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Role of insulin-like growth factor (IGF) axis in the development of tamoxifen resistance in breast cancer epithelial cells

Yousef Mohammedrabaa Hawsawi

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Leeds School of Dentistry, Faculty of Medicine and Health, University of Leeds and Leeds Institute of Cancer Studies and Pathology.

St. James`s University Hospital, Leeds

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Dedication

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Abstract

The development of tamoxifen resistance (TamR) in oestrogen receptor positive (ER$^+$) breast cancer is a major therapeutic challenge. Mechanisms suggested to account for this have mainly focussed on the activation of alternative growth factor pathways. The insulin-like growth factor (IGF) axis is a prime candidate for investigation in this area and the use of anti-IGF strategies in the clinical setting of tamoxifen resistance is under investigation. However such strategies, usually targeted to block the IGF-1 receptor (IGF-1R) have proved disappointing. The IGF axis is a multicomponent molecular system and the activity of IGF is modulated by the presence of six soluble high affinity IGF binding proteins (IGFBP 1-6). Given the potential role of the IGF axis in the development of tamoxifen resistance it is important to investigate whether the IGFBP family may play a role in this process opening up a route for alternative anti-IGF based therapies. Using the ER$^+$ MCF-7 cell line we demonstrated that five IGF axis genes (IGF-IR, IGF-2R, IGFBP-2, IGFBP-4 and IGFBP-5) were expressed by both parental wt and tamoxifen resistant (TamR) MCF-7 cells with the remaining genes (IGF-1, IGF-2, IGFBP-1, IGFBP-3 and IGFBP-6) either not expressed or expressed only at a very low level. IGFBP-5 expression was down-regulated by approximately 7-fold while IGFBP-2 was up-regulated by approximately 2-fold in TamR versus wt cells. These alterations in IGFBP-2 and IGFBP-5 gene expression were mirrored in protein levels measured in a conditioned medium by ELISA, Western and Ligand blot. Significantly, a knockdown of IGFBP-2 in TamR cells restored sensitivity to 4-hydroxytamoxifen (4-HT), reduced ERα expression to 45 ± 11.9% and enhanced cell migration. Knock down of IGFBP-5 in wt cells had no effect on sensitivity to 4-HT but enhanced cell migration. Exogenous IGFBP-2 had no effect on tamoxifen sensitivity which may suggest an intracellular mechanism of action for IGFBP-2. Immunohistochemical analysis of breast cancer tissue microarrays (TMAs) indicated that expression of IGFBP-2 was significantly associated with survival advantage in tamoxifen resistant patients.

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4-HT	4-hydroxytamoxifen
ALs	Aromatase inhibitors
BC	Breast cancer
BSA	Bovine serum albumin
Cdks	Cyclin-dependent kinases
cDNA	Complementary DNA
CSCs	Cancer stem cells
DCS	Dextran charcoal stripped
DFS	Disease free survival
E	Oestrogen
E_2	Oestradiol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF4E	Eukaryotic translation initiation factor 4E
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ER	Oestrogen receptor
FAK	Focal adhesion kinase

FBS	Foetal bovine serum
FFPE	Formalin-fixed, paraffin-embedded
FOX	Forkhead transcription factors
GAGs	Glycosoaminoglycans
GH	Growth hormone
GM	Growth medium
GnRH	Gonadotropin-releasing hormone
GPER	G protein-coupled ER
HBD	Heparin binding domain
HER2	Human Epidermal Growth Factor Receptor 2
HIF1α	Hypoxia-inducible factor 1α
HRP	Horseradish peroxidase
IGF	Insulin-like Growth Factor
IGFBPs	Insulin-like Growth Factor Binding Proteins
IHC	Immunosistochemistry
ILK	Integrin linked kinase
IP3	Inositol triphosphate
IR	Insulin receptor
IRS	Insulin receptor substrate
kDa	Kilodalton
Mab	Monoclonal antibody

MAPK	Mitogen activated protein kinase
MCF-7	Michigan Cancer Foundation-7 (Human BC cell line)
MECs	Mammary epithelial cells
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
NLS	Nuclear localisation signal
NSAIDs	Non-steroidal anti-inflammatory drugs
OS	Overall survival
P	Progesterone
PR	Progesterone receptor
p53	Protein 53 or tumour suppressor protein 53
PAPA-1	Pim-1 associated protein
PAPP-A	Pregnancy-associated plasma protein-A (PAPP-A)
PBS	Phosphate buffered saline
Pen/Strep	Penicillin/streptomycin
PI3K	Phosphatidylinositol-3-kinase
PP2A	Protein phosphorylase 2A
PRF	Phenol red free media
PRL	Prolactin
PTEN	Phosphatase and tensin homologue on chromosome 10

PTM	Post-translational modifications
qRT-PCR	Quantitative real-time PCR
RGD	Arg-Gly-Asp
ROC	Receiver Operating Characteristic
RT	Room temperature
RWD	Relative wound density
RXR	Retinoid X receptor (RXR)
nRXR	Nuclear retinoid X receptor (RXR)-α
S6K1	Ribosomal S6 kinases
SERDs	Selective oestrogen receptor down-regulators
SERM	Selective oestrogen receptor modulator
siRNA	short inhibitory RNA
SNPs	Single nucleotide polymorphisms
Tam-R	Tamoxifen resistant MCF-7 cells
Tam-S	Tamoxifen-sensitive MCF-7 Cells
TBS	Tris buffered saline
TGF-βVR	Transforming growth factor-beta V receptor
TMAs	Tissue microarrays
VEGF	Vascular endothelial growth factor
WT	Wild type (wt)
ZEB	Zinc-finger enhancer binding (ZEB) 1

Chapter 1 Introduction

1.1 General overview

Over the past decades, millions of women have died from breast cancer (BC) as a result of late diagnosis, disease relapse [1] and the development of resistance to endocrine and chemotherapeutic treatment regimens [2]. For several reasons the global incidence of BC has increased significantly in recent years [3]. Although this is partly a result of improved screening methodologies, in the developed world lifestyle factors (smoking, increased adiposity) have also had a bearing on BC incidence and BC remains the most common non-cutaneous female malignancy [4]. Currently BC is responsible for 15% of all female death caused by cancer and is the second most common form of cancer in women after lung cancer Fig 1.1 [5]. A conservative estimate suggests that worldwide there are approximately 1.38M cases of BC and around 458,000 deaths occur annually due to the disease [6]. In the United States, more than 232,670 estimated new cases of BC were reported in 2012 (Fig 1.1) with over 40,00 BC related deaths [7]. Currently, about 570,000 people are estimated to live with breast malignancy in the UK, and the rate is increasing http://publications.cancerresearchuk.org/downloads/Product/CS_REPORT_TOP10IN CMORT.pdf.

Despite recent breakthroughs in molecular biology and advances in the early detection of BC, about 30% of BC patients have recurrent disease [8] with a resulting impact on individual wellbeing and healthcare costs [9]. To tackle BC it is important to examine areas such as such as aetiology, risk factors and the molecular biology associated with the disease. In the next sections we briefly highlight these features and precede them with a short discussion of the anatomy of the breast.

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Estimated New Cases*

			Males	Females			
Prostate	233,000	27%			Breast	232,670	29%
Lung & bronchus	116,000	14%			Lung & bronchus	108,210	13%
Colorectum	71,830	8%			Colorectum	65,000	8%
Urinary bladder	56,390	7%			Uterine corpus	52,630	6%
Melanoma of the skin	43,890	5%			Thyroid	47,790	6%
Kidney & renal pelvis	39,140	5%			Non-Hodgkin lymphoma	32,530	4%
Non-Hodgkin lymphoma	38,270	4%			Melanoma of the skin	32,210	4%
Oral cavity & pharynx	30,220	4%			Kidney & renal pelvis	24,780	3%
Leukemia	30,100	4%			Pancreas	22,890	3%
Liver & intrahepatic bile duct	24,600	3%			Leukemia	22,280	3%
All Sites	**855,220**	**100%**			**All Sites**	**810,320**	**100%**

Estimated Deaths

			Males	Females			
Lung & bronchus	86,930	28%			Lung & bronchus	72,330	26%
Prostate	29,480	10%			Breast	40,000	15%
Colorectum	26,270	8%			Colorectum	24,040	9%
Pancreas	20,170	7%			Pancreas	19,420	7%
Liver & intrahepatic bile duct	15,870	5%			Ovary	14,270	5%
Leukemia	14,040	5%			Leukemia	10,050	4%
Esophagus	12,450	4%			Uterine corpus	8,590	3%
Urinary bladder	11,170	4%			Non-Hodgkin lymphoma	8,520	3%
Non-Hodgkin lymphoma	10,470	3%			Liver & intrahepatic bile duct	7,130	3%
Kidney & renal pelvis	8,900	3%			Brain & other nervous system	6,230	2%
All Sites	**310,010**	**100%**			**All Sites**	**275,710**	**100%**

Figure 1-1 Incidence and mortality rates for cancer (by gender) in US (2012).
Adapted from [7].

1.1.1 Anatomy of the breast

Mammary gland development in humans starts at the fifth week of embryogenesis where mammary epithelial cells (MECs) develop from an ectodermal bud [10]. After sixteen weeks of gestation, the embryonic glandular structures grow to become epithelial buds with some individual branch points. At thirty two weeks rudimentary lobule-alveolar composition develops surrounded by a compact stromal compartment [10]. During the neonate stage, the primitive branched composition contains one squamous epithelial layer and one basal cuboidal layer [11]. Extensive development then occurs during puberty under the regulation of ovarian secretion of oestradiol (E) and progesterone (P), both of which are essential for normal mammary gland ductal elongation and reproductive development. In addition mammary gland development is also controlled via pituitary-derived hormones, such as prolactin (PRL) and growth hormone (GH) [12].

At puberty, the adult female breast is completed as a tear-shaped gland which is composed mostly of adipose tissue and is situated between the third and sixth ribs [13]. It is enclosed within a thin fibrous envelope which separates it from the skin above and the muscle underneath. For clinical purposes, the breast has been divided into four quadrants: upper outer (where most cancers occur), upper inner, lower outer and lower inner. Anatomically, the mammary gland consists of 15-20 lobes which branch out from the nipple [14]. The size of each lobe varies greatly over a 20- to 30-fold range [13]. All lobes are enclosed by subcutaneous fat and fibrous connective tissue. These lobes are branched into small lobules [14], and each lobule is subdivided into 10 to 100 small branched alveoli. Each alveolus serves as a milk producing unit of the breast [15].

The mammary gland represents a unique organ where numerous rounds of lactation, differentiation, proliferation, and involution take place during reproductive life [16]. These complicated dynamic molecular, biochemical and structural alterations are regulated via ovarian sex steroid hormones E and P [17]. E is important for mammary epithelial cell (MEC) proliferation within the ducts and their elongation through puberty. Although the main source of E in the pre-menopausal woman is the ovary, E is also secreted in smaller amounts in other tissues such as subcutaneous adipose tissue. Several lines of evidence suggest a direct effect of E on cell proliferation not only in the different stages of normal breast growth but also as a mitogenic drive to BC cells. [11]. E controls cell proliferation and ductal elongation in the normal mammary gland mainly through activation of nuclear oestrogen receptors (ERs) [17], although the synchronized effects of other secreted growth factors are also involved. Although E can play a key role in BC cell growth and transformation the biological characteristics of individual tumours vary greatly and have an important bearing on treatment and prognosis of the disease (see Section 1.1.5).

1.1.2 Signalling

Binding of IGF-I to receptor leads to recruitment of adaptor proteins belonging to the insulin receptor substrate (IRS) family to auto-phosphorylated regions within the cytosolic domain of the receptor with subsequent activation of downstream signalling molecules including the protein kinase B (PKB), which also known as Akt, adapter protein Shc/Grb2, Ras/Raf-1, Mitogen-activated protein kinases (MAPK)/Erk and phosphoinositide 3-kinase (PI3K) resulting in the regulation of various cellular functions including mitogenesis, apoptosis, adhesion, migration and differentiation [18]. Since the IGFs may play a key role in BC progression, a clear understanding of such signalling mechanisms is important for a full understanding of this disease. Activation of PI3K and leads to increased inositol triphosphate (IP3) concentrations and stimulation of Akt protein kinase [19]. This regulates the activity of anti-apoptotic proteins, such as Mouse double minute 2 homolog (MDM2) [20] and the death promoter (BAD) protein [21]. Activated Akt also phosphorylates the forkhead transcription factors (FOX) [22], triggering the mammalian target of rapamycin (mTOR) to initiate protein synthesis and cell division. The targets of mTOR include the ribosomal S6 kinases (S6K1and S6K2) together with eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), which in concert act to increase translation, proliferation and cell growth. Moreover, phosphorylation of protein phosphorylase 2A (PP2A) via mTOR impedes the dephosphorylation of S6K1 and 4EBP1 [23]. The tumour suppressor genes tuberous sclerosis TSC1 and TSC2 regulate mTOR. AMPK phosphorylates TSC2, which increases GTPase activity of the G protein Ras homolog enriched in brain (RHEB) preventing mTOR activation and subsequent activation of protein synthesis [23]. Most of these genes (AKT, mTOR, PTEN and MAPK) have been associated directly with the development and progression of breast cancer. Indeed, activation of mTOR in malignant breast cells is linked with resistance to the chemotherapeutic agents tamoxifen

and trastuzumab [24]. Signalling by both IGF-I and -2 is negatively regulated by the tumour suppressor PTEN and dephosphorylation of Inositol trisphosphate (IP3) by PTEN inhibits IGF-1 induced Akt signalling [25]. Loss of PTEN causes an increase in IGF-2 signalling through the IGF-IR or IR-A [26, 27]. Therefore PTEN mutation could lead to increased IR and IGF-IR signalling resulting in stimulation of tumour growth. The involvement of the IGF axis in the signalling pathways discussed above and their importance in tumourigenesis has further stimulated research into the molecular details of IGF axis involvement in BC [28, 29]. An outline of IGF signalling pathways is shown in Fig 1.2.

Epithelial-mesenchymal transition (EMT) is a crucial mechanism involved in metastasis and invasion by cancer cells as it promotes migration and the separation of individual cells from the primary cancer and also enables the cells to access the lymphatic system promoting tumour dissemination. In this way tumour cells can easily migrate into secondary organs and create micro-metastases. Several extracellular and intracellular signalling pathways have been implicated in the initiation of EMT including the involvement of the IGF axis [30]. Cell adhesion to the neighbouring extracellular matrix (ECM) is mediated by cell-surface integrin receptors. Integrins mediate cell attachment, and modulate cell proliferation, apoptosis, migration and spreading. Integrin receptors are heterodimeric comprising a combination of one of 18 possible α subunits and 8β subunits. In total 24 different integrin receptors have been described in human cells where they act to regulate process such as focal adhesion, cell attachment and migration. For instance, the adaptor protein paxillin binds the β subunit of integrin dimers in the focal adhesion complex (FAK) where it recruits other signalling proteins to regulate the expression of genes involved in controlling cell structure and movement [29, 31]. Integrin receptors also bind ECM components such as fibronectin and vitronectin through distinct recognition motifs in the ECM proteins. Along with fo-

cal adhesions discussed above these interactions of integrins with ECM also regulate cell adhesion and motility and have clear relevance in the study of tumour spread. Some of the IGFBPs including the IGFBP-5 are found associated with the ECM and may play a role in cell adhesion [32].
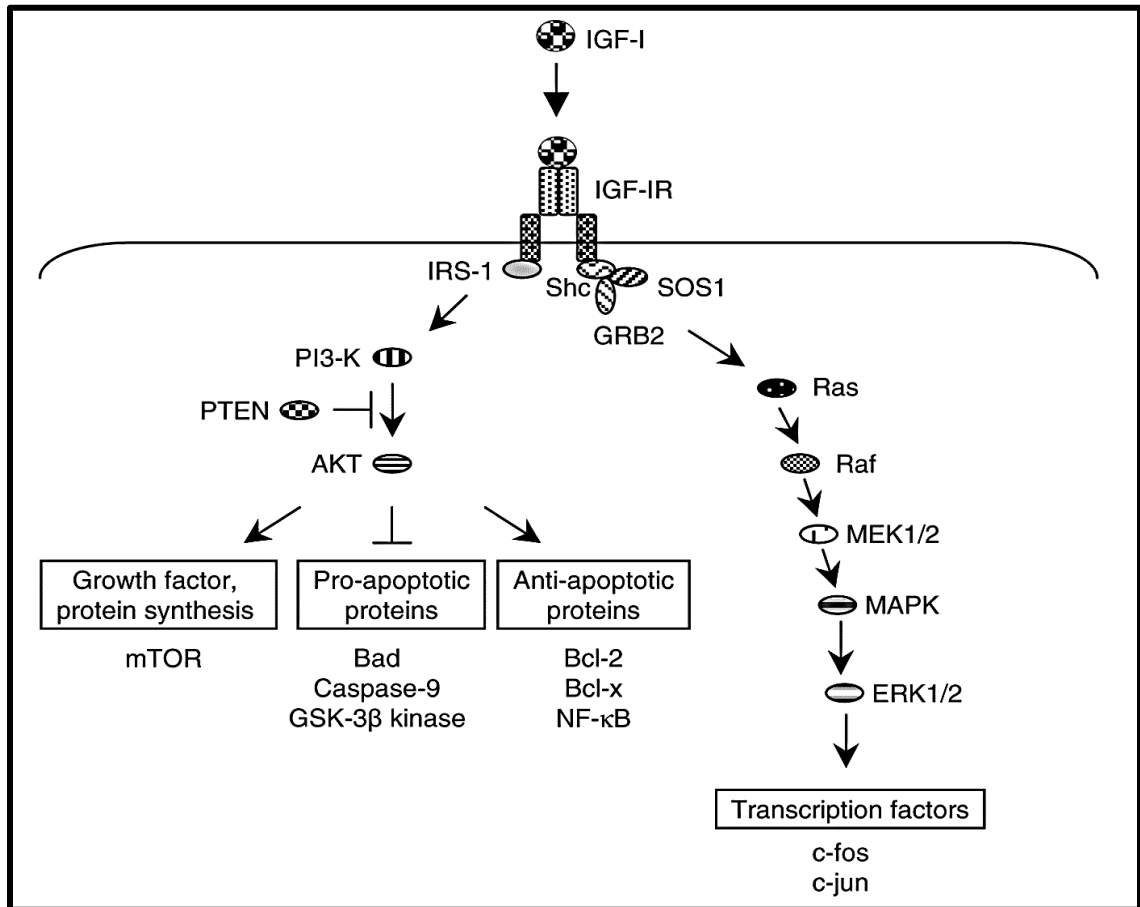
Figure 1-2 Schematic representation of IGF-1 signalling pathways.

Early events following IGF-1 binding to cognate receptor include recruitment of IRS and Shc-Sos-Grb2 adapter proteins to cytosolic domains of IGF-1R and subsequent activation of PI3-K/AKT and Ras/Raf/MAPK pathways. These in turn regulate downstream the activity of mTOR and families of both anti- and pro-apoptotic proteins together with trans acting transcription factors such as c-fos and c-jun. Some well characterised tumour suppressors (e.g. PTEN) interact with signalling pathways to regulate cell division. Adapted from [100].

1.1.3 BC – definitions, risk factors and treatment

BC has been defined as a malignant disease characterised by uncontrolled cell proliferation in the mammary gland with frequent subsequent spread to secondary sites [33]. Histologically, it is believed that the disease originates from a single cell whose division is deregulated essentially due to accumulated genetic and epigenetic alterations resulting in tumour growth and invasion of the surrounding tissues [34]. Clinically, there are two kinds of breast cancer: non-invasive and invasive. non-invasive tumours generally lack the capability of spreading and occur in well differentiated cells, whereas invasive cancers have the ability to spread within the body, and are usually characterised by a higher population of undifferentiated cells. Nearly all BC deaths are due to metastasising disease, which is resistant to conventional therapies. The metastatic progression consists of a series of events beginning with invasion of the primary tumour into the vascular system, extravasation, and ultimately the development of metastatic deposits in secondary target organs [35]. This migration of malignant cells is the crucial stage in the metastatic progression and many risk factors contribute in the initiation and progression of BC.

The single leading risk factor for BC is increasing age [36]. The majority of the cases (83%) are identified in women aged ≥50 [37], and men aged ≥60 [38]. Whilst BC is predominantly a disease of old women it can affect younger women even those less than 30 years of age. Genetic mutations (e.g. in *ATM*, *p53*, *CHEK2,* TSC2, PTEN, PI3K and AKT-1 genes) are found in 5% to 10% of breast malignancies [39]. Alterations in the *BRCA1* and *BRCA2* genes are the most common causes of hereditary breast malignancies, causing up to 80% of cases [40]. A range of other factors, such as low intakes of fibre and antioxidant vitamins (e.g. vitamin C, vitamin E, selenium and beta carotene), hyperglycaemia [41], and excessive alcohol and smoking [42, 43], and other issues directly connected with lifestyle and personal behaviour are im-

portant risk factors. Use of oral contraceptives, early age of menstruation, late meno-pause, lack of breast feeding and postmenopausal hormone therapy are also identi-fied risk factors [44, 45]. In addition, exposure to ionizing radiation, electromagnetic fields, artificial oestrogens and use of non-steroidal anti-inflammatory drugs (NSAIDs) are also described as risk factors [46]. Intriguingly, obesity is directly connected to an increased risk of cancer and cancer-related mortality and is estimated to be a factor in over 20% of cancer deaths in females and 14% in males [47]. In addition the level of fasting insulin was positively correlated with BC risk, regardless of obesity. An exten-sive survey reported increased risk of BC for postmenopausal women with type 2 dia-betes (T2D) [48] with a reported 20% increased risk of developing BC [49]. Epidemio-logic investigation reveals a clear link between BC and hyper-insulinemia [50]. Genet-ic background is also an additional risk factor for BC. In the US, Newman et al. 2006 pointed out that, the white American women are less likely to be diagnosed with early-onset BC compared to African American women although when comparing overall lifetime risk, white American women have a higher risk of developing BC compared to African American women [51].

Endocrine therapy is an effective approach to treat some BCs through removal or blockade of hormone action and subsequent inhibition of cancer cell growth. E and P are the most important steroid hormones involved in mammary gland physiology and several disruptors of this particular hormonal axis have been examined and used as to remove or ablate the mitogenic drive in breast tumours supplied by steroid hormones. These include the classes of agent such as selective estrogen receptor modulators (SERMs e.g. tamoxifen), selective estrogen receptor down-regulators (SERDs e.g. fulvestrant), gonadotropin-releasing hormone (GnRH) antagonists, aromatase inhibi-tors (e.g. anastrazole), anti-progestins and anti-androgens [52]. In a clinical context, endocrine therapy using tamoxifen is regularly given to patients with early-stages of

BC. The third-generation aromatase inhibitors (AIs) anastrozole, exemestane and let-rozole have also emerged as novel important agents for use in adjuvant endocrine therapy and work by reducing the level of oestrogen through blocking an aromatase catalysed conversion of testosterone into oestradiol [53]. Fulvestrant has been identi-fied as an oestrogen receptor down-regulator which is normally used as a third line endocrine therapy in postmenopausal women with advanced disease [54]. Interesting-ly in the context of the current thesis the mechanism of action of fulvestrant has been reported to be associated with increased expression of IGFBP-5 in BC cells [55]. Cur-rently, adjuvant treatment of BC (usually following surgical and/or radio therapeutic intervention) largely depends on the molecular profile presented by the tumour cells – next section.

1.1.4 Molecular biology of breast cancer

General considerations

Cancers in general and BC in particular display six main properties important for the multistep progress of carcinogenesis. These distinctive and complementary properties act together to create an organizing principle for explaining the complexities of neoplastic disease. They comprise resisting cell death, evading growth suppressors, sustaining proliferative signalling, inducing angiogenesis, enabling replicative immortality, and activating invasion and metastasis [56]. Kim and colleagues also report that genomic stability and cancer-related inflammation are supplementary features associated with tumourigenesis see Fig 1.3. To understand the molecular mechanisms involved in BC it is necessary to have some appreciation of the concept of the cell cycle which controls replication of DNA and cell division [57]. These events can be divided into five dynamic phases starting with the Interphase. Interphase is an essential phase which comprises three distinct successive stages. G1 phase called "monitor", and S phase – DNA synthesis followed by G2, where cells continue to grow and prepare for mitosis. Cells subsequently move to a critical phase called mitosis (M) phase [58]. In mitosis phase, four different stages take place including; telophase, anaphase, metaphase and prophase. Quiescence (G0) is a biochemically distinct state which cells can re-enter the cell cycle and go on to DNA replication and mitosis. The transitions between these phases are regulated by changes in the activity of specific cyclin-dependent kinases (CDKs), with Cdk1/Cdk2 and Cdk2/Cdk4/Cdk6 controlling the transitions from G2 to mitosis and G1 to S phase, respectively see Fig 1.4 [58].
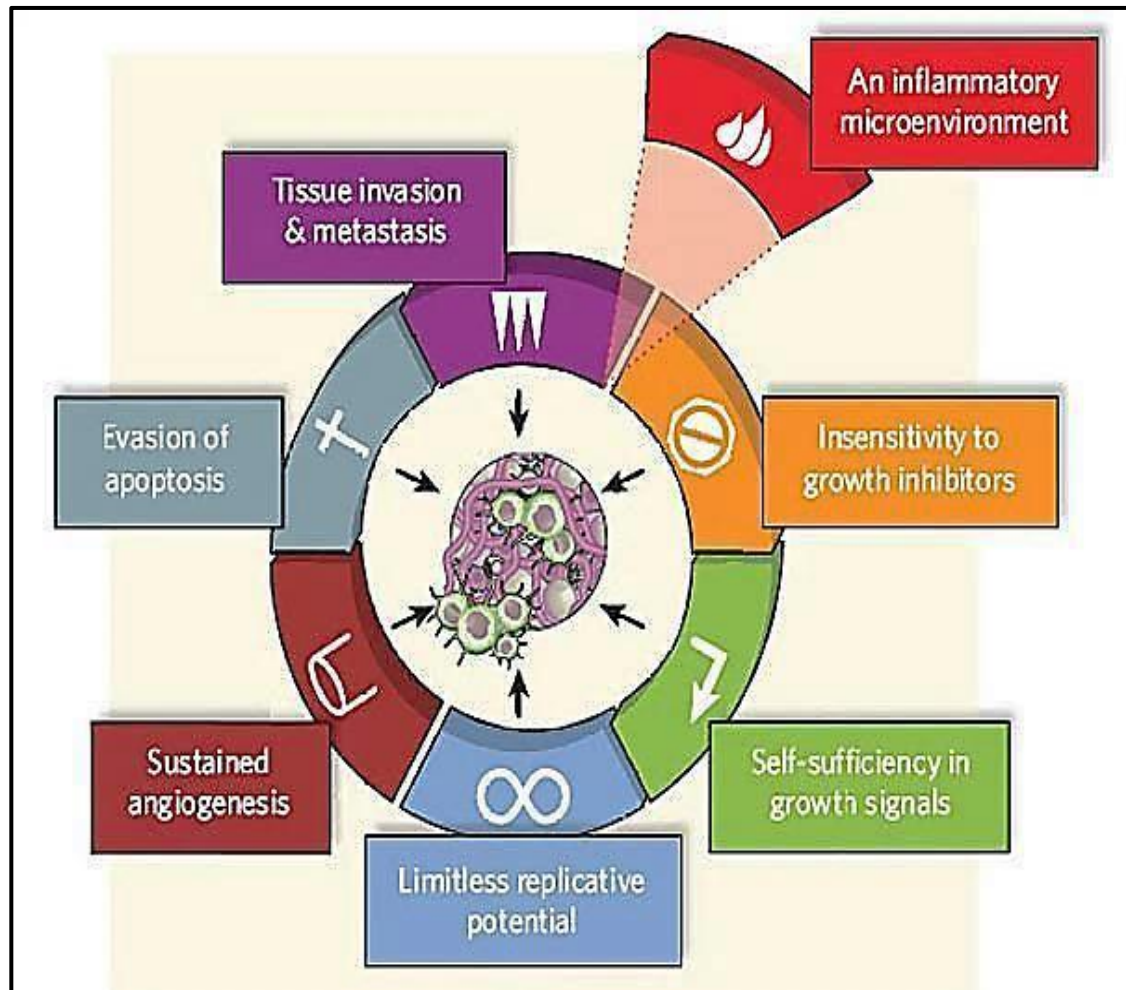
Figure 1-3 Characteristics of developing tumours.

Hallmarks include resistance to apoptosis, evasion of growth suppressors, sustained proliferative signalling, induction of angiogenesis, enabling replicative immortality, and activation of invasion and metastasis. Adapted from [59].
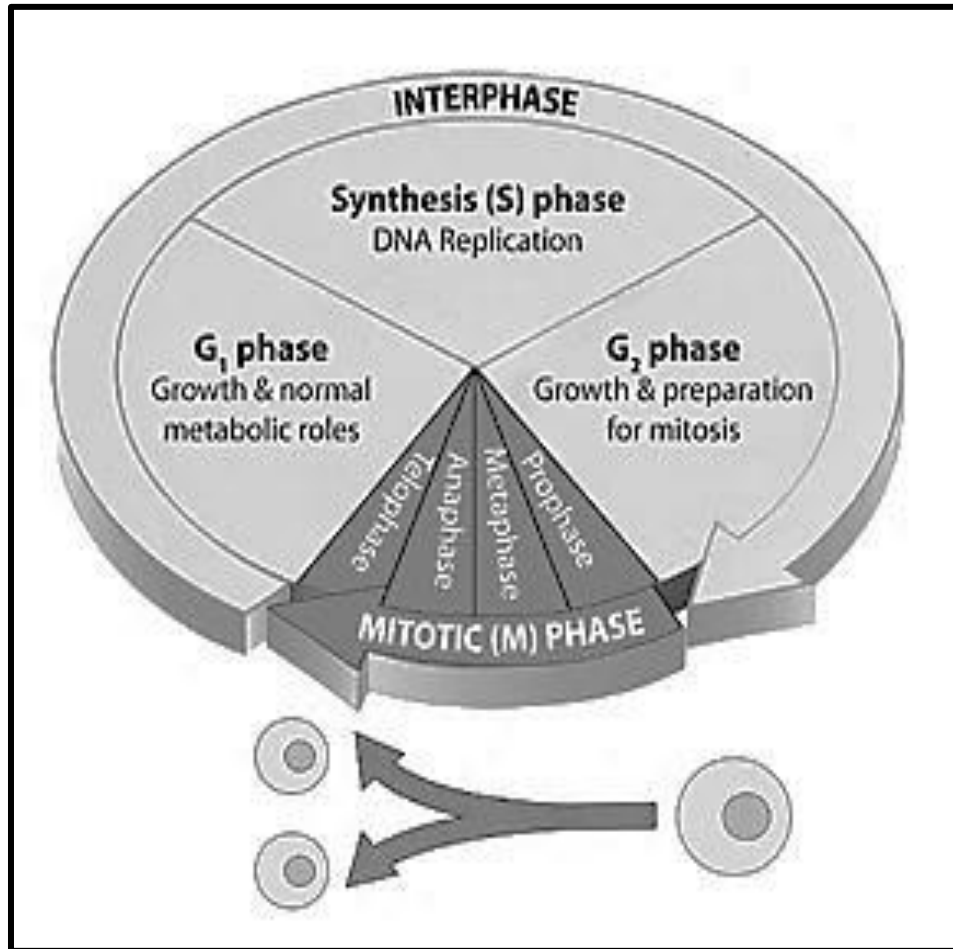
Figure 1-4 Representation of different phases of the cell cycle.

G1, S, G2 and M phases are depicted as described in text above. Adapted from

http://www.bing.com/images/search?q=Cell+Cycle+Diagram&Form=IQFRDR#view=detail&id=7534D1ECC0FD010AB643B5DB8AEC9BB260927FE1&selectedIndex=23

Breast epithelial cells receive signals from growth factors, steroid hormones, growth cytokines and cell matrix components which affect the activity of cyclins and CDK inhibitors, and thereby control the G_1/S transition [58]. Typically, the D-cyclins, comprising cyclin-D1, -D2 and –D3 are up regulated through initial stage of G_1 and trigger the transition from cell cycle arrest to S phase under the influence of agents such as epidermal growth factor (EGF), IGF-I and steroid hormones [57, 60]. Accordingly EGF and IGF-I play a potentially important role in development of BC. However BC is an extremely complex and heterogeneous disorder and disruption to the function of key proteins which regulate the processes of cell adhesion and cell cycle progression is often seen. [61]. In addition, cells undergo apoptosis as part of a normal regulatory process and often in tumour cells these mechanisms are subverted. For example, the tumour suppressor protein phosphate and tensin homologue on chromosome 10 (*PTEN*) is an important regulator of apoptosis and often within BC and other cancers mutations in this protein result in inhibition of apoptotic processes and uncontrolled cell division. Other important tumour suppressors include p53 and *BRCA1/2* which are involved in DNA repair. Mutation in these proteins can lead to disruption of cell cycle regulation with resulting malignancy and metastasis [39].

BC – subtyping

BC progression clearly depends on alteration of critical gene expression and a major recent breakthrough in BC research is the achievement of complementary DNA (cDNA) array analysis to evaluate the effect of a specific tumour signature on prognosis and this has opened a new window in molecular classification of breast malignancy. The development of microarray technology initially resulted in the identification of over 8,000 genes differentially expressed in different subtypes of BC [62]. In practical and clinical terms however immunohistochemical analysis has identified four different molecular subtypes of BC including normal breast, HER-2-positive, basal-like and luminal-like. The main subtype luminal has been further categorised into luminal A and luminal B [63] see Table 1.1 and Figure 1.5.

Table 1-1 Incidence of BC by basic molecular profile

No	Subtypes of BC	ER	PR	HER2	%
1	luminal A	+	+	-	44
2	luminal B	+	+	+	24
3	HER2	-	-	+	19
4	Basal-like	-	-	-	11
5	Normal-like				2
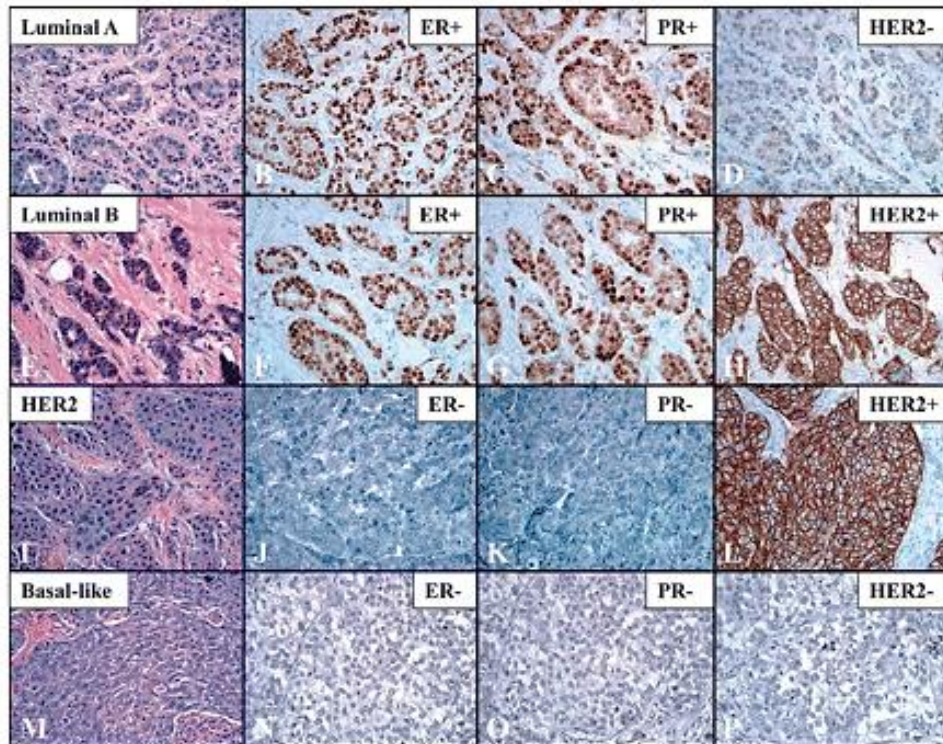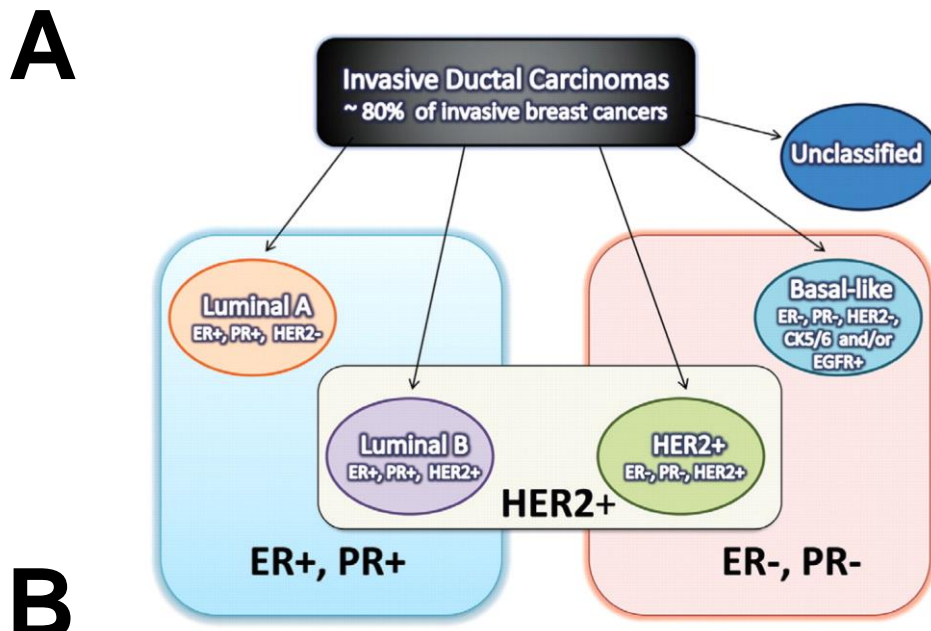
Figure 1-5 Histological classification of BC

A is a schematic showing different breast tumour subtypes. B shows typical histology of breast tumours based on differential immunohistochemical staining for ER, PR, and HER2. Adapted from [4]

[17]

Luminal A type BC is the most prevalent subtype followed by luminal B (Table 1.1). Both of these subtypes are ER+. Her-2 is expressed in around 40% of BC and is divided fairly equally between hormone receptor positive and negative subtypes. Basal-like BC tumours comprise a smaller (~10 %) group of tumours and due to their molecular profile are also termed triple negative (TN) BCs.

 BC prognosis is closely related to molecular subtype (see Fig 1.6). Generally ER+ subtypes which include luminal A and luminal B usually show a better prognosis with around 80%–85% 5-year survival rate. Luminal A neoplasms are associated with a significantly better prognosis than luminal B subtype. Prognosis of ER −ve subtypes is poorer with a 50%–60% 5-year survival. The better prognosis of ER+ cancers is largely due to the anti-oestrogen adjuvant strategies developed in the last few decades. Recently Her-2 +ve tumours have been targeted with the specific anti-receptor antibody trastuzumab (Herceptin). However the development of resistance to endocrine therapies (particularly tamoxifen) in ER+ BC and resistance to Herceptin in Her-2+ve tumours presents a major therapeutic challenge in the treatment and management of these BC subtypes.
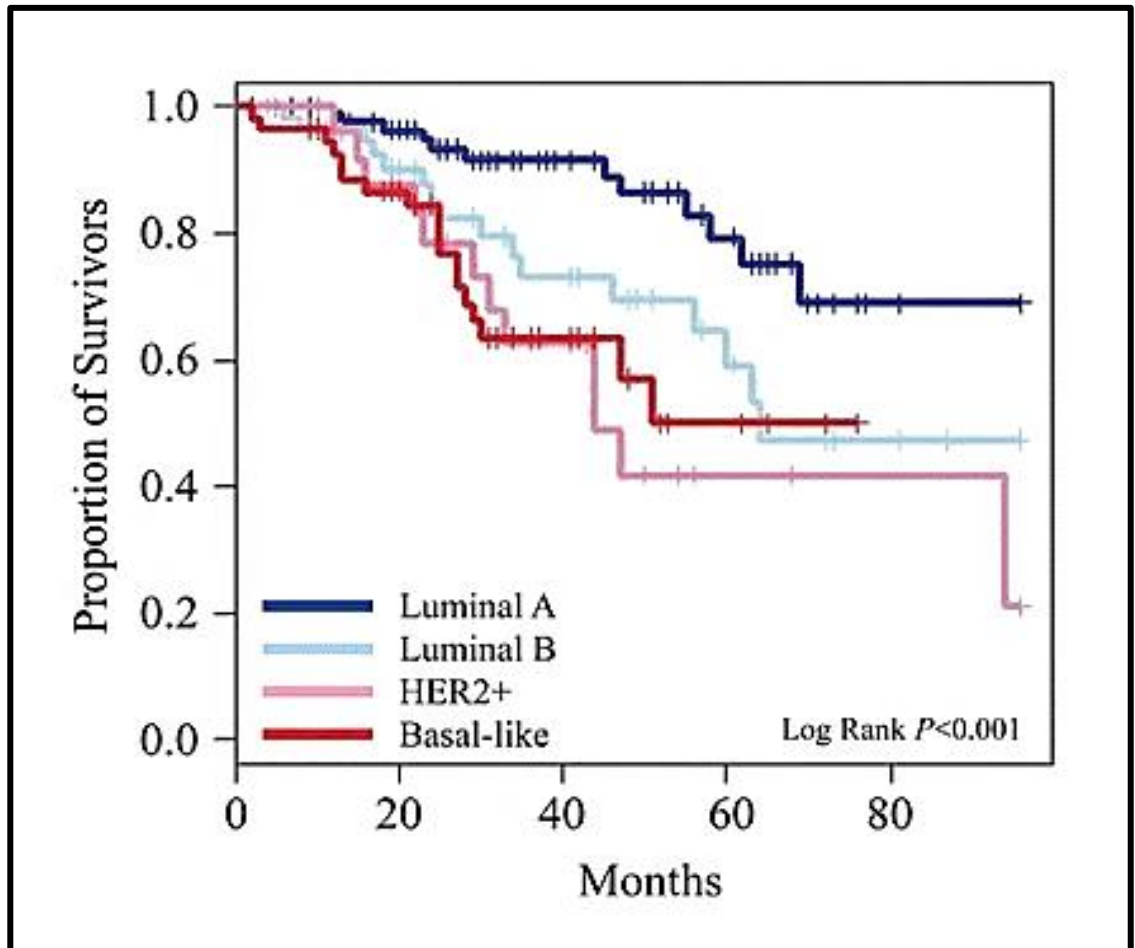
Figure 1-6 Overall survival rate (OSR) for BC by subtype classification.

Adapted from [4]

1.1.5 BC and tamoxifen resistance

The selective ER modulator (SERM) tamoxifen is a front line treatment for endocrine-responsive BC [64]. Tamoxifen displays mixed ER actions (agonist/antagonist) in a tissue specific manner although it acts to block 17β-estradiol activity as a growth promoter in E dependent breast tumours. Tamoxifen binds to ER in BC cells causing a conformational change in the receptor which results in an inactive trans acting complex at the promoter regions of E responsive genes. The other main route of endocrine therapy via aromatase inhibitors (AIs) depletes E from the tissues resulting in inactivated ERs. Around 50-60 % of ER+ BC patients initially respond to tamoxifen although resistance to the drug develops in a large cohort of these patients (70–80%) despite the continued expression of ERα [65, 66]. Many mechanisms have been postulated to account for continued cell division in the face of tamoxifen adjuvant therapy including alterations in tamoxifen metabolism [67], altered signal transduction pathways [68], modulation of ER activity [69] and alterations in binding co-activator and co-repressor molecules at the anti-oestrogen-ER complex [70].

A large body of evidence also indicates that the activation of alternative growth factor pathways may play a role in the development of tamoxifen resistance [71, 72]. The IGF axis is a prime candidate for such investigations as this molecular axis plays an important part in the development and differentiation of normal breast tissue and there is accumulating evidence that the IGF axis interacts with the E axis to regulate many aspects of mammary epithelial cell physiology including mitogenesis, apoptosis, adhesion, migration and differentiation [73-75]. IGFs have been well characterised as amongst the most potent mitogens for breast epithelial cells – reviewed in [76]. However so far the results of anti-IGF based strategies in clinical trials has been disappointing [66] . This may be due to the fact that the IGF axis is a multi-component molecular system consisting of growth factors (IGF-1 and –2), receptors (IGF-1R and

IGF-2R and insulin receptor (IR)), soluble growth factor binding proteins (IGFBP1-6) and several families of IGFBP proteases. IGFBPs modulate the activity of IGFs by various methods and also display growth factor independent activity. Given the potential role of IGF axis in the acquisition of tamoxifen resistance in breast cancer cell physiology it is important to investigate this molecular family especially with respect to expression and function of these genes in normal and cancerous breast tissue. As such studies form the basis of the experimental work presented in this thesis the second part of the introduction focuses on the structural and functional aspects of the components of the IGF axis with an introduction to their role in normal mammary gland function and in BC. We conclude the introduction with a short review of the limited literature related to the IGF axis and development of tamoxifen resistance before outlining the aims and objectives of our experimental work.

1.2 IGF Axis

1.2.1 Overview

The IGF axis is phylogenetically highly conserved and comprises 10 well-characterised gene products together with a few related accessory proteins. The axis plays a role in normal mammary gland biology and in some circumstances may be involved in the aetiology and metastasis of BC. Therefore a thorough understanding of the structure and function of the proteins which comprise this axis is essential both in the context of normal and abnormal mammary gland function.

1.2.2 IGF-I and IGF-2

IGF-I and -2 are small (~7.5kDa) polypeptides which show a structural and functional homology to insulin [77]. IGF-1 is mainly secreted by the liver and serum concentrations increase throughout postnatal development. [78]. Although liver is the major source of IGF-1 studies have shown that several tissues express IGF-I [79]. Expression and secretion of IGF-I and IGF-2 occurs in the adipocytes and stromal fibroblasts (but not epithelial cells) of the mammary gland and IGFs act as important regulators of cell physiology in this tissue [80]. IGF-I is regulated positively by pituitary growth hormone (GH) [81] while the mechanisms of IGF-2 regulation remain unclear [82]. IGF-I and IGF-2 share about 40% sequence and structural homology with insulin and both IGFs have been highly conserved through vertebrate evolution [83]. In humans the IGF-I gene is present as a single copy situated on chromosome 12q23.2 containing six exons [84]. Alternative splicing at exons 1, 2, 5 and 6 leads to four potential mRNA precursors [85]. IGF-I is synthesized as a larger molecule (propeptide) comprising a signal peptide and a carboxyl terminal extension peptide (E peptide). The E peptide and signal peptide are removed during processing of the mature IGF-1 [86]. Mature IGF-I is a 70 amino acid single peptide chain with three intra-molecular disulfide

bonds and differs from insulin by the presence of a carboxyl terminal D domain [87]. IGF-2 shares a similar structure and interestingly has been identified as an imprinted gene, expressed only from the paternal allele. IGF2 is also involved in development and growth although it may play a more important role in foetal than in adult life. In humans it is also a single copy gene located on chromosome 11p15.5. Nine alternative transcripts have been described although these are not represented at the protein level. IGF peptides contain four domains B-C-A-D [88]. The A and B domains of IGF-I and -2 show 50% homology to the A and B chains of insulin [89]. Figure 1.7 shows a representation of IGF-1, -2 and insulin highlighting the structural homology between these polypeptides.
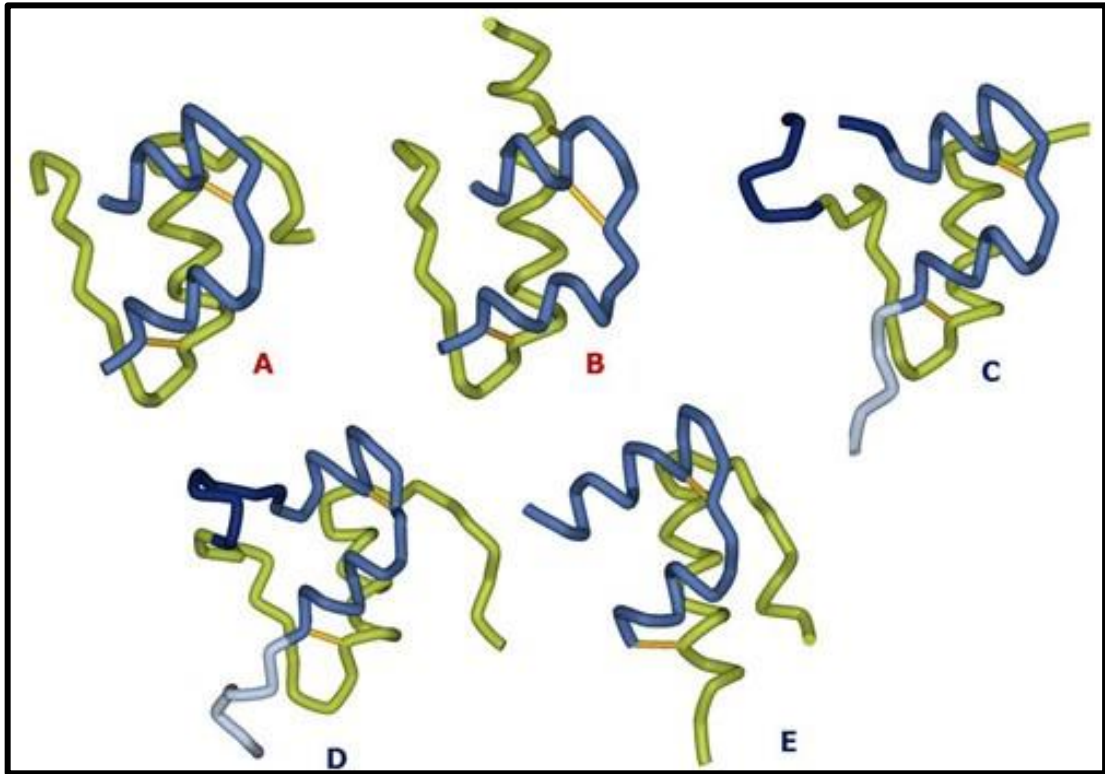
Figure 1-7 Structural homology in the insulin/IGF peptide family.

