Molecular cloning and functional studies of tenasin-C isoforms containing the fibronectin-type III repeat additional domain 1 (AD1)

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By

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Abstract

Tenascin-C is an extracellular matrix glycoprotein expressed at low levels in normal breast tissue and highly expressed in both the stroma and malignant cells of solid tumours. Multiple isoforms of TNC are generated by alternative splicing. The aim of this study was: 1) to investigate the expression of key high molecular weight (MW) TNC isoforms containing domains D, B/D, AD1 and AD2 in normal, benign and malignant breast and relate expression to histopathological features, 2) to investigate the functional significance of AD1 by molecular cloning and 2D invasion assays, 3) to perform differential gene expression analysis using GeneChip arrays and relate to expression of high MW TNC isoforms in MCF-7 cells.

AD1 and AD2 were detected in all TNC positive breast cell lines, normal and tumour breast tissues and isolated cells from normal breast tissue, with myoepithelial cells being the major source of AD1. In carcinomas, expression of high MW TNC was significantly associated with younger age (≤ 40 years; \( p = < 0.05 \) for all isoforms), negative ER (\( p = 0.011 \) for AD1 and 0.032 for AD1/AD2 respectively) and high grade (\( p = 0.017 \) and 0.019 respectively). Expression of total TNC, TNC-9/16 and TNC-14/16 was also associated with negative CK14 (\( p = 0.003 \) for all), and higher TNC-14/16 expression was associated with lobular carcinomas (\( p = 0.004 \)). Molecular cloning of AD1 and transfection studies using the TNC-14/AD1/16 isoform significantly increased MCF-7 cell invasion to a level comparable to TNC-9/14/16 (\( p = < 0.001 \)). Differential gene expression analysis showed that TNC-9/14/16 and TNC-14/AD1/16 significantly increased expression of interferon-inducible transmembrane protein 1 (IFITM1) and profilin-1 (PFN1) in transfected MCF-7 cells. However, quantitative RT-PCR analysis of tissue samples showed significant down-regulation of PFN1 in tumours, compared to normal breast (\( p = 0.02 \)), which was significantly associated with high TNC-14/16 expression (\( p = 0.002 \)).

In conclusion, high MW TNC isoforms including AD1 have been associated with more aggressive features of breast carcinomas and in-vitro with an invasive phenotype. Moreover, this study also identifies PFN1 as a novel gene target associated with tumours that express high levels of TNC-14/16.
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Declaration
I herewith declare that I autonomously carried out the PhD thesis entitled “Molecular cloning and functional studies of tenascin-C isoforms containing the fibronectin-type III repeat additional domain 1 (AD1)”. The following third party assistance has been enlisted: -

Dr Rachael Hancox – Extraction of RNA and preparation of 8/18 PCR products from breast reduction mammoplasties and invasive carcinoma tissue. Preparation, extraction and culture of myoepithelial cell and fibroblasts from reduction mammoplasties.
Dr Sinead Lambe – Preparation and extraction of RNA/cDNA from breast organoids and invasive carcinoma tissue.
Vasileos Modes – Preparation, extraction and culture of myoepithelial cells from normal breast reduction mammoplasties and non-involved tissue from cancer containing breasts. Preparation of RNA/cDNA from cells described above.
Lindsay Primrose – Generation of TNC-L, TNC-S, TNC-9/16 and TNC-9/14/16 sequences carried on the pCMV Script vector (previous Breast Cancer campaign grant).
Dr Graham Ball – Microarray data mining and regression analysis.

I did not receive any assistance in return for payment by consulting agencies or any other person. No-one has received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis. I conducted the project at the following institutions: -

Department of Cancer Studies and Molecular Medicine, University of Leicester.
The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

Signature.............................................................................................................................................
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# List of Abbreviations

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>AD1</td>
<td>Additional domain 1</td>
</tr>
<tr>
<td>AD2</td>
<td>Additional domain 2</td>
</tr>
<tr>
<td>ADH</td>
<td>Atypical ductal hyperplasia</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>Amp&lt;sub&gt;R&lt;/sub&gt;</td>
<td>Ampicillin resistance</td>
</tr>
<tr>
<td>AMV</td>
<td>Avian Myeloblastosis Virus</td>
</tr>
<tr>
<td>APAAP</td>
<td>Alkaline phosphatase anti-alkaline phosphatase</td>
</tr>
<tr>
<td>AREG</td>
<td>Amphiregulin</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BCC</td>
<td>Breast Cancer Campaign</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Ionic calcium</td>
</tr>
<tr>
<td>CALEB</td>
<td>Chicken acidic leucine-rich EGF like domain containing brain protein</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
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<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complimentary RNA</td>
</tr>
<tr>
<td>CK</td>
<td>Cytokeratin</td>
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<td>CM</td>
<td>Conditioned medium</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in-situ</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DiIC&lt;sub&gt;12&lt;/sub&gt;(3)</td>
<td>1,1′-didodecyl-3,3′,3′,3′-tetramethyldiocarbocyanine perchlorate</td>
</tr>
<tr>
<td>DKK1</td>
<td>Dickkopf-1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagles medium</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxy-adenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxy-cytidine triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxy-guanosine triphosphate</td>
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<tr>
<td>dTTP</td>
<td>deoxy-thymidine triphosphate</td>
</tr>
<tr>
<td>dUTP</td>
<td>deoxy-uridine triphosphate</td>
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<tr>
<td>dNTP</td>
<td>deoxy-nucleotide triphosphate</td>
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<td>dTTP</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E-Cad</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFL</td>
<td>EGF-like</td>
</tr>
<tr>
<td>EGFR</td>
<td>EGF-receptor</td>
</tr>
</tbody>
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EMA  Epithelial membrane antigen
EMT  Epithelial-Mesenchymal transition
ER   Oestrogen receptor
ERK 1/2 Extracellular signal-related kinase 1/2
FAK  Focal adhesion kinase
FBS  Foetal bovine serum
FCS  Foetal calf serum
FG   Fibrinogen
FGF  Fibroblast growth factor
FN   Fibronectin
FNIII Fibronectin-type III like domain
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
GCOS GeneChip Operating System
GIT  Gastro-intestinal tract
GMEM Gial/mesenchymal extracellular matrix protein
HBS  Homeodomain binding site
HBSS Hanks buffered salt solution
His  Histidine
HNSCC Head and neck squamous sarcoma
HXB  Hexabrachion
IDC  Infiltrating ductal carcinoma
IDG  Isoleucine-aspartic acid-glycine
Id2  Inhibitor of differentiation 2
IFITM1 Interferon induced transmembrane protein 1 (9 – 27)
IFN  Interferon
Ig   Immunoglobulin
IL   Interleukin
ILC  Invasive lobular carcinoma
Ile  Isoleucine
IMS  Industrial methylated spirit
iNOS Inducible nitric oxide
IVT  In-vitro transcription
Kan  Kanamycin
Kan^R Kanamycin resistance
kb   Kilobase
kDa  Kilodaltons
LB   Luria-Bertani
LCIS Lobular carcinoma in-situ
Leu  Leucine
Lif-1 Leukaemia inhibitory factor-1
LPA  Lysophosphatidic acid
LRI  Leicester Royal Infirmary
MAPK Mitogen-activated protein kinase
MEM Modified Eagles medium
Met  Methionine
min  Minutes
MMP  Matrix metalloproteinase
MPC  Magnetic particle concentrator
mRNA Messenger ribonucleic acid
MT-MMP Membrane-type matrix-metalloproteinase
MW  Molecular weight
NFκB Nuclear Factor Kappa B
NKC  Natural killer cell
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PDGF  Platelet-derived growth factor
PFN1  Profilin 1
PR  Progesterone receptor
Pro  Proline
PRTPβ  Protein-tyrosine phosphatase-β/ζ
(RT)-qPCR  Quantitative RT-PCR
RGD  Arginine-glycine-aspartate
RNA  Ribonucleic acid
rRNA  Ribosomal RNA
rpm  Revolutions per minute
RQ  Relative quantification
RT  Reverse transcription
RT-PCR  Reverse transcriptase polymerase chain reaction
SDS  Sodium dodecyl sulphate
sec  Seconds
SFM  Serum-free medium
SNP  Single nucleotide polymorphism
STAT1  Signal transducer and activator of transcription 1
T  Thymine
TA  Tenascin assembly domain
TAE  Tris acetic acid EDTA
Taq  Thermus aquaticus
TBS  Tris buffered saline
TCF/LEF-1  T-cell factor/lymphoid enhancer factor
TDLU  Terminal ductal lobular unit
TE  Trypsin/EDTA
TGF-β  Transforming growth factor beta
Thr  Threonine
TM1  Transmembrane-1
TN  Tenasin
TNC  Tenasin-C
TNR  Tenasin-R
TNW  Tenasin-W
TNX  Tenasin-X
TNY  Tenasin-Y
TNF-α  Tumour necrosis factor alpha
tPA  Tissue-type plasminogen activator
TPA  12-O-tetradecanoylphorbol-13-acetate
TPM1  Tropomyosin 1
SRE  Strain-responsive element
U  Units
uPA  Urokinase plasminogen activator
UV  Ultra violet
VBS  Veronal buffered saline
Vim  Vimentin
v/v  Volume by volume
w/v  Weight by volume
3'-UTR  3’-untranslated region
5'-UTR  5’-untranslated region
Chapter 1: Introduction
1.1 Breast cancer

Breast cancer is the most common female cancer in the Western world and the second most common cancer overall (behind lung cancer) with an estimated 1.15 million people diagnosed with the disease worldwide in 2002 and 4.4 million survivors up to 5 years after diagnosis. Half of these cases are in the industrialised countries, with 361,000 (27.3%) occurring in Europe. However, breast cancer ranks less highly (fifth) as a cause of death because of a relatively favourable prognosis (mortality to incidence ratio, 0.35) (Parkin et al., 2005). In the UK, currently, it is estimated that 46,000 new cases are identified each year with a mortality rate of more than 12,400 per annum (http://info.cancerresearchuk.org:8000/cancerstats/types/breast/).

Historically, breast cancer has been seen as a multi-step process encompassing progressive changes from normal, to hyperplasia with and without atypia, carcinoma in-situ, invasive carcinoma and metastasis. However, advances in immunohistochemistry and molecular genetics have paved the way for a more detailed model of cancer progression. It is no longer seen as a single pathway, but as a complex series of stochastic events leading to distinct and divergent pathways towards invasive breast cancer (reviewed in Simpson et al., 2005).

1.1.1 Pre-malignant changes

One of the earliest detectable changes is the loss of a normal regulation of cell number, resulting in epithelial hyperplasia, initially without atypia and subsequently with (Russo et al., 1991). Hyperplasia can occur in lobules, ducts and stroma. Epithelial hyperplasia occurs in a proportion of cases of fibrocystic change with the epithelial layer increasing to several layers deep. This is not associated with significant increased breast cancer risk until a disturbed (atypical) pattern of growth develops, which is considered an “at risk” lesion.

1.1.2 Carcinoma in-situ

Carcinoma in-situ is a term used to describe proliferation of epithelial cells that have undergone malignant transformation but remain at their site of origin, confined by a myoepithelial cell layer and basement membrane (Kalluri & Zeisberg, 2006). Non-invasive (in-situ) breast cancer comprises ductal carcinoma in-situ (DCIS) and lobular carcinoma in-situ (LCIS), which have distinct morphologies and clinical implications.
1.1.2.1 **Lobular carcinoma in-situ (LCIS)**

LCIS is generally considered to be a marker of increased risk of future malignancy rather than an anatomic precursor of invasive disease and has pathologic features characterised by proliferation of bland, homogeneous malignant cells within the terminal duct-lobular apparatus. The lobular architecture and investing basement membrane remain intact with no evidence of invasion into the surrounding stroma. The cells are typically of low histologic and nuclear grade, highly oestrogen receptor positive, lack E-cadherin and have tumour marker characteristics of indolent growth and good prognosis. This is very different from its non-invasive ductal counterpart, DCIS, which is typified by more aggressive cytologic and biologic characteristics (Afonso & Bouwman, 2008).

1.1.2.2 **Ductal carcinoma in-situ (DCIS)**

Pathologically, DCIS can be described as a heterogeneous entity with several morphologic variants (papillary, micropapillary, cribriform, solid, and comedo) that differ markedly in gross and histologic appearance, cellular characteristics, and clinical behaviour. DCIS has been reported to arise from ductal epithelium in the region of the TDLU and probably represents one stage in a continuum between atypical ductal hyperplasia and invasive carcinoma in the multi-step breast carcinogenesis (Sakorafas & Tsiotou, 2000). DCIS is graded low, intermediate or high, which is dependent upon a number of histological features, with high grade DCIS thought to develop a more aggressive phenotype due to 50-80% of all invasive carcinomas developing from ductal carcinoma (Weigelt et al., 2005).

Because there are no lymphatics or blood vessels in the epithelial layer, DCIS and LCIS offer no risk for metastatic spread until malignant cells cross the basement membrane (Sakorafas & Tsiotou, 2000).

1.1.3 **Invasive carcinoma**

1.1.3.1 **Invasive ductal carcinoma (IDC)**

Figure 1.1 exemplifies tumour development in the setting of mammary ductal carcinomas. Although the ductal epithelium and the underlying myoepithelial cells are separated from the surrounding connective tissue by a basement membrane in both normal female breast and DCIS, the basement membrane in DCIS, although intact, is slightly altered. During this process the basement membrane is degraded and the
activated stroma containing myofibroblasts, inflammatory infiltrate and newly formed capillaries comes into contact with the malignant cells (Kalluri & Zeisberg, 2006). IDC is the most common type of invasive carcinoma, which accounts for 73% of invasive breast cancers followed by infiltrating lobular carcinoma, which accounts for 10% (Ellis et al., 1992).

1.1.3.2 Invasive lobular carcinoma (ILC)
Invasive lobular carcinomas differ morphologically to IDCs and are characterised by single cell infiltrations that surround benign breast tissue in a targeted manner, are bland in appearance and have scant cytoplasm (Fisher et al., 1975; Martinez & Azzopardi, 1979). Typically, it does not provoke a substantial connective tissue response, and can fail to form distinct masses that can be easily diagnosed by needle core biopsy. ILCs also have a substantially increased predilection for multifocal and multicentric distribution as well as for bilaterality (Lesser et al., 1982).

1.1.3.3 Special types
Although rare in occurrence, there are several other types of breast carcinomas. They include mucoid or colloid (2.4%), tubular (1.2%), adenoid cystic (0.4%), cribriform (0.3%) and carcinosarcoma (0.1%). These percentages are the frequency of the tumour type of all invasive breast carcinomas (Joensuu & Toikkanen, 1995).
Figure 1.1: Tumour-stroma interactions during mammary ductal carcinoma progression

A) In the normal female breast, the ductal epithelium and the underlying myoepithelial cells are separated by a basement membrane (BM) from the surrounding ECM, capillaries and fibroblasts. B) In DCIS, the lumen contains carcinoma cells owing to the proliferation of transformed epithelia. C) During early invasion of carcinoma cells the BM is degraded by proteolytic enzymes and the cells invade into the surrounding stroma and ECM. Adapted from Kalluri & Zeisberg, (2006).
1.1.4 Breast cancer susceptibility genes

The process of breast carcinogenesis is thought to involve one or more distinct genetic mutations, which can be hereditary or sporadic. Hereditary breast cancer is characterised by inherited susceptibility to germline mutations (classed as high-, intermediate- and low-penetrance or “risk”) in genes such as BRCA1, BRCA2, TP53, CDH1 and PTEN (all high penetrance), ATM and CHEK2 (intermediate penetrance) and FGFR (low penetrance) (Easton et al., 2007). Sporadic breast cancer arises from somatic mutations of genes described above (although sporadic BRCA1 and BRCA2 mutations are very rare) as well as a number of other genes including PIK3 (for in-depth reviews see Wooster & Weber, 2003; Beggs & Hodgson, 2008).

1.1.4.1 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 and 2 encode for proteins whose function is the maintenance of global genome stability. Mutations in these genes account for 5 – 10% of all breast cancer cases and approximately 80% of all hereditary breast cancers (Reddeck et al., 1996). In familial breast cancer, germ-line mutations in the BRCA1 or BRCA2 genes are followed by somatic inactivation of the wild-type allele (Smith et al., 1992; Neuhausen and Marshall, 1994; Collins et al., 1995). They are thus thought to be tumour suppressors since loss of heterozygosity at either locus leads to retention of the mutant allele in tumours from heterozygous carriers. However, BRCA mutations are not associated with sporadic breast cancer and the wild-type allele is retained following loss of heterozygosity (Futreal et al., 1994; Lancaster et al., 1996). Although decreased levels of BRCA mRNAs and/or proteins have been observed in many sporadic breast cancers, the role of BRCA genes in such cancers is unclear (Taylor et al., 1998; Wilson et al., 1999; Egawa et al., 2001; Welch and King, 2001). However, hypermethylation of the BRCA1 promoter and loss of heterozygosity (LOH) of the regions of chromosome 17 and 13q12 are often seen in sporadic breast cancers (Cleton-Jansen et al., 1995; Dobrovic & Simpfendorfer, 1997; Esteller et al., 2000; Hanby et al., 2000; Johnson et al., 2002).

1.1.4.2 p53

The p53 protein plays a central role in modulating cellular responses to cytotoxic stresses by contributing to both cell-cycle arrest and programmed cell death. Loss of
p53 function during tumourigenesis can lead to inappropriate cell growth, increased cell survival, and genetic instability. Gene mutations of the p53 gene (TP53, located at 17p13) occur in approximately half of all malignancies from a wide range of human tumours (Kirsch and Kastan, 1998) and around 90% of carriers of the mutation develop cancer by the age of 70. TP53 mutations appear in 20-60% of sporadic breast cancers (Osborne et al., 1991; Deng et al., 1994). Germline mutations of the TP53 gene are responsible for Li-Fraumeni syndrome (Li & Fraumeni, 1984) and a significant proportion of these cancers harbour mutations of BRCA1. However, it is not yet known if p53 inactivation is a pre-requisite for the development of BRCA1 tumours to occur, or if the loss of BRCA1-associated DNA repair properties may explain, in some way, the high frequency of p53 mutations (Lacroix & Leclercq, 2005).

1.1.4.3 PTEN
The tumour suppressor PTEN (phosphatase with tensin homology), located on chromosome 10q23 was originally identified as a gene that is mutated in multiple sporadic tumour types as well as in patients with Cowden disease, a disorder characterised by multiple benign tumours. PTEN negatively regulates the phosphatidylinositol-3-kinase (PI3K) signalling pathway (Stambolic et al., 1998), and when deleted, mutated or otherwise inactivated, activation of PI3K effectors (such as protein kinase B) can occur and initiate tumourigenesis. PTEN mutations are associated with a 25-50% lifetime breast cancer risk, but accounts for a small proportion (<1%) of hereditary breast cancers (reviewed in Cully et al., 2006).

1.1.4.4 CDH1
E-cadherin (encoded by the CDH1 gene, located on chromosome 16q22) is a cell-cell adhesion protein that plays an essential role in the maintenance of cellular differentiation and epithelial architecture (reviewed in Roy and Berx, 2008). Mutations in this gene have been associated with hereditary diffuse gastric cancer syndrome, and have a 20-40% risk of developing breast cancer (Pharoah et al., 2001). Somatic mutations are usually found in LCIS and ILC, and are correlated with adverse cancer outcome (Parker et al., 2001).

1.1.4.5 ATM and ATR
Ataxia-telangiectasia, mutated (ATM) and ATM and Rad3-related (ATR) proteins are members of the phosphatidylinositol-3-kinase-like (PIKK) family and play an important
role in detecting the presence of double-stranded breaks in DNA induced by ionising radiation and UV as well as stalled replication forks and hypoxia (reviewed in Durocher & Jackson, 2001 and Yang et al., 2003). Carriers of ATM mutations suffer from ataxia-telangiectasia (AT); a radio-sensitivity and genome instability disorder characterised by progressive cerebellar degeneration and increased cancer incidence. The risk for developing breast cancer in ATM mutation carriers is about 11% at age 50, and 30% by age 70 (Easton, 1994). Carriers of ATR mutations suffer from Seckel syndrome, a heterogeneous recessive disorder that is characterized by dwarfism, developmental delay and severe microcephaly (O’Driscol et al., 2003). Furthermore, truncating ATR mutations in endometrial cancers are associated with biologic aggressiveness as evidenced by reduced disease-free and overall survival (Zighelboim et al., 2009).

1.1.4.6 FGFR

Fibroblast growth factor receptors (FGFRs) comprise a sub-family of receptor tyrosine kinases (RTKs) that regulate a broad spectrum of cellular and developmental processes, including apoptosis, proliferation, migration and angiogenesis (reviewed in Acevedo et al., 2009). The FGF signalling axis comprises 18 functionally defined ligands and four FGFRs, many of which have multiple isoforms that are made up mostly of alternative splicing in the third extracellular immunoglobulin-like domains (Powers et al., 2000; Ornitz and Itoh, 2001). Importantly, single nucleotide polymorphisms (SNPs) within intron 2 of the FGFR2 gene are associated with breast cancer through allelic FGFR2 up-regulation (Hunter et al., 2007). Mis-sense mutations or copy number gains of FGFR2 gene occur in breast cancer and gastric cancer to activate FGFR2 signalling. Aberrant FGFR2 signalling activation also induces proliferation and survival of tumour cells (reviewed in Katoh and Katoh, 2009).

1.1.5 Tumour invasion and metastasis

Metastasis is responsible for 90% of all human cancer deaths (Weigelt et al., 2005). The transition from in-situ tumour growth to metastatic disease is defined by the ability of tumour cells at the primary site to invade local tissue and to cross tissue barriers. The process of metastasis is highly inefficient, but not random (reviewed in Pantel & Brakenhoff, 2004). Instead, it is a cascade of linked sequential steps, each one involving multiple tumour-host interactions, which include adhesion; angiogenesis and proteolysis (Liotta, 1980; Folkman, 1986; Egeblad & Werb, 2002) (Figure 1.2).
To initiate the metastatic process, neoplastic cells must first penetrate the basement membrane and then invade the interstitial stroma by active proteolysis. Interaction with the host stroma also stimulates new blood vessel formation (angiogenesis), providing nutrition and access to the vascular compartment (Aznavoorian et al., 1993; Stetler-Stevenson et al., 1993). Subsequently, intravasation requires tumour cell invasion of the subendothelial basement membrane and disruption of the cell-cell junctions that seal their lumina. After reaching the bloodstream, either directly or through the lymphatic system, tumour cells often adhere to platelets and leukocytes, forming emboli that stop in microcirculation of target organs easier than isolated tumour cells (Guo & Giancotti, 2004). To successfully establish a metastatic colony, circulating neoplastic cells must survive immunologic surveillance, arrest at a distant vascular step (Mehlen & Puisieux, 2006), exit the bloodstream – by a process called extravasation – and undergo expansive growth within the parenchyma of the target organ. The proliferation of cells within a secondary organ must follow a process similar to that described for the primary site, in which several growth factors and expression of metastasis related genes play a key role (reviewed in Hanahan & Weinberg, 2000; Chiang & Massague, 2008; Joyce, 2009). It is these systemic metastases causing organ failure that result in death.
Changes in adhesion are prominent during the metastatic cascade. At the onset, carcinoma cells lose E-cadherin cell-cell adhesions, acquire a migratory phenotype, penetrate the BM and then invade the interstitial matrix. Tumour angiogenesis then allows the cancer cells to intravasate into the bloodstream either directly or via the lymphatic system. Once in circulation, tumour cells form small aggregates with platelets and leukocytes. Eventually, after transporting to distant organs, the tumour cells extravasate and either remain solitary (micrometastasis) or undergo local expansion through proliferation. Adapted from Thiery, (2002); Guo & Giancotti, (2004); Pantel & Brakenhoff, (2004) and Kalluri & Zeisberg, (2006).
1.2 Tumour-related prognostic factors

The number of tumour-related features available to predict the prognosis of patients with breast cancer has grown impressively in recent years, with extensive literature being available. Outlined here are some of the parameters used (for in-depth reviews of all prognostic factors, see Payne et al., 2008; Walker, 2008).

1.2.1 Histological grade

Histological grading of breast carcinomas is performed by a combined evaluation of three factors: gland/tubule formation; atypia/pleomorphism/nuclear size; and mitotic count, all of which are graded with a score of 1 to 3 and the total used as in Table 1.1. A low grade cancer is one where the cancer cells have a well-differentiated appearance with low mitotic count, whereas high grade cancers have cancer cells that show poor differentiation. Both the stage and grade are prognostic indicators, with a high stage and grade resulting in poor prognosis. The Nottingham Prognostic Index (NPI) incorporates tumour size, stage, and grade and is the most widely used tool to predict survival and the clinical course of the disease (Elston & Ellis, 2002). The formula being:

\[ \text{NPI} = (0.2 \times \text{tumour diameter in cm}) + \text{lymph node stage} + \text{tumour grade} \]

<table>
<thead>
<tr>
<th>Score</th>
<th>5-year survival</th>
</tr>
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<tr>
<td>( \leq 2.4 )</td>
<td>95%</td>
</tr>
<tr>
<td>2.4 – 3.4</td>
<td>85%</td>
</tr>
<tr>
<td>3.4 – 4.4</td>
<td>70%</td>
</tr>
<tr>
<td>4.4 – 5.4</td>
<td>50%</td>
</tr>
<tr>
<td>&gt; 5.4</td>
<td>20%</td>
</tr>
</tbody>
</table>

1.2.2 Lymph node stage

Internal mammary lymph nodes are a primary lymphatic drainage basin of the breast. They are not routinely examined for pathological staging, but they are involved in 9% of cases when no metastases are found in axillary nodes, illustrating a high-risk group of “node-negative” cases (Veronesi et al., 1985). However, metastasis to these nodes provides evidence that the tumour is locally confined. The presence of metastases to axillary lymph nodes (located in the armpit) shows that a tumour with the capacity to metastasise has done so and may have the capacity to metastasise to distant sites. This
is the most influential predictor of post-treatment recurrence and death (Donegan, 1997).

Essentially, there are two internationally recognised systems of staging: a simple numbering system (Table 1.2) and the TNM system, which describes the tumour, node status and whether the tumour has metastasised (Table 1.3).

### Table 1.2: Staging of breast cancers (Korkolis et al., 2004)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
</table>
| I     | Tumour up to 2 cm  
No lymph nodes affected  
No evidence of spread beyond the breast |
| II    | Tumour between 2 and 5 cm and/or  
Lymph nodes in armpit affected  
No evidence of spread beyond armpit |
| III   | Tumour more than 5 cm  
Lymph nodes in armpit affected  
No evidence of spread beyond armpit |
| IV    | Tumour any size  
Lymph nodes in armpit often affected  
Cancer has spread to other parts of the body |

### Table 1.3: The TNM system (Singletary et al., 2002)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
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<tbody>
<tr>
<td>T₁</td>
<td>Tumour 20 mm or less; no fixation or nipple retraction. Includes Paget’s disease</td>
</tr>
<tr>
<td>T₂</td>
<td>Tumour 20 – 50 mm or less than 20 mm without tethering</td>
</tr>
<tr>
<td>T₃</td>
<td>Tumour greater than 50 mm but less than 100 mm; or less than 50 mm but with infiltration, ulceration or fixation</td>
</tr>
<tr>
<td>T₄</td>
<td>Any tumour with ulceration or infiltration wide of it, or chest wall fixation, or greater than 100 mm in diameter</td>
</tr>
<tr>
<td>N₀</td>
<td>Node negative</td>
</tr>
<tr>
<td>N₁</td>
<td>Axillary nodes mobile</td>
</tr>
<tr>
<td>N₂</td>
<td>Axillary nodes fixed</td>
</tr>
<tr>
<td>M₀</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M₁</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>

### 1.2.3 Steroid-hormone receptors

Oestrogen receptor (ER) and progesterone receptor (PR) have been used for a number of years as indicators of prognosis and as guides for hormone and endocrine therapy. Individuals with receptor-positive tumours generally have a better prognosis, as indicated by a longer interval to disease recurrence and longer overall survival than patients with receptor-negative tumours (Veronese, 1995; Khoo, 1998). However, ER status can be used irrespective of PR as a prognostic factor (even though additional PR status significantly improves outcome prediction (Bardou et al., 2003)). ER-positive patients have been shown to have longer disease-free survival than patients with ER-
negative tumours (Knight et al., 1977). Furthermore, ER expression has also been associated with increasing age (Bentzon et al., 2008).

### 1.2.4 HER-2 expression

The human epidermal growth factor receptor 2 oncogene (HER-2/neu/c-erbB2) encodes a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases and is located on chromosome 17q21. It has an important role in cell differentiation, adhesion and motility (reviewed in Hanna et al., 1999). Amplification is the predominant mechanism of gene over-expression and abnormally high levels of the HER-2 protein are seen in 18 – 20% of breast cancers (Slamon et al., 1987). Furthermore, HER-2 positivity is associated with high-grade tumours, lymph node involvement (Burstein, 2005) and a poorer prognosis (Yamauchi et al., 2001).

Currently, HER-2 testing is performed using three methods in the UK: IHC, fluorescence *in-situ* hybridisation (FISH) and chromogenic *in-situ* hybridisation (CISH) (see Walker, 2007 and Payne et al., 2008 for in depth reviews on HER-2 testing as well as steroid hormone receptors).

### 1.2.5 Molecular profiling

In recent years, cDNA microarray and unsupervised analysis of gene expression patterns in breast cancer has been increasingly used to distinguish breast cancer subtypes with different prognoses (Sorlie et al., 2001; Sotiriou et al., 2003). Studies using this method have produced a signature of genes correlated with poor prognosis and regulate cell cycle, invasion, metastasis and angiogenesis (van ’t Veer et al., 2002; van de Vijver et al., 2002). Breast cancers are also classified into distinct subtypes (luminal A; luminal B; basal-like; Her-2 over-expressing and normal breast-like) (Perou et al., 2000; Sorlie et al., 2001; Hu et al., 2006; detailed review in Brenton et al., 2005), 2 of which are summarised below.

#### 1.2.5.1 Luminal breast cancers

Luminal breast cancers consist of the steroid-hormone receptor positive breast cancers and are reminiscent of the luminal epithelial component of the breast (Perou et al., 2000). They are the most common type of breast cancer (Carey et al., 2006) and are known to express cytokeratins 8/18, ER and genes associated with ER activation such as cyclin D1 (Perou et al., 2000; Sotiriou et al., 2003; Abd El-Rehim et al., 2004). Luminal cancers are also separated into two subtypes: luminal A and luminal B.
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general, luminal A cancers express higher amounts of ER-related genes but express proliferative genes at a lower level than luminal B (Sorlie et al., 2001). In addition, luminal cancers carry a good prognosis; however, luminal B cancers have a significantly worse prognosis than luminal A cancers (Sorlie et al., 2003).

1.2.5.2 Basal-like breast cancers

The basal subtype was so named due to the expression pattern mimicking that of basal epithelial cells of other parts of the body and normal breast myoepithelial cells (Perou et al., 2000). Approximately 15% of breast cancers are basal-like and are associated with high grade (Livasy et al., 2006), poor prognosis (Abd El-Rehim et al., 2004; Nielson, 2004; Carey et al., 2006), and younger patient age (Cheang et al., 2008) (reviewed in Rakha et al., 2008a; Rakha et al., 2008b). The definition of a basal-like tumour is of much debate (Gusterson, 2008); however, immunohistochemical panels that potentially identify basal-like tumours are ER-/PR-/Her2- (the “triple negative phenotype” or TNP) (Haffty et al., 2006), and/or expression of CK 5/6 (Nielson, 2004) and/or CK 14 (Carey et al., 2006) (for in depth review of all markers see Rakha et al., 2008b).

1.3 The extracellular matrix

Tissues are made up of an intricate network of cells and extracellular matrix (ECM), which is composed of a number of macromolecules such as fibrous structural proteins including collagens and elastins; adhesive glycoproteins such as fibronectin and laminin and a gel of proteoglycans and hyaluronan. These macromolecules are secreted locally, mainly by fibroblasts and form a significant proportion of the volume of any tissue (Cotran, 1999). The ECM and cell-cell interactions are essential for the regulation and development of multi-cellular organisms and not only function as a structural support, but also play an active role in differentiation (Hay, 1993), tissue repair (Midwood et al., 2004b) and cancer progression (Shekhar et al., 2003). There are both quantitative and qualitative modifications in ECM components in breast cancer, with ECM proteins forming a non-cellular compartment to the tumour microenvironment that is extensively modified and remodelled by proteases either secreted by neoplastic and non-neoplastic cells or localized at the surface of cells. As a result of the activity of these proteases, important changes in cell-cell and cell-ECM interactions occur, and new signals are generated from the cell surface. These signals affect gene expression and ultimately influence critical cell behaviour such as proliferation, survival, differentiation, and motility (reviewed in DeClerck et al., 2004). Recently, ECM-related genes have been
used to identify breast cancer subgroups which have different prognoses (Bergamaschi et al., 2008). This study found that ECM groups with the poorest prognosis consisted mainly of basal-like tumours, whereas ECM groups with good prognosis consisted mainly of luminal A tumours.

One ECM component that has been investigated as a marker for prognosis in human breast cancer is the glycoprotein tenasin-C (Jahkola et al., 1998b; Goepel et al., 2000), and is discussed in detail in section 1.4.
1.4  Tenascin-C

Tenascin-C (TNC) is now known to be one member of a growing family of ECM glycoproteins which play a morpho-regulatory role during tissue development, remodelling and disease. The other members of the family include tenascin-R (TNR) (Fuss et al., 1991; Rathjen et al., 1991), which is restricted to the CNS and is most prominent in development (Carnemolla et al., 1996). Tenascin-W (TNW) (Weber et al., 1998), which is found in the ECM of bone, muscle and kidney (Scherberich et al., 2004), has been postulated to be a marker of activated cancer stroma in low-grade breast cancer (Degen et al., 2007), with elevated levels seen in sera of patients with colorectal and breast cancer (Degen et al., 2008). Furthermore, TNW has also been shown to promote α8 integrin-dependent motility (Scherberich et al., 2005). Tenascin-X (TNX) (Bristow et al., 1993), the largest tenascin glycoprotein, has been found in foetal tissues and plays an essential role in the deposition of collagen fibres (Mao, 2002). Moreover, deficiency of this tenascin has been associated with Ehlers-Danlos syndrome (Burch, 1997). Finally tenascin-Y (TNY) (Hagios et al., 1996), which is secreted by differentiated fibroblasts in the connective tissue of muscles and differs from other tenascins in its domain structure.

TNC was discovered independently by several laboratories in the 1980s studying different aspects of cell, developmental, and tumour biology. Accordingly, it has been given a variety of names including glial/mesenchymal extracellular matrix protein (GMEM) (Bourdon et al., 1983), cytotactin (Grumet et al., 1985), J1220/200 (Kruse et al., 1985), hexabrachion (Erickson & Inglesias, 1984), tenascin (Chiquet-Ehrismann et al., 1986) and neuronectin (Rettig et al., 1989). In most cases TNC was discovered as a contaminant of cell surface fibronectin preparations and attracted interest due to its selective expression in developing and pathological tissues. The name “tenascin” was proposed because of its perceived role as an adhesion protein (from the Latin “tenere”, to hold) and its known association with a variety of “nascent” tissues (Chiquet-Ehrismann et al., 1986).

In early studies TNC in normal adult tissue was reported to be seldom expressed (Chiquet & Fambrough, 1984b; Chiquet-Ehrismann et al., 1986; Erickson & Bourdon, 1989) (only at a few sites around dermal papillae and larger vessels) and be absent from mature bone matrix (Chiquet-Ehrismann & Chiquet, 2003). However, more extensive studies have shown a distinctive pattern of distribution. TNC has been observed at
gastrulation and somite formation, and at later stages it is restricted to the rostral half of the somite associated with neural crest cell invasion (Crossin et al., 1986; Tan et al., 1987; Orend, 2005). It has also been shown to be expressed in parts of the developing CNS during neuronal migration in the cortex and cerebellum, in the peripheral nervous system during myelination, in non-neural tissues during branching morphogenesis of mammary gland and lung, the developing skeleton, cardiovascular system and connective tissues and in particular at sites of EMT (Ekblom & Aufderheide, 1989; Sakakura, 1991; Tucker, 1991; Yuasa, 1996; Orend, 2005). TNC has also been shown to be expressed during normal and pathological tissue remodelling, such as wound healing (Mackie et al., 1988; Whitby et al., 1991), where it is strongly and transiently induced both in remodelling bone and in the dermis adjacent to skin wounds (Webb et al., 1997), during pregnancy and lactation (Jones et al., 1995; Wirl et al., 1995; Bell et al., 1999) and in particular tumourigenesis (reviewed in Mackie, 1997; Jones & Jones, 2000a; Jones & Jones, 2000b; Chiquet-Ehrismann & Chiquet, 2003; Chiquet-Ehrismann, 2004; Orend, 2005; Orend & Chiquet-Ehrismann, 2006).

1.4.1 The tenasin-C knockout mouse

Initial studies into the function of TNC in-vivo using a TNC knockout mouse yielded disappointing results. Saga et al. (1992) were the first to generate a TNC knockout using homologous recombination. These mice were indistinguishable from their wild-type littermates: they were the same size, fertile and displayed no gross deficits in neuro-architecture or principal organ systems when cursory histological examinations were performed. This observation was confirmed by Forsberg et al. (1996) after independently constructing a second TNC knockout. This led to researchers suggesting that other tenascins were up-regulated to compensate, a notion that was quickly disproved by a number of studies (Saga et al., 1992; Steindler et al., 1995; Sakai et al., 1996). However, more detailed analysis of the behaviour of knockout mice and their response to trauma has suggested a function of TNC.

TNC knockout mice have been shown to have several neurological defects that result in abnormal behaviour. Fukamauchi et al. (1996; 1997a,b,c; 1998a,b) reported hyperactivity in an open field test, poor sensorimotor co-ordination, clinging and freezing behaviour during bridge-crossing tasks, and poor performance in passive avoidance learning tests. These changes in behaviour have been attributed to the altered level of transcripts encoding certain neurotransmitters – specifically a reduction in
tyrosine hydroxylase and an increase in the levels of preprotachykinin A and cholecystokinin. During myelination, however, findings are conflicting. Moscoso et al. (1998) concluded that TNC was not required for reinnervation after nerve crushing, whereas Cifuentes-Diaz et al. (1998) found that TNC is involved not only in myelination and axon outgrowth but also in the formation and stabilisation of the neuro-muscular junction. These findings indicated that TNC has a role in the development or maintenance of brain chemistry.

To investigate the role of TNC in regenerative processes Nakao et al. (1998) used habu snake venom to induce glomerulonephritis in knockout mice of three different genetic backgrounds and found striking differences. In the most extreme case (the GRS/A background), the progression of disease was irreversible and all knockout animals died of renal failure. This was attributed to early apoptosis and deficient proliferation of mesangial cells with subsequent excess deposition of ECM in the knockout kidney. Matsuda et al. (1999) performed linear perforation wounds and also placed sutures in the cornea of knockout mice. They noted that healing of the perforation wounds was indistinguishable from the wild-type mice. However, in the suture wounds, healing was greatly reduced with no induction of fibronectin expression observed at the wound edge in the knockout mice. Moreover, corneas from the wild-type mice were thickened at the suture site, whereas TNC-deficient corneas were compressed, with keratinocytes absent from the wound stroma.

