Molecular Differences in Sporadic Breast Cancer in Young Women (≤ 35-Year Old): Analysis of TGFBI, DDB2 and MCM5

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Molecular Differences in Sporadic Breast Cancer in Young Women (≤ 35-Year Old): Analysis of TGFBI, DDB2 and MCM5

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Abstract

The aim of this study was to investigate mRNA and protein expression of three genes (Transforming Growth Factor Beta Induced (*TGFBI*), Damaged-Specific DNA Binding (*DDB2*) and Minichromosomal Maintenance-5 (*MCM5*)) in breast cancers. Q-RT-PCR (36 cancers, 8 normal/benign tissues and 9 organoid samples), western blotting (6 cell lines, 6 cancers and 4 normal/benign tissues) and immunohistochemistry (67 breast cancers) were performed, and for TGFBI functional assays (viability, apoptosis and invasion) were carried out in two cell lines (ZR-75-1 and MDA-MB-468) after transient transfection with recombinant TGFBI and vector controls.

TGFBI showed reduced mRNA and protein expression in all cancer cell lines relative to HBL-100. The mRNA levels were also significantly lower in breast cancers compared to normal/benign tissues. Immunohistochemistry results showed that 46 / 67 breast cancers were negative or had <1% nuclear staining. There was a significant correlation between TGFBI mRNA levels and patient age; with lower levels expressed in younger women (p=0.04). Higher expression of DDB2 mRNA was observed in ER/PR positive (MCF-7, T47-D and ZR-75-1) compared to ER/PR negative (HBL-100, MDA-MB-231 and MDA-MB-468) cell lines. Higher DDB2 mRNA levels was .significantly correlated with ER positive (p=0.04) and grade II (p=0.02) tumours; lower levels were associated with younger patient age (p=0.025). In addition, higher DDB2 protein expression was associated with ER (p=0.001) and PR (p=0.004). Elevated MCM5 mRNA and protein levels were observed in MCF-7 and MDA-MB-231. MCM5 immunoreactivity was significantly correlated with low grade (p=0.02) and ER/PR positive (ER p=0.04 and PR p=0.01) tumours. Transfection with TGFBI had no effect on viability, apoptosis and invasion of ZR-75-1 and MDA-MB-468 cells.

In conclusion, the results fail to support the hypothesis of this study, namely that expression of TGFBI, DDB2 and MCM5 could contribute to the more aggressive features of sporadic breast cancers in younger women.

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List of Abbreviations

AJ Buffer	Alec Jeffreys Buffer
AMV-RT	Avian myeloblastosis reverse transcriptase
APS	Ammonium pyrosulphate
ATM	Ataxia telangiectasia mutated gene
BBD	Benign breast diseases
BMI	Body Mass Index
Bp	Base pair
BRAC1	Breast cancer associated gene 1
BRAC2	Breast cancer associated gene 2
BSA	Bovine serum albumin
CBP	Creb binding protein
cDNA	Complementary Deoxyribonucleic Acid
CHD1	Cadherin-1 gene
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma *in situ*
DDB1	DNA damage-binding protein 1
DDB2	DNA damage-binding protein 2
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
DPX	1,3-diethyl-8-phenylxanthine

ECL	Enhanced chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
ER	Oestrogen receptor
FAS I	Fasciclin I
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FFPE	Formalin Fixed Paraffin Embedded
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GRP	Gastrin releasing peptide
HBSM	Human bronchial smooth muscle
HCE	Human corneal epithelial
HER-1	Human epidermal growth factor receptor 1
HER-2	Human epidermal growth factor receptor 2
HPMC	Human peritoneal mesothelial cells
HPT	Hormone Replacement Therapy
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HRP	Horseradish peroxidise
IDC	Invasive Ductal Carcinoma
IGFBP3	Insulin-like growth factor binding protein 3
IHC	Immunohistochemistry
ILC	Invasive Lobular Carcinoma

IMS	Industrial methylated spirits T100
LB	Luria Bertani
LCIS	Lobular Carcinoma In Situ
LEC	Lymphatic endothelial cells
LOH	Loss of heterozigosity
MAPK	Mitogen-activated protein kinase
MCM	Minichromosomal maintenance
MCM5	Minichromosomal maintenance-5
$MgCl_2$	Magnesium chloride
ml	Mililiter
mM	Milimolar
mRNA	Messenger Ribonucleic acid
NER	Nucleotide excision repair
ng	nanogram
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog gene
Q-RT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RGD	"Arginine-Glycine-Aspartic acid"
RNA	Ribonucleic acid
ROC1	Ring finger-containing protein
RPMI	Rosewell park memorial institute
RT	Reverse transcriptase
R/T	Room temperature

SDS	Sodium dodecyl sulphate
PAGE	Polymerase acrylamide gel electrophoresis
SEM	Standard error of mean
SMC	Smooth muscle cells
Std	Standard deviation
TAE	Tris acetic acid EDTA
TBE	Tris borate EDTA
TBS	Tris buffered saline
TBS-T	Tris buffered saline tween
TE	Tris EDTA
TEMED	Tetramethylethylenediamine
TP53	Tumour Protein 53
TFRC	Transferrin receptor
TGF-β_1	Transforming Growth Factor Beta
TGF-β-I	Transforming growth factor beta induced
UNG	Uracil-N-Glycosylase
UP	Ultra pure
VSMC	Vascular smooth muscle cells
µm	Micrometer
µl	Microliter
µg	Microgram

Chapter 1

Introduction

1.1 Breast Cancer

Breast cancer is a major health problem worldwide. There have been considerable advances in the early detection, management and understanding of the biology of the disease. However, it is a heterogeneous disease with differences in the clinical presentation, pathological features and biological potential (Harris *et al.,* 2004).The age of the patient is one of these differences. It has been recognised for some time that breast cancer in younger women (≤ 35-years old) is more aggressive, and has a poorer outcome and survival; this has been associated with its pathobiological features (Adami *et al*., 1986; de la Rochefordiere *et al*., 1993; Chung *et al*., 1996; Walker *et al*., 1996). Two previous PhD students (Whyman, 2005 and Lambe, 2008) undertook microarray and molecular studies, which focused on breast cancer in younger women. This thesis will investigate three genes (*TGFBI, DDB2 and MCM5*) identified as being differentially expressed in breast cancer in younger women. This study will attempt to understand the differences in these genes in breast cancer with this age group.

1.2 Epidemiology

Breast carcinoma is the most prevalent malignancy in women worldwide. The highest incidences are in North America, North-west Europe and Australia, whilst the lowest rates are found in Africa and Asia (Cancer Research UK, 2009). In the United Kingdom (UK) breast carcinomas are one of the ten commonest cancers; 45,500 women are diagnosed with breast cancer each year and 12,000 women died from the disease in 2008. In contrast 277 males were diagnosed in 2007, with 70 male breast cancer deaths in 2008. The highest incidence of breast cancer is in the age group 55-69 (Figure 1.1, A), however, the number of deaths increases with age (Figure 1.1, B). A woman's risk of developing breast carcinoma is estimated to be 1 in 9 during her lifetime (Cancer Research UK, 2009).

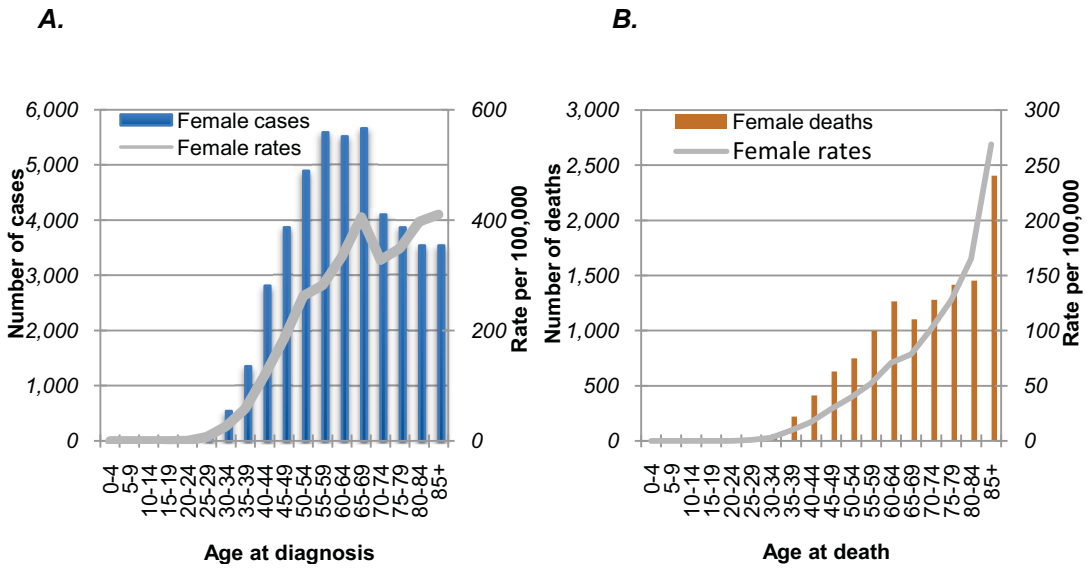


Figure 1.1 Breast Cancer Incidence and Death in United Kingdom

Number of breast cancer cases in UK in 2006 (A); taken from http://info.cancerresearchuk.org/cancerstats/types/breast/incidence/index.htm. Number of deaths in UK in 2007 (B); taken from http://info.cancerresearchuk.org/cancerstats/types/breast/mortality/index.htm.

1.3 Risk Factors

The causes of breast cancer are complex but many risk factors have been identified, which can influence the development of the disease. These relate to the genetic background of the woman, hormonal and reproductive factors, history of benign breast diseases (BBD) and lifestyle.

1.3.1 Genetic Background

It is recognised that there are genes that are associated with an increased risk of women developing breast cancer due to them carrying a mutation. The risk at which women will develop the disease in the future depends on the penetrance of the gene. These genes are categorized into three groups of high, intermediate and low penetrance (Byrnes *et al*., 2008; Ripperger *et al*., 2009).

1.3.1.1 High Breast Cancer Risk Associated with Cancer Predisposition Syndromes

Early onset familial breast cancers are attributable to inherited susceptibility to germline mutations in highly penetrant genes such as breast cancer associated gene 1 (*BRCA1*) and 2 (*BRCA2*). Mutation carriers have up to 60-85% risk of developing the disease (Ripperger *et al*., 2009; Easton *et al*., 2007; De Jong *et al*., 2002). Several other predisposition syndromes have been identified that also increase the risk of developing breast cancer (Table 1.1) (Ripperger *et al*., 2009). These include syndromes due to mutations in tumour protein 53 (*TP53*), phosphatase and tensin homolog gene (*PTEN*) and cadherin-1 (*CDH1*) (Table 1.1).

Table 1.1 High Breast Cancer Risk Associated with Predisposition Syndromes

Gene	Name	Syndrome	Breast Cancer Risk
TP53	Transformation-Related Protein 53	Li-Fraumeni Syndrome 1	50-60% by the age of 45
PTEN	Phosphatase and Tensin Homologue	Cowden Syndrome	30-50% by the age of 70
STK11/LKB1	Serine/threonine Protein Kinase 11	Peutz-Jegher Syndrome	45% by the age of 70
NF1	Neurofibromin	Neurofibromatosis Type I	SIR:3.5
NBN	Nibrin	Nijmegen Breakage Syndrome	2.8 for 657del5
CDH1	E-Cadherin	Hereditary diffused gastric cancer/ familial lobular breast cancer	52% by the age of 75 (2398 delC)

Taken from Ripperger *et al*., 2009
SIR: Standardized Incidence Ratio

1.3.1.1.1 BRCA1 and BRCA2

BRCA1 and *BRCA2* are highly penetrant tumour suppressor genes (Scully *et al*., 1999) located on chromosome 17q21 and 13q12-13 respectively (Hall *et al*., 1990; Wooster *et al*., 1994). Some similarity in the exons of *BRCA1* and *BRCA2* is present; however, no sequence homology is observed in the proteins (Chen *et al*., 1998). These proteins play a role in maintaining genome integrity by interacting with components of DNA repair machinery or by transcriptionally regulating genes involved in DNA damage

(Deng and Wang, 2003). Familial *BRCA2* mutation was also found to significantly increase the risk of developing breast cancer among men (Schlebusch *et al*., 2010).

1.3.1.1.2 TP53

TP53 is a tumour suppressor gene that is located on chromosome 17 (McBride *et al*., 1886). p53 protein plays an important role in controlling DNA damage in cells (Kastan and Bartek, 2004) and also in inducing cell cycle arrest by modulating the transcription of various genes (Whibley *et al*., 2009; Margolis *et al*., 2003). *TP53* is mutated in Li-Fraumeni syndrome 1 (Campeau *et al*., 2008), which is a disorder in which multiple neoplasms develop at an early age including soft tissue sarcomas, leukaemia and brain tumours; and it also increases the risk of developing breast cancer (Table 1.1) (Ripperger *et al*., 2009).

1.3.1.1.3 PTEN

The tumour suppressor gene *PTEN* is located on chromosome 10 (Steck *et al*., 1997), and its protein has a role in cell growth by inhibiting cell proliferation, survival, migration and apoptosis (Baker, 2007; Saal *et al*., 2007). *PTEN* mutation occurs in Cowden syndrome (Liaw *et al*., 1997), which is characterized by multiple hamartomas in skin, breast, thyroid and gastrointestinal tract (Ripperger *et al*., 2009). The heterozygous form increases the risk of breast cancer (Table 1.1). It was recently identified that loss of PTEN expression is significantly associated with the basal-like breast cancer subtype in human sporadic and *BRCA1* associated hereditary breast cancer (Saal *et al*., 2008). Deletion or mutation in the *PTEN* gene has also observed in a variety of human cancers (Stambolic *et al*., 2000).

1.3.1.1.4 CDH1

CDH1 is a tumour suppressor gene located on chromosome 16 and encodes E-Cadherin protein. This protein is very important for cell-cell adhesion, thus maintaining the architecture of epithelial tissues (Becker *et al*., 1994). Patients with mutation in this gene have familial diffuse gastric cancer, and subsequently they are at 50% risk of developing breast cancer (Ripperger *et al*., 2009).

1.3.1.2 Intermediate Penetrance Genes

Women with a heterozygous mutation in DNA repair genes such as: *ATM*, *CHEK2*, *BRIP1* or *PALB2* are at moderately increased risk of developing breast cancer (Table 1.2) (Ripperger *et al*., 2009).

Table 1.2 Intermediate Penetrance Genes Associated with Risk of Breast Cancer

Gene	***Name***	***Relative Breast Cancer Risk***
ATM	Ataxia Telangiectasia Mutated	2.4
CHEK2	Cell Cycle Checkpoint Kinase 2	2.0 for 1100delC
BRIP1	*BRCA1*-Interacting Protein 1	2.0
PALB2	Partner and Localizer of *BRCA2*	2.3

Taken from Ripperger *et al*., 2009

1.3.1.2.1 ATM

The *ATM* protein plays a role in signalling the presence of DNA damage, leading to the phosphorylation of key substrates such as *BRCA1* (Cortez *et al*., 1999). Homozygous mutation carriers present with progressive neurological disorders due to cerebellar degeneration; and also with immunodeficiency, translocation and growth retardation (Taylor and Byrd, 2005). Heterozygous mutation carriers show milder forms of neurological progression; however, they are at an increased risk of breast cancer compared to the general population (Table 1.2) (Easton, 1994; Stredrick *et al*., 2006; Ripperger *et al*., 2009).

1.3.1.2.2 CHEK2

CHEK2 is a DNA-damage signal transducer, which mediates the activation of downstream effectors that play a major role in the initiation of DNA repair, cell cycle arrest or apoptosis, depending on the activated effectors (Antoni *et al*., 2007). Heterozygous mutation carriers present with Li-Fraumeni syndrome 2, which is

characterized by early age cancer development (Bell *et al*., 1999), and also this disorder moderately increases the risk of breast cancer (Ripperger *et al*., 2009).

1.3.1.3 Low Penetrance Genes

A number of Single Nucleotide Polymorphisms (SNPs) have been shown to be associated with an increased risk of developing breast cancer (Table 1.3), for example in genes such as: *FGFR2*, *LSP1*, *MAP3K1*, *TGF-β_1*, *TOX3, COX11, CASP8* and *RAD51L1* (Ripperger *et al*., 2009; Mavaddat *et al*., 2010). The current prognostic investigators have their value; however, two very similar tumours may have quite different outcomes. Inherited genetics variants, particularly of low penetrance alleles may prove to be important in helping to more fully assess prognosis and plan treatment (Eccles and Tapper, 2010).

Table 1.3 Low Penetrance Genes Associated with Relative Risk of Breast Cancer

Gene	***Name***	***Relative Risk of Breast Cancer***	
		Homozygous	Heterozygous
FGFR2	Fibroblast Growth Factor Receptor	1.6	1.2
LSP1	Lymphocytes-Specific Protein 1	1.2	1.1
MAP3K1	Mitogen Activated Protein 3 Kinase 1	1.3	1.1
TGF-β_1	Transforming Growth Factor Beta 1	1.2	1.1
TOX3	High Mobility Group Box Family Member 3	1.6	1.3
COX11	Cytochrome c oxidase assembly protein COX11	0.95	------
CASP8	Caspase 8	0.88	------
RAD51L1	RAD51-Like 1	0.84	------

Taken from Ripperger *et al*., 2009 and Mavaddat *et al*., 2010

1.3.1.3.1 FGFRs

FGFRs play an important role in developmental processes including proliferation, differentiation and angiogenesis through the FGF/FGFR signalling (Knights and Cook, 2010). For example allelic specific up-regulation of *FGFR2* elevates susceptibility to breast cancer (Meyer *et al*., 2008). Four SNPs within intron 2 of *FGFR2* were shown to be associated with an increased risk of developing breast cancer (Hunter *et al*., 2007).

1.3.2 Reproductive and Hormonal Factors

Endogenous sex hormones particularly oestrogens have been associated with an increased breast cancer risk, mainly in post-menopausal women (Cauley *et al*., 1989). This might be due to the conversion of androgenic precursors to oestrogen in adipose cells (Simpson *et al*., 1990). Oestrogen is involved in the promotion of breast cancer, particularly for ER-positive tumours (Gupta and Kuperwasser, 2006).

1.3.2.1 Age at Menarche and Menopause

Short exposure to sex hormones due to late age at menarche and early age at menopause significantly reduces the risk of breast cancer (Kelsey e*t al.,* 1993; Russo *et al.,* 2006). The risk decreases by 22% per five year delay in menarche (Garcia-closas *et al*., 2006) but elevates by 3% for each year older at menopause (Collaborative Group on Hormonal Factors in Breast Cancer, 1997). However, a recent study of Japanese women found that none of the reproductive factors were significantly associated with the risk of breast cancer in premenopausal women, in contrast to the finding among postmenopausal women (Kawai *et al*., 2010).

1.3.2.2 Age at Full-Term Pregnancy

Both full-term pregnancy at a younger age (<30 years) and also additional pregnancies reduces the risk of breast cancer (Kelsey *at el.,* 1993; Vogel, 2000; Hulka *et al*., 2001; Kawai *et al* 2010). This risk is reduced by 7% for each full-term pregnancy (Collaborative Group on Hormonal Factors in Breast Cancer, 2002). This might be due to the cell differentiation that occurs in the breast during the woman's first full-term pregnancy (Singletary, 2002). In addition, full-term pregnancy after the age of 35

further increases the risk compared to nulliparous women (Kelsey *at el.,* 1993; Vogel, 2000; Hulka *et al*., 2001; Kawai *et al*., 2010).

1.3.2.3 Breastfeeding

Long duration of breastfeeding has been observed to reduce the risk of breast cancer by 4% for every 12 months of breastfeeding (Collaborative Group on Hormonal Factors in Breast Cancer, 2002; Hulka *et al*., 2001). This might be due to the lactation and high prolactin levels produced after pregnancy which results in less oestrogen exposure to the breast (Hulka *et al*., 2001).

1.3.2.4 Oral Contraceptive and Hormone Replacement Therapy (HRT)

Use of oral contraceptives is associated with a very small increase in the incidence of breast cancer but in the current and recent users; however, it is not a significant risk after 10 years of stopping use of the contraceptive (Collaborative Group on Hormonal Factors in Breast Cancer, 1997). The Woman's Health Initiative (WHI) (Anderson *et al*., 2004) demonstrated that older women who used oestrogens for at least 5 years had an increased breast cancer risk compared to women who reported no hormone used. Additionally, the use of conjugated oestrogen and progesterone HRT further increases the risk of breast carcinoma compared to not taking HRT (Beral, 2003; Anderson *et al*., 2004); and also increases the risk compared to the use of oestrogen only (Ross *et al*., 2000).

1.3.3 Breast Density and History of Benign Breast Diseases (BBD)

Women with a very low proportion of fat in their breasts are considered to have more dense breasts. These women are at five times higher risk of developing breast cancer compared to women with less dense breasts (McCormack and dos Santos Silva, 2006). A prior history of proliferative BBD particularly with atypical hyperplasia elevates the risk for future development of invasive breast carcinoma (Dupont *et al*., 1993; Bodian *et al*., 1993; Singletary, 2002) in post-menopausal women (Byrne *et al.,* 2000). A study carried out by Hartmann *et al*., (2005) demonstrated that women with atypical hyperplasia had a four-fold risk of developing breast cancer; while women with proliferative disease without atypia had a two-fold risk.

1.3.4 Lifestyle Factors

A woman's lifestyle is considered to have a significant influence on the risk of development of breast cancer. A number of lifestyle factors have been shown to increase the relative risk (Table 1.4). In addition, other lifestyle factors such as socioeconomic status are more likely to influence outcome once the disease has developed rather than development of breast cancer.

Table 1.4 Lifestyle Factors Associated with Relative Risk of Breast Cancer

Lifestyle Factor	***Description***	***Relative Risk Associated with Breast Cancer***
Diet	High consumption of dietary fat and calories (Vogel, 2000; Hulka *et al*., 2001); deficiency of folic acid, Vitamin B_{12}, Minerals (Rogers *et al.,* 1993), Vitamins (A, C, D and E) (Cui and Rohan, 2006), and low fruits, vegetables and fibre intake (Rogers *et al.,* 1993; Kellen *et al*., 2009) have all been shown to increase the risk of breast cancer.	A meta-analysis of 45 studies has shown that dietary fat appears to impart the highest risk; breast cancer risk increases by approximately 13% in women who have high fat intake (Boyd *et al*., 2003).
Height	Taller post-menopausal women are at increased risk of breast cancer; however, this association is unclear (Van den Brandt *et al*., 2000).	Relative risk of increasing height increases by 7% for each additional 5 centimetres (Van den Brandt *et al*., 2000).
Overweight/ Obesity	Multiple studies have found that postmenopausal obesity and being overweight moderately increase the risk of developing breast cancer (Reeves *et al*., 2007). This might be due to increased aromatization of androgens to oestrogens in adipose tissue and decrease in serum sex-hormone-binding globulin levels (Cauley *et al*., 1989). However, BMI was not a risk factor for breast cancer in premenopausal women (Kellen *et al*., 2009).	Postmenopausal women who are overweight have a 10-20% increased risk, while obese postmenopausal women have a 30% risk (Reeves *et al*., 2007).
Physical Activity	A strong protective effect for high physical activity was observed in post-menopausal women, in contrast to pre-menopausal women (Monninkhof *et al.,* 2007). However, inconsistent data were observed as a more recent study found that physical activity applies for both pre and post menopausal women (Howard *et al*., 2009).	20-40% reduced risk for women with high physical activity commitment (Howard *et al*., 2009; Maruti *et al*., 2008).

Alcohol Consumption	High alcohol consumption increases breast-cancer risk (Hulka *et al.,* 2001; Singletary, 2002; Tjonneland *et al.,* 2007).The mechanism might be related to the metabolism of carcinogens such as, ethanol (Rogers *et al.,* 1993).	Daily alcohol consumption increases the risk by 7-12% (Allen *et al*., 2009).
Shift Work	Recent studies found that night shift work (Megdal *et al*., 2005) and short sleeping duration (Kakizaki *et al*., 2008) are linked with increased risk of developing breast cancer. That might be due to reduction in the anti-carcinogenic melatonin hormone (Kakizaki *et al*., 2008).	High level of melatonin metabolite hormone reduces the risk of breast cancer by 38% (Schernhammer and Hankinson, 2009).
In-Utero Exposure	Higher birth weight or higher birth length or older maternal age at conception cause a slight increase in the risk of breast cancer that might be due to high oestrogen levels in the maternal body (Xue and Michels, 2007).	These factors increase the risk of breast cancer by 30% (Xue and Michels, 2007).
Medications	It was found that taking aspirin (Mangiapane *et al*., 2008) and non-steroidal anti-inflammatory drugs (Takkouche *et al*., 2008) reduces the risk of breast cancer.	Women regularly taking aspirin and NSAIDs reduce their risk of breast cancer by 25% (Mangiapane *et al*., 2008; Takkouche *et al*., 2008).
Medical Radiation Exposure	High doses of radiation might increase the risk of developing breast cancer (Vogel, 2000).	Diagnostic x-ray has an estimated increase risk by 0.1% (Berrington de Gonzalez and Derby, 2004).

1.4 Pathology of Breast Carcinoma

Breast carcinomas are divided into *in-situ* and invasive malignancies. All breast malignancies are considered to arise from cells of the terminal ductal lobular unit, irrespective of their particular type (Wellings *et al*., 1975).

1.4.1 In-Situ Breast Carcinoma

This refers to proliferation of malignant cells that are confined by basement membrane (Olivotto *et al*., 2001), and is subdivided into ductal and lobular, which have different morphological features.

1.4.1.1 Ductal Carcinoma In-Situ (DCIS)

There is proliferation of malignant cells which expand duct structures but do not invade through the basement membrane. It is divided into different types depending on the degree of pleomorphism and mitotic activity of cells: low, intermediate and high grade. Necrosis can be present, which can calcify (Silverstein 1998). Different architectural patterns (cribriform, micropapillary, solid) also occur (Walker, 2004). Patients with DCIS can present with a palpable mass but the increased frequency of DCIS is due to detection of associated calcification by mammographic screening. This early detection is one way of improving outcome from breast cancer, since if left DCIS can become invasive particularly the high grade form (Sakorafas and Tsiotou, 2000).

1.4.1.2 Lobular Carcinoma In-Situ (LCIS)

There is a proliferation of poorly cohesive cells that expand the size of acini but without alteration in lobular architecture (Walker, 2004). LCIS is considered to be a risk factor for the development of invasive cancer, but may also be a precursor lesion. LCIS has been more frequently diagnosed in women aged between 40 and 55 years (Simpson *et al*., 2003).

1.4.2 Invasive Breast Carcinoma

This is when malignant cells have broken through the basement membrane and invaded into the adjacent tissue.

1.4.2.1 Invasive (Infiltrating) Ductal Carcinoma (IDC)

IDC accounts for the majority of breast invasive carcinomas (75%) and occurs in both pre- and post menopausal women. In this type, tumour cells can be arranged in groups, cords and gland-like structure, of varying sizes with differing degrees of stroma present. Ductal carcinoma *in-situ* can also be present (Walker, 2004).

1.4.2.2 Invasive (Infiltrating) Lobular Carcinoma (ILC)

ILC accounts for 10-15% of invasive breast carcinoma. It is characterized by small and uniform cells, which are dispersed singly and the essential hallmark molecular feature is the loss or down-regulation of CDH1 (E-Cadherin), which explains their pattern of growth (Sarrio *et al*., 2003; Walker, 2004).

1.4.2.3 Special Types of Invasive Breast Carcinoma

There are various special types of invasive carcinomas, which are summarized in Table 1.5; women diagnosed with one of these special types show excellent prognosis and high survival rate (Harris *et al.,* 2004; Walker, 2004).

Table 1.5 Special Types of Invasive Breast Carcinomas

Special Carcinoma Type	***Description***
Tubular	* Accounts for 1-2% of the breast cancer * It is a well-differentiated tumour, with cells arranged as Tubules
Mucinous	* Accounts for 2-3% of the cases * Tumour cells present in small clusters in large amount of extracellular mucin
Medullary	* Accounts for less than 5% of the cases * Syncytial sheets of pleomorphic cells with associated lymphocytic infiltrate.
Papillary	* Accounts for 0.3% of the cases * Tumours present as a papillary structure

1.5 Breast Tumour Prognosis

There are various prognostic factors that are involved in determining the overall survival of patients such as: patient characteristics, tumour-related features, development of recurrence/metastasis, second breast cancer developing and tumour markers (Soerjomataram *et al*., 2008). The important determinants for predicting survival of breast cancer patients at the time of presentation (Volpi *et al.,* 2004; Singletary and Connolly, 2006) are tumour size (Galea *et al*., 1992), presence/absence of lymph node metastasis (Rampaul *et al*., 2001) and presence/absence of lymphovascular invasion (Pinder *et al*., 1994). Tumour grade is another important determinant (Elston and Ellis, 1991). This information can, along with tumour markers, be used to select the optimum treatment (Soerjomataram *et al*., 2008).

1.5.1 Tumour Histological Grading

Bloom and Richardson (1957) used a simple numerical system for grading, which depended on three histological factors including differentiation or tubule formation, pleomorphism, and mitoses and hyperchromatic nuclei. Elston and Ellis (1991) significantly amended the Bloom and Richardson grading system to form the Nottingham combined histologic grade. These modifications enhanced the reproducibility of scoring, and it is therefore the recommended method in the UK (National Coordinating Group for Breast Screening Pathology, 2005), and is recommended by the European Breast Screening Pathology Group, US Directors of Anatomic and Surgical Pathology (Connolly *et al*., 1996), the College of American Pathologists and the World Health Organization (Singletary *et al.,* 2002).

A good correlation has been found between histological grade and prediction of overall/disease-free survival of patients (Elston and Ellis, 1991; Simpson *et al*., 2000). Patients with grade 1 tumour show better prognosis with an 85% chance of 10-year survival; however, patients with grade 3 tumours have significantly poorer survival (45% 10-year survival) (Pinder *et al*., 2008).

1.5.2 Tumour Pathology Staging

The TNM (Tumour, Node, Metastases) staging system (Figure 1.2) is based on the size of the primary tumour (T), absence/presence of local lymph node involvement (N), and the presence/absence of distant metastases (M) (Singletary *et al*., 2006). The first

edition of TNM was published by the American Joint Committee of Cancer in 1977 and since that time regular revisions have been issued to reflect major advances in diagnosis and treatment (Singletary *et al*., 2006). TNM stage can provide important information about a patient's prognosis, outcome and survival rate (Singletary *et al.,* 2002).

Tumour size is one of the important prognostic factors for predicting long-term survival of the patient. Rosen and Groshen (1990) showed that women with tumours <10mm, 11-13mm, 14-16mm and 17-22mm in diameter have 20-year relapse free survival of 88%, 73%, 65% and 59% respectively. In addition, lymph node involvement is also considered to be essential in predicting the 10-year survival rate, which is 75% for node-negative compared to 25-50% for node positive patients (Rampaul *et al*., 2001). Hence, an increasing number of positive lymph nodes lead to poorer prognosis and 5-year survival (Jatoi *et al*., 1999).

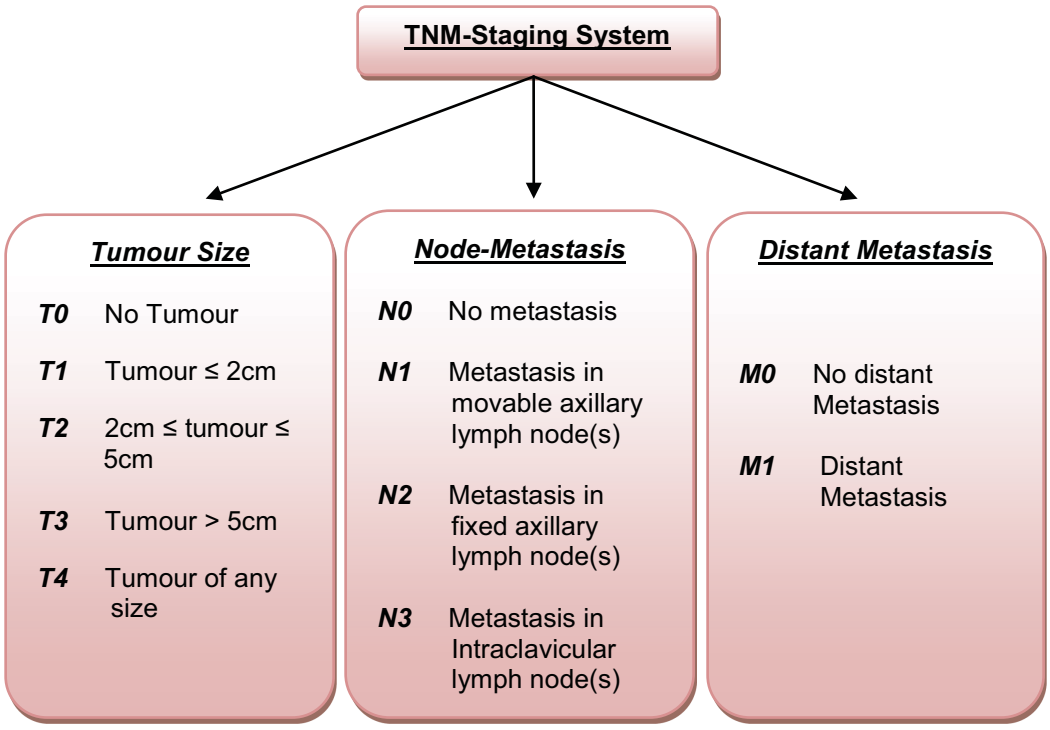


Figure 1.2 TNM-Staging System

Summary of the TNM-Staging System, which shows the ranges of the primary tumour size, lymph node involvement and distance metastasis. Adapted from Singletary *et al*., (2002).

1.5.3 Nottingham Prognostic Index (NPI)

The Nottingham Prognostic Index (NPI) was formulated by the Nottingham Breast Group, who combined three important prognostic factors together (tumour size, lymph node status and tumour grade). This index is the addition of lymph node stage (score as 1-3, 1: no node involvement, 2: three or fewer lymph node metastasis and 3: four or more lymph node metastasis) to the histological grade (score as in *1.5.1*, 1-3) and 0.2 x tumour size (cm) (Galea *et al*., 1992). These calculations provide information on the patient's outcome and survival (Table 1.6).

Table 1.6 Cut- off Points of NPI with their 5-Year Survival Rate

Score	***5-Year Survival Rate***
≤ 2.4	95%
2.4-3.4	85%
3.4-4.4	70%
4.4-5.4	50%
>5.4	20%

1.6 Predictive and Prognostic Markers of Breast Carcinoma

The most common biomarkers established in breast cancer are oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (*HER-2/neu*). Their main role is in the selection of treatment and predicting potential response to therapy, but they also provide prognostic information. Patients with ER and PR positive tumours have better prognosis and are more likely to be responsive to endocrine therapy (e.g. Tamoxifen and Aromatase Inhibitors) (Ciocca *et al*., 2006; Badve and Nakshatri, 2009). However, women with *HER-2/neu* over-expression have a shorter overall survival (Van de Vijver *et al*., 1988), suggesting an important biological role of HER-2/neu in the progression of breast cancer (Menard *et al*., 2000).

1.6.1 Oestrogen Receptors (ERs)

Two types of ERs have been recognized: ERα (~67kDa) and ERβ (~59kDa), which both have a highly homologous structure, similar oestrogen binding affinity and identical transcriptional activation (Jarvinen *et al*., 2000). The nuclear ERs play an important role in modulating the transcription of various genes (Bjornstrom and Sjoberg, 2005) through both direct and indirect ER binding (Badve and Nakshatri, 2009). The ERα positive and ERβ positive tumours are associated with better disease outcome and also increase the likelihood of response to endocrine therapy (Jarvinen *et al*., 2000; Murphy and Watson, 2006).

1.6.2 Progesterone Receptors (PRs)

PR is an oestrogen-regulated gene that is expressed as two isoforms PR-A and PR-B from a single gene (Kraus *et al*., 1993). Due to its regulation via oestrogen, ER-positive/PR-positive tumours are more common than ER-positive/PR-negative or ER-negative/PR-positive tumours (Cui *et al*., 2005).

1.6.3 HER-2/neu Receptors (C-erbB-2)

HER-2/neu proto-oncogene encodes a transmembrane glycoprotein of 185kDa, which is part of the EGFR family (Ross and Fletcher, 1998). There is no known ligand for HER-2/neu receptor; it heterodimerizes with one of its family members (Ross and Fletcher, 1998). This activates the mitogen-activated protein kinase (MAPK) pathway or phosphatidylinositol 3-kinase (PI3K) to increase the transcriptional activity of various genes (Menard *et al*., 2000). HER-2/neu over-expressing tumours have been associated with poor prognosis (Menard *et al*., 2001) due to their aggressive phenotype including high histological grade, ER^-/PR^- and reduced risk of overall survival (Ferrero-Pous *et al*., 2000).

1.7 Treatment of Breast Cancer

1.7.1 Surgery and Radiotherapy

Surgery is the first step in treating breast cancer. Its two main goals are removing the cancer and also defining the stage of the disease (Hammer *et al*., 2008). Breast Conservation Surgery (BCS) removes the tumour only, with lymph node surgery, and

this is followed by radiotherapy to ensure the ablation of tumour cells at the surgical excision margins (Hammer *et al*., 2008). This radiation post surgery plays an important role in reducing local recurrence (Cody *et al*., 2002; Young, 2001; Breast Disease Site Group of the Ontario Cancer Treatment and Research Foundation, 1998) but several studies found that it has no impact on the overall survival (Koukourakis, 2009). Mastectomy is the complete removal of breast and axillary lymph nodes (Hammer *et al*., 2008). The BCS approach followed by radiotherapy is optimal treatment for early breast cancers compared to simple mastectomy (SM) because it has equal recurrence rate and outcome to simple mastectomy (10-year survival for BCS and SM is 80.56% and 77.4% respectively (Mitov *et al*., 2006)) (Taucher and Jakesz, 2004; Danoff *et al*., 1985). Positive axillary lymph node and younger age patients have poorer local control and disease free survival post BCS and radiotherapy. Therefore, these women need more aggressive treatments (Kim *et al*., 2005).

1.7.2 Chemotherapy

There are two different forms of chemotherapy single-agent chemotherapy and poly-agent chemotherapy. Both treatments are beneficial in controlling any remaining deposits of disease, decreasing the recurrence rate and improving long-term survival (EBCTCG, 2005). Poly-agent chemotherapy reduces both recurrence and mortality rate from breast cancer while single-agent chemotherapy has an influence on the recurrence rate only (EBCTCG, 2005). Systemic adjuvant chemotherapy has a better outcome for younger women, showing improvement of 7-11% in 10-year survival for women <50 years old at diagnosis, while 2-3% for those 50-69 years (EBCTCG, 2002).

1.7.3 Endocrine Therapy

There are many forms of endocrine therapy including: ovarian ablation, luteinizing hormone-releasing hormone agonists, tamoxifen and aromatose inhibitors (Ciocca *et al*., 2006). All of these therapies reduce the activation of oestrogen on tumour cells either by inhibiting oestrogen from binding to their receptors (Tamoxifen) or by inhibiting their synthesis (Aromatose Inhibitors) (Mouridsen *et al*., 2003). Tamoxifen is a selective oestrogen receptor modulator drug, widely used for treating patients with ERα and PR positive tumours, because it improves 10-year survival of the patient and also shows better overall survival (Ciocca *et al*., 2006). Over-expression of HER-2 with low

hormone receptors is associated with low response to any type of endocrine therapy due to the resistance that occurs (Rastelli and Crispino, 2008).

1.8 Molecular Profiling and Breast Cancer

Although important information can be gained from the pathology, staging, ER/PR/HER-2 status of breast cancer there is a need for more detailed information about individual breast cancers. Microarray technology has become a useful method for allowing analysis of thousands of genes at the same time (Liu *et al*., 2008). It has led to a greater understanding of the genetic alterations underlying breast cancer development and progression, resulting in the identification of specific subgroups (molecular classification), and has extended knowledge of the molecular alterations that relate to good or poor prognosis, and response to different therapeutic agents (Weigelt *et al*., 2010).

1.8.1 Development and Progression of Breast Cancer

The development and progression of breast cancer depends on accumulations of genetic alteration events and also epigenetic changes that affect the function and regulation of various genes, and influence the controlling mechanism of cellular processes (Reis-Filho *et al*., 2005; Polyak, 2007). It had been proposed that breast cancer develops along two multi-step pathways similar to the Vogelstein colon cancer model (Vogelstein *et al*., 1988). These were based on morphological features, such as atypical ductal hyperplasia which is associated with a higher risk of developing breast cancer (See *1.3.3*) (Simpson *et al*., 2005). The first pathway was the progression from normal to invasive ductal carcinoma by passing through various stages: hyperplasia of usual type, atypical ductal hyperplasia and ductal carcinoma *in-situ* (DCIS). The second pathway was progression from normal breast to atypical lobular hyperplasia, followed by lobular carcinoma *in-situ* (LCIS) and then finally infiltrating lobular carcinoma (Reis-Filho *et al*., 2005; Simpson *et al*., 2005). However, molecular profiling and genomic technologies have extended understanding of breast cancer development and progression. Low grade (grade I) cancers and infiltrating lobular carcinoma (ILC) are ER^+/PR^+ and have frequent 16q loss with ILC having alterations of *CDH1* gene and gains of 1q and 16p (Natrajan *et al*., 2009; Melchor *et al*., 2008). However, high grade lesions (grade III) are ER^-/PR^-, may have *HER-2* over-expression, express 'basal' markers and have complex karotypes (Reis-Filho and Lakhani, 2003; Roylance *et al*.,

1999). These differences between low and high grades suggested that progression from a low lesion to a higher would be uncommon (Abdel-Fatah *et al*., 2007). However further studies, reviewed by Lopez-Garcia *et al*., 2010, indicate that ER^+ and ER^- breast cancer are distinct diseases. In ER^+ breast cancer grade is associated with the number and complexity of genetic aberrations. High grade ER^+ cancers can have genetic aberrations found in ER^+ low grade tumours, so a subgroup may originate from low grade. ER^- breast cancer is more complex and heterogenous and the genetic alterations differ from those in ER^+ disease. These differences are important in the study of breast cancer in younger women, since a high proportion of these cancers are ER^-.

1.8.2 Gene Expression Microarray

1.8.2.1 Classification of Breast Cancers

The first published major analysis of gene expression microarrays by the Stamford group resulted in the classification of breast cancer into four distinct subtypes, based on the variation in gene expression. The subtypes (luminal, normal-breast like, over-expression of HER-2 and basal-like) were obtained from Hierachical Cluster Analysis of cDNA microarrays (Perou *et al*., 2000). The luminal subtype was then subdivided into two different groups. luminal “A” and luminal “B”, which differ in their ER-activated and proliferation-related genes, with lower grade and better prognosis for luminal “A” compared to luminal “B” group (Sorlie *et al*., 2001; Sorlie *et al*., 2003). The normal-breast like, *HER-2* over-expression and basal-like subtypes are ER negative; however, the latter 2 subtypes have more aggressive features (Perou *et al*., 2000). Over-expression *HER-2* subtype has amplification of *HER-2* and also includes *HER*-pathway genes (Rouzier *et al*., 2005). Basal-like subtype express high molecular weight cytokeratins, P-cadherin, caveolins 1 and 2 (van de Rijn *et al*., 2002) and EGFR (Nielsen *et al*., 2004). This subtype is of high grade with high mitotic indices, and it is more common in younger women (Rakha *et al*., 2008). The different subtypes are associated with significant differences in relapse-free and overall survival (Hu *et al*., 2006a).

Three other subtypes have been recently described which are ER negative but have gene expression patterns distinct from each other: molecular apocrine (Farmer *et al*., 2005) and claudin-low (Herschkowitz *et al*., 2007).

1.8.2.2 Prediction of Prognosis and Response to Treatment

cDNA microarray studies have been found to predict breast cancer prognosis through genes that are involved with cell growth and proliferation, angiogenesis, apoptosis, signal transduction and transcription factors and regulators (van de Vijver *et al*., 2002; Li *et al*., 2009). There are several novel commercial assays, based on previously published microarray studies, that can be used to predict the patient's prognosis and response to therapies such as MammaPrint 70-gene signature (Van't Veer *et al*., 2002), Oncotype DX (Paik, 2007), Theros® (Ma *et al*., 2004) and Veridex® 76 gene signature (Wang *et al*., 2005). These assays differ in the tissue type that can be used (frozen/formalin fixed paraffin-embedded), ER and lymph node status of the patient.

MammaPrint is a microarray based assay that uses frozen breast cancer samples. It is for, patients <61 years old with stage I or II and lymph node negative disease (Van't Veer *et al*., 2002; Van de Vijver *et al*., 2002). It consists of 70 genes which are involved in proliferation, invasion, metastasis, stromal integrity and angiogenesis (Slodkowska and Ross, 2009). The 70 genes were identified by analyzing 78 frozen samples from breast cancer patients who were lymph-node negative and younger than 55 years (Van't Veer *et al*., 2002). Patients can be categorized into good or poor prognosis groups. Good prognosis patients are treated with endocrine therapy provided if their tumour is ER^+, while poor prognosis patients are treated with both chemotherapy and endocrine therapy depending on the tumour biology (Buyse *et al*., 2006; Bueno-de-Mesquita *et al*., 2009).

The Oncotype DX® is a quantitative RT-PCR assay that investigates 21 genes in formalin fixed paraffin embedded tissue and is for ER^+ breast cancers and lymph node negative patients (Paik *et al*., 2004; Paik, 2007). These genes were selected because they were found to be most strongly linked with distant recurrence of the 250 genes investigated (Paik, 2007; Ross *et al*., 2008). The Recurrence Score (RS) that is obtained from this assay ranges from 0-100 and predicts the risk of 10-year distant recurrence (Ross *et al*., 2008). In addition, the RS is important in predicting whether the patient will benefit from adjuvant systemic treatment (chemotherapy). Patients with low RS are treated with endocrine therapy, while high RS patients are treated with chemotherapy (Paik *et al*., 2006).

1.9 Breast Cancer in Younger Women

Breast cancer in younger women is strongly associated with a positive family history, particularly with *BRCA1* mutation. The relative risk of there being a germline *BRCA1* mutation is 33 and 32 in the women presenting with breast cancer in the 30-39 and 40-49 age groups respectively; however, it decreases with age thereafter (Antoniou *et al*., 2003). *BRCA1*, *BRCA2* and *TP53* mutations were found in half of the women diagnosed at age of 30 with a strong family history, while only 10% of the women identified with these mutations (*BRCA1*, *BRCA2* and *TP53)* were related to non-familial breast cancer (Lalloo *et al*., 2006).

Breast cancers in younger women tend to have more aggressive features (Maggard *et al*., 2003). A study that included 185 younger breast cancer patients (<35-years) out of 1427 undergoing surgery found that their tumours lacked oestrogen and progesterone receptors, tended to be of higher grade and vascular or lymphatic invasion was frequently presented compared to the 35-50 years group (Colleoni *et al*., 2002). Other studies have similar results, confirming that tumours in the younger age group are more likely to be higher grade, larger size, ER/PR negative and lymph node positive (Walker *et al*., 1996; Gnerlich *et al*., 2009; Anders *et al*., 2008), besides having over-expression of *HER-2* (Anders *et al*., 2008). All these characteristics tend to worsen the disease-free survival of the younger patients (Anders *et al*., 2008).

Younger age has been shown to be an independent predictor of poor outcome and survival compared to the older group. Chung *et al*., (1996) investigated 3,722 women diagnosed with invasive breast carcinoma, and found that the 210 younger women age group (<40-years) had the worst 5-year cancer specific survival (69.7%) compared to the other age groups (41-50 years, 51-60 years, 61-70 years, 71-80 years and >80 years). Another retrospective study was conducted, where 281 stage I and II breast cancer patients were reviewed, this found that the 5-year overall survival and 5-year relapse-free survival in the younger patients (<35-years) were 65% and 40% respectively compared to 98% and 80% in those over 35-years (Yildirim *et al*., 2000). Genomic analysis profiling has shown that there are differences between breast cancers from women at different age groups, at the molecular level. 367 genes were identified that could distinguish between breast cancers in women diagnosed at ≤ 45-years (200-cases) and cancers from women diagnosed at ≥65-years old (211-cases). A significant decrease in the mRNA levels of ERα, ERβ and PR was observed, with higher mRNA levels of the HER-2 and EGFR/HER-1 genes (Anders *et al*., 2008).

1.10 Previous Microarray Analysis of Breast Cancers in Younger Women (≤ 35-Years Old)

Research into sporadic breast cancer in younger women has been undertaken at the University of Leicester for several years (Walker *et al*., 1996; Johnson *et al*., 2002). In order to understand more about breast cancer occurring in young women (≤ 35-years old) a previous PhD student in the group used a cDNA microarray approach to try to identify novel gene differences in sporadic breast cancers in young women (Whyman, 2005).

Affymetrix GeneChips®, including 15,000 genes (Enzo® BioArray™ High Yield TM RNA Transcription Labelling Kit (Affymetrix, 900182)), were used to compare cDNA from 2 breast cancers from women aged 35 years, one normal breast organoid sample (isolated myoepithelial/epithelial aggregates) and the non-tumourgenic cell line HBL-100. Haematoxylin and Eosin staining of frozen sections of both cancers showed that the samples used comprised greater than 90% tumour cells. Both were infiltrating ductal carcinomas that were grade 3 and node positive.

In the first analysis the expression pattern of the cancers was compared with that of HBL-100 cells. 470 genes were identified that had higher expression in the cancers and 285 genes that had lower expression using Significance Analysis of Microarray (SAM). In the second analysis comparison was with the normal breast organoid and this identified 78 up-regulated genes and 372 down-regulated genes in the breast cancers. Since HBL-100 is an immortalised normal breast cell which shows tumourigenic features it was felt that comparison between the normal organoid and cancers was more relevant for identifying differences with normal breast. Following bioinformatic analysis 6 genes were found to have significant up-regulation in the breast cancers (Table 1.7), and they were further investigated using q-RT-PCR (SYBR green) by Whyman (Whyman, 2005). These 6 genes were considered suitable candidates for further study. None of the genes had been identified in previously published microarray-studies of breast cancer.

Table 1.7 Six Genes Identified as Up-Regulated in Breast Cancers Following Bioinformatic Analysis of cDNA Microarray Study

Gene Name	Gene ID	Fold Change Compared to Organoid	Gene Location	Assumed Function
Transforming Growth Factor Beta Induced (TGFBI) (68kD)	NM_000358	3.53	5q31	Adhesion protein
Retinoblastoma Binding Protein 4 (RBBP4)	X71810	2.04	1p35.1	Involved in histone acetylation and chromatin
A Kinase Anchor Protein 1 (AKAP1)	NM_003488	3.98	17q21-q23	Intracellular localisation of PKa and PP1
CCAAT/Enhancer Binding Protein, Alpha (CEBPA)	NM_004364	2.96	19q13.1	Transcription Factor
Damage-Specific DNA Binding Protein 2 (DDB2 48kD)	NM_000107	2.30	11p12-p11	DNA repair via NER
Granulin (GRN)	BC000324	5.90	17q21.32	Autocrine growth factor

In order to determine whether the up-regulation was specific to breast cancers occurring in younger women≤(35 -years old) another PhD student (Lambe, 2008) investigated the 6 genes (Table 1.7) and also selected 3 additional genes that also were shown to be up-regulated in breast cancer compared to normal (Table 1.8). All genes were investigated by analysing breast cell lines, normal organoids and cancers from women of different age groups.

Table 1.8 Three Further Genes that were Up-Regulated in Breast Cancers as Identified in the cDNA Microarray Study and Investigated by Sinead Lambe

Gene Name	Gene ID	Fold Change Compared to Organoid	Gene Location	Assumed Function
Acidic Protein Rich in Leucines (APRIL)	NM_006401	2.23	9q22.32	Regulating ribosomal RNA biosynthesis
Nuclear Receptor Coactivator 3 (NCOA3)	AI761748	5.33	20q12	Enhances transcriptional activator functions of nuclear receptors
Retinoic Acid Receptor Responder 3 (RARRES3)	NM_004585	3.42	11q23	Cell differentiation and growth suppression

The main results of Lambe's work were that both NCOA3 and RARRES3 were highly expressed at the mRNA level in breast cancers in women younger than 35 years compared to women > 35-years old. Her study also confirmed that NCOA3 protein is highly expressed in the ER^+ cell lines (MCF-7, ZR-75-1 and T-47D) compared to ER^-

(HBL-100 and MDA-MB-468). Immunohistochemistry found NCOA3 protein in cancers from both younger and older women regardless of ER status. RARRES3 protein was found in 4 breast cell lines (HBL-100, MDA-MB-468, MCF-7 and ZR-75-1) (Lambe, 2008).

Unlike the preliminary findings of Whyman, the Lambe study found TGFBI mRNA to be down-regulated in breast cancer, compared to normal, with no association with the age of the patient, and it was also found in low levels in cell lines except for HBL-100. DDB2 mRNA levels were higher in ER^+ compared to ER^- cell lines, and it was significantly reduced in cancers from the younger women group (mRNA level of 20 cancer cases). The other 5 genes investigated showed no statistical difference between normal and cancers≤ 35, or between breast cancers ≤ 35 years and those >35 years (Lambe, 2008).

For my thesis 3 genes which showed up-regulation in cancer in the microarray were selected (*TGFBI, DDB2* and *MCM5*). Two of these were studied by Lambe (2008) but more cases were needed for further investigations to confirm the results obtained. However, MCM5 was selected for further study as it showed fold change of 9.59 in the breast cancers compared to normal control (Table 1.9).

Table 1.9 Up-Regulated Genes in Breast Cancers Identified in the cDNA Microarray that were Investigated in this Study

Gene Name	***Gene ID***	***Fold Change Compared to Organoid***	***Gene Location***	***Assumed Function***
Transforming Growth Factor Beta Induced (TGFBI) (68kD)	NM_000358	3.53	5q31	Adhesion protein
Damage-Specific DNA Binding Protein 2 (DDB2)(48kD)	NM_000107	2.30	11p12-p11	DNA repair via NER
Minichromosomal Maintance 5 (MCM5)(82kD)	AA807529	9.59	22p13.1	G1 cell cycle regulator

TGFBI gene was selected to be further investigated in this thesis due to the different findings obtained by Whyman (2005) and Lambe (2008). The cDNA microarray data had shown significant up-regulation of *TGFBI* in breast cancer (fold change 3.53) from younger women (≤ 35-years old) (Whyman, 2005). In contrast Lambe's work showed down-regulation of TGFBI mRNA in breast cancers compared to normal controls regardless of the age of the patient. However, this work involved analysis of FFPE tumour tissues by q-RT-PCR, which may explain the differences due to sample heterogeneity and potential errors when analysing more degraded RNA from FFPE

tissues. The importance of this gene also relates to its activation and induction by TGF-β_1 (Table 1.11).

Transforming Growth Factor Beta (TGF-β_1) plays important roles in regulating many cellular processes such as: cell division, differentiation, motility, adhesion and death (Kretzschmar *et al*., 2000). It is a tumour suppressor gene that inhibits epithelial cell growth by phosphorylating pRB, through its influence on the transcriptional up-regulation of the cyclin dependent kinases inhibitors, which induce cell cycle arrest at G1 phase (Kretzschmar *et al*., 2000; Alexandrow and Moses, 1995). The main function of TGF-β_1 in cancer biology is complex but it is suggested that it has dual roles in breast cancer. TGF-β_1 acts as a tumour suppressor in the early stages of breast cancer; however, it acquires a proto-oncogenic activity essentially promoting tumour progression in the late stages of the disease (Kretzschmar *et al*., 2000; Benson, 2004). Resistance to TGF-β_1 response in the late stage can be due to limited or the absence of *TβRI, TβRII*, Smad 4, which in breast cancer cell lines is caused by mutations or aberrant expressions (Kalkhoven *et al*., 1995); however, mutations in *TβRI* and *TβRII* have not been documented in breast cancer (Benson, 2004). A recent study found that there was significant association between epigenetic suppression changes in the TGF-β_1 signalling pathway in human mammary epithelial cells (HMEC) and promotion of cell proliferation. It suggested that TGF-β_2, *TβRI* and *TβRII* are suppressed early in breast cancer due to epigenetic events mainly histone modifications (Hinshelwood *et al*., 2007). Thus, it was felt that further investigation of TGFBI would be appropriate due to the complexity of the role TGF-β_1 in breast cancer.

DDB2 gene was selected for further investigation in this thesis because of the findings in the two previous theses. The previous cDNA microarray data had shown significant up-regulation of DDB2 with a fold change of 2.30 in breast cancer (Whyman, 2005). This result was also confirmed by Lambe (2008) who found higher DDB2 mRNA levels in cancers from younger (<35-years) compared to older (>50-years) patients. However, Lambe only studied a small number of cancers and it was felt important to extend the study to a larger number of cancers and to include analysis of protein expression as well as mRNA analysis.

The importance of this gene is due to its association with DNA repair mechanisms. It has been suggested that DDB complex plays a role in the global genomic repair after binding to DNA lesions, to help in recruiting repair complexes to the region (Datta *et al*., 2001). Furthermore, its expression is linked with ER status in breast cell lines as was found by Lambe (2008) and Kattan *et al*., (2008). Thus, it was felt that further

investigation of DDB2 would be appropriate to examine its relationship to ER in a larger number of cancer cases.

MCM5 gene was selected for further analysis in this study because it showed a significant up-regulation with a 9.59 fold change in the breast cancers in young women (≤35 Years Old) compared to the normal control. This target had not been analysed previously by Whyman or Lambe but was highlighted as being worthy of investigation on re-review of the microarray data at the beginning of this study. MCM5 plays an important role in the initiation of DNA replication in eukaryotic cells (Burger, 2009), and it is considered to be a suitable biomarker for proliferating cells due to its dramatic increase during the cell cycle (Murphy *et al*., 2005). It has been considered to be a potential biomarker in various types of cancers (See Table 1.15) but no investigations were performed of breast cancer. Therefore, it was thought that *MCM5* was worthy of further investigation breast cancer.

1.11 Transforming Growth Factor-Beta-Induced (TGFBI)

TGFBI is a novel gene encoding a 68kDa secreted protein, which is composed of 683 amino acids (Skonier *et al*., 1992). It is known by several alternative names mainly Beta IG-H3; Keratoepithelin and BIGH3 (Thierry-Mieg, 2006). The TGFBI protein has been detected in different tissues such as corneal epithelial cells (Rawe *et al*., 1997), skin fibroblasts (LeBaron *et al*., 1995), mammary glands (Skonier *et al*., 1992), and smooth muscle cells of the bladder (Billings e*t al.,* 2000a).

1.11.1 TGFBI Gene

The *TGFBI* gene is located on chromosome 5q31.1 (Skonier *et al*., 1992; Stone *et al.,* 1994). The gene was first identified from human lung adenocarcinoma cells (A549) that had been treated with transforming growth factor Beta 1 (TGF-β_1) (Skonier *et al*., 1992). Additionally; it was first established to be 5q31-linked to corneal dystrophies in 1997 (Munier *et al*., 1997).

Sixteen different mRNA transcripts are produced by alternative splicing of the *TGFBI* gene. Four of these (b, c, d and e) and the other unspliced form (a) are presented in Table 1.10, which shows the encoding exons, number of amino acids and molecular weight of the translated protein (Thierry-Mieg, 2006).

Table 1.10 mRNA Transcripts of TGFBI

TGFBI Transcript	mRNA Length (bp)	Exons	Amino acid	Molecular Weight (kDa)
A	2926	17	683	74.7
B	2623	17	509	55.7
C	2991	14	474	51.8
D	3159	17	416	45.5
E	1797	6	-----	68

This is taken from (Thierry-Mieg, 2006).

1.11.2 Protein Structure

TGFBI protein (683aa/68-70kDa) consists of 4 repeat regions, each containing 140 amino acid repeats (Munier *et al*., 1997) with internal homology to the Drosophila Fasciclin I (FAS I) family (Skonier *et al*., 1994). Each FAS I domain has two conserved regions known as H1 and H2 (each 10aa). The second and fourth internal FAS I domains have two conserved sequences (Asn-Lys-Asp-Ile-Leu and Glu-Pro-Asp-Ile-Met respectively) known to mediate adhesion (Kim *et al*., 2000b; Ferguson *et al*., 2003). Two putative heparin-binding sequences are within the third FAS I domain, suggesting that this motif might be functional (Ferguson *et al*., 2003). In addition, TGFBI protein has NH_2 terminal secretory signal peptide and Arg-Gly-Asp (Arginine-Glycine-Aspartic acid sequence, RGD) motif at the COOH terminus, which serves as a ligand recognition sequence for several integrins (Skonier *et al*., 1992; Munier *et al*., 1997) (Figure 1.3).

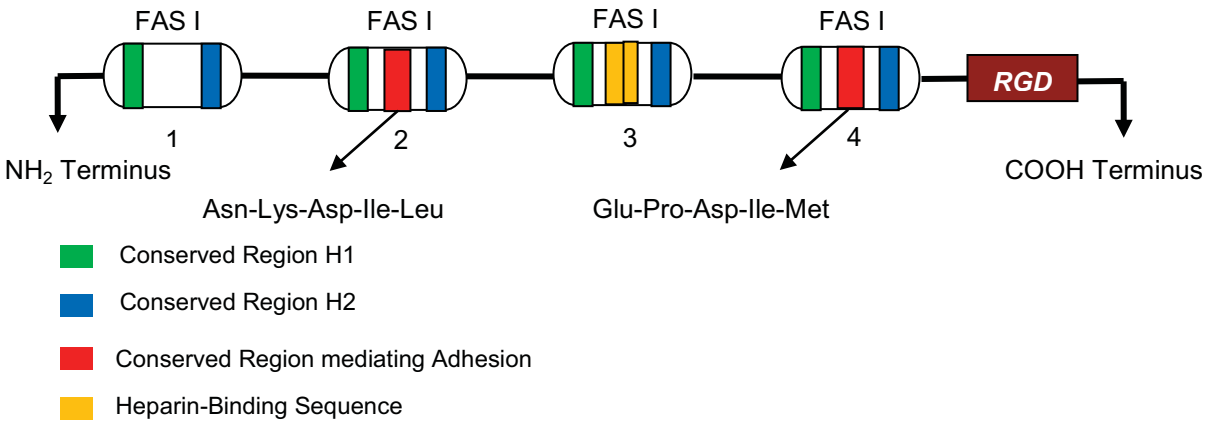


Figure 1.3 TGFBI Protein Structure

TGFBI protein consists of four FAS I domains and RGD at the C-terminal. Each FAS I 2 and 3 domain contains conserved H1, conserved H2 and conserved region mediating adhesion, while FAS I 1 and 4 domains are different from each other and also from the 2 and 4 domains.(Diagram adapted from Ferguson *et al*., 2003; Lee *et al*., 2006; Thapa *et al*., 2007).

1.11.3 Induction of TGFBI

Treatment by TGF-β_1 induces TGFBI expression in various cells types (Table 1.11), which showed very low or undetectable TGFBI expression before the treatment. Treatment of the human keratinocyte cell line (HaCaT) with TGF-β_1 loosened cell-cell contact, which led to cell spreading compared to untreated cells (Cho *et al*., 2004).

Table 1.11 TGF-β_1 Induces TGFBI Expression

Cell Type	***Reference***
Lung Adenocarcinoma (A549)	Skonier *et al*., 1992
Bladder Fibroblasts	LeBaron et al., 1995
Human Bronchial Smooth Muscle	Billings *et al*., 2000a
Bladder Smooth Muscle Cells	Billings *et al*., 2000b
Pancreatic Cancer Cell Lines (CAPAN-1 and PANC-1)	Schneider *et al*., 2002
Human Keratinocyte Cell Line (HaCaT)	Cho *et al*., 2004
Fibroblast-Like Synoviocytes (FLS)	Nam *et al*., 2006
Human Peritoneal Mesothelial Cells (HPMCs)	Park *et al*., 2008
Lymphatic Endothelial Cells (LEC)	Irigoyen *et al*., 2008
MG-63 Osteosarcoma Cells	Zamilpa *et al*., 2009

TGF-β_1, mainly the TGF-β_1 isoform, acts by binding to a heteromeric cell-surface complex. This complex is composed of two transmembrance serine/threonine kinases, named type “I” and type “II” receptors (Hirofumi *et al*., 1999). TGF-β_1 binding causes the phosphorylation of serine/threonine kinases, stimulating several intra-cellular signal transduction pathways, particularly the Smad pathway (Figure 1.4) (Akhurst and Derynck, 2001).

Post phosphorylation, Smad 2 and 3 interact with Smad 4 to form a stable complex that translocates to the nucleus, where it binds to various transcriptional coactivators such as CBP/P300. This connects the Smad complex with the general transcription factors of DNA promoters to regulate target gene expression (Derynck *et al*., 2001).

Any blockage or inactivation of the TGF-β_1 pathway would lead to a lack of response to TGF-β_1, and in turn would affect TGFBI expression (Shao *et al*., 2006) and might have

a role in the in the down-regulation of TGFBI expression (Shao *et al*., 2006). However, although TGF-β_1 signalling maybe inhibited by receptor and ligand methylation, mutation or deletion in neoplastic epithelial cells, stromal cells may still respond in a tumour tissue.

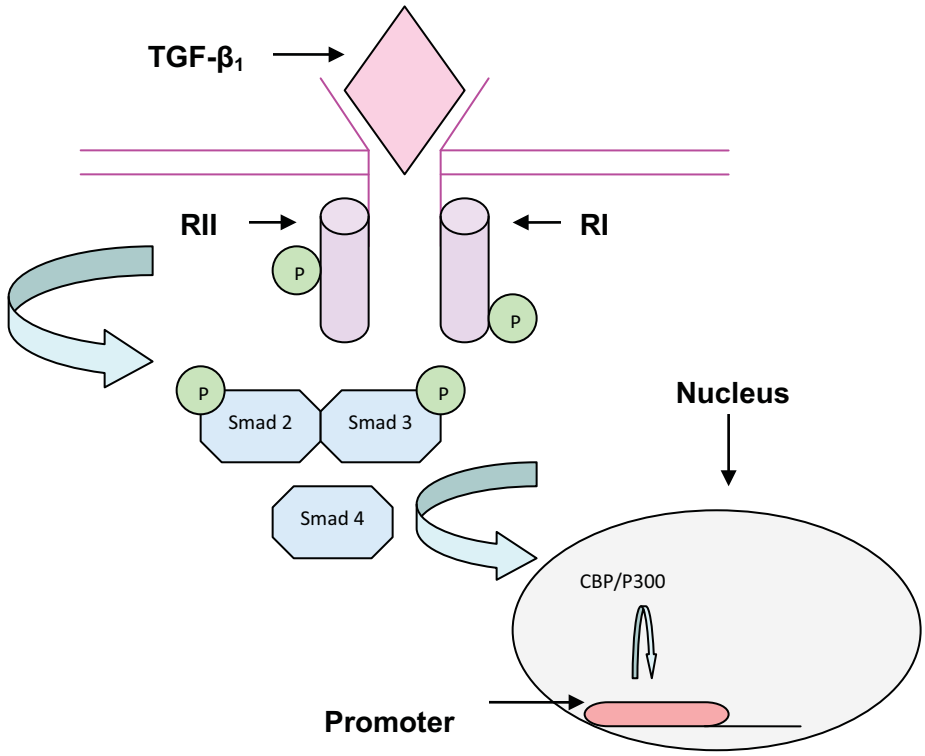


Figure 1.4 Smad Signalling Pathway of TGFBI

This diagram shows that the binding of TGF-β_1 to its receptor activates the Smad pathway, which plays an important role in promoting gene transcription and translation. It is adapted from (Derynck *et al*., 2001).

1.11.4 Protein Localization

TGFBI protein was identified to be a component of the extracellular matrix (ECM) (Skonier *et al*., 1994; Billing *et al*., 2000a, b). However, analysis of cultured primary human bronchial smooth muscle cells (HBSM) (Billing *et al*., 2000a), bladder fibroblasts and bladder smooth muscle cells (SMC) (Billing *et al*., 2000b) demonstrated the presence of TGFBI protein in the ECM but also in the nucleus and the cytoplasm. Additionally, immunohistochemical analysis of the mesothelium of parietal peritoneum (Park *et al*., 2008) and the lung cancer cell line (H522) (Zhao *et al*., 2006) revealed the presence of TGFBI protein in both intracellular and extracellular compartments. These studies demonstrate that although TGFBI protein is a component of the ECM of various

tissues, its nuclear localization suggests that it may have important regulatory functions (Billing *et al*., 2000a; Billings *et al*., 2000b). The nuclear localization of TGFBI can be a result of multiple isoforms of the mRNA or by post-translational proteolytic processing (Billings *et al*., 2000a).

1.11.5 Interaction with ECM Proteins

The precise functions of TGFBI protein are unknown but it has been suggested that it may act as an extracellular attachment protein (LeBaron *et al*., 1995), interconnecting different macromolecular components in the ECM to each other or to the cells. This is essential for the structural integrity and function of tissues and organs (Thapa *et al*., 2007). TGFBI protein binds to a number of ECM components including fibronectin (Billings *et al*., 2002), laminin (Kim *et al*., 2002) and glycosaminoglycans (Ferguson *et al*., 2003), suggesting that TGFBI might play a role in development processes and tissue modelling. In addition, TGFBI bound to collagens in vitro (Hashimoto *et al*., 1997). This association may be important for the modulation of collagen architecture (Hanssen *et al*., 2003).

1.11.6 Adhesion and Migration

TGFBI protein mediates adhesion and migration of different cell types via binding with various integrins (Table 1.12) (Lee *et al*., 2006), particularly $\alpha_3\beta_1$ in epithelial cells, $\alpha_v\beta_5$ in fibroblasts and $\alpha_v\beta_3$ in endothelial cells (Nam *et al*., 2006).

TGFBI protein mediates both adhesion and migration of aortic vascular smooth muscle cells (VSMC) through the interaction with the functional $\alpha_v\beta_5$ integrin (Lee *et al*., 2006). In addition, this integrin and $\alpha_v\beta_3$ play important roles in TGFBI mediating adhesion of osteoblasts through binding with the highly conserved tyrosine and histidine residues (YH motif) (Kim *et al*., 2002). TGFBI mediates adhesion and spreading of fibroblast-like synoviocytes (FLS) (Nam *et al*., 2006), human peritoneal mesothelial cells (HPMC) (Park *et al*., 2008) and lymphatic endothelial cells (LEC) (Irigoyen *et al*., 2008) through its binding to the functional receptor $\alpha v\beta_3$ integrin. In addition, $\alpha_3\beta_1$ integrin is responsible for the adhesion and migration of keratinocytes and renal tubular epithelial cells (Park *et al*., 2004), besides human corneal epithelial (HCE) (Morand *et al*., 2003).

