IGF-1 regulates Artemin expression and enhances Artemin signal transduction in human mammary carcinoma cells

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Abstract

Artemin (ARTN) is a neurotrophic signalling factor belonging to the glial-derived neurotrophic factor (GDNF) family of ligands. It acts as a survival, proliferation and migration factor for a number of neurological cell types, by signalling through the RET (rearranged during transfection) receptor and GDNF receptor (GFR)-α3 co-receptor complex. Since its discovery, a number of published studies have implicated Artemin as a potential oncogene and as a mediator of resistance to various cancer therapy agents. The study by Kang *et al.* (2010) demonstrating regulation of ARTN expression by estrogen, raised the possibility that ARTN expression and signal transduction might be regulated by other signalling pathways or cross-talk with them.

This thesis tested the hypothesis that insulin-like growth factor (IGF)-1, a growth factor known to cross-talk with other signalling pathways, including the estrogen receptor signal transduction pathway, might be a potential regulator of ARTN function. The evidence presented herein supports this hypothesis and demonstrated the induction of ARTN expression by IGF-1. Synergistic activation of downstream signalling mediators also raises the possibility of ARTN involvement in the development of resistance to IGF-1R inhibition in mammary carcinoma cells. As such, co-targeting of both ARTN and IGF-1 signalling pathways may potentiate drug inhibition of cancer growth.

The inevitable development of resistance to therapeutic interventions has been a major obstacle to curative cancer therapy. The ability of ARTN to confer *de novo* resistance to chemotherapy agents was also investigated. Conflicting results from cell function assays demonstrated the importance of cell line selection in *in vitro* modelling. These studies need further investigation to determine the context of ARTN function and the best course of action in targeting ARTN signalling for cancer treatment.

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List of Abbreviations

3D	Three Dimensional
AKT	v-akt Murine Thymona Viral Oncogene Homolog 1
APS	Ammonium Persulfate
ARTN	Artemin
ATCC	American Type Culture Collection
BCL-2	B-cell lymphoma 2
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
bp	Base Pair
cDNA	Complementary Deoxyribose Nucleic Acid
CLD	Cadherin-Like Repeat
CNS	Central Nervous System
CRD	Cysteine-Rich Domain
DEPC	Diethyl Pyrocarbonate
DMSO	Dimethyl Sulfoxide
dNTP	Deoxynucleotide Triphosphate
DRG	Dorsal Root Ganglion
DTT	Dithiothreitol
E-Cad	E-Cadherin
EDTA	Ethylenediaminetetra Acetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked immunosorbent Assay
EMT	Epithelial Mesenchymal Transition
ER	Estrogen Receptor
ERK	Extracellular Signal-Regulated Kinase
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GDNF	Glial Derived Neurotrophic Factor
GFL	GDNF Family of Ligands
GFRα	GDNF Family Receptor α
GH	Growth Hormone

GI	Gastrointestinal
GPI	Glycosyl phosphatidylinositol
GSK-3	Glycogen Synthase Kinase-3
HER-2	Human Epithelial Receptor 2
HIF	Hypoxia Inducible Factor
IGF-1	Insulin Growth Factor 1
IGF-1R	Insulin Growth Factor 1 Receptor
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
kDa	Kilodaltons
mAb	Monoclonal Antibody
M	Molar
MAPK	Mitogen Activated Protein Kinase
MEN2	Multpile Endocrine Neoplasia Type 2
mM	millimolar
mTOR	Mammalian Target of Rampamycin
MTT	3-[4,5-dimethyl-thiazol-2yl]-2.5-diphenyl tetrazolium bromide
NCAM	Neural Cell Adhesion Molecule
NSCLC	Non-small Cell Lung Cancer
NTN	Neuturin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide 3-kinases
PR	Progesterone Receptor
PSP	Persephin
PVDF	Polyvinylidene difluoride
RET	Rearranged during Transfection
rpm	Revolutions per Minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Deodecyl Sulfate
SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
siRNA	Small Interfering RNA
SCG	Superior Cervical Ganglion
TBS	Tris Buffered Saline
TEB	Terminal End Bud

TGF-β	Transforming Growth factor β
µg	Microgram
µL	Microlitre
µM	Micromolar
v/v	Volume per volume
VTA	Ventral Tegmental Area
w/v	Weight per volume

Chapter 1

General Introduction

1.1 Introduction

Nearly all known cancers are derived from single somatic cells and their descendants (Ponder, 2001). The clone cells accumulate a series of genetic and epigenetic changes that eventually lead to altered gene activity, and altered phenotypes which are selected upon. Ultimately, there evolves a cell population that disregard the normal controls of proliferation becoming cancerous. In their seminal review article in 2000, Hanahan and Weinberg identified six 'hallmark features' typical of, and essential to the cancer cell phenotype. These included a disregard of anti-proliferation signals and of signals to differentiate, a limitless proliferative ability, evasion of apoptosis, migration and invasion of distant tissues, and angiogenesis (Hanahan and Weinberg, 2000).

This concept of an entire cancerous tumour arising from a single altered somatic cell has been the backbone of the many insights into cancer initiation and progression that have been developed. The new ideas that develop are shaped by the assays on which they are based and although useful, our knowledge is at the same time constrained by what assays and techniques we have available to us and remains incomplete as a result (Ponder, 2001). But as this knowledge base has grown, the theories concerning the origins of cancer have evolved to encompass other aspects which were previously deemed to be inadequately explained. Examples include the vast heterogeneity observed in tumours and the relatively newly arisen cancer stem cell hypothesis (Tan

et al., 2006). In 2011 Hanahan and Weinberg released an updated list of cancer hallmarks which included several new entries, such as the reprogramming of cell energy metabolism and the evasion of immune destruction, in recognition of the advances in our understanding of cancer progression over the past decade (Hanahan and Weinberg, 2011).

The pathology of breast cancer, or mammary carcinoma, is much the same. It consists of a complex and incompletely understood process of successive genetic and epigenetic changes (Nowell, 1976; Polyak, 2001). Being the most prevalent cancer among women worldwide, it poses a significant public health problem (Parkin et al., 2005). According to the New Zealand Breast Cancer Foundation, a New Zealand woman's risk of developing breast cancer in her lifetime is now 1 in 9. A number of factors contribute to this risk, including age, lifestyle and reproductive history, family history, and genetic factors (Weir et al., 2007). Ultimately, these factors impact on multiple aspects including hormone profiles and gene expression.

Research into how the gene expression profile is affected could aid a better understanding of this disease and is a key step in eventually learning to treat and control it. Of particular interest are the changes that occur in the gene expression of the cancer cell, which has come under intense scrutiny by researchers. Identification of the crucial genetic and biochemical factors involved in carcinogenesis and tumour progression will allow for the development of novel approaches for treatment and possibly prevention (Smith et al., 2011; Steward and Brown, 2013; Tsao et al., 2004).

1.2 Mammary Gland Development

The mammary gland is an essential reproductive secretory organ and consists of several different cell types (Polyak, 2001; Watson and Khaled, 2008). It is responsible for the production and delivery of milk for the nutrition of young offspring, and it is considered to be unique in the way that it develops, because the majority of development takes place in the adult (Sternlicht, 2006). Unlike most other organs in multi-cellular organisms, which undergo a strictly linear developmental process, mammary gland instead undergoes separate and defined developmental stages: a linear growth stage during embryonic growth, a pubertal growth stage, and a cyclical development stage during adulthood in the events of pregnancy, lactation and involution (regression), all of which are strictly controlled by a range of hormones, growth factors and various signalling molecules (Neville et al., 2002).

In mice, where the mammary gland has been extensively studied, mammary development initially begins with the formation of mammary streaks or milk lines in the ectoderm at embryonic day (E)10 (Cowin and Wysolmerski, 2010; Veltmaat et al., 2006). This is the beginning of the embryonic growth stage. In the mouse, 5 ordered pairs of placodes form and invaginate into the mesenchyme to form the mammary bud. The positions where the placodes form in the mouse embryo are illustrated in Figure 1.1. The mammary bud continues to elongate, forming secondary branches at E16, until it embeds itself into the mammary fat pad. By E18, the embryonic growth stage is essentially complete and no further development occurs until the onset of puberty (Watson and Khaled, 2008).

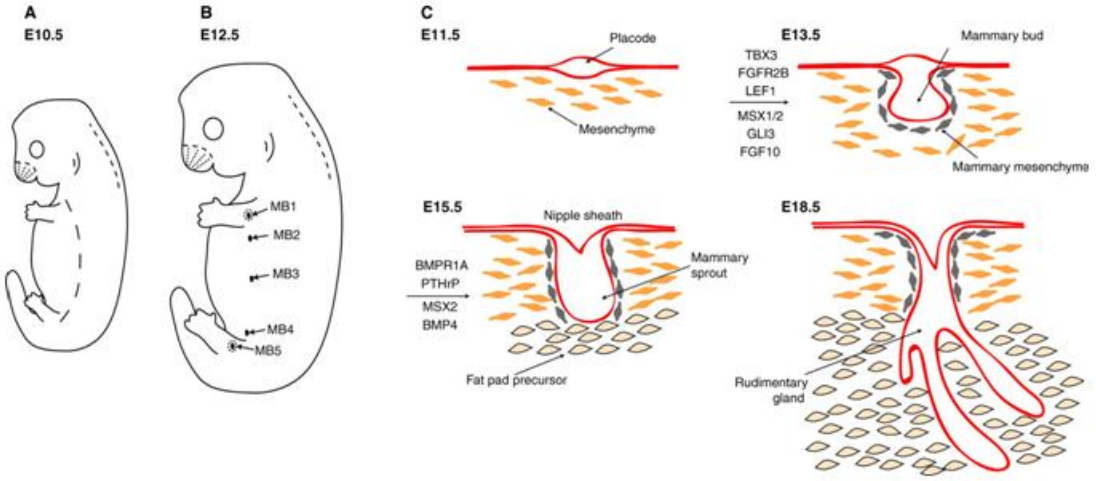

Figure 1.1- Diagram demonstrating the timeline of mammary gland formation in the mouse.

(A) Figure of the mouse embryo at E10.5, the dashed line indicates the position of the formation of the mammary streaks. **(B)** The relative positions where the placodes form. **(C)** Overview of mammary gland formation in the mouse from the initial formation of the mammary streaks and placodes until the time where development is arrested (Watson and Khaled, 2008).

In the period after birth and before puberty, the mammary gland grows in proportion to the rest of the body, but there is very little differentiation (Russo and Russo, 2004; Watson and Khaled, 2008). During this period, club-shaped structures form at the very ends of the ductal branches (Hinck and Silberstein, 2005; Medina, 2005). These are the terminal end buds (TEB), and consist of an outer layer of cap cells and an inner multilayered core of body cells that comprise various undifferentiated cell types (Figure 1.2) (Hinck and Silberstein, 2005).

At the commencement of puberty in the female, there is a substantial increase in the concentration of circulating estrogen (Cheng et al., 2004). This surge of estrogen serves as the impetus for recommencing development of the mammary gland (Cheng

et al., 2004). Rapid proliferation is initiated in the TEBs inducing elongation and branching of the mammary ducts until they reach the limits of the mammary fat pads and growth ceases (Hinck and Silberstein, 2005; Watson and Khaled, 2008). At this point, the TEBs regeress leaving an expansive network of undifferentiated ducts, which will remain relatively unchanged until the next stage of development – pregnancy (Hennighausen and Robinson, 2005; Hinck and Silberstein, 2005; Medina, 2005).

During pregnancy, the mammary gland undergoes further morphological changes to prepare for the onset of lactation. During this time, the hormones, progesterone and prolactin are released to induce alveologenesis (Hennighausen and Robinson, 2005). The differentiated alveoli are the mammary structures responsible for the production and delivery of milk at childbirth (Anderson et al., 2007). In this period, where the mammary gland is required to actively produce and deliver milk, the mammary gland has evolved a considerably different morphology to the gland in the virgin mammal (Atabai et al., 2007).

The mammary gland will retain this ability to produce milk for as long as it is required. The stimulus for continued milk production comes from the suckling action of the offspring on the nipple. Upon weaning, this suckling stimulus is removed, and involution begins, possibly influenced by the stasis of milk flow (Atabai et al., 2007; Watson, 2006). For the mammary gland, this is yet another period of extensive redevelopment. The secretory epithelial cells undergo widespread programmed cell death, and the breast stroma is repopulated by adipocytes (Atabai et al., 2007; Stein et al., 2007). Physical removal of these dead cells is also required and is carried out by the phagocytic cells of the immune system (Watson, 2006). This recruitment of the

immune system is necessary to prevent inflammation, which is often associated with such massive cell death. Anti-inflammatory cytokines are also secreted by the phagocytes for the prevention of inflammation of the breast, termed mastasis (Atabai et al., 2007).

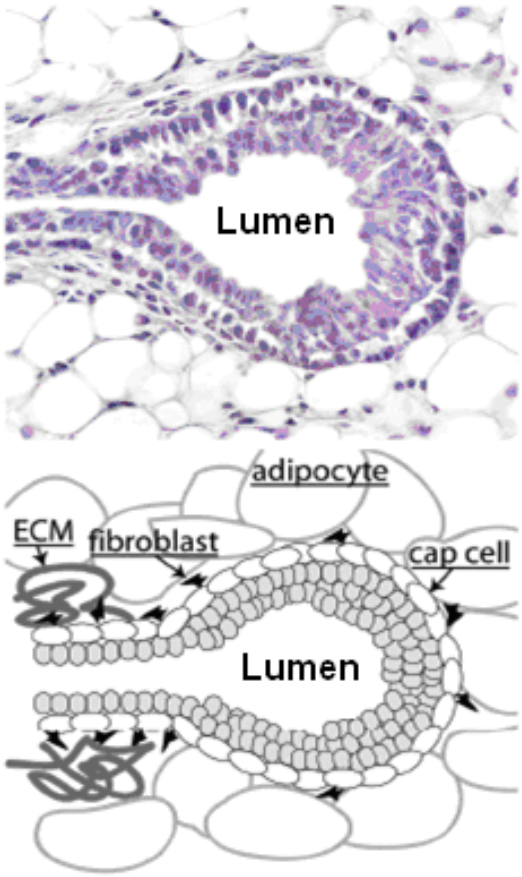

Figure 1.2- Stained cross section of a TEB and accompanying diagram depicting the multi-layered nature of the buds(Hinck and Silberstein, 2005).

1.3 Breast Cancer Etiology

According to the World Health Organization, breast cancer is the most prevalent female cancer in most developed and developing nations, and is one of the leading causes of cancer deaths. In the UK, the lifetime risk for developing breast cancer is 1 in 8 and accounts for 30% of diagnosed female cancers (www.cancerresearchuk.org). New Zealand is also home to one of the highest rates of breast cancer in the developed world (Hery et al., 2008) and a New Zealand woman's lifetime risk of developing breast cancer is now 1 in 9, with those aged over 50 at greatest risk (http://www.nzbcf.org.nz/).

Due largely to the public health issues and the heavy prevalence of breast cancer in women worldwide, the genetic and non-genetic causes of breast cancer have been studied extensively and continue to be. A number of genetic factors that contribute to breast cancer risk have been identified, predominantly in the breast cancer susceptibility genes *BRCA1* and *BRCA2*, but other less frequent mutations have been identified in genes such as *TP53, PTEN, FGFR2, and CHEK2*. In total, genome-wide association studies have identified over 70 loci associated with breast cancer susceptibility (Ghoussaini et al., 2013). Still, these genetic factors only account for 5-10% of cases (Chang-Claude, 2001). The remainder are considered sporadic cases and are likely affected by various lifestyle factors, including alcohol intake, obesity and use of hormone replacement therapy (Weir et al., 2007). A woman's reproductive history also has some influence on the breast cancer risk. Early age of menarche, late onset of menopause, and the age of first full term pregnancy are all suggested factors influencing the breast cancer risk, most likely because of the level of estrogen exposure that is involved (Feigelson and Henderson, 2000). However, it is late stage

invasive breast cancer that remains a major cause of cancer deaths in women, and our lack of understanding of the changes in gene expression involved is a significant barrier to effective management of the disease (Cardoso et al., 2012; Weigelt et al., 2005).

Because of their altered molecular and genetic profiles, cancer cells are characterized by many shared commonalities in phenotype and behaviour, and yet this is still a vastly heterogeneous set of diseases. Until recently, breast tumours had been classified into different clinical categories by receptor status, usually determined by immunohistological staining (Blows et al., 2010; Penault-Llorca and Viale, 2012). More recently, gene expression and genomic profiling has led to the identification of five main subtypes: luminal A, luminal B, HER2-enriched, basal-like and normal-like (Kao et al., 2009) and later ten subtypes through the METABRIC project (Curtis et al., 2012). These subtypes encompassed aspects of tumour grading and the different hormone receptor statuses, with luminal A/B tumours being largely ER+, HER2-enriched often exhibit elevated HER2 expression, and basal-like tumours are often also referred to as triple negative breast cancers as they are negative for the expression of ER, HER2 and progesterone receptor (PR) (Curtis et al., 2012).

But with the ever emerging technological advances in molecular and genomic profiling techniques, tumour classifications are evolving beyond these categories. Not only can the above five subtypes be further stratified (Prat et al., 2014), but greater profiling efforts have led to the identification of more unique subtypes with greater predictive values, including the claudin-low subtype (Engstrøm et al., 2013; Eroles et al., 2012). For this reason, continued in-depth research into the genetic alterations that occur in breast cancer cells is critical.

1.3.1 Treating Breast Cancer

Current treatment options for breast cancer include surgery, radiation therapy, chemotherapy, estrogen receptor antagonists/endocrine therapy, and targeted molecular therapy (Barton and Swanton, 2011; Ingle, 2013; Singletary et al., 2003). A combination of these therapies is often used to ensure the best patient outcome. The relatively new approach of targeted molecular therapy involves specific targeting of the phenotypic differences between cancer cells and normal cells (Kohn et al., 2004; Osborne et al., 2004; Widakowich et al., 2007). The successful development, and subsequent application of trastuzumab (Herceptin), a humanized recombinant monoclonal antibody against the epidermal growth factor receptor, HER2, reflected this shift in direction in the search for novel cancer therapies (Gajria and Chandarlapaty, 2011; Preston-Martin et al., 1990). Trastuzumab is used as an adjuvant treatment strategy in breast cancer with amplified HER2 receptor. However, it is only effective in 20-30% of breast cancer cases with HER2 over-expression and patients often suffer recurrence and develop refractory disease within 1 year (Gajria and Chandarlapaty, 2011; Preston-Martin et al., 1990). The reason for the lack of response and efficacy in these cases is unknown, illustrating the limits of our current understanding of cancer, and the need for further study.

One of the more commonly known reasons for treatment failure is resistance to treatment, both *de novo* and acquired (Lippert et al., 2008). Resistance to treatment often arises very quickly and limits the usefulness of a drug in the clinical management of cancers. In *de novo* resistance, the resistant trait is inherent to the cancer cell population, either genetically or through a favourable tumour

microenvironment, which can allow for the selection of this resistant phenotype once treatment commences (Gottesman, 2002; Hazlehurst et al., 2000; Liao et al., 2009). Acquired resistance, however, is developed over time when treatment begins and continues to exert selective pressure on the cancer cell population. Resistance in this population can be acquired through genetic or epigenetic means, such as gene amplification or mutation in the original drug target (Shoemaker, 2000), or the increased efflux of the chemotherapy agent from the cell, preventing it from interacting with its intended molecular target (Gottesman, 2002). The identification of the mechanisms behind treatment resistance can also provide new avenues for the identification of new targets and rational drug design and strategies for overcoming resistance. Identification of new targets can also serve to expand the current understanding of cancer biology and provide new tools for diagnosis, treatment and possibly prevention.

1.4 The GDNF Family of Ligands

The glial cell line-derived neurotrophic factor (GDNF) was originally isolated as a potent neurotrophic factor for dopaminergic neurons of the midbrain (Lin et al., 1993) and was the first identified member of what is now referred to as the GDNF family of ligands (GFL). The other members of this family include neurturin (NTN), persephin (PSP) and artemin (ARTN) (Airaksinen and Saarma, 2002; Airaksinen et al., 1999). NTN was later isolated by biochemical purification and identified by cDNA analysis, whereas PSP was identified by homology cloning and ARTN was identified through a database search for sequence homology (Baloh et al., 1998c; Saarma, 2000b).

The GFLs are also members of the Transforming Growth Factor (TGF)-β superfamily (Saarma, 2000a). Although, there is very low homology in the amino acid sequence of GLFs and TGF-β (less than 20%), there is a striking structural conservation between these proteins (Saarma, 2000b). Similar to the other members of the TGF-β family, GFLs contain a cysteine knot motif characterized by seven cysteine residues in a similar pattern of spacing within the amino acid sequence (McDonald and Hendrickson, 1993; Saarma, 2000b). These cysteine residues allow the formation of the active homodimers, which are held in conformation by disulfide bonds (Ibáñez, 1998; Lin et al., 1993).

Despite the structural homology between the GFL and the TGF-β families, the classification of GFL members as TGF-β family members was considered surprising for a number of reasons. Firstly, the other members of the TGF-β family signal through serine-threonine kinases, whereas the GFLs utilize the RET (rearranged during transfection) proto-oncogene receptor, which is a typical tyrosine kinase receptor (Murakumo et al., 2006). Secondly, GFL signalling also required the additional binding of a novel class of proteins called GDNF family receptor α (GFRα). These proteins are bound to the surface of the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (Murakumo et al., 2006; Saarma, 2000b). Further discussion of the GFL receptors can be found in Section 1.5 of the Introduction.

1.4.1 Developmental Function

The physiological roles of the GFLs have been extensively studied in *in vitro* cultures and in mouse models. As neurotrophic factors, GFLs have the ability to promote

proliferation and enhance cell survival for a range of neurological cell types, including the dopaminergic midbrain neurons, sensory neurons of the dorsal root ganglia (DRG), motoneurons, sympathetic and parasympathetic neurons and various other neurons in both the peripheral nervous and central nervous systems (Arenas et al., 1995; Durbec et al., 1996; Lin et al., 1993; Sariola and Saarma, 2003).

In particular, extensive study of GDNF has demonstrated the expansive role it plays in the developing nervous system. Mouse models of the developing midbrain show GDNF functioning to promote the survival and differentiation of dopaminergic neurons (Enomoto, 2005). ARTN is essential for the migration of superior cervical ganglion (SCG) sympathetic precursors as demonstrated in ARTN-deficient mice lacking SCGs when those neurons die due to their failure to migrate and project their axons for the appropriate trophic support (Andres et al., 2001; Baloh et al., 1998c; Rosenthal, 1999). GDNF and NRTN expression are essential for the proper establishment of sympathetic and parasympathetic innervation in various tissues, including the lungs, pancreas and intestinal tract (Airaksinen and Saarma, 2002; Takahashi, 2001a).

GDNF also plays a substantial role in the development of several tissues outside of the nervous system. In particular, GDNF is essential for kidney formation (Saarma, 2000b; Sariola and Saarma, 1999). In gene ablation studies, mice which were deficient for GDNF signalling often died at birth displaying renal agenesis or severe dysgenesis (Rosenthal, 1999). These mice also lacked enteric neurons and SCGs or only developed very small SCGs that were caudally displaced, as a result of the inability of the precursors to properly migrate and project their axons for innervation of the correct targets. In the developing embryo, GDNF is expressed by the

nephrogenic mesenchyme, activating the receptors on the tips of the ureteric bud initiating elongation and branching (Moore et al., 1996; Saarma, 2000b; Sariola and Saarma, 1999).

Proper GDNF signalling was also shown to be essential in male spermatogenesis (Airaksinen et al., 1999; Durbec et al., 1996; Enomoto et al., 2004). The Sertoli cells of the testis express GDNF to regulate the proliferation of undifferentiated spermatogonia stem cells in a paracrine fashion (Meng et al., 2000). Signalling stimulates DNA synthesis and delays differentiation in the spermatogonia cells. In mice over-expressing GDNF, the spermatogonia did not differentiate and the males were left infertile (Airaksinen and Saarma, 2002; Meng et al., 2001). On the other hand, inhibition of GDNF function, inhibited self-renewal of the spermatogonial progenitors, also causing infertility in males (Oatley et al., 2007). However, like the many aspects of GFL signalling, the processes regulating GDNF expression are still poorly understood.

1.4.2 Artemin

ARTN was the most recently discovered member of the GFLs and, like the other members, it is a potent promoter of the proliferation and survival of various neuronal cells types (Airaksinen et al., 1999; Andres et al., 2001; Ceyhan et al., 2007). The *ARTN* gene loci is located on chromosome 1p33-32, and is comprised of 5 exons. Five representative transcripts have been identified for *ARTN*, but cDNA sequences from across the available genome databases suggest that at least 11 splice variants could exist (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=human&c=Gene&l=ARTN). The ARTN protein is initially produced in the endoplasmic

reticulum as prepro-ARTN (Baloh et al., 1998d). Upon secretion, the signal peptide is cleaved to produce the active ARTN peptide. The mature peptide produced is 113 amino acids in size and alignment of the sequence with the other GFLs shows a fair amount of similarity, with ARTN exhibiting ~36% identity with GDNF and ~45% with NTN and PSP (Baloh et al., 1998d). The fully formed ARTN protein exists as both a monomer and a homodimer, although it is the dimeric form that is seen to be biologically active (Wang et al., 2006).

In fetal and adult mice, ARTN expression is present throughout the nervous system. It is most concentrated in the dorsal root ganglia (DRG) and immature Schwann cells, and but is only expressed very weakly in the brain (Ceyhan et al., 2006c). Studies of ARTN expression in relation to the expression of its receptors, GFRα3 and RET, suggests specific spatial and temporal expression patterns in embryonic and adult tissues (Masure et al., 1999). In the embryo, elevated GFRα3 (the preferential receptor for ARTN binding, see section 1.5.2) expression was detected throughout the sympathetic nervous system, illustrating the importance of ARTN signalling for its development. This is also where the neurotrophic capability of ARTN is primarily observed. Mice lacking ARTN exhibited a ptosis phenotype (drooping of the upper eye lid) due to a defect in sympathetic innervation, (Masure et al., 1999). Soon after birth, the level of GFRα3 expression was also greatly diminished.

ARTN expression was detected in the smooth muscle cells of the arteries in fetal and adult mice (Damon et al., 2007b). As vascular sympathetic innervation is an important determinant of blood pressure and blood flow, it has been proposed that the expression of ARTN by smooth muscle cells is responsible for developing

sympathetic innervation by promoting sympathetic axon growth and projection (Damon et al., 2007b).

Elsewhere in the adult, low level *ARTN* mRNA expression has been detected primarily in the placenta, pancreas, prostate, pituitary gland, trachea, testis, ovaries and intestinal tract (Baloh et al., 1998d; Bolon et al., 2004). However, the function of ARTN in these tissues has not been well defined.

1.5 GFL Receptors

GFL signalling is a complicated and poorly understood process. All of the ligands share a common receptor, RET, but binding and activation of this receptor requires the additional binding of a second co-receptor, GFRα (Arenas et al., 1995; Durbec et al., 1996). In addition, alternate pathways for GFL signalling exist which are independent of the RET and/or GFRα receptors.

1.5.1 RET

The RET (rearranged during transfection) receptor was originally discovered in 1985 as a proto-oncogene when a tranfection assay in NIH3T3 cells produced a fusion protein with transforming activity (Ibanez, 2013). The *RET* gene is located on chromosome 10q11.2 and contains 21 exons. Three splice variants of the receptor exist, *RET9*, *RET43* and *RET51*, arising from alternate splicing at the 3' terminal end. In most cells, *RET9* and *RET51* are the main products expressed (Anders et al., 2001; Murakumo et al., 2006).

RET is a typical tyrosine kinase receptor and can exist in monomeric or homodimeric form (Ibanez, 2013). It is a single pass, transmembrane protein with an extracellular ligand binding domain and an intracellular tyrosine kinase domain. The extracellular domain also contains four cadherin-like repeats (CLD1-4) and a cysteine-rich domain (CRD) (Anders et al., 2001; Ibanez, 2013).

Unsurprisingly, the expression of RET overlaps greatly with that of GDNF, with the greatest levels of expression are found in the developing peripheral and central nervous systems, indicating that it has an important role to play in the development and differentiation of the nervous system (Coulpier et al., 2002; Murakumo et al., 2006). In knockout mice models, RET-deficient mice exhibit renal agenesis or severe dysgenesis and a lack of enteric neurons, similar to that seen in GDNF knockout models (Golden et al., 2010).

In humans, RET mutations are associated with a number of familial disease syndromes. Loss-of-function RET mutations are known to cause Hirschsprung's disease (Iwashita et al., 1996). Also known as aganglionic megacolon, Hirschsprung's disease is characterised by severe constipation and obstruction due to a lack of nerve innervation in the large intestines (Takahashi, 2001a). Conversely, gain-of-function mutations in RET are the cause of multiple endocrine neoplasia type 2 (MEN2) (Jhiang, 2000). MEN2 is a familial syndrome passed in through germ line mutations. It has a high penetrance and is life-threatening (Brandi et al., 2001; Waguespack et al., 2011). Early diagnosis using DNA-based molecular testing is imperative for effective management and treatment. Depending on the risk level, prophylactic thyroidectomy may be recommended in children as young as only a few months after birth (Takahashi, 2001a).

1.5.2 GFRα

Activation of RET by GFL signalling requires additional binding of the GPI-anchored GFRα co-receptors (Peterziel et al., 2007; Peterziel et al., 2002). There are four isoforms of GFRα (GFRα1-4) generated by alternative splicing, and each GFL member has a binding preference for each isoform, illustrated in Figure 1.3 (Airaksinen et al., 1999; Saarma and Sariola, 1999). GDNF preferentially binds to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to GFRα4. However, some GFL members can bind to more than one co-receptor. For example, although ARTN preferentially binds to GFRα3, it can also bind to GFRα1 when GFRα3 is absent (Andres et al., 2001; Baloh et al., 1998c). The physiological significance of this is unclear.

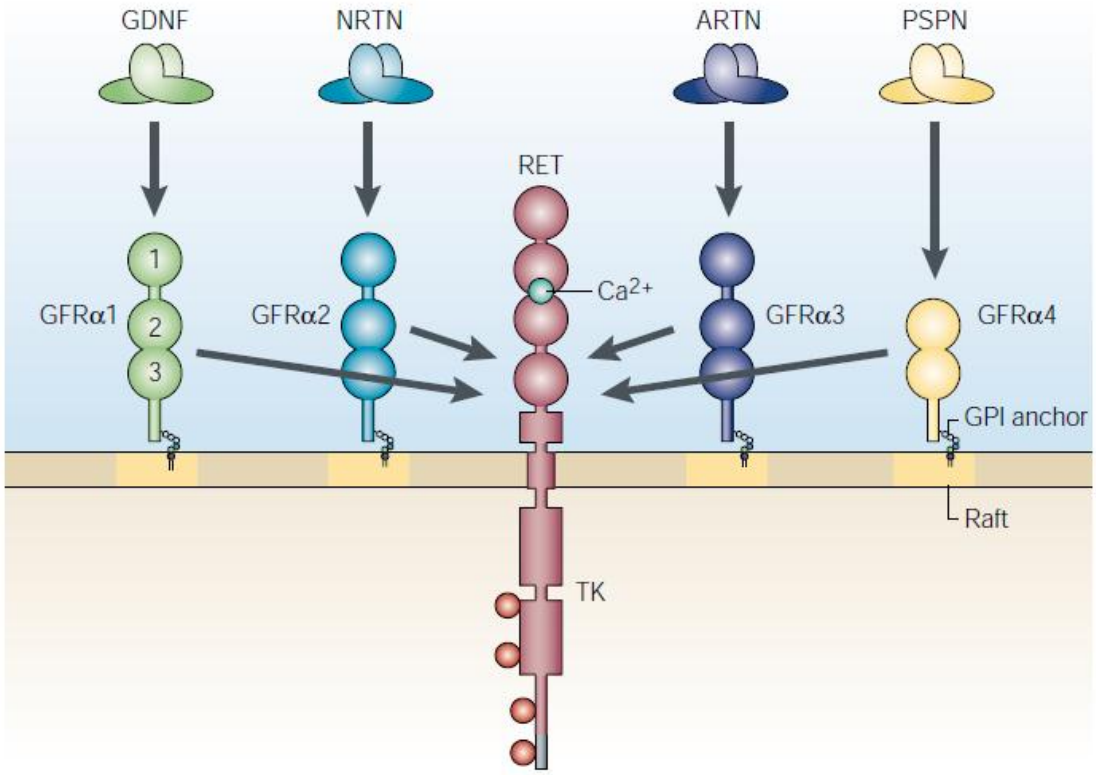

Figure 1.3- GFL and receptor interactions.

The canonical model of GFL signalling states that each ligand first binds to its own respective GFRα receptor and the GFL- GFRα complex then binds and recruits the common receptor, RET (Airaksinen and Saarma, 2002).

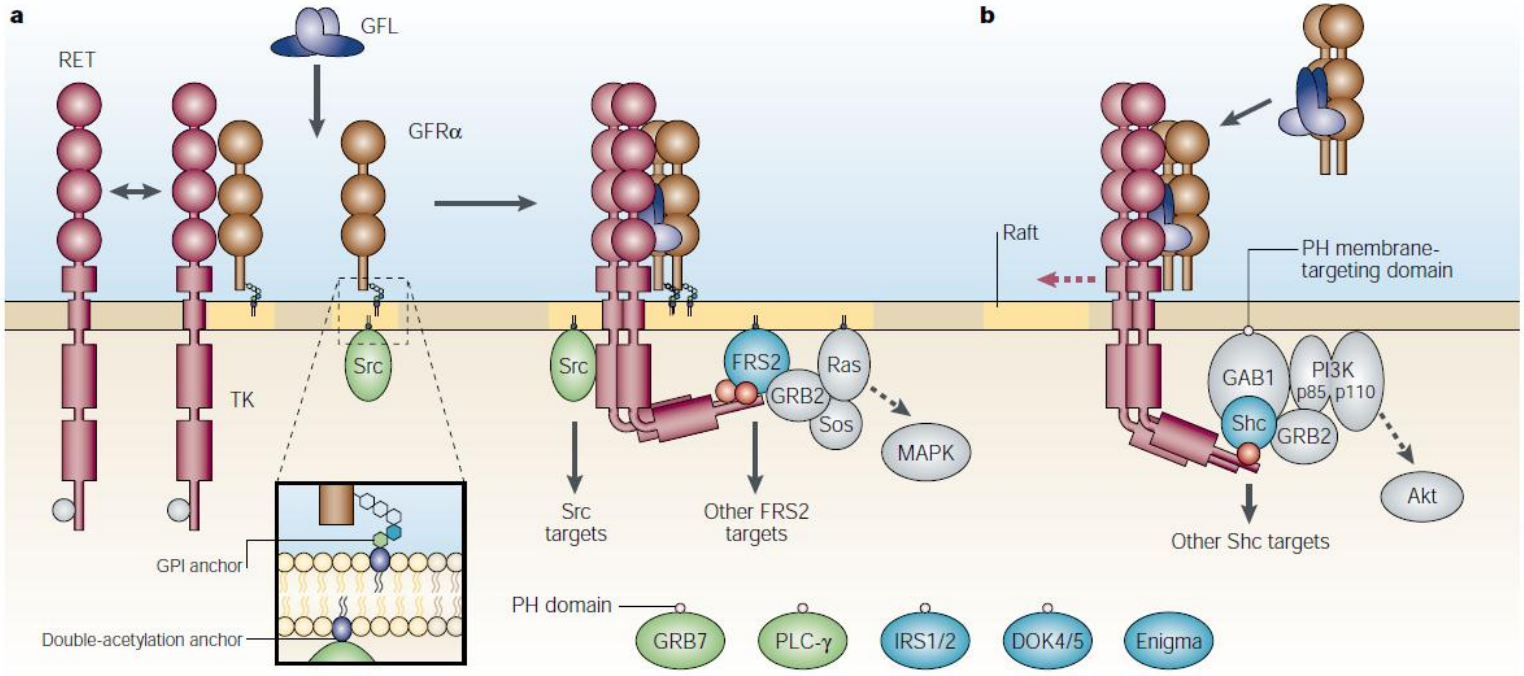

Figure 1.4- RET recruitment to the lipid rafts of the plasma membrane following GFL binding to its GFRα co-receptor.

(a) The inactive RET receptor normally resides outside of the lipid rafts where it interacts with a distinct set of signalling proteins. Recruitment to the lipid rafts by the GFL- GFRα complex not only induces dimerization and activation of RET, **(b)** but also exposes it to a different range of signalling proteins, and presumably allows RET to activate and signal through distinctly different pathways (Airaksinen and Saarma, 2002).

1.5.3 Signal Transduction

Functional GFLs exist as homodimers which bind first to their respective GFRα receptor and the RET receptor is later recruited to the complex, forming a heterohexamer on the cell surface (Murakumo et al., 2006; Sariola and Saarma, 1999). The schematic in Figure 1.4 is a somewhat simplified representation of this process, but illustrates how RET ligand specificity is determined by the GPRα co-receptors.

