The Expression and Distribution of Tenascin C in Breast Cancer Invasion

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By

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Abstract

The expression and distribution of tenascin C in breast cancer invasion

Tenascin C (TNC) is an extracellular matrix protein that is expressed at low levels in normal adult tissue but is highly expressed around breast cancers. TNC exists as multiple isoforms generated through alternative splicing. Isoform expression in benign and malignant breast disease was investigated.

Significant differences in TNC isoform profile were identified. Whilst all tissues expressed the fully truncated TNC, expression of two additional isoforms was significantly associated with the invasive phenotype ($p<0.001$). A subset of pre-invasive carcinomas also expressed these additional isoforms. Furthermore, expression correlated with the presence of additional protein isoforms in stroma, where they were produced by stromal fibroblasts in malignant tissue, and both periductal fibroblasts and residual myoepithelial cells in ductal carcinoma *in-situ* (DCIS).

In-vitro experiments indicated growth factor specific induction of TNC. Epidermal growth factor induced expression of higher molecular weight TNC isoforms but initial investigations with transforming growth factor β1 had no effect.

Two highly invasive breast cancer cell lines (MDA-MB 231 and MDA-MB 468) were found to produce TNC in contrast to tumour cells with a lower invasive capacity (MCF7 and T47D). TNC altered cell morphology and increased migration in cell lines MCF7, T47D and MDA-MB 468, but not MDA-MB 231. Effects on migration via alteration in fibroblast function was also seen in cell lines MCF7, T47D, and MDA-MB 468 , but not cell line MDA-MB 231. This indicates cell type specific direct and indirect effects of TNC on migration.

TNC had no effect on proliferation, or on the expression of a range of matrix metalloproteinases associated with breast carcinoma.

Primary tissue analysis demonstrates for the first time that specific TNC isoforms are expressed in invasive breast carcinomas and that these isoforms are identified in a subset of DCIS. These isoforms may predict invasion and thus provide appropriate targets for therapeutic intervention.

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List of Abbreviations

βME	β mercaptoethanol
ABC	Avidin/ biotin complex
AD	Additional FNIII Domain
ATCC	American Type Culture Collection
BM	Basement Membrane
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	Base Pair
BrdU	Bromo-deoxy uridine
BSA	Bovine Serum Albumin
CAM	Cell adhesion molecule
cDNA	Complementary DNA
CM	Conditioned Media
CNS	Central nervous System
DAB	Diaminobenzidene
DCIS	Ductal carcinoma *in-situ*
DEPC	Diethyl pyrocarbonate
DMEM	Dubeccos modified eagles medium
DMSO	Dimethyl sulphoxide
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
dATP	deoxy-adenosine triphosphate
dCTP	deoxy-cytidine triphosphate
dGTP	deoxy-guanosine triphosphate
dTTP	deoxy-thymidine triphosphate
dUTP	deoxy-uridine triphosphate
dNTP	deoxy-nucleotide triphosphate
dT	dTTP
DTT	Dithiothreitol
ECM	Extra-cellular matrix
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbemt assay
ELOSA	Enzyme linked oligonucleotide assay
FBS	Fetal bovine serum
FCS	Fetal calf serum
FG	Fibrinogen
FGF	Fibroblast growth factor
Fib.	Fibrinogen like domain of tenascin C
FNIII	Fibronectin type III like repeat
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
GGH	Glenfield General hospital
GMEM	Glioma mesenchymal extracellular matrix protein
Hi.C	High confluence cells
HXB	Hexabrachion
IAA	Iso-amyl alcohol
IDC	Infiltrating ductal carcinoma
ILC	Infiltrating lobular carcinoma
IMS	Industrial methylated spirit
ISH	*In-situ* hybridisation
kb	Kilo-base pairs

Kda	Kilo-dalton
LBB	Lysis binding buffer
LCIS	Lobular carcinoma *in-situ*
Lo.C	Low confluence cells
LMP	Low melting point
LRI	Leicester Royal infirmary
MAB	Maleic acid buffer
MABT	MAB plus Tween 20
MEM	Modified eagles medium
min.	Minutes
MMP	Matrix metalloproteinase
MPC	Magnetic particle concentrator
mRNA	Messenger RNA
MT-MMP	Membrane type MMP
NBT	Nitroblue tetrazoleum
OSCC	Oral SCC
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RGD	arginine-glycine-aspartic acid
RIA	Radio-immuno assay
RM	Reduction mammoplasty
RNA	Ribonucleic acid
RT	Reverse transcription
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulphate
sec.	Seconds
SSC	Standard saline citrate
SPARC	Secreted protein acidic and rich in cysteine
TA	Tenascin assembly domain
TAE	Tris acetic acid EDTA
Taq.	*Thermus aquaticus*
TBE	Tris boric acid EDTA
TBS	Tris buffered saline
TdT	Terminal deoxynucleotide transferase
TGFβ	Transforming growth factor β
TIMP	Tissue inhibitor of metalloproteinase
TNC	Tenascin C
TNR	Tenascin R
TNW	Tenascin W
TNX	Tenascin X
TNM	Tumour/ node status/ distal (metastatic) spread
yr	year

PCR reaction nomenclature

The tenascin C oligonucleotides are used in the current study in a variety of combinations, therefore due to the complex structure of tenascin C the following is an explanation of the PCR reaction nomenclature used throughout this study:

1. PCR reactions are described as cassette *x/y* with *x* being the forward primer and *y* being the reverse primer used in the reaction.
2. *x* and *y* give the exon number/designation in which they anneal.
3. A number of primers are designed across exon boundaries, these are designated *a-b* with *a* being the end of the exon before, and *b* being the beginning of the exon after the boundary.
4. Examples:
    a. 8/18 PCR – forward primer within exon 8 and reverse primer within exon 18.
    b. 14/16 PCR - forward primer within exon 14 and reverse primer within exon 16.
    c. 9-14/14-16 PCR - forward primer across the boundary of exons 9 and 14 and reverse primer across the boundary of exons 14 and 16.

Chapter 1 - Introduction

Tenascin C is an extra-cellular matrix glycoprotein which is highly expressed in situations associated with active cell migration and tissue remodelling. It is expressed in a site restricted fashion during embryogenesis and in adult tissue is neo-expressed in the wound healing response and a variety of tumours including breast carcinoma.

In order to understand the role of tenascin C in breast cancer it is first necessary to review overall structure of the breast and what changes occur with the progression of breast cancer. Following this is a detailed explanation of the structure and function of TNC with respect to cancer. The purpose of this study is then outlined with an explanation of objectives.

1.1 Introduction to breast cancer

Breast cancer is the most common cancer amongst women in the Western world. In the UK, currently, it is estimated almost 35,000 new cases will be identified annually. There is a high frequency of relapse in existing cases and a high mortality rate (15,000 PA). Incidence of breast cancer in the UK is amongst the highest in the developed world and is rising, with an age-standardised rate of 75 per 100,000 (Ferlay *et al* 1999; 2000) – age standardised rate is a measure a population would have if it had a standard age structure and is a necessary calculation given the affect age has on cancer incidence (McPherson *et al* 2000).

The major characteristics of cancer are unregulated cell growth and invasion of surrounding tissues, spread to distant organs and ultimately death. Cancer is thought to arise as a result of progressive alterations in the DNA of a cell leading to a malignant phenotype. The cause of these changes in breast cancer is unknown. Thus, identification of factors contributing to the development and progression of the disease is vital. It is hoped that an understanding of these factors will enable improvements to prevention, diagnosis and therapy.

1.1.1 Breast structure

The breast is a secretory organ composed of a network of glandular lobules and ducts contained in a stroma of supporting fat and fibrous tissue. Groups of terminal ducts form the lobules which are responsible for secreting milk during lactation. A system of ducts stem from these lobules and emerge on the surface of the nipple. This system of ducts is lined by a single layer of epithelial cells supported by an underlying layer of myoepithelial cells. The latter is in contact, and is surrounded by a basement membrane that forms a barrier between the ducts and the interstitial stroma which also contains fibroblasts. See figure 1.1.

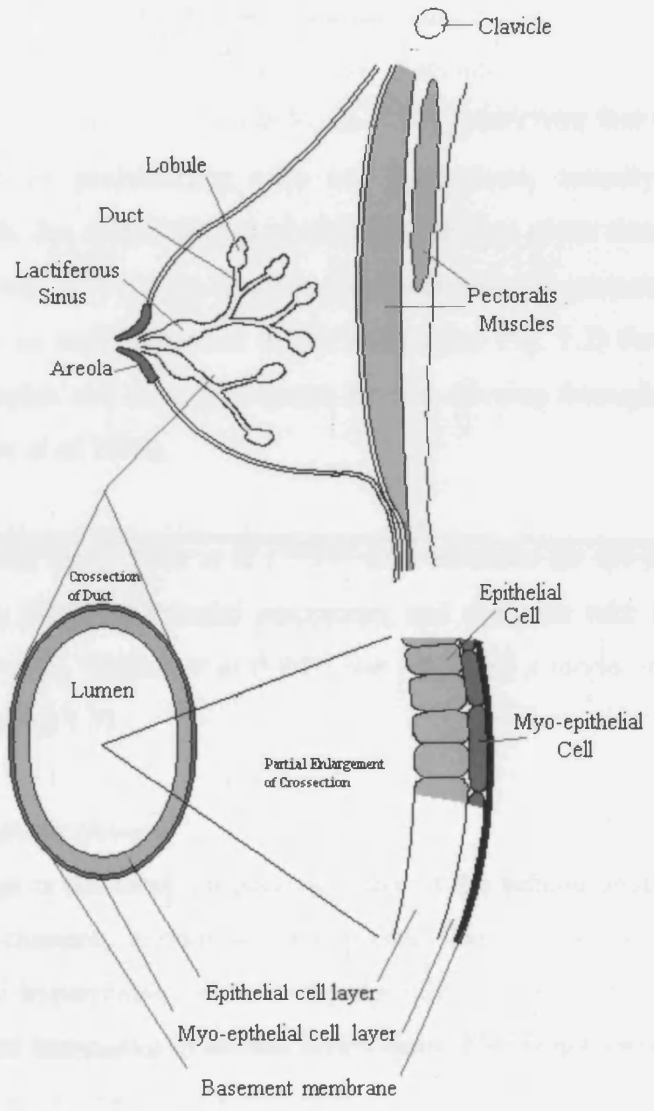


Figure 1.1: structure of the normal female human breast. The upper diagram shows a cross section of the whole breast with ducts and lobules (not to scale). The lower diagram shows a duct in cross section indicating the epithelial and myoepithelial cell layers surrounded by basement membrane.

1.1.2 Development and progression of breast cancer

Cancer is a multistep process in that the development of a malignant phenotype is dependent upon the sequential occurrence of specific events. This sequence is clearly defined in colorectal cancer (Kinzler & Vogelstein 1996), but this is not the case with breast cancer. During the evolution of breast cancer there is no defined benign-to-malignant sequence as a number of sub-clinical changes can arise which may contribute to the development of a malignant phenotype (Devilee *et al* 1994, Walker, 1997). A simplified model proposed by Russo & Russo (1991) postulates that an epithelial cell gives rise to a cohort of proliferating cells i.e. hyperplasia, initially without atypia and subsequently with. An accumulation of molecular events gives rise to neoplasia, initially an *in-situ* carcinoma followed by invasive disease and finally metastatic disease. However, as can be seen in an early depiction of this model (see Fig. 1.2) the progression of breast carcinoma is complex and there is potential for it to develop through a range of alternative pathways (Devilee *et al* 1994).

The model depicted by Devilee *et al* (1994) only accounts for the most common form of breast carcinoma, infiltrating ductal carcinoma, and does not take into account the other histological sub-types. Walker *et al* (1997) has proposed a model involving all the major sub-types (See Figure 1.3).

1.1.2.1 Pre-malignant change

Fibrocystic change is the most common disorder of the female breast and is characterised by proliferative changes, known as hyperplasia, that can occur in lobules, ducts and stroma. Epithelial hyperplasia occurs in a proportion of fibrocystic disease cases with the epithelial cell 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 (Dupont & Page, 1985, Walker 1997). It is not known whether such a condition regularly precedes the disease (Dupont & Page, 1985, McPherson *et al* 2000), but unsurprisingly, a history of breast cancer in one breast increases the risk in the other breast.

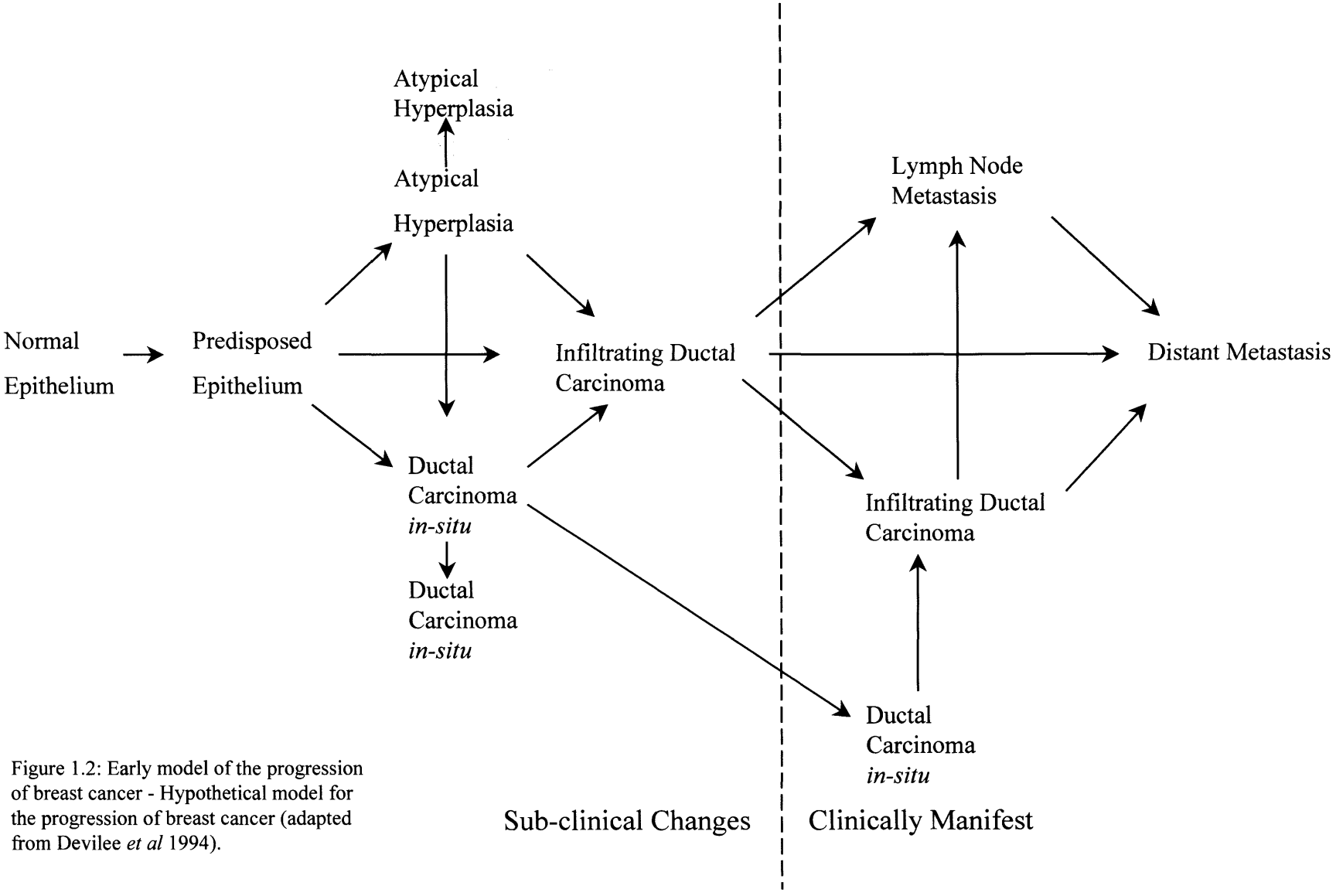


Figure 1.2: Early model of the progression of breast cancer - Hypothetical model for the progression of breast cancer (adapted from Devilee *et al* 1994).

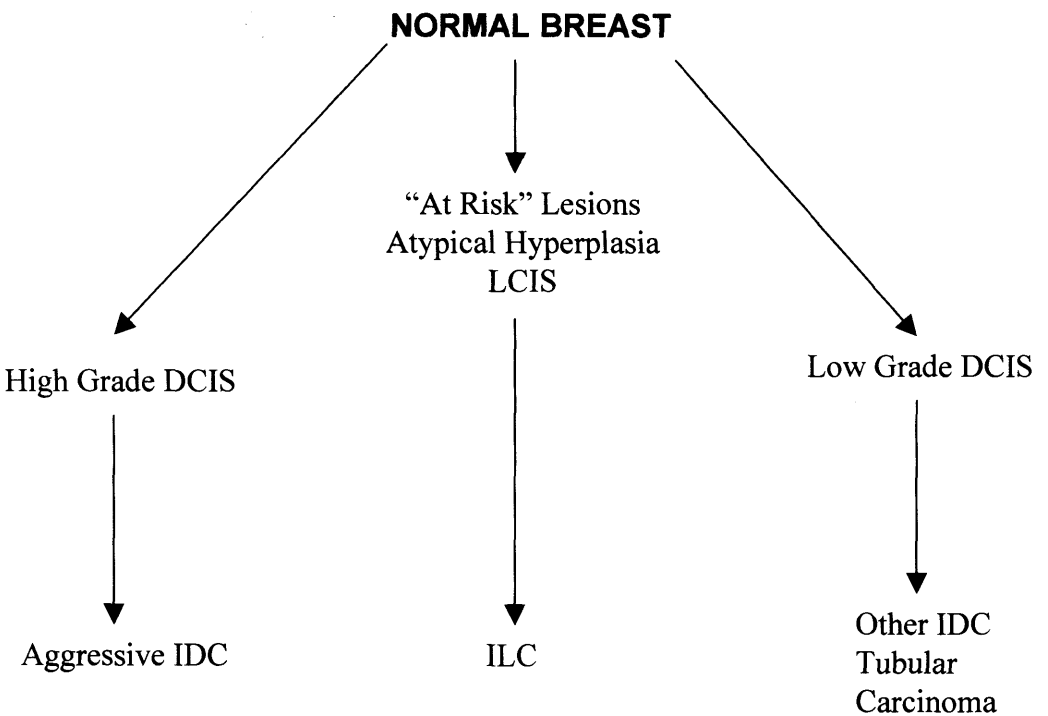


Figure 1.3: Later model for the progression of breast cancer – adapteed from Walker *et al* (1997)

1.1.2.2 In-situ carcinoma

A pre-invasive stage of breast cancer is well recognised in which the malignant cell remains within the confines of the duct and is surrounded by a myoepithelial cell layer. This is known as *in-situ* carcinoma which is histopathologically divided into two types, ductal or lobular carcinoma *in-situ*, which have distinct morphologies and clinical implications.

The malignant cells in Ductal Carcinoma *In-Situ* (DCIS) are largely confined to the ducts. It can be a precursor of infiltrating ductal carcinoma and evidence that *in-situ* carcinoma may progress to invasive carcinoma is provided by 30-50% of invasive tumours displaying an *in-situ* component (Lagios *et al* 1982). However it is recognised that not all DCIS has the same potential to progress to invasive carcinoma (see figs. 1.2 & 1.3). Loss of specific allelic markers in DCIS corresponds to loss in some invasive tumours which provides evidence that certain DCIS are precursor lesions (Chen *et al* 1992, Zhuang *et al* 1995, Fujii *et al* 1996). It is graded low, intermediate or high which is dependent upon a number of histological features (Elston & Ellis 1991, Bobrow *et al* 1995). High grade DCIS is thought to accumulate more genetic alterations than non-high grade DCIS and subsequently develop into a more aggressive disease (Walker 1997).

Lobular Carcinoma *In-situ* (LCIS) is regarded as a marker of increased breast cancer risk rather than a direct precursor of invasive carcinoma (Page & Anderson 1987, Walker 1997). LCIS may be a direct precursor of infiltrating lobular carcinoma (Zhuang *et al* 1995), but is more likely to signify epithelial instability. It indicates a risk of carcinoma in both breasts, not just the breast expressing the *in-situ* component, and either lobular or ductal carcinoma may develop.

1.1.2.3 Invasive carcinoma

As previously discussed, invasive carcinoma may arise on a background of benign breast disease, *in-situ* carcinoma or may arise *de novo*. The most common type of invasive carcinoma is infiltrating ductal carcinoma (IDC) which accounts for 73% of invasive breast cancers followed by infiltrating lobular carcinoma which accounts for 10% (Ellis *et al* 1992). A tumour is categorised as IDC of no special type if it does not fulfil criteria for other types of carcinoma. These rarer types include medullary, mucinous, tubular and invasive cribiform. Each type has a differing prognosis and ten-year survival rate (see Table 1.1).

Type	Frequency (%)	Ten year survival rate (%)
Ductal Carcinoma *In-situ*	-	92
Lobular Carcinoma *In-situ*	-	NA
Infiltrating Ductal	73	47
Infiltrating Lobular	10	54
Special Types		
Medullary	5	51
Mucinous	2	80
Tubular	3	-
Cribiform and Papillary	5	-
Mixed Tumours	2	-
Tubular-Cribiform	-	90

Table1.1 : Relative frequencies and ten year survival rate for breast cancer - different types of breast carcinoma (Ellis *et al* 1992).

1.1.3 Predicting the behaviour of breast cancer

Breast carcinoma is not just one disease with a defined outcome and factors relating to prognosis are of clinical importance. For example, histological grading is of high prognostic value and takes into account morphological features relative to clinical outcome. It is a crude measure of how much the tumour resembles normal tissue. The current system in use is a modification of the Bloom & Richardson system (1957) in which several microscopical features including tubule formation, nuclear pleomorphism and mitotic count are given numerical values that are combined to give a measurement of grade (Elston & Ellis 1991) (see tables 1.2 & 1.3). Low grade signifies well differentiated tumour which is of greater similarity to normal tissue than high grade tissue, which is poorly differentiated. There is a strong correlation between histological grade and patient survival (Elston & Ellis 1991).

Staging of breast carcinoma is also a useful tool in establishing prognosis. Several staging systems exist including the TNM method (Sobin & Wiittekind 1997) which, along with other systems, is based on tumour size (T), node status (N), and distal (metastatic) spread (M). It is hoped that knowledge of the molecular and biological aspects of breast cancer progression will lead to the identification of the processes leading to malignancy. However, further features which may be informative about the biology of individual lesions are required.

Parameter	Range	Score
Tubule formation	Majority of tumour, >75%	1
	Moderate amount, 10-75%	2
	Little or none, <10%	3
Nuclear pleomorphism	Mild	1
	Moderate	2
	Severe	3
Mitotic count *	0-5	1
	6-10	2
	>11	3

Table 1.3: Calculation of histological grade for breast cancer - scoring system for breast tumours allowing calculation of histological grade. * count per 10 high power fields

Total Score	Grade
3-5	1
6-7	2
8-9	3

Table 1.4: Histological grading system for breast cancer - based on scores (table 1.3) (Elston & Ellis 1991).

1.1.4 Invasion and metastasis

The ability to invade and metastasise are the features that distinguish malignant from benign tumours. Events occur which overcome the normal regulatory processes that control tissue morphology enabling tumour cells to cross tissue compartment boundaries and ultimately spread to distant sites to set up new growth.

Invasion is a complex process that requires several steps (Aznavoorian *et al* 1993). Initially cells become detached from each other by overcoming cell-cell contacts. Following this, carcinoma cells attach to the limiting basement membrane (BM), followed by expression or activation of proteolytic enzymes to degrade the basement membrane. This breach of the BM enables cells to enter the stromal space and interact with the extra-cellular matrix (ECM). In order to do this, cells need to overcome restrictions imposed by normal cell adhesions and also make and break bonds with specific ECM proteins. Interaction with the host stroma also stimulates new blood vessel formation (angiogenesis) which provides nutrition and access to the vascular compartment. Further BM barriers are overcome as malignant cells gain access to vascular and lymphatic channels and spread to distant sites

to set up new growth (Aznavoorian *et al* 1993). In the majority of cases it is these systemic metastases causing organ failure that results in death.

To summarise, at each stage of this process there are changes in adhesive interactions, both cell-cell and cell-matrix, breakdown and remodelling of the matrix and BM and multiple interactions with stromal proteins. The focus of this study is changes in the ECM, however, this influences cellular adhesive interactions and potentially matrix remodelling, therefore for completeness an overview of cell adhesion and proteolysis will be provided prior to focusing on the ECM.

1.1.4.1 Cell adhesion

Normal tissue structure is maintained by cell-cell and cell-matrix interactions. Epithelial cell adhesion is largely mediated by desmosomal cadherins and the classical cadherins (E- and P- cadherin) in adherens junctions (Takeichi *et al* 1990).

The major group of molecules mediating cell-matrix interactions are the integrins. As the major focus of this project is the effect of matrix on cell behaviour, the integrins will be considered in more detail.

1.1.4.2 Integrins

Cell-ECM interactions are mediated by the integrin family of cell adhesion receptors. Alterations in integrin expression and activity are associated with tumour progression (Ruoslahti 1992). Integrins are dimeric transmembrane receptors composed of dissimilar α and β sub-units and a range of at least 15 α and 9 β sub-units exist which combine to form over 20 different receptors (Hynes 1992). As well as providing adhesive functions, the transmembrane nature of the integrin complex provides a link between the ECM and the actin cytoskelton and associated proteins. Furthermore, a number of cell signalling molecules associate with the cytoplasmic domain of integrins (Miyamoto *et al* 1995).

The extracellular domain of the $\alpha\beta$ association determines specific ligand binding for ECM proteins, including laminin (Gehlsen *et al* 1988), fibronectin (Brown & Juliano 1985), and tenascin C (Yokosaki *et al* 1996). Ligand adhesion to the extracellular domain is mediated by short peptide sequences within the structure of those ligands that initially serve as a recognition system. Initiation of binding occurs prior to further binding to the same or other sites (Guan & Hynes 1990, Elices *et al* 1991). The most common recognition

sequence is the RGD (arginine-glycine-aspartic acid) that was initially identified in fibronectin and is present in a range of other proteins (Pierschbacher & Ruoslahti 1984). Commonly, integrins bind to several ligands (Takada *et al* 1989, Hynes 1992) and furthermore, several integrins may bind to the same ligand. This functional overlap creates diversity, however, such redundancy also reflects a role for integrins other than adhesion (Hynes 1992).

Further to providing anchorage to the ECM, integrins act as signalling molecules affecting various processes such as cell migration, proliferation, differentiation and apoptosis (Frisch & Francis 1994, Zutter *et al* 1995, Assoian 1997). Intracellular domains of integrins are mainly attached to the actin cytoskeleton or, in the case of $\alpha 6\beta 4$, the intermediate filament system (Horowitz *et al* 1986). The link between the cytoplasmic domain of integrins and the actin cytoskeleton is mediated via cytoskeletal proteins in the formation of focal adhesions or, in the case of $\alpha 6\beta 4$, hemidesmosomes. The $\alpha 6\beta 4$ integrin is unique in being a major component of the specialised cell-matrix adhesion complex, the hemidesmosome (Yamada & Miyamoto 1995, Borradori & Sonnennberg 1996). The variability in integrin receptor structure implicates binding of a single ligand in a range of cellular responses and integrins mediate signalling via two mechanisms. "Outside in" signalling mediates signals from the ECM resulting in the assembly of a focal adhesion plaques and the activation of focal adhesion kinases (Schwarz *et al* 1995, Borradori & Sonnennberg 1996). This ultimately leads to activation of signalling pathways leading to alterations in gene expression (Chen *et al* 1994, Zhu & Assoian 1995). "Inside out" signalling mediates the mechanism by which a cell regulates ligand affinity for its receptors (Schwarz *et al* 1995). This elicits a conformational change in the binding site in response to intracellular signals (O'Toole *et al* 1994).

Integrins are involved in a number of cellular processes, alterations of which are associated with the development of tumours (Zutter *et al* 1995, Varner *et al* 1995, Frisch *et al* 1996). An alteration in integrin expression is demonstrated in many epithelial malignancies and several integrins expressed by the normal breast have been shown to be altered in breast carcinoma (Sonnenberg *et al* 1991, Glukhova *et al* 1995). These are the collagen/laminin receptors $\alpha 2\beta 1$ and $\alpha 3\beta 1$, and the hemidesmosone component $\alpha 6\beta 4$ (Zutter *et al* 1995, Koukoulis *et al* 1991, Pignatelli *et al* 1992, Jones *et al* 1992). In the breast, epithelial and myo-epithelial cells have hemidesmosomes on their basal surfaces that are lost in invasive carcinomas (Bergstraesser *et al* 1995, Stahl *et al* 1997). This loss may favour tumour cell

migration by altering adhesion to the BM and the surrounding ECM. However, there is evidence that integrins may be involved in the control of other aspects of tumour invasion, namely proteolytic remodelling of the ECM (Seftor *et al* 1993).

1.1.4.3 Proteolysis

In order for a cell to invade the surrounding tissue and migrate through the ECM, a number of barriers need to be overcome. The stroma is not merely a passive barrier but itself changes and responds to the malignant cells to facilitate a local environment favouring invasion (Starkey 1990). This involves considerable remodelling of the stroma and initially breaching of the BM, the whole process facilitated by the production of proteolytic enzymes. Due to the complexity of the BM and the surrounding stroma, a broad spectrum of enzymes is required for its remodelling. Of these proteolytic enzymes, the matrix metalloproteinases are a focus of the current study and thus will be considered in detail.

Matrix metalloproteinases (MMPs), defined by their dependence on zinc for their catalytic status, are expressed during a variety of normal tissue remodelling processes, including changes in the mammary gland associated with the menstrual cycle and involution (Rudolph-Owen & Matrisian 1998). There are at least 20 MMPs which between them can degrade most, if not all the components of the ECM, including the components of the BM (Mignatti & Rifkin 1993). The MMPs can be broadly grouped according to their substrate specificity (see Table 1.4) as collagenases, gelatinases, stromelysins, and the membrane-type MMPs (MT-MMP) which are integral plasma membrane enzymes, and others which do not fit into these categories.

In terms of basic structure, the MMPs share similarity in their structural domains. Each enzyme consists of a signal peptide, a pro-peptide domain which plays a critical role in the activation of the enzyme (Springman *et al* 1990, Stetler-Stephenson *et al* 1991), a catalytic domain containing a zinc-ion binding site and a COOH terminal domain. The structure of the COOH terminal domain is thought to define substrate specificity and may associate with specific enzyme inhibitors (Marcy *et al* 1991, Murphy *et al* 1992, Fridman *et al* 1992). Individual MMPs may have variations outside this basic structure, for example, the MT-MMPs have their transmembrane domain at the COOH terminal (Sato 1994).

MMP activity is tightly controlled by several mechanisms. These include pro-enzyme activation, inhibition by specific inhibitors, and regulation of gene transcription.

The majority of MMPs are secreted as inactive zymogens that are subsequently activated under specific conditions. Activation is via a conformational change known as the cysteine switch mechanism (Van-Wart & Birkedal-Hansen 1990). Within the high molecular weight latent form of the enzyme is a cysteine zinc bond. Disruption of this bond by a variety of mechanisms results in autocatalytic cleavage converting the MMP to an active form. MMPs can also be activated via other families of proteolytic enzymes, such as the serine proteases (DeClerk & Laug 1996) as well as other, non-membrane bound, MMPs (Ogata et al 1995, Sang et al 1995). The MT-MMP sub group is responsible for the activation of a number of other MMPs (Sato et al 1994, Takino et al 1995, Pei 1999).

MMP activity is inhibited by specific tissue inhibitors of metalloproteinases (TIMPs) (see table 1.4). All TIMPs have a similar inhibitory action towards active MMPs (Stetler-Stephenson et al 1989) and bind at a 1:1 ratio, however, interaction with latent gelatinises has also been demonstrated and involves a binding site distinct from the enzyme active site (Kleiner et al 1992, Goldberg et al 1992).

Regulation of MMP gene transcription is complex and a variety of tumour promoters, growth factors and cytokines have been shown to have an effect (Brenner *et al* 1989, Kerr *et al* 1990). MMP promoter regions contain a range of responsive elements distinctive for each MMP (McDonnel *et al* 1990, Gutman & Wasylyk 1991) which provide a mechanism for a wide range in gene activity (Angel & Karin 1992) and a highly complex control system exists that appears to be specific to cell type and environment.

There is evidence for a role of MMPs in tumour invasion. Generally, enhanced expression is seen: MMP1 in colorectal cancer (Murray et al 1996), MMP2 & 9 in bladder cancer (Davies et al 1993), and also in breast carcinoma (this is reviewed in chapter 5). Many other factors have been related to MMP expression in carcinoma, including tenascin C (Tremble et al 1994). Thus, there is evidence of a role for MMPs at multiple levels in the evolution and progression of cancer. An understanding of the factors regulating aberrant expression is vital in the understanding of tumour progression.

Group	Enzyme	MMP	Substrate	Reference
Collagenases	Interstitial collagenase	1	Collagens I, II, III, VII, X	Goldberg *et al* 1986
	Polymorphonuclear collagenase	8	Collagens I, II, III	Hasty *et al* 1990
	Collagenase 3	13	Collagen II, denatured collagens, Aggrecan	Freije et al 1994, Knauper et al 1997
	Collagenase	18	Collagens I, II, III, VII, denatured collagens	Cossins et al 1996
Gelatinases	Gelatinase A, 72kDa type IV collagenase	2	Gelatins, Collagens I, IV, V, VII, X, elastin, fibronectin, laminin	Collier et al 1988, Seltzer et al 1989
	Gelatinase B, 92kDa type IV collagenase	9	Gelatins, Collagens IV, V, elastin	Wilhelm et al 1989
Stromelysins	Stromelysin 1	3	Proteoglycan core protein, fibronectin, laminin, denatured collagens, collagens IV, V, IX, X	Chin et al 1985
	Stromelysin 2	10	Denatured collagens, Collagens III, IV, V	Breathnach et al 1987, Muller et al 1988
	Stromelysin 3	11	Fibronectin, Laminin	Bassett et al 1990
Others	Matrilysin	7	Proteoglycan core protein, fibronectin, kaminin, denatured collagens	Quantin et al 1989, Miyazaki et al 1990
	Metalloelastase	12	Elastin	Senior et al 1982 & 1989
	MMP19	19	Stromelysin like	Pendas et al 1997
	Enamelysin	20	Amelogenin	Llano et al 1997
MT-MMP	MT1-MMP	14	Activates pro-MMP2 and pro-MMP13, Fibronectin, aggrecan, nidogen, Collagens I, III	Sato et al, 1994, Knauper et al 1996, dOrtho et al 1997
	MT2-MMP	15	Activates pro-MMP2, Laminin	Will & Hinzmann 1995, dOrtho et al 1997
	MT3-MMP	16	Activates pro-MMP2	Takino et al 1995
	MT4-MMP	17	May activate pro-MMP2	Puente et al 1996
	MT5-MMP	21	Activates pro-MMP2	Llano et al 1999, Pei 1999
TIMP	TIMP 1	-	Inhibits all active MMPs & latent MMP2	Docherty et al 1985
	TIMP 2	-	Inhibits all active MMPs & latent MMP9	Stetler-Stephenson et al 1989
	TIMP 3	-	Inhibits all active MMPs	Uria et al 1994, Apte et al 1995
	TIMP 4	-	Inhibits all active MMPs & latent MMP2	Greene et al 1996, Leco et al 1997, Bigg et al 1997

Table 1.4: The matrix metalloproteinases and their inhibitors - description of the different classes of MMPs and their inhibitors with their classifications, alternative names, substrates and key references.

1.1.5 The extra-cellular matrix

The ECM is composed of a network of macromolecules secreted by the stromal fibroblasts. This network is structurally and biochemically responsible for the integrity of a tissue. It provides an organised lattice within which cells interact with one another. The extra-cellular space, or the stromal compartment, primarily consists of fibrous proteins in a hydrated polysaccharide gel (Hynes 1985, Burgeson *et al* 1988). These macromolecules can be broadly classified as proteoglycans, and fibrous proteins which can be further classified as structural, adhesive and counter-adhesive.

1.1.5.1 Proteoglycans

Proteoglycans are formed by long, unbranched polysaccharide chains separately known as glycosaminoglycans. They are covalently linked to a protein residue which makes up less than 5% of the complete molecule (Iozzo *et al* 1994). Proteoglycans form negatively charged hydrated gels which act as selective filtration devices and permit the diffusion of nutrients, metabolites, and hormones. Certain types of proteoglycans are thought to facilitate cell migration during morphogenesis (Ruoslahti, 1991).

1.1.5.2 Collagens

Collagens are the major structural protein of the ECM. They exists in a variety of forms and several distinct families exist (Burgusen *et al* 1988). They usually form supramolecular aggregates i.e. fibrils, filaments or networks, alone or in conjunction with other ECM proteins interwoven with the collagen (Mecham *et al* 1991).The primary function of the collagens is to contribute to the structural integrity of the ECM and they are also involved in the anchorage of cells to the matrix. Collagen organisation is adapted to type of tissue and is thought to be mediated by fibroblasts (Linsenmeyer, 1991). Type IV collagens represent the major structural components of the basement membrane (BM) (Stanley *et al* 1982). This is a specialised adaptation of the ECM that occurs at the interface between tissue compartments. It acts as a barrier preventing epithelial cells interacting with stromal cells. In malignant tissue this barrier is broken down (Liotta *et al* 1986). In normal tissue the BM regulates many cellular functions including adhesion, migration and differentiation (Roskelly *et al* 1995, Slade *et al* 1999). Type IV collagens are atypical of the collagen family in that they form a sheet like lattice. This is interwoven with the other structural macromolecules of the BM such as laminin, and the proteoglycan perlocan (Stanley *et al* 1982, Relan & Schuger 1999) and specific interactions between

these components are responsible for maintaining the structure of the basement membrane, interactions with cells and interactions with other ECM proteins.

1.1.5.3 Fibronectin

Fibronectin is a major adhesive constituent of the ECM and is expressed in a cell specific manner to form a stable matrix (Hynes *et al* 1985). It is a dimeric modular protein that exists as a series of repeating units that form structural and functional domains. Thus it is capable of interacting with cell surface receptors, specifically integrins (Pierschbacher & Ruoslahti 1984, Aota *et al* 1994, Bowditch *et al* 1994) and other ECM proteins, including collagen (Schwarzbauer 1991) and tenascin C (Chiquet-Ehrismann *et al* 1991). It contains an easily accessible RGD site for multiple integrin binding which is further enhanced by a synergy site of the adjacent repeat domain (Main *et al* 1992, Dickinson *et al* 1994, Leahy *et al* 1996). Consequently, fibronectins promote the adhesion and spreading of many cell types, modulation of cytoskeletal structures within the cell, and may also trigger signal transduction cascades (Virtanen *et al* 1982, Vuori & Ruoslahti 1993, Tremble *et al* 1995). Fibronectin has a number of domains that undergo alternative splicing conferring functional diversity (Hynes *et al* 1985, Schwarzbauer 1991, Kar *et al* 1993). Expression of these domains is limited in normal adult tissues and they are developmentally regulated in a cell and tissue type specific manner (Oyama *et al* 1989, Pagani *et al* 1991). These alternatively spliced domains are thought to affect integrin binding and focal adhesions (Manabe *et al* 1997, Chen *et al* 1998). It has been demonstrated that fibronectin is upregulated in a range of carcinomas, and this is related to the appearance of alternatively spliced isoforms (Oyama *et al* 1993, Pujuguet *et al* 1996, Menzin *et al* 1998). In breast, these oncofetal forms of fibronectin, particularly containing an extra domain designated B, have been associated with tumour stroma (Gould *et al* 1990, Koukoulis *et al* 1993, Kaczmarek *et al* 1994). Specific alternative isoforms are thought to stimulate cell migration (Manabe *et al* 1997, Matsumoto *et al* 1999) and may have different sources such that cancer cells may express a different type to stromal cells.

1.1.5.4 Laminins

Laminins are major cell-adhesive proteins that are essential constituents of the BM (Martin & Timpl 1987). They consist of a trimer of sub-units, namely α, β, and γ, that exist in a number of different forms which combine to form at least 11 different laminins (Eckblom & Timpl 1996). Binding of cells to laminins is mediated by a range of integrin receptors and elicits multiple effects on cell morphology and function (Martin & Timpl 1987,

Kramer *et al* 1993). All major BMs contain laminin variants in a tissue specific manner (Paulsson *et al* 1991) and laminin 5 is the major BM associated laminin in the breast (Rouselle *et al* 1991). It exists within a complex of other laminins which localise to anchoring filaments at the junction that connects hemidesmosomes to the basement membrane (Champliaud *et al* 1996, Rouselle *et al* 1991). Laminin 5 has been demonstrated to be the specific ligand for the hemidesmosome integrin $\alpha 6\beta 4$ (Niessen *et al* 1994) and alterations in this interaction in breast carcinoma may mediate distinct signals in tumour progression (Tagliabue *et al* 1998). Loss of laminin 5 and hemidesmosomes has been reported in breast carcinoma (Henning *et al* 1999). Integrin-laminin interactions have also been implicated in control of MMP expression and it has been demonstrated that macrophages binding to laminin upregulate MMP9 (Khan & Falcone 1997). The function of laminins themselves may be altered by proteolytic cleavage, for example, laminin 5 is degraded by MMP 2 and this proteolysis is linked with increased epithelial cell migration (Gianelli *et al* 1999). Thus, binding of cells to laminins may have a complex role in tumour progression.

1.1.5.5 Counter-adhesive proteins

It is recognised that in both normal and pathological processes, the overcoming of adhesion is crucial in cell migration, hence tumour cell invasion. This is termed counter-adhesion (Prieto *et al* 1992) and enables transient binding of cells to specific components of the ECM. A number of established ECM proteins have been shown to exhibit counter-adhesive properties in relation to certain cell types and experimental conditions. These include thrombospondin (Lahav 1988, Murphy-Ullrich & Hook 1989), SPARC (Secreted Protein, Acidic & Rich in Cysteine) (Lane & Sage 1994), and the tenascins (Prieto *et al* 1992, Pesheva *et al* 1994). However, it is recognised that this counter-adhesive effect is domain and receptor specific and these proteins also exhibit adhesive effects (Prieto *et al* 1992, Stomski *et al* 1992).

Thrombospondins are a family of five related proteins that are involved with cellular proliferation and migration (Majack *et al* 1986, Yabkowitz *et al* 1993). They have been linked with malignancy, and thrombospondin 1 has been shown to be upregulated in breast carcinoma together with a potential cell surface receptor (Tuszynski *et al* 1992; 1993).

SPARC is an ECM glycoprotein thought to modulate adhesion to other components of the ECM (Lane & Sage 1994). It has been associated with breast adenocarcinoma cells (Howe

et al 1990) and increased expression has been found in several invasive tumours (Wewer *et al* 1988, Porter *et al* 1995).

Counter-adhesive properties have been suggested for a number of other ECM protein families including proteoglycans (Milev *et al* 1994, Yamagata & Kimata 1994), and also certain laminin isoforms (Calof *et al* 1994). However, a family of proteins with considerable counter-adhesive properties are the tenascins (Prieto *et al* 1992, Pesheva *et al* 1994), and tenascin C is the focus of this study.

1.2 Tenascin C

Tenascin-cytotactin (tenascin C) is one member of a growing family of extra-cellular matrix glyco-proteins which plays a morpho-regulatory role during tissue development, remodelling and disease. The other members of the family include TNR (Fuss *et al* 1991, Rathjen *et al* 1991), TNW (Weber *et al* 1998), TNX (Bristow *et al* 1993), and TNY (Hagios *et al* 1996).

