Exploring the impact of anti-oestrogen resistance on the capacity of breast cancer cells to modulate bone cell function and their sensitivity to bisphosphonates

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Every accomplishment starts

with the decision to try

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Abstract

Acquired resistance to endocrine therapy is a major limiting factor for their clinical effectiveness, resulting in disease relapse and an associated poor prognosis. Acquired resistance is also associated with the development of an invasive and migratory phenotype *in vitro* that may promote metastatic spread in vivo of which bone is the most frequent site. However, it is not currently known whether endocrine resistance affects the ability of breast cancer cells to modulate bone cell function important in establishing bone metastases or whether a resistant phenotype alters sensitivity to agents commonly used to treat bone metastasis such as bisphosphonates. Thus, this thesis aimed to explore the bone cell modulatory function of endocrine resistant and sensitive breast cancer cells along with their sensitivity to the bisphosphonate, zoledronic acid.

This thesis demonstrated that breast cancer cells were able to directly induce osteoclast differentiation from both murine and human precursor cells. Importantly, this effect was more prevalent in tamoxifen resistant and triple negative breast cancer subtypes. Our data also suggested that the breast cancer-mediated osteoclastogenic effect involved Src kinase, whilst bisphosphonates acted as anti-tumour agents in tamoxifen resistant cells through inhibition of EGFR/AKT/mTOR pathway.

In conclusion, this thesis suggests that acquisition of endocrine resistance confers a bone modulatory ability to breast cancer cells that may contribute to the development of bone metastases. However, this thesis reports the novel finding that acquired endocrine resistance augments the sensitivity of breast cancer cells to bisphosphonates, thus representing an opportunity to target resistant disease clinically.

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List of Abbreviations

AF	Activating function
AI	Aromatase Inhibitor
AIB1	Amplified in breast 1
AKT	v-akt murine thymoma viral oncogene homolog
ANOVA	Analysis of variance
AP-1	Activator protein 1
APS	Ammonium persulphate
ATAC	Anastrozole or Tamoxifen Alone in Combination
ATTC	American Type Cell Collections
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
BCMPG	Breast Cancer Molecular Pharmacology Group
cAMP	Cyclic adenosine monophosphate
CBS	Central Biotechnology Services
CD47	Cluster of differentiation 47
CM	Conditioned medium
DBD	DNA binding domain
DFS	Disease free survival
DNA	Deoxyribonucleic acid
DNTPs	Deoxynucleotide triphosphates
DPX	Mixture of Distyrene, a plasticizer and xylene
DTT	Dithiothreitol
E_2	Oestrogen/oestradiol
ECL	Enhanced chemiluminescence

EGFR	Epidermal growth factor receptor
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ER	Oestrogen receptor
ERE	Oestrogen response elements
EtBr	Ethidium bromide
FasR	Faslodex resistant cell line derived from MCF-7 cells
FCS	Foetal calf serum
FDA	Food and Drug Administration
GTPases	Guanosine-5'-triphosphate
HER2	Human epidermal growth factor receptor 2
IGF	Insulin-like growth factor
IGF1R	Insulin growth factor 1 receptor
IHC	Immunohistochemistry
kDa	Kilodaltons
LBD	Ligand binding domain
MAPK	Mitogen-activated protein kinases
MAS 5.0	Affymetrix Microarray Suite 5
MCF-7	Michigan Cancer Foundation 7
MCSF	Macrophage colony- stimulating factor
MgCl2	Magnesium chloride
MIF	Macrophage migration inhibitory factor
mRNA	Messenger RNA
MMLV	Molony-murine leukemia virus
mTOR	Mammalian target of rapamycin
MW	Molecular weight

NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
OPG	Osteoprotegerin
OS	Overall survival
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFS	Progression free survival
PI3K	Phosphatidylinositide 3-kinases
PKA	Protein kinase A
PKC	Protein kinase C
PR	Progesterone receptor
PTM	Post-translation modifications
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	Ribonucleic acid
rRPMI	RPMI 1640 with phenol red
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription PCR
Ser118	Serine 118
Ser167	Serine 167
SD	Standard deviation
SDS-PAGE	Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the means
SERD	Selective oestrogen receptor down regulator
SERM	Selective oestrogen receptor modulator
SFCS	Charcoal stripped foetal calf serum
SiRNA	Small interfering RNA

Src	Steroid receptor coactivator
SP-1	Specificity protein 1
TAE	Tris base, acetic acid and EDTA
TamR	Tamoxifen resistant cell line derived from MCF-7 cells
TBST	Tris-Buffered Saline-Tween buffer solution
TGFß	Transforming growth factor beta 1
TKI	Tyrosine kinase inhibitor
VEGF	Vascular endothelial growth factor
WB	Western blotting
wRPMI	Phenol-red-free RPMI 1640
ZOL	Zoledronic acid

1. General Introduction

1.1 Breast cancer epidemiology and risk factors

Breast cancer is the most frequently diagnosed malignancy in women and the second most common cause of death in patients with cancer, accounting for ~ 23% of all cancer cases (Ban & Godellas 2014). Specifically, it is estimated that one out of eight women will develop breast cancer during their lifetime (Li et al. 2017). Breast cancer represents a major global health concern accounting for nearly 1,700,000 new cases and approximately 460,000 deaths in 2008 worldwide (Druesne-Pecollo et al. 2012). According to GLOBOCAN statistics in 2012, an increase in breast cancer incidence rates by 18% it was observed compared to 2008 (Ferlay et al. 2014) and it is predicted that by 2050 the new breast cancer diagnoses will escalate to 3.2 million per year (Coughlin & Ekwueme 2009). Encouragingly, over the past few decades breast cancer survival rates have been increased and mortality rates decreased due to the advent of mammography screening that allows early detection, the implementation of adjuvant therapy and the development of targeted therapies for specific breast cancer subtypes (Baselga & Norton 2002).

A number of key factors have been reported that are associated with an increased risk of developing breast cancer. The strongest associations are seen with early menarche, late menopause and use of hormone replacement therapy (Verkooijen et al. 2009); the underlying cause in these cases is thought to be an increase in the duration of exposure of the mammary gland epithelium to estrogen (E_2) and/or progesterone (PG), thus demonstrating a link between breast cancer and estrogen exposure. Interestingly, there is a correlation between distinct risk factors and the propensity to develop specific breast cancer subtypes (Anderson et al. 2014). For instance, hormone receptor positive (HR+) breast cancer demonstrates an inverse association with parity

and breastfeeding, while late age of first pregnancy constitutes a risk factor for HR+ breast cancer (Rosato et al. 2013).

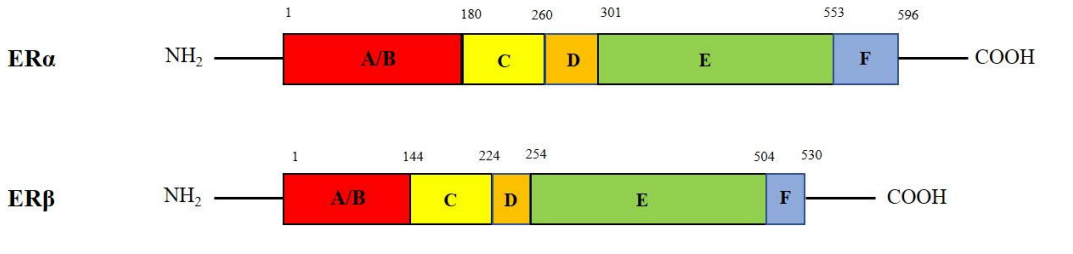
1.2 Oestrogen receptors

Oestrogens are steroid hormones that regulate proliferation, differentiation and function of multiple human tissues and play a key role in the maintenance of female reproductive system (Norman & Henry 2015). Oestrogens can easily diffuse across the cell membrane and once enter to the cell cytosol bind to and activate the oestrogen receptors (ERs), which in turn regulate several pathways (Bouman et al. 2005). The major oestrogen form in women is oestradiol (E_2) that presents the highest affinity with the ERs. There are also three other metabolites of E_2 known, the oestrone, oestriol and oestretrol, the latter is secreted only during pregnancy though (Lenz & McCarthy 2010).

ERs belong to the nuclear superfamily receptor family and appear in two different subtypes, designated as ER alpha (ERα) and ER beta (ERβ) (Nilsson & Gustafsson 2011). ERα was discovered in 1962 from observations that a characteristic protein regulated the E_2 levels in specific tissues (Jensen & Jacobsen 1962). It was assumed that ERα was the only ER until 1996, when ERβ was isolated from rat prostate and ovary (Kuiper et al. 1996). The ERα gene (ESR1) is located on chromosome 6 in humans and encodes a 596- amino acid protein, while ERβ gene (ESR2) is positioned on chromosome 14 and encodes a 530- amino acid protein (Bai & Gust 2009).

Although these two receptors are encoded by independent genes, they share similar structures; both are composed of 6 distinct regions, which are the N-terminal domain (NTD), the DNA-binding domain (DBD), the hinge region and the ligand-binding domain (LBD) (**Figure 1.1**). Two transactivation function (AF) domains AF1 and AF2

are located within the NTD and LBD respectively while region D plays the role of a link between C and E regions (Gilbert et al. 1992).



A/B	C	D	E	F
AF1 domain	DNA binding domain	Hinge domain	Ligand binding domain	AF2 domain

Figure 1.1: Domain structure of ERα and ERβ. The table describes the function of each domain.

AF1 is considered to be hormone-independent, while AF2 requires the presence of a steroid in order to function (Beato et al. 1987). Whilst AF1 and AF2 domains are capable of initiating transcription separately, it has been reported that full ER transcription activation is achieved by the collaboration between the AF1 and AF2 domains (Tzukerman et al. 1994). The highly conserved zinc-finger-containing DBD binds to oestrogen response elements (ERE), which are sequences that play a crucial role in the binding affinity of the ERs and the recruitment of co-activators (Brown & Sharp 1990; Glass & Rosenfeld 2000). The LBD is comprised of 12 helices, which fold to structure a compact three- layer helical cluster. The ligand binding pocket is located in the inner part of the domain, completely isolated from agonist ligands (Mueller-Fahrnow & Egner 1999). The hinge region contains nuclear localisation signals that are unveiled upon ligand binding and also serves as a flexible link between the DBD and the LBD regions (Kuiper et al. 1996).

ERα and ERβ possess high homology between their DBD (more than 95%), a moderate homology between their LBD (approximately 55% amino acids) and poor homology between their NTD (approximately 15%) due to the fact that ERβ is relatively shorter compared to ERα (Kumar et al. 2011; Kuiper et al. 1996; Cowley et al. 1997). The most considerable difference between the ERs is observed in their AF1 domains. Whilst ERα shows a high degree of AF1-mediated transcriptional activity, this region exhibits minor activity in ERβ (Hall & McDonnell 1999).

Both ERα and ERβ are expressed in various tissues including cardiovascular system, urogenital track, central nervous system, breast and bone. However, in the uterus and mammary gland ERα is the predominant isoform compared to ERβ. In addition, ERβ expression is present in the gastrointestinal track, whereas in the liver only ERα can be found (**Table 1.1**) (Gustafsson 1999).

Table 1.1: The distribution of the ERα and ERβ in the human body.

Tissue	Oestrogen Receptors
Central nervous system	ERα, ERβ
Breast	ERα, ERβ
Liver	ERα
Bone	ERα, ERβ
Cardiovascular system	ERα, ERβ
Gastrointestinal tract	ERβ
Urogenital track	ERα, ERβ

ERα has been tightly correlated with the development and progression of breast cancer as it is expressed in approximately 75% of breast tumours (Lim et al. 2016), however the exact role of ERβ still remains unclear and under investigation. Several studies have been reported that when ERβ is co-expressed with ERα the function of ERα is impaired (Matthews 2003) by ERβ-mediated ERα degradation and altering the binding patterns of key transcription factors, such as activator protein-1 (AP1), c-Fos and c-Jun (Matthews et al. 2006).

1.3 Mechanisms of ER action

1.3.1 Ligand-dependent ER mechanism

Specific proteins, such as heat shock protein 90 (hsp90), bound to the ER and maintain it in an inactive conformation and must be detached in order to enable ligand-dependent ER activity (Nilsson & Gustafsson 2011). Upon oestrogen binding in the LBD, the helix cluster undergoes a conformational change, which promotes the dissociation of ER from the binding proteins exposing the domains responsible for ER dimerisation, nuclear translocation and binding to EREs and other transcription factors (McKenna et al. 1999). By discarding hsp90 the ER is able to form stable homo- or hetero- dimers and subsequently translocate to the nucleus, where it binds to EREs located in the promoter of target genes (McKenna et al. 1999). Upon binding to DNA, co-factor proteins are recruited to further stabilise the transcription complex resulting in either activation or suppression of the downstream target gene (Hall et al. 2001).

1.3.2 Oestrogen-independent ER activation

ER activation can also be mediated by extracellular signals in a ligand-independent manner (Hall et al. 2001). Epidermal growth factor (EGF) and insulin-like growth

factor 1 (IGF1) are the major factors known to promote ER activation in the absence of E_2 resulting in the expression of ER target genes (Smith 1998). Although the exact mechanism of oestrogen-independent ER activation is not entirely understood, several studies have illustrated the importance of specific kinases, such as mitogen activated protein kinase (MAPK), protein kinase A (PKA) and p21, in this process (Katzenellenbogen & Katzenellenbogen 2000; Power et al. 1991). These kinases are able to phosphorylate the ER on several serine residues, thus promoting ER dimerisation, nuclear translocation and finally transcription of target genes in the absence of E_2 (Hall et al. 2001). Moreover, it has been observed that in each tissue phosphorylation of specific ER sites (e.g. Tyr537 and Ser118) is essential for E_2-independent activation of ER (Maggi 2011). This observation suggested that steroid-independent ER activation possibly fulfills a range of cell specific functions (Maggi 2011). However, some studies indicate that, even if is able to recruit co-factors, direct ER phosphorylation is not sufficient to initiate transcriptional activation by itself (Masuhiro et al. 2005).

1.3.3 ERE-independent ER action

Evidence that E_2-ER can lead to expression of genes that lack ERE-like sequences, led to the discovery that ER can promote gene activation without direct binding to DNA (Hall et al. 2001). This mechanism, known as 'transcriptional cross-talk' (Aranda & Pascual 2001), is estimated to underlie the expression of ~35% of all ER-mediated gene expression (O'Lone et al. 2004). ERE-independent mechanisms requires the binding of another transcription factor in order to mediate ER association with the DNA (O'Lone et al. 2004).

A major mediator of ER-DNA indirect binding is the stimulation protein-1 (Sp-1) (O'Lone et al. 2004) and a wide variety of important genes, such as cyclin D1 (Castro-Rivera et al. 2001) and low-density lipoprotein (LDL) receptors (Li et al. 2001), have been found to be regulated via this mechanism.

1.3.4 Non-genomic ER activity

The genomic ER mechanisms discussed so far, occur within 30 minutes to several hours following E_2 stimulation (Contrò et al. 2015). The observation that oestrogens can also stimulate rapid cytosolic signals that occur within seconds (Szego & Davis 1967) has led to the discovery of the non-genomic mechanisms of ER action. Upon E_2-ER binding a number of signalling pathways are stimulated and can be classified into four distinct categories: 1) Ras/Raf/MAPK (Marino et al. 2002), 2) phosphatidyl inositol 3 kinase (PI3K)/AKT (Acconcia et al. 2005), 3) cyclic adenosine monophosphate (cAMP)/PKA (Malyala et al. 2005) and 4) phospholipase C (PLC)/ protein kinase C (PKC) (Marino et al. 1998). Interaction of E_2-ER complex with the IGF-1 receptor (IGFR) results in activation of the latter and consequently MAPK signaling cascade (Kahlert et al. 2000). E_2- ER complex can also lead to EGF receptor activation resulting in PI3K/AKT signaling cascade events (Farach-Carson & Davis 2003; Kim & Bender 2005). These activated pathways can regulate the phosphorylation of several non-ER transcription factors resulting in initiation of gene transcription (Watters & Dorsa 1998). In addition, these pathways can also modulate ER transcription via phosphorylation of the nuclear ER and interaction with coregulatory proteins (Kow & Pfaff 2004).

It is worth mentioning that non-genomic regulation of ER by E_2, is cell type-specific. Indeed, the E_2-mediated activation of Src/PI3K/MAPK pathway was found

in late, but not early differentiated rat pre-adipocytes (Dos Santos et al. 2002). Similarly, E_2-mediated PKC cascade was evident in the preoptic area of female rat brain, however it was not observed such activity in the cortex or hypothalamus (Ansonoff & Etgen 1998).

Taken together, all these studies indicate that ERα is the major mediator of rapid E_2 action. However, little is known about the role of ERβ to trigger non-genomic E_2-mediated functions. It has been reported that ERβ-ER complex is able to activate p38 MAPK in human cancer cells, but no effect on ERK or AKT activation was observed (Acconcia et al. 2005; Marino et al. 2006). Moreover, ERβ was able to activate inositol tris-phosphate production, JNK and ERK phosphorylation in hamster ovary cells (Razandi et al. 1999). Although it is not entirely clear whether ERβ is able to promote E_2-mediated rapid signals, these findings imply that ERβ can regulate cell-specific signalling pathways.

1.4 Breast Cancer classification

Breast cancer is a heterogeneous disease, classified into a number of subtypes (**Table 1.2**) according to the molecular profile of the tumour (Yersal & Barutca 2014). Diagnostically, it is classified based on the expression of ERs and/or progesterone receptors (PR) as well as the overexpression or amplification of the human epidermal growth factor receptor 2 (HER2) (Yersal & Barutca 2014). Although initially categorised into two main groups dependent upon expression, or not, of the ER (Perou et al. 2000), more recent studies suggest that breast cancer is subdivided into at least 4 distinct clinical categories, the Luminal A and B, the HER2 and the basal-like (Dai et al. 2015) according to their molecular profile.

1.4.1 Luminal breast cancer

It is estimated that approximately 75% of breast tumours express the ER and/or PR and accordingly express ER-target genes and other genes that encode proteins for the luminal cell; such as cytokeratins, thus these cancers are denoted as having a Luminal phenotype (Ignatiadis & Sotiriou 2013). Luminal breast cancer are generally associated with a good prognosis and are likely to respond to endocrine treatments which selectively target the ER (Ignatiadis & Sotiriou 2013). Evolution and use of gene set analysis led to the design of three independent studies investigating breast gene expression (C Sotiriou et al. 2003; van 't Veer et al. 2002; Sørlie et al. 2001). Combination of these data resulted in the identification of two luminal subcategories, termed as Luminal A and Luminal B (Hu et al. 2006).

Luminal A represents the most common breast cancer subtype accounting for 50-60% of all breast cancer cases. It is characterised by low histological grade, low levels of the proliferation marker Ki67 and significantly high ER levels (Ciriello et al. 2013). Patients with Luminal A tumours usually have good prognosis and present low relapse rate compared to other breast cancer subtypes (Ignatiadis & Sotiriou 2013). Secondary tumour foci from Luminal A breast tumour are mostly observed in the bone, whereas lung and liver are less frequently observed sites of metastases (Guarneri & Conte 2009; Kennecke et al. 2010). The mainstay treatment for Luminal A tumours is the hormonal therapy (Ignatiadis & Sotiriou 2013).

Luminal B breast cancer accounts for the 15-20% of total breast cancers (Creighton 2012). It is characterised by high histological grade, Ki67 and ER expression (Cheang et al. 2009; Creighton 2012). Breast cancer patients with Luminal B tumours have worse prognosis, high recurrence possibility and lower survival rates compared to those with Luminal A subtype (Ades et al. 2014). The major difference between the

Luminal subtypes is considered to be the Ki67 expression. Unlike the Luminal A subtype, Luminal B tumours are not highly sensitive to endocrine therapy, however they respond well to neoadjuvant chemotherapy (Melisko 2005; Bhargava et al. 2010; Rouzier et al. 2005).

1.4.2 HER2+ breast cancer

HER2 positive (HER2+) breast cancer represents 15-20% of breast tumours. This breast cancer subtype is highly proliferative and overexpresses the HER2 oncogene, which is associated with the corresponding signalling cascade. HER2+ breast cancer usually exhibits a high histological grade and consequently demonstrates a more aggressive phenotype with poorer prognosis compared to Luminal tumours. It is estimated that approximately 50% of HER2+ breast cancers are positive for the ER. In some cases, HER2+ tumours can appear insensitive to the endocrine treatment, however they exhibit enhanced responsiveness to cytotoxic agents, such doxorubicin. Recently, the development of the translational science has led to the development of targeted therapies that have significantly improved HER2 prognosis and increased survival rates.

1.4.3 Basal-like breast cancer

The last group encompasses all the breast tumours that do not belong in the previous categories, designated as basal like breast cancers. These tumours are characterised by the expression of genes characteristic of the basally located region of the mammary gland, such as cytokeratins 5 and 17, thus termed as basal-like (Toft & Cryns 2011). Basal-like subgroup represents 8-37% of breast tumours and includes the triple negative breast cancer (TNBC) that do not express ER/PR/HER2 and the tumours that

carry mutated BRCA1 gene (Rakha et al. 2009). Since they lack a distinct therapeutic target, chemotherapy constitutes the only therapeutic approach for basal-like tumours (William D Foulkes et al. 2010). Whilst TNBCs exhibit enhanced sensitivity to chemotherapy, they are associated with an aggressive phenotype and a very poor prognosis compared to the other subtypes (Ismail-Khan & Bui 2010).

Table 1.2: Receptor status and frequency rates of breast cancer subtypes.

Subtypes	Receptor status	Frequency
Luminal A	ER^+ and/or PR^+, $HER2^-$low/absent Ki67	50-60%
Luminal B	ER^+ and/or PR^+, $HER2^+$ ($HER2^-$ with high Ki67)	15-20%
HER2	ER^-, PR^-, $HER2^+$	15-20%
Basal-like	Ki67, ER^-,PR^-, $HER2^-$,	8-37%

1.5 Endocrine Therapy

Endocrine therapy constitutes the oldest therapeutic approach for the treatment of breast cancer. Significant research effort over the past few decades, has led to significant advances in endocrine therapy and the development of new hormonal agents (Howell et al. 2002; Cole et al. 1971; Coombes et al. 1984; Baselga et al. 2012) with current therapies now representing an effective treatment for breast cancer patients whose tumours are ER+ (Palmieri et al. 2014). Endocrine treatments can be grouped into 4 distinct classes: luteinising-hormone-releasing-hormone (LHRH) analogues, aromatase inhibitors, selective oestrogen receptor modulators (SERMs) and selective oestrogen receptor down-regulators (SERDs).

1.5.1 LHRH agonists

LHRH agonists are synthetic peptide analogues of the natural hormone, LHRH. They are widely used in hormone-sensitive tumors, such as prostate and breast cancer. In pre-menopausal women, where oestrogen production is mainly supplied by the ovaries, LHRH agonists induce pharmacological oophorectomy effectively suppressing E_2 levels (Goel et al. 2009).

1.5.2 SERMs and Tamoxifen

SERMs are a class of compounds that compete with oestrogens for ER binding, thus preventing E_2-induced ER activity (Osborne et al. 2000). SERMs display both agonist and antagonist activities dependent on the target tissue (Osborne et al. 2000). In breast, SERMs exert antagonistic effects on the ER, whereas in endometrial tissue they act as partial agonists and thus their use is associated with a risk of endometrial

cancer development (Bland et al. 2009). The main representative of this class of hormonal treatment is Tamoxifen.

Tamoxifen was first synthesized in 1963 (Harper & Walpole 1967) and since then several clinical trials have confirmed the high benefit in patients with early or advanced breast cancer (Clemons et al. 2002). It was approved by the Food and Drug Administration (FDA) in 1977 for the treatment of advanced breast cancer (Cohen et al. 2001). Tamoxifen itself is a pro-drug with low affinity for the ER. However, it is metabolised in the liver via cytochrome P450 enzyme CYP2D6 (Ortiz de Montellano 2013), to its pharmacologically active metabolites endoxifen and 4-hydroxy-tamoxifen (4OH-Tam) (Bourassa et al. 2011). The latter displays up to 100 times higher affinity for the ER compared to the parental compound (Fan et al. 2000).

In breast cancer, Tamoxifen exert its antagonistic activity by competing with E_2 for binding to the ER. Upon binding tamoxifen induces conformational alterations in the ER that disrupt its interaction with co-activator proteins (Brzozowski et al. 1997). The ER-Tamoxifen complex further promotes the recruitment of suppressor proteins that impede oestrogen-mediated gene activation and also promotes cell cycle arrest in the G_o/G_1 phase (Renoir et al. 2008). In bone and in the endometrium Tamoxifen displays an agonistic profile accounting for the development of osteoporosis in pre-menopausal women and the risk of endometrial cancer development in patients that are exposed to Tamoxifen for a long period respectively (Bland et al. 2009). Overall, Tamoxifen discovery has significantly contributed to the treatment of breast cancer patients by prolonging the survival rates and reducing the risk of recurrence (Clemons et al. 2002).

1.5.3 Aromatase Inhibitors (AIs)

In post-menopausal women circulatory oestrogens are produced by the conversion of androgens in peripheral tissues such as adipose tissue, adrenal glands and liver (Brueggemeier et al. 2005). This process is known as aromatisation and requires the activity of the enzyme aromatase (Geisler et al. 1996). AIs are administered to post-menopausal patients with ER+ breast cancer and inhibit the function of the aromatase enzyme and hence oestrogen production (Smith & Dowsett 2003). Importantly, the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial revealed that the AI anastrozole exerted greater benefit in the adjuvant setting as first line treatment for ER+ postmenopausal women compared to tamoxifen (Baum et al. 2002). Due to the ATAC trial findings, tamoxifen is currently used as a second line treatment following relapse from AIs in ER+ breast cancer.

1.5.4 SERDs and Faslodex

This class represents a relatively new group of hormonal therapies for the treatment of patients with hormone-sensitive breast cancer and encompasses compounds with pure anti-oestrogenic activity. Biologically, SERDs exert anti-mitotic activity, pro-apoptotic actions and also result in the degradation of the ER itself. The main SERD used clinically is Faslodex (Fulvestrant).

Faslodex is an ER antagonist that exhibits 100 times higher affinity for the ER compared to Tamoxifen (Robertson 2007). It is a pure anti-oestrogen, since it does not exhibit any agonistic effects and consequently lacks the side effects of SERMs (Howell, C. Kent Osborne, et al. 2000). The mode of action of faslodex involves the binding, the inhibition and finally the degradation of the ER resulting in the suppression of ER signaling (Howell, C Kent Osborne, et al. 2000). Clinically,

Faslodex is generally used in patient's that have relapsed on prior anti-hormonal therapies (Tamoxifen or AIs) and its efficacy has been shown to be similar to that of the aromatase inhibitor, anastrozole (Morris & Wakeling 2002). There is also clinical data to suggest that it may be equivalent or marginally better than AIs as a first line treatment (Chia et al. 2008).

1.6 Endocrine resistance

Despite the benefits of endocrine therapy in ER+ disease, approximately 30% of early and almost all advanced breast cancers initially responsive to endocrine agents will acquire resistance (C. Kent Osborne & Schiff 2011). Moreover, a minority of breast cancers will fail to respond to endocrine treatments despite being ER+ (*de novo* resistance). As such, endocrine resistance represents one of the most challenging issues in the clinical setting as it is associated with disease progression and enhanced morbidity rates (Garcia-Becerra, Santos, Diaz and Camacho, 2013). Several studies have pointed to four major mechanisms of resistance: i) ER loss ii) differential activity of ER co-regulatory factors, iii) differential expression of cell cycle regulators and iv) ER cross-talk with receptor tyrosine kinases, such as EGFR and HER2 (Musgrove & Sutherland 2009).

1.6.1 Intrinsic (*de novo*) resistance

The main mechanism of *de novo* resistance to endocrine therapy is lack of ER expression and accounts for reduced responsiveness to endocrine therapy in approximately 15-20% of breast cancer patients (Dixon 2014). Another, mechanism of intrinsic resistance to the agent tamoxifen is the presence of inactive alleles of the cytochrome P450 isoenzyme, CYP2D6 (Hoskins et al. 2009). Patients carrying these

alleles fail to metabolise Tamoxifen to its active products and consequently their responsiveness to Tamoxifen is limited (Hoskins et al. 2009).

1.6.2 Acquired resistance

1.6.2.1 ER and co-regulatory factors

The ER modulates gene expression through interactions with co-regulatory proteins (co-activators and co-suppressors) and the differential expression of these factors significantly affects the equilibrium between the agonistic and antagonistic properties of SERMs as well as altering ligand-independent ER activity. Thus, these ER accessory proteins can represent critical determinants for responsiveness or resistance to endocrine agents (Schiff et al. 2003). The ER can also interfere with various molecules outside the nucleus that result in signaling pathway activation, which in turn enhance non-nuclear ER localisation and subsequently endorsement of its non-genomic activity (Schiff et al. 2004). This process generates a positive feedback loop between the ER and growth factors and it is important to note that non-nuclear ER mechanism can be activated by both E_2 and tamoxifen (Shou et al. 2004). In addition, hyper-expression of transcription factors, such as activator protein 1 (AP-1), specificity protein 1 (SP-1) and nuclear factor-κB (NF-κB), significantly predispose the development of endocrine resistance, especially to Tamoxifen (Zhou et al. 2007; Johnston et al. 1999). ER activity is highly regulated by post-translational modifications (methylation, phosphorylation and sumoylation) that notably affect ER responsiveness to endocrine therapy (Musgrove & Sutherland 2009; Prabhu et al. 2015). Furthermore, the expression of the recently discovered truncated splicing variant (ERα36) accounts for reduced responsiveness to endocrine agents (Shi et al. 2009).

1.6.2.2 Cell cycle regulators

The second possible mechanism of acquired resistance involves cell cycle regulators. Specifically, increased expression of molecules that positively regulate G1 phase progression have been found to impede the inhibitory effects of endocrine agents, resulting in resistance. Such molecules are the cyclins E1 and D1, whose overexpression can either activate cyclin-dependent kinases (CDK) or diminish the inhibitory effects of CDK suppressors p21 and p27 (Butt et al. 2005; Span et al. 2003). Similarly, down-regulation or reduced activity of cell cycle suppressor factors, such as p21 and p27, can lead to reduced sensitivity to endocrine agents (Chu et al. 2008). In addition, enhanced activity of growth factor receptors or other key signaling pathways such as AKT and Src negatively regulate cell cycle suppressor factor and consequently result in the development of endocrine resistance (C. Kent Osborne & Schiff 2011).

1.6.2.3 ER- cross talk with RTKs

ER cross-talk with RTKs is one of the most crucial mechanisms that can lead to the development of endocrine resistance. A number of studies have reported that elevated expression of RTKs and their downstream signaling cascades, primarily ERK and PI3K pathways, can promote the development of tamoxifen resistance (Cui et al. 2012; Fagan et al. 2012; Massarweh et al. 2008; Dourdin et al. 2008; deGraffenried et al. 2003; Faridi et al. 2003). Deregulation of ERK and PI3K cascades can also occur as a result of a variety of genetic and epigenetic alterations, including inactivation of the tumour suppressor phosphatase tensin homolog (PTEN) (Zhang et al. 2013), mutations in phosphatidylinositol 4,5-bisphospate 3-kinase catalytic subunit alpha isoform

(PIK3CA) that lead to constitutive PI3K activation (Campbell et al. 2004) and HER2 amplification (Meng et al. 2004).

EGFR and HER2 overexpression constitute the most widely studied mechanism of endocrine resistance in breast cancer. Evidence suggests that loss of HER2 suppressors including the fork-head box p3 (FOXp3) and the zinc finger transcription factor GATA4 (Zuo et al. 2007; Hua et al. 2009), play a key role in facilitating overexpression of this receptor. Elevated expression of EGFR and/or HER2 have been observed in MCF7-derived models of acquired tamoxifen or faslodex resistance, suggesting a prominent role of EGFR and HER in the development of endocrine resistance (McClelland et al. 2001)(Knowlden et al. 2003). Further investigation of these models demonstrated enhanced activation on MAPK and PI3K/AKT pathways that ultimately lead to cell growth and survival (Nicholson et al. 2007). In addition, immunohistochemical analysis from patient samples with both *de novo* and acquired tamoxifen resistance, revealed hyperexpression of EGFR, HER2 and MAPK (Gutierrez et al. 2005; Gee et al. 2005).

Src kinase interacts and regulates a number of proteins via phosphorylation on tyrosine residues. Previous studies have pointed to the involvement of Src in the development of endocrine resistance through its interaction with ER and p85 subunit of PI3K that ultimately leads to AKT and ERK1/2 activation and consequently cell proliferation (Vallabhaneni et al. 2011; Song et al. 2005; Cheskis et al. 2008). *In vitro* studies have demonstrated that Src substrate Cas is able to activate a number of different pathways, including those that regulate proliferation, invasion and survival. Elevated expression of Cas in breast cancer cells has been correlated with reduced responsiveness to tamoxifen (Dorssers et al. 1993). Since Cas is associated with

resistance, this indirectly implicates a role for Src in the development of endocrine resistance as an upstream regulator.

1.6.3 Src kinase

Src is a 60 kDa non-receptor tyrosine kinase (TK) that belongs to a nine-member family collectively termed Src family kinases (SFKs). This group of proteins is comprised of Src, Lyn, Fyn, Yes, Fgr, Hck, Lck, Blk and Frk (Finn 2008). Src is the oldest known oncogene, first identified in 1911 as the transforming product (v-Src) of the oncogenic Rous sarcoma retrovirus in chicken sarcomas (Thomas & Brugge 1997).

Src activity is primarily regulated by phosphorylation on tyrosine residues 527 (Y527) and 416 (Y416) (**Figure 1.2**). Phosphorylation of Y527 by tyrosine kinases Csk and its homolog Chk, reduces Src kinase activity enabling the molecule to take up an inactive conformation (Sen & Johnson 2011). The auto-phosphorylation site at Y416, promote the kinase activity by allowing the molecule to establish a more open structure that facilitates the interaction with downstream signalling molecules (Finn 2008). Thus, the auto-phosphorylation of Y416 constitutes the activation event of Src kinase. In addition, Src can be activated by surface receptors, such as EGFR (Thomas & Brugge 1997). Protein tyrosine phosphatases (PTP), such as PTPα, PTPγ, SHP-1,2 and PTP1B are able to regulate Src kinase function through dephosphorylation of Y527 (Sen & Johnson 2011). Specifically, PTP1B expression has been correlated with enhanced Src activation in breast cancer cell lines (Jeffrey D. Bjorge et al. 2000).

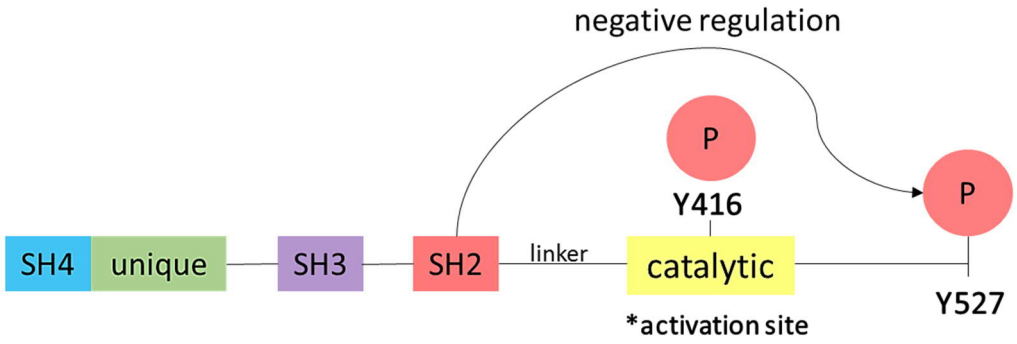


Figure 1.2: The structure of Src kinase.

Many studies have been conducted regarding the role of SFKs at a cellular level and have revealed that Src in particular regulates signalling pathways that control proliferation, migration, angiogenesis and differentiation (Christos Sotiriou et al. 2003; Summy & Gallick 2003). Given that Src and related members are implicated in cellular processes central to that of tumour development and progression, it is not surprising that Src has been implicated in cancer.

Src is over expressed or activated in many cancer types, including breast (Sen & Johnson 2011). Elevated Src expression and activity has been reported in cancer cells that display an aggressive, metastatic phenotype and resistance to anti-growth or endocrine treatments (Jackson et al. 2000; Irby & Yeatman 2002; Mao et al. 1997). Src inhibition in breast cancer resistant models has been found to diminish migration and invasion, indicating the pivotal contribution of Src in the metastatic behavior (Hiscox et al. 2007). In addition, Src plays a central role in bone cell metabolism and it is highly involved in bone metastasis as it is essential for osteoclast survival and ruffled border formation, which enables the osteoclast to attach the bone substrate during resorption (Boyce et al. 1992). Furthermore, recent evidence suggests that Src activation is tightly correlated with late-onset of bone metastasis in breast cancer, regardless of the subtype and the hormone receptor status (Zhang et al. 2009).

Src has been found to localise to the plasma membrane, where it can interact with growth factor receptors to regulate signalling pathways particularly those involved in cell proliferation (J D Bjorge et al. 2000). Whereas in normal breast cells Src is usually detected in cytoplasmic regions, in tumour cells it is primarily localised around the nucleus indicating a possible involvement of Src in nuclear-signal transduction events (Sen & Johnson 2011; Verbeek et al. 1996). In addition, Src participates in the non-genomic ER signalling. Through rapid E_2-mediated stimulation Src activates downstream signaling pathways that enhance tumour progression and metastasis (Silva et al. 2010).

Given the central role of Src in cancer progression and metastasis, a number of small molecule that target Src kinase have been developed. Src inhibitors are ATP-binding competitive inhibitors that do not selectively target Src, but are able to interfere and block other SFK members as well (Mayer & Krop 2010). Some of the most well studied Src kinase inhibitors include Dasatinib, Saracatinib and Bosutinib and their efficacy in breast and other cancers has been evaluated in large clinical trials (Sen & Johnson 2011).

Numerous *in vitro* studies have revealed that treatment with Src inhibitors was able to suppress migration and tumour growth in a variety of cancer cell types, including breast, prostate, lung, pancreatic and colon (Messersmith et al. 2009; Richard S. Finn et al. 2007; Park et al. 2008). Src inhibition results in reduced expression of various proteins that are regulated via Src, such as MAPK, AKT, and focal adhesion kinase (FAK), that subsequently leads to suppression of tumour growth (Jallal et al. 2007).

Due to the key role of Src in facilitating the non-genomic ER function and consequently the development of endocrine resistance (Varricchio et al. 2007), Src inhibitors may represent a potent agent for overcoming endocrine resistance. Src

inhibition in endocrine resistant cells resulted in reduced Src activity and decreased invasion (Hiscox, Morgan, Tim P Green, et al. 2006). In addition, treatment of androgen-independent prostate cancer cells with Saracatib, was found to inhibit tumour growth and to prevent the nuclear translocation of the androgen receptor (Yang et al. 2009).

The effectiveness of the combination of Src kinase inhibitors with other targeted agents or chemotherapy is also under investigation. Src inhibition has been found to enhance the chemosensitivity of human pancreatic cells to 5-fluororacil *in vitro* (Ischenko et al. 2008). In addition, simultaneous treatment of Saracatinib and RTK inhibitors displayed additive inhibition effect in MCF7-derived tamoxifen resistant cells compared to the single agents (Hiscox, Morgan, Tim P Green, et al. 2006). Overall these finding suggest that combination of targeted therapy or chemotherapy with concurrent Src inhibition demonstrates an additive benefit to cancer treatment by suppressing tumour growth and metastatic potential.

1.7 Breast cancer bone metastasis

It is estimated that approximately 85% of patients with advanced breast cancer will develop bone metastases at some point (A Lipton et al. 2009). This results in a low quality of life, mainly due to severe pain, pathological fractures and spinal cord compression (Theriault & Theriault 2012). The seed and soil theory that was introduced by Stephen Paget in 1889, suggests that cancer cells (seeds) can only establish metastatic foci in appropriate stromal environments (soils) (Ribatti et al. 2006). Consequently, not all types of cancer can form bone metastasis. There are two different types of bone metastasis; termed as osteolytic and osteoblastic. Osteolytic

metastasis are more frequent and result in bone breakdown, while osteoblastic are less uncommon (10-20%) and promote new bone formation (Chen et al. 2010a).

In order to understand how tumour cells can influence the bone microenvironment to support metastases, it is first important to consider how bone is remodeled physiologically. Bone is a metabolically dynamic tissue that undergoes consecutive remodelling and it is comprised of two distinct areas. The outer area mainly consists of hard-mineralised matrix in which reside several growth factors (Hauschka et al. 1986). The inner part is composed of bone marrow, which encompasses haematopoietic and stromal cells (Owen & Friedenstein 1988). Bone metabolism is dependent upon the well-orchestrated balance of the two main cell types that exist in the bone microenvironment, osteoblasts (OBs) and the osteoclasts (OCs) (Casimiro et al. 2009).

1.7.1 Osteoblasts and osteoblastic metastasis

Osteoblasts are large bone-producing cells. derived from mesenchymal stem cells (Komori 2006). Apart from bone synthesis, osteoblasts are major regulators of osteoclastogenesis as they produce macrophage-colony stimulating factor (MCS-F) and receptor activator for nuclear factor κB ligand (RANKL), which are key factors for osteoclast maturation (Khosla 2001). Moreover, osteoblasts secrete osteoprotegerin (OPG), which is a decoy receptor of RANKL and hence is able to inhibit osteoclast differentiation and consequently bone breakdown (Hofbauer 2004). The ratio of RANKL:OPG determines the extent of osteoclast activation and consequently bone degradation (Hofbauer 2004). In addition, on the osteoblast surface there are receptors for the parathyroid-related peptide (PTHrP). Once PTHrP binds to

its receptor, stimulates the secretion of RANKL with concurrent down-regulation of OPG, further regulating osteoclast activation (Datta & Abou-Samra 2009).

Osteoblastic bone metastases are stimulated by tumour-secreted factors and trigger the formation of new bone tissue. Due to the fact that osteoblastic metastases are relatively uncommon, the exact mechanism is yet poorly understood (Akhtari et al. 2008). Emerging evidence suggests that tumour-derived endothelin-1A (ET-1A) plays a central role in the development of osteoblastic metastases since ET-1A interaction with the ET-1A receptor (ETAR) stimulates signal transduction pathways and gene transcription involved in osteoblast proliferation and bone formation (Mohammad & Guise 2003); inhibition of ETAR subsequently reduces osteoblastic bone lesions (Yin et al. 2003).

1.7.2 Osteoclasts and osteolytic bone metastasis

Osteoclasts are large, multinucleated bone-resorbing cells that originate from haetopoietic cells of the bone marrow (Bar-Shavit 2007a). CD14 monocytes are considered to be osteoclast precursor cells that fuse together in order to form a multinucleated osteoclast (Udagawa et al. 1990). This process, known as osteoclast differentiation, occurs within 5-8 days and requires the contribution of MCS-F and RANKL factors (Asagiri & Takayanagi 2007).

RANKL itself is expressed by osteoblasts, stromal cells and T-cells all known to be present in the tumour microenvironment. Upon binding to its receptor RANKL, which is located on osteoclast precursor cell surface, osteoclast differentiation and activation triggered (Suda et al. 2001). RANKL is of fundamental importance in the regulation of osteoclastogenesis and osteoclast survival. *In vivo* studies have underlined the significance of RANKL in bone remodeling by demonstrating that lack

of RANKL and RANK genes in animal models leads to the development of osteopetrosis due to the absence of osteoclast differentiation and function (Dougall et al. 2014).