Activation of RET by GFLs triggers the dimerization of RET and autophosphorylation of specific tyrosine residues in the intracellular kinase domain. There are 12 tyrosines (Y) in total that are known sites for phosphorylation in RET, Y687, Y806, Y809, Y826, Y900, Y905, Y981, Y1015, Y1029, Y1062, Y1090 and Y1096 (Arighi et al., 2005; Murakumo et al., 2006). As shown in Figure 1.5, these residues provide the binding sites for numerous docking and adaptor proteins necessary for the activation of downstream signalling pathways, including RAS/ERK, PI-3K/Akt, p38MAPK and Rac/JNK (Besset et al., 2000; Veit et al., 2004; Worby et al., 1996). These pathways are important for the promotion of cell survival, differentiation, proliferation and motility. The specific residues required for each of these cellular activities depends on the cell type and also the isoform of RET that is expressed, although studies have demonstrated a requirement for the residues Y1062 and Y1096 and mutations at these sites abolished PI3K/AKT activation (Asai et al., 1996; Besset et al., 2000).

But the complexity of this signalling pathway does not end there. The different adaptor proteins involved mediate different cellular instructions, be it differentiation or proliferation. The specific GFL involved may trigger phosphorylation of different residues, but some residues are also capable of binding multiple different adaptor proteins (Murakumo et al., 2006). To illustrate this, the residue Y1062, when phosphorylated, binds the protein DOK4.

This is known to be an essential requirement in GDNF-dependent neutrite outgrowth. Mutations at this site result in the severe impairment of the enteric nervous system and in kidney hypoplasia. Intriguingly, phosphorylated Y1062 is known to also bind Shc, FRS2, IRS1/2 and enigma, activating other downstream signalling pathways for distinct biological outcomes (Murakumo et al., 2006; Salvatore et al., 2001).

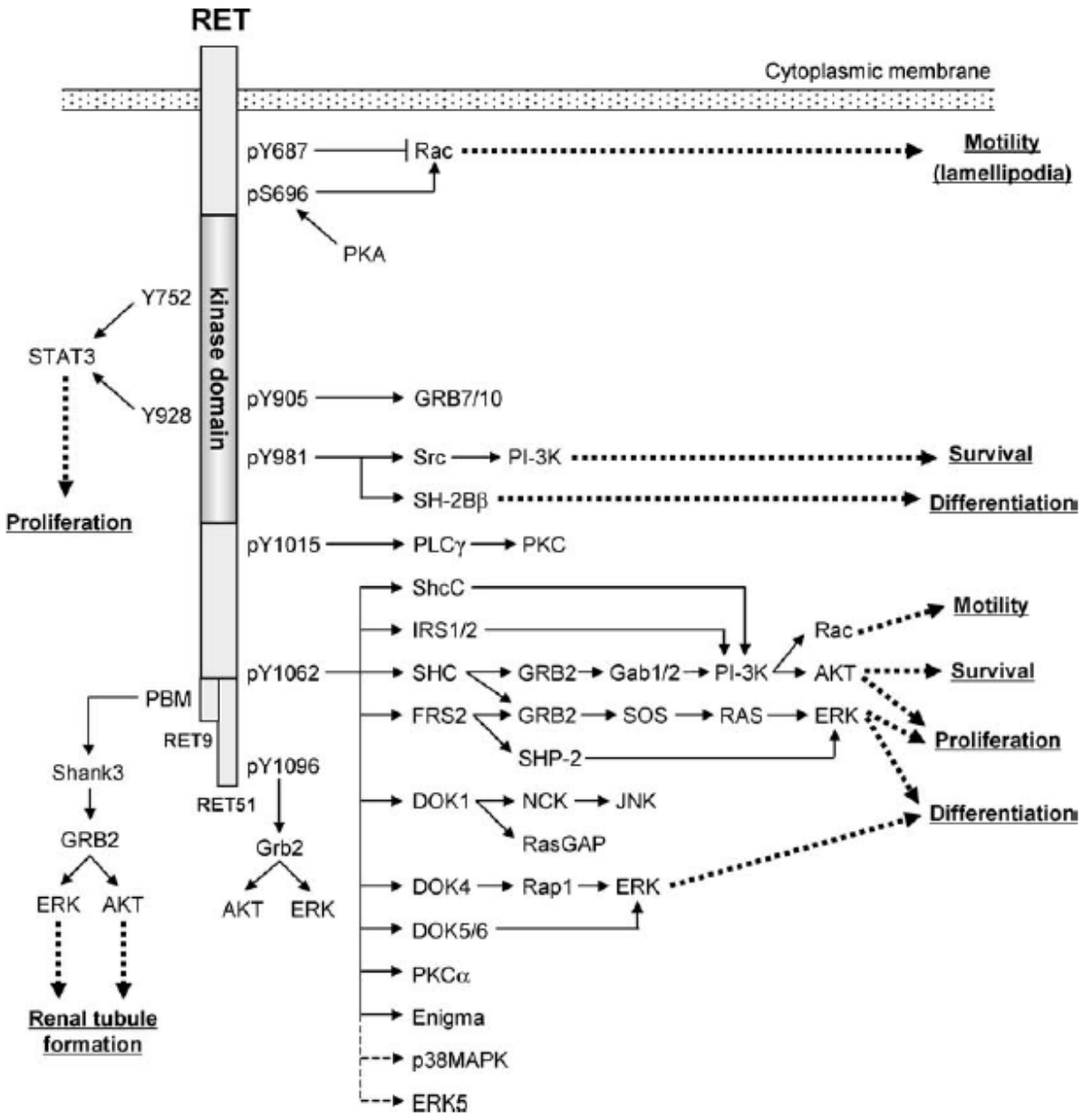

Figure 1.5- The multiple tyrosine phosphorylation sites of the RET receptor.
Each tyrosine residue binds to a unique set of docking and adaptor proteins which allow it to signal through different pathways, eliciting unique biological responses and outcomes (Murakumo et al., 2006).

Another interesting and confounding question that arises is that, as different GFLs signal through the same RET receptor, do these factors trigger qualitatively or quantitatively different signalling pathways and how? In cultured sympathetic neurons, GDNF, NRTN and ARTN, signalling through their cognate GFRα co-receptors, induce phosphorylation of the

same four key RET tyrosines (Y905, Y1015, Y1062 and Y1096) with similar kinetics, and may stimulate a similar profile of downstream signalling pathways (Airaksinen et al., 1999). Although, not all tyrosines in the RET cytoplasmic domain have been studied and other residues may be selectively recruited by each GFL. In addition, the GPI-anchor of the GFRα receptors localizes the GFL-GFRα complexes to the lipid rafts of the plasma membrane (Figure 1.4), whereas inactive RET resides outside these lipid rafts (Tansey et al., 2000). GFL-GFRα complex formation recruits the RET receptor to the lipid rafts, allowing the tyrosine kinase domain to interact with a potentially different set of adaptor docking proteins (Frêche et al., 2005).

1.5.4 Alternative Signalling Methods

Often in GFL signalling, RET and the respective GFRα receptors are not necessarily expressed in the same cell. This poses the question of how GFLs can successfully elicit the desired cellular response. One solution to this problem is the production of soluble GFRα receptors which do not contain the GPI anchor. The soluble receptor allows activation of GFL signalling in a *trans* fashion (Sariola and Saarma, 2003; Tansey et al., 2000).

There is also an increasing amount of evidence for RET-independent signalling. The canonical mechanism for GDNF signalling in cells is through binding to the RET-GFRα1 receptor complex (Airaksinen et al., 1999). However, in some neuronal cells, where GFRα1 is expressed, but RET is not, GDNF is still able to elicit a response, suggesting that GDNF is able to signal through GFRα alone. A completely novel receptor for GDNF was identified, allowing a RET-independent method of signalling. The neural cell adhesion molecule, NCAM, was demonstrated to bind GDNF and GFRα1, and activate downstream signalling

kinases (Paratcha et al., 2003). This led to decreased cell adhesion and increased cell motility. In support of this, NCAM knockout mice exhibit decreased GDNF-mediated neurite growth compared to wild-type. In addition, the transmembrane heparan sulfate proteoglycan, syndecan-3, was characterised in mice and demonstrated to interact with extracellular matrix immobilized GDNF, NTN and ARTN (Barnett et al., 2002; Bespalov et al., 2011). In *in vitro* and mouse models, syndecan-3 mediated signalling from immobilized, but not soluble GDNF, inducing cell migration and neutrite outgrowth. This alternative signalling model proposed binding of the GFLs by syndecan-3 creating a concentration effect of the immobile ligands, which were then presented to the classic GFRα-RET or -NCAM receptor complexes for downstream signalling activation (Bespalov et al., 2011).

1.5.5 Clinical Significance of GFL Signalling

As previously mentioned, RET mutations are the cause of a number of diseases in humans. Notably, constitutively active RET (gain-of-function mutations) are found in the majority of familial MEN2 cases and up to 50% of sporadic cases of thyroid cancers (Jhiang, 2000; Phay and Shah, 2010) and aberrant GFRα expressions can also manifest in various disease conditions (Wiesenhofer et al., 2000).

GFL misexpression in human diseases was less well known and initially, excitement over the discovery of GDNF lay in the neurotrophic factor's ability to enhance the survival of midbrain dopaminergic neurons in Parkinson's disease (Takahashi, 2001a). Parkinson's disease is characterised by massive degeneration of the dopaminergic neurons of the putamen and substania nigra (Backman et al., 2006). Most animal models recreate the condition with toxin-induced cell death in the concordant areas of the brain (Allen et al., 2013). GDNF has been tested in a range of rodent and primate models of Parkinson's disease, and was found to

have potent restorative effects. Patients treated with intraputamenal infusions of GDNF exhibited few side-effects and substantial improvement in their clinical condition (Gill et al., 2003).

Most follow-up studies to this have reported similar findings and efforts have largely changed to developing appropriate GDNF delivery systems (Allen et al., 2013; Patel and Gill, 2007). Due to the nature of the blood-brain barrier, proteins like GDNF cannot easily diffuse through it and clinical trials have involved direct infusions to the appropriate areas of the brain. Technical issues with delivery systems, such as catheter placement and design, are also issues that have affected GDNF bioavailability (Salvatore et al., 2006) and a lack of diffusion in the putamen has hampered the effectiveness of GDNF treatment for Parkinson's disease (Gimenez et al., 2011). Currently, several new methods are being trialled to overcome these issues including convection enhanced delivery, a form of direct infusion using microcatheters (Gimenez et al., 2011), and gene therapy using viral vectors, under trial in rhesus monkeys (Kells et al., 2010). The development of small molecule RET agonists and GDNF mimetics that can be administered systemically are also underway as another method of overcoming the issues with delivery (Bespalov and Saarma, 2007).

In terms of other neurological issues, it has been proposed that GFL-signalling could also be of use as a novel treatment for drug addiction (Messer et al., 2000; Takahashi, 2001a). In animal models, chronic administration of psychostimulant drugs, such as cocaine and morphine, induced neurobiological changes in the ventral tegmental area (VTA) of the brain. Delivery of GDNF into the VTA blocked these neuronal changes as well as blocking the rewarding effects of cocaine. However, the role of GDNF in this area is more complex, as delivery of GDNF to the VTA following cocaine withdrawal has also been shown to increase the time-dependent drug cravings in mice previously exposed to cocaine (Pickens et al.,

2011). Another notable effect of chronic cocaine and morphine administration was a decrease in the level of RET phosphorylation, suggesting a drug-mediated decrease in GDNF signalling in the VTA (Messer et al., 2000).

1.5.6 GFL signalling in Cancer

GFL signalling is becoming increasingly prominent in a wide spectrum of human diseases. In the field of cancer research, these signalling factors are gaining recognition as important participants in both tumourigenesis and tumour progression by driving cell transformation, growth and motility (Borrello et al., 2013; Jhiang, 2000; Phay and Shah, 2010). GFL members have been implicated in the development of various cancers of non-neuronal origins and considerable research efforts have been pledged to delineating their functions and effect on the cancer cell population.

The involvement of GDNF, RET and the GFRα1 receptors in MEN2, familial thyroid carcinoma and Hirschsprung's disease development have been well documented, and the dysfunction of this particular signalling pathway was identified as one of the genetic causes of these cancer syndromes (Jhiang, 2000). In human gliomas, GDNF and GFRα1 were shown to be significantly increased, with expressions levels up to 5 times greater that of normal glial cells (Wiesenhofer et al., 2000). GDNF has also been reported to promote the proliferation and survival of neuroblastoma and glioma cells, and GDNF activity was found to be directly correlated with the invasive capacity (Song and Moon, 2006; Veit et al., 2004). Increased GDNF activity is also identifiable in a number of different malignancies of non-neuronal origin, including pancreatic (Funahashi et al., 2005; Funahashi et al., 2007; Funahashi et al., 2003; Okada et al., 2003; Shimizu et al., 2004), mammary (Esseghir et al., 2007), small cell and non-small cell lung carcinomas (Garnis et al., 2005; Mulligan et al.,

1998), ovarian cancers (Aravindakshan et al., 2006), pituitary adenomas (Japon et al., 2002) and various other cancer types. A number of studies have been published on the effect GDNF has in promoting invasion and metastasis of pancreatic cancer cells, which occurs through an increase in the expression and activity of matrix metalloproteinase-9 (Okada et al., 2003), and urokinase-type plasminogen activator (Funahashi et al., 2007).

Similarly, ARTN has also been shown to have the same metastasis promoting effects in pancreatic cancer (Ceyhan et al., 2006c). However, unlike in neuronal cell lines and neuronal cancers, ARTN expression did not induce proliferation in pancreatic cancer cells. This could be due to innate differences between the different cell lines and suggests that ARTN may have a stronger influence on cell invasion and migration (Ceyhan et al., 2006c).

More recently, ARTN was also demonstrated to be oncogenic in mammary, endometrial and lung carcinoma cell lines (Banerjee et al., 2011; Kang et al., 2009; Pandey et al., 2010b; Tang et al., 2010). It was found to promote cell proliferation in some cell lines and enhance cell survival. ARTN also promoted a number of other aspects of cell behaviour that could encourage tumour progression, including cell motility and invasion, angiogenesis and *de novo* resistance to chemotherapeutic agents, radiotherapy, endocrine therapy and trastuzumab (Banerjee et al., 2012a; Banerjee et al., 2012b; Ding et al., 2014; Kang et al., 2010).

PSP was the most recent GFL to be implicated in cancer development. A recent study found *PSP* mRNA and protein to be significantly elevated in oral squamous cell carcinomas compared to normal oral keratinocytes (Baba et al., 2013). In this study, PSP expression correlated with increased cell proliferation *in vitro,* and immunohistochemical staining in clinical samples found PSP expression to correlate with tumour size. Increased PSP signalling in oral squamous cell carcinomas was also confirmed by increased RET receptor phosphorylation and activation of the MAPK signalling pathway (Baba et al., 2013).

Interest in the GFLs' mutual receptor, RET, and its activation in various cancers and cancer syndromes have led some groups to investigate the possibility of specific targeting of RET receptor function for cancer treatment (Mologni, 2011). Particularly in medullary thyroid cancers (MTC), where RET mutations account for a large proportion of cases, up to 50% of sporadic cases and virtually all genetically inherited cases (Phay and Shah, 2010). Trials of existing small molecule kinase inhibitors that have some specificity for RET have so far been somewhat successful, with a number of agents currently in early clinical trials. The inhibitor vandetinib, originally designed as a dual VEGF/EGFR inhibitor (Natale, 2008), was shown to be well tolerated and in a phase III trial increased the progression-free survival in MTC patients (Wells et al., 2011).

1.6 IGF-1 Signalling

Insulin-like growth factor 1 (IGF-1) is a potent mitogenic and anti-apoptotic signal for a wide range of cell types (Ahmed and Farquharson, 2010). There is ample evidence to indicate that IGF-1 is heavily involved in bodily growth of mammals. High serum IGF-1 levels in children and adolescents are strongly correlated with greater bodily growth and a larger body mass, whereas abnormally low serum IGF-1 is associated with diminished growth and dwarfism (Baserga et al., 1997; LeRoith et al., 1991).

As the name suggests, IGF-1 bears a high level of homology with insulin. The *IGF-1* gene is mapped to chromosome 12q23.2 (Hoppener et al., 1985). In humans, the majority of circulating IGF-1 is produced in the liver (Ahmed and Farquharson, 2010; Laron, 2001). The mature IGF-1 protein consists of 79 amino acids and is held in confirmation by 3 disulfide bonds (Baserga et al., 1997). In the bloodstream, IGF-1 is bound to IGF binding proteins, which modulate its bioavailability to receptors, but the exact function of which is not entirely clear (Baserga et al., 1997; Rubin and Baserga, 1995), although tumour suppressive functions have been proposed (Baxter, 2014; Subramanian et al., 2007). Signalling by IGF-1 is mediated largely by high affinity binding to the IGF-1 receptor (IGF-1R), although it is also capable of binding to the insulin receptor with considerably lower affinity (Baserga et al., 1997; Butler et al., 1998).

1.6.1 IGF-1R Structure and Function

The IGF-1R is a transmembrane receptor that exhibits high homology to the insulin receptor (approximately 70%), with the highest level of homology seen in the tyrosine kinase domain (Bähr and Groner, 2005; Baserga et al., 1997). In the inactive form, the receptor exists as a monomer. Each receptor monomer itself consists of an extracellular α subunit, which

contains the ligand-binding domain, and a transmembrane β subunit containing the intracellular tyrosine kinase and carboxy-terminal protein-interacting domains (Bähr and Groner, 2005).

The receptor binds to its primary ligand IGF-1 with very high affinity (Bähr and Groner, 2005). It is also able to bind insulin and insulin-like growth factor 2, but with considerably lower affinities (Bähr and Groner, 2005). When bound to IGF-1, the receptor monomers dimerize and undergo autophosphorylation at specific tyrosine residues. The first residues to be phosphorylated are Y1121, Y1135 and Y1136 (Butler et al., 1998). These three residues appear to be critical to the kinase activity of the IGF-1R, and deletion of any of these residues significantly diminishes the IGF-1R signalling capability (Butler et al., 1998). Phosphorylation of these tyrosines allows the carboxy-terminal to interact with its downstream signalling proteins through the recruitment of insulin-receptor substrates 1 and 2, Shc and Grb10 to the receptor complex (Bähr and Groner, 2005; Baserga et al., 1997; Richards et al., 1996). This inevitably leads to the activation of the mitogen activated protein kinase (MAPK) signalling pathway eventually leading to the up-regulation of Ras (Migliaccio et al., 1996). The MAPK pathway is critical to the proliferating cell and the MAPK enzyme is often over-expressed in mammary carcinoma cells (Sivaraman et al., 1997). Activation of this pathway results in an increase in the level of c-Myc and cyclin D1 protein expression, which are required for progression through the G1 checkpoint of the cell cycle and to enter S-phase, as shown in the overview of Figure 1.6 (Mawson et al., 2005).

Several other signalling pathways are also activated by IGF-1R signalling, including the phosphatidyl-inositol 3 -kinase (PI3K) pathway (Bähr and Groner, 2005; Kahlert et al., 2000b; Mawson et al., 2005). Activation of the PI3K pathway also up-regulates the expression of c-Myc and cyclin D1, further encouraging progression through the cell cycle

(Adesanya et al., 1999; Fresno Vara et al., 2004; Kahlert et al., 2000b; Mawson et al., 2005). Though similar to the MAPK pathway, which increases the cyclin D1 expression level, the PI3K pathway also has the ability to enhance the protein stability allowing them to persist for longer. Activated PI3K does this by inhibiting glycogen synthase kinase 3 (GSK-3), which under normal cell conditions phosphorylates cyclin D1 to increase protein degradation. Inhibiting GSK-3 action allows cyclin D1 to persist in the cell for longer to promote progression of the dividing cell through the cell cycle (Kahlert et al., 2000b; Mawson et al., 2005).

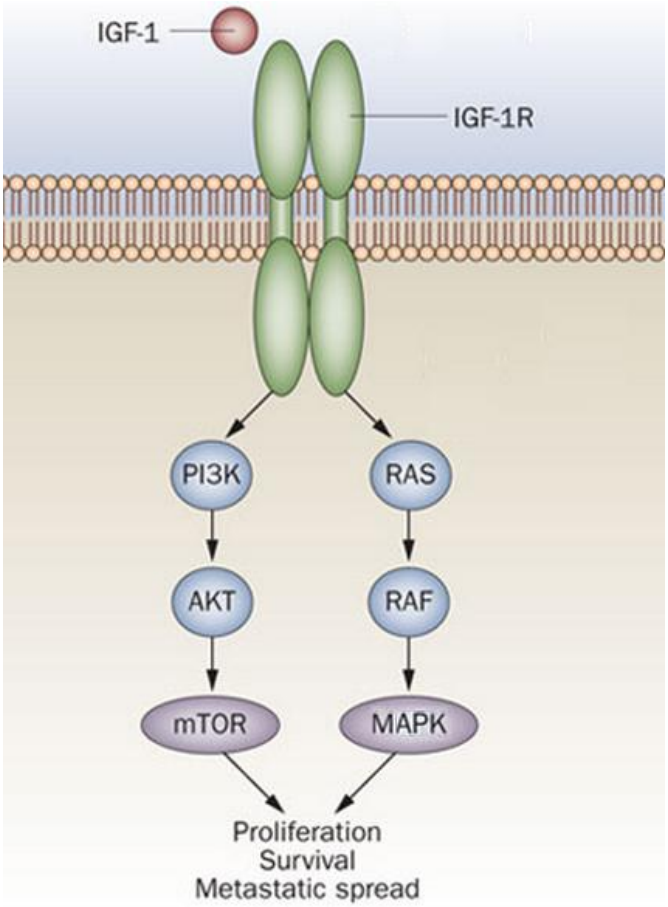

Figure 1.6- The IGF-1 signalling pathway.

Unbound IGF-1 is free to interact with the IGF-1R inducing dimerization and autophosphorylation of tyrosine residues. Subsequent binding and recruitment of adaptor proteins leads to activation of the MAPK and PI3K pathways and induction of the cellular responses depicted (Janku et al., 2010).

1.6.2 IGF-1R in Cancer Therapy

A great number of breast tumours exhibit up-regulation of IGF-1R, but unlike other growth factor receptors, such as ER or HER2, the prognostic significance of IGF-1R is less certain (Bähr and Groner, 2005; Bartucci et al., 2001; Creighton et al., 2008; Shimizu et al., 2004). Instead its significance is in the promotion of metastasis in breast tumours (Lopez and Hanahan), similar to what has been observed in ARTN over-expression. IGF-1 signalling is known to affect cell motility and migration *via* integrin signalling (Shen et al., 2006). The effect is increased E-cadherin and catenin phosphorylation, leading to decreased cell adhesion, and, increased cell mobility, which is necessary for metastasis. In addition to increasing cell motility, IGF-1R stimulation is also a regulator of angiogenesis (Oh et al., 2002; Shigematsu et al., 1999). It is able to up-regulate the expression of pro-angiogenic signalling molecules and growth factors, such as vascular endothelial growth factor and matrix metalloproteinase 2, which contribute greatly to cancer metastasis (Bähr and Groner, 2005) and the targeting of IGF-1R results in potent inhibition of angiogenesis *in vitro* (Bid et al., 2012). But most importantly, cells which do not express IGF-1R cannot be transformed (Baserga, 2004).

Many cancer cells also display aberrant IGF-1 signalling, through altered expressions of signalling components in the pathway, or mutations if the signalling mediators (Tognon and Sorensen, 2012), and both IGF-1 and IGF-1R expression are often found to be significantly elevated in cancerous cells and tissues (Rinaldi et al., 2006; Tamimi et al., 2011; Werner and Sarfstein). Quite simply, because IGF-1 signalling can be a powerful supporter of cell proliferation, growth and survival, gain of function mutations in this pathway are advantageous to cancerous development. The downstream mediators of IGF-1 signalling,

MAPK and PI3K are often found to contain mutations causing constitutive activation (Fresno Vara et al., 2004; Santen et al., 2002; Whyte et al., 2009).

A growing body of evidence also implicates IGF-1 signalling in mediating resistance to microenvironmental stresses (Peretz et al., 2002) and cytotoxic chemotherapy agents (Dunn et al., 1997; Gooch et al., 1999; Mitsiades et al., 2004; Weroha and Haluska, 2008). In the MCF-7 cell line, IGF-1 treatment increased the survival of cells exposed to doxorubicin and paclitaxel (Weroha and Haluska, 2008). In doxorubicin-treated cells, the addition of IGF-1 not only decreased the proportion of apoptotic cells, but also induce proliferation (Gooch et al., 1999). These powerful proliferative and anti-apoptotic signals are thought to be mediated through the induction of the PI3K pathway (Gooch et al., 1999). Similar results have been achieved using other cell lines, such as sarcomas and hepatocellular carcinoma, further indicating that IGF-1 signalling can promote resistance to cytotoxic agents (Weroha and Haluska, 2008).

Much of the current research into IGF-1R delves into its usefulness as a target for cancer therapy. Approaches using siRNA and antibodies targeting IGF-1R had shown considerable promise in preclinical trials (Bähr and Groner, 2005; Mitsiades et al., 2004). Inhibition of IGF-1R expression by these methods resulted in decreased proliferation, cell survival and metastasis (Girnita et al., 2008; Long et al., 1995). An increase in tumour sensitivity to conventional cancer treatments has also been reported and anti-IGF-1R antibodies reportedly elicited a reversion of the transformed phenotype in MCF-7 cells (Bähr and Groner, 2005; Baserga, 2004).

A number of those agents made their way to clinical studies, initially showing signs of promise and most were well tolerated. The most common toxicities reported have been mild to moderate hyperglycemia (20%), mild skin toxicities and fatigue (Rieder et al., 2011;

Rodon et al., 2008). However, the majority of these agents failed to demonstrate any significant effect improving the survival of treated patients, either as stand-alone therapeutic agents or in combination with traditional chemotherapy regimens (Chen and Sharon, 2013; Yee, 2012).

The most extensively studied agent is CP-751,871, a human, monoclonal antibody (mAb) developed by the pharmaceutical giant, Pfizer, to target IGF-1R (Lacy et al., 2008; Weroha and Haluska, 2008). Results from those clinical trials showed that CP-751,871 had little efficacy as a single agent; in squamous cell carcinoma. It had a relatively mild toxicity profile and appeared to have no dose-limiting toxicities. At the maximal dosage, a number of patients experience stabilization of disease (Weroha and Haluska, 2008). Encouragingly, early clinical trials combining CP-751,871 with carboplatin for the treatment of non-small cell lung cancer (NSCLC), the addition of CP-751,871 greatly increased the response rate to treatment compared to carboplatin alone (Weroha and Haluska, 2008). The agent was taken to stage III trials before the study was eventually terminated. The high response rates in the earlier trials could not be replicated and it was apparent that CP-751,871 did not improve treatment response rates compared to treating with carboplatin alone (Yee, 2012).

Other anti-IGF-1R mAbs were also trialled in combination with standard cancer treatments but the results were, again, disappointing (Chen and Sharon, 2013). Clinical trials of different mAbs in combination with tamoxifen for ER-positive breast cancers or EGFR for pancreatic cancer and NSCLC, failed to demonstrate any increase in endpoint improvement (Chen and Sharon, 2013; Karp et al., 2009; Rieder et al., 2011).

Small molecule inhibitors to IGF-1R kinase activity have also been developed, but since these are usually targeted to the tyrosine kinase domain, cross inhibition of the insulin receptor (IR) is a concern, due to the highly conserved structure of the region, shared by both

receptors (Chitnis et al., 2008). Several tyrosine kinase inhitiors (tyrphostins) with greater selectivity for IGF-1R have been developed and are making their way through preclinical trials. The inhibitor BMS-754807, manufactured by Bristol Myers Squibb demonstrated promising preclinical activity and is currently under evaluation in phase II clinical trials for the treatment of hormone responsive breast cancer (Lim et al., 2013).

1.6.3 IGF-1R signalling cross-talk with other pathways

As described in the previous sections, IGF-1 primarily signals by binding to the IGF-1R and inducing receptor dimerization, leading to autophosphorylation of the tyrosine residues in the β-subunit (Butler et al., 1998). The signal is then mediated through the recruitment of the downstream pathways- MAPK or PI3K/AKT (Kahlert et al., 2000a; Migliaccio et al., 1996). IGF-1/IGF-1R is prolific for its ability to cross-talk with other pathways, through the recruitment of the downstream MAPK and PI3K pathways, and also by heterodimerization with other tyrosine kinase receptors forming a hybrid receptor complex (Liu et al., 2014; Sachdev and Yee, 2007). Signalling pathways known to interact with IGF-1R include: EGFR (Morgillo et al., 2006), IR (LeRoith and Yakar, 2007; Sachdev and Yee, 2007), androgen receptors (Zhu and Kyprianou, 2008), ER (Fagan and Yee, 2008; Skandalis et al., 2013) and the human growth hormone receptor (Laron, 2001).

This heterodimerization can have profound effects on cell growth and resistance to endocrine therapy (Morgillo et al., 2006; Tognon and Sorensen, 2012). In the case of EGFR, hybridising with IGF-1R can allow receptor transactivation in the 2 pathways, allowing EGFR to be activated by IGF-1 ligand, and stimulating cell proliferation and survival processes in those cells (King and Wong, 2012). In addition, EGFR/IGF-1R hybrids can aid in circumventing specific inhibition by endocrine therapies, enhancing resistance to therapy.

Under normal circumstances, inhibition of EGFR by the small molecule inhibitor, erlotinib, results in cell cycle arrest and increased cell death (Huether et al., 2005). However, it has been demonstrated in cells over-expressing IGF-1R, the effectiveness of erlotonib inhibition is reduced (Camirand et al., 2005). Inhibition of EGFR by erlotinib has also lead to up-regulation of IGF-1R expression and phosphorylation in mammary carcinoma cell lines. Co-treatment with the IGF-1R inhibitor, AG1024, was shown to reverse this, enhancing the effects of inhibitory effects of erlotinib, decreasing cell proliferation and increasing apoptosis in those cells (Camirand et al., 2005).

IGF-1R is also known to interact and cross-talk with the ER, enhancing the mitogenic effects of estrogen and increasing resistance to anti-estrogens (Fox et al., 2013; Zhang et al., 2011). In mammary carcinoma cells, exogenous IGF-1 treatment can alleviate the inhibitory effects of tamoxifen or fulvestrant, through activation of the downstream MAPK and PI3K pathways (Zhang et al., 2011). In addition to collaborating with signalling, both receptors are also capable of direct transactivation allowing downstream signalling in both the IGF-1R and ER pathways despite the absence of either IGF-1 or estrogen (Kahlert et al., 2000a; King and Wong, 2012; Klotz et al., 2002).

1.7 Conclusion and Study Rationale

Breast cancer has become an increasingly serious threat to public health. It is a disease influenced by both genetic and environmental components, including a number of controllable lifestyle factors that can contribute to the breast cancer risk. However, our knowledge is far from complete and strategies for prevention are limited.

ARTN is a neurotrophic factor that is heavily involved in the development of the sympathetic and parasympathetic nervous systems. Its innate ability to rescue neurons from apoptosis has captured the attention of researchers for its potential as a novel treatment for psychiatric disorders, and its potent proliferative effect on neuroblastoma cells earmarked it as a potential new oncogene.

A number of published studies indicate that the over-expression of ARTN in mammary carcinoma and endometrial carcinoma cell lines could contribute greatly to cancer progression (Kang et al., 2009; Pandey et al., 2010b). In addition, preliminary results suggest that ARTN signalling in mammary carcinoma cells may be intimately involved with IGF-1 signalling as well. This project endeavours to dissect and uncover the nature of their signalling involvement.

The aim of the current project is to determine whether ARTN expression is regulated in response to IGF-1 stimulation and whether ARTN is able to mediate some of the transforming effects of IGF-1. This project will highlight the involvement of IGF-1 signalling in breast cancer development, and postulate a potential mechanism for ARTN-mediated oncogenic transformation/progression in mammary epithelial cells.

Ultimately, we hope to further the understanding of how such genetic processes perturb cellular functions and networks to give rise to cancer, and to provide novel tools for cancer diagnosis and the development of more effective cancer treatments.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

Material	Supplier
1kb plus DNA ladder	Invitrogen Life Technologies, Carlsband, CA, USA
3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)	Sigma Chemical Company, St Louis, MO, USA
Acrylamide/Bis solution (40%)	Bio-Rad laboratories, Inc., Hercules, CA, USA
Agarose (ultra pure)	Invitrogen Life Technologies, Carlsband, CA, USA
alamarBlue	Sigma Chemical Company, St Louis, MO, USA
Ammonium persulfate (APS)	Serva Electrophoresis GmbH, Heidelberg, Germany
Ampicillin	Sigma Chemical Company, St Louis, MO, USA
Anti-β-actin mouse monoclonal antibody (A5316)	Sigma Chemical Company, St Louis, MO, USA
Recombinant Artemin protein (2589-AR-025)	R&D Systems, Minneapolis, MN, USA
Bacto tryptone	BD Biosciences, Franklin lakes, NJ, USA
Bacto Yeast extract	BD Biosciences, Franklin lakes, NJ, USA
Bromophenol Blue	Sigma Chemical Company, St Louis, MO, USA
Bovine serum albumin (BSA)	Sigma Chemical Company, St Louis, MO, USA
Bradford reagent concentrate	Bio-Rad laboratories, Inc., Hercules, CA, USA
Chloroform	Scharlau Chemie SA, Barcelona, Spain
cOmplete mini, EDTA-free protease inhibitor cocktail tablets	Roche Diagnostics GmbH, Mannheim, Germany
Dimethyl-sulphoxide (DMSO)	Sigma Chemical Company, St Louis, MO, USA

Disodium Phosphate	Sigma Chemical Company, St Louis, MO, USA
Dithiothreitol (DTT)	Sigma Chemical Company, St Louis, MO, USA
DC protein assay reagents	Bio-Rad laboratories, Inc., Hercules, CA, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma Chemical Company, St Louis, MO, USA
Ethanol (absolute, analytical grade)	Scientific Supplies Ltd, Auckland, New Zealand
Ethidium bromide	Sigma Chemical Company, St Louis, MO, USA
FAST 3,3 diaminobenzidine hydrochloride (DAB) tablets	Sigma Chemical Company, St Louis, MO, USA
Fetal Bovine Serum (FBS)	Invitrogen Life Technologies, Carlsband, CA, USA
FuGENE 6 and FuGENE HD Transfection reagents	Roche Diagnostics GmbH, Mannheim, Germany
GAPDH AlphaScreen SureFire Assay kit	TGR BioSciences Pty Ltd, Adelaide, Australia
Geneticin/G418 (Gibco)	Invitrogen Life Technologies, Carlsband, CA, USA
Glutamax (Gibco)	Invitrogen Life Technologies, Carlsband, CA, USA
Glycerol	Sigma Chemical Company, St Louis, MO, USA
Glycine	Applichem GmbH, Darmstadt, Germany
Hoechst 33258	Sigma Chemical Company, St Louis, MO, USA
Human recombinant Insulin-like growth factor 1 (IGF-1)	Sigma Chemical Company, St Louis, MO, USA
Hydrochloric acid	Scientific Supplies Ltd, Auckland, New Zealand
Isopropanol	Scientific Supplies Ltd, Auckland, New Zealand
L-glutamine (Gibco)	Invitrogen Life Technologies, Carlsband, CA, USA
Lipofectamine 2000	Invitrogen Life Technologies, Carlsband, CA, USA
Magnesium Chloride	Scientific Supplies Ltd, Auckland, New Zealand
Matrigel	BD Biosciences, Franklin lakes, NJ, USA
Methanol	Scientific Supplies Ltd, Auckland, New Zealand
Mercaptoethanol	Sigma Chemical Company, St Louis, MO, USA
Paraformaldehyde	Sigma Chemical Company, St Louis, MO, USA
PathScan Phospho-IGF-1 Receptor β (Tyr1131) sandwich ELISA kit	Cell Signaling Technology, Inc, Danvers, MA, USA
PathScan Phospho-RET (pan Tyr) Sandwich EKLISA kit	Cell Signaling Technology, Inc, Danvers, MA, USA

Penicillin (1000U/ml)	Invitrogen Life Technologies, Carlsband, CA, USA
PVDF membrane	Bio-Rad laboratories, Inc., Hercules, CA, USA
Qiagen OneStep RT-PCR kit	Biolab Scientific Ltd, NewZealand and Australia
Qiagen plasmid mini and maxi prep kits	Biolab Scientific Ltd, NewZealand and Australia
RPMI 1640 medium (Gibco)	Invitrogen Life Technologies, Carlsband, CA, USA
Seeblue plus2 protein marker	Invitrogen Life Technologies, Carlsband, CA, USA
Sodium dodecyl sulfate (SDS)	Invitrogen Life Technologies, Carlsband, CA, USA
Sodium Phosphate	Scientific Supplies Ltd, Auckland, New Zealand
Streptomycin (1000µg/ml)	Invitrogen Life Technologies, Carlsband, CA, USA
Supersignal west Dura extended duration substrate	Pierce Biotechnology, Inc., Rockford, Illinois, USA
Tetramethyl-ethylendiamin (TEMED)	Sigma Chemical Company, St Louis, MO, USA
TRIzol reagent	Invitrogen Life Technologies, Carlsband, CA, USA
Tris(hydroxymethyl)aminomethane (Tris)	Serva Electrophoresis GmbH, Heidelberg, Germany
Triton X-100	Sigma Chemical Company, St Louis, MO, USA
Tryphan Blue (Gibco)	Invitrogen Life Technologies, Carlsband, CA, USA
Trypsin	Invitrogen Life Technologies, Carlsband, CA, USA
Tween-20	Serva Electrophoresis GmbH, Heidelberg, Germany
Vecta-Stain Elite ABC kit mouse IgG	In Vitro technologies, New Zealand

Table 2.1- List of chemicals and reagents, and their suppliers

2.1.2 Media, solutions and buffers

The formulations and final compositions of the different culture media, solutions, and buffers are outlined in the Appendix.