Tenascin C (TNC) was initially described by Bourdon (1983; 1985) as a novel extra-cellular matrix protein that was subsequently found to be associated with glioma and given the name glioma mesenchymal extra-cellular matrix protein (GMEM). Independently, a complex six-armed molecule was isolated as a contaminant of cell surface fibronectin preparations and it was termed a hexabrachion; a name used to describe the structure of the protein (Erickson & Iglesias 1984). Similar molecules were found in chickens: termed myotendinous antigen by Chiquet & Fambrough (1984), cytotactin by Grumet *et al* (1985) and in mice: J1 glycoprotreins by Kruse *et al* (1985).

Subsequent studies by Erickson & Taylor (1987) showed that myotendinous antigen was chicken hexabrachion and that GMEM was human hexabrachion indicating TNC is evolutionarily conserved (Gherzi *et* al 1997, Jones and Copertino 1996, Copertino *et* al 1997, Faustino *et* al 2002). Thus, all these proteins are now considered to be tenascin C, a name which takes into account early studies on tendon and derived from two Latin verbs: "tenere" (to hold - tendon) and "nasci" (to be born - growth) (Chiquet-Ehrismann *et al* 1986).

1.2.1 Tenascin C and Normal Tissue

Tenascin C is initially expressed during embryogenesis and appears in a site-restricted manner. It is found in mesenchymal tissue next to proliferating epithelia in the mammary gland, lung, kidney and gut (Aufderheide *et al* 1987, Aufderheide and Eckblom 1988, Karteenaho-Wiik *et al* 2001).

In normal human adult tissue the distribution of TNC is highly restricted, and early studies reported a complete absence of TNC (Chiquet & Fambrough 1984, Chiquet-Ehrissmann *et al* 1986, Erickson and Bourdon 1989). However, more extensive studies have shown a distinctive pattern of distribution. Two extensive studies showed weak expression at sites of epithelial-mesenchymal interactions in normal skin, breast, lung, liver and kidney tissue (Natali *et al* 1991, Koukoulis *et al* 1991). Alterations in distribution can be seen in a number

of tissues under hormonal control, and the menstrual cycle has a significant effect (Vollmer *et al* 1990, Ferguson *et al* 1990) as does pregnancy and lactation (Jones *et al* 1995, Wirl *et al* 1995, Bell *et al* 1999). There is evidence of transient alteration in TNC expression during wound healing (Mackie *et al* 1988, Whitby *et al* 1991, Wallner *et al* 2002) where it is strongly upregulated and appears in a distinct pattern along the wound edge where it remains throughout the healing process (Juhasz *et al* 1993). High levels of TNC can also be observed with scarring, particularly in keloid scars (Dalkowski *et al* 1999). Consequently, expression in normal tissue is complex.

1.2.2 Tenascin C and Neoplasia

Tenascin C was initially associated with neoplasia by Chiquet-Ehrismann *et al* (1986) who described TNC expression and distribution in carcinogen induced rat mammary tumours. Mackie *et al* (1987) identified upregulation of TNC expression associated with human malignant mammary gland lesions. This led to a number of extensive studies on a range of normal and malignant human tissues (Natali *et al* 1991, Koukoulis *et al* 1991, Shrestha *et al* 1996). TNC expression has subsequently been described in many different human tumours (see table 1.5). The pattern of distribution appears similar in all tumour types with a change from the BM zone to a diffuse stromal distribution. In most tissues TNC expression is also increased in certain non-neoplastic diseases and is associated with reparative and hyperplastic processes (Koukoulis *et al* 1991).

1.2.3 Tenascin C in the Breast

During normal breast development TNC is seen at times of cellular proliferation and reorganisation. Pre-natally, it is seen at high levels in the in the dense embryonic mesenchyme surrounding budding epithelia (Chiquet-Ehrissman *et al* 1986, Inaguma *et al* 1988). However, following birth, expression is reduced and low levels are seen in the mature mammary gland. Its distribution is also altered and it becomes localised to a discrete layer surrounding mammary ducts and blood vessels (Inaguma *et al* 1988, Howeedy *et al* 1990). TNC distribution also changes in relation to the menstrual cycle. Specifically TNC is upregulated in the later stages of the menstrual cycle when there is an increase in cell migration and proliferation (Ferguson *et al* 1990). 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, Tamura *et al* 1993). During pregnancy, however, the distinctive tissue remodelling associated with alveolar cell differentiation prior to lactation is concurrent with downregulation of TNC expression and

distribution (Jones *et al* 1995a, Wirl *et al* 1995). Following weaning, involution is initiated initially by massive apoptosis of the secretary epithelial cells, followed by proteolytic remodelling of the mammary gland by upregulation of MMPs (Lund *et al* 1996) which is associated with increased expression of TNC (Jones *et al* 1995a).

Tumour	References
Central nervous system	Bourdon *et al* 1983, Koukoulis *et al* 1991
Liver	Koukoulis *et al* 1991, Yamada *et al* 1992, Glubova and Vlaykova 2001
Lung	Oyama *et al* 1991, Koukoulis *et al* 1991, Wikman *et al* 2002
Oral SCC	Anzhabagan *et al* 1990, Harada *et al* 1994, Shrestha *et al* 1994, Regezi *et al* 2002
Salivary glands	Soini *et al* 1992
Colorectal	Sugawara *et al* 1991, Koukoulis *et al* 1991, Glubova and Vlaykova 2001
Kidney	Natali *et al* 1991, Koukoulis *et al* 1991
Prostate	Natali *et al* 1991, Ibrahim *et al* 1993
Urinary bladder	Natali *et al* 1991
Endometrium	Vollmer *et al* 1990, Sedele *et al* 2002
Ovary	Natali *et al* 1991, Wilson *et al* 1996
Lymphatic tissue	Soini *et al* 1992
Skin	Stamp 1989, Natali *et al* 1990
Vulva & Cervix	Tiita *et al* 1992, Buyukbayram & Arslan 2002
Endometrium	Vollmer *et al* 1990
Pancreas	Vaidya *et al* 1996, Linder *et al* 2001
Urinary Bladder	Tiita *et al* 1993
Larynx	Yoshida *et al* 1999, Uhlman & Niehmans 1999
Breast	See Section 1.2.3

Table 1.5: Examples of tumour types demonstrating alterations in tenascin C expression – with key references.

In contrast to normal tissue, high levels of TNC are seen in the stroma of both invasive and *in-situ* breast carcinoma (Mackie *et al* 1987, Howeedy *et al* 1990, Ferguson *et al* 1990, Koukoulis *et al* 1991) and TNC was initially considered as a stromal marker for epithelial malignancy in the mammary gland (Mackie *et al* 1987). However, subsequent studies identified enhanced TNC expression and a change in distribution associated with benign breast disease (Borsi *et al* 1992).

The strong and consistent overexpression of TNC in many cancers has stimulated interest in the potential role of this protein, and a need for a greater understanding of its structure, function and regulation.

1.2.4 Structure of Tenascin C

The human tenascin C gene has been localised to chromosome 9q33 and it consists of 28 exons spaced by 27 introns with gene transcription controlled by a single promoter (Gherzi *et al* 1995). The cDNA sequence is of a 2203 amino acid protein (Nies *et al* 1991) of around 1900 KDa with a sub-unit size ranging from 220-330 KDa (Erickson & Bourdon 1989).

The structure of the tenascin C protein was first deduced by Erickson & Iglesias (1984) after isolation from extracellular matrix preparations produced by human fibroblasts. Electron micrographs show a highly symmetrical six-armed hexabrachion composed of two trimers joined at a central core (Taylor *et al* 1989). Each arm is a sub-unit composed of four distinct structural domains (Gulcher *et al* 1991, Nies *et al* 1991, Mighell *et al* 1997) (see figure 1.4):

1. Globular amino terminal domain – tenascin assembly (TA) domain.
2. 14.5 epidermal growth factor (EGF) like repeats.
3. 8 - 17 fibronectin type III (FNIII) like repeats.
4. Carboxyl terminal sequence with homology to fibrinogen (FG).

Interestingly, whereas the four other members of the tenascin family share this same domain structure, they have not been seen as polypeptides (Jones & Jones, 2000). This may be crucial to function as monomers have been shown to have different activity to complete hexabrachions (End *et al* 1992).

The amino terminal region of the TNC monomer contains eight amino acid residues of cysteine plus a number of α-helical heptad repeats which link the individual TNC polypeptides to initially form a trimer (Nies *et al* 1991, Conway & Parry 1991). Hence, this is known as the tenascin assembly domain. The structure is stabilised by covalent disulphide bonds in the heptad region creating a trimer of TA domains that homophilically link with another trimer to form the hexabrachion (Kammerrer *et al* 1998). The hexabrachion is stabilised by further disulphide linkages (Erickson & Bourdon 1989). Further structural diversity may also occur as nonomers have been identified (Schenk & Chiquet-Ehrissmann 1994).

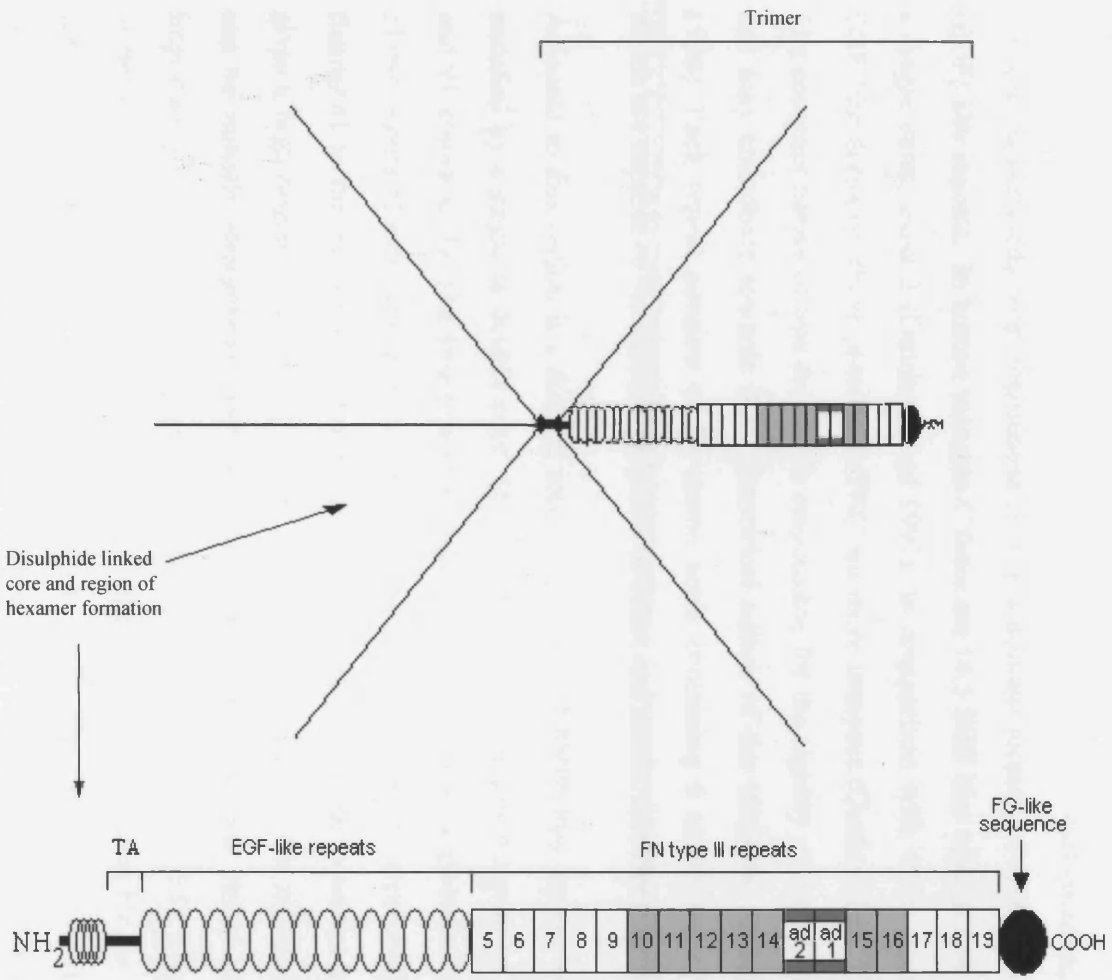


Figure 1.4: Structure of Tenascin C monomer and hexabrachion - The lower image is a single hexabrachion arm (or tenascin C sub-unit) showing relative positions of each domain. The numbers in boxes represent cDNA exon number with the shaded boxes representing the alternatively spliced FNIII repeats. The upper image represents the six-armed hexabrachion sructure composed of two trimers. TA = tenascin assembly domain.

Chiquet-Ehrissmann *et al* (1991) states that in each hexabrachion the monomers are identical, however, this may not be the case. In *in-vitro* experiments, heterotypic molecules have been seen composed of human and chicken trimers. Furthermore, it is possible trimers may contain different monomers. Erickson & Bourdon (1989) have observed hexabrachions with a mixture of long and short arms. Further evidence is provided by Kammerer *et al* (1998) who postulates that as the TA domains are identical, heterotypic molecules may arise, even between different members of the tenascin family.

Proceeding outwards from the central core is a domain formed of epidermal growth factor (EGF) like repeats. In human tenascin C there are 14.5 EGF like repeats that are encoded by a single exon, exon 2 (Gulcher *et al* 1991). In comparison with other proteins containing EGF like domains, those present in TNC are more compact (Cooke 1987, Handford 1990). The compact nature of this domain is responsible for the rigidity of the protein at this point and may contribute towards the symmetrical nature of the tenascins (Jones & Copertino, 1996). Each repeat consists of 31 amino acids containing 6 conserved cysteine residues which are capable of intrachain disulphide linkages and confer stability.

Adjacent to this region is a domain consisting of 8 - 17 FNIII like repeats each of which is encoded by a single or double exon. In the polypeptide chain each repeat varies between 87 and 91 amino acids. The structure of a single FNIII like repeat is globular and is composed of two sheets of anti-parallel β strands (Leahy *et al* 1992). This structure confers a degree of flexibility to the molecule, further enhanced by a lack of internal covalent bonds. This also gives a high degree of elasticity, in that under mechanical stress the zigzag array of repeats can be initially straightened and ultimately unravelled to several times its initial length. Importantly, this effect is completely reversible (Oberhauser *et al* 1998). The crucial feature of this domain is its variability in number of repeats which is responsible for the variation in sub-unit size. Early studies demonstrated that FNIII like repeats 6 -12 (exons 10 - 16) undergo alternative splicing in the primary transcript to produce a small and large isoform (Gulcher *et al* 1991). Results from a number of studies supported this. Western analysis has detected different sized polypeptides of 240 and 180 KDa (Gulcher *et al* 1991). Northern analysis has detected chicken TNC primary transcripts of between 6.4 and 8.3 kb (Jones *et al* 1988; 1989) and cDNA cloning of TNC transcripts identified two splice variants with a total of 8 or 15 FNIII like repeats (Gulcher *et al* 1991).

However, subsequent studies have shown the situation is far more complex with splicing producing a large number of alternative isoforms. Siri *et al* (1991) identified 8 variants and further studies have identified a total of 22 isoforms (Saga *et al* 1991, Borsi *et al* 1993, Sriramarao & Bourdon 1993, Dorries *et al* 1994, Zhao *et al* 1995, Mighell *et al* 1997, Wilson *et al* 1996, Saghizadeh *et al* 1998). This does not include all possible theoretical conformations and it is interesting to note that in mouse CNS development, 27 tenascin C isoforms have been identified from a molecule possessing less alternatively spliced repeats than human TNC (Joestner & Faisner, 1999). Two additional repeats have been identified which are thought to be tumour specific, designated additional domains 1 & 2 (ad1 & ad2) (Sriramarao & Bourdon 1993, Mighell *et al* 1997). Transcripts encoding ad1 have been found in a variety of human cancers (Derr *et al* 1997) whereas ad2 has only been found in oral carcinoma (Mighell *et al* 1997); however, homologues of both have been found in non-neoplastic avian tissues (Derr *et al* 1997). Different splice variants are seen at various stages of development and malignancy (Jones & Copertino 1996) with large variants expressed at times of active cell migration or tissue remodelling (Kaplony *et al* 1991, Ghert *et al* 2001). The variability in number of FNIII repeats has an effect on protein conformation. Amino acid composition gives a degree of uniqueness to each repeat in that specific peptide sequences convey an individual structure which affects the ligand-binding profile. Furthermore, assembly of repeats into an array creates further diversity in structure. This may have functional implications in that active sites may be lost or created (Leahy *et al* 1996).

The final domain is the carboxyl terminal sequence at the distal end of the hexabrachion arm. It consists of 210 amino acids and has approximately 32% homology with β and γ chain of human fibrinogen (Doolittle 1984, Nies *et al* 1991). Its structure is stabilised by 4 cysteine residues creating disulphide loops. The region is thought to bind calcium, an event which may have an effect on conformation (Jones & Copertino 1996).

1.2.5 Control of tenascin C expression

Increased TNC expression has long been associated with enhanced cell migration and tissue remodelling in both normal and malignant processes. Thus, the identification of molecules and regulatory pathways which govern local expression of tenascin has been a focus of research. Its coordinated expression is likely to be governed by local growth and differentiation factors and a number of events, acting alone or in conjunction, may influence TNC expression (Jones & Jones, 2000).

A number of studies have proposed that growth factors secreted by actively proliferating cells induce the synthesis of TNC in neighbouring cells, thus TNC induction relies on interactions with the local environment via these soluble factors (Inaguma *et al* 1988, Chiquet-Ehrismann *et al* 1989, Hiraiwa *et al* 1993). Both transforming growth factor β1 (TGFβ) and foetal bovine serum (FBS) have been shown to increase TNC expression. FBS is known to contain TGFβ but the greater increases seen with FBS indicate the presence of another factor (Pearson *et al* 1988, Chiquet-Ehrismann *et al* 1989). Increased expression of TGFβ is seen during the latter stages of the menstrual cycle and is concurrent with an increase in TNC expression (Knabbe *et al* 1987, Ferguson *et al* 1990). Fibroblast growth factor (FGF) has also been shown to stimulate TNC secretion (Rettig *et al* 1989, Suzuki *et al* 2002), as has epidermal growth factor (EGF). The effect of EGF is more potent than TGFβ in epidermoid carcinoma derived cell lines (Sakai *et al* 1995a). In terms of alternative splicing, both TGFβ and FGF have been shown to induce the larger TNC isoforms (Schwogler *et al* 1992, Tucker *et al* 1993). Borsi *et al* (1994) also suggests that different TNC isoforms may be associated with cell cycle regulation, and extra-cellular pH has also been shown to be a factor linked to expression of different splice variants (Borsi *et al* 1995).

It is thought that the growth factors act on the large number of regulatory elements flanking the TNC gene, including sequences that have been shown to confer a response to soluble factors. The high number of response elements indicates the presence of a diverse set of cell specific initiators of expression (Jones *et al* 1990). These regions are conserved during evolution (Copertino *et al* 1997, Gherzi *et al* 1995) indicating control of TNC expression is not species specific (Jones & Copertino, 1996). One of these sequences in the human TNC promoter has sequence similarity to a motif in a collagen gene known to be responsive to signals from the TGFβ growth factor family (Rossi *et al* 1988). A further element is responsive to changes in ECM composition. Denatured collagen elicits an integrin-mediated response via a mitogen activated protein kinase pathway to initiate TNC expression (Jones *et al* 1999). Mechanical stress has also been shown to play a role in induction of TNC with control at the level of transcription (Chiquet-Ehrissmann *et al* 1994). The TNC promoter does contain a stretch responsive element that is potentially activated by stretch alone or in conjunction with growth factors (Chiquet, 1999).

The promoter region also contains a number of elements that downregulate TNC expression. A glucocorticoid response element has been located in a consensus sequence of the chicken

TNC promoter and Eckblom *et al* (1993) found that glucocorticoids downregulate the expression of mouse TNC. During pregnancy, the presence of glucocorticoids in the breast induces lactation and the expression of milk proteins has been associated with the downregulation of TNC (Jones *et al* 1995a, Wirl *et al* 1995). Thyroid hormones and an active metabolite of vitamin D have also been shown to downregulate expression of TNC (Gonzalez-sancho *et al* 1998 & 1999), with the latter being successfully used in human trials for the treatment of breast cancer metastases (Bower *et al* 1991).

Overall, the presence of a range of responsive elements in the tenascin promoter has been investigated in a number of cell types via a variety of factors shown to influence the expression of TNC. However, these sometimes conflicting studies indicate that regulation of TNC expression is cell and tissue specific (Lightner *et al* 1994, Sakai *et al* 1995a, Chiquet-Ehrissmann *et al* 1995, Copertino *et al* 1997).

1.2.6 Function of tenascin C

The structure of tenascin C can be altered at a number of different levels, from individual peptide motifs up to alterations in the combination of monomers in the hexabrachion. This potentially leads to a wide range of unique ligand binding domains that could have diverse effects on cellular activity. The multifunctional activities attributed to TNC may occur through direct interactions with cell surface receptors or via indirect modulation of ECM proteins and cell adhesion molecules (Jones & Jones, 2000). The following sections discuss tenascin structure-function relationships in terms of overall effect on cell activity and also via direct associations with a range of ECM and cell surface ligands.

1.2.6.1 Cellular interactions with tenascin C

TNC supports the attachment of a number of primary cells and cell lines (Chiquet & Fambrough, 1984). However, in contrast, it has also been demonstrated that TNC interferes with cell attachment and spreading on otherwise adhesive substrates (Prieto *et* al 1992). Using recombinant TNC fragments, cell adhesive and counter-adhesive properties have been localised to specific regions on the molecule (see fig. 1.5) . At least four regions were found to interact with the cell surface, two in an adhesive manner and two in a counter-adhesive manner. The counter-adhesive sites span the EGF like repeat domain and the FNIII like repeats 7 - 8, whereas the adhesive sites span the FNIII like repeats 2 - 6 (including the variable region) and the C terminal FG like region (Prieto *et al* 1992, Fischer *et al* 1997,

Philips *et al* 1998). The predominance of adhesive or counteradhesive properties appears to be cell type specific and also depends on cell receptor profile (Crossin *et al* 1996).

The approach to functional studies has been the deletion of whole regions of the TNC molecule and as a result they concentrate on the action of single domains. TNC function is dependant on the concerted action of individual domains (Fischer *et al* 1997) and this approach may not give a true picture of function. Surprisingly, due to the complex nature of this molecule, no specific ligand binding properties have been associated with the EGF like region (Crossin *et* al 1996). A possible role for this domain stems from its similarity to EGF, a potent growth factor involved in the regulation of cell growth and differentiation. However, any mitogenic effects of TNC have been associated with the FNIII like repeat region and the effect is thought to act via a different mechanism to growth factors (End *et al* 1992).

1.2.6.2 Interactions of tenascin C with the extra-cellular matrix

The structural diversity of the FNIII like repeat region has functional implications, and this is illustrated by the number of functional studies on this domain. The variably spliced region between the fifth and sixth repeat confers a high degree of functional diversity (Chiquet-Ehrissmann *et al* 1990) and a number of ligands are targeted to this area (see figure 1.5). The third to sixth FNIII like repeats have been linked with an adhesive capacity whereas the seventh and eighth repeat are counteradhesive (Prieto *et al* 1992). These latter domains have previously been shown to have a cell binding site (Spring *et al* 1989) however, conflicting activities have been reported for this region depending on the method of investigation (Crossin *et al* 1996). The third to sixth repeat have been shown to be adhesive for a range of cell types including endothelial cells (Sriramarao *et al* 1993), fibroblasts (Prieto *et al* 1992) and neural cells (Crossin *et al* 1996). An integrin binding site has been localised to this area (Gulcher *et al* 1991, Bourdon & Ruoslahti, 1989) and the third repeat has been shown to have an RGD recognition sequence (Leavesley *et al* 1992, Ruoslahti 1996). A range of cell type specific integrins have since been identified as binding to this sequence (figure 1.5). Specifically, the integrins $\alpha_8\beta_1$, $\alpha_v\beta_3$, $\alpha_2\beta_1$, $\alpha_x\beta_1$ and $\alpha_v\beta_6$ bind to this tri-peptide. The integrin $\alpha_9\beta_1$ also binds to this domain of TNC, but not the RGD sequence (Yokasaki *et al* 1998). The sixth repeat has been shown to bind the $\alpha_x\beta_1$ integrin, and has been associated with neuron attachment and neurite outgrowth (Giese *et al* 1996, Philips *et al* 1998). Yokosaki *et al* (1996) considered the effect of individual integrins binding to the third FNIII like repeat and showed the interaction directly stimulated cell proliferation and that different integrins can induce quantitatively different proliferative responses. These responses are

based around the reorganisation of the actin cytoskeleton, a process involved in cell migration and proliferation. Studies on the $\alpha_v\beta_3$ integrin have identified a downstream pathway involving phosphorylation of specific receptor tyrosine kinases (Yokosaki *et al* 1996, Jones *et al* 1997). However, this contrasts with earlier work (End *et al* 1992), but it has been suggested that this is due to alternative growth activation pathways being implemented in normal and malignant cells (Jones & Jones, 2000).

A number of other cell surface receptors bind to the conserved FNIII like repeats. Namely, fibronectin (Hoffman *et al* 1988, Chiquet *et al* 1991) and contactin/F11 (Zisch *et al* 1992). Contactin is a cell surface adhesion molecule (CAM) of the immunoglobulin superfamily and is expressed by neurons during development (Ranscht 1988). It binds to TNC via an uninterrupted segment between the fifth and sixth repeat, thus will only bind to the smallest splice variant (Zisch *et al* 1992).

Like contactin, fibronectin also binds strongly to the truncated form of TNC. However, fibronectin will also bind to larger forms of TNC but the presence of alternatively spliced repeats weakens this binding (Chiquet-Ehrissman *et al* 1991, Ghert *et al* 2001). Other studies have consistently demonstrated that TNC interferes with the action of fibronectin (Chiquet-Ehrissmann *et al* 1988, Lightner & Erickson 1990) thus, exhibiting an important effect on cell behaviour, particularly migration and tissue remodelling. Tremble *et al* (1994) showed that a mixed TNC/fibronectin substrate upregulated a specific cohort of matrix degrading enzymes: collagenase, stromelysin and the 92kDa gelatinase. This effect was localised not to the alternatively spliced domain but to an FNIII like repeat in the distal conserved region of this domain, already associated with a counteradhesive effect (Prieto *et al* 1992). This link between TNC and the matrix metalloproteinases is crucial, as it indicates that TNC, possibly via other ECM components, alters the ECM to provide a modified tissue environment that is conducive to cell motility, growth, and proliferation (Jones & Jones, 2000).

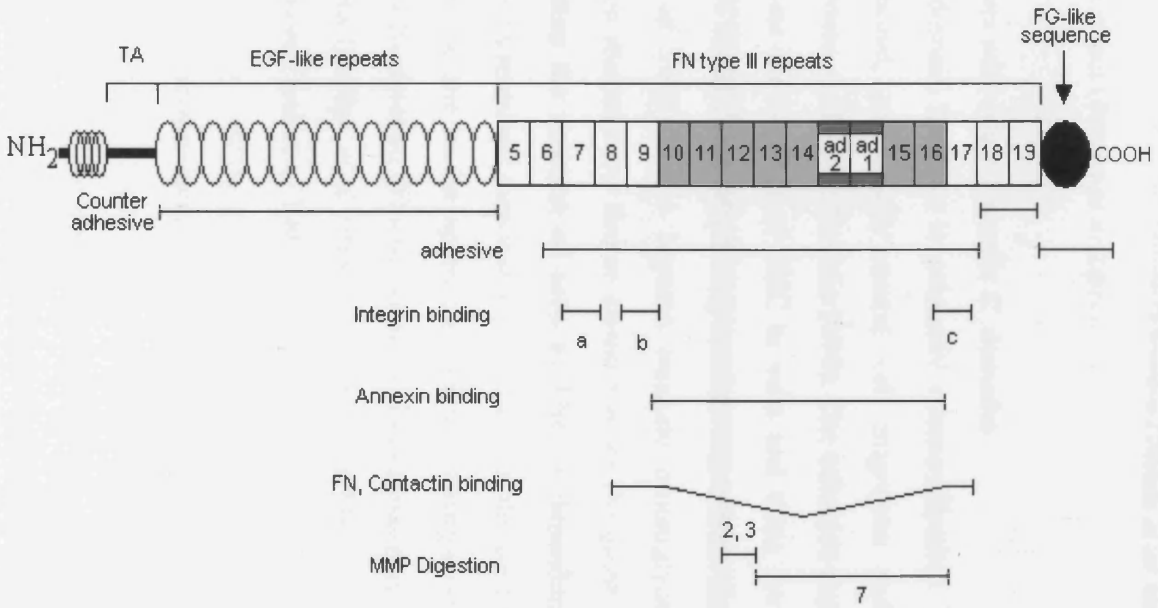


Figure 1.5: Functional domains of tenascin C monomer with examples of specific ligand binding sites - showing adhesive and counter-adhesive properties and examples of specific ligand binding associated with the FNIII-like domain. Fibronectin (FN) and contactin bind to the truncated form. Integrin binding regions are: a = $\alpha_v\beta_3$, $\alpha_2\beta_1$, $\alpha_v\beta_6$, $\alpha_9\beta_1$, $\alpha_x\beta_1$, $\alpha_8\beta_1$. b = $\alpha_x\beta_1$. c = $\alpha_x\beta_1$.

The presence of the alternatively spliced domain of TNC has been shown to have other effects on cell-substrate adhesions. Murphy-Ullrich *et al* (1991) showed that a recombinant form of this domain was a potent downregulator of preformed focal adhesions. Loss of focal adhesions is a characteristic of malignantly transformed cells and is associated with increases in cell motility (Bershadsky *et al* 1985) and may be accompanied by a mitogenic response (Schliwa *et al* 1984). It is suggested that the TNC induced loss of cell adhesion is via interaction with a cell surface molecule (Murphy-Ullrich *et al* 1991). Annexin II is a possible mediator of this effect (Emoto *et al* 2001).

1.2.7 Interactions with other tenascin C domains

The EGF like domain function is primarily counteradhesive. It has been associated with neural development, particularly neural cell migration and pathfinding during CNS development (Prieto *et al* 1992, Crossin 1994). The adhesive capacity of the FG like globe is thought to initiate the binding of TNC to cells and there are a number of potential cell surface receptors for this area which may mediate this effect. These include the integrin $\alpha_2\beta_1$ (Sriramarao *et al* 1993) and a heparan sulphate proteoglycan (Salmivirta *et al* 1991). Mutation deletion studies show that an absence of the FG globe stops the effects of the other domains indicating the reaction of cells to TNC is dependent upon the interplay of all domains. This is to an extent demonstrated by some of the conflicting functions attributed to TNC which may be due to the approach used to investigate function such as isolation of specific domains by deletion (Fischer *et al* 1997), recombinant expression of single repeats or whole domains (Philips *et al* 1998), plus effects of TNC monomers as well as studies on intact hexabrachions (End *et al* 1992).

To summarise, the complex nature of TNC structure/function relationships has been shown to rely on an interplay of domains in the native hexabrachion. Effects are further modulated by TNC itself and the complex nature of the ECM and cell receptor profile. Thus it can be stated that TNC is a highly structurally complex molecule and this is reflected in its apparent functional diversity. Finally, an important point is made by Jones & Jones (2000) who state that *in-vitro* functional studies may not accurately reflect the activities of the native TNC molecule.

1.3 Aims and objectives

The hypothesis of this study is that during the evolution and progression of breast cancer, key quantitative and qualitative changes occur in the expression of tenascin C. It is proposed that neoexpression of alternative TNC isoform variants can alter the microenvironment within the ECM and promote tumour cell invasion. Identification of these variants at a pre-invasive stage may allow the early detection of breast lesions with the potential for invasion.

To investigate this hypothesis, the specific aims of the project are:

1. To develop a technique for the identification of alternatively spliced isoforms of TNC for application to both cells and tissue samples.
2. To use this technique to identify the key changes in TNC isoform expression between normal, benign, *in situ* and invasive carcinoma. The aim is to identify, both qualitative and quantitative changes in expression in relation to disease progression.
3. To investigate TNC protein distribution in normal, benign, *in-situ* and invasive carcinoma and relate this to disease progression and TNC isoform expression.
4. To develop an *in-vitro* approach to study the control of TNC expression and its effects on cell behaviour using primary and established cell lines. The specific points that will be addressed are:
    a. The relationship between TNC expression and tumour cell behaviour in terms of proliferation and migratory capacity.
    b. The effect of exogenous TNC on tumour cell behaviour.
    c. The effect of growth factors on TNC expression.

It is hoped that the tissue study will identify specific isoforms associated with disease progression, and may provide useful markers predictive of invasive behaviour. The study of cellular interactions with TNC should help elucidate the functional significance of TNC.

Chapter 2 – Materials and Methods

2.1 Materials

2.1.1 Breast tissue and histology

All breast tissue was received fresh within 30 minutes of surgery at Glenfield Hospital, Leicester, or from Leicester Royal Infirmary (LRI). Samples approximately 8x5x3 mm were selected by a pathologist and frozen in liquid nitrogen, with subsequent storage in the vapour phase of a liquid nitrogen freezer. In most cases, parallel formalin fixed paraffin embedded blocks were taken. Normal human tonsil tissue was obtained from LRI.

The histological characteristics of the specimens were recorded by one of two breast pathologists (Prof. R. A. Walker or Dr. J. L. Jones). Tumours were classified according to the National Health Service Breast Screening Programme Reporting Guidelines (1995) and graded using the modified Bloom and Richardson System (Elston & Ellis, 1991). Details of the specimens used in the study are outlined in table 2.1.

Tissue Type	Number
Benign	25
Fad.	5
DCIS	16
LCIS	1
IDC I	10
IDC II	11
IDC III	14
ILC	7
Other*	2
Total	89

Table 2.1; Phenotype and number of primary breast tissues analysed in the current study - . Key: Fad. – Fibroadenoma, DCIS – ductal carcinoma *in-situ*, LCIS – lobular carcinoma *in-situ*, IDC – infiltrating ductal carcinoma (grades I, II, & III), ILC – infiltrating lobular carcinoma, * - other invasive tumours are one case of tubular carcinoma and one mixed infiltrating lobular/infiltrating ductal carcinoma.

2.1.2 Cell Lines

All cell lines were originally obtained from the American Type Culture Collection (ATCC, Rockville, MD.,USA.). Details of established cell lines are as follows:

MCF7: Derived from a malignant pleural effusion secondary to breast carcinoma. It retains characteristics of differentiated mammary epithelium, including expression of oestrogen receptor (Soule *et al* 1973).

T47D: Isolated from a pleural effusion in a 54 yr old female with an infiltrating ductal carcinoma. It shows epithelial differentiation and is oestrogen receptor positive (Freake *et al* 1981).

MDA-MB 231: Derived from malignant pleural effusion secondary to breast carcinoma. It forms poorly differentiated adenocarcinoma, consistent with grade III tumour, in nude mice (Cailleau *et al* 1974). The tumour cells are oestrogen receptor negative.

MDA-MB 468: Human breast adenocarcinoma derived from a pleural effusion. The tumour cells are oestrogen receptor negative (Cailleau *et al* 1978).

HBL100- Derived from the milk of a lactating mother, three days after delivery. Initially described as normal, although later shown to have abnormal features (Gaffney, 1982).

MCF10A: Derived from mastectomy tissue from a 36 yr old female with fibrocystic disease. This is a non-tumourigenic cell line, and is thought to have epithelial characteristics (Tait *et al*, 1990).

HT1080: A cell line derived from a fibrosarcoma in a 35 yr old male Caucasian. Established by Rasheed *et al* (1974).

Hfff2: Derived from a 14-18 week old human foetus. This is a fibroblast cell line obtained from foetal foreskin.

Sk-mel 28: Derived from malignant melanoma of a 51 yr old male and obtained from the Department of Obstetrics & Gynaecology, University of Leicester (Carey *et al,* 1976).

Primary Cell Cultures: Reduction Mammoplasty (RM) material was used as a source of primary fibroblasts, primary myoepithelial cells, and primary epithelial cells. Tissue was obtained from routine cosmetic RM procedures at Leicester Royal Infirmary, cell types separated, characterised, and donated to the current study by Dr. Louise Jones, Dr. Linda Gordon and Debbie Holliday, Breast Cancer Research Unit, Glenfield General Hospital.

2.1.3 Tissue Culture

2.1.3.1 Reagents

Dulbeccos minimum essential medium (DMEM), MEM α medium, dimethyl sulphoxide (DMSO), L-glutamine, insulin, hydrocortisone, non-essential amino acids and Fetal Bovine Serum (FBS) were all from Sigma. Trypsin/EDTA, Hams F12 medium, horse serum, and sterile phosphate buffered saline (PBS) were from GibcoBRL. Tissue culture flasks, both 25cm^2 and 75cm^2, 6 and 12 well plates and inserts (with 8μm pores) were all obtained from Falcon.

2.1.3.2 Cell Substrates and Growth Factors

Tenascin C protein was obtained from Chemicon, USA. Poly-D-lysine, Epidermal Growth Factor (EGF) and Transforming Growth Factor β1 (TGFβ) were obtained from Sigma.

2.1.4 Molecular Biology Materials

2.1.4.1 Enzymes

Taq. DNA polymerase was from Promega. Proteinase K and Expand Reverse Transcriptase (RT) were from Boehringer Mannheim. Terminal deoxynucleotidyl transferase (TdT) was from GibcoBRL.

2.1.4.2 Probes and Primers

All probes and primers were purchased from Genosys and supplied as lyophilised oligonucleotide pellets. All forward primers were supplied 5' biotinylated. For primer sequences and position data see tables 2.2 and 2.3.

Primer	Sequence (/ = exon boundary)	Exon	Position(bp)	Region	Fn Repeat*
EGFF	5' TCC TGC TGA CTG TCA CAA TC 3'	2	1266-1285	egf.	n/a
EGFR	5' TGC TCA CAT ACA CAT TTG CC 3'	2	1508-1489	egf.	n/a
T8F	5' CAA TCC AGC GAC CAT CAA CG 3'	8	3057-3076	con.	4
T11F	5' CAA TTT GGG AGA GGT CGT GG 3'	11	3633-3652	var.	7 (A2)
T11P	5' ACG ACC TCT CCC AAA TTG GG 3'	11	3650-3631	var.	7 (A2)
T12P	5' GCC TGG GAT TTC CAT GGA AC 3'	12	4080-4061	var.	8 (A3)
T13P	5' TCT CCC AGC TGT GGG AGA TC 3'	13	4190-4171	var.	9 (A4)
T14F	5' TCT GGT GCT GAA CGA ACT GC 3'	14	4595-4613	var.	10 (B)
T14P	5' GTT CGT TCA GCA CCA GAG AT 3'	14	4610-4591	var.	10 (B)
AD1P	5' GCA GTG AGT GAG CGT CAC TC 3'	AD1	130-111	var.	AD1
AD2F	5' ATC TCG TGG GAA GCT CAG G 3'	AD2	61-80	var.	AD2
AD2R	5' GAC AGA GCT GCG AGA CAC C 3'	AD2	177-159	var.	AD2
T15P	5' TTA GGT TTT CCA GAA GGG GC 3'	15	4741-4722	var.	11 (C)
T16P	5' GTT GTC AAC TTC CGG TTC GG 3'	16	5290-5271	var.	12 (D)
T18R	5' CGT CCA CAG TTA CCA TGG AG 3'	18	5410-5391	con.	13
T25F	5' TGA ACA AAA TCA CAG CCC AG 3'	25	6323-6342	Fib.	n/a
T26P	5' TGA TGG CTG AAT CTG TGT CC 3'	26	6541-6522	Fib.	n/a
T27R	5' CAG TGG AAC CAG TTA ACG CC 3'	27	6656-6637	Fib.	n/a
9/17F	5' CA TCC ACT G/CC ATG GGC TC 3'	9/17	n/a	n/a	n/a
9/16F	5' GC ATC CAC TG/A AGC CGA AC 3'	9/16	n/a	n/a	n/a
9/14F	5' GG CAT CCA CTG/CCA AAG AAC 3'	9/14	n/a	n/a	n/a
14/16R	5' TTC GGC TT/C TGT CGT GGC 3'	14/16	n/a	n/a	n/a

Table 2.2: TNC oligonucleotides used for PCR and Southern hybridization - key: Fn = fibronectin type III-like repeat, egf.= epidermal growth factor like repeat, con.=conserved Fn, var.=variably spliced FnIII-like repeat, Fib.=fibrinogen like domain, n/a = not applicable, * two terminologies are used to describe the FnIII- like repeats. Position (bp) taken from TNC cDNA sequence (Accession number NM_002160).

2.1.4.3 Membranes

Boehringer Mannheim supplied positively charged nylon membrane and nylon mesh. Nitrocellulose membrane supplied by Sartorius.

2.1.4.4 Electrophoreisis

Agarose was from ICN, and Nusieve Low Melting Point (LMP) agarose was from FMC. 100bp ladder was from GibcoBRL.

Primer set	Forward Primer	Reverse Primer	AT.(°C)
GAPDH	5' AGAACATCATCCCTGCCT CC 3'	3' GCCAAATTCGTTGTCATACC 5'	60
MMP1	5'CGACTCTAGAAACACAAGAGCAAGA3'	5'AAGGTTAGCTTACTGTCACACGCTT3'	58
MMP2	5'ATTGATGCGGTATACGAGGC3'	5'GGCACCCTTGAAGAAGTAGC3'	59
MMP7	5'GTTTAGAAGCCAAACTCAAGG3'	5'CTTTGACACTAATCGATCCAC3'	60
MMP9	5'CACTGTCCACCCCTCAGAGC3'	5'GCCACTTGTCGGCGATAAGG3'	58
MMP11	5'TAAAGGTATGGAGCGATGTGAC3'	5'TGGGTAGCCAAAGGTGTAGAAG3'	58
MMP13	5'GTGGTGTGGGAAGTATCATCA3'	5'GCATCTGGAGTAACCGTATTG3'	51
TIMP1	5'TGGGGACACCAGAAGTCAAC3'	5'CAGGGGATGGATAAACAGGG3'	59
TIMP2	5'AACGACATTTATGGCAACCC3'	5'ACCTGTGGTTCAGGCTCTTC3'	59
MT1-MMP	5'TCGCCAATGGAAAGACCTAC3'	5'TGATGATCACCTCCGTCTCC3'	60

Table 2.3: Non-tenascin C related oligonucleotides for PCR – showing forward and reverse primer sequences and annealing temperatures.

2.1.4.5 Photography and Autoradiography

Gel photographs were taken using a Sony Gel Documentation system 5000 and a UVP transilluminator, with Sony UPP-11-HD thermal paper. Chemiluminescent film was supplied by Amersham. Exposed autorads were developed using an automatic RP-X-OMAT Kodak processor.

2.1.4.6 Miscellaneous

Oligo dT paramagnetic beads, streptavidin paramagnetic beads (dynabeads) and Magnetic Particle Concentrator (MPC) obtained from Dynal, UK. Clear barrier wrap was supplied by Saran.

2.1.5 Immunohistochemical materials

2.1.5.1 Primary antibodies

Two antibodies to tenascin C were used:-

1. A monoclonal antibody to human tenascin C (clone BC-24) was obtained from Sigma, UK. The antibody recognises an epitope located in the EGF-like repeat region of human tenascin C, with its specificity confirmed.
2. A monoclonal antibody to human tenascin C (clone αIIIB) obtained from Chemicon, USA. The antibody recognises an epitope contained within domain B (corresponding to exon 14) in the alternatively spliced fibronectin type III like repeats.

