Effects of alternatively spliced tenascin-C isoforms on breast cancer cell behaviour

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Abstract

Aims: The tumour microenvironment plays a crucial role in the development of breast cancer. Tenascin-C (TNC), a matricellular protein and its high molecular weight (MW) isoforms have been shown to be over-expressed in the stroma of breast cancers and are associated with poor prognosis. The aim of this study was to investigate the effects of TNC knockdown in TNC expressing invasive breast cancer cell lines on gene expression and cell behaviour.

Methods: siRNAs targeting different exons in TNC (24, 14 and 14-AD1) were designed, synthesised and transfected into the highly invasive MDA-MB-231 breast cancer cell line. Down regulation of TNC was confirmed by Western blotting and RT-qPCR. The phenotypic alterations caused by TNC knockdown were analysed by 2D invasion assays and proliferation assays using the mitotic marker (pHH3). cDNA microarray and proteomics were used to analyse the effects of TNC knockdown at the mRNA and protein level. A novel polyclonal antibody was also generated for TNC-AD1 and expression of this and thrombospondin-1 (TSP-1), as a candidate TNC regulated gene was investigated in 36 breast cancer tissues using immunohistochemistry.

Results: The siRNA targeted cells showed significant down-regulation of both total TNC ($p < 0.001$) and high MW isoforms ($p < 0.001$) in MDA-MB-231 cells. Moreover, knockdown of total TNC and high MW TNC isoforms significantly decreased both cell invasion (total TNC $p < 0.001$, TNC-14 $p < 0.001$ and TNC-14-AD1 $p < 0.01$) and proliferation (total TNC $p < 0.001$ and TNC-AD1 $p < 0.05$). Microarray analysis following total TNC knockdown revealed significant changes in gene expression: *CREBL2, YWHAE, CDC14B* and *RRAS2* showed down regulation and *QKI* was specifically up-regulated by knock-down of both total TNC and high MW TNC isoforms. Proteomics and Western blot analysis showed increased levels of thrompospondin-1 (TSP-1) as result of total TNC knockdown as well as high MW TNC isoforms. TNC-AD1 expression in 36 breast cancer tissues was significantly associated with age (>40 years).

Conclusion: TNC knockdown significantly decreases proliferation and invasion in breast cancer cell lines, confirming its importance in breast cancer progression.

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List of Abbreviations

2D	2 Dimensions
AD1	Additional domain 1
AD2	Additional domain 2
ADH	Atypical ductal hyperplasia
ALH	Atypical lobular hyperplasia
AMV	Avian myeloblastosis virus
ATCC	American type culture collection
ATM	Ataxia-telangiectasia mutated
bp	Base pair
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
BSA	Bovine serum albumin
CDC14B	Cell division cycle 14 homolog B
cDNA	Complimentary DNA
CHEK2	CHK2 checkpoint homolog
CM	Conditioned medium
CNS	Central nervous system
CREBL2	cAMP responsive element binding protein-like 2
Ct	Cycle threshold
DAVID	Database for annotation, visualization and integrated discovery
DCIS	Ductal carcinoma *in-situ*
DISC1	Disrupted in schizophrenia 1
DKK1	Dickkopf-1
DMEM	Dulbeccos modified eagles medium
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
EGFL	EGF-like
EMT	Epithelial mesenchymal transition
ENDRA	Endothelial receptor type A
ER	Oestrogen receptor
ERK1/2	Extracellular signal-related kinase 1/2
FAD	food agriculture division
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FDR	False discovery rate
FF	Fresh frozen
FFPE	Formalin-fixed, paraffin-embedded

FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptors
FNIII	Fibronectin-type III like domain
HER-2	Human epidermal growth factor receptor 2
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1
HPRT1	Hypoxanthine guanine phosphoribysol transferase 1
HRT	Hormone replacement therapy
IDC	Invasive ductal carcinoma
IGFBP-7	Insulin growth factor binding protein-7
kDa	Kilodalton
KEGG	Kyoto encyclopedia of genes and genomes
LCIS	Lobar carcinoma *in-situ*
LC-MS	Liquid chromatography-mass spectrometry
MAPK	Mitogen-activated protein kinase
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MW	Molecular weight
n/k	Not known
PFN-1	Profilin-1
pHH-3	Phospho histone-3
PLGS	Protein lynx global server
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PTGS	Post transcriptional gene silencing
QKI	Quaking homolog, KH domain RNA binding
RMA	Robust multiarray average
rpm	Revolutions per minute
RPMI	Roswell park memorial institute
RQ	Relative quantitation
RRAS2	RAS viral (r-ras) oncogene homolog 2
RT	Reverse transcription
RT-qPCR	Real time-quantitative polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
siRNA	Small interfering RNA
SP	Side population
STK11	Theronine kinase 11
TA	Tenascin assembly
TAE	Tris Acetic acid EDTA
Taq	Thermus aquaticus
TBS	Tris-HCl buffered saline
TDLU	Terminal duct lobular units
TE	Trypsin/EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
TGF- α	Transforming growth factor-alpha
TGF- β	Transforming growth factor-beta

TNC	Tenascin C
TNC-L	TNC-Long
TNC-S	TNC-Short
TNF	Tumour necrosis factor
TNM	Tumour node metastases
TP53	Tumour protein 53
TSP-1	Thrombospondin-1
v/v	Volume by volume
Vim	Vimentin
w/v	Weight by volume
YWHAE	tryptophan 5-monooxygenase activation protein, epsilon polypeptide
ΔCt	Delta cycle threshold

Chapter 1. Introduction

1.1 Overview

Breast cancer is a common cancer in the Western world with high mortality and morbidity rates. Breast cancer is complicated by unique molecular signatures and diverse genetic alterations, each with distinct clinical outcomes. The microenvironment of the tumour includes cells and extracellular matrix (ECM). The interaction between tumour cells and the surrounding microenvironment is important for proliferation, survival, differentiation and migration. Alterations in the tumour microenvironment have been shown to play a crucial role in breast cancer progression. Although many ECM proteins are altered in breast cancer, one consistent finding was the up-regulation of the hexameric protein tenascin-C (TNC) in the tumour stroma. In this chapter, the epidemiology, risk factors, molecular phenotypes and genetic abnormalities in breast cancer will be reviewed. Subsequently, the pathogenesis and role of the microenvironment in breast cancer progression will be discussed, focussing on TNC and its role in breast cancer behaviour.

1.2 Breast cancer epidemiology

Breast cancer is the most common cancer diagnosed among women worldwide. In 2008, around 1.38 million women were diagnosed with breast cancer with high incidence seen in the developed countries and markedly lower incidence in developing countries. Globally, the mortality rate of breast cancer is almost 460,000 deaths in 2008 with variations across the regions in the world due to better survival (www.cancerresearchuk.org). Breast cancer is the most common cancer in the United Kingdom and accounts for approximately one third (31%) of all new cancer diagnoses in women. According to the 2010 breast cancer statistics, 49,961 women (99%) and 397 men (<1%) were newly diagnosed with breast cancer. The mortality rate of breast cancer is around 12,000 every year. The important risk factors for breast cancer are gender (female) and age (81% of breast cancers occur at > 50 years of age) (www.cancerresearchuk.org).

1.3 Anatomy of the breast

The mammary gland is of ectodermal origin and during embryogenesis the bud arises due to proliferation of basal cells of the epidermis (Anbazhagan et al. 1998). In contrast to other organs of the body, breast development occurs in the postnatal period and its completion occurs in adult life. The process of mammary epithelial differentiation also necessitates the completion of full time pregnancy, lactation and involution (Polyak, Kalluri 2010). The breast is located between the 2^{nd} and 6^{th} ribs of the chest wall and is divided into four quadrants: upper inner, upper outer, lower inner and lower outer quadrants. The upper outer quadrant of the breast is more prone to cancer compared to other lobes (Lee 2005). The breast is composed of 15 to 20 lobes rich in fat and fibrous connective tissue ramifying from the nipple and further divided into several lobules. The ductal network arises from the nipple and branches outwards while being supported

by fibrofatty stroma. The functional units of the breast are the terminal duct lobular units (TDLU) (Figure 1.1), which later terminate as ductules or acini arranged around the duct in the form of grapes (Pandya, Moore 2011). The terminal lobular duct is round in shape and comprises of an outer myoepithelial layer, which has contractile properties, and an inner-luminal epithelial layer (Arpino, Laucirica & Elledge 2005). The epithelial cells that line the terminal lobular duct are primarily involved in the development of breast cancer (Sainsbury, Anderson & Morgan 2000a).

http://www.breastcancer.org/pictures/breast_anatomy/image_1

Figure 1.1: Anatomy of the female breast and enlarged cross section of the normal duct. A) Ducts. B) Lobules. C) Dilated section of duct to hold milk. D) Nipple. E) Adipose tissue. F) Pectoralis major. G) Chest wall. Enlarged Cross section A) Normal duct cells. B) Basement membrane. C) Basement lumen (centre of duct). Picture taken from (www.breastcancer.org/pictures).

1.4 Risk factors

The aetiology of breast cancer development is very complex caused by interaction between genetic and environmental factors. Identified risk factors for breast cancer include age, reproductive factors including early menarche, late menopause, absence or short duration of breast feeding, exogenous hormones (e.g. hormone replacement therapy (HRT)), family history and life style.

1.4.1 Age

Breast cancer incidence increases with age. In the UK, women aged 50 years and older account for 81 % of all breast cancers. The chance of developing breast cancer doubles approximately 10 years after menopause. However, breast cancer predominantly affects younger women (≤ 40 years) when compared to lung cancer (McPherson, Steel & Dixon 2000).

1.4.2 Reproductive factors and breast density

During a woman's reproductive life, oestrogen exposure may increase breast cancer risk. In particular, women who start menarche at an early age and those who reach menopause at a late age are exposed to higher levels of oestrogen, and are more likely to develop breast cancer (Helmrich et al. 1983). These biological causes are due to the proliferative response of breast epithelial cells to oestrogen via the oestrogen receptor (ER) (Key, Pike 1988). Later age at first full-term pregnancy also increases the risk of breast cancer but the biological relationships are unclear (Helmrich et al. 1983). Exposure to exogenous oestrogen such as HRT (Porch et al. 2002) and oral contraceptives (Marchbanks et al. 2002) also increase the risk of breast cancer.

Breast density with high proportion of non fatty tissue increases the risk of breast cancer. Women with extremely dense breasts may have a 2-6 fold increased risk of breast cancer (McCormack, Silva 2006 ; Titus-Ernstoff et al. 2006). The density is

affected by weight, menopausal status, number of children and inheritance (Boyd et al. 2002).

1.4.3 Family history

In developed countries up to 10% of breast cancers are associated with genetic predisposition. The mode of inheritance of breast cancer is autosomal dominant. Two commonly identified genes for familial breast cancer are breast cancer susceptibility gene 1 and 2 (*BRCA1* and *BRCA2*), located on the long arm of chromosomes 17 and 13, respectively (McPherson, Steel & Dixon 2000).

1.4.4 Life style

Obesity has been shown to increase the risk of postmenopausal breast cancer (Hirose et al. 2001). After the menopause, the highest levels of oestrogen receptors are found in adipose tissue. Consumption of alcohol has also been associated with an increased risk of breast cancer (Atkinson 2003; Chen et al. 2002). This may be due to higher levels of sex hormones in the blood of alcohol consumers.

An increased risk of breast cancer occurrence has been suggested in night workers of >4.5 years shift work (Menegaux et al. 2013). Melatonin hormone is produced during the night (Snyder, Axelrod & Zweig 1967) and therefore artificial light suppresses its level, which then affects reproductive hormones and increases the proliferation of hormone-sensitive cells in the breast (Cohen, Lippman & Chabner 1978; Stevens 1987). A summary of the main risk factors for breast cancer is given in (Table 1.1).

Table 1.1 Risk factors for breast cancer (McPherson, Steel & Dixon 2000)

Factor	Relative risk	High Risk group
Age	>10	Elderly
Geographical location	5	Developed country
Age at menarche	3	Menarche before age 11
Age at menopause	2	Menopause after age 54
Age at first full pregnancy	3	First child in early 40s
Family history	≤ 2	Breast cancer in first degree relative when young
Previous benign disease	4-5	Atypical hyperplasia
Diet	1.5	High intake of saturated fat
Premenopausal	0.7	Body mass index >35
Postmenopausal	2	Body mass index >35
Alcohol consumption	1.3	Excessive intake
Exposure to ionising radiation	3	Abnormal exposure in young females after age 10
Hormonal replacement therapy	1.35	Use for ≥ 10 years
Diethylstilbestrol	2	Use during pregnancy

1.5 Breast cancer susceptibility genes

Breast carcinogenesis involves a multi-step process and is thought to involve one or more distinct genetic mutations (hereditary and/or sporadic). Much molecular research and linkage has led to the discovery of the high penetrance breast cancer susceptibility genes *BRCA1* and *BRCA2* (Hall et al. 1990; Wooster et al. 1994). The genes and loci involved in heredity breast cancer can be divided in to three groups depending on their risk for cancer development (Njiaju, Olopade 2012). Germ-line mutations with high penetrance (the proportion of individuals carrying a specific variation and also express a related phenotype) include *BRCA1, BRCA2, TP53, CDH1, STK11* and *PTEN* (relative risk of >5), intermediate penetrance genes include *CHEK2, ATM, PALB2* and *BRIP1*

(relative risk of >1.5 to <5) and low penetrance genes include *CASP8, FGFR2, MAP3K1* and *LSP1* (relative risk of >1.01 to < 1.5). The high penetrance genes will be discussed in detail below, whereas the intermediate and low penetrance genes are mentioned in (Table 1.2).

1.5.1 BRCA1 and BRCA2

Breast cancer susceptibility gene 1 (*BRCA1*), and 2 (*BRCA2*) are tumour suppressor genes located on chromosome 17q21 and 13q12-13, respectively (Hall et al. 1990; Wooster et al. 1994). *BRCA1* is composed of 24 exons and encodes for a protein with 1,863 amino acids, whereas *BRCA2* is composed of 27 exons and encodes a protein with 3,418 amino acids. Approximately 16% of the hereditary breast cancer can be attributed to germline mutations of *BRCA1* and *BRCA2* genes (van der Groep, van der Wall & van Diest 2011). The prevalence of *BRCA1* and *BRCA2* mutations is approximately one in 1,000 and one in 750, respectively (Antoniou et al. 2002).

BRCA1 and *BRCA2* are implicated in DNA repair and they form a complex that initiates repair of double strand breaks (DSBs) and activate homologous recombination (HR). *RAD51* along with *BRCA1* and *BRCA2* were co-localized at the site of DNA recombination and DNA damaged induced loci (van der Groep, van der Wall & van Diest 2011).

According to the database of Breast Cancer Information Core (BIC), 1,639 and 1,853 different mutations, variants and polymorphisms in the *BRCA1* and *BRCA2* genes have been recorded. The types of mutation include deletions or small frameshift insertions, mutations in splice sites or non-sense mutations resulting in a complete or partial loss of exons or incorporation of intronic sequences (van der Groep, van der Wall & van Diest 2011). *BRCA1* and *BRCA2* mutations are associated with increased risk of developing

breast, ovarian, prostate and pancreatic cancer. In breast and ovarian cancer, truncated and non-functional *BRCA* proteins were found (van der Groep, van der Wall & van Diest 2011). The histopathological features of *BRCA* associated breast cancers and sporadic breast cancers are different. *BRCA1* and *BRCA2* associated breast cancers are predominantly IDC in type (74% & 76% respectively), but *BRCA1* medullary carcinoma incidence is about 13%, which is significantly higher than in sporadic breast cancer (Lakhani et al. 1997; Lakhani et al. 1998).

1.5.2 TP53

The tumour protein 53 (*TP53*) gene is located on chromosome 17p13.1. TP53 is a nuclear phosphoprotein and functions as a transcription factor. TP53 was shown to regulate key physiological process such as the cell cycle, DNA repair, maintenance of genomic stability and apoptosis (Vogelstein, Lane & Levine 2000). Because of its tumour suppressor function *TP53* is also known as the "Guardian of the genome". Germline mutations of *TP53* are associated with Li-Fraumeni syndrome, which is associated with malignancies including sarcoma, breast cancer, brain tumours and tumour of the adrenal glands. The most common mutations in *TP53* are point mutations, which interfere with DNA binding and activation of TP53 dependent genes (Gasco, Yulug & Crook 2003; Sigal, Rotter 2000). Mutation of *TP53* is also frequently seen in *BRCA1* (56% - 100%) and *BRCA2* (29%) associated breast cancer (Holstege et al. 2009).

1.5.3 CDH1

The *CDH1* tumour suppressor gene encodes for cadherin 1 or E-cadherin, and is located on chromosome 16q22 (Rebbeck et al. 1996). E-cadherin is a cell adhesion glycoprotein and is crucial for cell to cell adhesion. Mutations in *CDH1* are associated with hereditary diffuse gastric cancer (HDGC) (autosomal dominant) and increased risk of

lobular breast cancer, with risk of breast cancer in women affected by HDGC being approximately 50% (Becker et al., 1994; Schrader et al., 2008). *CDH1* mutations also increase the risk of salivary gland cancer, lung cancer and colorectal cancer (Njiaju, Olopade 2012).

1.5.4 PTEN

Phosphatase and tensin homologue (*PTEN*) is a tumour suppressor gene located on chromosome 10q23.3 that encodes a phosphatidylinositol phosphate phosphatase (Rebbeck et al. 1996). The role of *PTEN* in cellular regulation is poorly understood. PTEN has the capacity to dephosphorylate both proteins and lipids (Teresi et al. 2008), and also act as a negative regulator of Akt phosphorylation (Zhou et al. 2003). It has been proposed that PTEN decreases tumour growth by inhibiting protein tyrosine kinase and regulates tumour invasion by interacting with focal adhesions (Li et al. 1997; Steck et al. 1997). Cowden's disease is caused by mutation of *PTEN* (autosomal dominant) and is associated with increased susceptibility to developing breast cancer (25-50%), thyroid cancer, endometrial neoplasm and benign hamartomas (Nelen et al. 1996).

1.5.5 STK11

The serine/theronine kinase 11 (*STK11/LKB1*) gene is located on chromosome 19p13.3 (Amos et al. 1997). This serine/theronine kinase has been shown to play an important role in cell polarisation, VEGF regulation, TP53 dependent apoptosis, and Wnt signal transduction (Alessi, Sakamoto & Bayascas 2006). Peutz-Jeghers syndrome (PJS) is caused by germline mutations of *STK11*. PJS increases the risk of cancer in diverse locations, which include the colon, breast, small intestine, oesophagus, stomach, breast, cervix and ovaries (Papp et al. 2010). The risk of breast cancer in PJS is around 45–54% (Hearle et al. 2006).

Table 1.2 Intermediate and low penetrance genes implicated in breast cancer

Gene	Name	Location	Function	Cancer syndrome	Associated tumours	References
ATM	Ataxia telangiectasia mutated	11q22-23	DNA damage recognition, protein kinase	Ataxia telangiectasia	Non-Hodgkin lymphoma, ovarian, breast (in heterozygous carriers).	(Swift et al. 1987)
CHEK2	checkpoint kinase-2	22q12.1	G2 checkpoint kinase that plays a central role in DNA repair	Li-Fraumeni syndrome	Breast	(Vahteristo et al. 2001)
FGFR2	Fibroblast growth factor receptor2	10q26	Regulate cellular and developmental processes, including apoptosis, proliferation, migration and angiogenesis	Craniosynostosis syndromes	Breast and gastric cancers	(Acevedo, Ittmann & Spencer 2009; Hunter et al. 2007; Jara et al. 2013)
NBS1	Nibrin	8q21	DNA repair	Nijmegen breakage syndrome	Microcephaly, growth restriction, immunodeficiency ,breast cancer	(Bogdanova et al. 2008)
CASP8	Caspase 8	2q33–q34	Apoptosis	--	Breast Cancer	(Cox et al. 2007)
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	5q11.2	Phosphorylation of enzymes, embryogenesis, T cell cytokine production and B cell antibody production keratinocyte migration.	--	Breast cancer	(Jara et al. 2013;Cancer Genome Atlas Network 2012)
LSP1	Lymphocyte-specific protein 1	11p15.5	Regulate Migration, motility and adhesion of lymphocytes	--	Breast Cancer	(Vachon et al. 2012)
BRIP/ FANCJ	BRCA1-interacting protein 1	17q22	DNA repair Interaction with BRCA1	Breast cancer	Foetal anomalies of skeleton kidneys, heart or any other major organ system Breast cancer	(Seal et al. 2006;Rahman et al. 2007)

1.6 Development of breast cancer

Breast cancers are derived from epithelial cells lining the terminal ductal-lobular unit (TDLU). Breast cancer is non-invasive (carcinoma *in situ*) when the lesion is limited to the basement membrane of the TDLU and draining duct. In the invasive type, the cancer cells are disseminated outside the basement membrane of the ducts and lobules and invade into the surrounding adjacent normal tissue. Based on the histological pattern, invasive and *in situ* breast cancers are classified as ductal or lobular, depending on their growth pattern and cellular morphology (Sainsbury, Anderson & Morgan 2000a).

1.6.1 Premalignant changes in breast cancer

The spectrum of premalignant breast lesions includes atypical ductal hyperplasia (ADH), atypical lobular hyperplasia (ALH), ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS) (Simpson et al. 2005; Wellings, Jensen & Marcum 1975). Atypical hyperplasia is characterised by increased numbers of cells and morphological abnormalities. DCIS and LCIS comprise malignant epithelial cells, but with an intact basement membrane and no evidence of invasion (Vecchione 1999).

1.6.2 Ductal carcinoma *in-situ* (DCIS)

In ductal carcinoma *in-situ*, epithelial cell proliferation is limited to within the basement membrane of the TDLU and the draining duct (Sainsbury, Anderson & Morgan 2000a), with metastasis being very uncommon (1 to 2%) (Fisher et al. 1995). The incidence of DCIS is around 20% of all breast lesions assessed by mammography (Ernster et al. 2002; Olivotto, Levine & Steering Comm Clin Practice Guidel 2001). There are several subtypes of DCIS based on morphology, which include papillary, micropapillary, cribriform, solid and comedo (Arpino, Laucirica & Elledge 2005). High grade DCIS is a common precursor for invasive breast carcinoma and recurrence in the affected breast is common (Burstein, Wong & Kaelin 2004).

1.6.3 Lobular carcinoma *in-situ* (LCIS)

Lobular carcinoma *in-situ* is a rare form of breast carcinoma (Foote, Stewart 1982). LCIS is not usually identified during mammography by signs of tumour mass enlargement or calcification, but identified incidentally during breast pathological examination (Rosen, Rosen 1997). LCIS is considered to be a sign of increased risk of malignancy progression rather than a precursor lesion and it can be localised or bilaterally extensive. LCIS is composed of a uniform population of small, polygonal, round or cuboidal cells, with a high nuclear to cytoplasmic ratio and a thin border of clear cytoplasm. Based on the extent of proliferation and distension of the lobular unit ALH is differentiated from LCIS (Simpson et al. 2003).

1.6.4 Invasive ductal carcinoma (IDC)

Invasive ductal carcinoma derives from ductal epithelial cells and accounts for 75% of invasive breast cancers (Yerushalmi, Hayes & Gelmon 2009). DCIS can progress to IDC and there is a high risk of metastasis to distant organs such as lymph nodes. Tumour cell invasion begins with degradation of the basement membrane (Mueller, Fusenig 2004). The malignant transformation of epithelial cells involves various epigenetic and genetic changes, as well as altered communication within the tumour microenvironment. The de-regulation of proliferation, differentiation, migration, impaired apoptosis and a pro-malignant stromal environment contributes to the progression of an invasive phenotype (Vargo-Gogola, Rosen 2007).

1.6.5 Invasive lobular carcinoma (ILC)

Invasive lobular carcinoma was first described in 1941 (Foote, Stewart 1982) and accounts for 5-15% of invasive breast carcinomas (Yerushalmi, Hayes & Gelmon 2009). The subtypes of ILC include alveolar, solid, histiocytoid, apocrine and signet ring (Martinez, Azzopardi 1979; Steinbrecher, Silverberg 1976). ILC cells are oestrogen

receptor (ER) and progesterone receptor (PR) positive with up - or down-regulation of the HER-2/neu gene (Frost et al. 1995; SastreGarau et al. 1996). Tumour size, axillary lymph node metastasis and absence of ER receptors are associated with poor prognosis (Orvieto et al. 2008).

1.6.6 Special types of invasive breast cancer

Special types of invasive carcinoma are classified on the specific patterns of growth and cellular morphology. These include tubular, cribriform, medullary, mucoid, papillary and classic lobular (Sainsbury, Anderson & Morgan 2000b).

1.6.7 Staging of breast cancer

The TNM staging is the most widely used system in the classification of tumour malignancy, which was created by the American Joint Committee on Cancer (AJCC). TNM staging system (Table 1.3) is a tool to stage tumour prognosis based on the extent of the primary tumour (T), lymph node status (N) and the presence of metastasis (M) (Singletary et al. 2002).

Table 1.3 The TNM system (Adapted and modified from Singletary et al. 2002)

Classification	Stage	Assessment
Primary tumour (T)	T_X	Primary tumour cannot be assessed
	T_0	No evidence of primary tumour
	Tis	Carcinoma *in situ*
	T_1	Tumour 2 cm or less in greatest dimension
	T_2	Tumour more than 2 cm but not more than 5 cm in greatest dimension
	T_3	Tumour more than 5 cm in greatest dimension
	T_4	Tumour of any size with direct extension to chest wall or skin
Lymph node (N)	N_x	Regional lymph nodes cannot be assessed
	N_0	No regional lymph node metastasis
	N_1	Metastasis in movable axillary node(s)
	N_2	Metastasis in axillary nodes fixed
	N_3	Metastasis in Infraclaviculara lymph node(s)
Metastases (M)	M_x	Distant metastasis cannot be assessed
	M_0	No distant metastasis
	M_1	Distant metastasis

1.7 Breast cancer molecular subtypes

The first research article regarding the classification of breast cancer subtypes based on gene expression prolifes was published by Perou and colleagues in 2000 (Perou et al. 2000). The group of intrinsic genes identified were obtained from cDNA microarray analysis of 38 breast cancer cases. Hierarchical cluster analysis revealed four molecular phenotypes, which included luminal, HER2-like, basal and normal breast. Similar analysis of a larger cohort of breast cancer patients have shown that the luminal group can be divided into at least two sub-types (luminal A and B). Recently, breast cancers have been subdivided into 10 molecular subtypes (Curtis et al. 2012); however, this chapter will only discuss the well-studied subtypes, based on experiments by Perou et al. (2000).

1.7.1 Luminal A

Luminal A breast cancer accounts for 50-60% of breast cancer and is the most common subtype, being histologically low in grade. The luminal epithelium in the mammary ducts is associated with expression of genes stimulated by the ER transcription factor and low expression of cell proliferation related genes (Perou et al. 2000; Eroles et al. 2012). Based on immunocytochemistry, luminal type A was associated with expression of ER, PR, Bcl-2 and cytokeratin CK8/18. The expression of HER2 was absent and Ki67 was minimally expressed. The expression of the transcription factor GATA3 was also significantly higher in the luminal A subgroup. The luminal A subtype includes all cases of lobular carcinoma *in situ* and most infiltrating lobular carcinomas (Eroles et al. 2012). The luminal A subtype has excellent outcomes and significantly reduced relapse rates (Sorlie et al. 2001). They are tamoxifen sensitive and aromatase inhibitors (AI) are used for treatment in postmenopausal women (Eroles et al. 2012).

1.7.2 Luminal B

The luminal B subtype accounts for 10-20% of breast cancers. In comparison with luminal A, luminal B subtypes are more aggressive, with higher histological grade and poorer outcomes than luminal A (Parker et al. 2009b; Kennecke et al. 2010). The main biological distinction between the two subtypes is expression of proliferation genes such as *CCNB1*, *MYBL2* and *MKI67*, which is expressed at higher levels in luminal B type tumours compared to luminal A (Hu et al. 2006). Luminal B tumours are also associated with increased expression of EGFR and HER2. The treatment of luminal B tumours is challenging as the mechanism related to survival, proliferation and metastasis is unclear. Luminal B tumours have poor prognosis despite treatment with tamoxifen (Paik et al. 2004) and pathological complete response (PCR) was significantly lower to neo-adjuvant chemotherapy compared to other subtypes (Parker et al. 2009a).

1.7.3 HER2 positive

The HER2 positive subtype accounts for 15-20% of all breast cancers. HER2 is a transmembrane tyrosine kinase receptor, which belongs to the family of EGF receptors and is structurally analogous to EGFR. HER2 is encoded by the *ERBB2/HER2* gene located on chromosome 17q21 (Thomas et al. 2002). The HER2 protein was found to be over-expressed in breast cancer and used as a prognostic marker (Cobleigh et al. 1999). HER2 positive breast cancers were shown to be highly proliferative; 75% are histologically high grade and the majority have *TP53* mutations (Eroles et al. 2012). HER2 positive breast cancer is associated with poor prognosis, but the advent of monoclonal antibody therapy with trastuzumab (Herceptin®) has significantly improved outcomes in both early and advanced stages (Piccart-Gebhart et al. 2005;Gianni et al. 2011).

1.7.4 Basal subtype or triple negative

The "basal" subtype accounts for 10-20% of all breast carcinomas. Basal types are common in young women of African origin with large tumour mass at the time of diagnosis. They are a higher histological grade and lymph nodes are frequently involved (Bosch et al. 2010). Basal subtype was associated with expression of genes characteristic of normal myoepithelial cells. The basal subtype was associated with expression of genes related to proliferation, inhibition of apoptosis and tumour invasion (Sorlie et al. 2006). The myoepithelial cells of basal subtype express high MW cytokeratins CK5 & CK17, P-cadherin, caveolin 1 & 2, EGFR, nestin and CD44; whereas, genes characteristic of luminal epithelial types include CK8/18 (Eroles et al. 2012). The ER, PR, HER2, EGFR and CK5/6 are the five markers used to identify the basal subtype with a sensitivity of 76% by immunohistochemistry and a specificity of 100% by microarray analysis (Nielsen et al. 2004). The basal subtypes are typically negative for ER, PR and HER2, and hence, overlap with "triple negative" (ER^-, PR^-, $HER2^-$) breast cancers (Eroles et al. 2012). Livasy et al 2006 evaluated the histologic and immunophenotypic properties of basal-like breast carcinomas with known microarray profile. The basal-like tumours were found to be grade 3 ductal or metaplastic carcinomas with geographical tumour necrosis, high mitotic count, expanding invasion margin and stromal lymphocytic response (Livasy et al. 2006). The visceral organs such as the central nervous system, lungs and lymph nodes are the main site of metastatic relapse (Smid et al. 2008).

1.7.5 Other types

The "normal-like" breast cancers account for 5-10% of all breast carcinomas and are a poorly characterised group. They express genes characteristic of adipose tissue and do not respond to neo-adjuvant chemotherapy. They are negative for ER, PR, HER2,

EFGR and CK5 (Eroles et al. 2012). Another intrinsic subtype named, claudin low was recently identified (12-14%). It is associated with low expression of genes involved in cell adhesion such as tight junctions, caludin 3-4-7, occludin, cingulin and E-cadherin. They express genes related to immune response and epithelial-to-mesenchymal transition (EMT) and have a poor prognosis (Prat et al. 2010; Hennessy et al. 2009). The features of breast cancer subtypes are listed in Table 1.4.

Table 1.4 Characteristics of breast cancer subtypes. Adapted and modified from (Eroles et al. 2012)

	Luminal A	**Luminal B**	**Basal-like**	**HER2-enriched**	**Normal breast like**	**Claudin-low**
Frequency	50-60%	10-20%	10-20%	10-15%	5-10%	12-14%
ER/PR/HER2	ER+ PR+ HER2-	ER+/- PR+/- HER2+/-	ER- PR- HER2-	ER- PR- HER2+	ER-/+ HER2-	ER- PR- HER2-
CK5/6 EGFR	-	-	+	-/+	+	-/+
Genes of Proliferation	Low	High	High	High	Low	High
Characteristics Genes	ESR1 GATA3 KRT8 KRT18 XBP1 FOXA1 TFF3 CCND1 LIV1	ESR1 GATA3 KRT8 KRT18 XBP1 FOXA1 TFF3 SQLE LAPTM4B	KRT5 CDH3 ID4 FABP7 KRT17 TRIM29 LAMC2	ERBB2 GRB7	PTN CD36 FABP4 AQP7 ITGA7	CDC44 SNAJ3
Histologic Grade	Low	Intermediate/ High	High	High	Low	High
TP53 Mutations	Low	Intermediate	High	High	Low	High
Prognostic	Excellent	Intermediate/ Bad	Bad	Bad	Intermediate	Bad

ER= Oestrogen; PR= Progesterone; HER2= Human epidermal growth factor receptor 2

1.8 Pathogenesis of breast cancer

Breast cancer progression from a pre-malignant lesion to an invasive cancer was previously considered as a multi-step process involving progressive changes from normal tissue to hyperplasia with or without atypia, carcinoma *in situ*, invasive carcinoma and metastasis (Figure 1.2 A) (Shackney, Silverman 2003). Advances in immunohistochemistry and molecular genetics have shown that cells undergo complex genetic changes and there are divergent pathways towards invasive breast cancer (Simpson et al. 2005). The accumulation of various epigenetic and genetic changes and abnormal interaction with the stromal microenvironment predisposes to the transformation of normal breast epithelial cells to malignant breast cancer cells. The process of malignant transformation of breast cancer is described in Figure 1.2. Alterations in the control of cell proliferation, survival, differentiation, cell migration and abnormal stromal microenvironment all contribute to an invasive phenotype (Vargo-Gogola, Rosen 2007). A normally differentiated and stratified epithelium is separated by a well-delineated basement membrane from the stromal compartment. The normal stroma contains collagen bundles that surround resting fibroblasts, mature blood vessels encircled by an uninterrupted basement membrane and leukocytes such as monocytes and macrophages. In transformation to pre-malignant dysplasia, the differentiation of epithelial cells is perturbed, leading to hyperplastic epithelium. The basement membrane separating the epithelium and stromal compartment remains intact, along with activated fibroblasts and an increase in the number of macrophages (Mueller, Fusenig 2004).

In the case of IDC, the tumour cells invade the surrounding microenvironment and the myoepithelial cells along with the basement membrane are degraded. In the advance stages of breast cancer, the myoepithelial cell layer and the basement membrane are

completely lost with invasion of epithelial cells, accumulation of stromal cells and angiogenesis (Khamis, Sahab & Sang 2012). The main sites of breast cancer metastasis are the lymph nodes. The process of metastasis involves invasion of cells through the basement membrane, intravasation of the vasculature or lymphatic circulation, survival in the vasculature without adhesion, extrusion from the vasculature or lymphatic system (extravasate), capture at distant sites and develop into a new tumour in a remote microenvironment (Figure 1.2 B) (Steeg 2006;Vargo-Gogola, Rosen 2007).

It has been proposed that breast cancer cells with stem cell-like characteristics (dormancy, self-renewal and differentiation) promote initiation, development and relapse (Tan et al. 2006). This is supported by the fact that long term (LT), short term (ST) and luminal or myoepithelial progenitor cells undergo several epigenetic and genetic alterations yielding several subtypes of tumours with different cells (Figure 1.2 C) (Vargo-Gogola, Rosen 2007).

http://www.nature.com/nrc/journal/v7/n9/fig_tab/nrc2193_F1.html

Figure 1.2: The schematic representation of cancer progression. The epigenetic and genetic alterations within the microenvironment and their interaction results in transformation of mammary epithelial cells. A) The process of breast cancer development contains several stages with de-regulation of proliferation, survival, differentiation and migration. B) The breast cells transformation is induced by abnormal tumour–stromal cell interactions, and the epithelial cells invade through the basement membrane and disseminate into a remote microenvironment and develop into a new tumour. The long term (LT), short term (ST) and luminal or myoepithelial progenitor cells undergo several epigenetic and genetic alterations yielding several subtype of tumours with different cells. These tumour subtypes have a unique gene expression pattern and have variable prognosis. Figure adapted from (Vargo-Gogola, Rosen 2007).

1.9 The role of the microenvironment in breast cancer

The epithelium and stroma are the two main cellular compartments of normal breast tissue. The stromal compartment includes the extracellular matrix (ECM) and stromal cells (Khamis, Sahab & Sang 2012). The basement membrane separates the stroma from the epithelial/myoepithelial cell bilayer (Figure 1.3) (Polyak, Kalluri 2010). The basement membrane is a unique part of the ECM, composed of various proteoglycans and glycoproteins that separate epithelial cells from the surrounding microenvironment (Kalluri 2003). The stromal cells include fibroblasts, endothelial cells, myofibroblasts and numerous immune cells and adipocytes (Sadlonova et al. 2007). The ECM is a 3D structure that surrounds these cells and comprises numerous proteins such as collagens, fibronectin and laminin. The ECM is also abundant in mediators of neoplastic remodelling such as matrix metalloproteinases (MMPs) and soluble growth factors (Khamis, Sahab & Sang 2012).

It has been reported extensively that the microenvironment plays a pivotal role in modulating important aspects of mammary epithelial cells such as proliferation, polarity, differentiation, survival and metastatic capacity of mammary epithelial cells (Figure 1.4) (Polyak, Kalluri 2010). The tumour microenvironment was proposed to be actively involved in breast cancer pathogenesis supported by a striking difference in molecular signatures between the stromal cells of tumours and normal breast tissue (Khamis, Sahab & Sang 2012). Cell to cell, and cell to microenvironment interactions are implicated in this process, but the mechanisms are poorly understood (Polyak, Kalluri 2010).

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2964182/figure/A003244F1/

Figure 1.3: The schematic representation of mammary duct and lobule. The basement membrane is indicated by a black line. The myoepithelial cells in the ducts form a nearly complete layer of luminal epithelial cells, whereas in the alveoli there are extra fenestrations. The basement membrane may have direct contact with luminal epithelial cells. The yellow arrows signify probable cell–cell and cell–matrix interactions. Adapted from (Polyak, Kalluri 2010).

1.9.1 Fibroblasts

Stromal fibroblasts play a crucial role in the pathogenesis of breast cancer. Cancer-associated fibroblasts (CAFs) secrete ECM proteins and mitogens, which are important for tumour growth and metastasis (Bauer et al. 2010). Fibroblasts have been shown to regulate several functions in tumours such as cell to cell interaction, adhesion, migration, ECM degradation, transcriptional regulation and paracrine signalling. Expression of fibulin1 (FBLN1), kruppel-like factor 4 (KLF4), TGFβ2, Wnt1 inducible signalling pathway protein 1 (WISP1), plasminogen activator inhibitor 2 (PAI2), and tissue plasminogen activator (PLAT) is deregulated in CAFs (Sadlonova et al. 2007; Bauer et al. 2010).

Recently, a co-culture model has compared the luminal cells, myoepithelial cells and stromal fibroblasts between normal and malignant breast tissue. The 3D heterotypic culture system containing mixed normal or tumour cells has shown that these cells arrange into structures recapitulating normal and DCIS breast, with myoepithelial cells oriented around the luminal population. The tumour cell co-units show an altered basement membrane with loss of β4-integrin corresponding to DCIS. The addition of CAFs compromised the co-unit organisation, which was prevented by MMP and/or c-met inhibitors (Holliday et al. 2009).

1.9.2 Myoepithelial cells

Myoepithelial cells have been shown to express various tumour suppressor proteins, angiogenesis inhibitors, proteinase inhibitors and ECM structural proteins (Khamis, Sahab & Sang 2012). The normal myoepithelial layer is an endogenous tumour suppressor in both a paracrine (bFGF, TGF-α, and IL-6) and an autocrine manner (resistance to transformation) (Nguyen et al. 2000; Teuliere et al. 2005). Because of their tumour suppressor function myoepithelial cells are also known as the "Cinderella" of the breast (Lakhani, O'Hare 2001).

1.9.3 Myofibroblasts

The stromal compartment of the tumour also includes myofibroblast, which are stimulated fibroblasts and express α-smooth muscle actin (α-SMA). The levels of VEGF, bFGF and Ki-67 were significantly up-regulated in myofibroblasts from human tissue sections of invasive breast cancer. The expression of the above mentioned factors in myofibrobaslt in breast cancer was shown to predict shorter overall survival and relapse-free survival (Surowiak et al. 2007). α-SMA-positive myofibroblasts were also shown to promote tumour invasion and angiogenesis in mouse gastric cancer (Guo et al. 2008).

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2964182/figure/A003244F2/

Figure 1.4: The perturbation of microenvironment during tumour progression. The schematic representation of DCIS, IDC and metastatic carcinoma. The basement membrane separates stroma from the layer of epithelial cells and the layers of myoepithelial cells. The stromal cells include cancer cells, fibroblasts, endothelial cells, myofibroblasts, numerous immune cells, adipocytes and their increase during tumour progression depends on the tumour subtype. In case if DCIS, there is an epigenetic and phenotypic alteration of myoepithelial cells and they reduce in numbers due to signalling from tumour epithelial cells and other stromal cells. Adapted from (Polyak, Kalluri 2010).

1.9.4 Endothelial cells

Endothelial cells are recruited to the tumour stroma where they play a critical role in angiogenesis, tumour growth and metastasis (Khamis, Sahab & Sang 2012). The presence of endothelial cells has been shown to promote the angiogenic activity of malignant mammary epithelial cells (Buchanan et al. 2012). Apart from transporting nutrients and oxygen, breast endothelial cells are important components of the microenvironment and provide growth signals to both the normal and malignant breast epithelium (Ingthorsson et al. 2010).

1.9.5 Adipocytes

Adipocytes have also been implicated in the pathogenesis of breast cancer and have been shown to promote the invasive capacity of breast cancer cells (Dirat et al. 2011; Walter et al. 2009). Co-culture of adipocytes with cancer cells increased their invasive capacity. Moreover, the modified adipocyte phenotype was characterised by low expression of adipocyte markers and lipids, with over-expression of MMPs and inflammatory markers (Dirat et al. 2011).

1.9.6 Bone marrow derived cells (BMDCs)

Bone marrow derived cells (BMDCs) such as mast cells, lymphocytes, macrophages and neutrophils are recruited by primary tumour cells to promote migration, angiogenesis and invasion (Khamis, Sahab & Sang 2012). These immune infiltrates are frequently observed at the site of degraded myoepithelial cell layers, suggestive of their role in invasion and metastasis (Man 2007).

1.10 Extracellular matrix (ECM)

The extracellular matrix (ECM) functions as a scaffold to maintain tissue and organ structure. The ECM is formed by the assembly of numerous proteins and polysaccharides forming an extensive meshwork within tissues, being primarily composed of fibrous structural proteins which include collagens, laminins, fibronectin, vitronectin and elastin. The ECM is also made up of some specialised proteins (growth factors, small matricellular proteins, small integrin binding glycoproteins) and proteoglycans. The composition of ECM components is variable depending on the type of tissue. Apart from providing mechanical support to the tissues, the ECM also regulates pivotal physiological processes such as cell proliferation, migration, and differentiation, alteration in cell shape, growth and survival (Daley, Peters & Larsen 2008). The ECM undergoes constant remodelling by degradation and reassembly and is intensified during development, wound repair, infectious diseases and in many other disease states. Integrins are heterodimeric trans-membrane receptors, which play an important role in the transduction of signal from the ECM to the cell interior. The signal transduction by integrins through ECM proteins significantly influences gene expression patterns and important cellular processes such as angiogenesis, cell proliferation and invasion. The ECM can be modified in response to signals transmitted by ECM receptors such as integrins, laminin receptors, syndecans and proteases such as

matrix metalloproteinases (MMPs) (Daley, Peters & Larsen 2008). In tumours, tissue homeostasis is deregulated due to diminished cell adhesion signalling, cytokine/growth factor independent growth and disrupted ECM signalling (Orend 2005b). Integrins have been shown to play an important role in transduction and integration of signals from the microenvironment. Integrins are heterodimeric transmembrane proteins and made up of α- and β- subunits. Approximately 24 integrins with various subunit combinations have been reported. The integrins were shown to regulate various carcinogenic processes such as proliferation, differentiation, survival and migration (Hynes 2002). Integrin α9β1 was implicated as a novel marker in basal breast cancers and it augments cell migration and invasion. Integrin α9β1 expression in breast cancer was associated with poor prognosis (Allen et al. 2011). It remains elusive how changes in ECM composition and signalling mechanisms contribute to tumour progression (Orend 2005b). TNC is one glycoprotein, which is highly expressed in the stroma of many solid tumours and in breast cancer in particular (Ishihara et al. 1995).

1.11 The Tenascin Family

The tenascins are oligomeric glycoproteins exclusively expressed in the ECM of vertebrate organisms (Orend 2005b). Currently, five tenascin types are observed in mammals: Tenascin-C, -R, -X, -W and -Y (Table 1.5). The primary structure of the tenascin family (Figure 1.5) includes consecutive amino-terminal heptad repeats, epidermal growth factor (EGF)-like repeats, fibronectin type III repeats, and a carboxyl-terminal fibrinogen-like globular domain (Hsia, Schwarzbauer 2005).

Figure 1.5: Tenascin family of proteins. The tenascin family shares an amino – terminal oligomerisation region followed by consecutively arranged domains of heptad repeats, EFG like repeats, FN type III repeats and a fibrinogen globe. The primary structure of tenascin-C and -R show a high degree of homology to each other. The mammalian protein similar to tenascin W may provide an alternative mechanism in mice with null deletions for other tenascins. There are several similarities between tenascin-X and -Y, such as in some tenascin- X variants there is an abundance of proline and serine residue-like tenascin-Y domains (modified from Hsia, Schwarzbauer 2005).

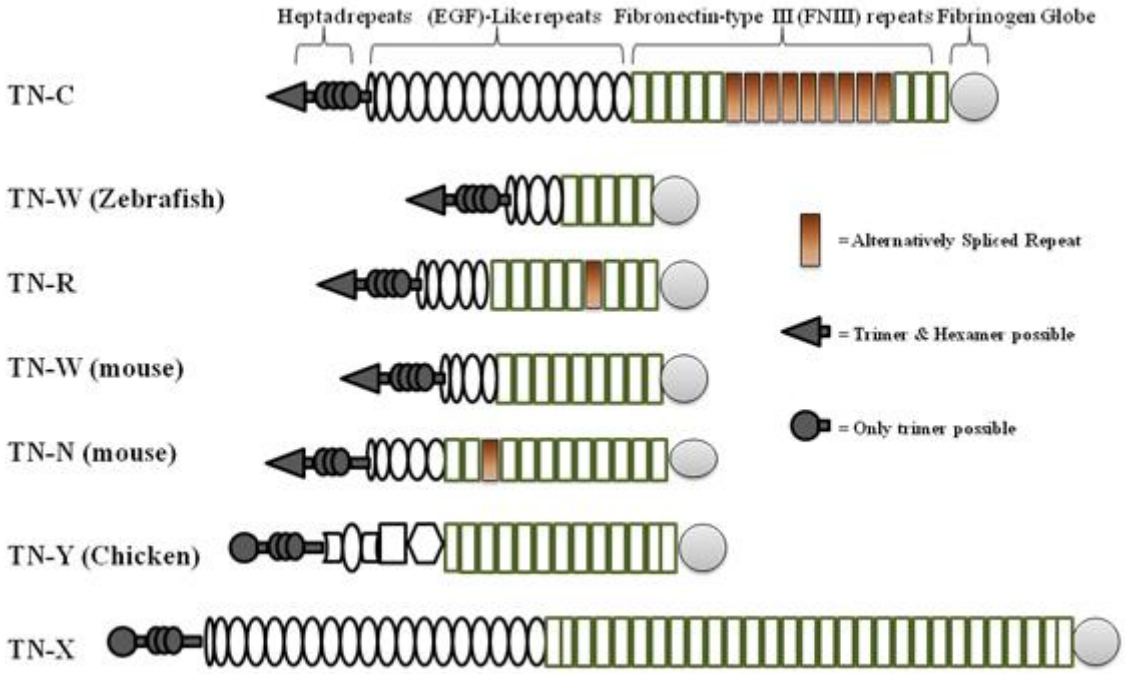


Table 1.5 Tenascins: structure, tissue expression, biological function and the associated pathological conditions.

Characteristics	*Tenascin-C*	*Tenascin-R*	*Tenascin-W*	*Tenascin-X*	*Tenascin-Y*
Molecular Weight	180-300 kDa	160-180 kDa	130 kDa	400 kDa	170-220 kDa
Protein Subunits	14.5 EGF-like repeats, 8 fibronectin type III repeats, assemble to form hexamers (Hsia, Schwarzbauer 2005).	4.5 EGF-like repeats and 8–9 fibronectin type III repeats (Hsia, Schwarzbauer 2005)	3.5 EGF-like repeats and 5 fibronectin type III repeats (Hsia, Schwarzbauer 2005)	18.5 EGF-like repeats and 29 or more fibronectin type III repeats (Hsia, Schwarzbauer 2005).	FibroneCtin type III repeats rich in serines and prolines (Hagios et al. 1996)
Alternative splicing regions	9 FNIII repeats (included or excluded diversity in isoform (Joester, Faissner 2001).	6^{th} FNIII repeat - two isoform (Pesheva, Probstmeier 2000).	Not reported	Humans – not reported, mouse splice variants reported (Speek, Barry & Miller 1996).	Humans – not reported, chicken splice variants reported (Chiquet-Ehrismann et al. 1994)
Tissues Expressed	Neural, skeletal, and vascular morphonogenesis, adult – skeletal tissues, tumour, inflammation, wound repair and neovascularisation, tumour (Hsia, Schwarzbauer 2005), mechanical stress (Chiquet-Ehrismann et al. 1994).	Central nervous system, Peripheral nervous system (Chiquet-Ehrismann 2004)	Zebrafish, developing skeleton, periostium, kidney and muscles (Chiquet-Ehrismann 2004) particularly expressed in breast cancer stroma (Degen et al. 2008).	Musculoskeletal, cardiac, and dermis tissue (Chiquet-Ehrismann 2004)	Chicken skeletal muscle and heart tissue (Hagios et al. 1996)
Biological Function	Inhibits cell adhesion to fibronectin, supports neurite growth (Tucker 2001)(Orend 2005a)	Influences neural pattern formation – adhesive and anti-adhesive action on cell matrix (Zacharias et al. 2002)	Promotes invasion in breast cancer cell (Degen et al. 2008) , inhibits proliferation and differentiation in osteoblasts (Kimura et al. 2007)	Regulate collagen fibrillogenesis, cell adhesion (Mao et al. 2002)	Establishment of connective tissue layers in and around skeletal, heart and smooth muscle in the chicken embryo (Hagios et al. 1996)
Pathological conditions	Pathological states such as infection, tumour , wound healing and angiogenesis (Chiquet-Ehrismann 2004)	CNS defects, abnormal behaviour (Weber et al. 1999)	Breast and colon cancer (Degen et al. 2008)	Ehler-Danlos Syndrome (Mao et al. 2002)	Not reported

1.12 Tenascin-C

1.12.1 Molecular structure

Tenascin-C (TNC) is an oligomeric ECM glycoprotein with a molecular mass of 180-300 kDa that contains six monomers linked by disulfide bonds at their N-termini. Each monomer subunit of TNC is made up of a globular amino-terminal domain, 14.5 epidermal growth factor-like repeats (EGF like repeats); up to 17 fibronectin type III-like repeats (FN III) and a carboxy-terminal sequence (Gulcher et al. 1991; Jones, Jones 2000). The TNC gene is located on chromosome 9 q32-q34 and it is made up of 30 exons separated by 29 introns (Midwood, Orend 2009). Alternative splicing at the pre-mRNA level of 9 FNIII repeats (exons 10-16, AD1 and AD2), which are included or excluded in a unique manner, allows TNC to show tremendous diversity in isoform expression with as many as 27 different mRNA variants having been identified in the developing mouse brain (Joester, Faissner 2001) (Figure 1.6).

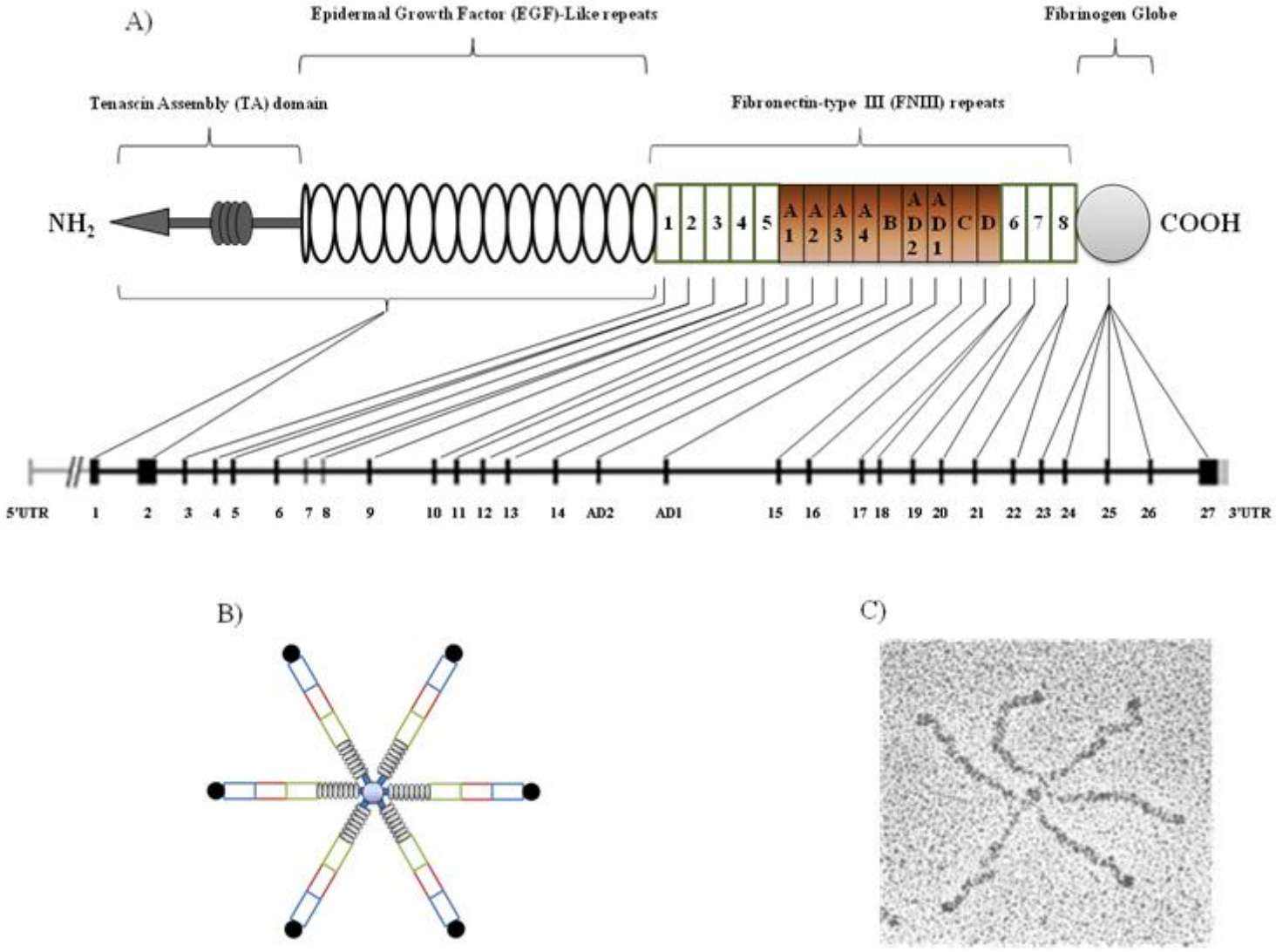


Figure 1.6: A) TNC Structure. Mammalian TNC polypeptides contain an additional repeat. Conserved and alternatively-spliced Fibronectin type-III repeats are represented by dark brown and light green boxes, respectively. The C-terminal is made up of a fibrinogen globe circle (Adapted from (Guttery et al. 2010a).

B) The tenascin assembly. A coiled interaction that occurs at the heptad repeat region allows the six polypeptides to form a hexamer. The oligomeric region is represented in the oval at the centre through interchain disulphide bonds. The different coloured domains of each TNC polypeptide are indicated as in (A).

C) Electron micrograph of rotary shadowed TNC. Adapted from (Erickson, Bourdon 1989).

1.12.1.1 Tenascin assembly (TA) domain

The TA domain is encoded by a single exon and is characterised by α-helical structure and cysteine residues to facilitate hexabrachion structure formation. TNC assembly has been shown to be mediated by two autonomous folding domains. Organisation into a functional hexamer involves the formation of a trimer intermediate by a short parallel α-helical coiled-coil domain, followed by the connection of two such triplets into a hexamer via the clustered TA domains N-terminal to the α-helical segment. Prior to hexabranchion formation, oligomerisation by a three-stranded coiled-coil domain is an essential step for clustering of three individual TA domains to provide a high local concentration of homophilic weak affinity sites, consequently resulting in binding affinity between TA domains (Kammerer et al. 1998).

1.12.1.2 Epidermal growth factor (EGF) - like repeats

The 14.5 Epidermal growth factor (EGF)- like repeats are encoded by a single exon and are involved in modulation of cell adhesion and cell motility (Midwood, Orend 2009). They are 31 amino acids in length and contain six cysteine residues that play a part in inter-chain disulphide bonding with the topology 1-3, 2-4, and 5-6 (Baron et al. 1992). The EGF-like regions are considered to be counter-adhesive for fibroblasts, neurons and glia, and may be involved in neuronal migration and axonal pathfinding during development (Spring, Beck & Chiquet-Ehrismann 1989; Prieto, Anderssonfisone & Crossin 1992;Crossin 1994;Krushel et al. 1994;Gotz et al. 1997; Fischer et al. 1997). This region also provides localised signals for growth and differentiation, presumably via interactions with EGF receptors (Thiery, Boyer 1992).

1.12.1.3 Fibronectin type III (FNIII) repeats

The fibronectin type III (FNIII) repeat region is the largest portion of the TNC protein and contains an array of repeats. Each repeat consists of 91 amino acids (except AD1, which has 92) (Sriramarao, Bourdon 1993b), with eight repeats being constitutively expressed (repeats 1-5 and 6-8 encoded by exons 3-9 and 17-22 respectively). In addition, 9 domains are susceptible to alternative splicing (A1-D , encoded by exon 10-16, AD1 and AD2) and are situated between domains 5 & 6 (Figure 1.7) (Jones et al. 1988;Gulcher et al. 1989;Jones et al. 1989;Spring, Beck & Chiquet-Ehrismann 1989;Gulcher et al. 1991;Nishi et al. 1991). FNIII arrays represent a highly elastic region of the TNC molecule and have been shown to undergo rapid stretching and refolding (Oberhauser et al. 1998; Marin et al. 2003), which has been postulated to modulate cell adhesion and downstream signalling by masking of the RGD motif within ECM ligands (Huang et al. 2001;Orend et al. 2003). Each domain of the alternatively spliced region is encoded by a single exon and can generate a number of different isoforms (Dorries, Schachner 1994).

http://www.sciencedirect.com/science/article/pii/S1357272504004261#gr1

Figure 1.7: TNC binding region. The alternative spliced region shaded in black above and the approximate location of ligand binding domains denoted by black bar. Taken from (Orend 2005a)

1.12.1.4 Fibrinogen globe

The fibrinogen globe of TNC consists of 210 amino acids encoded by five exons (Siri et al. 1991). Two parts of the molecule are bound together at the centre by disulphide bonds and non-covalent contacts. One side of the fragment consist of α-helix and β-sheet chains involved in a funnel-shaped domain formation with the hydrophobic cavity; whereas the other side involves the N-terminal chain which folds into a separate domain. The polypeptide loops formed by two consecutive intrachain disulphide bonds have been shown to bind Ca^{2+} (Jones et al. 1988). Moreover, the property of calcium-binding of the fibrinogen globe alters interactions with other proteins including collagen fibrils, integrins, heparin and a cell surface chondroitin sulphate proteoglycan called phosphocan (Jones, Jones 2000).

