Analysis of stromal changes in colorectal disease

Thesis submitted for the degree of
Doctor of Medicine
at the University of Leicester

by

Catherine Grierson BSc, MBChB, MRCS(Ed)

Department of Cancer Studies and Molecular Medicine, University of Leicester

2006

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Analysis of stromal changes in colorectal disease

Catherine Grierson BSc, MBChB, MRCS(Ed)

There is accumulating evidence that the micro-environment plays a pivotal role in the progression of malignancy and that key stromal changes can be identified which influence this process. This investigation focussed on two specific areas; fibroblast phenotype and the composition of the extracellular matrix, especially regarding the protein tenascin-C (TN-C).

The heterogeneity within colonic fibroblast populations is becoming increasingly apparent. Using immunohistochemical techniques this study defined specific populations and identified consistent phenotypic changes in these cells in malignancy. In particular CD34 expression was lost in sub-mucosal fibroblasts and α smooth muscle actin (αSMA) expression increased within tumour stroma. There was also evidence of progressive loss of high molecular weight caldesmon (hCD) expression by the pericryptal myofibroblasts with the development of malignancy. The stromal changes were specific to malignancy and were not demonstrated in pre-invasive adenomas.

TN-C is an important component of the extracellular matrix (ECM) which can occur as several different isoforms. Immunohistochemical techniques were used to demonstrate alterations in the distribution of TN-C between normal and malignant colon and the presence of isoforms containing exon 14 in both. The distribution of TN-C in pre-invasive adenomas was the same as that in normal colon but some sub-mucosal TN-C expression including exon 14 containing isoforms was observed in acute inflammation. Polymerase chain reactions (PCR) identified a total of 7 TN-C isoforms in colonic tissue with no obvious association between isoform profile and disease.

TN-C can also be expressed by tumour cells and PCR demonstrated multiple isoforms in the colorectal tumour cell lines SW480, SW620, HCT15 and HT29. However, TN-C expression did not appear to correlate with invasive capacity as demonstrated in preliminary in-vitro invasion assays.

These findings support a role for an altered stromal environment in invasive colonic malignancy but also emphasise the complexity of the relationship between a tumour and its microenvironment.

Acknowledgements

My main acknowledgment is to my supervisor Prof Louise Jones for her guidance and support throughout the preparation of this thesis and for moulding my scientific thinking. I am very grateful to her for her constant enthusiasm and experienced direction.

Sincere thanks are also due to the following people:
Dr Howard Pringle for his invaluable help and advice regarding the polymerase chain reaction work
Dr Deborah Holliday for her work on the invasion assays and for all her generous support and hospitality throughout my time in research
Mr John Jameson for making the project possible in the first place
Dr Cathy Richards for her friendly encouragement and assistance with the specimens
Mr Adam Scott and Dr Salli Muller for help in obtaining the specimens
The staff and students of the Dept of Cancer Studies and Molecular Medicine at Leicester University for all their practical advice and assistance

Finally, as promised, thanks go to Yannick

List of contents

List of figures

List of tables

List of abbreviations

αSMA	Alpha smooth muscle actin
bp	Base pairs
CM	Conditioned media
DAB	Diaminobenzidine
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's modified Eagles medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
FFPE	Formalin fixed paraffin embedded
FGF	Fibroblast growth factor
FN	Fibronectin
GAPDH	Glyceraldehye-3-phosphate dehydrogenase
GIST	Gastrointestinal stromal tumour
H&E	Haematoxylin and eosin
hCD	High molecular weight caldesmon
HGF	Hepatocyte growth factor
ICC	Interstitial cell of Cajal
ISH	In situ hybridisation
LBB	Lysis binding buffer
PBS	Phosphate buffered saline
PCMF	Pericryptal myofibroblast
PDGF	Platelet derived growth factor
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
TBS	Tris buffered saline
TGF-β	Transforming growth factor β
TN-C	Tenascin C
TNL	Long tenascin

TNM	Tumour Node Metastasis
tTN	Truncated tenascin
TV	Tubulovillous
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction: The role of the stroma in the colon and colorectal disease

1.1. STRUCTURE OF THE COLON AND RECTUM

The colon and rectum form the final portion of the gastrointestinal tract originating at the caecum and terminating at the start of the anal canal. Their functions are related to motility, absorption and protection and the structure reflects this. There are, however, several common pathologies which affect this area of the bowel and which are a significant cause of morbidity and mortality amongst Western populations.

Embryologically the large bowel is derived from the midgut, which gives rise to the caecum, ascending colon and proximal two thirds of the transverse colon, and from the hindgut, which produces the remaining third of the transverse colon, descending colon, sigmoid colon and rectum. The epithelium of the colon and rectum is glandular thus differentiating it from the anal canal which is composed of squamous epithelium. In this way it resembles the epithelium of the stomach and small intestine but the arrangement of the underlying structures is markedly different *(Day et al, 2003)*.

The large bowel mucosa consists of the epithelium and underlying lamina propria and is bounded by the muscularis mucosa. Beneath this, the submucosa and circular and longitudinal muscles compose the muscularis propria, outside which is the serosa *(Figure 1.1)*.

In the colon and rectum the epithelial cells are arranged in a single layer to form crypts which develop by the division and subsequent migration and maturation of stem cells at the base of the crypts. There are four types of epithelial cells; columnar epithelium, mucin secreting goblet cells, Paneth cells and endocrine cells. The area between the epithelial basement membrane and the muscularis mucosa is known as the lamina propria and contains fibroblasts, lymphocytes, plasma cells, mast cells, eosinophils and macrophages. The muscularis mucosa is a thin layer of smooth muscle which defines the mucosa and also contains foci of lymphoid tissue known as gastro-associated lymphoid tissue (GALT).

Beyond the muscularis mucosa is the submucosa which is made up of loose connective tissue such as collagen and reticulin and which also contains blood vessels, lymphatics and nerves. The major muscle layers in the large bowel are the circular and longitudinal smooth muscle layers of the muscularis propria and between these two layers lies the myenteric plexus which is important for sympathetic and

parasympathetic neurotransmission. Also between these layers are contained the Interstitial Cells of Cajal which are cells of uncertain derivation and function.
The serosa of the bowel wall lies on the extraluminal surface of the bowel and is a thin sheet of connective tissue containing blood vessels and lymphatics. It is covered in peritoneum *(Day et al, 2003) (Figure 1.1).*

1.2. DISEASES OF THE COLON AND RECTUM

1.2.1. **Adenocarcinoma of the colon and rectum**

Colorectal adenocarcinoma is a major cause of cancer related mortality in the UK causing 24.7 deaths per 100,000 in men and 14.9 deaths per 100,000 in women in 2002. It is the third commonest cause of cancer related death across both sexes. There were 79123 new diagnoses of colorectal cancer between 1996 and 1999 and the overall 5 year survival rates for cancers diagnosed during this period were 46.9% for men and 47.9% for women *(Office for National Statistics).* The prognosis is, however, significantly affected by the stage of the disease at diagnosis making early detection a priority in its management.
Invasive adenocarcinomas in the colon and rectum are thought to be derived most commonly from adenomatous polyps. These polyps are often asymptomatic, can be flat or pedunculated and occur throughout the large bowel. Histologically, they are classified as tubular, villous or tubulovillous with tubular adenomas consisting of closely packed branching tubules separated by varying amounts of lamina propria while a villous appearance is produced by folds in the mucosal surface. All adenomas are, by definition, dysplastic but this dysplasia can be classified as mild, moderate or severe with some polyps progressing to invasive malignancy.
The evidence that polyps give rise to carcinomas derives from several observations including molecular abnormalities common to both adenomas and adenocarcinomas *(Grady 2005).* In addition there is supporting epidemiological evidence such as the fact that that severe dysplasia is most common in populations at high risk of colorectal carcinoma *(Konishi et al, 1984).* The frequency of severe dysplasia increases with the size of the adenoma and is commonest in villous adenomas *(Muto et al, 1975).* The link between size and malignant potential is further supported by the finding that the percentage of adenomas displaying malignant change is 1% in adenomas <1cm but 50% in adenomas >2cm *(Muto et al, 1975).*

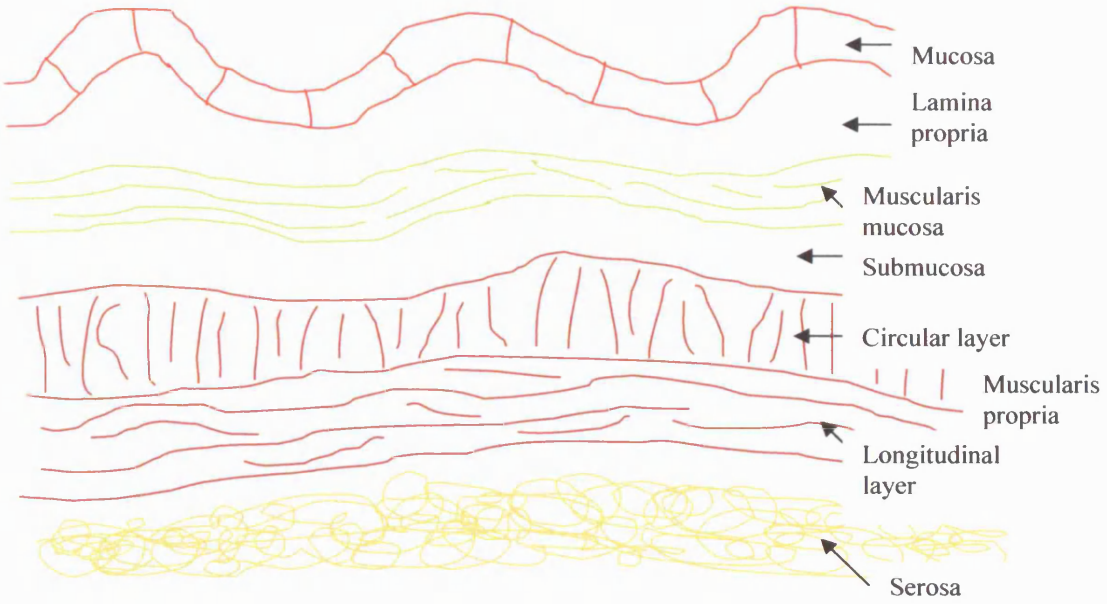


Figure 1.1. Schematic representation of the colon wall

Cancer syndromes and the adenoma-carcinoma sequence:

The sequence of genetic changes from adenoma to carcinoma was originally described by Fearon and Vogelstein and is the classical example of step-wise transformation within cancer biology *(Fearon & Vogelstein, 1990)*. The sequence includes four genetic changes, all of which are required to occur for development of invasive malignancy. Within the sequence there is sequential loss of the tumour suppressor genes APC, TP53 and DCC and mutation of the oncogene k ras.

There are several recognised familial forms of colorectal carcinoma which lead to the development of carcinoma at an early age. The most well known of these is Familial Adenomatous Polyposis Coli (or FAP) which is linked to germline mutation of the APC gene on chromosome 5q. Affected individuals thus inherit the first step in the adenoma carcinoma sequence and are therefore predisposed to developing large numbers of polyps and an increased likelihood that one will become malignant. The other well-described familial syndrome is Hereditary Non-Polyposis Colorectal Carcinoma (HNPCC) which leads to predominantly right sided tumours and does not appear to be associated with any increase in the number of adenomas. In this case the underlying genetic defect is loss of a DNA mismatch repair gene and it is thought that this leads to the accelerated growth of individual adenomas rather than a generalised increase in number *(Jass et al, 1994)*. This supports the idea that colorectal carcinomas are universally derived from adenomas, although they may not all share the same genetic pathway *(Jass, 1994)*.

Staging of colorectal carcinoma:

There are currently two major systems for staging colorectal carcinoma. Both are histological although radiological techniques are increasingly being used to estimate the stage preoperatively *(Heriot et al, 1998)*.

The original staging system was defined by Dukes in 1932 and, although it has been repeatedly modified since then, the prognostic implications have changed very little. The original classification identified three stages; A, B and C. An invasive tumour is defined as one which has breached the muscularis mucosa. A Dukes A tumour extends up to but not through the muscularis propria, a Dukes B tumour extends beyond the muscularis propria and a Dukes C tumour has lymph node involvement, regardless of the extent of the original tumour. Subsequent modifications include distinguishing between any lymph node involvement (C_1) and involvement of the

apical lymph node (C_2) *(Dukes et al, 1958)* and inclusion of the Dukes D stage indicating distal metastases *(Newland et al, 1987)*.

The other widespread system of classification is the Tumour Node Metastasis (TNM) system. This is defined by the American Joint Committee on Cancer (AJCC) and the Union Internationale Contre Cancer (UICC) and the most recent revision was in 1997. *(Fleming et al, 1997)*. The staging system is outlined below and relationship to survival indicated in Table 1.

Primary Tumour (T)

TX Primary tumour cannot be assessed

T0 No evidence of primary tumour

Tis Carcinoma *in situ* or invasion of lamina propria

T1 Tumour invades submucosa

T2 Tumour invades muscularis propria

T3 Tumour invades through the muscularis propria into the subserosa or into nonperitonealised pericolic or perirectal tissues

T4 Tumour directly invades other organs or structures and/or perforates visceral peritoneum

Regional Lymph Nodes (N)

NX Lymph nodes cannot be assessed

N0 No lymph node metastases

N1 Metastasis in one to three regional lymph nodes

N2 Metastasis in four or more lymph nodes

Distant Metastasis (M)

MX Distant metastasis cannot be assessed

M0 No distant metastasis

M1 Distant metastasis

Other systems of classification have attempted to give more accurate indicators of prognosis *(Sternberg et al, 1999; Compton et al, 2000)* but these have not so far impacted on clinical practice.

TNM Stage			% 5 year survival	
			Rectal Cancer	Colon Cancer
T1	N0	M0	>95	>95
T2	N0	M0	80	100
T3	N0	M0	65	90
T4	N0	M0	50	70
Any T	N1	M0	55	65
	N2	M0	30	40
	Any N	M1	<10	<10

Table 1: Five year survival for colorectal cancer by stage taken from Morris et al 2000

1.2.2. **Inflammatory diseases of the colon and rectum**

There are three major diseases involving inflammation of the colon and rectum and between them they are responsible for significant morbidity and mortality in the UK. There are also links with the development of malignancy.

Crohns Disease:

Crohns disease is a chronic idiopathic inflammatory condition which causes significant morbidity due to the variety of potentially affected sites and the recurrent nature of exacerbations, as well as the possible complications of stricturing, fistulation and perforation. It has an incidence of 38 per 100000 in those born in 1970 and 21 per 100000 in those born in 1958 *(Ehlin et al, 2003)*.

Crohns disease can involve any part of the gastrointestinal tract and was classically defined by Lockhart-Mummery and Morson in 1960. The lesions are not normally contiguous and cause skip lesions, while transmural involvement leads to fat wrapping. The disease can also produce fissures, fistula formation and abscesses. There are several classical pathological features including cobblestoning, rose thorn and serpiginous linear ulcer formation and the presence of granulomas. In the colon, granulomas are seen microscopically with transmural aggregates of chronic inflammatory cells and fissuring ulceration. All the layers of the bowel wall display connective tissue changes, including thickening and disruption of the muscularis

mucosa, muscularisation and fibrosis of the submucosa and hyperplasia of the muscularis propria and nerve plexuses.

Ulcerative Colitis:

Ulcerative colitis is also a chronic idiopathic condition and can cause a fulminant colitis which can lead to septicaemia or perforation but can also run a chronic relapsing course. The incidence per 100000 is reported as 30 and 27 in cohorts born in 1970 and 1958 respectively *(Ehlin et al, 2003)*.

Ulcerative colitis is a disease of the large bowel although there may be some 'back wash ileitis.' Involvement of the bowel is continuous and almost invariably involves the rectum. It is predominantly a mucosal disease except in fulminant colitis where ulceration and inflammation leads to loss of the mucosa and spread to the underlying muscularis propria. Fistulas and strictures are observed less commonly than in Crohns disease and the main histological feature is the formation of pseudoulceration produced by mucosal oedema with granulomas only occurring infrequently. In chronic disease shortening and branching of the crypts may be observed leading to an irregular mucosal surface while chronic inflammatory cells are limited to the mucosa.

Diverticular disease:

Diverticular disease is a very common condition and increases with age to affect 70% of people in their 80s. The formation of diverticulae is thought to be related to the Western diet due to a lack of fibre *(Painter, 1969; Gear et al, 1979)*. The disease is frequently asymptomatic but there are a variety of potentially serious complications of diverticular disease. These include diverticulitis, where a diverticulum becomes acutely inflamed, diverticular strictures, which develop subsequent to one or repeated episodes of inflammation, diverticular abscess formation and diverticular perforation. The latter complications were classified by Hinchey et al (1978).

The diverticulae referred to in diverticular disease are small outpouchings of large bowel mucosa, including the muscularis mucosa, which occur at the site of insertion of the mesenteric blood vessels. The diverticulae project through the circular smooth muscle layer to lie in the pericolic fat or appendices epiploicae.

Inflammatory bowel disease and malignancy:

Ulcerative colitis has long been known to be related to the development of carcinoma. The risk increases with the duration of the disease leading to a 1-2% annual risk of cancer developing in patients who have had disease involving the whole colon for 10 years, although risk is also related to the severity of the inflammation. *(Rutter et al 2004)*. Chronic inflammation has been observed to lead to dysplasia which can be graded as high or low grade. There is disagreement, however, about the prognostic significance of this grading as regards the development of neoplasia *(Lim et al, 2003; Ullman et al, 2003; Rutter et al, 2004)*.

Crohns disease is less strongly implicated in the development of carcinoma but studies have indicated an increased long term risk in patients with certain features, including presence of a rectal stump after resection, chronic severe anorectal disease and extensive colitis *(Freeman, 2001; Sjödahl et al, 2003)*.

The presence of diverticular disease does not increase the risk of developing adenocarcinoma but the disease is commonest in the group of people most likely to develop colorectal carcinoma and the overlap in symptoms presents diagnostic challenges and raises questions about the role of lifestyle factors in colorectal disease.

1.3. THE PROCESS OF TUMOUR INVASION

The process of invasion distinguishes benign from malignant disease and specifically colonic adenoma from carcinoma. It is important to try and understand the mechanisms modulating this transition in order to design strategies to predict and prevent the development of malignant disease. Invasion requires several barriers to be breached, including the basement membrane and, in the case of the colon, the muscularis mucosa. Loss of integrity of the basement membrane brings the tumour into contact with blood vessels and lymphatic vessels and hence enables the spread of metastases. Alterations in the expression of key molecules such as the proteolytic enzymes matrix metalloproteinases (MMPs) which degrade membrane barriers *(Zucker, 1988)* and cell adhesion molecules present on tumour cells *(Weaver & Bissell, 1999)* have been implicated in the process of tumour cell invasion and migration. While the tumour cell itself is ultimately the actively invading cell and there are many tumour derived factors which affect this movement, there is also

increasing evidence for a central role of the microenvironment in controlling tumour cell behaviour (Reviewed in *Micke & Östman 2004)*.

All cells lie within a protein rich scaffold known as the extracellular matrix (ECM). The proteins can be divided into structural or adhesive proteins and, in combination with blood vessels and fibroblasts, this matrix forms the stroma. The roles of the stroma are diverse and complex. It provides structural support but also transduces signals from the microenvironment. Such signals control proliferation, differentiation and apoptosis and include growth factors and proteolytic enzymes. The ECM is thus critical to maintenance of the normal environment and continuation of normal tissue function.

In particular, key roles have been identified for the stroma in the process of tumour invasion and progression and these include angiogenesis, inflammation and modulation of the ECM. The overwhelming importance of the microenvironment in supporting and promoting tumour development is illustrated by the process of angiogenesis. The very early stages of tumour growth are avascular but any significant increase in size requires a new blood supply *(Knighton et al 1977)*. Some of the factors stimulating angiogenesis derive from the tumour itself *(Sandler et al, 2004)* but stromal-derived factors such as transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and fibroblast growth factor-2 (FGF-2) can also stimulate angiogenesis (Reviewed in *Condon, 2005*).

Inflammation is also linked to the development of cancer with cells and mediators of chronic inflammation acting as tumour promoters at distinct phases of malignant progression. Many cancers arise on a background of chronic inflammation *(Houghton et al, 2004; Pikarsky et al, 2004)* but the chemical mediators regulating inflammation are also produced by cancer cells (Reviewed in *Balkwill et al, 2005)*. The inflammatory microenvironment of neoplastic tissues characteristically contains dendritic cells, mast cells and T cells *(Balkwill & Mantovani, 2001; Coussens & Werb, 2002; Nakayama et al, 2004)* and, most importantly, macrophages which produce factors leading to matrix remodelling, tumour proliferation and angiogenesis *(Bingle et al, 2002; Lin et al, 2001; Robinson et al, 2003)*.

The major cellular component of the microenvironment is the fibroblast which is responsible for the production of scaffolding extracellular matrix. As the key determinant of the ECM content, the fibroblast is of great potential importance in

disease processes involving the stroma and is therefore considered in some detail below.

1.4. FIBROBLASTS:

1.4.1. **Fibroblast function:**

Fibroblasts are mesenchymal cells which are pivotal in the formation and maintenance of the extracellular matrix (Reviewed in *Bhowmick et al, 2004)*. Fibroblasts are found in the stroma of all tissues and their role in inflammation, injury and repair has been well described (Reviewed in *Gabbiani, 2003*). The formation of a normal scar requires epithelialisation and formation of granulation tissue which both contracts the wound and provides tissue continuity. Fibroblasts are present within the granulation tissue but disappear by apoptosis to produce a final scar. In pathological situations such as fibrosis, the fibroblasts persist, resulting in a scar which retains large amounts of connective tissue *(Eckes et al, 2000; Gabbiani et al, 2003)*. Fibroblasts also secrete chemokines and cytokines important to immunomodulation which gives them a role in chronic inflammation *(Buckley et al, 2003)*. Other fibroblast derived factors include MMPs, urokinase plasminogen activator and related factors in the cascade and TGFß *(Bosman & Stamenkovic, 2003)*.

In addition to the normal fibroblast cell type, a fibroblast derivative resembling both fibroblasts and smooth muscle cells has been identified, termed the myofibroblast. Myofibroblasts are derived from fibroblasts *(Ronnov-Jessen et al, 1995)* but have developed characteristics resembling smooth muscle cells including the expression of α smooth muscle actin (αSMA) *(Richman et al, 1987)*. Early reports of myofibroblasts mainly concerned granulation tissue *(Feiner & Kaye, 1976)* but they have also been described in the stroma of a variety of malignancies *(Seemayer et al, 1979)* including colon carcinoma *(Balazs & Kovacs, 1981; Katenkamp et al, 1984)*. Acquisition of a myofibroblast phenotype is controlled by a variety of factors the most characteristic of which is $TGFß_1$ *(Ronnov-Jessen & Petersen, 1993; Roy et al, 2001)*. One proposed model is that conditions of mechanical stress induce an initial change in the fibroblast and leads to the production of and interaction with fibronectin (FN) fibres. Further differentiation takes place under the influence of TGFß and mechanical stress to result in a fully differentiated myofibroblast *(Tomasek et al, 2002)*. In the context of wound healing the presence of myofibroblasts within

granulation tissue enables the area to contract and therefore facilitates reepithelilisation.

1.4.2. **The role of fibroblasts in malignancy**

The contribution of fibroblasts to tumour development and progression has been shown in both animal models and in-vitro studies. It has been shown that fibroblasts from around cancers are required to maintain cancer cell growth *in vitro* while normal cells inhibit such growth *(Mukaida et al, 1991; Olumi et al, 1999)*. Irradiation of host stroma prior to the addition of unirradiated cells can lead to the development of tumours *(Barcellos-Hoff et al, 2000)* and specifically co-culture with irradiated fibroblasts increases the invasiveness of pancreatic cancer cells *(Ohuchida et al, 2004)*. Overexpression of $TGF\beta_1$ or hepatocyte growth factor (HGF) by fibroblasts has been shown to transform normal mammary organoids *(Kuperwasser et al, 2004)* and loss of TGFβ responsiveness in fibroblasts also leads to the development of specific tumours in experimental mice *(Bhowmick et al, 2004)*. These studies all demonstrate the importance of the stromal microenvironment in determining epithelial cell behaviour. More recently, the importance of fibroblasts in promoting tumour growth and angiogenesis has been demonstrated and related to the release of stromal cell-derived factor 1 (SDF-1) by these cells *(Orimo et al, 2005)*. It is widely regarded that the tumour promoting effect of fibroblasts is a response to tumour derived signals. For example, it was shown that fibroblasts respond to tumour-derived TGFβ by releasing HGF which then stimulates tumour cell invasion *(Nakamura et al, 1997)*. However, there is growing evidence that tumour associated fibroblasts may be intrinsically different to their normal counterparts.

1.4.3. **Diversity in fibroblast behaviour:**

Fibroblasts display different phenotypes in different tissues but it has been suggested that their behaviour may differ between physiological and pathological conditions and between individuals. Fibroblasts associated with tumours have been shown to exhibit abnormal behaviour including increased motility compared to fibroblasts from individuals without cancer *(Azzarone et al, 1984; Durning et al, 1984)*. Groups of fibroblasts can be defined by examining migratory responses to changes in cell density with normal and transformed cells forming two groups and fœtal fibroblasts forming an intermediate group *(Schor et al, 1985)*. In addition, normal skin

fibroblasts from individuals with malignancy display migratory behaviour which is distinct from that of fibroblasts from normal individuals and which is instead similar to fœtal fibroblasts *(Schor et al, 1985)*. This raises the possibility that fibroblast characteristics may indicate a predisposition to malignancy and this is further supported by the discovery that a proportion of asymptomatic individuals with a family history of breast cancer have fœtal- type fibroblasts *(Schor et al, 1986; Haggie et al, 1987)*. Further studies have shown that fibroblasts in breast cancer patients are more likely to maintain a persistent fœtal phenotype leading to the production of a soluble factor important in migration *(Schor et al, 1988)*. In addition non-breast fibroblasts from patients with breast cancer exhibit defective DNA repair, suggesting a global abnormality in fibroblasts from patients with malignancy *(Hannan et al, 2001)*.

Recent developments have suggested a further origin for the myofibroblast in malignancy. Studies involving both animal models and human subjects who had previously undergone bone marrow transplant have shown that a significant proportion of myofibroblasts are derived directly from the bone marrow. This has been shown to be the case in the colon *(Brittan et al, 2002)* and in tumour associated stroma *(Direkze et al, 2004)*. It is unclear from these studies however whether the presence of the bone marrow derived myofibroblasts represents a host defence mechanism or recruitment of the cells by the tumour itself.

1.4.4. **Fibroblast populations in the colon**

Fibroblasts are largely regarded as homogenous, relatively unspecialised cells, in contrast to the attention given to epithelial cell specialisation. However, there have been studies suggesting fibroblast subspecialisation *(Spanakis & Brouty-Boye, 1997)* and this is particularly evident in the colon. The colonic fibroblast is not a uniform cell type and the population is, in fact, very diverse with different cells occurring at all levels in the colon wall *(Powell et al 1999)*. This is important as different cells have different functions and fibroblasts essentially dictate the composition of the microenvironment.

Probably the best described group of fibroblasts in the colon are those which lie closest to the lumen; the *peri-cryptal myofibroblasts* (PCMFs). Peri-cryptal cells are closely related to the crypts, especially the bases and are visible as a single cell layer just below the basement membrane. They have been identified as myofibroblasts due

to shared characteristics with smooth muscle cells *(Richman et al, 1987)* as myofibroblasts are defined on the basis of αSMA expression. It is thought that these pericryptal cells control fluid absorption and ion permeability across the basement membrane *(Beltinger et al, 1999; Naftalin et al, 1999; Thiagarajah et al, 2000)*. They are also involved in secretion of factors such as the cyclooxygenase (COX) enzymes 1 and 2 *(Mahida et al, 1997)* and TGFß isoforms which are thought to influence epithelial restitution after injury *(McKaig et al, 1999)*. Other ECM proteins produced by peri-cryptal myofibroblasts include collagen type IV, laminin β_1 and γ_1 and fibronectin *(Mahida et al, 1997)*.

There are other fibroblasts present in the mucosa which are not myofibroblasts and do not stain with αSMA. These are the *non-pericryptal fibroblasts* and are found in the lamina propria. They are identified as fibroblasts by their morphology and the fact that they are vimentin positive but desmin negative *(Adeboyega et al, 2002)*.

Below the muscularis mucosa are the *submucosal fibroblasts* which often extend into the muscularis mucosa itself. These cells are not myofibroblasts and are, instead, identified by staining with CD34, *(Nakayama et al, 2000)* a sialomucin which is expressed on fibroblasts, *(Yamakazi & Eyden 1995)* endothelial cells *(Fina et al, 1990)* and haemopoietic stem cells *(Brown et al, 1991)*. These fibroblasts are clearly visible within the stroma as characteristic spindle shaped cells.

There is a further population of cells within the colon which may represent another fibroblast sub-population. These are the *Interstitial Cells of Cajal* (ICC) which lie between the circular and longitudinal layers of the muscularis propria. It had previously been thought that the cells were derived from neural crest cells but there has been increasing speculation that they actually have a mesenchymal origin *(Young et al, 1996)*. The possibility of a link with a fibroblast lineage has been raised as some investigators have found that they express CD34. The other distinctive cell marker present on these cells is c-kit, a membrane linked tyrosine kinase receptor *(Mazzia et al, 2000)*. There are conflicting studies concerning the extent to which positivity for CD34 and c-kit overlaps, with some authors maintaining that there are two distinct cell populations *(Vanderwinden et al, 1999)*. Others claim to have identified a group of cells expressing both markers *(Robinson et al, 2000)* but the prevalence of this overlap remains unclear *(Sircar et al, 1999)*.

The precise role of ICCs is almost as unclear as their origin but they are thought to act as gastrointestinal pacemaker cells *(Hanani & Freund, 2000)*. It has been

demonstrated that there is loss of c-kit $^{+}$ve and CD34 $^{+}$ve cells in pseudoobstruction *(Streutker et al, 2003)* and slow transit constipation *(Lyford et al, 2002)*. ICCs have also been suggested as the origin of gastrointestinal stromal tumours (GISTs) as these tumours are characteristically c-kit $^{+}$ve and CD34 $^{+}$ve *(Sircar et al, 1999)* but debate remains as to whether this represents malignancy deriving from a c-kit $^{+}$ve CD34 $^{+}$ve ICC or acquisition of the c-kit proto-oncogene in a CD34 $^{+}$ve population *(Hirota et al, 1998; Vanderwinden et al, 1999)*.

1.4.5. **Matrix metalloproteinases:**

One of the key ways in which fibroblasts affect the microenvironment is through the production of matrix metalloproteinases (MMPs) which are a group of enzymes which degrade the ECM. These enzymes are critical to a variety of physiological and pathological processes including tissue remodelling and repair *(Hulboy et al, 1997; Zhou et al, 2000)*, cell migration *(Giannelli et al, 1997)*, tumour invasion *(Nabeshima et al, 2000)* and metastasis formation *(Aparicio et al, 1999)*. There are atleast 20 MMPs which can be divided into several groups according to their substrate specificity. These groups include the collagenases, stromelysins, matrilysins and gelatinases and the membrane associated MMPs. Between them these enzymes can degrade collagen, laminin, fibronectin, tenascin, proteoglycans, gelatin and elastin *(Reviewed in Jones & Walker, 1997; Woessner & Nagase, 2000; McCawley & Matrisian, 2001)*. MMPs have been noted to increase markedly in a variety of inflammatory pathologies with raised levels found in coeliac disease *(Daum et al 1999)* and inflammatory bowel disease *(McKaig et al, 2003; Medina et al, 2003)* MMP levels have also been widely shown to increase in several malignancies *(Bramhall et al, 1997; Salmela et al, 2001)* and levels of specific MMPs have been linked to prognosis *(Zeng et al, 1996; Murray et al, 1998; Talvensaari-Mattila et al, 1998)*. MMPs are generally derived from the stroma although they can be produced by tumour cells *(Nabeshima et al, 2000)*. In the stroma, fibroblasts are a major source of MMPs, including MMP-1, MMP-2, MMP-3 and MMP-9 *(von Lampe et al, 2000; Roeb et al, 2001; McKaig et al, 2003)*. Tumour cells are also known to induce the production of MMPs in fibroblasts *(Mari et al, 1998; Ko et al, 2000)*.