A monoclonal antibody to the thymidine analogue, bromodeoxyuridine (BrdU) (clone Bu20a) was obtained from Dako, Denmark. The antibody recognises BrdU in single stranded DNA (produced by partial denaturation of double stranded DNA).

2.1.5.2 Secondary and tertiary antibodies

Biotinylated rabbit anti-mouse $F(ab)_2$ fragment, streptavidin and biotinylated horse-radish peroxidase obtained from Dako, Denmark.

2.1.5.3 Miscellaneous

Normal rabbit serum was from GibcoBRL, UK. Diaminobenzidene was from Sigma, UK.

2.1.6 *In-situ* hybridisation probes

Tenascin C probes were modified from PCR products (see methods). κ and λ immunoglobulin probes were obtained from Novocastra Laboratories.

2.1.7 Commonly used buffers and solutions

All chemical reagents were "analar" grade obtained from Sigma with the following exceptions:- Expand-RT buffer, 2'-deoxynucleoside 5'-triphosphates (dNTP's), digoxigenin-11-dUTP, cobalt chloride ($CoCl_2$), dithiothreitol (DTT), anti-digoxigenin alkaline Fab fragment, proteinase k, blocking reagent, CDP Star (all Boehringer Mannheim), RNasin

(Promega), TdT buffer (GibcoBRL), BSA (Advanced Protein Products), paraffin oil (Huddersfield Royal Infirmary), NBT/BCIP ready to use tablets (Roche).

Alkali denaturing solution	1.5M NaCl, 0.5M NaOH.
BCIP	0.5% 5-bromo-4-chloro-3-indolylphosphate w/v in dimethyl formamide (DMF).
Binding & Washing Solution (2x)	10mM Tris-HCl pH 7.5, 1mM EDTA, 2M NaCl.
Blocking solution (test strip & ISH)	3% BSA, 0.1% Triton X-100, in TBS & filtered before use. For test strip, incubated 37°C 2hr before filtering.
Detection buffer	100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$.
DEPC treated H_2O	0.1% DEPC incubated at 37°C 2 hours and autoclaved.
Diaminobenzidene solution	0.5mg/ml, 0.03% H_2O_2, in TBS.
dNTP mix (for asymmetric PCR)	13 mM dTTP, 20 mM each of dATP, dCTP, dGTP.
dNTP mix (PCR)	20 mM each of dATP, dCTP, dTTP dGTP.
Diluent (for test strip)	200ng of salmon sperm DNA denatured at 95°C, 10 min and placed on ice before diluting in 6xStandard Saline Citrate.
Eosin solution	0.5% Eosin,17μM calcium chloride ($CaCl_2.2H_2O$), 0.001% formaldehyde.

Freezing Medium (Breast cell lines)	50% DMEM, 2mM L-glutamine, 40% FBS, 10% DMSO.
Freezing Medium (Sk-mel 28)	50% MEM α, 40% FBS, 10% DMSO.
Gel Loading Buffer (5x)	1xTAE, 50% glycerol, 20μg/ml each of bromophenol blue and xylene cyanol.
Lysis Binding Buffer (LBB)	100mM Tris-HCl pH 8.0, 500mM LiCl, 10mM EDTA pH 8.0, 1% sodium dodecyl sulphate (SDS), 5mM dithiothreitol (DTT).
Maleic Acid Buffer (MAB)	0.1M maleic acid, 0.15M NaCl, pH 7.5.
MABT	MAB, 3% Tween 20.
Mayers Haemotoxylin	105mM aluminium potassium sulphate ($AlK(SO_4)_2.12H_2O$), 5mM citric acid ($C_6H_8O_7.2H_2O$), 303mM chloral hydrate ($C_2H_3Cl_3O_2$), 1mM sodium iodate ($NaIO_3$).
NBT	0.4mM nitroblue tetrazolium, 70% di-methyl formamide.
Neutralising solution	1.5M NaCl, 1M Tris-HCl, pH 8.
PCR reaction buffer (10x)	45mM Tris pH 8.8, 11mM $(NH_4)_2SO_4$, 4.5mM $MgCl_2$, 200mM dNTP's, 110μg BSA, 6.7mM β-mercaptoethanol (βME) and 4.4 mM EDTA pH 8.8

PCR reaction buffer (10x for assymetric PCR)	45mM Tris pH 8.8, 11mM $(NH_4)_2SO_4$, 4.5mM $MgCl_2$ 110µg BSA,6.7mM βME and 4.4 mM EDTA pH 8.8.
PE (10x)	Modified 0.5M Tris-HCl pH 7.5, 10% sodium Pyrophosphate, 20% polyvinylpyrolidone, 20% Ficoll, 0.5M EDTA.
Post-hybridisation buffer	Southern Blotting: 35% formamide, 2xSSC, 0.1% SDS. ISH: 50% or 30% formamide, 2xSSC.
Pre-hybridisation buffer	Southern Blotting: 35% formamide, 5xSSC, 30µg/ml denatured salmon sperm DNA, 2% w/v blocking reagent, 0.2% SDS, 3% "sarkosyl"NL30. ISH: 50% or 30% formamide, 0.6M NaCl, 150µg/ml denatured salmon sperm DNA, 10% dextran sulphate, 1xPE
20 x Phosphate Buffered saline (20xPBS)	2.6M NaCl, 60mM Na_2HPO_4, 140mM NaH_2PO_4.
RNase Buffer	2xSSC, 10mM $MgCl_2$, 1xPBS. Add 1mg/ 10ml RNase A 5 min. before use.
RNA gel loading buffer (10x)	96% formamide, 20mM EDTA pH 8.0.

Solution D	25mM sodium citrate pH 7.0, 0.5% sarkosyl, 4M Guanidinium thiocyanate - activated by addition 14.4 µl β mercaptoetanol.
Standard Saline Citrate (20x)	3M sodium chloride (NaCl), 0.3M trisodium citrate.
Stripping solution	0.2M NaOH, 1% SDS.
Total RNA extraction buffer	133mM sodium acetate pH 4.0, 67% phenol, 33% chloroform/iso- amyl alcohol (4% IAA).
Tris/acetic acid/EDTA(50xTAE)	2M Tris, 0.005M EDTA, 1M glacial acetic acid, pH8.
Tris Buffered Saline (20xTBS)	2.5M Tris, 7.5mM NaCl, pH to 7.65
Washing buffer	10mM Tris-HCL pH 8.0, 0.15M LiCl, 1mM EDTA.
Washing buffer/SDS	10mM Tris-HCL pH 8.0, 0.15M LiCl, 1mM EDTA, 0.1% SDS.

2.2 Methods

2.2.1 Cell lines and tissue culture

2.2.1.1 Maintenance

Sk-mel 28 - Maintained in 90% MEM α medium with 10% FBS in 25cm^2 flasks at 37°C and 5% CO_2/95% air atmosphere. Medium was completely changed every 48 hours.

Primary breast cells and other cell lines – All cell lines except MCF10A were maintained in 90% phenol red free DMEM, 2mM L-glutamine, 10% FBS in 25cm^2 or 75cm^2 flasks at 37°C and 5% CO_2/95% air atmosphere. Medium for HT1080 was supplemented with 1% non-essential amino acids. MCF10A cells were maintained in 1:1 Hams F12:DMEM plus 2mM L-glutamine, 5% heat inactivated horse serum, 10μg/ml insulin, 0.5μg/ml hydrocortisone and 20ng/ml EGF. Medium was completely changed every 48 hours.

2.2.1.2 Harvesting of cells

Cells were harvested when 90-100% confluent by trypsinisation. Cell monolayers were washed with sterile PBS followed by incubation at 37°C for 2-3 min with trypsin/EDTA to detach the cells. Following the addition of warm PBS, cells were removed from the flask and centrifuged at 1000rpm for 5min.

a) Passage of cells - cell pellet resuspended in an appropriate volume of warm culture medium and seeded into fresh flasks according to an appropriate split ratio.
b) Cell freezing - cell pellet resuspended in appropriate freezing medium. Ampoules initially stored at -70°C for 10 hrs before transfer to liquid nitrogen freezer for longer term storage.
c) Cell count for mRNA extraction - cells were centrifuged and resuspended in PBS. An aliquot was removed and diluted 1:1 with 0.4% trypan blue. The number of viable cells in 1mm^2 were counted using the improved Neubauer haemocytometer. The cell concentration was then calculated using the following formula where C = cell number, n = number of cells counted and d = dilution factor :

$$C = n \times d \times 10^4$$

2.2.2 Culture of cell lines under different conditions

2.2.2.1 Quiescent and proliferating cells

a) Quiescent cells - cells were grown under the previously described conditions until 90-100% confluent. These were classed as Quiescent, or high confluent (Hi.C.) cells.
b) Proliferating cells - cells were grown under the previously described conditions in 75cm^2 flasks until 30-50% confluent. These were classed as proliferating, or low confluent (Lo.C.) cells.

2.2.2.2 Serum supplemented or serum free medium

Cells were grown for 24hours, in six well plates, under serum supplemented conditions. Medium was then replaced with serum free DMEM and cells grown for a further 48 hours.

2.2.2.3 Transforming growth factor β (TGFβ) and epidermal growth factor (EGF)

Primary breast fibroblasts were grown for 24hours in six well plates under serum supplemented conditions, medium was then replaced with serum free DMEM and supplemented with either 5ng/ml TGFβ plus addition of 1% BSA, 20ng/ml EGF, or 100ng/ml EGF. Cells were then grown for a further 48hours.

2.2.2.4 Culture on tenascin C substrate

Breast cell lines and primary breast fibroblasts were grown on a range of concentrations of TNC in six well plates.

Six well plates were coated with TNC at coating concentrations of 1μg/ml, 2.5μg/ml and 20μg/ml in cold sterile PBS pH 7.5. 350μl TNC was added to each well plate and gently agitated for 45min. Excess fluid was removed and plates allowed to air dry for 1hour.

10^5 cells in 2ml serum supplemented medium were seeded into each well and cultured for 48hours. In parallel experiments, after 24hours medium was removed and replaced with serum free medium followed by a further 48 hours culture. Cells were then harvested by trypsinisation, cell counts performed, and appropriate numbers processed for mRNA extraction. In each case, cells grown on plastic under the same conditions were used as a negative control. All experiments were performed on three separate occasions.

2.2.2.5 Production of fibroblast conditioned medium

Fibroblast conditioned medium (CM) was generated from hfff2 cells grown on plastic, and from primary breast fibroblasts grown on plastic or on 20μg/ml TNC. Cells were grown under serum supplemented conditions until 60-70% confluent, then the medium was replaced with serum free medium and cells cultured for a further 48 hours. This CM was collected and stored in aliquots at –80°C.

2.2.3 Migration/invasion and proliferation assays

The direct effect of TNC on breast tumour cell migration and proliferation, and of TNC-primed fibroblast conditioned medium on tumour cell behaviour was analysed in a series of experiments using dual chamber migration assays combined with Bromo-deoxy Uridine (BrdU) incorporation.

2.2.3.1 Direct effect of TNC on breast tumour cells

The upper surface of 8μm pore cell culture inserts were each coated with either 100μl of 20μg/ml TNC or 5μg/ml poly-d-lysine. In each case, inserts were incubated for 45 minutes, the excess removed and inserts allowed to dry for 1 hour. 2.5 x 10^5 cells were seeded onto the inserts in serum supplemented medium for 24 hours, then changed to serum free medium. At this point, 1ml of 50% serum free DMEM: 50% hfff2 conditioned medium was added to the lower wells to provide a chemotactic stimulus. The cells were cultured for a further 48 hours. Three hours before the assays were terminated medium was removed from the insert and 0.5ml of medium supplemented with 5mM BrdU was added. All assays were performed on three separate occasions.

At the end of the assay, inserts were washed with PBS and cells removed from the upper surface of one insert and the lower surface of the other insert. To fix the cells, inserts were placed in cold acetone at 4°C for 10min. Inserts were then washed with PBS and processed for IHC detection of incorporated BrdU as described in section 2.2.3.3.

2.2.3.2 Effect of TNC-primed fibroblast conditioned medium on tumour cells

Inserts were coated with poly-d-lysine as described above, and tumour cells seeded on top, allowed to establish for 24 hours in serum supplemented medium, which was then replaced with serum free medium. To the lower chamber of test cases 1ml 50% serum free DMEM: TNC primed fibroblast CM was added, with 50% non-primed fibroblast CM to control cases. Assays were then carried out in an identical manner to that described above.

2.2.3.3 Immunohistochemical detection of incorporated BrdU

Immunohistochemistry for detection of BrdU was performed on cell culture inserts using the avidin-biotin complex (ABC) method described by Hsu et al (1981) because of the high sensitivity afforded. Partial denaturation of DNA was achieved by treating inserts with 2N HCl for 30min. followed by 3x 5min. washes in 0.1M sodium tetraborate before placing in TBS.

Inserts were covered in 20% normal rabbit serum (diluted in TBS) to block non-specific staining. Serum was removed and replaced with 1:200 dilution of the Anti-BrdU primary antibody and allowed to incubate at 4°C overnight.

Primary antibody was washed off with TBS and a 1:400 dilution of biotinylated rabbit anti-mouse $F(ab)_2$ fragment was applied, followed by incubation at room temperature for 30 min. At this point, avidin-biotin complex (ABC solution) was prepared, a mixture of 0.1% each of streptavidin and biotinylated horseradish peroxidase in TBS, and allowed to form complexes over a 30 minute period.

Secondary antibody was washed off in TBS followed by application of ABC solution to each insert and incubation at room temperature for 30 min. ABC solution was washed off with TBS for 5 min followed by application of diaminobenzidene solution for 10 min. The inserts were then washed with TBS, running tap water and counterstained with Mayers Haemotoxylin. Inserts were dehydrated to xylene, the membrane detached from the insert frame with a blade, and mounted cell side down using a resinous mountant.

2.2.3.4 Assessment of migration and proliferation

Following staining, migration and proliferation indices were calculated. Cell counts were performed on each insert by counting the number of cells in 10 high power fields using a $1cm^2$ eyepiece graticule. A migration index was calculated on the basis of the following formula:

$$\text{Migration index} = \frac{\text{cells on lower surface of membrane}}{\text{cells on lower surface + cells on upper surface}} \times 100 \text{ points}$$

A proliferation index was calculated from the following formula:

$$\text{Proliferation index} = \frac{\text{cells stained for BrdU}}{\text{total number of stained + unstained cells}} \times 100 \text{ points}$$

A minimum of 3 assays was evaluated under each condition, the mean migration and proliferation index calculated, and statistical analysis performed using SPSS version 14.

2.2.4 mRNA extraction

mRNA was extracted using Oligo-dT Dynabeads. Dynabeads are paramagnetic polymer beads to which a variety of molecules can be coupled, in this case an oligonucleotide consisting of 25 thymine residues. Thus, when mixed with a cell lysate, the poly dT tails complementarily bind to the polyadenylated tail which is added post-transcriptionally to the mRNA. The mRNA/bead complex can then be removed from the lysate with a magnet. Different methods were used to assess cell:bead ratio for cell lines and breast tissue:

2.2.4.1 Cell lines

Cell:bead ratio was optimised according to guidelines in the Dynal handbook such that mRNA from $2.5x10^5$ cells was extracted onto $1.32x10^7$ beads (in 40µl bead suspension). The cell suspension was centrifuged at 1000rpm for 5min. and the cell pellet resuspended in 250µl lysis binding buffer (LBB).

2.2.4.2 Breast tissue

A single 7µm section was cut from frozen tissue on a cryostat at -20°C onto a silane treated microscope slide. The section was then stained with haemotoxylin and eosin. Briefly, sections were air-dried for 5 min followed by fixation in 95% Industrial Methylated Spirit (IMS) for 30

secs. Sections were then rinsed in running tap water for 2 min followed by immersion in 0.1% Mayer's haematoxylin for 5secs and further rinsing. Finally sections were immersed in 0.5% eosin for 1-2 secs followed by rinsing in running tap water for 2 min and dehydration through 95% IMS, 99% IMS x 2, and xylene (each for 30 secs) before mounting from xylene in D.P.X. resinous mountant with coverslip.

Each section was examined under a light microscope (Leitz Wetzlab) and classified as high, medium, or low cellularity independently by two observers and 5, 10 or 15 x 5μm sections cut, respectively, into an eppendorf for mRNA extraction. 100μl of Lysis Binding Buffer containing 12.5μg of proteinase K was added to the eppendorf and was incubated at 37°C for 1hr following which $1.32x10^7$ oligo-dT beads were added and incubated at room temperature for 10 minutes. Bead/mRNA complex was then pelleted using MPC and washed twice with 200μl washing buffer/SDS and three times with washing buffer only, in each case discarding the supernatant following concentration of beads in the MPC. Bead/mRNA complex was resuspended in 20μl DEPC treated H_2O and stored at 4°C.

2.2.5 Reverse transcription (RT)

RT was performed using the Expand-RT kit according to manufacturers guidelines. For each extraction a positive RT and a negative RT reaction was performed.

Typically a 25μl reaction was performed containing 5μl Expand-RT buffer, 10mM DTT, 1mM dNTP's, 30U RNasin in DEPC treated H_2O. To the positive RT reaction 50U RT was added, to negative RT reaction an equivalent volume of DEPC treated H_2O was added. The reaction was performed in a Perkin Elmer Thermocycler at 42°C for 1 hr and the resultant cDNA was stored at 4°C.

2.2.6 Polymerase chain reaction (PCR)

2.2.6.1 PCR primer design

Unless otherwise stated, PCR primers were designed initially by Dr. Howard Pringle, Department of Pathology, University of Leicester, and subsequent primers with the help of Dr. Pringle. The Wisconsin Package (Genetics Computer Group) was used. For the tenascin C

primers, the program "bestfit" was used to establish areas of each exon which were not homologous to other regions. The "prime" program was then used with the default settings on all occasions. The "findpatterns" program was then used to ensure unique binding sites.

2.2.6.2 PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

In order to assess the efficiency of the mRNA extraction and RT-PCR procedure, a PCR reaction was performed using primers specific to GAPDH, designed and optimised by Dr. J. Shaw, Department of Pathology, University of Leicester. PCR was performed in a Perkin Elmer Thermocycler.

Typical reactions were in a final volume of 50µl using 1µl cDNA as a template (1µl resuspended bead complex), 5µl of 10xPCR reaction buffer and 2µl each of forward and reverse primers (10pmol/µl). Each reaction was overlaid with 1 drop of paraffin oil. Reactions were initially denatured at 94°C for 5 min. and held at the primer annealing temperature of 60°C for addition of 1U *taq.* polymerase i.e. "hot start", followed by an extension step of 72°C for 30 sec. Then 30 cycles of amplification was performed, comprising: denaturation at 95°C for 30 sec., primer annealing at 60°C for 30 sec, and an extension step of 72°C for 30 sec, followed by a final extension step of 72°C for 7min. PCR products were stored at 4°C and were visualised by running on a 2% gel containing ethidium bromide.

At this stage, if a signal was obtained from -RT samples, contamination with DNA was inferred, and the cDNA specimen was discarded. If -RT samples were negative, further PCR was carried out using only +RT samples, unless otherwise stated.

2.2.6.3 PCR Assessment of breast tissue mRNA integrity

Assessment of the integrity of mRNA utilised the previously described Dynabead extraction method. This was followed by PCR using primers specific to the EGF-like repeat region of TNC (the 5' end of the molecule) and the fibrinogen like region (the 3' end of the molecule).

Typical reactions were performed as previously described, however, using 1µl each of forward and reverse primers. The reactions then proceeded according to table 2.4.

	EGF PCR		Fib. PCR	
Step	Temp.(°C)	Time (min.sec.)	Temp.(°C)	Time (min.sec.)
Denaturing	94	0.30	94	1.0
annealing	60	0.30	58	0.45
Extension	72	0.30	72	1.0

Table 2.4: Tenascin C EGF repeat and fibrinogen domain PCR reaction conditions.

PCR products were run on a 3% gel and visualised as previously described. The intensity of product achieved with primers to the 3' and 5' end of the molecule were then compared, and since RNA is degraded in a 5' to 3' manner, a weaker EGF repeat signal indicated degradation.

2.2.6.4 PCR for tenascin C isoforms

PCR primers specific to tenascin C (TNC) were: forward strand primers for exons 8, 11, 14 and 25, and reverse strand primers for exons 11, 12, 13, 14, AD1, 15, 16, 17, 18, 26, and 27 (for position data see figure 2.1). Initial PCR reactions on cell lines used the primer cassettes 8/18, 8/14, and 14/18, which had previously been shown to be sufficiently informative (Bell *et al,*1999), plus cassette 8/AD1. PCR reactions on primary breast tissue used the cassettes 8/18 and 8/AD1 plus cassettes 11/16, and/or 14/16.

PCR was carried out as previously described as for fib. domain PCR. Products were visualised on a 1 or 2% gel.

TNC PCR was also performed with the primer cassettes 8/11, 8/12, 8/13, 11/AD1, 14/AD1, 8/15, and 8/16 using the previously described method.

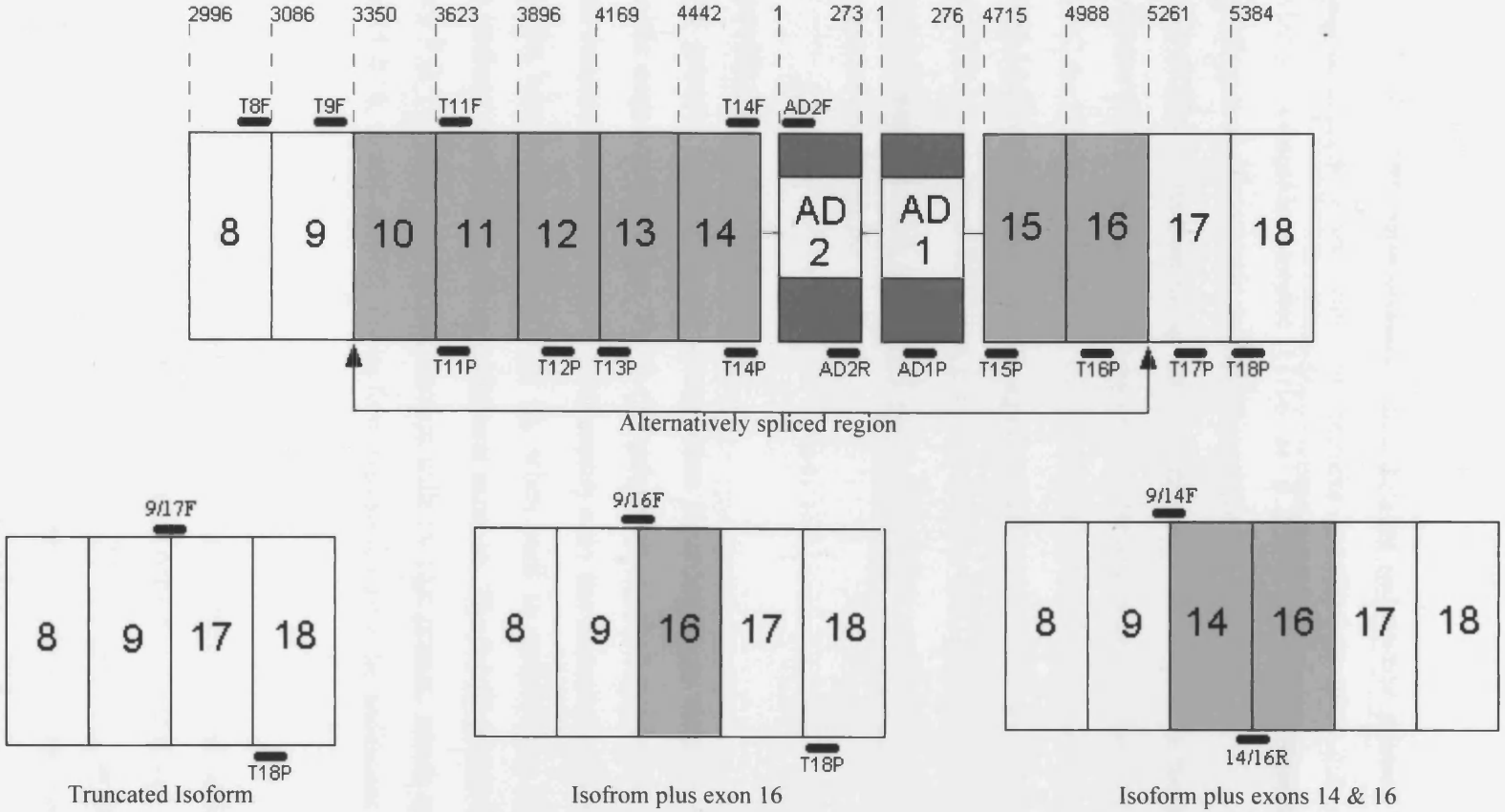


Figure 2.1: Annealing positions of TNC PCR primers and probes – Upper image shows positions in relation to the whole TNC variable region. The lower three images show positions of primers used to amplify variable regions of specific isoforms. Numbers in boxes refer to exon number, numbers above refer to nucleotide number.

2.2.6.5 PCR for AD2

PCR primers specific to domain AD2 were described by Mighell *et al* (1997), and used in this study in the following reactions:

1. *PCR for AD2 with other TNC exon primers - AD2* forward and reverse primers were used in the following combinations with the TNC primers described in table 2.2 :- 11/AD2, AD2/AD1, AD2/16 alongside cassette 11/16 as a positive control. Reactions were performed according to the previously described protocols.
2. *"Standard" AD2 PCR* - Performed using the cassette AD2F/AD2R under the previously described conditions with the following alterations:- A denaturation step for 30 sec, primer annealing at 64°C for 30 sec, an extension step of for 30 sec. PCR products were run on a 4% agarose (50% LMP agarose) gel with 2% agarose wells at 80-100 V for 2 hours.
3. *"Nested" AD2 PCR* - First round PCR was performed using TNC primer cassette 8/18 for 30 cycles. Second round PCR was performed using 1µl of first round PCR products with AD2F/AD2R cassette for 30 cycles.

2.2.6.6 PCR for specific tenascin C isoforms

A number of PCR primers were designed across exon boundaries, to amplify individual isoforms with specific exon combinations. The 9-17F primer across the boundaries of exons 9 & 17, when used in conjunction with T18R, would amplify only the truncated isoform. The 9-16F primer across the boundaries of exons 9 & 16, when used in conjunction with T18R, would amplify the isoform containing only an additional exon 16. The 9-14F primer across the boundaries of exons 9 & 14, when used in conjunction with 14-16R primer, which crosses the boundary of exons 14 &16, would amplify the isoform containing only the additional exons 14 & 16 (see figure 2.1).

PCR reactions were optimised using gel separated PCR products of the truncated isoform only, an isoform containing additional exon 16 only, and an isoform containing additional exons 14 & 16 only. Purified isoforms were obtained by performing TNC 8/18 PCR, as previously described, on two IDC samples known to produce the isoforms. PCR products were run an agarose gels and individual isoform PCR products extracted using a Hybaid Recovery Gel Extraction kit according to manufacturers instructions. This uses a modified silica matrix to

bind DNA in a melted agarose gel fragment, prior to high stringency washes and elution of DNA from the matrix.

To optimise primers, a number of PCR protocols were used:

1. *"Standard" PCR* - A denaturation step for 1 min, primer annealing for 45 sec (annealing temperatures of 58, 61, 65, 68, 69 & 72°C were investigated), and an extension step for 1 min, for 35 cycles of amplification. For the 9-14/14-16 and the 9-16/18 cassettes, PCR was performed at an annealing temperature of 70°C for 25 and 30 cycles.
2. *"Semi-touchdown" PCR* – for the 9-16/18 cassette, 10 cycles of denaturation for 1 min, primer annealing at 71°C for 45 sec, and an extension step for 1 min, followed by 20 cycles using an annealing temperature of 68°C, then a final extension step.
3. *"Nested" PCR* - First round PCR using TNC primer cassette 8/18 for 35 cycles. Second round PCR using 1µl of first round PCR products with 9-14/14-16 cassette for 30 cycles using an annealing temperature of 69°C. This protocol was then applied to a range of primary breast tissues.

2.2.6.7 Cycle sampling

In order to gain information on relative quantity of transcript, a cycle sampling technique was performed on a representative series of samples. This involved choosing 6 benign, 6 DCIS, 9 IDC, and 1 ILC sample and performing a GAPDH PCR at 25 and 30 cycles. 12 of these samples (4 benign, 5 DCIS, 4 IDC, and 1 ILC) were chosen for further investigation. Three PCR protocols were used:

1. *Fib. Domain PCR* – performed according to the previously described protocol with 5µl aliquots removed from the PCR reaction at 30, 35, and 40 cycles.
2. *9-14F/14-16R* - performed according to the previously described protocol with 5µl aliquots removed from the PCR reaction at 30, 35, 40 and 45 cycles.
3. *"Nested" PCR* - First round PCR using TNC primer cassette 8/18 with 5µl aliquots removed from the PCR reaction at 20, 25, 30, 35, and 40 cycles (each used as template for second round). Second round PCR using 1µl of first round PCR products with 9-14/14-16

cassette for 25 cycles using an annealing temperature of 69°C. This method was initially performed on 1 DCIS and 2 IDC samples before applying to the other samples.

PCR products were visualised as previously described.

2.2.6.8 PCR for matrix-metalloproteinase (MMP)

PCR primers were obtained that were specific for a range of MMPs implicated in breast carcinoma. The sequences for MMP 1, 9, 11 and 13 were obtained from Giambernardi *et al* (1998) and MMP7 from Yamashita *et al* (1999). The primers for MMP 2, TIMPs 1 and 2, and MT1-MMP were designed within the department of Pathology, University of Leicester.

PCR was carried out as previously described in a Perkin Elmer 9700 thermocycler using 30 second denaturing, 30 second annealing and 30 second extension steps. Reactions were either 30 or 35 cycles and were performed alongside a positive control of HT1080 cDNA for all reactions except MMPs 7 and 11 that used MDA-MB 468 cDNA. Products were run on 2% agarose gels and visualised as previously described.

For primary breast fibroblasts a cycle sampling protocol was used to achieve a semi-quantitative analysis. 10µl aliquots were removed at 25 and 30 cycles with the reaction allowed to run to completion at 35 cycles. 10µl aliquots were then mixed with 5x loading dye and run on 2% agarose gels as previously described.

2.2.7 Southern hybridisation

Southern analysis of TNC PCR generated fragments (from a variety of cassettes) was carried out using a method described by Southern (1975) modified to use digoxigenin labelled probes, anti-digoxigenin alkaline phosphatase conjugates and chemilluminescent visualisation.

2.2.7.1 Digoxigenin labelling of oligonucleotide probes

Digoxigenin is a derivative of the cardiac glycoside, digoxin. It is conjugated to deoxy-uridine triphosphate, which can be used for labelling of oligonucleotide probes. The P series of TNC primers (see figure 2.1 & table 2.2) were designed to use as oligonucleotide probes in Southern blotting. Probes were specific to exons 11, 12, 13, 14, AD1, 15, and 16.

An "End Labelling" protocol was used. This homopolymer tailing method uses recombinant TdT (TdT) to add nucleotides to the 3' hydroxyl terminus of an oligonucleotide in a template independent manner. Typically 1µg of probe was labelled in a 20µl reaction mixture containing 4µl 5xTdT buffer, 5mM $CoCl_2$, 0.085mM digoxigenin-11-dUTP, 0.425mM dATP, and 45U of TdT. Reactions were performed in a Perkin Elmer Thermocycler at 37°C for 15 min. The reaction was terminated by the addition of 0.5M EDTA and diluted to a volume of 50µl (20ng/µl). Labelled probes were stored at -20°C.

2.2.7.2 Test strip

The efficiency of the labelling reaction was assessed by the use of a test strip. Serial dilutions were prepared for each probe using a diluent. 1µl of each probe concentration (1000, 100, 50, 10, 5, 1, 0.5 & 0 pg/µl) was pipetted onto a nitrocellulose membrane and baked at 80°C for 2hr.

The test strip was then placed in a hybridisation chamber and immersed in a blocking solution at 37°C for 20 min with agitation. The strip was baked at 80°C for 20 min and rehydrated in blocking solution for 20 min. Immunological detection of the labelled probes was achieved by laying the test strip flat on perspex and covering with a 1:600 dilution of anti-digoxigenin alkaline phosphatase conjugate in blocking solution for 30 min. followed by three washes in TBS and two washes in dH_2O. The strip was then washed in detection buffer for 5 min, followed by incubation in detection buffer containing BCIP and NBT, in darkness for 1hr 30 min. The alkaline phosphatase catalyses the conversion of BCIP/NBT to a coloured precipitate. After incubation the strip was then washed twice in dH_2O for 5 min and allowed to air dry.

2.2.7.3 Blotting method and hybridisation

20µl of appropriate PCR product was added to 5µl of 5x loading buffer and denatured in a Perkin Elmer Thermocycler at 95°C for 5 min prior to loading on a 1 or 2% gel. Gels were run and visualised as previously described and were then pre-treated prior to transfer in alkali denaturing solution for 30 min, neutralising solution for 30 min and 20x SSC for 10 min. DNA

was transferred to a positively charged nylon membrane by capillary transfer in 20 x SSC for approximately 18 hr.

Following blotting, membranes were washed in 6 x SSC and wrapped in a clear barrier wrap. DNA was immobilised on the membrane by UV crosslinking on a transilluminator. Efficiency of transfer was assessed by restaining the gel in 1xTAE staining buffer and visualised using the previously described method. Membranes were stored flat at room temperature.

Membranes were pre-wetted in 6xSSC, placed between two nylon meshes in a hybridisation tube and pre-hybridised in 19 ml pre-hybridisation buffer in a Hybaid rotary oven at 37°C for a minimum of 1 hr. Membranes were then probed with 100ng of a single oligonucleotide (100ng digoxigenin labelled probe added to 1ml prehybridisation buffer which was then added to the 19ml in the tube) for approximately 18 hr at 37°C. Membranes were then washed in post-hybridisation buffer three times for 15 min at 37°C in Hybaid oven followed by a 5 min wash at room temperature with maleic acid buffer plus tween (MABT). Immunological detection of digoxigenin labelled hybrids was performed by blocking the membrane in 2% w/v blocking reagent for 30 min, followed by incubation with 1/10,000 dilution of anti-digoxigenin-alkaline phospatase conjugate with 2% w/v blocking reagent for 30 min. Membranes then underwent three 15 min washes in MABT followed by a 5 min wash in detection buffer. The chemilluminescent signal was produced by incubating the membrane flat on a piece of perspex with CDP-Star diluted 1/100 in detection buffer for 5 min followed by exposure to chemilluminescent film for 1-5 min at room temperature.

2.2.7.4 Stripping and storage of membranes

Prior to storage, membranes were stripped of probe by washing for 2 x 25 min at 37°C in stripping solution followed by washing in 2 x SSC 30 min at 37°C. Membranes were stored flat at room temperature.

2.2.8 Immunohistochemistry

Immunohistochemical localisation of tenascin C was performed on cryostat sections of benign and malignant breast tissue.

2.2.8.1 Section preparation

5-6μm cryostat sections were cut from cryofrozen tissue blocks onto aminopropyltriethoxysilane coated slides at -20°C and stored at -70°C in an airtight container, for a maximum of seven days, until required. Sections were air dried at RT for 20min prior to fixation.

2.2.8.2 Fixation

Sections were fixed in acetone for 10 min at 4°C and then allowed to equilibrate in tris buffered saline (TBS).

2.2.8.3 Immunological staining protocol

The ABC method was used as described previously but omitting the antigen retrieval step. Initial experiments determined the optimal dilution of primary antibody (see table 2.5) which was then employed throughout the study.

Antibody	Dilution
Anti-Tenascin C (clone BC24)	1:7,500
Anti-Tenascin C (clone αIIIB)	1:1,000

Table 2.5: Primary antibody dilutions for immunohistochemistry

2.2.8.4 Controls

For each section a negative control was performed alongside, which consisted of omission of primary antibody. To ensure consistency of results a mixture of benign and malignant samples were included in each experiment.

2.2.9 *In-Situ* Hybridisation (ISH)

Probes were generated for ISH using asymmetric PCR.

2.2.9.1 Asymmetric PCR

Asymmetric PCR was used to produce digoxigenin-11-dUTP labelled probes for *in- situ* hybridisation from PCR products. A reverse/antisense primer was used to produce an antisense oligonucleotide probe from the sense/coding strand of the PCR product. Digoxigenin-11-dUTP was used as a replacement for a proportion of the nucleotide dTTP to produce a labelled probe.

A standard TNC PCR protocol was performed and specific bands extracted from agarose gels using Hybaid Recovery Gel Extraction Kit as previously described. These products were used as a template in the generation of probes which used a standard PCR protocol adapted in the following way: reactions were performed in a Perkin Elmer Thermocycler in a final volume of 50µl using 1µl of appropriate PCR product as a template, 5µl of 10x asymmetric PCR reaction buffer, 0.5µl of 10x asymmetric dNTP mix, 3.5nmol of digoxigenin-11-dUTP and 10µl of the appropriate reverse primer (10pmole/µl). Each reaction was overlaid with 1 drop of paraffin oil. Reactions were initially denatured at 94°C for 5 min. and held at the appropriate primer annealing temperature for "hot start". The reactions then proceeded according the PCR protocols previously described, but without a final extension step.

Asymmetric probes were synthesized for β-actin, GAPDH, TNC EGF rpt., TNC fib. domain, TNC cassette 9-14/14-16 and also the smallest band from the 8/18 cassette and the largest band from the 14/18 cassette. For β-actin, a negative sense probe was generated using a forward primer in the asymmetric reaction and template prepared from RT-PCR using a biotinylated reverse primer.

2.2.9.2 Probe purification

For removal of biotinylated forward (sense) strands, 45µl asymmetric PCR product was mixed with 2µl Streptavidin Dynabeads (10µg/µl) and incubated at room temperature for 15 minutes. The bead complex was then pelleted using MPC, the supernatant removed and mixed with a

further 2µl Streptavidin Dynabeads. This was again pelleted in the MPC, the supernatant removed to a fresh tube, and stored at -20°C.

2.2.9.3 In-situ-hybridisation (ISH) protocol

To avoid degradation of RNA, all reagents used were treated with DEPC and glassware treated with 3% H_2O_2 to inactivate RNases.

4µm sections were cut from formalin-fixed paraffin embedded tissue blocks onto aminopropyltriethoxysilane coated slides. For each sample, at least three replicate slides were prepared. Sections were dewaxed in xylene and hydrated through serial alcohol dilutions (2x 99% IMS, 1x 95% IMS) to DEPC treated H_2O. Pre-treatment involved incubation with proteinase K (10µg/ml in 50 mM Tris HCl pH 7.6) for 1 hour at 37°C. Slides were then rinsed and washed in DEPC treated H_2O. Slides were placed in a humid chamber and each section was covered with 150µl of pre-warmed pre-hybridisation solution, an RNase free coverslip was placed over each section, and slides were incubated at 37°C for 1 hour. For hybridisation a range of probe dilutions and combinations were used (see table 2.6). Total TNC probes i.e. EGF rpt. & fib. domain were combined in pre-hybridisation solution. Probes to κ and λ immunoglobulin were used on tonsil tissue as a positive control. 8/18, 14/18 and fib. domain. probe were used individually.

Probe	**Dilution**
β-actin	1:40
GAPDH	1:40
TNC 14-16	1:40
TNC 8/18	1:40
TNC 14/18	1:40
Fib. domain.	1:40,1:80*
EGF. Rpt.	1:40,1:80*
κ immunoglobulin	#
λ immunoglobulin	#

Table 2.6: Probe dilutions used for ISH. * - combined mixture of EGF. rpt. and Fib. rpt. probes. # - 20µl of manufacturers pre-diluted solution added to each section.

As much pre-hybridisation solution as possible was removed from each section and replaced with 100µl of diluted probe. An RNase free siliconised coverslip was placed over each section and slides incubated at 37°C overnight.

Coverslips were removed and slides washed in dH_2O x 3 before 2 x 10 min. washes in pre-warmed post-hybridisation buffer at 37°C. Slides were then washed in dH_2O x 3 before incubation in blocking solution for 5 min. Immunological detection of the labelled probes was achieved by covering each section with a 1.25U/ml of anti-digoxigenin alkaline phosphatase conjugate in blocking solution for 30 min., followed by 2 x 5 min. washes in TBS. Slides were then washed in detection buffer for 5 min. Each section was then covered in 100µl detection buffer containing BCIP and NBT, a coverslip placed over each section and slides incubated in darkness for 6-18 hours. An alternative to the detection buffer/BCIP/NBT step was to use 2 x 5 min. washes in dH_2O, followed by incubation in NBT/BCIP ready to use tablets dissolved in dH_2O (1 tablet in 10ml).The alkaline phosphatase catalyses the conversion of BCIP/NBT to a coloured precipitate. The slides were then washed in running water for 5 min and mounted in an aqueous mountant.

2.2.9.4 Controls

1. For each sample a negative control was used in which no probe was added.
2. In order to check the efficiency of the total TNC probes, ISH was performed on umbilical cord tissue which is known to be highly abundant in TNC.
3. To control for the ISH technique, κ and λ immunoglobulin probes were utilised on normal human tonsil tissue.
4. To control for specificity sense β-actin probe was generated and used on parallel sections.

Chapter 3 –

Development of methods for detection and characterisation of tenascin C isoforms

3.1 Introduction

The initial approach to this study was to develop an RT-PCR based method coupled with Southern hybridisation analysis to identify specific TNC isoforms. There are many variables to consider when developing such a powerful technique to study primary breast tissue and during method development, an assessment has to be made concerning variables which indicate the appropriateness or not of this approach. An easily available source of tissue which produced tenascin C was required for method development, that could also be used as a positive control for TNC expression at each stage of the investigation.

Previous studies have identified a number of cell lines that produce large amounts of TNC. Natali *et al* (1991) and Carnemolla *et al* (1992) investigated TNC expression in a variety of human tumour cell lines. These included the breast carcinoma cell lines MDA-MB-231 and MCF7 which produced no TNC, the fibrosarcoma cell line HT-1080 which produced negligible amounts of TNC, in contrast to four melanoma cell lines, including Sk-mel 28, that produce very large amounts of TNC. Elevated serum levels of TNC have been reported in patients with a advanced degree of melanoma metastases (Herlyn *et al* 1991) and a many melanoma tumours are shown to be TNC immunopositive (Schnyder *et al* 1997). *In-vitro* studies have also shown that increased levels of TNC are secreted into the culture medium by advanced primary and metastatic melanomas (Herlyn *et al* 1991). The amount of TNC produced by sk-mel 28 cells is high at around 10μg per 10^6 cells, compared with a maximum of 4-5μg per 10^6 cells for a range of other cell lines (Carnemolla *et al* 1992). As such, sk-mel 28 is considered as an ideal source of large amounts of TNC (Saganiti *et al* 1992) and the company Chemicon, USA, has used it as a source of commercially available TNC. Finally, previous studies within the Department of Pathology, University of Leicester have used a similar approach to study TNC expression in foetal membranes, using this cell line as a positive control (Bell *et al* 1999). Thus, the current study employed sk-mel 28 for method development.

3.2 Results

Sk-mel 28 cells were grown to sub-confluence, harvested, and mRNA extracted followed by reverse transcription to yield cDNA.

3.2.1 PCR for housekeeping gene GAPDH on Sk-mel 28

This was used to assess efficiency of mRNA extraction and RT-PCR. Positive RT reactions yielded a single fragment of approximately 350bp, whereas PCR of negative RT reactions showed no amplified fragments. Intensity of signal was used to judge amount of cDNA template used in subsequent PCR reactions i.e. a weaker signal, indicating a less efficient extraction and RT step, would require more template for a signal equivalent to that from a more efficient extraction.