Table 1.12 Functional Integrins of TGFBI Protein in Various Cell Types

Cell Type	***Integrin***	***Reference***
Aortic Vascular Smooth Muscle Cells *Osteoblasts*	αvβ5	Lee *et al*., 2006 Kim *et al*., 2002 (a)
Fibroblast-like synoviocytes (FLS) *Mesothelial cells* *Lymphatic Endothelial Cells (LEC)* *Osteoblasts*	αvβ3	Nam *et al*., 2006 Park *et al*., 2008 Irigoyen *et al*., 2008 Kim *et al*., 2002 (a)
Keratinocytes *Renal Tubular Epithelial cells* *Human Corneal Epithelial (HCE)*	α3β1	Park *et al*., 2004; Park *et al*., 2004 Morand *et al*., 2003

1.11.7 Apoptosis

Several studies demonstrate that TGFBI plays an important role in apoptosis. Transfection of TGFBI plasmid in H522 lung cell line showed significant increase of apoptosis compared to the parental tumour cells, which may be as a result of IGFBP3 up-regulation (Zhao *et al*., 2006). Over-expression of TGFBI protein by transient transfection produced 2-fold increase in apoptosis of the MG-63 osteosarcoma cells, which might be dependent on the C-terminal fragmentation of TGFBI protein (Zamilpa *et al*., 2009).

1.11.8 Tumourigenesis

Microarray analysis of pancreatic cancer (Iacobuzio-Donahue *et al*., 2003), oral squamous cell carcinoma (OSCC) (Tomioka *et al*., 2006), colorectal tumours (Buckhaults *et al*., 2001) and human oesophageal squamous cell carcinoma (ESCC) (Hu *et al*., 2001) demonstrated that TGFBI mRNA is consistently elevated in neoplastic samples compared to normal specimens (Table 1.13). All of these studies performed RT-PCR with the same RNA used for microarray to validate findings, and confirmed the elevation of TGFBI in cancers compared to normal tissues. A previous study of pancreatic cancers (Schneider *et al.*, 2002) also showed high TGFBI mRNA levels in

26 out of 27 pancreatic cancer samples, compared to the normal samples. TGFBI is up-regulated in both colon carcinoma cells (Ma *et al*., 2008) and in highly invasive breast cancer cells (MDA-MB-435S, BT549, MDA-MB-231 and Hs578T) (Zajchowski *et al*., 2001). TGFBI expression was compared in tumour/normal RNA samples; 4 out of 10 renal, 5/7 pancreatic, 4/10 lung, 9/10 colon, 9/10 rectal and 4/7 small intestine cancer had high levels of TGFBI (Ivanov *et al*., 2008). Further studies were performed on renal clear cell carcinoma which demonstrated up-regulation of TGFBI as a result of the effect of of Kruppel-Like transcription factor 10 (KLF10) (Ivanov *et al*., 2008).

Although several studies demonstrated high levels of TGFBI mRNA/protein in a variety of cancers others found that TGFBI protein may be down-regulated in cancer (Skonier *et al*., 1994; Thapa *et al*., 2007), including lung, breast and prostate (Zhao *et al*., 2002). Shao *et al*., (2006) found TGFBI to be relatively expressed in normal and immortalized cell lines but down-regulated or undetectable in most of the tumour cell lines studied (prostate, kidney, lung and breast). An immunohistochemical study (Zhao *et al*., 2006) revealed the absence or reduction (> 2-folds) of TGFBI protein in 45 of 130 primary lung carcinomas compared to normal lung tissues. The mechanism suggested for the down-regulation of TGFBI is CpG hypermethylation of the promoter region; however, this is not the only mechanism because in two mammary tumour cell lines (MCF-7 and MDA-MB-231) there was no methylation of TGFBI promoter which suggested that it might be due to genomic loss at the 5q31 locus (Shao *et al*., 2006; Zhao *et al*., 2006). Skonier *et al*., (1994) showed reduced tumourigenicity of chinese hamster ovary cells, when transfected with TGFBI cDNA, in a nude mouse xenograft model. Transformed human fibroblasts (SV-40) exhibit down-regulation of TGFBI expression in comparison to normal fibroblasts, suggesting that TGFBI expression is reduced during the malignant transformation of this cell type (Schenker *et al*., 1998). It was also demonstrated that mice injected with H522 cell line containing TGFBI plasmid had significant suppression of tumour growth compared to mice injected with H522 vector only cells (Zhao *et al*., 2006). In addition, expression of TGFBI protein suppresses proliferation and invasion of neuroblastoma cells (Becker *et al*., 2006).

Conflicting evidence has been found as to whether TGFBI protein is involved in various aspects of tumourigenesis. Therefore, further research is required in this field. (Thapa *et al*., 2007).

Table 1.13 Details of Microarray Studies that Showed Over-Expression of TGFBI

Cancer Type	***Cell/Tissue***	***Normal Tissues/Cell***	***TGF-β1-I mRNA***	***Reference***
Pancreatic Cancer	AsPc1, BxPc3, CAPAN1, CAPAN2, CFPAC1, Hs766T, MiaPaca2, Panc-1, Su86.86, COLO357 Invasive pancreatic tissues	Normal pancreas samples	Over-expressed in pancreatic cell lines and invasive tissues compared to normal samples	Iacobuzio-Donahue *et al*., 2003
Oral Squamous Cell Carcinoma (OSCC)	Invasive OSSC (9 samples)	Normal matched oral mucosa	Up-regulated in OSSC cases compared to normal oral mucosa	Tomioka *et al*., 2006
Colorectal Carcinoma	Colorectal adenoma samples and carcinomas	Normal colon tissues	High levels in both adenoma and carcinoma	Buckhaults *et al*., 2001
Oesophageal Squamous Cell Carcinoma	HKESC-1 and HKESC-2	Normal oesophageal epithelium tissue specimen	Increased in both cell lines	Hu *et al*., 2001

1.12 Damage-Specific DNA Binding (DDB2)

DNA damage-binding (DDB) protein (also known as UV-DDB) is a heterodimer composed of the DDB1 (127kDa/p127) and DDB2 (48kDa/p48) subunits, which were first purified from HeLa cells (Fujiwara *et al*., 1999). This complex tightly binds to the hallmark of UV-induced mutagenesis (C to T or CC to TT transitions), which are known as Pyrimidine (6-4) pyrimidine photoproducts (6-4PP) and Cyclobutane pyrimidine dimers (CPD) (Brash, 1988). DDB complex plays a role in the global genomic repair after binding to the DNA lesions to help in recruiting the repair complexes to the region (Datta *et al*., 2001).

1.12.1 Gene and Protein

DDB1 (p127) maps to chromosome 11q12-q13, while *DDB2* (p48) is at 11p11-p12 (Dualan *et al*., 1995). The *DDB2* gene is 24-26kb and has 10 exons (Inoki *et al*., 2004), DDB2 protein is composed of an N-terminal segment, 5 bladed WD40 domains and a C-terminal helical domain (Inoki *et al*., 2004), while DDB1 protein consists of only 3 WD40 domains (Angers *et al*., 2006). Both DDB1 and DDB2 are glycosylated proteins; DDB2 protein is widely localized in the nucleus whereas DDB1 is mainly located in the cytoplasm but it moves to nucleus post irradiation (Rapic-Otrin *et al*., 2002; Wittschieben and Wood, 2003). Alternative splicing of DDB2 gene was found to produce 4 different DDB2 protein isoforms (Table 1.14) (Inoki *et al*., 2004).

Table 1.14 Alternative Splicing of DDB2

Alternative Splicing	***Symbol***	***mRNA Length (bp)***	***Omission of Exons***	***Number of aa***	***WD Motifs Present***
Wild-Type	WT	1284	-----	427	5
1	D1	717	4-7	238	1 and 5
2	D2	960	4 and 6	156	Only 1
3	D3	1092	3	363	2-5
4	D4	1106	6	244	1 and 2

1.12.2 Functions

It is suggested that the DDB complex acts as a damage recognition factor in the NER pathway (Datta *et al*., 2001). However, recent studies confirm the association of DDB complex with a number of other factors, which allow it to carry out different roles in mammalian cells. UV-DDB complex interacts with histone acetyltransferase cyclic AMP; DDB1 subunit binds with p300 and DDB2 subunit interacts with CBP (Rapic-Otrin *et al*., 2002), suggesting its involvement with chromatin remodelling during repair (Datta *et al*., 2001). The recruitment of these chromatin remodelling enzymes to the damaged region helps in releasing the DNA from the histone surface via mobilization of the nucleosomes (Rapic-Otrin *et al*., 2002), so allowing full access to the NER machinery repair proteins (Figure 1.5).

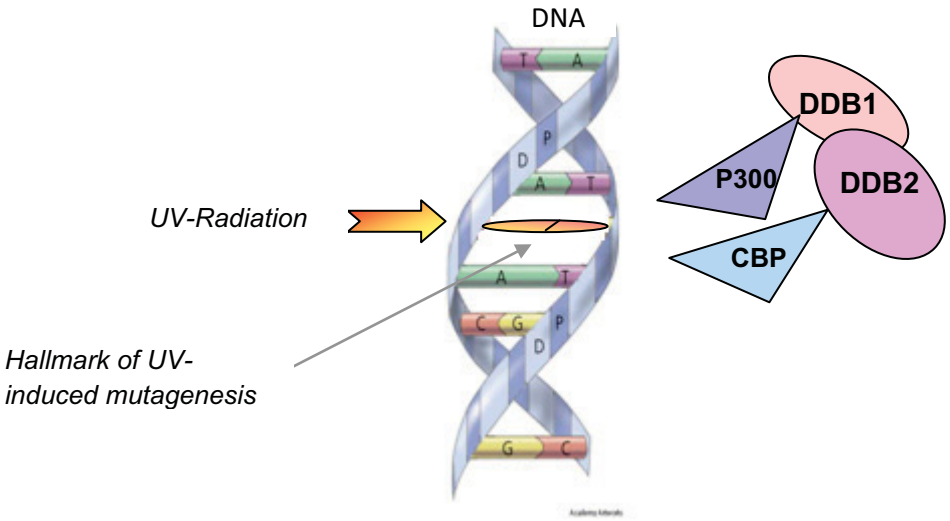


Figure 1.5 Role of DDB-Complex in NER Pathway

During NER, DDB subunits recruit various histone acetyl transferase proteins (P300 and CBP) to the UV-induced DNA damage in order to facilitate chromatin remodelling at the damaged site (Rapic-Otrin *et al.,* 2002*)*. (DNA: http://dictionary.reference.com/illus/illustration.html/ahd4/DNA/dna).

DDB2 is also activated by the E2F family, and in turn cooperates with DDB1 and E2F1 to transcriptionally activate genes required for DNA replication and G1/S transition in mice (Prost *et al*., 2007). DDB2 plays an important role in promoting cell cycle arrest post UV-irradiation due to the decrease in UV-DDB2-E2F1 complexes (Prost *et al*., 2007). In addition, DDB2 acts as a linker between p53 and NER proteins with a strong correlation found between the increased DDB2 mRNA levels and basal p53 expression post DNA damage (Hwang *et al*., 1999). Furthermore, UV-DDB play a role in modification or protolysis of DNA-binding and repair proteins as it binds to an ubiquitin ligase complex together with Cullin4A, ROC1 and DDB1 that becomes active after UV

irradiation of cells (Groisman *et al*., 2003). Cullin-4A, cytoplasmic ubiquitin-protein ligase, interacts with the Ring Finger-Containing Protein (ROC1) through its C-terminal domain, while the N-terminal binds to specific domains in the substrate such as DDB1, leading to the substrate degradation which is responsible for connecting the E1 ubiquitin-activating enzyme-E2 in order to degrade DDB2 (Chen *et al*., 2001).

1.12.3 Mechanism of Action

Following DNA damage by UV-irradiation, a dynamic nuclear accumulation of DDB1 (p127) was found, whereas DDB2 remains in the nucleus and becomes tightly bound to chromatin (Wakasugi *et al*., 2002). Within 4 hours of irradiation, a fraction (60%) of the chromatin-bound DDB2 is degraded (Rapic-Otrin *et al*., 2002), that might be due to the high Cull-4A levels which begins to dissociate from its negative regulators between 1-2 hours upon irradiation (Chen *et al*., 2001). During this time Cull-4A binds tightly to DDB1 in order to form the Cull-DDB-ROC1 complex, which has a role in weakening the interaction between histones (H3 and H4) and DNA (Chen *et al*., 2001), demonstrating the importance of DDB2 degradation in the recruitment of NER complexes in order to repair the DNA-damaged site (Rapic-Otrin *et al*., 2002) . After 8 hours, the DDB2 subunit is undetectable due to the Cull-4A degradation; however, its mRNA level increases (10-12 fold) and becomes several fold above the level in non-irradiated cells, post 48 hours of irradiation (Rapic-Otrin *et al*., 2002).

1.12.4 Tumourigenesis

DDB2 is considered as a protective factor against UV-induced skin cancer due to its role in enhancing NER of UV-induced photoproducts (Alekseev *et al*., 2005). It also functions as a tumour suppressor by controlling p53-mediated apoptosis after UV- DNA lesions (Itoh *et al*., 2004).

Enhanced expression of DDB2 in the mouse epidermis reduced UV-induced carcinogenesis both by delaying the onset of tumours and by reducing the number of tumours per mouse (Alekseev *et al*., 2005). It was also found that both homozygous and heterozygous DDB2 knockout mice exhibited enhanced skin carcinogenesis in response to chronic UV irradiation and have increased frequency of developing tumours notably lung and mammary adenocarcinomas (Yoon *et al*., 2005; Itoh *et al*., 2007).

Loss of DDB2 function in normal cells is related to susceptibility to tumour development (Alekseev *et al*., 2005). DDB2 protein is reduced in breast cancer leading to decrease in the UV-DDB activity, which can be as a result of the high expression of Cull-4A (Chen *et al*., 2001). It was also observed that DDB2 polymorphism might contribute in the aetiology of lung cancer (Hu *et al*., 2006b).

1.13 Minichromosomal Maintenance-5 (MCM5)

MCM5 is a member of the six MCM family genes (MCM2-7), which are involved in the initiation of DNA replication (origin licensing) in eukaryotic cells (Guida *et al.,* 2005; Burger, 2009), ensuring the integrity of the genome (Ohtani *et al.,* 1999). MCM5 was originally identified in yeast (*Saccharomyces cerevisiae)* (Ohtani *et al.,* 1999), then subsequently it was discovered in *Drosophila melanogaster*, *Xenopus laevis*, mice and humans (Guida *et al.,* 2005). MCM5 is considered as a fundamental biomarker for proliferating cells, due to the dramatic increase in its levels during the cell cycle (Murphy *et al.,* 2005), particularly, at the G1/S boundary (Ohtani *et al.,* 1999). However, MCM5 is usually lost at quiescence and differentiation (Murphy *et al.,* 2005).

1.13.1 MCM5 Gene and Regulation

The *MCM5* gene was identified on the short arm of the chromosome 22 (22p13.1) (Paul *et al*., 1996). *MCM5* gene has multiple E2F binding sites in the promoter, suggesting that the gene is regulated by this transcription factor (Ohtani *et al.,* 1999; Guida *et al.,* 2005).

1.13.2 Normal Mechanisms of MCM5 Action

The nuclear MCM5 protein appears to play an important role in the initiation of DNA replication; however, the precise controlling mechanisms remain unclear (Ohtani *et al.,* 1999). At G1 phase of the cell cycle, the pre-replication complex (pre-RC) forms in order to initiate DNA synthesis (Guida *et al.,* 2005). This complex consists of the Origin Recognition Complex (ORC) that binds to the chromatin (Stoeber *et al.,* 2002) and also to Cdc6 and cdt1, which cooperate together to attract the heterohexamer (ring-shaped) MCM proteins (Stoeber *et al.,* 2002, Guida *et al.,* 2005). The MCM proteins are helicases, involved in DNA elongation (Guida *et al.,* 2005, Stoeber *et al.,* 2002). Afterwards, the complex interacts with Dbf4/cdc7, which has kinase activity to attract

DNA polymerase. At this stage, the cell enters the S-phase (Guida *et al.,* 2005). After DNA replication, both MCM and Cdc6 are gradually dissociated from the chromatin (Korkolopoulou *et al.,* 2005, Guida *et al.,* 2005) allowing access of other factors that initiate DNA synthesis (Guida *et al.,* 2005).

Activation of MCM complex by cyclin-dependent kinases, such as Cdc6, Cdt1, and Dbf4/Cdc7, leads to initiation of DNA synthesis (Maiorano *et al*., 2006). They are tightly bound to chromatin in late mitosis and G1, while being removed in S and G2 phases. Once DNA replication is completed and all MCM proteins have been displaced from chromatin, they remain as a soluble nuclear pool during G2 phase and early mitosis (Tachibana *et al*., 2005). This regulation allows the control of replication origin firing, which is crucial to restrict the replication of the chromosome to only one round per cell cycle (Romanowski and Madine, 1997).

1.13.3 MCM5 in Tumours

MCM5 levels are up-regulated in a diversity of cancers (Table 1.15). However, MCM5 expression has not yet been investigated in breast cancer.

Table 1.15 MCM5 Elevated in Various Types of Cancers

Types of Cancer	***References***
Bladder Cancer	Stoeber *et al*., 2002
Oesophageal Cancer	Williams *et al.,* 2004
Anaplastic Thyroid Carcinoma	Guida *et al.,* 2005
Muscle Invasive Urothelial Bladder Carcinoma.	Korkolopoulou *et al.,* 2005
Cervical Squamous Epithelium Cancer	Murphy *et al.,* 2005

1.14 Aims of this Thesis

Background

As outlined in the introduction, breast cancers occurring in younger (≤35 yrs) women differ in that they have more aggressive features and poorer outcome, which may be due to differences in their molecular profiles. A previous PhD student used cDNA microarray and identified genes in breast cancers from this age group to be up or down regulated in relation to normal breast. Several genes were investigated by another former PhD student. The aim of my thesis is to examine three genes (TGFBI, DDB2 and MCM5, all up-regulated in cancer) that had been identified as showing differential expression and determine whether these changes are still present when more cancers are studied, and whether changes are specific to the younger age (≤35 yrs) group.

Hypothesis and Aims

The aim of this thesis is to test the hypothesis that the differential gene expression identified previously by cDNA microarray for TGFBI, DDB2 and MCM5 may contribute to more aggressive features of sporadic breast cancers in younger women.

Objectives

1- To investigate the RNA and protein expression of TGFBI, DDB2 and MCM5 in normal breast (tissues and isolated epithelial/myoepithelial cells), breast cancer cell lines and breast cancer tissues from patients of different age groups. This will be by quantitative reverse transcriptase polymerase chain reaction (q-RT-PCR), western blotting and immunohistochemistry with statistical analysis.
2- To determine the functional effect of TGFBI in breast cancer by using transient transfection and in vitro assays of viability, apoptosis and invasion.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Breast Tissue

The tissues studied were those previously investigated by Sinead Lambe for her PhD thesis (2008), but with additional cases having formalin-fixed, paraffin-embedded tissue. There were a total of 67 cancers originally selected for the study. A larger cohort was not considered necessary as statistically significant differences in expression had already been found by Lambe using fewer cases. Unfortunately, due to limiting frozen tissue, only 36 of these had both frozen and fixed tissue available for the study. 18 were from women aged ≤ 35 -years (10 with frozen tissue samples), 24 from women aged 36-49 years (14 with frozen samples) and 25 from women ≥ 50 years (12 with frozen tissue).

All specimens had been received fresh and sliced. Samples had been taken and rapidly frozen by immersion in liquid nitrogen and stored in a liquid nitrogen fridge. Larger samples were fixed in 10% formalin for 18-36 hours, followed by processing through graded alcohols, xylene and then embedded in paraffin wax. Data on tumour size, tumour type, grade, node status, oestrogen and progesterone receptor status and HER-2 status were provided by Professor RA Walker, and are shown in *Appendix I*. Frozen normal/benign tissue was available from 11 patients who had undergone reduction mammoplasties, with parallel tissues fixed normal tissues had been processed in the same manner as the cancer tissues.

There was ethical approval for the use of all tissues (Leicestershire, Northamptonshire, and Rutland REC (06/Q2502/70)).

Organoids, which are isolated aggregates of epithelial and myoepithelial cells, were provided by Sinead Lambe, a previous PhD student. These had been isolated from reduction mammoplasties from 8 women aged between 23 and 42 years.

2.1.2 Tonsil Tissue

Normal tonsil tissue was used as a positive control for the immunohistochemical analysis of *MCM5*. This was provided by the research laboratory, level 3, Department of Cancer Studies and Molecular Medicine and had been fixed and processed in the same way as the breast tissue.

2.1.3 Breast Cell Lines

All breast cell lines were originally purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). MCF-7, MDA-MB-231, MDA-MB-468 and T-47D were derived originally from pleural metastases. ZR-75-1 was derived from metastatic ascites. HBL-100 was derived from lactation sample of a healthy woman. All cancer cell lines used have epithelial morphological characteristics, except MDA-MB-231 which is related to the “stromal-like” group (Lacroix and Leclercq, 2004) (Table 2.1). All cultured cell lines were used at a low passage, up to a maximum of 20-22.

Table 2.1 Breast Cell Lines Used in this Study

Breast Cell Lines	***History of Derivation***	***ER***	***PR***	***HER-2***	***p53***	***E-Cad***	***References***
MCF-7	Human breast adenocarcinoma cell line that derived from a 69-year old caucasian woman with a malignant pleural effusion	POS	POS	NEG	Wt	POS	Brooks *et al*., 1973; Roetger *et al*., 1998; Hiraguri *et al.,* 1998
MDA-MB-231	Human breast adenocarcinoma cell line that derived from pleural effusion of a 51-year old caucasian woman	NEG	NEG	Low	M	NEG	Cailleau *et al*., 1978; Subik *et al*., 2010; Chao *et al*., 2010
MDA-MB-468	Human breast adenocarcinoma cell line that derived from 51-year old black woman with a pleural effusion	NEG	NEG	NEG	M	POS	Cailleau *et al*., 1978; Roetger *et al*., 1998; Moiseeva *et al*., 2007; Hiraguri *et al.,* 1998
T-47D	Human breast adenocarcinoma cell line that derived from pleural effusion of a 54-year old woman with infiltrating ductal carcinoma	POS	POS	Low	M	POS	Keydar *et al*., 1979; Offterdinger *et al*., 1999; Maemura *et al.,* 1995; Hiraguri *et al.,* 1998;
ZR-75-1	Human breast adenocarcinoma cell line that derived from malignant ascites of a 63-year old caucasian woman with breast cancer	POS	POS	POS	Wt	POS	Engel *et al*., 1978; Subik *et al*., 2010; Maemura *et al.,* 1995
HBL-100	An immortalised epithelial cell line derived from the milk of a 27-year old caucasian woman	NEG	NEG	NEG	Wt	NEG	Gaffney *et al*., 1982; Subik *et al*., 2010; Moiseeva *et al*., 2007

POS = Positive NEG = Negative Wt= Wild type M= Mutant p-53 Data taken from (Nigro *et al*., 1989)

2.1.4 Antibodies

All primary antibodies used are detailed in Table 2.2. Secondary antibodies and tertiary reagents used in immunohistochemistry and western blotting in Table 2.3.

Table 2.2 Primary Antibodies Used in this Study

Antibody	Clone Number	Company	City/Country	Product Number	Type	Description	Reference
α-Tubulin	B-7	Santa Cruz Biotechnology INC	Heidelberg Germany	Sc-5286	Mouse monoclonal IgG_2	It was raised against amino acids 149-448 representing full length α Tubulin of human origin	Song *et al*., 2007
β-Actin	AC-15	Sigma®	Welwyn Garden City United Kingdom	A 5441	Mouse monoclonal IgG_1	It was raised against β-cytoplasmic actin N-terminal peptide.	Sun *et al*., 2009
β-Tubulin	D66	Sigma®	Welwyn Garden City United Kingdom	T0198	Mouse monoclonal IgG_1	It was raised against the C-terminal region of β-tubulin	Gillardon, 2009
DDB2	S-16	Santa Cruz Biotechnology INC	Heidelberg Germany	SC-16295	Goat polyclonal IgG	It was raised against a peptide mapping near the C-terminus of DDB2 of human origin	Zolezzi and Linn, 2000
MCM5	CRCT5-1	AbCAM®	Cambridge/MA USA	Ab40093	Rabbit polyclonal IgG	It was raised against the N-terminal amino-acid(1-150) of human MCM5	Rizwani *et al*., 2009
TGFBI	10188-1-AP	ProteinTech Group, Inc	Chicago USA	10188-1-AP	Rabbit polyclonal IgG	It was raised against pure human TGFBI protein	Ma *et al*., 2008
Vinculin	hVIN-1	Sigma®	Welwyn Garden City United Kingdom	V 9131	Mouse monoclonal IgG	It raised against the clone hVIN-1 of human origin	North *et al*., 1993

Table 2.3 Secondary Antibodies and Tertiary Reagents Used in this Study

Antibody	Product Number	Company	City/Country	Description	Reference
ECL Anti-Mouse IgG/HRP (Raised in Sheep)	NXA931	GE Healthcare	Buckinghamshire United Kingdom	Affinity isolated mouse anti-sheep immunoglobulins tagged with HRP reporter provided in liquid form	Kischkel *et al*., 2001
ECL Anti-Rabbit IgG/HRP (Rasied in Donkey)	NA934V	GE Healthcare	Buckinghamshire United Kingdom	Affinity isolated rabbit anti-donkey immunoglobulins tagged with HRP reporter provided in liquid form	Narayan and Dragunow, 2010
Biotinylated Polyclonal Rabbit Anti-Goat	E 0466	Dakocytomation	Ely United Kingdom	Biotinylated, affinity-isolated rabbit anti-goat immunoglobulins provided in liquid form in 0.05 mol/L Tris/HCl, 0.1 mol/L NaCl, 15 mmol/L NaN3, pH 7.2	Rabie *et al*., 2003
Donkey anti-Goat IgG/HRP	SC-2020	Santa Cruz Biotechnology	Santa Cruz, CA USA	Affinity isolated donkey anti-goat immunoglobulins tagged with HRP reporter provided in liquid form	Staflin *et al*., 2009
StreptABComplex/HRP	K 0377	Dakocytomation	Ely United Kingdom	Reagent A 1ml of Streptavidin in 0.01mol/L Phosphate Buffer, 0.15mol/L Sodium Chloride, and 15mmol/L NaN3 (pH 7.2) Reagent B 1ml of Biotinylated Peroxidase in 0.01mol/L Phosphate Buffer, 0.15mol/L NaCl, and 15mmol/L NaN3, (pH7.2)	Rabie *et al*., 2003
StreptABComplex/HRP Duet, Mouse/Rabbit	K 0492	Dakocytomation	Ely United Kingdom	Reagent A 1mL Streptavidin in 0.01mol/L Phosphate Buffered Saline, 15mmol/L NaN_3 (pH 7.2) Reagent B 1mL Biotinylated Horseradish Peroxidise in 0.01 mol/L Phosphate-Buffered Saline, 15mmol/L NaN_3 (pH 7.2) Reagent C 1mL biotinylated, affinity-isolated goat antibody to mouse/rabbit immunoglobulins in 0.01mol/L phosphate-buffered Saline, 15mmol/L NaN_3 (pH 7.2)	Birgisdottir *et al*., 2006

2.1.5 Oligonucleotide Primers

Oligonucleotide primers (Table 2.4) were designed using the Primer_3 software programme (Rozen and Skaletsky, 2000). The sequence of each primer was tested for specificity using NCBI's Nucleotide Basic Local Alignment Search Tool (BLAST). Primers (Sigma®- Genosys, UK) were supplied as a lysophyolised pellet. They were centrifuged at 13000rpm at room temperature, and then resuspended in sterile water to a concentration of 200pm/µl. An aliquot was taken and diluted in sterile water to make up a working concentration of 10pm/µl.

Table 2.4 Oligonucleotide Primers Used in this Study

Gene	5' → 3' Sequence	Length (nt)	Tm	GC%	OD	Annealing Position (5' → 3')
GAPDH (350bp)	F AGAACATCATCCCTGCCTC R GCCAAATTCGTTGTCATACC	19 20	65.3 61.8	55 45	14.35 14.46	713 →731 1059 →1040
GAPDH (100bp)	F TCTGACTTCAACAGCGACAC R GCCAAATTCGTTGTCATACCAG	20 22	66.4 65.1	61.1 47.8	10.0 12.1	953 →971 1059 →1038
Oligo(dt)$_{20}$	TTTTTTTTTTTTTTTTTTTT	20	52.2	00	13.6	------
TGFBI Plasmid (pRC/CMV2)	F TTTCCAGGGCTTCCCAGAGGT R TGGTGCATTCCTCCTGTAGTGC	21 22	70 68	57 55	11.7 12.4	2025 → 2046 2127 → 2149
Vector (pRC/CMV2)	F CCCACTGCTTAACTGGCTTA R CAACAGATGGCTGGCAACTA	20 20	61.7 63.8	50 50	11.1 11.0	837 → 857 1050 → 1070

2.1.6 Q-RT-PCR TaqMan Probes

TaqMan inventoried FAM labelled probes were supplied by Applied Biosystem. The position of each assay was selected to span exon boundaries and to avoid splice variants (Table 2.5).

Table 2.5 Q-RT-PCR TaqMan Probes Used in this Study

Gene	Chromosome Location	Assay Location	Assay ID	Exon Boundary	Amplicon Length
GAPDH	12p13	633	Hs02758991_g1	7-8	93
HPRT1	Xq26.1	646	Hs99999909_m1	6-7	100
DDB2	11p12-p11	1423	Hs03044953_m1	9-10	88
MCM5	22q13.1	2197	Hs01052142_m1	16-17	70
TFRC	3q29	1609	Hs00174609_m1	13-14	79
TGFBI	5q31	1838	Hs00932734_m1	12-13	70

2.1.7 TGFBI DNA Plasmid

The TGFBI plasmid and empty vector was kindly provided by Dr Zhoa (University of Colombia, NY, USA). The map of the pRC/CMV2 is present in Figure 2.1

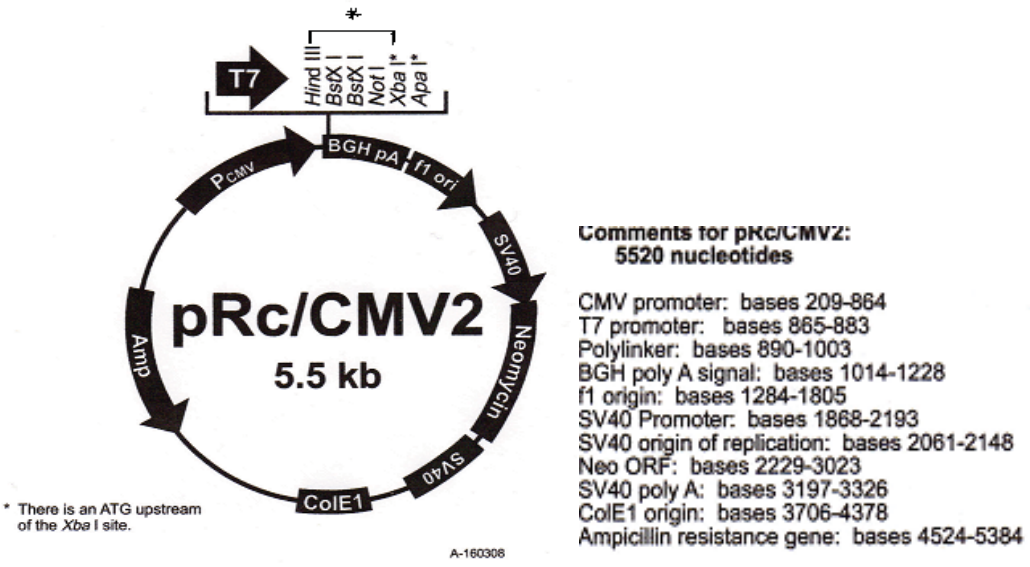


Figure 2.1 pRC/CMV2 Vector Map

This is taken from Invitrogen website: http://www.invitrogen.com/site/us/en/home.html. (*)= the region where TGFBI gene was cloned.

Media

Luria Bertani (LB) medium (*Appendix VI*)

Competent Cells

Subcloning efficiency DH5α (chemically competent E.Coli) (Catalogue Number: 18265-017, Invitrogen, Paisley/UK)

Reagents

Ampicillin (100mg/mL) (Cataloge Number: A5354, Sigma®-Aldrich, Dorset/UK)

Commercial Kits

QIAquick Spin Mini-Prep (Catalogue Number: 27104, Qiagen, Crawley/West Sussex/UK)

QIAquick Spin Maxi-Prep (Catalogue Number: 12163, Qiagen, Crawley/West Sussex/UK)

2.1.8 Cell Culture

Media

Dulbecco's modified eagle's medium (DMEM) without phenol red and L-Glutamine (Catalogue Number: 12491-015, GIBCO®, Paisley/UK), 10% foetal calf serum (FCS) (Catalogue Number: 10082147, GIBCO®, Paisley/UK), L-glutamine (Catalogue Number: G5792, Sigma®-Aldrich, Dorset/UK)

RPMI 1640 medium without phenol red and L-Glutamine (Catalogue Number: BE12-918F, Lonza, Basel/Belgium), 10% foetal calf serum (FCS) (Catalogue Number: 10082147, GIBCO®, Paisley/UK), L-glutamine (Cataloge Number: G5792, Sigma®-Aldrich, Dorset/UK)

Opti-MEM (Reduced Serum Medium) (Catalogue Number: 11058021, GIBCO®, Paisley/UK)

Washing Solutions

Dulbecco's phosphate buffered saline (DPBS) (Catalogue Number: H15-002, PAA, Pasching/Austria)

Reagents

Dimethyl sulphoxide (DMSO) (Catalogue Number: D-5879, Sigma-Aldrich, Dorset/UK)

Fugene HD Transfection Reagent (Catalogue Number: 04709705001, Roche Applied Sciences, West Sussex/UK)

BD Matrigel Matrix phenol-red free (Catalogue Number: 356237, BD-Biosciences, Beford/MA/USA)

$DiIC_{12}(3)$ lipophilic fluorescent tracer dye (Catalogue Number: 354218, BD-Biosciences, Beford/MA/USA)

CellTiter-Blue Reagent (Catalogue Number: G8080, Promega, Delta House/Southampton/UK)

Caspase- Glo® 3/7 Reagent (Catalogue Number: G8091, Promega, Delta House/Southampton/UK)

PmaxGFP (Catalogue Number: VCA-1003, Lonza (Amaxa),Basel/Belgium)

TGF-β_1 powder (Catalogue Number: 240-B, R&D Systems®, Abingdon/UK)

Enzymes

Trypsin/ EDTA (0.05%) (Catalogue Number: L11-659, PAA, Pasching/Austria)

Equipment

C-Chip disposable hemocytometer (Catalogue Number: PAA505050X, PAA, Pasching/Austria)

Tissue culture flasks (BD-Biosciences, Beford/MA/USA)

BD-Falcon: T_{25cm}^2 (Catalogue Number: 353109)

T_{75cm}^2 (Catalogue Number:353110)

T_{175cm}^2 (Catalogue Number: 353112)

6 and 24-well plates (Catalogue Number: 353934/353047, BD-Biosciences (BD-Falcon), Beford/MA/USA)

Fluoroblock cell culture inserts (with 8µm pores) (Catalogue Number: 353097, BD-Bioscience (BD-Falcon), Beford/MA/USA)

Amicon® Ultra-4 Centrifugal Filter Devices (Volumes up to 4mL) (UFC 800396, Millipore, Watford/UK)

Fluostar optima plate reader (BMG Labtech, Aylesbury/UK)

2.1.9 Western Blotting

Buffers

Gold protein lysis buffer (*Appendix VI*)

Resolving gel buffer (*Appendix VI*)

Stacking gel buffer (*Appendix VI*)

10% w/v Sodium dodecyl sulphate (SDS) buffer (*Appendix VI*)

Western loading buffer (*Appendix VI*)

Western running buffer (*Appendix VI*)

Western transfer buffer (*Appendix VI*)

Western washing buffer (1% and 3% TBS-T) (*Appendix VI*)

5% Western blocking solution (*Appendix VI*)

Reagents

10% w/v ammonium persulphate (Cataloge Number: A3678, Sigma-Aldrich®, Dorset/UK).

TEMED (Catalogue Number: T9281, Sigma-Aldrich®, Dorset/UK)

30% Acrylamide/Bis (Catalogue Number: A3699, Sigma-Aldrich, Dorset/UK)

Ponceau (Catalogue Number: 78376, Sigma-Aldrich, Dorset/UK)

Tween 20 (Catalogue Number: 63158, Sigma-Aldrich, Dorset/UK)

ECL Chemiluminescence Detection (Catalogue Number: RPN 2106, Amersham Bioscience, Little Chalfont/Buckinghamshire/UK)

30% Hydrogen Peroxide 100 volume (Catalogue Number: H/1750/15, Fisher Scientific, Loughborough/UK)

Protein Standard BSA (Catalogue Number: B8894, Sigma®-Aldrich, Dorset/UK)

Bio-Rad Protein Assay Dye Reagent concentrate (Catalogue Number: 500-0006EDU, BioRAD, Hertfordshire/UK)

BioRad Precision Plus Dual Marker (Catalogue Number: 161-0374, BioRAD, Hertfordshire/UK)

Protease Inhibitor Cocktail (Catalogue Number: P8340, Sigma®-Aldrich, Dorset/UK)

Membrane

Amersham Hyperfilm ECL (Catalogue Number: 28-9068-36, Amersham Bioscience, Little Chalfont/Buckinghamshire/UK)

Filter paper (Catalogue Number: SE1141, Amersham Bioscience, Little Chalfont/ Buckinghamshire/UK)

Hybond-XL nitrocellulose membrane (Catalogue Number: RPN2020S, Amersham Bioscience, Little Chalfont/Buckinghamshire/UK).

Equipment

1.5mm combs and 1.5mm plates (Catalogue Number: 170-4491/165-3312, BioRAD, Hertfordshire/UK)

2.1.10 H&E Staining and Immunohistochemistry

Reagents

DAB (Catalogue Number: D3939, Sigma®-Aldrich, Dorset/UK)

Eosin (*Appendix VI*)

Mayer's Haematoxylin (*Appendix VI*)

Vectabond (Catalogue Number: SP-1800, Vector Laboratories, Peteborough/ Leicestershire/UK)

Xylene (Catalogue Number: 215-5535-7, GENTA Medical, Tockwith/York/UK)

IMS (Catalogue Number: 200-58-6, GENTA Medical, Tockwith/York/UK).

DPX (Catalogue Number: 360294H, VWR, Lutterworth/Leicestershire/UK).

10% Normal Goat Serum (Catalogue Number: 50-062Z, Invitrogen, Paisley/UK)

10% Normal Rabbit Serum (Catalogue Number: 50-061Z, Invitrogen, Paisley/UK)

Equipment

Electric Pressure Cooker (Catalogue Number: S2800, Pascal/Dako, Ely/UK)

Buffer

High pH Buffer (pH=9) (Catalogue Number: S2367, Dako, Ely/UK)

2.1.11 Polymerase Chain Reaction (PCR)

Buffer

Avian Myeloblastosis Virus (AMV) reverse transcriptase buffer (X10) (Catalogue Number: A3561, Promega, Delta House/Southampton/UK)

AJ buffer (10X) (*Appendix VI*)

PCR gel loading buffer (*Appendix VI*)

Chemicals

Chloroform (99%) (Catalogue Number: 30911, Sigma-Aldrich®, Dorset/UK)

SeaKem agarose gel (Catalogue Number: 50005, Lonza, Basel/Belgium)

Isopropanol (Catalogue Number: P/7500/17, Fisher Scientific, Loughborough/UK)

Ethanol (Catalogue Number: E/0650DF/17, Fisher Scientific, Loughborough/UK)

Enzymes

Avian Myeloblastosis Virus Reverse Transcriptase Enzyme (AMV-RT) (Catalogue Number: M5101, Promega, Delta House/Southampton/UK)

Recombinant RNasin® RNase Inhibitor (Catalogue Number: N2611, Promega, Delta House/Southampton/UK)

Taq DNA polymerase (Catalogue Number: 10342-020, Invitrogen, Paisley/UK)

Reagents

RNeasy® Mini kit (Catalogue Number: 74104, Qiagen, Crawley/West Sussex/UK)

TRI® Reagent (Catalogue Number: 93289, Sigma-Aldrich®, Dorset/UK)

RNAqueous-Micro Kit (Catalogue Number: Am1931, Applied Biosystem (Ambion INC), Warrington/UK)

100bp DNA molecular weight marker (Catalogue Number: N3231L, New England BioLabs, Hertsfordshire/UK)

Hyperladder I (Catalogue Number: BIO-33025, Bioline, London/UK)

DNTP mix (Catalogue Number: U1511, Promega®, Delta House/Southampton/UK)

Magnesium Chloride (50mM) (Catalogue Number: BIO-37026, Bioline, London/UK)

Eithidium bromide 10 mg/ml (Catalogue Number: 46067, Sigma-Aldrich®, Dorset/UK)

Equipment

NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies INC, Thermo-Scientific, Wilmington/DE/USA)

Thermal Cycle (GeneAmp® PCR System 9700, Applied Biosystems, Warrington/UK)

2.1.12 Q-RT-PCR

Reagents

TaqMan® Universal PCR Master Mix without UNG (Uracil-N-Glycosylase) (Catalogue Number: 4352046, Applied Biosystems, Warrington/UK)

Equipment

MicroAmp® Fast Optical 96-well Reaction plates with Barcode (Catalogue Number: 4326659, Applied Biosystems, Warrington/UK)

MicroAmp® Optical Adhesive Film (Catalogue Number: 4313663, Applied Biosystems, Warrington/UK)

7500 Fast Real-Time PCR System (Catalogue Number: 4351106, Applied Biosystems, Warrington/UK)

2.1.13 General Buffers

Citrate Buffer (pH=6) (*Appendix VI*)

TE Buffer (pH=7) (*Appendix VI*)

TBE Buffer (pH=8.3) (*Appendix VI*)

TAE Buffer (pH=8.5) (*Appendix VI*)

Tris Buffered Saline (TBS) Buffer (pH 7.65) (*Appendix VI*)

2.2 Methods

2.2.1 Cell Culture

All breast cell lines were cultured and incubated in a humidified chamber at 37^{O}C, 5% CO_2 and 95% air. MCF-7, MDA-MB-231, MDA-MB-468, T-47D and HBL-100 were maintained in phenol red free Dulbecco's Modified Eagle's Medium (DMEM) containing 10% v/v Foetal Calf Serum (FCS) and 2mM L-Glutamine. ZR-75-1 cells were maintained in Rosewell Park Memorial Institute medium (RPMI) 1640 without phenol red, containing 10% v/v FCS and 2mM L-Glutamine.

2.2.1.1 Passaging Cells

Cell lines were grown in the appropriate flask size until they were semi- confluent (>70%). The medium was then aspirated, and cells washed twice with Dulbeccos Phosphate Buffered Saline DPBS. 0.05% Trypsin-EDTA was added and incubated for 2-3 minutes at 37^{O}C; the flask was gently tapped to encourage greater detachment of cells. Trypsin activity was terminated by the addition of medium, and the contents transferred to a 15mL Falcon tube and centrifuged at 1000rpm for 5 minutes. The supernatant was removed and the cell pellet resuspended in 3-5mL of growth media for subsequent culture.

2.2.1.2 Counting/Seeding Cells

Post passage, cells were counted using a C-Chip disposable hemocytometer. The mean number of cells present in the four large boxes was obtained to establish the total number of cells x 10^4 per ml (Figure 2.2). Cells were then seeded into either T25cm^2 flasks or 6-, 24- well plates, the numbers being dependent on both the optimum cell line density (Table 2.12) and the subsequent test.

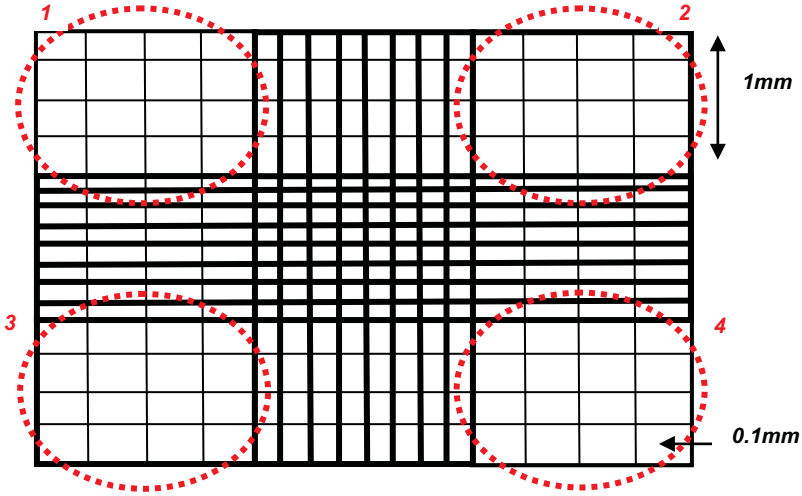


Figure 2.2 C-Chip Disposable Hemocytometer

The hemocytometer consists of 9 similar squares, each square 1x1mm, and the depth of the chamber is 0.1mm. Therefore, each square has a total volume of 10^{-4} cm^3.
The four red boxes were counted to obtain the mean number of the cells X 10^4 per ml.

2.2.1.3 Preservation and Storage

Cell lines were stored in liquid nitrogen (-196^{o}C). After passaging as described in *2.2.1.1*, cell pellets were resuspended in 5mL freezing medium (8mL of culture medium, 1mL FCS and 1mL of DMSO). Cells (1mL) were put into a cryotube, and stored at 4^{o}C for 1 hour, -20^{o}C for 1 hour, then -80^{o}C overnight before being placed in liquid nitrogen. Alternatively, cryotubes were placed in a unit containing 200µL of isopropanol and immediately incubated at -80^{o}C.

2.2.2 Western Blotting

2.2.2.1 Protein Extraction

Proteins were extracted from cells post harvesting as described in *2.2.1.1*. For frozen tissues 10-15 sections (each 5µm) were cut using a cryostat. Gold Protein Lysis buffer (Samuels *et al.*, 1993) was added (300-600µl) which contained 1:10 dilution of Protease Inhibitor Cocktail, vortexed briefly and incubated on ice for 10 minutes. Cell

pellets and tissues were disaggregated using a 22 gauge needle to ensure lysis and then centrifuged at 13,000rpm for 5 minutes. The supernatant containing soluble protein was removed and stored at -20^{O}C until analysis.

2.2.2.2 Concentrating Protein from Cell Conditioned Media

'Cell conditioned medium' describes the medium in which cells were cultured. This varied between experiments as cells were cultured at different densities and for different time periods. In order to have a higher concentration of the proteins released from the cells in a smaller volume an ultrafiltration method was used. Four ml of the conditioned media was added to an Amicon Ultrafilter device, which was then capped and placed into a 35^{O} fixed angle centrifuge rotor. It was spun at 7,500 x g for 20 minutes. The concentrated medium was withdrawn from the bottom of the filter, with a side-to-side sweeping motion being used to ensure total recovery. The concentrated medium was stored at -20^{O}C until required.

2.2.2.3 Protein Quantification

Protein concentrations were determined using Bio-Rad's protein quantification reagent (Bradford Assay) (Bradford, 1976). The protein lysate (5µl) was diluted in 795µl of distilled water, and 200µl of diluted (1:5) Bradford reagent added. Samples were mixed and left in the dark for 10 minutes to allow colour development. Subsequently, they were transferred to a 1ml cuvette to measure the absorbance at 595nm using a ThermoSpectronic Spectrophotometer. Protein quantity was calculated from a standard curve constructed from absorbance against a dilutional series of BSA (0.1µg/µl) (Table 2.6).

Table 2.6 Generation of BSA Standard Curve

Volume of BSA (µl)	*Volume of Water (µl)*	*Amount of Protein (µg)*
0	800	0
5	795	0.5
10	790	1
20	780	2
30	770	3
40	760	4
60	740	6
80	720	8

2.2.2.4 Gel Preparation

1.5mm discontinuous polyacrylamide gels were prepared for separation of proteins; table 2.7 shows the compositions of resolving and stacking gels for different percentage gels. 50µl of 10% APS and 5µl of TEMED were added to the resolving gel in order to initiate polymerization, prior to pouring. The stacking solution was poured after addition of 25µl of 10% APS and 5µl of TEMED, and then the gel was allowed to polymerize for approximately 30 minutes after inserting the 1.5mm comb.

Table 2.7 Composition of Various Percentages of SDS-PAGE Gels

Reagents	***6% (ml)***		***10% (ml)***		***12% (ml)***	
	R	***S***	**R**	***S***	***R***	***S***
H_2O	*5.4*	*2.7*	*4.1*	*2.05*	*3.4*	*1.7*
30% Acrylamide/Bis	*2*	*1*	*3.3*	*1.65*	*4*	*2*
Gel Buffer*	2.5	*1.25*	*2.5*	*1.25*	*2.5*	*1.25*
10% w/v SDS	0.1	*0.05*	*0.1*	*0.05*	*0.1*	*0.05*

R: Resolving Gel, S:Stacking Gel
*Resolving gel buffer-1.5mM Tris-HCL pH8.8, stacking gel buffer-0.5mM Tris-HCL pH 6.8. Composition referred to Laemmli, 1970.

2.2.2.5 Separation of Proteins by SDS-PAGE

Protein lysate or conditioned media (5-25 µg) was mixed with 1:10 western loading buffer, denatured at 99^{O}C for 5 minutes, quickly chilled on ice and then loaded into each well. Ten µl BioRad Precision Plus Dual (molecular weight marker) was loaded

into a separate well at one end of the gel. The tank was filled with running buffer and the gel run at 100V (BIORAD Power) for approximately 1-2 hours at room temperature until both samples and coloured marker reached the end of the resolving gel (Laemmli, 1970).

2.2.2.6 Transfer of Protein to a Nitrocellulose Membrane

Prior to transfer, nitrocellulose membrane and two pieces of supporting blotting filter paper were rinsed in transfer buffer for approximately 5 minutes. Nitrocellulose membrane was placed on the top of the gel, and then both filter paper and a wet sponge were positioned carefully on top of the membrane. The size markers were marked on the membrane using a ball point pen. Following this, another filter paper and wet sponge were placed onto the gel (Figure 2.3), and all air bubbles were removed. Wet blotting was used, with all components fully submerged in the transfer buffer. Transfer was performed using 100V for approximately 2 hours at room temperature (Towbin *et al*., 1992).

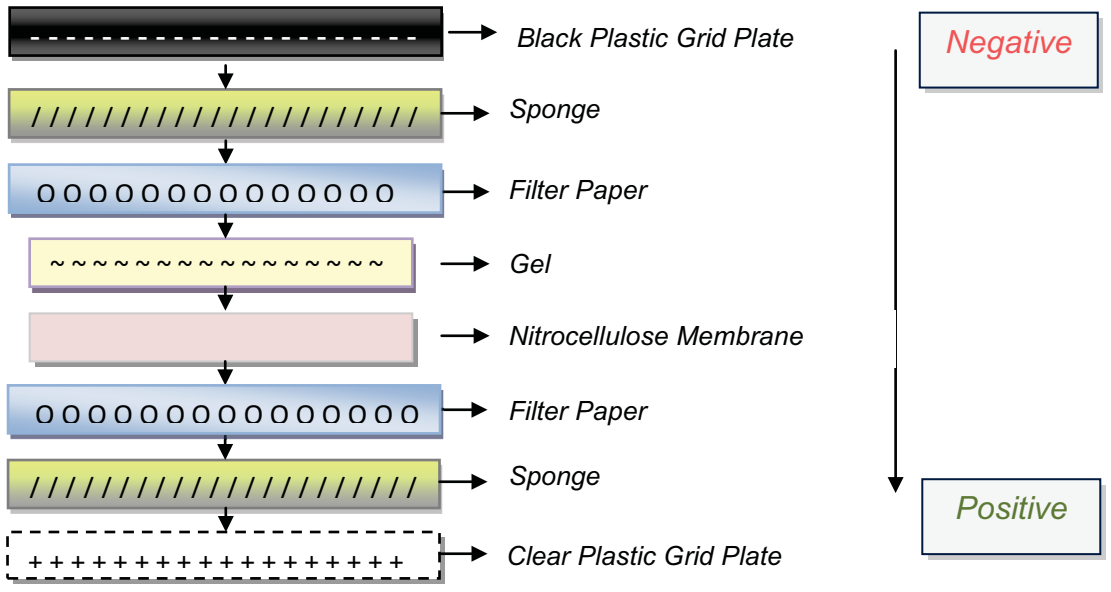


Figure 2.3 Order of the Components for Protein Transfer Procedure

2.2.2.7 Immuno-detection of Proteins

The gel was discarded after ensuring that proteins were transferred, by staining the membrane with Poinceau for 15 minutes. The membrane was rinsed with water followed by washing with TBS-T buffer to remove residual SDS. Non-specific binding was blocked by incubating the membrane with Marvel Dried Skimmed Milk solution (5%- with TBS-T) for 1 hour at room temperature (DenHollander and Befus, 1989). Membranes were incubated with diluted primary antibody for the protein of interest overnight at $4^{O}C$ using gentle agitation. Then the membrane was rinsed with various washing procedures depending on the nature of the primary antibody. Subsequently, secondary antibodies were added followed by washing.

2.2.2.8 Optimization of Methods for Western Blotting

The Western Blotting method was optimized for each of the antibodies using different secondary antibody dilutions and timings. Additionally, various gel percentages and washing procedures were assessed. For detection of DDB2 a range of washing procedures were also assessed (Table 2.8).

Table 2.8 Different Washing Procedures for DDB2 Antibody

	Washing Procedures for DDB2
1	Washing with 1% TBS-T
2	Washing the membrane with 5% milk, which was prepared from 3% TBS-T, and then wash with 3% TBS-T for three times
3	Washing the membrane with 5% milk, which was made in 3% TBS-T, then wash with 3% TBS-T followed with 1% TBS. This was repeated twice.

2.2.2.9 Chemiluminescence Detection

Reagents A and B were used from the Amersham Bioscience ECL detection system kit according to the manufacturer's instructions. These reagents were pipetted on the

membrane and incubated for 3-5 minutes (Figure 2.4). Excess liquid was removed and the membrane wrapped in Saran barrier wrap. The membrane was exposed to X-ray film for various times depending on the band intensity, and then the film developed using an AGFA Curix 60 film developer.

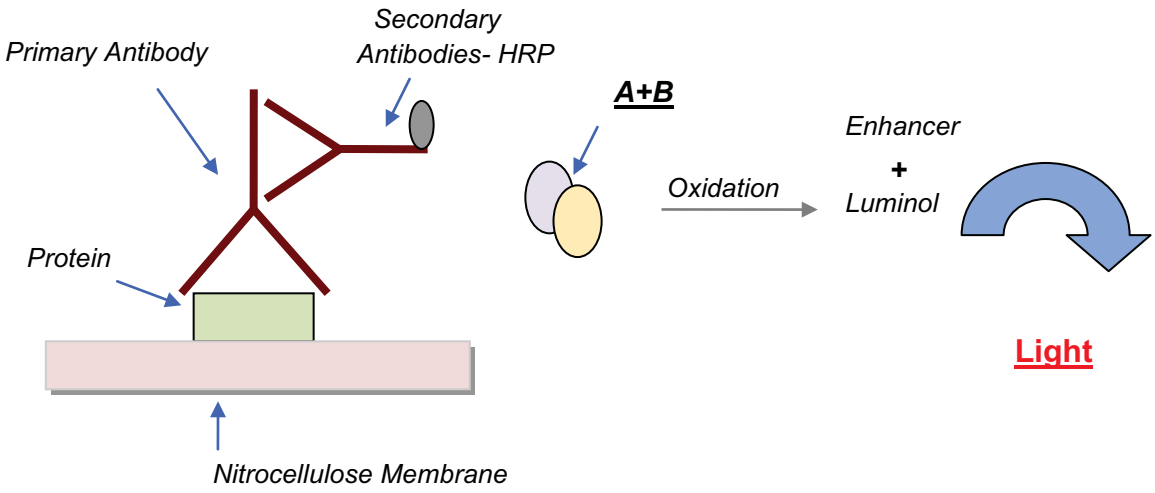


Figure 2.4 Reaction of Chemiluminescence Detection System

The chemical reaction between the reagents A and B and the secondary antibody-HRP produces an oxidized product, which emits visible light in various densities depending on the quantity of the binding secondary antibodies.

2.2.2.10 Stripping of the Membrane

Membranes were incubated with diluted 1:1 30% hydrogen peroxide for 45 minutes at room temperature to dissociate the bound antibodies previously used from the membrane. The membranes were washed three times with 1% TBS-T, each for 5 minutes, and then incubated with appropriate dilution of the primary antibody to be tested. The membrane was incubated with the appropriate dilution of the secondary antibody for the suitable time, and then results were detected as described in *2.2.2.9*.

2.2.3 Haematoxylin and Eosin (H and E) Staining

2.2.3.1 Fixed Tissue

Sections (4μm) were de-waxed by immersing twice in Xylene for 3 minutes and rehydrated twice in 99% IMS and once in 95% IMS each for 1 minute. The slides were equilibrated in running tap water for 1 minute; immersed in Haematoxylin for 5 minutes, and washed in running tap water for 1-2 minutes. The sections were immersed in Eosin for 3 minutes, washed with running tap water, and dehydrated through increasing IMS concentrations, cleared with Xylene, and then mounted with DPX.

2.2.3.2 Frozen Tissue

Frozen sections were cut at 5μm, placed on a glass slide and immersed in Haematoxylin for 20 seconds, then washed with running tap water for 1-2 minutes. Sections were then immersed in Eosin for 2 seconds and quickly dehydrated, cleared and mounted.

2.2.3.3 Assessment of Frozen Tissue Cellularity

H and E stained sections that were parallel to the frozen sections taken for RNA extraction were examined for all tissues to check for cellularity. This was undertaken with Professor RA Walker. For normal tissues the number of breast lobules and the amount of stroma were recorded. For the carcinomas the percentage of the section that was invasive cancer was assessed and the presence of *in-situ* carcinoma and/or normal tissue was noted. Although fixed and frozen tissues were available from the same patient, it was not always possible to closely match areas of tissue in these as the FFPF blocks have been used previously in other studies.

2.2.4 Immunohistochemistry (IHC)

2.2.4.1 De-waxing and Re-hydration

4µm sections were put onto slides coated with Vectabond, dried in an oven at 60^{o}C for one hour and de-waxed in Xylene twice for 5 minutes. Sections were then re-hydrated in graded alcohols and rinsed in running tap water for approximately 5 minutes.

2.2.4.2 Antigen Retrieval

Heat antigen retrieval was the technique of choice (Shi *et al*., 2001), with retrieval buffers of different pHs being assessed. An electric pressure cooker (Dako) was used, for which the temperature (120-125^{o}C) and duration of treatment (30-60Sec) could be adjusted to generate different Psi pressure. Pressure cooking is used within the Breast Research Unit, since microwave antigen retrieval has been found to result in variation in extent of staining of test sections (Rhodes *et al*., 2001; Walker, 2006). Different antigen retrieval buffers were assessed for each antibody: citrate (pH=6), TE (pH=7), TBE (pH=8.3) and high pH (pH=9) in order to break the protein cross-links formed by formalin fixation. The solutions were heated to 80^{o}C, slides inserted, lid closed tightly, subsequently the temperature, length of time and pressure optimum values of the pressure cooking determined for each antibody.

2.2.4.3 Immunohistochemistry Procedures

Immunohistochemistry has become an essential technique in histopathology. There have been method refinements from the time of its first introduction in the 1942 (Coon *et al*., 1942), with developments increasing from the 1970s (Delellis *et al*., 1979).

Endogenous peroxidase activity was blocked by immersing the sections in 2% hydrogen peroxide for 10 minutes. Slides were washed in running tap water for approximately 10 minutes followed by rinsing twice with TBS, each for 5 minutes. Sections were blocked for non-specific binding by incubating the tissue with normal serum diluted 1:5 in TBS for approximately 10 minutes. The type of the normal serum used depended on the animal species in which the primary antibody was raised and the detection system that was chosen (Table 2.9). Negative controls were left in normal

serum while positive control and test sections were incubated with 100µl of the relevant primary antibody overnight at 4^{O}C.

Slides were rinsed twice with TBS for 5 minutes each, and then incubated with 100µl of diluted biotinylated secondary antibody for 30 minutes at room temperature. The choice of the secondary antibody depended on the primary antibody and detection system used (Table 2.9).

2.2.4.4 Immunohistochemistry Detection Systems

Two different detection systems were used:

1- StreptABComplex/HRP (ABC)

The complex was prepared 30 minutes prior to use at room temperature by combining 1µl of solution A and 1µl of solution B in 998µl of TBS. Sections were incubated with 100µl ABC for 30 minutes then washed twice in TBS, for 5 minutes each (Figure 2.5).

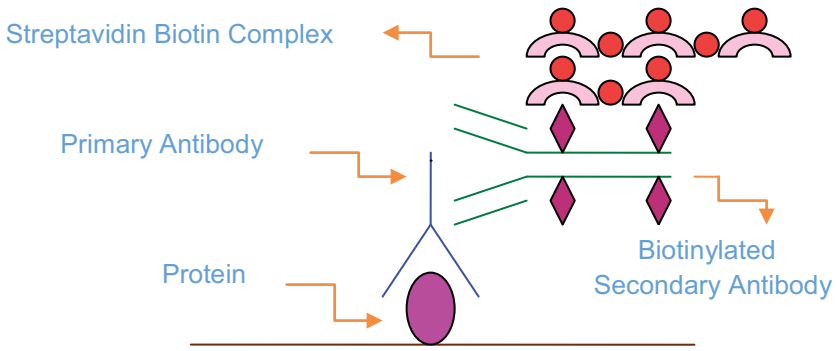


Figure 2.5 Streptavidin-Biotin Complex

The streptavidin-biotin complex interacts with the biotinylated secondary antibody (Adapted from Handbook Immunohistochemistry Staining Method, Edited by Thomas Boenisch, 2001).

2- StreptABComplex/HRP Duet, Mouse/Rabbit

The complex was prepared 30 minutes prior to use at room temperature by combining 1µl of solution A and 1µl of solution B in 98µl of TBS. Sections were incubated with 100µl of ABC/Duet for 30 minutes then washed twice in TBS, for 5 minutes each.

Table 2.9 Different Detection Systems Used for the Three Antibodies

Variables	***TGFBI***	***DDB2***	***MCM5***
Normal Serum	*Goat*	*Rabbit*	*Goat*
Secondary Antibody	*Biotinylated Goat Anti-Mouse/Rabbit*	*Rabbit Anti-Goat*	*Biotinylated Goat Anti-Mouse/Rabbit*
Tertiary Antibody	*StreptABComplex/HRP Duet*	*StreptABComplex/HRP*	*StreptABComplex/HRP Duet*

The tertiary and final amplification step is labelled with horseradish peroxidise, allowing chromogenic detection with 3,3'-Diaminobenzidine (DAB) substrate. DAB, which consists of 9.5ml of distilled water, 20µl DAB and 100µl of 3% hydrogen peroxide, was applied to sections for 5 minutes

After washing in running tap water for 5 minutes, sections were counterstained with Mayer's Haematoxylin for 20 seconds, and then washed again in water for 5 minutes. Subsequently, the sections were dehydrated through immersing in decreasing alcohol concentrations, and then cleared in xylene, before mounting in DPX.

2.2.4.5 IHC Controls

Two types of control were used for immunohistochemistry. A positive control tissue shown to stain well with the antibody was used in each batch of staining. This control checked the reproducibility of staining between batches allowing for comparison of staining assessment between runs. A negative control for each tissue section, was also incubated with 1:5 diluted normal serum (depending on the antigen) to ensure there was no non-specific staining.

2.2.4.6 IHC Assessment

Assessment of TGFBI and DDB2 was undertaken by myself initially, and then they were assessed jointly with Professor RA Walker, using a double-headed microscope. Any differences were discussed and the agreed result recorded.

For TGFBI both nuclear staining and extracellular matrix (ECM) staining were evaluated. Any normal breast tissue present was compared to the positive control,

which was normal breast, and categorised as no staining, weaker than control, similar to control. The extent of nuclear staining of ductal carcinoma *in-situ*, if present, and invasive carcinoma was assessed separately and categorised as negative (0% or <1%), low (1-5%), moderate (5-50%) and high (>50%). ECM staining in relation to carcinomas was evaluated as negative (0%), low (2-20%), moderate (20-50%) and high (>50%). The method of assessment was the same as had been used previously for TGF-β and stromal proteins (Walker *et al*., 1994).

For DDB2 nuclear staining of normal, ductal carcinoma *in-situ* and invasive carcinoma was evaluated as for TGFBI.

Assessment of MCM5 was undertaken by Professor RA Walker, since there was prominent staining of stromal cells between the tumour cells which made interpretation difficult. Between 500 and 1000 tumour cells were assessed and the percentage of nuclear reactivity of *in-situ* carcinoma, if present, and invasive carcinoma determined, as undertaken previously for cell cycle proteins (Walker *et al*., 1996; Walker, 2006).

2.2.4.7 IHC Statistical Analysis

Re-assessment of my ability to evaluate the staining was undertaken for both TGFBI and DDB2 using kappa statistical test according to Landis and Koch, 1977. This test was performed using SPSS version 16 to compare the repeatability of my values. TGFBI, DDB2 and MCM5 results were correlated with clinicopathological features using χ^2-squared (χ^2) test, and also Pearson correlation test was also used to compare the staining with patient age as being a continual variable instead of being categorized (*p*-value <0.05 was considered as significant for both tests). Follow-up data were not available, so analyses related to age, tumour size, grade, node status and receptor status was obtained. All analysis was performed using SPSS version 16.

2.2.5 Isolating RNA and synthesizing cDNA

2.2.5.1 RNA Extraction

1- Spin Technology Method

Total RNA was extracted from approximately $1x10^7$ cells or 15µm of 10 snap frozen tissue sections using the RNeasy® Mini Kit, according to the manufacturer's guidelines.

Both cell lines and breast frozen tissues were lysed by adding 400µl or 1000µl of RLT lysis buffer (as provided in kit) respectively, and then homogenized by passage for at least 5 times through a blunt 20-gauge needle to increase the RNA yield. Seven hundred µl of the homogenized sample was transferred to the RNeasy spin column and centrifuged for 15sec at 13,000rpm, after adding 1 volume of 70% ethanol for precipitation (cell lines 400µl; tissues 1000µl). The column membrane was washed once with 700µl of washing buffer RW1 (as provided in kit) and then twice with 500µl of washing buffer RPE (as provided in kit). Purified RNA was eluted into a 1.5ml collection tube after being centrifuged with 30µl of the RNase-free water (Figure 2.6). RNA was then ready for RNase treatment.

2- Tri-Reagent Method

The Tri-reagent method is an important technique for precipitating RNA from aqueous phase by the action of both isopropanol and high salt concentration (Chomczynski and Mackey, 1995). Organoids were placed into 1000µl of Tri-reagent, vortexed and left at room temperature for 5mins. Two hundred µl of chloroform was added to the samples and left for 3mins at room temperature before centrifugation at 13,000rpm for 15mins at 4^{O}C. The aqueous phase was removed to a clean tube and mixed well with 500µl of Tri-reagent and 100µl of chloroform, left for 3mins before centrifugation at the same speed. The aqueous phase was removed and placed in 500µl of isopropanol for 10mins. The supernatant was discarded after centrifugation (13,000rpm) for 15mins, and the pellet washed in 500µl of 70% ethanol. After spinning at the same speed, the supernatant was discarded and the pellet air dried, then resuspended in sterile ultrapure water (Figure 2.7). RNA was ready for DNase treatment.

2.2.5.2 DNase Treatment

RNA samples were treated with DNase using the DNAqueous-Micro Kit to eliminate genomic DNA contamination. Three µl of 10xDNase I buffer and 1µl of DNase were mixed gently with each RNA sample. Post incubation for 20mins at 37^{O}C, 3.4µl of thawed DNase Inactivation Reagent was added, vortexed once and then kept at room temperature for 2mins. The sample was centrifuged for 90sec at 13,000rpm to pellet the DNase and the supernatant RNA transferred to a fresh RNase-free tube and stored at -20^{O}C until required.

