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Lamin A/C as a prognostic biomarker in colorectal cancer

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Lamin A/C as A Prognostic Biomarker in Colorectal Cancer

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ABSTRACT

Lamins A and C (A-type lamins) are type V nuclear intermediate filament proteins which form a complex meshwork underlining the inner nuclear membrane termed the nuclear lamina. A-type lamins have been implicated in DNA replication, regulation of gene transcription, apoptosis and regulating the activity of several growth promoters. As such mutations in A-type lamins give rise to a diverse range of genetic diseases related to premature ageing and it has been speculated but not proven that expression of A-type lamins may influence tumour progression.
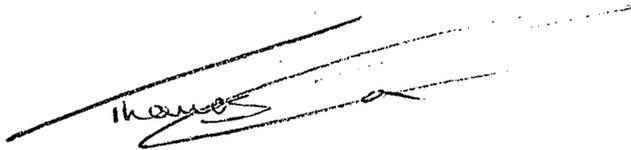
To test this hypothesis, a large (n=656) retrospective archive of colorectal cancer specimens from the Netherlands Cohort Study on Diet and Cancer was screened for the expression of A-type lamins. Data clearly show that patients lacking A-type lamin expression in their tumours have a significantly better prognosis compared to clinicopathologically similar patients expressing A-type lamins [HR = 0.59, (95% CI: 0.409 – 0.858), p=0.006].

Data also show that expression of lamin A in an *in vitro* colorectal cancer model leads to genome-wide changes and in particular promotes a significant down-regulation in Bone Morphogenic Protein 4 (BMP4) a member of the TGF-β superfamily which has already been linked to the pathogenesis of some solid tumours and also show that A-type lamin expression acts as a negative regulator of BMP4 mediated growth suppression. A-type lamin expression also results in a concomitant up-regulation in expression of the actin bundling protein T-plastin and down-regulation in expression of the cell adhesion molecule E-cadherin leading to a more motile, less adherent cellular phenotype in lamin A expressing cells.

Thus expression of A-type lamins may increase the risk of death in colorectal cancer because its presence gives rise to increased invasiveness due to the negative regulation of BMP mediated growth suppression. This is the first evidence directly linking the expression of A-type lamins to mechanisms promoting tumour progression.

DECLARATION

I hereby declare that this thesis is of my own composition, the results presented herein are of investigations conducted by myself at the School of Biological and Biomedical Sciences at Durham University under the supervision of Professor C.J. Hutchison. Work other than my own is clearly indicated by reference to the relevant workers or their publications. No material contained has been presented in any previous application for a higher degree at this or other University. The copyright of this thesis rests solely with the author. No quotation from it should be published in any format, including electronic and the internet without the author's prior written consent. All information derived from this thesis must be acknowledged correctly and in full.

Thomas Robert Cox

ACKNOWLEDGEMENTS

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ABBREVIATIONS

+ve	positive
5-FU	5-Fluorouracil
A	Adenine nucleotide
A	Absorbance
aa	Amino acid
Ab	Antibody
ACF	Aberrant crypt foci
ACVRII	Activin Receptor Type II
ACVRIIB	Activin Receptor Type IIB
AD	Autosomal Dominant
AD-EDMD	Autosomal Dominant - Emery Dreifuss muscular dystrophy
AJCC	American Joint Committee on Cancer
ALY	ALY Binding site
AMV-RT	Avian Myeblastosis Virus - Reverse Transcriptase
ANN	Approximate Nearest Neighbour
APC	Adenomatous Polyposis Coli
APS	Ammonium persulphate
AR	Autosomal recessive
AREG	Amphiregulin
BAF	Barrier to autointegration factor
BAX	*Bcl2* homolog
BCC	Basal cell carcinoma (of the skin)
Bcl-2	B-Cell Lymphoma/leukaemia-2
BMP	Bone Morphogenic Protein
BMP4	Bone Morphogenic Protein 4
BMPR1A	Bone Morphogenic Protein Receptor 1A
BMPR1B	Bone Morphogenic Protein Receptor 1B
BMPRII	Bone Morphogenic Protein Receptor Type II
bp	Base pair
BRB	Blot rinse buffer
BRR	Bannayan-Riley-Ruvalcaba
BSA	Bovine serum albumin
C	Cytosine nucleotide
C-	Carboxy terminal
C. elegans	*Caenorhabditus elegans*
χ^2	chi-square test
$CaCl_2$	Calcium Chloride
CCND1	Cyclin D1 gene
CD44	CD44 antigen
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)
cDNA	complementary DNA
cds	Coding sequence
CEA	Carcinoembryonic antigen
CI	Confidence Interval
CIN	Chromosome Instability
CKI	Casein Kinase I

CKII	Casein Kinase II
CMT1	Charcot-Marie-Tooth Disease Type 1
CMT2	Charcot-Marie-Tooth Disease Type 2
c-MYC	v-myc avian myecytomatosis viral oncogene homolog
c-NES	C-terminal Nuclear Export Signal
CNS	Central Nervous System
CO_2	Carbon Dioxide
CRC	Colorectal Cancer
CRDGF	Colorectum Derived Growth Factor
CREAM	Colorectal Epidemiology and Mutation
CRUK	Cancer Research UK
CS	Cowden Syndrome
CT	Computed Tomography
Da	Daltons
DAB	3,3'-diaminobenzidine tetrahydrochloride
DCC	Deleted in Colon Carcinoma
DCM1A	Dilated Cardiomyopathy type 1A
DEPC	Diethyl Pyrocarbonate
df	degrees of freedom - Student's t-test
DNA	DeoxyriboNucleic Acid
DNase	DeoxyriboNuclease
dNTP	Deoxynucleotide triphosphate
DoH	Department of Health
Dsh	Dishevelled
DTT	Dithiothreitol
DUSP5	Dual Specificity Phosphatase 5
ECACC	European Collection of Cell Cultures
ECL	Enhanced Chemiluminescence
EDMD	Emery-Dreifuss muscular Dystrophy
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
EGFP	Enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene glycol-bis[β-aminothyl ether]-N,N,N'N;-tetraacetic acid
EMD	Emerin Gene (see also *STA*)
EPIC	European Prospective Investigation into Cancer and Nutrition
ER	Endoplasmic Reticulum
ERBB	avian erythroblastosis oncogene B
ERK	Ras/extracellular signal-regulated kinase
EtBr	Ethidium Bromide
f	Female
FAK	focal adhesion kinase
FAP	Familial Adenomatous Polyposis
FBS	Foetal Bovine Serum
Fc	Fold Change
FOBt	Faecal Occult Blood testing
FOSL1	Fos-like Antigen 1
FPLD	Familial Partial Lipodystrophy
FTI	Farnesyl Transferase Inhibitor
Fz	Frizzled

FzR	Frizzled Receptor
G	Guanine nucleotide
GB	Great Britain
Gcl	Germ-cell-less
GCOS	GeneChip Operating System
GCT	Gene Class Testing
gDNA	genomic DNA
GFP	Green Fluorescent Protein
GI tract	Gastrointestinal tract
GO	Gene Ontology
Gro	Groucho
GSK3β	Glycogen Synthase 3β
H_2O_2	Hydrogen Peroxide
HCA	Heterocyclic Aromatic Amines
HCl	Hydrochloric Acid
HG U133	Human Genome U133 genechip [Affymetrix]
HGPS	Hutchinson-Gilford Progeria Syndrome
hMLH	human mutL homology
hMSH	human mutS homology
HNPCC	Hereditary Non-polyposis Colorectal Cancer
HR	Hazard Ratio
HRAS-VNTR	Harvey ras-1 Variable number tandem repeat polymorphism
HRP	Horse-radish Peroxidase
ICD	International Classification of Diseases
IF	Intermediate Filament
IgG	Immunoglobulin
IHC	Immunohistochemistry
INM	Inner Nuclear Membrane
JPS	Juvenile Polyposis Syndrome
kb	kilobase
KCl	Potassium Chloride
kDa	Kilo Dalton
KEGG	Kyoto Encyclopaedia of Genes and Genomes
Ki-ras	Kirsten Ras
KNN	K-Nearest Neighbour
L-15	Leibovitz growth media
LAP	Lamina Associated Protein
LBR	Lamin B receptor
Lef	Lymphoid enhancing factor
LEM	LAP2, Emerin, MAN1
LGMD1B	Limb Girdle Muscular Dystrophy type 1B
LMNA	Lamin A/C gene
LMNB1	Lamin B1 gene
LMNB2	Lamin B2 gene
LOH	Loss of Heterozygosity
Lrp	Low Density Lipoprotein Receptor related protein
LSM	Laser Scanning Microscope
m	Male
M	Molar
mA	milliAmps

mAb	Monoclonal Antibody
MAD	Mandibuloacral Dysplasia
MAPK	Mitogen-Activated Protein Kinase
MDT	Multidisciplinary team
mg	milligram
$MgCl_2$	Magnesium Chloride
MGED	Microarray and Gene Expression Data
MGMT	O^6-methylguanine-DNA methyltransferase
$MgSO_4$	Magnesium Sulphate
MIAME	Minimum Information About a Microarray Experiment
ml	millilitre
mM	milliMolar
MMP7	matrix metalloproteinase-7 (matrilysin)
MMR	MisMatch Repair
MOK2	Krüppel/TFIIIA-related zinc finger proteins
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
MSI	Microsatellite Instability
MTHFR	Methylene-Tetra-Hydro-Folate-Reductase
MUTYH	mutY homolog (E-coli)
Mw	Molecular Weight
N-	Amino terminal
N.I.C.E	National Institute of Clinical Excellence
NaCl	Sodium Chloride
NaOAc	Sodium Acetate
NaOH	Sodium Hydroxide
NCR	National Cancer Registry
NE	Nuclear Envelope
ng	nanogram
NHL	Non-Hodgkin's Lymphoma
NHS	National Health Service
NL	Nuclear Lamina
NLCS	Netherlands Cohort Study on Diet and Cancer
NLS	Nuclear Localisation Signal
nm	nanometre
NMSC	Non-melanoma Skin Cancer
n-NES	N-terminal Nuclear Export Signal
NPC	Nuclear Pore Complex
NSAID	NonSteroidal Anti-Inflammatory Drug
NUANCE	<u>N</u>ucleus and <u>A</u>cti<u>N</u> <u>C</u>onnecting <u>E</u>lement
OD	Optical Density
OMIM	Online Mendelian Inheritance in Man
ONM	Outer Nuclear Membrane
p	Page
p	Probability
$p21^{CIP1/WAF1}$	Cyclin-dependent kinase inhibitor 1A
PAH	Polycyclic Aromatic Hydrocarbons
PALGA	Pathologisch Anatomisch Landelijk Geautomatiseerd Archief
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis

PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PJS	Peutz-Jeghers Syndrome
PLAUR	plasminogen activator, urokinase receptor
PLS3	T-Plastin
pmol	picomole
PPARD	peroxisome proliferator activator receptor delta
pRb	Retinoblastoma protein
PSA	Prostate Specific Antigen
PTEN	Phosphatase and Tensin Homolog
PUFA	Polyunsaturated Fatty Acids
RD	Restrictive Dermopathy
RECQL2	DNA helicase-like / Werner syndrome ATP-dependent helicase
rhBMP	Recombinant Human BMP
RNA	RiboNucleic Acid
ROBO1	Roundabout, axon guidance receptor, homolog 1 (Drosophila)
rpm	Revolutions per minute
RT	Room Temperature
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
SCC	Squamous Cell carcinoma
SD	Standard Deviation
SDS	Sodium Dioecyl Sulphate
SDS-PAGE	Sodium Dioecyl Sulphate - Polyacrylamide Gel Electrophoresis
SLR	Signal Log Ratio
SMAD4	Mothers Against Decapentaplegic (Drosophila) Homolog 4
SOC	Super Optimal Broth [Catabolite Repression]
SOM	Self-organising Maps
SPSS	Statistical Package for the Social Sciences
SREBP-1	sterol regulatory element binding protein 1
STA	Emerin Gene (see also *EMD*)
STK11	Serine/Threonine Kinase 11
SUN	Sad1 / UNC-84 homology
SVD	Singular Value Decomposition
SVM	Support Vector Machines
T	Thymine nucleotide
t	Time
TAE	Tris-Acetate-EDTA
Taq	*Thermus aquaticus*
TBE	Tris-Borate-EDTA
TBS	Tris buffered saline
Tcf	T-cell factor
TE	Tris / EDTA
TEMED	N,N,N'N'-tetramethylethylenediamine
TGFα	Transforming Growth Factor α
TGFβ	Transforming Growth Factor β
TGFβIIR	Transforming Growth Factor β Receptor II
Tm	Melt temperature
TNM	Tumour-Node-Metastasis
TP53	Tumour Protein 53
Ub	Ubiquitin

UC	Ulcerative Colitus
UK	United Kingdom
US	United States
USA	United States of America
UV	Ultraviolet
V	Volts
v/v	volume / volume
-ve	negative
v-EGF	vascular endothelial growth factor
vs.	versus
w/v	weight / volume
Wg	Wingless
WHO	World Health Organisation
WISP3	WNT-1 inducible signalling pathway protein 3
XL	X-linked
yr	year
β-TrCP	β-Transducin repeat containing protein
δH2O	Distilled Water
δδH2O	Ultrapure Water
μg	microgram
μl	microlitre
μm	micrometre
μmol	micromole

CHAPTER 1 – Introduction

1.1 Colorectal Cancer

1.1.1 The national and international perspective

There are currently more than 200 different types of diagnosable cancer in the world today, but only four of them; lung, breast, prostate and large bowel (colorectal) account for over half of all new cases (Cancer Research UK 2007). Figure 1.1 shows the distribution of cancer subtypes diagnosed annually in the United Kingdom. Currently there are over a quarter of a million people diagnosed with cancer in the UK each year and more than 150,000 people die from the disease nationwide (Toms 2004). Figure 1.2 shows the most common causes of death by cancer annually in the UK. When considered globally, these figures reach somewhere in the region of 11 million diagnoses and 6 million deaths annually (Parkin, Bray et al. 2005).