Osteolytic metastasis constitutes the most frequent type of bone metastasis. Osteolysis occurs after enhanced tumour-mediated osteoclast activity, which is accompanied by restrained osteoblast function (Chirgwin J.M. and Guise T.A., 2000). Cancer cells promote osteoblast activation through the secretion of cytokines, including PTHrP, ILs. Osteoblasts in turn, produce RANKL that stimulates osteoclast differentiation and activation resulting in bone resorption (Gregory R. Mundy 2002). Bone breakdown releases factors that are stored in the bone microenvironment, such as transforming growth factor β (TGFβ), that further stimulate cancer cell proliferation perpetuating the co-called vicious cycle of bone metastasis (**Figure 1.3**) (Esposito & Kang 2014). In addition, cancer cells are able to stimulate osteoclastogenesis in a RANKL-independent manner. Specifically, breast cancer cells secrete PTHrP, IL-1, IL-6, IL-8, IL-11, IL-12 and tumor necrosis factor α (TNF-α) that can stimulate osteoclast differentiation, even in the absence of RANKL (Yoneda & Hiraga 2005).

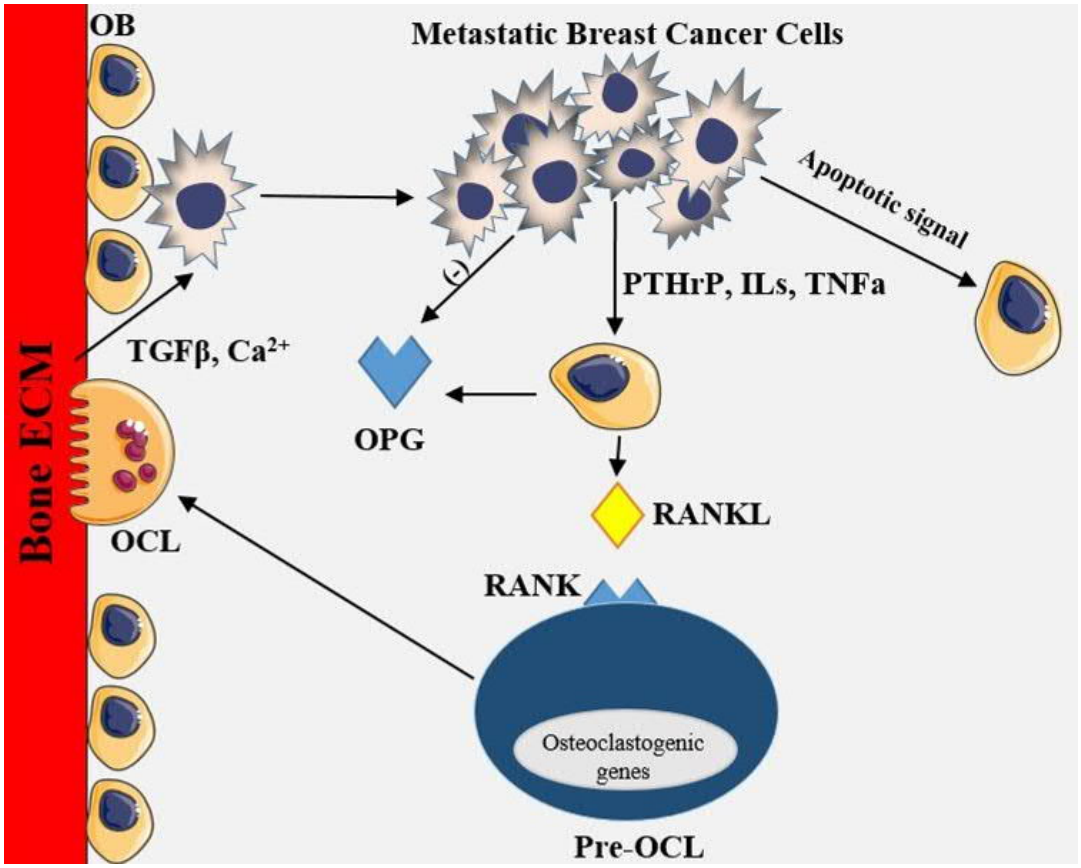


Figure 1.3: Molecular mechanism involved in osteolytic breast cancer bone metastasis. Metastatic breast cancer cells produce factors such as ILs and PTHrP that activate the osteoblasts. Osteoblastic cells in turn, produce RANKL promoting the differentiation of pre-osteoclasts to mature osteoclasts in a RANK/RANKL dependent manner. In addition, PTHrP can promote osteoclast maturation in a RANK/RANKL independent manner. Bone breakdown by osteoclasts, releases TGFβ, which further enhances the proliferation and growth of breast cancer cells, perpetuating the "vicious cycle" of bone metastasis. OB: osteoblast, OCL: osteoclast, IL: interleukin, TNFa: tumor necrosis factor alpha, PTHrP: parathyroid related peptide, OPG: osteoprotegerin, ECM: extracellular matrix, RANKL: receptor activator of NF-κB ligand, TGFβ: transforming growth factor β.

1.8 Bisphosphonates (BPs)

Bone loss is a problem in post-menopausal women and also in cancer patients with osteolytic bone metastasis. One therapy developed to help combat bone loss are the bisphosphonates. BPs are a class of compounds with anti-resorptive properties that are able to inhibit osteoclast-mediated bone degradation. They are characterised by two phosphonate groups (P) bound to same carbon atom (C) forming a P-C-P backbone that displays high affinity with hydroxyapatite crystals, known as bone mineral (Russell et al. 1999). BPs are synthetic analogues of pyrophosphate, in which the oxygen atom is replaced by the carbon, a key alteration that renders them resistant to enzymatic activity. They are classified in two subclasses: non-nitrogenous and the more recently developed nitrogen-containing compounds (N-BPs) (Russell et al. 1999).

Non-nitrogen-containing bisphosphonates (clodronate and etidronate) reside in the bone matrix and are digested by osteoclasts during osteolysis (Clézardin 2011). Due to the high similarity of these compounds with the pyrophosphate (PPi), they are incorporated into non-hydrolysable ATP analogues disrupting the function of ADP/ATP translocase that subsequently leads to osteoclast apoptosis due to a lack of energy and thus impaired metabolism (Lehenkari et al. 2002). Apart from osteoclast apoptosis induction, BPs block osteoclast progenitor recruitment inhibiting the generation and differentiation of new osteoclasts (Fleisch 1998). Importantly, BPs also impair osteoclast ability to form ruffled borders, which are essential for osteoclast attachment to the bony surface, thus preventing bone resorption (Colucci et al. 1998; Sato et al. 1991; Fleisch 1998).

N-BPs (alendronate, zoledronate, pamidronate, risedronate, ibandronate and minodronate) on the other hand represent second generation therapeutics that are also

able to disturb the mevalonate pathway by inhibiting farnesyl pyrophosphate synthase (FPPS), one the major enzymes of the mevalonate pathway that regulates cholesterol synthesis and protein prenylation. Agents such as zoledronic acid (ZOL) are able to inhibit FPPS that ultimately leads to mevalonate pathway disruption (**Figure 1.4**). FPPS inhibition results in accumulation of isopentenyl pyrophosphate, which in turn is converted into a cytotoxic molecule called ApppI. In addition, mevalonate pathway blockage prevents the prenylation of small GTPases, such as RhoA, Rac, Cdc42, RhoU, Arf6 and Rab, which are important for the formation of ruffled borders and sealing zone in osteoclasts (Itzstein et al. 2011). Importantly, impaired prenylation of small GTPases can also drive the osteoclast cell to apoptosis (Kavanagh et al. 2006; Mönkkönen et al. 2006).

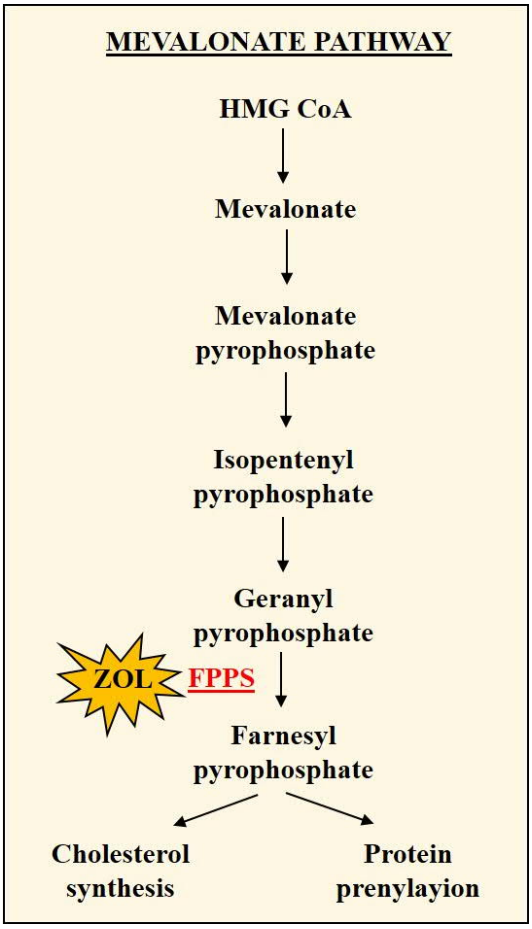


Figure 1.4: The mevalonate pathway and action of nitrogen-containing bisphosphonates.

1.8.1 Anti-tumour properties of N-BPs

Intriguingly, reports have emerged recently suggesting that N-BPs, such as ZOL, apart from their effectiveness in preventing bone resorption, may also exert anti-cancer properties in a variety of tumours, including breast (Coleman et al. 2014; Holen et al 2010; Winter et al. 2008). These anti-tumour properties are attributed to the direct action of ZOL on tumour cells themselves or other cells likely to be present in the bone microenvironment such as γδ T cells, endothelial cells and macrophages (Clézardin 2011).

BPs have been shown to directly inhibit proliferation, invasion and migration and promote programmed cell death in tumour cells through direct action (I. Holen 2010; Clézardin 2013). One potential mechanism through which they may achieve this is through disruption of the mevalonate pathway since BP-mediated inhibition of cell cycle progression can be partially counteracted by the addition of mevalonate pathway intermediates (Stresing et al. 2007). Since the mevalonate pathway is important for localisation of GTPases to the plasma membrane for signalling, this may thus represent a major mechanism of the anti-tumour action of BPs.

In the light of promising *in vitro* and pre-clinical data regarding the anti-tumour properties of BPs, particularly ZOL, three large cohort Phase III clinical trials were designed (ABCSG-12, ZO-FAST and AZURE) in order to evaluate potential benefit from ZOL in breast cancer patients (**Table 1.3**).

The ABCSG-12 trial evaluated tamoxifen or anastrozole efficacy with and without ZOL in pre-menopausal women that were treated with LHRH agonists. The addition of ZOL significantly improved disease-free survival (DFS) and overall survival (OS) rates after 76-month follow-up (Gnat et al. 2011).

The ZO-FAST trial involved post-menopausal women treated with aromatase inhibitor with and without ZOL. Immediate addition of ZOL to adjuvant endocrine treatment reduced the risk of DFS events by 34% compared to delayed addition of ZOL after a 60-month follow-up (De Boer et al. 2010)

The AZURE trial included pre- and post-menopausal patients receiving adjuvant chemotherapy and/or endocrine treatment alone or in combination with ZOL. AZURE outcomes revealed that ZOL did not improve DFS in the total population, however a statistically significant DFS benefit was found in patients that were at least 5 years after menopause. OS benefit was observed only in the subset of patients with age greater than 60 years (Coleman et al. 2010).

Table 1.3: **Zoledronic acid phase III clinical trials in breast cancer.** CT: chemotherapy, ST: standard treatment, ET: endocrine therapy, AI: aromatase inhibitor, DFS: disease-free survival OS: overall survival, PMW: post-menopausal women

	ABCSG-12	**ZO-FAST**	**AZURE**
Population	1803 Premenopausal, stage I/II, ER+, receiving LHRH agonists	1065 Postmenopausal stage I–IIIa, ER+, receiving AIs for 5 years	3360 pre- and postmenopausal, stage II/III, receiving standard CT and/or ET
Treatment	LHRH+ AI or Tam ± ZOL for 5 years	AI± ZOL for 5 years	ST(CT±ET) ± ZOL for 5 years
ZOL DFS benefit	Yes	only in the subset of PMW	Yes
ZOL OS benefit	Yes	only in patients <60 years	No

1.9 Aims

Acquired endocrine resistance in breast cancer results in disease relapse frequently at distant sites including bone. The central aim of this thesis was to explore whether endocrine resistance altered the ability of breast cancer cells to influence pre-osteoclast cells that play a major role in the vicious cycle of bone metastases. We further wished to investigate whether Src was involved in this process and to study the direct anti-tumour action of bisphosphonates on breast cancer cells, particularly in the context of endocrine resistance.

2. Materials and methods

2.1 Materials and reagents

The SRC inhibitor Saracatinib (AZD0530) (**Figure 2.1**) used for this study was a gift from AstraZeneca. Saracatinib act as an ATP-competitive inhibitor of SRC and Abl kinases (Liu et al. 2013). Stock solutions were prepared in DMSO at a concentration of 10mM and stored in 50ul aliquots at -20°C. Stocks were thawed before use and diluted in media to the appropriate concentration. Any thawed, unused stock solutions was stored at 4°C for a maximum of 2 weeks after which it were discarded.

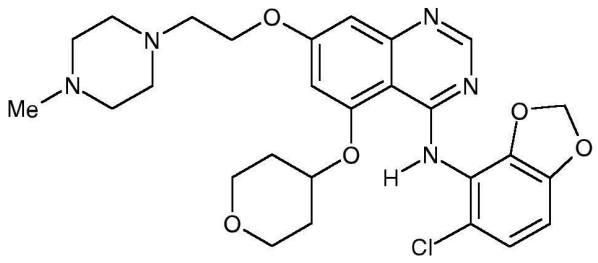


Figure 2.1: Chemical structure of Src inhibitor Saracatinib (Hennequin et al. 2006).

Zoledronic acid (ZOL) (**Figure 2.2**), a nitrogen containing bisphosphonate (N-BP) approved for the treatment of bone lesions in patients with advanced cancer (Clézardin 2013) was purchased from Sigma Aldrich and stock solutions were prepared in H_2O at a concentration of 5μM and stored in aliquots at -80°C until use.

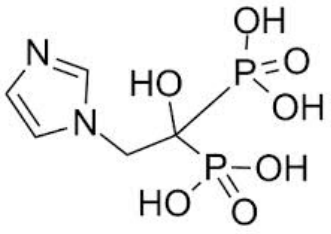


Figure 2.2: Chemical structure of Zoledronic acid.

Table 2.1: Materials and reagents used throughout the current study and their source of purchase.

Materials/Reagents	Supplier
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma Aldrich
5X siRNA buffer solution	Thermo Scientific
30% Acrylamide solution	Sigma Aldrich
Ammonium Persulphate (APS)	Sigma Aldrich
Fungizone	Invitrogen
Penicillin/Streptomycin	Invitrogen
Aprotinin	Sigma Aldrich
Leupeptin	Sigma Aldrich
BioRad Protein Assay Reagents A, B, S	BioRad Laboratories Ltd
Blue sensitive X-ray film	Photon Imaging Systems
Bovine Serum Albumin (BSA)	Sigma Aldrich
Bromophenol Blue	BDH Chemicals
Cell Culture Medium (RPMI 1640 and Phenol Red-free RPMI 1640)	Invitrogen
Chemiluminescence reagents (ECL, Dura, Femto,)	Fisher Scientific
Dimethyl sulphoxide (DMSO)	Sigma Aldrich
Di-thiothreitol (DTT)	Sigma Aldrich
Dharmafect Transfection Lipid	Thermo Scientific
Foetal Calf Serum	Gibco
Glycerol	Fisher Scientific
Glycine	Fisher Scientific
L-glutamine	Sigma Aldrich
NP40	Sigma Aldrich
Ponceau S solution (0.1% w/v in 5% acetic acid)	Sigma Aldrich

Materials/Reagents	Supplier
Phenylarsine oxide	Sigma Aldrich
Phenylmethylsulfonyl fluoride (PMSF)	Sigma Aldrich
Methanol	Fisher Scientific
Precision Plus Protein Blue marker	BioRad
siRNA buffer (1X) diluted in H20	Sigma Aldrich
Sodium Azide	Sigma Aldrich
Sodium dodecyl sulphate (SDS)	Sigma Aldrich
Sodium Fluoride	Sigma Aldrich
Sodium Molybdate	Sigma Aldrich
Sodium Orthovanadate	Sigma Aldrich
TWEEN20	Sigma Aldrich
Triton-X 100	Sigma Aldrich
Stripping buffer	Fisher Scientific
Tetramethylethylenediamine (TEMED)	Fisher Scientific
Trizma Base (Tris)	Fisher Scientific
2-propanol (isopropanol)	Sigma Aldrich
Acetic acid, glacial	Sigma Aldrich
Western Blocking Reagent	Roche Diagnostics
X-ray film developer solution (X-O-dev)	X-O- graph Imaging System
X-ray film fixative solution (X-O-fix)	X-O- graph Imaging System
Whatman qualitative filter paper, Grade 4 (diameter, 125 mm)	Sigma Aldrich
Ficoll Paque plus	GE Healthcare Life Sciences
Human recombinant MCSF	R&D Systems
Mouse recombinant RANKL	R&D Systems
Nitrocellulose membrane	Thermo Fisher Scientific
Foetal Calf Serum (FCS)	Gibco
Trypsin/EDTA 10x solution	Life Technologies

Table 2.2: List of primary and secondary antibodies used for Western blot analysis throughout the current project. Unless otherwise stated, primary antibodies were diluted 1:1,000 (actin 1:15,000) and secondary antibodies used at a 1:10,000 dilution.

Target protein	Species	Supplier
phospho-AKT(Ser473)	Rabbit	Cell Signalling
AKT (total)	Rabbit	Cell Signalling
phospho-42/44 MAPK	Rabbit	Cell Signalling
MAPK (total)	Rabbit	Cell Signalling
SRC (total)	Rabbit	Cell Signalling
phospho-SRC(Tyr416)	Rabbit	Cell Signalling
mTOR (total)	Rabbit	Cell Signalling
phospho-mTOR (Ser2448)	Rabbit	Cell Signalling
phospho-p70S6K (Thr389)	Rabbit	Cell Signalling
phospho-p70S6K (Ser371)	Rabbit	Cell Signalling
4EBP1	Rabbit	Cell Signalling
phospho-EGFR (Tyr1068)	Rabbit	Cell Signalling
EGFR (total)	Mouse	Cell Signalling
PARP	Goat	R&D
FAK (total)	Rabbit	Cell Signalling
phospho-FAK (Tyr397)	Rabbit	Cell Signalling
Anti-rabbit IgG	Goat	Cell Signalling
Anti-mouse IgG	Sheep	Santa Cruz
Anti-goat IgG	Rabbit	Santa Cruz
β-actin	Mouse	Santa Cruz

2.2 *In vitro* cell models and routine cell culture methods

2.2.1 Breast cancer cell models

The ER+, antihormone-sensitive breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (ATCC) and used as an *in vitro* model of Luminal A breast cancer. MCF-7 cells were routinely maintained in RPMI 1640 media containing phenol-red, glutamine (200 mM), 5 % (v/v) foetal calf serum (FCS), Fungizone (2.5 µg/ml) and penicillin/streptomycin (100 IU/ml and 100 µg/ml respectively). All experiments involving antihormones were performed in the above media but without phenol red (wRPMI).

To model triple-negative breast cancer, the MDA-MB-231 cell line was used (ATCC). These cells were maintained in DMEM supplemented with 5 % (v/v) FCS and 1 % antibiotics (Fungizone (2.5 µg/ml) and penicillin (100 IU/ml)/streptomycin (100 µg/ml).

2.2.1.1 Derivation of acquired-antihormone resistant cells

In vitro breast cancer models of acquired antihormone resistance were developed by the Breast Cancer Molecular Pharmacology Group (BCMPG) as follows: MCF-7 cells were continuously cultured in the presence of either 100nM 4-hydroxytamoxifen (‘tam’) or 100nM fulvestrant (faslodex, ‘fas’) with routine media changes every 3-4 days. The medium used for the development of the resistant models was wRPMI supplemented with 5% (v/v) charcoal-stripped foetal calf serum (SFCS) in order to provide a steroid-depleted environment. After a period of initial growth suppression (~3 months), cells began to regrow in the presence of these agents until full regain of growth capabilities in the presence of endocrine agent was acquired at ~ 8 months indicative of acquired resistance to these agents. These resistant cell lines were

designated TamR (tamoxifen resistant) and FasR (faslodex resistant) and maintained in wRPMI+5% (v/v) SFCS as has been previously characterised (Staka et al. 2005). Basic characteristics and culture conditions of all the breast cancer cell lines described above, are summarized in **Table 2.3**.

Table 2.3: Characterisation and molecular features of breast cancer cell models used and their respective culture conditions Adapted from (Neve et al., 2006)

Cell line	Molecular Subtype	ER & PR status	Culture conditions	Tumour Type
MCF-7	Luminal	ER+ PR+	rRPMI +5% FCS	Invasive ductal carcinoma
TamR	Luminal	ER+ PR+	wRPMI +5% SFCS	Invasive ductal carcinoma
FasR	Luminal	ER- PR-	wRPMI +5% SFCS	Invasive ductal carcinoma
MDA-MB-231	Triple negative	ER- PR-	DMEM +5% FCS	Adenocarcinoma

2.2.2 Mouse monocytic cells

RAW 264.7 cells were purchased from ATCC and is a murine monocytic cell line that is widely used as an osteoclast precursor model and can differentiate into osteoclasts upon exposure to RANKL (Collin-Osdoby & Osdoby 2012). RAW 264.7 cells were cultured in alpha Minimum Essential Medium (αMEM) supplemented with penicillin/streptomycin (100 IU/ml and 100 µg/ml respectively) and 10% (v/v) FCS.

2.2.3 Peripheral Blood Mononuclear Cells (PBMCs)

Blood from healthy volunteers was used as a source of PBMCs, which were cultured in αMEM supplemented with penicillin/streptomycin (100 IU/ml and 100 µg/ml respectively) and 10% (v/v) FCS. For blood collection, ethical approval was obtained by the Department of Medicine, Cardiff University (see Appendix B).

2.3 Routine cell culture, passaging and freezing

2.1 Cell culture

All cell lines were cultured in a 37 ˚C/ 5 % CO_2 incubator. Cell manipulations were carried out under sterile conditions in an MDH Class II laminar-flow safety cabinet and all equipment and consumables were either purchased sterile for single use or sterilised at 119 °C in a Denley BA825 autoclave.

2.2 Cell passaging

Once breast cancer cells reached ~70% confluency (by eye) media was aspirated and cells were detached following treatment for 3-5min with trypsin/EDTA solution (0.05%/0.02% in PBS respectively). The trypsin suspension containing the detached cells was centrifuged for 5min at 1000 rpm. After centrifugation, the supernatant was

discarded and the pellet was resuspended in growth media at 1:8 dilution and 10ml of cell suspension was transferred to a new T75 flask and returned to the incubator. Thereafter the media was replaced every 3-4 days unless otherwise stated. A similar process was followed for RAW 264.7, but for cell detachment a cell scraper was used instead of trypsin.

2.3 Cell freezing

The cell pellet from a 70% confluent T75 flask was resuspended in 2ml of maintenance media supplemented with 10% (v/v) serum. 800ul of cell suspension was then transferred into 1ml cryovials and 5% (v/v) of DMSO was added in each vial before they were stored in liquid nitrogen.

2.4 Cell proliferation assays

2.4.1 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The principle of the MTT assay is based the conversion of MTT into insoluble purple formazan crystals through the mitochondrial activity of viable cells. These formazan crystals can be solubilized and the absorbance of the resultant solution measured with a spectrophotometer. The absorbance reading is proportional to the number of viable cells.

Cells harvested by trypsinisation, pelleted and resuspended at $1.5x10^5$ cells/ml before they seeded into a 96-well plate (0.1ml/well) and left to adhere overnight. The following day the media was removed and fresh media was added containing the appropriate treatment. After 72h, 10µl of the MTT reagent (0.5µg/ml in PBS) was added to each well and cells were incubated for another 4h at 37°C in dark. Following

this incubation time, the formazan crystals that had formed were dissolved in 100µl of acidified isopropanol (isopropanol containing 0.04M HCl) and the absorbance of each well was measured at 540nm in a spectrophotometer.

2.4.2 Coulter counting assay

In some cases, a more sensitive method was required than the MTT assay that could more accurately detect subtle changes in cell number following treatment and so a coulter counter based method was employed. In these assays, cells were harvested and prepared as for the MTT assay but seeded into 24-well plates at a density of 10^5 cells/well and left to adhere overnight. The following day the media was aspirated and replaced with fresh media containing treatments as indicated in the respective results chapter. The plate was then returned to the incubator and cultured for the required time. Following this the media was aspirated from each well and replaced with trypsin solution (1ml 0.02% EDTA trypsin/well), left for two minutes and then all solution was drawn up into a 5ml syringe through a 25G needle to ensure that a single cell suspension was achieved. The cell suspension was then transferred into a Coulter counting cup containing 6ml of isoton. The wells were washed with 3x1ml isoton solution each time drawing up the well contents through a 25G needle and transferring the solution into the counting cup so that at the end of the procedure, the cup contained a total of 10ml volume. The final solution was placed on the Coulter Counter stage and the probe inserted approximately half way down the solution. Counting was performed automatically by the Coulter Multisizer II. Each treatment was performed in at least in triplicate and mean average was normalized to % of the corresponding control (set at 100%).

2.5 Microarray Analysis

Prior to this project, the BCMPG undertook a study to characterise the antihormone sensitive and resistant cell models at a gene level using Affymetrix gene-expression microarrays. Briefly, the approach taken to undertake the microarray study was as follows:

RNA samples from MCF-7, TamR and FasR cells were collected in triplicate and sent to a third-party biotechnology service, Cardiff University Central Biotechnology Services (CBS) to be microarrayed. RNA sample quality was assessed. Reverse transcription of RNA to cDNA, synthesis of cRNA to incorporate biotin (*in vitro* transcription), hybridisation, washing, streptavidin staining and scanning of chips to detect amount of transcript hybridised at each gene probe position was performed. Samples were hybridised to Affymetrix Human Genome U133A Arrays containing probe sets representing more than 39,000 transcripts derived from approximately 33,000 well-substantiated human genes. Data obtained from microarraying after normalisation and log2 transformation was uploaded to the commercial software GeneSifter Analysis Edition (Perkin Elmer) (https://login.genesifter.net/). Triplicate data were analysed using t-test or one-way Anova as appropriate. Boxplots for negative controls were generated and the median expression (~6.1) taken as an approximation of baseline expression above which gene expression was considered valid not background noise. In some cases, the gene chips contained more than one gene probe per gene and thus they were consequently placed on the 'Jetset' probe, which identifies the most reliable probe for the gene of interest (Li et al. 2011).

2.6 Gene expression detection by PCR analysis

2.6.1 RNA extraction

Cells were seeded into 60mm dishes and cultured until they reached ~70% confluency. Media was then aspirated and cells were washed twice with 1x PBS before TRI Reagent (Sigma Ltd; 0.75ml/60mm dish) was applied. After 2 minutes, cells were scraped using a disposable cell scraper and lysates were quickly transferred into a 1.5ml Eppendorf tube and stored at -80° overnight. Following this, lysates were thawed and allowed to equilibrate at room temperature prior to RNA extraction as follows:

All the following steps were performed on ice to minimize RNase activity. Chloroform ($CHCl_3$) was added to the cell lysates (0.2ml $CHCl_3$/ml TRI reagent), tubes vortexed for 15sec then left at room temperature for 15 min before centrifugation at 12000g for 15min, 4°C. After centrifugation, the cell lysates separated into three phases with the RNA being present in the upper aqueous phase. This phase was carefully pipetted without disturbing the interface layer and placed to a fresh Eppendorf tube. Isopropanol was added to the aqueous RNA-containing phase (0.75ml isopropanol/ml TRI reagent, and centrifuged at 12000g for 10min. The supernatant was gently removed and the resultant RNA pellet was air-dried and then dissolved in 20 μl of RNase-free water. Samples were stored in aliquots at -80°C until use.

2.6.2 RNA quantification and purification

RNA was quantified by diluting 1μl RNA in 499μl RNase-free water and evaluated in a spectrophotometer by measuring the absorbance at 260nm. The purification was assessed based on the formula: RNA= A_{260nm}x40 (dilution factor) and a ratio of 1.8-2 represented high RNA purity. To assess RNA integrity, samples were run in a 1%

agarose gel supplemented with ethidium bromide and intact RNA led to the formation of two bands indicative of the 18S and 28s ribosomal subunits.

2.6.3 cDNA synthesis

The extracted RNA was converted into its complementary DNA (cDNA) by reverse transcription. For each reaction a master mix solution, as described in **Table 2.4**, was added to 1µg (total volume 7µl) of RNA.

Table 2.4: The volume of the substrates required for master mix preparation for one reaction (1µg RNA)

Reagent	**Volume (µl)**	**Final concentration**
dNTPs	5	2.5mM
10x PCR Buffer	2	10 mM Tris-HCI, pH 8.3, 50 mM NH4, 0.001 % w.v gelatin
Dithiothreitol (DTT)	2	0.1M
Random Hexamers	2	100µM
$MgCl_2$	0.5	50mM

Samples were denatured at 95°C for 5min and then placed on ice for another 5min. To each reaction, 0.5µl RNase inhibitor and 1µl of Molony-murine leukemia virus (MMLV) was added (final volume 20µl) and samples were placed in a thermocycler for reverse transcrption using the following parameters:

Annealing at 22 ˚C for 10min

Reverse transcriptase extension at 22 ˚C for 40min

Denaturation at 95 ˚C for 5min.

The resultant cDNA was stored at -20 ˚C until required.

2.6.4 Oligonucleotide primer design

Gene sequences were obtained from the National Centre for Biotechnology database (see Appendix A) and oligonucleotide primers were designed using the OligoPerfect primer design tool (Invitrogen). In **Table 2.5** are shown the sequences, expected product size and conditions used for PCR analysis. The primer sequences were inputted into the Basic Local Alignment Search Tool (BLAST) to confirm their specificity for the gene of interest.

Table 2.5: The sequences, expected product size of the primers designed and used for RT-PCR analysis. Sizes of products are in base pairs (bp).

Gene target	Sequence	Expected product size	PCR conditions
PTHLH	**FW:** 5'-CCCTCTCCCAACACAAAGAA-3'	309 bp	55 ˚C, 29 cycles
	RV: 5'-GGAGGTGTCAGACAGGTGGT-3'		
RANKL	**FW:** 5'-AGAGCGCAGATGGATCCTAA-3'	140 bp	55 ˚C, 30 cycles
	RV: 5'-TTCCTTTTGCACAGCTCCTT-3'		
M-CSF	**FW:** 5'-ACCCCAGTTGTCAAGGACAG-3'	83 bp	55 ˚C, 29 cycles
	RV: 5'-TTCTGGGACCCAATTAGTGC-3'		
β-actin	**FW:** 5'-GGAGCAATGATCTTGATCTT-3'	204 bp	55 ˚C, 27 cycles
	RV: 5'-CCTTCCTGGGCATGGAGTCCT-		

2.6.5 Endpoint-PCR analysis

PCR analysis was performed by using 0.5µl cDNA and 25µl a mixture comprising of: 18.6µl RNase-free water, 2µl dNTPs, 0.75µl MgCl, 2.5µl PCR buffer, 0.625µl forward primer, 0.625µl reverse primer and 0.2µl TAQ polymerase. On the top of the reaction solution a drop of mineral oil was added to minimise evaporation. The samples were transferred in a thermocycler using the parameters described in **Table 2.6**:

Table 2.6: Thermocycle parameters used for Endpoint PCR amplification.

Phase	Step	Temperature (°C)	Time (min)	Cycle Number
1	denaturation annealing extention	95 dependent on target gene 72	2 1 2	1
2	denaturation annealing extention	95 dependent on target gene 72	1 0.5 1	dependent on target gene
3	denaturation	94	1	1
4	extention	60	7	1

PCR samples were electrophorised in 2% agarose gel [2g agarose in 100ml 1x Tris Acetate (TAE) buffer (50x TAE buffer: 242g Tris, 57.1 ml glacial acetic acid, 100ml 0.5% EDTA, 1L dH20, pH 8.3) and 1µl ethidium bromide). The gels were placed into an electrophoretic tank filled with 1x TAE buffer and samples were loaded into the gel wells (to 10µl PCR product was added 5µl loading buffer) and constant voltage of 75V was applied for approximately 1h. PCR products were visualised under a UV light and photographed using a Bio-Rad GS-690 Imaging Densitometer.

2.7 siRNA-mediated suppression of Src kinase

In order to investigate the potential contribution of Src kinase to breast cancer cell function, we used an siRNA based approach to suppress Src expression.

Cells were harvested by trypsinisation and seeded at 2 x 10^5 cells/dish into 35 mm dishes in their respective media, without the addition of antibiotics, and incubated at 37 ˚C until 60 % confluent. For transfection, 20 μM stock of the siRNA was diluted to 2μM in 1x siRNA buffer (5x siRNA buffer was diluted to 1x in sterile RNase free water). The constituents for each sample were assembled into two separate 1.5ml Eppendorf tubes (A and B) as stated in **Table 2.7**, mixed well by gentle pipetting and incubated at room temperature for 5min.

The two mixtures (A and B) were then combined, mixed by gentle pipetting and left to stand for 20 min at room temperature. Each sample was then diluted 1:5 in warm media (wRPMI, 5% SFCS, 2 % glutamine, without antibiotics), added to the cells and returned to the incubator for 72h to allow sufficient siRNA protein knockdown before harvesting.

Table 2.7: The volumes (μl) required for siRNA transfection of a 35mm dish.

	NT-siRNA		**SRC-specific siRNA**	
Tube	**A**	**B**	**A**	**B**
wRPMI	50	98.4	50	98.4
2μM siRNA	50	-	50	-
Dharmafect #1	-	1.6	-	1.6
Total volume	100	100	100	100

2.8 Western blotting analysis

Western blotting is a commonly used analytical technique that can detect and confirm the presence of specific proteins.

2.8.1 Protein Extraction

Cells were seeded into 60mm dishes and cultured ± treatments until 70-80% confluency. Before harvest, dishes were placed on ice and cells were washed twice in PBS. To each dish 120µl of lysis buffer were added [50mM Tris base (0.61g), 150mM (0.875g) NaCl, 5mM (0.19g) EDTA, 1ml TritonX-100 and 100ml dH_20 with final pH 7.4] containing freshly added protease/phosphatase inhibitors as stated in **Table 2.8**. Cells were scraped and the cell suspension was transferred into a 1.5ml Eppendorf tube and centrifuged in a pre-cooled centrifuge for 15min at 12000 rpm, 4°C to separate the insoluble cell debris. After centrifugation, the supernatant was transferred into a clean Eppendorf tube and stored at -20°C until use.

Table 2.8: Volume of protease/phosphatase inhibitors required for 1ml Lysis Buffer.

Inhibitor	Volume (µl) required for 1ml Lysis Buffer	Final Concentration
Sodium Fluoride	20	50mM
Sodium Orthovanadate	20	2mM
Sodium Molybdate	10	10mM
Phenylmethylsulfonyl	10	1mM
Phenylarsine	1	20µM
Leupeptin	2	10µg/ml
Aprotinin	4	8µg/ml

2.8.2 Protein quantification

The total protein concentration of cell lysates was measured using a Bio-Rad DC colorimetric assay based on a modified Lowry method. The principle of this assay relies on the reaction between the copper tartrate solution with the proteins that causes a reduction of Folin reagent and produces a blue colour. The optical density of the blue colour can be measured in a spectrophotometer and is proportional to the protein concentration in the sample.

Initially a standard curve was prepared by using a range of known BSA concentrations (0-1.45mg/ml) diluted in lysis buffer. At the same time, 10µl of each protein sample was placed into 1ml cuvettes. To both protein samples and BSA standards, 250µl of Bio-Rad 'reagent A' and 2ml of Bio-Rad protein 'reagent B' were added and mixed by vortexing. Following 5min incubation in the dark, the absorbance was measured at 750nm. Data were plotted to produce a standard curve from which

the protein concentration of the test samples could be calculated. 100µg of total protein from each sample was then mixed with an equal volume of 2X SDS-PAGE loading buffer (0.4 Tris, 10% SDS-pH 6.8, 50% glycerol and 0.05%bromophenol blue) containing 24 mg/ml DTT and heated to 90°C for 5 min prior to SDS-PAGE or, where samples were to be used later, stored at 4°C.

2.8.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gels for SDS-PAGE analysis consisted of the separating and the stacking gel, which were made between glass plates 1.5mm. The separating gel (lower) was poured to a height approximately 0.5cm below the gel combs and after 20min the stacking gel was added. The lower gel was usually 8- 12%, while, the upper was always 4% (**Table 2.9**). Gel combs were placed on the top and gel left to set.

Table 2.9: Volumes of the substrates required for resolving and stacking gel preparation.

Resolving gel (%)	**8%**	**10%**	**12%**	**Stacking gel (%)**	**4%**
Acrylamide	5.4ml	6.6	8	Acrylamide	1.67
dH_20	9.2ml	8	6.6	dH_20	5.83ml
Tris pH 8.8	5ml	5ml	5ml	Tris pH 6.8	2.5ml
10% SDS	0.2ml	0.2ml	0.2ml	10% SDS	0.1ml
Temed	0.2ml	0.2ml	0.2ml	Temed	25ul
10% APS	50ul	50ul	50ul	10% APS	50ul

Once set, protein samples were loaded into the wells 20ul/lane. Loaded samples were separated by application of a constant voltage of 120V for approximately 2h in the presence of Running Buffer (192mM glycine, 25mM Tris, 0.1% w/v SDS), which had been poured until the 1/3 of the height of the running tank.

2.8.4 Blotting and Blocking

After SDS-PAGE separated proteins were transferred from the gel onto a Nitrocellulose membrane using a semi-dry blotting system as depicted in **Figure 2.3** below. The cassettes were placed in a tank that was filled with Transfer Buffer (0.25M TRIS base, 1.92M Glycine, 20% methanol) together with an ice block for cooling. Transfer of proteins was performed at 100V for 60 minutes.

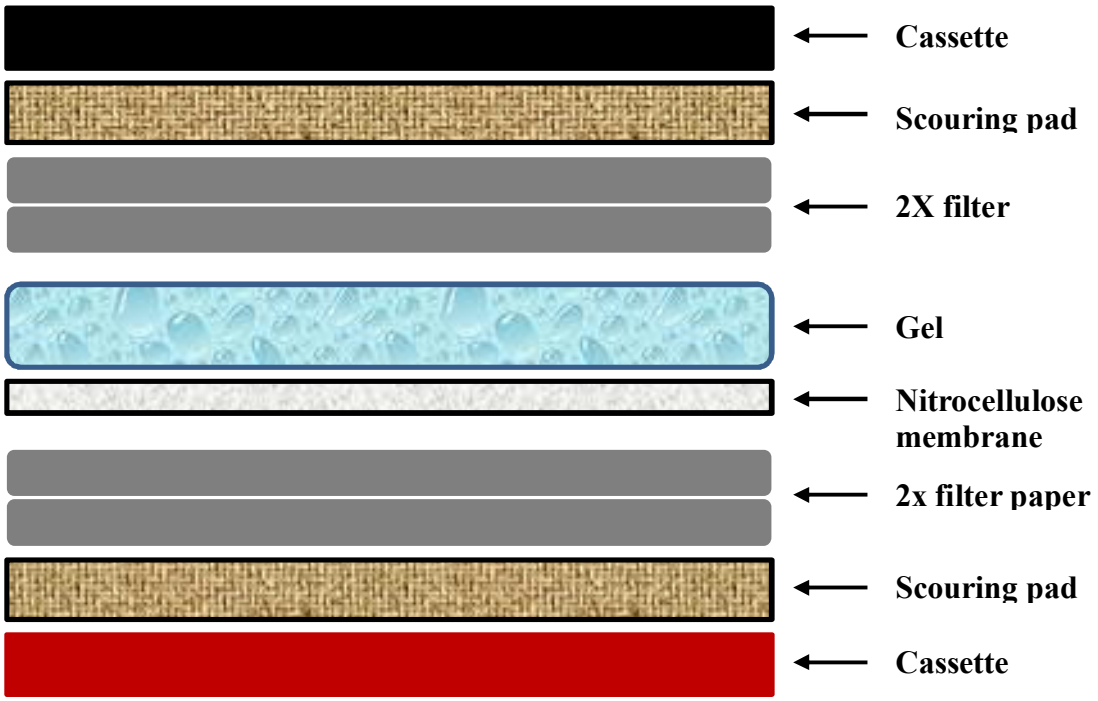


Figure 2.3: The assembly of the transfer *'sandwich'*. Proteins were transferred to nitrocellulose membranes using the semi-dry method assembled as shown. Black represents the cathode (-) and red the anode (+).

After transfer, Ponceau-S staining was performed to visualise transferred proteins in order to validate the transfer. The membranes were then rinsed in dH_2O to remove stain then blocked with 5% semi-skimmed milk powder in Tris-Buffered Saline Tween (TBS-T) for 1h.

Following blocking, membranes were washed in TBS-T (3x 5min) and then incubated with the appropriate primary antibody at the concentration indicated in Table 2.2, overnight at 4C° on a rocking platform. The following day the membrane was washed 3 times in TBS-T (5 min each) and incubated with secondary antibodies (horseradish peroxidase-conjugated IgG) used at 1/10000 for 1h at room temperature.

Finally, the membrane was washed 3 times in TBS-T (5 min each) and chemiluminesence detection reagent (ECL, Clarity or Femto) was applied. X-Ray film

was then exposed to the membrane and then processed with an automatic film developer/fixer machine.

2.9 ELISA

This assay was performed using the DuoSet ELISA kit for human RANKL (cat. # DY626 R&D systems). 96-well ELISA plates were coated with 1μg/ml mouse anti-human RANKL antibody (100λ/well) at RT overnight. On the next day plates were washed three times with PBS/0.05% Tween 20 and blocked with 1% BSA/PBS at RT for a minimum of 1h. Plates were washed three times with PBS/0.05% Tween 20 and either samples (conditioned media) or standards were applied to the ELISA plate (100λ/well) and incubated for 2h at RT. Plates were washed three times with PBS/0.05% Tween 20 and incubated with 9μg/ml goat anti-human RANKL antibody (100λ/well) for 2h at RT. Plates were washed, as previously described, and incubated with a streptavidin-HRP antibody (100λ/well) for 20min at RT. Following this, plates were washed and substrate solution was applied to each well (100λ/well) and incubated for 20min at RT in dark. Stop solution was then added (50λ/well H_2SO_4) and optical density was determined using an ELISA reader set to 450nm.

2.10 Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood

For blood collection from healthy donors, ethical approval was obtained by Cardiff University, School of Medicine with Reference Number: 14/25 (see Appendix B).