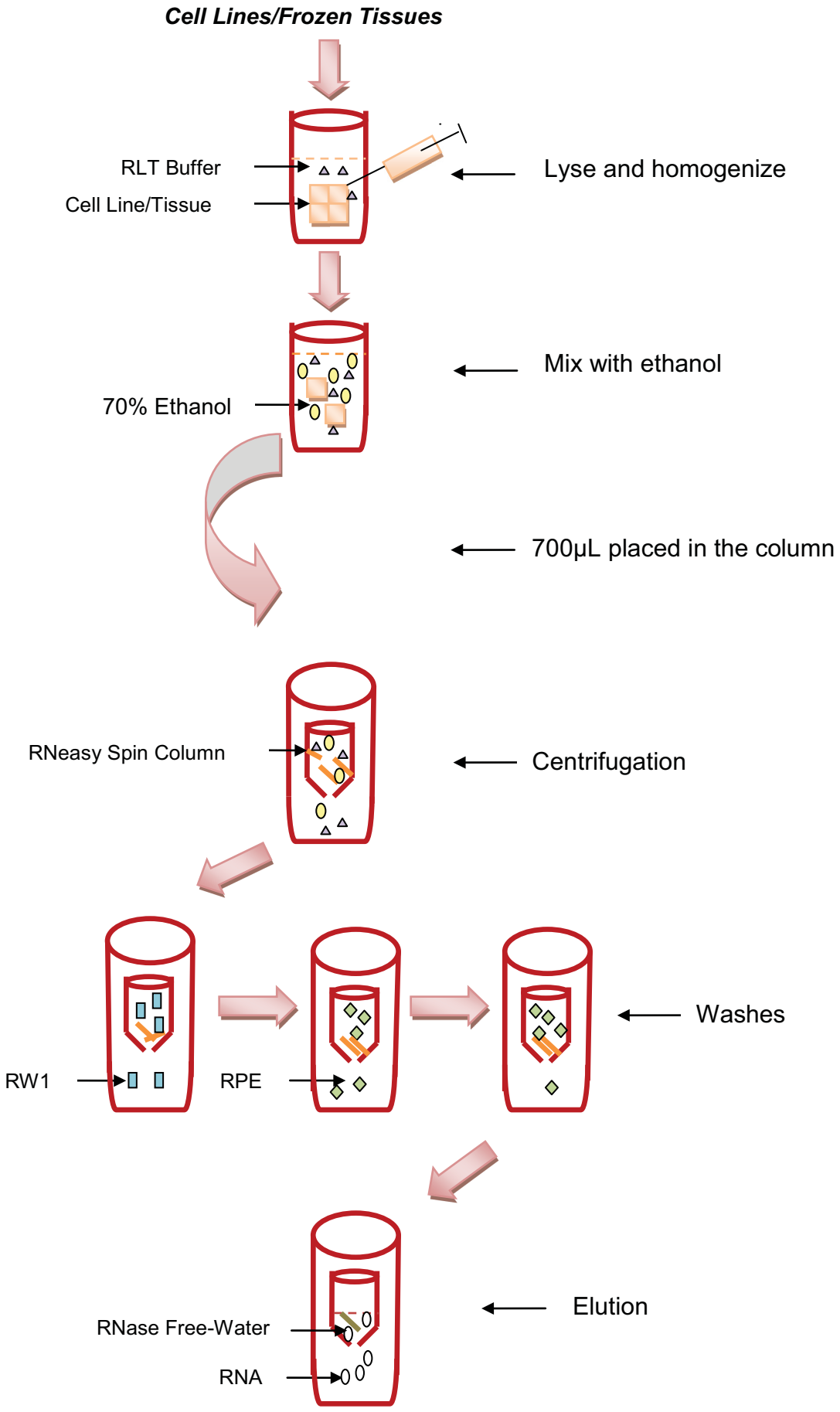


Figure 2.6 Diagram of the Spin Column Technology

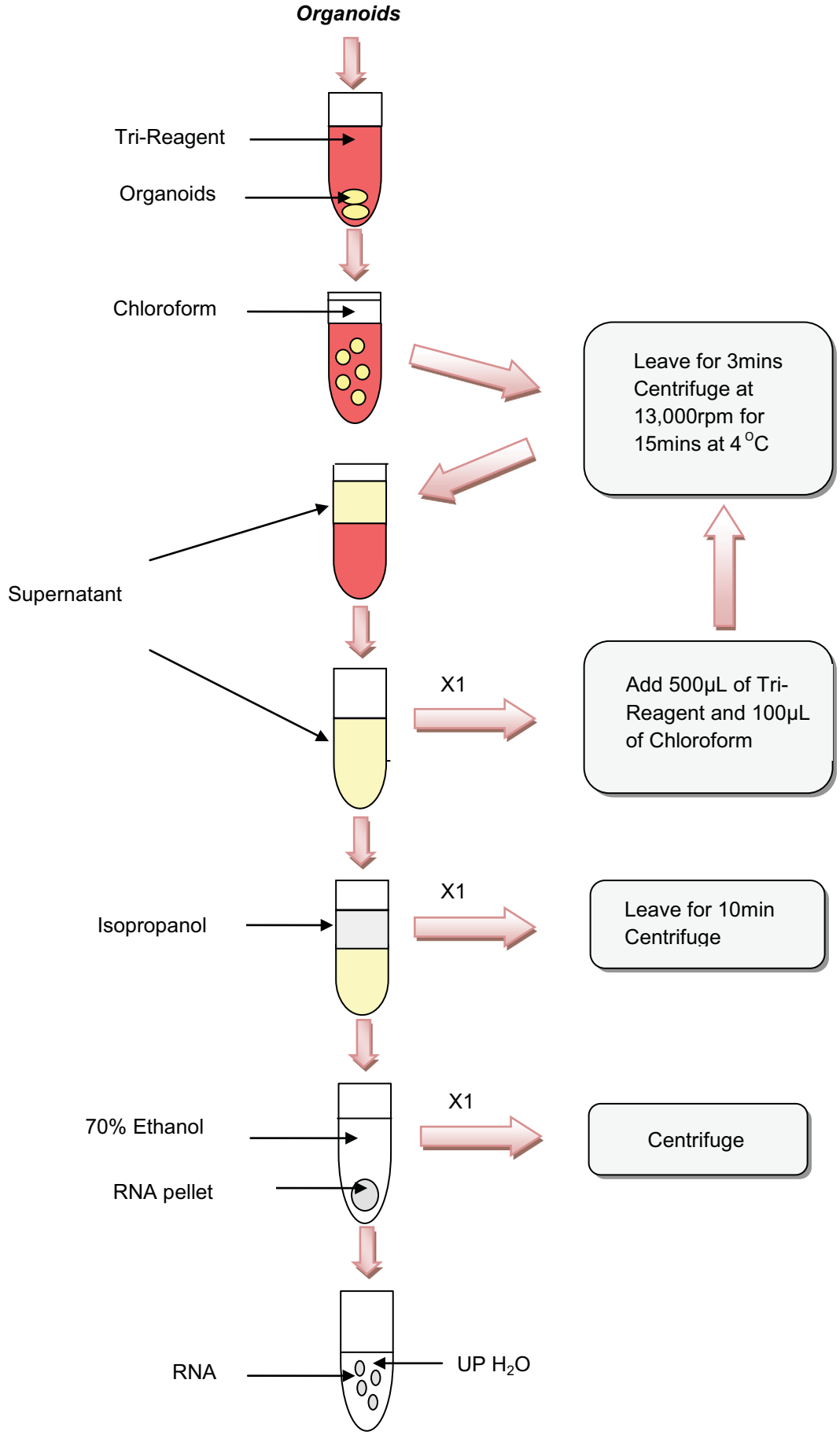


Figure 2.7 Diagram of the Tri-Reagent Method

2.2.5.3 RNA/DNA Quantification

RNA/DNA concentration was determined using the NanoDrop ND-1000 Spectrophotometer. The Spectrophotometer was calibrated using sterile water and a blank set using RNase-free water used for diluting RNA or TE buffer for diluting DNA. One µl of the RNA/DNA was loaded and absorbance measured at 260nm to give sample concentration in ng/µl. The ratio between absorbance at 260nm and 280nm was also measured to check the RNA/DNA purity. Pure RNA/DNA has ratio of 1.9-2.1.

2.2.5.4 Reverse Transcription (RT)

cDNA was synthesised using AMV-RT enzyme and Oligo-dt_{20} primer from 1µg of total RNA from cell lines and organoids. However, 150-250ng was used to reverse transcribe RNA from the frozen tissues. The master mix (9.7µl) (Table 2.10) was added to the RNA, which was diluted with sterile ultrapure H_2O to give a final volume of 20µl. Negative RT reactions, were RNA omitted, used to ensure that was no genomic DNA carry over from the RNA extraction. Both positive and negative RT reactions were incubated in the thermal cycler (Figure 2.8) for 20 minutes. The resulting cDNA was stored at $4^{O}C$ until required (D'Alessio and Gerard, 1988).

Table 2.10 Components of the RT Master Mix

Component	***Concentration***	***Volume (µl)***
AMV RT Buffer	5X	4
dNTP Mix	10 mM	2
$MgCl_2$	50 mM	2
Olig-dt_{20}	10pmol/µL	0.5
RNasin® RNase Inhibitor	40 U/µL	0.5
AMV Reverse Transcriptase	10 U/µL	0.7

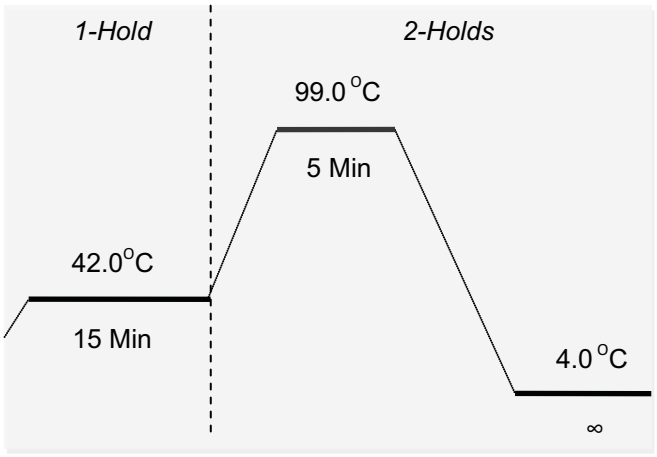


Figure 2.8 Incubation Times for cDNA Synthesis

2.2.5.5 Polymerase Chain Reaction (PCR)

One µl of the positive or negative RT reactions was added to the reaction and aliquoted with 47µl of the PCR master mix (Table 2.11). The samples were incubated in the thermal cycler at 98°C for 3mins, followed by 2-3mins at 60°C at which point 2µl of diluted Taq (1:1:8) (AJ Buffer: Taq: sterile water) was added, making a final reaction volume of 50µl. Approximately 25-30 cycles were performed for 30sec at 60°C (Figure 2.9), which was the annealing temperature for each primer used in this study. Amplified cDNA was stored at 4°C until further analysis (Mullis, 1990).

Table 2.11 The Components of PCR Reaction

Component	***Volume (µl)***
cDNA	1
AJ Buffer (10X)	5
Forward Primer (10pm/µl)	1
Reverse Primer (10pm/µl)	1
UP H_2O	40

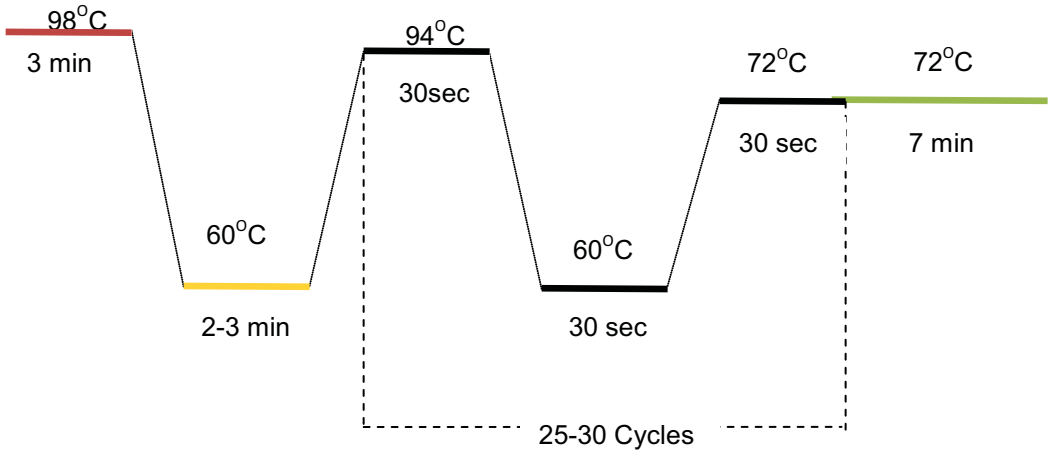


Figure 2.9 PCR Conditions for cDNA Amplification

2.2.5.6 Agarose Gel Electrophoresis

PCR products were separated using a SeaKem agarose gel prepared in 1XTAE buffer (% w/v) to semi-quantify levels of expression by band intensity. Ethidium Bromide was added to a final concentration of 2µg/ml to gel mixture to visualise PCR products. Ten µl of each sample was mixed with 2µl of 6 X PCR loading buffer, and loaded into wells. Additionally, 20µl of 100bp DNA ladder or Hyperladder I was loaded into separate well to determine the size of the products. The gel was run at 100V for approximately an hour and the PCR products visualised using UV transluminator. Results were digitally captured using FlashPoint 128 capture card and Vision Works 2.0.

2.2.6 Quantitative RT-PCR Method

2.2.6.1 General Conditions

There are several methodologies that can be used to investigate the quantity of mRNA levels in various samples. Inventoried TaqMan probes (Material *2.1.6*) were supplied by Applied Biosystems. Q-RT-PCR master mix was prepared by mixing both 0.5µl of TaqMan probe with 5µl of TaqMan® Universal PCR Master Mix without UNG (uracil-N-

glycosylase) for one sample. Thermal profile for all the assays used in this study is shown in Figure 2.11

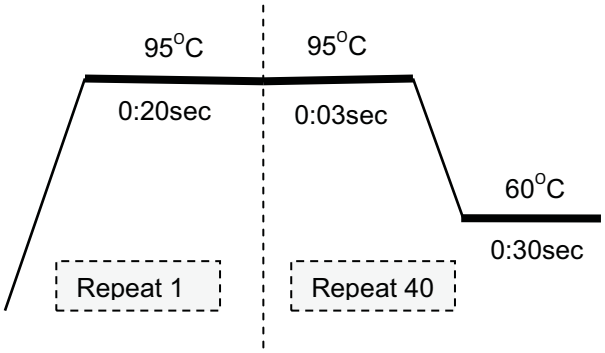


Figure 2.10 Thermal Profile of the q-RT-PCR

2.2.6.2 Standard Curves

Standard curves were generated by preparing serial dilutions (1:5) of cDNA from the HBL-100 cell line. Prepared q-RT-PCR master mix (5.5µl) was added to the wells and then 4.5µl of the diluted HBL-100 cDNA (1:20) was loaded in triplicate onto the wells plate, making final volume of 10µl. Negative controls were used, containing 4.5µl UP H_2O instead of HBL-100 cDNA. The plate was covered with optical adhesive film, centrifuged and placed in the Real-Time PCR System for 50 minutes. The results produced by the 7500 software version 2.0.1 were collected and manipulated in Excel.

2.2.6.3 Probe Efficiency

Standard curves were generated for each probe in order to determine the PCR efficiency. HBL-100 cDNA dilutions (1:5) were transformed into Log_2 values and plotted onto the x-axis, with Ct values on the y-axis. Slopes obtained from the linear curves generated were used in the PCR efficiency equation. A result between 90-110% indicates high efficiency of the probe. Additionally, the regression (R^2) value was calculated to determine how well the standard curve fitted the measured data and therefore reflected the reliability of the probe ($R^2 \geq 0.98$).

Calculation of PCR Efficiency from the Standard Curve

$$E = (2^{(-1/\text{slope})}-1)*100$$

2.2.6.4 Absolute Quantification (AQ)

The RNA levels of 6 genes were measured for 4 samples in each prepared plate. 4.5µl of the diluted cDNA (1:20) generated was added in triplicate to 5.5µl of master mix in the well. Negative controls were 4.5µl of UP H_2O instead of the cDNA. One of the cell lines (HBL-100) was used as positive control to ensure the experiment efficiency. Finally, the plate was covered with optical adhesive film, centrifuged and placed in the q-RT-PCR machine for approximately 50 minutes. The results produced by the 7500 software version 2.0.1 were collected and rearranged in Excel (Bustin, 2000).

2.2.6.5 Target Gene Analysis

Absolute quantification of each target gene used the cycle threshold (Ct), which was normalized to the mean Ct of three housekeeping genes (*GAPDH*, *HPRT1* and *TFRC*) in order to determine the ΔCt in the samples investigated.

To obtain the relative expression (ΔΔCt) for the different methods of RNA extractions (Tri-Reagent and RNeasy), ΔCt of Tri-Reagent was subtracted from ΔCt of RNeasy kit for each breast cancer tissue investigated.

The relative expression for breast cancers was calculated using two steps:

1- Subtracting the ΔCt value of each sample from the ΔCt of the control (mean of normal/benign tissues) to obtain the ΔΔCt.
2- Relative expression was calculated by using the equation: 2 to the power of -ΔΔCt. ($2^{-\Delta\Delta Ct}$).

2.2.6.6 Q-RT-PCR Statistical Analysis

One sample t-test was performed to determine whether there is significant difference between the two RNA extraction methods (Tri-Reagent and RNeasy kit). This test was selected because Shapiro-Wilk test confirmed that the results were normally distributed (p>0.05).

Kruskal-Wallis test with Bonferroni post-hoc correction was performed (data were not normal distributed, Shapiro-Wilk test $p<0.05$) to determine whether there was a significant difference between the three groups: normal organoids, normal/benign samples and breast cancers.

Mann Whitney test was performed to check correlations of mRNA expression of the three genes with the clinicopathological features including size, grade, lymph-node, ER and PR status and HER2. Patient age was correlated with TGFBI, DDB2 and MCM5 mRNA data using Pearson correlation, because the age was considered as continual variable and was also normally distributed (Shapiro-Wilk test, $p>0.05$). In order to test correlation between the IHC results and mRNA data for the breast cancer cases Spearman's rho correlation was used.

All statistical analysis was performed using SPSS version 16, and all tests showed $p<0.05$ was considered as significant result.

2.2.7 Preparation of Plasmid DNA

2.2.7.1 Transformation of XL-10 Gold E.Coli Cells

Transformation of XL-10 Gold ultra-competent *E. Coli* cells was performed using 100µl of thawed *E. Coli* cells. One Hundred pg of plasmid was added to a 15ml pre-chilled polypropylene Falcon tube plus 2µl of β-mercaptoethanol. Samples were incubated on ice for 10 minutes with gentle swirling every 2 minutes. Five µl of the plasmid and 1µl of pUC18 were added to separate tubes, mixed and incubated on ice for 30 minutes. Samples were heat-shocked at 42^{O}C for 20 seconds and then immediately held on ice for 2 minutes. NZY+ broth (*Appendix VI*), pre-heated to 42^{O}C was added (900 µl) to each sample which was then incubated at 37^{O}C for 1 hour with shaking at 200rpm (Graham *et al*., 1974).

2.2.7.2 Colony Subculture

One litre of pre-heated LB Agar (55^{O}C) was mixed with 10µl of Ampicillin (100mg/ml), which was then poured into petri dishes. When solidified 140µl of the transformation mixture was plated using aseptic technique and then incubated overnight at 37^{O}C. The

colonies that had grown after 16-18 hours were then sub-cultured onto individual Ampicillin agar plates and incubated overnight at 37^{O}C. Subsequently, single colonies were picked and transferred into LB medium and Ampicillin (50µg/µl) in conical flasks and incubated overnight at 37^{O}C with orbital shaking at 220rpm. In addition, 0.5ml of each sample was added to 0.5ml of 40% glycerol and stored at -80^{O}C until required for plasmid DNA purification.

2.2.7.3 Purification of Plasmid DNA

1- QIAGEN-Mini Prep

QIAGEN mini prep was performed to yield up to 20µg of plasmid DNA. After growing the colonies overnight (Method *2.2.7.2*) 3ml of bacterial culture volume was centrifuged at 13,000rpm for 15 minutes at 4^{O}C. The bacterial pellet was resuspended in 300µl of lysis buffer P1, vortexed until no cell clumps remained, followed by the addition of 300µl of buffer P2. Tubes were mixed gently by inversion 4-6 times and then incubated at room temperature for 5 minutes. The lysate was neutralized by the addition of 300µl chilled buffer P3 and mixed gently, with incubation on ice for 5 minutes to enhance precipitation. The clear supernatant containing plasmid DNA was transferred to a clean tube after centrifugation at 13,000rpm for 10 minutes at 4^{O}C. Again, supernatant was re-centrifuged at the same speed for 10 minutes while the QIAGEN-tip20 tube was equilibrated by applying 1ml of buffer QBT, allowing it to empty by gravity flow. The supernatant was loaded promptly onto the QIAGEN-tip20 tube allowing to entry into the resin by gravity flow. The QIAGEN-tip20 was washed twice with 2 ml of buffer QC, and subsequently plasmid DNA was eluted with 800µl of buffer QF. It was precipitated by adding 560µl room temperature isopropanol to desalt and concentrate the plasmid DNA; mixed well and centrifuged immediately at 13000rpm for 30 minutes at room temperature. The supernatant was discarded carefully; and the DNA pellet washed with 1ml of room temperature 70% ethanol. It was left for 5-10 minutes to air dry after spinning at 10,000rpm for 10 minutes at 4^{o}C, and then re-dissolved in a suitable volume of TE buffer (pH=8). Plasmid DNA concentration was measured (Method 2.2.5.3) and storage was at 4^{O}C until required.

2- QIAGEN-Maxi Prep

QIAGEN maxi prep and QIAGEN mini prep were performed to yield about 500µg and 20µg of plasmid DNA respectively. After growing the colonies overnight (Method *2.2.7.2*) 100ml of bacterial culture volume was centrifuged at 8000rpm for 15 minutes at 4^{O}C. The bacterial pellet was resuspended in 10ml of lysis buffer P1, vortexed until no cell clumps remained, followed by the addition of 10ml of buffer P2. Tubes were mixed gently by inversion 4-6 times and then incubated at room temperature for 5 minutes. The lysate was neutralized by the addition of 10ml chilled buffer P3 and mixed gently, with incubation on ice for 20 minutes to enhance precipitation. The clear supernatant containing plasmid DNA was transferred to a clean tube after centrifugation at 15000rpm for 30 minutes at 4^{O}C. Again, supernatant was re-centrifuged at the same speed for 15 minutes while the QIAGEN-tip500 tube was equilibrated by applying 10ml of buffer QBT, allowing it to empty by gravity flow. The supernatant was loaded promptly onto the QIAGEN-tip500 tube allowing to entry into the resin by gravity flow. The QIAGEN-tip500 was washed twice with 30ml of buffer QC, and subsequently plasmid DNA was eluted with 15ml of buffer QF. It was precipitated by adding 10.5ml room temperature isopropanol to desalt and concentrate the plasmid DNA; mixed well and centrifuged immediately at 13000rpm for 30 minutes at room temperature. The supernatant was discarded carefully; and the DNA pellet washed with 5ml of room temperature 70% ethanol. It was left for 5-10 minutes to air dry after spinning at 13000rpm for 10 minutes at 4^{O}C, and then re-dissolved in a suitable volume of TE buffer (pH=8). Plasmid DNA concentration was measured (Method 2.2.5.3) and storage was at 4^{O}C until required.

2.2.7.4 PCR Amplification from Colonies

Colonies from plates were picked using a P200 tip and placed into an eppendorf containing 200µl LB-broth and ampicillin. Ten microlitters were transferred into 0.5ml eppendorf and heated at 95^{O}C for 15 minutes. Subsequently, 40µl of PCR Master Mix was added and 28 cycles were performed for amplification using the TGFBI primers. The samples were run in agarose gel (Method 2.2.5.6).

2.2.8 Transient Transfection

2.2.8.1 Preparation of Cell Lines for Transfection

Cell lines were maintained as described in Method *2.2.1*. One day before transfection, the monolayer cell lines were washed with PBS, trypsinized, and counted for seeding at various densities (Table 2.12) depending on the chosen cell-culture vessel (mostly 6 well plates and T_{25} flasks) overnight to achieve a suitable transfection efficiency (Table 2.12).

Table 2.12 The Optimal Conditions for Transfection

Culture Vessel	*Surface Area (cm^2)*	*Total Volume of Media (ml)*	*Seeding Density*	*Amount of Transfection Complex (µl)*	*FuGENE Transfection Reagent (µl)*
6-Well Plate (1-well)	9.4	2	400,000	100	7
T-25 Flask	25	6	1,200,000	300	21

2.2.8.2 Optimization of Transfection

Cells were seeded into six-well culture dishes (Table 2.12) as described above (Method *2.2.1.2*), and then transfected (Method *2.2.8.3*) using 3:2, 4:2, 5:2, 6:2, 7:2 and 8:2 ratios of FuGENE® HD Transfection Reagent (µl) to DNA (µg) respectively.

2.2.8.3 Transfection

One µg of plasmid or vector with 1µg of GFP expression plasmid were diluted in Opti-MEM to a concentration of 0.02µg/µl. The seeding densities and volumes were adjusted in relation to the total surface area of the cell culture vessel being used (Table 2.12). The optimal ratio of FuGENE® HD Transfection reagent was added to the diluted DNA and incubated for 20 minutes at room temperature. The transfection complex was added to the cells after washing them twice with DPBS. Plates/Flasks were swirled, and then incubated for 6-7 hours. Media were discarded, cells washed twice with DPBS and fresh media added. Un-transfected cells were used as a negative control.

2.2.8.4 Transfection Efficiency

After transfection, cells were left in transfection media for 6-7 hours. Cells were washed with DPBS and incubated with complete media for a further 12-16 hours after transfection. At this time the transfection efficiency was determined microscopically using GFP reporter plasmid, which was added with the DNA plasmid during transfection. Green cells, which are assumed to contain the TGFBI plasmid, were counted in three different areas and calculated as a percentage of the total number of cells present in this area. The transfection efficiency was the mean of the percentage of the three areas that were counted.

2.2.9 Functional Assays

Cells were seeded into three T_{25} flasks one day prior to transfection, and then one flask was transfected with plasmid and one with empty vector (Method *2.2.8.3*). The third flask was untransfected control cells. New transfections were performed for each repeat experiment, at a specific confluence (70%). Equal densities were ensured by plating the same number of cells post counting. The same transfected cells were used for both viability and apoptosis assays in each experiment. The readings obtained at each time point were the times from replating cells in the appropriate well-plates with 10% FCS.

2.2.9.1 Optimization of Cell Density and Culture Conditions

In order to determine optimal cell density, cells were seeded at 5,000, 10,000 and 20,000 cells/cm^2 in triplicate and then incubated in 10% FCS media overnight. The complete media was replaced with serum free media (0% FCS) post washing with DPBS only for 3 wells of each density, and again incubated for 24 hours. One hundred µL of the CellTiter-Blue or 100µL of Caspase-Glo® 3/7 activity was added to each well for 3 hours or 30 minutes respectively before reading, with blanks containing complete media or serum free media for each cell line.

2.2.9.2 Viability Assay

The cells were prepared as in *2.2.9*. They were trypsinized; $1x10^4$ cells were seeded in triplicate in opaque-walled tissue culture plates compatible with the fluorometer and then incubated until the appropriate time point. Three hours before each time point (6, 12 and 24 hours) 100µl of CellTiter Blue was added to each well, and incubated at 37^OC. Negative controls were complete media alone. At the specific time points, the plate was shaken and then measured at wavelength of $560^{EX}/590^{EM}$ nm using the BMG Fluostar optimal plate reader.

2.2.9.3 Apoptosis Assay

Cells were prepared as in *2.2.9*. They were trypsinized; $1x10^4$ cells were seeded in triplicate in 96-well plate and then incubated for the appropriate time point. One hundred µl of the caspase 3/7 activity reagent was added to each well 30 minutes before assaying; the plate was placed again in the incubator. Positive and negative controls were used; positive control was un-transfected cells which were treated with Staurosporine (1µg/100µl). For negative control complete media was used to measure the background luminescence. At each time point (6 and 12 hours) the plate was shaken and the luminescence signals were measured using the BMG Fluostar optimal plate reader.

2.2.9.4 Invasion Assay

Two hundred microlitres of diluted Matrigel (1:1000 in Opti-MEM) was added to the FluoroBlok inserts (8µm pore), which were incubated at 37^OC for 2 hours. Diluted $DiIC_{12}(3)$ staining dye (1:10,000 in complete media) was then added to the flasks of the untreated cells and incubated for an hour. After incubation, excess Matrigel was removed and cells were typsinized.

For baseline invasion assays, cells were seeded in 3 inserts for each one of the three densities (1 X (10^4) and 10^5 cell/cm^2) in 100µl of complete media. Complete media (700µl) was also placed into each well of the plate. Cells were incubated to 4 hours, and then the complete media present in the insert was exchanged with 100µl Opti-MEM. The plate was then placed in a FLUOstar OPTIMA plate reader (BMG Labtech, UK) for 48 hours to measure the fluorescence from cells that had invaded through the matrigel.

To test the effect of media from transfected cells, cells were seeded into duplicate inserts in 100µl of complete media. Seven hundred microlitres of conditioned media that had been collected from cells transfected with TGFBI or empty vector after 72 hours of culture were placed into each well. Cells were incubated at 37^{O}C /5% CO_2 for 4 hours and media in the insert replaced with 100 µL of Opti-MEM. Cells were cultured at 37^{O}C /5% CO_2 for 48 hr in a FLUOstar OPTIMA plate reader (BMG Labtech, UK) with measurements taken every 2 hours.

2.2.9.5 Calculations and Statistical Analysis of Functional Assays

Both viability and apoptosis assays results were presented as mean of the three triplicate readings of TGFBI plasmid, empty vector and untreated cells in each experiment. The blank reading from the same column of the plate was subtracted from each reading in that column, and then the results were normalized to the readings at the 6 hour time point. This was normalizated by dividing the values of the various time points of TGFBI plasmid, empty vector and untreated cells by the mean of the three readings obtained at 6 hours after seeding transfected cells into the assay plate. The error bars represent the 95% confidence interval. The overall mean graphs present the mean of the three separate experiments after normalization and the error bars represent the 95% confidence interval.

The invasion results were presented as mean ±Standard Error of the Mean. The mean values for TGFBI conditioned media inserts were calculated as below for each time point:

(Reading 1 + Reading 2)/2 = Mean 1 (Reading 3 + Reading 4)/2 = Mean 2

(Blank Reading 1 + Blank Reading 2)/2 = Mean 3 (Blank Reading 3 + Blank Reading 4)/2 = Mean 4

Mean 1 – Mean 3 = Fluorescent 1

Mean 2 – Mean 4 = Fluorescent 2

Fluorescent 1 and Fluorescent 2 values were normalized to the mean of the empty vector conditioned media readings at 48 hour time point, after subtracting this mean from the mean of the blank. The Fluorescent 1 and Fluorescent 2 values of the same

experiment were averaged separately for each experiment preformed. As the experiment was repeated three times for ZR-75-1 and twice for MDA-MB-468, the final mean was derived from the means of the separate experiments, and the error bars represent the standard Error of the Mean.

Two-Way ANOVA (Analysis of Variance) with the Bonferroni correction were performed to analyze the viability, apoptosis and invasion assays, as all the data were normally distributed (Shapiro-Wilk test, $p>0.05$).

2.2.10 DAPI Staining

Cells were prepared and seeded as described in *2.2.8.1* and then transfected as in *2.2.8.3.* Transfected and untreated cells ($2x10^5$ seeding density was used) were seeded in 24 well-plate for different time points (6, 24 and 48 hours). At the appropriate time point complete media was removed, cells were washed with PBS twice then 2ml of acetone was added to each well and incubated for 20 minutes. After fixation, cells were equilibrated with PBS and then diluted DAPI (300nM in PBS) was added and left for 5 minutes. Cells were washed with PBS twice and viewed using a fluorescent microscope. The cells were counted in three different regions in each well and then results presented as described in *2.2.9.2.*

2.2.11 Synthesis and Secretion of TGFBI with Time

Cells were counted and seeded in 7 T_{25} flasks (Method *2.2.1.2*). Five of them were transfected with the TGFBI plasmid; one with vector alone and one remained untreated. After 6-7 hours of the transfection (Method *2.2.8.2*), all flasks were washed twice with PBS, fresh media was added and they were incubated at $37^{O}C$ overnight. The complete media was changed for serum free media after washing the cells twice with PBS, and cells have harvested after 6, 12, 24, 48 and 72 hours of culture. Cells were trypsinized and protein (Method *2.2.2.1*) and RNA (Method *2.2.5.1*, 1) extracted. Media from each time point was collected, purified and stored at $-20^{O}C$. Protein and media were subsequently analysed by western blotting (Method *2.2.2*) and RNA by q-RT-PCR (Method *2.2.6.4*).

2.2.12 Effect of TGF-β_1 on TGFBI Expression in MCF-7 Cells

$1.2x10^6$ MCF-7 cells were seeded in complete media in 5 T_{25} flasks and were incubated at 37^OC overnight. Cells were washed twice with PBS, and then incubated overnight with serum free media. Media were discarded, cells washed with PBS, and serum free media with different concentrations of TGF-β_1 (1, 2, 5, 25, 75 and 100ng/ml) added to the cells for 24 and 72 hours. Media was collected and cells trypsinized for protein extraction for subsequent western blotting (Methods *2.2.2*). In addition, microscopic images were captured using the computerized inverted microspore (Nikon Digital Camera) for the various TGF-β_1 concentrations and different time points.

Chapter 3

Expression of TGFBI, DDB2 and MCM5 in Breast Cancer

Introduction

Three genes *TGFBI*, *DDB2* and *MCM5* that were shown to be up-regulated in the previous microarray study (Whyman, 2005) were further investigated. There is conflicting evidence about the role of TGFBI in breast cancer (Thapa *et al*., 2007) but the literature suggests that both TGFBI and DDB2 could be of clinical interest as prognostic markers of breast progression (Itoh *et al*., 2007; Calaf *et al*., 2008). MCM5 has been shown to be a promising marker of proliferating cells due to its increase during the cell cycle (Murphy *et al*., 2005). MCM5 levels have been shown to be up-regulated in a range of cancers (Table 1.15) but it has not yet been investigated in breast cancer (Scarpini *et al*., 2008; Burger, 2009).

A number of studies have identified TGFBI protein in various human cell types (Billing *et al*., 2000b; Zhao *et al*., 2006; Park *et al*., 2008) and have also shown it to be a component of the ECM (Skonier *et al*., 1994; Billing *et al*., 2000a). It has been suggested that TGFBI may play an important role in a wide biological functions such as wound healing (LeBaron *et al*., 1995) and inflammation (Nam *et al*., 2006).

DDB2 plays an important role in nucleotide excision DNA-repair, mainly via its involvement with chromatin remodelling during repair (Datta *et al*., 2001). In addition, DDB2 has been found to be a tumour suppressor gene in normal cells (Kulaksiz *et al*., 2005). In MCF-7 breast cancer cells it is a transcriptional regulator of the SOD2 (Manganese Superoxide Dismutase) gene, indicating a possible role in breast cancer cell growth (Minig *et al*., 2009).

MCM5 is a member of the *MCM* gene family (*MCM2-7*), which are involved in the initiation of DNA replication (origin licensing), in eukaryotic cells (Guida *et al.,* 2005). Several studies have identified that it is highly expressed in various types of cancers (Introduction, Table 1.15). Therefore, MCM5 may be marker of proliferation and potential prognosis.

Aims

The aim of this chapter was to examine RNA and protein expressions of TGFBI, DDB2 and MCM5 in breast cell lines, normal breast tissues and tumour samples using q-RT-PCR, western blotting and immunohistochemistry. A second aim was to relate the findings to clinicopathological features of the cancers.

Results

3.1 Optimization

3.1.1 Q-RT-PCR Assay Optimization

HBL-100 cell line cDNA was synthesized for generation of all standard curves. Three strong bands were observed using manual GAPDH RT-PCR (Figure 3.1 (**+**)). No bands were seen in the –RT reaction and in the water blank demonstrating successful generation of cDNA and lack of genomic DNA contamination (Figure 3.1).

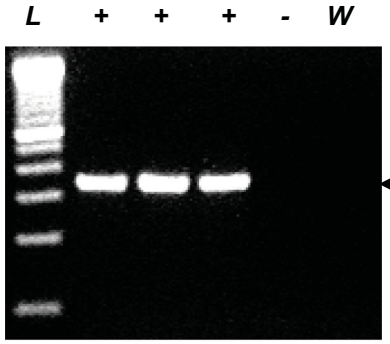


Figure 3.1 Generation of cDNA from HBL-100

HBL-100 cDNA was tested using manual GAPDH PCR (~350bp). (+) = +RT, (-) = -RT and (W) = Water Blank.

Standard curves were generated for 3-housekeeping genes (*GAPDH*, *HPRT1* and Transferrin Receptor (*TFRC*)) (Figure 3.2, A) and for the three 3-target genes (*TGFBI*, *DDB2* and *MCM5*) (Figure 3.2, B) using HBL-100 cDNA (Figure 3.1). All standard curves synthesised showed a strong and linear correlation between the concentration of cDNA template and the obtained cycle threshold (Ct) (Figure 3.2).

For the three housekeeping genes, GAPDH showed the lowest Ct values compared to the HPRT1 and TFRC although the same concentration of cDNA was used in all serial dilutions (Figure 3.2 A). GAPDH had also the highest efficiency compared to the rest of the assays, which was calculated from the slope of the linear curve generated (Table 3.1). Both HPRT1 and TFRC showed similar Ct values but with approximately two cycles higher in the TFRC assay in the various serial dilutions. The efficiency of the HPRT1 and TFRC assays were 87% and 88% respectively. All standard curves of the housekeeping genes showed $R^2 \geq 0.98$ (Table 3.1).

TGFBI showed the lowest Ct values in serial dilutions compared to DDB2 and MCM5 assays (Figure 3.2, B) using the HBL-100 cDNA. Both TGFBI and MCM5 assays showed similar efficiencies (Table 3.1). The DDB2 assay gave high Ct values (>35 cycles) at the various dilutions used, which might have influenced the efficiency

calculated from the standard curve (77%). Therefore, a different dilution range should have been used for this assay. All standard curves of the target genes showed $R^2 \geq 0.98$ (Table 3.1).

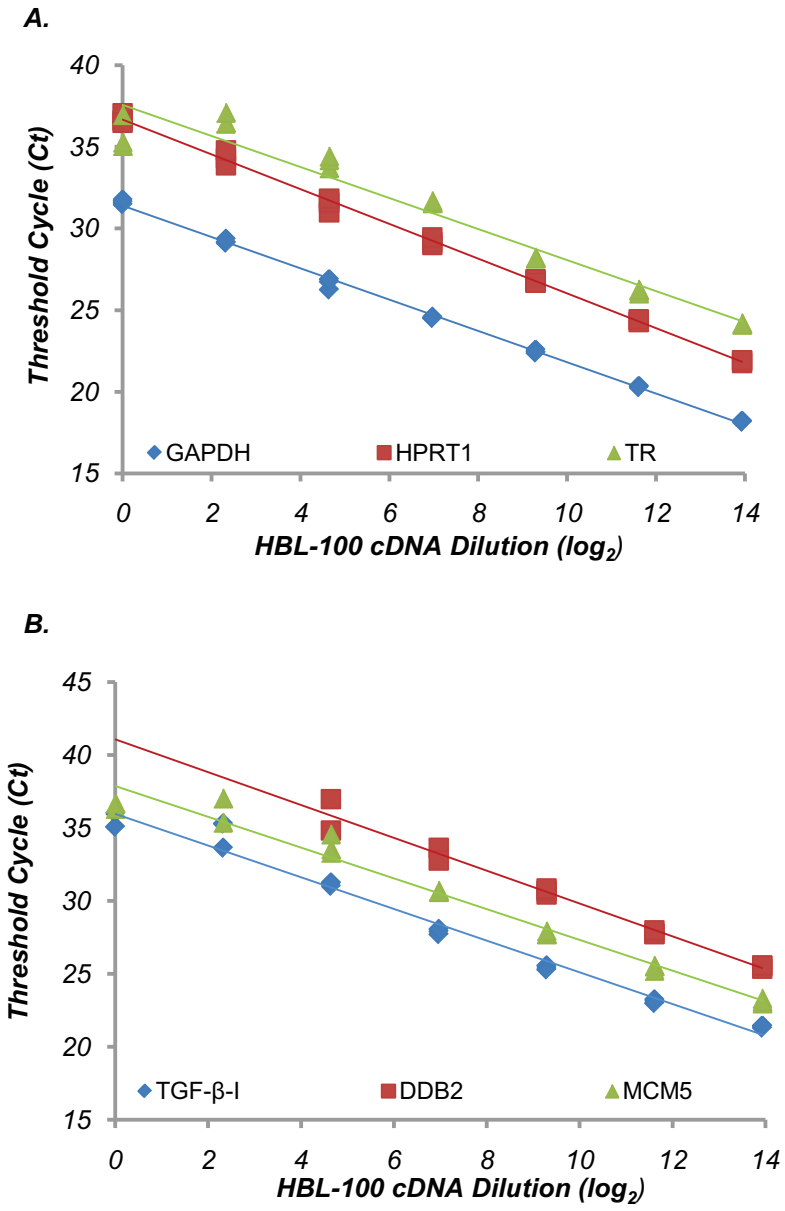


Figure 3.2 Examination of Assay Efficiency by Generation of a Standard Curve

The standard curves of the housekeeping genes (*GAPDH*, *HPRT1* and *TFRC*) (A) and the target genes (*TGFBI*, *DDB2* and *MCM5*) (B) using the q-RT-PCR were generated. The efficiency of the various assays was calculated from the slope of the linear curve. Standard deviation across the triplicate for each one of the dilutions was <1.05 (*Appendix II*).

Table 3.1 PCR Efficiency for Each Gene Assay

Assay	Standard Curve Equation	R^2	Efficiency (%)	
GAPDH	y = -0.9591x + 31.387	0.9977	110	Figure 3.2 A
HPRT1	y = -1.0642x + 36.656	0.9973	87	
TR	y = -1.0574x + 38.646	0.9839	88	
TGFBI	y = -1.0855x + 35.97	0.9843	83	Figure 3.2 B
DDB2	y = -1.1251x + 41.067	0.9826	77	
MCM5	y = -1.0537x + 37.876	0.9768	89	

3.1.2 Comparison of RNA Isolation

Two different methods (column based method (RNeasy) (Method *2.2.5.1*, 1) and Tri-Reagent (Method *2.2.5.1*, 2)) were used to extract total RNA from 6 frozen breast tissue samples. This was performed to check whether there were differences in the ΔCt value of the target genes investigated using RNA extracted by the different methods. The means of the triplicate Ct values for each of the housekeeping and target genes, ΔCt (mean of target genes – mean of housekeeping genes) and ΔΔCt (ΔCt of Tri-Reagent - ΔCt of the RNeasy kit) are presented in Table 3.2. To compare the 2 methods, each gene of interest was normalized relative to the mean of the 3 housekeeping genes for each sample (ΔCt) and then ΔΔCt values were obtained by subtracting ΔCt of Tri-Reagent method from the ΔCt of the RNeasy kit for each breast cancer tissue investigated.

Variations in the ΔΔCt values for TGFBI were observed among the different breast cancers investigated (Figure 3.3). Two samples showed ΔΔCt higher than 0 (Figure 3.3, C and E), while the other four showed ΔΔCt lower than 0 (Figure 3.3, A, B, D and F). This variation confirmed that there is no significant difference in the two RNA extraction methods when using TGFBI assay (One Sample t-test, $p>0.05$). The ΔΔCt of DDB2 expression was higher than 0 in all the breast cancers when total RNA was extracted using the Tri-Reagent method compared to the RNeasy kit (Figure 3.3, A-F) (One Sample t-test, $p=0.004$). The MCM5 ΔΔCt values for all the samples (Figure 3.3, A-F) were higher than 0, indicating that Tri-Reagent method for RNA extraction is better than RNeasy kit for frozen breast tissues (One Sample t-test, $p=0.03$).

All –RT samples were analysed using the GAPDH assay, and all were negative indicating the absence of DNA contamination. These results shows differences between the two methods; however, RNeasy kit was selected for extracting total RNA from breast cancers as it was an easier method, quicker and safer, since no phenol is used.

Table 3.2 The Means of Triplicate Ct values, ΔCt and ΔΔCt for each Gene in each Sample for the Two RNA Extraction Methods

Number of Case	***Method***	***GAPDH***	***HPRT1***	***TFRC***	***TGFBI***	***DDB2***	***MCM5***
A	Tri-Reagent	24.132	29.401	32.412	28.325	31.104	28.598
	RNeasy Kit	22.063	26.456	30.06	26.693	26.823	25.3833
	ΔCt(Tri-Reagent)				-0.32	2.45	-0.05
	ΔCt (RNeasy)				0.5	0.62	-0.81
	ΔΔCt (Tri-RNeasy)				**-0.82**	**1.82**	**0.75**
B	Tri-Reagent	24.449	28.256	31.851	28.354	30.139	29.567
	RNeasy Kit	26.651	29.380	35.628	30.917	30.614	31.616
	ΔCt(Tri-Reagent)				0.16	1.95	1.38
	ΔCt (RNeasy)				0.36	0.06	1.06
	ΔΔCt (Tri-RNeasy)				**-0.19**	**1.89**	**0.31**
C	Tri-Reagent	25.437	28.711	30.437	30.815	33.532	31.652
	RNeasy Kit	25.812	28.41	31.828	29.766	30.925	30.717
	ΔCt(Tri-Reagent)				2.62	5.33	3.45
	ΔCt (RNeasy)				1.08	2.24	2.03
	ΔΔCt (Tri-RNeasy)				**1.53**	**3.09**	**1.42**
D	Tri-Reagent	25.918	28.694	30.906	29.433	30.473	31.827
	RNeasy Kit	23.603	28.62	30.543	28.66	27.996	28.98
	ΔCt(Tri-Reagent)				0.92	1.96	3.32
	ΔCt (RNeasy)				1.07	0.40	1.39
	ΔΔCt (Tri-RNeasy)				**-0.14**	**1.55**	**1.92**
E	Tri-Reagent	26.647	29.520	35.105	30.729	31.380	31.663
	RNeasy Kit	25.781	27.885	33.443	28.763	29.593	30.004
	ΔCt(Tri-Reagent)				0.30	0.95	1.23
	ΔCt (RNeasy)				-0.27	0.55	0.96
	ΔΔCt (Tri-RNeasy)				**0.57**	**0.39**	**0.27**
F	Tri-Reagent	28.211	30.8771	34.988	31.251	33.276	33.792
	RNeasy Kit	23.9	27.856	30.066	27.503	27.52	29.326
	ΔCt(Tri-Reagent)				-0.11	1.91	2.43
	ΔCt (RNeasy)				0.22	0.24	2.05
	ΔΔCt (Tri-RNeasy)				**-0.33**	**1.67**	**0.38**

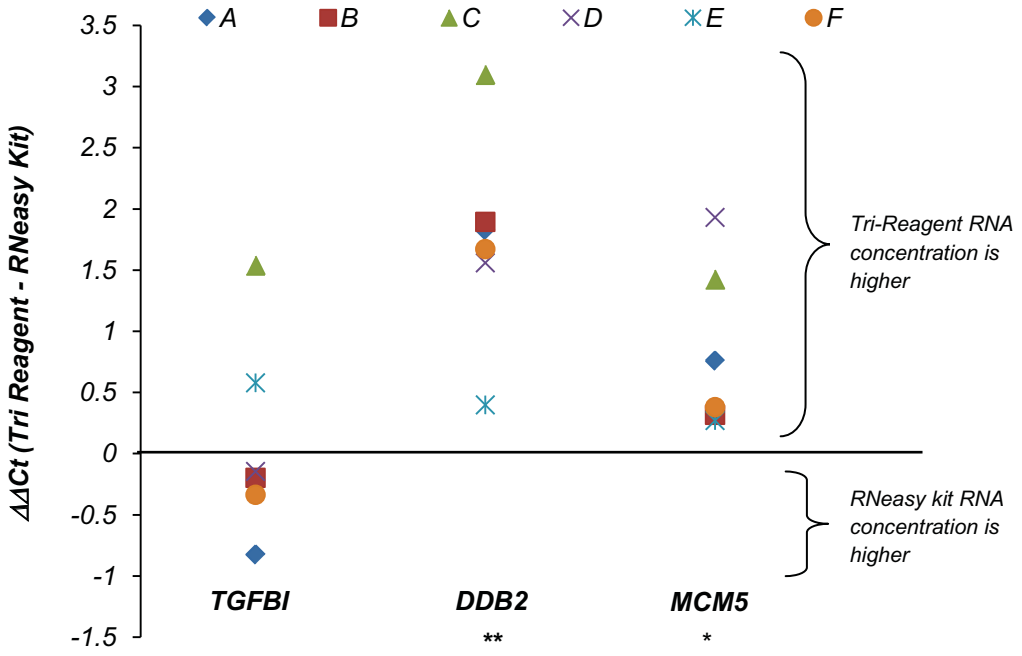


Figure 3.3 Comparing RNA Extraction Methods for Three Target Genes

Both Tri-Reagent and RNeasy kit method were used for extracting total RNA from 6 frozen breast cancer tissues (A, B, C, D, E and F). The ΔΔCt was obtained for each sample by first normalizing each one of the target genes (*TGFBI*, *DDB2* and *MCM5*) to the mean of the housekeeping genes (*GAPDH*, *HPRT1* and *TFRC*) and secondly by subtracting the ΔCt of Tri-Reagent method from ΔCt of RNeasy kit for each target gene. These results were checked for normality using Shapiro-Wilk test ($p>0.05$) and then one sample t-test was performed to confirm whether there is a significant difference between the two extraction methods (* $p<0.05$, ** $p<0.01$).

3.1.3 Loading Control for Western Blotting

Antibodies to several potential targets (β–Actin, α-Tubulin, β–Tubulin and Vinculin) were used to check the most suitable control for assessment of protein loading accuracy for each sample. A fixed amount of protein (20µg) was loaded for 6 breast cell lines and 3 normal breast organoids, and the signal intensity was compared for each of the protein loading controls (Figure 3.4).

All samples showed similar band intensities of the appropriate molecular weight (*42kDa*) for β –Actin (Figure 3.4) apart from HBL-100 (Figure 3.4, Lane 3), which could be due to unequal loading. Both cell lines and organoids showed bands at the expected size (*55kDa*) for α-Tubulin but extensive smearing was found and also 2 bands were observed for the organoids (Figure 3.4, Lane 7-9). There were three bands for β-Tubulin *(~50kDa*) for the breast cell lines but multiple strong bands for the organoid samples (Figure 3.4, Lane 7-9). Vinculin (*116kDa*) was also examined under the same conditions, and there were bands of the correct molecular weight for both cell

lines and organoids but there was considerably higher signal for organoids after short exposure (Figure 3.4).

The loading control of choice was therefore β-Actin due to similar intensity signals for both breast cell lines and organoids (Figure 3.4), while the other three proteins had multiple bands, with stronger and differential expression for the organoids.

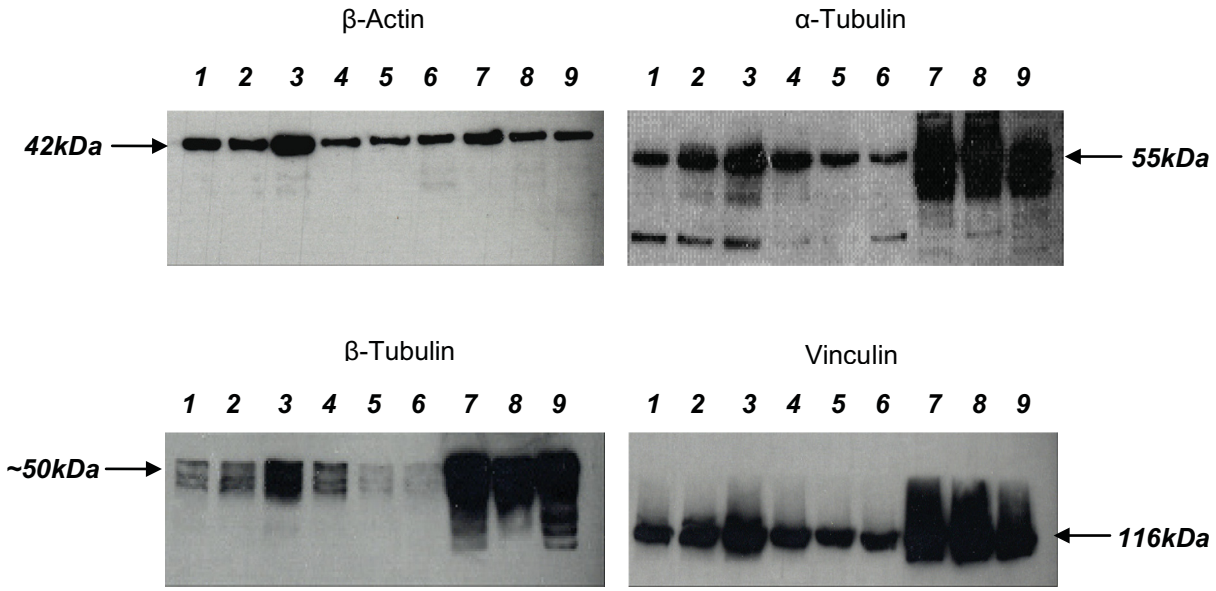


Figure 3.4 Comparisons of 4 Targets as Loading Controls

Lysates were obtained from 6 breast cancer cell lines (***1***: MCF-7, ***2***: MDA-MB-231, ***3***: HBL-100, ***4***: MDA-MB-468, ***5***: T-47D and ***6***: ZR-75-1) and 3 normal breast organoids (7-9), which were analyzed on a 12% SDS-PAGE gel. Various monoclonal antibodies against β–Actin , α -Tubulin, β-Tubulin and Vinculin were examined.

3.1.4 Optimization of Western Blotting Conditions

The Western Blotting method was optimized for each of the antibodies using different secondary antibody dilutions and timings. Additionally, various gel percentages and washing procedures were assessed. The optimization conditions examined are summarized in table 3.3.

Table 3.3 Optimization Conditions that Tested for Western Blotting

Variables	***TGFBI***	***DDB2***	***MCM5***
Gel Percentage	6%, 10%	10%,12%	10%
Primary Antibody Dilution	1:200	1:200	1:200
Washing Procedure	1% TBS-T	Method 2.2.2.8	1% TBS-T
Washing Time	3 times for 5 min	2 times for 5 min	3 times for 5 min
Secondary Antibody	Anti-Rabbit HRP	Anti-Goat HRP	Anti-Mouse HRP
Secondary Antibody Dilution	1:2000 (5%) Milk	1:2000 (5%) Milk 1:2000 (1%) 1:3000 (1%) 1:3000 (3%) TBS-T	1:2000 (5%) Milk
Secondary Antibody Incubation Time	30 min 45 min	30 min 45 min	45 min

Various percentages of SDS-PAGE gels were tested for TGFBI (6% and 10%) and DDB2 (10% and 12%). A 10% gel gave better protein separation and clearer band intensities. Longer secondary incubation time (45 minutes) was chosen for the TGFBI, due to clearer bands being obtained after detection. However, a shorter secondary incubation time (30 minutes) and lower secondary dilution (1:3000 in 3% TBS-T) was selected for DDB2 to minimize background problem. In addition, various washing procedures with different percentages of Tween-20 were investigated in order to decrease non-specific binding and thus reduce the background. The washing procedure that was selected for DDB2 is summarized in Table 2.9. The optimal conditions for western blotting for the three antibodies are summarized in Table 3.4.

Table 3.4 The Optimal Conditions Selected for Western Blotting

Variables	***TGFBI***	***DDB2***	***MCM5***
Gel Percentage	10%	10%	10%
Primary Antibody	Polyclonal Rabbit	Polyclonal Goat	Monoclonal Mouse
Primary Antibody Dilution	1:200	1:200	1:200
Washing Procedure	1% TBS-T	See Figure *3.4*	1% TBS-T
Washing Time	3 times for 5 min	2 times for 5 min	3 times for 5 min
Secondary Antibody	Anti-Rabbit HRP	Anti-Goat HRP	Anti-Rabbit HRP
Secondary Antibody Dilution	1:2000 (5%) Milk	1:3000 (3%) TBS-T	1:2000 (5%) Milk
Secondary Antibody Incubation Time	45 min	30 min	45 min

3.1.5 Optimization of Immunohistochemistry

Normal breast tissues were used for optimization of TGFBI and DDB2, while normal tonsil tissue was used for MCM5. Different dilutions of the primary antibody were selected on the basis of the manufactures data sheet. Antigen retrieval was achieved using pressure cooking with various temperatures, time and buffering conditions (pH). In order to increase the staining intensity, different secondary/tertiary kits were also tested (Table 3.5).

Table 3.5 Parameters Tested for Three Antibodies in Immunohistochemistry

Antibody	***Antigen Retrieval Temp/Time***	***Buffer***	***Dilution of Primary Antibody***	***Detection***
TGFBI	120, 122, 124^{O} 30sec	Citrate* TBE**	1:25 1:50 1:75 1:100	ABC/HRP Duet
DDB2	120, 123, 125^{O} 30, 45, 60 sec	Citrate TBE High pH***	1:20 1:30 1:40 1:50 1:60 1:70 1:75 1:80	ABC
MCM5	120, 123, 125^{O} 30, 45, 60sec	Citrate TBE High pH TE****	1:25 1:30 1:40 1:50 1:75 1:100	ABC Duet

* Citrate (pH=6) **TBE (pH=8.3) *** High pH (pH=9) **** TE (pH=7)

1- Transforming Growth Factor-Beta-Induced (TGFBI)

Various TGFBI antibody dilutions were tested on normal breast tissue to establish optimal staining. Strong nuclear staining was observed in the epithelial cells of both ducts and lobules of normal breast tissue at dilutions of 1:20 (A), 1:25 (B) and 1:50 (C), moderate staining was seen at 1:75 (D), and very weak staining was observed at 1:100 dilution (E) (Figure 3.5). Negative control showed no staining (Figure 3.5, F).

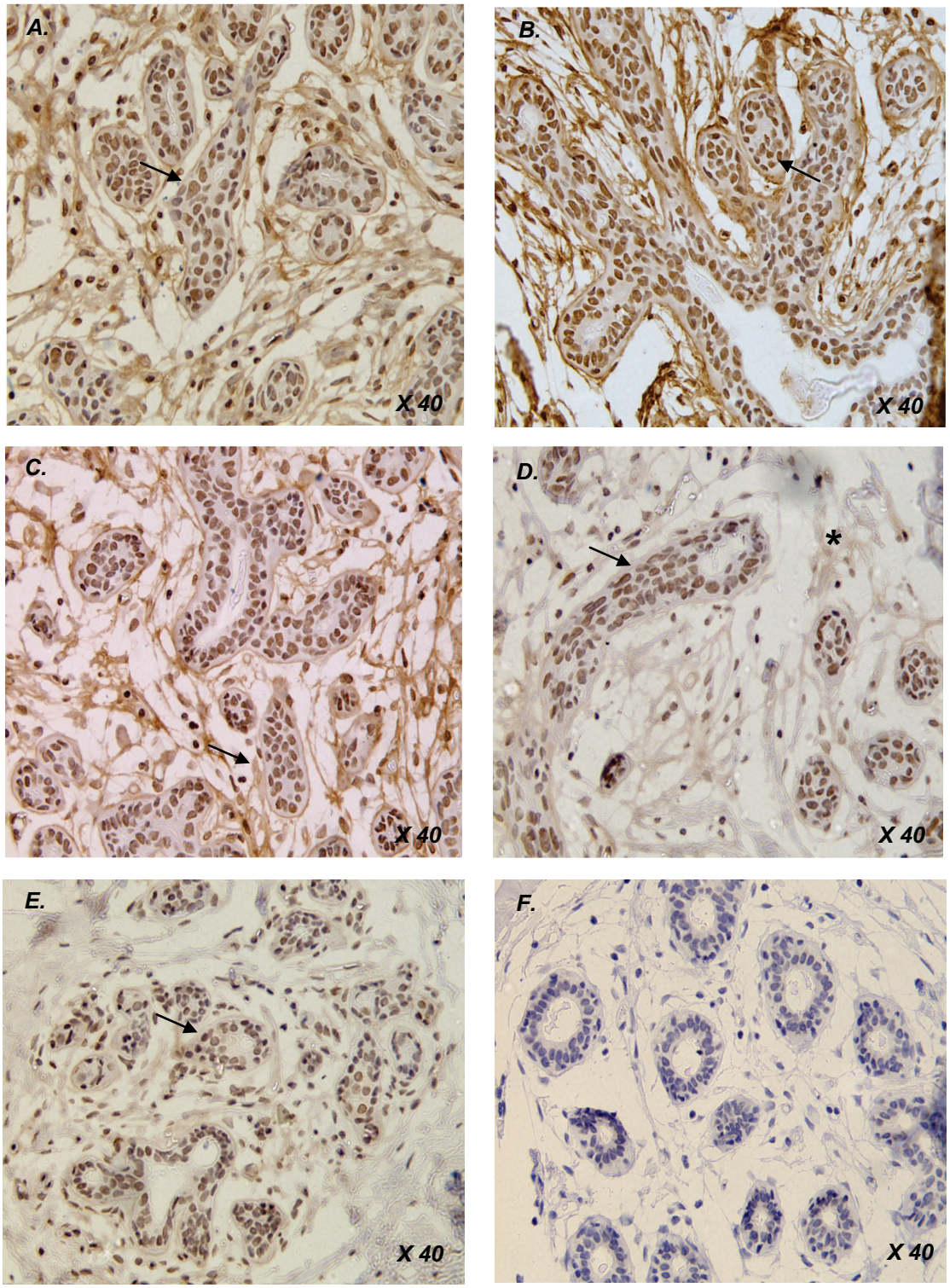


Figure 3.5 Example of TGFBI Optimization in Immunohistochemistry

Citrate buffer was used for antigen retrieval of normal breast tissue at pressure cooker at 120°C for 30sec. Tissues were incubated overnight with polyclonal rabbit TGFBI antibody at 1:20 (A), 1:25 (B), 1:50 (C), 1:75 (D), and 1:100 (E).Negative control, which is normal breast tissue was incubated with normal goat serum overnight (F). Images were captured using microscope at X40. (→ represents nuclear staining, (*) represents ECM staining.

2- DNA Damaged Binding-2 (DDB2)

Various antigen retrieval buffers citrate, TBE and high pH were used to optimize the DDB2 antibody. Strong nuclear staining of the normal epithelial cells was observed particularly when the tissue was treated with high pH buffer (Figure 3.6, C) compared to faint nuclear staining obtained when citrate (Figure 3.6, A) and TBE buffers (Figure 3.6, B) were used.

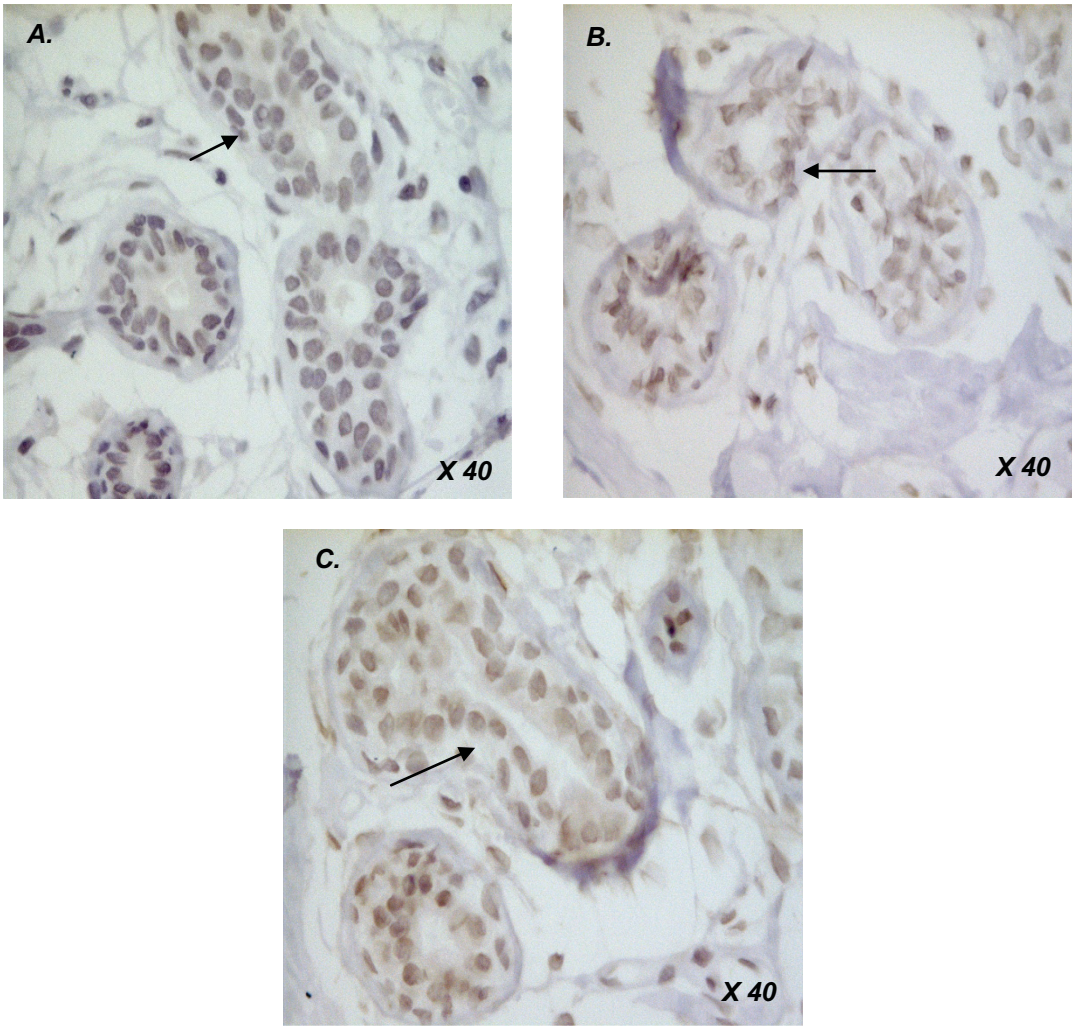


Figure 3.6 Example of DDB2 Optimization in Immunohistochemistry

Normal tissues were retrieved using citrate (A), TBE (B) and high pH (C) buffers with pressure cooker at 123°C for 30sec. Images were captured using microscope at X40. (→) represents nuclear staining.

3- Minichromosomal Maintenance-5 (MCM5)

Two different detection systems were used to optimize the MCM5 antibody on normal breast tissue sections. Faint nuclear staining was observed with both StreptABComplex/HRP Duet (Figure 3.7, A) and the StreptABComplex/HRP (Figure 3.7, B) kits. Tonsil tissue showed stronger nuclear staining particularly in the lymphocytes with pressure cooking at 125°C/45sec (Figure 3.7, C) compared to 125°C/60sec (Figure 3.7, D).

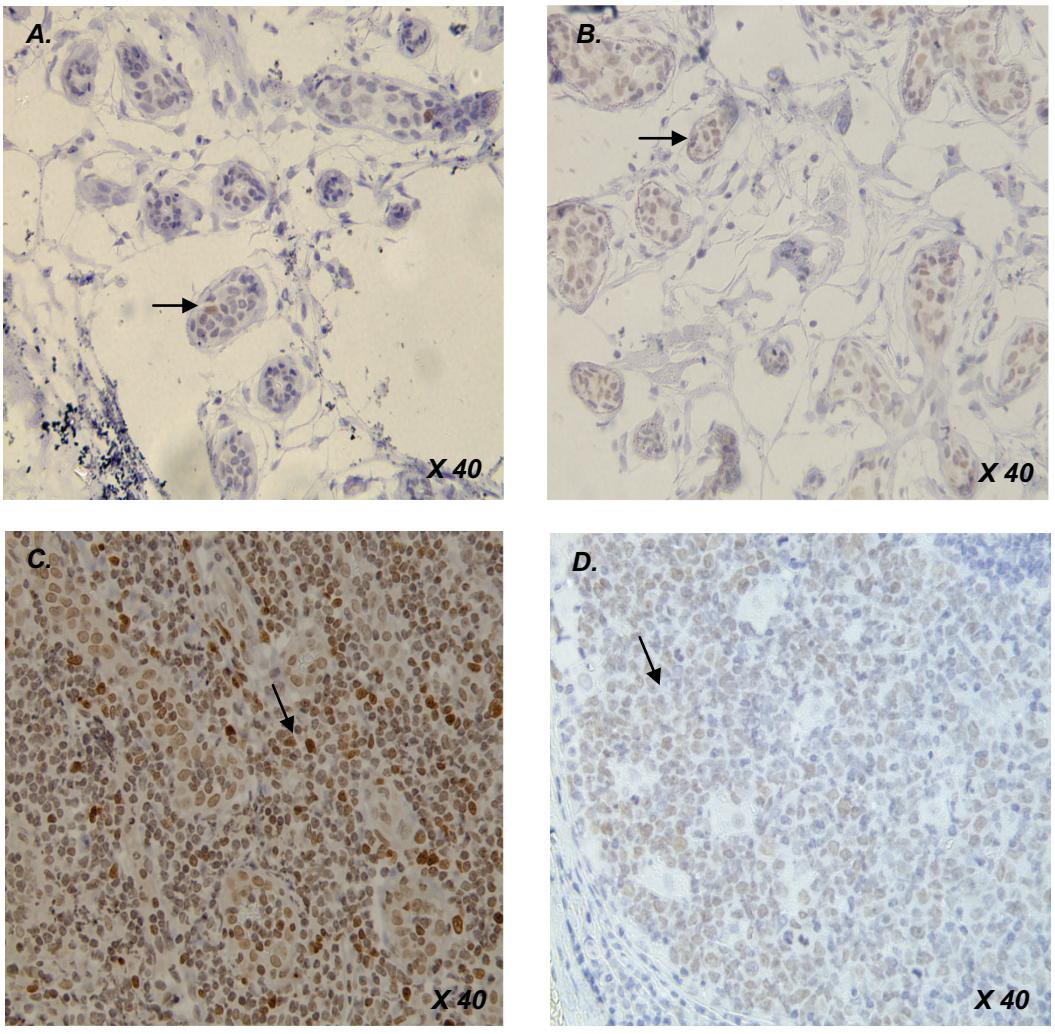


Figure 3.7 Example of MCM5 Optimization in Immunohistochemistry

Normal breast tissue (A and B) and tonsil tissue (C and D) using citrate buffer at pressure cooker of 125°C for 45sec (A, B, C) was examined, and tonsil tissue was tested at pressure cooker of 125°C for 60sec (D). Tissues were incubated overnight with polyclonal rabbit MCM5 antibody at 1:30 dilution. Images were captured using microscope at X40. (→) represents nuclear staining.

The final optimization conditions selected for the three antibodies are summarized in Table 3.6.

Table 3.6 Final Parameters Selected for Immunohistochemistry Using the 3 Antibodies

Variable	***TGFBI***	***DDB2***	***MCM5***
Pressure Cooker Temperature (°C)	*120*	*125*	*125*
Pressure Cooker Pressure (Psi)	*12*	*15*	*18.5*
Pressure Cooker Length of Time (Sec)	*30*	*30*	*45*
Antigen Retrieval Buffer	*Citrate**	*High pH***	*Citrate**
Dilution of Primary Antibody	*1:75*	*1:30*	*1:30*

* Citrate Buffer (pH= 6) **High pH (pH= 9)

3.2 Analysis of TGFBI, DDB2 and MCM5 Expression in Cell Lines

mRNA and protein expression of the three target genes (TGFBI, DDB2 and MCM5) were investigated in 6 breast cell lines (MCF-7, MDA-MB-231, HBL-100, MDA-MB-468, T-47D and ZR-75-1) using q-RT-PCR (Method *2.2.6*) and Western Blotting (Method *2.2.2*) respectively.

3.2.1 mRNA Analysis

GAPDH (~350bp) manual PCR was performed for 30 cycles in order to confirm RNA integrity in cell lines and also to ensure a lack of genomic DNA contamination (Figure 3.8). Similar intensity bands in the +RT reactions at 350bp (Figure 3.8, **1-6** (**+**)) confirmed that cDNA could be generated from 1µg of template. The –RT reactions were clear, indicating the absence of genomic DNA contamination (Figure 3.8, **1-6** (**-**)), and clear water blanks confirmed the lack of contamination in the PCR reaction (Figure 3.8 **W**).

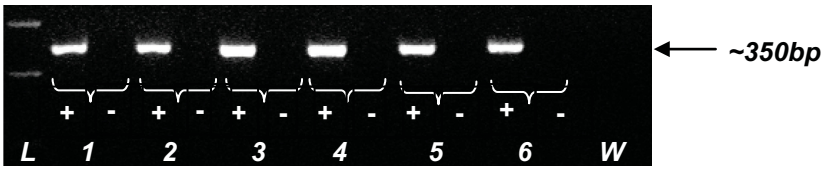


Figure 3.8 Confirmation of cDNA Generation for 6 Cell Lines Using GAPDH

cDNA for the 6 breast cell lines were tested using GAPDH manual PCR (***1***:MCF-7, ***2***: MDA-MB-231, ***3***:HBL-100, ***4***:MDA-MB-468, ***5***:T47-D and ***6***:ZR-75-1). (**+**) = +RT, (**-**) = -RT, (***L***) =100bp ladder and (***W***) = water blank.