2.2 General cell culture

2.2.1 Human cell lines

The human mammary carcinoma cell lines, MCF-7, T47D and ZR-75-1 were obtained from the American Type Culture Collection (Manassas, VA, USA). The MCF-7 and T47D cell lines over-expressing ARTN were generated by Dr. Jian Kang, during her PhD candidacy at the Liggins Institute, University of Auckland, NZ (Kang et al., 2009). All cell lines used were maintained using ATCC recommended conditions and cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, 100IU/ml penicillin, 100μg/ml streptomycin, and 2mM L-Glutamax. Penicillin and streptomycin were added to the media to discourage bacterial infection. Cells were grown in Greiner tissue culture treated flasks/plates at 37ºC in a humidified 5% CO_2 incubator. All tissue culture work was undertaken in a laminar flow hood under sterile conditions.

Cell Line	Description
MCF-7	Isolated by CM McGrath. Breast cancer cell line derived from a pleural effusion of an infiltrating ductal carcinoma. The cell line is well differentiated, epithelial, ER positive and non-invasive.
T47D	The T47D line was isolated by I. Keydar from a pleural effusion obtained from a 54 year old female patient with an infiltrating ductal carcinoma of the breast. This differentiated epithelial sub-strain is ER positive.
ZR-75-1	Deposited by LW Engel from ascites from the mammary duct of a 63 year old Caucasian patient with ductal carcinoma of the mammary gland. ER positive.

Table 2.2- Characteristics of human cell lines

2.2.1.1 Passaging and harvesting of human cell lines

All cell lines were grown in 75cm^2 culture flasks containing 10-20mL of recommended growth media until cells were 80-85% confluent, after which a portion was passaged into a new flask to allow continuation of stock cultures. Cells were passaged by first removing the media and subsequently rinsing the cells with PBS. Trypsin/EDTA (2mL) was then added and the flask and incubated at 37ºC/5% CO_2 for 2-5min. Cells were checked under the microscope to ensure all had detached from the flask and that the cells were monodisperse. 8ml of media was then added to the flask and the surface rinsed using a plastic pipette. The cells and media were subsequently transferred to a 10mL sterile tube and centrifuged for 5min at 1000rpm. Following centrifugation, the media was aspirated and the pellet resuspended in fresh media. For further culturing of stock cultures, an appropriate cell number was seeded into a fresh culture flask and approximately 10-20mL fresh media added, after which the cells were maintained in a 37ºC/5% CO_2 incubator.

Cell counting:

20µL of the cell suspension (total volume 10 mL media) was transferred to an Eppendorf tube and mixed with 180µL of trypan blue dye (0.4%). Cell counts were then carried out using a haemocytometer. The number of cells contained in 4 quadrants of 16 squares was counted (twice). The counted squares were then added together and averaged, upon which the resulting number was used to determine the amount of cells/mL using the following formulae:

(Cells per 4 quadrants/4) X 10000 X dilution factor = cells /mL

Cells/mL X final volume = total no. of cells

2.2.1.2 Storage of cell lines

Cells were trypsinised and resuspended in 10mL media, counted and then centrifugated. Following removal of the supernatant, cells were resuspended in freezing media. Aliquots of the cell suspension (1mL) were placed into each cryogenic vial. All vials were placed into an isopropanol containing freezing chamber and placed into a -80°C freezer for 24 hours to allow gradual cooling and freezing for cell preservation. Eventually, frozen cells were stored in the vapour phase of liquid nitrogen for long-term storage.

2.2.1.3 Revival of cell lines from liquid nitrogen storage

Cell aliquots from each cryogenic vial were thawed immediately in 10mL of 37°C serum-supplemented culture media, transferred into a $25cm^2$ tissue culture flask and cultured at 37°C (NAPCO Series 5400 CO_2 Incubator, Forma Scientific, Marietta, OH, USA). Media was changed the next day (to remove DMSO and dead cells) and every two days to allow growth of revived cells.

2.2.2 Stable Cell line Transfection

For stable transfection, cells were initially plated at approximately 60-70% confluence into a $75cm^2$ flask so that the cells would be in log phase growth at the time of transfection. ZR-75-1 cells (10^7cells) were transfected by electroporation using the Electro Square Porator™ ECM® 830 (BTX Harvard Apparatus, SanDiego, CA) in 400µL of PBS, with the following settings: 140V, 70msec, 4mm gap cuvette and 20µg of linearised plasmid. Cells were transfected with either the *ARTN*-containing plasmid (pIRESneo-ARTN) or an empty pIRESneo vector (pIRESneo-Vec) as a control. Following transfection, cells were plated in $75cm^2$ flasks in full serum RPMI and allowed to attach and recover for 48 hours. These cells

were respectively designated ZR751-ARTN and ZR751-Vec. Pooled stable transfectants were selected with RPMI media, containing 1000μg/mL G418 for 21-28 days. Initially when cell death was maximal, the media was changed every 2 days. Following that, the media was changed every 3-4 days. After 3-4 weeks, cells were trypsinised and then transferred into new 75cm^2 flasks. Cell lines were expanded and 10 vials of each cell line were frozen. Cells were maintained in culture for a maximum of four months, following which a new vial was revived to avoid clonal selection.

2.3 Cell function assays

Total cell number assay

Cells were trypsinised and seeded into 6-well cell culture plates at a density of 10,000cells/well. Cells were cultured in complete media for at least 8 hours at 37°C in 5% CO_2 to ensure that they attached to the culture surface. Cell culture media was then changed into 0.2% FBS (low serum) supplemented media and treated as described below. Assays were set up in triplicate and cells were counted every 2 days over a 10 day period. Cells in each well were trypsinised with 0.5% trypsin and collected in 15mL Falcon® tubes, separately. Cells were centrifuged at 1000rpm for 5min to remove the supernatant and collect the cell pellets. Cells were then resuspended in 500μL of serum-free media and counted using a haemocytometer.

2.3.1 MTT cell viability assay

Cells were plated into 96-well plates, at different concentrations (T47D-stables and wild-type cells 3000cells/well; ZR751 wild-type cells 5000 cells/well; MCF7-stables, and wild-type

cells 3000 or 5000cells/well) in serum-free RPMI media with treatment. After 2 days the cells were incubated with 0.45mg/mL MTT solution made up in serum free media at 37°C for 4h to allow formation of the blue fromazan crystals. Once formation of the formazan crystals was apparent, the MTT solution was aspirated and the cells solubilised in 100µL DMSO. The absorbance was read at 570nm and 690nm.

2.3.2 AlamarBlue assay for cell viability

MCF-7 cells were plated into 96-well plates at a density of 5000cells/well and allowed to attach and recover overnight in full RPMI media. The culture media was then carefully replaced with the appropriate treatment media and incubated at 37°C. At the indicated timepoints, the cells were incubated with 50µg/mL alamarBlue in RPMI media, for 3 hours and the fluorescence measured.

2.3.3 Bromodeoxyuridine (BrdU) assay

Entry into S-phase of the cell cycle was directly assayed by measuring the incorporation of 5-bromo-2-deoxyuridine.

BrdU Labelling: Cells were trypsinised and seeded in triplicate into 6-well cell culture plates at a density of 200,000-250,000cells/well. Cells were incubated overnight at 37°C in 5% CO_2, and after 12 hours the media was removed and the cells washed 3 times with PBS. Cells were then incubated in serum-free media for 18-20 hours. After incubation, 10µM BrdU (2µL of 10mM BrdU in 2mL of serum-free media) was added to each well of the 6-well plate. Following 30min incubation at 37ºC, media was removed and washed 3 times with 1X PBS.

Fixing: Cells were fixed in 4% formaldehyde diluted with PBS for 30min at 4ºC. Following 3 washes with PBS, 1mL of freshly made 3% H_2O_2 was added to each well and plates were incubated on a rocker at room temperature for 30min. At the end of incubation, H_2O_2 was removed and wells were washed with PBS. Cells were then incubated with 2N HCl (1mL per well) on a rocker at room temperature for 1 hour. After removing HCl, cells were washed twice with 0.1M borate solution followed by a 5min PBS wash.

Blocking and Incubation with Antibodies: Cells were incubated with 1mL per well blocking solution for 1 hour at room temperature on a rocker and washed with PBS again before the overnight incubation with 1mL per well of primary antibody (mouse anti-BrdU antibody ; NA20-100UG, Oncogene Research Products or Roche 11170376001) solution at 4ºC. The Vectastain® Elite® ABC Kit was used in the following steps that included an 8 hour incubation with 1mL per well of secondary antibody solution at 4ºC and an overnight incubation with 1mL per well of tertiary antibody solution at 4ºC. All these incubations with different antibodies were performed with gentle agitation followed by 3 × 15min washes with 1X PBS.

Staining: The substrate solution was prepared by dissolving FAST 3, 3-Diaminobenzidine hydrochloride (DAB) tablets in MilliQ water. 400µL was added to each well and colour development monitored. Staining was stopped with a 1X PBS wash. Finally, cells were fixed with ethanol and stored at 4ºC until counted.

Determination of the BrdU Labelling Index: For each well of 6-well culture plate, a total population of over 1000 cells was analysed in 10 arbitrarily chosen microscopic fields. Results were expressed as the percentage of cells synthesizing DNA (% BrdU positive nuclei). Stained cells were counted using an OLYMPUS® IX71 Inverted Laboratory System Microscope, Olympus Optical Co., Tokyo, Japan.

2.3.4 Apoptosis assay

Apoptotic cell death was measured by fluorescent microscopic analysis of cell DNA staining patterns with Hoechst 33258.

MCF-Vec and MCF-ARTN, MCF-siVec and MCF-siARTN, T47D-Vec and T47D-ARTN, and ZR-Vec and ZR-ARTN cells were plated at 2x 10^5cells/well in full serum media in 6-well plates and cultured for 24 hours. Cells were then washed with PBS and the media replaced with serum-free media, with and without treatment. 24 hours later the cells were fixed and permeabilised in 4% paraformaldehyde, 1% Triton-X-100 and stained with 4µg/mL of the karyophillic dye Hoeschst 33258 in PBS for 15mins at room temperature. Cells were washed with 1X PBS and apoptotic nuclear morphology was determined using an inverted UV fluorescence microscope (Olympus). Apoptotic cells were distinguished from viable cells by their nuclear morphology characterized by nuclear condensation and fragmentation as well as the higher intensity of the blue fluorescence of the nuclei. For statistical analysis, at least 200 cells were counted in eight random microscopic fields at 400X magnification.

2.3.5 Colony formation in soft agar

Cells were cultured to 70-80% confluence in 75cm^2 flask in complete media.

Each well of a 6-well plate (9.6cm^2) was covered with a base agar layer of 0.5% agarose in serum-free RPMI media. Dissolved DNA grade agarose at 0.5% (1.5mL/well of 6-well plate) was added and left to set at room temperature for 30mins.

DNA grade agarose (0.7%) was prepared in serum-free culture media and incubated in a water bath set at 41ºC. This temperature was used to maintaining cellular viability and to avoid polymerisation of the agarose. An equal amount of RPMI culture media, supplemented with 6% FBS was also warmed up to 41ºC. Cells were trypsinized, resuspended in complete media and pipetted up and down approximately 30 times to ensure a single cell suspension. $5x10^3$ cells were resuspended in 6% FBS RPMI media and warm 0.7% agarose via pipetting. This mixture (0.35% agarose) was added to each well on top of the base agarose (1.5mL/well for 6-well plates). All plates were left at room temperature for 1 hour to allow agarose to set and 2ml of 3% FBS RPMI media was then added to each well. The media was changed every second day for 14 days. Each experiment was performed in triplicate.

At day 14, the media was drained and then each well was washed once with ice-cold 1X PBS. All wells were stained with 0.5mL of 0.01% aqueous Crystal Violet reagent for 1 hour with gentle agitation followed by de-staining three times with 1X PBS for 5min. Sealed plates were stored in PBS for up to 4 weeks at 4°C. The total number of colonies in each well was counted at 40x magnification (OLYMPUS® IX70 Inverted Laboratory System Microscope).

2.3.6 Three-dimensional culture of cells in Matrigel™

Growth factor reduced Matrigel™ was purchased from BD Biosciences (BD No. 354230) and thawed overnight at 4°C. Once thawed, the matrigel was stored as 1mL aliquots at -20°C. The Matrigel™ was added to each well (50µL/well for 96 well plates) and the plate was placed in an incubator at 37ºC/5% CO_2 for 30min to allow the basement membrane extract to solidify. Concurrently, cells were trypsinised and resuspended in 2mL of complete media and centrifuged at 1000 rpm for 5min. Cells were then resuspended in 1mL of media and pipetted up and down several times to ensure a single-cell suspension. 25,000 cells were

mixed with 4% Matrigel™ in 5% serum media (4% Matrigel final concentration) with or without treatment. 200µL of the 4% Matrigel™ solution (1000cells/well) was pipetted onto the solidified Matrigel™ in each well. Cells were cultured in a 5% CO_2 humidified incubator at 37°C for 10 days. Every second day, 4% Matrigel™ in 5% serum media was added to the wells. The cells were treated with 50ng/mL IGF-1 or 2-4uM AG1024 throughout the course of the assay. After 10 day growth, 20µL of alamarBlue reagent was added to each well. The plate was incubated for 2-3 hours in a 5% CO_2 humidified incubator at 37°C following which the plate was read at 440nm (alamarBlue assay) using a Synergy2 multi-mode microplate reader and Gen5 data analysis software (Biotek).

2.4 Molecular biology methods

2.4.1 RNA isolation

Total RNA was isolated from exponentially growing cells (70% confluence) using TRIzol reagent. Cells were lysed by adding 1mL TRIzol/10cm^2 flask and lysates collected into 1.5mL eppendorf tubes. After 5min incubation at RT, 0.2mL of chloroform per ml of TRIzol reagent was added to each tube and mixed vigorously for 15sec. After centrifugation (at 12000g for 15min, 4ºC), the aqueous phase (RNA) was transferred to a fresh tube and precipitated by adding 0.7mL of isopropanol per mL of TRIzol and incubated at room temperature for 10min. A second centrifugation (at 12000g for 15min, 4ºC) was performed and the supernatant was discarded. The RNA pellet was washed (centrifugation at 12000g for 5min, 4ºC) in 1mL of 75% ethanol per ml TRIzol to remove salt. A third centrifugation was then performed (at 12000g for 5min, 4ºC) and the pellet was air dried at room temperature and resuspended in Diethylpyrocarbonate (DEPC) dH_2O. The concentration and

purity of RNA was determined spectrophotometrically using the ratios A260/A280nm and A260/A230nm.

2.4.2 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed by using a QIAGEN® OneStep RT-PCR Kit. A master mix was prepared according to the following protocol to avoid localised differences in salt concentration (Table 2.3). In order to keep the maximum enzyme activity, QIAGEN® OneStep RT-PCR Enzyme Mix was the last component added into the master mix.

RT-PCR reactions were carried out on a GeneAmp® PCR System 9700 (Applied Biosystems). A negative control, including every component similar to normal reaction except template RNA, was included in each experiment. Each reaction started with 30min reverse transcription at 50˚C followed by 15min at 95˚C. The PCR cycling consisted of 3 steps including denaturation at 94^{o}C for 30sec, annealing at specified temperature for 30sec, and extension at 72^{o}C for 1min for the indicated cycles. The specific annealing temperature and number of cycles as well as the primer sequences for individual reaction are listed in Table 2.4. Amplified β-Actin served as a control for RNA quantity and quality.

Components	Volume
RNase-free water	Variable
5X Qiagen OneStep RT-PCR buffer	5µL
Q solution*	5µL
dNTP mix (containing 10mM of each dNTP)	1µL
Forward Primer	400ng
Reverse Primer	400ng
QIAGEN OneStep RT-PCR enzyme mix	1µL
Template RNA	500ng
Total volume	25µL
* Only included in reactions for *ARTN* mRNA and its receptors	

Table 2.3- Reaction components for one-step RT-PCR

Amplified reactions were separated on a 1.5- 3.0% agarose gel prepared in Tris-Acetate-EDTA buffer (TAE 1X) (see Appendix) containing of 1% ethidium bromide solution (See Section 2.1.1). When the gel was polymerised, it was transferred into 1X TAE buffer. A defined amount (µL) of RT-PCR product was mixed with 3µL DNA loading dye (6X) (see Appendix) and then loaded into one well of the gel. A 1kb plus DNA ladder was used as a molecular weight marker. Electrophoresis was carried out at 80-100V for 20-40min. RT-PCR products were visualized under UV light, photographed and analyzed.

Gene	Primer sequence (5'-->3')	Product size (bp)	Annealing Temperature (^{o}C)	PCR cycles
IGF-1	F: TCCTCGCATCTCTTCTACC R: TGGCATGTCACTCTTCACT	413	55	40
IGF-2	F: GTGGCATCGTTGAGGAGT R: CTTGGGTGGGTAGAGCAATC	303	55	35
IGF-1R	F: TGGGGAATGGAGTGCTGTAT R: CGGCCATCTGAATCATCTTG	450	60	35
ARTN	F: TGCTGAGCAGCGTCGCAGAG R: GCTCTTCCACTGCACCAGCG	140	62	30-36
RET	F: CGTGAAGAGGAGCCAGGGTC R: TAACCATCATCTTCTCCAGGTCT	317	60	35
GFRα1	F: TTGCAGGACTCCTGCAAGACG R: GACCACAGCTTGGAGGAGCAG	595	60	35
GFRα3	F: CTGCTCACTTTCTTCGAGAAGG R: CAGGGTTTTCATTCTGGTGTGC	529	60	35
β-actin	F: ATGATATCGCCGCGCTCG R: CGCTCGGTGAGGATCTTCA	581	52	28

Table 2.4- Primers and reaction conditions for RT-PCR analysis.

2.4.3 Bacterial strains

The *Escherichia coli* (*E.Coli*) strain DH5α was obtained from Invitrogen. The genotype is DH5α: φ80dlacΔZM15, Δ(lacZY A-argF)U169, deoR, recA1, endA1, hsdR17(r-k, mk+), phoA, supE44, thi-1, gyr A96, rel A1 λ.

2.4.4 Growth of bacterial cultures

All bacterial cultures were grown aerobically in either liquid Luria-Bertani (LB) or on solid LB agar plates at 37ºC. Depending on the specific experiments, as indicated in appropriate Sections, relevant antibiotics and additives were further added for selective growth.

2.4.4.1 Bacterial transformation of plasmids

Plasmids were transformed into the *Escherichia coli* strain (*E. coli*) DH5α™ (Invitrogen). Heat-shock competent DH5α™ bacteria were mixed with the plasmid and incubated on ice for 30min. Transformation was performed using a heat-shock method by placing the mixed bacteria and DNA in a 42˚C water bath (Grant Instruments Ltd., Cambridge, UK) for 2min, immediately followed by cooling on ice for 5min. LB Broth (1mL) was added to each Eppendorf tube and incubated in a 37°C incubator (Sanyo Electric Co. Ltd., Japan) for 1 hour. Cultures were then spread on a LB agar plates. Due to the presence of a specific antibiotic resistance cassette in the plasmid, transformed bacteria were resistant to the corresponding antibiotic and this could be utilised as a selectable marker for positively transformed colonies. For instance, 50μg/mL Ampicillin is used in selection of bacteria transformed with the plasmid pIRESneo. After bacterial plates were incubated in the 37°C incubator for 20 hours, a single colony from each petri dish was picked and placed in 400ml of LB Broth containing 50μg/mL Ampicillin. Cultures were grown at 37°C with vigorous shaking (250rpm) in a Bioline® automatic incubator shaker (Edwards Instrument Co.) for a further 20 hours.

2.4.4.2 Plasmid DNA purification

Following a 20 hour culture (37°C) with vigorous shaking in antibiotic-supplied LB Broth, extraction and purification of plasmid DNA from transformed DH5α™ bacteria was performed using a QIAGEN® Plasmid Maxi Kit. The bacterial cells were harvested via centrifugation at 5000rpm for 10min at 4ºC using a Multifuge 3S-R centrifuge (Kendro Laboratory Products, Sorvall-Heraeus, Germany). The pellet was completely resuspended in 10ml of Buffer P1 by vortexing, followed by addition of 10mL of Buffer P2 and mixing thoroughly by gently inverting 4-6 times. After 5min incubation at RT, 10mL of chilled Buffer P3 was immediately mixed with the sample and incubated on ice for 20min. The compound solution was re-mixed by several gentle inversions and centrifuged at 8500g for 75min at 4ºC. The plasmid DNA containing supernatant was removed and centrifuged again at 8500g for 40min at 4ºC. In order to improve the level of purification, this step was repeated one more time. During this period, 10ml of Buffer QBT was applied to equilibrate the QIAGEN-tip 500 column and the DNA containing supernatant was loaded onto the equilibrated QIAGEN-tip 500. Columns were washed twice with 30mL of buffer QC following which the plasmid DNA was eluted with 15mL of Buffer QF. Isopropanol (10.5mL) was mixed with the eluate at room temperature then centrifuged at 8500g for 55min at 4ºC to precipitate the DNA. The DNA pellet was washed with 5mL of 70% ethanol at room temperature and then re-precipitated by centrifuging at 8500g for 20min. The supernatant was carefully decanted and the pellet allowed to air dry before being redissolved in a suitable volume (50-100μL) of ultrapure water. DNA was stored at -20ºC.

2.5 Protein methods

2.5.1 Protein extraction

Cells were plated at 60-70% confluence, changed into serum-free media or treatment media the following day. After treatment, cells were washed once with ice-cold PBS and harvested by the addition of lysis buffer (see Appendix) and mechanical scraping. The total cell lysates were then collected into 1.5mL Eppendorf tubes and held on ice. The total cell lysate was sonicated and centrifuged at 13,000rpm for 10mins at 10˚C.

The supernatants were transferred to new 1.5mL Eppendorf tubes and stored at -80ºC or used immediately in subsequent assays. The protein concentration was then measured in triplicate using DC protein assay (Bio-Rad laboratories, Inc.).

2.5.1.1 Protein extraction for detection by Phospho-specific antibodies

For western blots involving the specific detection of phosphorylated proteins, a phospho-specific lysis buffer (see Appendix) was ustilised. After the cells were harvested into 1.5mL Eppendorf tubes, the samples were kept on ice for 30mins and vortexed every 5mins or so, following which, the samples were centrifuged at 13,000rpm for 20mins at 4ºC.

The supernatants were transferred to new 1.5mL Eppendorf tubes and stored at -80ºC or used immediately in subsequent assays. The protein concentration was then measured in triplicate using DC protein assay.

2.5.2 Bio-Rad DC protein assay

Working reagent A was prepared by adding 20μL of reagent S to each ml of reagent A from the Bio-Rad Reagent package and mixing thoroughly. 10mg/mL BSA was diluted to make a series of standards containing from 0mg/mL to 5mg/mL protein with the lysis buffer used during the protein extraction, and used for the standard curve. Unknown protein samples (20μL) were also diluted 5 or 10 times with Lysis buffer 1 or 2. Standards and unknown samples (5μL) were pipetted into a 96-well microtiter plate. 25μL of reagent A+S was added into each well, followed by 200μL reagent B. The microtiter plate was incubated for 15min with gentle agitation in the dark at RT. After removing bubbles with clean, dry pipette tips, absorbances were read at 750nm using a Biotek Synergy 2 plate reader. Based on the absorbances of standards, the standard curve was plotted, and the absorbance of unknown protein sample was calculated from the standard curve.

2.5.3 Plate reading

The plate was placed onto the Biotek Synergy 2 plate reader with an appropriate absorbance wavelength setting. Standard curves were prepared by measuring the absorbance of BSA (wavelength 562nm) in serial dilutions. The calibration curves were linear (r^2 >0.99) over the concentration range tested (0 – 2000μg/mL) and the precision of all standards was acceptable (CV < 5%). The protein concentration was calculated using the Gen5 data analysis software (Biotek) by comparing the absorbance of the known protein BSA standards with those from the unknown samples.

2.5.4 Western Blotting

2.5.4.1 Polyacrylamide gel electrophoresis (PAGE)

A Laemmli SDS-polyacrylamide gel was cast using the Bio-Rad Mini-PROTEAN II system. The resolving gel was composed of 12-15% acrylamide (37.5: 1), 0.375M Tris-HCl, pH 8.8 and 0.1% SDS. The stacking gel was composed of 4% acrylamide, 0.125M Tris-HCl, pH 6.8, and 0.1% SDS. The gels were polymerized by the addition of freshly prepared 0.1% ammonium persulfate and 0.01% Tetramethylethylenediamine (TEMED). Protein samples were mixed with 2X sample buffer, supplemented with 0.1M DTT, and heated at 95ºC for 10min (to denature the proteins in the sample) before being loaded into the gel. Electrophoresis was carried out in 1X SDS-PAGE

After electrophoresis was completed, the gel was removed from the gel cassette sandwich. After cutting off the stacking portion, the gel was equilibrated in transfer buffer for 2min. Meanwhile, the Polyvinylidene Difluoride (PVDF) membrane, cut to the dimensions of the gel, was incubated in methanol for 1min and transferred into transfer buffer for 3min incubation. The filter paper and fibre pads were soaked in transfer buffer for 5min. The membrane and gel were sandwiched between 2 stacks of pre-soaked filter paper and sponges before being placed into the transfer apparatus, containing the transfer buffer. The separated proteins were transferred to the membrane at a constant 110V for 1.5-2 hours.

The PVDF membrane was then removed and incubated in 5% non-fat dry milk in TBS with 0.1% Tween-20 (TBST) overnight at 4˚C. Blots were then immunolabeled with the desired primary antibodies for 1h at room temperature or overnight at 4˚C (Table 2.5). Then the blots were washed three times and incubated with the secondary antibody for 1-2 hours at RT. Finally, the membrane washed again three times for 10mins before incubation with the chemoluminescence kit, according to the manufacturer's instructions, for 5min in the dark.

For reblotting, membranes were stripped for 5min in 0.2M NaOH. Blots were then washed for 10min with milliQ water, TBST at RT. The membrane was then blocked with 5% non-fat dry milk in TBST, overnight at 4ºC. Thereafter, membranes were immunolabeled as desired.

Antibody	Supplier	Catalogue number	Dilution factor	Secondary antibody	Band size (kDa)
β-actin	Sigma Aldrich	A5316	50,000	Sheep, anti-mouse	43
ARTN	R&D Systems	AF2589	500	Sheep, anti-goat	14 (monomer)
Phospho- p44/42 MAP kinase	Cell Signalling	9106	2000	Sheep, anti-mouse	44/42
p44/42 MAP kinase	Cell Signalling	9102S	2000	Sheep, anti-rabbit	44/42
Phospho-AKT	Cell Signalling	9271S	1000	Sheep, anti-rabbit	60
AKT	Cell Signalling	9272	1000	Sheep, anti-rabbit	60

Table 2.5- Antibodies used in western blot analysis.

2.5.5 Phospho-RET and phospho-IGF-1R ELISA

Both phospho-RET and phospho-IGF-1R ELISAs were conducted using PathScan Sandwich ELISA kits (Cell Signalling). Wild-type MCF-7 cells were treated with exogenous IGF-1 for the indicated times, then lysed with the 1X lysis buffer (supplied) and processed as indicted in the manufacturer's instructions.

The supplied sandwich ELISA wells were separated as needed. 100µL of diluted cell lysate (1:2 dilution for RET and 1:50 dilution for IGF-1R) was added per well and incubated overnight at 4˚C. The remainder of the assay was completed following the manufacturer's instructions, which were supplied with each kit.

The sample absorbances at 450nm were read on the Biotek Synergy 2 Plate reader. The results were normalized for the housekeeper protein, GAPDH, as measured by an AlphaScreen® assay.

2.5.6 GAPDH Alpha Screen® Assay

The expressions of the GAPDH protein as a housekeeping protein was used as a baseline for the Sandwich ELISA assays to ensure equal loading of the cell lysates and that the samples being tested and compared had equivalent protein concentrations. The AlphaScreen SureFire® GAPDH Assay Kit (TGR BioSciences) was used to quantify GAPDH levels in lysates. The AlphaScreen assay detects the target protein (in this case GAPDH) by sandwiching the target between antibodies conjugated to donor and acceptor beads. When brought together in close proximity, the streptavidin-coated donor bead upon excitation releases singlet oxygen molecules and a transfer of energy to the Protein A conjugated acceptor bead, resulting in the emission of light at the 520-620nm wavelength (http://www.perkinelmer.com/).

The MCF-7 cell lysates produced in the ELISA assays were diluted 1:25 with lysis buffer (this dilution factor was determined in previous studies). 4µL of diluted lysate was added to each well of a 384-well proxiplate plate and mixed with 5µL acceptor bead mix. The plate was then incubated for 2 hours at room temperature. 2µL of the donor bead mix was then added to each well and the plate was incubated in the dark, for a further 2 hours at room temperature. The AlphaScreen Signal was then read on an Envision plate reader (PerkinElmer).

2.5.7 Statistical analysis

All experiments were repeated at least three times and a representative result is presented in the results figures as appropriate. All numerical data are expressed as mean ± SEM (standard mean error) from a representative experiment performed in triplicate, and statistical analyses were done by an unpaired two-tailed t-test using Microsoft Excel or two-way ANOVA in GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA). Error bars on all graphs represent the standard error of the mean between measurements. Values of $p<0.05$ were considered significant.

Chapter 3

RESULTS: Regulation of endogenous Artemin expression and signalling by IGF-1 in wild-type mammary carcinoma cell lines

3.1 Introduction

As described in the Introduction Chapter, ARTN is a neurotrophic signalling factor belonging to the GDNF family of ligands (Baloh et al., 1998b; Masure et al., 1999). It exists as a dimeric protein and its distinctive 3-D helical structure has it classed as a member of the TGF-β superfamily (Airaksinen et al., 1999; Masure et al., 1999).

ARTN plays a pivotal role in neuronal development and is essential to neutrite outgrowth in the developing sympathetic and parasympathetic nervous systems (Airaksinen and Saarma, 2002; Damon et al., 2007a; Nishino et al., 1999). There is also evidence to suggest it is critical for neuronal plasticity and maintenance (Warnecke et al., 2010). Recently published studies have implicated ARTN involvement in neuropathic pain, mental health and drug addiction, as well as neural degeneration diseases (Di Cesare Mannelli et al., 2011; Gardell et al., 2003; Ossipov, 2011).

There is also a growing body of evidence for the involvement of ARTN in a range of human cancers. Early work demonstrated the ability of ARTN treatment to stimulate proliferation and cell survival of neuroblastoma cells, and to increase invasiveness of pancreatic cancer cells (Ceyhan et al., 2006a). In addition, recent studies have demonstrated the ability of elevated ARTN expression to promote migration and invasive ability in mammary, lung and

endometrial carcinoma cell lines. ARTN also acts as a mediator of chemo-resistance, radio-resistance and endocrine therapy resistance in these cell lines (Banerjee et al., 2011; Kang et al., 2009; Pandey et al., 2010b; Tang et al., 2010).

This research project has focused on the interaction of ARTN with the IGF-1 signalling pathway, and aimed to determine whether the two signalling pathways interact to modify one another's effects on cell behaviour and function. ARTN has already been shown to be under the regulation of estrogen signalling, a pathway notable for its interaction and cross-talk with the IGF-1 signalling pathway (Kang et al., 2010). IGF-1 is itself a potent stimulator of cell proliferation and survival, and implicated in tumour formation and progression (Anisimov and Bartke, 2013; Bartucci et al., 2001; Gooch et al., 1999; Rubin and Baserga, 1995). This project proposes that a level of interaction may exist between the ARTN and IGF-1 signalling pathways and that cross-talk between the two pathways may promote the oncogenic and tumour promoting effects. Herein, evidence is provided supporting that IGF-1 treatment up-regulates ARTN expression and the oncogenic effects of ARTN signalling. In addition, these results demonstrate that the two ligands signal by converging on the same downstream signalling pathways, resulting in greater activation of downstream signalling mediators.

3.1.1 Characterization of endogenous ARTN and ARTN receptor expression in wild-type mammary carcinoma cell lines

Mammary carcinoma cell lines were used to investigate the effect of IGF-1 treatment on ARTN signal transduction. Three cell lines (MCF-7, T47D and ZR-75-1) were selected for this study on the basis of expression of components of the ARTN and IGF-1 signalling pathways. According to the ATCC, all three cell lines are positive for ER and IGF-1R (but

negative for IGF-1 expression) and non-invasive, although their doubling times vary greatly, stated at 29, 32 and 80 hours for MCF-7, T47D and ZR-75-1 cells, respectively.

Initially, cell lines were characterized for endogenous expression of ARTN and the ARTN receptors, RET, GFRα1 and GFRα3. Endogenous mRNA expression was examined by semi-quantitative RT-PCR which was isolated following culture of cells in serum-free and full serum (10% FBS) culture conditions.

The endogenous mRNA expression of *ARTN, RET, GFRα1* and *GFRα3* was detectable in all three wild-type cells lines (Figure 3.1). The expression levels differed between the cell lines, with MCF-7 cells exhibiting consistently higher mRNA expression levels of the receptors compared to T47D and ZR-75-1 cell lines. Whereas T47D cells consistently expressed greater levels of ARTN compared to the other cell lines. The serum content of the culture medium had little effect on the mRNA expression levels across all three cell lines. Some minor differences in the expression levels between serum-free and full serum culture were also observed.

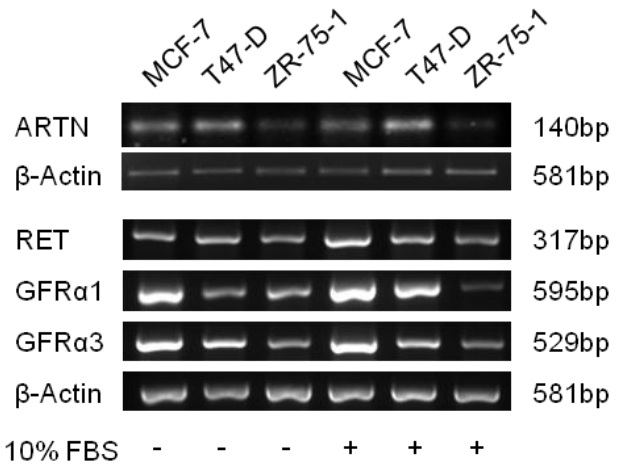

Figure 3.1- Endogenous expressions of ARTN and its receptors in wild-type mammary carcinoma cell lines.

Total cellular RNA was extracted from wild-type mammary carcinoma cells, cultured for 24 hours in serum-free and full (10%) serum conditions, and assayed for endogenous mRNA expression of ARTN and related receptors. A water only reaction was also included as a negative control (not shown).

3.1.2 Determination of IGF-1 dose responses in wild type mammary carcinoma cells

IGF-1 dose response studies were carried out using a cell viability assay to ascertain the optimal IGF-1 dose for use in subsequent experimental assays. These were performed in different serum conditions (serum-free, 0.2% FBS and 10% FBS (full serum)) for up to 8 days and the cell viability was measured by MTT assay every 2 days. Despite the EC_{50} of IGF-1 in mammary carcinoma cells having already been well-documented, the purpose of these assays were largely for confirmation that the results observed were consistent with previously published data and also to determine the best cell culture conditions for use in future assays.

It was evident from these results that the effect of IGF-1 is best observed in serum-free conditions or in low (0.2%) serum, particularly for longer culture durations (Figures 3.2-3.4). In 10% FBS, the effect of IGF-1 on cell viability was masked by the serum. For this reason, the following *in vitro* cell function assays were carried out in serum-free or low serum media, where possible.