** Non-Hodgkins Lymphoma
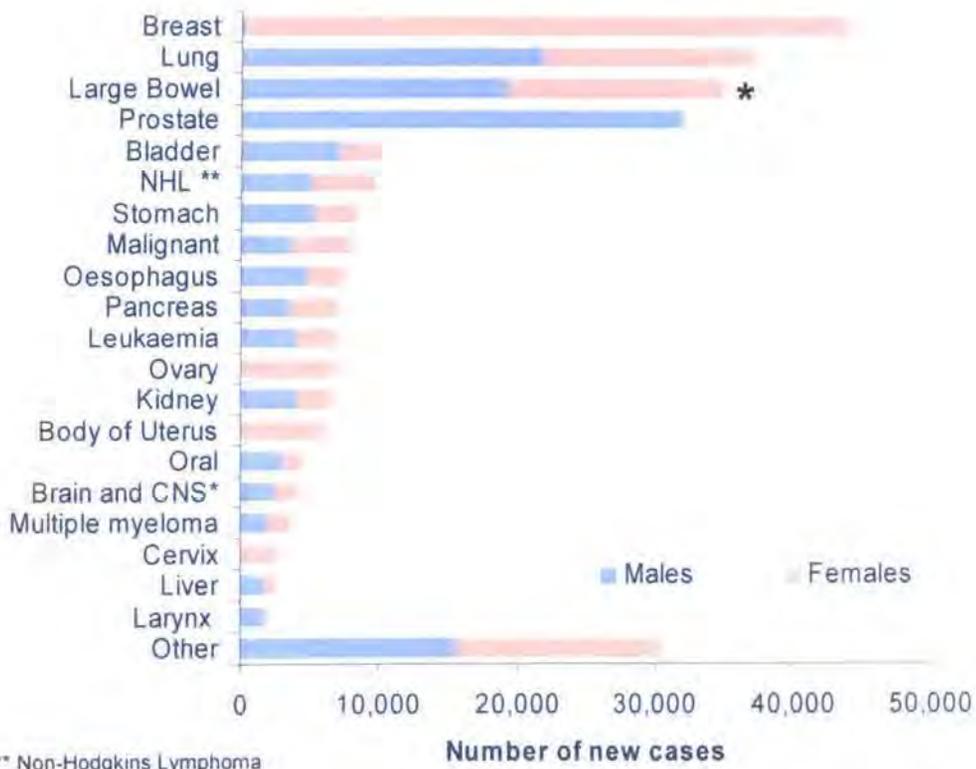
Figure 1.1 The 20 most commonly diagnosed cancers (excl. NMSC), UK, 2003

Source: Cancer Research UK (July 2007)

http://info.cancerresearchuk.org/cancerstats/incidence/commoncancers/
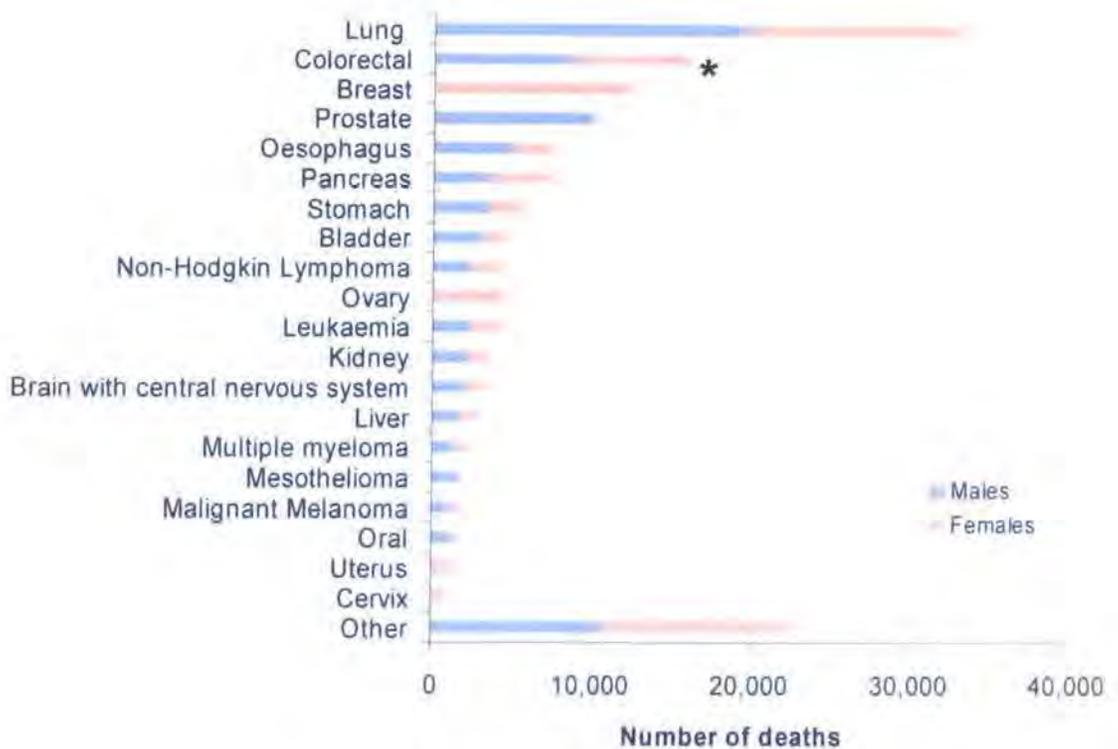
Figure 1.2 The 20 most common causes of death from cancer, UK, 2005

Source: Cancer Research UK (July 2007)

http://info.cancerresearchuk.org/cancerstats/mortality/cancerdeaths/

Colorectal cancer (CRC) is one of, if not the most common cancer malignancy in the UK and other developed countries . It is the second leading cause of cancer-related death, after lung cancer, and in the UK alone this accounts for approximately 13% of all new cancer cases and 10% of cancer deaths per year. Annually, this equates to approximately 35,000 new cases (~22,000 in the colon and ~13,000 in the rectum) and somewhere in the region of 16,000 deaths in the UK (Figure 1.1 and Figure 1.2) (Cancer Research UK 2007) which increases to 1 million diagnoses and 500,000 deaths when considered globally (Parkin, Bray et al. 2005). The lifetime risk within the general population is calculated to be approximately 5 to 6 percent with men and women being affected approximately equally, showing an incidence ratio of 1.2:1 (m:f) (Parkin, Bray et al. 2005). Whilst the survival rates for patients diagnosed with colorectal cancer are improving annually, of those diagnosed around half of them will ultimately die from the disease within 5 years .

The biggest problem with colorectal cancer is that symptoms often only present themselves after the transition from benign adenoma to malignant carcinoma (Cancer Research UK 2007) and at present, the risk factors associated with this progression remain largely enigmatic. There is an ever increasing plethora of evidence for the influence of a range of environmental and genetic factors, such as age, nutrition, low physical activity, obesity, smoking, inflammatory bowel disease and family history. However, the exact cause and progression of colorectal cancer, a vastly complex, multifactoral disease is still poorly understood, yet it has been estimated that less that 10% of all cases are 'truly genetic' and around 80% of cases can be attributed as a direct result of diet and lifestyle .

It is estimated that around 100 new cases of colorectal cancer are diagnosed every day in the UK alone (Toms 2004). The distribution of colorectal cancer cases throughout the large bowel is shown in figure 1.3. The left side of the colon is typically more affected than the right, with tumours in the sigmoid colon, rectosigmoid junction and rectum accounting for approximately half of all new cases.
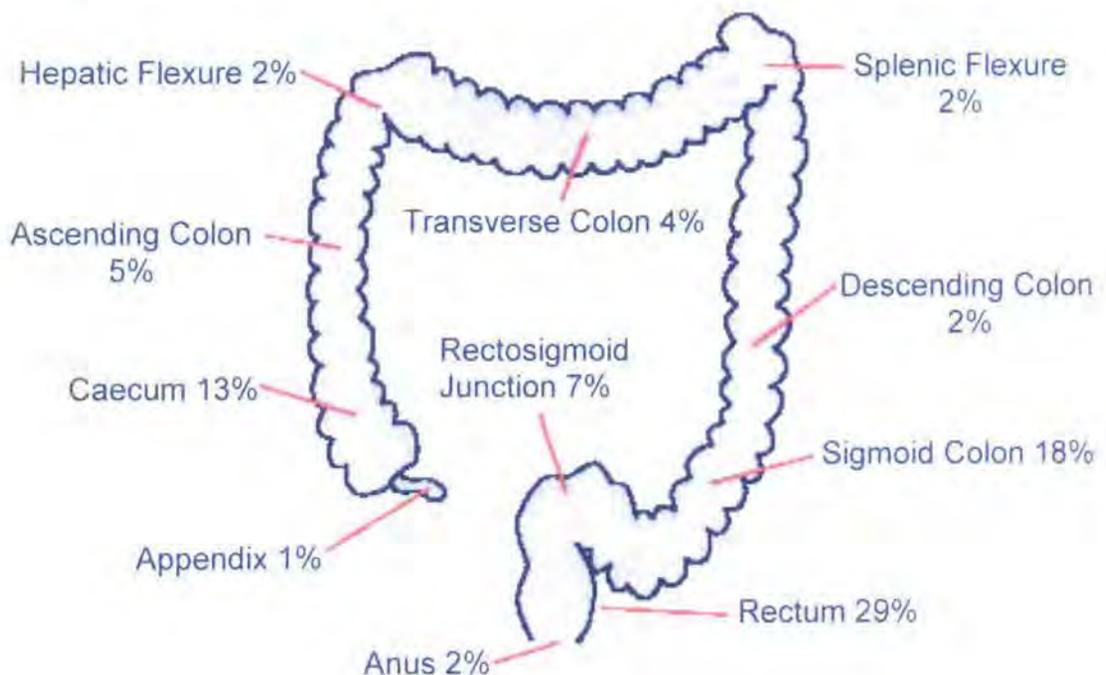
Figure 1.3 Distribution of case by site within the large bowel, England 1997-2000
Source: Cancer Research UK (July 2007)
http://info.cancerresearchuk.org/cancerstats/types/bowel/incidence/
(Proximal = Caecum to Hepatic Flexure, Distal = Hepatic Flexure to Rectum)

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1.1.2 Macroscopic and microscopic anatomy of the colon

1.1.2.1 Macroscopic organisation of the colon

The adult gastrointestinal tract is radially organised into four histologically distinct layers, the mucosa, submucosa, *Muscularis propria* and *Serosa* (Burkitt, Young et al. 1993) as shown in figure 1.4. The colonic mucosa is a simple columnar epithelium adjacent to the luminal surface of the colon and is underlined by layers of mesenchymal tissue and muscle. The microscopic organisation is discussed in the next section, but colorectal cancer as discussed in the context of this thesis constitutes tumours arising from the colonic mucosa itself. These are by far the most common and best studied cancerous lesion of the colorectum. Tumours arising in the smooth muscle and stromal layers of the colon and appendix do occur in the form of leiomyomas and leiomyosarcomas, although they are extremely rare in comparison to the prevalence of their epithelial counterparts (Hatch, Blanchard et al. 2000; Toms 2004).

1.1.2.2 Microscopic architecture of the colonic mucosa

The surface of the normal colonic mucosa is primarily composed of a mono-layer of columnar epithelial cells responsible for ion and water absorption, interspersed with goblet cells, responsible for synthesising and secreting mucin. Unlike the small intestine, the surface of the colon does not possess villi, but is studded with crypts, with numbers reaching over 100 million in adults. Each crypt is approximately 50 cells deep and at the base of each crypt, there is postulated to be somewhere in the region of 4-10 stem cells responsible for replenishing the crypt and intercrypt table throughout the whole of an individuals life .

These stem cells undergo mitotic, self-renewing divisions and the progeny then migrate unilaterally up the crypt (Twombly 2002). Directly above the proposed stem cell niche is a region of undifferentiated transiently amplifying cells which rapidly divide whilst continuing to migrate until they reach the upper third of the crypt. Here a switch occurs whereby cells stop dividing, and begin a path of differentiation which will

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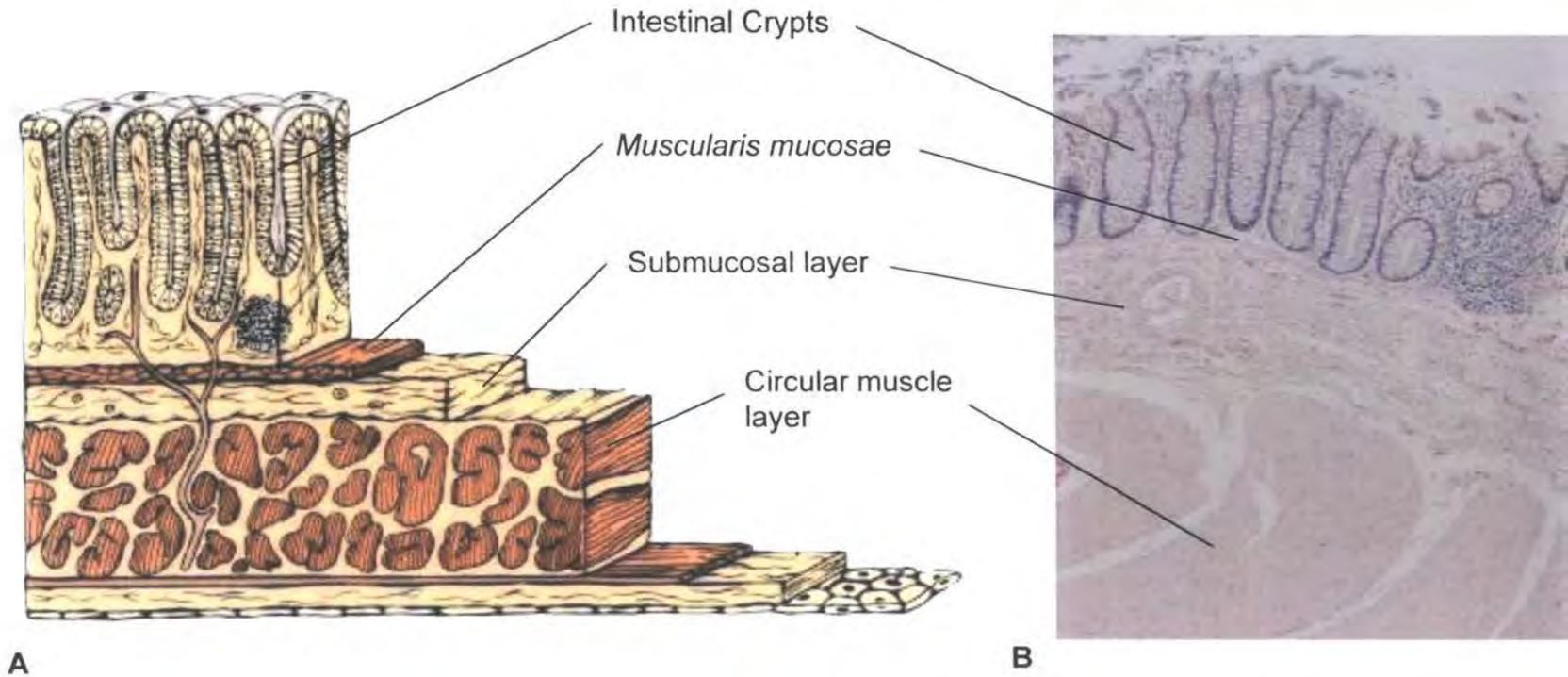
Figure 1.4. Diagrammatic representation (A) and immunohistochemical histology (B) of the colorectum. Staining is haematoxylin (purple) and Eosin (pink). (Modified from http://www.cancerline.com/ cancerlinehcp/)

Ultimately determine their fate. Upon reaching the lumen of the colon, the cells have undergone final differentiation and emerge into the lumen as fully differentiated, functional cells. They then undergo apoptosis and are sloughed off into the lumen with passing waste matter. The whole process of apoptosis, sloughing and extrusion from the mucosal surface at the apex, under normal conditions must exactly balance that of cellular proliferation at the base of the crypt for homeostasis to be maintained. This self-renewing process takes approximately four to six days in total. If the rate of cellular proliferation exceeds that of cellular loss, resulting in hyper-proliferation of the crypts, it ultimately leads to the formation of neoplasms within the gut lumen.

It is thought that the crypts most likely evolved to protect the crypt progenitor stem cells from the very mutagenic environment of the colonic lumen (Potter 1999). Under normal circumstances, interactions between colonic contents and replicating (progenitor) cells are practically nonexistent. By the time migrating crypt cells reach the luminal surface they are fully differentiated, non-replicating cells and imminently undergoing apoptosis. Thus any mutagenic event in these cells would have little or no impact on the integrity of the crypt cell population (Potter 1999).