Using q-RT-PCR, Ct triplicate repeats for the three targets and housekeeping genes were obtained for 6 breast cell lines. The values, means and standard deviations are shown in Table 3.7. For each individual cell line there was little variation in the values between the triplicates for each gene, confirmed by the low standard deviations obtained. Similar results were obtained for each housekeeping gene for each cell line for the three repeats, again with low standard deviations.

Table 3.7 Q-RT-PCR Data for the Six Breast Cell Lines

	MCF-7	MDA-MB-231	HBL-100	MDA-MB-468	T47-D	ZR-75-1
GAPDH						
Ct1	18.74	19.91	19.04	19.98	22.15	19.51
Ct2	18.8	19.85	19.50	19.89	22.11	19.60
Ct3	18.65	20.02	19.56	20.02	22.18	19.52
Mean	18.73	19.92	19.36	19.96	22.14	19.54
Std	0.075	0.086	0.284	0.066	0.035	0.049
HPRT1						
Ct1	23.17	22.88	22.03	23.42	25.43	23.34
Ct2	23.26	23.00	22.17	23.43	25.38	23.31
Ct3	23.18	22.99	22.10	23.64	25.35	23.33
Mean	23.20	22.95	22.1	23.49	25.38	23.32
Std	0.049	0.066	0.070	0.124	0.040	0.015
TFRC						
Ct1	25.38	23.54	23.55	23.17	20.71	22.08
Ct2	25.47	23.59	23.97	23.44	21.25	22.17
Ct3	25.33	23.57	23.91	23.69	21.10	22.14
Mean	25.39	23.56	23.81	23.43	21.02	22.13
Std	0.070	0.025	0.227	0.260	0.278	0.045
TGFBI						
Ct1	26.36	24.38	21.63	29.59	30.70	31.15
Ct2	27.05	24.32	21.56	30.35	30.68	30.32
Ct3	26.70	24.33	21.66	30.10	30.43	30.43
Mean	26.70	24.34	21.61	30.01	30.60	30.63
Std	0.345	0.032	0.051	0.387	0.105	0.450
DDB2						
Ct1	22.75	24.70	24.24	24.9	23.89	21.75
Ct2	22.67	24.73	24.24	24.97	23.50	21.36
Ct3	22.69	24.66	24.23	24.85	23.47	21.40
Mean	22.70	24.69	24.23	24.90	23.62	21.50
Std	0.041	0.035	0.005	0.060	0.234	0.214
MCM5						
Ct1	21.83	22.74	22.67	23.83	25.25	22.40
Ct2	22.30	22.77	22.77	24.40	25.20	23.16
Ct3	22.17	22.78	22.80	24.67	25.28	22.76
Mean	22.10	22.76	22.74	24.30	25.24	22.77
Std	0.242	0.020	0.068	0.428	0.040	0.380

The q-RT-PCR results for the breast cell lines were then analysed relative to the expression by the immortalised normal cell line, HBL-100 (Figure 3.9).

All the breast cancer cell lines showed that expression of TGFBI mRNA was lower than that of the HBL-100 cell line. The lowest TGFBI mRNA levels were observed in MDA-MB-468, T-47D and ZR-75-1 cell lines. There was a wide variation between the three results for MCF-7 (SD = 0.345) (Figure 3.9, A).

For DDB2, MCF-7, T47-D and ZR-75-1 cell lines showed higher mRNA levels relative to HBL-100, while MDA-MB-231 and MDA-MB-468 cells had similar levels to HBL-100 (Figure 3.9, B). The levels of DDB2 mRNA levels were higher in all of ER/PR positive cell lines.

MCF-7 cells had higher levels of MCM5 mRNA relative to HBL-100. MDA-MB-231 levels were slightly higher; MDA-MB-468 and T47-D levels lower than HBL-100. ZR-75-1 showed variation between triplicates, making determination of expression level difficult (Figure 3.9, C).

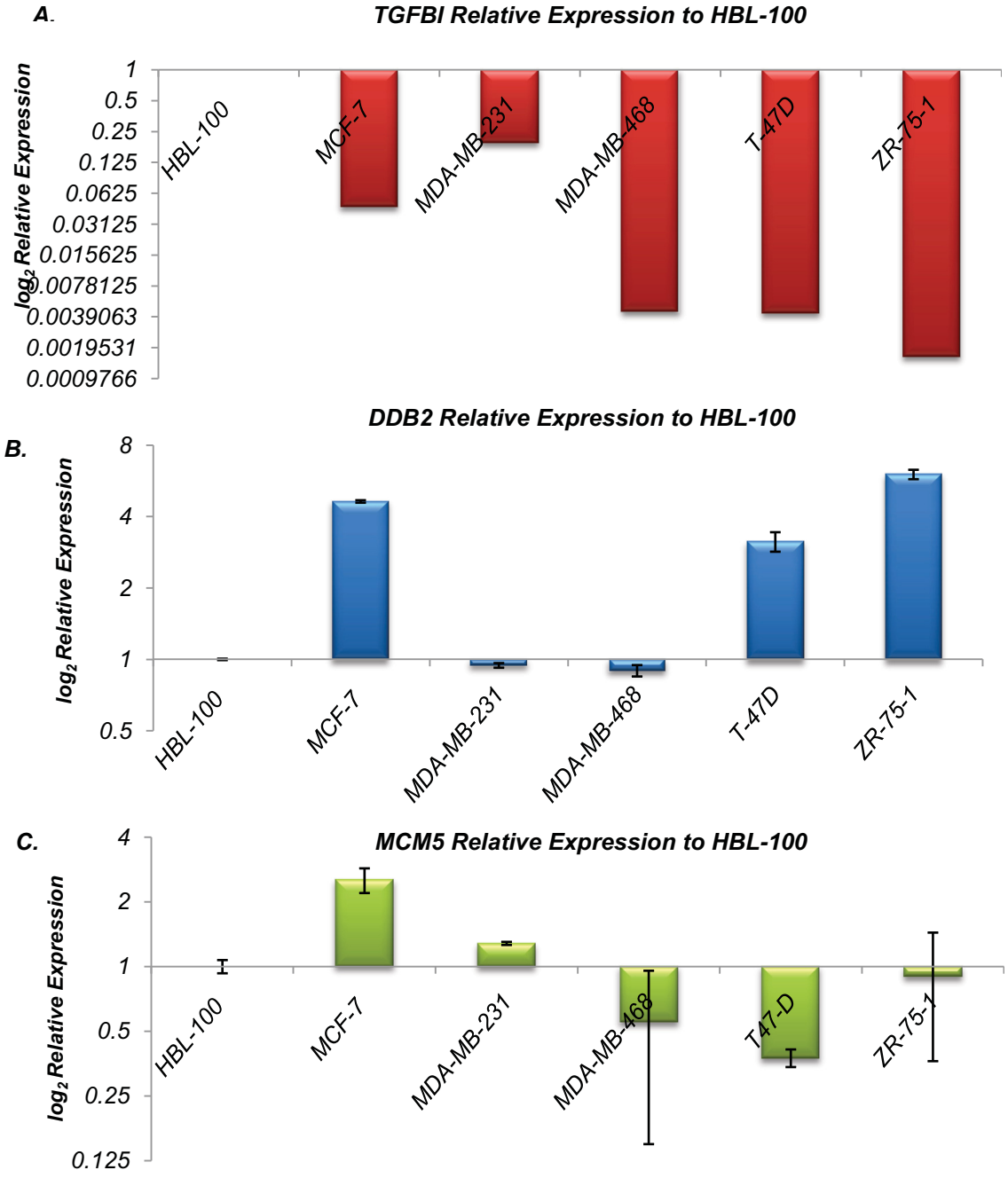


Figure 3.9 Relative Expression of TGFBI, DDB2 and MCM5 mRNA in 5 Breast Cell Lines

The TGFBI (A), DDB2 (B) and MCM5 (C) mRNA was calculated for 5 breast cell lines as a relative expression of HBL-100. The bars represent the standard deviation across the triplicate repeats. TGFBI bars could not be presented in the graph as negative or zero values cannot be plotted correctly on log charts; therefore, their standard deviation is shown in Table 3.7.

3.2.2 Western Blot Analysis of Cell Lines

Blots show representative examples of 3 repeats for each protein. TGFBI protein expression was investigated in 6 breast cell lines and 3 normal breast organoids, which demonstrated the presence of various TGFBI isoforms. Each Western Blot showed a lack of TGFBI of higher molecular weight (68kDa) form in both breast cancer cell lines and organoids. Three bands of different molecular weights (*55.7kDa, 51.8kDa* and *45.5kDa*) were observed in the organoids only, particularly for lanes 8 and 9 (Figure 3.10, A). These isoform sizes match with the human splice variants published (Thierry-Mieg, 2006). No bands were detected for the breast cancer cell lines.

DDB2 protein (*48kDa*) was only expressed in organoids and was absent in breast cell lines (Figure 3.10, B). However, a weaker band was seen in all breast cell lines at ~*51kDa* which was not expressed by organoids.

MCM5 (*82kDa*) was expressed in all breast cell lines at varying levels. Minimal expression of MCM5 was seen in one of the organoids (Figure 10, C, Lane: 7), but was absent in the other two (Figure 10, C, Lane: 8 and 9). However, these two samples showed a band of a lower molecular weight (~*70kDa*).

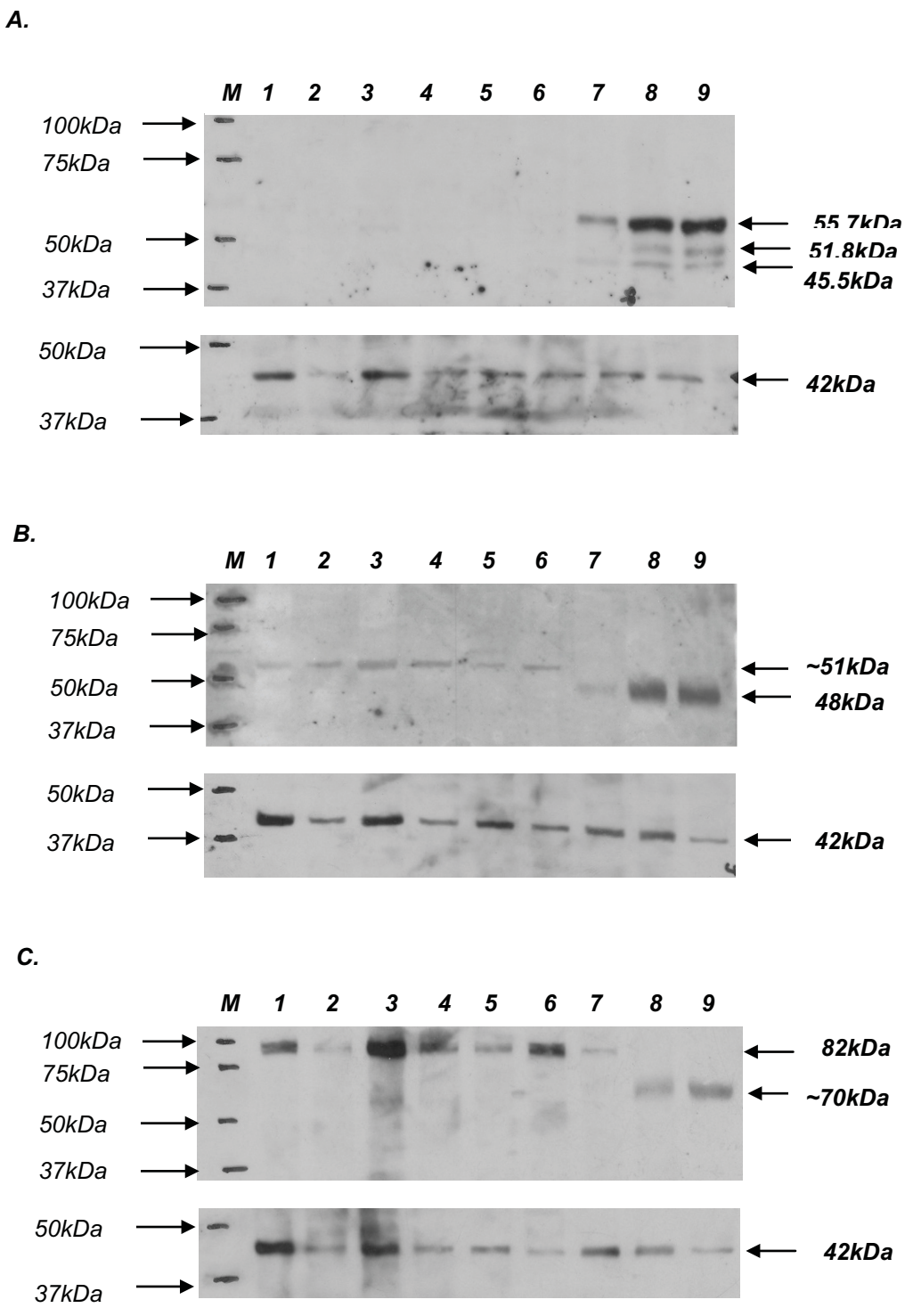


Figure 3.10 *Western Blotting Analysis of TGFBI, DDB2 and MCM5 Protein Expression*

The protein level of the three genes, TGFBI (A), DDB2 (B) and MCM5 (C) was investigated in the 6 breast cell lines (1: MCF-7, 2: MDA-MB-231, 3: HBL-100, 4: MDA-MB-468, 5: T-47D and 6: ZR-75-1) and 3 normal breast organoids (Lane 7-9). Lower panel (42kDa) is the β-Actin loading control, which was assessed for all proteins. (M)= 10µl BioRad Precision Plus Dual (molecular weight marker).

3.2.3 Summary of Cell Lines mRNA and Protein Results

The q-RT-PCR results showed that TGFBI mRNA is down-regulated in all cell lines examined relative to HBL-100, which was also confirmed by western blotting where TGFBI protein (68kDa) was absent from all the breast cell lines. HBL-100 expressed mRNA but showed no band at the 68kDa. The various molecular weights forms expressed by the organoids could be the result of alternative splicing of the *TGFBI* gene (Table 1.10).

Higher DDB2 mRNA expression was observed in ER/PR positive cell lines (MCF-7, T47-D and ZR-75-1), while lower expression was found in ER/PR negative cell lines (HBL-100, MDA-MB-231 and MDA-MB-468). However, western blotting showed the same band intensity at molecular weight of ~51kDa for all the cell lines examined. This molecular weight is different to the reported size (48kDa), which was present in the normal organoids. This size differences might relate to alternative splicing of the *DDB2* gene, which was been shown to produce four DDB2 protein isoforms (Table 1.14).

MCF-7 and MDA-MB-231 cell lines showed the highest MCM5 mRNA expression, while T47-D and MDA-MB-468 showed lower levels relative to HBL-100. The ZR-75-1 cell line had almost similar MCM5 mRNA expression to HBL-100 but wide variation between triplicates made determination of expression level difficult. Western blotting results were consistent with the mRNA data for ZR-75-1, T47-D and MDA-MB-468 but small differences were seen in the expression of the protein in MCF-7 and MDA-MB-231, which were expected to show stronger bands than HBL-100, but showed similar band intensity.

3.3 Analysis of TGFBI, DDB2 and MCM5 Expression in Breast Tissues

Expression of TGFBI, DDB2 and MCM5 was investigated in 8 normal breast organoids, 11 normal/benign breast tissues (only 9 analysed) and 43 breast cancers (only 36 analysed) using q-RT-PCR. In addition, TGFBI protein was assessed in 4 normal breast samples and 6 breast cancers using western blotting. These cases were selected from the q-RT-PCR analysed samples.

The frozen normal/benign tissues (Table 3.8) were stained with Haematoxylin (H&E) and Eosin before RNA analysis to check the nature and cellularity of the tissue (Examples shown in Figure 3.11).

Table 3.8 Assessment of Normal/Benign Breast Tissues

Number of Tissue	***Histopathological Features***
A1	Normal breast
B1	Benign changes and normal breast
C1	Normal breast
D1	Normal breast
E1	Benign changes
F1	Benign changes
G1	Benign changes and normal breast
H1	Normal breast
I1	Benign changes and normal breast
J1	Normal breast
K1	Normal breast

Gray samples not included in the q-RT-PCR analysis

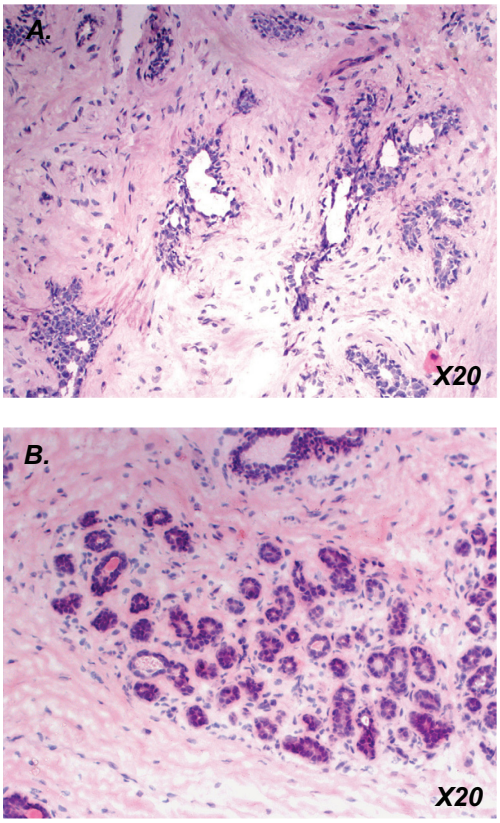


Figure 3.11 H&E Staining for Normal/Benign Tissues

Mild cystic change (A), and normal breast tissue (B). Images were taken at X20.

All frozen samples of breast cancers were also assessed prior to RNA extraction. For each sample the extent of the sample containing invasive cancer was determined (Table 3.9). Only those with 50% or more of the tissue containing invasive cancer were analysed by q-RT-PCR; 4 of these had a predominance of *In-Situ* carcinomas (Figure 3.12).

Table 3.9 Percentage of Invasive Areas in Breast Cancer Tissues

Number of Case	***Invasive (%)***
1 *	60
2	70
3	80
4	90
5 *	70
6	70
7 *	50
8	70
10	80
12	75
13	60
14	75
15	40
17	75
18	80
19	50
21 **	5
23	80
24	90
25	80
27	70
28	70
31 *	40
32	70
33	70
34	80
36	60
37	70
39 *	70
41	60
44	60
46	25
47	50
49	80
50	60
57	Little
58	30
59	70
60	60
62	80
63	80
65	30
67	70

Gray case not included in the q-RT-PCR analysis
*In-Situ area is present

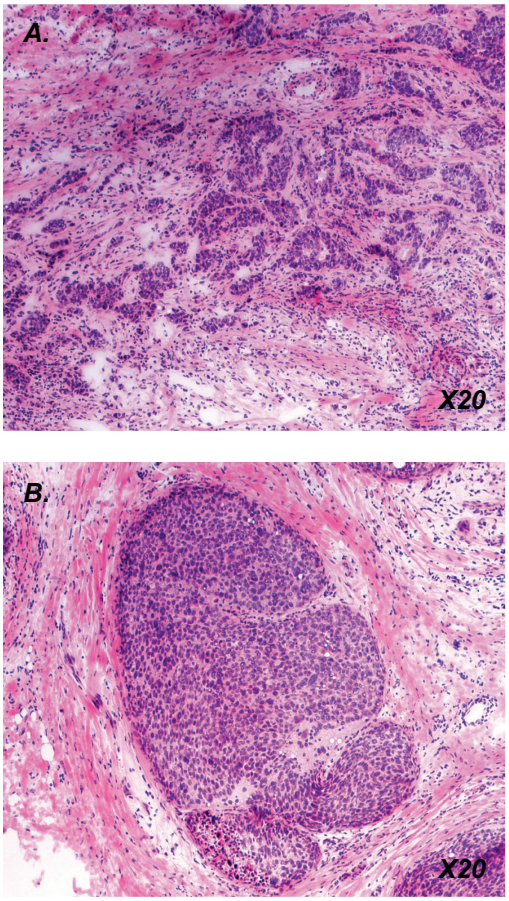


Figure 3.12 H&E Staining for Breast Cancers

Invasive breast cancer tissue (A) and invasive breast cancer with an *In-Situ* area (B) stained with H&E. The images were captured at X20.

3.3.1 mRNA Analysis

RNA was successfully isolated from all tissues and organoids as confirmed by manual GAPDH PCR. Strong bands were observed in organoids (100bp) (Figure 3.13, A1) and breast cancers (~350bp) apart from case number 7, 13 and 47 (Figure 3.13, B), confirming good cDNA quality. Eleven normal/benign tissues were investigated; the majority showed fainter bands (~350bp) particularly H1 and J1 (Figure 3.13, A2) due to low cellularity. Although the Tri-Reagent method was used for extracting RNA from benign and normal samples to increase the amounts of mRNA yield, less efficient cDNA was obtained compared to breast cancers and organoids. Therefore, these H1 and J1 were excluded from the further analysis. Absence of DNA contamination in –RT reactions and water blanks were confirmed (PCR=30 Cycles).

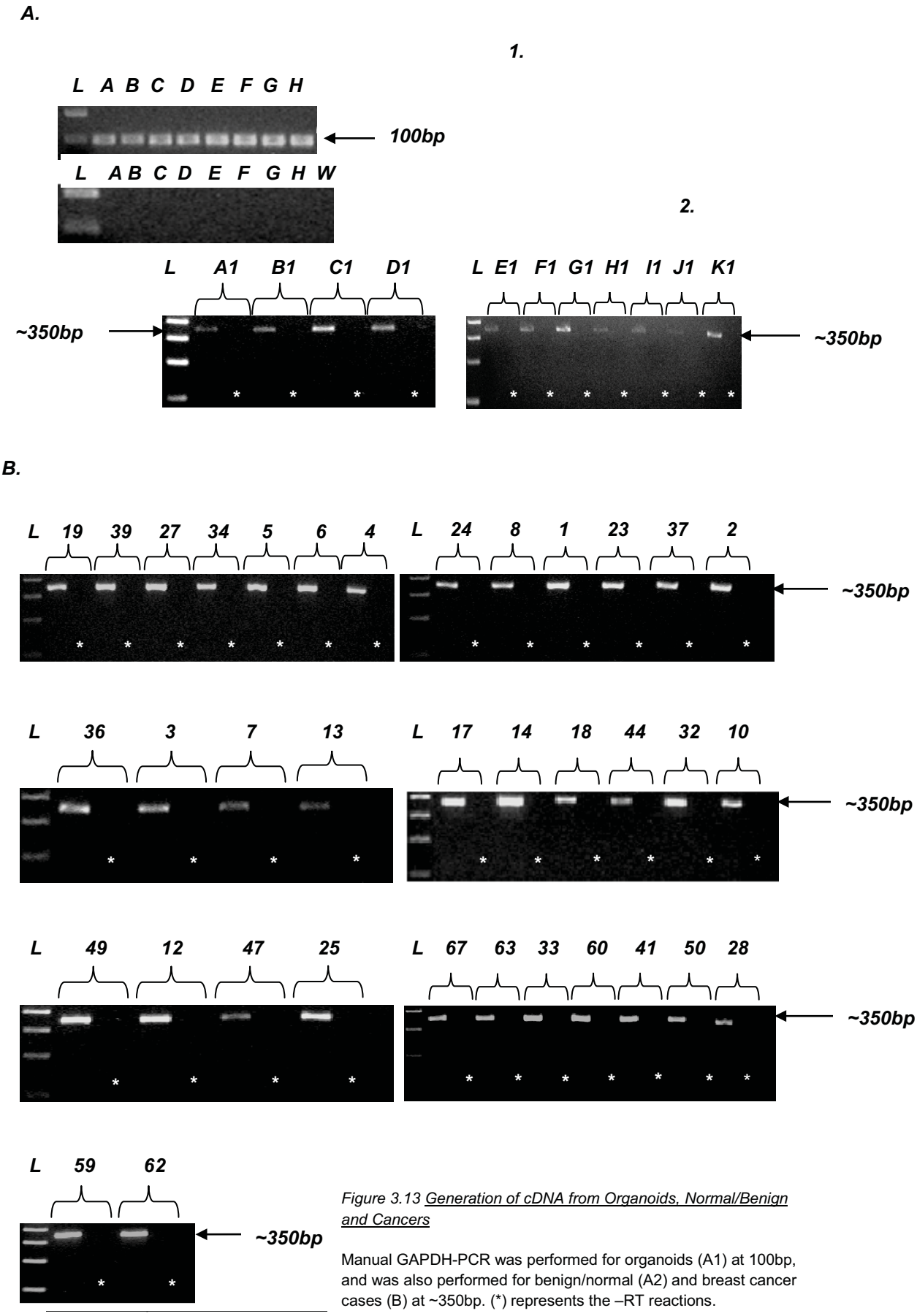


Figure 3.13 Generation of cDNA from Organoids, Normal/Benign and Cancers

Manual GAPDH-PCR was performed for organoids (A1) at 100bp, and was also performed for benign/normal (A2) and breast cancer cases (B) at ~350bp. (*) represents the –RT reactions.

The mean of q-RT-PCR triplicate readings for each of the breast cancers, normal/benign and organoids for three targets (*TGFBI, DDB2* and *MCM5*) and housekeeping (*GAPDH, HPRT1* and *TFRC*) genes are shown in Table 3.10.

For several samples Ct values were high for the endogenous controls, particularly TFRC assay, which might indicates either low concentration of RNA or poorer quality. However, no Ct values went beyond 35 cycles (limit for most q-RT-PCR analysis) except for few samples that showed high Ct value for TFRC assay only which were 3 breast cancers (5*, 7*, 10), 5 organoids (A, C, E, F and H) and 2 normal/benign tissues (B1 and D1). Only one breast cancer sample (44) showed Ct values higher than 35 for the three housekeeping genes. Thus, the q-RT-PCR results for all the samples were still included in the data analysis.

Table 3.10 Mean of Ct values for 6 Genes Analyzed in Cancers, Normal/Benign and Organoids

	GAPDH	*HPRT1*	*TFRC*	*TGFBI*	*DDB2*	*MCM5*
Breast Cancers						
1*	22.25	28.73	33.98	31.36	32.04	30.17
2	20.92	26.90	29.14	28.23	29.72	28.26
3	21.01	28.86	32.32	28.88	30.36	30.96
4	24.66	28.20	32.09	29.51	29.50	29.50
5*	26.20	31.67	35.83	31.33	33.77	31.30
6	20.42	26.08	29.87	25.54	29.95	27.40
7*	26.65	29.38	35.62	30.91	30.61	31.61
8	24.97	27.98	31.58	28.64	29.02	29.93
10	28.65	31.61	35.04	31.95	34.48	33.33
12	24.97	29.11	32.02	29.14	29.90	29.86
13	22.74	27.91	29.74	27.75	29.62	27.94
14	24.45	30.12	32.11	26.92	29.90	29.71
17	25.73	28.63	30.85	29.58	30.46	29.82
18	22.06	26.45	30.06	26.69	26.82	25.38
19	23.90	27.85	30.06	27.50	27.52	29.32
23	22.22	29.00	33.02	30.99	32.42	30.91
24	25.81	28.41	31.82	29.76	30.92	30.71
25	23.44	27.90	29.31	29.10	28.93	28.97
27	24.72	29.93	32.54	29.19	30.31	30.92
28	25.33	29.38	33.89	31.51	30.67	30.98
32	25.16	31.24	32.19	31.05	31.40	31.01
33	21.05	27.06	28.32	25.18	29.13	28.65
34	20.96	26.49	28.50	25.12	25.81	26.50
36	26.16	31.03	36.07	29.61	34.74	33.09
37	24.48	30.64	29.81	28.20	32.32	29.78
39*	22.40	27.52	29.64	27.50	27.69	27.12
41	21.71	27.01	28.76	26.34	28.87	29.00
44	30.52	35.18	38.79	35.95	35.09	36.69

47	27.98	31.73	34.83	27.67	33.07	32.55
49	20.94	26.43	28.17	27.41	28.09	27.16
50	24.68	30.30	34.15	29.08	30.65	30.90
59	28.39	32.75	35.40	32.29	34.49	33.98
60	24.52	28.86	32.08	29.18	29.32	30.92
62	26.84	28.60	33.30	28.89	30.61	30.93
63	25.14	28.88	32.85	29.67	30.17	30.62
67	25.33	29.38	33.89	31.51	30.67	30.98
Organoids						
A	28.72	32.30	36.12	30.68	32.06	31.54
B	28.22	27.54	33.63	30.64	30.25	30.69
C	32.97	34.96	35.24	34.92	36.07	36.05
D	26.78	27.08	28.86	29.28	29.65	30.39
E	29.34	30.44	36.73	34.55	34.71	32.32
F	28.95	31.33	35.80	34.02	33.11	31.74
G	26.78	27.22	31.65	30.75	32.41	30.60
H	31.31	33.78	39.02	36.88	35.73	35.54
Normal/Benign						
A1	29.86	32.16	33.21	32.60	33.45	33.87
B1	31.79	34.72	35.39	34.24	35.30	34.19
C1	28.75	30.23	34.22	32.24	32.29	33.26
D1	31.48	35.56	36.29	33.82	35.73	35.17
E1	25.04	30.25	31.88	27.59	31.21	30.64
F1	23.52	27.54	30.83	25.78	28.98	29.34
G1	25.29	30.29	32.03	27.68	30.91	30.73
I1	24.16	29.03	31.84	27.75	30.36	29.99
K1	23.55	26.90	30.31	26.16	28.68	28.77

(*) = In-Situ area is present

The majority of the breast cancers showed lower levels of TGFBI mRNA relative to the mean ΔCt of normal/benign tissues apart from 6 tumours, which 4 of them showed TGFBI over-expression (47, 14, 36 and 39*) while the other two (62 and 50) showed the same relative expression to the mean ΔCt of normal/benign tissues. The organoids revealed down-regulation expression of TGFBI mRNA relative to the normal/benign tissues except for organoid “A”, which showed over-expression of TGFBI (Figure 3.14, A).

Approximately equal number of breast cancers showed over (17 cases) and under (18 cases) expression of DDB2 mRNA relative to the mean ΔCt of normal/benign samples investigated. Only one breast tumour showed similar DDB2 mRNA expression with normal/benign tissues. Four of the organoids showed over-expression, 3 detected

under-expression and only one case revealed similar expression to normal/benign tissues (Figure 3.14, B).

Thirteen out of the 36 investigated breast cancers showed higher MCM5 mRNA relative to the mean ΔCt of normal/benign samples. Eighteen tumours showed low and five detected similar MCM5 mRNA levels relative to normal/benign tissues. Five of the organoids showed over and 3 under expression of MCM5 mRNA levels relative to normal/benign tissues (Figure 3.14, C).

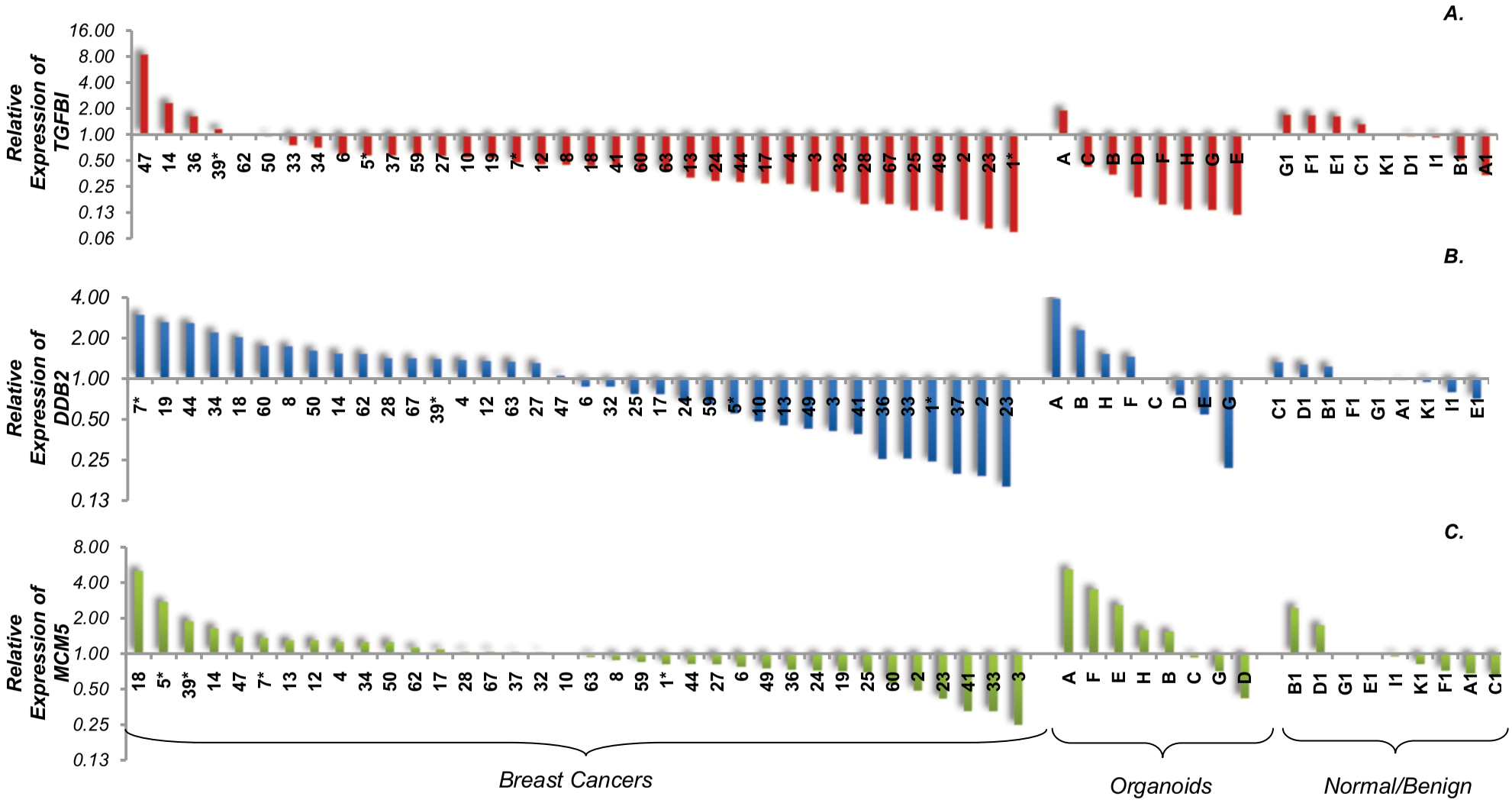


Figure 3.14 Relative Expressions of Breast Cancers and Organoids to Normal/Benign Samples for Three Target Genes

Results for breast cancers, organoid samples and normal/benign tissues are presented as ΔΔCt , which was calculated from sample ΔCt relative to mean ΔCt of the normal/benign tissue samples examined. This was carried out for the three target genes: TGFBI (A), DDB2 (B) and MCM5 (C).

Q-RT-PCR -ΔCt data for TGFBI, DDB2 and MCM5 are presented as box and whisker plots for the three groups: 9 normal/benign tissues, 36 breast cancers (invasive area≥ 50%) and 8 organoids. A kruskal-Wallis test was selected for analysis because -ΔCt values for the three groups showed no normal distributed (Shapiro Wilk test, $p<0.05$).

A kruskal-Wallis test (One-Way ANOVA, analysis of variance) showed a statistical significant difference for TGFBI between the three different groups (Figure 3.15, A). The median of TGFBI -ΔCt of the normal/benign tissues was significantly higher compared to both breast cancers and organoids samples using Bonferroni and post-hoc correction (kruskal-Wallis test, $p<0.05$) (Table 3.11). Although higher median -ΔCt was observed for breast cancers compared to organoids no significant difference was found between the two groups (kruskal-Wallis test, $p>0.05$) (Figure 3.15, A).

Both DDB2 and MCM5 showed no significant difference in the three median -ΔCt values (Table 3.11) between the three different groups (Figure 3.15, B and C).

Table 3.11 Data of -ΔCt of Whisker Plots for the Three Target Genes

	TGFBI			***DDB2***			***MCM5***		
Tissue	***N/B***	***C***	***O***	***N/B***	***C***	***O***	***N/B***	***C***	***O***
Median	*0.62*	*-0.48*	*-1.85*	*-1.70*	*-1.86*	*-1.38*	*-1.64*	*-1.70*	*-0.86*
Minimum	*-1.17*	*-3.04*	*-2.38*	*-2.15*	*-4.33*	*-3.86*	*-2.19*	*-3.56*	*-2.81*
Maximum	*1.53*	*3.84*	*1.69*	*-1.22*	*-0.061*	*0.33*	*-0.22*	*0.81*	*0.83*
Range	*2.70*	*6.88*	*4.08*	*0.93*	*4.27*	*4.19*	*1.96*	*4.37*	*3.65*

NB= Normal/Benign Tissues C= Breast Cancers O= Orangoids Samples

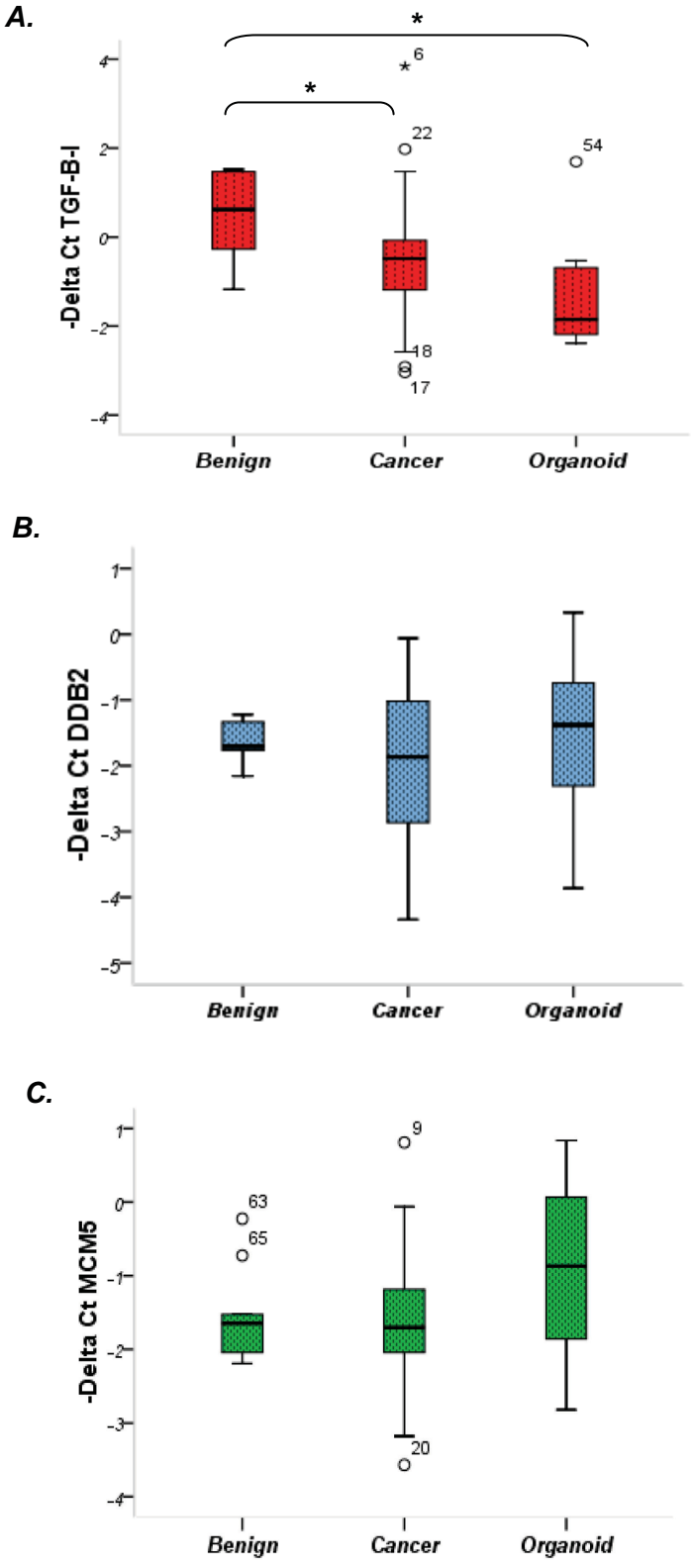


Figure 3.15 Box and Whisker Plots for the Three Target Genes

The -ΔCt of TGFBI (A), DDB2 (B) and MCM5 (C) for the three groups including benign tissue, cancer cancers and organoids are presented in box and whisker plots. (*) = $p<0.05$ of the Kruskal Wallis test with Bonferroni and post-Hoc tests. (o) = Outlier samples that present outside the range of the Whisker Plot.

The -ΔCts for all breast cancers were correlated with the clinicopathological variables including tumour size, tumour grade, lymph node metastasis, ER and PR status and HER-2 for the three target genes (Table 3.12).

The -ΔCt results for three genes were correlated with tumour size and grade (Table 3.12). TGFBI, DDB2 and MCM5 showed no differences in the -ΔCt between the two different sizes of the tumour (≤20mm and >20mm).

Both TGFBI and MCM5 showed no differences in the -ΔCt between grade III and grade II; however, there was a significant correlation between the -ΔCt of DDB2 and the grade of the tumour (Mann Whitney, $p<0.05$) (Table 3.12). Higher DDB2 mRNA levels were found in grade II tumours compared to grade III tumours (Figure 3.16, A).

Q-RT-PCR results for the three target genes for breast cancers were correlated to the lymph node status, ER, PR status and HER-2 (Table 3.12). TGFBI, DDB2 and MCM5 results showed no significant correlation between -ΔCt values and lymph node status. However, there was a significant correlation between the -ΔCt for DDB2 of the breast cancers and their ER status (Table 3.12), with higher levels of DDB2 in the ER-positive cancers compared to the ER-negative tumours using Mann-Whitney test ($p=0.04$) (Figure 3.16, B).

The three genes showed no relationship between their mRNA levels and both PR and HER-2 status (Mann-Whitney test, $p>0.05$).

Table 3.12 Correlation of Clinicopathological Features and -ΔCts of Breast Cancers for the 3 Genes

Clinicopathological Features		***Number of Cases***	***TGFBI***			***DDB2***			***MCM5***		
			M	***IR***	***p***	***M***	***IR***	***p***	***M***	***IR***	***p***
Size	≤20mm	12	-0.13	1.46	0.25	-2.09	2.83	0.34	-1.85	0.79	0.11
	>20mm	24	-0.84	1.25		-1.39	1.62		-1.45	0.87	
Grade	II	13	-0.51	1.31	0.52	-1.14	1.22	* ***0.02***	-1.66	0.76	0.87
	III	23	-0.50	1.44		-2.05	1.77		-1.57	0.87	
Lymph Node Metastasis	POS	19	-0.47	1.29	0.59	-1.87	1.65	0.40	-1.48	0.70	0.52
	NEG	17	-0.51	1.88		-1.18	2.25		-1.85	0.86	
ER	POS	25	-0.69	1.38	0.40	-1.20	1.68	* ***0.04***	-1.66	0.81	0.81
	NEG	10	-0.14	1.61		-2.63	2.37		-1.76	2.04	
PR	POS	26	-0.60	1.38	0.57	-1.21	1.69	0.05	-1.57	0.81	0.40
	NEG	9	-0.18	2.03		-2.72	2.60		-1.95	1.58	
HER-2	POS	3	-1.19	------	0.07	-1.18	------	0.51	-1.18	-----	0.52
	NEG	15	-0.23	0.88		-1.56	1.28		-1.66	0.88	

M = Median IR = Interquartile Range *p* = Mann-Whitney test *p* value (**p*<0.05)
ER=Oestrogen Receptor PR=Progesterone Receptor HER-2= Epidermal Growth Factor Receptor-2
NEG= Negative POS=Positive

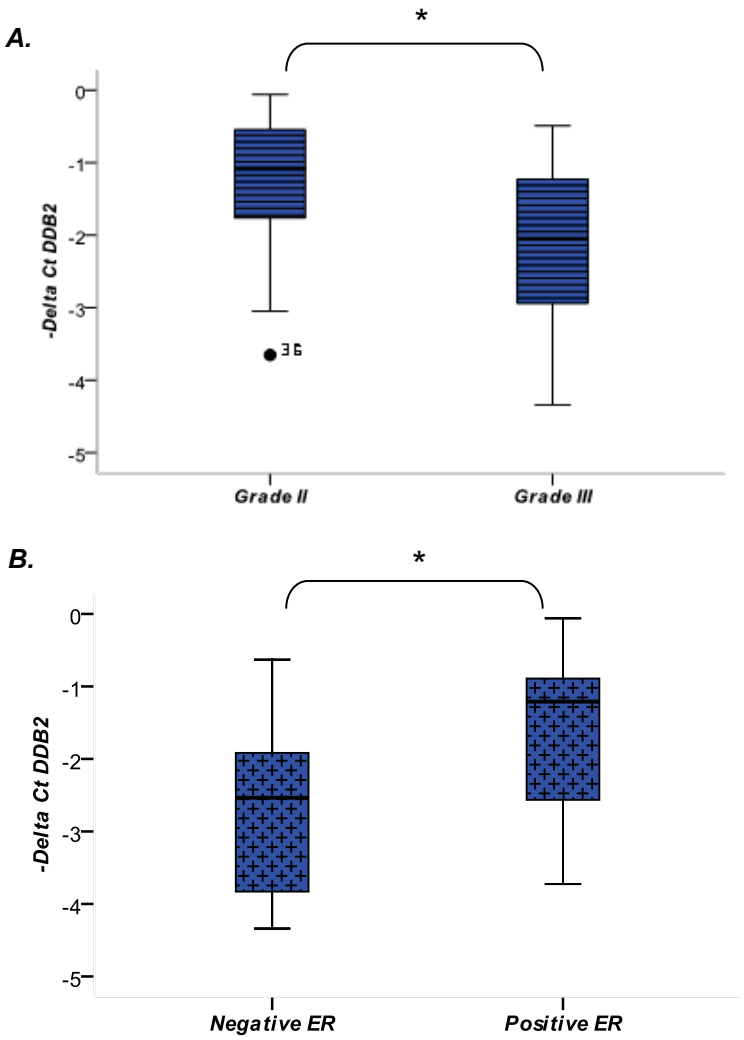


Figure 3.16 Summary of DDB2 -ΔCt Data Correlated with Tumour Grade and ER status

Summary of the DDB2 -ΔCt for all the breast cancers in relation to tumour grade (A) and ER status (B). (*) = Significant difference (p<0.05) using Mann Whitney test. (●) = outlier breast cancer case that are outside the whisker plot range.

The -ΔCt values for TGFBI, DDB2 and MCM5 were correlated with patient age (Figure 3.17). The Pearson correlation test was selected for analysis because the age variable was shown to be normally distributed (Shapiro-Wilk test, p>0.05).

There was significant correlation between both TGFBI and DDB2 mRNA expression levels and patient age (Pearson correlation test, one tailed p=0.04 (TGFBI) and p=0.025 (DDB2)), with higher mRNA levels of the two genes being associated with older patients (Figure 3.17). However, MCM5 mRNA levels were not associated with patient age (Pearson correlation test, one tailed p=0.18).

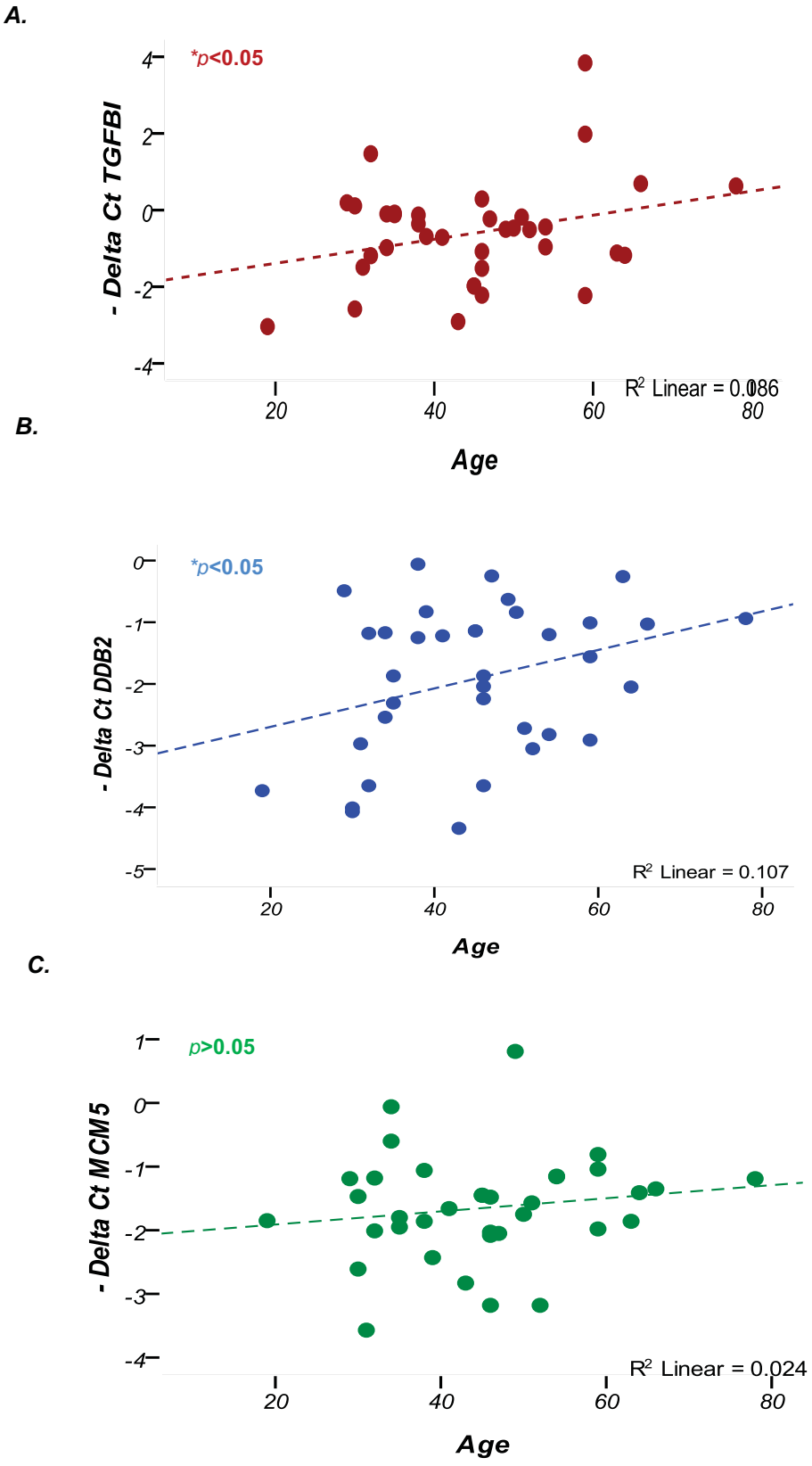


Figure 3.17 Summary of -ΔCt Data Correlated to Age for the Three Genes

The -ΔCt of all breast cancers for TGFBI (A), DDB2 (B) and MCM5 (C) were correlated with patient age using Pearson correlation test. *p<0.05 confirms that there was correlation between the patient age and the mRNA expression

3.3.2 Western Blotting for Breast Tissues

TGFBI protein expression was investigated in 6 frozen breast cancers and 4 frozen normal breast tissues. TGFBI protein was detected in all 6 breast cancers with different band intensities at molecular weight of 68kDa (Figure 3.18, A), while this isoform was absent in the organoid (***Org***) and ZR-75-1 cell line (***ZR***) controls. Two strong bands (*55.7kDa* and *45.5kDa*) and one much fainter band (*51.8kDa*) were observed in the organoid sample.

TGFBI protein was detected in the 4 normal breast tissues (***A1***, ***B1***, ***C1*** and ***D1***), with similar band intensity compared to the two tumours (***25*** and ***47***) apart from A1, which showed a fainter band intensity (Figure 3.18, B).

The β-Actin (42kDa) was assessed to ensure equal loading but no bands were seen in the normal tissues (Figure 3.18, B).

3.3.3 Correlation between TGFBI mRNA and Protein Expression by Western Blotting

The TGFBI -ΔCt for the breast cancers 5, 10 and 60 was -0.1, -0.2 and -0.7 respectively, indicating approximately similar -ΔCt. The cases numbers 5 and 10 showed moderate band intensities; however, case number 60 showed slightly stronger band compared to the rest (Figure 3.18, A).

Both breast cancers number 25 and 49 had the same -ΔCt; however, case number 49 showed a stronger band compared to case number 25. The case number 47 showed the highest -ΔCt compared to the rest of the tumours but western blotting detected fainter band compared to the rest of tumours apart from case number 25.

The normal tissues that were analyzed using western blotting showed similar band intensity compared to the two tumours apart from A1 and C1 in spite of obtaining different -ΔCt values (Figure 3.18, B).

Both ZR-75-1 and normal organoid showed absent of TGFBI protein at molecular weight of 68kDa, which was supported by the mRNA data, as -ΔCt was very low.

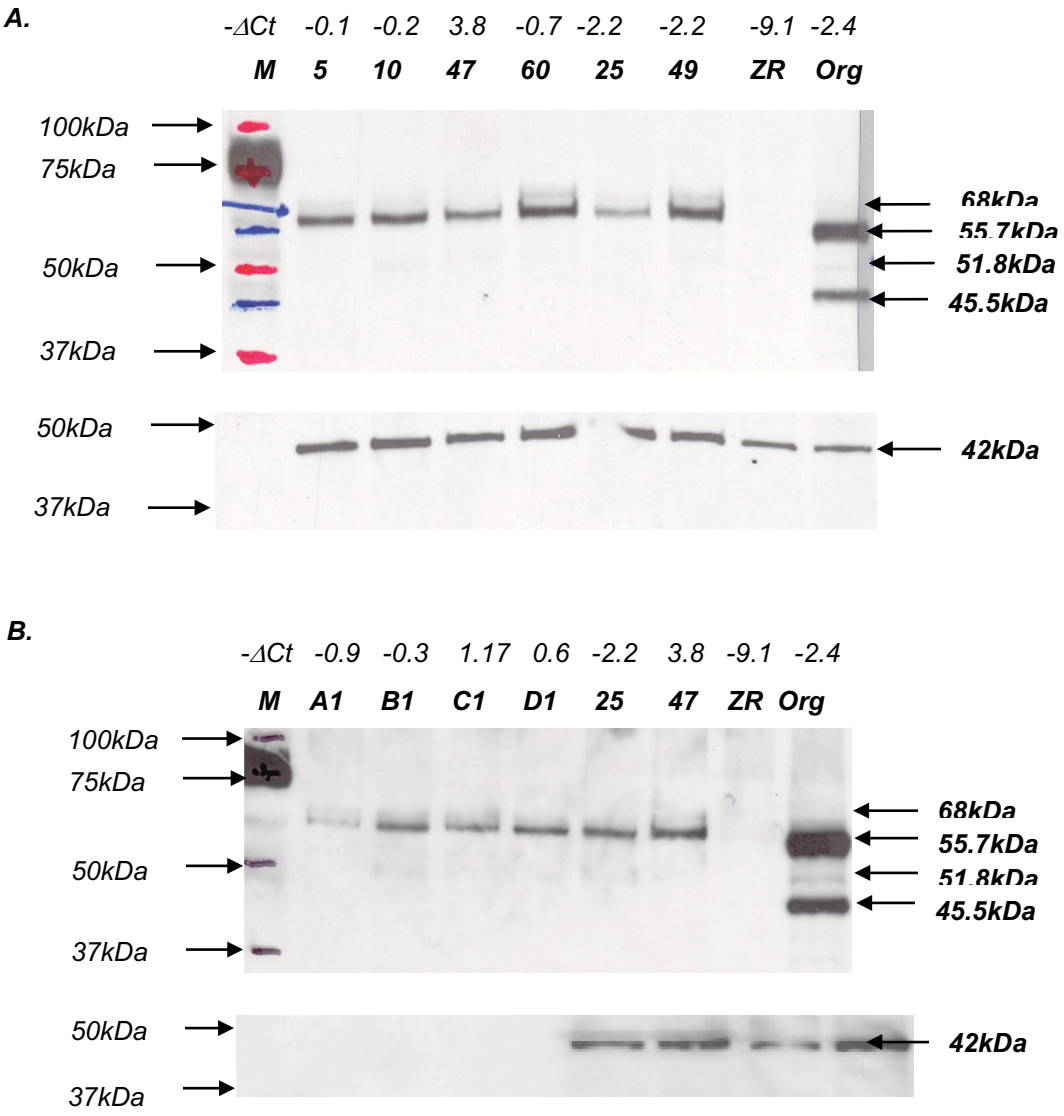


Figure 3.18 Western Blot Analysis of TGFBI Expression in Normal Tissues and Breast Cancers

The protein level of TGFBI was examined in 6 breast cancers (A) and in 4 normal tissues (B). The number of breast cancers and normal tissues presented according to table 3.9 and 3.8 respectively. (ZR) = ZR-75-1 cell line and (Org) = normal organoid. β-Actin, loading control, was assessed to ensure equal loading (42kDa). In addition, -ΔCt for each of the samples is also included to allow easier comparison between the two methods.

3.4 Immunohistochemistry Results

3.4.1 Assessment of Antibody Staining

Assessment of TGFBI and DDB2 was undertaken by myself initially, and then they were assessed jointly with Professor RA Walker. A re-assessment of my ability to assess the staining was undertaken. Staining for both proteins was scored by myself several months after the joint assessment, without knowledge of the first score. Kappa statistical test was performed according to Landis and Koch (1977) to compare the repeatability of my values. The agreement for TGFBI and DDB2 staining was moderate; kappa was found to be 0.45 and 0.57 respectively with $p<0.05$. Therefore, the values agreed with Professor Walker were used in the analyses. Assessment of MCM5 was undertaken by Professor Walker, since there was prominent staining of stromal cells between the tumour cells which made interpretation difficult.

1- *Transforming Growth Factor Beta Induced (TGFBI)*

Normal Breast Tissues

TGFBI expression was examined in 8 normal breast tissues. All of them showed variable nuclear staining in the epithelial cells of both ducts and lobules (Figure 3.19). In addition, weak ECM staining was observed in some samples. The strongest nuclear staining was observed in the normal breast tissue present in Figure 3.16; therefore, this tissue was chosen to be used as positive control in all the TGFBI IHC tests.

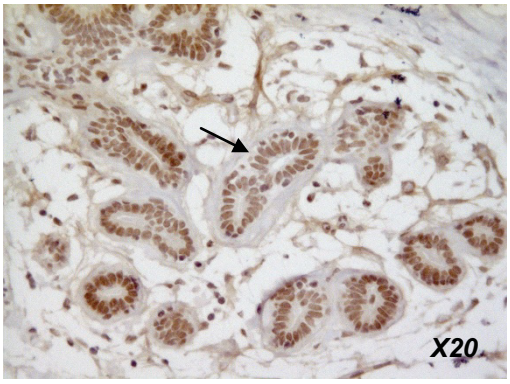


Figure 3.19 Normal Breast Tissues Tested for TGFBI Staining

Normal breast tissue was retrieved using citrate buffer at pressure cooker at 120°C for 30sec. Tissues were incubated overnight with polyclonal rabbit TGFBI antibody at 1:75. Image was captured at X20. (→) = nuclear staining.

Breast Cancer Tissues

All 67 breast cancer tissues were assessed together with Professor Rosemary Walker to determine both the intensity and extent of the nuclear staining in the normal, *in-situ* and invasive areas compared to the positive control; ECM staining was assessed in the cancers (*Appendix III*).

Normal tissue was present in 38 of the breast cancers were assessed. Twenty eight of the 38 cancers (73.7%) had no or weaker nuclear staining of normal cells compared to the positive control. In 10 cases (26.3%) the nuclear staining intensity was the same as in the positive control.

In-situ carcinoma was present in 30 of the breast cancers. Eighteen (60%) were completely negative or had <1% of the nuclei stained. Ten of the 30 cases (33.3%) had 5%, 10% or 11-50% nuclear staining in the *in-situ* carcinoma, whereas, only two cases (6.7%) showed nuclear staining in more than 50% of the *in situ* area (Figure 3.20, A).

Invasive cancer was present in all 67 cases. Forty six of the 67 (68.7%) had 0% or <1% nuclear staining. Sixteen (23.9%) of the cases had 5-50% nuclear staining in the invasive area, whilst 5 cases (7.4%) showed nuclear staining in >50% of the invasive cancer (Figure 3.20, B).

ECM staining was present in 48 out of the 67 cases (71.6%), with 19 (28.4%) of the cases lacking it (Figure 3.20, C and D).

Thirty breast cases had both *in-situ* and invasive areas. Seventeen of these cases (56.7%) had the same nuclear staining in both *in-situ* and invasive areas. Thirteen of the cases had 0% or had <1% nuclear staining; 3 had 5%, 10% or 11-50%, and only one case had >75% of the nuclear staining in both areas. Additionally, 8 of the 30 cases (26.7%) had less nuclear staining in the invasive compared to the *in-situ* carcinoma, and 5 (16.6%) had more nuclear staining.

TGFBI staining results for each of the breast cancer cases are shown in *Appendix III*. The findings for normal, *in-situ*, invasive and ECM staining in relation to patient age and pathological features are shown in Tables 3.13-3.16.

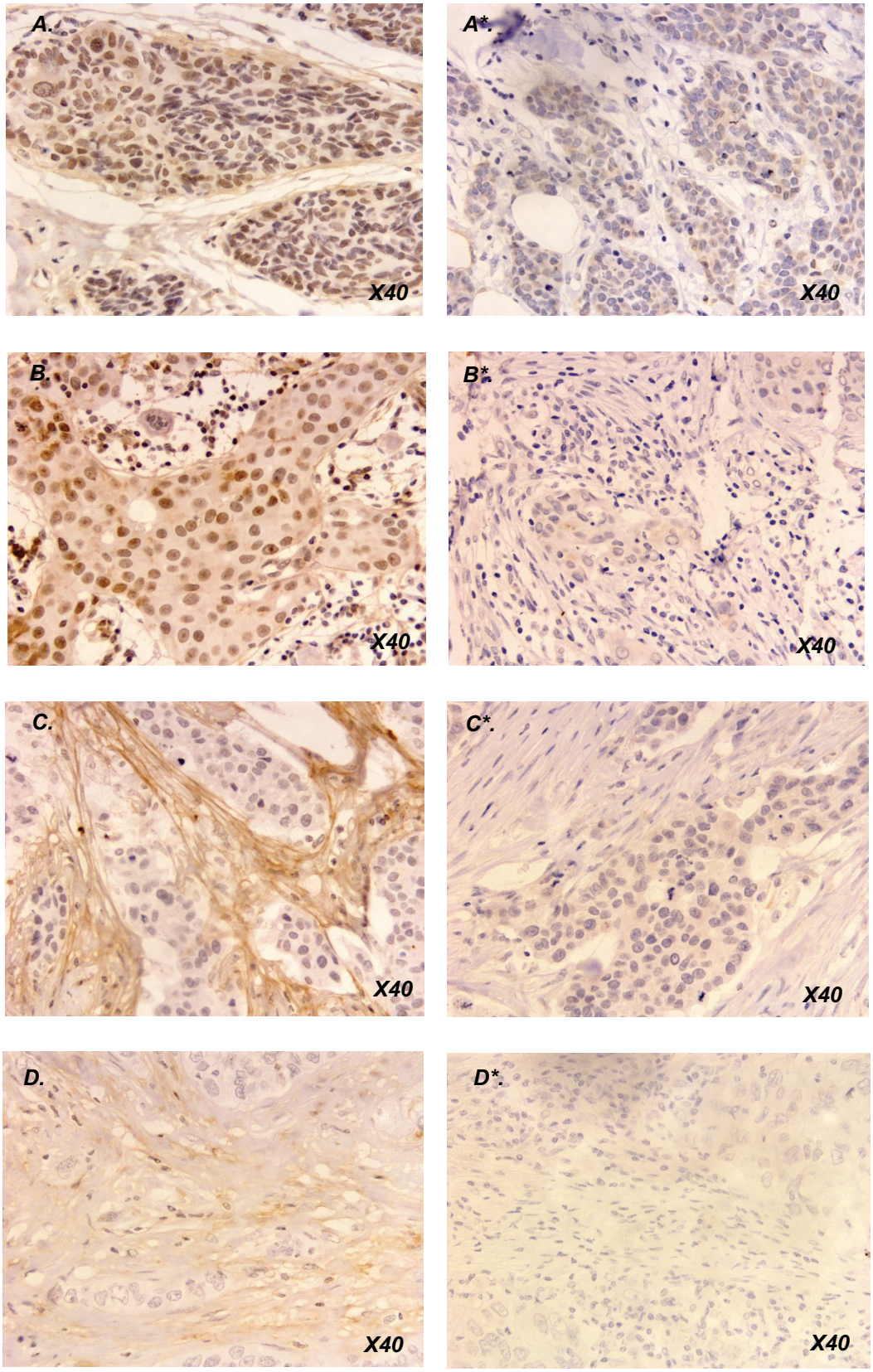


Figure 3.20 Examples of TGFBI Staining in Breast Cancers

Breast cancer cases were retrieved using citrate buffer at pressure cooker at 120°C for 30sec. Tissues were incubated overnight with polyclonal rabbit TGFBI antibody at 1:75 dilution. A= 10% nuclear staining of ductal carcinoma in-situ, B= 50-75% nuclear staining of invasive breast cancer, C and D= 75% and 5% Stromal Staining. Images were captured A-D using microscope at X40. Negative controls are also presented (A*-D*).

Table 3.13 Summary of the TGFBI Immunohistochemistry Results for the Normal Tissue Associated with Breast Cancers

		Nuclear Staining of Normal Area		
		Negative/Weaker than Control	***Similar to Control***	***Total***
Age	*≤35*	10	2	12
	36-49	10	4	14
	≥50	8	4	12
Size	≤2mm	14	4	18
	>2mm	14	6	20
Grade	I and II	14	7	21
	III	14	3	17
Lymph Node Metastasis	POS	17	4	21
	NEG	11	6	17
ER	NEG	3	1	4
	POS	25	9	34
PR	NEG	3	2	5
	POS	25	8	33
HER-2	NEG	10	3	13
	POS	1	1	2

Table 3.14 Summary of the TGFBI Immunohistochemistry Results for the *In-Situ* Tissue Associated with Breast Cancers

		In Situ Staining			
		Negative <1%	***5-50%***	***>50%***	***Total***
Age	*≤35*	6	3	0	9
	36-49	9	5	0	14
	≥50	3	2	2	7
Size	≤2mm	6	5	0	11
	>2mm	12	5	2	19
Grade	I and II	7	4	2	13
	III	11	6	0	17
Lymph Node Metastasis	NEG	8	6	1	15
	POS	10	4	1	15
ER	NEG	2	3	0	5
	POS	16	7	2	23
PR	NEG	3	3	0	6
	POS	15	7	2	24
HER-2	NEG	5	2	1	8
	POS	4	1	0	5

Table 3.15 Summary of the TGFBI Immunohistochemistry Results for the Invasive Tissue Associated with Breast Cancers

		Invasive Staining			
		Negative <1%	***5-50%***	***>50%***	***Total***
Age	*≤35*	12	4	2	18
	36-49	19	4	1	24
	≥50	15	8	2	25
Size	≤2mm	15	7	2	24
	>2mm	31	9	3	43
Grade	I and II	19	9	3	31
	III	27	7	2	36
Lymph Node Metastasis	NEG	26	8	2	36
	POS	20	8	3	31
ER	NEG	12	2	1	15
	POS	34	13	4	51
PR	NEG	13	2	1	16
	POS	33	13	4	50
HER-2	NEG	20	3	0	23
	POS	4	1	1	6

Table 3.16 Summary of the TGFBI Immunohistochemistry Results for the Stromal Staining

		Stromal Staining				
		Negative	***1-20%***	***20-50%***	***>50%***	***Total***
Age	*≤35*	3	10	3	2	18
	36-49	7	12	3	2	24
	≥50	8	7	5	5	25
Size	≤2mm	5	11	5	3	24
	>2mm	13	18	7	6	43
Grade	I and II	7	14	6	4	31
	III	11	15	5	5	36
Lymph Node Metastasis	NEG	11	16	3	6	35
	POS	7	13	8	3	32
ER	NEG	3	5	3	4	15
	POS	14	24	8	5	51
PR	NEG	2	7	3	4	16
	POS	15	22	8	5	50
HER-2	NEG	6	10	5	2	23
	POS	2	4	0	0	6

Statistical Analysis of TGFBI IHC Results

Chi-squared (X^2) test was performed to investigate whether there was a significant correlation between the clinic-pathological features of the breast carcinoma cases and the extent of the nuclear staining in normal, *in-situ* and invasive areas; the ECM staining was also tested.

Two different ways of grouping the extent of staining was conducted for *in-situ*, invasive and ECM areas to perform the chi-squared test. Statistical analysis was carried out using 3 groups (negative and <1%, 5-50% and >50%) of staining for the *in-situ* and invasive areas, while 4 groups (negative, 1-20%, 20-50% and >50%) of staining was categorized for the ECM (Table 3.17). Because of the small number of cases in the >50% staining category the statistical analysis was also performed for 2 categories; negative versus any staining (Table 3.18).

Both statistical analyses confirmed that there was no significant correlation (p<0.05) between the clinic-pathological features and the extent of nuclear staining of the normal, i*n-situ* and invasive areas. ECM staining also had no association with the clinic-pathological feature of the tumours.

Another statistical method, where patient age was considered as continuous variable was performed rather than using age groupings. The Pearson correlation test showed that the TGFBI protein results were not associated with patient age for the two categorization methods performed (Pearson correlation, one tailed, p<0.05).

Table 3.17 Statistical Analysis Results for TGFBI Using Individual Staining Categories

Variable	***Staining in the Normal Tissue (df), p-Value***	***Staining in the In Situ Tissue (df), p-Value***	***Staining in the Invasive Tissue (df), p-Value***	***ECM Staining (df), p-Value***
Age	(2), 0.632	(4), 0.131	(4), 0.630	(6), 0.547
Size	(1), 0.587	(2), 0.366	(2), 0.711	(3), 0.794
Grade	(1), 0.275	(2), 0.246	(2), 0.478	(3), 0.860
Lymph Node	(1), 0.258	(2), 0.733	(2), 0.736	(3), 0.249
ER	(1), 0.950	(2), 0.353	(2), 0.586	(3), 0.349
PR	(1), 0.456	(2), 0.535	(2), 0.490	(3), 0.321
HER-2	(1), 0.423	(2), 0.672	(2), 0.128	(3), 0.485

(df)= Degree of Freedom

Table 3.18 Statistical Analysis Results for TGFBI Using Negative and Positive Staining Categories

Variable	***Staining in the In-Situ Tissue (dt), p-value***	***Staining in the Invasive Tissue (dt), p-Value***	***ECM Staining (dt), p-Value***
Age	(2), 0.568	(2), 0.344	(2), 0.508
Size	(1), 0.643	(1), 0.417	(1), 0.405
Grade	(1), 0.547	(1), 0.228	(1), 0.463
Lymph Node	(1), 0.456	(1), 0.498	(1), 0.463
ER	(1), 0.317	(1), 0.323	(1), 0.562
PR	(1), 0.576	(1), 0.248	(1), 0.164
HER-2	(1), 0.506	(1), 0.241	(1), 0.724

(df)= Degree of Freedom

2- DNA Damaged Binding-2 (DDB2)

Normal Breast Tissues

Four normal breast tissues were tested for DDB2 staining. All the cases showed moderate nuclear staining in the epithelial cells of both ducts and lobules when using the optimized conditions (Table 3.6). The strongest nuclear staining was observed in the tissue shown in Figure 3.21; therefore, it was chosen to be used as positive control in all the DDB2 IHC tests.