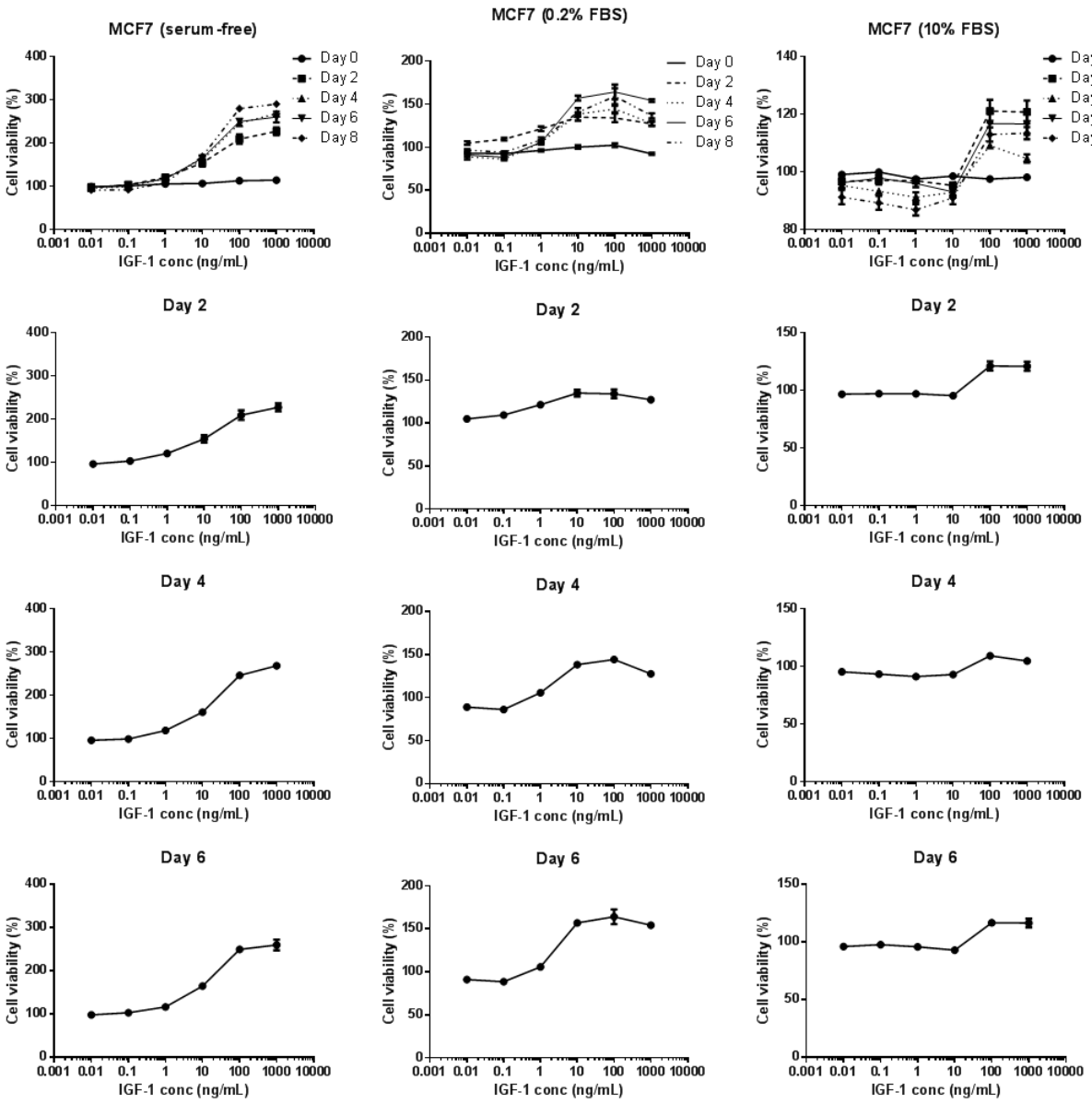

Figure 3.2- IGF-1 dose response in wild-type MCF-7 cells.

IGF-1 dose response assays were performed in wild-type MCF-7 cells by measuring cell viability following treatment with IGF-1 in serum-free, low serum (0.2% FBS) and full serum (10% FBS) conditions. The cell viabilities were standardised against the untreated control in each treatment set (100% viability: 0 ng/mL IGF-1).

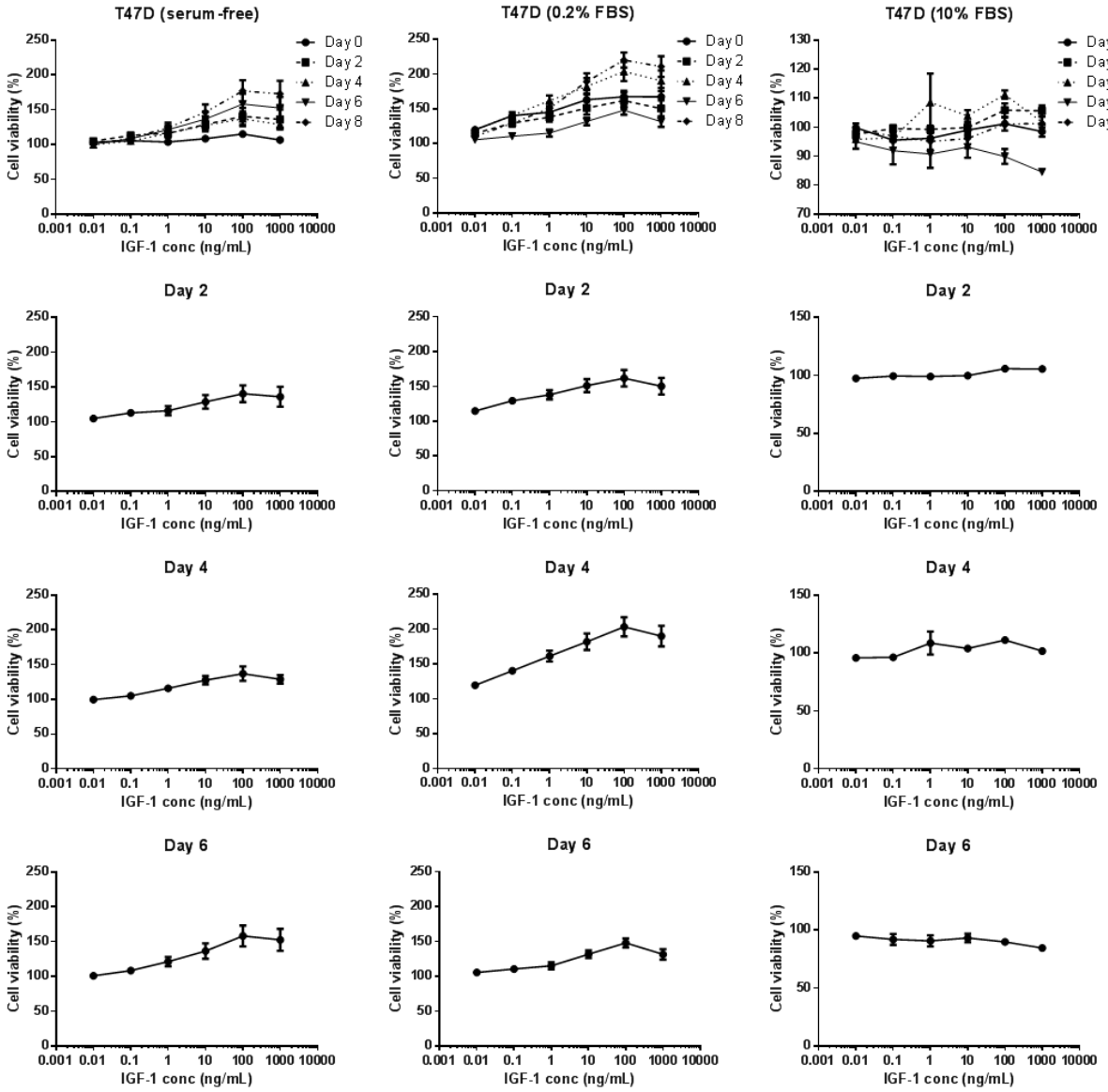

Figure 3.3- IGF-1 dose response in wild-type T47D cells.

IGF-1 dose response assays were performed in wild-type T47D cells by measuring the cell viability following treatment with IGF-1 in various serum conditions- Serum-free, low serum (0.2% FBS) and full serum (10% FBS). The cell viabilities were standardised against the untreated control in each treatment set (100% viability: 0 ng/mL IGF-1).

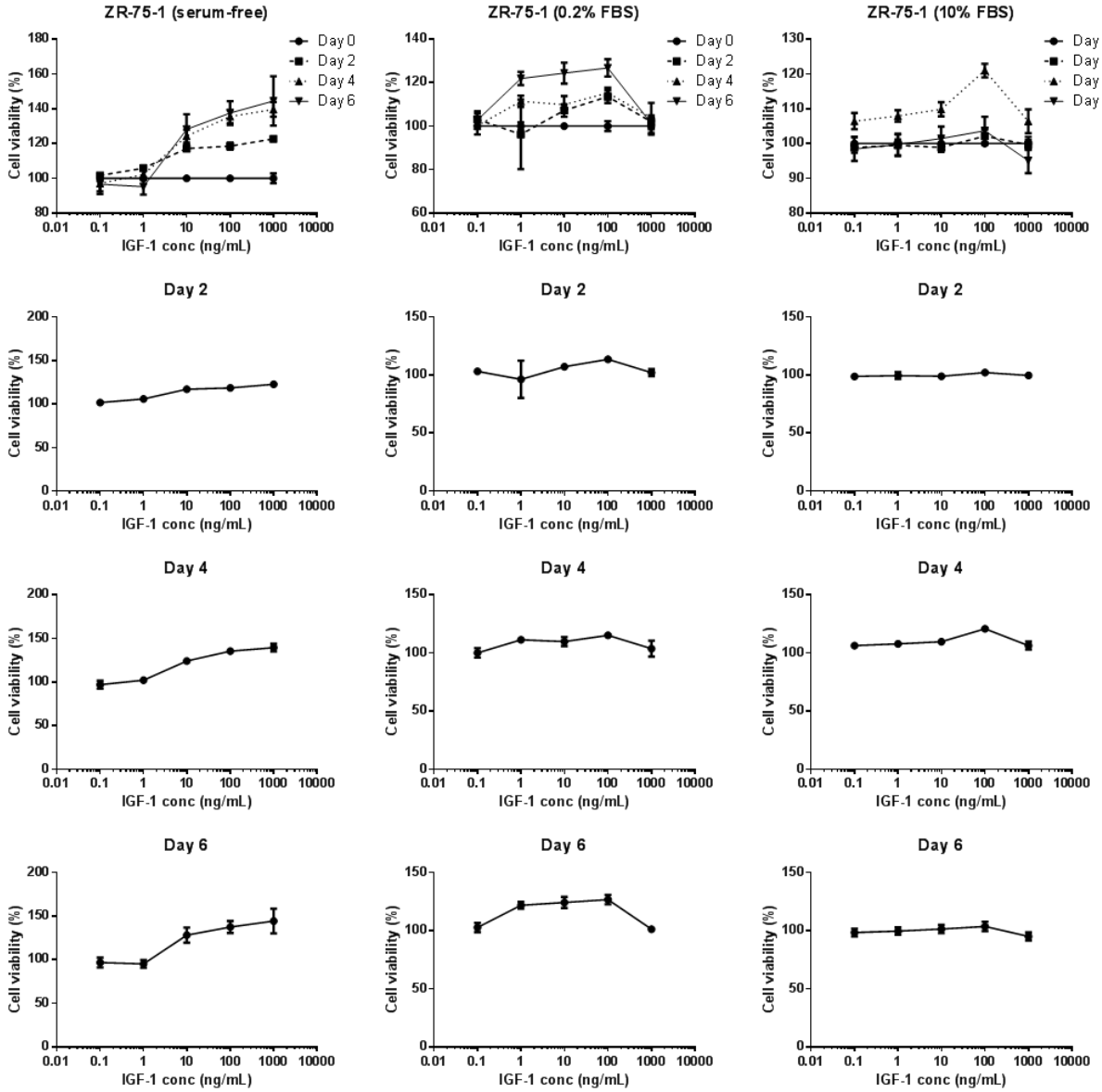

Figure 3.4- IGF-1 dose response in wild-type ZR-75-1 cells.

IGF-1 dose response assays were performed in wild-type ZR-75-1 cells by measuring the cell viability following treatment with IGF-1 in various serum conditions- Serum-free, low serum (0.2% FBS) and full serum (10% FBS). The cell viabilities were standardised against the untreated control in each treatment set (100% viability: 0 ng/mL IGF-1).

3.2 Insulin-like growth factor-1 increases the mRNA and protein expression levels of ARTN in wild-type mammary carcinoma cells

3.2.1 Induction of Artemin mRNA expression by IGF-1 treatment in wild-type mammary carcinoma cells

The effect of IGF-1 on *ARTN* mRNA expression in wild type mammary carcinoma cells was initially investigated by semi-quantitative RT-PCR. Wild-type cells were treated with 100ng/mL for up to 6 hours, the total cellular RNA was extracted at regular intervals and the *ARTN* mRNA expression was determined.

Over the treatment time-course, there was a small but consistent increase in *ARTN* mRNA expression. The increase in *ARTN* mRNA was most easily observable from 4 hours following IGF-1 treatment in MCF-7 (Figure 3.6a) and ZR-75-1 cells (Figure 3.6b). In T47D cells, the increase in *ARTN* is less clearly observed, possibly due to the comparatively high endogenous *ARTN* expression in this cell line (Figure 3.6c).

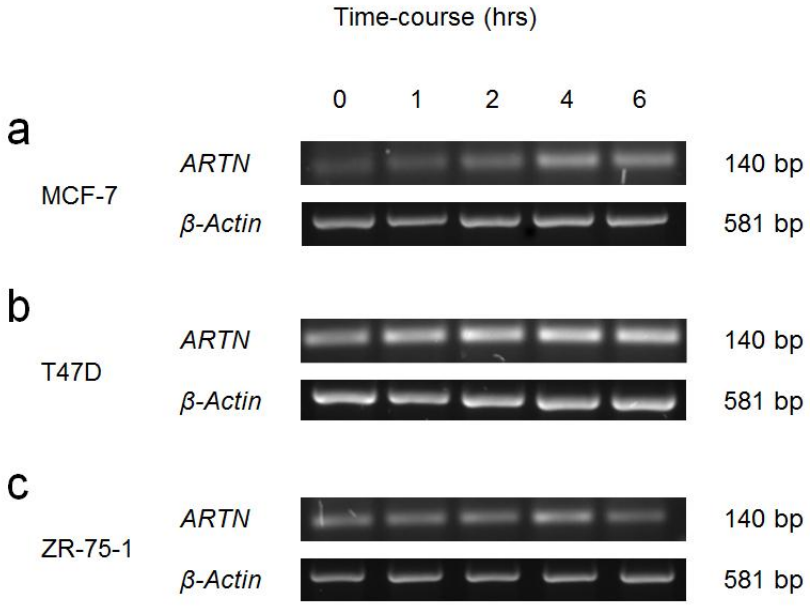

Figure 3.5- Timecourse RT-PCRs of ARTN mRNA expression in wild-type mammary carcinoma cell lines.

(a) MCF-7, (b) T47D and (c) ZR-75-1 cells were treated with 100ng/mL IGF-1 then assayed at discrete time points for *ARTN* mRNA expression. A small increase was observed in MCF-7, T47D and ZR-75-1 cells after 4 hours of treatment.

3.2.2 Induction of Artemin protein expression by IGF-1 treatment in wild-type mammary carcinoma cells

Following on from the semi-quantitative RT-PCR results demonstrating that IGF-1 increased *ARTN* mRNA levels, the effect of IGF-1 on ARTN protein expression was investigated following a similar treatment schedule.

MCF-7, T47D and ZR-75-1 cells were treated with 50 and 100ng/mL IGF-1 for up to 72 hours, and at regular intervals cell lysates were taken and assessed for ARTN protein by western blotting (Figure 3.6). Treatment with IGF-1 increased the levels of ARTN protein expression. In MCF-7 cells, the increase in ARTN protein was evident from 4 hours, onwards. In addition, the increase in expression appeared to be dose responsive given the greater increase in ARTN protein expression following 100ng/mL IGF-1 treatment compared to 50ng/mL IGF-1 treatment.

Similar results were also observed for the T47D and ZR-75-1 cell lines. The increase in ARTN protein expression was observed from as early as 2 hours following 50ng/mL IGF-1 treatment in MCF-7 and T47D cells, and at 4 hours in ZR-75-1 cells. However, assaying for the ARTN protein in total cell lysates was quite problematic. The ARTN protein is not very stable following extraction and deteriorated quickly. In addition, the anti-ARTN antibody required for the blots were difficult to handle and did not yield very consistent results (see Appendix for an example of a typical ARTN western blot in its entirety and for representation of the antibody specificity). A number of different conditions were tested to optimise the protocol. In the end this was remedied by avoiding the storage of lysates, but this meant that individual time points had to be assayed separately.

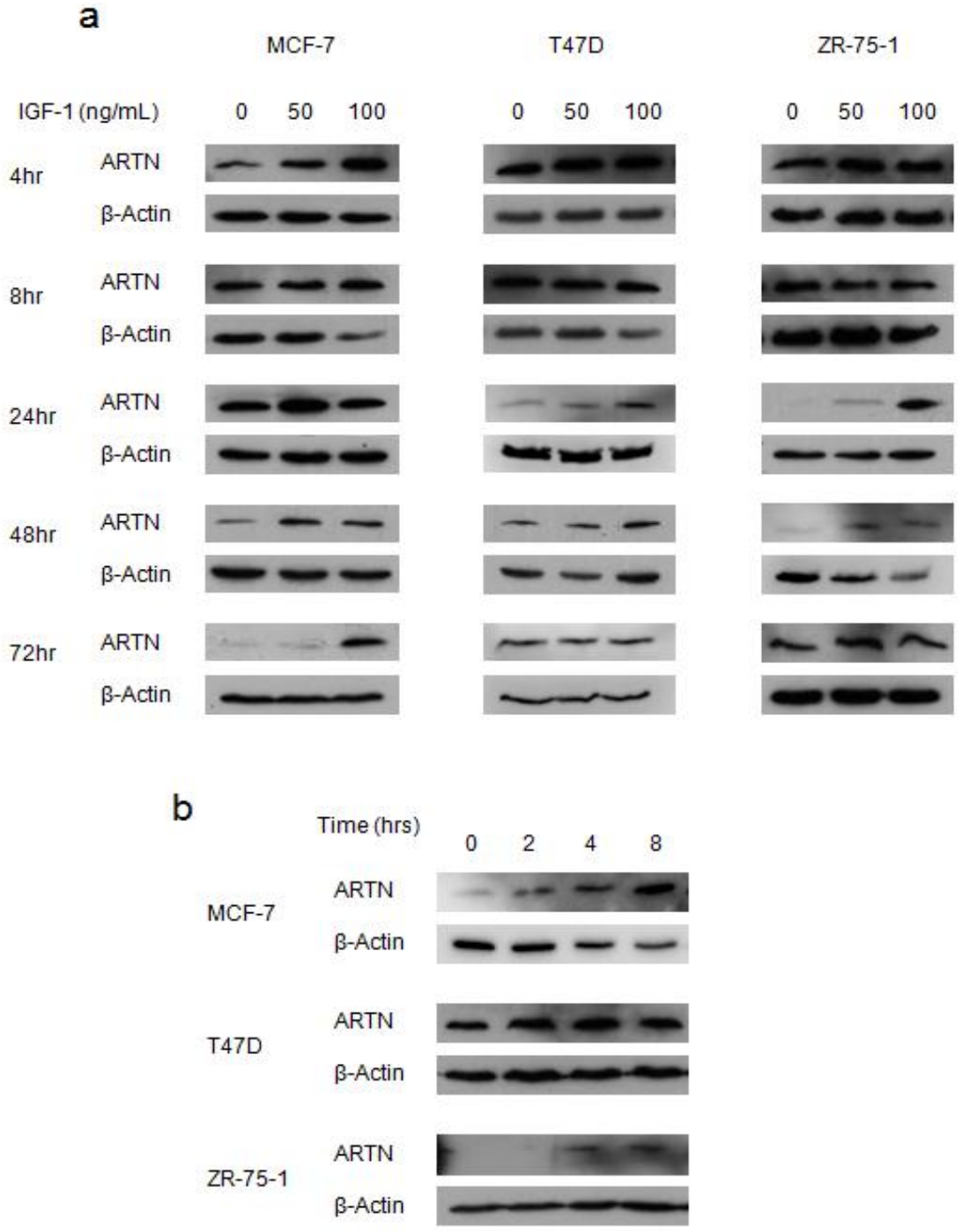

Figure 3.6- ARTN protein expression in mammary carcinoma cells increases following IGF-1 treatment.

All three cell lines exhibited increasing expression of the ARTN monomer following IGF-1 treatment. This response was observed with increasing (a) IGF-1 treatment dose and (b) duration of treatment with a single dose (50ng/mL IGF-1).

Please see Appendix for an example of ARTN western blots in its entirety.

3.2.3 Exogenous IGF-1 does not affect mRNA expression of the receptors for Artemin in mammary carcinoma cells

The mRNA expression levels of the ARTN receptors, *RET* and *GFRα3*, were also assayed by semi-quantitative RT-PCR following treatment with 100ng/mL IGF-1. *GFRα1* mRNA expression was also assessed in this assay. GFRα1 is the primary receptor for GDNF. However, ARTN can also bind it to some degree and elicit activation of signal transduction through recruitment of RET (Baloh et al., 2000).

In all three cell lines, the expression level of the receptors was mostly unchanged following culture with IGF-1 over a 6 hour time-course assay (Figure 3.7). *GFRα1* exhibited an increase in expression in ZR-75-1 cells after 1 hour of IGF-1 treatment, but there was no change in expression in *GFRα1* mRNA expression in the MCF-7 or T47D cell lines.

A longer time-course was carried out for the *RET* and *GFRα3* receptors (Figure 3.8). Due to the longer culture times in serum-free media, control samples of serum-free only culture samples (i.e. no IGF-1 treatment) at the corresponding time points were also included in the assay. The reason being that *ARTN* and *RET* have been documented to be estrogen-responsive genes and prolonged culture of mammary carcinoma cell in serum-free conditions has been shown to up-regulate ER activity (Jeng et al., 2000). Again, little change in receptor expression was observed in the longer time-course assay. There was a small increase in *GFRα3* mRNA expression in MCF-7 cells, but there was no effect on the expression of this gene in the other cell lines.

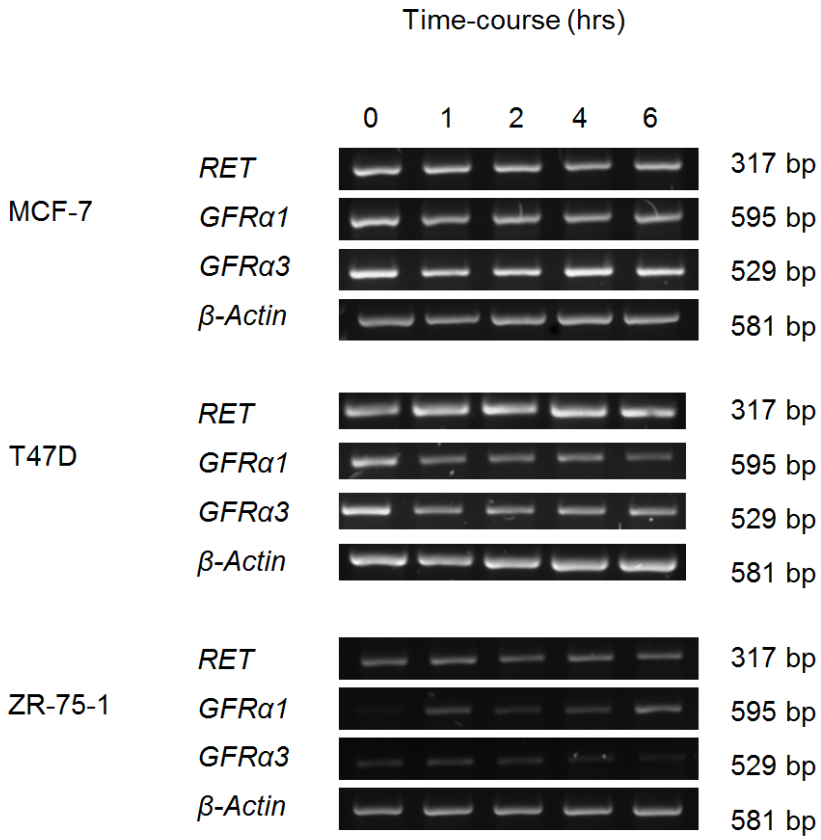

Figure 3.7- IGF-1 regulation of ARTN receptor mRNA expressions.

Total cellular RNA was extracted from wild-type mammary carcinoma cells treated with IGF-1 at the indicated time points. The cellular RNA was assayed for endogenous expression of the ARTN receptors. Overall, the receptors did not show any observable differences in expression following treatment with IGF-1.

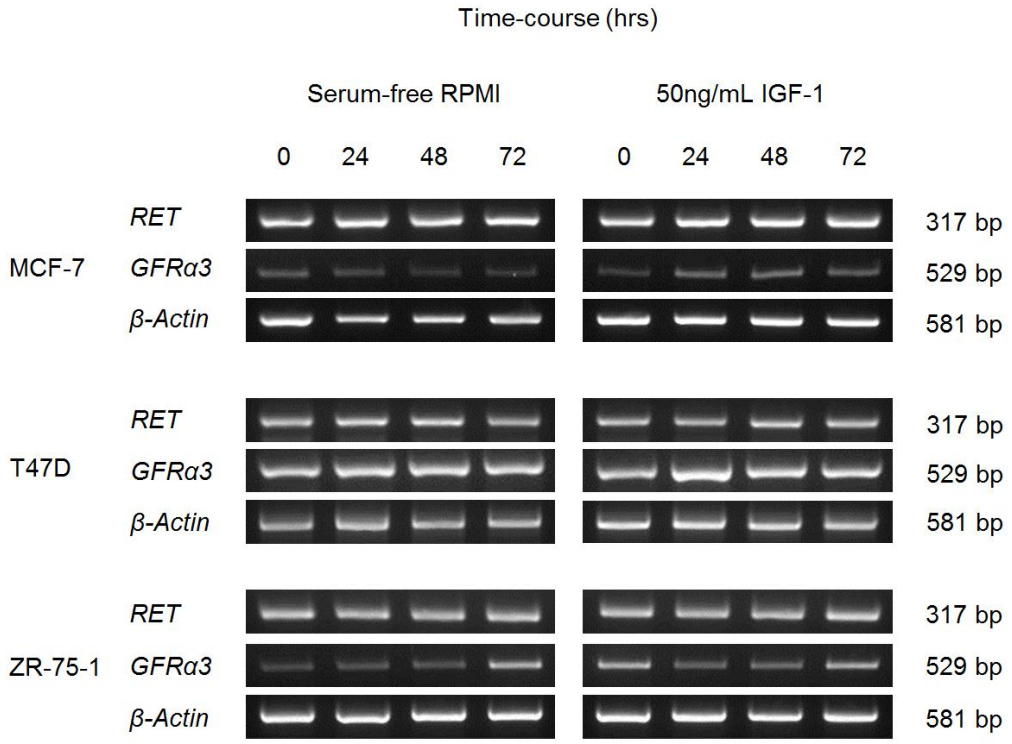

Figure 3.8- IGF-1 regulation of ARTN receptor mRNA expression.

Following prolonged culture of the wild-type mammary carcinoma cells in serum-free RPMI media, with and without the addition of IGF-1, the total cellular RNA was extracted and mRNA expression of the receptors for ARTN was assayed by semi-quantitative RT-PCR. We found no consistent or significant change in the receptor expression following treatment with IGF-1.

3.2.4 Activation of MAPK by exogenous IGF-1 and ARTN in wild-type MCF-7 cells

Activation of the MAPK pathway by tyrosine phosphorylation is one of the predominant mechanisms by which IGF-1 mediates downstream signal transduction. This is a well characterised pathway, and in IGF-1 signalling it is responsible for mediating the mitogenic effects on cell function and behaviour, including cell proliferation and survival (Migliaccio et al., 1996; Plotnikov et al., 2011; Zhang and Liu, 2002).

In recent studies, ARTN and the RET receptor have also been demonstrated to signal through the MAPK pathway (Baloh et al., 1998a), although its effects of RET signalling on cell function are cell-type specific (Santoro et al., 1994; Xing et al., 1998). Most notably, while ARTN signalling may increase cell proliferation in neuronal and neuroblastoma cell lines, in

pancreatic and breast cancer cells, there is little if any effect on proliferation (Ceyhan et al., 2006a).

In this section, the activation/phosphorylation of MAPK in wild-type MCF-7 cells by exogenous IGF-1 and ARTN was investigated. Wild-type MCF-7 cells were treated with increasing concentrations of IGF-1 and recombinant ARTN protein, then lysed and processed for the total protein content. The cell lysates were then assayed for the total and phosphorylated forms of the MAPK protein by western blotting.

Western blotting was used to confirm that treatment with IGF-1 induced phosphorylation of MAPK protein in the wild-type MCF-7 cells and to determine optimal experimental conditions. Consistent with the literature, IGF-1 induced an immediate increase in MAPK phosphorylation in MCF-7 cells (Figure 3.9a). Increasing the concentration of IGF-1 increased MAPK phosphorylation in a dose-dependent manner (Figure 3.9a) with maximal response observed following treatment with 100 and 500ng/mL IGF-1. Maximal activation of MAPK was observed at 5 and 10mins following the start of treatment, with the phosphorylation levels decreasing as treatment continued (Figure 3.9b). Treatment with IGF-1 did not affect the levels of total MAPK protein expressed across the samples.

Treatment with ARTN also increased MAPK phosphorylation but the maximal response was observed at 10mins. Maximal stimulation of MAPK phosphorylation was observed at 500ng/mL ARTN (Figures 3.9c and d).

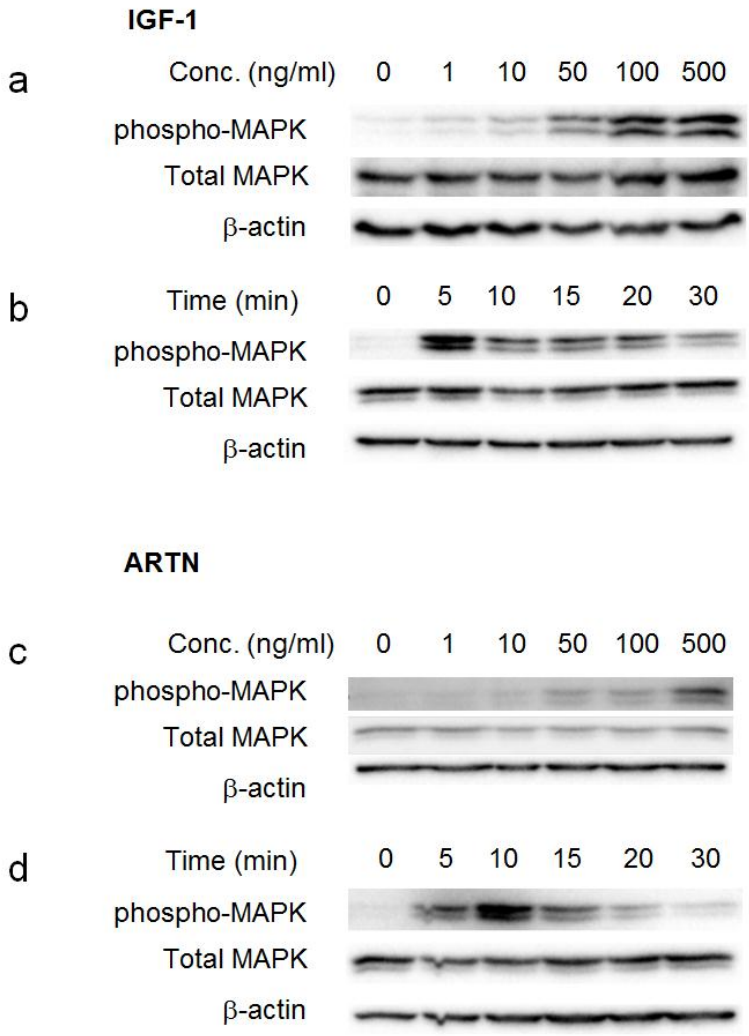

Figure 3.9- Regulation of MAPK phosphorylation by IGF-1 and ARTN.

Wild-type MCF-7 cells were treated with IGF-1 (**a** and **b**) or ARTN (**c** and **d**) for 10mins (**a** and **c**) or the time indicated (**b** and **d**). Lysates were assessed for the phosphorylated and non-phosphorylated forms of the MAPK protein by western blotting. The resulting blots demonstrated dose- and time-dependent activation of MAPK by both IGF-1 and ARTN treatment.

3.2.5 Activation of the PI3K signalling by phosphorylation of AKT protein

Further to the activation of the MAPK signal transduction pathway, IGF-1 also activates the PI3K/AKT pathway (Bähr and Groner, 2005). Similar to MAPK, the PI3K/AKT pathway is capable of mediating mitogenic signalling from receptors on the cell surface, but is more widely known for promoting cell survival. Although the mechanism by which it does this differs, activity of the AKT protein is also regulated by phosphorylation and dephosphorylation, which is readily assayed by western blotting.

To determine activation of the PI3K/AKT pathway, the level of AKT protein expression and phosphorylation induced by IGF-1 and ARTN was first assessed by Western blotting. As demonstrated in Figures 3.10a and b, IGF-1 increased AKT activation in a dose-dependent manner with maximal phosphorylation observed at 500ng/mL (Figure 3.10a). Maximal AKT phosphorylation was observed at 5 and 10mins following treatment with 100ng/mL IGF-1 (Figure 3.10b). Treatment with 1-500ng/mL IGF-1 did not affect the levels of total AKT protein in lysates.

It was interesting to note that although ARTN is known to activate the PI3K/AKT pathway in other cell lines, activation of AKT was not observed in MCF-7 cells treated with 1-500ng/mL ARTN, as determined by western blot analysis of AKT phosphorylation (Figures 3.10c and d).

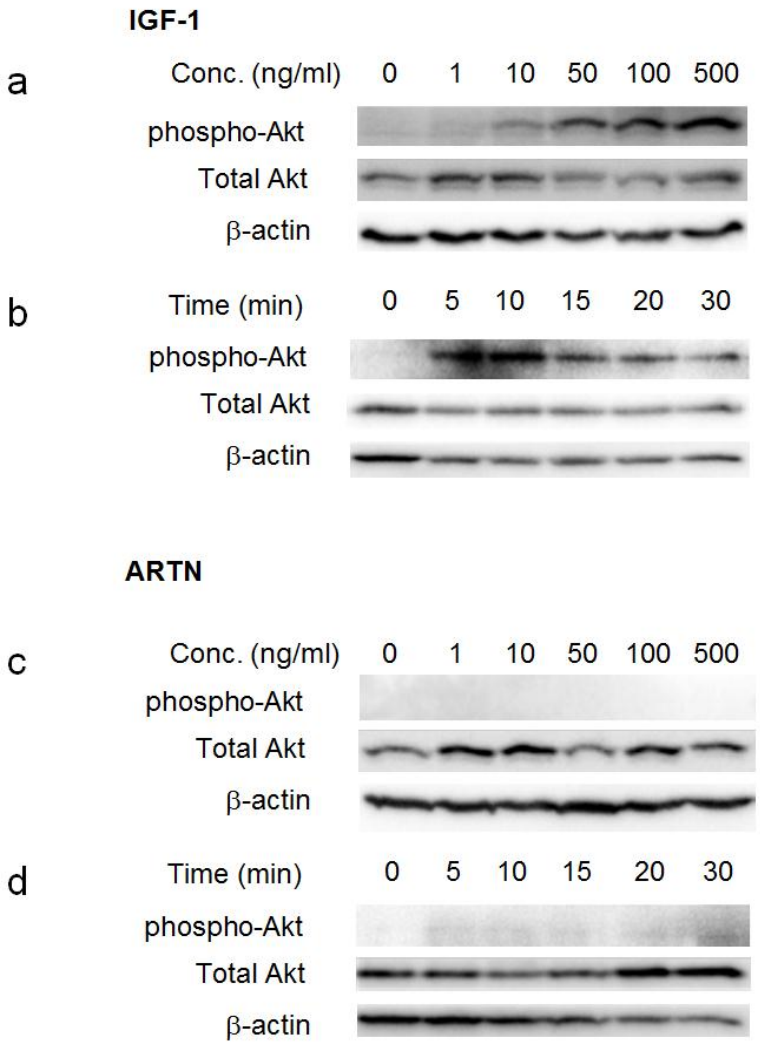

Figure 3.10- Regulation of AKT phosphorylation by IGF-1 and ARTN.

Wild-type MCF-7 cells were treated with IGF-1 (**a** and **b**) or ARTN (**c** and **d**) for 10mins (**a** and **c**) or the time indicated (**b** and **d**). Lysates were assessed for the phosphorylated and non-phosphorylated forms of the AKT protein by western blotting. The resulting blots demonstrated dose- and time-dependent activation of AKT by IGF-1, but not ARTN treatment.

3.2.6 Synergistic activation of MAPK and AKT by IGF-1 and ARTN

Treatment with IGF-1 and ARTN was combined to investigate the effect this would have on MAPK and AKT phosphorylation. A synergistic effect was observed for the phosphorylation of both MAPK and AKT proteins. Interestingly, for MAPK phosphorylation, this synergy was most readily observed at 60mins of co-treatment, and the level of protein phosphorylation was visibly greater in samples treated with both ARTN and IGF-1, than in samples treated with either ligand alone. However, at 10mins, this synergy was not evident (Figure 3.11a).

Similar results were found in western blotting for AKT protein phosphorylation. With combined treatment, there was synergistic activation of AKT after 10 and 60mins IGF-1 and ARTN treatment (Figure 3.11b).