Blood was collected from healthy volunteers in EDTA containing 9ml blood collection tubes, mixed well and allowed to stand for 10 minutes before use. An equal volume of αMEM medium, (serum free) was added. A volume of Ficoll was added that was equivalent to the total volume of blood/media. The addition of Ficoll was

carried out carefully with the use of a syringe and kwill underneath the blood mixture, trying not to disturb the blood layer. The samples were then centrifuged at 510g, 4°C for 25min with no brake. Following centrifugation, the blood cells separated according to their density. Using this technique and from bottom to top the phases were: red blood cells, Ficoll, a tiny layer containing the PBMCs and the plasma phase (**Figure 2.4**).

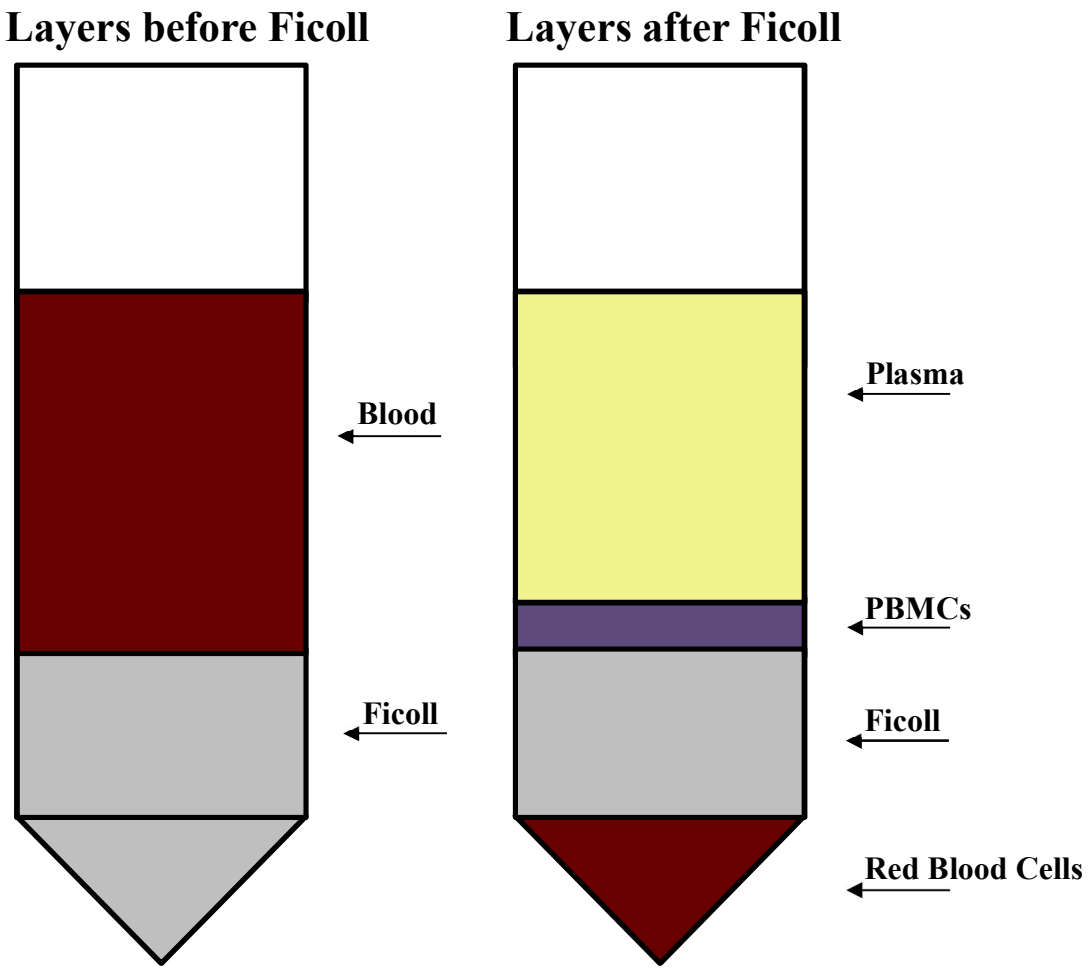


Figure 2.4: Density gradient centrifugation of whole blood with Ficoll.

The PBMC layer was carefully collected trying to minimise contamination with the adjacent layers of Ficoll and plasma. The samples were transferred to clean universals containing 10 ml cell culture medium and centrifuged at 510g, 4°C with no brake for another 10 min. Then, the supernatant was carefully removed and the pellet re-suspended in 10 ml of serum free αMEM and centrifuged at 510g, 4°C with no brake under for another 5min. Finally, the pellet was re-suspended in 2 ml of αMEM medium supplemented with serum and counted using a haemocytometer. Before the cells were placed into the haemocytometer, 10 µl of the sample was added to 90 µl of 2 % acetic acid in an Eppendorf tube and mixed gently to lyse any erythrocytes present in the sample.

2.11 PBMC differentiation

Sterile coverslips were placed into 96 well-plates and PBMCs were seeded at a density of $5x10^5$ cells per well. The differentiation of the human cells was triggered following treatment with 10ng/ml RANKL and 25ng/ml MCS-F for two weeks.

2.12 Tartrate-acid resistance phosphatase (TRAP) staining

Following incubation with RANKL, cells were washed twice in dH_20 and then fixed using a fixative solution containing 25ml citrate solution, 65ml acetone and 8ml of 37% formaldehyde, for 30 sec. The fixative solution was then aspirated and cells were rinsed with dH_2O before the staining solution for TRAP was applied. A TRAP staining solution was made as described in **Table 2.10**.

Table 2.10: Substrates and their corresponding quantities used for TRAP staining solution preparation.

Reagent	Quantity
pre-warmed (37°C) dH_20	5 ml
Fast Garnet GBC Solution	55 µl
Sodium nitrite solution	55 µl
Naphthol AS-BI phosphoric acid	55 µl
Acetate solution	222 µl
Tartrate solution	110 µl

Following incubation for 1h at 37°C in dark, the staining solution was removed and the cover slips were rinsed in dH_20. Finally, counterstaining was performed using acid haematoxylin solution for 2 min. When the counterstain solution was removed, coverslips were rinsed in tap water. This procedure resulted in the nuclei taking on a blue colour for easy identification, whilst TRAP-positive cells appeared as light pink.

2.13 Bone Resorption assay

Transverse dentine wafers, 0.13-0.16 mm thick were cut using a Buehler IsoMet Low Speed Saw with a diamond wafer blade (series 20 HC diamond) following which 6mm ivory discs were cut from the wafers using a paper hole punch. The discs were then subjected to sonication in dH_20 and left to dry before sterilising by immersion in 100% ethanol. The disks were then dried and then immersed in αMEM medium.

Following their isolation, PBMCs were seeded on ivory slices on a 96-well plate at $5x10^6$ cells/well and left to adhere overnight. The following day, cells were washed

twice in serum free media to remove the non-adherent cells and fresh media supplemented with 25ng/ml MCSF and 10ng/ml RANKL was added and cells returned to the incubator and cultured for 21 days in total with regular medium changes throughout. After 21 days the media was removed and ivory slices were washed in PBS for 2min. Once the PBS was removed 1% sodium hypochlorite solution was applied for 10min to ensure death of all the live cells. The slices were then washed twice in dH_20 (2min each) and vigorously rubbed in the palm of a gloved hand to remove all the cells from the slice surface. A subsequent staining step with 0.5% Toludine blue (0.5g toluidine blue, 0.5g boric acid, pH 7.3) for 2min was performed to visualise the resorption pits. Excess stain was then removed by washing the slices twice (2min each) in 70% ethanol solution. Finally, the ethanol was removed and slices were washed in tap water and allowed to dry. Resorption pits were viewed with an inverted microscope and images taken at x10 magnification.

2.14 Immunohistochemistry staining for Ki67

Cells were cultured on 0.13-0.17mm thick 3-aminopropyltriethoxysilane (TESPA) coated glass coverslips in 35mm dishes. Once they reached 70% confluency, media was aspirated and cells were fixed in 1ml formal saline for 5min. Cells were washed for 5min in 100% ethanol and then twice (5min each) in 0.02% PBS/Tween. To each coverslip MIB1 primary antibody at a concentration 1:50 diluted in PBS was added and incubated for 60min. Following that cells were washed twice (5min each) in 0.02% PBS/Tween and a secondary antibody detection was performed by adding 50µl/coverslip Dako mouse EnVision for 30min. Cells were washed twice (5min each) in 0.02% PBS/Tween and incubated with diaminobenzidine tetrahydrochloride (DAB) chromogen solution (50µl/coverslip) for 75min. Coverslips were washed in PBS and counterstained with 0.05% methyl green for 10min. Cells were finally washed three

times (5min each) in dH_20 and allowed to dry overnight. The following day coverslips were mounted onto glass microscope slides using di-butyl phthalate xylene (DPX).

Immunostaining was evaluated at 20× magnification using an Olympus BH-2 light microscope and representative photographs were taken. Ki67 expression was estimated by counting over 500 cells from at least 6 different fields of view and then calculating the percentage of cells deemed positive (brown cells) versus negative (blue cells) from three independent experiments.

2.15 Fluorescence-activated cell sorting (FACS) analysis

Cells were cultured in 60mm dishes ± treatments for times indicated in the relevant results section prior to trypinisation and pelleting by centrifugation at 1000 rpm for 5min. The supernatant was removed and cells were washed twice in PBS and centrifuged under the same conditions. The cell number was measured using a Coulter counter and 10^6 cells were fixed in 10ml ice-cold ethanol overnight at -20°C. The next day the cell suspension was centrifuged and cells were stained with propidium iodide solution [5.4ml PBS, 6µl Triton X100, 120µl Propidium iodide solution(2%v/v), 1.2mg DNAse-free RNAse]. Cell cycle distribution was then determined using BD FACSCanto II (BD Biosciences) flow cytometer.

2.16 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5 software and error bars were expressed as mean ±SEM. For comparing more than two groups of data, one-way analysis of variance tests (ANOVA) with Turkey multiple comparison test was used. For the comparison of pairs of data, a student's independent t-test was performed. Statistical significance is marked as follows in the results chapters: *$p<0.05$, ** $p<0.01$, ***$p<0.0001$.

3. Determining the impact of breast cancer cell conditioned media on RAW 264.7 cell differentiation to osteoclasts

3.1 Introduction

Breast cancer is the most common malignancy in women with over 70% of breast cancers being hormone receptor positive (Ignatiadis & Sotiriou 2013). The majority of breast cancers are thus likely to display estrogen-dependent growth (Yue et al. 2013) and sensitivity to ER modulatory agents such as tamoxifen. Despite the benefits observed with endocrine therapy, resistance remains a limiting factor with associated disease progression (Schiavon & Smith 2013).

For breast cancer, as with several other cancer types, the bone is the preferential site of metastasis and a high percentage of patients with advanced breast cancer present with skeletal metastases (Scully et al. 2012). Once in the bone microenvironment, breast cancer cells may cross-talk with, and modulate the function of bone cells (osteoblasts and osteoclasts), which can lead to either osteoblastic or osteolytic bone lesions (Ortiz & Lin 2012). In the latter case, breast cancer cells are known to stimulate osteoclastogenesis in both RANKL-dependent and independent mechanisms (Dougall 2012). Additional evidence also points to a role for Src kinase in the expression pathways that lead to the secretion of bone-modulatory proteins from cancer cells and also to mediate the function of osteoclasts themselves (Boyce et al. 2006). Interestingly, Src activity has been demonstrated to be elevated in acquired endocrine resistance where it promotes an invasive and highly metastatic phenotype (Hiscox, Morgan, Tim P. Green, et al. 2006).

This chapter sets out to investigate the hypothesis that acquisition of endocrine resistance in breast cancer influences the ability of these cells to promote osteoclastogenesis and that this occurs in a Src-dependent manner.

To investigate our hypothesis, we initially used the RAW 264.7 cell line (RAW cells). RAW cells are a mouse monocytic cell line that readily differentiate into

osteoclasts in response to RANKL and are a widely used model to study osteoclast differentiation *in vitro* (Collin-Osdoby & Osdoby 2012; Shevde et al. 2000; Lee et al. 2014; Hiken 2004). Mature and fully-differentiated osteoclasts are distinguished by two characteristics: (i) they are giant multi-nucleated cells and (ii) they stain positive for tartrate-resistant acid phosphatase (TRAP), a metalloprotein enzyme that is highly expressed in activated osteoclasts (Soysa et al. 2012).

In this chapter, the approach was to expose RAW cells to conditioned media from endocrine sensitive and resistant breast cancer cell models ±Src inhibition and determine the effects on osteoclastogenesis. Specifically, to achieve our aims the following objectives were set:

1. optimise a RAW cell-based assay in order to measure osteoclast differentiation
2. employ the RAW assay to investigate the ability of drug-responsive and drug-resistant breast cancer cell models to influence differentiation into osteoclasts
3. investigate whether Src kinase is involved in
    i) RANKL-mediated osteoclast differentiation
    ii) breast cancer conditioned media effects on RAW cell differentiation

3.2 Results

3.2.1 Optimisation of RAW 264.7 cells osteoclast differentiation assay

3.2.1.1 Creating a positive control- RANKL concentration curve

Optimisation of the RAW cell assay was performed using a method reported by (Hsu et al. 1999) that is depicted in **Figure 3.1**.

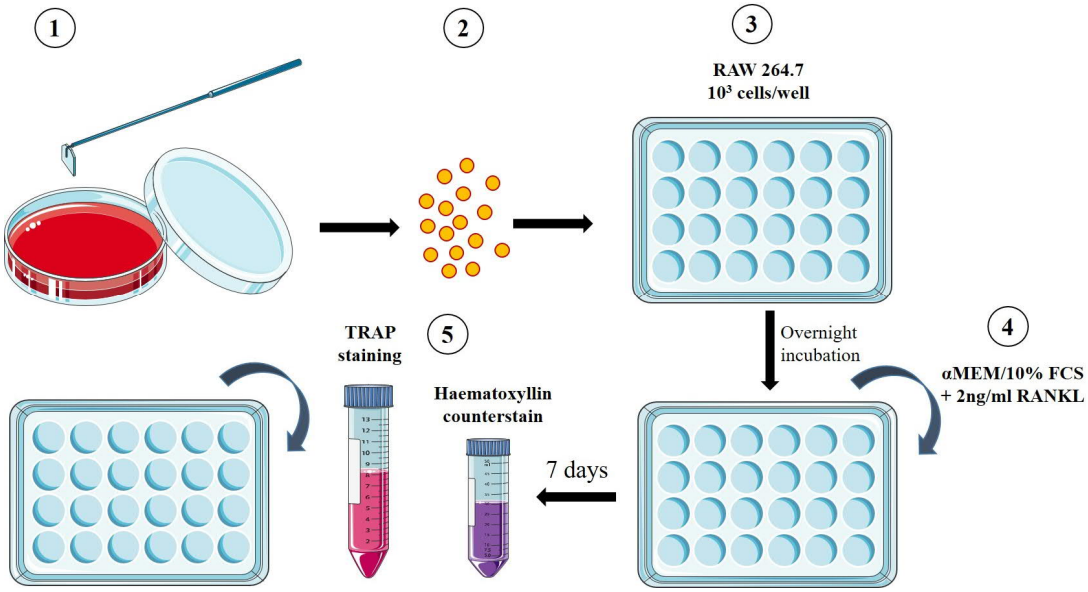


Figure 3.1: RAW 264.7 differentiation assay protocol. (1) Cells were scraped gently and pelleted following centrifugation. **(2)** Cell pellet was resuspended in αMEM/ 10% FCS media at a concentration of $2x10^3$ cells/ml. **(3)** Cells were seeded on 13mm glass coverslips at a density of 10^3 cells/coverslip and left to adhere overnight. **(4)** The following day the media was replaced with fresh media (αMEM/ 10% FCS) supplemented with 2ng/ml RANKL and cells were cultured for 7 days in total. Media supplemented with RANKL was replacing twice a week. **(5)** After a 7-day exposure to RANKL, cells were stained for TRAP to confirm their differentiation to osteoclasts and counterstained with haematoxylin to visualise their nuclei. Osteoclasts were assessed as differentiated when stained positive for TRAP and having more than 3 nuclei

Initially we wished to explore the optimum RANKL concentration for osteoclast differentiation. In order to achieve this, a range of different concentrations of RANKL (0.5-4ng/ml) were tried using the protocol described above. After a 7-day exposure to RANKL, cells were stained for TRAP and counterstained with haematoxylin to confirm their differentiation and to evaluate the number of their nuclei respectively. Multinucleation (> 3 nuclei) and TRAP+ staining were the criteria set for successful osteoclast differentiation. Treatment with the lowest concentration of RANKL (0.5 ng/ml) failed to induce differentiation of any osteoclasts. The effect of 1ng/ml RANKL was moderate in terms of number of osteoclasts identified in each sample, whereas when using either 2 or 4 ng/ml RANKL greatest numbers of osteoclasts were seen (**Figure 3.2**). Since both higher concentrations resulted in a similar effect it was decided the 2 ng/ml concentration was to be used for all the future experiments.

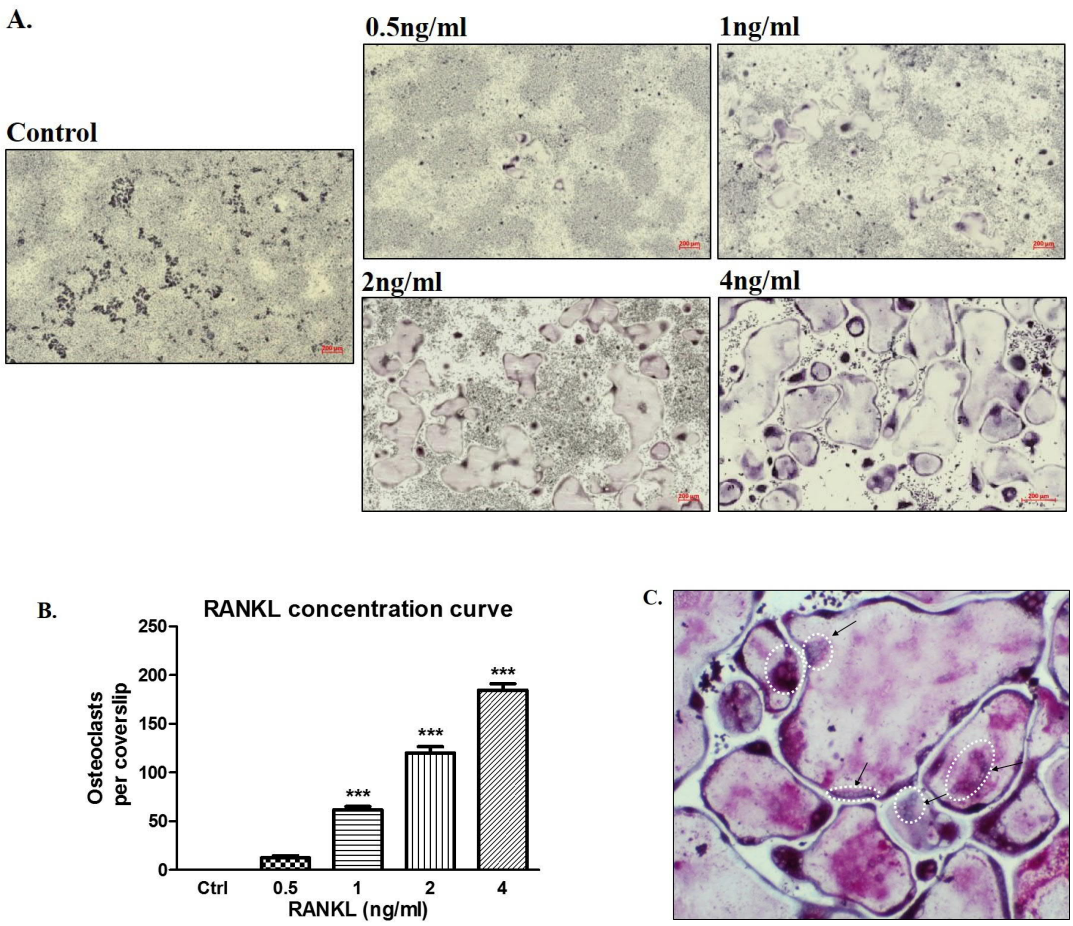


Figure 3.2: Optimisation of RANKL concentration to induce osteoclast differentiation. (A) TRAP staining and haematoxylin counterstaining of RAW 264.7 following treatment for 7 days with different concentrations of RANKL. The control did not contain any RANKL. Representative photographs were captured using an Olympus BH-2 light microscope at 40× magnification. **(B)** Quantitation based on the number of differentiated osteoclasts per coverslip. *** $p<0.001$ **(C)** High power image of differentiated osteoclasts following TRAP staining. Multi-nucleation is pointed with arrows.

3.2.1.2 Adjusting culture conditions to support osteoclast differentiation

As we wished to use our assay to determine the ability of endocrine-resistant breast cancer cells to promote osteoclastogenesis in RAW cells, we additionally had to consider the fact that these endocrine resistant models are routinely grown in a variety of media (see **Table 3.1**) ± phenol red and ± full or charcoal-stripped FCS (SFCS) as appropriate depending on the hormonal status of the cell line. Thus, a series of experiments were performed to establish whether RAW cells would be amenable to culture in these conditions and whether this would affect their ability to differentiate.

Table 3.1: Breast cancer cell lines hormone receptor status and their respective culture conditions. wRPMI: RPMI media without phenol-red, rRPMI: RPMI media containing phenol-red, TamR cells: MCF7-derived cells with acquired resistance to tamoxifen, FasR cells: MCF7-derived cells with acquired resistance to faslodex.

Cell line	**Hormone Receptor Status**	**Medium**	**Serum**
MCF-7	ER+, PR+	rRPMI	5% FCS
TamR	ER+, PR-	wRPMI	5% SFCS
FasR	ER-, PR-	wRPMI	5% SFCS
MDA-MB-231	ER-, PR-	DMEM	5% FCS

The RAW 264.7 differentiation assay was repeated using the culture conditions stated in **Table 3.1**. This investigated how RAW 264 cells respond to the RANKL treatment and whether osteoclast differentiation takes place under the culture conditions in which breast cancer cells are growing. Combinations of media/serum that are routinely used for MCF-7 cells (rRPMI/ 5% FCS) and MDA-MB-231 cells (DMEM/ 5% FCS) led to significant osteoclast yield in response to RANKL treatment after 7 days. TamR and FasR culture conditions (wRPMI/ 5% SFCS) resulted in poor osteoclast differentiation as far as the number and the size are concerned (**Figure 3.3**). These data were summarised in **Table 3.2**.

Based on these findings, we then wished to assess the RAW cell viability and growth under the aforementioned culture conditions using the MTT assay. Following a 7-day culture with RANKL, RAW cells demonstrated similar growth and proliferation with all the different media/serum combinations and no statistically significant difference was observed (**Figure 3.4**).

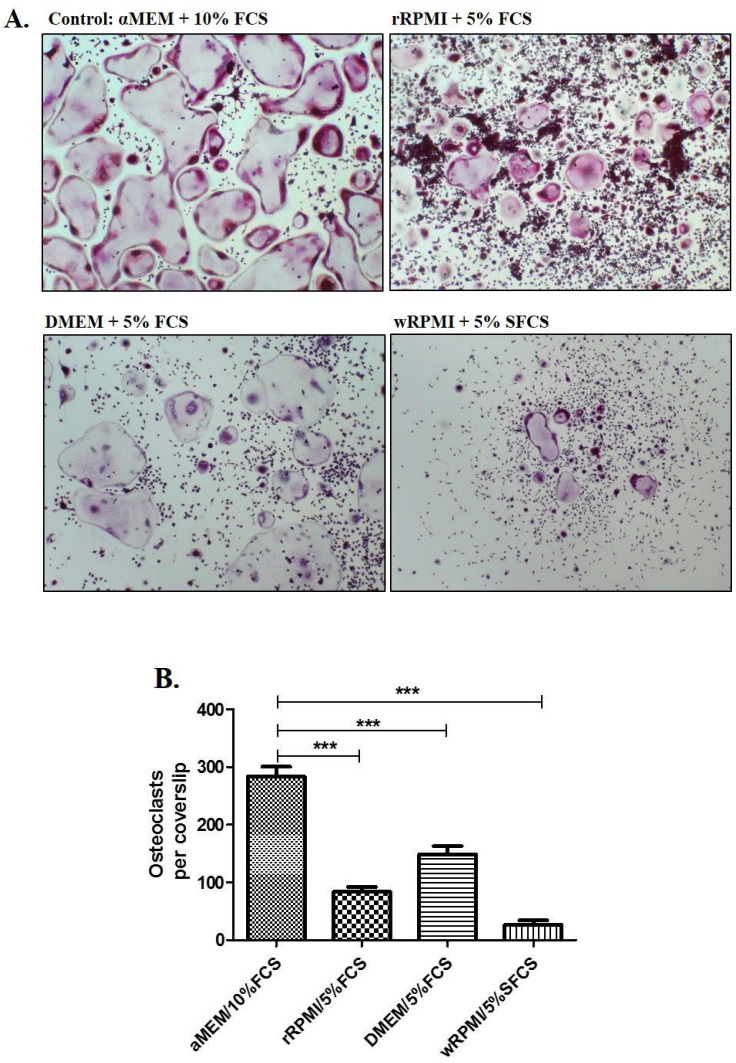


Figure 3.3: RAW cell osteoclast differentiation assay under various culture conditions. (A) TRAP staining and haematoxylin counterstain of RAW 264.7 cells following RANKL treatment in culture conditions that are routinely used for breast cancer cells lines. When RAW cells were grown in αMEM with 10% FCS, supplemented with 2ng/ml of mouse soluble recombinant RANKL for 7 days, giant TRAP positive osteoclasts were produced. When RAW cells were grown in either rRPMI or DMEM with 5% FCS in the presence of 2ng/ml RANKL, the osteoclast formation was reduced. When RAW cells were grown in either wRPMI with 5% SFCS in the presence of 2ng/ml RANKL, the osteoclast formation was significantly limited - only a few tiny osteoclasts were observed. Representative photographs were captured using an Olympus BH-2 light microscope at 40× magnification. **(B)** Quantitation based on the number of differentiated osteoclasts per coverslip. *** $p<0.001$

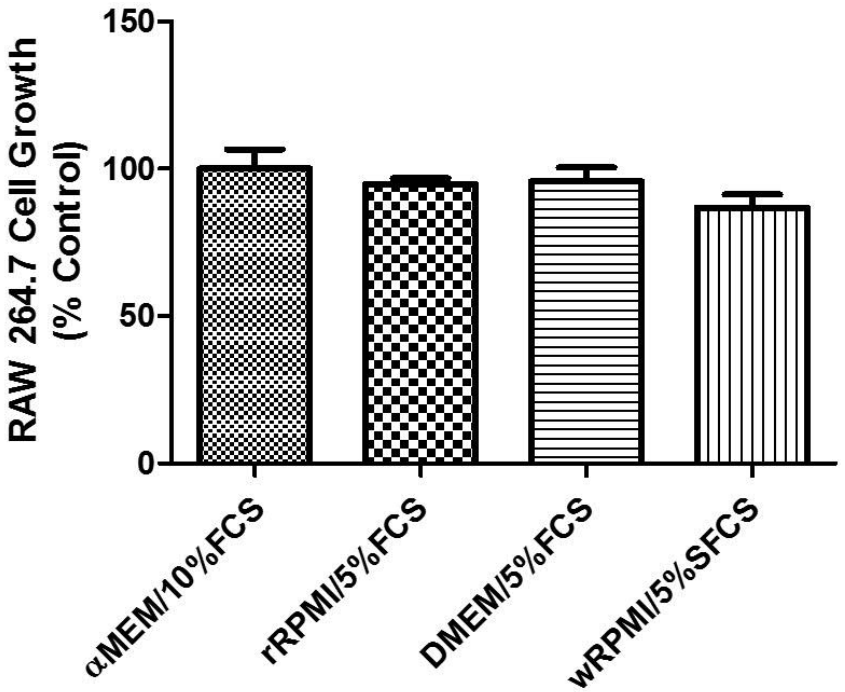


Figure 3.4: RAW cells viability assay under different culture conditions. RAW 264.7 cell number under different culture conditions was assessed using MTT assay and no significant difference was observed.

Due to the fact that the culture conditions of TamR and FasR cells resulted in an impaired osteoclast differentiation there was the need for further optimisation. This culture combination was supplemented with stripped serum, in which the steroids had been removed to prevent steroidal activation of the oestrogen receptor, but still retained growth factors and nutrients. Based on the poor yield of osteoclasts obtained from this combination, it was indicated that steroids might be fundamental for successful osteoclast differentiation. Therefore, we decided to collect the CM by maintaining the normal culture conditions of TamR and FasR cells and before CM was applied on RAW cells it was spiked with either 5% FCS or supplemented with 10^{-9}M oestradiol (E_2). After 7 days of treatment with RANKL under these culture conditions, cells were stained for TRAP in order to evaluate the osteoclast differentiation. The addition of both FCS and E_2 significantly enhanced the number of differentiated OC following RANKL treatment for 7 days (**Figure 3.5**). These data were summarised in **Table 3.2**.

The combination of wRPMI supplemented with SFCS was rejected due to the poor quality of osteoclasts produced. Although the addition of either E_2 or FCS significantly improved the number of differentiated osteoclasts, these conditions were not as effective as the αMEM/10%FCS. Osteoclast formation was also seen in RPMI and DMEM, however both media were not as effective as the αMEM. Therefore, it was decided for the following CM experiments to maintain both RAW and breast cancer cells in their normal culture conditions. For the positive and negative control, the same amount of media was added, but the media was not exposed to breast cancer cells.

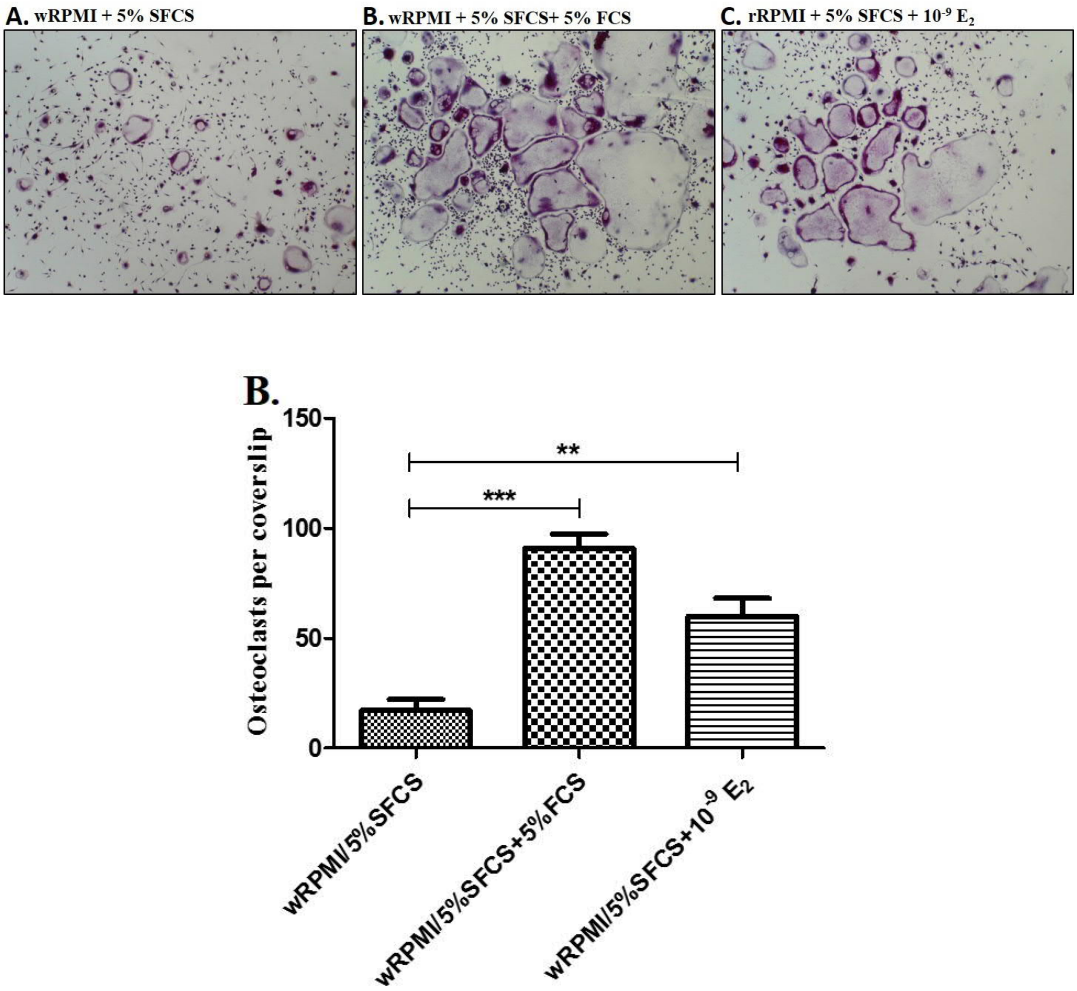


Figure 3.5: RAW differentiation assay under different culture conditions. (A) TRAP staining and haematoxylin counterstain of RAW 264.7 cells following RANKL treatment in different culture conditions. When RAW cells were grown in wRPMI with 5% SFCS in the presence of 2ng/ml RANKL, the osteoclast formation was limited - only a few, tiny osteoclasts were observed. When RAW cells were grown in the same medium spiked with 5% FCS the osteoclast formation was greater compared to case A. When RAW cells were grown in wRPMI with 5% SFCS in the presence of 2ng/ml RANKL, but supplemented with 10^{-9}M E_2, the osteoclast formation was greater compared to case A. Representative photographs were captured using an Olympus BH-2 light microscope at 40× magnification. **(B)** Quantitation based on the number of osteoclasts per coverslip. **p<0.01, *** p<0.001

Table 3.2: Summarising data of osteoclast differentiation assay under various culture conditions. Each different culture condition was assessed by performing at least three independent experiments, each of which consisted of 6 replicates.

Culture conditions	Osteoclast differentiation
Control: αMEM/ 10% FCS	successful
rRPMI + 5% FCS	adequate, relatively lower number and reduced size compared to the control
DMEM + 5% FCS	adequate, relatively lower number compared to the control
wRPMI + 5% SFCS	poor, low number and reduced in size compared to the control
wRPMI + 5% SFCS + 5% FCS	adequate, relatively lower number compared to the control
wRPMI + 5% SFCS + 10^{-9} E_2	adequate, relatively lower number compared to the control

3.2.2 Investigation of the breast cancer cell conditioned media effect on direct RAW cells differentiation without the exogenous addition of RANKL.

3.2.2.1 Effect of CM percentage on osteoclast formation

Initial observations on the ability of BC cells to induce RAW cell differentiation involved experiments using different breast cancer cell CM ratios given the reports in the literature that use a range of conditioned media concentrations from 10 to 40% (Tiedemann et al. 2009; Chen et al. 2013; Casimiro et al. 2013; Hussein et al. 2011). In order to confirm which CM percentage was more suitable to induce RAW differentiation, we decided to use CM derived from MCF-7, TamR, FasR and MDA-MB-231 cells in 3 different percentages: 10, 20 and 40% CM, keeping the collection time point consistent at 24h. The CM was collected by adhering to a strict protocol illustrated in **Figure 3.6**.

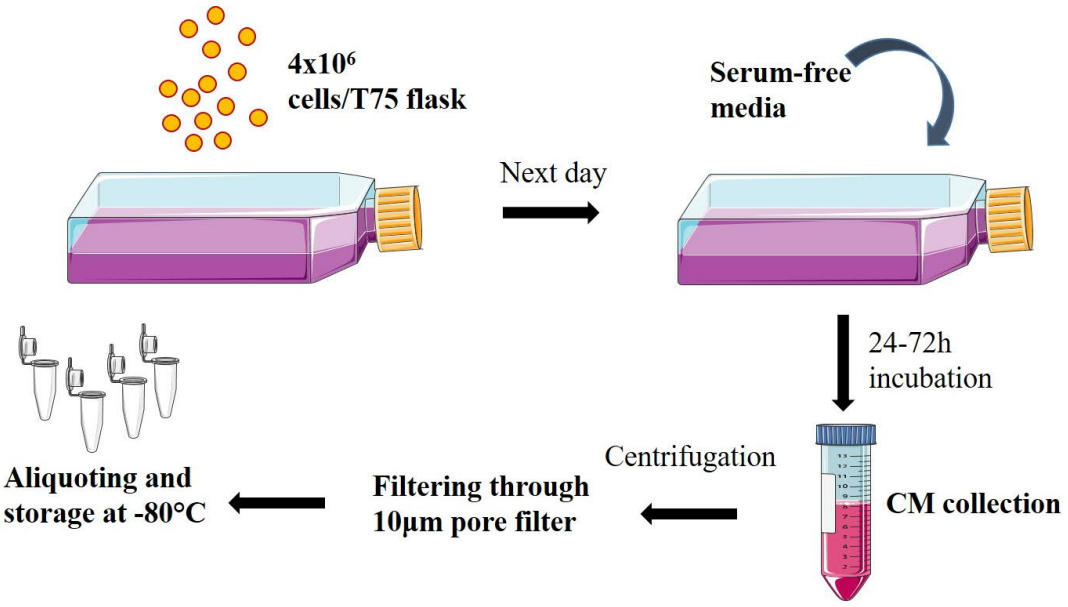
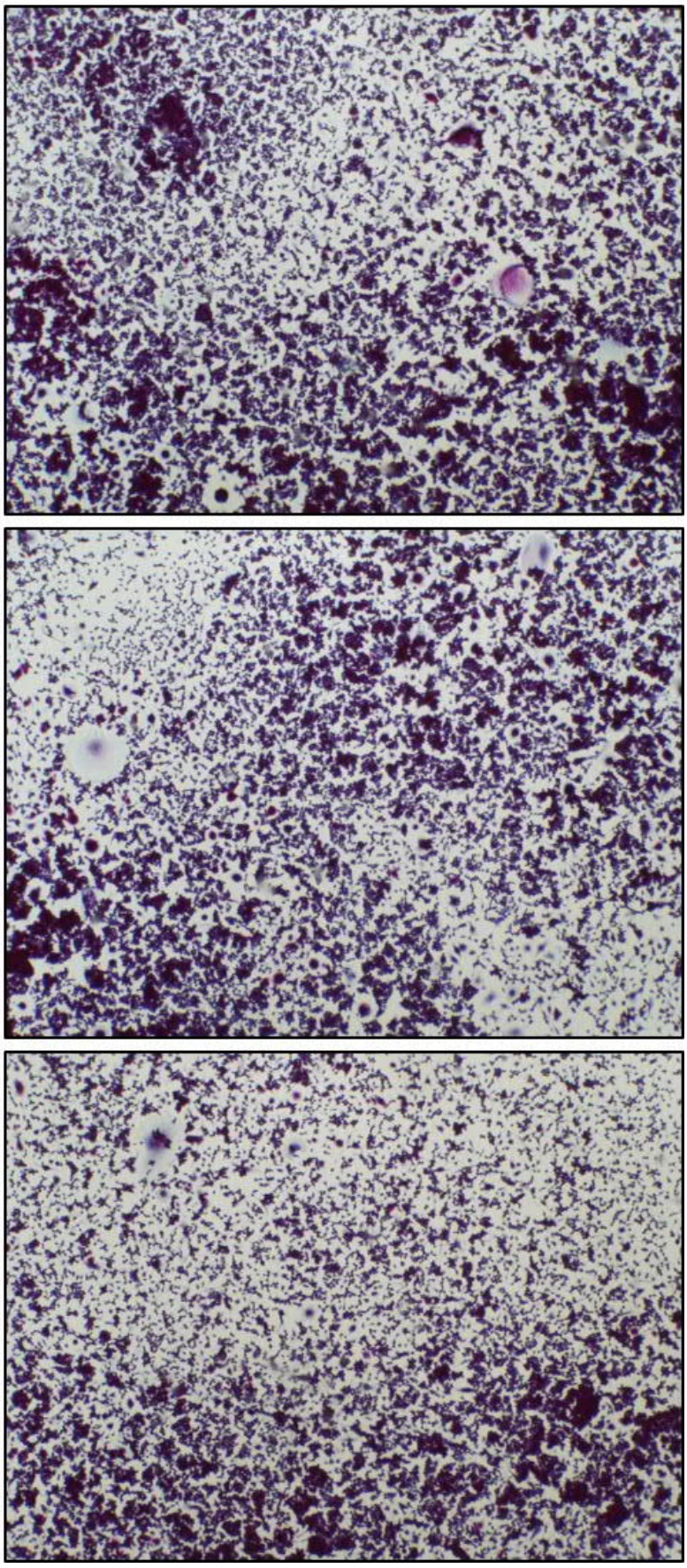


Figure 3.6: Breast cancer conditioned media collection protocol. Breast cancer cells were seeded into T75 flask at a density of $4x10^5$ cells/ml and left to adhere overnight. The following day the media was replaced with serum free culture media and incubated for 24 or 72h before collection. Once collected, media was centrifuged to remove cell debris and supernatant was filtered through a 10µm pore filter. Conditioned media were aliquoted into 1.5ml Eppendorf tubes and stored at -80 °C until use.

Both endocrine resistant (TamR and FasR) and triple negative breast cancer cell (MDA-MB-231) CM supported the differentiation of RAW cells to osteoclasts following a 7-day treatment with either 10 or 20% CM concentration. However, the osteoclasts produced following CM treatment were reduced in number and significantly smaller compared to those triggered by RANKL treatment. Interestingly, the luminal A breast cancer model, MCF-7, barely supported the formation of osteoclasts regardless of the CM concentration. A similar effect was observed with the highest concentration of 40% CM, which triggered the formation of a low number of osteoclasts regardless of the cell line the CM was derived from (**Figure 3.7**). Given the results obtained in these experiments it was decided to use the 10% CM concentration for all forthcoming experiments.

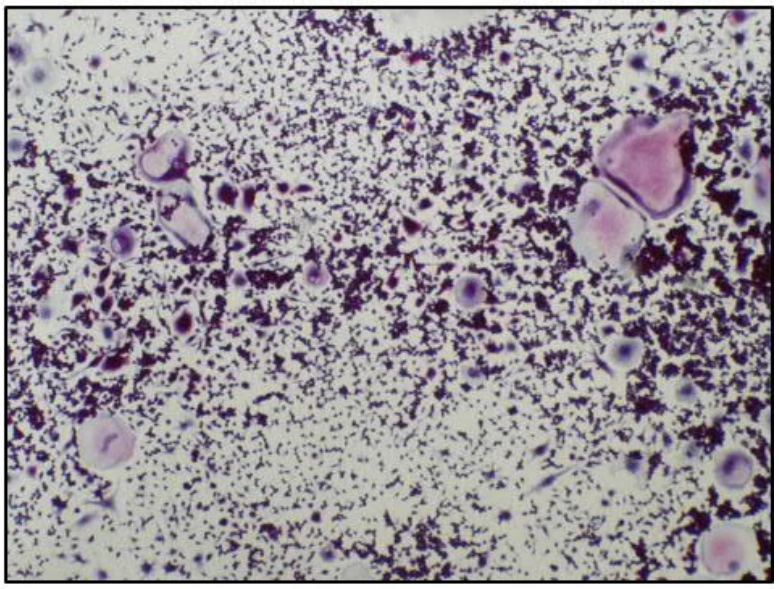
A. MCF-7



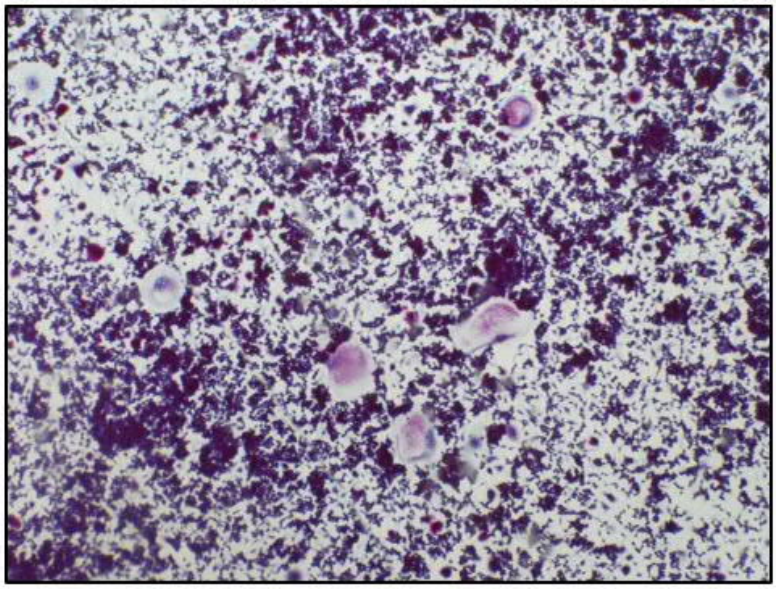
B.