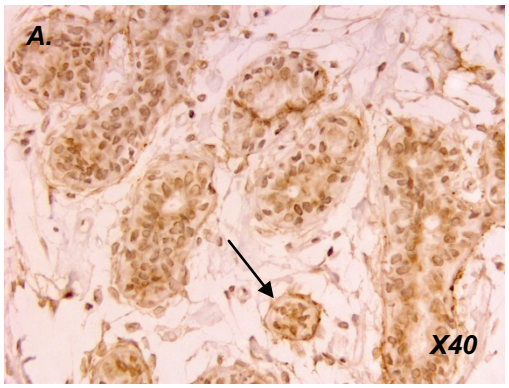


Figure 3.21 Normal Breast Tissue Tested for DDB2 Staining

Normal breast tissues was retrieved using high pH at pressure cooker at 125°C for 30sec. Tissues were incubated overnight with polyclonal goat DDB2 antibody at 1:30 dilution. Images were captured using microscope at X20. Strong nuclear staining in the positive control (→)

Breast Cancer Tissues

The results presented for the 67 breast cancers (*Appendix IV*) are from the assessment undertaken by myself and Professor Rosemary Walker, in which the intensity and distribution of nuclear staining in the normal, *in-situ* and invasive tissues was determined.

Normal breast was present in 39 cases of the 67 cancers. For 27 (69.2%) there was no or weaker nuclear staining in comparison to the positive control, while 12 (30.8%) cases had similar nuclear staining intensity as the positive control.

Twenty three of the 67 breast cases had *in-situ* carcinoma present. For 10 (43.5%), there was no or <1% nuclear staining. In 9 (39.1%) there was nuclear staining of 5-10% of cells, with only 3 (13%) cases and 1 (4.3%) case having nuclear staining between 11-50% and 51-74% respectively.

Thirty four (50.7%) of the 67 cases showed no or <1% cells with nuclear staining of the invasive component (Figure 3.22, A). Seventeen (25.4%) had 5% of cells with nuclear staining, 7 (10.4%) with 10% (Figure 3.22, B), 8 (12%) with between 20% and 50% of invasive area staining and only one (1.5%) with more than 50% of cell positive

For the 23 cases with both in-situ and invasive areas 12 (52.2%) showed similar staining for both components; 7 (30.4%) had greater staining of *in-situ* and 4 (17.4%) greater staining of the invasive component.

DDB2 staining of normal, *in-situ* and invasive cancer was also correlated to age and pathological features (Table 3.19-3.21).

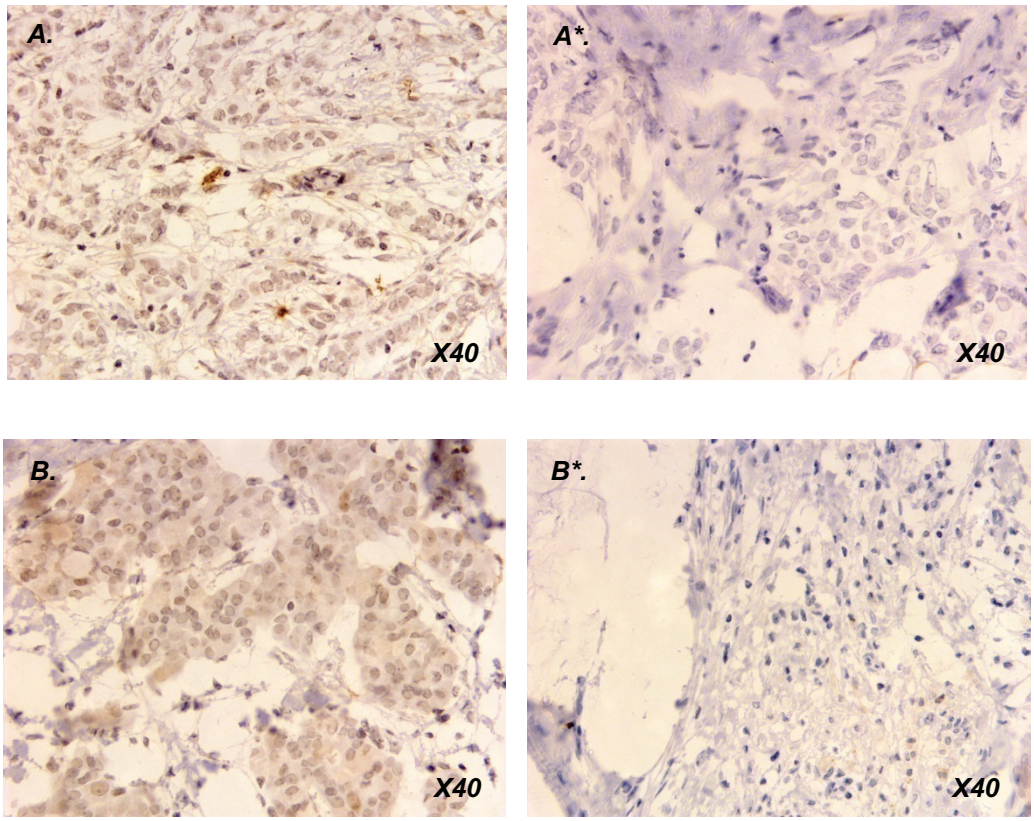


Figure 3.22 Examples of DDB2 Staining in Breast Cancers
Breast carcinoma cases were retrieved with high pH buffer at pressure cooker at 125°C for 30sec. Tissues were incubated overnight with polyclonal goat DDB2 antibody at 1:30 dilution. A= <1% nuclear staining in the invasive area of breast cancer, B= 10% nuclear staining in the invasive area of breast cancer. Images were captured for cases (A and B) using microscope at X40. Negative controls are also included (A* and B*).

Table 3.19 Summary of the DDB2 Immunohistochemistry Results in the Normal Tissue Associated with Breast Cancers

		Normal Nuclear Staining		
		Negative/Weaker than Control	***Similar to Control***	***Total***
Age	*≤35*	9	5	14
	36-49	8	3	11
	≥50	10	4	14
Size	≤2mm	11	8	19
	>2mm	16	4	20
Grade	I and II	15	6	21
	III	12	6	18
Lymph Node Metastasis	NEG	18	5	23
	POS	9	7	16
ER	NEG	2	2	4
	POS	25	10	35
PR	NEG	3	1	4
	POS	24	11	35
HER-2	NEG	10	3	13
	POS	2	0	2

Table 3.20 Summary of the DDB2 Immunohistochemistry Results in the *In-Situ* Tissue Associated with Breast Cancers

		In Situ Carcinoma Staining			
		Negative <1%	***5-50%***	***>50%***	***Total***
Age	*≤35*	2	4	0	6
	36-49	7	4	0	11
	≥50	1	4	1	6
Size	≤2mm	5	1	1	7
	>2mm	5	11	0	16
Grade	I and II	2	6	1	9
	III	8	6	0	14
Lymph Node Metastasis	NEG	4	6	1	11
	POS	6	6	0	12
ER	NEG	2	2	0	4
	POS	8	10	1	19
PR	NEG	2	2	0	4
	POS	8	10	1	19
HER-2	NEG	3	3	0	6
	POS	2	3	0	5

Table 3.21 Summary of the DDB2 Immunohistochemistry Results in the Invasive Tissue Associated with Breast Cancers

		Invasive Staining			
		Negative <1%	***5-50%***	***>50%***	***Total***
Age	*≤35*	8	10	0	18
	36-49	11	13	0	24
	≥50	15	9	1	25
Size	≤2mm	13	10	1	24
	>2mm	21	22	0	43
Grade	I and II	13	17	1	31
	III	21	15	0	36
Lymph Node Metastasis	NEG	18	17	1	36
	POS	16	15	0	31
ER	NEG	13	2	0	15
	POS	20	30	1	51
PR	NEG	13	3	0	16
	POS	20	29	1	50
HER-2	NEG	13	10	0	23
	POS	3	3	0	6

Statistical Analysis of DDB2 IHC Results

Chi-squared (X^2) test was conducted to test whether there was any relationship between the clinicopathological features and the staining of normal, *in-situ* and invasive for the 67 cancers.

Two different ways of grouping the extent of staining was conducted for *in-situ* and invasive areas to perform the chi-squared test. Statistical analysis was carried out using 3 groups (negative and <1%, 5-50% and >50%) of staining for the *in-situ* and invasive areas (Table 3.22). Because of the small number of cases in the >50% staining category the statistical analysis was also performed for 2 categories; negative versus any staining (Table 3.23).

Both methods confirmed that there was a significant correlation between DDB2 nuclear staining of the invasive carcinoma and the hormonal receptor status (Table 3.22 and 3.23). Positive ER and PR status was significantly associated with the presence of DDB2 nuclear staining, and the correlation with ER status was more powerful than for PR status. In addition, there was a significant association between larger size of the tumour and the extent of DDB2 in the *in-situ* carcinoma (Table 3.22) but this was not confirmed using the two category staining grouping (Table 3.23).

Patient age was also analyzed as being a continuous variable using Pearson correlation test. This test showed no significant correlation between DDB2 protein results and age of women with breast cancer using two different methods of categorization (Pearson correlation test, one tailed, $p>0.05$). Of note, there was a slight correlation between nuclear staining of DDB2 in the invasive tissue and patient age ($p=0.05$) using the 2 category staining grouping.

Table 3.22 Statistical Analysis Results of DDB2 Using Individual Staining Categories

Variables	***Staining in the Normal Tissue (df), p-Value***	***Staining in the In Situ Tissue (df), p-Value***	***Staining in the Invasive Tissue (df), p-Value***
Age	(2), 0.880	(4), 0.199	(4), 0.475
Size	(1), 0.135	***(2), 0.032****	(2), 0.338
Grade	(1), 0.748	(2), 0.158	(2), 0.266
Lymph Node	(1), 0.143	(2), 0.507	(2), 0.646
ER	(1), 0.379	(2), 0.877	***(2), 0.005****
PR	(1), 0.792	(2), 0.877	***(2), 0.016****
HER-2	(1), 0.448	(2), 0.740	(2), 0.775

*significant correlation between the two variables (df)= Degree of Freedom

Table 3.23 Statistical Analysis Results of DDB2 Using Two Categories

Variables	***Staining in the In Situ Tissue (df), p-Value***	***Staining in the Invasive Tissue (df), p-Value***
Age	(2), 0.148	(2), 0.503
Size	(1), 0.074	(1), 0.676
Grade	(1), 0.099	(1), 0.181
Lymph Node	(1), 0.510	(1), 0.895
ER	(1), 0.772	***(1), 0.001****
PR	(1), 0.772	***(1), 0.004****
HER-2	(1), 0.740	(1), 0.775

*significant correlation between the two variables (df)= Degree of Freedom

3- Minichromosomal Maintenance-5 (MCM5)

Normal Tissue

Eight normal breast tissues were tested for MCM5 using the optimized conditions. All the breast tissues showed only faint nuclear staining in the epithelial cells of the ducts and lobules, even with sensitive StreptABComplex/HPR Duet. Therefore, tonsil was chosen as the positive control since there was the strong nuclear staining of lymphocytes compared the normal breast tissues.

Breast Cancer Tissues

The 67 breast cancers that were stained for MCM5 were assessed by Professor Rosemary Walker to determine the extent of nuclear staining in the invasive tissues (*Appendix V*). The extent of MCM5 staining of the 67 breast cancers evaluated is summarized in Table 3.24. Forty one of the cancers showed nuclear staining between 1-10%, while 19 had nuclear staining between 11-50% and only three cancers had >50% staining. Examples of extent of staining in the cancers are shown in Figure 3.23.

The *In-situ* carcinoma was assessed in 5 cases and was higher in 3 and the same in 2 as the invasive carcinoma.

Table 3.24 Summary of the MCM5 IHC Results for Invasive Carcinoma

Extent	***Number of Cases***
Negative	4
2-5%	24
6-10%	17
11-20%	9
21-50%	10
>50%	3

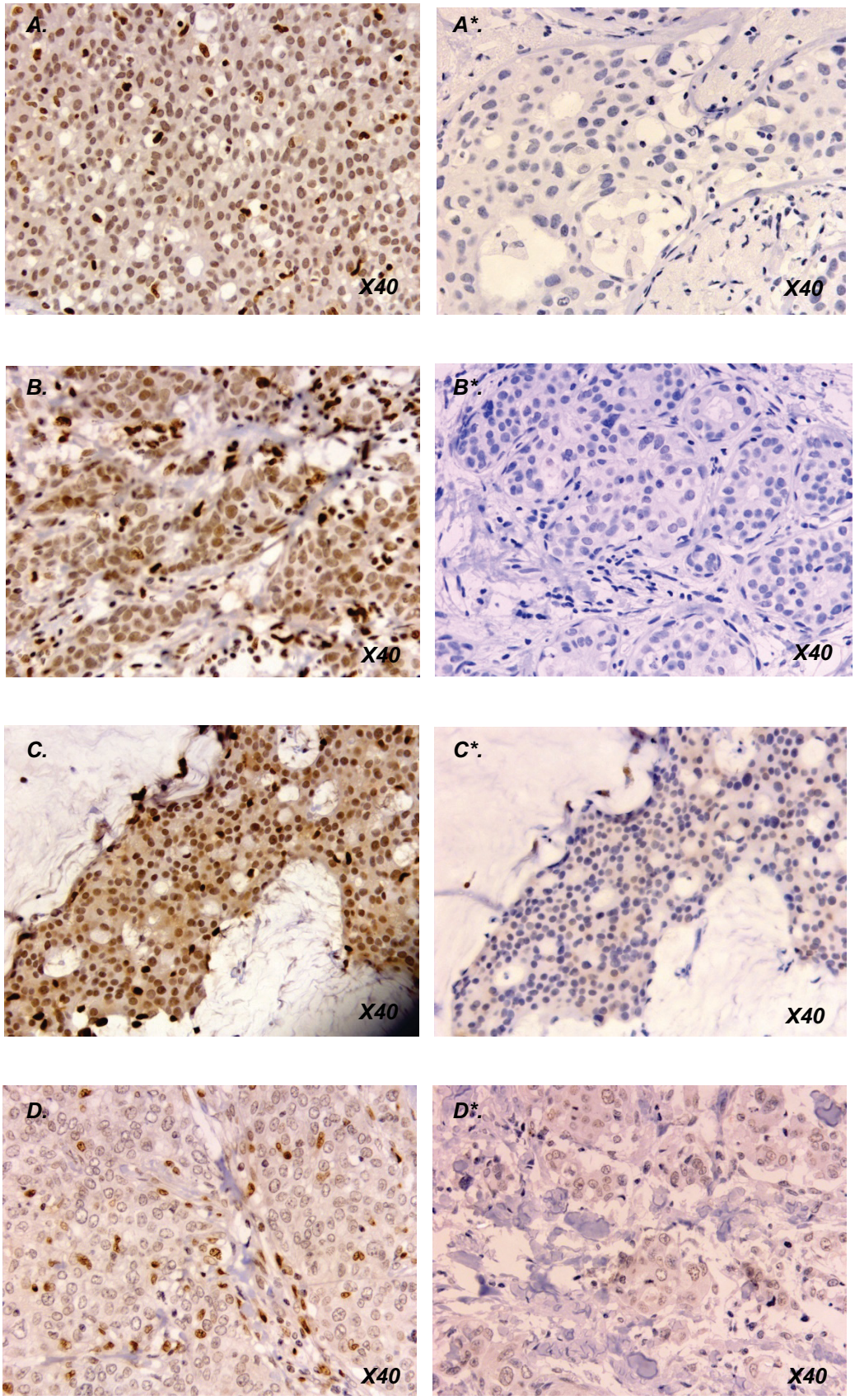


Figure 3.23 Examples of MCM5 Staining in Breast Cancers
Breast carcinoma were retrieved using citrate buffer at pressure cooker at 125°C for 45sec. Tissues were incubated overnight with polyclonal rabbit MCM5 antibody at 1:30 dilution. A= 50% unclear staining in the invasive area, B= 40% nuclear staining in the invasive area, C= 75% nuclear staining in the invasive area and D= 20% nuclear staining in the invasive area. Images were captured using microscope at X40. Negative Controls were also included (A*-D*).

Table 3.25 Summary of the MCM5 Immunohistochemistry Results for the Invasive Tissue Associated with Breast Cancers

		Invasive Staining			
		Negative <1%	***5-50%***	***>50%***	***Total***
Age	*≤35*	3	14	1	18
	36-49	6	17	1	24
	≥50	6	18	1	25
Size	≤2mm	4	19	1	24
	>2mm	11	30	2	43
Grade	I and II	3	25	3	31
	III	12	24	0	36
Lymph Node Metastasis	NEG	8	26	2	36
	POS	7	23	1	31
ER	NEG	6	9	0	15
	POS	8	40	3	51
PR	NEG	7	9	0	16
	POS	7	40	3	50
HER-2	NEG	4	18	1	23
	POS	3	3	0	6

Statistical Analysis of MCM5 IHC Results

Chi-squared (X^2) analysis was conducted to test whether there was any relationship between the clinicopathological features and the staining of invasive carcinoma for the 67 cancers.

Two different ways of grouping the extent of staining was conducted in order to perform the chi-squared test. Statistical analysis was carried out using 3 groups (negative and <1%, 5-50% and >50%) of staining for invasive areas (Table 3.25). Because of the small number of cases particularly the >50% staining category the statistical analysis was also performed on 2 categories; negative versus any staining (Table 3.25).

Both ways confirmed that there was a significant correlation between the MCM5 nuclear staining and the grade of the tumour (Table 3.26 and 3.26). Grade I and II tumours were significantly associated with higher MCM5 nuclear staining. In addition, there was a significant difference between the progesterone receptor status of the tumour and the extent of staining for MCM5 (Table 3.26), which was confirmed using the two different ways of grouping data (Table 3.26). When statistical analysis was conducted using two groups (negative versus any staining) a significant correlation was also found between ER and MCM5. Positive ER or PR status was significantly associated with high nuclear staining.

Pearson correlation test showed no correlation between MCM5 IHC results and patient age when considering age as continuous variable rather than being grouped for both staining categories (Pearson correlation test, one tailed, $p>0.05$).

Table 3.26 Statistical Analysis Results of MCM5 Using the Two Methods

Variables	*Staining in the Invasive Tissue Using three Groups (df), p-Value*	*Staining in the Invasive Tissue Using Two Groups (df), p-Value*
Age	(4), 0.973	(2), 0.790
Size	(2), 0.690	(1), 0.401
Grade	**(2), 0.017***	**(1), 0.021***
Lymph Node	(2), 0.9	(1), 0.972
ER	(2), 0.098	**(1),0.043***
PR	**(2), 0.031***	**(1), 0.011***
HER-2	(2), 0.237	(1), 0.096

*significant correlation between the two variables

3.4.2 Correlation between mRNA and Protein Expression in the Breast Cancers

For TGFBI, 22 out of 36 breast cancers showed similar findings for mRNA and protein expression (Table 3.27). However, the overall analysis showed that there was no correlation between TGFBI mRNA and protein expression (Spearman's rho correlation, 1-tailed test p=0.16).

For DDB2, 23 out of 36 breast cancers showed similar results for mRNA expression and immunohistochemistry (Table 3.27). There was a statistical correlation between the protein and mRNA levels (Spearman's rho correlation, 1-tailed, p=0.01).

For MCM5 the majority of carcinomas had low mRNA expression, and 45 of the cancers had no or 10% and less of cells staining. Statistical analysis showed that there was no correlation between mRNA and protein for MCM5 (Spearman's rho correlation, 1-tailed, p=0.37).

Table 3.27 Summary of mRNA and Protein Levels of each Breast Cancer for 3 Target Genes

Case Number	Protein IHC Results (Nuclear Staining %)			mRNA Expressions (-ΔCt)		
	TGFBI	DDB2	MCM5	TGFBI	DDB2	MCM5
1	50-75	5	1	-3.04	-3.73	-1.85
2	<1	0	2	-2.58	-4.07	-2.61
3	5	0	40	-1.49	-2.97	-3.57
4	0	5	2	-1.19	-1.18	-1.18
5	<1	10	2	-0.10	-2.54	-0.06
6	0	<1	10	-0.08	-1.87	-1.95
7	0	5	15	-0.36	-0.06	-1.06
8	0	5	5	-0.47	-0.84	-1.75
10	0	0	2	-0.18	-2.72	-1.57
12	5	5	20	-0.44	-1.20	-1.16
13	0	<1	40	-0.96	-2.82	-1.15
14	<1	<1	1	1.98	-1.01	-0.81
17	5	0	1	-1.81	-2.05	-1.41
18	0	0	1	-0.50	-0.63	0.81
19	10	5	40	-0.23	-0.25	-2.05
23	5	0	10	-2.91	-4.34	-2.83
24	0	0	2	-1.08	-2.24	-2.03
25	0	<1	5	-2.22	-2.04	-2.08
27	0	5	0	-0.13	-1.25	-1.86
28	0	5	20	-1.98	-1.14	-1.45
32	0	0	1	-1.52	-1.87	-1.48
33	0	0	0	0.29	-3.65	-3.18
34	25	20	5	0.19	-0.49	-1.19
36	<1	25	2	1.47	-3.65	-2.01
37	25-50	<1	0	-0.11	-4.01	-1.47
39	0	<1	5	0.98	-1.17	-0.60
41	25	0	0	-0.51	-3.05	-3.18
44	>75	5	40	-1.12	-0.26	-1.86
47	<1	0	50	3.84	-1.56	-1.04
49	<1	10	5	-2.23	-2.91	-1.98
50	>75	0	75	0.63	-0.94	-1.19
59	0	0	2	-0.21	-2.31	-1.80
60	0	10	5	-0.69	-0.83	-2.43
62	<1	0	15	0.69	-1.03	-1.35
63	20	20	30	-0.71	-1.22	-1.66
67	0	5	10	-1.98	-1.14	-1.45

3.4.3 Comparison between the Findings for mRNA and Protein with all the Clinic-pathological Features

Both TGFBI and DDB2 mRNA expressions were significantly correlated with patient age; lower level of mRNA was confirmed to be in younger women with breast cancer. However, MCM5 mRNA levels had no association with patient age, indicating that MCM5 might be of less importance in sporadic breast cancer in younger women. The TGFBI and MCM5 mRNA data were not confirmed with the protein expression, while DDB2 mRNA and protein showed the similar results.

Neither mRNA nor protein expressions of TGFBI showed a correlation with clinic-pathological features of the tumours.

The most important finding was the significant correlation of mRNA and protein levels of DDB2 with ER positivity in breast cancers; higher levels of DDB2 were found in ER positive tumours. In addition, mRNA levels of DDB2 were also associated with grade of the tumour but protein expression did not support this data. IHC results also showed relationship between DDB2 and PR status, which was not demonstrated by mRNA data.

MCM5 mRNA levels were not correlated with any of the clinic-pathological features; however, the protein expression confirmed its association with grade, ER and PR status confirming that higher levels present in grade II, ER and PR positive.

Discussion

This chapter considered the RNA and protein expression of three genes previously been found to be differentially expressed by a cDNA microarray, when comparing mRNA from two breast cancers from women aged 35-years with that from normal breast. My hypothesis is that the differential gene expression identified for these genes, *TGFBI*, *DDB2* and *MCM5*, could contribute to the more aggressive features of sporadic breast cancer in younger women. By evaluating normal breast, cell lines and breast cancers from women of different age groups it was anticipated that I could prove or disprove this theory.

Optimizations and Limitations

Q-RT-PCR

Q-RT-PCR is a highly sensitive technique that is commonly used for quantifying RNA levels in cells and tissues (Bustin, 2000 and Bustin, 2002). It provides accurate and reliable gene expression results, which can be influenced by the RNA quality of the investigated samples. Therefore, obtaining good RNA quality is important for achieving successful analysis (Valasek *et al*., 2005). In this study the synthesized cDNA was analyzed by 3% agarose gel electrophoresis to check its quality. All the breast cell lines and organoid samples studied showed high cDNA quality; however, it was variable for both normal/benign and cancer samples. There are several reasons for this variation. The cell lines and organoids are pure cell populations, whereas the tissue samples from which RNA was extracted consist of a mixture of stroma and cells. Within the cancers there can be normal tissue and stromal cells as well as tumour cells. Because of this variation in cellularity, haematoxylin and eosin stained sections, were evaluated for all tissues used, and those samples with low cellularity excluded. RNA integrity is particularly important and could be the major factor for the differences between the cells and the tissues. The tissue samples were frozen for some time and, although care was taken in the initial freezing, they were used for other studies, so they could have been subject to freeze/thaw changes. This would have resulted in damage to RNA.

The gene expression assays (3 target genes and 3 housekeeping genes) used in this study demonstrated high efficiency, apart from the DDB2 assay. This gene was expressed at lower levels compared to the other 2 target genes (*TGFBI* and *MCM5*) using the same serial dilutions and HBL-100 cDNA to generate the standard curves. High Ct values (>35 cycles) were obtained; therefore, it would have been worth using

different range of dilutions to improve the standard curve and subsequently the efficiency obtained.

Minus RT reactions were generated for all the samples that were investigated in this study. Negative RTs confirmed the specificity of the reaction and also the absence of DNA contamination. All samples used were normalized to the mean of 3 housekeeping genes (*GAPDH*, *HPRT1* and *TFRC*) that showed minimal variation in expression in various samples. This approach of averaging endogenous controls genes expression is recommended for normalization in q-RT-PCR experiments (Meller *et al*., 2005).

All breast cancers and organoid samples were included in the statistical analysis because relative expression (ΔCt), the way data were calculated and presented in this thesis, strongly suggests that the extent of degradation of the sample can increase the Ct value but it has no effect on the ΔCt expression (Antonov *et al*., 2005). Only two of the normal/benign tissues were excluded from the analysis because of the low cellularity and poor cDNA generated, which could not be detected on the 3% agarose gel using manual GAPDH PCR. In addition, the Ct value of housekeeping genes were less than 35 except for one tumour and a small number of other samples, which showed a high Ct value for the TFRC assay.

The q-RT-PCR was performed in triplicate, which showed relatively low variation for each sample. However, the cDNA used was from one synthesis for all the specimens and cell lines. Ideally cDNA synthesized on two to three separate occasions should have been compared. This is one of the limitations of the q-RT-PCR results in this study but is due in part to the small amounts of RNA that were obtained from the frozen tissues.

The cell lines data presented in this thesis are representative examples as these experiments were repeated several times using freshly isolated mRNA. However, this work was carried out at the start of the study and unfortunately due to software upgrades to the q-RT-PCR equipment it was not possible to retrospectively retrieves these data files. Therefore, it was not possible to statistically analyze the cell line data. However, it would have been worth repeating the cDNA synthesis for some of the tissue samples, where higher mRNA yields were obtained and then repeating the q-RT-PCR to check for any inter assay variation as well as intra assay variation by replicates.

Western Blotting

Western blotting was performed for 6 breast cell lines and 3 normal organoid samples for each one of the target genes (TGFBI, DDB2 and MCM5), and also 4 protein loading controls were examined (β-Actin, α-Tubulin, β-Tubulin and Vinculin). The western blotting data was repeated three times with the cell lines and organoids for each target gene, which confirmed the reliability and reproducibility of the data.

Differences were found in the loading controls examined except for β-Actin, which showed the least variation between the breast cell lines and organoids. Thus, it was selected to be the protein loading control in this study. Variation between cell lines and organoids, which are isolated aggregates of epithelial and myoepithelial cells, might be due to the isolation method used for the latter, which includes proteases. These could result in protein cleavage and be the reason that multiple bands were detected in the organoid samples for TGFBI protein. Thus, organoid samples were not suitable as controls for investigating TGFBI protein. It would have been beneficial to select a cell line as a positive control, which had high levels of TGFBI protein at the appropriate molecular weight (68kDa). This could have been cell lines treated with TGF-β_1 (Table 1.11).

Only a small number of breast cancers and normal tissues were investigated twice for TGFBI protein expression. Absence of the β-Actin loading control was observed in the frozen normal/benign tissues compared to frozen breast cancers. This was because of the low cell density present in these samples. To overcome this problem it would have been worthwhile loading a higher protein concentration for these samples or leaving the x-ray film for a longer period of time (Figure 3.18, 5 minutes exposure). In order to compare western blot results of cancers and normals it would be necessary to analyse different cell components, for example fibroblasts, myoepithelial cells, lymphocytes and tumour cells.

Immunohistochemistry

Several optimizations were carried out to give the best nuclear staining for the positive control that was used in each run to ensure consistency of staining of the breast cancer cases. Various primary antibody dilutions for TGFBI, DDB2 and MCM5 were examined. Antigen retrieval was achieved using a pressure cooker with various temperatures, time and buffering conditions. The optimal conditions selected for the three antibodies

are summarized in Table 3.6. In addition, the Duet Kit of secondary/tertiary was used to increase the staining intensity.

Heat antigen retrieval using a pressure cooker, was the technique of choice for examining the breast cancer cases, with retrieval buffers of different pHs being assessed (Shi *et al*., 2001). A pressure cooker was selected because it has been confirmed to result in more consistent staining compared to microwave antigen retrieval (Rhodes *et al*., 2001; Walker, 2006). The domestic microwave that was available within the laboratory can give variable results due to inconsistent heating (hot and cold spots), and has been found to give variable results of nuclear antigens such as oestrogen receptor (Rhodes *et al*., 2001).

The assessment of TGFBI and DDB2 was undertaken by myself initially, and then all sections were assessed jointly with Professor RA Walker, using a double-headed microscope. Any differences were discussed and the agreed result recorded. An assessment of my ability to assess staining was undertaken, in which I re-assessed sections several months later. Intra-observer agreement was performed using the generalized kappa-type statistics (Landis and Koch, 1977), which showed only a moderate agreement for TGFBI and DDB2 IHC categorical data (0.41-0.60). Hence the results agreed with Professor Walker were used in all statistical analyses. Assessment of MCM5 was undertaken entirely by Professor RA Walker, since there was prominent staining of stromal cells within and between the tumour cells which made interpretation difficult. Between 500 and 1000 tumour cells were assessed and the percentage of nuclear reactivity of *in-situ* carcinoma, if present, and invasive carcinoma determined, as undertaken previously for cell cycle proteins (Walker *et al*., 1996; Walker, 2006). Intra-observer variability on MCM5 was not performed. The percentage categorization used for TGFBI and DDB2 has been used previously for TGF-β_1 and stromal proteins (Walker *et al*., 1994). There are several scoring systems which are used to categorise immunohistochemical staining, which are based on either extent of staining, intensity of staining or a combination of the two. The latter includes the quick or Allred score (Harvey *et al*., 1999) and the H- (Histo-) score. The H-score involves counting the percentage of cells with different intensities of the staining (weak, moderate and strong) and summating to give a score from 0 to 300 (McCarty *et al*., 1985; Walker *et al*., 2006; Tang *et al*., 2009). Given sufficient time it would have been worth re-assessing the IHC results by this method but there was little variation in nuclear staining intensity, and I had problems with reproducibility using a simpler method. Researchers have suggested that experience in histopathology is required for determining the H-score (Barnes *et al*., 1996; Fisher *et al*., 2005).

Sixty seven breast cancer tissues were examined for the three target proteins TGFBI, DDB2 and MCM5 using a standard positive control for each run to overcome any problems in batch variation and allow comparisons. A limitation of the study was the number of cancer cases studied. The cases studied previously by Lambe (2008) were included; additional cases of formalin fixed paraffin embedded tissue were also investigated. The aim was to have relatively equal numbers of cases in the three age categories (≤ 35 years, 36 -49 years and ≥50 years) in order to avoid bias when analyzing the results. One problem is that the number of cancers occurring in the younger age group is much lower. Studying larger number of cases could have been beneficial. Tissue microarrays are an approach that allows many more cancers to be studied (Fergenbaum *et al*., 2004) but since only 2-3 small samples from each cancer were assessed, evaluation of markers that show low level heterogeneous staining may not be accurate.

Gene and Protein Expression

TGFBI

My overall results were similar to those of Lambe (2008) and did not support those of the microarray study of Whyman (2005). In the latter TGFBI was found to be expressed at a higher level in the two breast cancers of young women (35- Years Old) in comparison to normal breast samples (organoids). Whilst there was a trend for higher mRNA levels in breast cancers compared to organoids, this was not significant and expression was highest in normal/benign tissues. Analysis of TGFBI mRNA expression in relation to patient age showed that lower TGFBI mRNA levels were associated with younger patients (Pearson correlation test, $p<0.05$) (Figure 3.17), similar to the findings of Lambe (2008) but in contrast to Whyman's results (2005). Therefore TGFBI is unlikely to play a role in breast cancers in younger women.

Analysis of breast cell lines showed that HBL-100, an immortalised normal line, had the highest mRNA expression, but with low or undetectable expression in the cancer cell lines. Similar findings were obtained by a previous PhD student (Lambe, 2008). Western blotting confirmed the mRNA data, with only HBL-100 having a faint band at molecular weight of 55kDa, which is not the expected size but is one of the splice variant that were established (Thierry mieg, 2006). These results support a number of studies, which have found down-regulation of TGFBI in tumour cell lines such as prostate, kidney (293T), lung (H522, H810, and H1417), mammary, leukemia (K562, Jurkat) and stomach cancer (SNU16) (Shao *et al*., 2006). Transformed Human

Fibroblasts (SV-40) also exhibit down-regulation of TGFBI compared to the normal fibroblasts (Schenker *et al*., 1998). Down-regulation of TGFBI in MCF-7 and MDA-MB-231 breast cell lines has been suggested to be due to TGF-β signalling pathway blockage rather than being associated with DNA methylation (gene silencing) (Shao *et al*., 2006), although it is the most common epigenetic modification that is found to play a major role in the development and progression of cancer (Mani and Herceg, 2010).

Within the organoid samples several TGFBI protein isoforms (55.7kDa, 51.8kDa and 45.5kDa) were observed, although no band was detected at the molecular weight of 68kDa. The q-RT-PCR data showed low levels of TGFBI mRNA in the organoids because the TGFBI assay used could detect the 68kDa molecular weight (Materials *2.1.6*). The isoforms present in the organoids match with the human splice variants published (Thierry- Mieg, 2006) (Table 1.10).

The majority of the breast cancers showed lower levels of TGFBI mRNA relative to the mean ∆Ct of normal/benign tissues. However, higher or similar mRNA expression was obtained for a small number of the cancers compared to normal/benign samples. The 6 breast cancers selected for western blotting analysis showed bands at molecular weight of 68kDa of variable intensities; however, only 3 of the 6 investigated tumours were matched to their mRNA expression. The 4 normal/benign samples investigated using western blotting showed faint to moderate band intensity at molecular weight of 68kDa. According to these results, it seems that the molecular weight 68kDa is the predominant isoform of TGFBI protein in breast tissues, whilst it is absent from organoids, which are isolated aggregates of epithelial and myoepithelial cells. This difference might be due to the isolation method used for the latter, which includes proteases. These could result in protein cleavage and be the reason that multiple bands were detected in the organoid samples for TGFBI protein.

The TGFBI mRNA expression was lower in cancers from younger women (Pearson correlation test, $p<0.05$) (Figure 3.17). No other clinic-pathological relationships were identified. The finding of higher expression of TGFBI in normal breast compared to cancers differs from that found for cancers in other tissues such as renal, pancreatic, lung, colon, rectal and small intestine cancers (Ivanov *et al*., 2008). This suggests that its role in normal breast and breast cancer differs from that of other cell and tissue types.

The immunohistochemistry results showed that 46/67 of breast cancers had no or <1% nuclear staining, and 49/67 had ECM staining to varying degrees. Forty six of 67 of the breast cancers showed no or <1% nuclear staining, suggesting down-regulation of

TGFBI protein in the intracellular compartment. However, 48/67 of the same breast cancers showed variable of ECM staining. Twenty two of the cases had both nuclear and stromal staining, while 26 cases had only ECM staining. This immunohistochemistry finding is supported by other studies, which found the deposition of TGFBI protein in the ECM, cytoplasm and nucleus of various types of cells such as primary Human Bronchial Smooth Muscle (HBSM) cells (Billing *et al*., 2000a), bladder fibroblasts, and bladder Smooth Muscle cells (SMC) (Billing *et al*., 2000b). Both Billing *et al*., (2000a) and Billing *et al*., (2000b) confirmed that TGFBI is a component of the pulmonary ECM by using lung tissue from a 2-yr-old child, and also a component of bladder tissue ECM. These two studies found that TGFBI is localized to the nuclei, cytoplasm and ECM of some HBSM cells and bladder SMC depending on the antibodies used, which one generated against N-terminal while the other for the C-terminal peptide sequence of the TGFBI protein. In addition, TGFBI was shown to be localized in the three compartments of mesothelium of parietal peritoneum (Park *et al*., 2008). The presence of TGFBI protein in both intracellular and extracellular compartments can be as a result of multiple isoforms arising from alternative splicing or by post-translational proteolytic processing (Billings *et al*., 2000a). The immunohistochemistry results of this study were also supported by other studies, one of which revealed the absence or reduction (>2-fold) of TGFBI protein in 45 of 130 lung carcinoma compared to normal lung tissue using immunohistochemistry (Zhao *et al*., 2006). In addition, a decrease in the TGFBI protein expression was observed in the colloid carcinoma compared to benign breast tissue (Calaf *et al*., 2008). No correlation was found between the TGFBI protein levels and the clinic-pathological feature of the tumours in this study, this was similar to the findings obtained with the TGFBI mRNA data. Although lower levels of TGFBI mRNA were associated with breast cancer in younger women the IHC data showed no correlation with age.

No correlation was found between the IHC data and mRNA results for TGFBI. The difference between the mRNA and protein levels might be due to post translational modifications, another explanation might be intra-tumoural heterogeneity with different areas of the tumour being analyzed by q-RT-PCR and IHC.

DDB2:

In the cDNA microarray study (2005) DDB2 was found to be expressed at a higher level in the two breast cancers from young women compared to the organoid sample. However, I have not found DDB2 expression to be associated with younger age but the converse with higher DDB2 mRNA expression in breast cancers from older women (Pearson correlation test, $p<0.05$) (Figure 3.17).

Analysis of breast cell lines showed that HBL-100, MDA-MB-468 and MDA-MB-231, which are known to lack ER and PR, had lower DDB2 mRNA expression compared to MCF-7, T47-D and ZR-75-1, which are ER and PR positive. These findings are the same as Kattan *et al*., 2008 who identified over-expression of DDB2 in ER-positive MCF-7 and T47-D cell lines compared to low or undetectable levels in ER-negative breast cancer cells (MDA-MB-231, SKBR3). However, western blotting only identified a faint band at a molecular weight of 51kDa (not the expected molecular weight) in all the breast cell lines with no differences between the ER/PR positive and ER/PR negative cell lines. This result might be due to the alternative splicing of DDB2 gene, which was found to produce 4 different DDB2 protein isoforms (Table 1.14, Inoki *et al*., 2004). Although the DDB2 antibody used in this study was suitable for western blotting analysis according to the data sheet and other studies, detecting the appropriate molecular weight 48kDa, these different molecular weight bands observed might be due to non-specific binding of the antibody rather than alternative splicing. It would have been worth using different DDB2 antibody from another company for validation.

Q-RT-PCR results showed variation in the DDB2 mRNA levels of organoids relative to normal/benign tissues; 4 samples had higher DDB2 mRNA levels while 3 showed under-expression and only 1 showed the same mRNA level as mean ΔCt of normal/benign tissues. However, the western blotting detected a strong band of molecular weight of 48kDa in 2 out of 3 investigated organoids. Presence of different isoforms in the cell lines (51kDa) compared to organoids (48kDa) could be a result of alternative splicing (Inoki *et al*., 2004) (Table 1.14). There is a core promoter located within 220bp upstream of the putative transcription initiation sites of *DDB2* gene, which contains sp1, NF-1 and E2F sites that are important determinants of the promoter activity (Nichols *et al*., 2003).

I found there to be no statistically significant difference between the DDB2 mRNA expression of normal/benign tissue, breast cancers and organoid samples (Kruskal-Wallis, $p<0.05$), in contrast to the microarray data that showed significant increase in the DDB2 mRNA levels compared to the organoid samples. Differences in the results

might be due to the transcriptional heterogeneity of breast cancers, and could also refer to the small number of samples (only 2 breast cancers) used in the microarray study. As with TGFBI it may be that using organoid sample as a comparator in the microarray study was not appropriate (Whyman, 2005).

Immunohistochemistry showed that 34 out of 67 of breast cancers had no or <1% nuclear staining and 17 of them had only 5% of cells with nuclear staining, confirming the down-regulation of DDB2 in breast cancers. Besides, a fainter staining was observed in breast cancers compared to normal breast tissue. The IHC results significantly correlated with mRNA levels in the breast cancers (Spearman's rho correlations, 1-tailed, $p<0.05$).

There were interesting correlations with clinico-pathological features of the cancers for both q-RT-PCR and IHC. As with the breast cancer cell lines there was a significant correlation between DDB2 expression and the ER/PR status of the breast cancers. There was a significant correlation between the mRNA of DDB2 of the breast cancers and their ER status, with higher levels of DDB2 mRNA in the ER-positive cancers compared to the ER-negative tumours (Mann Whitney test, $p<0.05$). Furthermore, an association between the DDB2 and the grade of the tumour was found; higher DDB2 mRNA level was found in grade II tumours compared to grade III tumours. Immunohistochemistry findings also confirmed that positive ER and PR status was significantly associated with strong DDB2 nuclear staining in the invasive component. My study showed that DDB2 mRNA significantly correlated with patient age; higher levels were associated with the older age group. This is in keeping with the relationship between mRNA/protein levels and both ER and grade of the tumours, since cancers occurring in older age groups are more likely to be ER positive, and better differentiated cancers are ER positive.

Breast cancers occurring in younger women are more likely to be ER and PR negative (6 of 18 cases) but the original microarray found higher expression of DDB2 in the breast cancers from younger women in relation to the organoids. A microarray comparing cancers from younger and older patients may have given similar results to my findings. DDB2 expression would be worth exploring in relation to responsiveness of cancers to endocrine therapy, particularly if it could improve understanding of acquired resistance.

MCM5

In the microarray study (2005) MCM5 was expressed at a higher level in the two breast cancers than in the organoid samples. However, the findings of this study do differ from this.

Analysis of breast cell lines showed that MCF-7 and MDA-MB-231 had greater MCM5 mRNA levels relative to HBL-100, while lower expression was found in MDA-MB-468 and T47-D. The ZR-75-1 cell line showed variation between triplicates making determination of expression level difficult. Western blotting confirmed the mRNA data with detection of a faint band at molecular weight of 82kDa for both MDA-MB-468 and T47-D, while ZR-75-1 showed a similar level of expression to HBL-100. MCM5 protein expression for MCF-7 and MDA-MB-231 supported the mRNA data but band intensity was fainter when compared to HBL-100, which had relatively less MCM5 mRNA compared to the two cancer cell lines.

Low MCM5 mRNA levels were obtained for the three groups (normal/benign tissues, breast cancers and organoids samples), with no significant difference in the mRNA expression. Western blotting showed a strong band at different molecular weight (~70kDa) for the organoids compared to cell lines, suggesting the presence of various isoforms; therefore, more investigations on this aspect are needed as no information has been established yet.

Immunohistochemistry results showed that all breast cancers had a variable extent of nuclear staining, with only four cases being negative. A significant correlation was found between MCM5 nuclear staining and low tumour grade and ER/PR positivity. There was no significant correlation between MCM5 immunohistochemistry results and the mRNA data. A number of studies have demonstrated over-expression of MCM5 in cancers compared to normal tissues (Introduction, Table 1.8). My findings indicate that MCM5 could be a potential marker in less aggressive cancers (Grade II and ER/PR positive). It would have been worthwhile correlating MCM5 levels with proliferation as determined by Ki-67. Although a correlation was found with grade and ER status as was found for DDB2, there was no relationship between MCM5 mRNA and protein expression and age of the patient. There have been no previous published studies of MCM5 in breast cancer.

Summary

My results fail to support the hypothesis of the study, namely that expression of TGFBI, DDB2 and MCM5 could contribute to the more aggressive features of sporadic breast cancers in younger women.

TGFBI mRNA expression was down-regulated in breast cancer cell lines and also reduced in breast cancers compared to normal/benign tissues. Expression was lower in breast cancers in younger women than cancers from women of other ages. The majority of the breast cancer cases had no detectable nuclear staining. No correlation was found between TGFBI mRNA and protein expression and clinico-pathological features of the tumours (size, grade, ER/PR, and HER-2).

DDB2 mRNA levels were higher in ER/PR positive compared to ER/PR negative breast cell lines. These data are supported by one other published study (Kattan *et al*., 2008). A significant correlation was found between higher mRNA expression and older patient age. A significant correlation was also found between higher DDB2 expression (both mRNA and protein) and both ER positivity and lower grade of the tumour. Therefore, DDB2 expression does not relate to the more aggressive features found in breast cancer in younger women.

The cell line studied of MCM5 did not show any significant findings. There was no relationship between MCM5 expression and patient age. The main finding was that higher protein levels were found in better differentiated, ER positive cancers, which are more frequent in an older age group.

Chapter 4

Role of TGFBI in Breast Cancer

Introduction

The induction of TGFBI protein by the treatment of cells with TGF-β_1 has been demonstrated in a number of studies (Introduction, Table 1.4). Shao *et al*., 2006 proposed that the Smad pathway might play an important role in regulating the transcription of TGFBI gene in breast cancer cell lines. The MCF-7 cells have intact Smad pathway; however, lack of TbetaR-II protein and Smad 4 dysfunction was observed in ZR-75-1 and MDA-MB-468 respectively (Lynch *et al*., 2001). Therefore, MCF-7 cells were used for the induction studies.

TGFBI is an extracellular matrix protein that interacts with various ECM molecules (Thapa *et al*., 2007). It has also been identified in the cytoplasm and nucleus of cells such as human bronchial smooth muscle cells (Billings *et al*., 2000a) and the H522 lung cell line (Zhao *et al*., 2006). TGFBI protein plays an important role in cell adhesion, migration and invasion through attachment with various integrins (Thapa *et al*., 2007 and Table 1.5). TGFBI was also shown to significantly reduce proliferation and invasion of neuroblastoma cells *in vitro* and *in vivo*, which may be due to elevated Tissue Factor Pathway Inhibitor-2 (TFPI-2) (Backer *et al*., 2006). It was also shown that transfection of TGFBI protein into the H522 lung cancer cell line resulted in significant apoptotic induction compared to parental tumour cells (Zhao *et al*., 2006). In corneal dystrophy, human corneal epithelial (HCE) cells showed TGFBI mutations that are associated to high level of apoptosis (Morand *et al*., 2003).

Hypothesis and Aims

TGFBI is a tumour suppressor gene, which is reduced in breast cancers compared to normal/benign breast tissues. The aim of this chapter is to test the role of TGFBI by re-introduction of TGFBI plasmid into breast cancer cell lines. Up-regulation of TGFBI expression by transient transfection was performed to investigate whether it has an influence on the viability, apoptosis and invasion of ZR-75-1 and MDA-MB-468 breast cancer cell lines. These two cell lines were used because both of them showed low TGFBI RNA levels and absent TGFBI protein expression in the investigations in chapter 3. The three functional assays were compared between cells transfected with pRc/CMV2- TGFBI and a control pRc/CMV2 empty vector, which were kindly provided by Dr. Yongliang Zhao (University of Columbia, NY), and untreated cells. In addition, the effect of TGF-β_1 on the MCF-7 breast cancer cell line was investigated.

Results

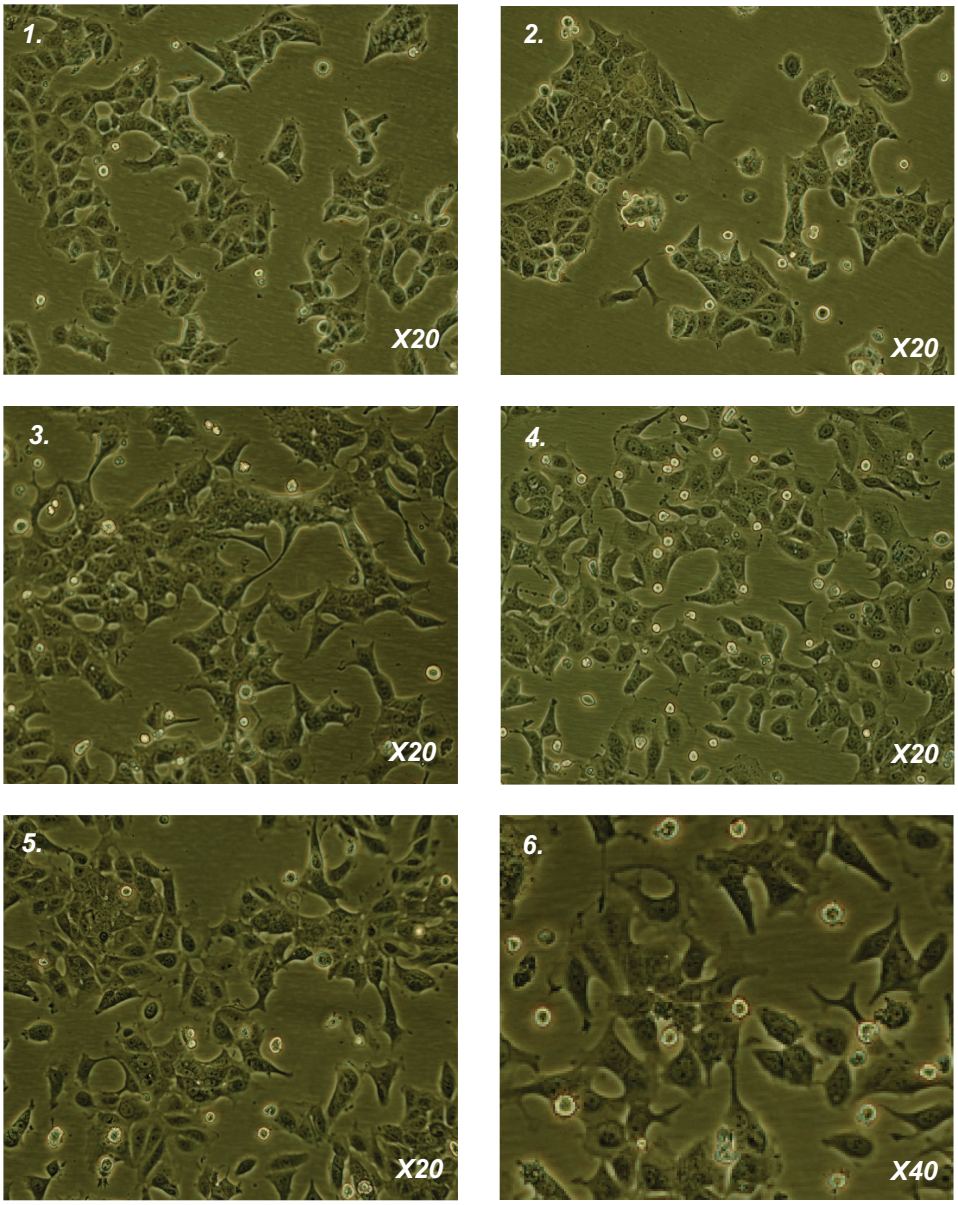
4.1 TGF-β_1 Induces TGFBI Protein in MCF-7 Cells

Human TGF-β_1 at concentrations of 0.5, 1, 5, 25 and 100ng/ml was added to the MCF-7 breast cell line and cultured for 24 and 72 hours. Microscopic images (X20 and X40) were taken at different time points and cell lysates and culture media collected for TGFBI protein examination using western blotting.

4.1.1 MCF-7 Microscopic Images

A more extended and elongated shape of MCF-7 cells was observed when they were treated with TGF-β_1 compared to the control cells (Figure 4.1, A and B). Essentially, high TGF-β_1 concentrations (25ng/ml and 100ng/ml) for 72 hours resulted in MCF-7 having irregular shaped structures and less adhesion between cells (Figure 4.1, B8 and B9) compared to control MCF-7 cells, which had no TGF-β_1 in the media (Figure 4.1, A6 and B10). More investigations are warranted to prove this that might be by staining with rhodamin phalloidin to detect F-actin bundles.

A.



B.

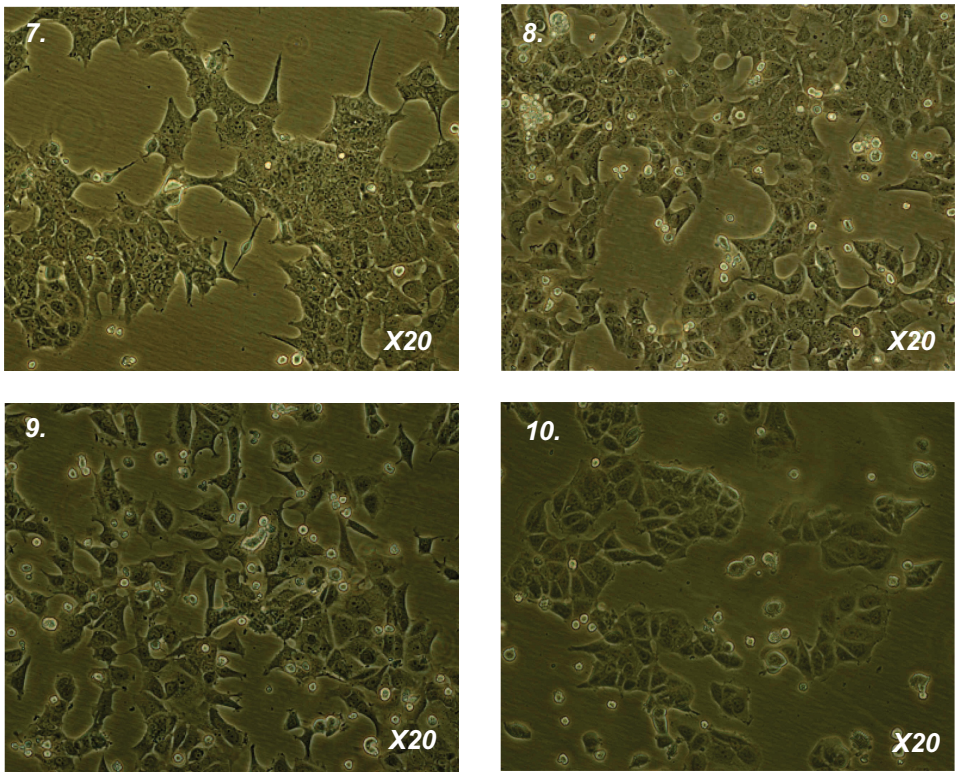


Figure 4.1 Morphological Changes of MCF-7 Cells exposed to Varying Concentrations of TGF-β_1

MCF-7 cells were incubated with serum free medium containing various concentrations of TGF-β_1 (0.5ng/ml (1), 1ng/ml (2), 5ng/ml (3), 25ng/ml (4), 100ng/ml (5) and TGF-β_1 free media (6)) for 24 hours (A). In addition, MCF-7 cells were incubated with various concentrations of TGF-β_1 (5ng/ml (7), 25ng/ml (8), 100ng/ml (9) and TGF-β_1 free media (10)) for 72 hours (B).

4.1.2 MCF-7 Western Blotting Post TGF-β_1 Culturing

Western blotting showed that TGFBI protein (68kDa) increased in the cell media after culturing MCF-7 cells with high concentration of TGF-β_1 (5, 25 and 100ng/ml) for 72 hours (Figure 4.2, C_5, C_{25} and C_{100}). No bands were observed in the cell lysate after treatment in both time points suggesting that TGFBI is secreted from the cells immediately and not retained in the nucleus (Figure 4.2, Lysate 24 hr and Lysate 72 hr). Conditioned media at 24 hours was not investigated as the yield of protein was too low.

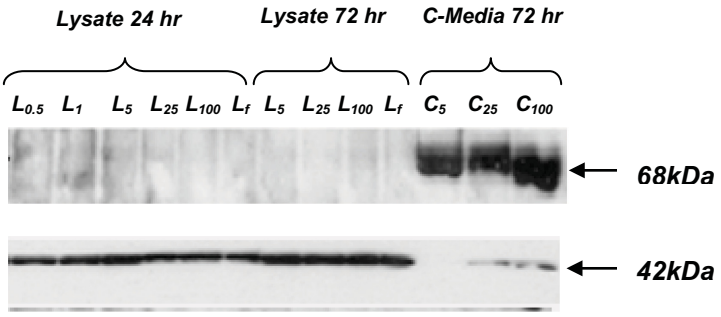


Figure 4.2 Western blotting Analysis of MCF-7 Cell Treated with TGF-β_1

Both lysate (L) and conditioned media (C) were collected after treating MCF-7 with different concentrations of TGF-β_1 (0.5, 1, 5, 25 and 100ng/ml for 24 hours time point, while 5, 25 and 100ng/ml for 72 hours time point). L_f represents the untreated control lysate.. No protein was detected in the conditioned media for the untreated control.

4.2 Preparation of TGFBI Plasmid DNA for Transfection

All TGFBI plasmids which were purified using the Mini-Prep kit showed predominantly supercoiled DNA (Figure 4.3, A, Lanes: ***P_1-P_4*** and ***P_6***). Faint bands of a higher size (Figure 4.3, A, Lane: ***P_1-P_c***) were observed, suggesting the presence of a small amount of open circular plasmid DNA (Figure 4.3, A).

The Maxi-Prep kit was used to prepare a higher concentration of plasmid DNA (Figure 4.3, B). Both TGFBI plasmid and the empty vector DNAs showed strong DNA bands, which were predominantly super-coiled (Figure 4.3, B, Lane: ***MP_1-MV_2***). Again, weak bands indicative of open circular DNA were also observed in both TGFBI plasmid (***MP_1-MP2***) and empty vector (***MV_1-MV_2***). Although there was a small amount of open circular DNA these samples were used for transfections as super-coiled DNA was predominant.

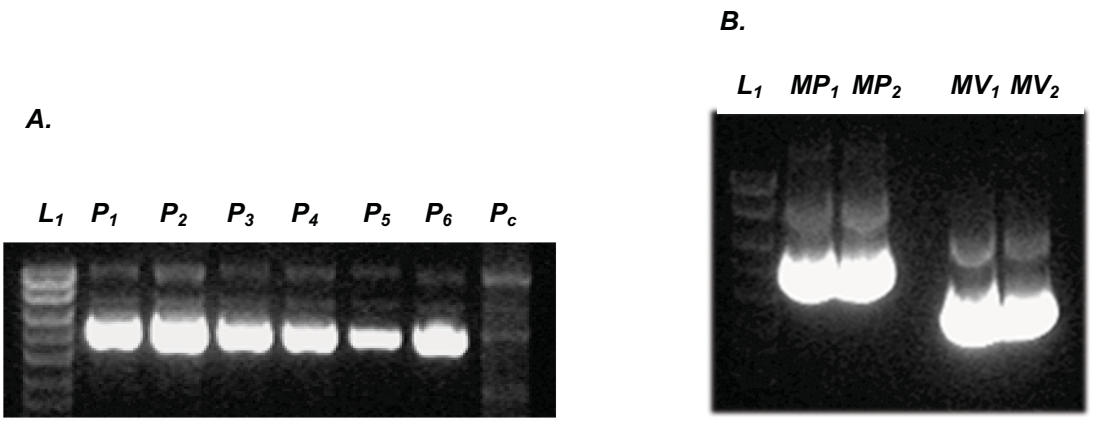


Figure 4.3 Super-coiled TGFBI Plasmid and Empty Vector for Transfection Studies

A) The Hyperladder I ***(L1)***, transformed TGFBI plasmids ***(P_1-P_6)*** that were purified using the Mini-Prep kit and positive control ***(P_C)*** were loaded into separate wells of 0.8% agarose gel. B) The plasmids ***(MP_1*** and ***MP_2)*** and empty vectors ***(MV_1*** and ***MV_2)*** DNA that were obtained using Maxi-Prep kit were also analysed using the same conditions.

PCR reactions were performed using two different pair of primers (Table 2.4), one of which was designed to detect the presence of the *TGFBI* gene in the transformed DNA plasmid, while the other confirmed the absence of the gene in the empty vector.

All the Mini-Prep TGFBI plasmids that were shown previously to be super-coiled had consistent bands (Figure 4.4, A, Lane: ***P_1-P_6***) at 103bp, similar to the original TGFBI plasmids (Figure 4.4, A, Lane: ***P_C***). This confirmed the presence of *TGFBI* gene in the purified DNA plasmid (Figure 4.4, A). Stronger bands of 103bp were observed for the Maxi-Prep TGFBI plasmids (Figure 4.4, B, Lane: ***MP_1*** and ***MP_2***), and intense bands at 214bp were seen for the Maxi-Prep empty vector DNAs (Figure 4.4, C, ***MV_1*** and ***MV_2***) (Figure 4.4, C). Clear water blanks demonstrated the lack of any contamination.

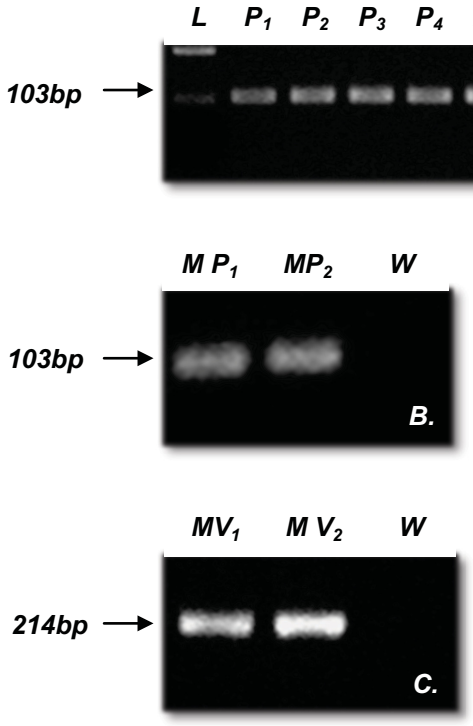


Figure 4.4 Presence/Absence of TGFBI Gene from Plasmid/Vector DNAs Using PCR

A 20µl of 100bp ladder ***(L)*** and PCR products of Mini-Prep TGFBI plasmids ***(A, P_1-P_C)***, Maxi-Prep TGFBI plasmids ***(B, MP_1*** and ***MP_2)*** and Maxi-Prep empty vector ***(C, MV_1 and MV_2)*** were loaded into separate wells of 3% agarose gel. Water blanks were also included ***(W)***.

4.3 Optimization of Transient Transfection

Two µg of DNA was used at a 1:1 ratio for each TGFBI plasmid to a GFP plasmid. Various transient transfection ratios were tested (3:2, 4:2, 5:2, 6:2, 7:2 and 8:2) to determine the suitable ratio for both cell lines (Figure 4.5, 1 and 2). 7µl of transfection reagent to 2µg of plasmid DNA was the ratio of choice for both ZR-75-1 (Figure 4.5, ***1***, A) and MDA-MB-468 (Figure 4.5, ***2***, A), due to the higher transfection efficiencies observed.

The transfection efficiency of the ZR-75-1 cell line ranged between 45-60% for all the transfection ratios. Higher efficiency was observed by increasing the transfection ratios; 3:2, 4:2, 5:2, 6:2, 7:2 and 8:2 showed efficiencies of 45%, 48%, 51%, 55%, 57% and 60% respectively. However, MDA-MB-468 generally showed low transfection efficiencies compared to ZR-75-1 cells, and ranged between 27-40%. The ratios 3:2, 4:2 and 5:2 gave 27%, 30% and 33% respectively, while with the 6:2 (37%), 7:2 (38%) and 8:2 (40%) ratios, slightly higher efficiencies were seen.

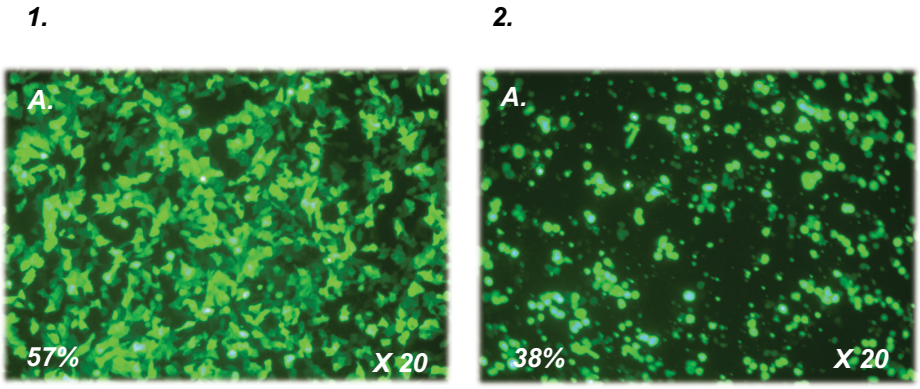


Figure 4.5 Optimization of the Transfection Ratios for Two Breast Cell Lines

7:2 transfection ratio was performed for testing the transfection efficiency of TGFBI plasmid for ZR-75-1 (1, A) and MD-AMB-468 (2, A) using 1µg of GFP and 1µg of plasmid DNA. Microscopic images (X20) were captured after overnight incubation (12-16 hours), and percentage of transfection efficiency is calculated.

ZR-75-1 and MDA-MB-468 cells were also transfected with 1µg of empty vector DNA and 1µg of GFP (1:1) using the same procedure that was used to optimize transfection with the TGFBI plasmid DNA. This was carried out to minimize differences between the cells transfected with TGFBI or empty vector. A ratio of 7:2 was selected as being optimal as it showed similar transfection efficeincy as obtained for the TGFBI plasmid DNA.

4.4 Transient Transfection of Breast Cell Lines: Confirmation of TGFBI Expression

Both ZR-75-1 and MDA-MB-468 cell lines were co-transfected separately with TGFBI plasmid and the empty vector plus GFP, at a 7:2 transfection ratio, and untreated cells

were included in each experiment. Cells were incubated overnight in complete medium to allow recovery from transfection, then cultured for 6,12, 24, 48 and 72 hours in serum free medium. Cells were harvested at each time point to investigate TGFBI RNA and protein levels using q-RT-PCR (Method *2.2.6*) and western blotting (Method *2.2.2*) respectively. Additionally, TGFBI protein levels were examined in the collected cell conditioned media.

4.4.1 TGFBI mRNA Levels in Transfected Cell Lines

A range of between 54-57% and 32-37% transfection efficiencies were obtained for ZR-75-1 and MDA-MB-468 respectively at each time point. The generated cDNAs from both ZR-75-1 (Figure 4.6, A) and MDA-MB-468 (Figure 4.6, B) were tested using manual PCR for GAPDH (350bp). Strong and consistent bands were observed for all the time points for TGFBI plasmid transfection (Figure 4.6, A and B Lane: $\boldsymbol{P_6}$-$\boldsymbol{P_{72}}$)(**+**)), empty vector transfection (Figure 4.6, A and B Lane: ***V***) and untreated cells (Figure 4.6, A and B Lane: ***U***). No bands were seen in the –RT controls (Figure 4.6, A and B Lane: ***P6-U*(-)**), additionally; clear water blanks were observed, indicating lack of contamination (Figure 4.6, A and B Lane: **W**).

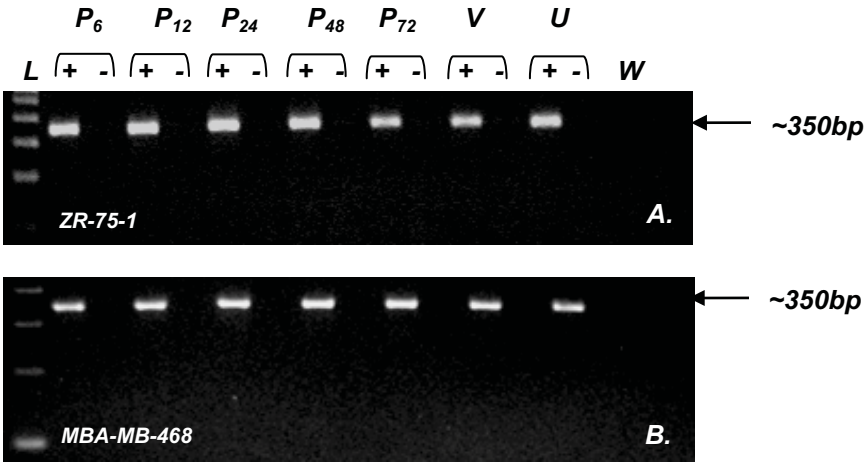


Figure 4.6 Isolated mRNA from Transfected Cells

PCR samples of the TGFBI plasmid transfection at different time points ($\boldsymbol{P_6}$-$\boldsymbol{P_{72}}$), 24-hours empty vector transfection (***V***), and the untreated cells (***U)*** of ZR-75-1 (A) and MDA-MB-468 (B). The time points represent the duration of cell culture in serum free media post incubation in complete media overnight to allow recovery from the transfection procedure (Method *2.2.11*). Twenty µl of 100bp ladder (***L***) were loaded into separate wells of 3% agarose gel examining GAPDH. Both (+) and (-) indicates the presence and absence of cDNA respectively from the PCR reactions. (***W***)= Water Blank.

Once successful mRNA isolation and generation of cDNA had been confirmed, the level of TGFBI mRNA was examined by q-RT-PCR. For ZR-75-1 (Figure 4.7, A), the lowest fluorescent cycle threshold (Ct~18.5) was observed in cells after 24 hours of culture in serum free media (Figure 4.7, ***A1***), while a Ct of 19 was demonstrated for both 6 and 12 hours time points (Figure 4.7, ***A2***). Following normalization to the mean of the 3-housekeeping genes (*GAPDH*, *HPRT1* and *TFRC*), the highest TGFBI mRNA levels (-∆Ct=3.8) were observed at 12 hours compared to 6, 24, 48 and 72 hours after culture in serum free media (Figure 4.8, (***P-12H***)). MDA-MB-468 showed the lowest cycle threshold (Ct~19.5) for both 6 and 12 hours time points (Figure 4.7, ***B1***); after normalization to the housekeeping genes. The highest level of TGFBI mRNA (-∆Ct=5.4) was at 12 hours compared to the rest of the time points (Figure 4.8, (***P-12H***)). MDA-MB-468 cells that were harvested after 24 hours of culture in serum free media showed a later Ct value (Ct~22) compared to ZR-75-1 cells. Post normalization, a slight decrease in the TGFBI mRNA (-∆Ct=4.1) was observed compared to the 6 and 12 hours time points (Figure 4.8, (***P-24H***)).