Figure 1.5 shows a diagrammatic representation of the crypt highlighting the stem cell niche, proliferative and differentiated zones alongside the histological view of the full crypt profile.
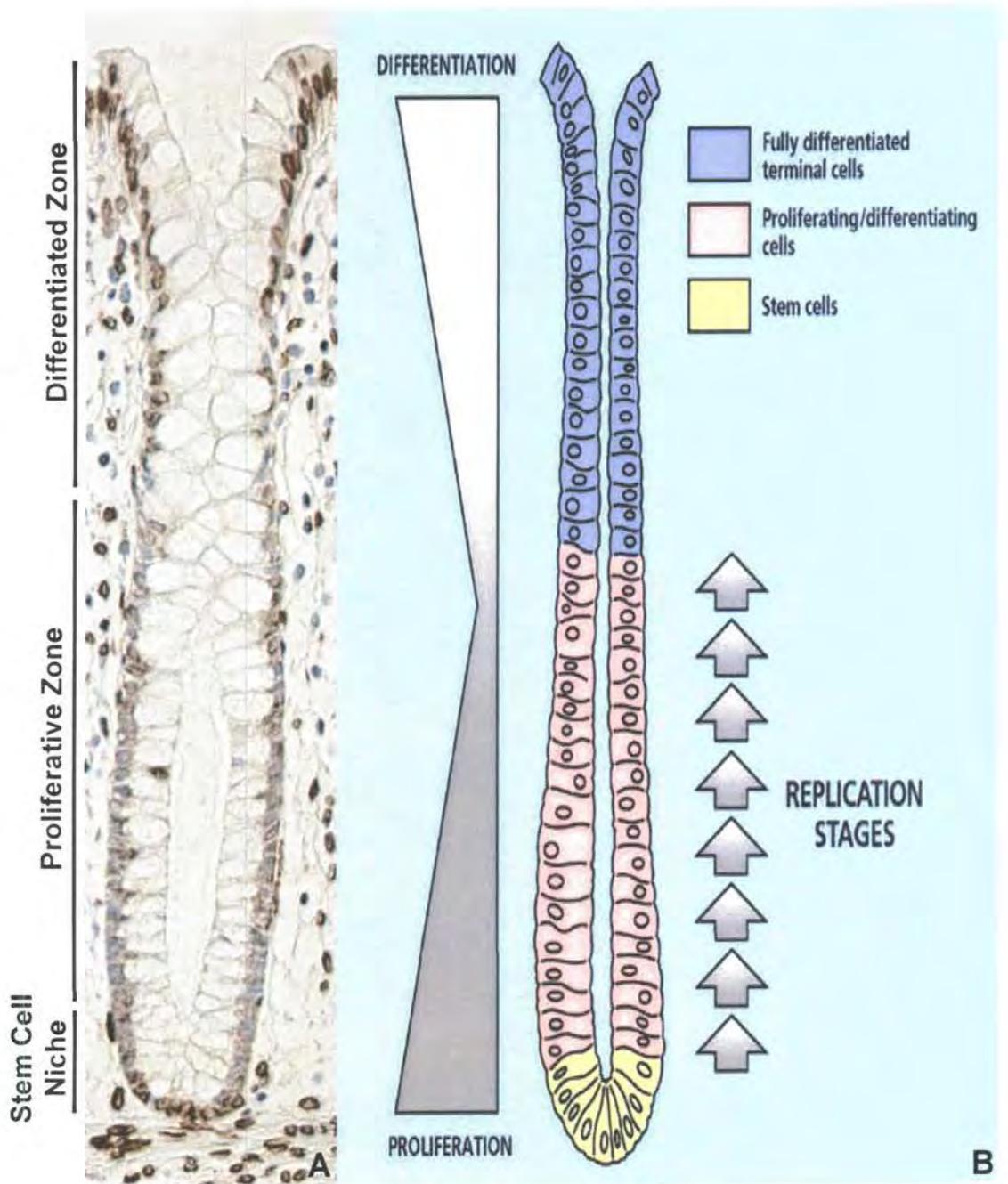
Figure 1.5

(A) Histological view of the crypt defined by immunoperoxidase staining with the Lamin A/C antibody JoL2 (Dyer, Kill et al. 1997) and counterstained with Mayers Haemalum.

(B) Diagrammatic representation of the crypt zones (modified from (Twombly 2002)).

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1.1.3 Clinicopathological staging of colorectal tumours

Colorectal tumours are solid, heterogeneous tumours and as such are classified, or staged clinicopathologically based on the degree of primary organ involvement and their metastatic spread to surrounding lymph nodes. There are 3 major staging systems in use across the world and this sub-staging is fundamental since it is used to determine the prognosis of newly diagnosed colorectal cancer patients and ultimately helps tailor their treatment regimen.

1.1.3.1 Dukes' staging as the Gold Standard in the UK

In the UK, the clinicopathological Dukes' staging system, devised some 75 years ago by Cuthbert Dukes and modified by Turnbull remains the "gold standard" for predicting prognosis in colorectal cancer patients. It was originally published for rectal cancers, but is now applied to all colorectal (colon and rectal) cancers. The staging system is relatively straightforward and when Dukes originally proposed his classification system it placed patients into one of three categories (Stages A, B, C). The system was subsequently modified to include a fourth stage (Stage D); and then again by subdividing Dukes' C into C1 and C2 to produce what is used nowadays and often termed the modified Dukes' staging system.

Dukes' A tumours are those in which the tumour has penetrated into the mucosa of the bowel wall, but have not penetrated and spread beyond the *muscularis mucosae*. If the tumour has spread beyond this and also penetrated the *muscularis propria* (muscle layers) but shows no pathological evidence of lymph node involvement, then the tumour is classified as Dukes' B. Once there is evidence of lymph node involvement, the tumour is either classified as Dukes' C1 in which 1-3 of the proximal lymph nodes are affected, or Dukes' C2 if 3 or more of the proximal lymph nodes are affected. Dukes' D patients are those in which the tumour has spread beyond the confines of the lymph nodes and are found to have distant metastases, which are commonly located in the liver, lungs, or bones. Figure 1.6 shows a cross-sectional view of the layers of the large intestine and the extend of tumour spread as defined by Dukes'
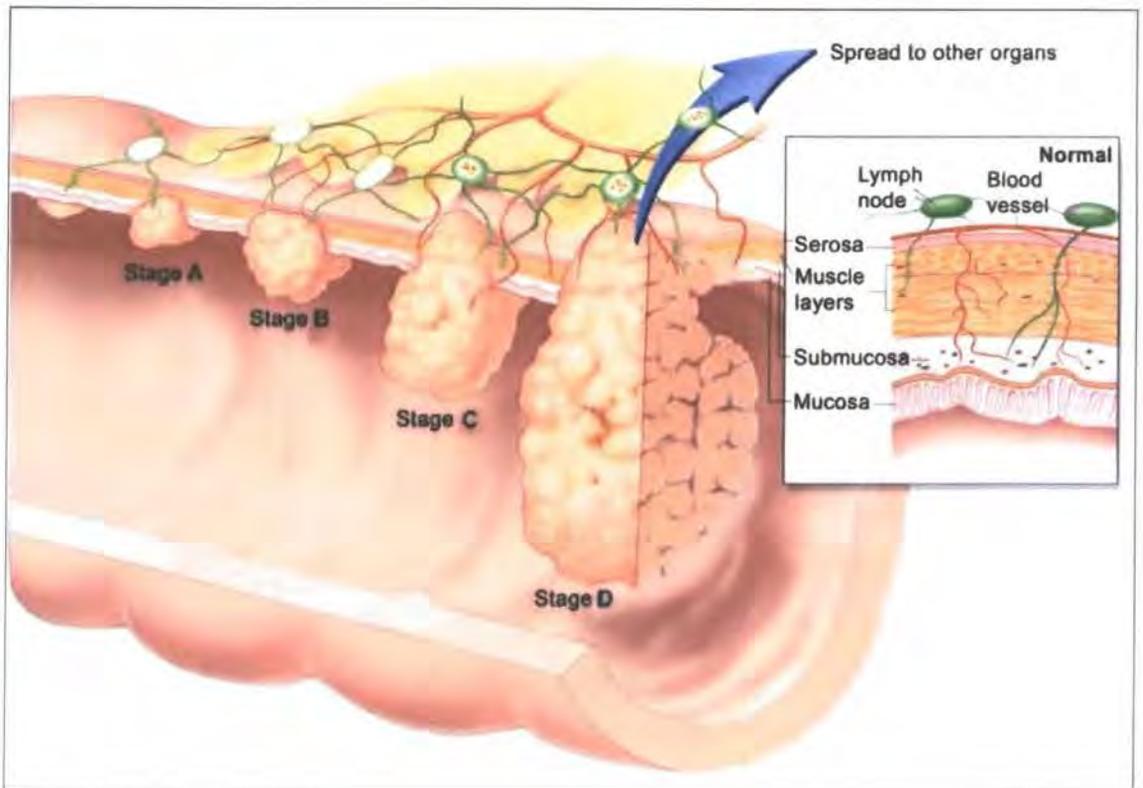
Figure 1.6 Diagrammatic representation of the cross section of the colon showing the extent of spread according to Dukes' classification (Modified from National Cancer Institute)

As has already been mentioned, the clinicopathological stage of the tumour is closely linked to patient survival and this was keenly observed by Dukes' in 1932. Table 1.1 shows the frequency of diagnosis and relative 5-year disease free survival (%) across the different Dukes' stages.

Table 1.1 Frequency and 5-year relative survival (%) by Dukes' stage

Dukes' Stage	Frequency of Diagnosis	5-year survival
A	11%	83%
B	35%	64%
C	26%	38%
D	28%	3%

Source: Cancer Research UK (2007)
http://info.cancerresearchuk.org/cancerstats/types/bowel/survival/

1.1.3.2 Astler-Coller and the American TNM staging systems

Although the original Dukes' system is useful in predicting survival outcome after surgical resection, it is often found that different patients within each stage have markedly different outcomes (Cass, Million et al. 1976). The Tumour – Node – Metastasis System (TNM) originally proposed by the American Joint Committee on Cancer (AJCC) is relevant to both clinical and pathologic staging and can be applied (albeit with slight modifications) to almost all solid tumours (AJCC 2002).

In terms of colorectal cancer, its advantages are that it has a more precise definition of the degree of primary tumour extension for lesions confined to the bowel wall, and defines node involvement by the number of nodes involved (N1, N1-3, N2, ≥N4). The sixth edition has also recently been refined by the addition of substaging for stage II and stage III lesions based on differential survival and relapse rates (Jessup, Gunderson et al. 2002). To that end, it is now currently being phased into use in the UK alongside the more traditional Dukes' system and is playing a key role in deciding patient treatment regimens. Table 1.2 shows the breakdown of the current TNM staging system for colorectal cancer. Only 10% of patients are diagnosed at stage 0 or stage I; the remainder are distributed equally between stage II, III and IV.

Table 1.2 The AJCC TNM staging system for Colorectal Cancer

AJCC/TNM	Description
Stage 0	Carcinoma *in situ*
Stage I	No nodal involvement, no distant metastasis
	Tumour invades submucosa (T1, N0, M0)
	Tumour invades *muscularis propria* (T2, N0, M0)
Stage II	No nodal involvement, no distant metastasis
	Tumour invades into *Subserosa* (T3, N0, M0)
	Tumour invades into other organs (T4, N0, M0)
Stage III	Nodal involvement, no distant metastasis
	1 to 3 regional lymph nodes involved (any T, N1, M0)
	4+ regional lymph nodes involved (Any T, N2, M0)
Stage IV	Distant metastasis (any T, any N, M1)

The pathological Astler-Coller staging system is another staging system still used in some parts of the world. This system was first proposed in 1954 and even today results in some confusion because it is often misinterpreted as related to the Dukes' system. The original scheme had five stages; A was limited to the mucosa, B1 involved the *muscularis propria* but did not penetrate it, B2 penetrated the *muscularis propria*, and C1 and C2 were counterparts of B1 and B2 with nodal metastases. Since then, later modifications have added three more stages. B3 represents involvement of adjacent structures, C3 is B3 with nodal metastasis, and D signifies presence of distant metastasis (Rosch 1998)

Table 1.3 shows a comparative analysis detailing the overlap between all three staging systems discussed, the extent of primary organ involvement and associated 5-year disease free survival.

Table 1.3 Comparison between classification systems

AJCC/ TNM	Dukes'	Astler-Coller	Extension To:	5-year Survival
I	A	A, B1	*muscularis mucosae*	93%
IIA	B	B2	*subserosa*/Pericolic Tissue	85%
IIB	B	B3	Visceral Per/Other Organs	72%
IIIA	C1	C1	N_1 1-3 lymph nodes	83%
IIIB	C1	C2, C3	N_1 1-3 lymph nodes	64%
IIIC	C2	C1, C2, C3	N_2 >3 lymph nodes	44%
IV	D	D	Metastatic Disease	8%

1.1.4 Screening for Colorectal Cancer

One of the main reasons that so many colorectal cancer patients present with more advanced, late-stage tumours is because of the relatively asymptomatic nature of the disease in the early stages and poor health seeking behaviour in the UK (Waters 2006). Yet, as with all cancers, treatment is markedly more effective if the disease is detected early. Currently the most cost-effective preventative measure is screening for early detection in what can be considered 'at risk' patients in order to prevent progression of benign polyps (adenomas) to malignant carcinomas. At present there are two main types of screening available to patients with suspected colorectal cancer; endoscopic examinations, (which include sigmoidoscopies and colonoscopies) and stool testing. Other older techniques are also still routinely used in some hospitals, such as barium enema X-ray. Colonoscopy is considered the gold standard for investigation but is somewhat more time-consuming and expensive than its sigmoidoscopy counterpart. Whilst both are simple and relatively painless to conduct, they are

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invasive procedures which must be carried out in the clinic. The other method is stool testing or faecal occult blood testing (FOBt) which is much simpler and can be carried out by the patient at home before being sent to the lab for analysis (Hardcastle and Pye 1989).

Several clinical trials have investigated the different methods of screening patients for colorectal cancer with the aim of identifying members of the population most at risk. In the UK to date, two screening programs have been piloted; FOBt screening and flexible sigmoidoscopy (Hardcastle, Chamberlain et al. 1996; Kronborg, Fenger et al. 1996; Atkin 2002; Alexander and Weller 2003). The first was a randomised controlled trial using FOBt screening which was undertaken in Nottingham (Hardcastle, Chamberlain et al. 1996) and aimed to look at all individuals between the ages of 45 and 75 to determine the effectiveness of detecting patients at risk of developing colorectal cancer. Patients were screened biennially using FOBt. Patients eliciting a positive result were then invited to undergo endoscopic investigation to confirm. A control group was also used in which patients were not screened.

The basis of FOBt screening is to detect non-visible or 'occult' blood in stools of patients which is often indicative of early colorectal neoplasia. It was found that FOBt screening was a good predictor of colorectal neoplasia in approximately 46% of cases. Of those tumours detected in patients, the majority were classified as Dukes' A (early stage tumours), thus indicating that FOBt screening could facilitate earlier colorectal cancer diagnosis. The most interesting finding however, was that the mortality rate of patients who underwent FOBt screening was reduced by approximately 39% compared to the control group (Hardcastle, Chamberlain et al. 1996).

The findings of Hardcastle and Chamberlain were then further supported by a similar study carried out in Minnesota in the US (Mandel, Bond et al. 1993; Mandel, Church et al. 1999). The work also aimed to make additional comparisons between the effectiveness of biennial and annual FOBt screening. They reported a similar decrease in the mortality rate of patients undergoing biennial FOBt screening compared to control, but also showed a further reduction in mortality rate of patients undergoing annual FOBt screening

compared to both the biennial FOBt screening and the unscreened control group. Both of these studies strongly supported the notion that FOBt screening could have huge potential benefits in reducing colorectal cancer mortality when applied to the population as a whole.

In order to determine the feasibility of such a proposed national screening program in the UK population, the UK Colorectal Cancer Screening Pilot was established and launched in 2000 with an independent report being commissioned by the UK Department of Health. Two sites were selected for a population-based trial: Coventry and Warwickshire in England, and Fife, Tayside and Grampian in Scotland. All men and women between the ages of 50 and 69 were invited to participate in the trials. It was reported that approximately 60% of those invited to participate took part in the FOBt screening program. The outcome was good and the results were positive which led the UK Colorectal Cancer Evaluation Team to conclude in their final commissioned report, that the benefits of FOBt screening at a national level to facilitate the early diagnosis of colorectal cancer and hence reduce mortality of patients, greatly outweighed the financial costs and logistical considerations of launching such an initiative (Alexander and Weller 2003). To this end, the National Bowel Cancer Screening Programme has been launched and is currently being rolled out over a two year period across the UK, (albeit slightly behind schedule) with the aim to achieve nationwide coverage by 2009 in order to screen and monitor all individuals in their sixth decade.

At the same time, the benefits of single flexible sigmoidoscopy screening was also investigated in a multi-centred, randomised controlled trial in the UK (Atkin 2002). Flexible sigmoidoscopy differs from FOBt screening in that it is the visual inspection of the rectum and sigmoid colon with a flexible endoscope. It has the advantage over FOBt screening in that it is able to detect pre-malignant lesions or polyps as well as early stage colorectal cancer. Endoscopic examinations however must be carried out in a clinic by trained staff and are considered highly invasive. There is also an associated risk of accidental perforation of the bowel which can occur during the procedure although this is rare. Atkins findings showed that approximately three quarters of cancers detected in the screened group could be considered as localised, early stage, i.e. Dukes' A or Dukes' B.

(Another advantage is that polyps detected during investigation can be removed by diathermy snare there and then without the need for further treatment). This was a much higher percentage than was detected by the FOBt screening studies indicating that single flexible sigmoidoscopy may be more sensitive. Additionally there was only a single case of a perforated bowel as a result of the procedure out of 40,000 patients in total, further supporting the notion that it is both safe and effective.

With this in mind, it would seem sensible to initiate a national screening program of endoscopic investigation for early diagnosis of colorectal cancer. Unfortunately, the estimated cost of such a procedure exceeds £125 whereas the associated cost of FOBt screening, including processing, is approximately £5 (Alexander and Weller 2003). Thus, screening by flexible sigmoidoscopy would have to deliver improvements in early diagnosis of colorectal cancer and a decrease in mortality rate much greater than that reported to date for the benefits of such a program to outweigh the cost and burden to the NHS.