TamR

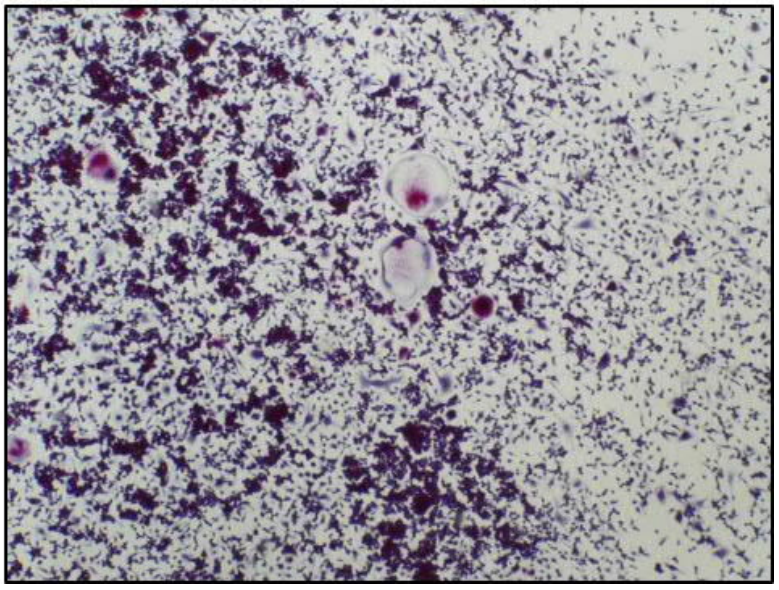
10%



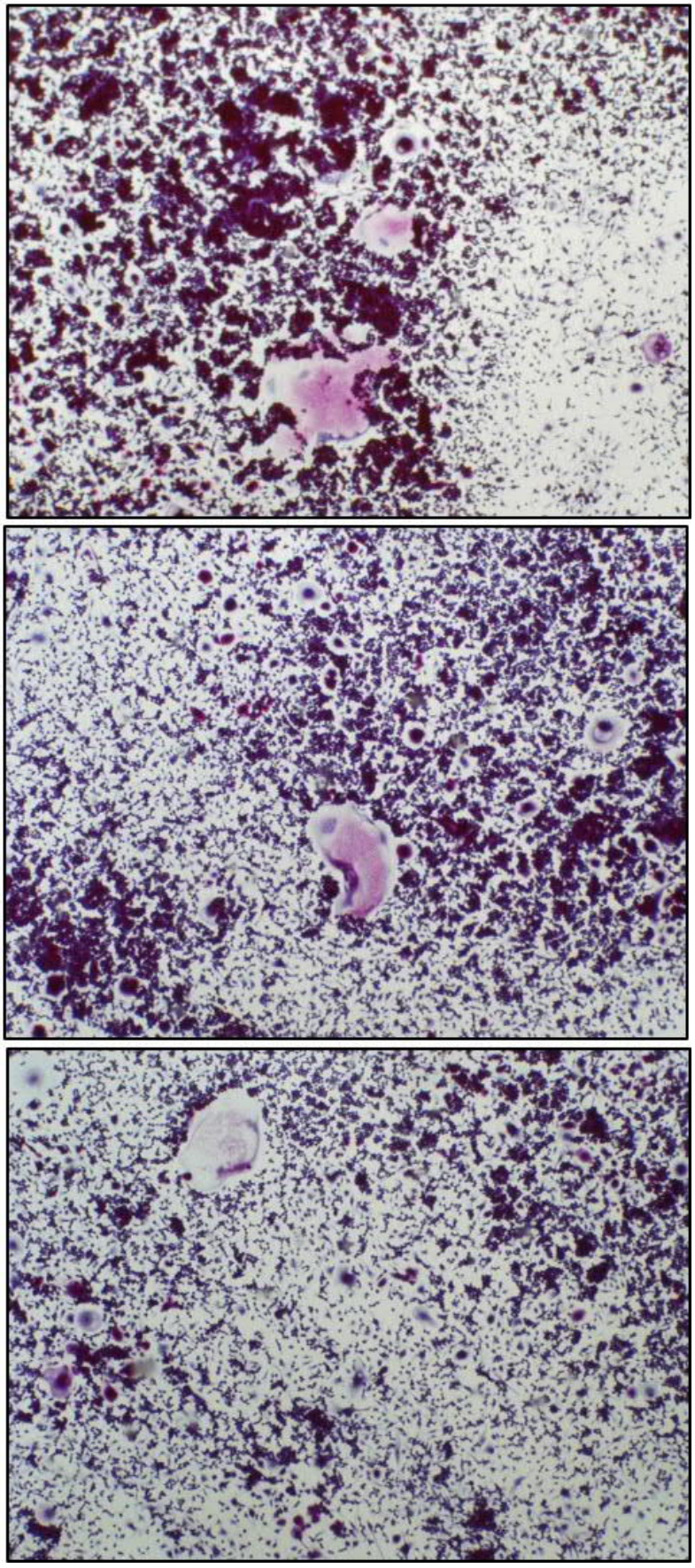
20%



40%

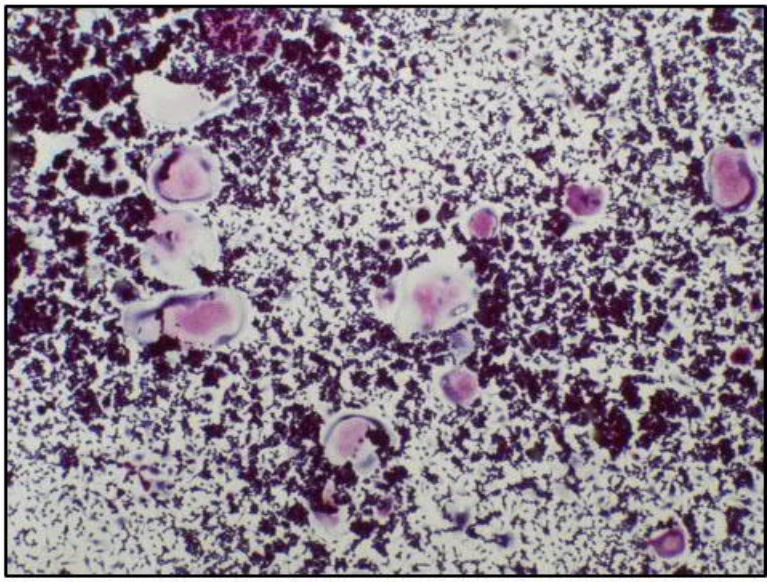


C. FasR

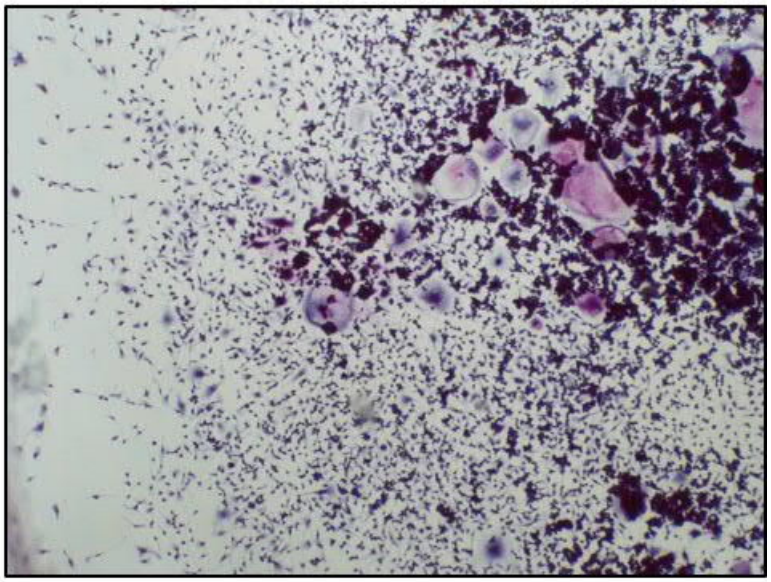


D. MDA-MB-231

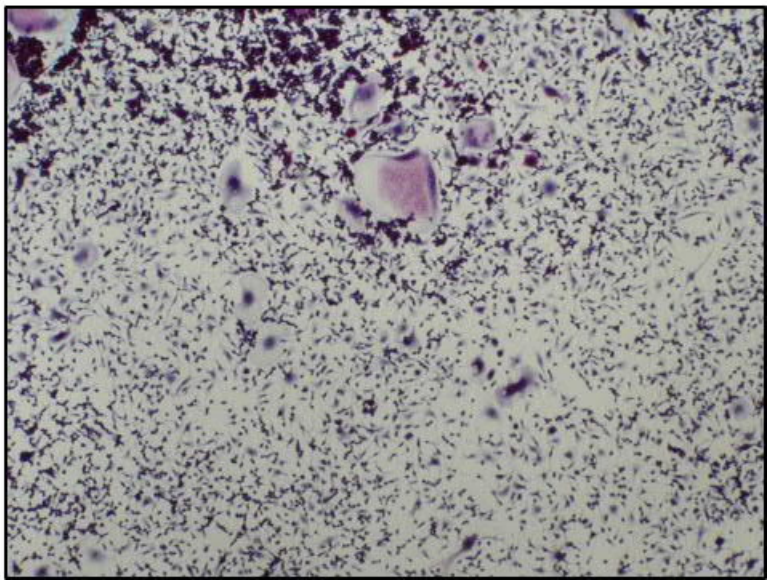
10%



20%



40%



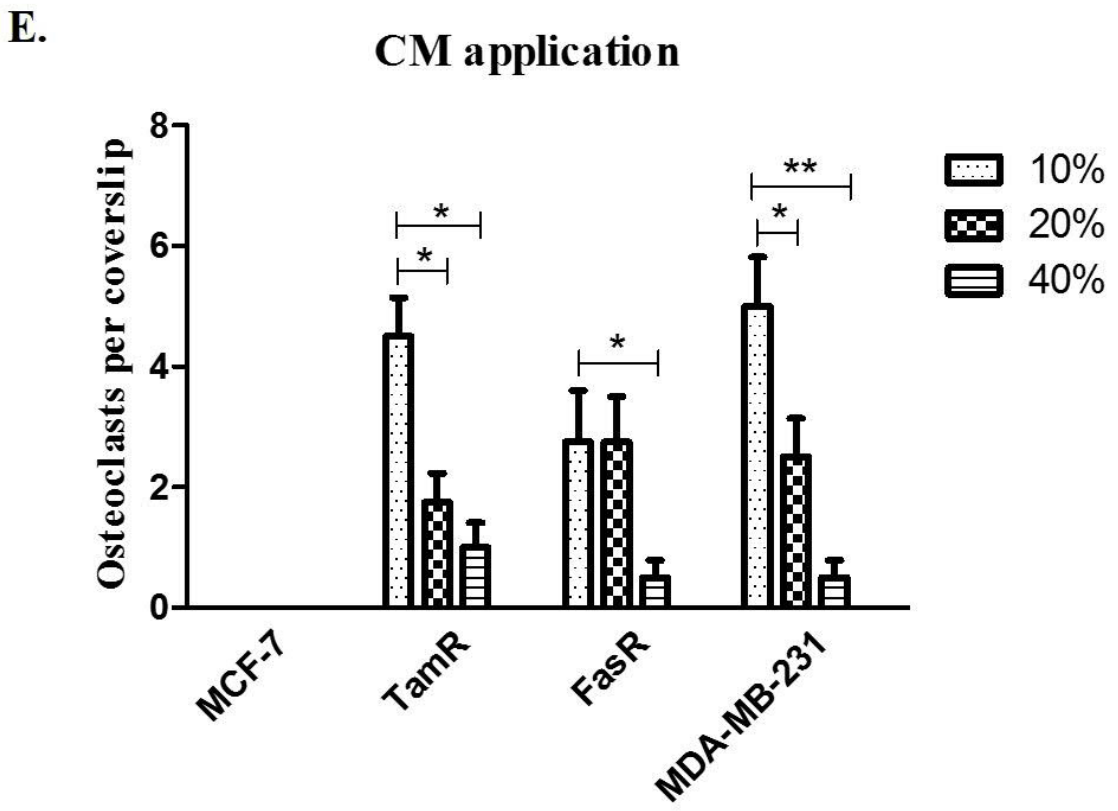
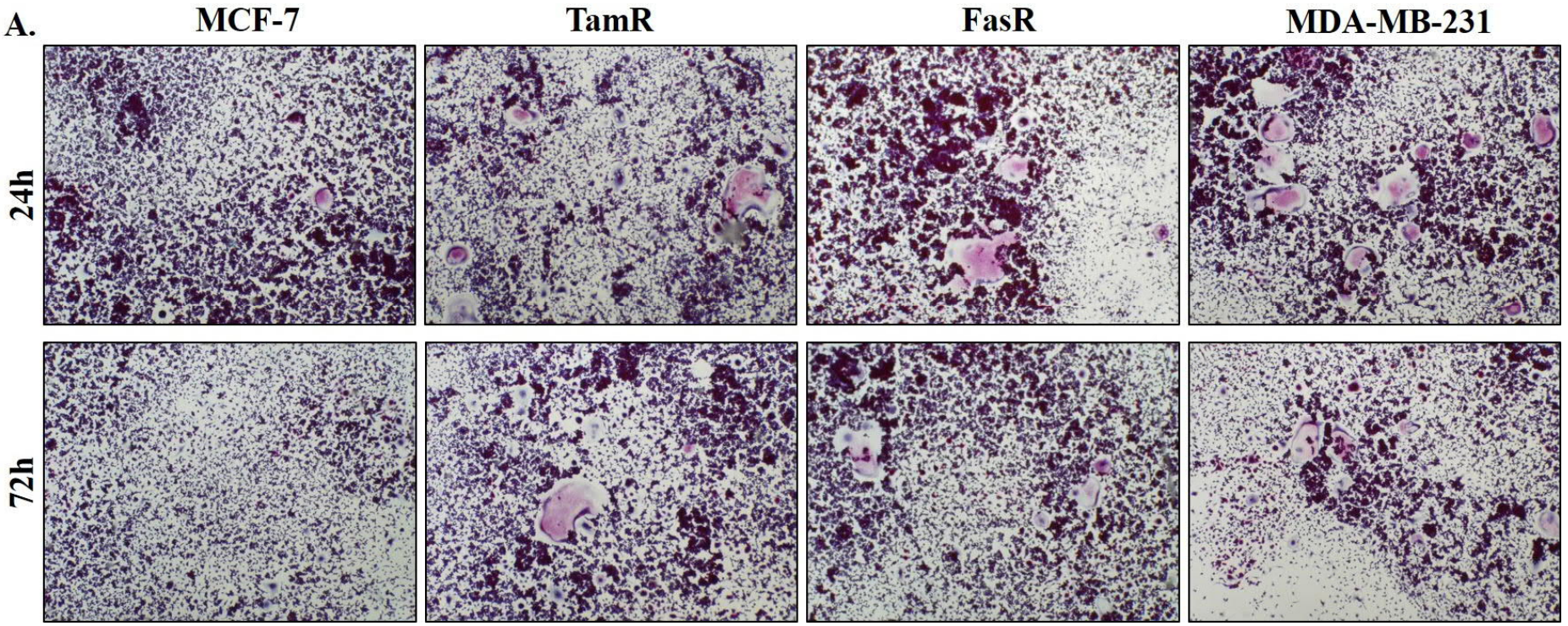


Figure 3.7: Breast cancer cell conditioned media collected at 24h triggers osteoclastogenesis. (A-D) TRAP staining with haematoxylin counterstain of RAW 264.7 cells following treatment with various percentages (10, 20 and 40%) conditioned medium, collected after 24h incubation, from four different breast cell lines (MCF-7, TamR, FasR and MDA-MB-231) for 7 days. **(E)** Quantitation based on the number of differentiated osteoclasts per coverslip. * $p<0.05$, ** $p<0.01$.

3.2.2.2 Effect of CM collection time on osteoclast formation

We next wished to explore the effects of CM collected at different time points whilst keeping the CM concentration in the osteoclast differentiation assay consistent. Thus, in addition to the 24h CM, 72h CM was also included. Quantitation of all the experiments performed with both 24 and 72h CM showed that the CM harvested at 24h was capable of inducing the highest number of osteoclasts compared to the CM collected at 72h. As far as the cell type is concerned, MDA-MB-231 and TamR cells exhibited the most robust osteoclastogenic effect among the cell lines resulting in the highest osteoclast yield, moderate osteoclast differentiation was observed when RAW cells were treated with FasR CM, while MCF-7 CM failed to induce the differentiation of any osteoclasts (**Figure 3.8**).


A.
MCF-7
TamR
FasR
MDA-MB-231
24h
72h

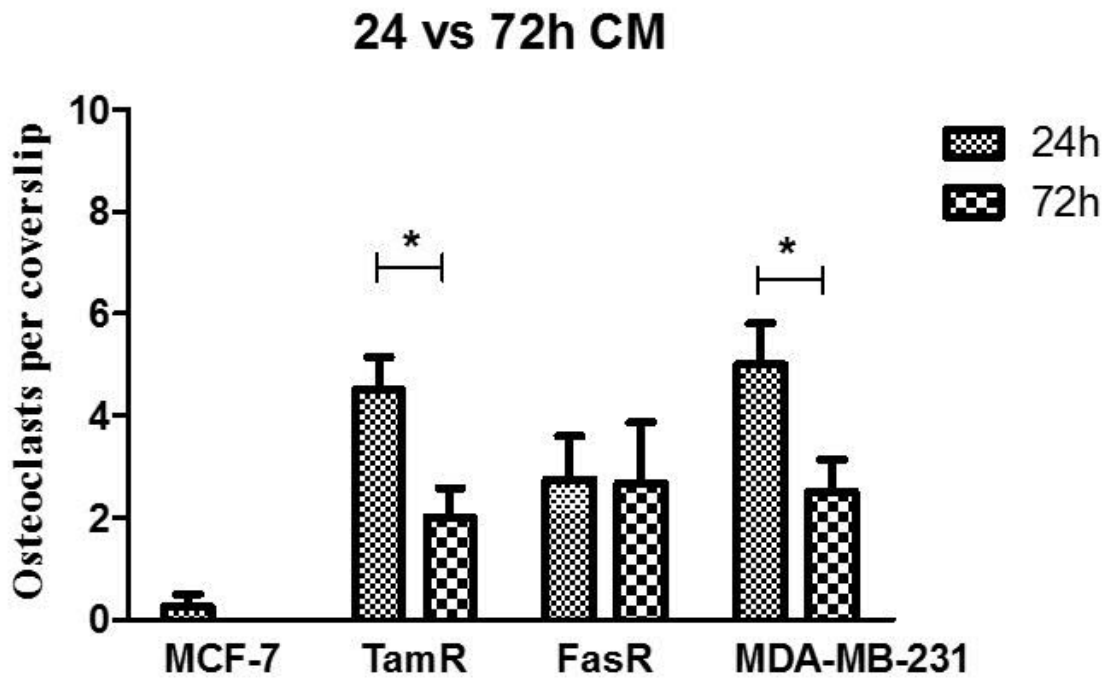
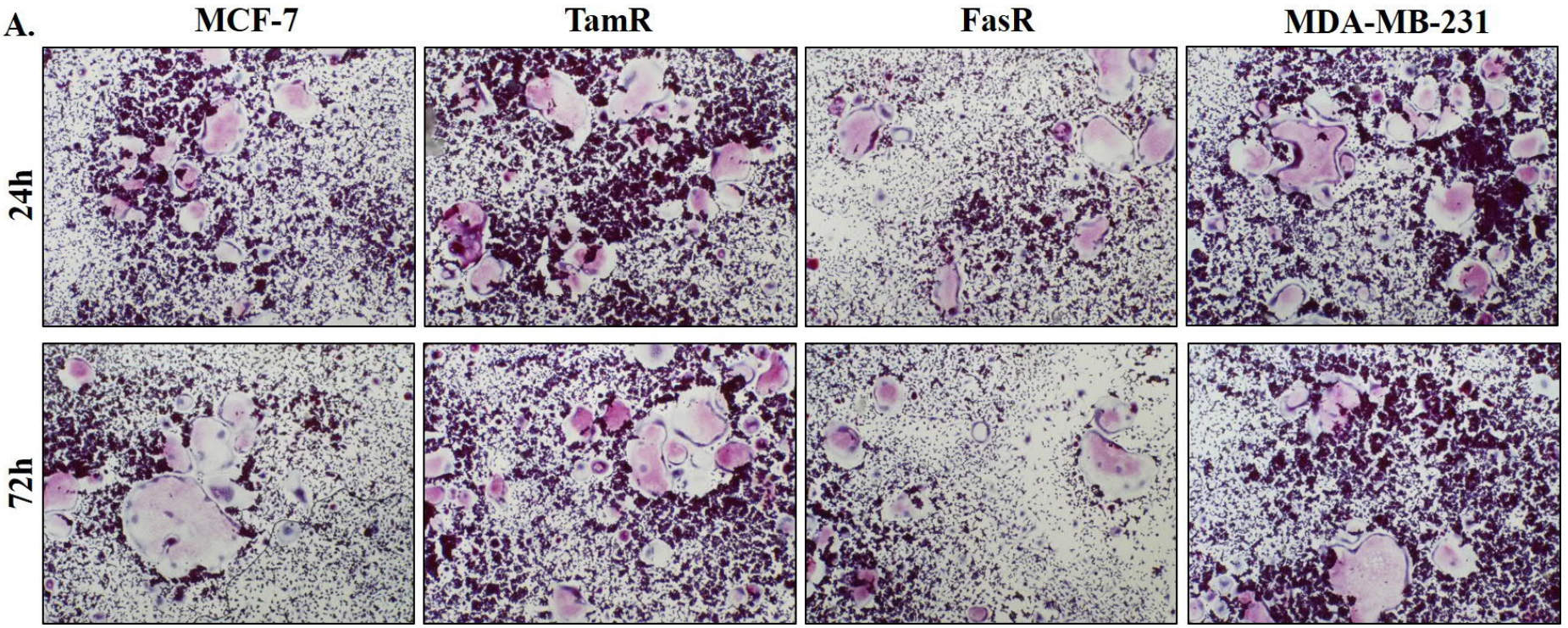


Figure 3.8: 24 vs 72h collected CM in RAW cell osteoclast differentiation. **(A)** RAW cells were treated for 7 days with 10% breast cancer cell CM collected at either 24 or 72h. Breast cancer cell CM harvested at 24h promoted the greater osteoclast yield compared to the CM harvested at 72h, especially in the case of TamR and MDA-MB-231 where statistical significance was observed between the two collection time points. Among the cell lines, MDA-MB-231 and TamR cells exhibited the most significant osteoclastogenic effect, FasR CM treatment resulted in a lower number of osteoclasts and MCF-7 CM failed to support osteoclast formation. **(B)** Quantitation based on the number of differentiated osteoclasts per coverslip. * $p<0.05$

3.2.2.3 Effects of RANKL priming on osteoclast formation

Although the CM treatment promoted osteoclast differentiation, the number of osteoclasts was very low. In an attempt to increase the osteoclast yield, RAW cells were primed with RANKL for 3d prior to the CM treatment and left in culture for 7d in total. Indeed, RANKL priming increased the number of differentiated osteoclasts, however there was no significant difference among the different breast cancer cell CM tried, as they all resulted in similar number of osteoclasts (**Figure 3.9**).

RANKL- primed



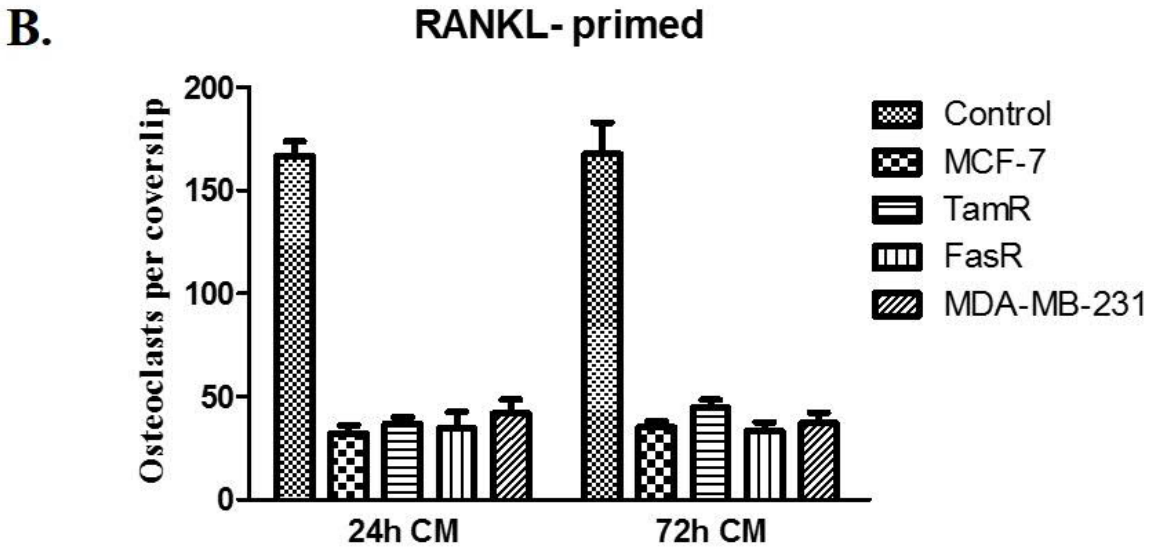


Figure 3.9: RAW differentiation assay with RANKL priming prior to CM treatment. (A) TRAP staining and haematoxylin counterstain of RAW 264.7 differentiation assays (n=4) primed with 2ng/ml RANKL for 3d and then treated for another 4d with 10% breast cancer cell CM collected at either 24 or 72h. Both breast cancer cell CM harvested at 24h and 72h resulted in a similar osteoclast number when RAW 264.7 were primed with RANKL prior to the CM treatment. The control was treated with 2ng/ml RANKL for 3d and left in culture for 7d in total. **(B)** Quantitation based on the number of differentiated osteoclasts per coverslip.

3.2.2.4 Low vs high RAW passage number on osteoclast formation

Several reports in the literature highlight the importance of using low passage number of RAW cells that do not exceed generation 20 (Berghaus et al. 2009; Marino et al. 2014; Collin-Osdoby & Osdoby 2012). There is evidence that high passage number (>20) has been correlated with reduced responsiveness and low osteoclast yield following RANKL stimulation (Collin-Osdoby & Osdoby 2012). In light of this, we then wished to explore whether CM-mediated osteoclast formation can be affected by a late generation of RAW cells. Thus, the differentiation assay was performed using a high passage batch of RAW cells (>40) treated with 10% CM from each breast cancer cell line for 7 days. TRAP staining and haematoxylin counterstain did not reveal the formation of any multinucleated and TRAP+ osteoclasts in response to CM treatment, while positive control that was stimulated with RANKL resulted in the differentiation of only a few osteoclasts (**Figure 3.10**).

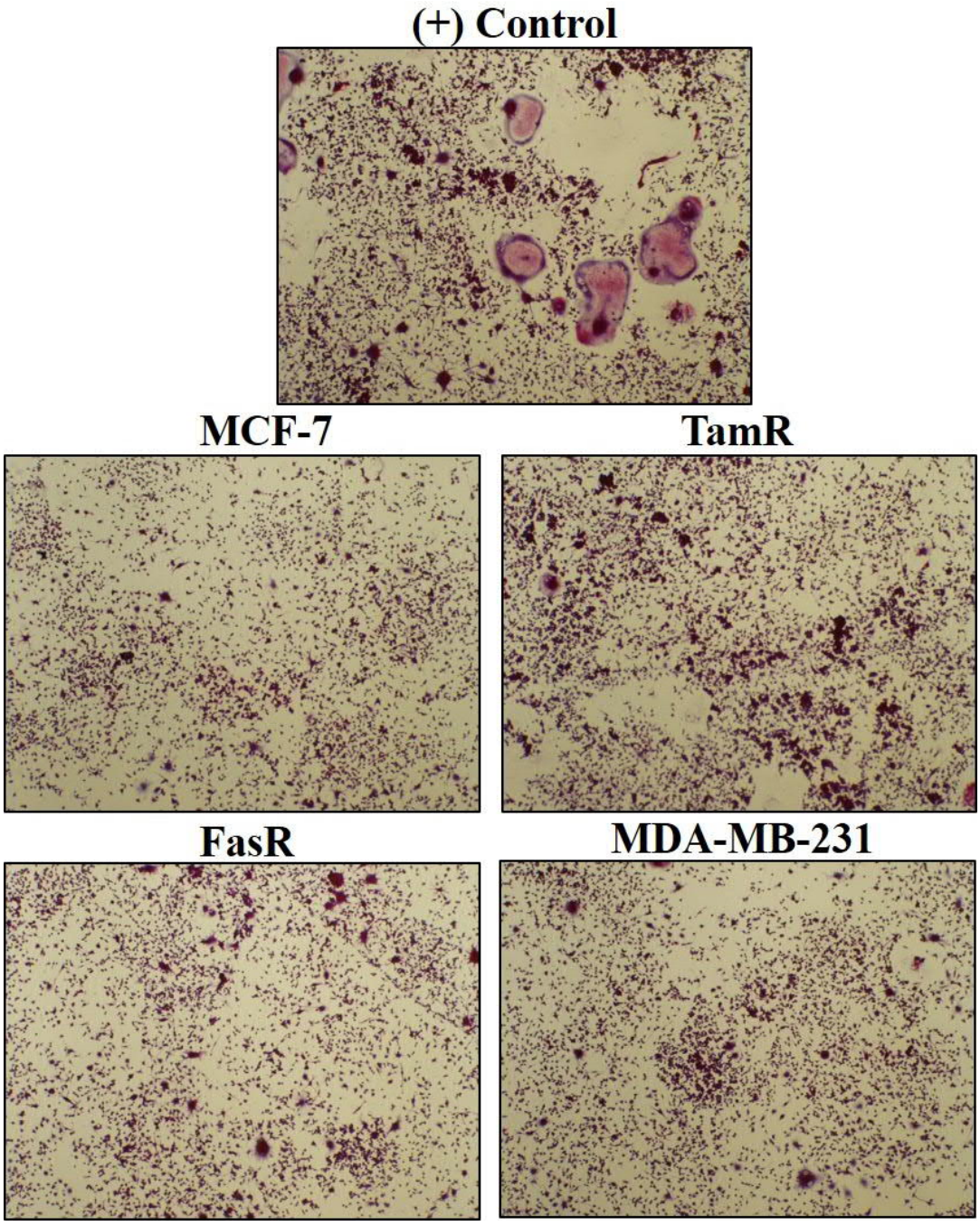


Figure 3.10: High passage number affects RAW cell responsiveness to RANKL and CM treatment. High passage (>40) RAW cells were treated for 7 days with 10% breast cancer cell CM collected at 24h. TRAP staining did not reveal the formation of any osteoclasts in response to the CM treatment, while RANKL stimulation resulted in a low number of differentiated osteoclasts. Positive control was treated with 2ng/ml RANKL for 7d.

3.2.3 Investigation of Src kinase involvement in osteoclast differentiation

3.2.3.1 Src-deficient breast cancer cell CM results in reduced number of osteoclasts

So far, our data suggested that breast cancer cell CM was able to induce osteoclast differentiation in RAW cells even in the absence of exogenous RANKL supporting our hypothesis that breast cancer cells secrete bone cell-modulatory factors that are responsible for the transformation of RAW 264.7 cells to mature osteoclasts. Src kinase plays a fundamental role in osteoclastogenesis and it is also expressed by breast cancer cells. Specifically, Src is highly expressed in TamR and MDA-MB-231 and CM from these cells was able to induce RAW cell differentiation to the greatest extent. Based on these findings and the fact that the literature highlights the importance of Src in osteoclast differentiation and survival we then wished to investigate whether CM from Src-deficient breast cancer cells were still able to trigger osteoclast differentiation.

siRNA-mediated Src knock-down was first confirmed using Western blotting (**Figure 3.11**), which revealed an almost complete loss of Src in siRNA-treated samples compared to samples treated with non-targeting (NT) siRNA. Application of CM from these Src-deficient cell models to the RAW differentiation assay resulted in reduced number of differentiated osteoclasts compared to the CM from un-treated breast cancer cell cultures. Specifically, in the case of TamR and MDA-MB-231 the number of differentiated osteoclasts generated by Src siRNA-treated CM was significantly lower ($p<0.05$) compared to the NT-CM (**Figure 3.12**).

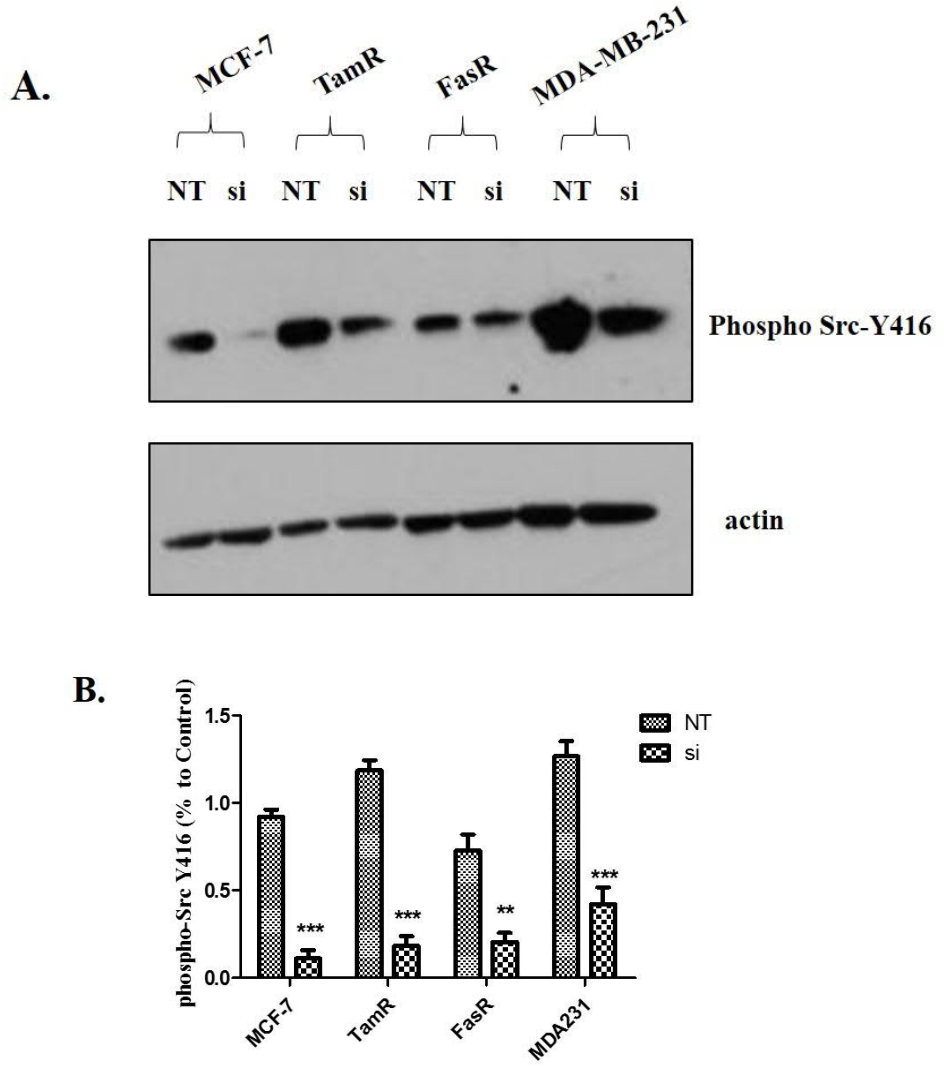
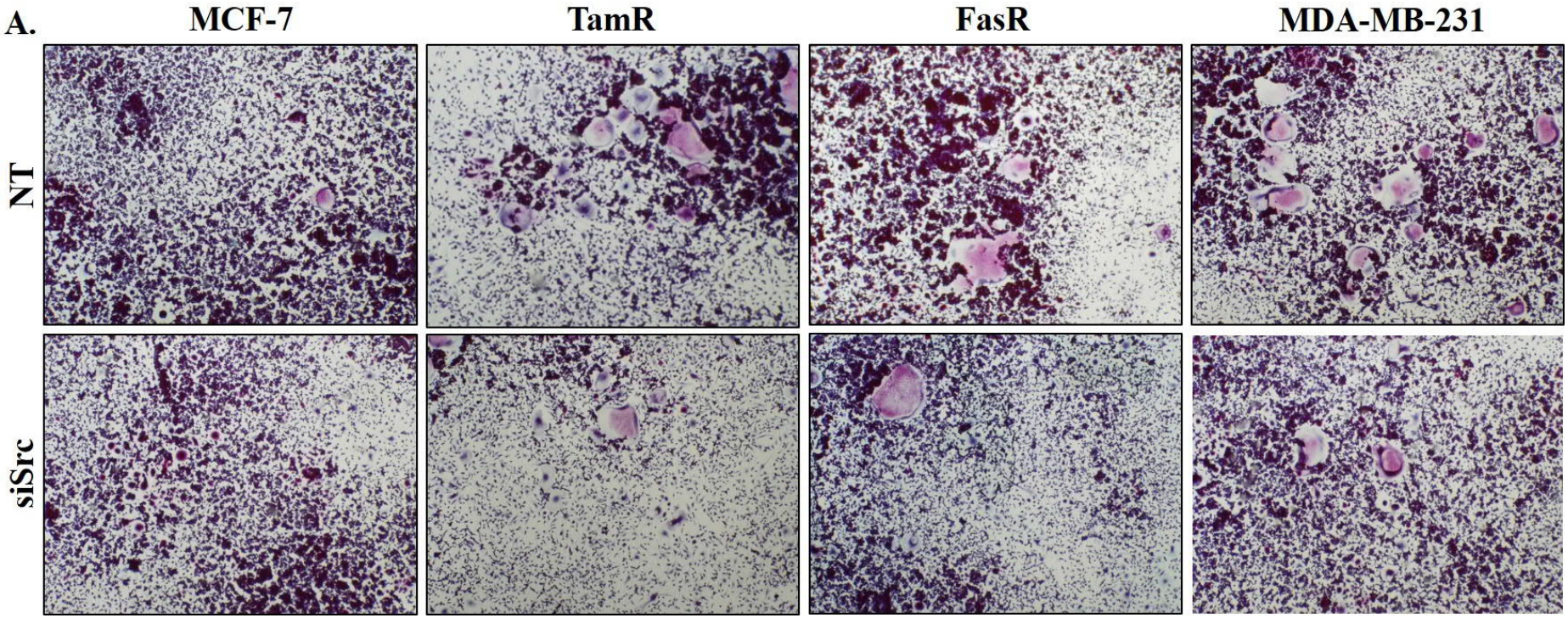


Figure 3.11: Src siRNA treatment suppresses activated Src protein in breast cancer cells. (A) Representative blot illustrates the levels of phospho-Src (Y416) following treatment with Src si-RNA and NT control. Protein expression of phospho-Src (Tyr-416) treated for 72h with either Src siRNA or NT control in MCF-7, TamR, FasR and MDA-MB-231 cells. All cells were grown to 50% confluence and treated with Src-siRNA at 25nM for 72h. Cell lysates were processed for Western Blotting and probed for phospho-Src Y416. Actin was used as a loading control. **(B)** Densitometry analysis. ** $p<0.01$, ***$p<0.001$


A.
MCF-7
TamR
FasR
MDA-MB-231
NT
siSrc

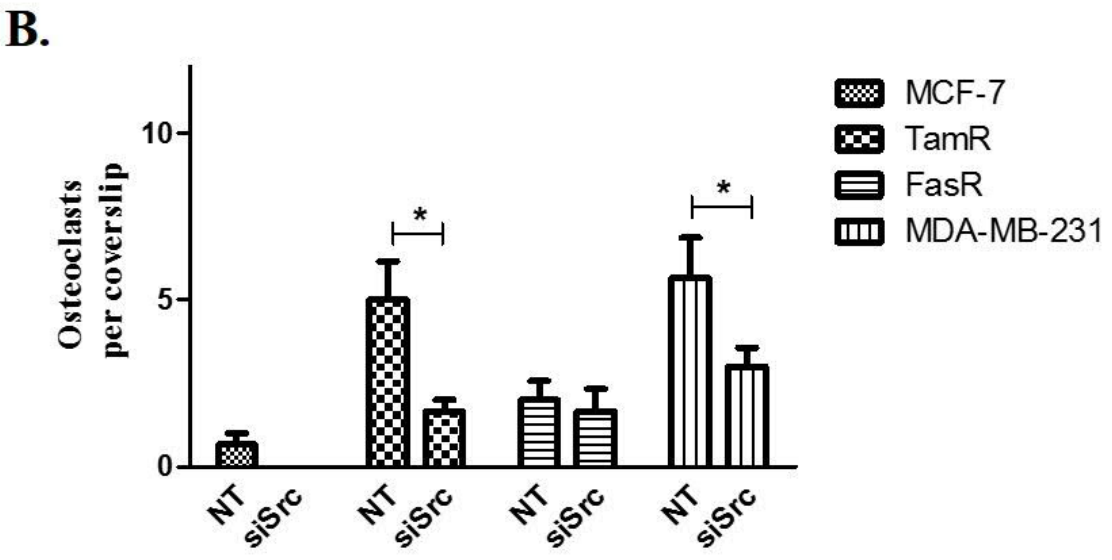


Figure 3.12: Src-deficient breast cancer cell CM results in a significantly lower number of osteoclasts compared to the untreated breast cancer cell CM. **(A)** CM was collected after 24h incubation with either Src-siRNA post-transfected or untreated cells, diluted at 10% with fresh medium and then added on to RAW cell culture. After 7 days, cells were fixed and stained for TRAP. Src- deficient CM induced lower number of differentiated osteoclasts compared to the corresponding NT control, especially in the case of TamR and MDA-MB-231. **(B)** Quantitation based on the number of differentiated osteoclasts per coverslip. * $p<0.05$

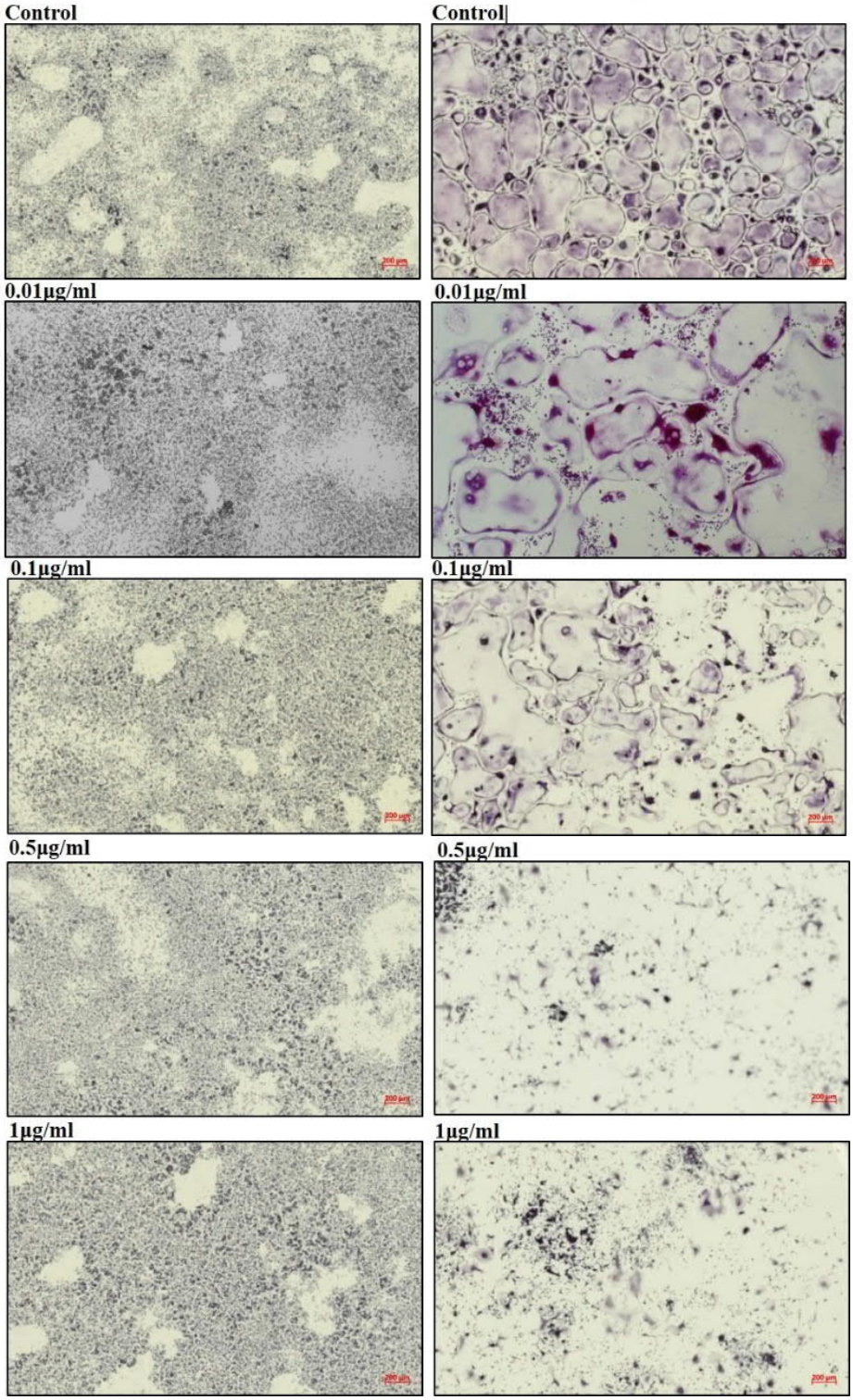
3.2.3.2 Exploring the cross- talk of RANKL and Src kinase

Based on the impaired osteoclastogenic effect seen following treatment with Src-deficient breast cancer cell CM and the importance of Src kinase involvement in osteoclast differentiation and survival that is widely highlighted in the literature (Miyazaki et al. 2004; Miyazaki et al. 2006; Izawa et al. 2012; Sgroi 2009; J. C. Edwards et al. 2006), we then wished to explore how RAW cells respond to RANKL treatment in the presence of the pharmacological Src inhibitor AZD0530, also known as Saracatinib. RAW cells were pre-treated with a range of AZD0530 concentrations (0-1μM) for 2h and then RANKL was added and left in culture for 7 days in total before TRAP staining. A negative control for each concentration of AZD0530 without RANKL was also included, to identify any effects that this drug might exert on RAW cells.