Insulin (T conformation). B. Insulin (R conformation) C. IGF-I. D. IGF-II E. Relaxin. For IGF-1 starting from the N-terminus, the B-domain (residues 1-29) is shown in green, the C-domain (residues 30-41) is shown in dark blue, A-domain(residues 42-62) is shown in blue, and the D-domain (residues 63-70) is shown in light blue. The disulphide bridges are shown in yellow. Note that in this Figure part of the C-domain of IGF-1 is unresolved. Adapted from http://www.fefchemicals.com/biopharm/scientific-information/articles/the-insulin-peptide-family/.

1.2.3 IGF Receptors

IGF-IR is a heterotetrameric tyrosine kinase receptor, containing two extracellular α subunits (~135 kDa) and two membrane-spanning β subunits (95 kDa each) [90]. IGF-IR is synthesized as a precursor which is glycosylated on the extracellular regions, dimerized and proteolytically processed to yield separate α and β chains [91]. The IGF-IR also shows a high degree of homology with the insulin receptor (IR) and hybrid IGF-IR/insulin receptor species have been reported for both the A and B isoforms of the insulin receptor (IR-A / IR-B) comprising an IGF-IR αβ dimer combined with an αβ dimer of either the IR-A or IR-B isoform. IR isoforms are derived from alternative splicing on exon 11 of the IR gene [92]. Although the physiological significance of their expression remains unknown such hybrid receptors have been reported in BC cells [93]. In term of gene structure, IGF-IR is a large gene comprising 21 exons and spanning nearly 100 kb [94]. This gene has been localized to chromosome 15q26.1 [95]. The 270 kDa IGF-2R gene has no structural homology to either IGF-IR or IR and is a single chain transmembrane protein identical to the cation-independent mannose-6-phosphate receptor. Killian and Jirtle (1999) revealed that the IGF-2R consists of 48 exons and spans nearly 136 kb [96], located at chromosome 6q25-q27 [97], In contrast to IGF-2 the IGF-2R gene is maternally imprinted. Figure 1.8 shows the structure of the IGF-IR and IR. The partial structural homology amongst the ligands and receptors in the IGF and insulin molecular family leads to a degree of promiscuity in binding between ligand and receptor and also results in a partial functional redundancy between the IGF-I, IGF-2 and insulin ligands [98].
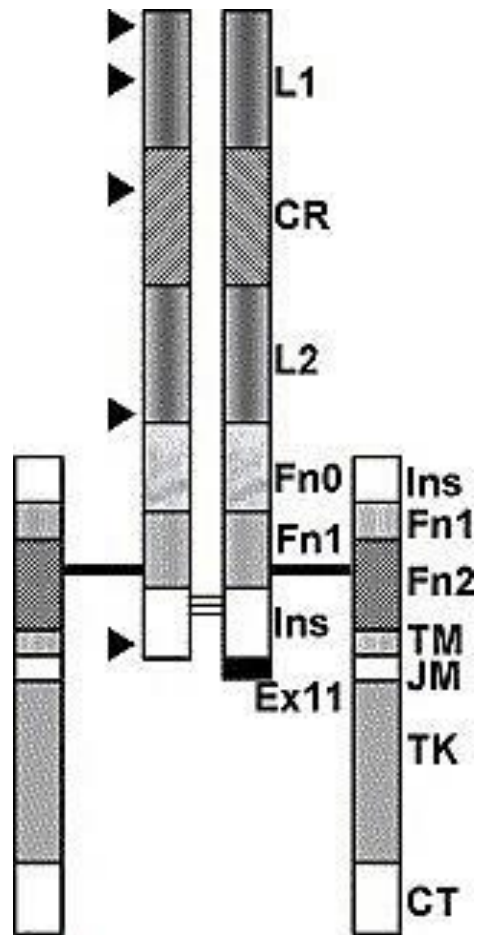
Figure 1-8 Domain structure of the IGF-IR/IR.

The schematic diagram represents the heterotetrameric IGF-IR and IR. L1 and L2 represent the large domains 1/2 with leucine-rich repeats. CR is the Cys-rich domain and Fn 0, 1 and 2represent fibronectin type III domains. Ins (insert) domain and Ex11 represent the residues encoded by exon11 of the IR. TM (transmembrane), JM (juxtamembrane), TK (tyrosine kinase domain) and CT(C-terminal domains). The left arrows represent the ligand binding regions. Adapted from [32].
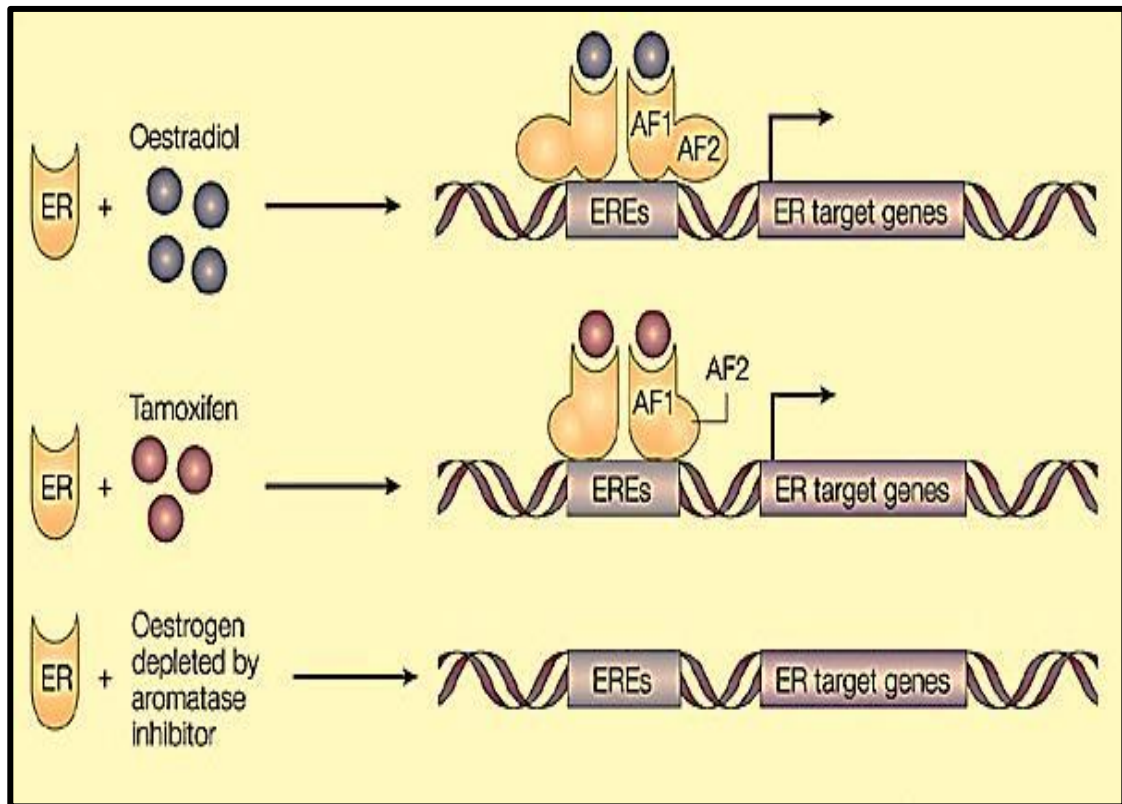
Figure 1-9 Mechanisms of action for anti-oestrogenic therapeutics.

Oestradiol bound to oestrogen receptors (ER) leads to conformational alteration and activation of the receptor through AF1 and AF-2 domains. Tamoxifen interaction with ER leads to an altered receptor confirmation such that AF domain function is inhibited. Aromatase inhibitors (AI) reduce oestradiol concentrations and reduce the abundance of hormone liganded ERs thus decreasing the hormone stimulated mitogenic drive for cell division. Adapted from [99].

1.2.4 IGFBPs

General

The six well-characterised IGFBPs (IGFBP1-6) share many structural and functional features including the ability to bind IGF-I and IGF-2 with high affinities (K_D 0.2 – 0.5 nM). Indeed the affinity of IGFs for IGFBPs exceeds that for cell surface IGF receptors and means that IGFBPs are important regulators of growth factor access to receptors. IGFBPs contain three highly conserved structural domains N-terminal, linker and C-terminal (Fig1.10) and are extensively disulphide bonded proteins. Structural information on IGFBPs is limited as no full length protein has yet been crystallised. Although IGFBPs bind IGF with high affinity and thereby sequester growth factor from cognate receptors they also provide a "reservoir" of IGF in tissues. Furthermore IGFBPs are subject to proteolysis which has the effect of releasing bound IGFs in tissue compartments to allow access of growth factors to receptors. IGFBPs also have extensive IGF independent effects which must be borne in mind for a full appreciation of the spectrum of their biological effects. All these features of the molecular physiology of the IGF axis are represented in the mammary gland and IGFBPs in particular have been shown to regulate growth, motility, apoptosis, adhesion and migration of both normal an malignant mammary epithelial cells [100] and see below.
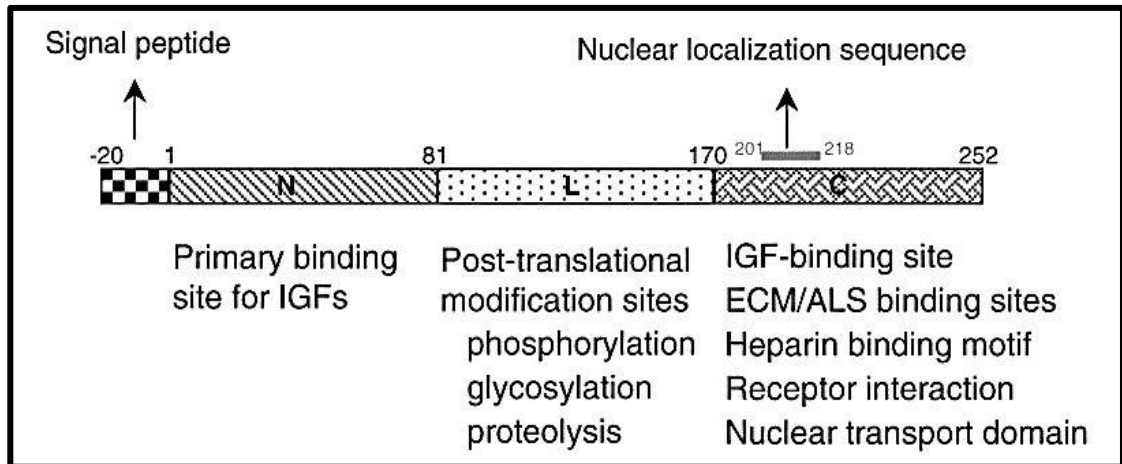
Figure 1-10 Schematic and generic structure for IGFBP family.

IGFBPs comprise N-terminal (N), linker (L) and C-terminal (C) domains. The primary IGF binding site lies in the N-terminal domain although there are also contributions from residues in the C-terminal domain. The C-terminal domain also contains binding sites for extracellular matrix (ECM) and acid labile subunit (ALS) together with a nuclear localisation signal (NLS) for some IGFBPs and structural motifs which may be important for some IGF independent actions of IGFBPs (receptor interaction). The L domain is the main site for post-translational modification of IGFBPs including phosphorylation, glycosylation and proteolysis. Adapted from [100].

1.2.4.1 Insulin-like growth factor binding protein-1

Insulin-like growth factor binding protein-1 (IGFBP-1) is expressed by several tissues including decidua, liver, kidney and endometrium. It is present as an extremely abundant 25 kDa protein in amniotic fluid. [101, 102]. It circulates in both phosphorylated and non-phosphorylated forms with the former displaying an approximately 5 fold higher affinity for IGF-I than the non-phosphorylated form [103]. Although the physiological significance of this remains unknown phospho-IGFBP-1 inhibits IGF-2 activity in fibroblasts and cultured vascular smooth muscle cells whereas dephosphorylated IGFBP-1 increases the activities of IGF-I in some cell types. IGFBP-1 and -2 have an Arg-Gly-Asp (RGD) sequence within their C-terminal domains and IGFBP-1 binds in an RGD-dependent manner to α5β1-integrins expressed in CHO cells. [104]. This ability of IGFBP-1 to simultaneously bind the α5β1 fibronectin receptor and present IGF-I at wound healing sites is believed to confer enhanced tissue repair properties of this IGFBP [105].

1.2.4.2 Insulin-like growth factor binding protein-2

The IGFBP-2 gene is located in region 2q33-q34 and consists of 4 exons containing three introns of lengths 27.0, 1.0, and 1.9 kb [106]. IGFBP-2 has a 2-fold lower affinity for IGF-I than IGF-2 [107]. IGFBP-2 is found in human, bovine, porcine and rat milk and serum, with particularly high concentrations in cerebrospinal fluid [108]. Interestingly, IGFBP-2 and -5 are located close together in a tail to tail orientation on chromosome 2 in humans suggesting that these two IGFBPs arose through an ancestral gene duplication event [109]. IGFBP-2 has been shown to mainly inhibit the action of IGFs. For example, Hoflich et al (1998) reported inhibition of human embryonic kidney fibroblasts proliferation when IGFBP-2 was over expressed [110]. This inhibitory ac-

tivity of IGFBP-2, as for other IGFBPs is subject to regulation by the action of IGFBP proteolytic enzymes [107].

1.2.4.3 Insulin-like growth factor binding protein-3

IGFBP-3 is the most abundant IGFBP in biological fluids binding over 90% of the circulating IGF-I in a 150-kDa ternary complex with an 80kDa acid-labile subunit (ALS). IGFBP-3 possesses both growth-inhibitory and -potentiating effects on cells that are independent of IGF action. IGFBP-3 binds the transforming growth factor-beta V receptor (TGF-βVR) on mammary epithelial cells (MECs) providing a potential mechanism for IGF-independent activities of IGFBP-3 [111]. Silha et al have suggested that IGFBP-3 may act as a tumour suppressor by binding IGF-I and IGF-2 to prevent the interaction of growth factor with the IGF-IR thereby decreasing mitogenic signalling through this receptor [112]. As a consequence, the cell cycle is interrupted with subsequent up-regulation of apoptotic signals [113]. IGFBP-3 has also been reported to inhibit the actions of IGF in other cell types [114].

1.2.4.4 Insulin-like growth factor binding protein-4

IGFBP-4 encodes a 258-amino acid polypeptide which has been mapped to chromosome 17q12-q21, in the same area as *BRCA1* [115]. IGFBP-4 contains 4 exons spanning about 15 kb of genomic DNA. In 1990, Shimasaki and co-workers identified IGFBP-4 as an inhibitor of IGF-induced bone cell proliferation [116] acting to inhibit IGF-1 binding to IGF-1R in muscle and bone [117]. IGFBP-4 activity is regulated by pregnancy-associated plasma protein-A (PAPP-A), a placental protease that is also secreted by fibroblasts, osteoblasts and granulosa cells [114]. Cleavage of IGFPB-4 releases IGF-I allowing interaction with cognate cell surface receptors.

1.2.4.5 Insulin-like growth factor binding protein-5

IGFBP-5 is a 35-kDa protein located on chromosome 2 in humans closely associated with IGFBP-2 [118]. Binding of IGFBP-5 to glycosoaminoglycans (GAGs) decreases its affinity to IGF-I by 17-fold whereas simultaneously inhibiting proteolysis of IGFBP-5 [119]. IGFBP-5 has been shown to stimulate the proliferation of osteoblast cells [120, 121]. It has also been reported to bind several other targets including vitronectin [122], thrombospondin, osteopontin [123], hydroxyapatite [124], plasminogen activator inhibitor-I (PAI-I) [125] and importin *β* [126] as well as the GAGs heparin, heparin sulphate and dermatan sulphate [119]. The production of IGFBP-5 by mammary epithelial cells has been reported to be increased dramatically throughout involution of the mammary gland [127] and transgenic mice expressing IGFBP-5 in the mammary gland show premature involution of the gland with increased MEC apoptosis [128]. Accordingly histological analysis showed reduction in alveolar end buds and decreased ductal branching. As well as compromising mammary development, over-expression of IGFBP-5 increased abundance of pro-apoptotic signalling components, increased plasmin generation and decreased expression of pro-survival molecules of the Bcl-2 family. IGFBP-5 has also been identified as a pro-apoptotic protein in other

tissues [129] and in the context of BC IGFBP5 shows pleiotropic effects inducing cell adhesion, increasing cell survival but inhibiting migration of MCF-7 human breast cancer cells [130]. Recently, Vijayan et al confirmed that IGFBP-5 enhances epithelial cell adhesion and also protected epithelial cells from TGFβ1-induced mesenchymal invasion suggesting that IGFBP-5 may act as a tumour suppressor [131]. Interestingly, Ahn et al 2010 reported that IGFBP-5 acts as a modulator of tamoxifen resistance in BC [132] and IGFBP5 expression correlates with increased survival.

1.2.4.6 Insulin-like growth factor binding protein-6

IGFBP-6 is a 23 kDa protein which located on chromosome 12q13.13 [133]. IGFBP-6 is secreted by human keratinocytes and acts as a growth inhibitor in these cells. IGFBP-6 also attenuates IGF induced cell migration, proliferation, adhesion and colony formation [134]. Uniquely amongst the IGFBPs IGFBP-6 has much higher affinity for IGF-2 than IGF-I (20-100 folds). IGFBP-6 is found in high concentrations in cerebrospinal fluid [135].

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1.2.4.7 Insulin-like growth factor binding protein -related proteins

Mac 25 is a tumour suppressor with 20-25 % homology to IGFBPs and is now termed IGFBP-7 [136]. IGFBP-7 is detectable in human breast, prostate, lung and colon cancer cell lines. It was reported that mac25 down-regulation was associated with BC progression [137]. Fig 1.11 below shows a cartoon representation of IGF axis components.
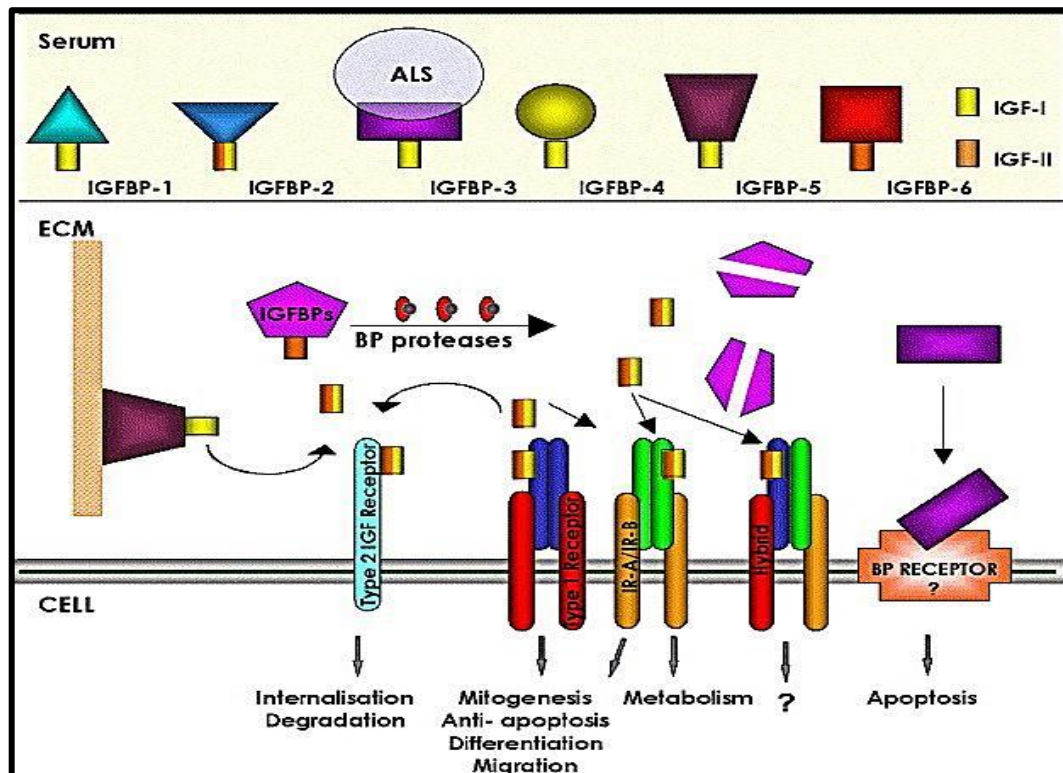
Figure 1-11 Diagrammatic representation of the IGF axis.

IGF-1 and -2 bind with high affinity to IGFBP1-6. IGFBP-6 binds IGF-2 with higher affinity than IGF-1. Affinities of the other IGFBPs for IGF-1 and -2 are similar. IGFBP-3 (and -5) is complexed with acid labile subunit (ALS) in biological fluids. IGFBPs subject to proteolysis to cause the release of growth factors. IGF-1 and -2 interact with varying affinities with IGF-1R, IGF-2R, IR-A, IR-B and hybrid IGF-1R/IR. Most IGFBPs (shown is IGFBP-5) interact with ECM molecules and there is evidence that specific IGFBPs (shown is IGFBP-3) display IGF-independent effects through interaction with specific cell surface receptors. Adapted from [138].

1.2.5 IGF Axis and BC

The IGF axis has long been known to play a role in tumourigenesis. In 1993, Sell et al. reported mouse embryonic fibroblasts deficient in IGF-IR could not be transformed by the SV40 large T antigen [139]. Subsequently Baserga et al. confirmed that IGF-IR expression was required for transformation by Ras activating oncogenes [140] and many studies now suggest a role for the IGF axis in malignant transformation and metastases [141]. BC cell proliferation is stimulated via insulin and IGF-I [142] and blocking the IGF-IR inhibits growth of breast tumour cells [143]. IGF-I also inhibits p53 induced apoptosis and stimulates angiogenesis through the induction of hypoxia-inducible factor 1α (HIF1α) and, vascular endothelial growth factor (VEGF) expression [144]. Major et al. (2010) highlighted that high serum IGF-I levels are linked to a higher risk of certain cancers although this is a controversial area with some conflicting reports [145]. Importantly in the context of BC, IGF-I increases the transcriptional activity of ERα, in addition to enhancing expression of oestrogen-inducible genes like the progesterone receptor [146]. Recent evidence also suggests that IGF-I up-regulates G protein-coupled ER (GPER) expression in BC cells [147]. This novel membrane-bound ER mediates rapid non-genomic effects of E$_2$ [148] independently of ERα and ERβ and may play an important role in the proliferative action of ovarian, endometrial and BC cells [149]. Interestingly, in BC cells, GPR30 may act via cross-talk with the epidermal growth factor receptor (EGFR) [150] and consequently provide a route of escape from anti-oestrogen adjuvant therapies [151].

The IGF axis has also been implicated in the maintenance of cancer stem cells (CSCs), and Kim et al (2002) indicated that IGF-I promoted epithelial- mesenchymal transition (EMT) via transforming growth factor (TGF)-β [152]. This occurs via up regulation of matrix metalloproteinase (MMP), which converts inactive TGF-β into its active form [153]. By degrading the extracellular matrix (ECM), MMPs also enhance

cell differentiation, motility, and invasion. Furthermore, the IGF axis plays a vital role in the activation of the transcription factors (TFs) involved in EMT including Snail [154] and zinc-finger enhancer binding (ZEB) 1 [155]. Finally activation of the IGF axis can save tumour cells from immune response-mediated attack and improve the survival of malignant cells [156]. Fig 1.12 highlights some of the mechanisms by which IGFs may act to promote tumourigenesis.
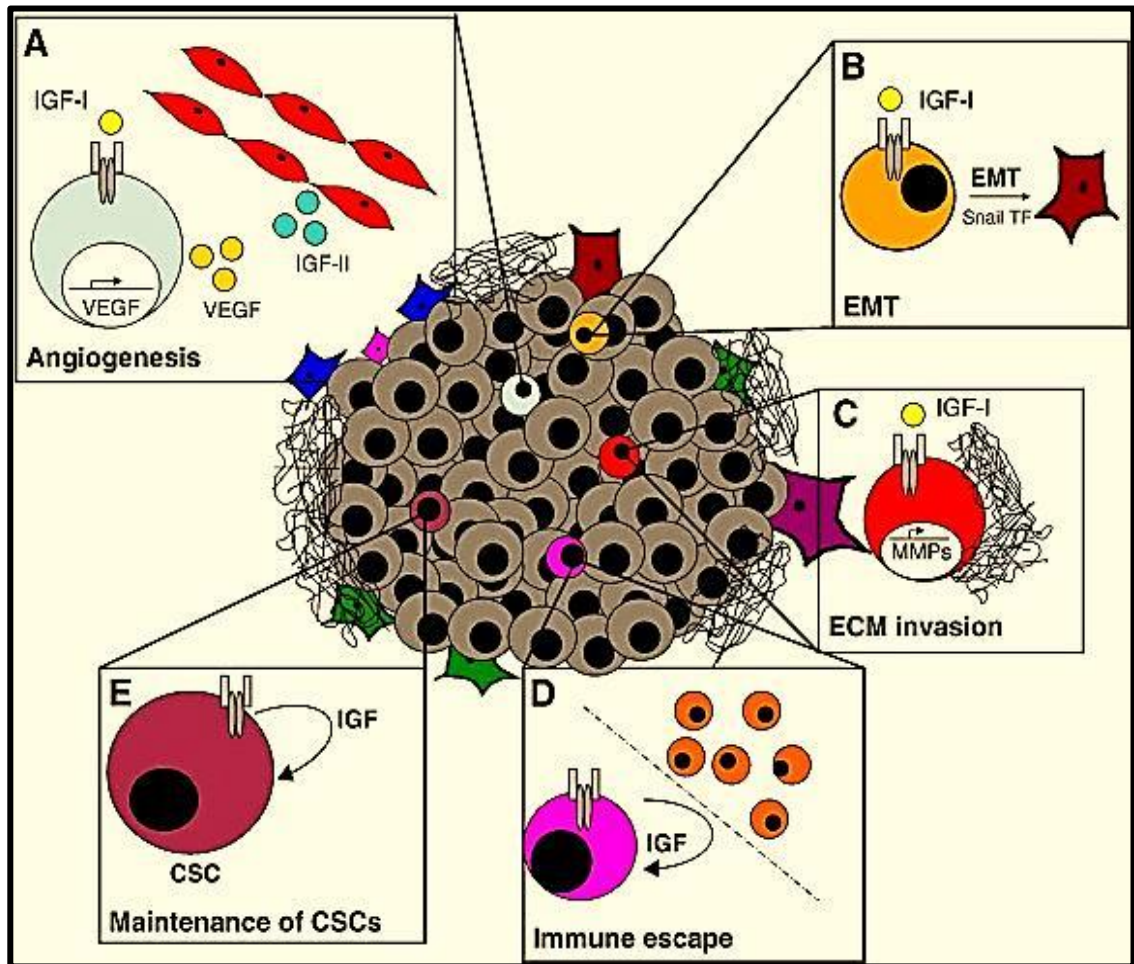
Figure 1-12 Aspects of IGF axis involvement in malignancy

The diagram shows the different routes through which the IGF axis may act to promote tumourigenesis. (A) Represents the activation of the expression VEGF by IGF-I, which thereby initiates angiogenesis. Also, IGF-2 serves as a chemo-attractant to draw the endothelial progenitor cells to ischemic sites. (B) Illustrates the induction of EMT (C) Demonstrates the activation of the expression of MMPs to promote cell motility and invasion by the degradation of ECM. (D) Shows the IGF axis can protect tumor cells from immune response-mediated attack and increase the survival of tumor cells by providing protection from apoptosis. (E) Indicates the IGF axis contribution to the maintenance of cancer stem cells (CSCs). Adapted from [156].

Following on from the signalling studies reported in 1.2.5, Ahmad et al (1999) reported high expression of AKT in various human breast carcinoma cell lines and confirmed that in the ER+ MCF-7 BC cell line AKT is modulated both by IGF-I and E_2[157]. Independent activation of AKT leads to E_2 and IGF-I independent proliferation of BC cells and activates several anti-apoptotic pathways. Baron et al highlighted that focal adhesion kinase (FAK) - a downstream signalling protein associated with integrin activation, is also a substrate for the IGF1R and IR [158]. IGFs also act as chemo attractants for BC cells possibly acting through this integrin – FAK mediated pathway [159] although others have shown that activation of the integrin signalling pathway inhibits the mitogenic action of IGF-I in BC cell lines [160]. In addition to FAK activation, IGF1R activation also regulates the transient tyrosine phosphorylation of other components of the focal adhesion complex including p130 Crk and paxillin in MCF-7 cells [161] which may be important for BC cell migration. In confirmation of this expression dominant-negative IGF1R inhibits metastasis and invasion of MDA-435 breast cancer cells *in vitro* and *in vivo* [162]. Clearly therefore the IGF axis may plays an important role in some aspects of BC metastasis.

1.2.6 IGFBPs

An extensive literature suggests that IGFBPs display many effects in different cancerous cells. On occasion these reports have been contradictory and this is an area of intense ongoing research. In the first instance the effects of IGFBPs are related to the influence they exert on the function of the highly mitogenic growth factors IGF-I and -2 which they bind with high affinity. These may be summarised as follows i) They serve as transporters in plasma and other biological fluids to control the efflux of IGF-I and IGF-2 into the tissues, ii) they extend the half-life of IGF-I and IGF-2 and thereby influence their metabolic clearance; iii) provide a means of tissue and cell-type specific localization; iv) they directly modulate interaction between IGF-I and the IGF-IR, thereby indirectly controlling its biological activities [82]. In addition to these IGF-dependent effects, IGFBPs in many instances display IGF-independent effects. In addition IGFBPs may show both IGF-dependent and IGF-independent effects within the same tissue. A further layer of complexity is added by the fact that IGFBPs can exert enhancing or inhibiting effects on IGF action and that such effects may show a tissue dependent specificity [163-167]. IGFBPs are also substrates for IGFBP proteases which cause the release of bound IGFs in the vicinity of cell surface receptors. BC cell lines and primary cultures secrete such proteases [168, 169] and it is suggested that the activity of these enzymes may regulate access of growth factors to malignant breast epithelial cells [82]. IGFBPs also have differential effects on initiation of apoptosis which appear to be regulated by the nature of extracellular matrix and other constituents in the vicinity of cells. The IGF-dependent and independent effects of IGFBPs are regulated via a complex sequence of post-translational modifications (PTM), including glycosylation [170], phosphorylation [171] and proteolysis [172]. All of these aspects of the molecular physiology of IGFBP activity make interpretation of data obtained from experiments with cancer cells in culture (including BC) difficult.

However, with these caveats in mind there follows a short discussion on the activity of IGFBPs in cancer cells with special reference to BC.

IGFBP-1 inhibits IGF-I action in breast cancer cells and causes and decreases IGF-1 stimulated cell motility [173, 174]. IGFBP-2 is over expressed in a wide variety of human malignancies, including BC [175], prostate cancer [176], ovarian cancer [177], and lung cancer [178]. Over expression of IGFBP-2 has also been reported in BC cell lines [26]. IGFBP-2 binds to integrins on the BC cell surface in a RGD dependent manner and up-regulates genes involved in apoptosis (e.g. TGF-β1 and NFκB) which in turn inhibits cancer cell migration [102]. Interestingly, single nucleotide polymorphisms (SNPs) in the 3' region of the IGFBP-2 gene have been associated with ER+ breast tumours carrying BRCA2 mutations [179]. Conversely, IGFBP-2 inhibited the tumour suppressor gene on chromosome 10 (PTEN) expression in MCF-7 BC cells [26]. PTEN inhibits protein synthesis and cell cycle progression and regulates phosphatidylinositol-3-kinase (PI3K) signalling and thus modulates IGF signalling pathways [23]. Therefore, IGFBP-2 over expression in this instance promoted tumour growth by inhibiting PTEN activity and enhancing IGF signalling. Other studies have reported the inhibition of cancer cell proliferation, migration, and invasion by IGFBP-2. Pereira and co-workers reported that, IGFBP-2 inhibited IGF-mediated tumour growth *in vivo* and cell migration *in vitro* via an αvβ3 integrin mediated mechanism [102]. Again in contradistinction, other studies show that, IGFBP-2 acts as a growth promoter in cancer. High levels of IGFBP-2 in serum were reported in several metastatic tumours including ovarian, adrenocortical, prostate cancer, colorectal cancer and some leukaemias [180, 181]. IGFBP-2 was also reported to be over expressed in invasive breast carcinoma and *in situ* breast carcinoma relative to the normal mammary gland [175]. In addition IGFBP-2 expression is regulated by epigenetic events such as DNA methyla-

tion and histone acetylation within the promoter region of the gene adding a further level of complication to the activity of this protein [182].

IGFBP-3 binds to IGF-I and –II to prevent interaction with the IGF-IR, thereby decreasing their signalling capacity [112] and inhibits the growth of Hs578T human BC cell line. IGFBP-3 initiates apoptosis in prostate cancer cells [183] and enhances radiation induced apoptosis [184], and paclitaxel induced apoptosis in breast cancer epithelial cells [185]. In mechanistic studies, IGFBP-3 was shown to interact with the nuclear retinoid X receptor (RXR)-α and modify its binding to the transcription factor Nur77; this in turn activates the apoptosis cascade [179]. Moreover, p53 dependent up regulation of IGFBP-3 interrupts cell cycle progression and up regulates apoptotic signals interacts in a p53 independent manner with the apoptotic Bcl-2 family [113]. Study showed that IGFBP-3 interacts with the 78-kDa glucose regulated protein (GRP78), causing tamoxifen resistance in BC [186]. IGFBP-3 initiates the dephosphorylation of FAK, in spite of lacking an RGD sequence [187]. Recently, a cell surface IGFBP-3 receptor has been reported to mediate the apoptotic effects of IGFBP-3 through caspase-8 activation [188]. In a clinical setting, Deal et al. (2001) highlighted some evidence that the circulating level of IGFBP3 is inversely associated with the risk of many tumours, in addition some anti-proliferative agents such as anti-oestrogens may act by up-regulating IGFBP-3 expression [189].

Little data is available on the expression and activity of IGFBP-4 in BC although over-expression of IGFBP-4 reduces proliferation in the malignant M12 prostate epithelial cell line in response to IGFs [190].

IGFBP-5 is expressed and secreted by multiple ER+ BC cell lines including MCF7, T47D, ZR751 and BT474 [191]. This positive correlation between ER expression and IGFBP-5 was confirmed following mRNA and protein analysis of IGFBP-5 in 47 human breast tumours [192]. This study also concluded that normal breast tissue produced significantly less IGFBP-5 compared to adjacent primary tumour cells, suggesting that IGFBP-5 expression is associated with the proliferative state. In a functional context Perks and colleagues reported that IGFBP-5 reduced ceramide-induced cell death in the Hs578T cell line [193]. IGF-I was reported to increase IGFBP-5 secretion in T47D cells although this was not accompanied by increased IGFBP-5 mRNA expression, suggesting that IGF-I influences IGFBP-5 synthesis in a post-translational manner perhaps by binding and inhibiting proteolysis of IGFBP-5 [194]. Another study in T47D cells revealed that progestins, anti-oestrogens and other growth inhibitory hormones reduced IGFBP-5 mRNA expression and protein secretion in T47D cells, also suggesting a role for IGFBP-5 in tumour cell proliferation [195]. IGFBP-5 has been used as a potential prognostic factor in BC diagnosis. [196] and Hao et al reported that, IGFBP-5 is among the genes that were up regulated in metastatic lymph node spread of primary breast tumours [197]. A separate study from Li et al., also showed that increased IGFBP-5 mRNA expression is positively associated with axillary lymph node invasion in ER+ BC [198]. Similarly in a study of ER+/PR+ patients BC patients low serum IGFBP-5 levels correlated with increased disease-free survival (DFS) [199]. Despite conflicting reports regarding the role of IGFBP-5 in normal mammary gland development and involution, there is a clear relationship between IGFBP-5 expression and breast tumour development and progression. Understanding this relationship may reveal IGFBP-5 as a predictive factor in patient survival.

Very little data has been published on the role of IGFBP-6 in BC physiology or even in normal mammary gland function. IGFBP-6 has been reported at low levels in breast cancer tissues [200] and levels are reported to be elevated in Her-2+ tumours which are resistant to trastuzumab although a causal role for IGFBP-6 in resistance has not been established [201]. IGFBP-6 has been reported to stimulate the migration of rhabdomyosarcoma cells in an IGF-independent manner [202].

1.2.7 IGF axis and tamoxifen resistance

The acquisition of resistance to SERMs in ER+ BC presents a major therapeutic challenge in the treatment and management of this type of cancer. The molecular phenotypes associated with such escape from SERM control are the subject of intense study through such techniques as gene array and proteomic analyses. The design of such studies has been predicated on the recruitment of alternative mitogenic signalling pathways by cancer cells resulting in continued tumour cell growth and metastasis. *In vitro*, BC cell lines can acquire tamoxifen resistance through growth in the presence of drug. The MCF-7 cell line is most commonly used in this research and there is a small literature which describes perturbations in IGF axis expression and function in such cells following the acquisition of tamoxifen resistance. Increased IGF-IR expression in tamoxifen resistant cells may enhance E_2 independent growth of the cells and an early study in wild type (wt) and TamR cells suggested that this may indeed occur [203] . However subsequent literature in this area is contradictory with reports describing both the up regulation [204-206] and down regulation [207-209] of IGF-IR expression in TamR cells and the significance of altered IGF-IR activity in the development of TamR remains under investigation. However there is evidence that in TamR cells which have also developed resistance to the EGF-R inhibitor AG 1478 subsequent cell growth is largely dependent on activation of the IGF-IR [210]. This

may provide a mechanism of escape of BC cells from both anti-oestrogen and anti-EGF-R (Herceptin) based therapy.

Some early studies reported altered IGFBP profiles on the acquisition of tamoxifen resistance by the MCF-7 and ZR-75-1 cell lines. However the species of IGBP reported in these studies were not identified immunologically but only by molecular weight on ligand blot [211]. Subsequently the same group identified IGFBP-2 as down regulated in TamR MCF-7 cell lines [212] although dexamethasone – a known regulator of IGFBP expression in mammary epithelial cell lines [213] - was present in these cultures. Recently, IGFBP-5 has been reported to be down regulated at both mRNA and protein levels in TamR MCF-7 cells. This may have biological significance as this binding protein has been shown to inhibit growth of BC cells [163] and IGFBP-5 treatment of BC tumour cells in a xenograft mouse model has been shown to restore tamoxifen sensitivity [132]. The mechanism of action of the SERD fulvestrant may be associated with increased expression of IGFBP-5 in BC cells [55]. However fulvestrant treatment of MCF-7 cells also enhances ERα association with IGF-IR in a Src dependent fashion and is associated with ERα translocation to the plasma membrane, phosphorylation of IGF-IR and MAPK activation [75]. These findings are unexpected for a drug whose principle use is in the inhibition of BC cell mitogenesis by enhancing ERα degradation. The findings of this particular study are important enough to require independent confirmation. Figure 1.12 summarises the main role of the IGF involvement in cancer.

Currently many approaches aimed at inhibition of inappropriate signalling activity through the IGF-IR are under investigation. Tyrosine kinase inhibitors and monoclonal antibodies (Mab) are the two most common types of targeted therapies used in clinical trials in this area [29], principally small molecule inhibitors of IGF-IR tyrosine kinase

activity [31] and humanised blocking monoclonal antibodies to the IGF-IR [214] see Fig 1.13. However other strategies have also been adopted including the expression of IGF1R-inhibitory proteins [215] along with antisense oligonucleotides [216] and RNA interference strategies aimed at reducing expression of IGF-IR [217].
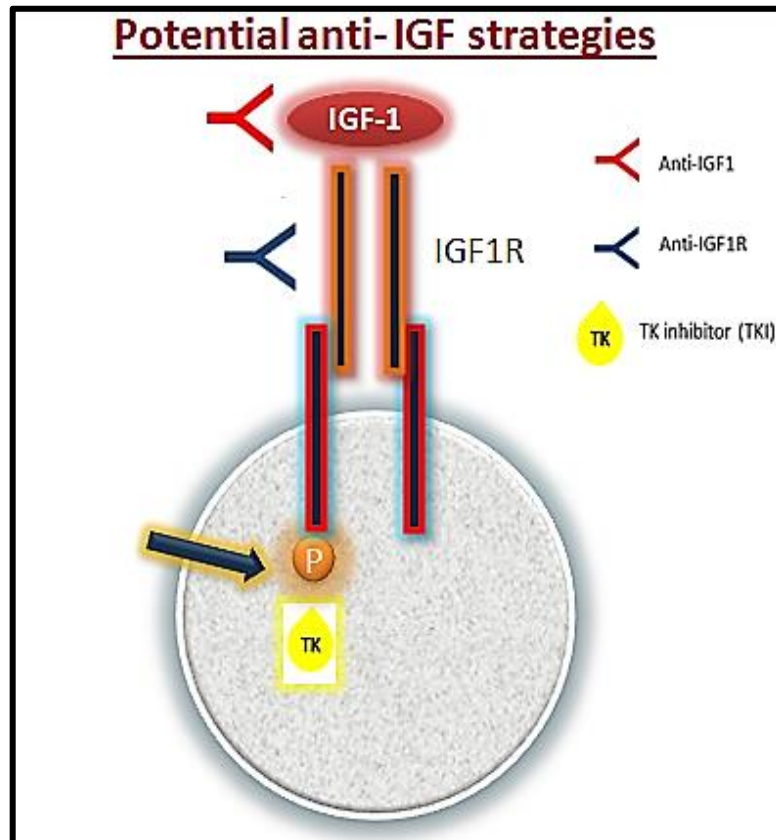
Figure 1-13 Schematic representation of anti-IGF directed therapies used in BC.

Shown are monoclonal antibodies (Mabs) directed to IGF-1 or IGF-1R and small molecule intracellular inhibitors of IGF-1R tyrosine kinase (TK) activity.

To date the most successful use of Mab in breast cancer has been the development of trastuzumab – a humanised Mab which blocks signalling through the HER2 receptor. Only about 25% of patients respond to trastuzumab reflecting essentially that cohort in whom the protein is present and active. In combination with more traditional chemotherapeutic strategies trastuzumab has been used to treat metastatic breast cancer patients. In a modified strategy the agent pertuzumab – an inhibitor of HER family dimerisation – is also used in combination with trastuzumab and with chemotherapy in some instances [218]. However it remains the case that anti-Her directed therapy is not appropriate in many ER+ BCs. Because of this anti-oestrogen directed treatment remains a cornerstone of therapeutic approach. Accordingly anti-IGF directed strategies have been applied either as mono-therapy or in combination with anti-oestrogen directed therapy (SERM, SERD or AI). Despite some initially encouraging data it is fair to say that the outcome of most clinical trials is not encouraging. The most recent of these is the reported lack of efficacy of the humanised anti-IGF-IR Mab in association with exemestane (AI) or fulvestrant (SERD) in increasing progression free survival compared to placebo with anti-oestrogen therapy [66]. Nevertheless, more effort along with some thoughts of potential explanations for the failure of such strategies could result in development of improved approaches [219]. For example In terms of the targeting the IGF-IR by Mabs, concern has been expressed that blockade of the IGF-1R may result in activation of the IR. This effect may occur secondarily to rises in pituitary growth hormone (GH) secretion because of only partial feedback by IGF-I at the pituitary level. Elevated GH levels will trigger an increase in serum insulin and subsequent activation of IR. Because blockade of the IGF-1R leads to a generalised hyperinsulinemia prophylactic treatment with metformin has been examined in an attempt to deal with this problem [220].

Furthermore BC cells typically present with a heterogeneous array of insulin/IGF receptors. As well as IGF-1R, IR-A and IR-B (the A and B isoforms of the insulin receptor) BC cells also express hybrid receptors containing the αβ subunits of IR-A or IR-B in association with the αβ subunits of IGF-IR. This variation in receptor presentation calls for a more sophisticated approach to the development of Mabs with appropriate blocking activities at individual receptors. If, as indicated by some studies above, the acquisition of tamoxifen resistance in MCF-7 cells is associated with decreased IGF-IR expression then the, lack of effect of anti-IGF1R antibodies may be explained by this phenomenon [221]. Such considerations are typically not so pressing in the development of small molecule inhibitors of receptor tyrosine kinase activity (TKIs) [222], and many such molecules are currently under study in clinical trials along with trials using a combination of Mab/TKI directed therapy. More recently development of specific inhibitors of the mTOR serine/threonine kinase in combination with anti-IGF directed therapies have shown encouraging preliminary data [223, 224].

Owing to some of the difficulties associated with the manipulation of IGF receptor and ligand activity discussed above, interest in the third main component of the IGF axis - the IGFBPs and their potential role in the progression of BC has received increased attention with a view to the investigation of this family of proteins as potential therapeutic targets in BC. It is this research area which is the subject of the current study.

1.3 Aim and Objectives of the Study

The overall aim of this report is to test this hypothesis – "**Acquisition of tamoxifen resistance by BC cells is associated with perturbations in the IGF axis and such perturbations play a causal role in the establishment of tamoxifen resistance**"

1.3.1 Objectives:

- examination of IGF axis gene expression in Tam-R vs. wt. MCF-7 cells using rt-PCR, protein blotting and ELISA methodologies

- use of gene knockout strategies to regulate the expression of candidate IGF axis genes in wt and TamR cells and subsequent examination of tamoxifen sensitivity in stably transfected cells

- To investigate whether candidate IGF axis genes regulate the phenotype of cell migration in wt and TamR cells

- to investigate the expression of candidate IGF axis genes in tamoxifen sensitive and tamoxifen resistant BC tissue microarrays

- to evaluate the prognostic and predictive value of candidate IGFBPs and their association with survival advantage in tamoxifen-resistant patients.