1.12.2 The tenascin-C knockout mouse

Knockout of TNC *in-vivo* was performed in the mouse using homologous recombination in embryonic stem cells. Mutant mice were produced in which the *TNC* gene was replaced with the lacZ gene and resulted in a complete loss of TN expression. These tenascin-C-knockout mice showed no anatomical or histological abnormalities compared to wild-type controls (e.g. same sizes; fertile; and no gross deficits in neuroarchitecture or principal organ systems (Saga et al. 1992). A similar phenotype was also observed in a second TNC mutant mouse (Forsberg et al. 1996). Researchers concluded that other tenascins may compensate by being up-regulated as a result of loss of TNC function (Saga et al. 1992; Steindler et al. 1995; Sakai et al. 1995). Subsequent studies have shown several abnormalities in behaviour and wound healing, and the function of TNC has been elucidated from behavioural studies of knockout mice and their response to trauma (Mackie, Tucker 1999). The phenotypes of TNC-knockout mice are summarised in Table 1.6.

Table 1.6 Summary of TNC-knockout mouse phenotypes (adapted and modified from Mackie and Tucker, 1999)

Study	Phenotype	Reference
Behaviour	Hyperlocomotion poor swimming abnormal circadian rhythm	(Fukamauchi et al. 1997;Fukamauchi et al. 1996)
Neurochemistry	Reduced tyrosine hydrolase, reduced neuropeptide Y, increased preprotachykinin A, increased cholecystokinin	(Fukamauchi et al. 1997;Fukamauchi, Aihara & Kusakabe 1998;Fukamauchi, Kusakabe 1997)
Peripheral nerves and nerve regeneration	Abnormal peripheral nerves, abnormal neuromuscular junction Reduced sprouting after Bo Tx-A	(Cifuentes-Diaz et al. 1998)
Wound healing	Reduced fibronectin (skin & cornea), wounds compressed with fewer migrating keratinocytes	(Forsberg et al. 1996; Matsuda et al. 1999)
Tumourigenesis	Increased monocytes/macrophages, altered tumour stroma, inhibition of tumour formation from xenograft	(Talts et al. 1999)
CNS injury	More astrocytes in glial scar	(Steindler et al. 1995)
Glomerulonephritis	Failure to regenerate	(Nakao et al. 1998)
Haemopoiesis	Reduced haemopoiesis in vitro	(Ohta et al. 1998)

1.12.3 TNC function and expression

TNC is expressed prominently during embryogenesis (specifically during neural, skeletal, and vascular morphogenesis) and transiently expressed during organ development, playing an important role in epithelial-mesenchymal interactions and differentiation of the gastrointestinal system, kidney and mammary glands. The expression of TNC declines during adulthood but reappears during pathological

conditions such as wound repair, neovascularisation, inflammation and tumourigenesis (Orend 2005b). TNC is also secreted by activated fibroblasts in response to mechanical stress (Chiquet-Ehrismann et al. 1994), and other pathological conditions such as tendon degeneration, synovitis, colitis, pathological bone marrow and interstitial pneumonia (Midwood, Orend 2009). TNC expression is induced by growth factors and cytokines such as transforming growth factor beta 1 (TGFβ-1), fibroblast growth factor (FGF), tumour necrosis factor alpha (TNF-α) and platelet derived growth factor (PDGF) (Chiquet-Ehrismann 1995). The addition of TGFβ-1 to cultured chick embryo fibroblasts has been shown to induce the expression of TNC, whereas FGF-2 is known to be an important factor in angiogenesis and induces expression of TNC in glial tumours (Rettig et al. 1994). PDGF has also shown to induce expression of TNC in smooth muscle cells and the central nervous system (CNS) (Rettig et al. 1994).

1.12.4 TNC in proliferation and cell adhesion

TNC has been shown to promote cell migration, inhibit focal adhesion formation, induce cell proliferation, and act as a cell survival factor. Moreover, TNC has also been shown to promote angiogenesis and remodelling of ECM components such as MMPs (Chiquet-Ehrismann, Chiquet 2003). TNC has been shown to inhibit cell adhesion to fibronectin and promote growth of tumours, as well as inhibiting binding of syndecan-4 to the XIIIth fibronectin-type III repeat of fibronectin. This repeat is essential for fibronectins interaction with syndecan-4 to promote full cell spreading (Huang et al. 2001), with inhibition resulting in increased growth of tumour cells and decreased proliferation of normal cells (Orend et al. 2003).

Furthermore, inhibition of syndecan-4 binding also activates the canonical Wnt pathway (via down-regulation of the Wnt inhibitor DKK1) and MAPK signalling activated due to an increased expression of PDGFRα and EDNRA, alters gene transcription and

stimulates tumour cell proliferation in glioma cells (Ruiz et al. 2004). There are many surface receptors for TNC, which include integrins, annexin II and syndecan-4. TNC also interferes with several cell signalling pathways and has been found to inhibit focal adhesion kinase and Rho-mediated signalling pathways (Midwood et al. 2004) (Figure 1.8).

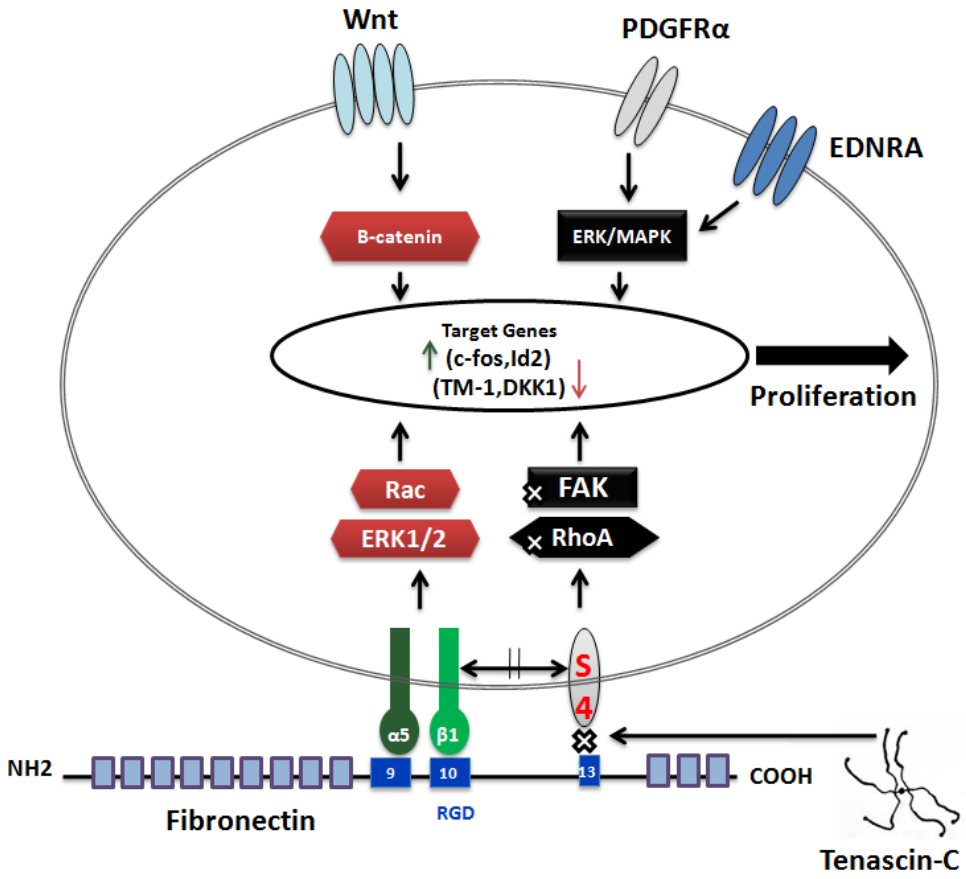


Figure 1.8: TNC inhibits syndecan-4 (S4) to 13th FN-III repeat of fibronectin. FAK inhibition via inhibited Rho resulting in compromised cell spreading due to assembly of actin stress fibres (Chiquet-Ehrismann, Chiquet 2003). However, ERK1/2 and Rac are still activated due to integrin binding. Subsequently, cellular signalling is stimulated and modifies transcription of target genes, leading to an increase in proliferation. MAPK signalling activated due to an increased expression of PDGFRα and EDNRA (Orend 2005a).

1.12.5 TNC expression in the normal breast

TNC is diferentially regulated at every stage of normal mammary gland development. For example, high levels of TNC appear in the dense embryonic mesenchyme at 14 days postgestation, but decreases in the postnatal period and adult stages (Chiquet-Ehrismann et al. 1986; Inaguma et al. 1988). However, low levels of TNC are detected in the mature mammary gland, which is localised to blood vessels, discrete layers surrounding the mammary ducts and acini (Inaguma et al. 1988; Howeedy et al. 1990). TNC is up-regulated in the stroma of normal breast as a consequence of the menstrual cycle. Immunohistochemistry analysis showed that the amounts of TNC during the menstrual cycle were increased gradually from the low levels at the first week, and reached maximal levels during the fourth week (Ferguson et al. 1990).

In pregnancy, the production of TNC is suppressed due to an increasing level of progestins, oestrogen and placental lactogen, which trigger the ability of mammary epithelial cells to proliferate and form the alveolar morphology (Voogt, 1978; Wirl et al. 1995).

1.12.6 TNC expression in breast cancer

TNC expression has been shown to be increased in cancers including carcinomas of the colon, breast, lung, prostate and brain (Orend 2005a). Expression of TNC is also associated with poor prognosis and has been shown to predict local and distant recurrence in breast cancer (Jahkola et al. 1998b), as well as being been correlated with high HER2, low oestrogen receptor expression and lymph node metastasis (Ishihara et al. 1995). The variability in expression of TNC exons has been shown to play a crucial role in the progression of breast cancer. Adams et al. (2002) examined in detail the pattern of TNC isoform expression in benign, pre-invasive and invasive breast lesions using reverse transcription-PCR (RT-PCR) and Southern blotting, with a distinct

diversity in the expression of TNC isoforms in breast cancer being observed. Although fully truncated TNC was identified in all breast cancer tissues, isoforms containing exons 14 + 16 (TNC14/16) were significantly associated with an invasive phenotype. Highly invasive breast cancer cell lines such as MDA-MB 231 and MDA-MB-436 were shown to express TNC, whereas less invasive, oestrogen receptor positive cell lines such as MCF-7 and T47D showed no detectable levels of TNC. Moreover, cell invasion and proliferation were promoted by over-expression of TNC16 and TNC 14/16 in MCF-7, T47D, MDA-MD-231, MDA-MB-468 and GI101 breast cancer cell lines via MMP dependent and independent mechanisms (Hancox et al. 2009).

Isoforms containing AD1 (TNC-AD1) may regulate changes in cell shape, adhesion, migration and proliferation (Fischer et al. 1997). Expression of isoforms containing AD1 were found to be significantly associated with high grade, ER negative tumours as well as younger patient age (<40 years) (Guttery et al. 2010a). TNC is expressed as different isoforms and its role in breast cancer metastasis is elusive. A deeper understanding of the patho-physiology of metastasis and TNC isoforms in breast cancer may help to find new targets for treatment and improve prognosis of patients with breast cancer.

1.12.7 Tamoxifen resistance and TNC

Tamoxifen is an oestrogen receptor modulator used commonly for the treatment of breast cancer. Most of the ER positive breast cancers do not benefit from tamoxifen therapy, and many developed tamoxifen resistance following an initial response (Pontiggia et al. 2012). Recently TNC was implicated in tamoxifen resistance in breast cancer. Helleman et al (2008) has examined expression of six ECM genes in 1,286 primary breast tumours which included TNC, collagen 1A1 (COL1A1), fibronectin 1(FN1), lysyl oxidase (LOX), secreted protein acidic cysteine-rich (SPARC) and tissue

inhibitor of metalloproteinase 3 (TIMP3). These genes were then related to tamoxifen response, prognosis and outcome. The expression of TIMP3, FN1, LOX, and SPARC were associated with distant metastasis-free survival (MFS) of lymph node negative patients who did not receive adjuvant systemic therapy (680 subjects). In patients who were positive for ER and lymph node metastasis with adjuvant tamoxifen therapy, high TNC expression was associated with shorter MFS and shorter progression free survival (PFS). There was no direct clinical benefit from first-line tamoxifen monotherapy (Shorter PFS and PRS) suggestive of a role for TNC in tamoxifen resistance (Helleman et al. 2008). Recently miR-335 was found to regulate the expression of TNC (Tavazoie et al. 2008). TNC can activate EGFR by interaction though their epidermal growth factor–like domains. Swindle et al. has shown that EGFR domains of TNC induce EGFR phosphorylation and activate MAPK signalling and cell division in the NR6 fibroblast cell line (Swindle et al. 2001). The Ten14 repeat of TNC was shown to bind to EGFR with low affinity and increase migration through stimulation of PLCγ and m-calpain (Iyer et al. 2008; Midwood, Orend 2009).

1.12.8 TNC and matrix metalloproteinases (MMPs)

The MMPs are a family of zinc-containing endopeptidases, which degrade ECM components (Overall, Lopez-Otin 2002) and have been implicated in tumour progression including invasive and metastatic phenotypes (Overall, Lopez-Otin 2002; Coussens, Fingleton & Matrisian 2002). Normally, MMPs are expressed during tissue remodelling such as embryonic development and wound healing (Woessner 1991). However, MMPs have been found to be up-regulated in pathological conditions, such as tumour cell invasion and metastasis (Woessner 1991; Egeblad, Werb 2002). In cancer tissues including breast cancer, MMPs produced by both tumour and stromal cells suggesting a link with tumour progression (Soini et al. 1994; Kumaki et al. 2001).

In breast cancer, both TNC and MMPs are up-regulated, suggesting TNC could induce MMP activity (Tremble, Chiquet-Ehrismann & Werb 1994). TNC up-regulates the expression of MMP-9 in mouse mammary cancer cells (Kalembeyi et al 2003), and enhances TGF-β-induced MMP-9 expression (Ilunga et al. 2004). In glioma cells, up-regulation of MMP-12 mediates the promoting effect of TNC (Sarkar et al. 2006). TNC-16 and TNC-14/16 mediate invasion through MMP-independent mechanisms (Hancox et al. 2009).

1.12.9 TNC exon additional domain 1 (AD1) and 2 (AD2)

The exon "additional domain 1" or AD1 was first identified by sequencing of two human tenascin cDNA clones from a U251 cDNA library (Sriramarao, Bourdon 1993a). It has been reported that the AD1 is expressed in the highly invasive quail fibrosarcoma cell line QT6, which is characterised by high motility and invasion illustrated by rapid passage through a fibronectin-coated filter in a blind well chamber assay. They are also known to predominantly express a high molecular weight form of TNC with three repeats in the variable domain (Derr et al. 1997). Derr et al (1997) also isolated AD1 transcripts from the human breast ductal carcinoma cell line Hs578T, suggesting that AD1 may play a role in progression and metastasis of human breast carcinoma. AD1 was also found to be expressed at sites of active tissue remodelling and fibronectin expression in the developing avian feather bud and the sternum. Furthermore, myoblasts expressing AD1 containing transcripts organise actin microspikes containing the actin bundling protein fascin and do not assemble focal contacts, possibly through inhibition of syndecan 4 binding of fibronectin (Orend et al. 2003). Previous findings by our group showed TNC-AD1 is associated with ER negative tumours and cancers from younger women (≤ 40 years) (Guttery et al. 2010a). Furthermore, the B/AD1/D (exon 14/AD1/16) isoform significantly increased cancer cell invasion and growth to a greater

extent than the B/D isoform. TNC-AD2 was found to be expressed in malignant oral cancers (Mighell et al. 1997). Both TNC-AD1 and AD2 were associated with ER negative breast cancers. Furthermore, AD2 expression was found in breast cell lines, normal breast and tumour tissue but at a lower level than AD1 (Guttery et al. 2010a).

1.12.10 TNC knockdown

Knockdown of TNC using short hairpin RNA (shRNA) showed a significant reduction invasion and metastasis of breast cancer cells to the lung (Tavazoie et al. 2008). In glioblastoma, TNC was down regulated by using of short interfering RNA (siRNA) and resulted in a significant decrease in metastasis (Zukiel et al. 2006), and decreased invasiveness and reactive change of peritumoral brain tissue (Hirata et al. 2009). In melanoma, TNC knockdown decreased the side population (SP) fraction in melanoma spheres and lowered their resistance to doxorubicin treatment (Fukunaga-Kalabis et al. 2010). Knowledge of TNC knockdown using siRNA technology is limited. However, previous findings suggest that knockdown of endogenous TNC using siRNA plays a crucial role in tumour invasion and metastases.

1.13 Background to the study

Studies in our group analysing expression of alternatively spliced isoforms of TNC showed that this may affect breast cancer progression. Adams et al. (2002) analysed high molecular weight TNC isoform expression in benign, pre-invasive and invasive breast lesions, and found exons 14 and 16 are associated with invasion (Adams et al. 2002). Furthermore, these isoforms were significantly associated with invasive and proliferative phenotypes (Hancox et al. 2009). High MW TNC isoforms including AD1 were then found to be significantly associated with aggressive features of breast carcinomas and *in-vitro* with an invasive phenotype (Guttery et al. 2010a).

1.14 Hypothesis

As specific TNC isoforms have been shown to influence breast cancer cell behaviour, knockdown of TNC isoform expression using siRNAs and subsequent effect on global gene expression will be analysed using cDNA microarray for mRNA and proteomics for protein alterations.

1.15 Aims and objectives

The aim of this study was to test the hypothesis that knockdown (by siRNA) of endogenously expressed TNC isoforms in breast cancer cell lines would affect global gene expression and modify tumour cell behaviour (invasion and proliferation). The specific objectives were:

1. To quantitatively analyse endogenous TNC mRNA and protein expression using RT-qPCR and Western blotting methods respectively, in order to obtain a profile of isoform expression in a number of breast carcinoma cell lines.
2. To generate a number of siRNA sequences to target exons present in all TNC transcripts and specific TNC isoforms for studies in breast cancer cell lines.
3. To transfect siRNA sequences against TNC isoforms into endogenously expressing breast adenocarcinoma cell lines.
4. To use cDNA microarrays and proteomics to compare differential gene expression and protein alterations; and to validate candidate genes using TaqMan RT-qPCR and Western blot methods in cell lines to address the following questions:

   A) Does knockdown of endogenous TNC expression differentially regulate specific genes associated with invasion, proliferation and migration?

   B) Which genes are specifically up-or down-regulated to the greatest extent by endogenous TNC knockdown?

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Cell culture

2.1.1.1 Reagents

Reagents and equipment used in tissue culture are listed in Table 2.1

Table 2.1 Details of tissue culture reagents and equipment

Reagents and equipment	Supplier
Dulbecco's modified Eagle's medium (DMEM)	Sigma-Aldrich, UK
Roswell Park Memorial Institute (RPMI)-1640	
L-Glutamine (200 mM)	
Trypsin/EDTA (TE)	Gibco, UK
Foetal bovine serum (FBS)	
Opti-MEM Reduced Serum Media (1-2% serum)	
Dulbecco's phosphate-buffered saline (DPBS)	Cambrex BioScience, USA
Lipofectamine 2000 transfection reagent	Invitrogen, UK
Tissue culture flasks (25 cm^2, 75 cm^2 and 150 cm^2)	BD Falcon, USA
Fluoroblok cell culture inserts	BD Biosciences, USA
24-well companion plates	
BD Matrigel matrix	
1, 1'-didodecyl-3, 3, 3', 3'- tetramethylindocarbocyanine perchlorate lipophilic fluorescent tracer dye (DiIC12 (3))	

2.1.1.2 Breast cancer cell lines

Breast cancer cell lines were originally collected from the American Type Tissue Culture Collection (ATCC, Rockville, MD., USA). Details of cell lines are in Table 2.2.

Table 2.2 Details of breast carcinoma cell lines used in this study

Cell line	TNC	ER	PR	Her2	E-Cad	Source
MDA-MB-231	+	-	-	Low	-	Human breast adenocarcinoma cell line obtained from a pleural effusion of a 51-year old Caucasian woman in 1973 (Cailleau et al. 1974)
MDA-MB-436	+	-	-	-	-	Human breast adenocarcinoma cell line obtained from a pleural effusion of a 43-year old Caucasian woman (Cailleau, Olive & Cruciger 1978)
MDA-MB-468	+	-	-	-	+	Human breast adenocarcinoma cell line obtained in 1977 from a 51-year old black woman with a pleural effusion (Cailleau, Olive & Cruciger 1978)
HBL-100	+	-	-	-	-	An immortalised epithelial cell line obtained from the milk of a 27-year old Caucasian nursing mother. Described as normal, however, contains a tandemly integrated SV40 virus genome (Gaffney 1982)
MCF-7	-	+	+	-	+	Human breast adenocarcinoma cell line derived from a 69-year old Caucasian woman in 1970 with a malignant pleural effusion secondary to breast carcinoma (Soule et al. 1973)
T-47D	-	+	+	Low	+	Human breast adenocarcinoma cell line derived in 1974 from a pleural effusion of a 54-year old woman with infiltrating ductal carcinoma (Keydar et al. 1979)

TNC= Tenascin C; ER = Oestrogen receptor; PR = Progesterone receptor; Her2= Human Epidermal Growth Factor Receptor 2; E-Cad= E-cadherin

2.1.2 Tissue samples

A total of 36 breast carcinoma tissues were obtained from the Department of Histopathology, University Hospitals of Leicester. All tissues were used under research ethics approval (06/Q2502/70). Details of the tissues are listed in Table 2.3.

Table 2.3 Clinicopathological features of breast carcinoma tissues

Clinicopathological Feature		Total
Tumour Type	**IDC**	31
	ILC	5
Tumour Grade	**I**	1
	II	18
	III	17
Patient Age	**≤ 40**	12
	> 40	24
ER Status	**ER^+**	25
	ER^-	7
	n/k	4
PR Status	**PR^+**	20
	PR^-	10
	n/k	6
Lymph Node Status	**LN^+**	19
	LN^-	16
	n/k	1

IDC = Invasive ductal carcinoma; ILC = Invasive lobular carcinoma;
ER = Oestrogen receptor; PR = Progesterone receptor; n/k = not known

2.1.3 RNA interference (RNAi)

All siRNA oligos were obtained from Sigma and Ambion, UK. Details of the siRNA oligos used in the study are in Table 2.4 below:

Table 2.4 Summary of siRNA used in the study

Target Name	Sense & Antisense Strands Sequences	Length	Region
Total TNC*	5'GGAAUAGAAUAAAGAAGAtt3' 5'UCUUCUUUAUUCAUAUUCCgg3'	21	Exon 3
Total TNC	5'CAGUUACAGAAUUAAGUAUUU3' 5'AUACUUAAUUCUGUAACUGUU 3'	21	Exon 7
Total TNC*	5'CGCGAGAACUUCUACCAAAtt3' 5'UUUGGUAGAAGUUCUCGCGtc3'	21	Exon 24
TNC- 14(I)	5'CCGAUGGGAUCUUCGAGACUU3' 5'AAGUCUCGAAGAUCCCAUCGG 3'	21	Exon 14
TNC- 14(II)	5'CCGAUGGGAUCUUCGAGACUU3' 5'AAGUCUCGAAGAUCCCAUCGG 3'	21	Exon 14
TNC- 14-16 (I)	5'AGCCACGACAGAAGCCGAAUU3' 5'UUCGGCUUCUGUCGUGGCUUU 3'	21	Exon 14-16
TNC- 14-16 (II)	5'CCACGACAGAAGCCGAACCUU3' 5'(Phos)GGUUCGGCUUCUGUCGUGGUU 3'	21	Exon 14-16
TNC-AD1 (I)	5'CCACAGUUGGGCACGCUAAUU3' 5'UUAGCGUGCCCAACUGUGGUU 3'	21	Exon AD1
TNC-AD1 (II)	5'ACGUCAGUGUGGCAGGAAC3(dT)(dT)' 5'(Phos)GUUCCUGCCACACUGACGU(dT)(dT) 3'	21	Exon AD1
TNC- 14-AD1(I)	5'UUGGGCACGCUAAUCUUUAUU3' 5'UAAAGAUUAGCGUGCCCAAUU 3'	21	Exon 14-AD1
TNC- 14-AD1(II)	5'CACGACAGAACCAAAGCCAUU3' 5'UGGCUUUGGUUCUGUCGUGUU 3'	21	Exon 14-AD1
Silencer Negative Control #1 siRNA *	Unknown	21	Unknown
Labelled Silencer negative control # 1 siRNA*	Unknown	21	Unknown

* siRNA commercially available ; other siRNA are custom designed

2.1.4 RT-PCR

All reagents and equipment used for RT-PCR are listed in Table 2.5. Primers and probes are listed in Table 2.6.

Table 2.5 Details of RT-PCR reagents and equipment used in the study

Reagents and equipment	Supplier
Total RNA Isolation (TRI) reagent	Sigma-Aldrich, UK
Qiagen RNeasy Mini Kit for RNA extraction	
Ethidium bromide	
Chloroform	Fisher Scientific, UK
Tris base , Acetate and EDTA (TAE)	
Magnetic Beads for Poly [A] + purification (Dynabeads Oligo (dT))	Invitrogen, UK
Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT)	Promega, USA
40 U/µL Ribonuclease Inhibitor (RNasin)	
10 mm 2´-deoxynucleoside 5´-triphosphates (dNTPs)	
10x Avian Myeloblastosis Virus (AMV) buffer	
TaqMan® Fast (2x) Universal PCR Master Mix	Applied Biosystems
MicroAmp 96-well plates	
TaqMan FAM-labelled probes assays	
SeaKem® LE Agarose gel	Cambrex BioScience, USA

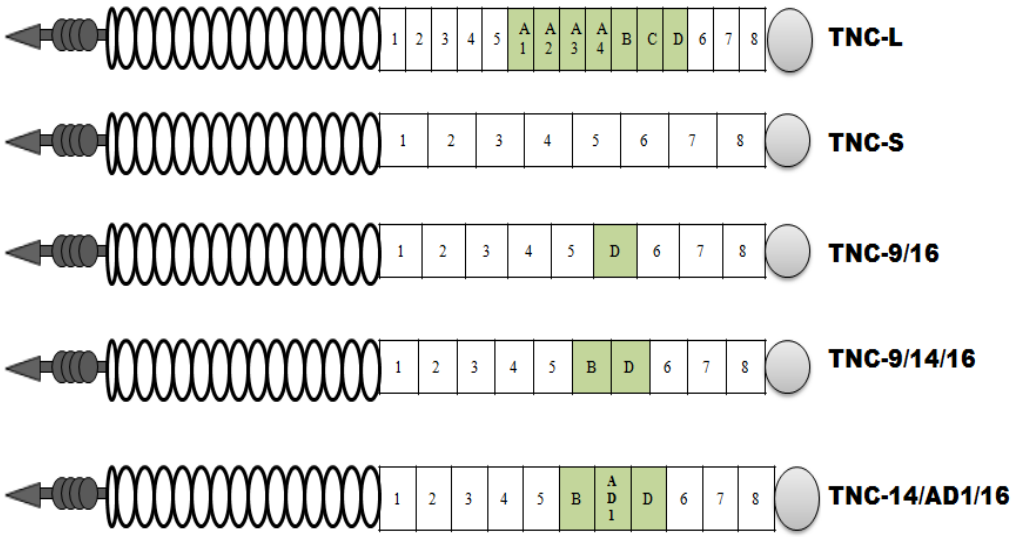
Table 2.6 Summary of primers and probes sequences

Primer	Sequence 5' → 3'	Length (nt)	Tm (ºC)	Annealing position (5' → 3')	Exon
qAD1 F	TGG TGG AGA ACA CTG GCT ATG AC	23	60.8	187 → 209	AD1
qAD1 R	GGG ATC CCC AGC CAA GGT	18	61.2	245 → 228	AD1
qAD1 Probe	CAG TGT GGC AGG AAC	15	70.1	212 → 226	AD1
qAD2 F	GAT CAC CCC CAT GAG ACC AT	20	58.6	121 → 140	AD2
qAD2 R	TGA TGA CAG AGC TGC GAG ACA	21	59.4	181 → 161	AD2
qAD2 Probe	TGC TGT CTG TGC CTG G	16	69.9	143 → 158	AD2
q14/16 F	TCC GGA CCA AAA CCA TCA GT	20	59.3	4679 → 4698	14
q14/16 R	TGA AAC CAG AAG GTT GTC AAC TTC	24	58.7	5022 → 4999	16
q14/16 Probe	ACG ACA GAA GCC GAA CC	17	70.3	4708 → 4714 and 4988 → 4997	14/16
q9/16 F	CAA GCC CGC ACA TGT GAA	18	59.6	3321 → 3338	9
q9/16 R	TGA AAC CAG AAG GTT GTC AAC TTC	24	58.9	5022 → 4999	16
q9/16 Probe	ATC CAC TGA AGC CGA AC	17	70.4	3342 → 3349 and 4988 → 4996	9/16
TNC-S F	GAGCAAGCCCGCACATG	17	58	3318 → 3334	9
TNC-S R	CCGAATTTTCAGTGATGTCTGAGA	24	59	5311 → 5288	17
TNC-S Probe	CATCCACTGCCATGGG	16	68	3341 → 3349 and 5261 → 5267	9/17
Total TNC	Applied Biosystems (Hs01115654_m1)	--	--	start 5380	17 → 18
HPRT1	Applied Biosystems (Hs99999909_m1)	--	--	start 648	6 → 7
CREBL2	Applied Biosystems (Hs00230923_m1)	--	--	start 634	3 → 4
YWHAE	Applied Biosystems (Hs00356749_g1)	--	--	start 415	2→ 3
CDC14B	Applied Biosystems (Hs00372920_m1)	--	--	start 1340	12→ 13
RRAS2	Applied Biosystems (Hs00273367_m1)	--	--	start 416	1→ 2
DISC1	Applied Biosystems (Hs00257791_s1)	--	--	start 707	1
QKI	Applied Biosystems (Hs03655538_s1)	--	--	start 2143	1

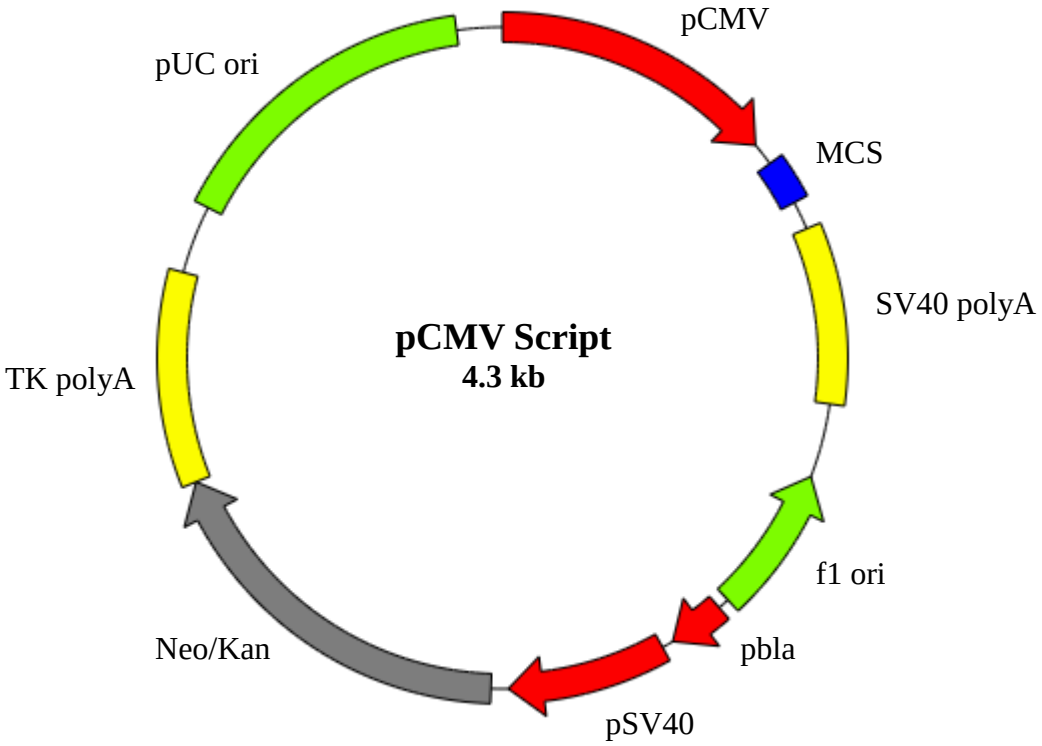
HPRT1 – hypoxanthine guanine phosphoribosyltransferase 1; **CREBL2** - cAMP responsive element binding protein-like 2; **YWHAE** - tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide; **CDC14B** - cell division cycle 14 homolog B ; **RRAS2** - related RAS viral (r-ras) oncogene homolog 2 ; **DISC1** - disrupted in schizophrenia 1 ; **QKI** - quaking homolog, KH domain RNA binding

Figure 2.1: A) Schematic representing the human TNC isoforms used in this study. All TNC isoforms were previously generated within our group. B) The pCMV Script mammalian expression vector used to carry TNC isoforms showing the multiple cloning site (MCS) under CMV promoter control and the kanamycin resistance gene (Kan), which was used for selection of successfully transformed *E. coli* cells (Guttery, 2009).

A)



B)



2.1.5 Immuno-staining

All reagents and equipment used for Western blot (WB), immunocytochemistry (ICC) and immunohistochemistry (IHC) are listed in Table 2.7. All antibodies used in these are listed also in Table 2.8.

Table 2.7 Details of immune-staining reagents and equipment used in the study

Method	Reagents and equipment	Supplier
WB	Hybond nitrocellulose membranes	Amersham Biosciences UK
	Enhanced Chemiluminescence	
	Bio-Rad coloured protein marker	BioRad Laboratories, USA
	Tween-20	Sigma-Aldrich, UK
	Protease inhibitor	
	30% acrylamide/bis-acrylamide	
	Ammonium persulphate	
	N, N, N', N'-tetramethylethylenediamine(TEMED)	
	β-mercaptoethanol	
	Methanol	Fisher scientific, UK
ICC/IHC	Novolink^{TM} Kit Polymer Detection System kit	Leica (Microsystem),UK
	Shandon Cytoblock kit	Thermo Scientific , UK
	Albumin Bovine Fraction	MP Biomedical, Germany
	Proteinase K (PK)	Roche Applied Science , UK

Table 2.8 List of antibodies used in immuno-staining

Antibody		Description	Host	Detection method	Dilution	Supplier
Primary	clone BC-24	TNC monoclonal antibody	Mouse	IHC/ICC	1:4000	Sigma-Aldrich, UK
	hVIN1	Vinculin monoclonal antibody	Mouse	WB	1:5000	
	clone H300	TNC polyclonal antibody	Rabbit	WB	1:1000	Santa Cruz, USA
	TSP-1 (A6.1)	Thrombospondin 1 monoclonal antibody	Mouse	IHC/ICC	1:1000	
	pHH3	Phospho-Histone 3 polyclonal antibodies	Rabbit	ICC	1:1000	Cell Signalling, UK
Secondary	Anti-rabbit IgG	Horseradish peroxidise (HRP)-linked whole antibodies	Donkey	WB	1:2000	GE Health Care Limited, UK
	Anti-mouse Ig	Horseradish peroxidise (HRP)-linked whole antibodies	Sheep	WB	1:2000	
	Anti-mouse/rabbit IgG	Anti-mouse/rabbit IgG-Poly-HRP, NovoLink™ Polymer reagent	mouse/ rabbit	IHC/ICC	-	Leica (Microsystems), UK

2.1.6 Proteomics

All reagents and equipment used for proteomics are listed in Table 2.9

Table 2.9 Details of proteomics reagents and equipment used in the study

Reagents and equipment	Supplier
Formic acid (FA)	Fisher Scientific, UK
Internal standard	Waters, UK
Ammonium Bicarbonate (ABC)	Sigma-Aldrich, UK
DL-Dithiothreitol (DTT)	
Iodoacetamide(IAA)	
HPLC water	

2.1.7 Reagents prepared for the study

Other reagents were made in house and used in this study are listed in Table 2.10

Table 2.10 Reagents made in-house

Gel electrophoresis	ICC/IHC	Western blot
Gel Loading Buffer (PCR) • 50% Glycerol • 0.21% Bromophenol Blue • 0.21% Xylene Cyanol • 200 mM EDTA (pH 8.0) • 170mL Pure Water **TAE Buffer** • 40 mM Tris-acetate • 2 mM EDTA	**Washing buffer (TBS)** • 10 mM Tris-HCL (pH 7.5) • 0.15 M Lithium Chloride • 1 mM EDTA (pH 8.0) **Blocking Solution** • 3% BSA (Bovine Serum Albumin) • 0.01% Triton • TBS	**Gold Lysis Buffer** • 1% Triton X-100 • 30 mM Tris (PH=8.0) • 137 mM Sodium Chloride (NaCl) • 15% Glycerol • 5mM EDTA **Running Buffer** • 25 mM Tris Base • 190 mM Glycine • 0.1% SDS **Transfer Buffer** • 25 mM Tris • 190 mM Glycine • 0.1% SDS (0.01%) • 20% Methanol **4x Loading Buffer** • 240 mM Tris HCL (pH=8.6) • 20% Mercaptoethanol • 40% Glycerol • 8% SDS • 0.2% Bromophenol Blue **Washing Buffer (TBS-T)** • 150 mM NaCl • 10 mM Tris-HCl (pH 7.5) • 0.1% Tween 20 **Blocking Solution** • 5% Marvel Milk powder • TBS-T

2.2 Methods

2.2.1 Cell culture

A cell line vial was taken from liquid nitrogen and thawed at room temperature for 1 minute. The cells were immediately transferred to a water bath at 37° C and thawed fully. In the laminar flow hood, 10% FCS and 2 mM L-glutamine were added to complete medium and cells were re-suspended in 10 ml of this. The cells were centrifuged at 1000 rpm for 5 mins at room temperature, and the resultant pellet was re-suspended in 10 ml of complete medium. A cell count was done and adequate numbers (3×10^5 - 5×10^5) of cells were seeded into a 25 cm^2 culture flask. The cells were incubated at 37º C and 5% CO_2 until passage at approximately 70-80% confluence.

2.2.1.1 Routine passaging of cells

Cells were routinely passaged in complete medium at 37° C in the presence of 5% CO_2. Cells were washed with DPBS 3 times when they were about 70-80% confluent. The cells were detached from the culture flask surface by the addition of 1x Trypsin/EDTA and incubating at 37° C for 5 mins. The flasks were gently tapped to augment detachment of cells. The action of Trypsin was then inhibited by the addition of 1 equal volume of pre-warmed complete medium. The cells were transferred to a new 15 ml Falcon tube and centrifuged at 1000 rpm for 5 mins. After centrifugation, the pellet was re-suspended in a complete medium and counted using a Neubauer haemocytometer. The cell concentration was calculated using the following formula:

(Total cell number = ((counted cell/4) x 10^4) x volume cells re-suspended in)

Having calculated the cell concentration, the required cell density was then seeded into a culture flask and maintained at 37°C in the presence of 5% CO_2.

2.2.2 siRNA design

SiRNAs were designed using the Dharmacon online RNAi design centre (http://dharmacon.com/designcenter/designcenterpage.aspx). In order to identify highly efficient and specific knockdown of TNC, several sequences targeting total TNC and TNC specific isoforms were uploaded into the Dharmacon online RNAi design centre. The siRNA sequences were chosen according to recommended parameters, such as G/C percentage and length of siRNA. The commonly recommended GC content for siRNA is between 30-64% (Birmingham et al. 2007; Amarzguioui et al. 2006). The length of siRNA is recommended to be 21-23 bases, allowing for a 19- to 21-base duplex region designed with two-base overhangs at each 3' end (Tilesi et al. 2009; Pei, Tuschl 2006).

2.2.2.1 Cell transfection by siRNA

Small interfering RNA was diluted in Opti-MEM to a concentration of 0.048 μg/μl and vortexed briefly. 5 μl of Lipofectamine 2000 transfection reagent was diluted in 250 μl Opti-MEM which then added into diluted siRNA. This was gently mixed by flicking the tube and incubated at room temperature for 20 mins. Cells were trypsinised, centrifuged at 1000 rpm for 5 mins and re-suspended in complete DMEM. An aliquot of cells were counted using a haemocytometer and the concentration of $2x10^5$ cells seeded into 6 well plates. The transfection complex was then mixed with cells and incubated at 37 °C/5% CO_2. 24 hrs post-transfection, cells were then washed three times in DPBS and incubated at 37 °C/5% CO_2 for 24 hrs in a complete media for RNA collection and for 48 hrs in Opti-MEM media for protein collection.

2.2.3 Total RNA extraction

Cell pellets were re-suspended in 1ml Tri Reagent. Samples were thawed, vortexed and left at room temperature for 5 mins. 200μl chloroform was added to the sample and left at room temperature for 3 mins. Cells were centrifuged at 13000 rpm for 15 mins at

4°C. The aqueous phase was transferred into a new Eppendorf. After measuring the volume of the aqueous phase, 1.25x absolute ethanol was added and vortexed. Total RNA was then isolated using the Qiagen RNeasy mini kit. According to manufacturer's instructions, the samples were applied to the silica base spin column and RNA was eluted using RNase-free water following a series of washes.

2.2.4 Nucleic acids quantification

A NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA) was used to determine the concentration of DNA and RNA in the aqueous solution. About 1.2 µl of DNA/RNA was loaded onto the pedestal and the absorbance measured (OD260 1 = 50 µg/ml dsDNA or 40 µg/ml RNA). By comparing the absorbance value at 260 and 280 nm, the purity was also measured.

2.2.5 Agarose gel electrophoresis

In order to analyse RNA quality, RNA was separated by agarose gel electrophoresis using 1% SeaKem® agarose in TAE buffer containing 2.5-5 µg/ml ethidium bromide. RNA was mixed with 3 µl 10x loading buffer. Electrophoresis was performed for 1 hour at 50 V. The samples were visualised using an ultra-violet light trans-illuminator after gel electrophoresis. An image of the gel was obtained using the program Alpha (Alpha computer systems).

2.2.6 Messenger RNA isolation using oligo-dT-linked Dynabeads

Messenger RNA (mRNA) extraction and processing was performed using oligo-dT-linked Dynabeads (Dynal). Briefly, RNA was placed at 65° C for 2 mins, left to cool at RT for 5 min and then incubated with 30 µl of Dynabeads mixed with 50 µl of binding buffer for 5 mins and placed in the magnetic rack. The supernatant was removed and washed twice with washing buffer and then re-suspended with 10 µl ultra-pure H_2O following the manufacturer's protocol.

2.2.7 Generation of cDNA by reverse transcription (RT)

Complementary DNA was synthesised from total RNA using AMV-Reverse transcription (AMV–RT). The reaction conditions are given in Table 2.11. The samples were incubated at 42° C for 60 mins in the GeneAmp 9700 96-well thermal cycler. Resulting cDNA was stored at 4° C until required.

Table 2.11 Summary of RT reaction conditions

Solutions	Volume (µl)
mRNA	10
5x AMV buffer	5
10 mm (dNTP)	2.5
AMV-RT	0.5
RNasin 40U/ µl	0.62
H_2O	6.38
Total	25

2.2.8 Quantitative RT- PCR

2.2.8.1 Primer design

Following the Applied Biosystems guidelines, all oligonucleotide primers were designed previously by our group except TNC short. Custom primers and FAM-labelled MGB probes were designed using the Primer Express software program (Applied Biosystems, UK). Primer and probe sequences were tested using the NCBI Nucleotide Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.gov/BLAST/). Lyophilised pellets were re-suspended in sterile UP H_2O to a concentration of 200 pm/µl (200 µM). An aliquot was then taken and diluted in sterile UP H_2O to a working concentration of 10 pm/µl (10 µM).

2.2.8.2 RT-qPCR reaction

The reactions were set up on ice and performed in triplicate on ABI 7500 Fast and Step-One thermal cycler. Before cycling, plates were centrifuged at 3000 rpm for 30 seconds. The custom-designed and commercial Applied Biosystems probes' reaction components are given in Table 2.12, and the reaction cycling steps are given in Figure 2.2.

Table 2.12 Summary of RT-qPCR reaction condition

Solutions	Volume (µl)	
	Applied Biosystems probes	Designed Probes
cDNA (1:10 dilutions)	4	3.6
2x TaqMan fast universal mastermix	5	5
FAM-MGB Probe	0.5	0.2
Forward primer	-	0.6
Reverse primer	-	0.6
H_2O	0.5	-
Total	10	10

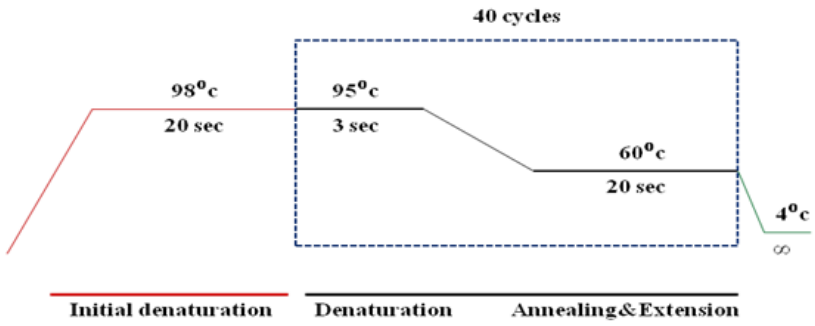


Figure 2.2: RT-qPCR reaction cycling conditions

2.2.8.3 Standard curves

Real time qPCR assay efficiencies were initially determined by standard curves. Standard curves were generated for all probes and primers. Briefly, a 1:5 dilution series of cell line cDNA was set up for each probe set and the efficiency calculated from the gradient of the slope. The standard curves for TNC primers and probes were previously produced by Dr. David Guttery for each primer/probe set using TNC isoform clone

templates and the slope of the standard curve was then utilised to calculate the number of molecules within each sample.

2.2.8.4 Calculation of number of molecules

Calculation of the number of molecules was performed previously for all TNC isoforms except TNC short (Guttery et al. 2010a). Briefly, based on the values obtained from the standard curves, the log number of molecules in each sample was calculated by:

$$x = \frac{m - c}{y}$$

Where: x = log number of molecules; m = cycle threshold; c = intercept of the graph; and y = slope of the graph.

The log value obtained was then used to calculate the number of molecules using the equation:

$$n = 2^x$$

Where: n = number of molecules and x = log number of molecules.

The value obtained was then normalised to the housekeeping gene to give the true number of molecules within each sample using the following equations:

$$\Delta Ct\ EC = Average\ Ct\ sample\ EC - Average\ Ct\ Control\ EC$$

$$RE = 2^{(-\Delta Ct\ EC)}$$

$$NE = n\ x\ RE$$

Where: ΔCt *EC* = difference in average Ct between EC of the sample versus EC in the control sample; *EC* = endogenous control; *NE* = normalised expression; *n* = number of molecules; *RE* = relative expression.

2.2.9 Western blotting

2.2.9.1 Sample Preparation

2.2.9.1.1 Cell lysates

Cells were harvested using trypsin-EDTA. After inhibition of trypsin activity with complete medium, cells were centrifuged at 1,000 rpm for 5 mins. Supernatant was discarded and the cell pellet was washed in PBS. The cell pellet was then re-suspended in 350 µl of gold lysis buffer and 35µl protease inhibitor, mixed thoroughly, and placed on ice for 10 mins. To ensure lysis, cells were passed through a 23 gauge needle 5 times. The lysed cells were then centrifuged at 13,000 rpm for 3 mins to remove cell debris and the resulting supernatant transferred to a fresh sterile eppendorf. Samples were stored at -20º C until subsequent analyses.

2.2.9.1.2 Conditioned media

Cell conditioned media (CM) was collected for analysis of secreted protein after incubation with Opti-MEM media for 48 hrs and centrifuged at 1000 rpm for 5 mins. 2 ml of CM was placed into the top of a Centricon column and then centrifuged at 4000 rpm in a bench top centrifuge (Jouan) for 30 mins at 4° C. The column was inverted into a clean collection tube and stored at -20° C until required.

2.2.9.2 Determination of protein concentration

Bio-Rad protein assay (Bradford assay) (Bradford 1976) was used to measure the protein concentration. Briefly, the mixture contained 795 µl sterile H_2O, 5 µl of sample and 200 µl of protein assay reagent (diluted 1:5 in sterile H_2O) and was allowed to develop in the dark for 10 mins. The absorbance was measured at 595 nm. Gold Lysis Buffer was used as a blank for cell lysates and the depleted media was used for CM.

Protein concentration was then calculated from the standard curve by measuring the absorbance against known protein concentrations for a series of BSA standards.

2.2.9.3 Gel preparation

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the separation of proteins using 6% resolving and 5% stacking gels. Acrylamide solution, Tris-HCl, SDS and water were mixed before polymerisation was initiated by addition of 10% ammonium persulphate (APS) and *N, N, N', N'*-tetramethylethylenediamine (TEMED). The resolving gel was pipetted into a cassette and then 1 ml of IMS layered over to form a seal and avoid the formation of air bubbles and evaporation. After 45 mins at room temperature the IMS was removed and the gel rinsed with sterile water. The stacking gel was loaded and the gel comb inserted and left for 45 mins at room temperature. Gels were wrapped in damp paper towels and Saran wrap, and then stored at 4 °C until required. A summary of gel preparation is shown in (Table 2.13).

Table 2.13 Summary of gel preparation

Solution	6% resolving gel (ml)	5% stacking gel (ml)
Sterile H_2O	5.40	2.85
30% acrylamide/bis acrylamide solution	2.00	0.85
Gel buffer (1.5M Tris-HCl, pH 8.8)	2.50	-
Gel buffer (0.5M Tris-HCl, pH 6.8)	-	1.25
10% w/v SDS	0.10	0.05
10% APS	0.05	0.025
TEMED	0.005	0.005

2.2.9.4 Loading protein to the gel

Five µg of total protein per sample was added to an Eppendorf. For conditioned media, an equal volume of Gold Lysis Buffer was added to each sample, and 3-5 µl Western loading buffer added to all samples. Samples were denatured at 99 °C for 5 mins, incubated on ice and loaded into each well. 20 µl of colour protein marker was also loaded into a separate well. The gel was run in running buffer at 100 V until the sample front had run the required distance.

2.2.9.5 Protein transfer

The gel was removed and washed in transfer buffer for 10 min. One piece of nitrocellulose paper and two pieces of Whatmann 3 mm filter paper (equal in size to the gel) were soaked in transfer buffer. The gel apparatus was transferred into a cassette and the protein electrophoresed in transfer buffer onto nitrocellulose membrane at 100 V for 1 hr at 4° C (Figure 2.3).

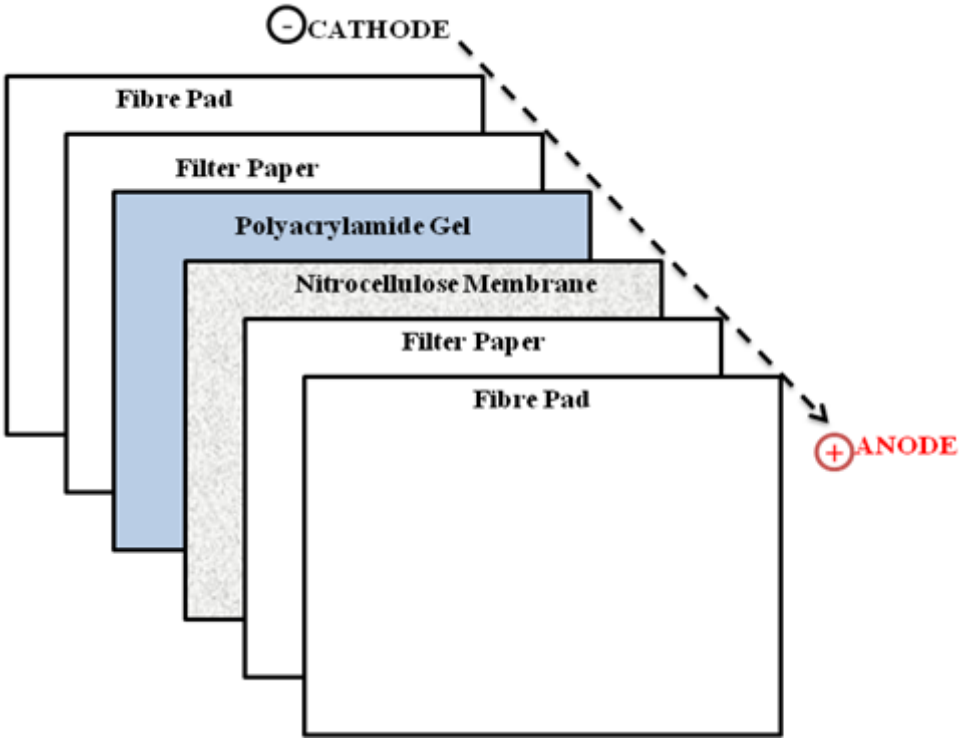


Figure 2.3: Schematic overview of Western blotting protein transfer system.

2.2.9.6 Western blot analysis

The nitrocellulose membrane was removed from the cassette and then washed in TBS-T, as follows: the membrane was first blocked by incubation in blocking solution for 1 hr at room temperature. Membranes were then probed with primary antibody in TBS-T overnight at 4 °C. Afterward, membranes were washed 3 times in TBS-T for 5 min at room temperature and incubated for 1 hr in TBS-T containing secondary antibody. An ECL kit was used for detection of protein according to manufacturer's instructions and visualised using X-ray film (Xerox, USA) with a variety of exposure times. (Figure 2.4)

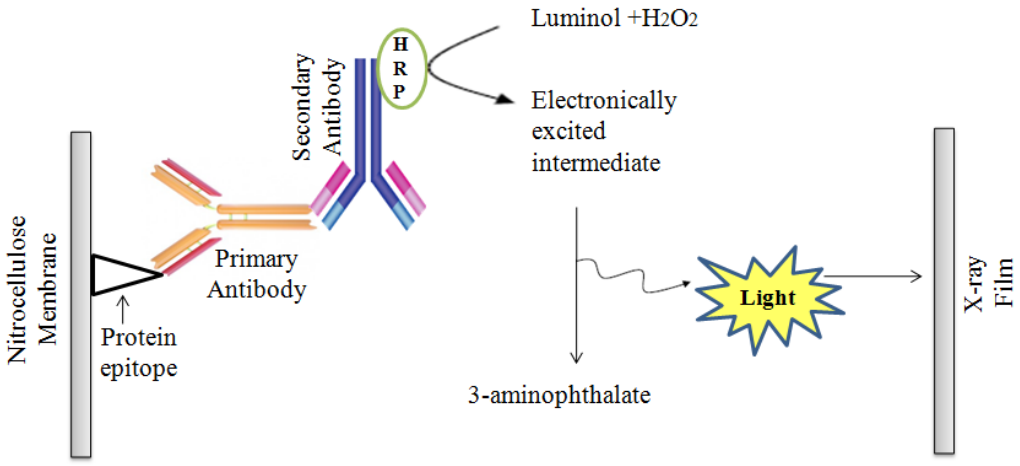


Figure 2.4: Schematic of the luminal ECL reaction. The haem-containing proteins, particularly horseradish peroxidises are able to catalyse the chemiluminescent reaction of luminol in the presence of hydrogen peroxide to produce nitrogen plus light (Marquette, Blum 2006).

2.2.10 Immunohistochemistry (IHC) / Immunocytochemistry (ICC)

2.2.10.1 Sample preparation

Cells transfected with siRNA were trypsinised at 72 hrs post-transfection and centrifuged at 1000 rpm for 5 mins. The cell pellets were fixed in 10% formalin for 3 hrs and then placed into a cytoblock cassette that was then centrifuged at 1500 rpm at low acceleration for 5 mins. The cassette was immersed in 70% ethanol until processing. Paraffin processing and embedding tissues in paraffin blocks were then

carried out by technical staff. Sections of formalin-fixed, paraffin-embedded (FFPE) cells and tissues were cut and mounted onto glass slides and left to dry at 37 °C overnight. Paraffin was melted at 65 °C in an incubator for 10 mins and then deparaffinised twice in xylene. Sections were then rehydrated in 99% and 95% IMS for 3 mins, with each followed by a subsequent wash in running tap water for 5 mins.

2.2.10.2 Optimisation of antigen retrieval

Two antigen retrieval methods were carried out in order to achieve the optimal antigen retrieval for IHC staining. These methods were heat-induced epitope retrieval and enzymatic retrieval.

2.2.10.2.1 Heat-induced epitope retrieval

Heat-induced antigen retrieval was performed using microwave. Briefly, slides were immersed in 1x 10mM citrate buffer (pH 6.0) and then microwaving at 750 watt for 20 mins (Tecnolec® Superwave 750). Slides were cooled down at room temperature and then washed in ultra-pure (UP) water.

2.2.10.2.2 Enzymatic retrieval

Enzymatic retrieval was performed using proteinase K (PK). Briefly, sections were covered with PK at a concentration of 1μg/ml for cyto-blocks and 7μg/ml for tissue-blocks, and then incubated at 37 °C for 1 hr. Slides were washed and rinsed with tap water.

2.2.10.3 IHC staining

Immuno-staining was performed via enzymatic antigen retrieval using PK followed by NovoLink™ Polymer Detection System. Briefly, peroxidase block was added into slide and incubated for 5 mins in order to inhibit the activity of endogenous peroxidase. Slides were then washed in TBS twice for 5 mins each wash. Protein block was then

added and incubated for 5 mins in order to reduce non-specific binding to the primary antibody. After washing slides in TBS twice for 5 mins, samples were incubated with primary antibody diluted in 3% BSA blocking solution overnight at 4°C. After overnight incubation, slides were again washed twice in TBS for 5 mins. Post-primary block was added and incubated for 30 mins in order to enhance the penetration of 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate buffer, and then slides were washed twice in TBS for 5 mins. NovoLink™ polymer (Anti-mouse/rabbit IgG-Poly-HRP reagent) was added to slides and incubated for 30 mins. Slides were washed in TBS twice for 5 mins. Peroxidise activity was developed with 100 µl DAB working solution (5 µl of DAB chromogen to 100 µl NovoLink™ DAB substrate buffer) and incubated for 5 mins. Slides were rinsed with tap water and stained with Mayer's Haematoxylin for 30 seconds. Sections were dehydrated with 95%, 99%, and 99% IMS for 3 mins each followed by xylene twice for 3 mins each. Finally, slides were demounted in aqueous mountant.

2.2.10.4 Assessment of IHC staining

Immunohistochemistry staining results were assessed using a Leitz Dialux® light microscope at different levels of magnification (10x, 20x and 40x). The expression of TNC-AD1 was evaluated according to the staining intensities of the localised AD1 protein in the cytoplasm of the malignant epithelial cells (Guttery et al. 2010a), and in the ECM; whereas, thrombospondon-1 (TSP-1) expression was evaluated according to staining intensities in the stroma of the tumour (Iochim et al., 2012). The evaluation of IHC staining was performed under the supervision of Dr. JH Pringle.

2.2.11 Proliferation associated marker

MDA-MB-231 cells transfected with siRNA targeting total TNC and TNC specific isoforms were cytospun at a cell density of 2 x 10^4 cells. The cells were fixed with 10% formalin for 10 mins. Immunocytochemistry was carried out using an antibody against Phospho-Histone H3 (pHH-3) as an immunomarker specific for cells undergoing mitosis (Veras et al. 2009). The analysis of pHH-3 staining was performed using the ImmunoRatio image analysis application, which calculates the percentage of positive staining (Tuominen et al. 2010). An example of ImmunoRatio is show in Figure 2.5.