In the colon several MMPs have been specifically linked with the development of colorectal carcinoma and with its progression. Overexpression of MMPs -1, -2, -3, -7, -9, -13 and MT1-MMP has been demonstrated in colorectal carcinoma (Reviewed in

Zucker & Vacirca, 2004) but the precise origin of each of these enzymes is less clear. Previous assumptions that the high levels of MMPs found in a variety of tumours derived from the malignant epithelial cells have been challenged by several studies demonstrating the presence of MMP mRNA in tumour associated fibroblasts, including those in colorectal tumours *(Polette et al, 1996; Nelson et al, 2000)*. In particular, both MMP-1 *(Shiozawa et al, 2000)* and MMP-2 *(Poulsom et al, 1992)* have been localised to stromal rather than epithelial tissues within tumours. The co-existing evidence for the presence of MMPs within colorectal cancer cells suggests that MMPs produced by fibroblasts and surrounding inflammatory cells may be binding to tumour cells, thus arming these cells with enzymes promoting invasion *(Zucker & Vacirca, 2004)* There is also evidence for a fibroblast origin for tissue inhibitors of metalloproteinases (TIMPs) *(Joo et al, 1999)* which suggests an additional modulatory role for the stromal cells.

1.5. EXTRACELLULAR MATRIX COMPOSITION

There are two groups of proteins within the ECM, the structural and the adhesive proteins. The collagens and laminins form the main groups of structural proteins while adhesive proteins include fibronectin and tenascin, which forms a major focus of this thesis.

1.5.1. **Structural proteins:**

Collagen:

Type I collagen is the most widely distributed collagen, occurring throughout the body. Like most other collagens it is synthesised by cells of the extracellular matrix such as fibroblasts, myofibroblasts, osteoblasts and chondrocytes. Some collagens are produced via an interaction between the stromal cells and the adjacent parenchymal or epithelial cells. Type IV collagen, which is the main component of the basement membrane, is one such example *(Stanley et al, 1982)*. The primary function of collagen is structural and the arrangement of the molecules reflects this. In areas requiring resistance to high pressures or forces, collagen is arranged in fibrils whereas in other areas it forms networks.

Laminin:

Laminin is also a structural protein and an important component of the basement membrane *(Stanley et al, 1982)*. It is synthesised by a wide variety of cells including epidermal cells, *(Stanley et al, 1982)* colonic epithelial cells and mesenchymal cells *(Perreault et al, 1998)*. Atleast 12 laminin isoforms have been identified *(Burgeson et al, 1994)* resulting from the combination of 5 α chains, *(Galliano et al, 1995; Miner et al, 1997)* 3 β chains and 3 γ chains *(Timpl et al, 1979; Koch et al, 1999)*. Laminins have a wide variety of functions and these are mainly mediated through integrins via an integrin binding domain in the α chain.

1.5.2. **Adhesive proteins:**

Fibronectin:

Fibronectin (FN) is a vital component of both the plasma and the ECM. Each FN monomer is composed of FN repeats (types I, II and III) which undergo alternative splicing *(Schwarzbauer et al, 1987; Norton & Hynes, 1990)* to produce up to 20 human fibronectin variants. In solution fibrin exists as a compact dimer but it is activated by a number of factors including alterations in pH or ionic strength *(Williams et al, 1982)* and the binding of cell surface receptors such as the integrin α5β1 *(Wierzbicka-Patynowski & Schwarzbauer, 2003)*. This induces conformational change in the molecule and enables it to interact with other FN molecules to form a fibrillar network in a process known as fibronectin matrix assembly.

FN interacts with a wide variety of integrins *(Plow et al, 2000)* but also binds to molecules such as heparin, collagen, heparan sulphate proteoglycans, fibrin and tenascin. These interactions enable FN to play a key role in cell adhesion, migration, growth and differentiation *(Pankov & Yamada, 2002)*.

1.6. TENASCIN

Since tenascin forms a major focus of this thesis, it will be discussed in greater detail in the following section.

1.6.1. **Structure of tenascin:**

One of the key stromal molecules mentioned above is the fibroblast derived protein tenascin. The molecule was first described in 1983 *(Bourdon et al, 1983)* and was originally known as glioma mesenchymal extra-cellular matrix protein (GMEM). It has also been known as hexabrachion, *(Erickson & Iglesias, 1984)* cytotactin *(Grumet et al, 1985)* and myotendinous antigen *(Chiquet & Fambrough, 1984)* with the name tenascin first appearing in 1986 *(Chiquet-Ehrismann et al, 1986)*. The first form to be identified and the one which has subsequently been most extensively investigated is tenascin-cytotactin (TN-C). There are, however, several other forms of the molecule, including TN-R, *(Fuss et al, 1991)* TN-W, *(Weber et al, 1998)* TN-X *(Bristow et al, 1993)* and TN-Y *(Hagios et al, 1996)*.

The human tenascin gene is located on chromosome 9q32-34 *(Gulcher et al, 1990)*. It consists of 27 exons and 26 introns *(Gulcher et al, 1991)* and transcription is controlled by a single promoter *(Gherzi et al, 1995)*. The resultant protein contains a maximum of 2203 amino acids *(Nies et al, 1991)* and is about 1900 KDa with individual sub-units of between 220 KDa and 330 KDa *(Erickson & Bourdon, 1989)*.

TN-C exists as a hexamer with the six arms linking together at the amino terminal via the 'tenascin assembly domain'. The proximal portions of the arms are thin and rigid whereas the distal portions are thicker and more flexible, terminating in a globular region *(Erickson & Iglesias, 1984)*. The N terminus contains a group of hydrophobic residues and eight cysteine residues which enables the association of the six monomers *(Spring et al, 1989; Siri et al, 1991)*. At the C terminus there are 14.5 epidermal growth factor (EGF) –like repeats, followed by a region which contains a number of fibronectin (FN) type III like repeats. The globular terminal portion resembles the carboxyl terminal portion of the β and γ chains of fibrinogen *(See Figure 1.2)*.

The size and nature of the FNIII repeat region in the TN-C molecule can be altered by alternative splicing of the mRNA *(Gulcher et al, 1991; Nies et al, 1991; Sriramarao et al, 1993)*. Early studies identified two forms of TN-C and concluded that the difference between the two forms lay in the FNIII repeat region *(Gulcher et al, 1989)*. Variable splicing of the exons encoding these repeats creates a variety of isoforms which are thought to be functionally significant *(Siri et al, 1995; Ghert et al, 2001; Tsunoda et al, 2003)*. Two systems of terminology are employed in the literature; exon numbers and fibronectin repeats as shown in Figure 1.3. The 7 FNIII repeats

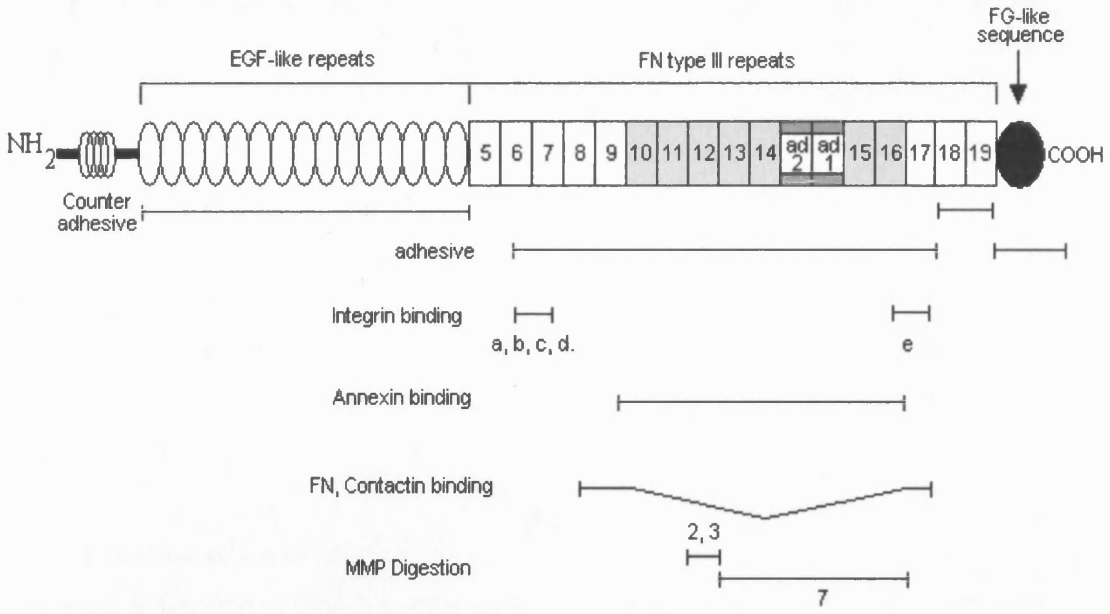


Figure 1.2 The structure of the tenascin molecule

9	A1 10	A2 11	A3 12	A4 13	B 14	ad2	ad1	C 15	D 16	17

Figure 1.3 Diagram correlating fibronectin repeats with coding exons

involved in the variable splicing lie within the FNIII region and are denoted as A_1-A_4, B, C and D. They are coded for by exons 10 to 16 with two more regions; so called 'additional domains' 1 and 2 (ad1 and ad2), occurring between 14 and 15 *(Sriramarao et al, 1993; Mighell et al, 1997) (See diagram)*. Atleast 9 different isoforms have been identified *(Mighell et al, 1997; Jones & Jones, 2000)* but this is far fewer than the total number of possible combinations which implies that splicing is not random. The most commonly identified forms are the 180 KDa and 240 KDa variants which represent 'short' (where none of the 7 FNIII repeats are included) and 'long' (where all the repeats are included) forms of the molecule *(Gulcher et al, 1989)*.

1.6.2. **Function of tenascin:**

The precise function of TN-C is complex as it has both adhesive and counter-adhesive properties. Its effects can occur via direct interactions with cell surface receptors or via modulation of other ECM modules contributing to the cellular microenvironment *(Jones & Jones, 2000)*. Its functions are further diversified due to variations in the composition of the FNIII repeat region.

The adhesive and counter-adhesive properties of TN-C can be localised to specific areas of the molecule with the counter adhesive sites being found in the EGF like repeat domain and FNIII like repeats 7-8 and the areas supporting cell adhesion localising to FNIII like repeats 2-6 (which includes the variable region) and the globular C terminal *(Prieto et al, 1992; Fischer et al, 1997; Philips et al, 1998)*. Few specific ligands have been demonstrated for the EGF like area *(Jones & Jones, 2000)* but this area has been shown to bind to EGF receptors under certain circumstances *(Swindle et al, 2001)*. Interestingly, most of these ligand binding sites reside in the conserved part of the molecule. However, their affinity could atleast theoretically be altered through alternative splicing due to resulting conformational changes of the whole molecule. A variety of ligands are known to bind to specific regions of the FNIII like repeats including fibronectin which binds to repeats 3-5 (*Ingham et al, 2004)* and several integrins which interact with repeats 2-6 and especially with 3 *(Prieto, 1993; Sriramarao et al, 1993)*. Studies have also shown that TN-C isoforms containing repeats A-D bind annexin II *(Matsuda et al, 1999)*.

The mechanism by which TN-C modulates cell function is both through direct and indirect cellular interactions. Direct interactions are mediated via integrin-integrin

binding. Atleast 5 integrins recognise TN-C as a ligand with 4 of these binding to the fibronectin type III repeat including α9β1, αvβ3 and αvβ6 *(Yokosaki et al, 1996)*. Indirect mechanisms have also been described such as inhibition of binding between fibronectin and syndecan-4 by TN-C. Syndecan-4 is a transmembrane heparin sulphate proteoglycan, which, in synergy with integrin α5β1, is required for cells to spread on FN *(Saoncella et al, 1999)*. Cells need to bind via both α5β1 and syndecan-4 to activate Rho signalling which leads to actin stress fibre assembly and cell spreading *(Midwood et al, 2002)*. Interference with the syndecan-4 binding site on FN via FNIII repeat 13 on TN-C has been demonstrated to reduce adhesion and increase proliferation of cancer cells in vitro *(Huang et al, 2001)*. However, as TN-C is also known to interact with syndecan-4 itself, it may bind to this as well as FN *(Salmivirta et al, 1991)*.

In combination with FN, TN-C has been shown to upregulate expression of collagenases, stromelysins, 92-kDa gelatinase and c-fos in vitro *(Tremble et al, 1994)*. It also upregulates MMP-9 in a breast cancer cell line and this effect is maximal in combination with TGFß *(Kalembeyi et al, 2003)*. TN-C is also thought to promote invasion in combination with scatter factor/hepatocyte growth factor (SF/HGF) and this is attributed to inactivation of the GTPase RHoA involving the EGF-like repeats *(De Wever et al, 2004)*. An increase in cell proliferation has also been observed in endometrial cancer cell lines and this was particularly related to FNIII repeats 6-8 *(Marselina et al, 1999)*.

The process of new vessel formation or angiogenesis within the stroma is increasingly held to be vital to development of the malignant process. TN-C has been shown to be expressed by migrating endothelial cells and also to promote endothelial cell adhesion, *(Zagzag et al, 2002)* suggesting that it may be involved in pathological angiogenesis. TN-C accelerates the migration of cardiac myofibroblasts and αSMA expression which is important in wound healing *(Tamaoki et al 2005)*. Mesenchymal TN-C has also been shown to be essential to the production of cancer cell derived VEGF, a factor which is widely observed at the site of angiogenesis in tumours *(Tanaka et al, 2004)*.

There are several regions located in the variable spliced part of the TN-C molecule which are of functional significance. As mentioned above, annexin II binds to repeats A-D *(Matsuda et al, 1999)*, FNIII repeat A3 is the only area susceptible to cleavage by MMP-2 and MMP-3 and the only MMP able to degrade short tenascin is MMP 7

which cleaves a site adjacent to the NH_2 terminal sequence *(Siri et al, 1995)*. In addition, long TN-C has been shown to inhibit T cell activation while short TN-C did not. Furthermore the region A1A2 was identified as the minimal additional regions required to produce this effect *(Puente Navazo et al, 2001)*. Differences have been shown in the ability of long and short TN-C variants to affect the adherence of chondrosarcoma cells to FN with the long variant failing to promote specific cell attachment in contrast to shorter variants *(Ghert et al, 2001)*. Thus it is clear that the precise splice variant of TN-C could be markedly influence the effect of the stromal microenvironment.

1.6.3. **Control of tenascin expression:**

TN-C is derived mainly from stromal cells although there are some carcinoma cells which have been shown to produce TN-C *(Yoshida et al, 1997; Vollmer et al, 1997; Yoshida et al, 1999)*. Production of TN-C by fibroblasts has, however, been shown to be greatly increased in the presence of cancer cells *(Wilson et al, 1999)*. Specific factors that have been implicated in this process include TGFß, insulin-like growth factor (IGF), progesterone and FGF *(Rettig et al, 1989)*. Of these, TGFß and FGF have been shown to promote production of the larger isoforms *(Schwogler et al, 1992; Tucker et al, 1993)*. In rat smooth muscle cells, TN-C production is stimulated by PDGF-BB and this was also found to affect production of the larger splice variants *(LaFleur et al, 1994)*. The nature and number of splice variants produced has also been shown to be affected by small variations in pH from 7.2 to 6.9, suggesting that changes in the acidity of the microenvironment may determine which isoforms are produced in pathological conditions *(Borsi et al, 1995)*. It is thought that there are a variety of regulatory elements flanking the TN-C gene and a diverse number of initiators of expression *(Jones et al, 1990)*. Mechanical stress has been shown to influence TN-C expression at the level of transcription *(Chiquet-Ehrismann et al, 1994)* and this is supported by the presence of a stretch responsive element in the promoter region *(Chiquet, 1999)*.

1.6.4. **Tenascin distribution:**

TN-C is prominent during embryogenesis and has been observed at the time of somite formation in chick embryos *(Crossin et al, 1986)*. TN-C subsequently features in development of the nervous, vascular and musculoskeletal systems *(Chiquet &*

Famborough, 1984) and is produced at points of epithelial-mesenchymal transition *(Ekblom & Aufderheide, 1989)*. Levels of TN-C fall in adult tissues and it is usually only expressed in areas of neo-vascularisation and wound healing *(Mackie et al, 1988)*. Higher levels are also found in pathological conditions, however, such as malignancy, inflammation and hypertensive disease *(Riedl et al, 1992; Laitinen et al, 1997; Jones & Rabinovitch, 1996)*.

TN-C is generally produced at low levels in the normal adult and is produced by the mesenchyme rather than mucosal or epithelial cells *(Perreault et al, 1998)*. The distribution of TN-C has been extensively studied in a variety of tissues. It is found in the basement membrane of some lung specimens *(Laitinen et al, 1997)* and is reported to be positive in the basement membrane and adjacent lamina propria in normal bladder mucosa *(Booth et al, 2002)*. In the uterine cervix it is present as a thin band within the basement membrane *(Iskaros & Koss, 2000)* and normal breast tissue expresses TN-C as a discontinuous band around ducts and acini and occasionally around vessels *(Ioachim et al, 2002)*. Some sinusoidal staining has been observed in liver *(Le Bail et al, 1997)*. Tenascin is also detectable in the smooth muscle cells of the media of normal aorta *(Satta et al, 1997)* and this reflects the findings with muscular blood vessels in other tissues *(Adams et al, 2002)*. TN-C mRNA has also been observed in significant amounts in normal brain *(Carnemolla et al, 1999)* where it is predominantly produced by astrocytes *(Ferhat et al, 1996)*.

1.7. TENASCIN IN DISEASE

1.7.1. **Inflammation:**

The level and distribution of TN-C expression is known to change in a variety of malignant and non-malignant pathological processes. In asthma, TN-C levels increase in the bronchial subepithelial basement membrane of subjects with chronic and seasonal asthma compared with controls *(Laitinen et al, 1997)*. TN-C expression is also noted to change in inflammatory diseases of the colon such as ulcerative colitis with an alteration in the magnitude and distribution of expression *(Riedl et al, 1992; Dueck et al, 1999)* although there is no evidence of any alteration in tenascin distribution in coeliac disease *(Korhonen et al, 2000)*. Benign breast lesions such as fibroadenomas show increases in TN-C and some stromal staining *(Yoshida et al, 1997; Adams et al, 2002)*. Similarly, in cervicitis, TN-C levels in the basement

membrane are increased and there is also tenascin present in the adjacent stroma *(Iskaros et al, 2000; Buyukbayram et al, 2002).*

In atherosclerotic vascular disease, increased TN-C expression is associated with the plaque characteristics of thrombus, angiogenesis and intraplaque haemorrhage *(Kajiwara et al, 2004)* and higher levels are present in those patients with acute coronary syndrome than stable angina. TN-C is also found at the interface between acutely infarcted and normal myocardium after myocardial infarction *(Imanaka-Yoshida et al, 2005).* TN-C is more prominent in abdominal aortic aneurysms than aortic occlusive disease but is higher in both compared to normal vessels and is specifically associated with inflammatory cells *(Satta et al, 1997).* This is in keeping with the finding that the level of TN-C expression in atherosclerotic plaques is related to the degree of inflammation rather than the plaque size *(Wallner et al, 1999).* As vascular disease is often thought to be a response to chronic injury, TN-C expression would be expected under these circumstances. TN-C has been co-localised with proliferating smooth muscle cells in the endothelial layer of pulmonary arteries in pulmonary hypertension *(Jones & Rabinovitch, 1996)* and it has also been demonstrated to increase following balloon catherisation *(Wallner et al, 2002).*

1.7.2. **Pre-invasive malignancy:**

Several studies have examined the change in TN-C distribution in *in situ* neoplasia to investigate the possibility of using stromal TN-C as a predictor of invasive disease. In cervical intraepithelial neoplasia (CIN) expression is increased in the basement membrane compared to normals and there are small amounts of stromal immunoreactivity in some cases although this is not related to grade *(Iskaros & Koss, 2000; Buyukbayram & Arslan, 2002).* The same pattern of increased basement membrane staining and related stromal staining is observed in vulval intraepithelial neoplasia (VIN) *(Goepel et al, 2003).* In cases of ductal carcinoma in situ (DCIS) in the breast there is also increased basement membrane staining around affected ducts and some localised stromal positivity *(Adams et al, 2002).* There is no true *in situ* disease state in the colon but studies examining adenomas have described loss of polarity in basement membrane staining with more generalised pericryptal staining than the usual localised staining at the top of the crypts *(Riedl et al, 1992).* A further study has reported TN-C mRNA in the stroma in a minority of adenomas *(Hanamura et al, 1997).*

1.7.3. **Invasive Malignancy:**

Most solid tumours that have been examined have shown an increase in TN-C expression and an alteration in distribution leading to increased stromal expression. Some of the tumours examined are summarised in Table 1.2

Tumour	**References**
Bladder	Titta et al 1993
Breast	Mackie et al 1987
Chondrosarcoma	Ghert et al 2001
Colon	Riedl et al 1992; Sakai et al 1993
Gastric	Ilunga & Iriyama 1995
Hepatocellular	Le Bail et al 1997
Lung	Soini et al 1993
Pancreas	Esposito et al 2006
Pleural mesothelioma	Kaarteenaho-Wiik et al 2003
Oesophageal	Salmela et al 2001
Oral squamous cell carcinoma	Harada et al 1994; Shrestha et al 1994
Ovary	Wilson et al 1996
Salivary gland tumours	Soini et al 1992
Thyroid	Tseleni-Balafouta et al 2006
Uterine cervix	Iskaros & Koss 2000

Table 1.2: Solid tumours expressing tenascin

Although TN-C expression is predominantly found in the stroma of solid tumours there is increasing evidence that some cancer cell lines will produce TN-C and also that the presence of such cells enhances TN-C expression in fibroblasts *(Wilson et al, 1999)*. Yoshida et al (1997) used in situ hybridisation to demonstrate TN-C mRNA within epithelial tumour cells and Dandachi et al (2001) subsequently described TN-C expression in the breast cancer cell lines HS 578T (carcinosarcoma), SK-BR-3, MDA-MB-231 (fibroblast-like) and HBL 100 (myoepithelial-like). Generally the production of TN-C by epithelial cells usually relates to the acquisition of some

mesenchymal characteristics such as those seen in epithelial-mesenchymal transition. The melanoma cell line SK-MEL 28 is known to produce a variety of TN-C isoforms *(Siri et al, 1991)* and there have also been reports of TN-C expression in laryngeal *(Yoshida et al, 1999)* and endometrial cancer cells *(Vollmer et al, 1997)*.

1.7.4. **Tenascin isoforms in disease:**

As well as a quantitative change in TN-C, it has been postulated that there may also be a qualitative change in the molecule with different isoforms occurring in pathological states compared to normal adult tissues. The presence of different TN-C isoforms in disease is well established *(Borsi et al, 1992)* but investigations into the nature and significance of these isoforms in specific pathologies is continuing. In particular, several studies have observed a switch from predominance of short TN-C to the long form. This has been clearly demonstrated in chondrosarcoma *(Ghert et al, 2001)* and breast *(Tsunoda et al, 2003)*. The precise nature of the isoforms expressed in breast carcinoma was examined in further detail by Adams et al (2002) who showed a significant association between the presence of exon 16 or exons 14 and 16 (14/16) and the malignant phenotype and also between exons 14/16 and the invasive phenotype. It was noted that some DCIS cases also expressed 14/16, raising the possibility that this represented a subset with greater invasive potential. The tenascin isoform profile in colon was examined in normal colon, ulcerative colitis, primary malignancy and metastatic disease by Dueck et al (1999). This study looked specifically at FN repeats A_3, A_4 and D (exons 12, 13 and 16) and demonstrated a switch in isoform profile with upregulation of all the isoforms in inflammation and a further increase in malignancy compared to normal colon. Interestingly, however, levels declined in metastatic disease. High grade astrocytomas have been shown to express higher levels of TN-C isoforms which contain FNIII repeat C, in contrast to normal tissues and other malignancies *(Carnemolla et al, 1999)*. Studies of oral malignancy led to the identification of a splice variant specific to malignancy; ad2 which was only detected in malignant samples *(Mighell et al, 1997)*.

There is also evidence that TN-C isoform profile alters in non-maligant conditions. Long TN-C isoforms are present in chronic pancreatitis but not in normal pancreas *(Esposito et al 2006)*. Following balloon catheterisation of the aorta in rats, smooth muscle cells were found to express TN-C isoforms containing A_1A_2 without any other FNIII repeats from the variable region. It was concluded that this isoform was

produced in response to arterial injury and that it promoted smooth muscle cell chemotaxis *(Wallner et al, 2002)*. In atherosclerotic plaques, macrophages produce only the short isoform of TN-C whereas smooth muscle cells produce both long and short isoforms *(Wallner et al, 1999)*. In combination, these results suggest that patterns of TN-C isoform expression may be both pathology and tissue specific.

1.7.5. **Therapeutic uses for tenascin:**

The prominent role of TN-C in a variety of pathologies has led to speculation about its therapeutic potential. As described above, TN-C expression is increased in pulmonary hypertension *(Wallner et al, 1999)* where it is induced by MMPs. By blocking MMPs and elastases, hypertrophy within rat pulmonary arteries was seen to regress and this was accompanied by a reduction in TN-C. Selective repression of TN-C with antisense/ribozyme constructs prevented further thickening but did not induce regression *(Cowan et al, 1999)*.

The only established therapeutic modality in current clinical practice is the use of radio-labelled anti-TN-C antibodies following surgical treatment of glioblastoma and anaplastic astrocytomas. The aim is to deliver well localised post-operative radiotherapy to the resection cavity with minimal effect on the rest of the brain. Several different antibodies have been utilised, some targeted against the variable region and some recognising all forms of tenascin. Those recognising an epitope in the variable region *(Akabani et al, 2000; Reardon et al, 2002)* are based on the principle that there is a switch in TN-C isoform expression from the short to long form in malignancy whereas use of an antibody against a common region (EGF repeats) attribute its efficacy to a general increase in TN-C levels around the tumour *(DeSantis et al, 2003)*. The identification of tumour-specific isoforms of TN-C and their potential effect on tumour behaviour make them attractive targets for therapy and this was the rationale behind focussing on this particular ECM protein for this study.

1.8. HYPOTHESIS AND AIMS

Fibroblasts play a central role in creating and maintaining the microenvironment and directing epithelial cell function. There are changes in fibroblast phenotype in malignancy which alter the microenvironment and contribute to disease progression. The colonic fibroblast population is particularly diverse making interpretation of phenotypic changes more difficult. However, to understand the potential contribution of the fibroblast population to colonic tumour progression these changes need to be accurately determined. One of the stromal changes frequently identified in solid tumours is an increase in TN-C expression and, in some tissues, specific tumour-associated TN-C isoforms have been identified. It is not well established whether there are TN-C isoforms specific to colorectal malignancy and, if so, whether these isoforms are present in pre-invasive malignancy and could contribute to tumour invasion and therefore possibly predict behaviour.

The hypothesis of this study is that changes occur in fibroblast populations which may enable tumour invasion and progression. We hypothesise that there are alterations in fibroblast phenotype which alter the tumour microenvironment and thus facilitate tumour invasion. We further hypothesise that such changes include the production of a specific TN-C isoform profile which facilitates tumour invasion.

In order to investigate these hypotheses the specific aims are:

-To characterise fibroblast sub-populations in normal colon and the phenotypic changes in pre-invasive and invasive malignancy

-To establish the expression pattern of TN in normal and diseased colon and relate changes to fibroblast phenotype

-To analyse in detail the TN isoform profile of normal and diseased colon and to establish whether there are tumour specific isoforms

-To address whether TN isoform profile relates to tumour invasive properties

Chapter 2

Materials and Methods

2.1. MATERIALS

2.1.1. **Immunohistochemistry**

Primary antibodies:

CD34: anti-CD34 mouse monoclonal antibody (Novocastra Laboratories Ltd); clone QBEnd10; recognises human endothelial cells *(Ramani et al, 1990)*.

CD31: anti-CD31 mouse monoclonal antibody (Dako); clone JC70A; recognises human endothelial cells *(Parums et al, 1990)*.

αSMA: anti-human smooth muscle actin mouse monoclonal antibody (Dako); clone 1A4; recognises α-smooth muscle isoform of actin *(Skalli et al, 1986)*.

Vimentin: anti-vimentin mouse monoclonal antibody (Dako); clone Vim 3B4; recognises vimentin *(Heid et al, 1988)*.

hCD: anti-human caldesmon mouse monoclonal antibody (Dako); clone h-CD; recognises 150kDa variant of high molecular weight human caldesmon *(Frid et al, 1992)*.

c-kit: (also designated CD117) anti c-kit polyclonal antibody (Dako); recognises c-kit protein (specifically amino acids 963 to 976 at c terminus) *(Matsuda et al, 1993)*.

Total tenascin: anti- human tenascin mouse monoclonal antibody (Sigma); clone BC-24; recognises epitope within EGF-like sequence.

Tenascin 14: anti-human mouse monoclonal antibody (Chemicon International); clone αIIIB; recognises epitope within domain B of alternatively spliced fibronectin repeats *(Balza et al, 1993)*.

Secondary antibodies:

Biotinylated rabbit anti-mouse immunoglobulins (Dako)

Biotinylated swine anti-rabbit immunoglobulins (Dako)

Tertiary antibody and visualisation:

Streptavidin-biotin peroxidase complex (ABC) (Dako)

Diaminobenzidine tetrahydrochloride (DAB) (Sigma)

Other reagents:

Acetone (CH_3COCH_3) (Fisher Scientific)

3- aminopropyltriethoxysilane (Acros)

Calcium chloride 2-hydrate ($Ca\ Cl_2.2H_2O$) (BDH)

Citric acid monohydrate ($C_6H_8O_7.H_2O$) (Fisher Scientific)

DPX mountant (BDH)

Eosin (VWR)

Haematoxylin (VWR)

Hydrochloric acid (HCl) (Fisher Scientific)

Hydrogen peroxidase (12%) (H_2O_2) (Fisher Scientific)

Industrial methylated spirit (99%) (Genta Medical)

Normal rabbit serum (Gibco)

Normal goat serum (Gibco)

OCT embedding medium (Raymond A Lamb Laboratory Supplies)

Silica gel (Self-indicating) (BDH)

Sodium hydroxide (NaOH) (Fisher Scientific)

Trypsin 250 (Becton Dickinson)

Vectabond

Xylene (Genta Medical)

2.1.2. **mRNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)**

Cell lines:

SKMel 28 was derived from malignant melanoma in a 51 year old male and obtained from the Department of Obstetrics and Gynaecology, University of Leicester

AMV 5x buffer (Promega)
100 Base pair ladder (Sigma)
dNTP mix (Invitro)
Oligo$(dT)_{25}$ dynabeads (Dynal)
RNAsin (Promega)
RT enzyme (Promega)
TAQ enzyme (Promega)
Primers: all primers were purchased from Genosys as lyophilised oligonucleotide pellets. (All forward primers supplied 5′ biotinylated). See Table 5:1 for sequences

Other reagents:

Chloroform ($CHCl_3$) (Fisher Scientific)
Ethanol (absolute) (CH_3COOH) (Sigma)
Ethidium bromide (Sigma)
Glycine (Invitrogen)
Isopropanol ($(CH_3)_2CHOH$) (Fisher Scientific)
Light liquid paraffin (South Devon Healthcare)
Seakem agarose (BioWhittaker Molecular Applications)
Tri-reagent (Sigma)
Tris (2-amino-2-(hydroxymethyl-1,3-propanediol) ($C_4H_{11}NO_3$) (Roche)

2.1.3. **Invasion Assays:**

Cell lines:

All cell lines were purchased from the American Type Culture Collection Rockville, Maryland
hfff2 human fœtal foreskin fibroblast cell line
HCT15 was isolated from a Dukes C colonic adenocarcinoma from an adult male patient.

HT-29 was isolated from a colonic adenocarcinoma from an adult female patient *(Trainer et al, 1988)*.
HCT116 was isolated from a colonic carcinoma from an adult male patient *(Brittain et al, 1981)*.
SW480 was isolated from a Dukes B colonic adenocarcinoma from an adult male patient *(Leibovitz et al, 1976)*.
SW620 was isolated from a lymph node metastasis from a Dukes C colonic adenocarcinoma from an adult male patient *(Leibovitz et al, 1976)*.

Media:
Complete medium: Dulbeccos Modified Eagles Medium (DMEM, Sigma) + 10% Fœtal Bovine Serum (FBS, Harlan Laboratories) + 2 mM L-Glutamine (Sigma).
Serum free medium: Dulbeccos Modified Eagles Medium (DMEM, Sigma) + 2mM L-Glutamine (Sigma).