After 48 and 72 hours of culture with serum free media, ZR-75-1 showed Ct values that fell mostly between 23 and 24 cycles (Figure 4.7, ***A3***). After normalization, higher TGFBI mRNA was shown for 48 hours compared to the 72 hours (Figure 4.8, (***P-48H***)). The same was seen with MDA-MB-468 cells, which showed Ct values between 24 and 26 for 48 and 72 hours respectively (Figure 4.7, ***B3*** and ***B4***), with TGFBI mRNA levels lowest at 72 hours after normalization (Figure 4.8, (***P-72H***)).

The vector transfection and the untreated cells for both cell lines (Figure 4.7, ***A4*** and ***B5***) showed very late cycle threshold 34-38 Ct for ZR-75-1 and 32-33 Ct for MDA-MB-468), confirming very low TGFBI mRNA expression (Figure 4.8, (***V-24H*** and ***U-24H***)). No amplification was observed in the water blank controls.

In summary, both transfected ZR-75-1 and MDA-MB-468 cell lines showed highest TGFBI mRNA levels at 12 hours after culture in serum free media (Method *2.2.11*), with a marked decrease at 72 hours (Figure 4.8, (***P-12H*) *and* (*P-72H***)). Additionally, the vector and untreated controls showed very low levels of TGFBI mRNA at all time points.

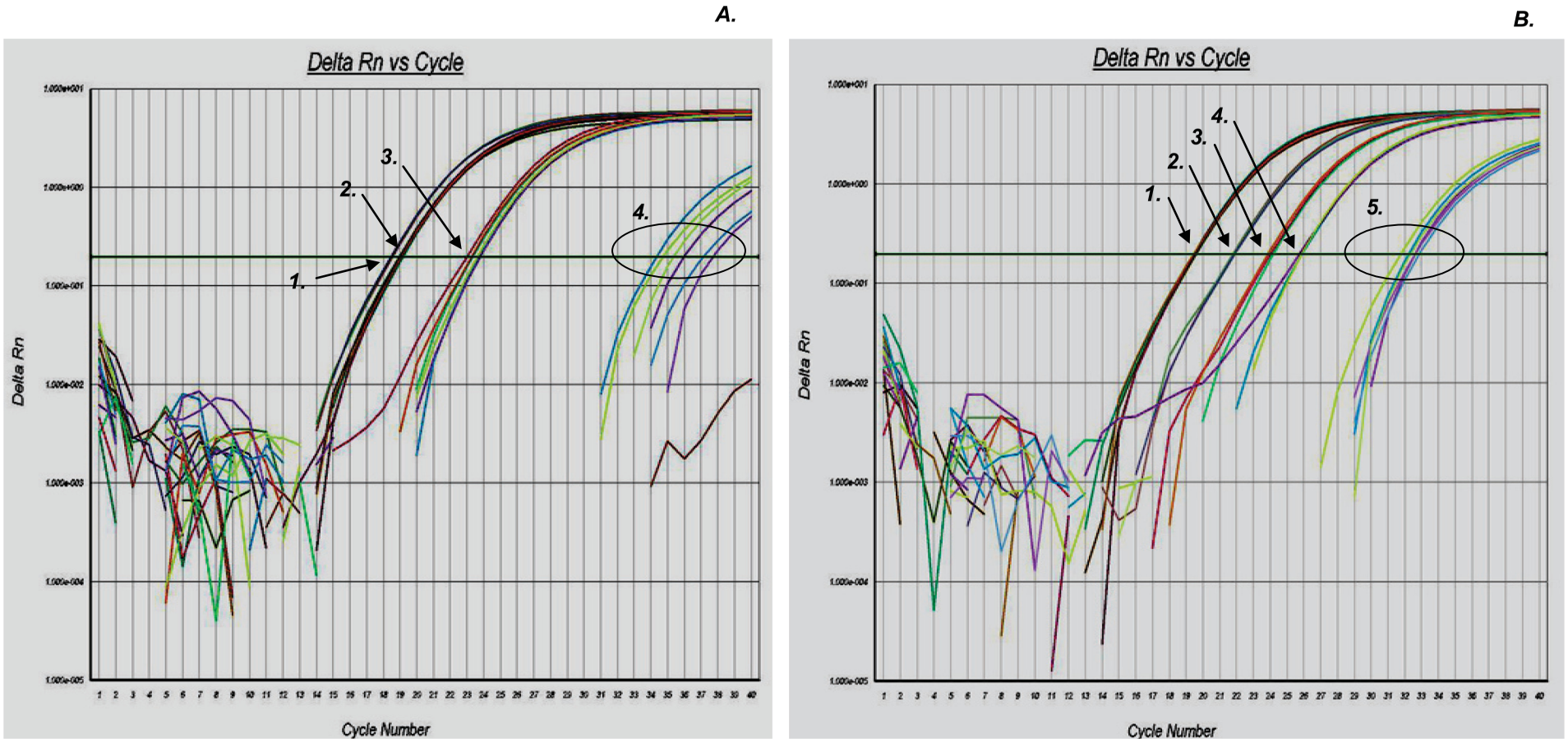


Figure 4.7 TGFBI Amplification Curves for Two Transfected Cell Lines

The ZR-75-1 q-RT-PCR amplification curve of TGFBI transfections was performed for various time points. The time points are period of culture in serum free media after overnight culture in complete media to allow recovery from transfection (***A***, 1: Ct of 24-hours, 2: Ct of 6 and 12 hours, 3: Ct of 48 and 72 hours, and 4: Ct of vector and untreated cells). The amplification curve of MDA-MB-468 cell line was also performed with the same time points described for ZR-75-1 cell line (***B***, 1: Ct of 18 and 24 hours, 2: Ct of 36 hours, 3: Ct of 60 hours 4: Ct of 84 hours and 5: Ct of vector and untreated cells).

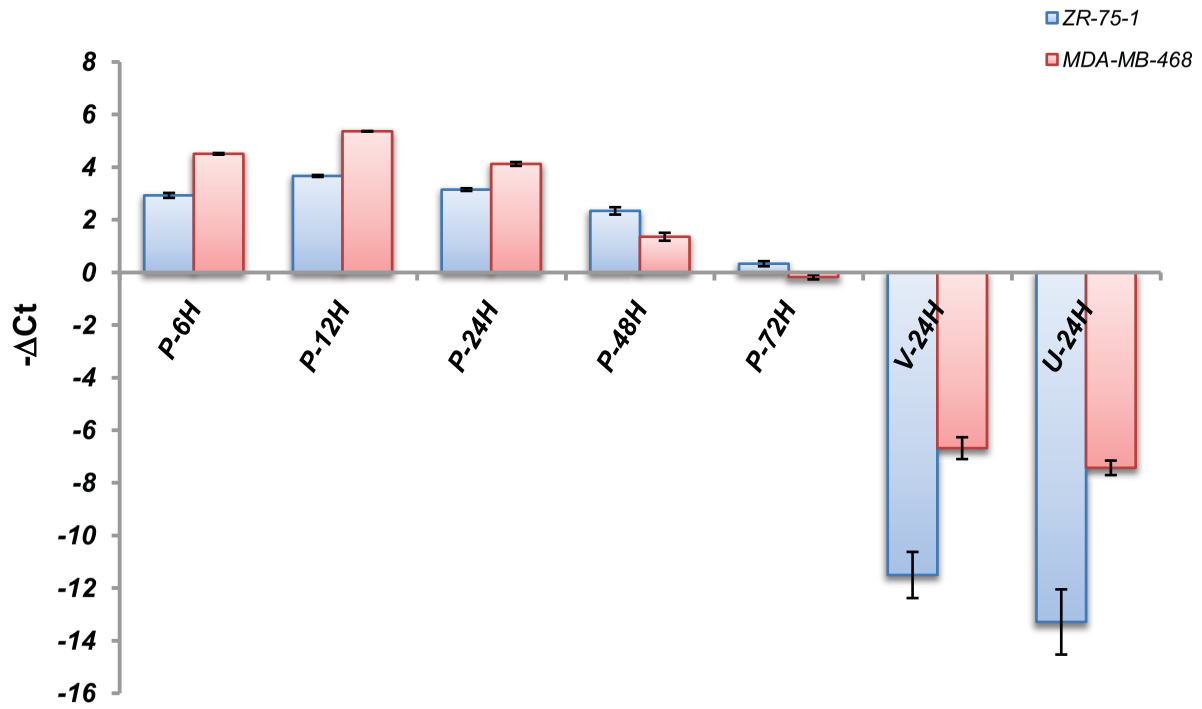


Figure 4.8 TGFBI mRNA Expression in Transfected ZR-75-1 and MDA-MB-468 Cells

TGFBI -ΔCt for both ZR-75-1 and MDA-MB-468 was obtained for different time points. The time points are the period of culture of transfected cells with serum free media post overnight recovery from transfection by culture in complete media. TGFBI plasmid transfection (6 hours: ***P-6H***, 12 hours: ***P-12H***, 24 hours: ***P-24H***, 48 hours: ***P-48H*** and 72 hours: ***P-72H***). Transfected cells with empty vector (***V-24H***) and untreated cells for 24 hours (***U-24H***) were also included. Error bars represents the standard deviation across the three triplicates.

4.4.2 TGFBI Protein Levels in Transfected Cell Lines

Western blotting was performed to investigate the TGFBI protein levels in both cells and conditioned media collected from the transfected (TGFBI plasmid and empty vector) and untreated cells (Methods *2.2.11*).

In ZR-75-1 cells, TGFBI protein (68kDa) was highly expressed after 12 hours of incubation with serum free media compared to the 6 and 24 hours time points (Figure 4.9, ***A1***), similar to the mRNA results. For MDA-MB-468 cells, similar bands were observed at both 6 and 12 hours (Figure 4.9, ***B1***). At 48 and 72 hours there were fainter bands for cell lysate analysis of both cell lines compared to stronger bands observed for the conditioned media for the same time periods (Figure 4.9, ***A1***, ***A2***, ***A3*** and ***B1***). No bands were detected in both empty vector and untreated cells and related conditioned media (Figure 4.9, ***A*** and ***B***, Lane: ***V - U and*** $\boldsymbol{M_V}$***-***$\boldsymbol{M_U}$), supporting q-RT-PCR data (Methods *2.2.11*).

Both positive (Figure 4.9, ***A1***, Lane: ***Org*** and ***B1***, Lane: $\boldsymbol{ZR_1}$) and negative (Figure 4.9, ***A1***, Lane: ***ZR*** and ***B1***, Lane: ***468***) controls were used to check the western blotting results. β-Actin (42kDa) (Figure 4.9, ***A4***, ***A5*** and ***B2***) was used to ensure equal loading (Figure 4.9, ***A4*** and ***B2***). In addition, high levels of TGFBI were detected in apoptotic MDA-MB-468 cells, which were collected from the cell culture media after 48 and 72 hours (Figure 4.9, ***A1***).

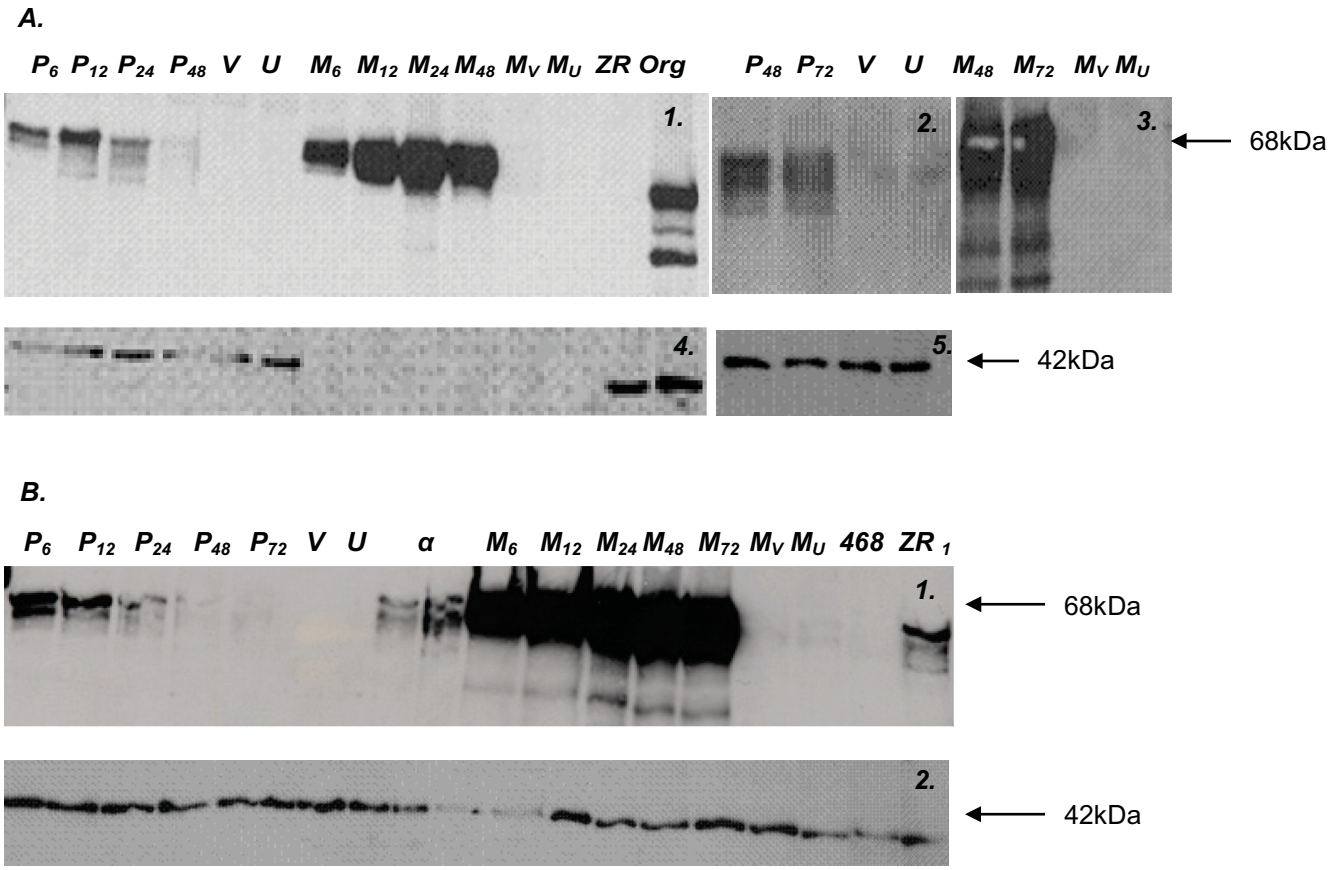


Figure 4.9 *Western Analysis of TGFBI Protein in Two Transfected Cell Lines*

The presence of TGFBI protein in the ZR-75-1 (***A1***, ***A2*** and ***A3***) and MDA-MB-468 (***B1***) was examined for the various transfection time points (***P_6-P_{72}***), empty vector (***V***) and untreated cells (***U***). The concentrated conditioned media was also tested (***M_6-M_{72}, M_V*** and ***M_U***). The time points presented are the time interval for culturing cells in serum free media after overnight recovery from transfection using complete media. Both positive (***A1***, ***Org:*** organoid and ***B1***, ***ZR1:*** 12 hours transfection) and negative controls (***A1***, ***ZR***: un-transfected ZR-75-1 and ***B1***, ***468***: un-transfected MDA-MB-468) were used. β-Actin was also tested for both ZR-75-1 (***A4*** and ***A5***) and MDA-MB-468 (***B2***). **α**: Represents two samples of cell pellet combined with floating cells in the cultured media after 48 and 72 hours time points.

4.5 Functional Studies

Three functional assays (viability, apoptosis (Caspase 3/7 activity) and invasion (TGFBI culture media)) were performed following transient transfection of TGFBI DNA plasmid into the two breast cell lines (ZR-75-1 and MDA-MB-468). The results of TGFBI plasmid was compared to the readings obtained for cells transfected with empty vector and also untreated cells.

The viability and apoptosis assays were performed three times for each cell line; for each experiment fresh transfection was undertaken. The two assays were conducted at the same time, using the same transfected or control cells to reduce variation. Readings were taken at 6, 12 and 24 hours for the viability assay, and at 6 and 12 hours for apoptosis assays. These time points are from the time of replating the cells in the appropriate plates, which was after overnight culture with complete media to allow transfection recovery. All results in both assays were normalized to the 6 hour time point to allow the data for repeat experiments to be combined. The statistical test Two Way ANOVA (Analysis of Variance) was used to check whether there was statistical difference between the transfections (TGFBI plasmid/empty vector) and time points. This test was selected because data showed normal distribution using the Shapiro-Wilk test (p>0.05).

Invasion assays were performed three times for ZR-75-1 and twice for MDA-MB-468, each in duplicate, using previously generated cell culture media from TGFBI plasmid and empty vector transfected cells (Figure 4.9). The results were normalized to the 48 hour time point and Two Way ANOVA (Analysis of Variance) statistical analysis was performed.

4.5.1 Viability Assay

Cell viability assays were performed for both ZR-75-1 and MDA-MB-468 breast cell lines (Method *2.2.9.2*). In this assay resazurin is converted to the fluorescent product resorufin by viable cells. Therefore, resorufin levels are proportional to the number of metabolically active and viable cells in the population.

4.5.1.1 Optimization of Cell Density and Culture Conditions

For MDA-MB-468 there was no variation between culture in 10% FCS and serum free media, apart from at 2x(10^4) cell density where higher fluorescent values were observed in the serum free media (0% FCS) (Figure 4.10, B). In contrast, ZR-75-1 demonstrated a noticeable reduction in the fluorescent values of the cells in serum free media compared to 10% FCS media (Figure 4.10, A). Therefore, 10% FCS media was used to perform the viability experiments for both cell lines to minimize stress related reduction in cell viability.

Various cell densities ($5x10^3$, $1x10^4$ and $2x10^4$) were tested for both ZR-75-1 (Figure 4.10, A) and MDA-MB-468 (Figure 4.10, B). Both $5x10^3$ and $1x10^4$ cell densities showed similar fluorescent values across the triplicates for both cell lines compared to the higher density ($2x10^4$) after 48 hours incubation. The $2x10^4$ cell density showed very high fluorescent values with wide variation for both cell lines. For MDA-MB-468 cells the variation between triplicates for both $5x10^3$ and $1x10^4$ cell densities were low (Figure 4.10, B). ZR-5-1 cells had a lower variation between triplicates at $5x10^3$ cell density but also low fluorescent values, so despite the variation seen at $1x10^4$ (Figure 4.10, A) this was selected since fluorescent values were higher.

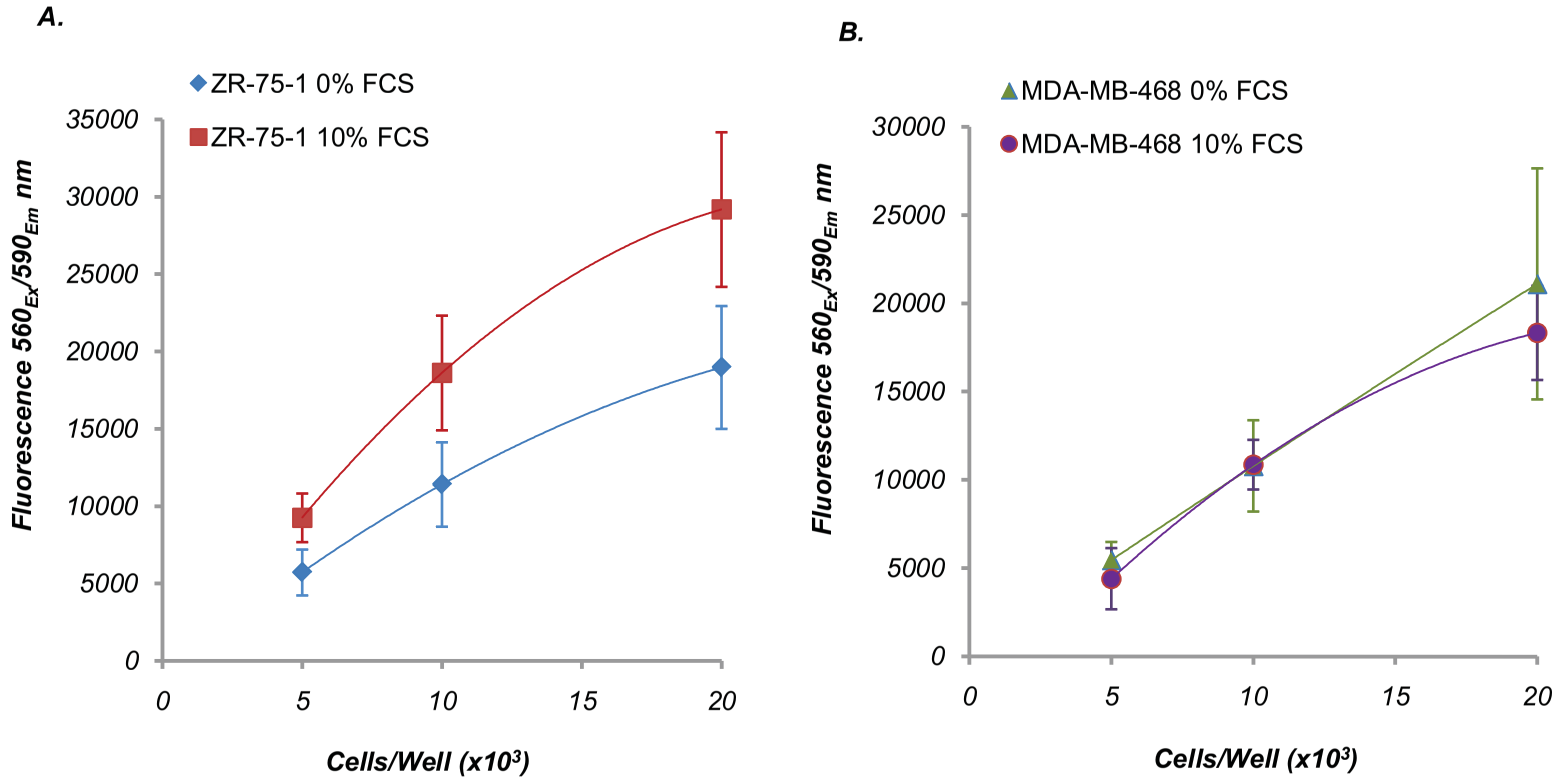


Figure 4.10 Effects of FCS and Various Densities on the Fluorescent Readings

Both cell lines ZR-75-1 (A) and MDA-MB-468 (B) were seeded in three different densities (5, 10 and 20 x(10^3)) in triplicates, with serum free (0% FCS) or complete media (10% FCS) to measure the resazurin that was converted to fluorescent resorufin product by viable cells. Readings were obtained after 48 hours of replating the cells. Results are presented as (mean ± standard deviation).

Cell viability assays were performed three times, with each experiment in triplicate. ZR-75-1 and MDA-MB-468 cells were transfected with TGFBI plasmid or empty vector, and untreated cells were included in each repeat. Readings were obtained at 6, 12 and 24 hours post replating the cells, following overnight culture with complete media to reduce changes due to the transfection (Method *2.2.9.2*). The fluorescent product resorufin was measured at fluorescence 560Ex/590Em nm.

4.5.1.2 ZR-75-1 Cell Viability Assays

The transfection efficiencies of the TGFBI plasmid DNA in the three separate experiments were 54%, 56% and 59% respectively. The three individual experiments (Figure 4.11, 1-3) showed that transfecting ZR-75-1 cells with TGFBI plasmid had no significant effect on the cell viability compared to cells transfected with the empty vector or untreated cells at the three time points investigated (6, 12 and 24 hours). The same was found for the mean of the three experiments (Figure 4.11, 4).

All three experiments and their mean showed an increase in the ZR-75-1 cell viability with time, irrespective of their treatment, particularly between 6 hour and 24 hour time points (Two Way ANOVA, $p<0.05$). Only the first experiment and the overall mean demonstrated a significant increase in cell viability between 12 and 24 hours time points (Figure 4.11, 1 and 4).

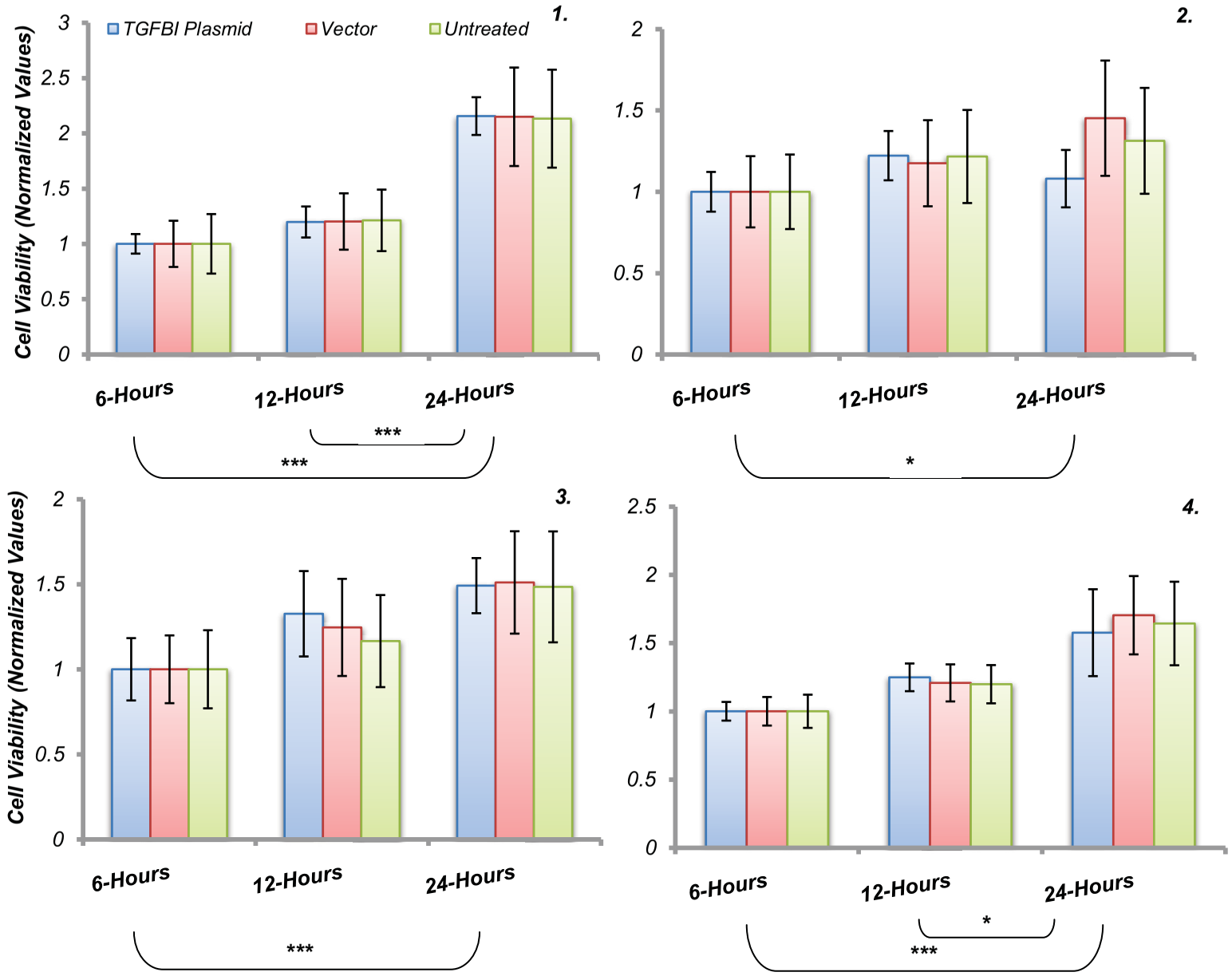


Figure 4.11 Viability Assays of Transfected ZR-75-1 Cell Line

Each experiment (1, 2 and 3) was carried out in triplicate with a new transfection. The time points presented are the times post replating cells in their appropriate plates. The cell replating procedure was performed after overnight recovery of cells from transfection (approximately 12 hours in complete media). The viability data is presented as normalized values to the mean readings at the 6-hour time point. The error bars in the three experiments (1-3) and in the overall mean (4) represent the 95% confidence interval.

Results were normally distributed according to the results obtained by Shapiro-Wilk test. Thus Two Way ANOVA (Analysis of Variance) with Tukey Post Hoc and Bonferroni tests were conducted. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

4.5.1.3 MDA-MB-468 Cell Viability Assays

Thirty percent, 40% and 32% transfection efficiency was obtained for the first, second and third experiment respectively. The three experiments showed no difference in the cell viability between cells transfected with TGFBI plasmid and transfected with empty vector, and also no difference to untreated cells (Figure 4.12, 1-3).

Only the third experiment showed a significant increase in the viability of MDA-MB-468 cells between 6 and 24 hours time points (Figure 4.12, 3).

The overall mean of the three experiments confirmed that there was no difference in cell viability between MDA-MB-468 cell transfected with TGFBI plasmid compared to cells transfected with empty vector and untreated cells at any time point. However, significant increase in the cell viability between 6 and 24 hour time points was confirmed (Figure 4.12, 4).

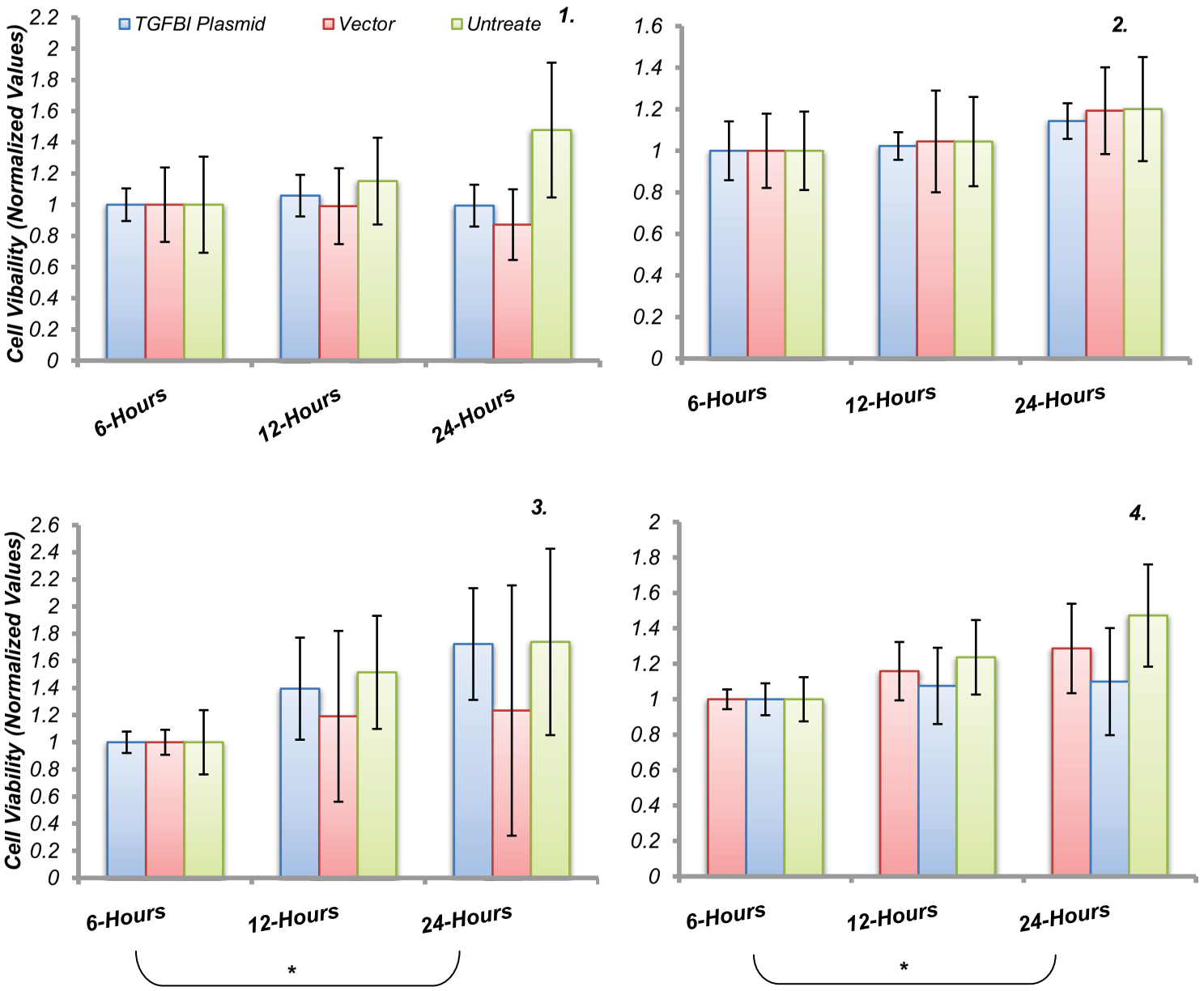


Figure 4.12 *Viability Assays of Transfected MDA-MB-468 Cell Line*

Each experiment (1, 2 and 3) was carried out in triplicate with a new transfection. The time points presented are the times post replating cells in their appropriate plates. The cell replating procedure was performed after overnight recovery of cells from transfection (approximately 12 hours in complete media). The viability data is presented as normalized values to the mean readings at the 6-hour time point. The error bars in the three experiments (1-3) and in the overall mean (4) represent the 95% confidence interval.

Results were normally distributed according to the results obtained by Shapiro-Wilk test. Thus Two Way ANOVA (Analysis of Variance) with Tukey Post Hoc and Bonferroni tests were conducted. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

4.5.1.4 DAPI Staining of Transfected Cell Lines

Because of the variability in the readings obtained across the triplicates in each experiment using the plate reader method, particularly for MDA-MB-468 cells, an alternative method (DAPI staining) was investigated as this method was established in our collaborators group (Dr E. Tulchinsky). DAPI staining showed no significant difference in the number of viable cells between cells transfected with TGFBI plasmid and empty vector, and also with untreated cells at all time points (6, 24 and 48 hours) (p>0.05, Two Way ANOVA) for both ZR-75-1 (Figure 4.13, A) and MDA-MB-468 (Figure 4.13, B). A significant elevation in cell viability with time was observed in both cell lines, particularly between 6 and 24 hours and also between 24 and 48 hours. This method includes several steps with the potential to introduce error. Different time points were also performed compared to the previous method and it was only carried out once; therefore, these results are only preliminary data.

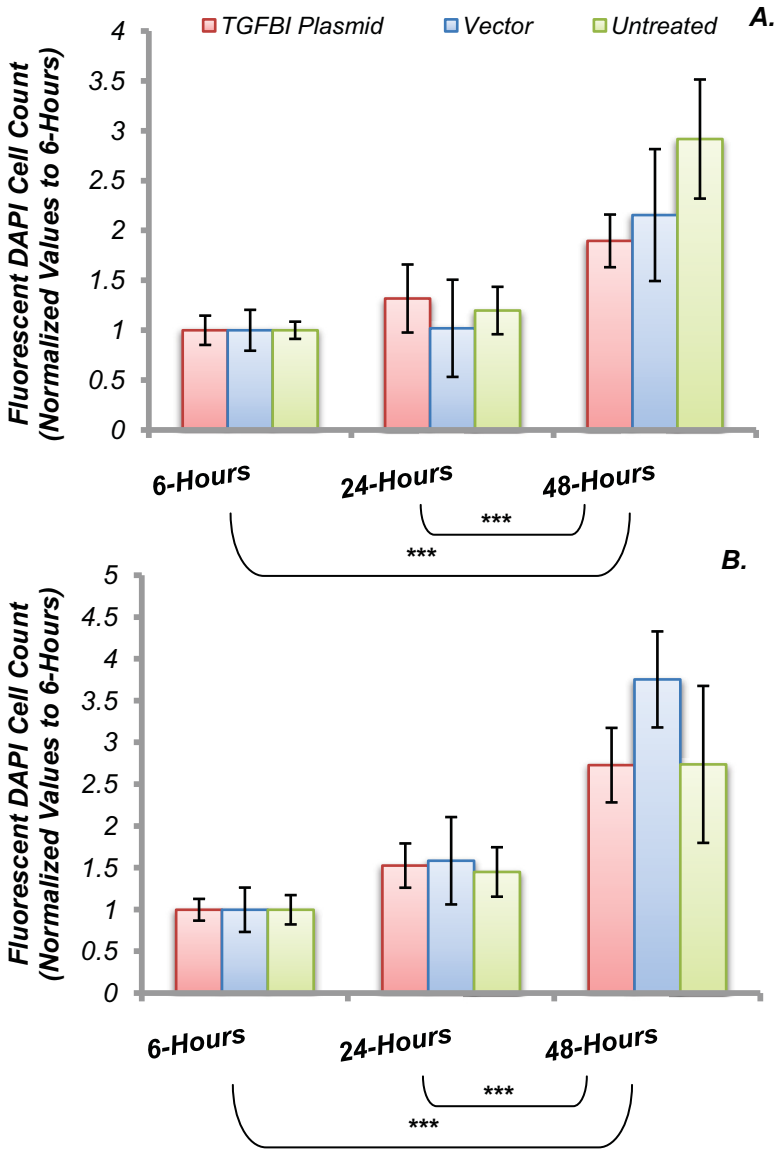


Figure 4.13 DAPI Staining for Two Cell Lines

ZR-75-1 (A) and MDA-MB-468 (B) cells were stained with DAPI at 6, 24 and 48 hours after replating cells in the appropriate plates. The cells replating was performed post overnight incubation in complete media after transfection. Stained cells were counted in three different fields, and then each value was normalized to the mean of 6-hour time point. The error bars represent the 95% confidence interval.

Results were normally distributed according to the results obtained by Shapiro-Wilk test. Thus Two Way ANOVA (Analysis of Variance) with Tukey Post Hoc and Bonferroni

4.5.1.5 Summary of Cell Viability Results

In summary, transfection with TGFBI appeared to have no significant influence on the viability of ZR-75-1 and MDA-MB-468 cells. Both cell lines, mainly ZR-75-1, showed significant increase of viability with time. These results were found with both the resorufin cell viability assay and DAPI staining.

4.5.2 Apoptosis Assay

The effect of transient transfection with TGFBI on apoptosis was performed, as in a previous study transfection with TGFBI in the H522 lung cell line had significantly increased apoptosis compared to un-transfected cells (Zhao *et al*., 2006). The Caspase-Glo® 3/7 (Promega) assay was used to investigate whether transient transfection with TGFBI plasmid altered levels of apoptosis in the breast cell lines (ZR-75-1 and MDA-MB-468) compared to transfected empty vector and untreated cells.

4.5.2.1 Optimization of Cell Densities and Culture Conditions

Various cell densities ($5x10^3$, $1x10^4$ and $2x10^4$) were examined for both ZR-75-1 (Figure 4.14, A) and MDA-MB-468 (Figure 4.14, B) cell lines. Low luminescent signals (Caspase-Glo® 3/7) were observed at $1x10^4$ compared to $2x10^4$, whereas values were slightly higher for the $5x10^3$ cell density for both ZR-75-1 and MDA-MB-468. The $1x10^4$ cell density was used for the rest of the experiments, as it showed consistency across the triplicates with low standard deviations for both cell lines.

The presence or absence of FCS in the media had no significant effect on the luminescent values for ZR-75-1 cells (Figure 4.14, A); however, a slight increase was found in apoptosis in the MDA-MB-468 cells in the absence of FCS (Figure 4.14, B). Media with 10% FCS was selected to carry out the rest of the apoptosis assays, to reduce additional stress of transfected cells induced by withdrawal of FCS.

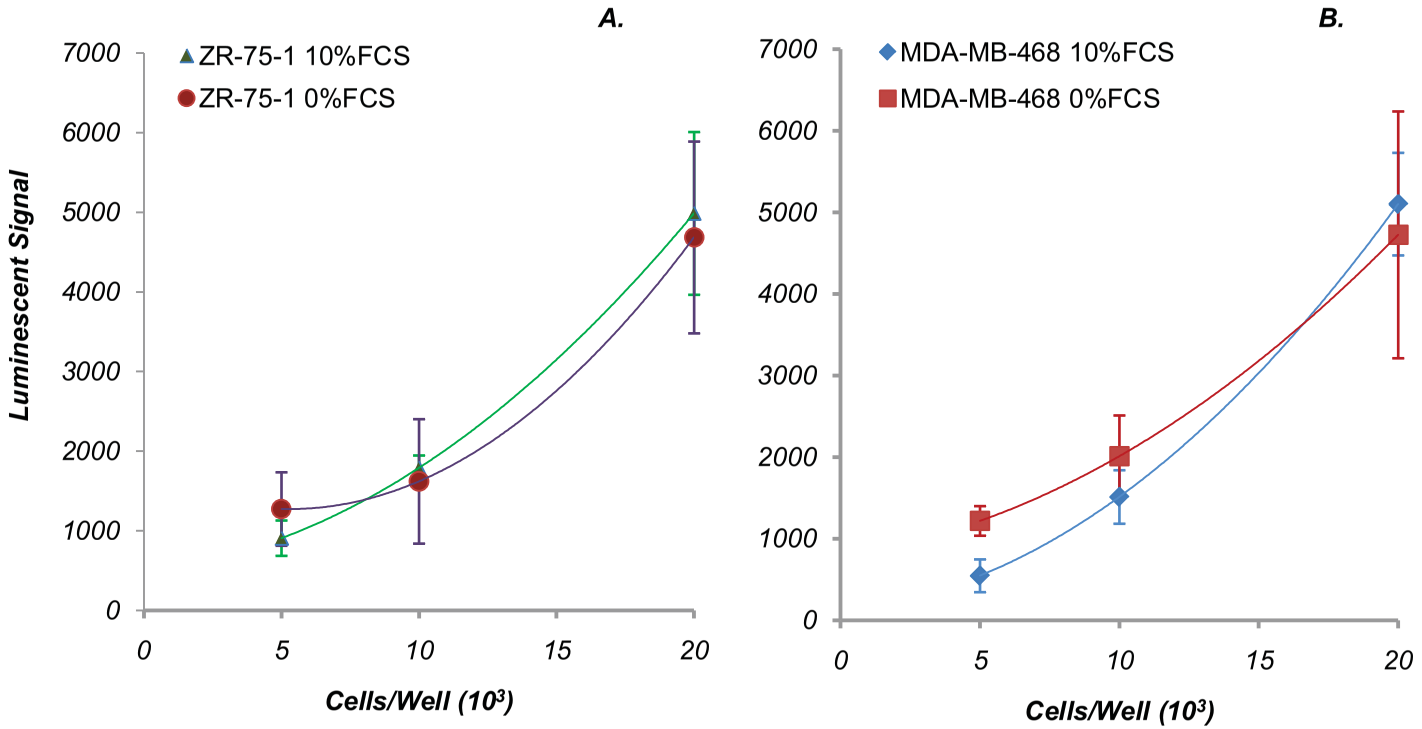


Figure 4.14 Effects of Cell Density and Various FCS on Caspase-Glu® 3/7 Luminescent Signal

Both cell lines ZR-75-1 (A) and MDA-MB-468 (B) were tested at three densities (5, 10, 20 x(10^3)) in triplicates with serum free (0% FCS) or complete media (10% FCS) to measure the luminescence signal generated, which is directly proportional to the amount of Caspase-Glu® 3/7 activity. Readings were obtained 48 hours post replating cells. Results are presented as Mean± Standard deviation across the triplicates.

Apoptosis assays were performed using the same transfected ZR-5-1 (Figure 4.11, 1-3) and MDA-MB-468 (Figure 4.12, 1-3) cells that were used in the viability assays. Two time points were compared in this assay (6 and 12 hours).

4.5.2.2 ZR-75-1 Apoptosis Assays

For the three experiments performed (Figure 4.15, 1 and 2), there was no significant influence on apoptosis of ZR-75-1 cells transfected with TGFBI plasmid compared to cells transfected with the empty vector and untreated cells (Two Way ANOVA, $p>0.05$). No statistically significant difference was found in the luminescent signal between the three groups of ZR-75-1 cells when the mean of the three experiments was analysed (Figure 4.15, 4).

The Staurosporine positive control included in the three experiments showed no increase in apoptosis of ZR-75-1 cells with time (Figure 4.15, 1-3). There was wide variability in the effect of Staurosporine between the triplicates in the second experiment at the 12 hour time point (Figure 4.15, 2).

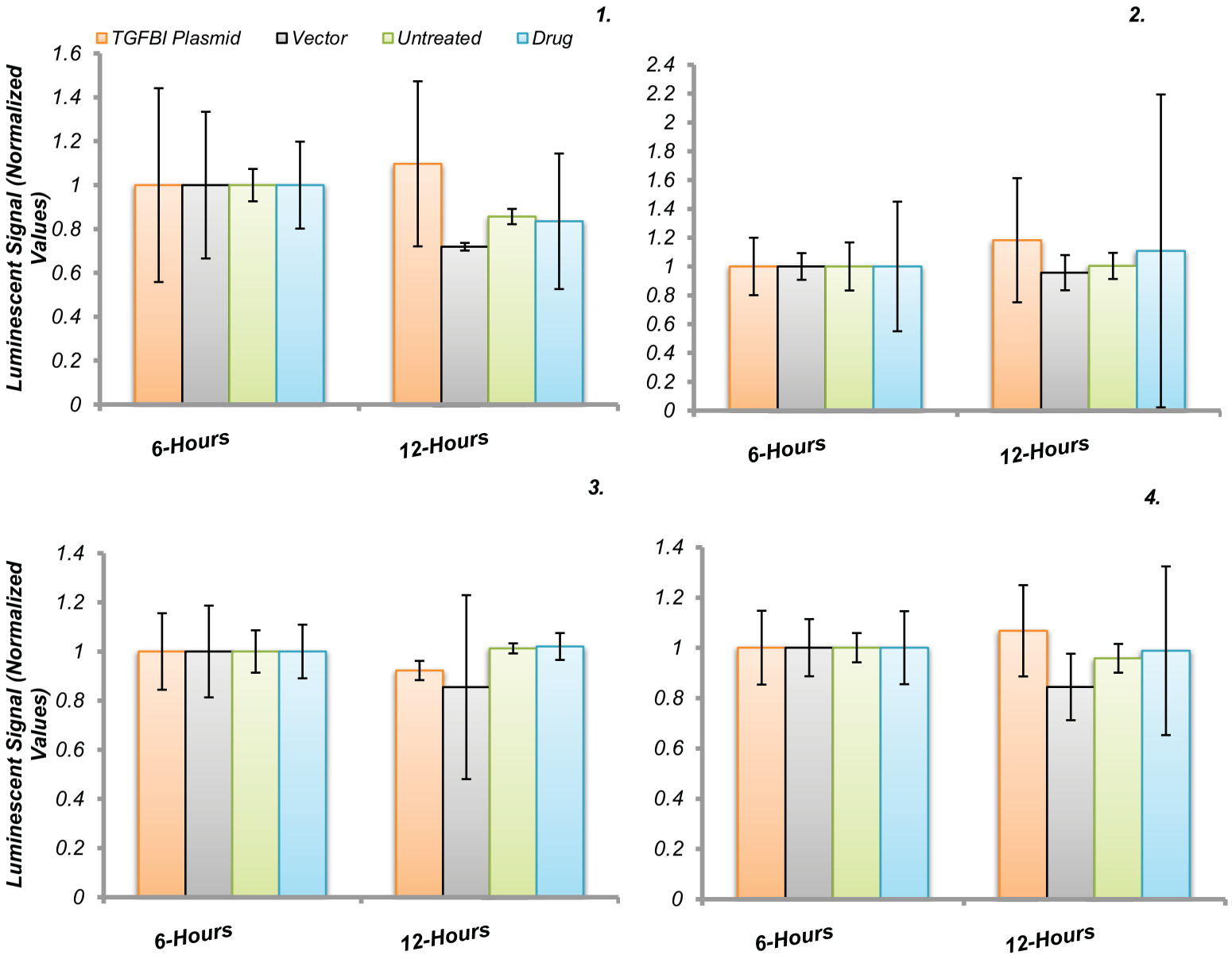


Figure 4.15 Investigating Apoptosis in TGFBI Transfected ZR-75-1

The apoptosis assays were independently performed three times (1, 2 and 3) to measure caspase 3/7 activity. Cells used in each experiment were the same transfected and control cells used in the viability assays (new transfection for each repeat). Time points represents the times post replating cells in their appropriate plates. The cells replating was performed after overnight recovery of cells from transfection (approximately 12 hours).

Positive control was included in each experiment and at each time point. This was un-transfected cells treated with Staurosporine drug (1µg/100µl). All data presented as normalized values to the mean of 6-hours time point. The error bars in three experiments (1-3) and in the mean of the experiments (4) represent the 95% confidence interval.

Results were normally distributed according to the results obtained by Shapiro-Wilk test. Thus Two Way ANOVA (Analysis of Variance) with Tukey Post Hoc and Bonferroni tests were conducted. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

4.5.2.3 MDA-MB-468 Apoptosis Assays

The three experiments (Figure 4.16, 1-3) showed no significant influence on caspase 3/7 activity in the MDA-MB-468 transfected with TGFBI plasmid in comparison to cells transfected with the empty vector and untreated cells (Two-Way ANOVA/Analysis of Variance, $p>0.05$). However, there was a significant increase in the luminescent signal between 6 and 12 hour time points in the three experiments but different levels of significance were found. The second experiment had the lowest significance (Two-Way ANOVA, $p<0.05$), whilst the first and third experiments showed strong correlation ($p<0.001$) between apoptosis and time (Figure 4.16, 1 and 3) for transfected and untreated cells.

The positive control cells treated with Staurosporine drug showed significant increase in apoptosis with time apart from experiment 1, where high 95% confidence interval was observed between the triplicates (Figure 4.16, 1).

The mean of the three experiments showed there to be a significant relationship between the increased number of apoptotic MDA-MB-468 cells and time, but with no effect of TGFBI on apoptosis (Figure 4.16, 4).

4.5.2.4 Summary of Apoptosis Results

Transfection with TGFBI had no influence in apoptosis of ZR-75-1 and MDA-MB-468 cells in the three replicate experiments. Apoptosis significantly increased with time for MDA-MB-468 cell line irrespective of TGFBI transfection.

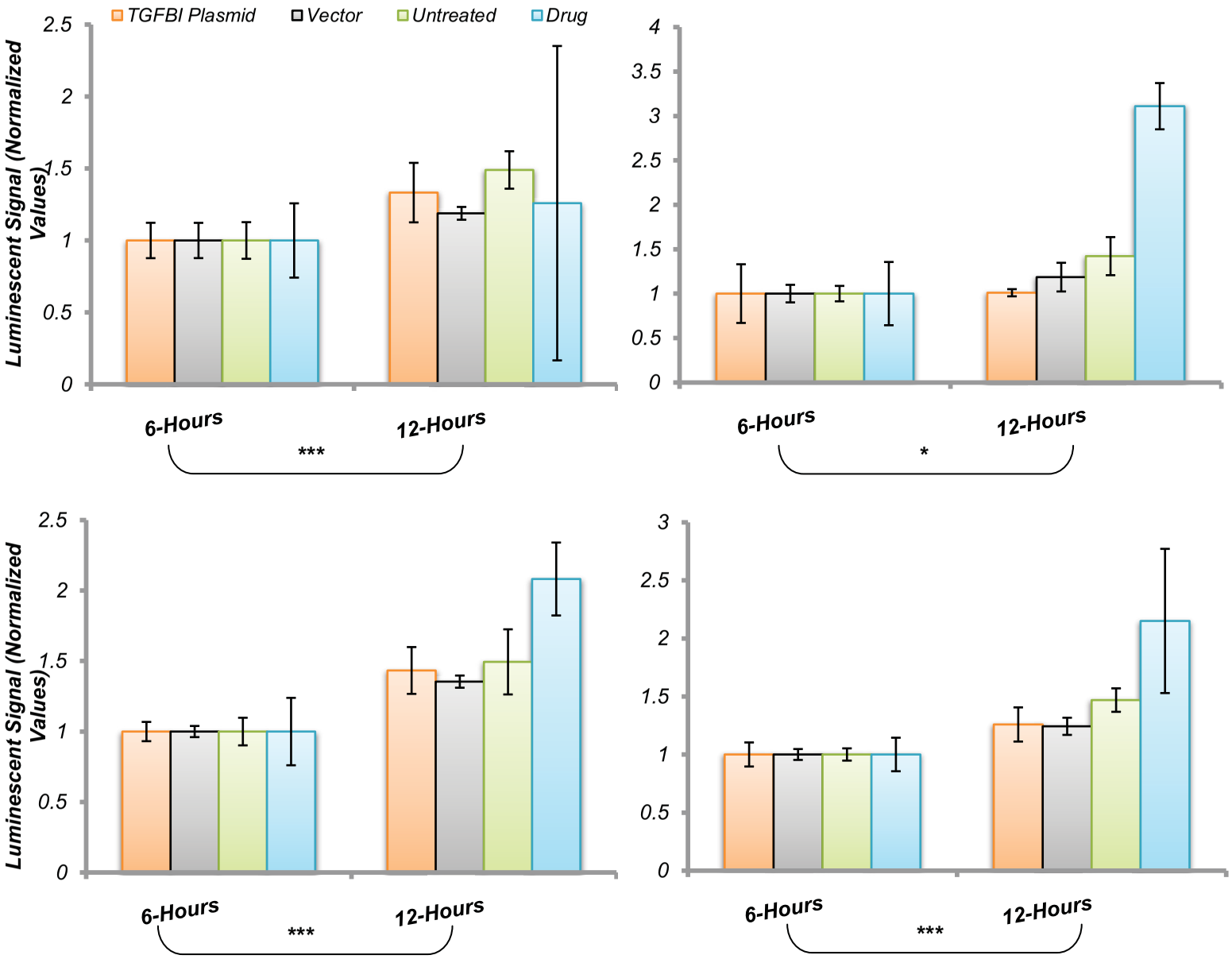


Figure 4.16 Investigating Apoptosis in TGFBI Transfected MDA-MB-468

The apoptosis assays were independently performed three times (1, 2 and 3) to measure caspase 3/7 activity. Cells used in each experiment were the same transfected and control cells used in the viability assays (new transfection for each repeat). Time points represents the times post replating cells in their appropriate plates. The cells replating was performed after overnight recovery of cells from transfection (approximately 12 hours).

Positive control was included in each experiment and at each time point. This was un-transfected cells treated with Staurosporine drug (1µg/100µl). All data presented as normalized values to the mean of 6-hours time point. The error bars in three experiments (1-3) and in the mean of the experiments (4) represent the 95% confidence interval.

Results were normally distributed according to the results obtained by Shapiro-Wilk test. Thus Two Way ANOVA (Analysis of Variance) with Tukey Post Hoc and Bonferroni tests were conducted. * $p<0.05$, ** $p<0.01$ and ***$p<0.001$.

4.5.3 Invasion Assays

Invasion assays were carried out to examine whether culture with TGFBI conditioned media has an influence on the invasion of the two breast cell lines (ZR-75-1 and MDA-MB-468), as demonstrated previously for colon cancer cells *in-vivo* (Ma *et al*., 2008). For optimization, three cell densities ($1x10^4$, $2x10^4$ and $1x10^5$) were assessed for invasion of both cell lines, each in triplicate, to determine the most appropriate density for subsequent experiments

4.5.3.1 Optimization of Cell Density

Three cell densities were compared ($5x10^3$, $2x10^4$ and $1x10^5$) for untreated ZR-75-1 (Figure 4.17, A) and MDA-MB-468 (Figure 4.17, B) cells.

The $2x10^4$ cell density was used for the rest of the invasion assays for both cell lines. This choice was based on the reliable and similar fluorescent values obtained for both cell lines, with in the best consistency across the triplicates, and low standard deviation for both cell lines (Figure 4.17, A and B).

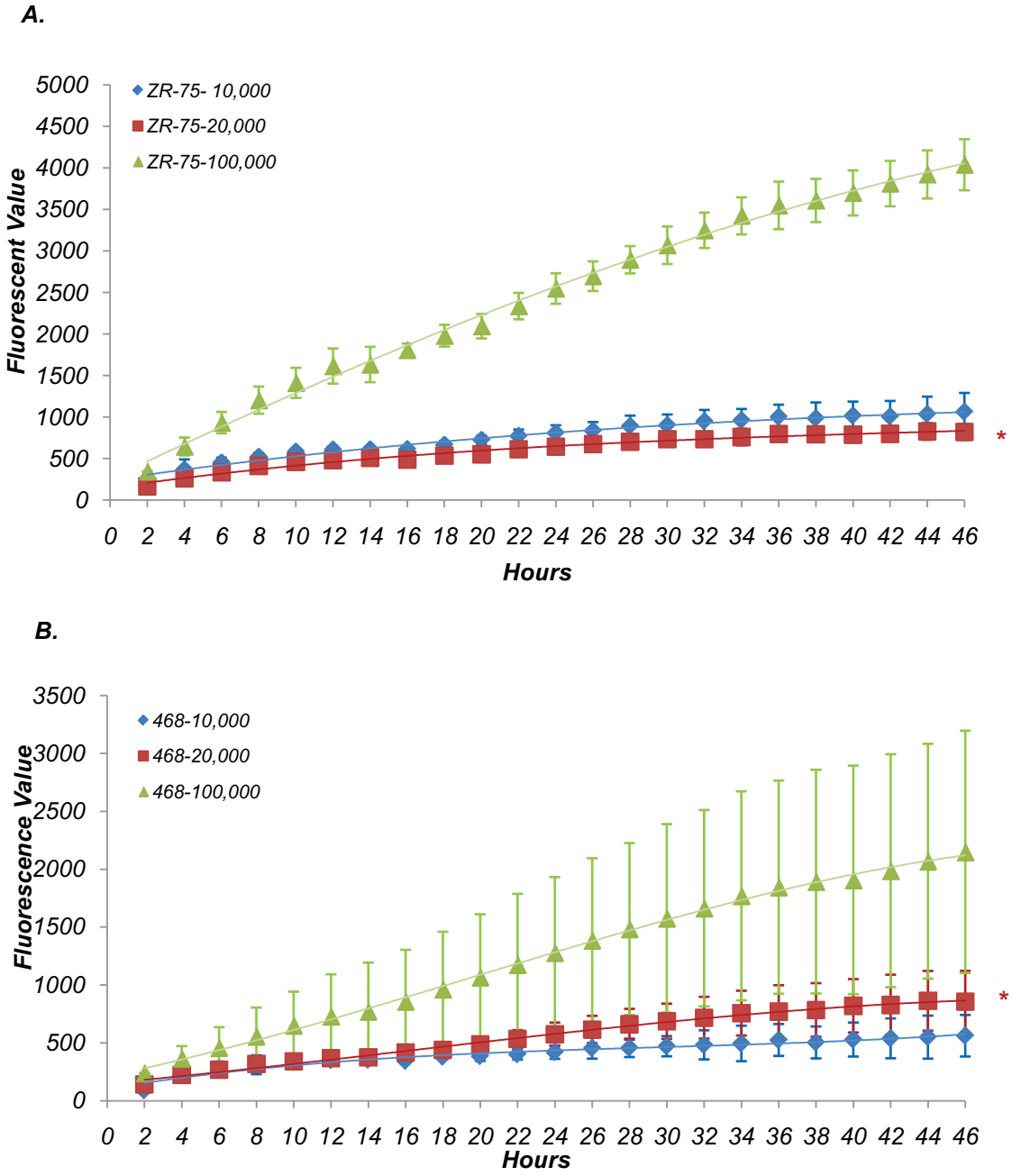


Figure 4.17 Optimization of Cell Density for Invasion Assays

Three different densities ($1x10^4$, $2x10^4$ and $1x10^5$) were tested for both ZR-75-1 (A) and MDA-MB-468 (B) to measure the invasion of cells through the matrigel. The invasion assay was performed for 48 hours, were readings taken every 2 hours. (*) the optimum density used in the rest of the invasion assays.

The effect of TGFBI conditioned media on invasion was monitored over 48 hours using the modified Boyden chamber assay, which is applicable to a plate reader. The invasion assay was performed three times for ZR-75-1 and twice for MDA-MB-468, each in duplicate using the TGFBI conditioned media collected 72 hours after transfection. This was used because TGFBI protein was shown to be at a high level in

the conditioned media at this time point (Figure 4.9). No TGFBI protein was found in the conditioned media of cells transfected with empty vector; therefore, it was used as a negative control in order to normalize the invasion results of TGFBI induced conditioned media in each repeat.

As, the conditioned media alone induced very low invasion; 1% FCS was added to enhance the invasion of both cell lines, as has been shown previously in the group (Guttery *et al*. 2010).

4.5.3.2 ZR-75-1 Invasion Assays

The three experiments were performed by using the conditioned media that was collected from TGFBI/empty vector transfected cells after incubating in serum free media for 72 hours (Figure 4.9). The TGFBI conditioned media resulted in a slight increase in cell invasion with time compared to the empty vector (Figure 4.18, A), however, this was not statistically significant (Two Way ANOVA, p=0.16).

4.5.3.3 MDA-MB-468 Invasion Assays

The mean of the two experiments, each performed in duplicate for the invasion assay of MDA-MB-468 cells are shown in Figure 4.18, B. TGFBI protein had no significant effect on the invasion of MDA-MB-468 cells (Two Way ANOVA, p=0.20). Both experiments showed variation across the duplicate readings, which resulted in wide SEM at all time points.

4.5.3.4 Summary of the Invasion Results

Culture of both ZR-75-1 and MDA-MB-468 cells with TGFBI cell conditioned media showed no statistically significant affect on invasion.

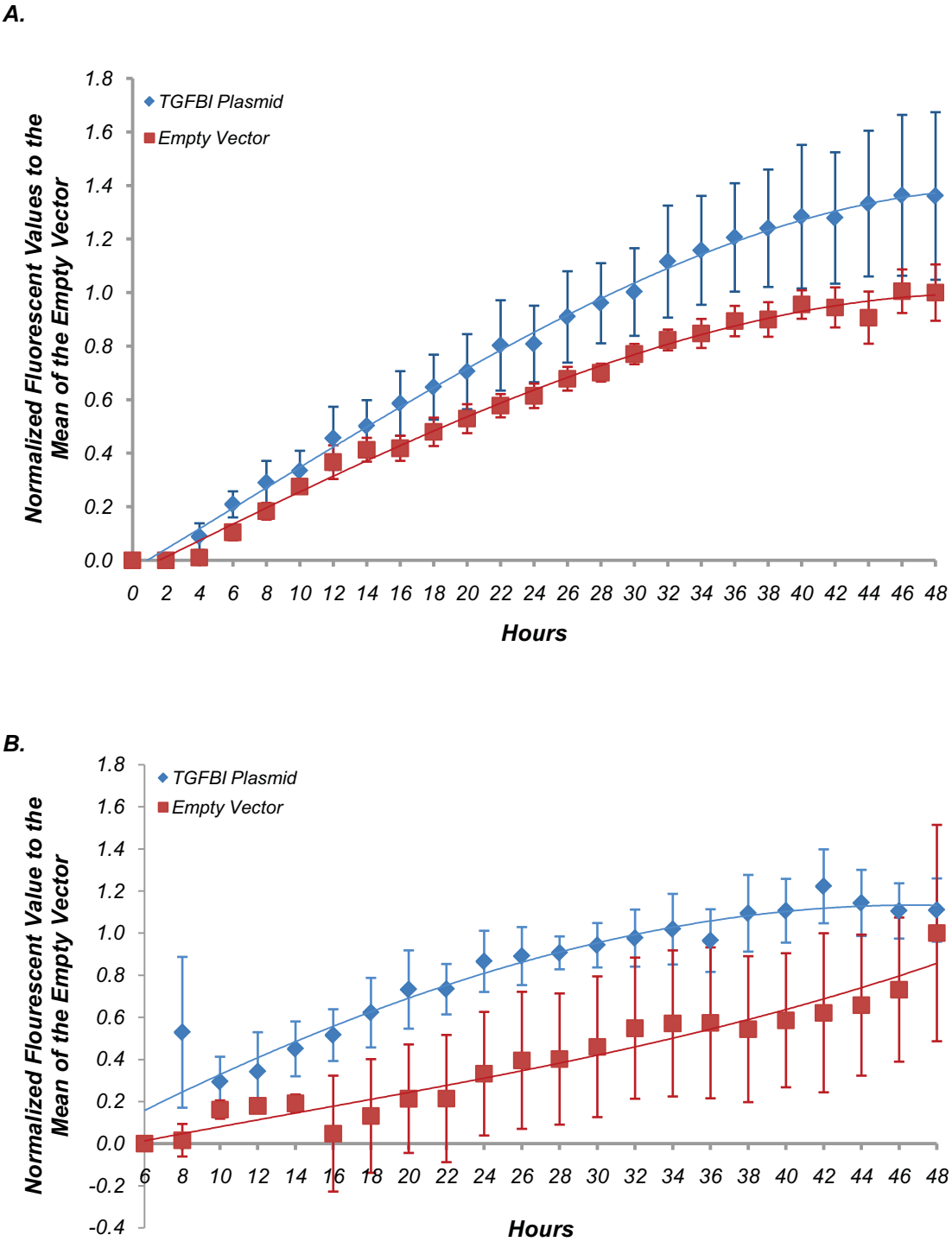


Figure 4.18 Invasion Assay Results Using TGFBI Conditioned Media

Three experiments of invasion for ZR-75-1 (A) and two experiments for MDA-MB-468 (B) cell lines were performed. Fluorescent value readings obtained from the presence of TGFBI in conditioned media were normalized to the mean value (mean of the duplicate readings) of the empty vector for each experiment (Exp 1, Exp2 and Exp3) at 48 hour time point. These graphs represent the mean of the experiments. The error bars represent the SEM across the experiments. Results were normally distributed; therefore, Two Way ANOVA was performed for statistical analysis.

Discussion

Optimizations and Limitations

Transfection

Both breast cancer cell lines MDA-MB-468 and ZR-75-1 were transfected with 7:2 ratio of Fugene and plasmid DNA using co-transfection with TGFBI plasmid plus GFP to check transfection efficiencies. In general, the MDA-MB-468 cell line showed a low and variable transfection efficiency (30-40%) compared to ZR-75-1 cells (50-60%), which might be a sign of cytotoxicity that occurs due to the high levels of transfected complex formation (Jacobsen *et al*., 2004). This variability might also be associated with the differences in characteristics and nature of cell lines, with ZR-75-1 cells being ER and PR positive and HER-2 negative but MDA-MB-468 cells being ER, PR and HER-2 negative (Table 2.1).

Functional Assays

Three functional assays were performed to determine the role of TGFBI in breast cancer cells. Both viability and apoptosis assays were carried out in triplicate in each experiment and 3 wells containing only media (background wells/negative controls) were also included to check for possible interference of media with assay chemistry. The invasion assay was conducted for ZR-75-1 and MDA-MB-468 twice each in duplicate.

Three groups of cells were used in the viability and apoptosis assays: transfected TGFBI plasmid cells, transfected empty vector cells and untreated cells. In the invasion assays un-transfected cells were used, with conditioned media that included or did not include TGFBI protein with 1% FCS. Equal cell density for all samples was confirmed by plating equal number of cells in each well post counting using the Hemocytometer (Method *2.2.1.2*).

Staurosporine is a potent inhibitor of cell growth of several human tumour cell lines, including breast cancer, by inducing cell cycle arrest at G1 phase (Pollack *et al*., 1996). This occurs as a result of an accumulation of dephosphorylated retinoblastoma protein, which has significant effect on increasing the apoptosis levels of cancer cells (Gescher, 1998; Abe *et al*., 2001). Staurosporine was used as a positive control in the apoptosis assays to ensure that the assays were working correctly and also to determine the ability to induce apoptosis in the two cell lines. ZR-75-1 cells showed resistance to the

drug at the 12 hour time point in the three experiments in contrast to MDA-MB-468 cells. This difference in response to Staurosporine might depend on the pRb function in ZR-75-1 and MDA-MB-468 cells; it has been suggested that cell lines with intact pRb are more likely to be resistant to Starosporine (Zhou *et al*., 2002). It has been demonstrated that MDA-MB-468 cells lack pRb, which make them more sensitive to UV radiation compared to cells with wild-type pRb such as ZR-75-1 (Carlson and Ethier, 2000; Billecke *et al*., 2002). Thus this might explain the reason behind the high levels of apoptosis observed in MDA-MB-468 compared to ZR-75-1 post treatment with Staurosporine. No positive control was used in the viability assays because un-transfected cells were grown in media with 10% FCS for both cell lines and so they were used as controls.

The results obtained for both viability and apoptosis at different time points were expressed relative to the 6 hour time point for the transfected and un-transfected cells to allow the data of the three experiments to be combined. The background (negative control) well result was subtracted from the test well results that were present in the same column. This was undertaken because it had been noticed that there was an increase in the readings across the columns, which might relate to a technical problems with the plate reader readings.

In some of the experiments conducted using the plate reader and DAPI, wide variations in the triplicates were observed. This could have been as a result of technical variations such as culture conditions, counting and seeding.

The invasion assays were performed based on a modified Boyden chamber assay to investigate whether TGFBI protein has a critical impact on the invasion of breast cancer cells. Both cell lines showed low levels of invasion. This result was also observed by Hancox *et al*., (2009), which showed very low Mean Invasion Index (MII 6%) for MDA-MB-468 using complete media. The lower levels of invasion than expected in this thesis might be due to the use of conditioned media with 1% FCS rather than complete media. Conditioned media with different percentages of FCS have a significant influence on invasion levels of breast cancer cell lines (Guttery et al. 2010).

TGFBI and Breast Cancer

Induction of TGFBI

Although the main focus of this chapter was the assessment of TGFBI function in breast cancer cells the induction of TGFBI by TGF-β_1 was considered in MCF-7 cells. It was found that a high concentration of TGF-β_1 induced TGFBI protein in MCF-7 cells after 72 hours of incubation in serum free media (See Method *2.2.11*), which was subsequently secreted into the culture medium, as detected by western blotting.

The induction of TGFBI protein by the treatment of cells with TGF-β_1 has been demonstrated in a number of studies (Introduction, Table 1.4). Shao *et al*., 2006 proposed that the Smad pathway might play an important role in regulating the transcription of TGFBI gene in breast cancer cell lines. Therefore, MCF-7 cells were selected for induction studies since their Smad pathway is intact (Pouliot and Labrie, 1999). It was observed that high TGF-β_1 concentrations resulted in an alteration in the morphology of the MCF-7 cells, changing from cohesive groups to cells with an extended, elongated shape and less adhesiveness to each other. This alteration in shape suggests that TGF-β_1 could have an effect on the cytoskeleton, either directly or indirectly. In order to analyse this rhodamin phalloidin F-actin bundles staining should have been performed. Phalloidin binds to actin particularly F-actin filaments, which are considered to be one of the important components of cytoskeleton (Low and Wieland, 1974; Steinmetz *et al*., 1998). The polymerization and depolymerisation of these filaments determine the generated forces that enable changes in the cell shape; highly branched filaments support changes in the cell shape while packed filaments cause no changes (Fletcher and Mullins, 2010). It would have been worth performing this test to confirm the changes that were observed in the shape of MCF-7 cells treated with TGF-β_1.