Regarding the role of TNC in tumourigensisis Talts et al. (1999) crossed TNC knockout mice with a strain that spontaneously develops mammary tumours upon induction of the polyoma middle T oncogene (PyTag) and observed smaller tumour cell-nests in the tumour stroma of the knockout animals. Moreover, there was higher infiltration of lymphocytes into the tumour stroma. Tanaka et al. (2004) also observed a significant inhibition of xenotransplanted tumour formation in nude mice lacking TNC, suggesting that TNC supports or enhances tumourigensisis in-vivo. Table 1.4 gives a summary of phenotypes in TNC-knockout mice (see Mackie and Tucker, 1999 for an in-depth review of TNC-knockout mice).
Table 1.4: Summary of TNC-knockout phenotypes (Mackie and Tucker, 1999)

<table>
<thead>
<tr>
<th>Study</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Behaviour</td>
<td>Hyperlocomotion, poor swimming, abnormal circadian rhythm</td>
<td>Fukamauchi et al., 1996a,b; 1997b</td>
</tr>
<tr>
<td>Neurochemistry</td>
<td>Reduced tyrosine hydrolase, reduced neuropeptide Y, increased preprotachykinin A, increased cholecystokinin</td>
<td>Fukamauchi et al., 1997a,b; 1998a</td>
</tr>
<tr>
<td>Peripheral nerves and nerve regeneration</td>
<td>Abnormal peripheral nerves, abnormal neuromuscular junction</td>
<td>Cifuentes-Diaz et al., 1998</td>
</tr>
<tr>
<td>CNS injury</td>
<td>More astrocytes in glial scar</td>
<td>Steindler et al., 1995</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>Failure to regenerate</td>
<td>Nakao et al., 1998</td>
</tr>
<tr>
<td>Wound healing</td>
<td>Reduced fibronectin (skin &amp; cornea), wounds compressed with fewer migrating keratinocytes</td>
<td>Forsberg et al., 1996; Matsuda et al., 1999</td>
</tr>
<tr>
<td>Tumourigenesis</td>
<td>Increased monocytes/macrophages, altered tumour stroma, inhibition of tumour formation from xenograft</td>
<td>Talts et al., 1999; Tanaka et al., 2004</td>
</tr>
</tbody>
</table>

1.4.2 The structure of tenasin-C

TNC is a polymorphic, high-molecular mass ECM glycoprotein with a highly symmetrical hexameric structure. TNC polypeptides are modular in structure and a number of variants can be produced through alternative splicing of primary transcripts. TNC, like other proteins of the ECM, binds to many different ligands, which include the integrin family of cell surface receptors, ECM proteins such as proteoglycans, fibronectin, collagen, and small molecules such as glycolipids (Jones, 1996).

TNC has remarkable symmetry with six arms radiating from a central core. The proximal portions of the arms are thin but at the distal portion the arms thicken and then terminate in an electron dense globular particle (Jones & Jones, 2000a). Early biochemical studies (Chiquet & Fambrough, 1984b; Kruse et al., 1984; Grumet et al., 1985; Friedlander et al., 1988; Hoffman et al., 1988) indicated TNC was a disulphide-linked oligomer containing subunits ranging from 180 – 300 kDa. The human TNC gene was localised to chromosome 9q32 – q34 (Gulcher et al., 1990; Rocchi et al., 1991), contains a single promoter (Gherzi, 1995) with the coding region spanning approximately 80 kb of DNA and consists of 27 exons separated by 26 introns (Gulcher et al., 1991). Partial or complete TNC sequences have been cloned from a variety of animal species including human (Gulcher et al., 1989; Nies et al., 1991; Siri et al., 1991), chicken (Jones et al., 1988; Pearson et al., 1988; Jones et al., 1989), mouse (Saga et al., 1991; Weller et al., 1991), rat (LaFleur et al., 1994), pig (Nishi et al., 1991) and
newt (Onda et al., 1991), from which a detailed model of the hexabrachion has been proposed (Jones et al., 1989; Spring et al., 1989) (Figure 1.3). This model is based on similarities to other proteins, the presentation and interaction of the polypeptides, and the elements that correspond to features seen in electron micrographs.

TNC monomers comprise an amino-terminal region containing many cysteine residues, which participate in oligomer formation via a tenascin assembly (TA)-domain, a series of 14.5 compact epidermal growth factor-like (EGFL) repeats, an array of up to 17 fibronectin type III (FNIII) domains, nine of which are susceptible to differential RNA splicing events, and a carboxy-terminal segment resembling the distal domain from the β and γ chains of fibrinogen.

1.4.2.1 The tenascin assembly (TA) domain
The TA-domain is encoded by a single exon producing eight cysteine residues and three to four α-helical heptad repeats that enable the amino termini of TNC polypeptides to be linked into oligomeric structures. Assembly of the TNC hexabrachion has been shown to be a sequential two-step process, mediated by the concerted interplay of two autonomous folding domains. The organisation of TNC into a functional hexamer includes the formation of a trimer intermediate by a short parallel α-helical coiled-coil domain followed by the connection of two such triplets to a hexamer by the clustered TA-domains interacting homophilically with the N-terminal to the α-helical segment. Oligomerisation by a three-stranded coiled-coil domain is an essential pre-requisite of TNC hexabrachion formation, and its principal function is the clustering of three individual TA-domains. This oligomerisation process provides a high local concentration of homophilic weak affinity sites, consequently resulting in an increase in the binding affinity between TA-domains. Three cysteine residues flanking the N-terminal end of the heptad repeats are then involved in the stabilisation of the structure by disulphide bonds, but they are not required for the formation of hexamers (Kammerer et al., 1998).
Figure 1.3: A model for the tenascin hexabrachion

A) Schematic representation of the domain structure of the human tenasin-C polypeptides and the exons that encode them. The line above the hexabrachion shows the amino acid number. The TA domain, EGFL repeats, FNIII repeats, and the globular domain with similarity to the β- and γ-chains of fibrinogen (FG) are indicated. B) Schematic representation of the assembly of six tenasin-C polypeptides into the hexabrachion. The oval at the centre represents the site for oligomerisation through interchain disulphide bonds. The different coloured domains of each TNC polypeptide are indicated as in (A). C) Electron micrograph of rotary shadowed TNC (from Erickson & Bourdon, 1989).
1.4.2.2 The epidermal growth factor-like (EGFL) domains

Proceeding outward from the central core, the next domain is formed by a contiguous group of epidermal growth factor-like (EGFL) repeats. The EGFL domain is one of the most frequently occurring building blocks in modular proteins and has been found in more than 300 human extracellular proteins (Pas, 2006). The EGFL repeats of TNC are encoded by a single exon (Jones & Jones, 2000b), are 31 amino acids in length and contain six cysteine residues that participate in interchain disulphide bonds with the topology 1-3, 2-4, and 5-6 (Baron et al., 1992b). They are exceptionally compact in nature and may provide rigidity to the polypeptide chain – a specific structural feature that may be responsible for the highly symmetrical radiation of the TNC arms from the central core of the hexabrachion. Unlike the EGFL repeats found in other proteins such as coagulation factors IX and X (Handford, 1990) and in the Notch, Delta, and Serrate receptors (Klein, 1998), the TNC repeats lack the acidic residues required for calcium binding. They also do not contain a large β-strand in the centre of the repeat, as is found, for example, in epidermal growth factor (EGF) (Cooke, 1987). In terms of function, the EGFL regions are considered to be counter-adhesive for fibroblasts, neurons and glia, and may be involved in neuronal migration and axon path finding during development (Spring et al., 1989; Prieto, 1992; Crossin, 1994; Krushel, 1994; Gotz, 1997; Fischer, 1997a). It has also been suggested that the EGFL repeats provide localised signals for growth and differentiation, presumably via interactions with EGF receptors (Thiery, 1992).

1.4.2.3 The fibronectin type III (FNIII) domains

A large portion of the TNC polypeptide consists of a linear array of fibronectin type III repeats encoded by either a single exon or by two exons interrupted by one intron. These repeats consist of approximately 91 amino acids (with the exception of AD1, which has 92) (Sriramarao, 1993) and map onto the distal portions of the hexabrachion arms, consisting of extended globular structures composed of seven anti-parallel β-strands arranged into two sheets (Dickinson et al., 1994). The hydrophobic residues of these sheets can bind to other fibronectin (FN) modules, promoting the assembly of FN fibrils (Pas, 2006). The type III domain has many features in common with a C-type immunoglobulin (Ig) domain (Patthy, 1990; Baron et al., 1992a; Leahy et al., 1992), and has also been described in several adhesion molecules of the Ig superfamily. FNIII arrays constitute 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).

The human form of TNC contains eight constitutively expressed FNIII domains (repeats 1 – 5 and 6 – 8 encoded by exons 3 – 9 and 17 – 22 respectively) along with nine additional FNIII domains situated between domains 5 & 6 (repeats A1 – A4, encoded by exons 10 – 13, repeats B – D encoded by exons 14 – 16, and the subsequently identified “additional domain 1” and “additional domain 2” encoded by exons AD1 and AD2 respectively), which are subject to alternative splicing (Jones et al., 1988; Gulcher et al., 1989; Jones et al., 1989; Spring et al., 1989; Gulcher et al., 1991; Nishi et al., 1991; Weller et al., 1991; Sriramarao, 1993; Dorries, 1994; Derr et al., 1997) (Figure 1.3). Each domain of the alternatively spliced region is encoded by a single exon and can be spliced independently to give rise to a number of different isoforms. For instance, RT-PCR analyses of TNC mRNA from the developing mouse brain detected four major splice variants with six (A1, A2, A4, B, C, D), five (A1, A2, A4, B, D), one (D) or no alternatively spliced type III repeats (Dorries, 1994). The variability of human TNC isoforms has mostly been studied in tumour tissues and cell lines, since TNC expression is up-regulated in a variety of tumours. Two RT-PCR approaches have been used involving either the amplification of the entire spliced region using primers hybridising adjacent to the splice site (Gulcher et al., 1989; Siri et al., 1991; Sriramarao, 1993; Vollmer et al., 1997; Saghizadeh et al., 1998; Bell et al., 1999), or the amplification of segments within the alternatively spliced FNIII domain (Mighell et al., 1997; Bell et al., 1999).

Different isoforms of TNC provide potential mechanisms for altering cell signalling and downstream functions by modulating binding interactions with different receptors, along with other ECM components. The large isoform of TNC (containing all of the alternatively spliced FNIII repeats) has been shown to bind with the calcium-dependent phospholipid-binding protein annexin-II (Chung & Erickson, 1994), which is considered to be counteradhesive due to its ability to disrupt focal adhesions (Jones & Jones, 2000b). Conversely, the fully truncated isoform of TNC differentially interacts with F11 contactin through the fifth and sixth FNIII domains and also binds FN with higher affinity than larger versions (Chiquet-Ehrismann, 1991). The expression of different isoforms of TNC depends on different stages during development, tissue type, cell-type, cell line (Prieto et al., 1990; Chiquet-Ehrismann, 1991; Kaplony et al., 1991;
Tucker, 1991; Weller et al., 1991), extracellular pH (Borsi et al., 1996), and by polypeptide growth factors including TGFβ1 (Jones & Jones, 2000a). However, at present the mechanisms that determine the splice pattern of TNC isoforms is unknown.

1.4.2.4 The additional domain 1

The exon “additional domain 1” or “AD1” was first reported in 1993 by Sriramarao and Bourdon (Sriramarao, 1993), identified by sequencing of two human tenascin cDNA clones from a U251 glioblastoma cDNA library. Using in-situ hybridisation, Derr et al. (1997) demonstrated that distribution of TNC transcripts containing AD1 is widespread in the chick embryo. Quantitative in-situ hybridisation revealed that AD1 containing mRNAs were a major component of TNC transcripts found at sites of tissue modelling, like scale mesenchyme and lung bronchioles. Similar results have also been described by Tucker (1991, 1998), and are consistent with a role for this repeat in modifying cell-fibronectin interactions or acting as part of a motility-promoting milieu. Derr et al. (1997) also showed that AD1 is expressed in the highly invasive quail fibrosarcoma cell line QT6. These cells have characteristics typical of motile, invasive cells: they pass rapidly through a fibronectin-coated filter in a blind well chamber assay, and they express mostly a high-molecular weight form of TNC with three repeats in the variable domain. Due to the highly invasive nature of these cells and the presence of the AD1 containing mRNA transcripts, it is not unreasonable to hypothesise that AD1 could be important in tumour cell invasion. Subsequent investigation into the human breast ductal carcinoma cell line Hs578T revealed the presence of AD1 transcripts, suggesting that TNC transcripts containing AD1 could play a role in human breast carcinoma progression and metastasis (Derr et al., 1997).

Fischer et al. (1997) showed that AD1 is present in mRNA transcripts that are expressed at sites of active tissue remodelling and fibronectin expression in the developing avian feather bud and sternum. However, they also showed that myoblastic cells expressing TNC transcripts containing AD1 organise actin microspikes that contain the actin-bundling protein fascin, and do not assemble focal contacts, possibly through inhibition of syndecan-4 binding of fibronectin (Huang et al., 2001; Orend et al., 2003). These findings suggest that TNC transcripts containing AD1 could provide a suitable microenvironment for regulating changes in cell shape, adhesion, migration and proliferation.
1.4.2.5 The fibrinogen globe

The fibrinogen globe of TNC consists of 210 amino acids encoded for by five exons (Siri et al., 1991) and shows approximately 32% identity with the β and γ chain of human fibrinogen (Doolittle, 1984; Nies et al., 1991). Two halves of the molecule are bound together at the centre using disulphide bonds and non-covalent contacts. One face of the fragment contains α-helix and β-sheet chains which form a funnel-shaped domain with the hydrophobic cavity; the other face contains 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, 1996). Moreover, the calcium-binding property of the fibrinogen globe influences interactions with other proteins including collagen fibrils, integrins, heparin and a cell surface chondroitin sulphate proteoglycan called phosphocan (Jones & Jones, 2000a).

1.4.3 Induction of TNC expression

1.4.3.1 The tenascin-C promoter

The site of transcription initiation was determined by S1 nuclease and primer-extension mapping (Jones et al., 1990). Sequencing of a 4.3 kb genomic DNA clone containing 3986 base pairs upstream of the RNA start site, the first exon, and part of the first intron revealed a number of sequence motifs implicated in the regulation and expression of eukaryotic genes. These include CCAAT boxes, phorbol ester-responsive elements, enhancer elements, and a consensus TATA box sequence located 24 bp upstream of the major RNA cap site. The flanking sequence also contains a number of regions of dyad symmetry and direct repeats unique to tenascin, as well as an array of A + T rich sequences resembling engrailed elements. A GATA-6 response element has also been mapped to position -467 to -460 of the TNC promoter (Ghatnekar & Trojanowska, 2007).

1.4.3.2 Growth factors, cytokines and transcription factors

Research over the last two decades has identified a number of regulatory pathways and molecules that regulate TNC expression. Certain growth factors have been proposed that are secreted by actively proliferating cells which induce TNC expression in neighbouring cells (Inaguma et al., 1988; Chiquet-Ehrismann et al., 1989; Hiraiwa et al., 1993; reviewed in Tucker & Chiquet-Ehrismann, 2008). Pearson et al. (1988) demonstrated rapid TNC secretion in chick embryo fibroblasts (CEF) exposed to TGFβ. Moreover, foetal bovine serum (FBS) was also shown to induce secretion of
TNC at an even greater rate. The induction of TNC secretion in CEFs by TGFβ was also described by Chiquet-Ehrismann et al. (1989), who cultured them in MCF-7 conditioned media and showed secretion at a similar level obtained using FBS. FBS is known to contain TGFβ (Childs et al., 1982); however, other factors within FBS must be able to induce TNC secretion due to the higher rate observed when CEFs are cultured in 10% FBS. In addition to enhancing secretion of TNC, TGFβ has also been shown to induce the expression of higher molecular weight isoforms via alternative splicing (Schwogler et al., 1992; Zhao & Young, 1995). Other growth factors that have been shown to induce secretion of TNC include epidermal growth factor (EGF) (Sakai et al., 1995), fibroblast growth factor (FGF) (Suzuki et al., 2002) and connective tissue growth factor (CTGF) (Gore-Hyer et al., 2002), a number of pro- and anti-inflammatory cytokines can also induce TNC expression including IL-1/4/6/8/13 (Chevalier et al., 1996; Makhluf et al., 1996; Chiquet-Ehrismann & Chiquet, 2003; Jinnin et al., 2006) as well as transcription factors including NfkB, cJun, Ets, Sp1 and Prx (Orend & Chiquet-Ehrismann, 2006).

1.4.4 Tenascin-C function

TNC possesses a number of unique ligand binding domains that have a diverse effect on cellular activity. Such activity is attributed to direct interactions with cell surface receptors or via indirect modulation of ECM proteins and cell adhesion molecules (Jones & Jones, 2000a). The following discusses structure-function relationships in terms of cell activity as well as interactions with ECM and cell surface ligands.

1.4.4.1 Cell attachment to tenascin-C

The effects of TNC on cell attachment are complex, in that TNC supports the attachment of some cell types but is counter-adhesive for others (Erickson & Taylor, 1987; Chiquet-Ehrismann et al., 1988; Bourdon & Ruoslahti, 1989; Spring et al., 1989; Prieto, 1992; Joshi et al., 1993). Different isoforms of TNC appear to have different roles, with the fully truncated isoform supporting the attachment of cells, whereas the full length isoform is counter-adhesive (Ghert et al., 2001). Specifically, the third to sixth FNIII-like repeats have been shown to have an adhesive capacity whereas the seventh and eight repeat are counter-adhesive (Prieto, 1992). The third to sixth repeats have been shown to be adhesive for a range of cell types including endothelial cells (Sriramarao et al., 1993), fibroblasts (Prieto, 1992) and neural cells (Crossin, 1996). By
using recombinant TNC fragments, cell adhesive and counter-adhesive properties have been localised to specific regions of the molecule and are summarised in Table 1.5.

<table>
<thead>
<tr>
<th>TNC fragment</th>
<th>Role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFL</td>
<td>Counter-adhesion; fibroblasts, neurons, glia, neurite repulsion</td>
<td>Spring et al., 1989; Prieto, 1992; Fischer et al., 1997; Gotz, 1997</td>
</tr>
<tr>
<td>FN 1 – 3</td>
<td>Cerebellar neuron attachment</td>
<td>Gotz, 1997</td>
</tr>
<tr>
<td>FN 1 – 5</td>
<td>Counter-adhesion; T-lymphocyte inhibition of β1 adhesion</td>
<td>Hauzenberger et al., 1999</td>
</tr>
<tr>
<td>FN 7 – 8</td>
<td>Counter-adhesion; fibroblasts</td>
<td>Spring et al., 1989; Prieto, 1992</td>
</tr>
<tr>
<td>FN 2 – 6</td>
<td>Cell attachment</td>
<td>Prieto, 1992</td>
</tr>
<tr>
<td>FN A – D</td>
<td>Attachment of uterine epithelia, reduced adhesion and implantation</td>
<td>Julian et al., 1994</td>
</tr>
<tr>
<td>Fibrinogen Globe</td>
<td>Cell attachment and spreading</td>
<td>Krushel, 1994</td>
</tr>
</tbody>
</table>

The main approaches used for functional studies have been to delete whole regions of the TNC molecule to concentrate on the action of single domains. However, studies using whole TNC molecules have shown that the alternatively spliced region is the region that produces the majority of the mitogenic effects.

1.4.4.2 Interactions of tenascin-C with the ECM and cell surface ligands

A number of functional studies have demonstrated the diversity of the FNIII domain, with a number of ligands being targeted to this area as well as the EGFL domain and the fibrinogen globe (Figure 1.4). Of the ECM proteins shown in Figure 1.4, most importantly TNC interacts strongly with fibronectin (Hoffman et al., 1988; Chiquet et al., 1991) through domains 1 – 5 and 7 – 8 (excluding the alternatively spliced region) (Hauzenberger et al., 1999). Although fibronectin will also bind to larger isoforms of TNC, the interaction is much weaker (Chiquet-Ehrismann, 1991; Ghert et al., 2001) and affects cell behaviour, particularly migration and tissue remodelling.
Figure 1.4: Ligand binding regions of TNC

Protein structure is shown with alternatively spliced region shaded in grey above and approximate location of ligand binding domains denoted by black bars. Ligands include EGFR (Swindle et al., 2001), PRTPβ (Milev et al., 1997), fibronectin (Chung & Erickson, 1997; Hauzenberger et al., 1999), a number of integrins (see text for refs), perlecan (Chung & Erickson, 1997), lecticans (Day et al., 2004), neurocan (Rauch et al., 2001), heparin (Fischer et al., 1995; Jang et al., 2004b), contactin (Zisch et al., 1992), annexin II (Chung & Erickson, 1994) and CALEB (Schumacher et al., 2001).
Like fibronectin, annexin II – a calcium dependent phospholipid binding protein – binds TNC. However, in contrast, annexin II is a high affinity ligand for the alternatively spliced region (Chung & Erickson, 1994) and its interaction with TNC induces loss of focal adhesions and mitogenesis as well as an increase in cell migration (Chung et al., 1996a). A number of integrins have been shown to bind TNC in an RGD-dependent manner (Prieto et al., 1993) including α8β1 (Denda et al., 1998) and αvβ3 (as well as the fibrinogen globe) (Sriramarao et al., 1993; Yokoyama et al., 2000). Other integrins also bind to the alternatively spliced region including α9β1, which also binds to the 3rd FNIII-like domain but not to the RGD sequence (Yokosaki et al., 1994) and α7β1, which binds to the D domain (Mercado et al., 2004). Two other integrins (α2β1 and αvβ6) have been shown to bind TNC but it is not known which domain they interact with (Sriramarao et al., 1993).

The presence of the alternatively spliced region has been shown to have other effects on cell-substrate adhesion. Murphy-Ullrich et al. (1991) showed that a recombinant form of this domain was a potent down-regulator of preformed focal adhesions. Loss of focal adhesion integrity is characteristic of malignantly transformed cells and is associated with increased cell motility (Bershadsky et al., 1985). The alternatively spliced region of TNC has been shown to induce proliferation of cells and compromise cell spreading by interacting with the 2nd heparin-binding domain of fibronectin and the integrin co-receptor syndecan-4 (Huang et al., 2001; Orend et al., 2003). Syndecan-4 is a heparin sulphated proteoglycan that serves as a co-receptor for integrin α5β1-mediated cell adhesion and signalling in a Rho-dependent manner (Saoncella et al., 1999). Inhibition of integrin α5β1/syndecan-4-dependent cell adhesion by TNC caused enhanced proliferation of glioma and breast carcinoma cells as well as loss of focal contacts that could be prevented by activation of syndecan-4 (Huang et al., 2001). Midwood et al. (2004a) showed that syndecan-4 inhibition is an important mechanism by which TNC inhibits cell spreading on a fibrin-based fibronectin matrix. This was modulated by transient phosphorylation of FAK and subsequently comprised activation of Rho (Huang et al., 2001; Midwood & Schwarzbauer, 2002; Orend et al., 2003), but no effect was seen on Rac or ERK 1/2 activation (Huang et al., 2001). A number of possible pathways have been proposed to be affected by interaction of TNC inhibition of cell attachment with fibronectin and syndecan-4. Ruiz et al. (2004) showed that plating of T98G cells on a FN/TNC milieu also triggered expression of ERK 1/2 and c-fos, as well as down-regulating expression of tropomyosin-1 (TPM1) and the Wnt signalling
inhibitor dickkopf-1 (DKK1). Recently, TNC has been implicated in a synergistic mechanism through simultaneous signalling by lysophosphatidic acid (LPA) and platelet-derived growth factor (PDGF) with down-regulation of TPM1, TPM2 and TPM3 initiating glioma cell spreading and migration when grown on a FN/TNC matrix (Lange et al., 2008), further implicating the alternatively spliced region in a tumour promoting role.

To summarise, TNC structure/function relationships are highly complex, with the alternatively spliced region playing a pivotal role in the modulation of TNC action. Effects are further enhanced by TNC itself and the highly complex nature of the ECM and cell receptor profile. Moreover, it is also clear that different domains of TNC are present at different stages of tumour progression, with certain domains having limited knowledge as to their function, and it is these domains that are of particular interest in this study.

1.4.5 Tenascin-C in normal breast

In normal breast development TNC is seen in periods of cellular proliferation and reorganisation. Prenatally, it is seen at high levels in the dense embryonic mesenchyme surrounding budding epithelia at 14 days post-gestation (Chiquet-Ehrismann et al., 1986; Inaguma et al., 1988). However, following birth expression is reduced and only low levels are found in the mature mammary gland. Its distribution is also altered and it becomes localised to a discrete layer surrounding the mammary ducts, acini and blood vessels (Inaguma et al., 1988; Howeedy et al., 1990). TNC distribution also changes in relation to the menstrual cycle. Specifically, TNC is up-regulated in the later stages of the menstrual cycle when there is an increase in cell migration and proliferation (Ferguson et al., 1990). Using immunohistochemistry, Ferguson et al. (1990) were able to show that during the first week of the menstrual cycle, TNC was present as a thin bright discontinuous line. In the second week, TNC appeared as a broad, paler and more complete band, and by the third week, there was an overall increase in the amount of TNC, which reached a maximum during the fourth week. Changes in TNC expression in relation to the menstrual cycle are also seen in the ovary and endometrium, at times of tissue remodelling and cellular proliferation (Vollmer et al., 1990).

During pregnancy, TNC is highly expressed in the mesenchymal villi, cell islands and cell columns in placental development (Castellucci et al., 1991). However, in the
breast, because of an increase in the amounts of progestins, oestrogen and placental lactogen (Voogt, 1978), TNC production is suppressed due to the proliferation of the mammary epithelium and the formation of alveolar morphology (in the mouse mammary gland) (Jones et al., 1995; Wirl et al., 1995). Subsequent to weaning, the process of involution is initiated, which is characterised by loss of milk production and massive apoptosis of the secretory epithelial cells, followed by proteolytic remodelling of the basement membrane by MMPs (Lund et al., 1996). At this point, high levels of myoepithelial derived TNC are observed surrounding the regressing epithelium (Jones et al., 1995), accompanied by a number of cell cycle regulators including TGFβ and p53 (Marti et al., 1999).

### 1.4.6 Tenascin-C in neoplasia

In contrast to normal tissue, high levels of TNC are seen in the stroma of both invasive and *in-situ* breast carcinoma (Mackie, 1987; Howeedy et al., 1990; Koukoulis et al., 1991; Jahkola et al., 1998a; Adams et al., 2002) and TNC has been proposed as a stromal marker for epithelial malignancy in the mammary gland (Mackie, 1987). However, studies relating to TNC and prognosis are conflicting, and have correlated expression with both good prognosis (Shoji et al., 1993; Sakakura & Kusakabe, 1994) and poor prognosis (Ishihara et al., 1995; Helleman et al., 2008).

During tumour progression, the cancer stroma becomes remodelled by both tumour cells and stromal cells, and protein components of the extracellular matrix (ECM) are dynamically changed by degradation and neosynthesis. Cellular interaction with the ECM strongly influences the behaviour of cancer and stromal cells, resulting in modulation of cell growth, migration, differentiation, and apoptosis (Donjacour & Cunha, 1991; Wernert, 1997; Liotta & Kohn, 2001). Compositional change of the ECM in cancer stroma is thus a key determinant of tumour growth and cancer progression.

Chiquet-Ehrismann *et al.* (1986) first outlined TNC expression and distribution in carcinogen-induced rat mammary tumours, where TNC was found in the stroma surrounding neoplastic epithelial cells. TNC has also been shown to be highly expressed by both transformed epithelial cells (Yoshida et al., 1997) and stromal cells (Hanamura et al., 1997; De Wever et al., 2004). Many studies have also shown both normal and malignant epithelial cells to be important sources of TNC (Prieto et al., 1990; Herlyn et al., 1991; Onda et al., 1991; Kawakatsu et al., 1992; Hiraiwa et al.,...
Mackie et al. (1987) first identified an up-regulation of TNC expression in human malignant mammary gland lesions, leading to a number of extensive studies into a variety of normal and malignant human tissue (Koukoulis et al., 1991; Natali et al., 1991; Shrestha et al., 1996). Subsequently, TNC has been described in a large number of human tumours (Table 1.6). TNC immunoreactivity is observed in all types of intraductal carcinoma, displaying strong and generally diffuse immunostaining around clusters of carcinoma cells and even around individual cells (Howeedy et al., 1990). The pattern of distribution appears similar in all tumour types with a change from the BM zone to a diffuse stromal distribution.

It has also been postulated that in different tumour tissue, the expression of different exons of TNC appear to play a role. In particular, TNC splice variants with extra repeats B/D are found in DCIS (Adams et al., 2002), $A_1/A_2/A_4$ are found in invasive colorectal carcinoma (Dueck et al., 1999), AD1 is found in malignant human melanoma and ductal breast carcinoma (Derr et al., 1997), AD2 is found in malignant oral cancers (Mighell et al., 1997), repeat C is found in malignant astrocytomas (Carnemolla et al., 1999), all alternatively spliced repeats are found in pancreatic ductal adenocarcinoma (Esposito et al., 2006) and several combinations are found in endometrial adenocarcinomas (Vollmer et al., 1990).
Table 1.6: Tumour types demonstrating TNC expression

<table>
<thead>
<tr>
<th>Organ</th>
<th>Tumour classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>Astrocytoma (Higuchi et al., 1993), glioblastoma multiforme (Higuchi et al., 1993), glioma (Bourdon &amp; Russlahti, 1989), gliomatosis cerebri (Higuchi et al., 1993), ependymoma (Korshunov et al., 2000), oligodendroglioma (McLendon et al., 2000), medulloblastoma (He et al., 1991), meningeioma (Castellani et al., 1995)</td>
</tr>
<tr>
<td>Breast</td>
<td>Adenosis (Yoshida et al., 1997), cystosarcoma phylloides (Howeedy et al., 1990), ductal carcinoma (Howeedy et al., 1990), fibroadenoma (Howeedy et al., 1990), fibrocystic disease (Howeedy et al., 1990), intraductal carcinoma (Howeedy et al., 1990), lobular carcinoma (Howeedy et al., 1990)</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>Anaplastic astrocytoma (Higuchi et al., 1993)</td>
</tr>
<tr>
<td>Female genital tract and ovaries</td>
<td>Cervical carcinoma <em>in-situ</em> and invasive carcinoma (Pilch et al., 1999), endometrial carcinoma (Sedele et al., 2002), hyperplastic endometrium (Sedele et al., 2002), ovarian tumours (Wilson et al., 1996), uterine sarcoma (Vollmer et al., 1993), vulvar carcinoma (Goepel et al., 2003)</td>
</tr>
<tr>
<td>Gastro-intestinal tract (GIT)</td>
<td>Adenocarcinoma of the colorectum and small intestine (Broll et al., 1995), adenocarcinomas of the stomach (Zirbes et al., 1999), carcinoma of papilla Vater (Vaidya et al., 1996), colorectal adenoma (Riedl et al., 1992), colorectal adenocarcinoma (Riedl et al., 1992), gastric carcinoma (Ikeda et al., 1995), squamous cell carcinoma of the oesophagus (Broll et al., 1995)</td>
</tr>
<tr>
<td>Kidney and bladder</td>
<td>Bladder carcinoma <em>in-situ</em> (Tiitta et al., 1993), bladder urothelial carcinoma (Tiitta et al., 1993), renal cell carcinoma (Lohi et al., 1995), oncocytoma (Sunardhi-Widyaputra &amp; Van Damme, 1993)</td>
</tr>
<tr>
<td>Lung</td>
<td>Adenocarcinoma (Kusagawa et al., 1998), pleural mesothelioma (Cai et al., 2002), squamous cell carcinoma (Kusagawa et al., 1998)</td>
</tr>
<tr>
<td>Liver</td>
<td>Fibrolamellar carcinoma (Scoazec et al., 1996), hepatocellular carcinoma (Yamada et al., 1992), intrahepatic cholangiocarcinoma (Aishima et al., 2003)</td>
</tr>
<tr>
<td>Skin</td>
<td>Basal cell carcinoma (Stamp, 1989), dermatofibroma (Franchi &amp; Santucci, 1996), Kaposi's sarcoma (Kaaya et al., 1996), melanoma (Natali et al., 1990), Merkel cell carcinoma (Koljonen et al., 2005), squamous carcinoma (Dang et al., 2006)</td>
</tr>
<tr>
<td>Soft tissues and lymphomas</td>
<td>Adipocytic tumours (Sis et al., 2004), B-cell non-Hodgkin's lymphoma (Vacca et al., 1996), malignant lymphoma (Soini et al., 1992), schwannoma (Schnyder et al., 1997), synovial sarcoma (Guarino &amp; Christensen, 1994)</td>
</tr>
</tbody>
</table>

1.4.7 Background to the study

Studies in our group analysing expression of high MW TNC isoforms in breast cancer have associated exons 14 and 16 with invasion (Adams et al., 2002). Furthermore, expression of these isoforms in breast adenocarcinoma cells has significantly increased their invasive capacity (Hancox et al., 2009). Recent studies focused on two less well
studied exons: AD1 and AD2. Experiments using nested RT-PCR have shown that AD1 containing transcripts are found frequently in carcinomas arising in young women, but were not related to tumour type, grade, receptor status or basal tumour phenotype (Rachael Hancox, personal communication). Using nested RT-PCR, AD1 was also found in isolated breast cell populations, where it was detected (by in-situ hybridisation) in myoepithelial cells lining larger ducts and also in the muscle layer of blood vessels. Moreover, AD1 containing transcripts were detected in breast carcinoma cell lines with high invasive/migratory capacity, being part of two novel intermediate-sized TNC isoforms. Sequencing of AD1 containing transcripts from two tumours localised the domain within intermediate sized TNC transcripts between exons 14 and either exons 15 or 16. Expression of AD2 was found in breast cell lines, normal breast and tumour tissue but was less frequent.

1.5 Aims and objectives

Hypothesis: The hypothesis to be tested in this thesis was that expression of key high MW isoforms of TNC, including AD1 and AD2, promote breast cancer development.

In order to test the hypothesis a number of key aims and objectives were investigated as follows:

1) Hypothesis: If TNC isoform expression promotes breast cancer development; changes in expression should be detectable at the mRNA and protein level.

Aims and objectives: In order to test this expression of TNC isoforms will be analysed using real-time (RT)-qPCR in cell lines, isolated normal breast populations and breast tissues and the data will be related to clinicopathological parameters.

2) Hypothesis: TNC isoforms containing AD1 promote tumour cell invasion in TNC null MCF-7 cells, in a similar way to other high molecular weight isoforms.

Aims and objectives: In order to test this, AD1 containing TNC-clones will be constructed by DNA recombination/nested PCR and the resulting clones used for transfection into TNC null cells. Following demonstration of successful expression, by RT-PCR and western blotting, the effects of AD1 containing isoforms on tumour cell invasion will be investigated using a 2D real-time invasion protocol to be developed as part of this thesis.
3) Hypothesis: Expression of high MW recombinant TNC isoforms in TNC null MCF-7 adenocarcinoma cell induces specific global gene expression changes that can be identified by molecular methods.

Aims and objectives: To transiently transfect MCF7 cells with TNC isoform clones carried on pCMV-Script and confirm isoform expression prior to recovery of mRNA for gene expression analysis using cDNA microarray. A secondary aim was to determine the optimum strategy for data analysis to identify the most significant gene expression changes. Given sufficient time, to investigate a number of candidate gene identified by cDNA microarray as novel gene targets for validation in breast cancer cell lines and tissues. The overall aim of the microarray studies was to address the following key questions:

a) Do AD1 containing isoforms effect global gene expression in a similar manner to other TNC isoforms, namely TNC-9/16 and TNC-9/14/16?

b) Do high MW isoforms affect expression of genes associated with invasion, proliferation and migration?

c) Are there any genes that are specifically up- or down-regulated by TNC isoforms containing AD1?
Chapter 2: Materials and Methods
2.1 Materials

2.1.1 Breast tissue

Breast tissue was available from reduction mammoplasties, breast organoids (isolated from reduction mammoplasties using a protocol adapted from Band and Sager (1989) and modified by Huper and Marks (2007) by overnight digestion to release small acini and separated by sedimentation to enrich for luminal and myoepithelial cells – in house term is “organoids”), breast cancer patients and women with cases of fibroadenoma from the NHS Trust. All tissue was taken with informed consent and local ethics approval. 22 reduction mammoplasties, 11 breast organoids, 14 fibroadenomas and 134 invasive carcinomas were used in this study. All pathological data was available from reports and checked by Prof. Walker and NHS BSP. Details of cases used are summarised in Table 2.1 - Table 2.4.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Invasive ductal carcinoma</th>
<th>Invasive lobular carcinoma</th>
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<tr>
<td>1-40</td>
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<tr>
<td>&gt;40</td>
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<td>25</td>
</tr>
<tr>
<td>n/k</td>
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<td>0</td>
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<tr>
<td>I</td>
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</tr>
<tr>
<td>II</td>
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<tr>
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<tr>
<td>-</td>
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<tr>
<td>n/k</td>
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<td>PR status</td>
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<tr>
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<tr>
<td>-</td>
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<td>n/k</td>
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</table>

Table 2.1: Clinicopathological features of invasive carcinomas

ER = Oestrogen receptor; PR = Progesterone receptor; CK = cytokeratin; n/k = not known;
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<thead>
<tr>
<th>Tumour Case</th>
<th>Age</th>
<th>Type</th>
<th>Grade</th>
<th>Node</th>
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<th>PR</th>
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<th>CK5/6</th>
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IDC = Invasive ductal carcinoma; ILC = Invasive lobular carcinoma; Pos = Positive; Neg = Negative; ER = Oestrogen receptor; PR = Progesterone receptor; CK = cytokeratins; n/k = not known, C = carcinoma
### Table 2.3: Summary of breast tumour tissue used from women > 40 years of age

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<td>49</td>
<td>IDC</td>
<td>I</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>n/k</td>
<td>n/k</td>
<td>C105</td>
<td>64</td>
<td>IDC</td>
<td>III</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
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</table>
### Table 2.4: Summary of reduction mammoplasty (RM), breast organoids (BO) and fibroadenoma (F/AD) cases

<table>
<thead>
<tr>
<th>RM Case</th>
<th>Age</th>
<th>Type</th>
<th>RM Case</th>
<th>Age</th>
<th>Type</th>
<th>BO case</th>
<th>Age</th>
<th>Type</th>
<th>F/AD Case</th>
<th>Age</th>
<th>Type</th>
<th>RW Case</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM32</td>
<td>n/k</td>
<td>RM</td>
<td>RM116</td>
<td>19</td>
<td>RM</td>
<td>1962</td>
<td>n/k</td>
<td>BO</td>
<td>F/AD1</td>
<td>n/k</td>
<td>F/AD</td>
<td>F/AD13</td>
<td>F/AD</td>
</tr>
<tr>
<td>RM33</td>
<td>n/k</td>
<td>RM</td>
<td>RM124</td>
<td>48</td>
<td>RM</td>
<td>1967</td>
<td>n/k</td>
<td>BO</td>
<td>F/AD2</td>
<td>n/k</td>
<td>F/AD</td>
<td>F/AD14</td>
<td>F/AD</td>
</tr>
<tr>
<td>RM34</td>
<td>n/k</td>
<td>RM</td>
<td>RM130</td>
<td>36</td>
<td>RM</td>
<td>1968</td>
<td>n/k</td>
<td>BO</td>
<td>F/AD3</td>
<td>n/k</td>
<td>F/AD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM35</td>
<td>n/k</td>
<td>RM</td>
<td>RM132</td>
<td>28</td>
<td>RM</td>
<td>1969</td>
<td>n/k</td>
<td>BO</td>
<td>F/AD4</td>
<td>n/k</td>
<td>F/AD</td>
<td></td>
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<tr>
<td>RM37</td>
<td>n/k</td>
<td>RM</td>
<td>RM133</td>
<td>26</td>
<td>RM</td>
<td>1970</td>
<td>n/k</td>
<td>BO</td>
<td>F/AD5</td>
<td>n/k</td>
<td>F/AD</td>
<td></td>
<td></td>
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<tr>
<td>RM40</td>
<td>n/k</td>
<td>RM</td>
<td>RM134</td>
<td>70</td>
<td>RM</td>
<td>1971</td>
<td>n/k</td>
<td>BO</td>
<td>F/AD6</td>
<td>n/k</td>
<td>F/AD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM42</td>
<td>n/k</td>
<td>RM</td>
<td>RM139</td>
<td>32</td>
<td>RM</td>
<td>1974</td>
<td>n/k</td>
<td>BO</td>
<td>F/AD7</td>
<td>n/k</td>
<td>F/AD</td>
<td></td>
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</tr>
<tr>
<td>RM51</td>
<td>n/k</td>
<td>RM</td>
<td>RM168</td>
<td>41</td>
<td>RM</td>
<td>1976</td>
<td>n/k</td>
<td>BO</td>
<td>F/AD9</td>
<td>n/k</td>
<td>F/AD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM54</td>
<td>n/k</td>
<td>RM</td>
<td>RM173</td>
<td>20</td>
<td>RM</td>
<td>1979</td>
<td>n/k</td>
<td>BO</td>
<td>F/AD10</td>
<td>n/k</td>
<td>F/AD</td>
<td></td>
<td></td>
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<tr>
<td>RM91</td>
<td>22</td>
<td>RM</td>
<td>RM179</td>
<td>40</td>
<td>RM</td>
<td>1982</td>
<td>n/k</td>
<td>BO</td>
<td>F/AD11</td>
<td>n/k</td>
<td>F/AD</td>
<td></td>
<td></td>
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<tr>
<td>RM95</td>
<td>25</td>
<td>RM</td>
<td>RM180</td>
<td>45</td>
<td>RM</td>
<td>1985</td>
<td>n/k</td>
<td>BO</td>
<td>F/AD12</td>
<td>n/k</td>
<td>F/AD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
- Norm = Normal; F/AD = Fibroadenoma; RM = Reduction mammoplasty; BO = Breast organoids; n/k = not known
2.1.2 Eukaryotic cell lines
All cell lines (apart from GI-101) were from the American Type Tissue Culture Collection (ATCC, Rockville, MD., USA). The GI-101 breast carcinoma cell line was kindly donated by Professor Louise Jones (Barts and the London). All cell lines were maintained at low passage as lab stocks. Details of cell lines are described in Table 2.5.