At 0.1μM or less, RAW cell differentiation into osteoclast cells did not appear to be significantly affected in terms of the osteoclast yield produced. Although the number of differentiated osteoclasts was relatively lower compared to the control, in terms of size the osteoclasts generated in the presence of AZD0530 were bigger and covered a similar surface compared to the control. At 0.5μM AZD0530 and above, RAW cell differentiation was notably impaired and no osteoclast formation was observed (**Figure 3.13**). Observation of the negative control that contained AZD0530 alone, did not reveal any toxic effects to be caused by the Src inhibitor on RAW 264.7 cells, as their cell number was not affected. In addition, MTT assay confirmed that RAW cell growth was not affected by the treatment with Saracatib (0-1μM), as no statistically significant alteration in RAW cell number was observed following treatment with the indicated doses of AZD0530 (**Figure 3.14**).

A.

AZD0530	+	+
RANKL	-	+



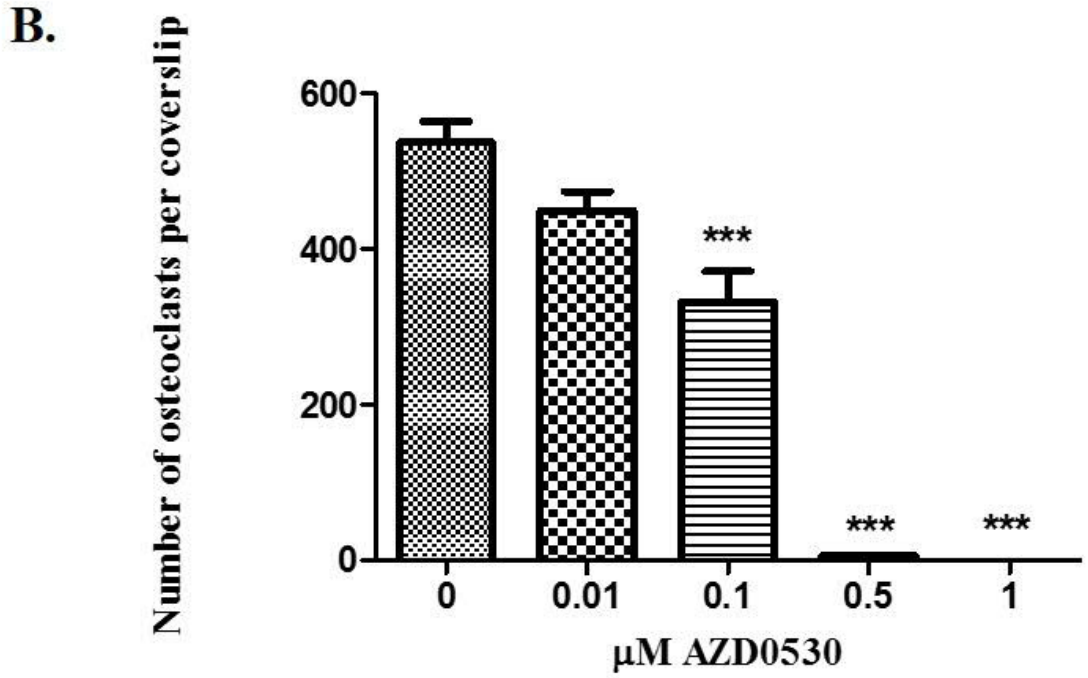


Figure 3.13: Src inhibition inhibits the differentiation of RAW 264.7 cells to osteoclasts in response to RANKL treatment in a dose-dependent manner. (A) TRAP staining and haematoxylin counterstain of RAW cells treated with various concentrations of AZD0530 (0-1µM) ± RANKL for 7 days. **(B)** Quantitation based on the number of differentiated osteoclasts per coverslip. *** $p<0.001$

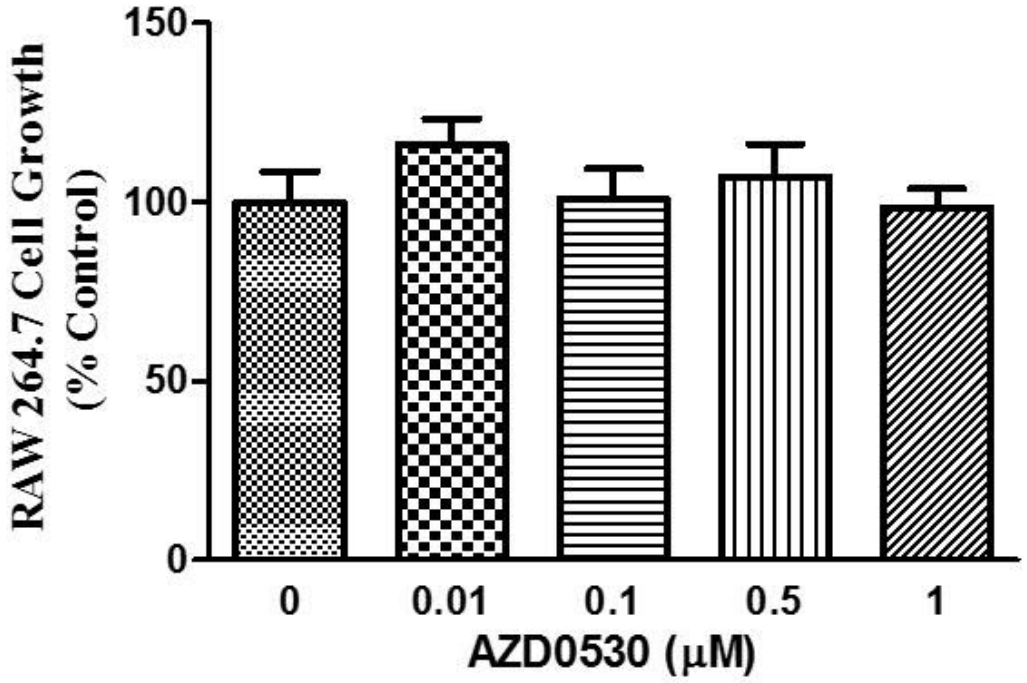


Figure 3.14: RAW cells numbers are unaffected following Src pharmacological inhibition with AZD0530. RAW 264.7 cells were treated with various AZD0530 concentrations (0-1μM) for 7 days and their number assessed using the MTT assay. Cell numbers were not significantly affected by the indicated doses of AZD0530.

Having found that Src kinase inhibition significantly impaired RANKL-mediated osteoclast differentiation, we next wished to explore the interplay of RANKL and Src kinase. In order to confirm that RANKL activates the Src pathway, a time-course experiment (0-24h) by treating RAW cells with RANKL ± AZD0530 was performed. Western blot for the activated form of Src kinase (Y416) revealed that 0.5μM AZD0530 suppressed suppress Src kinase levels after a 2h-treatment. However, this effect was attenuated by the addition of RANKL after 5min and this phenomenon was even more prevalent at 30min, when the highest expression level of Src was shown. Following that point Src kinase levels gradually started to reduce and at 24h returned to their initial low levels caused by the AZD0530 inhibition. RAW cell treatment with RANKL alone did not affect the expression levels of Src kinase, regardless of the duration of RANKL treatment (**Figure 3.15**).

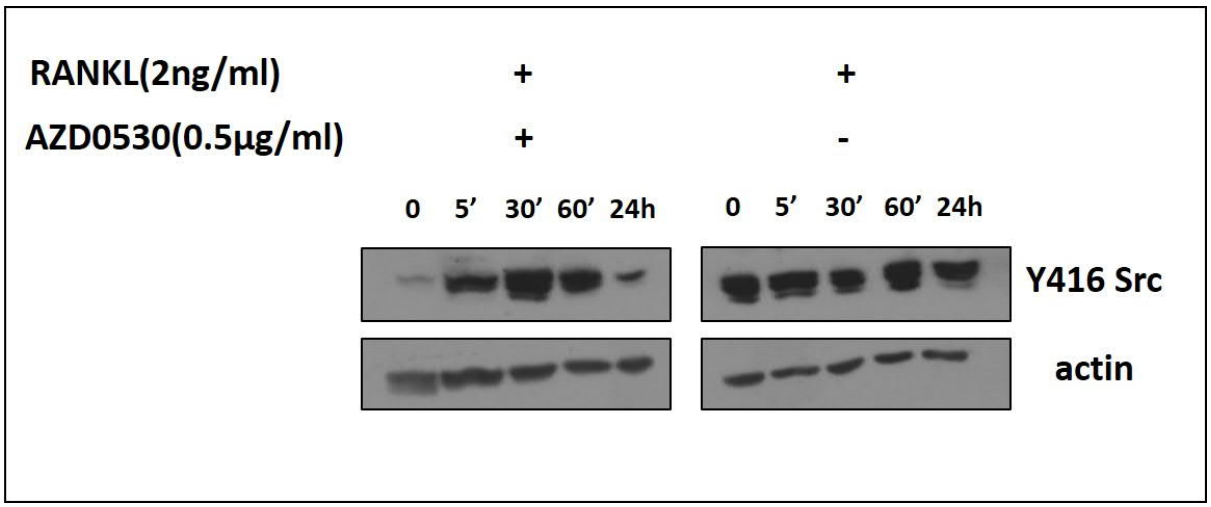


Figure 3.15: RANKL temporarily attenuates AZD0530-mediated Src inhibition in RAW cells. Cells were seeded on plates and cultured to 70% confluence and subsequently treated with RANKL (2ng/ml) ± pre-treatment with AZD0530 (0.5µM) for 2h. Samples were harvested at the indicated time-points (0-24h). Cells were lysed, processed for Western Blotting and probed for phospho-Y416 Src kinase as indicated. Actin was used as loading control. Representative blots shown from three independent experiments.

3.3 Discussion

Breast cancer is one of the most common malignancies in women. The evolution of our understanding of the pathophysiology underlying breast cancer has led to significant scientific breakthroughs and the development of new therapeutic agents that have increased the 10-year survival rate to around 80% for a large proportion of patients (Bodai & Tuso 2015). However, breast cancer bone metastasis still constitutes one of the most challenging topics to be faced in the clinical setting, since approximately 85% of patients with advanced breast cancer develop bone metastases (Harries et al. 2014). Resistance to endocrine treatment might also predispose to the development of bone metastasis as it is highly correlated with an aggressive and metastatic phenotype (Hiscox et al. 2004). Although significant developments have been made in the treatment of breast cancer, bone metastases still remain incurable (Ibrahim et al. 2013). Breast cancer cells secrete growth factors, such as PTHrP and interleukins, that promote osteoclast activation either indirectly or directly and subsequently lead to bone resorption (M. Bendre et al. 2003). In turn, bone breakdown releases factors that are stored in the matrix, such as TGFβ and VEGF, that further supply cancer cell growth and proliferation resulting in the so called 'vicious cycle of bone metastasis' (Chen et al. 2010b). Triple negative and endocrine resistant breast cancers represent the most aggressive breast tumour subtypes that are associated with disease progression and high morbidity rates (Anders & Carey 2008; Chang 2012). In the current chapter, we aimed to investigate whether the aggressive breast cancer phenotype, either due to the subtype or the development of endocrine resistance, predisposes the establishment of secondary tumour foci in the bone and, if so, a possible mechanism underlying this.

RAW cells are an accepted model for the study of osteoclast differentiation (Collin-Osdoby & Osdoby 2012) and have been found to respond to bone modulatory factors including RANKL and conditioned media from different cell models of solid tumours

(Tiedemann et al. 2009; Ouellet et al. 2011; Zhang et al. 2001; Araujo et al. 2009; Morrissey et al. 2010). Thus, RAW 264.7 cells were initially exposed for 7 days to various RANKL concentrations (0.5-4ng/ml), according to manufacturer's instructions, in order to determine the optimum concentration producing the highest osteoclast yield. Osteoclast differentiation was affected by RANKL in a dose-dependent manner, with the lowest concentration of 0.5ng/ml RANKL barely supporting osteoclastogenesis and the highest concentration of 4ng/ml RANKL leading to the highest number of osteoclasts formed. As such, in line with other reports all the RANKL concentrations 1-4ng/ml can be used to generate a positive differentiation control (Karlsson et al. 2016; Fowler et al. 2015; Mediero et al. 2015).

Several studies support that breast cancer cells secrete factors that facilitate the establishment of new tumour foci in the bone (Tiedemann et al. 2009; McCoy et al. 2013; Clohisy et al. 1996). Indeed, treatment of RAW cells with breast cancer cell CM resulted in the differentiation of multinucleated osteoclasts, in the absence of RANKL. Specifically, the breast cancer model with acquired resistance to tamoxifen (TamR) and the triple negative cells (MDA-MB-231) stimulated the highest number of differentiated osteoclasts. This might be explained by the fact that these subtypes clinically exhibit an aggressive and highly metastatic phenotype that is accompanied by reduced survival rates (Hiscox, Jiang, et al. 2006; Hiscox et al. 2004; William D Foulkes et al. 2010) The MCF-7-derived CM did not stimulate the formation of any osteoclasts and this is probably due to the low aggressive status that Luminal A breast cancers represent in the clinical setting compared with other subtypes (Ignatiadis & Sotiriou 2013), whilst the Faslodex resistant model (FasR) resulted in the formation of a moderate number of multinucleated osteoclasts.

Concentration and CM collection time constituted an important factor for the osteoclastogenic effect seen. Interestingly, the lowest percentage of CM tried (10%)

exerted the most robust impact on RAW cell differentiation to osteoclasts, while the highest CM concentration (40%) led to the lowest number of differentiated cells. Moreover, CM collected at 24h supported the formation of a higher osteoclast yield compared to the CM harvested at 72h. A possible interpretation of these findings might be the build-up of metabolic products by breast cancer cells that possibly impair RAW cell osteoclast differentiation. Although this observation still remains obscure, our findings were in agreement with the vast majority of the literature that suggests the use of 10% CM collected at 24h to induce RAW cell differentiation (Tiedemann et al. 2009; Araujo et al. 2009; Rafiei & Komarova 2013; Mourskaia et al. 2012).

Breast cancer cell CM treatment of RAW cells that have been previously primed with RANKL resulted in a similar number of differentiated osteoclasts regardless of the cell subtype the CM was derived from and no statistical differences were observed. This finding was in contrast with other studies suggesting that CM enhances the number of differentiated osteoclasts in RANKL-primed RAW cells. According to a recent study, soluble factors secreted by the prostate cancer cell line PC-3 are able to enhance osteoclast differentiation of RANKL-primed precursors (Rafiei & Komarova 2013), however this was not the case in our own system. This might be attributed to the fact that RANKL treatment possibly saturated the system and therefore any differential effect across different cell lines' CM was not revealed. Moreover, it is known that breast cancer cell lines express and secrete OPG that is a decoy receptor of RANKL and thus obstructs RANKL-mediated osteoclast differentiation (Thomas et al. 1999; Ney et al. 2012). Thus, an alternative explanation for this observation could be that differential expression of OPG levels in the CM possibly neutralised the effect of RANKL and masked the pure impact of CM on osteoclast differentiation and thus resulted in a similar number of differentiated cells.

The passage number of RAW cells constituted a critical factor for their responsiveness and induction of osteoclast differentiation. Such observation has been highlighted by several research groups, concluding that RAW cells should not be used over 20 passages (Watanabe et al. 2004). The reason underlying this is not fully clear, but it is possibly attributed to the fact that these cells are comprised of different subclones and suffer from genetic drift. Thus, RAW cells undergo genotypic alterations that result in reduced responsiveness over time in normal cell culture conditions (Cassady et al. 2003; Collin-Osdoby & Osdoby 2012). Evaluation of these observations in our system indicated that, indeed, CM treatment of high passage RAW cells failed to induce osteoclast differentiation. RANKL stimulation in these cells, revealed the formation of only a few osteoclasts further supporting the importance of using low passage RAW cells in the investigation of bone and cancer cells interactions.

Src kinase plays a key role in osteoclast differentiation and function (Kim & Kim 2016). In addition, Src is of fundamental importance in the formation of the ruffled borders in osteoclasts that enables them to attach the bony substrate during resorption (Boyce et al. 1992). Our data demonstrated that Src inhibition impaired RANKL-mediated osteoclast formation in a dose-dependent manner. In addition, RAW cell treatment with CM from Src-deficient breast cancer cells resulted in the formation of significantly lower number of osteoclasts compared to the CM that was derived from untreated breast cancer cells. These finding are in line with a relatively recent study revealed that Src inhibitor dasatinib impaired both RANKL- and PC-3 conditioned media- mediated RAW differentiation to osteoclasts (Araujo et al. 2009). Notably, in the case of TamR and MDA-MB-231 cells that exhibit an aggressive phenotype, the formation of osteoclasts was significantly lower implying that Src inhibition in the clinical setting might display a beneficial effect to patients with triple negative or tamoxifen resistant breast cancer.

It is known that RANKL activates Src to subsequently trigger AKT and MAPK activation (Mizukami et al. 2002). Western blot analysis for RAW cells that have been treated with RANKL ± AZD0530 for different times, revealed that RANKL did not seem to activate Src in the absence of AZD0530. However, this could be due to Src levels being saturated on the blot so that differences can't be seen. Nevertheless, Src activity was prolonged (>24hr) in absence of AZD0530, but when AZD0530 was present it diminished after 24hrs. Consequently, this effect may be important as RAW cell differentiation occurs over longer time points.

In summary, breast cancer cells secrete factors that can induce RAW cell differentiation to osteoclasts in a RANKL-independent manner. The highest number of differentiated cells generated following CM treatment derived from two highly aggressive subtypes; triple negative cells and breast cancer cells with acquired tamoxifen resistance. Furthermore, Src inhibition significantly impaired osteoclast differentiation and Src-deficient breast cancer cell CM resulted in a lower number of differentiated osteoclasts suggesting that Src inhibition might be a promising application into the clinical setting that would benefit breast cancer patients, especially those with triple negative or tamoxifen resistant breast cancer.

4. Tamoxifen-resistant breast cancer cells promote osteoclast differentiation in a Src-dependent manner

4.1 Introduction

The bone is a favoured site of breast cancer metastases and it has been demonstrated that breast cancers that spread to the bone are able to influence the balance between osteoclasts and osteoblasts in the bone microenvironment, promoting so-called osteolytic metastases (Zhang et al. 2010). Breast cancer patients with bone metastases frequently have skeletal-related co-morbidities such as spinal cord compression, fractures and severe pain leading to reduced quality of life (Gnant et al. 2012). Importantly, the data in the previous chapter suggested that breast cancer cells secrete factors that can influence the differentiation of murine monocytes into osteoclasts in the absence of RANKL. This was particularly the case with acquired endocrine resistant models that represent a range of clinical resistance phenotypes, both ER+ and ER-, along with a well-established model of triple-negative breast cancer (TNBC). Both endocrine-resistant and TNBC subtypes are associated with a poor prognosis clinically and frequently involve spread to the bone (Bianchini et al. 2016). Interestingly, previous studies by our group have identified a role for Src kinase in promoting the development of an aggressive phenotype in endocrine-resistant models (Hiscox, Morgan, Tim P. Green, et al. 2006), and a link between elevated Src activity and poor outcome on tamoxifen has been shown in translational studies (Morgan et al. 2009). Additionally, Src has been identified as playing a key role in TNBC growth and migration according to recent preclinical studies (Tryfonopoulos et al. 2011; Huang et al. 2007; Richard S Finn et al. 2007). This finding was of particular importance as TNBC treatment is limited to chemotherapy and therefore Src inhibition may represent another therapeutic option in a difficult-to-treat-population (Finn 2008).

Interestingly, as well as promoting migratory and invasive behaviour in cancer cells, Src kinase has also been shown to regulate the expression of cytokines and growth factors that themselves are implicated in osteoclastogenesis (Mundy 1993; Finn 2008). Given that both TNBC and relapsed disease (i.e. ER+ endocrine sensitive cancers that recur due

to acquired resistance) frequently associate with bone metastases, we wished to explore whether these types of breast cancer were able to influence the differentiation of osteoclast precursor cells into osteoclasts. Peripheral blood mononuclear cells (PBMC) were chosen as the pre-osteoclast model system with a view of making this research more translational and relevant to the human context. Thus, our central hypothesis under test here was that resistant and/or TNBC cell models that reflect clinically aggressive forms of breast cancer, are able to promote differentiation of PBMCs into osteoclasts and that this occurs through a Src-dependent mechanism.

To achieve our aims the following objectives were set:

1. optimise a PBMC-based assay in order to measure osteoclast differentiation
2. optimise a bone-degradation assay to measure osteoclast osteolytic function
3. employ the PBMC assay to investigate the ability of drug-responsive and drug-resistant breast cancer cell models to influence differentiation into osteoclasts
4. investigate whether Src kinase plays a role in breast cancer cell mediated PBMC differentiation to osteoclasts
5. elucidate the underlying mechanism(s) of breast cancer cell mediated osteoclast differentiation.

4.2 Results

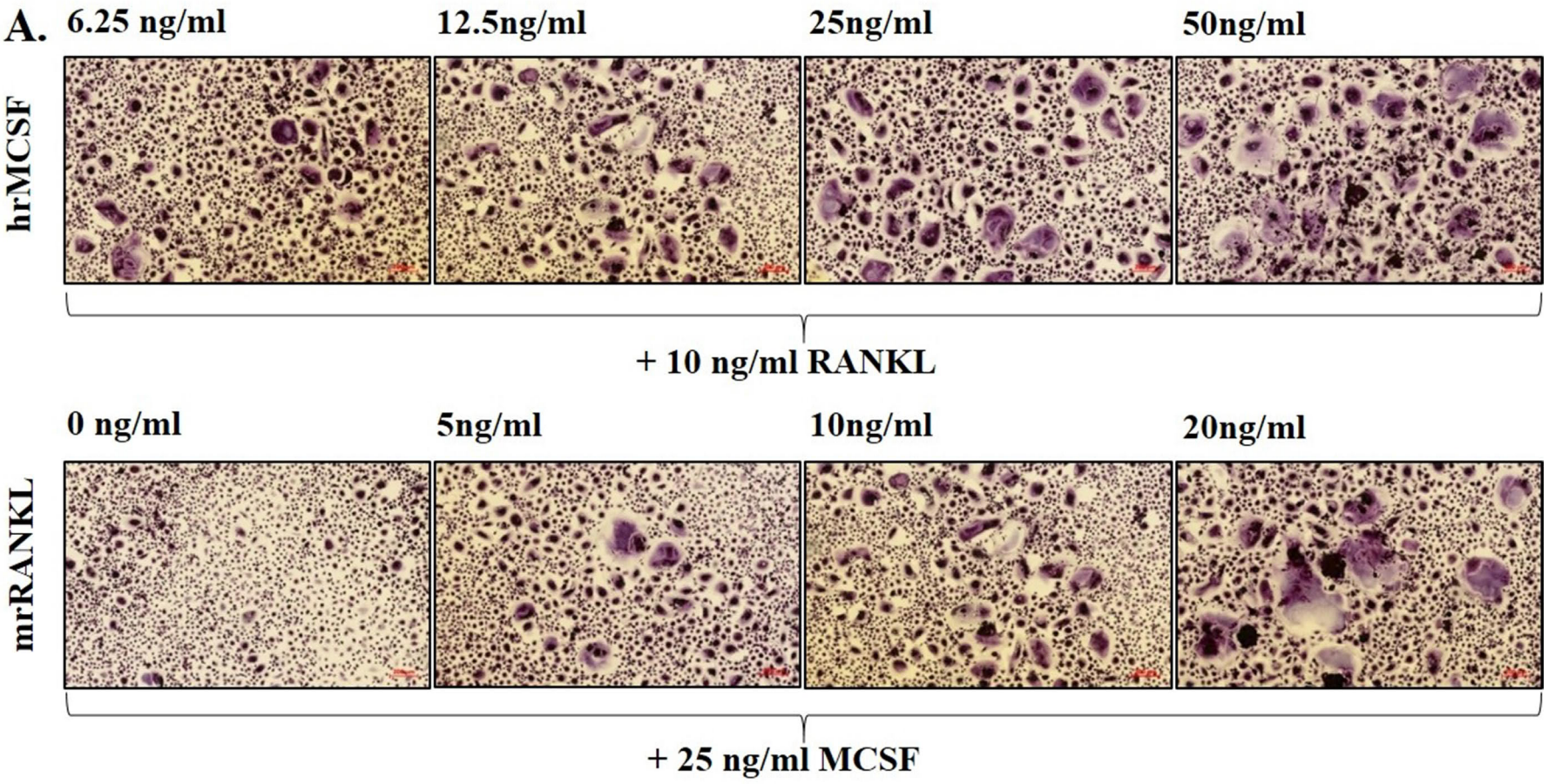
4.2.1 Optimisation of PBMC differentiation assay

To begin to explore the ability of breast cancer cell CM to promote differentiation of freshly-isolated PBMCs, it was first necessary to develop a standard protocol for PBMC differentiation which would subsequently be used as a positive, internal control in future experiments (Mabilleau & Sabokbar 2009). PBMCs were cultured in αMEM/10% FCS and treated for 14 days with MCSF, a factor essential for their proliferation, and RANKL which is necessary for their fusion and differentiation to large, multinucleated osteoclasts (Kim & Kim 2016). Optimisation involved treating PBMC cells with macrophage colony-stimulating factor (MCSF) and RANKL over the range 0-50ng/ml and 0-20ng/ml respectively, doses corresponding to the range of concentrations most frequently cited in the literature for human PBMC differentiation (Jevon et al. 2002; J. R. Edwards et al. 2006). Initially each MCSF concentration was used with a fixed RANKL concentration of 10ng/ml (Hirayama et al. 2002) and differentiation allowed to proceed for 14 days. Following that period cells were subject to TRAP staining and counterstained with haematoxylin (to aid visualisation).

Treatment of PBMC cells with RANKL (10ngml) and either 25ng/ml or 50ng/ml MCSF resulted in formation of TRAP-positive, multinucleated cells indicative of osteoclasts (**Figure 4.1**). Similarly, TRAP-positive, multinucleated cells were seen to develop following treatment with all concentrations of RANKL (5-25ng/ml) and a fixed MCSF dose (25ng/ml). For PBMCs isolation we recruited both male and female healthy donors with various ages in order to have a representative sample group (**Table 4.1**).

Table 4.1: Gender and age range of the blood donors recruited to study the effect of breast cancer cells on osteoclastogenesis

Donor number	Gender	Age
1	Female	50-59
2	Male	20-29
3	Female	20-29
4	Male	30-49
5	Female	40-49
6	Female	20-29
7	Male	20-29
8	Male	40-49
9	Female	30-39
10	Male	50-59


A.
6.25 ng/ml
12.5ng/ml
25ng/ml
50ng/ml
hrMCSF
+ 10 ng/ml RANKL
0 ng/ml
5ng/ml
10ng/ml
20ng/ml
mrRANKL
+ 25 ng/ml MCSF

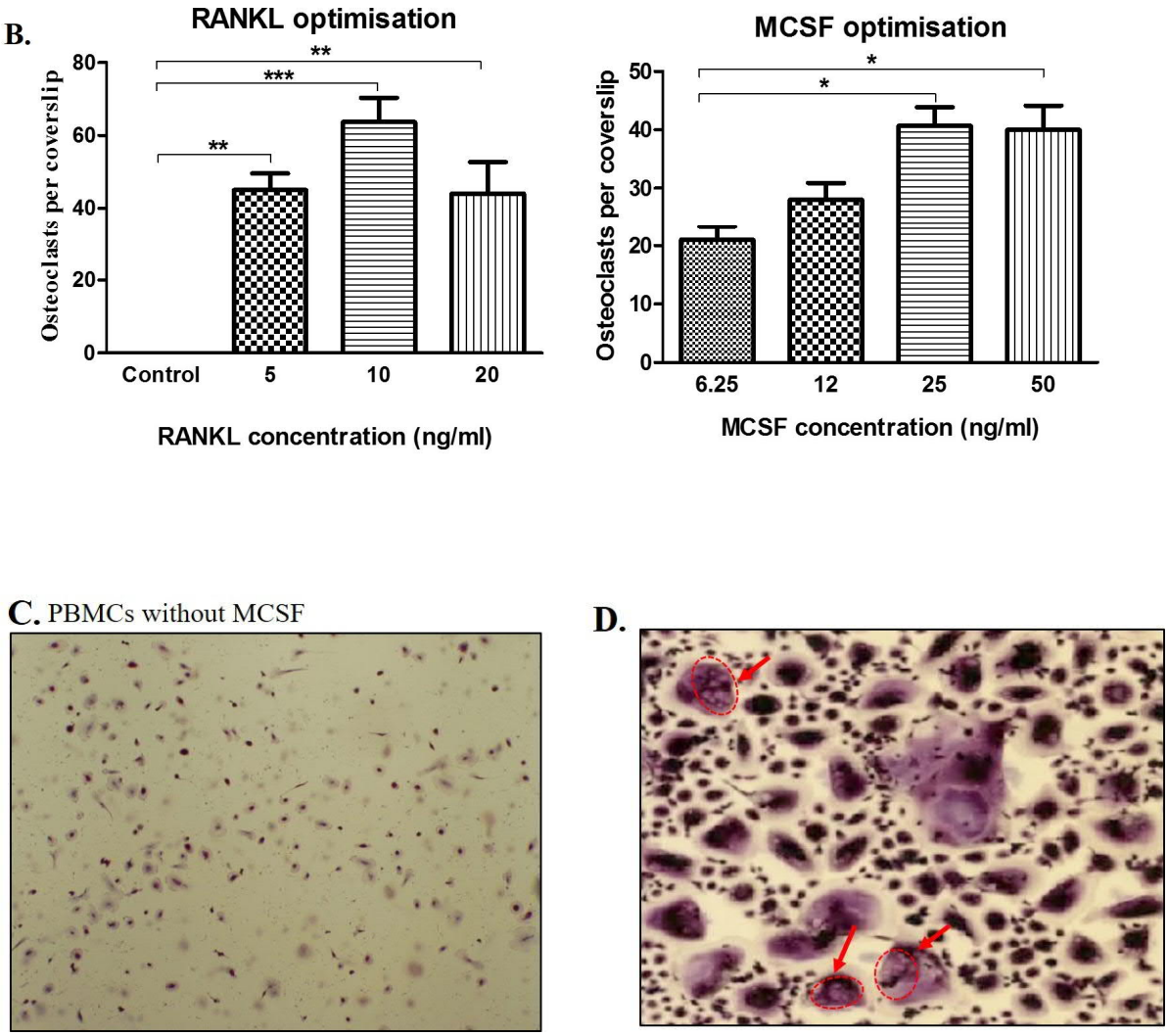


Figure 4.1: MCSF and RANKL promote PBMC differentiation into osteoclasts. Osteoclast differentiation assay was optimised by testing a range of MCSF and RANKL concentrations (RANKL: 0-20 ng/ml and MCSF: 6.25-50 ng/ml) on PBMCs for 14 days, in order to determine the optimal combination of ligands for osteoclast formation. **(A)** Representative pictures of TRAP staining and haematoxylin counterstain of differentiated osteoclasts were captured using 100x magnification. **(B)** Quantitation based on the number of the differentiated osteoclasts per coverslip. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ **(C)** PBMCs cultured in the absence of MCSF, most of the cells were dead. **(D)** High power (200x) osteoclast differentiation. Red arrows indicate the multinucleation.

4.2.2 Response of PBMC cells to breast cancer cell condition media (CM)

Having established a reproducible assay with which to investigate PBMC differentiation, essentially a positive control, we next wished to determine whether breast cancer cells were able to induce PBMC differentiation. We used conditioned media from breast cancer cells at a concentration of 10% based on our previous observations (see Chapter 3).

Freshly isolated PBMCs were treated with 10% breast cancer cell CM [i.e. a 1:9 dilution with fresh media (αMEM/10% FCS) that had not been used for cell culture] in the presence of MCSF for 14 days. Following that, cells were stained for TRAP and evaluated using a light microscope. Each assay had positive (RANKL and MCSF) and negative (MCSF only) controls. Treatment of PBMCs with conditioned media induced the formation of large, multinucleated and TRAP positive cells (**Figure 4.2**). For all donors tested, PBMC differentiation to multinucleated osteoclasts appeared greatest when stimulated with MDA-MB-231 and TamR cells CM; moderate differentiation was triggered by FasR CM, while the least amount of differentiation was observed following treatment with MCF-7 conditioned media (**Figure 4.2**).

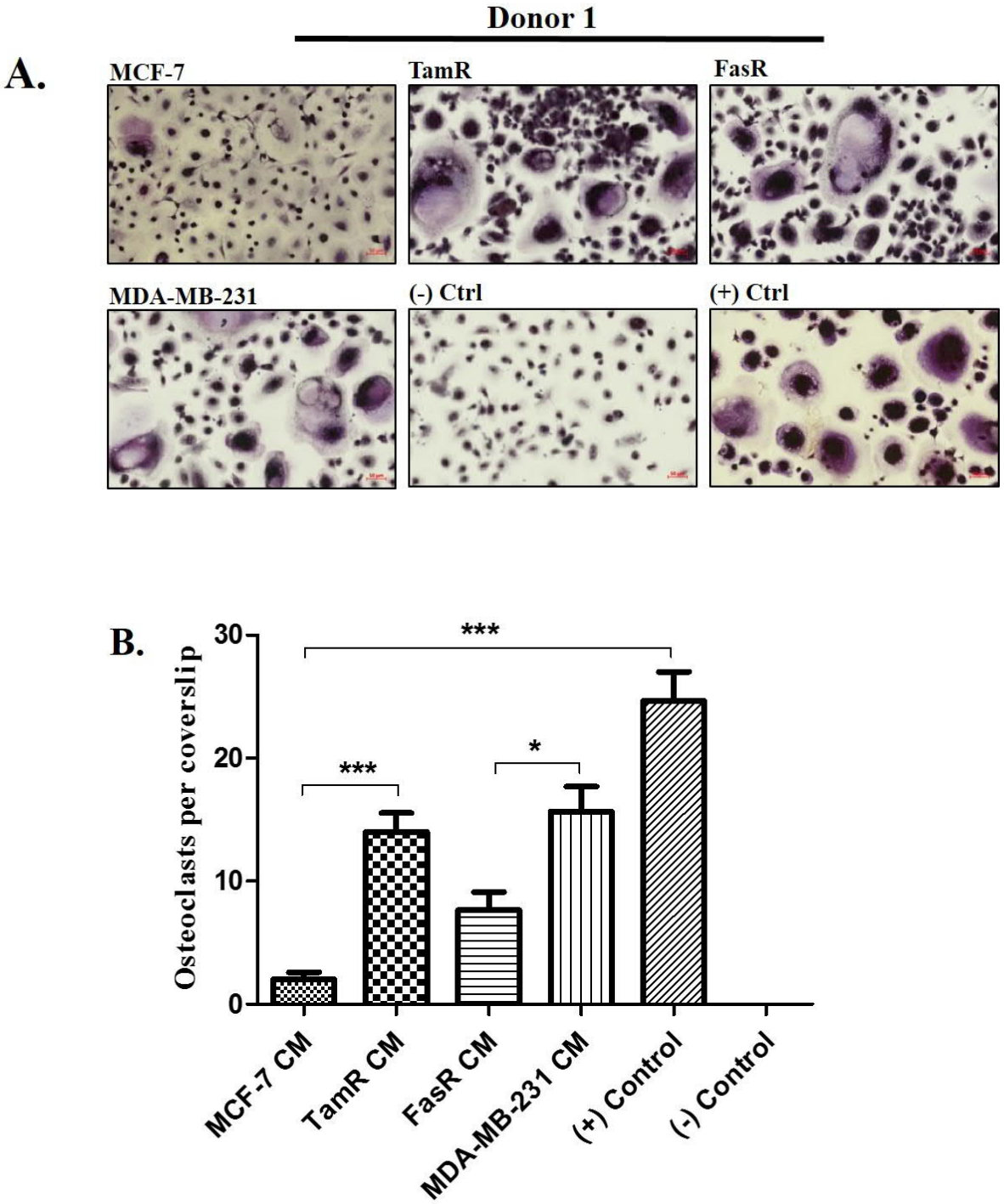


Figure 4.2: Breast cancer cell conditioned media supports osteoclast formation. (A) Representative image from Donor 1. CM was collected after 24h incubation with breast cancer cells, diluted 1:10 with fresh medium and then added on to PBMCs cultures. After 14 days, cells were fixed and stained for TRAP. Osteoclast formation was evaluated by counting the number of TRAP (+) cells that had 3 or more nuclei. **(B)** Quantitation of the number of osteoclasts per cover slip. * $p<0.05$, *** $p<0.001$ n=6 replicates for each condition

The experiment with the application of breast cancer cells CM was performed using PBMCs isolated from 10 different donors stated in **Table 4.1**, and the collective results of all the assays, normalised to results with MCF-7 CM, is shown below (**Figure 4.3)**.

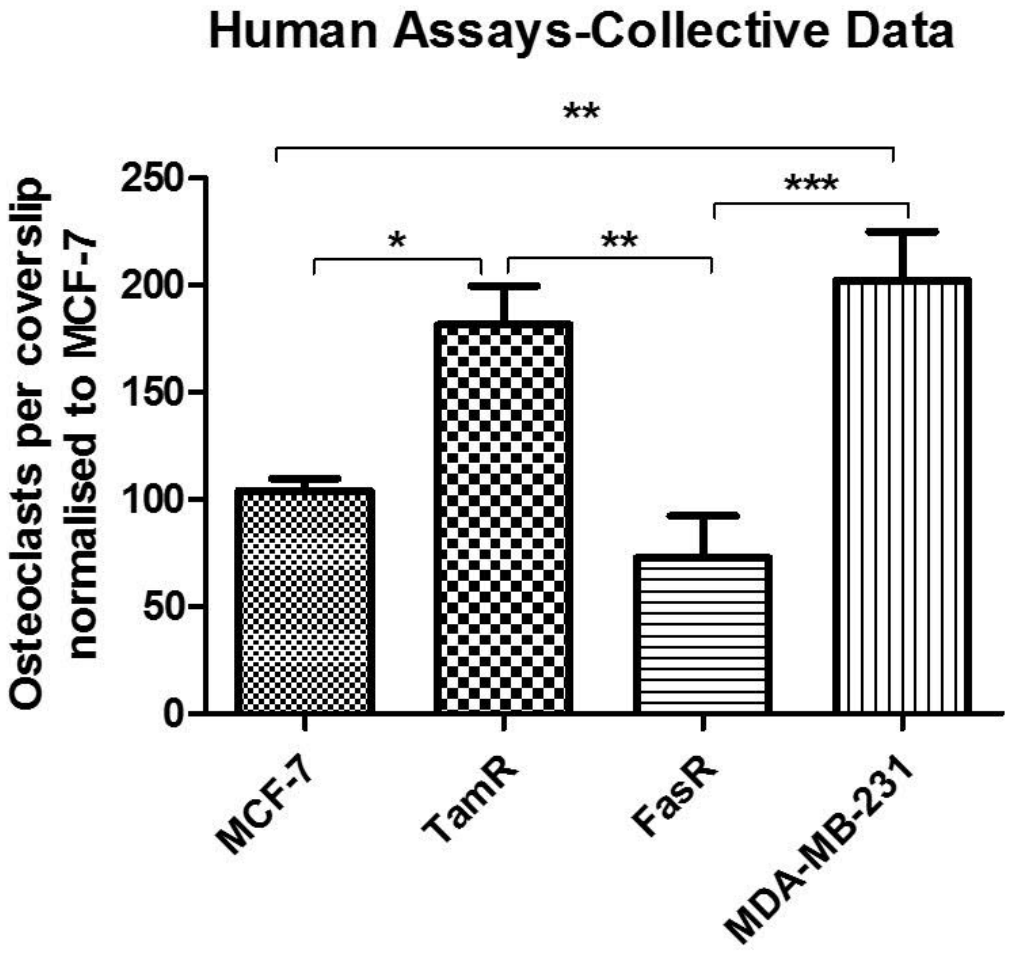


Figure 4.3: TamR and MDA-MB-231 cells CM exert significant osteoclastogenic effects compared to FasR and MCF-7 cell CM. Collective data of differentiated osteoclasts obtained following PBMC treatment from 10 different donors with breast cancer cells CM. Osteoclast number was normalised to MCF-7 data. n=6 replicates for each condition * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.2.3 Effect of breast cancer cell conditioned media on osteoclast bone resorptive activity

Having established that breast cancer cell CM promoted differentiation into cells appearing osteoclast-like, we then aimed to investigate whether these cells also possessed the functional features of bone-resorptive cells given that this is frequently underreported in the literature. To evaluate this, PBMCs were set up on ivory discs instead of coverslips and treated with CM for a period of 21 days. Cells were then removed from the discs and the discs stained with Toluidine Blue to visualise resorption pits. A positive (RANKL-stimulated, 10ng/ml) and a negative (no RANKL) control were also included in the experiment for assay validation. Ivory discs containing PBMCs stimulated by RANKL (positive control) stained positive for resorption pits whereas no such staining was observed in the negative control. Staining of PBMC after incubation with breast cancer conditioned media failed to show any discrete pit formation instead revealing a general blue stain over the ivory disk surface (**Figure 4.4**).