Chapter 2 Materials and Methods

2.1 Materials

Wild type (wt) MCF-7 and tamoxifen resistant MCF-7 (Tam-R) cells were generously provided by Prof Valerie Speirs (Leeds Institute of Cancer and Pathology) [225, 226]. RPMI-1640 media, RPMI-1640 phenol red free media (PRF), penicillin/streptomycin (Pen/Strep), 0.25% Trypsin-EDTA solution, foetal bovine serum (FBS) and dextran charcoal stripped (DCS)-FBS were from Invitrogen™, UK. Corning® 15 mL Centrifuge Tubes, T-75cm^2 TC Flasks, 6 and 96 well tissue culture plates were from Corning Life Sciences, UK. Other tissue culture plastic ware was from Scientific Laboratory Supplies Ltd, UK. Phosphate buffered saline (PBS) was from VWR-Lonza BioWhittaker®, UK. Trypan blue solution (0.4%) and dimethyl sulphoxide (DMSO) HYBRI-MAX$^®$ and β-mercaptoethanol were from Sigma, UK. The RNeasy® Mini Kit (cat no 74104) was from (Qiagen, UK). Ultrapure DNAse/RNase-free distilled water (cat no 10977035), DNase I (amplification Grade - cat 18068015) and RNase-Free Tubes (cat no AM 12400) were from InVitrogen UK. Optical Adhesive Seal (No.P3-0300) was purchased from Geneflow Ltd, UK. High Capacity RNA to cDNA kit (cat no 4387406), Taq-Man$^®$Gene and expression Master Mix (cat no 4369016) were purchased from Applied Biosystems (ABI). IGF axis and TaqMan probes were also obtained from ABI (the details of the TaqMan probes are summarised in table 8.1 in the Appendix 2). For enzyme linked immunosorbent assay (ELISA) IGFBP-2 DuoSet (cat no DY674) and IGFBP-5 DuoSet (cat no. DY875) were purchased from (R&D Systems, UK). The 96 well ELISA plates were purchased from Starlab, UK. Accessory reagents for ELISA 2N H_2SO_4 (cat no. DY994), Wash Buffer (cat no. WA126), Reagent Diluent (cat no. DY004), Streptavidin-HRP (cat no 890803), Substrate Solution Colour Reagent A (H_2O_2), Colour Reagent B (Tetramethylbenzidine) and normal goat serum (cat no

DY005) were also from (R&D Systems, UK). Absorbances were determined with a Thermo-Scientific Varioskan Flash type 300 spectrophotometer. Recombinant Human (rh) IGFBP2 cat no 674-B2-025,) rh IGFBP-5 (cat no 875-B5-025) and rh IGF-I (cat no 291-G1) were from (R&D Systems, UK). Absolute ethanol (200 Proof), Molecular Biology Grade, (cat no BP2818-500) was obtained from Scientific Laboratory Supplies Ltd, UK. Cell proliferation reagent WST-1 (cat no 11644807001) was from Roche, UK. The Cell-Lytic™ buffer (cat no. C2978), NP40 Cell lysis Buffer (cat no FNN0021), protease inhibitor tablets (cat no S8820), and phosphatase inhibitor cocktail 2 (cat no P5726) were from Sigma, (UK). BCA Protein Assay Kit (cat no PN23225) was from Pierce Biotechnology, UK). The Trans-Blot®Turbo Transfer System (Serial no 69 BR007547), Transfer pack mini format, 0.2 μm PVDF membranes (cat no 17-4156), 10x Tris-Buffered Saline (10x TBS) and Mini-protean®TGX Stain-Free™ Gels (cat no456-8083), Laemmli Sample Buffer (cat no 161-0737) and Precision Plus protein ™ Dual Colour Standards (cat no 161-0374) marker were from Bio-Rad, UK. Gel-Loading tips were from Sigma-Aldrich UK. Enhanced chemiluminescence (ECL) Dual-vue Western Blot markers (cat no FRPN810) were from Scientific Laboratory Supplies Ltd, UK. Restore™ Plus Western Blot stripping buffer (cat no 46430) was from Thermo-Scientific, UK. Bovine albumin Faction V (cat no 160069) was purchased from MP Biomedicals, UK. Tween® 20 (cat no C58H114O26) and Super-Signal® West Femto Maximum Sensitivity Substrates (cat no PN34095) were from Fisher Scientific Ltd, UK. Mouse monoclonal to IGFBP-2 (cat no MAB6741) and mouse monoclonal to IGFBP-5 (cat no MAB8751) were from R&D System, UK. Anti-mouse HRP secondary (ab97046) was from Abcam, UK. Polyvinylpyrroldone (cat no 9003-39-8) and Tergitol® Solution (cat no NP40S) were purchased from SGMA, UK. IGFBP-5 shRNA plasmid (cat no sc-39591-sh), IGFBP-5 shRNA plasmid control (cat no sc-108060), IGFBP-2 shRNA plasmid (cat no sc-37195-sh), IGFBP-2 shRNA plasmid control (cat

no sc-108060) puromycin (cat no CAY13884-25), shRNA plasmid transfection medium (cat no sc-108062) and transfection reagent (cat no sc-108061) were from Santa Cruz Biotechnology Inc., UK. Rabbit polyclonal anti-hIGFBP5 antibody (cat no ab 4255) and rabbit monoclonal anti-hIGFBP-2 antibody (cat no ab109284) were from Abcam, UK. Antibody diluent Reagent Solution (cat no 00321) and was from InVitrogen, UK. Antigen retrieval solution (cat no, MP-607-x500) was from A.MENARINI Diagnostics, UK. Novolink™ Max Polymer Detection System (Ref no RE7280-K), peroxidase enzyme (cat no RE7157), protein block (cat no RE7158), Novolink™polymer (cat noRE7161), Novolink™DAB Substrate Buffer (cat no RE7163), DAB Chromogen (cat no RE7162) and haematoxylin (cat no RE7164) were from Feica Biosystem, UK. Xylene and alcohol were from Scientific Laboratory Supplies Ltd, UK. Cover slip (cat no SAJ-2240-03A) was from Cell Path Ltd, UK. A Leica incubator Microscope was used for cell imaging. For the immunohistochemistry TMAs, the whole slides were scanned using the Scan scope T3 Scanner (Aperio Technologies, Leica, UK). For cell migration studies Image-Lock 96-well Plate (cat no 4379) were from Essen Bio Science, Ltd.UK and Incucyte instrumentation and software analysis packages were used (Essen Bio Science). Matrigel (Phenol Red Free cat no 734-0272 was from (VWR International, UK).

.

2.2 Methods

2.2.1 Tissue culture

For routine culture MCF-7 (Passage range between 23-36) and TamR cells Passage range between 2-8) were seeded in growth medium (GM) composed of RPMI-1640 media with phenol red supplemented with 5% (v/v) (FBS) 100U/ml penicillin, 100µg/ml streptomycin (PenStrep). Under experimental conditions Phenol Red free DCS medium was used to remove any oestrogenic stimulus provided by these additives. Tam-R cells were obtained through the continuous culture of MCF-7 cells (for 21 months) in phenol red free RPMI containing 5% DCS-FB and 100 nM 4-hydroxytamoxifen (4-HT) (SIGMA, UK). After 21 months, 4-HT was withdrawn from culture medium, and cells passaged in phenol red free RPMI (+5% DCS-FBS) as described previously [225, 226]. During routine tissue culture maintenance growth medium was changed every 3-5 days. At approximate by 70- 80% confluence, cells were washed with PBS and passaged (1:4) using 0.25 trypsin-EDTA. Cells were mixed with Trypan blue solution 0.4% (SIGMA, UK) in 1:1 v/v and counted using a haemocytometer. Cell numbers were recorded and cells were seeded at the appropriate densities as described in the relevant figure legends. In 1ml of freezing medium (10% DMSO, 40% DCS- and 50% RPMI1640) containing 1×10^6 cells/ml.

2.2.2 Real Time - Polymerase chain reaction

The RNeasy® Mini Kit (Qiagen, UK) was used to extract and purify mRNA exactly according to the manufacturer's protocol. Briefly, the buffer contains a high concentration of guanidine isothiocycanate, which supports the binding of RNA to the silica membrane (RLT) or RLT Lysis buffer was prepared by adding (1:100 v/v) 10µl of β-mercaptoethanol into 990 µl of RLT. 350 µl of the RLT buffer were added to cell pellets in 1.5 Eppendorf tubes. After vortexing for 1 min 350 µl of absolute ethanol were added. The mixture was transferred into the spin column and centrifuged at 10000g for 15 seconds. The flow-through was discarded and 700 µl of buffer RW1 contains a guanidine salt, as well as ethanol were added into the spin column and centrifuged at 10000g for 15s. The flow-through was again discarded and 500 µl of RPE wash buffer was added into the spin column and centrifuged at 10000g for 15s. This step was repeated with centrifugation for 2min. The spin columns were placed in new 1.5 eppendorf tubes and 30 µl of dH$_2$O were added and centrifuged at 10000g for one minute. The mRNA was collected and quantified using a NanoDrop™ 2000 Spectrophotometer. The A260/280 ratios along with mRNA concentration as ng/µl were recorded. This is to measure the purity of the RNA, however, there is a possibility of presence of DNA in RNA extracted which might have an impact on significance of the results. High Capacity RNA to cDNA kit (Applied Biosystems, UK) was used exactly according to the manufacturer's protocol to synthesise single stranded cDNA from mRNA. Briefly, 9µl of the RNA preparation (1µg RNA) was added to 10µl of 2x RT buffer followed by 1µl of 20x enzyme mix to formulate a 20µl reaction. The mixture was centrifuged and incubated in the PTC-100 Peltier thermal cycler at 37˚C for one hour, followed by 95˚C for 5 minutes and finally cooled to 4˚C, then stored at -20˚C until required for use. RT-PCR reactions were prepared under a highly sterile environment. Ultrapure

[53]

DNAse/RNase-free distilled RT-PCR master mix was prepared with cDNA and Taq-Man probes ABI (see Appendix 2 Table 8.1) in 20µl total reaction volume as follows:

Table 2-1 Summary of the RT-PCR reaction

	Reagent	volume (µl)
1	PCR Master Mix	10.0µl
2	cDNA Sample	1.0µl
3	Probes	1.0µl
4	dH$_2$O	8.0µl
5	Total volume	20.0µl

Expression of each gene was analysed in triplicate in 96 well PCR plates. After adding the appropriate volume of PCR Master Mix, cDNA Sample, Probes and dH$_2$O in each well, the plate was sealed, centrifuged for 20 seconds. Each plate contained a non-template control and a reverse transcriptase negative control. The Taqman® probes were amplified using the Roche 480 LightCycler®. The amplification protocol included; 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 2 step cycling; 15 sec at 95°C and 1 minute at 60°C. Quantification of the PCR results was estimated based on the threshold cycle (Ct). Ribosomal protein, large, P0 (RPLP0) was used as the house-keeping gene (HKGs) after validation. The data were analysed by using the compara-tive ΔCT method (ΔCt (target) – ΔCt HKG). For calculating relative changes in gene expression (wt MCF7 v TamR) the ΔΔCt method is used where the fold change in

gene expression is defined as $2^{-\Delta\Delta Ct}$ and is plotted as $\log_{10}$ on the ordinate axis in relevant figures.

2.2.3 Western and Ligand blot analysis

Cell conditioned media samples (1ml) containing secreted IGFBPs were freeze dried and stored at -20°C prior to analysis. For Western blot freeze dried media was dissolved in x1 SDS-PAGE sample buffer containing (1:20 v/v) β-mercaptoethanol and loaded onto the 15% SDS-polyacrylamide gel (45 μl /well) along with appropriate molecular weight markers. Gels were run at 120 V for 1 hr, and proteins were transferred to Immobilon-P semi dry membranes using the Trans-Blot Turbo device (BioRad) for 10 minutes. Subsequently, the membranes were blocked with 5% bovine serum albumin (BSA) in 0.1% TBST for at least 1hour on a shaker at room temperature. Then, the membranes were incubated overnight room temperature (RT) with either anti ER-α (1:1000 v/v) (Dako, Cambridge, MA – M704728-2), anti-ER-β (1:500 v/v) (Abcam, Cambridge, MA – ab288), (1:1000 v/v) anti-IGFBP‑2 (1:1000 v/v) (R&D System, Clone 92326‑ MAB6741), anti IGFBP‑5 (1:1000 v/v) (R&D System, Clone 145619‑ MAB8751), antibody or anti-actin (Santa Cruz Biotechnology) with 5% BSA containing tween 0.1% TBST. Membranes were washed x5 with TBST and incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody cat no ab97046 (1:100000) for 2 hours at room temperature in TBS. The membranes were washed x4 with TBST and x1 with TBS, membranes were developed with ECL substrate (1:1 v/v) (Pierce Rockford, IL) and images obtained on the Gel-Doc imager (Bio-Rad).

 For ligand blotting freeze dried medium was re-suspended in non-reducing SDS sample buffer (minus β-mercaptoethanol) and electrophoresis and blotting were performed as describe for Western blotting. Membranes were then blocked in 3% NP40

in TBS for 30 min, 3% BSA in TBS for 2hr and finally in 0.1% Tween 20 in TBS for 10 min on a shaker at RT. Filters were incubated overnight at 4°C with 20 ng mono-biotinylated IGF-2 (GroPep) in 1 ml 1%BSA/0.1% Tween in TBS. After washing 3 x 15 min in 1%BSA/0.1% Tween in TBS membranes were incubated in streptavidin-HRP conjugate (cat no ab97046) in 1%BSA/0.1% Tween (1:2000), washed 3 x 15 min in TBS and developed with ECL reagent.

2.2.4 Enzyme-Linked Immunosorbent Assay

IGFBP-5 and IGFBP-2 concentrations in conditioned media were determined by en-zyme-linked immunosorbent assay (ELISA) (R & D Systems, UK) according to the manufacturer's protocol. Briefly, diluted capture antibody at 4.0 µg/ml was plated in a 96-well microplate (100µl/well) and incubated overnight at room temperature. On the following day, the plate was washed with 400µl of diluted washing buffer (1:25 v/v) (0.05% Tween 20 in PBS). Plates were blocked with 300µl of blocking buffer (0.5% Tween 20 v/v in PBS with 0.05% NaN3 w/v) and incubated for 1 hour. After washing 100 µl of appropriately diluted standard along with samples were added and incubat-ed for 2 hours. Plates were washed and 100µl of detection antibody (1:200 v/v) were added and the plates were incubated for 2 hours. After washing 100µl of working dilu-tion (1:200 v/v) of streptavidin-HRP were added and the plates were incubated for 20 minutes. After washing 100µl of substrate solution (1:1 v/v) (colour reagent A H_2O_2 and colour reagent B Tetramethylbenzidine) were added and the plates were incubat-ed for 20 minutes. Finally, 50µl of stop solution (2N H2SO4) were added and absorb-ance was determined at 570nm.

2.2.5 Cell proliferation assay

Cell proliferation reagent WST-1 is a formazan dye used for spectrophotometric quantification of cell proliferation and viability. Cells were grown in Phenol Red (PR) free 5% DCS serum (PR-DCS) and then seeded in 96 well cell culture plates (TC) at 5×10^3 cells per well (100µl suspension). Control wells where no cells were seeded were included. Cells were left to attach overnight and then washed with PBS and treated with 0, 0.1, 1, 10 or 100 nM IGF-1 in a background of serum free PR free medium in triplicate for 48 hr. Following incubation 10µl of WST-1 reagent were added to each well and placed in an incubator for 0.5 – 4hr. The plate was placed on flat bed shaker for 1 min and then absorbance was determined at 450 nm. IGFBP-2 and IGFBP-5 were used at 0,1,10 or 100 nM or at a fixed concentration of 10 nM when co-incubated with varying concentrations of IGF-1. Details are given in the appropriate Figure legends. For experiments with 4-HT, cells were grown and seeded as above. To examine tamoxifen sensitivity of wt (MCF-7 cell passage no 35), TamR (TamR cell passage no 7), and IGFBP knock down (KO) cell lines stock tamoxifen was dissolved in absolute ethanol at 1mM and used at a final concentration of 0-1000 nM. At 0 – 5 day time points 10 µl of WST-1 were added to the wells and plates were read at 450 nm as described above.

2.2.6 IGFBP knockdown

IGFBP-2 and IGFBP-5 were knocked down in wt (passage no 35) and TamR cells (passage no 5) respectively using a short hairpin (sh) RNA based strategy. shRNA based knock down of gene expression occurs through stable integration of an shRNA containing plasmid. The subsequently transcribed shRNA product is processed to an si (short inhibitory) RNA product by the endoribonuclease Dicer. Further processing of the short inhibitory RNA (siRNA) species occurs ultimately leading to the association of siRNA–RISC (RNA induced silencing complex) with target mRNA and the hydroly-sis of the latter mRNA species. A diagram outlining the mechanism of action of shRNA based gene silencing is shown in Fig 2.1. Wt and TamR cells were seeded in Phenol Red (PR) free 5 %DCS serum (PRF-5% DCS) in two six well tissue culture plates at 5×10^5 cells per well. Cells were transfected according to the manufacturer's protocol. Briefly, cells were incubated at 37°C until 50-70% confluency. Cells were washed with 1 ml transfection media and incubated with 1ug IGFBP or control shRNA plasmids and 0.5-3% (v/v) transfection reagent in a final volume of 1ml for 6hr. After a further 6 hr, 1ml of 2x DCS-FBS were added to each well and incubation continued overnight. Then media were replaced with PR free 5% DCS serum containing 6 µg/µl puromycin (the appropriate selection agent) and changed every 3-5 days. Dead cells were removed with media changes and around 3-4 weeks, puromycin resistant cells began to grow. At 70% confluence the cells were expanded prior to performing an as-say for IGFBP-2 and IGFBP-5. Levels of IGFBP-2 and -5 in media conditioned by control and knock down cells together with untransfected wt and TamR cells were de-termined by ELISA as described above (2.2.4). Heterogeneous populations of shRNA IGFBP-5 and IGFBP-2 knockdown cells were cloned in 96-well plates by limiting dilu-tion. After 2 weeks individual colonies were identifiable in a significant proportion of microtitre wells. Typically 6 such clones were allowed to grow to confluence and me-

dium collected for IGFBP-2 or IGFBP-5 assay by ELISA. IGFBP concentrations in

conditioned medium were compared to those obtained in contemporaneously grown

wt or TamR cells. Clones which showed the highest level of knock down were subse-
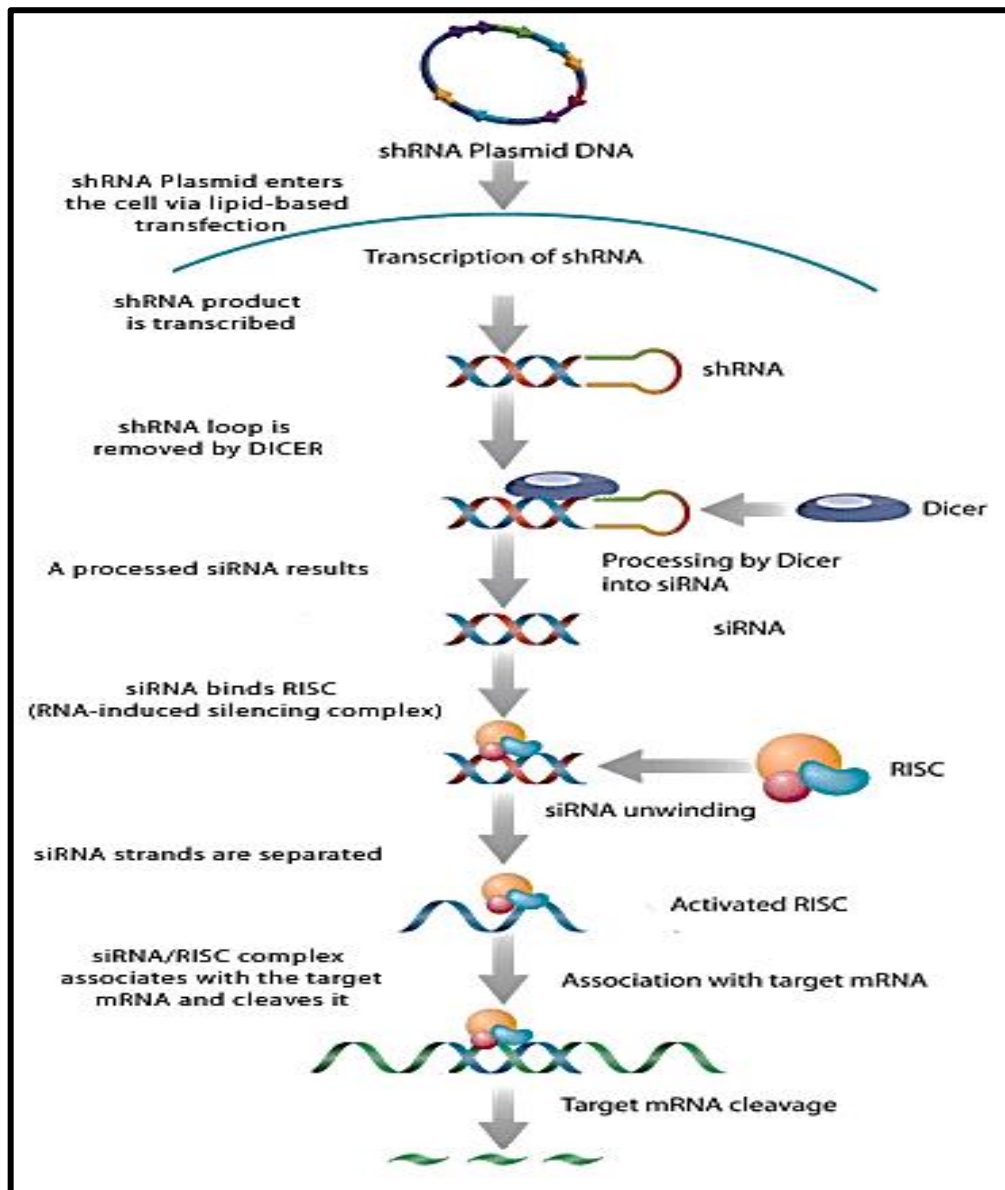
quently expanded and stored in liquid N2.

Figure 2-1 Mechanism of shRNA based gene silencing.

Adapted from

http://www.bing.com/images/search?q=shrna+&FORM=HDRSC2#view=detail&id=E7A805D8BB419386DDEB59A54F35667973B7AE05&selectedIndex=0

2.2.7 Cell migration

Matrigel (phenol red Free - PRF) was prepared by thawing overnight at 4 $^{\circ}$C and diluted in pre-cooled RPMI-1640 PRF media to provide a final protein concentration of 100µg/ml. 96-well Essen Image Lock plates were placed on ice and pre-cooled 200 µl yellow tips were used to load 50µl of the diluted matrigel into each well. Plates were then incubated at 37 °C CO_2 for 1h to allow solidification of matrix. RPMI-1640 PRF media with 5% DCS media was used to seed cells at concentrations of $5x10^5$ per well. The plates were incubated for 18hs to allow cells to attach to the matrix following which sterilised 96-well Wound-Maker pins were used to simultaneously generate precise and reproducible cell-free zones 700-800µm wide in cell monolayers. Plates were placed in the IncuCyte incubator at 37°C and equilibrated for a minimum of 15 minutes before the first scan. The software was set to scan the experiment every 1 hour for migration assays. The IncuCyte automatically acquires images for the entire duration of the assay (94h). IncuCyte integrated software quantifies cell migration using the metric relative wound density (RWD). In addition to experiments on Matrigel we also conducted experiments which monitored cell migration on plastic. Preparation of seeded 96-well Essen Lock plates was exactly as described above for Matrigel coated plates. Migration of six different cell lines was analysed using this technique – parental wt (passage no 36) and TamR MCF-7 cells (passage no 7); IGFBP-2 KO (clone F8) and scrambled shRNA control; IGFBP-5 KO (clone B4) and scrambled shRNA control. Images of wound healing were also captured at various time points and are presented along with plots of RWD v time. For each cell line 12 replicates were performed and data were generated as mean ± SD of RWD by the IncuCyte software. Data were subsequently imported to GraphPad Prism and analysed for significant differences in migratory activity using repeated measures ANOVA followed by Bonferroni's post-hoc test.

2.2.8 Immunohistochemistry

2.2.8.1 Breast cancer patients and ethical approval

Breast cancer samples used for tissue microarrays (TMAs) were collected from patients who had undergone initial surgery at Leeds Teaching Hospitals (LTH) NHS Trust, Leeds, UK, between 03 Jul 1975 to18 Nov 1997 (22 years). Before starting, ethical approval was gained from the Local Research Ethics Committee of the Leeds Teaching Hospitals (LTH) NHS Trust, Leeds, UK with REC reference number: 06/Q1206/180. A total of 424 patient samples divided into two subgroups, tamoxifen-resistant (TamR) and tamoxifen-sensitive (TamS). 289 patients had no evidence of acquired tamoxifen resistance. Therefore, this cohort was used as the control group in our investigation. On the other hand, 135 patients were confirmed to have acquired resistance to tamoxifen.

2.2.8.2 Samples collection and Tissue Microarray Preparation

Consented breast tissue samples were fixed in formalin, paraffin-embedded (FFPE) blocks. A 5µM section was taken and an H&E stain was conducted to identify tumour regions. A 0.6mm core was taken from the tumour area and transferred to a recipient paraffin TMA block in triplicate (Fig 2.2). During TMA construction a border and orientation cross of easily identifiable tissue is used to aid the analysis and scoring the stained TMA slide. 5µm sections were cut from the complete TMA and stained by Immunohistochemistry staining procedure (see section 2.2.8.4).
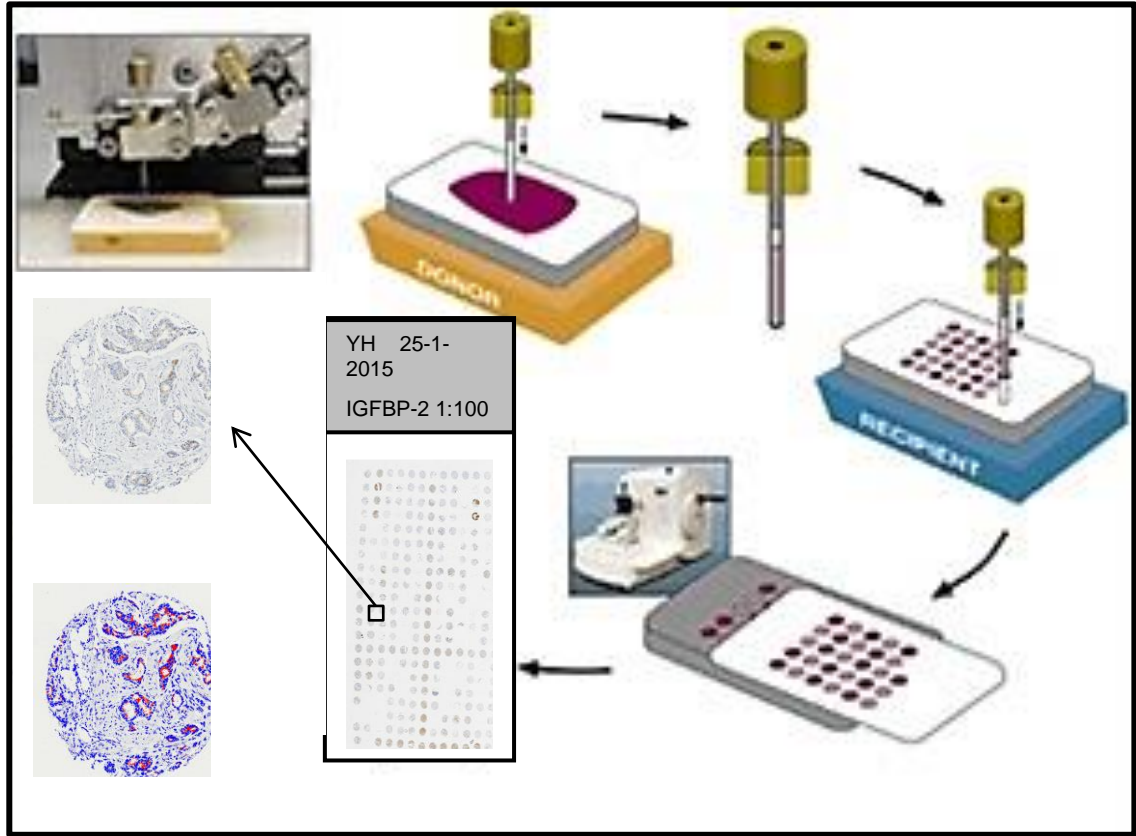
Figure 2-2 Diagram illustrates the TMAs constructions.

The arrays are collected by selecting core needle biopsies from a specific area of breast tumour in pre-existing paraffin-embedded tissue blocks and then re-embedding them into another arrayed "master" block, using an apparatus developed by Konenen et al. Each core is about 0.6mm and therefore, more than 600 specimens can be represented in a single paraffin block. 5μm sections are cut from the complete TMA and stained by Immunohistochemistry.

2.2.8.3 Antibody Optimisation

An online system called The Human Protein Atlas was used as a pilot to choose the appropriate tissues and specific IGFBP-2 antibody http://www.proteinatlas.org/. The anti-IGFBP-2 antibody was then initially optimised in kidney at 1:50, 1:100, 1:200 and 1:400 dilutions, and the 1:100 IGFBP-2 antibody concentration was chosen on the basis of its having superior cytoplasmic staining (see Appendix 3).

Multi-tissue slides containing tonsil, mucinous ovarian tumour, colon, muscle, colorectal tumour, oesophagus, placenta, desmoids type smooth muscle tumour and spleen were used for antibody optimisation. A staining was performed using polyclonal antibody against IGFBP-2 (Abcam) and IGFBP-5 (Santa Cruz) and was optimized by serial dilution. Figure 2.3 is representative of IGFBP-2 multi-tissue staining and the optimised images included in Appendix 3.
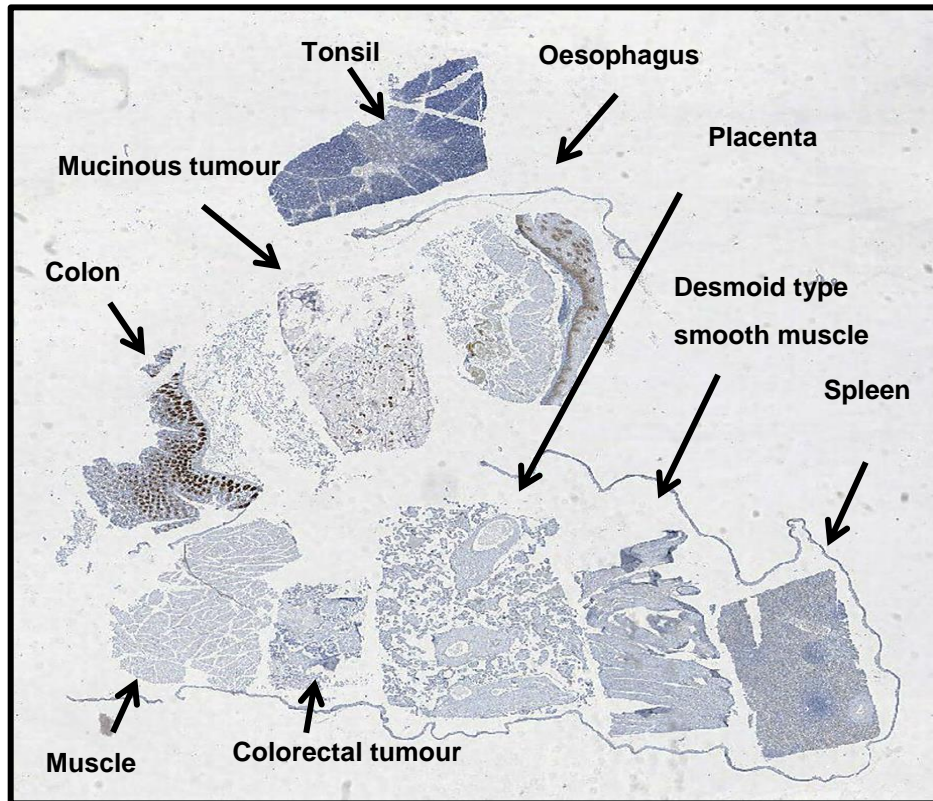
Figure 2-3 FFPE multi- tissue sections.

Multi-tissue slides containing tonsil, mucinous tumour, colon, muscle, colorectal tumour, oesophagus placenta, desmoids type smooth muscle tumour and spleen. Tissues were stained with IGFBP-2 at a dilution of 1:100.

2.2.8.4 Immunohistochemistry

To enhance detection, antigen retrieval was achieved after de-waxing using Mena Path Revelation buffer solution (cat no MP-607-X500) in the Mena Path pressure cooker containing 500 ml distilled water for 40 minutes. Immediately following this slides were immersed in PBST (1x TBS containing 0.2% Tween 20) buffer then distilled water. A liquid wax border was applied to the glass microscope slides around the tissue and TMAs to prevent reagent dispersion. Then the slides were washed in PBST and blocked with 100 µl Novacastra peroxidase (cat no RE7101) for 10 minutes at RT. Slides were then washed with PBST and 100 µl of protein blocking solution (cat no RE7102) was added and slides were incubated at RT for 2 minutes. Rabbit monoclonal antibodies against hIGFBP-2 (1:50 v/v) and rabbit polyclonal antibody against IGFBP-5 (1:800 v/v) were added and incubated at RT for 1 hour. These specific antibodies do not cross react with the other IGFBPs. Slides were washed 3 times for 5 minutes with PBST and 100µl of secondary antibody (Novolink polymer cat no RE7112) was added and incubated for 30 minutes. After washing 3 times in PBST for 5 minutes 100 µl of the diluted DAB chromogen (1:20 v/v) (cat no RE7105) was added to each slide and incubated at room temperature (RT) for exactly 5 minutes. Rehydration of slides was conducted in a series of alcohols, 100%, 75%, 50%, and 25% for 3 minutes in each concentration, and washed in running tap water. Endogenous peroxidase activity was blocked using 0.75% H_2O_2 for 20 minutes and then rinsed in running tap water. Slides were counter stained by immersing in Mayer's haematoxylin for 1 minute and washed in running tap water, then immersed in Scott's tap water for 2 minutes followed by a further wash in running tap water. The slides were then dehydrated in a series of ethanol (25% for 15 seconds, 50% for 2 minutes, 70% for 5 minutes, and 100% for 5 minutes) and were immersed in xylene 3 times for 3 minutes.

Finally, the sections were mounted in DPX and cover-slips applied. For negative controls, primary antibody was omitted.

2.2.8.5   Definition for cut-off point

After staining, slides were scanned (see Scope Scan® system, Aperio). Subsequently, the pixel intensity Aperio algorithm was used (Developed by A.Wright, Leeds Virtual Pathology Department) and the scanned slides were uploaded into a locally developed computational system by Alexander Wright called Tissue Microarray Informatics, to calculate the algorithmic score. Pixel intensity data were then exported to excel. To our knowledge this method had not been used for the antibodies studied in this thesis, therefore a cut-off point was required to enable dichotomisation of the data for downstream analysis. As specimens were triplicates, an average of scoring was taken. A Receiver Operating Characteristic Curve (ROC) was used to determine a point of dichotomisation. All these data were entered into an excel file, then saved as text file and uploaded into the cut-off finder online program (see http://molpath.charite.de/cutoff/) [227].

As an outcome, the cut off point was determined to be 0.1 for both cohorts with sensitivity of 66.7 % and specificity of 41.5%. Accordingly, TMA specimens were categorised into two categories; a positive group that represents strong staining (> 0.1) and the negative group with very weak or absent of staining (< 0.1). With help from clinicians at St James Teaching Hospital (Prof Christopher Twelves, Dr Maria Jove), clinical history and tumour characteristics were retrieved from the patients' clinical system. Survival durations in months and the event of 'death' was retrieved from the patients' clinical system and reported as 1 for 'alive' and 0 for 'dead'. Statistical Package for Social Sciences (SPSS) version 22 software and Log-rank test (Mantel–Cox test) hypothesis test was used to plot Kaplan-Meier graphs.

Chapter 3 Results

The IGF axis in wt and TamR MCF-7 cells

3.1 Introduction

We assessed expression of the IGF axis (IGF-1, IGF-2, IGF-1R, IGF-2R, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6) in TamR vs Wt MCF-7 cells. mRNA from both cell lines was extracted and used for first strand cDNA synthesis. TaqMan probes were used for qRT-PCR and eight biological repeats were performed for each cell line with triplicate technical replicates within each PCR microtitre reaction. RPLP0 was validated and used as a housekeeping gene (HKG).

3.2 RNA Quantification

Purity of RNA was determined using the ratio of A260/A280. RNA quality is generally considered adequate when the A260/A280 ratio value is > 1.85. A260/A280 ratios were typically 1.9-2.1 which is indicative that our RNA was mostly free of protein con-tamination. However, there is a possibility of presence of DNA in RNA extracted which might have an impact on significance of the results. Figure 3.1-A demonstrates the A260/A280 ratios in several RNA preparations from eight different batches of wt and TamR cells. RNA concentrations were between 200-640 ng/µl (Figure 3.1-B) and no reproducible differences were apparent between MCF-7 and TamR cells.
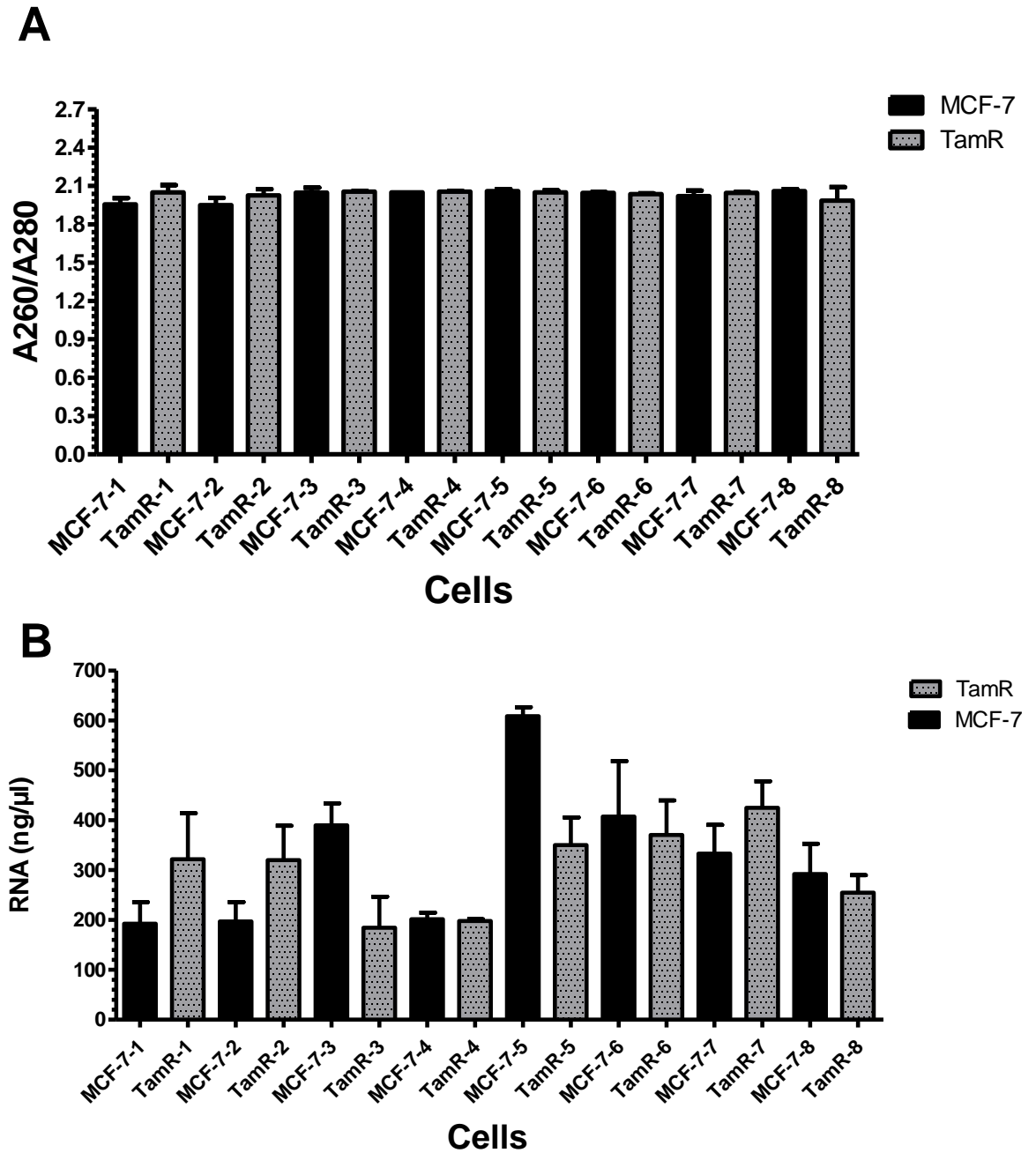
Figure 3-1 (A) A260/280 ratios and mRNA concentrations for eight different preparations of mRNA from wt MCF-7 and TamR cells.

Triplicate technical repeats were performed for each mRNA preparation. (B) Corresponding mRNA concentrations in preparations described in (A).

3.3 Validation of housekeeping gene.

The 36B4 gene encodes the acidic ribosomal phosphoprotein P0 (RPLP0) and pro-
vides a very reliable and consistent standard for use in gene expression analysis in
several tissues including, breast, brain and heart. Accordingly Ct values were deter-
mined for RPLP0 in wt and TamR MCF-7 cells. No statistical significance (Student's t-
test) was seen in Ct values for cDNA preparations from the two different cell lines wt
=20.01 ± 1.21; TamR 19.29 ± 0.82 (mean $\pm$ SD n=6; p=0.47) – Fig 3.2 and RPLP0 was
therefore used as a HKG for qRT-PCR experiments.
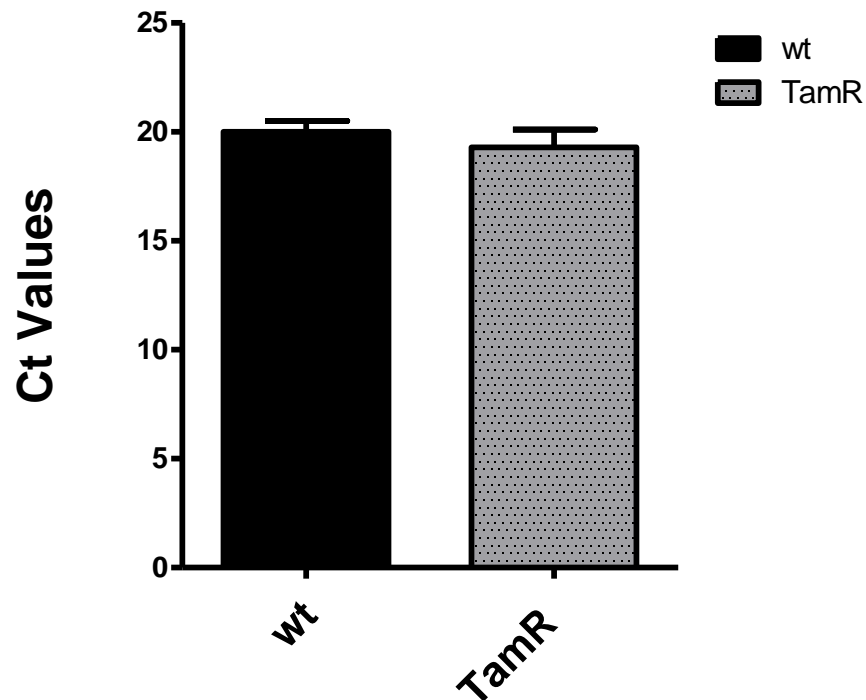
Figure 3-2 Validation of housekeeping gene (RPLP0).

Ct values for RPLP0 expression in wt and TamR cells. Data are presented as
mean+/-SD of Ct (n=6).

3.4 Raw Ct values for qRT-PCR

Four separate analyses of IGF axis gene expression in wt and TamR cells were performed. Ct values for a representative experiment are presented in Table 3.1. Based on these Ct values we were able to make some qualitative remarks about expression of the IGF axis genes in these cells. Consistently in our experiments IGF-I, IGF-2, IGFBP-1, IGFBP-3 and IGFBP-6 showed low abundance (mean Ct > 33). The other five IGF axis genes IGF-1R, IGF-2R, IGFBP-2, IGFBP-4 and IGFBP-5 were expressed at moderate to high levels (mean Ct < 26). An outlying value for a Ct obtained for IGF-2R expression in TamR cells (Ct3) is indicated in red although this value was included in the calculation of mean +/- SD for this gene.

Qualitative guide to Ct values in RT-PCR; –

> ➢ Ct > 40 almost certainly not expressed

> ➢ Ct 35-39 very low abundance

> ➢ Ct 30-34 low abundance

> ➢ Ct 25-29 moderate expression

> ➢ Ct 20-24 moderately high expression

Table 3-1 Individual Ct values for IGF axis expression in wt and TamR

	Wt					TamR				
Ct1	Ct2	Ct3	Mean	STD	IGF axis	Ct1	Ct2	Ct3	Mean	STD
20.83	20.8	20.28	**20.64**	0.31	RPLP0	19.23	18.91	19.06	**19.07**	0.16
41.92	41.87	41.21	**41.67**	0.40	IGF-I	41.36	N/A	40.93	**41.15**	0.30
36.47	36.78	36.61	**36.62**	0.16	IGF-2	N/A	40.27	N/A	**40.27**	N/A
23.86	23.87	23.66	**23.80**	0.12	**IGF-IR**	23.61	23.59	23.39	**23.53**	0.12
25.99	25.31	26.52	**25.94**	0.61	**IGF-2R**	25.21	25.17	17.62	**22.67**	4.37
35.45	35.58	35.19	**35.41**	0.20	IGFBP-1	42.28	N/A	39.86	**41.07**	1.71
25.66	25.51	25.19	**25.45**	0.24	**IGFBP-2**	22.69	22.7	22.64	**22.68**	0.03
33.79	32.74	33.97	**33.50**	0.66	IGFBP-3	34.2	34.39	34.4	**34.33**	0.11
23.7	23.33	23.36	**23.46**	0.21	**IGFBP-4**	24.20	23.23	25.52	**24.32**	1.15
24.04	23.7	23.48	**23.74**	0.28	**IGFBP-5**	28.2	26.45	26.52	**27.06**	0.99
35.25	34.91	34.77	**34.98**	0.25	IGFBP-6	32.71	32.85	32.49	**32.68**	0.18

Table 3.1 Individual Ct triplicate values (Ct1 – Ct3) for IGF axis expression in wt and TamR are indicated along with mean +/- SD. N/A no value obtained. This experiment was repeated four times and a representative data table is shown. Moderate to highly expressed genes are highlighted in bold red.

3.5 Expression of the IGF Axis in wt v TamR cells

A profile of IGF axis gene expression in wt v TamR cells indicated some differences in expression of these genes (Fig 3.3). Repeated analysis of expression of these 5 genes in wt v TamR cells indicated that IGFBP-5 was consistently down regulated whereas IGFBP-2 was consistently up regulated in TamR cells. In contrast no consistent changes in expression of IGF-1R, IGF-2R or IGFBP-4 between wt and TamR cells were evident (Fig 3.3). For IGFBP-2 consistent 2-4 fold increases in expression were seen in TamR cells v wt cells whereas IGFBP-5 expression was down regulated 5-7 fold in TamR v wt cells.
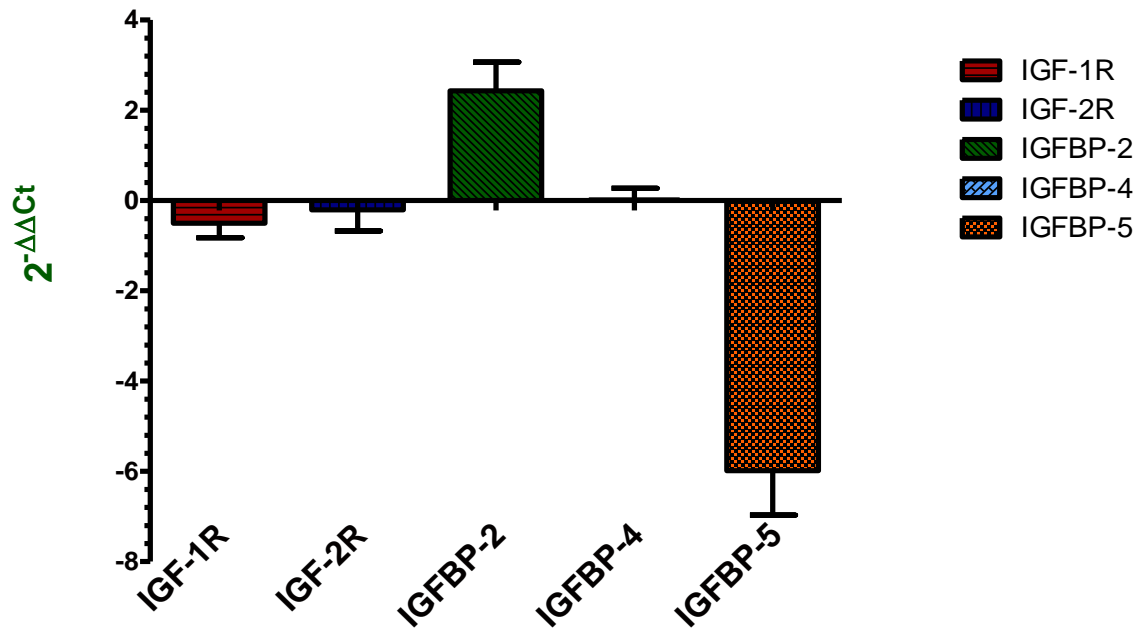
Figure 3-3 Fold changes in selected IGF axis genes in TamR v wt cells.