2.1.3 Cell culture reagents and supplements
Dulbeccos modified Eagles medium (DMEM) (containing 1000 mg/L D-Glucose, pyroxene HCl, NaHCO₃) without phenol red and L-Glutamine, dimethyl sulphoxide (DMSO) and L-Glutamine were from Sigma-Aldrich, UK. Opti-MEM reduced serum media; trypsin/EDTA (TE), foetal bovine serum and active geneticin (G418) antibiotic solution (50 mg/ml) were from Gibco, UK. Hanks buffered saline solution (HBSS) and Dulbeccos phosphate-buffered saline (DPBS) were from Cambrex BioScience, USA. Tissue culture flasks, 25 cm², 75 cm² and 150 cm², 6 and 24 well plates, individual Fluoroblok cell culture inserts (with 8 μm pores) and 24-well cell culture insert companion plates were all from BD Falcon, USA. BD Matrigel matrix and 1,1'-didodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiIC₁₂(3)) lipophilic fluorescent tracer dye were from BD Biosciences, USA. Fugene HD transfection reagent was from Roche Applied Sciences, UK.
### Table 2.5: Details of breast cell lines used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Activity in Boyden chamber assay</th>
<th>ER</th>
<th>PR</th>
<th>Her2</th>
<th>E-Cad</th>
<th>Vim</th>
<th>Notes</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Low</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>Human breast adenocarcinoma cell line originated in 1970 from a 69-year old Caucasian woman with a malignant pleural effusion secondary to breast carcinoma</td>
<td>Dickson et al., 1986; Sommers et al., 1989; Hall et al., 1990; Thompson et al., 1992; Hiraguri et al., 1998; Roetger et al., 1998; Tong et al., 1999</td>
</tr>
<tr>
<td>T-47D</td>
<td>Low</td>
<td>Pos</td>
<td>Pos</td>
<td>Low</td>
<td>Pos</td>
<td>Neg</td>
<td>Human breast adenocarcinoma cell line isolated in 1974 from a pleural effusion of a 54-year old woman with infiltrating ductal carcinoma</td>
<td>Keydar et al., 1979; Hall et al., 1990; Thompson et al., 1992; Hollywood &amp; Hurst, 1993; Maemura et al., 1995</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>Low</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Human breast adenocarcinoma cell line derived from an ascites of a 63-year old Caucasian woman</td>
<td>Thompson et al., 1992; Hollywood &amp; Hurst, 1993; Tong et al., 1999</td>
</tr>
<tr>
<td>Hs578T</td>
<td>High</td>
<td>Neg</td>
<td>Neg</td>
<td>Low</td>
<td>Neg</td>
<td>High</td>
<td>Human ductal carcinoma cell line obtained in 1976 from a 74-year old Caucasian woman with invasive ductal carcinoma</td>
<td>Hackett et al., 1977; Sommers et al., 1989; Thompson et al., 1992; Pierceall et al., 1995; Bouizer et al., 1999; Tong et al., 1999</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>High</td>
<td>Neg</td>
<td>Neg</td>
<td>Low</td>
<td>Neg</td>
<td>High</td>
<td>Human breast adenocarcinoma cell line derived from a pleural effusion of a 51-year old Caucasian woman</td>
<td>Cailleau et al., 1978; Sutherland et al., 1988; Sommers et al., 1989; Thompson et al., 1992; Hollywood &amp; Hurst, 1993</td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>High</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>Human breast adenocarcinoma cell line derived from a pleural effusion of a 43-year old Caucasian woman</td>
<td>Cailleau et al., 1978; Thompson et al., 1992; Suh et al., 2005</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Low</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td></td>
<td>Human breast adenocarcinoma cell line derived in 1977 from a 51-year old black woman with a pleural effusion</td>
<td>Cailleau et al., 1978; Thompson et al., 1992; Sheikh et al., 1993; Maemura et al., 1995; Hiraguri et al., 1998; Roetger et al., 1998</td>
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<tr>
<td>GI-101</td>
<td>n/k</td>
<td>Neg</td>
<td>Neg</td>
<td>n/k</td>
<td>n/k</td>
<td>n/k</td>
<td>Human breast adenocarcinoma cell line derived in 1993 from a 57 year old female with local first recurrence of an infiltrating ductal adenocarcinoma.</td>
<td>Hurst et al., 1993; Lacroix &amp; Leclercq, 2004</td>
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<td>HBL-100</td>
<td>High</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>An immortalised epithelial cell line derived from the milk of a 27-year old Caucasian nursing mother. Described as normal, however, contains a tandemly integrated SV40 virus genome.</td>
<td>Gaffney, 1982; Caron de Fromentel et al., 1985; Dandachi et al., 2001; Akkiprik et al., 2006; Lombaerts et al., 2006; Moiseeva et al., 2007</td>
</tr>
<tr>
<td>MCF-10A</td>
<td>Low</td>
<td>Neg</td>
<td>Neg</td>
<td>Low</td>
<td>Low</td>
<td></td>
<td>Derived from human fibrocystic mammary tissue of a 36-year old female. Exhibits characteristics of normal breast epithelium due to its lack of tumourigenicity in nude mice</td>
<td>Soule et al., 1990; Zhang et al., 2005; Akkiprik et al., 2006; Bindels et al., 2006</td>
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2.1.4 Chemicals and reagents
All chemicals were purchased from Sigma, Fisher, or Cambrex, unless otherwise stated. 100 bp DNA molecular weight markers were from New England Biolabs, USA. For sequencing reactions, Big Dye Terminator 3.1v and 5x sequencing buffer were from Applied Biosystems, UK. Centri-Sep columns were from Princetons Separations, Inc., USA.

2.1.5 Enzymes and reaction buffers
Restriction enzymes BClI, HindIII, NotI, SalI, and SfiI and associated buffers were from New England Biolabs, USA. AccuPrime Pfx DNA polymerase, Taq DNA polymerase and 10x AccuPrime PCR buffer were from Invitrogen, UK. 10x dephosphorylation buffer, 10x ligation buffer, T4 DNA ligase, shrimp alkaline phosphatase (SAP) and 100x BSA were supplied by Roche, UK. AMV-RT, AMV-RT 5x buffer and RNasin were from Promega, USA.

2.1.6 Nucleic acid manipulation kits
The RNeasy Mini kit, QIAquick gel extraction kit, QIAquick Spin Mini-prep, Midi-prep, Maxi-prep and the QIAquick PCR plasmid retrieval and purification kits were supplied by Qiagen, UK.

2.1.7 Oligonucleotide sequences
Oligonucleotide primers were supplied by Sigma-Genosys, UK. Sequences used in this study are summarised in Table 2.6 and Table 2.7.

2.1.8 Quantitative PCR reagents and materials
MicroAmp 96-well plates; adhesive film; 2x TaqMan Fast PCR mastermix; inventoried FAM-labelled TaqMan gene expression assay probe sets and custom FAM-labelled probes were from Applied Biosystems, UK. Custom designed primers were from Sigma-Genosys, UK.
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<tr>
<th>Primer</th>
<th>Sequence 5' → 3'</th>
<th>Length (nt)</th>
<th>Tm (°C)</th>
<th>Entrez Accession number</th>
<th>Annealing position (5' → 3')</th>
<th>Exon</th>
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<tbody>
<tr>
<td>TNCAD1 F</td>
<td>AAC CAA AGC CAC AGT TGG G</td>
<td>19</td>
<td>64.2</td>
<td>EU295718</td>
<td>1 → 19</td>
<td>AD1</td>
</tr>
<tr>
<td>TNCAD1 R</td>
<td>CTG TAA TGA CAA AGG CAG TGA GG</td>
<td>23</td>
<td>64.6</td>
<td>EU295718</td>
<td>276 → 254</td>
<td>AD1</td>
</tr>
<tr>
<td>AD1 F</td>
<td>CCA CAG TGG GGC ATG CTA AT</td>
<td>20</td>
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<td>M96686</td>
<td>10 → 29</td>
<td>AD1</td>
</tr>
<tr>
<td>AD1 R</td>
<td>GTG TCT TCC ACC AAG CCT GT</td>
<td>20</td>
<td>64.1</td>
<td>M96686</td>
<td>200 → 181</td>
<td>AD1</td>
</tr>
<tr>
<td>T8 F</td>
<td>CAA TCC AGC GAC CATCAA CG</td>
<td>20</td>
<td>68.8</td>
<td>EU295718</td>
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<tr>
<td>RT9 F</td>
<td>GCT ACC GCC TCA ATT ACA GTC TC</td>
<td>23</td>
<td>64.8</td>
<td>M55618</td>
<td>3176 → 3198</td>
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</tr>
<tr>
<td>T16 P</td>
<td>GTG TCT AAC TTT CGG TTC GG</td>
<td>20</td>
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<td>5290 → 5271</td>
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<tr>
<td>T18 R</td>
<td>CGT CCA CAG TTA CCA TGG AG</td>
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<tr>
<td>T7 F</td>
<td>TAA TAC GAC TCA CTA TAG GG</td>
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<td>AF028239</td>
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<tr>
<td>TOPO F</td>
<td>CCC AAG CAG GCT AGT TAA GC</td>
<td>20</td>
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<td>pcDNA3.1/V5-His-TOPO vector</td>
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<tr>
<td>TOPO R</td>
<td>AGG GGG TTT AAG CTC AAT GG</td>
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<tr>
<td>14-AD1 F</td>
<td>GCC ACG ACA GAA CCA AAG CCA CAG TGG G</td>
<td>29</td>
<td>78.8</td>
<td>M55618/EU295718*</td>
<td>4705 → 4714 and 1 → 18</td>
<td>14 and AD1</td>
</tr>
<tr>
<td>14-AD1 R</td>
<td>GCC TTT GGT TCT GTG GTG GTG GCA CTG</td>
<td>30</td>
<td>82.1</td>
<td>M55618/EU295718*</td>
<td>4714 → 4695 and 10 → 1</td>
<td>14 and AD1</td>
</tr>
<tr>
<td>AD1-16 F</td>
<td>GTC ATT ACA GAA GCC GCA GCG TCT GAC</td>
<td>30</td>
<td>74.9</td>
<td>M55618/EU295718*</td>
<td>4988 → 5007 and 267 → 276</td>
<td>AD1 and 16</td>
</tr>
<tr>
<td>AD1-16 R</td>
<td>GGT TCG TCT GTA ATG ACA AAG GCA GTG</td>
<td>30</td>
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<td>M55618/EU295718*</td>
<td>4997 → 4988 and 276 → 257</td>
<td>AD1 and 16</td>
</tr>
<tr>
<td>AD1-15 F</td>
<td>GTC ATT ACA GAG GCC CTG CCC CTT CTG G</td>
<td>28</td>
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<td>M55618/EU295718*</td>
<td>4715 → 4732 and 267 → 276</td>
<td>AD1 and 15</td>
</tr>
<tr>
<td>AD1-15 R</td>
<td>GCC AGG GCC TCT GTA ATG ACA AAG GCA GTG</td>
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<td>78.6</td>
<td>M55618/EU295718*</td>
<td>4724 → 4715 and 276 → 257</td>
<td>AD1 and 15</td>
</tr>
<tr>
<td>TN15-16 F</td>
<td>ATT GTT ACA GAA GCC CAG CCA GTT GAC</td>
<td>30</td>
<td>74.3</td>
<td>M55618</td>
<td>4978 → 5007</td>
<td>15 and 16</td>
</tr>
<tr>
<td>TN15-16 R</td>
<td>GGT GGC GTCT GTT GTA ACA ATC TCA GCC CTC</td>
<td>30</td>
<td>75.8</td>
<td>M55618</td>
<td>4993 → 4968</td>
<td>15 and 16</td>
</tr>
<tr>
<td>BCI2 ten2</td>
<td>GGT GAC CAC CAC AGC CCTT GGA TG</td>
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<td>2526 → 2548</td>
<td>5 → 6</td>
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<tr>
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<td>GGT GAT GAC CTG TGG TGA CCTA CAC</td>
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<td>5607 → 5587</td>
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<tr>
<td>BCI2 ten1</td>
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<td>pCMV MCS</td>
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<td>NM_002046</td>
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*For primers that span 2 exons – sequences show position relative to TNC (M55618) and AD1 (EU295718) respectively.
Table 2.7: Summary of primers used for quantitative (RT)-qPCR analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' → 3'</th>
<th>Length (nt)</th>
<th>Tm (ºC)</th>
<th>Entrez Accession number</th>
<th>Annealing position (5' → 3')</th>
<th>Exon</th>
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<td>60.8</td>
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<td>qAD1 R</td>
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<td>EU295718</td>
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<td>AD1</td>
</tr>
<tr>
<td>qAD1 Probe</td>
<td>CAG TGT GGC AGC AAC</td>
<td>15</td>
<td>70.1</td>
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</tr>
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<td>59.3</td>
<td>M55618</td>
<td>4679 → 4698</td>
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<tr>
<td>q14/16 R</td>
<td>TGA AAC CAG AAG GTT GTC AAC TTC</td>
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<td>58.7</td>
<td>M55618</td>
<td>5022 → 4999</td>
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<td>70.3</td>
<td>M55618</td>
<td>4708 → 4714 and 4988 → 4997</td>
<td>14/16</td>
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<tr>
<td>q9/16 F</td>
<td>CAA GCC CGC ACA TGT GAA</td>
<td>18</td>
<td>59.6</td>
<td>M55618</td>
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<tr>
<td>q9/16 R</td>
<td>TGA AAC CAG AAG GTT GTC AAC TTC</td>
<td>24</td>
<td>58.9</td>
<td>M55618</td>
<td>5022 → 4999</td>
<td>16</td>
</tr>
<tr>
<td>q9/16 Probe</td>
<td>ATC CAC TGA AGC CGA AC</td>
<td>17</td>
<td>70.4</td>
<td>M55618</td>
<td>3342 → 3349 and 4988 → 4996</td>
<td>9/16</td>
</tr>
<tr>
<td>qAD2 F</td>
<td>GAT CAC CCC CAT GAG ACC AT</td>
<td>20</td>
<td>58.6</td>
<td>U88892</td>
<td>121 → 140</td>
<td>AD2</td>
</tr>
<tr>
<td>qAD2 R</td>
<td>TGA TGA CAG AGC TGC GAG ACA</td>
<td>21</td>
<td>59.4</td>
<td>U88892</td>
<td>181 → 161</td>
<td>AD2</td>
</tr>
<tr>
<td>qAD2 Probe</td>
<td>TGC TGT CTG TGC CTG G</td>
<td>16</td>
<td>69.9</td>
<td>U88892</td>
<td>143 → 158</td>
<td>AD2</td>
</tr>
<tr>
<td>Total TNC</td>
<td>Applied Biosystems (Hs01115654_m1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Applied Biosystems (Hs02758991_g1)</td>
<td></td>
<td></td>
<td>NM_002046</td>
<td>start 628</td>
<td>6</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Applied Biosystems (Hs99999909_m1)</td>
<td></td>
<td></td>
<td>NM_000194</td>
<td>start 648</td>
<td>6</td>
</tr>
<tr>
<td>IFITM1</td>
<td>Applied Biosystems (Hs00705137_s1)</td>
<td></td>
<td></td>
<td>NM_003632</td>
<td>start 429</td>
<td>2</td>
</tr>
<tr>
<td>STAT1</td>
<td>Applied Biosystems (Hs00234829_m1)</td>
<td></td>
<td></td>
<td>NM_007315</td>
<td>start 1137</td>
<td>9</td>
</tr>
<tr>
<td>PFN1</td>
<td>Applied Biosystems (Hs00748915_s1)</td>
<td></td>
<td></td>
<td>NM_005022</td>
<td>start 647</td>
<td>3</td>
</tr>
<tr>
<td>CK8</td>
<td>Applied Biosystems (Hs02339473_g1)</td>
<td></td>
<td></td>
<td>NM_002273</td>
<td>start 1075</td>
<td>5</td>
</tr>
<tr>
<td>AREG</td>
<td>Applied Biosystems (Hs00155832_m1)</td>
<td></td>
<td></td>
<td>NM_001657</td>
<td>start 878</td>
<td>4</td>
</tr>
<tr>
<td>CCND1</td>
<td>Applied Biosystems (Hs00277039_m1)</td>
<td></td>
<td></td>
<td>NM_053056</td>
<td>start 625</td>
<td>2</td>
</tr>
</tbody>
</table>

GAPDH – glyceraldehyde-3-phosphate dehydrogenase; HPRT1 – hypoxanthine guanine phosphoribosyltransferase 1; IFITM1 – interferon induced transmembrane protein 1; STAT1 – signal transducer and activator of transcription 1; PFN1 – profilin 1; CK8 – cytokeratin 8; AREG – amphiregulin; CCND1 – cyclin D1
2.1.9 Plasmids, bacterial strains and growth media

Plasmid constructs pCMV TNC Long and pCMV TNC Short, derived from the pNUT TNC Long and pNUT TNC Short plasmids were generated from plasmids supplied by Harold Erickson (Aukhil et al., 1993). The pBlueScript KS (-) vector was from Stratagene, USA. The pCMV TNC-9/16, pCMV TNC-9/14/16 and pCMV TNC Vector Only plasmids were produced in house on a previous Breast Cancer Campaign (BCC) grant (Table 2.9 for full details). One ShotTOP10 and Subcloning Efficiency DH5α Chemically Competent E. coli were from Invitrogen. SCS110 Competent E. coli cells were from Stratagene, USA. The pcDNA3.1/V5-His TOPO TA expression kit was from Invitrogen. Luria-Bertani (LB) medium (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract and 0.5% w/v NaCl, pH 7.2) was made from core stocks. Antibiotics such as ampicillin (100 mg/ml) and kanamycin (50 mg/ml) were from Sigma-Aldrich, UK.

Table 2.8: Bacterial strains used in this study (Escherichia Coli)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>One Shot TOP10</td>
<td>F- mcrA Δmrr-hsdRMS mcrBC Δ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araE) 7697 galU galK rpsL (StrR) endA1 nupG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Subcloning Efficiency DH5α</td>
<td>F- Δ80lacZΔM15 ΔlacZYA-argF)U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ-</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>SCS110</td>
<td>rpsL (Str) thr leu endA thi-1 lacY galK galT ara tonA tss dam dcm supE44 Δ(lac-proAB) [F´ traD36 proAB lacI, ZAM15]</td>
<td>Stratagene</td>
</tr>
</tbody>
</table>

Table 2.9: Plasmid constructs and vectors used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1/V5-His TOPO</td>
<td>ori, AmpR, NeoR, SV40 ori, P_CMV</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pBlueScript KS (-)</td>
<td>ori, AmpR, NeoR, SV40 ori, P_T7</td>
<td>Stratagene</td>
</tr>
<tr>
<td>S/pBlueScript</td>
<td>Fully truncated TNC isoform carried on pBlueScript vector</td>
<td>p/BlueScript</td>
</tr>
<tr>
<td>pCMV Script</td>
<td>ori, KanR, NeoR, SV40 ori, P_CMV</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pCMV TNC Vector Only</td>
<td>TNC exons 20 – 27 (domains 7, 8 and fibrinogen globe) carried on pCMV Script Vector</td>
<td>Generated during previous BCC grant</td>
</tr>
<tr>
<td>pCMV TNC Long</td>
<td>Full length TNC isoform (excluding exons AD1 and AD2) carried on pCMV Script vector</td>
<td>Generated during previous BCC grant</td>
</tr>
<tr>
<td>pCMV TNC Short</td>
<td>Fully truncated TNC isoform carried on pCMV Script vector</td>
<td>Generated during previous BCC grant</td>
</tr>
<tr>
<td>pCMV TNC-9/16</td>
<td>Fully truncated TNC isoform + exon 16 (domain D) carried on pCMV Script vector</td>
<td>Generated during previous BCC grant</td>
</tr>
<tr>
<td>pCMV TNC-9/14/16</td>
<td>Fully truncated TNC isoform + exons 14 and 16 (domains B and D) carried on pCMV Script vector</td>
<td>Generated during previous BCC grant</td>
</tr>
<tr>
<td>mycHis TNC Long</td>
<td>Full length TNC isoform carried on mycHis vector (pCMV Script vector + mycHis tag)</td>
<td>pNut Long (Erickson)</td>
</tr>
<tr>
<td>mycHis TNC Short</td>
<td>Fully truncated TNC isoform carried on mycHis vector</td>
<td>pNut Short (Erickson)</td>
</tr>
<tr>
<td>mycHis TNC-9/14/16</td>
<td>Fully truncated TNC isoform + exons 14 and 16 (domains B and D) carried on mycHis vector</td>
<td>Generated during previous BCC grant</td>
</tr>
</tbody>
</table>
Figure 2.1: Map of the pcDNA3.1/V5-His-TOPO cloning vector

Showing the multiple cloning site (MCS) under CMV promoter control. The ampicillin resistance gene (Amp) is the selectable marker, which was used to select for successfully transformed *E. coli* cells. Map produced using Bio-Log Plasm (Bio-Log, Toulouse, France).
Figure 2.2: Map of the pBlueScript KS (-) vector

Showing the position of the multiple cloning site (MCS) under CMV promoter control. The ampicillin resistance gene (Amp) is the selectable marker, which was used to select for successfully transformed *E. coli* cells. Map produced using Bio-Log Plasm (Bio-Log, Toulouse, France).
Figure 2.3: Map of the pCMV Script mammalian expression vector

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. Also highlighted is the neomycin resistance gene (Neo), which can be utilised for stable clone selection. Map produced using Bio-Log Plasm (Bio-Log, Toulouse, France).
Figure 2.4: Schematic representation of in-house TNC isoforms

A) Schematic representation of the domain structure of the human TNC polypeptides highlighting the TA-; EGLF-; FNIII- and fibrinogen-like domains. B) A schematic representation showing the FNIII-like domain of the TNC isoforms used as a template for production of the recombinant AD1 plasmids. The vector only sequence is not shown as it only contains the 3'-end of the molecule starting within exon 20 through to exon 27.
2.1.10 Western blotting
The rabbit polyclonal antibody to TNC (clone H300), that recognises an epitope corresponding to amino acids 1601-1900 of human TNC was from Santa Cruz, USA. The monoclonal antibody to vinculin (hVIN1), raised against full length native vinculin of human origin was from Sigma-Aldrich, UK. The enhanced chemiluminescence (ECL) detection kit, donkey anti-rabbit and sheep anti-mouse horseradish peroxidise (HRP)-linked whole antibodies as well as Hybond nitrocellulose membranes were from Amersham Biosciences, UK. N, N, N’, N’-tetramethylethylenediamine (TEMED), protein standard bovine serum albumin (BSA), ammonium persulphate, 100x protease inhibitor, 30% acrylamide/bis-acrylamide electrophoresis solution and Tween-20 were from Sigma-Aldrich, UK. Protein assay dye reagent and precision plus dual colour protein size marker were both from BioRad Laboratories, USA. Reagents including Gold lysis buffer, transfer buffer, wash buffer, blocking solution, loading buffer, TBS-T, stacking buffer and resolving gel buffer were made from core stocks.

2.1.11 Immunocytochemical materials
The monoclonal antibody to human tenascin-C (clone BC-24), an antibody that recognises an epitope located in the EGF-like repeat region of human tenascin-C, and tertiary mouse alkaline phosphatase anti-alkaline phosphatase (APAAP – clone AP1B9) were from Sigma, UK. Secondary antibody rabbit anti-mouse F(ab)2 fragment was supplied by Dako, Denmark. Normal rabbit serum was from Gibco, UK. Tris-HCl buffered saline (TBS) and veronal buffered saline (VBS) were from in-house stocks. Acetone was purchased from Gibco, UK. Tris-buffered saline and veronal acetate buffer were from in-house stocks.

2.1.12 RNA bioanalysis
The RNA 6000 NanoChip, RNA 6000 Ladder and RNA 6000 Nano reagent kit were all from Agilent, UK.

2.1.13 Microarray kits and columns
The Eukaryotic Poly-A RNA Control Kit; One-Cycle cDNA Synthesis Kit; Sample Cleanup Module; GeneChip In-Vitro Transcription (IVT) Labelling Kit; GeneChip Eukaryotic Hybridization Control Kit and the GeneChip Human Genome U133 plus 2.0 arrays were all from Affymetrix, USA. Bovine Serum Albumin (50mg/ml) and PBS,
pH 7.2 were from Invitrogen, UK. Herring Sperm DNA was from Promega, USA. Dimethyl sulphoxide (DMSO) and goat IgG were from Sigma-Aldrich, UK. R-Phycoerythrin Streptavidin was from Molecular Probes; 20x SSPE was from Cambrex, USA; goat biotinylated anti-streptavidin antibody was from Vector Laboratories, USA and Tween-20 was from Pierce Chemical, USA. 100% and 80% ethanol, 12x MES stock buffer; 2x hybridization buffer; Wash buffers A and B as well as 2x stain buffer were made from core stocks.

2.2 Methods

2.2.1 Small, medium and large scale preparation of plasmid DNA
Plasmid DNA was transformed into competent *E. coli* cells (section 2.5.6) and extracted using either Mini, Midi or Maxi kits according to manufacturer’s instructions. Briefly, binding buffer was added to plasmid DNA and centrifuged through a silica membrane to bind DNA and remove RNA, cellular proteins and metabolites. The membrane was then washed using a low-salt buffer and centrifuged to remove salts and the plasmid DNA eluted using a 1x Tris-Cl elution buffer.

2.2.2 Preparation of total RNA
Total RNA was extracted from mammalian eukaryotic cells and breast tissue using the Qiagen RNeasy mini kit according to manufacturer’s instructions. Briefly, cells and tissue were re-suspended and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer and ethanol was added. Samples were then applied to a silica-base spin column. After a series of washes, RNA was eluted using RNase-free water.

2.2.3 Purification of PCR amplicons
PCR amplicons were purified using the QIAquick PCR purification kit according to manufacturer’s instructions. Briefly, PCR amplicons were diluted in a binding buffer and centrifuged through a silica-based membrane to remove oligonucleotide primers, enzymes and other reagents. The membrane was then washed with a low-salt wash buffer to remove salts and DNA was eluted using a 1x Tris-Cl buffer.
2.2.4 **Quantification of nucleic acids**

The concentration of DNA and RNA in aqueous solution and the total yield was determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). 1 µl of DNA/RNA was loaded onto the pedestal and the absorbance measured. The purity was determined by comparing the absorbance values at 260 and 280 nm, with pure DNA and RNA having a desired $A_{260/280}$ ratio of 1.8 and 2.1 respectively.

2.3 **Enzymatic manipulation of nucleic acids**

2.3.1 **Primer design**

Oligonucleotide primers (Table 2.6) were designed following recommendations outlined by Applied Biosystems, ensuring that they effectively and efficiently bound DNA sequences, allowing optimal amplification of the target DNA sequence. For primers used in molecular cloning, the primer_3 software program (http://www-genome.wi.mit.edu/genome_software/other/primer3.html) was utilised and the sequence of each primer confirmed using the NCBI’s Nucleotide Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.gov/BLAST). Lyophilized pellets were re-suspended in sterile UP H₂O to a concentration of 200 pm/µl (200 µM). An aliquot was then taken and diluted in sterile UP H₂O to a working concentration of 10 pm/µl (10 µM). For quantitative PCR assays, custom primers and FAM-labelled MGB probes (Table 2.7) were designed using the Primer Express software program (Applied Biosystems, UK). Primers and probe sequences were confirmed using the BLAST search tool.

2.3.2 **Polymerase chain reaction (PCR)**

PCR (Mullis et al., 1986) was performed on a GeneAmp 9700 96-well thermal cycler (Applied Biosystems, UK). Typically, reactions were performed using 1 µl template DNA; 5 µl 10x AJ buffer (45 mM Tris-HCl, pH 8.8; 11 mM (NH₄)₂SO₄; 4.5 mM MgCl₂; 200 mM dNTPs; 110 µg/ml BSA; 6.7 mM β-mercaptoethanol (β-ME) and 4.4 mM EDTA, pH 8.8); 1 µl forward primer; 1 µl reverse primer; 41 µl sterile UP H₂O and 1 µl (1 U) of Taq DNA polymerase (diluted 1:5 in 10x AJ buffer and sterile UP H₂O). Reactions were always set up on ice and a PCR mastermix prepared for each individual experiment. Annealing temperatures and extension times were adapted for different primer $T_m$ and amplicon size.
2.3.3 8/18 PCR

Primers annealing in exon 8 and 18 amplifying across the entire alternatively spliced region of TNC were used to amplify all TNC species. Typically, reactions were performed as described in section 2.3.2. A schematic is shown in Figure 2.5 using TNC-9/14/16 as an example.

![8/18 PCR Schematic](image)

Figure 2.5: Example of 8/18 PCR reaction

Schematic exons showing an 8/18 PCR reaction using the TNC-9/14/16 isoform as a template. Shaded exons represent the alternatively spliced exons.

2.3.4 High fidelity PCR amplification

AccuPrime Pfx DNA polymerase was used in order to ensure high specificity and fidelity of the target sequence for cloning. PCR was performed on a GeneAmp 9700 96-well thermal cycler using 1 μl DNA template; 5 μl 10x AccuPrime PCR buffer (200 mM Tris-HCl, pH 8.0; 500 mM KCl, 15 mM MgCl2, 2 mM dGTP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP, thermostable AccuPrime protein, 10% v/v glycerol); 1.5 μl forward and reverse primers; 40.6 μl sterile UP H2O and 0.4 μl (1U) AccuPrime Pfx DNA polymerase. Cycling conditions were as follows: -
Table 2.10: PCR cycling conditions for high-fidelity amplification

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature (°C)</th>
<th>Time (sec)</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>120</td>
<td>1</td>
</tr>
<tr>
<td>Denaturing</td>
<td>95</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>56</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>68</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>68</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

2.3.5 Generation of cDNA by reverse transcription (RT)

As one of the risks of experiments involving the use of RNA is contamination by RNases, all equipment including Gilsons were treated with RNAzap (Ambion) and filter-tipped pipette tips were used. Sterile gloves were worn and constantly treated with 70% IMS or changed regularly. cDNA was synthesised from total RNA by AMV-Reverse transcription (AMV-RT). 1 µg of total RNA (made up to a final volume of 10 µl in DEPC H₂O) was added to 1.5 µl of 10 µM oligo dT(25) primer and 4.88 µl DEPC H₂O. Samples were incubated at 70 °C for 5 min and allowed to equilibrate to room temperature. 5 µl of 5x AMV-RT buffer (250 mM Tris-HCl, pH 8.3; 250 mM KCl; 50 mM MgCl₂; 2.5 mM spermidine & 50 mM DTT); 2.5 µl 10 mM dNTPs; 0.62 µl RNasin; and 0.5 µl (1U) AMV-RT were added and reactions incubated at 42 °C for 60 min in a GeneAmp 9700 96-well thermal cycler. Samples were stored at 4 °C until required for PCR analysis (section 2.3.2).

Reactions were also performed without incubation with RT as a control for genomic DNA contamination of RNA extraction.

2.3.6 Agarose gel electrophoresis

DNA was mixed with 2 µl 10x loading buffer (0.21% w/v Bromophenol Blue; 0.21% v/v Xylene Cyanol FF; 200 mM EDTA, pH 8.0 & 50% v/v glycerol) and separated by agarose gel electrophoresis, typically using 0.8 – 1% w/v high gelling Seakem agarose in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) containing 2 µg/ml ethidium bromide. Gels were run for 1 hr at 100 V for analysis of PCR amplicons and 2 hr at 100 V for analysis of plasmid DNA integrity. After running, samples were visualised using an ultra-violet light trans-illuminator. An image of the gel was obtained using the program Alpha (Alpha Computer Systems, USA).
2.3.7 Quantitative PCR

Reaction conditions for custom designed probes were as follows: - 3.6 µl cDNA (diluted 1:10); 5 µl 2x TaqMan fast universal mastermix (No AmpErase UNG); 0.6 µl of forward primer; 0.6 µl reverse primer; 0.2 µl FAM-MGB probe.

For Applied Biosystems TaqMan assays, reaction conditions were as follows: - 4 µl cDNA; 5 µl 2x TaqMan fast universal mastermix (No AmpErase UNG); 0.5 µl FAM-MGB Probe set; 0.5 µl UP H2O. All reactions were set up on ice and performed in triplicate on ABI 7500 fast and Step-One thermal cyclers. Before cycling, plates were centrifuged at 3000 rpm for 30 sec. Cycling conditions were:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature (°C)</th>
<th>Time (sec)</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Annealing &amp; Extension</td>
<td>60</td>
<td>20</td>
<td>40</td>
</tr>
</tbody>
</table>

2.3.7.1 Standard curves

Standard curves were generated for each primer/probe set using appropriate TNC isoform clone templates. A 1:5 dilution series was set up for each probe set and the efficiency calculated from the gradient of the slope. The slope of the standard curve was then utilised to calculate the number of molecules within each sample.

2.3.7.2 Calculation of number of molecules

Values obtained from the standard curves were used to calculate the log number of molecules in each sample 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 \( x = \log \text{number of molecules} \).

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

\[
\Delta Ct\ EC = \text{Average Ct sample } EC - \text{Average Ct Control } EC
\]

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

then, \( NE = n \times RE \)

Where: - \( \Delta Ct\ EC \) = 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.

### 2.3.7.3 Relative quantification

For relative expression of target genes (\( \Delta \Delta Ct \) method), target genes were compared to an endogenous standard (GAPDH, beta-2-microglobulin (\( \beta 2M \)) or hypoxanthine guanine phosphoribysol transferase 1 (HPRT1)). Average threshold cycle (Ct) values were obtained and from this a \( \Delta Ct \) and \( \Delta \Delta Ct \) value was calculated do give a final RQ value from the following equations:

\[
\Delta Ct = \text{Average Ct sample} - \text{Average Ct endogenous control}
\]

then, \( \Delta \Delta Ct = \Delta Ct \text{ sample} - \Delta Ct \text{ sample reference} \)

then, \( RQ = 2^{(\Delta \Delta Ct)} \)

### 2.3.8 DNA sequencing

5x sequencing buffer (400 mM Tris-HCl, pH 9.0; 10 mM MgCl\(_2\)) was diluted 1:5 in sterile UP H\(_2\)O. Big Dye Terminator was then diluted 1:8 in 1x sequencing buffer. Each reaction contained: - 8 µl Big Dye Terminator mix; 0.5 µg template DNA; 3.5 µl (3.5 pmol) of sequencing primer and 6.5 µl sterile UP H\(_2\)O. Reactions were performed on a GeneAmp 9700 96-well thermal cycler as follows: -
Sequencing reactions were cleaned-up by the addition of 2 μl of 2.2% w/v SDS and thorough mixing. Samples were heated to 98 °C for 5 min and then incubated at 25 °C until required.

Big Dye Terminators were removed prior to sequencing using Centri-Sep columns according to manufacturer’s instructions. Samples were stored at 4 °C until required. Sequencing was performed by the Protein and Nucleic Acid Chemistry Laboratory (PNACL) at the MRC Toxicology Unit, Leicester on an ABI 377 sequencer and analysed using the Chromas software program (Technelysium Pty Ltd).

### 2.4 Western blotting

#### 2.4.1 Protein quantification

The protein concentration in each sample was determined using the Bio-Rad protein assay (Bradford assay). 5 μl of sample, 795 μl sterile H₂O and 200 μl of protein assay reagent (diluted 1:5 in sterile H₂O), were briefly vortexed, developed in the dark for 10 min and the absorbance measured at 595 nm. For ‘blanks’, depleted media was used for CM samples and Gold Lysis Buffer (1% w/v Triton X-100; 30 mM Tris, pH 8.0; 137 mM NaCl; 15% v/v glycerol; 5 mM EDTA) used for cell lysates. Absolute protein concentration in each sample was then determined from a standard curve, constructed from absorbance against known protein concentrations for a series of BSA standards.

#### 2.4.2 One dimensional SDS-polyacrylamide gel electrophoresis

Six percent polyacrylamide gels (1.5 mm thickness) were utilised for the separation of proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Table 2.13 shows the volumes of each solution required to achieve the desired percentage: -

| Table 2.12: Sequencing conditions |
|-------------------------------|--|--|--|
| **Stage** | **Temperature (°C)** | **Time (sec)** | **No. of cycles** |
| Denaturation | 96 | 10 | |
| Annealing | 50 | 5 | |
| Extension | 60 | 240 | |
| Final Extension | 4 | 420 | 1 |
| Hold | 4 | $\infty$ | |


Materials and Methods

2.4.2 Solutions used for SDS-PAGE

<table>
<thead>
<tr>
<th>Solution</th>
<th>6% resolving gel (ml)</th>
<th>5% stacking gel (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide/bis acrylamide solution</td>
<td>2.00</td>
<td>0.85</td>
</tr>
<tr>
<td>Gel buffer (1.5M Tris-HCl, pH 8.8)</td>
<td>2.50</td>
<td>1.25</td>
</tr>
<tr>
<td>10% w/v SDS</td>
<td>0.10</td>
<td>0.05</td>
</tr>
<tr>
<td>10% ammonium persulphate (APS)</td>
<td>0.05</td>
<td>0.025</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>Sterile H$_2$O</td>
<td>5.40</td>
<td>2.85</td>
</tr>
</tbody>
</table>

For the resolving and stacking gels, acrylamide solution, Tris-HCl, SDS and water were all mixed before polymerisation was initiated by addition of 10% APS and $N, N, N', N'$-tetramethylethylenediamine (TEMED). The resolving gel was pipetted into a Bio-Rad cassette and approximately 1 ml of IMS layered over to form a seal and prevent the formation of air bubbles and evaporation. After the gel had set the IMS was removed and the gel rinsed with sterile water. Any excess water was absorbed using Whatmann paper. The stacking gel was loaded, a gel comb inserted and left to set at RT. After setting, the gel comb was removed and the gel was stored at 4 °C wrapped in damp paper towels and Saran wrap until required.

25 µg of total protein per sample was added to fresh eppendorfs. For conditioned media, an equal volume of Gold Lysis Buffer was added to each sample, and an appropriate volume of 4x loading buffer (240 mM Tris-Cl, pH 6.8; 20% v/v β-mercaptoethanol; 8% w/v SDS; 40% v/v glycerol; 0.2% w/v bromophenol blue) also added to all samples (depending on the volume required to achieve 25 µg of protein). Samples were denatured at 99 °C for 5 min, incubated on ice and loaded into each well. 10 µl of BioRad Precision Plus Dual Color protein weight marker was also loaded into a separate well. The gel was run in running buffer (25 mM Tris; 190 mM glycine; 0.1% w/v SDS) at 100 V until the sample front had run the required distance.

2.4.3 Protein transfer and blotting

Transfer of polypeptides on a nitrocellulose membrane was performed as previously described (Towbin et al., 1979). The gel was removed and washed in transfer buffer (25 mM Tris; 190 mM glycine; 0.01% w/v SDS; 20% v/v methanol) 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. A BioRad blotting apparatus was then set up with as shown in Figure 2.6A. Air bubbles were removed, the gel apparatus transferred
into a cassette and the protein electrophoresed in transfer buffer onto nitrocellulose membrane at 100 V for 1 hr at 4 °C.

2.4.4 Enhanced chemiluminescence (ECL) detection

The nitrocellulose membrane was removed from the cassette and washed in TBS-T (0.1% v/v Tween-20; 1x TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.5)) to remove residual SDS. The membrane was blocked by incubation in blocking solution (5% w/v Marvel milk powder in TBS-T) for 1 hr at RT. Membranes were then probed with primary antibody to TNC and vinculin at a dilution of 1:1000 and 1:2000 respectively in TBS-T overnight at 4 °C. Membranes were washed 3 times in TBS-T at RT for 5 min and incubated for 1 hr in TBS-T containing secondary antibody donkey anti-rabbit horse radish peroxidise (HRP)-linked IgG (Amersham Biosciences, UK) for TNC and sheep anti-mouse HRP-linked IgG (GE Healthcare) at a dilution of 1:2000. An ECL kit (Amersham Biosciences, UK) was used for detection of protein according to manufacturer’s instructions (Figure 2.6B) and visualised using X-ray film (Xerox, USA) with a variety of exposure times.
Figure 2.6: The wet transfer system and detection of protein expression by ECL

A) Cartoon diagram of the wet transfer system showing a nitrocellulose membrane and resolving gel encased by two pieces of Whatmann paper. B) Schematic of the luminal ECL reaction. The bio-catalysis of the luminal chemiluminescence is considered as the most powerful way of triggering the light emission reaction. The heme-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).