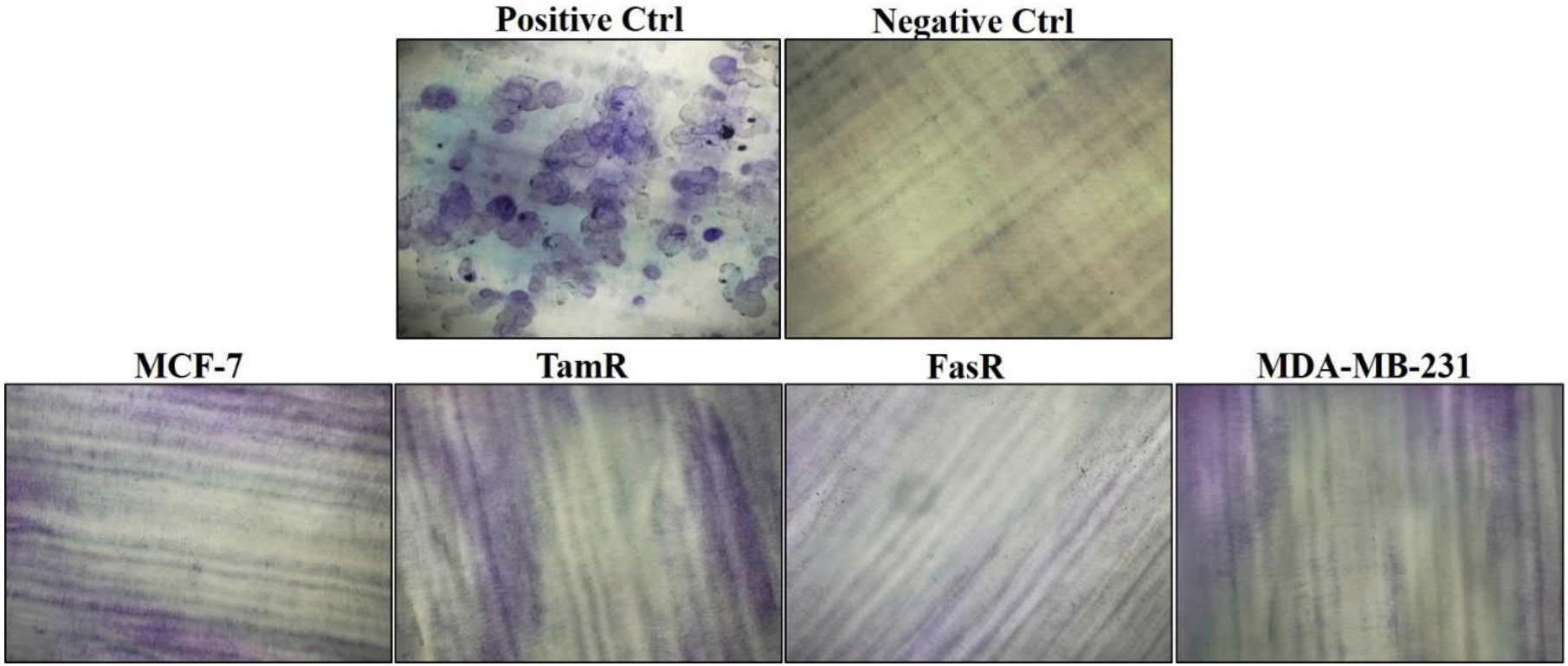


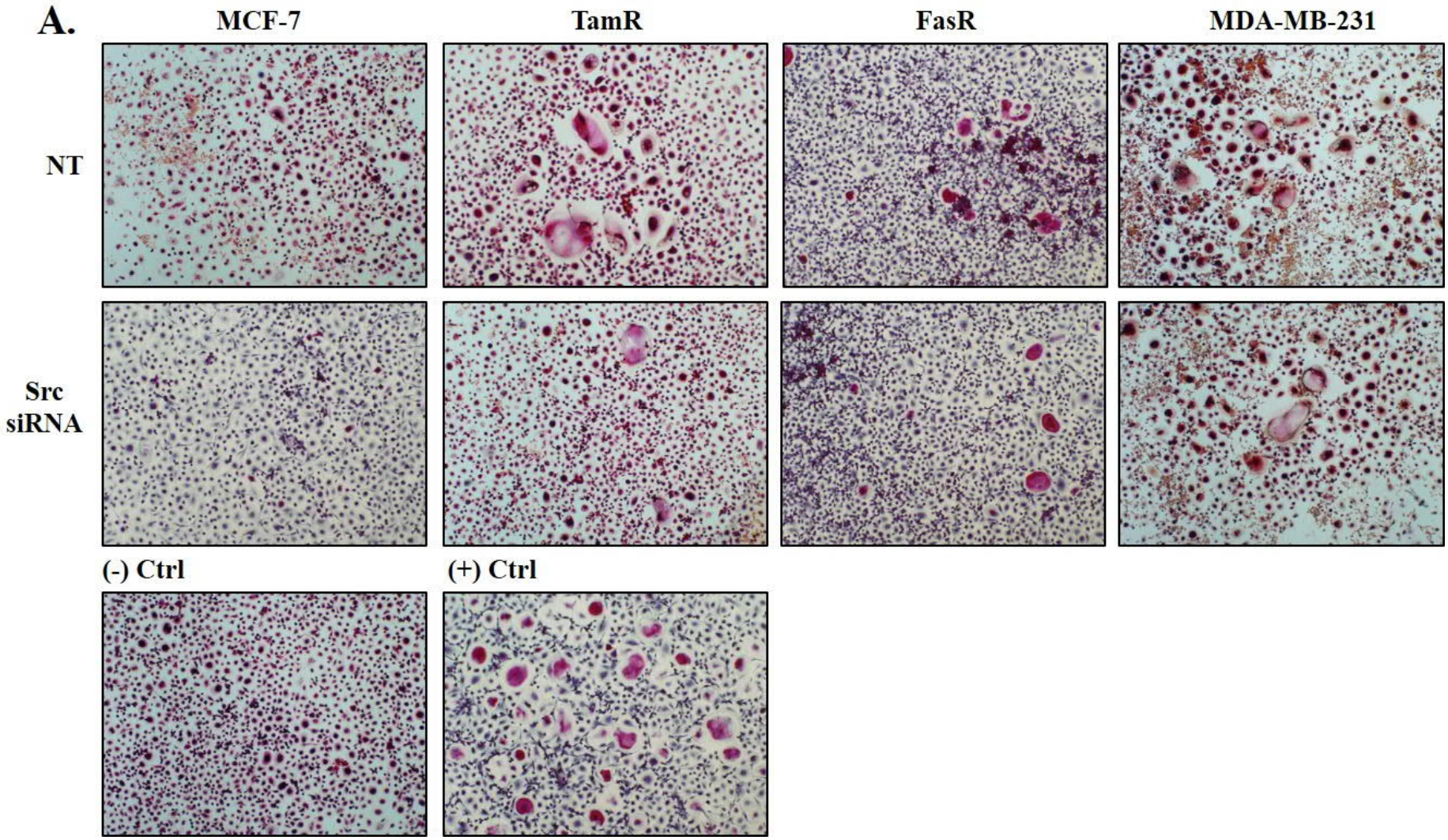
Figure 4.4: Osteoclasts generated in response to breast cancer cell CM do not possess resorptive features. Toluidine blue staining on ivory slices seeded with PBMCs and treated with MCSF alone (negative control), MCSF and RANKL (positive control) or MCSF and breast cancer cell CM for 21 days. Toluidine blue staining showed distinct resorption pits in positive control only.

4.2.4 Investigation into the role of Src kinase in breast cancer cell-mediated PBMC differentiation

Src kinase plays a key role in multiple signalling pathways that regulate the aggressive nature of breast and other cancer cells, promoting their invasion *in vitro* (Guarino 2010) and progression and spread in vivo (Elsberger 2014). Activation of Src can also promote expression of various factors some of which are known to regulate crosstalk with other cell types including bone cells. Given that tamoxifen-resistant MCF-7 cell models ('TamR cells') are known to have significantly elevated Src activity and also induced PBMCs differentiation, we hypothesised that this may occur in a Src-dependent manner. To explore this further, we suppressed Src expression using siRNA in our breast cancer models prior to collection of CM and used it in a PBMC differentiation assay.

siRNA-mediated Src knock-down was first confirmed using Western blotting (see Chapter 3, figure 3.11), which revealed an almost complete loss of Src in siRNA-treated samples compared to samples treated with non-targeting (NT) siRNA.

Application of CM from these Src-deficient cell models to the RAW assay resulted in reduced number of differentiated osteoclasts compared to the CM from un-treated breast cancer cell cultures (**Figure 4.5**)


A.
MCF-7
TamR
FasR
MDA-MB-231
NT
Src siRNA
(-) Ctrl
(+) Ctrl

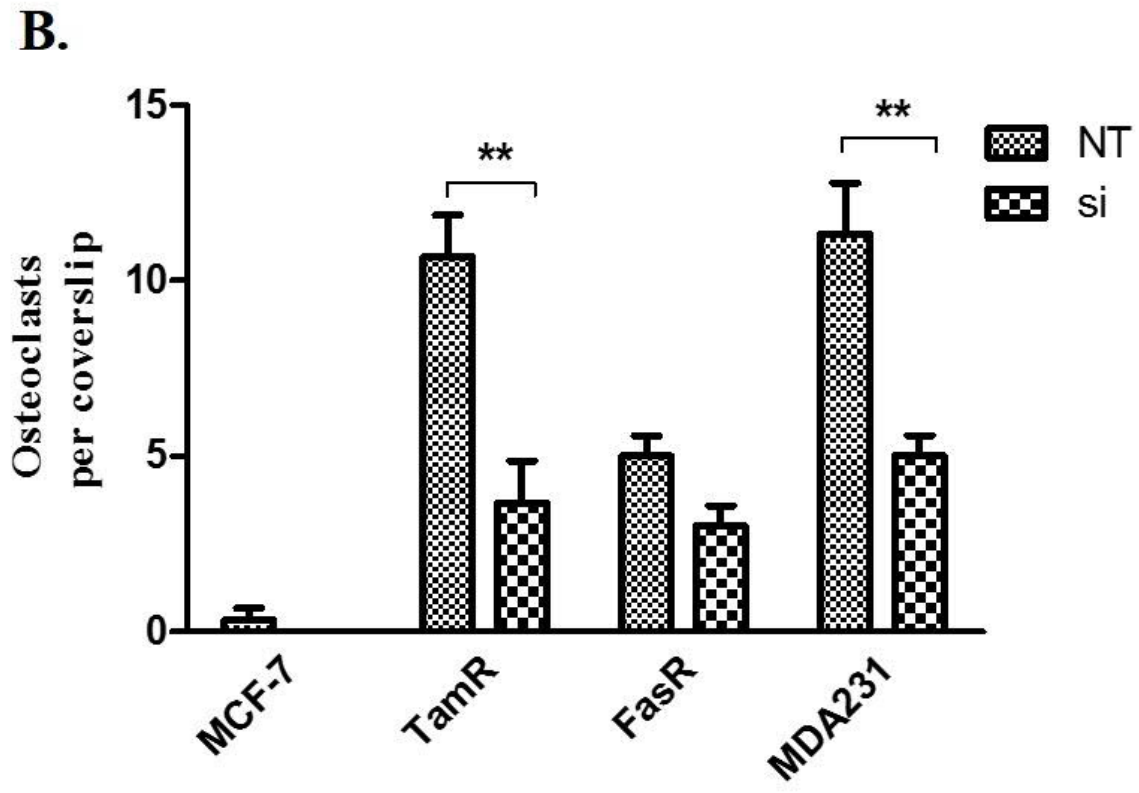
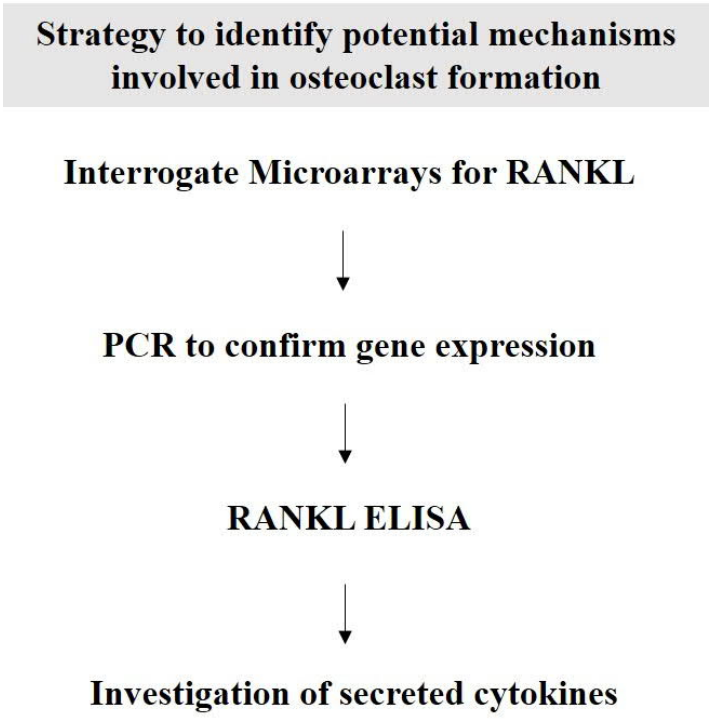


Figure 4.5: Src inhibition significantly reduces the osteoclastogenic effect seen by untreated breast cancer cells. (A) Representative figure from Donor 4. CM was collected after 24h incubation with untreated or Src siRNA post-transfected cells, diluted 1:10 with fresh medium and then added on to PBMCs cultures. After 14 days, cells were fixed and stained for TRAP. Osteoclasts formation was evaluated by counting the number of TRAP (+) cells that had 3 or more nuclei. **(B)** Quantitation based on the number of osteoclasts per cover slip. p values: ** $p<0.01$

4.2.5 Investigation of potential Src-dependent bone modulatory mechanisms in breast cancer cells

Having identified a potential role for Src kinase in driving PBMC differentiation into osteoclast cells, particularly in tamoxifen resistant and triple negative breast cancer cells, we next wished to begin to investigate the mechanism(s) underlying this. Based on the fact Src promotes breast cancer cell migration and invasion (Pohorelic et al. 2012) and in addition is essential for osteoclast function (Miyazaki et al. 2004) one hypothesis is that Src might regulate internal signalling pathways that in turn regulate the production and secretion of bone cell modulatory factors. To achieve this the following strategy plan was followed:



4.2.5.1 *Microarray interrogation*

The Breast Cancer Molecular Pharmacology Group have previously constructed an in-house microarray database for MCF-7 cells and their resistant counterparts. To explore a potentially novel link between Src and RANKL expression, we first interrogated our in-house microarray database and investigated the expression of probes corresponding to RANKL. This data suggested that RANKL was not expressed in any of the breast cancer cells (**Figure 4.6**).

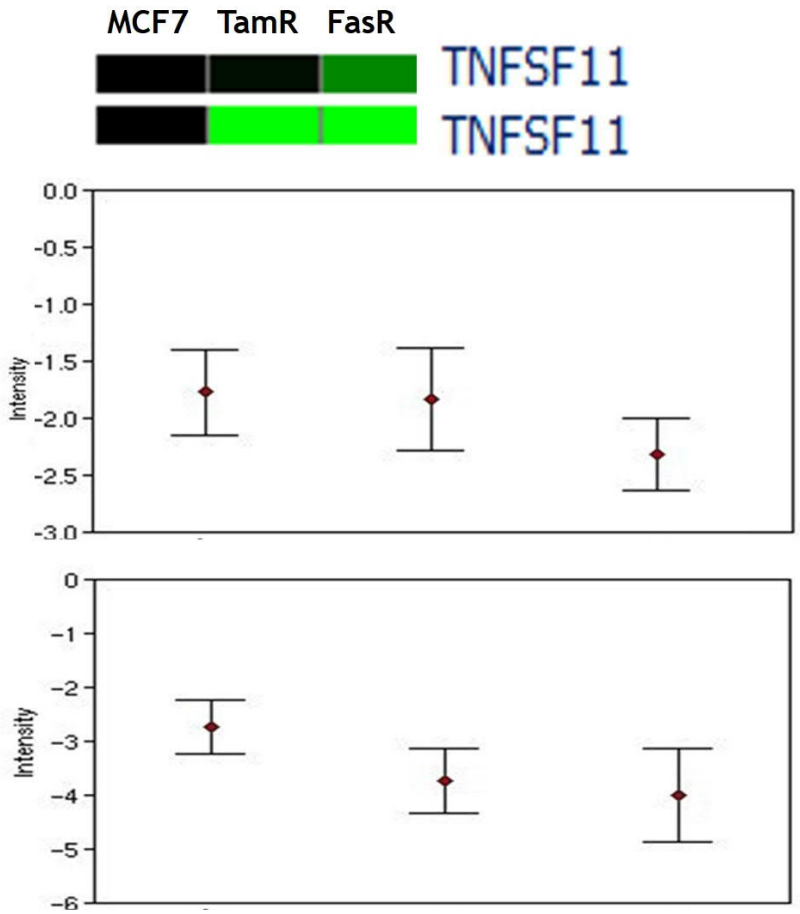
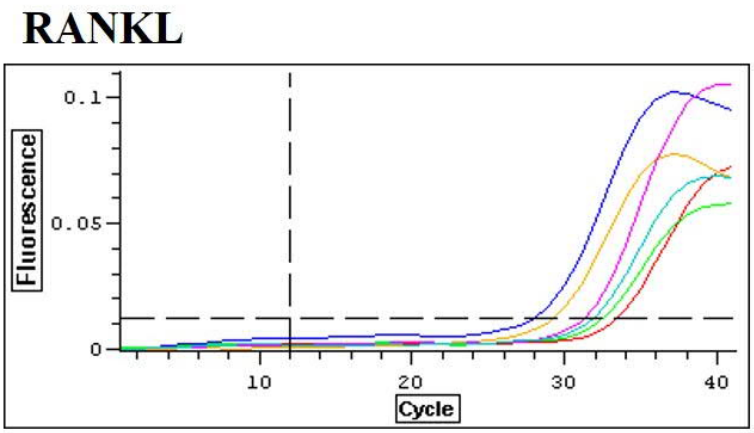


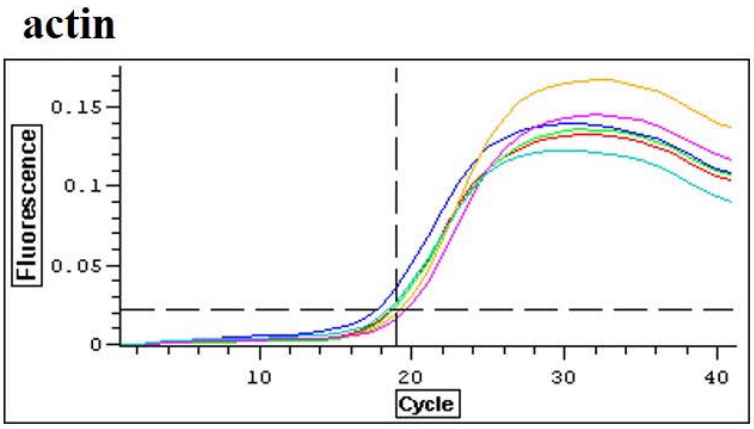
Figure 4.6: RANKL is not expressed by breast cancer cells. Microarray data for RANKL expression in MCF-7, TamR and FasR cells revealed that RANKL is not expressed (Intensity<0) TNFSF11: RANKL gene probe.

4.2.5.2 *Exploration of the RANKL gene levels in breast cancer cells*

Whilst the microarray data was in agreement with a number of published studies (Ney et al. 2012), one report does demonstrate RANKL expression in breast cancer cells (Owen et al. 2013). Thus, we wished to validate the microarray data using q-PCR analysis. This data suggested that RANKL was expressed in breast cancer cells (**Figure 4.7**), albeit only detectable at relatively high cycle number.



Label	C(T)
MCF RANKL	33.411
MCF RANKL	32.462
TAMR RANKL	28.017
TAMR RANKL	29.191
FASR RANKL	31.172
FASR RANKL	31.797



Label	C(T)
MCF actin	18.693
MCF actin	18.783
TAMR actin	17.637
TAMR actin	18.432
FASR actin	19.252
FASR actin	19.591

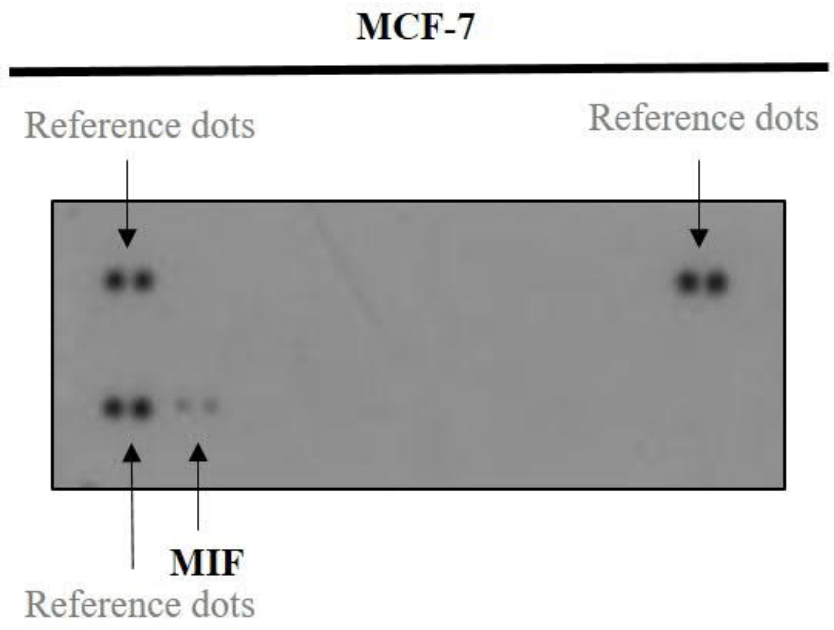
Figure 4.7: RANKL gene expression is detectable in breast cancer cells. qPCR investigation revealed that RANKL was expressed by breast cancer cells, however in a relative high cell number. Actin was used as a loading control.

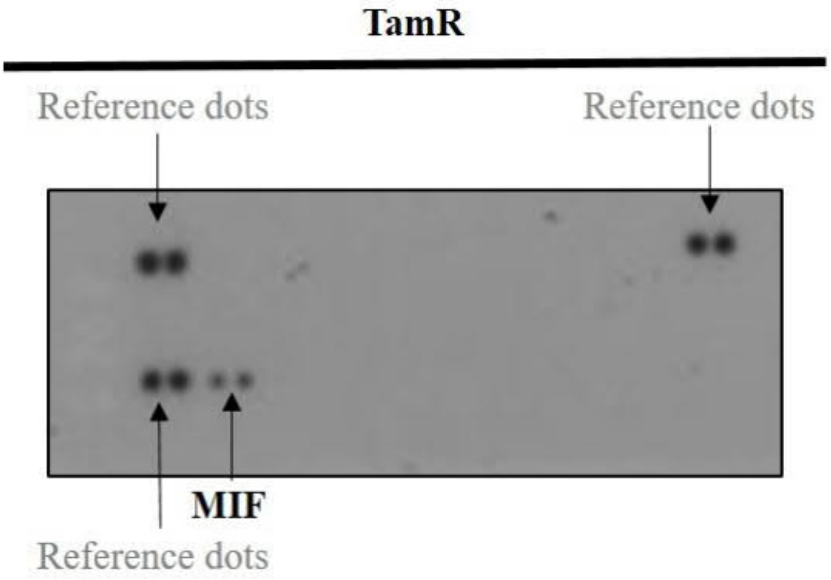
4.2.5.3 *Exploration of the RANKL secretion in breast cancer cells conditioned media*

Thus, to confirm RANKL abundance we next performed ELISA specific for RANKL to measure the levels of the human RANKL in the breast cancer cells conditioned media. A standard curve was generated using known concentrations of RANKL (0-5000 pg/ml) and then the samples of breast cancer cells were analysed compared to the standard curve. Conditioned media analysis of all the breast cancer cell lines did not detect any significant levels of RANKL and all the measurements were below the lowest value of the standard curve, confirming the microarray data (see Appendix C).

4.2.5.4 *Breast cancer cell line screening to identify potential bone-modulatory mechanisms*

Our research was equivocal regarding the role of RANKL in breast cancer-mediated PBMC differentiation and so to begin to explore potential mechanisms further we employed a limited screening strategy using a protein microarray using commercially available membranes for human cytokines (R&D Systems). These results revealed that MCF7 and both MCF7-derived resistant cell lines secreted Macrophage Migration Inhibitory Factor (MIF); this appeared higher in TamR cells versus MCF-7 and FasR cells. Cytokine profiling of MDA-MB-231 cells revealed that, including MIF, these cells also secreted CXCL1/GROa, CXCL12/SDF-1, GM-CSF, IL-8 and Serpin E1/PAI-1 (**Figure 4.8**). In **Table 4.2** is illustrated the cytokine expression in breast cancer cells along with their biological function.


MCF-7
Reference dots
Reference dots
MIF
Reference dots


TamR
Reference dots
Reference dots
MIF
Reference dots

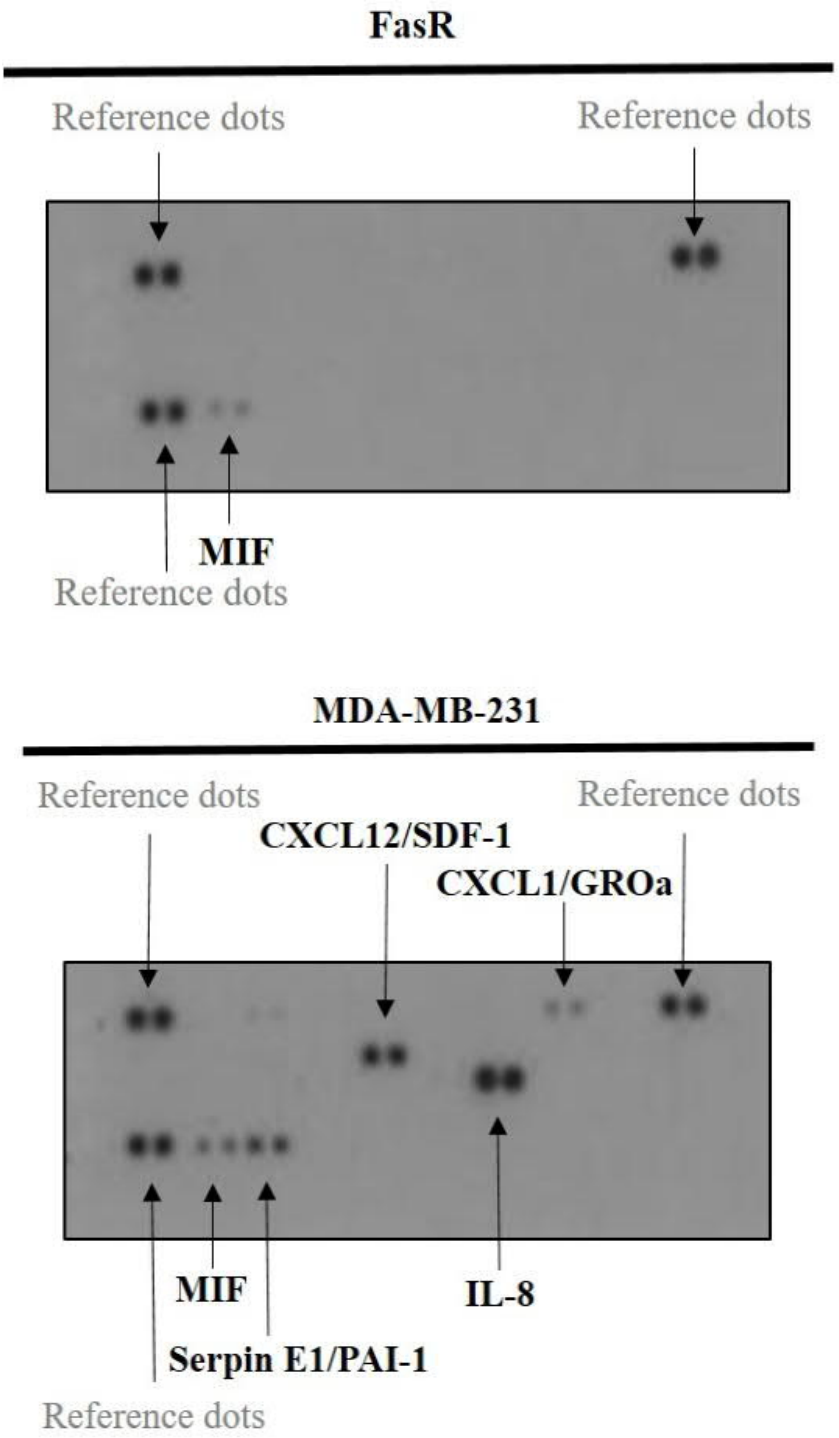


Figure 4.8: Expression of human cytokines in breast cancer cell models. An ELISA kit containing membranes with 46 different cytokines was employed to investigate the cytokines expressed by breast cancer cells. Analysis revealed that MCF-7, TamR and FasR cells secreted only the MIF cytokine, while MDA-MB-231 cells were found to secrete in addition to MIF, SeprinE1-PAI1, CXCL1/GROa, IL-8, SDF1.

Table 4.2: Cytokines secreted by breast cancer cells and their corresponding biological function.

	MCF-7	TamR	FasR	MDA-MB-231	Biological function
Cytokine expression	CXCL12/ SDF-1	CXCL12/ SDF-1	CXCL12/ SDF-1	CXCL12/SDF-1	increases recruitment of osteoclast precursors by upregulation of MMP-9 activity (Liao et al. 2005)
	MIF	MIF	MIF	MIF	possibly important for osteoclast differentiation and activation (Madeira et al. 2012)
				CCL2/MCP1	Stimulates osteoclastogenesis (Miyamoto et al. 2009)
				CXCL1/GROa	possibly affect osteoclast formation via its capacity to attract osteoclast precursors (Onan et al. 2009)
				GM-CSF	regulates fusion of mononuclear osteoclasts into bone-resorbing osteoclasts (Lee et al. 2009)
				Serpin E1/PAI-1	promotes tumour progression (Klein et al. 2012)
				IL-6	supports osteoclast differentiation (Roux & Orcel 2000)
				IL-8	promotes osteoclasts differentiation in a RANKL independent manner (Sabokbar et al. 2016)

4.3 Discussion

Bone represents a preferred site for breast cancer metastasis (Petrut et al. 2008) with approximately 85% of advanced breast cancer patients developing bone metastases resulting in reduced quality of life due to severe pain and pathological fractures (Gregory R Mundy 2002). A deeper understanding of the mechanisms underlying bone function and the pathophysiology of bone metastasis, has led to the development of bone-targeted therapies which aim to reduce the symptoms of bone disease and/or to prevent or delay its onset. However, it is currently poorly understood whether the development of endocrine resistance further predisposes the development of secondary tumour foci in the bone. Acquisition of resistance to endocrine therapies is a major limiting factor to their clinical effectiveness, resulting in disease relapse and poor prognosis (C Kent Osborne & Schiff 2011). This may be due in part to the observations that acquired resistance to agents such as tamoxifen is accompanied by a gain in aggressive cellular features likely to promote disease spread and survival at metastatic sites (Hiscox et al. 2004). In the present chapter, we aimed to investigate whether acquisition of an endocrine resistant phenotype conferred an ability on breast cancer cells to influence monocyte differentiation into osteoclasts.

The ability of breast cancer cells to influence the bone microenvironment was determined using a PBMC assay as this represents a widely used model to such investigations (Arrigoni et al. 2016; Morgan et al. 2004). One caveat of this assay is the issue of donor variability and heterogeneity, which can affect response. However, our data suggested that, whilst there was a variability among the different donors, a consistent trend was observed. Our findings revealed that breast cancer cells can promote PBMCs differentiation into TRAP+ multinucleated cells characteristic of osteoclasts. Morphologically, the osteoclasts generated by breast cancer cell stimulation were identical with those stimulated by RANKL, but the number of osteoclasts varied across

the different cell lines, with the major osteoclastogenic effect seen by MDA-MB-231 and TamR cells. Several reports have highlighted the aggressive phenotype that is exerted by triple negative breast cancer cells (William D. Foulkes et al. 2010; Dent et al. 2007b; Rakha & Chan 2011; Dent et al. 2009). In addition, previous studies by our group have demonstrated that breast cancer cells with acquired tamoxifen resistance exhibit a highly motile and invasive behaviour (Hiscox, Jiang, et al. 2006; Hiscox et al. 2004). Thus, our findings are compatible with the highly aggressive phenotype that is displayed by these cell models.

We also investigated the bone-resorptive ability of the osteoclasts, since this is a characteristic of such cells likely to be important in the in vivo context of osteolytic bone metastases. Intriguingly this is a little-reported aspect of osteoclast cells in the literature with the majority of studies focusing on osteoclast formation rather than bone lytic ability. Recent evidence suggests that CM from cancer cells is able to differentiate PBMCs into activated osteoclasts and subsequently result in bone erosion (Mizutani et al. 2009), however this could not be verified in our system. Our data indicated that osteoclasts induced by breast cancer cell conditioned media lacked an ability to degrade ivory disks, unlike RANKL-differentiated osteoclasts, which appeared to stimulate the production of resorptive pits. Although one might infer that our TRAP+, multinucleated cells may not be osteoclasts, other have reported a lack of bone-degrading activity in differentiated cells (Mabilleau & Sabokbar 2009) suggesting that differentiated osteoclasts are not necessarily activated and thus do not always possess bone collapsing properties such as formation of ruffled borders and Cathepsin K secretion (Bar-Shavit 2007b).

Src is a non-receptor tyrosine kinase, involved in many cellular processes such as growth, proliferation, migration and metastasis (Roskoski 2004), Src activation has been tightly correlated with breast cancer cell invasion and migration as well as the development of breast cancer bone metastasis (Sgroi 2009; Zhang et al. 2009; Pohorelic

et al. 2012). In addition Src is implicated in bone resorption due to its involvement in the formation of ruffled membranes that enable osteoclast attachment to the bony surface during resorption (Boyce et al. 1992) and osteoclast differentiation, activation and survival via PI3K pathway activation (Miyazaki et al. 2004; Kim & Kim 2016). Given the fundamental role of Src protein for breast and bone and the fact that it is highly expressed by the cell lines producing the highest number of osteoclasts (MDA-MB-231 and TamR), it was hypothesised that Src might be one of the key molecules that drove PBMCs fusion and subsequently osteoclast formation following CM treatment. Indeed, siRNA-mediated Src knockdown in TamR and MDA-MB-231 cells significantly reduced number of differentiated osteoclasts obtained with the conditioned media compared to breast cancer cells treated with non-targeting siRNA. These data implied that breast cancer cells may exert their osteoclastogenic effects in a Src-dependent manner. Although the fundamental role of Src in bone metabolism has been widely reported (Sgroi 2009; Aleshin & Finn 2010; Miyazaki et al. 2004), this is the first time that a largely Src-dependent correlation between the development of acquired resistance and osteoclastogenesis has been reported.

Breast cancer cells secrete various factors that may be involved in osteoclast formation the most well implicated of which is RANKL. RANKL is also known to be secreted by osteoblasts where it targets the RANK receptor located on the osteoclasts surface to initiate downstream signalling leading to osteoclast formation and ultimately the acquisition of bone-resorptive features (Liu & Zhang 2015). Our microarray data revealed that the RANKL gene is not expressed by our breast cancer cells, an observation further supported by others (Ney et al. 2012), and our own ELISA and qPCR data. We therefore concluded that it was very unlikely that our breast cancer cells triggered osteoclast formation in a RANKL-dependent manner.

Although the main osteoclastogenic pathway involves RANKL, there are also RANKL independent pathways that lead to osteoclast differentiation (Lau et al. 2007). TNFa has been reported to induce osteoclast formation in the presence of interleukin-1a in a RANKL-independent mechanism (Kim et al. 2005; Kobayashi et al. 2000). Moreover, there are studies supporting that interleukins enhance osteoclastogenesis, and, in some cases, support osteoclast formation in the absence of RANKL. IL-8 has been found to be one such molecule and it is known that it is highly expressed by MDA-MB-231 cells (M. Bendre et al. 2003). Additionally, in some cases, breast cancer cells secrete MCSF that is essential for osteoclast survival and differentiation or inhibit the osteoclast apoptosis (Mancino et al. 2001; Gallet et al. 2004). *Gallet et al.* managed to generate osteoclasts from a bone-marrow cell line that was supplied with RANKL, while CM from MDA-MB-231 cells was used as a source of MCSF.

In light of this evidence, we employed a human cytokine array panel of 36 different cytokines known to play a role in the bone microenvironment, in order to investigate whether the breast cancer cells secrete factors able to support osteoclastogenesis in the absence of RANKL. All the cell lines secreted MIF, with the highest expression in TamR cells. MIF is an inflammatory cytokine fundamental for innate immunity that is released in response to hypoxia, bacterial infection and other cellular processes, including carcinogenesis (Gu et al. 2015; Morand et al. 2006). MIF has a prominent role in bone breakdown as it has been reported in several studies that is involved in rheumatoid arthritis enhancing the bone erosion and disease development (Gu et al. 2015; Movila et al. 2016; Madeira et al. 2012). One study supports a role for MIF in osteoclastogenesis (Jacquin et al. 2009). Interestingly, a number of reports support that Src is a downstream molecule of MIF signalling. Specifically, MIF has been found to activate Src in a dose-dependent manner and has been suggested as a potential target for diseases characterised by high levels of cell adhesion molecules (Amin et al. 2006), including tumour spread. In

addition, literature suggests that MIF triggers Src-mediated MAPK and AKT signalling cascades and that this activation can be abolished by treatment with a kinase inhibitor (Lue et al. 2006; Mitchell et al. 1999; Lue et al. 2007). Taken together, this evidence implies that MIF might predispose the development of breast cancer bone metastasis in a Src dependent manner and that Src inhibition may thus attenuate this effect in cancers that overexpress MIF, such as tamoxifen resistant breast cancer.

Overall, this chapter illustrates the ability of breast cancer cells to influence osteoclastogenesis in a RANKL-independent manner. The resultant cells were TRAP+ and multinucleated but lacked any bone-resorptive activity *in vitro*. Furthermore, this process appeared to be dependent upon Src activity in breast cancer cells, specifically in the tamoxifen resistant and triple negative breast cancer models. Although no direct mechanism was elucidated, preliminary screening suggested that MIF may well represent a bone-modulatory protein that warrants further investigation in breast cancer cells.

5. Anti-tumour effects of zoledronic acid in endocrine sensitive and resistant breast cancer cell models

5.1 Introduction

Bone metastasis is one of the most frequent complications in patients with advanced breast cancer. It is estimated that nearly 85% of breast cancer patients with advanced disease will develop bone metastasis at some stage (Allan Lipton et al. 2009). Colonisation of bone by breast cancer cells leading to the development of secondary tumour foci is sustained by reciprocal interactions between cancer cells and bone cells (Weilbaecher et al. 2011). The result for the patient is a poor quality of life with severe pain, spinal cord compression (where the spine is involved) and pathologic fractures (Chen et al. 2010c). Although the duration of survival varies according to the primary tumour, bone metastases are generally incurable.

Bone is a dynamic tissue that is constantly undergoing remodelling based on the fine balance between osteoclasts, the cells that resorb bone substrate, and osteoblasts, the cells that generate new bone (Rucci 2008). The presence of cancer cells in the bone microenvironment can disturb this balance resulting in pathological conditions, the so-called bone metastasis (Chen et al. 2010c). Bone metastasis can be either osteolytic, when bone breakdown occurs as a result of osteoclast hyper-activation, or osteoblastic, when excess bone formation takes place through osteoblast hyper-activation (Papachristou et al. 2012).

General treatment options for patients with cancer-related bone lesions include bisphosphonate (BP) administration, such as Zoledronic acid (ZOL). Being a nitrogen containing BP, ZOL interferes with the mevalonate pathway resulting in reduced viability of cells that ingest it (Graham & Russell 2007; Roelofs et al. 2008). Additionally, N-BPs are able to impair the formation of the ruffled border in OCs, thus prevent their attachment to the bone surface and inhibit bone degradation (Colucci et al. 1998; Fleisch 1998).

Intriguingly, recent evidence also suggests that, apart from its bone cell-regulatory action, ZOL also exerts anti-tumour properties in various tumour types such as breast,

lung and prostate (Zekria et al. 2014) although the mechanism through which ZOL does this is not yet fully understood. Some studies claim that ZOL that directly interacts with cancer cells and drives them to apoptosis (Green & Guenther 2011), while others suggest that its role is indirect by either inhibiting angiogenesis or activating anti-cancer immune responses (Benzaïd et al. 2011).