The expression of IGF-1R, IGF-2R, IGFBP-2, -4 and -5 is compared between wt and TamR cells. Data is plotted as $2^{-\Delta\Delta Ct}$ and represents the fold change in expression in TamR v wt MCF-7 cells. This experiment was repeated at least six times and similar results were obtained in each case. The mean of the experiments is shown with data presented as mean ± SD.

3.6 Western blot

In order to confirm the changes evident in IGFBP-2 and -5 mRNA expression in wt v TamR cells at the protein level we used the techniques of Western and Ligand blotting. IGFBPs are essentially secreted proteins and accumulate in medium which is conditioned by cells in culture. Freeze dried conditioned medium of three wells of wt and TamR cells were reconstituted in sample buffer and analysed by Western blot for IGFBP-5 and IGFBP-2 expression (Fig 3.4 and 3.5). For IGFBP-5 it is clear that the protein shows increased abundance in medium conditioned by wt compared to TamR cells (Fig 3.4A). This agrees with the results obtained by qRT-PCR (Fig 3.3). Densitometric analysis of band intensity (Fig 3.4B) suggested a 6-fold decrease in protein expression in TamR compared to wt cells which also agrees closely with qRT-PCR data. Freeze dried conditioned medium was also reconstituted and analysed for IGFBP-2 protein by Western blot (Fig 3.5A). IGFBP-2 was expressed at a higher level in TamR cells compared to wt cells. Densitometric analysis of the data (Fig 3.5B) suggested that IGFBP-2 concentrations were approximately 3-fold higher in TamR cells than in wt cells. This data also agrees well with qRT-PCR results.

Figure 3-4A Western blot for IGFBP-5.

Expression of IGFBP-5 in wt and TamR cell conditioned medium. IGFBP-5 was clearly identified following enhanced chemiluminescence (ECL) detection. Images were typically captured after 1-15s of exposure. This experiment was repeated 3 times with similar results in each instance.

Figure3.4B Densitometric analysis of IGFBP-5 band intensity.

Triplicate lanes per blot were analysed for band intensity for both wt and TamR conditioned media using Image Lab software. Data are presented as mean +/- SD (n=3) of Arbitrary Unit (AU) intensity. This experiment was repeated 3 times with similar results in each instance. * P< 0.005 Students t-test; GraphPad Prism.
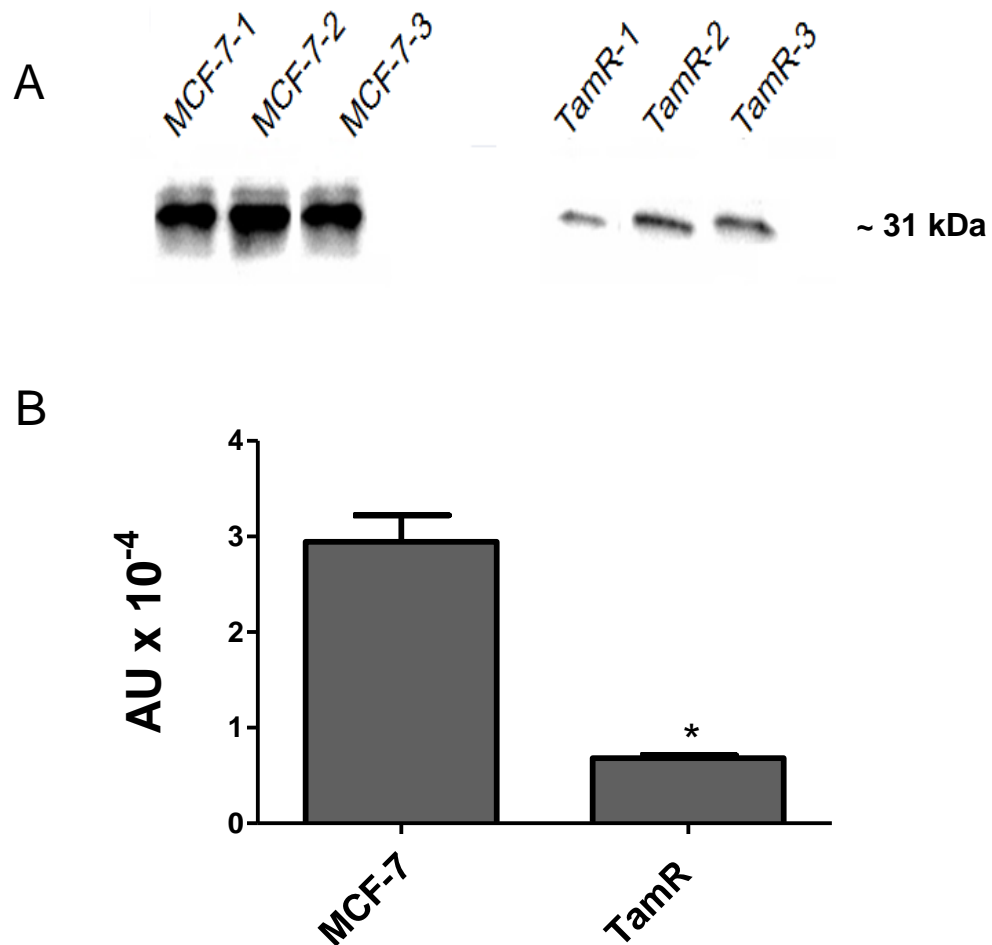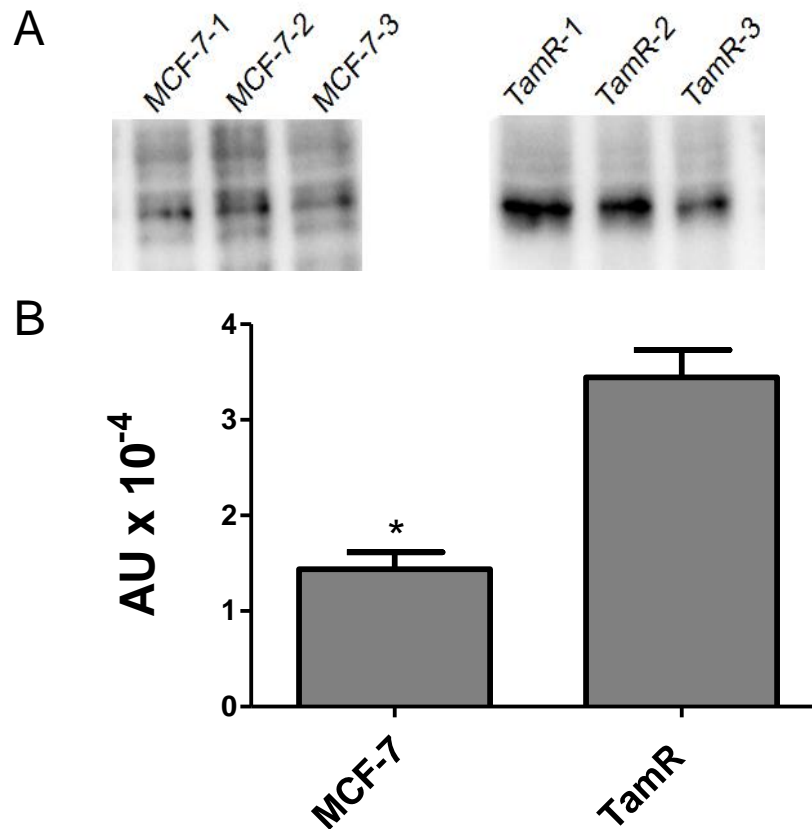
Figure 3-5A Western blot for IGFBP-2

Expression of IGFBP-2 in wt and TamR cell conditioned medium. IGFBP-2 was clearly identified following enhanced chemiluminescence (ECL) detection. Images were typically captured after 1-15s of exposure. This experiment was repeated 3 times with similar results in each instance.

Figure 3.5B Densitometric analysis of IGFBP-2 band intensity.

Triplicate lanes per blot were analysed for band intensity for both wt and TamR conditioned media using Image Lab software. Data are presented as mean +/- SD (n=3) of Arbitrary Unit (AU) intensity. This experiment was repeated 3 times with similar results in each instance. P< 0.005 Students t-test; GraphPad Prism 5.0.

3.7 Ligand blot

Ligand blotting using biotinylated IGF derivatives can be used to profile all of the IGF binding species in a particular biological sample (see methods Section 2.2.3). The results of such an experiment are shown in Fig 3.6. Two bands with Mr ~34kDa (IGFBP-2) and ~31 kDa (IGFBP-5) were detected. As identified by Western blotting the intensity of the IGFBP-2 band was higher in TamR than in wt cell conditioned media. Conversely the IGFBP-5 band was higher in the wt cell conditioned media than in TamR cells. In the latter cell conditioned media the level of IGFBP-5 was close to the lower detection limit of this technique. Although we did not expect to find IGFBP-1 or IGFBP-6 in either cell conditioned media, IGFBP-4 on PCR showed a similar Ct value for BP-2 and BP-5 in TamR and wt cells respectively (Table 3.1). However we did not detect IGFBP-4 at a typical Mr of 24 kDa in either wt or TamR conditioned cell media. There may be several reasons for this including proteolysis of IGFBP-4 or protein levels below the detection sensitivity of ligand blotting. However as we have no evidence that IGFBP-4 expression is altered in wt compared to TamR cells we did not investigate this matter further (see Discussion section 3.9).
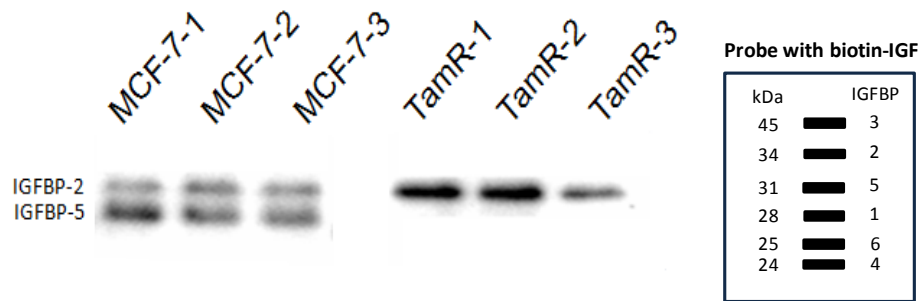
Figure 3-6 Ligand blot analysis of medium conditioned by wt and TamR MCF-7 cells.

Global IGFBP species secreted by wt and TamR MCF-7 cells were identified by blotting with biotinylated IGF-1 as described in the Methods section. IGFBP-2 and -5 are clearly identified in wt conditioned medium and IGFBP-2 in TamR medium. In these experiments exposure times of up to 30s were used. This experiment was repeated 3 times with similar results and a representative blot is shown. Expected migration positions of IGFBPs are indicated (inset).

3.8 Elisa

As a final confirmation of the alteration of IGFBP-2 and -5 expression in wt and TamR cells we undertook ELISA experiments using media conditioned by both cell types. For IGFBP-5 the standard curve for the ELISA was linear between 0.75 – 40 ng/ml and dilutions of conditioned media were appropriately adjusted to fall in this linear range. The results of such an experiment are shown below for IGFBP-5 Fig 3.7- upper panel and show that levels in wt cell conditioned media (~7 ng/ml) are around 5-fold higher than those in TamR cells (~1.4 ng/ml). This data agrees very closely to that obtained with qRT-PCR, Western and Ligand blot (Fig 3.3, 3.4 and 3.6). For IGFBP-2 ELISA the linear portion of the standard curve extended over the range 0.0625- 4 ng/ml and again samples of conditioned medium were appropriately diluted to fall in this region of the standard curve. Fig 3.7 (lower panel) indicates that IGFBP-2 concentration in TamR cell conditioned medium (~10 ng/ml) is around 7-fold higher than that in medium conditioned by wt cells (~1.4 ng/ml). Once again this data for IGFBP-2 expression is consistent with results obtained by qRT-PCR, Western and Ligand blotting (Fig 3.3, 3.5 and 3.6). It is also interesting that there is a correlation between absolute levels of IGFBP-2 and -5 as determined by ELISA and the raw Ct values obtained by qRT-PCR for both of these genes see Discussion section 3.9.
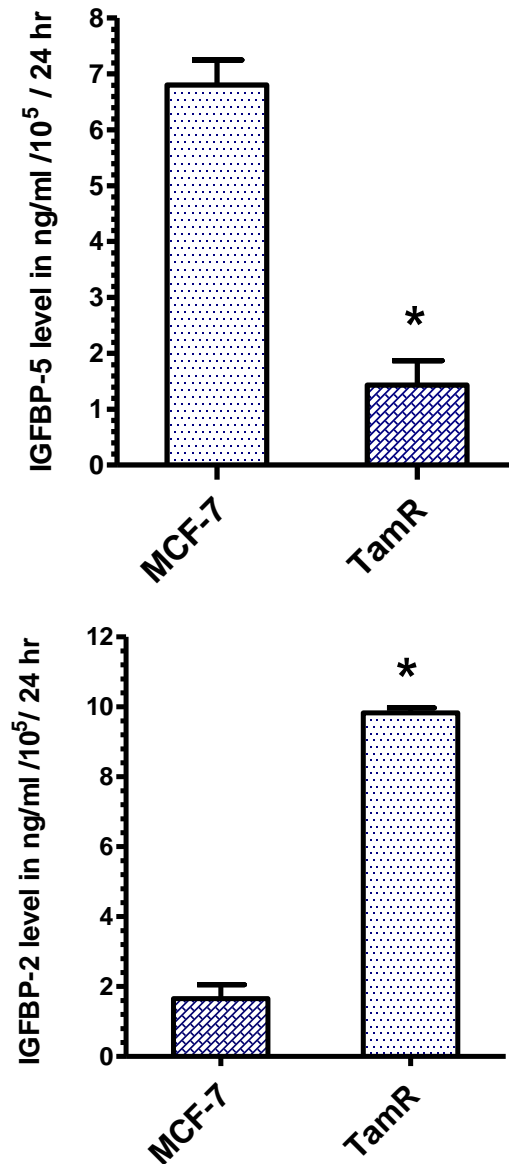
Figure 3-7 ELISA determination of IGFBP-5 (upper panel) and IGFBP-2 (lower panel) concentration in wt and TamR conditioned medium

IGFBP-5 and IGFBP-2 concentrations in medium conditioned by wt or TamR cells were determined by ELISA. Triplicate technical repeats were performed on media obtained from three separate wells for both wt and TamR cells. The data are expressed as mean ± SD (n=3) *p<0.01 TamR v wt (Student's t-test). This experiment was repeated 3 times with similar results in each case. A representative experiment is shown.

3.9 Discussion

Our initial screening studies of IGF axis expression in wt and TamR MCF-7 cells indicated that of the 10 genes examined 5 were expressed at moderate to high abundance (IGF-1R, IGF-2R, IGFBP-2, IGFBP-4 and IGFBP-5) and 5 were either not expressed or expressed at very low levels (IGF-1, IGF-2, IGFBP-1, IGFBP-3 and IGFBP-6). Essentially this expression profile agrees with that previously reported for MCF-7 cells [173, 191, 228] and indeed for other ER+ BC cell lines [229-231]. Early MCF-7 passage was used (passage no 25). There is some controversy with respect to IGFBP-3 expression with some studies reporting expression in MCF-7 cells [232-234]. This may be associated with the degree of confluence in cell cultures or cell senescence [235-237] and one study reported IGFBP-3 expression using confluent cultures of MCF-7 cells [233]. Our qRT-PCR studies were generally done in cells which had achieved 70-80 % confluence and this may provide an explanation for discrepant results. Another possibility is that IGFBP-3 expression may vary with passage number. We did not rigorously examine this possibility.

It is well known that breast epithelial cells do not secrete IGF growth factors. Early reports of IGF activity in BC epithelial cell conditioned medium were subsequently found to be the result of incomplete extraction of IGFBPs leading to cross reactivity in radioimmunoassay used at that time [238-241] and the well-established paradigm is that mammary stromal cells express IGF-1 and IGF-2 which then act as a mitogens [235-237] by binding to cognate receptors on the mammary epithelial cell surface. Our data confirm that IGF-1R and IGF-2R are expressed in both wt and TamR MCF-7 cells with IGF-1R more abundant at the mRNA level. We did not confirm if this is also the case at the level of protein expression in the cell membrane. In addition we did not examine insulin receptor (IR) expression in either wt or TamR cells although they have been reported to express this protein. Although IGF-2 is believed to exert mitogenic effects

via the IGF-1R this growth factor can also bind and signal through IR and future studies should include a report of IR expression in wt and TamR cells. This is increasingly important due to reports of hybrid IGF-R/IR species in BC cells [242] and thus information on the levels of both A and B isoforms of the IR would be informative. An important observation from our studies is that in repeat experiments neither IGF-1R nor IGF-2R expression was altered in TamR v wt cells (Table 3.1 and Fig 3.3). This is significant because increased IGF-1R expression has been mooted as possible mitogenic driver in tamoxifen resistant BC cells [203]. However the literature is contradictory in this area with reports that IGF-1R is either up regulated or down regulated in TamR cells [204-206]. Repeat analysis in our study indicated little change in IGF-1R expression at least at the mRNA level. Clearly it would be important to confirm such findings at the protein level.

Perhaps the most interesting results in this section of our work were the findings in relation to IGFBP expression. Raw Ct values (see Table 3.1) suggest that the relative abundance of the IGFBPs at the mRNA level was BP4 = BP5 > BP2 (wt cells) and BP2 > BP4 > BP5 for TamR cells. However while the expression of IGFBP-4 did not change between wt and TamR cells we repeatedly observed a reciprocal relationship between IGFBP-2 and IGFBP-5 in the two cell lines. Therefore in TamR cells repeat qRT-PCR experiments suggested that IGFBP-2 was up regulated 2-5 fold and IGFBP-5 was down regulated approximately 7-fold compared with wt cells (Fig 3.3). Because of the repeated robustness of our qRT-PCR findings we decided to investigate whether these changes in BP-2 and BP-5 mRNA expression were also evident at the protein level. Western blot analysis using specific anti-BP2 or anti-BP5 antibodies showed that the data obtained by qRT-PCR was confirmed in terms of protein abundance. Densitometry of repeat Western blots indicated a significant difference between BP-2 and BP-5 levels in wt and TamR cells (Fig 3.4A and 3.4B). Although we

show only that portion of the blot containing intact IGFBP protein(s) inspection of whole blots did not indicate a significant degree of proteolysis of either IGFBP-2 or IGFBP-5 in medium conditioned by either cell line. Western blotting data was confirmed using the technique of Ligand blotting which detects only IGF binding species following blotting with appropriately labelled IGF-1 or IGF-2. In our experiments we used biotinylated IGF-1 (see Materials & Methods section) and found both IGFBP-2 and IGFBP-5 in wt MCF-7 cell conditioned medium (Fig 3.5). As expected the signal for IGFBP-2 was stronger and the signal for IGFBP-5 was weaker in TamR cells. In fact in some experiments it was difficult to detect IGFBP-5 signal in TamR cell conditioned medium suggesting that the sensitivity of the ligand blot method was less than that of western blotting in this particular instance. Interestingly although IGFBP-4 showed an equivalent expression by qRT-PCR with IGFBP-5 in wt cells and was also robustly expressed in TamR cells we did not detect any signal for this binding protein which would be expected to run at around 24 kDa (Fig 3.6 – inset). Although we have no ready explanation for this it may be that BP-4 is proteolysed in MCF-7 cell conditioned medium and thus not detectable by Western blotting. In any case because IGFBP-4 expression was not altered when examined by qRT-PCR between wt and TamR cells and because the intact protein was not detected in either wt or TamR conditioned medium we did not pursue this area further. However differential proteolysis of IGFBP-4 in BC cells is a potentially important research area.

Because Western and Ligand blots are at best only semi-quantitative techniques we used ELISA methodology to get an accurate measurement of IGFBP-2 and IGFBP-5 levels in conditioned medium. Fig 3.7 shows the result of a typical ELISA experiment and the concentrations obtained are reported in Section 3.8. It is clear from inspection of Figs 3.4 and 3.7 that the fold changes in mRNA and protein expression are of the same order and that consequently changes in mRNA expression are accurately translated in terms of protein abundance. It is also apparent that the global protein concentrations for both IGFBPs – approximately 2-10 ng/ml lie within an order of magnitude. This raises the possibility that there is co-ordinate regulation of IGFBP-2 mRNA and protein expression and we return to this theme in the final chapter of this thesis.

 There is only a limited literature describing altered IGFBP expression profiles on the acquisition of tamoxifen resistance in BC cells. Early studies described altered IGFBP profiles after development of tamoxifen resistance in the MCF-7 and ZR-75-1 cell lines although the IGFBP species in this report were not identified immunologically but only assigned by molecular weight on ligand blot [211]. This group subsequently reported that IGFBP-2 was down regulated in tamoxifen resistant MCF-7 cells [212] which is contrary to our findings. However these authors included the synthetic corticosteroid dexamethasone in their cultures and this agent is a known regulator of IGFBP-2 expression in mammary epithelial cell lines [213]. Of more relevance is the study by Juncker-Jensen et al [243] who demonstrated IGFBP-2 up regulation in Tam R MCF-7 cells as well as cells resistant to the SERD fulvestrant and the pure anti-oestrogen R58,668. Although such observations are similar to those described in the current thesis Juncker-Jensen et al demonstrated that IGFBP-2 knock down using antisense oligonucleotides or siRNA did not affect the growth of resistant cells and concluded that IGFBP-2 may be a marker for resistance but had no causal role in this respect (see Chapter 4). In the context of IGFBP-5, the most relevant study with respect to

our findings is that of Ahn et al who used an RNA interference based screening methodology to identify IGFBP-5 as a determinant of tamoxifen sensitivity [132]. They demonstrated that shRNA based knockdown of IGFBP-5 expression in MCF-7 cells conferred tamoxifen resistance in these cells perhaps related to a concomitant loss of ERα expression. Further to this tamoxifen resistant MCF-7 cells showed decreased IGFBP-5 expression and addition of exogenous IGFBP-5 partly restored sensitivity to tamoxifen. We also found that ERα expression was down regulated by approximately 60% in TamR cells (see Fig 4.18). In the next chapter we examine further the hypothesis that IGFBP-2 and -5 play a causal role in the acquisition of tamoxifen resistance in MCF-7 cells.

Chapter 4 Results

Role of the IGF axis in development of tamoxifen resistance

4.1 Introduction

Since our results clearly confirmed that the acquisition of tamoxifen resistance in MCF-7 cells is associated with a down regulation of IGFBP-5 and up regulation of IGFBP-2 we next addressed the question of whether either of these changes in IGFBP profile was causally associated with the development of tamoxifen resistance. To investigate this possibility we decided to use a gene knock out strategy. Therefore we designed experiments to investigate whether IGFBP-2 knock down in TamR cells restored sensitivity of these cells to tamoxifen. In reciprocal experiments we also investigated whether knock down of IGFBP-5 in wt MCF-7 cells conferred tamoxifen resistance to these cells. We employed an shRNA based knock down strategy (see Methods section 2.2.6) with puromycin selection of stably transfected cell colonies.

4.2 Puromycin optimisation

Determination of the optimum puromycin concentration for selection of stably transfected clones was performed for both wt and TamR cells in 6 well plates and a range of puromycin concentrations of 0-10 ug/ml. We found that for both wt and TamR cells the minimal concentration of puromycin required for 100% cell death was 6 ug/ml and we therefore used this concentration in all of our subsequent experiments.

4.3 IGFBP-2 knockdown

We used the shRNA interference technique in attempt to knockdown IGFBP-2 ex-
pression in TamR cells. These experiments proved to be successful in TamR early
cell passage (passage no 7). The shRNA preparation is a pool of 3 target-specific len-
tiviral vector plasmids each encoding 19-25nt (plus hairpin) designed to knockdown
gene expression. In a six well plate transfections of target and control plasmids were
performed at a ratio of 1:1 to 1:6 where the concentration of plasmid is held constant
at 1ug and the volume of transfection reagent is varied from 1-6 ul. Such protocols
were performed essentially according to the manufacturer's instructions (see Methods
section 2.2.6 for further details). Under these conditions puromycin resistant colonies
were established in 6 well clusters after approximately 3 weeks. The results of these
IGFBP-2 knockdown experiments are shown in Fig 4.1. Scrambled shRNA was used
as control. IGFBP-2 ELISA of conditioned medium for each of these cultures indicated
statistically significant knockdown of IGFBP-2 expression in cell populations #1, #3,
#5 and #6 (p<0.005). For cell populations #2 and #4 significance was almost reached
with p=0.065 and 0.062 respectively. Levels of knockdown varied from 21% (#3) to
55% (#1) – see Table 4.1 To confirm knock down at mRNA level we conducted qRT-
PCR using cDNA prepared from all six heterogeneous cell populations. When data
were compared to control cells we found that levels of knockdown varied from 48% to
72% (see Fig 4.2.). In addition the level of IGFBP-2 knockdown in different cell popu-
lations as determined by qRT-PCR followed closely the data obtained by ELISA
(compare Figs 4.1 and 4.2). In experiments which knock down components of the IGF
axis it is important to establish that knock down of individual genes does not lead to
compensatory effects in the expression of other components of the axis. This is par-
ticularly important for IGFBPs where *in vivo* genetic knock out studies indicate there
may be some redundancy of function in this gene family. Therefore we used qRT-

PCR analysis to profile changes in the five IGF axis genes expressed in TamR cells following knock down of IGFBP-2. Profiling of gene expression confirmed that IGFBP-2 was knocked down in these cells (Fig 4.3). There was no change in IGF-1R or IGF-2R expression in knock down cells. In addition there was no compensatory increase in expression of IGFBP-4 or -5 in IGFBP-2 knockdown cells. Indeed data suggested a small fold decrease in expression of these IGFBPs in IGFBP-2 knockdown cells compared to parental TamR cells.

As the lowest absolute level of protein in knockdown cells was seen in cell population #6 – 3.45±0.34 ng/ml - (see Fig 4.1 and Table 4.1) we chose this cell population for cloning by limited dilution. The results of such an experiment are shown in Fig 4.4 where we established 6 different clones for IGFBP-2 shRNA transfected cells and assayed conditioned medium for IGFBP-2. For one of these clones (F8) the levels of IGFBP-2 in conditioned medium at 2.33 ng/ml were 66% decreased compared to parental TamR cells and approached the values seen for wt cells (see Fig 3.7). In all subsequent experiments clone F8 was used as a knockdown IGFBP-2 model and puromycin was included in all subsequent studies with the cell line.
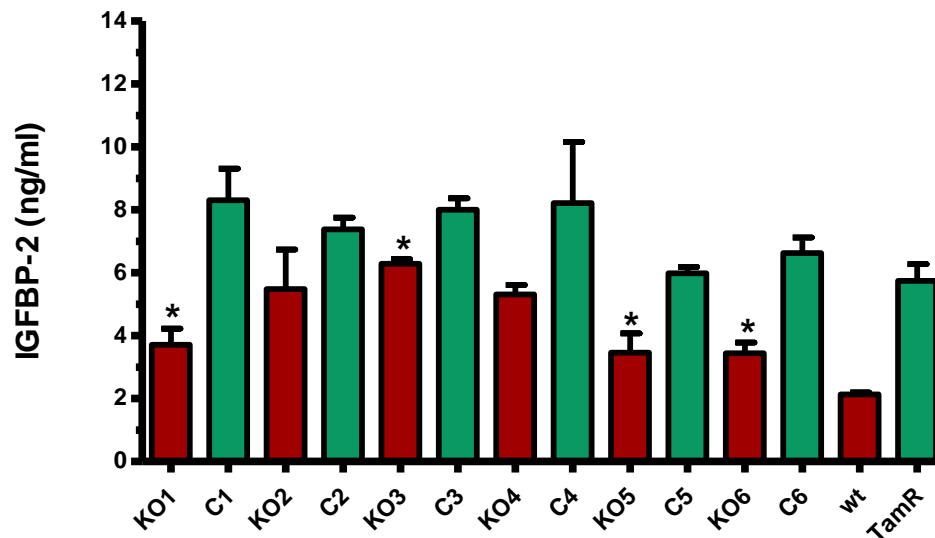
Figure 4-1 Knockdown of IGFBP-2.

Cells were treated with IGFBP-2 shRNA (KO – red bars) or control scrambled shRNA (C – green bars) at transfection reagent: plasmid ratios of 1-6. IGFBP-2 in conditioned medium was assayed by ELISA as described in Fig 3.7 IGFBP-2 was also assessed in medium conditioned by untransfected wt and TamR cells. Data are expressed as mean ± SD n=3 of triplicate technical repeats. * $p<0.005$ KO v control Student's test GraphPad Prism 5.0.

Table 4-1 Knockdown of IGFBP-2.

Cells	IGFBP-2 (ng/ml)	% KO
KO-1	3.7	55
Control-1	8.3	
KO-2	5.4	25
Control-2	7.3	
KO-3	6.2	21
Control-3	8.0	
KO-4	5.3	35
Control-4	8.2	
KO-5	3.4	42
Control-5	5.9	
KO-6	3.4	48
TamR	5.9	N/A
Wt	2.1	N/A

Table 4-1 IGFBP-2 knockdown in transfected cell populations. Mean ng/ml IGFBP-2 for medium conditioned by cell populations #1-6 (KO and control) are reported. % IGFBP-2 knockdown in each transfection is reported relative to controls. Values for TamR and wt cells are 5.9 and 2.15 ng/ml respectively.
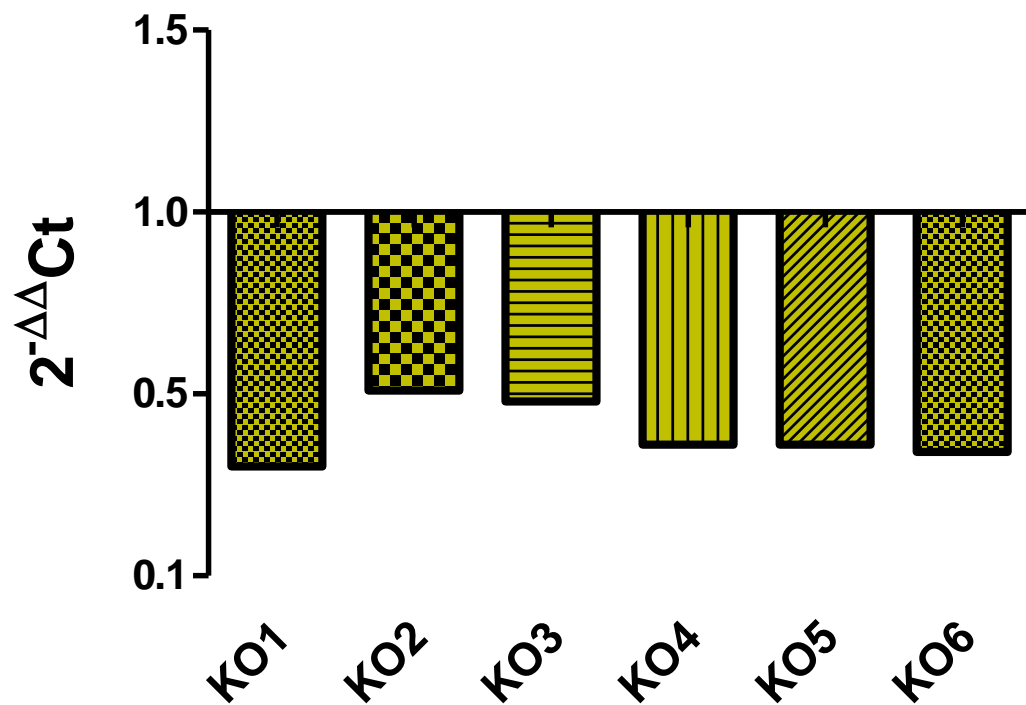
Figure 4-2 IGFBP-2 KO evaluation by qRT-PCR.

Confirmation by qRT-PCR of IGFBP-2 mRNA knockdown in transfected cell popula-
tions #1-6. Knockdown in IGFBP-2 shRNA transfected cells is expressed as 2-ΔΔCt
relative to scrambled shRNA transfected control – (C1 Fig 4.1) Data represent mean
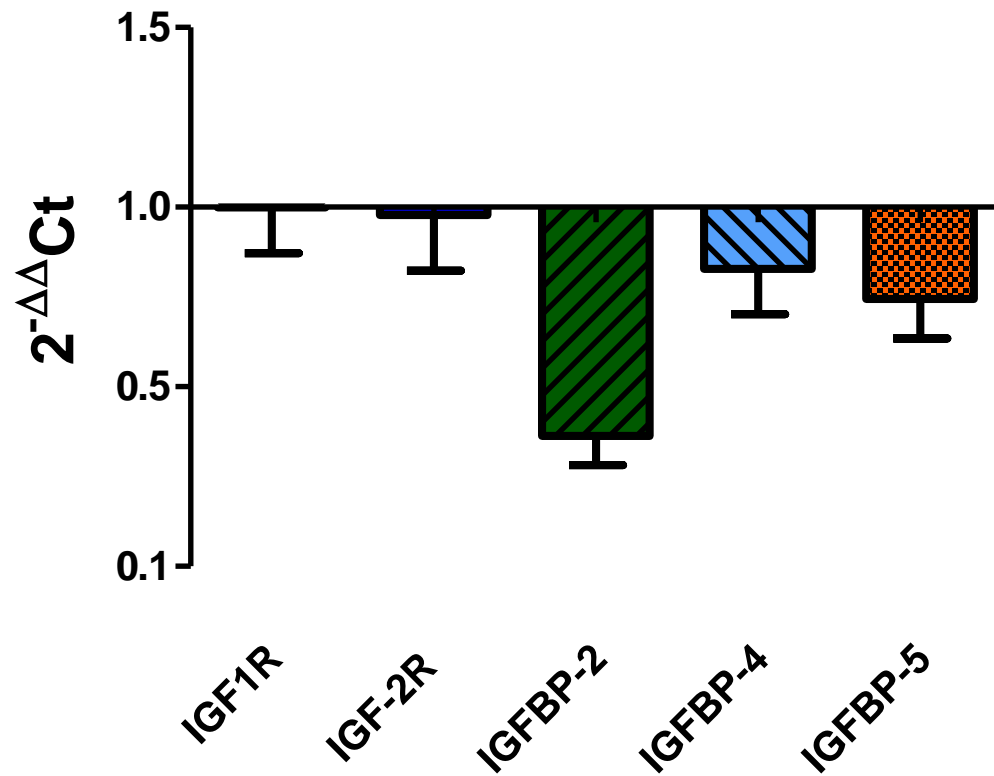levels of KO from duplicate technical repeats.

Figure 4-3 Profiling of IGF axis after Knockdown of IGFBP-2.

The levels of the five IGF axis genes expressed in TamR cells (IGF-1R, IGF-2R, IGFBP-2, -4 and -5) were examined by qRT-PCR following IGFBP-2 knockdown (KO6 Fig 4.1). IGFBP-2 levels were significantly altered in IGFBP-2 KO cells in comparison to control transfected cells (C6 Fig 4.1). Data are expressed as 2-ΔΔCt KO v control transfected TamR cells and represent mean ± SD (n=3).
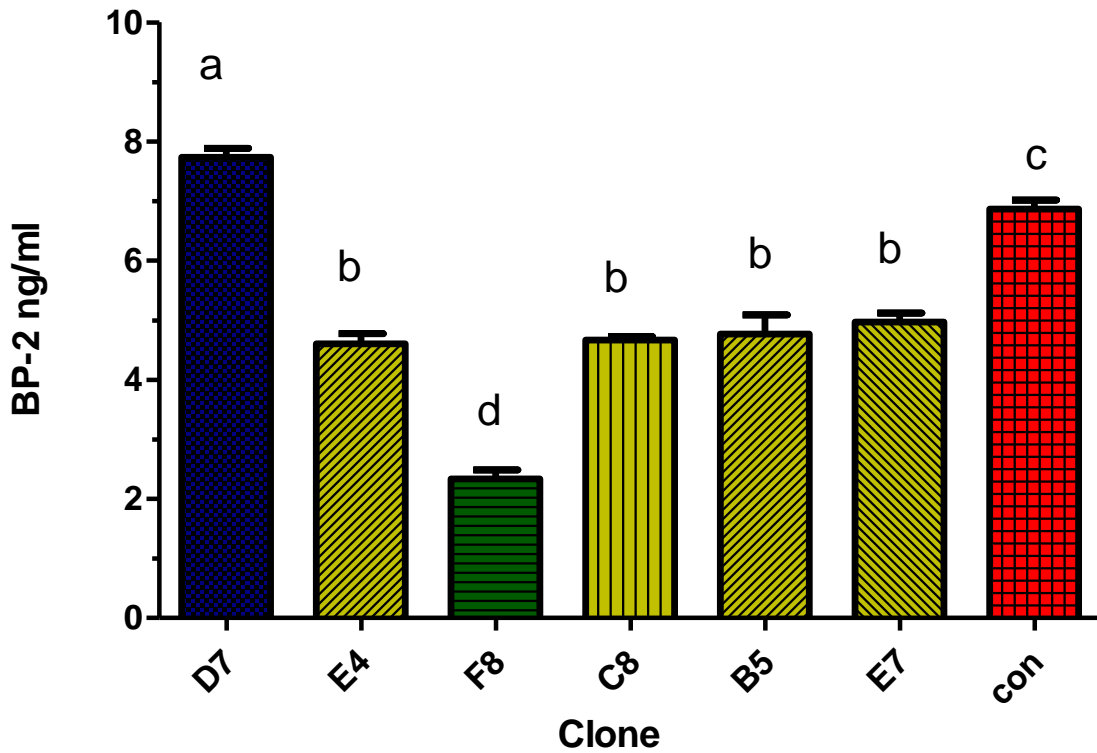
Figure 4-4 Limited dilution cloning of IGFBP-2 knockdown cells.

Heterogeneous cell population (KO6 Fig 4.1) was cloned by limiting dilution. Six different clones were selected and expanded. Conditioned medium from each was assayed by ELISA for IGFBP-2. The red column represents a scrambled shRNA control (C6 – Fig 4.1). Data are expressed as mean ± SD n=3; Columns with different superscripts are statistically different at $p < 0.05$ (One way Anova with Tukey's Multiple Comparison post-hoc test - GraphPad Prism 5.0).

4.4 IGFBP-5 knockdown

IGFBP-5 proved more difficult to knockdown in wt MCF-7 cells. However at the third attempt we were able to achieve some knockdown of IGFBP-5 in this cell population (see Fig 4.5). As for IGFBP-2 knockdown transfections of target and control plasmids were performed in 6 well plates at a ratio of 1:1 to 1:6 where the concentration of plasmid is held constant at 1ug and the volume of transfection reagent is varied from 1-6 ul. In contrast to IGFBP-2 transfections, cells transfected at a ratio of 1:1 and 1:2 did not grow through puromycin selection and therefore only data for cell populations #3-6 are shown. Figure 4.5 shows the results of ELISA analysis of IGFBP-5 protein in medium conditioned by each of the test and control transfected wt cells along with un-transfected wt and TamR cells. For cell population #3 IGFBP-5 concentration in knockdown cells was actually greater than that in putative knockdown cells. However cell populations #4, #5 and #6 all demonstrated significant knockdown of BP-5 in test v scrambled control transfected cells p< 0.005 Student's t-test. The concentration of IGFBP-5 in medium conditioned by KO #4 cells was not significantly different from that seen for wt cells and we did not work further with these cultures. Of the two re-maining cultures the greatest degree of knockdown was seen in cell population #6 although to some extent this was due to the higher scrambled control IGFBP-5 levels for cell population #6. Nonetheless in #6 KO cells IGFBP-5 levels were 4.9 ± 0.13 ng/ml v 10.8 ± 0.27 ng/ml (mean ± SD n=3; KO v scrambled control p< 0.0001) repre-senting a 55% level of knockdown in this cell population. This represents a similar de-gree of maximal knockdown as that achieved for IGFBP-2 knockdown (cell population #6) (see Fig 4.1 and Table 4.1). However of some concern was the evidence that IGFBP-5 knockdown in wt cells was also associated with down regulation of the other 4 IGF axis genes which are expressed in MCF-7 cells (Fig 4.6). Therefore expression of IGF-1R, IGF-2R, IGFBP-2 and IGFBP-4 was 76, 60, 34 and 23 % of that seen in

control transfected cells. In fact the level of knock down of BP-4 expression approached that seen for BP-5 itself (20%). We discuss this at more length in Section 4.7 and despite potential difficulties with interpretation of results we proceeded to clone out IGFBP-5 knockdown cells from population #6 using limiting dilution. The results of these experiments are shown in Fig 4.7 where we selected 11 clones which grew through puromycin selection in 96-well plates. Of these, 6 clones showed IGFBP-5 levels in conditioned medium which were significantly lower than those of a scrambled control transfected cells. Clone B4 had the lowest concentration of IGFBP-5 in conditioned medium 3.1 ± 1.5 ng/ml v 8.3 ± 0.81ng/ml n=3 ± SD; KO v control, p=0.003 representing a 63% knockdown of IGFBP-5 at the protein level. In all subsequent experiments clone B4 was used as a knockdown IGFBP-5 model and puromycin was included in all subsequent studies with the cell line.
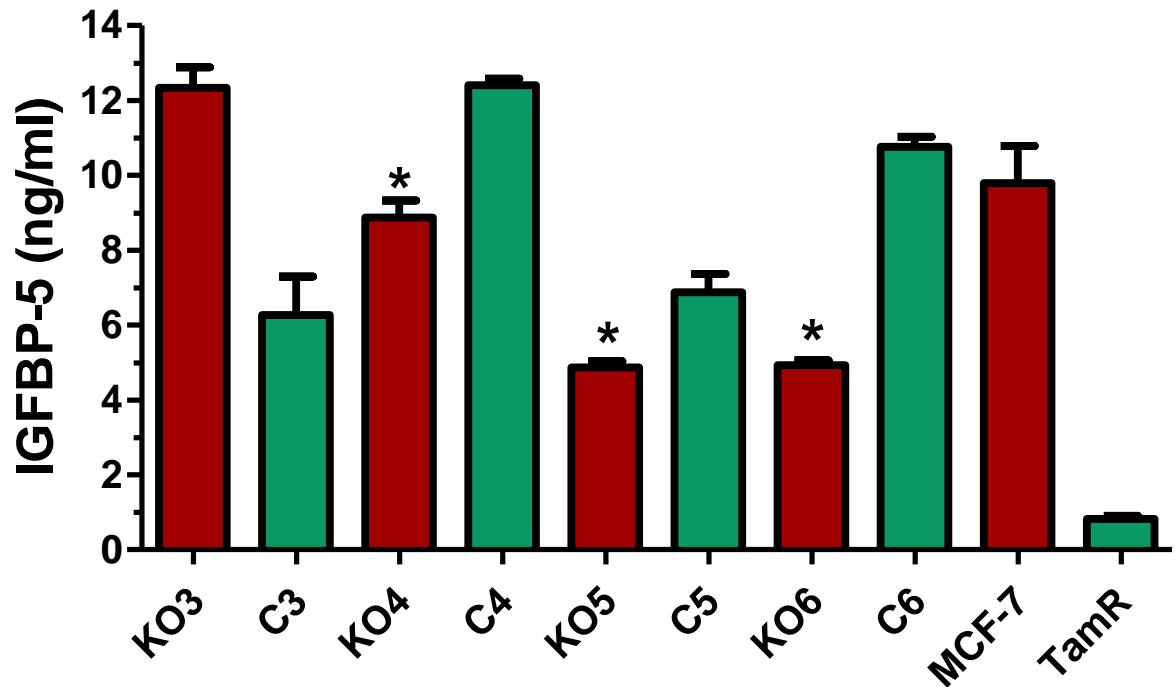
Figure 4-5 Knockdown of IGFBP-5 in wt MCF-7 cells.

Cells were treated with IGFBP-5 shRNA (KO) or control scrambled shRNA (C) at transfection reagent: plasmid ratios of 1-6. Cells transfected at ratios of 1 and 2 did not grow under puromycin selection and only the data from transfections 3-6 are shown. IGFBP-5 was assayed by ELISA as described in Fig3.7. Red bars IGFBP-5 shRNA transfected; green bars scrambled control transfected. IGFBP-5 was also assessed in untransfected wt and TamR cells as indicated. Data are expressed as mean ± SD n=3; * $p < 0.005$ KO v control (Student's t-test; GraphPad Prism 5.0.).
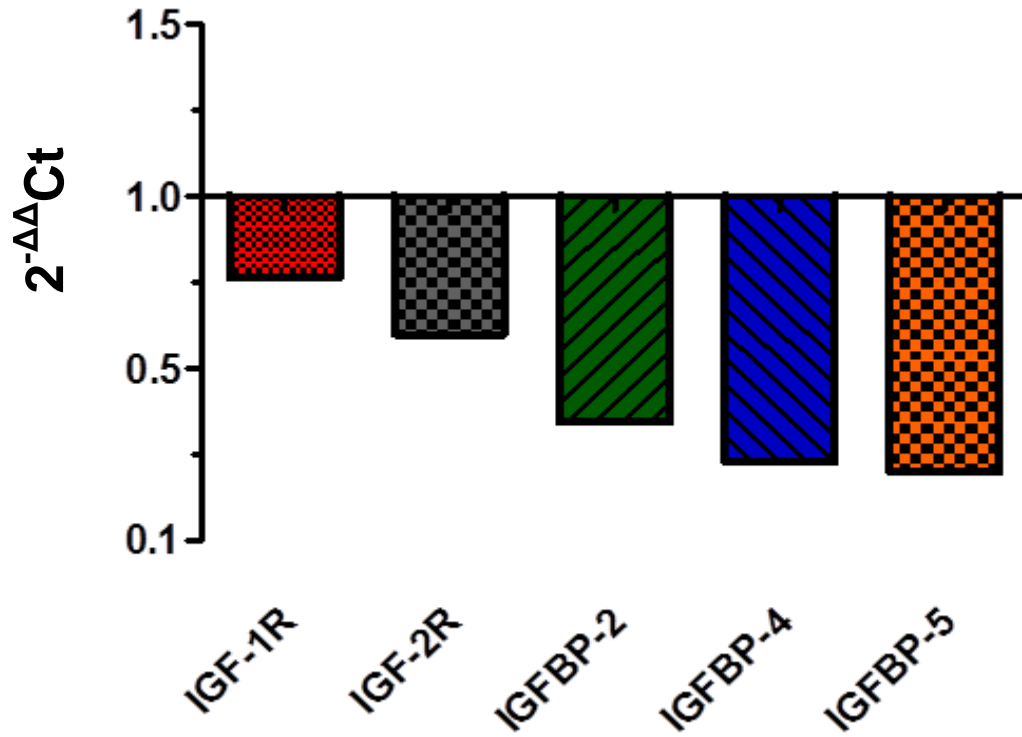
Figure 4-6 Profile of IGF axis after knockdown of IGFBP-5.