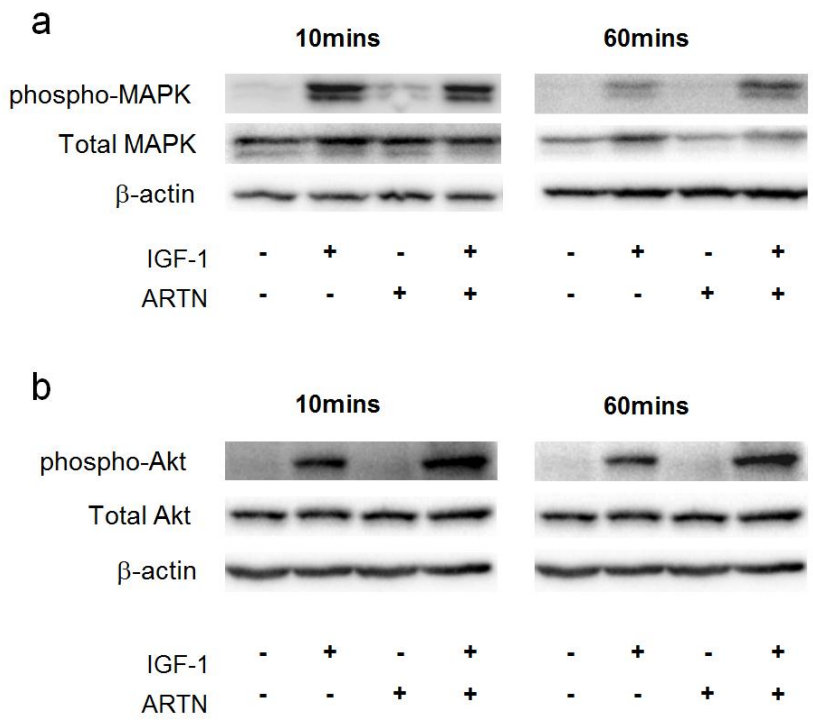

Figure 3.11- Synergistic activation of MAPK and AKT by ARTN and IGF-1.

Wild-type MCF-7 cells were treated with a combination of 100ng/mL each of both exogenous IGF-1 and ARTN before being lysed and assessed for **(a)** phospho-MAPK and total MAPK, **(b)** phospho-AKT and total AKT. The combined treatment in MCF-7 cells had a synergistic effect on MAPK and AKT phosphorylation compared to the effect of either treatment alone.

3.2.7 Treatment with exogenous IGF-1 induces activation the RET receptor in wild type mammary carcinoma cells

In order to further delineate the effect of IGF-1 treatment on ARTN signalling, the impact on activation of the RET receptor by receptor tyrosine phosphorylation was investigated in wild-type MCF-7 cells, and whether IGF-1 signalling affected activation of the RET receptor was determined. As described in section 1.5.3.3, the RET receptor is activated by phosphorylation at a number of tyrosine residues, although residues Y1062 and Y1096 are thought to be required for MAPK activation (Besset et al., 2000). The PathScan phospho-RET (pan Tyr) Sandwich ELISA kit (Cell Signalling Technology) which measures the general level of phosphorylation of the receptor was used for this purpose. Using the PathScan ELISA kit, phosphorylation and activation of the RET receptor was confirmed in wild-type MCF-7 cells following treatment with 100 and 200ng/mL recombinant ARTN protein (Figure 3.12a).

A time-course assay was conducted to determine the individual effects of exogenous ARTN and IGF-1 on the phosphorylation of RET. Wild-type MCF-7 cells were cultured in serum-free RPMI media, with 100ng/mL each of recombinant ARTN or IGF-1 added to the culture media. RET phosphorylation was evident for both ARTN and IGF-1 treatments at 5mins, with maximal stimulation with ARTN was observed at 30mins (Figure 3.12b). At 60mins post-ARTN treatment, RET phosphorylation began to decline. With IGF-1 treatment, a biphasic effect on RET activation was observed and 100ng/mL IGF-1 resulted in a small but significant ($p<0.01$) increase in RET phosphorylation. Activation of the RET receptor by IGF-1 has not been described previously. This low-level early activation indicates that IGF-1 may be directly stimulating RET activation, although weakly. After 4 hours of IGF-1 treatment a second larger increase in the amount of phospho-RET protein was observed (Figure 3.12b). This timing coincided with the IGF-1-induced increase in ARTN protein

levels observed in Section 3.2 and is likely to result from secondary activation of RET by ARTN.

Next, to determine whether ARTN and IGF-1 synergistically activated RET, MCF-7 cells were treated with 100ng/mL IGF-1 and/or recombinant ARTN for 10, 60, 120 and 240mins. At 60mins, co-treatment of the cells with IGF-1 and ARTN resulted in greater activation of RET compared to treatment with either ligand alone (Figure 3.12c). At this time point, combined ARTN and IGF-1 treatment resulted in RET phosphorylation 1.32-fold the level achieved by ARTN treatment alone ($p<0.05$), and 1.83-fold greater than IGF-1 alone ($p<0.01$). The additive effect on RET activation was observable for each of the time points assayed, but the effect at 240mins of treatment was less marked, likely due to the reduced activation by ARTN observed.

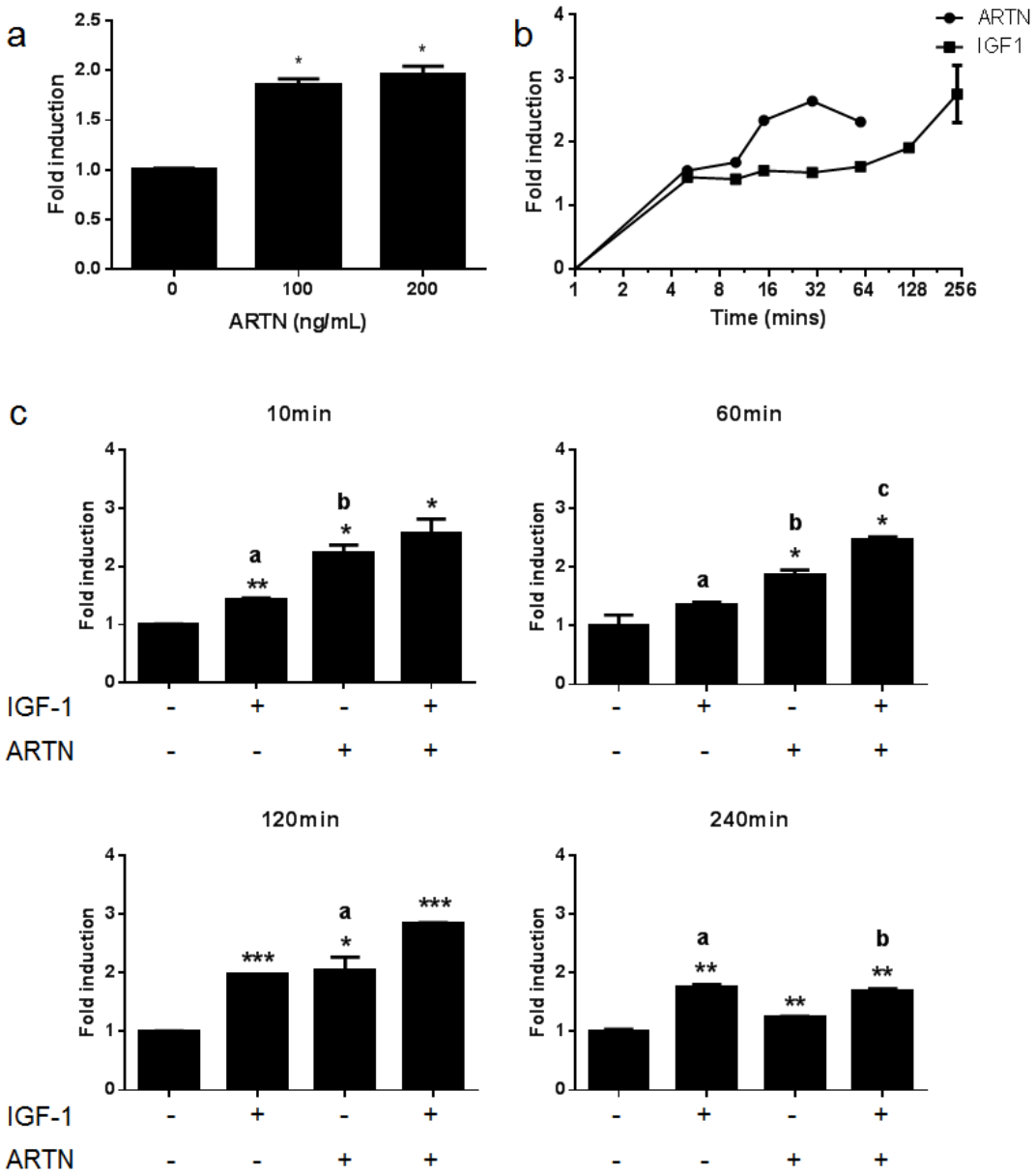

Figure 3.12- Activation of the RET receptor by ARTN and IGF-1.

Wild-type MCF-7 cells were cultured in serum-free RPMI media in the presence of 100ng/mL IGF-1 and 100ng/mL ARTN, or the combination of the two, for the indicated time periods. Cells were lysed and then assayed for phosphorylated RET receptor protein using a sandwich ELISA, as an indicator of RET receptor activation. **(a**) Dose responsiveness of RET phosphorylation to ARTN treatment and confirmation of receptor activation. **(b)** Timecourse of RET phosphorylation following ARTN and IGF-1 treatment, demonstrating the difference in activation profiles by the two ligands, particularly, the biphasic pattern of activation by IGF-1. **(c)** Additive induction of RET phosphorylation following combined ARTN and IGF-1 treatments. ELISA readings were normalised to the level of GAPDH protein in the lysates, as determined using an AlphaScreen assay. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (compared to non-treated control); **10min:** a, $p<0.05$(IGF-1 treatment *vs*. ARTN treatment); b, $p<0.05$ (IGF-1 treatment *vs*. Combined IGF-1/ARTN treatment); **60min:** a, $p<0.05$(IGF-1 treatment *vs*. ARTN treatment); b, $p<0.01$ (IGF-1 treatment *vs*. Combined IGF-1/ARTN treatment); c, $p<0.05$ (ARTN treatment *vs*. Combined IGF-1/ARTN treatment); **120min:** a, $p<0.01$ (IGF-1 treatment *vs*. Combined IGF-1/ARTN treatment); **240min:** a, $p<0.05$(IGF-1 treatment *vs*. ARTN treatment); b, $p<0.01$ (ARTN treatment *vs*. Combined IGF-1/ARTN treatment).

3.2.8 Treatment with recombinant ARTN protein does not activate IGF-1R signalling in wild-type mammary carcinoma cells

To determine whether the synergistic effect of ARTN/IGF-1 signalling on MAPK and AKT activation occurred through activation of IGF-1R, tyrosine phosphorylation of the of the β-subunit of the receptor was assessed by ELISA. The IGF-1R is a heterotetramer comprised of 2 transmembrane α-subunits and 2 β-subunits (Butler et al., 1998). The receptor is activated by autophosphorylation of the β-subunit at the tyrosine residues Y1121, Y1135 and Y1136 (Butler et al., 1998).

A PathScan phospho-IGF-1R Sandwich ELISA kit (Cell Signalling Technology) which measures phosphorylation at residue Y1136, was used to determine IGF-1R activation. The same treatment time-course as the RET ELISA was used. Confirmation of IGF-1R activation in wild-type MCF-7 cells, was carried out by assaying for receptor phosphorylation following treatment with 100 and 200ng/mL IGF-1 (Figure 3.13a).

A treatment time-course, similar to that conducted for the RET receptor, was carried out to determine the individual effects of exogenous ARTN and IGF-1 on the phosphorylation of IGF-1R. In the IGF-1 treatment time-course, a rapid induction of IGF-1R phosphorylation was observed at 5mins (Figure 3.13b). Receptor phosphorylation was sustained for almost the entirety of the assay treatment period and maximal receptor phosphorylation was achieved at 120mins of IGF-1 treatment. In contrast, ARTN did not induce IGF-1R phosphorylation during the treatment period. Additionally, combined ARTN and IGF-1 treatment in MCF-7 cells did not result in the additive or synergistic activation of the IGF-1R (Figure 3.13c). This is in contrast to the RET ELISAs, where additive activation of the RET receptor was clearly observed in some of the time-points used in this assay. We can therefore surmise that while treatment with exogenous IGF-1 results in the induction of ARTN/RET

signalling in MCF-7 cells, there is no reciprocal induction of the IGF-1 signalling pathway by ARTN. In addition, synergistic activation of MAPK and AKT signal transduction pathways is unlikely to occur through activation of RET or IGF-1R.

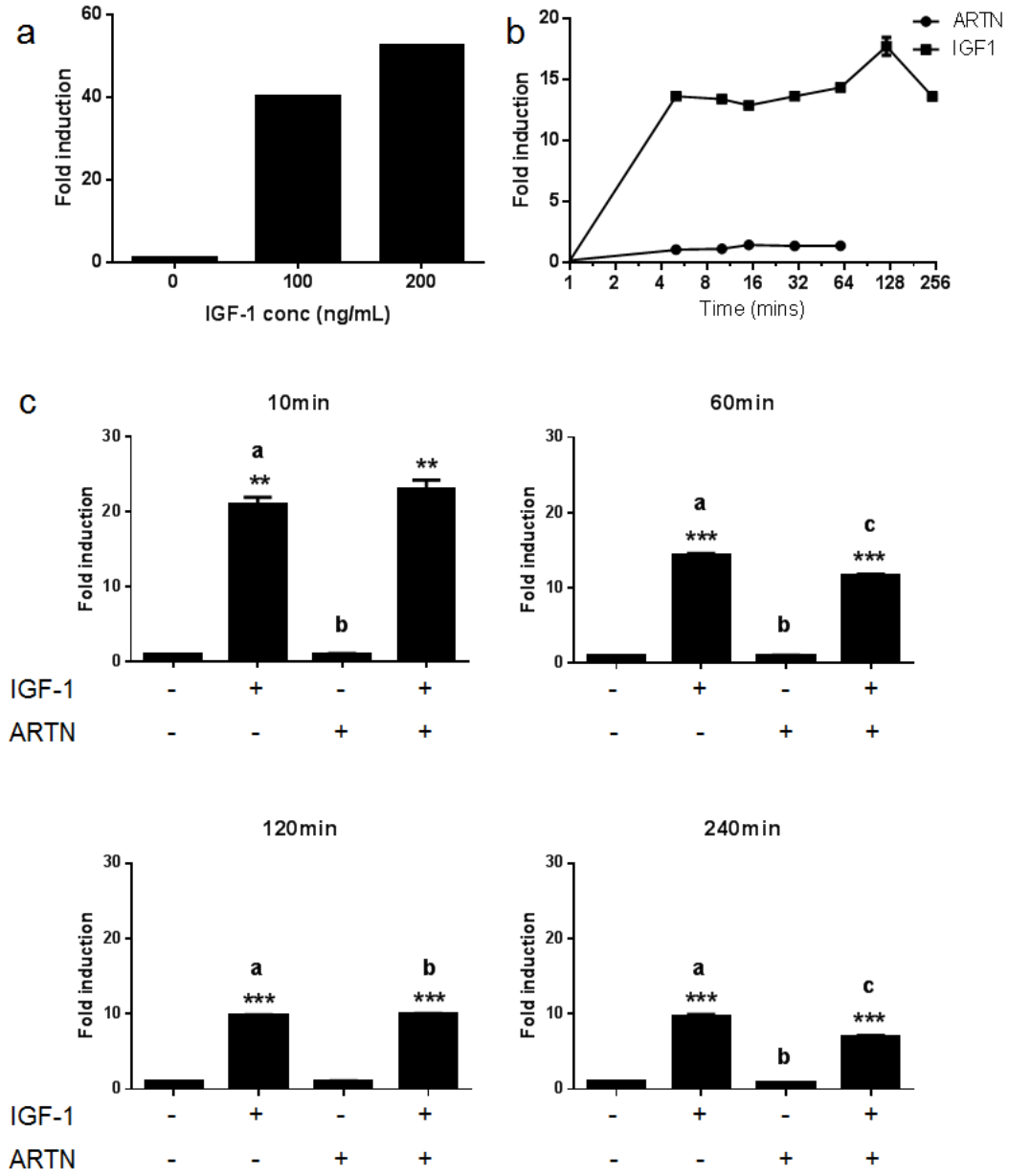

Figure 3.13- Activation of IGF-1R phosphorylation by IGF-1 and ARTN.

Wild-type MCF-7 cells were cultured in serum-free RPMI media in the presence of 100ng/mL IGF-1 and 100ng/ml ARTN, or the combination of the two, for the indicated time periods. Cells were lysed and then assayed for phosphorylated IGF-1R protein using a sandwich ELISA, as an indicator of IGF-1R activation. **(a)** Dose responsiveness of IGF-1R phosphorylation to IGF-1 treatment and confirmation of receptor activation. **(b)** Time-course of IGF-1R phosphorylation demonstrating sustained activation of IGF-1R by IGF-1, but not ARTN. **(c)** Co-treatment of MCF-7 confirmed the activation of IGF-1R by IGF-1 but not ARTN, across the time points assayed and confirming the lack of synergistic/additive activation by combining the treatment ligands. The results were normalised by GAPDH. ** $p<0.01$, *** $p<0.001$(compared to non-treated control); **10min:** a, $p<0.01$(IGF-1 treatment *vs*. ARTN treatment); b, $p<0.01$ (IGF-1 treatment *vs*. Combined IGF-1/ARTN treatment); **60min:** a, $p<0.001$ (IGF-1 treatment *vs*. ARTN treatment); b, $p<0.001$ (IGF-1 treatment *vs*. Combined IGF-1/ARTN treatment); c, $p<0.01$ (ARTN treatment *vs*. Combined IGF-1/ARTN treatment); **120min:** a, $p<0.001$ (IGF-1 treatment *vs*. ARTN treatment); b, $p<0.01$ (ARTN treatment *vs*. Combined IGF-1/ARTN treatment); **240min:** a, $p<0.001$(IGF-1 treatment *vs*. ARTN treatment); b, $p<0.05$ (IGF-1 treatment *vs*. Combined IGF-1/ARTN treatment); c, $p<0.001$ (ARTN treatment *vs*. Combined IGF-1/ARTN treatment).

3.3 Discussion

The evidence for the involvement of ARTN in cancer formation and progression is increasing as more published studies demonstrate the ability of ARTN to drive and encourage cancerous cell growth and cancer progression. Importantly, the cross-talking potential of RET with other signalling networks gives more significance to the interplay and interaction of ARTN signalling with other signalling molecules and pathways (Kuure et al., 2005; Peterziel et al., 2007; Tufro et al., 2007).

The results presented in this chapter demonstrated the regulation of ARTN by IGF-1 signalling in various mammary carcinoma cell lines. That IGF-1 signalling affected the mediators of ARTN signalling, possibly through modification of the RET receptor, provided evidence for the cross-talk between the two pathways. Exogenous IGF-1 was shown to increase both *ARTN* mRNA and protein expression in a time- and dose-dependent manner when assayed by semi-quantitative RT-PCR and western blotting, respectively. This regulation was more evident in MCF-7 cells compared to T47D and ZR-75-1, although the effect was universal across the mammary carcinoma cell lines we investigated. In contrast, expression of the ARTN receptors, *RET*, *GFRα1* and *GFRα3* were unaffected by IGF-1 when assayed by semi-quantitative RT-PCR. Similarly, Kang *et al* (Kang et al., 2010) previously demonstrated the regulation of ARTN expression and signalling by estrogen. In their study, ARTN expression in mammary carcinoma cells was up-regulated by the addition of exogenous estrogen to the culture medium.

Likewise, other studies on the subject of the GFLs and RET have also demonstrated functional interaction of these proteins with a variety of signalling pathways. Notably, the study by Boulay *et al.* (Boulay et al., 2008) demonstrated the interaction of the ERα and RET receptors in breast cancer cells. They demonstrated a substantial increase in RET and GDNF

expressions in the presence of estrogen and that RET signalling could potentiate estrogen-driven cell proliferation in MCF-7 and T47D cells.

When tyrosine phosphorylation of the RET receptor was investigated, ELISA results indicated strong activation in the presence of ARTN and IGF-1, both alone and in combination. RET activation by both ligands was almost immediate, at 5mins of treatment. However, the initial increase in RET phosphorylation induced by IGF-1 was small compared to activation by ARTN and would need to be confirmed by other, more sensitive, assays. In the presence of ARTN, the phosphorylation level of RET steadily increased for the majority of the treatment period assayed. Interestingly, RET activation by IGF-1 exhibited a biphasic pattern. IGF-1 treatment induced a notable second increase in RET phosphorylation that occurred after 2 hours of continuous treatment, coinciding with the IGF-1-stimulated increase in ARTN expression seen in the western blots presented earlier in this chapter. This biphasic pattern of RET activation, while unexpected, is not entirely unique and similar activation patterns have previously been reported. In promoting neutrite outgrowth, another GFL, NTN, has been shown to induce biphasic ERK 1/2 activation via cyclic AMP (Wan et al., 2011). GDNF was also shown to induce cell proliferation in a biphasic pattern. Orth *et al* (2000) found that at very low concentrations (2ng/mL), GDNF induced biphasic growth in human mesangial cells. This pattern was evident in its effects on cell number growth, DNA synthesis and proliferative rate. Interestingly, at higher doses of GDNF (10-50ng/mL), the biphasic pattern of activation was lost (Orth et al., 2000).

In the case of ARTN signalling, although the ligand binds and signals primarily through receptors RET and GFRα3, there is also some cross-binding and activation with the GFRα1 receptor (Airaksinen et al., 2006). It is therefore possible that the biphasic phosphorylation of

RET that we see with exogenous IGF-1 was due to the induction of ARTN expression and the activation of RET by the induced ARTN protein

No activation of IGF-1R by ARTN was observed in this study. Unlike results observed in the RET ELISAs, not only was there no activation by ARTN as a single agent, there was also no additional or synergistic activation of the receptor when used in combination with IGF-1. This demonstrated that the cross-talk in signalling between IGF-1 and ARTN, at the receptor level, is strictly one-way. While exogenous IGF-1 treatment in the mammary carcinoma cell lines was able to activate ARTN/RET signalling, no reciprocal activation of the IGF-1 pathway by ARTN was observed.

The activation of MAPK by IGF-1 was demonstrated by western blotting, and results were consistent with previously published studies (Zhang et al., 2011). ARTN has also been demonstrated to activate MAPK; however, the dose- and time-dependency of activation in MCF-7 cells had not been previously investigated in such detail. Concentrations as low as 20ng/mL induced MAPK phosphorylation, with maximal stimulation observed at 500ng/mL ARTN. In subsequent studies, 100ng/mL was used as this concentration is closer to the reported binding affinity of ARTN for GFRα3 measured on cells (K_d = 1-10 nM) (Wang et al., 2006) and is consistent with those used in other published studies (Ceyhan et al., 2006a; Meng et al., 2012). Treatment with 100ng/mL of ARTN induced rapid phosphorylation of MAPK at 5mins, with maximal stimulation at 10mins.

Activation of AKT was also investigated. AKT is a known signalling mediator in the IGF-1 signalling pathway (Zhang et al., 2011) and RET-mediated signal transduction (Banerjee et al., 2011; Pandey et al., 2010b). Activation of AKT by IGF-1 was demonstrated by western blotting, and results were consistent with previously published studies (Zhang et al., 2011). However, no phosphorylation of AKT in MCF-7 cells was detected with ARTN treatment

alone, even at the higher concentrations. This was a surprising finding given that ARTN has been shown to activate AKT in endometrial cancer cell lines (Pandey et al., 2010b) and triple negative breast cancer cell lines (Banerjee et al., 2011). Although a previous study by Li *et al.* (2009) demonstrated that autocrine Artn signalling in embryonic mice induced activation of MAPK mediators of signalling, but not PI3K (Li et al., 2009). Thus activation of this pathway by ARTN may be cell line specific. For example, the study by Shin *et al.* demonstrated that constitutively activated RET in papillary thyroid carcinomas did not correlate with constitutive phosphorylation of MAPK and AKT in patient tumours, when assessed by immunohistochemical staining (Shin et al., 2004). But in contrast, a few years prior to that, Specht *et al.* (Specht et al., 2001) demonstrated that the MAPK was constitutively phosphorylated in surgically resected papillary thyroid carcinoma compared to normal thyroid tissue.

Interestingly, following ARTN and IGF-1 treatment in MCF-7 cells, both MAPK and AKT demonstrated greater protein phosphorylation than when treated with either ligand alone. This increased protein phosphorylation is often correlated with greater signalling activation (Cargnello and Roux, 2011; Fresno Vara et al., 2004) and suggests that combining ARTN and IGF-1 treatment could produce an additive effect on mitogenic signalling in MCF-7 cells. Particularly with AKT, where ARTN treatment alone did not induce phosphorylation of the protein, but when combined with IGF-1, synergistic phosphorylation of AKT was observed. Further studies quantifying the increased MAPK and AKT phosphorylation observed in western blot analysis would be useful.

3.4 Conclusion

In the present study, exogenous IGF-1 was shown to regulate the expression of ARTN mRNA and protein in a dose-dependent manner, but had no effect on the expression of the main receptors for ARTN, RET and GFRα3. This effect on ARTN expression was observed in three different wild-type mammary carcinoma cell lines.

Although exogenous IGF-1 had no effect on RET receptor expression, it did induce activation of the receptor as indicated by increased tyrosine phosphorylation in ELISA assays; however, this would require confirmation in subsequent studies due to the low level of activity being induced. But overall, combined treatment in MCF-7 cells with recombinant ARTN and IGF-1 resulted in synergistic activation of downstream signalling components MAPK and AKT indicating some degree of crosstalk between the two pathways may exist.

Chapter 4

RESULTS: Constitutive forced expression of ARTN in mammary carcinoma cells enhances the mitogenic effects of IGF-1 signalling

4.1 Introduction

The results of Chapter 3 provided evidence for the regulation of ARTN expression by treatment with exogenous IGF-1. There was also evidence for the induction of ARTN/RET signalling by IGF-1 treatment in wild-type mammary carcinoma cells, including initial activation at the RET receptor and activation of the downstream signalling molecules.

IGF-1 is a potent mitogenic signalling molecule and its pleiotropic effects have been well documented (Anisimov and Bartke, 2013; Bähr and Groner, 2005; Baserga, 1997). Its promiscuous signalling is also well-described. Cross-talk can occur with a number of signalling pathways, including the estrogen and human growth hormone signal transduction pathways and, its namesake, insulin (Anisimov and Bartke, 2013). IGF-1 signalling governs a range of effects, ranging from linear growth, to regulation of metabolism and mediating resistance to radiotherapy and chemotherapy drugs in the treatment of various cancers (Bähr and Groner, 2005; Baserga, 1997).

Similarly, there is also a growing body of evidence for the involvement of ARTN in the progression of various cancers, of both neuronal and non-neuronal origins (Ceyhan et al., 2006a; Kang et al., 2009; Kapoor, 2012; Pandey et al., 2010b). In addition, recent studies have demonstrated that both ARTN and the RET receptor are over-expressed in certain types

of ER-positive breast cancers (Boulay et al., 2008; Gattelli et al., 2013; Kang et al., 2009; Mason, 2000; Murakumo et al., 2006). The newly documented cross-talk between the RET receptor and the estrogen receptor (ER) is becoming increasingly important in determining the effectiveness of endocrine therapies. Some of the current research now focuses on the inhibition of RET receptor functions, or that of its ligands, for the modulation of the response to endocrine cancer therapy (Boulay et al., 2008).

Given the propensity of both signalling pathways to interact with other signalling networks, a mechanism of cross-talk between the IGF-1 and ARTN/RET signalling pathways is proposed, similar to that seen between either of these molecules and estrogen signalling. These cross-talk mechanisms have the effect of enhancing the response to ligand elicited signalling and increasing the cell behavioural responses.

In this chapter, forced expression of ARTN in mammary carcinoma cell lines was found to cooperate with exogenous IGF-1 to enhance aspects of cell growth and survival. In addition, antagonism or inhibition of ARTN/RET signalling had the converse effect on cell behaviour and impaired mitogenic effects usually induced by IGF-1.

4.1.1 Establishment of ARTN forced-expression cell lines

To study the role of ARTN in regulating IGF-1 signalling in breast cancer, a cell line-based model was established in which MCF-7 and T47D cells were stably transfected with a pIRESneo3 vector containing the *ARTN* gene under the control of a cytomegalovirus (CMV) promoter. The pIRES-ARTN construct was previously generated by Dr Jian Kang during her PhD studies (Kang et al., 2009).

Mammary carcinoma cell lines were transfected with the pIRES-ARTN vector, or the empty pIRESneo3 vector for control purposes. Pooled stable transfectants were selected by culturing in RPMI media with G418 antibiotic. The resulting cell lines were designated with the suffix –ARTN and –Vec, respectively. The resulting cell lines were confirmed for *ARTN* forced expression by semi-quantitative RT-PCR for *ARTN* mRNA expression and by western blotting for protein expression (Figure 4.1).

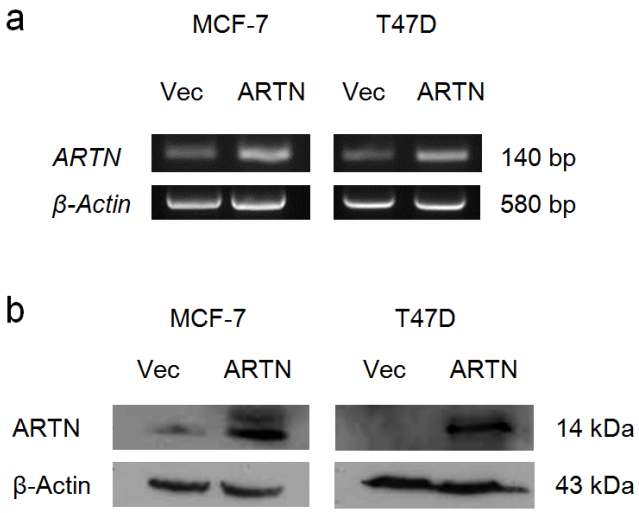

Figure 4.1- Establishment of ARTN forced expression stable cell lines.

Wild-type mammary carcinoma cell lines were stably transfected with an ARTN expression plasmid and forced expression was confirmed by **(a)** semi-quantitative RT-PCR and **(b)** western blot. β-Actin was used as a loading control.

4.2 ARTN forced expression in mammary carcinoma cells differentially impacts on IGF-1 signalling function and cell behaviour.

4.2.1 Characterisation of the mammary carcinoma cell lines for expression of IGF-1 signalling pathway components

The assays described in Chapter 3 demonstrated that IGF-1 treatment in MCF-7 cells increased ARTN protein and RET receptor activation. To investigate whether ARTN expression had a reciprocal effect on the IGF-1 signalling pathway, wild-type mammary carcinoma cell lines were characterised for expression of *IGF-1, IGF-2* and *IGF-1R.* In Figure 4.2, semi-quantitative RT-PCR confirmed that neither MCF-7 or T47D cell lines endogenously expressed *IGF-1* mRNA in any appreciable amount (Pacher et al., 2006). Low level expression of *IGF-2* mRNA was detected in both cell lines. *IGF-2* expression was also unaffected by the presence of FBS in the culture medium in MCF-7 and T47D cells (Figure 4.2). Finally, *IGF-1R* expression was also assayed, and mRNA was detected in both cell lines. In MCF-7 and T47D cells, culture in full serum conditions increased the level of *IGF-1R* mRNA suggesting regulation of the gene expression can be influenced by factors present in FBS.

Semi-quantitative RT-PCR was also used to determine whether ARTN affected expression levels of *IGF-1, IGF-2* and *IGF-1R.* MCF-Vec and MCF-ARTN, or T47D-Vec and T47D-ARTN cell lines were cultured in serum-free RPMI for 24 hours before total RNA was extracted. No change in the mRNA expression levels of these three genes was observed when assayed by semi-quantitative RT-PCR (Figure 4.3).

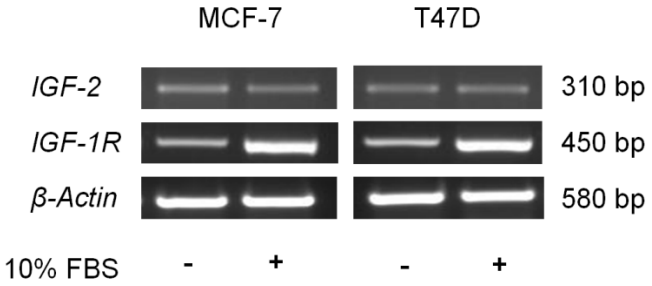

Figure 4.2- Endogenous expression of IGF-1 signalling components in wild-type mammary carcinoma cell lines.

Semi-quantitative RT-PCR analysis of IGF-2 and IGF-1R in MCF-7 and T47D cells. β-Actin was used as a loading control. *IGF-1* mRNA was also assayed, but there was no detectable level of expression and so is not shown.

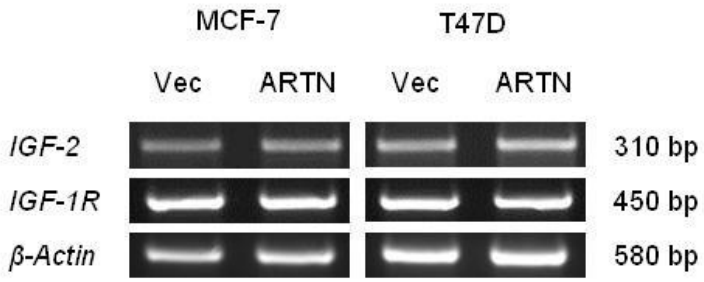

Figure 4.3- Endogenous expression of IGF-1 signalling components in ARTN forced expression stable cell lines.

The mRNA expression of the signalling components was not affected by forced expression of ARTN in MCF-7 and T47D wild-type cell lines, as assayed by semi-quantitative RT-PCR. *IGF-1* mRNA was also assayed, but there was no detectable level of expression and so it is not shown. β-Actin was used as a loading control.

4.2.2 ARTN forced expression combined with IGF-1 treatment increased MCF-7 cell number and cell viability

IGF-1 has been well documented as a potent signal for growth, both *in vivo* and *in vitro* (Laron, 2001). To investigate whether ARTN had any effect on the potential of IGF-1 to induce growth in MCF-7 cells, MCF-Vec and MCF-ARTN stable cell lines were treated with 50ng/mL IGF-1 in 0.2% FBS RPMI media, and the total cell number and relative cell viabilities for each cell line were recorded every 2 days during the assay period.

In the total cell number assay, the cell lines were seeded into 6-well plates at a density of 5,000cells/well. Over the 10 day assay period, these results indicated that forced expression of ARTN in MCF-7 cells did not significantly affect total cell number. Forced expression of ARTN slightly enhanced the growth response to IGF-1 treatment in MCF-ARTN cells compared with the control cell line (Figure 4.4a); however, the increases were marginal and only significant on day 8.

A total cell number assay was also attempted using the T47D-Vec and T47D-ARTN cell lines. However, the T47D cell lines had a much greater serum requirement for prolonged cell culture and could not be sustained in the low serum conditions required for this set of assays. In addition, at the serum level required to sustain the growth and survival of the T47D cells, the effect of IGF-1 treatment on the cells was lost.

The effect of IGF-1 on cell viability in the MCF-Vec and MCF-ARTN stable cell lines was tested by seeding the cells into 96-well plates at a density of 3,000 cells/well and treating with 50ng/mL IGF-1, in 0.2% FBS RPMI media. Cell viability was measured by MTT every 2 days, over a period of 6 days. In contrast to results from the total cell number assays, ARTN was found to significantly increase the viability of MCF-7 cells. On day 6 of the assay, forced expression of ARTN increased cell viability by 1.91-fold ($p<0.01$) (Figure

4.4b). IGF-1 treatment further increased MCF-ARTN cell viability at day 6 when compared with the control cell line, MCF-Vec (3.44-fold; $p<0.05$). These results suggest that ARTN forced expression additively cooperates with IGF-1 treatment to enhance MCF-7 cell viability beyond the increases induced by either ARTN forced-expression or IGF-1 treatment alone.

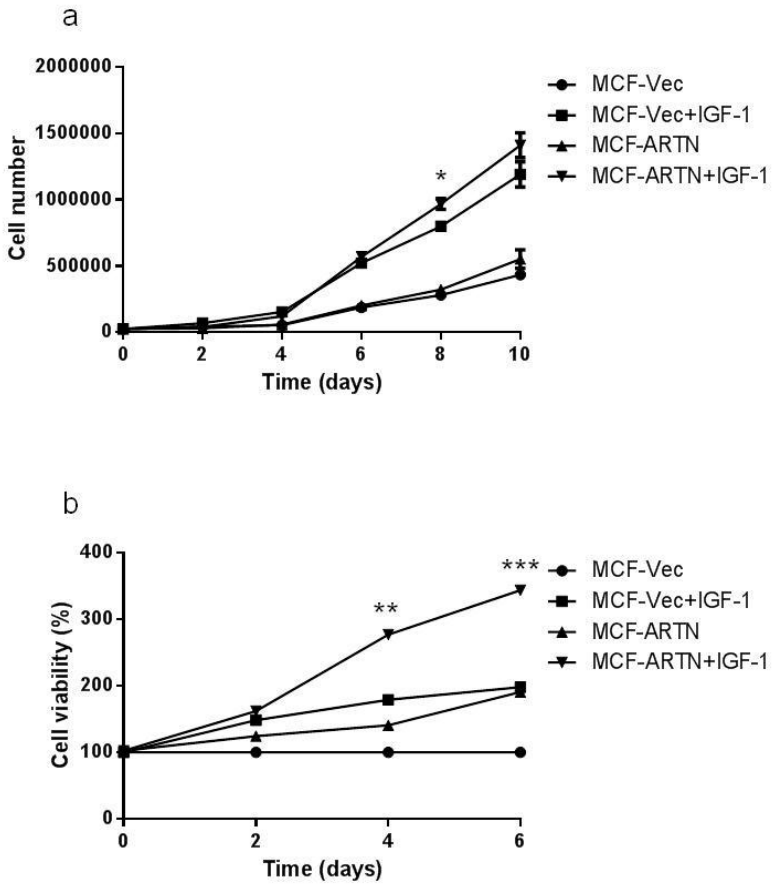

Figure 4.4- Total cell number and cell viability assays in MCF-Vec and MCF-ARTN.