A)
2.5 Molecular cloning

2.5.1 DNA recombination and nested PCR

Two rounds of DNA recombination and a final nested PCR round using oligonucleotide primers overlapping specific exons were used in order to produce the TNC isoform sequences for cloning. AccuPrime *Pfx* DNA polymerase was utilised to ensure a high-fidelity sequence during PCR amplification of the target sequence. Reactions consisted of: 1 μl DNA template (diluted to ~ 1ng for the flanking sequences and ~ 10ng for the target sequence); 5 μl 10x AccuPrime PCR buffer; 1.5 μl forward primer; 1.5 μl reverse primer; 39.6 μl sterile UP H2O; 0.4 μl (1U) AccuPrime *Pfx* DNA polymerase.

Cycling conditions for each round were as follows: -

Table 2.14: First round DNA recombination cycling

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temp (°C)</th>
<th>Time (sec)</th>
<th>No. of cycles</th>
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<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>120</td>
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<tr>
<td>Denaturing</td>
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<td>30</td>
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<tr>
<td>Annealing</td>
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<td>30</td>
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Table 2.15: Second round DNA recombination cycling

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<th>Time (sec)</th>
<th>No. of cycles</th>
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<td>Denaturing</td>
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<td>Extension</td>
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<tr>
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NB: * * = 50% ramp
Table 2.16: Nested PCR

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<th>Time (sec)</th>
<th>No. of cycles</th>
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<tr>
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<tr>
<td>Hold</td>
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</tbody>
</table>

NB: * = 50% ramp

After PCR cycling (Figure 4.7 and Figure 4.8 for a schematic representation), PCR products were purified (section 2.2.3) and quantified using the NanoDrop ND-1000 spectrophotometer (section 2.2.4).

### 2.5.2 TOPO cloning

5 μl of PCR products obtained in section 2.5.1 were incubated at 72 °C for 10 min with 1 μl Taq DNA polymerase (diluted 1:5 in 10x AJ buffer) to add a single deoxyadenosine (A) to the 3’-ends of the PCR products and allow effective ligation with the vector (which contains overhanging 3’- deoxythymidine (T) residues).

TOP10 Competent *E. coli* cells were thawed on ice in preparation for transformation. For TOPO cloning, reagents were mixed in a 0.5 ml eppendorf as follows: - 100 ng PCR product; 1 μl salt solution (1.2 M NaCl; 60 mM MgCl₂); UP H₂O to a final volume of 5 μl; 1 μl (10 ng) TOPO vector. The reaction was gently mixed and incubated at room temperature for 10 min. Samples were placed on ice in preparation for One Shot TOP10 Chemical Transformation (section 2.5.6).

For PCR analysis, colonies obtained from transformation were re-suspended in 200 μl of PBS and 10 μl incubated at 95 °C for 10 min to lyse the cells. PCR analysis was then performed using T8 F & T18 R primers. Transformed colonies were then streaked on an ampicillin selective LB agar plate to produce pure clones and analysed by PCR as described above. Fifty microlitres of PBS cell suspension containing successfully transformed cells was added to 5 ml of LB medium containing ampicillin (100 μg/ml)
and incubated at 37 °C overnight with shaking at 200 rpm in preparation for small scale synthesis (section 2.2.1).

2.5.3 Restriction digestion
Typically, a reaction consisted of 1 μg of DNA; 2 μl appropriate 10x NE buffer; 2 μl 10x BSA; 1 μl per restriction enzyme and sterile UP H₂O to a final volume of 20 μl. Samples were then incubated at the required temperature overnight. To check that restriction was successful, an aliquot was analysed on a 0.8% agarose gel.

2.5.4 SAP treatment of restricted plasmid constructs
A typical SAP reaction consisted of ~ 500 ng restricted cloning vehicle plasmid; 1.2 μl 10x dephosphorylation buffer (0.5 M Tris-HCl; 50 mM MgCl₂, pH 8.0) and 2 U of SAP. Samples were incubated at 37 °C for 1 hr and then at 65 °C for 10 min in order to inactivate the enzyme. Samples were then used immediately for ligation reactions (section 2.5.5).

2.5.5 Ligation
The quantity of sequence insert to use in the ligation reaction was calculated as follows:

\[
i = \left(\frac{n_v \times k_i}{k_v}\right) M
\]

Where: \(i\) = ng insert needed; \(n_v\) = ng vector; \(k_i\) = kb size insert; \(k_v\) = kb size of vector and \(M\) = molar ratio of insert to vector

The molar ratio of insert/vector was 3:1. A typical ligation reaction consisted of 50 ng restricted plasmid; X ng insert; 2 μl 10x ligation buffer (660 mM Tris-HCl, 50 mM MgCl₂, 50 mM DTT, 10 mM ATP, pH 7.5); 1 U T4 DNA ligase; UP H₂O to a final volume of 20 μl. Samples were centrifuged at 13,000 rpm for 20 sec and incubated at 15 °C overnight, then at 65 °C for 10 min to heat-inactivate the enzyme.

2.5.6 Transformation of chemically competent E. coli
Plasmid DNA was transformed into chemically competent TOP10, SCS110 or DH5α cells according to manufacturer’s instructions. Briefly, cells were thawed on ice, gently mixed with an aliquot of plasmid DNA and incubated on ice for 10 min with gentle
swirling every 2 min. Cells were then heat-shocked at either 37 °C or 42 °C (dependent on cell type) and immediately transferred to ice for 2 min. An aliquot of pre-warmed SOC medium (2% w/v tryptone; 0.5% w/v yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM MgCl₂; 10 mM MgSO₄; 20 mM glucose) was added and cells incubated at 37 °C for 1 hr with shaking at 200 rpm. An aliquot of transformation mixture was spread onto selective LB agar containing antibiotic and incubated at 37 °C overnight. Colonies formed were picked using a sterile pipette tip, re-suspended in 200 µl of PBS (145 mM NaCl; 7.5 mM Na₂HPO₄; 2.5 mM Na₂HPO₄·2H₂O) and stored at 4 °C until required.

2.6 Mammalian cell culture

2.6.1 Resuscitation of cells from frozen storage

Cells were retrieved from liquid nitrogen and left at room temperature for 1 min and immediately transferred to a 37 °C water bath for 1-2 min until fully thawed. In a class II safety cabinet the contents of the ampoule were removed and gently re-suspended in 10 ml of pre-warmed, phenol red free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS and 2 mM L-glutamine (complete DMEM). The cells were centrifuged at 1000 rpm for 5 min at room temperature and the pellet re-suspended in 10 ml of complete DMEM. Cells were counted and an appropriate number of cells seeded in a 25 cm² flask. Cells were incubated at 37 °C/5% CO₂ until passaging.

2.6.2 Routine cell maintenance

Cells were grown and routinely passaged in complete DMEM and incubated at 37 °C/5% CO₂. When cells had reached 70 – 80% confluency they were washed with PBS and the monolayer detached by the addition of 1x Trypsin/EDTA (TE) with incubation at 37 °C for 5-10 min. Flasks were gently tapped to encourage greater detachment of the cells. Trypsin activity was terminated by the addition of 1 volume of pre-warmed complete DMEM and cells were transferred to a fresh 15 ml Falcon tube. Cells were centrifuged at 1000 rpm for 5 min and the pellet was re-suspended in complete DMEM, seeded into culture flasks at the required density and maintained at 37 °C/5% CO₂ until required.

2.6.3 Long term storage

Cells in log phase growth were washed with PBS, trypsinised with 1x TE, centrifuged at 1000 rpm for 5 min and re-suspended in freezing medium (50% v/v DMEM; 2 mM L-
glutamine; 40% v/v FBS; 10% v/v DMSO). Cells were counted and 2 x 10^6 cells transferred to a cryotube and stored at -80 °C under isopropanol overnight. Cells were then transferred to liquid nitrogen for long term storage.

### 2.6.4 Calculation of cell number

Cells prepared for assaying were harvested and re-suspended in complete DMEM, serum free DMEM or DMEM containing 1% FCS, depending on which assay was to be performed. An aliquot was removed and the number of viable cells in 1 mm² was counted using a Neubauer haemocytometer and the cell concentration calculated using the following formula where C = Total cell number, v = volume cells re-suspended in and c = cell count:

\[ C = \left( \frac{c}{4} \right) \times 10^4 \times v \]

### 2.6.5 Fluorescent staining of cells

Cells were stained using the lipophilic fluorescent tracer dye 1,1'-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiIC_{12}(3)) at a concentration of 10 µg/ml in complete DMEM at 37 °C/5% CO₂ for 1 hr. Cells were then washed 3 times in DPBS, incubated for 1 hr in complete DMEM and harvested and counted in preparation for assaying.

### 2.6.6 Production of conditioned media

Cells maintained in complete DMEM were grown until 70 – 80% confluent, washed three times in DPBS and incubated at 37 °C/5% CO₂ for 48 hr in serum free DMEM or Opti-MEM. Conditioned media (CM) was then removed from cells and either stored at -20 °C for short term storage or -80 °C for long term storage. Before use, CM was thawed and centrifuged for 5 min at 1000 rpm to remove cell debris and transferred to a fresh Falcon tube.

### 2.6.7 Sample preparation for Western blotting

For protein quantification, CM was taken, centrifuged and concentrated using a Centricon Centrifugal Filter Device (Millipore, UK) according to manufacturer’s instructions. Concentrated protein samples and depleted media were stored at -20 °C until required. For cell lysates, cells were harvested (section 2.6.2) and centrifuged at
1000 rpm for 5 min. The supernatant was discarded and the cell pellet re-suspended in an appropriate volume of gold lysis buffer cocktail (Gold lysis buffer and 100x Protease Inhibitor), mixed thoroughly and incubated on ice for 10 min. The suspension was then passed through a 1 ml syringe fitted with a 25-gauge needle 5 times and centrifuged for 3 min at 13, 200 rpm. The resulting supernatant was transferred to a fresh eppendorf and placed on ice until required.

2.6.8 Transfection of cells by Fugene

Cells were trypsinised, centrifuged at 1000 rpm for 5 min and re-suspended in complete DMEM. An aliquot of cells were counted using a haemocytometer and the concentration of cells adjusted to be 65 – 70% confluent upon transfection. Cells were incubated at 37 °C/5% CO₂ for 24 hr. After incubation, cells were washed three times in DPBS and left at RT while the transfection complex was formed.

Supercoiled plasmid DNA was diluted in serum free media or Opti-MEM to a concentration of 0.02 µg/µl and vortexed briefly. Fugene HD transfection reagent was added to the media at a ratio of 6 µl Fugene:2 µg DNA, gently mixed by flicking the tube and incubated at RT for 20 min. During this time, complete DMEM was added to cells and then the transfection reagent added to the media. Cells were incubated at 37 °C/5% CO₂ for 24 hr in complete DMEM until required for assaying.

2.6.9 Culture of cells on a fibronectin (FN) milieu

Six well plates were coated with FN at a concentration of 1 µg protein/cm² (10 µg/ml) at 4 °C for 24 hr with gentle rocking. Wells were washed three times with cold DPBS and the non-coated plastic surface blocked with 1 ml of 1% heat-inactivated BSA in DPBS at 4 °C for 24 hr, giving rise to 10 µg/cm² of protein. Wells were then washed with cold DPBS and 2 ml of DMEM containing 1% v/v FCS added.

Before plating, cells were seeded at a density of 1.2 x 10⁶ in a T25 flask, transfected with TNC plasmids (section 2.6.8), serum starved for 24 hr, harvested, re-suspended in DMEM containing 1% v/v FCS and 4 x 10⁵ cells transferred to each well. Cells were cultured on the FN matrix at 37 °C/5% CO₂ for 18 hr and harvested for RNA (section 2.2.2). All cultures were performed in triplicate.
2.6.10 Invasion assays
The upper surface of FluoroBlok 8 μm pore cell culture inserts were each coated with 200 μl of Matrigel (diluted 1:100 in cold Opti-MEM) and incubated at 37 °C/5% CO₂ for 2 hr. After incubation, excess Matrigel was removed and an aliquot of fluorescently stained cells (section 2.6.5 for staining of cells) seeded into the insert in 100 μl complete DMEM. 700 μl of complete DMEM was then placed into each well. Cells were incubated at 37 °C/5% CO₂ for 4 hr and media in the insert replaced with 100 μl OptiMEM. Cells were cultured at 37 °C/5% CO₂ for 48 hr in a FLUOstar OPTIMA plate reader (BMG Labtech, UK) with measurements taken every 2 hr.

2.7 Differential gene expression analysis

2.7.1 BioAnalyser analysis of RNA integrity
Analysis of RNA integrity was performed using the Agilent Bio-Analyser and associated RNA NanoChip reagents according to manufacturer’s instructions. Briefly, after reconstitution of all reagents, the appropriate well of a NanoChip was loaded with 9 μl of gel-dye mix and pressurised in the priming station for 30 sec. A further 9 μl of gel-dye was then loaded into each of the wells marked “G”. In the remaining wells, 5 μl of RNA 6000 NanoMarker (including the ladder well) was added. 1 μl of RNA 6000 Ladder was then added into the appropriate well and then 1 μl of total RNA sample (previously incubated at 70 °C for 2 min) added into a sample well. The chip was vortexed for 1 min at 2400 rpm and analysed on an Agilent 2100 bioanalyser within 5 min of preparation.

2.7.2 One-cycle target labelling
Total RNA was extracted from MCF-7 cells transfected with different TNC isoforms and cultured on a fibronectin matrix (section 2.2.2 and 2.6.9) and the integrity analysed using the Agilent 2100 Bioanalyser (Agilent, UK) according to manufacturer’s instructions (section 2.7.1).

2.7.2.1 First-strand cDNA synthesis
First-strand cDNA synthesis was performed using the One-Cycle cDNA Synthesis Kit. This kit includes the T7-Oligo(dT) primer:

5’-GGCCAGTGATAGATACGACTCACTATAGGAGGCGG-(dT)_{24-3’}
A serial dilution of Eukaryotic Poly-A RNA spike-in controls (providing exogenous positive controls for the labelling process) containing several *B. Subtilis* genes (lys, phe, thr, and dap) at staggered concentrations was set up using Poly-A Control Dilution buffer at 1:20, then 1:50, then 1:5 giving a final ratio of copy number of each gene of 1:100,000; 1:50,000; 1:25,000 and 1:6,667 respectively.

A first-strand cDNA reaction was then performed using the following: - 1 μg total RNA; 2 μl diluted poly-A RNA controls; 2 μl of T7-Oligo(dT) primer (100 pmol) and RNase-free water to a final volume of 12 μl. Samples were then incubated at 70 °C for 10 min and incubated on ice for 2 min.

During incubation, a first-strand reaction mix was produced consisting of: - 4 μl 5x 1st Strand Reaction buffer; 2 μl dithiothreitol (DTT) (10 mM) and 1 μl dNTPs (500 μM each). This was then added to each RNA/T7-Oligo(dT) primer mix, mixed thoroughly and incubated at 42 °C for 2 min. After incubation, 200 U of SuperScript II reverse transcriptase was added to each sample and incubated at 42 °C for 1 hr, with a final incubation at 4 °C for at least 2 min.

### 2.7.2.2 Second-strand cDNA synthesis

During incubation of the first-strand synthesis reaction mixture, a second strand synthesis reaction mixture was produced consisting of: - 91 μl RNase-free water; 30 μl 5x 2nd Strand Reaction buffer; 3 μl dNTPs (200 μM each); 10 U *E. Coli* DNA ligase; 40 U *E. Coli* DNA polymerase I and 1 μl RNase H. This was then added to the first-strand reaction mixtures to a final volume of 150 μl. Samples were incubated at 16 °C for 2 hr and 10 U T4 DNA polymerase added to each sample with incubation at 16 °C for a further 5 min. 10 μl of EDTA (0.5 M) was added to the reaction mixture and the double stranded cDNA cleaned-up using the Sample Clean-Up Module according to manufacturer’s instructions.

### 2.7.2.3 Synthesis of biotin-labelled cRNA

Synthesis of biotin-labelled cRNA was performed using the GeneChip *in-vitro* transcription (IVT) Labelling Kit. This kit contains a mixture of the four natural ribonucleotides and one biotinconjugated nucleotide analogue at an optimized ratio.
All 12 µl of the cleaned-up double stranded cDNA mixture was added to an IVT reaction mix consisting of: - 8 µl RNase-free water; 4 µl 10x IVT Labelling buffer; 12 µl IVT Labelling NTP mix and 4 µl IVT Labelling enzyme. Reagents were mixed and incubated at 37 °C for 16 hr. Once the IVT reaction was complete, the biotin-labelled cRNA was cleaned-up using the Sample Clean-Up module according to manufacturer’s instructions and quantified using the NanoDrop. The adjusted cRNA yield was calculated using the following equation:

\[
\text{Adjusted cRNA yield} = \text{RNA}_m - (\text{total RNA}_i)(\text{Y})
\]

Where:
- \(\text{RNA}_m\) = amount of cRNA measured after IVT
- \(\text{Total RNA}_i\) = starting amount of total RNA
- \(\text{Y}\) = fraction of cDNA reaction used in IVT

### 2.7.2.4 Fragmentation of cRNA

Fragmentation of cRNA before target hybridisation is a critical step for optimal assay sensitivity. A fragmentation reaction was set up consisting of: - 10.5 µg adjusted cRNA; 4.2 µl 5x Fragmentation buffer and RNase-free water to a final volume of 20 µl. Samples were incubated at 94 °C for 35 min and incubated on ice following incubation. A 0.5 µg aliquot of fragmented cRNA was then analysed using the Agilent BioAnalyzer in order to ensure successful fragmentation. Samples were then used for target hybridisation.

### 2.7.3 Eukaryotic target hybridisation and detection

Fragmented cRNA was hybridised to a GeneChip Human Genome U133 plus 2.0 array and analysed using an Affymetrix GeneChip scanner to detect differential gene expression.

#### 2.7.3.1 Target hybridisation

A hybridisation cocktail was produced for each array consisting of: - 10 µg fragmented cRNA; 3.3 µl Control oligonucleotide (50 pM); 10 µl 20x Eukaryotic hybridisation controls (\(\text{bioB, bioC, bioD, cre}\)) (1.5, 5, 25 & 100 pM respectively); 2 µl Herring sperm DNA (0.1 mg/ml); 2 µl BSA (0.5 mg/ml); 100 µl 2x Hybridisation buffer (final conc. = 100 mM MES, 1 M [Na\(^+\)], 20 mM EDTA, 0.01% v/v Tween-20) ; 20 µl DMSO and UP H\(_2\)O to a final volume of 200 µl.
The hybridisation cocktail was incubated at 99 °C for 5 min, then 45 °C for 5 min and finally centrifuged at 13,200 rpm for 5 min to remove insoluble material from the cocktail. During incubation, the GeneChip arrays were filled with 1x hybridisation buffer and incubated at 45 °C for 10 min with rotation at 60 rpm. After incubation, 1x hybridisation buffer was removed from the arrays and replaced with 200 µl of the hybridisation cocktail. Arrays were incubated at 45 °C for 16 hr with rotation at 60 rpm.

2.7.3.2 Target washing, staining and scanning

Post hybridisation, the hybridisation cocktail was removed from each array and replaced with a low-stringency wash buffer (wash buffer A – 6x SSPE, 0.01% v/v Tween-20). The arrays were then subjected to a series of washing and staining cycles using a GeneChip Fluidics Station 400.

Briefly, arrays were washed with wash buffer A for 10 cycles (2 mixes per cycle) at 30 °C, then washed with a stringent wash buffer (wash buffer B – 100 mM MES, 0.1 M [Na⁺], 0.01% v/v Tween-20) for 6 cycles (15 mixes per cycle) at 50 °C. Targets were stained using SAPE solution (1x Stain buffer (100 mM MES, 1 M [Na⁺], 0.05% v/v Tween-20); 2 mg/ml BSA; 10 µg/ml Streptavidin phycoerythrin and UP H₂O) for 5 min at 35 °C and washed with wash buffer A (10 cycles, 4 mixes per cycle) at 30 °C. Arrays were then stained using antibody solution (1x Stain buffer, 2 mg/ml BSA, 0.1 mg/ml Goat IgG, 3 µg/ml biotinylated antibody) at 35 °C and stained again at 35 °C for 5 min using SAPE solution. Finally, arrays were subjected to a final wash with wash buffer A for 15 cycles (4 mixes per cycle) at 35 °C.

Post washing, array chips were scanned using the Affymetrix GeneChip scanner according to manufacturer’s instructions (Figure 2.7 for schematic of the labelling and hybridisation protocol).
Figure 2.7: Schematic for the labelling and hybridisation of cRNA for use on Affymetrix GeneChip arrays

First strand cDNA was produced from total RNA (1) and transcribed in-vitro into biotinylated cRNA (2). Biotinylated cRNA was then fragmented (3) and hybridised to a GeneChip (4). Chips were washed and stained with primary and secondary antibody (5) ready for scanning into GCOS (6).

2.7.3.3 Analysis of array data

The Affymetrix GeneChips were analysed initially using the open source dChip v 1.3 (Li & Wong, 2001) software programme in order to obtain normalised expression values. These values were then subjected to a regression analysis using Microsoft Excel 2007 (Microsoft Corp., USA) with a tolerance set to analyse genes with a > 2-fold difference in either direction. This part of the analysis was carried out in collaboration with Dr Graham Ball, Nottingham Trent University. From this, the 6 most significant genes were analysed further using (RT)-qPCR.

2.7.4 Statistical analysis

All statistical analyses were performed using SPSS 16 for Windows (SPSS Inc, USA). Analysis of TNC isoform expression in breast tissue utilised t-tests, non-parametric Kruskal-Wallis tests and $\chi^2$ (Chi-squared) tests unless otherwise stated. Once $\chi^2$ tests
had been performed, results were subjected to a two-tailed Fischer’s Exact test (http://statpages.org/ctab2x2.html). For analysis of tumour grade in relation to TNC expression, a Jonckheere-Terpstra test was employed.

Statistical analysis of (RT)-qPCR and microarray validation experiments employed a Two-Way Analysis of Variance (Two-way ANOVA) in order to test for significant variance between samples and assays.

2.7.5 Sequence clustering and alignment
DNA and amino acid sequences were aligned using the EMBL-EBI tools ClustalW sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw/index.html) (Thompson et al., 1994) in order to ascertain homologous regions within each DNA and amino acid sequence.
Chapter 3: Quantitative analysis of TNC isoform expression in breast cancer cell lines and tissue
3.1 Introduction

Although there is limited knowledge of TNC isoform expression in the breast (Borsi et al., 1992; Tsunoda et al., 2003), we have previously examined a limited repertoire in benign, pre-invasive and invasive breast disease (Adams et al., 2002). However, literature detailing expression of isoforms containing exons AD1 and AD2 is sparse. Derr et al. (1997) detected AD1 in the human intraductal carcinoma cell line Hs578T and human basal cell carcinoma, while Berndt et al. (1996) detected AD1 in two cases of urinary bladder carcinoma (by manual RT-PCR). However, to date AD2 has only been described in oral mucosae (Mighell et al., 1997).

In this study a (RT)-qPCR method was developed in order to compare levels of expression of key isoforms and relate this to clinicopathological data including patient age, tumour grade, tumour type, steroid hormone receptor status, CK5/6 and CK14 status, lymph node status and basal markers (section 1.2.5 for definitions) in breast cancer.

3.2 Aims

The aim of this chapter was to examine the expression of TNC isoforms TNC-AD1, TNC-AD2, TNC-9/16 and TNC-14/16 in breast cell lines, breast cancers and normal breast tissue. The specific objectives were:

1) To develop a (RT)-qPCR protocol using TaqMan probes to examine specific TNC isoform expression in 10 breast cell lines and 2 isolated populations to investigate whether phenotypical characteristics are linked to TNC isoform expression.

2) To calculate the expression levels of high MW TNC isoforms in a small cohort of normal breast organoids and cancers to quantitatively analyse whether there are any differences in isoform expression.

3) To expand the number of samples using 8/18 PCR products derived from reduction mammoplasties and tumours to identify relationships with clinicopathological features.

The analysis of high MW TNC isoform expression was performed in 3 stages as depicted by the flow diagram:
3.3 Results

3.3.1 Validation of (RT)-qPCR protocol

All TaqMan probes were validated by production of a standard curve using a serial dilution of a standard template, comprising 8/18 PCR products (Figure 2.5 for example and Figure 3.1 for amplicon positions) from isoforms containing exons 9, 14, 16, AD1, 17 and 18. For the TNC-AD2 probe, cDNA from the cell line MDA-MB-468 was used as our group has previously detected expression of AD2 in this cell line using manual PCR. The number of molecules was calculated from a known concentration and manipulated to give a Ct range of 22 – 35 (Figure 3.2).

![Amplicons of TaqMan probes](image1)

**Figure 3.1:** Amplicons of TaqMan probes

Schematic illustrating the amplicons of each TaqMan primer-probe combination and the exons they anneal to. The unshaded boxes represent the conserved exons and the shaded boxes represent the alternatively spliced exons. The total TNC probe detected all TNC isoforms, the TNC-9/16 probe detected only the 9/16 isoform, the TNC-14/16 probe detected all TNC isoforms with the 14/16 combination of exons, including the A1 – A4 domains (if expressed) and the TNC-AD1 and TNC AD2 probes detected all TNC isoforms containing AD1 and AD2.
3.3.1.1 Standard curves

Standard curves were produced for each primer/probe assays (total TNC (exons 17/18), TNC-AD1, TNC-AD2, TNC-9/16 and TNC-14/16). DNA/cDNA templates (obtained using a known concentration and length (in bp) of PCR template, calculating the number of moles of template and then calculating the number of molecules using Avogadro constant (6.02 x 10^{23} molecules/mol)) described in section 3.3.1 were used to assess efficiency of the PCR reaction as well as produce a quantitative measurement of TNC expression in terms of the number of molecules expressed. These were then used to calculate relative expression of each isoform (compared to the endogenous control (section 2.3.7.2)) as well as the percent of each transcript within the total population. For a probe/primer to be used for subsequent reactions, an R^2 value and PCR efficiency percentage of > 98.9% from the standard curve was desired, which all primer/probe assays analysed achieved (Figure 3.2 for example and Table 3.2), indicating efficient PCR kinetics. Concentrations of PCR templates used for the serial dilution are given in Table 3.1 below:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>No. of molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1 (40 fg/µl)</td>
<td>62,500,000</td>
</tr>
<tr>
<td>1:5 (8 fg/µl)</td>
<td>12,500,000</td>
</tr>
<tr>
<td>1:25 (1.6 fg/µl)</td>
<td>2,500,000</td>
</tr>
<tr>
<td>1:125 (320 attg/µl)</td>
<td>500,000</td>
</tr>
<tr>
<td>1:625 (64 attg/µl)</td>
<td>100,000</td>
</tr>
<tr>
<td>1:3125 (12.8 attg/µl)</td>
<td>20,000</td>
</tr>
<tr>
<td>1:15625 (2.56 attg/µl)</td>
<td>4,000</td>
</tr>
</tbody>
</table>

Note: fg/µl = femtograms/µl; attg/µl = attograms/µl

<table>
<thead>
<tr>
<th>TaqMan probe/primer set</th>
<th>R^2 value</th>
<th>PCR efficiency (%)</th>
<th>Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total TNC</td>
<td>0.9959</td>
<td>99.9</td>
<td>47.806</td>
</tr>
<tr>
<td>TNC-9/16</td>
<td>0.9927</td>
<td>99.0</td>
<td>48.905</td>
</tr>
<tr>
<td>TNC-14/16</td>
<td>0.9894</td>
<td>100.32</td>
<td>49.135</td>
</tr>
<tr>
<td>TNC-AD1</td>
<td>0.9981</td>
<td>98.94</td>
<td>48.715</td>
</tr>
<tr>
<td>TNC-AD2</td>
<td>0.9923</td>
<td>99.12</td>
<td>46.73</td>
</tr>
</tbody>
</table>
3.3.2 Expression of high MW isoforms of TNC is prevalent in highly invasive breast cell lines

Ten established breast cell lines and two isolated breast populations were analysed for their profile of TNC expression. The SK-Mel-28 malignant melanoma cell line was used as a positive control as it has been shown to express large amounts of TNC (Saginati et al., 1992) as well as a wide variety of high MW TNC isoforms (Bell et al., 1999). This experiment was performed using triplicate wells per sample and repeated twice.

Expression of total TNC, TNC-9/16, TNC-14/16, TNC-AD1 and TNC-AD2 varied widely from high to low and null. No TNC specific mRNA was detected in cell lines ZR-75-1, MCF-7 or T-47D. MDA-MB-436, HBL-100 and isolated fibroblasts were shown to express high levels of total TNC (Figure 3.3A). MDA-MB-231, MDA-MB-468, GI-101 and isolated myoepithelial cells expressed medium levels of total TNC (Figure 3.3B) and Hs578T and MCF-10A cells expressed total TNC at low levels (Figure 3.3C).

MDA-MB-436, MDA-MB-468, primary myoepithelial cells and fibroblasts all expressed TNC-9/16 at low levels, with MDA-MB-436 expressing the most. All of the
remaining cell lines were negative (Figure 3.3D). TNC-14/16 was expressed in all total TNC positive cell lines, with HBL-100s expressing the highest levels (Figure 3.3A – C).

All TNC positive cell lines and isolated normal cells expressed both TNC-AD1 and TNC-AD2, but TNC-AD2 was generally expressed at a much lower level. The non-tumourigenic HBL-100 cell line displayed the highest levels. Of the adenocarcinoma cell lines, MDA-MB-436 expressed the highest levels. Isolated breast myoepithelial cells were shown to express a similar level of TNC-AD1 and TNC-AD2 to HBL-100s, whereas fibroblasts expressed TNC-AD1 and TNC-AD2 at a much lower level (Figure 3.3A – D).

When the expression of TNC isoforms was expressed as a percentage of total TNC expression, TNC-9/16 transcripts were shown to be expressed at a very low percentage (< 1%) in MDA-MB-436, MDA-MB-468, primary myoepithelial cells and primary fibroblasts. In contrast, TNC-14/16 transcripts were shown to be in abundance in the total population of TNC transcripts for the majority of cell lines analysed. MDA-MB-468 and MCF-10A both expressed TNC-14/16 at relatively low percentages (< 10%), whereas cell lines with a more fibroblast-like phenotype, such as Hs578T and primary fibroblasts all expressed TNC-14/16 at a very high percentage (> 50% total TNC transcripts) (Figure 3.3E – F).

MDA-MB-231, MDA-MB-436, MDA-MB-468, MCF-10A and primary fibroblasts all expressed a low percentage of TNC-AD1 transcripts (< 10% of total TNC transcripts), whereas Hs578T, HBL-100 and primary myoepithelial cells expressed high percentages of TNC-AD1 (> 25% of total TNC transcripts). All TNC-AD1 positive cell lines expressed TNC-AD2 but at a much lower level with the exception of MCF-10A, which showed a similar expression level to TNC-AD1 (Figure 3.3E – F).
Figure 3.3: Expression of total TNC and high MW TNC isoforms in TNC positive cell lines and isolated normal breast populations

Normalised expression of total TNC, TNC-14/16 and TNC-AD1 in (A) high (> 4 x 10⁶ molecules total TNC), (B) medium (1 x 10⁶ – 4 x 10⁶) and (C) low (< 1 x 10⁶) TNC expressing cells. TNC-9/16 and TNC-AD2 are illustrated in graph (D) as they were expressed at a very low level. The percentage of each isoform compared to total TNC expression was also calculated (E & F). The number of molecules present was calculated from the Ct value and normalisation against the endogenous control. Error bars represent the standard error of the mean from two assays.
Quantitative analysis of TNC isoform expression

Chapter 3

C) Normalised expression of TNC isoforms in Hs578T and MCF-10A cell lines.

- **Total TNC**
- **TNC-AD1**
- **TNC-14/16**

D) Normalised expression of TNC isoforms in various cell lines and isolated normal breast populations.

- **TNC-AD2**
- **TNC-9/16**
E) Quantitative analysis of TNC isoform expression

Cell lines and isolated normal breast populations

F) Cell lines and isolated normal breast populations

Gene expression levels in different cell lines and normal breast populations.
### 3.3.3 Expression of TNC is down-regulated in benign tissue but TNC-14/16 is up-regulated in invasive carcinomas

Forty-two samples (11 organoids, 9 fibroadenomas and 22 carcinomas) were studied for which mRNA was isolated enabling direct relative measurement of all 5 TNC sequences by comparison to the endogenous control. GAPDH was used as an endogenous control and $-\Delta \text{Ct}$ values calculated for statistical analysis. Note that samples negative for TNC isoform expression were given a Ct value of 40 as this was the maximum cycle number in the reaction and this value was necessary to calculate $-\Delta \text{Ct}$. Furthermore, the percentage of each isoform in the total TNC population was calculated from the number of molecules present in each sample.

Analysis of variance revealed a significant increase in expression of total TNC, TNC-AD1, TNC-AD2 and TNC-9/16 in breast organoids ($p = 0.001$, $<0.001$, $0.001$ and $0.005$ respectively) and carcinomas ($p = <0.001$, $<0.001$, $0.002$ and $0.001$ respectively) compared to fibroadenomas. No significant increase in TNC-14/16 was found in breast organoids compared to fibroadenomas ($p = 0.390$); however, there was a significant decrease in expression in fibroadenomas compared to carcinomas ($p = <0.001$).

No significant differences in expression of total TNC, TNC-AD1, TNC-AD2 and TNC-9/16 were found in carcinomas compared to breast organoids ($p = 1.000$ for all). However, TNC-14/16 showed a significant increase in expression ($p = <0.001$) (Figure 3.4).
Figure 3.4: Box and whisker plots of total TNC and high MW TNC isoform expression in breast tissue

The boxes contain the \(-\Delta Ct\) lower and upper quartiles and the line represents the median. The whiskers connect the highest and lowest \(-\Delta Ct\) values that are not outliers. Outliers are indicated by ‘o’ and ‘*’ and p-values are indicated on the plots.

A) Total TNC

B) TNC-AD1

C) TNC-AD2

D) TNC-9/16

E) TNC-14/16
When the relative expression of TNC isoforms was expressed as a percentage of total TNC expression, TNC-9/16 levels in fibroadenomas and invasive carcinomas were shown to be low, with all samples expressing < 10% of the total population (all organoids were null). In contrast, expression of TNC-14/16 was shown to be high, particularly in the invasive carcinomas. TNC-14/16 was present in < 20% of TNC transcripts in breast organoids, whereas in most of the fibroadenomas and invasive carcinomas, > 20% of the transcripts was the TNC-14/16 isoform. Moreover, 2 of the fibroadenomas and 5 of the invasive carcinomas expressed TNC-14/16 in 30 – 40% of the population and 2 of the invasive carcinomas showed TNC-14/16 constituted > 50% of the TNC population of transcripts (Figure 3.5A).

TNC-AD1 was shown to be a major transcript in the total TNC population in breast organoids, with 14 – 40% of the TNC transcripts being TNC-AD1. TNC-AD2 transcripts were shown to be expressed at lower level in organoids; however, one isolated case showed expression of both TNC-AD1 and TNC-AD2 (> 20% for both). In fibroadenomas, TNC-AD1 transcripts were expressed at a low level (< 10%), and TNC-AD2 even lower (< 5%). Expression of TNC-AD1 in carcinomas varied (< 5 - > 20%), with only 3 cases containing TNC-AD1 in > 15% of the total TNC transcripts. As with expression in organoids and fibroadenomas, TNC-AD2 expression was found to be low, with < 7% of transcripts being TNC-AD2 apart from one case, in which TNC-AD2 constituted < 15% of the TNC transcripts (Figure 3.5B).
3.3.3.1 Expression of high MW TNC isoforms in non-involved tissue from cancer containing breasts (NTCCBs)

Evidence exists to suggest that the normal breast, in which cancer arises, differs morphologically to non-cancer containing breasts (Wellings et al., 1975; Alpers & Wellings, 1985; Sarnelli & Squartini, 1989). In particular, expression of integrin \( \alpha_6\beta_4 \) is lost in two-thirds of cases (Jones et al., 1992) and expression of EGFR is modified (Walker, 1992), whereas TNC expression in non-involved tissue from cancer-containing breasts (NTCCBs) is similar to normal breast tissue (Jones et al., 1992). This section describes the expression of TNC isoforms in NTCCBs. In total, 8 myoepithelial populations from normal breasts and 4 myoepithelial populations from NTCCBs were analysed from isolated mRNA kindly provided by Vasileos Modes (PhD student, CSMM department).

Expression of total TNC was detected in all samples. Expression of TNC-9/16 was detected in 3 myoepithelial populations from normal breasts and 2 myoepithelial populations from NTCCBs, whereas expression of TNC-14/16 was found in 5 of 8 normal samples and all 4 NTCCBs. In the majority of samples TNC-9/16 expression...
was always accompanied by expression of TNC-14/16; however, 1 normal sample showed TNC-9/16 expression only and not TNC-14/16. Expression of TNC-AD1 was detected in 2 normal samples and 2 NTCCBs, as was the case with expression of TNC-AD2. However, in the normal samples, expression of TNC-AD1 and TNC-AD2 appeared to be mutually exclusive, whereby either TNC-AD1 or TNC-AD2 was expressed, but not both. Co-expression of both AD1 and AD2 only occurred in 1 NTCCB sample.

No significant difference in expression of total TNC or any high MW TNC isoforms was found between normal samples and samples from NTCCBs. When expressed as a percentage, TNC-9/16 in breast organoids was very low in relation to total TNC expression (< 2% of total TNC transcripts). This was also the case in NTCCB samples. Expression of TNC-14/16 in normal samples was also < 2% of transcripts; however, in NTCCB samples – although there was an increase in TNC-14/16 expression – the increase was small, ranging from just over 2% to > 5% of transcripts (Figure 3.6A). Expression of TNC-AD1 was detected in > 10% of total TNC transcripts. Moreover, percent levels increased in NTCCBs, with ~ 20% of transcripts containing AD1. Expression of TNC-AD2 in all positive samples was very low (< 2% of transcripts) in normal and NTCCB samples (Figure 3.6B).
B)

No significant increase in percentage of TNC isoforms was detected in NTCCBs compared to myoepithelial cell from normal breasts. A slight increase was observed; however, this amounted to only a few percent. Normal = myoepithelial cells from normal breasts; NTCCB = myoepithelial cells from non-involved tissue from cancer containing breasts. A) TNC-9/16 and TNC-14/16. B) TNC-AD1 and TNC-AD2 expression.