For sk-mel 28 a strong signal was generally obtained (see figure 3.1), thus, 1µl of cDNA template was used in subsequent reactions.

3.2.2 PCR for tenascin C isoforms

3.2.2.1 PCR primer cassettes 8/18, 8/14, 14,18, and 8, AD1

PCR using TNC primer cassettes 8/18, 8/14, and 14/18 each yielded a specific pattern of bands indicating a particular pattern of isoform expression. The use of the 8/AD1 cassette indicates the presence of more than one AD1 containing isoform (see figure 3.2). On the basis of fragment size, the number of exons included in the variable FN type III-like repeat region can be deduced (summarised in table 3.1). A number of fragments can be seen which indicate previously undescribed exon boundaries, the 1090bp fragment produced by the 14/18 cassette, and the approximately 600bp band produced by the 8/18 cassette. It is interesting to note that these bands are not seen on a 1% agarose gel, whereas they are clearly visible on a 2% agarose gel (see figure 3.2).

For 8/18 and 11/16 TNC PCR, a "hot start" protocol was investigated. This was a successful approach in that when a hot start was used, a number of specific products were amplified that were not seen without "hot start" (see figure 3.3).

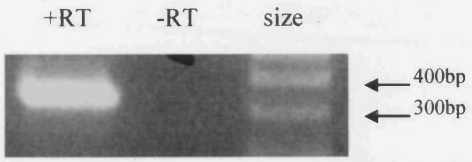


Figure 3.1: PCR for GAPDH on sk-mel 28 - showing positive 350bp product and negative reaction from reverse transcription plus size marker.

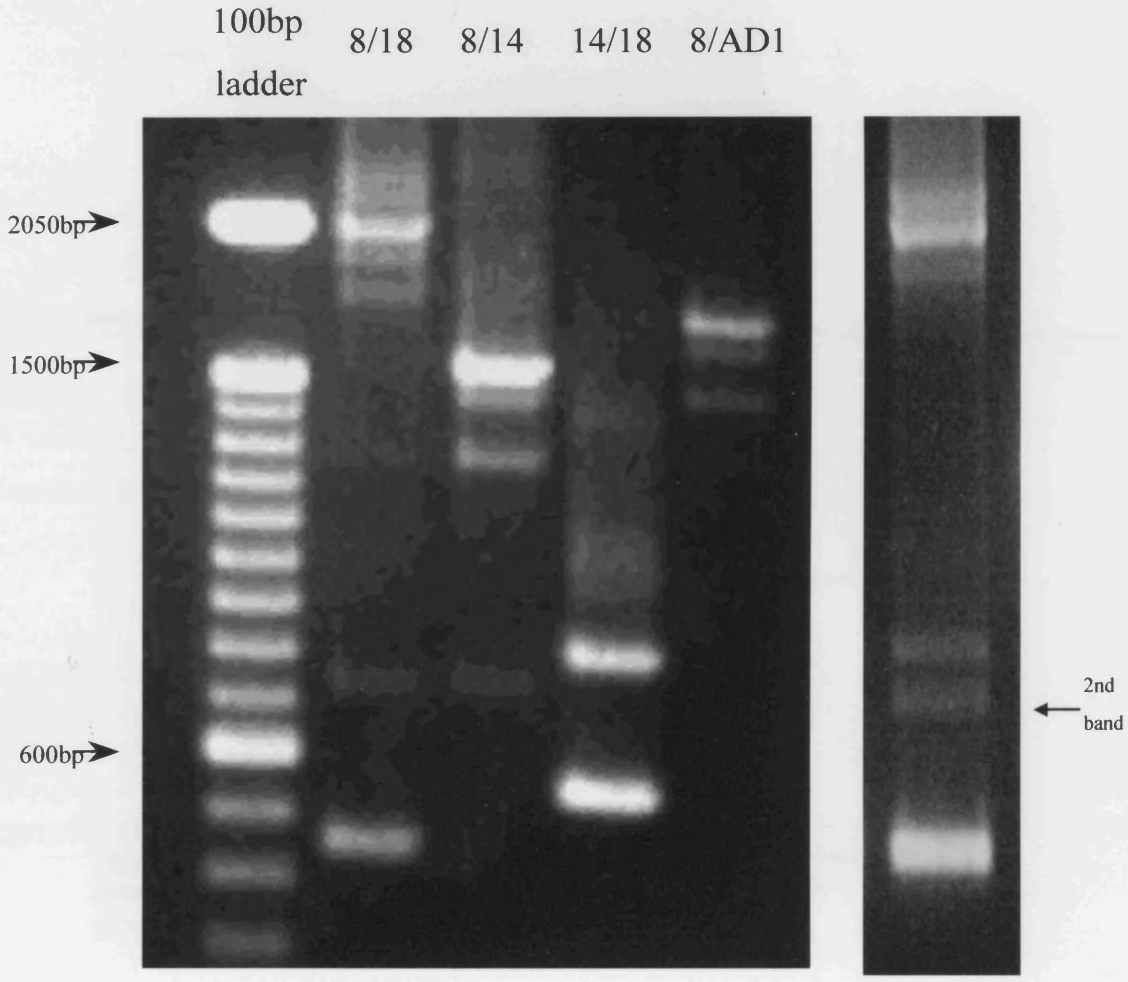


Figure 3.2: TNC PCR on Sk-mel 28 - using TNC primer cassettes 8/18, 8/14, 14/18 & 8/AD1(1% agarose gel). The second image shows 8/18 products although on a 2% gel, arrows indicate additional second band.

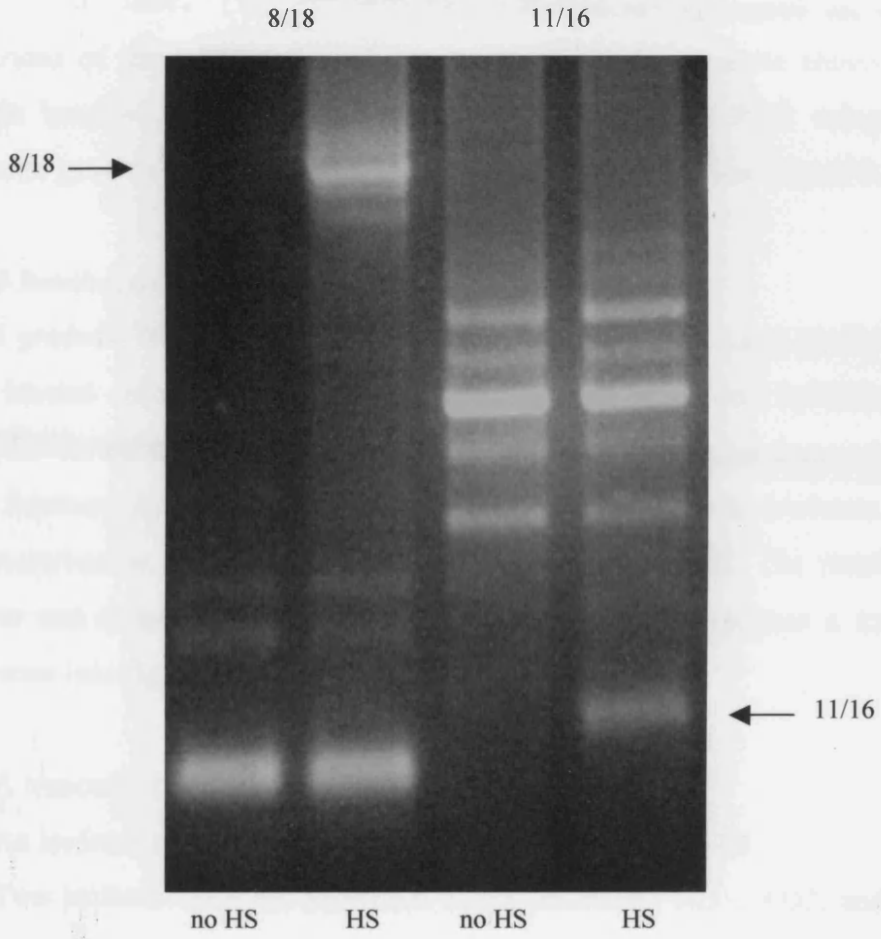


Figure 3.3 : Validation of "hot start" PCR - Proof of hot start (HS) using primer cassettes 8/18 and 11/16 on Skmel 28. Arrows show position of bands generated with HS but missing whwn HS is not used.

3.2.2.2 PCR using other tenascin C specific primer cassettes

PCR using TNC primer cassettes 8/11, 8/12, 8/13, 11/AD1, 14/AD1, 8/15, and 8/16 each yielded the predicted pattern of bands. The predicted number of exons included in the variable FN type III-like repeat region is indicated by fragment size.

AD2 primers were initially used in conjunction with other TNC primers, which gave a "ladder" of bands. This indicated the PCR was not optimised as, due to the relative positions of the AD1 and AD2 exons, the AD2/AD1 cassette should have generated a single band of approximately 330 bp. "Standard" AD2 PCR using the AD2F/AD2R cassette gave a single faint band of approximately 116 bp. (see Figure 3.4).

3.2.3 Southern hybridisation

PCR products from TNC primer cassettes 8/18, 8/14, 14,18 and 8/AD1 were run on a 1% gel, blotted onto a positive membrane which then underwent hybridisation using probes specific for exons 11, 12, 13, 14, 15, 16, and AD1. Figure 3.4 demonstrates how RT-PCR and Southern hybridization enables identification of specific isoforms. This allowed full characterisation of TNC isoforms produced by sk-mel 28. The results are summarised below and in table 3.1. It can be seen that sk-mel 28 produces a total of seven major isoforms (see figure 3.5 and table 3.1):

1. A truncated isoform with no additional exons.
2. An isoform with a single additional exon which is exon 10.
3. Two isoforms with six additional exons, excluding AD1, AD2, and either exon 12 or exon 15.
4. Two isoforms with seven additional exons, one excluding AD1 and AD2, and one excluding exon 12 and AD2.
5. An isoform with eight additional exons, including AD1 but excluding AD2.

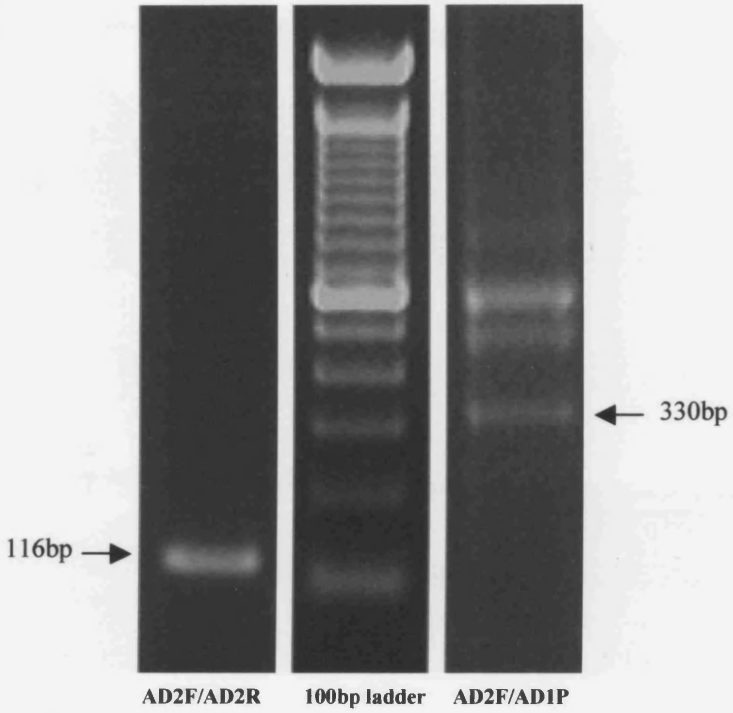


Figure 3.4: Additional domain (AD) PCR optimisation – the first image shows single band from AD2F/AD2R cassette. The third image shows multiple bands when primer AD2F used with primer AD1P (a single PCR product is predicted) indicating that primers are not compatible. Arrows indicate sizes of predicted PCR products.

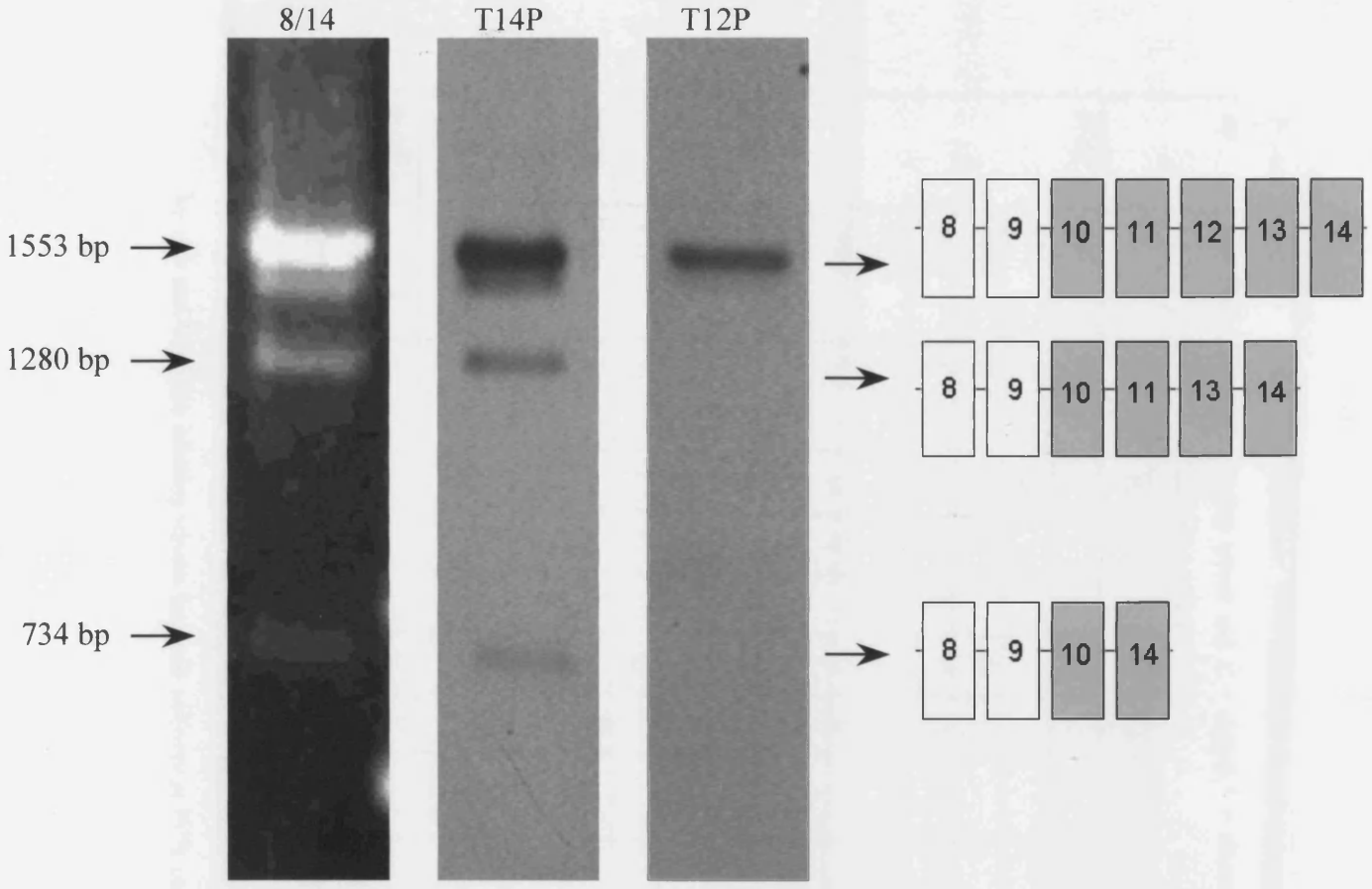


Figure 3.5: RT-PCR and Southern hybridisation enabling identification of TNC isoforms - How RT-PCR and Southern hybridisation enable identification of specific TNC isoforms. The first photograph is the banding pattern on a 2% agarose gel using the primer cassette 8/14 (the first half of the variable repeat region)showing three bands indicating the presence of two additional exons (734 bp), four additional exons (1280 bp) and five additional exons (1553 bp). The second photgraph shows an autorad from T14P hybridisation showing that all bands contain exon 14. However, the third photograph shows an autorad from T12P hybridisation showing that only the largest band contains exon 12. This technique enables the contruction of the partial isoform profile on the right. A detailed isoform profile for SK-mel 28 can be seen in figure 3.2.3.

Primer set	Band size (bp)	No. Exons	Southern Hybridisation signal using exon specific probe (exon no) □ = signal,* = doesn't conform to exon boundary								
			10	11	12	13	14	AD2	AD1	15	16
8/18	442	0									
	715	1	□								
	1807	6	□	□		□	□			□	□
	1807	6	□	□	□	□	□				□
	2080	7	□	□	□	□	□			□	□
	2080	7	□	□		□	□		□	□	□
	2353	8	□	□	□	□	□		□	□	□
8/14	734	2	□				□				
	1280	4	□	□		□	□				
	1553	5	□	□	□	□	□				
14/18	544	2					□				□
	817	3					□			□	□
	1090*	3*					□		□		□
	1120	4					□		□	□	□
8/AD1	1515	5	□	□		□	□		□		
	1788	6	□	□	□	□	□		□		

Table 3.1: Tenascin C PCR results from sk-mel 28 - showing PCR product size, number of exons indicated by size and Southern blotting results for each isoform or PCR product.

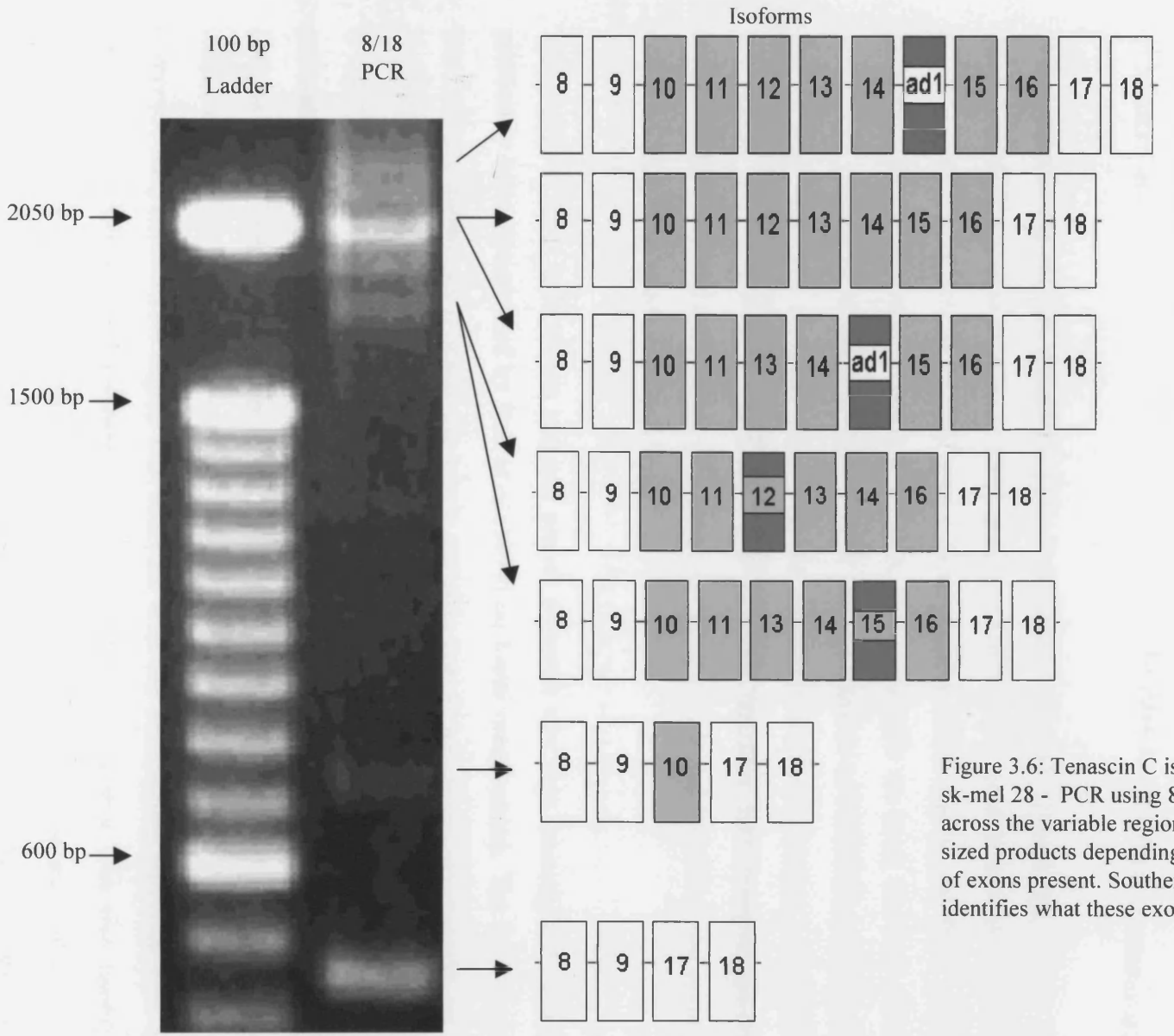


Figure 3.6: Tenascin C isoform profile of sk-mel 28 - PCR using 8/18 primers across the variable region give different sized products depending on the number of exons present. Southern blotting identifies what these exons are.

3.3 Discussion

3.3.1 Discussion of Methodology

The melanoma cell line Sk-mel 28 produces a variety of tenascin C isoforms (Natali *et al* 1991, Saginati *et al* 1992), hence it was considered an appropriate choice as positive control in order to develop an RT-PCR based method. However, before any analysis of TNC isoforms can take place a number of variables that could potentially affect results had to be taken into account.

When considering mRNA extraction procedures using oligo-dT Dynabeads, cell to bead ratio is vital. If too many cells are used, genomic DNA could be extracted alongside the mRNA. Any subsequent PCR may give a false positive result due to the PCR reaction generating fragments from contaminating DNA. If too few cells are used, this may yield an inadequate amount of mRNA for the RT-PCR and the number of PCR reactions required. Cell to bead ratio was assessed along with the efficiency of the extraction and RT-PCR procedures by performing a GAPDH PCR on both negative and positive RT-PCR reactions. This allowed assessment of a range of cell numbers and identified the optimum cell/bead ratio to be used on all cell lines.

The choice of most informative tenascin primer cassettes was then investigated (this had previously been investigated by Bell *et al* (1999) on foetal membranes). The 8/18 cassette was initially chosen as it spans the whole variable region and gives information on the complete range of isoforms. However, an inherent problem with PCR in this type of study is that the reaction preferentially amplifies the smallest fragments. This poses a problem, particularly with those high molecular weight isoforms thought to be of lower abundance such as the AD containing isoforms (Derr *et al* 1997, Mighell *et al* 1997). False negative results may be obtained in that amplification may not be sufficient to give a signal. To overcome this, the variable region was analysed with the overlapping cassettes 8/14 and 14/18 giving more detailed information. The use of these cassettes was also useful in clarifying certain anomalous results from the 8/18 cassette. The 1807bp and 2080bp bands produced from 8/18 PCR gave anomalous Southern blotting results that indicated these bands contained 2 species. The overlapping 8/14 and 14/18 cassette showed that these bands from 8/18 PCR contained two species based around alternative splicing of exons 12, 15 and AD1. This indicated the usefulness of this approach over strategies which had used

PCR alone (Siri *et al* 1991, Mighell *et al* 1997) and by limiting the number of PCR reactions, the amount of cDNA was conserved.

AD1 containing fragments were amplified from Sk-mel 28 cDNA using the 14/18 cassette, which were not seen using the 8/18 cassette. The 8/AD1 cassette was employed in this study to investigate the presence of AD1 containing isoforms. If a signal was obtained, this would provide evidence of AD1 containing isoforms. It is interesting to note that the band pattern obtained is similar to that from the 8/14 cassette with sizes indicating the addition of a single exon. This indicates a consistent pattern of splicing which confirms work by Bell *et al* (1999). The distribution of AD2 is considered to be more restricted (Derr *et al* 1997), however it has been identified in human tissues, specifically in oral SCC (Mighell *et al* 1997). However, at this stage of the current study, AD2 was not investigated extensively due to a lack of PCR primers or a probe for Southern blotting. This was not a problem when considering higher molecular weight isoforms as PCR primer cassette 14/AD1 gave a product size indicating a lack of exons between 14 and AD1 i.e. no AD2. Further evidence of a lack of AD2 was provided by Southern blotting of 14/15 PCR products identifying the additional exon between these as AD1. However a problem arose when attempting to identify the isoform containing the single additional exon. Southern blotting had excluded all exons except 10, for which there was also no probe for hybridisation, and AD2. At this stage of the study, investigation of expression of AD2 by Sk-mel 28 was not pursued as AD2 had only been seen previously in conjunction with other FNIII like repeats in higher molecular weight isoforms, and was also considered to have a highly restricted expression (Derr *et al* 1997, Mighell *et al* 1997). Sequencing of this isolated band would enable definite identification of this exon. Investigation of AD2 expression was given greater priority in relation to primary breast tissues and is considered in the next chapter of this report. In contrast, the lack of an exon 10 hybridisation probe was not as much of a problem in higher molecular weight isoforms as the use of 8/11 PCR indicated that exon 10 was included in all large isoforms and size of 8/14 products indicated the plus 1 exon isoform contained exon 10. At this stage, it is important to point out that a number of previous studies have confirmed the precise order of exons in the variably spliced region (Gulcher *et al* 1991, Siri *et al* 1991), and that the approach to the current study assumes this exon order is preserved and to an extent confirms this controlled splicing pattern.

The gel banding pattern obtained from sk-mel 28 provides evidence of a distinct, controlled pattern of splicing as opposed to more random mechanism. The number of

isoforms expressed by this cell line is limited to seven; this would not be the case if a random mechanism was employed as a ladder of bands at every possible size would be expected. RT-PCR indicates no expression of isoforms containing 2, 3, 4 or 5 exons and indicates a predominance of very high or very low molecular weight isoforms (see figure 3.5). Further evidence of a controlled mechanism is seen in the variable splicing of either exons 12 or 15 with no isoforms seen in which both exons are absent. A controlled splicing pattern may lead to expression of isoforms with specific functions and supports the concept that each repeat may contain specific functional motifs. A specific array of repeats may confer a specific function and indicates that the random inclusion of repeats, therefore an increase in size, is not the factor that conveys the functional difference between different isoforms. A distinct controlled splicing mechanism has been discussed previously by Bell *et al* (1999) who propose that individual exons have a limited number of other exons they can be adjacent to in an array of FNIII like repeats. A highly regulated isoform pattern has also been seen in chicken (Chiquet-Ehrissmann *et al* 1991) and mouse (Joestner & Faissner, 1999). The latter study also states that only the largest and smallest isoforms may be present in normal tissues and that the expression of intermediate sized isoform is more tightly regulated.

Southern analysis of 8/18, 8/14, 14/18 and 8/AD1 products was carried out using the method described by Southern *et al* (1975) but modified to use digoxigenin labelled probes, anti-digoxigenin alkaline phosphatase conjugates and chemilluminescent visualisation. This proved to be a particularly efficient method, primarily because the labelled probes could be re-used allowing easy confirmation of results.

The results obtained show that an efficient method has been developed which allows the characterisation of TNC isoforms produced by cells and could be potentially applied to other cells and tissues. It confirms other studies that have used similar methods (Gulcher *et al* 1991, Siri *et al* 1991, Sriramarao *et al* 1993, Wilson *et al* 1996). This approach displays certain advantages over other methods, for example direct sequencing of bands or cloning (Joestner & Faissner, 1999) that are far more labour intensive and lack the reproducible aspects of Southern blotting. Membranes can be stripped of probe and stored easily for long periods of time without noticeable deterioration. This lends itself to further investigations in that membranes can be reused with other probes, specifically a number of membranes were set aside for possible hybridisation studies with AD2 or exon 10 specific probes which have yet to be developed. Sequencing was used on a number of occasions

throughout the current study to confirm Southern blotting results, but the success of the hybridisation technique meant that sequencing was not the cornerstone of this investigation. Furthermore, sequencing would not be a useful approach for those PCR bands containing 2 species as a confusing double signal would be obtained for part of the sequence.

3.3.2 Presence of anomalous PCR banding pattern

Some specific TNC PCR reactions amplified fragments that do not fit with previously described exon boundaries. An example of these bands can clearly be seen in figure 3.2 from 8/18 PCR run on a 2% agarose gel. However, they were not seen during initial method development on sk-mel 28 where a single band of correct size is evident in figure 3.2 from 8/18 PCR run on a 1% agarose gel. The appearance of a second band coincided with an alteration in gel running conditions in that in order to increase resolution of the higher molecular weight bands, PCR products were routinely run on 2% agarose gels rather than 1% gels. It was possible that the appearance of this additional band may be a result of subtle changes in the cell line, an inherent problem with this type of *in-vitro* investigation. However, this was thought to be unlikely in that this extra band was seen in other cell lines and in primary tissues. An alternative is that this may be due to the presence of a pseudo, or half exon, a possibility suggested by Bell *et al* (1999) who used the same PCR primer sequences in the study of foetal membranes. A further explanation is that the anomalous bands could be due to PCR artefact specific to these primers. However, this pattern has also been seen by Wilson *et al* (1996) who used different primer sequences; an indication that this may be an inherent problem of this approach in the study of TNC. The presence of an anomalous band from 14/18 PCR may indicate a pseudo-exon occurs in this part of the reading frame with the anomalous bands being approximately 100bp smaller than a full exon. Exon 17, which encodes for only a part of the 13th FNIII like rpt. is 123bp in length (Gulcher *et al* 1991) thus is possible that exon 17 may be subject to alternative splicing, although this would need to be confirmed by sequencing the isolated band.

The nature of these "shadow" bands was however elucidated by Dr. Howard Pringle, Department of Pathology, University of Leicester. The PCR reactions, due to the nature of TNC expressing different sized isoforms, amplified different sized single stranded fragments. These fragments share a high degree of homology at either end of the molecule which consist of sequences for a part of exon 8, the whole of exons 9 and 17 and a part of exon 18. This is in excess of 600bp and may comprise the majority of the amplified

molecule. Denaturation during PCR creates a pool of single stranded products with a degree of ability to combine with any other sized strand. This creates double stranded molecules with internal single stranded loops which retard the movement of the product through the gel. This can be seen in figure 3.7.

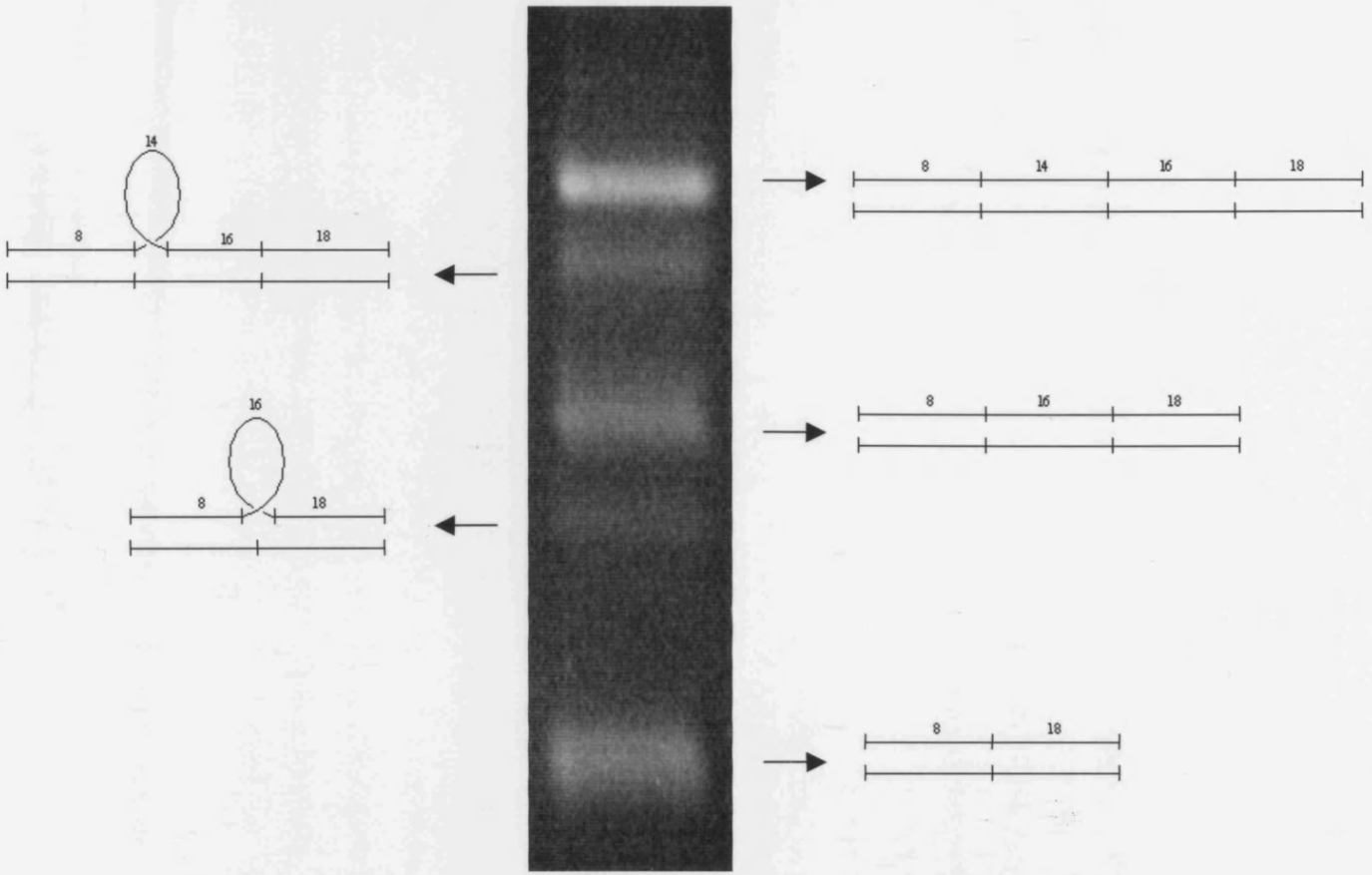


Figure 3.7: Explanation of anomalous PCR banding pattern - Diagram showing correct pairing on the right and incorrect pairing on the left of of pairs of single stranded TNC PCR products giving distinctive banding pattern on a 2 % agarose gel

Chapter 4 –

Analysis of tenascin C expression and distribution in primary breast tissue

4.1 Introduction

It is now well established that TNC is over-expressed in the stroma surrounding many solid tumours including breast cancer (Chiquet-Ehrissmann *et al* 1986, Mackie *et al* 1987). Studies have shown that expression of TNC changes at a pre-invasive stage (Jahkola *et al* 1998a) and changes are also associated with hyperplastic breast disease (Borsi *et al* 1992). However, although there has been limited investigation of different TNC isoforms in the breast (Borsi *et al* 1992), no detailed information is available concerning alterations in specific isoform profiles between benign, pre-invasive and invasive breast disease. This is of potential importance because of the functional implications of altered isoform expression. Identification of any changes in isoform profile may provide a marker of disease progression and focus further studies on the role of specific isoforms.

The approach to be used in this study is RT-PCR coupled with Southern hybridisation to identify specific isoform combinations, which has already successfully identified multiple isoform expression in the cell line sk-mel 28 in the current study (see Chapter 3 and also Bell *et al* (1999)). However, when applying this technique to primary tissue, it was evident that a number of problems associated with the long term storage of tissue had to be overcome and detailed analysis of RNA integrity was necessary. A distinct advantage of using cryo-frozen tissue is that for the tissue sections taken for RNA analysis, subsequent sections can be taken for immunohistochemistry studies. Thus, a detailed analysis of primary transcript could be linked with investigation of the protein distribution within a tissue. This and other techniques would allow identification of the specific cell population responsible for the production of TNC and would allow comparison with markers previously associated with the development of malignancy and invasion.

4.2 Results

4.2.1 PCR for housekeeping gene GAPDH

PCR of positive RT reactions yielded a single fragment of approximately 350bp, whereas PCR of negative RT reactions showed no amplified fragments. However, the signal obtained from breast tissue varied in intensity between samples, thus a range of 1-8μl of cDNA template was used in reaction for TNC isoforms. Weaker signals were generally obtained from benign samples; hence more template was used to obtain a signal adequate for comparison with malignant samples. This is detailed in table 4.1 and figure 4.1.

4.2.2 Analysis of tenascin C mRNA integrity: EGF rpt. & fib. domain PCR

PCR of positive RT reactions was performed using primers to the EGF repeat and the fibrinogen like region of TNC. EGF repeat products were run on 3% agarose gels, whereas fibrinogen repeat products were run on 2% agarose gels, and relative band intensities were compared (see fig. 4.2). This gave an indication of the degree of mRNA degradation in the tissue sample block and was also used along with GAPDH PCR as an indication of how much template should be included in subsequent PCR reactions (see table 4.1). Of the 91 samples initially investigated, 69 were considered suitable for TNC isoform analysis.

4.2.3 Amount of cDNA template used in tenascin C PCR

The PCR results with primers for GAPDH, EGF rpt., and the fib. domain allowed an assessment of the amount of template to be used in reactions for analysis of TNC isoform expression so that approximately equivalent amounts of TNC mRNA were being added to each reaction (see table 4.1).

Tissue Type	1μl template	2μl template	3-4μl template	≥5μl template	Total
Benign	0	2	4	9	15
Fad.	1	2	1	1	5
DCIS	6	3	2	2	13
Invasive	22	10	4	0	36

Table 4.1: Amount of cDNA template used in TNC PCR for each tissue type

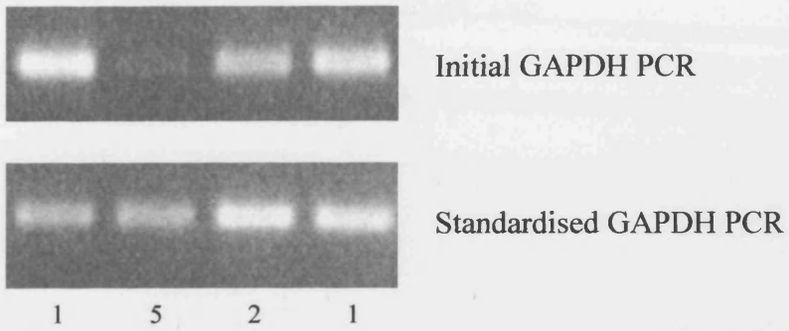


Figure 4.1: PCR for GAPDH primary breast tissue - Initial GAPDH signal in comparison with standardised GAPDH signal. The numbers below indicate the number of microlitres of cDNA template used to standardise result. The same volume of template was used in specific TNC PCR.

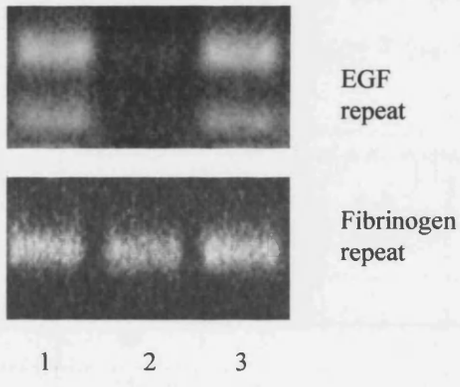


Figure 4.2: PCR using primers to the EGF repeat and fibrinogen domain of TNC - Examples of EGF PCR (exon 2) and Fibrinogen rpt PCR (exons 25-27). Lanes 1 & 3 show samples in which the mRNA is not degraded whereas lane 2 shows a sample which is degraded.

4.2.4 PCR for tenascin C isoforms using cassette 8/18

PCR with the 8/18 primer set on cDNA from the 69 suitable tissue samples indicated three distinct isoform profiles (see tables 4.2 & 4.3 and figs. 4.3 & 4.4).:

1. A 442bp band indicating expression of truncated isoform only.
2. Bands of 442 and 715bp indicating expression of the truncated isoform and a plus 1 exon isoform.
3. Bands of 442, 715, and 988bp indicating expression of the truncated isoform, a plus 1 exon isoform and a plus 2 exon isoform.

A band of 1261bp was seen in a single invasive tissue sample, indicating a plus 3 exon isoform.

In all samples the truncated isoform was seen. The truncated isoform and the plus 1 exon isoform (715bp) were seen in 25% of benign (n=20) and 70% of malignant samples (n=36). An isoform containing two additional exons (the 988bp band) was seen in 54% of DCIS (n=13) and 75% of invasive tumours (n=36), but only one benign sample (5%, n=20) which was a fibroadenoma. Southern hybridisation identified the single additional exon as exon 16, and the two additional exons as exons 14 & 16 (see figure 4.3and 4.4).

Type	Number	Number of additional exons		
		0	0 & 1	0, 1 & 2
Benign	15	12	3	0
Fad.	5	2	2	1
DCIS	13	3	4	6
IDC I	10	0	1	9
IDC II	10	2	1	7
IDC III	9	0	1	8
ILC	5	0	2	3
Other*	2	1	0	1

Table 4.2: Isoform expression in primary breast tissue indicated by TNC PCR of each tissue type - Results from 8/18 PCR on primary breast tissue samples showing the composition of the isoforms predicted in each case. * Other invasive tumours, one tubular carcinoma, one mixed infiltrating lobular/infiltrating ductal carcinoma.

Type	Number of additional exons			
	0	0 & 1	0, 1 & 2	Total
Benign	14	5	1	20
DCIS	2	4	7	13
Invasive	3	5	27	36
Total	19	15	35	69

Table 4.3: Isoform expression in benign, DCIS and invasive breast tissue indicated by TNC PCR of each tissue type - Summary of results from 8/18 PCR on primary breast tissue samples showing the composition of the isoform profiles seen in benign, *in-situ* and invasive carcinoma.

Statistical analysis showed a highly significant association between TNC isoform profile and tissue type (P<0.001).Further analysis revealed the nature of this association (see table 4.4).

Crosstabulation	Chi-squared test	Fisher's exact test
Benign/malignant X additional exons present/absent	P<0.0001	P<0.0001
Non-invasive/invasive X additional exons present/absent	P<0.0001	P<0.0001
Benign/malignant X exon 14 present/absent	P<0.0001	P<0.0001
Non-invasive/invasive X exon 14 present/absent	P<0.0001	P<0.0001

Table 4.4: Statistical analysis of TNC isoform expression and tissue type - Chi-squared test and Fishers Exact test results from testing tissue type against 8/18 PCR result under a number of criteria showed a highly significant association between TNC isoform profile and tissue type.

When the pattern of isoform expression was compared to tissue type a highly significant relationship was demonstrated between the presence of additional isoforms (either TN+16 or TN+14&16) and the malignant phenotype (either DCIS or invasive carcinoma) (P<0.0001).

