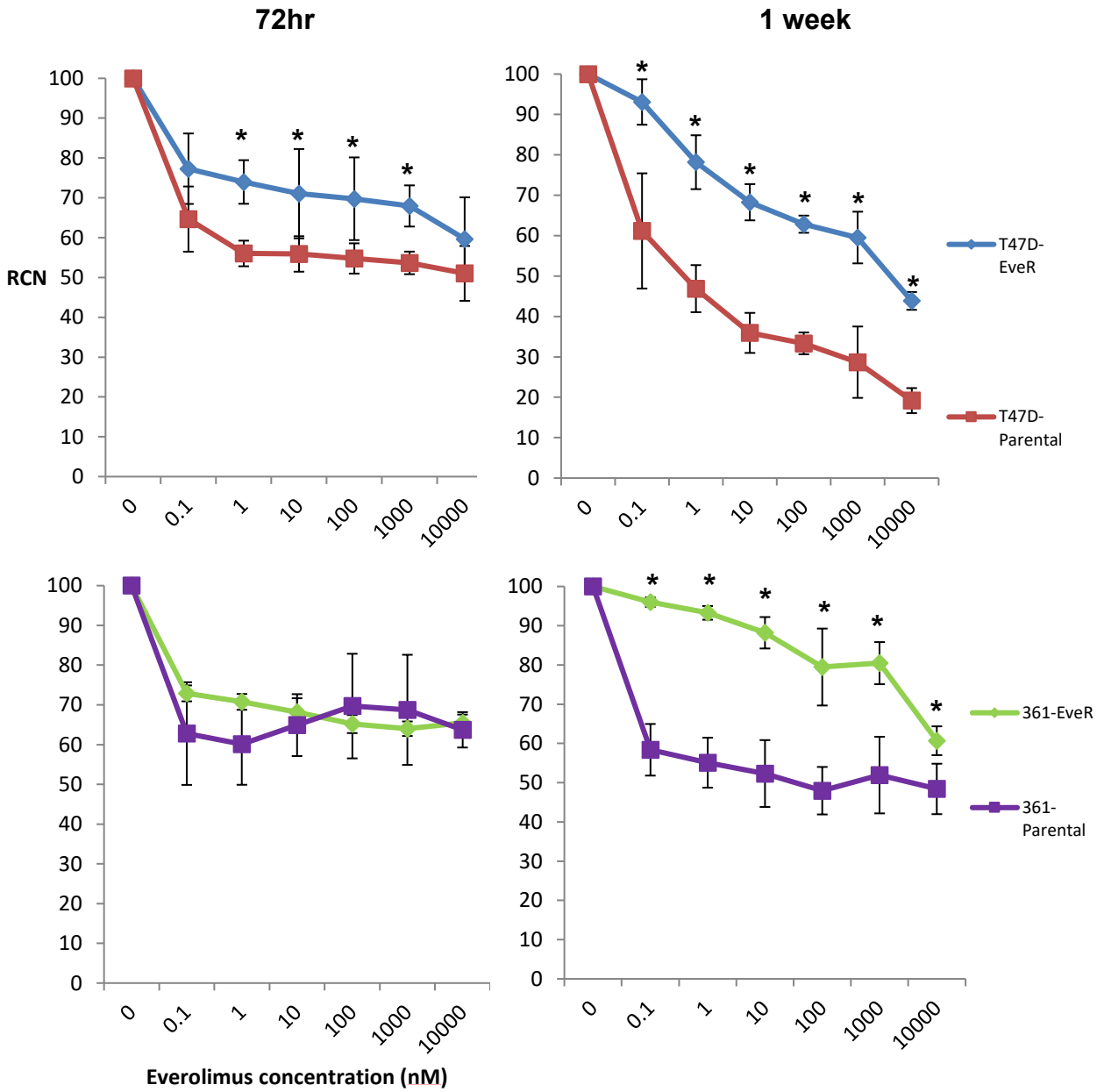


Figure 4.9: T47D and 361-EveR show significant resistance to everolimus. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant lines (EveR) v parental, treated with everolimus for either 72hr or 1 week. T47D-EveR line show significant resistance compared to the parental cells across nearly all does at both time points, whilst the 361-EveR cells showed significant resistance at 1 week but not 72hr. * P<0.05 compared to control (parental).*

4.3.6 - Everolimus and Calcitriol as a Combination Therapy to Overcome Everolimus Resistance

A major reason for developing these everolimus resistant cell lines was to test for drug combinations that may help re-sensitise the cells to everolimus once again. In the previous chapter, calcitriol as both a single agent and combination therapy with everolimus, was explored with a range of breast cancer cells. These experiments did not show much use for this combination therapy with these base breast cancer cells. However, since there was still good evidence that vitamin D affects the mTOR pathway. For example, breast cancer cells treated with a vitamin D analogue, show reduced mTOR signalling (O'Kelly et al, 2006) (as described in full detail in section 3.1.2) As such, we wanted to explore if this combination was now effective in the resistant cells.

Both EveR and parental were first tested with just calcitriol as shown in figure 4.10. As can be seen, neither EveR line was different in their calcitriol response compared to the parental cell lines; these values roughly match the data gathered for section 3.3.5 when the cell lines were originally treated with calcitriol. The combination of everolimus and calcitriol was then tested, with the addition of either 10nM or 1000nM calcitriol, with the data shown in figures 4.11-4.12. Considering that the 361-EveR cells do not show resistance to everolimus at 72hr, it is no surprise that no extra effect could be observed at this point, as seen from the data. Unfortunately the T47D-EveR also showed no added affect at this time point. The lower dose of calcitriol (10nM addition) also failed to elicit any extra response in either cell lines. However the addition for 100nM calcitriol for 1 week had a significant impact with both cell lines showing extra drug response here.

As we can see from figure 4.12, this addition all but ablated resistance, in terms of RCN/cell growth, in the 361-EveR cells. 5 out of the 7 tested concentrations of everolimus showed a significant difference with the addition of 1000nm calcitriol compared to everolimus alone, with the values for the combination treatments almost matching the 361-Parental when treated with everolimus alone. The T47D-EveR also had an extra response at the 1 week time point with the addition of 1000nm calcitriol, although this was far less exaggerated than the 361 cells. Although most points were

again significantly different from the everolimus alone, the difference did not bring the values back in line with that of the T47D-Parental treated with everolimus only, with about 50% of the resistance ablated.

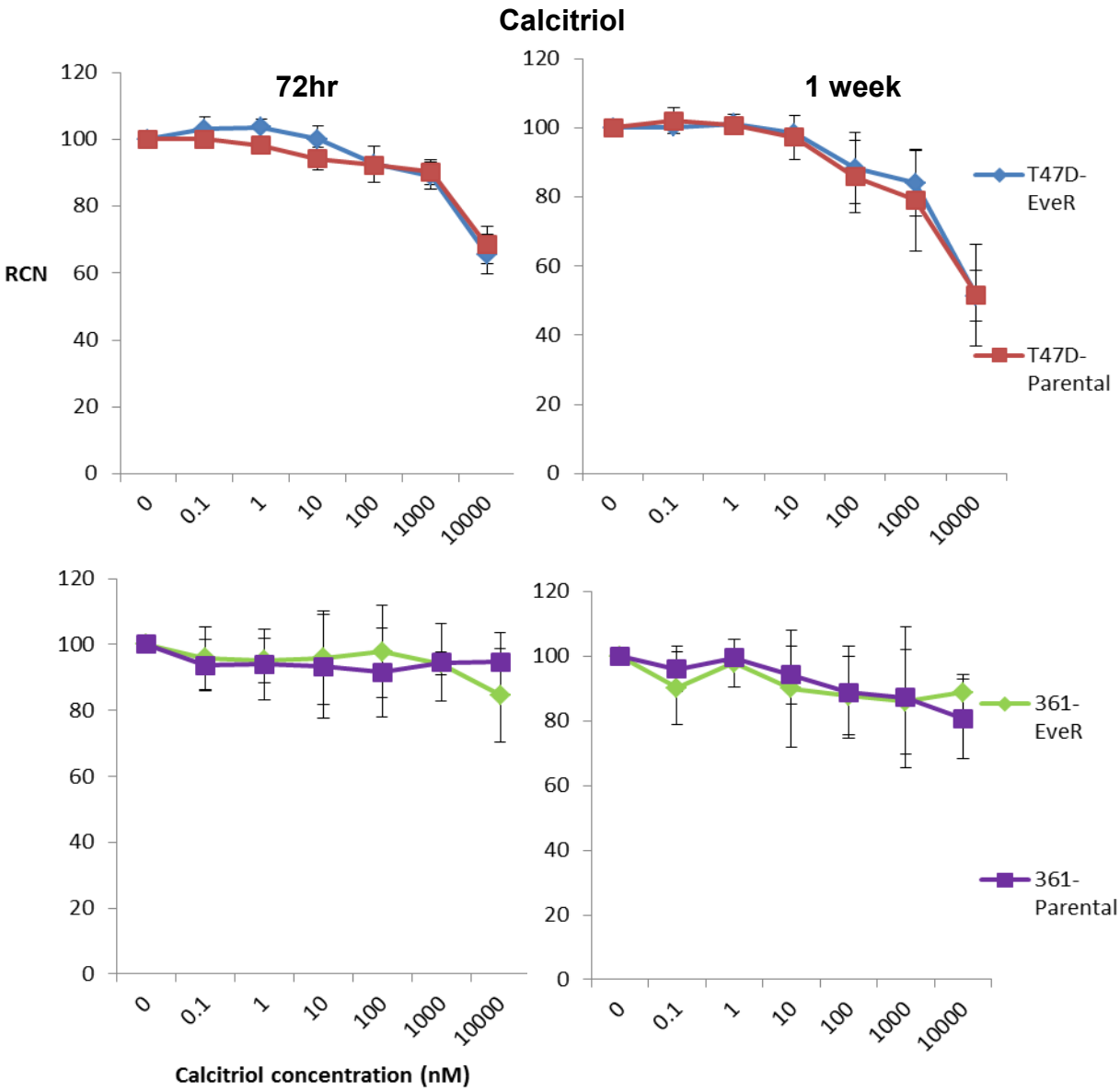


Figure 4.10: Response to calcitriol does not vary in EveR v parental lines. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant lines (EveR) v parental, treated with calcitriol for either 72hr (left) or 1 week (right).*

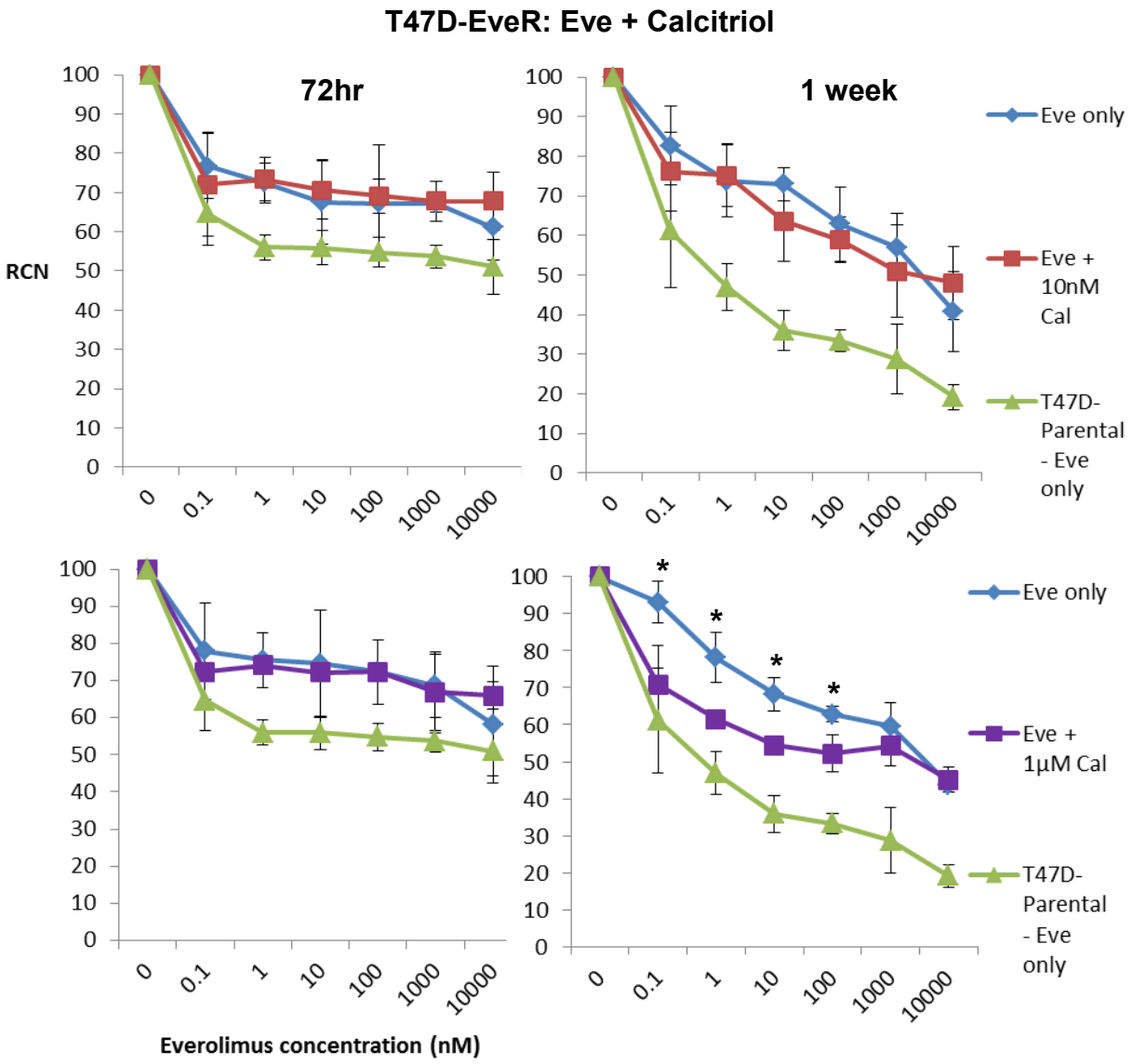


Figure 4.11: 1μM Calcitriol addition can significantly lower everolimus resistance in T47D-EveR cells*. SRB result (mean plus or minus standard deviation, n=3 independent experiments) for T47D-EveR treated for 72 hours or 1 week, with either everolimus (eve) or everolimus + 10nM or 1μM calcitriol (eve + cal). * P<0.05 compared to combination treatment. The addition of 1μM calcitriol for 1 week significantly lowered the growth of the T47D-EveR cells, reducing resistance by a varying amount, depending on the dose of everolimus.*

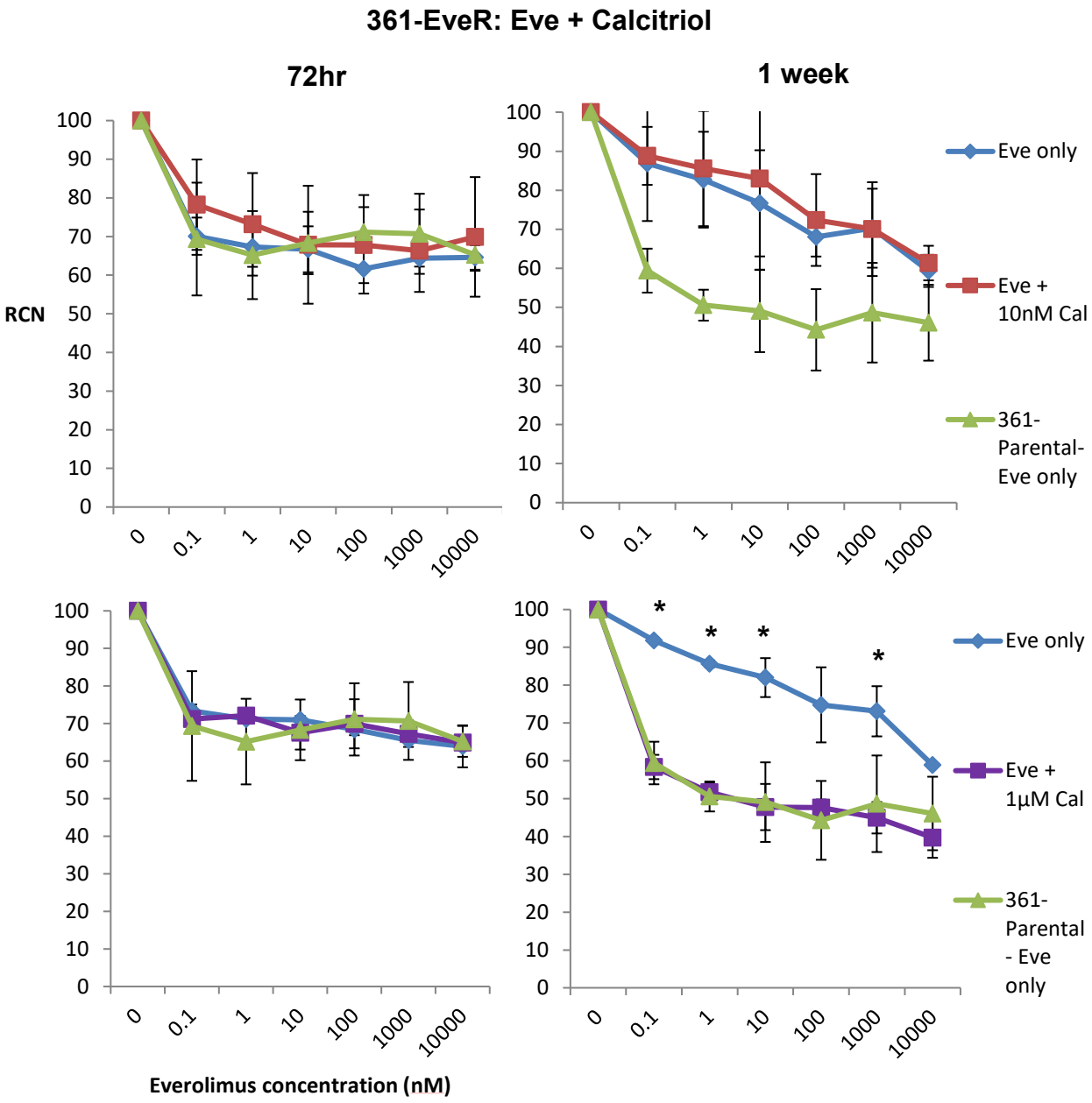


Figure 4.12: 1µM Calcitriol addition significantly lowers everolimus resistance in 361-EveR cells. *SRB result (mean plus or minus standard deviation, n=3 independent experiments) for 361-EveR treated for 72 hours or 1 week, with either everolimus (eve) or everolimus + 10nM or 1µM calcitriol (eve + cal). * P<0.05 compared to combination treatment. The addition of 1µM calcitriol for 1 week, significantly lowered the growth of the 361-EveR cells, all but ablating resistance, with the 361-EveR cells treated with both drugs growing at the same amounts as the 361-Parentalt treated with just everolimus.*

To test if this increased drug response related to an alteration of the VDR, western blotting was done to determine relative VDR levels in the EveR compared to the parental cell lines. Expression of the receptor did not vary when comparing the lines, suggesting that the added effect of calcitriol may not relate to its affects via the VDR (figure 4.13).

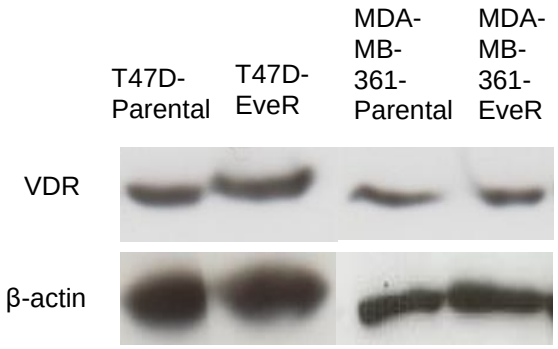


Figure 4.13: VDR expression did not vary in EveR v parental lines. *Representative western blots for T47D-EveR and Parental and MDA-MB-361-EveR and Parental cell lines. n=2 independent experiments. The expression of vitamin D receptor (VDR) was explored, with β-actin as a loading control. Neither resistant cell line had altered expression of VDR compared to the parental line.*

4.3.7 - Everolimus and Enzalutamide as a Combination Therapy to Overcome Everolimus Resistance

The androgen receptor (AR) may prove a useful new target in breast cancer therapeutics, with research showing its high expression across breast cancer subtypes (McGhan et al, 2014; McNamara et al, 2014; Mrklić et al, 2013). There is also now evidence that, similar to vitamin D discussed previously, androgens and signalling via the AR may in fact impact and alter mTOR signalling. For example, research has shown that whilst dihydrotestosterone activates the AR in triple negative breast cancer cells, it can also activate signalling via PI3K. On top of this, there is evidence in triple negative cells, that combining AR inhibition with HER-2 or mTOR inhibition, increased the treatment effect. (Gordon et al, 2017; Lehmann et al,

2014). As such, we wished to determine whether blocking/inhibiting AR could prove to be a viable way of re-sensitising the EveR cells to everolimus treatment, using the AR inhibitor, enzalutamide. As seen in figure 4.14, enzalutamide was first tested in both EveR and both parental lines, as a single agent therapy.

Enzalutamide on its own had little to no effect, in terms of cell growth; this is despite all lines being positive for the AR, as shown via western blotting, in figure 4.15. These blots showed that as well as all four cell lines expressing AR, 361-EveR and 361-Parental expressed in virtually equal amounts. Interestingly, T47D-EveR expressed higher levels of AR than their parental controls.

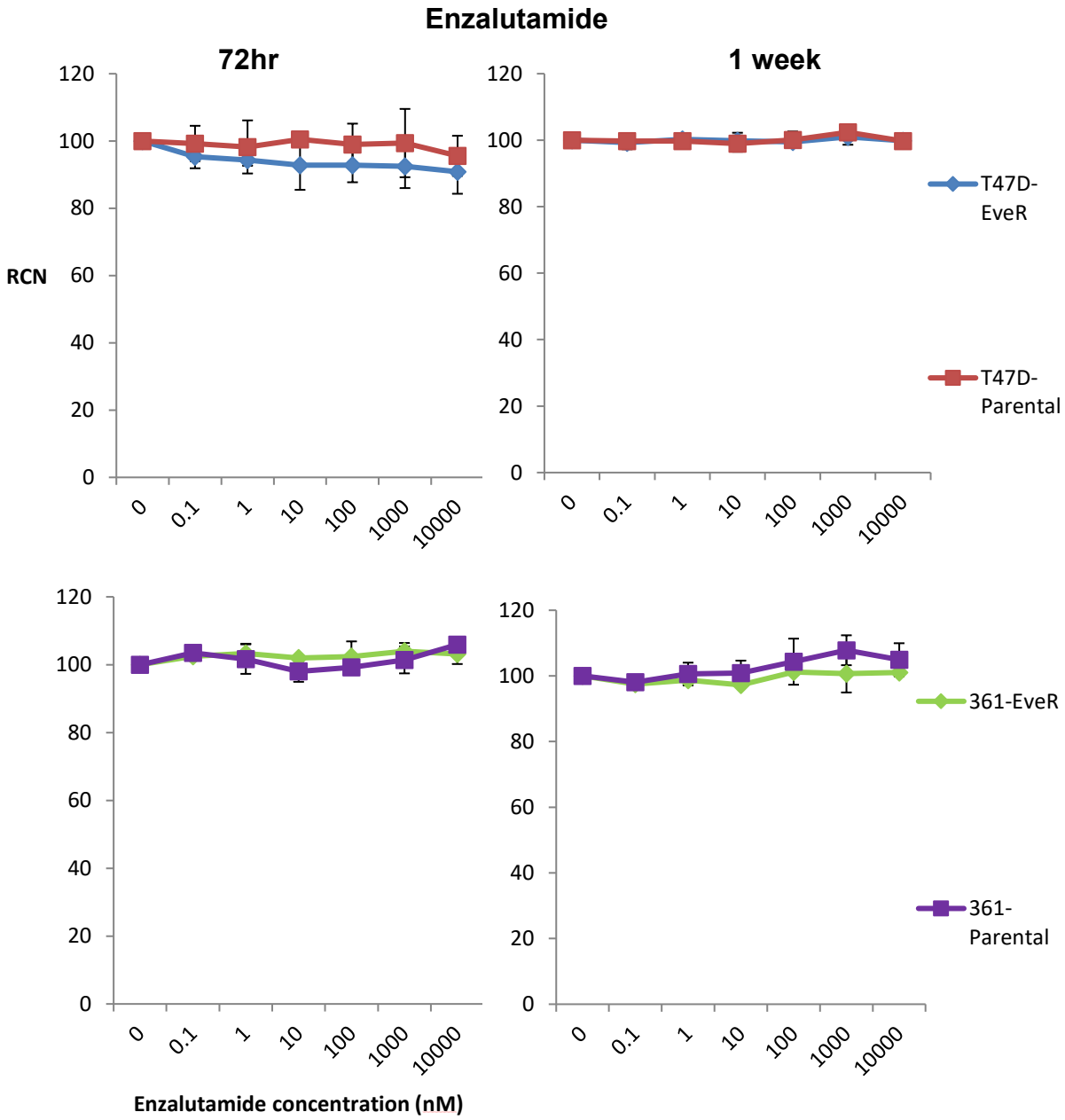


Figure 4.14: Enzalutamide shows very little growth inhibition as a single therapy. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant lines (EveR) v parental, treated with enzalutamide for either 72hr (left) or 1 week (right).*

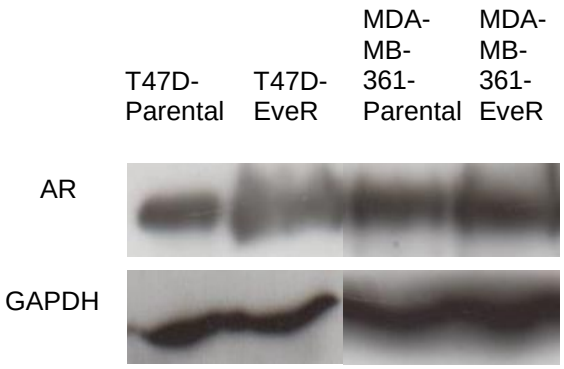


Figure 4.15: AR expression did not vary in EveR v parental lines. *Representative western blots for T47D-EveR and Parental and MDA-MB-361-EveR and Parental cell lines. n=2 independent experiments. The expression of androgen receptor (AR) was explored, with GAPDH as a loading control. T47D-EveR line showed slightly increased expression of AR compared to its parental line, but no difference was seen in the MDA-MB-361 cells.*

Enzalutamide was then tested as a combination therapy in conjunction with everolimus. SRB data for these experiments can be seen in figures 4.16-4.19. For all cell lines, the addition of enzalutamide to the everolimus regimen had no extra effect on RCN. This is including the addition of either 10nM or 1µM enzalutamide for either 72hr or 1 week.

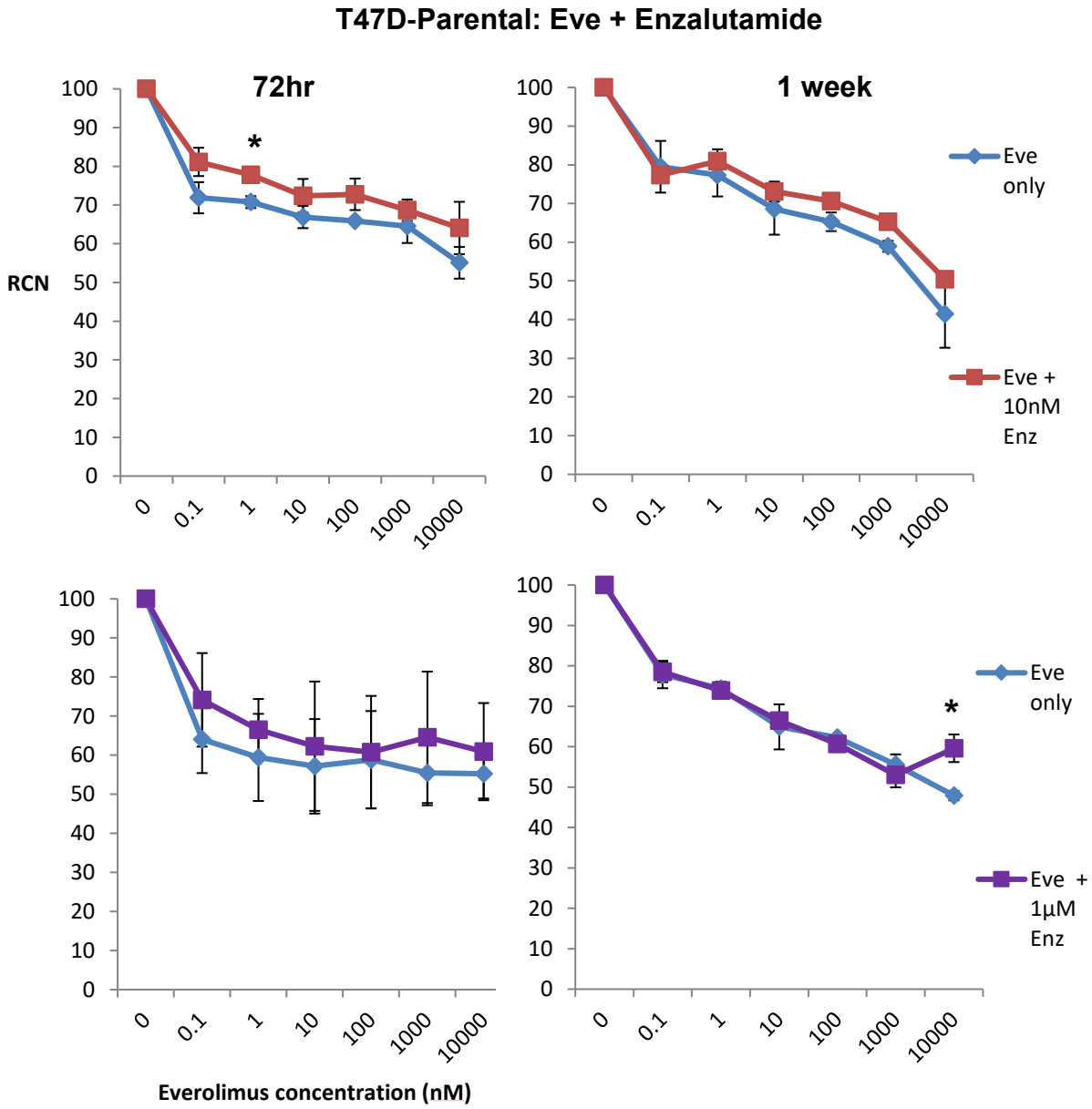


Figure 4.16: Everolimus+enzalutamide fails to significantly improve growth inhibition v everolimus only, in T47D-EveR cells. *SRB result (mean plus or minus standard deviation, n=3 independent experiments) for T47D-Parental treated for 72 hours or 1 week, with either everolimus (eve) or everolimus + 10nM or 1µM enzalutamide (Eve + Enz). * P<0.05 compared to control. In this cell line, the addition of enzalutamide with everolimus appeared to have very little extra effect, over everolimus only.*

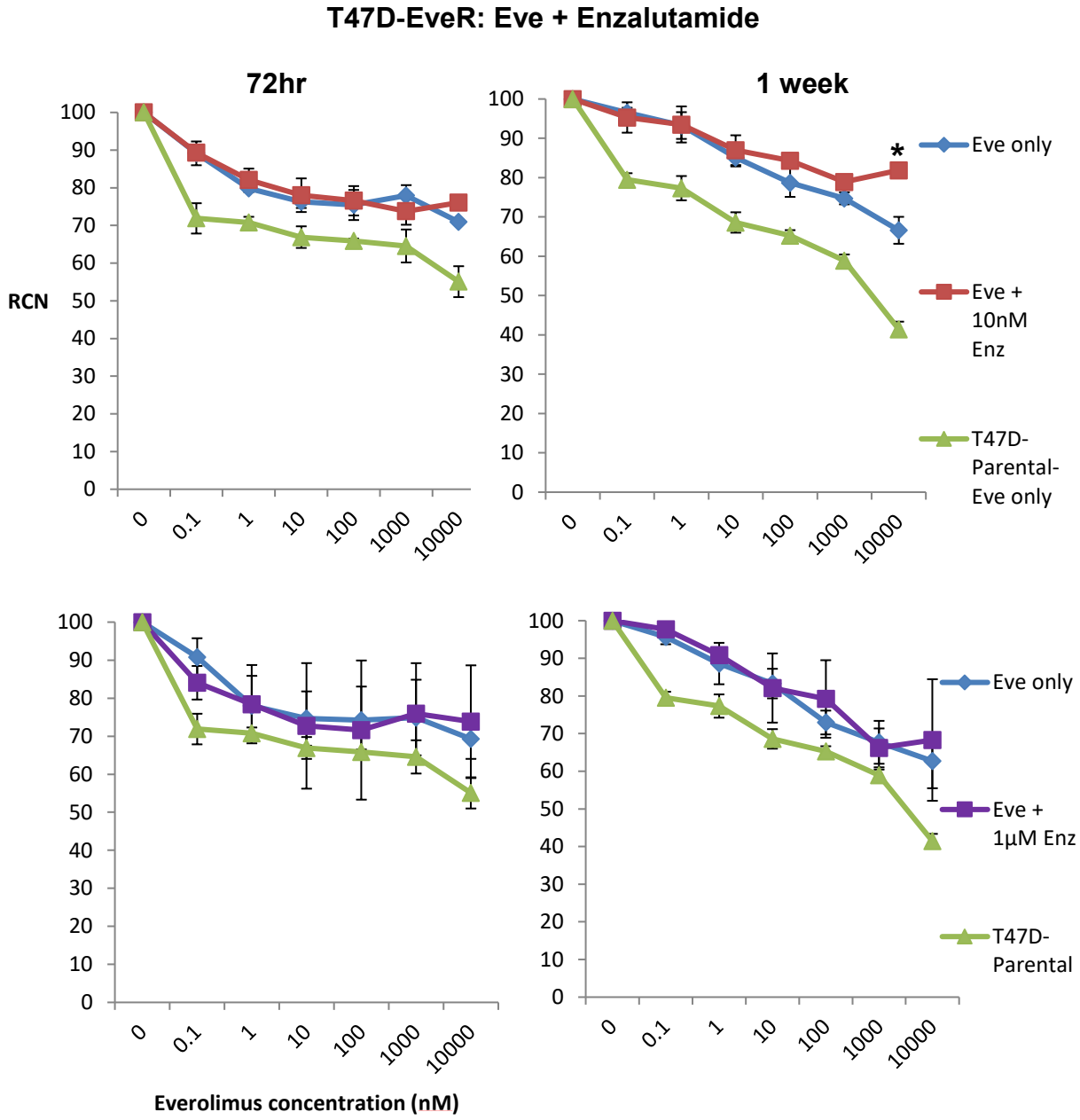


Figure 4.17: Everolimus+enzalutamide fails to significantly improve growth inhibition v everolimus only, in T47D-Parental cells. *SRB result (mean plus or minus standard deviation, n=3 independent experiments) for T47D-EveR treated for 72 hours or 1 week, with either everolimus (eve) or everolimus + 10nM or 1µM enzalutamide (Eve + Enz). * P<0.05 compared to combination treatment. In this cell line, the addition of enzalutamide with everolimus appeared to have very little extra effect, over everolimus only.*

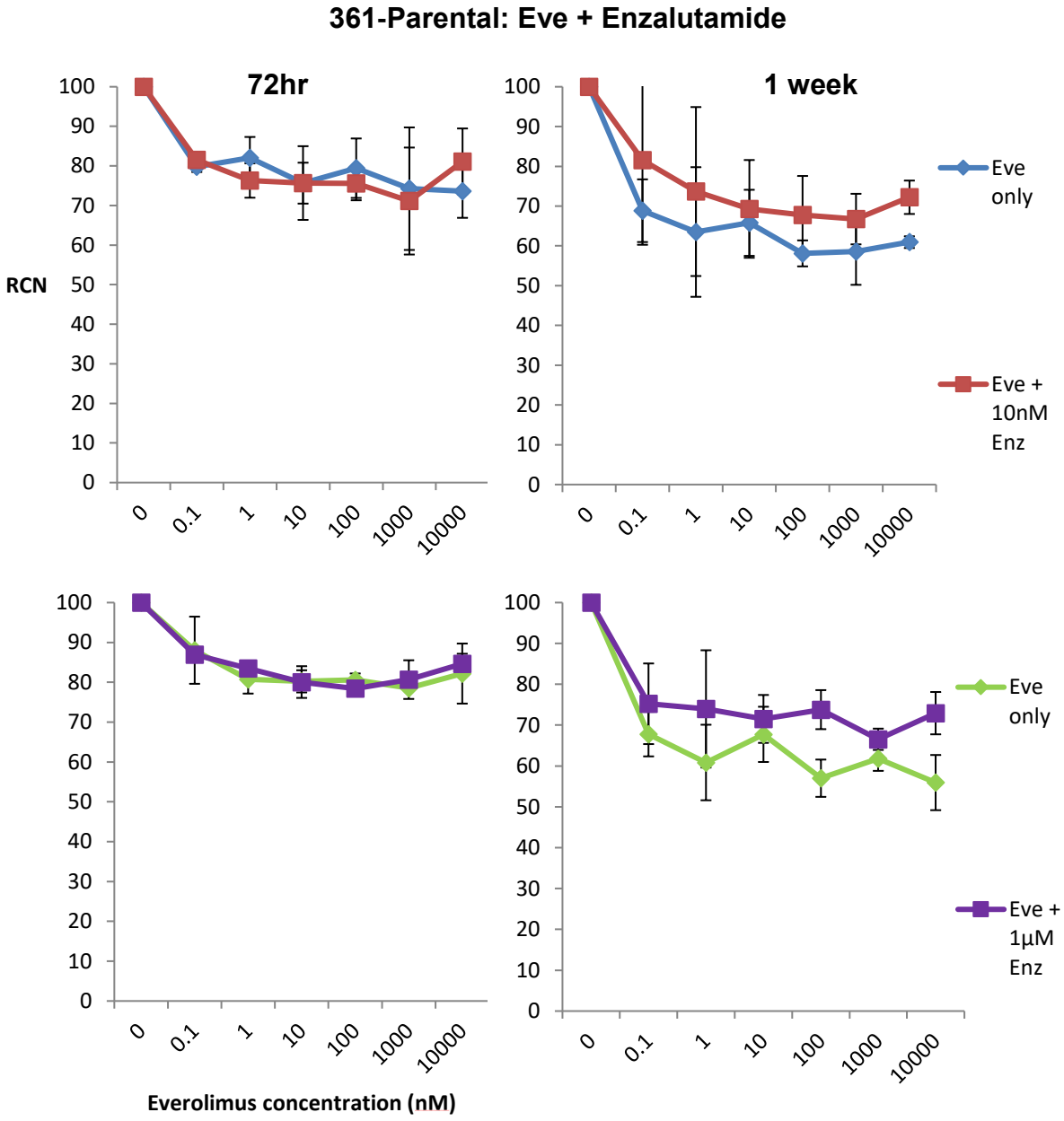


Figure 4.18: Everolimus+enzalutamide fails to significantly improve growth inhibition v everolimus only, in 361-Parental cells. *SRB result (mean plus or minus standard deviation, n=3 independent experiments) for 361-Parental treated for 72 hours or 1 week, with either everolimus (eve) or everolimus + 10nM or 1µM enzalutamide (Eve + Enz). In this cell line, the addition of enzalutamide with everolimus appeared to have very little extra effect, over everolimus only.*

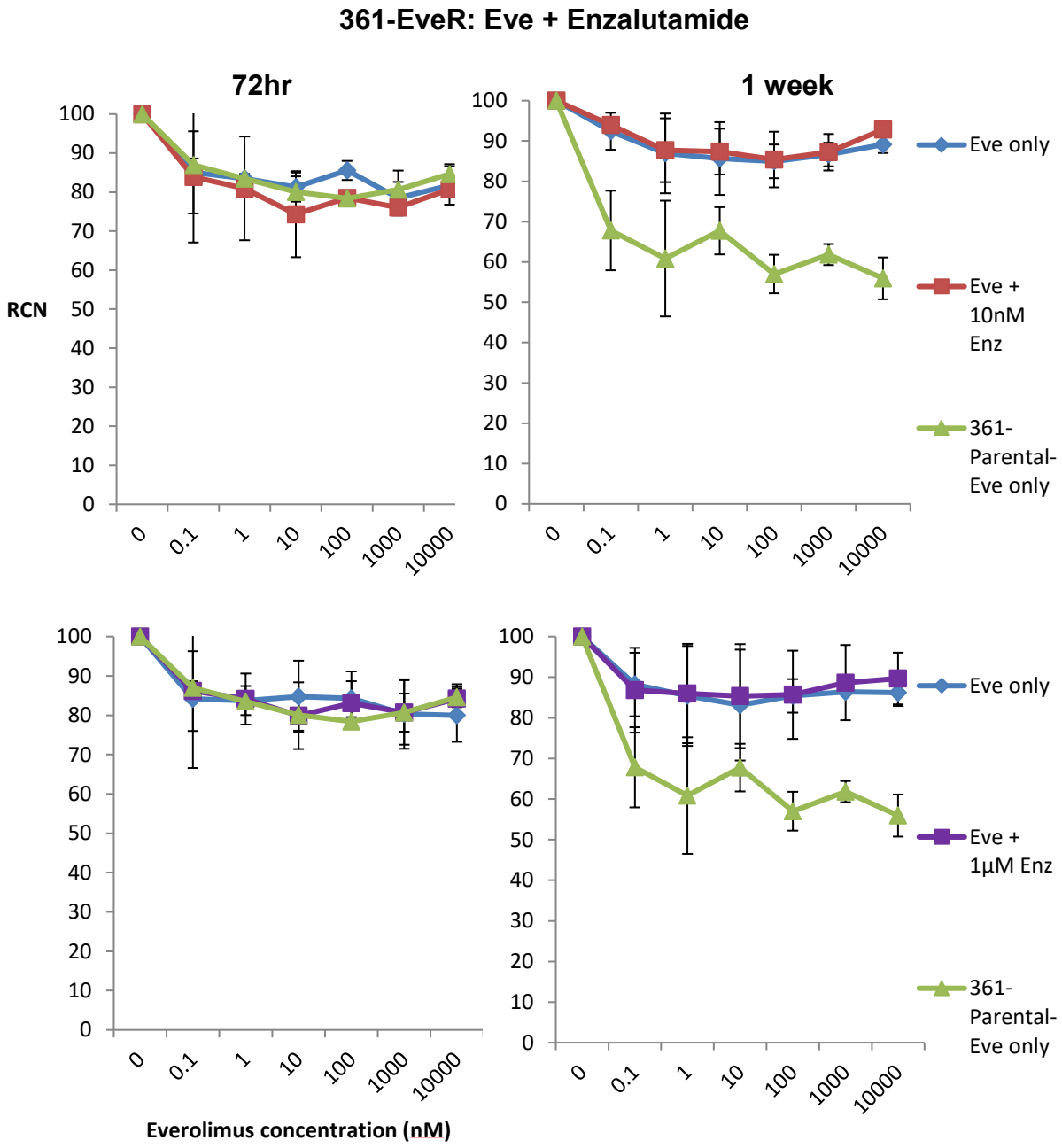


Figure 4.19: Everolimus+enzalutamide fails to significantly improve growth inhibition v everolimus only, in 361-EveR cells. *SRB result (mean plus or minus standard deviation, n=3 independent experiments) for 361-EveR treated for 72 hours or 1 week, with either everolimus (eve) or everolimus + 10nM or 1µM enzalutamide (Eve + Enz). In this cell line, the addition of enzalutamide with everolimus appeared to have very little extra effect, over everolimus only.*

4.4 - Discussion

This area of work looked to develop two everolimus resistant breast cancer cell lines, by treating them with the drug over a course of around 5-7 months. Overall this was achieved with both lines showing some resistance to everolimus. T47D-EveR cells were developed over the period of just under 6 months, encompassing 10 two week treatment cycles. T47D-EveR cells show a full drug resistant phenotype with significant resistance to everolimus after treatments of 72hr and 1 week. 361-EveR cells were developed over a slightly longer period of around 7 months and 14 two week treatment cycles. It is possible that the 361-EveR cells only showed a partial everolimus resistant phenotype, with no resistance seen at 72hr, but significant everolimus resistance (comparable to the data for the T47D-EveR cells) was seen after 1 week everolimus treatments. This will be further explored in subsequent chapters.