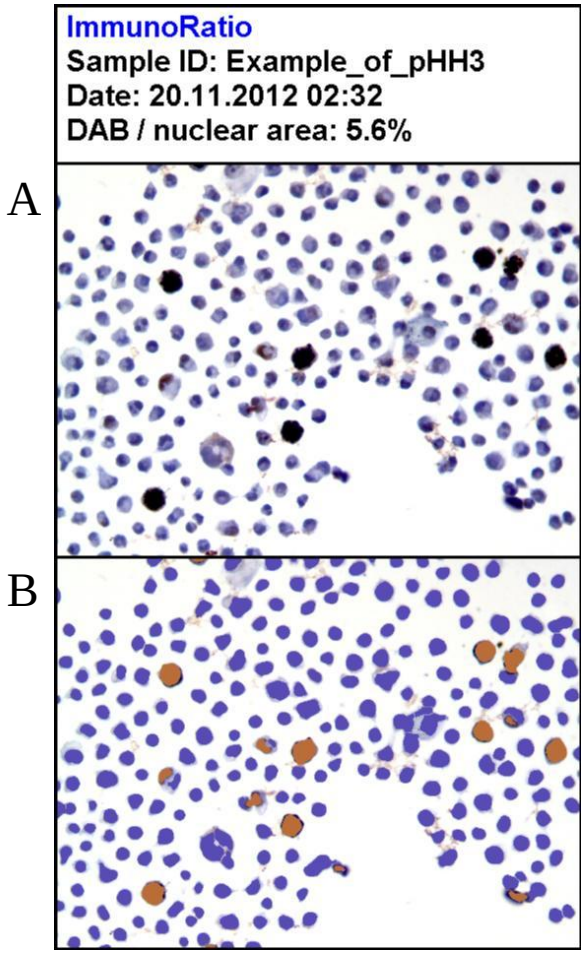


Figure 2.5: An example of pHH-3 stained image analysed with ImmunoRatio. The percentage of positively stained areas is shown in brown colour. A) The original image. B) Resulted image with staining components.

2.2.12 Invasion assays

The cell culture inserts (FluoroBlok 8 µm pore) were coated with 200 µl of Matrigel (1:100 dilutions in a cold Opti-MEM) and incubated at 37 °C/5% CO_2 for 2 hrs. Post-incubation, excess Matrigel was removed. Cells were stained with lipophilic fluorescent tracer dye 1,1'-didodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiIC12(3)) in complete DMEM at a concentration of 10 µg/ml and incubated for 1 hour at 37 °C/5% CO_2. Cells were washed 3 times in DPBS and incubated for 1 hour in complete DMEM. Cells were then harvested and counted. An aliquot of fluorescently-stained cells were seeded into the insert in 200 µl complete DMEM. 700 µl of complete DMEM was then placed into each insert. Cells were incubated for 4 hr at 37 °C/5% CO_2 and the media in the insert was replaced with 200 µl Opti-MEM (Guttery et al. 2010a). Cells were cultured for 48 hr at 37 °C/5% CO_2 in a FLUOstar OPTIMA plate reader (BMG Labtech, UK) with measurements taken every 2 hrs (Figure 2.6).

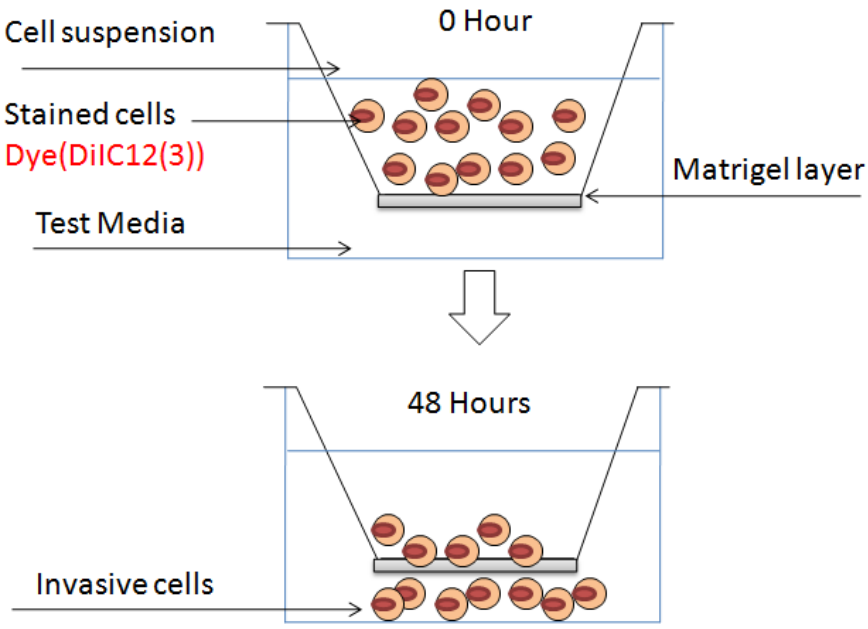


Figure 2.6: Schematic of cell invasion assay. The cell suspension of the transfected cells is placed in the top of the insert. The test media containing specific chemoattractants (5% FCS) is placed in the bottom chamber. Cell migration from the coated layer with Matrigel is counted using FLUOstar OPTIMA plate reader every 2 hrs.

2.2.13 Microarray studies

2.2.13.1 Analysis of RNA integrity

Gel electrophoresis and BioAnalyser (Almac Diagnostics, UK) were used in order to analyse the RNA integrity of the extracted RNA from the transfected cells with siRNAs. 0.5 µg RNA was mixed with 3 µl 10x loading buffer and loaded into 1% agarose gel. Gels were run for 1 hour at 50 V. Samples were visualised using an ultra-violet light trans-illuminator. In addition, the concentration and purity of the samples were also checked at the absorbance of 260 and 280 nm, and their A260/A280 ratios were between 1.68 and 2.08.

2.2.13.2 Microarray experiment

A total of 12 RNA samples were hybridised to Affymetrix Human U133 2.0 Plus arrays (service provided by Almac Diagnostics, UK). 1 µg of high quality total RNA from each sample was provided to Almac Diagnostics. Briefly, RNA was converted into double-stranded cDNA, which was then used as a template for the preparation of biotin-labelled cRNA. During in-vitro transcription, biotin-labelling and fragmentation were carried out to produce labelled cRNA. The fragmented cRNA of 12 samples was hybridised to Affymetrix Human U133 2.0 Plus array and analysed using an Affymetrix GeneChip® Scanner to produce a raw probe intensity CEL file. A general schematic of the microarray technology's labelling and hybridisation protocol is shown in Figure 2.7.

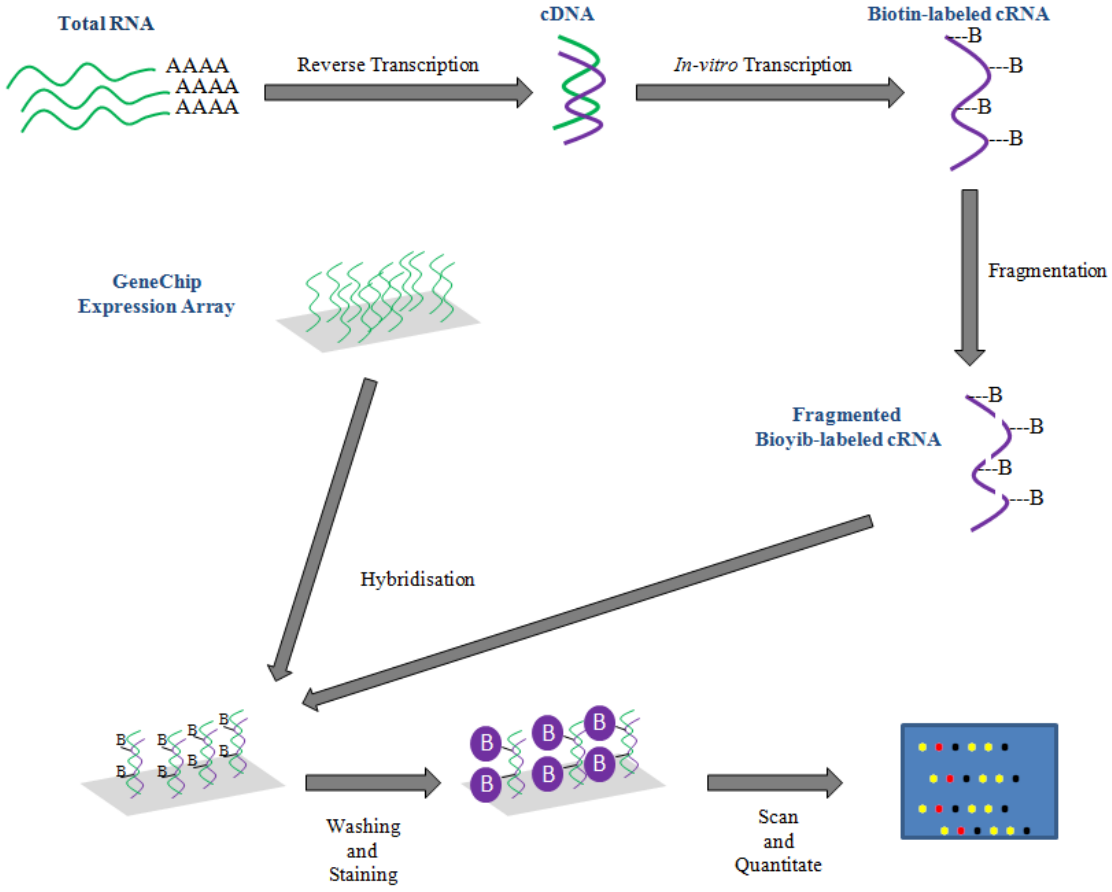


Figure 2.7: Schematic for Affymetrix GeneChip® arrays protocol (Taken and modified from www.affymetrix.com).

2.2.13.3 Microarray data analysis

2.2.13.3.1 Normalisation and filtration

The raw data (CEL files) were normalised and analysed using Partek® Genomics Suite™ 6.5, build 6.10.1129 (Partek Inc, USA, http://www.partek.com/). Raw data was normalised in order to remove artefactual data and to reduce any effects from both biological and non-biological sources (LaFramboise 2009). Prior to background correction, raw probe signal intensity values were pre-adjusted for target GC content and probe sequence. Background correction using the robust multiarray average (RMA) method (Bolstad et al. 2003) including quantile normalisation and median polish probe set summarisation was then performed. The effects of normalisation were then observed

through the comparison of un-normalised and normalised data using MA plots and Box-Plots. Following normalisation, the filtering of array data to deduce markers of significant difference was carried out according to a significance of $p \leq 0.05$ with false discovery rate (FDR) and thresholded with fold change ≥2. Array data analysis was carried out in collaboration with Dr Kevin Blighe, Cancer Studies and Molecular Medicine, Leicester University.

2.2.13.3.2 Analysis of gene expression

To identify the top lists of differentially expressed genes, the normalised and filtered data set was further analysed to deduce the most highly significant up- and down-regulated genes, and also to determine sample relationships based through hierarchical cluster (HCL). In addition, the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov) was used for pathway analysis (Dennis et al. 2003; Huang, Sherman & Lempicki 2009). To determine the number of overlapping genes between cell-line, time-point, and siRNA treatment, Venn diagrams were employed.

2.2.14 Proteomics

2.2.14.1 Sample preparation

Cell conditioned media (CM) of the cells transfected with TNC siRNAs was collected for the proteomic analysis. 2 ml of CM was placed into the top of a Centricon column and centrifuged at 4000 rpm in the bench top centrifuge (Jouan) for 30 mins at 4° C. The resultant protein solution (50 µl) was desalted by mixing with 2 ml of 250mM ammonium bicarbonate (ABC) and centrifuged using the same filter column at 4000 rpm for 30 mins at 4° C. This step was performed twice in order to make sure that the CM was desalted. The protein concentration was then measured using the Bio-Rad

protein assay (Bradford assay) (section 2.2.9.2). A digestion step was then performed. Briefly, 5 µl of 5mM DL-Dithiothreitol (DTT) was added into 100 µl of the sample and incubated for 30 mins at 37° C. 5 µl of 10mM iodoacetamide (IAA) was added into the sample and incubated for 15 mins in the dark at room temperature. 2 µl of 1µg/µl trypsin was added into the sample and incubated at 37° C overnight. The digestion was stopped by the addition of 1% formic acid (FA) into the sample. The samples were concentrated using a speed-vac to dry the sample supernatant and the resultant pellets were re-suspended in 15 µl 0.1% FA. Samples were stored at -80° C until required.

2.2.14.2 Protein quantification

In order to quantify the samples, the internal standard Alcohol Dehydrogenase (ADH) at a concentration of (50 fm/µl) was mixed with each sample in mass spectrometry vials at 1:1 ratio. Protein quantification was carried out by Mr Amirmansoor Hakimi in collaboration with Dr DJL Jones. Samples were analysed on LC-MS based on 110 mins run time.

2.2.14.3 Proteomics data analysis

The raw data obtained from LC-MS was analysed using the Protein Lynx Global server (PLGS) program version 2.3 for database searching. Raw data were calculated based on the amount of protein in (ng/µl), and normalised to the amount of (ADH) followed by a proteins online database search. The resultant data were filtered according to the exact match of proteins, common proteins in all replicates and statistical difference. The resultant proteins were exported into Microsoft Excel for subsequent analysis. The data were analysed and presented based on fold changes in expression relative to the control, and to the statistical significance (p values ≤ 0.05) between the siRNA treatments. Figure 2.8 represents the steps of proteomics data analysis.

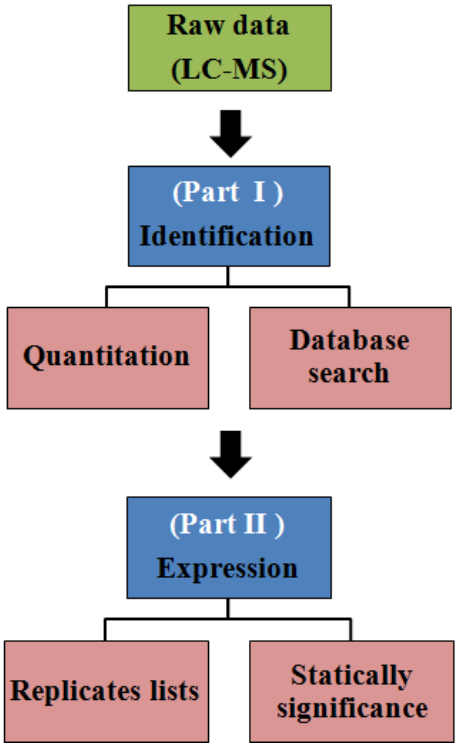


Figure 2.8: Schematic for proteomics data analysis using PLGS (v2.3) programme. Replicates data for each siRNA treatments were subjected into two steps of analysis (Identification and expression levels of regulated proteins in each sample).

2.2.15 Generation of TNC-AD1 antibody

In order to produce an antibody against TNC isoforms containing AD1 (TNC-AD1), four peptides corresponding to different regions within TNC-AD1 exon were chemically synthesised. The synthesised peptides were fused to an N-terminal with a maltose binding protein (MBP) tag, conjugated to keyhole limpet haemocyanin (KLH) and immunised in rabbits. These services were carried out by Genosphere Biotechnologies, France, and Euro Genetec, Belgium. Sera was taken from pre-immune rabbits and screened for reactivity against TNC-AD1 by Western blot analysis using the MDA-MB-231 and HBL-100 cell lines, which both express high levels of TNC-AD1, as described in section 2.2.9. The optimisation of the generated antibodies was performed on both the cell line models and the breast carcinoma tissues.

2.2.16 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 for Windows (GraphPad Software, Inc., USA). All assays used to determine the effects of siRNA employed a Two-Way Analysis of Variance (Two-way ANOVA) and Post-hoc Tukey's test in order to test the significant variance between cells transfected with TNC siRNAs compared to cells transfected to scrambled siRNA. Immunohistochemistry analysis for TNC-AD1 and TSP-1 expression employed a Fisher's Exact Test and Mann-Whitney Test in order to test the correlation between TNC mRNA profiles and the staining of TNC-AD1 and TSP-1.

Chapter 3. Analysis of TNC isoform knockdown in MDA-MB-231

3.1 Introduction

Previous studies in our group have shown that high molecular weight (MW) TNC isoforms (TNC-16, TNC-14/16 and TNC-14/AD1/16) are associated with invasion and proliferation in breast cancer (Adams et al. 2002; Hancox et al. 2009 ; Guttery et al. 2010a). Furthermore, TNC knockdown decreased the side population (SP) fraction in melanoma spheres and lowered their resistance to doxorubicin treatment (Fukunaga-Kalabis et al. 2010). Knockdown of endogenous TNC has also been shown to decrease glioblastoma cell invasiveness and reactive change of peritumoral brain tissue (Hirata et al. 2009). However, targeted silencing of TNC expression in highly invasive breast cancer cells has not been studied in detail. This thesis focused on down-regulation of endogenous TNC expression and its effects on breast cancer cell behaviour.

3.2 Aims and objectives

The aim of this chapter was to investigate the functional effects of knockdown of endogenously expressed high molecular weight TNC isoforms in the highly invasive breast cancer cell line (MDA-MB-231) using synthetic siRNA. The objectives were:

1- To profile the expression of endogenous TNC isoforms in four breast cancer cell lines using RT-qPCR and Western blotting.
2- To generate a number of siRNA sequences to target specific exons in high MW isoforms for studies in breast cancer cell lines.
3- To transfect siRNA sequences against total TNC and high MW TNC isoforms into the highly invasive MDA-MB-231 breast adenocarcinoma cell line.
4- To analyse the phenotypic consequence of TNC isoform knockdown using 2D invasion assays and Immunocytochemistry (by pHH-3 proliferation associated marker).

3.3 Results

Profiling of TNC expression in breast cancer cell lines

The native expression of TNC was first investigated in breast cancer cell lines. To achieve this, TNC mRNA expression was analysed in ER negative breast cell lines which endogenously express TNC (MDA-MB-231, MDA-MB-468, MDA-MB-436 and HBL-100). The expression of TNC was analysed using quantitative RT-PCR at the mRNA level and Western blot at the protein level.

3.3.1 RT-qPCR analysis of TNC isoforms expression in TNC positive cell lines

3.3.1.1 Assessment of TaqMan assay efficiency

TNC isoform mRNA expression in TNC positive cell lines was analysed using an inventoried TaqMan assay targeting the invariant exons 17-18, which detects all TNC isoforms (named "total TNC") and TaqMan assays designed and validated in our group (Hancox et al. 2009; Guttery et al. 2010a). Standard curves were produced for each assay using a known concentration and length of PCR template and calculating the number of moles and the number of molecules using Avogadro's constant (6.02×10^{23} molecules/mol) (e.g. TNC-S in Figure 3.1). All assays had a high efficiency and consistent R^2 values (Table 3.1).

Table 3.1 Quantitative PCR efficiency, R^2 values and intercept for each TaqMan probe set

TaqMan probe/primers	PCR efficiency %	R^2 values	intercept
Total TNC	99.90	0.9959	47.806
TNC-9/16	99.00	0.9927	48.905
TNC-14/16	100.32	0.9894	49.135
TNC-S	97.39	0.9901	48.686
TNC-AD1	98.94	0.9981	48.715
TNC-AD2	99.12	0.9923	46.73

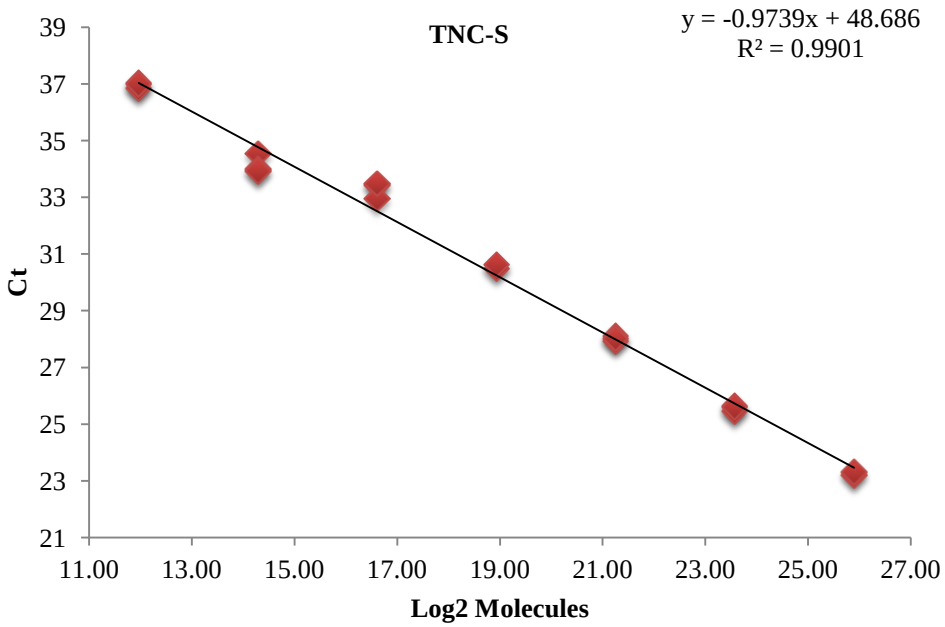


Figure 3.1: Taq Man assay standard curve for TNC short isoform (TNC-S).

3.3.1.2 Expression of total TNC and TNC isoforms in TNC positive cell lines

TNC positive breast cell lines were analysed for their profile of TNC expression. The SK-Mel-28 malignant melanoma cell line was used as a positive control; whereas the MCF-7 cell line was used as a negative control as it does not express TNC (Hancox et al. 2009; Guttery et al. 2010a). This experiment was performed twice using triplicate wells per sample and normalised to HPRT-1 as the endogenous control. A summary of cycle threshold (Ct) values, standard deviation (SD) and number of molecules is shown in (Table 3.2).

Table 3.2 Summary of Ct values for expression of all 6 TNC isoforms in TNC positive cell lines

Cell Line	TNC Isoforms	Repeats (Mean)		Ct Mean	SD	Number of Molecules
MDA-MB-231	Total TNC	28.87	29.43	29.15	0.14	5.5 E^{+06}
	TNC-S	32.44	31.90	32.17	0.12	1.7 E^{+06}
	TNC-AD1	34.02	33.79	33.90	0.13	4.3 E^{+05}
	TNC-AD2	32.02	33.91	32.96	0.48	2.4 E^{+05}
	TNC-9/16	35.46	34.99	35.22	0.36	1.9 E^{+05}
	TNC-14/16	32.17	33.12	34.64	0.13	1.2 E^{+06}
MDA-MB-436	Total TNC	26.88	27.81	27.34	0.08	2.9 E^{+06}
	TNC-S	32.44	31.90	32.17	0.12	2.4 E^{+05}
	TNC-AD1	30.03	30.79	30.41	0.13	7.2 E^{+05}
	TNC-AD2	32.03	33.91	32.97	0.48	3.4 E^{+04}
	TNC-9/16	35.47	35.00	35.23	0.36	2.7 E^{+04}
	TNC-14/16	31.05	29.89	30.47	0.07	8.2 E^{+05}
MDA-MB-468	Total TNC	28.13	28.66	28.39	0.28	1.6 E^{+06}
	TNC-S	29.38	30.67	30.02	0.16	1.9 E^{+06}
	TNC-AD1	34.45	33.55	34.00	0.53	7.2 E^{+04}
	TNC-AD2	35.10	35.98	35.54	0.75	6.0 E^{+03}
	TNC-9/16	33.31	34.31	33.81	0.20	9.5 E^{+04}
	TNC-14/16	32.58	31.87	32.23	0.15	3.9 E^{+05}
HBL-100	Total TNC	24.10	24.08	24.09	0.06	1.4 E^{+07}
	TNC-S	28.78	28.67	28.72	0.07	1.5 E^{+06}
	TNC-AD1	28.24	27.72	27.98	0.08	2.1 E^{+06}
	TNC-AD2	31.53	31.73	31.63	0.24	3.8 E^{+04}
	TNC-9/16	32.94	32.64	32.79	0.15	7.9 E^{+04}
	TNC-14/16	30.71	30.28	30.49	0.10	3.9 E^{+05}

Ct= Cycle threshold; SD= Standard Deviation

Expression of total TNC and TNC isoforms varied between cell lines. MDA-MB-231 and HBL-100 showed the highest level of total TNC, whereas MDA-MB-468 and MDA-MB-436 expressed lower levels of total TNC (Figure 3.2).

The fully truncated TNC isoform (TNC-S) was expressed in all TNC positive cell lines with MDA-MB-468 expressing the highest level and MDA-MB-436 the lowest.

Furthermore, TNC-S was the predominant isoform in all cell lines analysed (Figure 3.3).

All TNC positive cell lines expressed TNC-9/16 at low levels with MDA-MB-231 expressing the most (Figure 3.3 and Figure 3.5). TNC-14/16 was expressed in all total TNC positive cell lines, with MDA-MB-231 expressing the highest levels and MDA-MB-468 the lowest level (Figure 3.3).

All TNC positive cell lines also expressed both TNC-AD1 and TNC-AD2, but TNC-AD2 was generally expressed at a much lower level. HBL-100 cells expressed the highest level of TNC-AD1 (Figure 3.4 and Figure 3.5).

All TNC isoforms were expressed at high levels in the Sk-Mel-28 melanoma cell lines whereas the negative control MCF-7 cell lines did not express any TNC.

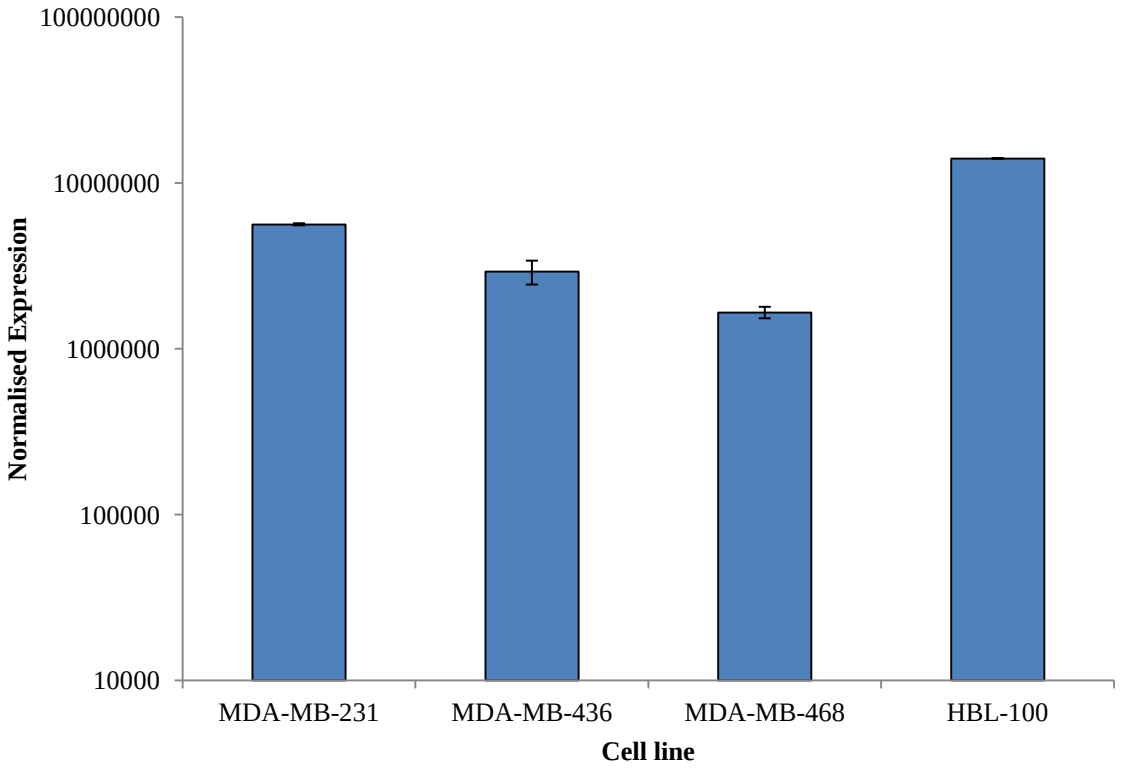


Figure 3.2: Expression of total TNC in TNC positive cell lines. The number of molecules was calculated from the Ct value and normalised against HPRT-1 as an endogenous control.

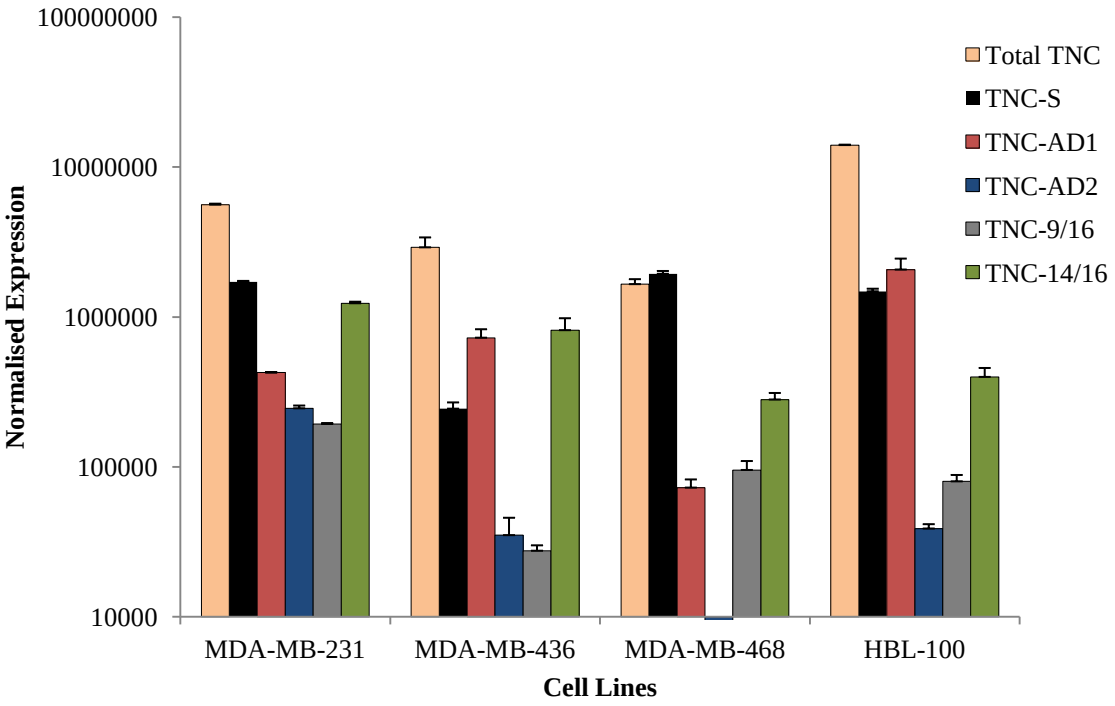


Figure 3.3: Expression of total TNC and 5 TNC isoforms in TNC positive cell lines. The number of molecules was calculated from the Ct value and normalised against HPRT-1 as an endogenous control.

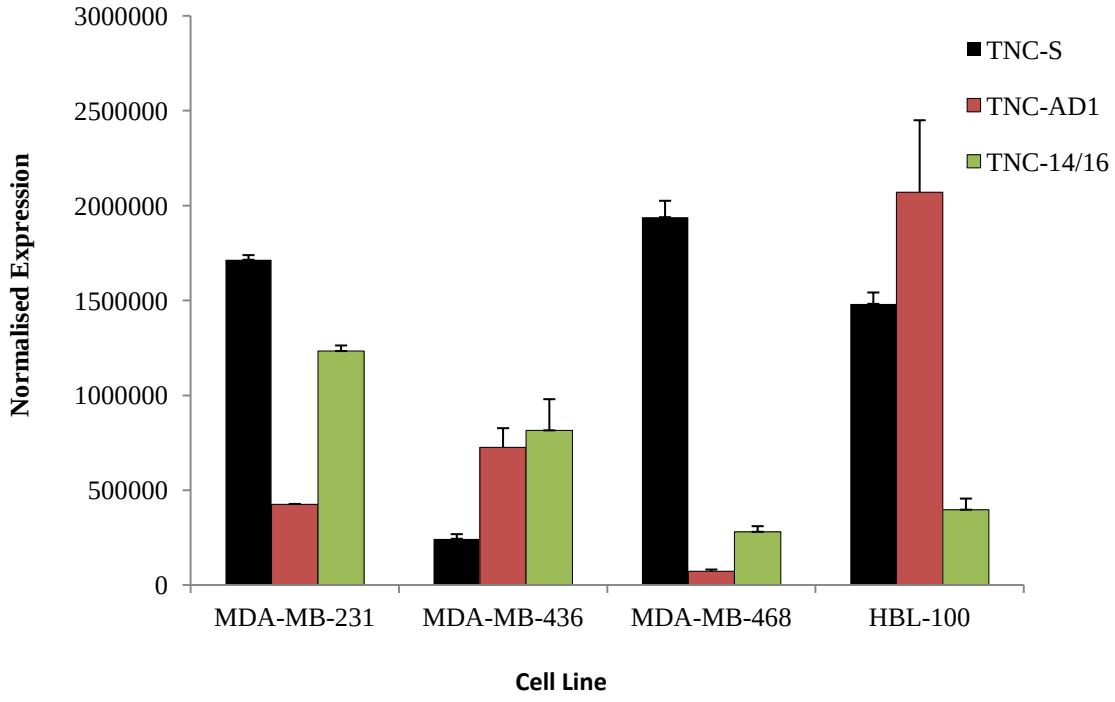


Figure 3.4: Expression of TNC-S, TNC-14/16 and TNC-AD1 isoforms in TNC positive cell lines. The number of molecules was calculated from the Ct value and normalised HPRT-1 as an endogenous control.

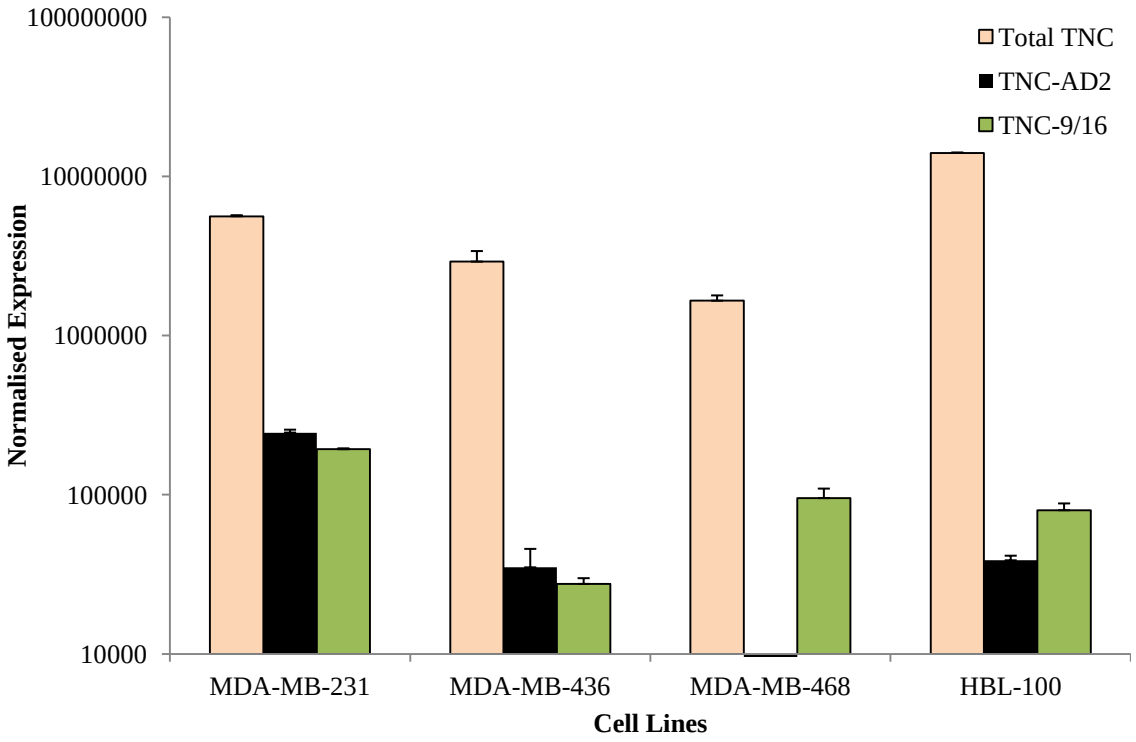


Figure 3.5: Expression of total TNC, TNC-9/16 and TNC-AD2 isoforms in TNC positive cell lines. The number of molecules was calculated from the Ct value and normalised against HPRT-1 as an endogenous control.

3.3.1.3 Western blot analysis

Western blot analysis of cell lysates and cell conditioned media from MDA-MB-231, MDA-MB-468, MDA-MB-436 and HBL-100 cell lines showed that two abundant isoforms of TNC were present (TNC-L and TNC-S), as assessed using the polyclonal H-300 anti-TNC antibody. TNC-L expression was predominant in the highly invasive MDA-MB-231 and MDA-MB-436 lines, and the immortalised HBL-100 cell line, while TNC-S expression was predominant only in the less invasive MDA-MB-468 line (Figure 3.6). Equal loading of protein was confirmed using an antibody targeting vinuclin (a membrane cytoskeleton protein).

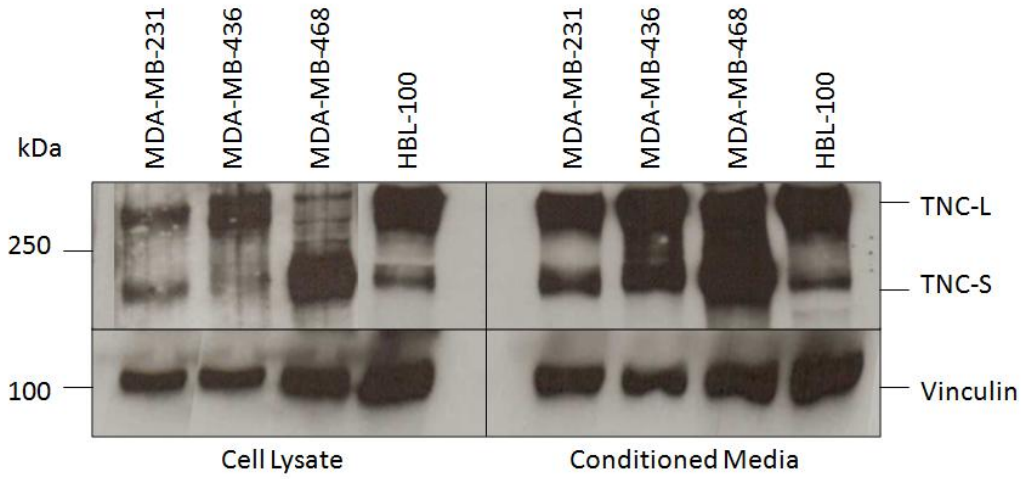


Figure 3.6: Western blot analysis of breast carcinoma cell lysates and conditioned media using anti-TNC H-300 antibody. Upper panel represents TNC isoforms (TNC-L and TNC-S).Lower panel represents loading control (Vinculin). The figure demonstrates the presence of TNC protein in all cell lines.

3.3.1.4 Correlation between mRNA and protein levels

Expression of TNC was present at both mRNA and protein levels. MDA-MB-468 predominantly expressed truncated TNC with high mRNA and protein levels; whereas, MDA-MB-436 predominantly expressed high MW TNC. MDA-MB-231 and HBL-100 cells expressed both high MW and truncated TNC.

3.4 Knockdown of TNC mRNA expression by siRNA

3.4.1 Generation and optimisation of TNC mRNA knockdown oligonucleotides

In order to identify a panel of siRNAs for total TNC and TNC specific isoforms, TNC oligonucleotide sequences were designed and synthesised specifically to target different exons of TNC. In total, eleven siRNAs were identified (Table 3.3) which targeted total TNC and exons present in high molecular weight TNC isoforms.

Table 3.3 siRNA targeting total TNC and high MW TNC isoforms

siRNA		Target exon	Target isoforms
Total TNC siRNA	TNC-3*	3	All TNC isoforms
	TNC-7*	7	All TNC isoforms
	TNC-24*	24	All TNC isoforms
High MW TNC siRNA	TNC-14(I)	14	TNC-14/16 and TNC-14/AD1
	TNC-14(II)*	14	TNC-14/16 and TNC-14/AD1
	TNC-14-16(I)	14-16	TNC-14/16
	TNC-14-16(II)*	14-16	TNC-14/16
	TNC-14-AD1(I)	14-AD1	TNC-14/AD1
	TNC-14-AD1(II)*	14-AD1	TNC-14/AD1
	TNC-AD1(I)	AD1	TNC-AD1
	TNC-AD1(II)	AD1	TNC-AD1

(*) = siRNA successfully knockdown TNC.

All generated siRNAs in (Table 3.3) were transfected into the highly invasive MDA-MB-231 cell line (Thompson et al. 1992) using Lipofectamine 2000 transfection reagent. The manufacturers (Invitrogen) recommendation for the assessment of RNA silencing using reverse transfection was 48 hrs post-transfection and 48-72 hrs for protein. Therefore, to achieve increased loss of TNC, cells were incubated for 48 hrs in a complete media post-transfection for mRNA analysis using RT-qPCR. The initial analysis by RT-qPCR showed a clear reduction for all TNC isoforms caused by siRNA targeting invariant exons (3, 7 and 24) (Figure 3.7 A). However, the high MW TNC isoforms were decreased by only 3 out of 8 siRNAs targeting TNC specific isoforms at specific exons (14, 14-16 and 14-AD1) (Figure 3.7 B), with an increased expression of the truncated TNC isoform (TNC-S). This experiment was performed once; however siRNAs that showed initial effects were subjected to downstream validation by RT-qPCR using more mRNA replicates and by Western blot using protein collected from cell lysates and conditioned media.

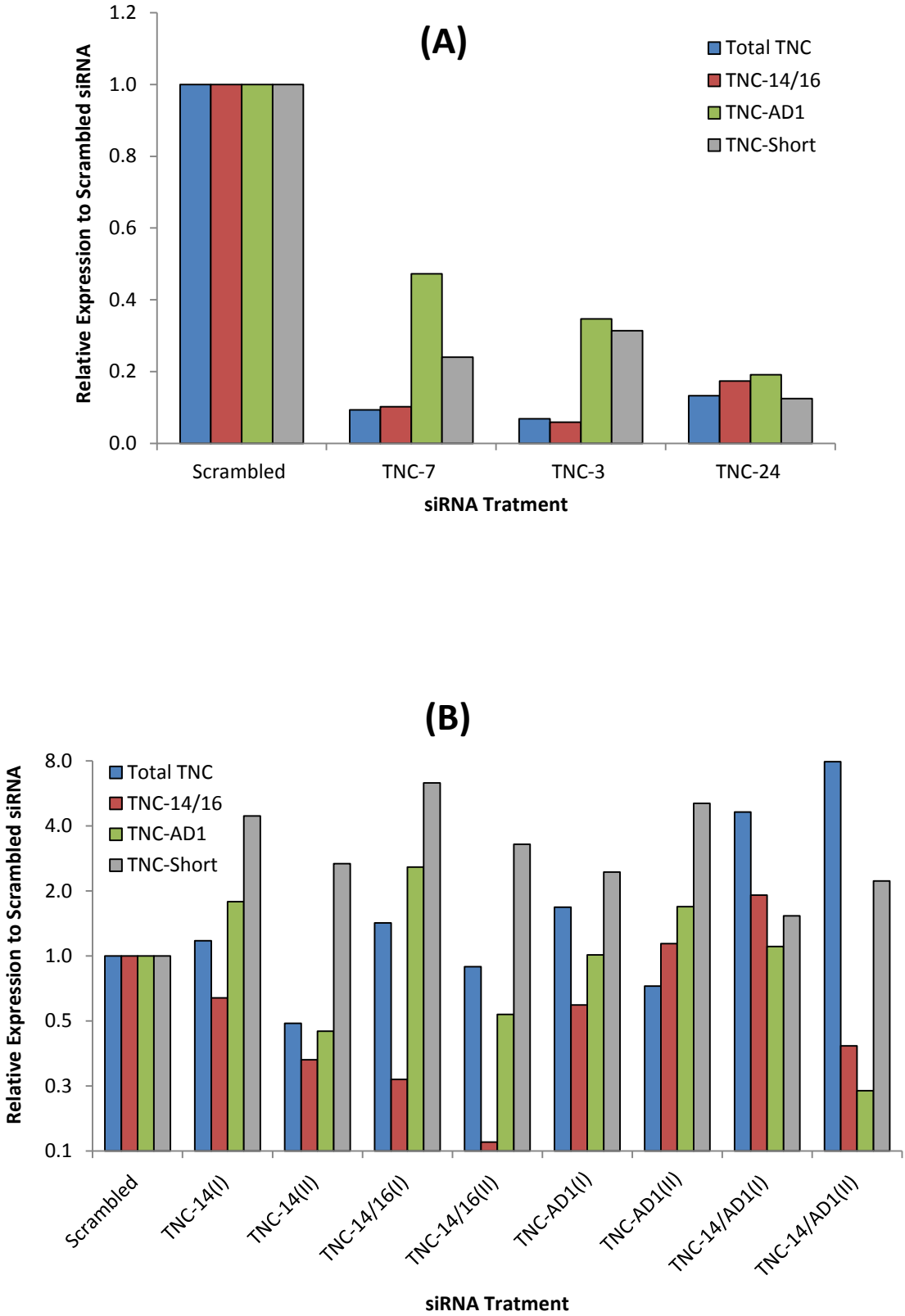


Figure 3.7: Relative Expression of total TNC and specific isoforms in transfected MDA-MB-231 cells with total TNC siRNAs (A) , and specific TNC isoforms siRNA (B) compared to scrambled siRNA.

3.4.2 Validation of TNC knockdown at mRNA and protein levels

Six siRNA were selected for further study based on the initial effects on TNC knockdown. MDA-MB-231 cells were transfected with these 6 siRNA with an incubation period of 48 hrs for mRNA and 72 hrs for protein collection. Total TNC and TNC isoform mRNA expression was then analysed using qRT-PCR and protein levels analysed by Western blot. A scrambled siRNA was used as a negative control, whereas a non-silencing fluorescently labelled siRNA was used to assess transfection efficiency (Figure 3.8). This experiment was performed three times using triplicate wells per sample.

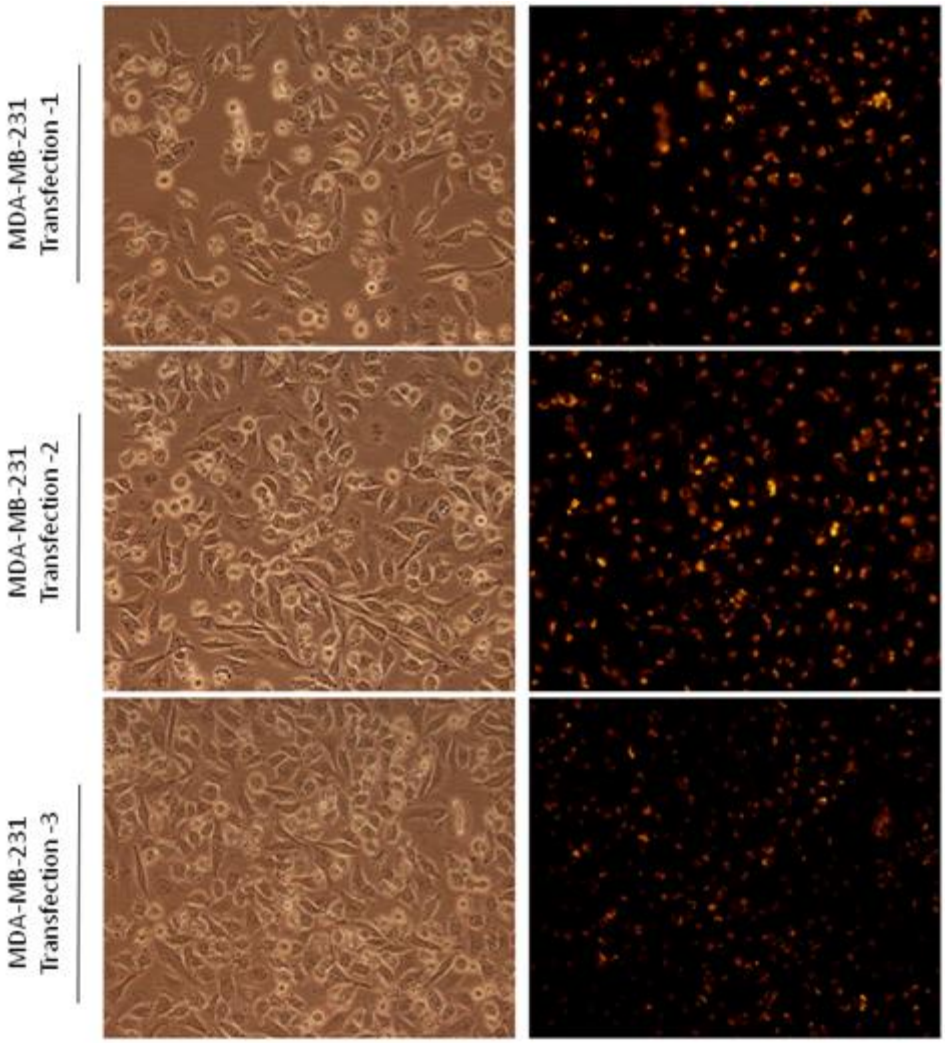


Figure 3.8: Fluorescence microscopy of MDA-MB-231 cells after transfection with non-silencing siRNA labelled fluorescent dyes. Fluorescence levels were examined 48 hrs after transfection with a result of >95 % cell fluorescence.

3.4.2.1 RT-qPCR validation of TNC knockdown mRNA

RT-qPCR analysis showed that the siRNAs tested were shown to significantly reduce TNC mRNA expression (Figure 3.9 and Figure 3.10). SiRNAs targeting TNC at invariant exon 24 gave the most statistically significant knockdown of total TNC ($p \leq 0.001$) (Figure 3.9). Highest knockdown of high MW TNC isoforms (TNC-14/16 and TNC-AD1) was achieved using siRNAs targeting exon 14 ($p \leq 0.01$ and $p \leq 0.001$) (Figure 3.10). Specifically, TNC-AD1 was down-regulated significantly by siRNA targeting exons 14-AD1 ($p \leq 0.001$) (Figure 3.10). Interestingly, in cells transfected with siRNAs targeting specific high MW isoforms, truncated TNC (TNC-S) was also shown to be significantly up-regulated ($p \leq 0.001$) (Figure 3.10).

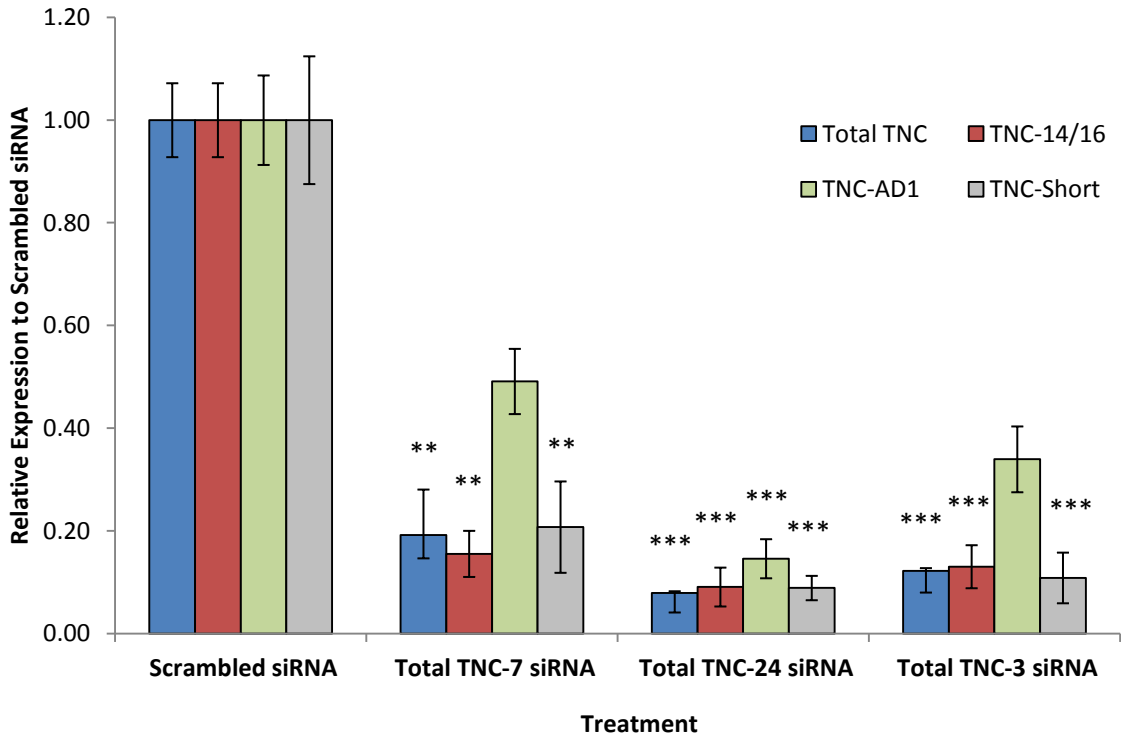


Figure 3.9: Relative expression means and (± SEM) of total TNC and specific isoforms in transfected MDA-MB-231 cells with total TNC siRNAs compared to scrambled siRNA. The significant differences between TNC expression and scrambled siRNA are indicated by asterisk (= p≤0.001; ***=p≤0.001).**

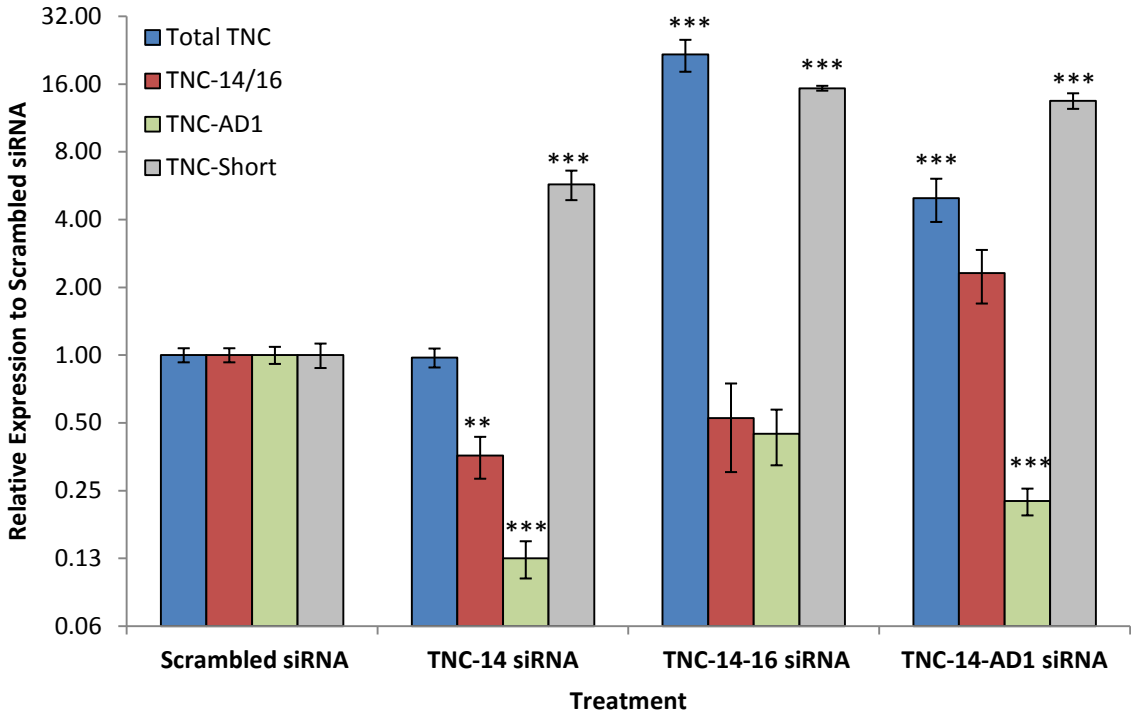


Figure 3.10: Relative expression means and (± SEM) of total TNC and specific isoforms in transfected MDA-MB-231 cells with high molecular weight TNC siRNAs compared to scrambled siRNA. The significant differences between TNC expression and scrambled siRNA are indicated by asterisk. (= p≤0.001; ***=p≤0.001).**

3.4.2.2 Western blot analysis of TNC knockdown proteins

Western blot analysis of cell lysates (Figure 3.11 A), and conditioned media (Figure 3.11 B), collected from cells transfected with siRNAs targeting total TNC and TNC high MW isoforms confirmed TNC down-regulation at the protein level. The reduction of TNC expression was more evident in the analysed cell lysate compared to the conditioned media, possibly because TNC is secreted into the conditioned media. In addition, there was a specific reduction of the high MW TNC level caused by targeting exon 14 with no effect on truncated TNC levels. There was no observable change in TNC protein levels in cells transfected with siRNAs against exons 14-16 and exon 14-AD1, as might be expected as TNC-L and TNC-S are the predominant isoforms detected.

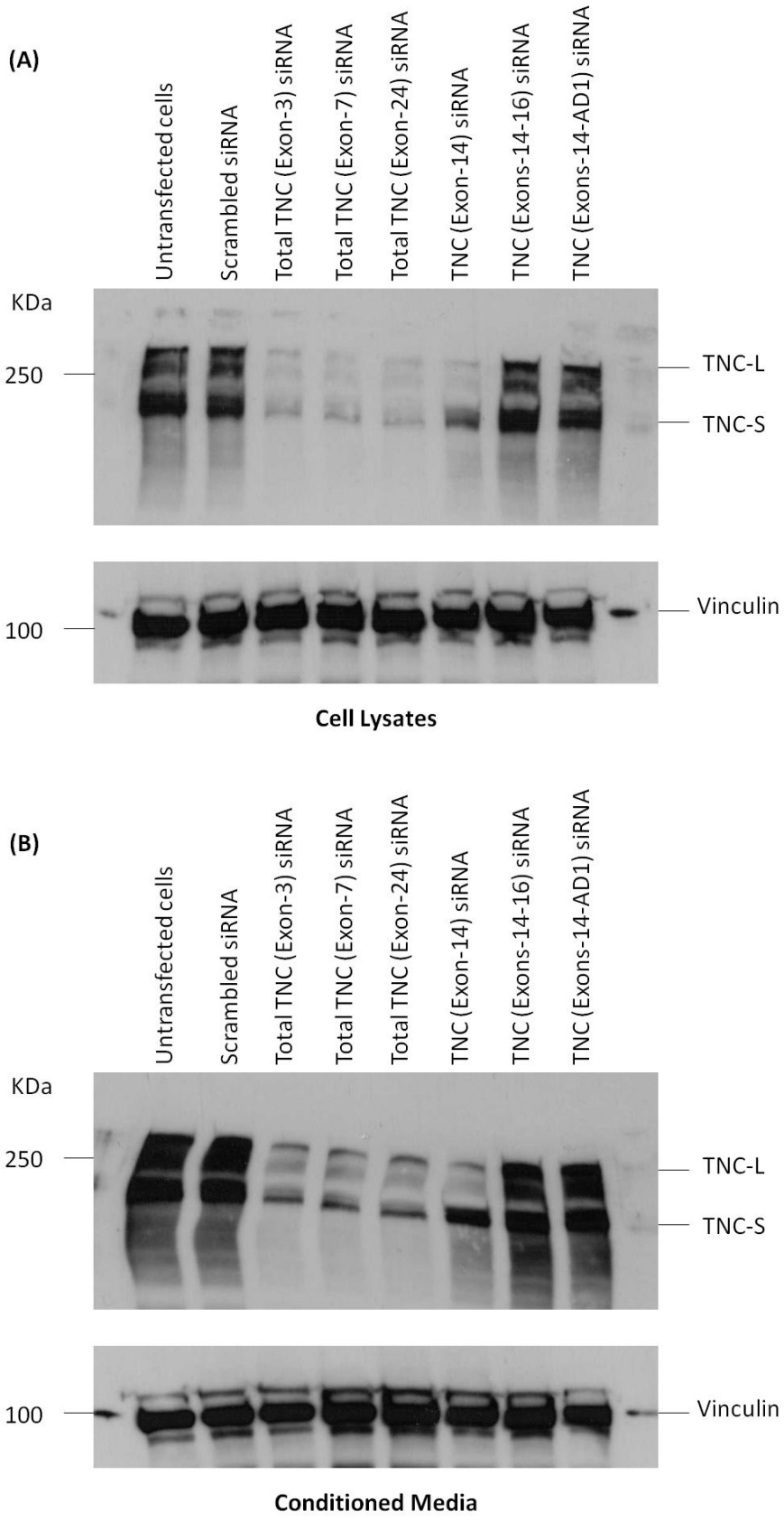


Figure 3.11: Western blot analysis of the transfected MDA-MB-231 breast cancer cell lines in both (A) cell lysate, and (B) conditioned media. siRNAs targeting different exons are represented in cell lysates and conditioned media. Upper panel represents predominant TNC isoforms (TNC-L and TNC-S). Lower panel represents loading control (Vinculin).