The levels of the five IGF axis genes expressed in TamR cells (IGF-1R, IGF-2R, IGFBP-2, -4 and -5) were examined by qRT-PCR following IGFBP-5 KO (KO6 Fig 4.5). Expression of all 5 IGF axis genes levels were altered in IGFBP-5 KO cells in comparison to control transfected cells (C6 Fig 4.1). Data are expressed as 2-ΔΔCt KO v control transfected wt cells. This experiment was performed once and means values of triplicate technical repeats are shown.
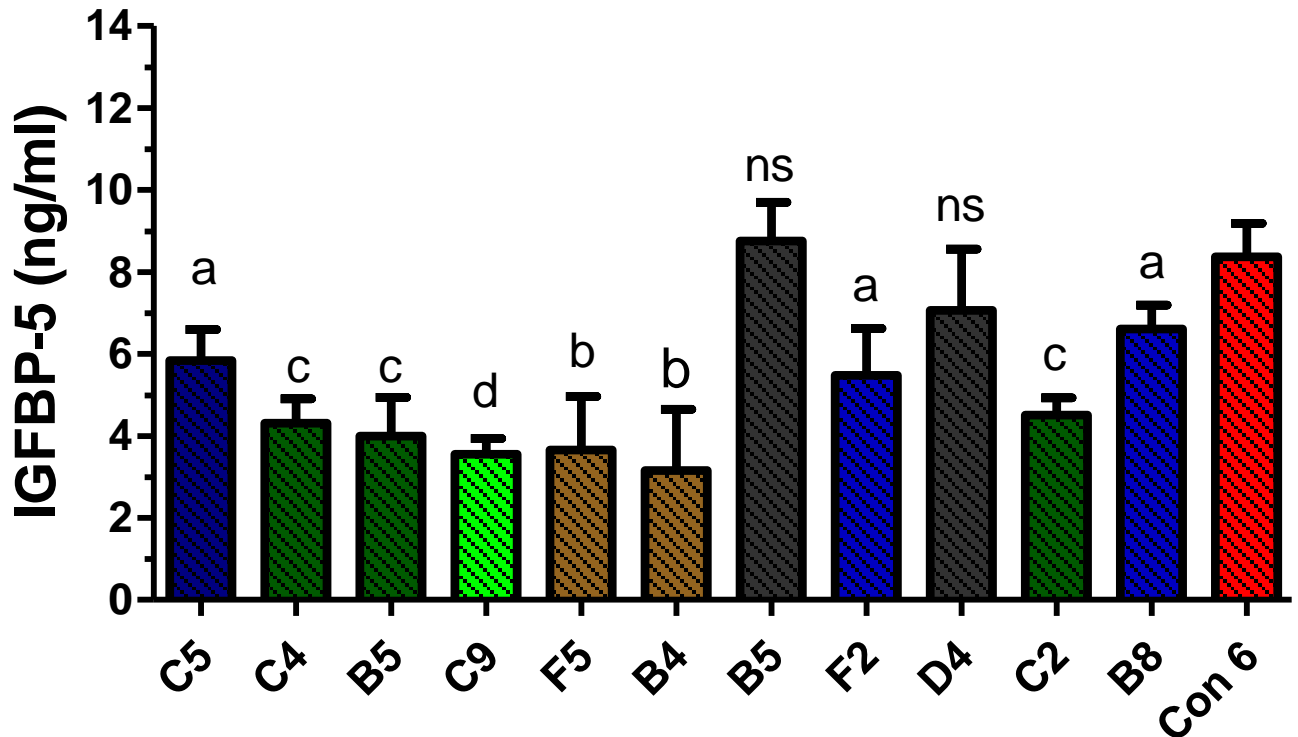
Figure 4-7 Limited dilution cloning of BP-5 KO cells.

Heterogeneous cell population KO6 – (Fig 4.5) was cloned by limiting dilution. Eleven different clones were selected and expanded. Conditioned medium from each was assayed by ELISA for IGFBP-5. The red column is scrambled shRNA control (C6 – Fig 4.5). Data are expressed as mean ± SD n=3; statistically significant v control a (p<0.05); b (p<0.01); c (p<0.005); d (p<0.001) ns not significant (Student's t-test; GraphPad Prism 5.0).

4.5 Cell proliferation and tamoxifen sensitivity

The purpose in establishing IGFBP-2 and IGFBP-5 knockdown cells was to test the hypothesis that either and/or both of these genes played a role in the development of tamoxifen resistance in MCF-7 cells. As a read out of tamoxifen resistance we adopted a well-established cell viability and proliferation assay which uses the formazan dye WST-1 (see Methods section 2.2.5). Such reagents assay both cell number and viability and are a convenient monitor of both cytostatic and cytotoxic activities displayed by test compounds. The shift in absorbance produced by cleavage of the tetrazolium salt WST-1 to its formazan product through the NADH- generating dehydrogenase(s) is considered to be directly proportional to the number of metabolically active cells. Such an assay format lends itself to reasonably high throughput with appropriate replication with respect to time points. Preliminary experiments identified an optimal seeding density of 5000 cells/well for these experiments which were conducted in 96 well plates. MCF-7 passage no 35 and TamR passage no 7 were used. In Fig 4.8 A and B we show the results of two replicated representative experiments which report the effect of 4HT (1uM) on the growth of wt and TamR cells over the time period 0-96hr. For Expt 1 described in Fig 4.8A in the absence of 4HT (upper panel) although the curves show a significant difference using a repeated measures ANOVA (p=0.0176) this is solely due to the 96 hr time point. No significant difference is apparent at any of the other time points (Bonferroni's post-hoc test). In contrast there is a highly significant difference be in the growth curves in the presence of 4HT Fig 4.8A (lower panel) (repeated measures ANOVA p<0.0001). In addition Bonferroni's post-hoc analysis indicates a significant difference between cell responses at the 48 (p<0.01), 72 and 96 hr time points (both p<0.001). Collectively these data suggest that the growth of wt MCF-7 cells is inhibited in 1uM 4HT whereas the growth of TamR cells, as expected, is largely unaffected. Therefore this WST-1 based assay combined

with the IGFBP knockdown cells represents an appropriate experimental design to examine whether IGFBPs play a causal role in the development of TamR.
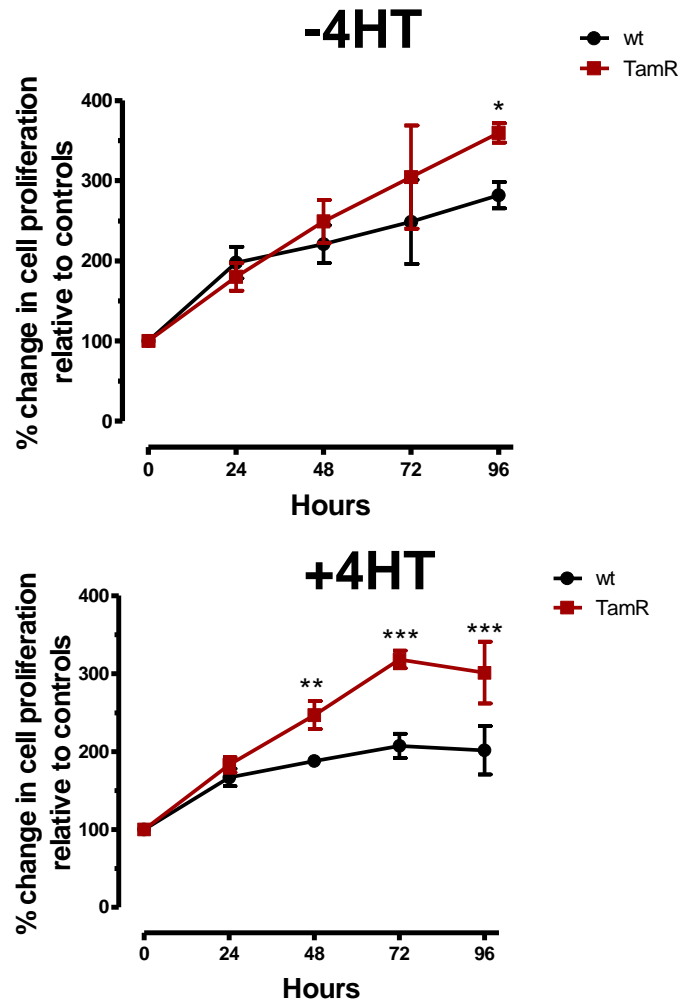

Figure 4-8A Growth of wt or TamR cells in 1uM 4HT

Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates in the absence (top panel) or presence (bottom panel) of 1uM 4HT. Cell growth was monitored over the period 0-96 hr. by WST-1 assay as described in Materials &Methods and was normalised to t=0 (100%). Each data point represents the mean +/-SD of triplicate technical repeats. Curves were analysed by repeated measures ANOVA followed by Bonferroni's post-hoc test *p< 0.05 ** p<0.01; ***p<0.001; GraphPad Prism 5.0. Raw OD450 values are reported in Appendix – Fig 4.8A (S).

Accordingly we used the cloned knockdown cell lines F8 (for IGFBP-2) and B4 (for IGFBP-5) prepared as described in Figs 4.4 and 4.7 and examined their sensitivity to growth in 4HT. Initially we used the TamR IGFBP-2 knockdown cells (clone F8) and reasoned that if increased IGFBP-2 expression in TamR cells (see Figs 3.3 – 3.7) was associated with development of TamR then knockdown of IGFBP-2 in these cells should restore sensitivity to 4HT. The result of such an experiment is shown in Fig 4.9. In the absence of 4HT (Fig 4.9 - upper panel) repeated measures ANOVA analysis followed by Bonferroni's post-hoc test indicates that only the 96hr time point shows significant difference between control and IGFBP-2 knockdown cells ($p<0.05$). No significant difference is apparent at any other time points. In contrast in the presence of 4HT (Fig 4.9 bottom panel) repeated measures ANOVA analysis indicates a highly significant difference between the growth curves for IGFBP-2 knockdown and control cells ($p<0.001$). In addition at 24, 48 and 72 hr time points there is a significant difference between the two cell lines ($p<0.001$). At the 96 hr time point these differences in cell growth are not seen. Therefore our data suggest that at least earlier time points knockdown of IGFBP-2 restores tamoxifen sensitivity to TamR cells as evidenced by compromised growth of the IGFBP-2 knockdown clone F8 compared with scrambled control transfected TamR cells. This data therefore partly supports the hypothesis that over expression of IGFBP-2 by TamR cells has a causal role in the acquisition of tamoxifen resistance. We also conducted parallel experiments with the wt IGFBP-5 knockdown clone B4 (see Fig 4.7). In this instance we reasoned that if increased expression of IGFBP-5 in wt cells in comparison to TamR cells was associated causally with tamoxifen sensitivity then knockdown of IGFBP-5 in wt cells may result in the acquisition of tamoxifen resistance. The results of such an experiment are shown in Fig 4.10. In line with previous experiments, in the absence of 4HT there was little difference in growth curves for IGFBP-5 knockdown and control

cells. Therefore repeated measures ANOVA followed by Bonferroni's post-hoc test showed no significant difference between the time points for control and knockdown cells ($p > 0.05$). For IGFBP-5 knockdown only the 24hr time point showed a significant difference between control and knockdown cells ($p < 0.001$) with no difference at any of the other time points. Therefore our data indicate that knockdown of IGFBP-2 in TamR cells restores sensitivity to 4HT in these cells perhaps indicating a causal role for IGFBP-2 in the development of tamoxifen resistance. In contrast, knockdown of IGFBP-5 in wt cells does not confer resistance to 4HT in these cells arguing that IGFBP-5 does not play a role in the development of tamoxifen resistance.
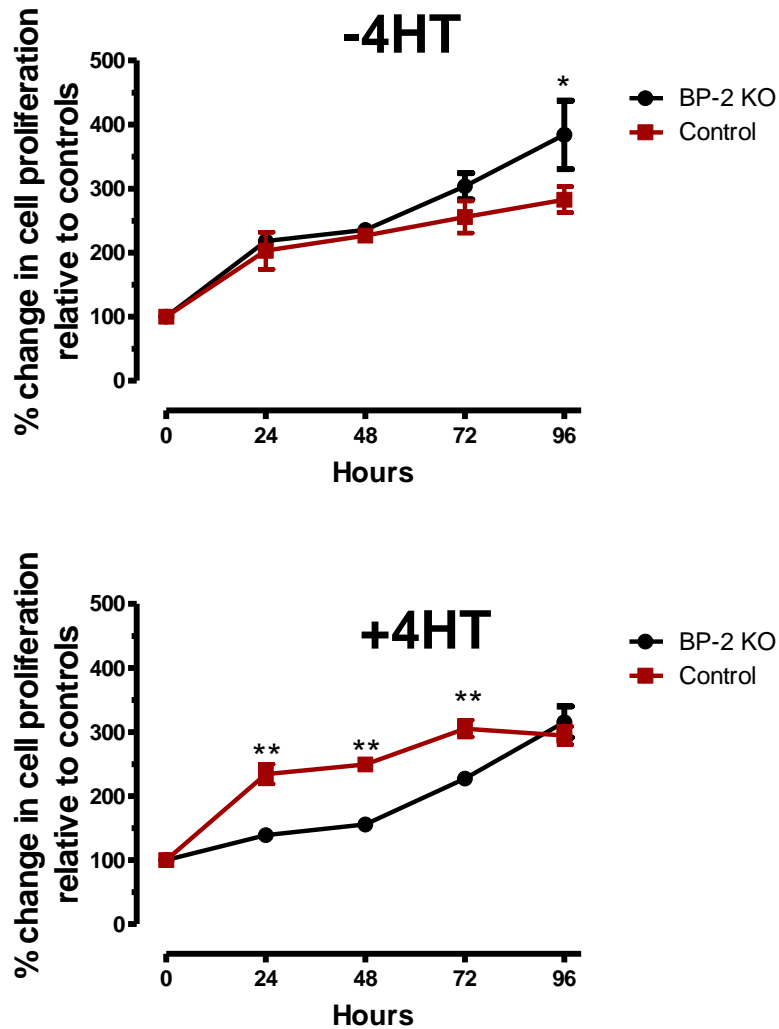
Figure 4-9 Growth of TamR BP-2 KO clone F8 or scrambled control transfected cells in 1uM 4HT.

Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates in the absence (top panel) or presence (bottom panel) of 1uM 4HT. Cell growth was monitored over the period 0-96 hr. by WST-1 assay as described in Materials &Methods and was normalised to t=0 (100%). This experiment was repeated three times with three technical repeats in each experiment and data is presented as mean ±SD (n=3). Curves were analysed by repeated measures ANOVA followed by Bonferroni's post-hoc test *p< 0.05 ** p<0.001; Raw OD450 values are reported in Appendix – Fig 4.9S.
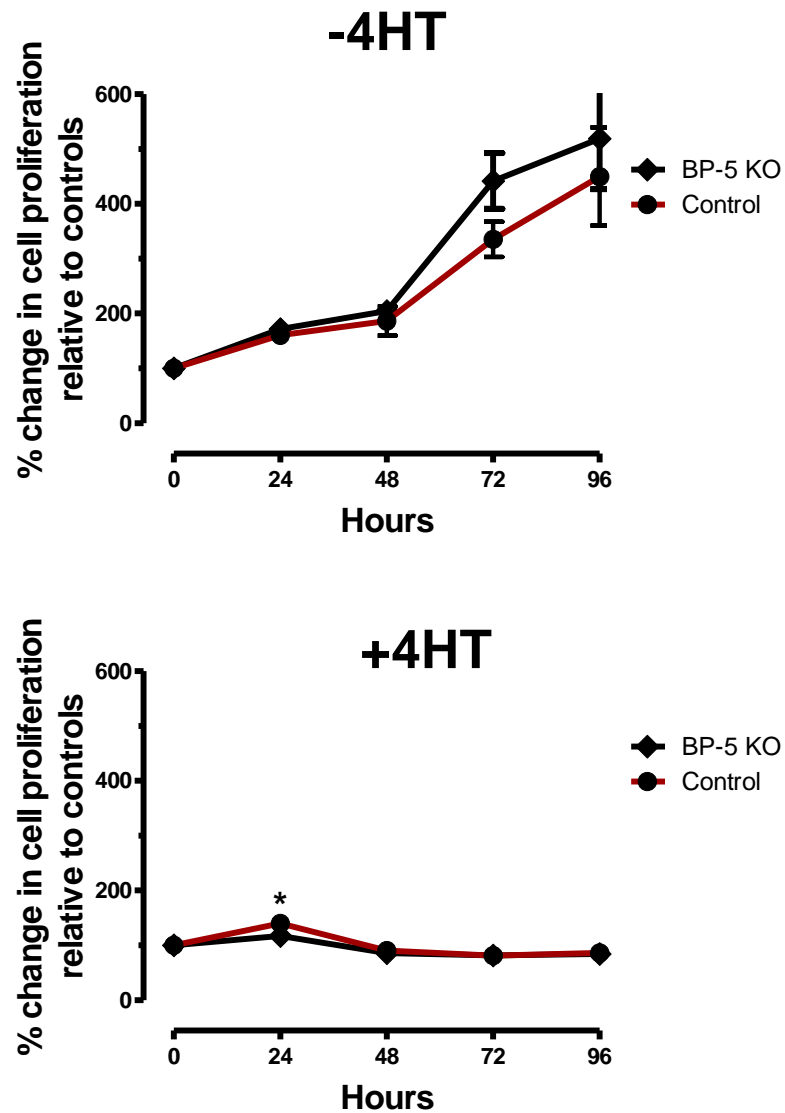
Figure 4-10 Growth of wt BP-5 KO clone B4 or scrambled control transfected cells in 1um 4HT.

Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates in the absence (top panel) or presence (bottom panel) of 1uM 4HT. Cell growth was monitored over the period 0-96 hr. by WST-1 assay as described in Materials &Methods and was normalised to t=0 (100%). This experiment was repeated three times with three technical repeats in each experiment results in each instance and data is presented as mean ±SD (n=3). Curves were analysed by repeated measures ANOVA followed by Bonferroni's post-hoc test *p <0.001; Raw OD450 values are reported in Appendix – Fig 4.10S.

4.6 Effects of exogenous IGFBP-2 and IGFBP-5 on wt and TamR cells.

Because the data obtained in the previous section using knockdown studies suggested that IGFBP-2 and IGFBP-5 could influence tamoxifen sensitivity in MCF-7 cells it was important to examine whether these effects could be replicated by the presence of exogenous IGFBPs. This hypothesis postulates that in the presence of increased exogenous IGFBP-2 wt cells would acquire tamoxifen resistance and that conversely in the presence of excess IGFBP-5 TamR cells would regain sensitivity to 4HT. These experiments also test the hypothesis that IGFBPs are able to exert these effects extracellularly. As a preliminary to this work we performed experiments to examine the effects of IGFBP-2 or IGFBP-5 alone or complexed with IGF-1 on wt and TamR cell growth and viability as determined by WST-1 assay. Such preliminary experiments are necessary for accurate interpretation of subsequent experiments which investigate the effects of extracellular IGFBPs in the presence of 4HT. We also included experiments which examined the effects of IGF-1 on MCF-7 cell growth. IGF-1 is a well characterised mitogen for MCF-7 cells and these experiments were designed to confirm that in our hands the mitogenic signalling pathways stimulated by IGF-1 were intact in our cell cultures. It should be noted that in order to maximise signal-to-noise ratio, these experiments were performed in a serum-free background. Figures 4.11, 4.12 and 4.13 show dose response curves for IGF-1, IGFBP-2 and IGFBP-5 in both wt and TamR cells. Wt and TamR MCF-7 cells responded in a dose-dependent fashion to IGF-1 (Fig 4.11) over the concentration range 0-100 nM. A maximum response was already evident at 10nM and cells responded to IGF-1 concentrations as low as 0.1 nM. There was some evidence that wt cells were more sensitive to IGF-1 stimulation than TamR cells. However the level of response of both cell types was limited and the standard deviations associated with technical replicates within this series of

experiments were large and this tended to compromise the quality of the data. This was particularly evident in experiments with TamR cells and Bonferroni post-hoc analysis indicated no significant difference between the response of wt and TamR cells at any IGF concentration. We discuss these findings in more detail elsewhere. For IGFBP-2 there was no consistent dose-dependent effect of binding protein in either wt or TamR cells. There was a trend for stimulation by IGFBP-2 at lower concentrations but there was a return to control values at higher concentrations of binding protein (Fig 4.12). Interestingly for IGFBP-5 there appeared to be a stimulatory effect on both cell lines as monitored by the WST-1 assay (p<0.001 repeated measures ANOVA). For wt MCF-7 cells effects were already evident at 0.1 nM and had plateaued at 1nM binding protein. For TamR cells maximum stimulation was apparent at 1 nM and the stimulation peaked at this concentration. However there was no statistically significant difference in the response of wt compared to TamR cells p>0.05 Bonferroni post-hoc test (Fig 4.13).

We then investigated the effect of co-incubation of IGF-1 ± IGFBP-2 or IGFBP-5 on the proliferation of wt and TamR MCF-7 cells. The result of such an experiment with wt cells is shown below in Fig 4.14. Again IGF-1 caused a dose dependent increase in wt MCF-7 cell proliferation with a 75% increase in cell proliferation at 10 nM IGF-1 where the effect of growth factor plateaued. In the presence of 10 nM IGFBP-2 the response to IGF-1 was significantly affected (p< 0.0 01; repeated measures ANOVA). Post-hoc Bonferroni analysis indicated a significant inhibition of IGF-1 activity at each concentration of growth factor in the presence of 10nM IGFBP-2 (p<0.001). For IGFBP-5 the data were somewhat less clear cut. Therefore while repeated measures ANOVA suggested a significant difference in response in the presence of IGF-1 + IGFBP-5 compared to IGFBP-5 alone post-hoc analysis indicated a significant differ-

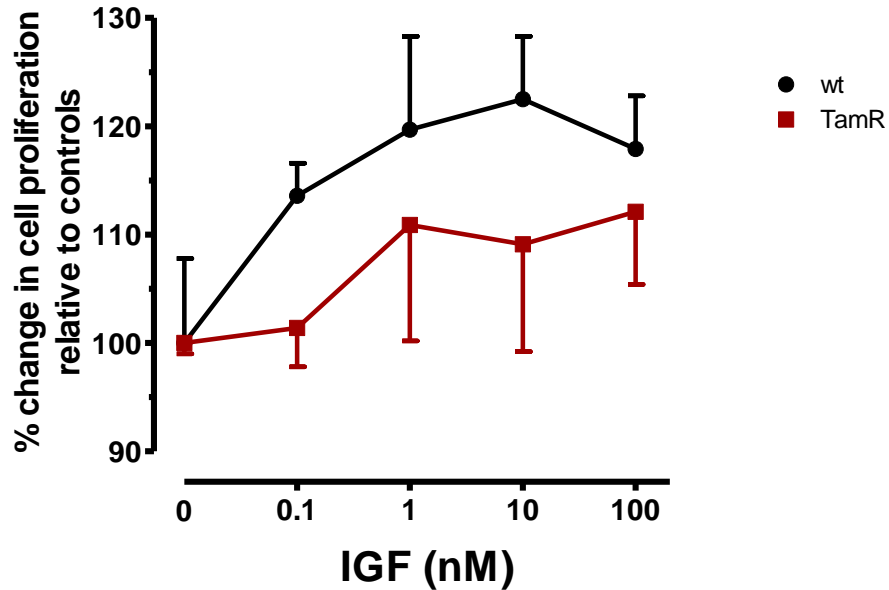ence (p< 0.05) only at the lowest (0.1 nM) and the highest (100nM) concentrations of

IGF-1.

Figure 4-11 Effect of IGF-1 on growth of wt and TamR MCF-7 cells.

Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates. After overnight attachment cells were treated with the indicated concentrations of IGF-1 in 100 ul of serum free PR free medium. After 48 hr WST-1 reagent (10ul) was added and A450 was determined after 30 min incubation at 37˚C. This experiment was repeated three times with triplicate technical repeats in each experiment. Data are presented as mean±SD (n=3) and are expressed as % control (0nM IGF-1). Curves were analysed by repeated measures ANOVA followed by Bonferroni's post-hoc test. Raw OD450 values are reported in Appendix – Fig 4.11S.
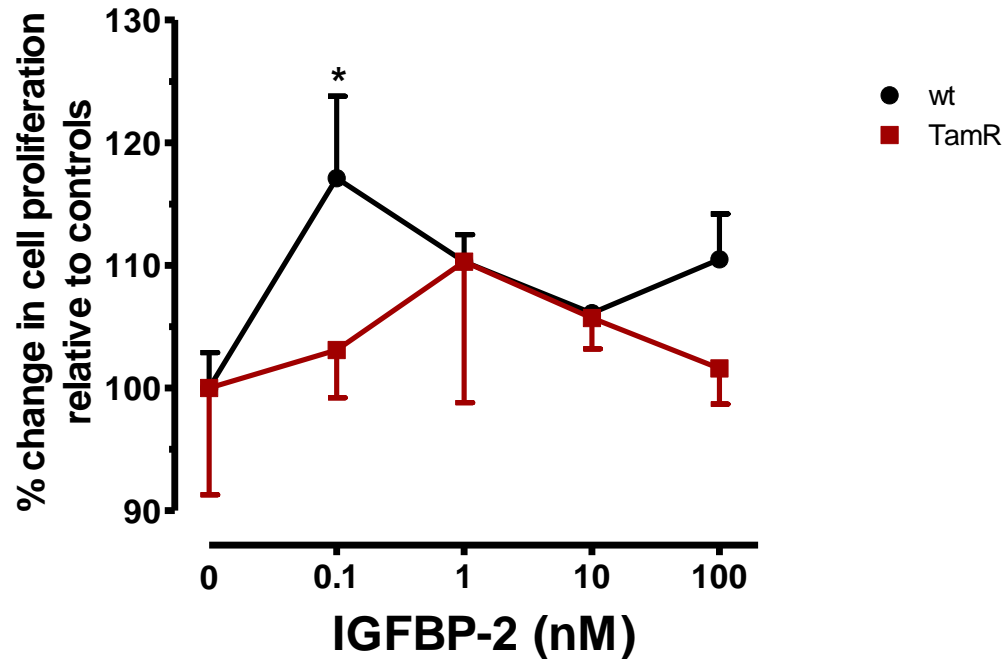
Figure 4-12 Effect of IGFBP-2 on growth of wt and TamR MCF-7 cells.

Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates. After overnight attachment cells were treated with the indicated concentrations of IGFBP-2 in 100 ul of serum free PR free medium. After 48 hr WST-1 reagent (10ul) was added and A450 was determined after 30 min incubation at 37˚C. This experiment was repeated three times with triplicate technical repeats in each experiment. Data are presented as mean±SD (n=3) and are expressed as % control (0nM IGFBP-2). Curves were analysed by repeated measures ANOVA followed by Bonferroni's post-hoc test. * $p < 0.05$ (wt v TamR) Raw OD450 values are reported in Appendix – Fig 4.12S.
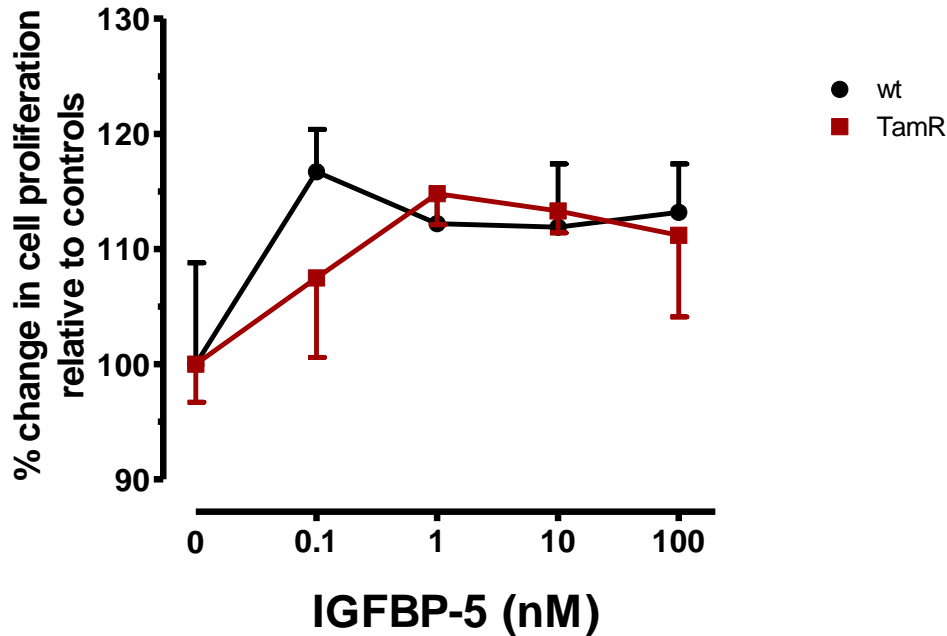
Figure 4-13 Effect of IGFBP-5 on growth of wt and TamR MCF-7 cells.

Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates. After overnight attachment cells were treated with the indicated concentrations of IGFBP-5 in 100 ul of serum free PR free medium. After 48 hr WST-1reagent (10ul) was added and A450 was determined after 30 min incubation at 37˚C. This experiment was repeated three times with triplicate technical repeats in each experiment. Data are presented as mean±SD (n=3) and are expressed as % control (0nM IGFBP-5). Curves were analysed by repeated measures ANOVA followed by Bonferroni's post-hoc test Raw OD450 values are reported in Appendix – Fig 4.13S.
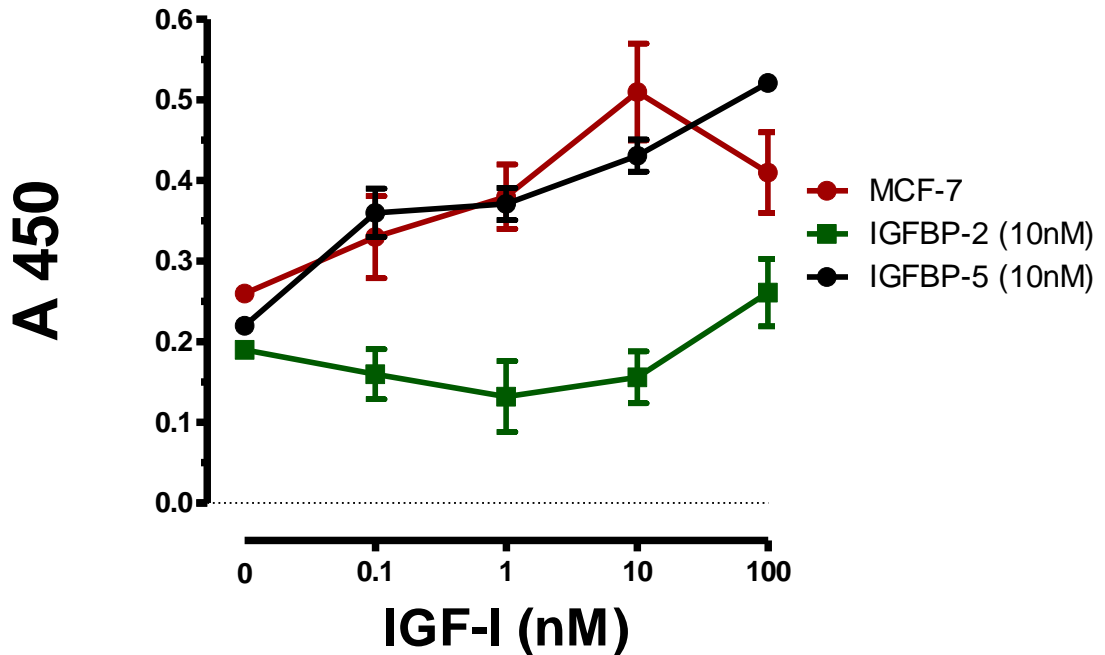
Figure 4-14 Effect of IGF-1 ± IGFBP-2 or IGFBP-5 on growth of <u>wt</u> cells.

Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates. After overnight attachment cells were treated with the indicated concentrations of IGF-1 (0-100nM) in the presence or absence of fixed concentrations (10 nM) of IGFBP-2 or IGFBP-5 in 100 ul of serum free PR free medium. After 48 hr WST-1 was added and A450 was determined after 30 min incubation at 37˚C. This experiment was repeated three times with triplicate technical repeats in each experiment. Data are presented as mean±SD (n=3) and are expressed as raw data (A 450) (0nM IGF-1) Curves were analysed by repeated measures ANOVA followed by Bonferroni's post-hoc test. * p < 0.05 (IGF-1 v IGF-1 + BP-5); ** p < 0.001 (IGF-1 v IGF-1 + BP-2). Raw OD450 values are reported in Appendix – Fig 4.14S.
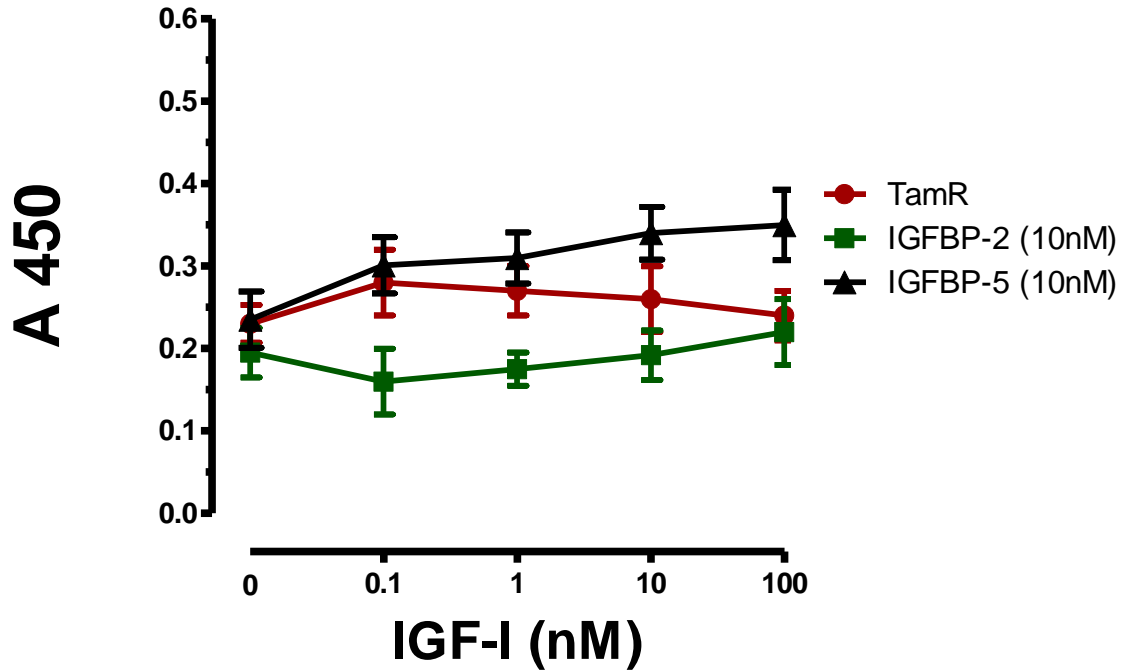
Figure 4-15 Effect of IGF-1 ± IGFBP-2 or IGFBP-5 on growth of TamR cells.

Cells were seeded in 100 ul of 5% DCS, PR free medium at 5000/well in 96-well microtitre plates. After overnight attachment cells were treated with the indicated concentrations of IGF-1 (0-100nM) in the presence or absence of fixed concentrations (10 nM) of IGFBP-2 or IGFBP-5 in 100 ul of serum free PR free medium. After 48 hr WST-1 was added and A450 was determined after 30 min incubation at 37˚C. This experiment was repeated three times with triplicate technical repeats in each experiment. Data are presented as mean±SD (n=3) and are expressed as raw data (A450) (0nM IGF-1). Curves were analysed by repeated measures ANOVA followed by Bonferroni's post-hoc test. ** p< 0.01, *** p<0.001 (IGF-1 v IGF-1 + BP-5) * p<0.01, **p<0.001 (IGF-1 v IGF-1 +BP-2). Raw OD450 values are reported in Appendix – Fig 4.15S.

When these experiments were repeated in TamR cells (Fig 4.15) IGF-1 stimulated cell proliferation although again the response to added growth factor in these cells was less robust than that seen for wt MCF-7 cells (compare redlines Fig 4.14 and 4.15). As for wt cells there was a trend for inhibition of IGF-1 action when IGF-1 was co-incubated with 10 nM IGFBP-2 ($p < 0.0001$ repeated measures ANOVA) and this reached statistical significance at IGF-1 concentrations of 0.1 and 1nM. This inhibitory effect of IGFBP-2 was overcome at higher IGF-1 concentrations. Co-incubation of IGFBP-5 increased the cell response to IGF-1 ($p < 0.0001$ repeated measures ANOVA) with a statistically significant difference seen at the two highest concentrations of IGF-1 ($p < 0.01$) an effect which replicated to some extent the results seen in wt cells (Fig 4.14). In fact the response to IGF-1 + IGFBP-5 (60 % increase compared to control at 100 nM IGF-1 + 10 nM IGFBP-5) is greater than that seen for either IGF-1 or IGFBP-5 alone in TamR cells (see Figs 4.11 and 4.13) suggesting a synergistic effect of IGF-1 + IGFBP-5 in these cells.

These preliminary experiments were conducted to establish the growth parameters of wt and TamR MCF-7 cells in response to IGF-1, IGFBP-2, IGFBP-5 and binary combinations of growth factor and binding proteins. However because our earlier observations clearly demonstrated that IGFBP-2 and IGFBP-5 were reciprocally regulated in wt and TamR cells it was important to examine how the presence of extracellular IGFBPs affected tamoxifen sensitivity. Accordingly we examined the tamoxifen sensitivity of wt cells in the presence of elevated extracellular concentrations of IGFBP-2 and also the tamoxifen sensitivity of TamR cells in elevated concentrations of extracellular IGFBP-5. The results of these experiments are shown in Figs 4.16 and 4.17. For wt cells 4HT again inhibited cell growth (Fig 4.16). Although in the presence of 50 nM IGFBP-2 there appeared to be a statistically significant increase in cell growth in comparison to wt cells grown in the absence of

extracellular IGFBP-2 this effect was not dramatic and the growth curves did not resemble those seen for parental TamR cells (see Fig 4.8 A and B).
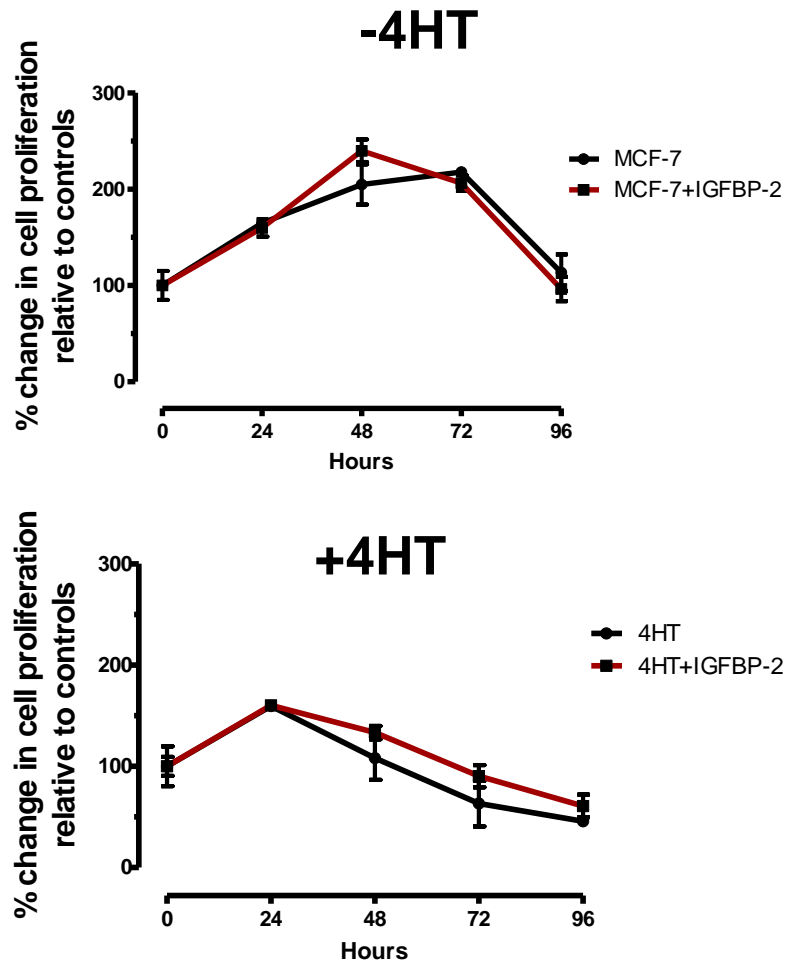
Figure 4-16 Effect of extracellular IGFBP-2 on Tamoxifen sensitivity of wt MCF-7 cells.

Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates in the absence or presence (+BP-2) of 50nM BP-2. Cell growth was monitored over the period 0-96 hr in the Absence (upper panel) or presence (lower panel) of 1uM 4HT using WST-1 assay as described in Materials &Methods and was normalised to t=0 (100%). Each data point represents the mean +/-SD of triplicate technical repeats. This experiment performed twice with similar results in each instance and a representative experiment is shown. Curves were analysed by repeated measures ANOVA followed by Bonferroni's post-hoc test. Raw OD450 values are reported in Appendix – Fig 4.16S.
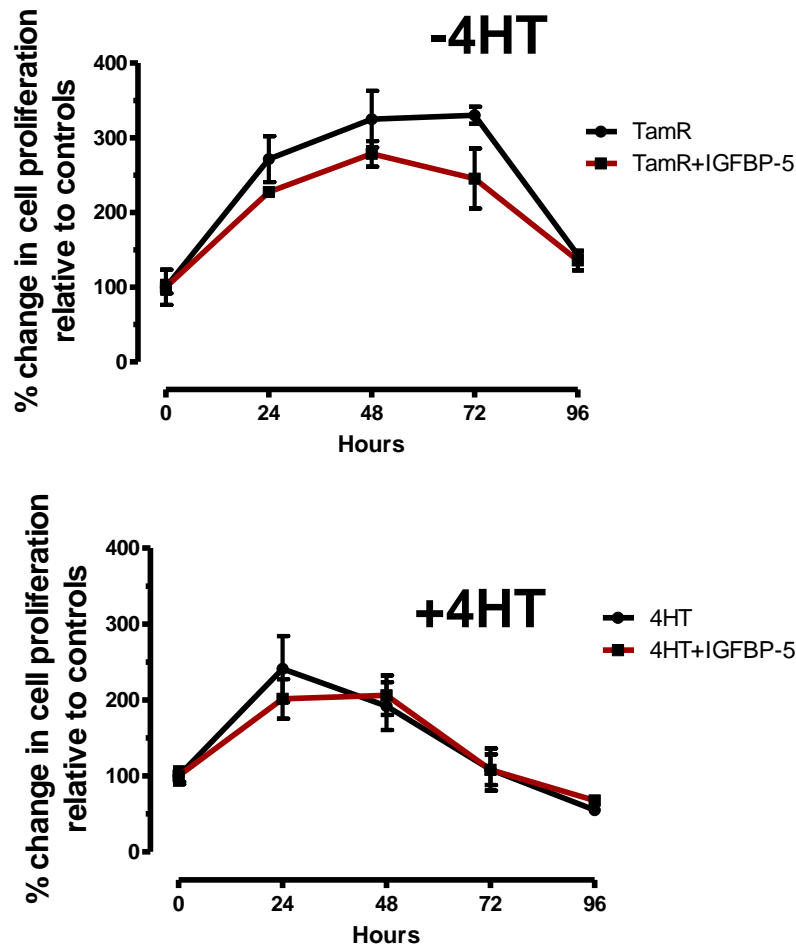
Figure 4-17 Effect of extracellular IGFBP-5.

Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates in the absence (TamR) or presence (+BP-5) of 50nM BP-5. Cell growth was monitored over the period 0-96 hr in the presence of 1uM 4HT using WST-1 assay as described in Materials &Methods and was normalised to t=0 (100%). Each data point represents the mean +/-SD of triplicate technical repeats. This experiment performed twice with similar results in each instance and a representative experiment is shown. Curves were analysed by repeated measures ANOVA followed by Bonferroni's post-hoc test. Raw OD450 values are reported in Appendix – Fig 4.17S.

4.7 Oestrogen Receptor (ER) expression

Because some reports associate lack of effect of tamoxifen with the absence or down regulation of ER expression in TamR cells we first of all analysed the expression of ER isoforms in wt and TamR MCF-7 cells. We investigated the expression of ERα, ERβ isoforms 1 and 2(ERβ1, ERβ2) as well as the recently described plasma membrane G protein linked GPR30. Ct values for expression of these genes as determined by qRT-PCR are shown in Table 4.2 ERα is expressed at a moderate to high abundance whereas all other receptor isoforms are only expressed at very low levels Ct >33. In four separate experiments with wt and TamR cells which examined the expression of ERα in triplicate technical repeats we found a 50% reduction in ERα expression in TamR cells compared to wt cells. Thus although ERα is down regulated in TamR cells it is still clearly expressed. These results are consistent with other reports in the literature. However of more interest for the current study is the recent report by Foulstone et al which shows that silencing of IGFBP-2 in MCF-7 cell leads to a loss of expression of ERα with consequent effects on cell growth. For this reason we also examined ERα expression in IGFBP-2 knockdown TamR cells and this data is presented in Fig 4.18. As indicated the expression of ERα in untransfected and control transfected TamR cells is similar at 62 ± 8.2% and 71 ± 16.6% of the levels seen in untransfected wt MCF-7 cells (mean ± SD n=3; p= 0.47). For IGFBP-2 knockdown cells the expression of ERα is further reduced to 45 ± 11.9% of untransfected wt MCF-7 cells. This level marginally fails to reach significance against both un transfected (p= 0.113) and control transfected (p=0.092) TamR cells. Nonetheless mRNA for ERα is clearly present in IGFBP-2 knockdown TamR cells.

Table 4-2 Raw Ct values after qRT-PCR of ER isoforms in wt and TamR MCF-7 cells.

Wild Type							Tamoxifen Resistant cells					
Ct1	**Ct2**	**Ct3**	**Mean**	**STD**	**ΔCt**	**ER**	**Ct1**	**Ct2**	**Ct3**	**Mean**	**STD**	**ΔCt**
20.19	20.16	19.88	**20.09**	0.17	N/A	RPLP0	18.49	18.44	18.21	**18.38**	0.15	N/A
22.9	23.58	22.87	**23.12**	0.40	3.04	**ESR1-α**	22.62	22.38	22.51	**22.50**	0.12	4.12
34.56	33.87	N/A	**34.22**	0.49	14.14	ESR2-β	33.92	34.49	32.66	**33.69**	0.94	15.31
34.57	32.47	34.18	**33.74**	1.12	13.66	ESR2-β1	36.52	33.99	34.30	**34.94**	1.38	16.56
38.66	36.54	32.80	**36.00**	2.97	15.92	ESR2-β2	36.31	36.23	35.98	**36.17**	0.17	17.79
32.59	32.47	32.35	**32.47**	0.12	12.39	GPR30	33.56	33.39	33.34	**33.43**	0.12	15.05

Table 4.2 Raw Ct values after qRT-PCR of ER isoforms in wt and TamR MCF-7 cells. ERα, Rβ1, ERβ2 and GPR 30 expression was analysed in wt and TamR MCF-7 cells Individual triplicate are reported. ΔCt represents Ct target – Ct HKG (RPLP0).
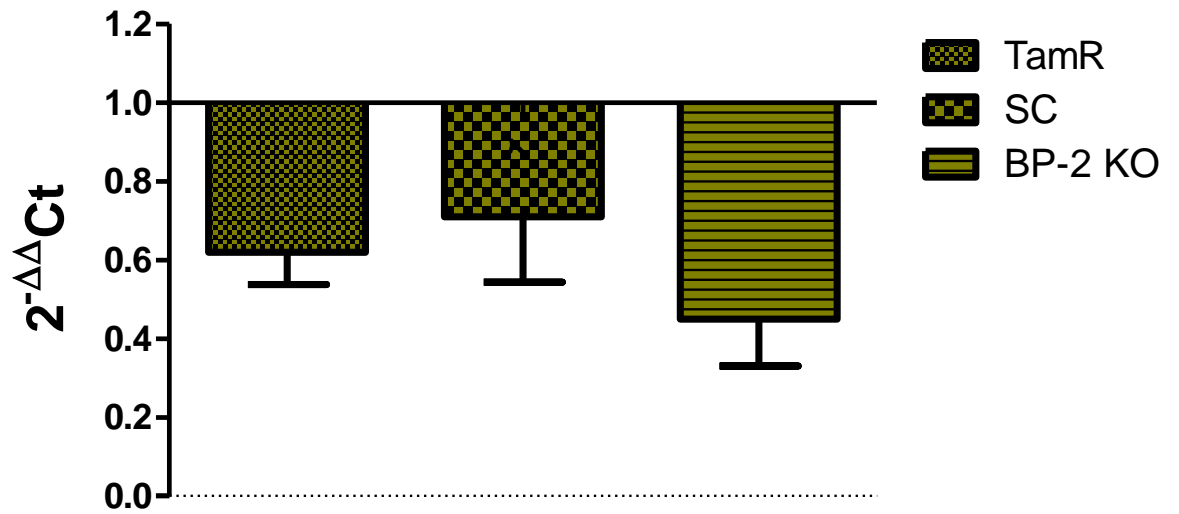
Figure 4-18 Expression of ERα in untransfected TamR cells control transfected TamR cells (sc) and IGFBP-2 KO TamR cells.