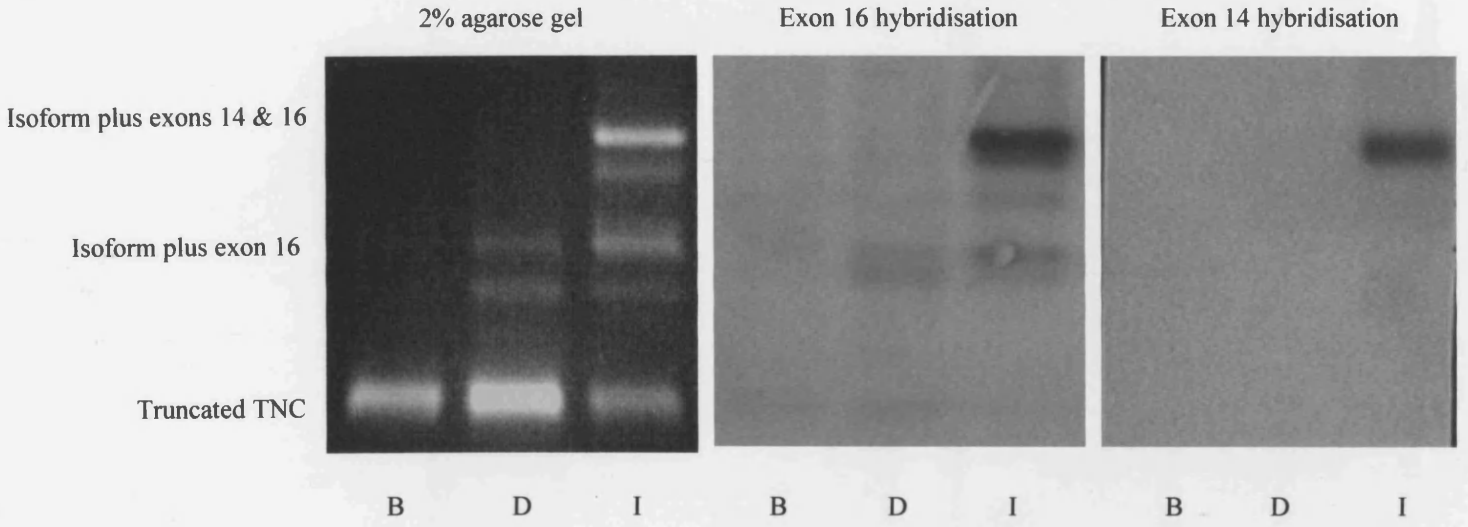


Figure 4.3: Typical isoform profile of benign, DCIS and invasive breast tissue - for benign (B), DCIS (D) and invasive (I) tissue showing isoform profile for each tissue type. The first image shows a 2% agarose gel showing the complete isoform profile, the second image shows an autoradiograph from an exon 16 Southern hybridisation and the third image shows an autoradiograph from an exon 14 Southern hybridisation thus allowing identification of specific isoform profiles.

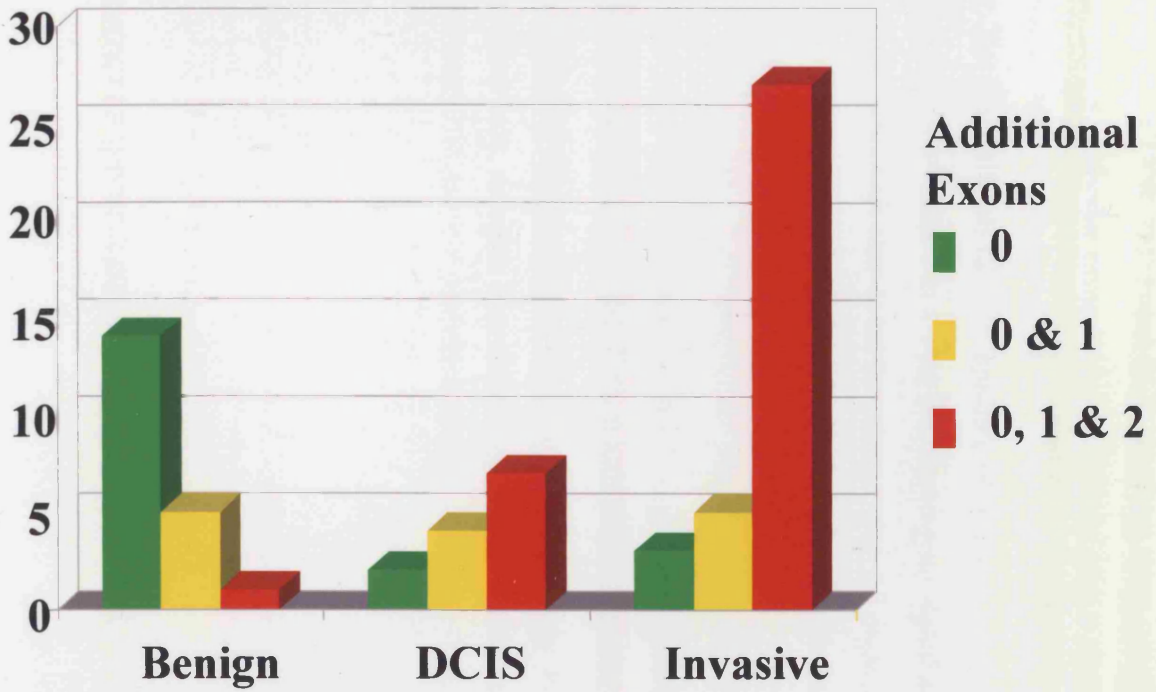


Figure 4.4: Graph indicating relationship between TNC isoform profile and tissue type - Graph showing relative proportions of each isoform in benign, DCIS & invasive breast carcinoma.

4.2.5 Isoform specific PCR

4.2.5.1 Optimisation of primers

A range of PCR protocols were investigated for the primer cassettes 9-17/18 (specific for the truncated isoform), 9-16/18 (specific for the isoform plus exon 16) and 9-14/14-16 (specific for the isoform plus exons 14 & 16). These PCR reactions were initially performed on single isoform templates, thus a particular primer set should only give a signal from the isoform for which it was designed with no product from the other two isoforms which, therefore, act as negative controls. The results from isoform specific PCR and the subsequent cycle sampling, were assigned a numeric value from 0 to 4 with 0 indicating no signal and 4 indicating a signal from a saturated band. Although this is an inaccurate way of depicting PCR results, since reactions were performed on specific templates, and results were from strong bands of equal size, it was considered useful to do this for ease of analysis.

PCR with the 9-17/18 and 9-16/18 primers sets failed to generate specific signals. Both gave a product with the specific template, but also at least one of the negative controls (table 4.5 and figure 4.5).The 9-14/14-16 PCR gave a single band of approximately 300bp, the expected size, with the positive control only, using an annealing temperature of 69°C for 35 cycles.

Primer Set	Annealing Temp. (°C)	Size (bp)	Intensity (0-4)** Isoforms		
			0	+1	+2
9-17/18	58	150	4	4	4
	61	150	4	4	4
	65	150	4	4	4
	68	150	4	4	4
	69	150	4	4	4
	70*	150	4/4	4/4	4/4
	72	150	4	4	4
9-16/18	58	450	3	4	4
	61	450	3	4	4
	65	450	3	4	4
	68	450	2	4	4
	69	450	1	4	4
	70*	450	0/0	1/2	0/1
	71	450	0	1	1
	72	450	0	0	0
	ST	450	0	3	3
9-14/14-16	58	300	3	4	4
	61	300	3	4	4
	65	300	2	3	4
	68	300	0	1	3
	69	300	0	0	3
	72	300	0	0	0

Table 4.5: Products generated from optimisation of isoform specific TNC PCR - Results from isoform specific PCR under a variety of PCR reaction conditions. ** intensity of signal recorded when each individual isoform used as a template, * performed for 25 and 30 cycles, ST = semi-touchdown PCR.

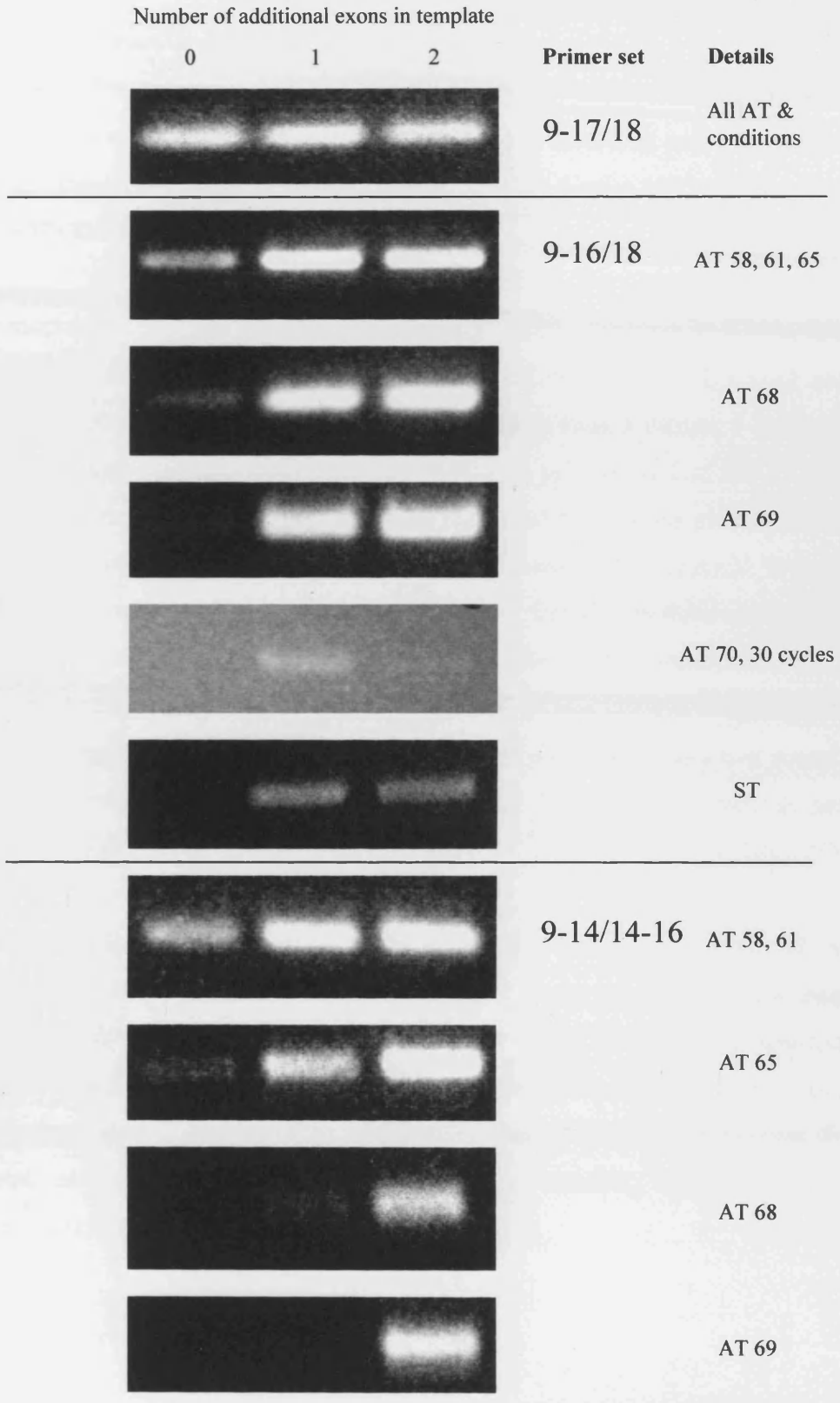


Figure 4.5 : Products generated from optimisation of isoform specific PCR - Results from isoform specific PCR optimisation showing conditions, AT = annealing temp.in degrees C, ST = semi-touchdown PCR. For more details see section table 4.5.

4.2.5.2 Analysis of primary breast tissue

As only the 9-14/14-16 primer set could be optimised, this was used to analyse expression of the TNC14-16 isoform in the breast tissue samples. A cycle sampling protocol was used in order to assess whether alterations in isoform profile between benign and malignant samples reflected a quantitative or qualitative change.

In order to standardise signal and limit the inherent inter-sample variation in cDNA, GAPDH PCR was initially performed on 22 samples (6 benign, 6DCIS, 9 IDC, 1 ILC). 12 samples were chosen which gave a similar strong GAPDH signal, these were 3 benign, 4 DCIS, 4 IDC and 1 ILC. An amplicon was generated with the 9-14/14-16 primers in 5 of the 12 samples, but in no specific pattern i.e. the reaction pattern did not correlate with that predicted from the 8/18 PCR. Thus, the methodology was reassessed and a nested PCR protocol investigated using 8/18 PCR products as a template for 9-14/14-16 PCR, initially on three samples known to express the plus 2 exon isoform. This generated a specific product from 8/18 PCR at 25 cycles and a second round using 9-14/14-16 at 25 cycles. This nested/cycle sampling protocol was then applied to the full cohort of 12 samples with cycle sampling on the first round PCR reaction. For comparison, a fib. domain cycle sampling protocol was used, to provide information on expression levels of total TNC.

Fib.domain PCR yielded a specific product for all samples from 30, 35, and 40 cycles, however, results from 30 cycles were considered most informative as a difference in intensity was observed between samples. In most cases a weaker signal was seen in non-invasive tissues. However, with 9-14/14-16 PCR a different pattern was generated such that all invasive samples gave a strong signal, whereas, at an informative level, 6 non-invasive samples did not give any signal, and even with a total number of cycles reaching 65, no product was demonstrated in 4 samples (see table 4.6 and figure 4.6).

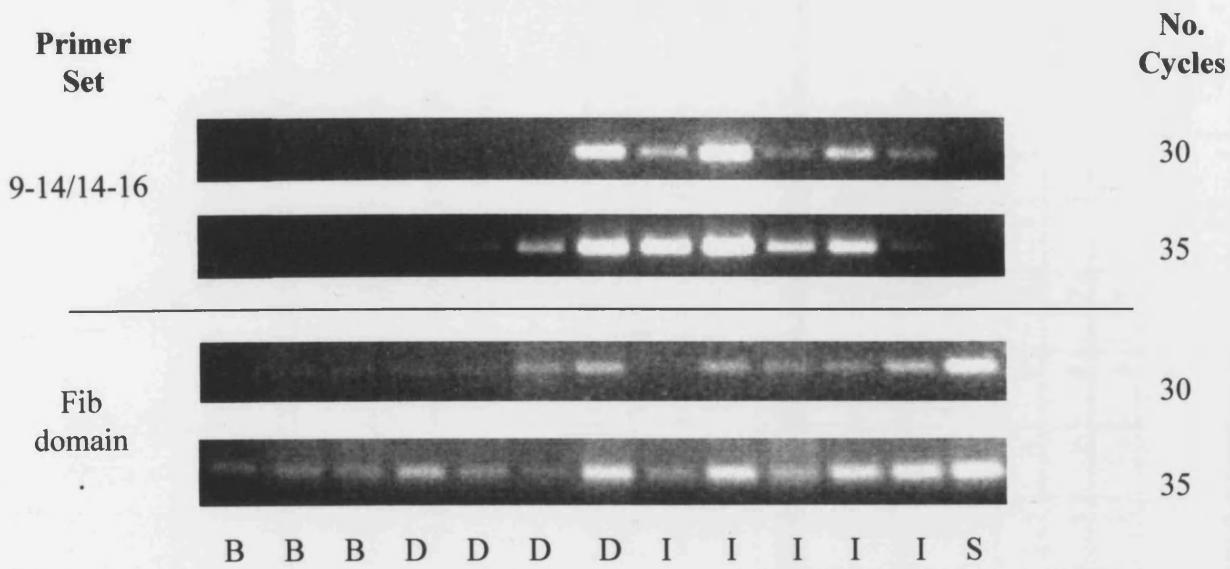


Figure 4.6: Cycle sampling PCR – cycle sampling PCR on a range of primary breast tissues using 9-14/14-16 PCR in comparison with Fib. domain PCR. 9-14/14-16 gel photographs indicate quantitative change i.e. increase in 14-16 isoform expression between benign (B), DCIS (D) and invasive (I) tissue as no signal is obtained from benign and some DCIS cases but a strong signal from invasive cases. Fib domain indicates levels of total TNC expression. S = Sk-mel 28.

Type	8/18 Result	Fib. domain. intensity (No.cycles)			9-14/14-16 intensity (No. 1st round cycles)				
		30	35	40	20	25	30	35	40
B1	0	1	2	4	0	0	0	0	0
B2	F*(0,1)	1	2	3	0	0	0	0	0
B	0	1	2	3	0	0	0	0	0
D1	0	1	3	4	0	0	0	0	0
D2	0,1	1	2	4	0	0	0	1	2
D3	0,1	2	2	4	0	0	1	2	4
D4	F*(0,1,2)	2	4	4	0	2	4	4	4
I1	0,1,2	0	3	3	0	0	2	3	4
I2	0,1,2	2	4	4	0	1	4	4	4
I3	0,1,2	2	3	4	0	0	2	3	4
I4	0,1,2	2	4	4	0	0	3	3	4
I5	0,1,2	3	4	4	0	0	2	2	2

Table 4.6: Cycle sampling results from arrange of primary breast tissues - Intensity of signal was graded 0-4 (see previous explanation). B = benign, D = DCIS, I = invasive, F* indicates this 8/18 PCR failed, thus the 9-14/14-16 PCR also failed (previous 8/18 result in brackets). nr indicates 8/18 PCR not performed due to lack of template.

The 9-14/14-16 PCR was then applied to the cDNA of 58 tissue samples. 50% (n=14) of normal/benign breast samples yielded a product for the plus 2 exon isoform, compared to 73% (n=11) of *in-situ* carcinoma, and 86% (n=28) of invasive carcinoma. See table 4.7:

Type	negative	positive	Total
Benign	7	7	14
Fad.	1	4	5
DCIS	3	8	11
Invasive*	4	24	28
Total	15	43	58

Table 4.7: Expression profile from 9-14/14-16 PCR on primary breast tissue - Results from 9-14/14-16 PCR on primary breast tissue samples showing frequencies of positive and negative signals * all invasive tumours combined.

As with initial analysis of 8/18 PCR, a chi-squared test was unsuitable as a number of the frequencies were too small, hence a Fisher's Exact test was performed. Tissue types were grouped as previously described (see section 4.2.4), however the test was performed on benign samples with and without inclusion of results from fibroadenoma samples and results summarised in table 4.8.

Crosstabulation	Fisher's exact test	
	2 sided	1 sided
Benign/malignant X PCR signal present/absent	P<0.033	P<0.027
Non-invasive/invasive X PCR signal present/absent	P<0.060	P<0.035
Benign/malignant X PCR signal present/absent*	P<0.062	P<0.051
Non-invasive/invasive X PCR signal present/absent*	P<0.073	P<0.049

Table 4.8: Statistical analysis of the relationship between tissue type and expression profile on 9-14/14-16 PCR - Fisher's exact test results from testing tissue type against 9-14/14-16 PCR result under a number of criteria. * Fibroadenoma data included.

These results demonstrate a significant relationship between expression of TNC 14 & 16 and the malignant (i.e. DCIS and invasive) phenotype compared to benign tissue. However, this significant relationship is lost when fibroadenoma are included in the benign group.

4.2.6 Other TNC PCR cassettes

4.2.6.1 11/16

A range of tissues (3 benign, 2 DCIS and 5 invasive carcinomas) were investigated using this cassette. Four different phenotypes were observed (see figure 4.7):

1. No signal, indicating that any isoforms expressed do not contain these exons.
2. A 1085bp band indicating the presence of isoforms containing 3 exons between exons 11 and 16.
3. Bands of 1085 and 812bp indicating the presence of isoforms containing 3 or 2 exons between exons 11 and 16.
4. Bands of 1085, 812, and 536bp indicating the presence of isoforms containing 3, 2, or 1 exon between exons 11 and 16.

A comparison of isoform profile with 11/16 primers (see figure 4.7) and 8/18 primers is shown in table 4.9.

Tissue	B1	B2	B3	D1	D2	I1	I2	I3	I4	I5
11/16	ns	ns	3	3	3	3,2	ns	3,2,1	3,2	3,2,1
8/18	0	0	0	ns	0	0,1	0	0,1,2	0,1,2	0,1,2

Table 4.9: Comparison of isoform expression profile using 11/16 and 8/18 PCR on a range of breast tissues - 11/16 and 8/18 PCR results for 3 benign (B), 2 DCIS (D), and 5 invasive carcinomas (I). ns = no signal, numbers refer to the number of exons between primers indicated by band size on gel.

The results showed that all cases generating a signal yielded a product indicating the presence of 3 exons but only the invasive specimens exhibited bands with 1 or 2 additional exons. Whilst this potentially important change was identified at an early stage in the study, it was thought that changes detected using the 8/18 PCR cassette were possibly more informative and therefore efforts were concentrated on investigating the 8/18 cassette further.

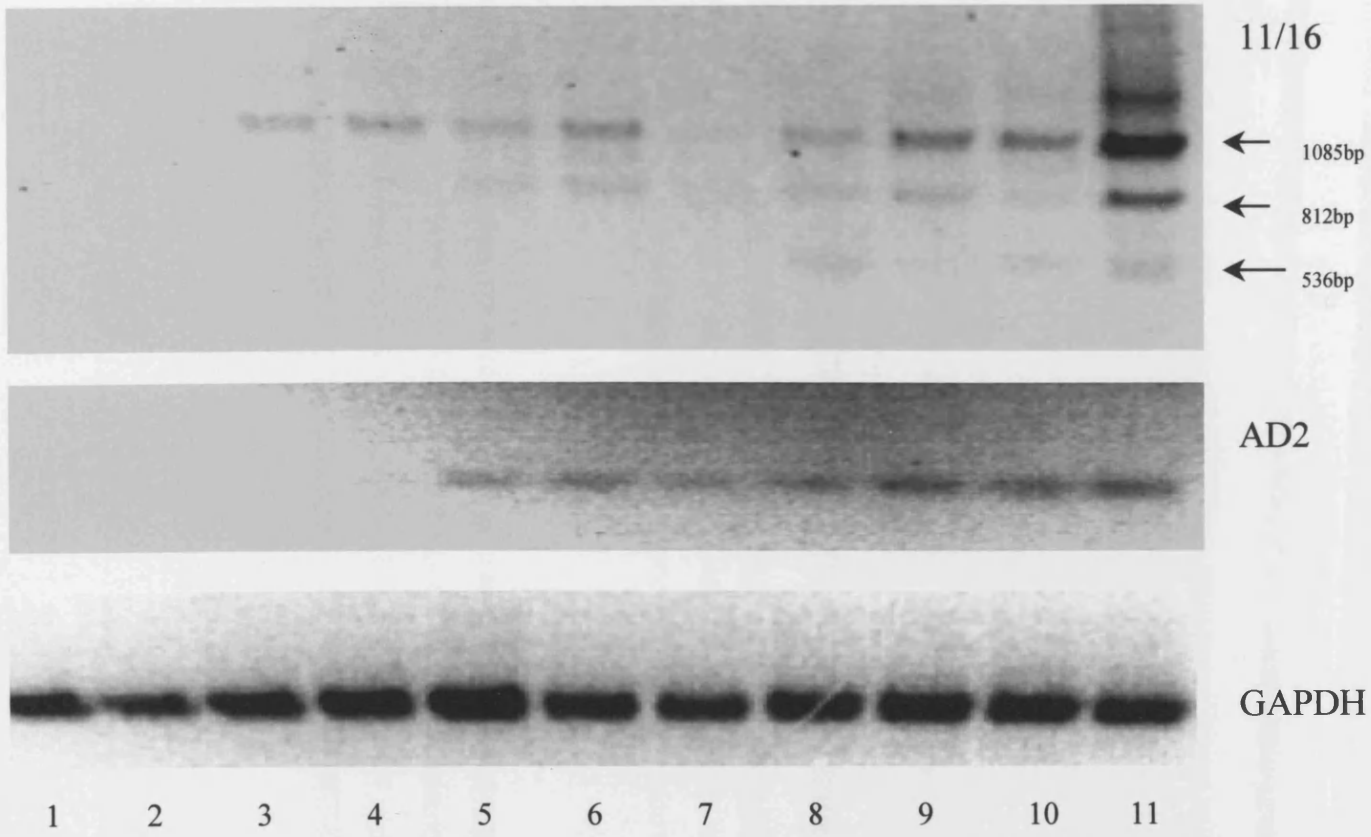


Figure 4.7: PCR for the tenascin C cassettes 11/16 and AD2 - TNC cassettes shown are 11/16, AD2F/AD2R along with GAPDH PCR results for the same samples. Arrows indicate size of PCR products of interest. Samples 1-3 benign, 4-5 DCIS, 6-10 IDC (6 & 7 grade I, 8 grade II, 9 & 10 grade III), 11 Sk-mel-28.

Further 11/16 PCR analysis was performed approximately 18 months after initial mRNA extraction, however, only a few cases gave a readable signal with the majority exhibiting smearing. This unfortunately precluded further investigation such as screening a larger proportion of samples in order to perform any statistical analysis for significance, or using Southern blotting/sequencing to identify which exons are variably spliced.

4.2.6.2 8/AD1

12 benign, 7 DCIS and 16 invasive carcinoma tissues were investigated using this cassette. No signal was seen in any case.

4.2.6.3 AD2

A "standard" AD2 PCR was performed on 3 benign, 2 DCIS, and 5 IDC (2 IDC I, 1 IDC II, 2 IDC III) samples. This initially produced no product in benign and 2 DCIS samples, but produced a single band of approximately 150 bp in 1 DCIS and all IDC samples (see fig.4.7). However, when the PCR was repeated using the same samples inconsistent and conflicting results were obtained. Samples previously giving a negative result, subsequently gave a positive result, and *vice versa*. A pattern of inconsistent results was also seen when "nested" AD2 PCR was used on the same samples.

4.2.6.3 14/16

In the 13 samples investigated, the 14/16 cassette generated products of approximately 150 bp indicating the presence of both exons 14 and 16. Products of approximately 400 bp were also generated indicating isoforms containing an additional exon between 14 and 16 (see fig. 4.8).

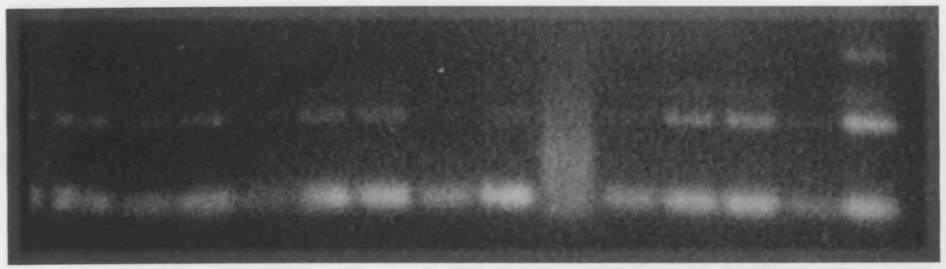


Figure 4.8: PCR for the tenascin C cassette 14/16 on a range of primary breast tissues - Examples of tenascin PCR using the 14/16 cassette.Lanes 1-3:benign, lanes 4-6:DCIS, lanes 7-13:IDC, lane 14: SK-mel-28

4.2.7 Immunohistochemistry

4.2.7.1 Immunohistochemical localisation of total tenascin C (clone BC-24)

A number of patterns of reactivity were detected using an antibody specific to the EGF rpt region of TNC i.e. for total TNC.

Strong, discrete staining was localised to the basement membrane zone in all normal/benign and DCIS samples. In invasive tumours, this basement membrane associated pattern of staining was not evident, but diffuse staining was evident in the peri-tumoural stroma. The pattern of reactivity observed in the stromal compartment could be categorised into one of four groups (see figure 4.9):

1. No stromal staining.
2. Diffuse periductal stromal staining, not completely surrounding the duct.
3. Diffuse periductal stromal staining, completely surrounding the duct and consisting of a pattern of striations of varying depth, regions of which are partly parallel with basement membrane.
4. Diffuse peri-tumoural strong stromal staining, very extensive.

This system of analysis was applied to each tissue type and the expression pattern in relation to tissue type is summarised in table 4.10. A number of sections contained two or more different tissue phenotypes e.g. both invasive tumour and DCIS tissue, and certain sections taken from supposed tumour tissue did not contain any tumour tissue and can be considered as surround tissue, as discussed below.

The trends observed were that benign/normal tissue showed no stromal staining (70%, n=10) or focal diffuse periductal stromal staining (30%) with just occasional ducts or acini showing staining in surrounding stroma. All DCIS tissue showed stromal staining with non-high grade samples showing a diffuse pattern and high grade samples showing a very distinctive pattern of diffuse periductal stromal staining. All IDC tissue showed a pattern of extensive stromal staining. Benign tissue and DCIS within invasive carcinoma displayed the same pattern of reactivity seen in pure benign and DCIS cases. Any blood vessels seen in sections also stained strongly for total TNC.

Tissue Type	None	Diffuse	Diffuse with striations	Extensive Stromal	Total
Benign	7*[2]	3*[1]	0	0	10
Fad.	0	0	0	6	6
DCIS	0	4	9	0	13
IDC	0 (1)	0	0 (4)	17*[3]	17

Table 4.10: Extent and distribution pattern of total TNC protein in arrange of primary breast tissues - extent and pattern of stromal staining of different tissue types using total TNC antibody. Details of sections containing more than one tissue type - *[1] 2 sections identified as partial fibroadenoma, *[2] 2 sections identified as normal/benign were from surround areas of DCIS, *[3] 4 sections also contained DCIS tissue and 1 section also contained benign/normal tissue, these areas showed a different staining pattern and are indicated in brackets.

Five ILC tumours were analysed, three of which contained areas of DCIS which exhibited diffuse stromal staining with laminations, and one case which contained benign tissue, that showed distinct basement membrane staining. There was no peri-tumoural staining in relation to the ILC in four of the five cases, however the fifth case which contained pure ILC showed diffuse peri-tumoural staining.

4.2.7.2 Localisation of tenascin C containing domain B (Exon 14) (Clone IIIB)

Applying an antibody specific to domain B of the FNIII like repeat region of TNC gave rise to a number of distinctive staining patterns. The majority of normal/benign tissues displayed no reactivity with this antibody. In a small number of benign samples diffuse BM associated staining was seen localised to only the occasional duct. In contrast, the pattern of staining in DCIS was comparable to that seen with total TNC staining, with the same cases exhibiting the distinctive periductal stromal reactivity. The pattern seen with IDC was also similar to that seen with total TNC, however the stromal staining was weaker. As with the antibody to total TNC any blood vessels demonstrated strong reactivity with this antibody. See figure 4.9.

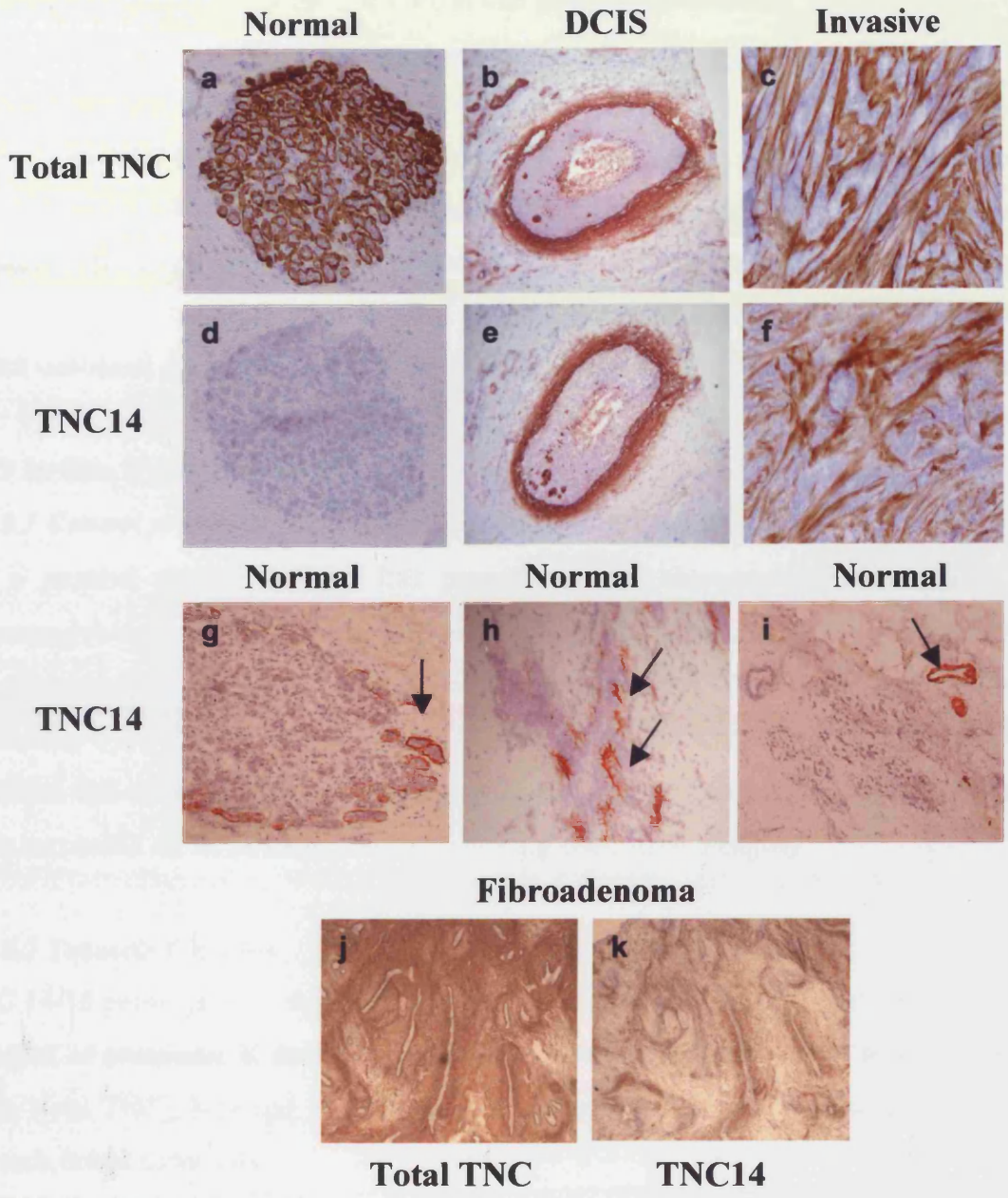


Figure 4.9: Expression of tenascin C protein in a range of primary Breast Tissue - Immunohistochemistry - Using an antibody that recognises all TNC isoforms (Total TNC), strong linear staining is seen in the basement membrane (BM) region of normal ducts and acini (a), in contrast to the diffuse stromal reactivity around invasive carcinomas (c). Stromal reactivity is also present around ducts containing DCIS (b). With the antibody specific to exon-14 of TNC (TNC14), most normal breast ducts and acini did not exhibit staining (d), with only occasional ducts and acini in 7 cases revealing BM reactivity (g,h). Blood vessels in normal breast displayed consistent reactivity with Ab14 antibody (i). A similar pattern of staining as that seen for total TN was present in DCIS (e) and invasive carcinomas (f), indicating the presence of exon-14-containing higher molecular weight species in tumour-associated stroma. Total TNC staining of fibroadenoma (j) shows a diffuse stromal stain, whereas staining with TNC14 shows a weak diffuse stromal stain, unlike that seen in DCIS, associated with the ducts.

4.2.8 Correlation of tenascin C RT-PCR and protein distribution

Those benign samples demonstrating a degree of diffuse stromal staining were those samples showing expression of plus exon 16 TNC isoform. The DCIS samples giving a plus 14-16 result from 8/18 PCR were all high grade DCIS and demonstrated the striated pattern of stromal staining, whereas the three non-high grade DCIS gave neither a plus 14-16 result nor a striated stromal staining pattern. Furthermore, those invasive tissues demonstrating only expression of truncated or plus 16 TNC isoform were those, when examined histologically, which contained a substantial degree of normal/benign or DCIS surround tissue (Figure 4.11).

4.2.9 In-Situ Hybridisation (ISH)

4.2.9.1 Control probes (κ and λ immunoglobulin, GAPDH, β-actin)

As a positive control for the ISH procedure, well characterised probes to κ and λ immunoglobulin light chains were used on tonsil tissue. These gave a dark blue/purple stain in the cytoplasm of lymphocytes after approximately 2-3 hours. These positive results indicated successful hybridisation. GAPDH probe gave no signal when used at a range of probe dilutions and up to 20μg/ml of proteinase K during pre-treatment, however β-actin probes were successful on all 18 cases studied indicating good RNA integrity.

4.2.9.2 Tenascin C probes

TNC 14-16 probe gave no signal on umbilical cord using a range of probe dilutions and up to 20μg/ml of proteinase K during pre-treatment. However the other TNC probes, EGF repeat alone (total TNC), 8/18 and 14/18, gave a consistent staining pattern with umbilical cord and for each breast tissue type.

Fibroblasts in umbilical cord gave a strong signal with total TNC probes, truncated TNC (8/18 probe), and 14/18 probes after approximately six hours. With the 18 breast cases studied, a signal was seen after 12-18 hours. In normal/benign breast no signal was generated with any TNC probe, whereas a strong signal was seen in invasive carcinoma localised to the peritumoural stroma. No convincing signal was localised to the tumour cell population. In DCIS cases a signal was demonstrated localised to myoepithelial cells around affected ducts and associated periductal fibroblasts (see figure 4.10). Tissue incubated with sense probes or in the absence of probe exhibited no signal, confirming the specificity of the reaction.

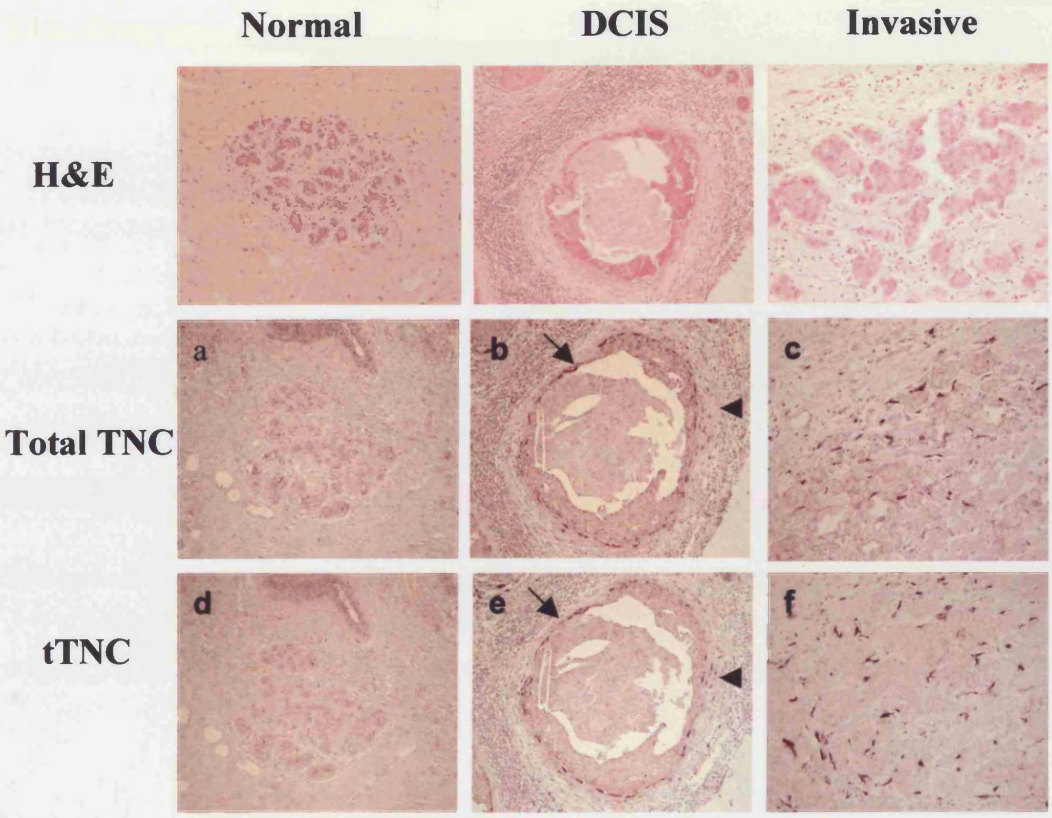


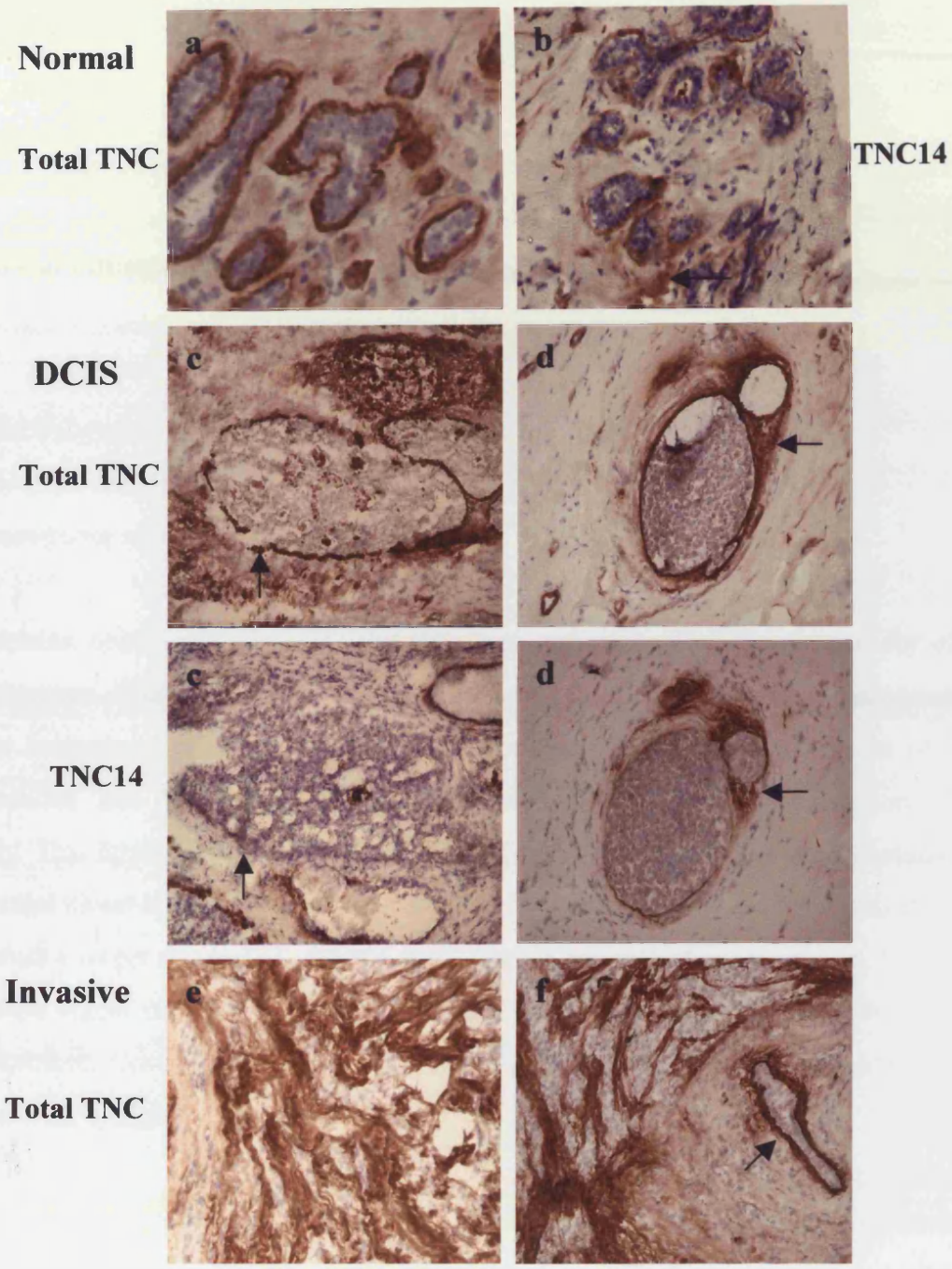
Figure 4.10: Expression of tenascin C mRNA in a range of primary breast tissues – results from ISH using a variety of different probes. No signal was generated in normal breast tissue using probes to total TNC or tTNC (a & d). In contrast strong signals were generated in the fibroblasts surrounding invasive tumour groups (f & c). In DCIS, a signal with each probe is evident in residual myoepithelial cells surrounding affected ducts (arrow b & e) and in peri-ductal fibroblasts (arrow head b & e). An identical pattern of reactivity was seen with probes to TNC14-17 (not illustrated).