3.4.3 Effects of TNC knockdown on breast cancer cell invasion and proliferation

From the panel of siRNAs analysed, three siRNA were chosen for further validation and subsequent functional studies (Total TNC (exon 24), TNC (exon 14) and TNC (exons 14-AD1)) (Figure 3.12).

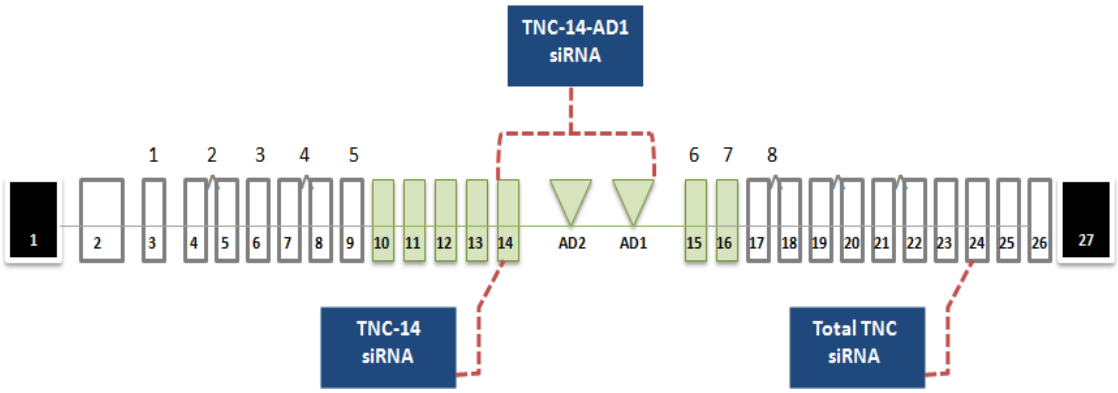


Figure 3.12: Schematic shows siRNAs targeting TNC exons. The exons in alternative spliced region are shaded in green.

Our research group has shown that the high MW TNC isoforms (TNC-16, TNC-14/16 and TNC-14/AD1/16) are implicated in breast cancer invasion and proliferation (Adams et al. 2002, Hancox et al. 2009; Guttery et al. 2010a). Therefore, siRNA targeting exon 14 and exons 14-AD1 were selected as they significantly down-regulated the high MW TNC isoforms. In addition, down-regulating all TNC isoforms is also important in order to find out the variations between individual and all TNC isoforms. Therefore, a siRNA targeting the invariant exon 24 was selected as it showed the most significant down-regulation of total TNC.

Validating the efficiency of the selected siRNA in another cell background was an important step prior to the functional studies. Therefore, MDA-MB-436 cell lines were transfected along with MDA-MB-231 cells with the selected siRNAs, and the protein was collected after 72 hrs post transfection. Western blot analysis of the cell lysate and

conditioned media confirmed the effects of the selected siRNA by the down-regulation of TNC expression in a similar pattern in both cell lines (Figure 3.13).

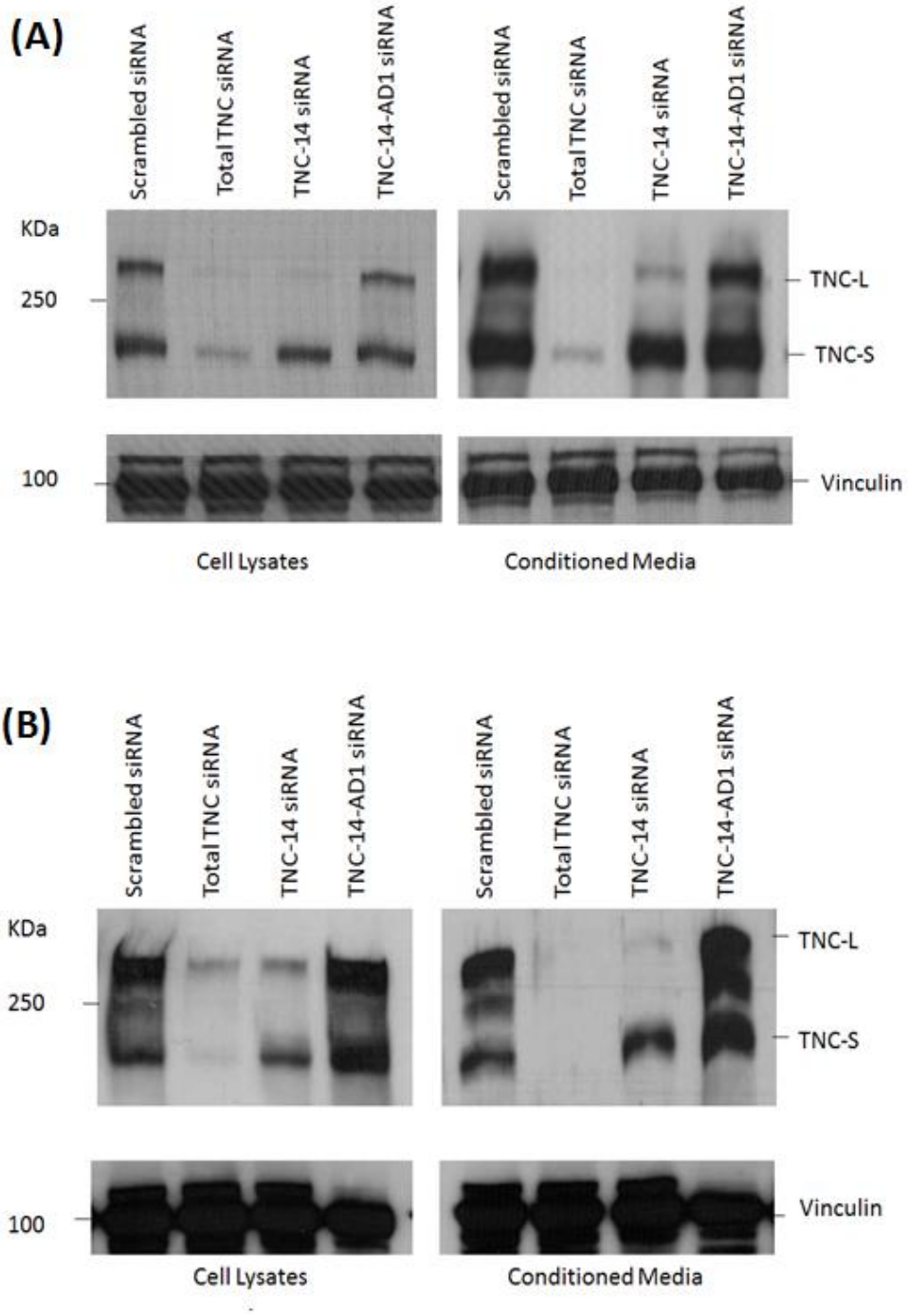


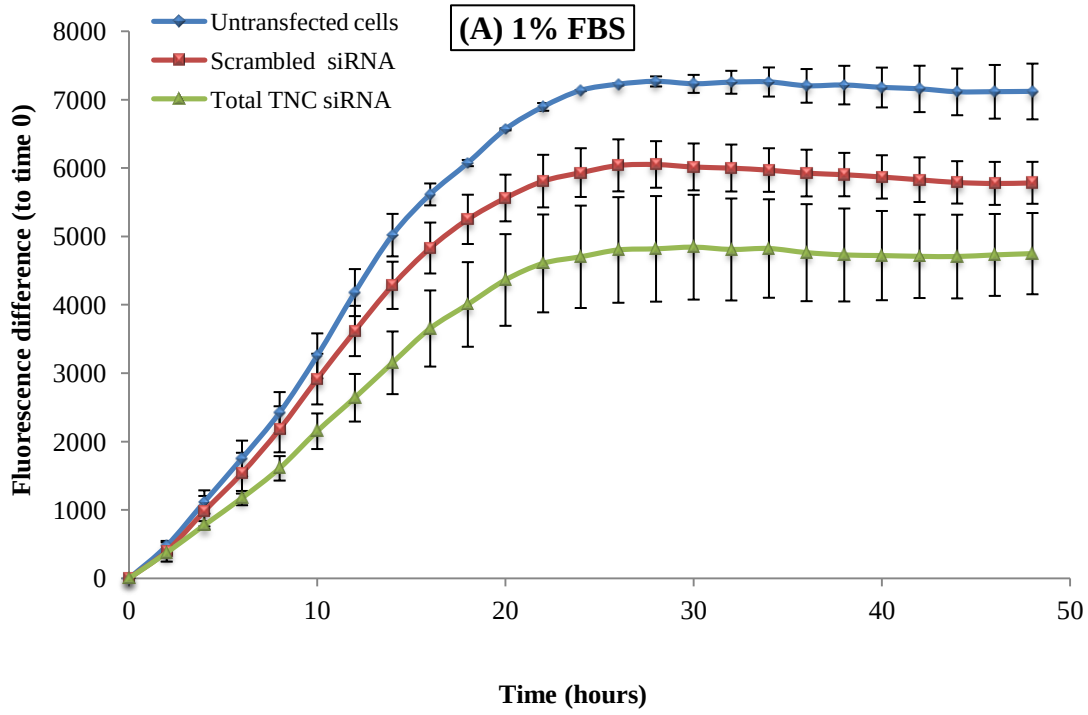
Figure 3.13: Western blot analysis of transfected MDA-MB-231 (A) and MDA-MB-436 (B) breast cancer cell lines in both cell lysate and conditioned media. siRNA treatment targeting different exons represented in cell lysates and conditioned media. Upper panel represents TNC isoforms (TNC-L and TNC-S). Lower panel represents loading control (Vinculin).

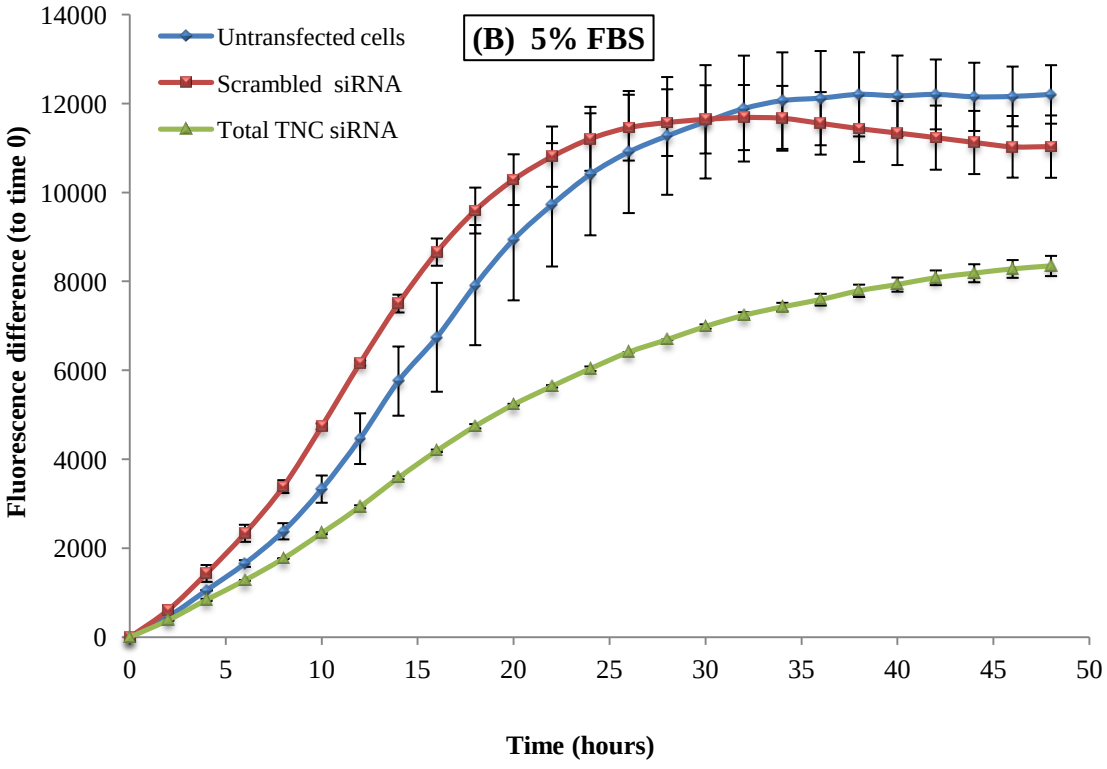
3.4.3.1 2D invasion assay

3.4.3.1.1 Optimisation of the invasion assay

A real-time 2D invasion assay using the BMG FLUOstar plate reader (Guttery et al. 2010a) was performed in order to investigate the effects of TNC knockdown on breast cancer cell invasion. Firstly, optimisation of the 2D invasion assay was performed using MDA-MB-231 cells transfected with total TNC exon 24 siRNA and the scrambled siRNA as a control. Previous studies in our group have shown that MDA-MB-231 cells invade optimally at a concentration of 5 x 10^4 cells/insert and a Matrigel concentration of 10 μg/ml/insert (Hancox et al. 2009;Guttery et al. 2010a). Complete DMEM with variable concentrations of FBS (1% and 5%) was used as the chemotactic source. MDA-MB-231 cells showed less difference in invasion with 1% FBS concentration in the chemotactic source compared to scrambled siRNA (Figure 3.14 A), whereas, 5% FBS showed a clear difference in invasion between treated cells with siRNA compared to the control (Figure 3.14 B). This experiment was performed once using duplicate wells.

Figure 3.14 Optimisation of 2D Invasion assay for MDA-MB-231 cell line transfected with total TNC siRNA using (1% FBS (A) and 5 % FBS (B)) DMEM as the chemotactic source. Fluorescence values were measured every 2 hrs for 48 hrs. Graphs represent the difference in fluorescence from time 0.





3.4.3.1.2 Effects of TNC knockdown on cancer cell invasion

MDA-MB-231 cells were transfected with siRNAs targeting total TNC, TNC-exon 14 and TNC-exon 14-AD1 and incubated for 24 hrs in transfection media. The next day, cells were fluorescently stained with (DiIC12(3)) and 2D invasion assays performed using 5% FBS in the chemotactic source, with a Matrigel barrier at a concentration of 10 μg/ml and readings taken every 2 hrs for a total of 48 hrs. After 24 hrs of transfection, MDA-MB-231 cells transfected with all TNC siRNA showed a significant decrease in invasion compared to untransfected cells and cells transfected with a scrambled siRNA (Total TNC siRNA $p \leq 0.001$, TNC-14 siRNA $p \leq 0.001$ and TNC-14-AD1 siRNA $p \leq 0.01$). This experiment was performed in triplicate using two wells per parameter giving consistent results (Figure 3.15).

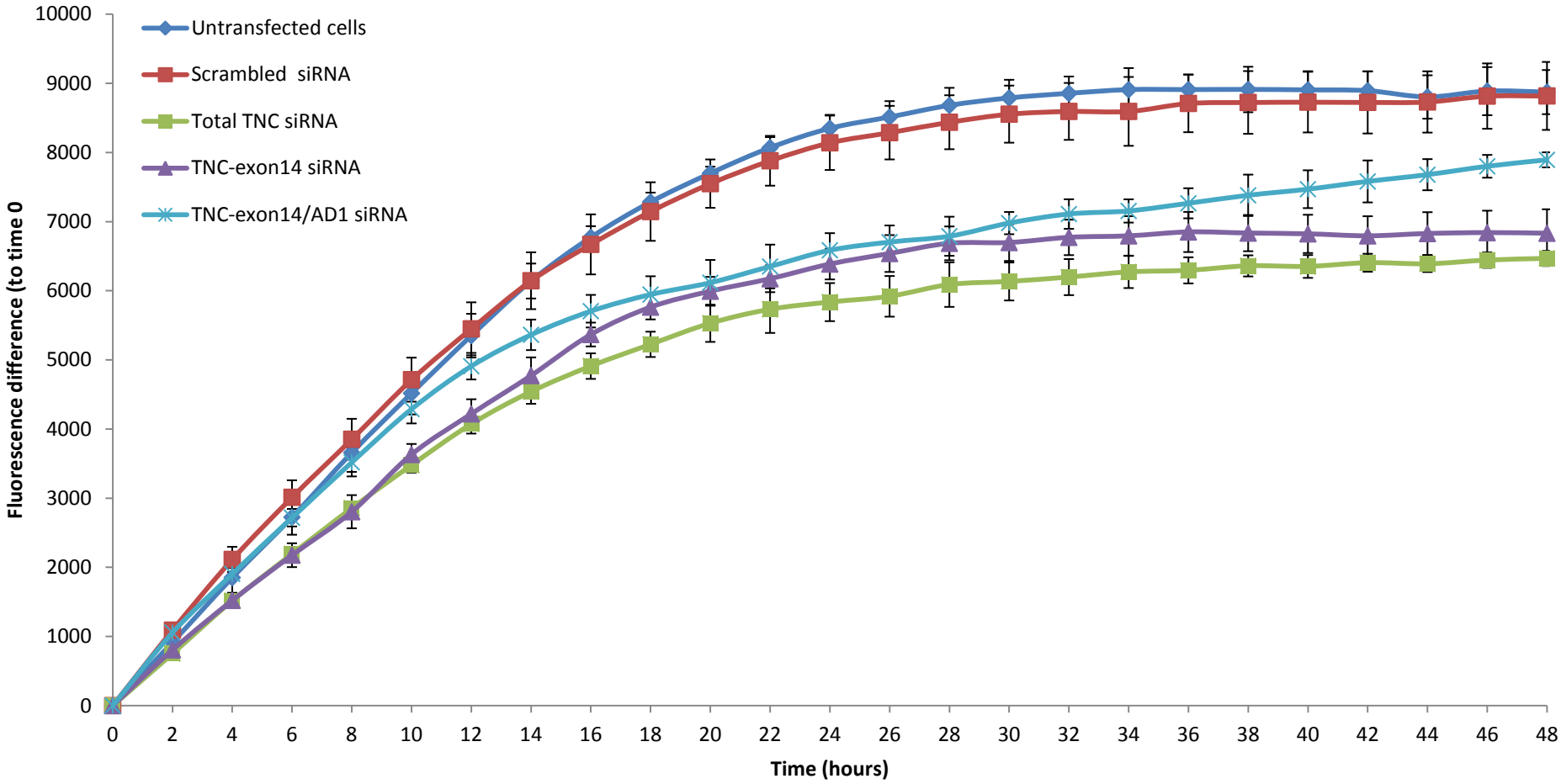


Figure 3.15: Invasion assay for MDA-MB-231 cell line transfected with specific isoforms siRNA using 5% FCS DMEM as the chemotactic source. Fluorescence values were measured every 2 hrs for 48 hrs. Graphs represent the difference in fluorescence from time 0. Error bars represent standard error of the mean (6 replicates). Statistical tests used were Two-way ANOVA and Post hoc Tukey test between siRNA treatments and time in cells transfected with TNC siRNAs compared to cells transfected to scrambled siRNA.

3.4.3.2 Analysis of proliferation associated marker

Phospho-Histone 3 (pHH-3), a mitotic division marker (Veras et al. 2009) was used to analyse the effect of TNC knockdown on cancer cell proliferation using immunocytochemistry. As before, cells were transfected with siRNAs targeting total TNC, TNC-exon 14 and TNC-exon 14-AD1 and incubated for 24 hrs in transfection media. Cells were then incubated with fresh complete media for 48 hrs. The expression of pHH-3 was analysed by immunocytochemistry using the Novolink polymer detection system and anti-pHH-3 antibody at a dilution of 1:1000. MDA-MB-231 cells transfected with TNC siRNAs showed a significant decrease in pHH-3 expression compared to cells transfected with scrambled siRNA (total TNC siRNA $p \leq 0.001$, TNC-14-AD siRNA $p \leq 0.05$) (Figure 3.16). The graph below was generated based on the ImmunoRatio image analysis application, which calculates the percentage of pHH-3 positive staining within five fields (Appendix 1). This experiment was performed in triplicate (Figure 3.17).

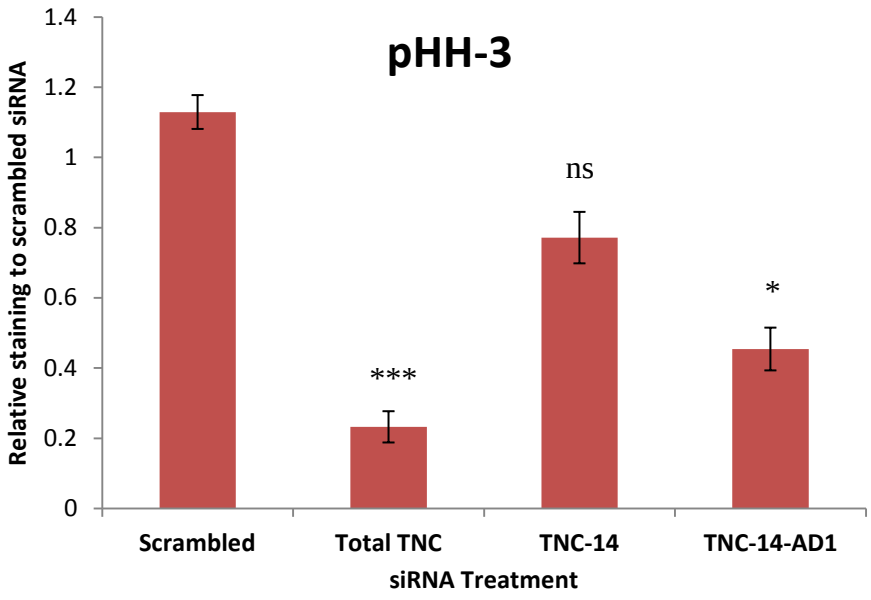
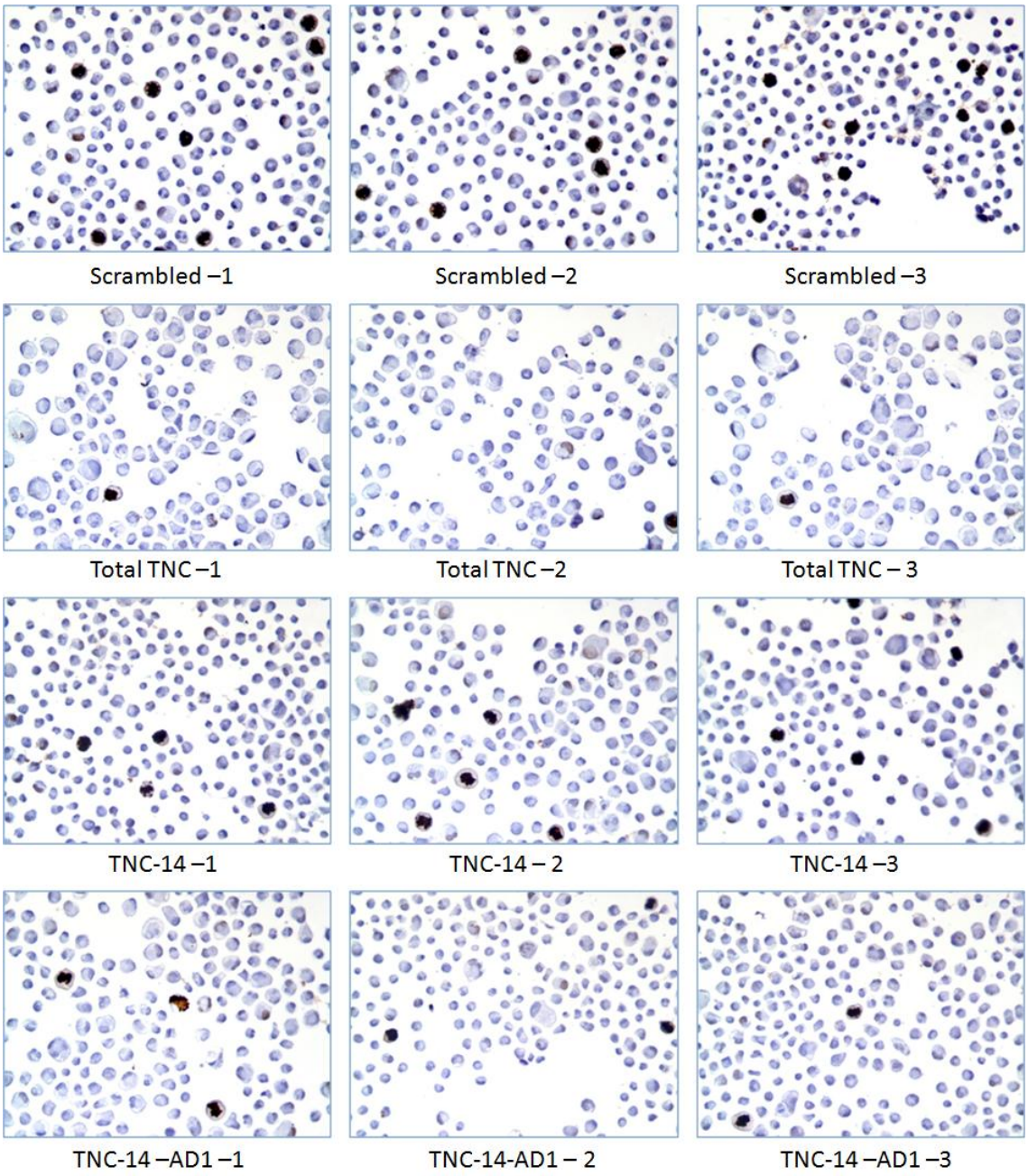


Figure 3.16: Analysis of Phospho-Histone 3 (pHH-3) staining on MDA-MB-231 transfected with total TNC and specific isoforms siRNAs. The significant differences between TNC siRNA and scrambled siRNA are indicated by asterisk (* = p≤0.05; ***=p≤0.001).

Figure 3.17: Immunocytochemistry analysis of Phospho-Histone 3(pHH-3) expression. MDA-MB-231 cells were transfected in triplicate with total TNC and specific isoforms siRNAs . Magnification is x40.



3.5 Discussion

3.5.1 Expression of endogenous TNC expression in ER negative breast carcinoma

The ECM plays an important role in tumour progression through extensive remodelling by proteolytic degradation and regulation of important physiological process such as cell growth, differentiation and apoptosis (Jones 2001). The ECM of breast cancer stroma is altered compared to normal ECM, and one significant change is the over-expression of TNC in the cancer-associated stromal matrix (Ishihara et al. 1995, Jahkola et al. 1998a). TNC exists in different isoforms due to alternative splicing of FNIII repeats at the pre-mRNA stage. In the developing mouse brain, TNC isoforms show a restricted and developmentally regulated distribution (Erickson 1993).

In this chapter, TNC mRNA profiles were obtained for four ER negative breast cell lines that endogenously express TNC (MDA-MB-231, MDA-MB-468, MDA-MB-436 and HBL-100). RT-qPCR confirmed the expression of total TNC and specific TNC isoforms (TNC-S, TNC-14/16, TNC-9/16, TNC-AD1 and TNC-AD2) at the mRNA level, and Western blot confirmed TNC expression at the protein level, with both the full length and truncated TNC protein being expressed in all TNC positive breast cell lines analysed in this study. Previous studies have considered these the only isoforms in breast cancer and illustrated a switch from the truncated to the long isoform throughout tumour progression (Borsi et al. 1996, Ghert et al. 2001). The results obtained in this study confirm that high MW TNC isoforms are predominantly expressed in ER negative breast cell lines, which are also known to be highly invasive (Thompson et al. 1992). TNC 14/16 was expressed at higher levels in cells with a highly invasive phenotype, whereas TNC-9/16 was expressed at lower levels. However, TNC-9/16 and TNC-14/16 showed low levels in HBL-100s, which have myoepithelial characteristics (Gordon et

al. 2003). These findings correlate with previous studies, as do the findings that HBL-100 cells express the highest levels of TNC-AD1 and TNC-AD2 (Guttery et al. 2010a).

3.5.2 Analysis of siRNA efficiency

The use of small interfering RNA (siRNA) to knockdown gene expression has become a powerful tool in functional and medical genomic research through directed post-transcriptional gene silencing (Park et al. 2008). One of the limitations of effective targeted silencing using siRNA is the design of the oligonucleotide itself. The efficiency of siRNA-mediated suppression of gene expression depends on a number of factors, including not only the chosen siRNA sequence but also the structure of the siRNA, and the receptiveness of the cell type to siRNA uptake (Siolas et al. 2005, Kim et al. 2005). In addition, the half life of the target protein needs to be considered in order to achieve optimal silencing (Park et al. 2008). Empirical testing of a number of siRNAs in this study showed that the recommended conventions for siRNA design do not ensure effective silencing of the target gene. In spite of this, a number of siRNAs were shown to significantly reduce specific and targeted TNC isoform expression in this study.

3.5.3 TNC knockdown decreases cancer cell invasion and proliferation

TNC plays a pivotal role in tumour progression, proliferation, invasion, metastasis and angiogenesis (Guttery et al. 2010b). In breast cancer, TNC expression is associated with poor outcome and is also shown to predict local and distant recurrences (Ishihara et al. 1995, Jahkola et al. 1998a).

Previous studies in our group have shown differences in the pattern of TNC isoform expression between benign, pre-invasive and invasive lesions using RT-qPCR and Southern blotting. Furthermore, all types of lesions were shown to express truncated tenascin (tTN), and TNC isoforms containing exon 16 (TNC16) and exon 14/16 (TNC14/16) (Adams et al. 2002). In this study, siRNAs targeting different TNC exons

were transfected into the highly invasive MDA-MB-231 cells and the results obtained showed a significant down regulation of TNC expression at both the mRNA and protein levels, as demonstrated by RT-qPCR and Western blotting, respectively. Previous studies by our group have demonstrated that high MW isoforms of TNC facilitate tumour invasion and proliferation in breast cancer (Adams et al. 2002, Hancox et al. 2009; Guttery et al. 2010a), with an increase in invasion seen in MDA-MB-231, MCF-7, T-47D and GI-101 cell lines over-expressing TNC 9/16 and TNC 9/14/16 (Hancox et al. 2009). In this regard, MDA-MB-231 cell lines were selected and transfected with TNC siRNAs in order to assess the effects of TNC knockdown on cell invasion and proliferation. The results obtained in this study correlate with previous findings by confirming that TNC plays a significant role in the pathogenesis of breast cancer by the significant reduction in tumour invasion and proliferation when TNC expression is down-regulated. TNC-16 and TNC 14/16 have been associated with tumour cell malignancy, but TNC 14/16 was specific for invasion (Adams et al. 2002), as well as promotion of tumour cell proliferation and invasion in a MMP independent and dependent mechanism (Hancox et al. 2009). This study showed that knocking down expression of isoforms containing exon 14 decreases cancer cell invasion and proliferation, which substantiates previous studies by suggesting that high MW isoforms are strongly associated with invasion and proliferation. The importance of the full length TNC isoform in the progression of breast cancer has been previously shown to be at invading sites of intra-ductal cancers and in the stroma of invasive ductal cancers (Tsunoda et al. 2003). Moreover, additional exons (exon 15 in domain C) have been reported to be associated with vessels of anaplastic glioma and considered as a marker of vascular proliferation (Viale et al. 2002). Despite all this, the role of TNC containing AD1 repeats in breast cancer progression is not clear. TNC containing novel AD1 and AD2 repeats were first identified in chicken embryo and later identified in normal and

malignant human oral mucosa (Sriramarao, Bourdon 1993a; Mighell et al. 1997). The AD1 repeat was also identified in fibrosarcoma cell line, malignant melanoma and ductal breast carcinoma (Derr et al. 1997). Extensive RT-qPCR analysis of breast tissue from high grade and hormone sensitive breast cancer in young women has shown novel TNC isoforms containing AD1 repeats. Furthermore, 2D invasion assays have shown that AD1 containing isoforms influence cell invasion in proportion to the TNC14/16 isoform (Guttery et al. 2010a). The results obtained here correlate with previous studies in that siRNAs targeting total TNC, TNC-14 and TNC-14-AD1 resulted in a significant decrease in cancer cell proliferation and invasion.

3.6 Conclusion

Targeted silencing of TNC expression using siRNA technology leads to an efficient reduction of TNC expression with significant effects on invasion and proliferation in breast cancer cell lines endogenously expressing TNC. This study showed that high MW TNC isoforms may play an important role in breast cancer progression and warrants further investigation to elucidate the pathways on which they act.

Chapter 4. Investigation of mRNA and protein changes in response to TNC knockdown by siRNA in breast cancer cells

4.1 Introduction

In vitro studies have shown that TNC modulates cell signalling pathways involving Wnt, mitogen-activated protein kinase (MAPK), focal adhesion kinase (FAK) and Rho (Orend et al. 2003; Ruiz et al. 2004; Midwood et al. 2004). By analysing the effects of TNC on global gene expression using cDNA microarray, Ruiz et al. (2004) found that TNC stimulates tumour cell proliferation in glioma cells by down-regulating the expression of tropomyosin-1, subsequently leading to cell spreading by destabilisation of actin stress fibres. They also found that TNC differentially regulates genes associated with several pathways, such as endothelial receptor type A (ENDRA), MAPK, and the Wnt inhibitor dickkopf-1 (DKK1).

Previous cDNA microarray studies in our group demonstrated that over-expression of two TNC isoforms (TNC-9/14/16 and TNC-14/AD1/16), associated with invasion in MCF-7 breast adenocarcinoma cells resulted in a down-regulation of expression of the tumour-suppressor gene profilin-1 (PFN-1) at the mRNA level. This finding suggested that up-regulation of TNC could increase breast tumour cell motility and invasion by down regulation of PFN-1 (Guttery 2009, PhD thesis).

The interaction of TNC with other genes associated with tumorigenesis is crucial and warrants further studies on the effect of TNC on several genes deregulated in crucial stages of breast cancer development such as proliferation, adhesion, invasion, apoptosis and angiogenesis. However, knowledge of genes expression differentially expressed by knockdown of endogenous TNC and its high MW isoforms is limited.

Microarray and proteomics have become powerful approaches in biological applications. In this chapter, analysis of TNC knockdown at mRNA and protein levels was performed using microarray and proteomics. Subsequent data analysis using

bioinformatics software packages including, normalisation, data filtering, statistical analysis, biological pathway analysis and selection of the candidate genes will also be discussed. Following this, the major part of this chapter will be dedicated to the validation of the candidate genes expression and proteins changes caused by the silencing effect of endogenous TNC and its high MW isoforms.

4.2 Aims and objectives

The aim of this chapter was to investigate the effects of knockdown of TNC by siRNAs at the mRNA and protein level in 2 cell lines (MDA-MB-231 and MDA-MB-436).

cDNA microarray specific objectives were:

- To transfect MDA-MB-231 and MDA-MB-436 cells with validated siRNAs against total TNC, confirm TNC knockdown, extract RNA and assess RNA quality using a BioAnalyzer.
- To analyse the extracted RNA from the transfected cells by Affymetrix Human U133 Plus 2 arrays to select candidate genes for further analysis using bioinformatics approaches.
- To validate candidate gene expression on RNA extracted from cell lines and breast cancer tissues using real time-qPCR to address the following questions:

    I) Which genes show the most significant up - or down-regulation in response to endogenous TNC knockdown by siRNA?

    II) Does knockdown of endogenous TNC expression differentially regulate specific genes associated with invasion, proliferation and migration?

TNC isoforms knockdown specific objectives were:

- To transfect MDA-MB-231and MDA-MB-436 cells with siRNAs targeting specific TNC isoforms (TNC-14 and TNC-14-AD1).

- To analyse candidate gene expression on the transfected cells using real time-qPCR to assess whether candidate gene expression is affected by knockdown of TNC isoforms associated with malignancy.

Proteomics specific objectives

- To transfect MDA-MB-231 with siRNAs targeting total TNC.
- To analyse the conditioned media from the transfected cells by Liquid Chromatography-Mass Spectrometry (LC-MS), to select candidate proteins for further analysis using Western blot and immunocytochemistry.
- To analyse candidate protein expression on breast cancer tissues using immunohistochemistry, and correlate with TNC expression at the mRNA level.

4.3 Results

To determine the effect of TNC knockdown on global gene expression using cDNA microarray and protein alterations using proteomics, the siRNAs targeting total TNC and high MW TNC isoforms (Table 4.1) were transfected into two breast cancer cell lines, and cells incubated for 24 and 48 hrs. Note that siRNAs targeting specific TNC isoforms were not used for the microarray and proteomics investigation studies due to the high cost per sample. Therefore, a total TNC siRNA (the invariant exon 24) was used to measure differential gene expression and protein changes resulting from total TNC knockdown, and then candidate genes were examined in specific siRNA experiments (TNC-14 and TNC-14-AD1 siRNAs). A summary flow diagram of the specimens and replicates used in microarray and proteomics is shown in Figure 4.1.

Table 4.1 Summary of total TNC and specific high MW siRNAs

siRNA	Binding exon	Target TNC isoform	RT-qPCR detection assay
Total TNC	Invariant exon (24)	All TNC isoforms	Total TNC probe
TNC-14	Exon (14)	TNC-14/16 and TNC-14/AD1	TTNC-14/16 and TNC-AD1 probes
TNC-14-AD1	Exons (14-AD1)	TNC-14/AD1	TNC-AD1 probe

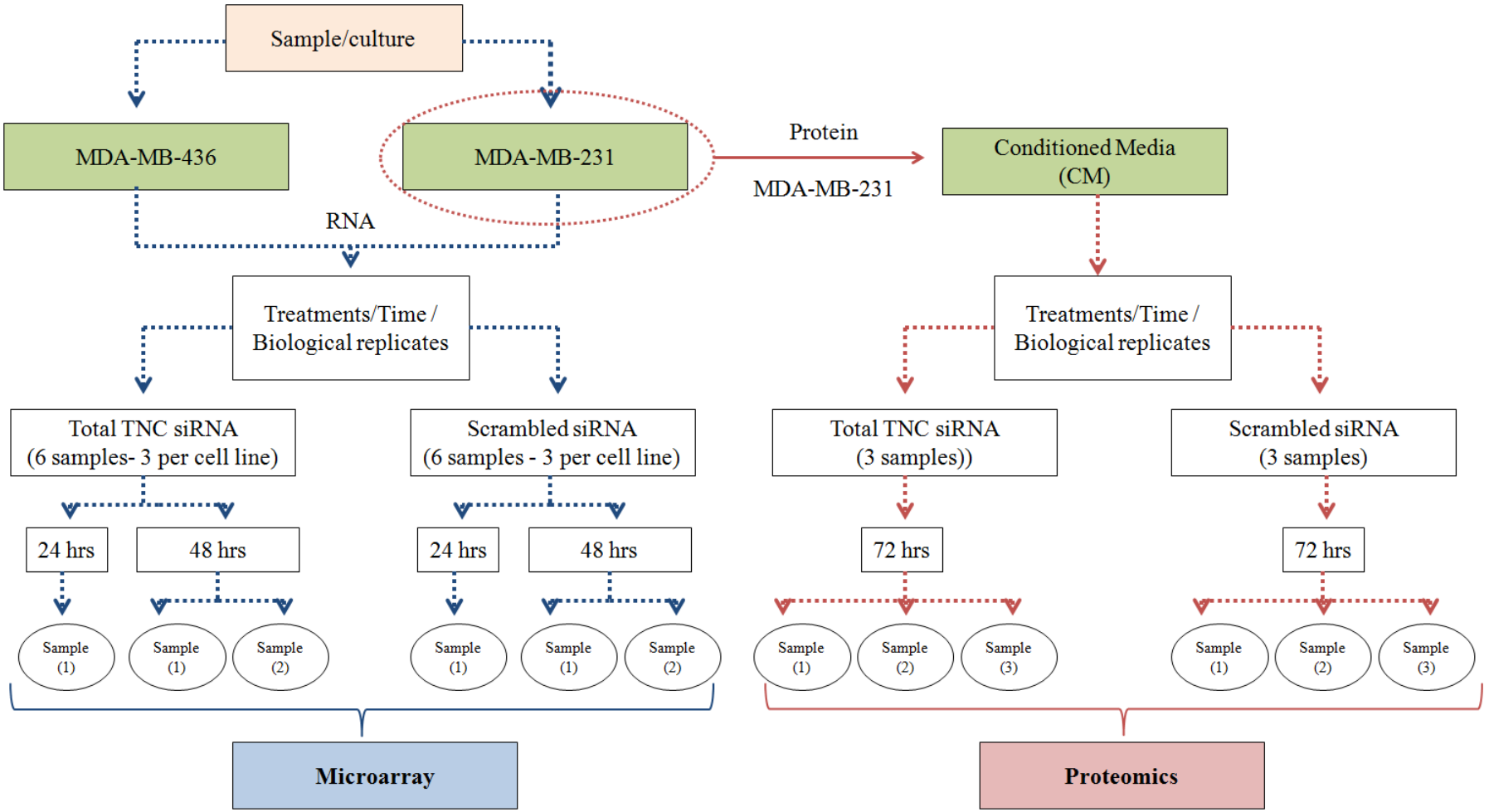


Figure 4.1: Summary flow chart for microarray and proteomics, including cell lines, siRNA treatments and time points.

4.3.1 The efficiency of total TNC siRNA transfection on breast cancer cell lines

To validate the efficiency of total TNC siRNA compared to the scrambled siRNA control, RNA collected from the transfected cell lines at two time points post transfection (24 and 48 hrs) was analysed using RT-qPCR. Analysis by RT-qPCR showed that expression of total TNC, as well as the specific isoforms analysed (TNC-14/16 and TNC-AD1), was down regulated by > 80 % in both cell lines at both 24 and 48 hrs (Figure 4.2). Real time qPCR analysis of assays specific to total TNC (targeting exon 17-18), TNC-14/16 and TNC-AD1, showed statistically significant down regulation (p<0.001) of all mRNA species at both 24 and 48 hrs. It also showed that the knockdown affect was slightly more at 48 hrs post transfection. A summary of Ct mean values, standard error of the mean and relative expression is shown in (Table 4.2).

Table 4.2 Summary of Ct values for total TNC siRNAs transfected in breast cancer cells

Cell lines	siRNAs	TNC Probes	Time Point									
			24 hrs					48 hrs				
			Ct Mean	ΔCt	RQ	SEM	*P value*	Ct Mean	ΔCt	RQ	SEM	*P Value*
MDA-MB-231	Scrambled	Total TNC	24.6	2.9	1.00	0.02	-	25.7	4.3	1.00	0.04	-
		TNC-14/16	27.1	5.4	1.00	0.07	-	29.1	7.7	1.00	0.21	-
		TNC-AD1	28.2	6.5	1.00	0.06	-	29.4	8.0	1.00	0.27	-
	Total TNC	Total TNC	27.4	5.7	0.15	0.01	**<0.001**	28.7	7.2	0.14	0.03	**<0.001**
		TNC-14/16	29.8	8.1	0.16	0.04	**<0.001**	32.7	11.2	0.09	0.01	**<0.001**
		TNC-AD1	30.0	8.3	0.29	0.03	**<0.001**	32.7	11.2	0.13	0.05	**<0.001**
MDA-MB-436	Scrambled	Total TNC	24.3	2.4	1.00	0.09	-	25.9	4.1	1.00	0.05	-
		TNC-14/16	27.5	5.6	1.00	0.15	-	28.3	6.5	1.00	0.03	-
		TNC-AD1	27.7	5.9	1.00	0.24	-	27.1	5.4	1.00	0.04	-
	Total TNC	Total TNC	27.0	5.2	0.16	0.04	**<0.001**	28.6	6.9	0.15	0.03	**<0.001**
		TNC-14/16	29.7	7.9	0.21	0.03	**<0.001**	31.2	9.6	0.12	0.01	**<0.001**
		TNC-AD1	30.1	8.2	0.21	0.06	**<0.001**	29.6	7.8	0.19	0.04	**<0.001**

Ct= Cycle threshold; SEM= Standard Error of the Mean; ΔCt= Delta Ct; RQ= Relative Quantitation

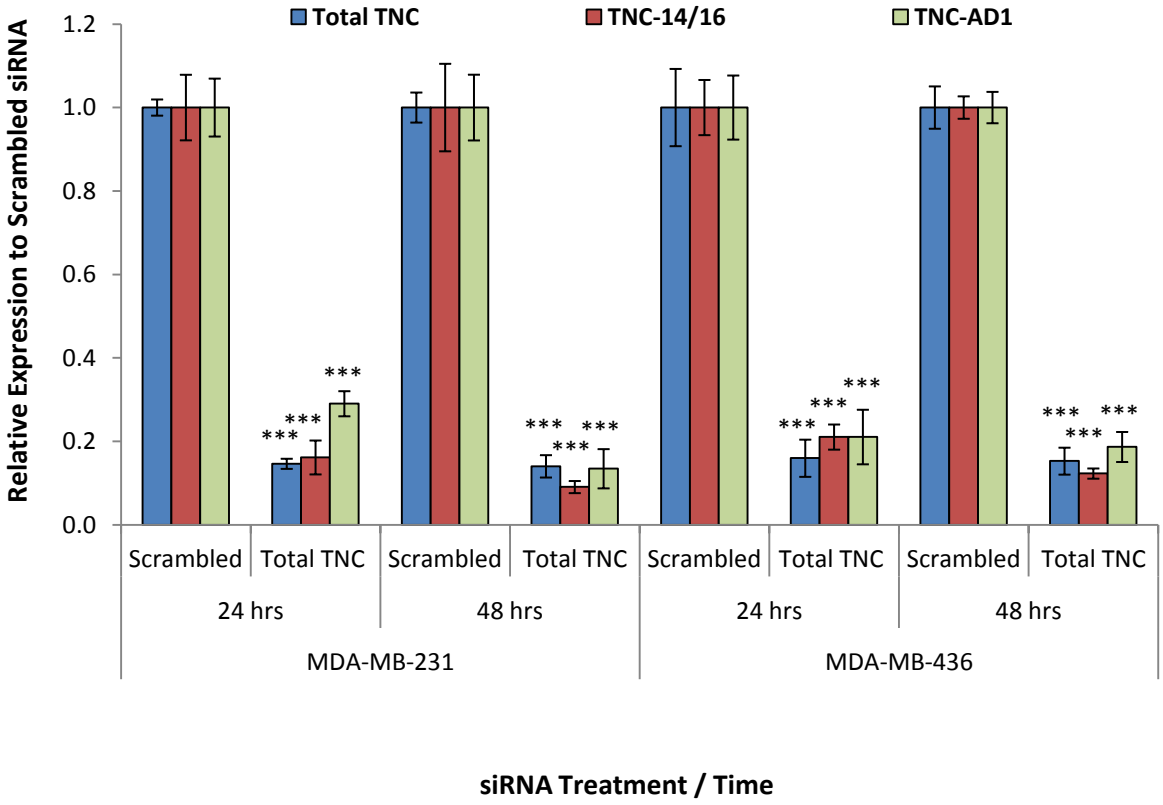


Figure 4.2: Relative expression means and (± SEM) of TNC between samples in breast cancer cell lines transfected with total TNC siRNA at 24 and 48 hrs post transfections compared to scrambled siRNA. Blue columns are the expression of all TNC isoforms, red and green are high MW TNC isoforms. All results are highly significant as indicated by the stars (*=p≤0.001).**

4.3.2 Preparation of mRNA for cDNA microarray

Breast cancer cell lines were transfected with siRNA targeting total TNC and scrambled siRNA as a control; and RNA was isolated representing six samples from each cell line (12 samples in total). Cells transfected with total TNC and scrambled siRNAs were lysed at 24 and 48 hrs post transfection and the quality of the extracted RNA assessed using gel electrophoresis and a BioAnalyzer. The extracted RNA was shown to be of high quality, with low amounts of degradation as shown on the electrophoresis gel with two visible bands corresponding to 28S and 18S rRNA (Figure 4.3). In addition, Bioanalyzer (Figure 4.4) also showed that all samples contained higher 28S than 18S peaks and have high RNA integrity number (RIN), confirming high RNA quality.

Having confirmed high quality RNA, the concentration was determined and each sample was hybridised to Affymetrix Human U133 Plus 2 arrays (service provided by Almac Diagnostics, UK).

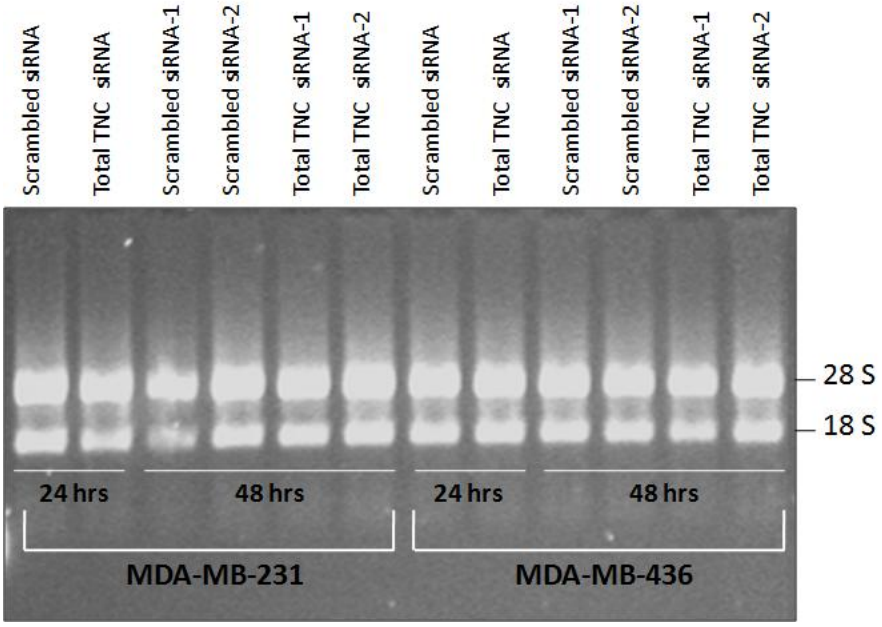


Figure 4.3: Agarose gel electrophoresis for RNA quality verification. All samples transfected with scrambled and total TNC siRNAs in both cell lines at 24 and 48 hrs showed a clear appearance of 28S and 18S rRNA bands confirming high quality RNA.

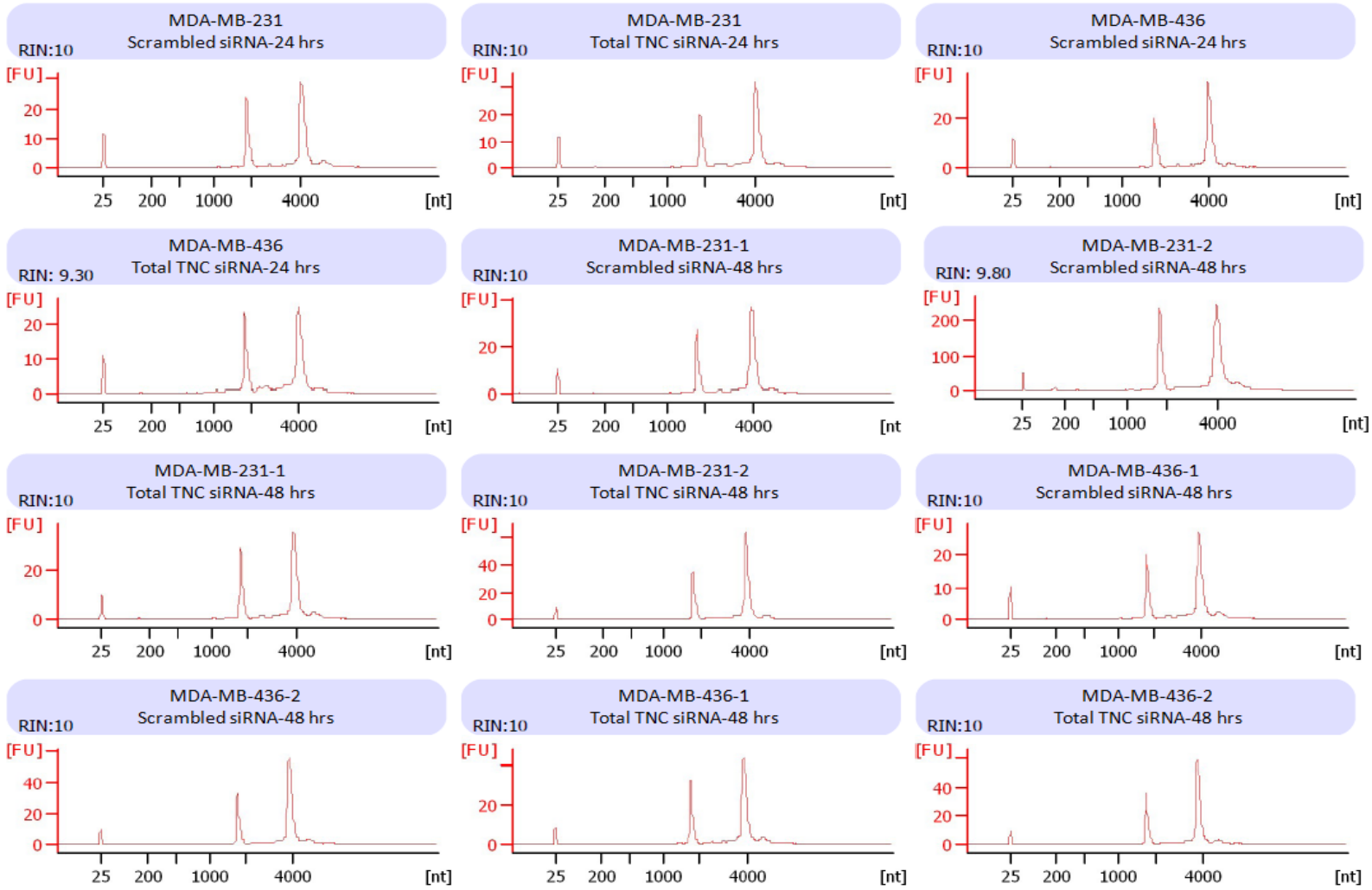


Figure 4.4: Bioanalyzer analysis for RNA quality. 5S, 28S and 18S ribosomal RNA peaks are observed in all samples. RNA integrity number (RIN) value for all samples were high (RIN >9), which represent high quality RNA.

4.3.2.1 cDNA expression array data analysis

4.3.2.1.1 Normalisation

The raw data obtained from the Affymetrix chips was analysed and normalised using the Partek® Genomics Suite™ 6.5, build 6.10.1129 with help from Dr. Kevin Blighe (Department of Cancer Studies, University of Leicester). Prior to background correction, raw probe signal intensity values were pre-adjusted for GC content and probe sequence. Background correction using the robust multiarray average (RMA) method (Bolstad et al. 2003), including quantile normalisation and median polish probe set summarisation, was then performed in order to remove artfactual data and noise. In order to minimise the affect of sample bias on the results, further analysis were performed before and after quantile normalisation using the MA scatter plots and Box-plots.

MA scatter plots, which graphically represent the differences in intensities between two samples (normalised and un-normalised), are shown in (Figure 4.5) to highlight the effect of normalisation on the data-set. For non-normalised samples, the curvature present in the MA plot indicates the intensity dependant bias effects on the array, which were then removed after normalisation was performed (Figure 4.5). Box-plots which identify the intensity distribution across the probe values for each sample pre and post normalisation is shown in (Figure 4.6). There were variations in the distribution of intensity signals among the array before normalisation, which were then corrected and became equal for all samples (Figure 4.6).

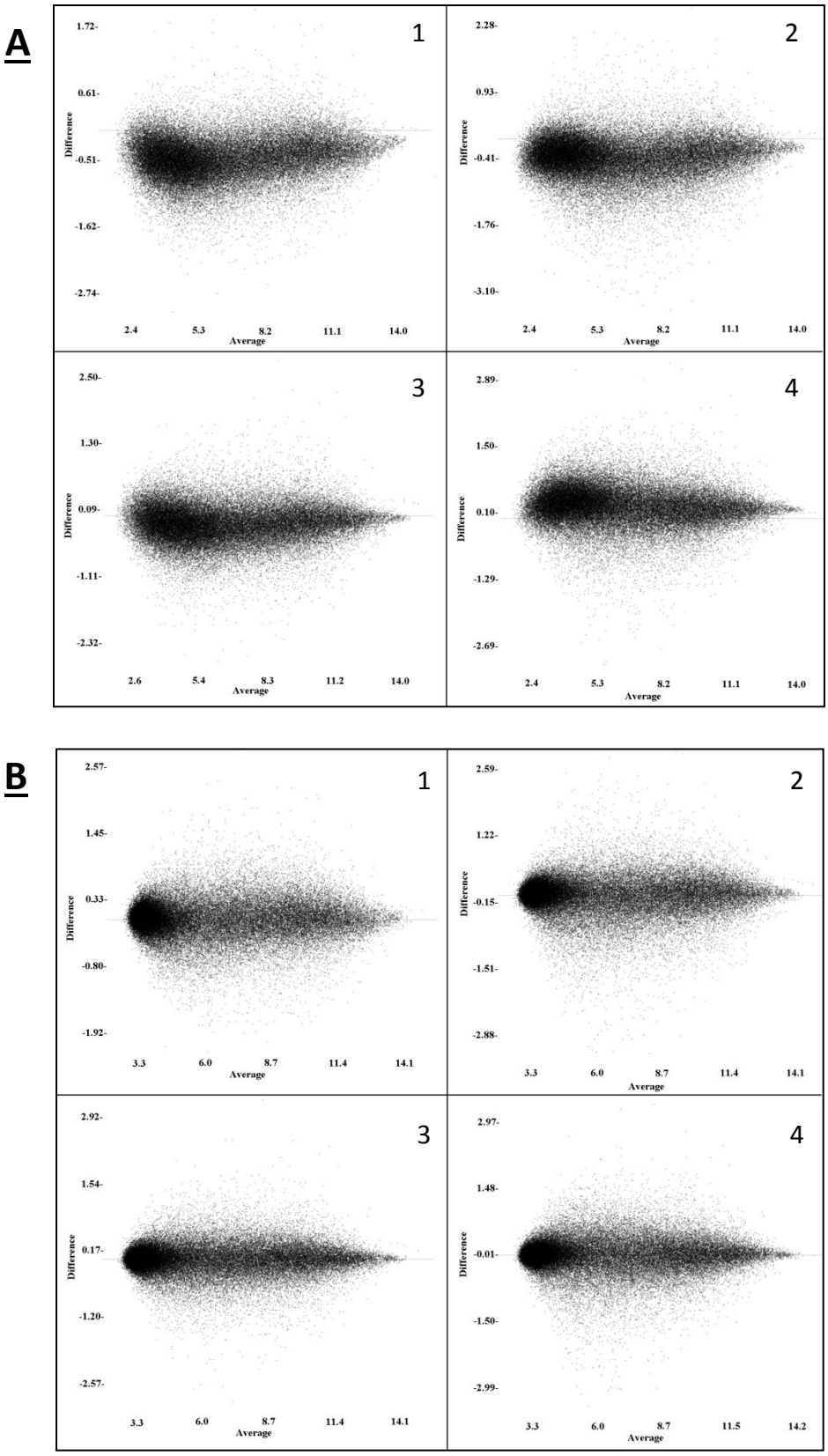


Figure 4.5: MA scatter plots of the two cell-lines at two time points reveals a skew in the distribution of marker values, which is corrected by quantile normalisation. A) Before normalisation. B) After normalisation. Each plot represents scrambled siRNA vs TNC siRNA for: 1, MDA-MB-231 at 24 hrs; 2, MDA-MB-231 at 48 hrs; 3, MDA-MB-436 at 24 hrs; and 4, MDA-MB-436 at 48 hrs. X-axis represents the average log_2 value between corresponding markers; Y-axis represents the fold-change between corresponding markers.