Three experiments were performed with each cell line with triplicate technical replicates within each experiment. Data are presented relative to ERα expression in wt untransfected cells in the form 2-ΔΔCt and indicate the decrease in ERα expression in each cell line relative to untransfected wt cells. Mean ± SD (n=3).

4.8 Discussion

In order to examine whether alterations in IGFBP-2 or -5 expression had any causal role in the acquisition of TamR in MCF-7 cells we used an shRNA knockdown strategy. We reasoned that if over expression of IGFBP-2 by TamR cells were associated with TamR then knockdown of IGFBP-2 should result in restoration of tamoxifen sensitivity. Conversely if decreased IGFBP-5 expression by TamR cells is associated with TamR then knockdown of IGFBP-5 in wt cells should impart tamoxifen resistance. The use of stably expressed shRNA from an appropriately designed and integrated vector is a well-established route for gene knockout studies (Fig 2.1) and http://link.springer.com/protocol/10.1007%2F978-1-4939-1538-5_1. We found it relatively straightforward to reduce IGFBP-2 expression in TamR cells using this strategy with success achieved at all transfection reagent: plasmid ratios examined (Fig 4.1and Table 4.1). In addition we were able to fairly quickly establish clonal cell lines form initial heterogeneous cell populations with the former growing through puromycin selection in about 3 weeks after cloning by limiting dilution.

 One difficult issue relates to the degree of target knock down and whether it is sufficient to allow observable biological effects. This can only be addressed experimentally and may only be relevant to carefully defined biological end points. In our case we provide clear cut evidence that attenuation of IGFBP-2 expression in TamR cells is associated with the partial reversal of 4HT resistance supporting a role for IGFBP-2 in the development of tamoxifen resistance (Fig 4.9). We can find no exact parallel studies reported in the literature using TamR MCF-7 cells although there are some studies which report IGFBP-2 KO in wt MCF-7 cells. Foulstone et al used an siRNA based strategy to transiently transfect MCF-7 cells [71]. They reported that loss of IGFBP-2 expression induces cell death. However they also reported that attenuation of IGFBP-2 using this strategy resulted in increased sensitivity of MCF-7 cells to paclitaxel and

doxorubicin induced cell death. Perhaps of more relevance to our studies, in supplementary material published with this report they indicate that IGFBP-2 expression is greater in TamR cells than in wt MCF-7 cells. Although the degree of fold increase in their study approximately 2.2 v 3.5 ng/10^5 cells/24 hr is somewhat less than we report, their observations agree qualitatively with data in this current thesis (see Fig 3.9) and indeed basal values of IGFBP-2 protein as determined by ELISA are very similar in both reports. Although these studies are not exactly comparable with ours the increased sensitivity to chemotherapeutic agents and increased cell death in IGFBP-2 KO and wt MCF-7 cells are not inconsistent with an increased sensitivity to 4HT in IGFBP-2 knockdown cells reported in our study. The design and aim of our work meant that we did not undertake knockdown of IGFBP-2 in wt MCF-7. Nonetheless we believe that our data and that reported by Foulstone et al support a tumour promoter function for IGFBP-2 in BC.

IGFBP-5 knockdown proved more problematical and we did not achieve successful and stable attenuation of IGFBP-5 expression until our third attempt. We have no ready explanation for this although it should be noted that BP-5 knockdown studies were performed in wt cells passage no 35 whereas IGFBP-2 knockdown studies were conducted in TamR cells passage no 7 and perhaps cells may show differential efficiency of plasmid uptake. The late passage no of MCF-7 may have a significance effect on the results. This is perhaps confirmed by the observation that in our successful IGFBP-5 knockdown experiment the lower transfection reagents: plasmid ratios (1 and 2) did not allow the establishment of puromycin resistant colonies (Fig 4.5). Against this in our early failures we did achieve selection in puromycin but no attenuation of IGFBP-5 expression. Although successful knockdown is sequence dependent and specific with respect to the shRNA vector we used the same reagent (s) throughout our IGFBP-5 transfection studies and have no evidence that the manufacturer

changed the targeted sequences for successive batches of the product. It is possible that the appropriate cellular machinery required to process the IGFBP-5 shRNA transcript (see Mats & Methods Fig 2.1) is dependent on cell passage number but we did not rigorously investigate this possibility. Of equal concern was our observation that the expression of the other 4 IGF axis genes examined were also inhibited in IGFBP-5 knockdown cells v transfected controls (Fig 4.6). This was not seen for our IGFBP-2 knockdown studies and it argues that there may be "off target" effects following transfection with the IGFBP-5 shRNA construct or even widespread non-specific effects. Whatever the case we were eventually able to create both heterogeneous and clonal cell populations with attenuated IGFBP-5 expression (Fig 4.5 and 4.7). We found that knock down of IGFBP-5 in wt MCF-7 cells did not influence tamoxifen sensitivity. These generally negative effects meant that we did not pursue qRT-PCR experiments with the IGFBP-5 knockdown clone further – for example to establish whether IGF axis was generally down regulated in this clone as was described in the heterogeneous population of cells from which it was derived (#6 – Fig 4.5). As for IGFBP-2 studies it is difficult to find similar studies of IGFBP-5 KO in the literature especially with respect to tamoxifen resistance as a biological end point. An early study using antisense oligonucleotide directed attenuation of BP-5 expression in wt MCF-7 cells reported an ablation of sensitivity to the SERD fulvestrant [244]. Our hypothesis was that inhibition of IGFBP-5 expression through a stably transfected shRNA strategy would induce tamoxifen resistance in wt MCF-7 cells which is somewhat at odds with the findings of Huynh et al [55]. However these findings may be explained by differences in transfection strategy and anti-oestrogen challenge.

As well as examining the effects of IGFBP-2 and IGFBP-5 knockdown we also considered it important to investigate the consequences of over expression of IGFBP-2 and IGFBP-5 in wt and TamR cells respectively. As a prelude to this we undertook

some preliminary experiments to examine the effects of IGFBPs in both TamR and wt cells in the absence of 4HT. These are required in order to interpret accurately the results of experiments subsequently conducted in 4HT. In general terms we found that wt and TamR cells responded mitogenically to IGF-1 and that this effect was inhibited by co-incubation with IGFBP-2 but was enhanced on co-incubation with IGFBP-5. On its own IGFBP-5 also appeared to have a stimulatory effect in both cell types. The effects of IGF-1 in our cultures were not large and this may reflect the insensitivity of the WST-1 assay relative to other mitogenic read outs such as ^{3}H-thymidine incorporation. In addition these experiments were done under serum free conditions to avoid any potential interference from endogenous IGFs or IGFBPs and under these conditions cell responses may be blunted. However there is evidence in the literature to support both the inhibitory effects of exogenous IGFBP-2 on IGF-1 activity in MCF-7 cells [245] and also to confirm the enhancing activity of IGFBP-5 on IGF-1 action in this cell line [165].

However the main thrust of this experimental work was not to confirm or refute previous studies on IGF and IGFBP effects on the growth of MCF-7 cells but to examine the role of extracellular IGFBP-2 and IGFBP-5 on tamoxifen resistance in these cells and in the absence of expression vectors for IGFBP-2 or IGFBP-5 we used addition of exogenous IGFBPs in our cell cultures to examine the effects of these binding proteins with respect to tamoxifen resistance. When we conducted these experiments we found that neither extracellular IGFBP-2 nor IGFBP-5 affected tamoxifen sensitivity in either wt or TamR cells (Fig 4.16 and 4.17). There may be many explanations for this but possibly the two most important are as follows. Firstly, in these experiments it is assumed that it is extracellular effects of IGFBPs which are being examined. The establishment of stably transfected MCF-7 cell lines over expressing IGFBPs would theoretically allow for both intracellular and extracellular

effects of IGFBPs to be examined (although a strategy to differentiate between these effects would have to be adopted). It may be that the effects of IGFBPs on tamoxifen sensitivity are cell compartment specific and there is evidence that for both IGFBP-2 and -5 this is the case in many cell types including BC cells [163, 246-248]. The second caveat is that the development of tamoxifen resistance typically takes place over a period of several months during which cells are exposed to the drug with resistant cells eventually growing through. This suggests that such a process involves perhaps long term reprogramming of gene expression and it may therefore be unrealistic to expect that the acute treatment of tamoxifen sensitive wt MCF-7 cells would result in the rapid development of TamR. Whatever the case experiments with stably transfected over expressing wt and TamR cells may help at least partly address this issue.

Chapter 5 Results

Cell migration

5.1 Introduction

One way in which tamoxifen resistant cells can display a more aggressive phenotype is by acquiring increased migratory activity [249]. For this reason we decided to undertake experiments to investigate whether in our hands TamR cells displayed increased migratory capacity and whether this property could be manipulated by the knock down of IGFBP-2 or IGFBP-5 using the stably transfected cell lines described and characterised elsewhere in this thesis (see Figs 4.4 and 4.7). We had access to the Incucyte™ automated 96-well wound creation and real time monitoring instrumentation and used this for the experiments described in this Chapter (see also materials and Methods for further details section 2.2.7 on page 60). MCF-7 cells are relatively non-invasive [250] therefore only cell migration and not invasion experiments were performed. In our laboratory MCF-7 cells did not achieve the required density when seeded onto Matrigel to perform robust migration experiments and therefore all migration experiments were performed on plastic.

5.2 Migration of parental wt and TamR cells

The cell migration assay was designed to examine the effect of IGFBP-2 and IGFBP-5 KO on cell migration. However in preliminary experiments we first of all examined the migration of parental wt MCF-7 cells. Cells were seeded and allowed to attach overnight. Subsequently 700-800µm wounds were made using the Incucyte instrumentation as described in Methods section 2.2.7. Cell migration was measured by following wound closure in real time. Images were captured every hour for 94 h and we present images for three different time points, (0, 48, and 72h after wounding) quantitative da-

ta was collected each hour over the 94hr time course. Fig 5.1 shows representative images of wound closure for parental wt and TamR MCF-7 cells and the metric of relative wound density (RWD) is used to analyse and quantify the cell migration data over the 94 h time course of the experiment. Analysis of such data in Fig 5.2 shows that TamR cells show more rapid wound closure (migration) than wt MCF-7 cells (P< 0.0001; 2-way ANOVA) thus confirming data published by other laboratories [26] and providing a base from which to study the effects of IGFBP-2 KO in TamR cells and IGFBP-5 KO in wt cells.

5.3 Migration of IGFBP-2 KO cells

Figure 5.3 shows images of wound closure in IGFBP-2 KO and scrambled (sc) control KO cells. In Fig 5.4 this data is analysed quantitatively and indicates that although IGFBP-2 KO is associated with a significant inhibition of migration of TamR cells (repeated measures ANOVA followed by Bonferroni's post-hoc test) the migration profile of the sc control cells is not statistically different from the IGFBP-2 KO TamR cells (p> 0.05). It is not possible therefore to draw any conclusions with respect to the effect of IGFBP-2 knock down on TamR cell migration.

5.4 Migration of IGFBP-5 KO cells

More encouraging data was obtained following analysis of IGFBP-5 KO in wt MCF-7 cells (Figs 5.5 and 5.6). Therefore analysis showed that knock down of IGFBP-5 in wt MCF-7 cells resulted in a statistically significant increase in cell migration compared to either parental untransfected MCF-7 cells or scrambled (sc) control transfected cells (p < 0.0001) repeated measures ANOVA. Importantly in this instance no statistically significant differences were evident between parental untransfected MCF-7 cells and sc control transfected cells (Bonferroni's post hoc test p>0.05 at all time points). Clos-

er inspection of the data indicated that statistically differences in wound closure rates between IGFBP-5 KO and parental/control transfected cells were apparent at 9 and 27 hr within the time course and remained significant until the end of the experiment (94 hr).
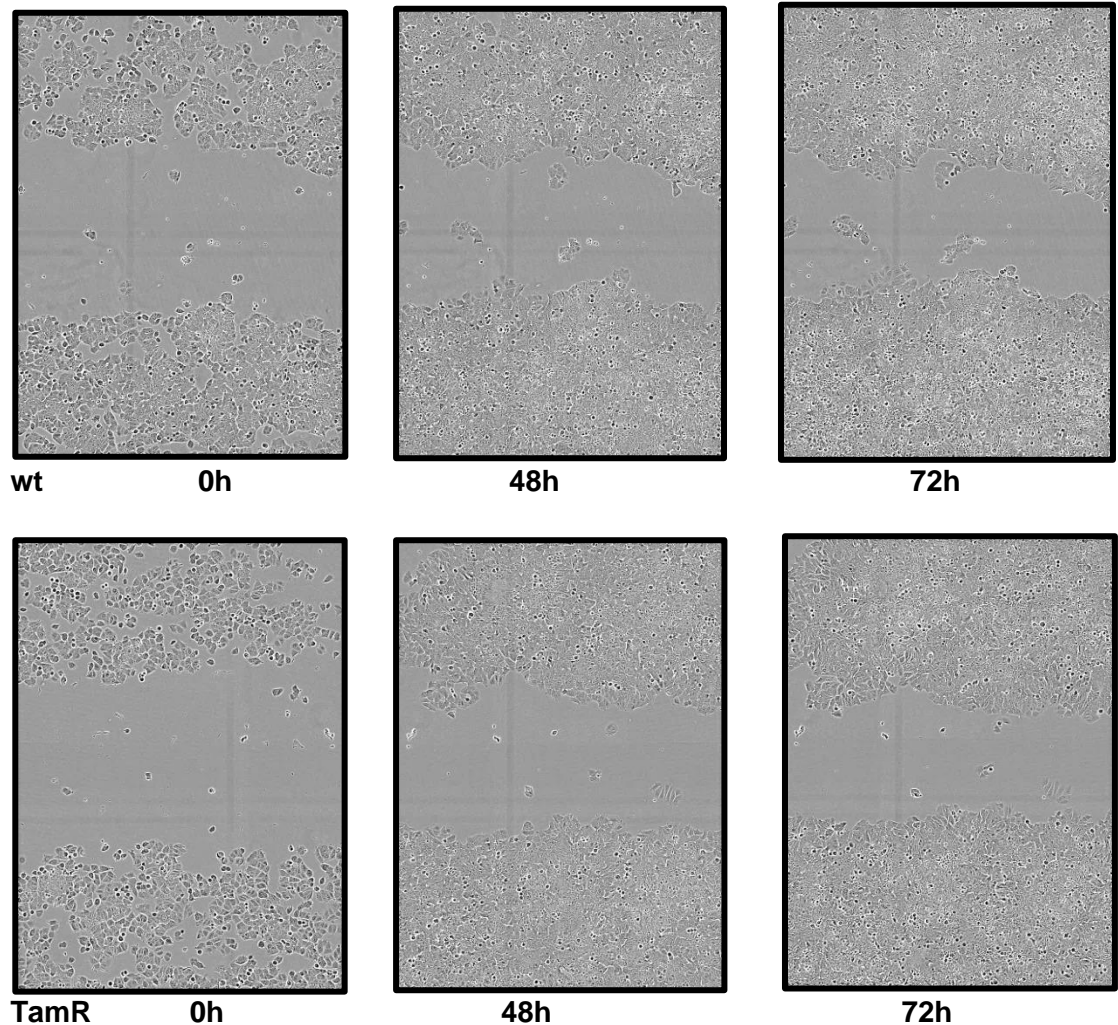

Figure 5-1 Migration of wt and TamR MCF-7 cells

Images represent cell migration (wound closure) at three different time points. Cells were plated at 5 x 10^4 cells per well directly on plastic. After overnight incubation Incucyte was used to create uniform width scratches and wound closure images were subsequently captured after 0, 48, and 72 h after wounding at 10 x magnifications by the IncuCyte™ software. Top panels wt MCF-7 cells; bottom panels TamR cells.
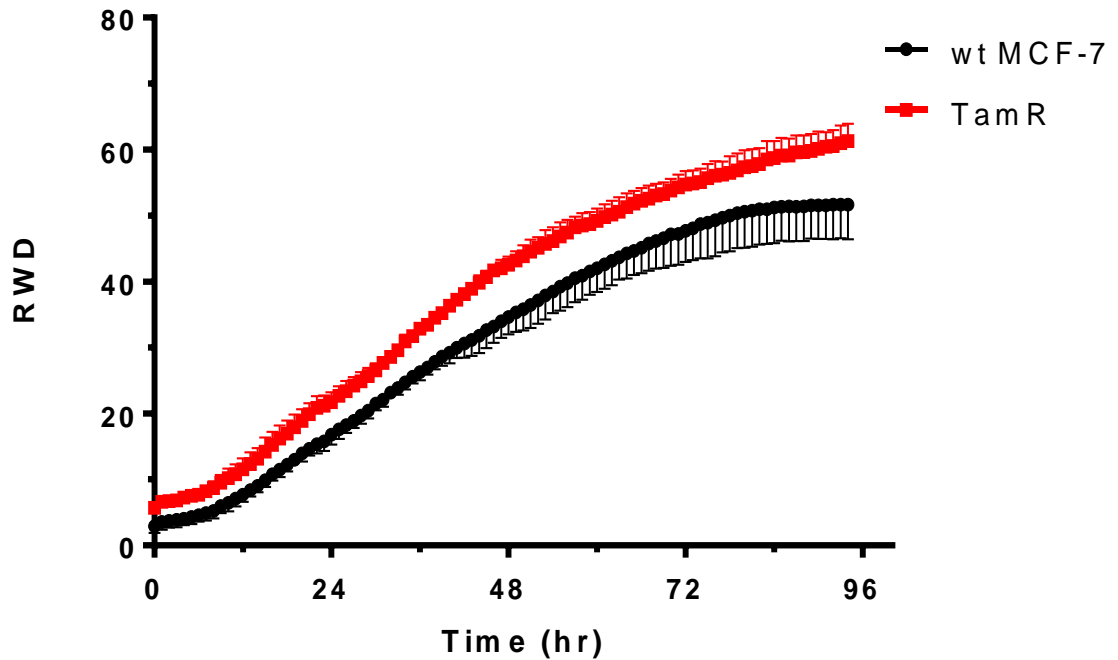
Figure 5-2 Migration of wt and TamR cells plotted RWD vs time.

Real time analysis of relative wound density (RWD) for MCF-7 and TamR. Cell migration was monitored over the period 0-94 hr. The black curve represents wt and the red curve represents the TamR MCF-7 cells. Data are presented as mean ± SD; n=12 for each time point. This experiment was performed twice with similar results in each instance and a representative experiment is shown. Curves were analysed by repeated measures ANOVA followed by Bonferroni's post-hoc test. p< 0.0001 wt v TamR GraphPad Prism 5.0.
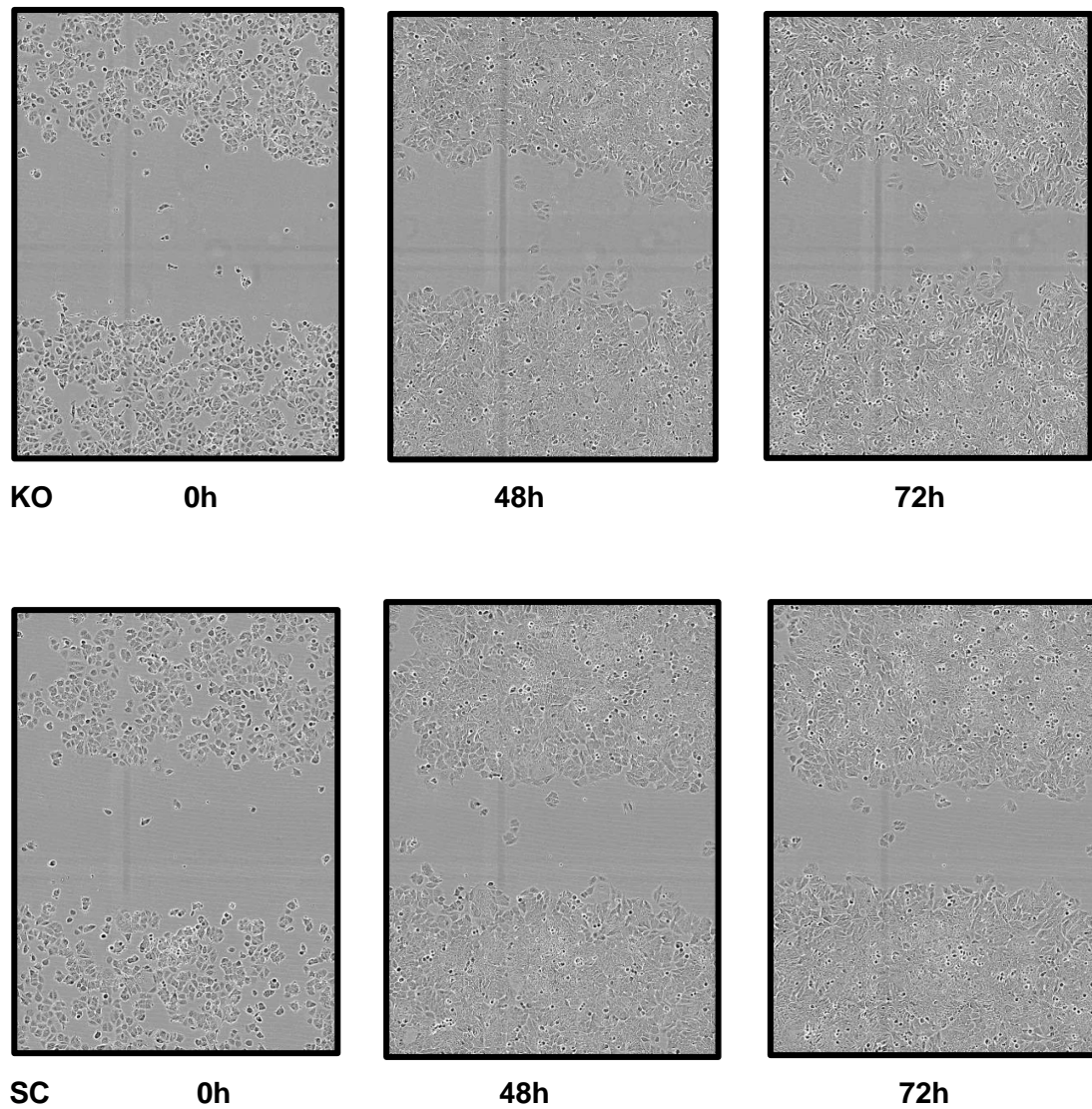
KO   0h   48h   72h

SC   0h   48h   72h

Figure 5-3 Migration of IGFBP-2 KO clone F8 and scrambled control (sc) cells.
The images represent cell migration (wound closure) at three different time points. Cells were plated at 5 x 10^4 cells per well directly on plastic. After overnight incubation Incucyte was used to create uniform width scratches and wound closure images were subsequently captured after 0, 48, and 72 h after wounding at 10 x magnifications by the IncuCyte™ software. KO - IGFBP-2 KO TamR cells; sc scrambled control TamR cells.
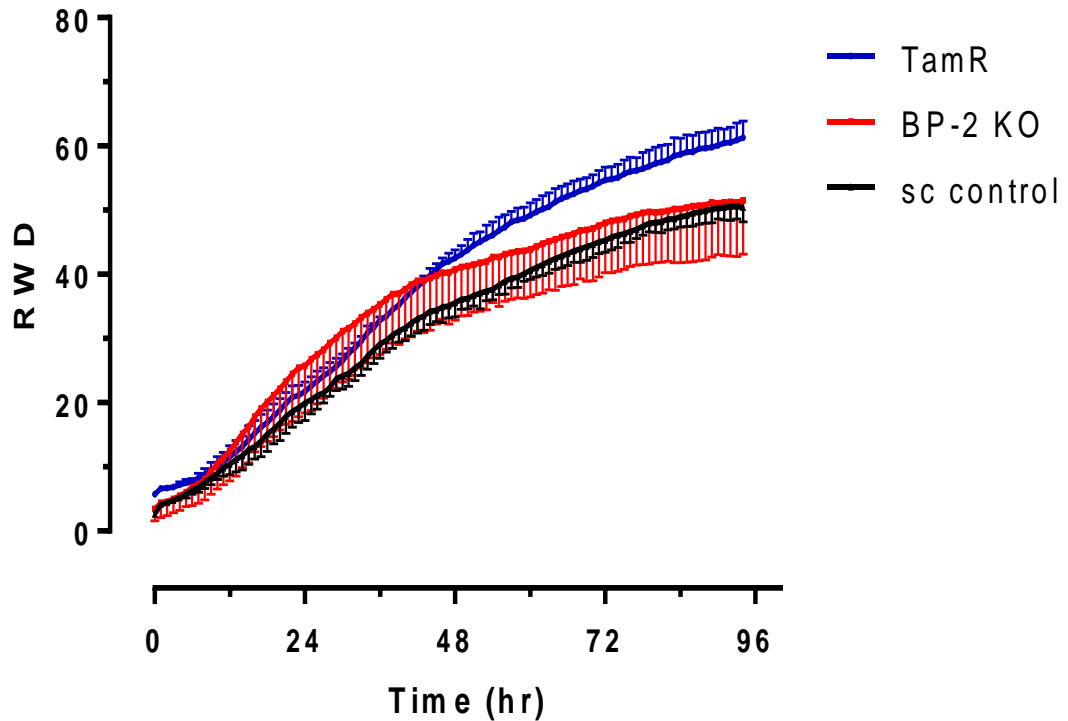
Figure 5-4 Migration of IGFBP-2 KO clone F8 and scrambled control (sc) cells plotted as relative wound density v time.

Cells were seeded at a concentration of 5 X 10^4 cell per well on plastic. The graph demonstrates the relative wound density for IGFBP-2 Ko and scrambled control over time. Cell migration was monitored over the period 0-94 hr. The black curve represents the scrambled control (sc) and the red curve represents the IGFBP-2 KO clone F8. Data are presented as mean ± SD; n=12 for each time point. This experiment was performed twice with similar results in each instance and a representative experiment is shown. Curves were analysed by two way ANOVA followed by Bonferroni's post-hoc test. p< 0.0001 wt v TamR GraphPad Prism 5.0.
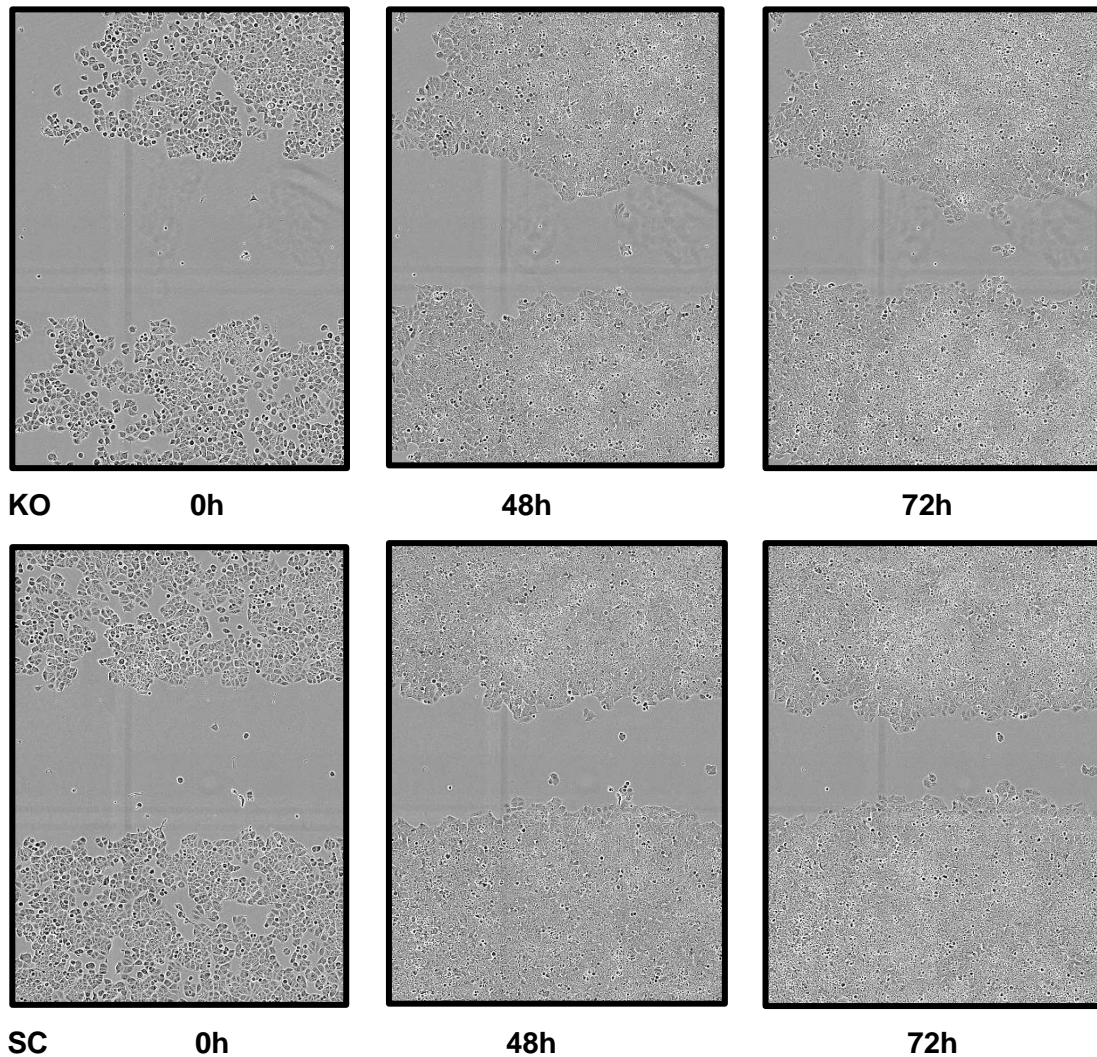
KO   **0h**   **48h**   **72h**

SC   **0h**   **48h**   **72h**

Figure 5-5 Migration of IGFBP-5 KO clone B4 and scrambled control (sc) cells.

The images represent cell migration (wound closure) at three different time points. Cells were plated at 5×10^4 cells per well directly on plastic. After overnight incubation Incucyte was used to create uniform width scratches and wound closure images were subsequently captured after 0, 48, and 72 h after wounding at 10 x magnifications by the IncuCyte™ software.
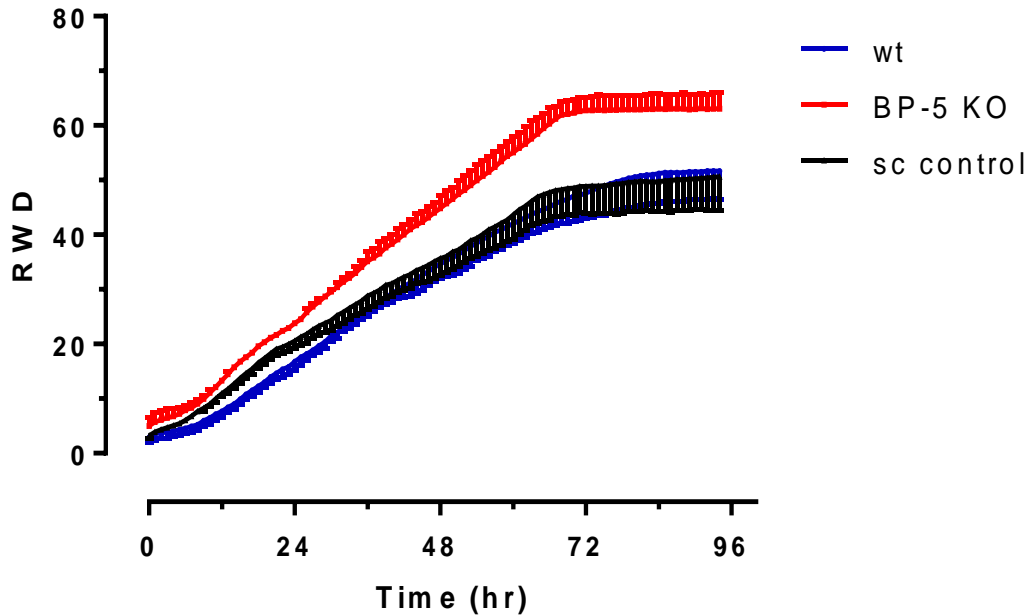
Figure 5-6 Migration of IGFBP-5 KO clone B4 and scrambled control (sc) cells plotted as relative wound density v time.

Cells were seeded at a concentration of 5 X 10^4 cell per well on plastic. The graph demonstrates the relative wound density for IGFBP-5 KO and scrambled control over time. Cell migration was monitored over the period 0-94 hr. The black curve represents the scrambled control (sc) and the red curve represents the IGFBP-5 KO clone B4. Data are presented as mean ± SD; n=12 for each time point. This experiment was performed twice with similar results in each instance and a representative experiment is shown. Curves were analysed by two way ANOVA followed by Bonferroni's post-hoc test. p< 0.0001 wt v TamR GraphPad Prism 5.0.
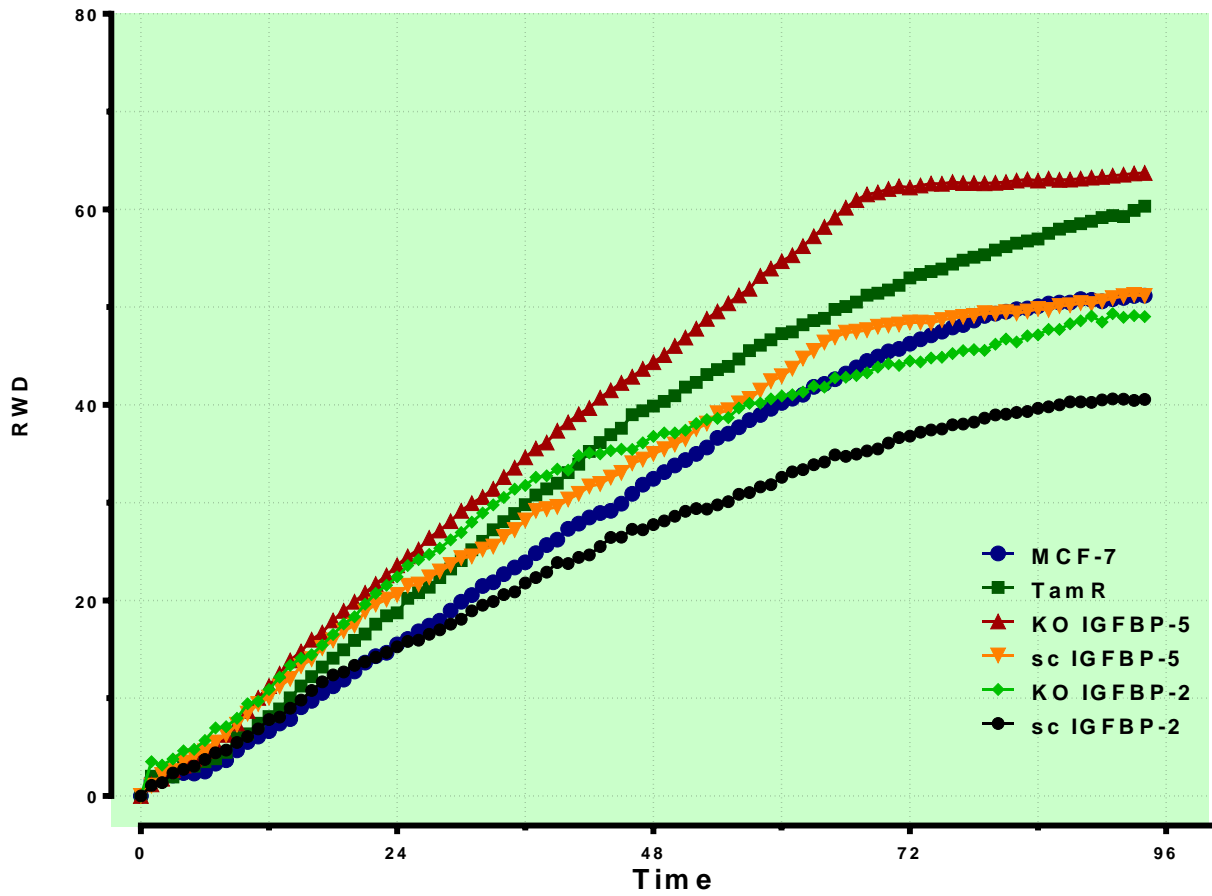
Figure 5-7 Composite data from Figs 5.2, 5.4 and 5.6 representing migration of all six cell lines studied.

Note that for clarity standard deviations have been omitted.

5.5 Discussion

In this chapter we aimed to investigate the effect of knock down of IGFBP-2 and IGFBP-5 on cell migration. We initially demonstrated that TamR cells had a greater migratory activity than wt cells and that therefore this IncuCyte based protocol could be used to monitor the effects of IGFBP-2 knock down in TamR cells and IGFBP-5 knockdown in wt cells on cell migration. Earlier work in this thesis showed that knock down of IGFBP-2 restored tamoxifen sensitivity to TamR MCF-7 cells (Fig 4.9). As such we tested the hypothesis that this restoration of tamoxifen sensitivity may be associated with a diminished cell migratory activity i.e. a restoration of wt MCF-7 phenotype. Although the stably transfected IGBP-2 knock down clone F8 did indeed show reduced migratory activity with respect to untransfected TamR cells (Fig 5.4) we noted that migratory potential was also diminished in the scrambled shRNA stably transfected clone. Although it is unclear why this should be the case it may be that there are some off target effects of the control shRNA which effect the expression or activity of genes closely associated with cell migration. Whatever the case we cannot conclude based on our current evidence that knock down of IGFBP-2 restores a phenotype of reduced migration in clonal cell line F8.

There is a literature which supports a role for IGFBP-2 in the regulation of MCF-7 cell migration and associated phenotypes although it is in part conflicted. For example IGFBP-2 has been shown to down-regulate PTEN expression by an integrin-mediated mechanism in MCF-7 cells culminating in a marked increase in cell proliferation [26]. However there are also reports which suggest that IGFBP-2 may inhibit tumourigenesis *in vitro* and *in vivo* and in MCF-7 cells over expressing integrin β3, IGFBP-2 associated with the αvβ3 complex and inhibited IGF-1 or -2 mediated cell migration. Reduced tumour growth in these transfected cells was associated with integrin mediated localisation of IGFBP-2 to the cell surface [102]. Similarly an engineered protease re-

sistant IGFBP-2 inhibits MCF-7 tumour cell growth as a xenograft in a female nude Balb/c mouse model illustrating the importance of post-translational modification on the activity of IGFBPs. [245]. On occasion pleiotropic effects of IGFBP-2 are evident. Therefore in the ER -ve Hs578T cell line, although IGFBP-2 promoted de-adhesion of cells it also inhibited proliferation through an α5β1 integrin binding mechanism [251]. As this cell line lacks a functional IGF1R such effects were postulated to occur in an IGF-1 independent fashion and indeed subsequent studies using microarray analysis in Hs578T cells demonstrated that exogenous IGFBP-2 regulated the expression of several genes associated with cell proliferation, adhesion and apoptosis [252].

In contrast to the results for IGFBP-2 knock down we found clear evidence that knock down of IGFBP-5 in wt MCF-7 cells was associated with increased cell migration and therefore resembled the phenotype of TamR cells where IGFBP-5 expression is re-duced. In these experiments sc control transfected cells showed a migratory activity which was not significantly different from untransfected wt cells. In fact an analysis of the composite data for cell migration (Fig 5.7) indicates that IGFBP-5 knock down cells show the highest migratory activity of all lines examined including the TamR cells. As IGFBP-5 protein expression levels in B4 knockdown cells approached the low levels seen in TamR cells but were not less than TamR cells (see Figs 4.5 and 4.7) this suggests that IGFBP-5 is not the sole determinant of cell migration in this ex-perimental system. It is also interesting that IGFBP-5 knock down Clone B4 did not shown any indication of a tamoxifen resistant phenotype arguing that tamoxifen re-sistance and cell migration in our experimental may be dissociated with respect to IGFBP-5 action.

In analogy with IGFBP-2 there is also a somewhat conflicted literature with respect to the activity of IGFBP-5 in the regulation of BC cell migration and related phenotypes.

[133]

Our data are consistent with previous findings from our group which report that IGFBP-5 inhibits cell migration in MCF-7 cells [130] and therefore any decrease in IGFBP-5 expression may be associated with increased migratory potential. In fact IGFBP-5 also enhances adhesion of MCF-7 cells to mesenchymal cell derived matrix and may play a role in the inhibition of epithelial-mesenchymal transition (EMT) a process closely associated with tumour cell development and metastasis [131]. In contrast to an inhibitory effect of IGFBP-5 on cell migration, Kricker et al reported that IGFBP-5 enhanced IGF-1 stimulation of MCF-7 cell migration when cultures were grown on vitronectin [253]. However, as always, a distinction must be made between IGF-dependent and independent effects of IGFBPs.

Chapter 6 Clinical significance of IGFBP2 & IGFBP-5 expression in tamoxifen-resistant breast cancer

6.1 Introduction

It has been reported that insulin-like growth factor-binding protein (IGFBP)-2 is associated with breast tumour progression [254] and antiestrogen resistant breast tumour [255]. This association indicates that the up-regulation of IGFBP-2 in breast cancer (BC) can potentially be an important event in breast neoplasia. Moreover, as presented in previous chapters, our results showed some evidence that knocking out IGFBP-2 restores tamoxifen sensitivity. Therefore, an evaluation of the prognostic value of IGFBP-2 and IGFBP-5 is necessary in order to identify their association with survival advantage in tamoxifen-resistant patients as these may have potential to be use as predictive markers in these individuals.

This chapter details an investigation into the potential prognostic value of IGFBP-2 and IGFBP-5 expression in BC. Prognostic value relates to the measurement, at the time of diagnosis, of a biomarker that indicates the predictability of disease-free survival (DFS) or overall survival (OS) of a patient prior to administration of a treatment. Therefore, the aim of the investigation described in this chapter was to assess the prognostic values of IGFBP-2 and IGFBP-5 in a cohort of 424 tumours obtained from women with oestrogen receptor (ER) + BC.

Follow-up data covering a 22-year period were available, and a pathology database was used to identify pathological parameters. In missing cases, individual patient case notes were reviewed. Immunohistochemistry (IHC) was performed using tissue microarrays (TMAs) to evaluate whether IGFBP-2 or IGFBP-5 expression observed in clinical samples was predictive of tamoxifen resistance in BC patients, as described in the Methods section 2.2.8. In order to conduct such an evaluation, it is essential to choose an appropriate IGFBP-2 or IGFBP-5 antibody (Ab). According to the Human Protein Atlas, IGFBP-5 has been detected with weak cytoplasmic positivity in 14 of 80 tissues http://www.proteinatlas.org/ENSG00000115461-IGFBP5/tissue. Our initial attempt to optimise IGFBP-5 showed inconsistent IGFBP-5 IHC staining (see Appendix 3). As a result of several difficulties with IGFBP-5 Ab optimisation, including identification of the correct primary IGFBP-5 Ab and suitable tissue for optimisation, as well as time limitations, we focused on IGFBP-2, and therefore optimised the IGFBP-2 Ab.

6.2 Antibody optimisation

An online system called The Human Protein Atlas was used as a pilot to choose the appropriate tissues and specific IGFBP-2 antibody http://www.proteinatlas.org/. The anti-IGFBP-2 antibody was then initially optimised in kidney at 1:50, 1:100, 1:200 and 1:400 dilutions, and the 1:100 IGFBP-2 antibody concentration was chosen on the basis of its having superior cytoplasmic staining (see Appendix 3). Following optimisation of the IGFBP-2 antibody, the BC TMAs were stained with IGFBP-2 antibody at a 1:100 dilution. The slides were scanned at x20 magnification using the ScanScope™ system, and were visualised for scoring using the ImageScope™ pixel (Px) intensity Aperio algorithm. There are currently no established criteria for determining IGFBP-2 positivity, so definition of the cut-off point was essential.

6.3 Definition of cut-off point for scoring

Our initial cohort consisted of 493 people with BC. However, 69 patients were excluded from the final analysis due to missing clinical data (n = 12) or a core missing TMA (n = 57). Therefore, algorithm scoring was carried out in the remaining 424 patients (289 tamoxifen-sensitive and 135 tamoxifen-resistant), as shown in Figure 6.1.
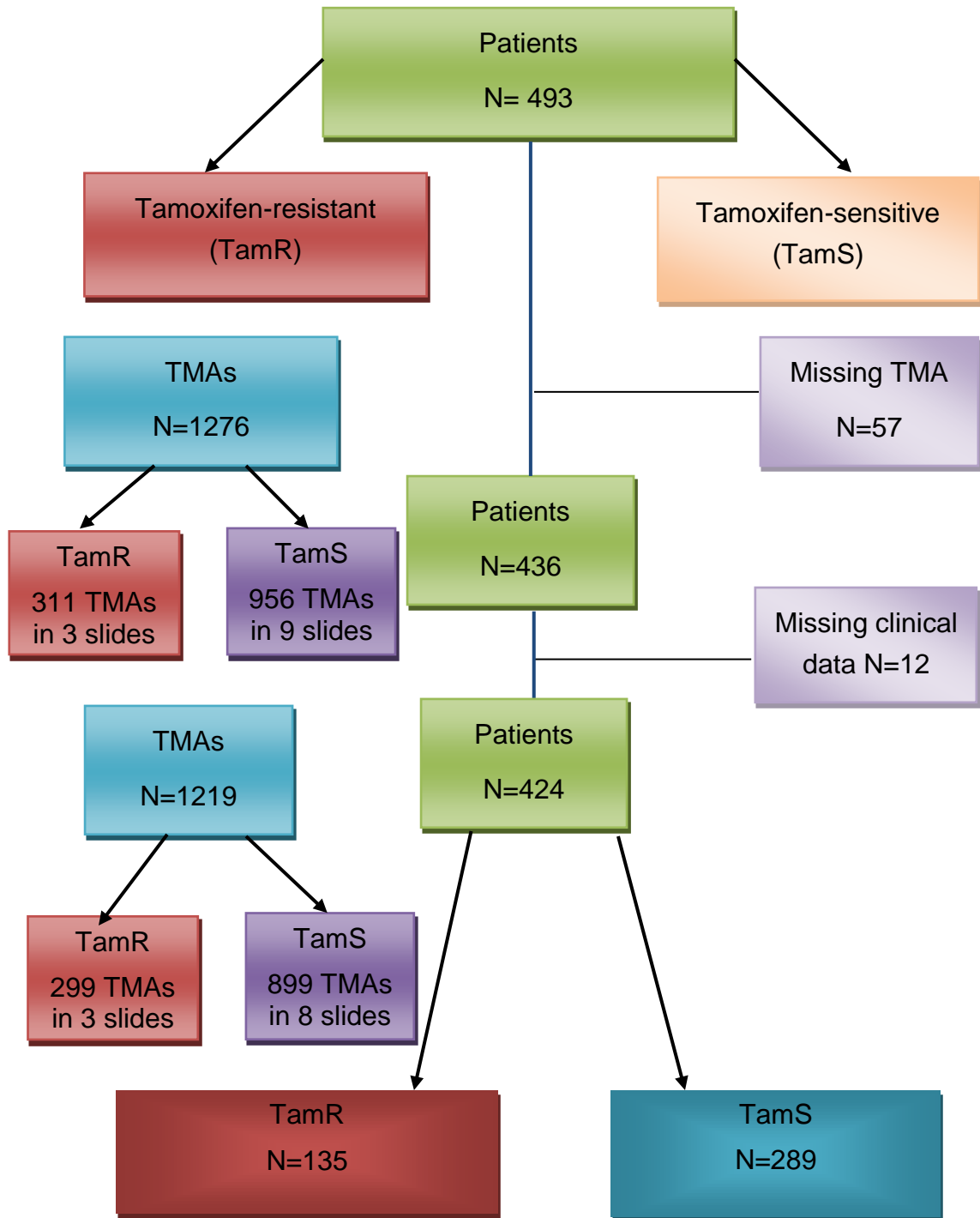
Figure 6-1 Consort diagram representing the patient samples used in the study.