There are several other methods in existence for colorectal cancer screening. Barium enema X-ray has already been mentioned, and is a more traditional, albeit invasive technique. The drive however, is towards non-invasive methods. Magnetic resonance imaging (MRI) scans are now frequently used to stage tumours and plan treatment and a relatively new technique being investigated is Computed tomography (CT) colonography (virtual colonoscopy) and guidelines on its use have already been issued by the National Institute of Clinical Excellence (N.I.C.E. 2005a). Currently, a randomised multicentre prospective trial (SIGGAR1) is comparing CT colonography to visual colonoscopy and barium enema and should report its findings in the near future (The NHS Health Technology Assessment Programme 2006).

1.1.5 Treatment and long-term prospects for patients

As discussed earlier, patient prognosis is closely linked to staging of the tumour. The prognosis for Dukes' A and Dukes' B patients is promising and surgical resection of the tumour and surrounding lymphatics alone is often highly effective

at curing the disease. In Dukes' B patients, there is penetration of the tumour through and beyond the submucosa, but at the time of surgery there is no evidence of either regional or distant lymph node involvement in the specimen removed. The average 5-year disease free survival rate for this group of patients is approximately 65 to 70% following surgical resection. However, just like their tumours, this patient subgroup is heterogeneous and a subset of patients exhibit a much poorer outcome which more closely resembles that more typically associated with Dukes' C patients where regional and distant lymph node involvement has been detected. Within this group, the average 5-year disease free survival falls to around 40%

The presence of lymph node involvement in Dukes' C patients predicts a 60% chance of recurrence within 5 years and as such, adjuvant chemotherapy and sometimes radiotherapy (in rectal cancer patients) is routinely administered to patients following surgery if they are well enough to tolerate it. However, in Dukes' B patients, such adjuvant therapy is not routinely administered unless it is deemed necessary by the surgical team or pathologist. For Dukes' D patients, where distant metastases have been found, or in patients harbouring locally inoperable primary tumours adjuvant therapy is usually only administered in a palliative capacity. For patients with advanced (Dukes' D) colorectal cancer the median survival is around six months and it has been shown that chemotherapy can improve this median survival by three to four months (Simmonds 2000).

1.1.5.1 The effects of Adjuvant and Neo-adjuvant therapy on patient outcome

There is evidence to suggest that a 6-month course of intravenous adjuvant chemotherapy following surgery significantly reduces the chance of colon cancer recurrence and improves five year disease free survival across the board by approximately 5 to 6% (Dube, Heyen et al. 1997). Evidence for the benefits of adjuvant chemotherapy for rectal cancer patients is less clear but results from randomised clinical trials do show an improvement in disease free survival (Lee, Ahn et al. 2002). Combined radiotherapy and chemotherapy delivered before surgery (so-called 'neo-adjuvant therapy') has also been shown to reduce the

chance of recurrence of rectal cancer and may also improve the overall patient survival (Schaffer, Thoma et al. 2002).

Across the Dukes' staging system, clinical trials have shown that treatment with a post-operative course of adjuvant chemotherapy in Dukes' C patients can reduce the recurrence rate from 60% to between 40% and 50% and has also been shown to reduce the overall death rate by 16-33% . Hence, in light of this and other such evidence, it has now made this the standard of care for all Dukes' C patients in the UK.

However, results are less clear with regard to Dukes' B patients where only 30% to 40% will develop a recurrence and ultimately die from the disease within 5 years. Adjuvant post-operative chemotherapy is not routinely administered, since its benefits in Dukes' B patients has been harder to determine and remains controversial. Data from trials undertaken and statistical reviews have shown that insufficient numbers of patients are frequently used in trials and the ability to detect treatment benefits in these patients is heavily influenced by experimental approach. That said, adjuvant chemotherapy is becoming more widely used in daily practice worldwide to treat certain Dukes' B patients that it is thought requires or may benefit from such therapy. However, the eligibility of a Dukes' B patient for adjuvant chemotherapy is still based on clinical and histopathological factors associated with recurrence. Among them are low-grade tumour differentiation; infiltration of perineurium, veins or lymphatics; intestinal obstruction; bowel wall perforation at time of surgery and young age. Interestingly, the combined use of radiotherapy in association with chemotherapy has also been evaluated (Wolmark, Wieand et al. 2000) yet showed no measurable beneficial effect on disease survival in rectal cancer patients [Comprehensively reviewed (Sanghera, Wong et al. 2008)]. Radiotherapy however is not used for cancer of the colon since the morbidity associated with its use on the abdomen is prohibitive.

Mainstream administration of adjuvant chemotherapy usually includes a combination of 5-Fluorouracil with either high or low does of leucovorin in weekly or monthly doses over a 6-month period. There is also the option of adding irinotecan and oxaliplatin (N.I.C.E. 2005b) or irinotecan may also be given alone

as a second-line treatment. Several other drugs, including bevacizumab (trade name Avastin) which is a monoclonal antibody against vascular endothelial growth factor (v-EGF) and cetuximab (trade name Erbitux) which is a chimeric monoclonal antibody against epidermal growth factor receptor (EGFR), are currently being evaluated in clinical trials as single agents or in combination, for both first and second line treatment (N.I.C.E. 2005c). Other clinical trials are investigating the optimum dose schedules and how best to administer adjuvant therapy, and results from these trials may have implications for current treatment protocols and may result in changes in drugs routinely used to treat colorectal cancer. Thus, the optimal treatment of patients and decision to administer adjuvant chemotherapy currently depends on establishing accurate diagnoses through a combination of clinical staging and histopathological data that is ascertained post-operatively. It is now mandatory in the NHS for each patient's management to be planned by multidisciplinary teams (MDT), which is resulting in a rising proportion of patients (Dukes' B especially) being referred for adjuvant chemotherapy.

1.2 Colorectal Carcinogenesis – Aetiology and Pathogenesis

1.2.1 Maintenance of the Crypt – Wnt signalling and its implications in colorectal cancer

As already mentioned, the turnover of cells at the intercrypt table is extremely high, therefore the differentiated population of cells at the apex of the crypt must be perpetually replenished via the unidirectional transit amplification and lineage specific differentiation of the multipotent stem cells which are postulated to be located at the base of the crypt (Gordon and Hermiston 1994; Booth and Potten 2000; Marshman, Booth et al. 2002). The whole transitional process from proliferation to differentiation in the healthy crypts of the colon constitutes what is termed the crypt axis. This highly defined axis is maintained by the canonical Wingless-Int (Wnt) signalling pathway which is considered to be the most significant regulator of both normal crypt homeostasis and also colorectal cancer development (Bienz and Clevers 2000; Gadal and Nehrbass 2002; van de Wetering, Sancho et al. 2002; Pinto and Clevers 2005).

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The Wnt factors are a family of approximately twenty signalling molecules which are involved in virtually every developmental process in humans. Biochemically, they are secreted glycoproteins and in the case of the colon, they are thought to emanate from the base of the intestinal crypts and/or the underlying mesenchymal tissue close to the basement membrane, although the exact location of their origin in this tissue has not been fully elucidated (Batlle, Henderson et al. 2002; Pinto and Clevers 2005).

The secreted Wnt factors perfuse the colonic epithelium and impose a proliferative phenotype on the lower regions of the crypt (Barker and Clevers 2000). This is done through a signalling cascade which leads to the stabilisation of the cytoplasmic protein β-catenin, allowing it to transit to the nucleus and engage DNA binding proteins of the T-cell factor / lymphoid enhancing factor (Tcf/Lef) family (van de Wetering, Sancho et al. 2002). The interaction of Tcf/Lef factors with β-catenin transiently converts them from transcriptional repressors to transcriptional activators and initiates transcription of Wnt target genes (Pinto, Gregorieff et al. 2003; Logan and Nusse 2004; Pinto and Clevers 2005).

For a Wnt factor to trigger this canonical signalling cascade it is required to interact simultaneously with a Frizzled (Fz) receptor and a co-receptor of the Low Density Lipoprotein receptor (Lrp) family, Lrp-5 or Lrp-6 located at the cell surface membrane. As a means of control, this whole process is negatively regulated by the adenomatous polyposis coli (APC) protein (Korinek, Barker et al. 1997). Through its many functional domains, APC interacts with numerous proteins and is involved in cell migration, adhesion, chromosome stability and cytoskeletal organisation (Abdel-Rahman and Peltomaki 2004), but in this instance, its key role is as an essential component of what is known as the multiprotein degradation complex, responsible for directly regulating the activity of cytoplasmic β-catenin.

The multiprotein destruction complex consists of several proteins including but not limited to: APC; a scaffold protein named Axin; and two serine/threonine kinases, glycogen synthase 3β (GSK3β) and casein kinase I (CKI). The role of the multiprotein destruction complex is to de-activate β-catenin by

phosphorylation, which consequently targets it for ubiquitination by β-transducin repeat containing protein (β-TrCP) and then leads to its subsequent degradation by the proteasome. Under normal circumstances this process occurs constitutively in the absence of a Wnt signal (Bienz and Clevers 2000). Upon activation of the canonical Wnt signalling pathway [comprehensively reviewed by (Logan and Nusse 2004)], interaction of Wnt ligands with their membrane spanning co-receptors, Fz and Lrp5/6, results in the recruitment of Axin and another protein, Dishevelled (Dsh), to the plasma membrane. Consequently, the multiprotein complex is then dissociated, and β-catenin is no longer phosphorylated, thus leaving it free to translocate to the nucleus and initiate transcription of target genes (see Figure 1.7).

In the absence of a Wnt signal, β-catenin levels in the nucleus are low and Tcf/Lef transcription factors are bound to the transcriptional repressor Groucho (Gro) (Cavallo, Cox et al. 1998). In the presence of a Wnt signal, stabilised nuclear β-catenin is able to overcome this repression by direct association with Tcf/Lef factors, namely Tcf-4 in the colon, thus transactivating the transcription of downstream target genes which includes c-MYC, CCND1, MMP7, CD44, PLAUR and PPARD (Jo and Chung 2005). These downstream target genes are typically associated with initiating and driving cellular proliferation, an example being c-MYC which represses the cell cycle inhibitor $p21^{CIP1/WAF1}$ and allows cells to leave G1 and enter S phase of the cell cycle.

Therefore, it would follow that β-catenin/Tcf-4 driven gene transcription could be responsible for preserving the stem cell and proliferating progenitor population in the lower regions of the crypt which are essential in sustaining the crypt axis and replenishing the continual turnover of differentiated cells at the crypt lumen (van de Wetering, Cavallo et al. 1997; van de Wetering, Sancho et al. 2002). Since Wnt signalling appears to be switched off higher up the crypt in the differentiated zone, it would appear that it is a key component in the proliferation/differentiation switch associated with crypt regeneration.

With that in mind, Wnt signalling has been shown to be reinitiated in the earliest stages of colorectal tumour development. Immunohistochemical studies of aberrant crypt foci (ACF), the earliest and usually benign progenitors of

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colorectal cancer (van de Wetering, Sancho et al. 2002) have shown high nuclear accumulation of β-catenin. Mutational analysis of colorectal cancer specimens has also shown that in 85% of all patients, there are key truncating mutations in the APC protein (Miyoshi, Nagase et al. 1992; Powell, Zilz et al. 1992; Kinzler and Vogelstein 1996). This same protein is also mutated in the hereditary form of colorectal cancer, Familial Adenomatous Polyposis (FAP) syndrome. Such truncations are known to disrupt the multiprotein destruction complex and lead to constitutively active β-catenin/Tcf-4 signalling (Korinek, Barker et al. 1997; Morin, Sparks et al. 1997).

Hence it can be considered that the APC protein is central to colorectal tumourigenesis and may be considered the dominant switch in the malignant transformation of normal colonic epithelial cells by imposition of a constitutively proliferative phenotype at a very early stage (Fodde, Smits et al. 2001; van de Wetering, Sancho et al. 2002).
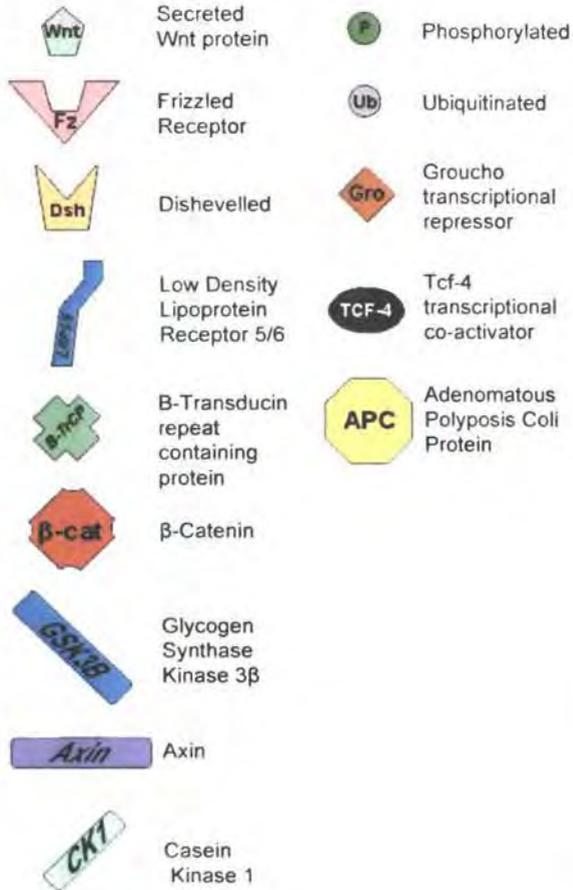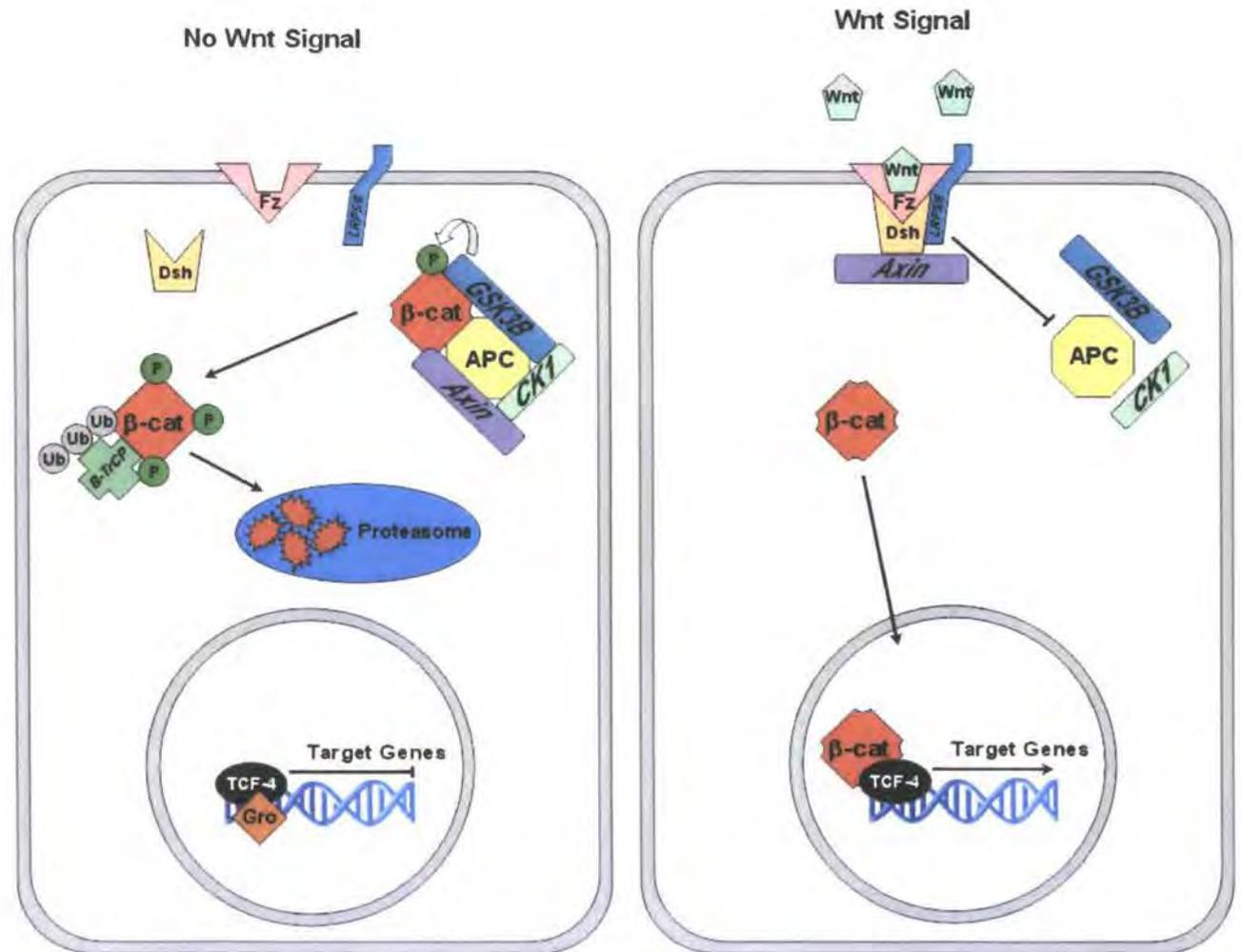
Figure 1.7 Diagrammatic representation of the canonical Wnt Signalling pathway

1.2.2 Molecular basis of tumour initiation and subsequent progression

As I have already discussed, in order to maintain the surface lining of the colon, the cellular behaviour is strictly regulated and there is normally a perfect homeostatic balance between proliferation and apoptosis. As with all developmental processes, this regulation relies on a complex system of signals and as such, any disruption of this homeostasis has the potential to allow cells to escape the tight constraints used to control them. Tumourigenesis is a complex, multi-step process in which this delicate balance is upset, producing cells with significant survival advantages and ultimately leading to their often aggressive clonal expansion. The sequence in which the steps of tumourigenesis occur are reflected in the rate-limiting stages of tumour progression.