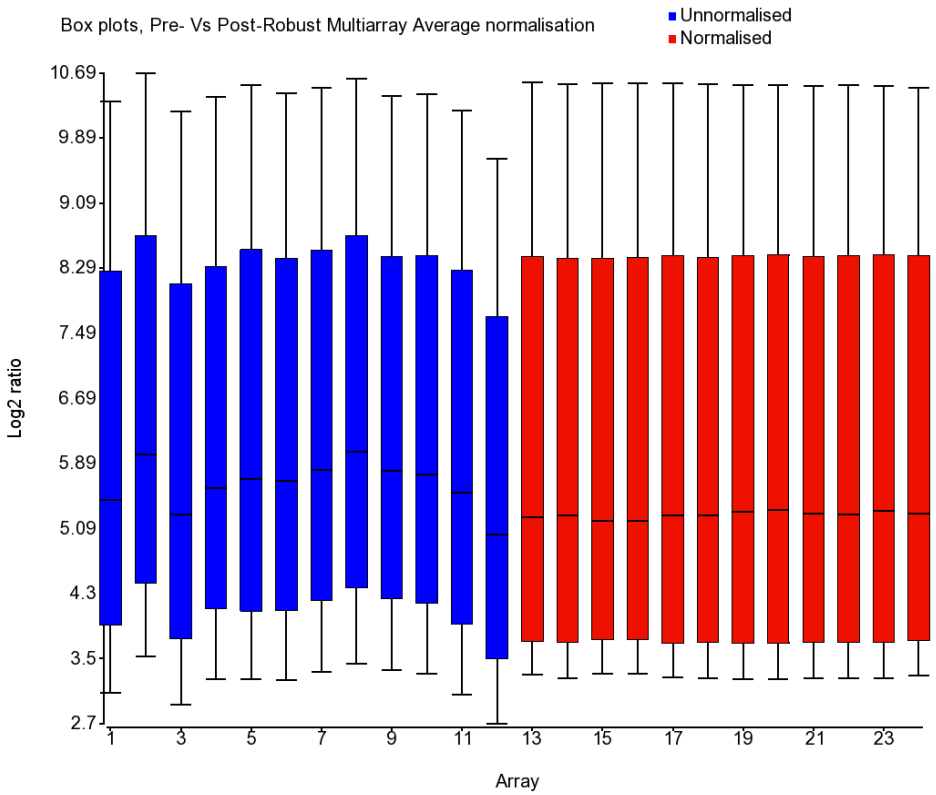


Figure 4.6: Box plot of all 12 samples pre and post normalisation. Each sample represented by one box plot. The blue box plots represent log_2 ratio for raw intensity values, and the red box plots represent log_2 ratio for normalised data.

Hierarchical clustering was then performed on all samples using complete linkage and incorporating the log2 ratios for all markers (54,680 individual markers) on the chip. Firstly, this revealed a large natural difference in gene expression profile between the two native cell lines (Figure 4.7). Secondly, cDNA derived from the two cell lines also separated based on siRNA treatment and time point. Importantly, sample replicates at 48 hrs in each cell line and treatment also gave good reproducibility clustered together in individual clusters (Figure 4.7).

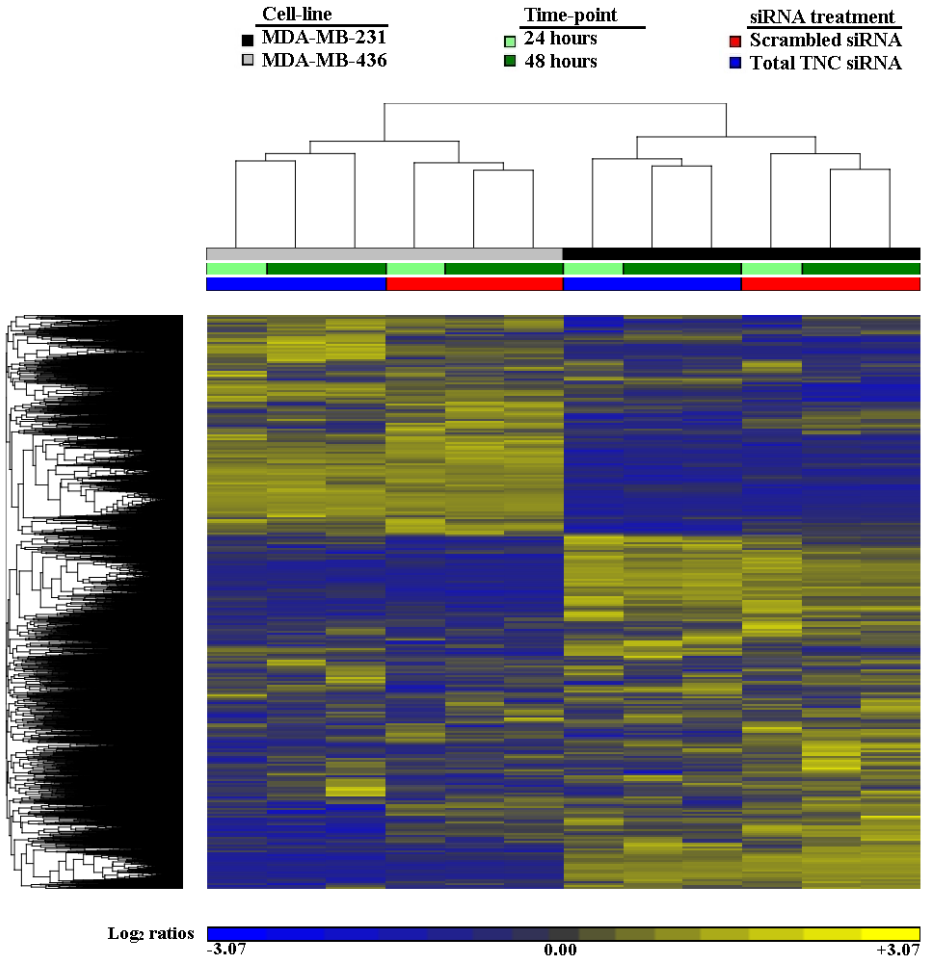
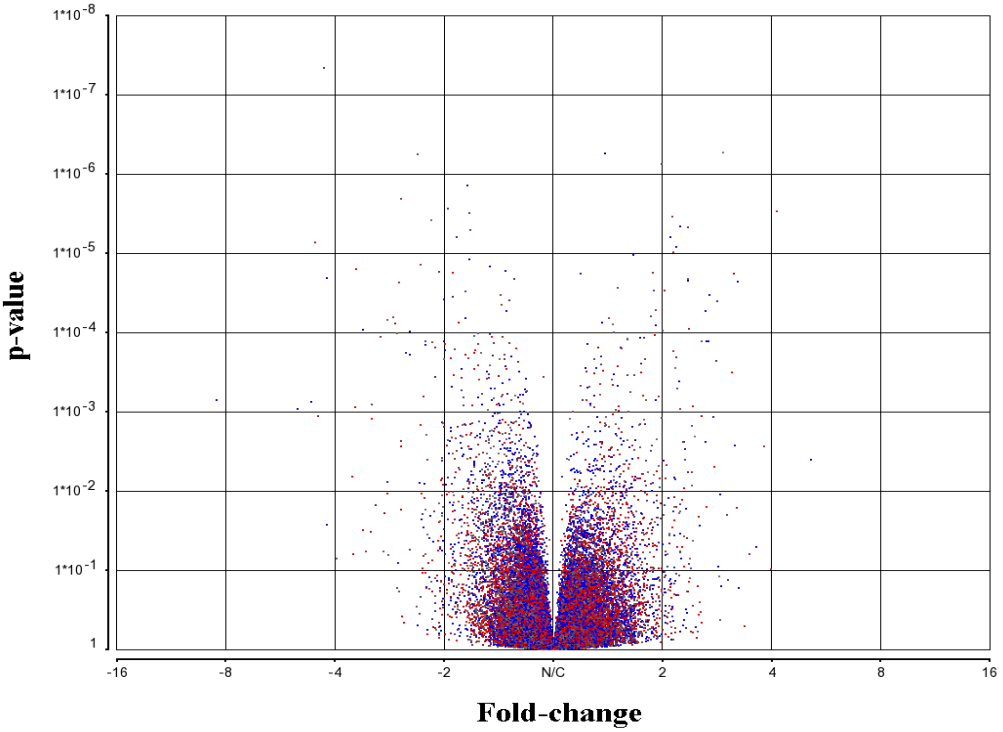


Figure 4.7: Hierarchical clustering of samples identifies two main clusters, representing the two different cell lines. Within each cluster, samples were again divided by siRNA treatment and time point. Yellow, positive log_2 ratio; blue, negative log_2 ratio.

4.3.2.1.2 Filtering of data

The normalised data was then filtered and a threshold applied according to a fold change in expression of 2 (up or down) in cells treated with total TNC siRNA compared to the scrambled siRNA. In addition, statistical analysis was used to filter genes with a p value ≤ 0.05 with false discovery rate (FDR) using ANOVA. The relationship between the significance level and fold change between both cell line and time point according to a FDR p-value < 0.05 is represented by volcano plots in Figure 4.8. This revealed that more markers increased in significance and absolute fold-change between the two time points.

A) 24 hrs, Scrambled siRNA Vs Total TNC siRNA.



B) 48 hrs, Scrambled siRNA Vs Total TNC siRNA.

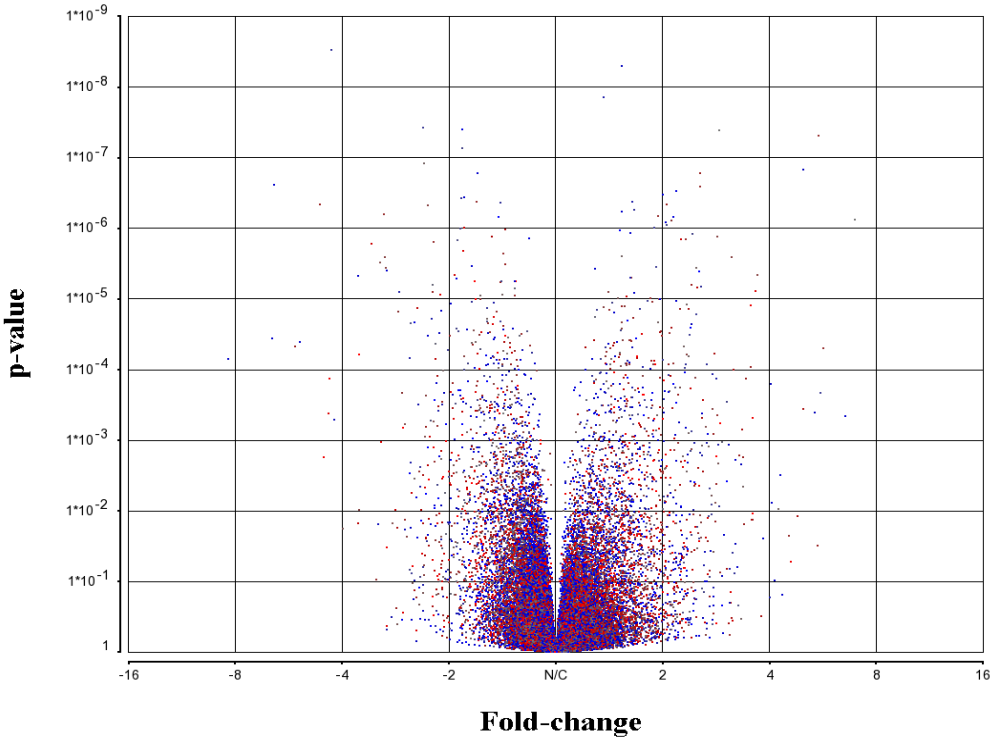


Figure 4.8: Volcano plot showing the significance and fold change in gene expression due to total TNC knockdown in breast cancer cell lines at two time points (A, 24 hrs; B, 48 hrs). Scrambled siRNA treatment is shown in red and TNC siRNA treatment is shown in blue. Cell lines are grouped for the purposes of this plot. At 48 hrs, more markers achieved a greater significance and higher absolute fold-change than at 24 hrs.

When taking up-regulated and down-regulated genes into account post-filtering, Venn diagrams reveal the level of overlapping genes amongst these breast cancer cell lines that are differentially regulated as a result of total TNC knockdown at two time points. There were a total of 13 (5 up and 8 down) overlapping genes that were differentially regulated in both cell lines at 24 hrs and 156 (106 up and 48 down) genes at 48 hrs (Figure 4.9). There were 9 (7 up-regulated and 2 down-regulated) genes that were unique to the MDA-MB-436 cell-line at 24 hrs, while 11(7 up-regulated and 4 down-regulated) were unique to MDA-MB-231. At 48 hrs, however, there were 329 and 417 up- or down-regulated genes unique to each cell line. The difference between the unique number of affected genes in each cell line and at each time point is perhaps a reflection of the alternate effects of TNC knockdown on each and the subsequent coping mechanisms adopted in response to this knockdown. In addition, the large general difference in number of affected genes between 24 and 48 hrs could be a reflection of TNC knockdown's increasing affect over time.

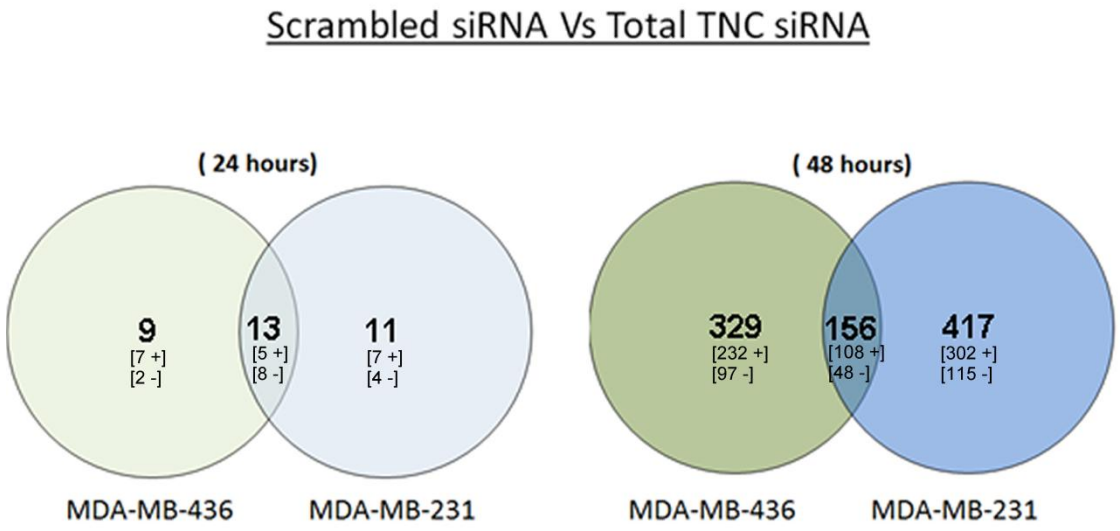


Figure 4.9: Venn diagram reveals overlapping genes amongst breast cancer cell lines that are differentially regulated as a result of total TNC knockdown in MDA-MB-436 and -231 cells at 24 and 48 hrs.

4.3.2.1.3 Pathway analysis

A functional annotation cluster and pathway analysis was performed on 902 (642 up and 260 down) genes identified after 48 hrs of siRNA transfection in both cell lines using the Database for Annotation, Visualization and Integrated Discovery (DAVID). A total of 220 differentiated genes were identified in sixteen KEGG pathways (Kanehisa et al., 2002) (Table 4.3). A number of pathways involved in the progression of cancer such as cell cycle, focal adhesion, ECM-receptor interaction and apoptosis were identified. In addition 32 genes were found to be regulated in pathways in cancer, and 29 genes have differential expression in pathways associated with other cancers including small cell lung cancer, colorectal cancer and pancreatic cancer.

Table 4.3 Pathway analysis of the regulated genes by the knockdown of TNC

Pathway	Number of identified genes	p-value
Pathways in cancer	32	$1.5\ E^{-03}$
ECM-receptor interaction	13	$1.7\ E^{-03}$
Small cell lung cancer	12	$5.3\ E^{-03}$
Focal adhesion	21	$5.8\ E^{-03}$
Cell cycle	7	$7.2\ E^{-04}$
Neurotrophin signalling pathway	15	$6.9\ E^{-03}$
Basal cell carcinoma	9	$9.0\ E^{-03}$
TGF-beta signalling pathway	11	$1.9\ E^{-02}$
Chondroitin sulfate biosynthesis	5	$2.9\ E^{-02}$
Endocytosis	17	$4.0\ E^{-02}$
ErbB signalling pathway	10	$4.5\ E^{-02}$
Apoptosis	10	$4.5\ E^{-02}$
Axon guidance	13	$4.6\ E^{-02}$
Cytokine-cytokine receptor interaction	21	$7.5\ E^{-02}$
Hedgehog signalling pathway	7	$8.1\ E^{-02}$
Colorectal cancer	9	$8.4\ E^{-02}$
Pancreatic cancer	8	$9.3\ E^{-02}$

4.3.2.1.3.1 Effects of TNC knockdown on genes associated with ECM and tumour progression

The KEGG pathway analysis by DAVID (Table 4.4) identified 72 genes associated with different pathways involved in the development of cancer, in particular signalling pathways associated with the interactions between cells and ECM. However, these 72 genes were subjected to further analysis using DAVID and Coexpressdb (Obayashi, Kinoshita 2011), to identify the correlations between pathways and to determine the overlapping genes. Looking at the genes that were up-regulated or down-regulated when analysed by DAVID and Coexpressdb, there were 23 overlapping genes found in more than one pathway (Figure 4.10), which reflect their importance in the development of breast cancer as a result of TNC knockdown. In addition, 21 genes were associated with focal adhesion, with 17 overlapping genes in different pathways and a network of 42 genes co-expressed.

Table 4.4 Pathway analysis in of the regulated genes associated with cancer progression

Pathway	Genes count	p-value	Genes identified
Pathways in cancer	32	1.4 E^{-03}	BID, E2F2, FGFR3, WNT5B, XIAP, ARNT2, EGLN3, TCF7L2, MMP1, SUFU, TGFB2, LAMB2, PIK3CA, PIK3R5, CCNA1, AXIN2, FN1, BMP4, COL4A4, FZD8, COL4A2, BMP2, RET, TGFBR2, CDK6, SMAD2, BAD, BIRC3, COL4A6, WNT2B, PIAS2, WNT9A
ECM-receptor interaction	13	1.7 E^{-03}	COL4A4, COL4A2, TNC, COL5A2, COL4A6, COL5A1, HMMR, ITGA9, LAMB2, ITGB8, RELN, TSP-1, FN1
Focal adhesion	21	5.8 E^{-03}	COL4A4, COL4A2, FLT1, XIAP, TNC, BAD, BIRC3, COL5A2, COL5A1, COL4A6, PAK6, ITGA9, LAMB2, ITGB8, PIK3CA, PIK3R5, PDGFC, RELN, TSP-1, SHC4, FN1
Cell cycle	7	7.2 E^{-04}	YWHAH, CDC14B, SMAD2, ANAPC10, CCNA1, YWHAE, TGFB2
TGF-beta signalling pathway	11	1.9 E^{-02}	INHBB, BMP4, NOG, BMP2, SMAD6, FST, GDF5, TGFBR2, SMAD2, TSP-1, TGFB2
ErbB signalling Pathway	10	4.5 E^{-02}	PAK6, ERBB4, EREG, ERBB3, PIK3CA, PIK3R5, BAD, AREG, NRG1, SHC4
Apoptosis	10	4.5 E^{-02}	BID, XIAP, PRKAR1A, PIK3CA, PIK3R5, BAD, PRKACB, BIRC3, IL1A, NGF

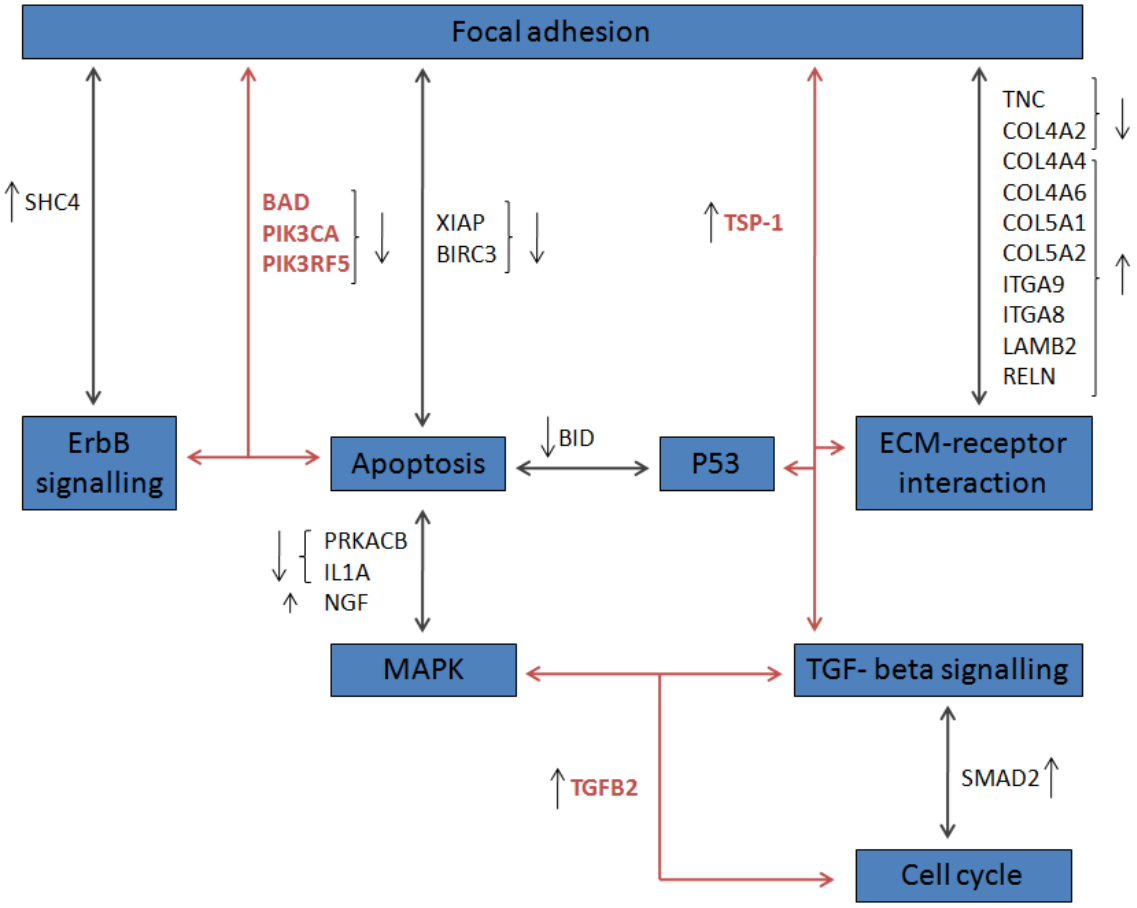


Figure 4.10: Differential genes expression that overlapped in multiple pathways. Overlapping genes in 3 pathways or more are highlighted in red, whereas genes in only one pathway are highlighted in black. Up- or down-regulated genes are shown by small arrows.

Next, we sought to determine the most significant gene changes by looking at the top 20 up- and down-regulated genes based on average fold change common across both cell lines. These are listed in Table 4.5 and Table 4.6.

Table 4.5 The top 10 up-regulated genes resulting from TNC knockdown in transfected breast cancer cell lines with total TNC siRNA compared to scrambled siRNA

Gene Symbol	Average fold change	Fold Change			
		MDA-MB-231		MDA-MB-436	
		24 hrs	48 hrs	24 hrs	48 hrs
DISC1	5.5	3.3	4.1	7.7	7.0
OLFML3	4.8	2.4	5.6	2.5	8.6
EMP2	4.4	3.6	5.2	3.9	4.8
ANAPC10	3.4	3.1	3.0	3.1	4.2
YWHAH	2.9	3.1	2.5	3.2	2.8
SAR1B	2.8	2.6	2.7	2.8	2.1
SEMA6D	2.6	2.2	2.7	2.1	3.3
SEMA4F	2.6	2.2	3.4	2.4	2.4
QKI	2.5	2.3	2.0	3.0	2.5
TGFB2	2.5	2.5	3.4	2.0	2.2

Table 4.6 The top 10 down-regulated genes resulting from TNC knockdown in transfected breast cancer cell lines with total TNC siRNA compared to scrambled siRNA

Gene Symbol	Average fold change	Fold Change			
		MDA-MB-231		MDA-MB-436	
		24 hrs	48 hrs	24 hrs	48 hrs
CREBL2	-6.6	-4.3	-6.9	-5.0	-10.2
YWHAE	-4.3	-3.9	-4.1	-4.5	-4.7
PLEKHA3	-3.7	-2.5	-4.3	-3.0	-5.0
NSA2	-3.5	-3.0	-3.1	-3.7	-4.2
CDC138	-3.4	-3.7	-3.9	-3.3	-2.8
GPATCH4	-2.9	-3.1	-2.7	-2.5	-3.3
KIF3B	-2.9	-2.3	-2.8	-3.0	-3.6
RNF138	-2.5	-2.3	-2.7	-2.6	-2.4
CDC14B	-2.5	-2.1	-2.6	-2.4	-3.0
UBE2Q1	-2.5	-2.3	-2.2	-2.9	-2.8

4.3.3 RT-qPCR analysis of differential gene expression

Candidate genes (*CREBL2, YWHAE, CDCD14B, DISC1* and *QKI*) were selected for further validation from the top 20 overlapping genes in both breast cell lines transfected with total TNC siRNA compared to the scrambled siRNA. In addition, the selection was also according to their role in cancer, particularly those associated with cancer cell invasion and proliferation. One additional gene (*RRAS2*) was not in the top 20 regulated genes and also selected for subsequent validation. *RRAS2* was significantly down-regulated as a result of TNC knockdown ($p = 0.00058$) with > 2 fold down regulation compared to cells transfected with the control scrambled siRNA. In addition, previous findings of the effects of *RRAS2* in breast cancer, hepatocellular carcinoma (HCC), oesophageal tumorigenesis and skin cancers showed that the up-regulation of *RRAS2* was implicated in the tumour growth and invasive phenotype (Keely et al. 1999; Luo et al. 2010;Lee et al. 2011; Sharma et al. 2005). All candidate genes showed ≥ 2 fold up or down regulation compared to cells transfected with the control scrambled siRNA (Table 4.7). Figure 4.11 shows hierarchical clustering based on the number of probes of the 6 genes selected for further validation by quantitative RT-PCR.

Table 4.7 Genes selected for validation by (RT)-qPCR

Gene Symbol	No. of probes	Average fold change	Non-adjusted p-value	FDR p-value	Function and role in cancer
CREBL2	3	-5.6	9.0 E^{-06}	6.5 E^{-04}	Up-regulation plays an important role in multiple steps of breast cancer bone metastasis (Son et al. 2010). It plays a critical role in adipogenesis and lipogenesis (Ma et al. 2011).
YWHAE	1	-4.0	1.9 E^{-06}	8.9 E^{-04}	Belongs to the 14-3-3 family of proteins. Binding to MK5 inhibits cell migration (Tak et al. 2007).
CDC14B	3	-2.5	1.7^{-05}	8.5 E^{-04}	Depletion causes severe mitotic defects by altered regulation of mitotic exit (Tumurbaatar et al. 2011).
RRAS2	1	-2.4	7.2 E^{-06}	5.8 E^{-04}	Increases the growth properties of breast carcinoma cells (Keely et al. 1999). Promotes motility of hepatocellular carcinoma (HCC) cells in vitro and intrahepatic metastasis in vivo (Luo et al. 2010). Up-regulated in highly aggressive skin tumours (Lee et al. 2011). Is associated with early stages of eosophageal tumorigenesis (Sharma et al. 2005).
DISC1	1	4.0	1.6 E^{-04}	2.3 E^{-03}	Plays a critical role in foetal and adult brain development and neuronal function (Bradshaw, Porteous 2012).
QKI	1	2.2	1.7 E^{-04}	2.5 E^{-03}	Over expression causes reduced proliferation and tumorigenesis (Yang et al. 2010;Bian et al. 2012). Reduced expression correlates with poor differentiation status, depth of invasion, gastric lymph node metastasis, distant metastasis, advanced TNM stage, and poor survival (Bian et al. 2012)

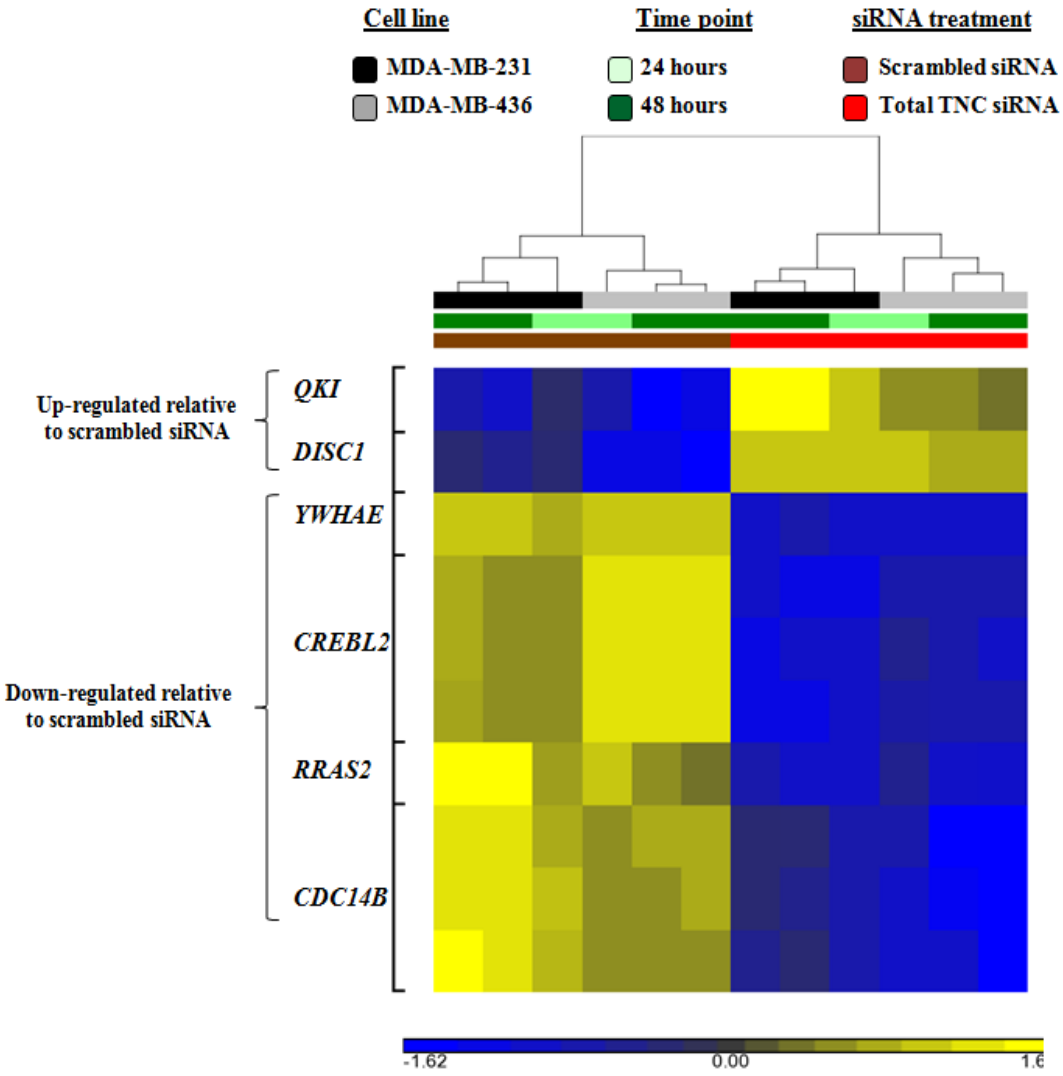


Figure 4.11: Hierarchical clustering of the 6 selected candidate genes. It show the differences based on different probes comparing scrambled siRNA with total TNC siRNA. Up-regulated expression is represented in yellow, whereas down regulated expression is represented in blue.

4.3.3.1 Optimisation of Taq Man real time qPCR assays

Next, expression of the 6 candidate genes was investigated in the transfected MDA-MBA-231 and MDA-MB-436 cells using real-time qPCR. Standard curves were produced for each assay using mRNA isolated from various cell lines. All assays showed high efficiencies and consistent R^2 values (Table 4.8 and Figure 4.12). Detection of *DISC1* was stopped at this stage due to failed standard curve generation on different mRNA templates.

Table 4.8 Quantitative PCR efficiency, R^2 values, slopes and cell lines for the gene assays

Taq Man assay probes	PCR efficiency %	R^2 values	Cell line
CREBL2	96.494	0.970	MDA-MB-436
RRAS2	95.359	0.957	MDA-MB-436
QKI	98.157	0.994	MDA-MB-231
YWHAE	99.751	0.998	SK-MEL-28
CDC14B	107.193	0.988	SK-MEL-28

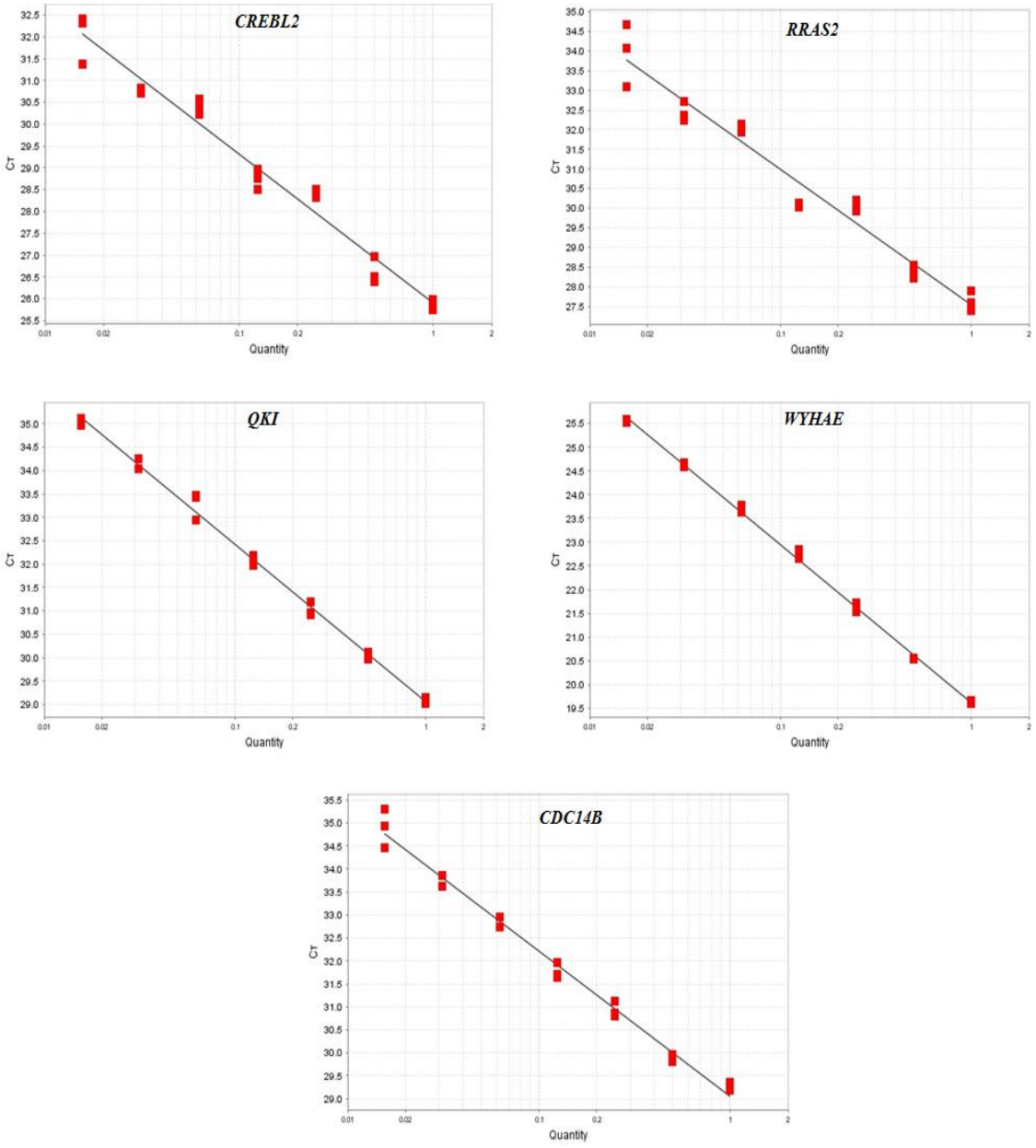


Figure 4.12: Taq Man assay standard curves for the candidate genes.

4.3.4 Analysis of candidate gene expression in TNC positive and negative cell lines

Expression of 5 candidate gene profiles was initially analysed in different breast cell lines: Four cell lines, which endogenously express TNC (MDA-MB-231, MDA-MB-436, MDA-MB-468 and HBL-100), and three TNC null cell lines (MCF-7, T-47D and ZR-75-1). The down regulated genes (*CREBL2, YWHAE, CDC14B* and *RRAS2*) were expressed slightly more in TNC positive cells compared to TNC null cells when normalised to *HPRT1*. *QKI* was expressed at low levels in TNC positive cells except MDA-MB-468, which predominantly expresses truncated TNC when analysed by RT-qPCR and Western blot; whereas TNC null cells did not express *QKI* except for MCF-7. In general, no clear pattern of candidate gene expression was present between TNC positive and TNC null cell lines. ΔCt values for each candidate gene in all cell lines are shown in Table 4.9.

Table 4.9 Average ΔCt values of candidate genes in TNC positive and negative cell lines.

Cell line		*CREBL2*	*YWHAE*	*CDC14B*	*RRAS2*	*QKI*	TNC
TNC Null cell lines	**MCF-7**	4.45	-0.34	7.37	4.82	5.28	Un det
	T-47D	5.34	-2.04	8.59	4.22	Un det	Un det
	ZR-75-1	6.30	-0.36	9.93	6.13	Un det	Un det
TNC Positive cell lines	**MDA-MB-231**	3.62	-2.04	7.08	3.54	7.34	2.91
	MDA-MB-436	3.09	-0.65	8.10	4.18	7.81	2.41
	MDA-MB-468	5.88	0.36	9.51	5.91	Un det	4.11
	HBL-100	6.75	0.42	7.91	4.38	7.83	1.21

Un det = Un determined

4.3.4.1 Analysis of the five candidates in siRNA treated MDA-MB-231 and MDA-MB-436 cells

Following candidate gene identification, RT-qPCR analysis of mRNA using inventoried TaqMan assays was performed. However, the expression of all candidate genes at the protein level was not performed due to time limitations.

The expression profile of the five candidate genes was analysed using the same cDNA template as used for the microarray, generated from MDA-MB-231 and MDA-MB-436 cells transfected with the total TNC siRNA. Relative quantitation (RQ) values were calculated using the mean Ct value obtained from three replicates normalised to the mean of an endogenous control (*HPRT-1*). All assays were performed in triplicate.

Note, the level of gene expression is different between both cell lines, and the treatment with scrambled siRNA is not expected to cause changes on gene expression. Therefore, in order to show a clear difference between siRNA treatments, the graphs for all candidate gene expression were presented based on the relative quantitation to cells transfected with scrambled siRNA (means ± SD for three independent experiments).

4.3.4.1.1 Validation of the down regulated genes

Analysis of *CREBL2*, *YWHAE* and *RRAS2* in cells transfected with total TNC siRNA confirmed the results obtained from the Gene Chip Microarray analysis. Significant down-regulation was observed in both cell lines at both time points ($p < 0.001$ for both) (Figure 4.13 and Table 4.10). However, analysis of *CDC14B* also showed down regulated expression with no statistical significance at some time points. In MDA-MB-231 cells, *CDC14B* was significantly down regulated at 24 hrs ($p < 0.01$), but not at 48 hrs. In MDA-MB-436 cells, it was only down regulated at 48 hrs ($p < 0.01$) (Figure 4.13 and Table 4.10).

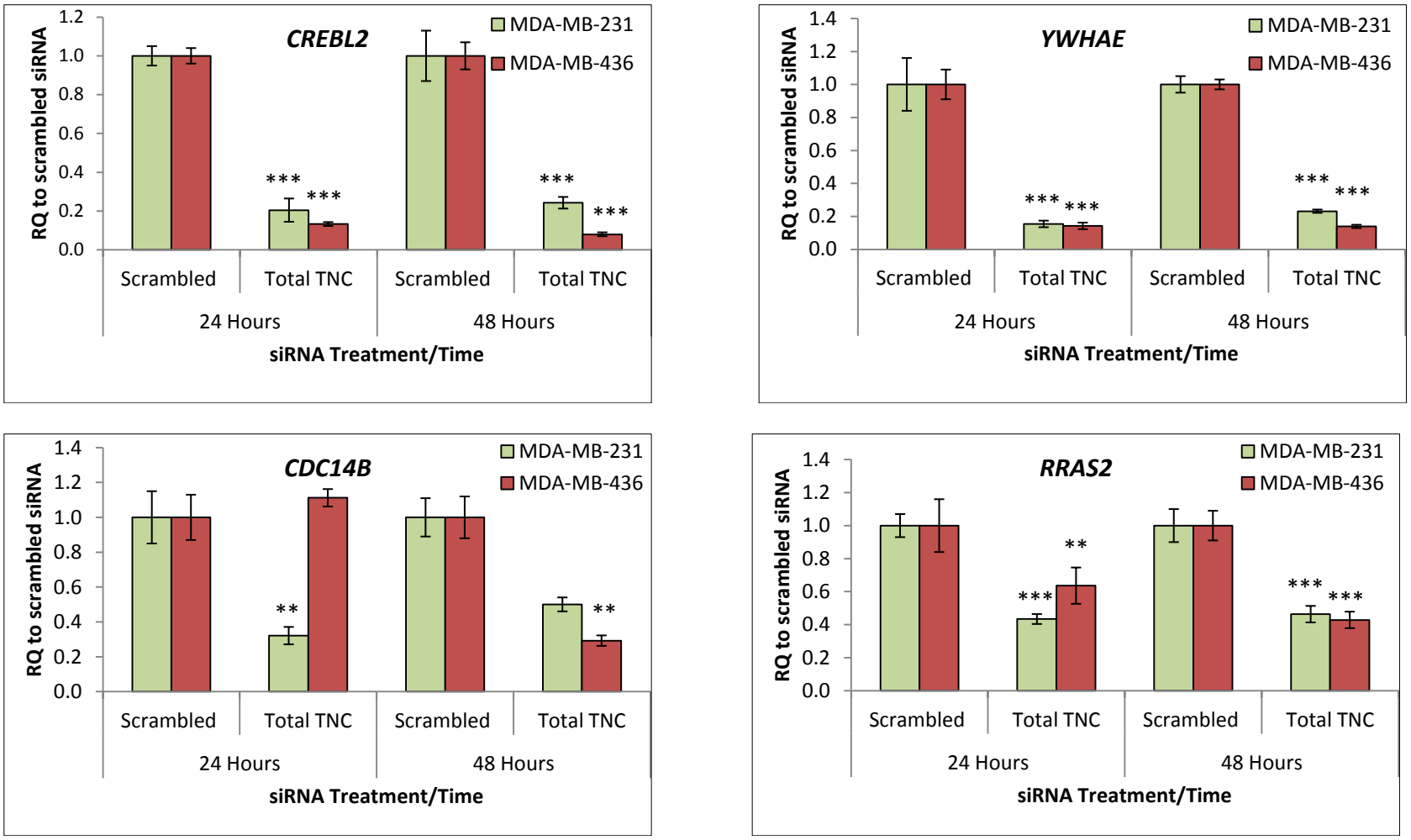


Figure 4.13: Relative expression values of down regulated genes in transfected MDA-MB-231 and MDA-MB-436 breast cancer cell line with total TNC siRNA compared to scrambled siRNA control. The graph shows the mean RQ value (± SEM) between samples compared to scrambled siRNA. Significant differences (= p≤0.01; ***=p≤0.001) are indicated by asterisks.**

Table 4.10 RT-qPCR validation of down regulated genes expression in transfected cell lines with total TNC siRNAs.

Gene	Cell line	Time point	siRNA	RT-qPCR data					Microarray data	
				Mean Ct	ΔCt	RQ	SEM	*P* value	RQ	*P* value
CREBL2	MDA-MB-231	24 Hrs	Scrambled	25.57	4.25	1.00	0.21	-	-	-
			Total TNC	27.71	6.55	0.20	0.13	<0.001	0.23	<0.0001
		48 Hrs	Scrambled	23.83	3.43	1.00	0.11	-	-	-
			Total TNC	26.05	5.5	0.24	0.07	<0.001	0.14	<0.0001
	MDA-MB-436	24 Hrs	Scrambled	21.55	-0.03	1.00	0.11	-	-	-
			Total TNC	24.38	2.6	0.13	0.12	<0.001	0.20	<0.0001
		48 Hrs	Scrambled	22.22	4.32	1.00	0.13	-	-	-
			Total TNC	25.93	7.8	0.08	0.20	<0.001	0.09	<0.0001
YWHAE	MDA-MB-231	24 Hrs	Scrambled	21.34	0.03	1.00	0.06	-	-	-
			Total TNC	23.92	2.77	0.15	0.09	<0.001	0.26	<0.0001
		48 Hrs	Scrambled	21.21	1.06	1.00	0.07	-	-	-
			Total TNC	23.99	3.17	0.14	0.19	<0.001	0.24	<0.0001
	MDA-MB-436	24 Hrs	Scrambled	21.46	-0.6	1.00	0.08	-	-	-
			Total TNC	23.72	2.21	0.14	0.16	<0.001	0.22	<0.0001
		48 Hrs	Scrambled	18.81	1.00	1.00	0.14	-	-	-
			Total TNC	21.71	3.85	0.14	0.08	<0.001	0.21	<0.0001
CDC14B	MDA-MB-231	24 Hrs	Scrambled	30.16	8.84	1.00	0.05	-	-	-
			Total TNC	31.67	10.52	0.32	0.17	<0.01	0.48	<0.0001
		48 Hrs	Scrambled	32.09	10.11	1.00	0.13	-	-	-
			Total TNC	32.27	11.12	0.50	0.09	ns	0.38	<0.0001
	MDA-MB-436	24 Hrs	Scrambled	30.51	10.11	1.00	0.12	-	-	-
			Total TNC	31.67	10.50	1.11	0.15	ns	0.45	<0.0001
		48 Hrs	Scrambled	31.84	13.83	1.00	0.13	-	-	-
			Total TNC	33.68	15.62	0.29	0.09	<0.01	0.33	<0.0001
RRAS2	MDA-MB-231	24 Hrs	Scrambled	26.08	4.77	1.00	0.05	-	-	-
			Total TNC	27.14	5.98	0.43	0.04	<0.001	0.48	<0.0001
		48 Hrs	Scrambled	25.98	5.58	1.00	0.09	-	-	-
			Total TNC	27.25	6.68	0.46	0.14	<0.001	0.43	<0.0001
	MDA-MB-436	24 Hrs	Scrambled	26.51	4.68	1.00	0.05	-	-	-
			Total TNC	27.12	5.32	0.64	0.11	<0.01	0.50	<0.0001
		48 Hrs	Scrambled	26.61	8.63	1.00	0.13	-	-	-
			Total TNC	27.9	10.05	0.43	0.11	<0.001	0.40	<0.0001

Ct= Cycle threshold; SEM= Standard Error of the Mean; ΔCt= Delta Ct; RQ= Relative Quantitation; ns= not significant

4.3.4.1.2 Validation of the up-regulated genes

Analysis of *QKI* in cells transfected with total TNC siRNA confirmed the results obtained from the Gene Chip analysis. Significant up-regulation was observed in both cell lines transfected with total TNC siRNA at 48 hrs ($p < 0.001$ and $p < 0.01$) (Table 4.11 and Figure 4.14).

Table 4.11 RT-qPCR analysis of *QKI* expression in transfected breast cancer cell line with total TNC siRNAs.

Gene	Cell line	Time point	siRNA	RT-qPCR data					Microarray data	
				Mean Ct	ΔCt	RQ	SEM	*P* value	RQ	*P* value
QKI	MDA-MB-231	24 Hrs	Scrambled	30.65	9.34	1.00	0.02	-	-	-
			Total TNC	30.54	9.38	0.98	0.08	ns	2.30	<0.0001
		48 Hrs	Scrambled	31.84	11.43	1.00	0.12	-	-	-
			Total TNC	29.12	8.57	7.48	1.20	<0.001	2.00	<0.0001
	MDA-MB-436	24 Hrs	Scrambled	33.28	11.43	1.00	0.09	-	-	-
			Total TNC	32.84	11.02	1.34	0.28	ns	3.00	<0.0001
		48 Hrs	Scrambled	34.33	16.53	1.00	0.14	-	-	-
			Total TNC	33.32	15.46	2.18	0.45	<0.01	2.50	<0.0001

Ct= Cycle threshold; SEM= Standard Error of the Mean; ΔCt= Delta Ct; RQ= Relative Quantitation; ns= not significant

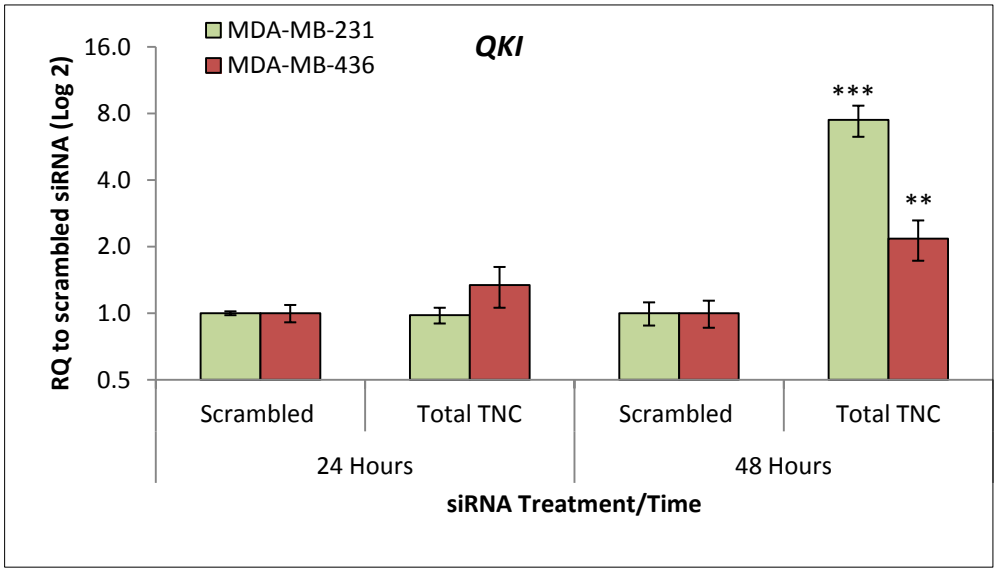


Figure 4.14: Relative expression values of Q*KI* gene in transfected MDA-MB-231 and MDA-MB-436 breast cancer cell line with total TNC siRNA compared to scrambled siRNA control. The graph shows the mean RQ value (± SEM) between samples compared to scrambled siRNA. Significant differences (= p≤0.01; ***=p≤0.001) are indicated by asterisks.**

4.3.4.1.3 Summary of RT-qPCR analysis of transfected cells with total TNC siRNA

RT-qPCR analysis of the candidate genes confirmed the effects of total TNC knockdown on candidate gene expression and correlated with GeneChip analysis of both breast cancer cell lines (MDA-MB-231 and MDA-MB-436) transfected with total TNC siRNA at both time points. Furthermore, RT-qPCR analyses of *CREBL2, YWHAE, RRAS2* and *QKI* showed similar results with GeneChip analysis; whereas there was some variance found with *CDC14B*. For example, *CDC14B* expression in MDA-MB-436 at 24 hrs was shown to be up-regulated by RT-qPCR analysis; whereas it appeared down-regulated by GeneChip analysis. In addition, there were no significant changes shown in *QKI* expression in both cell lines at 24 hrs post transfection. The correlation summary between GeneChip and RT-qPCR relative quantitation is shown in Table 4.12.

Table 4.12 Comparison of RQ between RT-qPCR analysis and GeneChip analysis

Genes	MDA-MB-231				MDA-MB-436			
	RT-qPCR (RQ)		GeneChip (RQ)		RT-qPCR (RQ)		GeneChip (RQ)	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
CREBL2	0.20***	0.24***	0.23***	0.14***	0.13***	0.08***	0.20***	0.09***
YWHAE	0.15***	0.14***	0.26***	0.24***	0.23***	0.14***	0.22***	0.21***
CDC14B	0.32**	0.50 ^{ns}	0.48***	0.38***	1.11 ^{ns}	0.29**	0.45***	0.33***
RRAS2	0.43***	0.46***	0.48***	0.43***	0.64**	0.43***	0.50***	0.50***
QKI	0.98 ^{ns}	7.48***	2.30***	2.00***	1.34 ^{ns}	2.18**	3.00***	2.50***

** = p≤0.001; ***=p≤0.001; ns= not significant

4.3.4.2 Effect of specific TNC isoform knockdown

Next, the effect of specific TNC isoform knockdown was examined with respect to the candidate genes. MDA-MB-231 and MDA-MB-436 cells were transfected with siRNAs targeting two high MW TNC isoforms (TNC-14 and TNC-14-AD1) and incubated for 24 and 48 hrs. RT-qPCR was performed to test the efficiency of TNC high MW isoform knockdown, and to analyse candidate gene expression in order to assess whether there were different effects on expression of the candidate genes as a result of specific TNC isoform knockdown. TNC expression was down regulated by >80 % at both 24 and 48 hrs using TNC-14 siRNA. Whereas, using TNC-14-AD1 siRNA caused down regulation by > 50 % at 24 hrs, and by > 80 % at 48 hrs (Figure 4.15).

Real time qPCR analysis showed statistically significant down regulation of TNC high MW isoforms (TNC-14/16 and TNC-AD1) caused by siRNA targeting TNC-14 (P<0.001), in both cell lines at both time points. TNC-14-AD1 siRNA showed a significant down regulation on TNC-AD1 in both cell lines at 24 and 48 hrs post transfection, and also showed a significant up-regulation of total TNC in both cell lines at both time points (Figure 4.15). A summary of Ct mean values, standard deviation and relative expression is shown in Table 4.13

Table 4.13 Summary of Ct values for TNC specific isoforms siRNA transfected in breast cancer cell lines

Cell lines	siRNAs	TNC Probes	Time Point									
			24 hrs					48 hrs				
			Ct Mean	ΔCt	RQ	SEM	*P value*	Ct Mean	ΔCt	RQ	SEM	*P value*
MDA-MB-231	Scrambled	Total TNC	24.6	2.9	1.00	0.02	-	25.7	4.3	1.00	0.04	-
		TNC-14/16	27.1	5.4	1.00	0.07	-	29.1	7.7	1.00	0.21	-
		TNC-AD1	28.2	6.5	1.00	0.06	-	29.4	8.0	1.00	0.27	-
	TNC-14	Total TNC	24.7	3.7	0.60	0.06	ns	26.9	5.3	0.51	0.04	ns
		TNC-14/16	28.9	7.8	0.20	0.03	<**0.001**	33.2	11.5	0.07	0.02	<**0.001**
		TNC-AD1	29.7	8.7	0.22	0.04	<**0.001**	32.1	10.4	0.22	0.08	<**0.001**
	TNC-14/AD1	Total TNC	23.4	2.0	1.86	0.21	<**0.05**	24.5	3.0	2.54	0.35	<**0.01**
		TNC-14/16	27.2	5.8	0.87	0.27	ns	29.3	7.8	0.94	0.11	ns
		TNC-AD1	28.7	7.3	0.59	0.07	<**0.05**	30.9	9.4	0.38	0.08	<**0.01**
MDA-MB-436	Scrambled	Total TNC	24.3	2.4	1.00	0.09	-	25.9	4.1	1.00	0.05	-
		TNC-14/16	27.5	5.6	1.00	0.15	-	28.3	6.5	1.00	0.03	-
		TNC-AD1	27.7	5.9	1.00	0.24	-	27.1	5.4	1.00	0.04	-
	TNC-14	Total TNC	24.5	2.9	0.73	0.09	ns	26.5	4.8	0.63	0.05	ns
		TNC-14/16	30.1	8.5	0.14	0.01	<**0.001**	32.9	11.3	0.04	0.01	<**0.001**
		TNC-AD1	28.6	7.1	0.44	0.05	<**0.01**	30.9	9.2	0.08	0.03	<**0.001**
	TNC-14/AD1	Total TNC	23.7	1.6	1.88	0.28	<**0.05**	24.2	2.5	3.09	0.32	<**0.01**
		TNC-14/16	27.9	6.0	0.74	0.03	ns	29.1	7.4	0.54	0.07	ns
		TNC-AD1	28.7	6.8	0.59	0.16	<**0.05**	30.1	8.5	0.13	0.04	<**0.001**

Ct= Cycle threshold; SEM= Standard Error of the Mean; ΔCt= Delta Ct; RQ= Relative Quantitation; ns= not significant

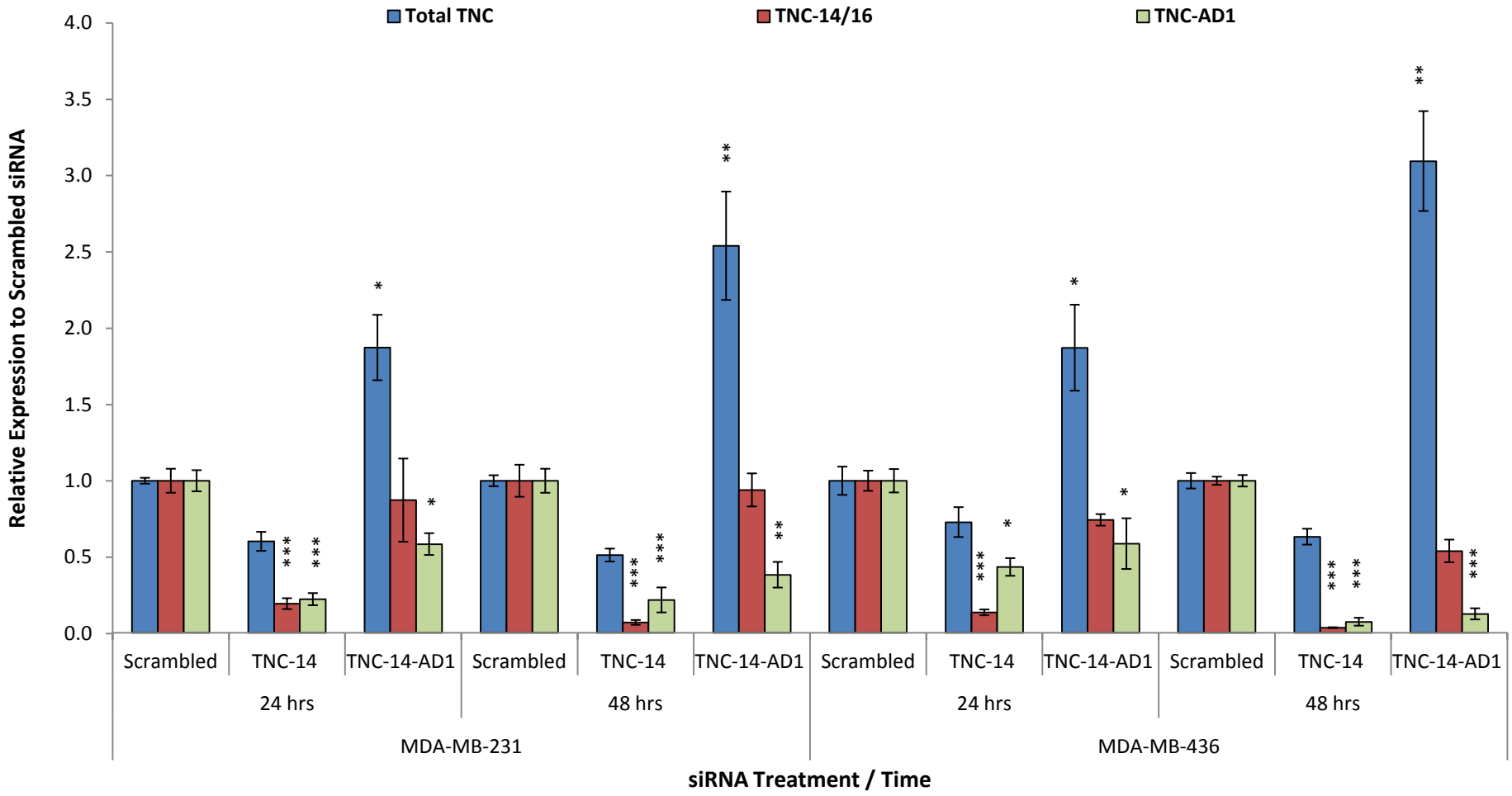


Figure 4.15: Relative expression of TNC on transfected breast cancer cell lines with TNC specific isoforms siRNA at 24 and 48 hrs post transfections. The graph shows the mean RQ value (± SEM) between samples compared to scrambled siRNA. Significant differences (* = p≤05; ** = p≤0.01; ***= p≤0.001) are indicated by three asterisks. Blue columns are the expression of total TNC probe, red and green are the high MW TNC isoforms.