Other reagents:
Aquamount (BDH)
Cotton buds
Fibronectin from bovine plasma diluted to 1μg/ml in dPBS (Sigma)
Growth factor depleted Matrigel diluted to 10μ/ml in SF DMEM (basement membrane-like matrix derived from the Engelbroth-Holm-Swarm (EHS) mouse sarcoma. Major components: laminin with Collagen IV, heparin sulphate proteoglycans, entactin and nidogen) *(Kleinman et al, 1982)* (Sigma)
Phosphate buffered saline (PBS) (Sigma)
12 well inserts with 8μm pore (Falcon, Becton Dickinson)

2.2. METHODS

2.2.1. **Source of tissue**

Immunohistochemistry:

Colorectal tissue was collected as part of a new, prospectively consented colorectal tissue bank. Informed consent for retention of tissue for research purposes was obtained from patients undergoing colorectal resections in accordance with local ethical guidelines. The specimens were collected fresh from the operating theatre and inspected by a consultant histopathologist who authorised removal of a portion of tissue for research purposes when appropriate. Initially pieces of tissue measuring approximately 10 x 5mm were fixed to a sliver of cork with embedding medium (OCT) and snap frozen in liquid nitrogen but later larger specimens measuring about 10mm in diameter fixed onto entire cork discs were used. Usually atleast two pieces of diseased tissue were taken from each patient, accompanied by the equivalent number of normal pieces of tissue from the same patient (normal being defined as atleast 5cm from the tumour). The smaller sections were then transferred to screw cap eppendorf tubes while the larger pieces were wrapped in aluminium foil for long term storage at -196°c in a liquid nitrogen storage dewar (Statebourne Cryogenics BioRack). Frozen sections were cut from this tissue and stained. In addition, dedicated formalin fixed paraffin embedded (FFPE) research blocks were taken after the whole specimen had been processed, again with the agreement of the consultant histopathologist. Sections from these blocks were also used for immunohistochemistry.

In addition to the prospective tissue bank material, some immunohistochemical studies also included analysis of archival tissues, these cases comprised patients who had had colonic resection for adenocarcinoma (Dukes stage A, B or C) or removal of an adenoma (villous, tubulovillous or tubular; mild, moderate or severe dysplasia). The FFPE blocks were retrieved from the archive at Glenfield Hospital in Leicester then cut and irreversibly coded by a third party before immunohistochemistry was performed on sections.

Reverse transcription- polymerase chain reaction (RT-PCR):

mRNA was retrieved from fresh frozen tissues in the consented colorectal tissue bank. In addition, cDNA was supplied on four colorectal cell lines which had been supplied by ATCC and grown up in the laboratory by technical staff.

2.2.2. **Immunohistochemistry**

Tissue sectioning:

Formalin fixed paraffin embedded blocks:

4μm sections were cut and floated on a water bath at 50°c and then transferred onto glass slides treated with either 2% 3-aminopropylethoxysilane or vectabond, depending upon the proposed method of antigen retrieval. Slides were kept at 37°c overnight then stored at room temperature. The slides were generally used within two weeks of sectioning as a deterioration in staining with some antibodies, especially those for TN-C, was demonstrated after this time. Immediately prior to staining, the sections were dewaxed in xylene then rehydrated using a series of alcohols (2x 99% IMS; 95% IMS), returned to water and equilibrated in tris buffered saline (TBS).

Cryopreserved samples:

4μm sections were cut using a cryostat at -20°c and transferred onto plain glass slides. The slides were kept at -20°c in a sealed container with silica gel for up to 48 hours before use. Prior to staining slides were air dried at room temperature for 20 minutes, fixed in acetone for 10 minutes at 4°c then equilibrated in TBS.

Haemotoxylin and eosin staining:

All the blocks, both frozen and FFPE, had a section cut for haematoxylin and eosin (H&E) staining to enable optimal histological evaluation of the sections. The FFPE sections were firstly dewaxed in xylene and rehydrated by immersion in graded alcohols (99% 2x 2 mins; 95% 1x 2 minutes) with periodic agitation and were then washed in running tap water for 1 minute. The frozen sections were fixed in 95% alcohol then washed in running water. Both types of section were then immersed in Meyers haematoxylin (FFPE sections 2 mins; Frozen sections 30 secs), washed in water for 2 minutes and then placed in 0.5% eosin (FFPE sections 30 secs; Frozen sections 10 secs). Sections were washed in running water to remove excess stain and transferred to 95% alcohol. The sections were then dehydrated through successive

alcohols and xylene then mounted using DPX mountant and covered with an appropriately sized coverslip.

Antigen retrieval:

Pressure cooking:

A solution of 1.5l of 10mM citric acid buffer pH 6.0 was prepared and heated in a pressure cooker until it boiled. The slides were then placed in a rack in the solution and cooked for 1, 2 or 3 minutes at pressure. After this time the pressure cooker was cooled using cold water and the slides were removed and washed in water for 5 minutes before being returned to TBS.

Trypsin antigen retrieval:

A solution of 0.1% trypsin and 0.1% calcium chloride pH 7.8 was prepared and warmed to 37°c in a waterbath. The solution was then applied directly to the slides and incubated at 37°c for 5, 10 or 15 minutes after which time the slides were washed in water for 5 minutes and returned to TBS.

Microwave antigen retrieval:

Slides were placed in racks in a plastic container filled with 10mM sodium citrate pH 6.0. The container was loosely covered and microwaved at full power for 5, 10 or 15 minutes after which time the container was removed and allowed to cool for 20 minutes at room temperature. The slides were then placed in TBS.

Water bath antigen retrieval:

A solution of 10mmol/l Tris and 1mmol/l EDTA pH 9.0 was prepared and heated to 95-97°c in a water bath. When the required temperature had been attained, the slides were added and incubated for 15 minutes. Slides were then removed, washed in water and returned to TBS.

Streptavidin-biotin complex peroxidase technique:

Following antigen retrieval and incubation in TBS, the slides were placed in a humid chamber and covered with 100µl 20% normal serum (normal rabbit serum for monoclonal antibodies; normal goat serum for polyclonal antibodies) for 10 minutes. 100µl of appropriately diluted primary antibody prepared in 20% normal serum was

then added to test sections while serum was left on the negative controls. The sections were incubated at room temperature for 1 hour then rinsed in water and washed twice in TBS for 5 minutes. 100µl of secondary antibody (rabbit anti-mouse diluted 1:400 in TBS for monoclonal antibodies; swine anti-rabbit diluted 1:600 for polyclonal antibodies) was then applied for 30 minutes and the slides were then rinsed in water and washed twice in TBS for 5 minutes. The tertiary antibody had been prepared at the same time as the secondary antibody and was composed of 1µl streptavidin and 1µl biotinylated horseradish peroxidase in 1000µl TBS. This was applied to the slides for a further 30 minutes after which they were rinsed in water and washed in TBS for 5 minutes then covered in diaminobenzidine (DAB) solution for 5 minutes. A 0.5ml aliquot of DAB solution was made up to 10mls with TBS and 100µl 3% hydrogen peroxide. The DAB was washed off with TBS and running tap water, after which the slides were counterstained with Mayers haematoxylin for 20 seconds, washed again and dehydrated through a series of alcohols and xylene. The slides were mounted using DPX mountant and appropriately sized cover slips.

Optimisation:

The optimum method of antigen retrieval and primary antibody dilution was determined for each antibody in turn and this is described in the relevant results chapters. Some antibodies did not require antigen retrieval, in some cases the method used was not that recommended on the data sheet and some of the methods listed above were not used at all in the final assessments.

Controls:

As indicated above, for each antibody a negative control was included to assess non-specific staining. In addition, where possible, a suitable positive control known to stain with the antibody in question was included with each batch of slides. In most cases this was a breast carcinoma specimen although a colon carcinoma previously demonstrated to be positive was employed for some of the tenascin staining and small bowel was used for CD117.

Evaluation of immunohistochemistry:

All slides were examined independently by two observers, one of whom is a consultant histopathologist. In most cases, staining was simply recorded as being present or absent as the staining, where present, was generally homogenous. However, when assessing αSMA staining in the lamina propria, the intensity of staining was recorded as this varied between cases.

2.2.3. **mRNA extraction and RT-PCR**

Frozen sections for mRNA extraction:

4μl sections were cut using a cryostat and placed in sterile eppendorf tubes. Generally about 15 sections were added to each tube. 1000μl tri-reagent was then added to each of the tubes which were kept at -80°c until required.

mRNA extraction and reverse transcription:

Tissue specimens:

The tubes containing tri-reagent and tissue were returned to room temperature and agitated to ensure good mixing. 200μl chloroform was added, mixed and left for 5 minutes at room temperature then the tubes were centrifuged at 13000rpm at 4°c for 15 minutes. The clear aqueous layer was carefully transferred to another tube to which 500μl tri-reagent and 100μl chloroform were also added. After mixing, tubes were left for 3 minutes then centrifuged for 15 minutes. The aqueous layer was again removed and transferred then 500μl isopropanol and 1μl glycine was added. The mixture stood for 10 minutes at room temperature before being centrifuged for 10 minutes. The supernatant was then removed and discarded to leave a pellet which was resuspended in 500μl 70% ethanol and centrifuged at 4°c for 15 minutes. The supernatant was again discarded and the pellet was air dried before resuspension in 20μl PCR grade water to which 80μl lysis binding buffer (LBB) was then added.

At this stage, the quantity of total RNA present could be assessed using a spectrophotometer (Genesys™ 10 series spectrophotometer). 1μl of each RNA sample was diluted in 99μl sterile water and the absorbency at 260nm and 280nm was measured to assess DNA and RNA content and ratio. If the Promega kit was being used, this could then be used to assess how much total RNA to add to the reaction.

Cell line specimens:

The cells were stored as lysates in 100µl lysis binding buffer so were defrosted and then incubated with 5µl proteinase K (1mg/ml) for about 60 minutes at 37°c. The lysates were then sheared through 21 gauge and 25 gauge needles before proceeding with the mRNA extraction.

Oligo $(dT)_{25}$ dynabeads:

If oligo $(dT)_{25}$ dynabeads were being used, an aliquot containing 30µl per reaction was washed in LBB and resuspended in the original starting volume using LBB. This was done by pelleting the beads using a magnetic particle concentrator (MPC). 30µl beads was added to each RNA sample then mixed and incubated at room temperature for 5 minutes. This mixture was then pelleted and the supernatant was removed and discarded. The beads were resuspended in 60µl wash buffer with SDS and the process was repeated twice. After these two washes the beads were washed twice in wash buffer without SDS then finally resuspended in 30µl PCR grade water.

For the samples used in the final TN-C assessments, the RNA was specifically primed at this stage to optimise transcription of the area of interest. 4.5µl (10pmol/µl) of primer to the conserved fibrinogen domain T27R (See Chapter 5 for sequence) was added to each 30µl RNA specimen to produce a concentration of approximately 15nmols per reaction. The mixture was then heated to 70°c for 5 minutes.

A mastermix of reagents required for the reverse transcription reaction were prepared in the ratios shown below and kept on ice. Each 30µl RNA sample could be used to produce 2 positive reactions and 1 negative reaction with 15µl mastermix being added to 10µl bead suspension to produce a 25µl reaction.

	+ RT (µl)	**-RT (µl)**
5x AMV buffer	5	5
DNTP mix	2.5	2.5
H_2O	6.38	6.88
RNAsin	0.62	0.62
RT enzyme	0.5	-
Total	15	15

The tubes were incubated in the thermocycler (either GeneAmp PCR system 9700 Applied Biosystems or Perkin Elmer) at 42°c for 1 hour. The resulting cDNA was kept at 4°c until required.

RT Promega kit:

When this kit was being used to produce cDNA from total RNA, the volume containing 1µg RNA was calculated from the spectrophotometry measurements by the equation:

$$\frac{1}{260\text{nm absorbency} \times 4}$$

A 20µl reaction was then prepared using the following reagents:

Reagent	**Volume (µl)**
AMV-RT buffer	2
$MgCl_2$	4
dNTPs	2
RNAsin	0.5
dT primer	1.0
RNA	variable*
H_2O	variable**
RT enzyme	0.5***
Total	20

* According to spectrophotometer reading

** Varied to compensate for RNA volume

*** Omitted and replaced with H_2O in $^-$RT

The tubes were then placed in the thermocycler and incubated using the following cycle:

42°c for 15 minutes

99°c for 5 minutes

4°c for ∞

The resulting cDNA was stored at 4°c until required. Before the RT $^{+}$ve samples were used in a PCR reaction, the equivalent RT^{-}ve samples were checked for contamination using a PCR reaction for GAPDH. Once the lack of contamination had been established, only RT $^{+}$ve samples were used in the PCR reactions.

Polymerase chain reaction:

A mastermix of reagents for the PCR reaction was prepared and the required volume of cDNA was added (1-5µl) to produce a 50µl reaction. A standard reaction is shown below. Prior to use the AJ buffer was spun before use to precipitate bovine serum albumin (BSA) which inhibits the reaction.

	Volume (µl)
10x AJ buffer	5
Primer (forward)	1
Primer (reverse)	1
Sterile water	42*
cDNA	1**
Total	50

* This volume would be adjusted to compensate if increased volumes of cDNA were used

** This volume could be varied

One tube in each reaction would contain an equivalent volume of sterile water instead of cDNA to run a 'water blank' control. The 50µl reagents in the PCR were overlaid with 50µl light liquid paraffin and placed in the thermocycler. All PCR reactions were 'hot start' so, after the reaction had reached the annealing temperature for the first time, 1µl TAQ enzyme was added. (TAQ was diluted 1:5 in 1xAJ buffer to produce 1 unit e.g. for 20 tubes requiring 1µl TAQ each make up 4µl TAQ, 1.6µl AJ and 14.4µl

H_2O) The PCR primers and cycles used are shown in Chapter 5. PCR products were stored at 4°c until required.

Agarose gel electrophoresis:

Agarose gels were made up in 1, 2, 2.5 or 3% concentrations. Depending on the size of the gel to be run, the required weight of agarose was added to 50ml or 100ml 1x TAE and heated in the microwave until the agarose had dissolved. The solution was then cooled slightly and 10μg/ml ethidium bromide was added to produce a concentration of 1μl/100mls. The solution was poured into the gel plate which had previously been sealed at both ends with autoclave tape and which contained a comb to create the wells. It was allowed to cool until set. The tape and comb were then removed and the plate was placed in a gel electrophoresis tank containing enough 1xTAE (with 1μl/100mls ethidium bromide) to cover the plate. PCR products were added to the wells mixed with loading buffer in the quantities of 15μl product with 3μl loading buffer. 5μl of a 100 base pair DNA ladder was run alongside products of each primer set. Electrodes were attached to each end of the gel tank and the voltage was set between 70v and 100v. Once the dye had migrated the required distance through the gel, the power was disconnected and the gel was removed and photographed using an ultraviolet transilluminator. Images were recorded using the UVP VisionWorks 3.1™ software.

2.2.4. **Invasion assays**

These assays were undertaken by an experienced colleague in the laboratory and methods are included here for completeness.

Production of conditioned media:

Fibroblasts were plated into a $25cm^2$ tissue culture flask and grown to 60% confluency, after which the medium was removed. The cells were washed with dPBS, 3ml serum free (SF) DMEM was added and the fibroblasts were cultured for a further 48 hours. The media was then removed, centrifuged to remove any cell debris and the supernatant transferred into a clean tube and stored at -80°c until required.

For the reproducibility study, hfff2 cells were cultured in T75 tissue culture flasks. This produced 15ml of conditioned media (CM) enabling the invasion assays to be performed in triplicate using the same batch of media.

Conditioned media invasion assays:

200µl of fibronectin was coated onto the underside of the membrane of an 8µm pore 12 well insert and incubated for 30 minutes at room temperature, after which excess fibronectin was removed. The inserts were dried at room temperature for 1 hour before being plated into the 12 well plates. The topside of the membrane was then covered with Matrigel and incubated for 2 hours at 37°c. Excess Matrigel was then removed and the insets washed with SF DMEM. 4 x 10^5 cells from the relevant cell line in complete DMEM added to the insert and 1 ml complete DMEM was placed in the well to surround the insert. The cells were left overnight at 37°c to enable attachment. The media was then removed from the wells and inserts which were washed with SF DMEM. 0.5ml SF DMEM was added to the insert and 1ml 50:50 conditioned media: SF DMEM to the base of the insert. The assays were run for 48 hours.

Processing of invasion assays:

Supernatant was removed and inserts were washed with PBS and one side was scraped gently with a cotton bud to remove the cells. This allowed the other side of the insert to be counted easily. Inserts were fixed in acetone (8 minutes; 4°c), washed in PBS followed by tap water then stained in haematoxylin (1 minute) and washed again in tap water. At this stage the membrane base was carefully removed from the rest of the insert using a scalpel blade and mounted using Aquamount.

All assays were performed in duplicate, with 2 inserts processed for counting of the upper membrane and 2 inserts for analysis of the lower membrane surface. For assessment of reproducibility, the same CM and fibroblast population (hfff2 cell line) was analysed in triplicate in 3 separate experiments.

Analysis of invasion assays:

For each membrane, 20 representative fields were photographed on a Zeiss microscope at x200 magnification. These images were loaded into a Paint Shop Pro™ and the cells counted. The invasion index was counted as shown below:

% Invasion = (Total number of cells on top surface of 2 membranes ÷ Total number on top + bottom surface of 2 membranes) × 100

Chapter 3

Characterisation of colonic fibroblast phenotypes in health and disease

3.1. INTRODUCTION

The fibroblast is the key cell embedded in the ECM, producing many of the critical proteins and enzymes which remodel, repair and maintain the microenvironment that modulates epithelial function *(Braun et al, 2004)*. The colon contains a particularly diverse population of fibroblasts with different phenotypes and functions and this has potential significance for identifying processes amenable to modulation in pathological inflammation and malignancy. Several fibroblast markers have been highlighted as having putative importance, both in the characterisation of fibroblast sub-groups and for examining changes in the fibroblast phenotype in inflammation and malignancy. Such markers include CD34, αSMA, c-kit and high molecular weight caldesmon (hCD) *(Nakayama et al, 1999; Nakayama et al, 2000; Adeboyega et al, 2002)* but no study to date has systematically compared the markers CD34, αSMA and hCD across normal colon, adenomas and adenocarcinomas to include all degrees of dysplasia and stages of tumour progression. In addition no study has yet investigated the origin of the αSMA positive cells which have been previously reported to occur in the lamina propria of adenomas *(Adeboyega et al, 2002)* and whether these cells represent proliferation of the existing myofibroblast population, transformation of a different fibroblast group or recruitment of a further population of cells such as bone marrow derivatives.

The hypothesis of this study is that changes occur in fibroblast phenotypes in malignancy and that this altered phenotype contributes to disease progression. It is not yet clear whether such changes exist prior to invasion, although there is a precedent from other systems such as breast that this might occur and may be important, not only functionally but as a predictive marker. The aim of this part of the study therefore was to characterise fibroblast sub-populations in normal colon and phenotypic changes in pre-invasive and invasive malignancy using the fibroblast markers CD34, αSMA and hCD. Any changes between the tissues may indicate possible mechanisms by which the stroma initiates or facilitates invasion and progression of malignancy.

3.2. RESULTS

3.2.1. **Optimisation of immunohistochemistry:**

Each of the antibodies employed was optimised for use on FFPE colon tissue before use on the chosen sections. Modes of antigen retrieval included pressure cooking, trypsin antigen retrieval, microwave treatment and water bath treatment using the methods described in Chapter 2. Some antibodies did not require antigen retrieval to achieve optimal staining. The optimal dilution for each antibody was assessed, including that recommended by the manufacturers, although often a lower concentration could be used successfully. At all times, negative controls were also included for every case and these were all negative. The optimal conditions used for the remainder of the study are summarised in Table 3.1

Antibody	**CD34**	**CD31**	**αSMA**	**hCD**	**c-kit**
Ag retrieval	None	Trypsin (10 mins)	None	Pressure cook (2 mins)	None or Pressure cook (2 minutes)
Blocking serum	Normal rabbit serum	Normal rabbit serum	Normal rabbit serum	Normal rabbit serum	Normal swine serum
Primary Ab dilution	1:100	1:30	1:600	1:100	1:50
Secondary antibody	Rabbit anti-mouse	Rabbit anti-mouse	Rabbit anti-mouse	Rabbit anti-mouse	Swine anti-rabbit
Dilution	1:400	1:400	1:400	1:400	1:600
Buffer	TBS	TBS	TBS	TBS	TBS

Table 3.1 Optimised immunohistochemistry methods employed for five antibodies on colorectal specimens

A total of 101 cases were examined including 14 normal colon, 52 carcinomas including 18 Dukes stage A, 18 Dukes stage B and 16 Dukes stage C and 45

adenomas including tubular, tubulovillous (TV) and villous adenomas with mild, moderate and severe dysplasia.

Optimal staining for each case was not always achieved and the total number of cases analysed for each antibody is summarised in Tables 3.2, 3.3 and 3.4.

The pattern of staining for each antibody was highly consistent in relation to the histological characteristics as described below and in Tables 3.2, 3.3 and 3.4.

3.2.2. **Patterns of marker expression**

CD34

In normal colon CD34 stained sub-mucosal fibroblasts and this was also observed in adenomas where there was sufficient sub-mucosa to assess this. In tumours, CD34 expression was lost in the peri-tumoral stroma but this effect was very localised, as demonstrated by the Dukes A specimens *(See Table 3.2 and Figure 3.1)*. In addition, CD34 was expressed by cells lying in the region between the two layers of the muscularis mucosa as well as by blood vessels throughout the bowel wall, which served as an internal positive control.

αSMA

αSMA was not expressed in the sub-mucosal fibroblasts of normal or adenomatous tissues although it was visible in the lamina propria staining the peri-cryptal myofibroblasts *(See Figures 3.1 and 3.2)*. It was, however, strongly expressed in the peri-tumoral stroma in carcinomas of all stages *(See Table 3.3)*. There was also increased expression of αSMA in the lamina propria of all types of adenoma and in the normal mucosa adjacent to invasive malignancy *(See Figure 3.2)*. There was no clear relationship between extent of αSMA expression and adenoma type or degree of dysplasia.

h Caldesmon

h Caldesmon stained muscularis mucosa and muscularis propria throughout the colon wall and thus acted as an internal positive control for each of the sections examined. Staining with this antibody was inconsistent and therefore staining was only scored if the internal control was positive. hCD was seen to stain the pericryptal myofibroblasts in normal colonic mucosa but did not stain fibroblasts elsewhere in the colon. hCD staining was observed around the crypts in adenomas but there was less staining than

in the normal section. In invasive malignancy, hCD expression was not present around neoplastic glands and was not observed in the peritumoral stroma although expression was maintained in the pericryptal myofibroblasts of adjacent normal tissue *(See Figure 3.3)*.

CD31

This was included as a control to facilitate the process of distinguishing fibroblasts from endothelial cells when both stained with CD34 as fibroblasts do not stain with CD31 *(Ashman et al, 1991; Miettinen M et al, 1994)*. The quality of staining with CD31 was sub-optimal, even after several adjustments had been made to the process of antigen retrieval but positivity was observed in blood vessels in some specimens. As the assessment progressed, it became easier to distinguish the two cell types on a morphological basis.

c-kit

This marker was included to try and investigate further the population of CD34 positive cells found within the muscularis propria. Small bowel was used as a positive control since it is richer in interstitial cells of Cajal than colon. Local protocols advised that antigen retrieval led to over-detection of the antibody so attempts were made to perform staining without this step. However, no staining was demonstrated in normal colon or small bowel specimens without antigen retrieval. Antigen retrieval (pressure cooking for 2 minutes using an antibody dilution of 1:50) produced consistent staining of mast cells and some faint staining which might be attributed to ICCs but this was not convincing and therefore formal analysis was not carried out.

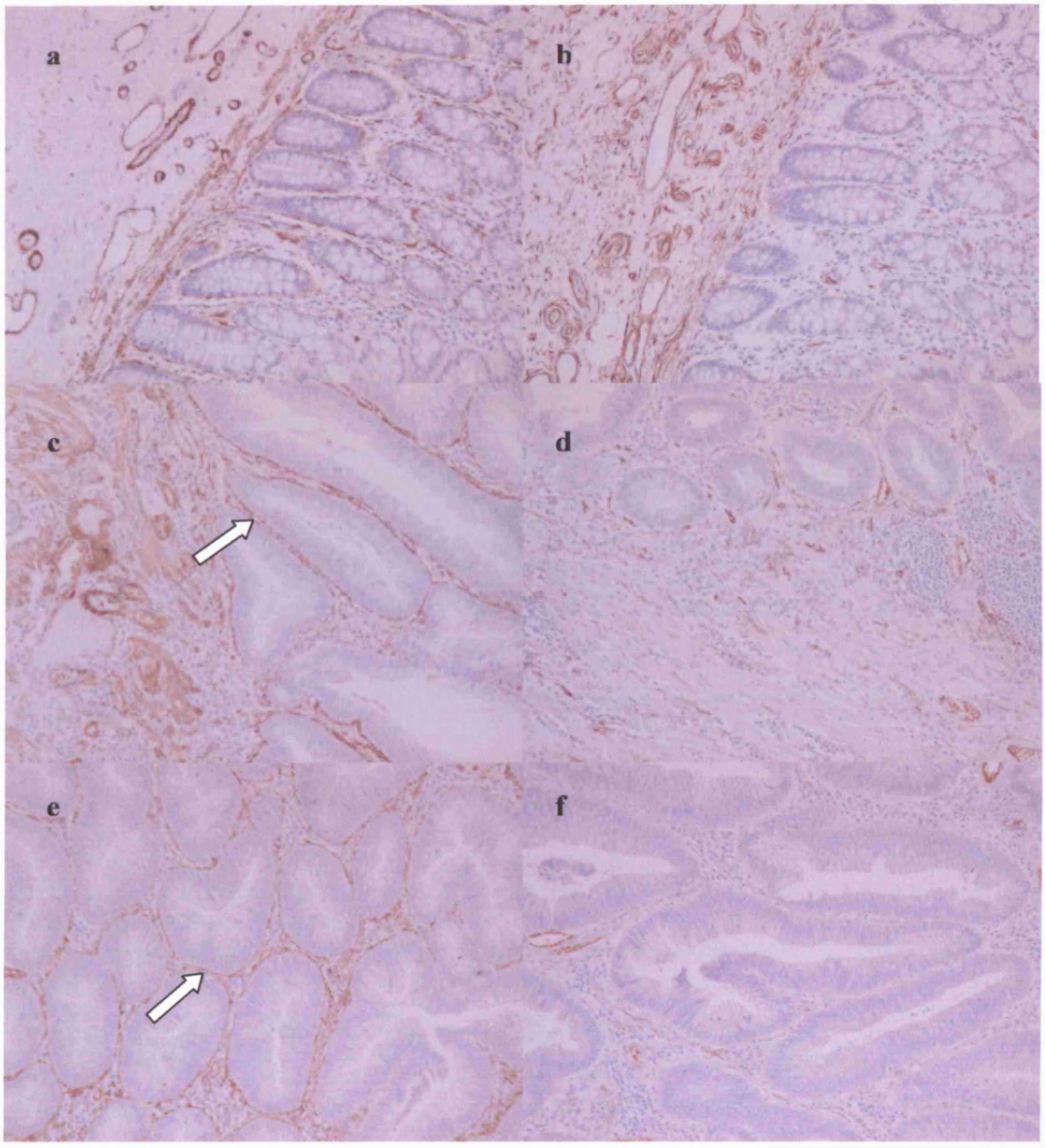


Figure 3:1 Immunohistochemistry for αSMA and CD34 in normal colon and colonic adenoma

The left panel (a, c and e) is stained for αSMA and the right panel (b, d and f) is stained for CD34. αSMA is detected in the pericryptal myofibroblasts (indicated by arrow) in normal (a) and adenomatous (c and e) tissue, in addition to staining the muscularis mucosa and the walls of blood vessels in the lamina propria.
Staining for CD34 is seen in the fibroblasts of the submucosa of the normal colon (b) and adenomas (d and f) as well as in blood vessel walls throughout the mucosa and submucosa (b, d and f).

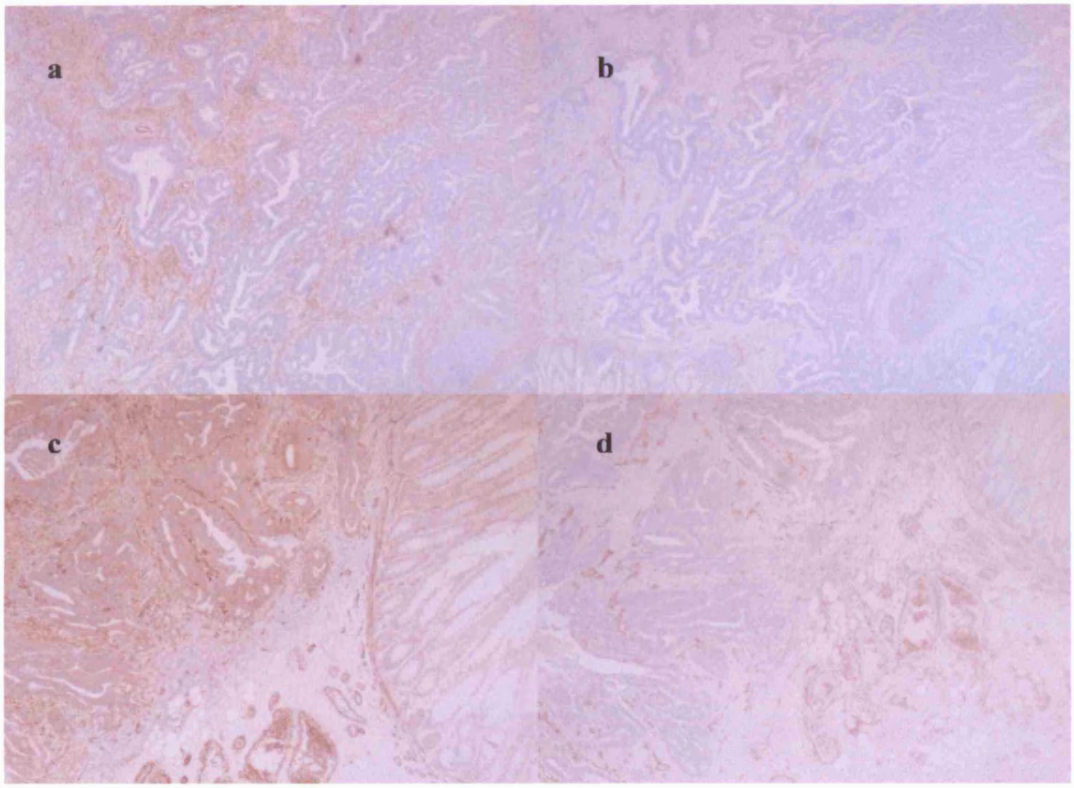


Figure 3.2 Immunohistochemistry for αSMA and CD34 in colorectal adenocarcinoma

The left panel (a and c) is stained for αSMA and the right panel (b and d) is stained for CD34. αSMA is strongly expressed throughout the peri-tumoral stroma (a and c) with c demonstrating the localised nature of this expression.
CD34 expression is absent in peri-tumoral stroma but this phenomenon is again very localised with d illustrating the presence of the CD34 staining in normal sub-mucosa adjacent to the tumour.