The total time for development of each EveR cell line roughly matched the time taken to develop drug resistant lines in similar research. In work by Jordan and Colleagues (2014) tamoxifen resistance emerged from around 6 months onwards in MCF-7 cells. In research by Box and colleagues (2013), from which our resistance development method was based, resistance to gefitinib was noted 2-5 months into the process. The MDA-MB-361 cells took slightly longer to become everolimus resistant than both these previously described methods (around 7 months), in part possibly due to slow-proliferative rates.

Some adaptation of the development methodology was done over the course of these 7 months to accommodate changes in the cells and other factors. This mainly relates to including the parental cell line in the SRB assay from treatment cycle 8/9 onwards. This allowed for more accurate assessment of the developing resistant phenotype. These were not included in data sets of the earlier cycles, despite being grown alongside the EveR lines. In retrospect it may well have been worth including these cells for all SRB assays. It can also be noted that resistance was only measured using 1 week treatments and not 72hr as well. This was partially due to limited number of cells available for testing after each treatment cycle and also due

to the 1 week treatment allowing for 1 week rest period for EveR cells to recover after everolimus treatments.

It is worth noting that only one plate/SRB test was run after each treatment cycle to test for a new GI60. Because of this it is possible that small variations in responsiveness, seen in the early phases of development, were actually within the range of baseline responsiveness, rather than a true alteration in phenotype. Whilst this is a small issue in this method of developing resistant cells, it is for this reason that we looked to get a minimum of 2-3 treatment cycle's data showing stable resistance, before we concluded that a genuine resistant phenotype has been reached. Whilst multiple plates may allow for this sort of error to be corrected, this strategy is highly unfavourable and impractical due to the low number of cells available after each treatment and the increased weeks and months this would potentially add to the development process.

An important part of developing drug resistant cell lines is using them to explore possible drug combinations that may be effective in reducing, or overcoming the resistance phenotype. Separate evidence suggests that both androgens/signalling via the AR, and vitamin D, can alter mTOR signalling in breast cancer (Gordon et al, 2017; Lehmann et al, 2014; O'Kelly et al, 2006). As such drugs relating to these biological aspects were combined with everolimus, to study if they were at all effective in helping to overcome the resistance phenotype, due to the supposed interplay between mTOR signalling and AR or vitamin D.

Everolimus treatment was combined with the AR inhibitor, enzalutamide, to test this drug combination in the EveR models. Unfortunately, as the data shows here, enzalutamide had virtually no effect on either T47D or MDA-MB-361 cells (EveR or parental lines) in terms of inhibiting cell growth and showed absolutely no use as an additive to an everolimus regimen, to overcome resistance. The majority of research into the use of enzalutamide and other AR inhibitors, in breast cancer, is currently focused on studying their effectiveness in treating triple negative breast cancers. Many papers have shown that AR inhibition may prove to be a very useful method of treating this cancer type, with good responses to these therapies, leading to the possibility that AR may one day be a biomarker alongside ER, PR and HER-2

(Barton et al, 2015; Gucalp et al, 2013; Traina et al, 2018). It is possible that the presence of ER or HER-2 signalling may actually override the usefulness of targeting AR in the T47D and MDA-MB-361 cells (T47D express ER and MDA-MB-361 both ER and HER-2), as despite the fact that both express the AR, blocking AR had no effect on their growth. It is also possible that non-genomic actions of androgens could play a role in properly blocking the effect of androgens (especially in the cell lines studied here) and that is why inhibiting the AR directly has had little effect in this setting (Foradori et al, 2008).

The combination of everolimus and calcitriol/vitamin D itself was also tested to study if it could re-sensitise the cells to everolimus treatment. In chapter 3 of this thesis this same combination was tested to look for increases in the efficacy of everolimus, to mostly no success. However it is evident from the data displayed here that the combination may in fact be of some use in everolimus resistance, albeit only with the higher doses of calcitriol, over a longer period helping to partially or fully overcome resistance. As has been discussed previously, vitamin D has been suggested to have an effect on mTOR signalling, possibly being able to down-regulate and inhibit it (Lisse et al, 2011; O'Kelly et al, 2006) Work proceeding this thesis in our research group showed that vitamin D's effect on the cell lines used here, is mediated away from the VDR (Harvey, unpublished). The data from this thesis backs this evidence up as VDR levels did not vary between the cell lines and their parental controls, but the effect of vitamin D combined with everolimus, varied greatly.

The successful combination treatments presented here lends further evidence that vitamin D does indeed interact and interplay with mTOR signalling in some form. This data also suggests that calcitriol is impacting on mTOR signalling, rather than just having an additive effect, due to the relative weak effect calcitriol had as a single agent therapy, especially concerning the 361-EveR cell line. Work that could elucidate exactly how vitamin D interacts with the mTOR axis in breast cancer would be useful to help determine how we could implement the use of vitamin D with mTOR inhibitors at a clinical level. Furthermore it could shed light on exactly why the EveR cells are now resistant, if the vitamin D targets an area of the mTOR axis which is involved in everolimus resistance.

Chapter 5: Characterisation of Everolimus Resistant Cell Lines

5.1 - Introduction

5.1.1 - The Role of Canonical Wnt Signalling in Cancer Stem Cells and Breast Cancer

Canonical Wnt signalling is an extremely important in cell biology, including a key role in controlling the appropriate development and pattern of cells/ tissues in the embryo, as well as being important in helping stem cells to maintain their properties of self-renewal (Clevers and Nusse, 2012). However, its dysregulation can also play a role in the development of breast cancer, with Wnt pathway over-activation, showing that it can contribute to tamoxifen resistance *in vitro* (Loh et al, 2013). However it is also noted to be more activate in populations of cells identified as breast cancer stem cells (CSC) (Lamb et al, 2013), having been shown to help regulate breast CSCs. In breast cancer cell cells, Wnt signalling helps promote treatment resistance as well as epithelial-mesenchymal transition (EMT), via Snail activity, with EMT believed to be a central process in CSC formation and activity (Guo et al, 2014; Kotiyal and Bhattacharya, 2014). Disseminated cells can often lie dormant at metastatic sites, until promoted to grow, with many of these promoting signals also being involved in CSC regulation, such as TGF-β1 (Guo et al, 2014; Yeh and Ramaswamy, 2015). It is this drug resistance, that activate Wnt signalling can be involved in, that makes Wnt inhibitors in combination with an mTOR inhibitor, and attractive candidate for a drug combination.

Developing reliable inhibitors of the Wnt ligands themselves have proven difficult, and thus drugs here tend to aim to down-regulate Wnt signalling either through one of the other Wnt signalling components or indirectly via other pathways. Tankyrase inhibition is one such method, with its inhibition causing stabilisation of axin, leading to the degradation of β-catenin and thus pathway inhibition (Afifi et al, 2014). Drugs to inhibit tankyrase, such as XAV-939 or direct down-regulation of it via siRNA, has

shown to down-regulate Wnt signalling and the activation of Wnt target genes, in MDA-MB-231 breast cancer cells (Bao et al, 2012). Other drugs that inhibit Wnt pathway activity in some format (and have shown some efficacy in breast cancer cells) include γ-tocotrienol (Ahmed et al, 2016) and pyrvinium pamoate (Xu et al, 2016). Clearly research exploring the use of Wnt inhibitors in breast cancer is at a very early stage, however due to the importance of Wnt signalling in CSC regulation, and the proven effectiveness of many of these drugs *in vitro*, it is very likely that in future years work may move to a clinical level.

5.2 – Aims

- This chapter aimed to characterise everolimus resistant (EveR) breast cancer cells developed in previous chapters, against their parental counter parts by:
    - Determining the response of these resistant cells, to other mTOR inhibitors and breast cancer therapies.
    - Examining the expression and activation of mTORC1 and mTORC2 signalling components in EveR cell lines.
    - Determining whether everolimus resistant cells have any changes in proliferation rate, ability to migrate and oncogenic potential.

5.3 - Results

5.3.1 - Response of Everolimus Resistant Cells to Other mTOR Inhibitors

The EveR cell lines were tested for their response with several other mTOR inhibitors. This included other rapalogues such as rapamycin and temsirolimus and dual mTORC1/2 inhibitors like BEZ-235 (which also inhibits PI3K). Since the rapalogues all act using the same mechanism, by inhibiting mTOR through binding to FKBP12 (Brown et al, 1994), it was expected that the EveR cells would also be resistant to treatment with rapamycin or temsirolimus. As can be seen from figures 5.1 and 5.2, the response of the cells to these two drugs was in fact very varied, compared to their response to everolimus.

Both EveR cell lines showed significant resistance to rapamycin (figure 5.1) when treated for 1 week, with around 30-40% growth difference at each concentration tested; however, at 72hr there was little difference between either EveR cell line and its parental control. Only T47D-EveR showed some small, but significant differences, at 2 of the concentrations.

When treated with temsirolimus (figure 5.2) the difference between EveR cells and parental controls was even less pronounced, with the 361-EveR cells showing no resistance at 72hr and very little of significance at 1 week (a difference of around 10% RCN was only seen at 100nM). T47D-EveR also showed less resistance to temsirolimus than they did everolimus, with only 1nM treatment at 72hr, being significantly different compared to the control cells. Resistance to temsirolimus was observed at 1 week, at concentrations of 100nM or less.

Treatment of the cell lines with the mTOR/PI3K inhibitor, BEZ-235 (figure 5.3) for 1 week or 72 hr, showed that neither everolimus resistant cell line had any resistance to BEZ-235.

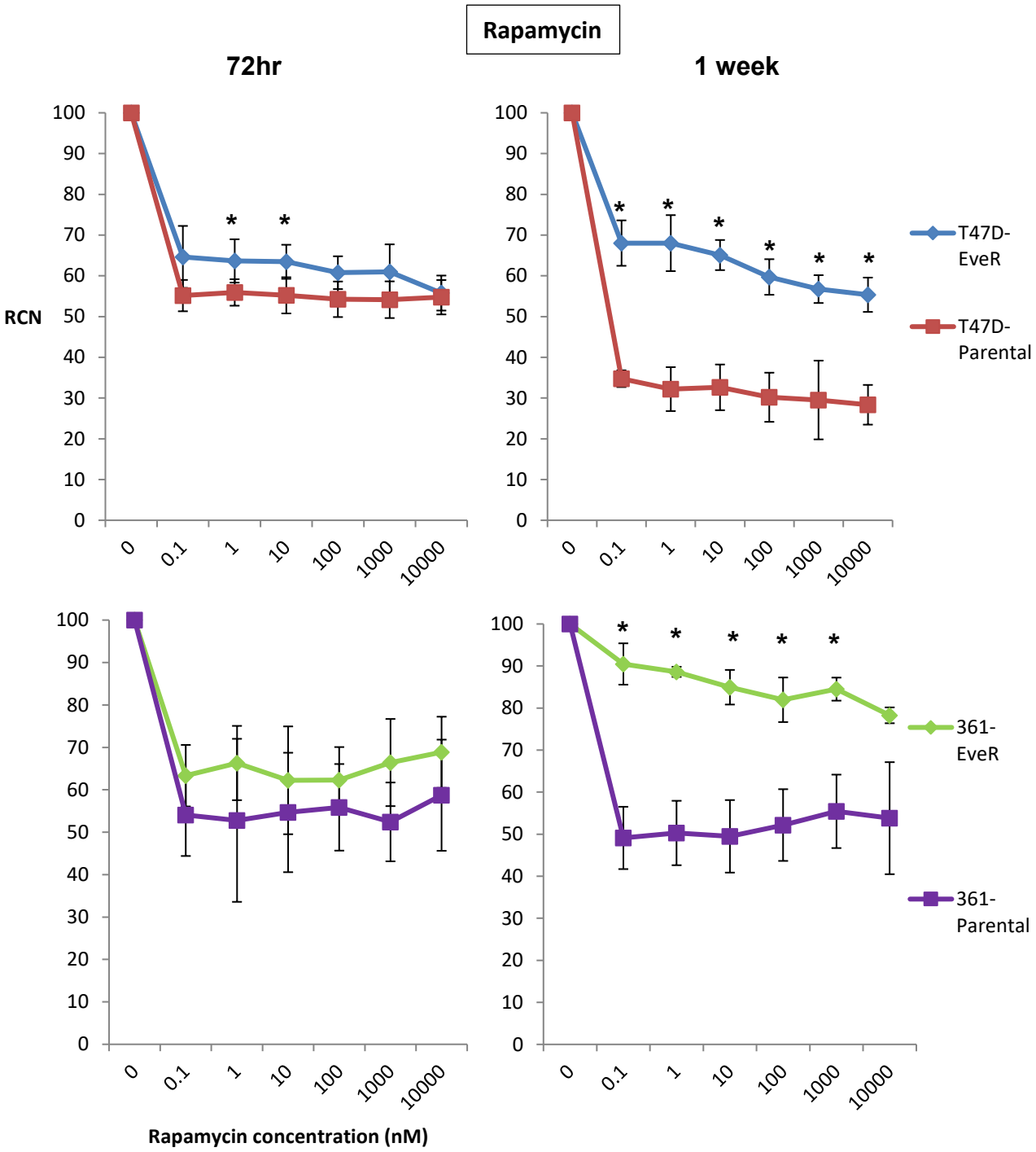


Figure 5.1: EveR cells are resistant to rapamycin at a 1 week time point. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant lines (EveR) v parental, treated with rapamycin for either 72hr (left) or 1 week (right). Both EveR lines show significant resistance after a 1 week treatment but little no resistance at 72hr. * P<0.05 compared to control.*

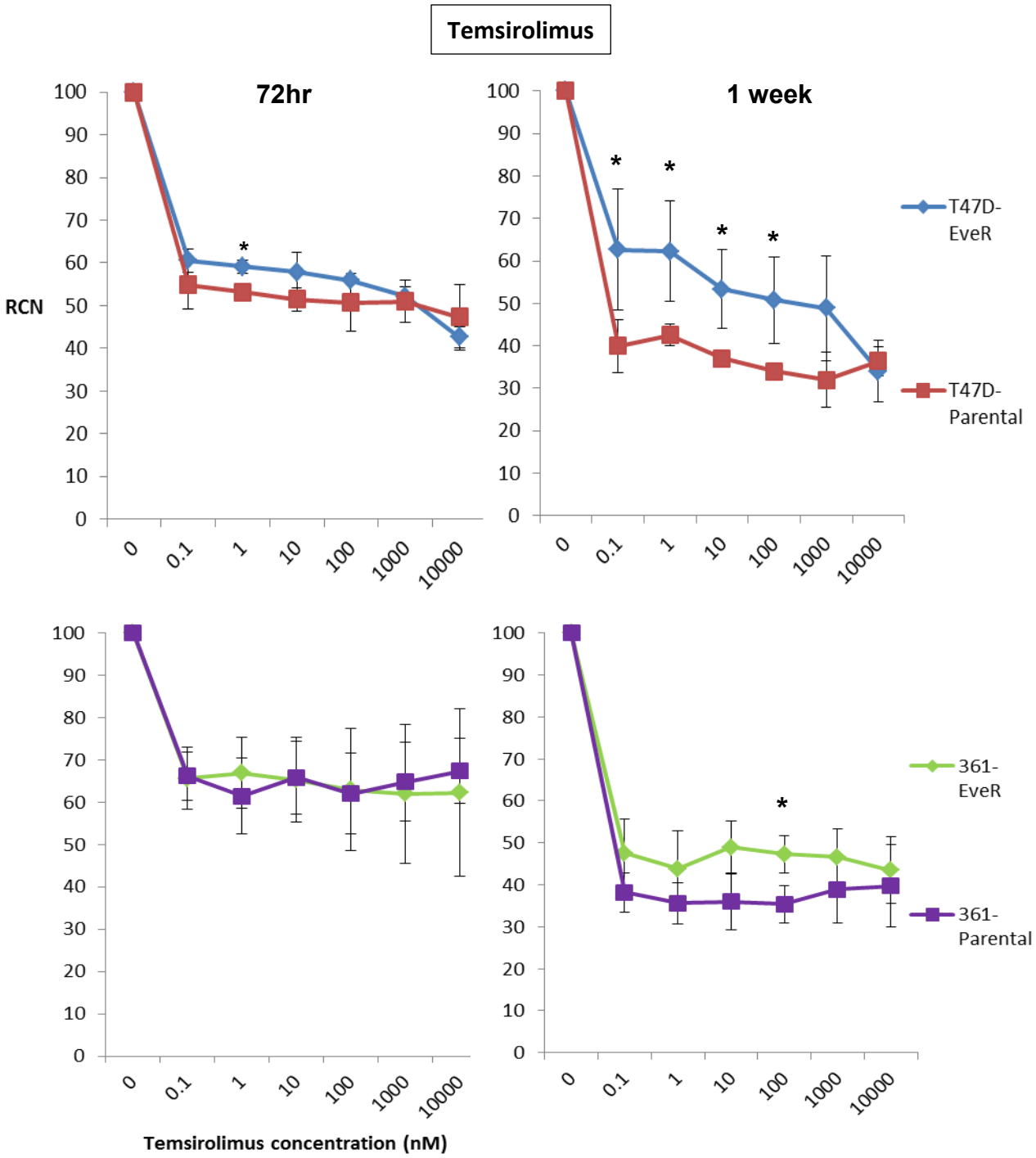


Figure 5.2: T47D-EveR show resistance to temsirolimus at a 1 week time point. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant lines (EveR) v parental, treated with temsirolimus for either 72hr (left) or 1 week (1 week). T47D-EveR show significant resistance after a 1 week treatment but little no resistance at 72hr, with 361-EveR showing little resistance at both time points. * P<0.05 compared to control.*

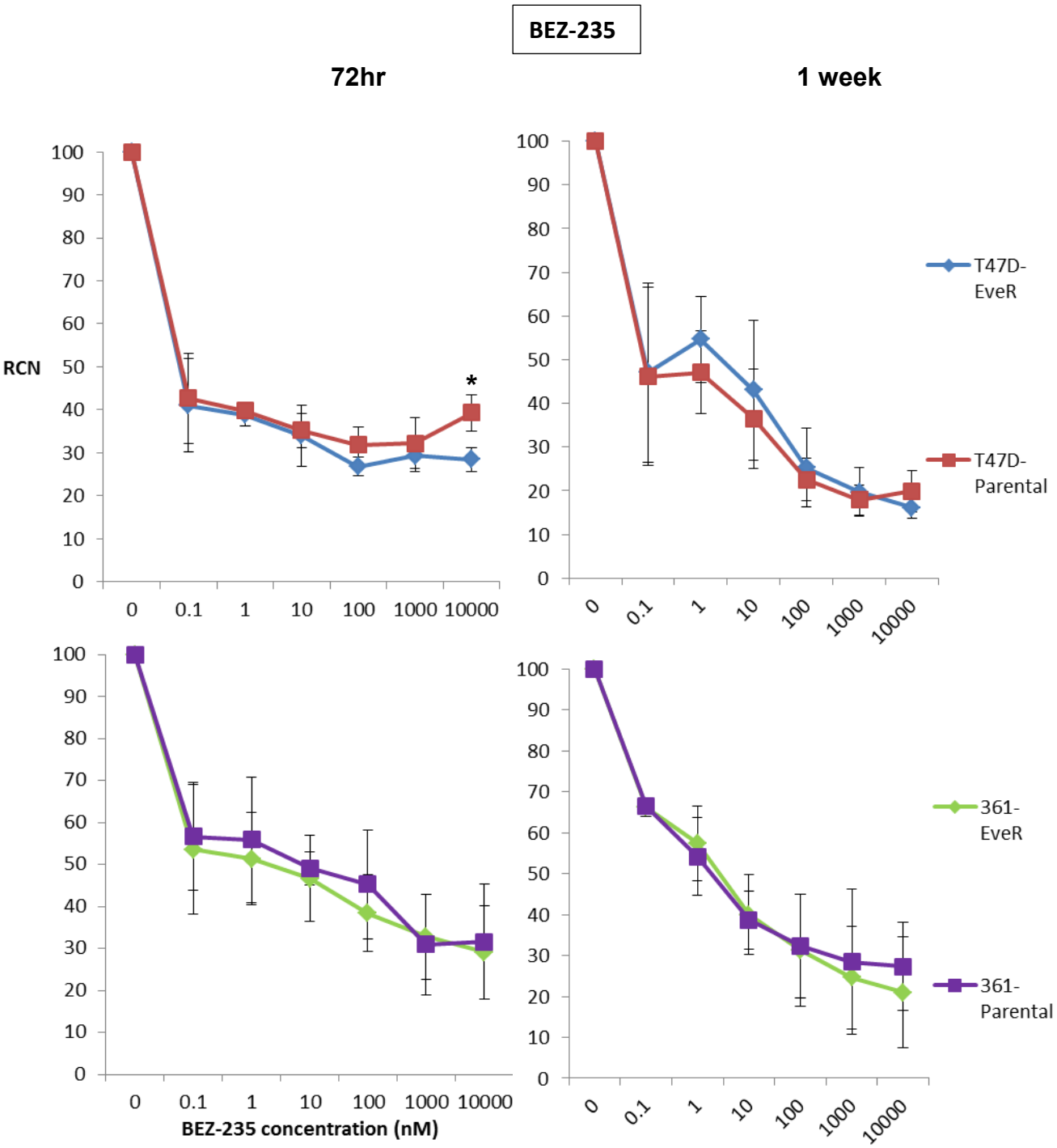


Figure 5.3: EveR cells are not resistant to BEZ-235. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant lines (EveR) v parental, treated with BEZ-235 for either 72hr (left) or 1 week (right). Neither EveR line showed any resistance to BEZ-235 treatment. * P<0.05 compared to control.*

5.3.2 - Confirming the Expression of mTOR Pathway Components at a Protein Level in Everolimus Resistant Cells

The status of mTOR pathways proteins was checked using western blotting. Since everolimus and other rapalogues specifically target the mTORC1 arm of this pathway, we hypothesised that everolimus resistance may result in an alteration in the expression of certain mTOR components (figure 5.4).

Several of the proteins studied here showed no altered expression between the EveR and parental cell lines. Perhaps most importantly, the expression of mTOR itself had not been altered in either resistant line. Also, accompanying this, no difference in p-mTOR at Ser2448 was observed. The expression of both total and p-Akt was unchanged when comparing EveR cells to their parental controls, with all cell lines still expressing high levels of both.

The T47D-EveR cells showed several noticeable changes in important mTOR related proteins. In the EveR cells, an-upregulation of both rictor and p-mTOR (Ser 2481) were seen, along with a slight down-regulation of raptor, suggesting an increase in active mTORC2 complexes. This was accompanied by small but noticeable increase in downstream targets of mTORC2 including the total and phospho-forms of both SGK1 and PKCα. Despite the down-regulation of raptor, no change in p-S6K was seen. Smaller changes were observed in the 361-EveR cells compared to their parental counter-parts. As seen in figure 5.4, there was some change in raptor and DEPTOR, with 361-EveR expressing slightly more of these. There was also an extremely small increase in the expression of phospho and total PKCα.

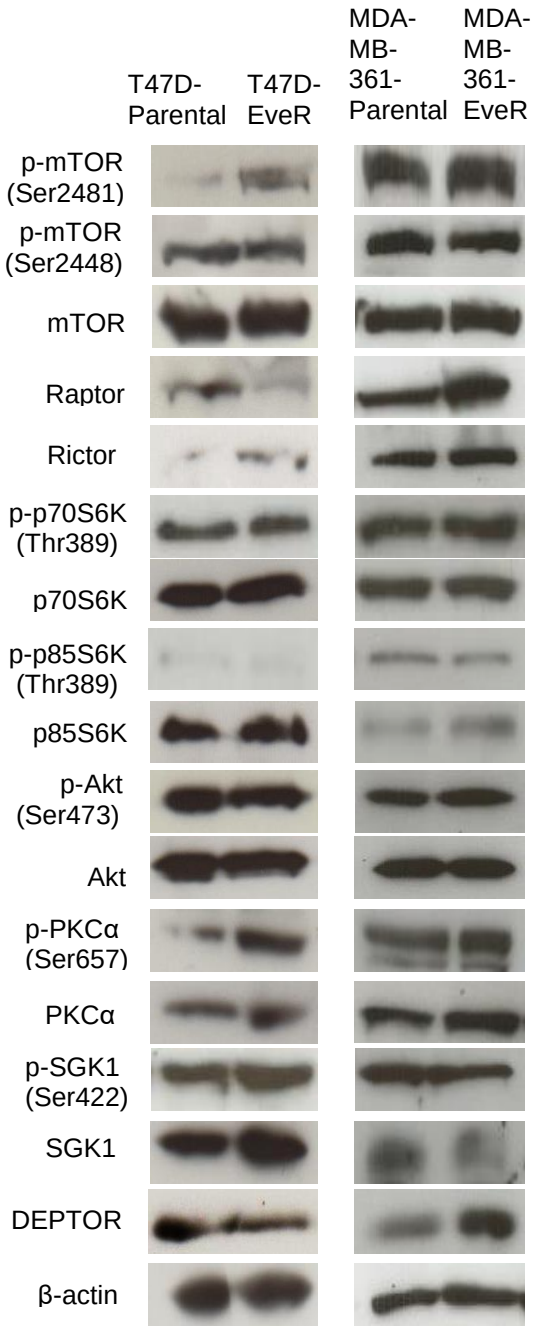


Figure 5.4: mTORC2 proteins are up-regulated in T47D-EveR. *Representative western blots for T47D-EveR and Parental and MDA-MB-361-EveR and Parental cell lines. n=2 independent experiments. Target proteins included major mTOR signalling pathway components to give an overview of the status of mTOR signalling in these breast cancer lines, with β-actin as a loading control. T47D-EveR showed up-regulation of mTORC2 related proteins including rictor and phospho and total SGK1 and PKCα, compared to T47D-Parental cells.*

The status of the major breast cancer markers, HER-2, ERα and PR were also examined to see if everolimus resistance had majorly affected their expression (figure 5.5). No major changes were seen in either the 361 or T47D-EveR cell lines compared to their respective parental controls.

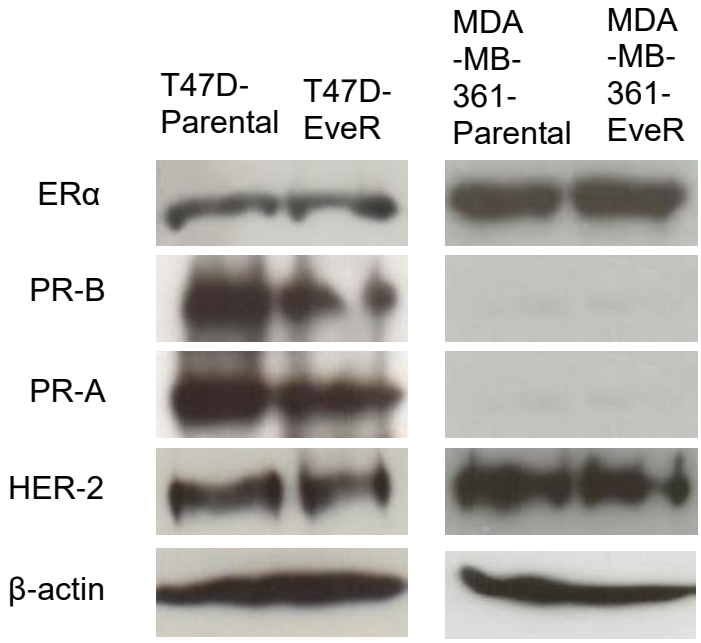


Figure 5.5: EveR cells show no major change in breast cancer markers. *Representative western blots for T47D-EveR and Parental and MDA-MB-361-EveR and Parental cell lines. n=2 independent experiments. Target proteins were key markers of breast cancer that included oestrogen receptor α (ERα) and progesterone receptor (PR) expression. HER-2 was also tested, with breast cancer cells classified as '+' for HER-2 if the cells over-express it. Little change was noted between the EveR lines and their respective parental lines.*

5.3.3 - Confirming the Expression of mTOR Pathway Components at a Gene Level in Everolimus Resistant Cells

The status of major mTOR components was also determined using qPCR analysis. Before this was done, housekeeping genes were selected using a geNorm primer kit (Primer Design) and qbase+ (Biogazelle) software. Both EveR and both parental cell lines were assessed together to find the most stable genes to use for later mTOR qPCR experiments. The most stable genes from this analysis were *SDHA* and *UBC* (figure 5.6). However, *TOP1* and *YWHAZ* were selected for use as reference genes. This was because not only was their stability (M) values were very similar *SDHA/UBC*, but we had already used primers for *TOP1* and *YWHAZ* previously in section 3.3.4.

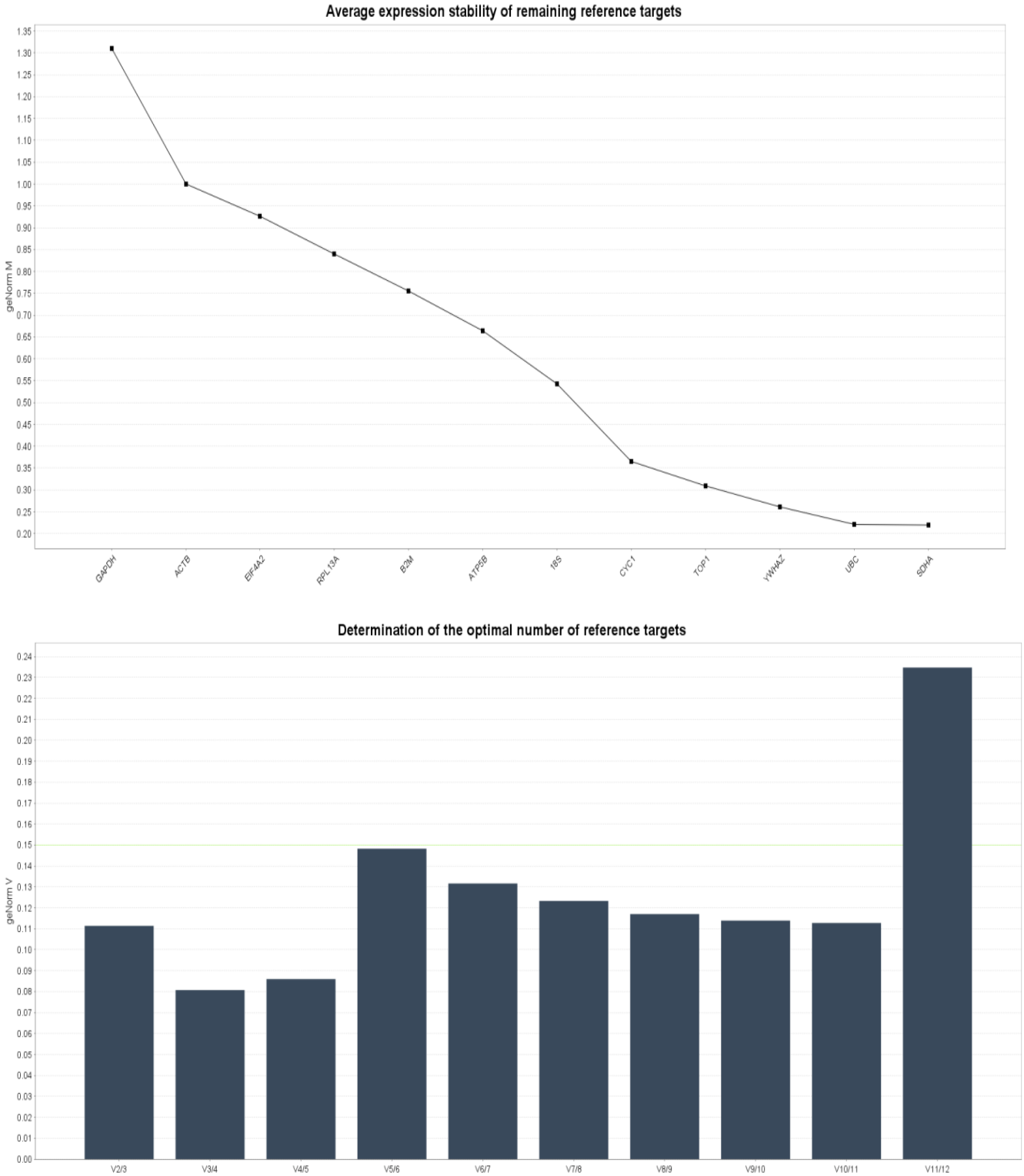


Figure 5.6: TOP1 and YWHAZ are recommended reference genes. *geNorm analysis, using qbase+ (Biogazelle), with the stability of the potential housekeeping genes ranked in graph A, using the 'M' value, with the most stable on the right with the lowest value. Graph B shows the ideal number of reference genes, with any 'V' value lower than 0.15 suitable. The use of 2 genes fit these criteria, with TOP1 and YWHAZ, being selected.*

The expression of *MTOR, RAPTOR, RICTOR* and *DEPTOR* were then determined using the same qPCR method, with the RQ used to compare expression across all cell lines, shown in figures 5.7. Some differences were seen between EveR and parental lines with *DEPTOR* expression higher in T47D-EveR compared to parental cells and *MTOR* and *RAPTOR* expression lower in 361-EveR compared to the parental control.

When comparing the qPCR data here (figure 5.7) to the western blotting data for the expression of the equivalent proteins (figure 5.4), the different experimental methods did not conform well to one another, in terms of the interpretation of the data. For example, the differential expression of rictor protein between the cell lines, was not reflected in the qPCR data. Equally, the qPCR data showed some alterations in *MTOR* gene expression that was not reflected at a protein expression level. Within these cells, this may represent some difference in the ratio of translated protein to transcribed RNA, for some mTOR components.

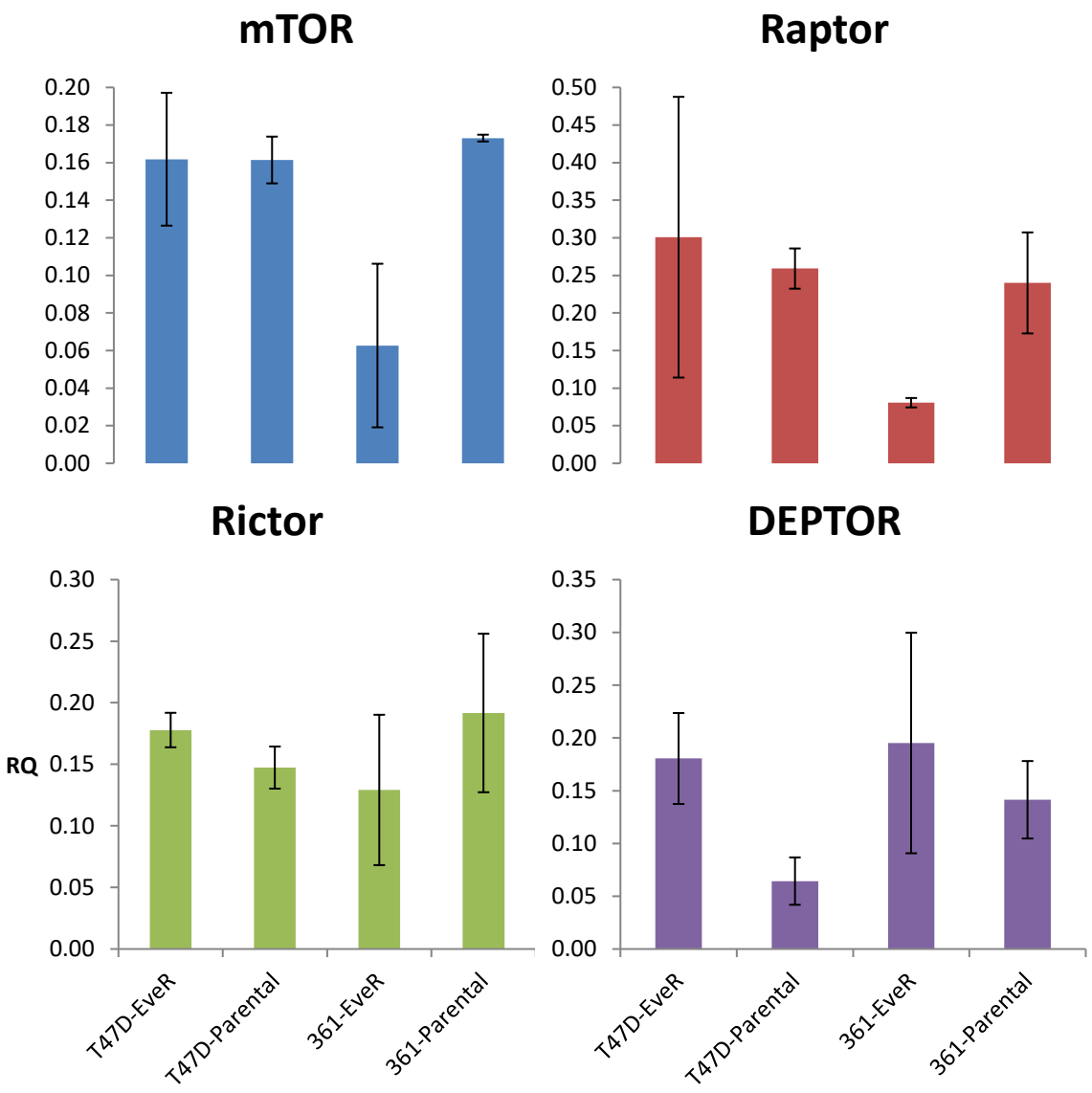


Figure 5.7: Gene expression for key mTOR components in EveR cells. *qPCR results for individual genes (mean plus or minus standard deviation, n=2 independent experiments) studying gene expression of MTOR, RAPTOR, RICTOR and DEPTOR, with data displaying RQ (relative quantity).*

5.3.4 - Studying Proliferative Differences in Everolimus Resistant Cells

Since changes in growth rate had been noted (whilst growing under normal culture conditions), during the development of the resistant lines, the proliferative capacity of both EveR lines and the parental lines were studied by plotting growth curves and

using flow cytometry to analyse cell cycle progression. Growth curves for cell lines can be seen in figures 5.8 and 5.9.

All 4 cell lines were also stained with trypan blue to analyse any change in dead cells and thus any change in apoptosis that may have occurred during the development process. No change in dead cell number was observed, when comparing the EveR to its parental line, in either the T47D or MDA-MB-361 cells. However, both cell lines did show small but noticeable changes in the number of live cells, with proliferation in both parental lines, over taking the EveR lines at day 5, with a statistically significant (*P*=<0.05) difference, at day 7 for T47D and day 6 and 7 for MDA-MB-361.

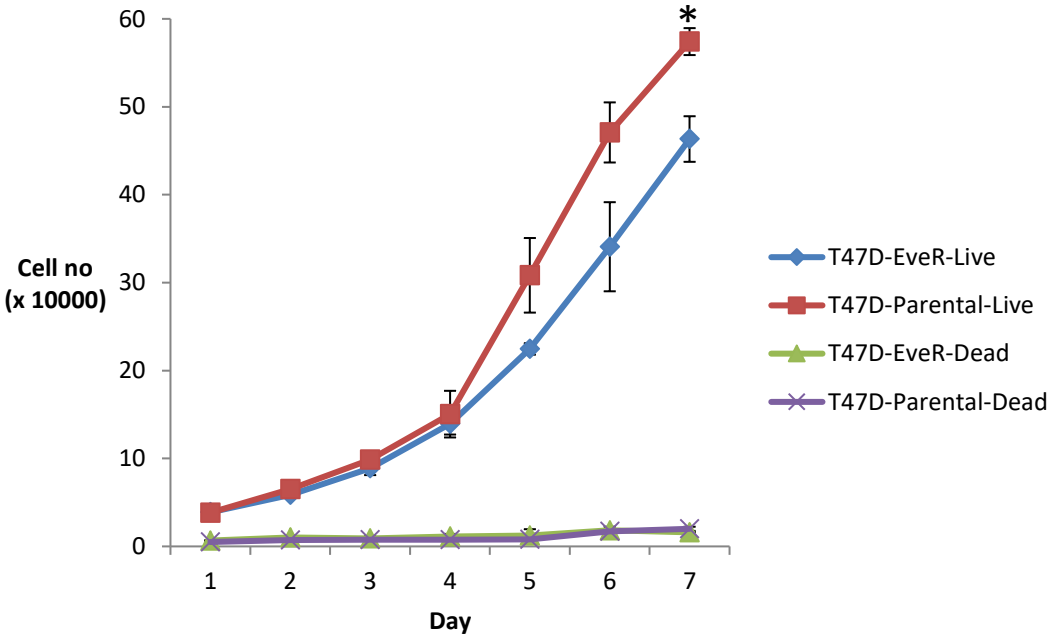


Figure 5.8: T47D-EveR proliferate significantly slower than parental cells. *1 week growth curve data (mean plus or minus standard deviation, n=2 independent experiments) for T47D-EveR v T47D-Parenatals, plotting both live and dead cells. No change in dead cells was noted across the week. T47D-EveR grew generally slower than the parental cells after day4. * P<0.05, EveR compared to parental line.*

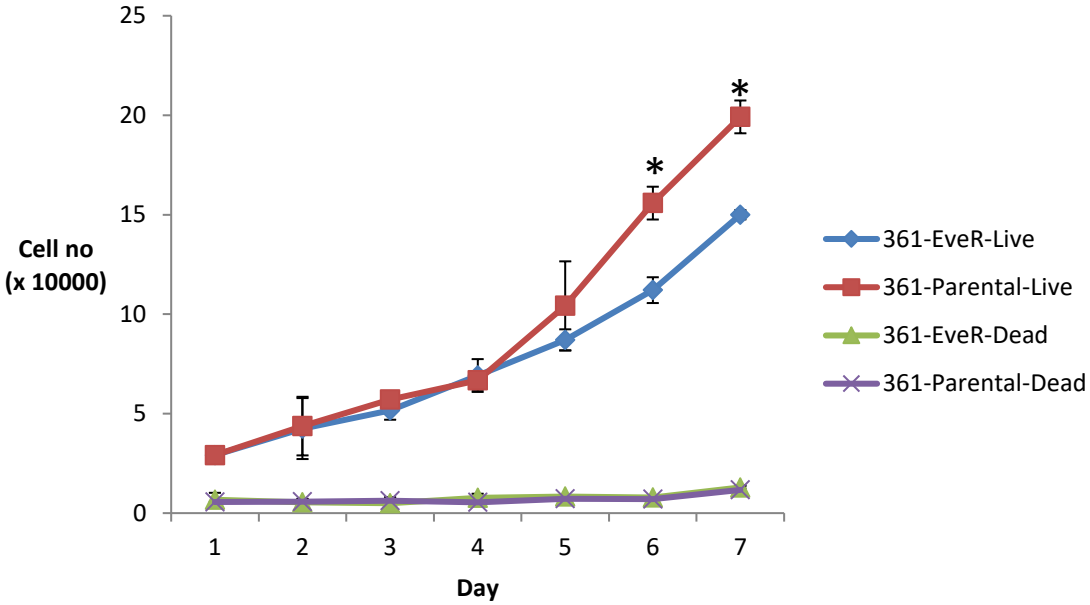


Figure 5.9: 361-EveR proliferate significantly slower than parental cells. *1 week growth data (mean plus or minus standard deviation, n=2 independent experiments) for 361-EveR v 361-Parentals, plotting both live and dead cells. No change in dead cells was noted across the week. 361-EveR grew generally slower than the parental cells after day 4. * P<0.05, EveR compared to parental line.*

Propidium iodide (PI) staining and subsequent flow cytometry analysis was used to show the cell cycling profile of these cells, and thus further analyse the observed growth differences. An example flow cytometry plot is shown in figure 5.10. The ACEA NovoCyte flow cytometer cell cycle analysis parameters were used to determine the proportion of cells in each phase of the cell cycle, with G2 having roughly the double amount of PI signal to G1 cells and S-phase cells judged to be in between.