TGFBI was shown to be secreted by MCF-7 cells after 72 hours of treatment with TGF-β_1. As demonstrated in the immunohistochemistry studies, TGFBI is present in the stroma of breast cancers, and the secreted form could represent the stromal form. It is well recognised that the stroma is important in controlling cell behaviour in both normal tissues and cancer (Walpita and Hay, 2002). In order to determine whether secreted/stromal TGFBI could modify cell morphology and adhesion further experiments would be needed in which MCF-7 cells are cultured either on purified TGFBI or in culture medium that has a high level of TGFBI.

Role of TGFBI

The precise function of TGFBI protein in tumourigenesis remains unknown but a number of studies have demonstrated it to be an important molecule that plays a role in tumour progression, angiogenesis and metastasis (Zhao *et al*., 2006; Becker *et al*., 2006; Zhang *et al*., 2009; Zamilpa *et al*., 2009). In order to investigate the potential role of TGFBI in breast cancer two cell lines were used, ZR-75-1 and MDA-MB-468. These had shown very low TGFBI mRNA levels by q-RT-PCR (Figure 4.8), and also absence of TGFBI protein was confirmed by western blotting (Figure 4.9) hence it was possible to up-regulate TGFBI levels by transient transfection. They also differ in relation to their ER status, growth characteristics and invasion capability (Gordon *et al*., 2003).

Transient transfection of both cell lines with TGFBI showed that mRNA expression was highest after 12 hours of exchanging the complete media, left overnight post transfection, to serum free media (Figure 4.8) with a parallel high level of protein within the cells at the same time point (Figure 4.9, A and B). Secretion of TGFBI into the culture medium was detected at 12 hours but this increased with time and was maximal at 72 hours, whereas the cellular protein decreased from 12 hours. The differences in the protein levels with time and location probably mirror those detected by immunohistochemistry with the cell (nuclear) location and the secreted form in the extracellular matrix. Previous studies have identified TGFBI in both intracellular and extracellular components (Billing *et al*., 2000a; Billings *et al*., 2000b; Zhao *et al*., 2006; Park *et al*., 2008). It has also been suggested that the presence of TGFBI in various locations maybe due to multiple isoforms arising from alternative splicing in the 5' region of the mRNA, or by post-translational proteolytic processing (Billings *et al*., 2000a). My experiments suggest that the extracellular form is the secreted 68kDa TGFBI form; and lower molecular weight forms were also detected but only in the normal organoids.

The viability assays of ZR-5-1 cells transfected with TGFBI showed no significant difference in the number of metabolically active and viable cells compared to the ZR-75-1 cells transfected with the empty vector and to ZR-75-1 untreated cells (Two Way ANOVA, Analysis of Variance, $p>0.05$). MDA-MB-468 cell line gave the same finding; no difference in cell viability was demonstrated between the three groups of cells. However, a significant increase in cell viability with time was confirmed in ZR-75-1 for transfected TGFBI, transfected empty vector and untreated cells particularly between 6 and 24 hours time points. This finding indicates fast growth of this cell line in contrast to MDA-MB-468, which showed no significant increase in growth over this time period. DAPI staining also suggested that TGFBI had no significant effect on the proliferation

of ZR-75-1 and MDA-MB-468 cell at 6, 24 and 48 hours time points (Method *2.2.10*). The DAPI staining are preliminary data, as this experiment was only performed once due to the fact that the protocol had many steps, each with the potential to introduce error and also technical difficulties in accurately counting the DAPI stained cells. It would have been of interest to examine the effect of TGFBI on proliferation and on S-phase of the breast cells by analyzing Ki67 and BrDU incorporation, respectively. A recent study concluded that TGFBI is a tumour suppressor gene since mice lacking TGFBI had retarded growth and were prone to spontaneous tumour formation (Zhang *et al*., 2009). Furthermore, TGFBI reconstitution in TGFBI $^{-/-}$ mouse embryonic fibroblasts (MEFs) cells led to the suppression of proliferation by suppression of Cyclin AMP-Responsive Element Binding protein (CREB) and Cyclin D1 expression (Zhang *et al*., 2009). TGFBI expression was also demonstrated to significantly reduce proliferation of neuroblastoma cells *in vitro* and *in vivo* (Becker *et al*., 2006). Apart from examining proliferation, investigation of CREB and Cyclin D1 expression in the TGFBI transfected breast cell lines and controls would have been worth doing.

The apoptosis assay in this study showed that TGFBI has no significant influence on apoptosis of both cell lines compared to cells transfected with the empty vector and untreated controls. Both ZR-75-1 and MDA-MB-468 do not seem to be particularly sensitive to apoptosis via the TGFBI pathway, confirming that not every cell type appears to be sensitive to apoptosis mediated by TGF-β and the TGFBI pathway (Kim *et al*., 2005). A study by Zamilpa *et al*., 2009 suggested that absence of apoptosis in cells transfected with TGFBI might be due to the blockage of C-terminal fragmentation (RGD peptide release) of TGFBI. This mechanism might prevent the soluble RGD peptide from directly activating caspase-3 in the cell cytoplasm (Buckley *et al*., 1999). It was suggested that both cell lines have similar apoptotic index under normal culture conditions (% apoptosis approximately 15%), which was shown to be very low (Yang *et al*., 2007). This low apoptosis rate could be the reason behind detecting low changes, which might have been due to the sensitivity of the assay used in this study.

This study showed no influence of TGFBI on apoptosis of ZR-75-1 and MDA-MB-468 cell lines when measuring caspase 3/7 activity. Despite this TGFBI protein was over-expressed in the MDA-MB-468 floating cells collected from the conditioned media after 48 and 72 hours of incubation with serum free media, post overnight culture with complete media to recover from the transfection process. These floating cells were combined with cell pellets obtained at the same time point, and then analyzed using western blotting (Figure 4.9). Over-expression of TGFBI protein in these cells confirms that TGFBI protein might have a role in apoptosis at a later time point post transfection.

The apoptosis findings obtained using the plate reader do not correlate with previous studies, which showed that over-expression of TGFBI protein significantly increased apoptotic induction in HeLa, Human Corneal Epithelial (HCE) cells, H522 (lung cell line) and MG-63 (osteosarcoma cells) (Morand *et al*., 2003; Zhao *et al*., 2006; Zamilpa *et al*., 2009). This might relate to the differences between the cells of different origins.

In my study, it was found that TGFBI protein had no influence on the invasion of ZR-75-1 and MDA-MB-468 cells. These results are not supported by a previous study, which showed that expression of TGFBI protein enhanced the aggressiveness and changed the metastatic features of colon cancer cells *in vivo* (Ma *et al*., 2008). However, as this study was performed *in-vivo* instead this could explain the difference in the results obtained as my study was carried out *in vitro* using breast cell lines.

In conclusion, transient transfection with TGFBI had no effect on cell viability and apoptosis of both cell lines (ZR-75-1 and MDA-MB-468). No statistically significant effects were observed on invasion in response to culture with TGFBI conditioned media. Therefore, the role of TGFBI down-regulation in breast cancers (as identified in chapter 3) on enhancing breast cancer development and progression could be due to other mechanisms, or be of no significance. The data obtained could be due to the simple culture systems used in this study, which do not reflect the complexity of tissues. Cell culture is a very important method; however, the cells are not provided with the same normal physiological environment. Multiple micro-environmental parameters are involved in affecting cell growth and function such as ECM composition and cell-cell contact (Lelievre and Bissell, 1998). Disruption of tissue architecture which is considered to be an essential regulator of epithelial function can lead to loss of biochemical information between the cells and their environment (Hagios *et al*., 1998). The use of 3 dimensional culture systems, with growth of cancer cells in different matrices, would have been of value in my study (Holliday *et al*., 2009).

Chapter 5

Conclusion and Future Studies

The aim of my thesis was to examine three genes (*TGFBI*, *DDB2* and *MCM5*) which had previously been found in a cDNA microarray to be up-regulated in two breast cancers from women aged 35 years in comparison to normal breast; to determine whether the up-regulation was still present when more cancers were studied; whether the changes were specific to cancers in the younger age (≤35 yrs) group; and whether this could contribute to the more aggressive nature of sporadic breast cancer in this age group. In order to do this breast cell lines, normal/benign tissues, organoid (epithelium and myoepithelium) samples and breast cancers, stratified by age, were investigated for these target genes using q-RT-PCR, western blotting and immunohistochemistry. In order to clarify the role of TGFBI in breast cancer *in-vitro* functional studies of its effects on cell viability, apoptosis and invasion were undertaken in two breast cancer cell lines (ZR-75-1 and MDA-MB-468).

Conclusions

TGFBI

This gene was considered worthy of further investigation for several reasons. It has been found to be up-regulated in several different cancer types (Ivanov *et al*., 2008). TGF-β_1, which induces the synthesis of TGFBI, has an important role in breast cancer, in both development and progression of the disease (Akhurst and Derynck, 2001; Derynck *et al*., 2001). However, my findings do no support an obvious role for TGFBI in breast cancer, and certainly not for cancers in the younger age group.

In line with the findings of a previous PhD student (Lambe, 2008) TGFBI mRNA levels were found to be lower in breast cancer cell lines relative to the non-tumourigenic HBL-100, and there was no evidence of protein expression at expected molecular weight of 68kDa. TGFBI mRNA levels were also lower in breast cancers compared to normal/benign tissues. However, there was a trend of higher mRNA expression in the cancers when compared to normal organoids (epithelial/myoepithelial aggregates), which would correlate with the findings of the microarray. This suggests that the conclusions drawn from the microarray do not support findings in normal breast tissues. The use of dissociated normal breast as the comparator for the array, rather than intact normal breast tissue appears to be the cause of this. Western blotting data supported the mRNA results for 3 of the 6 tumours investigated but similar band intensities at a molecular weight of 68kDa were observed in normal/benign tissues and cancers in spite of the differences in their mRNA levels. Immunohistochemistry results showed that the majority of the breast cancers (46/67) had lacked nuclear staining in the invasive tissues

but ECM staining was observed to varying degrees in 48 of the 67 tumours. Neither TGFBI mRNA and protein expression correlated with the clinicopathological features of the tumours; however, a significant correlation between mRNA levels and the patient age was confirmed (Pearson correlation, one tailed, p=0.04), with lower mRNA levels correlating with breast cancer in younger women.

Functional assays were performed to determine the role of TGFBI in breast cancer using *in-vitro* cell culture systems. The two cell lines (ZR-75-1 and MDA-MB-468) that were transiently transfected with TGFBI showed that TGFBI had no obvious role in regulating viability and apoptosis in these cell lines compared to cells transfected with the empty vector and untreated cells. In addition invasion assays, which utilised conditioned medium from cells transfected with TGFBI and vector alone showed that in this system TGFBI had no effect on the invasion of ZR-75-1 and MDA-MB-468 cells. Although the main focus of the functional studies was the assessment of the role of TGFBI in breast cancer cell lines the induction of TGFBI by TGF-β_1 was also considered in MCF-7 cells. It was found that a high concentration of TGF-β_1 induced TGFBI protein in MCF-7 cells after 72 hours of incubation in serum free media, which was subsequently secreted into the culture medium as detected by western blotting.

DDB2

This gene was considered worthy of further investigation for several reasons. It has been suggested that loss of DDB2 function in normal cells is related to susceptibility to tumour development (Alekseev *et al*., 2005). This might be due to its important role in DNA repair mechanism after binding to DNA lesions, to help in recruiting repair complexes to the region (Datta *et al*., 2001). DDB2 expression was linked with ER status in breast cell lines as was found by Lambe (2008) and Kattan *et al*., (2008); therefore, it was of interest investigating its relationship to ER status in a larger number of breast cancers.

DDB2 mRNA expression was lower in negative ER/PR (HBL-100, MDA-MB-468 and MDA-MB-231) when compared to ER/PR positive cell lines (MCF-7, T47-D and ZR-75-1). Western blotting detected similar band intensities at ~51kDa for all cell lines examined; however, different molecular weight form was observed in the organoid samples (48kDa). No significant difference was found between the mRNA expressions of the three groups examined: normal/benign tissues, breast cancers and organoid samples. In contrast to the microarray data, no significant difference was found between the mRNA levels of breast cancers and normal organoid samples, which could relate to

the larger number of cases used in the analysis in this study compared to the 2 breast cancers used in the microarray.

Immunohistochemistry results showed approximately equal number of tumours that had negative and positive staining, therefore, no critical conclusion could be drawn from these results but they were significantly correlated with the mRNA data. Both mRNA and protein expression of DDB2 were shown to be significantly correlated with ER and PR status of the tumour, indicating that high mRNA and protein levels were associated with positive ER and PR status. Besides, a significant correlation was also found between high mRNA expression of DDB2 and grade II rather than grade III tumours. Of note, a significant correlation (Pearson correlation, one tailed, p=0.025) between mRNA levels and patient age was found, showing lower levels in younger women.

MCM5

MCM5 has been found to be up-regulated in a number of cancers (Introduction, Table 1.15), and it was also shown to be highly expressed with a 9.59 fold change in the breast cancers in young women (≤35 Years Old) compared to normal control in the microarray that was performed by Whyman (2005). This target had not been analysed previously by Whyman (2005) or Lambe (2008) but it was considered worthy of investigation on re-review of the microarray data.

MCF-7 and MDA-MB-231 showed the highest mRNA expression of MCM5 relative to HBL-100, while the rest of the cell lines showed lower mRNA levels. Western blotting results were consistent with the mRNA data for ZR-75-1, T47-D and MDA-MB-468 but small differences were seen in the expression of the protein in MCF-7 and MDA-MB-231. No significant difference in the mRNA levels of MCM5 was found between the three groups investigated: normal/benign tissues, breast cancers and organoid samples, whereas the immunohistochemistry results showed that 63 of the 67 breast cancers had a variable extent of nuclear staining.

Only MCM5 protein expression was found to correlate with grade and ER/PR status of the tumour; with high protein levels being associated with grade II and ER/PR positive tumours. Unfortunately, both mRNA and protein levels of MCM5 showed no significant correlation with patient age.

Limitations and Suggested Improvements of this Study

The major limitation in the q-RT-PCR results was generating cDNA only once from all the specimens and cell lines investigated. This was essentially due to the small amounts of RNA that were obtained from the frozen tissues, and also due to the software upgrades to the q-RT-PCR equipment which prevented retrospective retrieval of the cell lines data, which had been replicated early on in the study. Q-RT-PCR was performed in triplicate for each cDNA synthesized, which checks intra-assay variation but ideally cDNA synthesised on two to three separate occasions should have been compared. A small number of tissue samples used showed high Ct values but they were included in the analysis because it was suggested that even though the sample can increase the Ct value, it has no effect on the ∆Ct expression (Antonov *et al*., 2005). These high values can be due to poor RNA integrity, which can arise as a result of freeze/thaw changes. This would result in damage to RNA.

Organoid samples may not have been the best positive control to be used for analysing western blotting results. This is because of the differences in molecular weight size observed between the cell lines and organoid samples. The multiple bands that were detected in the organoids particularly when assessing TGFBI protein could be due to the proteases used in isolation the organoid samples. These different forms made the interpretation of the results more difficult. It would have been beneficial to select different cell lines with the target protein to be examined. For example a positive control for TGFBI would be cell lines, which had been treated with TGF-β_1 (Table 1.11).

The H-score, which is a commonly used method for assessing immunohistochemistry results, was not used in this study. Instead we selected a system using the extent of staining as used in previous studies of TGF-β_1 (Walker *et al*., 1994) as being more appropriate since there was little variation in the staining intensity. Also, the system used was simpler and intra-observer assessment showed that I would need more experience in order to be able to interpret staining as required for the H-score. However, given sufficient time it would have been worth re-assessing the immunohistochemistry results by this method.

Investigating only 67 breast cancer cases for immunohistochemistry and 36 frozen tumours for mRNA was a major limitation of the study. The frozen tissues were only available from a small number of cases since it had been used for many other studies in the group. The aim of this study was to have relatively equal numbers of cases in the three age categories ≤ 35 years, 36 -49 years and≥50 years) in order t o avoid bias when analyzing the immunohistochemistry results, where grouping was used. In addition

breast cancers are less frequent in the younger age group so finding larger numbers is difficult, as shown by a previous study of Walker *et al*., 1996, which included 48 cases from women 35 years and less. Also, only cancers from women who did not receive treatment prior to surgery were used, which excluded those younger women who had received neo-adjuvant therapy.

The main limitation of the functional assays is the wide variations in the readings across the triplicate in viability and apoptosis assays in both cell lines (ZR-75-1 and MDA-MB-468), which influenced the overall results. To overcome this problem additional replicates should have been included or more than 3 repeats for each experiment should have been performed. It would have been also beneficial to investigate the role of TGFBI using different methods instead of relying on the plate reader for example investigating the role of TGFBI on proliferation instead of viability by using proliferation markers (Ki67) before and after transient TGFBI transfection. It would also have been worth examining apoptosis by using immunocytochemistry with M30 antibodies for the three groups of cells: transfected TGFBI plasmid, transfected empty vector and untreated cells. Using staurosporine to induce ZR-75-1 apoptotic cells was not an appropriate positive control for the apoptosis assays performed because ZR-75-1 cells showed resistance to it at the 12 hour time point. Therefore, using a different drug or a higher staurosporine concentration might improve and confirm the apoptosis data.

The invasion assays were performed only twice in duplicate, which affected the overall results. Conditioned media with 1% FCS was used instead of complete media. This minimized the invasion rate of both cell lines; therefore, it would have been worth repeating this experiment with 5% FCS instead of 1% in the conditioned media, which might increase the invasion that was observed.

Future Studies

Some interesting data from this thesis was observed; however, more cancer cases should be used for validation of the changes seen in expression of the three genes for example by using tissue microarray (TMAs) and correlating with other markers. More IHC and q-RT-PCR would be worthwhile for looking at in larger numbers of breast cancer cases, if possible matching between the frozen and FFPE tissues.

TGFBI

It would be of interest to examine the mechanisms behind the TGFBI down-regulation by investigating DNA methylation (gene silencing) or TGF-β pathway signalling in tissues and cell lines (Shao *et al*., 2006). I demonstrated that MCF-7 cells induced TGFBI protein after being treated with TGF-β_1, confirming a relationship between active TGF-β signalling pathway and induction of TGFBI. It would be worthwhile examining other breast cancer cell lines after ensuring that the signaling pathway is intact (Introduction *1.11.3*).

This thesis showed no role for TGFBI in viability, apoptosis and invasion of breast cell lines (ZR-75-1 and MDA-MB-468); therefore it would be worth looking at proliferation in greater details than just viability. For example, investigating Cyclin D1 before and after transfection would be interesting to determine whether TGFBI has a direct effect on the cell cycle in breast cancer as suggested by Zhang *et al*., (2009).

DDB2

It might be interesting to explore further the different molecular form that was observed for DDB2 in cells lines compared to organoids by using RNA studies, for example, selecting a combination of primer for RT-PCR to discriminate between splice variant or by cDNA sequencing. This approach might help to determine the difference between the two molecular weights detected in cell lines and organoids samples, as evidence of presence of various splicing variants in DDB2 has been published by Inoki *et al*., (2004). Analyzing the DDB2 protein level in frozen breast cancer tissues using western blotting is warranted to determine the size in cancers compared to normal breast tissue. It might also be worth investigating whether DDB2 is associated with response to endocrine therapies and overall patient survival as this was expressed at highest levels in ER^+ cell lines and tissues. In addition, it would be of interest to examine the role of DDB2 in the development and progression of breast cancer using functional studies.

MCM5

MCM5 is considered to be a worthy potential marker in all age groups of breast cancers. Therefore, it might be of interest to investigate MCM5 further by using a larger number of breast cancer cases. In addition, knock-out MCM5 from breast cell lines, which are

confirmed in this study to express it will be worth investigating to determine the functional role of this gene in breast cancer.

In conclusion, two of the three investigated genes (*TGFBI and DDB2*) were significantly down-regulated in sporadic breast cancer in younger women. Both DDB2 and MCM5 were identified as potential markers for ER+ and grade II breast cancers. This might explain why DDB2 mRNA expression is lower in cancers from younger women, since they are more frequent ER and PR negative. Functional studies with TGFBI transient transfection showed that there was no influence of TGFBI on viability, apoptosis and invasion of ZR-75-1 and MDA-MB-468 cells. Further studies in other cells are warranted in order to confirm these preliminary findings.

Appendices

Appendix I Summary of the Clinicopathological Features of Fixed Breast Carcinomas Used in this Study

Number of Case	Age	Size	Grade	Lymph Node	ER	PR	HER-2
× 1	19	1	3	Neg	High	Low	-----
× 2	30	2	3	Neg	Neg	Neg	-----
× 3	31	1	3	Pos	High	High	-----
× 4	32	2	3	Neg	High	High	Pos
× 5	34	2	3	Pos	Neg	Low	-----
× 6	35	1	3	Neg	Neg	Neg	Neg
× 7	38	2	2	Neg	High	High	-----
× 8	50	1	3	Pos	High	High	-----
9	50	2	2	Neg	High	High	-----
× 10	51	2	3	Pos	Neg	Neg	Neg
11	51	2	2	Pos	High	High	-----
× 12	54	2	3	Pos	High	High	-----
× 13	54	2	3	Pos	High	Low	Neg
× 14	59	1	3	Neg	Neg	Neg	-----
× 15	62	2	3	Neg	High	High	-----
16	64	2	3	Neg	Neg	Neg	-----
× 17	64	2	3	Pos	-----	-----	-----
× 18	49	2	3	Neg	Neg	Neg	Pos
× 19	47	2	2	Neg	Low	High	Neg
20	49	1	2	Neg	High	High	-----
× 21	46	1	3	Neg	High	High	-----
22	40	2	3	Neg	Low	Low	Pos
× 23	43	1	3	Pos	Neg	Neg	-----
× 24	46	2	3	Neg	Neg	Neg	Neg
× 25	46	2	3	Pos	High	High	Pos
26	47	1	3	Pos	High	High	Neg
× 27	38	2	3	Pos	Low	Neg	Pos
× 28	45	2	2	Neg	High	High	-----
29	56	1	2	Neg	High	High	-----
30	45	1	3	Neg	Neg	Neg	-----
× 31	41	2	3	Pos	High	High	Neg
× 32	46	2	3	Pos	High	High	Neg
× 33	46	2	3	Pos	Neg	Neg	Neg
× 34	29	1	3	Neg	High	Low	-----
35	30	1	3	Pos	High	High	-----
× 36	32	1	2	Neg	High	High	-----
× 37	30	1	3	Pos	Neg	Neg	-----
38	35	1	2	Neg	High	High	-----
× 39	34	2	2	Pos	High	Low	Neg
40	54	2	2	Neg	High	High	Neg
× 41	52	2	2	Neg	High	High	-----
42	69	2	3	Pos	Neg	Neg	-----
43	42	2	3	Pos	High	High	Neg
× 44	63	2	1	Pos	High	High	-----
45	54	2	2	Neg	High	High	-----
× 46	60	2	2	Pos	High	Low	-----
× 47	59	3	3	Pos	High	High	Neg
48	64	2	1	Neg	High	High	-----
× 49	59	2	3	Neg	High	High	-----
× 50	78	2	2	Pos	High	High	-----
51	35	2	3	Pos	Neg	Neg	Pos
52	34	1	3	Neg	High	Low	Neg
53	31	1	2	Neg	High	Low	Neg
54	35	2	3	Pos	Neg	Neg	Neg

Number of Case	Age	Size	Grade	Lymph Node	ER	PR	HER-2
55	33	2	2	Pos	High	High	Neg
56	49	2	2	Neg	High	High	Neg
× 57	46	1	2	Neg	High	High	-----
× 58	43	2	2	Pos	High	Neg	Neg
× 59	45	1	2	Neg	High	Low	Neg
× 60	39	1	2	Neg	High	Low	Neg
61	33	1	2	Neg	High	High	-----
× 62	66	2	2	Pos	High	High	Neg
× 63	41	2	2	Pos	High	Low	Neg
64	58	1	2	Pos	High	High	-----
× 65	64	2	1	Neg	High	High	-----
66	59	1	2	Neg	High	High	-----
× 67	45	2	2	Neg	High	High	-----

All the cases are Infiltrating Ductal Carcinoma (IDC). × Frozen carcinoma tissues that used for q-RT-PCR

Tumour Size:
1: < 20mm diameter
2: ≥ 20mm diameter

Tumour Grade:
1: Well differentiation
2: Moderate differentiation
3: Poor differentiation

Lymph node Involvement:
Neg: no lymph node metastasis
Pos: there is lymph node metastasis

Oestrogen (ER) and Progesterone (PR) status:
Neg: receptors not present
Pos: there is presence for the receptor, which was divided into two categories **Low** and **High**

HER-2 Receptors:
Neg: No HER-2 Receptors
Pos*:* there is HER-2 Receptors

Appendix II Q-RT-PCR Raw Reading Data for the Standard Curves

	Readings						
Dilution	15625	3125	625	125	25	5	1
Log_2 Dilution	13.93	11.61	9.29	6.97	4.64	2.32	0.00
GAPDH	18.17	20.32	22.57	24.51	26.25	29.12	31.64
	18.21	20.34	22.39	24.58	26.74	29.16	31.48
	18.14	20.26	22.41	24.52	26.89	29.36	31.77
Std	0.035	0.041	0.098	0.037	0.334	0.128	0.145
HPRT1	21.76	24.33	26.78	28.99	30.99	34.29	36.62
	21.91	24.26	26.89	29.17	31.60	33.87	37.06
	21.94	24.44	26.67	29.45	31.83	34.78	36.48
Std	0.095	0.091	0.106	0.232	0.434	0.454	0.300
TFRC	24.12	26.19	28.23	31.57	33.70	36.43	36.96
	24.20	26.02	28.17	31.56	34.20	36.47	35.07
	24.12	26.26	28.19	31.69	34.42	37.10	35.28
Std	0.047	0.123	0.033	0.074	0.367	0.374	1.037
TGFBI	21.44	23.25	25.43	27.91	31.02	35.30	36.01
	21.30	23.19	25.58	27.71	31.21	33.64	35.08
	21.44	22.99	25.30	28.07	31.26		
Std	0.080	0.133	0.139	0.179	0.124		
DDB2	25.57	27.76	30.46	33.63	34.83		
	25.37	27.74	30.69	32.77	36.96		
	25.58	27.98	30.85				
Std	0.118	0.129	0.196				
MCM5	22.98	25.56	27.72	30.63	33.55	37.03	36.70
	23.31	25.53	27.74	30.71	34.57	35.37	36.32
	23.13	25.22	27.93	30.62	33.32		
Std	0.163	0.188	0.113	0.047	0.664		

Appendix III Immunohistochemistry Results of the 67 Cases for TGFBI

Number of Case	***Nuclei Staining in Normal Areas***	***Nuclei Staining in the In Situ Areas (%)***	***Nuclei Staining in the Invasive Areas (%)***	***ECM Staining in the Tissue (%)***
1	Weaker	-------	50-75	5
2	-------	-------	<1	75
3	Weaker	-------	5	50
4	-------	0	0 *	0
5	Weaker	<1	<1 *	0
6	Negative	-------	0	20
7	-------	-------	0	10
8	Negative	0	0 *	5
9	Weaker	-------	20	5
10	-------	-------	0	50
11	Weaker	-------	0	0
12	Weaker	-------	5	75
13	-------	-------	0	0
14	-------	-------	<1	0
15	Negative	0	0 *	0
16	-------	-------	0	75
17	-------	-------	5	0
18	-------	-------	0	0
19	Same	10	10 *	0
20	Weaker	-------	>75	0
21	-------	10	<1 **	0
22	Weaker	0	10 ***	20
23	-------	10	5 **	75
24	-------	-------	0	10
25	-------	0	0 *	5
26	Weaker	0	0 *	0
27	Same	0	c *	20
28	Weaker	5	0 **	0
29	-------	50	25 **	75
30	Weaker	-------	0	5
31	-------	0	0 *	5
32	-------	-------	0	0
33	-------	0	0 *	50
34	Weaker	25	25 *	5
35	Weaker	0	0 *	5
36	Weaker	0	<1 ***	50
37	Same	20	25-50 ***	10
38	Weaker	0	0 *	0
39	-------	0	0 *	5
40	Weaker	75	50 **	0
41	-------	25	25 *	0
42	-------	-------	0	50
43	Same	<5	0 **	5
44	-------	>75	>75 *	20
45	Weaker	<1	0 **	20

46	Same	-------	50	50
47	Weaker	-------	<1	0
48	-------	-------	0	20
49	-------	-------	<1	5
50	Same	-------	>75	50
51	-------	50	50-75 ***	5
52	Weaker	-------	<1	50
53	-------	-------	0	10
54	-------	-------	0	75
55	Weaker	-------	0	10
56	Same	-------	0	100
57	-------	0	0	5
58	Weaker	0	<1	10
59	Weaker	-------	0	5
60	Weaker	-------	0	50
61	Same	-------	50	5
62	-------	-------	<1	0
63	Weaker	-------	20	25
64	Same	-------	5	25
65	-------	-------	<1	75
66	Same	-------	0	100
67	Weaker	0	0	10

* Distribution of nuclei staining is the **SAME** in both in situ and invasive areas
** Distribution of nuclei staining is **LOWER** in the invasive compared to the in situ area
*** Distribution of nuclei staining is **HIGER** in the invasive compared to the in situ area
----- No normal tissue present

NEGATIVE: No nuclei staining present
WEAKER: Nuclei staining is weaker compared to the positive control
SAME: Nuclei staining appear the same as the positive control

0%: No nuclei staining in the area
<1%: There are few nuclei stained in the area
5%-10%: There is nuclei staining in less than quarter of the area
11-50%: There is nuclei staining in one quarter to half of the area
51-74%: There is nuclei staining in half to three quarter of the area
≥75%: There is nuclei staining mostly in all the area

Appendix IV Summary of the DDB2 Immunohistochemistry Results

Number of Case	***Nuclei Staining in Normal Areas***	***Nuclei Staining in the In Situ Areas***	***Nuclei Staining in the Invasive Areas***
1	Weaker	-------	5%
2	-------	-------	0%
3	Weaker	-------	0%
4	Weaker	20%	5% **
5	Same	10%	10% *
6	Weaker	-------	<1%
7	-------	-------	5%
8	Weaker	<1%	5% ***
9	Weaker	-------	<1%
10	-------	-------	0%
11	-------	-------	5%
12	Same	-------	5%
13	-------	-------	<1%
14	-------	-------	<1%
15	Weaker	5%	5% *
16~	-------	-------	0%
17	-------	-------	0%
18	-------	-------	0%
19	Weaker	-------	5%
20	Weaker	5%	5% *
21	Weaker	<1%	<1% *
22	Weaker	0%	0% *
23	-------	-------	0%
24	-------	-------	0%
25	-------	<1%	<1% *
26	Same	-------	5%
27	-------	5-10%	5% **
28	-------	20%	5% **
29	Same	51-74%	51-74% *
30	Weaker	-------	<1%
31	-------	10%	10% *
32	-------	-------	0%
33	-------	0%	0% *
34	Weaker	-------	20%
35	Same	0%	20-25% ***
36	Weaker	-------	25%
37	Same	0%	<1% **
38	Weaker	-------	<1%
39	Weaker	5	1 **
40	Negative	5%	0 **
41	Weaker	-------	0
42	-------	-------	0
43	Weaker	0	5 ***
44	-------	5	5 *
45	Weaker	20	10 **
46	Same	-------	20
47	Weaker	-------	0
48	-------	-------	0

49	-------	-------	10
50	Weaker	------	0
51	-------	10	10 *
52	Same	-------	5
53	------	------	0
54	------	------	0
55	Negative	------	10
56	Weaker	-------	20-50
57	--------	-------	0
58	Weaker	-------	5
59	Same	0	0 *
60	-------	-------	10
61	Similar	------	40
62	Weaker	-------	0
63	-------	-------	20
64	Same	-------	0
65	-------	-------	20-50
66	Weaker	-------	0
67	Same	0	5 ***

* Distribution of nuclei staining is the **SAME** in both in situ and invasive areas
** Distribution of nuclei staining is **LOWER** in the invasive compared to the in situ area
*** Distribution of nuclei staining is **HIGER** in the invasive compared to the in situ area
----- No normal tissues present

NEGATIVE: No nuclei staining present
WEAKER: Nuclei staining is weaker compared to the positive control
SAME: Nuclei staining appear the same as the positive control

0%: No nuclei staining in the area
<1%: There are few nuclei stained in the area
5%-10%: There is nuclei staining in less than quarter of the area
11-50%: There is nuclei staining in one quarter to half of the area
51-74%: There is nuclei staining in half to three quarter of the area
≥75%: There is nuclei staining mostly in all the area

Appendix V Summary of the MCM5 Immunohistochemistry Results

Number of Case	***Nuclei Staining in the Invasive Areas***
1	1
2	2
3	40
4	2
5	2
6	10
7	15
8	5
9	1
10	2
11	10
12	20
13	40
14	1
15	2
16~	1
17	1
18	1
19	40
20	20
21	5
22	1
23	10
24	2
25	5
26	1
27	0
28	20
29	50
30	40
31	2
32	1
33	0
34	5
35	5
36	2
37	0
38	20
39	5
40	40
41	0
42	5
43	2
44	40
45	1
46	5
47	50
48	15

49	5
50	75
51	50
52	10
53	4
54	1
55	2
56	70
57	15
58	20
59	2
60	5
61	80
62	15
63	30
64	10
65	10
66	2
67	10

0%: No nuclei staining in the area
<1%: There are few nuclei stained in the area
5%-10%: There is nuclei staining in less than quarter of the area
11-50%: There is nuclei staining in one quarter to half of the area
51-74%: There is nuclei staining in half to three quarter of the area
≥75%: There is nuclei staining mostly in all the area

Appendix VI ***Recipes of Reagents***

- 1 X Gold Protein Lysis Buffer (1L)
10mL of 1% Triton X-100
30mL of 30mM Tris (PH=8)
8g of 137mM Sodium Chloride
150mL of 15% Glycerol
1mL of 5mM EDTA

- Gel Buffer
Tris, SDS and HCL
A. 1.5mM Tris-HCL pH=8.8 (Resolving)
B. 0.5mM Tris-HCL pH=6.8 (Stacking)

- 10% Sodium Dodecylsalphate Buffer
100g Sodium Dodecyl Sulphate in 1L of distilled water

- Western Gel Loading Buffer (4X) (pH=6.8) (10mL)
2.608mL of 1M Tris HCL (pH=8.6)
2mL β-Mercaptoethanol
4mL Glycerol
0.80g SDS
0.01g Bromophenol Blue
1.397mL Pure Water

- 10X Western Running Buffer (pH=8.3) (500mL)
54g Tris Base
72g Glycine
5g SDS

- 10X Western Transfer Buffer (pH=8.3) (1L)
30.2g of 25mM Tris
144.4g of 192mM Glycine
10mL of 10% SDS (0.01%)
Methanol

- Western Washing Buffer (1%TBS-T) (1L)
100mL of 20 x TBS
10mL Tween 20
890mL Distilled Water

- Western Washing Buffer (3%TBS-T) (1L)
100mL of 20 x TBS
30mL Tween 20
870mL Distilled Water

- 5% Western Blocking Solution (1L)
5g of Marvel Dried Skimmed Milk
100mL of distilled water

- Mayer's Haematoxylin (2L)
1800mL Distilled Water
100g Aluminium Potassium Sulphate
2g Haematoxylin dissolve in 10mL 99% IMS
400mg Sodium Iodate
400mg Citric Acid
100g Chloral Hydrate

- Eosin (2L volume at 1%)
20g Eosin dissolve in 1800mL of ordinary tap water
1mL of 1% Calcium Chloride
2mL of 38-40% Formaldehyde

- 20 X Citrate Buffer (pH=6) (1L)
42g Citric Acid in 500mL ultrapure water
Sodium Peroxide to adjust pH
5M Sodium Peroxide for final adjustment

- 100 X Tris/EDTA (TE) Buffer (pH=7) (2L)
242.3g Tris
74.4g EDTA

- 10 X TBE Buffer (pH=8.3) (1L)
108g Tris
55g Boric Acid
40mL of 0.5M EDTA

- 20 X Tris Buffered Saline (TBS) (pH=7.65) (2L)
242.3g of 0.05M Tris (Hydroxymethyl Mehtylamine)
350.6g of 0.15M Sodium Chloride

-10X AJ Buffer (PCR Buffer) (100mL)
3.73g KCL
10mL of 1M Tris-HCL (pH=9.0)
0.143g $MgCl_2$
1mL Triton-X 100
89mL Pure Water

- 50X TAE Buffer (pH=8.5) (1L)
242g of Tris
57.1mL of Glacial Acid
100mL of EDTA (pH=8.0)
843mL Pure Water

- 6X PCR Gel Loading Buffer (200mL)
30mL Glycerol
0.5g Bromophenol Blue
0.5g Xylene Cyanol
170mL Pure Water

-NZY^+ broth (pH=7.5) (1L)
5g NaCl
2g $MgSO_4*7H_2O$
5g Bacto-Yeast Extract
10g NZ amine
1L of Pure Water

- Luria-Bertani (LB) Medium
1% w/v bactro-tryptone
0.5% w/v bactro-yeast extract
0.5% w/v NaCl
Adjusted to pH=7.2 with NaOH

References

*Cancer Research UK*2009, May-last update. Available: CancerStats Report-Breast Cancer UK, Cancer Research UK.

"Breast radiotherapy after breast-conserving surgery. The Steering Committee on Clinical Practice Guidelines for the Care and Treatment of Breast Cancer. Canadian Association of Radiation Oncologists", 1998, *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne,* vol. 158 Suppl 3, pp. S35-42.

"Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer", 1997, *Lancet,* vol. 350, no. 9084, pp. 1047-1059.

"Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer", 1997, *Lancet,* vol. 350, no. 9084, pp. 1047-1059.

"Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer", 1997, *Lancet,* vol. 350, no. 9084, pp. 1047-1059.

"Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer", 1997, *Lancet,* vol. 350, no. 9084, pp. 1047-1059.

Abdel-Fatah, T.M., Powe, D.G., Hodi, Z., Lee, A.H., Reis-Filho, J.S. & Ellis, I.O. 2007, "High frequency of coexistence of columnar cell lesions, lobular neoplasia, and low grade ductal carcinoma in situ with invasive tubular carcinoma and invasive lobular carcinoma", *The American Journal of Surgical Pathology,* vol. 31, no. 3, pp. 417-426.

Abe, S., Kubota, T., Otani, Y., Furukawa, T., Watanabe, M., Kumai, K., Akiyama, T., Akinaga, S. & Kitajima, M. 2001, "UCN-01 (7-hydoxystaurosporine) inhibits in vivo growth of human cancer cells through selective perturbation of G1 phase checkpoint machinery", *Japanese journal of cancer research : Gann,* vol. 92, no. 5, pp. 537-545.

Adami, H.O., Malker, B., Holmberg, L., Persson, I. & Stone, B. 1986, "The relation between survival and age at diagnosis in breast cancer", *The New England journal of medicine,* vol. 315, no. 9, pp. 559-563.

Adami, H.O., Malker, B., Holmberg, L., Persson, I. & Stone, B. 1986, "The relation between survival and age at diagnosis in breast cancer", *The New England journal of medicine,* vol. 315, no. 9, pp. 559-563.

Akhurst, R.J. & Derynck, R. 2001, "TGF-beta signaling in cancer--a double-edged sword", *Trends in cell biology,* vol. 11, no. 11, pp. S44-51.

Alekseev, S., Kool, H., Rebel, H., Fousteri, M., Moser, J., Backendorf, C., de Gruijl, F.R., Vrieling, H. & Mullenders, L.H. 2005, "Enhanced DDB2 expression protects mice from carcinogenic effects of chronic UV-B irradiation", *Cancer research,* vol. 65, no. 22, pp. 10298-10306.

Alexandrow, M.G. & Moses, H.L. 1995, "Transforming growth factor beta and cell cycle regulation", *Cancer research,* vol. 55, no. 7, pp. 1452-1457.

Allen, N.E., Beral, V., Casabonne, D., Kan, S.W., Reeves, G.K., Brown, A., Green, J. & Million Women Study Collaborators 2009, "Moderate alcohol intake and cancer incidence in women", *Journal of the National Cancer Institute,* vol. 101, no. 5, pp. 296-305.

Anders, C.K., Hsu, D.S., Broadwater, G., Acharya, C.R., Foekens, J.A., Zhang, Y., Wang, Y., Marcom, P.K., Marks, J.R., Febbo, P.G., Nevins, J.R., Potti, A. & Blackwell, K.L. 2008, "Young age at diagnosis correlates with worse prognosis and defines a subset of breast cancers with shared patterns of gene expression", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 26, no. 20, pp. 3324-3330.

Anders, C.K., Hsu, D.S., Broadwater, G., Acharya, C.R., Foekens, J.A., Zhang, Y., Wang, Y., Marcom, P.K., Marks, J.R., Febbo, P.G., Nevins, J.R., Potti, A. & Blackwell, K.L. 2008, "Young age at diagnosis correlates with worse prognosis and defines a subset of breast cancers with shared patterns of gene expression", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 26, no. 20, pp. 3324-3330.

Anderson, G.L., Limacher, M., Assaf, A.R., Bassford, T., Beresford, S.A., Black, H., Bonds, D., Brunner, R., Brzyski, R., Caan, B., Chlebowski, R., Curb, D., Gass, M., Hays, J., Heiss, G., Hendrix, S., Howard, B.V., Hsia, J., Hubbell, A., Jackson, R., Johnson, K.C., Judd, H., Kotchen, J.M., Kuller, L., LaCroix, A.Z., Lane, D., Langer, R.D., Lasser, N., Lewis, C.E., Manson, J., Margolis, K., Ockene, J., O'Sullivan, M.J., Phillips, L., Prentice, R.L., Ritenbaugh, C., Robbins, J., Rossouw, J.E., Sarto, G., Stefanick, M.L., Van Horn, L., Wactawski-Wende, J., Wallace, R., Wassertheil-Smoller, S. & Women's Health Initiative Steering Committee 2004, "Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial", *JAMA : the journal of the American Medical Association,* vol. 291, no. 14, pp. 1701-1712.

Angers, S., Li, T., Yi, X., MacCoss, M.J., Moon, R.T. & Zheng, N. 2006, "Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery", *Nature,* vol. 443, no. 7111, pp. 590-593.

Antoni, L., Sodha, N., Collins, I. & Garrett, M.D. 2007, "CHK2 kinase: cancer susceptibility and cancer therapy - two sides of the same coin?", *Nature reviews.Cancer,* vol. 7, no. 12, pp. 925-936.

Antoniou, A., Pharoah, P.D., Narod, S., Risch, H.A., Eyfjord, J.E., Hopper, J.L., Loman, N., Olsson, H., Johannsson, O., Borg, A., Pasini, B., Radice, P., Manoukian, S., Eccles, D.M., Tang, N., Olah, E., Anton-Culver, H., Warner, E., Lubinski, J., Gronwald, J., Gorski, B., Tulinius, H., Thorlacius, S., Eerola, H., Nevanlinna, H., Syrjakoski, K., Kallioniemi, O.P., Thompson, D., Evans, C., Peto, J., Lalloo, F., Evans, D.G. & Easton, D.F. 2003, "Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies", *American Journal of Human Genetics,* vol. 72, no. 5, pp. 1117-1130.

Antonov, J., Goldstein, D.R., Oberli, A., Baltzer, A., Pirotta, M., Fleischmann, A., Altermatt, H.J. & Jaggi, R. 2005, "Reliable gene expression measurements from degraded RNA by quantitative real-time PCR depend on short amplicons and a proper normalization", *Laboratory investigation; a journal of technical methods and pathology,* vol. 85, no. 8, pp. 1040-1050.

Badve, S. & Nakshatri, H. 2009, "Oestrogen-receptor-positive breast cancer: towards bridging histopathological and molecular classifications", *Journal of clinical pathology,* vol. 62, no. 1, pp. 6-12.

Badve, S. & Nakshatri, H. 2009, "Oestrogen-receptor-positive breast cancer: towards bridging histopathological and molecular classifications", *Journal of clinical pathology,* vol. 62, no. 1, pp. 6-12.

Baker, S.J. 2007, "PTEN enters the nuclear age", *Cell,* vol. 128, no. 1, pp. 25-28.

Barnes, D.M., Harris, W.H., Smith, P., Millis, R.R. & Rubens, R.D. 1996, "Immunohistochemical determination of oestrogen receptor: comparison of different methods of assessment of staining and correlation with clinical outcome of breast cancer patients", *British journal of cancer,* vol. 74, no. 9, pp. 1445-1451.

Becker, J., Erdlenbruch, B., Noskova, I., Schramm, A., Aumailley, M., Schorderet, D.F. & Schweigerer, L. 2006, "Keratoepithelin suppresses the progression of experimental human neuroblastomas", *Cancer research,* vol. 66, no. 10, pp. 5314-5321.

Becker, K.F., Atkinson, M.J., Reich, U., Becker, I., Nekarda, H., Siewert, J.R. & Hofler, H. 1994, "E-cadherin gene mutations provide clues to diffuse type gastric carcinomas", *Cancer research,* vol. 54, no. 14, pp. 3845-3852.

Bell, D.W., Varley, J.M., Szydlo, T.E., Kang, D.H., Wahrer, D.C., Shannon, K.E., Lubratovich, M., Verselis, S.J., Isselbacher, K.J., Fraumeni, J.F., Birch, J.M., Li, F.P., Garber, J.E. & Haber, D.A. 1999, "Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome", *Science (New York, N.Y.),* vol. 286, no. 5449, pp. 2528-2531.

Benson, J.R. 2004, "Role of transforming growth factor beta in breast carcinogenesis", *The lancet oncology,* vol. 5, no. 4, pp. 229-239.

Beral, V. & Million Women Study Collaborators 2003, "Breast cancer and hormone-replacement therapy in the Million Women Study", *Lancet,* vol. 362, no. 9382, pp. 419-427.

Berrington de Gonzalez, A. & Darby, S. 2004, "Risk of cancer from diagnostic X-rays: estimates for the UK and 14 other countries", *Lancet,* vol. 363, no. 9406, pp. 345-351.

Billecke, C.A., Ljungman, M.E., McKay, B.C., Rehemtulla, A., Taneja, N. & Ethier, S.P. 2002, "Lack of functional pRb results in attenuated recovery of mRNA synthesis and increased apoptosis following UV radiation in human breast cancer cells", *Oncogene,* vol. 21, no. 29, pp. 4481-4489.

Billings, P.C., Herrick, D.J., Howard, P.S., Kucich, U., Engelsberg, B.N. & Rosenbloom, J. 2000, "Expression of betaig-h3 by human bronchial smooth muscle cells: localization To the extracellular matrix and nucleus", *American journal of respiratory cell and molecular biology,* vol. 22, no. 3, pp. 352-359.

Billings, P.C., Herrick, D.J., Kucich, U., Engelsberg, B.N., Abrams, W.R., Macarak, E.J., Rosenbloom, J. & Howard, P.S. 2000, "Extracellular matrix and nuclear localization of beta ig-h3 in human bladder smooth muscle and fibroblast cells", *Journal of cellular biochemistry,* vol. 79, no. 2, pp. 261-273.

Billings, P.C., Whitbeck, J.C., Adams, C.S., Abrams, W.R., Cohen, A.J., Engelsberg, B.N., Howard, P.S. & Rosenbloom, J. 2002, "The transforming growth factor-beta-inducible matrix protein (beta)ig-h3 interacts with fibronectin", *The Journal of biological chemistry,* vol. 277, no. 31, pp. 28003-28009.

Birgisdottir, V., Stefansson, O.A., Bodvarsdottir, S.K., Hilmarsdottir, H., Jonasson, J.G. & Eyfjord, J.E. 2006, "Epigenetic silencing and deletion of the BRCA1 gene in sporadic breast cancer", *Breast cancer research : BCR,* vol. 8, no. 4, pp. R38.

Bjornstrom, L. & Sjoberg, M. 2005, "Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes", *Molecular endocrinology (Baltimore, Md.),* vol. 19, no. 4, pp. 833-842.

Bland, K.I., Menck, H.R., Scott-Conner, C.E., Morrow, M., Winchester, D.J. & Winchester, D.P. 1998, "The National Cancer Data Base 10-year survey of breast carcinoma treatment at hospitals in the United States", *Cancer,* vol. 83, no. 6, pp. 1262-1273.

BLOOM, H.J. & RICHARDSON, W.W. 1957, "Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years", *British journal of cancer,* vol. 11, no. 3, pp. 359-377.

Bodian, C.A., Perzin, K.H., Lattes, R., Hoffmann, P. & Abernathy, T.G. 1993, "Prognostic significance of benign proliferative breast disease", *Cancer,* vol. 71, no. 12, pp. 3896-3907.

Boyd, N.F., Stone, J., Vogt, K.N., Connelly, B.S., Martin, L.J. & Minkin, S. 2003, "Dietary fat and breast cancer risk revisited: a meta-analysis of the published literature", *British journal of cancer,* vol. 89, no. 9, pp. 1672-1685.

Bradford, M.M. 1976, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", *Analytical Biochemistry,* vol. 72, pp. 248-254.

Brash, D.E. 1988, "UV mutagenic photoproducts in Escherichia coli and human cells: a molecular genetics perspective on human skin cancer", *Photochemistry and photobiology,* vol. 48, no. 1, pp. 59-66.

Brody, L.C. & Biesecker, B.B. 1998, "Breast cancer susceptibility genes. BRCA1 and BRCA2", *Medicine,* vol. 77, no. 3, pp. 208-226.

Brooks, S.C., Locke, E.R. & Soule, H.D. 1973, "Estrogen receptor in a human cell line (MCF-7) from breast carcinoma", *The Journal of biological chemistry,* vol. 248, no. 17, pp. 6251-6253.

Buchholz, T.A., Weil, M.M., Ashorn, C.L., Strom, E.A., Sigurdson, A., Bondy, M., Chakraborty, R., Cox, J.D., McNeese, M.D. & Story, M.D. 2004, "A Ser49Cys variant in the ataxia telangiectasia, mutated, gene that is more common in patients with breast carcinoma compared with population controls", *Cancer,* vol. 100, no. 7, pp. 1345-1351.

Buckhaults, P., Rago, C., St Croix, B., Romans, K.E., Saha, S., Zhang, L., Vogelstein, B. & Kinzler, K.W. 2001, "Secreted and cell surface genes expressed in benign and malignant colorectal tumors", *Cancer research,* vol. 61, no. 19, pp. 6996-7001.

Buckley, C.D., Pilling, D., Henriquez, N.V., Parsonage, G., Threlfall, K., Scheel-Toellner, D., Simmons, D.L., Akbar, A.N., Lord, J.M. & Salmon, M. 1999, "RGD peptides induce apoptosis by direct caspase-3 activation", *Nature,* vol. 397, no. 6719, pp. 534-539.

Bueno-de-Mesquita, J.M., Linn, S.C., Keijzer, R., Wesseling, J., Nuyten, D.S., van Krimpen, C., Meijers, C., de Graaf, P.W., Bos, M.M., Hart, A.A., Rutgers, E.J., Peterse, J.L., Halfwerk, H., de Groot, R., Pronk, A., Floore, A.N., Glas, A.M., Van't Veer, L.J. & van de Vijver, M.J. 2009, "Validation of 70-gene prognosis signature in node-negative breast cancer", *Breast cancer research and treatment,* vol. 117, no. 3, pp. 483-495.

Burger, M. 2009, "MCM2 and MCM5 as prognostic markers in colon cancer: a worthwhile approach", *Digestive diseases and sciences,* vol. 54, no. 2, pp. 197-198.

Burger, M. 2009, "MCM2 and MCM5 as prognostic markers in colon cancer: a worthwhile approach", *Digestive diseases and sciences,* vol. 54, no. 2, pp. 197-198.

Bustin, S.A. 2002, "Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems", *Journal of Molecular Endocrinology,* vol. 29, no. 1, pp. 23-39.

Bustin, S.A. 2000, "Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays", *Journal of Molecular Endocrinology,* vol. 25, no. 2, pp. 169-193.

Buyse, M., Loi, S., van't Veer, L., Viale, G., Delorenzi, M., Glas, A.M., d'Assignies, M.S., Bergh, J., Lidereau, R., Ellis, P., Harris, A., Bogaerts, J., Therasse, P., Floore, A., Amakrane, M., Piette, F., Rutgers, E., Sotiriou, C., Cardoso, F., Piccart, M.J. & TRANSBIG Consortium 2006, "Validation and clinical utility of a 70-gene prognostic signature for women with node-negative breast cancer", *Journal of the National Cancer Institute,* vol. 98, no. 17, pp. 1183-1192.

Byrne, C., Connolly, J.L., Colditz, G.A. & Schnitt, S.J. 2000, "Biopsy confirmed benign breast disease, postmenopausal use of exogenous female hormones, and breast carcinoma risk", *Cancer,* vol. 89, no. 10, pp. 2046-2052.

Byrnes, G.B., Southey, M.C. & Hopper, J.L. 2008, "Are the so-called low penetrance breast cancer genes, ATM, BRIP1, PALB2 and CHEK2, high risk for women with strong family histories?", *Breast cancer research : BCR,* vol. 10, no. 3, pp. 208.

Cailleau, R., Olive, M. & Cruciger, Q.V. 1978, "Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization", *In vitro,* vol. 14, no. 11, pp. 911-915.

Calaf, G.M., Echiburu-Chau, C., Zhao, Y.L. & Hei, T.K. 2008, "BigH3 protein expression as a marker for breast cancer", *International journal of molecular medicine,* vol. 21, no. 5, pp. 561-568.

Campeau, P.M., Foulkes, W.D. & Tischkowitz, M.D. 2008, "Hereditary breast cancer: new genetic developments, new therapeutic avenues", *Human genetics,* vol. 124, no. 1, pp. 31-42.

Carlson, C.A. & Ethier, S.P. 2000, "Lack of RB protein correlates with increased sensitivity to UV-radiation-induced apoptosis in human breast cancer cells", *Radiation research,* vol. 154, no. 5, pp. 590-599.

Cauley, J.A., Gutai, J.P., Kuller, L.H., LeDonne, D. & Powell, J.G. 1989, "The epidemiology of serum sex hormones in postmenopausal women", *American Journal of Epidemiology,* vol. 129, no. 6, pp. 1120-1131.

Chandler, J.R., Guillamondegui, O.M., Sisson, G.A., Strong, E.W. & Baker, H.W. 1976, "Clinical staging of cancer of the head and neck: a new "new" system", *American Journal of Surgery,* vol. 132, no. 4, pp. 525-528.

Chao, Y.L., Shepard, C.R. & Wells, A. 2010, "Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition", *Molecular cancer,* vol. 9, pp. 179.

Chen, P.L., Chen, C.F., Chen, Y., Xiao, J., Sharp, Z.D. & Lee, W.H. 1998, "The BRC repeats in BRCA2 are critical for RAD51 binding and resistance to methyl methanesulfonate treatment", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 95, no. 9, pp. 5287-5292.

Chen, X., Zhang, Y., Douglas, L. & Zhou, P. 2001, "UV-damaged DNA-binding proteins are targets of CUL-4A-mediated ubiquitination and degradation", *The Journal of biological chemistry,* vol. 276, no. 51, pp. 48175-48182.

Cho, H.R., Hong, S.B., Kim, Y.I., Lee, J.W. & Kim, N.I. 2004, "Differential expression of TGF-beta isoforms during differentiation of HaCaT human keratinocyte cells: implication for the separate role in epidermal differentiation", *Journal of Korean medical science,* vol. 19, no. 6, pp. 853-858.

Chomczynski, P. & Mackey, K. 1995, "Substitution of chloroform by bromo-chloropropane in the single-step method of RNA isolation", *Analytical Biochemistry,* vol. 225, no. 1, pp. 163-164.

Chung, J., Grammer, T.C., Lemon, K.P., Kazlauskas, A. & Blenis, J. 1994, "PDGF- and insulin-dependent pp70S6k activation mediated by phosphatidylinositol-3-OH kinase", *Nature,* vol. 370, no. 6484, pp. 71-75.

Chung, M., Chang, H.R., Bland, K.I. & Wanebo, H.J. 1996, "Younger women with breast carcinoma have a poorer prognosis than older women", *Cancer,* vol. 77, no. 1, pp. 97-103.

Ciocca, D.R., Gago, F.E., Fanelli, M.A. & Calderwood, S.K. 2006, "Co-expression of steroid receptors (estrogen receptor alpha and/or progesterone receptors) and Her-2/neu: Clinical implications", *The Journal of steroid biochemistry and molecular biology,* vol. 102, no. 1-5, pp. 32-40.

Ciocca, D.R., Gago, F.E., Fanelli, M.A. & Calderwood, S.K. 2006, "Co-expression of steroid receptors (estrogen receptor alpha and/or progesterone receptors) and Her-2/neu: Clinical implications", *The Journal of steroid biochemistry and molecular biology,* vol. 102, no. 1-5, pp. 32-40.

Cody, H.S.,3rd 2002, "Current surgical management of breast cancer", *Current opinion in obstetrics & gynecology,* vol. 14, no. 1, pp. 45-52.

Collaborative Group on Hormonal Factors in Breast Cancer 2002, "Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease", *Lancet,* vol. 360, no. 9328, pp. 187-195.

Colleoni, M., Rotmensz, N., Robertson, C., Orlando, L., Viale, G., Renne, G., Luini, A., Veronesi, P., Intra, M., Orecchia, R., Catalano, G., Galimberti, V., Nole, F., Martinelli, G. & Goldhirsch, A. 2002, "Very young women (<35 years) with operable breast cancer: features of disease at presentation", *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO,* vol. 13, no. 2, pp. 273-279.

Connolly JL, Fechner RE, Kempson RL et al., Recommendations for the reporting of breast carcinoma. Human Pathol 1996; 27:220-4

Coons, A.A., *et al*. (1942) *J. Immunol.* **45**, 159-170.

Cortez, D., Wang, Y., Qin, J. & Elledge, S.J. 1999, "Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks", *Science (New York, N.Y.),* vol. 286, no. 5442, pp. 1162-1166.

Cui, X., Schiff, R., Arpino, G., Osborne, C.K. & Lee, A.V. 2005, "Biology of progesterone receptor loss in breast cancer and its implications for endocrine therapy", *Journal of*

clinical oncology : official journal of the American Society of Clinical Oncology, vol. 23, no. 30, pp. 7721-7735.

Cui, Y. & Rohan, T.E. 2006, "Vitamin D, calcium, and breast cancer risk: a review", *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* vol. 15, no. 8, pp. 1427-1437.

D'Alessio, J.M. & Gerard, G.F. 1988, "Second-strand cDNA synthesis with E. coli DNA polymerase I and RNase H: the fate of information at the mRNA 5' terminus and the effect of E. coli DNA ligase", *Nucleic acids research,* vol. 16, no. 5, pp. 1999-2014.

Danoff, B.F., Haller, D.G., Glick, J.H. & Goodman, R.L. 1985, "Conservative surgery and irradiation in the treatment of early breast cancer", *Annals of Internal Medicine,* vol. 102, no. 5, pp. 634-642.

Datta, A., Bagchi, S., Nag, A., Shiyanov, P., Adami, G.R., Yoon, T. & Raychaudhuri, P. 2001, "The p48 subunit of the damaged-DNA binding protein DDB associates with the CBP/p300 family of histone acetyltransferase", *Mutation research,* vol. 486, no. 2, pp. 89-97.

de Jong, M.M., Nolte, I.M., te Meerman, G.J., van der Graaf, W.T., Oosterwijk, J.C., Kleibeuker, J.H., Schaapveld, M. & de Vries, E.G. 2002, "Genes other than BRCA1 and BRCA2 involved in breast cancer susceptibility", *Journal of medical genetics,* vol. 39, no. 4, pp. 225-242.

de la Rochefordiere, A., Asselain, B., Campana, F., Scholl, S.M., Fenton, J., Vilcoq, J.R., Durand, J.C., Pouillart, P., Magdelenat, H. & Fourquet, A. 1993, "Age as prognostic factor in premenopausal breast carcinoma", *Lancet,* vol. 341, no. 8852, pp. 1039-1043.

DeLellis, R.A., Sternberger, L.A., Mann, R.B., Banks, P.M. & Nakane, P.K. 1979, "Immunoperoxidase technics in diagnostic pathology. Report of a workshop sponsored by the National Cancer Institute", *American Journal of Clinical Pathology,* vol. 71, no. 5, pp. 483-488.

Deng, C.X. & Wang, R.H. 2003, "Roles of BRCA1 in DNA damage repair: a link between development and cancer", *Human molecular genetics,* vol. 12 Spec No 1, pp. R113-23.

DenHollander, N. & Befus, D. 1989, "Loss of antigens from immunoblotting membranes", *Journal of immunological methods,* vol. 122, no. 1, pp. 129-135.

Derynck, R., Akhurst, R.J. & Balmain, A. 2001, "TGF-beta signaling in tumor suppression and cancer progression", *Nature genetics,* vol. 29, no. 2, pp. 117-129.

Dirier, A., Burhanedtin-Zincircioglu, S., Karadayi, B., Isikdogan, A. & Aksu, R. 2009, "Characteristics and prognosis of breast cancer in younger women", *Journal of B.U.ON.: official journal of the Balkan Union of Oncology,* vol. 14, no. 4, pp. 619-623.

Dualan, R., Brody, T., Keeney, S., Nichols, A.F., Admon, A. & Linn, S. 1995, "Chromosomal localization and cDNA cloning of the genes (DDB1 and DDB2) for the p127 and p48 subunits of a human damage-specific DNA binding protein", *Genomics,* vol. 29, no. 1, pp. 62-69.

Dupont, W.D., Parl, F.F., Hartmann, W.H., Brinton, L.A., Winfield, A.C., Worrell, J.A., Schuyler, P.A. & Plummer, W.D. 1993, "Breast cancer risk associated with proliferative breast disease and atypical hyperplasia", *Cancer,* vol. 71, no. 4, pp. 1258-1265.

Early Breast Cancer Trialists' Collaborative Group 2002, "Multi-agent chemotherapy for early breast cancer", *Cochrane database of systematic reviews (Online),* vol. (1), no. 1, pp. CD000487.

Early Breast Cancer Trialists' Collaborative Group (EBCTCG) 2005, "Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials", *Lancet,* vol. 365, no. 9472, pp. 1687-1717.

Easton, D.F. 1994, "The inherited component of cancer", *British medical bulletin,* vol. 50, no. 3, pp. 527-535.

Easton, D.F., Pooley, K.A., Dunning, A.M., Pharoah, P.D., Thompson, D., Ballinger, D.G., Struewing, J.P., Morrison, J., Field, H., Luben, R., Wareham, N., Ahmed, S., Healey, C.S., Bowman, R., SEARCH collaborators, Meyer, K.B., Haiman, C.A., Kolonel, L.K., Henderson, B.E., Le Marchand, L., Brennan, P., Sangrajrang, S., Gaborieau, V., Odefrey, F., Shen, C.Y., Wu, P.E., Wang, H.C., Eccles, D., Evans, D.G., Peto, J., Fletcher, O., Johnson, N., Seal, S., Stratton, M.R., Rahman, N., Chenevix-Trench, G., Bojesen, S.E., Nordestgaard, B.G., Axelsson, C.K., Garcia-Closas, M., Brinton, L., Chanock, S., Lissowska, J., Peplonska, B., Nevanlinna, H., Fagerholm, R., Eerola, H.,

Kang, D., Yoo, K.Y., Noh, D.Y., Ahn, S.H., Hunter, D.J., Hankinson, S.E., Cox, D.G., Hall, P., Wedren, S., Liu, J., Low, Y.L., Bogdanova, N., Schurmann, P., Dork, T., Tollenaar, R.A., Jacobi, C.E., Devilee, P., Klijn, J.G., Sigurdson, A.J., Doody, M.M., Alexander, B.H., Zhang, J., Cox, A., Brock, I.W., MacPherson, G., Reed, M.W., Couch, F.J., Goode, E.L., Olson, J.E., Meijers-Heijboer, H., van den Ouweland, A., Uitterlinden, A., Rivadeneira, F., Milne, R.L., Ribas, G., Gonzalez-Neira, A., Benitez, J., Hopper, J.L., McCredie, M., Southey, M., Giles, G.G., Schroen, C., Justenhoven, C., Brauch, H., Hamann, U., Ko, Y.D., Spurdle, A.B., Beesley, J., Chen, X., kConFab, AOCS Management Group, Mannermaa, A., Kosma, V.M., Kataja, V., Hartikainen, J., Day, N.E., Cox, D.R. & Ponder, B.A. 2007, "Genome-wide association study identifies novel breast cancer susceptibility loci", *Nature,* vol. 447, no. 7148, pp. 1087-1093.

Eccles, D. & Tapper, W. 2010, "The influence of common polymorphisms on breast cancer", *Cancer treatment and research,* vol. 155, pp. 15-32.

Ellis, I.O., Galea, M., Broughton, N., Locker, A., Blamey, R.W. & Elston, C.W. 1992, "Pathological prognostic factors in breast cancer. II. Histological type. Relationship with survival in a large study with long-term follow-up", *Histopathology,* vol. 20, no. 6, pp. 479-489.

Elston, C.W. & Ellis, I.O. 2002, "Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up", *Histopathology,* vol. 41, no. 3A, pp. 154-161.

Elston, C.W. & Ellis, I.O. 1991, "Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up", *Histopathology,* vol. 19, no. 5, pp. 403-410.

Emmert-Buck, M.R., Bonner, R.F., Smith, P.D., Chuaqui, R.F., Zhuang, Z., Goldstein, S.R., Weiss, R.A. & Liotta, L.A. 1996, "Laser capture microdissection", *Science (New York, N.Y.),* vol. 274, no. 5289, pp. 998-1001.

Engel, L.W., Young, N.A., Tralka, T.S., Lippman, M.E., O'Brien, S.J. & Joyce, M.J. 1978, "Establishment and characterization of three new continuous cell lines derived from human breast carcinomas", *Cancer research,* vol. 38, no. 10, pp. 3352-3364.

Farmer, P., Bonnefoi, H., Becette, V., Tubiana-Hulin, M., Fumoleau, P., Larsimont, D., Macgrogan, G., Bergh, J., Cameron, D., Goldstein, D., Duss, S., Nicoulaz, A.L.,

Brisken, C., Fiche, M., Delorenzi, M. & Iggo, R. 2005, "Identification of molecular apocrine breast tumours by microarray analysis", *Oncogene,* vol. 24, no. 29, pp. 4660-4671.

Fergenbaum, J.H., Garcia-Closas, M., Hewitt, S.M., Lissowska, J., Sakoda, L.C. & Sherman, M.E. 2004, "Loss of antigenicity in stored sections of breast cancer tissue microarrays", *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* vol. 13, no. 4, pp. 667-672.

Ferguson, J.W., Mikesh, M.F., Wheeler, E.F. & LeBaron, R.G. 2003, "Developmental expression patterns of Beta-ig (betaIG-H3) and its function as a cell adhesion protein", *Mechanisms of development,* vol. 120, no. 8, pp. 851-864.

Ferrero-Pous, M., Hacene, K., Bouchet, C., Le Doussal, V., Tubiana-Hulin, M. & Spyratos, F. 2000, "Relationship between c-erbB-2 and other tumor characteristics in breast cancer prognosis", *Clinical cancer research : an official journal of the American Association for Cancer Research,* vol. 6, no. 12, pp. 4745-4754.

Fisher, E.R. 1984, "The impact of pathology on the biologic, diagnostic, prognostic, and therapeutic considerations in breast cancer", *The Surgical clinics of North America,* vol. 64, no. 6, pp. 1073-1093.

Fisher, E.R., Anderson, S., Dean, S., Dabbs, D., Fisher, B., Siderits, R., Pritchard, J., Pereira, T., Geyer, C. & Wolmark, N. 2005, "Solving the dilemma of the immunohistochemical and other methods used for scoring estrogen receptor and progesterone receptor in patients with invasive breast carcinoma", *Cancer,* vol. 103, no. 1, pp. 164-173.

Fisher, E.R., Gregorio, R.M., Fisher, B., Redmond, C., Vellios, F. & Sommers, S.C. 1975, "The pathology of invasive breast cancer. A syllabus derived from findings of the National Surgical Adjuvant Breast Project (protocol no. 4)", *Cancer,* vol. 36, no. 1, pp. 1-85.

Fletcher, D.A. & Mullins, R.D. 2010, "Cell mechanics and the cytoskeleton", *Nature,* vol. 463, no. 7280, pp. 485-492.

Fujiwara, Y., Masutani, C., Mizukoshi, T., Kondo, J., Hanaoka, F. & Iwai, S. 1999, "Characterization of DNA recognition by the human UV-damaged DNA-binding protein", *The Journal of biological chemistry,* vol. 274, no. 28, pp. 20027-20033.

Gaffney, E.V. 1982, "A cell line (HBL-100) established from human breast milk", *Cell and tissue research,* vol. 227, no. 3, pp. 563-568.

Galea, M.H., Blamey, R.W., Elston, C.E. & Ellis, I.O. 1992, "The Nottingham Prognostic Index in primary breast cancer", *Breast cancer research and treatment,* vol. 22, no. 3, pp. 207-219.

Garcia-Closas, M., Brinton, L.A., Lissowska, J., Chatterjee, N., Peplonska, B., Anderson, W.F., Szeszenia-Dabrowska, N., Bardin-Mikolajczak, A., Zatonski, W., Blair, A., Kalaylioglu, Z., Rymkiewicz, G., Mazepa-Sikora, D., Kordek, R., Lukaszek, S. & Sherman, M.E. 2006, "Established breast cancer risk factors by clinically important tumour characteristics", *British journal of cancer,* vol. 95, no. 1, pp. 123-129.

Gatti, R.A., Boder, E., Vinters, H.V., Sparkes, R.S., Norman, A. & Lange, K. 1991, "Ataxia-telangiectasia: an interdisciplinary approach to pathogenesis", *Medicine,* vol. 70, no. 2, pp. 99-117.

Gescher, A. 1998, "Analogs of staurosporine: potential anticancer drugs?", *General pharmacology,* vol. 31, no. 5, pp. 721-728.

Gillardon, F. 2009, "Leucine-rich repeat kinase 2 phosphorylates brain tubulin-beta isoforms and modulates microtubule stability--a point of convergence in parkinsonian neurodegeneration?", *Journal of neurochemistry,* vol. 110, no. 5, pp. 1514-1522.

Gnerlich, J.L., Deshpande, A.D., Jeffe, D.B., Sweet, A., White, N. & Margenthaler, J.A. 2009, "Elevated breast cancer mortality in women younger than age 40 years compared with older women is attributed to poorer survival in early-stage disease", *Journal of the American College of Surgeons,* vol. 208, no. 3, pp. 341-347.

Goodwin, P.J., Ennis, M., Pritchard, K.I., Trudeau, M.E., Koo, J., Hartwick, W., Hoffma, B. & Hood, N. 2002, "Insulin-like growth factor binding proteins 1 and 3 and breast cancer outcomes", *Breast cancer research and treatment,* vol. 74, no. 1, pp. 65-76.