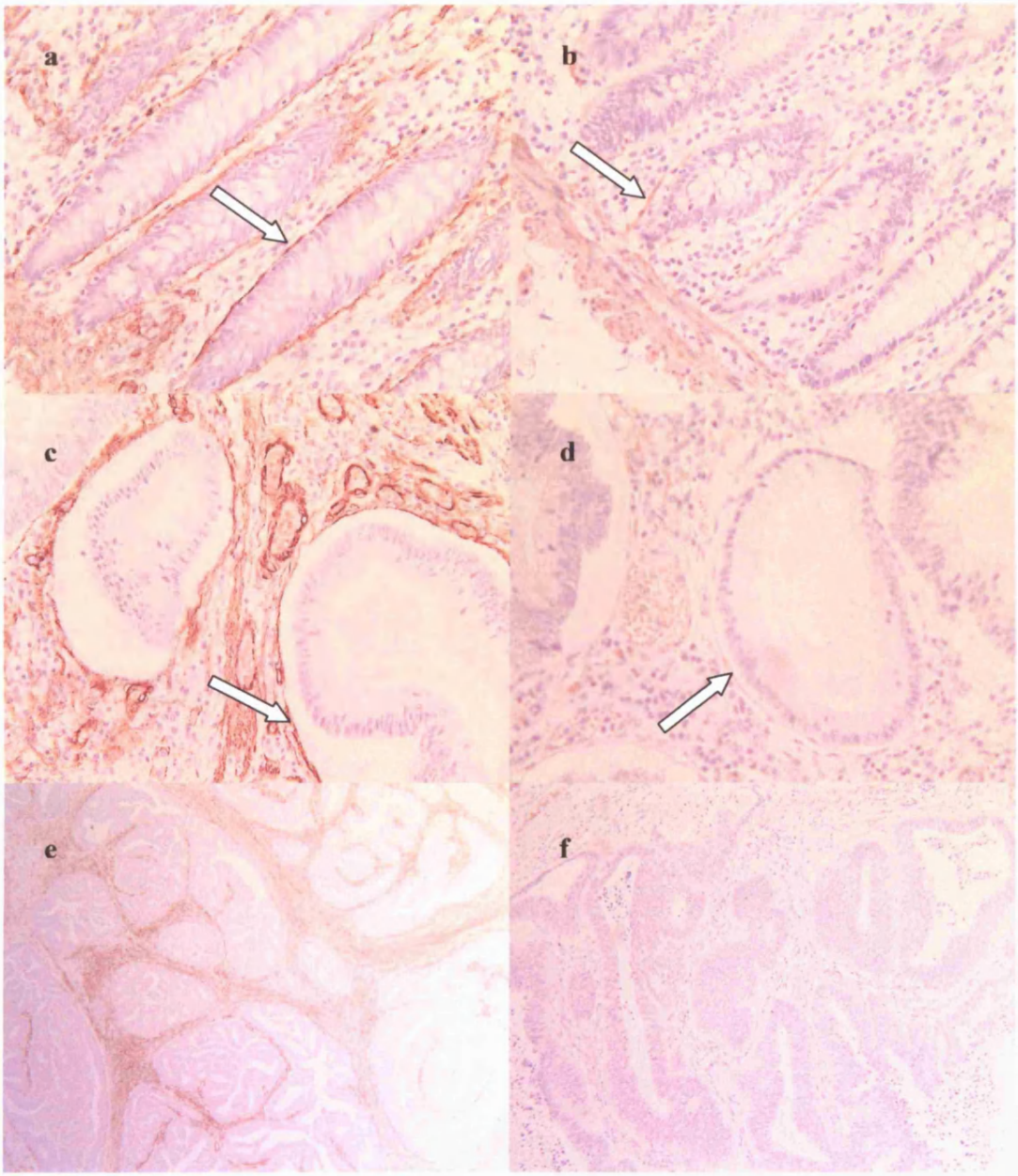


Figure 3.3 Immunohistochemistry for αSMA and hCD in normal colon, adenoma and adenocarcinoma

The left panel (a, c and e) is stained with αSMA and the right panel (b, d and f) is stained with hCD.
The peri-cryptal myofibroblasts are positive for αSMA in normal (a) and adenomatous (c) colon (as indicated by the arrowheads) and there is strong stromal staining with αSMA in adenocarcinoma (e).
The peri-cryptal myofibroblasts are positive for hCD in normal colon (b) but staining is weaker in adenomas (d) as indicated by the arrowheads. No hCD staining is detected in adenocarcinoma (f).

Table 3.2 Presence of CD34 staining in the submucosa of normal, adenomatous and malignant colon

Histology (Type and Degree of dysplasia)	Submucosal fibroblasts CD34 $^{+}$ve	Submucosal fibroblasts CD34 $^{-}$ ve	Inadequate submucosa to assess
Normal	**14**	**0**	/
Tubular Mild	3	0	2
Tubular Mod	6	0	0
Tubular Severe	1	0	1
TV Mild	1	0	1
TV Mod	5	0	2
TV Severe	6	0	1
Villous Mild	2	0	1
Villous Mod	6	0	0
Villous Severe	7	0	0
All adenomas	**37**	**0**	**8**
Dukes A	0	18	/
Dukes B	0	18	/
Dukes C	0	16	/
All carcinomas	**0**	**52**	/

Table 3.3 Presence of αSMA staining in the submucosa of normal, adenomatous and malignant colon

Histology	Submucosal fibroblasts αSMA $^{+}$ve	Submucosal fibroblasts αSMA $^{-}$ve	Inadequate submucosa to assess
Normal	**0**	**14**	/
Tubular Mild	0	4	2
Tubular Mod	0	4	0
Tubular Severe	0	3	1
TV Mild	0	1	1
TV Mod	0	4	2
TV Severe	0	3	1
Villous Mild	0	2	1
Villous Mod	0	6	0
Villous Severe	0	10	0
All adenomas	**0**	**37**	**8**
Dukes A	18	0	/
Dukes B	16	0	/
Dukes C	15	0	/
All carcinomas	**49**	**0**	/

Table 3.4 Presence of hCD staining in the lamina propria of normal, adenomatous and malignant colon

Histology (Number of specimens)	**Presence of PCMF staining**
Normal (1)	+
Tubular Mild (3)	+
Tubular Mod (2)	+
TV Mild (1)	+
TV Mod (1)	+
TV Severe (1)	+
Villous Mild (1)	+
Dukes A (2)	-
Dukes B (1)	-
Dukes C (2)	-

3.3. DISCUSSION

This study aimed to examine several characteristic fibroblast markers to investigate how expression of these markers might alter through the development of malignancy. Fibroblast populations have previously been examined in breast malignancy where the acquisition of αSMA positivity has been noted by several studies *(Sappino et al, 1988; Lazard et al, 1993; Chauhan et al, 2003)*. However, fibroblasts around areas of benign change such as fibroadenomas and radial scarring have also been noted to express αSMA to varying degrees *(Sappino et al, 1988; Chauhan et al, 2003)*. CD34 has also been examined in the same tissue and it was found that loss of CD34 occurs in malignancy and around some areas of ductal carcinoma in situ (DCIS) *(Chauhan et al, 2003)*. These changes are, therefore, not specific to malignancy and there was some suggestion that there was variability in expression of the markers around pre-invasive lesions. In the lung, CD34 stromal cells are seen in normal tissue and in the stroma of adenocarcinomas but in no other types of malignancy *(Nakayama et al, 2003)*. Similarly, in gastric carcinomas, the expression of CD34 in the stroma has been linked to tumour type with 'intestinal' type showing no CD34 expression in the tumour stroma whereas 'diffuse' type did have CD34 positivity in the stroma but only in advanced tumours *(Nakayama et al, 2001)*. Like CD34 expression, the expression of αSMA in the stroma of gastric adenocarcinomas appears to vary with tumour type and degree of invasion *(Nakayama et al, 2002)*.

Normal colon contains several discrete fibroblast populations, as defined by previous studies *(Powell et al 1999)* and by the findings with the markers in this study. The mucosa contains αSMA $^{+}$ve, CD34 $^{-}$ve, hCD $^{+}$ve cells, which are pericryptal myofibroblasts, arranged around the base of the crypts. No fibroblasts are demonstrated in the lamina propria of normal colon on the basis of αSMA, CD34 and hCD staining but previous reports have demonstrated a vimentin $^{+}$ve group of fibroblasts in this area *(Adeboyega et al, 2002)*. Below the muscularis mucosa are the αSMA $^{-}$ve, CD34 $^{+}$ve, hCD $^{-}$ve submucosal fibroblasts.

The findings in this study of loss of CD34 in the stroma of colorectal adenocarcinoma concurs with the findings of previous, more limited studies *(Nakayama et al, 2000)* although the retention of CD34 expression in the sub-mucosa of adenomas has not previously been reported. The marked overexpression of αSMA in colorectal tumour stroma has not been reported before, although the presence of αSMA positive cells in

the lamina propria of adenomas has been observed. Our results suggest that there is no relationship between αSMA expression and histological type which supports a previous report *(Adeboyega et al, 2002)* but contrasts with other studies which have suggested that αSMA expression is related both to histological type and degree of dysplasia *(Sappino et al, 1989; Li et al, 1999)*. The reason for this contradiction is unclear as αSMA is employed as the marker in all three studies, although the authors of the latter two studies were focussed on investigating colonic PCMFs. It is asserted that levels of αSMA expression decline around glands with increasing evidence of dysplasia and malignant change, results that directly contradict the findings of this study.

The changes in submucosal colonic fibroblast phenotype demonstrated in this study are both sensitive and specific in that all malignancies, regardless of their size or stage exhibited the same submucosal changes and the changes were detected only where invasive malignancy was observed. The adenomas did contain variable amounts of submucosa which sometimes made it difficult to assess this population of fibroblasts but, in those polyps that could be assessed, there was no suggestion that the profile differed from that of normal tissue, across all adenoma types and degrees of dysplasia. It was also interesting to note the localised nature of the change and this was particularly well demonstrated in some of the Dukes A tumours where there was a sharp transition in expression of fibroblast markers around the invasive margins of the tumour. These results imply a submucosal fibroblast reaction to malignancy rather than a facilitative change prior to invasion.

The changes in the lamina propria were more complex, however. Here the pericryptal fibroblasts were clearly demonstrated with αSMA and hCD, thus confirming the findings of Nakayama et al *(1999)* that, although hCD has previously been reported not to stain myofibroblasts, *(Lazard et al, 1993)* the PCMFs are an exception. The lamina propria did exhibit an increase in αSMA staining in the adenomas but this change was not reflected on staining with hCD, in fact, the level of hCD staining decreased in the lamina propria of the adenomas in comparison with normal mucosa. Furthermore, there was complete absence of hCD staining in the stroma of the carcinomas. These observations are based on a relatively small number of cases, especially regarding normal colon, but are highly consistent within those sections that stained successfully. The implication of the combined results of the αSMA and hCD staining is that, although there is an overall increase in the number of myofibroblasts

observed in adenomas and adenocarcinomas, this population of myofibroblasts does not derive from PCMFs. Furthermore, PCMF actually appear to decline in number during malignant progression in the colon. This supports the findings of previous studies which indicate a decline in PCMF numbers with increasing dysplasia *(Yao & Tsuneyoshi, 1993; Yao & Talbot, 1996; Li et al, 1999)* although the markers employed in these studies were common to all myofibroblasts and therefore it is unclear how altered fibroblasts were distinguished from pericryptal myofibroblasts. The identification of myofibroblastic response in the lamina propria in adenomas indicates a stromal response prior to the development of invasive malignancy. There is evidence from other systems that myofibroblasts release pro-invasive factors such as hepatocyte growth factor/scatter factor *(Lewis et al, 2004)* which could induce tumour invasion. Studies to examine differences in factors secreted by myofibroblasts from non-malignant colon and adenomatous polyps have not shown any difference in the effect of conditioned media from these two cell groups on proliferative activity of colon cancer cells. There was however an early difference in proliferation when the tumour cells were cultured with the groups of myofibroblasts with the adenoma myofibroblasts promoting earlier proliferation and there was also evidence of high levels of a specific protein fragment secreted by the polyp myofibroblasts and not present in the myofibroblasts from normal colon *(Chen et al, 2005)*. Since all invasive carcinomas are characterised by an intense myofibroblastic response, it is interesting to speculate whether these non-pericryptal myofibroblasts are involved in mediating tumour invasion. In addition, the progressive loss of PCMFs with the development of invasive malignancy may indicate a protective function for intact PCMFs. Once the barrier is lost, local invasion and migration can progress unchecked.

The quality of the immunohistochemistry in these cases was generally good. With two antibodies, however, less than optimal staining was achieved. CD31 only stained endothelial cells weakly, despite careful optimisation of the antigen retrieval techniques. This antibody had been included as a control due to the ability of CD34 to stain endothelial cells and the aim was to ensure that the areas visualised with CD34 were, indeed, fibroblasts and not small blood vessels. Antibody to CD31 gave some staining of the lamina propria endothelial cells but this was unreliable. Ultimately, the fibroblasts were sufficiently distinctive morphologically and the endothelial cells recognisable as lining blood vessels for the observers to be confident that the staining observed was not just endothelial.

c-kit (CD117) staining was of interest due to the existence of colonic cells which are positive for both this marker and CD34 *(Robinson et al, 2000)*. Unfortunately this antibody was also difficult to optimise as it was a polyclonal antibody and the aim was to use it without antigen retrieval as this has previously been suggested to result in artefactual staining. There is one previous example of the antibody being successfully used on FFPE colon without antigen retrieval *(Streutker et al, 2003)* but most studies describe use on small bowel or with antigen retrieval. Mast cells within the colon are known to express c-kit *(Ikuta & Weissman, 1992; Hamann et al, 1994)* and these cells did show staining, both with and without antigen retrieval but there was little evidence of staining of the ICCs. The most convincing control would have been a GIST but unfortunately it was not possible to obtain a specimen at the time.

CD34 positive cells were observed in the region of the muscularis propria and it is likely that these were ICCs or the related cells described by Vanderwinden et al *(1999)*. The lack of successful c-kit staining made it impossible for these results to contribute to the discussion surrounding the origin of ICCs but these cells were located away from the mucosal and submucosal fibroblasts which were the main focus of this study and almost certainly represent a separate population.

The localised and specific changes in the submucosal fibroblasts are unlikely to have any diagnostic value as the quantity of submucosa required for visualisation of the fibroblasts would normally mean that invasion would be evident histologically. It is, however, possible that loss of CD34 expression in the submucosal fibroblasts might confirm invasion when it is difficult to decide exactly whether the tumour has breached the muscularis mucosa.

One area which was not examined by this study is the relationship between inflammation and alteration in fibroblast phenotype. This is important as many of the changes surrounding a tumour are reactive rather than a specific tumour response. In a similar study in breast tissue, fibroblasts in radial scars and areas of reactive fibrosis exhibited the same changes as those in areas of malignancy *(Chauhan et al, 2003)*. The picture is further complicated by the possibility of malignancy developing in areas of chronic inflammation, as with ulcerative colitis. To establish the specificity of the loss of CD34 and acquisition of αSMA for malignancy in the colon, it would be important to repeat the study using both acutely and chronically inflamed colonic tissue. There is no evidence in the literature of this having been performed but it

would be important to do so to establish the specificity of the phenotypic alterations demonstrated.

The area that has greater potential for future study is the behaviour of the cells in the lamina propria as these results suggest that a specific population of non-pericryptal myofibroblasts is being upregulated prior to invasion. This may represent increased expression of an existing cell population or it may represent transformation of another fibroblast population into myofibroblasts. Alternatively it may represent an example of recruitment of a bone marrow derived population of myofibroblasts rather than transformation of a local population. In view of their presence in pre-invasive lesions it remains to be concluded whether they represent a response to mucosal changes or exert an influence over the changes themselves. If, however, these cells are specifically related to dysplasia and early malignant processes then selective inhibition may eventually be possible.

Chapter 4

Localisation of tenascin proteins in the colon

4.1. INTRODUCTION

Tenascin C (TN-C) is a stromal protein which is generally expressed at low levels within the adult. Expression is upregulated in areas of tissue remodelling including wound healing, and neoplasia *(Mackie et al, 1988; Howeedy et al, 1990)*. TN-C has multiple functions, including adhesive and counter-adhesive properties and can promote cell migration *(Philips et al, 1998; Wilson et al, 1999)*, induce cell proliferation and angiogenesis *(Chung et al, 1996; Schenk et al, 1999)*, inhibit focal adhesion formation *(Murphy-Ullrich et al, 1991)* and induce expression of genes which can further modify the ECM, such as MMPs *(Tremble et al, 1994)*. These features suggest that expression of TN-C in peri-tumoral stroma may contribute to an environment which promotes cell motility and growth. There are also indications that alterations in the distribution of TN-C expression may occur in pre-invasive malignant lesions *(Adams et al, 2002; Buyukbayram et al, 2002)* but there has only been limited investigation of this in the colon *(Hanamura et al, 1997)*.

TN-C exists as multiple isoforms due to variable splicing of the FN repeats in the variable region. This region is involved in several functions of relevance to tissue breakdown and repair and the presence and relative prevalence of different isoforms may therefore be highly significant in pathological processes. TN-C isoforms have been examined in several tissues including breast, *(Adams et al, 2002; Tsunoda et al, 2003)* ovary, *(Wilson et al, 1996)* prostate, *(Katenkamp et al, 2004)* and oral mucosa *(Mighell et al, 1997)* in investigations which have predominantly focussed on short and long TN-C. These investigations, and those examining the colon, *(Sakai et al, 1993; Hauptmann et al, 1995; Dueck et al, 1999)* have used mainly molecular techniques and to date there have been no immunohistochemical studies looking at the differential expression of isoforms within the colon. Immunohistochemistry has the advantage of demonstrating not only the presence but also the location of tenascin isoforms which is important given that a variety of cell types are known to express TN-C *(Ghert et al, 2002; Leins et al, 2003; Jones & Rabinovich, 1996)*.

This study hypothesised that a variety of TN-C isoforms would be present in the peri-tumoral stroma and that these would be distinct in nature and distribution to those present in normal colon. It was also hypothesised that alteration in the isoforms present may occur prior to invasion and so be demonstrated in adenomas. The aim of this part of the study, therefore, was to establish the expression pattern of TN-C in

normal and diseased colon and relate changes to fibroblast phenotype using immunohistochemical techniques.

4.2. RESULTS

4.2.1. **Antibody optimisation:**

Immunohistochemistry was performed on sections of normal, adenomatous and malignant colon from the colorectal tissue bank. For these cases both frozen and FFPE samples were used. As there were no adenomas available in the tissue bank, anonymised samples from the tissue archive were used and therefore no frozen tissue was available.

Two antibodies were used: the BC-24 clone (Sigma) which recognises an epitope within the EGF-like sequence of TN-C and therefore stains all isoforms of TN-C or 'total tenascin' (total TN) and the αIIIB clone (Chemicon International) which is specific for FN repeat B, coded for by exon 14 (TN14). This antibody therefore only identifies those isoforms of TN-C which contain this FN repeat. The total TN antibody had previously been used successfully on frozen and FFPE tissue but the only published reports of TN14 antibody referred to its use on frozen tissue *(Adams et al, 2002)*. The aim therefore was to optimise both antibodies for use on both types of material. Antibody dilutions for the frozen sections were satisfactorily employed as per previous published work *(Adams et al, 2002) (See Table 4.1)* but the technique for FFPE sections required further optimisation with assessment of optimal dilution factor and antigen retrieval *(See Table 4.2)*. Total TN antibody produced optimal staining with trypsin antigen retrieval while pressure cooking was found to give the best results for TN14 antibody. Both antibodies were tested at a variety of dilutions *(See Tables 4.1 and 4.2)*. Positive and negative controls were included in all runs and were positive and negative respectively.

4.2.2. **Distibution of tenascin expression**

Tenascin distribution in normal colon:

In the sections of normal colon, distribution of staining with total TN antibody was confined to the basement membrane, muscularis mucosa and muscularis propria *(See Table 4.3)*. The TN-C staining in the basement membrane was continuous throughout the basement membrane but was strongest at the top of the crypts, closest to the lumen. Staining of the muscularis mucosa was also strong and continuous and there was strong staining of the muscularis propria.

Staining using the TN14 antibody was not identified in the basement membrane of normal colon and but was observed in the muscularis mucosa in the frozen sections. Staining with TN14 was seen in the muscularis propria although this was much more marked on the frozen specimens than the FFPE sections where 17/26 frozen stained positive compared to 4/23. In both types of sample, however, the staining was not as extensive as that observed for total TN *(See Figure 4.1)*.

Tenascin distribution in colonic adenomas:

All the 18 adenoma specimens exhibited the same distribution of TN-C with both antibodies as seen in normal colon, although it was often difficult to assess the submucosa as some adenomas contained little of this area and there was no evidence of TN-C in the lamina propria. There was no evidence of any alteration in TN-C distribution in relation to degree of dysplasia or type of adenoma.

Tenascin distribution in colonic adenocarcinoma:

The 15 tumour samples stained all demonstrated the presence of TN-C in the tumour stroma with the antibody to total TN and also showed that TN14 containing isoforms were among those present using the TN14 antibody *(See Table 4.4)*. Again the staining for TN14 was not as extensive as that for total TN but it was consistently and convincingly present in all samples *(See Figure 4.2)*.

Tenascin distribution in inflamed colon:

The inflammatory specimens were more varied than any other type of specimen and included inflammation due to Crohns disease, UC and diverticulitis *(See Tables 4.5 and 4.6)*. The staining pattern for TN-C in the majority of specimens was indistinguishable from normal colon but there was a small group of acutely inflamed specimens (2 for frozen sections; 6 FFPE) which demonstrated stromal staining beneath the area of greatest mucosal inflammation (2/2 and 6/6 respectively) using the antibody to total TN *(See Figure 4.3)*. In addition, these areas were found to contain TN14 containing isoforms, as seen on both frozen and FFPE sections (2/2 and 4/6 respectively).

Tenascin distribution in blood vessels:

Since previous reports had indicated that blood vessels may contain TN-C isoforms other than short TN-C, *(Adams et al, 2002)* any muscular walled blood vessels were assessed for TN-C staining. As not all the specimens contained large calibre blood vessels it was not possible to do this on all specimens. There was, however, consistent staining of blood vessels with total TN antibody and 2/26 of the frozen specimens of normal colon showed blood vessel staining with TN14 antibody.

Table 4.1 Optimised immunohistochemistry methods employed for two antibodies to TN-C on frozen colorectal samples

Antibody	**Total Tn**	**Tn 14**
Ag retrieval	None	None
Blocking serum	Normal rabbit serum	Normal rabbit serum
Primary antibody dilution	1:7500	1:1000
Secondary antibody	Rabbit anti-mouse	Rabbit anti-mouse
Dilution	1:400	1:400
Buffer	TBS	TBS

Table 4.2 Optimised immunohistochemistry methods employed for two antibodies to TN-C on FFPE colorectal samples

Antibody	**Total Tn**	**Tn 14**
Ag retrieval	Trypsin (10 mins)	Pressure cook (2 mins)
Blocking serum	Normal rabbit serum	Normal rabbit serum
Primary antibody dilution	1:2000	1:200
Secondary antibody	Rabbit anti-mouse	Rabbit anti-mouse
Dilution	1:400	1:400
Buffer	TBS	TBS

Table 4.3 Distribution of tenascin staining in normal colon

Frozen tissue stained with total TN

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	26	20	18	0	16	23
Negative		6	8	26	10	3

FFPE tissue stained with total TN

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	24	7	15	0	10	22
Negative		17	9	24	14	2

Frozen tissue stained with TN14

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	26	1	12	0	2	17
Negative		25	14	26	24	9

FFPE tissue stained with TN14

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	23	0	0	0	0	4
Negative		23	23	23	23	19

Table 4.4 Distribution of tenascin staining in colonic adenocarcinoma

Frozen tissue stained with total TN

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	29	N/A	N/A	29	7	12
Negative		N/A	N/A	0		

FFPE tissue stained with total TN

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	15	N/A	N/A	15	2	7
Negative		N/A	N/A			

Frozen tissue stained with TN14

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	29	N/A	N/A	29	6	8
Negative		N/A	N/A	0		

FFPE tissue stained with TN14

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	15	N/A	N/A	15		
Negative		N/A	N/A			

Table 4.5 Distribution of tenascin staining in acutely inflamed colon

Frozen tissue stained with total TN

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	2	1	1	2	0	2
Negative		1	1	0	2	0

FFPE tissue stained with total TN

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	6	2	3	6	3	6
Negative		4	3	0	3	0

Frozen tissue stained with TN14

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	2	1	0	2	0	1
Negative		1	2	0	2	1

FFPE tissue stained with TN14

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	6	0	0	4	0	1
Negative		6	6	2	6	5

Table 4.6 Distribution of tenascin staining in chronically inflamed colon

Frozen tissue stained with total TN

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	4	0	2	1	2	4
Negative		4	2	3	2	0

FFPE tissue stained with total TN

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	7	3	6	1	3	5
Negative		4	1	6	4	2

Frozen tissue stained with TN14

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	4	0	1	1	0	3
Negative		4	3	3	4	1

FFPE tissue stained with TN14

Staining	Total samples	Basement Membrane	Muscularis Mucosa	Stroma	Blood Vessels	Muscularis Propria
Positive	7	0	0	0	0	2
Negative		7	7	7	7	5

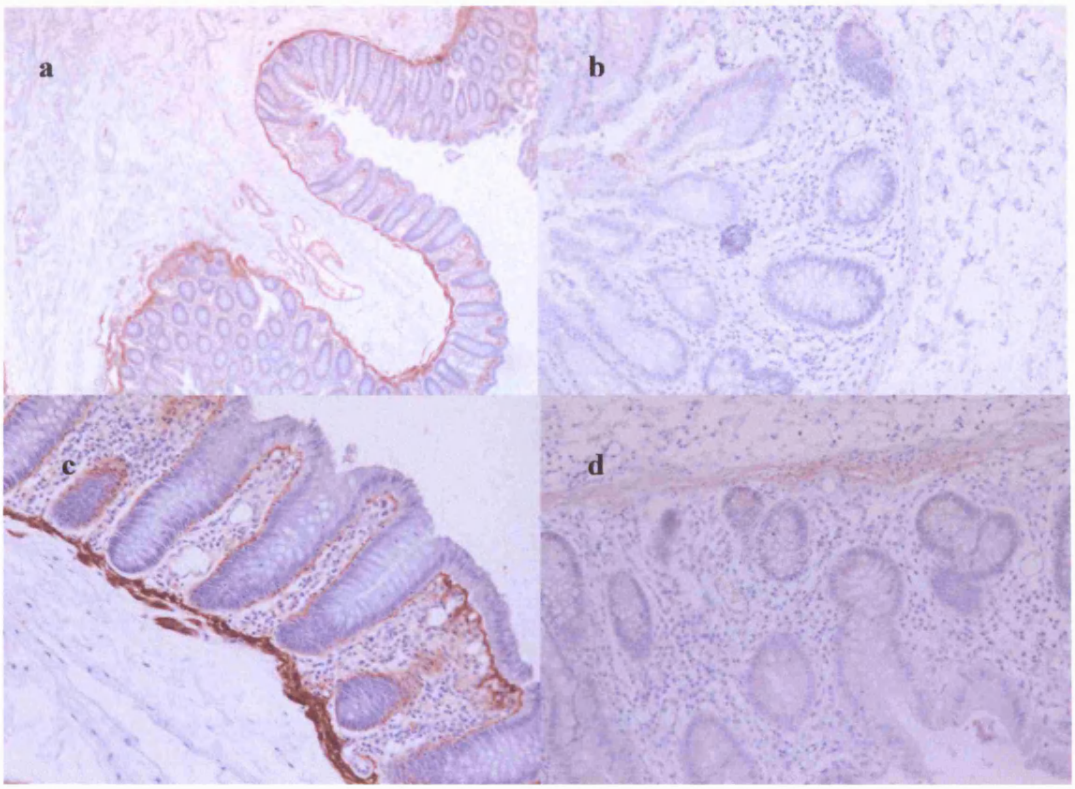


Figure 4:1 Immunohistochemistry for total tenascin and tenascin 14 in normal colon

The left panel (a and c) are stained for total tenascin and the right panel (b and d) are stained for tenascin 14.
Total tenascin is detected in the basement membrane (c) and muscularis mucosa (a and c) and in the walls of large blood vessels (a).
Tenascin 14 is detected in the muscularis mucosa (b and d).

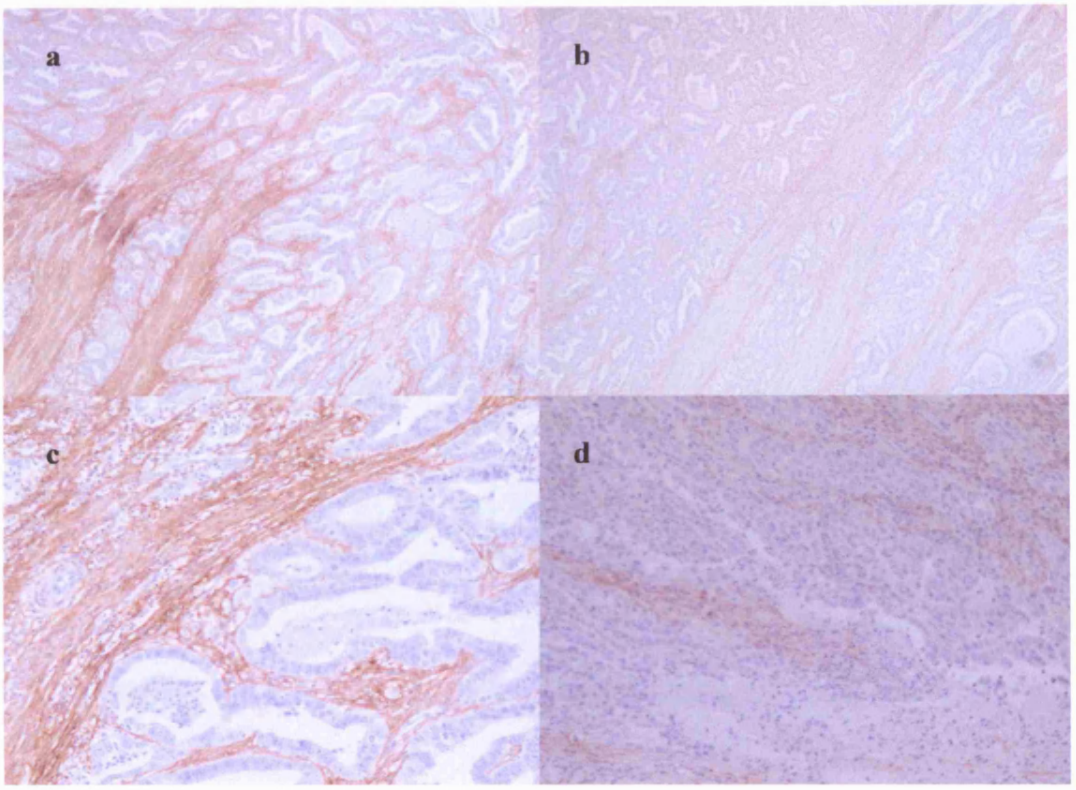


Figure 4.2 Immunohistochemistry for total tenascin and tenascin 14 in colorectal adenocarcinoma

The left panel (a and c) is stained for total tenascin and the right panel (b and d) is stained for tenascin 14.
Total tenascin is detected throughout the peritumoral stroma (a and c).
Tenascin 14 is also detected in the peritumoral stroma (b and d) but staining is less extensive.

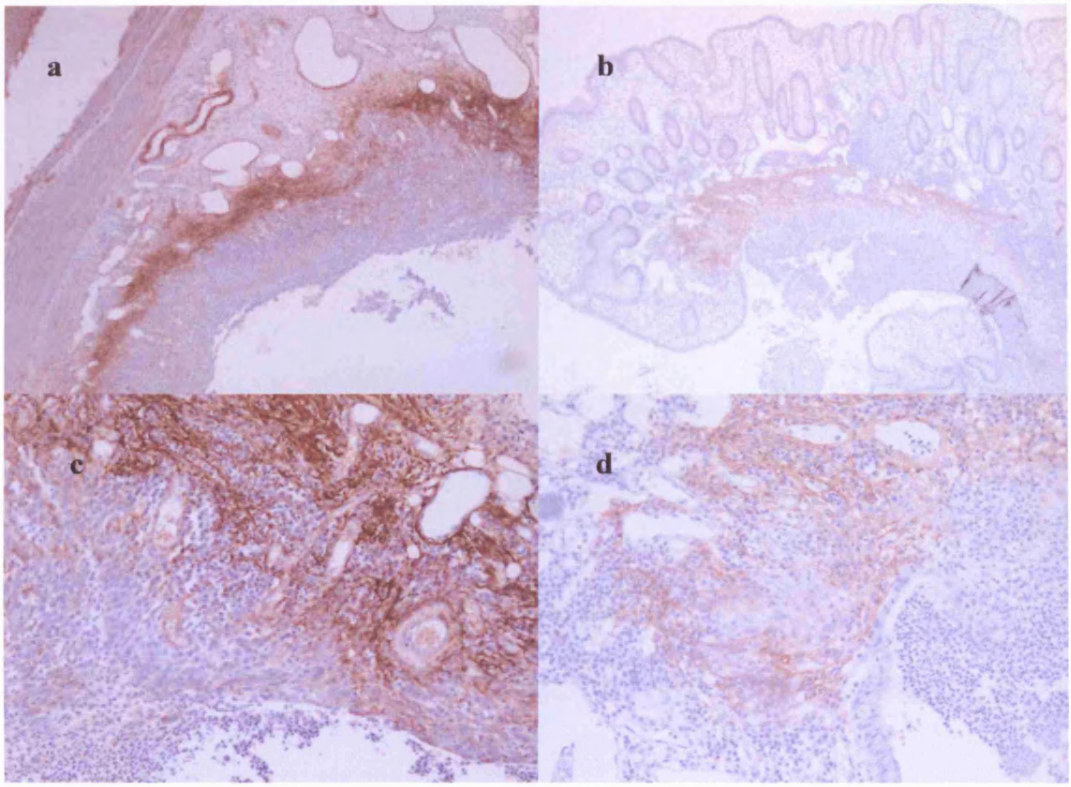


Figure 4.3 Immunohistochemistry for total tenascin and tenascin 14 in acutely inflamed colon

The left panel (a and c) shows staining with total tenascin and the right panel (b and d) shows staining with tenascin 14.
Total tenascin is detected in the basement membrane, muscularis mucosa, blood vessels and muscularis propria of the colon but is also present in the stroma beneath area of mucosal inflammation (a and c).
Tenascin 14 is detected in the muscularis mucosa and also in the stroma related to areas of acute mucosal inflammation (b and d).