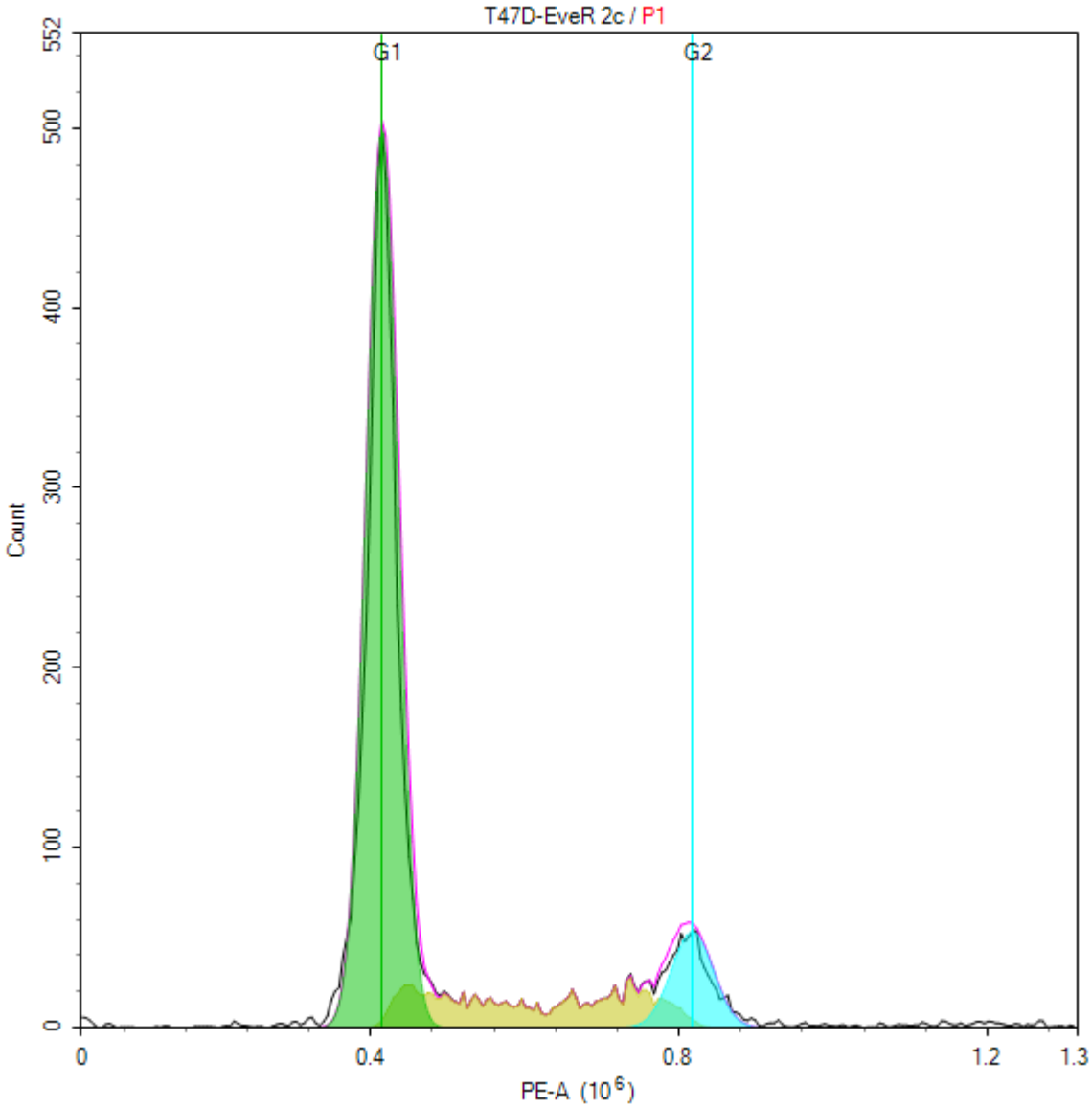


Figure 5.10: Example flow cytometry readout from the ACEA NovoCyte flow cytometer cell cycle analysis function*. Propidium iodide staining is represented along the x-axis (PE-A) with cell count along the y-axis. Using propidium iodide staining as a marker for DNA content, the function counts the cells in G1 phase (green), S-phase (yellow) and G2 phase of the cell cycle (blue).*

Although there were some very small percentage differences, with the 361-Parental having slightly more cells in S phase, and fewer in G1, than the EveR cells, this was non-significant (figure 5.11). T47D-Parental cells did show more, with the T47D-Parental having a higher proportion of cells in G2 and S phase, and less in G1 (the latter significantly different), than the T47D-EveR cells. This suggests that, the T47D-

EveR cells are cycling more slowly than the parental cell line, as previously suggested by the growth curve data.

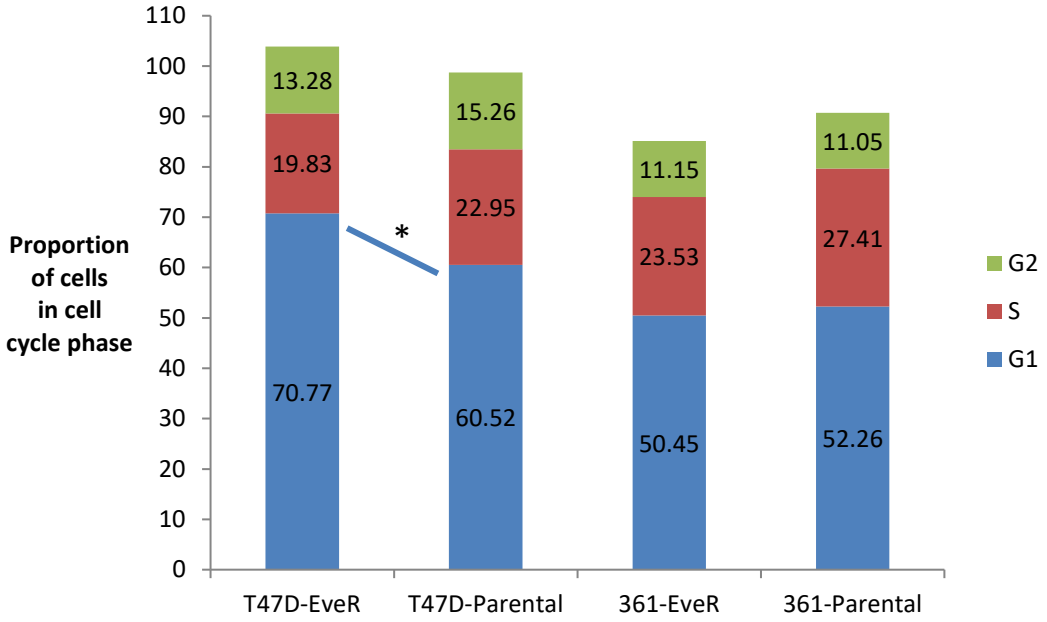


Figure 5.11: T47D-EveR show a significantly greater proportion of cells in G1 phase. *Flow cytometry data for T47D-EveR, 361-EveR and parental control cells, using propidium iodide staining to study cell cycle profile of each line. T47D-Parental generally showed significantly fewer cells in G1 phase (* P<0.05) and more in S and G2 than the EveR line. 361-EveR cells showed no significant difference to their parental controls.*

5.3.5 - Survival of Everolimus Resistant Cells in Suspension

A cell suspension assay was used to determine if the EveR cell lines showed an altered capacity for survival in suspension culture. Figure 5.12 shows the data for the cell suspension assay. There was no difference in either dead or live cells when comparing the T47D-EveR or 361-EveR with their respective parental controls.

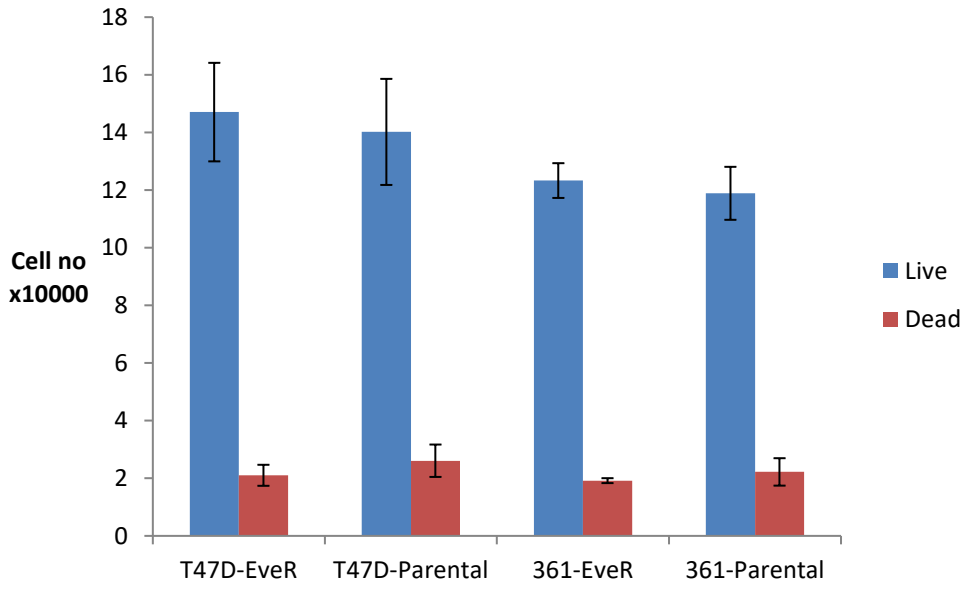


Figure 5.12: EveR cells have similar survivability in suspension, to parental lines. *Cell suspension data (mean plus or minus standard deviation, n=3 independent experiments) for EveR and parental cell lines. Cells were seeded into poly(HEMA) coated plates and incubated for 24hrs. Viability was then assessed by trypan blue exclusion. Data shows the live and dead cells counted in each line after the incubation. No difference can be seen when comparing the live or dead cells of the EveR v Parental of each cell line.*

5.3.6 - Migratory Capacity of Everolimus Resistant Cells

The rate of cell migration was in the EveR cell lines were compared to the parental cells, using a wound healing assay. Cells were treated with mitomycin C before being scratched, to inhibit cell proliferation and allow for the study of migration alone. Experiments were done a total of 3 times with the end point pictured at 48hrs post-scratch. The % wound closure was then estimated by comparing pictures from the start of the experiment to the end. Examples of this are shown in figures 5.13-5.14. It is worth noting from these figures, that wound closure was highly irregular across the wound front, making it extremely difficult to assess precisely (in exact distance) how much the wound had closed. Therefore the distance closed as a whole was estimated for each replicate and expressed as a percentage (0, 25, 50, 75 or 100% closure).

Data for all three experiments for all cell lines can be seen in table 5.1. The 361-EveR and parental cells showed very low rates of migration with virtually all replicates either showing 25% wound closure or no closure at all; both 361 cell lines showed the same amount of wound closure as the other, in each individual experiment.

The T47D lines showed a much greater level of migration, with wound closure often being between 25-75%. A small difference was observed, with the T47D-EveR cell line having a slightly increased closure over the parental cells. However due to the high variability and small variation between the lines, this difference was not statistically significant (P=0.48).

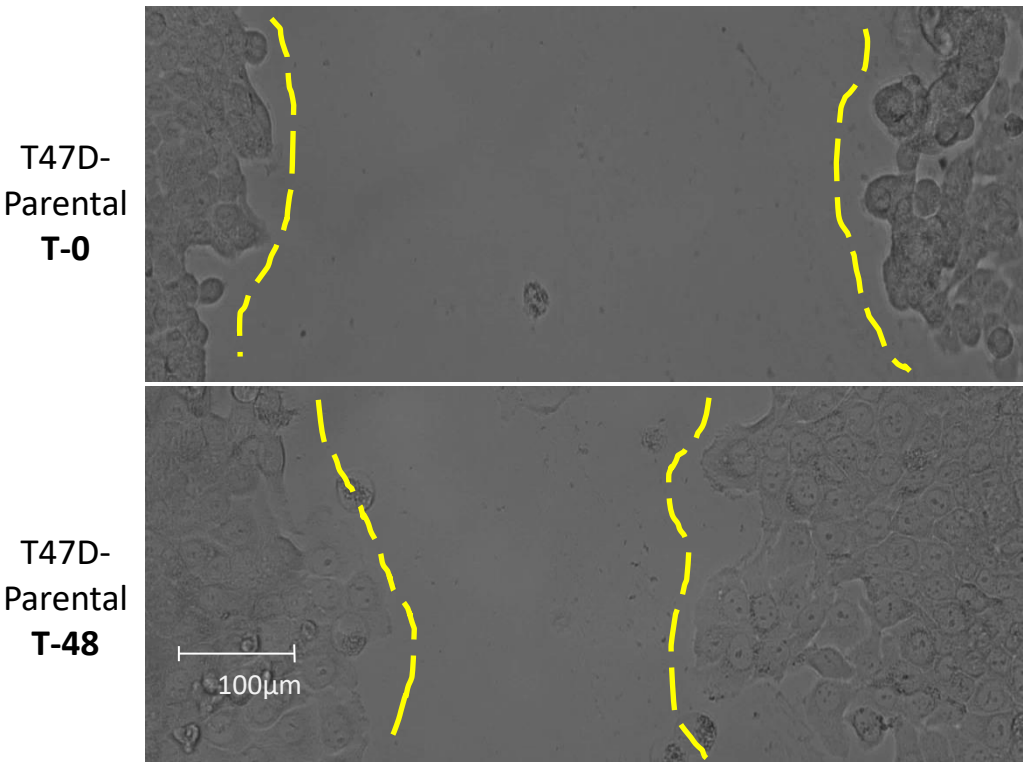


Figure 5.13: Example microscope images (x20 mag) for the wound healing assay for the T47D cells. *Pictures were shown were taken at the point of scratching (T-0) and 48hrs later (T-48). Yellow dashed line represents the cells at the front of the wound. For these example images, wound closure was estimated to be 50%. Please note these are examples of how the wound closure was estimated, and are not directly reflective of the data shown in table 5.1.*

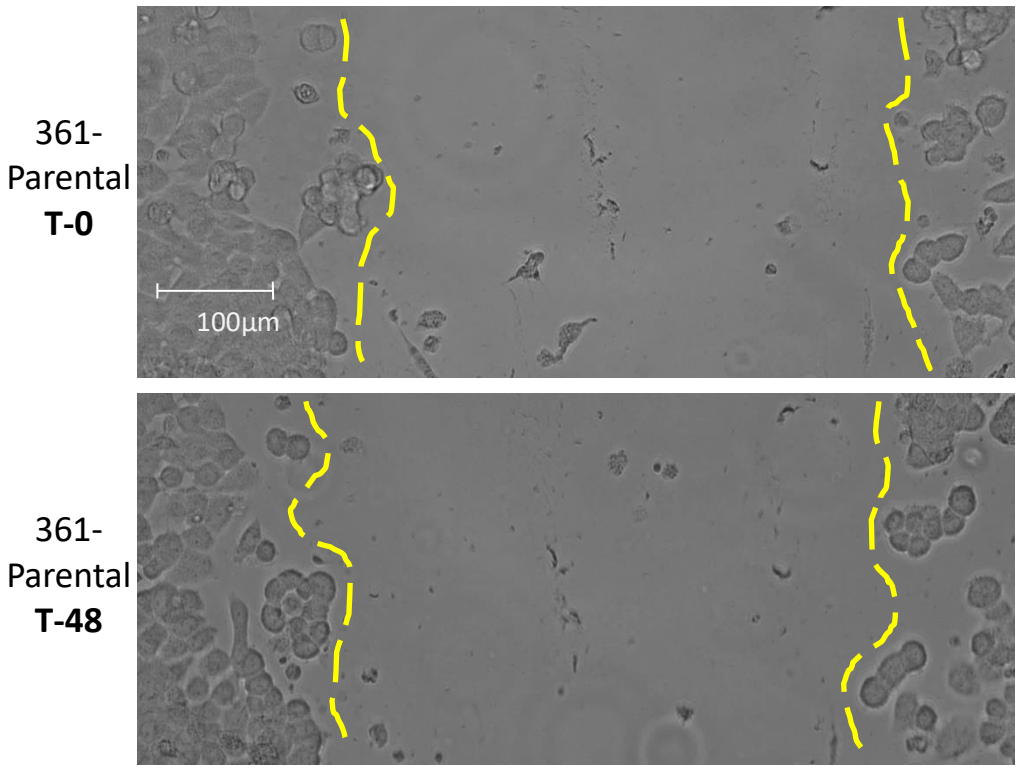


Figure 5.14: Example microscope images (x20 mag) for the wound healing assay for the 361 cells. *Pictures were shown were taken at the point of scratching (T-0) and 48hrs later (T-48). Yellow dashed line represents the cells at the front of the wound. For these example images, wound closure was estimated to be 0%. Please note these are examples of how the wound closure was estimated, and are not directly reflective of the data shown in table 5.1.*

	Wound closure %			
	Experiment 1	**Experiment 2**	**Experiment 3**	**Average**
T47D-EveR	58.3 (SD+8.96)	75 (SD+10.4)	33 (SD+14.4)	55.5
T47D-Parental	41.7 (SD+14.4)	58.3 (SD+16.07)	33 (SD+5.7)	44.4
361-EveR	8.3 (SD+7.2)	25 (SD+0)	8.3 (SD+7.2)	13.9
361-Parental	8.3 (SD+14.4)	25 (SD+25)	8.3 (SD+7.2)	13.9

Table 5.1: EveR cells show no significant difference in wound healing ability. *Wound healing assay data. Experiments were done 3 times with each experiment containing 3 technical replicates. % of wound closure for all technical replicates per run, for each cell line, was averaged and displayed here. No difference could be seen in the 361 cells. A small, but non-significant (P=0.48) difference could be detected in the T47D lines.*

5.3.7 - Antibody Arrays to Study Global Changes in Protein Expression

Phospho-kinase and oncology proteome arrays were used to give a wider picture of how the EveR cells might have changed, compared to parental cells; in terms of signalling pathways that could of have been changed and changes in oncogenic markers. Results for these arrays for both the T47D and MDA-MB-361, EveR and parental lines, can be seen in in figures 5.15-5.30.

Overall, of the nearly 130 proteins tested (of which, in the phospho-kinase array, many were tested for multiple phosphorylation sites), EveR cells pre-dominantly showed a down regulation of proteins and phosphorylated proteins compared to the parental lines. Although some proteins and phospho-proteins were up-regulated, this was generally in a much smaller number and by a smaller margin, in terms of relative change in expression, than the number and relative amount of down-regulated proteins.

Figures 5.15, 5.17 and 5.19 show the phospho-kinase array data for the T47D lines. As we can see from the data most of the phospho-targets studied showed down-regulation, with only some STATs and p53 showing a very small increase in

expression and β-catenin and AMPKα2 showing around 1.5-2 x relative expression of protein. Small down-regulations of the mTOR related proteins p-Akt (Ser473) (also noted in the 361-EveR cells) and p-p70S6K (Thr389) were noted in the T47D-EveR line, that was not similarly observed from western blotting data (figure 5.4). These arrays also suggested a 1.5 relative decrease in p-mTOR (Ser2448) whilst western blotting data showed no change. Along with FAK and Chk2, the largest set of down-regulated proteins in the T47D-EveR line in the phospho-kinase array was the Src family kinases, which all showed very large (2.5-3.5 relative expression) changes in their phosphorylation; this includes Src, Lyn, Lck, Fyn, Yes, Fgr and Hck.

The 361-EveR data for the phospho kinase array, with the data displayed in figures 5.16, 5.18 and 5.20, showed less down regulation in phospho-targets, with a more even spread of down and up-regulated targets. However, compared to the T47D array, the changes were far less dramatic, with most alterations having a relative expression change of less than 1.5 (unlike the down-regulations of over 1.5-2 relative expression in the T47D-EveR cells). Some exceptions can be seen however, with PRAS40, ERK and JNK all having altered relative expression of over 1.5.

Some interesting similarities can be seen when comparing the two sets of phospho kinase data. Both EveR lines showed down-regulation of key phospho-proteins in MAPK pathways including ERK1/2, JNK1/2/3 and MSK1/2, suggesting the EveR lines have a decreased reliance on MAPK pathways. Both EveR cell lines also showed an up-regulation of β-catenin (total protein) and a down-regulation of GSK3α/β phosphorylation.

The oncology array provided a more uniform picture for both EveR cell lines, with an almost universal down-regulation of the majority of proteins studied, as shown in figures 5.21-5.30. In both EveR cell lines, the majority of proteins tested in this array showed a decrease in relative expression by around 1.2-1.8. Both EveR lines also only showed 3 up-regulated proteins each, of the dozens investigated, with the up-regulation being of a very small relative change and with little conformity between the two EveR lines. These were BCL-x, CapG and ErbB4/HER-4 in T47D-EveR cells and EGFR/HER-1, CCL20 and p53 in 361-EveR cell. Of note is the down-regulation of amphiregulin, which whilst down-regulated in the T47D-EveR relatively by a factor

of around 1.6, it was down-regulated nearly 8 times in the 361-EveR cells, compared to their parental counter parts.

Protein	Phosphorylation site
p38α	T180/Y182
ERK1/2	T202/Y204/T185/Y187
JNK1/2/3	T183/Y185/T221/Y223
GSK-3α/β	S21/S9
p53	S392
EGFR	Y1086
MSK1/2	S376/S360
AMPKα1	T183
AKT1/2/3	S473
AKT1/2/3	T308
p53	S46
mTOR	S2448
CREB	S133
HSP27	S78/S82
AMPKα2	T172
β-Catenin	Total protein
p70S6K	T389
p53	S15
c-Jun	S63
Src	Y149
Lyn	Y397
Lck	Y394
STAT2	Y689
STAT5a	Y694
p70S6K	T421/S424
RSK1/2/3	S380/S386/S377
eNOS	S1177
Fyn	Y420
Yes	Y426
Fgr	Y412
STAT6	Y641
STAT5B	Y699
STAT3	Y705
p27	T198
PLC-γ1	Y793
Hck	Y411
Chk2	T68
FAK	Y397
PDGFRβ	Y751
STAT5a/b	Y694/Y699
STAT3	Y727
WNK1	T60
PYK2	Y402
PRAS40	T246
HSP60	Total protein

Table 5.2: Table of phosphorylation sites tested for on each protein in the phospho -kinase array. β-catenin and HSP60 were tested for total protein and not the phosphorylated form.

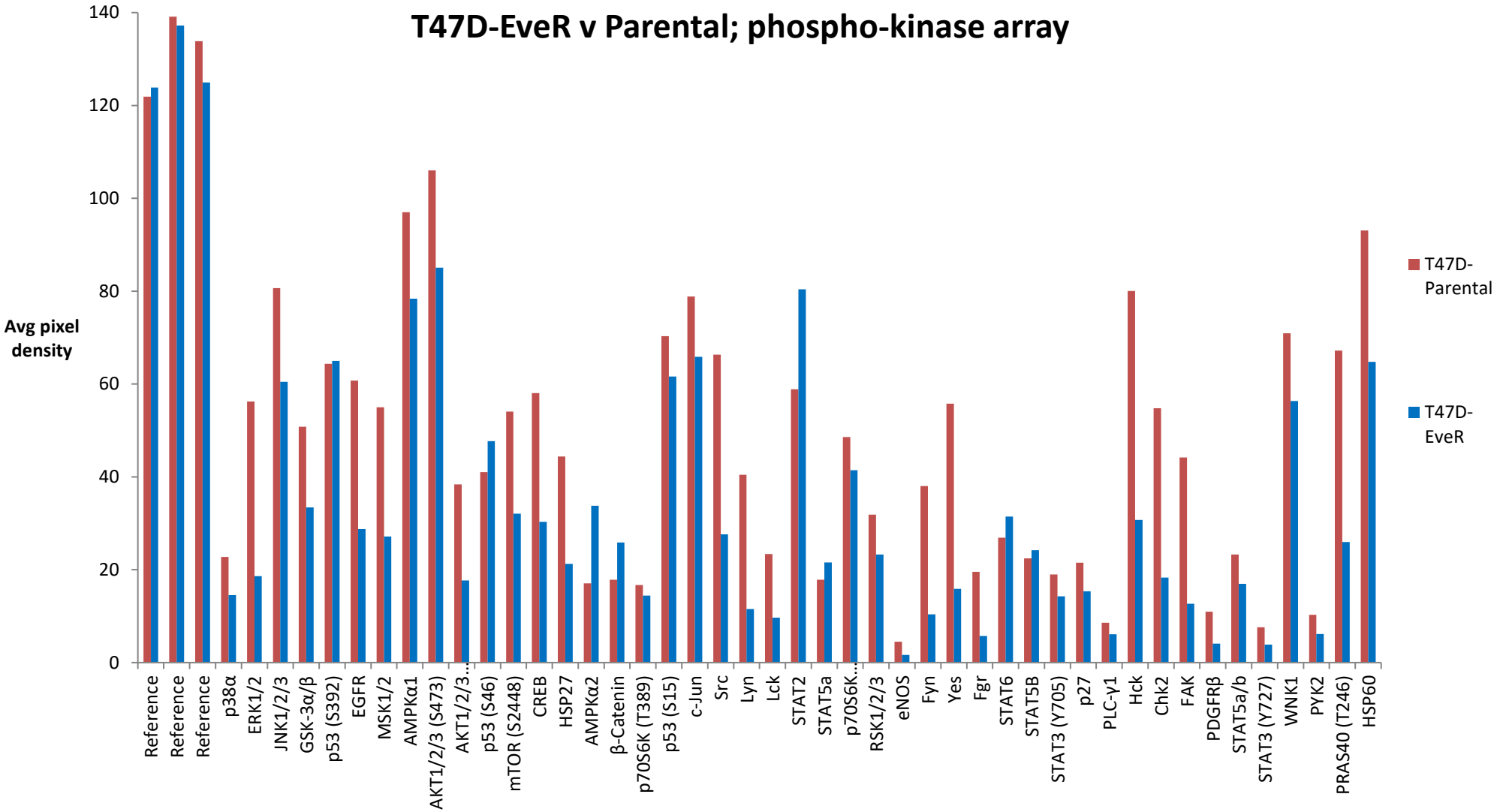


Figure 5.15: Phospho-kinase proteome array data for T47D-EveR v parental cells. *Average pixel density shown for each phosphorylated target.*

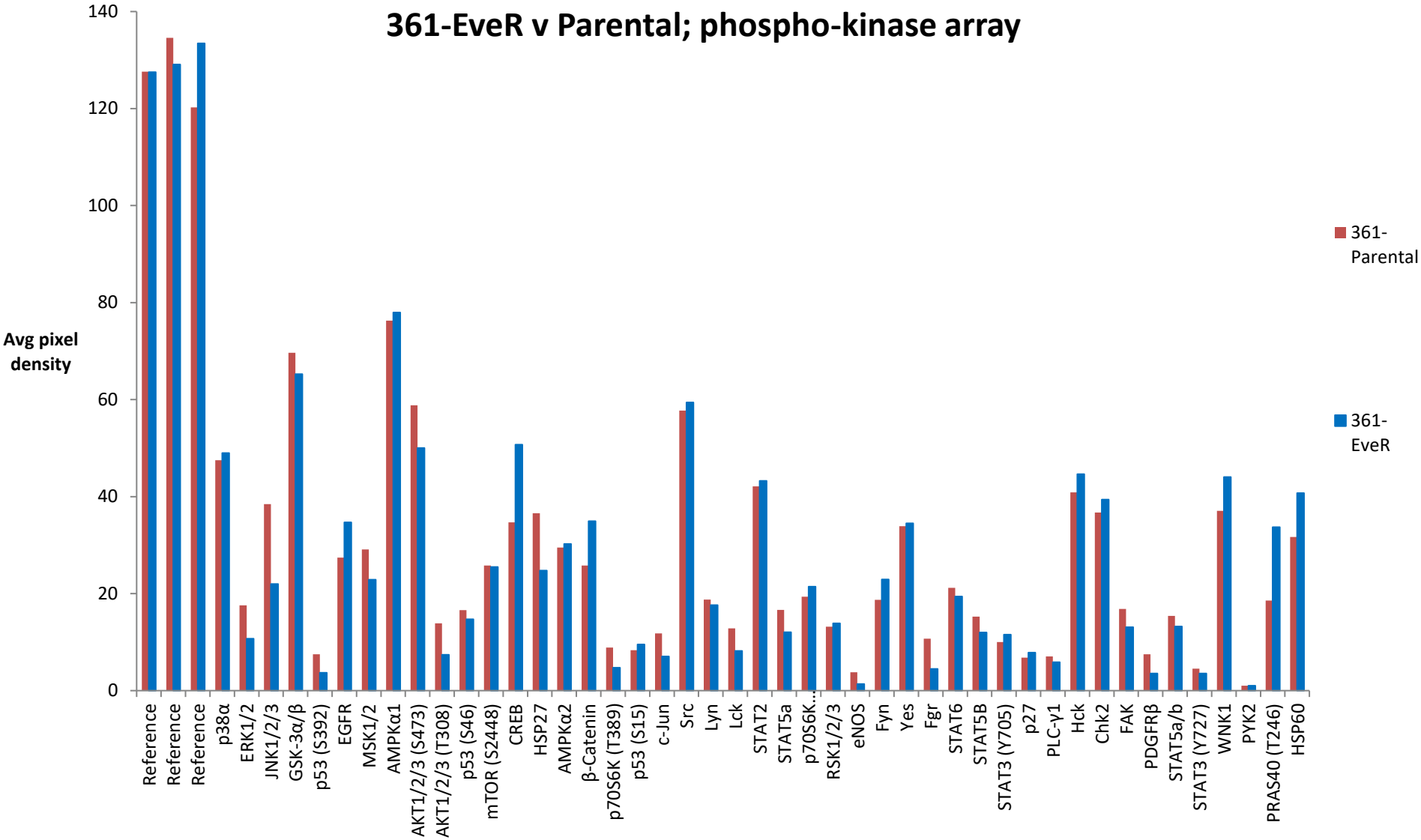


Figure 5.16: Phospho-kinase proteome array data for 361-EveR v parental cells. *Average pixel density shown for each phosphorylated target.*

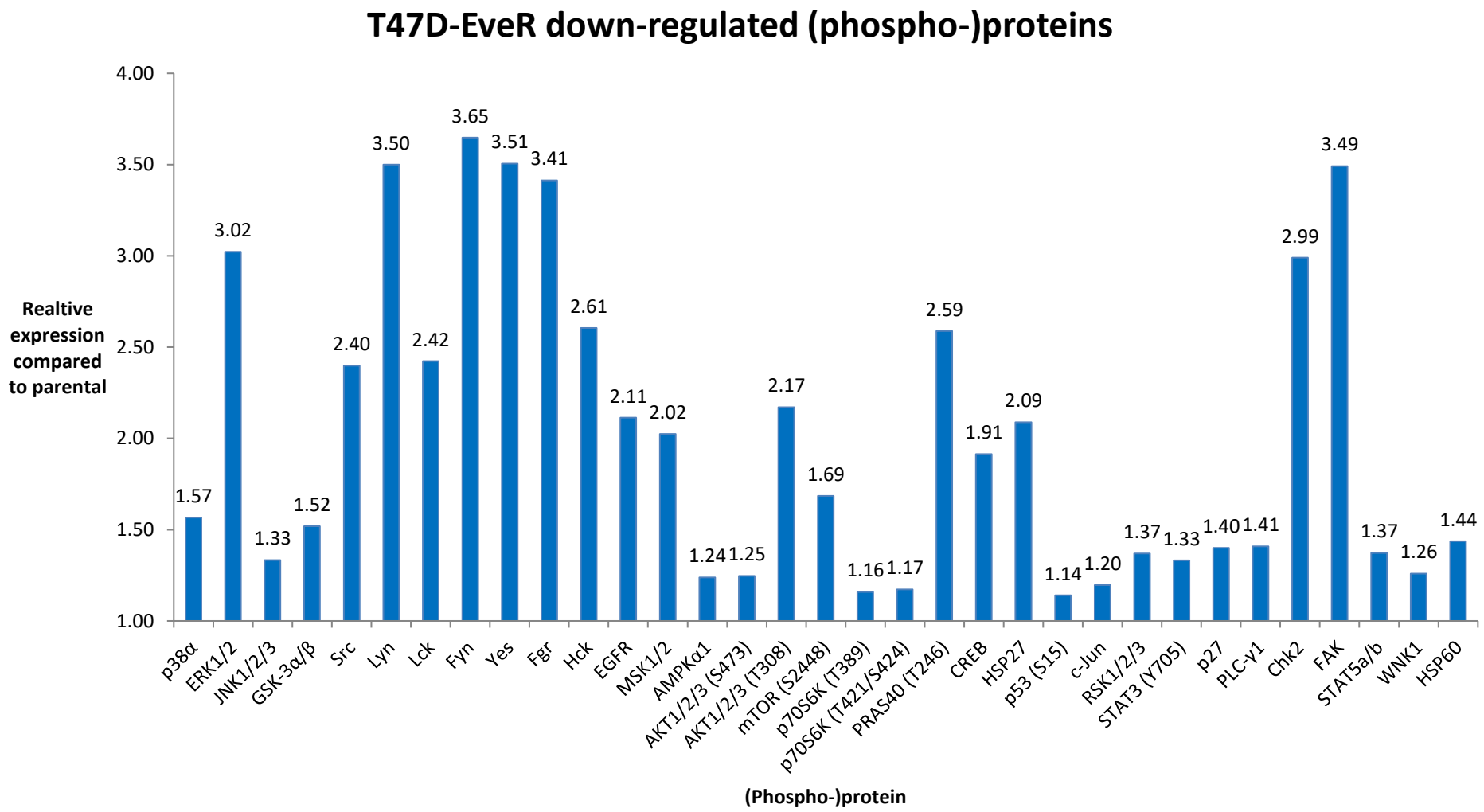


Figure 5.17: T47D-EveR show a host of down-regulated phospho proteins. *Phospho-kinase proteome array data for T47D-EveR v parental cells, showing relative expression in the EveR cells of any down-regulated proteins, compared to the parental line. Relative expression of 1.0 = even expression of protein in EveR and parental lines.*

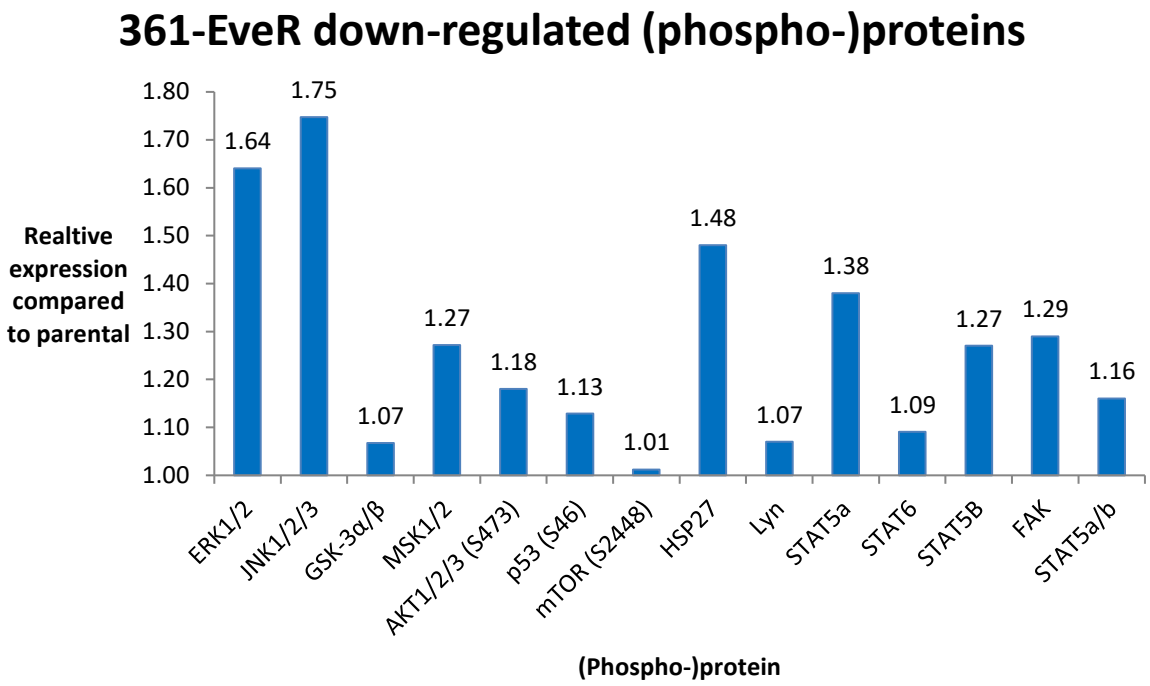


Figure 5.18: 361-EveR show a set of down-regulated phospho-proteins. *Phospho-kinase proteome array data for 361-EveR v parental cells, showing relative expression in the EveR cells of any down-regulated proteins, compared to the parental line. Relative expression of 1.0 = even expression of protein in EveR and parental lines.*

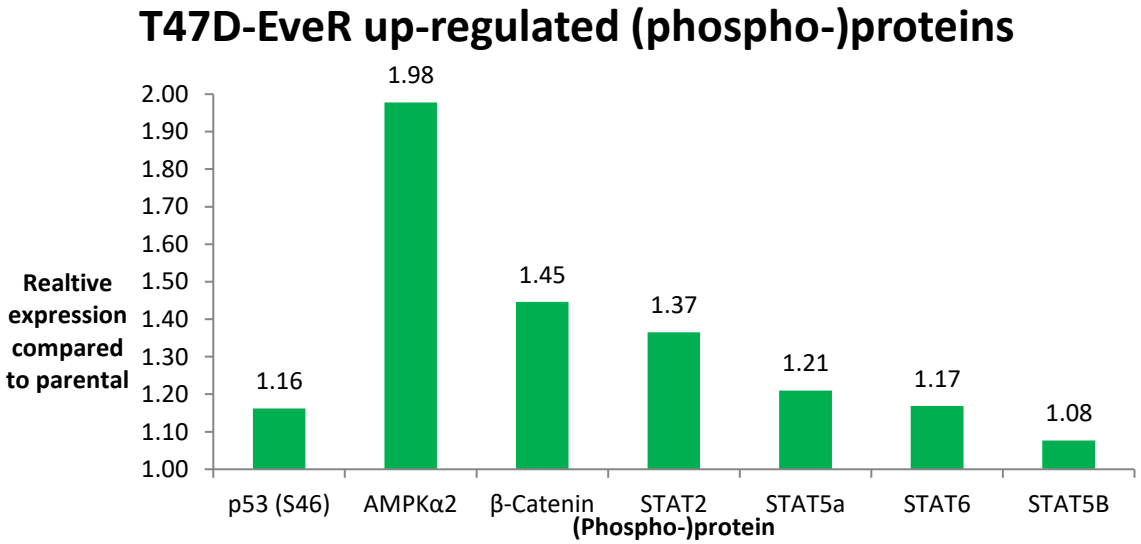


Figure 5.19: T47D-EveR show a small set of marginally up-regulated phospho-proteins. *Phospho-kinase proteome array data for T47D-EveR v parental cells, showing relative expression in the EveR cells of any up-regulated proteins, compared to the parental line. Relative expression of 1.0 = even expression of protein in EveR and parental lines.*

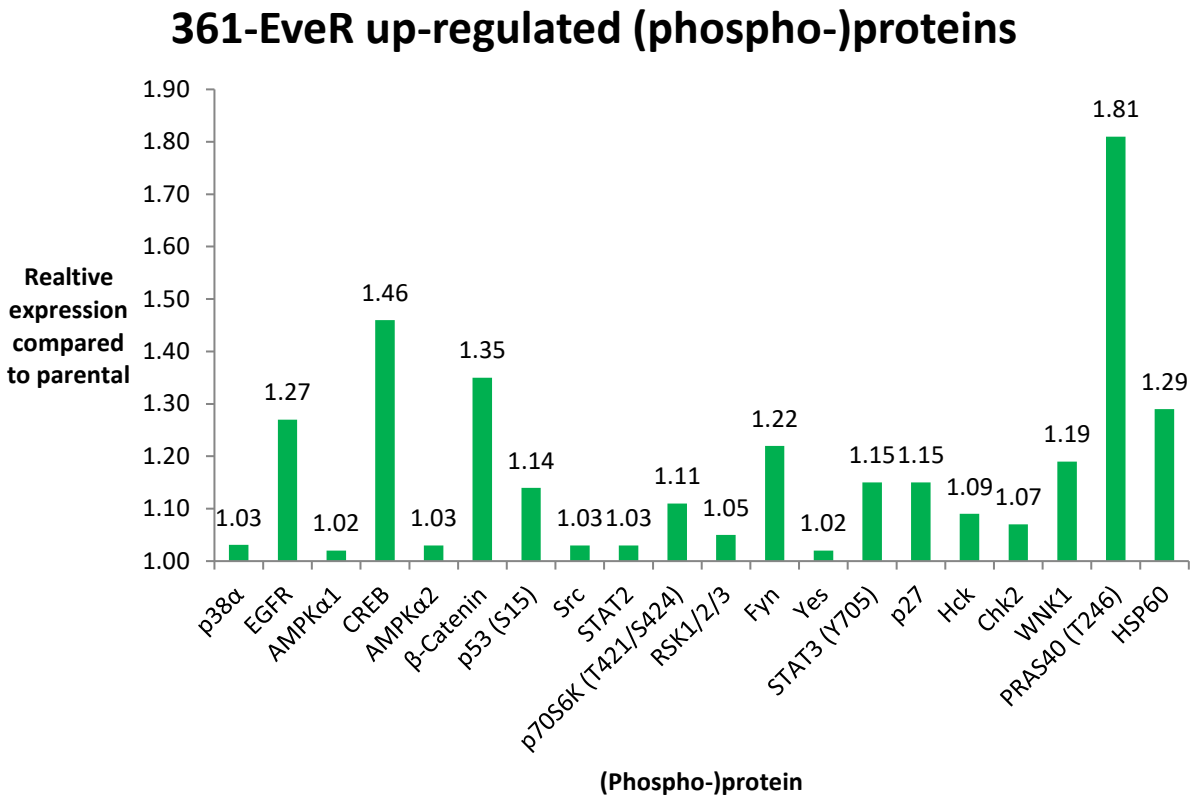


Figure 5.20: 361-EveR show a set of up-regulated phospho-proteins. *Phospho-kinase proteome array data for 361-EveR v parental cells, showing relative expression in the EveR cells of any up-regulated proteins, compared to the parental line. Relative expression of 1.0 = even expression of protein in EveR and parental lines.*