A total of 289 samples from TamS patients and 135 samples from TamR patients were used, giving a total of 424 samples.

After application of the algorithm, the results ranged between 0.00297±Px and 0.96279±Px, with an average of 0.214718± Px for the tamoxifen-sensitive (TamS) cohort and 0.21797± Px for the tamoxifen-resistant (TamR) cohort. This data range necessitated a dichotomisation point that categorised positive and negative staining groups. Scores provided by the Aperio algorithm were then exported to Excel. The data were saved as a text file and uploaded into the cut-off finding system (see http://molpath.charite.de/cutoff/). Receiver Operating Characteristic (ROC) curve was generated [227], and the cut-off point was determined to be 0.1, with 66.7% sensitivity and 41.5% specificity (see Appendix 4). Therefore, any data above 0.1 were considered positive, and any data below 0.1 were considered negative, accordingly. Figure 6.2 illustrates the ROC curve.
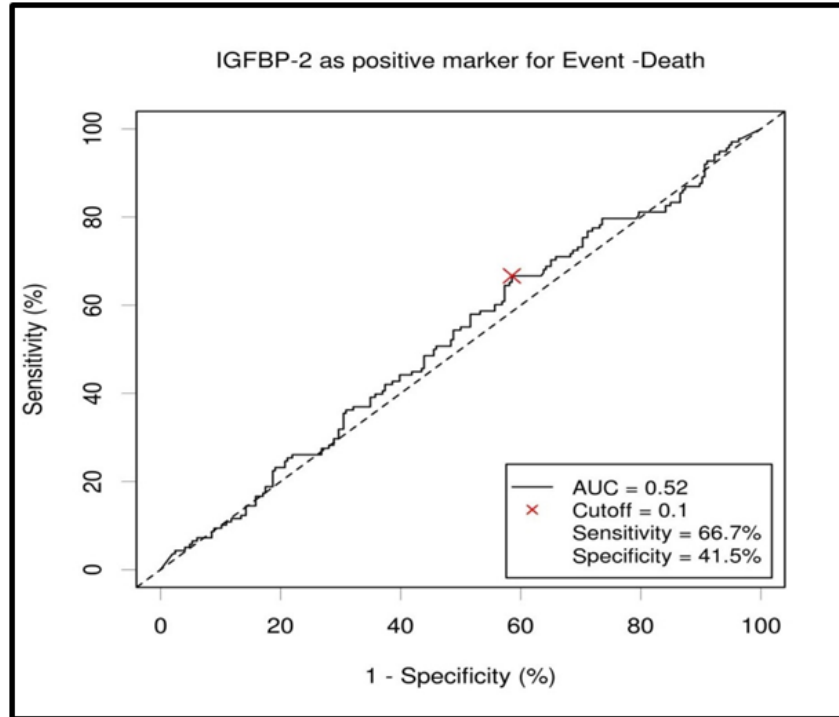
**Figure 6-2 ROC curve to determine optimum cut-off point.**

Using an online cut-off finding system (see http://molpath.charite.de/cutoff/), the cut-off point shown is 0.1 (red cross), with 66.7% sensitivity and 41.5% specificity. A total of 424 samples were included, and the algorithm results ranged between 0.00297± Px and 0.96279± Px.

6.4 Scoring algorithm

Scoring was conducted on the basis of the cut-off point, using the ImageScope™ pixel intensity Aperio algorithm. On IHC staining before application of the algorithm, the blue stain represented the negative, while the brown stain represented the positive. After application of the algorithm, the blue still represented the negative, while the brown stain turned to red to represent positive staining. An example of IGFBP-2 staining before and after application of the algorithm is shown in Figure 6.3.

Following the scoring, the event of 'death' was retrieved from the patients' clinical system and reported as 1 for 'alive' and 0 for 'dead'. The data were analysed using Statistical Package for Social Sciences (SPSS) version 22 software, and a log-rank test (Mantel–Cox test) hypothesis test was used. All statistical tests were two-sided, with a p value of less than 0.05 considered statistically significant.
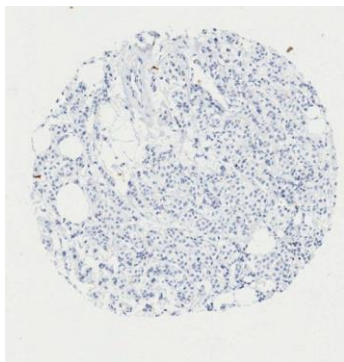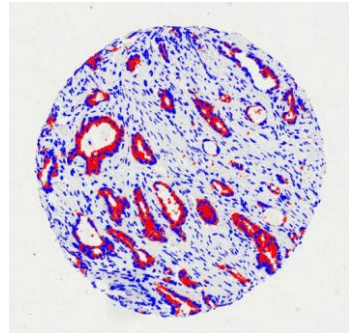
IHC staining before analysis
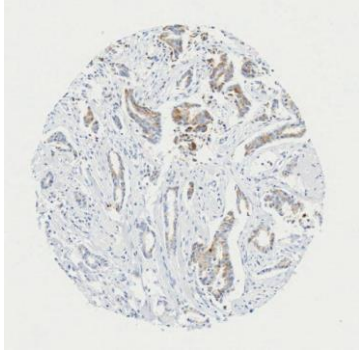
IHC staining after analysis

Weak positive
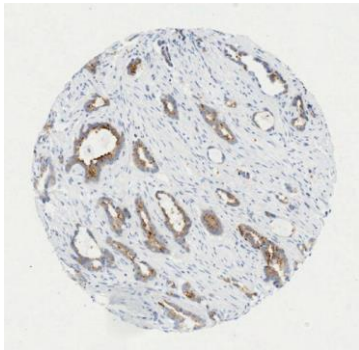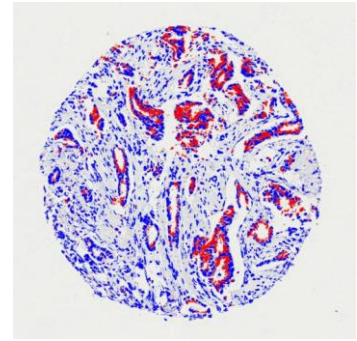
Positive

Negative
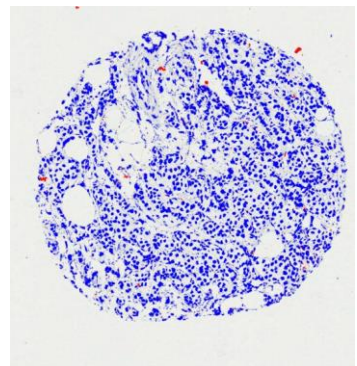
Figure 6-3 IGFBP-2 immunohistochemistry in breast TMA cores

Representative tissue cores showing cytoplasmic staining that represents expression of IGFBP-2 in breast cancer before (left) and after (right) image analysis. The slides were scanned at x20 magnification using the ScanScope™ system, and visualised for scoring using the ImageScope™ pixel intensity Aperio algorithm. Before application of the algorithm, the blue indicates the negative and the brown represents the positive. After application of the algorithm, the blue indicates the negative and the red represents the positive.

Over two thirds of the samples (71.5%) showed positive IGFBP-2 expression, while only approximately a third (29.5%) of the samples were negative for IGFBP-2. The data were dichotomised on the basis of the cut-off point; positive data were represented as 1 and negative data were represented as 0. Survival duration data were retrieved from the clinical data system, and entered into SPSS version 22 software, and survival was calculated for the TamS cohort. Intriguingly, the Kaplan-Meier graph showed that a non-significant trend for higher expression of IGFBP-2 was associated with worse OS in the TamS cohort (Fig. 6.4).

Figure 6-4 Kaplan-Meier survival curve showing the relative association between IGFBP-2 and survival in TamS patients.

The relationship between positive and negative expression of IGFBP-2 was investigated in TamS patient cohorts. Data from 289 TamS patients were entered into the SPSS and a graph was generated. The green indicates positive expression of IGFBP-2, while the blue represents negative expression. The statistical log-rank test (Mantel–Cox test) test used was two-sided, with a p value of 0.127 considered non-significant.

In addition, IGFBP-2 expression was also examined in the TamR cohort, of which 51% showed positive expression of IGFBP-2, while 49% showed negative expression. To study the relationship between positive and negative IGFBP-2 expression and OS in the TamR cohort, the data were entered into the SPSS and the survival curve was plotted. It was found that IGFBP-2 expression was non-significantly (p=0.771) correlated with OS (Figure 6.5).
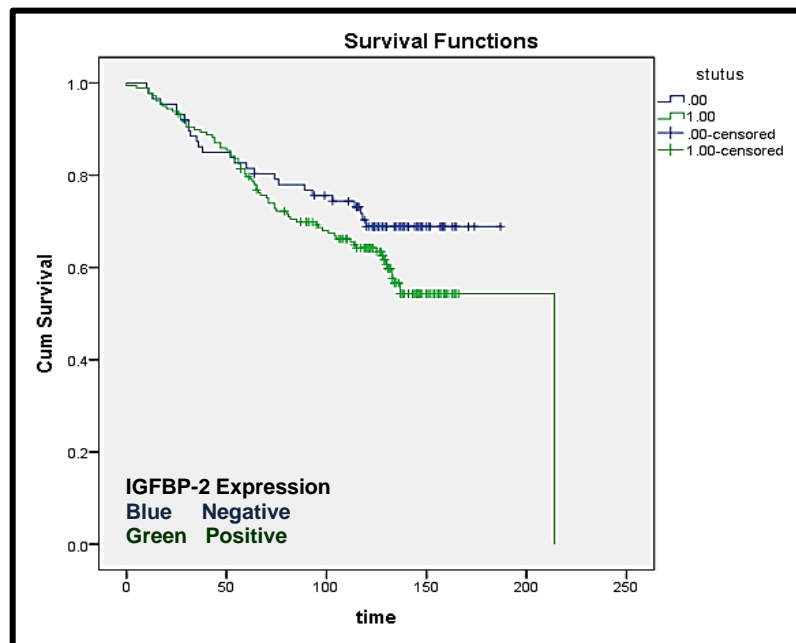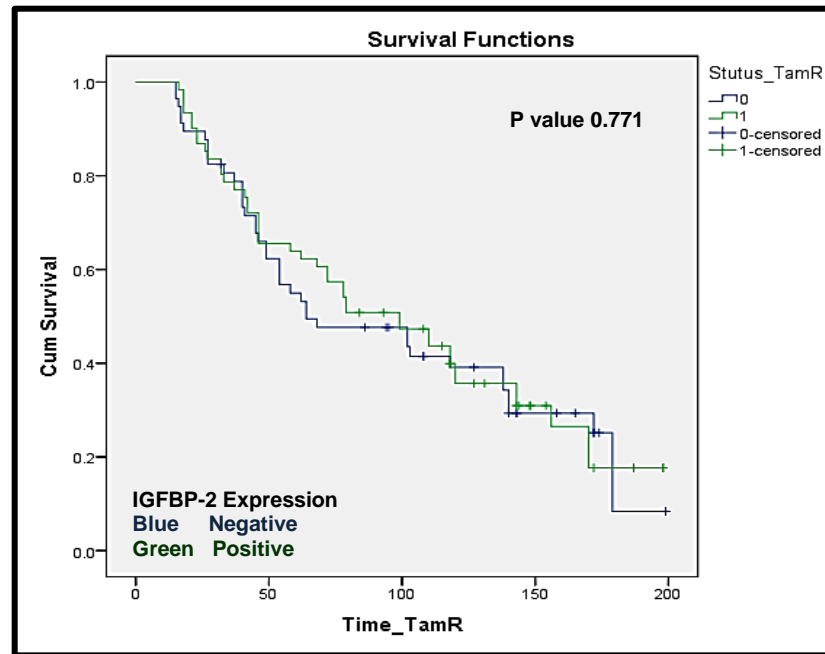

Figure 6-5 Kaplan-Meier survival curve showing the relative association between IGFBP-2 and survival in the TamR cohort.

The relationship between the positive and negative IGFBP-2 expression was investigated in the TamR patient cohort using data from 135 patients. Data were entered into the SPSS and a graph generated. The green indicates positive expression of IGFBP-2, while the blue represents negative expression. The statistical log-rank test (Mantel–Cox test) test used was two-sided, with a p value of 0.771 considered non-significant.

6.5 Positive expression of IGFBP-2 is significantly associated with a survival advantage in TamR patients.

The group expressing positive IGFBP-2 levels represented 71% of the TamS cohort (178 patients) and 51% of the TamR cohort (62 patients). To study the relationship between IGFBP-2 expression and survival rates between these two cohorts, a Kaplan Meier survival analysis was performed. The TamS cohort had significantly (p<0.001) worse survival compared to the TamR cohort. Approximately two thirds of the patients who expressed IGFBP-2 in the TamS patient cohort were dead at 10 years of follow-up, compared to one third of the TamR patient cohort at the same time point, suggesting that IGFBP-2 expression was significantly (p<0.001) associated with improved survival in TamR patients. Therefore, IGFBP-2 expression might be useful as a predictive biomarker for TamR in BC. Figure 6-6 illustrates the relationship between high IGFBP-2 expression and OS in both cohorts.
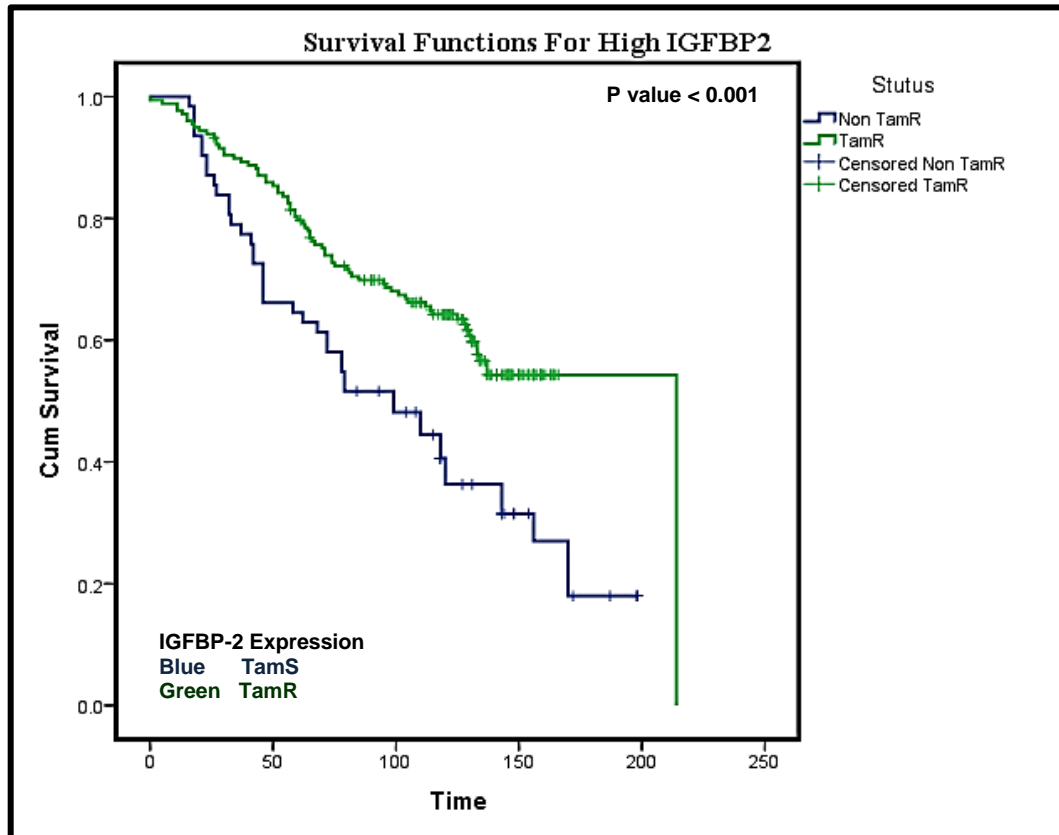
Figure 6-6 Kaplan-Meier survival curve showing the analysis of positive IGFBP-2 expression in non-TamR vs TamR patients.

The curve represents the relative association between IGFBP-2 expression and survival in TamS and TamR cohorts. Green indicates TamR, while blue represents TamS. The statistical log-rank test (Mantel–Cox test) test was used. The difference is significant (p< 0.001).

6.6 Negative expression of IGFBP-2 is significantly associated with poor survival in TamR patients

Negative IGFBP-2 expression was shown in 29% of the TamS cohort (87 patients) and 49% of the TamR cohort (57 patients). Therefore, we compared the patients with negative IGFBP-2 expression in both cohorts; a total of 144 patients. The TamR cohort had a significantly ($p<0.001$) higher correlation with poor survival than the TamS group. Figure 6-7 demonstrates the comparison of negative expression of IGFBP-2 in both cohorts.
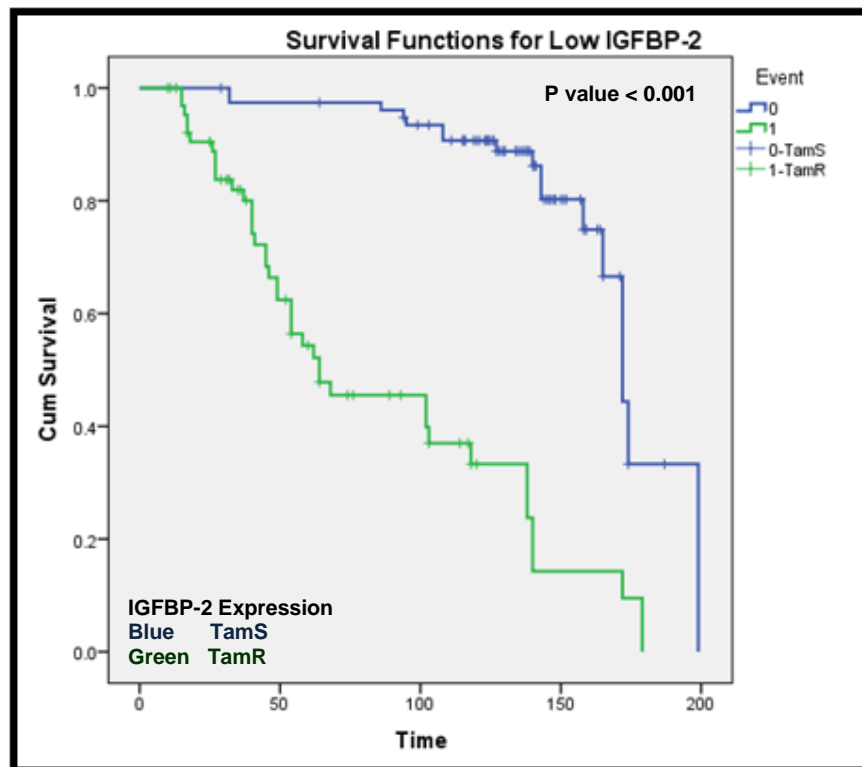
Figure 6-7 Kaplan-Meier survival curve showing negative IGFBP-2 expression in non-TamR vs. TamR cohorts.

The curve represents the relative association between negative IGFBP-2 expression and survival in the TamS and TamR cohorts. Data from 87 non-TamR patients and 57 TamR patients were entered into SPSS version 22 software and used to plot the graph. Green indicates TamR, while blue represents TamS. The statistical log-rank test (Mantel–Cox test) test was used, and the difference is significant ($p<0.001$).

As previously mentioned, we were unable to evaluate the relationship between IGFBP-5 expression and OS because of difficulties in optimising the IGFBP-5 Ab. Therefore we used an alternative online KM-plotter system to study the prognostic value of IGFBP-5.

6.7 Mining a public data set for IGFBP-2 and IGFBP-5

6.7.1 IGFBP-2 assessment in ER+ BC patients treated with tamoxifen

The Kaplan-Meier (KM)-plotter was developed to assess the survival and prognostic value of biomarkers online [256]. This system is capable of evaluating the effect of 22,277 genes on the survival of 4,142 people with BC, 1,648 with ovarian cancer, 2,437 with lung cancer and 765 with gastric cancer, and the aim is to conduct a meta-analysis for in silico biomarker assessment [256]. We used this online tool to compare our results with microarray data from 4,142 ER+ BC patients (Fig 6.8). A log-rank test (Mantel–Cox test) hypothesis test was used to plot the OS and recurrence-free survival (RFS) curves.
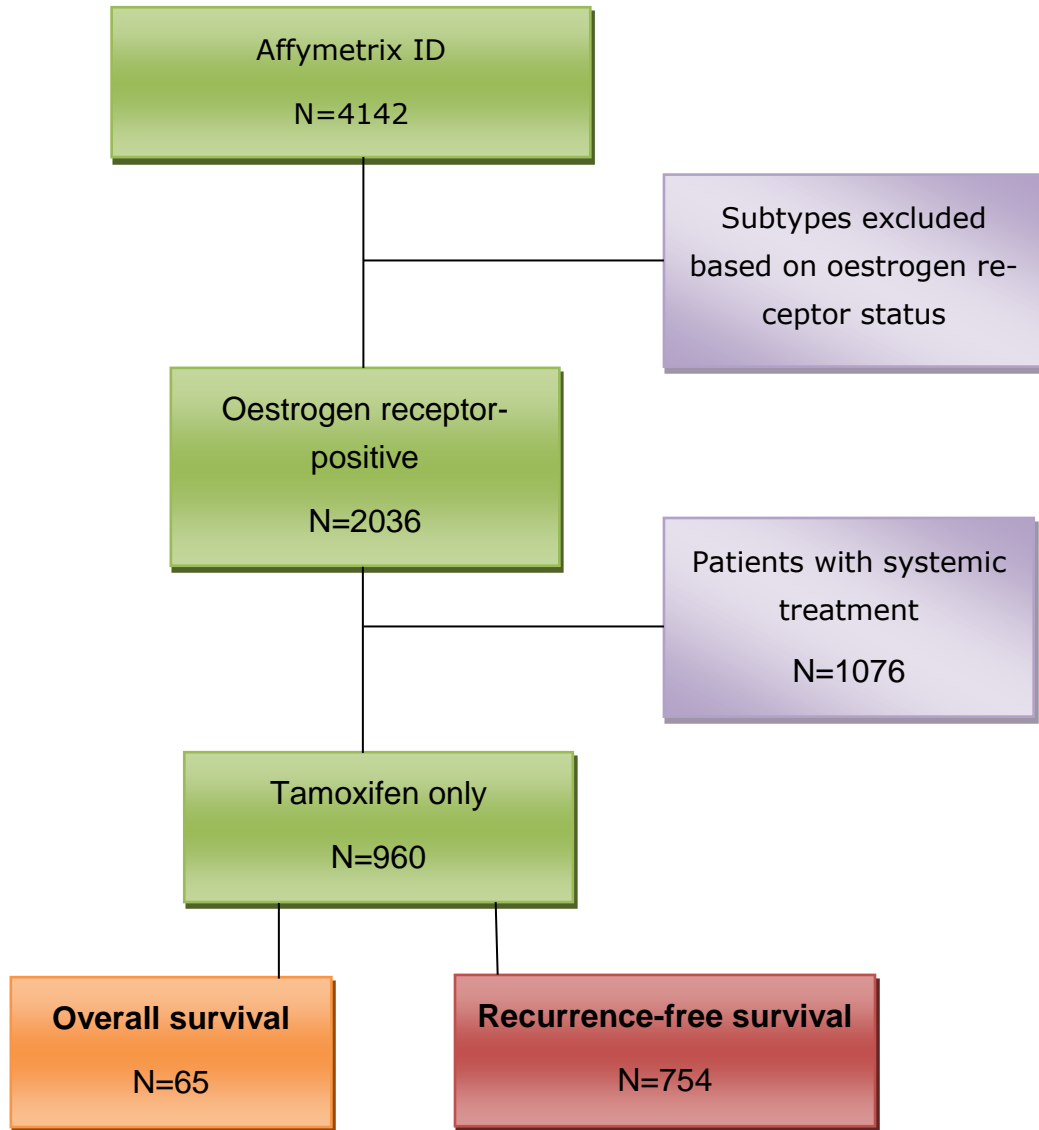
Figure 6-8 Consort diagram representing the patient samples used in the study.

Data from 754 patients were used for RFS and data from 65 patients were used for OS

The ER+ patient samples were split into two groups by median and compared using a Kaplan-Meier survival plot to analyse the potential prognostic value of IGFBP-2. The RFS and OS rates indicated no association between IGFBP-2 expression and TamR in BC. Data from 700 patients were used for RSF, and data from 65 patients were used for OS. Figure 6-9 shows the relative association between IGFBP-2 and survival
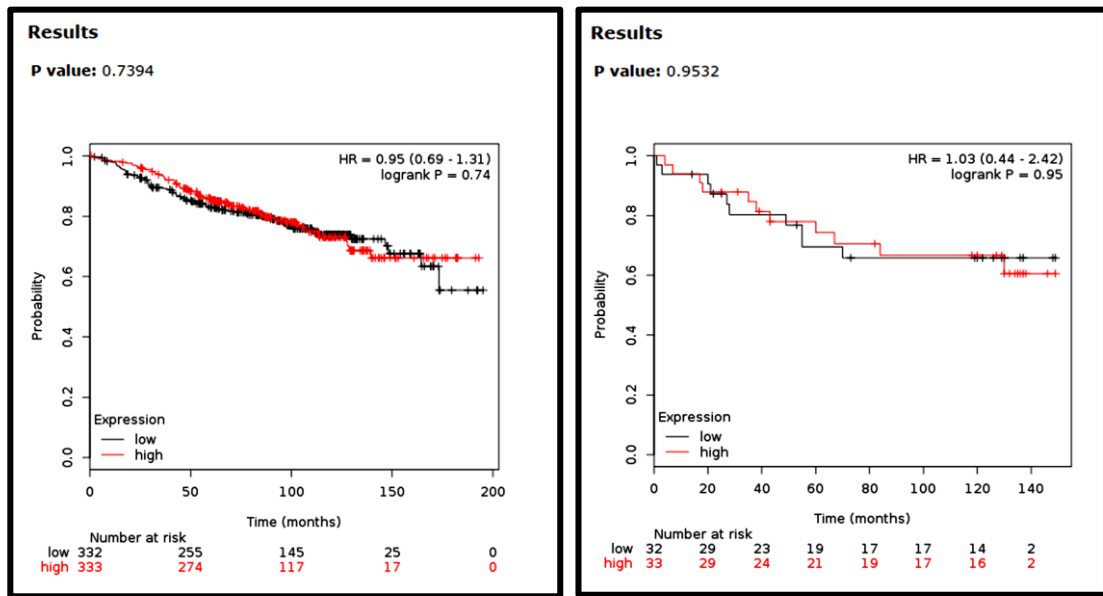
Recurrence free survival (RFS)   **overall survival (OS)**

Figure 6-9 Meta-analysis of IGFBP-2 expression in ER+ patients treated with tamoxifen.

The RFS (left, p = 0.74) and OS (right, p = 0.95) rates are non-significant. The values shown in red represent IGFBP-2 expression, and those shown in black denote no IGFBP-2 expression.

6.7.2 IGFBP-5 assessment in ER+ BC patients treated with tamoxifen

Data from 700 patients for RFS and data from 65 patients for OS were used to evaluate the prognostic value of IGFBP-5. We applied the KM-plotter system, which showed that IGFBP-5 expression was unrelated to RFS and OS (Fig. 6-10). The log-rank test (Mantel–Cox test) hypothesis test was used. The RFS (p=0.3663) and OS (p=0.3595) were non-significant.

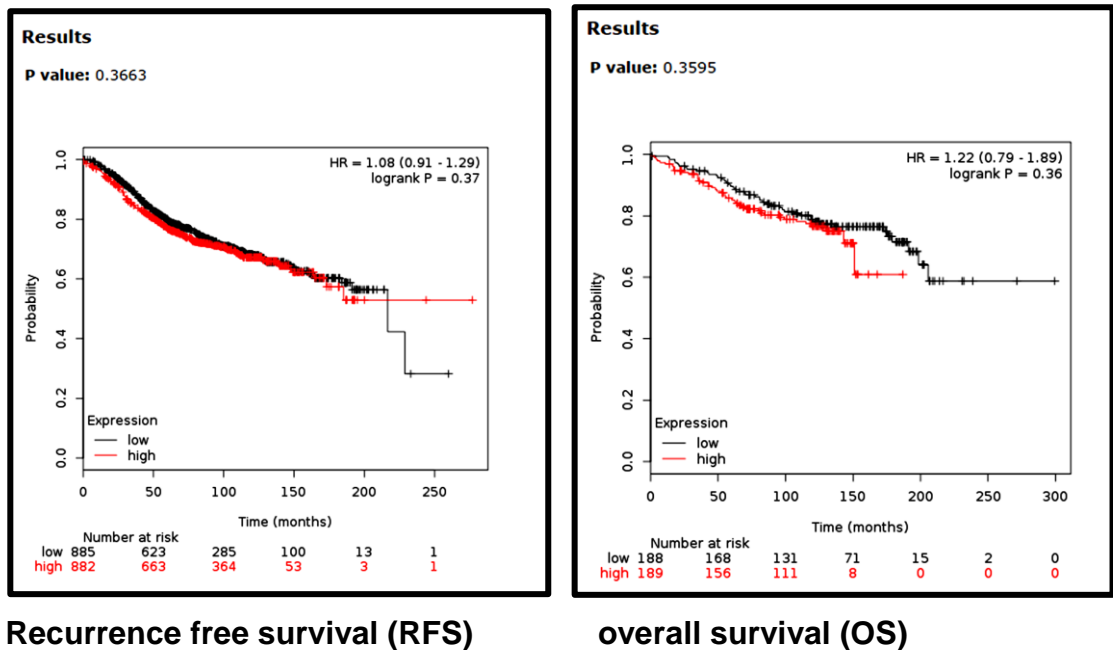**Recurrence free survival (RFS)**   **overall survival (OS)**

Figure 6-10 Meta-analysis of IGFBP-5 expression in ER+ patients treated with tamoxifen.

The RFS (p=0.3663) and OS (p=0.3595) are non-significant. The values shown in red and black denote high and low IGFBP-5 expression, respectively.

6.8 Discussion

To date, clinical data have provided ambiguous evidence as to whether IGFBP-2 and IGFBP-5 serve as valuable prognostic biomarkers for BC [257]. A well-documented finding is that IGFBP-2 serum levels in ER+ BC patients significantly increases. Moreover, IGFBP-2 is associated with tumour progression in several different cancers, including prostate cancer [258], ovarian cancer [259], colon cancer [260], lung cancer [261], adrenocortical cancer [262], breast cancer [254] and leukaemia [263], as well as in drug-resistant tumours [255]. This association indicates that the up-regulation of IGFBP-2 in different tumours can potentially be an important event in breast neoplasia. Accordingly, we examined the prognostic value of IGFBP-2 and IGFBP-5.

An IGFBP-2 specific Ab was used to evaluate IGFBP-2 expression. Since we used an automated algorithm scoring system, definition of a cut-off point was necessary in dichotomising the data for downstream analysis. An ROC (Fig 6-2) was used to determine the point of dichotomisation by an online cut-off finder programme (see http://molpath.charite.de/cutoff/) [227].

Our results showed that high IGFBP-2 expression represented 71% and 51% of the TamS (178 patients) and TamR (62 patients) cohorts, respectively. These findings are supported by the results of Park et al. (2008), who found that 40% of BC patients exhibited increased IGFBP-2 expression in their tumours [264]. Nonetheless, the positive IGFBP-2 expression in the present study was correlated with poor survival prospects for the people with TamS BC. Conversely, IGFBP-2 expression exhibited no association (p=0.771) with the OS of the TamR cohort in the present study.

Taylor et al. (2010) suggested that the IGFBP-2 and IGFBP-5 genes can potentially provide prognostic or predictive value for BC [265]. Interestingly, the authors also reported that such genes can be used to predict responses to different therapeutic regimens, including selective oestrogen-receptor modulators, selective oestrogen-receptor disruptors SERDs and aromatase inhibitors [132, 244, 266].

An interesting IHC study conducted in Norwegian women used 120 BC resections; the results showed a gradual elevation in IGFBP-2 expression from atypical hyperplasia through to carcinoma *in situ* and invasive carcinoma [175]. Similarly, a TMA analysis of over 4,000 primary invasive breast tumours revealed IGFBP-2 over-expression, and also indicated that an adverse survival outcome is correlated with IGFBP-2 expression in ERα-tumours [267]. Another study revealed that, combined with the cell adhesion protein, β-catenin, the expression of IGFBP-2 is linked with lymph-node metastasis in BCs [268]. Similarly, high levels of IGFBP-2 expression, together with loss of PTEN expression, are associated with triple negative BC and poor survival rates [269]. In research using the SKBR3 cell line, IGFBP-2 was up-regulated in an *in vitro* model [270]. Such up-regulation may be postulated as occurring via an ErbB2 signalling mechanism, which provides a route of escape from the anti-EGF-based therapeutic strategy. Other interesting recent reports have suggested that IGFBP-2 is hypothetically a target for an immune-based route for BC treatment; multiple antigenic peptides comprising IGFBP-2 epitopes have been used to block tumour progression in a transgenic mouse model of BC [264, 271]. Therefore, we adopted a Kaplan–Meier-based comparison of the high and low IGFBP-2 expression in both cohorts (TamS and TamR) in the present study. The positive and negative IGFBP-2 expression in the TamS group was significantly associated (p<0.001) with poor survival (Figs. 6-6 and 6-7, respectively). In contrast, Wang et al. (2008) reported that although IGFBP-2 expression is high in ER+ tumours, such expression is unrelated to OS [272].

The online KM-plotter showed no association between IGFBP-2 expression and TamR in BC (Figs. 6.9) [256]. However, the analysis was restricted to gene expression not protein, as assessed in BC TMAs. In addition, the KM-plotter also showed no association between IGFBP-5 gene expression and TamR in BC. Therefore, no evidence was obtained with regard to the potential role of IGFBP-5 in breast malignancy, at least in terms of gene expression. However, despite this deficiency, it has been reported that IGFBP-5 mRNA is up-regulated relative to a normal mammary gland in breast tumour tissue, although IGFBP-5 expression and tumour grade are unrelated [273]. Several reports have suggested that IGFBP-5 is either elevated [274, 275] or decreased in lymph-node metastases [273]. In a study using samples from 116 patients, Mita et al. (2007) found that high IGFBP-5/IGFBP-4 mRNA expression ratio is related to decreased survival with a poor prognosis [199]. Consequently, the authors evaluated IGFBP-5 mRNA expression as a factor for poor prognosis in BC. This is consistent with our KM-plotter system result, which showed that IGFBP-5 mRNA expression was unrelated to RFS and OS. Similarly, Becker et al. (2012) identified IGFBP-5 as being specifically expressed in invasive BC tissue, using IHC based on 76 BC samples. Nevertheless, the study confirmed that an elevation in IGFBP-5: IGFBP-4 expression ratio was adversely associated with RFS and DFS in the studied cohort [276]. In contrast, a study of 153 BC biopsies from tamoxifen-treated patients showed that high IGFBP-5 expression is related to increase OS [132]. Plant et al. (2014) recently carried out an IHC-based study and identified reduced IGFBP-5 protein levels in the stroma surrounding aggressive metastatic BC tissues [248]. However, the authors did not specify the source of the association between the stroma and IGFBP-5 expression. Altogether, the aforementioned findings reinforce the hypothesis that IGFBP-5 plays a role in BC progression. Some obstacles in the optimisation of IGFBP-5, such as choice of the appropriate IGFBP-5 Ab, choice of the suitable tissue

for Ab optimisation and lack of information on the Human Protein Atlas web site, as well as time limitations, meant that the prognostic and predictive value for IGFBP-5 in BC were not assessed in the present study.

In summary, positive expression of IGFBP-2 in TamS was associated with significantly ($p<0.001$) worse survival than in the TamR cohort, suggesting that IGFBP-2 expression was significantly associated with improved survival in TamR patients. Conversely, negative expression of IGFBP-2 in TamR was associated with significantly ($p<0.001$) worse survival compared to the TamR cohort. Therefore, a high level of IGFBP-2 in clinical samples may be used as a predictive biomarker for tamoxifen resistance in BC. Although the present research was not conducted on IGFBP-5, the KM-plotter system showed that its expression, in terms of mRNA, was unrelated to RFS and OS. Further investigations could confirm our results.

Chapter 7 General Discussion

The most striking feature in our studies was the consistently observed reciprocal regulation of IGFBP-2 and -5 expression seen between wt and TamR cells whereby IGFBP-2 was up regulated approximately 2-5-fold in TamR v wt cells whilst IGFBP-5 was down regulated to approximately the same level in TamR cells. This may argue for a co-ordinate regulation of IGFBP-2 and -5 expression and indeed we have previously reported a reciprocal regulation of IGFBP-2 and -5 expression during differentiation of both mammary epithelial cell lines and primary cultures [277]. It is interesting in this context to note that IGFBP-2 and -5 are located very close together in a tail-to-tail configuration on chromosome 2 in humans separated by approximately 30 kbs of genomic sequence [118]. Such architecture suggests that IGFBP-2 and -5 may have arisen via a gene duplication event and that a common cis or trans acting regulatory mechanism of reciprocal gene expression may exist. Clearly further experimentation is required to test this hypothesis but such a co-ordinate and reciprocal regulation would provide a novel mechanism of gene regulation in the IGFBP family.

There are some studies which describe mechanism (s) by which IGFBP-2 and IGFBP-5 may influence tumourigenesis in BC although the literature in this area is somewhat contradictory and further experimental clarification would be welcome. Nonetheless these reports may have some relevance to the findings presented in the current study. For example IGFBP-2 was reported to act via an integrin based mechanism to suppress PTEN activity in MCF-7 cells thus prolonging PI3K activity to provide a pro-tumourigenic signal [26]. Further studies suggested a pro-survival action of IGFBP-2 through an ERα dependent mechanism. Interestingly knock down of IGFBP-2 ablated ERα expression and this effect was reversed by addition of exogenous IGFBP-2 [71]. A related study from this group confirmed decreased ERα expression when IGFBP-2

secretion was inhibited by the flavinol EGCG in MCF-7 cells. Under these conditions cell growth was inhibited and the expression of the p53 and p21 tumour suppressors was enhanced [278]. Similarly analysis of shRNA based IGFBP-2 knockdown in the BC cell line BT474 indicated regulation of numerous pro-tumourigenic pathways. In a different experimental model using the neuroblastoma cell line SK-N-SHEP over expression of IGFBP-2 stimulated the proliferation and migration of cells through ECM suggesting a mitogenic and metastatic action of this protein [279] . From a protein structure perspective, site directed mutagenesis studies suggest that these pro-tumourigenic effects of IGFBP-2 are dependent on an intact heparin binding domain (HBD) in the protein. However in another model of neurological tumourigenesis IGFBP-2 was reported to bind integrin α5 in an RGD-dependent manner to promote JNK mediated migration of glioblastoma cells [202, 280] and subsequent studies also suggested the involvement of integrin β1 activation of integrin linked kinase (ILK) and the NF-κB pathway in this process. IGFBP-2 has also been reported to have tumour-igenic activity in the prostate and yeast two hybrid analysis using a human prostate cDNA library identified Pim-1 associated protein (PAPA-1) as an IGFBP-2 binder and suggested that this nucleolar protein may act to inhibit the growth promoting activity of IGFBP-2 in the prostate [281]. An alternative tumour promoting function of IGFBP-2 may be through activation of VEGF expression and subsequent stimulation of angio-genesis [282, 283]. In a very recent study such activity was found to be dependent on the nuclear translocation of IGFBP-2 through a canonical importin-α based mecha-nism and IGFBP-2 was identified in both nuclear and cytoplasmic compartments by cell lysate and immunofluorescent analysis. Accordingly neuroblastoma cells express-ing a nuclear localisation signal (NLS) mutant of IGFBP-2 fail to stimulate angiogene-sis in vivo in comparison to wt IGFBP-2 transfectants.[247].

Any strategy which aims to reduce pericellular IGFBP concentrations e.g. via sh/si/mi RNA methodologies may result in increased local abundance of IGF growth factors resulting in an increased IGF dependent mitogenic stimulation [284].These pleiotropic effects of IGFBPs are typical in many cell lines and primary cultures and mean that caution must be exercised in the interpretation of experimental findings. We have clearly presented evidence that the over expression of IGFBP-2 may be associated with the development of TamR in MCF-7 BC cells. In this context it is important to note that the expression of IGFBP-2 in MCF-7 cells is regulated through the PI3K/Akt/mTor pathway to up regulate mRNA expression through a trans acting Sp1 activation on the IGFBP-2 promoter [285, 286]. We did not examine whether this pathway was up regulated in TamR cells where IGFBP-2 expression is increased although such studies would clearly be of merit. In addition, whether any of the mechanisms discussed above pertain in tamoxifen resistant tissues are clearly worthy of further study as they may provide a route for therapy in such cases.

There is also a limited literature on the potential role (s) of IGFBP-5 in BC – reviewed recently in [100] although again much of it is contradictory in nature. In the normal mammary gland IGFBP-5 may regulate the involution phase of the lactation cycle and may also play a role in differentiation and morphogenesis of the gland [127, 277, 287, 288]. IGFBP-5 induces cell adhesion but inhibits migration in MCF-7 cells [130, 131] and Butt et al report inhibition of MDA-MB-231 and Hs578T cell line growth following stable or adenovirus based transfection of IGFBP-5 [163]. An interesting observation is that intracellular location of IGFBP-5 can differentially regulate the activity of the protein in BC cells. Therefore despite the fact that IGFBPs in general are viewed as secreted proteins, in MDA-MB-435 BC cells nuclear location of IGFBP-5 is associated with a growth inhibitory action whereas accumulation of IGFBP-5 in the cytoplasm is associated with a growth stimulatory activity and is a poor prognostic factor for BC

[246].In Hs578T cells IGFBP-5 inhibits ceramide or RGD induced apoptosis [289] and subsequent studies suggested that IGFBP-5 may regulate apoptosis and cell survival through sphingosine kinase and PKC mediated survival signals [166]. However effects of IGFBP-5 show BC cell line specificity. Thus both wt and a non-IGF binding mutant of IGFBP-5 inhibit ceramide induced apoptosis in Hs578T cells but only the wt IGFBP-5 was effective in MCF-7 cells. In addition mutant IGFBP-5 ablated the pro-survival effects of IGF-1 in MCF-7 cells (an IGF responsive cell line) whereas the wt protein enhanced IGF-1 survival properties [290]. Such exquisite regulation of IGF and IGFBP-5 activity has obvious significance in a tissue such as the mammary gland where IGF-IGFBP affinity can be regulated by mechanisms such as IGFBP-ECM association and post-translational modification of IGFBP (including proteolysis). Although it is difficult to set our observations of reduced IGFBP-5 expression in TamR cells in context with the above studies it may be that those reports of other workers describing growth inhibitory effects of IGFBP-5 may have relevance and some of the associated mechanisms may operate when IGFBP-5 levels are decreased in TamR cells [130, 131, 163, 246]. In a similar vein our group recently reported that IGFBP-5 is involved in maintenance of epithelial-mesenchymal cell barriers and thus may inhibit the process of epithelial-mesenchymal transition (EMT) [131] . Such processes are generally believed to play an important role in the local and systemic dissemination of tumour cells (including BC) [30]. Therefore in tamoxifen resistant BC reduced IGFBP-5 expression may promote EMT leading to tumour metastasis.

In a clinical context, IGFBP-2 and/or IGFBP-5 is may have predictive or prognostic value in BC [265] and may also be used to predict responses to different therapeutic regimes including SERMs, SERDs and AIs [132, 244, 266]. In general terms increased expression of IGFBP-2 in BC tissues is associated with poorer survival rates. An IHC based study in Norwegian women using 120 breast resections reported a

gradual increase in IGFBP-2 expression from atypical hyperplasia through to carci-noma in situ and invasive carcinoma [175]. Similarly a TMA analysis of over 4000 pri-mary invasive breast cancers identified over expression of IGFBP-2 in tumour tissue and an adverse survival outcome correlated with IGFBP-2 expression in ERα negative cancers [267]. Interestingly over expression of IGFBP-2 in the MDA-MB-231 cell line was associated with increased chemotherapeutic resistance *in vitro* and *in vivo* and this effect could be ablated by down regulation of IGFBP-2 expression using anti-sense directed oligonucleotides. A subsequent study reported that expression of IGFBP-2 in association with the cell adhesion protein β-catenin is associated with lymph node metastasis of BCs [268] and high levels of IGFBP-2 expression together with loss of PTEN expression were associated in triple negative (TN) BC along with poorer survival rates [269]. For IGFBP-5 in the clinical setting of BC there is less evi-dence for a potential role in tumourigenesis and the literature is still occasionally con-flicted. For example, IGFBP-5 mRNA was reported to be up regulated in breast can-cer tissue relative to normal gland although there was no correlation between tumour grade and IGFBP-5 expression [273]. Similarly there is evidence that IGFBP-5 is ele-vated [274, 275] or decreased in lymph node metastases [273]. An analysis of 116 patient samples identified that a high IGFBP-5/IGFBP-4mRNA ratio was related to poorer prognosis and a decreased period of disease free survival [199] and therefore high expression of IGFBP-5 mRNA was defined as a poor prognostic factor in BC. However a more recent tissue microarray analysis (TMA) of 153 BC biopsies from ta-moxifen treated patients suggested that high expression of IGFBP-5 was associated with increased overall survival [132]**.** An IHC based study of 76 BC samples indicated BP-5 expression in invasive BC tissue. This same study also reported that an in-creased BP-5: BP-4 expression ratio was negatively associated with recurrence free survival (RFS) and disease free survival (DFS) in this cohort of patients [276]. In con-

trast to this a very recent IHC based study reduced IGFBP-5 protein in the stroma surrounding metastatic BC tissues[248]. Although it is difficult to resolve these contrasting observations it should be noted that in the latter study the cellular source of stromal associated IGFBP-5 was not clear.

Interestingly single nucleotide polymorphisms (SNPs) in the 3' end of both *IGFBP-2* and *IGFBP-5* were reported to be associated with an increased risk of BC in a cohort American-African women age< 40 and similar findings were confirmed in a population of Nigerian women [291]. These observations have been developed by the publication of an extensive SNP analysis in the 5'region of the IGFBP-5 gene (at 2q35 in humans) which describes an SNP close to an enhancer region of the IGFBP-5 promoter. The authors provide evidence that expression of this allele is associated with down regulation of IGFBP-5 and increases risk of ER+ breast cancers [292]. Whether this SNP is associated with TamR BC is unknown but the association of TamR with decreased IGFBP-5 expression highlighted in this study suggests that this is worthy of further investigation.

<u>Conclusions</u>

1. Both wt and TamR MCF-7 cells express 5 of the IGF axis genes – IGF-1R, IGF-2R, IGFBP-2, IGFBP-4 and IGFBP-5.

2. In TamR cells IGFBP-2 and -5 are reciprocally regulated such that BP-2 is up regulated and BP-5 is down regulated with respect to wt cells at both the mRNA and protein level.

3. Attenuation of IGFBP-2 expression in TamR cells partially restores sensitivity to 4HT suggesting a causal role for this binding protein in the development of TamR

4. Attenuation of IGFBP-5 expression in wt cells has little effect on sensitivity to 4HT.

5. Extracellular IGFBP-2 or IGFBP-5 has no effect on the sensitivity of either wt or TamR cells suggesting an intracellular mechanism of action for IGFBP-2.