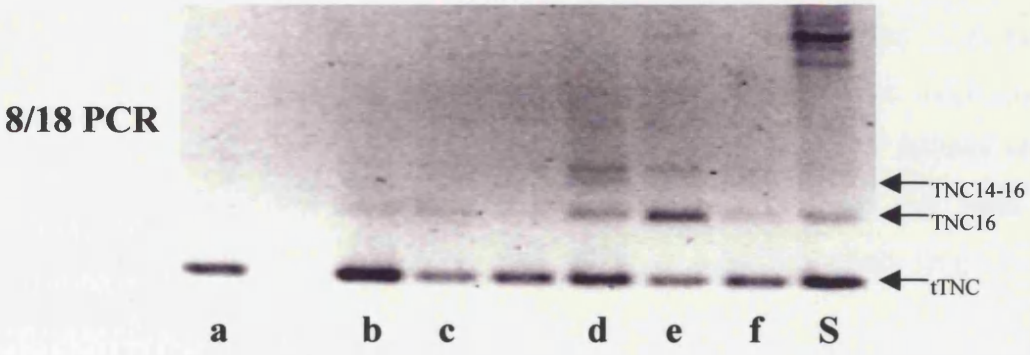
Figure 4.11: Correlation of Tenascin C RT-PCR and protein distribution – The upper images show TNC protein distribution in various typical primary tissues using a total TNC antibody in all tissues and an antibody specific to exon 14 containing TNC in some tissues. The lower image illustrates corresponding TNC 8/18 PCR result on the same samples.

The first normal sample (a) demonstrates basement membrane (BM) TNC with no diffuse stromal staining and 8/18 PCR showing expression of truncated TNC (tTNC) only. The second normal sample (b) shows a degree of BM associated stromal staining (arrow) and an RT-PCR result showing expression of tTNC and plus 16 TNC (TNC16).

The first DCIS sample (c), an example of low grade DCIS, shows BM staining with the total TNC antibody but no stromal staining with either antibody (arrow), plus RT-PCR showing expression of tTNC and TNC16. In contrast, the high grade DCIS sample (d), the distinctive striated stromal staining is seen with both antibodies (arrow) and RT-PCR shows expression of tTNC and TNC16, plus strong expression of TNC14-16.

The first invasive sample (e) consists exclusively of tumour tissue and gives an RT-PCR result including expression of TNC14-16. Whereas the second invasive sample (f) contains a degree of normal/benign tissue (arrow) and gives an RT-PCR result of tTNC and TNC16.


Normal
Total TNC
a
b
TNC14
DCIS
Total TNC
c
d
TNC14
c
d
Invasive
Total TNC
e
f


8/18 PCR
TNC14-16
TNC16
tTNC
a
b
c
d
e
f
S

4.3 Discussion

4.3.1 Adaptations of methodology for primary breast tissue

When applying this method to primary breast tissue a number of complications arise which are not relevant in cell line studies. In order to achieve the aims of the study a number of inter-sample variables needed to be standardised to validate results. These were:

1. Establishment of appropriate Cell:Bead ratio for mRNA extraction.
2. Adequate mRNA yield.
3. Assessment of mRNA integrity.

Initial problems concerned the establishment of an appropriate cell/bead ratio for efficient mRNA extraction. With cell lines, a cell count is easily done, however, this is not possible in tissues. An assessment of the cellularity of each sample was made on the basis of H & E stained sections and the number of sections included for mRNA extraction adjusted accordingly. This approach proved successful in its high mRNA yield without contamination. Benign/normal tissue samples consistently exhibited lower cellularity than malignant samples, and as a result a larger number of sections were used in the extraction procedure. Despite this, a less intense signal on GAPDH PCR was frequently obtained, indicating lower levels of mRNA, therefore, more template was used in TNC PCR in order to standardise signal for comparison with malignant samples.

As well as low tissue cellularity, a weaker GAPDH signal may also be due to an inadequate extraction. Proteins within the cell/tissue lysate interfere with the extraction procedure and including a large number of sections in an extraction increases the relative proportions of these contaminants. Proteinase k was added to the extraction to degrade these extra-cellular components and aid cell lysis. This proved successful in some cases, however, large amounts of extra-cellular matrix debris may have contributed to the large number of failures seen in this group (10 of 25 attempted extractions failed). This problem was not seen with fiboadenomas as, although they are benign, the tissue contains a comparatively larger number of cells, hence fewer sections were used in extractions.

Certain aspects of the tissue collection procedure and section cutting method may affect integrity of the RNA. During collection, tissue is unavoidably left at room temperature, furthermore, during section cutting the cryo-frozen tissue blocks are partially thawed. The nature of RNA means it is very susceptible to degrading enzymes (RNases) which are highly abundant in the environment and in the tissue itself. To reduce this effect it is necessary to cryo-freeze the tissue blocks as soon as possible which stops the activity of these enzymes. Any increase in temperature will reactivate the RNases and lead to a loss of RNA. Possible degradation was particularly considered to be a problem with older tissue blocks used in previous studies. This was demonstrated by performing several extractions on the same tissue block, and mRNA degradation was evident in subsequent extractions, thus previously unused blocks were used if available. With benign samples, this was particularly problematic due to the limited numbers available and the majority of benign samples had been used in previous studies. As a result of the current study, collection procedures have altered such that a separate tissue block is taken from each case solely for the purpose of RNA extraction.

It was considered important to have a method of analysing mRNA integrity, since degradation could lead to false negative results from TNC PCR. Thus a PCR based method of mRNA assessment was developed to fit in with existing protocols. The oligo-dT Dynabeads take advantage of the polyA tail of nucleotides added to the end of the primary transcript following transcription. The polyA tail complementarily binds to the dT tail on the bead leaving the beginning of the transcript exposed. PCR primers were designed for either end of the resultant TNC cDNA strand, specifically, the EGF repeat region (exon 2 at the exposed end of the molecule) and the fibrinogen like region (a cassette spanning exons 25 - 27 at the end of the molecule closest to the bead). The rationale of this is based on degrading enzymes specifically acting on single stranded nucleic acids, thus the 3' end of the transcript is protected following binding to the poly-dT tail. As a result, degradation will only occur from the 5' to 3' end of the transcript. If a weaker signal from the EGF repeat PCR was evident, compared to the fibrinogen domain PCR, RNA degradation was indicated (see figure 4.2). If further investigations indicated that RNA was compromised, that sample was removed from the study. However, if degradation was minimal, more template was used and it was found that specific PCR primers still gave an adequate readable signal. This PCR based method was used alongside GAPDH signal to assess amount of template to be used in more specific TNC PCR.

Furthermore, it served to eliminate RNA compromised tissues from the study and thus eliminate false negative results.

Problems also occurred with storage of the cDNA template. cDNA was not eluted from the dynabeads but left as a bead/cDNA complex and stored at 4°C as per manufacturers instructions. However, when further analysis was attempted after approximately six months a weaker or no signal was seen with TNC PCR. Degradation was confirmed by GAPDH PCR yielding a weaker signal. This ruled out a number of more detailed studies of TNC isoform profile, particularly using the 11/16 primer set.

4.3.1.1 Choice of tenascin C PCR primer cassette

For tissue analysis the 8/18 cassette, which spans the entire alternatively spliced region, was initially used. In addition, the 11/16 primer cassette was used since this was thought to be more informative than 8/14 and 14/18 as exons 11 and 16 are considered to be highly conserved (see Chapter 3 of the current study and also Sagizadeh *et al* 1998, Bell *et al* 1999). A further advantage of this cassette is that it reduced the amount of cDNA template used, particularly in those samples requiring more template to give adequate amplification. The 11/16 cassette gives information on those isoforms in which the alternatively spliced region has been included and excludes the truncated isoform, possibly the most abundant form in normal breast tissue (Borsi *et al* 1993). The 8/AD1 cassette was also used in some cases for analysis of possible AD1 and AD2 containing isoforms.

Forward and reverse primer sequences for AD2 were designed by Mighell *et al* (1997). These were applied to Sk-mel 28 cDNA in a variety of combinations with other TNC primers across a range of annealing temperatures. The banding pattern obtained, particularly from the AD2/AD1 cassette, was not concurrent with expected results and cast doubt on the compatibility of these AD2 primers with the other TNC primers. Further investigations using these AD2 primers alone on Sk-mel 28 yielded a single band of the correct size and this was then applied to primary breast tissues. An amplified product was seen in a proportion of DCIS and all IDC tissues, but not in benign samples (see fig 4.7). It was initially concluded from this that AD2 could be a tumour marker which had been suggested by previous studies (Mighell *et al* 1997, Derr *et al* 1997). However, when this was repeated on the same samples, results were not consistent. PCR primers probing internally on a single exon and not across an exon

boundary have a tendency to bind elsewhere non-specifically, which may be exaggerated when the primers are targeted to repeat regions. The attempt to increase specificity using "nested" PCR produced similarly inconsistent results. It was therefore concluded that consistent and dependable results could not be obtained with this primer set which was not investigated further. However, given previous studies it would be interesting to pursue an investigation of AD2 as a tumour marker, possibly by designing further primers compatible with the current TNC primers or utilising ISH.

4.3.1.2 Optimisation of isoform specific PCR

A number of primers were designed to amplify individual isoforms. They were designed across exon boundaries, thus would anneal only if that boundary occurred. The 9-17 forward primer was designed across the boundary of exons 9 and 17 which only occur alongside each other in the truncated isoform, hence, when used in conjunction with the T18R reverse primer should only give a signal with the truncated isoform. The 9-16 forward primer was designed across the boundary of exons 9 and 16 which only occur alongside each other in the plus exon 16 isoform, hence, when used in conjunction with the T18R reverse primer should only give a signal with the plus exon 16 isoform. As exon 16 is only ever situated prior to the conserved exons 17 and 18, no isoform specific reverse primer is required. This is not the case with the primers designed for the plus exon 14 & 16 isoform. The 9-14 forward primer was designed across the boundary of exons 9 and 14 which occur alongside each other in the plus exons 14 & 16 isoform. However, a number of different exons may follow exon 14, thus the 14-16 reverse primer was designed across the boundary of exons 14 and 16. When the 9-14 forward primer and the 14-16 reverse primer were used together, a signal should only be obtained from the plus exons 14 & 16 isoform (see figure 4.8 and table 4.5).

Since the exons in the variable region are of repeat sequences, they are highly homologous, making cross-reactivity a particular problem. This was addressed by optimising each primer set on a pure template of the isoform for which they were designed. Each primer set should only generate a product with its specific template, and if products are generated with other templates then these must be considered as non-specific. When the primer sets were applied to the purified isoforms, the 9-16/18 cassette gave a signal from the plus exon 16 isoform fragment and the plus exons 14 & 16 isoform fragment signal, also the 9-17/18 cassette gave a signal with all templates. This non-specific annealing is a result of the highly homologous

nature of these exons, which is particularly exacerbated at the exon borders. Only the 9-14/14-16 primer cassette could be optimised to generate a product with the TNC 14-16 isoform alone and was then applied to primary tissue.

4.3.1.3 Cycle sampling

The initial purpose of the isoform specific PCR primers was to study relative amounts of each isoform in benign and malignant tissue and establish whether changes were qualitative or quantitative. Only the primer cassette specific for the plus exons 14 & 16 isoform was optimised, but this was the isoform specifically expressed in malignant/invasive tissue. Hence a semi-quantitative comparison could be made with total TNC using the fibrinogen domain primers and a cycle sampling protocol. PCR reactions were performed over a range of cycles and run on a gel. If the observed alterations in TNC expression were a quantitative factor, both reaction signals would become saturated at a similar rate independent of sample type, whereas if changes in expression were a qualitative factor, the rate of saturation would be dependent on sample type. If a qualitative change was occurring it could be predicted, that in non-invasive samples, fib. domain PCR would become saturated before a signal was seen with isoform specific PCR, whereas in invasive samples the rate of saturation would be similar.

Cycle sampling is a simplistic approach towards quantifying PCR products and more sophisticated methods were considered. These include competitive PCR and ELOSA (Enzyme Linked Oligonucleotide Assay). However, this semi-quantitative method was chosen as it fitted in with the existing, and well established, TNC PCR protocols. The alternative methods were considered unsuitable given the high level of specificity required for this particular investigation, and furthermore will have involved periods of optimisation. This factor was crucial due to time constraints and limited amounts of sample availability.

4.3.2 Discussion of Results

4.3.2.1 Different tenascin C isoforms are expressed in breast carcinoma

Differences were seen in the mRNA splicing pattern between benign/normal breast, *in-situ*, and invasive carcinoma. In all tissues with adequate RNA integrity the 8/18 PCR cassette was used which spans the whole variably spliced region and thus may represent the number of highly abundant isoform permutations expressed in that tissue (Bell *et al* 1999). All samples produced a product of 442 bp which corresponds to the truncated form i.e. total excision of the

variable region. This was confirmed by the absence of a hybridisation signal using oligonucleotide probes specific for exons in the variable region. This appears to be the most common isoform in the breast, and is also thought to be the most common in the majority of tissues (Borsi *et al* 1993, Sagizadeh *et al* 1997, Bell *et al* 1999). However, in some tissues, fragments of 715 bp and 988 bp were generated. These fragments hybridised with internal oligonucleotide probes, therefore they were not considered to be PCR artefacts and size indicated they correspond to addition of one and two exons respectively. The single additional exon was identified as exon 16 and the second additional exon was identified as exon 14. Results from the developmental stages of this study show that exons 14 and 16 are highly conserved in Sk-mel 28. Other studies have also shown these exons to be highly conserved with 20 of the 22 previously reported isoforms containing exons 14, 16 or both (Mighell *et al* 1997, Borsi *et al* 1993, Sriramarao & Bourdon 1993, Saga *et al* 1991, Dorries *et al* 1994, Zhao *et al* 1995, Wilson *et al* 1997, Saghizadeh *et al* 1998). The current study has indicated that the isoform profile may alter as the breast acquires malignant and invasive phenotypes. The truncated isoform was seen in all samples and can be considered as the "normal" form (Borsi *et al* 1993), whereas the plus exon 16 isoform was only seen in a small proportion (25%) of benign samples in comparison to 70% of malignant samples (includes both *in-situ* and invasive carcinoma). Furthermore, the plus exons 14 & 16 isoform was seen in 54% of DCIS and 75% of invasive carcinoma, but only one benign sample (5%) which was a fibroadenoma. Thus it appears there is a progressive change in isoform profile as the breast acquires a malignant and then invasive phenotype.

Higher molecular weight (MW) TNC mRNA transcripts have previously been associated with neoplastic human breast disease and coincide with neo-expression of higher MW protein isoforms (330 kDa sub-units) against a background of smaller MW proteins (190kDa sub-units) (Borsi *et al* 1992). Increased expression of higher MW isoforms has also been associated with lung carcinoma (Kusagawa *et al* 1998), ovarian carcinoma (Wilson *et al* 1996), and oral squamous cell carcinoma (Hindermann *et al* 1999). These higher MW forms are not exclusive to malignant tissue and are seen in a range of normal tissues at comparatively low levels (Borsi *et al* 1993) and also in normal cell lines (Kawakatsu *et al* 1992). Tenascin C expression has long been associated with cellular proliferation during embryonic development (particularly in the mesenchyme) and normal tissue remodelling, such as the menstrual cycle and wound healing (Aufderhide & Eckblom 1988, Anbazhagan *et al* 1990). It was initially

theorised that TNC expression was a response to tumour cell proliferation and a marker for epithelial malignancy (Chiquet-Ehrissmann *et al* 1986, Mackie *et al* 1987). However, the increase of TNC, particularly high molecular weight isoforms has been seen in hyperplastic breast disease (Borsi *et al* 1992). This is borne out by the current study identifying the 14-16 isoform in a single fibroadenoma and 2 of the 5 fibroadenomas showing expression of the plus 16 isoform. This contributes towards the conclusion that TNC is not purely a marker of malignancy.

The majority of previous studies have considered only the very small (fully truncated), or very large TNC isoforms (inclusion of at least six FNIII like repeats) and suggest a transition from small to large TNC in malignancy (Kusagawa *et al* 1988, Borsi *et al* 1993, Wilson *et al* 1996, Hindermann *et al* 1999, Ghert *et al* 2001). The size of transcripts indicated in the current study would correspond to the truncated protein and intermediate sized isoforms and suggests that the intermediate sizes are associated with invasion. This confirms previous work identifying intermediate sized isoforms in relation to a cohort of invasive breast tumours (Borsi *et al* 1992), although at a much lower frequency than the current study. This may be due to the relative insensitivity of the Northern blotting technique employed in that study compared to RT-PCR. Other studies indicate that Northern blotting lacks the sensitivity to detect intermediate sized isoforms (Sagizadeh *et al* 1998). The approach of Northern blotting uses means it is unable to decipher exact isoform *composition* and only gives an indication of *size*. The small and large isoforms seen may not correspond to total exclusion/inclusion of the alternatively spliced region if they are not the major isoforms produced by a tissue as in foetal membranes (Bell *et al* 1999). This may account for some of the discrepancies seen in the reported sizes of particularly the small, supposedly truncated, isoform ranging from 190 to 220 KDa (Erickson & Bourdon 1989, Lightner *et al* 1994) which becomes significant in view of the fact that a FNIII like repeat is approximately 10Kda (Erickson & Bourdon 1989, Chiquet-Ehrissmann *et al* 1991). Few studies have employed a similar approach to this project, but these support the nature and spectrum of isoforms identified. In foetal membranes, the plus 16 isoform is the most common (Bell *et al* 1999) as is the case in normal rat neural tissue (Joestner & Faissner, 1999). The isoform profile of foetal membranes also contains the 14-16 isoform which has also been seen by Siri *et al* (1991). Importantly, this isoform is upregulated in invasive ovarian carcinoma (Wilson *et al* 1996) which may indicate a specific function of this isoform in some types of tumours.

It was important to establish whether the changes in isoforms detected were truly qualitative changes, or were detected because of the increased amount of TNC known to occur in the majority of invasive carcinomas (Natali *et al* 1991, Koukoulis *et al* 1991) including breast (Chiquet-Ehrissmann *et al* 1986). It was conceivable that these isoforms were present in benign tissue at the same ratio to the truncated isoform as in malignant tissue. Thus, because of the lower amounts of total TNC mRNA in benign breast, the larger isoforms were not detected. A quantitative change involving increases of both low and high MW forms of TNC has been reported in prostate carcinoma (Ibrahim *et al* 1993) and the detection of the plus 2 exon on the gel in the current investigation may have been due to an overall increase in TNC making the signal more detectable. This difference may have been further exaggerated by PCR preferentially amplifying smaller fragments, effectively giving an exaggerated false positive result for the truncated isoform and exaggerated false negative results for the additional isoforms. However, previous studies utilising an RT-PCR technique have demonstrated more intense signals from higher MW TNC mRNA (Sagizadeh *et al* 1998, Bell *et al* 1999, Joestner & Faissner, 1999) and this was also identified in the current study using sk-mel 28 (see chapter 3). Many of the breast samples exhibited weaker intensity bands for the larger isoforms compared to truncated TNC, however, in some cases stronger intensity bands for larger isoforms were demonstrated (Figure 4.3). This suggests a switch in dominance of the isoforms present. This was further supported by the cycle sampling experiment using primers specific to the plus 14-16 isoform which showed that even when samples exhibited similar levels of total TNC, the expression of the 14-16 isoform was lower or absent in many benign samples (Figure 4.6). Relative amounts of high MW TNC have been shown to increase by tenfold in malignant compared to normal breast (Borsi *et al* 1992) and this may account, at least in part, for the overall increase in amount of TNC.

The current study has identified changes in isoform expression not only in the majority of invasive carcinoma but also in a subset of pre-invasive DCIS. Overexpression of total TNC protein in DCIS has previously been reported (Howeedy *et al* 1990) and detection has been suggested to be useful in predicting behaviour (Xue *et al* 1998, Goepal *et al* 2000).The expression of additional isoforms in some DCIS but not others may reflect the potential for invasive behaviour as it is recognised that some, but not all DCIS will progress to invasion.

When the tissues were screened with the 9-14/14-16 specific primers a greater proportion of benign samples gave a signal (although of weaker intensity than in tumour tissue), in comparison to the pattern observed using the 8/18 PCR cassette. There are a number of possible reasons for this. This PCR cassette is designed to give a single band, therefore there is no competition from smaller fragments. However, being a nested protocol, any template is amplified for 60 cycles which renders the reaction very sensitive, hence, the significance of a weak signal obtained under these conditions can be questioned. Thus, the results from the 8/18 cassette are probably more representative as the 9-14/14-16 PCR would detect even trace amounts of this isoform. The one group of tissue that consistently exhibited a band with the 9-14/14-16 cassette was the fibroadenomas, and this may be attributed either to increases in total amount of TNC or to lower level changes in isoform expression pattern not detected using 8/18 PCR.

A number of other primer cassettes were used in conjunction with the 8/18 cassette. The results from sk-mel 28 (chapter 3) and previous studies (Bell *et al* 1999) predicted that the 11/16 cassette would be particularly informative in evaluating higher molecular weight isoforms. It would circumvent problems associated with the high expression levels of the truncated isoform. However, given that the 8/18 cassette predicted isoforms containing a maximum of two exons, the 11/16 cassette gave apparently discrepant results. Fragment size and banding pattern indicated a variable region containing 3 - 5 additional exons (see fig. 4.7). Similarly discrepant results were obtained using the 14/16 PCR cassette and it was thought this cassette would yield a single band in invasive and some pre-invasive samples only, and thus be a useful screening tool. This was not the case as results indicated multiple isoforms containing exons 14 and 16 were also present in benign samples (see fig. 4.8). This apparent inconsistency confirms the presence of small amounts of higher MW isoforms in benign tissue. The pattern that appears to be emerging is that both truncated and higher molecular weight isoforms are expressed in benign tissue, with intermediate sized isoforms, for example the isoform containing additional exons 14 & 16, being more highly expressed in malignancy. Keeping with this, Borsi *et al* (1992) demonstrated the presence of very low levels of high MW isoforms in normal tissues. A further possibility is that different TNC isoforms are expressed in other components present in the breast tissue sections, for example blood vessels and there is some evidence to support this from IHC as discussed below. Increased expression of other intermediate sized isoforms has also been reported in ovarian tumours (Wilson *et al*

1996) and indicated in the current study by 8/18 PCR showing expression of a plus 3 exon isoform in a single invasive tumour.

The consistency observed in splicing patterns allow speculation of what the isoforms seen in 11/16 PCR may be (Bell *et al* 1999). Cell line work, particularly with Sk-mel 28 (Siri *et al* 1991, Bell *et al* 1999 and the current study) indicate that exons 12 and 15 are very susceptible to splicing events which is responsible for many of the differences between isoform composition. Thus, it can be speculated from size of 11/16 PCR bands (see figure 4.7) that the largest isoform contains exon 14 and both exons 12 and 15. The band below this is lacking in either exon 12 or 15 and the band below this is lacking in both exons 12 and 15. Thus the smallest band containing a single exon between 11 and 16 may also contain 14 and this would be adjacent to 16. This 14-16 sequence is the variant which has been associated with invasive tumours in this study. It can be further speculated that the plus 3 exon isoform seen in a single case using 8/18 PCR may be an isoform containing exon 11 in addition to exons 14 and 16. This may indicate a specific consistent pattern of splicing associated with malignancy. Unfortunately this could not be addressed in the present study owing to degradation of cDNA.

As exon 14 adjacent to 16 has been associated both with the invasive phenotype and pre-invasive lesions in the current study and also associated with invasion in ovarian tumours (Wilson *et al* 1996) it is possible that this array of repeats conveys a specific function associated with the progression of tumours towards invasion. If this is the case, this is the first time a specific array of repeats has been associated with a specific function in an *in-vivo* situation. It has been speculated that different arrays of repeats are associated with different functions (Chiquet-Ehrissmann *et al* 1991), demonstrated by *in-vitro* studies utilising recombinant TNC fragments (Philips *et al* 1998) but specific isoform compositions have not been investigated. To address the functional significance of these isoforms and specifically whether they have a role in promoting invasion, it would be essential to express them in a model system.

In summary, the RT-PCR and Southern blotting analysis has identified a specific alteration in TNC expression in DCIS and invasive breast carcinoma with up-regulation of an isoform containing exons 14 & 16. This isoform may play a role in the development and progression of invasion, though, *in-vitro* studies would be necessary to address this. This may not be the

only change as results have indicated that other isoforms containing 3 or 4 additional exons may also be associated with the invasive phenotype.

4.3.2.2 Tenascin C Protein expression and distribution is altered in breast carcinoma

Immunohistochemistry (IHC) and ISH was used to study TNC protein distribution and expression in normal/benign, *in-situ*, and invasive carcinoma. Initially, an antibody which was specific to a conserved region of the protein (the EGF repeat region), was used that would indicate the distribution of all TNC proteins. Serial sections on the same cases were investigated using an antibody specific to the B domain (exon 14) of the FnIII like variable region. Since RT-PCR studies had identified this was a potentially important region associated with invasion, this was a very useful antibody to possess.

In normal breast tissue, total TNC was localised close to the myoepthelial cell layer in the basement membrane. This suggests that the myoepithelial cells are the source of the TNC in normal and benign breast as has previously been suggested (Lightner *et al* 1994, Yoshida *et al* 1997). However, BM and stromal proteins could be expressed by stromal fibroblasts. Using ISH in the current study, no signal was detected in any cell population. This probably reflects the very low level of TNC synthesis in normal breast (Koukoulis *et al* 1991, Natali *et al* 1991, Borsi *et al* 1992).

In a proportion of benign/normal tissue there was a slight diffuse periductal stromal staining pattern around occasional ducts and acini. This correlates with PCR result in that these benign samples gave a positive signal with the plus exons 14 & 16 isoform specific PCR, and also a plus exon 16 signal from 8/18 PCR. Furthermore, blood vessel staining within a tissue with both TNC antibodies also correlated with expression of larger isoforms indicated by PCR. The diffuse stromal staining in normal/benign tissue may be due to changes in TNC distribution during the menstrual cycle. Similar patterns have previously been associated with an increase in TNC expression and a change in TNC distribution in the breast during the 4^{th} week of the menstrual cycle (Ferguson *et al* 1990). Other organs, including the ovary (Tamura *et al* 1993) and endometrium (Vollmer *et al* 1990), also exhibit changes in amount and distribution of TNC depending on stage of menstrual cycle, although no link has previously been made with larger forms of TNC.

In DCIS, total TNC antibody demonstrated basement membrane associated staining, as seen in benign tissue. Furthermore, a distinctive pattern of stromal staining was seen in high grade DCIS with both antibodies. The appearance was of a pattern of striations, areas of which are partially parallel with the basement membrane. A pattern which has been previously reported (Gould *et al* 1990). The majority of cases analysed were high grade DCIS, but three cases were non-high grade. Interestingly the non-high grade did not exhibit this striated pattern indicating that alteration of TNC in DCIS may be related to grade; this has been previously suggested (Jahkola *et al* 1998a). The IHC pattern correlates directly with pattern of isoform expression detected by RT-PCR with the high grade cases demonstrating expression of the 14-16 isoform. This suggests that altered TNC isoform expression and distribution may distinguish a sub-group of DCIS more likely to progress to invasion. ISH in DCIS identified the source of TNC synthesis as both the myoepithelial cell layer and associated periductal stromal fibroblasts, but only at discrete foci. This may be indicative of local environmental changes that may promote micro-invasion (Jahkola *et al* 1998a). Similar focal gaps have been associated with BM degradation prior to invasion in breast (Howeedy *et al* 1990, Gould *et al* 1990) and prostate carcinoma (Xue *et al* 1998). Thus it is possible TNC may be the trigger for invasion (Lagios *et al* 1989, Jahkola *et al* 1996 & 1998a).

In tissue from infiltrating ductal carcinoma (IDC) TNC is visible in the stroma around tumour sheets and groups. ISH showed that stromal fibroblasts were the source of the protein confirming previous work done on carcinoma of the breast (Ferguson *et al* 1990, Lightner *et al* 1994, Yoshida *et al* 1994 & 1997) ovary (Wilson *et al* 1996), cervix (Pilch *et al* 1999) and prostate (Xue *et al* 1998). In contrast to previous studies (Lightner *et al* 1994, Yoshida *et al* 1994 & 1997), there was no evidence of tumour cell synthesis of TNC in this study. Tumour cell production of TNC has been associated with a poor prognosis (Ishihara *et al* 1994) and also metastases and recurrence (Jahkola *et al* 1996, 1998a & b). Although ISH on tissues did not locate signal to tumour cells, analysis of breast cancer cell lines has identified some tumours that produce TNC (Chapter 5), thus the lack of ISH signal in primary tumour tissue most likely reflects selection bias in the cases studied. A wider study is indicated, not only to confirm expression of TNC by tumour cells, but also to address whether tumour cells synthesise a different TNC isoform compared to fibroblasts, thus influencing tumour behaviour.

The alterations in TNC profile and distribution in the breast are consistent with carcinoma of other organs and generally there is an increase in stromal TNC (Koukoulis *et al* 1991, Natali *et al* 1991, Shrestha *et al* 1996). In some tumour types, such as bladder (Tiitta *et al* 1993), liver (Jaskiewicz *et al* 1993), and prostate carcinoma (Xue *et al* 1998)), there is an association with grade and level of tumour differentiation, unlike the breast (Tokes *et al* 1996), and cervix (Pollanen *et al* 1996). In the prostate TNC amount inversely correlates with an increasing grade, and this is related to metastases and poor prognosis. This indicates that TNC may have some sort of protective effect which has also been suggested for skin carcinoma (Anbazhagan *et al* 1990), colorectal carcinoma (Sugawara *et al* 1993), and the breast (Ishihara *et al* 1995). This effect may be isoform specific, and some forms of TNC may maintain tissue integrity. In the breast, the protective effect may be elicited by the truncated isoform, which may be negated by the neo-expression and relative increase in high MW forms. This also may be the case for other organs as the truncated form is the most common form seen in normal tissue (Borsi *et al* 1992) and high MW forms are associated with other carcinomas (Verstraeten *et al* 1992, Wilson *et al* 1996, Kusagawa *et al* 1998). Disappearance, or a lack of TNC being associated with poor prognosis, may also be due to degradation of the protein, and protein fragments have been linked with metastases in lung cancer (Kusagawa *et al* 1998). Conversely, high levels of TNC in the breast are associated with metastases (Jahkola *et al* 1996; 1998b).

Although a change in isoform profile is associated with tumour invasion in this investigation, the change is not consistent between different organs. The 14-16 form (FNIII like repeats B and D) identified here been seen in ovarian carcinoma (Wilson *et al* 1996). However, in renal carcinoma, the truncated form predominates (Lohi *et al* 1995), in colorectal carcinoma downregulation of isoforms containing repeat D is associated with metastases (Dueck *et al* 1999) and repeat C is associated with glioma and cavernoma (Viale *et al* 2002). This points towards changes in isoform profile being tissue specific (Ghert *et al* 2001) which may have functional consequences (Philips *et al* 1998). Furthermore, effect of TNC may also be dependant upon the receptor profile of a cell (Yokosaki *et al* 1996) and TNC receptors have been seen to change as carcinoma, particularly SCC develops (Ramos *et al* 1997, Uhlman & Niehans 1999).

4.3.3 Summary

In summary, this study has demonstrated a change in TNC isoform profile in malignant compared to normal/benign tissue with expression of two additional isoforms, TNC plus 16 and TNC plus 14 & 16. A proportion of DCIS also exhibit the same change in isoform expression. IHC and ISH demonstrated that this is associated with a change in distribution of TNC synthesis and localisation from the BM zone to the stromal compartment. The detection of these changes in DCIS suggests that this altered TNC profile precedes invasion and could therefore be implicated in the initiation of invasion. This is in keeping with *in-vitro* studies that have shown TNC to promote tumour migration, cell proliferation, and down-regulation of focal adhesions (Murphy-Ullrich *et al* 1991,End *et al* 1992, Yokasaki *et al* 1996).

On the basis of the current study and previous work, a hypothetical model for the role of TNC in breast cancer invasion has bee proposed. It is suggested that neoplastic epithelia releases signals that stimulate TNC expression in stromal cells, including expression of intermediate sized isoforms. Release of TNC proteins then alters fibroblast behaviour through altered cell-matrix interactions and this altered fibroblast phenotype creates a micro-environment that promotes tumour cell invasion.

Investigation of this hypothesis is the basis of chapter 5.

Chapter 5 – *In-vitro* Tenascin C Investigation

5.1 Introduction

Analysis of primary breast tissue has demonstrated a significant relationship between up-regulation of specific TNC isoforms and the malignant and invasive phenotype. Furthermore, this altered TNC isoform profile occurs at a pre-invasive stage, suggesting that these TNC isoforms may contribute to the initiation of the invasive process.

Expression of TNC in peri-ductal stroma could influence tumour progression in a number of ways. Interaction of tumour cells may directly influence cell migration and/or proliferation. Previous studies have indicated that culture of cells on TNC can promote migration (Kiernan *et al* 1996, Chung *et al* 1996, Deryugina & Bourdon 1996, Herold-Mende *et al* 2002, Wallner *et al* 2002) and proliferation (Chiquet-Ehrissmann 1986, End *et al* 1992, Tan *et al* 1999, Huang *et al* 2001, Herold-Mende *et al* 2002). TNC may also influence tumour progression via an indirect effect on fibroblasts, altering their behaviour and further contributing to the creation of a pro-invasive microenvironment. One of the most consistent changes identified in peri-tumoural fibroblasts, in both breast and other carcinomas, is the expression of the matrix metalloproteinases (MMPs). More than 20 MMPs have now been identified, however, those most frequently associated with breast carcinoma and tumour behaviour are MMP 1, 2, 7, 9, 11, 13, and MT1-MMP. The majority of these MMPs are synthesised primarily by the stromal fibroblasts surrounding tumours, rather than the tumour cells themselves (Bassett *et al* 1990, Davies *et al* 1993, Okada *et al* 1995, Heppner *et al* 1996). MMP2 and MT1-MMP are expressed in the majority of breast carcinomas (Ueno *et al* 1997, Jones *et al* 1999), and high levels of expression correlate with the presence of local and distant metastases (Ueno *et al* 1997, Mimori *et al* 2001). MMP11 has been shown to be exclusively expressed by peri-tumoural fibroblasts in the breast (Bassett *et al* 1990, Hahnel *et al* 1993, Heppner *et al* 1996). Expression has also been associated with high grade DCIS (Wolf *et al* 1993, Philips *et al* manuscript submitted). MMP13 is also localised predominantly to stromal cells of invasive carcinomas (Uria *et al* 1997). In a study including DCIS and cases with microinvasion, MMP13 was identified in the peri-ductal stroma of the majority of cases exhibiting microinvasion, but not in cases without (Nielsen *et al* 2001), suggesting it may be involved in the transition of DCIS to invasive disease. This data, together with work in our own laboratory indicates that many MMPs are expressed by fibroblasts around invasive and pre-invasive

breast disease, co-localising with TNC expression. There is evidence from different sources to suggest that cellular interactions with matrix proteins can induce MMP expression. Thus, it is possible that following expression of TNC isoforms, there is an interaction of fibroblasts with TNC, via integrin mediated adhesions, leading to the induction of MMPs.

A critical point is evidently what induces expression of TNC in peri-tumoural fibroblasts. Proliferating carcinoma cells produce a range of factors, some of which may alter TNC expression in neighbouring cells (Inaguma *et al* 1988, Chiquet-Ehrismann *et al* 1989, Hiraiwa *et al* 1993). A number of factors have been linked with induction of TNC including EGF, FGF, PDGF, TGFβ IGFII, gastrin and progesterone (Rettig *et al* 1989, Wilson *et al* 1996, Makhluf *et al* 1996, Vollmer *et al* 1997, Chimal-Monroy & Diaz de Leon 1999, Kucharczak *et al* 2001, Suzuki *et al* 2002). Of these soluble factors, both EGF and TGFβ are strongly implicated in breast carcinoma (Travers *et al* 1988, Mizukami *et al* 1991, Walker & Dearing 1992). TGFβ is strongly linked with ECM remodelling during development (Osin *et al* 1998, Barcellos-Hoff & Ewan 2000) and malignancy (Ignotz & Massague 1986, Kahari *et al* 1991, Zhao 1999, Hagedorn *et al* 1999, Berking *et al* 2001). One of a number of ECM components it regulates is TNC (Pearson *et al* 1988, Chiquet-Ehrismann *et al* 1989, Sakai *et al* 1995a, Vollmer *et al* 1997, Chimal-Monroy & Diaz de Leon 1999) and it has also been shown to have an effect on TNC alternative splicing (Zhao & Young 1995). EGF is involved in mammary epithelial cell differentiation (Wirl *et al* 1995) via modulation of ECM components (Baley *et al* 1990, Chammas *et al* 1994). It is associated with tumour progression (Stampfer 1985) and has been shown to have a more potent effect on TNC induction than TGFβ (Sakai *et al* 1995a) an effect itself regulated by steroid hormones (Sakai *et al* 1995b). The current study has focussed on these two growth factors.

TNC has also been shown to be induced by physical factors such as stretch (Chiquet-Ehrismann *et al* 1994, Trachslin *et al* 1999, Theilig *et al* 2001, Altman *et al* 2001). This could be particularly relevant in the situation of DCIS where proliferation of neoplastic cells leads to distension of the beast duct and would exert tensile forces on the surrounding stroma.

From this experimental data, a model for the potential role of TNC in promoting tumour progression can be hypothesised (see figure 5.1). This suggests that in DCIS, neoplastic cells proliferate and expand the abnormal duct. As a response to either soluble factors released by

tumour cells or mechanical stress, peri-ductal fibroblasts switch on TNC expression. Specifically truncated TNC, TNC16 and TNC14-16. These isoforms are laid down in the stroma and through integrin mediated interactions stimulate fibroblasts to produce MMPs. These MMPs then promote tumour invasion either directly via breakdown of BM components, or indirectly through release of matrix-bound growth factors. Tumour cells may then interact directly with the TNC matrix resulting in promotion of migration and proliferation.

In order to investigate this hypothesis a number of *in-vitro* models have been established, and these have been used to address the specific aims of this chapter, which are:

- Does tumour cell interaction with TNC promote tumour cell migration and invasion?
- Do fibroblast and tumour cell interactions with TNC lead to induction of MMP expression?
- Are soluble factors such as TGFβ and EGF involved in the control of TNC isoform expression?

Thus, the effect of TGFβ and EGF on primary fibroblasts was investigated to assess their potential as inducible factors. Also, the effect of a commercially available form of TNC on both primary and established breast cell lines was investigated. Specifically an analysis of cell motility and proliferation, alongside alterations in MMP expression.

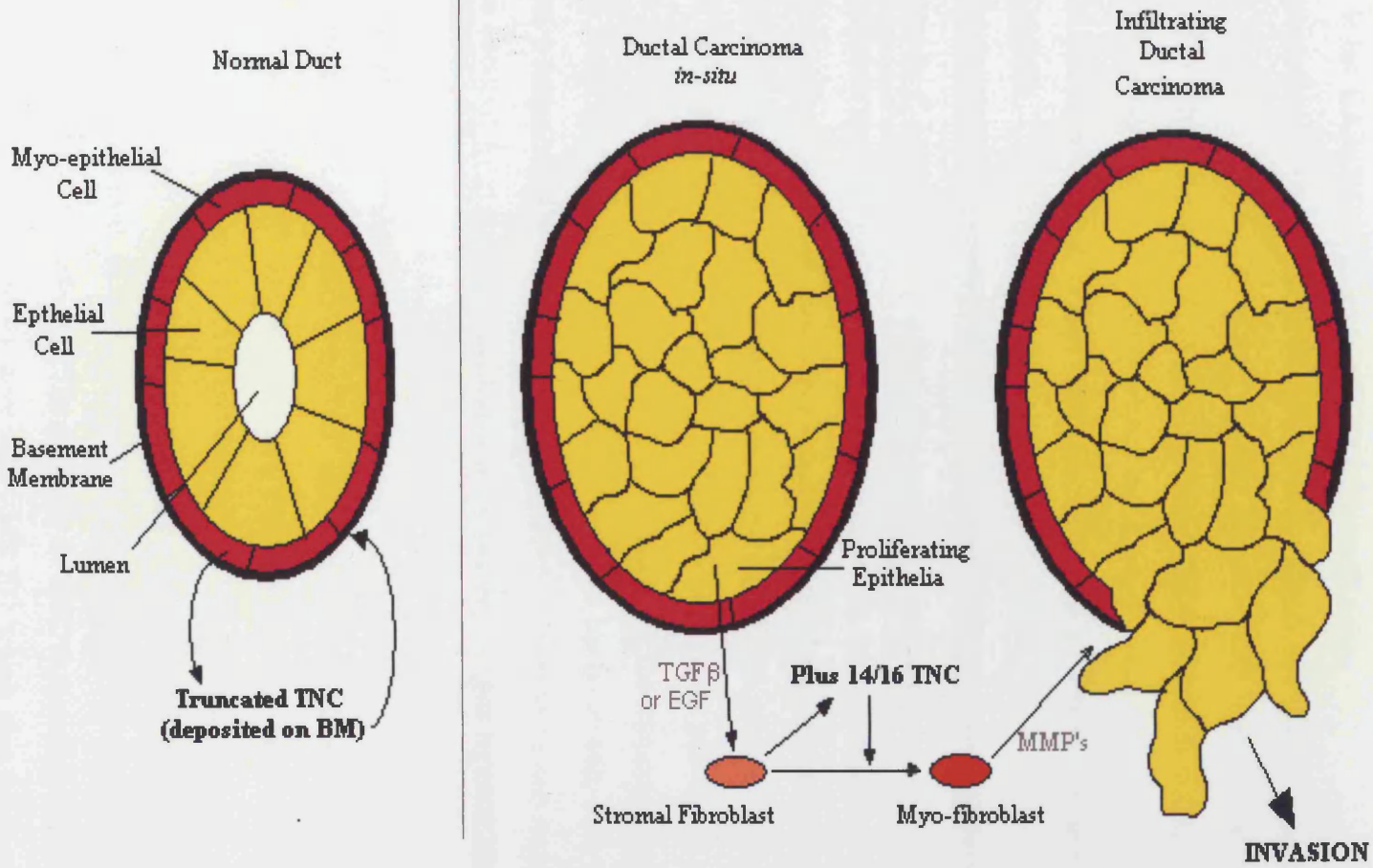


Figure5.1: Proposed model for the action of tenascin C in the normal breast and breast cancer invasion.

5.2 Results

All results in this section are typical examples of consistent and reproducible observations seen on a minimum of three separate occasions. Figures also illustrate typical results.

5.2.1 PCR for GAPDH on fibroblasts and breast cell Lines

A strong signal was obtained from cultured primary fibroblasts (figure 5.2) and all established cell lines (figure 5.3). GAPDH signal was not altered in cell lines treated with TGFβ, EGF (figure 5.4) or TNC (figures 5.8 - 5.12). Primary cells from which mRNA was extracted on day 0 gave weaker signals, possibly due to cell/tissue debris contaminants during cell separation, thus, up to 3μl cDNA template was used to obtain an equivalent signal in subsequent PCR.

5.2.2 Tenascin C expression by cultured primary fibroblasts

TNC expression was investigated in untreated fibroblasts from normal breast (post isolation, pre-cultured cells), in order to establish a baseline in comparison to cultured cells. Growth of primary fibroblasts in cell culture medium alters the expression profile of tenascin C. Cassette 8/18 PCR performed on cDNA from day 0 fibroblasts prior to placing in culture medium, amplified a single band at 442bp indicating expression of the truncated isoform. However, 8/18 PCR performed on day 4 quiescent cells amplified bands at 442, 600, 1807, 2080, and 2353bp i.e. a range of higher molecular weight isoforms appear to be neo-expressed. A similar result was obtained from quiescent cells on day 14 and day 21 (see figure 5.2).