3.3.4 Expression of high MW TNC isoforms is associated with younger patient age, higher grade, negative ER and negative CK14 status

A total of 181 samples were analysed for expression of total TNC, 9/16, 14/16, AD1 and AD2. For 139 samples (22 reduction mammoplasties, 5 fibroadenomas and 112 carcinomas) a first round 8/18 PCR product was available from a previous study. This served as a template for detection of all 5 amplicons by nested-qPCR. In these samples a nested PCR approach was necessary as no additional cDNA was available for analysis. In order to relate data from the 8/18 analyses to direct expression it was necessary to estimate an average difference in detection between the two assays using 8/18 PCR products from HBL-100, MDA-MB-231, Hs578T and SK-Mel-28 cell lines (from a previous study) as calibrators (Table 3.3).
### Table 3.3: Average Ct values and average differences in Ct between 8/18 and direct amplification in cell lines

<table>
<thead>
<tr>
<th>Isoform</th>
<th>HBL-100</th>
<th>MDA-MB-231</th>
<th>Hs578T</th>
<th>SK-Mel-28</th>
<th>Average Ct</th>
<th>Average Ct difference compared to direct amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total TNC</td>
<td>16.34</td>
<td>13.89</td>
<td>19.53</td>
<td></td>
<td>16.59</td>
<td>10.51</td>
</tr>
<tr>
<td>TNC-9/16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>22.79</td>
<td>8.02</td>
</tr>
<tr>
<td>TNC-14/16</td>
<td>22.74</td>
<td>21.46</td>
<td>26.41</td>
<td></td>
<td>23.54</td>
<td>5.62</td>
</tr>
<tr>
<td>TNC-AD1</td>
<td>23.95</td>
<td>26.65</td>
<td>28.39</td>
<td></td>
<td>26.33</td>
<td>3.93</td>
</tr>
<tr>
<td>TNC-AD2</td>
<td>26.58</td>
<td>28.09</td>
<td>28.46</td>
<td></td>
<td>27.71</td>
<td>2.50</td>
</tr>
</tbody>
</table>

### 3.3.4.1 Detection of TNC expression in breast tissue

Total TNC expression was detected in all reduction mammoplasties and normal breast organoids, fibroadenomas and 98.5% (132 of 134) of invasive carcinomas. TNC-9/16 expression was detected in 66.7% (22 of 33) of normal breast tissue samples (however, all organoids were negative); 57.1% (8 of 14) of fibroadenomas and 85% (114 of 134) of invasive carcinomas. TNC-14/16 expression was detected in 78.8% (26 of 33) of normal samples; all fibroadenomas and 97.7% (131 of 134) of invasive carcinomas. Thirty-one percent (42 of 134) of invasive carcinomas, 28.5% (4 of 14) of fibroadenomas and 36.4% (12 of 33) of normal samples expressed AD1-containing isoforms; 23% (31 of 134) of invasive carcinomas, 28.5% (4 of 14) of fibroadenomas and 12% (4 of 33) of normal samples expressed AD2-containing isoforms and 14% (19 of 134) of invasive carcinomas; 7% (1 of 14) of fibroadenomas and 3% (1 of 33) normal samples expressed isoforms containing both AD1 and AD2. Table 3.4 shows the total number of positive samples for both 8/18 PCR products and direct amplification from mRNA.
Table 3.4: Detection of total TNC and high MW TNC isoforms by 8/18 nested PCR and direct amplification from isolated mRNA

<table>
<thead>
<tr>
<th>Samples</th>
<th>Normal</th>
<th>Fibroadenomas</th>
<th>Invasive Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total TNC</td>
<td>+</td>
<td>33</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TNC-9/16</td>
<td>+</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>TNC-14/16</td>
<td>+</td>
<td>26</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>TNC-AD1</td>
<td>+</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>21</td>
<td>10</td>
</tr>
<tr>
<td>TNC-AD2</td>
<td>+</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>29</td>
<td>10</td>
</tr>
<tr>
<td>TNC-AD1 &amp; AD2</td>
<td>+</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>32</td>
<td>13</td>
</tr>
<tr>
<td>Total Samples</td>
<td>33</td>
<td>14</td>
<td>134</td>
</tr>
</tbody>
</table>

Figure 3.7: Venn diagram of high MW TNC isoform expression in invasive carcinomas

Sizes of rectangles are not indicative of sample number. Samples negative for high MW TNC isoform expression are represented by the “negative” total. U = total number of samples (the “universe” number).
The Venn diagram (Figure 3.7) shows that TNC-14/16 was expressed exclusively in 10 of 134 samples, whereas all other samples expressed high MW isoforms, but TNC-14/16 was always present when these were expressed. Furthermore, TNC-9/16 and TNC-14/16 were co-expressed in nearly half of the samples, with all high MW isoforms being present in 17 of 134 samples.

3.3.4.2 Expression of TNC in carcinomas, fibroadenomas and normal tissues
Analysis of variance showed that expression of total TNC, TNC-9/16 and TNC-14/16 was significantly increased in invasive carcinomas compared to normal tissues ($p = 0.004$, $p = 0.004$ and $p = 0.002$ respectively). Expression of TNC-9/16 was also significantly increased in invasive carcinomas compared to fibroadenomas ($p = 0.044$). No significance was found in expression of TNC-AD1 or TNC-AD2. Furthermore, no significant difference was found in fibroadenomas compared to normal tissue for total TNC, TNC-14/16, TNC-AD1 or TNC-AD2 (Figure 3.8).
Figure 3.8: Expression of TNC in normal tissues, fibroadenomas and invasive carcinomas according to Ct values from (RT)-qPCR

Box and whisker plots showing the Ct value lower and upper quartiles and the line represents the median. The whiskers connect the highest and lowest Ct values that are not outliers. The ‘o’ and ‘*’ above and below each box represents outliers and p-values are indicated on the plots.

A) Total TNC  
B) TNC-AD1  
C) TNC-AD2  
D) TNC-9/16  
E) TNC-14/16

p = 0.004  
p = 1.000  
p = 0.563  
p = 1.000  
p = 1.000  
p = 1.000  
p = 1.000  
p = 1.000  
p = 1.000  
p = 1.000  
p = 0.004  
p = 1.000  
p = 0.044  
p = 1.000  
p = 0.002  
p = 0.248  
p = 1.000
3.3.4.3 *Comparison of TNC expression with clinicopathological features of carcinomas*

Expression levels of total TNC and high MW TNC isoforms were related to clinicopathological features of the carcinomas including tumour grade, patient age, lymph node status, steroid hormone receptor and cytokeratin 5/6 and 14 status, and basal phenotype. In this study, a range of putative basal markers were analysed and samples defined as either triple negative (ER-/PR-/Her2- (termed “basal 1”)) or CK 5/6 and/or CK14 positive (termed basal 2). For total TNC, TNC-9/16 and TNC-14/16 *t* tests were used for statistical analysis. For TNC-AD1 and TNC-AD2, non-parametric Kruskal-Wallis analysis was performed as the data was not normally distributed. For co-expression of AD1 and AD2, Fischer’s \( \chi^2 \) Exact tests were performed. For analysis of TNC expression and tumour grade, Jonckheere-Terpstra analysis was performed.

Expression of total TNC, TNC-9/16, TNC-14/16, TNC-AD1, TNC-AD2 and co-expression of AD1 and AD2 was significantly associated with younger age (\( \leq 40 \) years of age) \( (p = 0.003; p = 0.002, p = < 0.001 p = < 0.001, p = 0.001 \) and \( p = < 0.001 \) respectively) (Figure 3.9). Expression of TNC-AD1 and co-expression of AD1 and AD2 was also significantly associated with negative ER status \( (p = 0.011 \) and 0.032 respectively) and higher grade \( (p = 0.017 \) and 0.019 respectively) and although expression of TNC-14/16 was not found to be significantly associated with ER status, there was a trend towards significance for negative ER status \( (p = 0.053) \).

Expression of total TNC, TNC-9/16 and TNC-14/16 was also significantly associated with negative CK14 status \( (p = 0.003 \) for all) (Figure 3.10). Furthermore, expression of TNC-14/16 was found to be significantly increased in ILCs compared to IDCs \( (p = 0.004) \), whereas co-expression of AD1 and AD2 was found to be significantly associated with IDCs \( (p = 0.025) \).

No significance was found comparing TNC expression with other conventional prognostic parameters such as lymph node status, PR status or either basal phenotype. Table 3.5 - Table 3.7 give detailed summaries of expression of TNC and high MW isoforms and clinicopathological parameters.
Figure 3.9:  Expression of TNC isoforms in relation to patient age

The boxes contain the Ct value lower and upper quartiles and the line represents the median. The whiskers connect the highest and lowest Ct values that are not outliers. The ‘o’ and ‘*’ above and below each box represents outliers and p-values are indicated on the plots.

A) Total TNC

B) TNC-9/16

C) TNC-14/16

D) TNC-AD1

E) TNC-AD2

F) TNC-AD1 and AD2

p = 0.003

p = < 0.001

p = < 0.001
Figure 3.10  Expression of TNC isoforms in relation to ER, CK14 and tumour type

Box and whisker plots illustrating expression of TNC and high MW isoforms of TNC with negative ER and CK14 status as well as increased expression in ILCs. Only significant differences are shown. (A) and (B) show expression compared to ER status, (C) – (E) shows expression compared to CK14 status and (F) shows expression of TNC-14/16 compared against tumour type. The ‘o’ above each box represents outliers and p-values are indicated on the plots.

A) TNC-AD1  

B) TNC-14/16  

C) Total TNC  

D) TNC-9/16  

E) TNC-14/16  

F) TNC-14/16
3.3.4.4  *TNC isoform expression stratified by age*

Expression of TNC isoforms was stratified into 5 year age groups and expression of total TNC, TNC-9/16 and TNC-14/16 was defined as either high, low, or negative. For each isoform, the median Ct value was calculated and samples with Ct values below the median were defined as high, samples with Ct values above the median were defined as low. Samples with Ct values > 40 were negative. Median values were 25.87706591 for total TNC, 32.35777424 for TNC-9/16 and 31.11918291 for TNC-14/16. For TNC-AD1 and TNC-AD2 the median Ct value was 40; therefore, expression was deemed to be either positive or negative.

The majority of high total TNC, TNC-9/16 and TNC-14/16 expressing carcinomas were from patients 31 – 35 years of age. The majority of TNC-AD1 and TNC-AD2 positive samples were from patients aged 26 – 35 (Figure 3.11A – D).

A)  **Total TNC**

![Bar chart showing total TNC expression stratified by age](chart)

- **High expression**
- **Low expression**
- **Negative**
B) TNC-9/16

C) TNC-14/16
D) TNC-AD1 and TNC-AD2

Figure 3.11: Expression of TNC isoforms stratified into 5 year age groups

The majority of high total TNC, TNC-9/16 and TNC-14/16 samples were from patients aged between 31 – 35 years. Similarly, the majority of TNC-AD1 and TNC-AD2 cases were found in patients aged 26 – 35 years. A) total TNC, B) TNC-9/16, C) TNC-14/16, D) TNC-AD1 and TNC-AD2.
### Quantitative analysis of TNC isoform expression | Chapter 3

<table>
<thead>
<tr>
<th>Clinicopathological Feature</th>
<th>TNC isoform expression</th>
<th>P values</th>
</tr>
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Table 3.5: Summary of total TNC, TNC-9/16 and TNC-14/16 expression in carcinomas and clinicopathological features

T-tests were used for statistical analysis of TNC isoform expression and clinicopathological features except tumour grade, which utilised Jonckheere-Terpstra analysis. P-values were calculated for expression levels of each isoform. Light grey shading shows clinicopathological parameters comparing high, low and negative expression (defined in section 3.3.4.4). **p ≤ 0.01; ***p ≤ 0.001. n/k = not known.
### Quantitative analysis of TNC isoform expression

#### Chapter 3

TNC isoform expression and clinicopathological features are summarized in Table 3.6. The table shows TNC-AD1 and TNC-AD2 expression in carcinomas and p-values. Statistical tests used were Kruskal-Wallis test for distribution for TNC-AD expression and all clinicopathological parameters except grade, which was determined by Jonckheere-Terpstra analysis; Fischer’s Exact $\chi^2$ tests were used for analysis of AD1/AD2 positive/negative tumours.

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<th>Clinicopathological Feature</th>
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Table 3.6: Summary of TNC-AD1 and TNC-AD2 expression in carcinomas and clinicopathological features.

Table shows TNC-AD1 and TNC-AD2 expression in carcinomas and p-values. Statistical tests used were Kruskal-Wallis test for distribution for TNC-AD expression and all clinicopathological parameters except grade, which was determined by Jonckheere-Terpstra analysis; Fischer’s Exact $\chi^2$ tests were used for analysis of AD1/AD2 positive/negative tumours. *$p = \leq 0.05$; **$p = \leq 0.01$; ***$p = \leq 0.001$. 

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Table 3.7: Expression of TNC isoforms compared to basal markers

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3.4 **Discussion**

**3.4.1 Limitations of TaqMan assays**

An initial limitation to this study was the design of appropriate TaqMan probes for amplification of specific TNC isoforms. The inventoried total TNC probe (crossing the boundary between exon 17 and 18) was a good choice as this amplifies a portion of the constitutively expressed exons 17 and 18, and will therefore detect all TNC isoforms. Similarly, analysis of TNC-9/16 transcripts only required the design of a probe that would anneal across the boundary of exons 9 and 16, with the forward primer annealing in exon 9 and the reverse primer annealing in exon 16. However, in order to amplify the TNC-14/16 isoform exclusively, one primer needed to anneal across the 9/14 exon boundary and the other needed to anneal across the 14/16 boundary, with the probe annealing in exon 14. This would result in amplification across the whole of exon 14 and produce an amplicon at least 289 bp in length. It is recommended that amplicons for TaqMan amplification are no longer than 150 bp. Moreover, previous studies have shown that amplification of PCR products greater than 270 bp greatly reduces PCR efficiency (Karen Page – personal communication; Niederstätter, 2006). Therefore this approach was not used. Instead, the probe was designed to anneal across the boundary of exons 14 and 16, with the forward primer annealing in exon 14 and the reverse primer annealing in exon 16. This would ensure that transcripts containing exons AD1, AD2 and 15 were not detected. However, the primer/probe set does not discriminate between transcripts containing only exons 14/16 and transcripts containing exons 14/16 as well as exons 10 – 13 (the ‘A’ domains). Previous studies have shown that exons
encoding the ‘A’ domains of the alternatively spliced region are indeed expressed along with exons 14 and 16 (Sriramarao, 1993; Mighell et al., 1997; Bell et al., 1999; Joester & Faissner, 1999; von Holst, 2007) and the possibility that transcripts containing exons 10 – 13 are also amplified using this primer/probe set cannot be ignored, even though there are no other reports confirming this in breast cell lines or breast tissue.

Ideally, this study would have analysed expression of the two novel transcripts containing AD1 (14/AD1/16 and 14/AD1/15/16). However, as with amplification of TNC-14/16, the size of the amplicon produced for each isoform would be too large for efficient PCR. Therefore, it was decided to design a probe that would anneal within AD1. This would show amplification of all AD1 containing transcripts, possibly containing domains previously described (Sriramarao, 1993). Furthermore, there is currently no evidence in the literature showing exons that are expressed along with AD2 in the breast; however, a number of isoforms containing AD2 have been described in oral cancer (Mighell et al., 1997), which include exons 13, 14, AD1, 15 and 16. Therefore it was decided – as with AD1 – to design a primer/probe set that would anneal within AD2.

3.4.2 Expression of high MW isoforms is prevalent in highly invasive breast cell lines

Real-time (RT)-qPCR confirmed the hypothesis that high MW TNC isoforms are prevalent in breast cell lines that have a more invasive capacity. In particular, highly invasive cell lines expressed high levels of transcripts containing exons 14/16, with 9/16 being less prevalent. Analysis of isolated normal breast cells showed that fibroblasts are a major source of TNC-9/16 and TNC-14/16, a finding that confirms previous studies in the group (Adams et al., 2002). In contrast, isolated myoepithelial cells showed low levels of TNC-9/16 and TNC-14/16. Interestingly, the major source of TNC-AD1 and AD2 was myoepithelial, with fibroblasts showing lower levels, not detectable by in-situ hybridisation (Hancox et al. – in preparation). This profile of expression was also seen in HBL-100s, which have myoepithelial characteristics (Gordon et al., 2003). These data show that there are differences in isoform distribution between different cell types as well as during progression. This has not been reported previously; however, other studies have illustrated a change in total TNC distribution during the menstrual cycle (Ferguson et al., 1990) and in other organs, including ovary (Tamura et al., 1993), endometrium (Vollmer et al., 1993) and high levels of TNC in the
myoepithelium are observed during involution and a regressing epithelium (Jones et al., 1995).

Expression of TNC-AD1 in myoepithelial cells isolated from normal breast and some invasive breast cancers and cell lines raises new questions as to its role in normal and malignant tissue. Myoepithelial cells are situated in an ideal location to control luminal epithelial cell function including polarity, electrolyte and fluid flow, and response to endocrine and/or paracrine signals (Lakhani & O'Hare, 2001; Deugnier et al., 2002) as well as to contribute significantly to basement membrane production by expression and deposition of fibronectin, collagen IV, nidogen, and the bioactive laminin (Gusterson et al., 1982; Warburton et al., 1982; Gudjonsson et al., 2002). They have been suggested to have a tumour suppressive role due to their negative effect on tumour cell growth, invasion, and angiogenesis (Sternlicht & Barsky, 1997; Sternlicht et al., 1997; Lakhani & O'Hare, 2001; Deugnier et al., 2002; Jones et al., 2003). Therefore, the expression of TNC-AD1 in myoepithelial cells in the normal breast could play an important regulatory role in normal mammary gland development. However, TNC-AD1 could increase cell invasiveness when expressed by malignant epithelial cells by interfering with cell adhesion via interaction with the integrin co-receptor syndecan-4 (Huang et al., 2001) as part of high MW TNC isoforms.

The previous suggestion by Adams et al. (2002) that TNC-9/16 and TNC-14/16 are exclusively expressed in the stroma was not confirmed in this study. Expression of all four TNC isoforms analysed was identified in several breast adenocarcinoma cell lines, suggesting an epithelial origin and these data correlated with previous studies (Kawakatsu et al., 1992; Lightner et al., 1994; Ishihara et al., 1995; Yoshida et al., 1995; Yoshida et al., 1997; Tokes et al., 2000; Gordon et al., 2003). The differences seen compared to the study by Adams et al. (2002) could be due to either culture conditions of the adenocarcinoma cells (cultured in 10% DMEM, known to contain TGFβ and FCS – see section 1.4.3.2), or to differences in sensitivity of in-situ hybridisation and (RT)-qPCR. Furthermore, although TNC isoform expression was detected at the mRNA level, this does not necessarily imply that it will be translated into protein. Lightner et al. (1994) and Yoshida et al. (1997) showed similar results as chromogenic in-situ hybridisation experiments showed an epithelial origin of TNC mRNA in IDC samples, whereas IHC revealed a diffuse stromal staining of TNC protein, particularly in fibroblasts. However, studies by Tokes et al. (2000) and Ishihara et al. (1995) have
shown TNC protein to be abundant in carcinoma cells at the invasion front of a tumour and can be related to prognosis.

The percentage of high MW TNC isoforms containing exons 14/16 appeared to correlate with the invasive capacity of the cell. Previous studies in our group associated TNC-14/16 and TNC-9/16 with invasion by increasing the invasive capacity of MCF-7 and MDA-MB-231 cells (Hancox et al., 2009). This study supports the previous findings in that high MW isoforms (particularly TNC-14/16) are prevalent in highly invasive cell lines. The breast adenocarcinoma cell lines Hs578T, MDA-MB-231 and MDA-MB-436 have all been shown to have a high invasive capacity (Thompson et al., 1992) and all exhibited high levels of TNC-14/16. Co-expression of TNC-9/16 appeared to have a synergistic effect on tumour cell invasion, as the most invasive of the breast cell lines co-expressed this isoform (i.e. MDA-MB-436 and HBL-100).

Domain D (exon 16) has been shown to dramatically increase neurite outgrowth (Meiners et al., 2001), suggesting that in carcinoma cell migration TNC-9/16 could be important in lamellopodia and filopodia formation, as suggested by Wenk et al. (2000) on a three-dimensional fibrin matrix containing FN/TNC. HBL-100 cells are highly invasive (Gordon et al., 2003) and express TNC-AD1 and AD2 at high levels, but low levels of TNC-14/16. Hs578T cells show expression levels of TNC-14/16 comparable to MDA-MB-231 cells – another highly invasive cell line (Thompson et al., 1992; Gordon et al., 2003) – but also show a high level of AD1 expression (Derr et al., 1997) and invade rapidly through a collagen matrix (Thompson et al., 1992; Tong et al., 1999). Literature available on AD2 is sparse; however, this study has revealed for the first time that TNC-AD2 isoforms are present in the breast and could contribute to a motility promoting environment, as suggested by studies in chicken (Derr et al., 1997; Fischer, 1997a), but due to its low expression in carcinoma cells it may not be as significant as other exons.

3.4.3 Expression of total TNC, TNC-9/16 and TNC-14/16 is up-regulated in breast cancer

The majority of previous studies considered only the fully truncated (short) or large TNC isoforms in breast tissues, and showed that there is a transition from the short to the large isoform during tumour progression (Borsi et al., 1993; Wilson et al., 1996; Kusagawa et al., 1998; Hindermann et al., 1999; Ghert et al., 2001). Previous studies
have also shown that exons 14 and 16 are expressed in different carcinomas (Saga et al., 1991; Borsi et al., 1993; Dorries, 1994; Mighell et al., 1997; Saghizadeh et al., 1998; Adams et al., 2002; Berndt et al., 2006), with an increase in these high MW isoforms being associated with lung carcinoma (Kusagawa et al., 1998), ovarian carcinoma (Wilson et al., 1996), oral squamous carcinoma (Hindermann et al., 1999) and urothelial carcinoma (Berndt et al., 2006). However, these high MW isoforms are not exclusive to malignant tissue and are seen in a range of normal tissues at lower levels (Borsi et al., 1993) and also in normal cell lines (Kawakatsu et al., 1992). TNC isoforms containing exons AD1 and AD2 have been postulated to be tumour specific (Derr et al., 1997; Mighell et al., 1997; Berndt et al., 2006), and along with TNC-9/16 and TNC-14/16, AD1 may contribute to tissue remodelling and a motility promoting environment (Tucker et al., 1994; Fischer et al., 1997). This study showed good correlation with previous studies; however, there were some differences.

This study confirmed the hypothesis that high MW TNC isoforms are up-regulated in breast cancer tissue. Expression of total TNC, TNC-9/16 and TNC-14/16 was shown to be significantly up-regulated in invasive carcinoma tissue compared to control samples when a larger cohort of samples was analysed. However, in a smaller cohort where comparisons could be made to an endogenous control (section 3.3.3), expression of all isoforms was down-regulated in benign tissues relative to normal and carcinoma tissues (except TNC-14/16). This has not been described previously, and could purely be due to the majority of benign samples being negative for expression of TNC-AD1, TNC-AD2 and TNC-9/16 and the use of 40 cycles in order to calculate a –ΔCt. This study showed good correlation with previous studies when using Ct values in a larger cohort of samples. Here, expression of total TNC, TNC-9/16 and TNC-14/16 was up-regulated in invasive carcinomas. High levels of TNC are seen in the stroma of invasive breast carcinoma (Mackie, 1987; Howeedy et al., 1990; Koukoulis et al., 1991), in particular high MW isoforms containing exons 14 and 16 (Adams et al., 2002; Tsunoda et al., 2003). Analysis of the percentage levels of each isoform was also in agreement with previous studies in that high MW isoforms were prevalent within the tissue (Chiquet-Ehrißmann et al., 1986; Koukoulis et al., 1991; Natali et al., 1991; Borsi et al., 1992) as well as a change in isoform profile (Adams et al., 2002); however, no significant increase in the expression of TNC-AD1 or TNC-AD2 was observed. The expression of high MW TNC isoforms in the breast is consistent with carcinoma of other organs, where generally there is an increase in stromal TNC (Koukoulis et al., 1991; Natali et
al., 1991; Shrestha et al., 1996). Moreover, high levels of TNC in the breast are associated with metastases (Jahkola, 1996; Jahkola et al., 1998b). From this evidence it would appear that up-regulation of TNC-9/16 and TNC-14/16 expression has a significant role in the progression of breast cancer.

### 3.4.4 Expression of high MW TNC is associated with younger age, tumour grade, CK14 and steroid-hormone receptor status

Evidence in the literature regarding the prognostic value of TNC is conflicting; however, in this study expression of high MW splice variants was not cancer specific, and in invasive carcinomas they were more commonly detected in younger women (≤ 40 years of age). Due to all of the high MW TNC isoforms analysed in this study being associated with younger age, this would suggest that the TNC molecule is highly susceptible to alternative splicing in younger women with invasive carcinoma, and it is the expression of high MW isoforms that is associated with younger patient age rather than the inclusion of specific domains. Expression of high MW TNC isoforms did not relate to tumour prognostic features such as lymph node status; however, expression of TNC-AD1 was associated with high grade, which is also a feature associated with young patient age (Nixon et al., 1994; Xiong et al., 2001), and negative ER status. Dandachi et al. (2001) associated epithelial TNC expression with high tumour grade and negative ER status (as well as vimentin and Her-2 over-expression), whereas Ioachim et al. (2002) showed an inverse correlation with TNC expression and receptor status but a positive correlation with tumour grade. In some tumour types, such as bladder (Tiitta et al., 1993), liver (Jaskiewicz et al., 1993) and prostate (Xue et al., 1998) there is an association with grade and tumour differentiation, unlike the breast (Tokes et al., 1999) and cervix (Pollanen et al., 1996). In the prostate TNC level inversely correlates with increasing grade, and is related to metastases and poor prognosis. This indicates that TNC may have a protective effect which has also been suggested for skin carcinoma (Anbazhagan et al., 1990), colorectal carcinoma (Sugawara et al., 1991) and the breast (Ishihara et al., 1995). This effect may be isoform specific, with expression of high MW isoforms eliciting any effect produced by the fully truncated isoform. This study was also in agreement with Sholi et al. (1993), who found no correlation with lymph node status. However, they reported no correlation with ER, as did Tokes et al. (1999).
The association of TNC-AD1 and co-expression of AD1 and AD2 with negative ER status, high grade and younger age raises the possibility that these isoforms are indicative of a poor prognosis. Negative ER tumours are more likely to be of higher histological grade, and the patients to have a decreased overall survival depending on age and lymph node status (Parl et al., 1984). Furthermore, BRCA1-related breast cancers are more likely to be ER negative and arise in women under the age of 45 (Loman et al., 1998; Foulkes et al., 2004; Eerola et al., 2008), suggesting that TNC-AD1/AD2 expression could also be indicative of BRCA1 germline mutations in hereditary cancers. Also, due to the high level if TNC-AD1 present in myoepithelial cells (section 3.3.2) and the association of TNC-AD1 with clinicopathological features previously described, TNC-AD1 expression could be indicative of a myoepithelial tumour phenotype as they have been frequently associated with aggressive tumour behaviour (Cattoretti et al., 1988; Domagala et al., 1990a; Domagala et al., 1990b; Peralta Soler et al., 1999) and negative ER status (Perou et al., 2000; Kesse-Adu & Shousha, 2004).

The association of TNC-9/16 and TNC-14/16 expression and negative cytokeratin-14 status suggests that expression of these two isoforms could be predominantly of stromal origin, particularly from fibroblasts. CK14 is an epithelial marker (Chu et al., 2001; Kurzen et al., 2001), furthermore, TNC-9/16 and TNC-14/16 have been shown to be expressed at high levels in the peritumoural stroma of invasive carcinomas by both immunohistochemistry and in-situ hybridisation (Adams et al., 2002). Therefore, it is reasonable to suggest that these isoforms are indicative of a mesenchymal tumour phenotype.

3.5 Conclusion

In conclusion, expression of high MW isoforms of TNC including exons 14, 16, AD1 and AD2 were described in breast carcinomas, normal breast tissues and isolated normal breast cell populations. The expression of high MW isoforms in breast carcinoma cell lines and isolated normal breast cells suggested that the epithelium is an important source of TNC and is not tumour specific. Furthermore, a difference in isoform distribution was seen with stromal fibroblasts being the major source of TNC-9/16 and TNC-14/16 and myoepithelial cells being the principal source of TNC-AD1 and TNC-AD2. In breast cancers, expression of high MW isoforms was significantly associated with younger patient age, negative ER and CK14 status, and high tumour grade but was
not associated with other conventional prognostic indices including lymph node status. A significant up-regulation of TNC-9/16 and TNC-14/16 was also seen in tumour tissue compared to control samples, enhancing the hypothesis that tumours arising in younger women are biologically distinct and expression of TNC is enhanced in tumour tissue.
Chapter 4: Molecular cloning of the additional domain 1 (AD1) and functional studies
4.1 Introduction

Previous studies have associated increased expression of TNC isoforms with cancer progression (Borsi et al., 1992; Tsunoda et al., 2003). Moreover, investigations within our group have shown that two isoforms (9/16 and 9/14/16) in particular are associated with invasion (Adams et al., 2002; Hancox et al. – manuscript submitted). However, AD1 and AD2 (Sriramarao, 1993; Mighell et al., 1997) have not been studied in detail. This thesis focused around AD1, which has been found to be localised to isolated breast cell populations by in-situ hybridisation, where it was detected in myoepithelial cells lining larger ducts and also in the muscle layer of blood vessels (Hancox et al. – in preparation).

4.2 Aims

The aim of this chapter was to investigate the functional significance of TNC isoforms containing AD1 using molecular cloning and transfection studies. The specific objectives were:

1) To identify cell lines that express AD1 by RT-PCR and use high-fidelity polymerases to amplify AD1 from genomic DNA, sequence the exon and clone it into a suitable cloning vector.

2) To use high fidelity DNA recombination/nested PCR methods in order to produce AD1 isoforms identified in established cell lines and use molecular cloning methods to produce mammalian expression plasmids containing TNC-AD1 isoforms.

3) To analyse the function of AD1 containing isoforms by transfection and 2D invasion assays to assess cell invasiveness.

4.3 Results

4.3.1 Isolation and sequencing of AD1

4.3.1.1 RT-nested PCR

RT-nested PCR was used to amplify AD1 containing mRNA transcripts from both HT1080 and MDA-MB-231 cell lines. Nested 8/18 amplification (Figure 4.1) produced a strong band at ~450 bp, corresponding to the truncated isoform of TNC, and weak bands at ~2000 bp, corresponding to the long isoform containing all of the alternatively spliced exons (excluding AD1 and AD2). An ~750 bp amplicon was also detected.
suggesting the fully truncated isoform plus one other exon (possibly exon 16) (Figure 4.2A). Re-amplification of this mixed template using primer combinations RT9 F/AD1R, AD1 F/T16P and AD1 F/AD1 R showed that two isoforms containing AD1 were present. Nested PCR with primer pair AD1 F/AD1 R gave a strong band at 200 bp, confirming the presence of AD1 within the amplicons (Figure 4.2B). Secondly, primer pair RT9 F/AD1 R gave a strong band at ~650 bp, suggesting the presence of an additional exon after exon 9 and before AD1 (Figure 4.2C). Thirdly, primer pair AD1 F/T16 P gave two amplicons at ~300 bp and ~600 bp, suggesting one amplicon containing exons AD1 and 16, and another amplicon containing exons AD1, 15 and 16 (Figure 4.2D).

Figure 4.1: Amplification of AD1 containing isoforms from cell lines

Nested PCR from an 8/18 PCR product was used to amplify AD1 containing isoforms. Schematic shows the method used in order to obtain TNC-AD1 isoforms expressed in cell lines. A) 8/18 amplification of TNC transcripts. B) Nested PCR using primers T9 F/AD1 R and AD1 F/T16 P. C) PCR amplicons from nested PCR primer combinations.
Figure 4.2: RT-nested PCR of cDNA from MDA-MB-231 breast adenocarcinoma and HT-1080 fibrosarcoma cells.

A) PCR amplification using primer combination T8 F/T18 R. Note that HT-1080 4R and 6R are HT-1080 cells stably transfected with the mycHis TNC Vector Only plasmid. B) PCR amplification using primer pair AD1 F/AD1 R to confirm the presence of AD1 within the transcripts. C) PCR amplification using primer pair RT9 F/AD1 R to elucidate which exons are present after exon 9 and before AD1 within the mRNA transcript. D) PCR amplification using primer pair AD1 F/T16 P to elucidate which alternatively spliced exons are present within each transcript after exon AD1.
4.3.1.2 Sequencing of PCR amplicons

Sequencing of the RT9 F/AD1 R PCR amplicon using the AD1 R primer revealed the presence of exon 14 before AD1, as well as the 14:AD1 exon boundaries. Sequencing of the ~300 bp PCR amplicon corresponding to the 14/AD1/16 isoform using primer AD1 F revealed the presence of exon 16 after AD1. Sequencing of the ~600 bp PCR amplicon corresponding to the 14/AD1/15/16 isoform using primer T16 P confirmed the adjacent location of exons AD1 and 15 (Figure 4.3).

Sequencing from primers RT9 F and AD1 F identified the exon boundaries of each terminal of the AD1 sequence (Figure 4.3A – C). In each case, the exon boundaries for AD1 differ from the sequence on the GenBank database (accession number M96686). The sequences identified showed a 1bp deletion at the 5’ terminal and an insertion at the 3’ terminal, with the 5’ most base being an adenine (A) residue, not a guanidine (G) residue and the 3’ most base being a guanidine (G) residue not an adenine (A) residue (as shown in GenBank). This was shown to be the case for sequences derived from both HT1080 and MDA-MB-231 cell lines (Figure 4.4 for consensus sequence and translation). However, the sequence obtained was in agreement with both the genomic alternative and reference assemblies (accession numbers NT_008470 and NW_924573 respectively).

Sequencing of AD1 was repeated twice and this confirmed that the exon is 276 bp in length, encoding 92 amino-acids with a T → C mismatch at position 22 of the AD1 sequence compared to M96686 (GenBank). Analysis using the SNP predictor program (http://www.ncbi.nlm.nih.gov/SNP/snp_blastByOrg.cgi) identified a C → T single nucleotide polymorphism (SNP) present at position 22 (reference SNP 11794797), confirming that AD1 sequences obtained from both cell lines contain the wild-type sequence.

Two mismatches were identified in comparison to exon 15 of the M55618 sequence (GenBank), revealing a deletion at position 4933 and an addition at position 4860 of the M55618 sequence. However, comparison with the genomic Celera sequence showed that the sequences were correct and also in agreement with the consensus mRNA sequence (accession number NM_002610), which results in a 9 amino acid change within domain C compared to the M56618 sequence of LWLHPRASN to SGFTQGHQT from amino acid residue 74 → 82.
Figure 4.3: Sequencing of gel purified PCR amplicons from MDA-MB-231 cells

A) Exon boundary of exon 14 and AD1. Primer used for sequencing was RT9 F. B) Exon boundary of AD1 and exon 15. Primer used for sequencing was AD1 F. C) Exon boundary of AD1 and exon 16. Primer used for sequencing was AD1 F.
Figure 4.4: The consensus sequence of AD1

The consensus sequence of exon AD1 was obtained by sequencing of PCR amplicons derived from MDA-MB-231 breast adenocarcinoma and HT-1080 fibrosarcoma cell line cDNA. The sequence shows some slight differences to the sequence submitted by Sriramarao et al. (1993), with the first nucleotide being an adenine residue instead of a guanidine residue, and the last nucleotide being a guanidine residue. Note that the ‘G’ nucleotide at the start of the sequence (highlighted in blue) is taken from the 3’ end of exon 14 in order to translate the first glutamic acid residue.

```
1  GAAACAAAGCCACACGTTGGGCACGCTAATCTTTAGCAATATTACTCCAAAAAGCTTCAAC
   E P K P Q L G T L I F S N I T P K S F N
59

60  ATGTCATGGGACCACGCTCAAGCTGGGCTTTTTGAAGAGTTATCTAATGAGTGAGTCGCT
   M S W T T Q A G L F A K I V I N V S D A
119

120  CACTCACTGAGTCGTCAGCAATTCACAGTCTCAGGAGATGCAAAGCAAGCTCACATC
   H S L H E S Q Q F T V S G D A K Q A H I
179

180  ACAGGTTCATGAGGAGAACACTTGCTATGCAGTCAGTGCTGGCCAGGACGCACTTGGCAG
   T G L V E N T G Y D V S V A G T T L A G
239

240  GATCCACTCAGAGACCTCACTGCTTTGTCATTACAG
   D P T R P L T A F V I T
276
```
4.3.1.3  AD1 sequence alignment

Alignment of AD1 with all other FNIII-like domains using the EMBL-EBI tools ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2) revealed that AD1 had highest DNA identity with exon 15, and > 50% identity with all other exons of the alternatively spliced region. At the amino acid level, AD1 showed highest identity with domain C (exon 15), with identities ranging from 26 – 35% with all the other domains (Figure 4.5 and Table 4.1).

Table 4.1: Summary of sequence identity between AD1 and other FNIII domains of TNC

<table>
<thead>
<tr>
<th>Exon</th>
<th>Domain</th>
<th>DNA identity (%)</th>
<th>Amino-acid identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>A₁</td>
<td>54.13</td>
<td>27.17</td>
</tr>
<tr>
<td>11</td>
<td>A₂</td>
<td>51.23</td>
<td>26.09</td>
</tr>
<tr>
<td>12</td>
<td>A₃</td>
<td>53.90</td>
<td>31.52</td>
</tr>
<tr>
<td>13</td>
<td>A₄</td>
<td>50.34</td>
<td>30.43</td>
</tr>
<tr>
<td>14</td>
<td>B</td>
<td>55.12</td>
<td>33.70</td>
</tr>
<tr>
<td>AD2</td>
<td>AD2</td>
<td>52.40</td>
<td>29.35</td>
</tr>
<tr>
<td>15</td>
<td>C</td>
<td>56.18</td>
<td>35.87</td>
</tr>
<tr>
<td>16</td>
<td>D</td>
<td>52.82</td>
<td>29.35</td>
</tr>
</tbody>
</table>

Additional, analysis using the BLAST algorithm showed that the amino acid sequence was highly conserved, and shares 59.78% identity with chicken AD1. BLAST analysis also showed sequence conservation with a number of similar sequences in a variety of species including chimpanzee, canine, pig and mouse.
Figure 4.5: Alignment of the amino acid sequences of the FNIII-like alternatively spliced region of tenasin-C

Amino acid sequences of the nine alternatively spliced domains of tenasin-C are aligned with homologous regions in order of percentage identity. Residues identical in at least four of the proteins are shown as black letters on a grey background. The positions of conserved proline (P), tryptophan (W), leucine (L), glycine (G) and threonine (T) residues are shown as white letters on a black background. The additional leucine amino acid residue (L) present in AD1 is represented in bold type with an arrow above.
4.3.2 Step-wise molecular cloning of recombinant AD1 isoforms

4.3.2.1 Amplification of exon AD1 and cloning into TOPO

Amplification of AD1 from genomic DNA using AccuPrime Pfx high fidelity polymerase and TNC AD1 F/R primers was successful and produced a single specific band 276 bp in length. The AD1 sequence was then ligated into the pcDNA3.1/V5-His-TOPO cloning vector using methods described in section 2.5.1. PCR using TOPO F/TOPO R primers confirmed the presence of AD1 in the TOPO vector with bands of expected sizes produced on an agarose gel (Figure 4.6). This plasmid was subsequently named TOPO AD1.

![Image of gel showing amplification and cloning into TOPO vector]

**Figure 4.6:** Amplification of AD1 and cloning into TOPO vector

A) AD1 amplified from genomic DNA. B) PCR analysis showed successful ligation of AD1 into TOPO. DNA ladder sizes are indicated on the left hand side of the gel.
4.3.2.2 DNA recombination/nested PCR to produce TNC-14/AD1/16 and TNC-14/AD1/15/16

A DNA recombination/nested PCR strategy was employed using AccuPrime Pfx DNA polymerase in order to generate the TNC sequences 14/AD1/16 and 14/AD1/15/16 (Figure 4.7 and Figure 4.8 for schematic).

Generation of the TNC sequences described above by DNA recombination/nested PCR was successful (Figure 4.9) with primers producing PCR amplicons 1935 bp and 2208 bp in length respectively.
Figure 4.7: DNA recombination/nested PCR for TNC-14/AD1/16 generation

A schematic showing the part FNIII-like exons of the constructs used as a template for DNA recombination/nested PCR in order to incorporate AD1 in between exons 14 and 16. Predicted annealing positions of primers utilised to produce the sequence in each round of PCR are indicated by arrows with primer names given.

(Continued on page 121)
(Continued from page 120)

C) $BC/I_{ten1}$

D) $S/I_{ten1}$

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Figure 4.8: DNA recombination/nested PCR for TNC-14/AD1/15/16 generation

A schematic showing part of the FNIII-like exons of the constructs used as a template for DNA recombination/nested PCR to incorporate AD1 in between exons 14 and 15. Predicted annealing positions of primers utilised to produce the sequence in each round of PCR are indicated by arrows and primer names are given.

(Continued on page 123)
(Continued from page 122)

C) $BC/1\text{ ten1}$

D) $TNC-14/AD1/15/16$
Figure 4.9: Production of recombinant AD1 isoforms by ligation/nested PCR

A DNA recombination/nested PCR strategy was undertaken to generate recombinant AD1 sequences. Products were visualised by agarose gel electrophoresis. Each lane shows the PCR products amplified by the BClI ten1 and SfiI ten1 primer pairs. DNA ladder marker sizes on the left hand side of the gel picture. A) Illustrates the 14/AD1/16 PCR amplicon and B) Illustrates the 14/AD1/15/16 PCR amplicon.
4.3.2.3 **TOPO cloning of TNC-14/AD1/16 and TNC-14/AD1/15/16 sequences**

TNC-14/AD1/16 and TNC-14/AD1/15/16 were cloned into the pcDNA3.1/V5-His-TOPO vector and transformed into TOP10 competent *E. coli* cells.