Generally, the genes implicated in tumourigenesis are divided into three main categories: oncogenes, tumour-suppressor genes and DNA stability genes. In the general sense, oncogenes promote and drive cellular proliferation and growth. Mutations in oncogenes will result in constitutively active gene products and therefore mutations in a single oncogene allele are sufficient to initiate or promote tumourigenesis. As of yet, there have been no reports of inherited oncogenic mutations leading to colorectal cancer. Tumour-suppressor genes on the other hand are responsible for down-regulating the growth-stimulatory pathways. Thus, a mutation in both alleles of a tumour-suppressor gene is required in order to inactivate gene function completely, known as the two-hit hypothesis (Knudson 1971). In sporadic colorectal cancer, both of these "hits" can be somatically acquired through a variety of mechanisms, e.g. promoter hypermethylation, point mutation, deletion or chromosomal rearrangement. In autosomal dominant hereditary colorectal cancer syndromes, such as Familial Adenomatous Polyposis (FAP), one allele is mutated in the germline and the second is somatically mutated. Such a condition drastically increases the chances of loss of heterozygosity (LOH) [discussed by (Muller, Heinimann et al. 2000)]. The mutated tumour-suppressor genes inherited in other hereditary colorectal cancer syndromes include, Bone Morphogenic Protein Receptor 1A (BMPR1A) and Mothers Against Decapentaplegic (Drosophila) Homolog 4 (SMAD4) in Juvenile Polyposis Syndrome (JPS), and Serine/Threonine Kinase

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11 (STK11) in Peutz-Jeghers Syndrome (PJS). The role of DNA stability genes is to help maintain the integrity of the genome by repairing DNA replication errors, inhibiting recombination between non-identical DNA sequences and eliciting cellular responses to DNA damage. When these systems are dysfunctional, deleterious mutations can accumulate throughout the genome rapidly increasing tumour progression. Examples of DNA stability genes mutated in hereditary colorectal cancer syndromes include the MisMatch Repair (MMR) genes in Lynch syndrome and mutY homolog (E.coli) (MUTYH) in recessive adenomatous polyposis syndrome.

Hence, with this in mind, one of the key molecular steps, not only in colorectal cancer, but in all types of cancer formation is the loss of genomic stabilisation (Lengauer, Kinzler et al. 1998). When considering colorectal cancer, this can occur in one of two ways. In the classic adenoma-carcinoma sequence pathway (see Figure 1.8), the major driving force is chromosome instability (CIN) leading to loss of heterozygosity. More recently though, another mechanism known as Microsatellite Instability (MSI) has been attributed to a small subset of colorectal cancer cases, which whilst sharing some similar mechanisms as CIN, the main driving force behind neoplasia in these cases is the loss of MMR gene function.

Underlying the whole process of colorectal tumourigenesis is a series of successive, cumulative genetic alterations. The most common genetic alterations in sporadic colorectal cancers are activating mutations in the oncogene Kirsten-ras (*Ki-ras*) and mutation or loss of the tumour suppressor genes; APC, SMAD4 and *TP53*. It is widely accepted that colorectal adenomas typically develop from the normal stem cells at the base of the colorectal crypts through acquisition of such mutations . The vast majority of colorectal tumours then progress through a series of histopathological stages starting with the formation of dysplastic crypts leading to adenomas or polyps. The first somatic mutation is thought to cause clonal expansion of progenitor cells that initiates the neoplastic process producing the polyp . Hence, colorectal polyps are essentially hyperplastic growths that project from the lining of the colon or rectum. They can be sessile or pedunculate, single or multiple, but are usually benign. Histologically they are divided into hamartomatous, serrated and adenomatous polyps but they are seldom symptomatic. Their significance however lies in their potential for

malignant transformation. Polyps will then go on to form benign adenomas through a series of successive somatic mutations resulting in additional rounds of clonal expansion. Adenomas can vary in size from less than 1cm up to and above 4cm in size (see figure 1.9 B-D), but size is by no means a deciding factor in undergoing further malignant transformation to produce invasive tumours with metastatic properties. Hence the progression of a dysplastic crypt into a malignant tumour is the result of a series of cumulative genetic and sometimes epigenetic changes involving the activation of oncogenes and inactivation of tumour suppressor genes controlling cell growth, differentiation and apoptosis . To put it into perspective, this chain of events is thought to occur by chance and can take anywhere from 5 to 40 years.

Full blown adenocarcinomas themselves can be both polypoid vegetating masses (see figure 1.9 E) and flat, infiltrating lesions, which are often ulcerated (see figure 1.9 F). They have the potential to reach large dimensions, especially when located in the caecum or ascending colon, but most colorectal malignancies are typically located distal to the splenic flexure, in the rectum, rectosigmoid junction and sigmoid colon. Colorectal malignancies will grow both longitudinally and laterally, often aggressively infiltrating surrounding organs, although at present, there is little consistent evidence to suggest that either the actual size or degree of differentiation of the tumours is tightly associated with the clinical outcome (Ponz de Leon and Di Gregorio 2001).

Figure 1.8 shows a diagrammatic representation of the classical adenoma-carcinoma sequence of events highlighting genetic alterations in both the CIN and MSI pathways of colorectal tumourigenesis. Figure 1.9 shows the stages of development of colorectal adenocarcinomas from normal colon to malignant carcinoma as viewed by colonoscopic investigation.
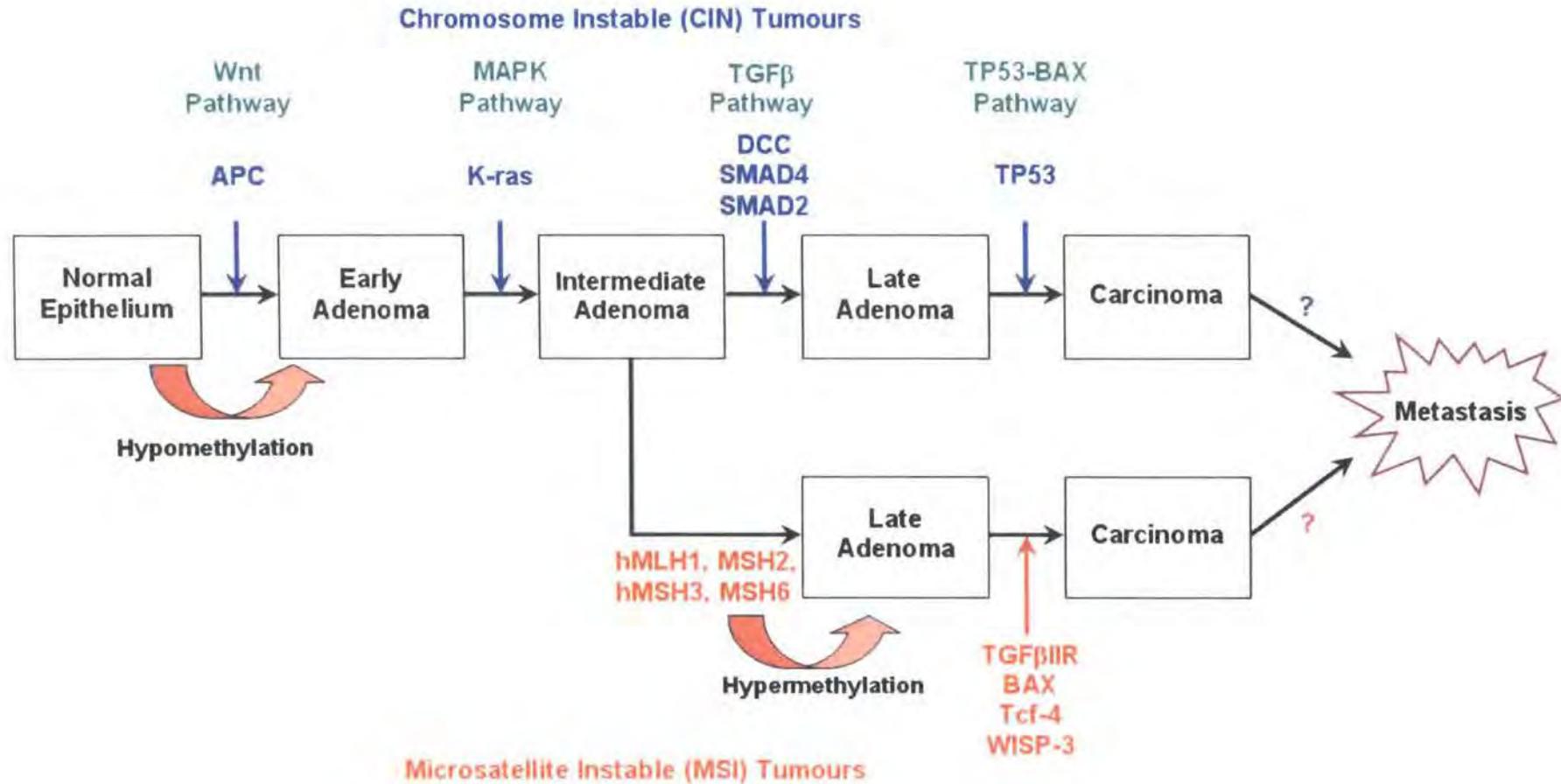
Figure 1.8 Key molecular changes during colorectal carcinogenesis for both chromosome instable (CIN) and Microsatellite Instable (MSI) tumours
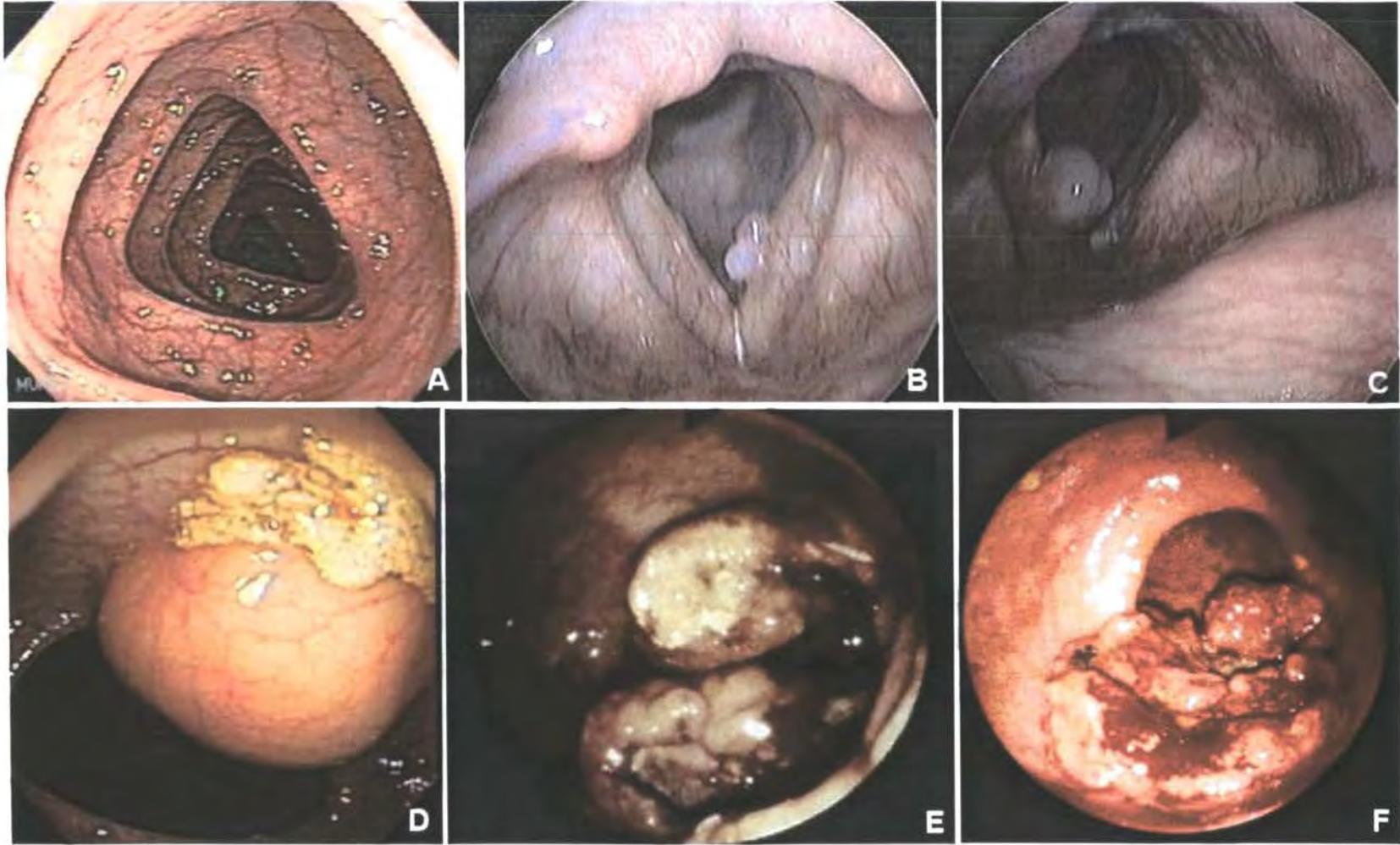
Figure 1.9 Colonoscopic views of **(A)** Normal colon **(B-D)** Colorectal Adenomas of increasing size **(E)** Polypoid vegetating adenocarcinoma **(F)** Flat infiltrating adenocarcinoma

1.2.3 Environmental factors associated with colorectal cancer

It is estimated that the contribution of genetic factors alone to the development of colorectal cancer is somewhere in the region of 35%, and hence the remaining 65% is due to environmental factors (Lichtenstein, Holm et al. 2000). There is a vast amount of research that has been and is still being undertaken concerning the role of lifestyle and diet in the development of colorectal cancer. This has been comprehensively reviewed for instance by Potter (Potter 1999); and more recently the final, international, commissioned report from the World Cancer Research Fund in collaboration with the American Institute for Cancer Research (World Cancer Research Fund 2007). Whilst Potter discusses the increased colorectal cancer risk associated with intake of processed red meat and animal fat, alcohol and smoking, the World Cancer Research Fund report systematically reviews all factors of diet, nutrition and lifestyle in relation to not just colorectal but all major cancers worldwide.

It has more recently been shown too that Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) have been consistently associated with reductions in colorectal cancer risks, as have hormone replacement therapies. In addition, patients with inflammatory bowel disease, both ulcerative colitis and Crohn's disease, are widely accepted to be at an increased risk of developing colorectal cancer (Itzkowitz and Yio 2004), with the colorectal cancer risk in these cases being related to the chronic inflammation.