In light of this, the aims of this chapter were to investigate the effect of ZOL in the breast cancer context, focusing on any differential effects between endocrine-sensitive and endocrine-resistant models. In addition, we wished to determine the mechanism of action underlying any tumour-suppressive activities produced by ZOL. Specifically, to achieve our aims the following objectives were set:

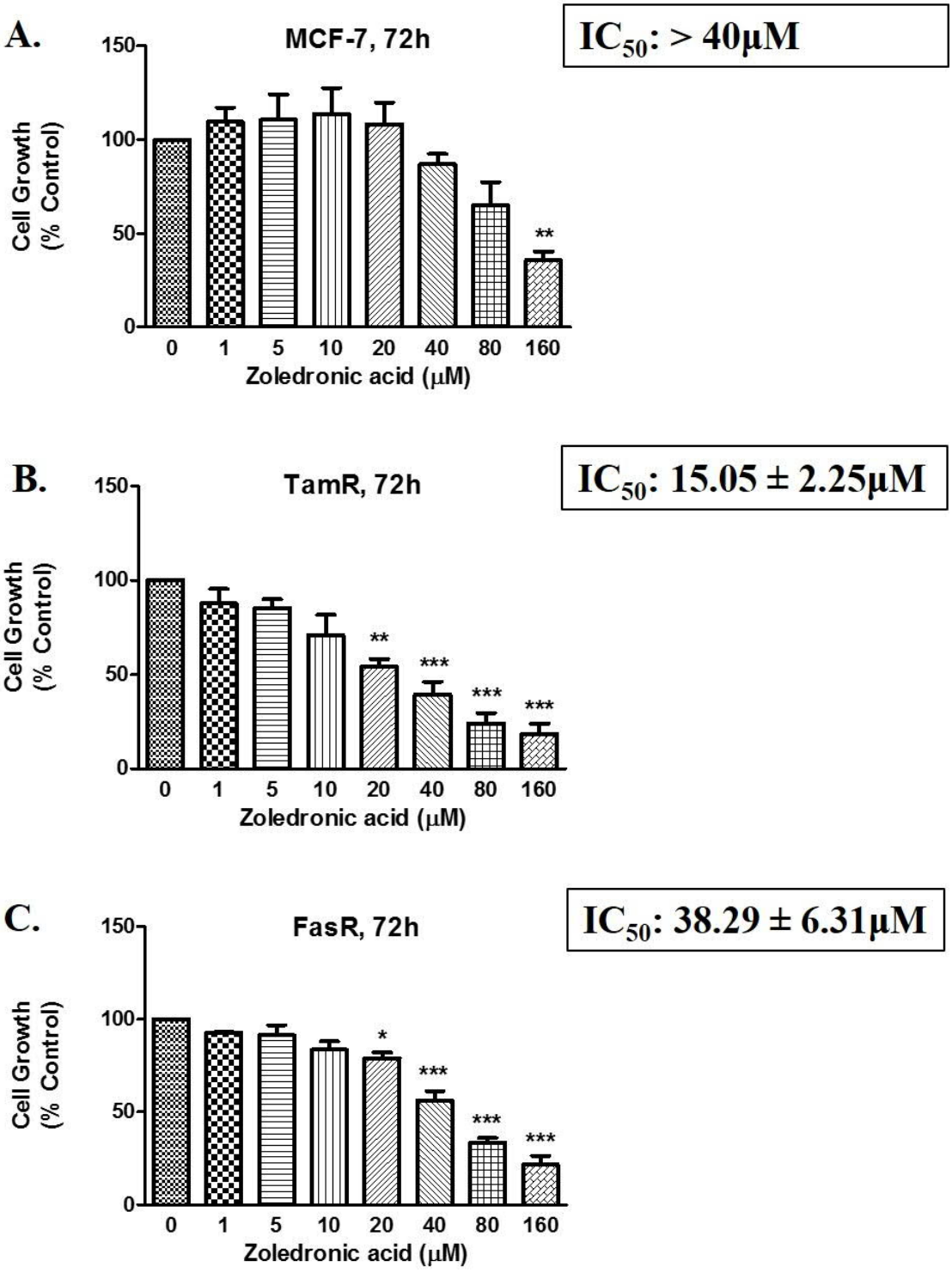
1. Investigate ZOL effects on breast cancer cell proliferation and Ki67 expression
2. Explore ZOL effects on breast cancer cell cycle progression
3. Investigate which pathways and protein molecules are affected by ZOL treatment in endocrine sensitive and resistant breast cancer cell models

5.2 Results

5.2.1 Zoledronic acid suppresses breast cancer cell proliferation

Initially we explored the effect of Zoledronic acid on breast cancer cell proliferation over a range of doses (0-160μM) at 72h. This dose range reflected what widely reported in the literature from other groups (Kars et al. 2007; Insalaco et al. 2012; Ibrahim et al. 2012). MCF-7 cell proliferation was slightly induced when treated with lower doses of ZOL, while a weak suppressive effect at higher doses was observed (**Figure 5.1 A**). FasR cell proliferation was modestly suppressed at 20-160μM and IC_{50} value was at 38.29 ± 6.31μM (**Figure 5.1 C**). In contrast, TamR cells exhibited enhanced sensitivity to ZOL compared to the other two cell models, even at low concentrations, in a dose-dependent manner, which was reflected by the IC_{50} value at 15.05 ± 2.25μM (**Figure 5.1 B).** Superimposing the ZOL dose response curves illustrate the superior inhibitory effect of ZOL on TamR cells compared to the other two cell lines (**Figure 5.1 D**).

This data revealed that TamR cells were much more sensitive to ZOL, particularly at lower concentrations, versus MCF-7 and FasR cells. To study the TamR response further, we performed a time course using 20μM ZOL. ZOL-mediated growth suppression occurred from 48h with maximal effects seen at 96h when almost all cells were dead (**Figure 5.2**).


A.
MCF-7, 72h
IC50: > 40µM
Cell Growth (% Control)
150
100
50
0
**
0 1 5 10 20 40 80 160
Zoledronic acid (µM)
B.
TamR, 72h
IC50: 15.05 ± 2.25µM
Cell Growth (% Control)
150
100
50
0
**
***
***
***
0 1 5 10 20 40 80 160
Zoledronic acid (µM)
C.
FasR, 72h
IC50: 38.29 ± 6.31µM
Cell Growth (% Control)
150
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0 1 5 10 20 40 80 160
Zoledronic acid (µM)

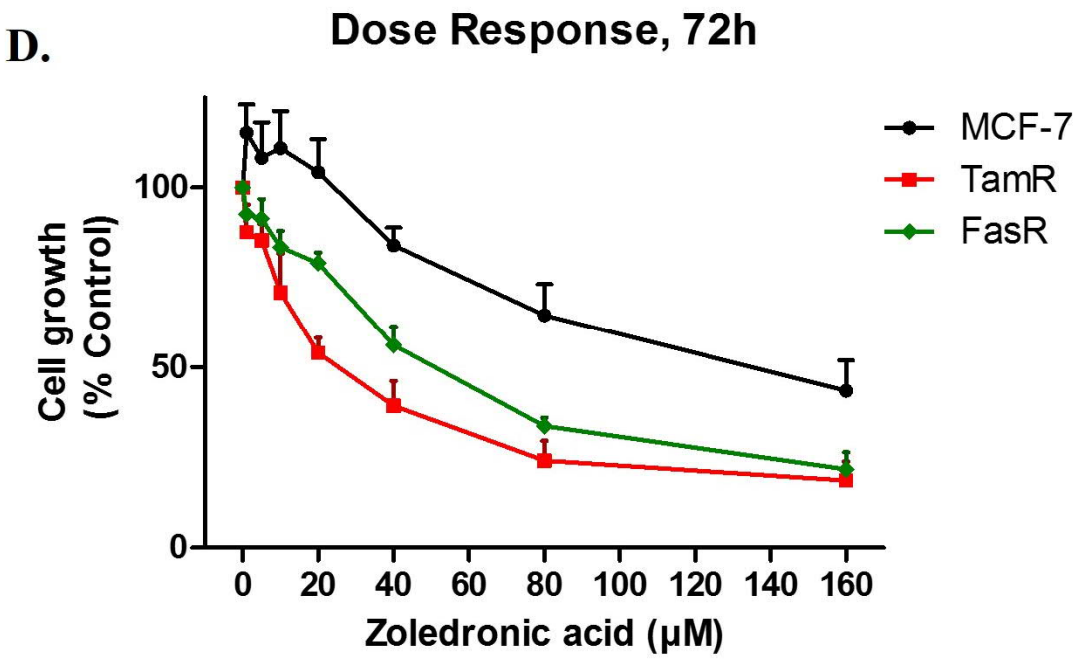


Figure 5.1: Effect of Zoledronic acid on breast cancer cell proliferation. (A) MCF-7 cell growth was not significantly suppressed in response to ZOL (IC_{50} > 40µM). **(B)** TamR cell growth was greatly inhibited following ZOL treatment (IC_{50}: 15.05 ± 2.25µM). **(C)** FasR cell growth was modestly suppressed by ZOL treatment (IC_{50}: 38.29 ± 6.31µM). **(D)** Combination of the dose response curves for all the cell lines clearly illustrates the superior inhibitory effect of Zoledronic acid in Tam R cells. The cell growth in response to Zoledronic acid (0–160 µM) over a period of 72h was assessed using MTT assay. Data are mean cell proliferation values ± SEM (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

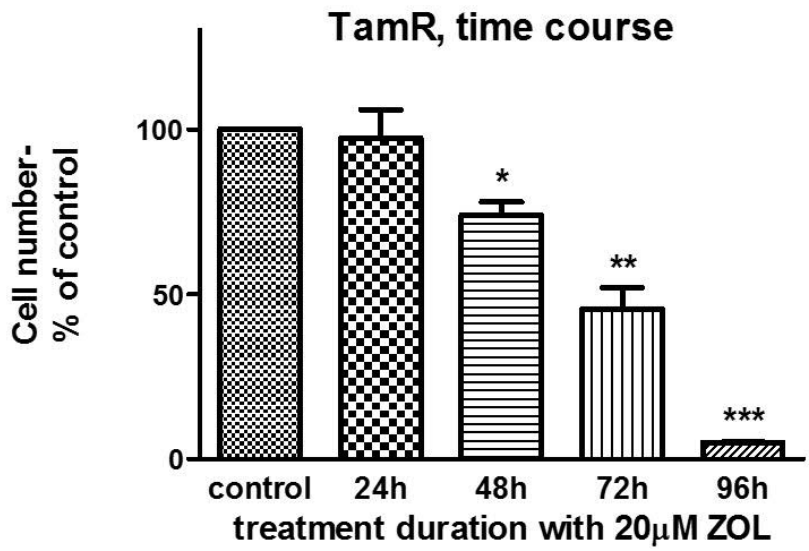


Figure 5.2: Zoledronic acid suppresses TamR cell proliferation in a time-dependent manner. Time-course treatment of TamR cells with 20μM Zoledronic acid (0-96h). Zoledronic acid suppressed cell proliferation in a time-dependent manner with the first significant effect shown at 48h. Data are mean cell proliferation values ±SEM (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

5.2.2 Zoledronic acid significantly suppresses Ki67 expression in endocrine-sensitive and resistant models

The data so far suggested that ZOL exerts a potent anti-proliferative effect on TamR cells. To further investigate ZOL-mediated suppression of proliferation we performed IHC staining of the proliferation marker Ki67. These data revealed that ZOL suppressed Ki67 in both MCF7 and TamR cells with the effects being greatest in the latter. No significant changes were seen with FasR cells (**Figure 5.3**).

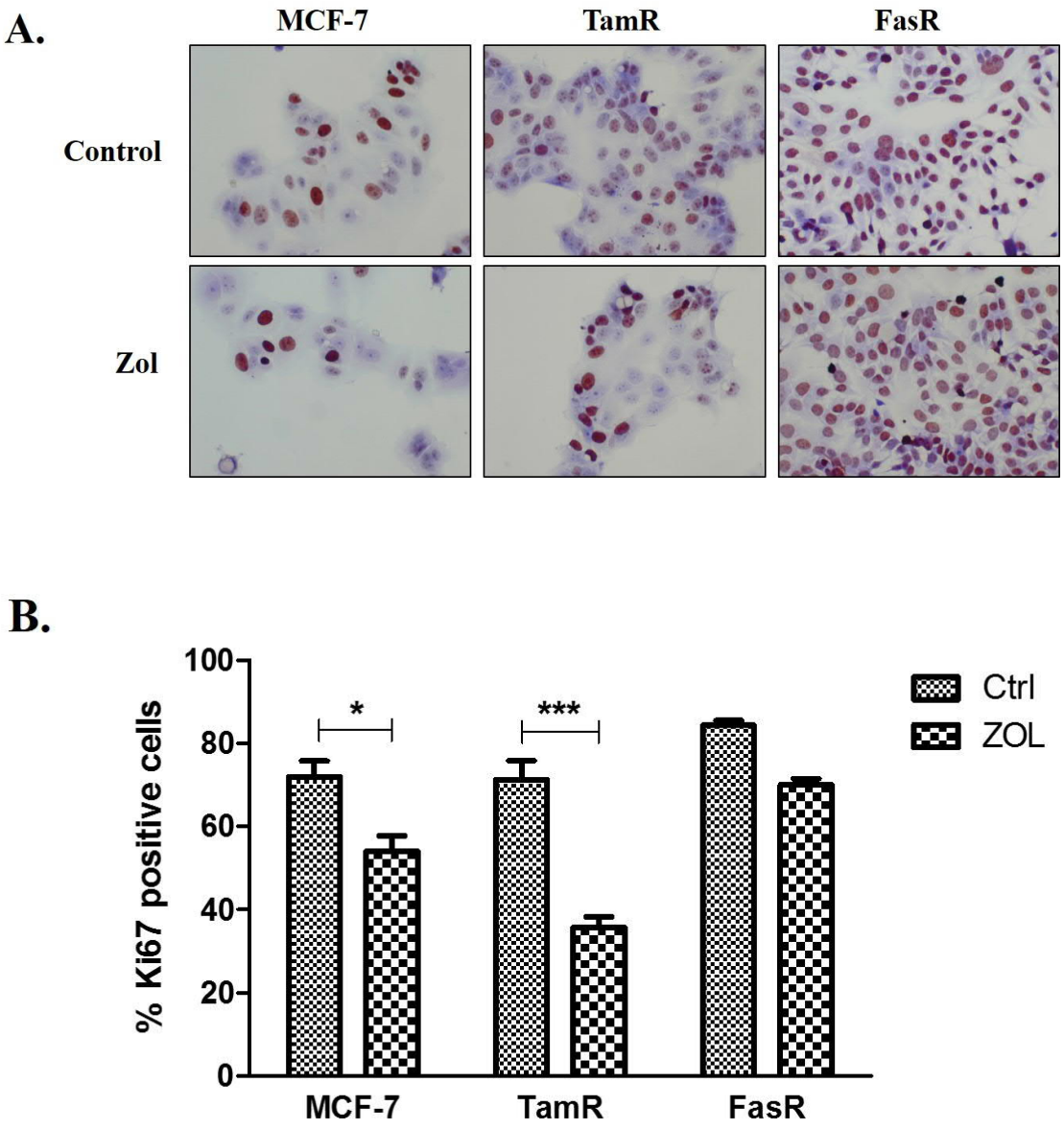
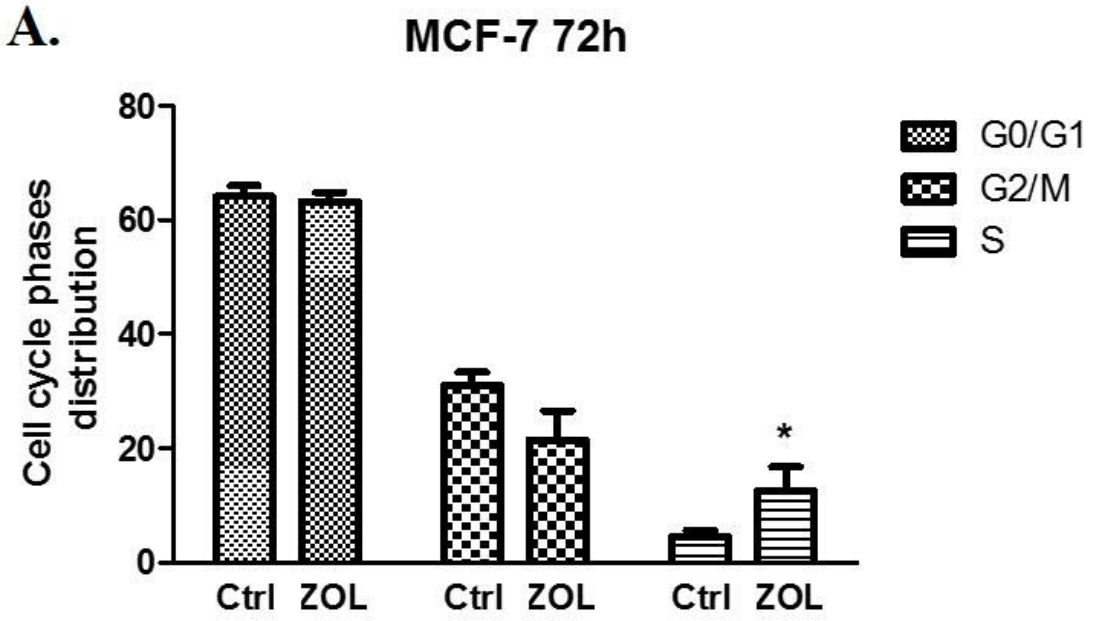
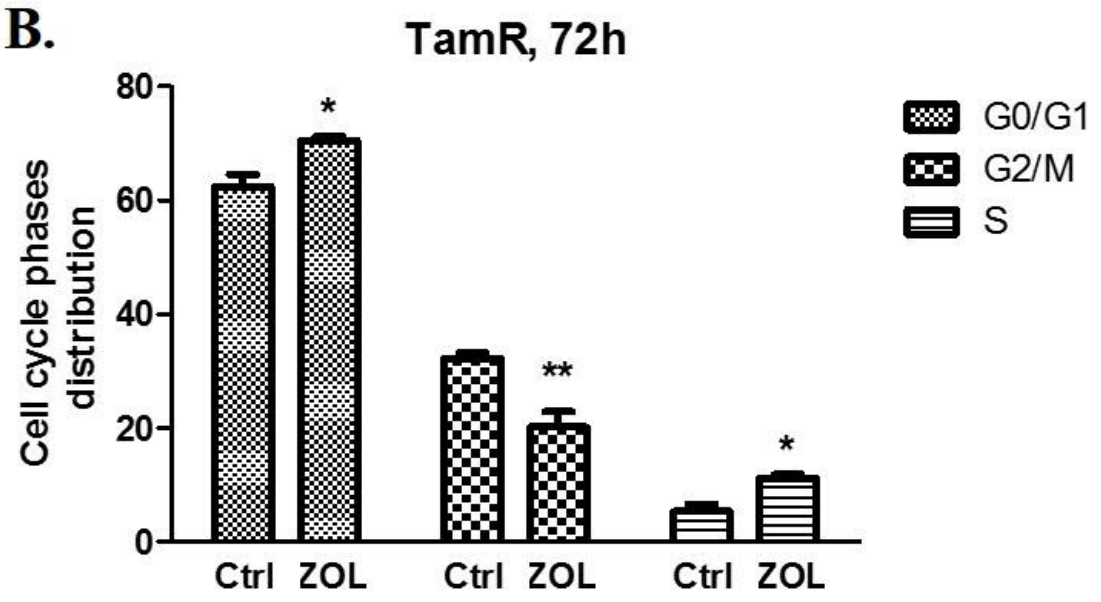


Figure 5.3: Zoledronic acid significantly suppresses proliferation marker Ki67 in MCF-7 and TamR cells, but not in FasR cells. Ki67 detection with immunohistochemistry demonstrated that treatment of breast cancer cells with 20μM ZOL for 72h was able to suppress Ki67 expression, more notably in the case of TamR cells. Data are mean values ±SEM (n=3). * $p<0.05$, *** $p<0.001$

5.2.3 Zoledronic acid affects the cell cycle in Tamoxifen resistant cells

Having found that ZOL suppressed cell growth in breast cancer cell lines, we then wished to explore whether this might be due to ZOL-mediated changes in the cell cycle. Control (0 uM ZOL) and cells treated with 20μM ZOL were analysed with propidium iodide FACS analysis. Whilst MCF7 cells displayed an increase in S-phase cells following ZOL treatment, TamR cells displayed an increase in G1 and S-phase cells and a decrease in M-phase cells following treatment with ZOL **(Figure 5.4 A & B**). In contrast, treatment with ZOL did not appear to affect cell cycle phase distribution in FasR cells (**Figure 5.4 C**).


A.
MCF-7 72h
Cell cycle phases distribution
80
60
40
20
0
Ctrl
ZOL
Ctrl
ZOL
Ctrl
ZOL
*
G0/G1
G2/M
S


B.
TamR, 72h
Cell cycle phases distribution
80
60
40
20
0
Ctrl
ZOL
Ctrl
ZOL
Ctrl
ZOL
*
**
*
G0/G1
G2/M
S

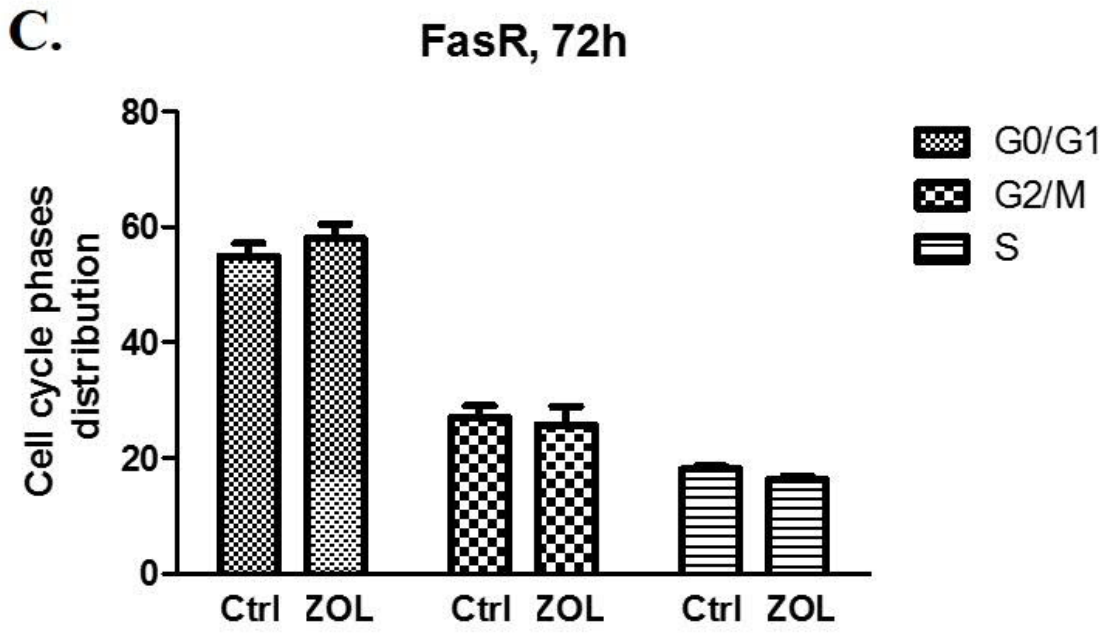


Figure 5.4: Cell cycle analysis using FACS in breast cancer cell lines. Cells treated with 20μM ZOL for 72h and compared with untreated cells to determine any differences in cell cycle phase distribution. Treatment with ZOL led to a slight increase in Go/G1 and S phases with concurrent decrease of G2/M phase in MCF-7 and TamR cells. FasR cell cycle was not affected by ZOL treatment. Data are mean cell cycle values ±SEM (n=3). * $p<0.05$, ** $p<0.01$

5.2.4 Exploration of the mechanism of action of Zoledronic acid

Our data thus far reveals that ZOL exerts an anti-proliferative effect on endocrine sensitive and resistant cells with the TamR cells being most sensitive to ZOL. As such, we next sought to investigate the mechanism underlying the enhanced sensitivity of these cells. Recent studies have reported that ZOL may bind the EGFR and erbB2 receptors and subsequently inhibit downstream signalling pathways resulting in an altered cellular phenotype (Agnes Stachnik et al. 2014; Yuen et al. 2014). Since TamR cells are known to overexpress EGFR and HER2 compared to their endocrine-sensitive MCF-7 counterparts and also the FasR resistant model **(Figure 5.5)** (Knowlden et al. 2003), we hypothesised that this might represent a mechanism of action for ZOL. If this was the case, we further hypothesised that the anti-proliferative effects of ZOL might be attenuated in the presence of an EGFR activator such as TGFa.

To investigate this hypothesis, we first selected an appropriate stimulatory concentration for TGFa by performing an MTT assay using a range of TGFa doses (**Figure 5.6**). These data pointed to 10nM as being a dose that produced a small but significant growth-promoting effect over this time point (72h).

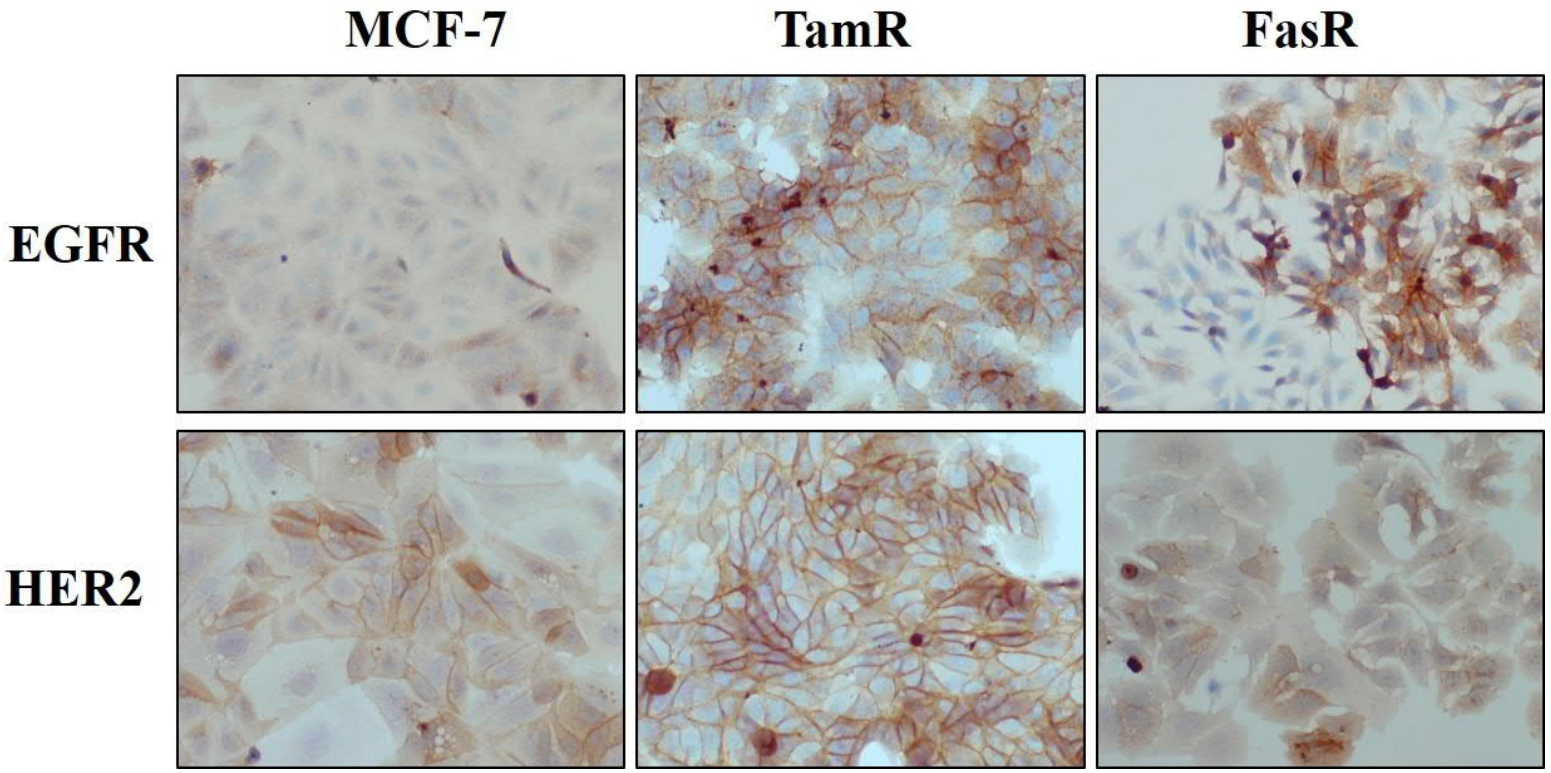


Figure 5.5: Immunohistochemical staining for EGFR and HER2 in MCF-7, TamR and FasR breast cancer cell models. Both EGFR and HER2 expression is elevated in TamR cells compared to their endocrine sensitive counterparts (MCF-7) and Faslodex resistant models (FasR).

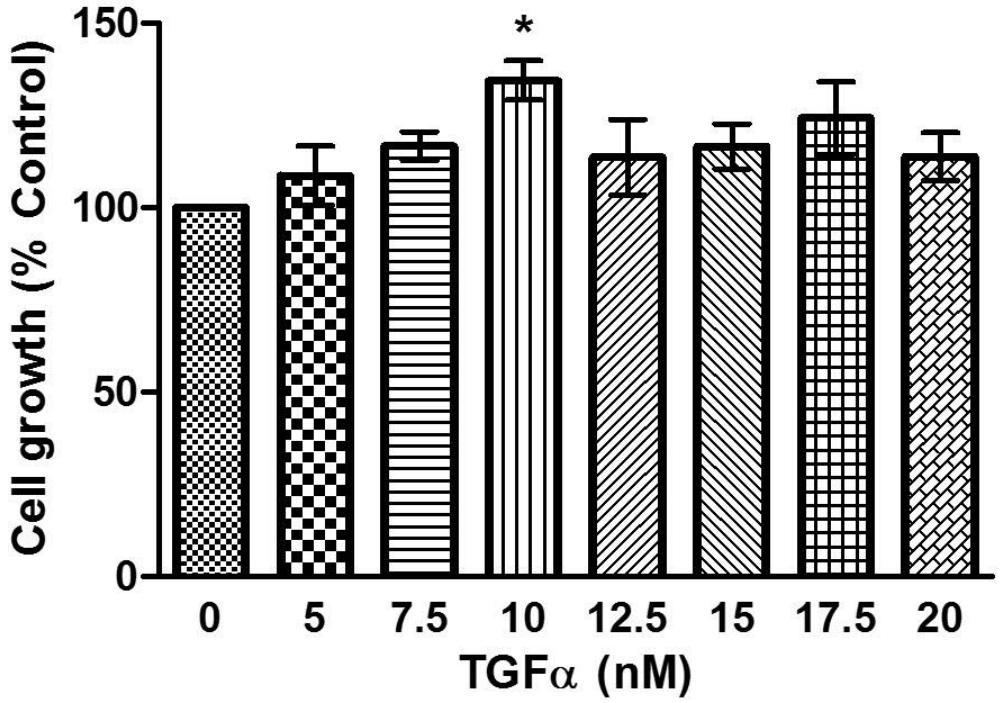


Figure 5.6: Stimulatory effect of TGFα on the growth of TamR cells. The cell growth in response to TGFα (0–20 nM) over a period of 72h was assessed using MTT assay. Data are mean cell proliferation values ±SEM (n=3). * $p<0.05$

We next investigated whether ZOL + TGFa was as effective as ZOL alone in suppressing the proliferation of TamR cells. These data revealed that the anti-proliferative effects of ZOL were reduced in the presence of TGFa at all concentrations tested (**Figure 5.7**).

To further investigate the importance of the EGFR in ZOL-action, we investigated the hypothesis that dual treatment with ZOL and a Tyrosine Kinase Inhibitor (TKI) of the EGFR (gefitinib) would result in an additive effect in terms of proliferation suppression. Analysis of cell growth data demonstrated that combination treatment led to a greater cell growth inhibition compared to either agent alone (**Figure 5.8**).

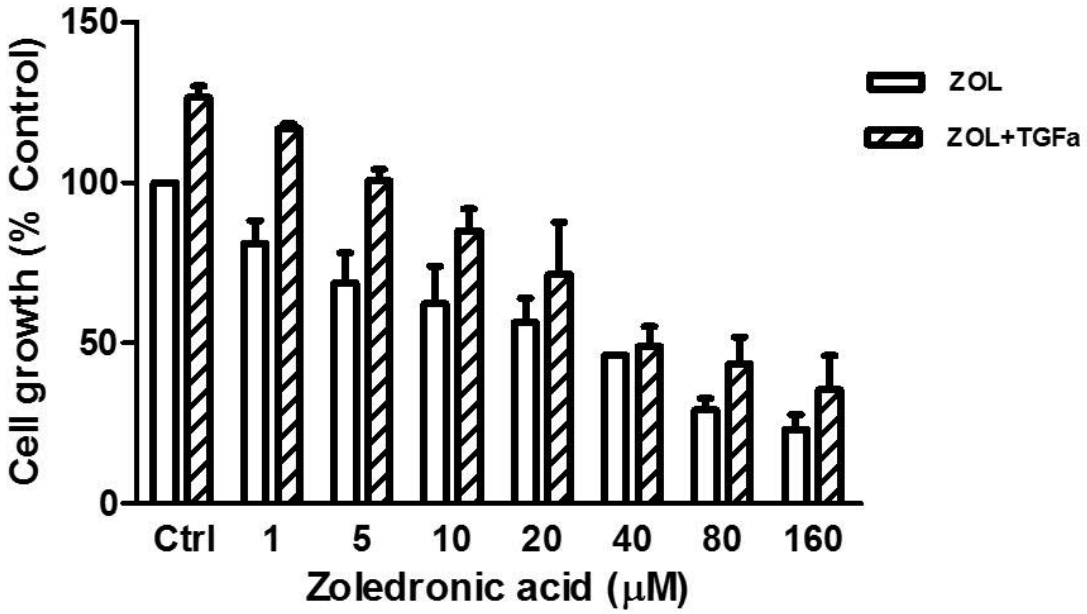


Figure 5.7: ZOL alone is more effective in suppressing TamR cell proliferation than ZOL + TGFα.

Comparison of the inhibitory effect of Zoledronic acid on TamR cells and TamR cells that have been pre-treated with 10nM TGFα for 2h. The cell growth in response to Zoledronic acid (0–160 μM) over a period of 72h was assessed using MTT assay. Data are mean cell proliferation values ±SEM (n=3).

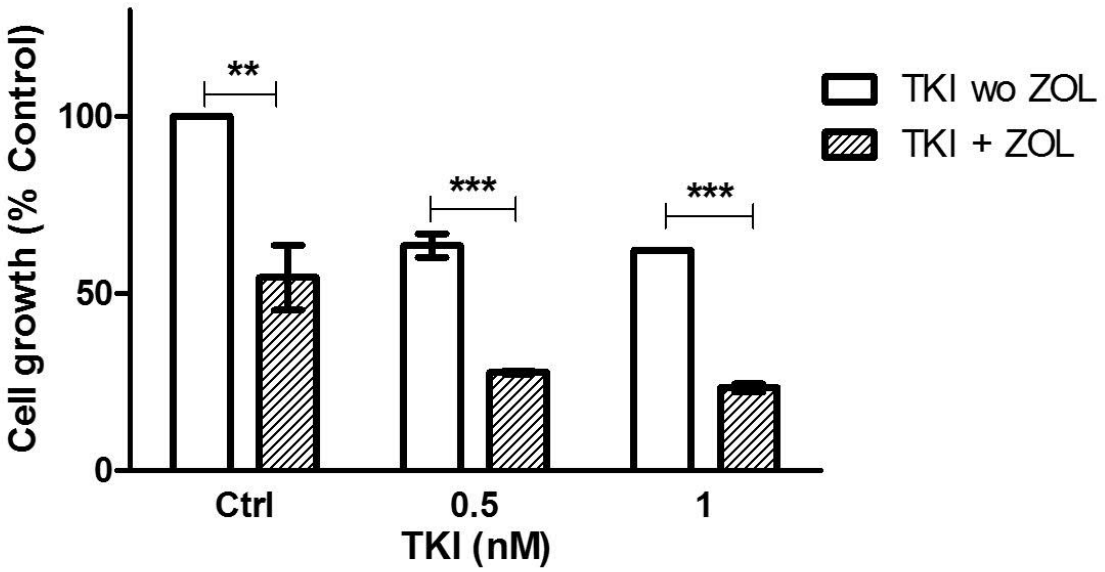


Figure 5.8: Combination of Zoledronic acid and TKI results in a further improved suppression of TamR cells. Cells were pre- treated with 20μM ZOL for 2h and then added TKI (0–1nM) and compared with TKI alone. Pre-treatment of TamR cells with ZOL resulted in enhanced sensitivity of cells in TKI treatment compared to TKI alone. The cell growth in response to treatment over a period of 72h was assessed using MTT assay. Data are mean cell proliferation values ±SEM (n=3). ** $p<0.01$, *** $p<0.001$

5.2.5 Zoledronic acid suppressed EGFR in endocrine sensitive and resistant cells

Results so far indicated that ZOL possibly exerts an inhibitory effect on RTKs. To confirm that we performed Western blot analysis for EGFR and HER2 in TamR samples treated with various concentrations of ZOL (0-40μM). Results revealed that ZOL was able to suppress activated EGFR levels in TamR cells following 72h treatment with the highest dose of ZOL (40μM). In contrast, HER2 levels were not found to be affected by any of these doses of ZOL treatment. (**Figure 5.9**).

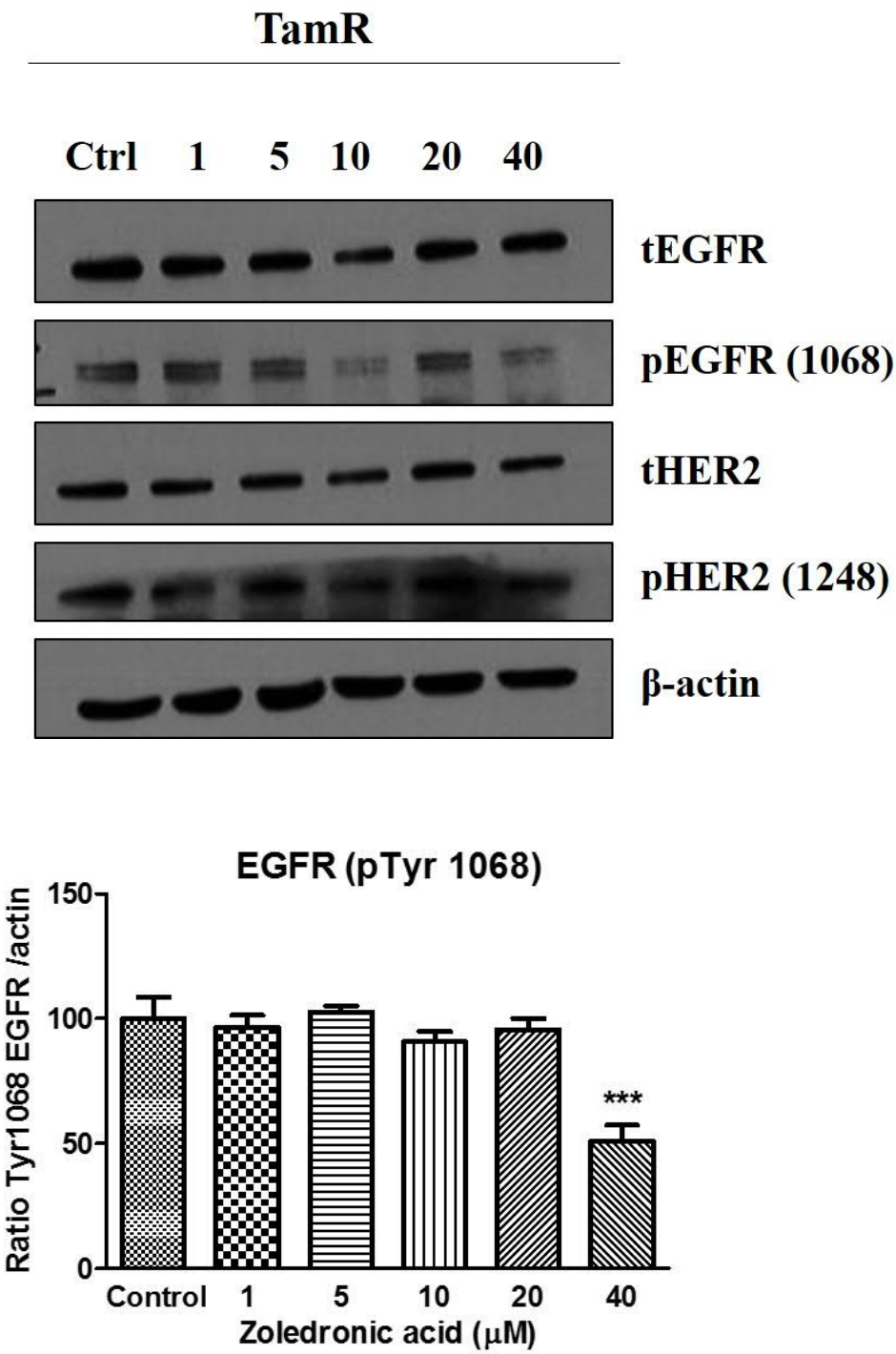
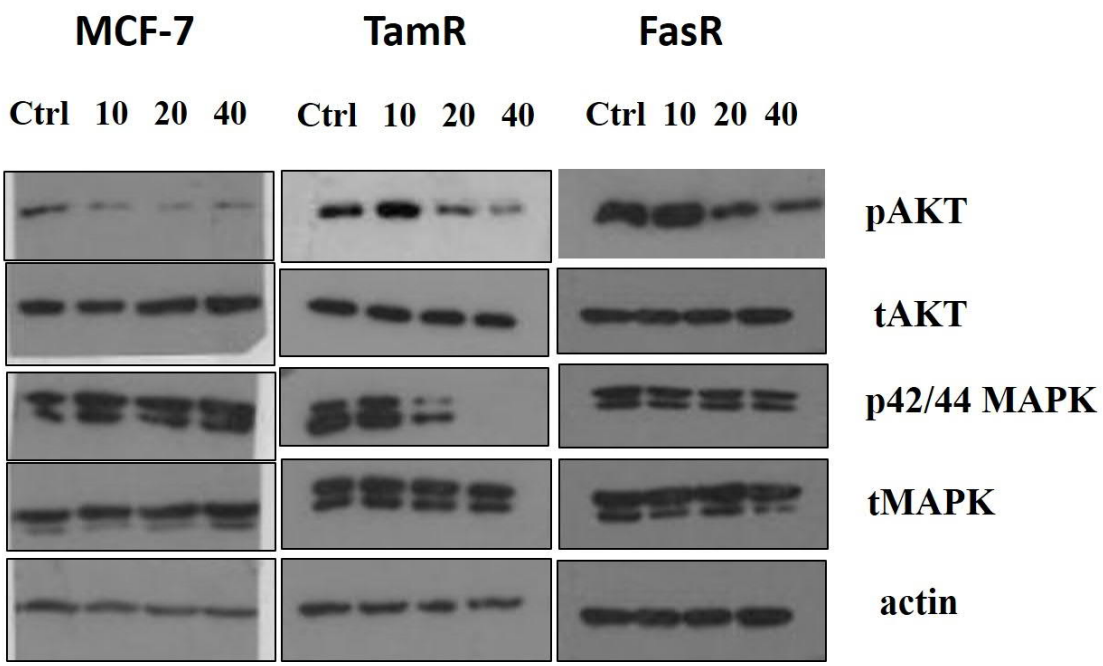
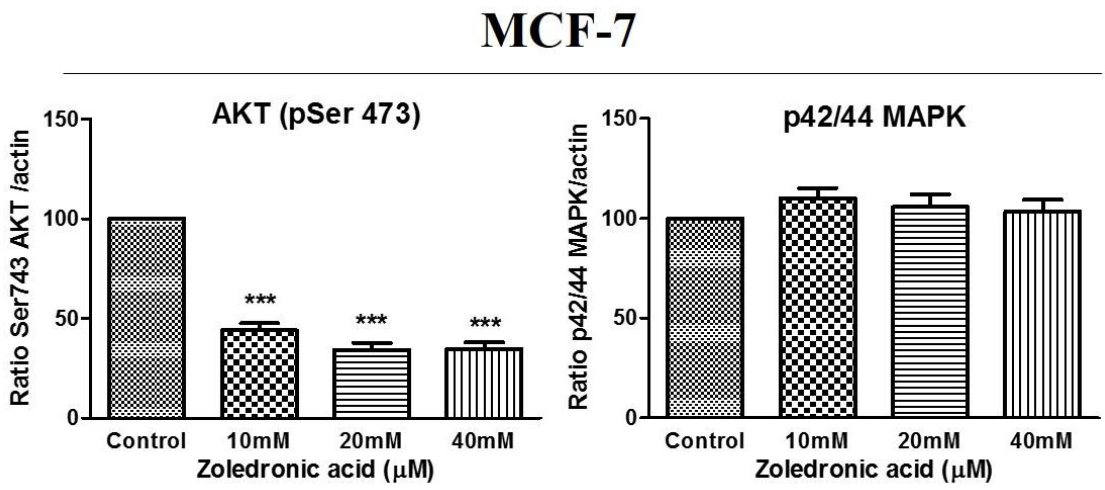


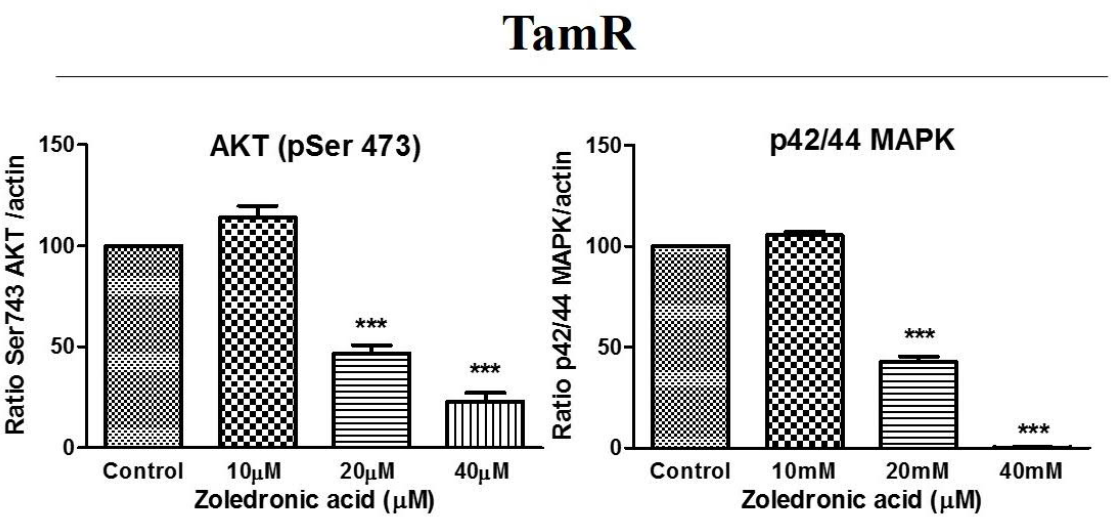
Figure 5.9: Zoledronic acid suppresses EGFR but not HER2 levels in TamR cells. Treatment of TamR cells with a range of ZOL concentrations (0-40μM) for 72h revealed that ZOL was able to suppress the activated levels of EGFR, whereas HER2 level were unaffected. Densitometry analysis for EGFR levels indicated that the highest dose of ZOL tried (40μM) induced a statistically significant suppression effect. ***$p<0.001$

5.2.6 Zoledronic acid inhibits AKT in endocrine sensitive and resistant cells

To further investigate the mechanism of action of ZOL in TamR cells, we next explored whether key signalling molecules involved in pathways known to regulate proliferation were affected by ZOL treatment. Western blot analysis revealed that ZOL treatment resulted in a dose dependent suppression of AKT activity in both endocrine sensitive and resistant models. In TamR cells, MAPK was reduced in a similar manner. ZOL did not affect the total levels of these proteins (**Figure 5.10**).