(a) Forced expression of ARTN combined with IGF-1 treatment slightly enhanced cell growth in a total cell number assay conducted in 0.2% FBS (Day 8: *p<0.05; MCF-Vec *vs* MCF-ARTN. *p<0.05; MCF-Vec+IGF-1 *vs* MCF-ARTN+IGF-1) **(b**) Cell viability was determined using alamarBlue in ARTN forced expression stable cell lines following treatment with 50ng/ml IGF-1 in 0.2% FBS (Day 4: **p<0.01; MCF-Vec *vs* MCF-ARTN, **p<0.01; MCF-Vec+IGF-1 *vs* MCF-ARTN+IGF-1; Day 6: ***p<0.001; MCF-Vec *vs* MCF-ARTN, ***p<0.001; MCF-Vec+IGF-1 *vs* MCF-ARTN+IGF-1).

4.2.3 ARTN forced expression combined with IGF-1 treatment had no significant impact on the rate of cell proliferation

The rate of cell proliferation in the stably transfected cell lines was determined by measuring the rates of BrdU incorporation. Following 18-20 hours treatment with 50ng/mL IGF-1, in serum-free RPMI media, the stable cell lines were cultured for 30mins in RPMI media with BrdU (a pyrimidine analogue) to ascertain the rate of DNA synthesis and thereby cell

proliferation. The results of this set of assays differed greatly depending on the cell line being tested.

The rate of cell proliferation between MCF-Vec and MCF-ARTN cells was not significantly different, consistent with previously published findings (Kang et al., 2009) (Figure 4.5a). Treatment with IGF-1 increased MCF-Vec BrdU incorporation by 5.9-fold ($p<0.001$) and MCF-ARTN by 7.06-fold ($p<0.001$). Although a consistent trend for increased BrdU incorporation was observed in MCF-ARTN *versus* MCF-Vec cells over repeat experiments, this was not statistically significant.

In contrast, in T47D cells, forced expression of ARTN increased the rate of cell proliferation by 1.5-fold in T47D-ARTN cells compared to T47D-Vec ($p<0.05$). When treated with IGF-1, however, the differences were more modest. IGF-1 treatment increased the proliferative rate of T47D-Vec cells by 1.72-fold ($p<0.01$) and the rate of the T47D-ARTN cells by 1.35-fold ($p<0.01$).

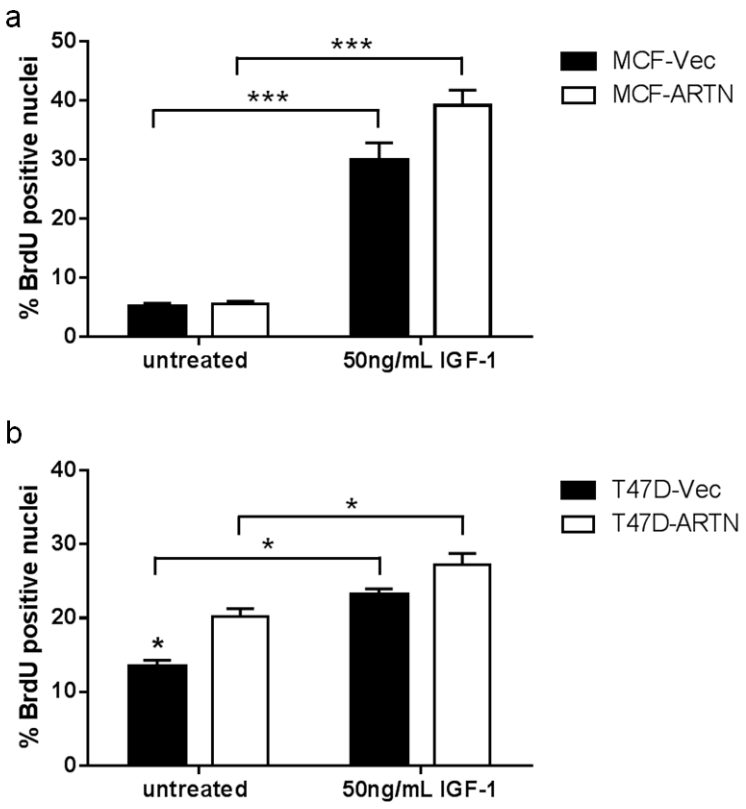

Figure 4.5- Cell proliferation as determined by BrdU incorporation

Cells were treated with IGF-1 in serum-free RPMI media for 18-20 hours before being labelled with BrdU and fixed for subsequent staining. Proliferating cells were identified by light microscopy.

* $p<0.05$, *** $p<0.001$

4.2.4 Forced expression of ARTN reduced stress-induced apoptotic cell death following treatment with IGF-1

To complement the results of the cell proliferation assays, the rate of apoptosis was investigated in the cell lines stably transfected with ARTN. Apoptosis was induced by culturing the cells in serum-free RPMI media for 24 hours, with or without 50ng/mL IGF-1 treatment, and apoptotic cells were identified by changes in nuclear morphology following staining with the fluorescent DNA dye, Hoechst 33258.

ARTN forced expression reduced the apoptotic rate in MCF-ARTN cells by 2.52-fold compared to MCF-Vec ($p<0.001$) (Figure 4.6a). In the MCF-7 stables, IGF-1 treatment reduced MCF-Vec apoptotic cell death by 1.95-fold, with a difference of 1.69-fold in MCF-Vec *versus* MCF-ARTN following treatment ($p<0.05$).

In T47D, cells forced expression of ARTN reduced the rate of apoptosis by 1.64-fold in T47D-ARTN cells compared to T47D-Vec ($p<0.05$). IGF-1 treatment reduced the apoptotic rate in T47D-Vec cells by 1.36-fold ($p<0.05$) and the rate of the T47D-ARTN cells by 1.44-fold ($p<0.05$), with a difference between the two IGF-1 treated cell lines of 1.74-fold ($p<0.01$) (Figure 4.6b).

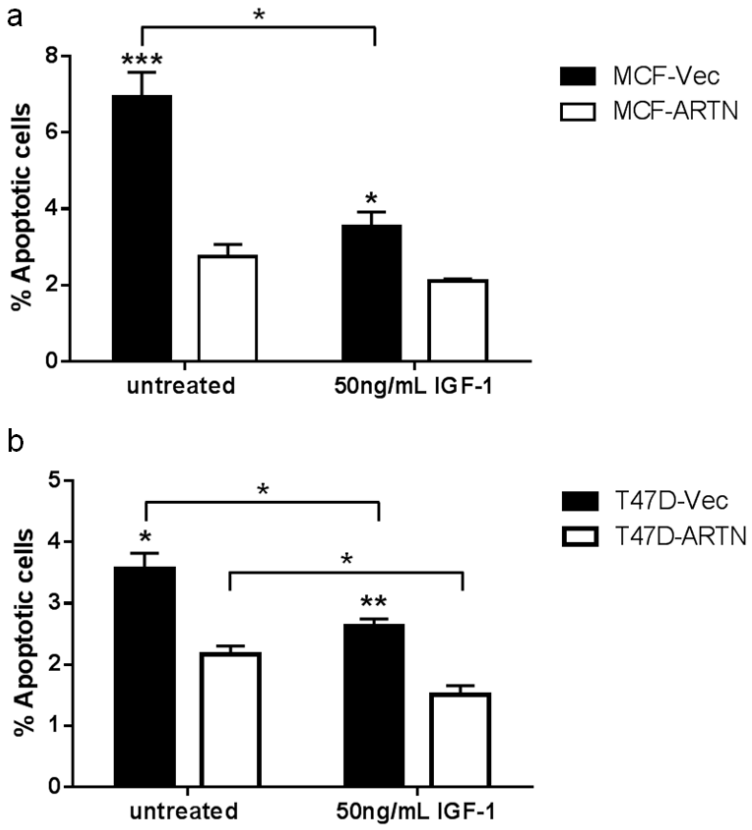

Figure 4.6- Apoptotic cell death determined by Hoechst 33258 staining.

Cell lines were all cultured in serum-free RPMI media for 24 hours before staining with Hoechst 33258. Apoptotic cells were identified by nuclear condensation and morphology under fluorescent microscopy. * $p<0.05$, ** $p<0.01$

4.2.5 Forced-expression of ARTN in MCF-7 cells cooperates with IGF-1 signalling to enhance colony formation in soft agar

Anchorage-independent growth is often an indicator of cancer cell oncogenicity. A soft agar colony formation assay was used to measure anchorage independence in the MCF-Vec and MCF-ARTN cells lines. Cells were embedded into soft agar as single cells and were grown in three-dimensional colonies, while supplemented with RPMI media and 3% FBS. The culture media was replaced every 2-3 days, and 50ng/mL exogenous IGF-1 was also added to the culture media and agar for the duration of the assay. After 14 days of culture, the culture plates were stained with 0.1% crystal violet solution and the colonies counted by light microscopy.

Consistent with previous findings (Kang et al., 2009), forced ARTN expression increased MCF-7 cell colony number more than 1.47-fold ($p<0.05$) in the soft agar assays when compared with MCF-Vec cells (Figure 4.7). IGF-1 treatment also significantly increased the number of colonies formed in the control cell line, MCF-Vec (1.39-fold, $p<0.01$). Combining ARTN forced expression and IGF-1 treatment increased the colony number further in these assays and additively increased the MCF-ARTN colony number 1.3-fold ($p<0.001$) compared to IGF-1 treated MCF-Vec cells. T47D cells did not grow in soft agar under reduced serum conditions so colony formation was not determined for T47D-Vec and T47D-ARTN.

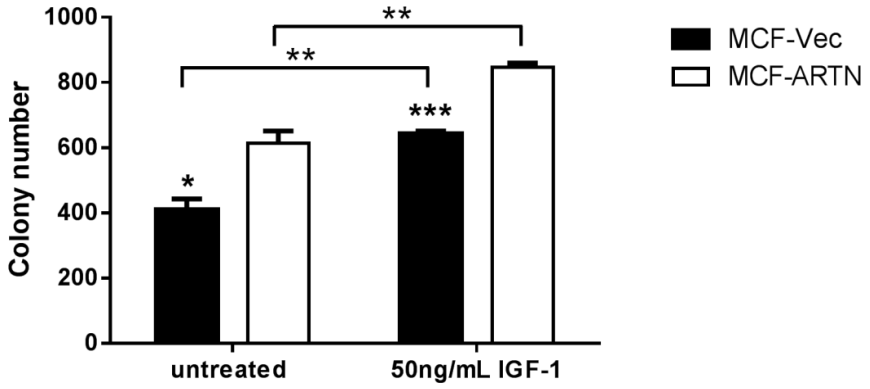

Figure 4.7- Soft agar colony formation in ARTN forced expression stable cell lines.
IGF-1 treatment increased the number of colonies formed for both cells lines, but there were significantly more colonies formed by IGF-1 treated MCF-ARTN cells than MCF-Vec cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.2.6 ZR-75-1 cells demonstrated different responses to the forced expression of ARTN

In addition to the ARTN forced expression cell lines previously established in MCF-7 and T47D, forced expression of ARTN was also established in ZR-75-1 cells. ZR-75-1 cells were stably transfected with the pIRES-ARTN vector of control vector (pIRES-neo3) described above, by electroporation. Stable transfectants were selected for by culturing in 10% FBS RPMI medium, containing 1000 µg/mL G418, for 21-28 days. Forced expression of ARTN in the stable cell lines was confirmed by semi-quantitative RT-PCR and western blotting, for mRNA and protein expression, respectively (Figures 4.8a and b). The new cell lines were designated ZR-Vec (vector transfected control) and ZR-ARTN (ARTN forced expression cell line) and were sustained in culture with 10% FBS supplemented RPMI media.

The stably transfected ZR-75-1 cell lines were used in growth assays for total cell number and cell viability. However, these assays were mostly unsuccessful. Similar to the problems experienced with the T47D stable cell lines, the growth of ZR-75-1 cells was not sustained in culture under low serum conditions. In addition, the higher serum content required for

prolonged growth in culture masked the effects of IGF-1. However, assays requiring shorter culture periods (i.e. cell proliferation and apoptosis) more readily provided results under the required culture conditions.

Surprisingly, BrdU incorporation assay demonstrated that ARTN reduced proliferation in ZR-75-1 cells (Figure 4.8c). The ZR-ARTN cell line exhibited a lower rate of cell proliferation than ZR-Vec cells (1.33-fold reduction, $p<0.05$). In addition, the proliferative rate in the IGF-1 treated cells was also significantly lower in the ZR-ARTN cells than in ZR-Vec (1.38-fold reduction, $p<0.01$), contrary to results obtained in the MCF-7 and T47D cell lines. IGF-1 did not significantly affect the cell proliferation rate of either cell line.

The results of the apoptosis assays using ZR-Vec and ZR-ARTN cells followed a similar trend to results obtained in MCF-7 and T47D cell lines. However, the actual figures for apoptotic rate were particularly low and neither ARTN nor IGF-1 alone had any significant effect on the apoptotic rates (Figure 4.8d). Though notably, IGF-1 signalling reduced the apoptotic rate in MCF-ARTN by 1.50-fold compared to MCF-Vec following IGF-1 treatment ($p<0.05$).

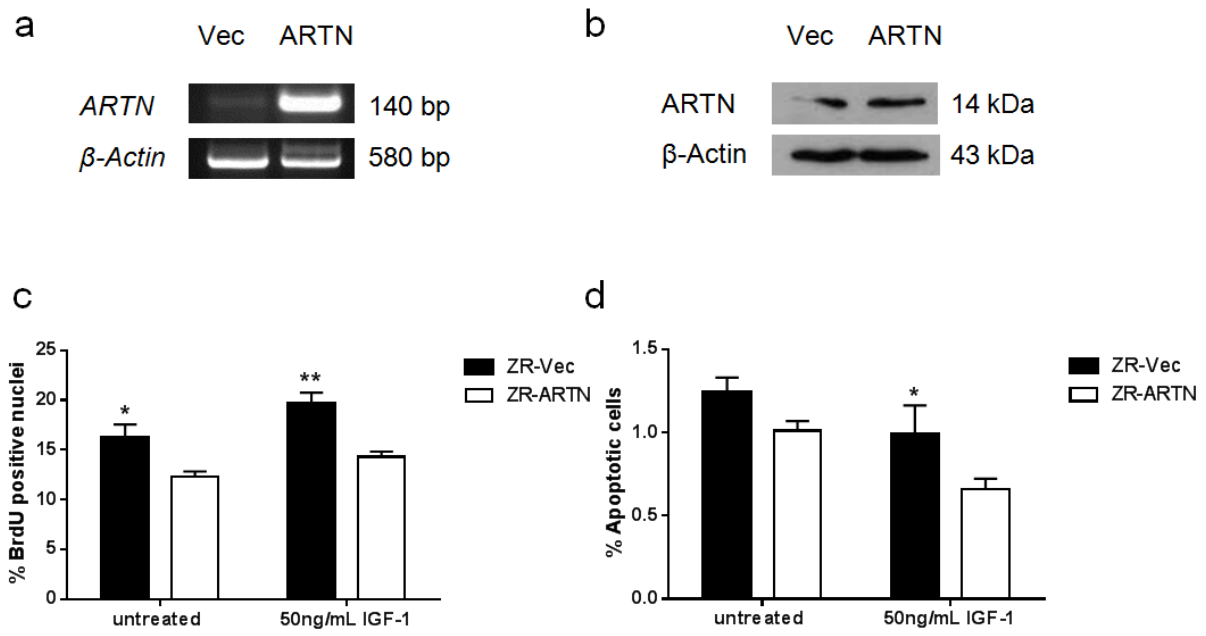

Figure 4.8- Effect of ARTN forced expression on ZR-75-1 cells.

ARTN forced expression cell lines were established, and verified by **(a)** semi-quantitative RT-PCR and **(b)** western blotting. β-Actin was used as loading control for both assays. **(c)** BrdU incorporation assays showed a reduced in the rate of proliferation in ZR-ARTN cells compared to ZR-Vec. **(d)** Apoptosis in ZR-Vec and ZR-ARTN cells were not significantly affected by IGF-1 treatment. *p<0.05, **p<0.01

4.3 Depletion of ARTN expression by siRNA in mammary carcinoma cells reduces the cellular response to IGF-1 treatment

4.3.1 Establishment of the siRNA knockdown cell lines in MCF-7

To study the effect of ARTN knockdown on IGF-1 signalling, MCF-7 cells were stably transfected with an siRNA knockdown plasmid containing an siRNA that targets *ARTN* mRNA. The siARTN sequence was cloned into the vector pSilencer 3.1 under the regulation of an H1 promoter, a polymerase III promoter. MCF-7 cell lines stably transfected with pSilencer3.1-siARTN (designated MCF-siARTN) or a control vector containing a scrambled

siRNA control sequence (designated MCF-siVec) were previously generated by Dr. Jian Kang for the use in her aforementioned PhD thesis (Kang et al., 2009)

Semi-quantitative RT-PCR was used to assay the mRNA expression of components of the IGF-1 signalling pathway. Consistent with results observed in Section 4.2, siRNA knockdown of ARTN had no observable effect on the mRNA expression of *IGF-2* or *IGF-1R* (Figure 4.9).

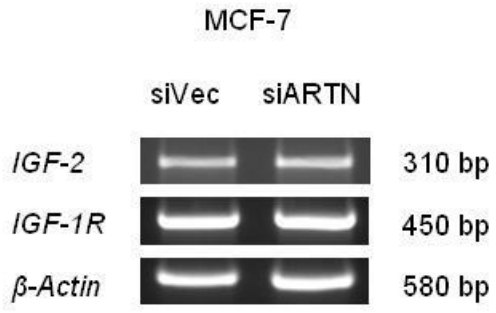

Figure 4.9- Endogenous expression of IGF-1 signalling components in ARTN siRNA knockdown cell lines.

Expression of *IGF-2* and *IGF-1R* mRNA was determined by semi-quantitative RT-PCR. β-actin was used as a loading control. *IGF-1* mRNA was not assayed as there is no expression in MCF-7 cells, as described above.

4.3.2 siRNA-mediated depletion of ARTN expression in MCF-7 cells reduced IGF-1-induced cell growth and cell viability

MCF-siVec and MCF-siARTN stable cells were treated with 50ng/mL IGF-1 in 0.2% FBS RPMI media. Each cell line was assayed for total cell number and cell viability every 2 days during the assay period.

For the total cell number assays, each cell line was seeded into 6-well plates, at a density of 5,000cells/well and the cell number recorded over 10 days. In Section 4.2.2, it was demonstrated that forced expression of ARTN in MCF-7 cells enhanced the growth response to IGF-1, but only marginally. Conversely, siRNA knockdown of ARTN expression reduced the response of MCF-7 cells to IGF-1 (Figure 4.10a). siRNA-mediated depletion of ARTN did not affect total cell number. Treatment with IGF-1 increased the total cell number in MCF-siVec cells by 4.56-fold on day 10 ($p<0.001$), whereas treatment with IGF-1 increased total cell number in the MCF-siARTN cell line by 2.78-fold ($p<0.001$), when compared to the untreated cells.

Next, the IGF-1 effect on cell viability was tested in MCF-siVec and MCF-siARTN cells. The cells were seeded into 96-well plates at 3,000cells/well and treated with 50ng/mL IGF-1. Cell viability was measured using alamarBlue over a 6 day period. Similar to the total cell number assay, siRNA depletion of ARTN alone did not significantly affect the viability of MCF-7 cells. However, it did affect the response to IGF-1 treatment (Figure 4.10b). IGF-1 treatment of MCF-siARTN cells increased cell viability by 2.69-fold on day 6 ($p<0.001$), while IGF-1 treatment increased MCF-siVec cell viability by 3.11-fold ($p<0.001$), relative to the untreated cell lines.

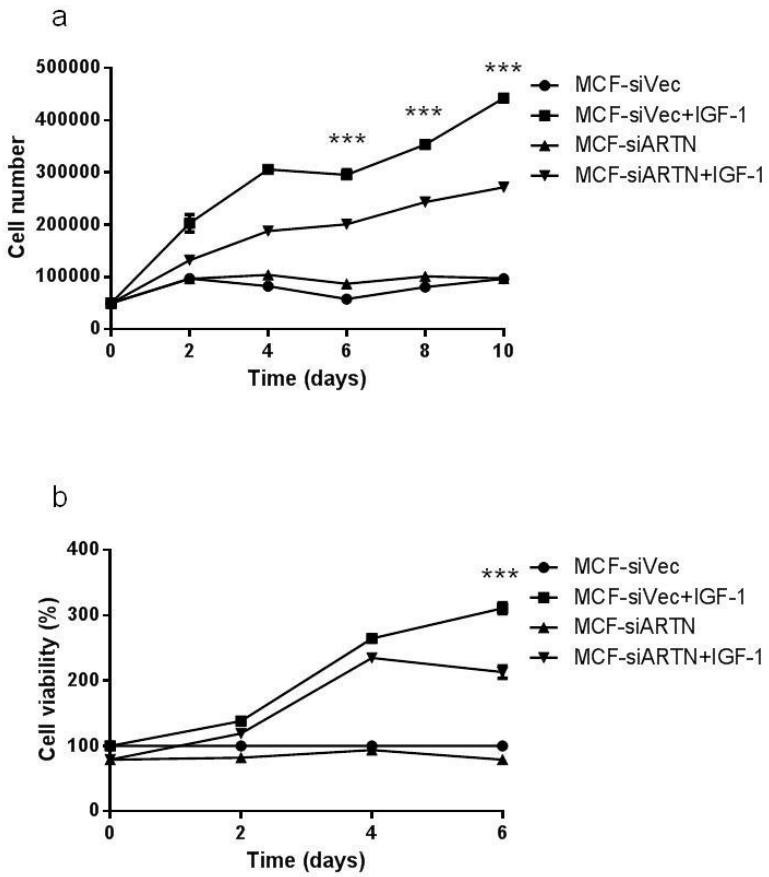

Figure 4.10- Depletion of ARTN expression by siRNA in MCF-7 cells reduces IGF-1-induced cell growth and viability

(a) Total cell number assay. siRNA-mediated depletion of ARTN reduced total cell number of MCF-7 cells when treated with IGF-1 (Day 10: *** p<0.001; MCF-siVec *vs* MCF-siVec+IGF-1. *** p<0.001; MCF-siARTN *vs* MCF-siARTN+IGF-1. *** p<0.001; MCF-siVec+IGF-1 *vs* MCF-siARTN+IGF-1.) (b) Cell viability assay. siRNA-mediated depletion of ARTN reduced the viability of MCF-7 cells when treated with IGF-1 . (Day 6: *** p<0.001; MCF-siVec *vs* MCF-siVec+IGF-1. *** p<0.001; MCF-siARTN *vs* MCF-siARTN+IGF-1. *** p<0.001; MCF-siVec+IGF-1 *vs* MCF-siARTN+IGF-1).

4.3.3 Knockdown of ARTN expression in MCF-7 cells reduced IGF-1-induced cell proliferation and survival

To investigate the contribution of cell proliferation and survival to overall growth, MCF-7 cells with depleted ARTN were assayed for proliferation and apoptotic cell death. The rate of cell proliferation in MCF-siVec and MCF-siARTN cells following treatment with 50ng/mL IGF-1 was determined using a BrdU assay. Consistent with assay results obtained following

ARTN forced expression, there was no significant difference between the rate of cell proliferation in MCF-siVec and MCF-siARTN cell lines. However, in the presence of IGF-1 the proliferative response of the cell lines was significantly different (Figure 4.11a). Although the proliferative rate of both cell lines were greatly increased with IGF-1 treatment, the extent of the increase was much greater in MCF-siVec cells (4.67-fold, $p<0.001$) compared to MCF-siARTN (2.41-fold, $p<0.05$). Here, the depletion of ARTN expression synergistically reduced the proliferative response to IGF-1 treatment to almost half that of the MCF-siVec control (4.67-fold *versus* 2.41-fold, $p<0.05$, 2-way ANOVA).

Next, cell survival in MCF-siVec and MCF-siARTN cells was assessed. Cells were cultured for 24 hours in serum-free RPMI media with or without 50ng/mL IGF-1 treatment, then fixed and stained with Hoechst 33258. Consistent with assay results obtained above following ARTN forced expression, siRNA-mediated depletion of ARTN expression significantly reduced cell survival conferred by exogenous IGF-1 treatment. Depletion of ARTN expression alone had little observable effect on the rate of cell survival. IGF-1 treatment reduced the apoptotic cell death in MCF-siVec by 1.77-fold (reduction, $p<0.01$) and MCF-siARTN by 1.26-fold ($p<0.05$) (Figure 4.11b).

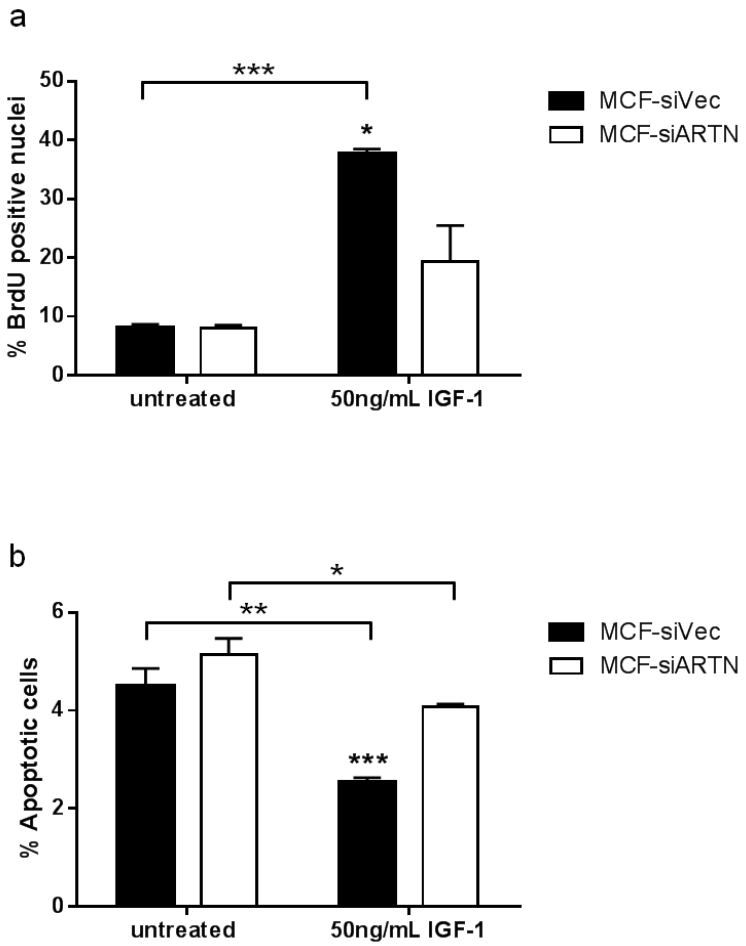

Figure 4.11- Depletion of ARTN expression by siRNA in MCF-7 cells reduces IGF-1 enhancement of cell proliferation and survival.

(a) The rate of cell proliferation assayed by BrdU incorporation was unaffected by siRNA knock down of ARTN expression in MCF-siARTN, but the IGF-1-induced proliferative response was reduced, compared to MCF-siVec. **(b)** Apoptotic cell death in untreated cells was similar when assayed by Hoechst staining. siRNA depletion of ARTN reduced the response of MCF-siARTN cells to IGF-1, when compared to the control cell line, MCF-siVec. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.3.4 siRNA-mediated depletion of ARTN expression in MCF-7 cells differentially affected the ability of MCF-7 cells to form three-dimensional colonies in soft agar and Matrigel®.

To measure the oncogenicity of the ARTN knock down cell lines, cells were cultured in soft agar and assessed for their ability to form colonies in the semi-solid medium. In addition, 3D growth was assessed in the basement membrane extract, Matrigel®.

To assess colony formation in soft agar, MCF-siVec and MCF-siARTN cells were seeded into 6-well plates at 5000cells/well and cultured for 14 days in 3% serum media. The plates were then stained with 0.1% crystal violet solution and the colonies counted by light microscopy. siRNA-mediated depletion of ARTN expression in MCF-7 cells reduced colony formation by 2.4-fold ($p<0.001$) when compared with MCF-siVec cells (Figure 4.12a). IGF-1 treatment increased the number of colonies formed by 1.31-fold in MCF-siVec cells ($p<0.05$) and by 2.3-fold in the MCF-siARTN cell line ($p<0.01$).

In the Matrigel assay, cell lines were embedded in Matrigel in 96-well plates as single cells and grown in 3D culture over a period of 10-14 days. Viable colony growth was measured by incubation with alamarBlue. siRNA-mediated depletion of ARTN in MCF-7 cells reduced the growth of MCF-7 cells in Matrigel by 1.91-fold ($p<0.01$) (Figure 4.12b). However, the cells retained responsiveness to IGF-1 treatment with MCF-siARTN cells exhibiting a 2.0-fold increase in colony viability following IGF-1 treatment ($p<0.01$) compared to MCF-siVec cells, which exhibited a 1.5-fold increase in viability ($p<0.01$).

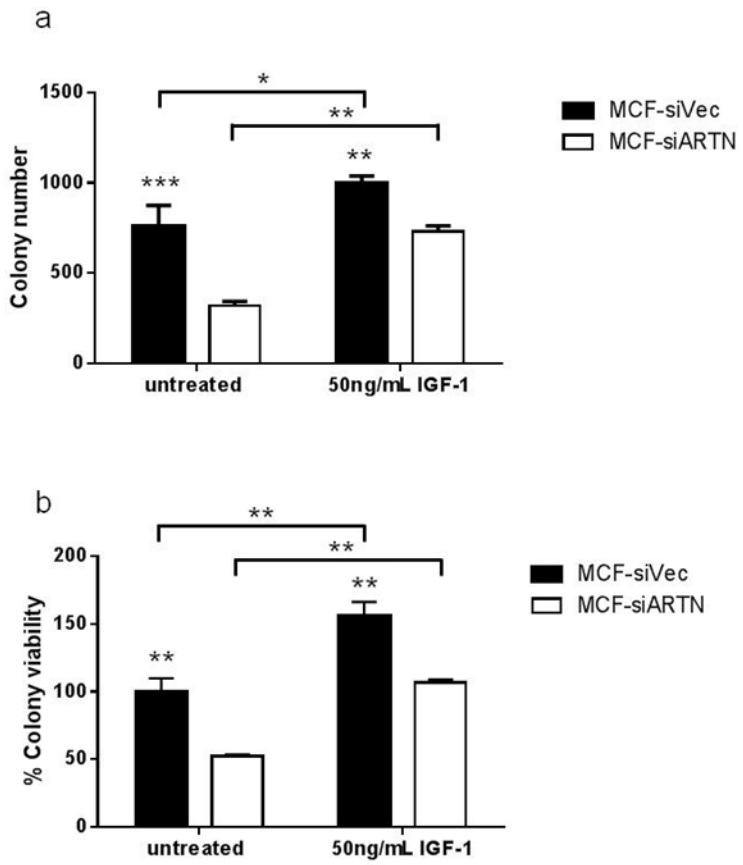

Figure 4.12- Soft agar colony formation and 3D growth in Matrigel™ in MCF-7 cells following siRNA-mediated depletion of ARTN expression.

(a) MCF-siVec and MCF-siARTN colony formation in soft agar. siRNA-mediated depletion of ARTN did not demonstrate any significant effect on colony growth following IGF-1 treatment. **(b)** 3D culture in Matrigel. siRNA-mediated depletion of ARTN resulted in greater reductions in the amount of viable colonies formed in 3D culture. In both treated and untreated conditions, MCF-siARTN cells formed significantly fewer viable colonies compared to the MCF-siVec cell line. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.4 Discussion

Chapter 3 provided evidence of interaction between the ARTN and IGF-1 signalling pathways. The work in the current chapter aimed to expand those previous findings and to determine whether the signalling interaction between the IGF-1 and ARTN pathways also affected mammary carcinoma cell function and behaviour. Overall, in the assays used to assess the cell behaviour, the forced expression of ARTN cooperated with exogenous IGF-1 treatment to produce measureable and observable differences in cell function and behaviour which could lead to increased cell oncogenicity.

The standard hallmarks of caner have been extensively reviewed, with some of those properties being investigated in this study (Hanahan and Weinberg, 2000). Although ARTN forced expression alone was found to promote a number of these attributes, many others were also unaffected until in the presence of IGF-1. As demonstrated, the forced expression of ARTN alone was not always sufficient to induce a change in cell behaviour, such as in the assays for cell number growth, cell proliferation and survival. But the presence of IGF-1 in the culture medium resulted in increases in growth that were greater than expected in the ARTN forced expression cell lines, and combining ARTN forced expression with IGF-1 treatment also enhanced cell survival to a greater degree than either ligand alone, in MCF-7 and T47D cells. In MCF-7, this cooperation also extended to the assays for anchorage independent growth, colony formation in soft agar.

This cooperative effect was observable in cell viability assays as measured by MTT but not in the total cell number assays, possibly due to inherent differences in the assay techniques. While total cell number assays measure the overall growth in cell population, MTT assays are oxidation-reduction reactions that are mediated by cytosolic enzyme activity, reducing the tetrazolium dye to insoluble formazan crystals (Vistica et al., 1991). These differences in

endpoint measurement could account for some of the discrepancies in the results. Furthermore, while the differences between cell lines in the total cell number assays were not large, there were significant differences between MCF-Vec and MCF-ARTN cell lines. This could be attributed to the fact that the apoptosis assays were set up in serum-free culture conditions, whereas and the total cell numbers were performed in 0.2% FBS.

These factors make ARTN signalling and its components an attractive target for inhibition, either alone or in combination with other treatment options to potentiate and increase the effectiveness of more standard treatments. Studies involving depletion and knockdown of ARTN demonstrate the possibility of antagonising ARTN signalling as a therapeutic strategy for the treatment of cancer. Recently, a study in esophageal carcinoma demonstrated the utilisation of miRNA to target ARTN expression (Li et al., 2011). Oesophageal carcinomas had higher expressions levels of ARTN than the surrounding tissues, and miRNA targeting of ARTN in oesophageal cancer cells inhibited the capacity for cell migration and invasion, as well as inhibiting wound healing.

Other studies have demonstrated that depletion of ARTN signalling can reduce the oncogenicity of carcinoma cells. *In vitro* studies demonstrated that antagonising ARTN signalling, by siRNA depletion or by functional inhibition using antibodies, can reduce colony formation in soft agar and impair 3D growth in Matrigel in a variety of cell lines including ER+ and ER- breast cancer cells, lung and endometrial cancer cells (Kang et al., 2009; Pandey et al., 2010b; Tang et al., 2010).

Many of the cell behaviour and function assays were also assessed in MCF-siVec and MCF-siARTN cells. In this series of assays, depletion of ARTN alone in MCF-7 cells had little effect on cell proliferation and apoptosis in the current study, but was effective in reducing colony formation in soft agar and 3D growth in Matrigel.

When combined with IGF-1 treatment, depletion of ARTN expression significantly impeded the ability of IGF-1 to elicit a mitogenic/survival response. In both cell viability and total cell number assays, ARTN significantly enhanced the response of MCF-7 cells to IGF-1, and the growth of MCF-siARTN cells in the presence of IGF-1, was reduced to approximately half that of the MCF-siVec cell line. In addition, depletion of ARTN abrogated the effect IGF-1 on cell proliferation and cell survival with significantly reduced proliferation and increased apoptotic death in MCF-siARTN cells compared to controls.

It is important to note, however, the effects of ARTN signalling varied greatly between cell lines. Although the results obtained in MCF-7 and T47D cells were mostly in agreement, when similar assays were carried out in ZR-75-1 cells, there were differences in response to ARTN forced expression with some results consistently contrary to the results obtained in MCF-7 and T47D cells. Most notably, the rate of cell proliferation decreased with forced expression of ARTN in ZR-75-1 cells, which is contrary to results in MCF-7 and T47D cells. It is possible that the reversed effect of ARTN in ZR-75-1 cells could be due to the phenomena of insertional mutagenesis, however, by using mixed clones when establishing these expression cell lines, this possibility should be minimised. In addition, correct ARTN mRNA and protein expression was confirmed by semi-quantitative RT-PCR and western blot prior to their use in later assays.

These differences in the effect of ARTN on cell function illustrate that the cellular context is vitally important in the study of oncogenes. That these assay results can differ so greatly between cell lines suggests that there are inherent genetic differences important in determining the functional responses to ARTN signalling which warrant further investigation.

4.5 Conclusion

This chapter extended findings from Chapter 3 demonstrating that ARTN can potentially interact with IGF-1 signalling, affecting some aspects of cancer cell behaviour. In MCF-7 cells, forced expression of ARTN enhanced the mitogenic effects of IGF-1 to increase cell growth, survival and oncogenicity, as measured by anchorage-independent growth. However, the mechanism involved in the interaction between these two pathways has not been fully elucidated.

The effects observed in MCF-7 and T47D cells were not consistent across other cell lines. ZR-75-1 cells did not share all of the same responses to ARTN forced expression, indicating that inherent differences between the cell lines also play a large part in regulating the cellular response to ARTN signalling.