5.2.3 Tenascin C expression by established breast cell lines

No PCR products were generated using TNC primer cassettes 8/18, 8/14, 14,18 and 8/AD1 for cell lines T47-D and MCF7. In contrast, using the 8/18 cassette, each of the breast cell lines HBL-100, MCF10A, MDA-MB 231, and MDA-MB 468, yielded a series of PCR products indicating a range of isoform expression (see figure 5.3). On the basis of fragment size, the number of exons included in the variable FN type III-like repeat region for those cells producing TNC can also be deduced. The majority of TNC expressing cell lines produced the truncated form only. The exceptions were MDA-MB 231 which also produced a plus 1 exon isoform, and HBL-100 which also produced a distinctive triplet of fragments around 2kb.

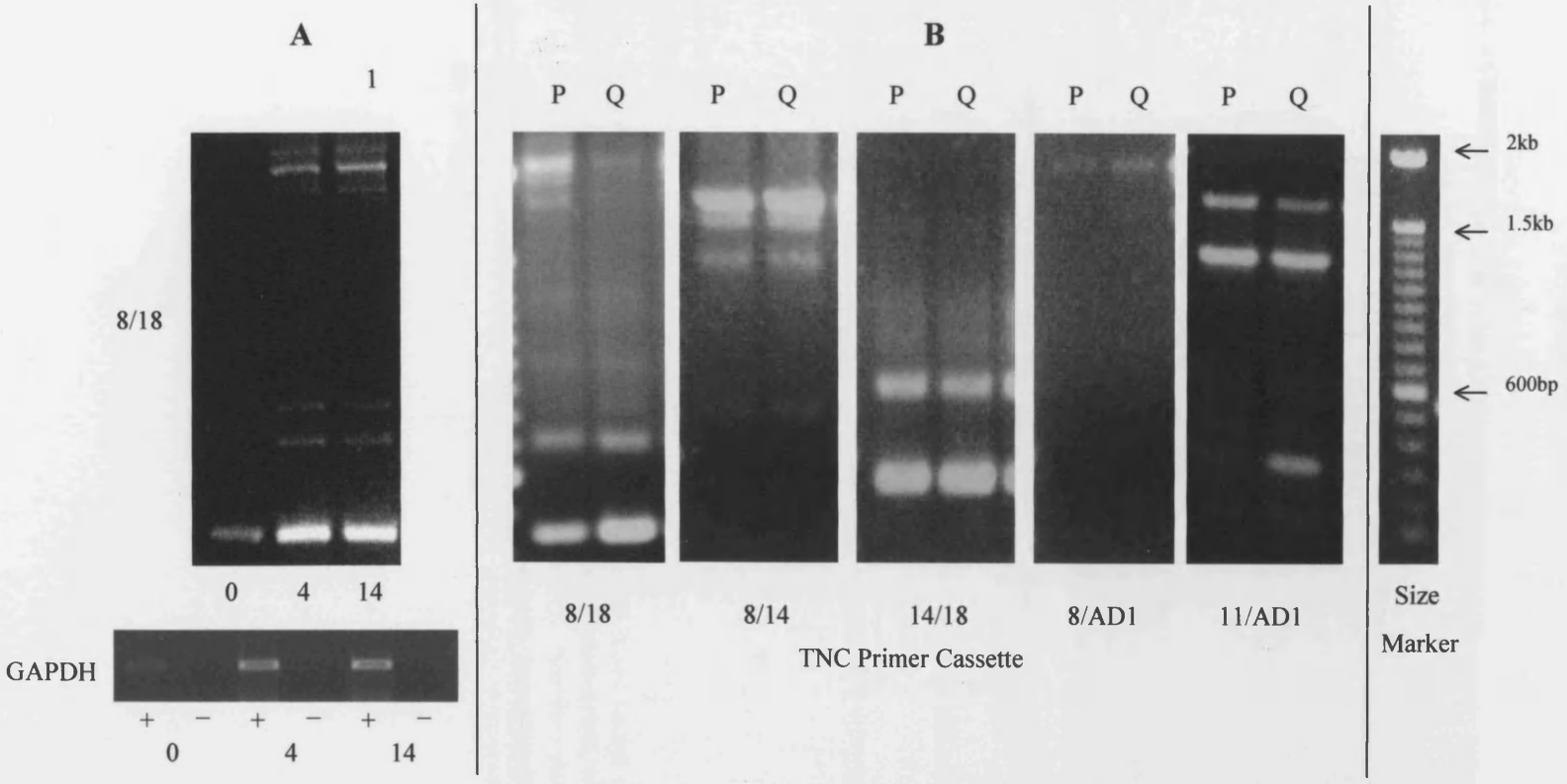
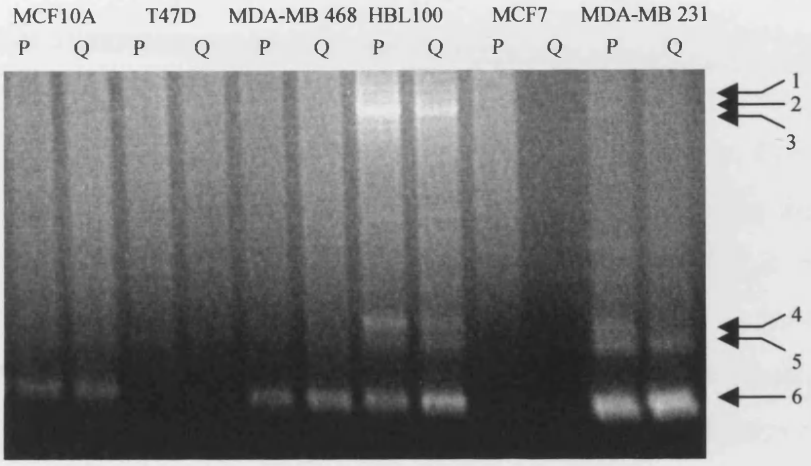


Figure 5.2 : Expression of tenascin C by primary cultured fibroblasts: A. Showing GAPDH PCR results (on Positive (+) and negative (-) reverse transcription product) and 8/18 PCR results prior to placing in culture (day 0), and 4 and 14 days after growing in culture medium, B. Various other TNC primer cassettes. P = proliferating cells, Q = quiescent cells.



Arrow No.	Product size (bp)	No. exons
1	2353	8
2	2080	7
3	1807	6
4	715	1
5	approx. 600*	na
6	442	0

Figure 5.3 : Expression of tenascin C by established breast cancer cell lines - TNC 8/18 PCR results from breast cell lines listed along top of photograph, which also indicates whether cells were proliferating (P) or quiescent (Q) . Numbers along side of photograph indicate bands of interest, see above table. Indicating PCR product size and predicted number of exons. * does not fit with previously described exon boundaries, na- not applicable.

5.2.4 Tenascin C expression by proliferating and quiescent cells

Alterations in TNC expression have previously been associated with proliferating cells (Aufderheide *et al* 1987, Aufderheide & Eckblom, 1988), thus expression patterns in quiescent and proliferating cells were analysed. The pattern obtained from proliferating and quiescent cells altered in terms of intensity of specific bands. Increased expression of higher molecular weight isoforms was associated with proliferation. In some cases, this was associated with reduced expression of lower MW forms, particularly the truncated isoform. This is observed in MDA-MB 231 cells, HBL-100 cells and primary cultured fibroblasts using the 8/18 cassette and very clearly in fibroblasts using the 11/AD1 cassette (see figures 5.2 & 5.3). A change occurred in isoform composition such that the 1212bp band was weaker in the quiescent cells and a 393bp band in the quiescent cell line was not present in the proliferating cells, thus larger isoform are more abundantly expressed by proliferating cells. Southern hybridisation identified the additional exons present in the 1212bp fragment were exons 12, 13, and 14, as expected for this sized fragment using these primers.

5.2.5 Effect of epidermal growth factor and transforming growth factor β on tenascin C expression by fibroblasts

The effect of recombinant soluble EGF or TGFβ on TNC isoform expression in fibroblasts was examined. A change in banding pattern was observed with primary fibroblasts treated with EGF, but not with TGFβ (figure 5.4). An increased expression of higher molecular weight isoforms is associated with addition of EGF to the growth medium.

5.2.6 Effect of tenascin C on tumour cell morphology and behaviour

A commercially available TNC isoform was used to assess the effects of exogenous TNC on tumour cell morphology and behaviour. When cells were grown on 20μg/ml TNC a noticeable effect on cell morphology was observed such that an increase in lamellopodia and filopodia was seen for cell lines T47D, MCF7, and MDA-MB 231, but not MDA-MB 468 or primary breast fibroblasts (see figure 5.5).

Migration and proliferation assays were performed for each cell line grown on poly-D-lysine (PDL), or 20μg/ml TNC under serum free conditions. Fibroblast conditioned media was used as a chemo-attractant. To assess whether effects of TNC were mediated via alteration of

fibroblast function, migration and proliferation assays were performed on tumour cells grown on PDL, but in the presence of conditioned media from fibroblasts grown on 20 μg/ml TNC. Each assay was performed in triplicate, and invasion and proliferation indices calculated. A mean and standard deviation was established for each index and can be seen in table 5.1 and figures 5.6 & 5.7.

Mean values were analysed for independence on SPSS version 10.1 using an independent samples t-test. TNC had no significant effect on proliferation, however significant direct and indirect effects were seen on migration . For the non-TNC producing cell lines MCF7 and T47D, a significant effect ($P<0.001$) on migration was seen with cells grown on TNC such that migration index was increased by 6 and 17 points respectively. For the TNC producing cell line MDA-MB 468 grown on TNC a significant increase ($P<0.001$) was seen in the migration index of 30 points. However, no effect on migration was observed for the TNC producing cell line MDA-MB 231 grown on TNC. Effects on migration via alteration in fibroblast function were also seen. The conditioned media from fibroblasts grown on TNC significantly ($P<0.001$) increased migration in cell lines MCF7 (8 points), T47D (5 points), and MDA-MB 468 (11 points), but not cell line MDA-MB 231. Furthermore, a significant difference ($P<0.001$) was seen between migration indices of cells grown on TNC and cells grown with TNC primed CM for T47D and MDA-MB 468 cells but not MCF7 or MDA-MB 231 cells.

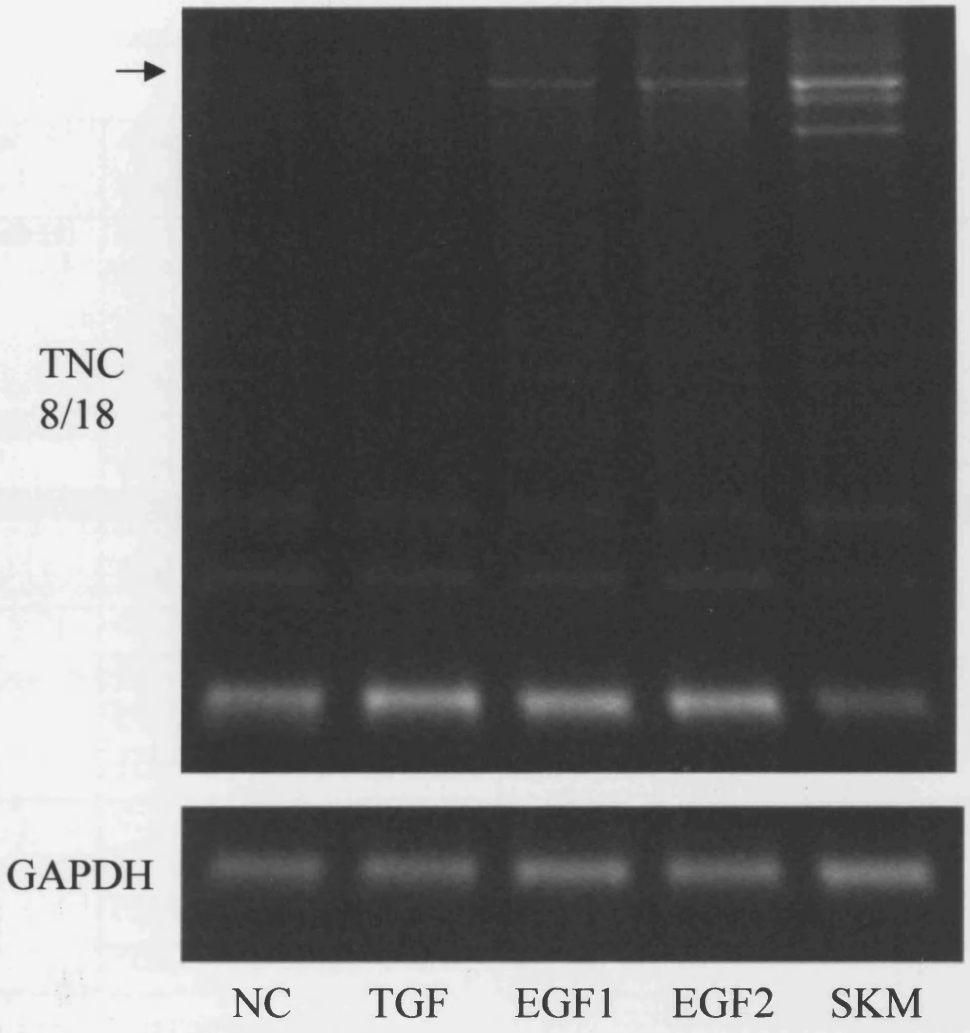


Figure 5.4: Expression of tenascin C by primary breast fibroblasts grown in medium supplemented with TGF beta or EGF - key: NC - negative control of serum free medium, TGF - medium supplemented with 5ng/ml TGF beta, EGF - medium supplemented with 20ng/ml EGF (EGF1) or 100ng/ml EGF (EGF2), SKM - positive control of Sk-mel 28. Upper image shows representative result from 8/18 PCR illustrating neo-expression of higher MW isoforms by EGF but not TGF beta (arrow indicates position of new band on gel). The lower image illustrates GAPDH PCR from the same samples.

Cell Line	Assay Conditions	Migration Index		Proliferation Index	
		Mean	SD	Mean	SD
MDA-MB 231	C	32.7	1.2	37.6	2.3
	T	36.7	2.3	39.1	2.2
	CTCM	34.7	3.0	37.6	1.4
	TCM	36.9	2.8	39.0	2.5
MDA-MB 468	C	11.6	2.7	35.0	3.3
	T	41.4	3.6	36.4	2.7
	CTCM	11.0	1.4	34.2	4.0
	TCM	22.4	3.0	36.1	4.2
T47D	C	3.3	0.5	30.1	3.6
	T	19.4	1.7	26.9	2.2
	CTCM	2.5	0.6	32.4	1.1
	TCM	7.4	0.6	28.6	1.1
MCF7	C	4.4	0.5	26.5	2.3
	T	10.7	1.7	24.3	2.0
	CTCM	3.4	0.4	23.0	2.5
	TCM	12.0	0.8	23.7	2.1

Table 5.1: Effect of TNC on breast tumour cell migration and proliferation - mean and standard deviation of migration and proliferation indices for each breast cancer cell line. C = PDL control, T = TNC, CTCM = PDL control for TNC conditioned medium, TCM = TNC conditioned medium.

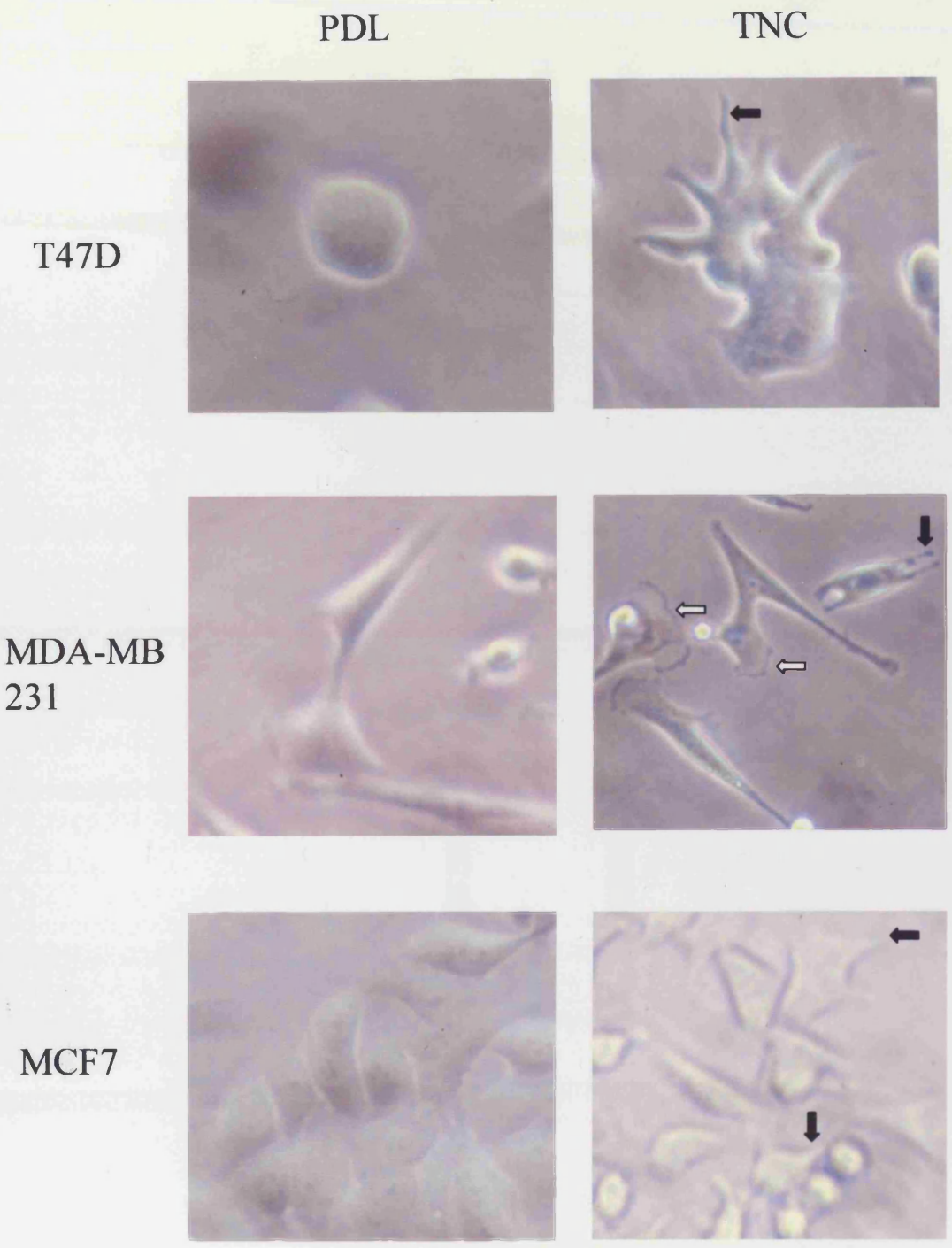
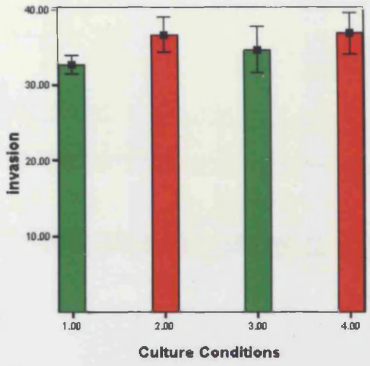
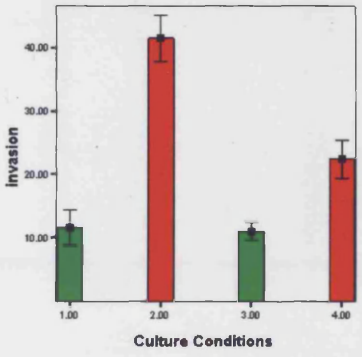


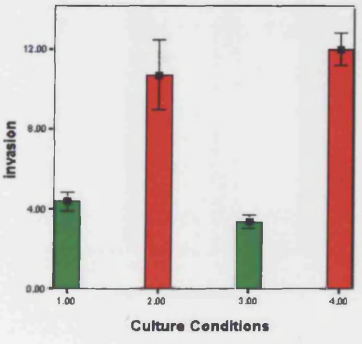
Figure 5.5: Alterations in cell morphology in cells grown on tenascin C substrate - the photographs on the left depict cells grown on PDL or plastic showing morphology of T47D cells (top), MDA-MB 231 cells (middle) and MCF7 cells (bottom). On the right the photographs depict cells grown on TNC substrate. Note the alterations in call morphology with the formation of filopodia (black arrows) and lamellopodia (white arrows).



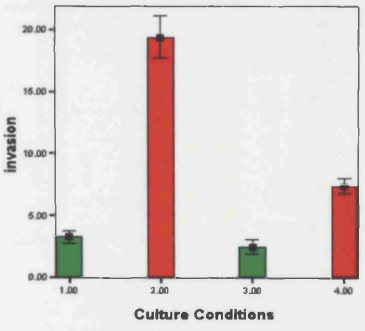
MDA-MB 231



MDA-MB 468

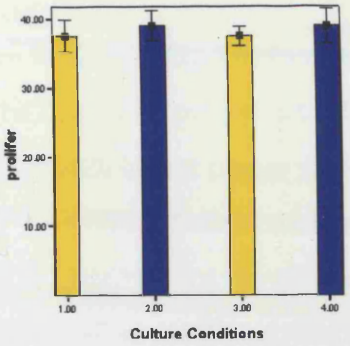


MCF7

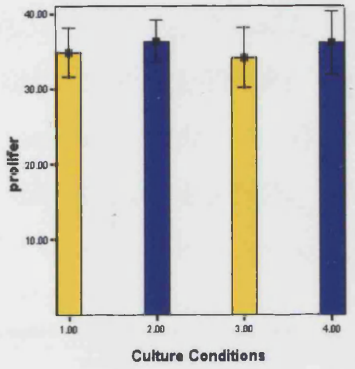


T47D

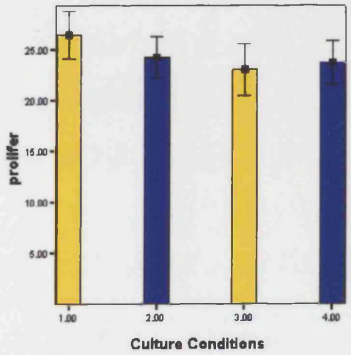
Figure 5.6: Graph depicting invasion/migration indices for breast cancer cell lines grown on TNC substrate - Proliferation index on the x axis, culture conditions on the y axis. Culture conditions: 1- cells grown on PDL with hfff2 cm, 2- cells grown on TNC with hfff2 cm, 3 - cells grown on PDL with primary breast fibroblast cm, 4 cells grown on PDL with TNC primed primary breast fibroblast cm.



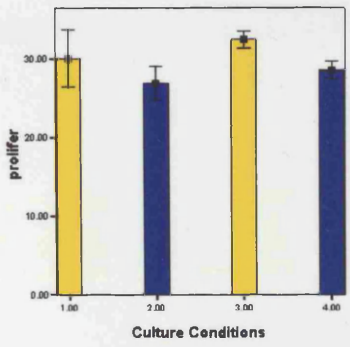
MDA-MB 231



MDA-MB 468



MCF7



T47D

Figure 5.7: Graph depicting proliferation indices for breast cancer cell lines grown on TNC substrate - Proliferation index on the x axis, culture conditions on the y axis. Culture conditions: 1- cells grown on PDL with hfff2 cm, 2- cells grown on TNC with hfff2 cm, 3 - cells grown on PDL with primary breast fibroblast cm, 4 cells grown on PDL with TNC primed primary breast fibroblast cm.

5.2.7 Effect of tenascin C on matrix metalloproteinase expression

Given the observed effect of TNC on cell motility, the effect of TNC on expression of a range of proteolytic enzymes associated with breast cancer invasion was investigated. The products of each MMP PCR were all of the predicted sizes (Giambernardi *et al* 1998, Yamashita *et al* 1998) using positive controls of HT1080 cDNA for all reactions except MMPs 7 and 11 which used MDA-MB 468 cDNA. The baseline profile of MMP expression for each cell line, under serum free conditions, is as follows: MCF7 and T47D cells express only MMP13 (weak expression by cell line T47D), TIMP1 and TIMP2. MDA-MB 231 cells express MMP1, MMP7 (weak expression), MMP11, MMP13, TIMP1, TIMP2 and MT1-MMP. MDA-MB 468 cells express MMP2 (weak expression), MMP7, MMP9 (weak expression), MMP13, TIMP1, TIMP2, and MT1-MMP. Fibroblasts express MMP1, MMP2, MMP13, TIMP1, TIMP2, and MT1-MMP. Alongside this cells were grown under serum supplemented conditions. This had no effect on cell lines MCF7, T47D, and fibroblasts, however, serum supplemented MDA-MB 231 cells additionally showed weak expression of MMP2 and MMP9, and serum supplemented MDA-MB 468 cells exhibited a slightly increased level of MMP2 and MMP13 expression. All these results are summarised in table 5.2. No change in MMP expression was observed from cells grown on any concentration of TNC under both serum free and serum supplemented conditions (see figures 5.8 – 5.12).

PCR Reaction	MCF7		T47D		MDA-MB231		MDA-MB468		Fibroblasts	
	ss	sf	ss	sf	ss	sf	ss	sf	ss	sf
MMP 1	x	x	x	x	✓	✓	x	x	✓	✓
MMP 2	x	x	x	x	**0.5**	x	**1**	**0.5**	✓	✓
MMP 7	x	x	x*	x*	**0.5**	**0.5**	✓	✓	x	x
MMP 9	x	x	x	x	**0.5**	x	**1**	**1**	x	x
MMP 11	x	x	x	x	✓*	✓*	x	x	x	x
MMP 13	✓*	✓*	**0.5***	**0.5***	✓*	✓*	**1***	**0.5***	✓	✓
TIMP 1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
TIMP 2	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
MT1-MMP	x	x	x	x	✓	✓	✓	✓	✓	✓

Table 5.2: Baseline MMP PCR profile of breast cancer cell lines and primary breast fibroblasts - grown on 20 μg/ml TNC under serum supplemented (ss) and serum free (sf) conditions. ✓ indicates a strong signal, a number indicates intensity of signal graded from 0 to 4, x indicates no signal, * indicates signal disagrees with previous studies (Giambernardi *et al* 1988, Yamashita *et al* 1998).The results for primary fibroblasts were obtained using a cycle sampling PCR protocol. With a negative signal (MMPs 7, 9, & 11), a maximum of 35 cycles was used. The signals obtained for MMP1, MMP2, TIMP1 and TIMP2 was from a 25 cycle PCR, the signal obtained for MT1-MMP was from a 30 cycle PCR, and the signal obtained for MMP13 was from a 35 cycle PCR.

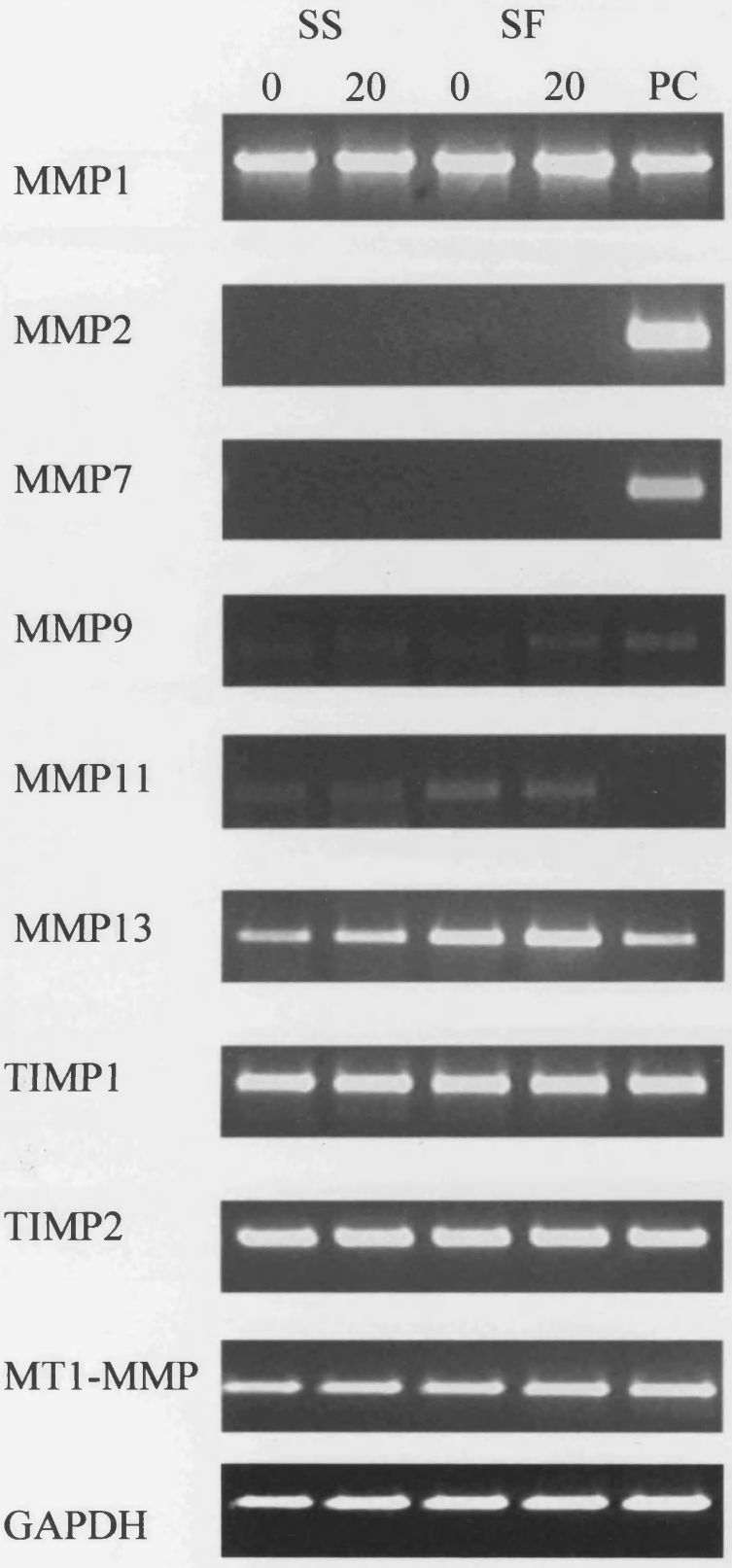


Figure 5.8: Matrix metalloproteinase expression profile for cell line MDA-MB 231 grown on tenascin C - Expression of various MMPs and TIMPs by breast cancer cell line MDA-MB 231 grown on plastic as a control and TNC as a substrate (20μg/ml coating concentration) for 48 hours under serum supplemented (SS) and serum free (SF) conditions. PC = positive control.

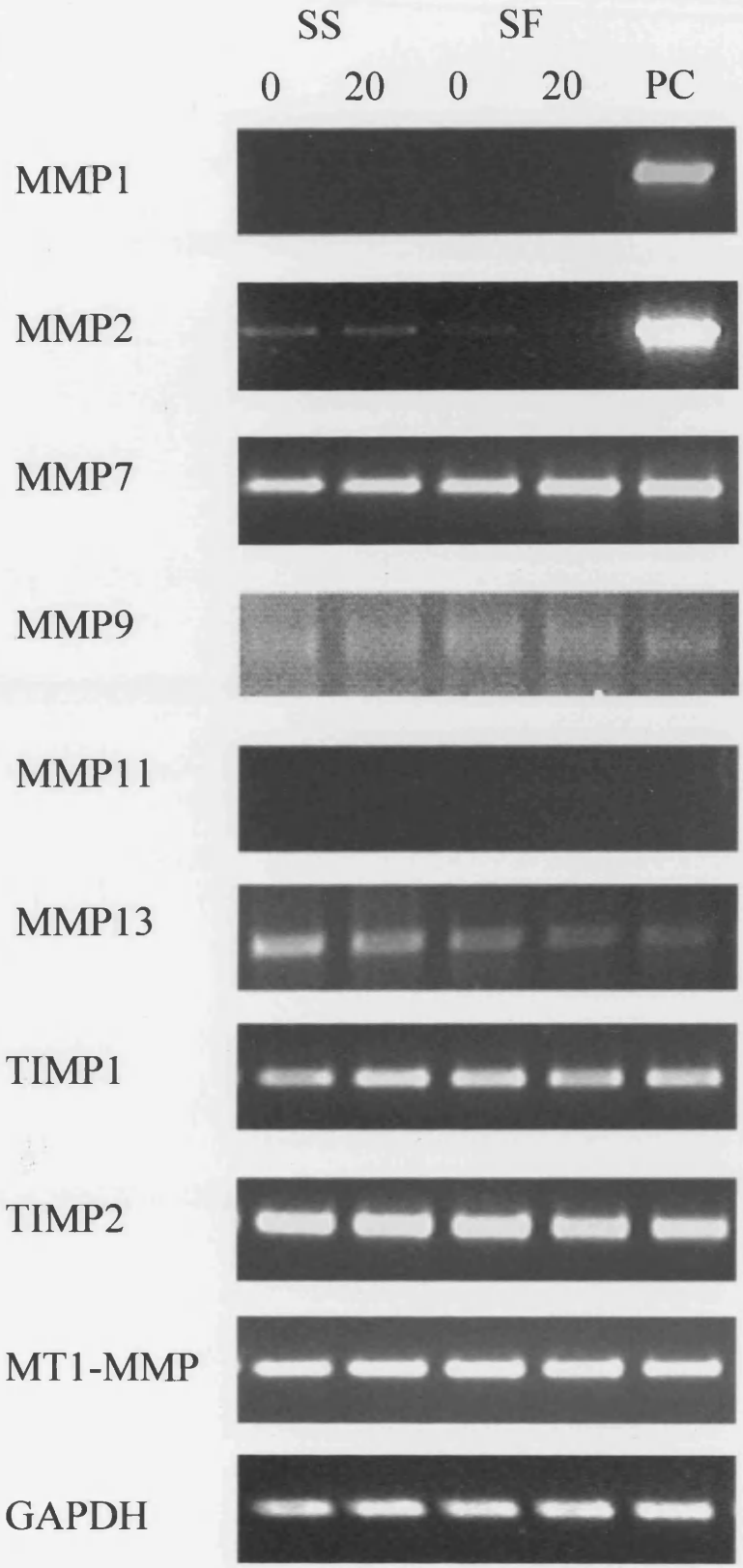


Figure 5.9: Matrix metalloproteinase expression profile for cell line MDA-MB 468 grown on tenascin C - Expression of various MMPs and TIMPs by breast cancer cell line MDA-MB 468 grown on plastic as a control and TNC as a substrate (20μg/ml coating concentration) for 48 hours under serum supplemented (SS) and serum free (SF) conditions. PC = positive control.

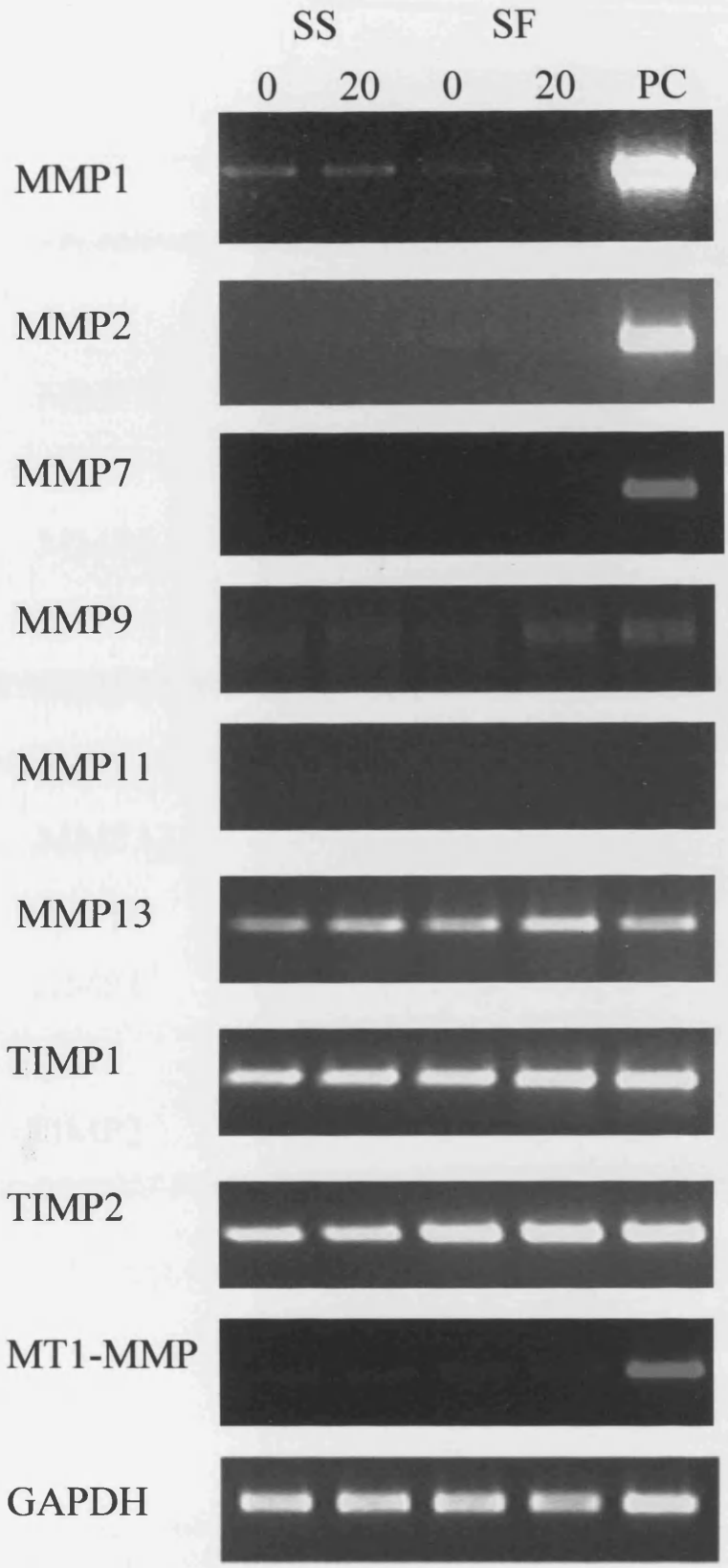


Figure 5.10: Matrix metalloproteinase expression profile for cell line MCF7 grown on tenascin C - Expression of various MMPs and TIMPs by breast cancer cell line MCF7 grown on plastic as a control and TNC as a substrate (20μg/ml coating concentration) for 48 hours under serum supplemented (SS) and serum free (SF) conditions. PC = positive control.

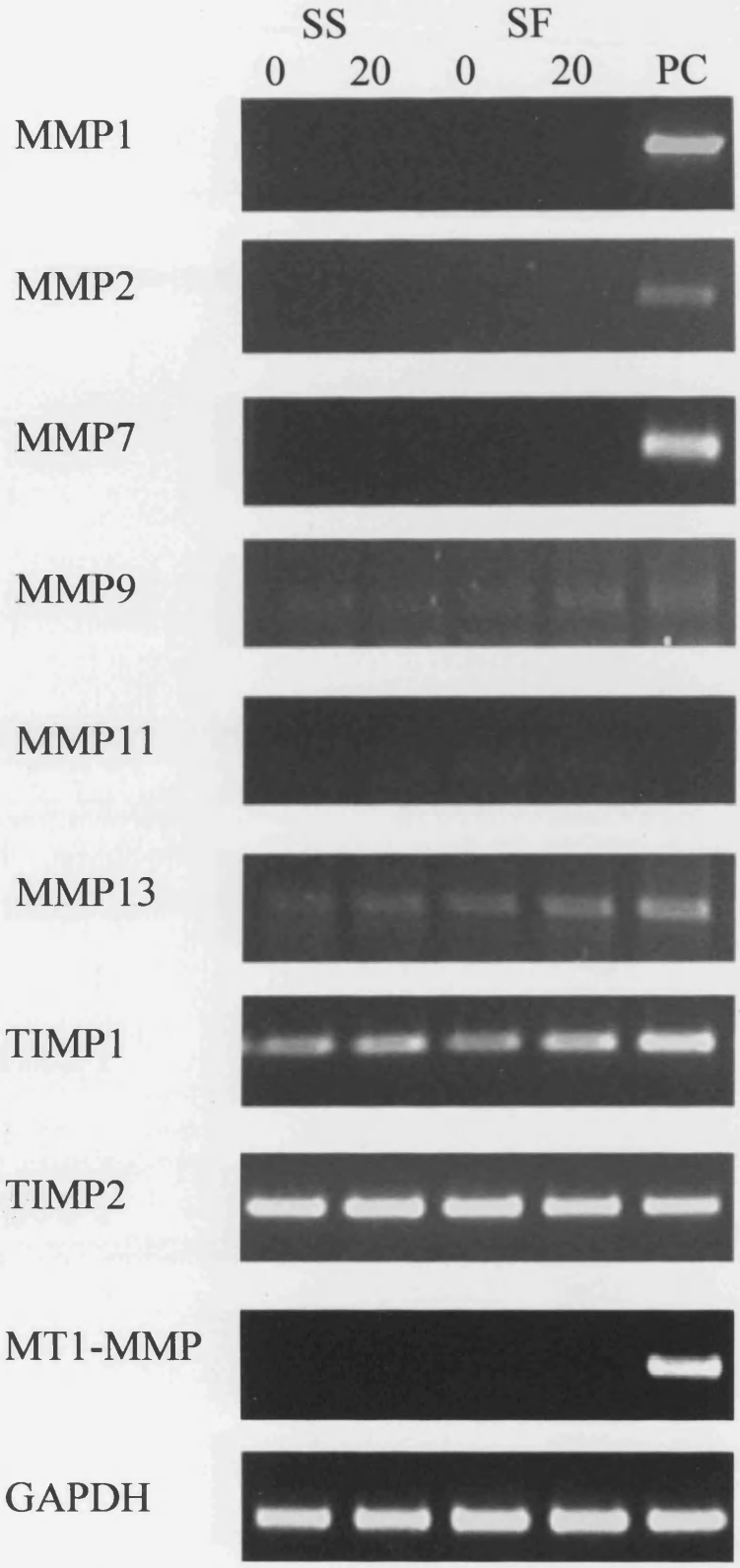


Figure 5.11: Matrix metalloproteinase expression profile for cell line T47D grown on tenascin C - Expression of various MMPs and TIMPs by breast cancer cell line T47D grown on plastic as a control and TNC as a substrate (20μg/ml coating concentration) for 48 hours under serum supplemented (SS) and serum free (SF) conditions. PC = positive control.