4.3. DISCUSSION

TN-C is found in a variety of tissues in the adult but only at low levels. It has, however, been widely demonstrated to be linked to both cancer and inflammation through its role in regeneration and repair *(Reviewed in Chiquet-Ehrismann & Chiquet, 2003)*. Its expression in both situations, however, is complex and varies between tissues. Most reports have found very little TN-C expression in normal colon *(Iskaros et al, 1997)*. In those cases where TN-C has been observed, it is reported to be distributed in the smooth muscle of the muscularis mucosa or muscular blood vessel walls *(Gulubova & Vlaykova, 2001)*. TN-C has also been demonstrated in the sub-basement membrane zone in the upper part of the crypts *(Hanamura et al, 1997)* but has not been reported in the stroma of normal tissues. This study has clearly shown widespread and consistent TN-C expression in the normal colon demonstrating that tenascin is present throughout the colon wall under normal conditions. Strong staining was obtained throughout the basement membrane and not just in the upper parts of the crypts. Similarly, there was widely observed staining of muscular structures including blood vessels and the muscularis mucosa and propria. The reason for the differing results may lie in the use of antigen retrieval as Iskaros et al *(1997)* make no reference to antigen retrieval whereas Hanamura et al *(1997)* whose results most closely resemble those of this study employed a pepsin antigen retrieval technique. This does not, however, explain the lack of staining described by Gulubova and Vlaykova *(2001)* as this study used frozen sections. Some groups have suggested that antigen retrieval techniques may result in artefactual staining *(Reviewed in Leong, 2004)*. It is unlikely that this was the case in the current study as a similar pattern of staining was seen on frozen sections where antigen retrieval was not required.

The distribution of TN-C in malignancy has been examined in a large number of tissues. From this, two common observations arise. The first is that TN-C expression increases just below the basement membrane in pre-invasive disease in some tissues *(Iskaros et al, 2000; Adams et al, 2002; Goepel et al, 2003)*. The second is that TN-C is expressed strongly in the peri-tumoral stroma of most solid tumours, regardless of tumour cell type *(Mackie et al, 1987; Booth et al, 2002; Regezi et al, 2002; Leins et al, 2003; Raitz et al, 2003)*. Colorectal adenocarcinoma does not have a true *in situ* phase but severely dysplastic polyps are known to be at increased risk of becoming

invasive. The lack of significant sub-mucosal tissue in the majority of the adenomas examined made it difficult to evaluate precisely whether there was any increase in TN-C expression in this area but, in cases where sub-mucosa was present, there did not appear to be any increase in staining.

The lack of any significant TN-C in the lamina propria of the adenomas is interesting in the light of the results in Chapter 3 which indicated the presence of a large number of myofibroblasts in this area. As TN-C is known to derive from myofibroblasts *(Hanamura et al, 1997; De Wever et al, 2004)* this provides further evidence for the functional diversity of myofibroblasts. Diversity in properties such as TN-C production may be an innate characteristic or may be acquired. While there are multiple factors regulating the production of TN-C *(Jones & Jones, 2000)* it is becoming increasingly clear that stretch can significantly influence TN-C expression *(Chiquet-Ehrismann et al, 1994; Wallner et al, 1999)*. This may explain differences in TN-C distribution between tissues as it can be postulated that, whereas an early colonic epithelial tumour can expand into the bowel lumen and so create less disruption to the surrounding stroma, a comparable tumour in the breast for example would stretch surrounding tissue and so stimulate TN-C production in the myofibroblasts. In this way it can be seen that, while αSMA and TN-C may frequently be co-expressed they are, in fact, independently regulated.

In keeping with previous studies *(Riedl et al, 1992; Sakai et al, 1993)* TN-C was found to be consistently present in the stroma around invasive carcinomas. The results for the frozen and FFPE sections generally supported each other with most discrepancies relating to level of staining. In particular, in the normal, higher levels of staining were observed in the frozen sections, however, the tissue architecture was much better preserved in the FFPE sections enabling clearer identification of the submucosa. This was of particular importance in the acutely inflamed samples where the muscularis mucosa is often difficult to discern. Although antibodies identifying the long variant of TN-C *(Ghert et al, 2001)* and TN14 containing isoforms *(Adams et al, 2002)* have been used on other tissues, there has been no published work describing their use in the colon. In addition, the successful use of the antibody on FFPE as well as frozen tissue enabled increased clarity in defining the distribution of these isoforms, especially in the acutely inflamed samples.

Using the TN14 antibody, TN14 containing isoforms were consistently demonstrated in the muscle layers of the normal colon on the frozen sections, indicating that TN-C

isoform production is site specific but also that there is functional diversity between different mesenchymal cell populations. This is important because it shows the TN-C isoform expression profile of normal tissues to be more complex than previously thought. As the muscle has a very specific function within the bowel wall and is again related to stretch this may explain the large amounts of TN-C present within it, including the longer isoforms.

The discovery of TN14 containing isoforms in the tumour stroma in a less extensive distribution than the total TN suggests that peritumoural TN-C expression involves more than one isoform. The antibody for TN14 does not distinguish between all the possible TN14 containing isoforms but previous reports indicate that long TN-C and molecules expressing exons 14 and 16 in combination are likely to be among those expressed in malignancy *(Wilson et al, 1996; Dueck et al, 1999; Ghert et al, 2001; Adams et al, 2002)*. The lack of any stromal changes or TN14 expression in the adenomas would suggest that, in the colon atleast, TN-C is not useful as a predictor of future invasive behaviour.

The identification of staining with the TN14 antibody in the stroma of the most acutely inflamed samples suggests that the presence of TN-C molecules other than short TN-C is not confined to malignancy. Such stromal TN-C expression in acute inflammation may reflect its well described role in wound healing. However, the large range of possible exon 14 containing molecules does not preclude the possibility of tumour specific isoform containing exon 14, and the lack of discrimination between TN14 containing isoforms in the inflamed and malignant specimens does not permit the conclusion that the two pathologies are demonstrating the same response. To further clarify the nature of the isoforms present in normal, inflamed and malignant colon, molecular techniques were therefore employed as described in Chapter 5.

Chapter 5

Molecular analysis of tenascin isoforms in the colon

5.1. INTRODUCTION

The highly restricted temporo-spatial expression pattern of TN-C, especially during development *(Joester & Faissner, 1999)* implies a tight structure-function relationship. Previous reports have suggested that specific TN-C isoforms may be associated with certain disease processes, for example TN14/16 in breast carcinoma *(Adams et al, 2002)* and TN15 in glioblastoma *(Bourdon et al, 1983)* and this raises the possibility of exploiting these isoforms as therapeutic targets. For any molecule to have diagnostic or therapeutic usefulness it must be present in sufficient quantities to be reliably detected and be biologically relevant to the pathological process. Most previous work in the TN-C field has used specific antibodies or probes to identify particular isoforms in benign and malignant tissue *(Dueck et al, 1999; Katenkamp et al, 2004)* but in this study the aim was to survey the entire variable region of TN-C to establish the profile of isoforms expressed in the colon and whether this pattern varies with pathological changes in the bowel. In this way, less studied isoforms and those for which no antibody or probe exist can be identified and the overall pattern of isoform expression established.

Having already established by immunohistochemistry the presence of different TN-C isoforms in the colon in the normal and diseased state, this part of the study aimed to use RT-PCR to analyse in detail the TN-C isoform profile of normal and diseased colon and to establish whether there are tumour specific isoforms. To do this primers were selected which would cover large portions of the variable region to assess the number and size of isoforms present, as well as primers which would flank areas of particular interest.

5.2. RESULTS

5.2.1. **Assessment of sample quality**

As the frozen colon specimens had not been used for any previous work and the optimum methods of storage and retrieval had not yet been established in our laboratory, it was important to assess the quality of the cDNA being produced. Some of the samples were assessed for DNA/RNA content using the spectrophotometer but it was also necessary to measure the quantity of the housekeeping gene present in each of the samples. Several GAPDH PCR programmes were evaluated before deciding on a 28 cycle regime *(See Table 5.1)*. At the time of preparing the samples attempts were made to ensure that roughly equivalent amounts of mRNA were used by adding the same number of sections for lysis. cDNA was generated from these sections and GAPDH assessment was then used to choose the best samples for analysis. A total of 37 RT $^{+}$ve and RT $^{-}$ve samples underwent PCR using GAPDH primers and 28 cycles of amplification. The strength of the bands at 350 base pairs (bp) was then graded 1, 2 or 3 *(Figure 5.1)*. Only samples scoring 3 were used for isoform profiling and this was subsequently validated by a reaction which showed that the strength of the bands produced using 8/18 primers correlated well with those produced by GAPDH primers.

From this analysis 21 samples were selected and these were used in all the subsequent experiments examining TN-C expression.

The selected samples comprised: 10 tumour specimens
3 inflammatory specimens
8 normal specimens

5.2.2. **Optimisation of RT-PCR**

8/18 primer set

Although this primer set had been used previously in our laboratory the programme described no longer produced successful results. The annealing temperature was therefore reassessed and optimised. The programme used in the final assessment is described in *Table 5.1.* During optimisation the sensitivity and specificity of the reaction was checked using cDNA from the cell lines T47D and MCF7 transiently transfected with a PCMV flag-tagged construct of several isoforms of TN-C. These

consisted of genes containing none of the variable region (truncated TN-C (tTN)), molecules containing all of the FN repeats in the variable region (long TN-C (TNL)), molecules containing only FN repeat D, coded for by exon 16 (TN16) and molecules containing FN repeats B and D coded for by exons 14 and 16 (TN14-16) *(See Figure 5.3)*. For the final assessments the positive control was cDNA extracted from SK-MEL 28 human melanoma cell line which is known to express a variety of TN isoforms *(Carnemolla et al, 1992)*. 5µl of colonic cDNA and 1µl positive control cDNA were used for each of the TN-C assessments.

11/16 primer set

This primer set worked well at the temperatures used previously in our laboratory so further optimisation was not required *(See Table 5.1)*. SK-MEL 28 was again used as a positive control in the final assessments.

ad1 primer set

This primer set worked well at the temperatures used previously in our laboratory so further optimisation was not undertaken *(See Table 5.1)*. The specificity of the reaction was tested using cDNA derived from a topo vector transfected with an ad1 clone which had been amplified in bacteria and transformed in DH5α and this was subsequently used as a positive control.

9-14/14-16 primer set

This primer set did not produce any product at the temperatures previously used in our laboratory using T47D and MCF7 cells transfected with TN9-14-16. The annealing temperature, number of cycles and specificity was therefore reassessed using the same template. The annealing temperature was lowered to 57, 58 and 59°c, all of which gave a strong band of the expected size (300 bp). The annealing temperature of 58°c was therefore selected and the colorectal samples were assessed using the primer set. Due to concern about the specificity of the reaction, the primer set was further evaluated using cDNA from the T47D and MCF7 cells transfected with TN14-16, TN16 and TNL. This showed some cross reactivity between TN14-16 and TN16 at this temperature but very strong cross reactivity between TN14-16 and TNL. The annealing temperature was increased and 67°c was identified as the optimum annealing temperature as 68°c led to loss of all bands and 66°c did not

Table 5.1 Primer sets and PCR cycles

GAPDH

Forward primer 5′ AGAACATCATCCCTGCCTCC

Reverse primer 5′ GCCAAATTCGTTGTCATA

PCR programme:
98°c for 3 minutes
94°c for 30 seconds
69°c for 30 seconds* } 28 cycles
72°c for 30 seconds
4°c for ∞

Tenascin 8/18

Forward primer (8F) 5′ 7CAATCCAGCGACCATCA

Reverse primer (18R) 5′ CGTCCACAGTTACCATGG

PCR programme:
98°c for 3 minutes
94°c for 30 seconds
63°c for 30 seconds* } 35 cycles
72°c for 2 minutes
4°c for ∞

Tenascin 11/16

Forward primer (11F) 5′ 7CAATTGGGAGAGGTCG

Reverse primer (16P) 5′ GTTGTCAACTTCCGGTTC

PCR programme:
98°c for 3 minutes
94°c for 30 seconds
60°c for 30 seconds* } 35 cycles
72°c for 1 minute
4°c for ∞

Tenascin ad1

Forward primer (AD1F) 5′ CCACAGTTGGGCATGCTAAT

Reverse primer (AD1R) 5′ GTGTTCTCCACCAAGCCTGT

PCR programme:

98°c for 3 minutes

94°c for 30 seconds
61°c for 30 seconds* } 35 cycles
72°c for 30 seconds

4°c for ∞

Tenascin 9-14/14-16

Forward primer (9-14F) 5′ 7ATCCACTGCCAAAGAAC

Reverse primer (14-16P) 5′ TTCGGCTTCTGTCGTGGC

PCR programme:

94°c for 5 minutes

94°c for 30 seconds
69°c for 30 seconds* } 1 cycle
72°c for 30 seconds

94°c for 30 seconds
68°c for 30 seconds } 3 cycles
72°c for 30 seconds

94°c for 30 seconds
67°c for 30 seconds } 3 cycles
72°c for 30 seconds

94°c for 30 seconds
66°c for 30 seconds } 3 cycles
72°c for 30 seconds

94°c for 30 seconds
65°c for 30 seconds } 30 cycles
72°c for 30 seconds

4°c for ∞

Tenascin 27R (for specific priming)

Reverse primer (27R) 5' CAGTGGAACCAGTAACG

* denotes addition of TAQ enzyme at start of part of cycle indicated

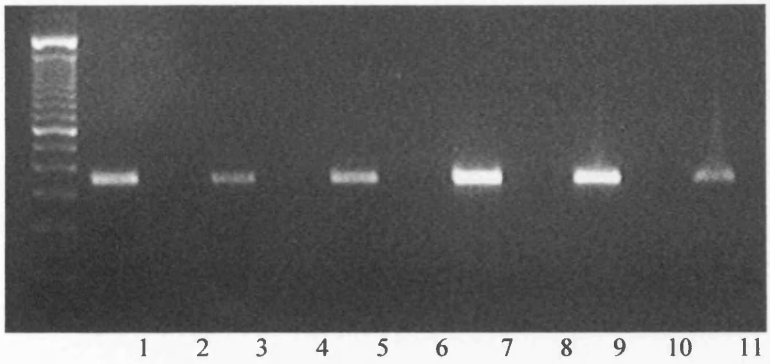


Figure 5.1 Example of relative GAPDH expression in colorectal samples

The odd numbered lanes contain RT $^{+}$ve samples and the even numbered lanes contain RT $^{-}$ve samples. Bands in lanes 7 and 9 were judged to be strength 3

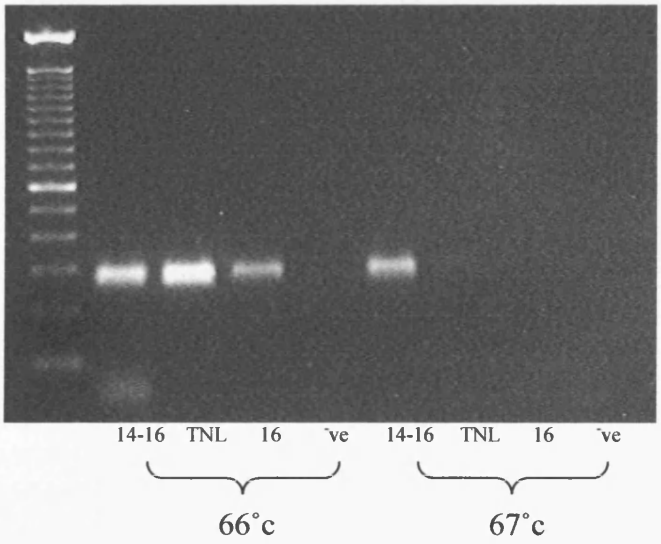
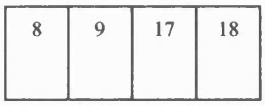


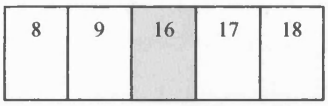
Figure 5.2 Optimisation of annealing temperatures for primer set 9-14/14-16

The controls used contained tenascin isoforms including exons 14 and 16 (14-16), all of the variable region (TNL) and exon 16 (16) as well as a negative control.

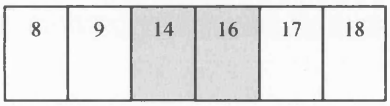
tTN



TN16



TN14-16



TNL

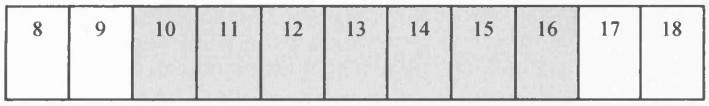


Figure 5.3 Diagram showing structure of tenascin isoforms used as positive controls

The exons present are denoted by numbers. The variable region is indicated in blue.

demonstrate differential specificity for TN14-16 and TNL *(Figure 5.2)*. However, at this temperature there was still a detectable band in the TNL sample so the cycle was further modified to use a 'touchdown' method of PCR reducing the temperature from 69°c to 65°c and concluding with 30 cycles at this temperature (35 cycles led to detection of TNL). This was demonstrated to be specific for TN14-16. Both TN14-16 and TNL were included as positive controls in the final assessments to ensure continuing specificity.

5.2.3. **Tenascin isoform expression in colon samples**

Isoforms detected using 8/18 primer set

When used to assess the colon samples, this primer set produced up to 5 bands on the gel *(Figure 5.4)*. The size of these bands was correlated with those bands in previously published work to estimate the number of additional exons present *(Bell et al, 2001)*. The lowest molecular weight band appeared around 442 bp and is likely to represent truncated TN (tTN) where no additional exons from the variable region are included *(See Table 5.2)*. The next band appears around 715 bp and represents the inclusion of one additional exon (tTN+1). Further bands were seen, around 1261 bp, consistent with the presence of three additional exons (tTN+3) and also around 1807, which is likely to show the presence of five additional exons (tTN+5). There was one further band well above the 2000 bp level which suggests the inclusion of all exons, possibly including ad1 and ad2 (TNL+ad1/ad2). The strength of the bands varied.

Correlating the isoform expression profile to the tissue type, tTN was seen in all 21 samples, tTN+1 in 19/21 (9 tumours, 3 inflammatories, 7 normals), tTN+3 in 9/21 (3 tumours, 2 inflammatories, 4 normals), tTN+6 in 8/21 (3 tumours, 2 inflammatories and 3 normals) and TNL+ad1/ad2 in 2/21 (0 tumours, 0 inflammatories, 2 normals). There was no detectable difference in pattern or level of expression between the three types of sample. Results are summarised in Table 5.3.

Isoforms detected using 11/16 primer set

The results of this primer set were less easy to interpret than those using primer set 8/18 as the bands were not as strong. There was a maximum of 4 bands but these were seen in fewer of the samples than with the 8/18 primer set. The position of the primers means that only isoforms expressing atleast 3 additional exons (i.e. 10, 11 and 16) would be detected (given that 10 is consistently expressed with 11 in previous

models). Using this primer set, bands were found at the levels of 285 bp indicating that no extra exons were present between the two primers and thus corresponding to tTN+3, 558 bp with one additional exon (tTN+4), 831 bp with two additional exons (tTN+5) and 1104 bp with three additional exons (tTN+6) *(See Table 5.2 and Figure 5.4)*.

In those samples where bands could be clearly defined, 7/21 samples expressed tTN+3 (1 tumour, 2 inflammatories, 4 normals), 7/21 expressed tTN+4 (0 tumours, 3 inflammatories, 4 normals), 5/21 expressed tTN+5 (0 tumours, 3 inflammatories, 2 normals) and 5/21 expressed tTn+6 (1 tumour, 2 inflammatories, 1 normal). This shows good correlation with the 8/18 results, with the detection of some additional bands.

Isoforms detected using ad1 primer set

ad1 was found to be present in 13/21 samples (4 tumours, 2 inflammatories, 7 normals) *(See Table 5.5 and Figure 5.6)* as confirmed by the presence of the band at 276bp *(Sriramarao & Bourdon 1993)* and the positive control.

Isoforms detected using 9-14/14-16 primer set

Only one sample demonstrated a very weak band specific for TN14-16 *(See Figure 5.7)*. This was a tumour sample. The band did not occur with 35 cycles at 65°c.

Table 5.2 Isoforms represented by bands present on gels using 8/18 and 11/16 primer sets

Level of band (bp)	**No. of additional exons from variable region**	**Likely sequence** (exons from variable region in bold)
8/18 primer set		
440	0	8-9-17-18
715	1	8-9-**16**-17-18
1261	3	8-9-**10-11-16**-17-18
1807	5	8-9-**10-11-13-14-16**-17-18
>2000	>6	8-9-**10-11-12-13-14-ad1-ad2-15-16**-17-18
11/16 primer set		
285	3	8-9-**10-11-16**-17-18
558	4	8-9-**10-11-13-14-16**-17-18
831	5	8-9-**10-11-12-13-14-16**-17-18
1104	6	8-9-**10-11-12-13-14-16**-17-18

Precise base pair sizes and the most likely corresponding sequence taken from *Bell et al, 1999.*

Table 5.3 Relative expression of different isoforms between types of colon sample using 8/18 primer set

Tenascin isoform	Number of samples containing isoform		
	Tumour samples	Inflammatory samples	Normal samples
tTN	10	3	8
tTN+1	9	3	7
tTN+3	3	2	4
tTN+6	3	2	3
tTN(+ad1/ad2)	0	0	2

Table 5.4 Relative expression of different isoforms between types of colon sample using the 11/16 primer set

Tenascin isoform	Number of samples containing isoform		
	Tumour samples	Inflammatory samples	Normal samples
TN+3	1	2	4
TN+4	0	3	4
TN+5	0	3	2
TN+6	1	2	1

Table 5.5 Relative expression of ad1 exon in colon samples

Tenascin isoform	Number of samples containing isoform		
	Tumour samples	Inflammatory samples	Normal samples
ad1	4	2	7

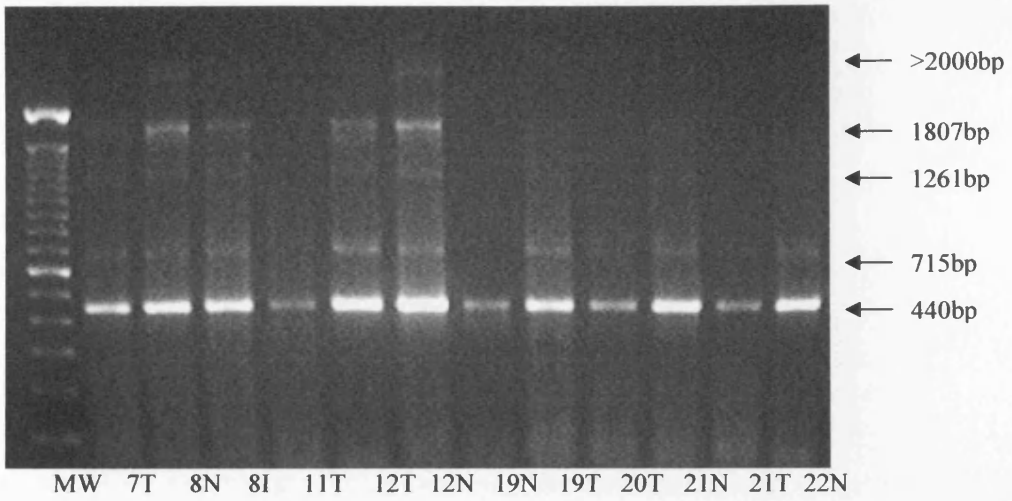


Figure 5.4 Patterns of tenascin isoform expression in representative colorectal samples using primer set tenascin 8/18.

T denotes a tumour sample and N denotes a normal sample. The bands indicated demonstrate the expression of products of 440, 715, 1261, 1807 and >2000 base pairs. MW denotes molecular weight marker.

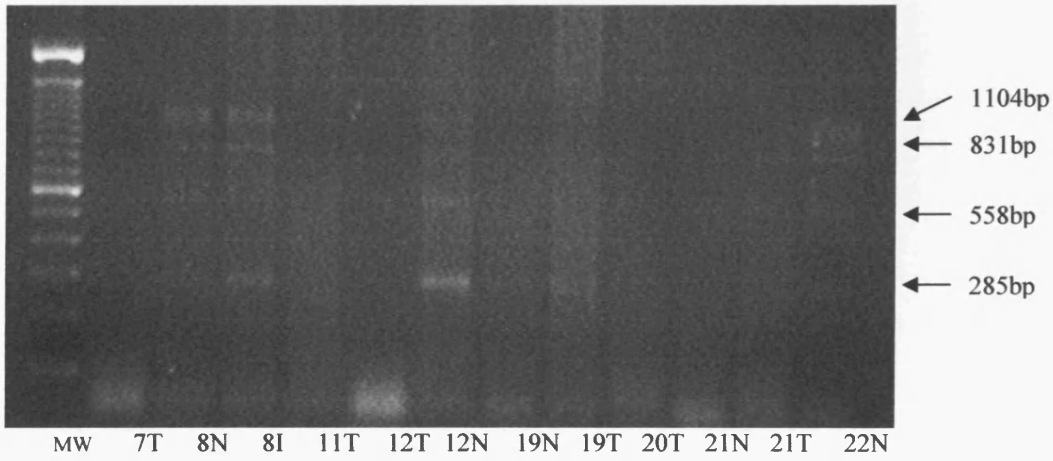


Figure 5.5 Patterns of tenascin isoform expression in representative colorectal samples using primer set tenascin 11/16

The bands indicated demonstrate the expression of products of 285, 558, 831 and 1104 base pairs.

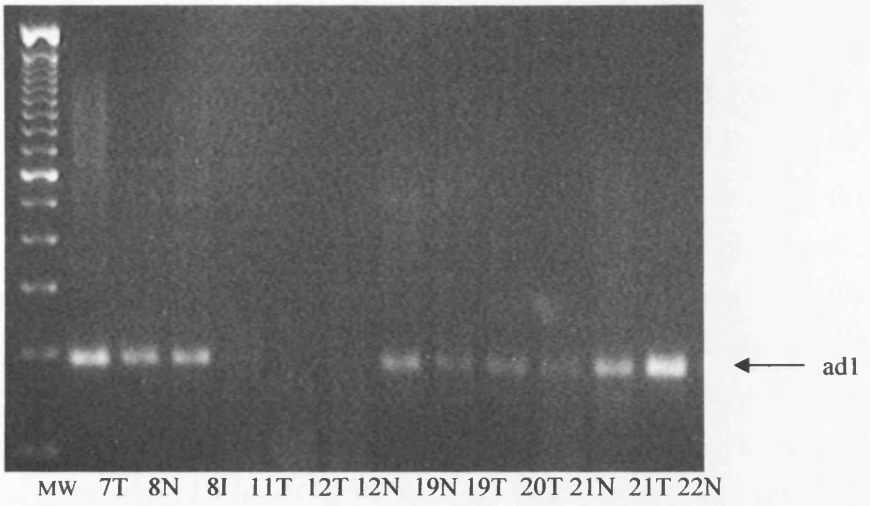


Figure 5.6 Pattern of expression of tenascin isoforms containing ad1 exon in representative colorectal samples using primer set ad1

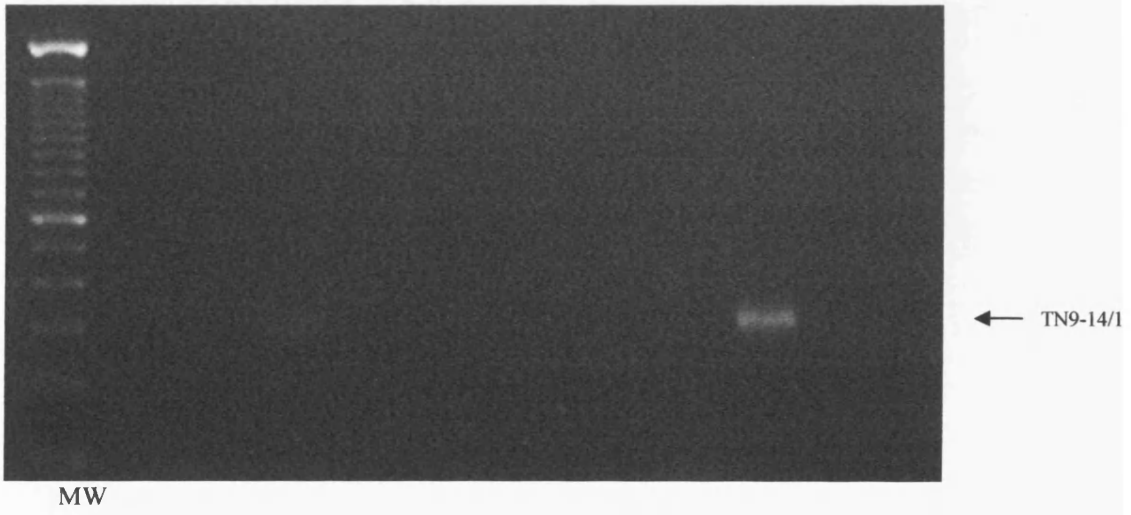


Figure 5.7 Pattern of expression of tenascin isoform TN14-16 in representative colorectal samples using primer set tenascin 9-14/14-16

A single tumour sample demonstrated a faint band consistent with the presence of TN14-16.

5.3. DISCUSSION

The first issue in interpreting the results of the PCR experiments was the quality of the mRNA extracted from the colorectal resection specimens. The process of snap freezing pieces of colon wall for mRNA extraction had not been performed in our laboratory before and, although the technique is well established, several issues were encountered as the tissue bank developed. The first issue is that the samples retrieved included the full thickness of the colon wall. This was thought to be important to ensure that a reasonable amount of submucosa was contained within the specimen but did mean that the presence of the muscle layer made the samples much thicker than the colonic biopsies which have been used in previous work *(Dueck et al, 1999)*. The second issue was the time taken to freeze the specimen. Although the time between removal of the specimen from the patient and freezing was reduced as much as possible by collection of the specimen from theatre, the specimen had already been devascularised within the patient for some considerable time before removal. During this time the bowel is extensively handled and is warmed under the theatre lights. This is unavoidable due to the way in which a bowel resection is performed but does differentiate it from other tissues where the ischaemic time is much less. This time may account for the significant loss of good quality mRNA in many of the specimens. It is, however, difficult to alter any of these factors without compromising the operative technique or other aspects of patient care.

As indicated above, the size of the specimen being preserved altered during the investigation. The frozen specimens taken from the first 8 patients were smaller than those used subsequently. Of these specimens, 6/13 (46.2%) were used in the final analysis while 15/24 (62.5%) of the larger, later specimens produced GAPDH bands of sufficient strength to be used. There did not therefore appear to be any difference in quality between methods of preparation. The advantage of the larger tissue sections was that the frozen sections were generally of better quality for histological analysis.

Between them, the primer sets used in this work demonstrated a maximum of 7 different isoforms present in the colon. Use of the 8/18 primer set enabled the whole of the variable region to be surveyed, rather than priming for portions of the area as other investigations have done *(Wallner et al, 2002; Katenkamp et al, 2004)*. Some of the products generated by this method are large, making precise identification of their nature difficult but the number of bands detected in some of the samples shows,

firstly, that it is possible to detect the larger isoforms in this way and, secondly, that there are a greater variety of TN-C isoforms present in the colon than previously shown. Previous reports have indicated that splicing patterns are highly conserved and that not all of the theoretically possible isoforms will be produced *(Mighell et al, 1997)*. Using the patterns identified by Bell et al *(1999)* who utilised the 8/18 primer set on fœtal membranes, parallels were drawn with the results obtained here *(See Table 5.2)*. To establish the precise nature of the isoforms, the bands could have been sequenced or Southern blotting employed to identify specific exons with probes but at this stage the study was primarily concerned with identifying differences in isoform expression pattern between normal and diseased colon.