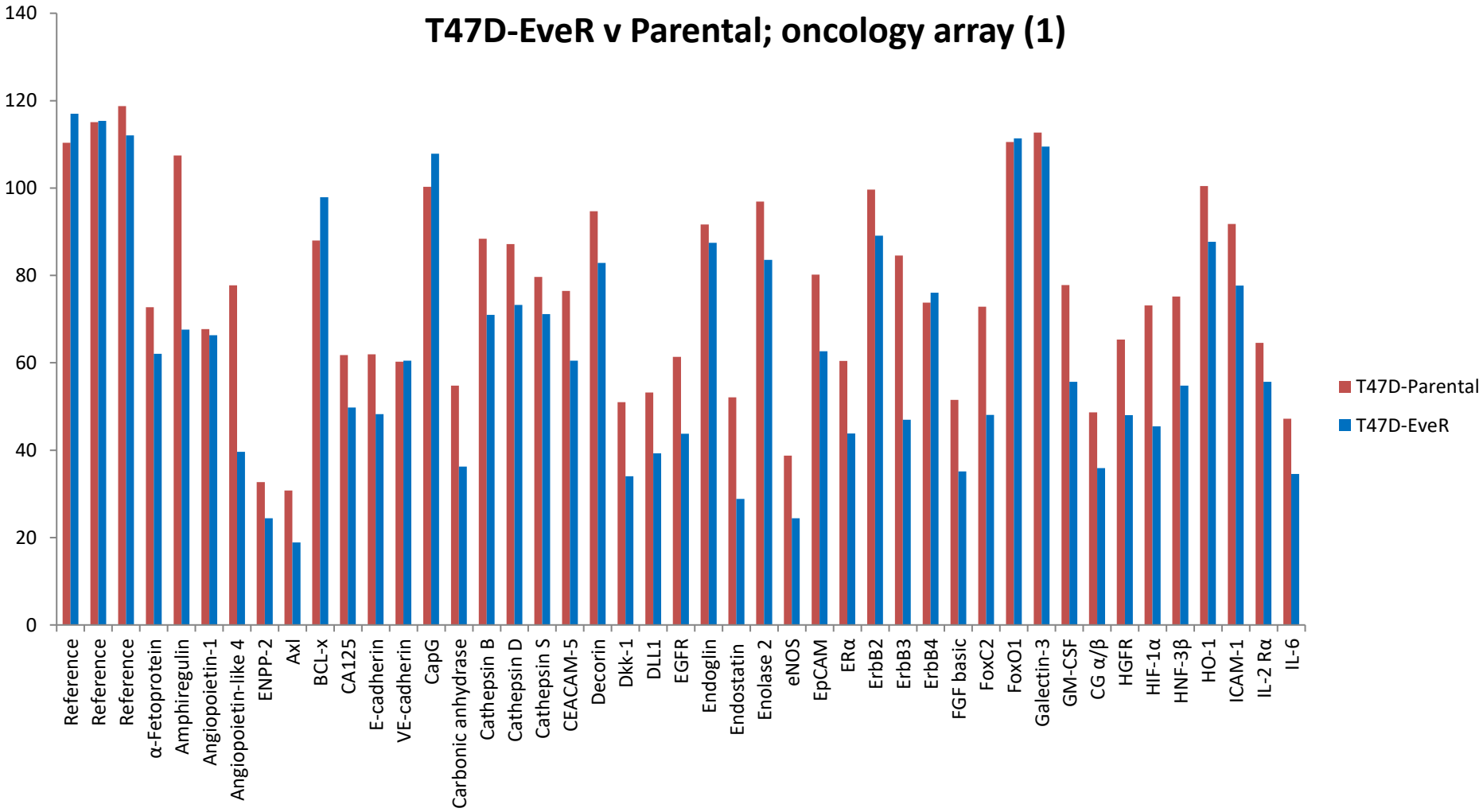


Figure 5.21: Oncology proteome array data (part 1) for T47D-EveR v parental cells*. Average pixel density shown for each phosphorylated target.*

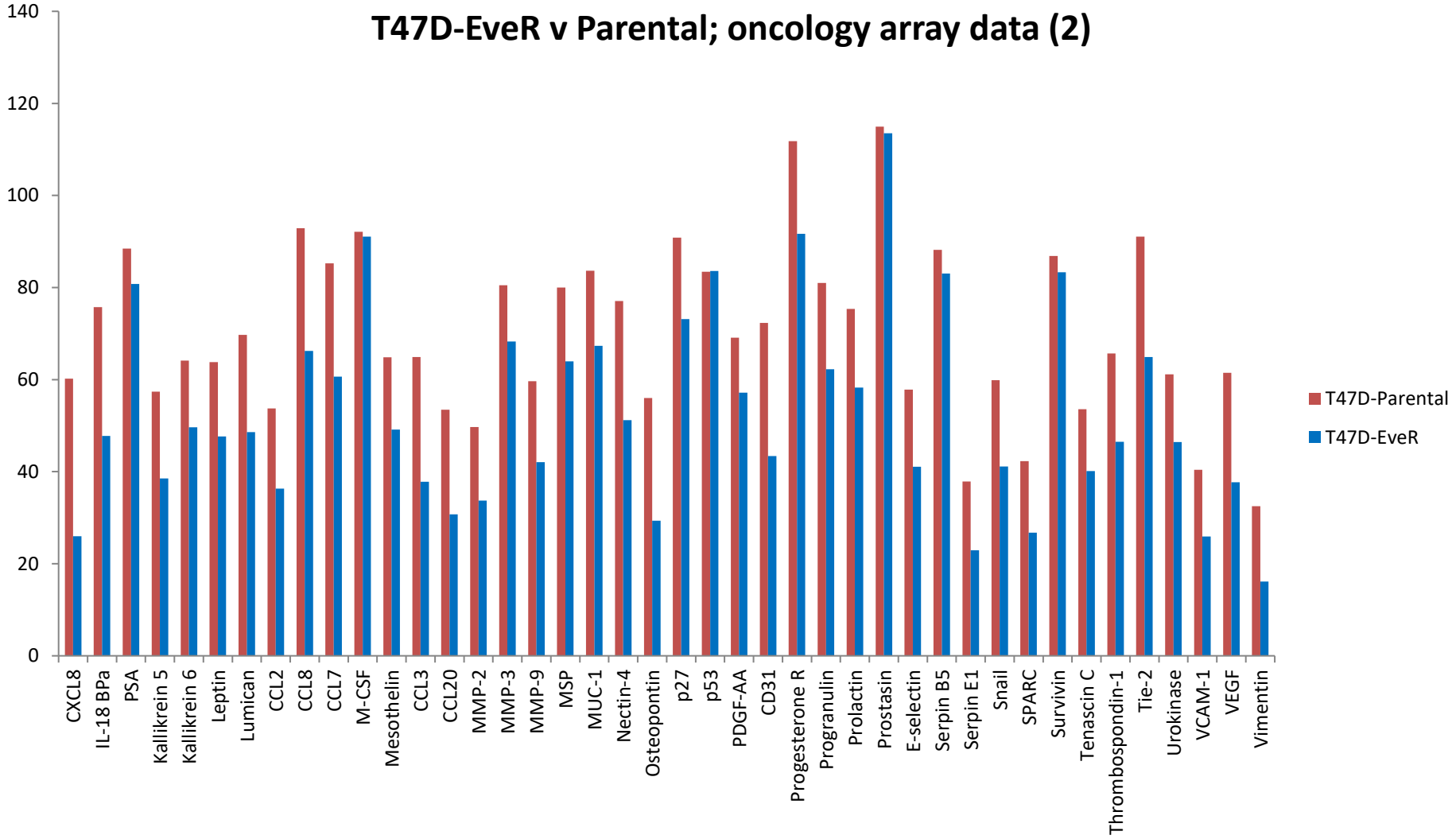


Figure 5.22: Oncology proteome array data (part 2) for T47D-EveR v parental cells. *Average pixel density shown for each phosphorylated target.*

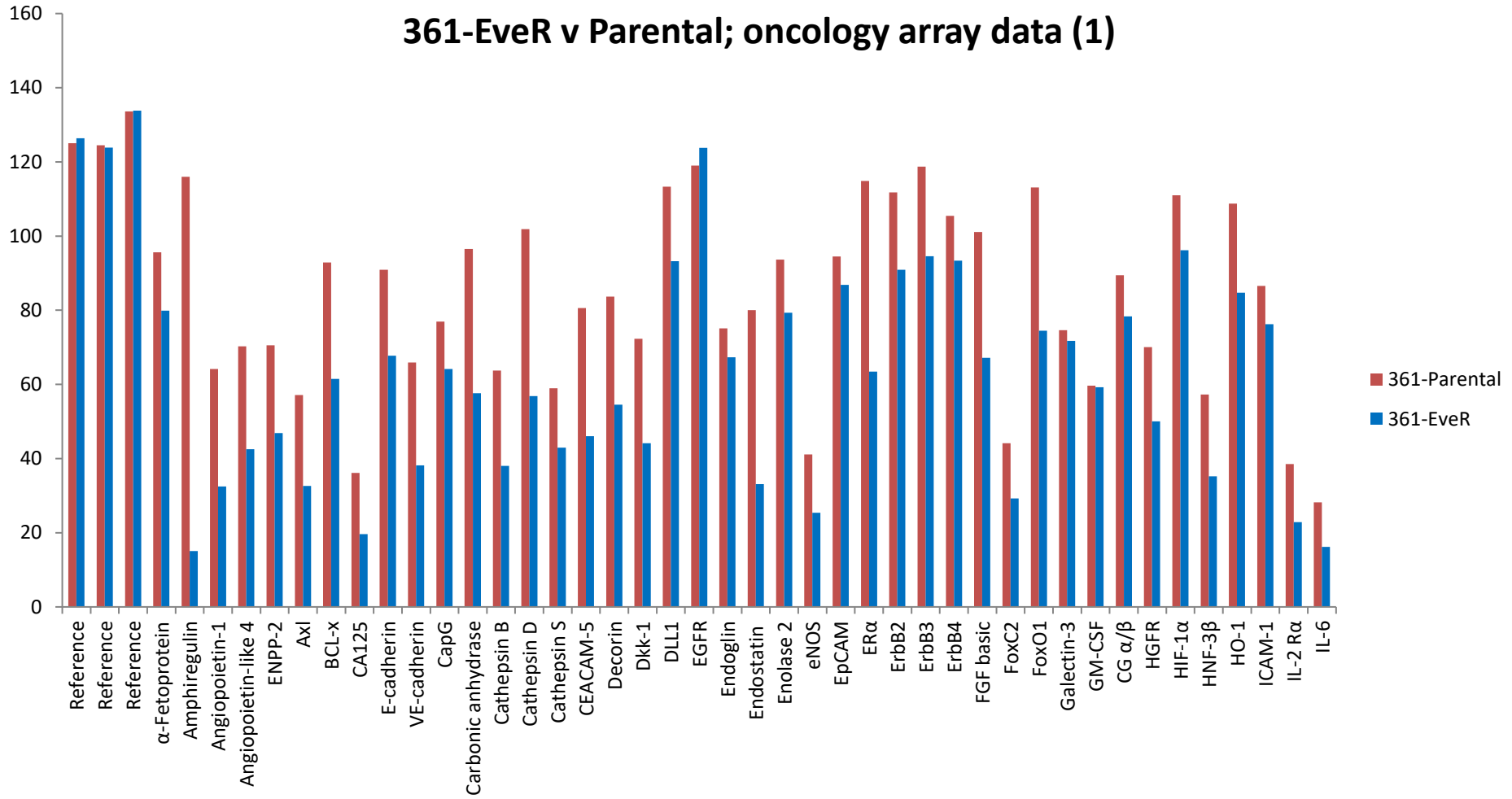


Figure 5.23: Oncology proteome array data (part 1) for 361-EveR v parental cells. *Average pixel density shown for each phosphorylated target.*

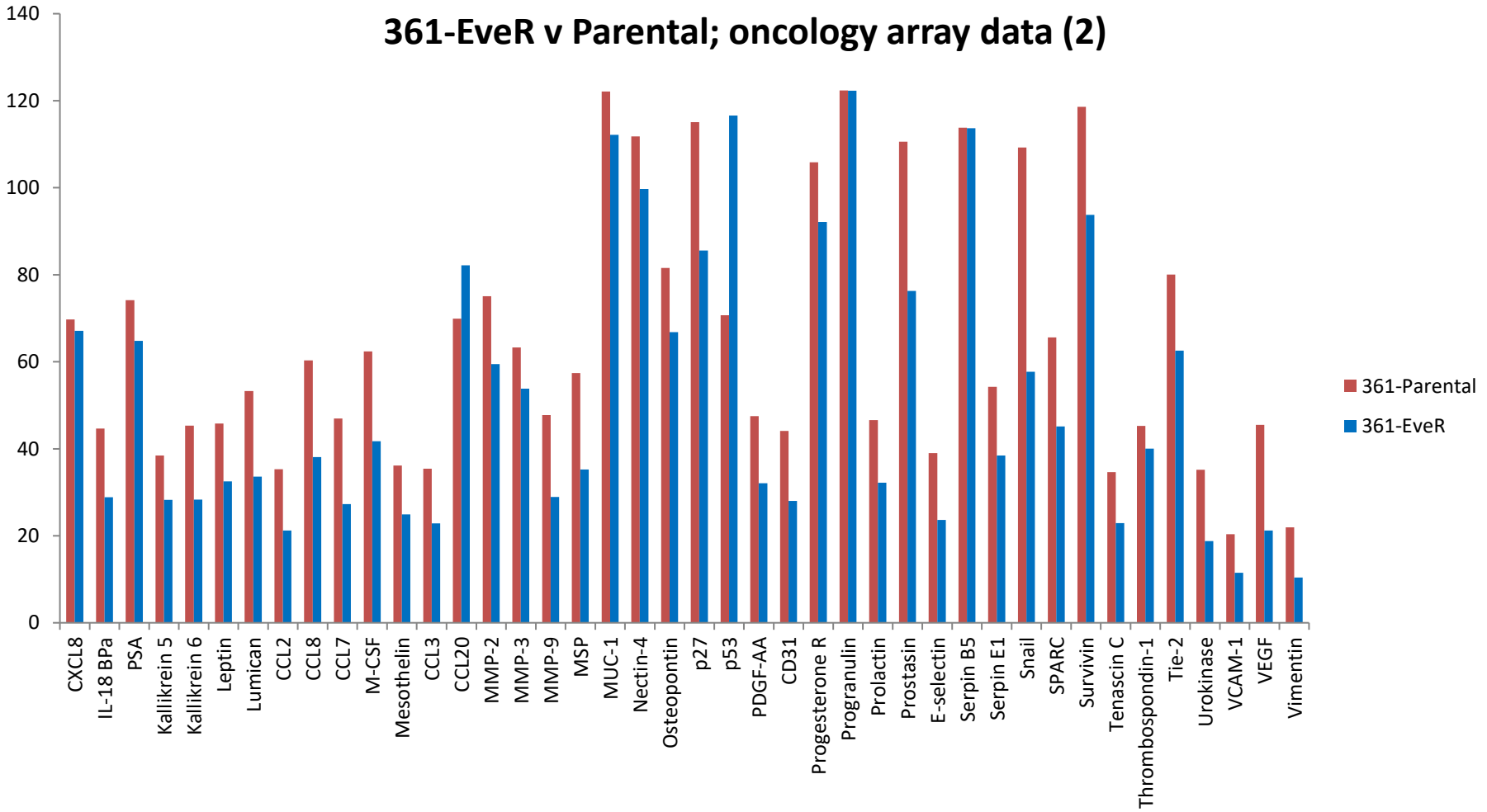


Figure 5.24: Oncology proteome array data (part 2) for 361-EveR v parental cells. *Average pixel density shown for each phosphorylated target.*

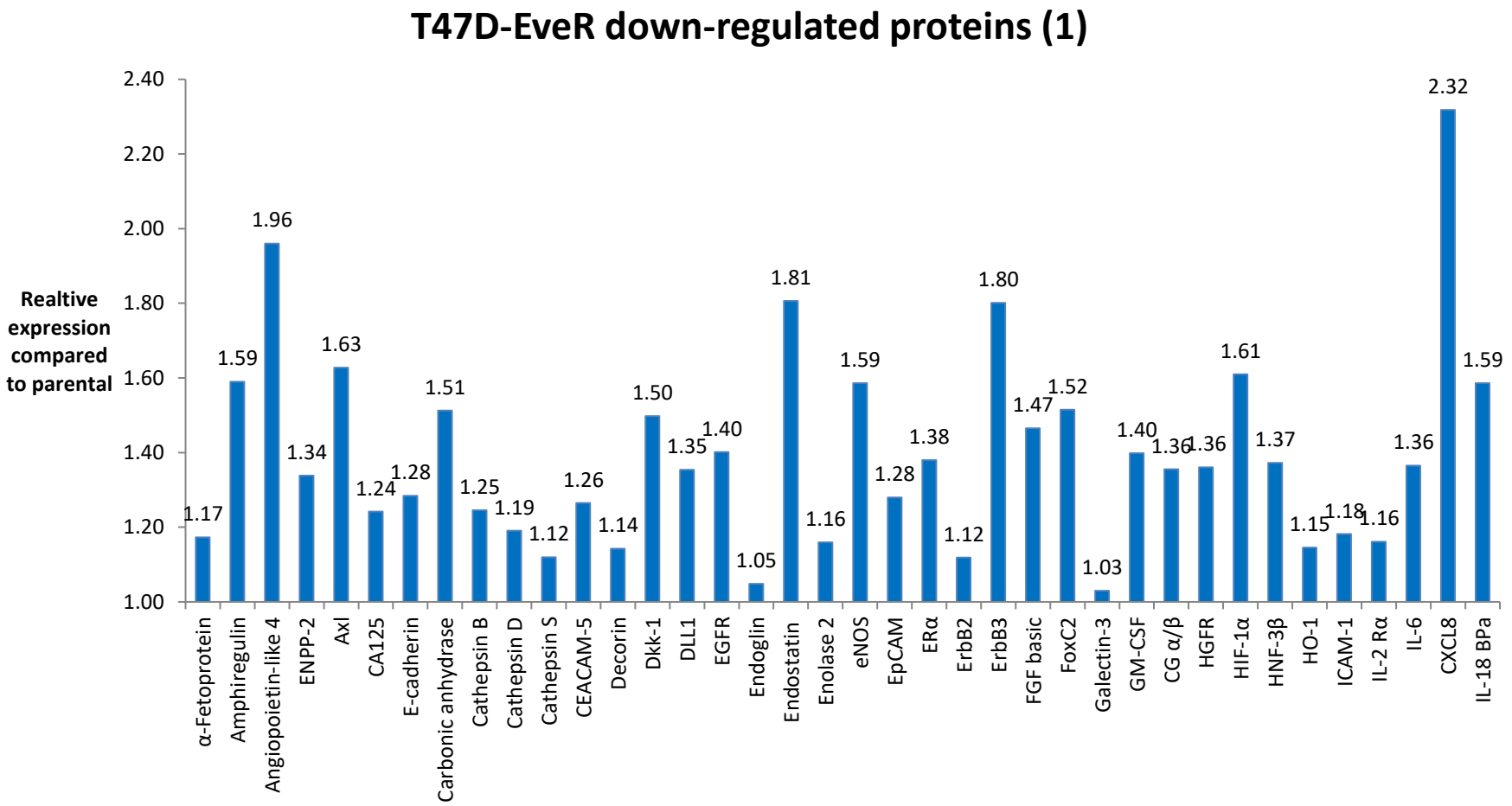


Figure 5.25: T47D-EveR show a wide range of down-regulated proteins. *Oncology proteome array data (part 1) for T47D-EveR v parental cells, showing relative expression in the EveR cells of any down-regulated proteins, compared to the parental line. Relative expression of 1.0 = even expression of protein in EveR and parental lines.*

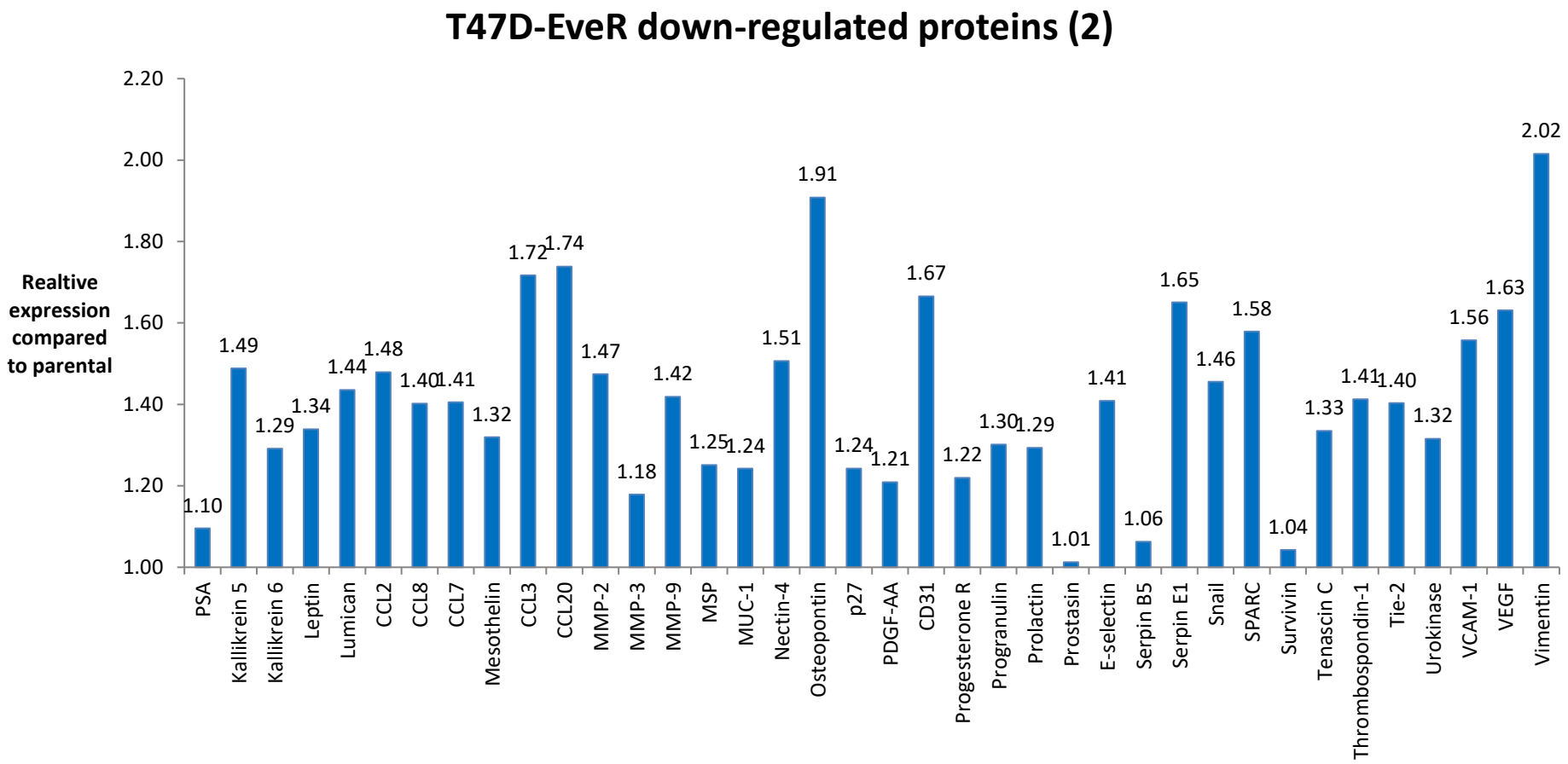


Figure 5.26: T47D-EveR show a wide range of down-regulated proteins. *Oncology proteome array data (part 2) for T47D-EveR v parental cells, showing relative expression in the EveR cells of any down-regulated proteins, compared to the parental line. Relative expression of 1.0 = even expression of protein in EveR and parental lines.*

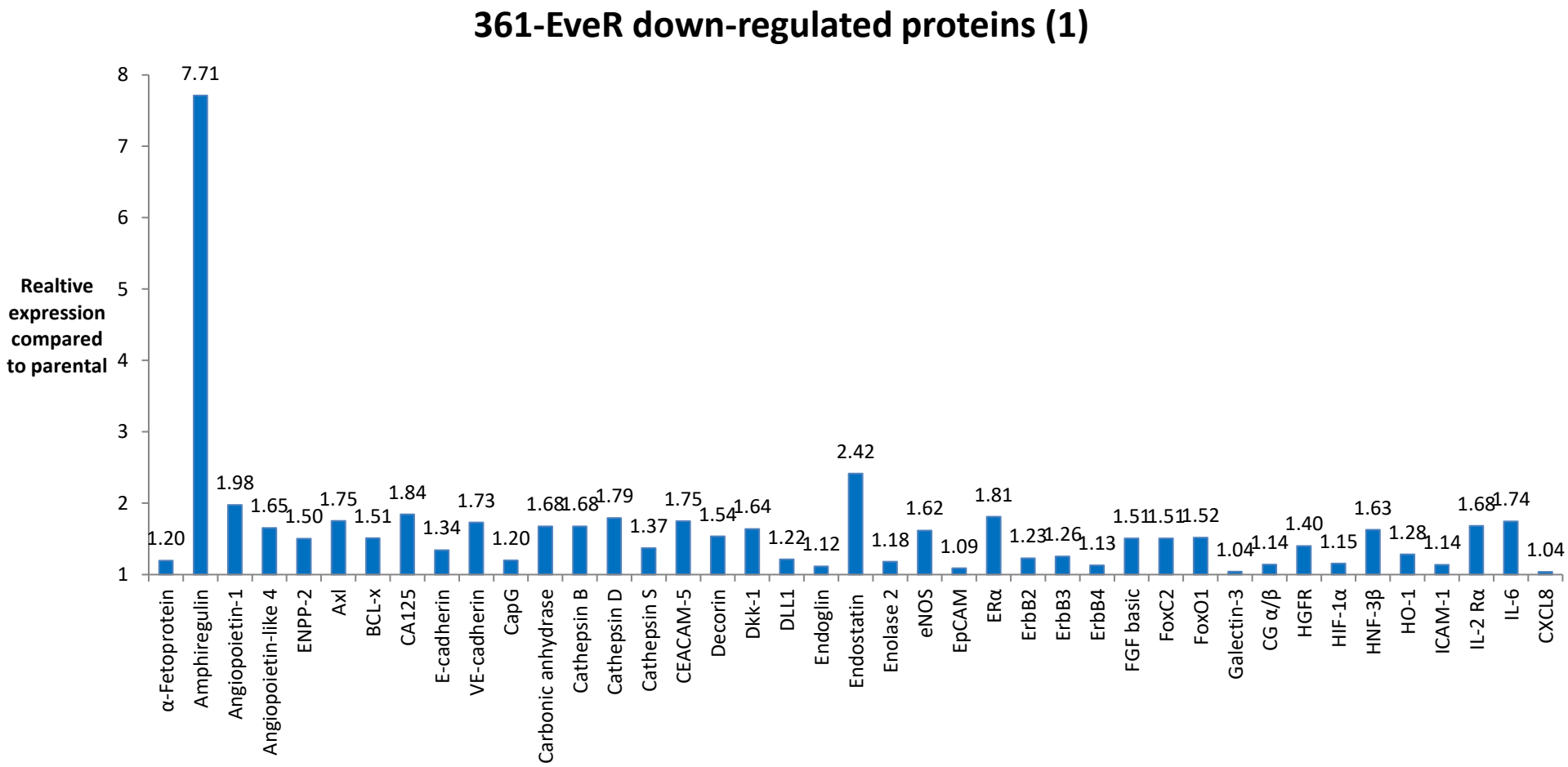


Figure 5.27: 361-EveR show a wide range of down-regulated proteins. *Oncology proteome array data (part 1) for 361-EveR v parental cells, showing relative expression in the EveR cells of any down-regulated proteins, compared to the parental line. Relative expression of 1.0 = even expression of protein in EveR and parental lines.*

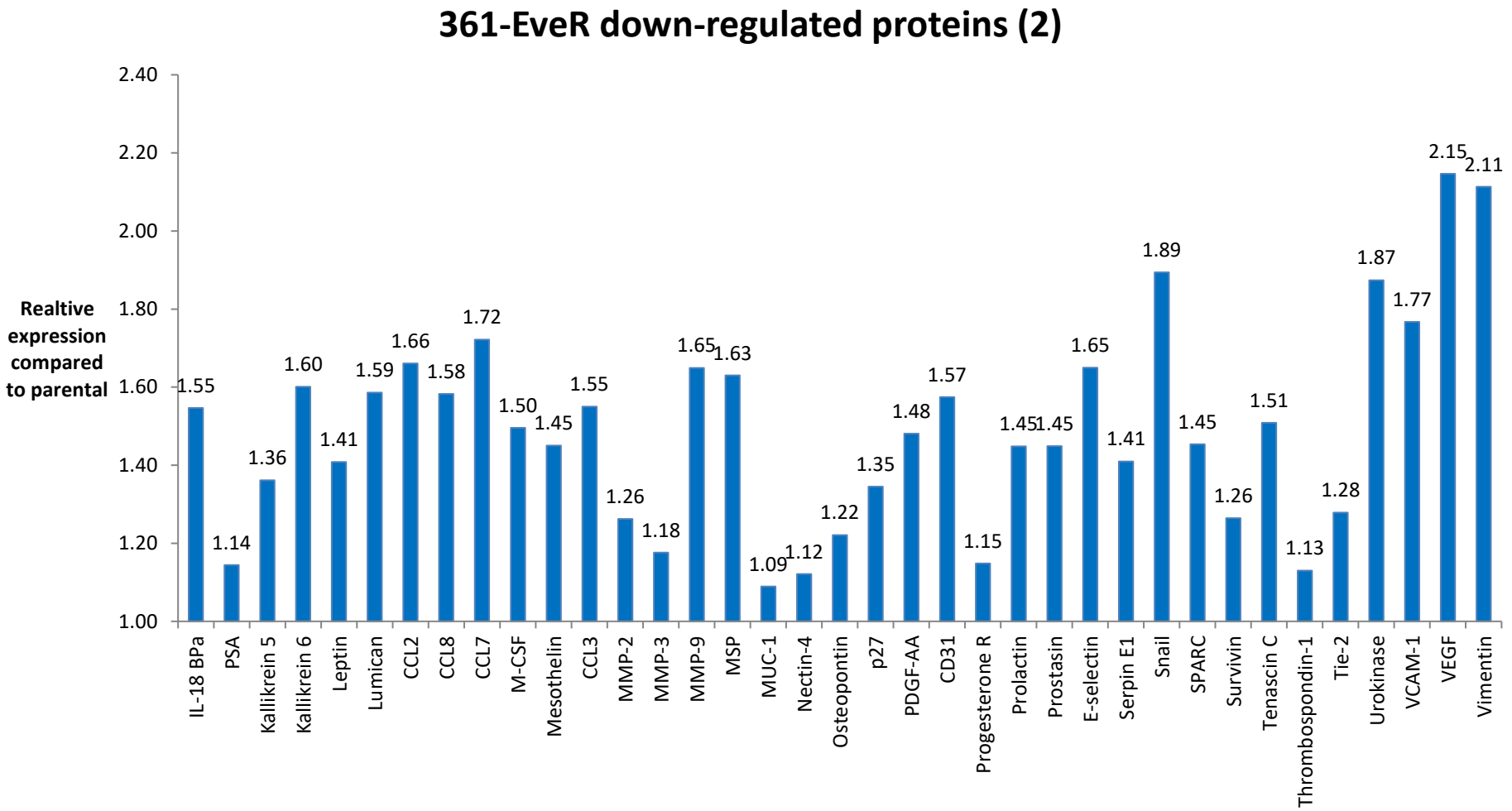


Figure 5.28: 361-EveR show a wide range of down-regulated proteins. *Oncology proteome array data (part 2) for 361-EveR v parental cells, showing relative expression in the EveR cells of any down-regulated proteins, compared to the parental line. Relative expression of 1.0 = even expression of protein in EveR and parental lines.*

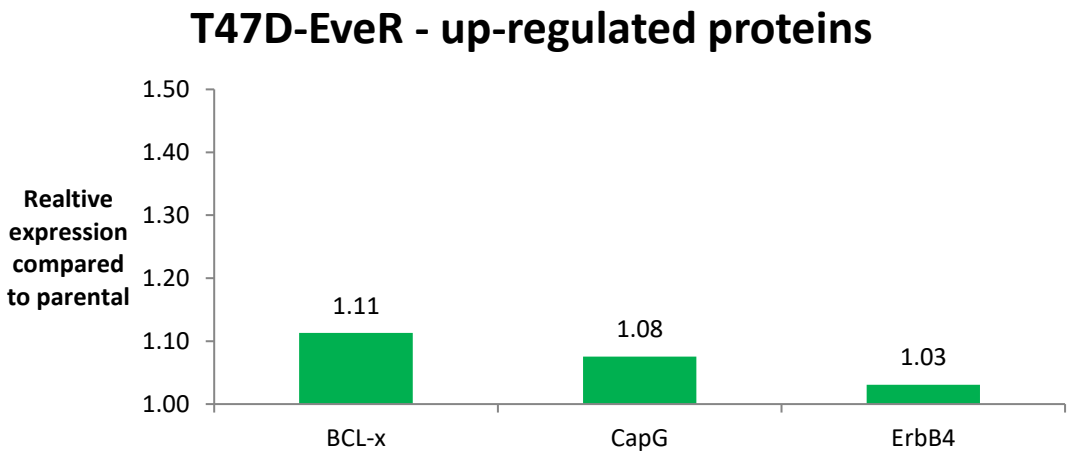


Figure 5.29: T47D-EveR show very few marginally up-regulated proteins. *Oncology proteome array data for T47D-EveR v parental cells, showing relative expression in the EveR cells of any up-regulated proteins, compared to the parental line. Relative expression of 1.0 = even expression of protein in EveR and parental lines.*

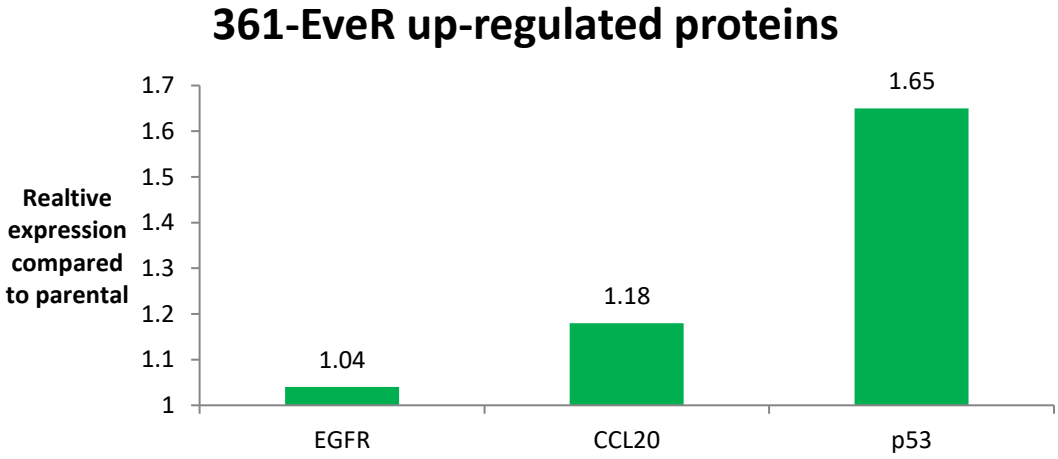


Figure 5.30: 361-EveR show very few marginally up-regulated proteins. *Oncology proteome array data for 361-EveR v parental cells, showing relative expression in the EveR cells of any up-regulated proteins, compared to the parental line. Relative expression of 1.0 = even expression of protein in EveR and parental lines.*

5.3.8 - Everolimus and Canonical Wnt Inhibition as a Combination Therapy to Overcome Everolimus Resistance

As mentioned in section 5.3.7, both EveR cell lines showed an up-regulation of β-catenin and a downregulation of GSK-3β; suggesting a possible increase in canonical Wnt signalling. Wnt signalling is an important pathway in growth and development, having a key role in stem cell maintenance, and it's up-regulation in sub-populations of breast cancer cells is linked to an increased stem cell-like phenotype (Clevers and Nusse, 2012; Lamb et al, 2013).

To test whether this may relate to the resistance phenotype, the inhibitor XAV-939 was selected to carry out further drug treatments. This is an indirect inhibitor of Wnt signalling, via tankyrase inhibition, which causes stabilisation of axin; this ultimately leads to degradation of β-catenin and thus pathway inhibition (Afifi et al, 2014). The combination of everolimus and XAV-939 was also tested to see if this could overcome the resistant phenotype.

Figure 5.31 shows how the cells responded to the addition of just XAV-939. Cells generally showed a very weak response to XAV-939, with very little growth inhibition occurring across the board. The T47D's showed small responses at 72hrs, but not 1 week, with the only noticeable difference between EveR and parental being at 10,000nM where the T47D-Parental responded by a greater margin than the EveR.

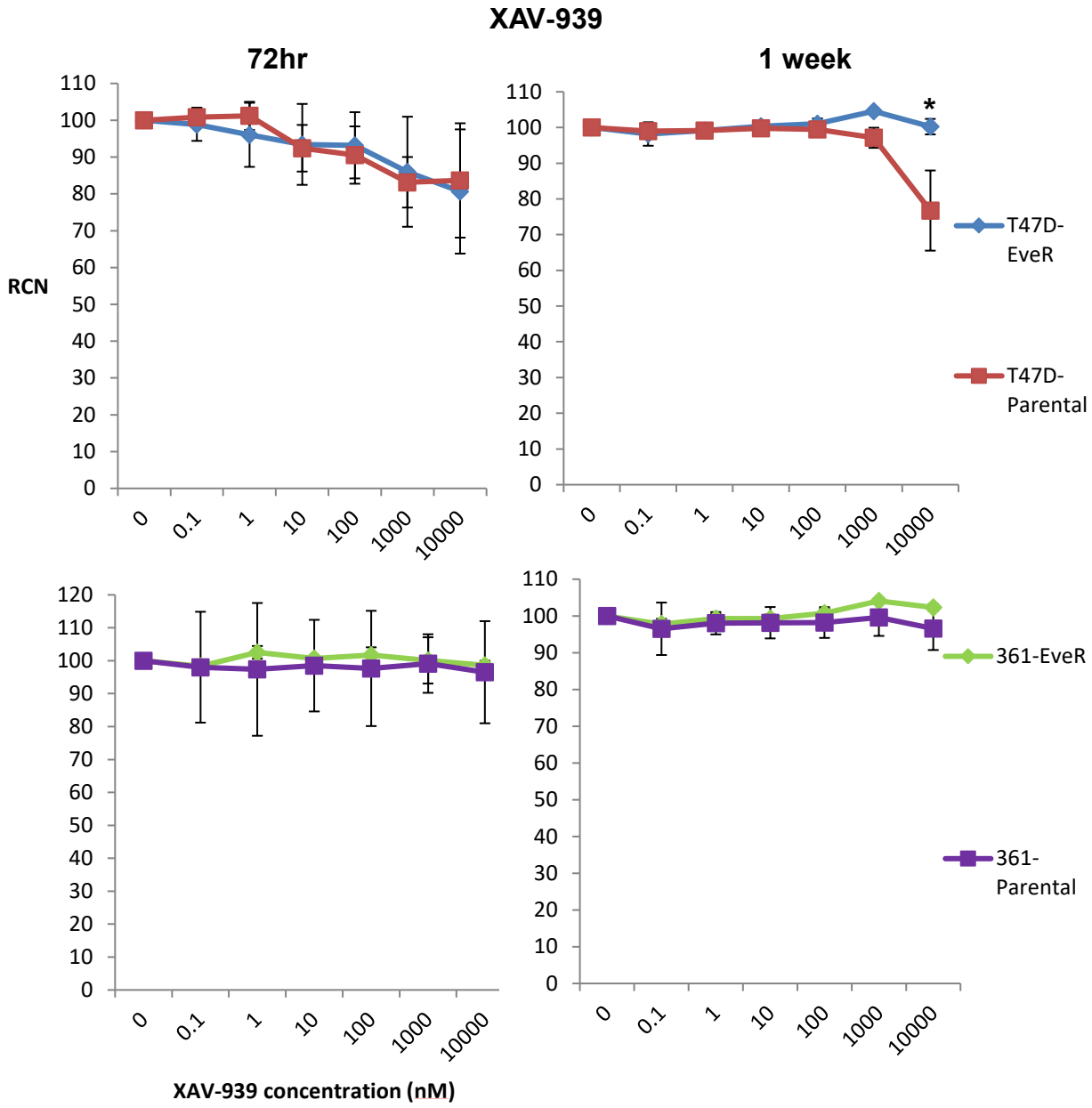


Figure 5.31: XAV-939 shows very little growth inhibition as a single therapy. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant lines (EveR) v parental, treated with XAV-939 (an indirect canonical Wnt inhibitor) for either 72hr (left) or 1 week (right). * P<0.05 compared to control.*

The combination of everolimus and XAV-9393 was then tested, with this data shown in figures 5.32-5.35. Across the board there was very little extra response with the addition of XAV-939 to everolimus. Both parental lines showed virtually no difference in terms of responsiveness, with the addition of XAV-939. The addition of two different concentrations of XAV-939 were tested with the lower addition of 10nM having no added effect on everolimus responsiveness, in the EveR lines.

The addition of the higher 10,000nM had no real added effect when tested after 72hrs but did appear to have some very modest added effects at the 1 week time point. With both EveR lines, these experiments showed decreased cell growth averages compared to the everolimus on its own, although neither came close to truly ablating resistance. Despite these partial decreases in cell growth average, for this combination (especially in the T47D-EveR), values for these differences in both cell lines were not significant. This was mostly due to a great variation in response, with some repeats showing little to no extra response, whilst some showed a modest extra response.