Chapter 5

RESULTS: ARTN expression levels affect the susceptibility of mammary carcinoma cells to chemotherapy drug treatments

5.1 Introduction

Chemotherapy is one of the most important avenues of treatment for patients of varying cancer types. The variety and differences in the modes of action make it possible to combine chemotherapy drugs in treatment regimes, while keeping toxicity profiles in check. However, one of the greatest barriers to effective treatment is the eventual development of resistance to therapy, which can allow cancers to continue to grow in spite of continued treatment (Garraway and Jänne, 2012; Shoemaker, 2000). Both *de novo* and acquired resistance to therapy are intrinsic aspects of cancer development that prevent the elimination of residual disease and promote repopulation by cancerous cells (Borst, 2012). Resistance can be conferred to a cell population by numerous mechanisms, including genetic or epigenetic modification and a favourable tumour microenvironment. Control of these mechanisms often involve numerous druggable molecular targets and can offer new methods for more effective disease elimination and better clinical outcome (Ocaña and Pandiella, 2008).

More recently, the direction of research in cancer treatment has moved from the more broad action chemotherapy agents, such as platinum derivatives or nucleoside analogues, that rely on the (usually) more rapid proliferation of cancerous cells compared to non-cancerous cells, to focussing on more targeted agents that exploit the molecular differences between the two,

such as imatinib, a kinase inhibitor specific for the mutant BCR-ABL protein in chronic myelogenous leukaemia (Horne et al., 2013; Widakowich et al., 2007; Zoubir et al., 2010). In theory, exploiting molecular targets expressed exclusively by cancerous cells but not by normal tissue, should lead to greater specificity and less treatment side effects and toxicity (Gottesman, 2002). This targeted approach has led to an upsurge in the number of studies whose aim is to uncover molecular targets and expression profiles that are unique to cancerous cells. However, despite the change in approach, the inevitable development of resistance remains a major barrier to effective treatment.

ARTN and RET expression have been shown to correlate with poor clinical outcome in breast cancer patients, possibly through mediation of cancer drug resistance (Kang et al., 2010; Pandey et al., 2010a). It has become apparent that ARTN plays an important role in promoting cancer development, as well as numerous other disease states, particularly as a potent survival factor for a range of cell types (Andres et al., 2001; Baloh et al., 1998b; Nishino et al., 1999; Takahashi, 2001b). It was originally identified as a survival factor for sensory and sympathetic neurons in *in vitro* culture (Warnecke et al., 2010). Later studies found that ARTN also had a potent effect on the growth and survival of neuroblastoma cells (Ceyhan et al., 2006a), and recently, it was also shown to encourage metastasis in numerous cancer types, including pancreatic (Ceyhan et al., 2006a), lung (Tang et al., 2010), endometrial (Pandey et al., 2010b) and breast (Banerjee et al., 2011; Kang et al., 2009).

The role of ARTN in mediating resistance to various cancer therapies and treatment methods makes it a tempting target for the development of targeted cancer therapies. Kang *et al.* (2010) demonstrated the ability of ARTN to increase resistance of MCF-7 and T47D cells to tamoxifen and fulvestrant, two anti-estrogen agents widely used in the treatment of ER-positive breast cancers. Whereas Banerjee *et al.* (2012) demonstrated that ARTN also

increased resistance in MDA-MB-231 and BT549 cells to the chemotherapy drug, paclitaxel (Banerjee et al., 2012a). Both studies went further to prove that the knockdown of ARTN function, by siRNA or neutralizing antibody, restored the cell sensitivity to these therapeutic drugs. Very recently, ARTN forced expression was shown to mediate resistance to trastuzumab in the HER2-posistive cell lines, BT474 and SKBR3, by promoting stem cell-like behaviour (Ding et al., 2014). Conversely, siRNA-mediated depletion of ARTN in these cell lines sensitised the cells to trastuzumab treatment.

Similarly, IGF-1 signalling has also been linked to increasing resistance to a number of chemotherapy agents and is another attractive option for the development of targeted cancer treatments, particularly by antagonism of the receptor, IGF-1R (Chen and Sharon, 2013; Jones et al., 2005; Litzenburger et al., 2011). However, the effort to design viable *in vivo* antagonists has had limited success (Baserga, 2004; Chen and Sharon, 2013). Many agents have shown great promise and effectiveness during preclinical testing, but few have made it through to the later stages of clinical trials (Baserga, 2004; Weroha and Haluska, 2008). The development of IGF-1R antagonists for the treatment of cancer has been further hampered by the eventual development of resistance to these agents (Weroha and Haluska, 2008). Both *de novo* and acquired resistance to IGF-1R antagonists present a problem in current clinical trials.

This chapter aimed to investigate the role of ARTN in the resistance to chemotherapy agents routinely used in frontline breast cancer therapy. In addition, this chapter also presents evidence for the involvement of ARTN in mediating the acquired resistance to IGF-1R antagonists, to further solidify the evidence for interaction between the two signalling pathways.

5.1.1 5-Fluorouracil and Paclitaxel dose responses in wild-type MCF-7 cells

5-Fluorouracil (5FU) and paclitaxel are routinely used in the clinic for first-line adjuvant treatment of several cancer types, including breast, testicular, lung and endometrial (Bunnell and Winer, 1998; Jover et al.; Mekhail and Markman, 2002; Sparano et al., 2008). Both agents disrupt mitosis in rapidly proliferating cells, but through very different mechanisms of action. 5FU is a pyrimidine analogue and acts to inhibit thymidine synthesis (a nucleoside required for DNA synthesis) and disrupt DNA synthesis during cell division (Bunnell and Winer, 1998; Jover et al.). Paclitaxel, on the other hand, is an anti-microtubule agent that specifically targets the action of the mitotic spindle to prevent separation of the chromosomes at telophase of cell division (Mekhail and Markman, 2002).

To investigate the effect of ARTN expression on the MCF-7 cell response to chemotherapy drug treatments, the dose response profiles to 5FU and paclitaxel in wild-type MCF-7 cells were first established. In these assays, wild type MCF-7 cells were seeded into 96-well plates at a density of 10,000cells/well and cultured in RPMI medium, and a range of drug concentrations were later added to the medium. The assays were carried out in different serum conditions, with or without a 24 hour wash-out period after treatment, to determine the best conditions for use in the cell viability assays that followed. The intention of the 24 hour wash-out period was to allow the remaining viable cells to recover after chemotherapy drug treatment, modelling the effect of residual disease in clinical settings.

These assays were carried out over a 3 day treatment period and the cell viabilities were measured by incubation with alamarBlue. For 5FU (Figure 5.1), it was decided on low-serum culture conditions followed by a wash-out period, whereas, the paclitaxel assays (Figure 5.2) were carried out in full serum, also with a wash-out period.

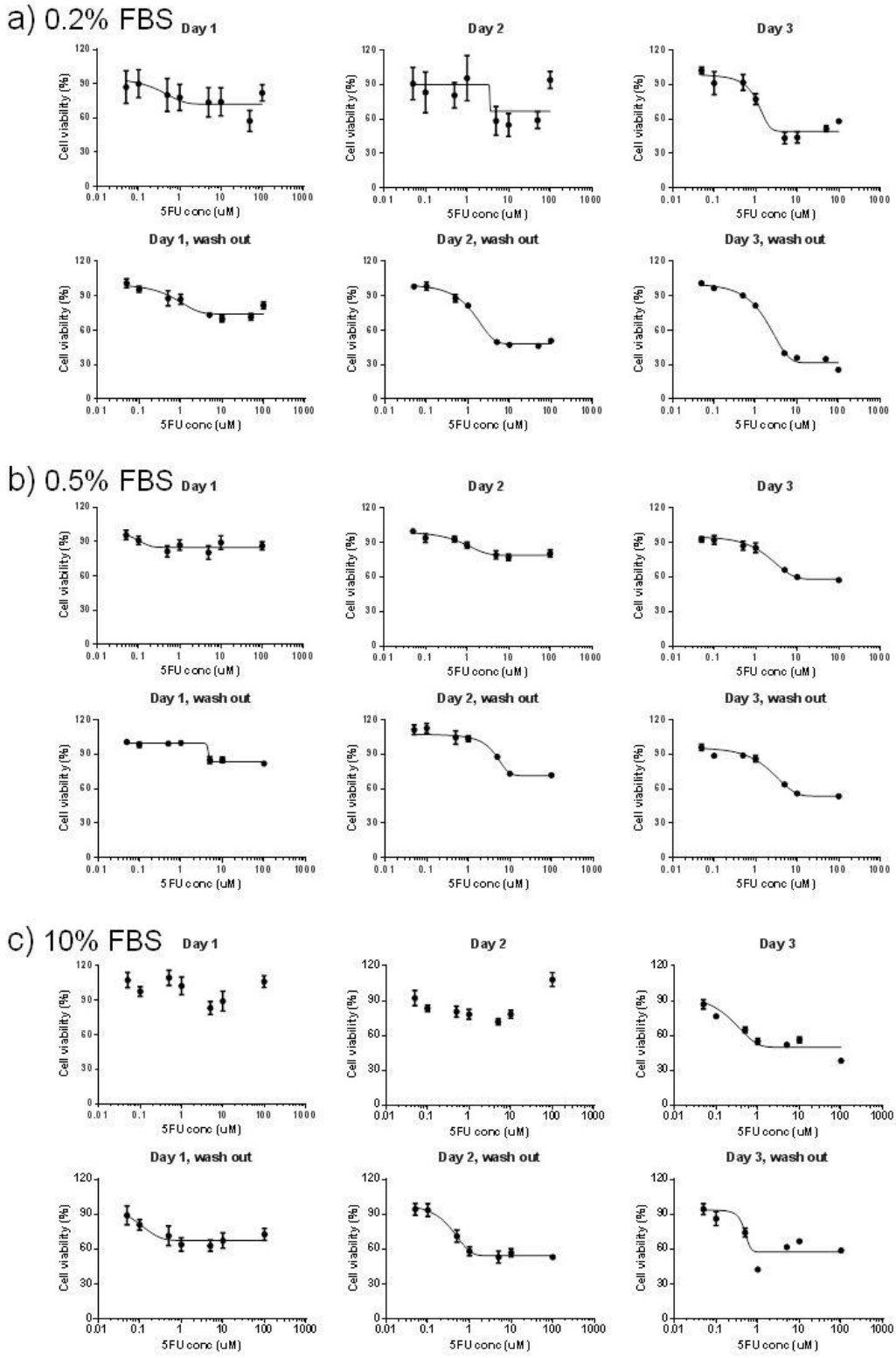

Figure 5.1- 5FU dose response in wild type MCF-7 cells.

Each set of dose response experiments were performed in the stated serum conditions, and carried out with continuous drug treatment (top row of each set) or followed by a 24 hour wash-out period at the end of the treatment period, where the cells were cultured in normal media, without 5FU (bottom row of each set).

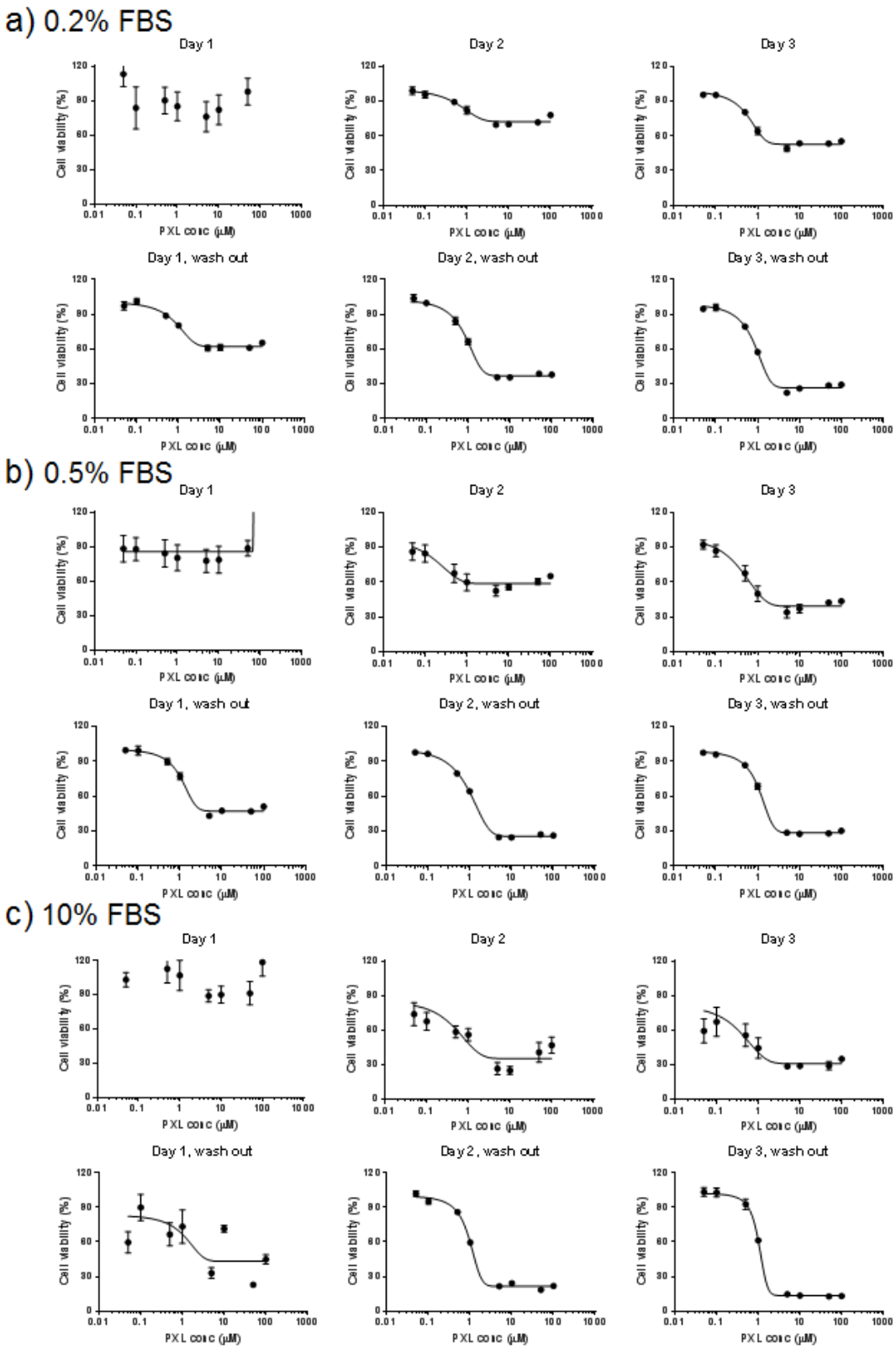

Figure 5.2- Paclitaxel (PXL) dose response in wild type MCF-7 cells.

Each set of dose response experiments were performed in the stated serum conditions, and carried out with continuous drug treatment (top row of each set) or followed by a 24 hour wash-out period at the end of the treatment period, where the cells were cultured in normal media, without paclitaxel (bottom row of each set).

5.2 ARTN expression levels affected the responsiveness of MCF-7 cells to treatment with chemotherapy agents

5.2.1 Stable forced expression of ARTN affected the dose response to the chemotherapeutic agents, 5FU and paclitaxel

As mentioned in the Introduction of this chapter, 5FU and paclitaxel are chemotherapy agents routinely used as front-line adjuvant therapy in patients with solid tumours. The effectiveness of 5FU and paclitaxel in disrupting cell growth was investigated in the MCF-Vec and MCF-ARTN cell lines to determine whether increased ARTN expression could confer resistance to this drug treatment.

MCF-Vec and MCF-ARTN cells were seeded into 96-well plates at 10,000cells/well and cultured under the conditions set out in the previous section. The assays were carried out over a period of 6 days, and following a 24 hour wash-out period, the cell viability was determined by incubating with alamarBlue every 2 days. In MCF-7 cells, forced expression of ARTN shifted the MCF-ARTN dose response curves to the left, indicating that the ARTN increased the sensitivity of the cells to both drug treatments (Figure 5.3). On day 6, the IC_{50} values for 5FU were 4.572μM in MCF-Vec *vs* 1.00μM in MCF-ARTN cells ($p<0.001$), while the IC_{50} for paclitaxel was 1.206nM in MCF-Vec *vs* 0.478nM in MCF-ARTN cells ($p<0.001$).

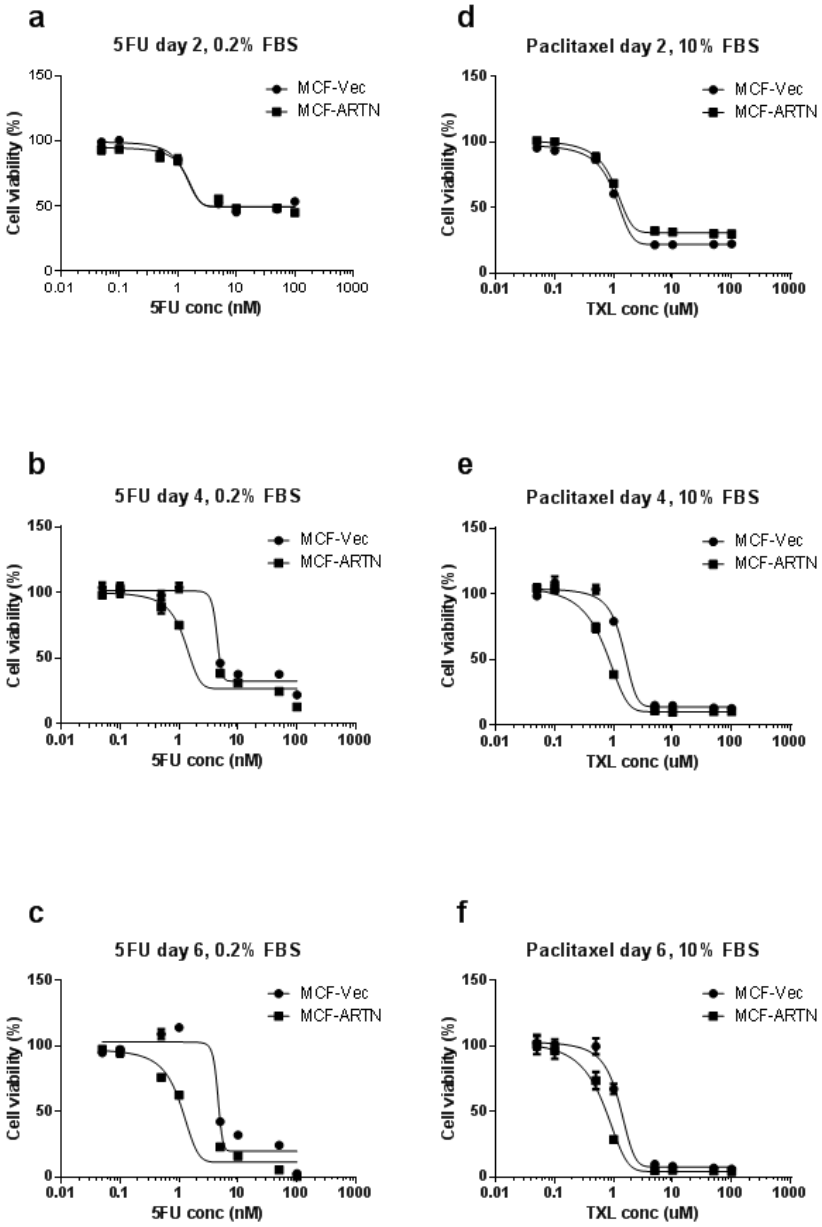

Figure 5.3- Drug dose responses in ARTN stable cell lines.

Under the conditions tested, MCF-ARTN cells exhibited greater sensitivity to treatment with the chemotherapy agents, 5FU (**a, b** and **c**) and paclitaxel (**d, e** and **f**), compared to the MCF-Vec cells, indicated by the leftward shift of the MCF-ARTN dose response curves.

5.2.2 IGF-1 treatment did not rescue MCF-7 cells from inhibitory effects of 5FU and paclitaxel

To investigate the effect of combining IGF-1 treatment on the cell viabilities of the chemotherapy treated cells, MCF-Vec and MCF-ARTN cells were seeded into 96-well plates at 10,000 cells/well and cultured in 0.2% FBS. The cells were treated with 50ng/mL IGF-1 in combination with 2nM 5FU or 2µM paclitaxel for 3 days, followed by a 24 hour wash-out period. The cell viabilities were then measured using alamarBlue.

Combining IGF-1 treatment with the chemotherapy agents in the MCF-Vec and MCF-ARTN stable cell lines provided some protection to the inhibitory drug effects on cell viability (Figure 5.4). The effect of IGF-1 treatment on the drug response was relatively small, but it provided slightly better protection from the drug inhibitory effects in MCF-ARTN than in MCF-Vec cells. Following 5FU treatment in MCF-Vec, there was no significant difference in viability between cells treated with 5FU only and cells treated with both 5FU and IGF-1. However, MCF-ARTN, cells that were treated with both 5FU and IGF-1 exhibited a 1.69-fold increase in viability compared to cells treated with 5FU only ($p<0.001$). Similarly, following paclitaxel treatment in MCF-Vec, the cell viability of cells treated with both IGF-1 and paclitaxel was 1.64-fold that of those treated with paclitaxel only ($p<0.001$). Whereas in MCF-ARTN, cells treated with both IGF-1 and paclitaxel exhibited 1.83-fold greater cell viability than cells treated with paclitaxel only ($p<0.001$).

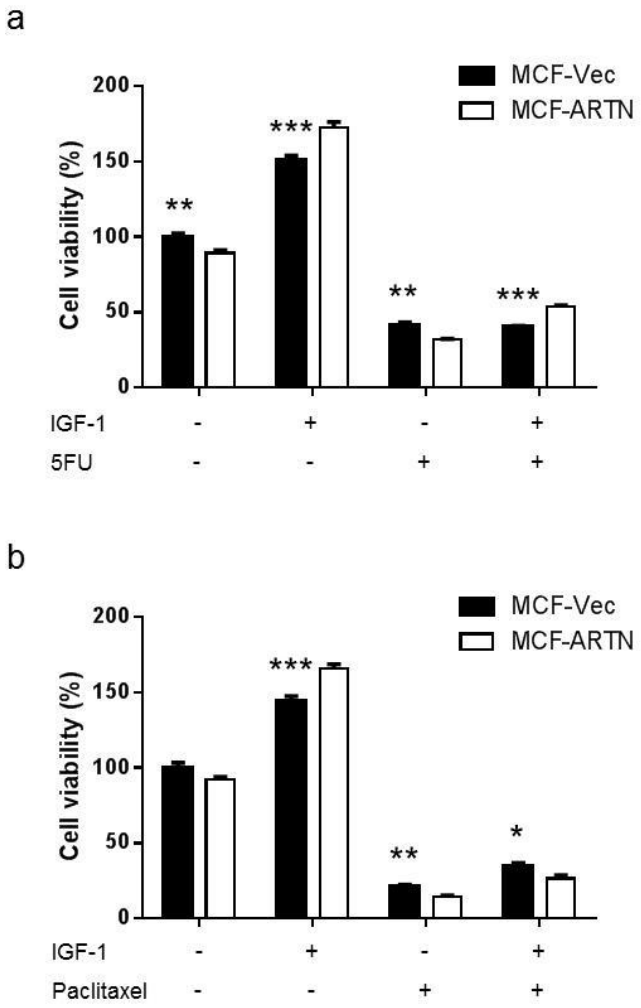

Figure 5.4- IGF-1 treatment offers little protection from the effects of 5FU and paclitaxel in MCF-7 cells.

After 3 days in IGF-1(50ng/mL) and drug treatment, both MCF-Vec and MCF-ARTN cells exhibited greatly reduced cell viability with **(a)** 5FU (2nM) or **(b)** paclitaxel (2µM). Neither ARTN forced expression nor IGF-1 treatment protected the cells from the chemotherapy drugs' inhibitory effects.

5.2.3 Depletion of ARTN expression by siRNA did not significantly affect the response to 5FU and paclitaxel in MCF-7 cells

Following on from the dose responses carried out in the ARTN forced expression stable cell lines, the converse was also investigated by repeating the drug dose response assays in the ARTN knock down cell lines; MCF-siVec and MCF-siARTN. This set of assays followed the same conditions as the previous dose response viability assays.

Knock down of ARTN expression by siRNA shifted the dose response curves to the right, when the MCF-siARTN curve was compared to the MCF-siVec (Figure 5.5). This occurred for the responses to both 5FU and paclitaxel and was consistent with the overall trend of the results from the forced expression assays. However, the differences between IC_{50} values calculated for the two cell lines were not statistically significant.

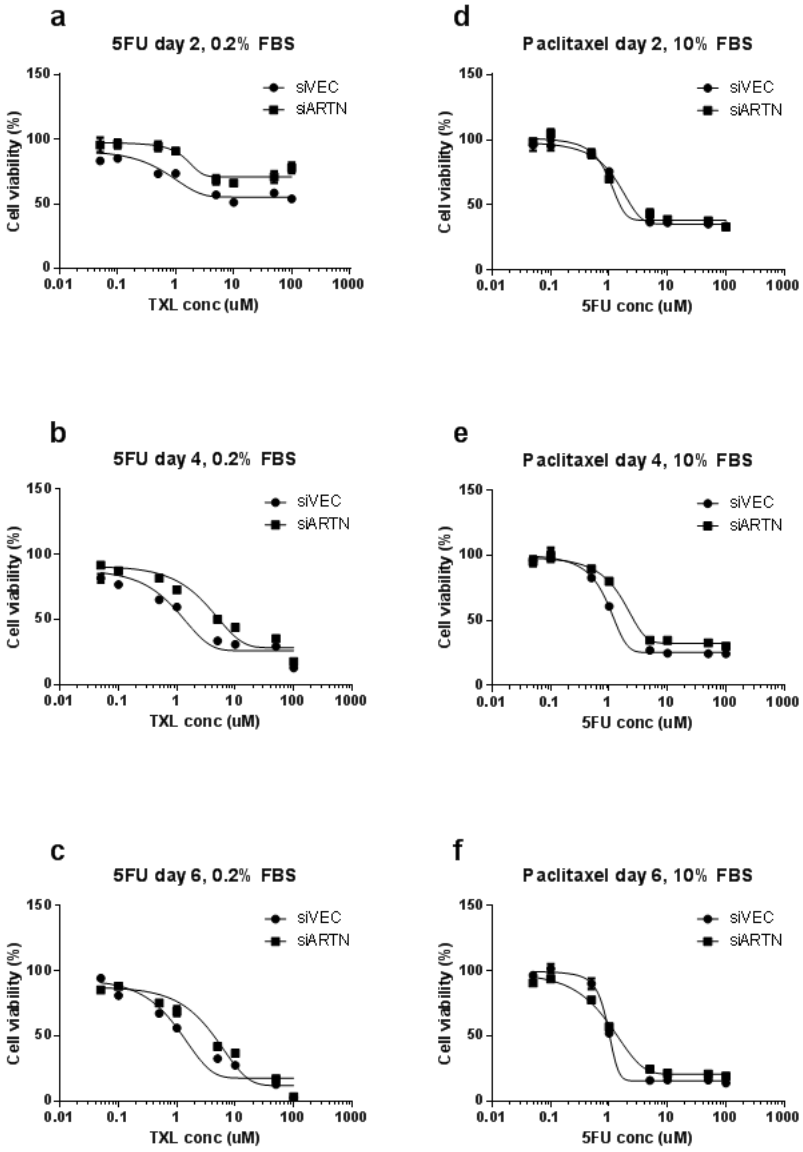

Figure 5.5- Drug dose responses in ARTN knockdown of expression cell lines.

Under the conditions tested, the MCF-siARTN cells response to treatment with the chemotherapy agents, 5FU (**a, b** and **c**) and paclitaxel (**d, e** and **f**), trended towards increased resistance to treatment compared to MCF-siVec cells, indicated by the rightward shift of the MCF-siARTN dose response curves.

5.3 ARTN expression levels affected the responsiveness of mammary carcinoma cells to antagonism of the IGF-1R by AG1024

5.3.1 Forced expression of ARTN in MCF-7 cells affects the cellular response to the IGF-1R antagonist, AG1024

Chapters 3 and 4 describe the interaction between ARTN and IGF-1-mediated signal transduction. As ARTN and IGF-1 synergistically activate signal transduction in MCF-7 cells, it was hypothesised that elevated ARTN may promote resistance to inhibitors of the IGF-1R pathway.

AG1024 is a commercially available selective small molecule inhibitor of IGF-1R. It is a substrate competitive inhibitor that prevents receptor activation by binding the intracellular tyrosine kinase domain (Parrizas et al., 1997). This imposes a conformational change to prevent ATP binding and inhibiting receptor autophosphorylation (Camirand et al., 2005). AG1024 has an IC_{50} of 0.4-10μM depending on cell type. 4 μM was used in these studies, which is consistent with previously published studies in MCF-7 cells (Camirand et al., 2005; Wang et al., 2008). A number of the cell function assays were used to investigate the effect of ARTN on the efficacy of IGF-1R antagonism by AG1024 in MCF-7 cells. The results that are presented here, suggest that ARTN forced expression reduces sensitivity to AG1024 treatment in this cell line.

In a BrdU assay, ARTN enhanced cell proliferation following treatment with AG1024 in MCF-ARTN cells compared to the MCF-Vec cell line. In this assay, the proliferative rate in MCF-ARTN cells decreased by 1.78-fold ($p<0.01$) with 18-20 hours of AG1024 treatment, whereas the proliferative rate in MCF-Vec cells decreased by 2.22-fold ($p<0.001$). A comparison of the both cell lines also indicated that MCF-ARTN cells had 1.43-fold higher

proliferation following treatment with AG1024 ($p<0.01$), when compared to MCF-Vec cells (Figure 5.6a).

In an apoptosis assay, forced expression of ARTN conferred protection from AG1024-induced apoptosis and enhanced the survival of MCF-7 cells (Figure 5.6b). After 24 hours of AG1024 treatment, the rate of apoptosis in MCF-ARTN cells was 1.86-fold greater than in the rate in untreated MCF-ARTN cells ($p<0.05$) and in MCF-Vec cells, the rate of apoptosis was 3.12-fold greater than in untreated MCF-Vec cells ($p<0.001$). The results of this assay generally followed a similar trend to that in the forced expression cell lines.

Anchorage-independent growth was assessed using a colony formation assay in soft agar. AG1024 treatment in MCF-Vec cells resulted in a 2.67-fold reduction ($p<0.01$) in the number of colonies formed, while treatment in MCF-ARTN cells resulted in a 1.49-fold reduction ($p<0.05$). Overall, the number of colonies formed in soft agar by MCF-ARTN after AG1024 treatment, was also significantly greater than MCF-Vec, with an increase of 2.33-fold colonies ($p<0.05$) (Figure 5.6c).

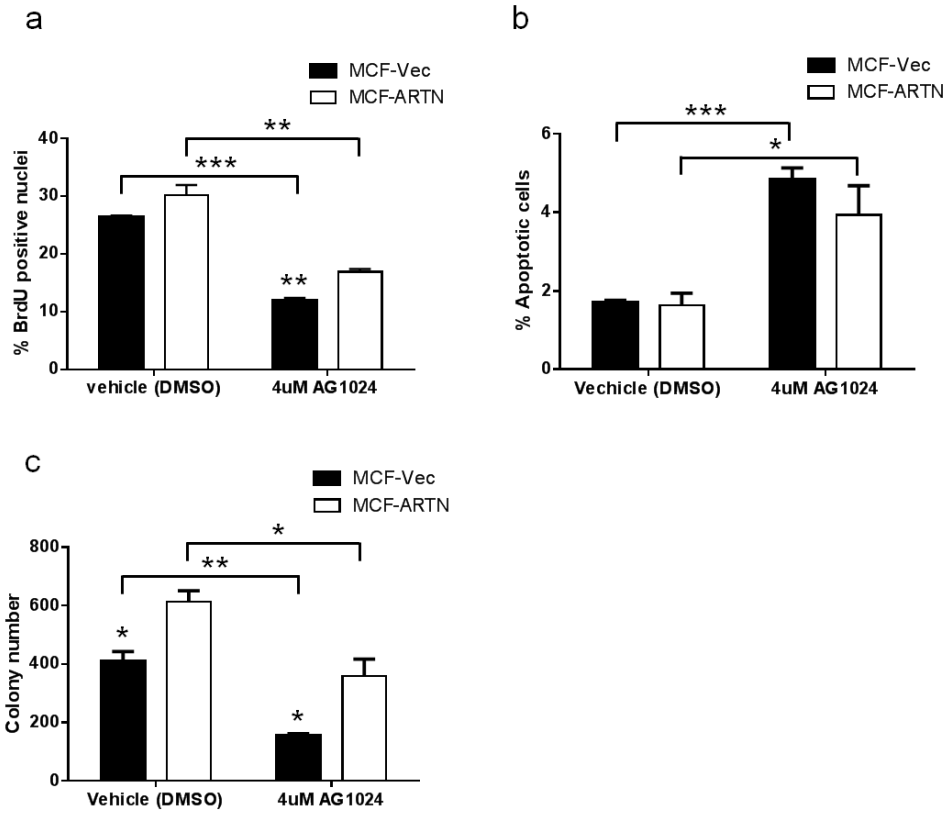

Figure 5.6- ARTN forced expression protected MCF-7 cells from some of the effects of AG1024 treatment.

(a) ARTN forced expression protected the cells from AG1024-induced reduction in cell proliferation, **(b)** but had no significant effect on cell apoptosis. **(c)** ARTN forced expression protected the cells from AG1024-induced reduction in MCF-7 colony formation in soft agar. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.3.2 Acquired resistance to AG1024 in mammary carcinoma cells increased cell viability and endogenous ARTN expression

To investigate whether ARTN contributes to acquired AG1024 resistance in mammary carcinoma cells, a set of cell lines resistant to AG1024 treatment was established in MCF-7 and ZR-75-1 cell lines. This was achieved by prolonged exposure (6-8 weeks) of the wild-type cells to low doses of AG1024 in 10% FBS RPMI media. The dosage of AG1024 was gradually increased until the cells were able to be maintained at the AG1024 IC_{50} (4μM) without any observable reduction in their doubling time. The resulting resistant cell lines were denoted with the suffix –R, whereas the original wild-type cell lines were denoted with the suffix –WT. The resistance of MCF7-R and ZR751-R cells to AG1024 was confirmed by measuring the cell viability following 24 hours of treatment with AG1024 in 0.2% FBS RPMI medium (Figure 5.7). The results of the assays indicated that the viability of the resistant cell lines were significantly higher than that of the wild-type cell lines, both before and after AG1024 treatment. Untreated, the MCF7-R cell viability was over 3-fold greater than the observed for the MCF7-WT cell line ($p<0.001$). While the MCF7-R cells retained some sensitivity to AG1024, as demonstrated by the reduction in cell viability following treatment, MCF7-R cell viability was 1.55-fold greater following AG1024 treatment compared with AG1024-treated wild type cells ($p<0.001$). By comparison, resistance in the ZR751-R cell line was more robust with no significant decrease in viability following AG1024 treatment.

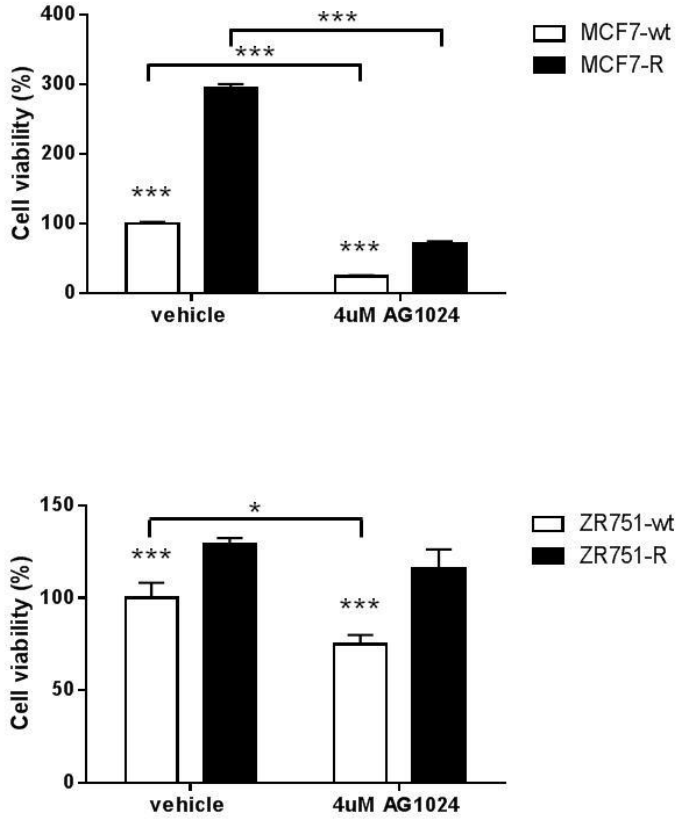

Figure 5.7- Establishment of mammary carcinoma cell lines with acquired resistance to the IGF-1R antagonist, AG1024.

MCF-7 and ZR-75-1 cells were cultured in the presence of increasing AG1024 dose over a prolonged period (6-8 weeks) until the cell growth was stabilised and there was no observable cell death under normal culture conditions. The resulting cell lines were tested for resistance to AG1024 treatment by cell viability assays, using incubation with alamarBlue and were designated MCF7-R (top) and ZR751-R (bottom). In both sets of cell lines, there was a significant increase in viability in the untreated resistant cells. Both wild-type and resistant cells were responsive to AG1024 treatment, but the resistant cells exhibited a reduced response to AG1024 treatment in reduced (0.2%) serum conditions * $p<0.05$, *** $p<0.001$.