Using the 8/18 primer set atleast 5 isoforms can be identified. These are of sizes consistent with tTN, tTN+1 exon, tTN+3 exons, tTN+5 exons and a larger isoform. This last isoform is greater in size than the 2080 bp molecule identified as tTN+6 in previous reports and it is therefore likely that this isoform contains all of the exons in the variable region with the addition of ad1 and possibly ad2. The primer set 11/16 produced 4 bands which corroborate the findings with 8/18 and indicate the presence of a further two isoforms, tTN+4 and tTN+6, making a total of 7 different isoforms detected in the colon. The 11/16 primers detect fewer bands in total compared to 8/18 but this probably represents lower efficiency of the PCR cycle which, unlike that for 8/18, was not reoptimised for this work. As well as surveying the variable region for all the isoforms present, primers were employed to look for specific isoforms. The presence of ad1 has not previously been investigated or established in colon but this study demonstrated it in 13/21 samples. The high rate of detection of ad1 in the colon samples, despite the fact that the longest form of TN-C was only found in two of the samples using 8/18, highlights the greater efficiency of isoform specific primers over those which span a long area such as the 8/18 set.

In contrast to previous work *(Wilson et al, 1996; Dueck et al, 1999)* there did not appear to be any difference in the pattern or magnitude of expression of tenascin isoforms between normal, inflamed and malignant tissue. This was supported using both the 11/16 and 8/18 primer sets. The immunohistochemistry had already demonstrated an increase in TN14-containing isoforms in both inflammation and malignancy compared to normal tissue but on RT-PCR there was no visible difference in the strength of the bands present in these samples compared to the normals. This was also in contrast to previous studies on other tissues which have shown a switch to

TNL in malignancy *(Ghert et al, 2002; Tsunoda et al, 2003)*. In addition, there was no evidence of additional bands indicating the presence of new isoforms in the diseased samples. The additional domain band ad1 has been cited as a potential tumour specific exon but these results demonstrate that it is present in all types of colonic tissue. The ad2 domain was not examined specifically in this study although this has also been linked with malignancy *(Mighell et al, 1997)* but the possible presence of both ad1 and ad2 in the highest band using 8/18 may contradict this as both the samples expressing this band were normal.

Other investigators have used the 8/18 primer set on adult human tissues *(Wilson et al, 1996; Adams et al, 2002)* but have identified predominantly low molecular weight isoforms, especially in breast where the largest product seen was tTN+3. This difference in findings may be explained by the large amount of muscle present in the colon and this may also explain the apparent discrepancy between the immunohistochemistry and PCR findings. Although there was almost no TN14 expression in the normal mucosa or stroma using immunohistochemistry, TN14 containing isoforms were present in the muscularis mucosa, muscularis propria and blood vessels. It is likely, therefore, that many of the larger isoforms seen are derived from the muscle and are not therefore found in tissues containing less muscle. This raised the possibility that the large amount of TNL present in the muscle was masking the presence of smaller amounts of other isoforms. There were two main options available to counter this problem. The first was to remove the muscle layer and the second was to use specific primer sets to look for the presence of known isoforms. The option of removing the muscle layer by laser capture microdissection was explored but was not considered to be practical for several reasons. Firstly the longer TN-C isoforms are present throughout the bowel wall due to the variety of muscular structures present. Removal of the muscularis propria alone would probably not have been sufficient to ensure detection of less common isoforms and removal of the muscularis mucosa and muscular walled blood vessels would have been technically challenging. Secondly, a colorectal tumour is only defined as malignant once it has breached the muscularis mucosa and most of the resected specimens had also invaded the muscularis propria. Separation of the tumour, tumour stroma and muscle would have only been possible on a very small number of specimens and may even have distorted the results if invasion induced expression of new isoforms in the muscle itself.

As an alternative strategy, it was decided to investigate the possible presence of the TN14-16 isoform as this had been shown to be strongly associated with invasion in breast *(Adams et al, 2002)*. During the process of optimisation of the primer set 9-14/14-16, issues of both sensitivity and specificity had to be overcome. Although a band could successfully be obtained at a variety of temperatures, it disappeared very sharply when the annealing temperature was raised from 66°c to 67°c. When positive controls for both TN14-16 and TNL were used, however, bands were obtained for both samples at all temperatures, although the TNL band at 66°c was significantly fainter than the TN14-16 band. As TNL was likely to be present in much larger quantities than TN14-16 in the colorectal samples, it was necessary to be sure of the absolute specificity of the PCR reaction. The issue of specificity arose due to the large amounts of homology between TN14-16 and TNL as the variable region is composed of FN repeats, enabling the primers to bind and extend at several points on the molecule if the conditions are not sufficiently stringent. The technique which was finally adopted was touchdown PCR which produced a band specific to TN14-16. Interestingly the gel that was run under conditions which enabled detection of TN16, TN14-16 and TNL produced strong bands in most of the samples except a number of the tumour samples. This is likely to indicate the muscle layer as a strong source of TNL as not all the tumour samples included the muscle layer as the tumour had often replaced the muscle layer.

Use of the 9-14:14-16 primer set did not produce any convincing bands in marked contrast to the results in breast adenocarcinoma where the TN14-16 isoform was detected in 86% invasive carcinomas and 73% DCIS cases *(Adams et al, 2002)*. The discrepancy in the colon and breast results may lie in the source of TN14-16. Although the commonest carcinomas affecting the colon and breast are glandular in nature, the surrounding structures are markedly different and it may be that, in malignancy, a stromal protein such as TN-C differs in behaviour between the two tissues. The absence of this isoform in the normal and inflamed colon samples is still of significance, however, as it indicates that the isoform is indeed tumour specific, albeit not in colorectal cancer, and this is highly important for any future therapeutic targeting of this isoform as toxicity and cross-reactivity would be a major consideration. The other consideration is that a greater portion of the TN-C may be tumour derived than has previously been appreciated and that the isoform profile is actually tumour rather than tissue dependent. For this reason the investigation

proceeded to analyse colorectal cell lines for the expression of TN-C as described in *Chapter 6.*

Chapter 6

Relationship between tenascin isoform expression and tumour behaviour

6.1. INTRODUCTION

Although tenascin is predominantly expressed by stromal fibroblasts, in situ hybridisation studies have indicated that epithelial tumour cells are the source of TN in some cases *(Yoshida et al 1997; Dandachi et al 2001)*. Both breast and prostate adenocarcinomas are known to express tenascin and mRNA for both long and short isoforms has been detected in prostate carcinoma cells *(Katenkamp et al, 2004)*. Malignant cells are, however, genetically diverse and cell lines can have widely differing phenotypes, even when derived from tumours affecting the same organ. A number of colorectal cell lines have been generated and the genotypes and functional characteristics of these have been defined to varying degrees *(Nimmrich et al 2000; Williams et al 2003)*. In view of the finding of multiple TN-C isoforms in malignant colorectal tissue, it was decided to examine a variety of colorectal cell lines for the presence of tenascin expression and any differences in isoform expression. This part of the study therefore aimed to address whether TN isoform profile relates to tumour behaviour. To begin this process of correlating tenascin isoform profile with cell function, invasion assays were utilised to examine one feature of tumour behaviour.

6.2. RESULTS

6.2.1. **Tenascin isoform expression in colorectal tumour cell lines**

The majority of PCR was carried out on previously prepared stocks of cDNA from four colorectal tumour cell lines (HT29, HCT116, HCT15 and SW480) using the primer sets 8/18, 11/16, ad1 and 9-14/14-16. Due to some discrepant results (as detailed below) the 8/18 PCR reaction was repeated on freshly prepared cDNA from the cell lines HT29, HCT116, SW480 and SW620. The invasion assays were also performed on this second group of cell lines. The primer sets were utilised as described in Chapter 5 *(See Table 5.1)* and GAPDH reactions and the use of positive and negative controls were also employed as in Chapter 5.

Isoforms detected using 8/18 primer set

The reaction using the stored cDNA only showed convincing bands in SW480 where two bands representing tTN and tTN+1 were demonstrated *(See Figure 6.1)*. When the reaction was repeated using freshly prepared cDNA, three out of the four cell lines (SW480, SW620 and HT29) were shown to express TN-C mRNA with only HCT116 failing to express TN-C *(See Figure 6.5)*. In those cell lines which did express TN-C, several isoforms were present. SW620 and HT29 contained tTN, tTN+1 and tTN+3 while SW480 contained tTN and tTN+1 (See Table 6.1 and 6.2 and Table 5.2 for isoform sequences).

Isoforms detected using 11/16 primer set

Only SW480 generated amplicons with this primer set and this reaction indicated the presence of the isoforms tTN+3, tTN+4 and tTN+5 *(See Table 6.1)*. No bands were generated for HT29, HCT15 or HCT116 with this primer set *(See Figure 6.2)*.

Isoforms detected using ad1 primer set

This showed the presence of ad1 in HT29, HCT15 and SW480 but not HCT116 *(See Figure 6.3 and Table 6.1)*.

Isoforms detected using 9-14/14-16 primer set

None of the cell lines contained the TN-C isoform 9-14-16 *(See Figure 6.4 and Table 6.1)*.

6.2.2. **Invasion assays**

These results were provided by my colleague Dr Deborah Holliday and used techniques well established in the laboratory. SW480 and HCT116 showed invasion of 26% and 15% respectively. The other two cell lines, SW620 and HT29, both showed less than 5% invasion. Since HCT116 does not express TN-C and has the highest invasion index in this assay, there is no clear relationship between tumour expression of TN-C and invasive capacity using this system.

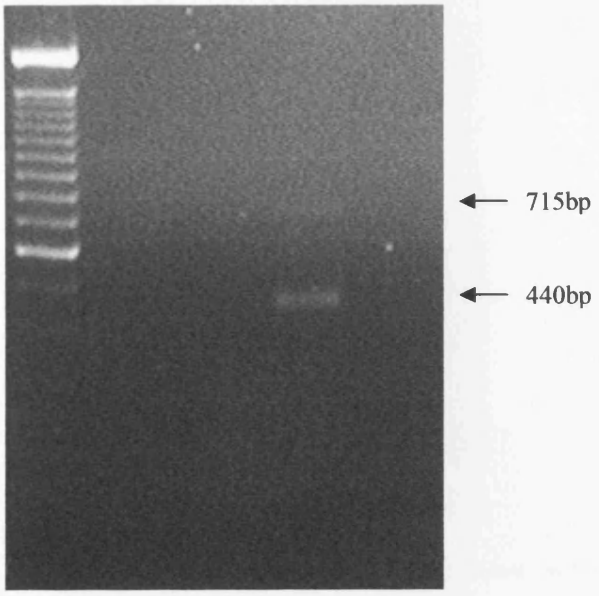


Figure 6.1 Pattern of tenascin isoform expression in four colorectal carcinoma cell lines using primer set tenascin 8/18

The lane containing products from SW480 demonstrated bands of 440 and 715 base pairs consistent with tTN and tTN+1 exon.

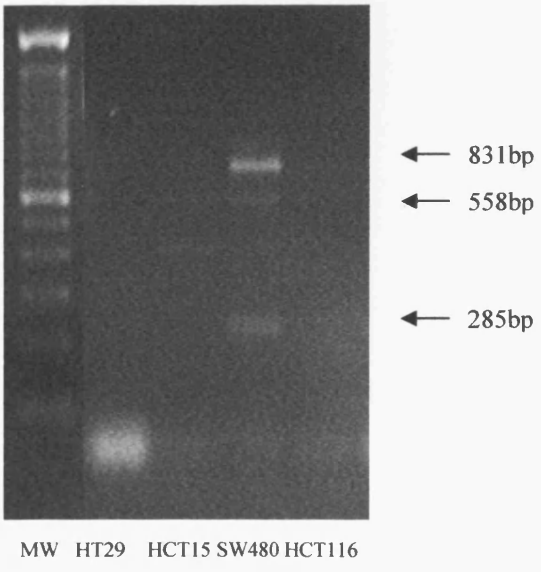
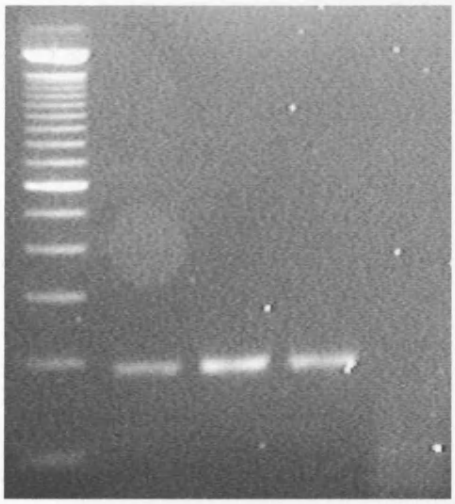


Figure 6.2 Patterns of tenascin isoform expression in four colorectal carcinoma cell lines using primer set tenascin 11/16.

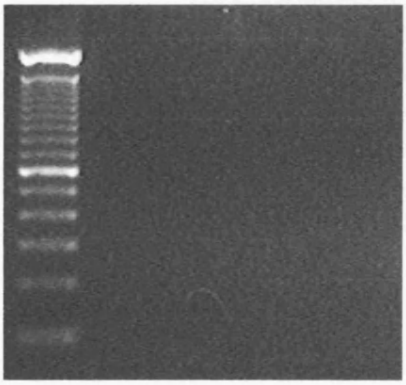
The cell line SW480 demonstrated products containing 285, 558 and 831 base pairs consistent with the tenascin isoforms tTN+3, tTN+4 and tTN+5.



MW HT29 HCT15 SW480 HCT116

Figure 6.3 Pattern of expression of tenascin isoforms containing exon ad1 in four colorectal carcinoma cell lines using primer set tenascin ad1.

Four of the cell lines demonstrated bands consistent with the expression of isoforms containing exon ad1.



Mol wgt marker HT29 HCT15 SW480 HCT116

Figure 6.4 Patterns of expression of the tenascin isoform TN14-16 in four colorectal carcinoma cell lines using primer set tenascin 9-14/14-16

None of the cell lines demonstrated bands consistent with the expression of TN14-16

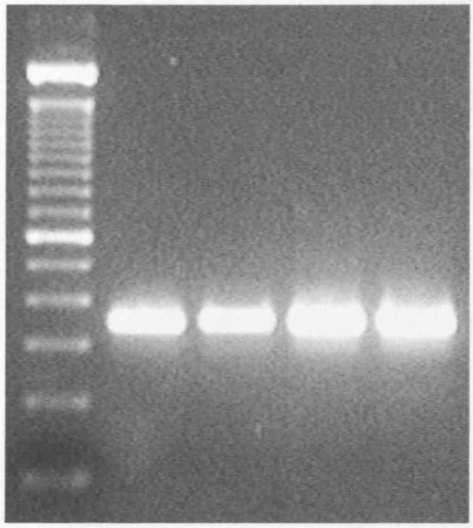


Figure 6.5 Expression of GAPDH in four colorectal cell lines using GAPDH primer set. The relative strength of the bands was compared to assess quality of the mRNA.

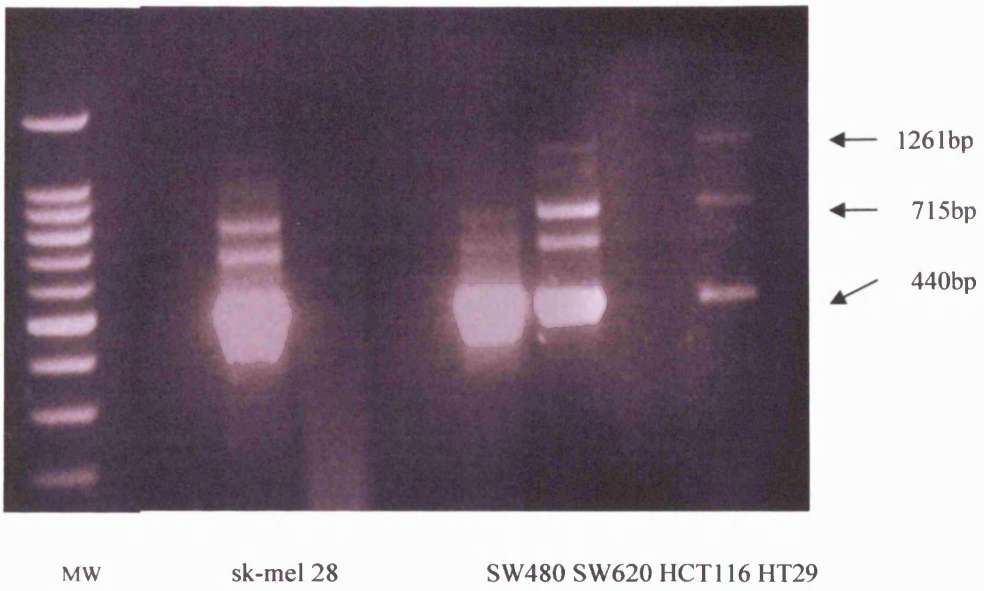


Figure 6.6 Patterns of expression of tenascin isoforms in four cultured colorectal carcinoma cell lines using tenascin 8/18 primer set

Cell lines SW480, SW620 and HT29 demonstrate products of sizes 440, 715 and 1261 base pairs consistent with tTN, tTN+1 and tTN+3

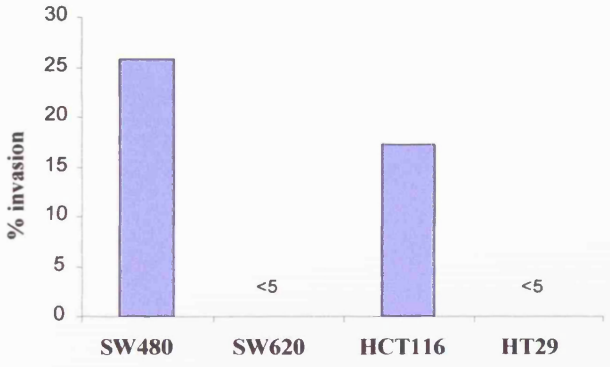


Figure 6.7 Graph showing percentage invasion of four colorectal cell lines

SW480 and HCT116 demonstrated 26% and 15% invasion respectively.

Table 6.1 Isoforms represented by bands present on gels using tenascin primer sets on stored colorectal cell line cDNA

Primer set	Cell line	Level of band	Tn isoforms present
8/18	HT29	Nil	
	HCT116	Nil	
	HCT15	Nil	
	SW480	440bp	tTN
		715bp	tTN+1
11/16	HT29	Nil	
	HCT116	Nil	
	HCT15	Nil	
	SW480	285bp	tTN+3
		558bp	tTN+4
		831bp	tTN+5
ad1	HT29	276bp	ad1 present
	HCT116	276bp	ad1 present
	HCT15	276bp	ad1 present
	SW480	276bp	ad1 present
9-14/14-16	HT29	Nil	
	HCT116	Nil	
	HCT15	Nil	
	SW480	Nil	

Table 6.2 Isoforms represented by bands present on gels using tenascin primer sets on freshly prepared colorectal cell line cDNA

Primer set	Cell line	Level of band	Tn isoforms present
8/18	HT29	440bp	tTN
		715bp	tTN+1
		1261bp	tTN+3
	HC116	Nil	
	SW620	440bp	tTN
		715bp	tTN+1
		1261bp	tTN+3
	SW480	440bp	tTN
		715bp	tTN+1

6.3. DISCUSSION

TN-C is an extracellular matrix protein and the enhanced expression demonstrated in malignancy occurs predominantly in the stroma *(Iskaros et al 2000; Gulubova & Vlaykova 2001)*. Some studies, however, have also demonstrated TN-C production in benign and malignant epithelial lesions *(Yoshida et al, 1997; Katenkamp et al, 2004)* and in some non-epithelial tumour cell lines *(Ghert et al, 2002)*. TN-C expression has been linked to the acquisition of a mesenchymal phenotype by breast carcinoma cells and correlates with increased malignancy *(Dandachi et al, 2001)*. The isoform profile of TN-C produced by malignant cells has been less extensively investigated. Thyroid carcinoma cell lines have been shown to express TN-C with the majority displaying a predominance of long isoforms while medullary carcinomas mainly express short isoforms *(Tseleni-Balafouta et al, 2006)*. Examination of TN-C mRNA expression in prostatic adenocarcinoma revealed that tumour cells expressed predominantly longer TN-C isoforms containing multiple exons while the stromal cells were expressing truncated TN-C *(Katemkamp et al 2004)*. The expression of TN-C by some colorectal tumour cells has been demonstrated by in situ hybridisation (ISH) studies *(Hanamura et al, 1997)* but there have been no studies to establish the nature of the isoforms produced by colorectal adenocarcinoma cells.

The results shown in this study clearly demonstrate that colorectal cell lines can be the source of TN-C and that frequently more than one isoform is generated. The combined results from the various primer sets show that only HCT116 does not express TN-C. SW480 has been demonstrated to produce atleast five isoforms while SW620 and HT29 both produce atleast three. The presence of ad1 in SW480, HT29 and HCT15 indicates that larger isoforms may also be present at low levels, although it is possible that ad1 may be incorporated into smaller isoforms as well.

There is an obvious discrepancy between the 8/18 primer set results for HT29 using the two batches of cDNA with the second reaction indicating the presence of three isoforms of TN-C although the first reaction does not indicate the presence of any TN-C mRNA. In addition, the presence of ad1in HT29 and HCT15 as well as SW480 indicates that TN-C mRNA is indeed expressed by these two cell lines although no isoforms were demonstrated using either the 8/18 or 11/16 primer sets. For organisational reasons, the PCR reactions using stored and freshly prepared cDNA were undertaken in different laboratories using different PCR machines with the

resultant implications for reproducibility. The cells from which the mRNA was extracted had been thawed and re-frozen several times potentially affecting mRNA quality and the cells from which the mRNA derived may have reached different levels of confluency in culture. Alterations in extracellular pH have been demonstrated to influence the isoform profile of fibroblast derived TN-C *(Borsi et al 1995)* making the pH of the culture media a further consideration. In addition, the stored cDNA utilised in the first experiments had been used previously by other individuals in the laboratory and may have degraded as a result of prolonged storage or freeze-thawing. The presence of these multiple variables necessitated repetition of the PCR reaction using the 8/18 primer set prior to performing the invasion assays with the discrepant results described above.

The overall quality of the cDNA was assessed using a GADPH reaction which produced strong bands for all the samples but in view of the differing results it may be that this is not a sufficiently sensitive indicator of mRNA quality. The lack of bands for HT29 using the prepared cDNA may indicate that less TN-C was present overall due to differences in the condition of the original cells, or that the quality of the cDNA was insufficient to obtain a good reaction. The presence of ad1 in HCT15 when the 8/18 and 11/16 primer sets did not demonstrate the presence of TN-C may indicate that TN-C is only present in low amounts and can only be demonstrated using a specific primer to a short sequence.

Results from earlier in this study have shown TN-C to be present in the tumour stroma in sufficient quantities to be clearly demonstrated using immunohistochemical techniques. The epithelial tumour cells did not demonstrate any staining but, as the TN-C produced by these cells is deposited in the stroma, TN-C from the cells would be better demonstrated by ISH. The RT-PCR studies described in previous chapters do not demonstrate the origin of the various isoforms present in the tumour specimens. With the demonstration of multiple TN-C isoforms within the tumour cell lines it remains possible that some of the isoforms present were solely tumour in origin rather than deriving from the stroma. Recent work in the laboratory has demonstrated a subset of breast carcinomas in which a particular ad1 containing TN-C isoform is produced by the tumour epithelial cells *(JL Jones personal communication)* These in situ probes were not available at the time of this study, however, future studies could employ ISH to demonstrate the precise cellular origin of TN-C and the isoform distribution within normal and diseased colonic tissue.

The four cell lines exhibited differing levels of invasion, with SW480 and HCT116 being highly invasive and HT29 and SW620 showing low levels of invasion. The results support previous findings that SW480 is more invasive than HT29 *(Sclaeppi et al, 1997)* but the lack of a direct correlation between invasive capacity and TN-C expression suggest that the invasive capacity of a malignant epithelial cell is not solely related to TN-C production. The cell lines used are known to differ in a variety of genetic and behavioural features and SW480 and HCT116 have been shown to have differential transcription of a number of genes *(Nimrich et al, 2000)* which could therefore account for the difference in invasion between the four cell lines. The invasion assay utilised in this study has been adapted from the method published by Hendrix et al *(1989)* and within our laboratory it has been demonstrated to produce highly reproducible results *(D Holliday personal communication)*. The fibroblast conditioned media is included to produce conditions optimal for invasion. These assays do not, however, account for the influence of interactions between tumour and stromal cells, resulting for example in enhanced MMP expression *(Rösmann et al 2002)* or upregulation of the plasminogen activating system *(Baker et al 2000)*. Previous studies have demonstrated that both TN-C and myofibroblast derived scatter factor hepatocyte growth factor (SF/HGF) are necessary to induce invasion by colon carcinoma cells in culture *(De Wever et al, 2004)*. More recent work has also shown that the addition of stromal cells to colorectal tumour cell cultures can induce the expression of TN-C in HT29 and Caco-2 tumour cell lines *(Mukaratirwa et al, 2005)*. This study also indicates that this is a reciprocal relationship and is modulated by TGFß. The situation in vivo may, therefore, be significantly different from that represented here both in quantity of TN-C expressed and in isoform profile. Further work, including blocking TN-C antibody approaches and more extensive utilisation of co-cultures could help indicate whether tumour-derived TN-C contributes to invasive capacity.

This chapter presents results from very preliminary experiments investigating the relationship between TN-C isoform expression and invasive behaviour in the context of colorectal malignancy. More extensive studies are clearly required to establish the cellular origin of TN-C isoforms and their contribution to the process of invasion.

Chapter 7

Final discussion and future directions

This study set out to investigate further the complex relationship between tumour and microenvironment in the development and progression of invasive malignancy in the colon, focussing specifically on the areas of fibroblast phenotype and the ECM protein TN-C.

The colonic fibroblast population is diverse and this was confirmed by our immunohistochemical findings. Using immunohistochemical studies and the markers CD34, αSMA and hCD, the colonic peri-cryptal myofibroblast (PCMF) (CD34 $^{-}$ve, αSMA^{+}ve, hCD^{+}ve) and sub-mucosal fibroblast (CD34^{+}ve, αSMA^{-}ve, hCD^{-}ve) groups were delineated. There was a change in fibroblast phenotype in the presence of malignancy as the tumour stroma was CD34^{-}ve, αSMA^{+}ve, hCD^{-}ve and the loss of sub-mucosal CD34 was specifically localised around areas of invasive malignancy. The submucosal changes were not observed in pre-invasive adenomas although increased amounts of αSMA were observed in the lamina propria. There was also the suggestion that these myofibroblasts did not arise from the pericryptal myofibroblasts but from another fibroblast population as the PCMFs are hCD^{+}ve and hCD staining decreased with the development of malignancy whereas expression of αSMA increased.

Although the specificity of the alterations in invasive malignancy was striking, so was the absence of any sub-mucosal changes preceding the development of invasive malignancy. This emphasises the importance of the process of invasion, both in terms of the implications for overall disease progression but also as a key biological determinant. The absence of pre-invasive alterations in cell markers reduces the clinical significance of these markers as possible predictors of future invasion as the discovery of such a marker would be invaluable in guiding the management of colonic polyps. There was however the suggestion of pre-invasive alterations in the laminia propria of the adenomas with increased αSMA expression and decreased hCD expression. As the hCD was specifically expressed by the PCMFs this may represent loss of a key barrier to invasion as the PCMFs are known to perform a number of functions within the colon.

Tenascin-C (TN-C) is an important component of the ECM and important in migration and adhesion. Immunohistochemical techniques demonstrated the presence of TN-C in the basement membrane, muscularis mucosa and muscularis propria of normal colon and also showed that isoforms containing exon 14 were amongst those expressed in the muscularis mucosa and muscularis propria. There was extensive

expression of TN-C in the tumour stroma and exon 14 containing isoforms were present. The distribution of TN-C in pre-invasive adenomas was the same as that in normal colon but some sub-mucosal TN-C expression was observed in acutely inflamed colon and, again, exon 14 containing isoforms were present. Polymerase chain reaction (PCR) was then employed to investigate the nature of the isoforms present in normal, malignant and inflamed colon. This demonstrated that a total of 7 different isoforms were present but there was no clear association between isoform profile and disease.

The presence of large amounts of TN-C within the muscle was intriguing in that it confirmed that long isoforms occur in significant amounts in normal tissues, but this also presented practical challenges as large amounts of the longer isoforms could mask the presence of smaller isoforms detected using PCR. The use of specific probes enabled targeted investigation of isoforms such as 9-14-16 but primers were not available for all the isoforms. In addition, the close involvement of the muscle layers with most invasive colorectal tumours makes it difficult to elucidate the precise source of the TN-C isoforms in this case. ISH may offer some solutions to this problem, especially if probes to the smaller isoforms were available. The lack of convincing TN 9-14-16 in the colon is interesting as it implies that the presence of this isoform in breast malignancy *(Adams et al, 2002)* is not only tumour specific but tissue specific, thus implying a specialised stromal response to malignancy in different carcinomas. In this case the absence of TN 9-14-16 expression in normal colon would be an advantange as it would reduce side-effects of any therapy specifically targeting the isoform on other tissues.

The complexity of the balance between tumour and host response was emphasised by the findings using the tumour cell lines. This identified TN-C expression by 4 out of 5 colorectal carcinoma cell lines and demonstrated that more than one isoform was present in each case. The fact that TN-C expression did not appear to correlate with invasion capacity serves to emphasise the multi-factorial nature of the process of invasion *(De Wever et al, 2004)*. Again, ISH in tissue sections may clarify the precise contributions of the tumour and stroma to TN-C content of the tumour stroma both in quantity and isoform composition.

The absence of clear pre-invasive changes or tumour specific isoforms in these results may preclude a simple clinical application but the existing evidence as to the extent of stromal involvement in the progression of malignancy does not indicate a single

switch mechanism applying to all tissues. Instead, a picture is emerging of a complex network of factors which are both tissue and tumour specific and which can both limit and permit tumour development. Against this background, the findings of colon specific fibroblast heterogeneity, multiple TN-C isoforms in both normal and malignant colon and TN-C production by the colorectal carcinoma cells themselves should prove useful details in elucidating the full mechanism of invasion in colorectal malignancy.