4.3.4.2.1 Down regulated genes

In MDA-MB-436 cells transfected with siRNAs targeting high MW TNC isoforms (TNC-14 and TNC-14-AD1) *CREBL2* showed significant down-regulation at 24 and 48 hrs ($p < 0.001$ and < 0.05). However, in MDA-MB-231 cells transfected with TNC-14-AD1 siRNA, there was a significant up-regulation at 48 hrs, suggesting that *CREBL2* is not regulated by these two specific TNC isoforms (Figure 4.16 and Table 4.14).

Analysis of *YWHAE* in cells transfected with siRNAs targeting high MW TNC isoforms (TNC-14 and TNC-14-AD1) showed a significant down regulation in MDA-MB-436 cells ($p < 0.001$ and < 0.01), but no significant changes was achieved in MDA-MB-231, again suggesting that *YWHAE* is not regulated by specific TNC isoforms in this cell background (Figure 4.16 and Table 4.14).

Both MDA-MB-231 and MDA-MB-436 cells transfected with siRNAs targeting high MW TNC isoforms (TNC-14 and TNC-14-AD1), neither showed a significant change in *CDC14B* expression at either time point - although it increases in MDA-MB-231 cells at 48 hrs (Figure 4.16 and Table 4.14).

In both cells transfected with siRNAs targeting TNC-14, there was a significant down regulation of *RRAS2* at 48 hrs ($p < 0.01$ and $p < 0.001$). But no significant up-or down-regulation was observed in either cell line transfected with TNC-14-AD1 siRNA (Figure 4.16 and Table 4.14). The summary of RT-qPCR data of the down regulated gene expression in transfected breast cancer cell line with high MW TNC siRNAs is shown in Table 4.14.

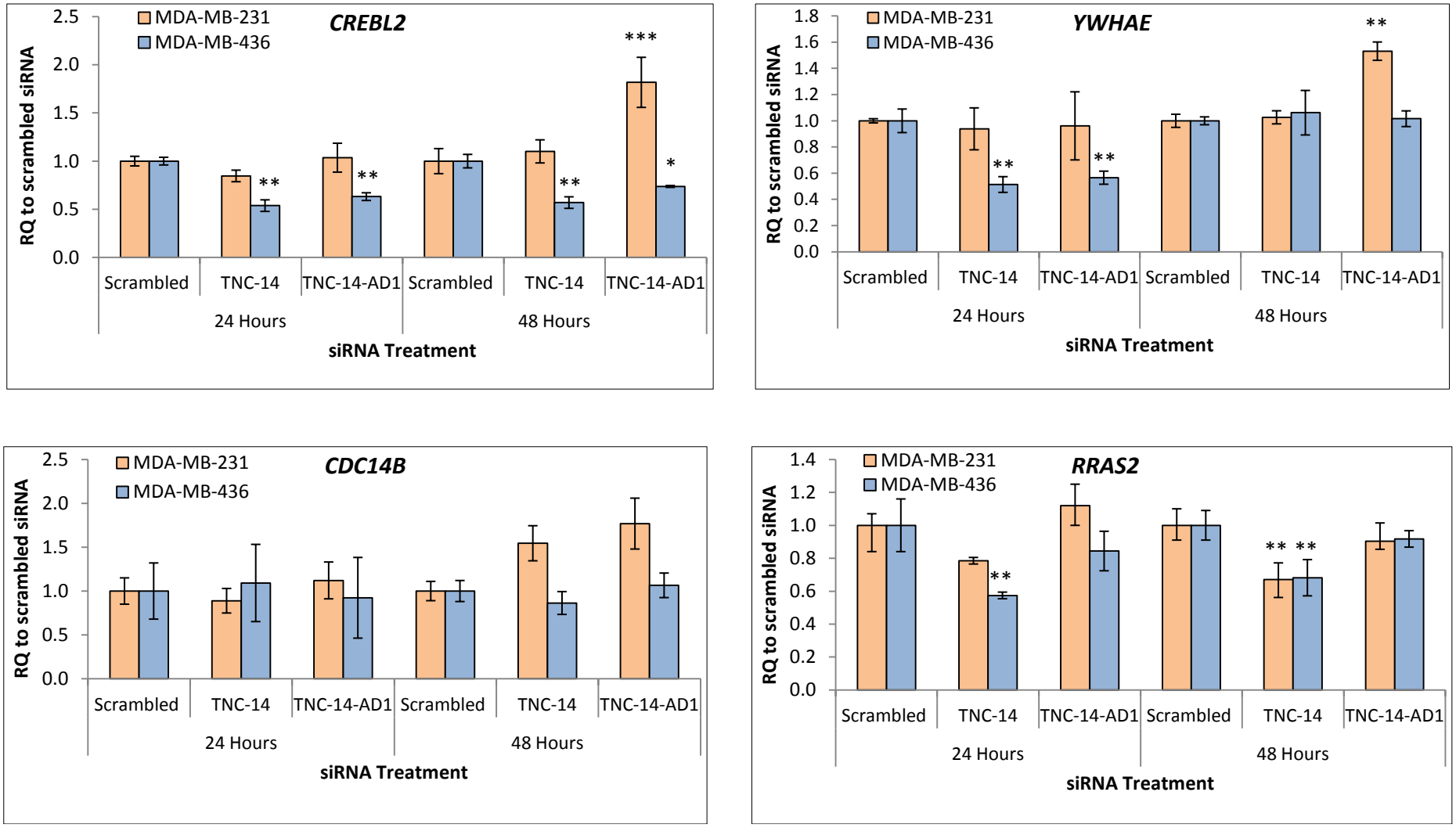


Figure 4.16: Relative expression values of down regulated genes in transfected MDA-MB-231 and MDA-MB-436 breast cancer cell lines with high MW TNC siRNAs compared to scrambled siRNA control. The graph shows the mean RQ value (± SEM) between samples compared to scrambled siRNA. Significant differences (= p≤0.01; ***=p≤0.001) are indicated by asterisks.**

Table 4.14 RT-qPCR analysis of down regulated genes expression in transfected breast cancer cell line with high MW TNC siRNAs

Gene	Time point	siRNA	MDA-MB-231					MDA-MB-436				
			RT-qPCR data					RT-qPCR data				
			Mean Ct	ΔCt	RQ	SEM	*P* value	Mean Ct	ΔCt	RQ	SEM	*P* value
CREBL2	24 Hrs	Scrambled	25.6	4.2	1.00	0.05	-	21.6	-0.3	1.00	0.04	-
		Total TNC	27.7	6.6	0.20	0.06	<0.001	24.4	2.6	0.13	0.01	<0.001
		TNC-14	25.8	4.5	0.85	0.06	ns	22.2	0.6	0.54	0.06	<0.01
		TNC-14-AD1	25.6	4.2	1.04	0.15	ns	22.3	0.4	0.63	0.04	<0.01
	48 Hrs	Scrambled	23.8	3.4	1.00	0.13	-	22.2	4.3	1.00	0.07	-
		Total TNC	26.1	5.5	0.24	0.03	<0.001	25.9	7.8	0.08	0.01	<0.001
		TNC-14	23.7	3.3	1.10	0.12	ns	23.1	5.2	0.57	0.06	<0.001
		TNC-14-AD1	23.4	2.6	1.82	0.26	<0.001	22.8	4.7	0.74	0.01	< 0.05
YWHAE	24 Hrs	Scrambled	21.3	0.3	1.00	0.02	-	21.2	-0.6	1.00	0.09	-
		Total TNC	23.9	2.8	0.15	0.02	<0.001	24.0	2.2	0.14	0.02	<0.001
		TNC-14	21.5	0.2	0.94	0.16	ns	21.9	0.3	0.51	0.06	<0.001
		TNC-14-AD1	21.6	0.4	0.96	0.26	ns	22.1	0.3	0.56	0.05	<0.01
	48 Hrs	Scrambled	21.5	1.0	1.00	0.05	-	18.8	1.0	1.00	0.03	-
		Total TNC	23.7	3.2	0.23	0.01	<0.001	21.7	3.8	0.14	0.03	<0.001
		TNC-14	21.5	1.0	1.03	0.05	ns	18.8	1.3	1.06	0.17	ns
		TNC-14-AD1	21.3	0.4	1.53	0.07	<0.01	19.0	0.8	1.02	0.6	ns
CDC14B	24 Hrs	Scrambled	30.2	8.8	1.00	0.15	-	32.1	10.2	1.00	0.32	-
		Total TNC	31.7	10.5	0.32	0.05	<0.01	32.3	10.5	1.11	0.05	ns
		TNC-14	30.4	9.1	0.89	0.14	ns	31.9	10.4	1.09	0.44	ns
		TNC-14-AD1	30.1	8.7	1.12	0.21	ns	32.7	10.7	0.92	0.46	ns
	48 Hrs	Scrambled	30.5	10.1	1.00	0.11	-	31.8	13.8	1.00	0.12	-
		Total TNC	31.7	11.1	0.50	0.04	ns	33.7	15.6	0.29	0.03	<0.01
		TNC-14	29.9	9.5	1.54	0.20	ns	32.1	14.4	0.86	0.13	ns
		TNC-14-AD1	30.1	9.3	1.77	0.29	ns	31.9	13.9	1.07	0.14	ns
RRAS2	24 Hrs	Scrambled	26.1	4.8	1.00	0.07	-	26.5	4.7	1.00	0.16	-
		Total TNC	27.1	5.9	0.43	0.03	<0.001	27.1	5.3	0.64	0.05	<0.01
		TNC-14	26.4	5.1	0.78	0.02	ns	27.0	5.4	0.57	0.02	<0.01
		TNC-14-AD1	26.0	4.6	1.12	0.13	ns	26.9	4.9	0.84	0.12	ns
	48 Hrs	Scrambled	26.0	5.6	1.00	0.10	-	26.6	8.6	1.00	0.09	-
		Total TNC	27.3	6.7	0.46	0.05	<0.001	27.9	10.1	0.43	0.11	<0.001
		TNC-14	26.6	6.2	0.67	0.10	<0.01	27.2	9.3	0.68	0.11	<0.01
		TNC-14-AD1	26.6	5.7	0.90	0.11	ns	26.9	8.9	0.92	0.05	ns

Ct= Cycle threshold; SEM= Standard Error of the Mean; ΔCt= Delta Ct; RQ= Relative Quantitation; ns= not significant

4.3.4.2.2 Up regulated genes

In both cell lines, siRNAs targeting high MW TNC isoforms (TNC-14 and TNC-14-AD1) there was a significant up-regulation in *QKI* at both time points ($p < 0.001$ for both) (Table 4.15 and Figure 4.17).

Table 4.15 RT-qPCR analysis of *QKI* expression in transfected breast cancer cell line with high MW TNC siRNAs

Gene	Time point	siRNA	MDA-MB-231 RT-qPCR data					MDA-MB-436 RT-qPCR data				
			Mean Ct	ΔCt	RQ	SEM	*P* value	Mean Ct	ΔCt	RQ	SEM	*P* value
QKI	24 Hrs	Scrambled	30.7	9.3	1.00	0.01	-	33.3	11.4	1.00	0.09	-
		Total TNC	30.5	9.4	0.98	0.08	ns	32.8	11.0	1.34	0.28	ns
		TNC-14	28.8	7.5	3.58	0.59	<0.001	31.7	10.2	2.50	0.48	<0.001
		TNC-14-AD1	28.9	7.5	3.63	0.56	<0.001	32.2	10.3	2.38	0.64	<0.001
	48 Hrs	Scrambled	31.8	11.4	1.00	0.12	-	34.3	16.5	1.00	0.14	-
		Total TNC	29.1	8.6	7.48	1.20	<0.001	33.3	15.5	2.18	0.14	<0.01
		TNC-14	27.7	7.2	18.90	0.70	<0.001	31.3	13.5	8.71	0.60	<0.001
		TNC-14-AD1	28.0	7.2	19.25	0.50	<0.001	32.2	14.2	5.08	0.50	<0.001

Ct= Cycle threshold; SEM= Standard Error of the Mean; ΔCt= Delta Ct; RQ= Relative Quantitation; ns= not significant

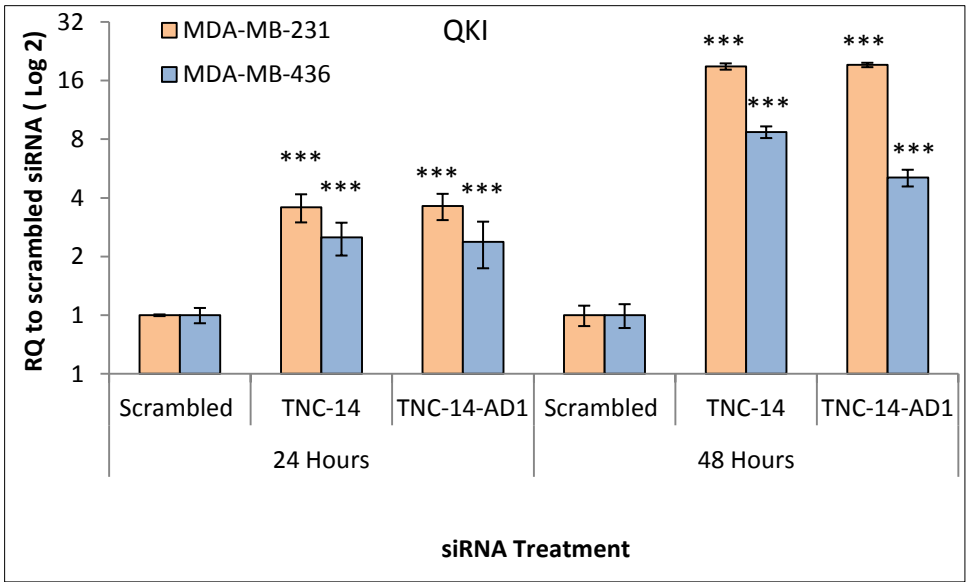


Figure 4.17: Relative expression values of *QKI* gene in transfected MDA-MB-231 and MDA-MB-436 breast cancer cell line with high MW TNC siRNA compared to scrambled siRNA control. The graph shows the mean RQ value (± SEM) between samples compared to scrambled siRNA. Significant differences (*=p≤0.001) are indicated by asterisks.**

4.3.4.2.3 Effects of specific TNC knockdown by siRNA on expression of candidate genes identified by microarray

In summary, RT-qPCR analysis of cells transfected with specific TNC siRNA at both time points showed variable effects caused by the knockdown of specific TNC isoforms. Expression of *QKI* was much higher when analysed by RT-qPCR compared to GeneChip analysis in both cell lines transfected with total TNC at 24 and 48 hrs, confirming that knockdown of specific TNC isoform significantly up-regulated *QKI* expression. In addition, the most significant affect on *QKI* was seen after 48 hrs of transfection in both cell lines. Down regulation of other candidate genes (*CREBL2, YWHAE* and *RRAS2*) was shown to be altered by siRNA targeting TNC-14 in transfected MDA-MB-436 cells. In addition, most of the statistical affects were seen in MDA-MB-436 transfected with both siRNA at 24 hrs post-transfection. *CDC14B* expression was not affected by knockdown of both TNC isoforms in both cell lines at both time points. There was some variance found in gene expression. For example, siRNA targeting TNC-AD1 showed a significant increase in expression of *CREBL2* and *YWHAE* in MDA-MB-231. A summary of the relative expression of candidate genes in cells transfected with specific TNC isoform is shown in Table 4.16.

Table 4.16 Relative expression summary for the 6 candidate genes in cells transfected with high MW TNC siRNA

Cell line	Time point	siRNA	*CREBL2*	*YWHAE*	*CDC14B*	*RRAS2*	*QKI*
MDA-MB-231	24 Hrs	TNC-14	0.85^{ns}	0.94^{ns}	0.89^{ns}	0.78^{ns}	3.59 ***
		TNC-14-AD1	1.04^{ns}	0.96^{ns}	1.12^{ns}	1.12^{ns}	3.64 ***
	48 Hrs	TNC-14	1.10^{ns}	1.03^{ns}	1.54^{ns}	0.67 **	18.91 ***
		TNC-14-AD1	1.82 ***	1.53*	1.77^{ns}	0.90^{ns}	19.25 ***
MDA-MB-436	24 Hrs	TNC-14	0.54***	0.51 ***	1.09^{ns}	0.57***	2.51 ***
		TNC-14-AD1	0.63 ***	0.57**	0.92^{ns}	0.84^{ns}	2.38 ***
	48 Hrs	TNC-14	0.57 ***	1.06^{ns}	0.86^{ns}	0.68 **	8.71 ***
		TNC-14-AD1	0.74 *	1.02^{ns}	1.07^{ns}	0.92^{ns}	5.08 ***

* = p≤05; ** = p≤0.01; ***=p≤0.001, ns = Not significant

4.4 Proteomics

4.4.1 Transfection of MDA-MB-231 cell lines

Next, the effect of TNC knockdown on global protein expression was investigated in MDA-MB-231 cells. Three replicate transfections were performed using the siRNA targeting exon 24 of TNC and scrambled siRNA. The transfected MDA-MB-231 cells were cultured at 37° C in a complete media with 10% FBS for 24 hrs. For the purpose of cell conditioned medium (CM) analysis, the complete media was replaced with Opti-MEM reduced serum media (2% FBS) and incubated for 48 hrs at 37° C. The CM from three wells per siRNA treatment (each well containing 2.5 x10^5 cells with 2 ml CM) was collected and concentrated using centricon columns at 4000 rpm for 30 minutes at 4° C. The transfection efficiency of total TNC knockdown was confirmed by Western blot using the H-300 anti-TNC antibody. Western blot analysis of CM collected from cells transfected with siRNAs targeting total TNC confirmed down regulation of TNC at the protein level compared to cells transfected with scrambled siRNAs (Figure 4.18), as shown by the absence of the two predominant isoforms (TNC-L and TNC-S).

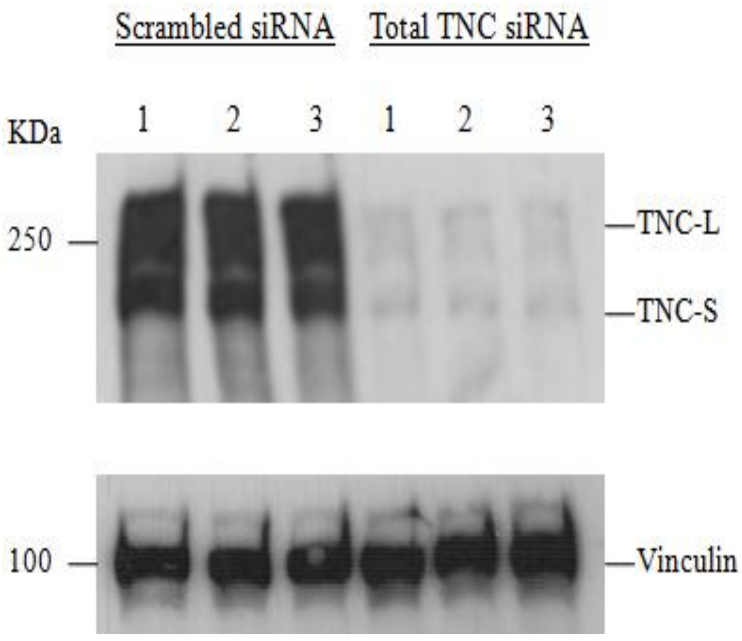


Figure 4.18: Western blot analysis of conditioned media from MDA-MB-231 breast cancer cell lines transfected with an siRNA targeting total TNC and a scrambled siRNA.

The upper panel shows loss of expression of the predominant TNC isoforms (TNC-L and TNC-S). The lower panel shows the loading control (Vinculin).

4.4.2 Proteomics data analysis

Following incubation for 72 hrs, conditioned media was collected and analysed using Liquid Chromatography-Mass Spectrometry (LC-MS) to measure post-translational changes in protein levels resulting from siRNA knockdown of TNC.

This part of the study was a pilot investigation as LC-MS had not been previously performed using conditioned media, and was carried out toward the end of my studentship. LC-MS was performed in collaboration with Dr Don Jones and Mr. Amirmansoor Hakimi, Department of Cancer Studies and Molecular Medicine, University of Leicester, UK.

The raw data obtained from LC-MS was analysed using the Protein Lynx Global server (PLGS) program version 2.3 for protein database search. Data was analysed at two levels: identification and expression levels. Prior to the identification of proteins through the online database, the quantitation of proteins in the tested samples were normalised to the amount of the internal control - Alcohol Dehydrogenase (ADH), which was loaded at a concentration of (50 fm/µl). The identification of protein sequences was performed against the database proteins using PLGS.

Next, the expression level was performed on the normalised data based on two filtrations steps. Firstly, filtration based on the exact match between proteins in the tested samples and the online database protein sequences which resulted in 298 differentially regulated proteins. Secondly, subsequent filtration was performed according to the common proteins detected in all replicates with a p value of ≤ 0.05, which resulted in 72 regulated proteins (14 up-regulated and 58 down-regulated).

Finally, the fold change in protein amounts of cells treated with TNC siRNA was calculated relatively to the amounts protein in cells treated with the scrambled control

siRNA, and the most significant protein changes (top 20 up- and down-regulated proteins) based on the average fold changes was determined Table 4.17.

Table 4.17 The top 20 proteins regulated by TNC knockdown in transfected MDA-MB-231 breast cancer cell lines with total TNC siRNA compared to scrambled siRNA

Regulated proteins		Scrambled siRNA treatment		TNC siRNA treatment		RQ to scrambled siRNA	
		Amount (ng)	SEM	Amount (ng)	SEM	Fold change	P value
Up-regulated proteins	TSP-1	23.46	0.04	69.82	0.10	2.98	≤0.001
	IGFBP7	2.09	0.05	5.21	0.04	2.5	≤0.001
	LGALS3BP	3.41	0.01	6.5	0.01	1.91	≤0.001
	APP	5.05	0.07	7.96	0.05	1.58	≤0.001
	CTSD	1.43	0.05	2.2	0.11	1.54	≤0.01
	SRGN	1.74	0.06	2.56	0.06	1.47	≤0.01
	PRSS23	0.92	0.05	1.34	0.04	1.45	≤0.01
	B2M	1.41	0.06	1.99	0.11	1.41	≤0.05
	CST3	1.06	0.08	1.47	0.08	1.39	≤0.05
	TIMP1	1.43	0.03	1.91	0.09	1.34	≤0.05
Down-regulated proteins	PFN1	2.83	0.09	1.49	0.13	0.53	≤0.05
	ENO1	7.07	0.02	3.35	0.16	0.47	≤0.05
	YWHAZ	5.29	0.14	2.47	0.17	0.47	≤0.05
	VIM	24.32	0.14	11.02	0.22	0.45	≤0.05
	HSP90AB1	9.28	0.05	3.89	0.17	0.42	≤0.01
	ACTN4	6.69	0.05	2.78	0.25	0.41	≤0.01
	HSPA8	8.05	0.04	2.94	0.17	0.36	≤0.001
	ACTB	18.77	0.08	6.13	0.14	0.33	≤0.001
	PPIA	3.03	0.04	0.91	0.17	0.30	≤0.001
	HNRNPA1	2.67	0.06	0.68	0.04	0.26	≤0.001

SEM= Standard error of the mean, RQ= Relative Quantitation; ng = Nanogram

4.4.3 Secretome analysis

To determine differentially secreted proteins in the conditioned media between breast cancer cells treated with siRNA targeting total TNC and cells treated with the scrambled control siRNA, proteins identified by LC-MS were uploaded into Ingenuity Pathways (IPA) (Ingenuity Systems, Redwood City, CA), to distinguish protein localisation. 60 proteins were identified from the data search based on their location within the cell (Table 4.18), with the majority located in the cytoplasm and extracellular space: 75% of proteins were identified as being intracellular (42 proteins in the cytoplasm and 3 in the nucleus), while 18% (11 out of 60) were found to be secreted into the extracellular space (Figure 4.19).

Table 4.18 The secretomes localisation. Ingenuity Pathways (IPA) showed a number of 60 secretomes based of their locations within the cell.

Location	Proteins Account	Identified proteins
Cytoplasm	42	ACTA1,ACTA2,ACTB,ACTC1,ACTG1,ACTG2,ACTN4,CALR,CTSD,EEF1A1, ENO1, GAPDH, HSP90AA1,HSP90AB1, SPA2, HSPA8, NME1, PFN1, PGK1, PPIA, PRDX1, QSOX1, S100A6, SFN, STMN1, UBA1A, TUBA1B, TUBA1C, TUBA3E, TUBA4A, TUBA8, TUBB, TUBB2A, TUBB2B, TUBB3, VIM, YWHAB, YWHAE, YWHAG, YWHAH, YWHAQ,YWHAZ
Extracellular Space	11	CST3, HPX, IGFBP4, IGFBP7, MMP1, PRSS23, PSAP, SRGN, TF, TSP-1, TIMP1
Nucleus	3	HNRNPA1, HNRNPA2B1, NME2
Plasma Membrane	4	APP,B2M,LGALS3BP,MSN

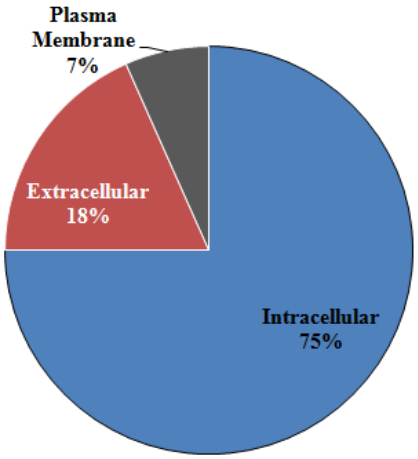


Figure 4.19: Cellular localisation of secretomes. Pie chart shows the distribution of detected proteins with sub-cellular localisation identified in conditioned media of MDA-MB-231 cell line.

Lawlor et al. (2009) classified gene expression profiles based on the molecular breast cancer subtypes using two breast cell lines: MCF-7 to represent the luminal breast subtype, and MDA-MB-231 to represent the basal-like subtype (Lawlor et al. 2009). MCF-7 is a TNC null cell line, whereas MDA-MB-231 is TNC positive (Hancox et al. 2009; Guttery et al. 2010a). Therefore, secreted proteins resulting from knockdown of TNC in MDA-MB-231 in this study were compared with the gene profile study of (Lawlor et al. 2009).

Of the 60 secretomes imported from Ingenuity Pathways (IPA), 24 secreted proteins showed a correlation with this data (4 up-regulated and 20 down-regulated) (Table 4.19). Most of the identified proteins were down-regulated, suggesting that TNC silencing leads to the suppression of proteins associated with tumour progression.

Table 4.19 List of the regulated proteins in the breast cancer secretomes

Secretome	Number of proteins	Gene profiles			TNC silencing
		MCF-7 (TNC null)	MDA-MB-231 (High TNC)	Reference	MDA-MB-231 (Low TNC)
B2M,CTSD, PRSS23,SRGN,	4	Up-regulation	Down-regulation	(Lawlor et al. 2009)	Up-Regulation
CTSD,ACTN4, CALR,EEF1A1, ENO1,HNRNPA1, HSP90AB1,MSN, NME1,NME2, PFN1,PGK1,PPIA, PSAP,VIM, YWHAE,YWHAG, YWHAQ,ACTB,	20	Down-regulation	Up-regulation	(Lawlor et al. 2009)	Down-regulation

4.4.4 Validation of proteomics data by Western blot

Due to the time limitation, only one candidate was selected for further analysis. Thrombospondin 1 (TSP-1) was selected as it was most significantly altered by TNC knockdown, with a 2.98 fold up-regulation compared to cells treated with scrambled siRNA. Conversely, previous microarray studies (Ruiz et al. (2004)) found that the level of TSP-1 was significantly down-regulated in T98G glioblastoma cells cultured on a TNC milieu compared to cells cultured on fibronectin. Moreover, TSP-1 was shown to be differentially up-regulated at the RNA level confirmed by the microarray, following TNC knockdown at the mRNA level.

Western blot analysis of TSP-1 expression was carried out using the same cell conditioned media used for the proteomic investigation, collected from three independent biological transfections of MDA-MB-231 cells with both total TNC and scrambled siRNAs. Western blot analysis showed increased expression of TSP-1 after TNC knockdown, confirming proteomic data (Figure 4.20).

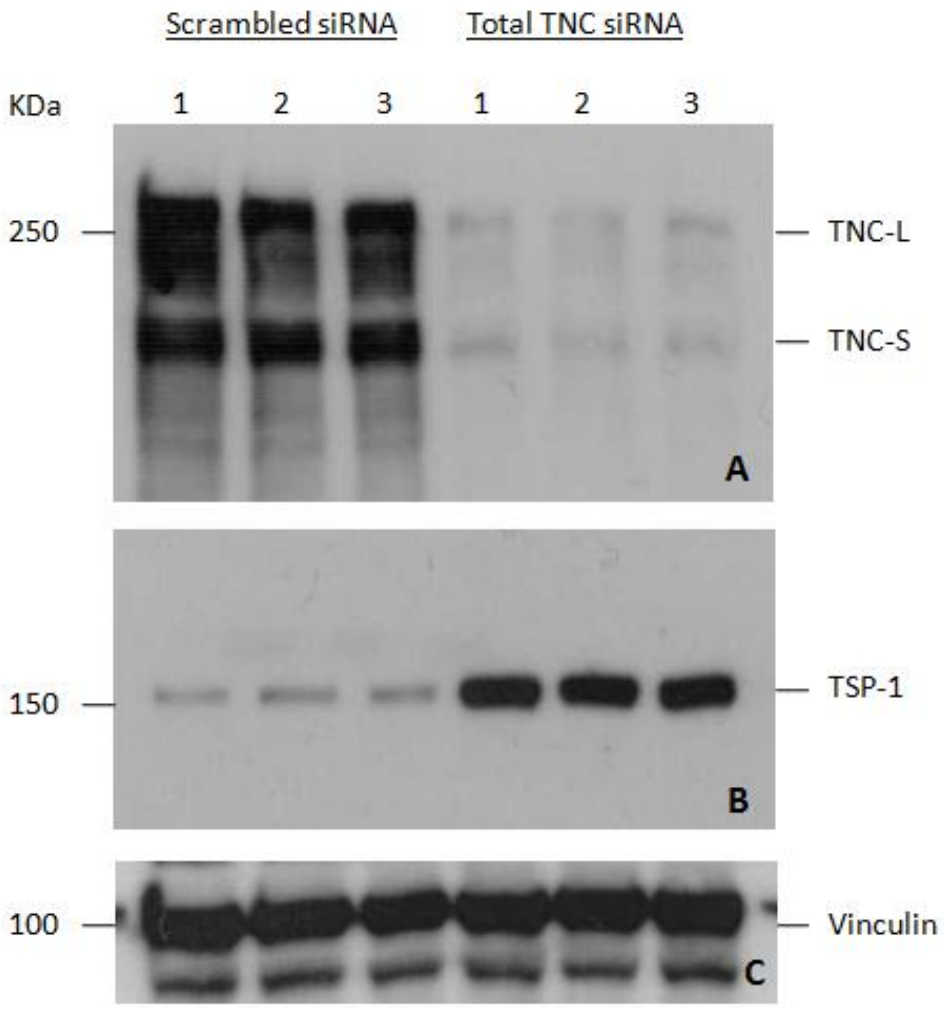


Figure 4.20: Western blot analysis in conditioned media of MDA-MB-231 breast cancer cell lines transfected with siRNA targeting total TNC and scrambled siRNA. A) H300 anti-TNC antibody reactivity confirmed the down-regulated expression of predominant TNC isoforms (TNC-L and TNC-S). B) Anti-TSP-1 reactivity showed the up-regulated expression of TSP-1 in cells transfected with total TNC siRNA. C) Loading control (Vinculin).

4.4.5 Effects of TNC isoform knockdown on TSP-1 expression

To analyse the effect of specific TNC isoform knockdown on TSP-1 expression, MDA-MB-231 cells were transfected with siRNAs targeting high MW TNC isoforms (TNC-14 and TNC-14-AD1) and incubated for 72 hrs. Western blot was performed to test the efficiency of TNC high MW isoform knockdown, and assess whether there are different effects on TSP-1 expression as a result of specific TNC isoform knockdown. Western blot analysis of conditioned media collected from cells transfected with siRNAs

targeting total TNC and TNC high MW isoforms confirmed TNC down-regulation at the protein level using H-300 anti-TNC antibody. However, there was a specific reduction of the high MW TNC level caused by siRNA targeting exon 14 with no effect on truncated TNC levels. There was no observable change in TNC protein levels in cells transfected with siRNAs against exon 14-AD1 (Figure 4.21) as might be expected, TNC-L and TNC-S were the predominant bands detected. In addition, Western blot analysis of TSP-1 expression in cells transfected with total TNC and high MW isoforms confirmed TSP-1 up-regulation in cells transfected with siRNAs targeting total TNC and TNC high MW isoforms compared to cells transfected with scrambled siRNA (Figure 4.21)

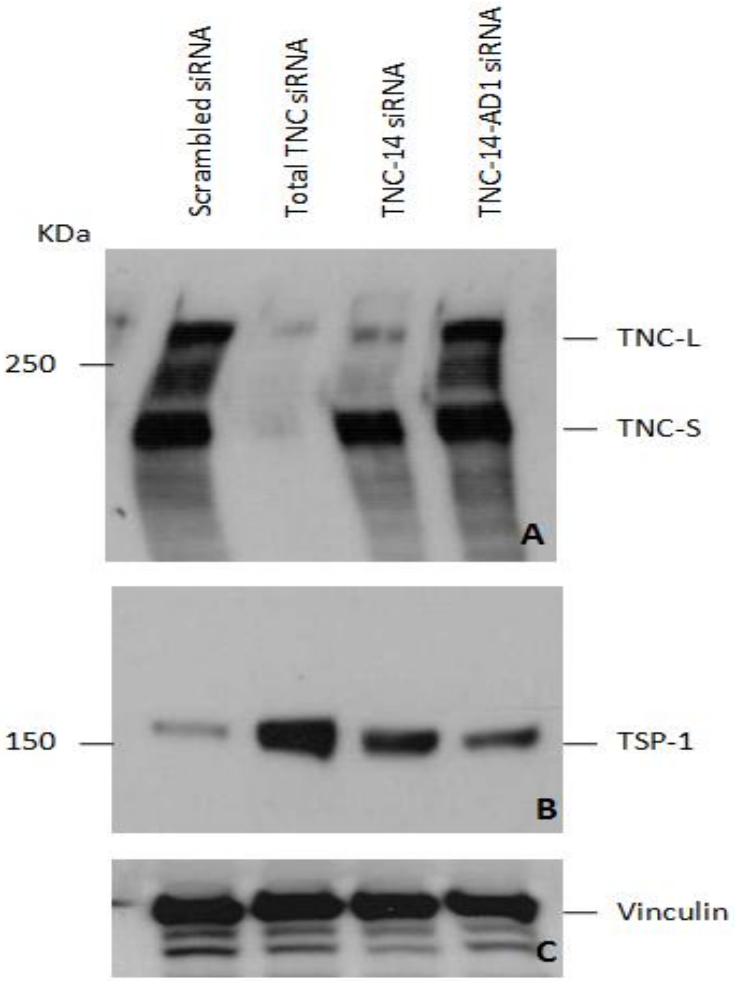


Figure 4.21: Western blot analysis in conditioned media of MDA-MB-231 breast cancer cell lines transfected with siRNA targeting high MW TNC isoforms, total TNC and scrambled siRNA.

A) H300 anti-TNC antibody reactivity confirmed the down-regulated expression of total TNC and high MW TNC isoforms.

B) Anti- TSP-1 reactivity showed the up-regulated expression of TSP-1 in cells transfected with total TNC and high MW TNC isoforms siRNAs.

C) Loading control (Vinculin).

4.4.6 Immunocytochemistry analysis of TSP-1

Further validations were also performed to test the effect of total TNC and specific TNC isoforms on TSP-1 expression by immunocytochemistry (ICC), using MDA-MB-231 cells transfected with total TNC and TNC isoform-specific siRNAs. ICC analysis using an anti-TSP-1 antibody showed increased levels of TSP-1 in cells transfected with total TNC siRNA (36.7%) compared to cells transfected with scrambled siRNA (9.5 %). Cells transfected with TNC-14 and TNC-14-AD1 siRNAs also showed increased TSP-1 (22.5% and 19.1%) (Figure 4.22). Expression percentage in all samples was calculated based on the positive staining average of five fields using immunoRatio (http://153.1.200.58:8080/immunoratio/).

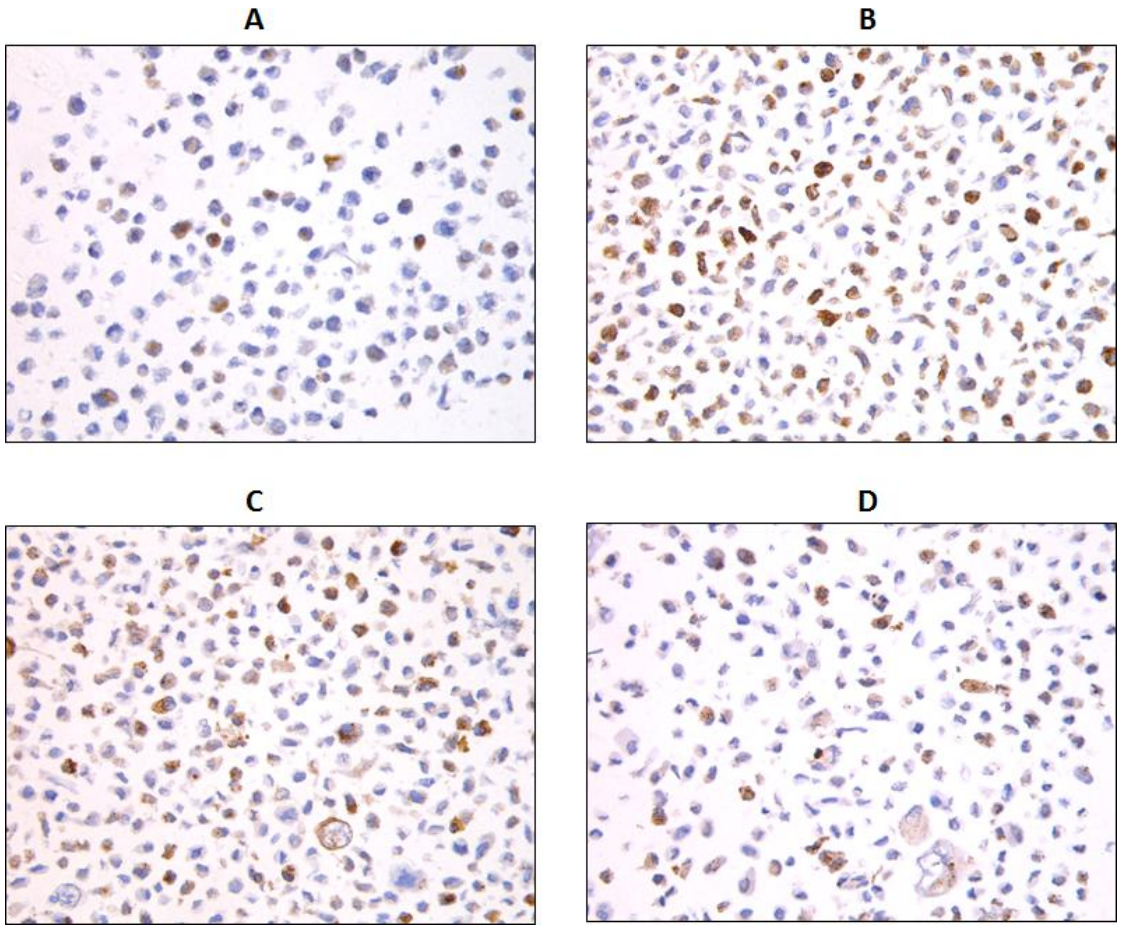


Figure 4.22: ICC analysis of TSP-1 expression in MDA-MB-231 breast cancer cell lines transfected with siRNA targeting total TNC and scrambled siRNA. A) Cells transfected with scrambled siRNA. B) Cells transfected with total TNC siRNA. C) Cells transfected with TNC-14 siRNA. D) Cells transfected with TNC-14-AD1 siRNA.

4.4.7 Expression of TSP-1 in breast cancer tissue

In order to analyse the expression of TSP-1 in breast carcinomas, the TSP-1 antibody was first optimised for use in tissue, using dilutions initially at three concentrations (1:100, 1:300 and 1:500). 1:300 showed the optimal dilution for specific staining with minimal background (Figure 4.23).

In addition, prior to the analysis of TSP-1 expression in breast cancer tissues, IHC was carried out in normal breast tissues, which showed no staining for TSP-1 (Figure 4.24).

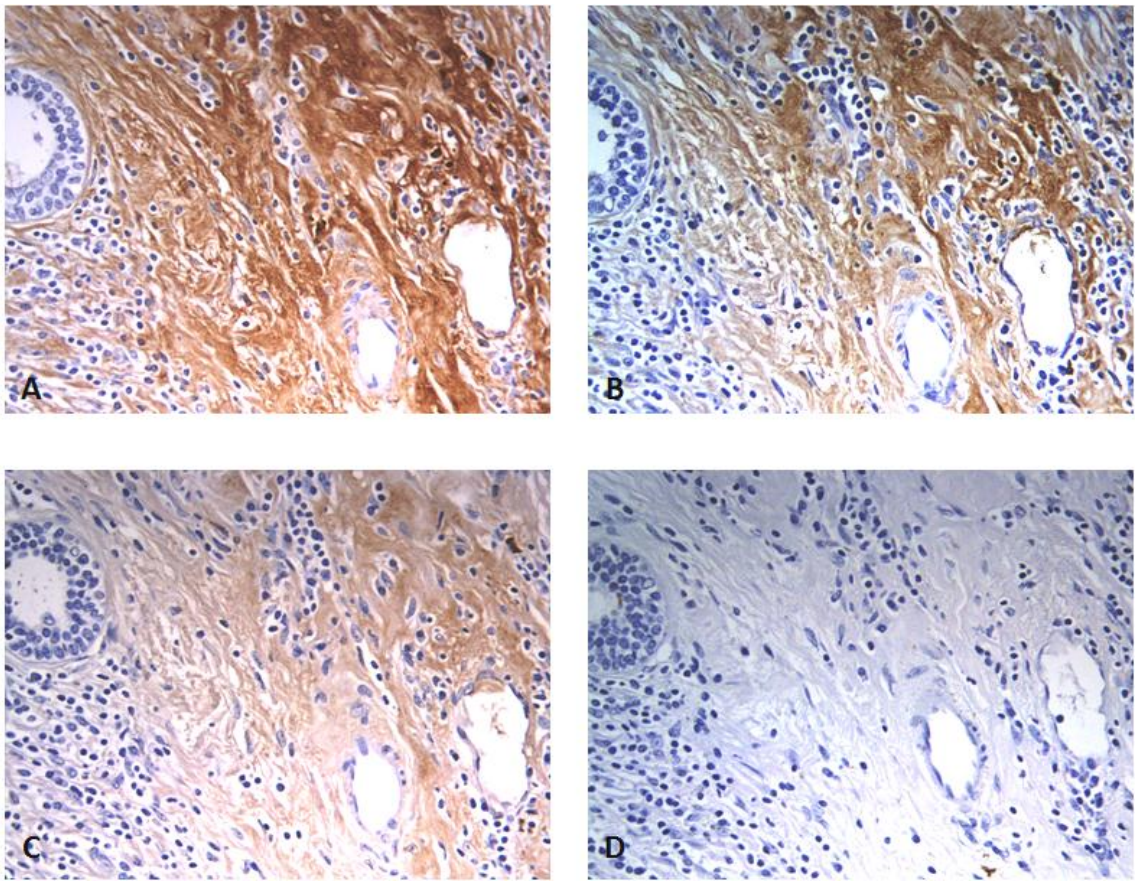


Figure 4.23: Optimisation of TSP-1 antibody dilutions. A) TSP-1 antibody at 1:100. B) TSP-1 antibody at 1:300. C) TSP-1 antibody at 1:500. D) No primary antibody.

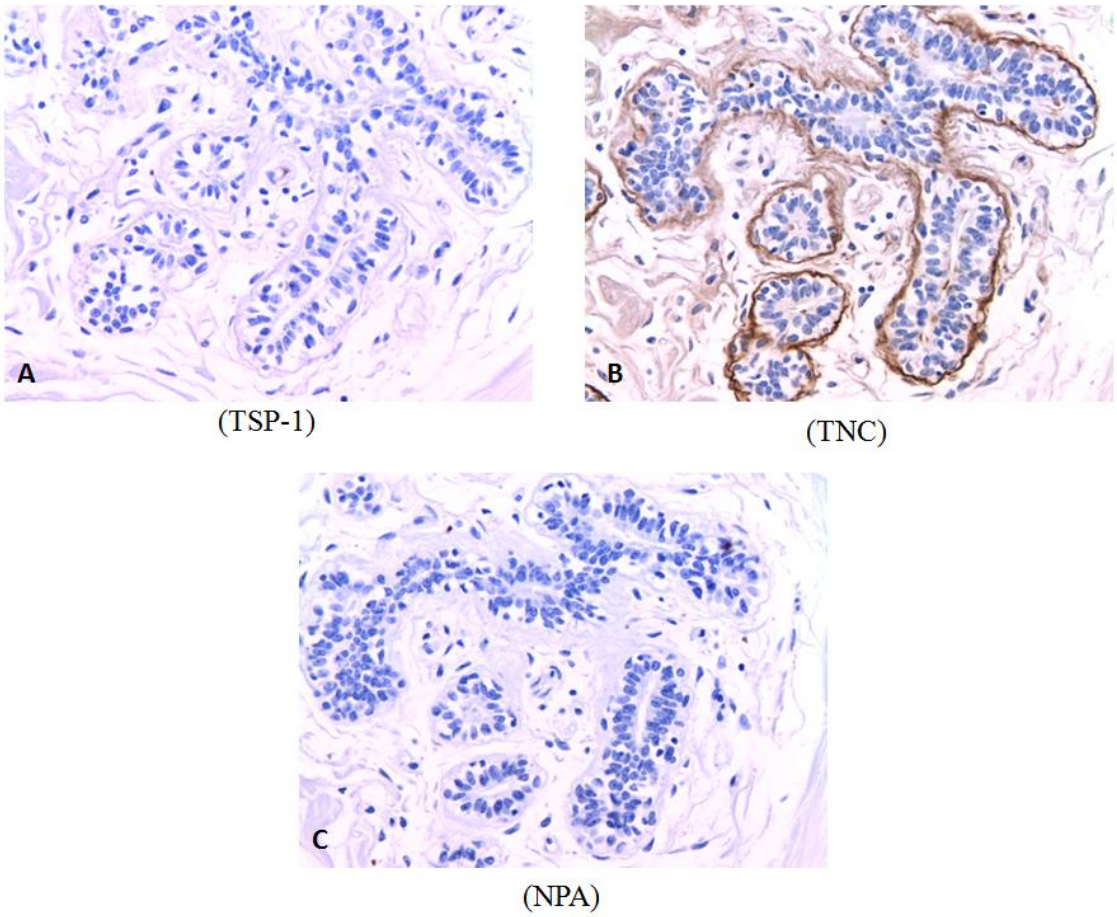


Figure 4.24: TSP-1 and TNC staining in normal breast tissue. A) No TSP-1 expression found in normal breast tissue. B) Expression of TNC in normal breast tissue using BC-24 antibody. C) No primary antibody staining control. Magnification 40x.

A total of 36 tissue samples previously analysed for TNC expression by our group using RT-qPCR were also used in this study. All samples showed expression of total TNC, which varied widely from high to low levels (Guttery et al. 2010a).

In order to correlate the expression of TNC with TSP-1 at the mRNA and protein level, IHC analysis was carried out on matched tissues (FFPE) from the same cases using the anti TSP-1 antibody. Next, the staining location of was noted, as either stroma or in the basement membrane around the ducts (Figure 4.25).

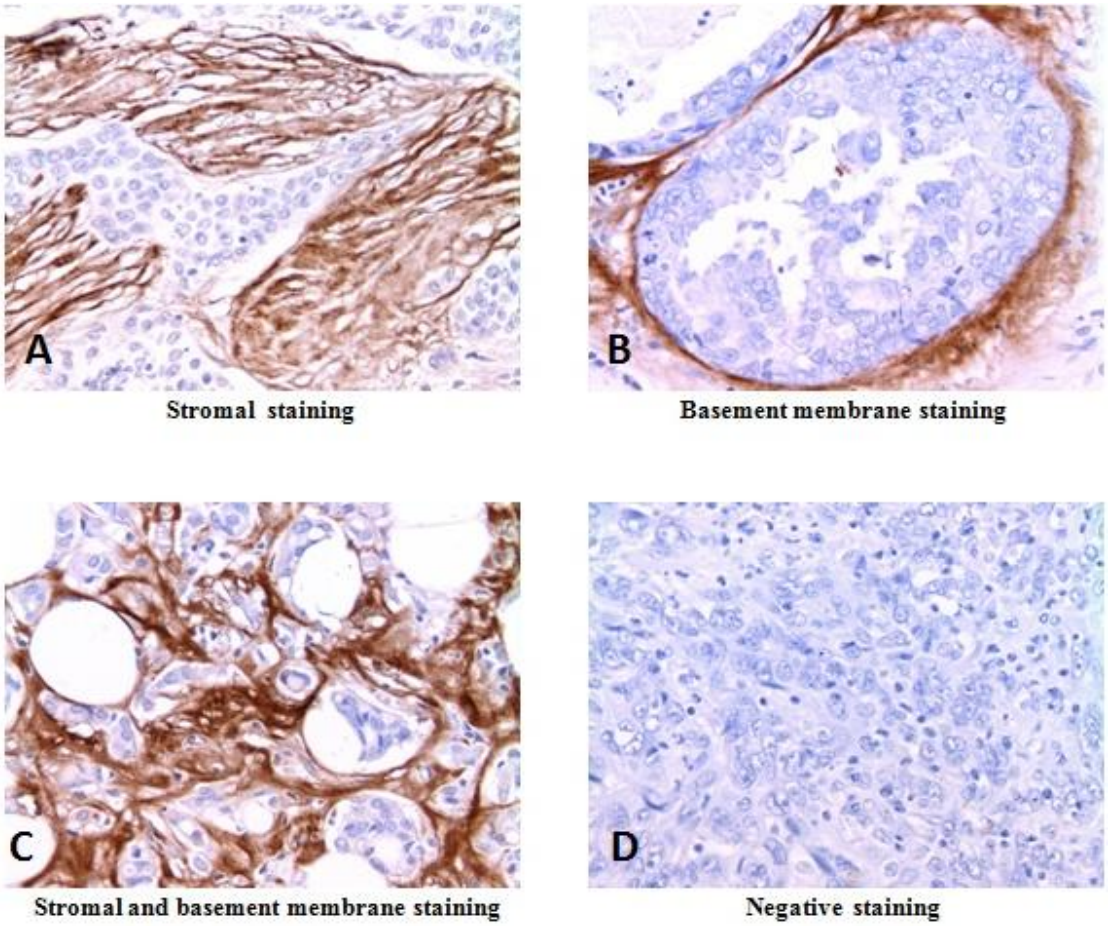


Figure 4.25: The localisation of TSP-1 protein in breast carcinoma tissue. A) The expression of TSP-1 in cancer stroma. B) The expression of TSP-1 around the breast duct in the basement membrane. C) The expression of TSP-1 in both the stroma and the basement membrane around the ducts. D) Negative staining for TSP-1. Magnification 40x.

IHC analysis identified 14 out of 36 (38.9%) positive cases for TSP-1 and 22 (61.1%) were negative. Nine (64.3%) of the 14 cases positive for TSP-1 showed staining in the stroma, two (14.3%) showed expression around ducts, and 3 (21.4%) showed expression in both the stroma and around the duct. The summary of TNC expression based on the number of molecules and TSP-1 staining are shown in Table 4.20.

Table 4.20 The summary of TNC and TSP-1 expression in breast carcinoma tissues

cases	TNC mRNA expression (RT-qPCR)						TSP-1 protein expression (IHC)	
	Number of molecules			Level of expression			(Immuno-staining)	
	TNC	AD1	TNC 14/16	TNC	AD1	TNC 14/16	Stroma	BM
C-1	1.5E+09	182237	5.1E+08	Mod	High	High	-	-
C-2	1E+13	542004	3E+10	High	High	High	+ (30%)	-
C-3	8.3E+07	284907	3678336	Low	High	Low	+ (50%)	-
C-4	3.5E+07	131747	2660401	Low	High	Low	-	+ (20%)
C-5	1.4E+09	195491	6.5E+07	Mod	High	High	+ (50%)	-
C-6	2E+11	299581	1.3E+10	High	High	High	+ (10%)	-
C-7	2.4E+08	171349	1.2E+07	Mod	High	High	+ (60%)	-
C-8	6.4E+08	688136	4.5E+07	Mod	High	High	+ (15%)	+ (25%)
C-9	1.1E+10	129625	905464	Mod	High	Low	-	+ (50%)
C-10	576942	104164	180982	Low	High	Low	+ (10%)	-
C-11	602221	114384	204825	Low	High	Low	-	-
C-12	1324118	148676	481671	Low	High	Low	-	-
C-13	1935513	173818	682821	Low	High	Low	-	-
C-14	4.7E+09	23762.1	2361116	Mod	low	Low	+ (15%)	+ (15%)
C-15	4.3E+10	50445.2	7.1E+07	Mod	low	High	+ (10%)	-
C-16	5.7E+09	36558	9.8E+07	Mod	low	High	+ (40%)	+ (20%)
C-17	7.5E+09	46750	4.5E+07	Mod	low	High	-	-
C-18	2.4E+10	39588.7	123477	Mod	low	Low	+ (10%)	-
C-19	355220	19025.5	78737.5	Low	low	Low	-	-
C-20	2640252	41782.7	457194	Low	low	Low	-	-
C-21	1.6E+11	0	5.7E+09	High	Neg	High	-	-
C-22	1.3E+11	0	1.6E+08	High	Neg	High	-	-
C-23	4781458	0	0	Low	Neg	Neg	-	-
C-24	6.3E+08	0	6.4E+07	Mod	Neg	High	-	-
C-25	2.8E+09	0	486554	Mod	Neg	Low	-	-
C-26	9.7E+09	0	2133279	Mod	Neg	Low	-	-
C-27	1.1E+10	0	194500	Mod	Neg	Low	-	-
C-28	9576555	0	449405	Low	Neg	Low	-	-
C-29	5.8E+09	0	1.2E+07	Mod	Neg	High	+ (20%)	-
C-30	7.1E+09	0	395783	Mod	Neg	Low	-	-
C-31	5.3E+09	0	705221	Mod	Neg	Low	-	-
C-32	2.1E+09	0	294514	Mod	Neg	Low	-	-
C-33	3.4E+12	0	7.1E+09	High	Neg	High	-	-
C-34	555774	0	35327.2	Low	Neg	Low	-	-
C-35	2428436	0	29181.6	Low	Neg	Low	-	-
C-36	5.4E+09	0	1.2E+09	Mod	Neg	High	-	-

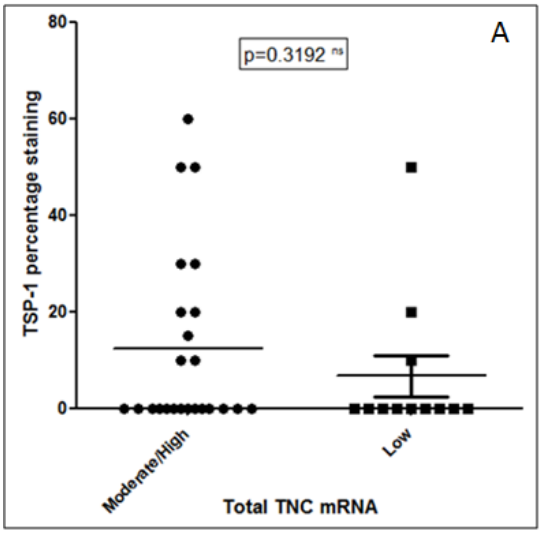
BM= Basement Membrane Neg= Negative; Mod= Moderate; (+) Positive staining; (-) No staining; C= case

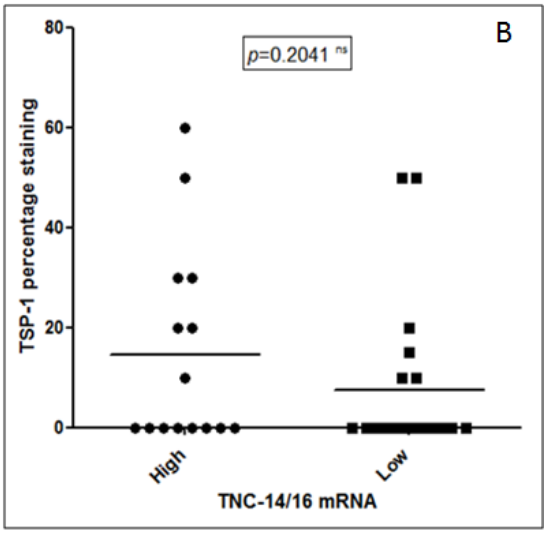
Correlation between TSP-1 and TNC expression (Table 4.21) showed 14 cases expressing TSP-1 were also positive for total TNC and TNC high MW isoforms at the mRNA level. A Mann-Whitney test was performed to correlate TSP-1 staining and TNC mRNA expression (number of molecules). This identified a significant correlation between TNC-AD1 and TSP-1 ($p = 0.0013$) (Figure 4.26 C), but there was no significant correlation between total TNC or TNC-14/16 mRNA and TSP-1 immuno-staining (Figure 4.26 A & B).