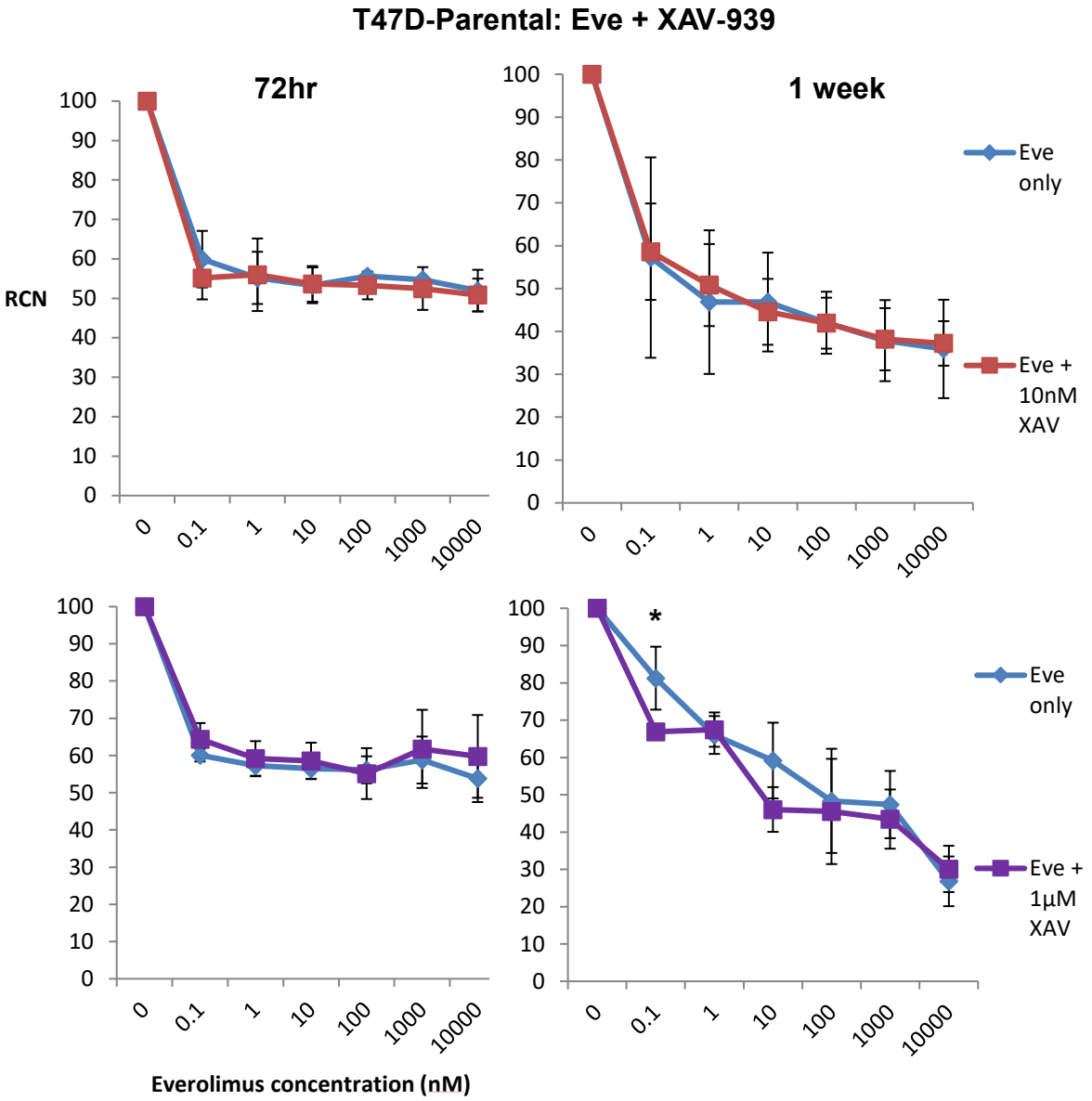


Figure 5.32: Everolimus+XAV-939 fails to improve growth inhibition v everolimus only, in T47D-Parental cells*. SRB result (mean plus or minus standard deviation, n=3 independent experiments) for T47D-Parental treated for 72 hours or 1 week, with either everolimus (eve) or everolimus + 10nM or 1µM XAV-939 (eve + XAV). * P<0.05 compared to combination treatment. In this cell line, the addition of XAV-939 with everolimus appeared to have very little extra effect, over everolimus only.*

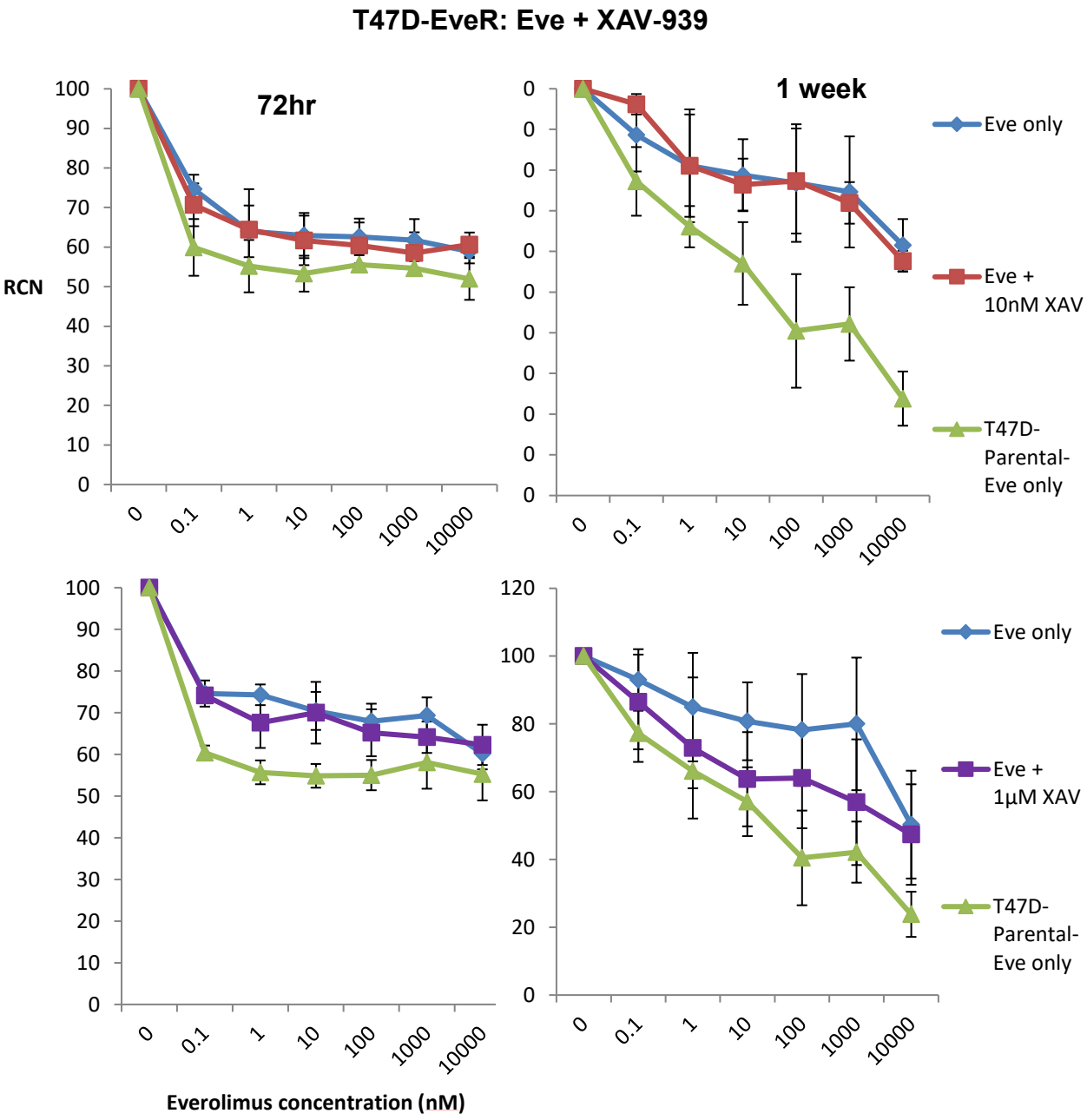


Figure 5.33: Everolimus+XAV-939 fails to significantly improve growth inhibition v everolimus only, in T47D-EveR cells*. SRB result (mean plus or minus standard deviation, n=3 independent experiments) for T47D-EveR treated for 72 hours or 1 week, with either everolimus (eve) or everolimus + 10nM or 1µM XAV-939 (eve + XAV). Whilst the addition of 1µM XAV-939 did decrease the average RCN at 1 week for the T47D-EveR's, this was not significant at any point, due to variation in the results.*

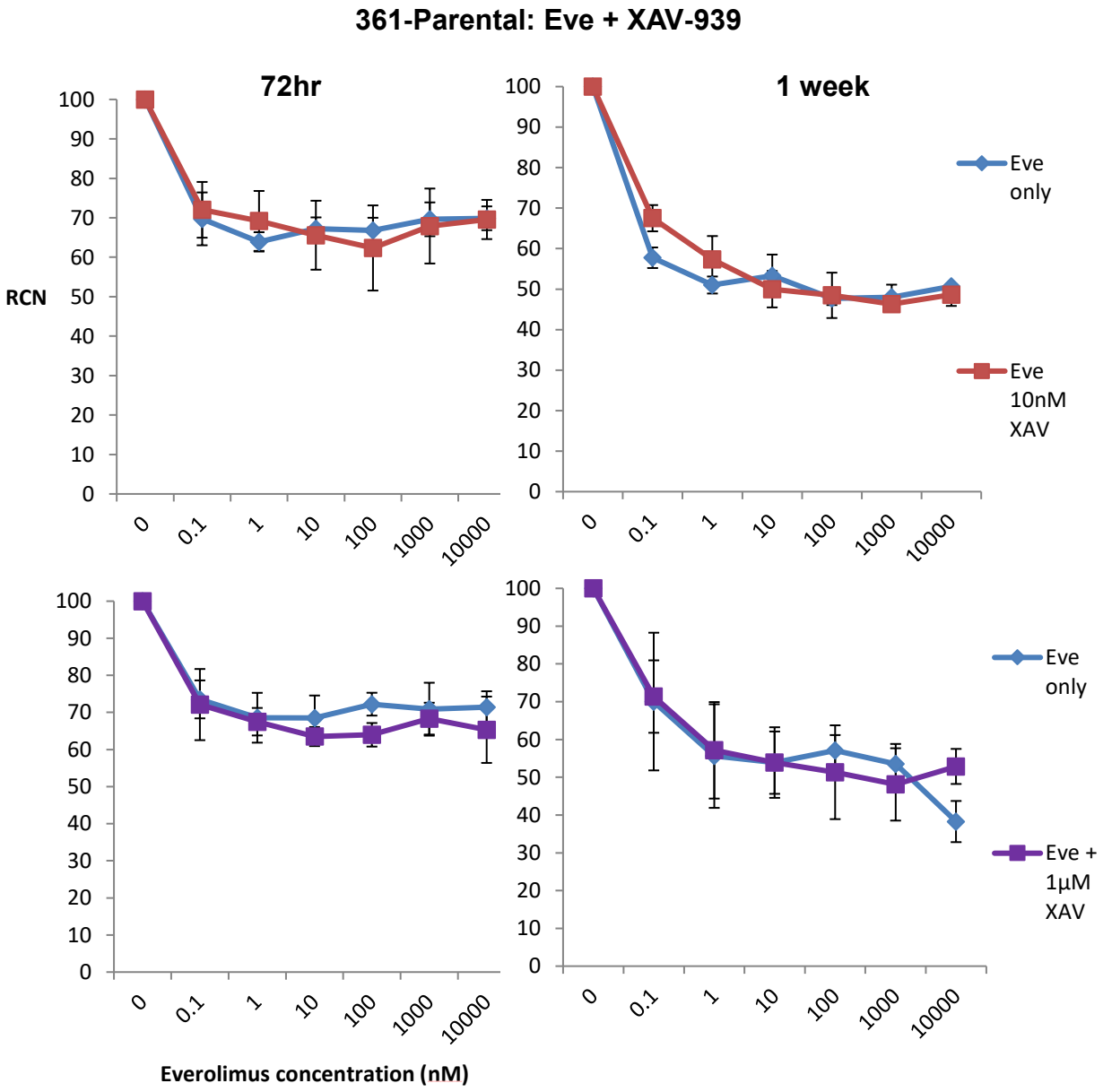


Figure 5.34: Everolimus+XAV-939 fails to improve growth inhibition v everolimus only, in 361-Parental cells. *SRB result (mean plus or minus standard deviation, n=3 independent experiments) for 361-Parental treated for 72 hours or 1 week, with either everolimus (eve) or everolimus + 10nM or 1µM XAV-939 (eve + XAV). In this cell line, the addition of XAV-939 with everolimus appeared to have very little extra effect, over everolimus only.*

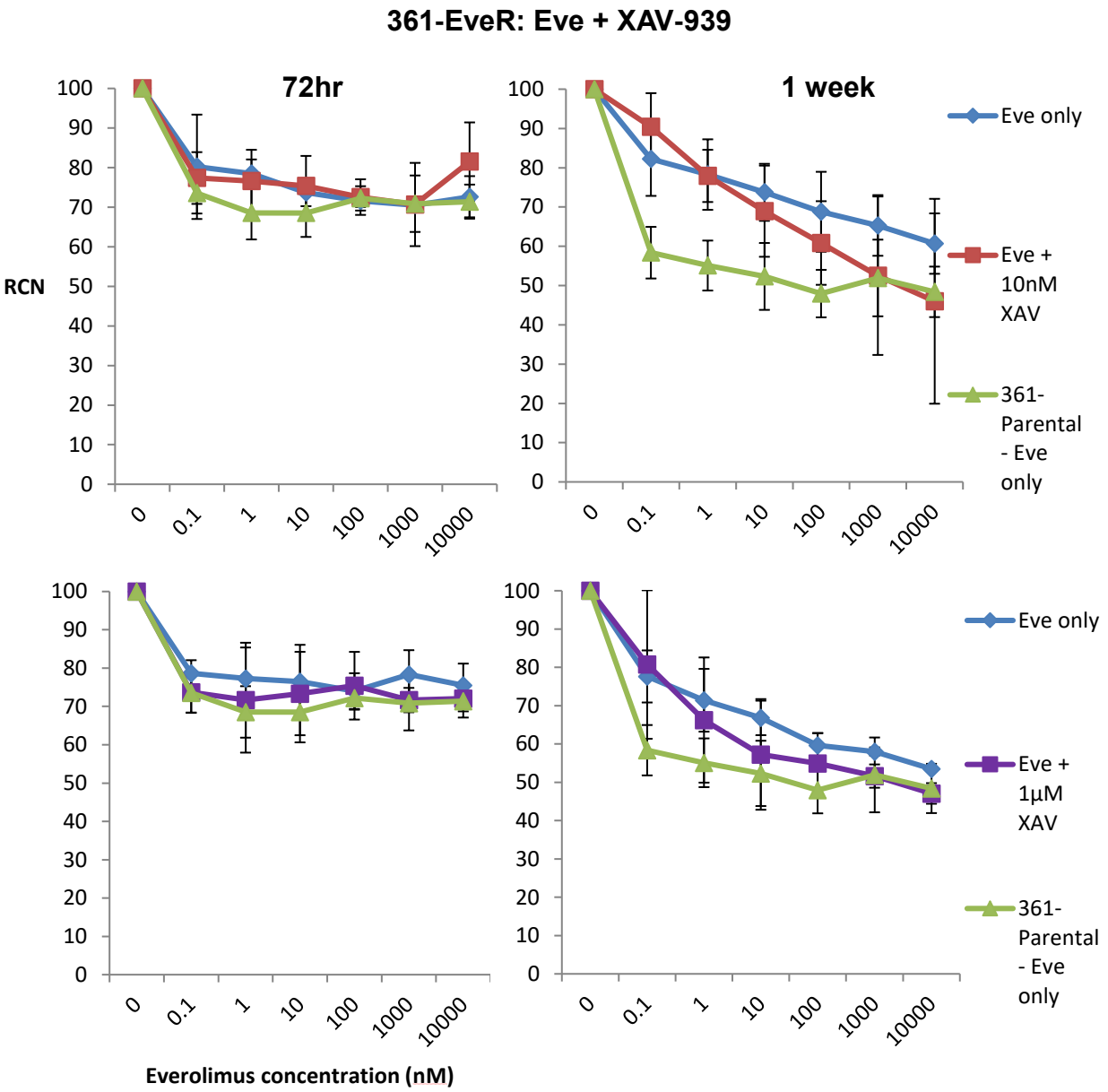


Figure 5.35: Everolimus+XAV-939 fails to significantly improve growth inhibition v everolimus only, in 361-EveR cells. *SRB result (mean plus or minus standard deviation, n=3 independent experiments) for 361-EveR treated for 72 hours or 1 week, with either everolimus (eve) or everolimus + 10nM or 1µM XAV-939 (eve + XAV). Whilst the addition of 1µM XAV-939 did decrease the average RCN slightly at 1 week, this was not significant at any point.*

5.4 - Discussion

The work presented in this chapter sought to characterise the everolimus resistant breast cancer cell lines in comparison to their parental counter-parts. By looking at a variety of important cellular and molecular characteristics, we aimed to reveal any major differences between the EveR and parental lines, thus shedding light on potential mechanisms of resistance which would lead to further avenues of investigation.

Initially, the resistant models response to rapalogues, other than everolimus was tested, since in theory the response would be predicted to be similar, due to a virtually identical molecular mechanism of action of these drugs. Very interestingly, both the T47D and 361-EveR lines showed varying resistance to both rapamycin (sirolimus) and temsirolimus, with both lines especially showing a far lower resistance to temsirolimus but were highly resistant to rapamycin. As previously described, all rapalogues act via binding intracellularly to FKBP12, with the complex of rapalogue-FKBP12 then binding to mTOR to inhibit it (Chen et al, 1995; Choi et al, 1996; Yang et al, 2013). One explanation for this variation in response could be the presence of mutations in either FKBP12 or mTOR. Hypothetically, a mutation in either one could then differentially affect the binding of the rapalogues, since they all have different side-groups attached to the basic rapamycin structure. This theory is supported by published evidence which has revealed mutations in *MTOR* that can effect rapalogue action and binding, inclduing work by Hassan and colleagues (2014) and Wagle and colleagues (2014). Sequencing of *FKBP1A* (FKBP12) and *MTOR* in our *in vitro* model of resistance, could reveal such mutations, that help explains both the mechanism of everolimus resistance and the mechanisms behind the differential response to rapalogues.

A key area that was found to be different between EveR and parental lines, was concerning mTORC2, in both western blotting results and drug treatments. Western blotting data revealed that noticeable changes had occurred concerning mTORC2 related proteins, with the T47D-EveR cells showing up-regulation of rictor and p-mTOR (Ser2481) as well as the mTORC2 down-stream proteins p-/PKCα and to a lesser extent p-/SGK1. It is worth noting that the 361-EveR cells did not show these

same changes. Since the 361-EveR cells are only resistant to everolimus at the 1 week time point (and not the 72hr), these cells are loosely deemed to only achieved a partial phenotype of resistance. This therefore could explain why the 361-EveR cells did not show so many differences to their parental controls in this data set.

Alongside this, neither cell line showed any resistance to BEZ-235, which inhibits PI3K as well as mTOR in either complex. These results together lead us to the possibility that mTORC2 up-regulation (at both a protein complex level and an activation of downstream target level) is involved in everolimus resistance; since mTORC2 up-regulated and is both connected to and adjacent to mTORC1 signalling and when we inhibit mTOR in both complexes, we see no resistance. Since mTORC2 is far less characterised than mTORC1, it is possible that mTORC2's up-regulation serves a cellular purpose that allows for everolimus resistance. Whilst mTORC2 is technically up-stream of mTORC1 via Akt, it is unlikely that this is part of the everolimus mechanism (i.e. because more mTORC2 could activate more mTORC1 thus reducing everolimus effect) since there was no change in either total Akt or p-Akt, specific for mTORC2 activation. Further testing by down-regulating mTORC2 proteins may help to elucidate if this up-regulation is involved in the everolimus mechanism.

A lack of resistance to BEZ-235 also suggests the increased effectiveness of inhibiting the PI3K axis over just inhibiting mTORC1. Since PI3K axis inhibition has been shown to overcome rapalogue resistance in other cancers (Vandamme et al, 2016), these results further suggest that PI3K inhibitors may offer a logical route forward over rapalogues in future clinical settings.

Antibody arrays were done on the EveR and parental cells, as a method of broadly comparing the cells for any noticeable changes in a wide variety of signalling pathways, and key oncogenic proteins. This was done in the hope that we could discover changes between the cells, possibly away from mTOR signalling, that would reveal new characteristics of the EveR cells from the signalling pathways altered, or help elucidate the mechanisms behind their resistance. Whilst we expected to see differences between the lines, the array data showed that most proteins (and phospho-proteins) studied were down-regulated in the EveR lines.

There were of course some up-regulations, such as the up-regulation of β-catenin, suggesting that Wnt signalling maybe involved in EveR resistance; although as the combination treatment data with XAV-9393 and everolimus showed, Wnt inhibition appeared not to significantly reduce resistance to everolimus.

Whilst they may be some commonality to the targets that are down-regulated in the arrays, the vast number and diverse nature of the targets suggest that the EveR cells are down regulating multiple signalling pathways, across the board. This suggests that the EveR cells may have entered (or show the early signs of) a possible state of cellular dormancy. This theory is given greater weight by the fact that both EveR cell lines have slowed down their proliferation rate, especially the T47D-EveR, which show the full, not just partial, resistant phenotype. Breast cancer cell dormancy is a phenomenon which is now being highly researched, in various groups due, to its important implications in metastasis and mortality in patients. It has now been established that a specific subset of cells from a tumour can escape to distant sites of metastasis, called disseminating tumour cells (DSTs). Once at their preferred 'soil', often the bone in the case of breast cancer, these DSTs can receive signals from the soil to become dormant. These dormant cells effectively enter a state of quiescence, where they barely divide, and thus escape the attention of the body and any chemotherapy (Bliss et al, 2014; Patel et al, 2011; Quayle et al, 2015). These cells can then be roused from their dormancy, by factors such as oestrogen or progesterone in the case of luminal tumours (Ogba et al, 2014), to then form new tumours in these metastatic sites, often months or years after their dissemination.

These breast DSTs have a distinctive fingerprint that can help identify this cell subset. This can include the increased/decreased expression of certain proteins and use of various signalling pathways involving such molecules as CXCR4 (Nobutani et al, 2015), TGFβ2, p38α/β (Bragado et al, 2013), cyclin D1, JNK and CDK (Quayle et al, 2015). If the EveR cells have entered or are showing signs of dormancy (such as decreased cell growth), then further study involving these dormancy markers in the EveR cells may determine their exact dormancy status. Increased dormancy may also help explain the development of resistant phenotype since everolimus slows cell growth rather than killing the cancer cells. Therefore the dormancy may act as a defence, against the treatment/everolimus, by the cancer cell.

On top of this possible increased dormancy, it is also possible that this is sign of increased ‘stemness’, with the two phenotypes seemingly related in breast cancer biology. CSCs can help propagate a tumour and are often treatment resistant, with CSCs often being far more dormant than the majority of the cancer cells in a tumour (Yeh and Ramaswamy, 2015). The EveR cells also show a possible up-regulation of Wnt signalling, which is a key pathway in breast CSC regulation (Lamb et al, 2013). Therefore further testing to see if the EveR cells have a greater stem-like phenotype, may be useful in explaining some observations from this chapter, relating to dormancy and decreased proliferation.

Chapter 6: Exploration of Everolimus Resistance Mechanism

6.1 - Introduction

6.1.1 - Multidrug Resistance in Breast Cancer

Resistance is clinically observed to both standard chemotherapy and more targeted treatments such as trastuzumab and tamoxifen. This can be related to multidrug resistance (MDR); a set of mechanisms in cancer cells allowing them to overcome a broad spectrum of drugs, including to both targeted and non-targeted treatments. One of the most commonly observed mechanisms to give rise to the MDR phenotype is the over-expression and dysregulation of ATP-binding cassette (ABC) transporters. These transporters bind a wide range of drugs including standard chemotherapy agents of the taxane family (e.g. paclitaxel), to cause an efflux of the drugs out of the cell. Commonly over-expressed ABC transporters giving rise to the MDR phenotype in breast (and other) cancers include P-glycoprotein (PGP/MDR1), multidrug resistance-associated protein 1 and 2 (MRP1/2) and breast cancer resistance protein (BCRP) (Chen et al, 2013; Choi and Yu, 2014; Kovalev et al, 2013; Marquette and Nabell (2012); Martin et al, 2014).

6.1.2 - Mutations in mTOR and Associated Rapalogue Resistance

Over-activation of the mTOR pathway predominately derives from up-stream activation/alteration of various growth factor pathways. However since mTOR is the centre of the eponymous signalling pathway, it is inevitable that mutations arise at this point, in human cancers. Research by Grabiner and colleagues (2014) documented a wealth of mutations that had arisen in mTOR, with a high clustering in the FAT and FATC domains; many of the mutations observed in this research altered both the activity of mTOR complexes as well as the binding to various key mTOR components such as DEPTOR.

Whilst the mutations observed in this particular piece of research did not show any evidence of causing reduced binding to rapalogues, logic suggests that mutations in the FRB domain, which binds rapalogues (along with FKBP12) would have the potential to cause insensitivity to rapalogues, with other pieces of research giving weight to this hypothesis. Wagle and colleagues (2014) detected a mutation in the FRB domain, in a thyroid cancer patient who had shown resistance to everolimus. This mutation (F2108L) was shown to sterically hinder the binding of rapalogues, thus giving clinical evidence of FRB mutations causing rapalogue resistance. Whilst no clinical evidence of these types of mutations exists in breast cancer yet, an *in vitro* model with BT4747 breast cancer cells that are rapamycin resistant, has also shown the presence of mutations in the FRB domain (Hassan et al, 2014). The mutation observed in this piece of research (S2035L), is similar to mutations studied in previous work by Chen and Colleagues (1995), who demonstrated that these mutations lower mTOR's ability to bind rapalogues.

6.1.3 - Breast Cancer Stem Cells

Cancer stem cells (CSCs) have emerged in the last two decades as both a shakeup of the traditional thinking of cancer biology, and as a new possibility for therapeutic options. It had long been proposed (in the clonal evolution model) that tumours did not have a hierarchy, and that the genetic instability of cancer led to the formation of different clonal populations, thus acquiring new characteristics such as increased metastatic potential, via this heterogeneity. This model has been challenged by the emerging evidence of CSCs and the rise in popularity of the cancer stem cell model, for the development of tumours. In this proposed system, tumour heterogeneity is related to a hierarchy, with a subset of cells, able to drive the growth and spread of the cancer. CSCs have similar properties to normal stem cells in that they can self-renew and thus maintain a tumour from this subset of cells, but also have tumorigenic capacity and plasticity. They have been identified in malignancies such as AML and breast, brain (glioblastoma), melanoma and prostate cancers (Dawood et al, 2014; Vlashi and Pajonk, 2015).

Breast CSCs are presently well characterised and have defined biomarkers to separate them from normal cancer cell populations. Perhaps the most reliable

markers for breast CSCs, is the expression of high aldehyde dehydrogenase (ALDH) activity/ALDH+/ALDH1+ (Ginestier et al, 2007; Liu et al, 2014b) and/or the presence CD44 and lack of CD24 (CD44+/CD24-) (Al-Hajj et al, 2003; Qiu et al, 2016). Breast CSCs are known to have an up-regulation of pathways commonly associated with CSCs such as increased Wnt and notch signalling, with these pathways contributing to the stemcell-like phenotype (Kotiyal et al, 2014; Zhao et al, 2014). Interestingly, the expression of these key markers is seemingly involved in a major biological feature of these CSCs; namely their plasticity. Research has shown that breast CSCs have the ability to transition between various states, usually reflecting either an epithelial or mesenchymal phenotype; with CD44+/CD24- populations bearing mesenchymal characteristics whilst ALDH+ bear more epithelial characteristics. This ability to perform EMT or MET (mesenchymal to epithelial transition) means they can, for example, proliferate at will or detach and spread to new niches and form new metastasis. Equally these properties allow these cells to slow down their rate of proliferation, becoming more dormant, and avoid the effects of chemotherapy (Liu et al, 2014b; Luo et al, 2015b). Partly due to the wealth of evidence showing the importance of breast CSCs in maintaining and propagating a tumour (coupled with evidence of an up-regulation of the targeted pathways), drugs are being tested that inhibit pathways promoting stem-like phenotypes and CSCs, such as Wnt inhibitors. In breast cancer, these include the pre-clinical testing of the tankyrase inhibitor XAV-939 (Bao et al, 2012) and γ-tocotrienol (Ahmed et al, 2016).

6.2 – Aims

- The specific aim of this chapter was to elucidate the mechanisms behind everolimus resistance in the previously developed everolimus resistant cells.
- This was done by:
    - Studying whether multi-drug resistance may play a role in everolimus resistance, by treating everolimus resistant cells with a range of standard chemotherapy agents and then looking for any resistance
    - Developing the current everolimus resistant cells into long term everolimus resistant models and study any further changes that may arise.
    - Studying whether mTORC2 up-regulation and/or activity can contribute or cause everolimus resistance, by down-regulating mTORC2 targets using siRNA, and then treating the transfected cells with everolimus, to look for changes in resistance.
    - Determining whether everolimus resistant cells have an increase in stem-like phenotype using ALDH activity as a marker.
    - Examining FKBP12 and the FRB domain of mTOR for mutations, via sequencing, to determine if these may explain both the everolimus resistance mechanism and/or the differential response to rapalogues, observed in everolimus resistant cells.

6.3 - Results

6.3.1 - Determining Whether Everolimus Resistant Cells are Resistant to Standard Chemotherapy Agents

As has been established in previous chapters, the EveR cells show varying resistance to everolimus, rapalogues and other mTOR inhibitors (section 3.35 and 5.3.1). I now wished to explore whether the EveR cells expressed resistance to a wider range of drugs, with varying mechanisms of action, which broadly can be described as standard chemotherapy agents. Research has shown that over-expression of some ABC transporters, such as P-glycoprotein, can cause resistance to standard chemotherapy drugs like paclitaxel, and give rise to a, MDR phenotype (Chen et al, 2013; Kovalev et al, 2013; Martin et al, 2014). Thus by testing a variety of chemotherapy drugs on the EveR cells, we would reveal if they too expressed a similar phenotype, which would help inform us whether this could be a possible mechanism for everolimus resistance. Both the T47D-EveR and 361-EveR (and the respective parental cells) were treated with 5-fluorouracil, methotrexate, cyclophosphamide, doxorubicin and paclitaxel for either 72hrs or 1 week.

SRB data gathered in these experiments (figures 6.1-6.5) showed almost uniformly, that there was no difference in sensitivity between EveR and parental cell lines, to these chemotherapy drugs. Across virtually all drug concentrations, at both time points and in both cell lines, very little difference in drug responsiveness could be seen, although a few small exceptions were noted. 361-EveR cells showed a very marginal, significant difference in doxorubicin response at 1 week when treated with 100nm doxorubicin. However this was only observed at one time point and one drug concentration and is most likely due to the sigmoidal nature of this graph/data and the fact that 100nM falls at the point where doxorubicin starts to have a very radical effect on growth. Other differences observed, included some of the data points for the parental cells, showing more resistance to the drugs than the EveR cell lines. For example, some differences were seen with the T47D-Parental when treated with methotrexate for 72hr. However, the significant differences here were by very small margins and tended not to represent overall trends in the data.

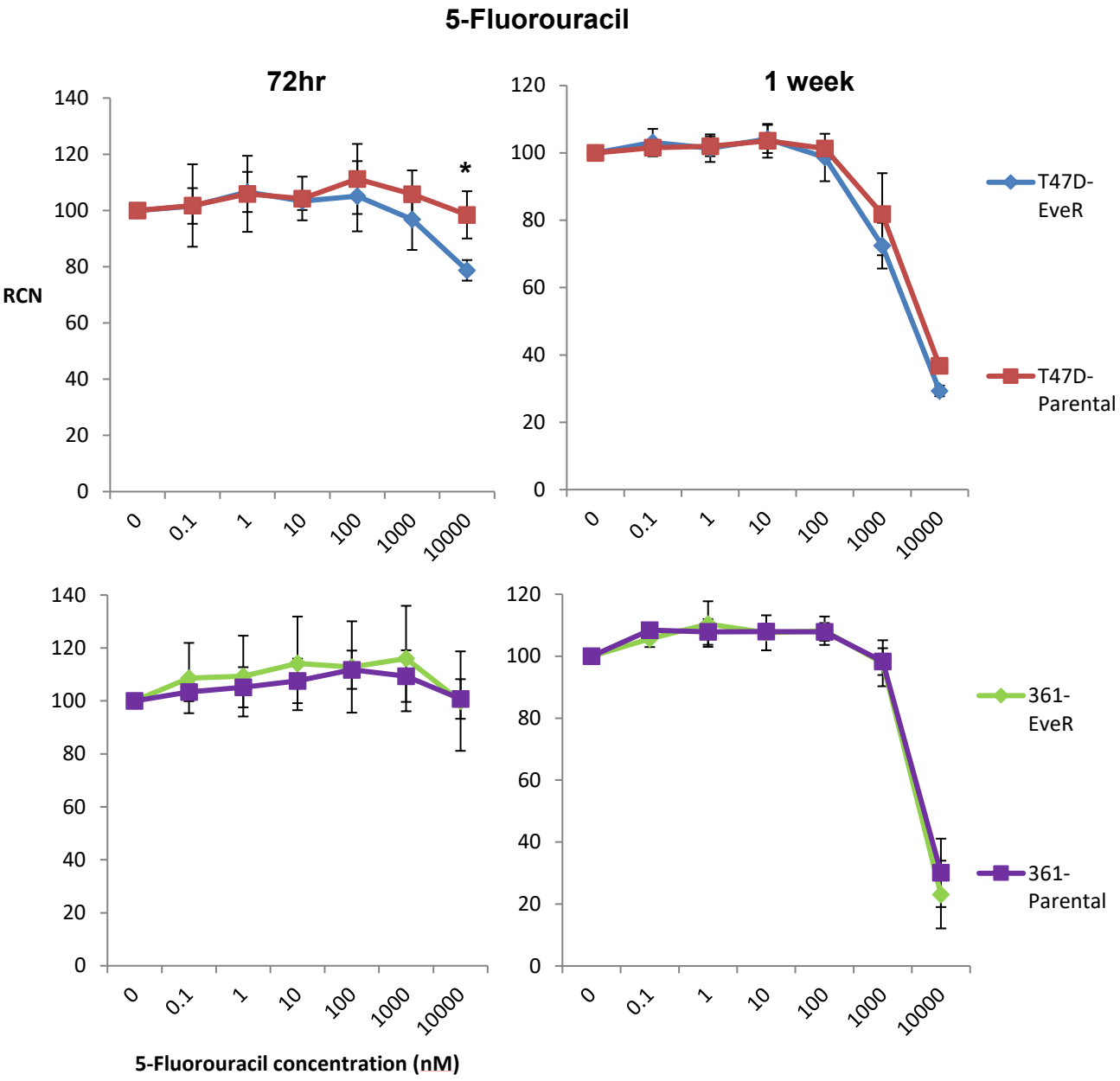


Figure 6.1: EveR and parental cells show no difference in sensitivity to 5-fluorouracil. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant lines (EveR) v parental, treated with 5-fluorouracil for either 72hr (left) or 1 week (right). * P<0.05 compared to control. No difference in sensitivity to 5-fluorouracil was observed.*

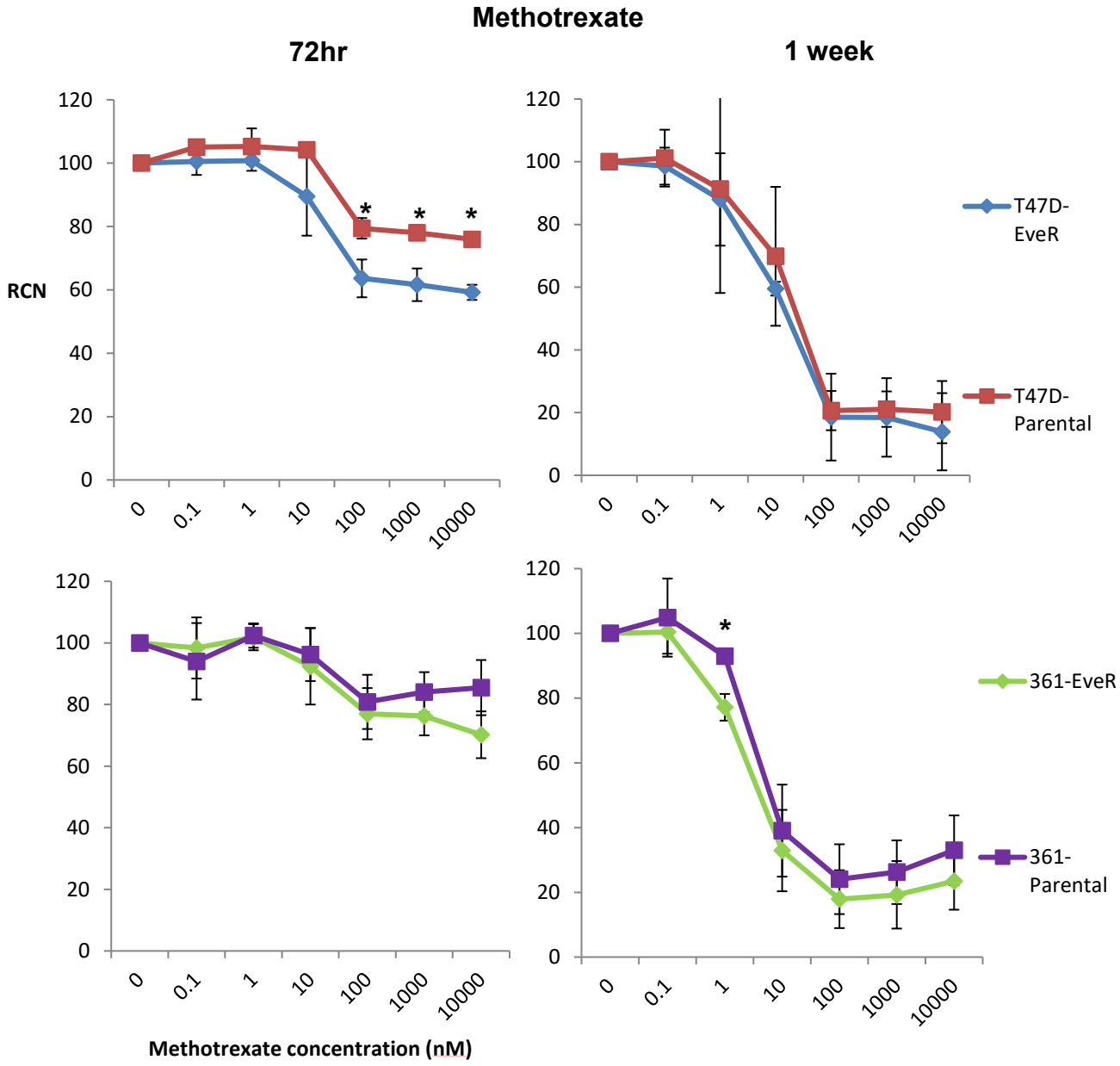


Figure 6.2: T47D cells are weakly resistant to methotrexate. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant lines (EveR) v parental, treated with methotrexate for either 72hr (left) or 1 week (right). * P<0.05 compared to control. No resistance to methotrexate was seen in the EveR lines, although the T47D-Parental did have some small, but significant difference at 72hr compared to the T47D-EveR, with the parental being slightly more resistant.*

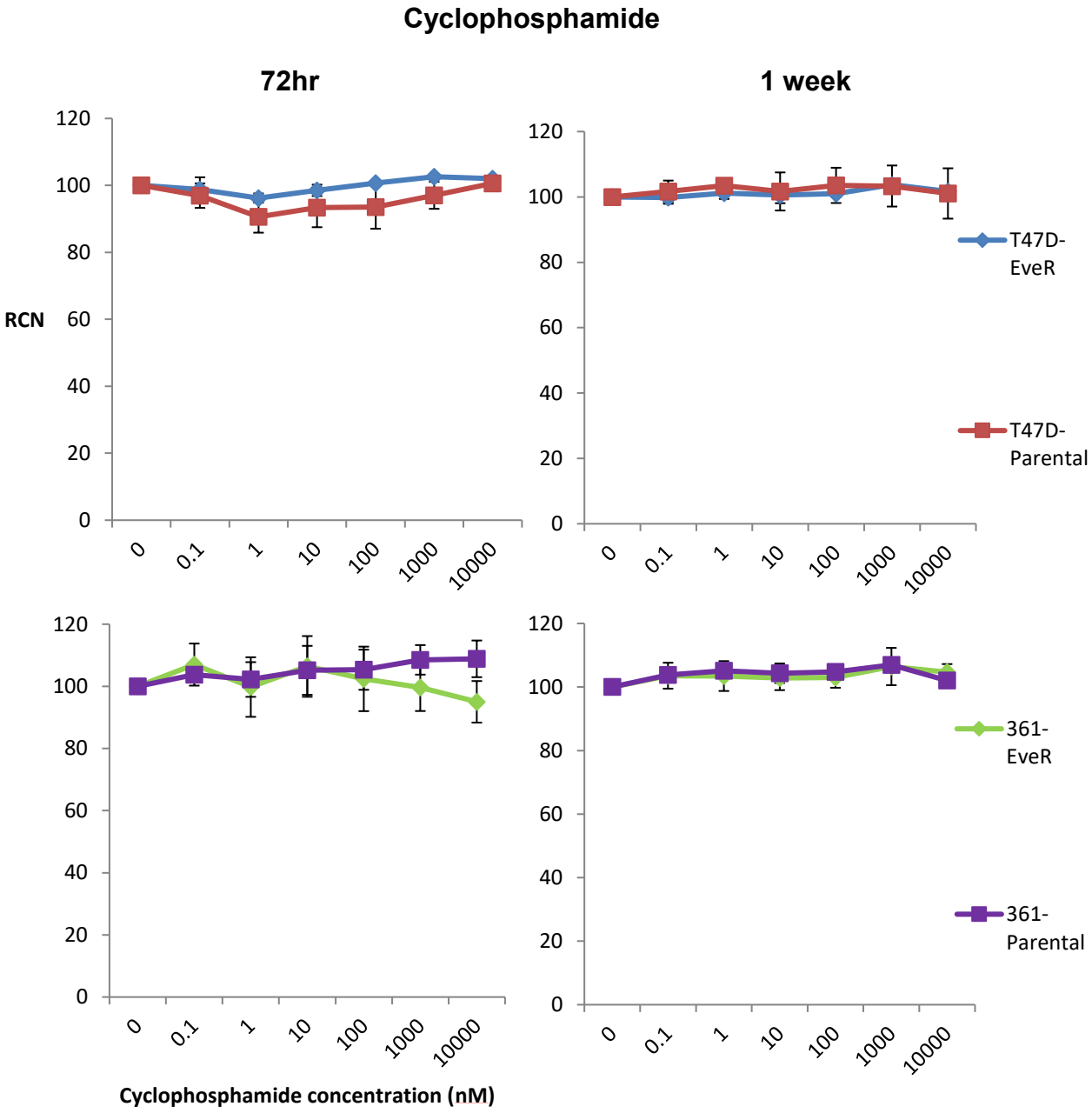


Figure 6.3 EveR and parental cells show no difference in sensitivity to cyclophosphamide. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant lines (EveR) v parental, treated with cyclophosphamide for either 72hr (left) or 1 week (right). * P<0.05 compared to control. No difference in sensitivity to cyclophosphamide was observed.*

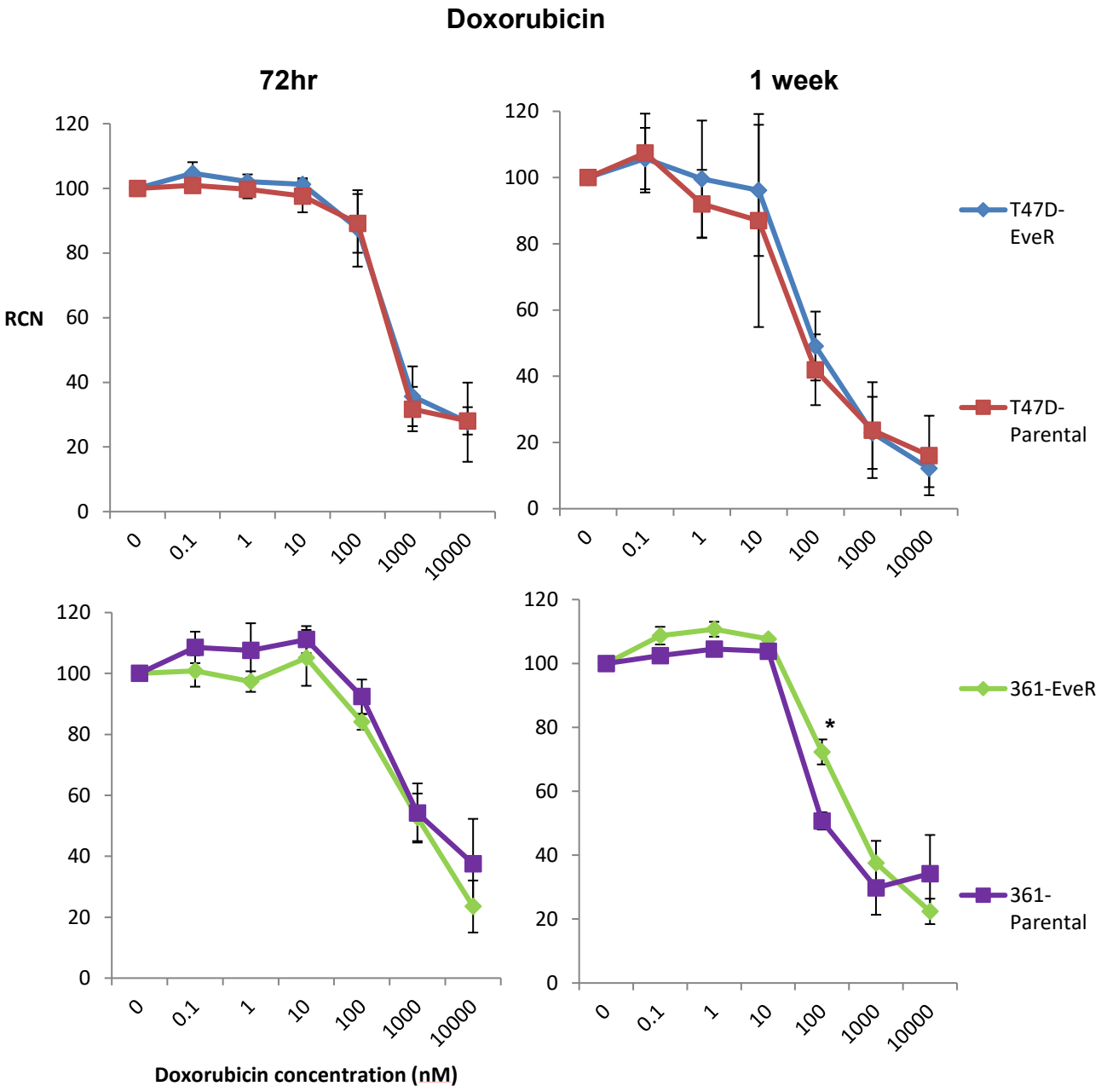


Figure 6.4: EveR and parental cells show no difference in sensitivity to doxorubicin. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant lines (EveR) v parental, treated with doxorubicin for either 72hr (left) or 1 week (right). * P<0.05 compared to control. No difference in sensitivity to doxorubicin was observed.*

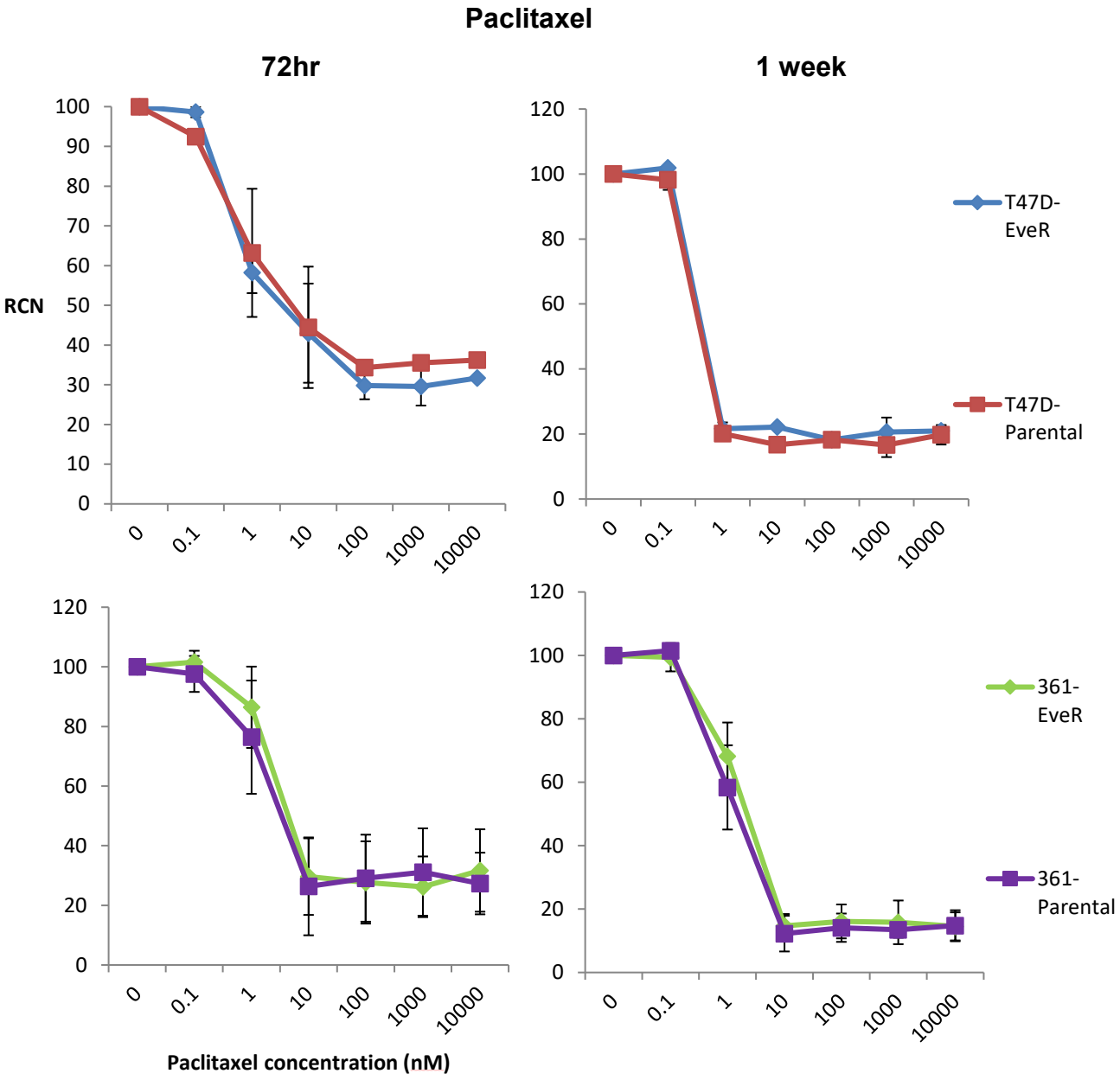


Figure 6.5: EveR and parental cells show no difference in sensitivity to paclitaxel. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant lines (EveR) v parental, treated with paclitaxel for either 72hr (left) or 1 week (right). * P<0.05 compared to control. No difference in sensitivity to paclitaxel was observed.*

6.3.2 - Development of Long Term Everolimus Resistant Models

Having now characterised the EveR cell lines as described in the previous chapters, I wished to further develop these lines into a more 'long-term' resistant model. This entailed taking the existing T47D/361-EveR and parental control lines and culturing them further using the exact same method detailed to develop the lines originally. However some variation from the original methodology was implemented. Firstly, the concentration of everolimus, during the treatment week, was not varied unlike before, and was kept constant at 100nM. This was chosen as both EveR lines were resistant to this concentration, but it was equally high enough to match possible concentrations seen in patients (Kirchner et al, 2004), and thus would potentially push cells further along their development of resistance-based phenotypes.