MCF-7
TamR
FasR
Ctrl 10 20 40
Ctrl 10 20 40
Ctrl 10 20 40
pAKT
tAKT
p42/44 MAPK
tMAPK
actin


MCF-7
AKT (pSer 473)
Ratio Ser743 AKT /actin
150
100
50
0
***
***
***
Control
10mM
20mM
40mM
Zoledronic acid (μM)
p42/44 MAPK
Ratio p42/44 MAPK/actin
150
100
50
0
Control
10mM
20mM
40mM
Zoledronic acid (μM)


TamR
AKT (pSer 473)
Ratio Ser743 AKT /actin
150
100
50
0
***
***
Control
10μM
20μM
40μM
Zoledronic acid (μM)
p42/44 MAPK
Ratio p42/44 MAPK/actin
150
100
50
0
***
***
Control
10mM
20mM
40mM
Zoledronic acid (μM)

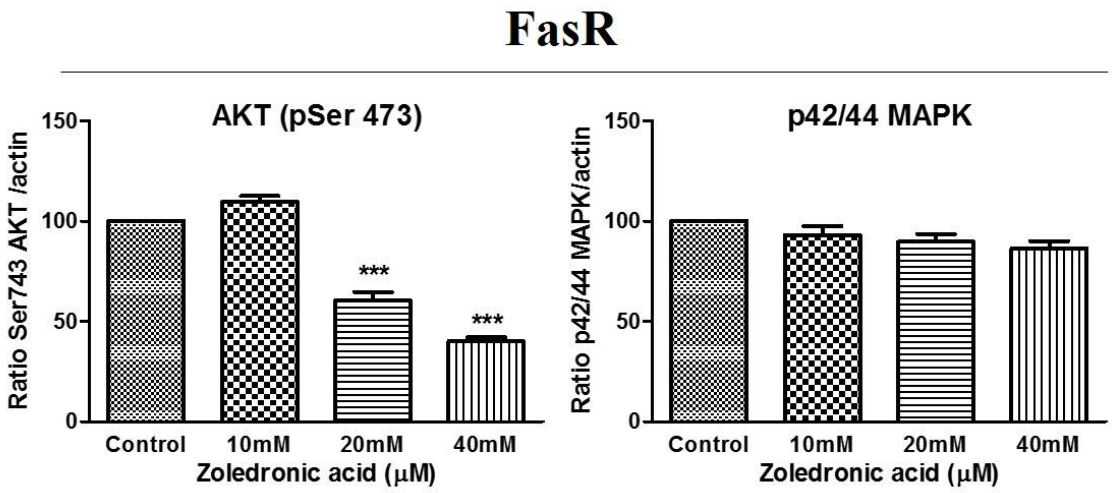
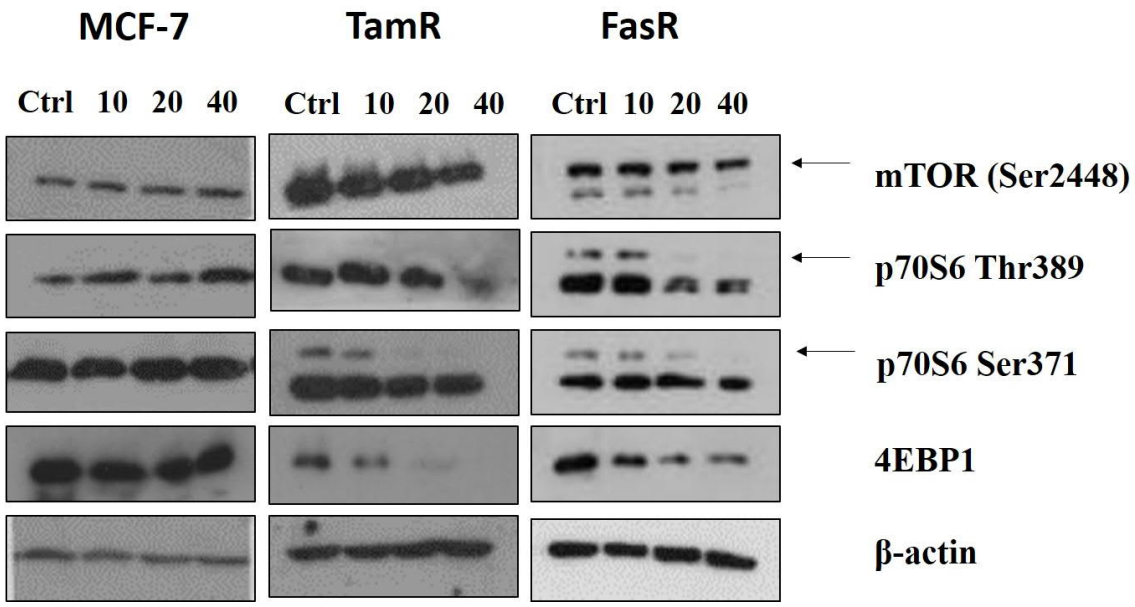


Figure 5.10: Zoledronic acid treatment results in a loss of AKT in MCF-7, TamR and FasR cells and loss of MAPK only in the case of TamR cells. Cells were grown to 70% confluence and treated with ZOL at the indicated concentrations for 72h. Subsequent cell lysates were processed for Western Blotting and immuno-probed for proteins as indicated (β-actin was used as loading control). Representative blots shown from three independent experiments. *** $p<0.001$

5.2.7 Zoledronic acid inhibits the mTOR pathway in endocrine resistant cells

Having found that a generic consequence of ZOL treatment was the reduction of AKT activity, we next wished to investigate the consequences of this for the mTOR pathway, an important element of AKT-mediated signalling that controls cellular proliferation. Western blot analysis revealed that mTOR pathway components were inhibited in both TamR and FasR cells in a dose-dependent manner; no effects were observed in MCF-7 cells (**Figure 5.11**). Densitometry analysis of these data showed that ZOL treatment resulted in a significant inhibition of mTOR components in TamR and FasR cells



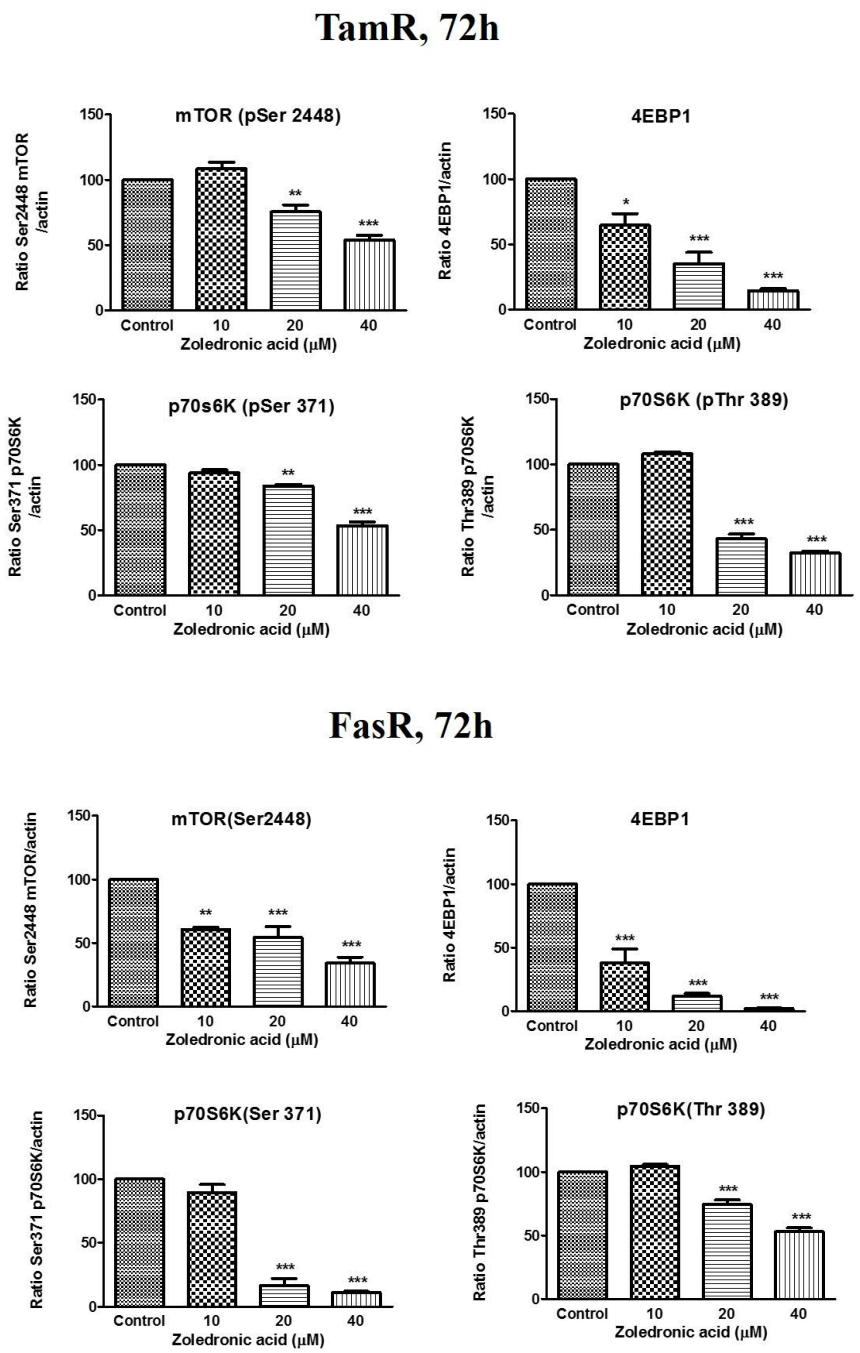


Figure 5.11: Zoledronic acid inhibits the mTOR pathway in endocrine resistant cells. Cells were grown to 70% confluence and treated with ZOL at the indicated concentrations for 72h. Subsequent cell lysates were processed for Western Blotting and immuno-probed for proteins as indicated (β-actin was used as loading control). Representative blots shown from three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

5.3 Discussion

Bisphosphonates (BP) are the mainstay of treatment for patients with osteoporosis and cancer-related bone disease. Two classes of BPs are used: non-nitrogen containing and nitrogen containing BPs. Non nitrogen-containing BPs are digested by osteoclasts and form a non-functional ATP analogue that competes with ATP, depriving the cell of the energy required for its metabolism resulting in initiation of apoptosis and cell death (Lehenkari et al. 2002). Nitrogen-containing BPs cause mevalonate pathway disruption by interfering with farnesyl pyrophosphate (FPPS) and geranylgeranyl pyrophosphate synthases (GGPPS). FPPS inhibition results in accumulation of isopentenyl pyrophosphate, which in turn is converted into a cytotoxic molecule called ApppI. In addition, mevalonate pathway blockage prevents small GTPases prenylation inhibiting their function in osteoclasts and thus drive the cell to apoptosis (Kavanagh et al. 2006; Mönkkönen et al. 2006).

Apart from their therapeutic effectiveness in protecting bone tissue, emerging clinical and preclinical studies indicate that N-BPs also possess anti-tumour properties that may be attributed to their interaction with cells other than osteoclasts including the tumour cells themselves (Clézardin 2011). Despite potential anti-tumour properties of ZOL in malignancies such as breast, prostate and lung being reported by numerous studies (A. Stachnik et al. 2014; Marra et al. 2009; Mathew & Brufsky 2015) their direct anti-tumour mechanism of action is poorly understood. In the present chapter, we aimed to explore whether ZOL exerts anti-tumour effects on endocrine sensitive and resistant breast cancer models and, if so, to attempt to elucidate the mechanism of this.

Whilst ZOL inhibited proliferation of both endocrine sensitive and resistant models, the effects were significantly greater in the tamoxifen resistant cells. Our data suggests that one consequence of ZOL treatment may be deregulation of the cell cycle and growth arrest. Our observations support S-phase arrest in TamR and MCF7 cells consistent with

a previous study demonstrating that ZOL induced apoptosis and S-cycle arrest in human fibrosarcoma cells through suppression of Topoisomerase II (Topo II) (Okamoto et al. 2014). Topo II requires ATP for its catalytic activity and, as it has already been mentioned, ZOL forms a non-functional ATP analogue that competes with ATP. Another mechanism by which ZOL can promote apoptosis via S-cycle arrest is by modulating the cyclins or cell cycle regulatory molecules as S-phase arrest is associated with caspase-dependent and independent apoptotic pathways (Okamoto & Kawamura 2012).

Compatible with proliferation and cell cycle findings are the Ki67 expression data, which again showed a reduction in Ki67-positive cells following ZOL treatment in TamR cells, other cells did not show a significant difference between control or ZOL-treated cells.

Recent preclinical studies have been reported that the anti-tumour properties of ZOL are shown in tumours that express or hyper-express the EGFR supporting that ZOL directly binds to this receptor and thus inhibits the downstream pathway (Yuen et al. 2014; Agnes Stachnik et al. 2014). Our own studies here also suggest an involvement of the EGFR in ZOL action since stimulation of the EGFR attenuated ZOL response, whist EGFR inhibition augmented it. This observation was in concordance with a previous study demonstrated that gefitinib augmented the anti-tumour effects of ZOL in non-small cell lung cancer with mutated EGFR (Chang et al. 2009).

The data of this thesis revealed that treatment of TamR cells with ZOL was able to suppress activated EGFR level. However, it is not clear whether ZOL acts only on the EGFR or also affects one or more of the many downstream elements governed by the EGFR and other RTKs. Either hypothesis is supported by our data, which shows a dose-dependent decrease in the activity of AKT and MAPK following ZOL action in EGFR-overexpressing TamR cells.

Having found there was strong evidence of anti-tumour activity of ZOL to breast cancer cells, and more notably advanced responsiveness of TamR cells to ZOL, we then sought to elucidate the mechanism underlying this effect. Protein expression analysis of key molecules that regulate cell growth, proliferation and migration, such as AKT and MAPK, were found to be decreased in response to ZOL. Specifically, ZOL was able to reduce AKT expression in MCF-7, TamR and FasR cells and also managed to down-regulate the expression of MAPK in TamR cells only. That was consistent with other studies that have demonstrated that ZOL can cause disruption of AKT and MAPK signaling pathways in a variety of tumours, such as breast and prostate (Mognetti et al. 2014; Fournier et al. 2002; Clyburn et al. 2010). In an attempt to further investigate the downstream pathway that is affected by the AKT and MAPK down-regulation we identified that ZOL restrained the mTOR signaling in TamR and FasR cells, while this pathway appeared to be unaffected in MCF-7 cells. It has already been mentioned that ZOL interferes with the mevalonate pathway and disrupts protein prenylation of GTPases. Therefore, a possible explanation of the enhanced anti-tumour activity of ZOL in TamR cells would be the indirect inhibition of the mTOR pathway by ZOL, through prenylation inhibition of the Ras homologue enriched in brain protein (RHEB) that activates the mTOR.

In conclusion, our data suggests that zoledronic acid exerts anti-tumour effects in breast cancer cell models through modulation of RTK signalling pathway intermediates and that this is particularly evident in the context of acquired endocrine (tamoxifen) resistance.

6. General Discussion

Bone is a frequent site for breast cancer metastasis, with around 85% of patients with advanced breast cancer developing metastatic bone lesions. The vast majority of bone metastases are of the osteolytic type, arising as a result of enhanced differentiation of precursor cells into osteoclasts and augmented activation of bone-resident osteoclast cells. Osteolytic bone metastases are usually accompanied by chronic and severe bone pain with pathological fractures and which significantly impact on a patients' morbidity, quality of life and mortality. Interestingly, the type of breast cancer most likely to spread to the bone is the Luminal A subtype, which also represents the most frequent form of this disease (~75% of all breast cancers) and is characterised by presence of hormone receptors. Although such tumorous are routinely treated with endocrine agents, in many instances resistance can be acquired with associated disease relapse.

The development of endocrine resistance is accompanied by the acquisition of aggressive features such as tumour cell invasion and motility that are likely to facilitate the establishment of secondary tumour foci at distant sites (Chang 2012; Ali et al. 2016; C Kent Osborne & Schiff 2011; Hiscox et al. 2004). Moreover, endocrine resistance is usually accompanied by enhanced RTK signalling that induces the activation of downstream signalling cascades, such as Ras/MAPK and PI3K/AKT, that contribute to the aggressive phenotype of these models (Kurokawa et al. 2000). In support of this, Hiscox et al. (2012) reported that breast cancer cells with acquired tamoxifen resistance exhibited an invasive and aggressive phenotype *in vitro*. To date, however, little is known whether or how the development of endocrine resistance may contribute to formation of bone metastases.

Triple negative is another aggressive subtype of breast cancer, known to be associated with highly metastatic phenotype and poor prognosis (Bosch et al. 2010; Anders & Carey 2009; Dent et al. 2007b). Several studies have demonstrated the motile and invasive phenotype of triple negative breast cancer cells (Ferrari-Amorotti et al. 2014; Bailey et

al. 2012; Sánchez-Bailón et al. 2012). In addition, clinical evidence revealed that TNBC demonstrated the most aggressive clinical course compared to the all other breast cancer subtypes (Dent et al. 2007a).Given the aggressive phenotype of the endocrine resistant and triple negative breast tumours, part of the aims of this thesis were to investigate whether these subtypes further facilitate the development of bone metastasis compared to the Luminal A breast cancers.

Using a murine monocytic cell line, the data showed that conditioned media from cell models of both acquired tamoxifen resistance and triple negative breast cancer were able to induce osteoclastogenesis. This data was further validated using a human PBMC model. Indeed, breast cancer cells in the bone microenvironment are known to release soluble factors, such as PTHrP and cytokines, that promote development and activation of osteoclasts and osteoblasts. The result of this is a destruction of bone matrix, which can release factors, such as TGFβ, that in turn perpetuate breast cancer proliferation. This bi-directional interplay is known as the vicious cycle of bone metastasis.

Src kinase is known to play a critical role in breast cancer as a mediator of key signaling pathways that regulate cell proliferation, angiogenesis, invasion and metastasis. Elevated Src expression has also been correlated with an aggressive phenotype in breast cancer. In support of this, several studies have indicated that endocrine resistant models that are associated with aggressive cell behaviours, such as invasion and metastasis, have differential expression of Src compared to the endocrine sensitive models (Hiscox, Morgan, Tim P. Green, et al. 2006; Hiscox et al. 2010; Guest et al. 2016). Our data implicated Src as a mediator of breast cancer cell ability to influence osteoclastogenesis, while this effect was attenuated by treatment of the Src kinase inhibitor Sracatinib. Similar observation has been made by Araujo et al. 2009, where it was demonstrated that Src pharmacological inhibition with Dasatinib of PC-3 prostate cancer cells, significantly impaired the ability of conditioned media to differentiate RAW cells to osteoclasts.

Numerous *in vitro* studies have shown that treatment with Src inhibitors is able to suppress migration and tumour growth in a variety of cancer cell types, including breast, prostate, lung, pancreatic and colon (Messersmith et al. 2009; Richard S. Finn et al. 2007; Park et al. 2008). Src inhibition is also reported to promote reduction in expression of various proteins, such as MAPK, AKT, and focal adhesion kinase (FAK), that subsequently leads to suppression of tumour growth (Jallal et al. 2007). Importantly, emerging data from early phase clinical trials indicate the efficacy of Src-inhibitors in preventing bone lesions and decreasing levels of bone resorption markers in serum and urine (Hannon et al. 2010). Taken together, this data suggests that Src inhibition owns a central role in the development of breast cancer bone metastasis, especially in tamoxifen resistant and triple negative breast cancer where Src levels are elevated, and this effect can be attenuated by Src inhibition.

The use of RAW cells as osteoclast precursor model assisted in the preliminary investigation of this thesis. However, the limitation of this model was the murine origin of these cells and thus may react slightly differently compared to human cells. Another limitation of this model was that a cell line does not completely simulate the primary cells' behaviour. Thus, to further endorse and validate the findings from RAW cell studies, a peripheral blood mononuclear cell (PBMC) model was introduced as osteoclast precursors.

PBMC treatment with breast cancer cell conditioned media resulted in osteoclast differentiation in support of the findings observed by using RAW cells. Specifically, tamoxifen resistant and triple negative breast cancer cell conditioned media led to the highest number of differentiated cells possibly due to the aggressive nature of these models as it was previously detailed. This was in accordance with previous studies that have shown the ability of MDA-MB-231 conditioned media to induce osteoclast differentiation (Tiedemann et al. 2009), however it was the first time reported such effect

for tamoxifen resistant cells. In addition, this thesis revealed that breast cancer cells influenced PBMCs osteoclast differentiation in a Src-dependent manner.

RANKL has been widely implicated as a regulator of osteoclast differentiation. However, our data revealed that RANKL was not expressed by any of the breast cancer models used in this study, despite the ability of some of these models to induce differentiation. Thus, our observations implied that breast cancer cells were able to promote osteoclast differentiation in a RANKL-independent manner. RANKL-independent pathways for osteoclast differentiation have been previously reported in the literature and involve the participation of a number of molecules, including interleukins and other cytokines (Lau et al. 2007; Dougall 2012; McCoy et al. 2013). Indeed, investigation of 36 different cytokines for the breast cancer cells lines involved in this study demonstrated that all breast cancer models secrete the macrophage migration inhibitory factor (MIF), with the highest expression level observed in tamoxifen resistant cells. MIF is a cytokine secreted in pathological conditions, such as inflammation and cancer. In addition, it has been reported that MIF possibly participates in osteoclast differentiation (Madeira et al. 2012; Movila et al. 2016). MIF signalling in breast cancer cells is triggered following binding to its receptor CD74, which in turn activates the AKT pathway with the involvement of Src and PI3K. Given the pivotal role of Src in MIF signalling, studies have shown that this MIF-mediated AKT activation can be abolished by Src inhibitors (Lue et al. 2006; Mitchell et al. 1999; Lue et al. 2007). Taken together the observations that Src knockdown reduces breast cancer cells' ability to induce osteoclast differentiation and the fact that MIF signalling is regulated in a Src-mediated manner, these findings suggest that the osteoclastogenic effect seen by breast cancer cells might be attributed to a synergistic effect between Src and MIF. Consequently, Src and MIF possibly represent target molecules for triple negative and tamoxifen resistant breast

cancer, however further research needs to be done by either using MIF siRNA or inducing MIF overexpression in breast cancer cells in order to confirm this assumption.

Although breast cancer bone metastasis constitutes a major challenge in the clinical setting, the onset of bisphosphonates, which are agents with anti-resorptive properties, has significantly contributed to the alleviation of symptoms and the delay of bone disease progression. Interestingly, the new generation N-BPs have been found that apart from their effectiveness in preventing bone breakdown, also exert anticancer properties that target the tumour itself (Clézardin 2013). Three major clinical trials have scrutinised the anti-tumour properties of Zoledronic acid in pre- and post-menopausal women. ABCSG phase III clinical trial involved 1803 pre-menopausal patients that had been treated with LHRH agonists and aromatase inhibitors or tamoxifen with or without Zoledronic acid. This trial revealed benefit in disease free survival (DFS) and overall survival (OS) compared to endocrine treatment alone (Gnant et al. 2011). ZO-FAST clinical trial was primarily designed to evaluate Zoledronic acid activity in preventing aromatase inhibitor-associated bone loss, however the anticancer properties of Zoledronic acid were assessed as secondary endpoints. This trial involved 1065 post-menopausal patients treated with aromatase inhibitor with or without Zoledronic acid. Although this study did not reveal any benefit in OS, upfront zoledronic acid conferred a 34% reduction in the risk of disease recurrence or death compared to the delayed Zoledronic acid (de Boer et al. 2010). AZURE clinical trial involved 3360 pre- and post-menopausal patients and explored the anticancer effects of Zoledronic acid in combination with adjuvant chemotherapy and endocrine therapy. In AZURE, Zoledronic acid failed to show any benefits in DFS and OS in the mixed population, however it was significantly reduced the risk of bone metastasis development. In addition, Zoledronic acid was found to improve disease outcome only for patients with established menopause (>5 years) (Coleman et al. 2014). Apart from its anti-tumour properties, observational studies have suggested that

Zoledronic acid reduces by 20-30% the risk of breast cancer development (Mathew & Brufsky 2012).

The data in this thesis demonstrated a potential anti-cancer activity for bisphosphonates against tamoxifen resistant subtypes of breast cancer. Zoledronic acid significantly suppressed the proliferation marker Ki67 in TamR cells, while this effect was not that prevalent in the case of faslodex resistant and endocrine sensitive breast cancer cells. In support of these observations, Zoledronic acid was found to induce S-phase arrest in TamR cells. This was in accordance with previous studies reported that Zoledronic acid interferes in the cell cycle of a variety of cancer cell models, including breast, by promoting S-phase arrest (Okamoto et al. 2014; Okamoto & Kawamura 2012; Schech et al. 2013). This constitutes an interesting finding as it is the first time that acquired tamoxifen resistance has been identified as a mechanism of sensitisation to bisphosphonates.

These findings may suggest that Zoledronic acid exhibits a differential inhibitory effect in TamR cells, however further investigation is essential before firm conclusions are drawn. Recent studies have reported that Zoledronic acid may bind the EGFR and HER2 receptors and subsequently inhibit downstream signalling pathways (Agnes Stachnik et al. 2014; Yuen et al. 2014). Thus, enhanced sensitivity to this agent might be observed by tumours that overexpress these receptors. Among the cell lines used for this thesis, TamR cells expressed the highest levels of EGFR. In light of this, it was hypothesised that stimulation of EGFR expression by TGFα in TamR cells would possibly amplify the inhibitory effect seen by Zoledronic acid. Although such response was not shown in this study, it was found that dual treatment of TamR cells with Zoledronic acid and the Tyrosine Kinase Inhibitor gefitinib significantly restricted the growth of TamR compared to each single agent. This finding suggests that combined treatment of gefitinib and Zoledronic acid might confer an additional benefit for patients

with tamoxifen resistant breast cancer. Moreover, this observation was in line with previous studies reporting that dual treatment with these agents represents a more effective treatment for non-small cell lung cancer with EGFR mutations (Chang et al. 2009).

This thesis provided evidence that Zoledronic acid reduced EGFR activation, but not HER2, confirming Yuen et al. 2014 study that bisphosphonates target EGFR and/or HER2 in order to exert their anti-tumour effects. Further investigation to identify which downstream signalling pathways were affected by the EGFR downregulation, demonstrated that Zoledronic acid reduced AKT and MAPK signalling cascades and consequently mTOR pathway in TamR cells. Downregulation of the mTOR pathway by Zoledronic acid has been previously reported by other studies (Lan et al. 2013; Moriceau et al. 2010; Broom et al. 2015; Kato et al. 2016), however this thesis revealed the novel finding that Zoledronic acid inhibits growth and proliferation of TamR cells in an mTOR-mediated manner.

The work presented here suggests that acquired tamoxifen resistant breast cancer and the TNBC subtype are both able to promote osteoclast differentiation in a RANKL-independent manner via a mechanism that potentially involves Src kinase and the cytokine, MIF. In addition, our data revealed an important anticancer activity of Zoledronic acid which may occur through modulation of the mTOR pathway in these two breast cancer contexts. Zoledronic acid might therefore offer a potential benefit for patients with tamoxifen resistant breast cancer or the TNBC subtype.

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Appendices

Appendix A. Gene and primer sequences

PTHLH (V1)

ATGCAGCGGAGACTGGTTCAGCAGTGGAGCGTCGCGGTGTTCCTGCTGAGC

TACGCGGTGCCCTCCTGCGGGCGCTCGGTGGAGGGTCTCAGCCGCCGCCTC

AAAAGAGCTGTGTCTGAACATCAGCTCCTCCATGACAAGGGGAAGTCCATC

CAAGATTTACGGCGACGATTCTTCCTTCACCATCTGATCGCAGAAATCCACA

CAGCTGAAATCAGAGCTACCTCGGAGGTGTCCCCTAACTCCAAGCCCTCTCC

CAACACAAAGAACCACCCCGTCCGATTTGGGTCTGATGATGAGGGCAGATA

CCTAACTCAGGAAACTAACAAGGTGGAGACGTACAAAGAGCAGCCGCTCA

AGACACCTGGGAAGAAAAAGAAAGGCAAGCCCGGGAAACGCAAGGAGCA

GGAAAAGAAAAAACGGCGAACTCGCTCTGCCTGGTTAGACTCTGGAGTGAC

TGGGAGTGGGCTAGAAGGGGACCACCTGTCTGACACCTCCACAACGTCGCT

GGAGCTCGATTCACGGTAA

M-CSF

ATGACCGCGCCGGGCGCCGCCGGGCGCTGCCCTCCCACGACATGGCTGGGC

TCCCTGCTGTTGTTGGTCTGTCTCCTGGCGAGCAGGAGTATCACCGAGGAGG

TGTCGGAGTACTGTAGCCACATGATTGGGAGTGGACACCTGCAGTCTCTGC

AGCGGCTGATTGACAGTCAGATGGAGACCTCGTGCCAAATTACATTTGAGT

TTGTAGACCAGGAACAGTTGAAAGATCCAGTGTGCTACCTTAAGAAGGCAT

TTCTCCTGGTACAAGACATAATGGAGGACACCATGCGCTTCAGAGATAACA

CCCCCAATGCCATCGCCATTGTGCAGCTGCAGGAACTCTCTTTGAGGCTGAA

GAGCTGCTTCACCAAGGATTATGAAGAGCATGACAAGGCCTGCGTCCGAAC

TTTCTATGAGACACCTCTCCAGTTGCTGGAGAAGGTCAAGAATGTCTTTAAT

GAAACAAAGAATCTCCTTGACAAGGACTGGAATATTTTCAGCAAGAACTGC

AACAACAGCTTTGCTGAATGCTCCAGCCAAGATGTGGTGACCAAGCCTGAT

TGCAACTGCCTGTACCCCAAAGCCATCCCTAGCAGTGACCCGGCCTCTGTCT

CCCCTCATCAGCCCCTCGCCCCCTCCATGGCCCCTGTGGCTGGCTTGACCTG

GGAGGACTCTGAGGGAACTGAGGGCAGCTCCCTCTTGCCTGGTGAGCAGCC

CCTGCACACAGTGGATCCAGGCAGTGCCAAGCAGCGGCCACCCAGGAGCAC

CTGCCAGAGCTTTGAGCCGCCAGAGACCCCAGTTGTCAAGGACAGCACCAT

CGGTGGCTCACCACAGCCTCGCCCCTCTGTCGGGGCCTTCAACCCCGGGATG

GAGGATATTCTTGACTCTGCAATGGGCACTAATTGGGTCCCAGAAGAAGCC

TCTGGAGAGGCCAGTGAGATTCCCGTACCCCAAGGGACAGAGCTTTCCCCC

TCCAGGCCAGGAGGGGGCAGCATGCAGACAGAGCCCGCCAGACCCAGCAA

CTTCCTCTCAGCATCTTCTCCACTCCCTGCATCAGCAAAGGGCCAACAGCCG

GCAGATGTAACTGGTACCGCCTTGCCCAGGGTGGGCCCCGTGAGGCCCACT

GGCCAGGACTGGAATCACACCCCCCAGAAGACAGACCATCCATCTGCCCTG

CTCAGAGACCCCCCGGAGCCAGGCTCTCCCAGGATCTCATCACTGCGCCCCC

AGGGCCTCAGCAACCCCTCCACCCTCTCTGCTCAGCCACAGCTTTCCAGAAG

CCACTCCTCGGGCAGCGTGCTGCCCCTTGGGGAGCTGGAGGGCAGGAGGAG

CACCAGGGATCGGAGGAGCCCCGCAGAGCCAGAAGGAGGACCAGCAAGTG

AAGGGGCAGCCAGGCCCCTGCCCCGTTTTAACTCCGTTCCTTTGACTGACAC

AGGCCATGAGAGGCAGTCCGAGGGATCCTTCAGCCCGCAGCTCCAGGAGTC

TGTCTTCCACCTGCTGGTGCCCAGTGTCATCCTGGTCTTGCTGGCCGTCGGA

GGCCTCTTGTTCTACAGGTGGAGGCGGCGGAGCCATCAAGAGCCTCAGAGA

GCGGATTCTCCCTTGGAGCAACCAGAGGGCAGCCCCCTGACTCAGGATGAC

AGACAGGTGGAACTGCCAGTGTAG

RANKL isoform 1,2

ATGCGCCGCGCCAGCAGAGACTACACCAAGTACCTGCGTGGCTCGGAGGAG

ATGGGCGGCGGCCCCGGAGCCCCGCACGAGGGCCCCCTGCACGCCCCGCCG

CCGCCTGCGCCGCACCAGCCCCCTGCCGCCTCCCGCTCCATGTTCGTGGCCC

TCCTGGGGCTGGGGCTGGGCCAGGTTGTCTGCAGCGTCGCCCTGTTCTTCTA

TTTCAGAGCGCAGATGGATCCTAATAGAATATCAGAAGATGGCACTCACTG

CATTTATAGAATTTTGAGACTCCATGAAAATGCAGATTTTCAAGACACAACT

CTGGAGAGTCAAGATACAAAATTAATACCTGATTCATGTAGGAGAATTAAA

CAGGCCTTTCAAGGAGCTGTGCAAAAGGAATTACAACATATCGTTGGATCA

CAGCACATCAGAGCAGAGAAAGCGATGGTGGATGGCTCATGGTTAGATCTG

GCCAAGAGGAGCAAGCTTGAAGCTCAGCCTTTTGCTCATCTCACTATTAATG

CCACCGACATCCCATCTGGTTCCCATAAAGTGAGTCTGTCCTCTTGGTACCA

TGATCGGGGTTGGGCCAAGATCTCCAACATGACTTTTAGCAATGGAAAACT

AATAGTTAATCAGGATGGCTTTTATTACCTGTATGCCAACATTTGCTTTCGA

CATCATGAAACTTCAGGAGACCTAGCTACAGAGTATCTTCAACTAATGGTGT

ACGTCACTAAAACCAGCATCAAAATCCCAAGTTCTCATACCCTGATGAAAG

GAGGAAGCACCAAGTATTGGTCAGGGAATTCTGAATTCCATTTTTATTCCAT

AAACGTTGGTGGATTTTTTAAGTTACGGTCTGGAGAGGAAATCAGCATCGA

GGTCTCCAACCCCTCCTTACTGGATCCGGATCAGGATGCAACATACTTTGGG

GCTTTTAAAGTTCGAGATATAGATTGA

Appendix C. Scanned data Elisa hRANKL

C:\PROGRAM FILES\REVELATION\Plate Data\hRANKL15july16.lPrinted on 7/15/16 at 5:02:56 PM
Plate ID: hRANKL15july16 Maddi 450/540

Page 1 of

DYNEX TECHNOLOGIES REVELATION 4.25

Name :
Address :
Phone :
FAX :

TEST NO.	:	W/L MODE	: DUAL	DATE	: 7/15/16
TEST NAME	: Maddi 450&570	TEST FILTER	: 450 nm	TIME	: 5:02:31 PM
PLATE	: hRANKL15july16	REF. FILTER	: 570 nm	OPERATOR	:

OVER Limit : 3.500
Calculation mode : Endpoint

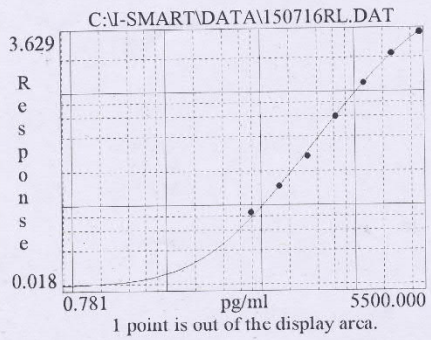
DATA MATRIX/TABLE : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.016	0.019	0.004	0.004	0.001	0.003	0.001	0.003	0.003	0.004	0.004	0.003
B	0.090	0.083	0.009	0.001	0.003	0.003	0.002	0.003	0.003	0.003	0.003	0.004
C	0.149	0.147	0.011	0.007	0.002	0.003	0.002	0.001	0.006	0.004	0.003	0.001
D	0.265	0.276	0.010	0.001	0.003	0.003	0.002	0.004	0.003	0.005	0.004	0.005
E	0.604	0.592	1.484	0.003	0.002	0.003	0.003	0.004	0.004	0.003	0.005	0.002
F	1.187	1.189	-0.015	0.001	0.003	0.004	0.004	0.000	0.004	0.004	-0.000	0.002
G	2.049	2.261	-0.008	0.003	0.002	0.003	0.003	0.004	0.003	0.003	0.003	0.002
H	3.280	3.322	-0.005	0.001	0.003	0.002	0.002	0.005	0.004	0.003	0.001	0.001

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

Protocol #: 2 Assay Name: human RANKL User: Denia
Data File: C:\I-SMART\DATA\150716RL.DAT
Curve Fit: 4 PL



1 point is out of the display area.

Y=(A-D)/(1+(X/C)^B)+D
A = 0.019
B = 1.157
C = 4301.374
D = 6.072

Min(X) = 0.000
Max(X) = 5000.000
Min(Y) = 0.019
Max(Y) = 3.309

Standard Weighting function is used.
SSE = 1.064E-04
RMS = 0.005

[Replicate Data Report]

Label	Response	SD	%CV	Def. Conc.	Calc. Conc.	SD'	%CV'	%Diff.
STD1	0.016			0.000	off crv: lo			----
STD1	0.019			0.000	off crv: lo			----
	0.018	**0.002**	**12.12**	**0.000**	**off crv: lo**	----	----	----
STD2	0.090			78.125	92.926			-18.9
STD2	0.083			78.125	84.831			-8.6
	0.087	**0.005**	**5.72**	**78.125**	**88.891**	**5.724**	**0.06**	**-13.8**
STD3	0.149			156.250	158.351			-1.3
STD3	0.147			156.250	156.193			0.0
	0.148	**0.001**	**0.96**	**156.250**	**157.273**	**1.526**	**0.01**	**-0.7**
STD4	0.265			312.500	279.782			10.5
STD4	0.276			312.500	291.050			6.9
	0.270	**0.008**	**2.88**	**312.500**	**285.419**	**7.968**	**0.03**	**8.7**
STD5	0.604			625.000	623.361			0.3
STD5	0.592			625.000	611.133			2.2
	0.598	**0.008**	**1.42**	**625.000**	**617.245**	**8.647**	**0.01**	**1.2**
STD6	1.187			1250.000	1249.103			0.1
STD6	1.189			1250.000	1251.394			-0.1
	1.188	**0.001**	**0.12**	**1250.000**	**1250.248**	**1.620**	**0.00**	**0.0**
STD7	2.049			2500.000	2381.752			4.7
STD7	2.261			2500.000	2719.495			-8.8
	2.155	**0.150**	**6.96**	**2500.000**	**2546.934**	**238.878**	**0.09**	**-1.9**
STD8	3.280			5000.000	4918.563			1.6
STD8	3.322			5000.000	off crv: hi			----
	3.301	**0.030**	**0.90**	**5000.000**	**4978.289**	----	----	**0.4**
SMP1	0.004				off crv: lo			
	0.004	----	----		**off crv: lo**	----	----	
SMP2	0.009				off crv: lo			
	0.009	----	----		**off crv: lo**	----	----	
SMP3	0.011				off crv: lo			
	0.011	----	----		**off crv: lo**	----	----	

Label	Response	SD	%CV	Def. Conc.	Calc. Conc.	SD'	%CV'	%Diff.
SMP4	0.010				off crv: lo			
	0.010	----	----		**off crv: lo**	----	----	