Once plasmid DNA had been extracted, PCR using T8 F/T18 R primers confirmed the presence of recombinant AD1 sequences with bands of expected sizes at 1265 bp and 1538 bp in length for TNC-14/AD1/16 and TNC-14/AD1/15/16 respectively (Figure 4.10). The sequences were named TOPO 14/AD1/16 and TOPO 14/AD1/15/16.

Sequencing of TOPO 14/AD1/16 and TOPO 14/AD1/15/16 revealed that AD1 had been incorporated at the correct position with no mismatches at any point compared to the M55618 GenBank sequence and exon 15 was in agreement with the genomic reference sequence (accession number NW_924573).
Figure 4.10: Generation of 14/AD1/16 and 14/AD1/15/16 clones in TOPO vector
Schematic of the 8/18 PCR reaction and successful generation of TOPO 14/AD1/16 and TOPO 14/AD1/15/16 as analysed by agarose gel electrophoresis. Figure shows part of the FNIII alternatively spliced domain present in the construct. DNA molecular weight marker indicated on the left of the gel.
4.3.2.4 Production of unmethylated TOPO 14/AD1/16 and TOPO 14/AD1/15/16

In order to introduce the recombinant AD1 sequences into the fully truncated isoform of TNC, it was essential to transform both recombinant AD1 sequences in TOPO and also the S/pBlueScript plasmid (containing the entire fully truncated TNC coding sequence carried on the pBlueScript vector) into dam/dcm (-) SCS110 chemically competent *E. coli* cells. This step was necessary as the sequences were to be restricted out of the TOPO vector using unique *Bcl*I and *Sfi*I sites and *Bcl*I is known to be sensitive to DNA methylation by dam methylase.

Transformation of cells with these plasmids produced a large number of colonies for all constructs. PCR using T8 F/T18 R primers confirmed successful transformation with expected band sizes at 1265 bp and 1538 bp for TOPO sequences 14/AD1/16 and 14/AD1/15/16 respectively and 440 bp for S/pBlueScript (Figure 4.11).

![Figure 4.11: 8/18 PCR amplification of unmethylated TOPO and S/pBlueScript plasmids](image_url)

An 8/18 PCR was performed on plasmid DNA that had been transformed into SCS110 cells in order to produce unmethylated DNA. A) PCR confirmed the presence of the fully truncated isoform in pBlueScript. B) PCR also confirmed the presence of TOPO 14/AD1/16 and TOPO 14/AD1/15/16. DNA ladder sizes are indicated on the left hand side of the gel.
4.3.2.5 Generation of TNC-14/AD1/16/pBlue and TNC-14/AD1/15/16/pBlue plasmids

In order to introduce recombinant AD1 sequences into the fully truncated TNC sequence carried on the pBlueScript vector, unmethylated recombinant AD1 plasmid DNA and the unmethylated S/pBlueScript plasmid were restricted using the BClI and SfiI restriction endonucleases. These enzymes restrict the TNC gene at unique sites within the gene (BClI – 2594/2598; SfiI – 3572/3569, located in exons 6 and 19 of the fully truncated sequence respectively). Moreover, the pBlueScript vector was used as it does not contain the BClI or SfiI restriction sites; therefore, this vector was ideal for efficient cloning.

Analysis of restriction by agarose gel electrophoresis revealed that restriction of each plasmid was successful. The restricted sequences were then purified and the S/pBlueScript fragment was treated with shrimp alkaline phosphatase (SAP). Restricted TOPO 14/AD1/16 and TOPO 14/AD1/15/16 sequences were then ligated into the restricted S/pBlueScript plasmid. PCR using T8 F/T18 R and T25 F/T7 R primers revealed that ligation was successful with bands of expected sizes (Figure 4.12). These plasmids were hence called TNC-14/AD1/16/pBlue and TNC-14/AD1/15/16/pBlue respectively (see Appendix I for complete TNC sequences containing AD1).
Figure 4.12: Ligation of TNC-AD1 sequences into S/pBlueScript for cloning

PCR was performed on transformed DH5α cells to confirm successful ligation of the TNC AD1 sequences into the S/pBlueScript expression plasmid. **A)** PCR using T8 F/T18 R primers confirmed the presence of the AD1 sequences. **B)** PCR using T25 F/T7 R primers confirmed the AD1 sequence had successfully ligated into the S/pBlueScript sequence due to the presence of bands amplified from exon 25 of the TNC sequence and the T7 promoter sequence of the plasmid. DNA molecular weight markers are indicated on the left hand side.
4.3.2.6 **Generation of pCMV TNC-14/AD1/16 and pCMV TNC-14/AD1/15/16 plasmids**

*Not*I and *Sal*I fragments of TNC-14/AD1/16/pBlue and TNC-14/AD1/15/16/pBlue were recloned into S/pCMV Script (see Table 2.9 for plasmid detail) to generate the final expression constructs for both 14/AD1/16 and 14/AD1/15/16.

PCR using T8 F/T18 R and pCMV-MCS F/TN 5’ primers revealed successful ligation with bands of expected sizes (Figure 4.13). These plasmids were named pCMV TNC-14/AD1/16 and pCMV TNC-14/AD1/15/16 respectively. Figure 4.14 summarises the step-wise cloning process and Figure 4.15 illustrates the FNIII domains of the final constructs.
PCR was performed on transformed DH5α cells to confirm successful ligation of the TNC AD1 sequences into the S/pCMV Script expression plasmid.  

A) PCR using T8 F/T18 R primers confirmed the presence of the TNC-14/AD1/16 sequence.  

B) PCR using T8 F/T18 R primers confirmed the presence of the TNC-14/AD1/15/16 sequence.  

C) PCR using pCMV/MCS and TN 5’ primers confirmed the AD1 sequences had successfully ligated into the S/pCMV Script.
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Figure 4.14: Summary of step-wise molecular cloning of TNC-14/AD1/16 and TNC-14/AD1/15/16 isoforms

Schematic representation of the step-wise molecular cloning and production of tenascin-C sequences containing exon AD1. AD1 isoforms were introduced into the TOPO vector (1) and restricted using BClI and SfiI (2), the fully truncated (short) isoform was also restricted using the same nucleases (3). The TNC-AD1 sequence was ligated into the restricted S/pBlueScript (4) and (5) using T4 DNA ligase and then restricted by NotI and SalI (6) to introduce the sequence into the pCMV Script vector (7).
Figure 4.15: AD1 containing TNC sequences generated by molecular cloning

TNC-AD1 sequences were produced by recombination/nested PCR in order to introduce exon AD1 at the correct position of the sequence. A) A schematic representation of the complete domain structure of the human TNC polypeptides highlighting the TA-; EGLF-; FNIII- and FN like domains. B) Schematic diagram of the TNC FNIII-like repeats of TNC-14/AD1/16 and TNC-14/AD1/15/16 produced within this project illustrating the position of AD1 within the sequence.
4.3.3 Functional studies of cloned constructs

The function of AD1 containing transcripts was investigated in MCF-7 cells (TNC null) using 2D invasion assays and compared to other TNC isoforms (TNC Short, TNC-9/16, TNC-9/14/16) and a vector only control (TNC Vector Only) (see Figure 2.4 for schematic of these isoforms).

4.3.3.1 Expression of pCMV TNC-14/AD1/16 and pCMV TNC-14/AD1/15/16 in-vitro

TNC-14/AD1/16 and TNC-14/AD1/15/16 carried on pCMV Script (hence called TNC-14/AD1/16 and TNC-14/AD1/15/16) were transfected into MCF-7 human breast adenocarcinoma cells and RNA and protein expression confirmed by RT-PCR, immunocytochemistry and Western blotting. For detection of TNC protein by immunocytochemistry, the monoclonal BC-24 anti-TNC antibody was used along with Fast Red detection. For Western blotting, the polyclonal H-300 anti-TNC antibody was used along with ECL detection. Figure 4.16 shows the binding domains of both antibodies. RT-PCR was performed using T8 F/T18 R primers.

Transfection of MCF-7s with TNC plasmids was performed using Fugene HD transfection reagent, with cells incubated in transfection media for 6 hrs and then incubated O/N in complete media. For production of conditioned media, cells were washed and incubated in serum free DMEM for 48 hrs. Cell lysates and conditioned media were then analysed for protein by Western blot and an aliquot of cells were also taken for RNA and immunocytochemistry.

RT-PCR confirmed expression of each isoform in transfected MCF-7 cells at the RNA level, with PCR amplicons of expected size demonstrated (Figure 4.17A). At the protein level, Western blot analysis of conditioned media and cell lysates detected TNC-14/AD1/16 but not TNC-14/AD1/15/16 (Figure 4.17B). Repeat transfections detected
TNC-14/AD1/16 but not TNC-14/AD1/15/16. Conversely, immunocytochemistry revealed that transfected cells were positive for both TNC-14/AD1/16 and TNC-14/AD1/15/16 (Figure 4.17C).

Figure 4.17: Transfection of MCF-7 cells with recombinant AD1 isoforms

Recombinant AD1 plasmids were transfected into MCF-7 cells to examine mRNA transcription and protein translation. A) RT-PCR using T8F/T18 R primers. B) Western blot analysis of protein in conditioned media (left panel) and cell lysates (right panel). C) Immunocytochemistry of transfected MCF-7s using BC-24 antibody and Fast Red detection.
4.3.3.2 Optimisation of 2D invasion assays

In order to analyse the effects different TNC isoforms have on carcinoma cell invasion, a real-time 2D invasion assay using the BMG FLUOstar plate reader was performed instead of the traditional 2D invasion where cells are fixed after 48 hrs, stained and counted. This protocol uses fluorescence detection of fluorescently stained cells to give raw, quantitative data which is then manipulated to produce an invasion curve from time 0. Cells were transfected with TNC plasmids and incubated for 6 hrs in transfection media. Cells were then incubated in complete DMEM O/N before being used the next day for the invasion study (see section 2.6.10 for method). Readings were taken every 2 hrs for a total of 48 hrs.

First, the 2D invasion assay had to be optimised. This was done using highly invasive MDA-MB-231 cells (Thompson et al., 1992) and MCF-7 cells. Previous studies in our group have shown that MDA-MB-231 and MCF-7 cells invade optimally at a concentration of $5 \times 10^4$ cells/insert and $1 \times 10^4$ cells/insert respectively, and these concentrations were used throughout. Furthermore, although hfff$_2$ conditioned media has been used previously in 2D invasion assays, which used a Boyden chamber (Gordon et al., 2003), fluorescence decreased when conditioned media was used as the chemotactic source in the plate reader assay (Rachael Hancox – personal communication, Figure 4.18).

![Figure 4.18: MCF-7 invasion using hfff$_2$ conditioned media and 10% DMEM as the chemotactic source (supplied by Rachael Hancox)](image-url)
Complete DMEM plus FCS (the concentration of which was optimised in this study) was used instead. In addition, in order to obtain reproducible results an initial incubation period of 4 hrs after seeding of cells into the upper chamber was performed with no concentration gradient present. This gave the cells time to settle upon the Matrigel barrier so that all cells were ready to invade once the concentration gradient was introduced (Rachael Hancox – personal communication).

Also of importance when using the protocol was the fluorescent dye used for detection. Previous optimisation experiments in our group showed that the use of the nucleic acid binding dye SYTO 82 was not suitable for this assay (Rachael Hancox – personal communication), unlike other studies (Hart, 2006) as this caused cell death when used at the recommended concentration, possibly through inhibition of DNA replication (data not shown). This was surprising as SYTO 82 has been shown to have little or no effect on DNA amplification in a real-time PCR environment (Gudnason et al., 2007). Furthermore, SYTO 82 labelling is inconsistent, and extensive optimisation using this dye is required in order to find the optimum concentration and method of staining. Therefore, DiIC(12)$_3$ was used for cell staining as it is less cytotoxic, incorporates into the lipid membrane easily, is highly photostable and highly fluorescent. Moreover, once applied to cells, the dye diffuses laterally within the plasma membrane (http://www.invitrogen.com) and has been used successfully in a number of studies (Ledley et al., 1992; Kawasaki et al., 2000).

2D invasion assays using MDA-MB-231 and MCF-7 breast adenocarcinoma cells were first optimised by varying the concentration of Matrigel (200 µg/ml, 50 µg/ml and 10 µg/ml) and FBS in the chemotactic source (10%, 5%, 1% and serum free (0%)).

An initial experiment using MDA-MB-231 cells showed highest invasion with an FBS concentration in the chemotactic source of 10%, and the Matrigel barrier at a concentration of 10 µg/ml. Unfortunately due to time limitations, this experiment was performed once using only one well per parameter. Invasion of the cells decreased with increasing concentrations of Matrigel and decreasing concentrations of FBS within the chemotactic source (Figure 4.19A – C). A further experiment using MDA-MB-231 and MCF-7 cells gave similar results; however, invasion of MDA-MB-231 cells was much greater than the optimisation experiments, even though the variation between replicates was very small. Invasion of MCF-7 cells through the Matrigel barrier gave a similar
profile of invasion using different concentrations of FCS in that 10% FCS produced the highest invasion levels, but was less pronounced than MDA-MB-231 cells (Figure 4.20). Therefore, subsequent experiments used a Matrigel barrier concentration of 10 µg/ml and 10% FCS in the chemotactic source.
Figure 4.19: 2D invasion of MDA-MB-231 cells through a Matrigel barrier

Increasing concentrations of Matrigel and FBS were used to assess cell invasion and determine optimal conditions for the invasion protocol. Fluorescence values were measured in real time every 2 hours. Graphs represent the difference in fluorescence from time 0. A – C show invasion of MDA-MB-231 cells through a Matrigel barrier of varying concentrations and FCS at varying concentrations within the chemotactic source.

A) 10 μg/ml Matrigel

B) 50 μg/ml Matrigel
C) 200 µg/ml Matrigel
Figure 4.20: Optimised 2D invasion of MDA-MB-231 and MCF-7 cells through a 10 µg/ml Matrigel barrier

Increasing concentrations of FBS were used with 10 µg/ml Matrigel as the invasion barrier to assess cell invasion and determine optimal conditions for the invasion protocol. Assay was performed in duplicate wells. Fluorescence values were measured in real time every 2 hours. Graphs represent the difference in fluorescence from time 0.

A) MDA-MB-231

B) MCF-7
Previous experiments in our group have significantly associated TNC isoforms containing exons 16 and 14/16 with increased carcinoma cell invasion (Hancox et al., 2009). MCF-7, T-47D and MDA-MB-231 cells transfected with TNC isoforms TNC Short, TNC Long, TNC-9/16 and TNC-9/14/16 showed that cells transfected with TNC-9/16 and TNC-9/14/16 significantly increased cell invasion (using a traditional 2D invasion assay) compared to an untransfected control, an effect that was abrogated by addition of BC-24 anti-TNC antibody. Furthermore, fibroblasts transfected with TNC isoforms described above and co-cultured in the lower chamber of invasion assays with MCF-7 cells showed that TNC-9/16 and TNC-9/14/16 significantly increased invasion. This effect was also abrogated by addition of BC-24 blocking antibody.

In order to extend these data, TNC isoforms described above (with the exception of TNC-Long) were transfected into MCF-7 cells and analysed using the optimised protocol described in section 4.3.3.2. In addition, cells were transfected with the newly generated TNC-14/AD1/16 clone to assess whether this isoform has a similar effect on invasion as TNC-9/16 and TNC-14/16.

4.3.3.3 Direct effect of TNC isoforms on breast carcinoma cell invasion

Immunocytochemistry using the BC-24 anti-TNC antibody showed that > 30% of cells were successfully transfected (by visual estimation) and all TNC isoforms were transfected at a similar level (Figure 4.21). In the 2D invasion assays Vector Only controls showed a similar level of invasion to untransfected MCF-7s; however, Generalised Univariate analysis revealed that TNC Vector Only significantly increased cell invasion ($p = 0.006$). Cells transfected with TNC-9/14/16 and TNC-14/AD1/16 significantly increased cell invasion compared to untransfected cells ($p < 0.001$), showed the highest level of invasion and displayed similar profiles that were not significantly different from each other ($p = 1.000$). Cells transfected with TNC Short showed slightly higher invasion than untransfected cells (though this was significant ($p = < 0.001$)), with cells transfected with TNC-9/16 significantly increasing cell invasion ($p = < 0.001$) and showing higher invasion than with TNC Short but lower than with TNC-9/14/16 and TNC-14/AD1/16 (Figure 4.22). This experiment was performed once using duplicate wells per isoform. Transfection of cells was performed once.
Figure 4.21: Transfection efficiency of MCF-7 breast carcinoma cells transfected with cloned TNC isoforms

MCF-7 breast adenocarcinoma cells were transiently transfected with TNC isoforms TNC-Short, TNC-9/16, TNC-9/14/16, TNC-14/AD1/16 and TNC Vector Only and stained using the BC-24 anti-tenascin-C antibody. Red cells represent TNC positive cells. Magnification is x40.
Figure 4.22: Real-time analysis of TNC transfected MCF-7 cell invasion

MCF-7 cells were transfected with TNC isoforms and utilised in a modified Boyden chamber assay. The graphs shows real-time analysis of increasing invasion compared to time 0. Error bars represent standard error of the mean (2 replicates).
4.4 Discussion

4.4.1 Molecular cloning of AD1 and sequence analysis

Sriramarao and Bourdon (1993) have shown that unlike all other FNIII-like repeats in the alternatively spliced region, AD1 was 276 bp in length, instead of 273 bp, a finding confirmed by this study. Moreover, previous studies in our group have shown that there are two intermediate sized isoforms containing AD1 expressed in breast cancers (14/AD1/16 and 14/AD1/15/16) and cell lines (Hancox et al. – in preparation). Sequence analysis of AD1 from both MDA-MB-231 and HT-1080 cells revealed slight differences in the sequence compared to the GenBank AD1 sequence. BLAST analysis confirmed that the sequence obtained in this study was wild-type AD1. Initial cloning experiments using the sequence submitted to GenBank (M96686) by Sriramarao and Bourdon (1993) resulted in a stop codon being produced within AD1 due the presence of a guanidine residue at the 5’ end of the sequence, significantly hindering progression of the functional studies due to the synthesis of clones based on this sequence. Further analysis of the AD1 sequence obtained in this study revealed the presence of a SNP, 22 bases from the 5’ end. This results in a non-synonymous Thr \( \rightarrow \) Met amino acid change. This could affect the hydrophobic properties of the domain due to the presence of a non-polar sulphur-containing side chain on methionine. Methionine prefers to be buried in hydrophobic cores and is fairly non-reactive, thus is rarely involved in protein function but can be quite susceptible to oxidation by reactive oxygen species (Hoshi & Heinemann, 2001) and could affect TNC potency during inflammation. Although there is no literature available regarding the functional significance of this SNP, 62 SNPs have been discovered within the TNC gene and one coding SNP within domain D (Leu1677Ile) of the alternatively spliced region has been shown to be associated with adult asthma (Matsuda et al., 2005).

Sequence alignment of AD1 showed that this domain has highest identity to exons 15 and 14 at both the DNA and protein level. Furthermore, BLAST analysis showed that the exon is conserved across a variety of species from mouse to man. The high sequence identity to exon 15 may suggest that these exons have similar function. Previous studies have shown that splicing of exon 15 in normal tissue is rare (Carnemolla et al., 1999; El-Karef et al., 2007), but exon 15 is strongly expressed as part of higher MW isoforms (Bell et al., 1999; Joester & Faissner, 1999; Silacci, 2006). Moreover, domain C (exon 15) has been shown to direct neurite elongation (Meiners et
al., 1999; Liu et al., 2005) as well as being regarded as a marker of vascular proliferation in cerebral cavernomas (Viale et al., 2002) and piecemeal necrosis activity in patients with chronic hepatitis C (Tanaka et al., 2006). This suggests that domain C has a role in the development of a tumour promoting environment, and due to AD1’s high identity with this exon this further enhances the hypothesis that AD1 plays a similar role. AD1 also showed high sequence identity with exon 14, and is expressed at high levels in invasive carcinomas (Adams et al., 2002).

Sequencing of AD1 revealed no RGD sequence within the domain. The RGD sequence motif is an essential cell attachment site in fibronectin and is critical in integrin binding (Akiyama et al., 1995). TNC shows RGD-dependent cell adhesion activity (Bourdon & Ruoslahti, 1989), mediated by the 3rd FNIII-like repeat (Joshi et al., 1993; Jang et al., 2004a). The alternatively spliced region of TNC has also been shown to inhibit cell attachment by down-regulation of focal adhesions (Murphy-Ullrich et al., 1991; Huang et al., 2001), suggesting that higher MW isoforms of TNC are involved in cell migration, and invasion. However, when higher MW TNC isoforms are expressed integrin binding is not inhibited, and the down-regulation of focal adhesions has been shown to be mediated by blockage of a co-receptor, syndecan-4 (Huang et al., 2001; Orend et al., 2003). This suggests that AD1 could play a role in the down-regulation of focal adhesions.

Molecular cloning of TNC-14/AD1/16 was successful with Western blotting showing that recombinant protein was being released into the media. However, although sequencing of TNC-14/AD1/15/16 appeared to show no mismatches at any point in the sequence (compared to the reference assembly), the protein did not appear to be translated and released into the media. However, an alternative explanation may be that the antibody used for detection of this particular isoform was not suitable and further optimisation using different antibodies is needed. The BC-24 antibody used for immunocytochemistry has been shown to be unsuitable for Western blotting (Rachael Hancox – personal communication), therefore this antibody cannot be considered. Immunocytochemistry (using the BC-24 antibody that recognises the EGFR region of TNC) showed that the EGFR region part of the TNC molecule was translated but did not show whether the full molecule was translated. Further immunocytochemical experiments using BC-24 in parallel with other antibodies could elucidate whether the entire coding sequence was being translated. Currently, there is no antibody available...
raised specifically against AD1 and the H-300 anti-TNC antibody is not suitable for immunocytochemistry (Rachael Hancox – personal communication). However, there are many other alternative antibodies that could be tested in future studies, such as T2H5 (raised against the full length isoform from a mammary tumour of human origin), E-9 (raised against amino acids 1601 – 1900) and F-17 (raised against the C-terminus) (all from Santa Cruz, USA).

4.4.2 Optimisation of the 2D invasion assay

Previous studies in our group used a modified Boyden chamber assay to obtain an end-point analysis after 48 hr and immunocytochemistry (Gordon et al., 2003) to assess cell invasion. This method only surveyed a single time point and is limited due to potential errors occurring as a result of manual counting of cells. This study used automated fluorescence detection to assess tumour cell invasion in real-time and to confirm whether the results obtained using the end-point assay correlated with findings obtained in this study. The protocol developed in this study was able to take multiple readings over time at a user defined temperature and when enclosed in a gas tight bag provides an environment conducive to cell proliferation, migration and invasion. The BMG FLUOstar OPTIMA fluorescence microplate reader has been used previously (Gubbels et al., 2003; Payne et al., 2005) and provides a reproducible method to assess tumour cell invasion (Hart, 2006). Initial optimisation experiments gave good reproducibility using this method in duplicate wells - but not between assays - with MDA-MB-231 and MCF-7 cells showing similar invasion levels compared to previous studies in our group (Hancox et al., 2009). The use of FBS within this study proved to be a good chemotactic source for induction of tumour cell invasion. Furthermore, the initial high invasion of cells within the first 2 hrs after the introduction of a concentration gradient suggests that this is caused purely by the gradient and any effect TNC has appears to be apparent after a longer period.

In summary, the protocol developed in this study for the assessment of tumour cell invasion gave results comparable to the traditional 2D invasion assay, as TNC-9/16 and TNC-9/14/16 showed the highest levels of invasion in both assays. There was no need for time consuming counting which is vulnerable to observer variation. However, inter-assay variation appeared to be a problem. Compared with traditional invasion systems involving washing, staining and drying steps the protocol is fast, saving time and labour, with statistical analysis being much more powerful due to the possibility of
using multiple time points. However, the protocol requires further optimisation in order to obtain reproducible results between replicate assays.

4.4.3 TNC-14/AD1/16 increases carcinoma cell invasion

Previous studies in our group have shown that TNC-9/16 and TNC-9/14/16 significantly enhanced tumour cell proliferation and invasion both directly and indirectly as a response to TNC transfected fibroblast expression. This effect was dependent on tumour cell interaction with TNC, being abrogated by blocking antibodies to TNC (Hancox et al., 2009). This study confirmed that expression of higher MW isoforms in MCF-7 cells increased their invasive capacity, as did expression of TNC-14/AD1/16. Exogenous TNC has been shown to have an effect on oligodendrocyte motility (TNC-9/14/16 in particular (Kiernan et al., 1996)) glioma cell migration (Deryugina & Bourdon, 1996; Herold-Mende et al., 2002), endothelial cell migration (Chung et al., 1996a) and smooth muscle cell motility (Wallner et al., 2002) as well as breast carcinoma cell invasion (Chiquet-Ehrismann et al., 1989). The current study has shown that different isoforms elicit different effects on cell invasion, an observation that has been discussed by others (Chiquet-Ehrismann, 1990, 1991; Murphy-Ullrich et al., 1991; Chung & Erickson, 1994; Ghert et al., 2001; Puente Navazo et al., 2001; Adams et al., 2002; Herold-Mende et al., 2002). TNC-9/14/16 and TNC-9/16 showed effects in keeping with their role as tumour promoting isoforms (Adams et al., 2002). Moreover, the fully truncated isoform (TNC Short) had no significant effect on tumour cell invasion, consistent with its role as a component of normal basement membrane in many tissues (Jones & Jones, 2000b). Importantly, this study has shown AD1 for the first time to increase carcinoma cell invasion to a level comparable with TNC-9/14/16.

Previous studies in our group have shown that the extent of the effect exogenous TNC expression has on tumour cell invasion is dependent upon the cell type it is expressed in, with MDA-MB-231 showing the greatest increase in invasion when transfected with TNC-9/16 and TNC-9/14/16 (Hancox et al., 2009) (Figure 4.23). The study by Hancox et al. (2009) showed that cell invasion was significantly increased in MDA-MB-231, MCF-7, T-47D and GI-101 cell lines by both TNC-9/16 and TNC-14/16; whereas proliferation of these cells was only influenced by TNC-9/16 and TNC-14/16 in MCF-7s. This suggests that high MW isoforms of TNC greatly influence cell behavior and particularly invasion. A parallel invasion experiment using MDA-MB-231 cells transfected with high MW TNC isoforms was performed in this study; however, due to
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Technical difficulties with the computer linked to the BMG FLUOstar machine it was not possible to retrieve the results and due to time constraints it was not possible to repeat the experiment.

A) Cell invasion

B) Cell proliferation

Figure 4.23: Invasion and proliferation of breast cell lines transfected with TNC isoforms (Hancox et al., 2009)

A) Invasion of MDA-MB-231, MCF-7, T-47D, MDA-MB-468 and GI-101 cell lines transfected with 4 different TNC isoforms constructs and the vector alone. MDA-MB-231 and T-47D cells transfected with TNC-L show higher invasion than controls ($p < 0.05$). All cell lines exhibited significantly higher invasion with TNC-9/16 and TNC-9/14/16 compared to the vector alone (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), other than GI101, which showed significantly higher invasion with TNC-9/14/16 only ($p = 0.01$). B) Proliferation of MDA-MB-231, MCF-7 and T-47D cell lines transfected with 4 different TNC isoforms constructs and the vector alone. MBA-MB-231 cells exhibit no significant changes in proliferation when transfected with any of the isoforms. MCF-7 and T-47D both showed increased proliferation when transfected with TNC-9/14/16 compared to vector alone ($p < 0.05$). MCF-7 exhibited an increase in proliferation with TNC-9/16 compared with vector alone, whereas T-47D’s did not show any changes with TNC-16 but did have a significant ($p < 0.05$) increase in proliferation with TNC-L.

The inclusion of alternatively spliced domains of TNC may alter its biological function through a number of mechanisms. Domain D (exon 16) has been shown to increase neurite outgrowth via interaction with integrin α7β1 (Mercado et al., 2004), whereas domains B/D (exons 14/16) have been shown to interact with the cell adhesion molecule F3/contactin (Rigato et al., 2002). There is no literature regarding the interaction of AD1 with any other ligands, cell adhesion molecules or receptors, though it is unlikely to replicate the action of domain D on neurites, as MCF-7s do not express integrin α7. However, the alternatively spliced region of TNC has been heavily implicated with MMP activity (Jian et al., 2001; Ilunga et al., 2004; Westernoff et al., 2005; Sarkar et al., 2006; Galoian et al., 2007), and could provide a mechanism for AD1 activity.
4.5 Conclusion

In conclusion, RT-PCR analysis of isoform expression in human carcinoma cells confirmed previous reports within our group suggesting the presence of two intermediate sized isoforms containing AD1 situated in between either exons 14 and 16, or in between exons 15 and 16. Molecular cloning of these isoforms was successful; however only TNC-14/AD1/16 was shown to be translated and released into the media. AD1 was confirmed as a FNIII-like domain of TNC which is conserved across a variety of species and has highest identity with the neighbouring domains B and C. This observation suggests that AD1 may have a similar role to these domains in promoting invasion. Furthermore, a SNP was discovered in the AD1 exon that produces a non-synonymous amino-acid change of Thr → Met, which could have functional significance. Moreover, functional studies using 2D invasion assays showed that TNC-14/AD1/16 increases cell invasion to a level comparable to TNC-9/14/16, with invasion of TNC-9/14/16 and TNC-9/16 transfected MCF-7s showing similar invasion to previous reports in our group. The 2D invasion assays, although very preliminary, gave encouraging results, suggesting that AD1 could enhance tumour cell invasion. However, it is important to stress that these data are only preliminary and much more work is needed to optimise the protocol and also to use other breast adenocarcinoma cell lines for comparison to MCF-7s, such as T-47D, MDA-MB-231 and MDA-MB-468.
Chapter 5: Effects of TNC isoforms on differential gene expression in MCF-7 cells
5.1 Introduction

A previous study by Ruiz et al. (2004) analysed global changes of transcript levels in tumour cells grown on a fibronectin milieu in the presence or absence of the long isoform of TNC. They determined that TNC down-regulated tropomyosin 1 (TPM1), which is involved in cell spreading via the formation of actin stress fibres and focal adhesions. Moreover, cells grown on a TNC substratum differentially expressed several genes that have an impact on mitogen-activated protein kinase (MAPK) and Wnt signalling and their pathways such as endothelin receptor type A (ENDRA) and the Wnt inhibitor dickkopf-1 (DKK1). (RT)-qPCR and Western blot analysis revealed TNC up-regulates a number of other proteins involved in signalling pathways and transcription, including c-fos and inhibitor of differentiation (Id2).

5.2 Aims

The aim of this chapter was to test the hypothesis that cells transiently transfected with cloned TNC isoforms (TNC Short, TNC-9/16, TNC-9/14/16 and TNC-14/AD1/16) and cultured on a fibronectin milieu alter global gene expression in different ways. The specific objectives were:

1) To transfec “TNC null” MCF-7 breast adenocarcinoma cells with cloned TNC isoforms TNC Short, TNC-9/16, TNC-9/14/16 and TNC-14/AD1/16 as well as a vector only control, culture the transfected cells on a fibronectin milieu, extract RNA and assess quality by BioAnalysis.
2) To produce cRNA from total RNA using the Affymetrix one-cycle strategy and hybridise to a GeneChip Human Genome U133 plus 2.0 array.
3) To use a regression analysis approach on the data produced to select candidate genes for further analysis.
4) To validate candidate genes using TaqMan (RT)-qPCR methods in cell lines, isolated breast populations and tumour tissue.

5.3 The MIAME criteria

Due to the explosion of studies involving microarray analysis and initial inconsistencies between laboratories and experiments (Van’t Veer et al., 2002; Wang et al., 2005), as well as differences between microarray platforms (reviewed in Maraqa et al., 2006), the proposition of the minimal information about a microarray experiment (MIAME) criteria was proposed (Brazma et al., 2001) by members of the Microarray Gene
Expression Data Society. These criteria are used to produce data in a standard format that enables comparisons of similar experiments and is now being implemented as a requirement for publication by many journals to ensure that high-quality microarray data are available. The six components that should be addressed when publishing data are shown below.

MIAME criteria

- Overall experimental design
  number of hybridisations, replicates and type of comparison being assessed
- Array design
  the commercial name of the array or a detailed description of the platform, nature of probes and names of each probe
- Samples
  source of target, extraction of the nucleic acids and process of labelling
- Hybridisations
  amount of labelled target used, hybridisation buffer, time, volume and temperature plus washing procedures
- Measurements
  original images, raw data and final results following normalisation and comparison between replicates
- Normalisation controls
  details of the type of normalisation performed

5.4 Results

5.4.1 Transfection of MCF-7s

MCF-7 breast adenocarcinoma cells were used in this study as in-vitro they are deemed to be TNC null (Lightner, 1994) and are considered a useful model for analysis of TNC expression.

MCF-7s were transfected and manipulated as described in the flow diagram shown on the following page.
Transfection efficiency was analysed by immunocytochemistry with the BC-24 anti-TNC antibody and H-300 anti-TNC antibody for Western blotting. Immunocytochemistry showed that > 30% of cells were positive for TNC expression (Figure 5.1). Western blot analysis of MCF-7 cell lysates and conditioned media showed that all transfected cells were expressing the desired TNC isoform; however, cell lysates showed lower levels of TNC-9/16 and TNC-14/AD1/16 (Figure 5.2).

Note that TNC Long was not used in this study due to the limited number of GeneChips available. Furthermore, previous studies in our group have shown that TNC-9/16 and TNC-9/14/16 have the greatest effect on MCF-7 invasion in a Boyden chamber assay (Hancox et al., 2009); therefore, the mechanisms by which these isoforms elicit their effects were of great interest.
Figure 5.1: Immunocytochemical analysis of transfection efficiency in MCF-7 cells

MCF-7 cells were transfected with TNC isoforms in preparation for analysis by microarray and (RT)-qPCR validation. A large number of stably transfected cells were expressing TNC isoforms and cells transiently transfected were transfected at >30% efficiency. Magnification is x20.
Figure 5.2:  Western analysis of MCF-7 cells transfected with TNC isoforms

MCF-7 breast adenocarcinoma cells transfected with TNC isoforms were analysed by Western blotting for protein expression. The blot shows extraction from cell lysates (A) and conditioned media (B). Protein molecular weights (kDa) are indicated on the left hand side of the picture. Note that for both blots, the top panel represents the anti-TNC H300 antibody and the bottom panel represents the anti-vinculin antibody used as a loading control.
5.4.2 cDNA microarray

5.4.2.1 BioAnalysis of total RNA

Total RNA extracted from transfected cells was analysed for RNA integrity using the Agilent BioAnalyser 2100. Of the samples analysed by BioAnalysis, all 3 TNC Vector Only samples; 2 TNC Short samples; 2 TNC-9/16 samples; all 3 TNC-9/14/16 samples and all 3 TNC-14/AD1/16 were of sufficient quality to proceed, with all samples giving clear 18S and 28S rRNA peaks on the capillary electrophoresis trace (Appendix II).

5.4.2.2 One-cycle amplification, target labelling and hybridisation

Production of double stranded cDNA by first strand and second strand reverse transcription, as well as in-vitro transcription into cRNA was successful. Fragmentation of 10 µg of cRNA also proved to be successful, with all samples showing RNA species 30 – 200 bp in length. Hybridisation of fragmented cRNA also proved to be successful, with scanned images of the chips giving clear intensity values for spike-in and hybridisation controls when analysed in the GeneChip Operating System (GCOS - Affymetrix, UK) (Appendix II).

Analysis of differential gene expression in MCF-7 breast cells transfected with TNC isoforms was performed using two methods. A regression analysis was performed as outlined in Figure 5.3 in order to obtain the top 200 genes up- or down-regulated in order of relative expression compared to the Vector Only control. The second approach was to use the open source Multiple Experiment Viewer v4.2 software program (TM4, USA) in order to perform statistical analysis and hierarchical clustering of genes.

5.4.2.3 Regression analysis of normalised expression data

Data obtained from the Affymetrix GCOS was initially analysed using the open source software program dChip in order to normalise expression values from .CEL files generated by GCOS. After normalisation, expression values were analysed using the DataAnalysis pack in Microsoft Excel 2007, whereby standard residuals were produced by comparing each chip against each other. Figure 5.3 represents this process, where TNC Vector Only 1 was compared against all other samples (TNC Short, TNC-9/16, TNC-9/14/16 and TNC-14/AD1/16). The process was then repeated using TNC Vector Only 2 and finally TNC Vector Only 3.
Figure 5.3: Schematic representation of regression analysis

Each of the TNC Vector Only expression values was compared against every other chip and a standard residual calculated. An average value was then taken and used as a fold change in expression value.

There are 38,500 genes represented by 54,676 probe sets on the Affymetrix U133 plus 2.0 GeneChips, regression analysis gave fold change data for every single probe on the GeneChip. Overall, expression of genes was similar in cells transfected with TNC-9/14/16 and TNC-14/AD1/16. Table 5.1 and Table 5.2 show the top 10 genes up- and down-regulated by TNC isoform expression using regression analysis and fold change in MCF-7 cells transfected with TNC-14/AD1/16 to determine the ranks.
Table 5.1: Regression analysis data for the top 10 genes up-regulated by MCF-7s transfected with TNC isoforms relative to Vector Only controls

<table>
<thead>
<tr>
<th>Rank</th>
<th>Gene name</th>
<th>Gene ID</th>
<th>Average fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TNC Short</td>
</tr>
<tr>
<td>1</td>
<td>ISG15 ubiquitin-like modifier (ISG15)</td>
<td>NM_005101</td>
<td>1.65</td>
</tr>
<tr>
<td>2</td>
<td>Interferon-induced transmembrane protein 1 (IFITM1)</td>
<td>NM_003641</td>
<td>-0.09</td>
</tr>
<tr>
<td>3</td>
<td>Nuclear casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1)</td>
<td>NM_022731</td>
<td>5.55</td>
</tr>
<tr>
<td>4</td>
<td>Interferon-induced transmembrane protein 3 (IFITM3)</td>
<td>NM_021034</td>
<td>-0.54</td>
</tr>
<tr>
<td>5</td>
<td>Interferon-induced transmembrane protein 2 (IFITM2)</td>
<td>NM_006435</td>
<td>-0.47</td>
</tr>
<tr>
<td>6</td>
<td>Interferon, alpha-inducible 27 (IFI27)</td>
<td>NM_005532</td>
<td>0.05</td>
</tr>
<tr>
<td>7</td>
<td>Metallothionein 2A (MT2A)</td>
<td>NM_005953</td>
<td>1.47</td>
</tr>
<tr>
<td>8</td>
<td>Interferon-induced transmembrane protein 1 (IFITM1)</td>
<td>NM_003641</td>
<td>-0.42</td>
</tr>
<tr>
<td>9</td>
<td>Brain abundant, membrane attached signal protein 1 (BASP1)</td>
<td>NM_006317</td>
<td>-0.53</td>
</tr>
<tr>
<td>10</td>
<td>Beta-2-Microglobulin (β2M)</td>
<td>NM_004048</td>
<td>-2.13</td>
</tr>
</tbody>
</table>

Some of the up-regulated genes outlined in Table 5.1 appear to play an important role in breast cancer. Tissue microarrays have shown ISG15 to be up-regulated in breast carcinoma (Bektas et al., 2008), whereas over-expression of MT2A has been shown to predict chemo-resistance (Yap et al., 2009). The IFITM family is highly inducible by both type I (IFNA/IFNB) and type II (IFNG) interferons (Lewin et al., 1991). Although literature regarding their function in breast cancer is sparse, they have been regarded as a molecular marker in human colorectal tumours (Andreu et al., 2006). Furthermore, over-expression of IFITM1 has been shown to render SNU-216 gastric cells more resistant to natural killer cells (Yang et al., 2005) and promote invasion at early stage of head and neck cancer progression (Hatano et al., 2008). Currently, there is very little literature regarding the function of NUCKS1 and no literature associating it with breast
cancer. Similarly, there is very little literature regarding the function of IFI27. β2M – although used frequently as an endogenous control in quantitative PCR experiments – is believed to be associated with tumour status in many cancers. Strong expression of β2M in oral cavity squamous cell carcinoma has been significantly correlated with a relatively advanced tumour stage, positive nodal status, TNM stage, poor survival and increased cell migration and invasion (Chen et al., 2008).