Gordon, L.A., Mulligan, K.T., Maxwell-Jones, H., Adams, M., Walker, R.A. & Jones, J.L. 2003, "Breast cell invasive potential relates to the myoepithelial phenotype", *International journal of cancer.Journal international du cancer,* vol. 106, no. 1, pp. 8-16.

Graham, F.L., van der Eb, A.J. & Heijneker, H.L. 1974, "Size and location of the transforming region in human adenovirus type 5 DNA", *Nature,* vol. 251, no. 5477, pp. 687-691.

Groisman, R., Polanowska, J., Kuraoka, I., Sawada, J., Saijo, M., Drapkin, R., Kisselev, A.F., Tanaka, K. & Nakatani, Y. 2003, "The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage", *Cell,* vol. 113, no. 3, pp. 357-367.

Guida, T., Salvatore, G., Faviana, P., Giannini, R., Garcia-Rostan, G., Provitera, L., Basolo, F., Fusco, A., Carlomagno, F. & Santoro, M. 2005, "Mitogenic effects of the up-regulation of minichromosome maintenance proteins in anaplastic thyroid carcinoma", *The Journal of clinical endocrinology and metabolism,* vol. 90, no. 8, pp. 4703-4709.

Gupta, P.B. & Kuperwasser, C. 2006, "Contributions of estrogen to ER-negative breast tumor growth", *The Journal of steroid biochemistry and molecular biology,* vol. 102, no. 1-5, pp. 71-78.

Guttery, D.S. April 2009; University of Leicester, " Molecular cloning and functional studies of tenascin-C isoforms containing the fibronectin-type III repeat additional domain 1 (AD1) ", .

Guttery, D.S., Hancox, R.A., Mulligan, K.T., Hughes, S., Lambe, S.M., Pringle, J.H., Walker, R.A., Jones, J.L. & Shaw, J.A. 2010, "Association of invasion-promoting tenascin-C additional domains with breast cancers in young women", *Breast cancer research : BCR,* vol. 12, no. 4, pp. R57.

Hagios, C., Lochter, A. & Bissell, M.J. 1998, "Tissue architecture: the ultimate regulator of epithelial function?", *Philosophical transactions of the Royal Society of London.Series B, Biological sciences,* vol. 353, no. 1370, pp. 857-870.

Hall, J.M., Lee, M.K., Newman, B., Morrow, J.E., Anderson, L.A., Huey, B. & King, M.C. 1990, "Linkage of early-onset familial breast cancer to chromosome 17q21", *Science (New York, N.Y.),* vol. 250, no. 4988, pp. 1684-1689.

Hammer, C., Fanning, A. & Crowe, J. 2008, "Overview of breast cancer staging and surgical treatment options", *Cleveland Clinic journal of medicine,* vol. 75 Suppl 1, pp. S10-6.

Hanby, A.M. 2005, "The pathology of breast cancer and the role of the histopathology laboratory", *Clinical oncology (Royal College of Radiologists (Great Britain)),* vol. 17, no. 4, pp. 234-239.

Hancox, R.A., Allen, M.D., Holliday, D.L., Edwards, D.R., Pennington, C.J., Guttery, D.S., Shaw, J.A., Walker, R.A., Pringle, J.H. & Jones, J.L. 2009, "Tumour-associated tenascin-C isoforms promote breast cancer cell invasion and growth by matrix metalloproteinase-dependent and independent mechanisms", *Breast cancer research : BCR,* vol. 11, no. 2, pp. R24.

Hanssen, E., Reinboth, B. & Gibson, M.A. 2003, "Covalent and non-covalent interactions of betaig-h3 with collagen VI. Beta ig-h3 is covalently attached to the amino-terminal region of collagen VI in tissue microfibrils", *The Journal of biological chemistry,* vol. 278, no. 27, pp. 24334-24341.

Harris Jay R, Lippman Marc E, Morrow Monica, Osborne Kent C 2004, *Diseases of the Breast,* Third Edition edn, .

Hartmann, L.C., Sellers, T.A., Frost, M.H., Lingle, W.L., Degnim, A.C., Ghosh, K., Vierkant, R.A., Maloney, S.D., Pankratz, V.S., Hillman, D.W., Suman, V.J., Johnson, J., Blake, C., Tlsty, T., Vachon, C.M., Melton, L.J.,3rd & Visscher, D.W. 2005, "Benign breast disease and the risk of breast cancer", *The New England journal of medicine,* vol. 353, no. 3, pp. 229-237.

Harvey, J.M., Clark, G.M., Osborne, C.K. & Allred, D.C. 1999, "Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 17, no. 5, pp. 1474-1481.

Hashimoto, K., Noshiro, M., Ohno, S., Kawamoto, T., Satakeda, H., Akagawa, Y., Nakashima, K., Okimura, A., Ishida, H., Okamoto, T., Pan, H., Shen, M., Yan, W. & Kato, Y. 1997, "Characterization of a cartilage-derived 66-kDa protein (RGD-CAP/beta ig-h3) that binds to collagen", *Biochimica et biophysica acta,* vol. 1355, no. 3, pp. 303-314.

Herschkowitz, J.I., Simin, K., Weigman, V.J., Mikaelian, I., Usary, J., Hu, Z., Rasmussen, K.E., Jones, L.P., Assefnia, S., Chandrasekharan, S., Backlund, M.G., Yin, Y., Khramtsov, A.I., Bastein, R., Quackenbush, J., Glazer, R.I., Brown, P.H., Green, J.E., Kopelovich, L., Furth, P.A., Palazzo, J.P., Olopade, O.I., Bernard, P.S., Churchill, G.A., Van Dyke, T. & Perou, C.M. 2007, "Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors", *Genome biology,* vol. 8, no. 5, pp. R76.

Hinshelwood, R.A., Huschtscha, L.I., Melki, J., Stirzaker, C., Abdipranoto, A., Vissel, B., Ravasi, T., Wells, C.A., Hume, D.A., Reddel, R.R. & Clark, S.J. 2007, "Concordant epigenetic silencing of transforming growth factor-beta signaling pathway genes occurs early in breast carcinogenesis", *Cancer research,* vol. 67, no. 24, pp. 11517-11527.

Hiraguri, S., Godfrey, T., Nakamura, H., Graff, J., Collins, C., Shayesteh, L., Doggett, N., Johnson, K., Wheelock, M., Herman, J., Baylin, S., Pinkel, D. & Gray, J. 1998, "Mechanisms of inactivation of E-cadherin in breast cancer cell lines", *Cancer research,* vol. 58, no. 9, pp. 1972-1977.

Hoang, M.L., Leon, R.P., Pessoa-Brandao, L., Hunt, S., Raghuraman, M.K., Fangman, W.L., Brewer, B.J. & Sclafani, R.A. 2007, "Structural changes in Mcm5 protein bypass Cdc7-Dbf4 function and reduce replication origin efficiency in Saccharomyces cerevisiae", *Molecular and cellular biology,* vol. 27, no. 21, pp. 7594-7602.

Holliday, D.L., Brouilette, K.T., Markert, A., Gordon, L.A. & Jones, J.L. 2009, "Novel multicellular organotypic models of normal and malignant breast: tools for dissecting the role of the microenvironment in breast cancer progression", *Breast cancer research : BCR,* vol. 11, no. 1, pp. R3.

Howard, R.A., Leitzmann, M.F., Linet, M.S. & Freedman, D.M. 2009, "Physical activity and breast cancer risk among pre- and postmenopausal women in the U.S. Radiologic Technologists cohort", *Cancer causes & control : CCC,* vol. 20, no. 3, pp. 323-333.

Hu, Y.C., Lam, K.Y., Law, S., Wong, J. & Srivastava, G. 2001, "Profiling of differentially expressed cancer-related genes in esophageal squamous cell carcinoma (ESCC) using human cancer cDNA arrays: overexpression of oncogene MET correlates with tumor differentiation in ESCC", *Clinical cancer research : an official journal of the American Association for Cancer Research,* vol. 7, no. 11, pp. 3519-3525.

Hu, Z., Fan, C., Oh, D.S., Marron, J.S., He, X., Qaqish, B.F., Livasy, C., Carey, L.A., Reynolds, E., Dressler, L., Nobel, A., Parker, J., Ewend, M.G., Sawyer, L.R., Wu, J., Liu, Y., Nanda, R., Tretiakova, M., Ruiz Orrico, A., Dreher, D., Palazzo, J.P., Perreard, L., Nelson, E., Mone, M., Hansen, H., Mullins, M., Quackenbush, J.F., Ellis, M.J., Olopade, O.I., Bernard, P.S. & Perou, C.M. 2006, "The molecular portraits of breast tumors are conserved across microarray platforms", *BMC genomics,* vol. 7, pp. 96.

Hu, Z., Shao, M., Yuan, J., Xu, L., Wang, F., Wang, Y., Yuan, W., Qian, J., Ma, H., Wang, Y., Liu, H., Chen, W., Yang, L., Jin, G., Huo, X., Chen, F., Jin, L., Wei, Q., Huang, W., Lu, D., Wu, T. & Shen, H. 2006, "Polymorphisms in DNA damage binding protein 2 (DDB2) and susceptibility of primary lung cancer in the Chinese: a case-control study", *Carcinogenesis,* vol. 27, no. 7, pp. 1475-1480.

Hulka, B.S. & Moorman, P.G. 2001, "Breast cancer: hormones and other risk factors", *Maturitas,* vol. 38, no. 1, pp. 103-13; discussion 113-6.

Hunter, D.J., Kraft, P., Jacobs, K.B., Cox, D.G., Yeager, M., Hankinson, S.E., Wacholder, S., Wang, Z., Welch, R., Hutchinson, A., Wang, J., Yu, K., Chatterjee, N., Orr, N., Willett, W.C., Colditz, G.A., Ziegler, R.G., Berg, C.D., Buys, S.S., McCarty, C.A., Feigelson, H.S., Calle, E.E., Thun, M.J., Hayes, R.B., Tucker, M., Gerhard, D.S., Fraumeni, J.F.,Jr, Hoover, R.N., Thomas, G. & Chanock, S.J. 2007, "A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer", *Nature genetics,* vol. 39, no. 7, pp. 870-874.

Hwang, B.J., Ford, J.M., Hanawalt, P.C. & Chu, G. 1999, "Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 96, no. 2, pp. 424-428.

Iacobuzio-Donahue, C.A., Maitra, A., Olsen, M., Lowe, A.W., van Heek, N.T., Rosty, C., Walter, K., Sato, N., Parker, A., Ashfaq, R., Jaffee, E., Ryu, B., Jones, J., Eshleman, J.R., Yeo, C.J., Cameron, J.L., Kern, S.E., Hruban, R.H., Brown, P.O. & Goggins, M. 2003, "Exploration of global gene expression patterns in pancreatic adenocarcinoma using cDNA microarrays", *The American journal of pathology,* vol. 162, no. 4, pp. 1151-1162.

Inoki, T., Yamagami, S., Inoki, Y., Tsuru, T., Hamamoto, T., Kagawa, Y., Mori, T. & Endo, H. 2004, "Human DDB2 splicing variants are dominant negative inhibitors of UV-damaged

DNA repair", *Biochemical and biophysical research communications,* vol. 314, no. 4, pp. 1036-1043.

Irigoyen, M., Anso, E., Salvo, E., de las Herrerias, J.D., Martinez-Irujo, J.J. & Rouzaut, A. 2008, "TGFbeta-induced protein mediates lymphatic endothelial cell adhesion to the extracellular matrix under low oxygen conditions", *Cellular and molecular life sciences : CMLS,* vol. 65, no. 14, pp. 2244-2255.

Itoh, T., Cado, D., Kamide, R. & Linn, S. 2004, "DDB2 gene disruption leads to skin tumors and resistance to apoptosis after exposure to ultraviolet light but not a chemical carcinogen", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 101, no. 7, pp. 2052-2057.

Itoh, T., Iwashita, S., Cohen, M.B., Meyerholz, D.K. & Linn, S. 2007, "Ddb2 is a haploinsufficient tumor suppressor and controls spontaneous germ cell apoptosis", *Human molecular genetics,* vol. 16, no. 13, pp. 1578-1586.

Ivanov, S.V., Ivanova, A.V., Salnikow, K., Timofeeva, O., Subramaniam, M. & Lerman, M.I. 2008, "Two novel VHL targets, TGFBI (BIGH3) and its transactivator KLF10, are up-regulated in renal clear cell carcinoma and other tumors", *Biochemical and biophysical research communications,* vol. 370, no. 4, pp. 536-540.

Jacobsen, L.B., Calvin, S.A., Colvin, K.E. & Wright, M. 2004, "FuGENE 6 Transfection Reagent: the gentle power", *Methods (San Diego, Calif.),* vol. 33, no. 2, pp. 104-112.

Jarvinen, T.A., Pelto-Huikko, M., Holli, K. & Isola, J. 2000, "Estrogen receptor beta is coexpressed with ERalpha and PR and associated with nodal status, grade, and proliferation rate in breast cancer", *The American journal of pathology,* vol. 156, no. 1, pp. 29-35.

Jatoi, I., Hilsenbeck, S.G., Clark, G.M. & Osborne, C.K. 1999, "Significance of axillary lymph node metastasis in primary breast cancer", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 17, no. 8, pp. 2334-2340.

Johnson, S.M., Shaw, J.A. & Walker, R.A. 2002, "Sporadic breast cancer in young women: prevalence of loss of heterozygosity at p53, BRCA1 and BRCA2", *International journal of cancer.Journal international du cancer,* vol. 98, no. 2, pp. 205-209.

Kakizaki, M., Kuriyama, S., Sone, T., Ohmori-Matsuda, K., Hozawa, A., Nakaya, N., Fukudo, S. & Tsuji, I. 2008, "Sleep duration and the risk of breast cancer: the Ohsaki Cohort Study", *British journal of cancer,* vol. 99, no. 9, pp. 1502-1505.

Kalkhoven, E., Roelen, B.A., de Winter, J.P., Mummery, C.L., van den Eijnden-van Raaij, A.J., van der Saag, P.T. & van der Burg, B. 1995, "Resistance to transforming growth factor beta and activin due to reduced receptor expression in human breast tumor cell lines", *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research,* vol. 6, no. 9, pp. 1151-1161.

Kalluri, R. & Zeisberg, M. 2006, "Fibroblasts in cancer", *Nature reviews.Cancer,* vol. 6, no. 5, pp. 392-401.

Kannabiran, C. & Klintworth, G.K. 2006, "TGFBI gene mutations in corneal dystrophies", *Human mutation,* vol. 27, no. 7, pp. 615-625.

Kastan, M.B. & Bartek, J. 2004, "Cell-cycle checkpoints and cancer", *Nature,* vol. 432, no. 7015, pp. 316-323.

Kattan, Z., Marchal, S., Brunner, E., Ramacci, C., Leroux, A., Merlin, J.L., Domenjoud, L., Dauca, M. & Becuwe, P. 2008, "Damaged DNA binding protein 2 plays a role in breast cancer cell growth", *PloS one,* vol. 3, no. 4, pp. e2002.

Kawai, M., Minami, Y., Kuriyama, S., Kakizaki, M., Kakugawa, Y., Nishino, Y., Ishida, T., Fukao, A., Tsuji, I. & Ohuchi, N. 2010, "Reproductive factors, exogenous female hormone use and breast cancer risk in Japanese: the Miyagi Cohort Study", *Cancer causes & control : CCC,* vol. 21, no. 1, pp. 135-145.

Kellen, E., Vansant, G., Christiaens, M.R., Neven, P. & Van Limbergen, E. 2009, "Lifestyle changes and breast cancer prognosis: a review", *Breast cancer research and treatment,* vol. 114, no. 1, pp. 13-22.

Kelsey, J.L., Gammon, M.D. & John, E.M. 1993, "Reproductive factors and breast cancer", *Epidemiologic reviews,* vol. 15, no. 1, pp. 36-47.

Kern, S.E., Pietenpol, J.A., Thiagalingam, S., Seymour, A., Kinzler, K.W. & Vogelstein, B. 1992, "Oncogenic forms of p53 inhibit p53-regulated gene expression", *Science (New York, N.Y.),* vol. 256, no. 5058, pp. 827-830.

Keydar, I., Chen, L., Karby, S., Weiss, F.R., Delarea, J., Radu, M., Chaitcik, S. & Brenner, H.J. 1979, "Establishment and characterization of a cell line of human breast carcinoma origin", *European journal of cancer,* vol. 15, no. 5, pp. 659-670.

Kim, J.E., Jeong, H.W., Nam, J.O., Lee, B.H., Choi, J.Y., Park, R.W., Park, J.Y. & Kim, I.S. 2002, "Identification of motifs in the fasciclin domains of the transforming growth factor-beta-induced matrix protein betaig-h3 that interact with the alphavbeta5 integrin", *The Journal of biological chemistry,* vol. 277, no. 48, pp. 46159-46165.

Kim, J.E., Kim, E.H., Han, E.H., Park, R.W., Park, I.H., Jun, S.H., Kim, J.C., Young, M.F. & Kim, I.S. 2000, "A TGF-beta-inducible cell adhesion molecule, betaig-h3, is downregulated in melorheostosis and involved in osteogenesis", *Journal of cellular biochemistry,* vol. 77, no. 2, pp. 169-178.

Kim, J.E., Kim, S.J., Lee, B.H., Park, R.W., Kim, K.S. & Kim, I.S. 2000, "Identification of motifs for cell adhesion within the repeated domains of transforming growth factor-beta-induced gene, betaig-h3", *The Journal of biological chemistry,* vol. 275, no. 40, pp. 30907-30915.

Kim, K.J., Huh, S.J., Yang, J.H., Park, W., Nam, S.J., Kim, J.H., Lee, J.H., Kang, S.S., Lee, J.E., Kang, M.K., Park, Y.J. & Nam, H.R. 2005, "Treatment results and prognostic factors of early breast cancer treated with a breast conserving operation and radiotherapy", *Japanese journal of clinical oncology,* vol. 35, no. 3, pp. 126-133.

Kischkel, F.C., Lawrence, D.A., Tinel, A., LeBlanc, H., Virmani, A., Schow, P., Gazdar, A., Blenis, J., Arnott, D. & Ashkenazi, A. 2001, "Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8", *The Journal of biological chemistry,* vol. 276, no. 49, pp. 46639-46646.

Knights, V. & Cook, S.J. 2010, "De-regulated FGF receptors as therapeutic targets in cancer", *Pharmacology & therapeutics,* vol. 125, no. 1, pp. 105-117.

Korkolopoulou, P., Givalos, N., Saetta, A., Goudopoulou, A., Gakiopoulou, H., Thymara, I., Thomas-Tsagli, E. & Patsouris, E. 2005, "Minichromosome maintenance proteins 2 and 5 expression in muscle-invasive urothelial cancer: a multivariate survival study including proliferation markers and cell cycle regulators", *Human pathology,* vol. 36, no. 8, pp. 899-907.

Koukourakis, G. 2009, "Radiation therapy for early breast cancer", *Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico,* vol. 11, no. 9, pp. 596-603.

Kraus, W.L., Montano, M.M. & Katzenellenbogen, B.S. 1993, "Cloning of the rat progesterone receptor gene 5'-region and identification of two functionally distinct promoters", *Molecular endocrinology (Baltimore, Md.),* vol. 7, no. 12, pp. 1603-1616.

Kretzschmar, M. 2000, "Transforming growth factor-beta and breast cancer: Transforming growth factor-beta/SMAD signaling defects and cancer", *Breast cancer research : BCR,* vol. 2, no. 2, pp. 107-115.

Kulaksiz, G., Reardon, J.T. & Sancar, A. 2005, "Xeroderma pigmentosum complementation group E protein (XPE/DDB2): purification of various complexes of XPE and analyses of their damaged DNA binding and putative DNA repair properties", *Molecular and cellular biology,* vol. 25, no. 22, pp. 9784-9792.

Lacroix, M. & Leclercq, G. 2004, "Relevance of breast cancer cell lines as models for breast tumours: an update", *Breast cancer research and treatment,* vol. 83, no. 3, pp. 249-289.

Laemmli, U.K. 1970, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4", *Nature,* vol. 227, no. 5259, pp. 680-685.

Lalloo, F., Varley, J., Moran, A., Ellis, D., O'dair, L., Pharoah, P., Antoniou, A., Hartley, R., Shenton, A., Seal, S., Bulman, B., Howell, A. & Evans, D.G. 2006, "BRCA1, BRCA2 and TP53 mutations in very early-onset breast cancer with associated risks to relatives", *European journal of cancer (Oxford, England : 1990),* vol. 42, no. 8, pp. 1143-1150.

Lambe, S.M. 2008, *The analysis of novel gene targets in breast cancer in women ≤35 years*, University of Leicester.

Landis, J.R. & Koch, G.G. 1977, "The measurement of observer agreement for categorical data", *Biometrics,* vol. 33, no. 1, pp. 159-174.

Landis, J.R. & Koch, G.G. 1977, "The measurement of observer agreement for categorical data", *Biometrics,* vol. 33, no. 1, pp. 159-174.

LeBaron, R.G., Bezverkov, K.I., Zimber, M.P., Pavelec, R., Skonier, J. & Purchio, A.F. 1995, "Beta IG-H3, a novel secretory protein inducible by transforming growth factor-beta, is present in normal skin and promotes the adhesion and spreading of dermal fibroblasts in vitro", *The Journal of investigative dermatology,* vol. 104, no. 5, pp. 844-849.

Lee, B.H., Bae, J.S., Park, R.W., Kim, J.E., Park, J.Y. & Kim, I.S. 2006, "Betaig-H3 Triggers Signaling Pathways Mediating Adhesion and Migration of Vascular Smooth Muscle Cells through Alphavbeta5 Integrin", *Experimental & molecular medicine,* vol. 38, no. 2, pp. 153-161.

Lelievre, S.A. & Bissell, M.J. 1998, "Communication between the cell membrane and the nucleus: role of protein compartmentalization", *Journal of cellular biochemistry.Supplement,* vol. 30-31, pp. 250-263.

Li, L.F., Xu, X.J., Zhao, Y., Liu, Z.B., Shen, Z.Z., Jin, W.R. & Shao, Z.M. 2009, "Integrated gene expression profile predicts prognosis of breast cancer patients", *Breast cancer research and treatment,* vol. 113, no. 2, pp. 231-237.

Liaw, D., Marsh, D.J., Li, J., Dahia, P.L., Wang, S.I., Zheng, Z., Bose, S., Call, K.M., Tsou, H.C., Peacocke, M., Eng, C. & Parsons, R. 1997, "Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome", *Nature genetics,* vol. 16, no. 1, pp. 64-67.

Liu, H., Li, X., Yoon, V. & Clarke, R. 2008, "Annotating breast cancer microarray samples using ontologies", *AMIA ...Annual Symposium proceedings / AMIA Symposium.AMIA Symposium,* , pp. 414-418.

Lopez-Garcia, M.A., Geyer, F.C., Lacroix-Triki, M., Marchio, C. & Reis-Filho, J.S. 2010, "Breast cancer precursors revisited: molecular features and progression pathways", *Histopathology,* vol. 57, no. 2, pp. 171-192.

Low, I. & Wieland, T. 1974, "The interaction of phalloidin. Some of its derivatives, and of other cyclic peptides with muscle actin as studied by viscosimetry", *FEBS letters,* vol. 44, no. 3, pp. 340-343.

Lynch, M.A., Petrel, T.A., Song, H., Knobloch, T.J., Casto, B.C., Ramljak, D., Anderson, L.M., DeGroff, V., Stoner, G.D., Brueggemeier, R.W. & Weghorst, C.M. 2001, "Responsiveness to transforming growth factor-beta (TGF-beta)-mediated growth

inhibition is a function of membrane-bound TGF-beta type II receptor in human breast cancer cells", *Gene expression,* vol. 9, no. 4-5, pp. 157-171.

Ma, C., Rong, Y., Radiloff, D.R., Datto, M.B., Centeno, B., Bao, S., Cheng, A.W., Lin, F., Jiang, S., Yeatman, T.J. & Wang, X.F. 2008, "Extracellular matrix protein betaig-h3/TGFBI promotes metastasis of colon cancer by enhancing cell extravasation", *Genes & development,* vol. 22, no. 3, pp. 308-321.

Ma, X.J., Wang, Z., Ryan, P.D., Isakoff, S.J., Barmettler, A., Fuller, A., Muir, B., Mohapatra, G., Salunga, R., Tuggle, J.T., Tran, Y., Tran, D., Tassin, A., Amon, P., Wang, W., Wang, W., Enright, E., Stecker, K., Estepa-Sabal, E., Smith, B., Younger, J., Balis, U., Michaelson, J., Bhan, A., Habin, K., Baer, T.M., Brugge, J., Haber, D.A., Erlander, M.G. & Sgroi, D.C. 2004, "A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen", *Cancer cell,* vol. 5, no. 6, pp. 607-616.

Maemura, M., Akiyama, S.K., Woods, V.L.,Jr & Dickson, R.B. 1995, "Expression and ligand binding of alpha 2 beta 1 integrin on breast carcinoma cells", *Clinical & experimental metastasis,* vol. 13, no. 4, pp. 223-235.

Maggard, M.A., O'Connell, J.B., Lane, K.E., Liu, J.H., Etzioni, D.A. & Ko, C.Y. 2003, "Do young breast cancer patients have worse outcomes?", *The Journal of surgical research,* vol. 113, no. 1, pp. 109-113.

Maiorano, D., Lutzmann, M. & Mechali, M. 2006, "MCM proteins and DNA replication", *Current opinion in cell biology,* vol. 18, no. 2, pp. 130-136.

Mangiapane, S., Blettner, M. & Schlattmann, P. 2008, "Aspirin use and breast cancer risk: a meta-analysis and meta-regression of observational studies from 2001 to 2005", *Pharmacoepidemiology and drug safety,* vol. 17, no. 2, pp. 115-124.

Mani, S. & Herceg, Z. 2010, "DNA demethylating agents and epigenetic therapy of cancer", *Advances in Genetics,* vol. 70, pp. 327-340.

Mann, M. & Jensen, O.N. 2003, "Proteomic analysis of post-translational modifications", *Nature biotechnology,* vol. 21, no. 3, pp. 255-261.

Margolis, R.L., Lohez, O.D. & Andreassen, P.R. 2003, "G1 tetraploidy checkpoint and the suppression of tumorigenesis", *Journal of cellular biochemistry,* vol. 88, no. 4, pp. 673-683.

Maruti, S.S., Willett, W.C., Feskanich, D., Rosner, B. & Colditz, G.A. 2008, "A prospective study of age-specific physical activity and premenopausal breast cancer", *Journal of the National Cancer Institute,* vol. 100, no. 10, pp. 728-737.

Mavaddat, N., Antoniou, A.C., Easton, D.F. & Garcia-Closas, M. 2010, "Genetic susceptibility to breast cancer", *Molecular oncology,* vol. 4, no. 3, pp. 174-191.

McBride, O.W., Merry, D. & Givol, D. 1986, "The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm (17p13)", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 83, no. 1, pp. 130-134.

McCarty, K.S.,Jr, Miller, L.S., Cox, E.B., Konrath, J. & McCarty KS, S. 1985, "Estrogen receptor analyses. Correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies", *Archives of Pathology & Laboratory Medicine,* vol. 109, no. 8, pp. 716-721.

McCormack, V.A. & dos Santos Silva, I. 2006, "Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis", *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* vol. 15, no. 6, pp. 1159-1169.

Megdal, S.P., Kroenke, C.H., Laden, F., Pukkala, E. & Schernhammer, E.S. 2005, "Night work and breast cancer risk: a systematic review and meta-analysis", *European journal of cancer (Oxford, England : 1990),* vol. 41, no. 13, pp. 2023-2032.

Melchor, L., Honrado, E., Garcia, M.J., Alvarez, S., Palacios, J., Osorio, A., Nathanson, K.L. & Benitez, J. 2008, "Distinct genomic aberration patterns are found in familial breast cancer associated with different immunohistochemical subtypes", *Oncogene,* vol. 27, no. 22, pp. 3165-3175.

Meller, M., Vadachkoria, S., Luthy, D.A. & Williams, M.A. 2005, "Evaluation of housekeeping genes in placental comparative expression studies", *Placenta,* vol. 26, no. 8-9, pp. 601-607.

Menard, S., Fortis, S., Castiglioni, F., Agresti, R. & Balsari, A. 2001, "HER2 as a prognostic factor in breast cancer", *Oncology,* vol. 61 Suppl 2, pp. 67-72.

Menard, S., Tagliabue, E., Campiglio, M. & Pupa, S.M. 2000, "Role of HER2 gene overexpression in breast carcinoma", *Journal of cellular physiology,* vol. 182, no. 2, pp. 150-162.

Meyer, K.B., Maia, A.T., O'Reilly, M., Teschendorff, A.E., Chin, S.F., Caldas, C. & Ponder, B.A. 2008, "Allele-specific up-regulation of FGFR2 increases susceptibility to breast cancer", *PLoS biology,* vol. 6, no. 5, pp. e108.

Minig, V., Kattan, Z., van Beeumen, J., Brunner, E. & Becuwe, P. 2009, "Identification of DDB2 protein as a transcriptional regulator of constitutive SOD2 gene expression in human breast cancer cells", *The Journal of biological chemistry,* vol. 284, no. 21, pp. 14165-14176.

Mitov, F.S. & Molov, V.V. 2006, "Breast-conserving surgery in early-stage breast cancer (indications, local recurrences, survival, cosmetic results)", *Folia medica,* vol. 48, no. 1, pp. 23-30.

Moiseeva, E.P., Heukers, R. & Manson, M.M. 2007, "EGFR and Src are involved in indole-3-carbinol-induced death and cell cycle arrest of human breast cancer cells", *Carcinogenesis,* vol. 28, no. 2, pp. 435-445.

Monninkhof, E.M., Elias, S.G., Vlems, F.A., van der Tweel, I., Schuit, A.J., Voskuil, D.W., van Leeuwen, F.E. & TFPAC 2007, "Physical activity and breast cancer: a systematic review", *Epidemiology (Cambridge, Mass.),* vol. 18, no. 1, pp. 137-157.

Morand, S., Buchillier, V., Maurer, F., Bonny, C., Arsenijevic, Y., Munier, F.L. & Schorderet, D.F. 2003, "Induction of apoptosis in human corneal and HeLa cells by mutated BIGH3", *Investigative ophthalmology & visual science,* vol. 44, no. 7, pp. 2973-2979.

Mouridsen, H.T., Rose, C., Brodie, A.H. & Smith, I.E. 2003, "Challenges in the endocrine management of breast cancer", *Breast (Edinburgh, Scotland),* vol. 12 Suppl 2, pp. S2-19.

Mullis, K.B. 1990, "Target amplification for DNA analysis by the polymerase chain reaction", *Annales de Biologie Clinique,* vol. 48, no. 8, pp. 579-582.

Munier, F.L., Korvatska, E., Djemai, A., Le Paslier, D., Zografos, L., Pescia, G. & Schorderet, D.F. 1997, "Kerato-epithelin mutations in four 5q31-linked corneal dystrophies", *Nature genetics,* vol. 15, no. 3, pp. 247-251.

Murphy, L.C. & Watson, P.H. 2006, "Is oestrogen receptor-beta a predictor of endocrine therapy responsiveness in human breast cancer?", *Endocrine-related cancer,* vol. 13, no. 2, pp. 327-334.

Murphy, N., Ring, M., Heffron, C.C., Martin, C.M., McGuinness, E., Sheils, O. & O'Leary, J.J. 2005, "Quantitation of CDC6 and MCM5 mRNA in cervical intraepithelial neoplasia and invasive squamous cell carcinoma of the cervix", *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc,* vol. 18, no. 6, pp. 844-849.

Nam, E.J., Sa, K.H., You, D.W., Cho, J.H., Seo, J.S., Han, S.W., Park, J.Y., Kim, S.I., Kyung, H.S., Kim, I.S. & Kang, Y.M. 2006, "Up-regulated transforming growth factor beta-inducible gene h3 in rheumatoid arthritis mediates adhesion and migration of synoviocytes through alpha v beta3 integrin: Regulation by cytokines", *Arthritis and Rheumatism,* vol. 54, no. 9, pp. 2734-2744.

Narayan, P.J. & Dragunow, M. 2010, "High content analysis of histone acetylation in human cells and tissues", *Journal of neuroscience methods,* vol. 193, no. 1, pp. 54-61.

National Coordinating Group for Breast Screening Pathology. Pathology Reporting of Breast Disease. NHSBSP Publication No 58. Sheffield: NHS Cancer Screening Programmes and The Royal Collage of Pathologists, 2005.

Natrajan, R., Lambros, M.B., Rodriguez-Pinilla, S.M., Moreno-Bueno, G., Tan, D.S., Marchio, C., Vatcheva, R., Rayter, S., Mahler-Araujo, B., Fulford, L.G., Hungermann, D., Mackay, A., Grigoriadis, A., Fenwick, K., Tamber, N., Hardisson, D., Tutt, A., Palacios, J., Lord, C.J., Buerger, H., Ashworth, A. & Reis-Filho, J.S. 2009, "Tiling path genomic profiling of grade 3 invasive ductal breast cancers", *Clinical cancer research : an official journal of the American Association for Cancer Research,* vol. 15, no. 8, pp. 2711-2722.

Nichols, A.F., Itoh, T., Zolezzi, F., Hutsell, S. & Linn, S. 2003, "Basal transcriptional regulation of human damage-specific DNA-binding protein genes DDB1 and DDB2 by Sp1, E2F, N-myc and NF1 elements", *Nucleic acids research,* vol. 31, no. 2, pp. 562-569.

Nielsen, T.O., Hsu, F.D., Jensen, K., Cheang, M., Karaca, G., Hu, Z., Hernandez-Boussard, T., Livasy, C., Cowan, D., Dressler, L., Akslen, L.A., Ragaz, J., Gown, A.M., Gilks, C.B., van de Rijn, M. & Perou, C.M. 2004, "Immunohistochemical and clinical

characterization of the basal-like subtype of invasive breast carcinoma", *Clinical cancer research : an official journal of the American Association for Cancer Research,* vol. 10, no. 16, pp. 5367-5374.

Nigro, J.M., Baker, S.J., Preisinger, A.C., Jessup, J.M., Hostetter, R., Cleary, K., Bigner, S.H., Davidson, N., Baylin, S. & Devilee, P. 1989, "Mutations in the p53 gene occur in diverse human tumour types", *Nature,* vol. 342, no. 6250, pp. 705-708.

North, A.J., Galazkiewicz, B., Byers, T.J., Glenney, J.R.,Jr & Small, J.V. 1993, "Complementary distributions of vinculin and dystrophin define two distinct sarcolemma domains in smooth muscle", *The Journal of cell biology,* vol. 120, no. 5, pp. 1159-1167.

Offterdinger, M., Schneider, S.M., Huber, H. & Grunt, T.W. 1999, "Expression of c-erbB-4/HER4 is regulated in T47D breast carcinoma cells by retinoids and vitamin D3", *Biochemical and biophysical research communications,* vol. 258, no. 3, pp. 559-564.

Ohtani, K., Iwanaga, R., Nakamura, M., Ikeda, M., Yabuta, N., Tsuruga, H. & Nojima, H. 1999, "Cell growth-regulated expression of mammalian MCM5 and MCM6 genes mediated by the transcription factor E2F", *Oncogene,* vol. 18, no. 14, pp. 2299-2309.

Olivotto, I., Levine, M. & Steering Committee on Clinical Practice Guidelines for the Care and Treatment of Breast Cancer 2001, "Clinical practice guidelines for the care and treatment of breast cancer: the management of ductal carcinoma in situ (summary of the 2001 update)", *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne,* vol. 165, no. 7, pp. 912-913.

Paik, S. 2007, "Development and clinical utility of a 21-gene recurrence score prognostic assay in patients with early breast cancer treated with tamoxifen", *The oncologist,* vol. 12, no. 6, pp. 631-635.

Paik, S., Shak, S., Tang, G., Kim, C., Baker, J., Cronin, M., Baehner, F.L., Walker, M.G., Watson, D., Park, T., Hiller, W., Fisher, E.R., Wickerham, D.L., Bryant, J. & Wolmark, N. 2004, "A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer", *The New England journal of medicine,* vol. 351, no. 27, pp. 2817-2826.

Paik, S., Tang, G., Shak, S., Kim, C., Baker, J., Kim, W., Cronin, M., Baehner, F.L., Watson, D., Bryant, J., Costantino, J.P., Geyer, C.E.,Jr, Wickerham, D.L. & Wolmark, N. 2006, "Gene expression and benefit of chemotherapy in women with node-negative,

estrogen receptor-positive breast cancer", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 24, no. 23, pp. 3726-3734.

Park, S.H., Choi, S.Y., Kim, M.H., Oh, E.J., Ryu, H.M., Kim, C.D., Kim, I.S. & Kim, Y.L. 2008, "The TGF-beta-induced gene product, betaig-h3: its biological implications in peritoneal dialysis", *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association,* vol. 23, no. 1, pp. 126-135.

Park, S.W., Bae, J.S., Kim, K.S., Park, S.H., Lee, B.H., Choi, J.Y., Park, J.Y., Ha, S.W., Kim, Y.L., Kwon, T.H., Kim, I.S. & Park, R.W. 2004, "Beta ig-h3 promotes renal proximal tubular epithelial cell adhesion, migration and proliferation through the interaction with alpha3beta1 integrin", *Experimental & molecular medicine,* vol. 36, no. 3, pp. 211-219.

Paul, R., Hu, B., Musahl, C., Hameister, H. & Knippers, R. 1996, "Coding sequence and chromosome mapping of the human gene (CDC46) for replication protein hCdc46/Mcm5", *Cytogenetics and cell genetics,* vol. 73, no. 4, pp. 317-321.

Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S.X., Lonning, P.E., Borresen-Dale, A.L., Brown, P.O. & Botstein, D. 2000, "Molecular portraits of human breast tumours", *Nature,* vol. 406, no. 6797, pp. 747-752.

Pharoah, P.D., Guilford, P., Caldas, C. & International Gastric Cancer Linkage Consortium 2001, "Incidence of gastric cancer and breast cancer in CDH1 (E-cadherin) mutation carriers from hereditary diffuse gastric cancer families", *Gastroenterology,* vol. 121, no. 6, pp. 1348-1353.

Pinder, S.E., Ellis, I.O., Galea, M., O'Rouke, S., Blamey, R.W. & Elston, C.W. 1994, "Pathological prognostic factors in breast cancer. III. Vascular invasion: relationship with recurrence and survival in a large study with long-term follow-up", *Histopathology,* vol. 24, no. 1, pp. 41-47.

Pinder S, Harris G and Elston C 2008. “The role of the pathologist in assessing prognostic factors for breast cancer. Prognostic and predictive factors in breast cancer” (2nd Edition) edited by Rosemary A Walker and Alastair M Thompson. Chapter 2 6-17.

Pollack, I.F., Kawecki, S. & Lazo, J.S. 1996, "Blocking of glioma proliferation in vitro and in vivo and potentiating the effects of BCNU and cisplatin: UCN-01, a selective protein kinase C inhibitor", *Journal of neurosurgery,* vol. 84, no. 6, pp. 1024-1032.

Polyak, K. 2007, "Breast cancer: origins and evolution", *The Journal of clinical investigation,* vol. 117, no. 11, pp. 3155-3163.

Pouliot, F. & Labrie, C. 1999, "Expression profile of agonistic Smads in human breast cancer cells: absence of regulation by estrogens", *International journal of cancer.Journal international du cancer,* vol. 81, no. 1, pp. 98-103.

Prost, S., Lu, P., Caldwell, H. & Harrison, D. 2007, "E2F regulates DDB2: consequences for DNA repair in Rb-deficient cells", *Oncogene,* vol. 26, no. 24, pp. 3572-3581.

Rabie, A.B., She, T.T. & Harley, V.R. 2003, "Forward mandibular positioning up-regulates SOX9 and type II collagen expression in the glenoid fossa", *Journal of dental research,* vol. 82, no. 9, pp. 725-730.

Rakha, E.A., Reis-Filho, J.S. & Ellis, I.O. 2008, "Basal-like breast cancer: a critical review", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 26, no. 15, pp. 2568-2581.

Rampaul, R.S., Pinder, S.E., Elston, C.W., Ellis, I.O. & Nottingham Breast Team 2001, "Prognostic and predictive factors in primary breast cancer and their role in patient management: The Nottingham Breast Team", *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology,* vol. 27, no. 3, pp. 229-238.

Rapic-Otrin, V., McLenigan, M.P., Bisi, D.C., Gonzalez, M. & Levine, A.S. 2002, "Sequential binding of UV DNA damage binding factor and degradation of the p48 subunit as early events after UV irradiation", *Nucleic acids research,* vol. 30, no. 11, pp. 2588-2598.

Rastelli, F. & Crispino, S. 2008, "Factors predictive of response to hormone therapy in breast cancer", *Tumori,* vol. 94, no. 3, pp. 370-383.

Rawe, I.M., Zhan, Q., Burrows, R., Bennett, K. & Cintron, C. 1997, "Beta-ig. Molecular cloning and in situ hybridization in corneal tissues", *Investigative ophthalmology & visual science,* vol. 38, no. 5, pp. 893-900.

Reeves, G.K., Pirie, K., Beral, V., Green, J., Spencer, E., Bull, D. & Million Women Study Collaboration 2007, "Cancer incidence and mortality in relation to body mass index in the Million Women Study: cohort study", *BMJ (Clinical research ed.),* vol. 335, no. 7630, pp. 1134.

Reis-Filho, J.S. & Lakhani, S.R. 2003, "The diagnosis and management of pre-invasive breast disease: genetic alterations in pre-invasive lesions", *Breast cancer research : BCR,* vol. 5, no. 6, pp. 313-319.

Reis-Filho, J.S. & Pinder, S.E. 2007, "Non-operative breast pathology: lobular neoplasia", *Journal of clinical pathology,* vol. 60, no. 12, pp. 1321-1327.

Reis-Filho, J.S., Simpson, P.T., Gale, T. & Lakhani, S.R. 2005, "The molecular genetics of breast cancer: the contribution of comparative genomic hybridization", *Pathology, research and practice,* vol. 201, no. 11, pp. 713-725.

Rhodes, A., Jasani, B., Balaton, A.J., Barnes, D.M., Anderson, E., Bobrow, L.G. & Miller, K.D. 2001, "Study of interlaboratory reliability and reproducibility of estrogen and progesterone receptor assays in Europe. Documentation of poor reliability and identification of insufficient microwave antigen retrieval time as a major contributory element of unreliable assays", *American Journal of Clinical Pathology,* vol. 115, no. 1, pp. 44-58.

Ripperger, T., Gadzicki, D., Meindl, A. & Schlegelberger, B. 2009, "Breast cancer susceptibility: current knowledge and implications for genetic counselling", *European journal of human genetics : EJHG,* vol. 17, no. 6, pp. 722-731.

Rizwani, W., Alexandrow, M. & Chellappan, S. 2009, "Prohibitin physically interacts with MCM proteins and inhibits mammalian DNA replication", *Cell cycle (Georgetown, Tex.),* vol. 8, no. 10, pp. 1621-1629.

Roetger, A., Merschjann, A., Dittmar, T., Jackisch, C., Barnekow, A. & Brandt, B. 1998, "Selection of potentially metastatic subpopulations expressing c-erbB-2 from breast cancer tissue by use of an extravasation model", *The American journal of pathology,* vol. 153, no. 6, pp. 1797-1806.

Rogers, A.E., Zeisel, S.H. & Groopman, J. 1993, "Diet and carcinogenesis", *Carcinogenesis,* vol. 14, no. 11, pp. 2205-2217.

Romanowski, P. & Madine, M.A. 1997, "Mechanisms restricting DNA replication to once per cell cycle: the role of Cdc6p and ORC", *Trends in cell biology,* vol. 7, no. 1, pp. 9-10.

Rosen, P.P. & Groshen, S. 1990, "Factors influencing survival and prognosis in early breast carcinoma (T1N0M0-T1N1M0). Assessment of 644 patients with median follow-up of 18 years", *The Surgical clinics of North America,* vol. 70, no. 4, pp. 937-962.

Ross, J.S. & Fletcher, J.A. 1998, "The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy", *Stem cells (Dayton, Ohio),* vol. 16, no. 6, pp. 413-428.

Ross, J.S., Hatzis, C., Symmans, W.F., Pusztai, L. & Hortobagyi, G.N. 2008, "Commercialized multigene predictors of clinical outcome for breast cancer", *The oncologist,* vol. 13, no. 5, pp. 477-493.

Ross, R.K., Paganini-Hill, A., Wan, P.C. & Pike, M.C. 2000, "Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin", *Journal of the National Cancer Institute,* vol. 92, no. 4, pp. 328-332.

Rouzier, R., Perou, C.M., Symmans, W.F., Ibrahim, N., Cristofanilli, M., Anderson, K., Hess, K.R., Stec, J., Ayers, M., Wagner, P., Morandi, P., Fan, C., Rabiul, I., Ross, J.S., Hortobagyi, G.N. & Pusztai, L. 2005, "Breast cancer molecular subtypes respond differently to preoperative chemotherapy", *Clinical cancer research : an official journal of the American Association for Cancer Research,* vol. 11, no. 16, pp. 5678-5685.

Roylance, R., Gorman, P., Harris, W., Liebmann, R., Barnes, D., Hanby, A. & Sheer, D. 1999, "Comparative genomic hybridization of breast tumors stratified by histological grade reveals new insights into the biological progression of breast cancer", *Cancer research,* vol. 59, no. 7, pp. 1433-1436.

Rozen, S. & Skaletsky, H. 2000, "Primer3 on the WWW for general users and for biologist programmers", *Methods in molecular biology (Clifton, N.J.),* vol. 132, pp. 365-386.

Russo, G., Zegar, C. & Giordano, A. 2003, "Advantages and limitations of microarray technology in human cancer", *Oncogene,* vol. 22, no. 42, pp. 6497-6507.

Russo, J., Fernandez, S.V., Russo, P.A., Fernbaugh, R., Sheriff, F.S., Lareef, H.M., Garber, J. & Russo, I.H. 2006, "17-Beta-estradiol induces transformation and tumorigenesis in human breast epithelial cells", *The FASEB journal : official publication of the*

Federation of American Societies for Experimental Biology, vol. 20, no. 10, pp. 1622-1634.

Saal, L.H., Gruvberger-Saal, S.K., Persson, C., Lovgren, K., Jumppanen, M., Staaf, J., Jonsson, G., Pires, M.M., Maurer, M., Holm, K., Koujak, S., Subramaniyam, S., Vallon-Christersson, J., Olsson, H., Su, T., Memeo, L., Ludwig, T., Ethier, S.P., Krogh, M., Szabolcs, M., Murty, V.V., Isola, J., Hibshoosh, H., Parsons, R. & Borg, A. 2008, "Recurrent gross mutations of the PTEN tumor suppressor gene in breast cancers with deficient DSB repair", *Nature genetics,* vol. 40, no. 1, pp. 102-107.

Saal, L.H., Johansson, P., Holm, K., Gruvberger-Saal, S.K., She, Q.B., Maurer, M., Koujak, S., Ferrando, A.A., Malmstrom, P., Memeo, L., Isola, J., Bendahl, P.O., Rosen, N., Hibshoosh, H., Ringner, M., Borg, A. & Parsons, R. 2007, "Poor prognosis in carcinoma is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 104, no. 18, pp. 7564-7569.

Sakorafas, G.H. & Tsiotou, A.G. 2000, "Ductal carcinoma in situ (DCIS) of the breast: evolving perspectives", *Cancer treatment reviews,* vol. 26, no. 2, pp. 103-125.

Samuels, M.L., Weber, M.J., Bishop, J.M. & McMahon, M. 1993, "Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase", *Molecular and cellular biology,* vol. 13, no. 10, pp. 6241-6252.

Sarrio, D., Moreno-Bueno, G., Hardisson, D., Sanchez-Estevez, C., Guo, M., Herman, J.G., Gamallo, C., Esteller, M. & Palacios, J. 2003, "Epigenetic and genetic alterations of APC and CDH1 genes in lobular breast cancer: relationships with abnormal E-cadherin and catenin expression and microsatellite instability", *International journal of cancer.Journal international du cancer,* vol. 106, no. 2, pp. 208-215.

Scarpini, C., White, V., Muralidhar, B., Patterson, A., Hickey, N., Singh, N., Mullerat, J., Winslet, M., Davies, R.J., Phillips, M.L., Stacey, P., Laskey, R.A., Miller, R., Nathan, M. & Coleman, N. 2008, "Improved screening for anal neoplasia by immunocytochemical detection of minichromosome maintenance proteins", *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* vol. 17, no. 10, pp. 2855-2864.

Schenker, T. & Trueb, B. 1998, "Down-regulated proteins of mesenchymal tumor cells", *Experimental cell research,* vol. 239, no. 1, pp. 161-168.

Schernhammer, E.S. & Hankinson, S.E. 2009, "Urinary melatonin levels and postmenopausal breast cancer risk in the Nurses' Health Study cohort", *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* vol. 18, no. 1, pp. 74-79.

Schiff, R., Massarweh, S.A., Shou, J., Bharwani, L., Mohsin, S.K. & Osborne, C.K. 2004, "Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance", *Clinical cancer research : an official journal of the American Association for Cancer Research,* vol. 10, no. 1 Pt 2, pp. 331S-6S.

Schlebusch, C.M., Dreyer, G., Sluiter, M.D., Yawitch, T.M., van den Berg, H.J. & van Rensburg, E.J. 2010, "Cancer prevalence in 129 breast-ovarian cancer families tested for BRCA1 and BRCA2 mutations", *South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde,* vol. 100, no. 2, pp. 113-117.

Schneider, D., Kleeff, J., Berberat, P.O., Zhu, Z., Korc, M., Friess, H. & Buchler, M.W. 2002, "Induction and expression of betaig-h3 in pancreatic cancer cells", *Biochimica et biophysica acta,* vol. 1588, no. 1, pp. 1-6.

Scully, R., Ganesan, S., Vlasakova, K., Chen, J., Socolovsky, M. & Livingston, D.M. 1999, "Genetic analysis of BRCA1 function in a defined tumor cell line", *Molecular cell,* vol. 4, no. 6, pp. 1093-1099.

Shao, G., Berenguer, J., Borczuk, A.C., Powell, C.A., Hei, T.K. & Zhao, Y. 2006, "Epigenetic inactivation of Betaig-h3 gene in human cancer cells", *Cancer research,* vol. 66, no. 9, pp. 4566-4573.

Shi, S.R., Cote, R.J. & Taylor, C.R. 2001, "Antigen retrieval techniques: current perspectives", *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society,* vol. 49, no. 8, pp. 931-937.

Silverstein, M.J. 1998, "Ductal Carcinoma In Situ of the Breast: Controversial Issues", *The oncologist,* vol. 3, no. 2, pp. 94-103.

Simpson, I.A., Cushman, S.W., Egan, J.J., Habberfield, A.D., Londos, C., Nishimura, H. & Saltis, J. 1990, "Hormonal regulation of glucose transport in rat adipose cells", *Biochemical Society transactions,* vol. 18, no. 6, pp. 1123-1125.

Simpson, J.F., Gray, R., Dressler, L.G., Cobau, C.D., Falkson, C.I., Gilchrist, K.W., Pandya, K.J., Page, D.L. & Robert, N.J. 2000, "Prognostic value of histologic grade and proliferative activity in axillary node-positive breast cancer: results from the Eastern Cooperative Oncology Group Companion Study, EST 4189", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 18, no. 10, pp. 2059-2069.

Simpson, P.T., Gale, T., Fulford, L.G., Reis-Filho, J.S. & Lakhani, S.R. 2003, "The diagnosis and management of pre-invasive breast disease: pathology of atypical lobular hyperplasia and lobular carcinoma in situ", *Breast cancer research : BCR,* vol. 5, no. 5, pp. 258-262.

Simpson, P.T., Reis-Filho, J.S., Gale, T. & Lakhani, S.R. 2005, "Molecular evolution of breast cancer", *The Journal of pathology,* vol. 205, no. 2, pp. 248-254.

Singletary, S.E. 2003, "Rating the risk factors for breast cancer", *Annals of Surgery,* vol. 237, no. 4, pp. 474-482.

Singletary, S.E., Allred, C., Ashley, P., Bassett, L.W., Berry, D., Bland, K.I., Borgen, P.I., Clark, G., Edge, S.B., Hayes, D.F., Hughes, L.L., Hutter, R.V., Morrow, M., Page, D.L., Recht, A., Theriault, R.L., Thor, A., Weaver, D.L., Wieand, H.S. & Greene, F.L. 2002, "Revision of the American Joint Committee on Cancer staging system for breast cancer", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 20, no. 17, pp. 3628-3636.

Singletary, S.E. & Connolly, J.L. 2006, "Breast cancer staging: working with the sixth edition of the AJCC Cancer Staging Manual", *CA: a cancer journal for clinicians,* vol. 56, no. 1, pp. 37-47; quiz 50-1.

Skonier, J., Bennett, K., Rothwell, V., Kosowski, S., Plowman, G., Wallace, P., Edelhoff, S., Disteche, C., Neubauer, M. & Marquardt, H. 1994, "beta ig-h3: a transforming growth factor-beta-responsive gene encoding a secreted protein that inhibits cell attachment in vitro and suppresses the growth of CHO cells in nude mice", *DNA and cell biology,* vol. 13, no. 6, pp. 571-584.

Skonier, J., Neubauer, M., Madisen, L., Bennett, K., Plowman, G.D. & Purchio, A.F. 1992, "cDNA cloning and sequence analysis of beta ig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor-beta", *DNA and cell biology,* vol. 11, no. 7, pp. 511-522.

Soerjomataram, I., Louwman, M.W., Ribot, J.G., Roukema, J.A. & Coebergh, J.W. 2008, "An overview of prognostic factors for long-term survivors of breast cancer", *Breast cancer research and treatment,* vol. 107, no. 3, pp. 309-330.

Song, H., Hollstein, M. & Xu, Y. 2007, "p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM", *Nature cell biology,* vol. 9, no. 5, pp. 573-580.

Sopori, M. 2002, "Effects of cigarette smoke on the immune system", *Nature reviews.Immunology,* vol. 2, no. 5, pp. 372-377.

Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Thorsen, T., Quist, H., Matese, J.C., Brown, P.O., Botstein, D., Eystein Lonning, P. & Borresen-Dale, A.L. 2001, "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 98, no. 19, pp. 10869-10874.

Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J.S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., Demeter, J., Perou, C.M., Lonning, P.E., Brown, P.O., Borresen-Dale, A.L. & Botstein, D. 2003, "Repeated observation of breast tumor subtypes in independent gene expression data sets", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 100, no. 14, pp. 8418-8423.

Staflin, K., Zuchner, T., Honeth, G., Darabi, A. & Lundberg, C. 2009, "Identification of proteins involved in neural progenitor cell targeting of gliomas", *BMC cancer,* vol. 9, pp. 206.

Stambolic, V., Tsao, M.S., Macpherson, D., Suzuki, A., Chapman, W.B. & Mak, T.W. 2000, "High incidence of breast and endometrial neoplasia resembling human Cowden syndrome in pten+/- mice", *Cancer research,* vol. 60, no. 13, pp. 3605-3611.

Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D.H. & Tavtigian, S.V. 1997, "Identification of a candidate tumour suppressor gene, MMAC1,

at chromosome 10q23.3 that is mutated in multiple advanced cancers", *Nature genetics,* vol. 15, no. 4, pp. 356-362.

Steinmetz, M.O., Stoffler, D., Muller, S.A., Jahn, W., Wolpensinger, B., Goldie, K.N., Engel, A., Faulstich, H. & Aebi, U. 1998, "Evaluating atomic models of F-actin with an undecagold-tagged phalloidin derivative", *Journal of Molecular Biology,* vol. 276, no. 1, pp. 1-6.

Stoeber, K., Swinn, R., Prevost, A.T., de Clive-Lowe, P., Halsall, I., Dilworth, S.M., Marr, J., Turner, W.H., Bullock, N., Doble, A., Hales, C.N. & Williams, G.H. 2002, "Diagnosis of genito-urinary tract cancer by detection of minichromosome maintenance 5 protein in urine sediments", *Journal of the National Cancer Institute,* vol. 94, no. 14, pp. 1071-1079.

Stone, E.M., Mathers, W.D., Rosenwasser, G.O., Holland, E.J., Folberg, R., Krachmer, J.H., Nichols, B.E., Gorevic, P.D., Taylor, C.M. & Streb, L.M. 1994, "Three autosomal dominant corneal dystrophies map to chromosome 5q", *Nature genetics,* vol. 6, no. 1, pp. 47-51.

Stredrick, D.L., Garcia-Closas, M., Pineda, M.A., Bhatti, P., Alexander, B.H., Doody, M.M., Lissowska, J., Peplonska, B., Brinton, L.A., Chanock, S.J., Struewing, J.P. & Sigurdson, A.J. 2006, "The ATM missense mutation p.Ser49Cys (c.146C>G) and the risk of breast cancer", *Human mutation,* vol. 27, no. 6, pp. 538-544.

Subik, K., Lee, J.F., Baxter, L., Strzepek, T., Costello, D., Crowley, P., Xing, L., Hung, M.C., Bonfiglio, T., Hicks, D.G. & Tang, P. 2010, "The Expression Patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by Immunohistochemical Analysis in Breast Cancer Cell Lines", *Breast cancer : basic and clinical research,* vol. 4, pp. 35-41.

Sun, S., Yi, X., Poon, R.T., Yeung, C., Day, P.J. & Luk, J.M. 2009, "A protein-based set of reference markers for liver tissues and hepatocellular carcinoma", *BMC cancer,* vol. 9, pp. 309.

Tachibana, K.E., Gonzalez, M.A. & Coleman, N. 2005, "Cell-cycle-dependent regulation of DNA replication and its relevance to cancer pathology", *The Journal of pathology,* vol. 205, no. 2, pp. 123-129.

Takkouche, B., Regueira-Mendez, C. & Etminan, M. 2008, "Breast cancer and use of nonsteroidal anti-inflammatory drugs: a meta-analysis", *Journal of the National Cancer Institute,* vol. 100, no. 20, pp. 1439-1447.

Tang, S.W., Parker, H., Winterbottom, L., Hassell, K., Ellis, I.O., Morgan, D.A. & Cheung, K.L. 2009, "Early primary breast cancer in the elderly - Pattern of presentation and treatment", *Surgical oncology,* .

Taucher, S. & Jakesz, R. 2004, "Survival after surgical treatment of breast cancer", *Langenbeck's archives of surgery / Deutsche Gesellschaft fur Chirurgie,* vol. 389, no. 2, pp. 75-82.

Taylor, A.M. & Byrd, P.J. 2005, "Molecular pathology of ataxia telangiectasia", *Journal of clinical pathology,* vol. 58, no. 10, pp. 1009-1015.

Terry, P.D. & Rohan, T.E. 2002, "Cigarette smoking and the risk of breast cancer in women: a review of the literature", *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* vol. 11, no. 10 Pt 1, pp. 953-971.

Thapa, N., Lee, B.H. & Kim, I.S. 2007, "TGFBIp/betaig-h3 protein: a versatile matrix molecule induced by TGF-beta", *The international journal of biochemistry & cell biology,* vol. 39, no. 12, pp. 2183-2194.

Thierry-Mieg, D. & Thierry-Mieg, J. 2006, "AceView: a comprehensive cDNA-supported gene and transcripts annotation", *Genome biology,* vol. 7 Suppl 1, pp. S12.1-14.

Thomas Boenisch. 2001, “Handbook Immunohistochemistry Staining Method”. Third Edition.

Tjonneland, A., Christensen, J., Olsen, A., Stripp, C., Thomsen, B.L., Overvad, K., Peeters, P.H., van Gils, C.H., Bueno-de-Mesquita, H.B., Ocke, M.C., Thiebaut, A., Fournier, A., Clavel-Chapelon, F., Berrino, F., Palli, D., Tumino, R., Panico, S., Vineis, P., Agudo, A., Ardanaz, E., Martinez-Garcia, C., Amiano, P., Navarro, C., Quiros, J.R., Key, T.J., Reeves, G., Khaw, K.T., Bingham, S., Trichopoulou, A., Trichopoulos, D., Naska, A., Nagel, G., Chang-Claude, J., Boeing, H., Lahmann, P.H., Manjer, J., Wirfalt, E., Hallmans, G., Johansson, I., Lund, E., Skeie, G., Hjartaker, A., Ferrari, P., Slimani, N., Kaaks, R. & Riboli, E. 2007, "Alcohol intake and breast cancer risk: the European Prospective Investigation into Cancer and Nutrition (EPIC)", *Cancer causes & control : CCC,* vol. 18, no. 4, pp. 361-373.

Tomioka, H., Morita, K., Hasegawa, S. & Omura, K. 2006, "Gene expression analysis by cDNA microarray in oral squamous cell carcinoma", *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology,* vol. 35, no. 4, pp. 206-211.

Towbin, H., Staehelin, T. & Gordon, J. 1992, "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979", *Biotechnology (Reading, Mass.),* vol. 24, pp. 145-149.

Valasek, M.A. & Repa, J.J. 2005, "The power of real-time PCR", *Advances in Physiology Education,* vol. 29, no. 3, pp. 151-159.

van de Rijn, M., Perou, C.M., Tibshirani, R., Haas, P., Kallioniemi, O., Kononen, J., Torhorst, J., Sauter, G., Zuber, M., Kochli, O.R., Mross, F., Dieterich, H., Seitz, R., Ross, D., Botstein, D. & Brown, P. 2002, "Expression of cytokeratins 17 and 5 identifies a group of breast carcinomas with poor clinical outcome", *The American journal of pathology,* vol. 161, no. 6, pp. 1991-1996.

van de Vijver, M.J., He, Y.D., van't Veer, L.J., Dai, H., Hart, A.A., Voskuil, D.W., Schreiber, G.J., Peterse, J.L., Roberts, C., Marton, M.J., Parrish, M., Atsma, D., Witteveen, A., Glas, A., Delahaye, L., van der Velde, T., Bartelink, H., Rodenhuis, S., Rutgers, E.T., Friend, S.H. & Bernards, R. 2002, "A gene-expression signature as a predictor of survival in breast cancer", *The New England journal of medicine,* vol. 347, no. 25, pp. 1999-2009.

van de Vijver, M.J., Peterse, J.L., Mooi, W.J., Wisman, P., Lomans, J., Dalesio, O. & Nusse, R. 1988, "Neu-protein overexpression in breast cancer. Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer", *The New England journal of medicine,* vol. 319, no. 19, pp. 1239-1245.

van den Brandt, P.A., Spiegelman, D., Yaun, S.S., Adami, H.O., Beeson, L., Folsom, A.R., Fraser, G., Goldbohm, R.A., Graham, S., Kushi, L., Marshall, J.R., Miller, A.B., Rohan, T., Smith-Warner, S.A., Speizer, F.E., Willett, W.C., Wolk, A. & Hunter, D.J. 2000, "Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk", *American Journal of Epidemiology,* vol. 152, no. 6, pp. 514-527.

van 't Veer, L.J., Dai, H., van de Vijver, M.J., He, Y.D., Hart, A.A., Mao, M., Peterse, H.L., van der Kooy, K., Marton, M.J., Witteveen, A.T., Schreiber, G.J., Kerkhoven, R.M.,

Roberts, C., Linsley, P.S., Bernards, R. & Friend, S.H. 2002, "Gene expression profiling predicts clinical outcome of breast cancer", *Nature,* vol. 415, no. 6871, pp. 530-536.

Vendrell, J.A., Robertson, K.E., Ravel, P., Bray, S.E., Bajard, A., Purdie, C.A., Nguyen, C., Hadad, S.M., Bieche, I., Chabaud, S., Bachelot, T., Thompson, A.M. & Cohen, P.A. 2008, "A candidate molecular signature associated with tamoxifen failure in primary breast cancer", *Breast cancer research : BCR,* vol. 10, no. 5, pp. R88.

Vogel, V.G. 2000, "Breast cancer prevention: a review of current evidence", *CA: a cancer journal for clinicians,* vol. 50, no. 3, pp. 156-170.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M. & Bos, J.L. 1988, "Genetic alterations during colorectal-tumor development", *The New England journal of medicine,* vol. 319, no. 9, pp. 525-532.

Volpi, A., Bacci, F., Paradiso, A., Saragoni, L., Scarpi, E., Ricci, M., Aldi, M., Bianchi, S., Muretto, P., Nuzzo, F., Simone, G., Mangia, A., Schittulli, F. & Amadori, D. 2004, "Prognostic relevance of histological grade and its components in node-negative breast cancer patients", *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc,* vol. 17, no. 9, pp. 1038-1044.

Wakasugi, M., Kawashima, A., Morioka, H., Linn, S., Sancar, A., Mori, T., Nikaido, O. & Matsunaga, T. 2002, "DDB accumulates at DNA damage sites immediately after UV irradiation and directly stimulates nucleotide excision repair", *The Journal of biological chemistry,* vol. 277, no. 3, pp. 1637-1640.

Walker, R.A. 2006, "Quantification of immunohistochemistry--issues concerning methods, utility and semiquantitative assessment I", *Histopathology,* vol. 49, no. 4, pp. 406-410.

Walker, R.A., Dearing, S.J. & Gallacher, B. 1994, "Relationship of transforming growth factor beta 1 to extracellular matrix and stromal infiltrates in invasive breast carcinoma", *British journal of cancer,* vol. 69, no. 6, pp. 1160-1165.

Walker, R.A., Lees, E., Webb, M.B. & Dearing, S.J. 1996, "Breast carcinomas occurring in young women (< 35 years) are different", *British journal of cancer,* vol. 74, no. 11, pp. 1796-1800.

Walpita, D. & Hay, E. 2002, "Studying actin-dependent processes in tissue culture", *Nature reviews.Molecular cell biology,* vol. 3, no. 2, pp. 137-141.

Wang, Y., Klijn, J.G., Zhang, Y., Sieuwerts, A.M., Look, M.P., Yang, F., Talantov, D., Timmermans, M., Meijer-van Gelder, M.E., Yu, J., Jatkoe, T., Berns, E.M., Atkins, D. & Foekens, J.A. 2005, "Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer", *Lancet,* vol. 365, no. 9460, pp. 671-679.

Weigelt, B., Baehner, F.L. & Reis-Filho, J.S. 2010, "The contribution of gene expression profiling to breast cancer classification, prognostication and prediction: a retrospective of the last decade", *The Journal of pathology,* vol. 220, no. 2, pp. 263-280.

Wellings, S.R., Jensen, H.M. & Marcum, R.G. 1975, "An atlas of subgross pathology of the human breast with special reference to possible precancerous lesions", *Journal of the National Cancer Institute,* vol. 55, no. 2, pp. 231-273.

Whibley, C., Pharoah, P.D. & Hollstein, M. 2009, "P53 Polymorphisms: Cancer Implications", *Nature reviews.Cancer,* vol. 9, no. 2, pp. 95-107.

Whyman, G.J. 2005, *Sporadic breast cancer in young women a microarray investigation*, University of Leicester.

Williams, G.H., Swinn, R., Prevost, A.T., De Clive-Lowe, P., Halsall, I., Going, J.J., Hales, C.N., Stoeber, K. & Middleton, S.J. 2004, "Diagnosis of oesophageal cancer by detection of minichromosome maintenance 5 protein in gastric aspirates", *British journal of cancer,* vol. 91, no. 4, pp. 714-719.

Wittschieben, B.O. & Wood, R.D. 2003, "DDB complexities", *DNA repair,* vol. 2, no. 9, pp. 1065-1069.

Wooster, R., Neuhausen, S.L., Mangion, J., Quirk, Y., Ford, D., Collins, N., Nguyen, K., Seal, S., Tran, T. & Averill, D. 1994, "Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13", *Science (New York, N.Y.),* vol. 265, no. 5181, pp. 2088-2090.

Xue, F. & Michels, K.B. 2007, "Intrauterine factors and risk of breast cancer: a systematic review and meta-analysis of current evidence", *The lancet oncology,* vol. 8, no. 12, pp. 1088-1100.

Yang, S., Liu, J., Thor, A.D. & Yang, X. 2007, "Caspase expression profile and functional activity in a panel of breast cancer cell lines", *Oncology reports,* vol. 17, no. 5, pp. 1229-1235.

Yildirim, E., Dalgic, T. & Berberoglu, U. 2000, "Prognostic significance of young age in breast cancer", *Journal of surgical oncology,* vol. 74, no. 4, pp. 267-272.

Yoon, T., Chakrabortty, A., Franks, R., Valli, T., Kiyokawa, H. & Raychaudhuri, P. 2005, "Tumor-prone phenotype of the DDB2-deficient mice", *Oncogene,* vol. 24, no. 3, pp. 469-478.

Young, A.E. 2001, "The surgical management of early breast cancer", *International journal of clinical practice,* vol. 55, no. 9, pp. 603-608.

Zajchowski, D.A., Bartholdi, M.F., Gong, Y., Webster, L., Liu, H.L., Munishkin, A., Beauheim, C., Harvey, S., Ethier, S.P. & Johnson, P.H. 2001, "Identification of gene expression profiles that predict the aggressive behavior of breast cancer cells", *Cancer research,* vol. 61, no. 13, pp. 5168-5178.

Zamilpa, R., Rupaimoole, R., Phelix, C.F., Somaraki-Cormier, M., Haskins, W., Asmis, R. & LeBaron, R.G. 2009, "C-terminal fragment of transforming growth factor beta-induced protein (TGFBIp) is required for apoptosis in human osteosarcoma cells", *Matrix biology : journal of the International Society for Matrix Biology,* vol. 28, no. 6, pp. 347-353.

Zhang, Y., Wen, G., Shao, G., Wang, C., Lin, C., Fang, H., Balajee, A.S., Bhagat, G., Hei, T.K. & Zhao, Y. 2009, "TGFBI deficiency predisposes mice to spontaneous tumor development", *Cancer research,* vol. 69, no. 1, pp. 37-44.

Zhao, Y., El-Gabry, M. & Hei, T.K. 2006, "Loss of Betaig-h3 protein is frequent in primary lung carcinoma and related to tumorigenic phenotype in lung cancer cells", *Molecular carcinogenesis,* vol. 45, no. 2, pp. 84-92.

Zhao, Y.L., Piao, C.Q. & Hei, T.K. 2002, "Downregulation of Betaig-h3 gene is causally linked to tumorigenic phenotype in asbestos treated immortalized human bronchial epithelial cells", *Oncogene,* vol. 21, no. 49, pp. 7471-7477.

Zhou, W., Lin, Y., Wersto, R., Chrest, J. & Gabrielson, E. 2002, "Staurosporine-induced G(1) arrest in cancer cells depends on an intact pRB but is independent of p16 status", *Cancer letters,* vol. 183, no. 1, pp. 103-107.

Zolezzi, F. & Linn, S. 2000, "Studies of the murine DDB1 and DDB2 genes", *Gene,* vol. 245, no. 1, pp. 151-159.