6. IGFBP-5 knock down in wt MCF-7 cells increases cell migration whereas IGFBP-2 knock down in TamR cells has no effect.

7. High IGFBP-2 expression is significantly ($P< 0.001$) associated with survival advantage in tamoxifen resistant patients cohort.

Further Studies

The work currently reported used a KO strategy to investigate the role of IGFBP-2 and -5 in the development of TamR in MCF-7 cells. In the absence of vectors over expressing BP-2 or BP-5, we manipulated extracellular concentrations of IGFBPs as a surrogate for the creation of cell lines over expressing BP-2 or BP-5. However this is somewhat unsatisfactory on a number of counts (see Section 4.7) and ideally we would like to produce wt MCF-7 cells over expressing BP-2 and TamR cells over expressing BP-5. The acquisition of TamR by the former cells and the restoration of tamoxifen sensitivity in the latter would provide direct confirmation of a role for each of these IGFBPs in the phenotype of TamR. We hope to create these cell lines in the near future.

Earlier work from our group has suggested that IGFBP-5 may play a role in the regulation of MCF-7 cell adhesion and migration [130, 131]. In this model BP-5 acts through an $\alpha 2\beta 1$ integrin mediated mechanism involving activation of the Rho GTPase family member Cdc42 with subsequent increased adhesion and decreased migration on a mesenchymal extracellular matrix. In the current work we report that knock down of IGFBP-5 increases cell migration and this is consistent with our earlier studies. It would be interesting to confirm whether this also involves a Rho GTPase/Cdc42 based mechanism. Investigations into the potential mechanisms associated with IGFBP-2 involvement in TamR are also worthy of further study. Some of these are already being elucidated [71] and it is an important research area.

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291. Garner, C.P., et al., *Genetic variation in IGFBP2 and IGFBP5 is associated with breast cancer in populations of African descent.* Hum Genet, 2008. **123**(3): p. 247-55.

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Appendixes

1- Supplementary Figures 4.7S -4.16S
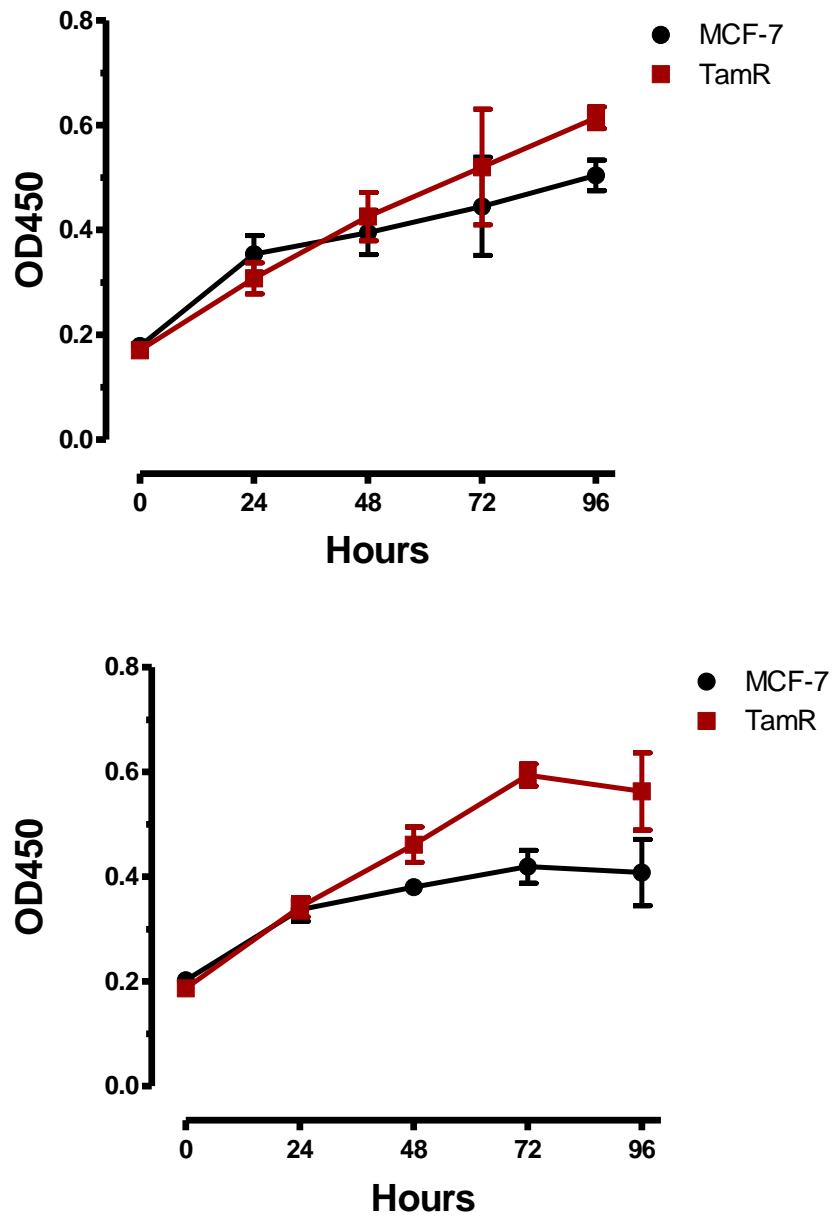
Figure 4.8A(S) Growth of wt or TamR cells in 1uM 4HT. Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates in the absence (top panel) or presence (bottom panel) of 1uM 4HT. Cell growth was monitored over the period 0-96 hr. by WST-1 assay as described in Materials &Methods
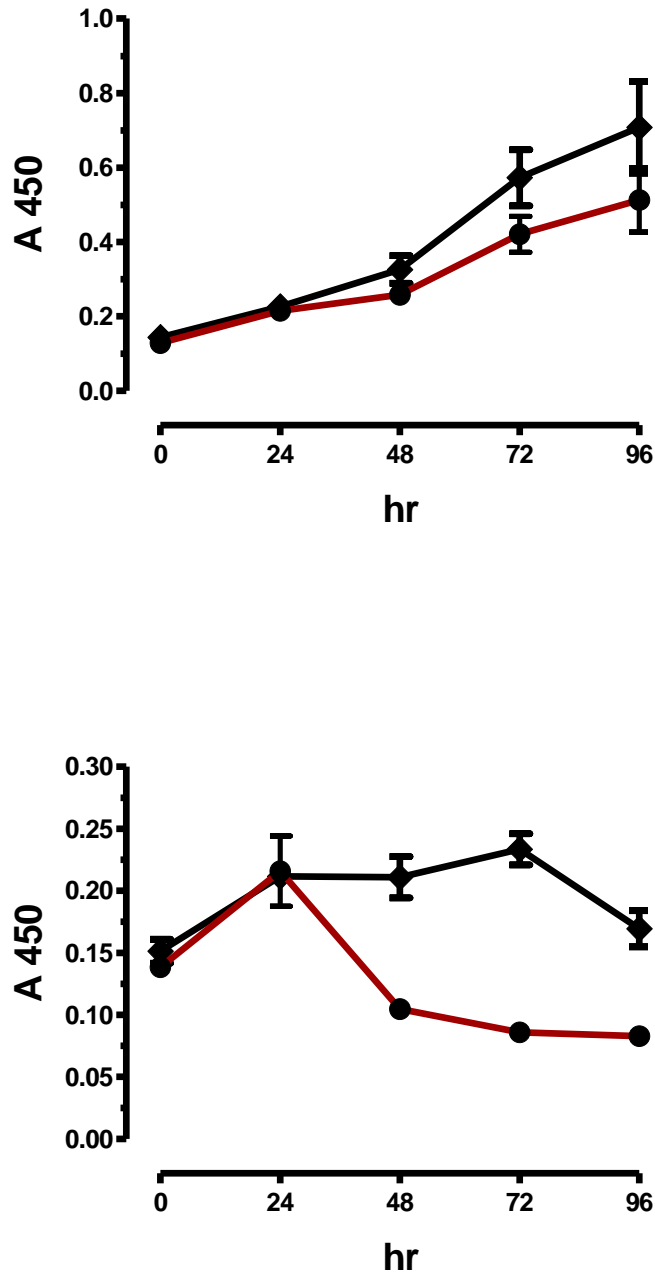
Figure 4.8B (S) Growth of wt or TamR cells in 1uM 4HT. Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates in the absence (top panel) or presence (bottom panel) of 1uM 4HT. Cell growth was monitored over the period 0-96 hr. by WST-1 assay as described in Materials &Methods
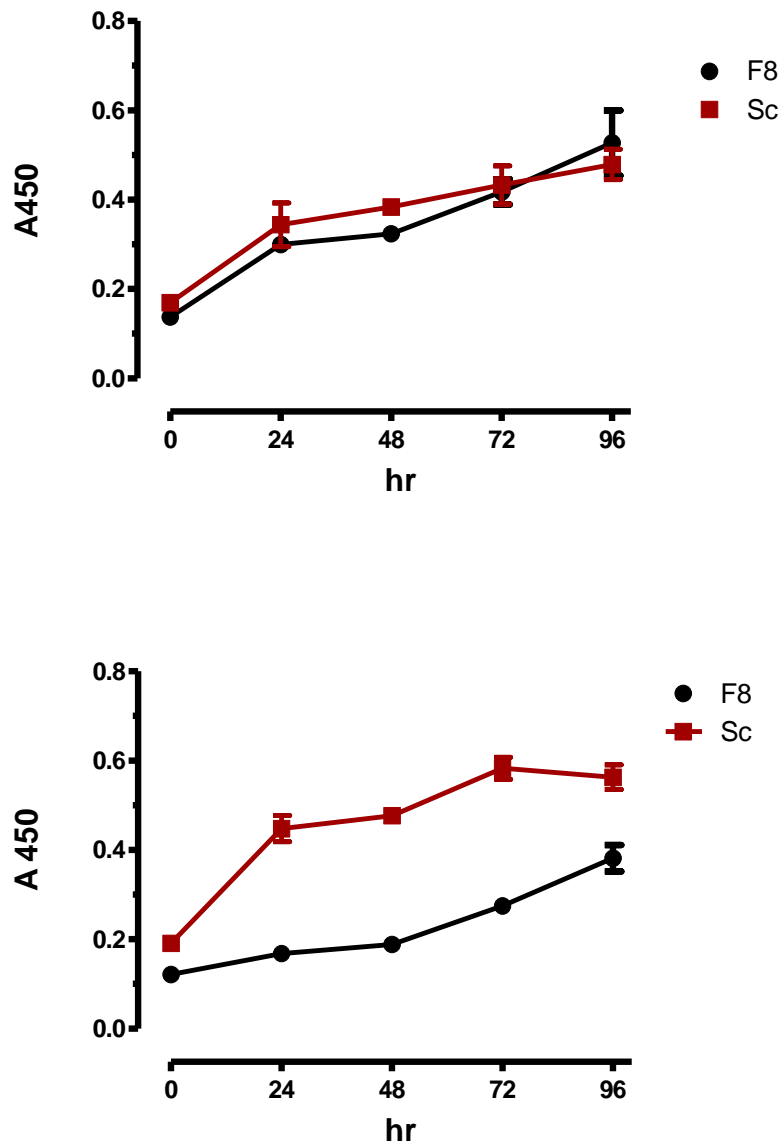
Figure 4.9S Growth of TamR BP-2 KO clone F8 or scrambled control transfected cells in 1uM 4HT. Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates in the absence (top panel) or presence (bottom panel) of 1uM 4HT. Cell growth was monitored over the period 0-96 hr. by WST-1 assay as described in Materials &Methods
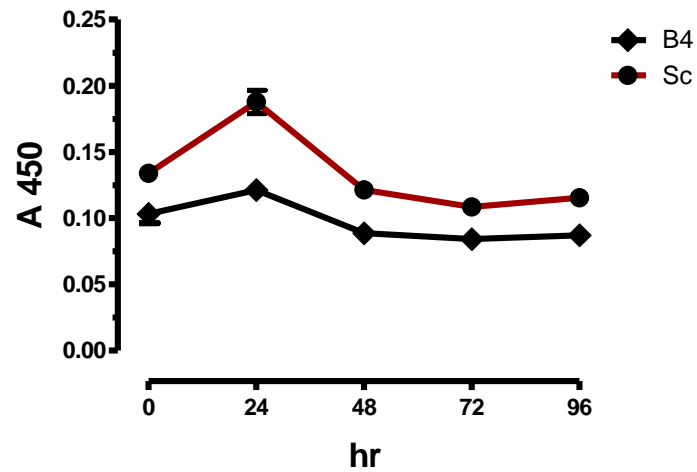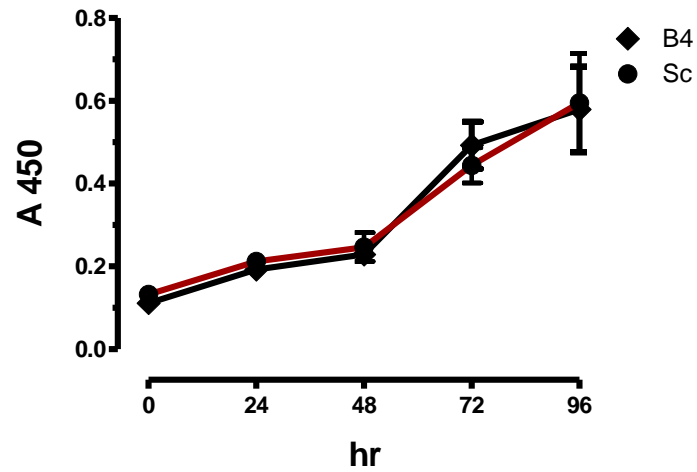
Fig 4.10S Growth of wt BP-5 KO clone B4 or scrambled control transfected cells in 1um 4HT. Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates in the absence (top panel) or presence (bottom panel) of 1uM 4HT. Cell growth was monitored over the period 0-96 hr. by WST-1 assay as described in Materials &Methods
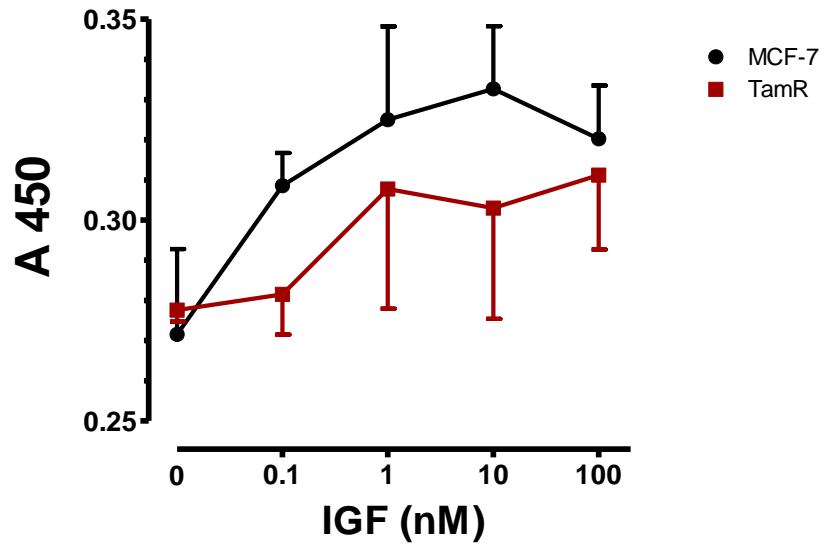
Fig 4.11S Effect of IGF-1 on growth of wt and TamR MCF-7 cells. Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates. After overnight attachment cells were treated with the indicated concentrations of IGF-1 in 100 ul of serum free PR free medium. After 48 hr WST-1 reagent (10ul) was added and A450 was determined after 30 min incubation at 37C.
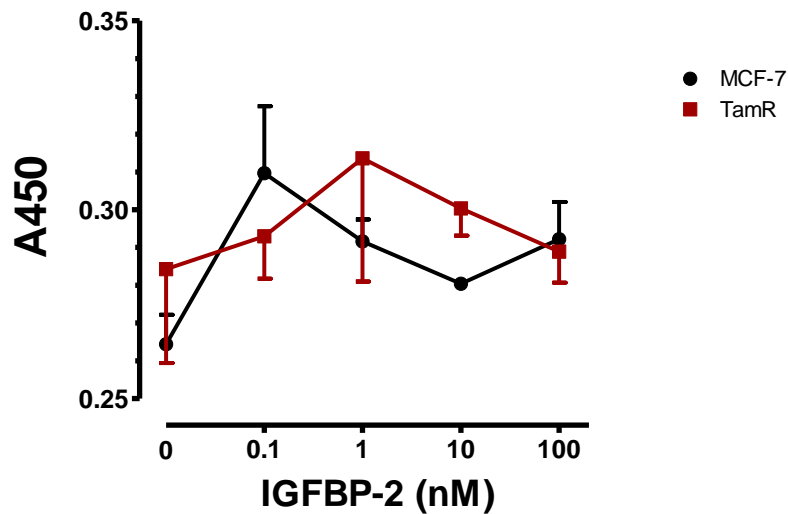
Fig 4.12S Effect of IGFBP-2 on growth of wt and TamR MCF-7 cells. Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates. After overnight attachment cells were treated with the indicated concentrations of IGFBP-2 in 100 ul of serum free PR free medium. After 48 hr WST-1 reagent (10ul) was added and A450 was determined after 30 min incubation at 37C.
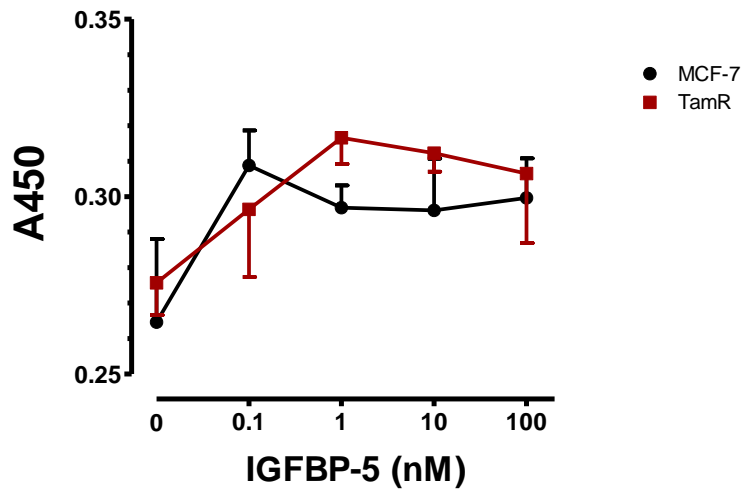
Fig 4.13S Effect of IGFBP-5 on growth of wt and TamR MCF-7 cells. Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates. After overnight attachment cells were treated with the indicated concentrations of IGFBP-5 in 100 ul of serum free PR free medium. After 48 hr WST-1reagent (10ul) was added and A450 was determined after 30 min incubation at 37C.
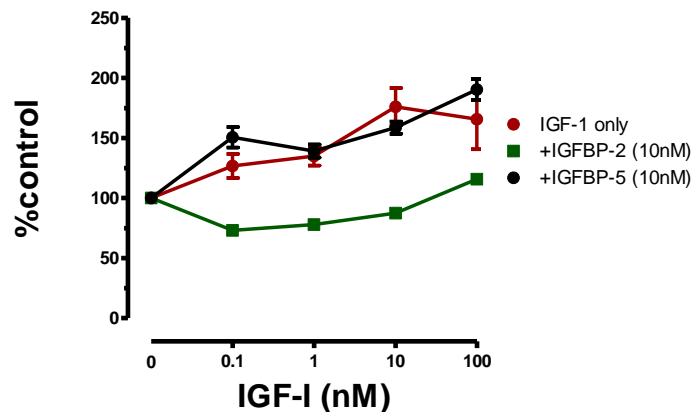
Fig 4.14S Effect of IGF-1 ± IGFBP-2 or IGFBP-5 on growth of wt cells. Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates. After overnight attachment cells were treated with the indicated concentrations of IGF-1 (0-100nM) in the presence or absence of fixed concentrations (10 nM) of IGFBP-2 or IGFBP-5 in 100 ul of serum free PR free medium. After 48 hr WST-1 was added and A450 was determined after 30 min incubation at 37C.
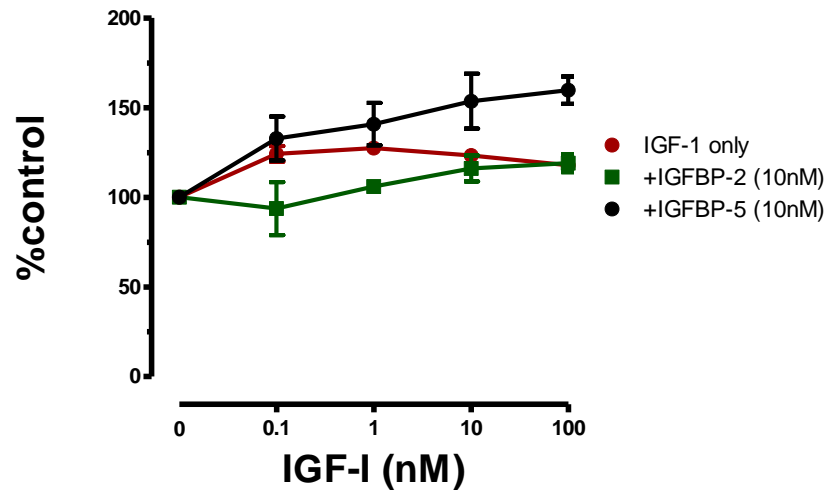
Fig 4.15S Effect of IGF-1 ± IGFBP-2 or IGFBP-5 on growth of TamR cells. Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates. After overnight attachment cells were treated with the indicated concentrations of IGF-1 (0-100nM) in the presence or absence of fixed concentrations (10 nM) of IGFBP-2 or IGFBP-5 in 100 ul of serum free PR free medium. After 48 hr WST-1 was added and A450 was determined after 30 min incubation at 37C.

MCF-7 +/- BP2

MCF-7 +/- BP2 + 4HT
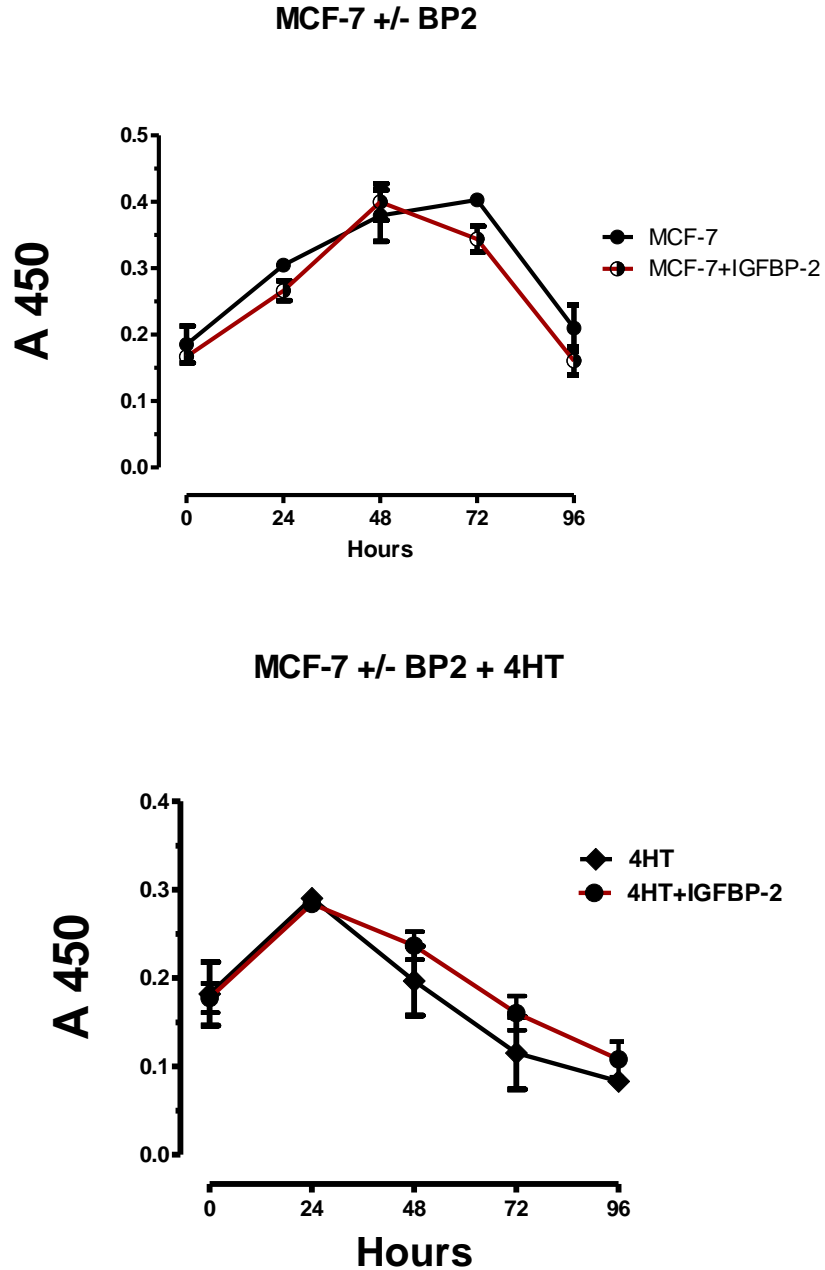
Fig 4.16S Fig 4.15 Effect of extracellular IGFBP-2 on Tamoxifen sensitivity of wt MCF-7 cells. Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates in the absence (wt) or presence (+BP-2) of 50nM BP-2. Cell growth was monitored over the period 0-96 hr in the presence of 1uM 4HT using WST-1 assay as described in Materials &Methods
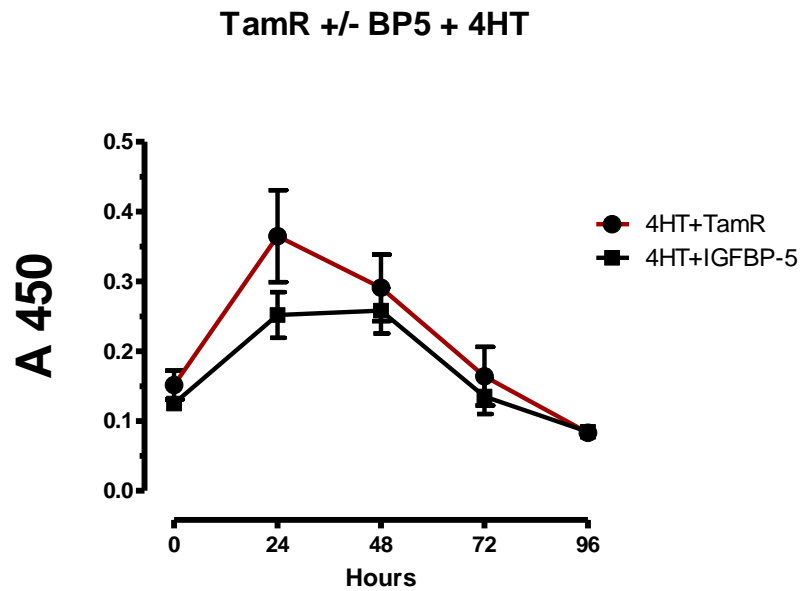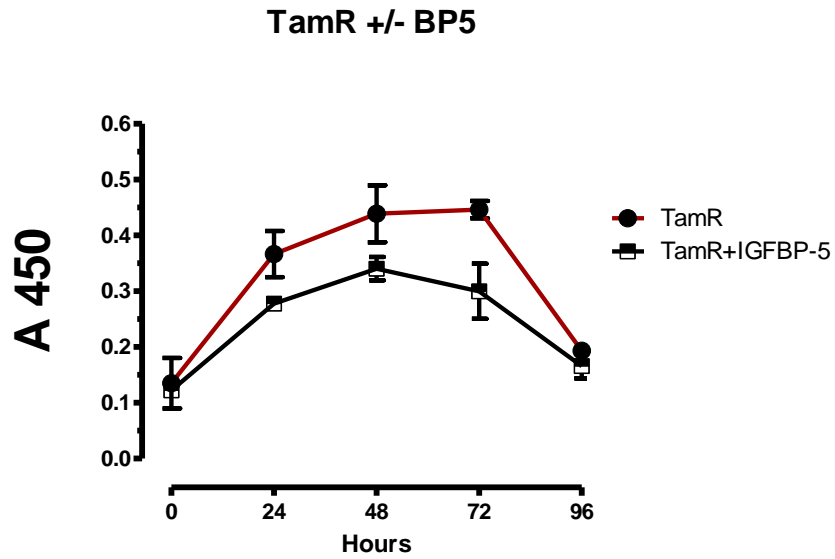
Fig 4.17S Fig 4.16 Effect of extracellular IGFBP-5 on tamoxifen sensitivity of TamR MCF-7 cells. Cells were seeded in 100 ul of 5% DCS PR free medium at 5000/well in 96-well microtitre plates in the absence (TamR) or presence (+BP-5) of 50nM BP-5. Cell growth was monitored over the period 0-96 hr in the presence of 1uM 4HT using WST-1 assay as described in Materials &Methods.

2- Taqman assay identifiers

No		Reverse Primer Seq	Assay ID	Lot Number	Amplicon Length
1	GAPDH	GGGCGCCTGGTCACCAGGGCTGCTT	Hs99999905_m1	1127396	124
2	RPLP0	TGTTTCATTGTGGGAGCAGACAATG	Hs99999902_m1	1159813	105
3	IGF1	TTATTTCAACAAGCCCACAGGGTAT	Hs01547656_m1	1127281	68
4	IGF2	GGCCATGCAGACACCAATGGGAATC	Hs04188276_m1	4351372	83
5	IGF1R	CCATCTTCGTGCCCAGACCTGAAAG	Hs00609566_m1	1128743	64
6	IGF2R	TGTCAGAGTGGAAGGGGACAACTGT	Hs00974474_m1	1110170	59
7	IGFBP1	CAGCAGACAGTGTGAGACATCCATG	Hs00236877_m1	1139777	69
8	IGFBP2	ACAACCTCAAACAGTGCAAGATGTC	Hs01040719_m1	1111303	54
9	IGFBP3	AGACGCCTGCCGCAAGGTTAATGTG	Hs00426289_m1	1109668	84
10	IGFBP4	CCCCAAGCAGTGTCACCCAGCTCTG	Hs01057900_m1	4351372	81
11	IGFBP5	GCAAGTCAAGATCGAGAGAGACTCC	Hs00181213_m1	1134543	85
12	IGFBP6	GCCCGCGCGCCTGCTGTTGCAGAGG	Hs00181853_m1	1115870	145
13	ESR1	TGATGAAAGGTGGGATACGAAAAGA	Hs00174860_m1	1113405	62
14	ESR2	ACCTGTAAACAGAGAGACACTGAAA	Hs01100353_m1	1110864	73

3- Optimisation of IGFBP-2 (kidney)
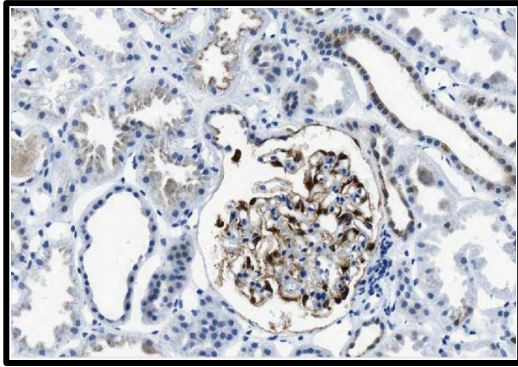
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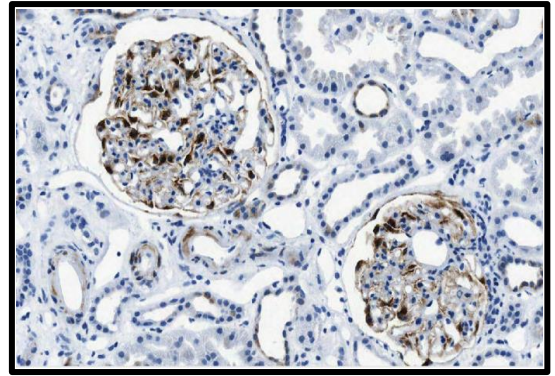
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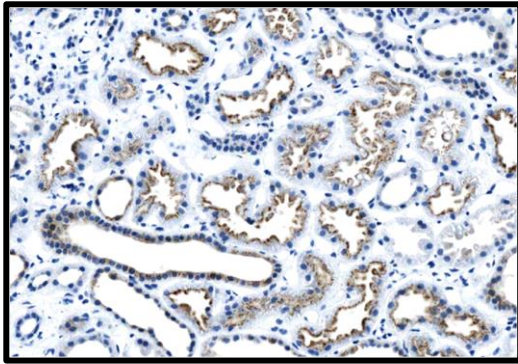
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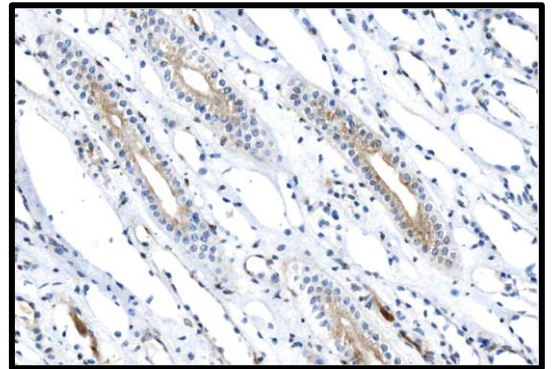
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Optimisation of IGFBP-2 in multi-tissues (1:100)
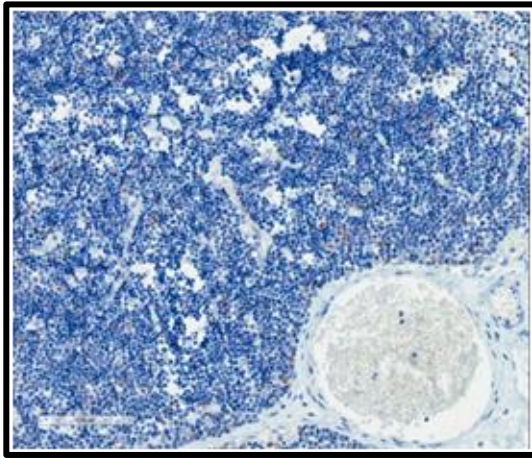
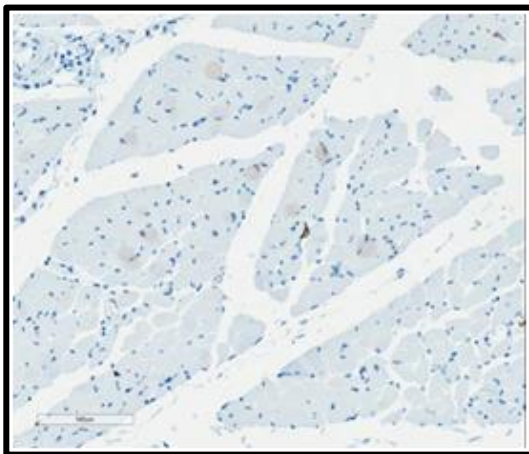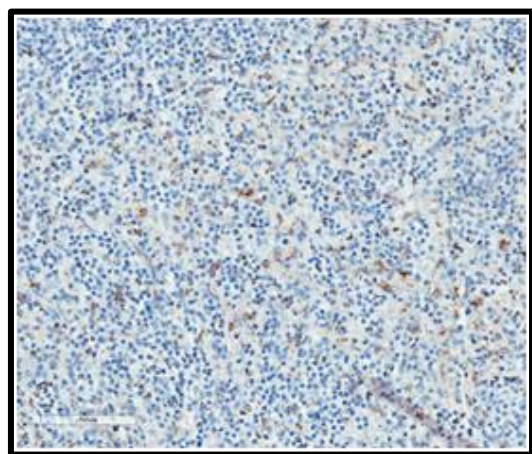Tonsil
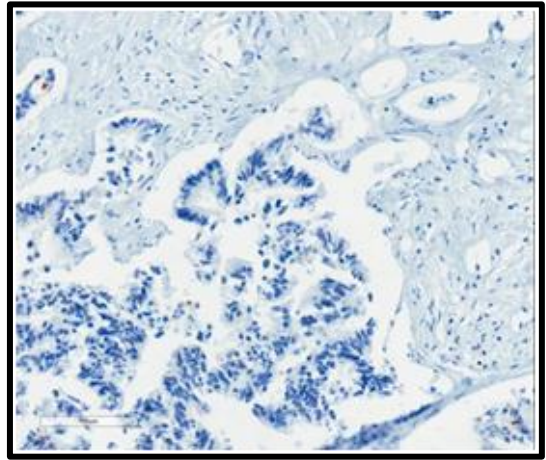
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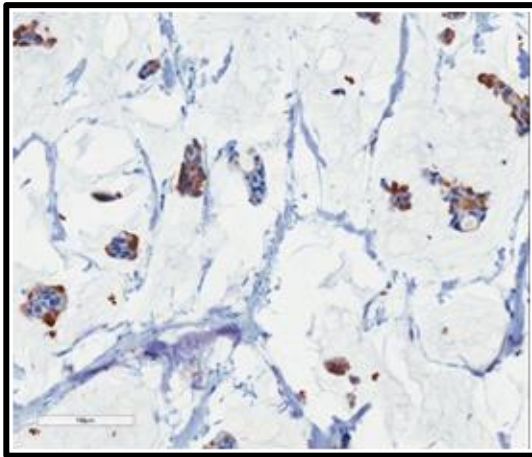
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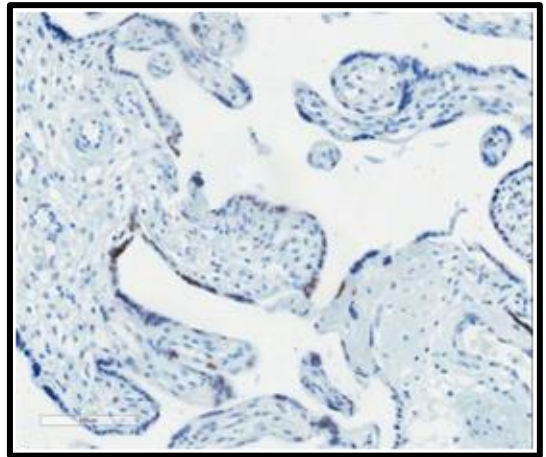
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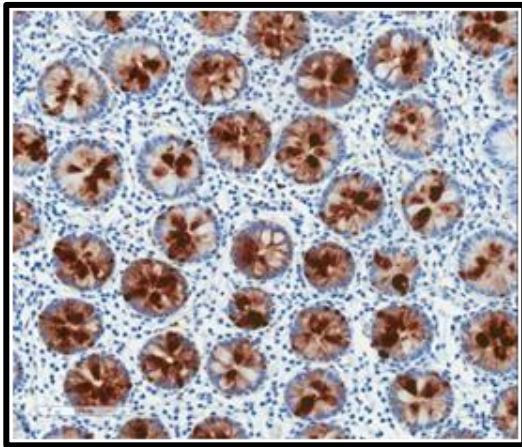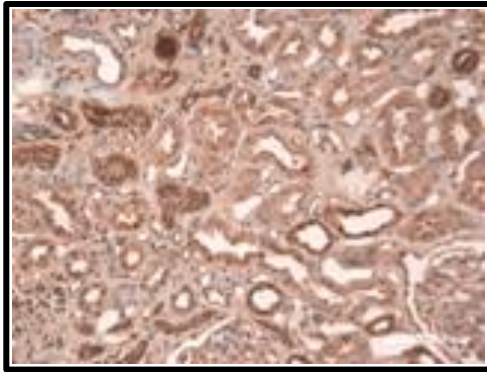
Colon
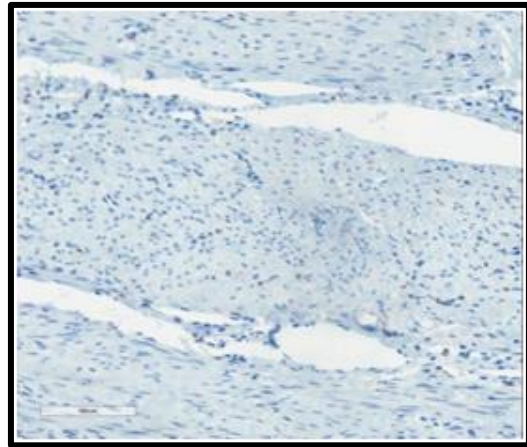
Desmoid type smooth muscle tumour

Muscle
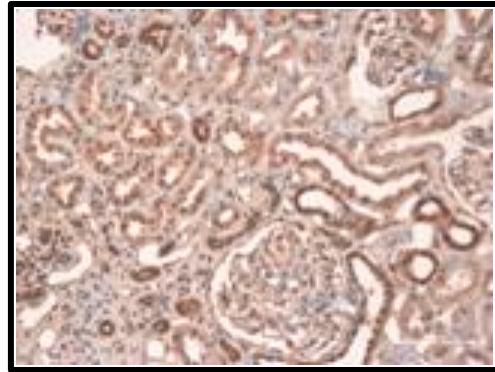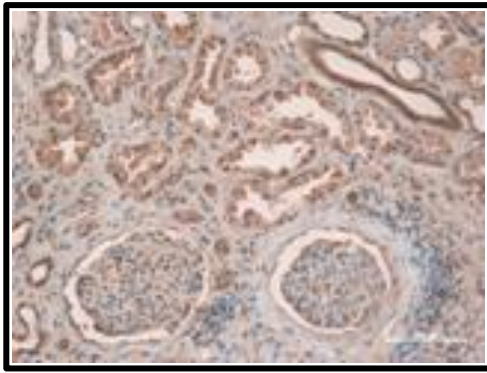
Spleen
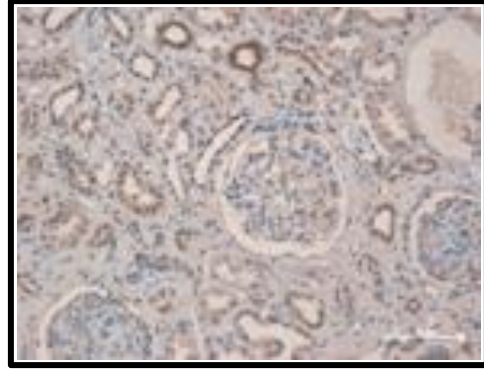
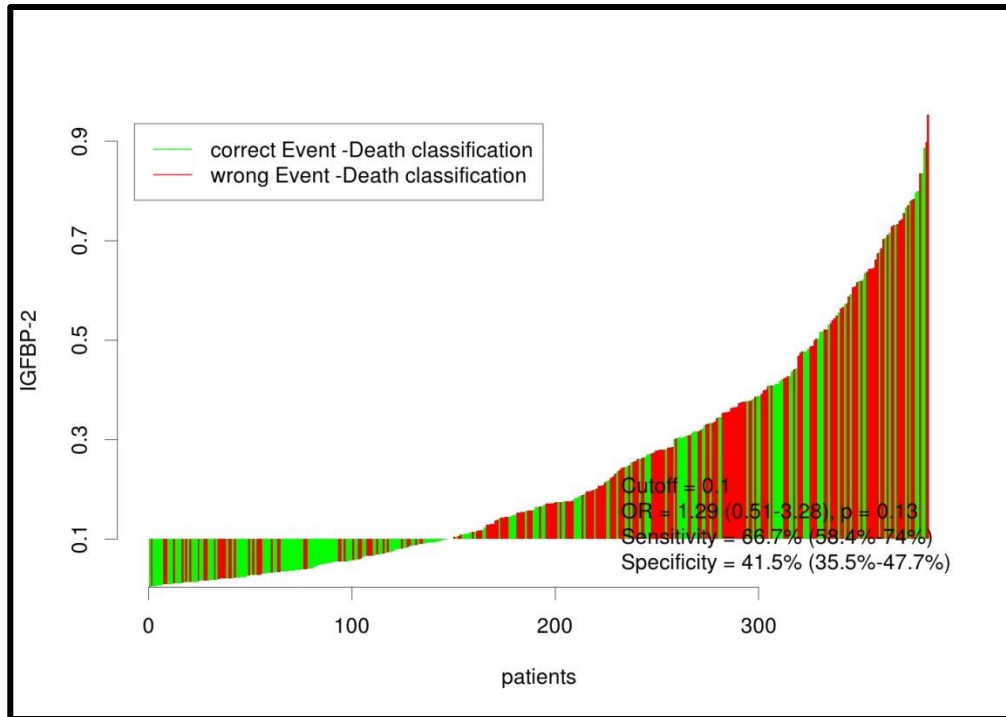**4- Optimisation of IGFBP-5 (kidney)**


1:50

1:100

1:200

1:400

5- Definition of cut-off point for scoring

ROC curve to determine optimum cut-off point.

Using an online cut-off finding system (see http://molpath.charite.de/cutoff/), the cut-off point shown is 0.1, with 66.7% sensitivity and 41.5% specificity. A total of 424 samples were included, and the algorithm results ranged between 0.00297± Px and 0.96279± Px. The red cross represents the cut-off point
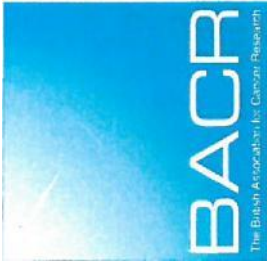
6- Awards

for Cancer Research
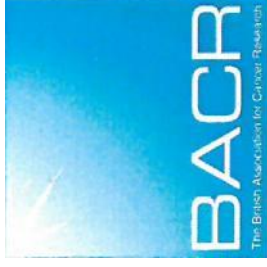
The BACR Hamilton Fairley
Poster Prize

Awarded to

Yousef Hawsawi

at the **2014 NCR** Cancer Conference

pool on 2nd to 5th November 2014

Appendix 5: Publication

Publications

1) **Hawsawi, y**., El-gendy, R., Twelves, C., Speirs, V. & Beattie, J. 2013. **Insulin-like growth factor - oestradiol crosstalk and mammary gland tumourigenesis**. *Biochim Biophys Acta,* 1836**,** 345-53.

2) Beattie, J., Hawsawi, y., Alkharobi, H., El-Gendy, R., 2015. **IGFBP-2 and -5: important regulators of normal and neoplastic mammary gland physiology**. *J. Cell Commun. Signal,* DOI 10.1007/s12079-015-0260-3

Awards

I was awarded the British Association for Cancer Research (BACR) - **Hamilton-Fairley Young Investigator Award** at the 10th National Cancer Research Institute (NCRI) Conference held in Liverpool (2-5 Nov 2014).

Published Abstracts

1) **Insulin like Growth Factor Binding Proteins and Tamoxifen Resistance in Breast Cancer**

Hawsawi[3], Reem El-Gendy[1], Valerie Speirs[3], Christopher Twelves[2], James Beattie[1].

1- University of Leeds School of Dentistry, Leeds, UK,

2- BHRC Biomedical Health Research Centre,

3- Section of Pathology, Anatomy and Tumour Biology, Leeds Institute of Cancer and Pathology, University of Leeds, Lees, UK, NCRI 20147

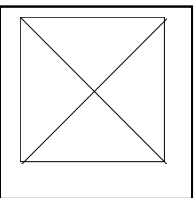2) **The insulin like growth factor axis and development of tamoxifen resistance in breast cancer.**

Hawsawi YM, Beattie J, El-Gendy R, Speirs V, Twelves C. UT Health Science Center at San Antonio

1- University of Leeds School of Dentistry, Leeds, UK

2- Leeds Institute of Cancer and Pathology, Leeds, West Yorkshire, United Kingdom;

3- Biomedical Health Research Centre, Leeds, West Yorkshire, United Kingdom;

4- King Faisal Specialist Hospital and Research Centre, Jeddah, Western, Saudi Arabia SABCS2014.

3) **Role of Insulin like Growth Factor Binding Proteins and Tamoxifen Resistance in Breast Cancer Epithelial Cells.**

Hawsawi, Y.M[1].   ; Jove, M[2].   ; Humphries, M[3].   ; Wright, A[3].   ; El-Gendy, R[1]. ; Twelves, C[2]. ; Speirs, V[3]. ; Beattie, J[1].

1- University of Leeds School of Dentistry, Leeds, United Kingdom.

2- St James's Institute of Oncology, Leeds, United Kingdom.

3- Leeds Institute of Cancer and Pathology, University of Leeds, Leeds, United Kingdom - Dublin Pathology 2015 23-25 June

[4]