### Table 5.2: Regression analysis data for the top 10 genes down-regulated by MCF-7s transfected with TNC isoforms relative to Vector Only

<table>
<thead>
<tr>
<th>Rank</th>
<th>Gene name</th>
<th>Gene ID</th>
<th>Average fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TNC Short</td>
<td>TNC-9/16</td>
</tr>
<tr>
<td>1</td>
<td>Solute carrier family 39 (zinc transporter), member 6 (SLC39A6)</td>
<td>AI635449</td>
<td>-12.77</td>
</tr>
<tr>
<td>2</td>
<td>Solute carrier family 39 (zinc transporter), member 6 (SLC39A6)</td>
<td>NM_012319</td>
<td>-9.45</td>
</tr>
<tr>
<td>3</td>
<td>Poly(A) binding protein, cytoplasmic 1 (PABPC1)</td>
<td>AI734929</td>
<td>0.68</td>
</tr>
<tr>
<td>4</td>
<td>Melanophilin (MLPH)</td>
<td>NM_024101</td>
<td>-2.98</td>
</tr>
<tr>
<td>5</td>
<td>Heat shock 70kDa protein 8 (HSPA8)</td>
<td>AB034951</td>
<td>-0.79</td>
</tr>
<tr>
<td>6</td>
<td>Ribosomal protein L36a (RPL36A)</td>
<td>NM_021029</td>
<td>-1.17</td>
</tr>
<tr>
<td>7</td>
<td>Activated leukocyte cell adhesion molecule (ALCAM)</td>
<td>AA156721</td>
<td>-12.54</td>
</tr>
<tr>
<td>8</td>
<td>Achaete-scute complex homolog 1 (Drosophila) (ASCL1)</td>
<td>BC001638</td>
<td>-14.50</td>
</tr>
<tr>
<td>9</td>
<td>Adhesion molecule with Ig-like domain 2 (AMIGO2)</td>
<td>AC004010</td>
<td>5.71</td>
</tr>
<tr>
<td>10</td>
<td>SERPINE1 mRNA binding protein 1 (SERBP1)</td>
<td>AF131807</td>
<td>-5.45</td>
</tr>
</tbody>
</table>

Many of the down-regulated genes outlined in Table 5.2 appear to play important roles in breast cancer. SLC39A6 belongs to the LIV-1 sub-family of ZIP proteins that show
structural characteristics of zinc transporters (reviewed in Taylor & Nicholson, 2003). Furthermore, LIV-1 has been previously associated with ER-positive breast cancer (Manning et al., 1995) and metastatic spread to the regional lymph nodes (Manning et al., 1994). PABPC1 is a highly conserved protein involved in mRNA stabilization and translation (Gray et al., 2000; Grosset et al., 2000) and has been shown to interact with BRCA1 (Dizin et al., 2006). Melanophilin is a carrier protein which in humans is encoded by the MLPH gene (Matesic et al., 2001; Strom et al., 2002). There is no literature currently outlining its role in breast cancer; however, MLPH has a role in melanosome transport and genetic mutations in this gene cause Griscelli syndrome in humans that manifests as pigmented dilution of the skin and the hair and immunodeficiency (Izumi et al., 2003). Hsp70 proteins function as ATP-dependent molecular chaperones that assist the folding of newly synthesized polypeptides (reviewed in McKay, 1993). In breast cancer, its expression correlates with increased cell proliferation, poor differentiation, lymph node metastases, and poor therapeutic outcome (Ciocca et al., 1993; Lazaris et al., 1997; Vargas-Roig et al., 1997; Vargas-Roig et al., 1998). ALCAM is a cell surface immunoglobulin reported as a good prognostic marker in breast cancer (King et al., 2004) as well as suppressing breast cancer cell invasion (Jezierska et al., 2006). There is little or no literature regarding the role of ASCL1, AMIGO2 and SERBP1 in breast cancer; however, ASCL1 is highly expressed in small cell lung cancer (Westerman et al., 2002), whereas AMIGO2 down-regulation by RNAi results in altered morphology, increased ploidy, chromosomal instability, decreased cell adhesion/migration in the AGS gastric adenocarcinoma cell line and a nearly complete abrogation of tumourigenicity in nude mice (Rabenau et al., 2004). In ovarian cancer, SERBP1 is significantly over-expressed in tumour epithelial cells and correlates with advanced disease stage (Koensgen et al., 2007).

5.4.2.4 Statistical analysis and hierarchical clustering
Genes significantly up- or down-regulated were filtered using ANOVA statistical analysis with a significance threshold of $\leq 0.0003$ (Ruiz et al., 2004). This identified 229 genes which were differentially expressed between Vector Only and the TNC isoforms, including all genes outlined in Table 5.1 and Table 5.2. Figure 5.4 shows the heat map and hierarchical clustering of samples using this type of analysis.
Figure 5.4: Hierarchical clustering of samples comparing Vector Only controls with TNC isoforms
5.4.3 (RT)-qPCR analysis of candidate gene expression

Candidate genes were selected from the top 200 up-regulated genes in the Vector Only vs. other TNC isoforms data set when analysed by regression analysis. This involved first investigating candidate genes roles in breast cancer through an extensive literature search. Based on their potential significance in breast cancer and evidence of differential gene expression by both regression and statistical analysis, 6 genes were selected for further study (Table 5.3). All of these genes showed a > 5-fold up-regulation by regression analysis in cells transfected with TNC-14/AD1/16 compared to Vector Only controls.

Table 5.3: Genes selected for further analysis by (RT)-qPCR

<table>
<thead>
<tr>
<th>Candidate gene</th>
<th>Gene ID</th>
<th>Average fold change (by regression analysis)</th>
<th>Function in breast cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TNC Short</td>
<td>TNC-9/16</td>
</tr>
<tr>
<td>Interferon-induced transmembrane protein 1 (IFITM1)</td>
<td>NM_003641</td>
<td>-0.09</td>
<td>0.86</td>
</tr>
<tr>
<td>Signal transducer and activator of transcription 1 (STAT1)</td>
<td>NM_007315</td>
<td>-0.34</td>
<td>-0.45</td>
</tr>
<tr>
<td>Profilin 1 (PFN1)</td>
<td>NM_005022</td>
<td>0.68</td>
<td>12.08</td>
</tr>
<tr>
<td>Amphiregulin (Schwannoma-derived growth factor) (AREG)</td>
<td>NM_001657</td>
<td>9.31</td>
<td>-0.98</td>
</tr>
<tr>
<td>Cytokeratin 8 (CK8)</td>
<td>NM_002281</td>
<td>-0.22</td>
<td>0.38</td>
</tr>
<tr>
<td>Cyclin D1 (CCND1)</td>
<td>NM_053056</td>
<td>-0.36</td>
<td>2.57</td>
</tr>
</tbody>
</table>
5.4.3.1 Validation of TaqMan assays

Inventoried TaqMan assays were used for analysis of candidate gene expression in transfected MCF-7 cells as well as 10 established breast cell lines, 2 isolated populations from normal breast, breast cancers and breast organoids were validated by production of standard curves using mRNA isolated from MDA-MB-231 cells (Table 5.4 and Figure 5.5). Once validated, assays were used for analysis of candidate gene expression.

### Table 5.4: \( R^2 \) values, PCR efficiency and intercept values for candidate gene assays

<table>
<thead>
<tr>
<th>TaqMan assay/primer set</th>
<th>( R^2 ) value</th>
<th>PCR efficiency (%)</th>
<th>Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFITM1</td>
<td>0.9973</td>
<td>98.80</td>
<td>36.548</td>
</tr>
<tr>
<td>STAT1</td>
<td>0.9951</td>
<td>99.81</td>
<td>38.741</td>
</tr>
<tr>
<td>PFN1</td>
<td>0.9954</td>
<td>99.65</td>
<td>32.898</td>
</tr>
<tr>
<td>AREG</td>
<td>0.9977</td>
<td>103.55</td>
<td>38.796</td>
</tr>
<tr>
<td>CK8</td>
<td>0.9964</td>
<td>101.84</td>
<td>38.254</td>
</tr>
<tr>
<td>CCND1</td>
<td>0.9987</td>
<td>98.39</td>
<td>39.217</td>
</tr>
<tr>
<td>GAPDH</td>
<td>0.9988</td>
<td>98.25</td>
<td>34.316</td>
</tr>
</tbody>
</table>

**Figure 5.5:** Example standard curve for TaqMan assays

PCR efficiency and \( R^2 \) values of > 98% were required for a probe to be considered for use in expression analysis. Example given is IFITM1.
5.4.4 Expression of candidate genes in transfected MCF-7s

5.4.4.1 Total TNC

It was important to demonstrate similar levels of each recombinant isoform in transfected MCF-7 cells. RQ analysis (using TNC Short as the control sample) showed no significant variation in TNC mRNA expression either between replicates or isoforms (Table 5.5 and Figure 5.6). Note that average RQ values were calculated using the mean ΔCt value obtained from 2 replicates of the endogenous control (GAPDH).

Table 5.5: Average RQ values for total TNC expression in TNC transfected cells

<table>
<thead>
<tr>
<th>TNC isoform</th>
<th>Mean Ct</th>
<th>SD (%)</th>
<th>Average RQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC Short</td>
<td>17.42</td>
<td>0.19 (1.06%)</td>
<td>1.00</td>
</tr>
<tr>
<td>TNC-9/16</td>
<td>18.17</td>
<td>0.34 (1.87%)</td>
<td>0.94</td>
</tr>
<tr>
<td>TNC-9/14/16</td>
<td>17.00</td>
<td>0.33 (1.96%)</td>
<td>1.26</td>
</tr>
<tr>
<td>TNC-14/AD1/16</td>
<td>16.61</td>
<td>0.32 (1.95%)</td>
<td>1.52</td>
</tr>
</tbody>
</table>

Where: - Ct = Cycle threshold value; SD = Standard Deviation; RQ = Relative Quantification

Figure 5.6: Relative expression of total TNC in transfected cells compared to TNC Short
The graph shows the mean RQ value (± standard error of the mean (SEM)) of each sample.
5.4.4.2 IFITM1

Expression of IFITM1 confirmed results obtained from the GeneChip analysis, with cells transfected with TNC-9/14/16 and TNC-14/AD1/16 both significantly up-regulating expression ($p = < 0.001$ for both). No significant up- or down-regulation was found in cells transfected with TNC-9/16 and TNC Short ($p = 1.000$ for both) (Table 5.6 and Figure 5.7).

Table 5.6: Average RQ values for IFITM1 expression in TNC transfected cells

<table>
<thead>
<tr>
<th>TNC isoform</th>
<th>Mean Ct</th>
<th>SD (%)</th>
<th>Average RQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC Vector Only</td>
<td>27.04</td>
<td>0.49 (1.81%)</td>
<td>1.01</td>
</tr>
<tr>
<td>TNC Short</td>
<td>26.86</td>
<td>0.44 (1.64%)</td>
<td>1.26</td>
</tr>
<tr>
<td>TNC-9/16</td>
<td>28.21</td>
<td>0.43 (1.52%)</td>
<td>0.74</td>
</tr>
<tr>
<td>TNC-9/14/16</td>
<td>21.46</td>
<td>0.50 (2.31%)</td>
<td>46.54</td>
</tr>
<tr>
<td>TNC-14/AD1/16</td>
<td>22.87</td>
<td>0.54 (2.38%)</td>
<td>16.15</td>
</tr>
</tbody>
</table>

Figure 5.7: Relative expression of IFITM1 in transfected MCF-7 cells compared to TNC Vector Only

The graph shows the mean RQ value (± SEM) between samples compared to TNC Vector Only. Significant differences ($p = < 0.001$) are indicated by three asterisks.
5.4.4.3 STAT1

Expression of STAT1 in transfected cell lines differed in MCF-7 cells transfected with TNC-14/AD1/16 compared to the results obtained by regression analysis. MCF-7 TNC-14/AD1/16 cells showed no significant up- or down-regulation compared to TNC Vector Only control \( (p = 1.000) \), but cells transfected with TNC-9/14/16 showed a significant up-regulation \( (p = 0.026) \). Cells transfected with TNC-9/16 also correlated with the microarray data, with STAT1 being significantly down-regulated \( (p = 0.033) \), but to a much greater level. TNC Short transfected cells also down-regulated STAT1 but this was not found to be significant \( (p = 0.598) \) (Table 5.7 and Figure 5.8).

Table 5.7: Average RQ values for STAT1 expression in TNC transfected cells

<table>
<thead>
<tr>
<th>TNC isoform</th>
<th>Mean Ct</th>
<th>SD (%)</th>
<th>Average RQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC Vector Only</td>
<td>26.37</td>
<td>0.37 (1.40%)</td>
<td>1.00</td>
</tr>
<tr>
<td>TNC Short</td>
<td>27.30</td>
<td>0.29 (1.06%)</td>
<td>0.56</td>
</tr>
<tr>
<td>TNC-9/16</td>
<td>28.70</td>
<td>0.54 (1.88%)</td>
<td>0.33</td>
</tr>
<tr>
<td>TNC-9/14/16</td>
<td>24.77</td>
<td>0.70 (2.82%)</td>
<td>3.03</td>
</tr>
<tr>
<td>TNC-14/AD1/16</td>
<td>26.21</td>
<td>0.62 (2.35%)</td>
<td>1.02</td>
</tr>
</tbody>
</table>

Figure 5.8: Relative expression of STAT1 in transfected MCF-7 cells compared to TNC Vector Only

The graph shows the mean RQ value \( \pm \) SEM) between samples compared to TNC Vector Only. Significant differences \( (p < 0.05) \) are indicated by one asterisk.
5.4.4.4 PFN1

(RT)-qPCR analysis of PFN1 expression did not correlate with data obtained by microarray. GeneChip analysis showed that in cells transfected with TNC-9/16; TNC-9/14/16 and TNC-14/AD1/16, PFN1 was up-regulated 12 – 16 fold. However, analysis by (RT)-qPCR showed that PFN1 was down-regulated significantly in cells transfected with TNC-9/14/16 and TNC-14/AD1/16 ($p = 0.001$ and $< 0.001$ respectively). PFN1 expression in cells transfected with TNC-9/16 was also down-regulated but this was not deemed significant ($p = 0.512$). A slight up-regulation in cells transfected with TNC Short was found but this was not significant ($p = 1.000$) (Table 5.8 and Figure 5.9).

Table 5.8: Average RQ values for PFN1 in TNC transfected cells

<table>
<thead>
<tr>
<th>TNC isoform</th>
<th>Mean Ct</th>
<th>SD (%)</th>
<th>Average RQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC Vector Only</td>
<td>19.09</td>
<td>0.57 (2.97%)</td>
<td>1.00</td>
</tr>
<tr>
<td>TNC Short</td>
<td>18.93</td>
<td>0.67 (3.51%)</td>
<td>1.19</td>
</tr>
<tr>
<td>TNC-9/16</td>
<td>20.32</td>
<td>0.64 (3.14%)</td>
<td>0.71</td>
</tr>
<tr>
<td>TNC-9/14/16</td>
<td>20.32</td>
<td>0.22 (1.06%)</td>
<td>0.41</td>
</tr>
<tr>
<td>TNC-14/AD1/16</td>
<td>20.58</td>
<td>0.36 (1.75%)</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Figure 5.9: Relative expression of PFN1 in transfected MCF-7 cells compared to TNC Vector Only

The graph shows the mean RQ value ($\pm$ SEM) between samples compared to TNC Vector Only. Significant differences ($p = \leq 0.001$) are indicated by three asterisks.
5.4.4.5 \textit{AREG}

Expression of AREG in cells transfected with TNC isoforms correlated with data obtained from GeneChip analysis. Cells transfected with TNC Short significantly up-regulated AREG expression ($p = 0.038$), whereas cells transfected with TNC-9/16 down-regulated AREG and cells transfected with TNC-9/14/16 and TNC-14/AD1/16 up-regulated AREG, although all failed to reach statistical significance ($p = 0.332, 0.084$ and $1.000$ respectively) (Table 5.9 and Figure 5.10).

Table 5.9: Average RQ values for AREG expression in TNC transfected cells

<table>
<thead>
<tr>
<th>TNC isoform</th>
<th>Mean Ct</th>
<th>SD (%)</th>
<th>Average RQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC Vector Only</td>
<td>21.75</td>
<td>0.46 (2.11%)</td>
<td>1.02</td>
</tr>
<tr>
<td>TNC Short</td>
<td>20.42</td>
<td>0.56 (2.72%)</td>
<td>2.71</td>
</tr>
<tr>
<td>TNC-9/16</td>
<td>23.39</td>
<td>0.47 (2.01%)</td>
<td>0.54</td>
</tr>
<tr>
<td>TNC-9/14/16</td>
<td>20.54</td>
<td>0.51 (2.48%)</td>
<td>2.25</td>
</tr>
<tr>
<td>TNC-14/AD1/16</td>
<td>21.15</td>
<td>0.50 (2.35%)</td>
<td>1.35</td>
</tr>
</tbody>
</table>

Figure 5.10: Relative expression of AREG in transfected MCF-7 cells compared to TNC Vector Only

The graph shows the mean RQ value ($\pm$ SEM) between samples compared to TNC Vector Only. Significant differences ($p = < 0.05$) are indicated by one asterisk.
5.4.4.6 CK8

Expression of CK8 in cells transfected with TNC isoforms correlated with data obtained from GeneChip analysis, with slight differences in the relative expression compared to TNC Vector Only control. Cells transfected with TNC Short significantly down-regulated CK8 expression ($p = 0.006$), whereas cells transfected with TNC-9/16; TNC-9/14/16 and TNC-14/AD1/16 significantly up-regulated CK8 expression ($p = 0.001$, $< 0.001$ and $0.013$ respectively). Gene expression in cells transfected with TNC Short and TNC-9/16 was greater by (RT)-qPCR analysis than GeneChip analysis, whereas expression in cells transfected with the remaining isoforms was less when analysed by (RT)-qPCR (Table 5.10 and Figure 5.11).

Table 5.10: Average RQ values for CK8 expression in TNC transfected cells

<table>
<thead>
<tr>
<th>TNC isoform</th>
<th>Mean Ct</th>
<th>SD (%)</th>
<th>Average RQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC Vector Only</td>
<td>20.98</td>
<td>0.21</td>
<td>(1.02%)</td>
</tr>
<tr>
<td>TNC Short</td>
<td>22.48</td>
<td>0.28</td>
<td>(1.25%)</td>
</tr>
<tr>
<td>TNC-9/16</td>
<td>19.66</td>
<td>0.28</td>
<td>(1.40%)</td>
</tr>
<tr>
<td>TNC-9/14/16</td>
<td>18.99</td>
<td>0.34</td>
<td>(1.77%)</td>
</tr>
<tr>
<td>TNC-14/AD1/16</td>
<td>19.67</td>
<td>0.31</td>
<td>(1.56%)</td>
</tr>
</tbody>
</table>

Figure 5.11: Relative expression of CK8 in transfected MCF-7 cells compared to TNC-Vector Only

The graph shows the mean RQ value (± SEM) between samples compared to TNC-Vector Only. Significant differences are indicated by one asterick ($p = < 0.05$) two asterisks ($p = < 0.01$) or three asterisks ($p = < 0.001$).
5.4.4.7 CCND1

Expression of CCND1 in cells transfected with TNC isoforms correlated with data obtained from GeneChip analysis; however, the effects were less significant when analysed by (RT)-qPCR. CCND1 was significantly up-regulated in cells transfected with TNC-9/16; TNC-9/14/16 and TNC-14/AD1/16 ($p = 0.005$, $< 0.001$ and $0.05$ respectively). No significant difference in CCND1 expression was shown in cells transfected with TNC Short ($p = 1.000$) (Table 5.11 and Figure 5.12).

<table>
<thead>
<tr>
<th>TNC isoform</th>
<th>Mean Ct</th>
<th>SD (%)</th>
<th>Average RQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC Vector Only</td>
<td>28.03</td>
<td>0.47 (1.69%)</td>
<td>1.00</td>
</tr>
<tr>
<td>TNC Short</td>
<td>28.00</td>
<td>0.43 (1.52%)</td>
<td>1.08</td>
</tr>
<tr>
<td>TNC-9/16</td>
<td>27.35</td>
<td>0.20 (0.73%)</td>
<td>2.70</td>
</tr>
<tr>
<td>TNC-9/14/16</td>
<td>26.21</td>
<td>0.30 (1.16%)</td>
<td>3.62</td>
</tr>
<tr>
<td>TNC-14/AD1/16</td>
<td>27.01</td>
<td>0.52 (1.91%)</td>
<td>1.84</td>
</tr>
</tbody>
</table>

**Figure 5.12:** Relative expression of CCND1 in transfected MCF-7 cells compared to TNC Vector Only

The graph shows the mean RQ value ($\pm$ SEM) between samples compared to TNC Vector Only. Significant differences are indicated by one asterisk ($p = \leq 0.05$), two asterisks ($p = < 0.01$) or three asterisks ($p = < 0.001$)
5.4.4.8 Summary of (RT)-qPCR analysis of transfected cells

In summary, for the majority of samples (RT)-qPCR confirmed that the 6 candidate genes correlated with GeneChip analysis of MCF-7 cells transfected with different TNC isoforms and the changes in expression caused by these isoforms. However, the levels of expression were understated by (RT)-qPCR analysis compared to GeneChip, with the exception being IFITM1 in cells transfected with TNC-9/14/16, which was much higher when analysed by (RT)-qPCR. Furthermore, the (RT)-qPCR showed a complete contradiction in expression of PFN1, which was down-regulated by (RT)-qPCR analysis but up-regulated by GeneChip analysis. Table 5.12 summarises the relative expression of candidate genes in MCF-7 cells transfected with each TNC isoform and Table 5.13 summarises the correlation between GeneChip regression analysis and (RT)-qPCR analysis.

Table 5.12: Summary of relative expression for the 6 candidate genes in TNC transfected cells

<table>
<thead>
<tr>
<th>TNC isoform</th>
<th>IFITM1</th>
<th>STAT1</th>
<th>PFN1</th>
<th>AREG</th>
<th>CK8</th>
<th>CCND1</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC Vector Only</td>
<td>1.01</td>
<td>1.00</td>
<td>1.00</td>
<td>1.02</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>TNC Short</td>
<td>1.26</td>
<td>0.56</td>
<td>1.19</td>
<td>2.71*</td>
<td>0.37**</td>
<td>1.08</td>
</tr>
<tr>
<td>TNC-9/16</td>
<td>0.74</td>
<td>0.33*</td>
<td>0.71</td>
<td>0.54</td>
<td>4.22***</td>
<td>2.70**</td>
</tr>
<tr>
<td>TNC-9/14/16</td>
<td>46.54***</td>
<td>3.03*</td>
<td>0.41**</td>
<td>2.25</td>
<td>3.81***</td>
<td>3.62***</td>
</tr>
<tr>
<td>TNC-14/AD1/16</td>
<td>16.15***</td>
<td>1.02</td>
<td>0.32***</td>
<td>1.35</td>
<td>2.21*</td>
<td>1.84*</td>
</tr>
</tbody>
</table>

*p = ≤ 0.05; **p = ≤ 0.01; ***p = ≤ 0.001

Table 5.13: Summary of regression GeneChip analysis and (RT)-qPCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>TNC Short</th>
<th>TNC-9/16</th>
<th>TNC-9/14/16</th>
<th>TNC-14/AD1/16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GeneChip</td>
<td>qRT</td>
<td>GeneChip</td>
<td>qRT</td>
</tr>
<tr>
<td>IFITM1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>STAT1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>--</td>
</tr>
<tr>
<td>PFN1</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>AREG</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CK8</td>
<td>-</td>
<td>--</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>CCND1</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

* = ≤ 2-fold increase; ++ = > 2-fold increase; +++ = ≥ 10-fold increase
- = ≤ 2-fold decrease; -- = > 2-fold decrease; --- = ≥ 10-fold decrease

5.4.5 Expression of candidate genes in cell lines and isolated normal breast populations

Expression of candidate genes was analysed in 10 established breast cell lines and isolated myoepithelial and fibroblast populations from normal breast tissue. In general, IFITM1 expression was slightly increased in cells expressing TNC; however, the increase was not as significant as in MCF-7 cells transfected with TNC-9/14/16 and
TNC-14/AD1/16. Generally STAT1, AREG, CK8 and CCND1, were all downregulated in TNC positive cells compared to TNC null cells, with the most striking down-regulation seen in AREG and CK8 expression. PFN1 expression varied widely, with no clear pattern of expression apparent. –ΔCt values for each candidate gene are shown in Table 5.14.

Table 5.14: Average –ΔCt values of candidate genes in cell lines and normal cell populations

<table>
<thead>
<tr>
<th>Cells</th>
<th>TNC-14/16</th>
<th>TNC-AD1</th>
<th>IFITM1</th>
<th>STAT1</th>
<th>PFN1</th>
<th>AREG</th>
<th>CK8</th>
<th>CCND1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Undet.</td>
<td>Undet.</td>
<td>-6.21</td>
<td>-5.37</td>
<td>1.71</td>
<td>-1.28</td>
<td>-0.51</td>
<td>-8.12</td>
</tr>
<tr>
<td>T-47D</td>
<td>Undet.</td>
<td>Undet.</td>
<td>-3.89</td>
<td>-4.76</td>
<td>0.76</td>
<td>-1.33</td>
<td>-0.84</td>
<td>-8.14</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>Undet.</td>
<td>Undet.</td>
<td>-6.37</td>
<td>-5.35</td>
<td>-1.95</td>
<td>-5.20</td>
<td>-1.67</td>
<td>-7.59</td>
</tr>
<tr>
<td>Hs578T</td>
<td>-3.07</td>
<td>-5.81</td>
<td>-7.55</td>
<td>-6.94</td>
<td>4.54</td>
<td>-12.89</td>
<td>-9.77</td>
<td>-8.10</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>-5.49</td>
<td>-8.49</td>
<td>-5.93</td>
<td>-7.22</td>
<td>-0.48</td>
<td>-8.65</td>
<td>-6.45</td>
<td>-7.80</td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>-5.07</td>
<td>-7.67</td>
<td>-6.80</td>
<td>-8.41</td>
<td>5.20</td>
<td>-7.31</td>
<td>-8.18</td>
<td>-11.38</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>-7.43</td>
<td>-6.94</td>
<td>-4.41</td>
<td>-8.02</td>
<td>-1.35</td>
<td>-12.25</td>
<td>-4.09</td>
<td>-10.81</td>
</tr>
<tr>
<td>GI-101</td>
<td>-6.22</td>
<td>-7.35</td>
<td>-8.87</td>
<td>-7.98</td>
<td>-0.28</td>
<td>-7.96</td>
<td>-4.07</td>
<td>-8.98</td>
</tr>
<tr>
<td>HBL-100</td>
<td>-4.16</td>
<td>-3.43</td>
<td>-5.27</td>
<td>-8.65</td>
<td>-0.83</td>
<td>-11.84</td>
<td>-6.65</td>
<td>-12.45</td>
</tr>
<tr>
<td>MCF-10A</td>
<td>-10.42</td>
<td>-8.32</td>
<td>-4.41</td>
<td>-8.17</td>
<td>2.32</td>
<td>-1.72</td>
<td>-6.10</td>
<td>-11.29</td>
</tr>
<tr>
<td>P-ME</td>
<td>-6.53</td>
<td>-4.98</td>
<td>-5.03</td>
<td>-7.52</td>
<td>2.09</td>
<td>-3.48</td>
<td>-11.38</td>
<td>-10.71</td>
</tr>
<tr>
<td>P-Fib</td>
<td>-4.95</td>
<td>-10.21</td>
<td>-2.64</td>
<td>-5.42</td>
<td>4.40</td>
<td>-14.31</td>
<td>-9.93</td>
<td>-10.34</td>
</tr>
</tbody>
</table>

P = Primary; ME = Myoepithelial cells from normal breast; Fib = Fibroblasts from normal breast tissue

Pearson correlation showed an inverse correlation with AREG and TNC-14/16 expression (ρ = -0.705, p = 0.034). No further correlations were found with any other candidate genes or with TNC-AD1 expression. Table 5.15 gives a detailed summary of ρ (rho) and p values for expression of TNC-14/16 and TNC-AD1 compared to candidate gene expression. Figure 5.13 shows a scatter graph of TNC-14/16 vs. AREG expression.

Table 5.15: Pearson correlation ρ and p values for candidate gene expression in cell lines

<table>
<thead>
<tr>
<th>Isoform</th>
<th>ρ value</th>
<th>IFITM1</th>
<th>STAT1</th>
<th>PFN1</th>
<th>AREG</th>
<th>CK8</th>
<th>CCND1</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC-14/16</td>
<td>-0.196</td>
<td>0.232</td>
<td>0.079</td>
<td>-0.705*</td>
<td>-0.293</td>
<td>-0.136</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.614</td>
<td>0.547</td>
<td>0.840</td>
<td>0.034</td>
<td>0.444</td>
<td>0.727</td>
<td></td>
</tr>
<tr>
<td>TNC-AD1</td>
<td>-0.282</td>
<td>-0.560</td>
<td>-0.286</td>
<td>0.024</td>
<td>-0.086</td>
<td>-0.309</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.462</td>
<td>0.117</td>
<td>0.456</td>
<td>0.952</td>
<td>0.826</td>
<td>0.418</td>
<td></td>
</tr>
</tbody>
</table>

* p = ≤ 0.05
5.4.6 Expression of candidate genes in breast tissue

Expression of candidate genes was analysed in 8 breast organoids and 12 breast cancers. The carcinomas comprised 6 cases ≤ 40 years and 6 cases > 40 years of age, each age group containing 3 cases that were positive for TNC-AD1 expression.

Expression of candidate genes in tumour tissue showed a significant down-regulation of PFN1 and AREG compared to breast organoids ($p = 0.017$ and $p = 0.037$ respectively), whereas expression of CK8 was significantly up-regulated in tumour tissue ($p = 0.021$). No significant difference in expression was found in IFITM1, STAT1 or CCND1 ($p = 0.247$, $p = 0.247$ and $p = 0.090$ respectively) (Figure 5.14).
Figure 5.14: Expression of candidate genes in breast organoids and invasive carcinomas

Box and whisker plots showing $-\Delta \text{Ct}$ values of candidate genes in breast organoids and tumour tissue. Bars within the data sets represent the median $-\Delta \text{Ct}$ value. The ‘o’ represents outliers. P-values are indicated on the box plots.

A) IFITM1  

B) STAT1

C) PFN1  

D) AREG

E) CK8  

F) CCND1

$p = 0.247$  
$p = 0.247$  
$p = 0.017$  
$p = 0.021$  
$p = 0.037$  
$p = 0.090$
Pearson correlation showed a significant inverse correlation between expression of TNC-14/16 and PFN1 in invasive carcinomas ($\rho = -0.785; \ p = 0.002$). No other correlations were found for the remaining candidate genes, even though PFN1 showed a highly negative correlation with total TNC expression ($\rho = -0.544; \ p = 0.067$). Pearson correlations could not be performed for TNC-9/16, TNC-AD1 or TNC-AD2 expression due to the low number of samples that were positive. Table 5.16 gives a summary of all Pearson correlation values and Figure 5.15 shows the scatter plot for PFN1 vs. TNC-14/16 expression.

### Table 5.16: Pearson correlation values for PFN1 vs. TNC expression

<table>
<thead>
<tr>
<th>Isoform</th>
<th>$\rho$ value</th>
<th>IFITM1</th>
<th>STAT1</th>
<th>PFN1</th>
<th>AREG</th>
<th>CK8</th>
<th>CCND1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total TNC</td>
<td>$\rho$ value</td>
<td>0.139</td>
<td>0.172</td>
<td>-0.544</td>
<td>0.134</td>
<td>-0.340</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>$p$ value</td>
<td>0.665</td>
<td>0.593</td>
<td>0.067</td>
<td>0.677</td>
<td>0.279</td>
<td>0.929</td>
</tr>
<tr>
<td>TNC-14/16</td>
<td>$\rho$ value</td>
<td>-0.186</td>
<td>0.219</td>
<td>-0.786**</td>
<td>-0.087</td>
<td>-0.365</td>
<td>-0.030</td>
</tr>
<tr>
<td></td>
<td>$p$ value</td>
<td>0.563</td>
<td>0.492</td>
<td>0.002</td>
<td>0.787</td>
<td>0.244</td>
<td>0.926</td>
</tr>
</tbody>
</table>

**$p \leq 0.01$**

### Figure 5.15: Correlation of PFN1 and TNC expression

$-\Delta$Ct values are plotted on the graph showing a highly significant inverse correlation between increasing TNC-14/16 expression and a down-regulation of PFN1. Rho ($\rho$) value is indicated on the graph.
5.4.7 Summary of (RT)-qPCR in breast cell lines and tissues

In summary, (RT)-qPCR validation of candidate gene expression did not correlate to GeneChip analysis in MCF-7 cells transfected with TNC isoforms. PFN1 expression as analysed by (RT)-qPCR was in complete contradiction to the GeneChip analysis with expression down-regulated when analysed by (RT)-qPCR. Moreover, (RT)-qPCR results appeared to understate the level of gene expression compared to GeneChip analysis, which was usually greater. Also, results obtained from transfected cells could not be correlated with expression in cell lines and isolated normal cell populations. The levels of candidate gene expression varied greatly in the majority of cell lines compared to transfected cells, with TNC positive cells showing a down-regulation of most candidate genes compared to TNC null cells. Importantly, down-regulation of AREG expression was significantly correlated with an increase in TNC-14/16 expression, and although not shown to be significant, TNC-14/16 expression was also correlated with PFN1 down-regulation.

Results in tumour tissue cannot be completely correlated with results obtained in transfected cells. However, PFN1 was shown to be significantly down-regulated in invasive carcinoma tissue, which was associated with high levels of TNC-14/16 expression. AREG was also significantly down-regulated in carcinoma tissue and CK8 significantly up-regulated; however, this was not correlated with TNC expression.

5.5 Discussion

5.5.1 Affymetrix GeneChip analysis

All selected RNA samples from cells transfected with TNC isoforms were successfully labelled and subsequently hybridised to the Affymetrix GeneChips. Of the top 200 genes found to be up-regulated compared to cells transfected with Vector Only controls, there were many that showed potential as markers. Of the 6 candidate genes that were investigated further PFN1 and IFITM1 appeared to be the most significant. Each of the candidate genes will be discussed in greater detail separately.

The considerations of experimental design are of critical importance if statistically and biologically valid conclusions are to be drawn from the data. Ideally, all samples should be produced in quadruplicate, with artificial neural networks utilised for the analysis. There are now a vast array of software programs for analysing microarray
data, including Significance Analysis of Microarrays or SAM (http://www-stat.stanford.edu/~tibs/SAM), GeneSpring (Agilent, UK), the Microarray Multi-Experiment Viewer (TM4, USA – a very useful, free java application) and dChip (another free software package). All of these software packages use a variety of statistical analysis tools, including t-tests and ANOVAs in order to analyse the data and are very useful in their own right. Moreover, regression analysis using Microsoft Excel also proved to be an extremely useful tool as it analysed each chip individually against every other sample, thus “smoothing out” any major discrepancies between replicates.

Regarding the use of breast tissue for validation, the use of organoids from normal breast tissue was used due to the lack of fresh reduction mammoplasty samples. This is not ideal as breast organoids are extracted by collagenase digestion from reduction mammoplasties (Gomm et al., 1995) and are a concentrated source of myoepithelial and luminal cells, with stromal cells (i.e. fibroblasts) and endothelial cells filtered out. This could affect the global gene expression levels and hence yield different results to RNA extracted that has been extracted directly from normal breast tissue. However, they are considered to be a “normal” control sample and have been used in other studies (Taylor-Papadimitriou et al., 1989; Simian et al., 2001).

5.5.2 Gene expression profiling by (RT)-qPCR

GeneChip analysis showed that TNC-9/14/16 and TNC-14/AD1/16 shared similar profiles when exogenously expressed in TNC null cells. TNC-9/16 – although associated with invasion (Adams et al., 2002) – appears to have a similar effect on carcinoma cells compared to TNC-9/14/16; however, this effect was not as pronounced. TNC Short appeared to agree with other studies in that it does not affect gene expression levels of cancer related genes compared to TNC-9/14/16 (with the exception of AREG). Validation of these findings using (RT)-qPCR was in agreement with the majority of the GeneChip analysis. However, one major difference was the complete contradiction of PFN1 profiling in cells expressing TNC-9/14/16 and TNC-14/AD1/16, with (RT)-qPCR showing a down-regulation in expression, a finding confirmed by profiling in tumour tissue. The difference in (RT)-qPCR and tumour tissue results could be due to a “false-positive” being produced, perhaps by contamination of the chip or due to the array probe detecting different isoforms of PFN1 compared to the TaqMan assay. However, the discovery of PFN1 expression being significantly associated with TNC-14/16 expression shows promise for this gene as a candidate for further study.
5.5.2.1 Interferon-inducible trans-membrane protein 1 (IFITM1)

Interferons (IFNs) are multifunctional cytokines that exhibit anti-proliferative and differentiating activities that play a critical role in the defence against viral and parasite infection, as well as immune tumour surveillance. The 9-27 (IFITM1) cDNA was first identified as an IFN inducible gene (Ackrill et al., 1991; Lewin et al., 1991), encoding a 17 kDa membrane protein (Deblandre et al., 1995), whose expression is induced by type I and II IFNs in immune cells, epithelial cells, and fibroblastic cells, which are sensitive to IFNs. Furthermore, 9-27 is known to be a component of several membrane proteins including CD19, CD21 (CR2) (Matsumoto et al., 1991) and CD81 (TAPA-1) (Bradbury et al., 1992), all of which are key molecules in B-lymphocyte signal transduction and strongly influence the regulation of cell growth and cellular adhesion (Takahashi et al., 1990; Mahmoud et al., 1999). However, the effect of IFITM1 appears to be dependent upon cell type, as recent studies have shown that IFITM1 plays and essential role in interferon-γ (IFN-γ) mediated anti-proliferative and anti-virus responses, immune surveillance and tumour suppression (Yang et al., 2007). Conversely, Yang et al. (2005) reported that exogenous expression of IFITM1 in SNU-216 gastric cells rendered them more resistant to natural killer cells (NKCs). Moreover, over-expression of IFITM1 in these cells and also SNU-484 and SNU-638 gastric cells increased their invasive capacity in a modified Boyden chamber assay, which has been reproduced in head and neck squamous carcinoma cells (HNSCCs) (Hatano et al., 2008).

Currently there is little evidence of IFITM1 expression in the breast, the only report showing an increase in IFITM1 (as well as STAT1) expression in MCF-7s exposed to fractioned radiation (Tsai et al., 2007). Microarray analysis and (RT)-qPCR validation of IFITM1 expression in MCF-7 cells transfected with TNC-9/14/16 and TNC-14/AD1/16 showed a significant increase in IFITM1 expression, suggesting a role in the invasion of tumour cells via MMP activation (Hatano et al., 2008) and evasion of tumour cells from the immune system. The alternatively spliced region of TNC is known to have an immunosuppressive effect on T-lymphocyte activation and proliferation (Puente Navazo et al., 2001; Parekh et al., 2005); however, this is the first evidence of TNC isoforms potentially aiding tumour cell proliferation and invasion by suppression of NKC activity.
5.5.2.2 Signal transducers and activators of transcription 1 (STAT1)

Signal transducers and activators of transcription (STAT) proteins are a family of latent transcription factors involved in cytokine, hormone, and growth factor signal transduction (Schindler & Darnell, 1995; Bromberg, 2001). Since their discovery in interferon (IFN)-regulated gene transcription in the early 1990s (Sadowski et al., 1993; Shuai et al., 1993) a number of family members have been identified in mammalian cells: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6, all of which share a common ancestral origin (Copeland et al., 1995). STAT1 has been associated with malignant transformation induced by various oncoproteins (Bowman et al., 2000).

In the breast (Clevenger, 2004), STAT1 is activated almost exclusively by interferon-γ and mice that lack STAT1 have no innate response to either viral or bacterial pathogens because defence against these pathogens usually requires response to interferon (Durbin et al., 1996; Meraz et al., 1996). Furthermore, STAT1-deficient mice develop spontaneous and chemically induced tumours over time (Kaplan et al., 1998); hence, STAT1 can be described as a tumour suppressor. Work done in tissue culture systems on STAT1-deficient cells (Bromberg et al., 1996; Kumar et al., 1997) demonstrated an important role for STAT1 in ligand-mediated growth arrest and apoptosis. Thus, there is growing evidence that STAT1 activation frequently leads to anti-proliferative and pro-apoptotic events, and may partly explain why the lack of this molecule in vivo leads to increased tumour formation.