Secondly, the cells were not tested for a GI60 (using the SRB assay) after each treatment. Since we were not necessarily looking for small shifts in responsiveness, they were instead tested once a month, to look to see if any radical changes in resistance had occurred. As previously done, the parental control cells for each resistant cell line were cultured alongside.

The objective of this work was to study the long-term resistant models and look to see if further alterations in the phenotypes of the cells had occurred, such as further changes in proliferative rate or further alteration in expression of mTOR related proteins. This would then help shed light on how cells become resistant to everolimus but also how they may develop if exposed to everolimus for multiple treatment cycles, months apart.

As a result a T47D long term resistant model was developed dubbed 'T47D-EveR-LT', along with its parental counter-part, 'T47D-Parental-LT'. These cells were cultured for a further 4 month period, roughly doubling their passage number from the point when they initially became resistant. The development was stopped at this 4 month mark as no major changes in everolimus responsiveness had occurred (discussed further below). At this point the cells were then characterised for alterations in their phenotype. Unfortunately a long term resistant 361-EveR model could not be developed over the course of this time, due to infection in cell stocks

during the long term development process and subsequent restrictions on time and resources.

6.3.3 - Confirming the Expression of mTOR Pathway Components at a Protein Level in Long Term Everolimus Resistant Cells

Like the EveR and parental control cells originally developed, the T47D-EveR-LT and parental controls were characterised for a number of mTOR related proteins. For this section, we focused predominantly on the proteins that were up-regulated in the T47D-EveR line, which were connected to mTORC2. Since we are exploring mTORC2 as a mechanism for everolimus resistance (section 6.3.6), it was desired to examine whether further alterations in this area of the mTOR pathway had occurred.

mTOR, rictor, PKCα and SGK1 were studied for their expression using western blotting (figure 6.6). Very little difference in the levels of these proteins could be discerned, when comparing EveR-LT to the parental cells. This is of a great contrast to the characterisation of these proteins in the original T47D-EveR lines (compared to their parental). In the T47D-EveR-LT compared to parental cells, a roughly even expression of mTORC2 components, rictor and p-mTOR (Ser2481) and even expression of downstream targets of mTORC2, PKCα and SGK1 (and their phosphorylated forms), was observed.

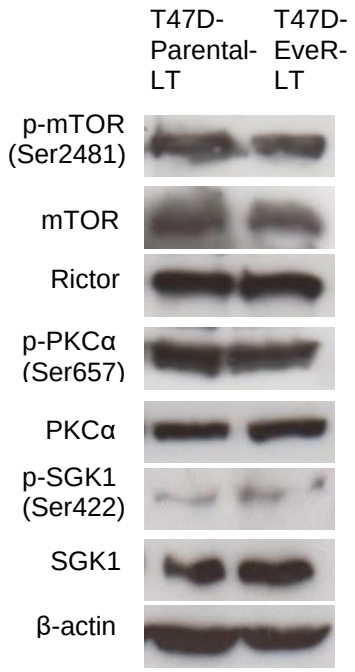


Figure 6.6: Expression of key mTOR signalling proteins has not altered in the EveR-LT model. *Representative western blots for T47D-EveR-LT and Parental. n=2 independent experiments. Target proteins included components of mTORC2 and those downstream of the complex, with β-actin as a loading control. No discernible difference could be seen between these proteins, when comparing the EveR-LT to the Parental-LT.*

6.3.4 - Studying Proliferative Differences in Long Term Everolimus Resistant Cells

When studied previously, the T47D-EveR cells showed some proliferative differences to the parental controls, with the resistant line decreasing their proliferative rate relatively. The long-term resistant cells were therefore studied in a similar manner to see if these growth changes remained. Cell cycle profile in the T47D-EveR-LT and Parental-LT cells was examined using PI staining and subsequent flow cytometry analysis.

The flow cytometry data (figure 6.7) showed no significant difference when comparing the two lines for the proportion of cells in G1, S or G2 phase. This suggests that any changes in proliferative rate that had existed between the EveR and parental cell lines (figure 5.11) were now no longer present, when comparing the respective LT versions of these lines.

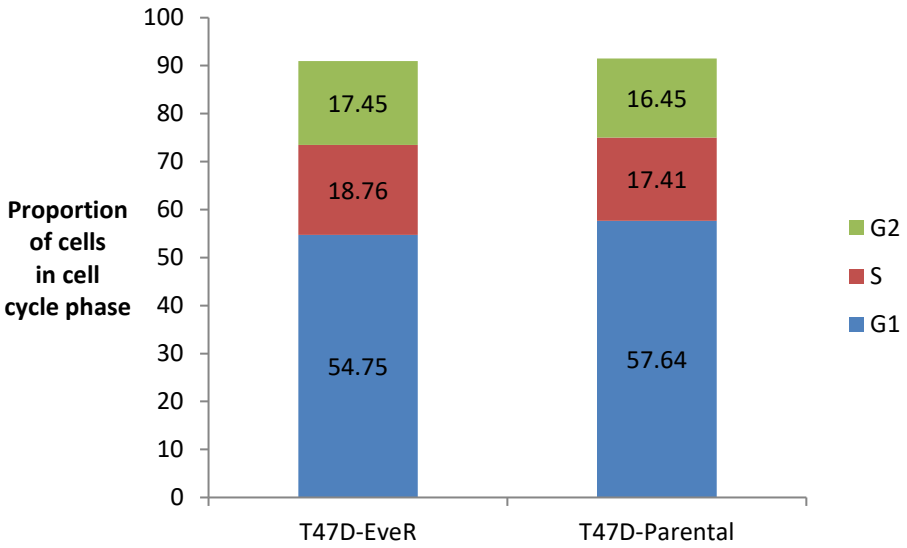


Figure 6.7: T47D-EveR-LT cells show no difference in cycling profile compared to parental cells. *Flow cytometry data for T47D-EveR-LT and parental cells, using propidium iodide staining to study cell cycle profile of each line. n=3 independent experiments. Little difference was noted in profile between each line.*

6.3.5 - Characterisation of Response to Everolimus and Calcitriol in Long Term Everolimus Resistant Cells

It was desired to see if the long term resistance model cells had become more or less resistant to everolimus. Figure 6.8 shows the data for the LT cells when treated with everolimus for 72hr or 1 week. When comparing these data to the same results after the first development process (figure 4.9), we can see that the EveR-LT cells response to everolimus is very similar to the original EveR cells, with very little change in responsiveness, despite the extra weeks exposure, on/off, to everolimus.

The response to calcitriol and calcitriol in combination with everolimus (shown in figures 6.9 and 6.10) was also checked, as the combination had shown significant results when tested previously with the original EveR cells (section 4.3.6). The response to calcitriol was relatively unchanged from the previous work, with calcitriol having only a small impact on the growth of either cell line. Everolimus combined with 1μM calcitriol (treated for 1 week) produced a similar pattern of results to the original EveR cells, with the addition of calcitriol decreasing cell growth but not ablating resistance entirely. However, this was by a smaller margin and results were

more variable, than before, with only the highest concentration of everolimus showing a significant difference between the everolimus only and combination treatment data points.

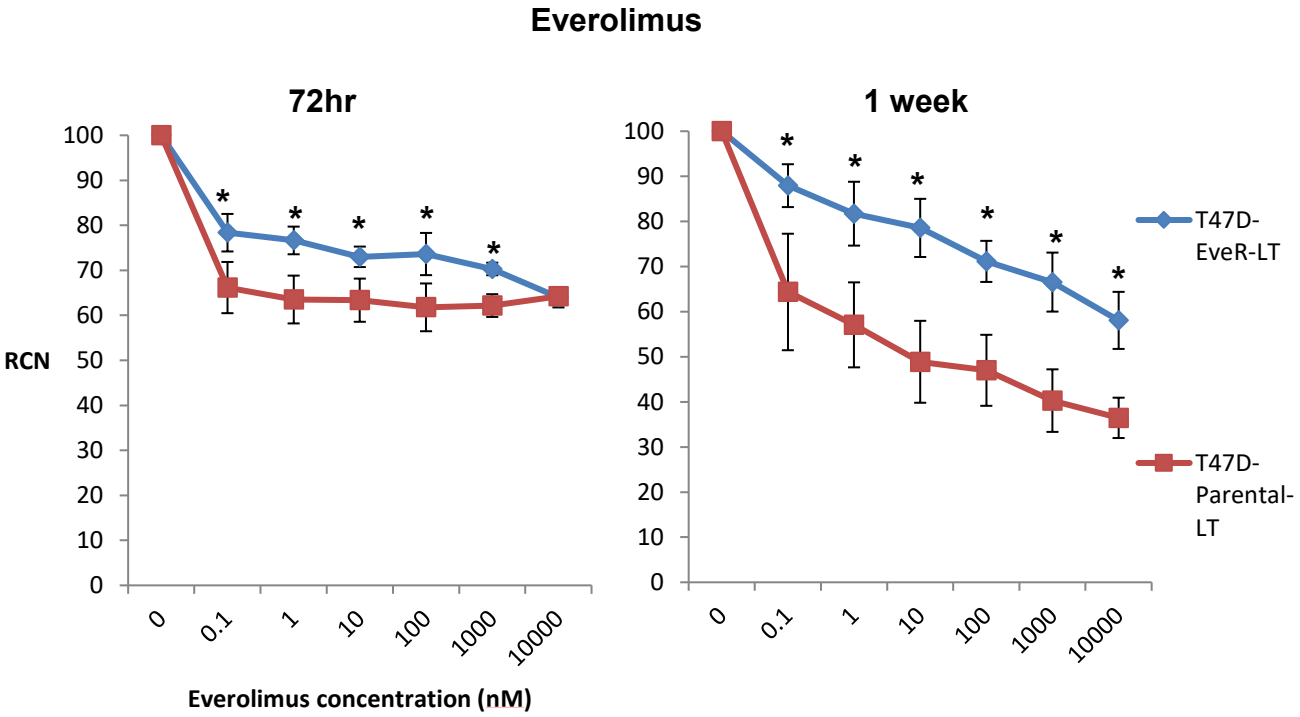


Figure 6.8: T47D-EveR-LT still show significant resistance to everolimus. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant-Long term lines (EveR-LT) v parental, treated with everolimus for either 72hr (left) or 1 week (right). * P<0.05 compared to control. The T47D-EveR-LT line showed similar levels of resistance as did previous results with the T47D-EveR line.*

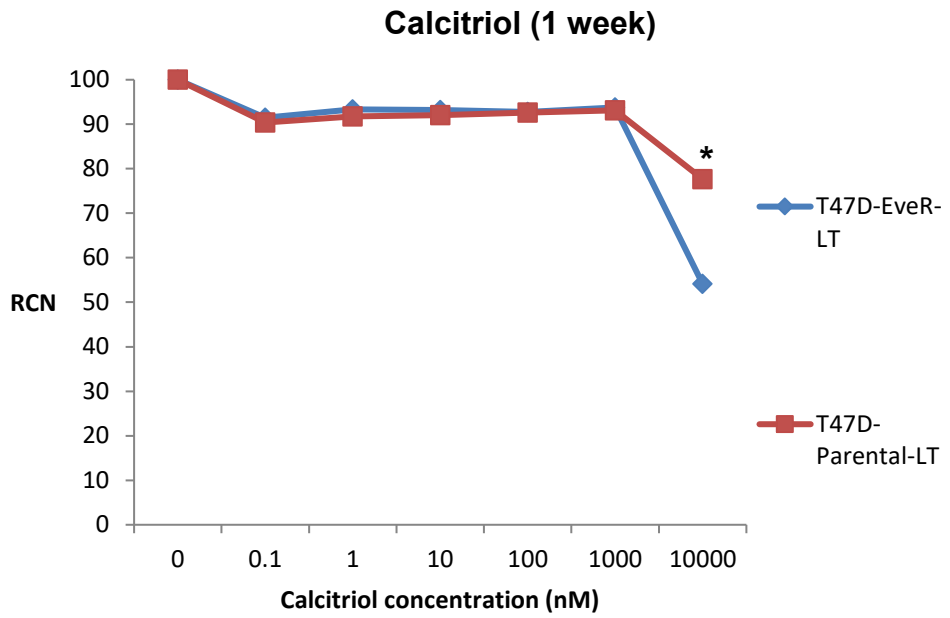


Figure 6.9: LT model show weak growth inhibition by calcitriol. *SRB results (plus or minus standard deviation, n=3 independent experiments) for everolimus resistant-Long term lines (EveR-LT) v parental, treated with calcitriol for 1 week.*
** P<0.05 compared to control.*

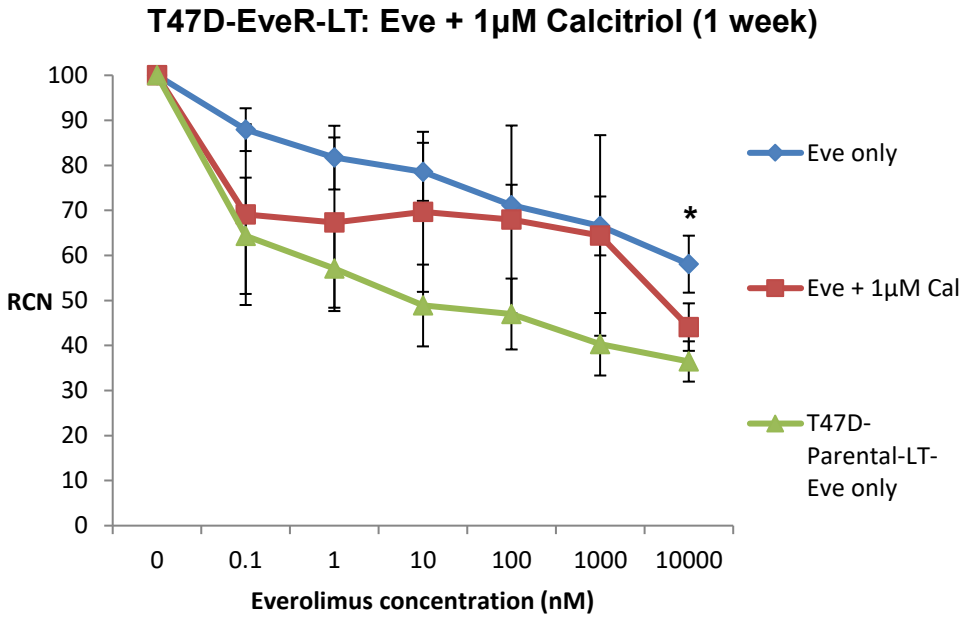


Figure 6.10: Everolimus+calcitriol fails to significantly improve growth inhibition v everolimus only, in LT-EveR cells. *SRB result (mean plus or minus standard deviation, n=3 independent experiments) for T47D-EveR-LT treated for 1 week, with either everolimus (eve) or everolimus + 1µM calcitriol (eve + cal). * P<0.05 compared to combination treatment.*

6.3.6 - Optimisation of siRNA Transfections in Breast Cancer Cells

To test whether the observed increases in the protein levels of mTORC2 components and downstream effectors (figure 5.4), was part of the everolimus resistance mechanism, siRNA transfection were used to knockdown key areas of mTORC2 signalling. Transfections were done with Dharmacon transfection reagents and siRNA's, with siGLO transfection indicator (Dharmacon) first used to test concentrations needed for efficient transfections in these cell lines. Both T47D-Parental and 361-Parental were seeded at 10, 20 or $30x10^3$ cells per well and then transfected with the siGLO using 0.25, 1, 1.75 or 2.5µL Dharmafect 1 transfection reagent. Cells were then viewed using a microscope, in both normal light and green fluorescence channels, to study siGLO uptake into the cells, by visual comparison.

Transfection efficiency was nearly identical between the two cells lines (depending on dharmafect 1 concentration), with both showing a good uptake of the siGLO indicator (good transfection defined by most cells showing multiple transfection complexes of siGLO). Efficiency also did not vary between different concentration of cells per well. For these reasons example images shown (figures 6.11-6.14) are taken from both cell lines and different cell concentrations. 2.5µL of Dharmafect 1 per well was quickly excluded as a viable method, after preliminary testing showed a high level of cytotoxicity at this concentration.

Figures 6.11 and 6.12 show T47D-Parental and 361-Parental cells transfected with siGLO indicator and then observed after 24hrs. Transfections worked at all concentrations of Dharmafect 1 tested, although a small difference could be noted between 0.25 and 1µL per well, with the former being observed to have a less efficient uptake of siGLO; 1 and 1.75µL achieved similar rates of transfection, especially in 361 cells. All three concentrations of Dharmafect 1 were well tolerated, although 1.75µL per well did have slightly more dead cells than the two lower volumes of Dharmafect tested. Cells were also observed for any variation in siGLO uptake 48hrs after transfection. Example images for this can be seen in figure 6.13. In terms of both cell health and transfection rate between Dharmafect volumes, this had not changed from the previous day.

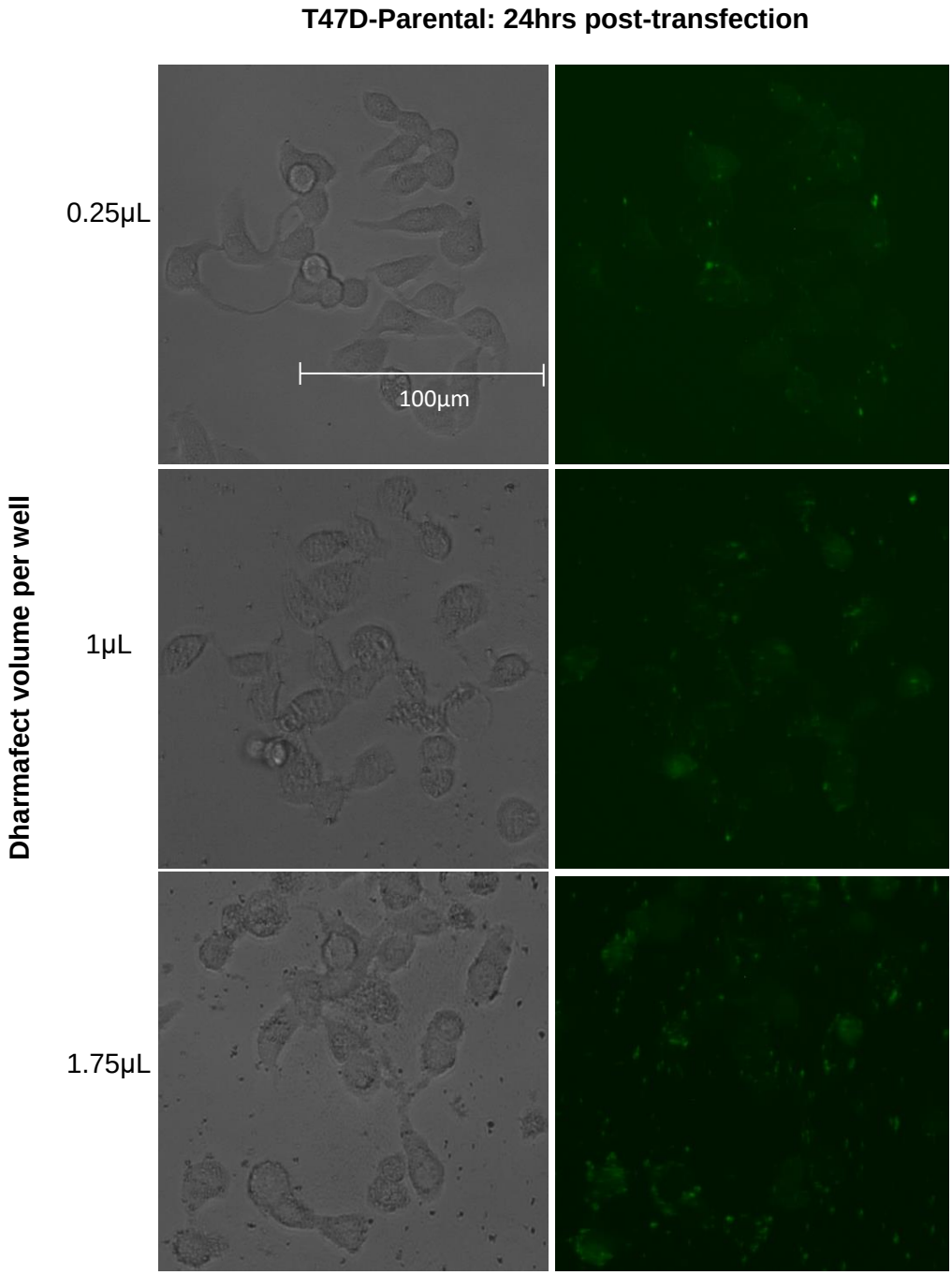


Figure 6.11: ≥1 dharmafect per well gives good transfection rates in T47D cells. *Example images of T47D-Parental cells, taken 24hrs post transfection using siGLO transfection indicator (green) and varying volumes of Dharmafect 1 transfection reagent. siGLO uptake can be seen in the green fluorescence pictures, with an increase in transfection efficiency in increasing Dharmafect concentrations but, higher levels of cell death and unhealthy cells in the 1.75µL volume wells. Magnification = x20. Good transfection defined as most cells showing multiple transfection complexes.*

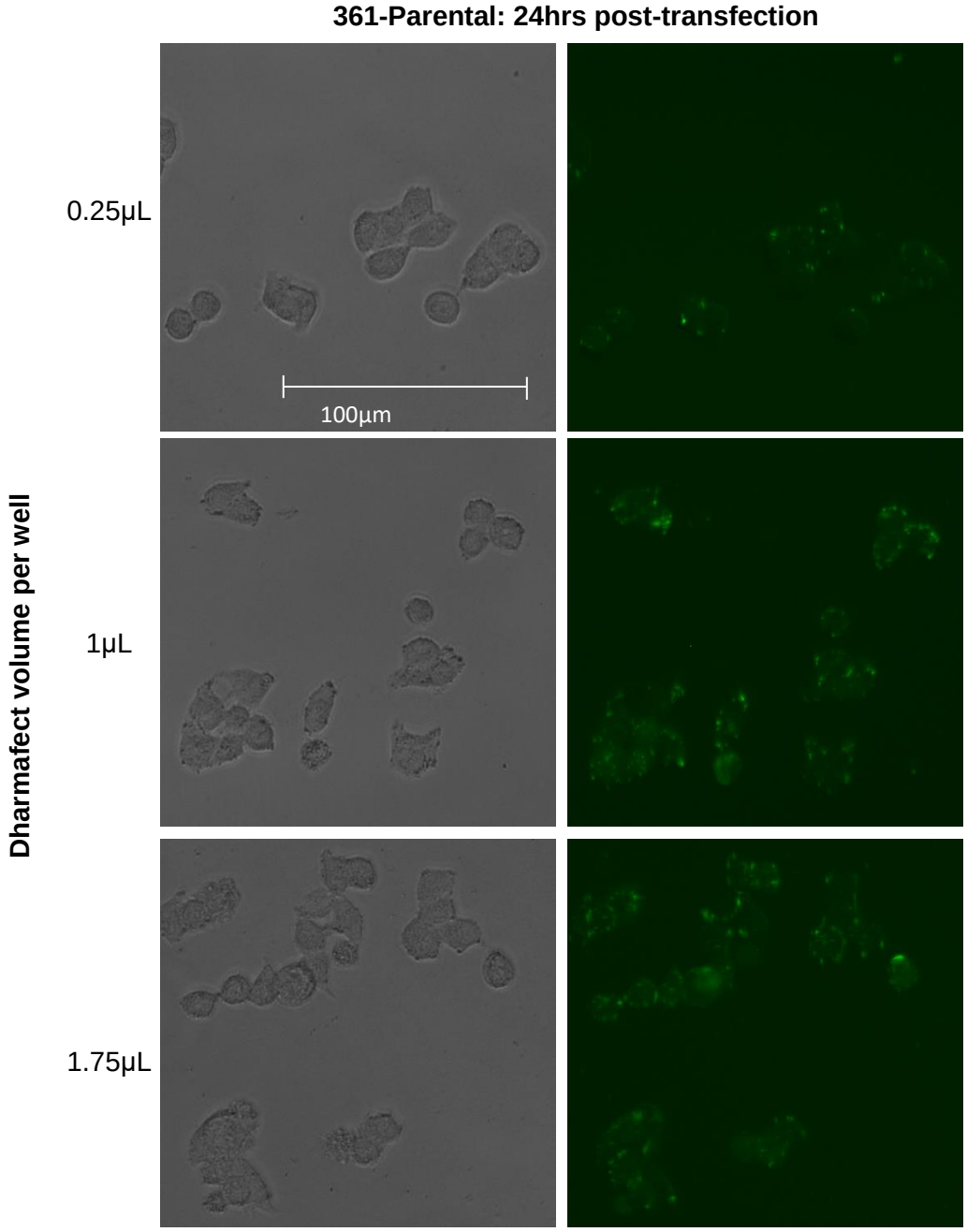


Figure 6.12: ≥1 dharmafect per well gives good transfection rates in 361 cells. *Example images of 361-Parental cells, taken 24hrs post transfection using siGLO transfection indicator (green) and varying volumes of Dharmafect 1 transfection reagent. siGLO uptake can be seen in the green fluorescence pictures, with an increase in transfection efficiency in 1µL Dharmafect per well, compared to 0.25µL. More dead and unhealthy cells were observed in the 1.75µL wells compared to lower volumes. Magnification = x20. Good transfection defined as most cells showing multiple transfection complexes.*

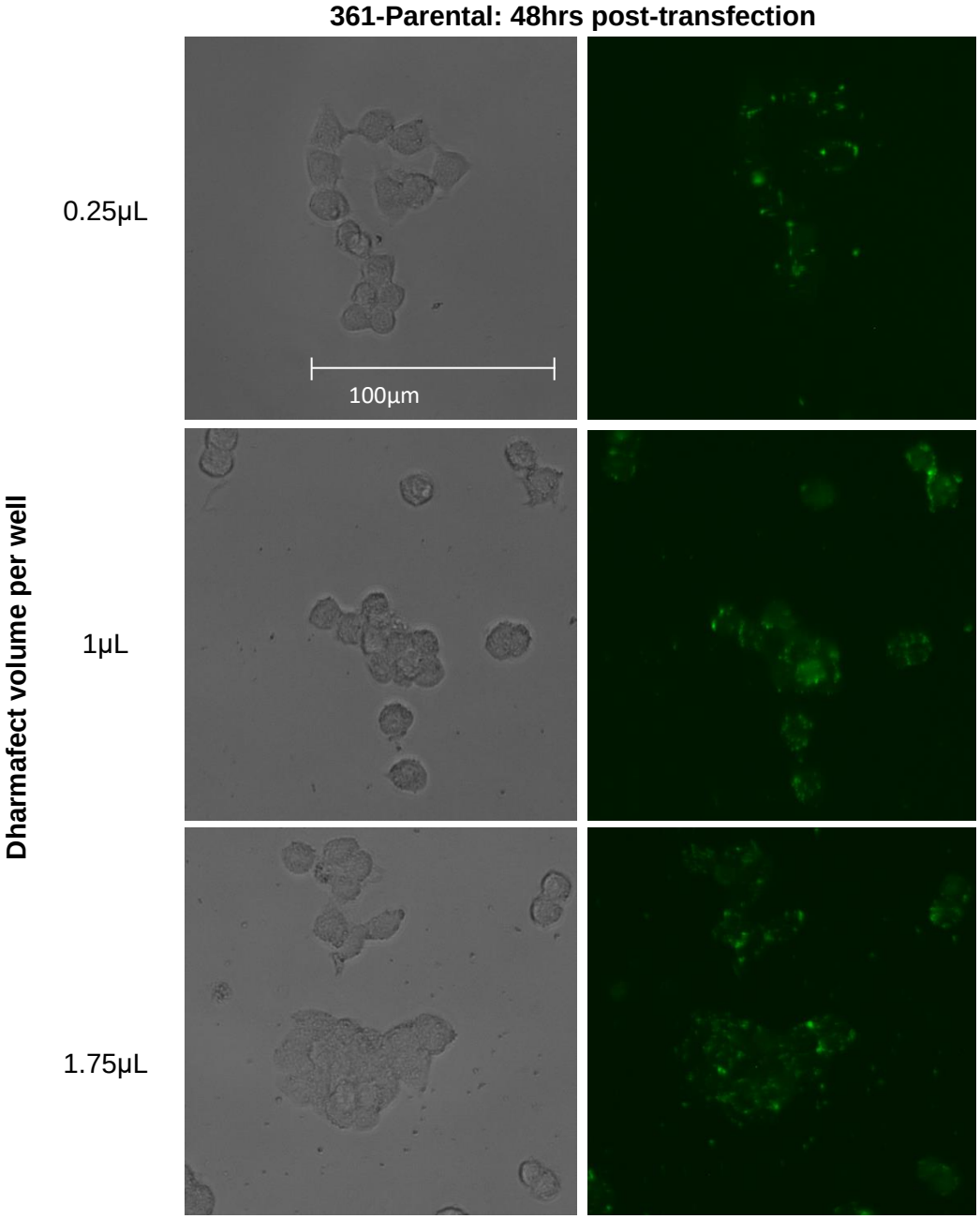


Figure 6.13: ≥1 dharmafect per well gives good transfection rates in 361 cells. *Example images of 361-Parental cells, taken 48hrs post transfection using siGLO transfection indicator (green) and varying volumes of Dharmafect 1 transfection reagent. siGLO uptake can be seen in the green fluorescence pictures, and similarly to 24hrs post transfection, an increase in transfection efficiency was observed in wells with 1µL Dharmafect, compared to 0.25µL. Magnification = x20. Good transfection defined as most cells showing multiple transfection complexes.*

A subsequent re-transfection was also tested to see if this could both boost transfection efficiency at lower volumes and if this procedure could be tolerated by these cell lines. Example images for this can be seen in figure 6.14. At 24hr following the second transfection, the re-transfection did appear to slightly increase siGLO uptake at the 0.25µL volume, but this was still less efficient than the 1µL volume. Cell health when using the double transfection method was an issue, with the 1.75µL Dharmafect per well resulting in an increased number of floating and stressed cells. Much like the single transfection, siGLO uptake and cell health had not changed dramatically 48hrs post-transfection, compared to 24hrs.

For these reasons the 1µL Dharmafect 1 volume per well was selected to go forward from this point, as it showed a good transfection efficiency in both cell lines and when using a single or double transfection, while maintaining relatively healthy cells.

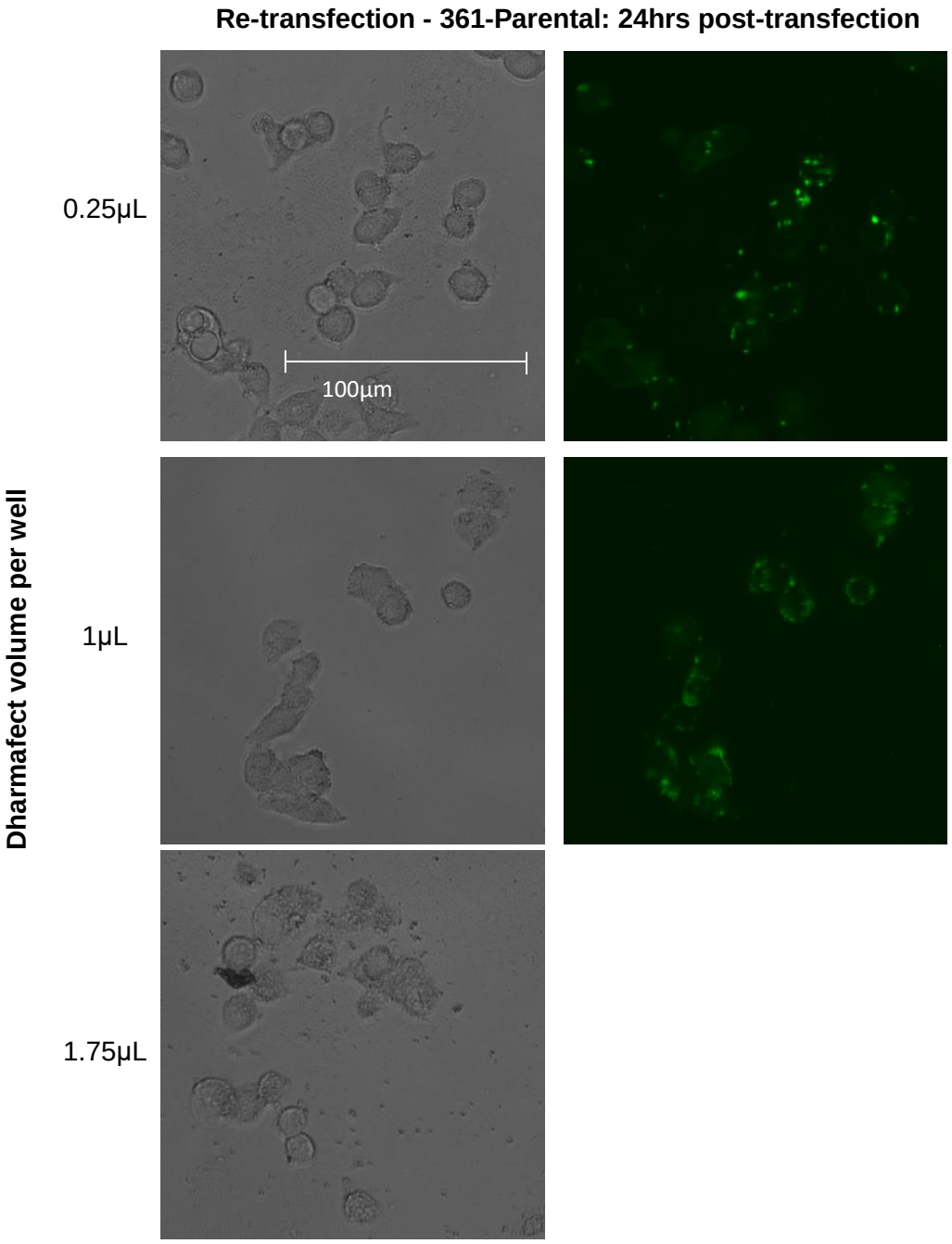


Figure 6.14: Re-transfection with dharmafect increases efficiency but is also more toxic to cells. *Example images of 361-Parental cells, taken 24hrs post re-transfection using siGLO transfection indicator (green) and varying volumes of Dharmafect 1 transfection reagent. siGLO uptake can be seen in the green fluorescence pictures. Re-transfection slightly increased siGLO uptake in 0.25 and 1µL Dharmafect volumes per well, compared to the single transfection shown previously. However cell healthiness was decreased compared to the single transfection, with 1.75µL proving too much in a double transfection, culminating in vastly deceased cell growth and increased dead cells. Magnification = x20.*

6.3.7 - Optimising siRNA Knockdown of *PRKCA* and *SGK1*

Once the volumes and concentrations to be used for the transfection reagents had been decided, the siRNAs to be used were tested for their efficiency in knocking down the desired target. T47D-Parental and T47D-EveR were selected for this process, to test the siRNAs on one resistant and one parental control line. A single transfection was first tested using either the PRKCA (PKCα) or SGK1 siRNA, with the non-targeting siRNA used as a negative control, and GAPDH used as a loading control for the western blots. In relation to the knockdown of PRKCA/PKCα (figure 6.15), it can be seen from these figures that a good and stable knockdown was achieved 72hr post-transfection, with these levels holding even after 5 days. Unfortunately, stable knockdown of SGK1 was not achieved using a single transfection (figure 6.16), with no noticeable decrease in SGK1 protein seen at any time point post-transfection.

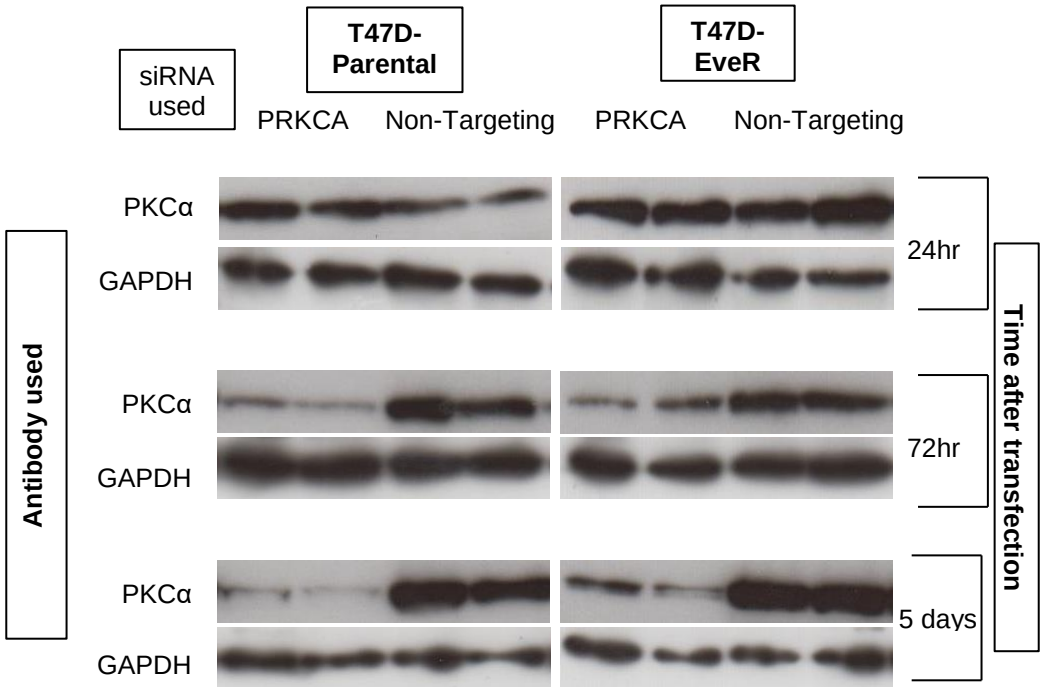


Figure 6.15: siRNA transfection down-regulates PKCα expression after 72hrs. *Western blots for T47D-Parental and EveR, run in duplicate. Cells were transfected with either PRKCA siRNA or a non-targeting control, with lysates taken at intervals post-transfection, with these then studied via western blotting for PKCα, with GAPDH as a loading control. Decreased levels of PKCα protein were clearly seen at 72hr and 5 days post-transfection, in both cell lines, compared to the non-targeting control.*

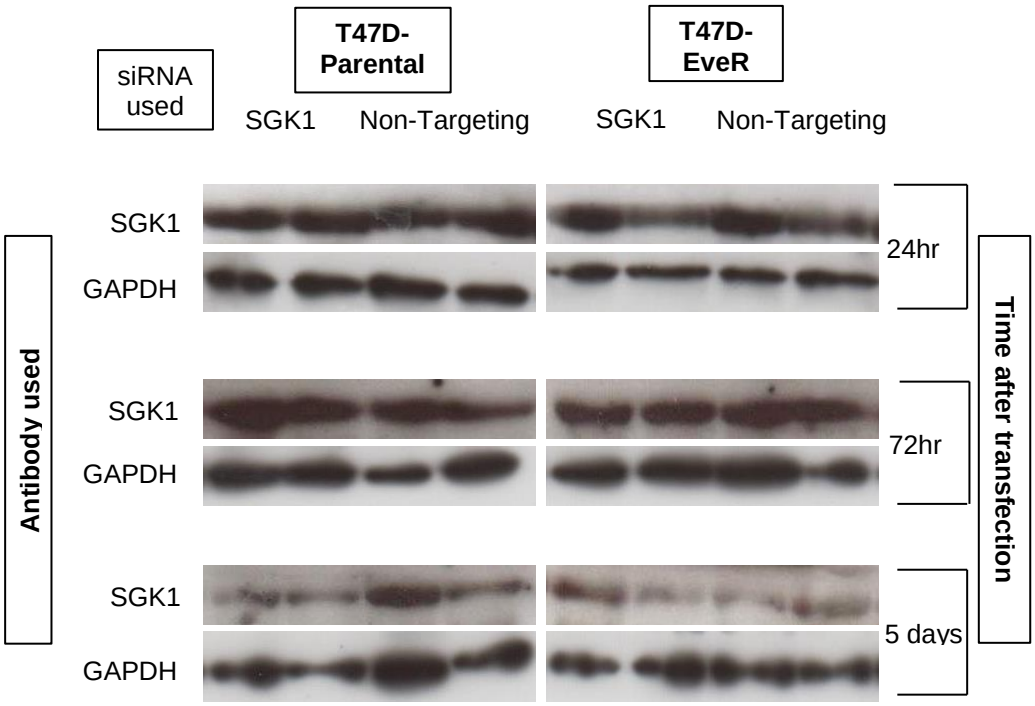


Figure 6.16: Single siRNA transfection does not noticeably down-regulate SGK1 expression. *Western blots for T47D-Parental and EveR, run in duplicate. Cells were transfected with either SGK1 siRNA or a non-targeting control, with lysates taken at intervals post-transfection, with these then studied via western blotting for SGK1, with GAPDH as a loading control. No decrease, relative to control, was seen in SGK1 protein in the SGK1 siRNA samples.*

Due to the lack of SGK1 knockdown after a single transfection method, a subsequent re-transfection was then tested to see if this could achieve the desired effect, with results for this seen in figure 6.17. This re-transfection also failed to achieve any form of significant knockdown for SGK1. Due to the fact that previous results (figure 5.4) had shown only small changes in SGK1 expression (in only 1 of the EveR lines), combined with the issues in using siRNA to knock it down and various time-constraints, work in this area only carried forward with down-regulating PKCα.