To determine whether acquired resistance to AG1024 affected the expression of ARTN, mRNA and protein expression was investigated in the resistant cell lines. *ARTN* mRNA expression of the resistant cells was assayed by semi-quantitative RT-PCR. Cells were seeded into 6-well plates, and cultured in serum-free RPMI media for 24 hours, before the total cellular RNA was extracted. For both MCF-7 and ZR-75-1 cell lines, there was a

significant increase in the level of *ARTN* mRNA expressed (Figure 5.8a). Interestingly, 24 hours of treatment with AG1024 also increased the mRNA expression of *ARTN* in both the wild type and resistant cell lines (Figure 5.8b).

The RT-PCR results lead to investigation of the ARTN protein expression by western blotting. ARTN protein expression was increased in MCF7-R cells when compared to MCF7-WT cells. Following AG1024 treatment, a small increase in ARTN protein expression was observed in both cell lines (Figure 5.9). A small increase in ARTN protein expression was observed in ZR751-R cells when compared to ZR751-WT cells by western blot analysis. AG1024 treatment increased the level of ARTN protein expression in both wild-type and AG1024-resistant ZR-75-1 cell lines.

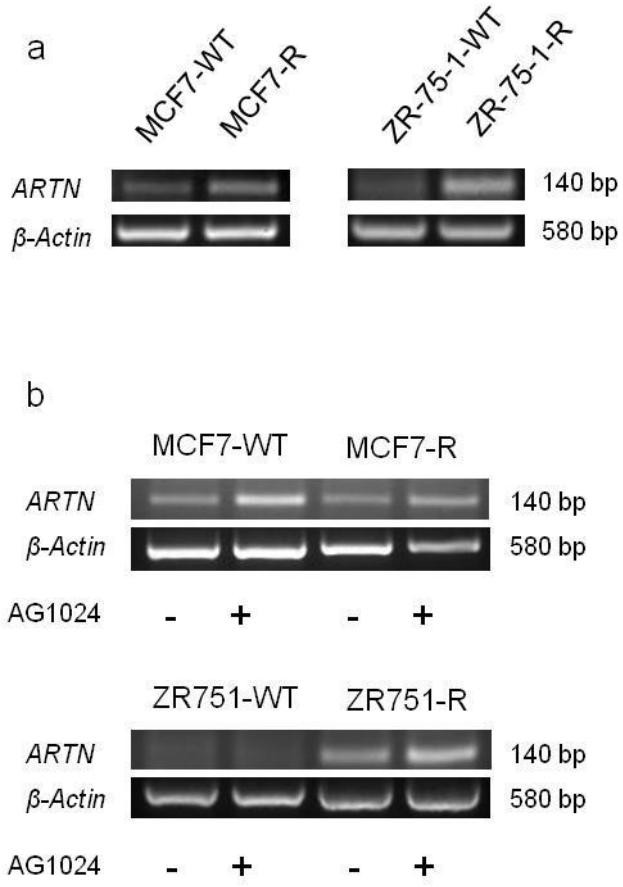

Figure 5.8- *ARTN* mRNA expression in wild-type and AG1024-resistant cell lines.

(a) Semi-quantitative RT-PCR for *ARTN* mRNA demonstrating an increase in ARTN expression in the resistant cell lines compared to the original wild-type cell lines. **(b)** 24 hour AG1024 treatment of the cells also increased the *ARTN* mRNA expression, although in ZR751-WT cells, this was not definitive, as the endogenous expression level of *ARTN* was inherently very low

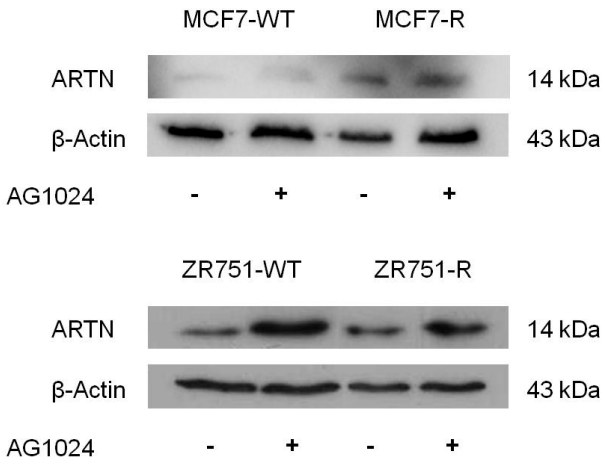

Figure 5.9- ARTN protein expression in wild-type and AG1024-resistant cell lines.

Western blot assays for the expression of ARTN in AG1024-resistant cell lines revealed an increase in the ARTN protein expression in cells treated with AG1024 for 24 hours

5.4 Discussion

Overall, these results demonstrated that ARTN expression can affect the cell response/sensitivity to drug treatment in mammary carcinoma cell lines. In conjunction with other published studies, the effect that ARTN expression and signalling has on the drug sensitivity differed considerably between various carcinoma cell lines.

The importance of the GFLs and their receptors in promoting oncogenesis and resistance to therapy is becoming increasingly prominent (Burmi et al., 2006; Kang et al., 2010; Pandey et al., 2010a). The family member, ARTN, has been shown to be oncogenic in mammary carcinoma cell lines, as well as mediating resistance to a number of chemotherapy, such as paclitaxel, and endocrine therapy agents, such tamoxifen and fulvestrant (Banerjee et al., 2012b; Kang et al., 2009).

However, as mentioned above, the effects of ARTN on cell function are contextual and can vary significantly depending on the cell line investigated. This chapter has indicated that the forced expression of ARTN in MCF-7 cells decreased the 5FU and paclitaxel IC_{50}, resulting in cells that were more sensitive to the effects of the drugs. Interestingly, Banerjee *et al.* (2012) demonstrated that ARTN increased the IC_{50} for paclitaxel treatment in the ER- cell lines, MB-MDA-231 and BT549 (Banerjee et al., 2012a).

In this chapter, the reverse situation also demonstrated that siRNA knockdown of ARTN made MCF-7 cells more resistant to the drugs' effects. Even when combined with IGF-1 treatment, the MCF-ARTN cells had significantly reduced viability compared to the IGF-1 treated MCF-Vec cells. Again, this is contradictory to the previously published studies by Kang et al (2010) and Banerjee et al (2012) (Banerjee et al., 2012a; Kang et al., 2010).

It is unclear why ARTN forced expression in MCF-7 resulted in a response that was contradictory to the results of previous studies, in BT549 and MDA-MB-231 cells, despite having followed similar experimental conditions. The mechanism of action for ARTN/RET signalling is largely unknown, and it is possible that the inherent differences between cell lines could be responsible for the differences in response given that the aforementioned studies utilised different cell lines for their demonstrations of drug resistance. In addition, the results presented in Chapter 4 of this thesis also demonstrated the importance of the cell line and their differences in response to ARTN forced expression depending on the context of the cell.

Continuing on the theme of targeted cancer therapy, IGF-1 and IGF-1R antagonists have emerged as a new avenue of interest and as potential agents for targeted cancer therapy (Baserga, 2004; King and Wong, 2012). Numerous studies have demonstrated the transforming effects of IGF-1 and IGF-1R, and its promotion of oncogenesis, *in vitro* and *in vivo*, while prospective clinical studies have demonstrated the inverse correlation between circulating IGF-1 and breast cancer patient survival (Rinaldi et al., 2006; Tamimi et al., 2011). However, many of these new IGF-1R targeted agents have gone through to clinical trials only to fail to show enough effectiveness in front line therapy (Chen and Sharon, 2013). The rapid development of resistance to IGF-1R antagonists is one of the major concerns (Rodon et al., 2008).

In an effort to further study the interaction of the ARTN and IGF-1 signalling pathways, two different cell lines were used to investigate the acquired resistance to AG1024, a small molecule IGF-1R antagonist and tyrosine kinase inhibitor. AG1024 is a synthetic tyrphostin which selectively binds to, and inhibits activation of IGF-1R, but can also bind the insulin receptor (IR), although with much lower affinity (Parrizas et al., 1997). It has been

extensively studied in preclinical trials and the effectiveness of AG1024 treatment has been demonstrated *in vitro,* reducing cell proliferation and cell survival, as well as sensitising some cell lines to radiation and chemotherapy (Li et al., 2013; von Willebrand et al., 2003; Wen et al., 2001).

The effect of treatment in the ARTN forced expression cell lines reinforced the hypothesised cross-talking between ARTN and IGF-1 signalling. The forced expression of ARTN was shown to enhance *de novo* resistance to AG1024 when the MCF-ARTN cell line treated with the inhibitor was shown to exhibit greater levels of cell proliferation, cell survival and greater 3D colony growth in soft agar assay, compared to MCF-Vec cells. This aspect of ARTN function could be worth investigating and whether inhibition of ARTN signalling could potentiate the effects of IGF-1R inhibition and in preventing the development of resistance to inhibition.

For further study of resistance to IGF-1R inhibition, cells with acquired resistance to AG1024 treatment was simulated in both MCF-7 and ZR-75-1 by prolonged culture of wild-type cells in the presence of the inhibitor. The newly resistant cell lines were tested for their resistance to AG1024 inhibition of IGF-1R by measuring the cell viability following treatment. Both resistant cell lines exhibited increased cell viability compared to their respective wild-type cell lines, and while not immune to the suppressive effects of AG1024 treatment, the proportion of viable cells was significantly greater in the resistant cell lines compared to the wild-type.

As ARTN is upregulated in breast cancer cells lines which are resistant to other therapeutic drugs such as tamoxifen or trastuzumab (Banerjee et al., 2012a; Kang et al., 2010), the expression of ARTN in the AG1024-resistant cell lines was investigated. Semi-quantitative RT-PCR performed in both MCF-7 and ZR-75-1 cells demonstrated that the mRNA

expression of *ARTN* was greater in AG1024-resistant cell lines compared to the wild-type cells. In addition, when cells were treated with AG1024 for 24 hours, the expression of *ARTN* was again increased in both the wild-type and resistant cell lines. This increase in expression following AG1024 treatment was also evident at the protein level when the difference in ARTN expression was confirmed by western blotting.

It is increasingly evident that the IGF-1 and ARTN signalling pathways are intricately linked and a significant amount of interaction occurs between the two pathways. However the mechanism underlying this interaction is unclear. Interestingly, the results presented in this chapter imply that both activation and inhibition of IGF-1 signalling can increase the expression of ARTN in mammary carcinoma cells. However, treating with IGF-1 and AG1024 still exerted the expected effects of stimulation and inhibition, respectively, on cell behaviour and function. The fact that ARTN expression seems to be so readily induced by such opposing forces on the same signalling pathway is interesting and warrants further study to delineate a mechanism.

5.5 Conclusion

The results of this chapter indicate that ARTN forced expression in specific cell lines may sensitise cells to certain chemotherapeutic agents, but also promote resistance to others. This presents an interesting conundrum that serves to further highlight the importance of understanding the mechanisms of action responsible for ARTN and RET signalling. Particularly since ARTN antagonism has been earmarked as a potential strategy for cancer treatment. The increasingly complex relationship between IGF-1 and ARTN interaction was also solidified in the assays implicating the involvement of ARTN in *de novo* and acquired

resistance to IGF-1R antagonism by AG1024 with increased expression observed in cell lines resistant to this drug.

Chapter 6

General Discussion

Overall, the work presented in this thesis has demonstrated the existence of cross-talk between the signalling pathways of the GFL member, ARTN, and IGF-1, a potent growth factor and mitogen, and the importance of their signalling co-operation in mammary carcinoma cells. IGF-1 is a growth factor with wide ranging pleiotropic effects so it is not surprising to find that IGF-1 signalling also interacts with a number of other signalling factors (Liu et al., 2014). ARTN itself is also becoming an increasingly important player in the promotion of oncogenesis in cell lines of both neuronal and non-neuronal origins (Ceyhan et al., 2006b; Damon et al., 2007a; Honma et al., 2002; Parkash and Goldman, 2009). Alone, the over-expression of ARTN has been shown to increase cell motility and migration, as well as enhance cell survival and inhibit cell apoptosis (Kang et al., 2009; Meng et al., 2012). While in combination with IGF-1, a potent promoter of oncogenesis itself, both ligands work in a cooperative manner to increase cell viability, proliferation and enhance the anchorage-independent growth of cells cultured in a 3D matrix.

The discovery that ARTN/RET signalling cross-talks with other signalling pathways has made it an attractive area of research (Boulay et al., 2008; Esposito et al., 2008; Kuure et al., 2005; Tufro et al., 2007). IGF-1 and IGF-1R also frequently cross-talk with other pathways (Baserga, 1997; Mawson et al., 2005), and both the ARTN and IGF-1 signalling pathways have since been linked to the mediation of resistance to several modes of cancer therapy (Gooch et al., 1999; Kang et al., 2010; Pandey et al., 2010a). However, the research presented here does not entirely agree with some of the published literature and emphasises the importance in ensuring that multiple cell lines are used in this type of research.

6.1 ARTN and IGF-1 function in mammary carcinoma: oncology recapitulates ontology

The literature has provided an abundance of detailed work describing the function of ARTN and IGF-1 in development. As a neurotrophic factor, ARTN is responsible for the development of vital neural networks (Enomoto et al., 2001; Nishino et al., 1999). Knockout studies demonstrated the requirement of ARTN for the migration of sympathetic neurons, when mice lacking ARTN also lack sympathetic and parasympathetic innervations (Andres et al., 2001). Being a developmental factor, ARTN classically illustrates the old adage that "oncology recapitulates ontology". IGF-1, on the other hand, mediates much of the linear growth that happens later in development, particularly during childhood and puberty (Ahmed and Farquharson, 2010; Anisimov and Bartke, 2013). Importantly, IGF-1 expression is also affected by lifestyle and diet to a greater degree than many other hormones and signalling factors, and is more universally expressed than ARTN (Anisimov and Bartke, 2013; Giovannucci, 2003).

ARTN is a secreted neurotrophin expressed primarily by the developing nervous systems and the smooth muscle in blood vessels (Honma et al., 2002). In adult breast tissue, ARTN and its receptors are not normally expressed (Yang et al., 2006). Given that ARTN expression is inducible by hormones, such as estrogen (Kang et al, 2009) or IGF-1, increases in the expression of these hormones could theoretically lead to induction of ARTN expression in adult tissues and promote oncogenesis.

Moreover, the re-expression of ARTN in solid breast tumours and most mammary carcinoma cell lines was demonstrated by immunostaining assays and led to many hypothesising its involvement in cancer development (Kang et al., 2009; Quartu et al., 2007). Although, many

of the early studies demonstrated a wide variation of responses to ARTN signalling in different cell lines that were used. This variation was demonstrated in Chapter 4. The behavioural responses to ARTN forced expression differed not only in extent, but in the case of ZR-75-1 cells, some responses were reversed compared to the responses of MCF-7 and T47D cells, despite the effect of IGF-1 treatment being comparatively constant in each of the cell lines. The phenotypic differences that might account for the varied responses to ARTN were not immediately apparent. Further molecular or genotypic profiling would be needed to help differentiate between these ER+ mammary carcinoma cell lines.

Overall, there have been several breast cancer cell lines which have exhibited increases in cell survival and invasiveness in response to ARTN forced expression (Kang et al., 2009). The increased survival is likely due to ARTN up-regulation of several anti-apoptotic factors, including BCL-2, whereas the up-regulation of TWIST1 might account for the increased motility and invasiveness of otherwise non-invasive carcinoma cell lines (Banerjee et al., 2011; Kang et al., 2009).

6.2 Downstream signalling mediators of ARTN and IGF-1 function in mammary carcinoma cells

When the studies of Boulay *et al* (2008) and Kang *et al* (2010) demonstrated the regulation of RET and ARTN expressions by estrogen treatment, they also demonstrated the cross-talk abilities of GFL signalling and highlighted the implications that came with it (Boulay et al., 2008; Kang et al., 2009). Little is still known about downstream signalling mechanism of the receptor complexes, but the classic mediators of downstream signalling, MAPK and PI3K/AKT, are purported to be involved and could provide ready targets for intervention (Mograbi et al., 2001; Song and Moon, 2006; Takaku et al., 2013).

The MAPK pathway is a protein kinase cascade (Cargnello and Roux, 2011). It relies on the recruitment of adapter molecules to the original activated receptor and the activation of downstream effector molecules by phosphorylation, such as RAS, RAF and MEK. This leads to the eventual activation of MAPK, which in turn activates transcription factors in the nucleus to effect gene transcription and encourage cell proliferation.

The PI3K/AKT pathway operates parallel to MAPK and its activation also encourages cell proliferation and cell survival (Fresno Vara et al., 2004; Fruman and Rommel, 2014). The activation of this pathway differs slightly to that of MAPK, where the initial step of activation requires the production of inositol phospholipids at the cell membrane. This pathway eventually leads to the activation of AKT by phosphorylation, which in turn activates mTOR and inactivates the pro-apoptotic protein, BAD.

The results of this study allude to the cooperative co-activation of the MAPK pathway by both IGF-1 and ARTN signalling. There was no activation of AKT, by ARTN in wild-type MCF-7 cells. This was interesting as a previous study by Banerjee *et al* (2012) demonstrated that ARTN forced expression in the ER- mammary carcinoma cell lines, BT549 and MB-MDA-231, did increase phosphorylation of AKT (Banerjee et al., 2011) indicating that this pathway is not utilised by ARTN in all cell lines. In support of this, an *in vivo* study also demonstrated that Artn signalling in mice did not require activation of PI3K/AKT pathway (Li et al., 2009). However, although ARTN treatment alone did not activate AKT, synergistic activation of this pathway with combined ARTN and IGF-1 treatment was observed in MCF-7 cells, suggesting that both growth factors can cooperate to enhance activation of this pathway. Elucidating the mechanism involved will be of interest. The results presented in Chapter 3 suggested that both signalling pathways converge onto the same downstream signalling mediators to affect cell function. Additionally, it is possible that

IGF-1 also interacts directly or indirectly with the RET receptor as IGF-1 treatment resulted in a small but rapid increase in the phosphorylation of the RET receptor. This observation and the mechanism involved warrants further investigation.

Currently, little is still known about the precise signalling mechanisms that are employed by ARTN in mammary carcinoma cells. The main signalling pathways that are currently known, involve the activation of MAPK or AKT proteins by phosphorylation, upon receptor activation. The results obtained in this study demonstrated synergistic activation of MAPK and AKT, by ARTN and IGF-1 co-treatment. However, the receptor activation assays indicated that this synergy was most likely not through the activation of RET or IGF-1R, but through alternate mechanisms. With regards to ARTN signalling, it is possible that the synergy observed in MAPK and AKT phosphorylation was due to activation through other receptors for ARTN, such as NCAM or GFRα1/3. In future studies, investigation of these alternative pathways for ARTN signalling could be helpful in providing additional insight into the activated mechanisms and downstream pathways.

One of the main limitations of this project has been the low serum conditions necessary for assessing IGF-1 function in the behavioural assays with longer incubation times, particularly in the total cell number assays and in 3D cultures for anchorage-independent growth. This has meant that useful results could only be achieved in the MCF-7 cell lines, as both T47D and ZR-75-1 cells had much greater serum requirements for maintenance of the cell population and could not be sustained in the low serum (0.2% FBS) conditions required for some of these assays. This was unfortunate since, as discussed earlier in this chapter, the responses to treatment can vary significantly between cell lines. Ideally, the longer assays would also be performed in multiple different cell lines in order to verify the existence of

similar effects on the cell behaviour and growth in response to both ARTN and IGF-1 treatments.

6.3 The etiology of chemotherapy drug resistance

Resistance to therapy has become an integral part of cancer research. The development of resistance can severely limit one's treatment options with regards to clinical application. Because normal cells do not exhibit the same kind of adaptation to treatment that cancer cells do, and patient tolerances to drug toxicities do not improve with prolonged drug exposure, this is an aspect of cancer biology that needs to be overcome (Gottesman, 2002; Hazlehurst et al., 2000). There are a number of theories and hypotheses that have arisen to account for cancer cell resistance to chemotherapy, each of which provides unique methods for solving the problem.

Treatment resistance in cancer cells is typically categorised as *de novo* or acquired (Gottesman, 2002; Hazlehurst et al., 2000). In *de novo* resistance, it is a trait that is present in the cancer cell population before exposure to drug treatment. The mechanisms of *de novo* resistance may be inherent to the cancer cell's genetics, or it can be contributed to by a protective tumour microenvironment that favours cell survival (Shoemaker, 2000). This type of resistance can potentially affect the efficacy of treatment preventing the elimination of residual disease, or facilitate the progression of acquired resistance to therapy. In contrast, acquired drug resistance, normally develops after prolonged exposure to drug treatment, resulting in clonal selection of the cancer cell population for a more favourable phenotype (Little et al., 2013). This can result from a mutation in the original molecular drug target or through other compensatory mechanisms in the cellular machinery, through genetic or epigenetic means.

IGF-1 and IGF-1R over-expression have been linked to the development of drug resistance by promoting cell survival and facilitating the selection of therapy resistant cells (Gottesman, 2002; Peyrat et al., 1990; Schiff et al., 2003). Likewise, ARTN and RET signalling have also been shown to promote cell survival and the development of resistance to therapy (Banerjee et al., 2012a; Kang et al., 2010; Pandey et al., 2010a; Plaza-Menacho et al., 2010). ARTN's connection to tamoxifen resistance is of particular clinical importance to frontline treatment of ER-positive breast cancer (Kang et al., 2010).

These previous studies have also investigated the contribution of ARTN expression to *de novo* resistance to chemotherapy agents in mammary carcinoma cells (Banerjee et al., 2012a; Ding et al., 2014; Kang et al., 2010). Those studies demonstrated that ARTN conferred greater resistance to tamoxifen, paclitaxel and trastuzumab in the mammary carcinoma cell lines studied. However, the *de novo* resistance that was demonstrated in BT549 and MDA-MB-231 cells with stable forced expression of ARTN, was not observed in MCF-7 cells. Instead, ARTN forced expression sensitised MCF-7 cells to treatment with 5FU and paclitaxel.

Again, this points to a gap in the understanding of ARTN and its functions outside of its normal developmental role, but it also highlights the importance of the selection of cell lines for study. Differences between cell lines could result in wide differences in response to ligand signalling. Since the nature of ARTN signalling is largely unknown, one can only speculate as to the reason for the differences in cellular response to ARTN and its effect on *de novo* drug resistance. Here, the differences could be due to the basic categorisation of the mammary carcinoma cell lines that are used and the types of breast cancer they most closely imitate. Gene profiling studies carried out on some of the most commonly used cancer cell

lines had sought to more clearly define those cell lines and determine their place in *in vitro* modelling of actual tumours (Kao et al., 2009).

Traditionally, carcinomas have been clinically divided into 5 main catetgories: luminal A or B, basal-like, ERBB2 and normal-like, based on their molecular profiles (Curtis et al., 2012; Kao et al., 2009). The gene profiling study carried out by Kao *et al* (2009) used microarray information on the cell gene expressions to stratify the most commonly used cell lines into 3 categories of basal A or B, and luminal (Kao et al., 2009). From their study, the cell lines that were used for this project, MCF-7, T47D and ZR-75-1, are categorised as luminal cell lines, which are all positive for ER and most resemble luminal tumours. Whereas the MDA-MB-231 and BT549 cell lines that showed increased *de novo* resistance in previous studies, were categorised by that study as basal B type cell lines. These two cell lines are negative for ER expression and most resemble ERBB2 and basal tumours, respectively.

These basic differences in the genotypic profile of the carcinoma cell lines could account for the great dependency on cell line that have governed the results of the ARTN studies. It becomes obvious that one must be careful with their choices for study, and a greater understanding of the fundamentals of cell behaviours and gene functions is needed to achieve the progression that is needed in this field.

The results in the latter parts of Chapter 5 utilised the IGF-1R antagonist, AG1024, to model *de novo* and acquired resistance in wild-type mammary carcinoma cell lines. In the ARTN forced expression cell lines, AG1024 inhibition of IGF-1R resulted in reduced cell proliferation, reduced cell survival and decreased anchorage-independent colony growth. While inhibition of function was observed in both cell lines, ARTN forced expression protected MCF-ARTN cells from some of its effects. This would suggest that ARTN might play a role in promoting *de novo* resistance to drug-mediated inhibition of IGF-1R.

To investigate this theory, AG1024-resistant cells were established in the MCF-7 and ZR-75-1 cell lines and then assayed for changes to ARTN expression by semi-quantitative RT-PCR and western blotting. Interestingly, ARTN expression was found to be increased in the resistant cell lines compared to the sensitive wild-type cell lines, suggesting the involvement of ARTN in mediating the acquired resistance to AG1024. Given the results from Chapter 3, which indicated that IGF-1 signalling lead to an increase in the level of ARTN expression, we had expected that inhibition of IGF-1 signalling by AG1024 to reduce ARTN expression in the short-term. Whereas long-term IGF-1 signalling inhibition by AG1024 would be subject to selection pressures and produce an increase in ARTN expression. However, this was not the case and under the conditions tested, both short-term and long-term treatment with AG1024 resulted in increased ARTN expression; implicating that AG1024 was also capable of inducing ARTN up-regulation.

While it is not unusual for some molecules to have dual antagonist and agonist actions on cell targets, human growth hormone, for example, is agonistic at low concentrations but is inhibitory at higher concentrations (Anisimov and Bartke, 2013). However, this is an unlikely attribute for small molecule inhibitors, like tyrphostins. In the case of AG1024, it is a selective tyrosine kinase inhibitor which prevents auto-phosphorylation in the β subunit of IGF-1R, preventing downstream signalling activation (Levitzki, 1992; Wen et al., 2001). Although AG1024 is selective for IGF-1R, there is some cross-inhibition of the IR.

Interestingly, long-term culture of mammary carcinoma cell lines in serum-free conditions can also result in an increase in the ARTN expression, as explained in Chapter 3, attributed to an up-regulation of ER activity, which in turn increased the ARTN expression (Fox et al., 2013). Given this knowledge, it is possible that the effects of dual IGF-1R and IR inhibition by AG1024 could mimic the effects of serum-free culture in the mammary carcinoma cell

lines, leading to the up-regulation of ARTN expression, via ER activation. Although, due to time restraints, this hypothesis was not tested for in the scope of this project, it could be an interesting point for future study.

In conclusion, the data presented in this thesis support the hypothesis for an IGF-1 and ARTN signalling cross-talk mechanism. Considering the individual roles of each ligand in the promotion of cancer development, this could have important clinical implications. In addition, the differential effects of ARTN signalling on various cell lines highlights the importance of cell line selection in *in vitro* modelling of disease for results as well as advocating the need for personalised treatment regimes. Continued research is needed to properly decipher the role that ARTN plays in mammary carcinoma, whether it should be targeted in developing cancer treatments, and in which contexts it would be most useful.

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Appendix

Buffers and solutions	Composition
Bacterial culture	
LB medium	10g Bacto^{TM}Tryptone, 5g Bacto^{TM}Yeast extract, 5g NaCl, 1L Milli-Q H_2O, autoclaved and stored at room temperature
LB plate	15g agarose was added to 1L liquid LB medium and sterilized by autoclaving. After the medium was cooled down to 50ºC, ampicillin was added at the concentration of 100µg/mL. After gently mixing, the medium was poured into the sterile plates. Upon the medium was solidified, the dishes were stored at 4ºC in the inverted position.
Cell culture	
RPMI Medium	10.39g/L RPMI powder, 2g/l $NaHCO_4$; Dissolve it by adding autoclaved H_20, adjust the pH to 7.2 by adding HCl or NaOH, adjust volume to 1L with additional autoclaved H_20 and filter sterilise.
Complete RPMI medium	450mL RPMI medium, 50ml heat-inactivated fetal bovine serum, 5mL glutamine, 5mL penicillin/streptomycin
10 X PBS	80g NaCl, 2g KCl, 14.4g Na_2HPO_4, 2.4g KH_2PO_4, 1L Milli-Q H_2O. Adjust pH to 7.4
10X trypsin/EDTA solution (0.25%)	2.5g Trypsin, 0.372g EDTA, 0.35g $NaHCO_3$, 1L autoclaved H_2O. Adjust pH to 7.2.
Freezing medium	0.5mL DMSO, 3ml heat inactivated fetal bovine serum, 6.5mL RPMI medium
0.4% Trypan blue	0.4g Trypan blue, 100mL 1X PBS.
RNA extraction	
DEPC water	1mL DEPC, 1L Milli-Q H_2O. Solution was made in the fume hood and stirred overnight at room temperature before being autoclaved.
DNA electrophoresis	
50X TAE buffer	242g Tris, 37.2g $Na_2EDTA.2H_2O$, 57.2mL glacial acetic acid, 1L Milli-Q H_2O
1-3% agarose gel	1-3g agarose, 100mL 1X TAE buffer

1% Ethidium bromide solution	0.2g ethidium bromide, 20mL Milli-Q H_2O
DNA loading dye 6x	250 mg bromophenol, 30mL glycerol, 70mL H_2O
Protein extraction	
Lysis buffer	2% (w/v) SDS, 20% glycerol, 0.06M Tris-HCl pH6.8, 1 mini EDTA-free complete protease inhibitor tablet
Phospho-specific lysis buffer	10 mL (final concentration): 500µL (1 M Tris-HCL pH 7.4 (121.1 g/mol)); 1mL (10% Nonidet P-40); 300 µL (5 M NaCl (58.44 g/mol)); 50µL (0.2 M EDTA (452.24 g/mol)); NaF 1mM; PMSF 1mM; 100µL (100 mM Na_3VO_4); 1 mini EDTA-free complete protease inhibitor tablet, 6.62mL H_20
SDS-PAGE	
4% Stacking gel	500µL 40% acrylamide, 1.26mL 0.5M Tris-HCl pH 6.8, 50µL 10% SDS, 3.18mL Milli-Q H_20, 5µL TEMED, 25µLl 10% APS
12% Separating gel	3mL 40% acrylamide, 2.5mL 1.5M Tris-HCl pH 8.8, 100µL 10% SDS, 4.35mL Milli-Q H_20, 5µL TEMED, 50µL 10% APS
15% Separating gel	3.6mL 40% acrylamide, 2.5mL 1.5M Tris-HCl pH 8.8, 100µL 10% SDS, 3.85mL Milli-Q H_20, 5µL TEMED, 50µL 10% APS
6X SDS loading dye	6mL glycerol, 3mL 1M Tris-HCl, pH 6.8, 1.2g SDS and 5mg bromophenol blue
1X SDS running buffer	3.03g Tris, 14.41g glycine, 10mL10% SDS, 1L Milli-Q H_20
1X Transfer buffer	3.03g Tris, 14.41g glycine, 200mL MeOH, 800mL Milli-Q H_20
Western Blot	
10X TBS	24g Tris, 88g NaCl, 1L Milli-Q H_20, pH 7.4
0.1% TBST	100ml 10x TBS, 900mL MilliQ H_20, 1mL Tween-20
Blocking buffer	5g non-fat dry milk powder or BSA, 100mL 0.1% TBST
BrdU incorportation	
Blocking solution	1X PBS; horse serum 3 drops
Primary Antibody Solution	Anti-BrdU antibody 50µL; 1X PBS 10mL; BSA 200µg
Secondary Antibody Solution	Secondary Antibody 1 drop; 1X PBS 10mL; horse serum 2 drops; Tween-20 2µL
Tertiary Antibody solution	Reagent A 1 drop; reagent B 1 drop; 1X PBS 10mL
Substrate solution	1 DAB tablet; 1 H_20_2 tablet; 1mL milliQ H_20

Other	
1.5 M Tris-HCl	27.3g Tris; 80mL Milli-Q H_20 ; Adjust pH value to 8.8 with 2N HCl and make up to 150mL with MilliQ H_20
0.5M Tris HCL	9.1 g Tris; 80 mL Milli-Q H_20 ; Adjust pH value to 6.8 with 2N HCl and make up to 150mL with MilliQ H_20

Whole ARTN western blots

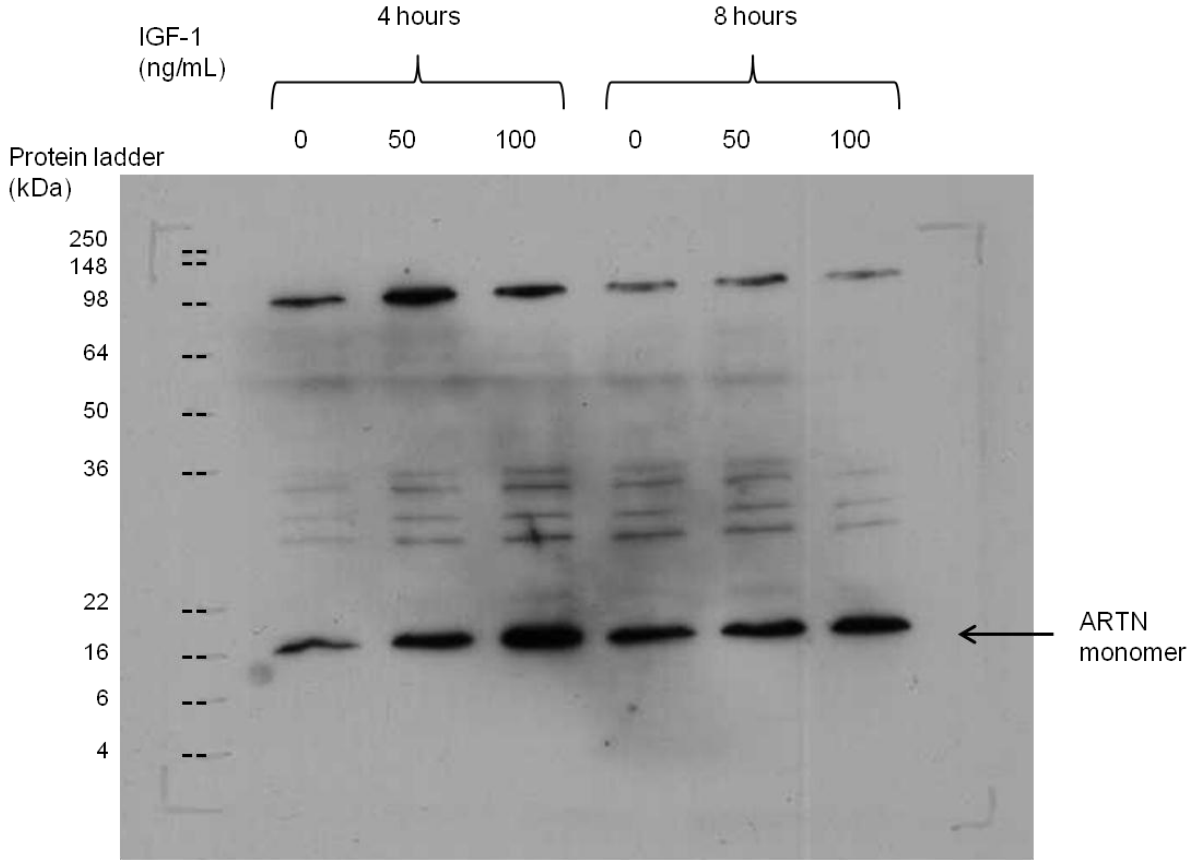

Figure 8.1- Whole ARTN western blot using wild-type MCF-7 cells.

The above is an example of a typical ARTN western blot performed using total cell lysates from wild-type MCF-7 cells and developed on x-ray film. The ARTN monomer is distinct and readily identified from its low molecular weight. However, identification of the dimer is not possible due the number of similar looking non-specific bands that are seen at similar molecular weights.

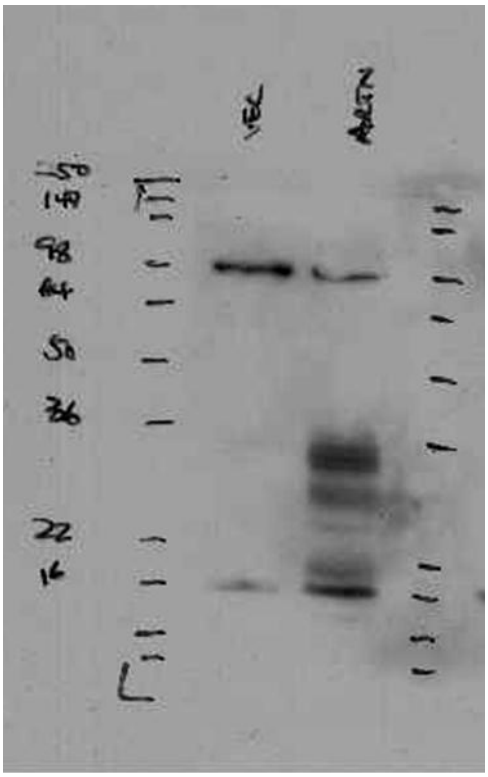

Figure 8.2- Whole ARTN western blot in MCF-Vec and MCF-ARTN stable cell lines.

The above is an example of a typical ARTN western blot performed using total cell lysates from MCF-Vec and MCF-ARTN stable cell lines cultured in serum-free RPMI for 24 hours. The ARTN monomer is readily identified from its low molecular weight, and the dimer is more prominent and demonstrates a significant increase in expression in the MCF-ARTN cell line compared to MCF-Vec.