Table 4.21 The correlation between TNC and TSP-1 expression in breast carcinoma tissues

TNC expression at mRNA		Total (N=36)	TSP-1 staining		*P* value
			positive	negative	
Total TNC	Low	12	3	9	0.3192 ^{ns}
	Mod/High	24	11	13	
TNC-14/16	Low	21	6	15	0.2041^{ns}
	Mod/High	15	8	7	
TNC-AD1	positive	20	13	7	0.0013***
	negative	16	1	15	

ns = not significant





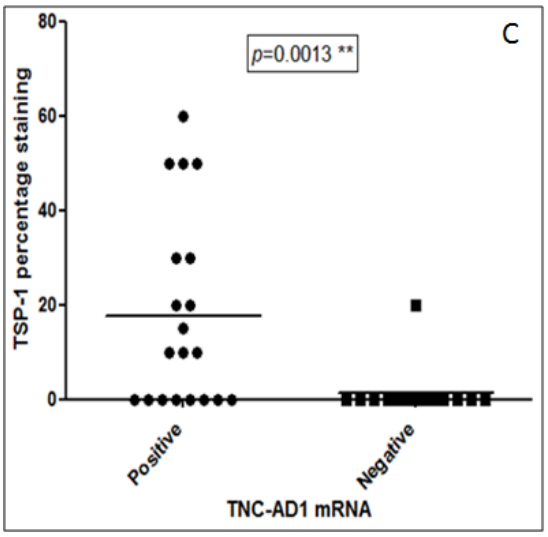


Figure 4.26 The Mann-Whitney test shows the correlation of expression levels between total TNC and high MW TNC isoforms expression at mRNA and TSP-1 staining in breast cancer tissues.

A) Correlation between TSP-1 and total TNC expressions.
B) Correlation between TSP-1 and TNC-14/16 expressions.
C) Correlation between TSP-1 and TNC-AD1 expressions.

Next, comparing expression of TSP-1 and clinicopathological features showed that all positive cases for TSP-1 were IDC (38.9%). 41.7 % of the cases >40 years old were positive for TSP-1; 28 % were ER+, 30% were PR+ and 26.3% were LN+.

A contingency table using a Fisher's Exact Test showed no significant correlation between TSP-1 expression and clinicopathological features, probably due to small numbers (Table 4.22).

Table 4.22 The correlation summary between TNC-AD1 and TSP-1 expression and clinicopathological features. Fisher's exact test was used for statistical analysis of TNC-AD1 expression comparison with clinicopathological features.

Clinicopathological features		Total (N=36)	TNC-AD1 mRNA expression (RT-qPCR)			TSP-1 protein expression (IHC)		
			+ ve	-ve	P value	+ ve	-ve	P value
Tumour Type	IDC	31	18	13	0.1380 ns	14	17	0.0546 ns
	ILC	5	2	3		0	5	
Tumour Grade	(I & II)	19	9	10	0.3351 ns	7	12	0.7900 ns
	(III)	17	11	6		7	10	
Patient Age	≤ 40	12	6	6	0.7295 ns	4	8	0.7272 ns
	> 40	24	14	10		10	14	
ER Status	ER^+	25	12	12	0.4123 ns	7	17	0.0917 ns
	ER^-	7	6	2		5	3	
	n/k	4	2	2		2	2	
PR Status	PR^+	20	22	9	0.6979 ns	6	14	0.2839 ns
	PR^-	10	6	4		5	5	
	n/k	6	3	3		3	3	
Lymph Node Status	LN^+	19	9	10	0.3064 ns	5	14	0.0717 ns
	LN^-	16	11	5		9	7	
	n/k	1	0	1		0	1	

IDC = Invasive ductal carcinoma; ILC = Invasive lobular carcinoma; ER = Oestrogen receptor; PR = Progesterone receptor; n/k = not known; + ve = Positive; -ve = Negative; ns = Not significant

4.5 Discussion

4.5.1 Microarray analysis

Microarray analysis of total TNC knockdown (at invariant exon 24) in breast carcinoma cells (MDA-MB-231 and MDA-MB-436) identified a number of target genes, which were differentially regulated at the mRNA level. Although the vast majority of genes that were significantly altered in cells transfected with total TNC siRNA were unique to each cell line, 156 genes were commonly altered in both. Furthermore, the majority of changes were achieved at 48 hrs post-transfection in both cell lines.

Pathway analysis showed the effects of TNC silencing on differential gene expression was associated with several pathways, which relate to the development of cancer, such as focal adhesion, cell cycle, ECM-receptor interaction, TGF-beta signalling, MAPK, ErbB signalling, TP53 and apoptosis. However, the majority of the identified genes were associated with focal adhesion pathways.

Cell adhesions play a fundamental role in cell biological signatures including proliferation, motility, differentiation and survival (Petit, Thiery 2000). In this study, focal adhesion was the most common pathway regulated by TNC silencing, and most of the differentially regulated genes were up-regulated, suggesting that TNC silencing stimulates cell adhesion and inhibits cell proliferation and tumour progression. For example, increased level of integrin α9 (ITGA9) is associated with reduced breast cancer cell proliferation and migration (Mostovich et al. 2011), and integrin α8 (ITGA8) was found to be a biomarker for ovarian cancer (Cai et al. 2007).

4.5.1.1 RT-qPCR expression profiling of candidate genes

Six genes were selected for validation by RT-qPCR as a result of knockdown of total TNC using microarray technology. Validation of total TNC knockdown showed consistent results for *CREBL2, YWHAE, RRAS2* and *QKI* in both breast cancer cell lines (MDA-MB-231 and MDA-MB-436). In addition, of the candidate genes that were investigated further for the effect of specific TNC isoforms knockdown, *QKI* appeared to be the most significant. *QKI* was significantly up-regulated by the knockdown of both total TNC and specific TNC isoforms suggesting this is a good candidate for further study.

In order to obtain meaningful gene expression data, using intact RNA with high quality is a critical step in the successful use of molecular biology methods such as qRT-PCR or microarray analysis (Fleige et al. 2006). Therefore, validation of candidate gene

expression was not performed in breast tissue due to low detection (high Ct) of the *HPRT-1* housekeeping gene, which was too low to warrant proceeding with analysis of the candidate genes.

4.5.1.1.1 cAMP responsive element binding protein-like 2 (CREBL2)

Microarray analysis and RT-qPCR validation of *CREBL2* expression in both cell lines transfected with total TNC siRNA showed a significant decrease in C*REBL2* expression; whereas knockdown of specific TNC isoforms resulted in its down-regulation in MDA-MB-436 cells, suggesting that regulation of *CREBL2* expression is not isoform-specific.

CREBL2 [*CREB* (cAMP-response-element-binding protein) - like 2] was first recognized in a frequently deleted region on chromosome 12p13, which are commonly associated with haematopoietic malignancies, breast cancer, ovarian cancer and non-small cell lung cancer (Wlodarska et al. 1996).

The information related to the role of *CREBL2* in cancer is limited; however, a few studies have suggested that *CREBL2* may have tumour suppressor activity in malignancies (Andreasson et al. 1997; Hoornaert, Marynen & Baens 1998). *CREB* was found to be highly expressed in metastatic MDA-MB-231 breast cancer cells compared to non-metastatic MCF-7 cell line. In MDA-MB-231 cells, *CREB* signalling positively regulated proliferation, migration, and invasion. Furthermore, during the development of breast cancer, over expression of *CREB* was found to play a crucial role in breast cancer bone metastasis (Son et al. 2010).

Recently Ma et al. (2011) have shown that *CREBL2* is a critical regulator of adipogenesis. *CREBL2* was found to be up-regulated during preadipocyte differentiation and *CREBL2* was co-localized to *CREB* under physiological conditions and during

over-expression. PPARγ (peroxisome-proliferator-activated receptor γ) and C/EBPα (CCAAT/enhancer-binding protein α) expression were increased by *CREBL2*, thereby enhancing adipogenesis and lipogenesis by interacting with glucose transporter 1 (GLUT1). Knockdown of *CREBL2* suppressed both adipogenesis and lipogenesis. Knockdown of *CREB* reduced *CREBL2* levels and vice versa, suggesting their interaction was pivotal in adipogenesis (Ma et al. 2011).

4.5.1.1.2 Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide (YWHAE)

Microarray analysis and RT-qPCR validation of *YWHAE* expression in both cells transfected with total TNC siRNA showed a significant decrease in *YWHAE* expression. Suggesting, the knockdown of total TNC may decrease the oncogenic activity of *YWHAE*.

The tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein family (*YWHA*/*14-3-3*) are highly conserved and expressed ubiquitously in all eukaryotic cells. *14-3-3* proteins regulate several intracellular processes such as phosphorylation dependent switching and protein-protein interactions (De et al. 2012).

Chuthapisith et al. (2007) identified 20 proteins differentially expressed in chemosensitive, paclitaxel- and adriamycin - resistant MCF-7 breast cancer cell lines using 2D gel electrophoresis and MALDI-TOF peptide mass fingerprinting. *14-3-3* epsilon was differentially expressed in both adriamycin- and paclitaxel-resistant tumour cells, suggestive of a potential biomarker for chemotherapy resistance in breast cancer treatment (Chuthapisith et al. 2007). In the microarray analysis of breast cancer tissues from patients with different clinical outcomes (relapse and without relapse after 72 months from surgery), 6 genes including *YWHAE* were up-regulated and found to be associated with disease free and overall survival. The expression of *YWHAE* was

associated with shorter disease free and early death confirmed by using covariates of significant factor such as oestrogen receptor (ER^-) and lymph node status (LN^+) (Cimino et al. 2008).

4.5.1.1.3 Cell division cycle 14 homolog B (CDC14B)

Microarray analysis and RT-qPCR validation of *CDC14B* expression in both cell lines transfected with total TNC siRNA showed a significant decrease in *CDC14B* expression. No significant change in expression was observed resulting from knockdown of TNC specific isoforms. These findings suggest other TNC species or combinations of isoforms may regulate *CDC14B* expression and its subsequent effects on the cell cycle and reduce cell morphological changes and malignant transformation.

Protein phosphorylation in cells plays a critical role in various biological processes such as the cell cycle, metabolism, gene transcription, translation, cytoskeletal rearrangements, apoptosis, protein interactions, protein stability and cell movements (Mocciaro, Schiebel 2010). *CDC14* is a dual-specificity phosphatase (DUSP), which has the potential to dephosphorylate both phosphoserine/phosphothreonine and phosphotyrosine residues from substrates (Mocciaro, Schiebel 2010). *CDC14* was first discovered in the budding yeast *Saccharomyces cerevisiae* by Hartwell as an essential regulator of the cell cycle (Hartwell et al. 1974). *CDC14* positively regulates mitotic exit in budding yeast (Jaspersen et al. 1998) and coordinates cytokineses in the fission yeast (Trautmann et al. 2001). *CDC14* is not indispensible for cell division, indicating the role of redundant phosphatases for mitotic exit (Mocciaro, Schiebel 2010).

In mammals, two *CDC14* homologues are expressed, *CDC14A* and *CDC14B*, respectively (Mocciaro, Schiebel 2010). *CDC14A* was found to be involved in mitosis, meiosis, cytokinesis and DNA repair (Mocciaro, Schiebel 2010). *CDC14B* plays an important role in regulating the G1 phase length of the cell cycle (Rodier et al. 2008).

Silencing of *CDC14B* has lowered the degradation of the Skp2 ubiquitin ligase and accelerated the entry length to S-phase (Rodier et al. 2008).

Recent studies suggest that *CDC14B* may be deregulated in breast cancer, prostate cancer, mantle cell lymphoma and acute myeloid leukemia. *CDC14B* was shown to control centriole duplication by attenuating centriole amplification in S-phase arrested cells and cells treated with proteasome inhibitor (Z-L_3VS-induced centriole duplication system) (Wu et al. 2008). *CDC14B* was also involved in DNA damage response by generating the G2 checkpoint through activation of ubiquitin ligase (APC/C^{Cdh1}) and the resultant degradation of the prominent mitotic kinase (Plk1), and thus plays an imperative role in efficient DNA repair (Bassermann et al. 2008).

The oncogeneic activity of *CDC14B* was mediated by the Ras-Mek pathway. Over-expression of mammalian *CDC14B* in transfected NIH-3T3 fibroblasts resulted in morphological changes and malignant transformation. The F-actin organisation in the fibroblasts was deranged with depletion of actin stress fibres and vinculin adhesions. The transfected fibroblast with *CDCl4B* showed tumorigenic potential and the alterations in the transcriptional profiles were analogous to those transfected with H-RasV12 (plasmid of a mutant active H-RAS). Interestingly, the pharmacological inhibition of Ras-Mek was shown to reverse the morphological changes induced by *CDC14B* (Chiesa et al. 2011). In addition, *CDC14B* also regulates the function of the tumour suppressor protein *TP53* by dephosphorylation (Li, Ljungmann & Dixon 2000).

4.5.1.1.4 RAS viral (r-ras) oncogene homolog 2 (RRAS2)

Microarray analysis and RT-qPCR validation of *RRAS2* expression in both cells transfected with total TNC siRNA showed a significant decrease in *RRAS2* expression. No significant changes in expression were observed as a result of specific TNC isoform

knockdown, suggesting that TNC exerts its effect on *RRAS2* activity in a non-isoform specific manner.

The R-RAS subfamily belongs to RAS-related proteins, which includes GTPases such as RRAS1, RRAS2 (TC21) and RRAS3 (M-Ras). *RRAS2* (*TC21*) has been shown to transform epithelial and fibroblast cell lines (Cox et al. 1994; Graham et al. 1999). *RRAS2 (TC21)* was shown to induce malignant transformation of NIH3-T3 fibroblast cells, without causing any morphological changes (Cox et al. 1994). The activation of *RRAS2* has induced transformation of RIE-1 rat intestinal epithelial cells as well as terminal differentiation of PC12 pheochromocytoma cells (Graham et al. 1999). Mutations of the *RRAS2* gene was observed in breast cancer and human leiomyosarcoma cell lines (Huang et al. 1995). *RRAS2* was also found to be over-expressed in tumours such as breast, lymphomas, skin carcinomas, oral cavity and oesophageal cancers (Alarcon, Martinez-Martin 2012). *RRAS2* (TC21) was shown to regulate key physiological processes in tumour cells such as proliferation, epithelial mesenchymal transition (EMT), migration, anoikis and chemotherapy resistance (Alarcon, Martinez-Martin 2012; Larive et al. 2012).

RRAS2 (TC21) was also implicated in the pathogenesis of breast cancer. Mutations in *RRAS2* (TC21) were found in breast cancer cell lines such as CAL51; however, the mutations were absent in breast carcinomas *in vivo* (mutation free in 9 primary and 15 metastatic carcinoma samples) (Barker, Crompton 1998). Transfection of *RRAS2* in MCF-10A human breast epithelial cell lines altered its cellular morphology with deranged cell-cell adherens junctions and enhanced motility, and formed colonies in soft agar (Barker, Crompton 1998). *RRAS2* (TC21) was found to be over expressed in 7 out of 9 breast tumour cell lines, suggesting the ability of TC21 over-expression in the contribution of breast cancer development (Clark et al. 1996).

Tamoxifen is a widely used treatment for ER positive breast cancer. The *RRAS2* (TC21) -582C>T promoter polymorphism was shown to predict the outcome of breast cancer patients treated with Tamoxifen. Carrier patients of the -582T allele have higher expression of cytoplasmic *RRAS2* (TC21), increasing recurrence rates, and thereby plays a key role in chemotherapy resistance (Rokavec et al. 2008). *RRAS2* was found to be imperative for proper mammary gland development. *RRAS2* (TC21) knockout mice showed no alterations in cardiovascular parameters, growth rates, viability or fertility. However, there was a significant deficiency in the development of the mammary glands during puberty. The paucity of *RRAS2* (TC21) in mammary glands led to a reduced number of terminal end buds (TEBs) and ductal branches, resulting in poor development of the gland tree in mammary fat pads (Larive et al. 2012).

4.5.1.1.5 Disrupted in schizophrenia 1 (DISC1)

Microarray analysis showed *DISC1* expression was significantly increased in both cell lines transfected with total TNC siRNA. But validation of this by RT-qPCR was not performed due to low efficiency of the *DISC1* probe. The *DISC1* gene was originally identified in a Scottish family with a spectrum of major mental illness (Stclair et al. 1990). In particular, a t b(1; 11) (q42;q14.3) translocation of the *DISC1* gene was correlated with psychiatric illnesses such as schizophrenia, bipolar disorder, recurrent major depression, neurophyschological, cognitive and structural traits (Blackwood et al. 2001; Ekelund et al. 2001). The two major splice variants of human *DISC1* were L form and Lv isoforms which differ by approximately 22 amino acids within the C terminus (James et al. 2004).

DISC1 plays a critical role in foetal and adult brain development and neuronal function (Bradshaw, Porteous 2012). *DISC1* is regarded as an imperative 'hub' protein connecting multiple functional systems including migration of neurons, neurite

development, cAMP signalling, and cytoskeletal modulation within the brain (Chubb et al. 2008; Bradshaw, Porteous 2012). *DISC1* is vital for neural progenitor proliferation in the dentate gyrus of the hippocampus in adults and during foetal brain development (Meyer, Morris 2009).

In murine models the *DISC1-GSK3* pathway was shown to modulate the proliferation of neural progenitor cells in the embryonic murine cortex and the mature dentate gyrus (Brandon et al. 2009). *DISC1* was found to regulate neurogenesis by positively regulating Wnt signalling via inhibition of GSK3-β. *DISC1* stabilizes the β-Catenin levels by binding to GSK3-β through their N-terminal region and inhibiting their action (Mao et al. 2009). *DISC1* was also shown to bind to proteins in the centrosome such as NDEL1, PCM1 and BBSs. *DISC1* stabilizes these proteins by interaction with the dynein motor complex and the centrosome, and thereby modulates microtubules involved in neurogenesis (Brandon et al. 2009; Ozeki et al. 2003; Kamiya et al. 2008).

4.5.1.1.6 Quaking homolog, KH domain RNA binding (QKI)

The *QKI* protein belongs to the Signal Transduction and Activation of RNA (STAR) family and contains a RNA binding domain named KH. KH domains of *QKI* selectively bind to cellular mRNAs containing *QKI* response element (QRE), and are found to be conserved among various species (Chen et al. 1997). The *QKI* locus encodes for different protein sets owing to alternative splicing, which include *QKI-5*, *QKI-6*, and *QKI-7*. *QKI* has shown to regulate various key cellular process such as RNA transportation, mRNA stability, nuclear retention and translational modulation through dimerisation and binding to QREs located in the UTR of the target mRNA. *QKI* has been shown to regulate the cell cycle, apoptosis and angiogenesis, which are impaired in breast cancer. Some of the validated targets of QKI include p53, MBP, p27, MAG and CTNNB1 (Fu et al. 2012).

QKI was suggested to have a tumour suppressor action by which it was found to be significantly reduced in tumours such as breast, bladder, testis, ovary, cervix and colon cancer with abnormal reduction in the histone variant (macro H2A1) (Novikov et al. 2011). *QKI* was one of the top 50 genes inversely associated with ER positive breast tumours which found to be significantly down regulated in ER positive breast cancers (stage 1-3) (Pusztai et al. 2003). However, in this study analysis of the MCF-7 breast cell line (which is ER positive) and has a TNC null phenotype showed low expression for *QKI*. In addition, T47-D and ZR-75 are ER positive and TNC null and showed no expression of *QKI*, whereas MDA-MB-231 (ER negative and TNC positive) cells showed lower expression of *QKI* compared to MCF-7, but *QKI* was significantly up-regulated upon knockdown of total TNC and high MW TNC isoforms.

QKI expression was also significantly reduced in gastric cancer tissues, predominantly due to promoter hyper-methylation (Bian et al. 2012). Down regulation of *QKI* expression was associated with impaired differentiation, invasion, gastric lymph node metastasis, distant metastasis, advanced TNM stage and poor prognosis (Bian et al. 2012). Recently *QKI* was identified as a novel tumour suppressor in glioblastoma multiforme by interacting with p53 and thereby stabilizing miR-20a, which in turn, controls TGF-β receptor 2 (TGF-βR2) and the TGF-β signalling network (Chen et al. 2012).

Microarray and RT-qPCR analysis *QKI* expression showed a significant increase in expression in cells transfected with siRNA targeting total TNC. Further analysis by RT-qPCR in cells transfected with TNC specific isoform siRNAs showed greatest increase in *QKI* expression, suggesting that the effect of TNC on *QKI* expression is isoform specific, and supports the hypothesis that *QKI* is a tumour suppressor gene. So our hypothesis would be that TNC is modulating *QKI* expression in breast cancer cells.

4.5.2 Proteomics analysis

Proteomics analysis in MDA-MB-231 cells transfected with siRNAs targeted total TNC versus cells transfected with scrambled siRNA allowed a pilot investigation to identify the effects of TNC silencing on secreted proteins. 72 proteins were found to be regulated and differentially secreted in conditioned media. Correlation of the secreted proteins with previous studies showed agreement in 24 proteins associated with tumour progression including proliferation, EMT, migration, apoptosis, angiogenesis and invasion (e.g. HNRNPA1, VIM, PFN-1, YWHAE, IGFBP-7 and TSP-1).

TNC silencing caused a significant down-regulation of vimentin (vim) expression confirmed by proteomics analysis. Vimentin is associated with EMT and metastatic transformation of epithelial cells (Steinert, Roop 1988). Increased expression of vimentin is associated with increased cancer cell migration and invasion (Korsching et al. 2005). The genes involved in EMT were found to be regulated by the co-expression of TNC and vimentin in mammary carcinoma cells such as Hs578T, SK-BR-3, MDA-MB-231, and in the myoepithelial cell line HBL-100 (Dandachi et al. 2001).

Up-regulation of insulin growth factor binding protein-7 (IGFBP-7) was also identified due to TNC silencing. IGFBP-7 plays an important role in cancer and showed a suppressive function in prostate and breast cancer (Jiang et al. 2008). Elevated expression of IGFBP-7 reduced the growth and migration of triple negative breast cancer cell lines (Benatar et al. 2012).

Heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) was found to be over-expressed in proliferating and transformed cells (Biamonti et al. 1993), and in cancers including breast, lung and colon (Zerbe et al. 2004). Down-regulation of HNRNPA1 has induced apoptosis in various cancer cell lines in both human and mice, with no changes detected in normal fibroblast and epithelial cell lines (Patry et al. 2003). Interestingly,

secretome analysis showed that silencing of TNC significantly reduced the expression HNRNPA1.

Profilin-1 (PFN-1) plays an important role in the regulation of F-actin dynamics in cells (Schluter, Jockusch & Rothkegel 1997). In breast cancer cells, PFN-1 has shown to have tumour suppression effects (Zou et al. 2007). PFN-1 down-regulation by siRNAs reduced the formation of actin filaments and focal adhesions in human umbilical vein endothelial cells (Ding et al. 2006). Furthermore, previous microarray findings by our research group have shown that MCF-7 cells transfected with high MW TNC isoforms induced the expression of PFN-1. In this study, proteomics analysis of cells treated with TNC siRNA showed that knockdown of TNC leads to a significant down-regulation of PFN-1 expression.

4.5.2.1 Expression profiling of candidate proteins

Of the candidates proteins identified by proteomic analysis of MDA-MB-231 cells transfected with siRNAs targeted total TNC, thrombospondin-1 (TSP-1) was selected for further analysis and showed consistent results by Western blot. In addition, TSP-1 was tested further for the effect of specific TNC isoform knockdown and showed that TSP-1 was also up-regulated upon silencing of these isoforms. Further validations for the effect of total TNC and TNC specific isoforms knockdown were also performed using ICC in cells transfected with siRNA targeting both total TNC and high MW TNC isoforms which also appeared to be consistent with both proteomics and Western blot.

After confirming the effects of TNC knockdown on TSP-1 levels using cell line models, validation of TSP-1 in breast tissue was performed using 36 breast carcinomas with known TNC isoform expression at the mRNA level using RT-qPCR. In this study, the majority of TSP-1 staining was found in the tumour stroma of invasive carcinomas,

correlated significantly with TNC-AD1 at the mRNA level and was more commonly detected in women older than 40 years.

The relationship between TSP-1 and TNC-AD1 is unclear. In this study, there was clear evidence of the effects of TNC-AD1 silencing on TSP-1 up-regulation, which was confirmed by staining of TSP-1 on breast cancer cell lines. However, previous findings have shown that strong staining of TSP-1 in the stroma of breast cancer tissues also express high levels of TNC. This contradictory relationship is likely due to the small cohort of cases. Therefore, a larger series is required to further validate the findings here.

TSP-1 has pro-angiogenic activity in breast cancer and circulating plasma levels of TSP-1 has been suggested as a marker of breast cancer aggressiveness (Byrne et al. 2007). Although TSP-1 was found to be low in tumour cells, fibroblasts in the stroma secrete higher levels of TSP-1 and inhibit angiogenesis (Hanamura et al. 1997; Fontana et al. 2005). Stromal TSP-1 expression was positively correlated to the ECM expression of tenascin, laminin, fibronectin, syndecan-1 and collagen type IV (Ioachim et al. 2012). In this study, IHC staining of TSP-1 showed staining in the tumour stroma of invasive breast cancer and was lacking in normal breast tissues, which correlates with theses previous findings.

Ioachim et al (2012) examined tissue sections of 124 breast carcinomas for TSP-1 expression and compared this to clinical parameters, angiogenesis and ECM protein expression (i.e. tenascin). In the survival analysis, lower TSP-1 tumour expression was associated with increased risk of recurrence, whereas higher TSP-1 expression was found in invasive lobular breast cancer (Ioachim et al. 2012). In this study, the

expression of TSP-1 was up-regulated as a result of TNC knockdown, suggesting that TSP-1 up-regulation may reduce the risk of recurrence.

The relationship between TSP-1 expression and hormone receptor status is ambiguous. A recent study showed that high plasma levels of TSP-1 were more prominent in oestrogen receptor negative and progesterone receptor negative patients (Suh et al. 2012). Whereas, in this study, the tissue expression of TSP-1 has a tendency to be expressed in ER^+ and PR^+ patients; however, it was not statistically significant.

TSP-1 up-regulates the integrin alpha-6 subunit in human breast cancer cells, thereby augmenting cell adhesion to laminin and assists tumour cell invasion (John, Rothman & Tuszynski 2010). The transition from resting endothelial cells to a sprouting phenotype was promoted by tenascin, whereas TSP-1 inhibits sprout formation (Canfield, Schor 1995). TSP-1 was also synthesised and secreted by tumour cell lines such as melanoma, squamous carcinoma, osteosarcoma and glioma (Suh et al. 2012).

4.6 Conclusion

This study has confirmed that TNC knockdown by siRNA affects global gene expression at the mRNA (CREBL2, YWHAE, RRAS2, CDC14B and QKI) level and (TSP-1) protein level in conditioned media. By comparing candidate gene expression in transfected cells and siRNA treatments, the data showed that the effect of TNC expression depends on the cell background. In addition, it also showed the major effect was exerted by knockdown of all TNC isoforms (total TNC), rather than knockdown of specific TNC isoforms. However, the expression of QKI was up-regulated to a greater level by knockdown of TNC specific isoforms. LC-MS, Western blot and ICC have shown an up-regulation of TSP-1 protein expression in cells transfected with total TNC and TNC isoform specific siRNA. In addition, TSP-1 expression was also found to be

associated with invasive carcinomas and TNC-AD1 expression. These findings could provide a new mechanism of TNC action in tumorigenesis.

Chapter 5. Generation of a TNC-AD1 domain specific antibody

5.1 Introduction

Previous studies in our group have identified TNC transcripts containing the additional domain 1 (AD1) expressed in tumour cells of DCIS and associated myoepithelial cells lining larger histologically normal breast ducts. AD1 containing transcripts were also identified in carcinomas and correlated with high grade and hormone insensitive breast cancers in younger women (< 40 years of age) (Guttery et al. 2010a). However, analysis of TNC isoforms containing the AD1 domain at the protein level is lacking due to the absence of an available anti-AD1 antibody.

Although a number of TNC specific antibodies are available, which target different domains (Table 5.1), none are specific to the AD1 domain. Generation of antibodies targeting AD1 would be extremely useful for applications such as Western blotting and IHC to analyse its expression and localisation in breast tumour tissue, as well as for functional studies.

Table 5.1 Available antibodies for the detection of TNC

Antibody	Binding region	Host	Application
BC-24	Epidermal growth factor (EGF) like repeat	Mouse	IHC/ICC
H-300	Domain 6 and 7 of FNIII repeat	Rabbit	Western blot
Clone IIIB	Domain B of FNIII repeat	Mouse	IHC/ICC

5.2 Aims and objectives

The aim of this chapter was to generate antibodies targeting the AD1 domain for use in tissue and functional studies. The specific objectives were:

1- To use bioinformatics to select sequences within the AD1 domain for targeting with anti-peptide antibodies.
2- To ascertain the optimum antibody concentration for use in Western blot, ICC and IHC.
3- To validate the antibody specificity by analysing the effect of siRNA-mediated knockdown of AD1-containing isoforms of TNC.
4- To use the successful antibody for the detection of TNC-AD1 expression in breast cancer tissue.

5.3 Results

5.3.1 Bioinformatics analysis of AD1 protein structure and sequence

5.3.1.1 AD1 sequence alignment

Firstly, the amino acid sequences of AD1 from different species were aligned to assess their similarity and identify the correct species and protein sequence prior to antibody production. In addition, comparisons were performed between the AD1 DNA and protein sequences and other exons within the TNC FNIII-like domains in order to avoid any potential cross reactivity.

5.3.1.1.1 The sequence of TNC-AD1

The consensus sequence of AD1 was generated previously by sequencing of PCR amplicons derived from MDA-MB-231 breast adenocarcinoma and HT-1080 fibrosarcoma cell line cDNA (Guttery 2009). The TNC-AD1 exon is 276 bp in length, encoding 92 amino-acids with a C → T single nucleotide polymorphism (SNP) present at position 22 (Guttery 2009) (Figure 5.1.).

```
1                                                           59
GAACCAAAGCCACAGTTGGGCACGCTAATCTTTAGCAATATTACTCCAAAAAGCTTCAAC
E  P  K  P  Q  L  G  T  L  I  F  S  N  I  T  P  K  S  F  N
60                                                          119
ATGTCATGGACCACTCAAGCTGGGCTTTTTGCAAAGATTGTTATCAATGTGAGTGACGCT
M  S  W  T  T  Q  A  G  L  F  A  K  I  V  I  N  V  S  D  A
120                                                         179
CACTCACTGCATGAGTCCCAGCAATTCACAGTCTCAGGAGATGCAAAGCAAGCTCACATC
H  S  L  H  E  S  Q  Q  F  T  V  S  G  D  A  K  Q  A  H  I
180                                                         239
ACAGGCTTGGTGGAGAACACTGGCTATGACGTCAGTGTGGCAGGAACCACCTTGGCTGGG
T  G  L  V  E  N  T  G  Y  D  V  S  V  A  G  T  T  L  A  G
240                                 276
GATCCCACCAGACCCCTCACTGCCTTTGTCATTACAG
D  P  T  R  P  L  T  A  F  V  I  T
```

Figure 5.1: The consensus sequence of AD1

5.3.1.1.2 Alignment of the FNIII-like alternatively spliced region of TNC

Alignment of FNIII-like domains including AD1 was performed using the EMBL-EBI tools ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2). Domain C (exon 15) showed highest identity with AD1 at both the protein and DNA levels (56.18%) (Table 5.2 and Figure 5.2A).

Table 5.2 Summary of TNC-AD1 sequence identity compared to other FNIII domains of TNC

Domain	Exon	Amino acid identity %	DNA identity %
A_1	10	27.17	54.13
A_2	11	26.09	51.23
A_3	12	31.52	53.90
A_4	13	30.43	50.34
B	14	33.70	55.12
C	15	35.87	56.18
D	16	29.35	52.82
AD2	AD2	29.35	52.40

5.3.1.1.3 Alignment of the human AD1 sequence with AD1 from other species

The Basic Local Alignment Search Tool (BLAST) was used to analyse TNC-AD1 similarity across species including mouse, rat and chicken. BLAST analysis showed that the amino acid sequence of TNC-AD1 is conserved with 33% identity with rat AD1 (Table 5.3 and Figure 5.2.B)

Table 5.3 Summary of TNC-AD1 sequence identity in other species compared to the human TNC-AD1 sequence

Species	Length	Amino acid identity %	DNA identity %
Mouse	91	32	32
Rat	91	33	32
Pig	91	30	27
Chicken	91	29	34

A

```
Exon_AD1-FNIII AD1   EPKPQLGTLIFSNITPKSFNMSWTTQAGLFAKIVINVSDAHSLHESQQFTVSGDAKQAHI 60
Exon_AD2-FNIII AD2   VAKPLLSHLTVSNMTWGSVSISWEAQESAFDSFLIEVSNSDHPHETMVLSVPGVSRSSVI 60
Exon_16-FNIII D      EAEPEVDNLLVSDATPDGFRLSWTADEGVFDNFVLKIRDTKKQSEPLEITLLAPERTRDL 60
Exon_15-FNIII C      EALPLLENLTISDINPYGFTVSWMASENAFDSFLVTVVDSGKLLDPQEFTLSGTQRKLEL 60
Exon_14-FNIII B      AKEPEIGNLNVSDITPESFNLSWMATDGIFETFTIEIIDSNRLLETVEYNISGAERTAHI 60
Exon_13-FNIII A4     EDLPQLGDLAVSEVGWDGLRLNWTAADNAYEHFVIQVQEVNKVEAAQNLTLPGSLRAVDI 60
Exon_12-FNIII A3     EEVPDMGNLTVTEVSWDALRLNWTTPDGTYDQFTIQVQEADQVEEAHNLTVPGSLRSMEI 60
Exon_11-FNIII A2     GETPNLGEVVVAEVGWDALKLNWTAPEGAYEYFFIQVQEADTVEAAQNLTVPGGLRSTDL 60
Exon_10-FNIII A1     EQAPELENLTVTEVGWDGLRLNWTAADQAYEHFIIQVQEANKVEAARNLTVPGSLRAVDI 60

Exon_AD1-FNIII AD1   TGLVENTGYDVSVAGTTLAGDPTRPLTAFVIT 92
Exon_AD2-FNIII AD2   TNLKASSNYTAHLHGLI-GGQRAQTLMVQATT 91
Exon_16-FNIII D      TGLREATEYEIELYGIS-KGRRSQTVSAIATT 91
Exon_15-FNIII C      RGLITGIGYEVMVSGFT-QGHQTKPLRAEIVT 91
Exon_14-FNIII B      SGLPPSTDFIVYLSGLA-PSIRTKTISATATT 91
Exon_13-FNIII A4     PGLEAATPYRVSIYGVI-RGYRTPVLSAEAST 91
Exon_12-FNIII A3     PGLRAGTPYTVTLHGEV-RGHSTRPLAVEVVT 91
Exon_11-FNIII A2     PGLKAATHYTITIRGVT-QDFSTTPLSVEVLT 91
Exon_10-FNIII A1     PGLKAATPYTVSIYGVI-QGYRTPVLSAEAST 91
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B

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Human                EPKPQLGTLIFSNITPKSFNMSWTTQAGLFAKIVINVSDAHSLHESQQFTVSGDAKQAHI 60
Mouse                EAEPEVDNLLVSDATPDGFRLSWTADEGIFDSFVIRIRDTKKQSEPQEISLPSPERTRDI 60
Rat                  EAEPEVDNLLVSDATPDGFCLSWTADEGIFDSFVIRIRDTKKQSEPQEITLPSPDRTRDI 60
Pig                  EAEPEVDNLLVSDATPDGFRLSWTADEGVFDSFVLKIRDTKKQSEPLEITLLASERTRDI 60
Chicken              EAEPEVDNLLVSDATPDGFRLSWTADDGVFDSFVLKIRDTKRKSDPLELIVPGHERTHDI 60

Human                TGLVENTGYDVSVAGTTLAGDPTRPLTAFVIT 92
Mouse                TGLREATEYEIELYGIS-RGRRSQPVSAIATT 91
Rat                  TGLREATEYEIELYGIS-RGRRSQPVSAIATT 91
Pig                  TGLREATEYEIELYGIS-SGKRSQPVSAIATT 91
Chicken              TGLKEGTEYEIELYGVS-SGRRSQPINSVATT 91
```

Figure 5.2: Alignment of the amino acid sequences of TNC-AD1.

A) Alignment of TNC-AD1exon with other TNC exons within the FNIII-like alternatively spliced region of TNC.

B) Alignment of human TNC-AD1amino acids sequence with other species AD1 sequences.

Residues identical in at least four of the proteins are shown as black letters on a grey background. The positions of conserved amino acids are shown as white letters on a black background. The additional leucine amino acid residue (L) present in AD1 is represented as yellow letter on a red background.

5.3.1.2 Hydrophobicity plot

It is generally accepted that the ideal protein sequence for potential antigenic epitopes is hydrophilic, surface-oriented, and flexible. This is due to the most natural environment where the hydrophilic region resides on the surface, whereas the hydrophobic region more likely resides hidden in the interior (Rose et al. 1985). Therefore, hydrophobic screening of the AD1 amino acid sequence was performed using the EXPASY tool (http://web.expasy.org/protscale) to determine the hydrophilic regions within the sequence. Hydrophobic analysis showed that there were four sequences containing hydrophilic amino acids (Figure 5.3).

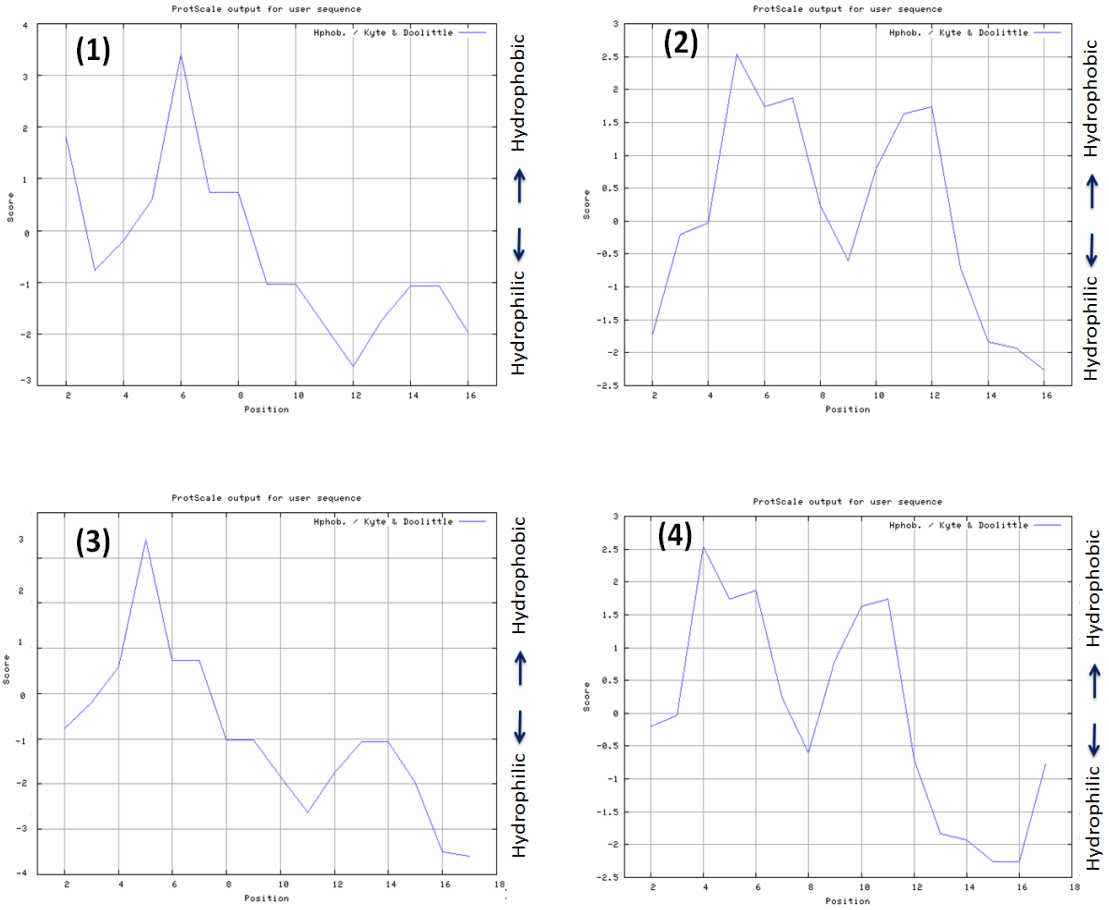

Figure 5.3: Hydrophobicity plot of TNC-AD1 amino acids sequences using the Kyte-Doolittle scale. The positive values represent the hydrophobic regions on the sequence

TNC-AD1 amino acid sequences (Table 5.4) were sent for peptide antigen synthesis and immunisation programs as described in section 2.2.15 (materials and methods). Two sequences were sent to Genosphere Biotechnologies, France, and two sequences were sent to Eurogentec, Belgium for antibody generation. Table 5.4 contains the selected sequences for antibody generation and **Error! Reference source not found.** shows the location of each sequence within the TNC-AD1 domain.

Table 5.4 Summary of the selected TNC-AD1 sequences for antibody generation

Company	Sequence	Amino acid Sequence	Amino acids length	Start position
Genosphere Biotechnologies (GB)	GB 1	CKIVINVSDAHSLHE	15	32
	GB 2	CVSVAGTTLAGDPTR	15	70
Eurogentec (E)	E 1	FAKIVINVSDAHSLH	15	30
	E 2	YDVSVAGTTLAGDPT	15	69

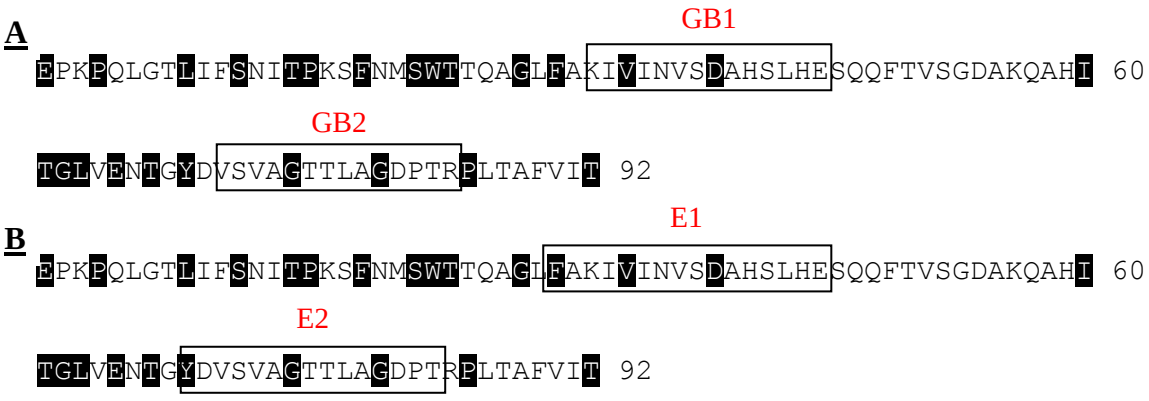

Figure 5.4: Amino acid sequences selected for antibody production. A) The sequences selected for the Genosphere Biotechnologies program. B) The sequences selected for the Eurogentec program. Conserved amino acids are shown as white letters on a black background.

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5.3.2 Optimisation of TNC-AD1 antibodies

5.3.2.1 Eurogenetic program

Prior to immunisation, sera from 5 rabbits were screened by Western blot to test the pre-immune reactivity to TNC-AD1 from breast cancer cells expressing high levels ofTNC. Rabbits 1 and 5 were selected according to their low background reactivity (Figure 5.5). Two peptide antigens were synthesised by Eurogenetic and conjugated with keyhole limpet hemocyanin (KLH) carrier protein. Two different rabbits were injected with the synthetic antigens and subjected to four cycles of immunisation and bleeding. Periodically, four sera were collected from the two immunised rabbits and tested for the detection of AD1 expression using both Western blot and immunocytochemistry (ICC).

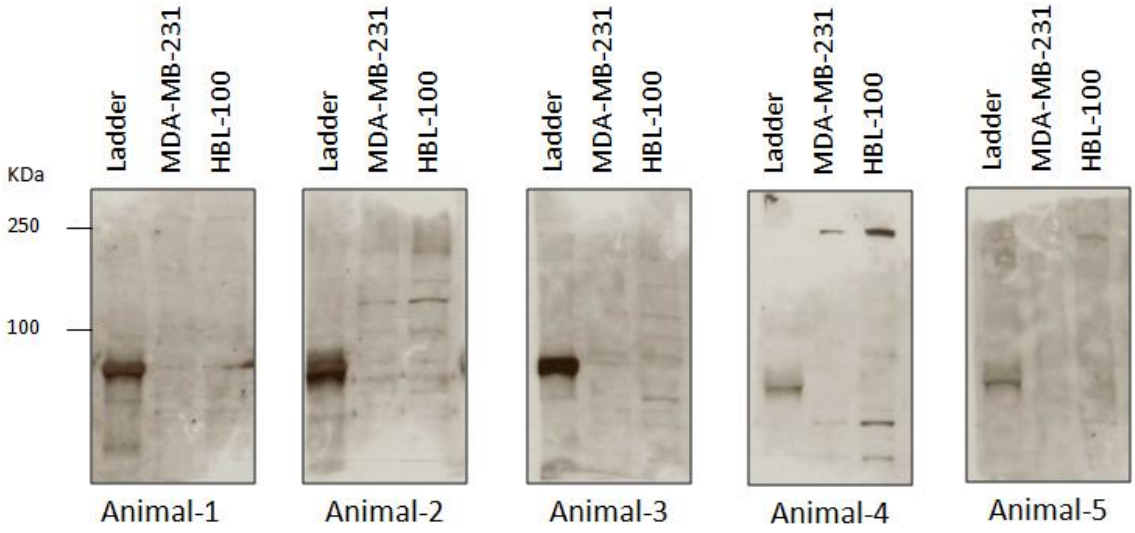


Figure 5.5: Western blot analysis of pre-immune sera. Pre-immune sera from 5 rabbits were tested for TNC reactivity with human protein from breast cell lines expressing high levels of TNC-AD1.

Western blot analysis was carried out using sera from immunised rabbits on conditioned media from MDA-MB-231 and Sk-Mel-28 cells, which endogenously express TNC isoforms containing AD1. TNC null MCF-7 breast adenocarcinoma cells were used as negative controls; whereas MCF-7s transiently transfected with two isoforms: TNC-Long and TNC-14/AD1/16 were used as positive controls. Western blot analysis of transiently transfected MCF-7 conditioned media using the H-300 anti-TNC antibody confirmed successful transfection; whereas no detectable TNC was present in non-transfected MCF-7 cells. It also confirmed the expression of TNC protein in MDA-MB-231 and Sk-Mel-28 cells (Figure 5.6 A). Unfortunately, no immunoreactivity was detected for TNC-AD1 expression using any sera by Western blot in MDA-MB-231s, Sk-Mel-28 or MCF-7 cells transiently transfected with TNC-14/AD1/16 (Figure 5.6 B&C).

Optimisation of the AD1 antibody for use in ICC was then carried out on cells that endogenously express TNC (MDA-MB-231 and Sk-Mel-28). TNC null MCF-7 cells were used as a negative control. ICC analysis showed no detection of TNC-AD1 in cells endogenously expressing isoforms containing AD1 or MCF-7 cells (Figure 5.7).

The above optimisations confirmed that the both sera were not suitable due to a lack of specific signal against TNC-AD1. Therefore, the serum was not subjected to affinity purification of the specific antibody.

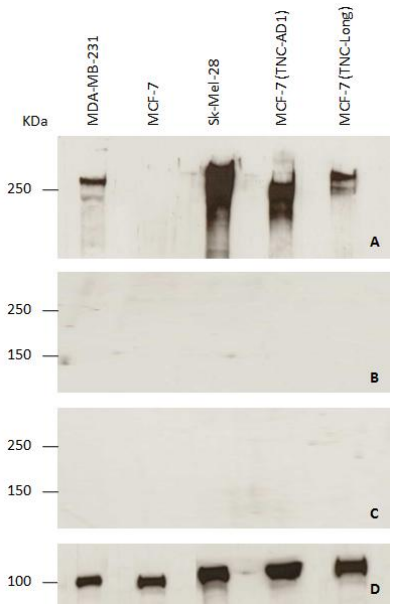


Figure 5.6: Western blot analysis of TNC expression. A) Immunobloting using H-300 anti-TNC antibody. B+C) immunobloting using the final bleedings of two immunised animals for the detection of TNC-AD1. D) Loading control using an anti-vinculin antibody.

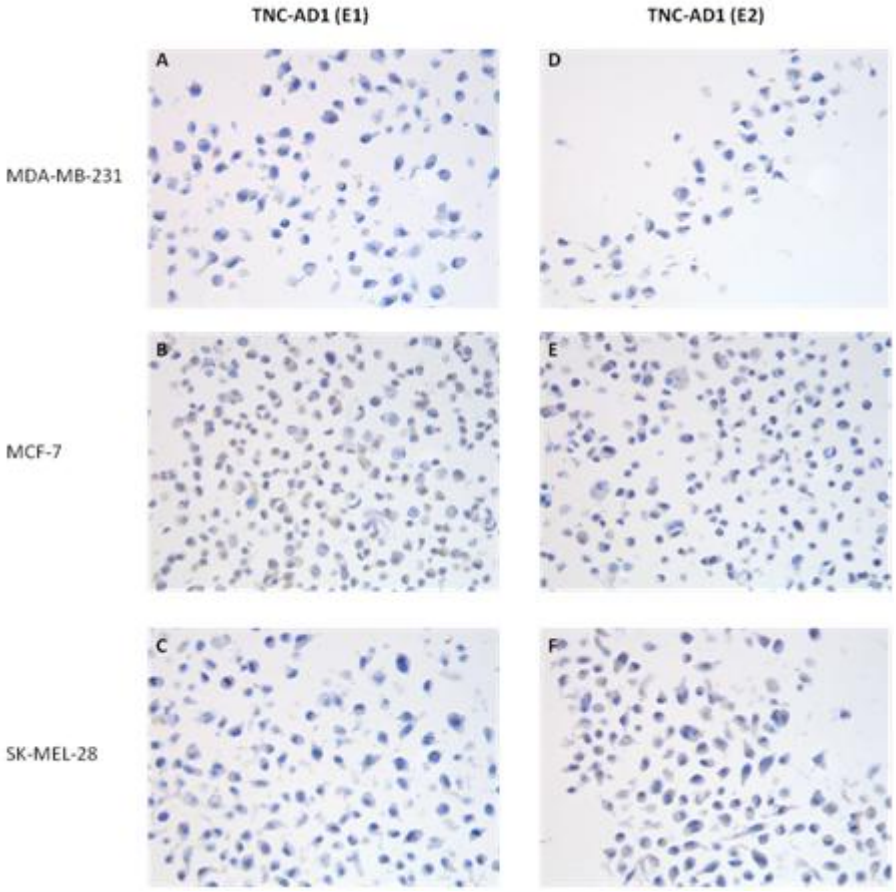


Figure 5.7: ICC staining for TNC-AD1 expression in cell lines. MDA-MB-231(A&D) and SK-Mel-28(C&F) cells endogenously express TNC-AD1, whereas MCF-7 cells are TNC null (B&E). There was no signal for TNC-AD1 using the final bleedings of the the two immunised animals (Animal-1 (A,B and C); Amimal-2 (D,E and F)).

5.3.2.2 Genosphere Biotechnologies program

Due to the failure of the first two sera to detect AD1 expression, a second screen of two polyclonal anti-peptide antibodies generated and purified by Genosphere Biotechnologies was performed. Two peptide antigens were synthesised and conjugated with KLH carrier protein, which were then injected into two different rabbits and subjected to four cycles of immunisation. In this program, Genosphere Biotechnologies offered a complete service including total IgG purification post-immunisation. Optimisation of the purified polyclonal antibodies against TNC-AD1 was performed using both Western blot and ICC.

5.3.2.3 Anti-TNC-AD1 optimisation on cell lines

5.3.2.3.1 Immunocytochemistry (ICC)

To assess the optimal antibody concentration, a serial dilution was performed (Table 5.5). The different antibody dilutions were tested on cytospun HBL-100 cells (which express high levels of TNC-AD1 (Guttery et al. 2010a)). Analysis of TNC-AD1 staining at a concentration of 0.5 µg/ml showed the optimal dilution and revealed clear specific cytoplasmic staining, whereas other dilutions showed strong nuclear and cytoplasmic localisation (Figure 5.8).

Table 5.5 Serial dilution of the purified TNC-AD1 antibody (GB1 sequence)

Antibody dilutions	Nuclear staining	Cytoplasmic staining
1:625 (4 µg/ ml)	+	+
1:1250 (3 µg/ ml)	+	+
1:2500 (2 µg/ ml)	+	+
1:5000 (1 µg/ ml	+	+
1:10,000 (0.5 µg/ ml)	-	+
1:20,000 (0.25 µg/ ml)	-	-

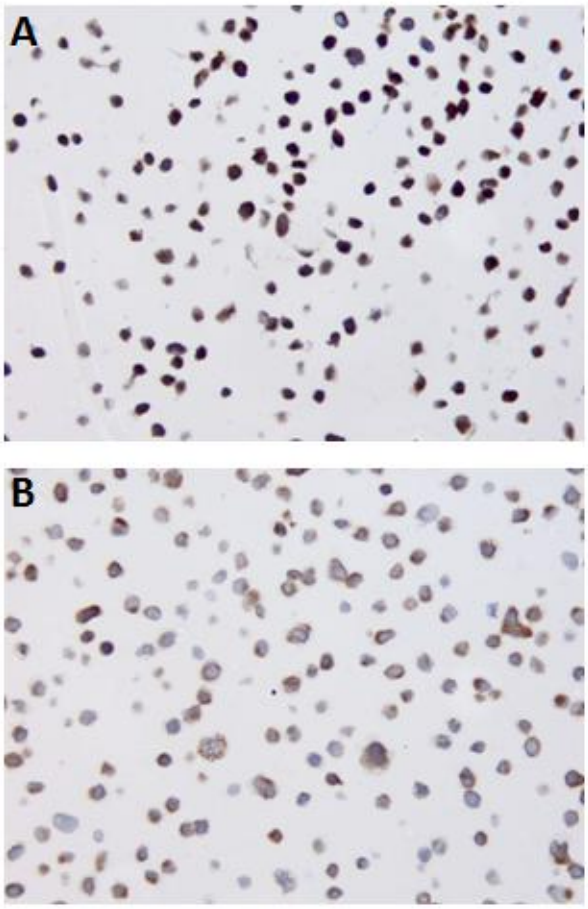


Figure 5.8: ICC optimisations of TNC-AD1 antibody concentration. A) TNC-AD1 antibody concentration at 1µg/ml. A) TNC-AD1 antibody concentration at 0.5µg/ml.

ICC optimisation of TNC-AD1 expression was also carried out on cells that endogenously express TNC (MDA-MB-231 and Sk-Mel-28) and a TNC null cell line (MCF-7) was used as a negative control.

ICC analysis of anti-AD1 polyclonal antibodies generated from animal-1 showed a clear detection of TNC-AD1 in cells endogenously expressing TNC-AD1 (Figure 5.9. A&C), with no TNC-AD1 expression detected in MCF-7 cells (Figure 5.9 B). Conversely, there was no detection for TNC-AD1 expression obtained from anti-AD1 polyclonal antibodies generated from animal-2 in cells endogenously expressing TNC-

AD1 (Figure 5.9. D&F), and non-specific staining was detected in MCF-7 cells (Figure 5.9. E)

The above optimisations confirmed that one of the two raised anti-AD1 polyclonal antibodies was suitable due to the detection of a signal, which was more likely against TNC-AD1. Therefore, the successful anti-AD1 polyclonal antibody was subjected to further optimisation in knockdown experiments and tissue studies.

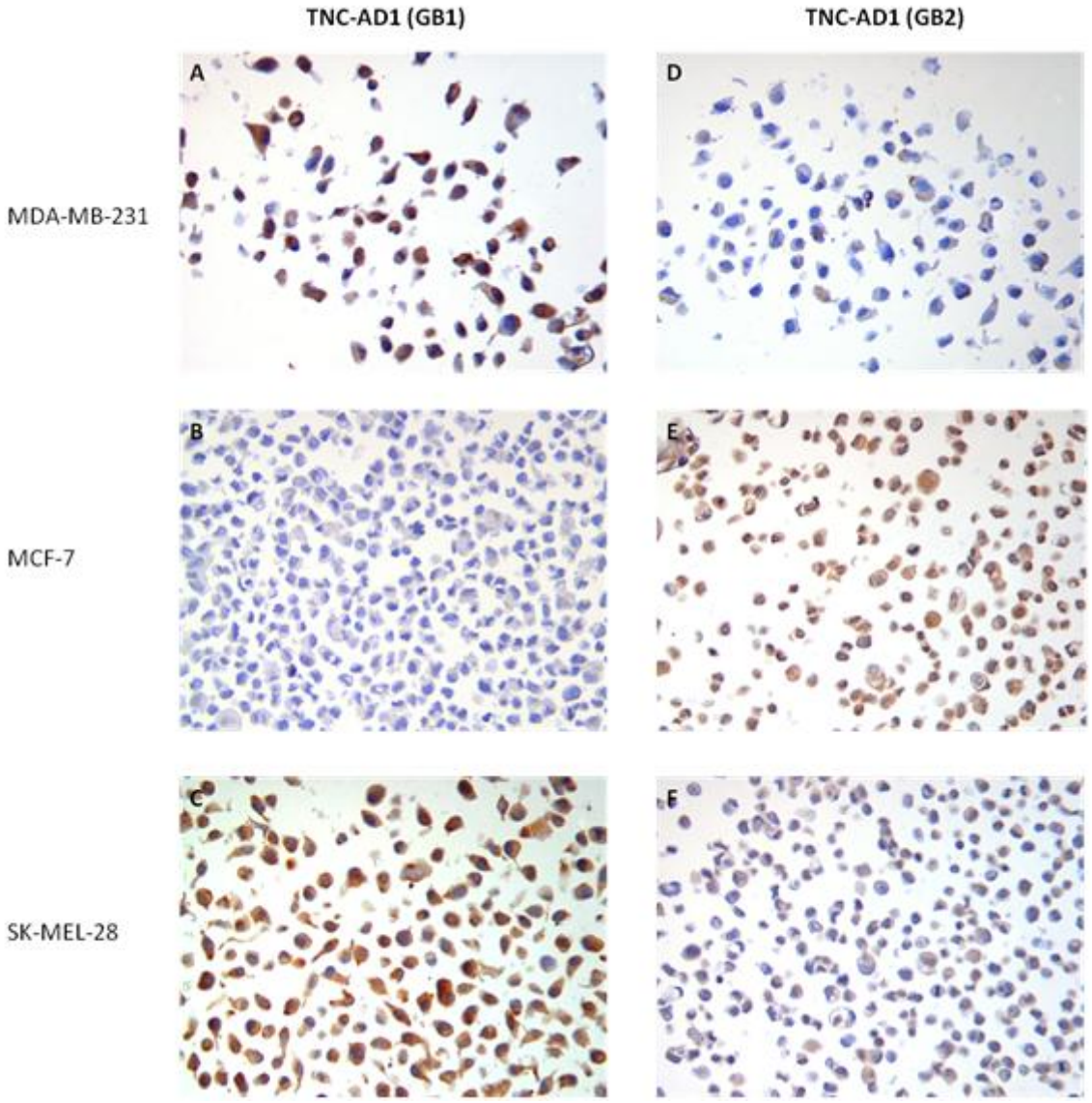


Figure 5.9: ICC staining for TNC-AD1 expression in cell lines. MDA-MB-231(A&D) and SK-Mel-28(C&F) cells endogenously express TNC-AD1, whereas MCF-7 cells are TNC null (B&E). The purified antibodies generated from two immunised animals (GB1 (A,B and C); GB2 (D,E and F)) were tested for the expression of TNC-AD1. GB1 showed a clear detection of TNC-AD1, whereas GB2 did not show any expression of TNC-AD1.