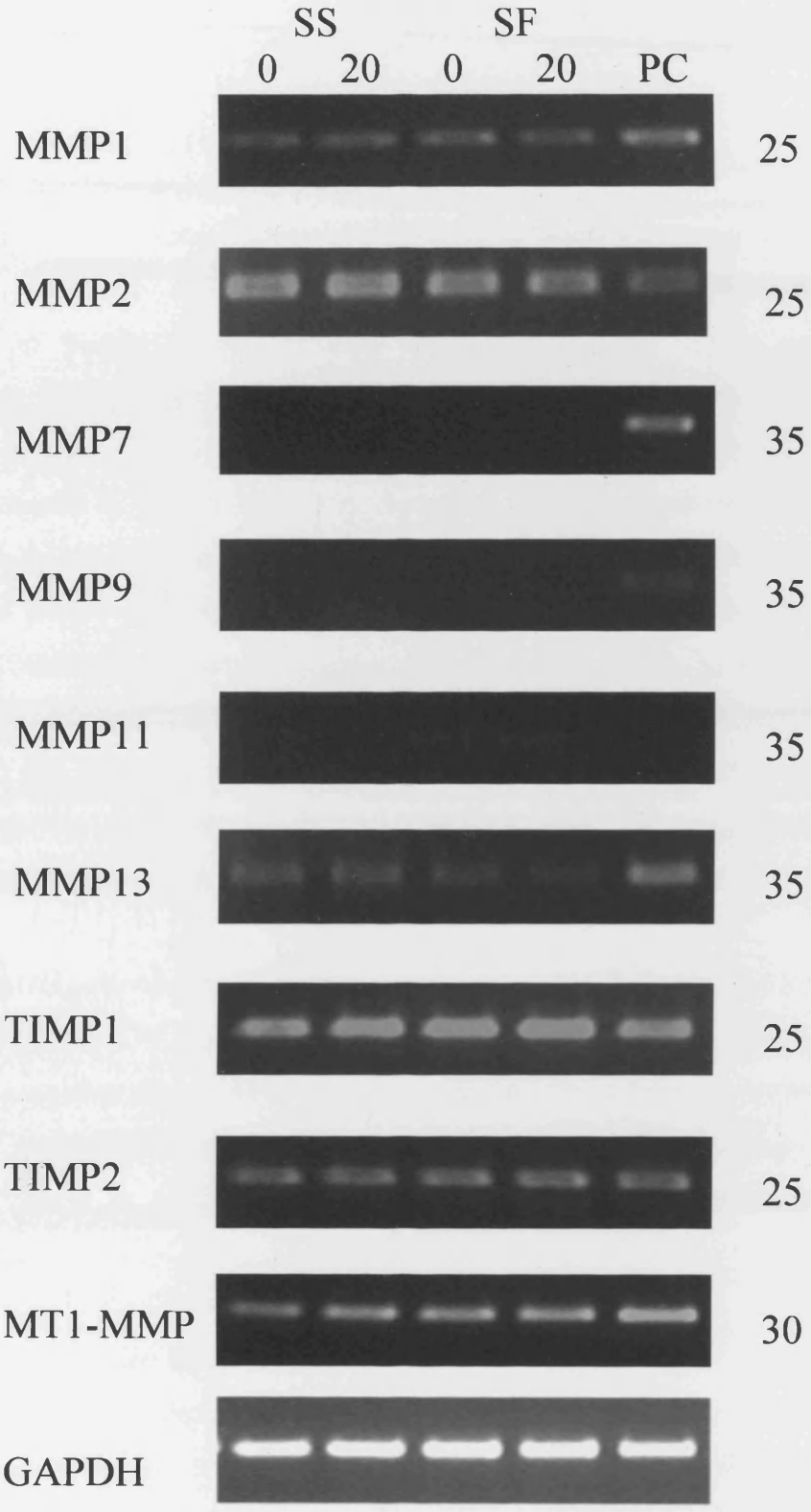


Figure 5.12: Matrix metalloproteinase expression profile for fibroblasts grown on tenascin C - Expression of various MMPs and TIMPs by primary breast fibroblasts grown on plastic as a control and TNC as a substrate (20μg/ml coating concentration) for 48 hours under serum supplemented (SS) and serum free (SF) conditions. The numbers on the right refer to the number of cycles at which the PCR reaction was informative. PC = positive control.

5.3 Discussion of Results from Cell Culture

5.3.1 *In-vitro* expression of tenascin C

An important issue to consider when working in cell culture systems is the effect of the culture system itself on the parameter of interest. To evaluate this, TNC isoform expression was analysed in freshly isolated primary fibroblasts prior to culture compared to cultured fibroblasts. Results from pre-cultured (day 0) primary fibroblasts differ significantly from cultured primary fibroblasts. The 8/18 cassette indicates the truncated isoform is the major type expressed in day 0 fibroblasts, however, cultured fibroblasts (from day 4 onwards) produce a high molecular weight triplet of bands around 2kbp. A further consideration is that, using the 8/AD1 PCR cassette, primary tissues do not appear to contain the AD1 exon, however, cultured primary fibroblasts give a strong signal. In the current study, day 0 fibroblasts have not been investigated using this cassette, although studies of Sk-mel 28 have identified the larger of the 2kbp triplet of bands (2353bp), also seen in fibroblasts, as containing AD1. This indicates that larger variants, particularly those containing AD1, may be induced by the cell culture process.

Anchorage of cells on plastic introduces a degree of tensile stress on the cells which has been shown to induce TNC expression (Traschlin *et al* 1999). Furthermore, cell culture medium contains a number of undefined factors, specifically FCS which is known to contain TGFβ1 and EGF, both of which are known to induce TNC in carcinoma derived cell lines (Sakai *et al* 1995a, Pearson *et al* 1989) and in primary breast fibroblasts investigated within this laboratory.

Normal primary fibroblasts are not thought to be a source of TNC (Lightner *et al* 1994, Yoshida *et al* 1997). However, *in-vitro* culturing of cells introduces a number of factors which may affect phenotype. During the separation process, the fibroblasts are extracted into 1% FCS, which may be responsible for the induction of TNC (Pearson *et al* 1989). However, when grown under serum free conditions, these cells still express TNC, but a change in isoform profile is seen. Expression of higher MW isoforms is reduced, a similar pattern to that seen in quiescent cells. Other factors are also implicated in TNC induction. Mechanical stress has long been associated with upregulation of ECM proteins (Leung *et al* 1976) and more

recent studies have shown upregulation of TNC in fibroblasts under stressed conditions in collagen gels (Chiquet-Ehrissmann *et al* 1994, Chiquet *et al* 1996). Trachslin *et al* (1999) speculates that when cells, particularly fibroblasts, are grown on plastic, the attachment of cells to plastic confers a degree of tensile stress and initiates the upregulation of TNC and other ECM proteins. The expression of TNC by normal serum starved fibroblasts may be caused by this tensile stress. Early TNC reports associated its expression with tissues bearing high tensile stress such as tendons, hence its initial name of myotendinous antigen (Chiquet & Fambrough 1984).However, other work in this laboratory has also shown upregulation of other tumour-associated factors by these apparently normal fibroblasts (Philips *et al* unpublished). It is possible that normal cells under culture conditions acquire characteristics otherwise associated with cells from tumour stroma.

Changes in isoform profile were also detected in relation to the proliferative state of the fibroblasts. PCR with both 8/18 and 11/AD1 cassettes demonstrated that higher MW isoforms were expressed in proliferating fibroblasts with predominance of smaller MW isoforms in quiescent cells. These findings were confirmed with 8/18 cassette results from the MDA-MB 231 cell line which indicate a plus 1 exon isoform is linked with proliferating cells and is not present in quiescent cells (see figure 5.3).

The current study has demonstrated that proliferating tumour cells express TNC which confirms previous work (Chiquet-Ehrismann 1986). However, growth on TNC does not appear to induce proliferation. This would suggest that expression of TNC is a consequence of proliferation rather than a cause. High MW isoforms are seen, thus the variable FN III like repeat region may be of particular importance. This region has previously been linked with a mitogenic effect by End *et al* (1992) who proposed tenascin C as a modulator of cell growth and concluded that expression of the large molecular weight variants stimulates proliferation. Thus, specific isoform composition may also be crucial which may account for the lack of proliferative response by cells grown on the form of TNC used in the current study.

5.3.2 Modulation of tenascin C expression by growth factors

TNC is induced in breast carcinoma (Mackie et al 1987) with initiation of induction occurring at a pre-invasive stage as indicated in the current study, and by others (Jahkola et al 1998a, Adams *et al* 2002). The transcriptional factors regulating this expression of TNC are unknown

(Shirasaki et al 1999), however, a number of possibilities exist. Both biochemical and physical signals associated with growth, migration and progression of carcinoma have been shown to have an effect on TNC expression, both alone and in conjunction (Jones & Jones 2000). The regulatory mechanisms controlling TNC expression can be elucidated when considering the structure of the TNC promoter.

The mouse TNC promoter has been shown to contain a number of regulatory elements which contribute to different aspects of gene regulation in different cell lines, furthermore, these elements are evolutionarily conserved (Gherzi *et al* 1995, Copertino *et al* 1997). Characterisation of these regulatory sites in primary human fibroblasts has revealed they bind specific transcription factors that mediate the transcription of TNC (Shirasaki *et al* 1999). These transcription factors have been linked with cellular migration, tumour formation and metastases and importantly for this study, they have been associated with high levels of growth factors (Gilles *et al* 1996).

A range of growth factors and cytokines have been associated with up-regulation of TNC. These are transforming growth factor β1 (TGFβ), nerve growth factor (NGF), epidermal growth factor (EGF), interleukins 1 & 4, TNFα (tumour necrosis factor α) and fibroblast growth factor (FGF) (Pearson *et al* 1988, Yavin *et al* 1991, Sharifi *et al* 1992, Meiners *et al* 1993, Sakai *et al* 1995a, Rettig *et al* 1994, Suzuki *et al* 2002). TGFβ and EGF were chosen for study because of their well-established link with progression of breast cancer. TGFβ stimulates secretion of ECM (Ignotz & Massague 1986, Kahari *et al* 1991, Zhao 1999), and has been shown to affect TNC expression in fibroblasts (Pearson *et al* 1988, Chiquet-Ehrissmann *et al* 1989), one of our proposed sources of TNC. TGFβ itself is upregulated in relation to breast carcinoma at a pre-invasive stage (Walker & Dearing 1992) and co-expression of TGFβ and TNC has also been seen during the menstrual cycle (Knabbe *et al* 1987, Ferguson *et al* 1990). It has also been shown to influence TNC expression in carcinoma cell lines, however, the same study indicated that EGF was a more potent factor (Sakai *et al* 1995a). This effect of EGF however, appears to be cell type specific (Wirl *et al* 1995).

TGFβ does not appear to affect expression of TNC in primary breast fibroblasts which under control conditions (serum free medium) express predominantly the truncated form of TNC. This contrasts with recently reported results (Zhao 1999) in which TGFβ markedly increased

amounts of both high and low MW forms of TNC protein in primary lung fibroblasts. Surprisingly, increased amounts of TNC protein in the matrix of the same breast fibroblasts under the influence of TGFβ has been demonstrated in this laboratory (Philips *et al* unpublished observations). This anomaly suggests that the RT-PCR cycle sampling method utilised in the current study may not be sensitive enough to detect relatively small changes in the amount of TNC mRNA message, but it may be sufficient to detect the reported more potent effect of EGF (Sakai *et al* 1995a). This pattern of a change in amount of protein without a concurrent change in mRNA has been reported previously for certain cell lines (Lightner *et al* 1994) and was thought to be caused by down regulation of TNC at the time of RNA harvesting. The current study has shown that TNC mRNA is affected by how long cells have been in culture and whether or not they are proliferating or quiescent. However the observations concerning TGFβ not inducing TNC mRNA expression were consistent and reproducible. It is possible that TGFβ does not increase expression of TNC, but may stabilise the TNC protein. This would confirm previous reports that the effect of TGFβ was via modulation of the ECM (Ignotz & Massague 1986, Kahari *et al* 1991).

However, further studies into the effect of TGFβ on TNC expression has been performed in the laboratory by a colleague. These reveal inconsistent results with some experiments demonstrating upregulation of TNC – including upregulation of plus 1 and plus 2 exon isoforms from 8/18 PCR – but other experiments showing no effect. This is part of an ongoing project, however the possible reason for such inconsistencies being considered is patient specific differences in sensitivity to growth factors. If true, this has potentially important implications for the role of fibroblasts, and differences in individual fibroblasts, in the generation and progression of breast cancer.

This study has demonstrated that EGF enhances expression of higher MW TNC isoforms. EGF had previously been shown to increase TNC in a range of carcinoma cell lines (Sakai *et al* 1995a), but no specific change in isoform profile, nor a change in expression, have been reported in primary breast fibroblasts. Thus EGF may be one of the factors resulting in expression of higher MW TNC isoforms in neoplasia. Higher molecular weight (MW) TNC mRNA transcripts have been associated with neoplastic human breast tissues in the current study, and by others (Borsi *et al* 1992, Adams *et al* 2002). An increase in message under the

influence of EGF coincides with increased expression of higher MW protein isoforms (Borsi *et al* 1992) and by Philips *et al* (unpublished observations) using the same batch of breast fibroblasts as the current study. Increased expression of higher MW isoforms has also been associated with lung carcinoma (Kusagawa *et al* 1998), ovarian carcinoma (Wilson *et al* 1996), and oral squamous cell carcinoma (Hindermann *et al* 1999). Interestingly, what is identified here is a change in expression rather that neo-expression of TNC isoforms. Faint signals can be seen on the gel which indicates the presence of higher MW isoforms in unstimulated cells. This contributes to the conclusion that these higher MW isoforms are always present (Borsi *et al* 1992) but that the relative proportions of isoforms is altered in neoplasia (discussed in chapter 4).

The current study and work in this laboratory (Philips *et al*, unpublished) has provided evidence that these two growth factors do not have the same effect on TNC induction. The growth factor specific response on breast fibroblasts may be a consequence of specific receptor profile and both these growth factors have been shown to have alternative effects, linked with tumourigenesis, dependent upon receptor profile (Bacus *et al* 1993, Zhao 1999). A lack of effect of TGFβ on TNC expression suggests an alternative role and TGFβ has been shown to be a potent tumour suppressor in the early stages of carcinoma (Roberts *et al* 1985). Two types of receptors are required for TGFβ mediated production of ECM proteins (Zhao 1999) and loss of one of these, TGFβ type II receptor, contributes to the loss of the tumour suppressor activity of this growth factor (Ko *et al* 1998). This alteration in receptor profile leads to carcinoma cell proliferation and tumour progression and may lead to a change in the ECM, possibly including alteration in TNC expression. There is a large family of receptors for EGF, the HER family of receptors (Klapper *et al* 2000). High levels of various members of this family have been described in primary human breast cancers (Bacus *et al* 1993). Increased signalling via these receptors has been seen to have a range of effects which are associated with tumour progression (Stampfer 1985). One of these effects is increased expression of ECM proteins including TNC (Sakai *et al* 1995a).

Thus, there are two specific growth factor mediated responses involving TNC seen in primary breast fibroblasts. However, the responses are not consistent with changes reported in primary breast tissue. This reflects an inherent problem with *in-vitro* studies misrepresenting the situation in primary tissues and although induction of higher MW forms is seen, these are not

the 14-16 isoform that has been associated with breast carcinoma in the current study. The isoforms induced by EGF are the very largest sizes. Speculation can be made as to what these isoforms are due to the consistent pattern of splicing discussed in the current study (Chapter 4) and by Bell *et al* (1999). The size of the two bands indicates the presence of messages containing 7 and 8 exons in the variably spliced region. When compared with exon combinations observed in Sk-mel 28, it is possible that the largest induced band contains AD1 whereas the band below this is lacking in AD1. The distinctive triplet of bands seen in Sk-mel-28 that is caused by the variable splicing of exons 12 and 15 is not seen in the EGF induced fibroblasts which indicates a more specific change in expression profile. Hence, a distinct qualitative change, not an increase in all forms of TNC is observed.

5.3.3 Expression of tenascin C in breast cancer cell Lines

The current study has examined six established breast cell lines – MCF7, T47D, MDA-MB 231, MDA-MB 468, MCF10A and HBL100 which have been extensively characterised in our laboratory (Gordon *et al*, manuscript submitted). The current study has shown that only MCF7 and T47D cell do not express TNC, with both MDA-MB 231, MDA-MB 468 and the non-tumourigenic cell lines yielding a product with TNC PCR. Furthermore, isolated myoepithelial cells strongly express TNC (Gordon *et al* manuscript submitted).

These expression patterns closely resembles those seen in previous studies (Natali *et al* 1991, Carnemolla *et al* 1992, Kawakatsu *et al* 1992, Lightner *et al* 1994). Low levels of TNC expression by MDA-MB 231 and MDA-MB 468 are seen (Kawakatsu *et al* 1992) which corresponds to low levels of the protein (Lightner *et al* 1994), whereas, the HBL100 cells express more than one form of TNC (Kawakatsu *et al* 1992). These studies also confirm the lack of TNC expression by both the MCF7 and T47D cell lines. However, expression of isoform profile using RT-PCR in the current study does not correspond to the isoform profile indicated by immuno-precipitation studies of conditioned medium (Kawakatsu *et al* 1992). Specifically, MDA-MB 231 and MDA-MB 468 cells were shown to produce a 250kDa form of the protein, but not a 190kDa isoform. As previously discussed (Chapter 4) these forms may not correspond to the very largest, or very smallest protein (Bell *et al* 1999). However, this does not totally explain the discrepancy. A flaw when considering soluble TNC expression is that larger isoforms are released into the medium and smaller isoforms are retained in the ECM. With certain cell lines, in excess of 75% of total TNC is retained in the

matrix, thus an analysis of conditioned medium alone only considers soluble TNC (Carnemolla *et al* 1992). Furthermore, mRNA is examined at a single point, near confluence, whilst examination of protein corresponds to protein synthesised the entire time in culture (Lightner *et al* 1994). This is demonstrated in that mRNA from quiescent cells indicates a different isoform profile than proliferating cells.

The established breast cell lines can be classified as myo-epithelial-like, or luminal-like, depending on their phenotype. The non-TNC expressing MCF7 and T47D breast carcinoma cells are considered to have a luminal epithelial cell like profile. They are oestrogen receptor (ER) positive and grow as tightly cohesive epitheloid cell colonies. They express E-cadherin and Epithelial Membrane Antigen (EMA), but lack vimentin and β4 integrin. The TNC expressing MDA-MB 231 carcinoma cells are considered to have a more myo-epithelial cell like profile. They are ER negative, lack E-cadherin and EMA, but strongly express β4 integrin. They also have a different growth pattern and grow as single elongated cells with a fibroblastoid morphology. MDA-MB 468 carcinoma cells have a more intermediate phenotype, as do the non-tumourigenic cell lines MCF10A and HBL100. A myoepithelial phenotype is frequently associated with a more aggressive tumour (Cattoretti *et al* 1988, Wetzels *et al* 1989, Domagala *et al* 1990a, Domagala *et al* 1990b, Peralta Soler *et al* 1999) and these cell lines can be considered to be characteristic of different degrees of disease. Thus TNC expression is associated with a more aggressive phenotype indicative of a poor prognosis which confirms previous findings (Ishihara *et al* 1995). Furthermore, TNC could be considered another marker associated with the myoepithelial phenotype (Gordon *et al* manuscript submitted).

Alternatively TNC expression may be indicative of an undifferentiated phenotype as seen in mesenchymal cells. Mesenchymal cells specifically express TNC during normal embryonic development and during tumour progression (Chiquet & Fambrough, 1984, Aufderheide *et al* 1987). A loss of cell differentiation occurs with tissue acquiring more mesenchymal, or stromal, characteristics. This is known as the epithelial-mesenchymal transition (EMT) (Birchmeier & Birchmeier, 1993) . A change in morphology leads to a loss of intercellular adhesion and increased cell motility. It has been linked with more aggressive features in terms of invasion, poor prognosis and has recently been linked with upregulation of TNC (Dandachi

et al 2001). It is thought that the transition occurs due to the loss of the functional integrity of adherens junctions. Studies has shown that inhibition of E-cadherin, an integral component of adherens junctions, disrupts the normal E-cadherin/catenin interactions, thus the loss of the junctions necessary for continuing epithelial morphology (Kintner, 1992). Loss of E-cadherin expression is associated with the progression of tumours towards invasion (Jones *et al* 1996).

5.3.4 Effect of tenascin C on breast cancer cell behaviour

TNC was used as a substrate on which cells were grown, rather than using soluble TNC in the cell culture medium. This choice was made as TNC in primary tissue forms part of the ECM, thus its use as a cell substrate was considered the more physiologically relevant option.

Initial observations were that the TNC used did affect cell morphology with an increase in lammellopodia and filopodia. These features are indicative of an increase in cell motility (Sheetz *et al* 1998) and allow speculation of an effect of TNC on cell migration. The TNC was obtained from Chemicon, USA and was an intermediate sized isoform. Ideally the 14-16 isoform identified in primary breast tissue would be preferred, but this was not available. In house production was considered, however, given an appropriate source, any extraction protocol may not yield enough functional isoform for any useful investigation and as a result the commercial form was used. This was problematic in itself, as the exact sequence of FNIII-like repeats was not known for any commercial TNCs, however, size was. The TNC chosen was provided as a predominantly 250kDa monomer. Given the reported size range of the TNC monomer (Erickson & Bourdon 1989), this size indicated an intermediate sized isoform which the current study and others had associated with tumour progression (Wilson *et al* 1996, Adams *et al* 2002).

5.3.5 Effect of tenascin C on cell migration and proliferation

This study has identified a correlation between breast cancer cell invasive ability and presence of TNC in the matrix. However, it is not established whether TNC actually promotes tumour invasion.

As part of the characterisation of cell lines, baseline invasion assays have been performed on all cells (Gordon *et al* manuscript submitted). This data shows MCF7 and T47D have a low

invasive capacity whereas MDA-MB 231 and MDA-MB 468 cells have a higher invasive capacity. This pattern of invasive behaviour has been confirmed in the current study albeit using a modification of the previously employed invasive assay. For each of the breast cancer cell lines grown on the TNC substrate, a consistent change in cell morphology was observed which was indicative of an increase in cell migratory activity. Thus, the assay conditions used in the current study were altered from those described by Gordon *et al* (manuscript submitted) in order to replicate this change in morphology.

To address the effect of TNC, the invasive capacity of the breast cell lines was analysed with the cells grown on TNC and also in the presence of conditioned medium taken from fibroblasts cultured on TNC. The effect on proliferation was also assessed.

These experiments demonstrated different effects on migration depending upon cell line, however no effect on proliferation was seen. Enhanced migration of MCF7, T47D and MDA-MB 468 cells was demonstrated by both direct and indirect interaction with TNC, but not for MDA-MB 231 cells. Exogenous TNC has previously been shown have an effect on oligodendrocyte motility (Kiernan *et al* 1996), glioma cell migration (Deryugina & Bourdon 1996, Herold-Mende *et al* 2002), endothelial cell migration (Chung *et al* 1996), and smooth muscle cell motility (Wallner *et al* 2002) and the current study confirms a direct effect. The lack of effect of this particular TNC isoform on the MDA-MB 231 cell line may indicate these cells normally express this isoform, thus addition of this isoform may be expected to have little or no effect. The current study has shown that proliferating MDA-MB 231 cells express intermediate sized isoforms and the form used as a substrate is of intermediate size. It has also been demonstrated that both T47D and MCF7 cells do not express TNC, however, as MDA-MB 468 do it may suggest that this cell line does not produce this isoform, and provides evidence that different isoforms have different effects, as has been discussed in the current study and by others (Chiquet-Ehrissmann *et al* 1990, Chiquet-Ehrissman *et al* 1991, Murphy-Ullrich *et al* 1991, Chung & Erickson 1994, Siri *et al* 1995, Philips *et al* 1998, Puente-Navazo *et al* 2001, Herold-Mende *et al* 2002, Adams *et al* 2002).

As well as enhancement of migration via direct cell-matrix interactions with TNC, migration was also increased by indirect mechanisms. Furthermore, as there is no significant difference between controls from the direct and indirect investigations of TNC on migration and there *is*

a significant difference between direct and indirect effect, two independent mechanisms of TNC action on migration are indicated. One via direct action on tumour cells and the other indirectly via stromal fibroblasts. This is the case for T47D and MDA-MB 468 cells, but not MCF7 . The presence of alternative modes of action suggests that TNC has alternative effects for different types of breast tumour, however as a different situation exists for each of the non TNC producing cell lines, it is possible this may not be TNC isoform specific. It is likely cell receptor profile is a factor, and as previously discussed in chapter 1, there are many possible TNC receptors (Barnea *et al* 1994, Chung & Erickson 1994). In this context however integrins may be of vital importance. A range of integrins bind to TNC which potentially elicit a range of responses depending on which specific integrin interacts with TNC (Yokasaki *et al* 1996; 1998). Furthermore, Huhtala *et al* (1995) speculates about a co-operative interaction of integrins such that signalling via one integrin may inhibit signalling via another integrin. This may be the case with MCF7 cells.

The lack of a proliferative response to TNC conflicts with earlier reports (Chiquet-Ehrissmann 1986, End *et al* 1992, Tan *et al* 1999, Huang *et al* 2001, Herold-Mende *et al* 2002), however these were not investigating breast carcinoma cell lines. This is a further indication that response to TNC may be receptor profile specific. Yokosaki *et al* (1996) demonstrates cellular response to binding of TNC depends on specific TNC-receptor interactions; β3 elicits a different proliferative response to β1 or β6. It is possible the cell lines investigated in the current study do not express those integrins associated with a high proliferative response. A possible alternative association of TNC with proliferating cells has been discussed previously.

To summarise, TNC increases invasion in some breast tumour cell lines. Therefore, to investigate potential mechanisms involved, the current study proceeded to analyse expression of members of the MMP family known to be important in tumour invasion.

5.3.6 Effect of tenascin C on expression of matrix metalloproteinases

Cell-matrix interactions are a well recognised mechanism for the induction of MMPs. For example, cells grown on fibronectin demonstrate upregulation of MMPs 1, 3, 9 and MT1-MMP (Werb *et al* 1989, Stanton *et al* 1998). Furthermore, growth on a collagen matrix (the major component of the ECM) induces expression of MMPs 1 and 9 (Larjara *et al* 1993, Sudbeck *et al* 1994, Rikonen *et al* 1995). Thus, as TNC is proposed to be involved in the initiation of invasion (Jahkola *et al* 1998a), MMPs may be the agents responding to changes in TNC expression which has been previously reported (Tremble *et al* 1994, Strestha *et al* 1996). The range of MMPs chosen for analysis reflects the evidence from tissue studies that they may play a role in breast cancer progression (discussed in the introduction to this chapter). MMPs are implicated in breast carcinoma genesis, growth and also metastases. Evidence is provided from a range of sources. For example, MMP11 has been linked to induction of mammary tumours in mice (Rio *et al* 1996), and is over-expressed in human breast tumours (Bassett *et al* 1990). However, some of the strongest evidence is provided by MMP inhibition resulting in a reduction in tumour formation, invasion, and metastases (DeClerk *et al* 1991, Sledge *et al* 1995, Eccles *et al* 1996).

However, in the current study, there appears to be no effect of TNC on the expression of these MMPs and their inhibitors. There are a number of possible reasons, both technical and physiological, as to why a negative result is seen.

The TNC used is unlikely to be the abnormal 14-16 isoform expressed in the breast and ovary (Wilson *et al* 1996, Adams *et al* 2002). Thus there may be functional differences associated with the TNC used here that may be unrelated to the secondary structure required for the induction of MMPs.

The RT-PCR method used is not a quantitative technique, although cycle sampling does convey a degree of quantitation that has previously been used to assess relative amounts of TNC (Ljubimov *et al* 2001) and MMPs (Giambernardi *et al* 1998). However, this method may not be sensitive enough to detect small, subtle, changes in expression which may be more physiologically realistic.

Any attempt to purify intact hexabrachion may disrupt any secondary protein structure and negate any function. A possibility in this case, when considering the TNC is supplied as a monomer which indicates some part of the processing has disrupted structure enough to reduce the hexabrachion.

MMPs are expressed as inactive zymogens (Van Wart & Birkedal Hansen 1990), therefore TNC may not increase MMP expression, but may be responsible for increased activation. Activation of MMP2 may be a consequence of the expression of a package of genes involved with the epithelial-mesenchymal transition (MacDougal & Matrisian 1999) as is TNC (Dandachi *et al* 2001).

The tissue remodelling associated with changes in TNC and MMPs may be via another pathway. Firstly, the action of TNC on MMP expression may not be a direct response and may be mediated via another component of the ECM. The upregulation of MMPs seen by Tremble *et al* (1994) was observed with the addition of TNC to a fibronectin rich substrate.

The action of MMPs may affect TNC (Thomasset *et al* 1998). MMP mediated remodelling of the basement membrane in transgenic mice results in the formation of high levels of TNC in the reactive stroma. MMPs themselves regulate the action of TNC in that certain domains including the FNIII-like domain are susceptible to degradation by MMPs (Siri *et al* 1995). The inclusion of the alternatively spliced region introduces a new site for proteolytic cleavage and the resultant proteolytic fragments may have different effects. Single FNIII like repeats have a different function compared with an array of repeats (Philips *et al* 1998), as has the TNC monomer compared with intact hexabrachion (End *et al* 1992). TNC fragments have also been linked with metastases in lung cancer (Kusagawa *et al* 1998, Cai *et al* 2002). Therefore, the TNC/MMP association may be reciprocal under certain circumstances (Jones & Jones 2000), and under certain conditions TNC may not upregulate MMPs.

Finally, the lack of MMP response seen in the current study may be that TNC induces activity of other proteolytic enzymes in the progression of breast carcinoma. TNC is associated with the expression of cathepsin D and PAI-1 (Jahkola *et al* 1999). Cathespin D and TNC are both associated with distant metastases (Jahkola *et al* 1996; 1998b; 1999). PAI-1 and TNC are both associated with local and distant recurrence (Jahkola *et al* 1996; 1998b; 1999) and with poor prognosis (Christensen *et al* 1996).

Chapter 6 – Conclusions and Future Work

An overview of the effects of tenascin C in breast carcinoma can be split into three parts, its induction, its expression, and its effects. The following discusses these aspects in turn, the conclusions made from the results of the current study, and highlights the implications for future research. A number of investigations are already underway.

The induction of TNC in the breast appears to be, unsurprisingly, regulated by a number of independent factors which work alone, or in combination (Jones & Jones 2000). The two growth factors investigated here, EGF and TGFβ, have both been implicated in the initiation of TNC expression (Pearson *et al* 1988, Sakai *et al* 1995a). However, they appear to affect TNC expression in different ways. Initially results indicated TGFβ may not increase levels of expression, but affect stability of the TNC message. Evidence for this was provided by a lack of effect on amount, or isoform profile, of the TNC transcript, accompanied by an increase in both small and large protein isoforms in the fibroblast matrix (Philips *et al* unpublished). This contrasts with early work on TGFβ and TNC that demonstrated an increase in both transcript and protein (Pearson *et al* 1988). However, subsequent work performed by a colleague has revealed inconsistent results. Possibly due to patient-specific variability in response to growth factors. Effect of TGFβ on other ECM components such as fibronectin (Ignotz & Massague 1986) hints at a common activation mechanism for ECM proteins. As well as in fibroblasts (Pearson *et al* 1988, Borsi *et al* 1994), TGFβ has been seen to affect TNC expression in other mammary cell types and is associated with acquisition of an undifferentiated tissue phenotype (Wirl *et al* 1995). The effect of TGFβ may be a common mechanism in other carcinomas and increases have been seen in primary ovarian fibroblasts (Wilson *et al* 1999) and melanoma cultures (Herlyn *et al* 1991). Investigations of the effect of fetal calf serum (FCS) show greater increases than TGFβ alone, which indicates action of other growth factors that may be specific for each ECM protein (Pearson *et al* 1988).

Turning to EGF, effect of this growth factor on TNC expression is thought to be more sensitive to cell receptor profile as induction is only seen under specific conditions and has not been previously reported in fibroblasts. Upregulation has been seen in carcinoma cells (Sakai *et al* 1995a), but down-regulation has been reported in highly differentiated mammary epithelial cells (Wirl *et al* 1995). EGF alters expression profile of TNC in breast fibroblasts such that it increases relative expression of the highest molecular weight (MW) isoforms. This contrasts with isoforms expressed by carcinoma cells which were intermediate sized (Sakai *et al* 1995a). This is the reason why EGF was investigated as

intermediate sized forms are indicated to be the abnormal forms expressed in breast carcinoma. This is not borne out by *in-vitro* studies on breast cells.

Induction of the 14-16 isoform was not seen in the *in-vitro* investigations performed in the current study and isoform profile was not considered during *in-vitro* studies on primary ovarian fibroblasts (Wilson *et al* 1999). This is significant as ovarian tumours had also been shown to express this isoform (Wilson *et al* 1996). Thus, an important focus of future work needs to be which particular factors induce expression of which particular isoforms. The current study has indicated that specific factors may induce particular isoforms with subsequent analysis suggesting TGFβ has a crucial role . It is possible that an individual factor may not be solely responsible (Jones & Jones 2000). Mechanical stress is an alternative mechanism for TNC induction (Chiquet-Ehrissmann *et al* 1994, Chiquet *et al* 1996, Trachslin *et al* 1999). Investigation is performed in relaxed (un-stressed) and fixed (stressed) collagen gels and collagen gels are currently being investigated in this laboratory in order to establish an *in-vitro* 3D model of breast carcinoma (Gordon *et al* unpublished). The expression of TNC in these models was planned as part of the current study, however time limitations meant this was not possible. Various situations could be investigated: the effect of tensile stress alone, or in combination with various growth factors. Other lines of investigation could include the effect of denatured collagen, which has also been shown to affect TNC expression (Jones *et al* 1997 & 1999) as well as interactions with basement membrane proteins.

Alteration of expression of TNC in primary tissue has been demonstrated at both the level of the primary transcript and a corresponding change in protein expression. The current study is the first to report that expression of the 14-16 isoform is upregulated in relation to breast malignancy and invasion (Adams *et al* 2002), which has also been seen in ovarian tumours (Wilson *et al* 1996). It can be speculated from analysis of various RT-PCR cassettes that adjacent 14-16, domains B and D, may also be expressed in a number of tumour related isoforms. This indicates a consistent change in isoform profile, and a subsequent consistent change in protein secondary structure, which may have a specific function in the progression of breast carcinoma. Changes in protein secondary structure of the alternatively spliced repeats have demonstrated a structure-function relationship (Philips *et al* 1998). The presence of these tumour related isoforms alters the ECM and may provide a substrate on which tumour cells can migrate (Prieto *et* al 1992). Ligand binding in this domain will change and various structures, receptors and ECM proteins that are altered in invasion have binding sites in this area, including fibronectin (Chiquet-

Ehrissmann *et al* 1991), focal adhesions (Murphy-Ullrich *et al* 1991), integrins (Yokosaki *et al* 1996), and annexin II (Chung *et al* 1996). Individual isoforms may also promote matrix proteolysis (Tremble *et al* 1994).

The protein distribution also changes as breast carcinoma progresses such that the neo-expressed higher molecular weight isoforms, which contain domain B, become stromally related. This, along with the distribution of all forms of TNC, leads to speculation that the truncated form, which is the most common form in the breast (Borsi *et al* 1992), is present in relation to the basement membrane and confirms that different sized TNCs have different distributions (Chiquet-Ehrissmann *et al* 1991). The truncated form may have a protective effect in that its function is to maintain tissue integrity (Anbazhagan *et al* 1990). The presence of other isoforms in the stroma may overcome this as it relates to the breakdown of normal tissue structures. The changes in isoform profile may not only be tumour related and TNC is reported to be a marker of stromal proliferation in benign breast disease (Borsi *et al* 1992) and the menstrual cycle (Ferguson *et al* 1990). Confirmation of this is seen in the current study and relates to the presence of the of the B domain. Presence of these forms is seen in fibroadenoma, and very low levels in normal breast, however, no menstrual cycle data exists for these cases. A study is planned of a large cohort of tissues for which menstrual cycle data is available using domain specific antibodies where available. Alongside this a more detailed study of individual domain expression in tumour tissue would be beneficial as it is appreciated that the tumour related isoform seen here may not be the only one. Primarily it is planned to investigate whether detection of these abnormal isoforms represents a sub-set of pre-invasive lesions with a higher capacity for invasion and metastases. A study of the source of TNC on the same cases would also be useful as it has been suggested by a number of studies that different isoforms are produced by different cell types. The current study has indicated that both the myoepithelial cells and the stromal fibroblasts express TNC. However, expression by the tumour cells themselves was not seen. This conflicts with previous reports indicating a sub-set of tumours in which the tumour cells express TNC, and this is linked with poor prognosis (Yoshida *et al* 1995, Tokes *et al* 1999, Melis *et al* 1997, Goepal *et al* 2000). Evidence of this is provided by the current study in that more mesenchymal breast carcinoma cell lines express TNC whereas the more epithelial cell lines do not. These TNC producing cell lines also have higher migration indices. This suggests that in a proportion of tumours an epithelial-mesenchymal transition is occurring (Birchmeier & Birchmeier, 1993). Associated with this is a loss of cell differentiation and tissues acquiring more mesenchymal, or stromal,

characteristics. Mesenchymal cells are well known to express TNC in normal and malignant processes (Chiquet & Fambrough, 1984, Aufderheide *et al* 1987). That no tumour cells in the current study express TNC suggests this sub-set to be small which contrasts with more extensive work (Melis *et al* 1997). However, antibody sensitivity may be a factor.

The preliminary *in-vitro* studies, concerning TNC function, performed here have shown no direct effect on a cohort of proteolytic enzymes related to breast carcinoma. This contrasts with previous reports (Tremble *et al* 1994), but may be due to a number of reasons:

1. The TNC isoform used may not effect MMP expression.
2. The secondary structure required for this function may not be intact.
3. The technique used may not be sensitive enough to detect subtle changes in MMP expression.
4. TNC may act indirectly via other ECM components.
5. TNC may act via other proteolytic enzymes.
6. TNC may not increase expression of an enzyme but alter its activity.

However, TNC has been shown to induce certain morphological changes associated with invasion and has both direct and indirect effects on migration. The focus of much of the future work is based around developing more appropriate *in-vitro* methods of studying TNC function that will overcome the problems seen thus far. Overall, due to the complex multiple interactions with the surrounding environment *in-vivo* a more appropriate *in-vitro* environment is required to begin to accurately assess TNC function.

Expression of TNC in human cells would ensure appropriate processing of the TNC hexabrachion. Therefore, the development of clones of specific TNC isoforms, particularly the 14-16 isoform, is in progress. Transfection is planned into primary and established fibroblasts cell lines that already express native TNC, which will be vital in determining function against a background of normal TNC functions. Consequently this may result in the expression of heterotypic molecules that may be more physiologically relevant (Kammerer *et al* 1998). The intention is to express TNC as a defined matrix, hence a number of 2D and 3D models are planned to assess the effect of different isoforms on tumour invasion, matrix remodelling and growth. As well as incorporation of different TNCs into these models, effects will also be investigated in relation to fibronectin,

different laminin isoforms and other factors related to TNC function. The transfected fibroblasts will also be introduced into these various models to assess alterations in expression and activity of a number of proteolytic enzyme cascades. Furthermore, the effect of TNC isoforms on tumour cell-fibroblasts interactions will also be investigated in these models. Finally, a model of DCIS has been developed in this laboratory, therefore by incorporation of different TNCs into this model, their effects on the initiation of invasion can be studied.

Final Summary and Conclusions

Throughout this study, a model for the action of TNC in breast cancer invasion and progression has been developed. At various points during this study, different factors have been included in this model and the final model can be seen in figure 6.1.

To summarise, under normal conditions the truncated form of TNC is expressed by the myoepithelial cell layer and is deposited on the basement membrane. In the initial stages of tumour development malignant transformation occurs in the epithelial cells resulting in disordered growth and proliferation. This leads to *in-situ* (non-invasive) carcinoma. In a sub-set of DCIS various factors are produced that alter TNC expression in the stroma. Although the factor leading to the upregulation of the 14-16 form has not been positively identified, particular growth factors have been shown to affect production of other specific isoforms, and mechanical stress induced by the expanding duct may be a contributory event. Upregulation of the 14-16 isoform is seen at a pre-invasive stage, which may initiate invasion. High levels of TNC are seen in the stroma of invasive tumours, and it is possible the 14-16 form may be a tumour marker, but upregulation of domain B (exon 14) containing forms is seen in normal processes. The effect of tumour related TNCs is that they promote tumour cell migration, and possibly proliferation, via an indirect mechanism related to the expression of proteolytic enzymes. This may promote tumour cell migration, hence invasion and metastases.

Work is currently underway which may clarify these, and may establish other, relationships in the complex role of tenascin C in breast cancer invasion.

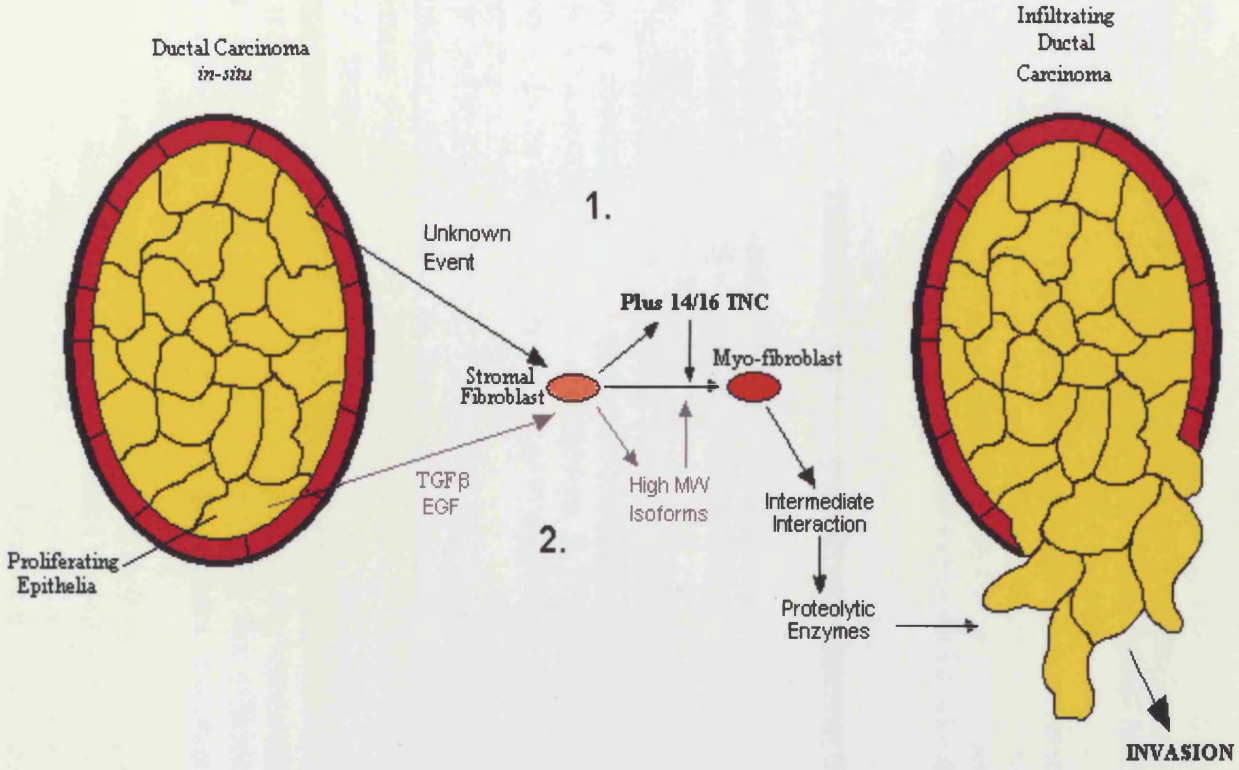


Figure 6.1: Final Proposed model for the action of Tenascin C in Breast Cancer - Pathway 1. (black arrows) depicts the induction of the 14-16 isoform via an, as yet, unknown factor. Pathway 2. (grey arrows) depicts the induction of higher molecular weight isoforms via EGF or TGF beta.

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Appendix – Related publications

Adams M. Jones JL. Walker RA. Bamford M. Bell SC. Pringle JH. (2000) An altered tenascin C isoform profile is expressed in *in-situ* and invasive breast carcinoma compared to normal/benign breast – a role in tumour invasion. *J. Pathology*. **192**. 3A.. Abstract.

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