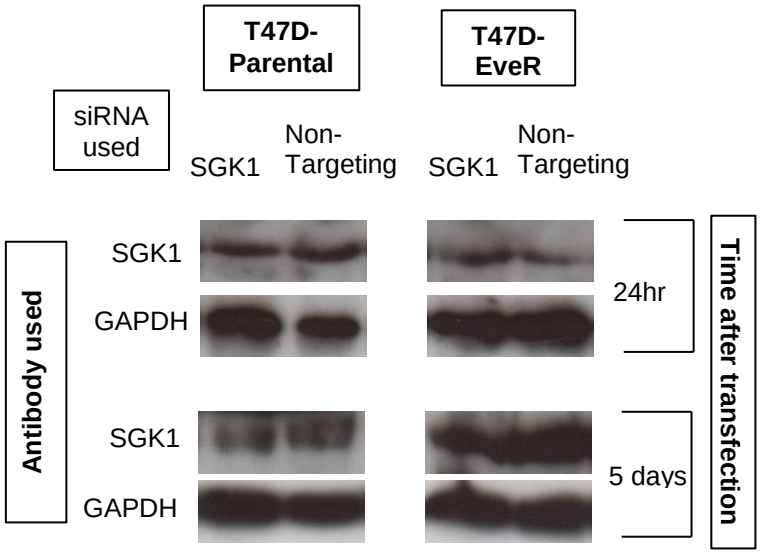


Figure 6.17: siRNA re-transfection does not noticeably down-regulate SGK1 expression. *Western blots for T47D-Parental and EveR using a subsequent re-transfection method. Cells were transfected, twice, two days in a row with either SGK1 siRNA or a non-targeting control, with lysates taken at intervals post-transfection, with these then studied via western blotting for SGK1, with GAPDH as a loading control. No decrease, relative to control, was seen in SGK1 protein in the SGK1 siRNA samples.*

6.3.8 - *PRKCA* Knockdown Combined with Everolimus Treatment

Once all preliminary testing and optimisations had been completed, both EveR and parental cell lines were used to test the effect of everolimus (5 day treatment) on cells treated with PRKCA siRNA, with the non-targeting (NT) siRNA used as a negative control. The SRB assay was used to assess the drugs effect as with previous experiments, and western blotting was used to check the efficiency of each knockdown, alongside each drug treatment. Results for these drug treatments can be found in figure 6.18 and 6.19.

Overall, neither EveR cell line showed a reduction in resistant phenotype with *PRKCA* (PKCα) down-regulated. Both T47D-EveR and 361-EveR (and their respective controls) showed no significant difference in their response to everolimus, when treated when PRKCA was *PRKCA* was down-regulated compared to the NT control.

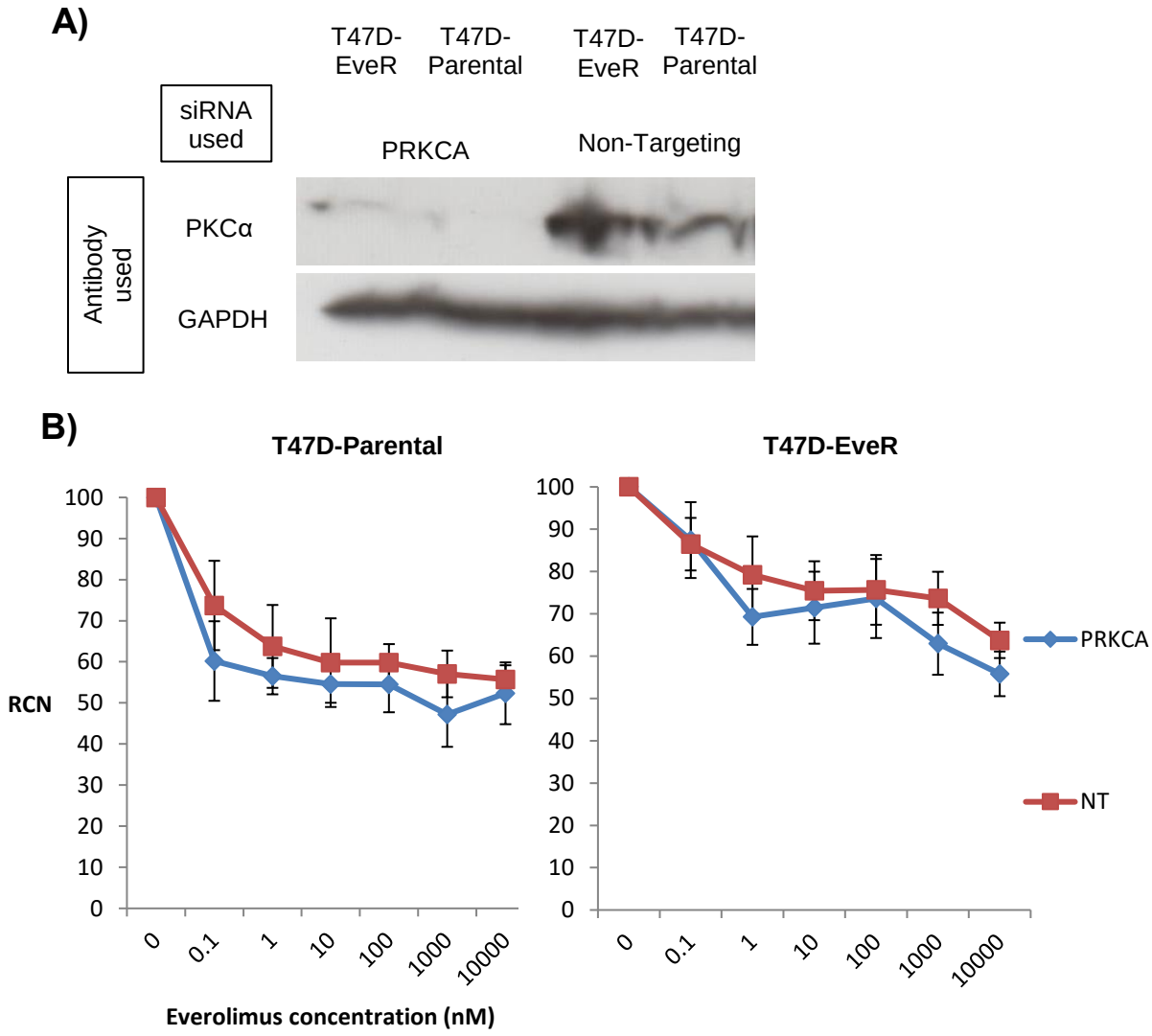


Figure 6.18: PKCα down-regulation does not reduce resistance to everolimus in T47D cells. *A) Representative western blots for T47D-EveR and parental. Cells were grown alongside side drug treatment plates with lysates harvested 5 days after transfection, to check individual knockdown efficiency for each set of plates. B) SRB results (plus or minus standard deviation, n=3 independent experiments) for T47D-EveR and parental cells, treated with everolimus for 5 days after transfection with either PRKCA siRNA or a non-targeting (NT) control siRNA. PRKCA knockdown using siRNA failed to reduce everolimus resistance in the T47D-EveR line.*

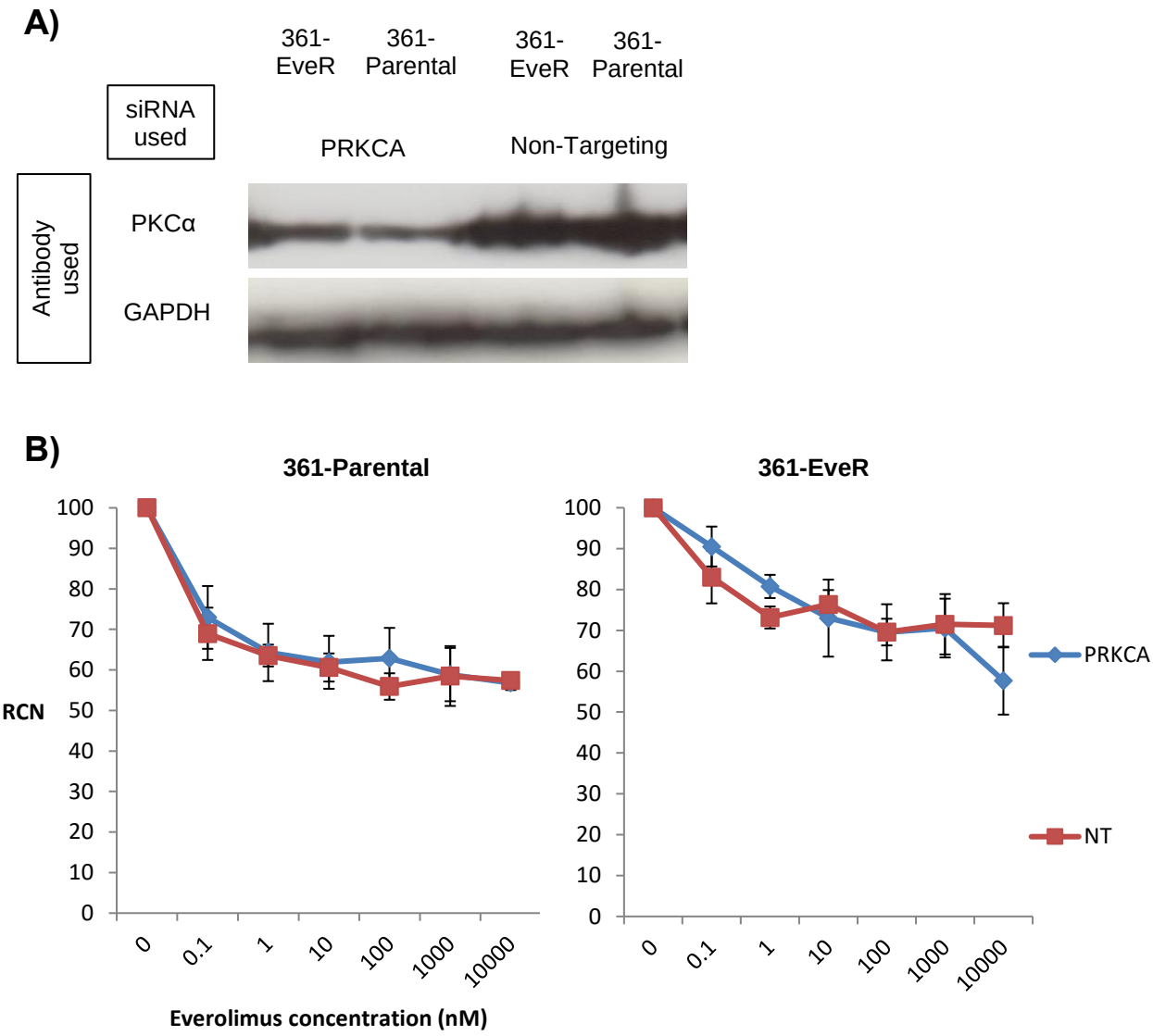


Figure 6.19: PKCα down-regulation does not reduce resistance to everolimus in 361 cells. *A) Representative western blots for 361-EveR and parental. Cells were grown alongside side drug treatment plates with lysates harvested 5 days after transfection, to check individual knockdown efficiency for each set of plates. B) SRB results (plus or minus standard deviation, n=3 independent experiments) for 361-EveR and parental cells, treated with everolimus for 5 days after transfection with either PRKCA siRNA or a non-targeting (NT) control siRNA. PRKCA knockdown using siRNA failed to reduce everolimus resistance in the 361-EveR line.*

6.3.9 - Determining Aldehyde dehydrogenase Activity in Drug Resistant Cells using an ALDEFLUOR Assay

In chapter 5, it was noted that EveR cells showed some characteristics of cells that were more dormant and more stem-like, including reduced proliferation, down-regulated signalling across various cascades/pathways and an increase in Wnt signalling. To determine if the cells had increased in stem-like phenotype, an ALDEFLUOR kit (Stemcell Technologies) was used to measure aldehyde dehydrogenase (ALDH) activity; a key biomarker for breast cancer stem cells (Ginestier et al, 2007; Liu et al, 2014b). Following staining, cell staining patterns were analysed by flow cytometry. Cells were first gated using forward and side scatter to eliminate cellular debris, and then further gated with side scatter and FITC, to detect ALDH activity, based on manufacturer's recommendations. Example flow cytometry plots for this gating method can be seen in figure 6.20.

Data from the ALDFELUOR flow cytometry experiments can be found in figure 6.21. Overall, ALDH activity did not significantly vary between T47D-EveR and T47D-Parental cells. T47D-Parental had a higher proportion of cells with ALDH activity, with 43.08% of cells having ALDH activity, compared to the T47D-EveR with an average of 33.59%. Both 361 cell lines showed a greater average activity than the T47D cells, with the 361-EveR having significantly higher activity than both T47D-EveR and T47D-Parental. Interestingly, the 361-EveR line had a significantly higher activity of ALDH compared to their parental controls; an average of 75.35% 361-EveR cells showed ALDH activity, compared to the 361-Parental cells having 57.85% average activity.

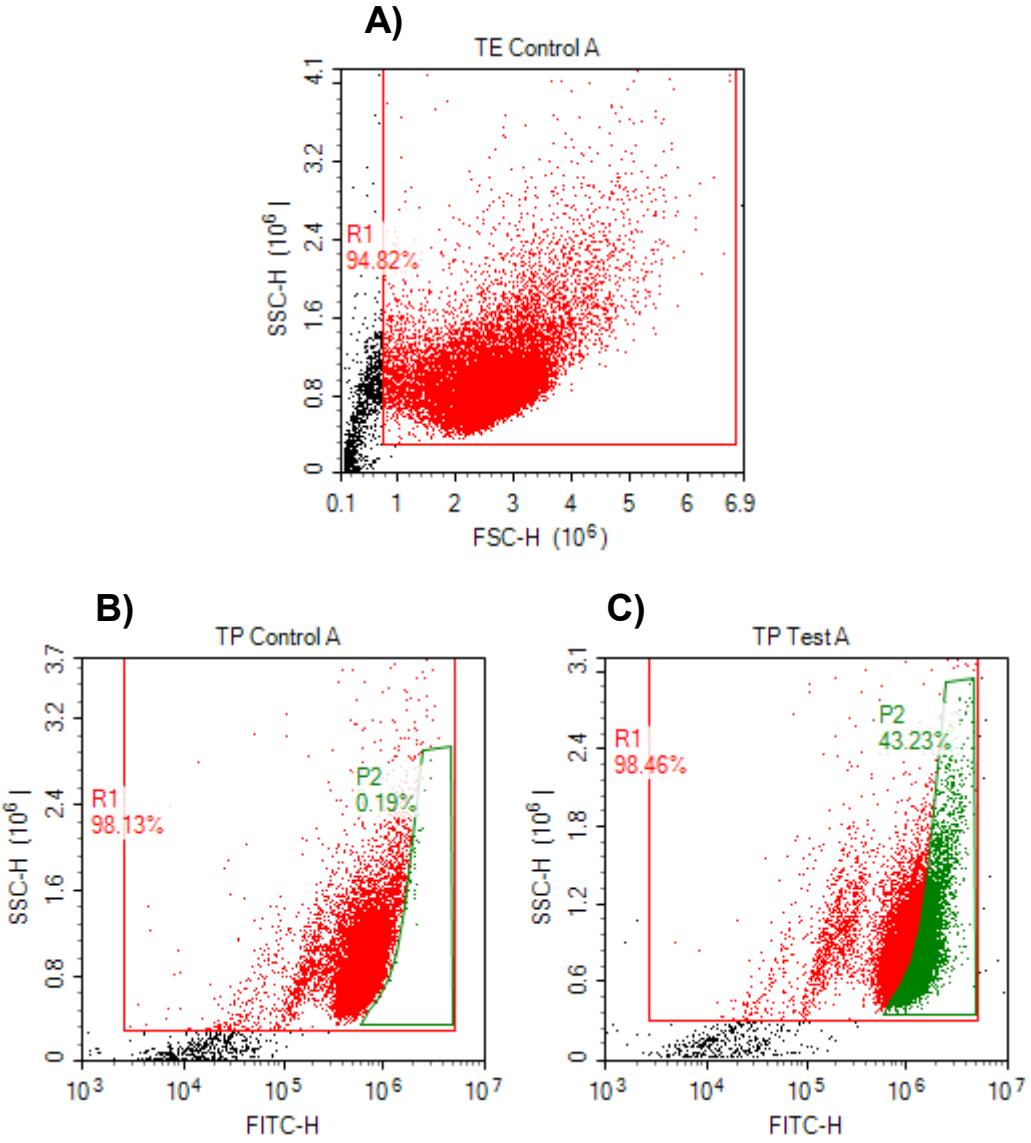


Figure 6.20: Example flow cytometry readout and gating method, for ACEA Novocyte flow cytometer, when using the ALDEFLUOR kit. *A) Using a control tube (containing an ALDH inhibitor) Cells were first gated (R1) using side and forward scatter, to gate out the majority of debris. B) Cells in gate R1 were then gated again (P2) using side scatter and FITC, to create a gate encompassing less than 1% of the cells in gate R1, and that was to the right of the majority of the cells. C) A sample's equivalent test tube (lacking the ALDH inhibitor) was then measured and the gates drawn from the control tube, copied onto the readouts for the test tube. The % cells in gate P2 (green) was then used as a measure of ALDH activity in that sample.*

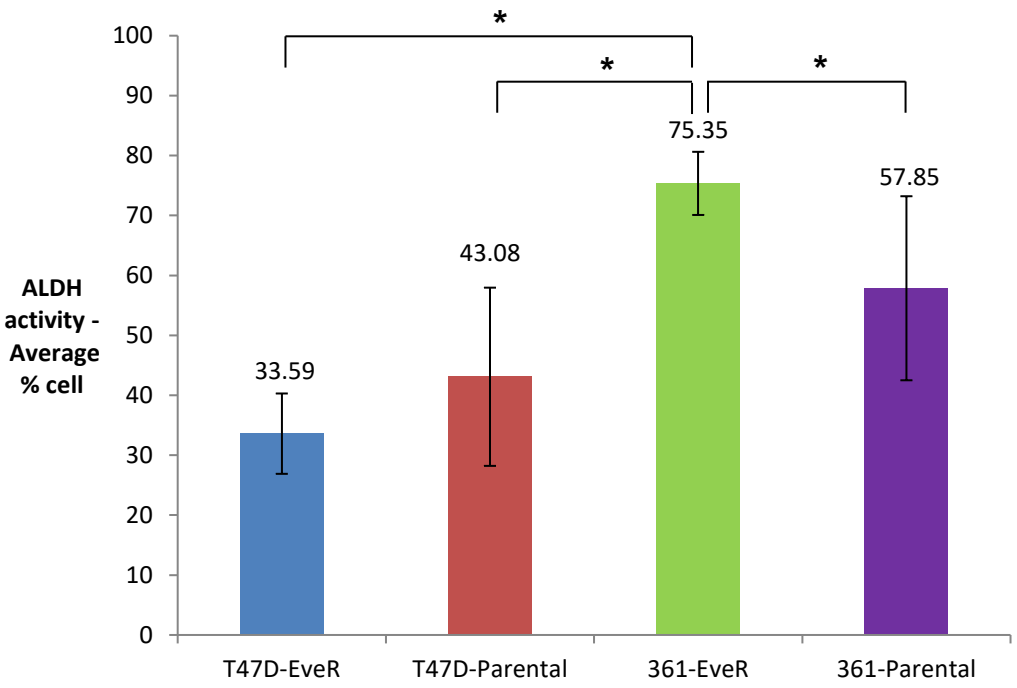


Figure 6.21: 361-EveR cells show significant increase in ALDH activity. *Flow cytometry data for T47D EveR and parental and 361-EveR and parental cells, using an ALDEFLUOR kit to measure % cells positive for ALDH activity (mean plus or minus standard deviation), n=3 independent experiments. * P<0.05. 361-EveR cells showed significantly more ALDH activity than the 361-Parental cells, suggesting a possible increase in stem-like phenotype in these cells. T47D-EveR and parental were not significantly different in terms of their ALDH activity.*

6.3.10 - Sanger Sequencing to Study Mutations in the FRB domain of mTOR and FKBP12

The dose-response to the rapalogues, everolimus, temsirolimus and rapamycin, was observed to be highly varied, in data gathered in sections 4.3.5 and 5.3.1; both EveR cell lines were resistant to everolimus, slightly less resistant to rapamycin and had very little resistance to temsirolimus. Since these drugs all act via the same mechanism, we theorised that mutations may have arisen in either *FKBP1A* (FKBP12) (which is the protein that rapalogues bind to before binding mTOR, to inhibit it) or the FRB domain of *MTOR* (which binds the complex of rapalogue-FKBP12) thereby affecting binding of the individual drugs. Sanger sequencing was used to analyse both regions, with the EveR cells compared to their parental controls, as well as published sequences (NCBI, 2018a; NCBI 2018b; UniProt, 2018)

Sequence data for the FRB domain (figure 6.22) revealed no mutations along the length of it (399bp/133amino acids) in either EveR or parental control lines. This included not only no differences in protein sequence, but also no difference in any base pairs (see appendix for full sequence), compared to the published sequence (wt) (NCBI, 2018a; UniProt, 2018). Sequencing was successful along the entire FRB domain for all samples, apart from 361-Parental, in which the first 48 nucleotides (16 amino acids) failed to sequence properly.

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---------+---------+---------+---------+---------+---------+---------+---------+
  10      20      30      40      50      60      70      80
---------+---------+---------+---------+---------+---------+---------+---------+
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FRB domain (wt) VSEELIRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKS
T47D-EveR VSEELIRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKS
T47D-Parental VSEELIRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKS
361-EveR VSEELIRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKS
361-Parental _____HEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKS

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---------+---------+---------+---------+---------+---
  90      100     110     120     130
---------+---------+---------+---------+---------+---
```

FRB domain (wt) GNVKDLTQAWDLYYHVFRRISKQLPQLTSLELQYVSPKLLMCRDLELAVPGTY
T47D-EveR GNVKDLTQAWDLYYHVFRRISKQLPQLTSLELQYVSPKLLMCRDLELAVPGTY
T47D-Parental GNVKDLTQAWDLYYHVFRRISKQLPQLTSLELQYVSPKLLMCRDLELAVPGTY
361-EveR GNVKDLTQAWDLYYHVFRRISKQLPQLTSLELQYVSPKLLMCRDLELAVPGTY
361-Parental GNVKDLTQAWDLYYHVFRRISKQLPQLTSLELQYVSPKLLMCRDLELAVPGTY

Figure 6.22: FRB domain of all cell lines matches published data. *Sanger sequencing data for the MTOR FRB domain (133 amino acids) in T47D-EveR, T47D-Parental, 361-EveR and 361-Parental cell lines. Sequences were generated and then aligned using DNASTAR Lasergene (Megalign) and the clustal W method. Nucleotides were then converted to protein sequences. No change of any amino acid was observed in any cell line, compared to the published sequence (wt) (NCBI, 2018a; UniProt, 2018).*

The full length of FKBP12 (*FKBP1A*) was studied next for any mutational changes that could affect rapalogue binding, since this acts essentially as an intracellular receptor for rapalogue. Similar to sequencing done on the FRB domain, sequences for the samples were compared to one another, and to published sequences of FKBP12 (NCBI 2018b). T47D-EveR, T47D-Parental and 361-Parental showed no variation in sequence from the published sequence of FKBP12 (figure 6.23), with all base pairs matching in these samples (full sequences in the appendix).

Initial sequencing of 361-EveR showed great variation from the published sequence (figure 6.23 for protein sequence and 6.24 for nucleotide sequence). This initial sample showed a total of 24 nucleotide alterations across the length of the gene, plus a deletion of a cysteine residue at position 132 (bp) and a 13 nucleotide addition at position 216 (bp). As a result of these mutations, including the cysteine deletion, a stop codon was now found 56 codons in to the sequence; suggesting that in this sample a truncated version of FKBP12 could be translated.

To confirm whether these mutations were present in multiple experimental repeats in the 361-EveR cell line, a second set of cDNA was analysed, taken at a similar point to the previous set, but from a different cell culture flask. This second sequence generated for 361-EveR showed no nucleotide variation from the published sequence (figure 6.25).

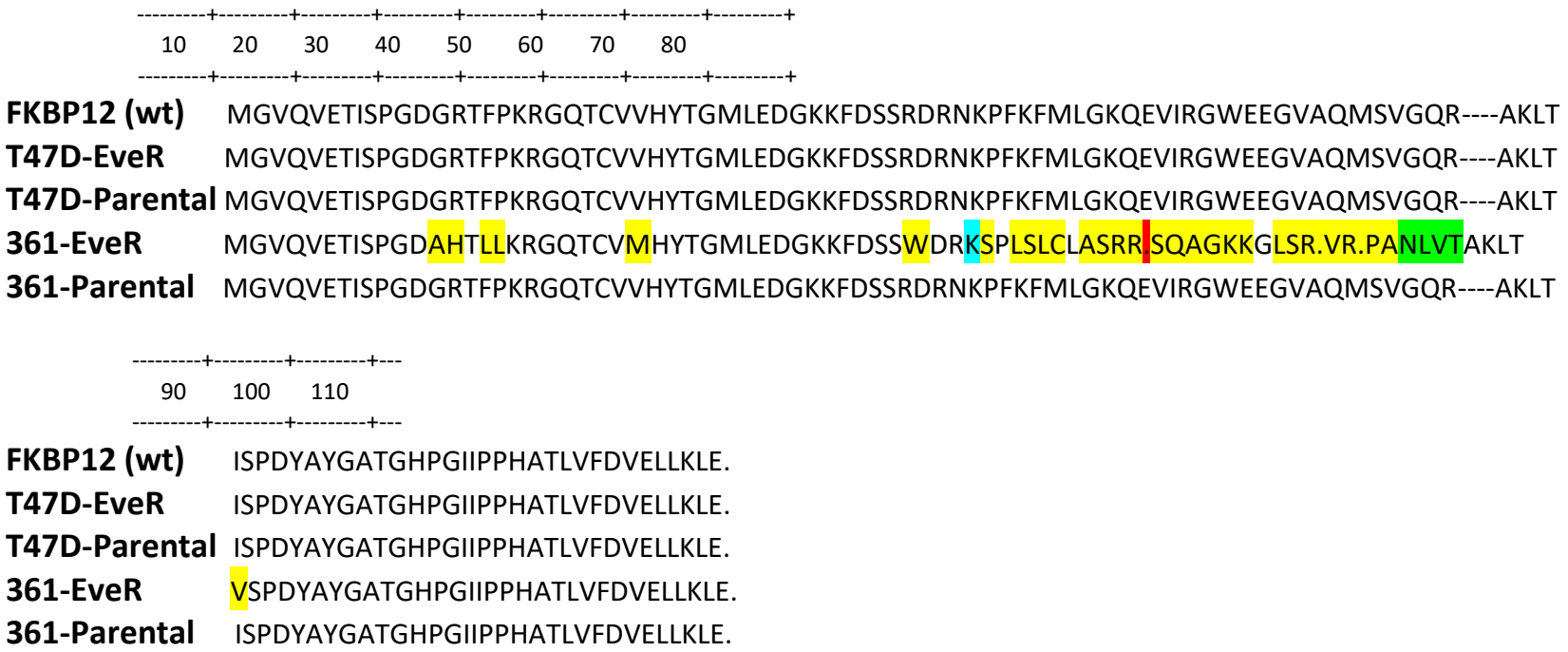

Figure 6.23: FKBP12 of 361-EveR shows mutations. *Sanger sequencing data for FKBP12 (FKBP1A) (109 amino acids) in T47D-EveR, T47D-Parental, 361-EveR and 361-Parental cell lines. Sequences were generated and then aligned using DNASTAR Lasergene (Megalign) and the clustal W method. Nucleotides were then converted to protein sequences. No change of any amino acid was observed in T47D-EveR and parental and 361-Parental, compared to the published sequence (wt) (NCBI 2018b). 361-EveR varied from the wt with multiple changes across the sequence in terms of amino acid differences (yellow), including the addition of new nucleotides to the sequence (green). A single deleted nucleotide (C) was detected, creating a frameshift from the lysine (K) (blue), introducing a stop codon after 55 amino acids (red).*

```
---------+---------+---------+---------+---------+---------+---------+---------+
   10      20      30      40      50      60      70      80
---------+---------+---------+---------+---------+---------+---------+---------+
```

FKBP12 (wt) ATGGGAGTGCAGGTGGAAACCATCTCCCCAGGAGACGGGCGCACCTTCCCCAAGCGCGGCCAGACCTGCGTGGTGCACTA

361-EveR ATGGGAGTGCAGGTGGAAACCATCTCCCCAGGAGACGCGCACACCCTCCTGAAGCGCGGCCAGACCTGCGTGATGCACTA

```
---------+---------+---------+---------+---------+---------+---------+---------+
   90     100     110     120     130     140     150     160
---------+---------+---------+---------+---------+---------+---------+---------+
```

FKBP12 (wt) CACCGGGATGCTTGAAGATGGAAAGAAATTTGATTCCTCCCGGGACAGAAACAAGCCCTTTAAGTTTATGCTAGGCAAGC

361-EveR CACCGGGATGCTTGAAGATGGAAAGAAATTTGATTCCTCCTGGGACAGAAA_AAGCCCTTTAAGCTTATGCTTGGCAAGC

```
---------+---------+---------+---------+---------+---------+---------+---------+
   170     180     190     200     210     220     230     240
---------+---------+---------+---------+---------+---------+---------+---------+
```

FKBP12 (wt) AGGAGGTGATCCGAGGCTGGGAAGAAGGGGTTGCCCAGATGAGTGTGGGTCAGAGA----------GCCAAACTGACT

361-EveR AGGAGGTGATCCCAGGCTGGGAAGAAAGGGTTGTCCAGATGAGTGCGGTGACCCGCCAATTTAGTGACAGCCAAACTGACT

```
---------+---------+---------+---------+---------+---------+---------+---------+
   250     260     270     280     290     300     310     320
---------+---------+---------+---------+---------+---------+---------+---------+
```

FKBP12 (wt) ATATCTCCAGATTATGCCTATGGTGCCACTGGGCACCCAGGCATCATCCCACCACATGCCACTCTCGTCTTCGATGTGGA

361-EveR GTATCTCCAGATTATGCCTACGGTGCCACTGGGCACCCAGGCATCATCCCACCACACGCCACTCTCGTCTTCGATGTGGA

```
          ---------+---------
              330
          ---------+---------
FKBP12 (wt)  GCTTCTAAAACTGGAATGA
361-EveR     GCTTCTAAAACTAGAATGA
```

Figure 6.24: FKBP12 of 361-EveR shows mutations. *Sanger sequencing data for FKBP12 (FKBP1A) (327 nucleotides) in 361-EveR cells, compared to the published sequence (wt) (NCBI 2018b). Sequences were generated and then aligned using DNASTAR Lasergene (Megalign) and the clustal W method. 361-EveR cells show multiple mutations across its length, compared to the wt, including 24 nucleotide changes (yellow), deletion of a single cysteine at position 132bp (blue), and a 13 nucleotide addition at position 216bp (green).*

```
---------+---------+---------+---------+---------+---------+---------+---------+
   10      20      30      40      50      60      70      80
---------+---------+---------+---------+---------+---------+---------+---------+
```

FKBP12 (wt) ATGGGAGTGCAGGTGGAAACCATCTCCCCAGGAGACGGGCGCACCTTCCCCAAGCGCGGCCAGACCTGCGTGGTGCACTA
361-EveR ATGGGAGTGCAGGTGGAAACCATCTCCCCAGGAGACGGGCGCACCTTCCCCAAGCGCGGCCAGACCTGCGTGGTGCACTA

```
---------+---------+---------+---------+---------+---------+---------+---------+
   90      100     110     120     130     140     150     160
---------+---------+---------+---------+---------+---------+---------+---------+
```

FKBP12 (wt) CACCGGGATGCTTGAAGATGGAAAGAAATTTGATTCCTCCCGGGACAGAAACAAGCCCTTTAAGTTTATGCTAGGCAAGC
361-EveR CACCGGGATGCTTGAAGATGGAAAGAAATTTGATTCCTCCCGGGACAGAAACAAGCCCTTTAAGTTTATGCTAGGCAAGC

```
---------+---------+---------+---------+---------+---------+---------+---------+
   170     180     190     200     210     220     230     240
---------+---------+---------+---------+---------+---------+---------+---------+
```

FKBP12 (wt) AGGAGGTGATCCGAGGCTGGGAAGAAGGGGTTGCCCAGATGAGTGTGGGTCAGAGAGCCAAACTGACTATATCTCCAGAT
361-EveR AGGAGGTGATCCGAGGCTGGGAAGAAGGGGTTGCCCAGATGAGTGTGGGTCAGAGAGCCAAACTGACTATATCTCCAGAT