It is now widely accepted that many environmental factors may exert direct effects on colorectal carcinogenesis by means of induction of DNA damage (Young 2007). The result of such mutations subsequently affects the function of the encoded proteins and as a result may drive the selection of tumours harbouring specific mutations. A few molecular epidemiological studies have included the mutation status of the tumour (i.e. *Ki-ras* oncogene, APC and *TP53* status, as well as microsatellite instability) and have observed differences in associations between lifestyle and diet and colorectal tumours with and without such aberrations.

1.2.3.6 Obesity

There is increasing evidence that the dysregulation of energy homeostasis in the body is associated with colorectal carcinogenesis (Cancerbackup 2008). Epidemiological data have consistently demonstrated a positive relation between increased body size and colorectal malignancy from as early as 1988 (Klatsky, Armstrong et al. 1988) and almost every year throughout the last two decades [for example (Suadicani P 1993; Schoen, Tangen et al. 1999; Shimizu, Nagata et al. 2003; Pischon, Lahmann et al. 2006)] [systematically reviewed by (World Cancer Research Fund 2007)].

The phenomenon of "insulin resistance" or the impaired ability to normalise plasma glucose levels is considered the root cause, but other mechanisms have also been advanced (Gunter and Leitzmann 2006). Obesity-induced insulin resistance leads to elevated levels of plasma insulin, glucose and fatty acids. Exposure of colonic crypt cells to heightened concentrations of insulin is thought to induce a mitogenic effect within these cells, whereas exposure to glucose and fatty acids may induce metabolic perturbations, alterations in cell signalling pathways and oxidative stress (Gunter and Leitzmann 2006).

Family based studies have shown that the propensity to become obese is a heritable trait, but it typically only manifests in situations of excess energy intake over expenditure. Therefore, inheritance of a genetic profile that predisposes to increased body size may also be predictive of colorectal cancer (Abate 2000).

To date, some sixty cohort studies and 86 case-control studies investigated body fatness (as measured by BMI) and cancers of the colon and rectum [extensively reviewed by (World Cancer Research Fund 2007)]. Most of the cohort studies showed increased risk with increased body fatness which was statistically significant in approximately half of the studies. When stratified according to cancer site, the World Cancer Research Fund report also suggests a larger and more consistent increased risk for colon cancer than for rectal cancer or for colorectal cancer as a whole.

1.2.3.3 Dietary Fat

It is thought that dietary fats may exert direct effects on colorectal tumourigenesis by augmenting bile acid production and subsequently resulting in increased colonic cell proliferation, ultimately increasing the risk of endogenous mutations. This is as well as the obvious associations of high dietary fat intake and increased BMI. Systemic effects of high dietary fat intake include the effect of specific types of fat on membrane characteristics of cells. Such changes in membrane characteristics are thought to increase the risk of insulin resistance and hence survival in tumour cells, decrease the protective effect of n-3 fatty acids by decreasing prostaglandin E2 production, and alter the effects of essential polyunsaturated fatty acids on inflammatory responses .

Previously there has been an emphasis on the quantity of total fat in the diet; however research is now suggesting that it is in fact the fat quality in combination with quantity which is more important in predisposing an individual to colorectal cancer (World Cancer Research Fund 2007). For example, n-3 polyunsaturated fatty acids (PUFA) seem to have protective properties at different stages of colorectal carcinogenesis , whereas n-6 PUFAs are though to increase the risk of colorectal cancer. A plausible explanation of the potentially carcinogenic properties of the n-6 PUFA lies in the peroxidation products of malondialdehyde and 4-hydroxynonenal that can readily form adducts with DNA and induce G $\rightarrow$ T and G $\rightarrow$ A transitions .

Investigations into the direct effect of dietary fat on specific mutations associated with colorectal tumourigenesis have shown there is an increased risk of tumours harbouring mutant *Ki-ras* with low dietary fat intake and low mono-unsaturated fatty acid intake . Similarly, Slattery et al showed strong associations between the quantity of dietary fat, saturated fats and mono-unsaturated fatty acids and tumours harbouring G$\rightarrow$T mutations in the *Ki-ras* gene. This was then further supported in 2004 when it was observed that there was an increased risk of *Ki-ras* mutations associated with high consumption of polyunsaturated fatty acids and linoleic acid .

With regard to mutations in the APC gene, a recent case-control study showed, that a high intake of dietary fat seemed to increase the risk of adenomas without truncating APC mutations . However, in terms of colorectal cancer, positive associations were observed for high dietary fat intake and tumours that did harbour truncating APC mutations .

1.2.3.5 Alcohol

Irrespective of the type of drink, alcohol (ethanol) is classified by the International Agency for Cancer Research as a human carcinogen (IACR 2008). The evidence that alcoholic drinks are a cause of cancers of the mouth, pharynx, and larynx, oesophagus, colorectum, and breast is convincing (World Cancer Research Fund 2007). Early reports concerned with food, nutrition, and the prevention of disease have often excluded alcohol. This is because alcohol is also a drug, the impact of which is behavioural and social, as well as biological. More recently, alcoholic drinks have been included in such reports because of the evidence that low to moderate consumption protects against coronary heart disease (but not cerebrovascular disease), and also because of the evidence on cancer, given that ethanol is a human carcinogen.

In terms of colorectal cancer, to date 37 cohort studies have investigated alcoholic drinks and colorectal cancer and 41 case-control studies have investigated ethanol intake and colorectal cancer. Of these, 18 of the cohort studies showed an increased risk for the highest intake group when compared to the lowest, which was statistically significant in four. One study showed a non-significant increased risk in men and non-significant decreased risk in women. Two studies reported no effect on risk and three studies reported a decreased risk; none was statistically significant (World Cancer Research Fund 2007).

Despite the evidence being weaker than was initially assumed, it has led the World Cancer Research fund to conclude in their 2007 report that "There is ample and generally consistent evidence from cohort studies. A dose-response is apparent. There is evidence for plausible mechanisms. The evidence that consumption of more than 30 g/day of ethanol from alcoholic drinks is a cause of colorectal cancer in men is convincing, and probably also in women"

1.2.3.1 Meat and Fish

The EPIC study (European Prospective Investigation into Cancer and Nutrition), based on about 1,300 colorectal cancer cases, has shown a significant (55%) increase in colorectal cancer risk associated with a 100g/day increase in consumption of red and processed meat (Norat, Bingham et al. 2005).

This is supported by results of two meta-analyses, one of which found a significant risk increase for colorectal cancer with higher consumption of red meat and processed meat (Norat, Lukanova et al. 2002), and another showing significant positive associations with all meat, red meat, and the strongest increase for processed meat (49% risk increase for a 25 g/day serving) (Sandhu, White et al. 2001).

A large US case-control study has also reported that the highest risks are associated with fried meat and the lowest for baked or boiled, which may be related to formation of heterocyclic aromatic amines produced during the cooking process (Butler, Sinha et al. 2003). The EPIC study has also shown a significant risk reduction of approximately 50% with increased intakes of fish, including fresh, canned, salted and smoked (Norat, Bingham et al. 2005).

1.2.3.2 Dietary Fibre

Concomitantly, the EPIC study has shown a lower risk of colorectal cancer with higher fibre intake, (after adjustment for a number of potential confounding factors, including folate) (Bingham, Norat et al. 2005). There was about a 20% reduction in risk of colorectal cancer in men and women in the highest quintile of fibre consumption, compared to the lowest. The association was strongest for left-sided colon cancer (sigmoid colon, rectosigmoid junction), with a greater than 40% risk reduction for the highest quintile of fibre intake. However, as with many other studies, there was no significant risk reduction shown for rectal cancer.

Recently however, a new meta-analysis study, excluding the results of the aforementioned EPIC study, showed no significant reduction in risk of colorectal cancer with higher than normal fibre intake, but did show that very low fibre

intake (less than 10 g/day) significantly increases the risk of colorectal cancer (Park, Hunter et al. 2005).

One of the biggest problems however, is that in some studies published, the apparent null findings may be due to flawed experimental design such as an insufficient range of fibre intake or other methodological problems. Alternatively, it could be considered that other immeasurable features of such a high fibre diet (i.e. a plant-based diet rich in fruits, vegetables, and whole grains) could be responsible for the protective effects observed. Despite the apparent lack of consensus, investigators agree that fibre should generally be eaten as part of a healthy diet because of its potential to prevent colorectal cancer as well as its beneficial effects on other chronic diseases, such as heart disease and diabetes.

1.2.3.4 Cigarette smoking

Aside from the obvious implications associated with lung cancer and heart disease, it is also postulated that the carcinogenic compounds present in tobacco smoke may cause irreversible damage to the colorectal mucosa. The burning of tobacco produces numerous genotoxic compounds including polycyclic aromatic hydrocarbons (PAH), heterocyclic aromatic amines (HCA), and N-nitrosamines. The colon and rectum may become exposed to these carcinogens either directly by ingestion of tobacco smoke or indirectly, through contact with blood or bile that may contain potential carcinogens. In rodent studies, benzo(a)pyrene was found to induce G→T transversions and N-nitrosamines to induce G:C→ A:T transitions in the *ras* family of oncogenes, both of which are major constituents of cigarette smoke. Cigarette smoking has also been associated with microsatellite instable colorectal tumours in three case-control studies

Thus it would seem that environmental and genetic influences go hand in hand throughout every step of colorectal tumourigenesis. Where genetic alterations are not hereditary, environmental factors provide the causative and also catalytic effect.

1.2.4 Differences in sporadic and hereditary cases

Colorectal cancer is traditionally classified as either sporadic or familial, but these concepts are rapidly becoming less tenable in respect of the ever increasing understanding of the underlying mechanisms of tumourigenesis. A seemingly familial occurrence of cancer may not be due to genetic inheritance, but due to chance or more likely a shared environment. Conversely, colorectal cancer that appears to be sporadic may in fact be part of an unrecognised or undocumented familial syndrome, concealed by a small family size, reduced penetrance, or poor diagnostics.

Sporadic cases of colorectal cancer are currently estimated to account for approximately 75% of all colorectal cancer cases and are most strongly associated with the aged population (Toms 2004) due to long-term exposure to risk factors such as those previously discussed (Potter 1999; Terry, Giovannucci et al. 2001). The majority (~85%) of colorectal cancer patients are diagnosed in the sixth decade of life or later. It is thought that colorectal cancer causing genetic alterations may start accumulating approximately 10 years prior to the development of the tumour. Cases of colorectal cancer in much younger individuals are almost always likely to be the result of an inherited predisposition to the disease.

As already mentioned, genetic predisposition to colorectal cancer greatly increases the chance of polyps forming and leads to the earlier onset of the disease. Hereditary forms of the disease are usually autosomal dominant and categorised according to symptoms, tumour pathology and age of onset. Certain 'Cancer families' have been identified in which members carry hereditary colorectal cancer-causing mutations, and the first such family to be diagnosed with hereditary bowel cancer in Britain originated from West Cornforth, County Durham (Dunstone and Knaggs 1972).

Three types of hereditary colorectal cancer syndrome are recognised and described below. In most cases, the predisposing germ-line aberration has been identified.

1.2.4.1 Familial Adenomatous Polyposis Syndrome

The familial adenomatous polyposis (FAP) syndrome is a rare autosomal dominant disease with a prevalence of approximately 1/8000 (Abdel-Rahman and Peltomaki 2004) and is marked by a germline mutation in the Adenomatous Polyposis Coli (APC) gene that predisposes individuals to developing large numbers (often thousands) of benign adenomas (polyps) throughout the length of the colon. These polyps have the potential to eventually develop into malignant carcinomas . FAP currently accounts for approximately 1% of all colorectal cancers in the west; with the average age of polyp appearance of affected individuals being 16 years of age. Since the penetrance of this syndrome is 100%, the average age of colorectal cancer diagnosis in these patients if the polyps are left untreated is 40 years, but inter- and intra-familial variation is common.

The gene for FAP, the APC tumour suppressor gene is located on chromosome 5q21-q22 (Bodmer, Bailey et al. 1987). The mutation spectrum is very wide, and more than 800 different germline mutations have been found. The different mutation sites within the gene are associated with varying severities of the disease, though people with the same germline mutation may also show different disease manifestations. Typically, the germline mutations associated with FAP are confined to the 5' half of the APC gene, whereas somatic mutations which are responsible for 85% of sporadic cases are clustered to the central region of the open reading frame, spanning codons 1,286 to 1,513 of exon 15 . Although this mutation cluster region represents only 8% of the 8,535 bp APC gene, 68% to 77% of somatic mutations in APC are found in this region . A few recurrent mutations are known, but there are no hotspots which account for more than 10% of the total known mutations. Interestingly, approximately 20-25% of mutations are *de novo*, suggesting that other factors may act as modifiers.

1.2.4.2 Hereditary Non-polyposis Colorectal Cancer

HNPCC, more commonly known as Lynch syndrome, is estimated to be responsible for approximately 2–5% of all colorectal cancer cases. It is the most commonly inherited form of colorectal cancer and increases the lifetime risk of a

patient to approximately 80-85% (Aarnio, Mecklin et al. 1995; Dunlop, Farrington et al. 1997; Lynch and de la Chapelle 1999; de la Chapelle 2004). HNPCC is characterised by early onset of colorectal cancer, but is also associated with non-colorectal tumours, including cancers of the endometrium, ovaries, stomach, pancreatico-biliary system, brain and urinary tract.

HNPCC is caused by a fault in DNA mismatch repair (MMR) genes; with faults in the MMR genes *hMSH2* and *hMLH1* accounting for over 90% of detectable mutations in HNPCC patients (Liu, Parsons et al. 1996). Pilot studies in colorectal cancer patients under 30 years of age have shown that 41% are carriers of MMR gene mutations (Farrington, Lin-Goerke et al. 1998). Such mutations allow deleterious changes in the mainstream cancer genes (APC, *Ki-ras* and *TP53*) to persist and lead to development of colorectal cancer (Leach, Nicolaides et al. 1993; Thibodeau, Bren et al. 1993).

Genetic testing for predisposing mutations in people with a strong family history of these cancers enables screening and prevention to be targeted to those most at risk. For people with a known mutation, especially young patients from HNPCC families, prophylactic surgery is often recommended, and currently a study in Scotland is screening all newly diagnosed colorectal cancer patients under the age of 55 years for MMR gene mutations.

1.2.4.3 Hamartomatous Polyposis Syndromes

Apart from FAP and HNPCC, hereditary factors are estimated to account for approximately 15-20% of colorectal cancer cases and a national collaborative study is currently underway to identify other genes involved in familial colorectal cancer (Penegar, Wood et al. 2007).

Currently hamartomatous polyposis syndromes can be subdivided into four distinct syndromes; Cowden Syndrome (CS), Bannayan-Riley-Ruvalcaba Syndrome (BRR), Juvenile Polyposis Syndrome (JPS) and Peutz-Jeghers Syndrome (PJS) (Muller, Heinimann et al. 2000; Fearnhead, Wilding et al. 2002) All of them predispose the sufferer to the development of multiple hamartomatous polyps and subsequent increased risk of gastrointestinal

malignancy. Germ-line mutations in the tumour-suppressor gene Phosphatase and Tensin homolog (*PTEN*) have been implicated in CS, BRR and JPS (Li, Yen et al. 1997; Marsh, Coulon et al. 1998) whilst germ-line mutations in serine/threonine kinases *LBK1* and *STK11* have been implicated in PJS (Hemminki, Markie et al. 1998; Jenne, Reimann et al. 1998). In addition to these high-risk predispositions, polymorphisms with moderate risk effects have also been described, and include variants in the APC, Harvey *ras-1* variable number tandem repeat polymorphism (HRAS1-VNTR) and Methylene-Tetra-Hydro-Folate-Reductase (MTHFR) genes (Houlston and Tomlinson 2001). Interestingly, the mutations behind these syndromes are rarely if at all seen in sporadic colorectal cancer.

Other conditions which have also been linked to an increased risk of colorectal cancer include ulcerative colitis (UC) and Crohn's disease. UC is estimated to be responsible for around 1% of cases (Winawer 1999). Recent research suggests that environmental factors related to long-term inflammation of the bowel may contribute more to the increased cancer risk in UC than the associated inherited susceptibility (Wong and Harrison 2001).