5.3.2.3.2 Knockdown of TNC isoforms containing AD1

In order to test the specificity and efficiency of the purified anti-AD1 antibody to detect any loss of endogenously expressed AD1 containing TNC isoforms, MDA-MB-231 cells were transfected with specific siRNAs to total TNC and high MW isoforms containing AD1. The expression of total TNC and AD1 containing isoforms was analysed by ICC using the BC-24 anti-TNC and TNC-AD1 antibodies. Total TNC staining was less intense when stained by both antibodies in cells transfected with total TNC siRNA compared to cells transfected with a scrambled siRNA negative control (Figure 5.10 B&E). Interestingly, staining intensity using the anti-AD1 antibody was reduced in cells transfected with the TNC-AD1 siRNA compared to cells transfected with the scrambled sequence (Figure 5.10 F); whereas total TNC expression appeared to remain unchanged when stained with the BC-24 antibody (Figure 5.10 C).

These findings suggest that the purified TNC-AD1 antibodies were able to specifically detect the endogenous AD1 containing isoforms.

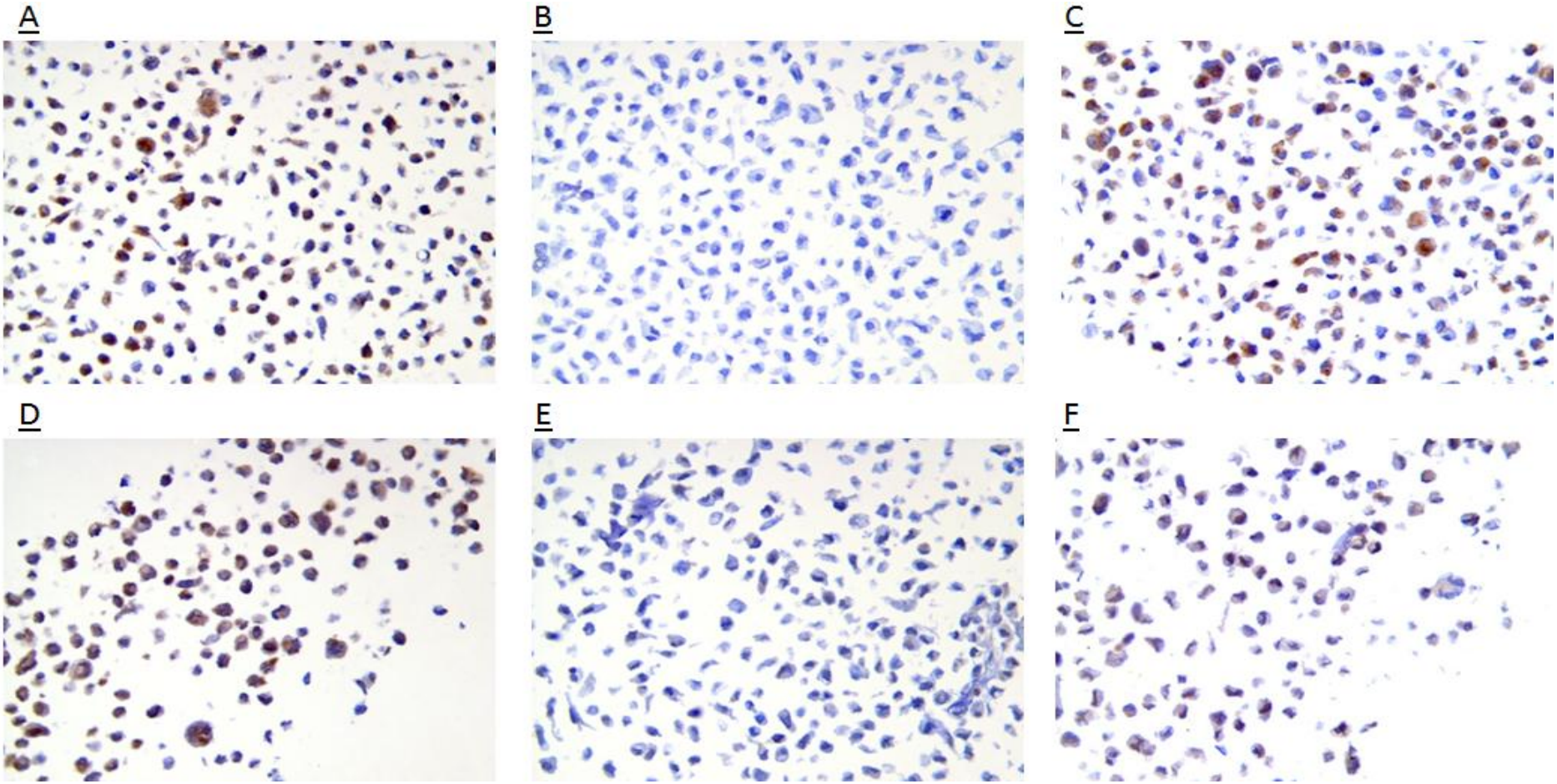


Figure 5.10: The knockdown of total TNC and TNC-AD1 in MDA-MB-231 using siRNA. A + D) MDA-MB-231 transfected with scrambled siRNA. B+ E) MDA-MB-231 transfected with total TNC siRNA. C+ F) MDA-MB-231 transfected with TNC-14-AD1 siRNA IHC staining using BC-24 monoclonal anti-TNC antibody is shown in A, B and C; whereas IHC staining using the polyclonal anti-TNC-AD1 antibody is shown in D, E and F.

5.3.2.3.3 Western blot

Western blot analysis was performed using the polyclonal anti-AD1 antibodies on conditioned media from MDA-MB-231 cells and TNC null MCF-7 cells transiently transfected with recombinant TNC isoforms TNC-Short, TNC-Long, TNC-9/14/16, TNC-14/AD1/16 and an empty vector. Western blot analysis of MCF-7 conditioned media using the H-300 anti-TNC antibody confirmed successful transfection (Figure 5.11A), as well as expression of TNC protein in MDA-MB-231 cells and an absence in non-transfected MCF-7 cells (Figure 5.11B); however, no immunoreactivity was detected for TNC-AD1 expression using the anti-AD1 antibodies by Western blot, in MDA-MB-231s or MCF-7 cells transiently transfected with TNC-14/AD1/16 (Figure 5.11).

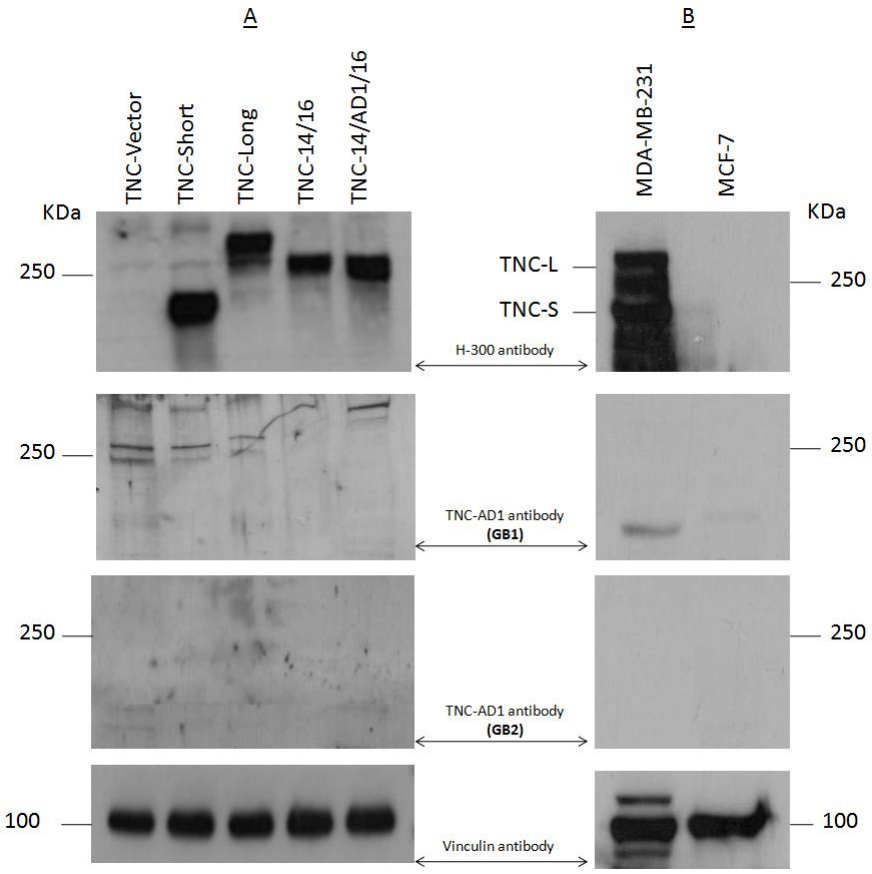


Figure 5.11: Western blot analysis of TNC expression using H-300 anti-TNC and two anti-AD1 antibodies. A) Expression of exogenous TNC isoforms transfected in MCF-7. B) Expression of endogenousTNC using MDA-MB-231 cell line compared to MCF-7 TNC null cell lines. Anti-vinculin antibody was used as loading control.

5.3.2.4 Anti-TNC-AD1 optimisation on tissues

Following successful immunoreactivity of the purified anti-AD1 polyclonal antibody using cell lines, subsequent analysis of the anti-AD1 polyclonal antibody (GB1) was performed on breast tissue by IHC. Optimal conditions for IHC using the anti-AD1 antibody were determined by testing different antigen retrieval methods.

5.3.2.4.1 Antigen retrieval

Two antigen retrieval methods were tested to allow efficient binding of the antigenic sites. These methods were heat-induced epitope retrieval using a microwave and enzymatic retrieval using proteinase K (see section 2.2.10.2, materials and methods). FFPE tissue from DCIS samples positive for TNC-AD1 mRNA by RT-qPCR (Guttery, 2009) were analysed by IHC with the anti-AD1 polyclonal antibody, and BC-24 anti-TNC monoclonal antibody was used as a positive control for total TNC staining. Antigen retrieval using proteinase K showed clear expression of TNC-AD1 in the tumour stroma as well as in the myoepithelial layer of the breast duct and blood vessels (Figure 5.12.B&D). However, considerable non-specific staining was observed in the tumour cells using anti-AD1 antibody when compared to BC-24 anti-TNC. In contrast, heat-induced epitope retrieval using a microwave only revealed weak staining intensity (Figure 5.12 A&C).

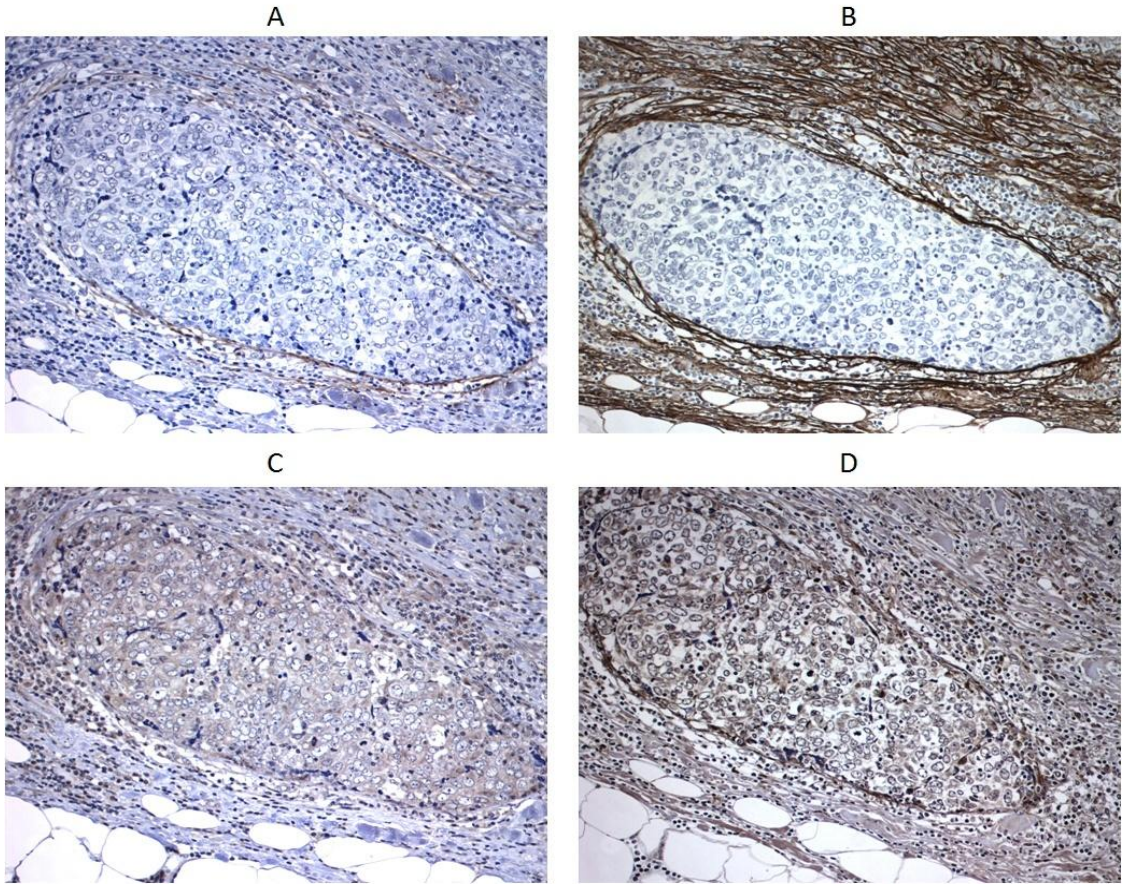


Figure 5.12: IHC optimisation of antigen retrieval methods. Heat-induced epitope retrieval is shown in (A and C), enzymatic retrieval is shown in (B and D). TNC-AD1 antibody staining is shown in (C and D), BC-24 anti TNC antibody staining is shown in (A and B).

5.3.2.4.2 Expression of AD1 in breast tissue

Having validated the specificity of the anti-AD1 antibody by ICC in cell lines, the TNC-AD1 antibody was used to analyse AD1 protein expression in normal breast and breast carcinoma tissues. Analysis of TNC expression using the BC-24 TNC antibody showed TNC to be localised to myoepithelial cells in normal breast tissue, which is likely to be the fully truncated TNC isoform (Figure 5.13A); whereas AD1 was not detected in any normal breast tissue analysed using the anti-AD1 antibody (Figure 5.13 B). Figures 5.13 C, D and E are examples of breast cancer tissues, which were previously screened for mRNA expression of AD1 containing TNC isoforms using RT-qPCR (Guttery, 2009) and were also found to be consistent with AD1 protein expression in this study using IHC and the anti-AD1 antibody.

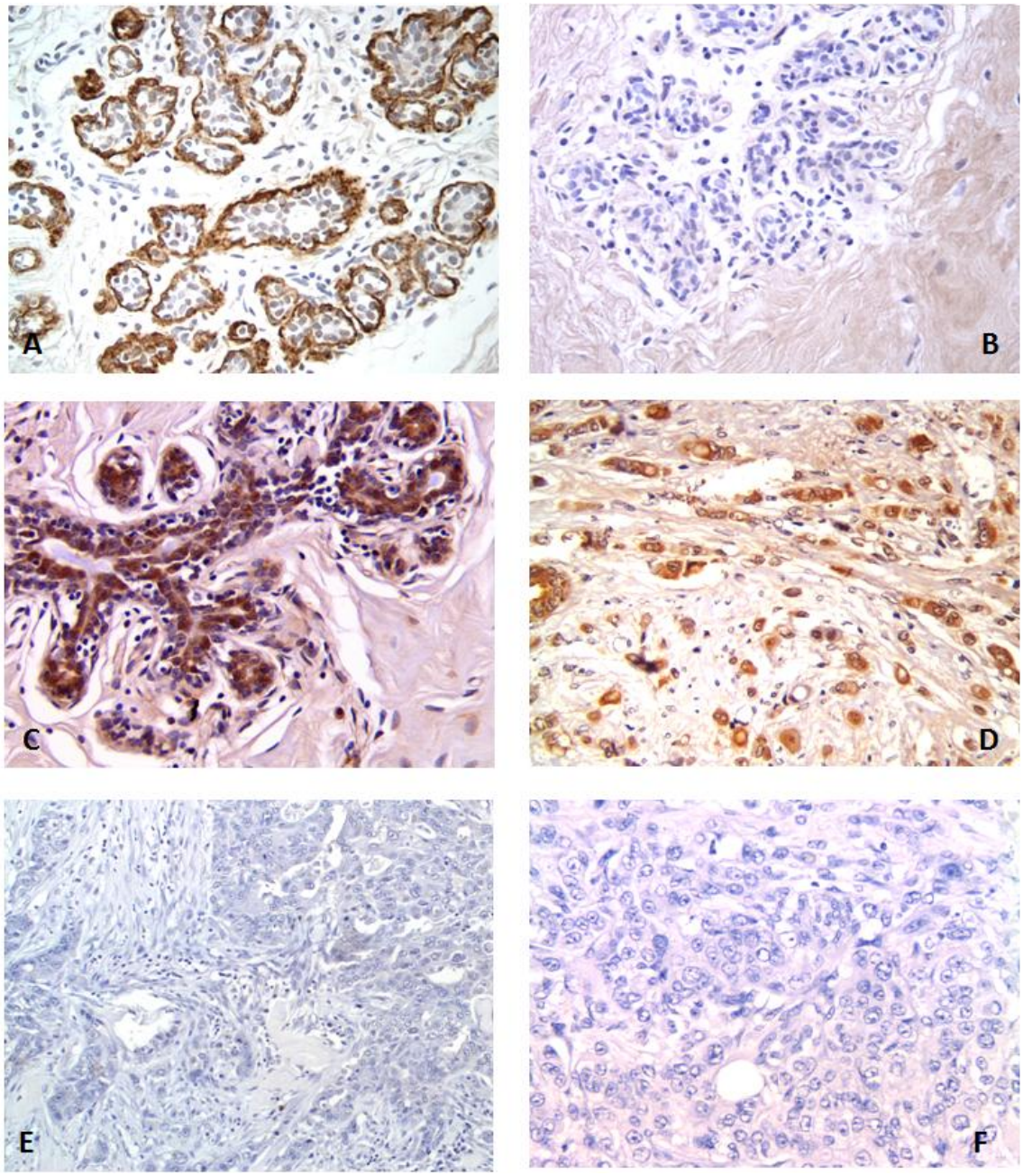


Figure 5.13: IHC staining of breast tissues using BC-24 anti-TNC antibody (A), and anti-AD1 antibody (B; C; D and E). A) Truncated TNC expression in normal breast. B) TNC-AD1 negative in normal breast tissue. C) TNC-AD1 positive in fibroadenoma breast tissue breast tissue. D) TNC-AD1 positive in lobular carcinoma breast tissue. E) TNC-AD1 negative breast tissue. F) No primary antibody control.

5.3.2.5 Optimisation summary

In summary, the immunohistochemistry (IHC) and immunocytochemistry (ICC) experiments suggest that the affinity-purified TNC-AD1 antibody is specific for TNC-AD1 protein due to the fact that TNC-AD1 immunoreactivity was only detected in breast tissue samples known to be AD1 positive by RT-qPCR and not in any AD1 negative samples. In addition, tissues negative for AD1 were positive for total TNC mRNA, confirming the specificity of the AD1 antibody. The optimal conditions for IHC analysis were shown to be enzymatic retrieval using proteinase K and an antibody concentration of 0.5 µg/ml. However, the TNC-AD1 antibody was shown to be unsuitable for use in Western blotting.

5.3.3 Tissue study of TNC-AD1 expression in breast cancer patients

A total of 36 cancers were analysed previously by our group for TNC-AD1 mRNA by RT-qPCR (Guttery et al. 2010a). RT-qPCR analysis showed that the expression of TNC-AD1 varied widely from high to low and negative. Total TNC expression was detected in all cases and TNC-AD1 expression was detected in 20 of 36 samples.

Next, IHC analysis using anti-AD1 antibody was carried out on matched FFPE tissue from the same breast cancers. The breast tissues positive for TNC-AD1 expression showed clear detection for AD1 with localisation of TNC-AD1 protein in the ECM and cytoplasm or both (Figure 5.14 A, B & C); whereas, no TNC-AD1 expression was detected in breast tissues negative for TNC-AD1 (Figure 5.14 D). TNC-AD1 expression was evaluated and scored according to the localisation of TNC-AD1 protein in the ECM and cytoplasm.

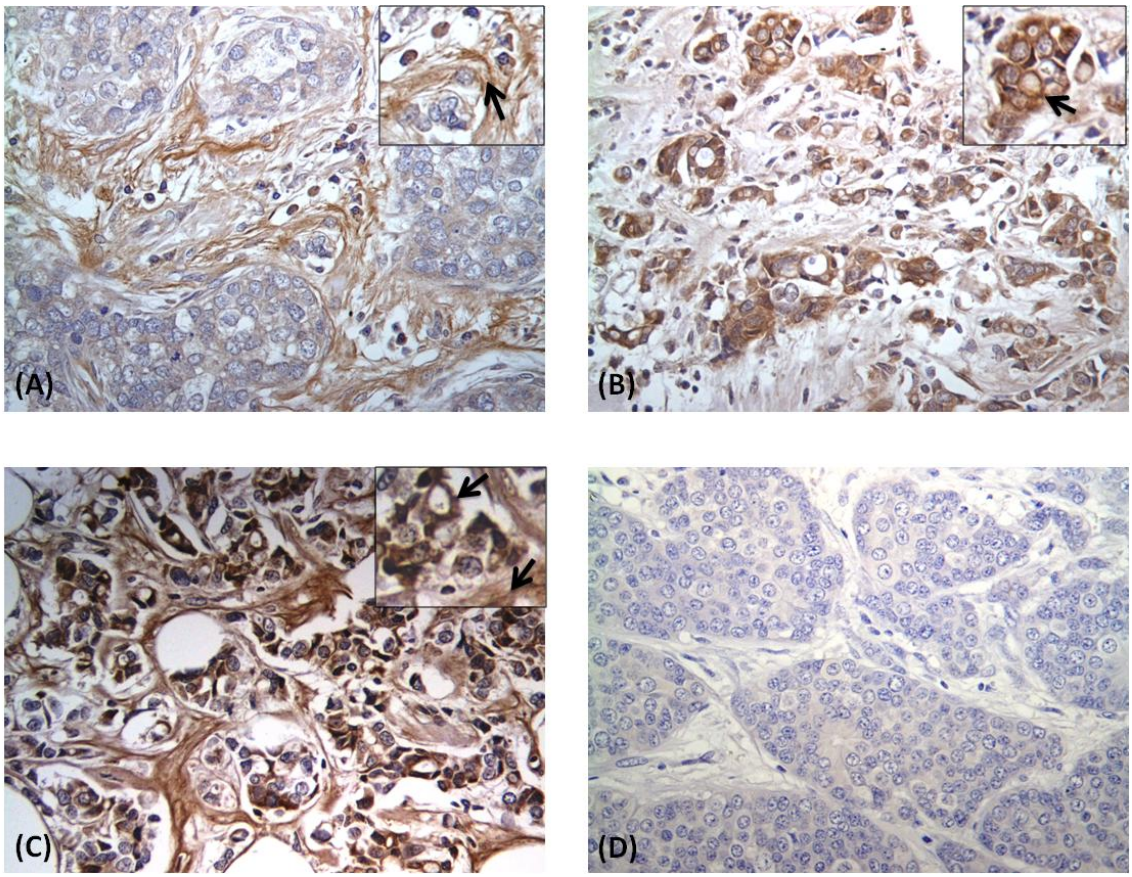


Figure 5.14: IHC staining for TNC-AD1 expression in breast tissues. A) Breast tissue positive to TNC-AD1 with ECM staining (shown inset with arrow). B) Breast tissue positive to TNC-AD1 with cytoplasmic staining (shown inset with arrow). C) Breast tissue positive to TNC-AD1 with both cytoplasmic and ECM staining (shown inset with arrows). D) Breast tissue negative to TNC-AD1.

IHC analysis showed that of the 36 breast cancers, 15 (41.7%) were positive for TNC-AD1 by IHC, and 21(58.3%) showed no staining. Five (26.6%) of the 15 cases positive for TNC-AD1 showed staining in both the ECM and cytoplasm, and 10 (66 %) showed only cytoplasmic expression. A summary of TNC-AD1 mRNA expression (from fresh frozen tissue) and TNC-AD1 staining (from matched FFPE tissue) is shown in Table 5.6.

Table 5.6 Summary of TNC-AD1 expression at both mRNA and protein levels

cases	TNC mRNA expression (RT-qPCR)				TNC-AD1 protein expression (IHC)	
	Number of molecules		Level of expression		(Immuno-staining)	
	TNC	AD1	TNC	AD1	Cytoplasm	ECM
C-1	1.5E+09	182237	Mod	High	+ (50%)	+ (50%)
C-2	1E+13	542004	High	High	-	-
C-3	8.3E+07	284907	Low	High	+ (20%)	-
C-4	3.5E+07	131747	Low	High	+ (15%)	-
C-5	1.4E+09	195491	Mod	High	-	-
C-6	2E+11	299581	High	High	+ (25%)	-
C-7	2.4E+08	171349	Mod	High	+ (10%)	-
C-8	6.4E+08	688136	Mod	High	+ (70%)	+ (90%)
C-9	1.1E+10	129625	Mod	High	-	-
C-10	576942	104164	Low	High	+ (20%)	-
C-11	602221	114384	Low	High	+ (25%)	-
C-12	1324118	148676	Low	High	-	-
C-13	1935513	173818	Low	High	-	-
C-14	4.7E+09	23762.1	Mod	Low	-	-
C-15	4.3E+10	50445.2	Mod	Low	-	-
C-16	5.7E+09	36558	Mod	Low	+ (50%)	+ (05%)
C-17	7.5E+09	46750	Mod	Low	+ (70%)	+ (10%)
C-18	2.4E+10	39588.7	Mod	Low	+ (40%)	-
C-19	355220	19025.5	Low	Low	+ (60%)	-
C-20	2640252	41782.7	Low	Low	-	-
C-21	1.6E+11	0	High	Neg	-	-
C-22	1.3E+11	0	High	Neg	-	-
C-23	4781458	0	Low	Neg	-	-
C-24	6.3E+08	0	Mod	Neg	-	-
C-25	2.8E+09	0	Mod	Neg	-	-
C-26	9.7E+09	0	Mod	Neg	+ (10%)	-
C-27	1.1E+10	0	Mod	Neg	-	-
C-28	9576555	0	Low	Neg	-	-
C-29	5.8E+09	0	Mod	Neg	-	-
C-30	7.1E+09	0	Mod	Neg	-	-
C-31	5.3E+09	0	Mod	Neg	-	+ (90%)
C-32	2.1E+09	0	Mod	Neg	-	-
C-33	3.4E+12	0	High	Neg	-	-
C-34	555774	0	Low	Neg	-	-
C-35	2428436	0	Low	Neg	-	-
C-36	5.4E+09	0	Mod	Neg	+ (40%)	-

ECM= Extracellular matrix; (+) Positive staining; (-) No staining; C= case

Correlation between TNC-AD1 at the mRNA and protein levels was examined (Table 5.7), which showed 60% of cases expressing TNC-AD1 protein were also positive at the mRNA level.

A Mann-Whitney test showed a significant correlation between TNC-AD1 staining and mRNA expression (p = 0.0208) (Figure 5.15 B). However, there was no significant relationship between TNC-AD1 staining and total TNC (Figure 5.15 A).

Table 5.7 The correlation between TNC mRNA and TNC-AD1 staining expression in breast carcinoma tissues.

TNC expression at mRNA		Total (N=36)	TNC-AD1 staining		P value
			Positive	Negative	
Total TNC	Low	12	5	7	0.3650 ^{ns}
	Mod/High	24	10	14	
TNC-AD1	positive	20	12	8	0.0208*
	negative	16	3	13	

ns = not significant

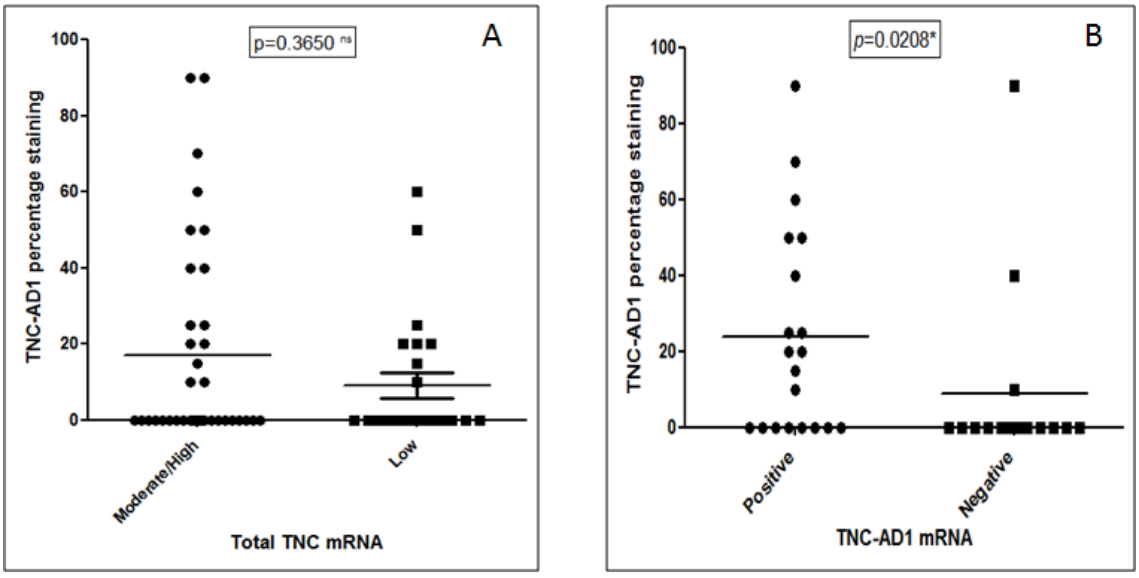


Figure 5.15: The Mann-Whitney test shows the correlation of expression levels between total TNC and TNC-AD1 expression at mRNA and TNC-AD1 staining in breast cancer tissues. A) Corelation between total TNC mRNA and TNC-AD1 staing. B) Corelation between TNC-AD1 at mRNA and proetein.

Next, comparing expression of TNC-AD1 and clinicopathological features showed that 30.5% of IDC cases were positive for AD1 at both mRNA and protein levels, 36.1% were >40 years old (p = 0.0399), 30.5% were ER^+ and 25% were LN^+. The summary contingency table using a Fisher's Exact Test between TNC-AD1 expression and clinicopathological features is shown in Table5.8.

Table5.8 The correlation summery between TNC-AD1 expressions and clinicopathological features. Fisher's exact test was used for statistical analysis of TNC-AD1 expression comparison with clinicopathological features.

Clinicopathological features		Total (N=36)	TNC-AD1 mRNA expression (RT-qPCR)			TNC-AD1 protein expression (IHC)		
			+ ve	-ve	P value	+ ve	-ve	P value
Tumour Type	**IDC**	31	18	13	0.1380 ^{ns}	11	20	0.0610^{ns}
	ILC	5	2	3		4	1	
Tumour Grade	**(I & II)**	19	9	10	0.3351 ^{ns}	9	10	0.4632^{ns}
	(III)	17	11	6		6	11	
Patient Age	**≤ 40**	12	6	6	0.7295 ^{ns}	2	10	0.0399*
	> 40	24	14	10		13	11	
ER Status	**ER^+**	25	12	12	0.4123 ^{ns}	11	13	0.6807^{ns}
	ER^-	7	6	2		3	5	
	n/k	4	2	2		1	3	
PR Status	**PR^+**	20	22	9	0.6979 ^{ns}	10	10	0.2974^{ns}
	PR^-	10	6	4		3	7	
	n/k	6	3	3		2	4	
Lymph Node Status	**LN^+**	19	9	10	0.3064 ^{ns}	9	10	0.5567^{ns}
	LN^-	16	11	5		6	10	
	n/k	1	0	1		0	1	

IDC = Invasive ductal carcinoma; ILC = Invasive lobular carcinoma; ER = Oestrogen receptor; PR = Progesterone receptor; n/k = not known; + ve = Positive; -ve = Negative; ns = Not significant; *= $p \leq 0.05$

5.4 Discussion

5.4.1 Generation of TNC-AD1 polyclonal antibodies

Previous findings on TNC-AD1 expression were limited to the mRNA levels (Derr et al. 1997; Guttery et al. 2010a), but attempts to study human AD1 expression at the protein level were hampered by the lack of TNC-AD1 isoform specific antibodies. Therefore, generation of rabbit polyclonal antibodies against AD1 was performed as part of this thesis.

Of the generated polyclonal antibodies, only one successfully recognised TNC-AD1 when tested by ICC and IHC, but not by Western blot. The antibody may not work in a particular assay, which may be that the peptide sequence corresponds to a non-exposed region of the native protein (Harlowet et al. 1988; Rosenberg et al. 1996) or the epitope for antibody recognition was destroyed during the denaturation stage of the Western blot protocol. Furthermore, commercially available anti-TNC antibodies are also known to only work when tested by Western blot or ICC/IHC (e.g. H-300 and BC-24, respectively).

In this study, the expression of TNC-AD1 was found to be cytoplasmic in cell lines endogenously expressing TNC-AD1, and both the cytoplasm and ECM in breast cancer tissues. In addition, ICC analysis of AD1 protein showed consistency with mRNA profiles in cells endogenously expressing TNC-AD1. This was also the case for TNC null cell lines. Furthermore, breast cancer tissues with known profiles for total TNC and TNC-14-AD1-16 also showed consistent expression of AD1 at the protein level. The findings here are consistent with previous studies of AD1 localisation using *in situ* hybridisation in that protein localisation of AD1 containing isoforms was found to be in the malignant epithelial cells of invasive tumours (Guttery et al. 2010a). However,

protein localisation was not found in the myoepithelium surrounding larger normal ducts in this study, but may be found if using a larger cohort of control specimens.

Guttery et al. (2010) also analysed RNA isolated from 134 carcinomas using RT-qPCR and found AD1 expression in 42 (31%) carcinomas. In addition, AD1 was significantly associated with grade 3 tumours, ER negative status and with younger patient age (≤40 years). Contrary to this, staining of TNC-AD1 in breast cancer tissues was significantly associated with patient age over 40 years. Therefore, the findings in this chapter correlate with the previous findings only regarding the presence of AD1 expression in invasive breast cancer, but there was no relationship between women younger than 40 years with breast cancer and TNC-AD1 expression at the protein level.

The IHC method is commonly used in diagnostic applications despite its weaknesses including the sensitive variations between fields, fixation time and processing, antigen retrieval, staining procedure and interpretations (O'Leary 2001). On the other hand, the sensitivity of quantitative RT-PCR is capable of detecting low abundance mRNA (Bustin 2000). Therefore, the correlation between the expressions of TNC-AD1 using both methods was not identical, which may be due to variations in these methods and sample heterogeneity due to the use of fresh frozen (FF) and FFPE tissues.

Hence, detecting specific TNC-AD1 isoforms using antibody targeting was achieved, and in future this novel polyclonal antibody could be used in addition to commercially available antibodies (ie BC24, H300 and clone IIIB) for TNC detection in normal and malignant tissues.

5.5 Conclusion

The generated polyclonal antibody against TNC-AD1 successfully recognised TNC-AD1 and was used in a pilot study to evaluate expression of TNC-AD1 protein in human breast tissues. The AD1 antibody may serve as a specific tool for further functional studies of the pathological role of TNC-AD1 in breast cancer.

Chapter 6. Conclusions and Future Direction

The matricellular protein TNC is expressed transiently during embryonic development, wound healing, regeneration and also at sites of dynamic tissue remodelling. TNC is also highly expressed in the stroma of solid tumours, including breast cancers (Ishihara et al. 1995). Expression of TNC in breast cancer is associated with poor clinical outcome (Ishihara et al. 1995; Tokes et al. 1999; Goepel et al. 2000). However, the role of TNC and its isoforms in breast cancer development and progression is less well studied.

6.1 The effect of TNC knockdown on invasion and proliferation

MDA-MB-231 cells transfected with siRNA targeting endogenous total TNC and high MW TNC isoforms showed a significant reduction in invasion compared to cells transfected with a scrambled siRNA. Invasion was reduced when endogenous TNC was down regulated and this effect was more significant than for individual isoforms (total TNC>TNC-14>TNC-14-AD1). The siRNA targeting total TNC and TNC-AD1 also decreased cell proliferation, identified by the mitosis marker (pHH-3). This mirrors previous research in our group, where transfection of recombinant TNC-16, TNC 14/16 and TNC-14/AD1/16 isoforms into TNC null MCF-7 cells significantly increased invasion and tumour cell growth (Guttery et al. 2010a). TNC was also shown to increase proliferation and invasion in various cell lines such as glioblastoma (Hirata et al. 2009), melanoma (Fukunaga-Kalabis et al. 2010) and pancreatic cancer (Paron et al. 2011). Together data presented in this thesis following siRNA targeting of TNC clearly supports these previous studies, which show that TNC promotes tumour cell invasion and proliferation.

6.2 The effect of TNC knockdown on global gene expression

In the microarray study, when TNC was targeted by siRNA in MDA-MB-231 and MDA-MB-436 cells, multiple genes were shown to be either up or down regulated, but with little overlap between the two cell backgrounds. Six genes, which were common to both cell backgrounds (*CREBL2, YWHAE, RRAS2, CDC14B, DISC1*and *QKI)* were selected for further study, but only five of these were validated by RT-qPCR (*CREBL2, YWHAE, CDC14B, RRAS* and *QKI)* as the *DISC1* assay consistently showed no expression.

Of the six genes selected for further study, *QK1* was the only gene that was up-regulated by siRNA targeting of TNC. This study is the first to show that down regulation of TNC and its high MW isoforms up-regulates *QKI* in breast cancer cell lines. This supports, previous data which showed that expression of *QK1* was inversely related to breast cancer and suggests that *QKI* is a tumour suppressor gene (Novikov et al. 2011). Similarly, tropomyosin 1 (*TM1*) is a tumour suppressor gene that has shown to decrease the growth of MCF-7 breast cancer cells (Zhu et al. 2007). TNC has been shown to decrease the expression of *TM1* by interacting with syndecan-4 (Lange et al. 2008). Based on results presented in this thesis, one could hypothesise that TNC helps to drive cancer progression by decreasing the levels of tumour suppressor genes such as *QKI* and *TM1*. Further research should be focussed on mechanisms involving TNC mediated suppression of QKI or other tumour suppressor genes in breast cancer.

6.3 Proteomic analysis of TNC knockdown in breast cell lines

Proteomics is a powerful tool for large scale detection and analysis of proteins and post-translational modifications. Protein structures and dynamics have been measured quantitatively with high sensitivity (Lamond et al. 2012). In the pilot proteomic analysis TSP-1 was found to be significantly increased in breast cancer cell lines transfected

with siRNA targeting total TNC, which was confirmed by Western blotting. Furthermore, Western blot analysis of conditioned media obtained from cells transfected with siRNAs targeting specific high MW TNC isoforms also showed an increase in TSP-1 expression.

The matricellular protein TSP-1 is known to be a potent inhibitor of angiogenesis, and is associated with cancer, wound healing and inflammation (Lopez-Dee, Pidcock & Gutierrez 2011). Moreover, low TSP-1 was shown to increase the risk of invasion and metastasis in human breast cancer (Ioachim et al. 2012). Interestingly, in a previous study, TSP-1 was identified in the stroma of malignant breast cancers and correlated with levels of TNC in the ECM (Wang et al. 1996). Subsequently, it was shown that TSP-1 was expressed at low levels in tumour cells, whereas stromal fibroblast showed high levels of TSP-1 (Hanamura et al. 1997).

The process of angiogenic regulation by TNC and TSP-1 is not clear. In this study, tissue sections from invasive ductal carcinomas known to be positive for TNC-AD1 at the mRNA level showed significantly stronger staining for TSP-1 protein (13 of 20 positive for TSP-1). Interestingly, knockdown of total TNC and high MW TNC isoforms up-regulated TSP-1 protein levels in MDA-MB-231 cells. This is the first study to show that TNC regulates the endogenous expression of TSP-1 in invasive breast cancer cell lines. Recently, it has been proposed that activation of the PI3K/Rho pathway by activated RAS can increase MYC phosphorylation and inhibit TSP-1 gene expression (Chong et al. 2012); hence it would appear there are several potential routes to TSP-1 activation.

6.4 TNC-AD1 expression in breast cancer patients

Attempts to generate an antibody to TNC-AD1 were successful, in that a polyclonal sera was generated, which showed specific staining in cell model experiments (AD1+ve vs. AD1 –ve cells). Importantly, a pilot study in breast cancer tissue sections also showed AD1 positive staining, which correlated with previous mRNA data. In 36 carcinomas, TNC-AD1 staining was found in either tumour cell cytoplasm, ECM or both, with most common expression found in women over the age of 40 years. The results from immunohistochemistry experiments support our previous studies of AD1 localisation by *in situ* hybridisation, in that protein localisation of AD1 containing isoforms was in the malignant epithelial cells of invasive tumours (Guttery et al. 2010a). However, in the mRNA study *AD1* expression was significantly associated with breast cancers in women < 40 years of age. In this analysis, TNC-AD1 protein expression was related to hormone receptor status and metastasis (ER, PR and LN positive, but not statistically significant). It has been previously shown that TNC was associated with tamoxifen resistance and poor prognosis (Helleman et al. 2008). Future studies should be focussed on TNC-AD1 and other TNC high MW isoforms on prognosis, relapse and treatment resistance in breast cancer, for example by tissue microarray.

6.5 Limitations of the study

It is important to briefly consider the limitations of this study, imposed by technical procedures, sample size and other variables. Firstly, RNA interference is a useful tool for gene silencing; however, it is subject to technical difficulties such as incomplete loss of gene function and differences in visible phenotype by partial silencing compared to the genetically null phenotype. This study yielded up to 90% efficiency for TNC silencing and hence, we believe that the phenotypes produced are relevant. Moreover, in this study, we selected three siRNAs targeting different exons of total TNC to minimise

non-target effects. However, high MW isoforms of TNC were targeted by a single siRNA and we do not know whether selecting multiple sites within the same exon of interest may yield a different phenotype (Jackson et al. 2006).

Secondly, technical difficulties are known as the 'Achilles heel' of microarray experiments. Due to cost constraints the microarray study was limited to knockdown of total TNC only and other high MW TNC isoforms were not investigated. Because of degraded mRNA, false microarray results are possible, and the microarray experiment has to be repeated for many times to reduce the error. Therefore we used multiple replicates for each cell line.

In most of the tissue sections analysed by immunohistochemistry, there was a significant correlation between mRNA level by RT-qPCR and TNC-AD1 proteins by IHC. We have analysed only 36 samples of breast cancer at different clinical stages; therefore, a larger cohort is required to make significant judgements.

6.6 Conclusion

This study showed clearly that knockdown of total TNC and high MW TNC isoforms significantly reduced proliferation and invasion in highly invasive breast cancer cell lines. In addition, knockdown of total TNC showed a marked change in global gene expression. Of note, transcripts of *QKI* were specifically up-regulated by silencing of high MW TNC isoform as well as total TNC silencing; whereas, by proteomic and Western blot analysis, thrombospondin-1 (TSP-1) was significantly up-regulated in response to knockdown of both total TNC and high MW TNC isoforms. Finally, a polyclonal antibody to detect AD1 was generated as part of this thesis for use in tissue studies.

In conclusion, results of this thesis have shown that TNC and its isoforms play an important role in breast cancer, influencing proliferation, invasion and angiogenesis.

This warrants further investigation to evaluate whether TNC and its signalling pathways might be a suitable drug target for breast cancer treatment.

6.7 Future perspectives

TNC has been shown to regulate various critical stages of breast cancer progression such as proliferation, invasion and angiogenesis. It is not known whether TNC is the main mediator of tumourigenesis in breast cancer. Future studies should be focussed on TNC in stromal cells such as fibroblasts, endothelial cells and ECM signalling pathways. The effect of specific high MW TNC isoforms in breast cancer pathogenesis is elusive and requires further research.

The tissue based studies with the TNC-AD1 antibody and TSP-1 need to be extended to a larger cohort to confirm our findings. The effects of age, ethnicity, drug treatment and adiposity on TNC expression are not clear and warrant further study. Finally, TNC was recently implicated in drug resistance in breast cancer; in the future drugs may be developed targeting TNC and its high MW isoforms to modulate tumour cell proliferation, invasion, angiogenesis, treatment resistance and hence clinical outcome in breast cancer.

Appendices

Appendix 1: Staining percentage of pHH3 in transfected MDA-MB-231 with siRNA targeting total TNC and high MW TNC isoforms.

Staining area	pHH-3 staining %											
	Scrambled siRNA			Total TNC siRNA			TNC-14 siRNA			TNC-14-AD1 siRNA		
	T-1	T-2	T-3	T-1	T-2	T-3	T-1	T-2	T-3	T-1	T-2	T-3
Field (1)	4.9	5.6	4.2	1.2	0.6	1.5	3.1	3.1	4.3	2.3	1.7	2.1
Field (2)	4	4.5	6.8	1.3	0.9	1.1	3.3	3.5	4.5	2.9	1.8	1.8
Field (3)	6.3	4.5	6.7	1.5	0.9	1.4	3	3.2	3.9	2	2.1	2.1
Field (4)	5.7	4.9	6.3	0.9	0.8	1.2	2.6	4	4.7	2.1	1.2	2.1
Field (5)	4.7	4.5	6.6	1.1	0.9	1.2	3.4	3.6	4.8	2.3	2.8	2.7
Average staining %	5.12	4.8	6.12	1.2	0.82	1.28	3.08	3.48	4.44	2.32	1.92	2.16
Total %	5.34			1.10			3.66			2.13		

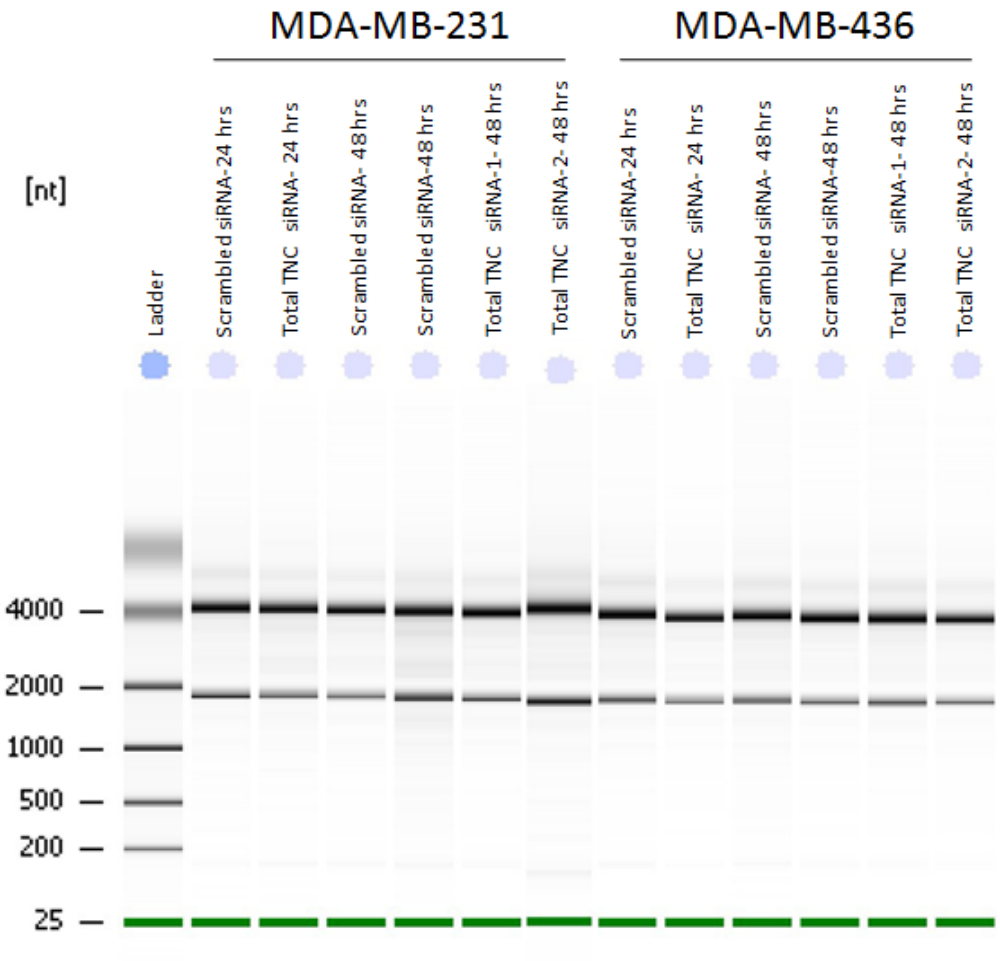
T= Transfection

Appendix 2: Statistical significance of the invasion assay resulted from the transfected MDA-MB-231 with siRNA targeting total TNC and high MW TNC isoforms.

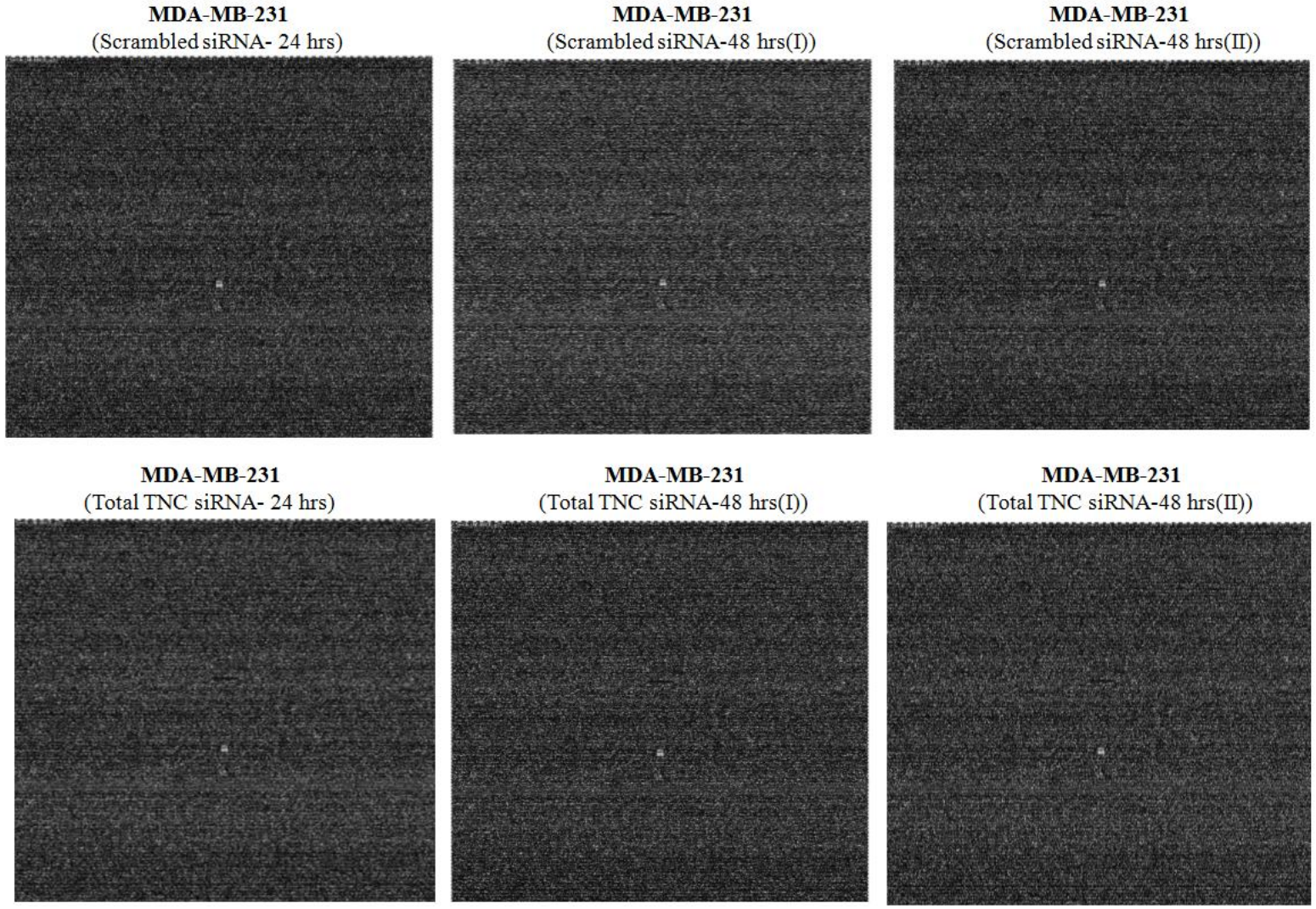
Time point	Total TNC siRNA	TNC-14 siRNA	TNC-14-AD1
2	ns	ns	ns
4	ns	ns	ns
6	ns	ns	ns
8	ns	ns	ns
10	ns	ns	ns
12	*	*	ns
14	***	***	ns
16	***	***	*
18	***	***	**
20	***	***	***
22	***	***	***
24	***	***	***
26	***	***	***
28	***	***	***
30	***	***	***
32	***	***	***
34	***	***	***
36	***	***	***
38	***	***	***
40	***	***	***
42	***	***	***
44	***	***	**
46	***	***	**
48	***	***	**

(* = P<0.05; ** = P<0.01;*** = P<0.001)

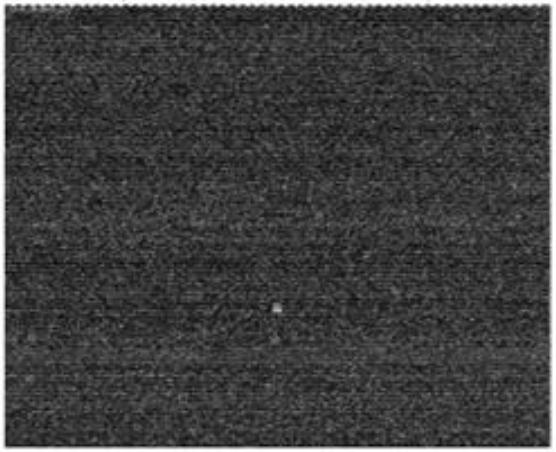
Appendix 3: Electrophoresis automated run of all sample's total RNA



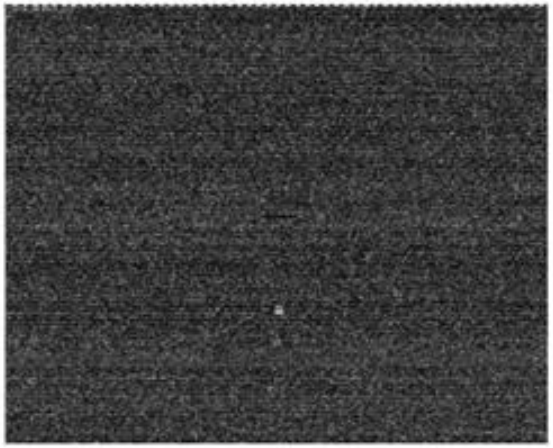
Appendix 4: images of the scanned GeneChip arrays for the trasfected breast cell lines with total TNC and scrambled siRNAs.



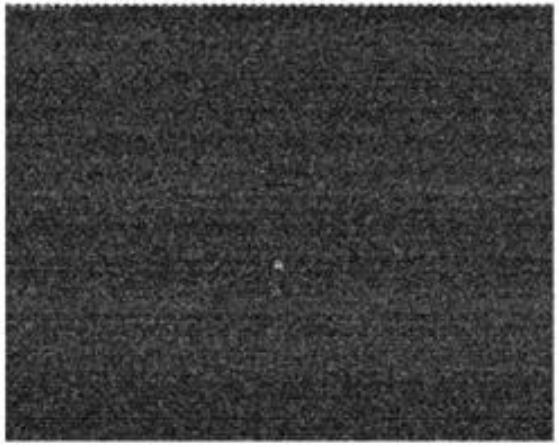
MDA-MB-436
(Scrambled siRNA- 24 hrs)



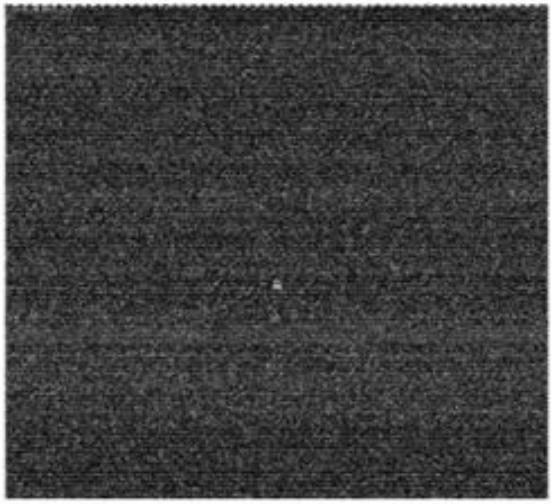
MDA-MB-436
(Scrambled siRNA-48 hrs(I))



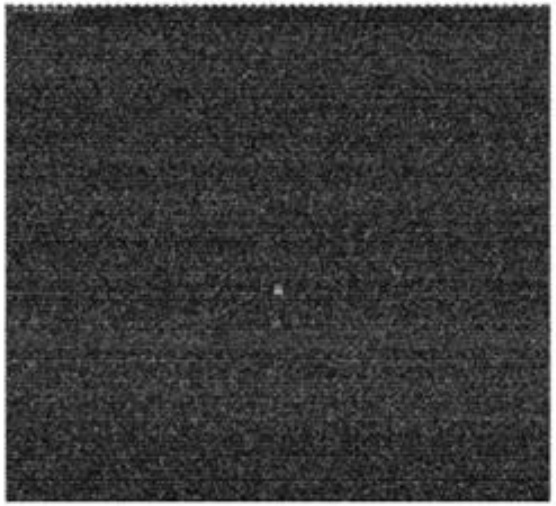
MDA-MB-436
(Scrambled siRNA-48 hrs(II))



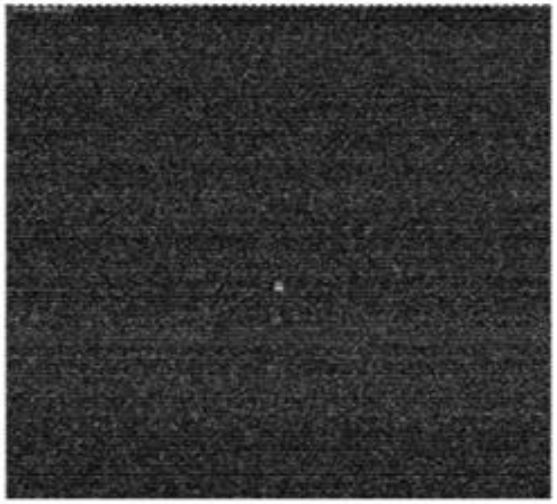
MDA-MB-436
(Total TNC siRNA- 24 hrs)



MDA-MB-436
(Total TNC siRNA-48 hrs(I))



MDA-MB-436
(Total TNC siRNA-48 hrs(II))



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Due to third party copyright restrictions, the following figures have been removed from the introduction of the electronic version of this thesis:

Figure 0.1: Anatomy of the female breast and enlarged cross section of the normal duct.

http://www.breastcancer.org/pictures/breast_anatomy/image_1

Figure 0.2: The schematic representation of cancer progression.

http://www.nature.com/nrc/journal/v7/n9/fig_tab/nrc2193_F1.html

Figure 0.3: The schematic representation of mammary duct and lobule.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2964182/figure/A003244F1/

Figure 0.4: The perturbation of microenvironment during tumour progression.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2964182/figure/A003244F2/

Figure 0.5: TNC binding region.

http://www.sciencedirect.com/science/article/pii/S1357272504004261#gr1