Chapter 8
References

Adams M, Jones JL, Walker RA, Pringle H and Bell SC. Changes in Tenascin-C isoform expression in invasive and pre-invasive breast disease. *Can Res* 2002; **62**: 3289-3297

Adeboyega PA, Mifflin RC, DiMari JF, Saada JI and Powell DW. Immunohistochemical study of myofibroblasts in normal colonic mucosa, hyperplastic polyps and adenomatous colorectal polyps. *Arch Pathol Lab Med* 2002; **126**: 829-836

Akabani G, Cokgor I, Coleman RE, Trotter DG, Wong TZ, Friedman HS, Friedman AL, Garcia-Turner A, Herndon JE, DeLong D, McLendon RE, Zhao X-G, Pegram CN, Provenzale JM, Bigner DD and Zalutsky MR. Dosimetry and dose-response relationships in newly diagnosed patients with malignant gliomas treated with iodine-131-labeled anti-tenascin monoclonal antibody 81C6 therapy. *Int J Radiation Oncology Biol Phys* 2000; **46**: 947-958

Aparicio T, Kermorgant S, Dessirier V, Lewin MJM and Lehy T. Matrix metalloproteinases inhibition prevents colon cancer peritoneal carcinomatosis development and prolongs survival in rats. *Carcinogenesis* 1999; **20**: 1445-1451

Ashman LK, Aylett GW, Cambareri AC and Cole SR. Different epitopes of the CD31 antigen identified by monoclonal antibodies: cell type-specific patterns of expression. *Tissue Antigens* 1991; **38**: 199-207

Azzarone B, Mareel M, Billard C, Scemama P, Chaponnier C and Marciera-Coelho A. Abnormal properties of skin fibroblasts from patients with breast cancer. *Int J Cancer* 1984; **33**: 759-764

Baker EA, Bergin FG and Leaper DJ. Plasminogen activator system, vascular endothelial growth factor and colorectal cancer progression. *J Clin Pathol: Mol Pathol* 2000; **53**: 307-312

Balazs M and Kovacs A. Electron microscopic study of carcinoma of the colon. *Exp Pathol* 1981; **20**: 203-214

Balkwill F and Mantovani M. Inflammation and cancer: Back to Virchow. *Lancet* 2001; **357**: 539-545

Balkwill F, Charles KAand Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* 2005; **7**: 211-217

Balza E, Siri A, Ponassi M, Caocci F, Linnala A, Virtanen I and Zardi L. Production and characterization of monoclonal antibodies specific for different epitopes of human tenascin. *FEBS* 1993; **332**: 39-43

Barcellos-Hoff MH and Ravani SA. Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. *Cancer Res* 2000; **60**: 1254-1260

Bell SC, Pringle JH, Taylor DJ and Malak TM. Alternatively spliced tenascin-C mRNA isoforms in human fetal membranes. *Mol Hum Reprod* 1999; **5**: 1066-1076

Beltinger J, McKaig BC, Makh S, Stack WA, Hawkey CJ and Mahida YR. Human colonic subepithelial myofibroblasts modulate transepithelial resistance and secretory response. *Am J Physiol Cell Physiol* 1999; **277**: C271-C279

Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, Washington MK, Neilson EG and Moses HL. TGF-beta signalling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* 2004; **303**: 848-851

Bingle L, Brown NJ and Lewis CE. The role of tumour associated macrophages in tumour progression: Implications for new anticancer therapies. *J Pathol* 2002; **196**: 254-265

Booth C, Harnden P, Selby PJ and Southgate J. Towards defining roles and relationships for tenascin-C and TGFß-1 in the normal and neoplastic urinary bladder. *J Pathol* 2002; **198**: 359-368

Borsi L, Carnemolla B, Nicolo G, Spina B, Tanara G, Zardi L. Expression of different tenascin isoforms in normal, hyperplastic and neoplastic human breast tissues. *Int J Cancer* 1992; **52**: 688-692

Borsi L, Balza E, Gaggero B, Allemanni G and Zardi L. The alternative splicing pattern of the tenascin-C pre-mRNA is controlled by the extracellular pH. *J Biol Chem* 1995; **270**: 6243-6245

Bosman FT and Stamenkovic I. Functional structure and composition of the extracellular matrix. (Review) *J Pathol* 2003; **200**: 423-428

Bourdon MA, Wikstrand CJ, Furthmayr H, Matthews TJ and Bigner DD. Human glioma-mesenchymal extracellular matrix antigen defined by monoclonal antibody. *Cancer Res* 1983; **43:** 2796-2805

Bramhall SR, Neoptolemos JP, Stamp GWH and Lemoine NR. Imbalance of expression of matrix metalloproteinases (MMPs) and tissue inhibitors of the matrix metalloproteinases (TIMPs) in human pancreatic carcinoma. *J Pathol* 1997; **182**: 347-355

Braun S, auf dem Keller U, Steiling H and Werner S. Fibroblast growth factors in epithelial repair and cytoprotection. *Phil Trans R Soc Lond* 2004; **539**: 753-757

Bristow J, Tee MK, Gitelman SE, Mellon SH and Miller WL. Tenascin-X: a novel extracellular matrix protein encoded by the human XB gene overlapping P450c21B. *J Cell Biol* 1993; **122**: 265-278

Brittan M, Hunt T, Jeffrey R, Poulsom R, Forbes SJ, Hodivala-Dilke K, Goldman J, Alison MR and Wright NA. Bone marrow derivation of pericryptal myofibroblasts in the mouse and human small intestine and colon. *Gut* 2002; **50**: 752-757

Brittain MG, Fine WD, Khaled FM, Thompson J and Brittain DE. Heterogeneity of malignant cells from a human colonic cell carcinoma. *Cancer Res* 1981; **41**: 1751-1756

Brown J, Greaves MF and Molgaard HV. The gene encoding the stem cell antigen CD34 is conserved in mouse and expressed in haemopoietic progenitor cell lines, brain and embryonic fibroblasts. *Int Immunol* 1991; **3**: 175-184

Buckley CD. Why does chronic inflammatory joint disease persist? *Clin Med* 2003; **3**: 361-366

Burgeson RE, Chiquet M, Deutzmann R, Ekblom P, Engel J, Kleinman H, Marti GR, Meneguzzi G, Paulsson M and Sanes J. A new nomenclature for the laminins. *Matrix Biol* 1994; **14**: 209-211

Buyukbayram H and Arslan A. Value of tenascin-C content and association with clinicopathological parameters in uterine cervical lesions. *Int J Cancer* 2002; **100**: 719-722

Carnemolla B, Borsi L, Bannikov G, Troyanovsky S and Zardi L. Comparison of human tenascin expression in normal, simian-virus-40-transformed and tumor derived cell lines. *Eur J Biochem* 1992; **205**: 561-567

Carnemolla B, Castellani P, Ponassi M, Borsi L, Urbini S, Nicolo G, Doraratto A, Viale G, Winter G, Neri D and Zardi L. Identification of a glioblastoma-associated tenascin-C isoform by a high affinity recombinant antibody. *Am J Pathol* 1999; **154**: 1345-1352

Chauhan H, Abraham A, Phillips JRA, Pringle JH, Walker RA and Jones JL. There is more than one kind of myofibroblast: analysis of CD34 expression in benign, in situ and invasive breast lesions. *J Clin Pathol* 2003; **56**: 271-276

Chen AL, Kizhake VS, Rychachou PG, Luxon BA and Evers BM. Proteomic analysis of colonic myofibroblasts and effect on colon cancer cell proliferation. *Surgery* 2005; **138**: 382-390

Chiquet M and Fambrough DM. Chick myotendinous antigen. I. A monoclonal antibody as a marker for tendon and muscle morphogenesis. *J Cell Biol* 1984; **98**: 1926-1936

Chiquet M and Fambrough DM. Chick myotendinous antigen. II. A novel extracellular glycoprotein complex consisting of large disulphide-linked subunits. *J Cell Biol* 1984; **98**: 1937-1946

Chiquet M. Regulation of extracellular matrix gene expression by mechanical stress. *Matrix Biol* 1999; **18**: 417-426

Chiquet-Ehrismann R, Mackie EJ, Pearson CA and Sakakura T. Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell* 1986; **47**: 131-139

Chiquet-Ehrismann R, Tannheimer M, Koch M, Brunner A, Spring J, Martin D, Baumgartner S and Chiquet M. Tenascin-C expression by fibroblasts is elevated in stressed collagen gels. *J Cell Biol* 1994; **127**: 2093-2101

Chiquet-Ehrismann R and Chiquet M. Tenascins: regulation and putative functions during pathological stress. *J Pathol* 2003; **200**: 488-499

Chung CY, Murphy-Ullrich JE and Erickson HP. Mitogenesis, cell migration and loss of focal adhesions induced by tenascin-C interacting with its cell surface receptor annexin II. *Mol Biol Cell* 1996; **7**: 883-892

Compton C, Fenoglio-Preiser CM, Pettigrew N and Fielding LP. American Joint Committee on Prognostic Factors Consensus Conference. *Cancer* 2000; **88**: 1739-1757

Condon MS. The role of the stromal microenvironment in prostate cancer. *Semin Cancer Biol* 2005; **15**: 132-137

Coussens LM and Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860-867

Cowan KN, Jones PL and Rabinovitch M. Elastase and matrix metalloproteinase inhibitors induce regression and tenascin-C antisense prevents progression of vascular disease. *J Clin Invest* 2000; **105**: 21-34

Crossin KL, Hoffman S, Grumet M, Thiery JP and Edelman GM. Site-restricted expression of cytotactin during development of the chick embryo. *J Cell Biol* 1986; **102**: 1917-1930

Dandachi N, Hauser-Kronberger C, Moré E, Wiesener B, Hacker GW, Dietze O and Wirl G. Co-expression of tenascin-C and vimentin in human breast cancer cells indicates phenotypic transdifferentiation during tumour progression: correlation with histopathological parameters, hormone receptors and oncoproteins. *J Pathol* 2001; **193**: 181-189

Daum S, Bauer U, Foss H-D, Schuppan D, Stein H, Riecken E-O and Ullrich R. Increased expression of mRNA for matrix metalloproteinases-1 and -3 and tissue inhibitor of metalloproteinases-1 in intestinal biopsy specimens from patients with coeliac disease. *Gut* 1999; **44**: 17-25

Day DW, Jass JR, Price AB, Shepherd NA, Sloan JM, Talbot IC, Warren BF and Williams GT. Morson and Dawson's Gastrointestinal Pathology. 4th edition *Blackwell* 2003

DeSantis R, Anastasi AM, D'Alessio V, Pelliccia A, Albertoni C, Rosi A, Leoni B, Lindstedt R, Petronzelli F, Dani M, Verdoliva A, Ippolito A, Campanile N, Manfredi V, Esposito A, Cassani G, Chinol M, Paganelli G and Carminati P. Novel antitenascin antibody with increased tumour localisation for pretargeted antibody-guided radioimmunotherapy (PAGRIT). *Br J Cancer* 2003; **88**: 996-1003

De Wever O, Nguyen Q-D, Van Hoorde L, Bracke M, Bruyneel E, Gespach C and Mareel M. Tenascin-C and SF/HGF produced by myofibroblasts in vitro provide convergent proinvasive signals to human colon cancer cells through RhoA and Rac. *FASEB J* 2004;

Direkze NC, Forbes SJ, Brittan M, Hunt T, Jeffery R, Preston SL, Poulsom R, Hodivala-Dilke K, Alison MR and Wright NA. Multiple organ engraftment by bone-marrow-derived myofibroblasts and fibroblasts in bone-marrow-transplanted mice. *Stem Cells* 2003; **21**: 514-520

Dukes CE. The classification of cancer of the rectum. *J Pathol Bacteriol* 1932; **35**: 323

Dukes CE and Bussey HJR. The spread of cancer and its effect on prognosis. *Br J Cancer* 1958; **12**: 309

Dueck M, Riedl S, Hinz U, Tandara A, Moller P, Herfarth C and Faissner A. Detection of tenascin-C isoforms in colorectal mucosa, ulcerative colititis, carcinomas and liver metastases. *Int J Cancer* 1998; **82**: 477-483

Durning P, Schor SL and Sellwood RA. Fibroblasts from patients with breast cancer show abnormal migratory behaviour in vitro. *Lancet* 1984; **2**: 890-892

Eckes B, Zigrino P, Kessler D, Holtkötter O, Shephard P, Mauch C and Krieg T. Fibroblast-matrix interactions in wound healing and fibrosis. *Matrix Biol* 2000; **19**: 325-332

Ehlin AGC, Montgomery SM, Ekbom A, Pounder RE and Wakefield AJ. Prevalence of gastrointestinal disease in two British national birth cohorts. *Gut* 2003; **52**: 117-1121

Ekblom P and Aufderheide E. Stimulation of tenascin expression in mesenchyme by epithelial-mesenchymal interactions. *Int J Dev Biol* 1989; **33**: 71-79

Erickson HP and Iglesias JL. A six-armed oligomer isolated from cell surface fibronectin preparations. *Nature* 1984; **311: 267-269**

Erickson HP and Bourdon MA. Tenascin: an extracellular matrix protein prominent in specialized embryonic tissues and tumors. *Annu Rev Cell Biol* 1989; **5**: 71-92

Esposito I, Penzel R, Chaib-Harrireche M, Barcena U, Bergmann F, Riedl S, Kayed H, Giese N, J Kleeff, Friess H and Schirmacher P. Tenascin C and annexin II expression in the process of pancreatic carcinogenesis. *J Pathol* 2006; **208**: 673-685

Fearon ER and Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759

Feiner H and Kaye GI. Ultratructural evidence of myofibroblasts in circumscribed fibromatosis. *Arch Pathol Lab Med* 1976; **100**: 265-268

Ferhat L, Cheassus-Au-Louis N, Khrestchatisky M, Ben-Ari Y and Rep A. Seizures induce tenascin-C mRNA expression in neurons. *J Neurocytol* 1996; **25**: 535-546

Fina L, Molgaard HV, Robertson D, Bradley NJ, Monaghan P, Delia D, Sutherland DR, Baker MA and Greaves MF. Expression of CD34 gene in vascular endothelial cells. *Blood* 1990; **175**: 2417-2426

Fleming ID, Cooper JS, Henson DE, Hutter RVP, Kennedy BJ and Murphy GP. American Joint Committee on Cancer cancer staging manual 5th edition. *Philadelphia: Lippincott-Raven* 1997

Fischer D, Brown-Ludi M, Schulthess T and Chiquet-Ehrismann R. Concerted action of tenascin-C domains in cell adhesion, anti-adhesion and promotion of neurite outgrowth. *J Cell Sci* 1997; **110**: 1513-1522

Freeman HJ. Colorectal cancer complicating Crohns disease. *Can J Gastroenterol* 2001; **15**: 213-236

Frid MG, Shekhonin BV, Koteliansky VE and Glukhova MA. Phenotypic changes o human smooth muscle cells during development: late expression of heavy caldesmon and calponin. *Dev Biol* 1992; **153**: 185-193

Fuss B, Pott U, Fischer P, Schwab ME and Schachner M. Identification of a cDNA clone specific for the oligodendrocyte-derived repulsive extracellular matrix molecule J1-160/180. *J Neurosci Res* 1991; **29**: 299-307

Gabbiani G. The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 2003; **200**: 500-503

Galliano M-F, Aberdam D, Aguzzi A, Ortonne JP and Meneguzzi G. Cloning and complete primary structure of the mouse laminin α3 chain. *J Biol Chem* 1995; **270**: 21820-21826

Gear JSS, Ware A, Fursdon P, Mann JI, Nolan DJ, Brodribb AJM and Vesey MP. Symptomless diverticular disease and intake of dietary fibre. *Lancet* 1979; **8115**: 511-514

Ghert MA, Jung ST, Qi W, Harrelson JM, Erikson HP, Block JA and Scully SP The clinical significance of tenascin-C splice variant expression in chondrosarcoma. *Oncology* 2001; **61**: 306-314

Ghert MA, Qi W, Erickson HP, Block JA and Scully SP. Tenascin-C splice variant adhesive/anti-adhesive effects on chondrosarcoma cell attachment to fibronectin. *Cell Struct Func* 2001; **26**: 179-187

Ghert MA, Qi WN, Erickson HP, Block JA and Scully SP. Tenascin-C expression and distribution in cultured human chondrocytes and chondrosarcoma cells. *J Orthop Res* 2002; **20**: 834-841

Gherzi R, Carnemolla B, Siri A, Ponassi M, Balza E and Zardi L. Human tenascin gene. Structure of the 5'-region, identification and characterization of the transcription regulatory sequences. *J Biol Chem* 1995; **270**: 3429-3434

Goepel C, Stoerer S and Koelbl H. Tenascin in proinvasive lesions of the vulva and vulval cancer. *Anticancer Res* 2003; **23**: 4587-4591

Grady WM. Epigenetic events in the colorectum and in colon cancer. *Biochem Soc Trans* 2005; **33**: 684-688

Grumet M, Hoffman S, Crossin KL and Edelman GM. Cytotactin, an extracellular matrix protein of neural and non-neural tissues that mediates glia-neuron interaction. *Proc Natl Acad Sci USA* 1985; **82**: 8075-8079

Gulcher JR, Nies DE, Marton LS and Stefansson K. An alternatively spliced region of the human hexabrachion contains a repeat of potential N-glycosylation sites. *Proc Natl Acad Sci USA* 1989; **86**: 1588-1592

Gulcher JR, Alexos MJ, Le Beau MM, Lemons RS and Stefansson K. Chromosomal localization of the human hexabrachion (tenascin) gene and evidence for recent reduplication within the gene. *Genomics* 1990; **6**: 616-622

Gulcher JR, Nies DE, Alexakos MJ, Ravikant NA, Sturgill ME, Marton LS and Stefansson K. Structure of the human hexabrachion (tenascin) gene. *Proc Natl Acad Sci USA* 1991; **88**: 9438-9442

Gulubova MV and Vlaykova T. Tenascin immunoreactivity in the large bowel and liver in patients with colorectal cancer. *Histochem J* 2001; **33**: 111-120

Haggie JA, Sellwood RA, Howell A, Birch JM and Schor SL. Fibroblasts from relatives of patients with hereditary breast cancer show fetal-like behaviour in vitro. *Lancet* 1987; **1**: 1455-1457

Hagios C, Koch M, Spring J, Chiquet M and Chiquet-Ehrismann R. Tenascin-Y: a protein of novel domain structure is secreted by differentiated fibroblasts of muscle connective tissue. *J Cell Biol* 1996; **134**: 1499-1512

Hamann K, Grabbe J, Welker P, Haas N, Algermissen B and Czarnetzki BM. Phenotypic evaluation of cultured human mast and basophil cells and of normal human skin mast cells. *Arch Dermatol Res* 1994; **286**: 380-385

Hanamura N, Yoshida T, Matsumoto E, Kawarada Y and Sakakura T. Expression of fibronectin and tenascin-C mRNA by myofibroblasts, vascular cells and epithelial cells in human colon adenomas and carcinomas. *Int J Cancer* 1997; **73**: 10-15

Hanani M and Freund HR. Interstitial cells of Cajal- their role in pacing and signal transmission in the digestive system. *Acta Physiol Scand* 2000; **170**: 177-190

Hannan MA, Siddiqui Y, Rostom A, Al-Ahdal MN, Chaudhary MA and Kunhi M. Evidence of DNA repair/processing defects in cultured skin fibroblasts from breast cancer patients. *Cancer Res* 2001; **61**: 3627-3631

Harada T, Shinohara M, Nakamura S and Oka M. An immunohistochemical study of the extracellular matrix in oral squamous cell carcinoma and its association with invasive and metastatic potential. *Virchows Arch* 1994; **424**: 257-266

Hauptmann S, Siegert A, Berger S, Denkert C, Kobel M, Ott S, Siri A and Borsi L. Regulation of cell growth and the expression of extracellular matrix proteins in colorectal adenocarcinoma: a fibroblast-tumor cell coculture model to study tumor-host interactions in vitro. *Eur J Cell Biol* 2003; **82**: 1-8

Heid HW, Moll I and Franke WW. Patterns of expression of trichocytic and epithelial cytokeratin in mammalian tissues. I. Human and bovine hair follicles. *Differentiation* 1988; **37**: 137-157

Hendrix MJC, Seftor EA, Seftor REB, Misiorowski RL, Saba PZ, Sundareshan P and Welch DR. Comparison of tumour-cell invasion assays- human amnion versus reconstituted basement membrane barriers. *Inv Met* 1989; **9**: 278-297

Heriot AG, Grundy A and Kumar D. Preoperative staging of rectal carcinoma. *Br J Surg* 1999; **86**: 17-28

Higaki S, Tada M, Nishiaki M, Mitani M, Yanai H and Okita K. Immunohistochemical study to determine the presence of pericryptal myofibroblasts and basement membrane in colorectal epithelial tumours. *J Gastroenterol* 1999; **34**: 215-220

Hinchey EJ, Schaal PG and Richards GK. Treatment of perforated diverticular disease of the colon. *Advances in Surgery* 1978; **12**: 85-109

Hirota S, Isozaki K, Moriyama Y. Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Tunio GM, Matsuzawa Y, Kanakura Y, Shinomura Y and Kitamura Y. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumours. *Science* 1998; **279**: 577-580

Houghton J, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H, Cai X, Fox JG, Goldenring JR and Wang TC. *Science* 2004; **306**: 1568-1571

Howeedy AA, Virtanen I, Laitinen L, Gould NS, Koukoulis GK and Gould VE. Differential distribution of tenascin in normal, hyperplastic and neoplastic breast. *Lab Invest* 1990; **63**: 798-806

Huang W, Chiquet-Ehrisman R, Moyano JV, Garcia-Pardo A and Orend G. Interference of tenascin-C with syndecan-4 binding to fibronectin blocks cell adhesion and stimulates tumor cell proliferation. *Cancer Res* 2001; **61**: 8586-8594

Hulboy DL, Rudolph LA and Matrisian LM. Matrix metalloproteinases as regulators of reproductive function. *Mol Hum Reprod* 1997; **3**: 27-45

Ikuta K and Weissman IL. Evidence that haemopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc Natl Acad Sci USA* 1992; **89**:1502-1506

Ilunga K and Iriyama K. Expression of tenascin in gastric carcinoma. *Br J Surg* 1995; **82**: 948-951

Imanaka-Yoshida K, Hiroe M, Nishikawa T, Ishiyama S, Shimojo T, hta Y, Sakakura T and Yoshida T. Tenascin-C modulates adhesion of cardiomyocytes to extracellular matrix during tissue remodelling after myocardial infarction. *Lab Invest* 2001; **81**: 1015-1024

Ingham KC, Brew SA and Erickson HP. Localization of a cryptic binding site for tenascin on fibronectin. *J Biol Chem* 2004; **279**: 28132-28135

Ioachim E, Charchanti A, Briasoulis E, Karavasilis V, Tsanou H, Arvanitis DL, Agnantis NJ and Pavlidis N. Immunohistochemical expression of extracellular matrix components tenascin, fibronectin, collagen type IV and laminin in breast cancer: their prognostic value and role in tumour invasion and progression. *Eur J Cancer* 2002; **38**: 2362-2370

Iskaros BF, Tanaka KE, Hu X, Kadish AS and Steinberg JJ. Morphologic pattern of tenascin as a diagnostic biomarker in colon cancer. *J Surg Oncol* 1997; **64**: 98-101

Iskaros BF and Koss LG. Tenascin expression in intraepithelial neoplasia and invasive carcinoma of the uterine cervix. *Arch Pathol Lab Med* 2000; **124**: 1282-1286

Jass JR. Colorectal adenoma progression and genetic change; is there a link? *Am J Med* 1994; **27**: 301

Jass JR, Stewart SM, Stewart J and Lane MR. Hereditary non-polyposis cancer; morphologies, genes and mutations. *Mutate Res* 1994; **290**: 125

Joester A and Faissner A. Evidence for combinatorial variability of tenascin-C isoforms and developmental regulation in the mouse central nervous system. *J Biol Chem* 1999; **274**: 17144-17151

Jones FS, Crossin KL, Cunningham BA and Edelman GM. Identification and characterization of the promoter for the cytotactin gene. *Proc Natl Acad Sci* 1990; **87**: 6497-6501

Jones FS and Jones PL. The tenascin family of ECM proteins: structure, function and regulation during embryonic development and tissue remodeling. *Dev Dyn* 2000; **218**: 235-259

Jones JL and Walker RA. Control of matrix metalloproteinase activity in cancer *J Pathol* 1997; **183**: 377-379

Jones PL and Rabinovitch M. Tenascin-C is induced with progressive pulmonary vascular disease in rats and is functionally related to increased smooth muscle cell proliferation. *Circ Res* 1996; **79**: 1131-1142

Joo YE, Seo KS, Kim HS, Rew JS, Park CS and Kim SJ. Role of tissue inhibitors of matrix metalloproteinases (TIMPs) in colorectal carcinoma. *J Korean Med Sci* 1999; **14**: 417-423

Kaateenaho-Wiik R, Soini Y, Pöllänen R, Pääkkö P and Kinnula VL. Over-expression of tenascin-C in malignant pleural mesothelioma. *Histopathol* 2003; **42**: 280-291

Kajiwara K, Ueda H, Yamamoto H, Imazu M, Hayashi Y and Kohno N. Tenascin-C is associated with coronary plaque instability in patients with acute coronary syndromes. *Circ J* 2004; **68**: 198-203

Kalembeyi I, Inada H, Nishiura R, Imanaka-Yoshida K, Sakakura T and Yoshida T. Tenascin-C upregulates matrix metalloproteinase-9 in breast cancer cells: direct and synergistic effects with transforming growth factor β1. *Int J Cancer* 2003; **105**: 53-60

Katenkamp D, Berndt R, Perevoshchikov A and Raikhlin NT. Ultrastructural observations of the stroma of human colon carcinomas with special regard to myofibroblasts. *Arch Geschwulstforsch* 1984; **54**: 301-308

Katenkamp K, Berndt A, Hindermann W, Wunderlich H, Haas KM, Borsi L, Zardi L and Kosmehl H. mRNA expression and protein distribution of the unspliced tenascin-C isoform in prostatic adenocarcinoma. *J Pathol* 2004; **203**: 771-779

Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K and Martin GR. Isolation and characterization of type IV procollagen, laminin and heparan sulphate proteoglycan from the EHS sarcoma. *Biochem* 1982; **21**: 6188-6193

Knighton D, Ausprunk D, Tapper D and Folkman J. Avascular and vascular phases of tumour growth in the ckick embryo. *Br J Cancer* 1977; **35**: 347-356

Ko K, Yazumi S, Yoshikawa K, Konda Y, Nakajima M, Chiba T and Takahashi R. Activation of fibroblast-derived matrix metalloproteinase-2 by colon-cancer cells in non-contact co-cultures. *Int J Cancer* 2000; **87**: 165-171

Koch M, Olson PF, Albus A, Jin W, Hunter DD, Brunken WJ, Burgeson RE and Champliaud MF. Characterization and expression of the laminin γ3 chain: a novel, non-basement membrane-associated laminin chain. *J Cell Biol* 1999; **145**: 605-617

Konishi F, Muto T and Kanyia J. Histopathologic comparison of colorectal adenomas in English and Japanese patients. *Dis Colon Rectum* 1984; **27**: 515

Korhonen M, Ormio M, Burgeson RE, Virtanen I and Savilahti E. Unaltered distribution of laminins, fibronectin, and tenascin in celiac intestinal mucosa. *J Histochem Cytochem* 2000; **48**: 1011-1020

Kupperwasser C, Chavarria T, Wu M, Magrane G, Gray JW, Carey L, Richardson A and Weinberg RA. Reconstruction of functionally normal and malignant human breast tissue in mice. *Proc Natl Acad Sci USA* 2004; **101**: 4723-4724

LaFleur DW, Fagin JA, Forrester JS, Rubin SA and Sharif BG. Cloning and characterization of alternatively spliced isoforms of rat tenascin. *J Biol Chem* 1994; **268**: 20757-20763

Laitinen A, Altraja A, Kämpe M, Linden M, Virtanen I and Laitinen LA. Tenascin is increased in airways basement membrane in asthmatics and decreased by an inhaled steroid. *Am J Respir Crit Care Med* 1997; **156**: 951-958

Lazard D, Sastre X, Frid MG, Glukhova MA, Thiery J-P and Koteliansky VE. Expression of smooth muscle-specific proteins in myoepithelium and stromal fibroblasts of normal and malignant breast tissue. *Proc Natl Acad Sci USA* 1993; **90**: 999-1003

Le Bail B, Faouzi S, Boussarie L, Balabaud C, Bioulac-Sage P and Rosenbaum J. Extracellular matrix composition and integrin expression in early hepatocarcinogenesis in human cirrhotic liver. *J Pathol* 1997; **181**: 330-337

Leibovitz A, Stinson JC, McMombs WB, McCoy CE, Mazur KC and Maboy ND. Classification of human colorectal adenocarcinoma cell lines. *Cancer Res* 1976; **36**: 4562-4569

Leins A, Riva P, Lindstedt R, Davidoff MS, Mehraein P and Weis S. Expression of tenascin-C in various human brain tumors and its relevance for survival in patients with astrocytomas. *Cancer* 2003; **98**: 2430-2439

Leong AS. Pitfalls in diagnostic immunohistology. *Adv Anat Pathol* 2004; **11**: 86-93

Lewis MP, Lygoe KA, Nystrom ML, Anderson WP, Speight PM, Marshall JF and Thomas GJ. Tumour-derived TGF-β1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. *Br J Canc* 2004; **90**: 822-832

Li A, Hasui K, Yonezawa S, Tanaka S and Sato E. Immunohistochemical analysis of pericryptal fibroblast sheath and proliferating epithelial cells in human colorectal adenomas and carcinomas with adenoma components. *Path Int* 1999; **49**: 426-434

Lim CH, Dixon MF, Vail A, Forman D, Lynch DAF and Axon ATR. Ten year follow up of ulcerative colitis patients with and without low grade dysplasia. *Gut* 2003; **52**: 1127-1132

Lin EY,Nguyen AV, Russell RG and Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumours to malignancy. *J Exp Med* 2001; **193**: 727-739

Ljubimiv AV, Saghizadeh M, Spirin KS, Khin HL, Lewin SL, Zardi L, Bourdon MA and Kenney MC. Expression of tenascin-C splice variants in normal and bullous keratopathy human corneas. *Invest Ophthalmol Vis Sci* 1998; **39**: 1135-1142

Lockhart-Mummery HE and Morson BC. Crohn's disease (regional enteritis) of the large intestine and its distinction from ulcerative colitis. *Gut* 1960; **87**: 105

Lohi J. Laminin-5 in the progression of carcinomas. *Int J Cancer* 2001; **94**: 763-767

Lyford GL, He C-L, Soffer E, Hul TL, Strong SA, Senagore AJ, Burgart LJ, Young-Fadok T, Szurszewski JH and Farrugia G. Pan-colonic decrease in interstitial cells of Cajal in patients with slow transit constipation. *Gut* 2002; **51**: 496-501

McCawley LJ and Matrisian LM. Matrix metalloproteinases: they're not just for matrix anymore. *Curr Opin Cell Biol* 2001; **13**: 534-540

McKaig BC, Makh SS, Hawkey CJ, Podolsky DK and Mahida YR. Normal human colonic subepithelial myofibroblasts enhance epithelial migration (restitution) via TGF-β3. *Am J Physiol Gastroinest Liver Physiol* 1999; **276**: G1087-G1093

McKaig BC, McWilliams D, Watson SA and Mahida YR. Expression and regulation of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinases by intestinal myofibroblasts in inflammatory bowel disease. *Am J Pathol* 2003; **162**: 1355-1360

Mackie EJ, Chiquet-Ehrismann R, Pearson CA, Inaguma Y, Taya K, Kawarada Y and Sakakura T. Tenascin is a stromal marker of epithelial malignancy in the mammary gland. *Proc Natl Acad Sci USA* 1987; **84**: 4621-4625

Mackie EJ, Halfter W and Liverani D. Induction of tenascin in healing wounds. *J Cell Biol* 1988; **107**: 2757-2767

Mahida YR, Beltinger J, Makh S, Gőke M, Gray T, Podolsky DK and Hawkey CJ. Adult human colonic subepithelial myofibroblasts express extracellular matrix proteins and cyclooxygenase-1 and -2. *Am J Physiol Gastrointest Liver Physiol* 1997; **273**: G1341-G1348

Mari BP, Anderson IC, Mari SE, Ning Y, utz Y, Kobzik L and Shipp MA. Stromelysin-3 is induced in tumor/stroma cocultures and inactivated via a tumor-specific and basic fibroblast growth factor-dependent mechanism. *J Biol Chem* 1998; **1**: 618-626

Marselina IT, Strunck E, Scholzen T, Gerdes J and Vollmer G. Extracellular matrix regulates steady-state mRNA levels of the proliferation associated protein Ki-67 in endometrial cancer cells. *Cancer Letts* 1999; **140**: 145-152

Matsuda A, Tagawa Y, Yamamoto K, Matsuda H and Kusakabe M. Identification and immunohistochemical localization of annexin II in rat cornea. *Curr Eye Res* 1999; **19**: 368-375

Matsuda R, Takahashi T, Nakamura S, Sekido Y, Nishida K, Seto M, Seito T, Sugiura T, Ariyoshi Y and Takahashi T. Expression of the c-kit protein in human solid tumours and in corresponding fetal and adult normal tissues. *Am J Pathol* 1993; **142**: 339-346

Mazzia C, Porcher C, Julé Y, Christen M-O and Henry M. Ultrastructural study of relationships between c-kit immunoreactive interstitial cells and other cellular elements in the human colon. *Histochem Cell Biol* 2000; **113**: 401-411

Medina C, Videla S, Randomski A, Randomski MW, Antolin M, Guarner F, Vilaseca J, Salas A and Malagelada J-R. Increased activity and expression of matrix metalloproteinases-9 in a rat model of distal colitis. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G116-G122

Micke P and Östman A. Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? *Lung Cancer* 2004; **45**: S163-S175

Midwood KS and Schwarzbauer JE. Tenascin-C modulates matrix contraction via focal adhesion kinase- and Rho- mediated signalling pathways. *Mol Cell Biol* 2002; **13**: 3601-3613

Miettinen M, Lindenmayer AE and Chaubal A. Endothelial cell markers CD31, CD34 and BNH9 antibody to H- and Y-antigens- evaluation of their specificity and sensitivity in the diagnosis of vascular tumours and comparison with von Willebrand factor. *Mod Pathol* 1994; **7**: 82-90