Microarray analysis and (RT)-qPCR validation of STAT1 expression showed a significant decrease in expression in cells transfected with the TNC-9/16 isoform, and – surprisingly – a significant increase in expression in cells transfected with TNC-9/14/16. TNC positive breast cancer cell lines showed a significant decrease in expression compared to TNC null cells, suggesting that TNC does indeed have an effect on STAT1 expression. However, it does not appear to be isoform specific. Although TNC-9/14/16 increases expression of STAT1 in MCF-7 cells, it appears that only this isoform can induce the increase in expression. Cell lines that do not display TNC-9/16 expression have lower levels of STAT1 mRNA, but do express a number of other isoforms that could mask the effect of TNC-9/14/16.

The variation of STAT1 expression in MCF-7 cells transfected with isoforms of TNC raises intriguing questions as to the method of action of each isoform. TNC-9/16 transfected cells appear to show similar results to reports in the literature; suggesting
that this isoform could exert its effects on tumour progression by playing a role in tumour surveillance and apoptotic pathways. However, the increase in expression of STAT1 in MCF-7s expressing TNC-9/14/16 isoforms suggests that each isoform executes their effects in specific ways. STAT1 elevation in tumour-associated macrophages has been linked to the suppression of T-cell mediated immune responses in tumours (Kusmartsev & Gabrilovich, 2005). This could be responsible for an up-regulation of inducible nitric oxide synthase (iNOS) – which is known to be expressed in basal-like tumours (Glynn, 2007) – and in turn enhance suppression of T-cells, providing a link with TNC-9/14/16 expression and host immune system evasion by tumour cells.

5.5.2.3 Profilin 1 (PFN1)

Profilins are small (14 – 17 kDa) ubiquitous proteins that are important regulators of F-actin dynamics in cells (Theriot & Mitchison, 1993; Schlüter, 1997). They bind monomeric actin (G-actin) and, depending on the conditions, may either inhibit or promote actin filament assembly (Carlsson et al., 1977; Pantaloni & Carlier, 1993; Kang et al., 1999). PFN1 has been shown to have a tumour suppressing effect on breast cancer cells (Janke et al., 2000; Roy & Jacobson, 2004; Zou et al., 2007) via dephosphorylation of AKT and post-transcriptional up-regulation of PTEN (Das et al., 2009). Furthermore, silencing of PFN1 expression in human umbilical vein endothelial cells using siRNAs leads to a significant reduction in the formation of actin filaments and focal adhesions (Ding et al., 2006), and exogenous expression of PFN1 causes human breast cancer cells to adopt a non-tumourigenic phenotype (Zou et al., 2007).

Microarray analysis showed that TNC-9/14/16 and TNC-14/AD1/16 isoforms up-regulated expression of PFN1 in transfected MCF-7s. Conversely, validation by (RT)-qPCR showed that expression of PFN1 was significantly down-regulated. This disparity between microarray and (RT)-qPCR data highlights one important limitation of microarray data: sequence specificity. This (probably) false positive result could reflect an error in the sequences of the GeneChip. Other possibilities include cross-hybridization by splice variants, related genes and/or pseudo-genes. However, (RT)-qPCR validation showed great correlation with studies in the literature, suggesting PFN1 to be a tumour-suppressor and down-regulation of this gene increases cell motility and invasion, characteristics shown by cells expressing TNC-9/14/16 in particular. Roy et al. (2004) showed by stably transfecting PFN1 into the BT474 breast...
cancer cell line that migration of these cells was significantly reduced. Janke et al. (2000) showed that transfection of PFN1 cDNA into the CAL51 breast carcinoma cell line drastically reduced its growth rate, encouraged cell anchorage and spreading as well as forming structures reminiscent of glandular organisation in 3D-ECM assays. Breast cell lines (as described in Chapter 3) expressing high levels of TNC-14/16 showed lower PFN1 expression (the exception being Hs578T and isolated fibroblasts), and these cells also show a high invasive capacity. Moreover, high expression shown in isolated myoepithelial cells further enhanced the PFN1 “tumour suppressor” theory (Sternlicht et al., 1997). (RT)-qPCR confirmed that PFN1 was down-regulated in breast carcinomas. Furthermore, this was significantly associated with an increase in TNC-14/16 expression, thereby providing a link for the first time between TNC and PFN1 expression and could provide a possible mechanism by which TNC exerts its effects.

**5.5.2.4 Amphiregulin (AREG)**

Amphiregulin (AREG) is a heparin-binding, glycosylated protein originally identified as an EGFR ligand in MCF-7s (Shoyab et al., 1988) and is expressed in many tissues, including placenta, ovary, testis, heart, pancreas, spleen, kidney, lung, ovary, colon and breast (Plowman et al., 1990). AREG is evolutionarily and structurally related to EGF and TGF-α: the C-terminal 40 amino acid segment of the human protein (residues 44–84) has 38% and 32% sequence identity with human EGF and TGF-α, respectively (Shoyab et al., 1989). Like other members of the EGF ligand family, AREG mediates its action by binding to and activating its respective cell surface receptor. In addition to EGFR activation, AREG has also been shown to activate ErbB2, ErbB3 and ErbB4 in an EGFR-dependent manner, thus promoting receptor heterodimerisation (Johnson et al., 1993; Beerli & Hynes, 1996; Riese et al., 1996). Under normal conditions, expression of AREG is tightly regulated (Plowman et al., 1990); however, expression is increased after liver injury (Berasain et al., 2005b) and is involved in cell proliferation during liver regeneration (Berasain et al., 2005a). In the developing breast (McBryan et al., 2008), AREG has been proposed as a paracrine regulator of oestrogen-induced ductal morphogenesis (LaMarca & Rosen, 2007), whereas in cancer, protein expression of AREG is generally higher in invasive ductal carcinomas than in DCIS or in normal, non-involved mammary epithelium (Salomon et al., 1995). Moreover, AREG is expressed by tumour epithelium, not stroma and is associated with the aggressiveness of breast cancer cell lines (Ma et al., 1999).
Microarray analysis showed AREG expression was increased in cells transfected with TNC Short, TNC-9/14/16 and TNC-14/AD1/16. Validation of this by (RT)-qPCR correlated with the microarray results, but showed TNC Short to have the greatest effect on AREG expression. The fact that TNC Short also up-regulated AREG expression (as did TNC-9/14/16 and TNC-14/AD1/16) would suggest that the effect TNC has is not isoform specific.

Results obtained in TNC transfected MCF-7s correlated in part with observations in the literature. TNC-9/14/16 expressing cells were shown to up-regulate AREG expression, a finding consistent with the invasive phenotype seen in mammary carcinomas (LaMarca & Rosen, 2007). Moreover, the high expression of AREG seen in carcinoma cells lines such as MCF-7, T-47D as well as isolated myoepithelium and very low expression in isolated fibroblasts in this study further confirms the suggestion that AREG is expressed by tumour epithelium. AREG expression has been postulated to be induced by oestrogen receptor. *In-vitro* experiments using the oestrogen positive MCF-7 cell line have shown that treatment with oestrogen can lead to enhanced expression of AREG at both the mRNA and protein levels (Martinez-Lacaci et al., 1995). This study has shown that ER negative cell lines do not show high levels of AREG expression, further enhancing the hypothesis that AREG expression is driven by ER and also shown in this study by analysis of breast cancer cell lines, which showed a significant correlation between TNC-14/16 expression in ER negative cell lines and a down-regulation of AREG. One mechanism by which AREG expression is postulated to be increased is through the canonical Wnt/β-catenin pathway (Katoh & Katoh, 2006). The canonical Wnt pathway has been shown to be induced by exposure to TNC (Ruiz et al., 2004) (section 5.4.4.6 for further details) and this could provide a mechanism by which AREG is up-regulated by TNC. The down-stream effect of AREG up-regulation could be an increase in expression of cyclin D1 (CCND1). Shin et al. (2003) showed that treatment of human thoracic aortic smooth muscle cells (HTASMCs) with 100 ng/ml of AREG induces phosphorylation of ERK 1/2 and Elk1, which in turn up-regulates CCND1 expression. However, the suggestion that AREG is associated with the aggressiveness of breast cancer cell lines (Ma et al., 1999) was not confirmed due to the very low levels of expression seen in highly invasive cell lines such as MDA-MB-231, Hs578T and HBL-100.
5.5.2.5 Cytokeratin 8 (CK8)

Cytokeratin 8 (also known as keratin 8) is a type II keratin (Moll et al., 1982) of approximately 53 kDa and along with cytokeratin 18 (CK18), forms the major components of the intermediate filaments of simple or single layered epithelial tissues from which many carcinomas arise (Oshima et al., 1996). The expression of CK8 and CK18 in adult tissues has been well documented (Moll et al., 1983b; Debus et al., 1984; Quinlan et al., 1985); with normal mammary cells (i.e. myoepithelia) expressing CK5 and CK14 while luminal cells express epithelial cytokeratins 8, 18 and 19 (Nagle et al., 1986; Dairkee et al., 1988; Taylor-Papadimitriou et al., 1989). Hence, tumours expressing CKs 8, 18 and 19 are defined as “luminal” (Rejthar & Nenutil, 1997; Malzahn et al., 1998). Furthermore, expression of the luminal cytokeratins has been associated with a favourable prognosis (Takei et al., 1995; Schaller et al., 1996; Abd El-Rehim et al., 2004), with CK8/18 being inversely associated with tumour grade, better overall survival and negative ER status, and can be used as an independent prognostic factor of relapse-free survival (Takei et al., 1995).

Expression of CK8 in cells transfected with TNC isoforms was significantly up-regulated with TNC-9/16, TNC-9/14/16 and TNC-14/AD1/16. This was surprising as TNC positive breast carcinoma cell lines all showed low level expression of CK8, suggesting that expression of these isoforms within “luminal-like” cells further enhances CK8 expression. It is well documented that the loss of luminal cytokeratins (i.e. 8, 18 and 19) is significantly associated with a higher tumour grade, high mitotic index, negative oestrogen/progesterone-receptor status (Willipinski-Stapelfeldt et al., 2005) and poor clinical outcome (Woelfle et al., 2004). Moreover, reduced expression of CK8, 18 and 19 in primary breast carcinomas is associated with the presence of micro-metastatic tumour cells in bone marrow (Woelfle et al., 2003). Another factor which could explain the low level expression of CK8 in the TNC positive cells lines is the co-expression of vimentin. It has been previously reported that TNC is co-expressed along with vimentin (Dandachi et al., 2001), and that the loss or down-regulation of CK8 is also synonymous with vimentin expression (Guelstein et al., 1988), which is associated with an epithelial-to-mesenchymal transition (Thiery, 2002). The highly invasive breast cancer cells used in this study were all positive for vimentin expression, perhaps explaining why these cells express CK8 at such low levels. Also, expression of CK8 in isolated myoepithelial cells correlates with previous studies, and are more likely to express the basal cytokeratins CK5, 6 and 14 (Walker et al., 2007).
MCF-7 cells express CK8 at a high level (Moll et al., 1982) and with a further increase due to TNC isoform expression, this could suggest that the effect TNC has on CK8 expression is dependent upon the cell type it is expressed in. Moreover, expression of cytokeratins is known to be conserved during tumour progression (Moll et al., 1983a), therefore a sudden loss of CK8 expression within MCF-7 cells is unlikely. Expression of CK8 in tumours showed no significant difference compared to breast organoids. CK8 is known to be highly expressed within the inner glandular epithelium in normal breast acini (Bankfalvi et al., 2004). Furthermore, the majority of breast tumours analysed in this validation study were luminal cancers (i.e. expressed ER and/or PR), which as described earlier express CK8. Therefore, this could explain why no significant down-regulation in CK8 expression was seen in this study.

5.5.2.6 Cyclin D1 (CCND1)

Cyclin D1 belongs to a family of three closely related D-type cyclins, termed cyclin D1, D2 and D3. These proteins are expressed in an overlapping, redundant fashion in all proliferating cell types, and control cell-cycle progression by activating their respective cyclin-dependent partners CDK4 and CDK6, resulting in the phosphorylation of retinoblastoma protein and advancement through the G1 phase of the cell cycle (Sherr & Roberts, 1999). Amplification of the cyclin D1 gene occurs in 20% of human breast cancers (Dickson et al., 1995), whereas CCND1 protein is over-expressed in > 50% of human mammary carcinomas (Gillett et al., 1994). Such over-expression could result from a trans-acting regulatory disturbance or from a clonal somatic regulatory mutation in one allele of the gene. Using mutational studies Hosowaka et al. (1995) identified a polymorphic NciI site within the coding region of the CCND1 gene and also showed that in ZR-75-1 cells, allele specific amplification of CCND1 is approximately 6-fold (Hosowaka, 1998). Moreover, over-expression is maintained during all stages of disease progression including metastatic lesions (Gillett et al., 1996) and appears to have a causative role in breast cancer formation (Wang et al., 1994); with ablation of CCND1 expression resulting in resistance to breast cancers induced by neu and ras oncogenes (Yu et al., 2001).

Microarray analysis and (RT)-qPCR validation showed a significant increase in CCND1 expression in MCF-7 cells transfected with TNC isoforms TNC-9/16, TNC-9/14/16 and TNC-14/AD1/16. These results are consistent with findings by Boudreau et al. (1996) in fully differentiated mouse mammary epithelial cells (CID-9), who showed that
CCND1 is up-regulated in the presence of TNC in alveolar structures. Interestingly, Zwijsen et al. (1996) showed that over-expression of CCND1 in MCF-7 cells (under low-serum conditions) controls the transition between proliferation and quiescence by stimulating cells to remain in the cell cycle in the absence of growth factors. This is one avenue that could be explored in future studies.

In breast cell lines and isolated populations CCND1 expression was highly variable, with the majority of highly invasive cells showing a low level of CCND1 expression. However, expression of CCND1 in ZR-75-1 was in concordance with observations in the literature (Hosokawa & Arnold, 1998), and showed a high level of CCND1 expression caused possibly by allele-specific amplification (Lammie et al., 1991; Buckley et al., 1993; Zuckerberg, 1995). Furthermore, results obtained in this study also correlated with reports in the literature showing high levels of CCND1 expression in MCF-7, T-47D (Kaabinejadian, 2008) and MDA-MB-231 (Im et al., 2008) cell lines and lower expression in MDA-MB-468 cells (Kaabinejadian, 2008). Expression of CCND1 in HBL-100s was very low and correlated with results shown by Lin et al. (2000a). Interestingly, Lin et al. (2000a) showed that the level of CCND1 expression correlated with nuclear β-catenin activity on its promoter and that high levels of β-catenin in MCF-7 cells correlated with high levels of CCND1 expression. β-catenin expression is enhanced when T98G cells are grown on a TNC substratum (Ruiz et al., 2004), enhancing the canonical Wnt signalling cascade. However, expression of CCND1 was not induced in these cells, suggesting that β-catenin targets different genes depending upon the cell it is activated in. Recently, the canonical Wnt/β-catenin signalling pathway has been shown to up-regulate CCND1 in IDC (Prasad et al., 2007), and with the observation that TNC activates the canonical Wnt pathway in T98G cells, this could provide a mechanism by which TNC isoforms up-regulate CCND1 expression.

This study showed that transfected cells expressing the fully truncated isoform of TNC did not show any up-regulation in CCND1 expression, suggesting that the alternatively spliced domains of the molecule have an effect on its expression. The full length isoform of TNC (TNC Long) has been shown to interfere with cell adhesion to a fibronectin milieu and stimulate proliferation via interaction with the integrin α5β1 co-receptor syndecan-4 (Huang et al., 2001). This in turn enhanced the canonical Wnt signalling pathway by repression of the Wnt inhibitor dickkopf-1 (DKK1). DKK1
expression did not increase in this study (by regression analysis); however, the activation of the canonical Wnt signalling pathway by TNC isoforms via alternative mechanisms cannot be ruled out.

5.6 Conclusion

This study has demonstrated that TNC isoforms TNC-9/14/16 and TNC-14/AD1/16 affect global gene expression with a similar profile of changes and that the profiles differ to those for TNC-9/16 and TNC Short. By comparing candidate gene expression in transfected cells and established cell lines, the data demonstrate that the effect of TNC isoform expression is dependent upon the cell background it is expressed in. This study has shown that PFN1 is down-regulated in cells transfected with TNC-9/14/16 and TNC-14/AD1/16 (by (RT)-qPCR analysis) and that down-regulation of PFN1 in invasive carcinoma tissue is significantly associated with TNC-14/16 expression. This association could therefore provide a novel mechanism by which TNC isoforms affect tumour cell invasion.
Chapter 6: Discussion
6.1 Expression of higher MW TNC isoforms in cell lines and breast tissue

6.1.1 Expression in cell lines and isolated populations

Malignant epithelial cells have been shown to be an important source of TNC in intraductal and invasive breast carcinomas (Lightner et al., 1994; Yoshida et al., 1997). Data generated in this thesis has shown a switch in isoform distribution, with isolated fibroblasts and myoepithelial cells showing distinct differences in the level of isoform expression. Myoepithelial cells have been shown to be the major source of TNC-AD1 and fibroblasts the major source of TNC-14/16, suggesting that they have different modes of action. Moreover, the expression of high MW TNC isoforms appears to be indicative of cell phenotype. The established breast cell lines can be classified as “myoepithelial-like”, “luminal-like” or “fibroblast-like”, depending on their phenotype. TNC null cells (i.e. MCF-7, T-47D and ZR-75-1) can be considered to exhibit a luminal phenotype as they are ER and PR positive (Hall et al., 1990; Thompson et al., 1992; Hollywood & Hurst, 1993) and grow as tightly cohesive epitheloid cell colonies (also known as having a “cobblestone” morphology). They are also positive for E-cadherin (Hall et al., 1990; Hollywood & Hurst, 1993) and epithelial membrane antigen (EMA) (Gordon et al., 2003) but lack vimentin (Thompson et al., 1992; Sommers et al., 1994). Conversely, MDA-MB-231, MDA-MB-436 and HBL-100 exhibit a more myoepithelial-like phenotype. They are ER and PR negative, lack E-cadherin and EMA, but strongly express vimentin (Gordon et al., 2003). A myoepithelial phenotype has been frequently associated with aggressive tumour behaviour (Cattoretti et al., 1988; Domagala et al., 1990a; Domagala et al., 1990b; Peralta Soler et al., 1999). Due to the finding in this study that myoepithelial cells are a major source of TNC-AD1, this particular isoform can be implicated in tumour development and progression. Furthermore, TNC is associated with a more aggressive phenotype indicative of poor prognosis (Ishihara et al., 1995; Yoshida et al., 1995; Tokes et al., 1999; Goepel et al., 2000) and can be considered as another marker associated with the myoepithelial phenotype (Gordon et al., 2003). One alternative to this theory is that TNC expression could be indicative of an undifferentiated phenotype such as that seen in mesenchymal cells. Mesenchymal cells specifically express TNC during normal embryonic development and tumour progression (Chiquet & Fambrough, 1984a; Aufderheide et al., 1987). A loss of cell differentiation occurs within tissue acquiring more mesenchymal, or stromal, characteristics, also known as epithelial-to-mesenchymal
transition (EMT) (Hay, 1995; Thiery, 2002; Huber et al., 2005) and has been observed in cells expressing TNC (Dandachi et al., 2001; Maschler et al., 2004; Beiter et al., 2005), suggesting that TNC may be linked to EMT.

The invasive capacity of a cell cannot be exclusively attributed to the expression of TNC and its many isoforms. As described earlier the expression (or lack) of certain cell markers including ER, PR, E-cadherin, vimentin and certain MMPs in conjunction with certain TNC isoforms appears to endow the cell with a more invasive phenotype (Thompson et al., 1992; Dandachi et al., 2001; Gordon et al., 2003; Kalembeyi et al., 2003; Ilunga et al., 2004), even though studies in our group suggest that TNC does not up-regulate expression of MMPs directly (Hancox et al., 2009). It is well established that MMPs play a crucial role in tumour metastasis (Duffy et al., 2000), in particular, expression of TNC up-regulates expression of the collagenase, MMP-9 (Kalembeyi et al., 2003). This enzyme plays an important role in the degradation of gelatine and type IV collagen (Somari et al., 2006) - the main components of basement membrane (Liotta et al., 1980) – and the expression of TNC-14/16 could be a major influence in the up-regulation of this protease. Moreover, expression of PR on the cell membrane also appears to have an influence on tumour cell invasiveness. Lin et al. (2001) showed that exogenous expression of PR in MDA-MB-231 cells increased attachment to ECM proteins (via induction of focal adhesions (Lin et al., 2000b)) and also down-regulated urokinase plasminogen activator (uPA) and up-regulated tissue-type PA (tPA). uPA and tPA are known to be related to cell invasiveness and metastatic potential, with low levels of uPA and high levels of tPA in breast cancer tissue being associated with overall disease-free survival and a lower relapse rate (de Witte et al., 1999; Nielsen et al., 2007). With all of these factors taken into account, the expression of high MW isoforms of TNC could have synergistic effects on tumour cell invasion and proliferation.

6.1.2 High MW TNC isoforms and tumour phenotype

A range of prognostic markers have been investigated previously (reviewed in Esteva & Hortobagyi, 2004). In this study TNC-AD1 and co-expression of AD1 and AD2 was associated with negative ER. ER and PR status has been used since the 1970s for the management of breast cancer both as an indicator of endocrine responsiveness and as a prognostic factor for early recurrence. Moreover, patients with systemically untreated ER-positive/PR-positive tumours have better clinical outcomes compared with women
with ER-negative/PR-negative tumours, affirming the prognostic significance of the receptor-positive phenotype (Bardou et al., 2003). Although PR status is used as an indicator of a functionally intact oestrogen response pathway (Horwitz & McGuire, 1975), ER status is very important clinically as it mediates the growth-stimulating effects of circulating oestrogen and tumours that do not express ER are much less likely to respond to hormonal therapies such as tamoxifen and aromatase inhibitors (Elledge, 2004). Moreover, even if PR status is negative, many tumours are still positive for ER and respond to treatment (although the efficacy is reduced (Rhodes & Jasani, 2009)) and could be a more useful indicator of response to treatment due to the recent studies questioning the validity of ER-/PR+ tumours (De Maeyer et al., 2008; Rhodes & Jasani, 2009). In addition, ER negative tumours have also been shown to have high expression of c-erbB2 and EGFR (Putti et al., 2005) as well as high incidence of BRCA1 mutation and high grade (Robson et al., 1998; Foulkes et al., 2004). With this in mind, TNC-AD1 could potentially predict functional ER and hence influence clinical management.

In this study, all high MW TNC isoforms investigated were significantly associated with younger age. Breast carcinomas in younger women exhibit a more aggressive phenotype compared to tumours arising in post-menopausal women (Adami et al., 1986; Yildirim et al., 2000; Arnes et al., 2005; El Saghir et al., 2006). This may in part be attributed to the greater frequency of high-grade tumours in young women (Walker et al., 1996). However, there is evidence that tumours in younger women are biologically distinct (Johnson et al., 2002), being associated with poorer survival independent of tumour grade and stage (Chung et al., 1996b) and displaying a higher frequency of loss of heterozygosity (LOH) compared to grade- and stage-matched postmenopausal cancers (Pillers, 1992). The association of high MW TNC isoforms with young age, independent of tumour grade further supports the hypothesis that these tumours are biologically distinct.

6.2 Effects of high MW TNC isoforms on cell invasion

Previous studies in our group have shown that high MW isoforms of TNC (in particular TNC-9/16 and TNC-14/16) increased the invasive capacity of carcinoma cells both directly (through transient transfection of the carcinoma cells) and in-directly (through transient transfection of fibroblasts and 2D invasion assays showing their effect on carcinoma cells) (Hancox et al., 2009). In order to extend these experiments, a new protocol was performed measuring invasion in real-time and also assess for the first
time whether high MW isoforms containing AD1 increase the invasive capacity of carcinoma cells as was suggested by Derr et al. (1997). Initial cloning experiments to produce the two intermediate-sized isoforms containing AD1 identified in this study and previously within the group (TNC-14/AD1/16 and TNC-14/AD1/15/16) were successful but the larger of the two isoforms (TNC-14/AD1/15/16) was not detected by Western blotting after being transfected in-vitro. Different antibodies to TNC could be used in order to detect expression (if any) and if the result is still negative then further cloning of this isoform is therefore required for any functional studies to be performed.

This study showed for the first time that AD1 containing isoforms significantly increase cell invasiveness compared to TNC Vector Only controls and to a similar extent as TNC-9/14/16. The results obtained in this study also showed that the protocol used gives results for invasion comparable to the conventional 2D invasion assay but is more robust and less labour intensive, suggesting that this method could be used in the future for more extensive studies. However, further optimisation of this protocol is required, possibly using a variety of breast adenocarcinoma cell lines to further assess its efficiency and robustness.

The discovery that AD1 increases cell invasiveness raises important questions as to its role in cancer progression. With the notion that it increases invasion comparable to TNC-9/14/16, as well as being highly expressed in tumour epithelium (Hancox et al., 2009), this would suggest that expression of high MW TNC isoforms in both the epithelium and stromal compartments are important for tumour progression. Currently, there is no commercially available antibody for AD1 detection; however, the molecular cloning of AD1 containing isoforms raises the possibility of antibody production. Although production of such an antibody would be time consuming and costly, immunohistochemical studies using an antibody against these isoforms would provide valuable information regarding their distribution and expression in-vivo. Furthermore, this antibody could also be used to analyse an array of cell lines to assess whether mRNA levels observed in this study correlate with protein expression. Finally, repeat experiments using stably transfected cells would reduce any inconsistencies produced through transient transfection.
6.3 Differential gene expression produced by TNC isoforms in cancers

The cDNA microarray analysis proved to be a useful tool in the elucidation of differential expression caused by high MW TNC isoform expression, with analysis of the candidate genes in breast tissue showing that PFN1 is a candidate gene for further analysis due to its association with TNC-14/16 expression. However, questions could be raised regarding the use of breast organoids as a control sample due to the concentrated nature of myoepithelial and luminal cells within the sample and lack of stromal fibroblasts. Further studies of these candidate genes should be performed using tissue from reduction mammoplasties that has not been manipulated to give a more realistic control sample. That said, PFN1 did show significant differences due to TNC-14/16 expression, with increased expression in samples positive for TNC-14/16 showing decreased expression of PFN1. This discovery in breast cancer tissue and cell lines is novel, although down-regulation of PFN1 has been shown to increase cancer cell invasion (Roy & Jacobson, 2004). This is the first study to relate TNC isoform expression to a down-regulation of PFN1 expression and to implicate TNC isoforms to immune system evasion by tumour cells. However, it is important to note that the data are preliminary, and further confirmatory studies are needed. Additionally, the contradiction of expression in PFN1 isoform expression between cDNA microarray studies and (RT)-qPCR analysis highlights the importance of microarray validation and also illustrates the possibility of false positives, further exemplifying the importance of (RT)-qPCR validation.

Unfortunately, due to the extensive use of cDNA from both breast organoids and tumour tissue for (RT)-qPCR studies in Chapter 3, samples for validation of cDNA microarray experiments were limited. Consequently, although enough samples were available for statistical analysis comparing candidate gene expression in breast organoids vs. tumour tissue, there were not enough samples for a statistically significant analysis of differential gene expression in relation to TNC-AD1 isoform expression. To address this, a new, larger cohort of breast tissue samples is required. Moreover, markers described in section 6.1 could be used to assess whether these candidate genes could also have prognostic value.
6.4 Conclusion

In conclusion, this study has extended previous findings by the research group and has generated novel data to support the hypothesis that high MW isoforms of TNC are associated with a number of clinicopathological features of breast cancer, tumour progression and tumour cell invasion. The most significant findings of this thesis have resulted from extensive (RT)-qPCR studies, and have shown associations with negative ER, high grade and young patient age. Two intermediate sized TNC isoforms containing AD1 were discovered and successfully cloned, although one isoform failed to be translated into protein. Moreover, preliminary 2D invasion assays using AD1-containing isoforms showed an increase in carcinoma cell invasion to a similar level as TNC-9/14/16. Microarray analysis of differential gene expression was successful for MCF-7 cells transfected with a number of TNC isoforms and a novel gene (PFN1) was associated with breast cancers expressing TNC-14/16 and warrants further investigation.

6.5 Future work

Future work using a larger cohort of samples from both normal breast (i.e. from reduction mammoplasties) and tumour tissue from various stages of disease is needed. Moreover, although direct amplification of TNC isoforms from cDNA was a useful method for quantitative analysis of isoform expression in breast tissue, again a larger cohort of samples should be used for more powerful statistical analysis to be performed. In addition, histopathological profiling involving a larger number of markers – including c-erbB2, BRCA1 mutation and EGFR – would also be useful to assess whether these changes in tumour pathology are associated with high MW TNC isoform expression. A more extensive analysis of the basal markers (including CK 5/6 and CK 14) would also be useful to assess whether TNC expression is associated with basal phenotype, even though this study suggests otherwise. Therefore, markers associated with a luminal phenotype (including CK 7/8, CK 18 and CK 19 (Abd El-Rehim et al., 2004)) could be analysed for this purpose using tissue microarrays (TMAs).

In relation to the transfection studies, there are a number of other candidate genes worthy of investigation. A new cohort of breast cancer cases is again required to fully determine any significant correlations with TNC isoform expression. Further work is currently being undertaken in the group in order to further validate the results obtained.
in this study by transiently transfecting MCF-7, T-47D and ZR-75-1 cells with high MW TNC isoforms and culturing them on a FN milieu. Candidate gene analysis using (RT)-qPCR will then be performed to assess whether the effects seen in this study are common among different TNC null cell lines.

One final aspect of this study that warrants further investigation is to more fully elucidate the effect of TNC-14/AD1/16 on tumour cell invasion. Additional studies using the BMG FLUOstar plate reader and a fully optimised protocol would be useful to assess whether inter-assay reproducibility can be attained in order to give more statistically significant results. Also, re-cloning of TNC-14/AD1/15/16 would provide an excellent opportunity to investigate whether exon 15 increases the effect of AD1 on tumour cell invasion. If these studies are successful then it would be worth elucidating the mechanism by which the specific isoforms promote tumour cell invasion using, for example, blocking antibodies and siRNA approaches.

6.6 Therapeutic potential of TNC and its isoforms

The targeted delivery of bioactive molecules (e.g. antibody constant regions, cytokines, drugs, radionuclides, photosensitizers, pro-coagulant factors, etc.) to the tumour environment by means of ligands specific to good-quality tumour-associated antigens and endowed with suitable pharmacokinetic properties is a promising avenue for the therapy of disseminated cancers (Carter, 2001; Neri and Bicknell, 2005). Due to the expression of TNC correlating with tumourigenesis-enhancing events, this seems a natural step in a therapeutical approach to cancer treatment.

Currently, the most promising approach is to target TNC with anti-TNC-directed antibody fragments (Merlo et al., 1997; Silacci et al., 2005) or aptamers (Daniels et al., 2003; Schmidt et al., 2004) coupled to cytotoxic reagents, such as radioactive iodine (\(^{131}\)I). Petronzelli et al. (2005) reported improved tumour targeting through the use of a monoclonal anti-TNC antibody mixture, whereas aptamers offer an attractive alternative due to being short DNA or RNA sequences that can adopt a specific and stable three-dimensional shape \textit{in-vivo}. Therefore, they provide tight, specific binding to protein targets (reviewed in Ireson and Kelland, 2006).

The use of radio-labelled anti-TNC antibodies is currently being investigated during clinical trials in glioblastoma patients. Reardon et al. (2002; 2006) used \(^{131}\)I-labelled
anti-TNC in a phase-II trial and showed that patients treated with the $^{131}$I-labelled 81C6 anti-TNC antibody injected directly into the surgically created resection cavity (SCRC) followed by conventional external-beam radiotherapy and a year of alkylator-based chemotherapy displayed a longer median survival term than patients treated with conventional radio- and chemotherapy. They also showed that tumours in patients treated with $^{131}$I-labelled 81C6 anti-TNC antibody shrunk considerably. Subsequent trials showed that injection of $^{131}$I-labelled 81C6 anti-TNC antibody into the SCRC at a specific dosage (44 Gy) was the optimal dosage and produce longer patient survival (Akabani et al., 2005; Reardon et al., 2008). Other studies have investigated the use of chimeric anti-TNC antibodies in the treatment of gliomas. Zalutsky et al. (2008) used a $^{211}$At-labelled chimeric 81C6 anti-TNC antibody ($^{211}$At-ch81C6) administered directly into the SCRC and then treated with salvage chemotherapy. They showed that use of this antibody was feasible, safe and associated with a promising anti-tumour benefit in patients with malignant central nervous system tumours (i.e. longer patient survival). It is important to note that direct injection of these antibodies into the SCRC was necessary to produce the anti-tumourigenic effects as intra-tumoural penetration of the antibody is limited when administered intravenously or intra-arterially (Zalutsky et al. 1989).

Currently, there are no clinical trials involving the use of anti-TNC antibodies to treat patients with breast cancer. Furthermore, the number of antibodies available for specific regions of the TNC molecule is very limited. However, the potential for use of radio-labelled anti-TNC antibodies in breast cancer should not be ignored. As shown in patients with malignant gliomas (see above), the use of radio-labelled anti-TNC antibodies injected directly into the tumour shows great promise in reducing both the size of the tumour and subsequent prognosis. If antibodies to specific isoforms of TNC can be generated (namely TNC-9/16 and TNC-9/14/16) this could provide therapy for specific isoforms associated with invasion and proliferation (Adams et al., 2002; Hancox et al., 2009).
Appendices
Appendix I: Recombinant TNC sequences containing AD1

A) TNC-14/AD1/16:

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601  ATTGATGGGCCAGATGACATGCTGAGACAGGCCTTACCGGCCAGAGCTGCCACGGCCTTC
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3641  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
3701  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
3761  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
3821  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
3881  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
3941  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
4001  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
4061  AATATCTCTGCTGAGGATTTGGAAGTCTTGCTGAGGGAGAACCTACCCATCGATCTCACAGG
4121  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
4181  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
4241  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
4301  AATATCTCTGCTGAGGATTTGGAAGTCTTGCTGAGGGAGAACCTACCCATCGATCTCACAGG
4361  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
4421  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
4481  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
4541  AATATCTCTGCTGAGGATTTGGAAGTCTTGCTGAGGGAGAACCTACCCATCGATCTCACAGG
4601  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
4661  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
4721  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
4781  AATATCTCTGCTGAGGATTTGGAAGTCTTGCTGAGGGAGAACCTACCCATCGATCTCACAGG
4841  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
4901  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
4961  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
5021  AATATCTCTGCTGAGGATTTGGAAGTCTTGCTGAGGGAGAACCTACCCATCGATCTCACAGG
5081  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
5141  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
5201  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
5261  AATATCTCTGCTGAGGATTTGGAAGTCTTGCTGAGGGAGAACCTACCCATCGATCTCACAGG
5321  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
5381  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
5441  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
5501  AATATCTCTGCTGAGGATTTGGAAGTCTTGCTGAGGGAGAACCTACCCATCGATCTCACAGG
5561  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
5621  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
5681  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
5741  AATATCTCTGCTGAGGATTTGGAAGTCTTGCTGAGGGAGAACCTACCCATCGATCTCACAGG
5801  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
5861  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
5921  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
5981  AATATCTCTGCTGAGGATTTGGAAGTCTTGCTGAGGGAGAACCTACCCATCGATCTCACAGG
6041  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
6101  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
6161  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
6221  AATATCTCTGCTGAGGATTTGGAAGTCTTGCTGAGGGAGAACCTACCCATCGATCTCACAGG
6281  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
6341  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
6401  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
6461  AATATCTCTGCTGAGGATTTGGAAGTCTTGCTGAGGGAGAACCTACCCATCGATCTCACAGG
6521  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
6581  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
6641  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
6701  AATATCTCTGCTGAGGATTTGGAAGTCTTGCTGAGGGAGAACCTACCCATCGATCTCACAGG
6761  GACGTGCCAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
6821  CAAAAGGAGACCCTTCCACCCATCGATCTCACAGAGGACGAGAACCTACTCCATC
6881  CTGACCCTGCTCTGGAAGACACCGTTGGCCAAATTTGACCGCTACCGCCTCAATTACG
6941  AATATCTCTGCTGAGGATTTGGAAGTCTTGCTGAGGGAGAACCTACCCATCGATCTCACAGG
6999 | 209 | Page
E N L T I S D I N P Y G F T V S W M A S
GAAAACCTAACATTTCCGACATTAATCCCTACGGGTTCACAGTTTCCTGGATGGCATCG
E N A F D S F L V T V V D S G K L L D P
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Q E F T G Q R K L E L R G L I T G
CAGGAATTCACATTTCCGACATTAATCCCTACGGGTTCACAGTTTCCTGGATGGCATCG
I G Y E V M V S G F T Q G H Q T K P L R
ATTTGGCTATGAGGTATAGTGCTCTGCTCTACCCAGAGGACATCAAACAGGCCCCGAG
A E I V T E A E P E V D N L L V D S A T
GCTGAGATTGGTTGACAGAACCGGAAAGCTGGAGCTCGGACCAACTTTTGTCTC
P D G F R L S W T A D E G V F D N F V L
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K I R D T K Q S E P L E I T L A P E
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R T R D L T G L R E A T E Y E I L Y G
CGTACCAGGGACTTTACAGGCTCAGAGAGGCTACTGAATACGAAACTTGACATAGG
S M V T V D G T K T Q T R L V K L I P G
TCCATGTTAACTGTGGAGCCGAGAAGCTCGAGCTGATGACAGGTGGCCCA
P K E V I F S D I T E N S A T V S W R A
CCAAAGGAAGTCATTTTTTCATCAGACATCATCAGAAAATTCGCTACTGTGAGCTGAGGCGA
P T A Q V E S F R I T Y V P I T G G T P
CCCACTACGGCCAGCTGAGGCTCAGGCTTACCTATGTGCCATGACAGAGGTGATACCC
S M V T V D G T K T Q T R L V K L I P G
TCCATGTTAACTGTGGAGCCGAGAAGCTCGAGCTGATGACAGGTGGCCCA
V E Y L V S I I A M K G F E E S E P V S
GTGGAGTACCCATTGTGACACTCATCAGCCATGAAATTCGCTACTGTGAGCTGAGGCGA
G S F T T A L D G P S G L V T A N I T D
GGGTCACTACCCAGGCTCTGGATGCGCCCTATGCGTCTGAGCAAGCTATGACAGG
S E A L A R W Q P A I A T V D S Y V I S
TCAGAAGGCTTGGGAGCAAGCAGCCAGCCATGTGGAAGCTGATGACAGGTGGCCCA
Y T G E K V P E I T R T V S G N T V E Y
TACACAGGGCAGAAAGCGGTGCCAAGTATACAGCAGGCGGTGCGGGAACACAGGTGGAGAT
A L T D E P A T E Y T L R I F A E K G
GCTCTGAGGCACTGCGGAGCTGGGCAACAGAATACACACTGAAGATCTGCGAGAAGGG
P Q K S S T I T A K F T T D L D S P R D
CCCCAGAGGAGGCTCAACCATCATCCTGGCAGAAATCTGCGTCTGACCTACCCAAAGGAC
L T A T E V Q S E T A L L T W R P R A
TTGACTGCTACTGGAGTGTCAGGGAACACCTGCCCCTTACCTGGCCGACAGCCCACTACAGC
S V T G Y L L V Y E S V D G T V K E V I
TCAGTCCAGGTTACCTCGCTGGTCTATGAAACTCGTGAGAGTGACATGCAAGGAGGAGTCATT
V G P D T T S Y S L A D L S P S T H Y T
GCTGGCTCAGATACCCACCTCTACAGGCTTGGGAGCAAGCAGCCAGCCATGTGGAAGCTGATGACAGGTGGCCCA
Appendix II: BioAnalyser traces

Total RNA integrity

Ladder

TNC Vector Only 1

TNC Vector Only 2

TNC Vector Only 3

TNC Short 1

TNC Short 2

TNC-9/16 1

TNC-9/16 2
Graphical representation of total RNA gel
BioAnalysis of fragmented cRNA
Graphical representation of fragmented cRNA
Images of stained GeneChips from GCOS

TNC Vector Only 1
TNC Vector Only 2
TNC Vector Only 3
TNC Short 1
TNC Short 2
TNC-9/16 1
TNC-9/16 2
Appendix III: Publications arising from this thesis

The following publications and sequences arising from this thesis are either in print, online or in preparation: -


**Appendix IV: Ethical Approval**

Fresh and/or formalin fixed tissue was obtained from patients undergoing breast surgery in accordance with Ethics Approval from Leicestershire LREC and North East London LREC held by Professors RA Walker and JL Jones respectively.
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