```
---------+---------+---------+---------+---------+---------+---------+---------+-------
   250     260     270     280     290     300     310     320
---------+---------+---------+---------+---------+---------+---------+---------+-------
```

FKBP12 (wt) TATGCCTATGGTGCCACTGGGCACCCAGGCATCATCCCACCACATGCCACTCTCGTCTTCGATGTGGA GCTTCTAAAACTGGAATGA
361-EveR TATGCCTATGGTGCCACTGGGCACCCAGGCATCATCCCACCACATGCCACTCTCGTCTTCGATGTGGA GCTTCTAAAACTGGAATGA

Figure 6.25: Sequencing of a 2nd set of cDNA for 361-EveR reveals no mutations in FKBP12. *Sanger sequencing data for FKBP12 (FKBP1A) (327 nucleotides) in 361-EveR cells, compared to the published sequence (wt) (NCBI, 2018b). Sequences were generated and then aligned using DNASTAR Lasergene (Megalign) and the clustal W method. This 2nd set of sequencing for 361-EveR revealed no mutations compared to wt.*

6.3.11 - Determining Expression Patterns of FKBP12

As sequencing had given varying results as to whether 361-EveR cells did show mutations in *FKBP1A*, expression studies were then carried out. qPCR and western blotting were used to determine expression levels of *FKBP1A*/FKBP12 and therefore determine if the truncated FKBP12 predicted in one of the 361-EveR sequences generated, but not the other, was present.

qPCR data (figure 6.26) showed small but non-significant differences in gene expression of *FKBP1A*. Both T47D-EveR cells compared to their parental controls (P=0.36) and 361-EveR cells compared to their parental controls (P=0.09), showed a slightly higher expression of *FKBP1A* at a gene level. It should be noted that gene expression levels of *FKBP1A* for all samples were quite low, compared to the expression of other mTOR related genes (see section 5.3.3). For example, the lowest expression of *RAPTOR* in all four cells lines showed an RQ value of around 0.05, whilst the highest expression of *FKBP1A* was around 0.01 RQ value.

Western blotting for FKBP12 (figure 6.27) revealed a similar trend, with both EveR cell lines expressing noticeably higher levels of the protein compared to parental controls. Despite increasing exposure times, no experimental repeats for 361-EveR (or any other cell line) revealed any second band to show the presence of a truncated version of FKBP12, with all 361-EveR samples showing expression of the full length protein (12kDa), suggesting the mutations seen in the first 361-EveR sample sequenced were merely a one off set of mutations in that sub culture, rather than in the stock for that cell line.

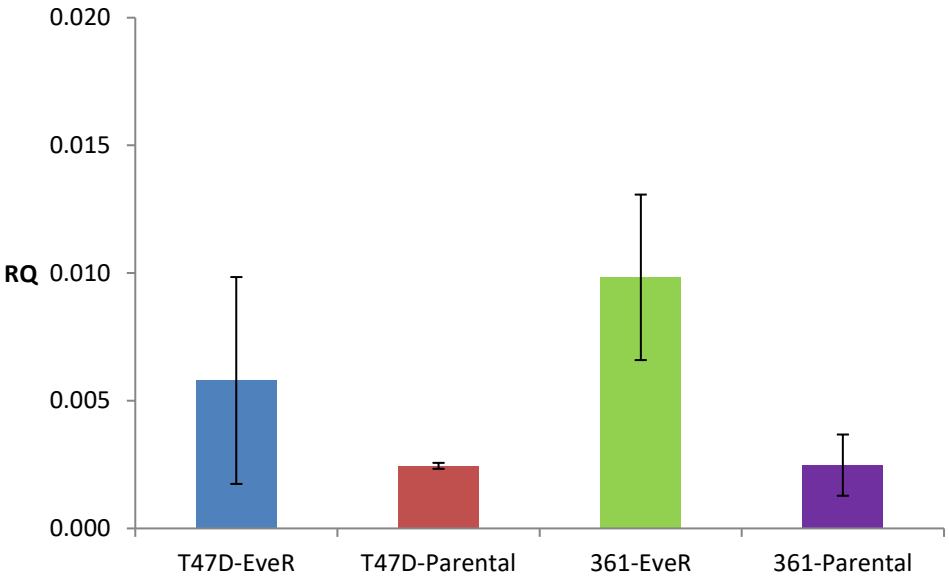

Figure 6.26: EveR cells show greater gene expression of FKBP12. *qPCR results for FKBP1A (FKBP12) expression (mean plus or minus standard deviation, n=2 independent experiments), with data displaying RQ (relative quantity). Both EveR cell lines show small (but not significant) increases in gene expression of FKBP1A over their parental controls. T47D-EveR compared to parental cells, P=0.36, 361-EveR compared to parental cells, P=0.09.*

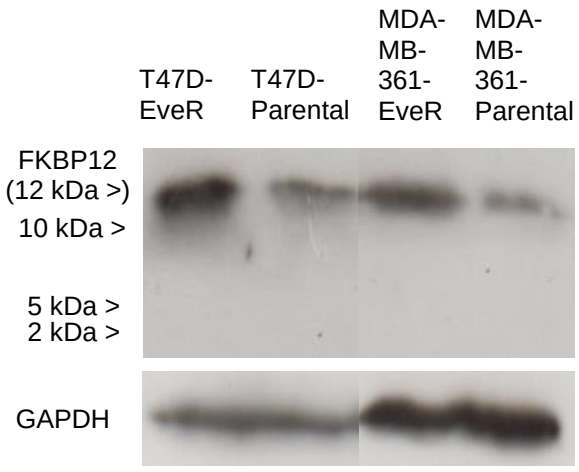

Figure 6.27: EveR cells show greater protein expression of FKBP12. *Western blots for T47D-EveR, T47D-Parental, 361-EveR and 361-Parental cell lines. n=2 independent experiments FKBP12 expression levels were studied with GAPDH as a loading control. All cell lines showed FKBP12 expression at the expected size (12 kDa) but no extra or alternative bands were seen at lower weights. Both EveR cell lines showed increased levels of FKBP12 protein.*

6.4 - Discussion

The work presented in this chapter sought to continue on from the previous section of research, by exploring key characteristics of the EveR cells, and testing these aspects further to discern if they can relate to a mechanism for everolimus resistance.

Firstly, a range of standard chemotherapeutic drugs were tested on the EveR cells to look for resistance in drugs acting on targets away from the mTOR pathway. If resistance was discovered, this would suggest that the EveR cells may be using expression of multi-drug resistance genes, such as P-glycoprotein (Choi and Yu, 2014), to cause resistance to everolimus. However, as the data revealed, both EveR cell lines showed similar, and in most cases identical, sensitivity to these drugs, as their parental counter-parts. Overall this suggests that the resistance observed to some rapalogues, is probably not related to multi-drug resistance, and that the likelihood of there being an alteration of a common MDR protein (e.g. p-glycoprotein), is very low. Therefore the mechanism is more likely to be related to the mTOR pathway. Research studying rapalogue resistance in breast cancer has not yet found MDR phenotypes to cause resistance to these drugs. Our data, along with the previous body of work, therefore suggests that MDR phenotypes may not be as relevant to rapalogue resistance in future research.

The up-regulation of mTORC2 signalling was explored as possible mechanism for everolimus resistance in these cell models. As had been shown in the previous chapter, T47D-EveR cells showed an up-regulation of components needed for mTORC2 signalling and phospho-/proteins that were downstream of mTORC2, mainly PKCα and SGK1 (although no up-regulation of Akt was observed). I hypothesised that since mTORC1 and 2 signalling are closely related, it was possible that mTORC2 signalling was possibly compensating for the dampening of mTORC1 signalling, caused by continual everolimus treatment. For this reason, siRNA transfections were used to test this hypothesis, with the down-regulation of PKCα combined with everolimus treatment. Unfortunately, this data set revealed that cells with down-regulated PKCα were not more susceptible to everolimus treatment, with the resistance phenotype unchanged.

Since phospho-/PKCα was noticeably up-regulated in the T47D-EveR cells, it came as some surprise that this up-regulation did not appear to be involved in the everolimus resistant phenotype, especially considering how closely related the two pathways of mTORC1 and 2 signalling are. Unfortunately I was unable to successfully down-regulate SGK1 using the siRNA transfection method that we employed, meaning that we could not test whether SGK1 was more related to everolimus resistance than PKCα. However, the data gathered in the previous chapter showed that SGK1 was only slightly up-regulated in terms of protein expression level, and this was only in the T47D-EveR cell line. This suggested that PKCα was far more likely to be related to the resistance mechanism, rather than SGK1.

In light of the siRNA data gathered here, the question remains as to why the EveR cells have up-regulated mTORC2 components and related signalling molecules. The T47D-EveR cells had shown a relatively clear alteration of mTORC2 signalling, with these cells expressing greater levels of rictor and p-mTOR (Ser2481), with the former being integral for mTORC2 activity and the latter shown by Copp and colleagues (2009) to be a good biomarker for mTORC2 complexes. Since the exact role of mTORC2 signalling in the cell is not as well defined as mTORC1 (with mTORC1 being shown to be clearly involved in various signalling pathways), it is possible that mTORC2 targets/pathways away from the AGC kinases (Akt, SGK1, PKCα) could be partially responsible for the resistance phenotype.

mTORC2 has been shown in some research to have roles with other proteins and pathways. For example, research has shown that mTORC2 can phosphorylate various HDACs, which in turn has been shown in glioblastomas to promote the Warburg effect, via eventual regulation of c-myc (Masui et al, 2013). Since the Warburg effect and the metabolic reprogramming of cancer cells is important for producing many building blocks for cell growth in tumours, mTORC2 activity may prove to be more important in carcinogenesis as future work elaborates on its role in this effect (Courtnay et al, 2015; Masui et al, 2013; Masui et al, 2014). Research by DeStefano and Jacinto (2013) has also shown that mTORC2 can phosphorylate Fbw8/Fbxw8 (on Ser86), which itself is a subunit of a ubiquitin ligase complex. This

piece of research showed that this interaction helped control the levels of IRS-1, which suggests that both mTOR complexes are involved in regulating IRS-1 (Magnuson et al, 2012). Further research with our *in vitro* models of everolimus resistance could help discern if mTORC2 has an exact role in everolimus resistance, if any at all. siRNA down-regulation of rictor (combined with everolimus treatment), could help to establish if the resistance is related to the up-regulation of mTORC2 components. If this hypothesis was proven correct, further exploration of other mTORC2 targets could then be explored. Previous research has already suggested that myc may be involved in everolimus resistance (Bihani et al, 2015), therefore this may be a logical next target for siRNA transfection testing, once western blotting has established if its expression is altered in these cells.

As suggested by the data gathered here, mTORC2 up-regulation may in fact not be related to everolimus resistance. In fact experiments using the LT everolimus resistance model, also suggests that mTORC2 may not be important for long-term everolimus resistance in this particular model. All key mTORC2 components and down-stream targets that were up-regulated in the T47D-EveR cells, were again studied using western blotting in the T47D-EveR-LT cell line, comparing them to a parental control cell line. These results showed that despite being up-regulated in the T47D-EveR cell line, the major mTORC2 proteins and targets studied, were no longer over-expressed in the T47D-EveR-LT cell line (compared to parental controls). These changes had occurred at some point during the development of the LT cell line, despite the fact that their responsiveness to everolimus had not changed along the process, suggesting the phenotype and possibly the original mechanism of resistance was also unchanged. Since the long term resistance model had retained its everolimus resistance but lost the up-regulation of mTORC2, this would suggest that mTORC2 up-regulation may not be critical for the everolimus resistance phenotype and gives further evidence that it is indeed not part of the resistance mechanism. Alternatively, it could suggest that the up-regulation of mTORC2 relates to a transitory phenotype that is involved in the development of everolimus resistance, but is not required for its overall maintenance.

CSCs in breast cancer are now relatively well studied, with their role within cancer progression becoming more clarified as research progresses. Since CSCs can help

a tumour to survive after chemotherapy, propagate a tumour and allow it to disseminate and metastasise (Liu et al, 2014b; Luo et al, 2015b), the presence of cells possessing a CSC-like phenotype (stemness) is extremely important. In the previous chapter the EveR cells had shown a number of characteristics which suggested that they may have gained a more stem-like phenotype, including the down-regulation of numerous proteins/pathways, a decrease in proliferative rate (suggesting they may have a more dormant phenotype than before) and the increase of proteins associated with Wnt signalling. One of the most readily studied biomarkers for breast CSCs is ALDH, so the activity of this enzyme was checked in the EveR cells and compared to the parental controls using an ALDEFLUOR kit, provided by Stemcell Technologies.

Results for these experiments revealed that whilst the T47D-EveR cell line did not show a significant difference in ALDH activity from the controls, the 361-EveR cell line did, showing significantly higher activity than all other cell lines studied. This adds evidence to our hypothesis that the development of a resistant phenotype was accompanied with changes that have made the cells more stem-like, giving them a phenotype closer to that of CSCs. Whilst the T47D-EveR cells were not positive for the increase in this biomarker, this does not necessarily mean that they are not more stem-like as well. The expression of CD44+, CD24- is the other set of key biomarkers for breast CSCs and future experiments studying these in the EveR models, using techniques such as flow cytometry (Liu et al, 2014b; Yenigun et al, 2013) would further help determine how stem-like these lines are. Since breast CSCs have been shown to sometimes express either ALDH+ or CD44+/CD24-, reflecting the ability to transition from an epithelial to mesenchymal phenotype and vice versa (Liu et al, 2014b; Luo et al, 2015b), it is possible that the T47D-EveR cells are low in ALDH activity but express CD44+/CD24-, and are thus still stem-like. If this is indeed the case it would further indicate, along with the results in this (and the previous) chapter, that everolimus resistance can be accompanied with cellular and molecular changes that make the cells overall more dangerous and harder to treat as they have gained greater stemness. Beyond this point, future studies looking at the stemness of everolimus resistant patient samples would help determine if the changes noted in our *in vitro* models, are also clinically relevant.

Finally, in relation to the observed differential response to the varying rapalogues, mutations in both FKBP12 and the FRB domain of mTOR were checked using sequencing. Rapalogues generally first bind to FKBP12, before the complex of drug and FKBP12 binds to the FRB domain of mTOR, to inhibit mTORC1 activity (Brown et al, 1994; Choi et al, 1996). We hypothesised that mutations may exist in either of these protein regions since they mediate the rapalogues biological response, thus explaining why drugs with the same mechanism of action, show such a different response in our EveR models. Sanger sequencing of the mTOR FRB domain and the entire length of FKBP12 found no mutations in these regions in either T47D-EveR or parental cell line and none in the 361-Parental cells. 361-EveR cells showed no mutations in mTOR but for 1 of the 2 samples studied, did show mutations in FKBP12, which predicted a truncated protein. Since western blotting, with samples taken at a similar time to the cDNA used for sequencing, revealed no truncated protein, it was determined that mutations in the lone sample were most likely isolated (in only that sub-culture used for that particular set cDNA) and were not reflective of the stock of 361-EveR cells.

Whilst no mutations were observed overall, western blotting and qPCR results showed that both T47D-EveR and 361-EveR cell lines had up-regulated FKBP12, compared to parental controls. This was to a very noticeable degree at protein level, and to a lesser extent at a transcriptional level. This result was contrary to expectation, as logically it had been expected that FKBP12 may be down-regulated in the resistant lines, which would have explained why they were more resistant, as they would possess less of the receptor needed to mediate rapalogue response. The role this up-regulation plays in the EveR cells is therefore to be debated.

One objective of sequencing and studying the expression levels of FKBP12, had been to determine if this area of the pathway had been altered and could relate to the mechanism of resistance in these cells. With the outcome lacking any mutations in the two areas and there being an up, not down-regulation of FKBP12, it would seem that this set of data does not help elucidate all or part of the resistance mechanism. As the receptor/FKBP12 is still present, in its full length, everolimus and other rapalogues can still act via it to inhibit mTORC1, therefore this alteration does not help explain resistance. Previous studies have shown that FKBP12 tends not to

bind to the FRB domain of mTOR, unless in the presence of a rapalogue (Chen et al. 1995). Rapamycin has been shown to bind mTOR without FKBP12, but the affinity for mTOR is around 2000 fold lower than when rapamycin is bound to FKBP12, therefore FKBP12 helps facilitate rapalogue binding, rather than acting as a traditional receptor (Banaszynski et al, 2005). One hypothesis could be that resistance to rapalogues could relate to the extra FKBP12 binding to mTOR, without rapalogue present, however these previous studies would indicate that this is unlikely.

Whilst this data does not help determine the everolimus resistance mechanism, it may help explain why there was a differential response to the three different rapalogues studied here. As shown in the previous chapter, the EveR cells of course show significant resistance to everolimus, and similar/slightly lower levels of resistance to rapamycin. However resistance to temsirolimus was extremely limited and muted by comparison. The binding affinities (or dissociation constants/ K_d) of each rapalogue to FKBP12 is different. Rapamycin shows the greatest affinity for FKBP12 of the three tested, with a K_d=0.8nM (Banaszynski et al, 2005), whilst everolimus shows a slightly lower affinity with a K_d=1.8-2.6nM (MacKeigan and Krueger; Schuler et al, 1997). The affinity for temsirolimus to FKBP12 was calculated from work presented by Hoy and McKeage (2010), and was calculated to be K_d=4.95nM, giving it the lowest affinity for FKBP12, of the three. This makes the affinity for FKBP12 just over 6 fold lower for temsirolimus, compared to rapamycin, and 2-3 fold lower compared to everolimus.

Because of temsirolimus' far lower binding affinity, increased FKBP12 levels would in theory favour this drug to bind more FKBP12, thus possibly explaining its increased effectiveness over the other rapalogues. However it seems unlikely that the increased levels are detrimentally affecting the binding or action of everolimus. It is possible that the increased FKBP12 is in fact aiding us in countering the unknown mechanism of everolimus resistance; in that the increased FKBP12 is allowing an increased temsirolimus response due to its lower binding affinity, (now there is more receptor) despite the unknown mechanism being in place that is still holding back the response to everolimus (and rapamycin). If FKBP12 up-regulation is common in everolimus resistant breast cancer cells then then temsirolimus could be tested as a

useful alternative to everolimus. Future work studying everolimus resistance in breast cancer patients would help to determine if the features noted in these models are present at a patient level, such as studying FKBP12 levels or temsirolimus response in samples from everolimus resistant patients.

Chapter 7: Discussion

7.1 - mTOR Signalling and Inhibition in Breast Cancer and the Implications of Everolimus Resistance

The scope of this research was to explore mTOR signalling in breast cancer, in relation to the use of everolimus, as this is currently the only mTOR inhibitor approved for patients with this disease. Overall, this thesis aimed to explore resistance to everolimus using *in vitro* models.

mTOR signalling is a critical regulator of growth in the cell, with the down-stream actions of mTORC1 centrally involved in the control of translation and autophagy (Laplante and Sabatini, 2012). Because of this, mTOR signalling up-regulation has been shown to be advantageous towards the growth of a tumour (Chiang and Abraham, 2007; Xu et al, 2014). In breast cancer, mTOR signalling is often over-active, primarily due to changes up-stream of mTOR itself. Growth factor receptors and pathways that are frequently mutated or altered in breast cancer, including proteins in PI3K signalling and receptors of the HER/ErbB family (e.g. HER-2), lie directly up-stream of mTOR (Dibble and Cantley, 2015; Margariti et al, 2011; Wiza et al 2012). mTOR signalling is also involved in resistance to therapies that target areas such as HER-2 signalling (Margariti et al, 2011).

Since mTOR has such a central position in many critical signalling pathways in breast cancer, it is of course a valuable target for therapeutics to treat this disease. As such, drugs targeting mTOR are well explored in breast cancer, with clinical trials testing various rapalogues having been explored. Everolimus has been approved for use in breast cancer since 2012; with this being the first mTOR targeted therapy used in breast cancer (European Medicines Agency, 2016a). Whilst many drug targets are kinases like mTOR, almost all use of such therapies is eventually associated with drug resistance. Currently, resistance to everolimus in patients has not yet been reported; however *in vitro* evidence is beginning to suggest that everolimus treatment will most likely be associated with this undesired effect (Bihani et al, 2015; Sarbassov et al, 2004; Sun et al, 2005). It is from this basis that this

research looked to build, by creating our own everolimus resistant cell models, and characterising them in order to understand what may be contributing to resistance and thereby allowing for the testing of strategies to overcome resistance.

7.2 – Confirming and characterising mTOR Signalling in Breast Cancer Cells

Whilst mTOR signalling is relatively well understood in breast cancer cells, the base expression of many mTOR components has not wholly been explored in various cell lines, including some of the key scaffolding proteins. On top of this, few papers have consolidated this information and looked to characterise the levels and activity of the two mTOR complexes in a range of breast cancer cells. This information would not only benefit breast cancer research into mTOR signalling, but it may also help to understand in what types of cancer cells mTOR inhibitors act best against.

mTOR functions in two distinct complexes, mTORC1 and mTORC2. Both of course have the kinase mTOR, mLST8 (believed to be needed for kinase function) and the inhibitor DEPTOR. From this point the two complexes differ with raptor being a key scaffolding protein for mTORC1 whilst rictor is the equivalent protein in mTORC2 (Huang and Fingar, 2014; Laplante and Sabatini, 2012). Downstream, mTORC1's primary role is in controlling translation which it does via the phosphorylation of S6K and 4E-BP (Gingras et al, 2001; Ma and Blenis, 2009). mTORC2 does not sit perfectly in a pre-defined signalling pathway, but does however have the ability to phosphorylate and help control AGC kinases like Akt (Sarbassov et al, 2005), PKCα (Angliker and Ruegg, 2013; Jacinto et al, 2004) and SGK1 (Mori et al, 2014). The various proteins mentioned here are key in both activating mTOR signalling and in carrying out its effects, and their expression and activation was examined in this research.

Data from this research indicated that all breast cell lines studied had active mTOR signalling, but that the protein levels of various mTOR components and phosphorylated mTOR targets (and thus active components), varied dramatically between each cell line. Variation in active mTOR signalling is to be expected in cell lines of varying breast cancer subtype, and does not necessarily reflect how effective an mTOR inhibitor will be. However contrary to our initial hypothesis, the levels of

active mTOR targets (e.g. p-Akt), correlated negatively with the relative levels of scaffolding proteins but positively with the levels of the inhibitory component DEPTOR; this effect being most noticeable with the mTORC2 scaffold protein rictor and mTORC2 target Akt. It was initially believed that the greater levels of rictor would equate to an increase in mTORC2 signalling, as seen by p-Akt levels, but these data proved this was not the case. In the context of mTOR signalling, this piece of information sheds new light on the intricacies of mTOR complex formation and active signalling.

It is well established that mTORC1 negatively feeds back to IRS-1 to perturb its own signalling (Magnuson et al, 2012; Saran et al, 2015) and research has also shown that mTORC2 can perform a similar function and help lower levels of IRS-1 via control of the Fbw8/CUL7 ubiquitin ligase complex (DeStefano and Jacinto, 2013). Our research now adds the possibility of a greater level of complexity to mTOR activation and negative feedback. The data suggests that for a yet unknown reason, an increase in mTOR scaffold proteins could lower downstream signalling, instead of increasing it, meaning there are there could be fewer active mTOR complexes, not more, despite the fact that there are more complex components available. Previous work by Peterson and colleagues (2009) has already indicated that increased DEPTOR can increase signalling by ablating negative feedback, and it would appear that this research adds to this hypothesis, suggesting it may extend also to mTORC2.

Our work establishes a clear link between active mTORC2 signalling and the presence of fewer complex components. We hypothesise that there may exist an ‘ideal ratio’ of complex components to allow for maximal signalling from mTOR, as a slight decrease in rictor level, resulted in vastly more active Akt. This may in turn be due to mTOR complexes activating a greater number of negative feedback pathways, as previously mentioned, and we speculate that other feedback loops may exist that help explain the effects we have observed. More work would need to be done studying the levels of all/more mTOR targets in relation to mTOR component levels, to establish the exact extent of this effect and to see if our thinking must be altered on how a signal is transmitted from top to bottom through the mTOR pathway.

7.3 - The Development of Everolimus Resistant Breast Cancer Cells and the Determination of Mechanisms Behind this Phenotype

The primary focus of this research was to develop everolimus breast cancer cells (EveR) and characterise them with the goal of discerning useful biological features that everolimus resistant cells possess, possible mechanisms behind this resistance and methods of overcoming the resistance.

As previously mentioned, published research investigating everolimus resistance has not yet reached the stage at which they are investigating this phenotype within breast cancer patients, due in part to the relative recentness in which everolimus was approved for use in breast cancer. As such the primary method of exploring drug resistance here was to develop *in vitro* models, in a similar manner to research previously published in this field (Bihani et al, 2015; Jordan 2014).

Our methodology for developing these cells was similar to that of previously published work on everolimus/rapalogue resistance in breast cancer (Bihani et al, 2015; Hassan et al, 2014; Jordan 2014), with our primary source of technical information for the development process taken from research by Box and colleagues (2013). Whilst this research did not utilise a breast cancer model it did follow a system that was easily applicable the breast cancer cells of our choice. This system essentially revolved around testing the cells, using the SRB toxicology assay, for the drug concentration that inhibited growth by 60-70% (GI60/70), then treating the cells for around a week, before they were reassessed for their drug responsiveness. This process allowed a continued monitoring and tracking of the development of any resistance phenotype, with the development stopped once a stable GI60 was achieved. This process was overall relatively successful, with the generation of two EveR breast cancer cell lines, along a similar time frame to the original research (5-6 months). Some draw backs were noted however. Cells were generally cultured in T25 flasks, to lower the amount of drug needed for each treatment cycle. This reduced the amount of cells available for testing in between treatment cycles. Future use of this method may want to employ the use of larger culture flasks, if the

drug/cost benefit would allow so, but overall our development of these models was deemed a success.

This thesis begins to build upon the small base of research already present in the field of everolimus resistance in breast cancer. The main published research on this field consists of work done by Bihani and colleagues (2015), who studied acquired resistance to everolimus in various breast cancer cells, research by Jordan and colleagues (2014), who studied acquired everolimus resistance in tamoxifen resistant MCF-7 cells and finally research by Hassan and colleagues (2014), who looked at acquired resistance to rapamycin in BT474 breast cancer cells. These pieces of research are among the first to explore everolimus/rapalogue resistance in breast cancer cell models with acquired resistance, although only the work by Bihani and colleagues (2015) runs along a very similar line to this thesis, being one of the only pieces of research to construct breast cancer cell lines with everolimus resistance as the intended phenotype.

The research by Bihani and colleagues (2015) and Jordan and colleagues (2014), both gave evidence towards mechanisms for everolimus resistance. The research by Jordan and colleagues (2014) presents an interesting case since the breast cancer cells used were not developed with everolimus resistance in mind, rather tamoxifen resistance. This work gave evidence that signalling through the ER may help contribute to everolimus resistance, since dual ER/mTOR proved extremely effective in inhibiting the growth of these cells. This is perhaps less of a surprise since clinically, everolimus has proved most useful in ER+ patients, especially those who are HER-2 - (Andre et al, 2014; Baselga et al, 2012; Beaver and Park, 2012; Dorris and Jones, 2014; Hurvitz et al 2015a).

In the piece of research that perhaps most closely matches this thesis in terms of the *in vitro* model used, Bihani and colleagues (2015) found that myc up-regulation can help confer resistance to everolimus in breast cancer cells, with *MYC* expression inhibitors combined with everolimus treatment proving very effective at inhibiting cell growth. This thesis did not focus on either ER inhibition or myc as everolimus resistant mechanisms, despite the good evidence presented by these pieces of research. Since everolimus resistance is poorly understood in breast cancer and

currently research is beginning to explore this concept, this thesis aimed to explore other avenues of potential resistant mechanisms. Since in all likelihood multiple mechanisms are possible and probable in this setting, we aimed to look at novel explanations for everolimus resistance to help expand what is a currently a very narrow and new field. This thesis ultimately did not show any new definite mechanisms for everolimus resistance, with the avenues of PKCα down-regulation, multi-drug resistance and mutations in mTOR and FKBP12 giving negative results.

However there is some evidence that the mechanisms explored in those previous pieces of research would be worth exploring in the *in vitro* models constructed for this thesis. The role of mTORC2 is expanding, with it being shown not only to phosphorylate AGC kinases like PKCα, but also having the ability to phosphorylate HDACs, which has been shown to lead to the up-regulation of myc (Masui et al, 2013; Masui et al, 2014). This provides a possible link between our EveR cells and the work by Bihani and colleagues (2015), as the EveR models showed up-regulation of mTORC2 but PKCα down-regulation did not affect resistance, suggesting that another target, such myc via HDACs, could be a better fit. Future work exploring whether myc could play a role in the resistant phenotype in our EveR cells could help not only to further characterise them but also give more weight to the role of myc and mTORC2 in everolimus resistance.

Overall, the *in vitro* models discussed here are currently the best way of exploring the issue of everolimus resistance; however, like all cell models they have their limitations. We have already discussed the variation in possible mechanisms presented by only three/four models, and this appears to be an intrinsic problem when using cell models, due in part to the differences in culture methods and the cell lines used. The models in this thesis were developed from T47D and MDA-MB-361 breast cancer cells, which have currently not been used before to look at everolimus resistance, using this method, with much of the previous work using cell lines such as the MCF-7 cells (Bihani et al, 2015; Jordan et al, 2014). Our two cell lines were chosen in part due to their ER+ status and thus it was decided they would be good candidates and hopefully representative of the type of cancer which might be treated with everolimus. Since they had not been used for these types of models before, this would also help widen the field when studying phenotypes in everolimus resistant

cells. Until studies enrolling large cohorts of everolimus resistant breast cancer patients emerge, it will be difficult to track which phenotype, trends and characteristics are representative of both patients and cell models, rather than just of individual cell models.

7.4 - Alternate Drugs to Everolimus and Drug Combinations to Overcome Everolimus Resistance

Potential mechanisms are of course extremely important in characterising drug resistant cell lines. However, discerning methodologies and drug combinations to combat this resistant phenotype is also of the upmost importance. This was another primary objective of this piece of research, since work by our group and others on these possible drug combinations can lay the foundation for future testing at a clinical level.

Many of the cell lines used here were selected due to previous work studying their responsiveness to calcitriol/vitamin D (Harvey, unpublished). Evidence suggests that in many cell and cancer cell types, vitamin D can alter and regulate the mTOR pathway, and thus shows potential as a combinatory drug, with an mTOR inhibitor. For example, in T-cells, keratinocytes and breast cancer cells, vitamin D (or an analogue) treatment was shown to reduce Akt activation, often accompanied by a reduction in overall mTOR activation (Datta-Mitra et al, 2013a; Datta-Mitra et al, 2013b; O'Kelly et al, 2006). Initial evidence for the use of mTOR inhibition combined with vitamin D comes from work by Yang and colleagues (2010). Their work treating AML cells both *in vitro* and *in vivo* with a combination of everolimus and calcitriol (the active metabolite of vitamin D), showed a potentiating effect when the two drugs were used together. Currently, there is no published evidence of the use of this type of drug combination in breast cancer cells.

The data presented in this thesis is therefore initial evidence of extending this concept to breast cancer cells. Both of the EveR cell lines developed here showed an increased response to the combination of calcitriol and everolimus, either reducing everolimus resistance significantly or reducing it entirely to the point where cell responsiveness matched that of the parental controls. Going forward, everolimus

usefulness could be bolstered with the addition of the relatively cheap and available vitamin D/calcitriol. However more work from this point is required to study exactly how calcitriol affects the mTOR pathway, with tests such as western blotting of key mTOR proteins, after calcitriol or combination treatment, needed to help construct a clearer view of calcitriol's effect the mTOR pathway in breast cancer cells. This is especially important since western blotting data suggests the effect calcitriol had, did not relate to the presence of the VDR, since VDR levels did not vary between resistant cells and their parental controls. Looking further afield, testing with other breast cancer cell lines, work *in vivo* and eventually testing clinically, would help to establish calcitriol as working, breast cancer therapeutic agent.

The most obvious and well tested use of alternate agents to inhibit mTOR signalling, are drugs that act to inhibit mTOR via an ATP-competitive mechanism. Rapalogues were of course the first discovered set of drugs to inhibit mTOR, but many ATP-competitive inhibitors have since been developed that target mTOR or mTOR and PI3K. Targeting in this way has shown to be quite effective pre-clinically, in cancers such as breast and neuroendrocrine tumours. BEZ-235, a dual mTOR/PI3K inhibitor has shown effective use against a variety of cancer cells (Britten et al, 2014; Dey et al, 2016). Research with mTOR specific ATP-competitive inhibitors such as MLN0128, CC-223 and AZD2014 has shown their effectiveness in inhibiting the growth of cancer cells such as breast (Hassan et al, 2014; Guichard et al, 2015; Wilson-Edell et al, 2014). Additionally both MLN0128 and AZD8055 (the latter very similar to AZD2014), have shown effectiveness in reducing the growth of rapalogue resistant breast cancer cells, in the previously mentioned research by Hassan and colleagues (2014) and Jordan and colleagues (2014). This gives them even more relevance to our own research since not only have they shown effectiveness in inhibiting mTOR, but they have shown use in the context of rapalogue resistance.

The use of ATP-competitive inhibitors is becoming more popular due to the various issues associated with rapalogue treatment, which they appear to help circumnavigate. Research has already shown that rapalogue treatment may help activate Akt by shifting the burden of signalling towards mTORC2 (Sarbassov et al, 2004; Sun et al, 2005). Therefore the use of these types of drugs helps avoid these issues by targeting both mTOR complexes via mTOR itself, rather than just

mTORC1. Also, whilst our research did not yet show a direct link between mTORC2 up-regulation and everolimus resistance, as previously mentioned, this is still a possibility due to the up-regulation of indirect mTORC2 targets like myc. Thus, these drugs would also avoid these potential problems.

The data presented here adds to this body of evidence suggesting the use of an ATP-competitive inhibitor of mTOR. We showed that everolimus resistant cells are not resistant to BEZ-235 in any way, backing up the data given by work such as that of Hassan and colleagues (2014) and Jordan and colleagues (2014). Our work taken together with the other research mentioned here, displays the need to explore this class of drugs further at a clinical level, and supports the hypothesis that they may be more effective than rapalogues and may encounter less resistance. So far CC-223 has been explored in a phase I trial in breast cancer patients (Bendell et al, 2015), whilst AZD2014 is currently being explored in a phase II trial in advanced breast cancer patients (who have relapsed after treatment with an aromatase inhibitor), either as a single therapy or in conjunction with fulvestrant (Cancer Research UK, 2016; NIH, 2017).

Finally, in the context of drugs that may help combat the everolimus resistant phenotype, data presented here shows that we may wish to explore the use of alternative rapalogues to everolimus. SRB data gathered here revealed that despite being heavily resistant to everolimus, our *in vitro* models were not very resistant to the use of temsirolimus, although were still almost as resistant to rapamycin.

A core principle of the rapalogue set of drugs is that they all act via the same mechanism. This revolves around the immunophilin, FKBP12, which away from its role with rapalogues, is involved in modulating the immune system through its binding of FK506 (Cameron et al, 1995; Lee et al, 2014). FKBP12 also acts effectively as the intracellular ‘receptor’ for rapalogues. Despite the fact that rapalogues such as rapamycin can bind mTOR without FKBP12, the affinity for mTOR is increased radically when the drug is bound to FKBP12 (Banaszynski et al, 2005). From this point, the complex of rapalogue and FKBP12 binds mTORC1 to inhibit its action (Choi et al, 1996; Yang et al, 2013), with research suggesting this binding affects the interaction between mTOR and raptor (Oshiro et al, 2004).

Our testing revealed that FKBP12 levels had increased in the everolimus resistant cells, with this being the current basis for our hypothesis as to why temsirolimus shows this differential effect compared to everolimus. This centres on the fact that temsirolimus appears to have a much weaker binding affinity for FKBP12, than either everolimus or rapamycin. It must be considered that the binding affinity concerning temsirolimus to FKBP12 was calculated from only one other source (Hoy and McKeage, 2010), so future work may wish to confirm the nature of this interaction, to help confirm this hypothesis.

Overall this suggests that temsirolimus may prove useful in cases where cells have become resistant to everolimus. As previously discussed, work looking at if FKBP12 is regularly over expressed in either other rapalogue resistant cells or rapalogue resistant patients would be vital in confirming the usefulness of this effect; for example if FKBP12 over-expression if regularly associated everolimus resistance, temsirolimus may be a genuinely useful alternative option. Currently temsirolimus has shown relatively limited use in breast cancer patients during clinical trials. In a small phase II trial only 9.2% of patients showed even a partial response to the drug (Fleming et al, 2012), whilst the phase III HORIZON trial showed a lack of benefit overall to temsirolimus treatment combined with letrozole, in post-menopausal women (Wolff et al, 2013). The data shown in this thesis therefore present a whole new use for temsirolimus; however more work is clearly needed to confirm the frequency of FKBP12 over-expression, before this idea can be tested further.

7.5 - Biological Impact of Everolimus Resistance and an Increase in Cancer Stemness and Dormancy Phenotype

The development of everolimus resistance in these cells was associated with multiple changes in phenotype and characteristic, when comparing them to their parental counter parts. Of these changes, a trend appeared to emerge, with the EveR cells gaining a more dormant phenotype. This was noted due to a decrease in progression through the cell cycle and the down-regulation in expression of dozens of different proteins studied in the antibody arrays, with these scattered across a variety of signalling pathways and processes. Accompanying this, in one of the few

increases in protein expression, were elevated levels of canonical Wnt signalling proteins. An apparent increase in dormancy as well as increase in Wnt signalling in these cells points towards them gaining greater stemness; in that they share more characteristics with breast CSCs, with this cell subset in tumours often possessing these characteristics (Kotiyal et al, 2014; Liu et al, 2014b; Luo et al, 2015; Yeh and Ramaswamy, 2015; Zhao et al, 2014).

At this current stage, our data suggests an increase in dormancy and stemness in the EveR cell model, however more work will be needed to confirm this. One of the EveR cell lines already showed an increase in ALDH activity, a key marker of breast CSCs (Ginestier et al, 2007; Liu et al, 2014b), so studying the expression of CD44 and CD24 would be the next logical targets, due to their importance as CSC markers (Al-Hajj et al, 2003; Qiu et al, 2016). As well as this, markers for dormancy would help clarify their phenotype, with markers such as CXCR4, cyclin D1 and CDK serving as good markers (Nobutani et al, 2015; Quayle et al, 2015). As stated previously, studying patients who are everolimus resistant (once these samples are available) would again help to determine if this phenotype persists clinically. If everolimus resistance is making these cells more stemcell-like, it would have large implications for the use of everolimus in treating breast cancer patients.

CSCs present a challenge for clinicians, which could potentially add a more dangerous paradigm to the emergence of everolimus resistance, beyond the ineffectiveness of everolimus. In the cancer types they have been detected in, CSCs have demonstrated the ability key ability of self-renewal , thus they are able to propagate a tumour and show a far greater capacity for drug resistance and survival after chemotherapy (Dawood et al, 2014; Guo et al, 2014; Kotiyal and Bhattacharya, 2014; Vlashi and Pajonk, 2015; Yeh and Ramaswamy, 2015). Breast CSC have also shown great versatility, with the ability to switch between an epithelial or mesenchymal state when required (Liu et al, 2014b; Luo et al, 2015). Overall, their presence makes a cancer much harder to treat. If everolimus resistant cells are more stemcell-like in their phenotype, greater attention may be needed to study everolimus resistance and its effects, as this therapy may actually cause a cancer to progress in this instance. This observed effect may give further evidence towards the

use of ATP-competitive and dual mTOR inhibitors, if they do not cause the rise of this subset of more dangerous cells.

7.6 - Concluding Remarks

Breast cancer now has one of the highest survival rates of any cancer, due in part to screening techniques and the amount of targeted and effective therapies used in the treatment of this disease. However, despite the effectiveness of these therapies, drug resistance will inevitably be an issue, forcing research in this field to continue to adapt and deploy counter-measures against them. Since everolimus has only been used since 2012 in breast cancer, drug resistance is not well studied here. By studying *in vitro* models of everolimus resistance in breast cancer, we aimed to contribute to the small amount of information circulating on this subject, and study everolimus resistance before it has arisen as a major clinical issue.

In this thesis, a range of interesting characteristics of the everolimus resistant cells were determined. As well as this, we have discovered novel potential approaches for treating these drug resistant cells. Primarily, this has shown the potential effectiveness of combining vitamin D with everolimus and the effectiveness of the ATP-competitive inhibitor BEZ-235. In respect to BEZ-235, this thesis helps give weight to the already growing number of papers showing a lack of resistance to these types of drugs. There are already a number of clinical trials testing these drugs, and this thesis along with the greater body of work already published, will in theory contribute to pushing forward new clinical trials, with the scope of offering an alternative to rapalogues. In respect to the vitamin D/everolimus combination data presented here, it is among the first of its kind to show that vitamin D can be effective in combating everolimus resistance. Future work from this project may look to test this combination within *in vivo* models and if this is also successful, this drug combination could offer an exciting avenue for future testing in a clinical setting.

This project also determined that everolimus resistant cells can show novel properties and phenotypes that are similar to that of CSCs. Future work from this thesis may also look to fully clarify the exact nature of this phenotype by studying a greater number of CSC markers and properties in our EveR models. If everolimus

treatment can make a cell more stem-like, this could be vital knowledge for a clinical setting and may help add caveats to using rapalogues, thus in the long run helping to guide treatment use and subsequent patient safety.

Ultimately, as more research is conducted in this area, clearer trends for mechanisms of everolimus resistance will hopefully emerge, allowing researchers to more selectively target and effectively treat in this scenario. This may in all likelihood be due to the increase in the use of ATP-competitive inhibitors which can in theory circumnavigate certain biological issues. As more time passes, studies will most likely be published looking at patient examples of everolimus resistance, with this helping to conclusively elucidate the issues presented here.

Chapter 8: Bibliography

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Chapter 9: Appendix

```
---------+---------+---------+---------+---------+---------+---------+---------+
      10      20      30      40      50      60      70      80
---------+---------+---------+---------+---------+---------+---------+---------+
```

FRB domain (wt)
GTGAGCGAGGAGCTGATCCGAGTGGCCATCCTCTGGCATGAGATGTGGCATGAAGGCCTGGAAGA
GGCATCTCGTTTGTA
T47D-EveR
GTGAGCGAGGAGCTGATCCGAGTGGCCATCCTCTGGCATGAGATGTGGCATGAAGGCCTGGAAGA
GGCATCTCGTTTGTA
T47D-Parental
GTGAGCGAGGAGCTGATCCGAGTGGCCATCCTCTGGCATGAGATGTGGCATGAAGGCCTGGAAGA
GGCATCTCGTTTGTA
361-EveR
GTGAGCGAGGAGCTGATCCGAGTGGCCATCCTCTGGCATGAGATGTGGCATGAAGGCCTGGAAGA
GGCATCTCGTTTGTA
361-Parental ----------
GGCATGAAGGCCTGGAAGAGGCATCTCGTTTGTA

```
---------+---------+---------+---------+---------+---------+---------+---------+
      90     100     110     120     130     140     150     160
---------+---------+---------+---------+---------+---------+---------+---------+
```

FRB domain (wt)
CTTTGGGGAAAGGAACGTGAAAGGCATGTTTGAGGTGCTGGAGCCCTTGCATGCTATGATGGAACG
GGGCCCCCAGACTC
T47D-EveR
CTTTGGGGAAAGGAACGTGAAAGGCATGTTTGAGGTGCTGGAGCCCTTGCATGCTATGATGGAACG
GGGCCCCCAGACTC
T47D-Parental
CTTTGGGGAAAGGAACGTGAAAGGCATGTTTGAGGTGCTGGAGCCCTTGCATGCTATGATGGAACG
GGGCCCCCAGACTC
361-EveR
CTTTGGGGAAAGGAACGTGAAAGGCATGTTTGAGGTGCTGGAGCCCTTGCATGCTATGATGGAACG
GGGCCCCCAGACTC
361-Parental
CTTTGGGGAAAGGAACGTGAAAGGCATGTTTGAGGTGCTGGAGCCCTTGCATGCTATGATGGAACG
GGGCCCCCAGACTC

```
---------+---------+---------+---------+---------+---------+---------+---------+
      170     180     190     200     210     220     230     240
---------+---------+---------+---------+---------+---------+---------+---------+
```

FRB domain (wt)
TGAAGGAAACATCCTTTAATCAGGCCTATGGTCGAGATTTAATGGAGGCCCAAGAGTGGTGCAGGA
AGTACATGAAATCA
T47D-EveR
TGAAGGAAACATCCTTTAATCAGGCCTATGGTCGAGATTTAATGGAGGCCCAAGAGTGGTGCAGGA
AGTACATGAAATCA
T47D-Parental
TGAAGGAAACATCCTTTAATCAGGCCTATGGTCGAGATTTAATGGAGGCCCAAGAGTGGTGCAGGA
AGTACATGAAATCA
361-EveR
TGAAGGAAACATCCTTTAATCAGGCCTATGGTCGAGATTTAATGGAGGCCCAAGAGTGGTGCAGGA
AGTACATGAAATCA
361-Parental
TGAAGGAAACATCCTTTAATCAGGCCTATGGTCGAGATTTAATGGAGGCCCAAGAGTGGTGCAGGA
AGTACATGAAATCA

```
---------+---------+---------+---------+---------+---------+---------+---------+
      250     260     270     280     290     300     310     320
---------+---------+---------+---------+---------+---------+---------+---------+
```

FRB domain (wt)
GGGAATGTCAAGGACCTCACCCAAGCCTGGGACCTCTATTATCATGTGTTCCGACGAATCTCAAAGC
AGCTGCCTCAGCT
T47D-EveR
GGGAATGTCAAGGACCTCACCCAAGCCTGGGACCTCTATTATCATGTGTTCCGACGAATCTCAAAGC
AGCTGCCTCAGCT
T47D-Parental
GGGAATGTCAAGGACCTCACCCAAGCCTGGGACCTCTATTATCATGTGTTCCGACGAATCTCAAAGC
AGCTGCCTCAGCT
361-EveR
GGGAATGTCAAGGACCTCACCCAAGCCTGGGACCTCTATTATCATGTGTTCCGACGAATCTCAAAGC
AGCTGCCTCAGCT
361-Parental
GGGAATGTCAAGGACCTCACCCAAGCCTGGGACCTCTATTATCATGTGTTCCGACGAATCTCAAAGC
AGCTGCCTCAGCT

```
---------+---------+---------+---------+---------+---------+---------+---------
      330      340      350      360      370      380      390
---------+---------+---------+---------+---------+---------+---------+---------
FRB domain (wt)
CACATCCTTAGAGCTGCAATATGTTTCCCCAAAACTTCTGATGTGCCGGGACCTTGAATTGGCTGTGC
CAGGAACATAT
T47D-EveR
CACATCCTTAGAGCTGCAATATGTTTCCCCAAAACTTCTGATGTGCCGGGACCTTGAATTGGCTGTGC
CAGGAACATAT
T47D-Parental
CACATCCTTAGAGCTGCAATATGTTTCCCCAAAACTTCTGATGTGCCGGGACCTTGAATTGGCTGTGC
CAGGAACATAT
361-EveR
CACATCCTTAGAGCTGCAATATGTTTCCCCAAAACTTCTGATGTGCCGGGACCTTGAATTGGCTGTGC
CAGGAACATAT
361-Parental
CACATCCTTAGAGCTGCAATATGTTTCCCCAAAACTTCTGATGTGCCGGGACCTTGAATTGGCTGTGC
CAGGAACATAT
```

Figure 9.1: Sanger sequencing of mTOR FRB domain reveals no mutations. *Sanger sequencing data for the FRB domain of mTOR (399 nucleotides) in T47D-EveR, T47D-Parental, 361-EveR and 361-Parental cell lines, compared to the published sequence (wt) (NCBI, 2018a; UniProt, 2018). Sequences were generated and then aligned using DNASTAR Lasergene (Megalign) and the clustal W method. No mutations were revealed in any sample compared to the published sequence.*

```
---------+---------+---------+---------+---------+---------+---------+---------+
      10       20       30       40       50       60       70       80
---------+---------+---------+---------+---------+---------+---------+---------+
FKBP12 (wt)
ATGGGAGTGCAGGTGGAAACCATCTCCCCAGGAGACGGGCGCACCTTCCCCAAGCGCGGCCAGACC
TGCGTGGTGCACTA
T47D-EveR
ATGGGAGTGCAGGTGGAAACCATCTCCCCAGGAGACGGGCGCACCTTCCCCAAGCGCGGCCAGACC
TGCGTGGTGCACTA
T47D-Parental
ATGGGAGTGCAGGTGGAAACCATCTCCCCAGGAGACGGGCGCACCTTCCCCAAGCGCGGCCAGACC
TGCGTGGTGCACTA
361-Parental
ATGGGAGTGCAGGTGGAAACCATCTCCCCAGGAGACGGGCGCACCTTCCCCAAGCGCGGCCAGACC
TGCGTGGTGCACTA
```

```
---------+---------+---------+---------+---------+---------+---------+---------+
      90      100     110     120     130     140     150     160
---------+---------+---------+---------+---------+---------+---------+---------+
```

FKBP12 (wt)
CACCGGGATGCTTGAAGATGGAAAGAAATTTGATTCCTCCCGGGACAGAAACAAGCCCTTTAAGTTT
ATGCTAGGCAAGC
T47D-EveR
CACCGGGATGCTTGAAGATGGAAAGAAATTTGATTCCTCCCGGGACAGAAACAAGCCCTTTAAGTTT
ATGCTAGGCAAGC
T47D-Parental
CACCGGGATGCTTGAAGATGGAAAGAAATTTGATTCCTCCCGGGACAGAAACAAGCCCTTTAAGTTT
ATGCTAGGCAAGC
361-Parental
CACCGGGATGCTTGAAGATGGAAAGAAATTTGATTCCTCCCGGGACAGAAACAAGCCCTTTAAGTTT
ATGCTAGGCAAGC

```
---------+---------+---------+---------+---------+---------+---------+---------+
     170     180     190     200     210     220     230     240
---------+---------+---------+---------+---------+---------+---------+---------+
```

FKBP12 (wt)
AGGAGGTGATCCGAGGCTGGGAAGAAGGGGTTGCCCAGATGAGTGTGGGTCAGAGAGCCAAACT
GACTATATCTCCAGAT
T47D-EveR
AGGAGGTGATCCGAGGCTGGGAAGAAGGGGTTGCCCAGATGAGTGTGGGTCAGAGAGCCAAACT
GACTATATCTCCAGAT
T47D-Parental
AGGAGGTGATCCGAGGCTGGGAAGAAGGGGTTGCCCAGATGAGTGTGGGTCAGAGAGCCAAACT
GACTATATCTCCAGAT
361-Parental
AGGAGGTGATCCGAGGCTGGGAAGAAGGGGTTGCCCAGATGAGTGTGGGTCAGAGAGCCAAACT
GACTATATCTCCAGAT

```
---------+---------+---------+---------+---------+---------+---------+---------+-------
      250      260      270      280      290      300      310      320
---------+---------+---------+---------+---------+---------+---------+---------+-------
FKBP12 (wt)
TATGCCTATGGTGCCACTGGGCACCCAGGCATCATCCCACCACATGCCACTCTCGTCTTCGATGTGG
AGCTTCTAAAACTGGAATGA
T47D-EveR
TATGCCTATGGTGCCACTGGGCACCCAGGCATCATCCCACCACATGCCACTCTCGTCTTCGATGTGG
AGCTTCTAAAACTGGAATGA
T47D-Parental
TATGCCTATGGTGCCACTGGGCACCCAGGCATCATCCCACCACATGCCACTCTCGTCTTCGATGTGG
AGCTTCTAAAACTGGAATGA
361-Parental
TATGCCTATGGTGCCACTGGGCACCCAGGCATCATCCCACCACATGCCACTCTCGTCTTCGATGTGG
AGCTTCTAAAACTGGAATGA
```

Figure 9.2: Sanger sequencing of FKBP12 reveals no mutations. *Sanger sequencing data for FKBP12 (FKBP1A) (327 nucleotides) in T47D-EveR, T47D-Parental and 361-Parental cell lines, compared to the published sequence (wt) (NCBI, 2018b). Sequences were generated and then aligned using DNASTAR Lasergene (Megalign) and the clustal W method. No mutations were revealed in these samples compared to the published sequence.*