1.3 Biomarkers – risk factors and indicators

The biggest limitation of all the current clinicopathological diagnostic staging systems is that they fail to discriminate the innate biological behaviour of tumours. It has been clear for some time now that in order to address this problem, measurable biomarkers are needed, which can be used not in place of, but in conjunction with the current staging systems to allow more accurate predictions of patient prognosis in situations where clinical staging alone may be problematic and potentially misleading. This would for example, aid identification of high-risk Dukes' B patients with resected, lymph node-negative tumours that are likely to experience relapse. Such stratification could be carried out on pre-operative samples which would then allow more informed planning for patients in terms of surgery and/or postoperative adjuvant therapies. It would also provide an innovative approach for designing targeted trials to assess the benefits of adjuvant chemotherapy.

A biomarker is typically any substance that can be measured biologically and is associated with an increased or decreased risk of the disease. Biomarkers can either be present in the serum, or be genetic testing factors, and currently both are being studied acutely to elucidate markers that may be of use at any stage during the treatment of cancer.

1.3.1 Serum Biomarkers

Serum biomarkers are produced by the body's organs and in the case of cancer, by the tumours themselves (Maurya, Meleady et al. 2007). Changes in the level of such serum biomarkers may be indicative of tumour activity, but many of the current biomarkers are non-specific for cancer and are produced by normal organs as well, so their application must be scrutinised.

One serum biomarker which is widely used today, and currently one of the few used consistently in primary care is Prostate Specific Antigen (PSA). PSA is produced by normal prostate cells in relatively small amounts, but it has been shown that elevated levels of PSA in the serum can be correlated with the existence of prostate cancer. However, there are also several reasons other than cancer that can cause a rise in PSA. For example, infection in the prostate (prostatitis), and even vigorous physical examination by a doctor can cause a PSA rise (Cancerbackup 2008). Instead, it is important to monitor factors such as the degree of elevation, the rapidity of increase, and the fraction of free non-bound PSA (higher in benign causes) together in order to determine the next step. While no treatment is ever based solely on PSA levels, alterations above normal can spur further diagnostic testing to catch the disease at an early stage.

In terms of colorectal cancer, carcinoembryonic antigen (CEA) is another biomarker that is routinely used in patient treatment (Cancerbackup 2008). CEA levels are elevated in patients with colorectal cancer; however, it is also elevated in patients with breast, lung, and pancreatic cancer as well as by other environmental factors. As a screening test, its uses are limited as it can also be elevated by other factors such as smoking for instance. However, its use following surgery is more informative. CEA levels can be monitored both pre and

post-operatively. CEA levels typically fall after surgical resection, and elevation following surgery may be indicative of the need for additional adjuvant therapies, whereas elevation in the long term may be indicative of local or distant relapse.

1.3.2 Genetic Biomarkers

So called genetic biomarkers for colorectal cancer fall into roughly six categories; oncogenes and tumour suppressor markers such as *TP53*, *Bcl-2*, *Ki-ras* and *DCC*; proliferation markers such as Proliferating Cell Nuclear Antigen (PCNA*)*, *Ki67* and *Mib-1*; DNA mismatch repair such as *hMLH1*; metastatic markers such as matrix metalloproteinases; angiogenesis markers such as *v-EGF*; and other biochemical markers such as thymidylate synthase. The use of immunohistochemical methods to detect these tumour-associated antigens is preferred in the clinical setting to the more complex and time-consuming real time techniques such as flow cytometry.

Most immunohistochemical analysis of oncogenes and tumour suppressor genes relies on detecting aberrant expression of the protein within the cell. Increased or decreased levels of the protein can potentially be used to predict prognosis in patients, but there still exists a controversy over its reliability. For example, mutations in *TP53* can be easily detected as a nuclear accumulation of the protein. Whilst this is a simple and effective technique it relies on a simple assumption that a genetic change in the *TP53* protein will always lead to it's over expression. Similarly, it also assumes that an observed increase in *TP53* expression is a result of mutation within the gene. Nevertheless, there is a strong correlation between *TP53* gene status and *TP53* staining although there is a great deal of concern in light of so many conflicting studies as to whether this marker can provide significant prognostic information at present [reviewed (Joerger and Fersht 2007)].

Like *TP53*, studies into the use of *Ki-ras* as a prognostic marker for colorectal cancer show conflicting data, even within the international RASCAL studies (Andreyev, Norman et al. 1998; Andreyev, Norman et al. 2001). Whilst *Ki-ras* mutations occur in approximately 30% of colorectal cancers, mutational analysis has shown that not all *Ki-ras* mutations lead to a more aggressive tumour and

subsequently poorer prognosis (Andreyev, Norman et al. 2001). For this reason alone, *Ki-ras* mutational status does not currently seem to be the best candidate for predicting patient prognosis.

PCNA, *Ki67* and *Mib-1* are the favourite and most promising proliferative biomarkers being investigated at the moment, but to date most of the studies have shown little prognostic significance. A study by Palmqvist (Palmqvist, Sellberg et al. 1999) has however shown prognostic significance in Dukes' B, node-negative patients for *Ki67* when determined at the invasive edge alone.

With little evidence to support large-scale prospective studies in current biomarkers, attention has more recently been focussed on looking for new proliferative biomarkers and as such, cyclins involved in cell cycle regulation have been targeted. Such studies have looked at the prognostic significance of Cyclin A and *p27/Kipl* (a putative tumour suppressor) but have yet to provide conclusive evidence. For a comprehensive review of many of these markers see (Graziano and Cascinu 2003).

The identification of other individual biomarkers such as Osteopontin; Neuregulin and B-cell lymphoma/leukaemia-2 (*Bcl-2*) to name a couple, have been investigated for several years now. More recently however, work in cell lines has shown that transforming growth factors *TGF-α* and *TGF-β1* have mitogenic properties in promoting the growth of colorectal cancer *in vitro*. This has been substantiated in recent investigations in patients linking poor survival in early stage colorectal cancer and is emerging as an independent prognostic factor. However, since many of these biomarkers are yet to be investigated in large-scale prospective studies, these apparently promising markers cannot at present be adopted as the basis of routine prognostic or diagnostic assays.

1.3.3 The emerging concept of DNA methylation in colorectal cancer

As discussed earlier, the loss of DNA stability genes is one pathway by which colorectal tumourigenesis can occur. Typically, this loss of function is through hypermethylation in the promoter regions of affected genes, and it is well

documented that silencing of the *hMLH1* gene is an early event in colorectal cancer characterised by microsatellite instability (MSI) (Kane, Loda et al. 1997).

Aberrant DNA methylation patterns have also been demonstrated in the colonic mucosa affected by ulcerative colitis (Issa, Ahuja et al. 2001). Similarly, it has been described that the O^6-methylguanine-DNA methyltransferase (MGMT) gene promoter is methylated in the normal-appearing colorectal mucosae adjacent to colorectal cancer (Shen, Kondo et al. 2005) and that similar MGMT promoter hypermethylation occurs in 20% – 40% of colorectal cancers (Nagasaka, Sharp et al. 2003). The biological consequence of loss of MGMT expression through hypermethylation is that the MGMT protein removes mutagenic adducts from the O^6-guanine base residue in DNA. When left unrepaired, O^6-methylguanine is read by DNA polymerase as adenine, resulting in multiple G→A transitions (Pegg 1990; Pegg and Byers 1992). Such MGMT silencing has been strongly associated with an increase in G→A mutations in the *Ki-ras* and *TP53* genes in colorectal tumours (Esteller, Toyota et al. 2000; Esteller, Risques et al. 2001). However, what is still unclear is whether MGMT promoter methylation in apparently normal mucosa is associated with an increased risk of colorectal cancer, although it would be reasonable to postulate that a neoplastic lesion harbouring MGMT promoter methylation is more likely to progress to malignancy than one which does not.

1.3.4 Biomarkers and the pharmacodynamic potency of chemopreventative therapy

Over the past ten years the number of treatment options for colorectal cancer has increased with many more therapeutic agents going into clinical development. New targeted therapies have been designed whose action is directed against particular molecular targets isolated through such biomarker studies. Examples include the already mentioned bevacizumab targeted against vascular endothelial growth factor (v-EGF) and cetuximab targeted against epidermal growth factor receptor (EGFR) (Vanhoefer 2005). These new and emerging adjuvant therapies are currently being tested in combination with chemotherapy for both early stage and advanced colorectal cancer.

However, one of the greatest challenges in treating colorectal cancer is the problem of tumour resistance to chemopreventative therapies. Large-scale DNA microarray analysis is currently proving to be a powerful tool in identifying biomarkers that will help to predict the best treatment options for individual patients (Boyer, Allen et al. 2006).

1.3.5 The future of Biomarkers

It is widely accepted that colorectal cancer progression is a function of multiple genetic alterations. As such, many individuals believe that isolating single biomarkers will not be reliable enough to be routinely adopted as prognostic or diagnostic tools. Instead, a more comprehensive approach should be implemented to investigate the molecular events on a wider scale in order to fully understand the disease mechanism and be able to predict prognosis in a disease progression of this complexity. The simultaneous testing of multiple molecular predictors of survival may supply major information, however in such investigations, large multivariate models must be applied which require large patient groups for statistical reliability. For now at least, confirmatory investigations of single biomarkers in prospective studies of large-scale patient groups is the way forward. Such studies can then be implemented into clinical trials to identify high and low risk patients. As we move into the 21st century, the use of such individual biomarkers in predicting patient prognosis and determining the administration of adjuvant therapies will inevitably improve and prolong the lives of patients with surgically resected colorectal cancer.

1.4 Architecture of the Nucleus and implications in disease

1.4.1 The Nucleus

At the heart of almost all eukaryotic cells is the complex and vastly dynamic structure known as the nucleus. This multifunctional organelle is responsible for storing the genetic information of the cell in the form of chromatin, and concomitantly regulating the expression of the genes contained within the

chromatin throughout all stages of the cell cycle. It is also a key player in controlling the overall dynamics of the mitotic process (Lamond and Earnshaw 1998; Mizuno 1999).

1.4.2 The Nuclear Envelope

The contents of the nucleus are segregated from the rest of the cell by the presence of the nuclear envelope, a double membranous structure constructed from both phospholipids and proteins (Dessev 1992). As well as providing a static, structural barrier role responsible for defining the boundaries of the nucleus, it also plays a dynamic role in gene regulation acting as an anchor point for the attachment of chromatin and other transcriptional regulators thus controlling gene transcription and expression (Pfaller and Newport 1995; Marshall, Dernburg et al. 1996)

Additionally, the nuclear envelope is studded with channels known as nuclear pore complexes (NPCs) which are responsible for governing the passage of mRNA out of and other small signalling molecules into the nucleus, thus regulating the transmission of information between cytoplasm and nucleoplasm (Macara 2001). Complex signalling pathways which originate at the periphery of the cell via cell surface protein receptors and other signalling events often cascade in such a manner to direct information to the nucleus (Leonard, Imada et al. 1999; Roberts 1999). The convening of these signalling pathways at the nucleus then triggers the appropriate cellular response to an event which can include, but is not limited to responses modulating the timing of mitosis, altering gene expression patterns, and/or initiating an apoptotic program.

During interphase, the dual membranous structure of the nuclear envelope surrounds the nucleus and is comprised of an inner nuclear membrane (INM) and an outer nuclear membrane (ONM), the outer being contiguous with the endoplasmic reticulum (Worman and Courvalin 2000). The pore membranes, where the NPCs reside, form a membranous connection between the INM and the ONM. (Ostlund and Worman 2003) as shown in figure 1.10.
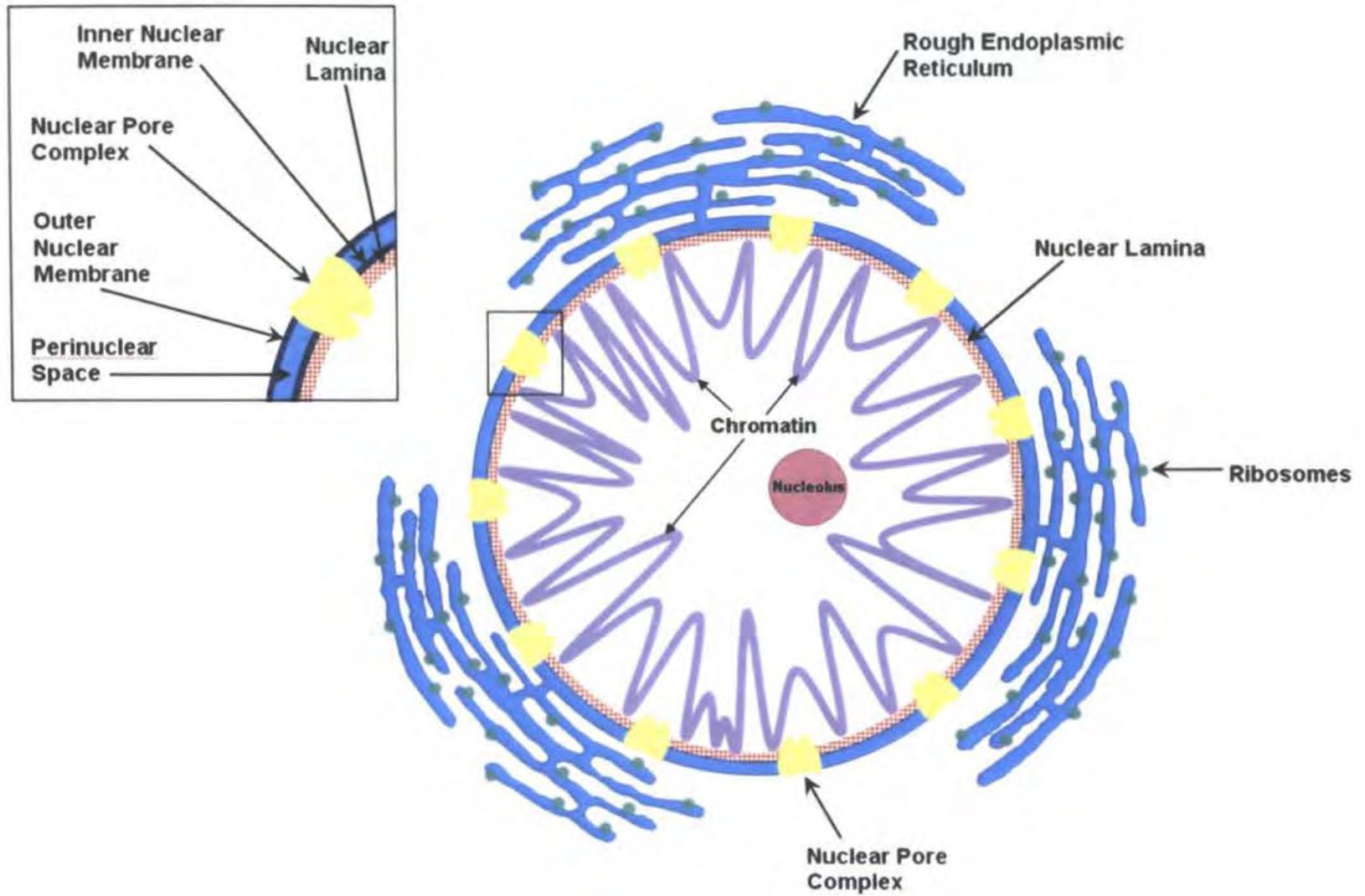
Figure 1.10 Diagrammatic representation of the major structural components of the eukaryotic nucleus

1.4.3 The Nuclear Lamina

In a manner very similar to that of the plasma membrane, the structure and stability of the nuclear envelope is responsive to a complex meshwork of intermediate filament proteins that underlie the inner nuclear membrane (Fawcett 1966; Fisher, Chaudhary et al. 1986). Collectively, the components of this meshwork are known as the nuclear lamins and the structure they form is more formally known as the nuclear lamina (NL) (Stuurman, Heins et al. 1998). The nuclear lamina was first isolated from rat liver nuclei in 1976 (Dwyer and Blobel 1976) and reports vary as to its exact thickness which can range from between 10 and 100nm (Fawcett 1966; Aaronson and Blobel 1975; Dwyer and Blobel 1976; Scheer, Kartenbeck et al. 1976; Hoger, Grund et al. 1991). Stabilisation of the nuclear lamina is mediated through complex interactions with various integral membrane proteins of the inner nuclear membrane, and the best characterised binding partners of the nuclear lamins are the lamina associated proteins (LAPs); emerin, MAN1 and lamin B receptor (Hutchison 2002).