Mighell AJ, Thompson J, Hume WJ, Markham AF and Robinson PA. Human tenascin-C: Identification of a novel type III repeat in oral cancer and of novel splice variants in normal, malignant and reactive oral mucosae. *Int J Cancer* 1997; **72**: 236-240

Miner JH, Patten BL, Lentz SI, Gilbert DJ, Snider WD, Jenkins NA, Copeland NG and Sanes JR. The laminin alpha chains: expression, developmental transitions and chromosomal locations of alpha 1-5, identification of heterotrimeric laiminis 8-11 and cloning of a novel alpha 3 isoform. *J Cell Biol* 1997; **137**: 685-701

Morris PJ and Wood WC. Oxford Textbook of Surgery. 2nd edition *Oxford University Press* 2000

Mukaida H, Hirabayashi N, Hirai T, Iwata T, Saeki S and Toge T. Significance of freshly cultured fibroblasts from different tissues in promoting cancer cell growth. *Int J Cancer* 1991; **48**: 423-427

Mukaratirwa S, Koninkx JF, Gruys E and Nederbragt H. Mutual paracrine effects of colorectal tumour cells and stromal cells: modulation of tumour and stromal cell

differentiation and extracellular matrix component production in culture. *Int J Exp Pathol* 2005; **86**: 219-229

Murphy-Ullrich JE, Lighter VA, Aukhil I, Yan YZ and Erickson HP. Focal adhesion integrity is down-regulated by the alternatively spliced domain of human tenascin. *J Cell Biol* 1991; **115**: 1127-1136

Murray GI, Duncan ME, O'Neill P, McKay JA, Melvin WT and Fothergill JE. Matrix metalloproteinase-1 is associated with poor prognosis in oesophageal cancer. *J Pathol* 1998; **185**: 256-261

Muto T, Bussey HJR and Morson BC. The evolution of cancer of the rectum. *Cancer* 1975; **36**: 2251

Nabeshima K, Inoue T, Shimao Y, Okada Y, Yoshifumi I, Seiki M and Koono M. Front-cell-specific expression of membrane-type 1 matrix metalloproteinase and gelatinase A during cohort migration of colon cancer cells induced by hepatocyte growth factor/ scatter factor. *Cancer Res* 2000; **60**: 3364-3369

Naftalin RJ, Zammit PS and Pedley KC. Regional differences in rat large intestinal crypt fuction in relation to dehydrating capacity in vivo. *J Physiol* 1999; **514**: 201-210

Naftalin RJ and Pedley KC. Regional crypt function in rat large intestine in relation to fluid absorption and growth of the pericryptal sheath. *J Physiol* 1999; **514**: 211-227

Nakamura T, Matsumoto K, Kiritoshi A, Tono Y and Nakamura T. Induction of hepatocyte growth factor in fibroblasts by tumour derived factors affects invasive growth of tumour cells: in vitro analysis of tumor-stromal interactions. *Cancer Res* 1997; **57**: 3305-3313

Nakayama H, Miyazaki E and Enzan H. Differential expression of high molecular weight caldesmon in colorectal pericryptal fibroblasts and tumour stroma. *J Clin Pathol* 1999; **52**: 785-786

Nakayama H, Enzan H, Miyazaki E, Kuroda N, Naruse K and Hiroi M. Differential expression of CD34 in normal colorectal tissue, peritumoural inflammatory tissue and tumour stroma. *J Clin Pathol* 2000; **53**: 626-629

Nakayama H, Enzan H, Miyazaki E, Kuroda N, Naruse K, Kiyoku H, Toi M and Hiroi M. CD34 positive stromal cells in gastric adenocarcinoma. *J Clin Pathol* 2001; **54**: 846-848

Nakayama H, Enzan H, Miyazaki E and Toi M. α Smooth muscle actin positive stromal cells in gastric carcinoma. *J Clin Pathol* 2002; **55**: 741-744

Nakayama H, Enzan H, Yamamoto M, Miyazaki E, Hidaka C, Okumichi T, Okumichi A and Kajihara H. CD34-positive stromal cells in primary lung carcinomas. *Oncol Rep* 2003; **10**: 1313-1316

Nakayama T, Yao L and Tosato G. Mast cell derived angiopoietin-1 plays a critical role in the growth of plasma cell tumours. *J Clin Invest* 2004; **114**: 1317-1325

Nelson AR, Fingleton B, Rothenburg ML and Matrisian LM. Matrix metalloproteinases: Biologic activity and clinical implications. *J Clin Oncol* 2000; **18**: 1135-1139

Newland RC, Chapius PH and Smyth EJ. The prognostic value of substaging colorectal carcinoma A prospective study of 1117 cases with standardised pathology. *Cancer* 1987; **60**: 852-857

Nies DE, Hemesath TJ, Kim JH, Gulcher JR and Stefansson K. The complete cDNA sequence of human hexabrachion (tenascin) A multidomain protein containing unique epidermal growth factor repeats. *J Biol Chem* 1991; **266**, 2818-2823

Nimrich I, Erdmann S, Melchers U, Finke U, Hentsch S, Moyer MP, Hoffman I and Müller O. Seven genes that are differentially transcribed in colorectal tumour cell lines. *Cancer Letts* 2000; **160**: 37-43

Norton PA and Hynes RO. In vitro splicing of fibronectin pre-mRNAs. *Nuc Acid Res* 1990; **18**: 4089-4097

Ohuchida K, Mizumoto K, Murakami M, Qian L-W, Sato N, Nagai E, Matsumoto K, Nakamura T and Tanaka M. Radiation to stromal fibroblasts increases invasiveness of pancreatic cancer cells through tumor-stromal interactions. *Cancer Res* 2004; **64**: 3215-3222

Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD and Cunha GR. Carcinoma-associated fibroblasts direct tumour progression of initiated human prostatic epithelium. *Cancer Res* 1999; **59**: 5002-5011

Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naemm R and Carey VJ. Stromal fibroblasts present in invasive breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 2005; **121**: 335-348

Painter NS. Diverticular disease of the colon A disease of this century. *Lancet* ii; 586-588

Pankov R and Yamada KM. Fibronectin at a glance. *J Cell Sci* 2002; **115**: 3861-3863

Park CC, Bissell MJ and Barcellos-Hoff MH. The influence of the microenvironment on the malignant phenotype. *Mol Med Today* 2000; **6**: 324-329

Parums DV, Cordell JL, Micklem K, Heryet AR, Gatter KC and Mason DY. JC70: a new monoclonal antibody that detects vascular endothelium associated antigen on routinely processed tissue sections. *J Clin Pathol* 1990; **43**: 752-757

Perreault N, Herring-Gillam E, Desloges N, Bélanger I, Pageot L-P and Beaulieu J-F. Epithelial vs mesenchymal contribution to the extracellular matrix in the human intestine. *Biochem Biophys Res Comm* 1998; **248**: 121-126

Philips GR, Krushel LA and Crossin KL. Domains of tenascin involved in glioma migration. *J Cell Sci* 1998; **111**: 1095-1104

Pikarsky E, Porat RM, Stein I, Abramovitch R. Amit S, Kasem S, Gutkovich-Pyest E, Uriell-Shoval S, Galun E and Ben-Neriah Y. NF-κB functions as tumour promoter in inflammation associated cancer. *Nature* 2004; **431**: 4461-4466

Plow EF, Haas TA, Zhang L, Loftus J and Smith JW. Ligand binding to integrins. *J Biol Chem* 2000; **275**: 21785-21788

Polette M, Nawrocki B, Gilles C, Sato H, Seiki M, Tournier JM and Birembaut P. MT-MMP expression and localisation in human lung and breast cancer. *Virchows Arch* 1996; **428**: 29-35

Poulsom R, Pignatelli M, Stetler-Stevenson WG, Liotta LA, Wright PA, Jeffrey RE, Longcroft JM, Rogers L and Stamp GW. Stroma expression of 72 Kda type IV collagenases (MMP-2) and TIMP-2 mRNAs in colorectal neoplasia. *Am J Pathol* 1992; **141**: 389-396

Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI and West AB. Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol Cell Physiol* 1999; **277**: C1-C9

Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI and West AB. Myofibroblasts. II. Intestinal subepithelial myofibroblasts. *Am J Physiol Cell Physiol* 1999; **277**: C183-C201

Prieto AL, Andersson-FisoneC and Crossin KL. Characterization of multiple adhesive and counteradhesive domains in the extracellular matrix protein cytotactin. *J Cell Biol* 1992; **119**: 663-678

Prieto AL, Edelman GM and Crossin KL. Multiple integrins mediate cell attachment to cytotactin/tenascin. *Proc Natl Acad Sci USA* 1993; **90**: 10154-10158

Puente Navazo MD, Valmori D and Rüegg C. The alternatively spliced domain TnFnIII A1A2 of the extracellular matrix protein tenascin-C suppresses activation-induced T lymphocyte proliferation and cytokine production. *J Immunol* 2001; **167**: 6431-6440

Raitz R, Martins MD and Araújo VC. A study of the extracellular matrix in salivary gland tumours. *J Oral Pathol Med* 2003; **32**: 290-296

Ramani P, Bradley NJ and Fletcher CD. QBEND/10, a new monoclonal antibody to endothelium: assessment of its diagnostic utility in paraffin sections. *Histopathology* 1990; **17**: 237-242

Reardon DA, Akabani G, Coleman E, Friedman AH, Friedman HS, Herndon JE, Cokgar I, McLendon RE, Pegram CN, Provenzale JM, Quinn JA, Rich JN, Regalado LV, Sampson JH, Shafman TD, Wikstrand CJ, Wong TZ, Zhao X-G, Zalutsky MR and Bigner DD. Phase II trial of murine ^{131}I- labelled antitenascin monoclonal antibody 81C6 administered into surgically created resection cavities of patients with newly diagnosed malignant gliomas. *J Clin Oncol* 2002; **20**: 1389-1397

Regezi JA, Ramos DM, Pytela R, Dekkar NP and Jordan RCK. Tenascin and β6 integrin are overexpressed in floor of mouth in situ carcinomas and invasive squamous cell carcinomas. *Oral Oncol* 2002; **38**: 332-336

Rettig WJ, Triche TJ and Garin-Chesa P. Stimulation of human neuronectin secretion by brain-derived growth factors. *Brain Res* 1989; **487**: 171-177

Richman PI, Tilly R, Jass JR and Bodmer WF. Colonic pericrypt sheath cells: characterisation of cell type with new monoclonal antibody. *J Clin Path* 1987; **40**: 593-600

Riedl SE, Faissner A, Sclag P, Von Hebay A, Koretz K and Moller P. Altered content and distribution of tenascin in colitis, colonic adenoma and colorectal carcinoma. *Gastroenterology* 1992; **103**: 400-406

Riedl S, Kadmon M, Tandara A, Hinz U, Moller P, Herfarth C and Faissner A. Mucosal tenascin C content in inflammatory and neoplastic diseases of the large bowel. *Dis Colon Rectum* 1998; **41**: 86-92

Robinson SC, Scott KA, Wilson J, Thompson RG, Proudfoot AEL and Balkwill F. A chemokines receptor antagonist inhibits experimental breast tumor growth. *Cancer Res* 2003; **63**: 8360-8365

Robinson TL, Sircar K, Hewlett BR, Chorneyko K, Riddell RH and Huizinga JD. Gastrointestinal stromal tumors may originate from a subset of CD34-positive interstitial cells of Cajal. *Am J Pathol* 2000; **156**: 1157-1163

Roeb E, Dietrich CG, Winograd R, Arndt M, Breuer B, Fass J, Schumpelick V and Matern S. Activity and cellular origin of gelatinases in patients with colon and rectal carcinoma. *Cancer* 2001; **92**: 2680-2691

Ronnov-Jessen L and Petersen OW. Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia. *Lab Invest* 1993; **68**: 696-707

Ronnov-Jessen L, Peterson OW, Koteliansky VE and Bissell MJ. The origin of myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. *J Clin Invest* 1995; **95**: 859-873

Rősmann S, Hahn D, Lottaz D, Kruse M-N, Stőcker W and Sterchi EE. Activation of human meprin-α in a cell culture model of colorectal cancer is triggered by the plasminogen-activating system. *J Biol Chem* 2002; **277**: 40650-40658

Rowley DR. What might a stromal response mean to prostate cancer progression? *Cancer Metast Rev* 1999; **17**: 411-49

Roy SG, Nozaki Y and Phan SH. Regulation of α-smooth muscle actin gene expression in myofibroblast differentiation from rat lung fibroblasts. *Int J Biochem Cell Biol* 2001; **33**: 723-734

Rutter M, Saunders B, Wilkinson K, Rumbles S, Schofield G, Kamm M, Williams C, Price A, Talbot I and Forbes A. Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. *Gastroenterol* 2004; **126**: 451-459

Sakai T, Kawakatsu H, Hirota N, Yokoyama T, Sakakura T and Saito M. Specific expression of tenascin in human colonic neoplasms. *Br J Canc* 1993; **67**: 1058-1064

Salmela MT, Karjalainen-Lindsberg M-L, Puolakkainen P and Saarialho-Kere U Upregulation and differential expression of matrilysin (MMP-7) and metalloelastase (MMP-12) and their inhibitors TIMP-1 and TIMP-3 in Barrett's oesophageal adenocarcinoma. *Br J Cancer* 2001; **85**: 383-392

Salmivirta M, Elenius K, Vaino S, Hofer U, Chiquet-Ehrismann R, Thesleff I and Jalkanen M. Syndecan from embryonic tooth mesenchyme binds tenascin. *J Biol Chem* 1991; **266**: 7733-7739

Sandler AB, Johnson DH and Herbst RS. Anti-vascular endothelial growth factor monoclonals in non-small cell lung cancer. *Clin Cancer Res* 2004; **10**: 4258s-4262s

Saoncella S, Echtermeyer F, Denhez F, Nowlen JK, Mosher DF, Robinson SD, Hynes RO and Goetinck PF. Syndecan-4 signals co-operatively with integrins in a Rho-dependent manner in the assembly of focal adhesions and actin stress fibres. *Proc Natl Acad Sci USA* 1999; **96**: 2805-2810

Sappino AP, Skalli O, Jackson B, Schurch W and Gabbiani G. Smooth-muscle differentiation in stromal cells of malignant and non-malignant breast tissues. *Int J Cancer* 1988; **41**: 707-712

Sappino AP, Dietrich PY, Skalli O, Widgren S and Gabbiani G. Colonic pericryptal fibroblasts. Differentiation pattern in embryogenesis and phenotypic modulation in epithelial proliferative lesions. *Virchows Arch A Pathol Anat Histopathol* 1989; **415**: 551-557

Satta J, Soini Y, Pöllänen R, Pääkkö P and Juvonen T. Tenascin expression is associated with a chronic inflammatory process in abdominal aortic aneurysms. *J Vasc Surg* 1997; **26**: 670-675

Schenk S, Chiquet-Ehrismann R and Battegay EJ. The fibrinogen globe of tenascin-C promotes basic fibroblast growth factor-induced endothelial cell elongation. *Mol Biol Cell* 1999; **10**: 2933-2943

Schlaeppi M, Ruegg C, Tran-Thang C, Chapuis G, Tevaearai H, Lahm H and Sordat B. Role of integrins and evidence for two distinct mechanisms mediating human colorectal carcinoma cell interaction with peritoneal mesothelial cells and extracellular matrix. *Cell Adhes Commun* 1997; **4**: 439-455

Schor SL, Schor AM, Rushton G and Smith L. Adult, foetal and transformed fibroblasts display different migratory phenotypes on collagen gels: evidence for a isoformic transition during foetal development. *J Cell Sci* 1985; **73**: 221-234

Schor SL, Schor AM, Durning P and Rushton G. Skin fibroblasts obtained from cancer patients display fœtal migratory behaviour on collagen gels. *J Cell Sci* 1985; **73**: 235-244

Schor SL, Haggie JA, Durning P, Howell A, Smith L, Sellwood RA and Crowther D. Occurrence of a fetal fibroblast phenotype in familial breast cancer. *Int J Cancer* 1986; **37**: 831-836

Schor SL, Schor AM and Rushton G. Fibroblasts from cancer patients display a mixture of both foetal and adult-like phenotypic characteristics. *J Cell Sci* 1988; **90**: 401-407

Schwarzbauer JE, Patel RS, Fonda D and Hynes RO. Multiple sites of alternative splicing of the rat fibronectin gene transcript. *EMBO* 1987; **6**: 2573-2580

Schwogler S, Odenthal M, Meyer zum Buschenfelde KH and Ramadori C. Alternative splicing products of the tenascin gene distinguish liver storing cells from arterial smooth muscle cells and skin fibroblasts. *Biochem Biophys Res Commun* 1992; **185**: 768-775

Seemayer TA, Lagace R, Schurch W and Tremblay G. Myofibroblasts in the stroma of invasive and metastatic carcinoma: a possible host response to neoplasia. *Am J Surg Pathol* 1979; **3**: 525-533

Shiozawa J, Ito M, Nakayama T, Nadashima M, Kohno S and Sekine I. Expression of matrix metalloproteinase-1 in human colorectal carcinoma. *Modern Pathol* 2000; **13**: 925-933

Shrestha P, Sakamoto F, Takagi H, Yamada T and Mori M. Enhanced tenascin immunoreactivity in leukoplakia and squamous cell carcinoma of the oral cavity: an immunohistochemical study. *Oral Oncol, Eur J Cancer* 1994; **30**: 132-137

Sircar KMD, Hewlett BR, Huizinga JD, Chorneyko K, Berezin I and Riddell RH. Interstitial cells of Cajal as precursors of gastrointestinal stromal tumors. *Am J Surg Pathol* 1999; **23**: 377-389

Siri A, CarnemollaB, Saginati M, Leprini A, Casari G, Baralle F and Zardi L. Human tenascin: primary structure, pre-mRNA splicing patterns and localisation of the epitopes recognised by two monoclonal antibodies. *Nucleic Acids Res* 1991; **19**: 525-531

Siri A, Knäuper V, Veirana N, Caocci F, Murphy G and Zardi L. Different susceptibility of small and large human tenascin-C isoforms to degradation by matrix metalloproteinases. *J Biol Chem* 1995; **270**: 8650-8654

Sjödahl RI, Myrelid P and Söderholm JD. Anal and rectal cancer in Crohn's disease. *Colorectal Dis* 2003; **5**: 490-405

Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D and Gabbiani G. A new monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol* 1986; **103**: 2787-2796

Soini Y, Paako P, Virtanen I and Veli-Pekka L. Tenascin in salivary gland tumours. *Virchows Arch Pathol Anat Histopathol* 1992; **421**: 417-222

Soini Y, Pääko P, Nuorva K, Kamel D, innala A, Virtanen I and Lehto VP. Tenascin immunoreactivity in lung tumours. *Am J Clin Pathol* 1993; **100**: 145-150

Spanakis E and Brouty-Boye D. Discrimination of fibroblast subtypes by multivariate analysis of gene expression. *Int J Cancer* 1997; **71**: 402-409

Spring J, Beck K and Chiquet-Ehrismann R. Two contrary functions of tenascin: dissection of the active sites by recombinant tenascin fragments. *Cell* 1989; **59**: 325-334

Sriramarao P and Bourdon MA. A novel tenascin type III repeat is part of a complex of tenascin mRNA alternative splices. *Nucleic Acids Res* 1993; **21**: 163-168

Stanley JR, Hawley-Nelson P, Yaar M, Martin GR and Katz SI. Laminin and bullous pemphigoid antigen are distinct basement membrane proteins synthesized by epidermal cells. *J Invest Dermatol* 1982; **78**: 456-459

Stanley JR, Woodley DT, Katz SI and Martin GR. Structure and function of basement membrane. *J Invest Dermatol* 1982; **79**: 69s-72s

Streutker CJ, Huizinga JD, Campbell F, Ho J and Riddell RH. Loss of CD117 (c-kit) and CD34-positive ICC and associated CD34-positive fibroblasts defines a subpopulation of chronic intestinal pseudoobstruction. *Am J Surg Pathol* 2003; **27**: 228-235

Sternberg A, Sibirsky O, Cohen D, Blumenson LE and Petrelli NJ. Validation of a new classification system for curatively resected colorectal adenocarcinoma. *Cancer* 1999; **86**: 782-792

Swindle CS, Tran KT, Johnson TD, Banerjee P, Mayes AM, Griffith L and Wells A. Epidermal growth factor (EGF)-like repeats of human tenascin-C as ligands for EGF receptor. *J Cell Biol* 2001; **154**: 459-468

Talvensaari-Mattila A, Pääkkö P, Höyhtyä M, Blanco-Sequeiros G and Turpeenniemi-Hujanen T. Matrix metalloproteinase-2 immunoreactive protein A marker of aggressiveness in breast carcinoma. *Cancer* 1998; **83**: 1153-1162

Tamaoki M, Imanaka-Yoshida K, Yokoyama K, Nishioka T, Inada H, Hiroe M, Sakakura T and Yoshida T. Tenascin-C regulates recruitment of myofibroblasts during tissue repair after myocardial injury. *Am J Pathol* 2005; **167**: 71-80

Tanaka K, Hiraiwa N, Hashimoto H, Yamazaki Y and Moriaki K. Tenascin-C regulates angiogenesis in tumor through the regulation of vascular endothelial growth factor expression. *Int J Cancer* 2004; **108**: 31-40

Thiagarajah JR, Gourmelon P, Griffiths NM, Lebrun F, Naftalin RJ and Pedley KC. Radiation induced cytochrome c release causes loss of rat colonic fluid absorption by damage to crypts and pericryptal myofibroblasts. *Gut* 2000; **47**: 675-684

Timpl R, Rhode H, Robey PG, Rennard SI, Foidart JM and Martin GR. Laminin- a glycoprotein from basement membranes. *J Biol Chem* 1979; **254**: 9933-9937

Titta O, Wahlstrom T, von Smitten K, Blomqvist C and Virtanen I. Tenascin in inflammatory conditions and neoplasms of the urinary bladder. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1993; **63**: 283-287

Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C and Brown RA. Myofbroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 2002; **3**: 349-363

Trainer DL, Kline T, McCabe FL, Faucette LF, Field J, Chaikin M, Anzano M, Riernan D, Hoffstein S and Li DJ. Biological characterization and oncogene expression in human colorectal cancer cell lines. *Int J Canc* 1988; **41**: 287-296

Tremble P, Chiquet- Ehrismann R and Werb Z. The extracellular matrix ligands fibronectin and tenascin collaborate in regulating collagenases gene expression in fibroblasts. *Mol Biol Cell* 1994; **5**: 439-453

Tseleni-Balafouta S, Gakiopoulou H, Fanourakis G, Voutsinas G, Balafoutas D and Patsouris E. Tenascin-C protein expression and mRNA splice variants in thyroid carcinoma. *Exp Mol Pathol* 2006; **80**: 177-182

Tsunoda T, Inada H, Kalembeyi I, Imanaka-Yoshida K, Sakakibara M, Okada R, Katsuta K, Sakakura T, Majima Y and Yoshida T. Involvement of large tenascin-C splice variants in breast cancer progression. *Am J Pathol* 2003; **162**: 1857-1867

Tucker RP, Hammarback JA, Jenrath DA, Mackie EJ and Xu Y. Tenascin expression in the mouse: in situ localization and induction in vitro by bFGF. *J Cell Sci* 1993; **104**: 69-76

Ullman T, Croog V, Harpaz N, Sachar D and Itzkowitz S. Progression of flat low-grade dysplasia to advanced neoplasia in patients with ulcerative colitis. *Gastroenterol* 2003; **125**: 1311-1319

Vanderwinden J-M, Rumessen JJ, De Laet M-H, Vanderhaegen J-J and Schiffmann SN. $CD34^+$ cells in human intestine are fibroblasts adjacent to but distinct from Interstitial cells of Cajal. *Lab Invest* 1999; **79**: 59-65

Vogelstein B, Fearon ER and Hamilton SR. Genetic alterations during colorectal tumour development. *N Eng J Med* 1988; **319**: 525

Vollmer G, Tan MI, Wunsche W and Frank K. Expression of tenascin-C by human endometrial adenocarcinoma and stroma cells: heterogeneity of splice variants and induction by TGF-beta. *Biochem Cell Biol* 1997; **75**: 759-769

Von Lampe B, Barthel B, Coupland SE, Riecken E-O and Rosewicz S. Differential expression of matrix metalloproteinases and their tissue inhibitors in colon mucosa of patients with inflammatory bowel disease. *Gut* 2000; **47**: 63-73

Wallner K, Chen L, Prediman KS, Fishbein MC, Forrester JS, Kaul S and Sharifi BG. Tenascin-C is expressed in macrophage-rich human coronary atherosclerotic plaque. *Circ* 1999; **99**: 1284-1289

Wallner K, Shah PK and Sharifi BG. Balloon catheterization induces arterial expression of new tenascin-C isoform. *Atherosclerosis* 2002; **161**: 75-83

Weaver VM and Bissell MJ. Functional culture models to study mechanisms governing apoptosis in normal and malignant mammary epithelial cells. *J Mamm Gland Biol Neoplasia* 1999; **4**: 193-201

Weber P, Montag D, Schachner M and Bernhardt RR. Zebrafish tenascin W a new member of the tenascin family. *J Neurobiol* 1998; **35**: 1-16

Whitby DJ and Ferguson MW. The extracellular matrix of lip wounds in fetal, neonatal and adult mice. *Development* 1991; **112**: 651-668

Wierzbicka-Patynowski I and Schwarzbauer JE. The ins and outs of fibronectin matrix assembly. *J Cell Sci* 2003; **116**: 3269-3276

Williams EC, Janmey PA, Ferry JD and Mosher DF. Conformational states of fibronectin. Effects of pH, ionic strength and collagen binding. *J Biol Chem* 1982; **257**: 14973-14978

Williams NS, Gaynor RB, Scoggin S, Verma U, Gokaslan T, Simmang C, Fleming J, Tavana D, Frenkel E and Becerra C. Identification and validation of genes involved in the pathogenesis of colorectal cancer using cDNA microarrays and RNA interference. *Clin Canc Res* 2003; **9**:931-946

Wilson KE, Langdon SP, Lessells AM and Miller WR. Expression of the extracellular matrix protein tenascin in malignant and benign ovarian tumours. *Br J Cancer* 1996; **74**: 999-1004

Wilson KE, Bartlett JMS, Miller EP, Smyth JF, Mullen P, Miller WR and Langdon SP. Regulation and function of the extracellular matrix protein tenascin-C in ovarian cancer cell lines. *Br J Cancer* 1999; **80**: 685-692

Woessner JF and Nagase H. Matrix metalloproteinases and TIMPs. 1st Ed. *Oxford University Press* 2000

Yamazaki K and Eyden BP. Ultrastructural and immunohistochemical observations on intralobular fibroblasts of human breast with observations on the CD34 antigen. *J Submicrosc Cytol Pathol* 1995; **27**: 309-323

Yao T and Tsuneyoshi M. Significance of pericryptal fibroblasts in colorectal epithelial tumours: A special reference to the histologic features and growth patterns. *Hum Pathol* 1993; **24**: 525-533

Yao T and Talbot IC. The demonstration of pericryptal fibroblasts in background mucosa and dysplasia complicating ulcerative colitis. *Histopathology* 1996; **28**: 325-331

Yokosaki Y, Monis H, Chen J and Sheppard D. Differential effects of the integrins $\alpha 9^{\beta}1$, $\alpha v^{\beta}3$ and $\alpha v^{\beta}6$ on cell proliferative responses to tenascin. *J Biol Chem* 1996; **271**: 24144-24150

Yoshida T, Matsumoto E-I, Hanamura N, Kalembeyi I, Katsuta K, Ishihara A and Sakakura T. Co-expression of tenascin and fibronectin in epithelial and stromal cells of benign lesions and ductal carcinomas in the human breast. *J Pathol* 1997; **182**: 421-428

Yoshida T, Yoshimura E, Numata H, Sakakura Y and Sakakura T. Involvement of tenascin-C in proliferation and migration of laryngeal carcinoma cells. *Virchows Arch* 1999; **435**: 496-500

Young HM, Ciampoli D, Southwell BR and Newgreen DF. Origin of interstitial cells of Cajal in the mouse intestine. *Developmental Biology* 1996; **180**: 97-107

Ye S. Polymorphism in matrix metalloproteinase gene promoters: implication in regulation of gene expression and susceptibility of various diseases. *Matrix Biol* 2000; **19**: 623-629

Zagzag D, Shiff B, Jallo GI, Greco MA, Blanco C, Cohen H, Hukin J, Alle JC and Friedlander DR. Tenascin-C promotes microvascular cell migration and phosphorylation of focal adhesion kinase. *Cancer Res* 2002; **62**: 2660-2668

Zeng ZS, Huang Y, Cohen AM and Guillem JG. Prediction of colorectal cancer relapses and survival via tissue RNA levels of matrix metalloproteinase-9. *J Clin Oncol* 1996; **14**: 3133-3140

Zhou Z, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, Wang J, Cao Y and Tryggvason K. Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I. *Proc Natl Acad Sci USA* 2000; **97**: 4052-4057

Zucker S. A critical appraisal of proteolytic enzymes in cancer invasion: Emphasis on tumor surface proteinases. *Cancer Invest* 1988; **6**: 219-231

Zucker S and Vacirca J. Role of matrix metalloproteinases (MMPs) in colorectal cancer *Cancer Met Rev* 2004; **23**: 101-117

Chapter 9
Appendix

Appendix: Solutions listed in methods

Immunohistochemistry

Tris buffered saline (TBS 20x)	Tris 50mM; NaCl 150mM; pH 7.65
Citric acid buffer (20x)	Citric acid monohydrate 10mM; pH 6.0
DAB solution	Diaminobenzidine 10mg/ml
Mayers haematoxylin	Haematoxylin 2g; distilled H_20 2000ml; aluminium; potassium sulphate 100g; citric acid 2g; chloral hydrate 100g; sodium iodate 400mg to make 2l solution
Eosin (1%)	Eosin 20g; 1% CaCl 1ml; 40% formaldehyde 2mls

mRNA extraction, reverse transcription and polymerase chain reaction

Lysis binding buffer

1M Tris/HCl pH 8.0	20ml	*Final concentration*	0.5M
1.5M LiCl	57ml		0.5M
0.5M EDTA	400µl		0.01M
0.1M DTT	10ml		0.005M
Make up to 200ml with DEPC H_2O			
2g SDS			1%

Dynabeads wash buffer

1M Tris/HCl pH 8.0	2ml	*Final concentration*	0.01M
1.5M LiCl	20ml		0.15M
0.5M EDTA pH 8.0	40µl		0.001M
Make up to 200ml with DEPC H_2O			
+/- 0.2g SDS			

AMV reverse transcriptase 5x buffer	250mM Tris-HCl pH 8.3; 250mM KCl; 50mM $MgCl_2$; 2.5mM spermidine; 50mM DTT

Promega Reverse Transcription System

AMV reverse transcriptase high concentrate	Potassium phosphate 0.1M pH 7.2; Triton X®-100 0.2%; DTT 2mM; glycerol 50%
Recombinant RNAsin ribonuclease inhibitor	
Oligo(dT) primer	0.5mg/ml
dNTP mix	10mM each of 4 dNTPs pH 7.0
Reverse transcription 10x buffer	Tris-HCl 100mM pH 8.0; KCl 500mM; Triton®X-100 1%
$MgCl_2$	25mM

AJ Buffer

(PCR buffer is designated 'AJ buffer' as it is based on the recipe used in Prof Alec Jefferies laboratory)

1M Tris/HCl pH 8.8	*For 5ml 10x AJ*	2.25ml	*1x AJ* 45mM
1M $(NH_4)_2SO_4$		500µl	11mM
1M $MgCl_2$		225µl	4.5mM
100mM dNTPs		100µl each	200µM each
20mg/ml BSA		275µl	110µg/ml
14.3M β Mercaptoethanol		23.4µl	6.7mM
10mM EDTA pH 8.0		2.2µl	4.4µM

Tris-Acetate-EDTA (TAE)	Tris 2M; glacial acetic acid (CH_3COOH) 1M; EDTA 0.05M; pH 7.5-7.8

Gel loading buffer

5x buffer	2mg bromophenol blue
	8mls H_2O
	2mls 50x TAE
	10mls glycerol

10x buffer *(working concentration)*	2mls 5x buffer
	2mls 50x TAE
	12mls glycerol
	4mls H_20