Nuclear lamins are classified as type V intermediate filament family proteins because of their close similarities in secondary structural organisation and moreover the striking sequence homology of their characteristic α-helical rod domain, to that of other intermediate filaments within the cell such as desmin, neurofilaments and vimentin (Fisher, Chaudhary et al. 1986; McKeon, Kirschner et al. 1986).

The common secondary structure of intermediate filament proteins comprises a non-helical amino-terminal (N-terminal) head domain and a carboxyl-terminal (C-terminal) tail domain interspersed with a characteristic central coiled-coil α-helical rod domain. This rod domain is then further subdivided into four distinct helical regions, termed 1A, 1B, 2A and 2B. Each helical region is characterised by heptad repeats of apolar residues and they are separated by three non-helical linker segments (Fuchs and Weber 1994).

However, despite close similarity to vertebrate cytoplasmic filaments, the nuclear lamins differ in that they possess an extended rod domain by virtue of a 42

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amino acid (six heptad repeat) insertion within coil 1B (Erber, Riemer et al. 1998) and a somewhat shorter head domain (Fisher, Chaudhary et al. 1986). Similarly, in order to facilitate the targeting of nuclear lamins to the nucleus they also contain a nuclear localisation signal (NLS) in their tail domain (Loewinger and McKeon 1988). Other differences also include the possession of a C-terminal CaaX motif (C, Cysteine; a, any aliphatic residue; X, any amino acid) which is extremely important for localisation of the nuclear lamins at the inner nuclear membrane (Nigg, Kitten et al. 1992). Figure 1.11 shows a diagrammatic representation of the intermediate filament structure of nuclear lamins.
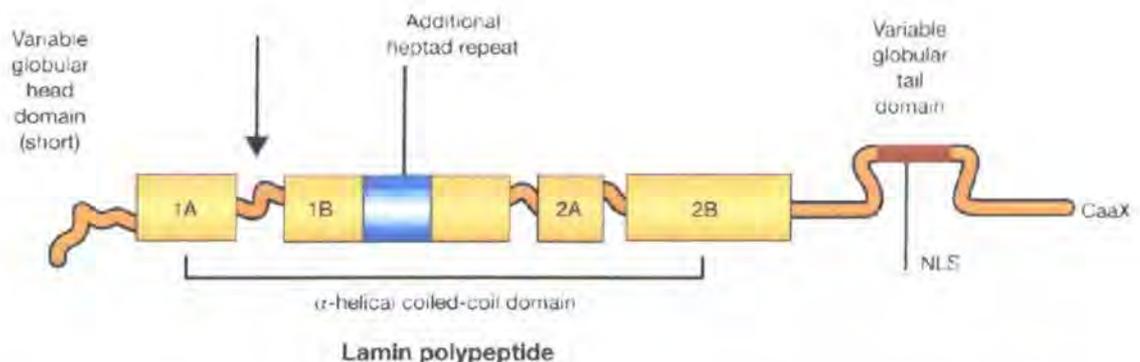
Figure 1.11 Intermediate filament structure of the nuclear lamins (Adapted from (Hutchison and Worman 2004)

If we look across the animal kingdom, there appears to be a general conservation of nuclear lamin structure across organisms. As with other protein families, one can observe an increase in the number of genes encoding nuclear lamins and also the number of splice isoforms as organism complexity increases. For example, in the nematode worm *Caenorhabditus elegans*, there is only a single lamin (LMN-1) gene (Liu, Rolef Ben-Shahar et al. 2000). In the fruit fly *Drosophila melanogastor* there are reported to be two lamin subtypes (Dm0 and lamin C) (Gruenbaum, Landesman et al. 1988; Bossie and Sanders 1993; Riemer, Stuurman et al. 1995). As we increase in complexity further, we can identify three nuclear lamins in birds (Lehner, Stick et al. 1987) and five in amphibians (Benavente, Krohne et al. 1985; Stick 1988; Hofemeister, Kuhn et al.

2002). When we look at mammalian cells, there are seven nuclear lamins in total, which are sub-divided into two groups; the A-type and B-type lamins. This sub-classification is based on their differing biochemical and physiochemical properties (i.e. isoelectric point), their ultra structural characteristics, their tissue expression specificity and mitotic fate (Gerace and Blobel 1980; Gerace, Comeau et al. 1984; Krohne and Benavente 1986; Gerace and Burke 1988; Broers, Machiels et al. 1997)

Lamins A, AΔ10, C and C2 are classified as A-type lamins and are alternative splice variants of the *LMNA* gene (Fisher, Chaudhary et al. 1986; McKeon, Kirschner et al. 1986; Furukawa, Inagaki et al. 1994; Machiels, Zorenc et al. 1996). The *LMNA* gene has been mapped and shown to be located on chromosome 1q21.1 - 21.3 (Lin and Worman 1993; Wydner, McNeil et al. 1996). Lamins B1, B2 and B3 are classified as B-type lamins. Lamin B1 is the only product of the *LMNB1* gene, located on chromosome 5q23.3 - 31.1 (Lin and Worman 1995; Wydner, McNeil et al. 1996), whilst both lamins B2 and B3 are alternative splice variants of the *LMNB2* gene (Pollard, Chan et al. 1990; Biamonti, Giacca et al. 1992; Furukawa and Hotta 1993) found on chromosome 19p13.3 (Biamonti, Giacca et al. 1992). To date, both lamin C2 and B3 have only been detected in male germ cells (Furukawa and Hotta 1993; Furukawa, Inagaki et al. 1994). Conversely, lamins A, C AΔ10 are developmentally regulated and expressed in most differentiated somatic cells (Rober, Weber et al. 1989; Machiels, Zorenc et al. 1996), with the most notable exceptions being the early embryo, embryonic stem cells, stem cells of the immune and haematopoietic systems as well as in cells of the neuroendocrine system (Goldman, Gruenbaum et al. 2002; Mounkes, Kozlov et al. 2003). Lamins B1 and B2 on the other hand are expressed in almost all cells since all vertebrate cells express at least one B-type lamin (Hutchison and Worman 2004).

The *LMNA* gene is composed of 12 exons and it is the alternative splicing of these codons which gives rise to the isoforms previously mentioned. The alternative splice site for prelamin A (the precursor to lamin A) and lamin C is located at codon 566, situated in exon 10. The alternative splicing gives rise to a common 3' end found in lamin A and Lamin C (Lin and Worman 1993) and alternate 5' ends. In the lamin C splice variant, codons 567 to 572 are lamin C

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specific, and hence transcription terminates at codon 572. The prelamin A splice variant contains the additional 743 nucleotides of exon 11 and 12 which adjoin the 3'-end of codon 566, resulting in both exons 11 and 12 being lamin A specific. The lamin AΔ10 variant also retains the lamin A specific tail domain, but consequently misses all 30 amino acids (90 nucleotides) which are encoded by exon 10 (Machiels, Zorenc et al. 1996). Thus lamin A; lamin C and lamin AΔ10 only differ in their 5' tail domains, and this alternative splicing of the *LMNA* gene is diagrammatically represented in Figure 1.12.

The main difference between A-type and B-type lamins is that A-type lamins are developmentally regulated and their synthesis appears to accompany cellular differentiation with tissue expression patterns being primarily driven by the developmental programs controlling embryogenesis and organ differentiation (Rober, Weber et al. 1989). Lamins A and C are generally not found in nascent embryonic cells, and do not become significantly expressed until after birth (Stewart and Burke 1987). In the adult, expression of lamins A and C has been reported to be completely absent in haematopoietic, immune and epithelial stem cells, however in their progeny, prelamin A expression is observed after differentiation (Rober, Gieseler et al. 1990). Interestingly though, the expression of lamin A has very recently been shown to be high in the epithelial stem cells of the colon located at the base of the intestinal crypts (Willis, Cox *et al.* unpublished data).

Conversely, B-type lamins are essential for cell survival and consequently, at least one B-type lamin is expressed at every stage of development in all cell types including adult stem cells (Stick and Hausen 1985; Lebel, Lampron et al. 1987; Stewart and Burke 1987; Paulin-Levasseur, Giese et al. 1989; Rober, Weber et al. 1989; Coates, Hobbs et al. 1996; Broers, Machiels et al. 1997; Harborth, Elbashir et al. 2001; Steen and Collas 2001).

Looking more specifically, A-type lamins are expressed predominantly in highly differentiated cell types; they are considered neutral and become soluble during disassembly of the nuclear envelope during mitotic division. This is in contrast, to the B-type lamins, which are expressed ubiquitously in all cell types, are acidic in nature and remain associated with membranous structures during lamina

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depolymerisation and mitotic breakdown of the nucleus. Such observations are clearly indicative that nuclear lamins have a broad spectrum of tissue specific effects, the implications of which are not as yet fully understood.

The *LMNA* gene encodes the lamin A, C and AΔ10 splice variants
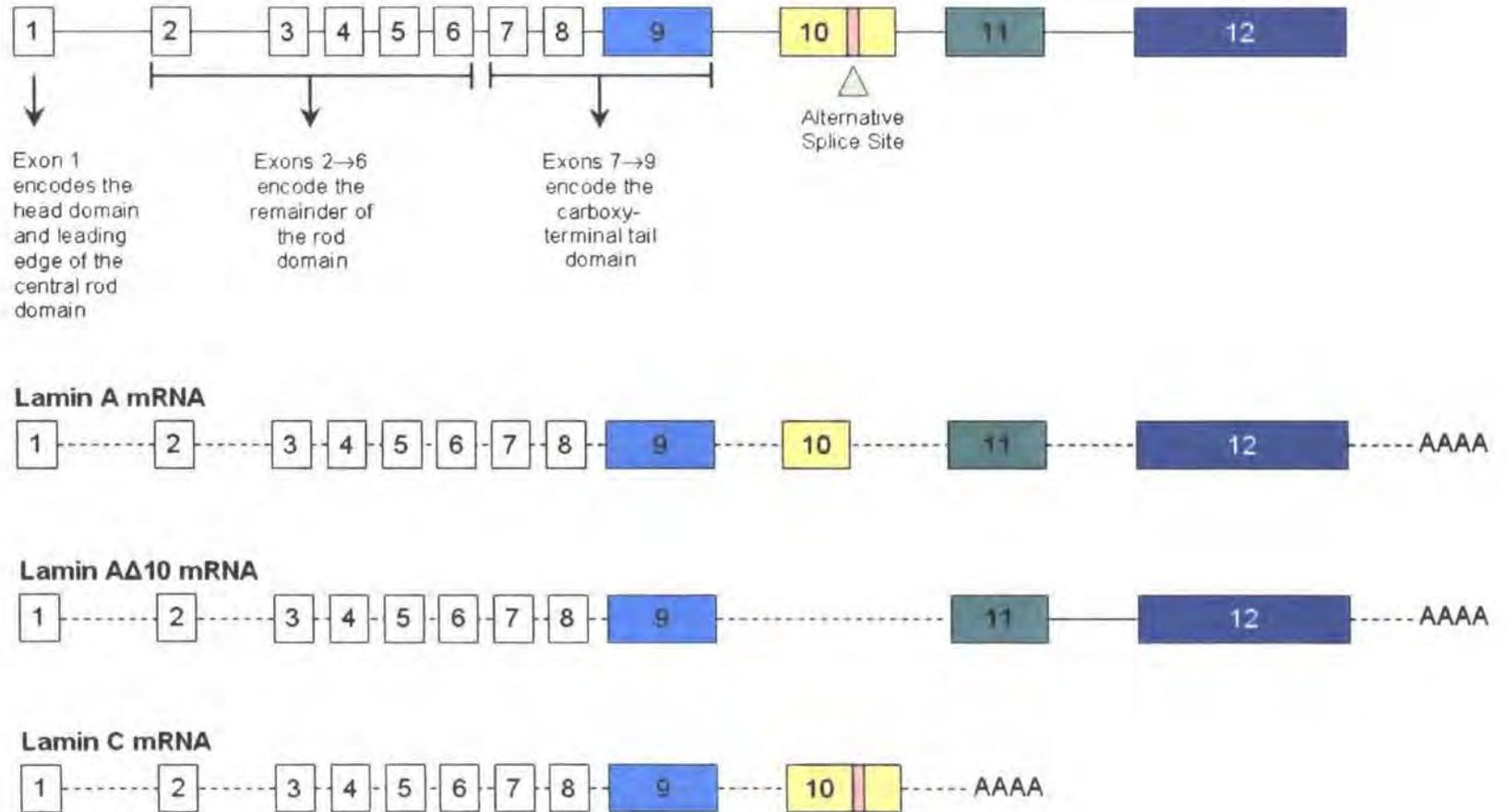
Figure 1.12 Splice variants of the LMNA gene (modified from (Willis 2005)

1.4.4 Nuclear Envelope Dynamics

During cell division the process of mitosis in vertebrate cells is considered 'open'. That is to say that there is a complete disassembly of the nuclear envelope and reformation around the two sets of daughter chromosomes. In 'closed' mitosis, the nuclear envelope remains intact and chromosomes migrate to opposite poles of a spindle within the nucleus. During this process of open mitosis, the entire nuclear lamina and associated nuclear pore complexes must reversibly disassemble and this is first observed in prometaphase (Georgatos, Pyrpasopoulou et al. 1997). The highly coordinated step-wise reassembly of the nuclear lamina then occurs during late anaphase/telophase (Chaudhary and Courvalin 1993).

1.4.4.1 Filament behaviour and assembly

Models of nuclear lamina assembly have been proposed and modified over the past two decades based on both *in vitro* and *in vivo* observations. The formation of lamin filaments is thought to result from the following series of sequential steps. First, obligate parallel unstaggered dimers form through interaction at the coiled-coil interface involving the α-helical central rod domains, similar to that observed in cytoplasmic intermediate filament assembly (Aebi, Cohn et al. 1986; Heitlinger, Peter et al. 1991; Heitlinger, Peter et al. 1992). Subsequently, lamin dimers associate head-to-tail and then in anti-parallel geometry to form higher-order intermediate filaments having a diameter of between 10-13 nm (McKeon, Kirschner et al. 1986). Unlike vertebrate cytoplasmic intermediate filaments however, lamin filaments then extend longitudinally to form long protofilaments before arranging by lateral associations to produce the two-dimensional matrix which underlies the inner nuclear membrane and interconnects the nuclear pore complexes (Aebi, Cohn et al. 1986; Hutchison 2002).

Evidence to support this was observed *in vitro*, where lateral growth of protofilaments resulted in the formation of paracrystals with a 24-25 nm transverse banding repeat. This was interpreted to represent the anti-parallel, half-staggered associations of lamin head-to-tail polymers (protofilaments) which gives rise to ~10nm wide mature filaments. So far, the higher-order organisation

of these filaments has only been elucidated in *Xenopus* oocytes where they were observed to be arranged in a two-dimensional orthogonal lattice with an average crossover spacing of 52nm (Aebi, Cohn et al. 1986; Heitlinger, Peter et al. 1991; Moir, Donaldson et al. 1991; Heitlinger, Peter et al. 1992; Stuurman, Heins et al. 1998; Goldberg, Harel et al. 1999).

In 2001, Hutchison and co-workers (Hutchison, Alvarez-Reyes et al. 2001) proposed a model by which lamins A and C are incorporated into the nuclear lamina once B-type lamins have associated with the inner nuclear membrane. Since lamin C does not possess an isoprenylation signal, it was therefore suggested that it becomes incorporated into the nuclear lamina on the back of lamin A. It was also proposed that lamin A and lamin C form anti-parallel, half-staggered tetramers which can then make head-to-tail associations with lamin B tetramers already associated subjacent to the inner nuclear membrane. At the same time, Vaughan an co-workers (Vaughan, Alvarez-Reyes et al. 2001) reported that lamin C and emerin are both dependent on lamin A for localisation at the nuclear envelope, and went on to postulate that the presence of lamin C and emerin may be essential to further stabilise the nuclear lamina (Vaughan, Alvarez-Reyes et al. 2001).

1.4.5 Functions of the Nuclear Lamina

The nuclear lamina is responsible for tethering chromatin to the nucleoplasmic face of the inner nuclear membrane hence maintaining overall nuclear architecture (Gotzmann and Foisner 1999). Recently it has also become known as a tensegrity element since it maintains the overall stability of the nucleus by resisting tensile and mechanical deformation exerted on the cell (Hutchison and Worman 2004). At the same time, correct localisation of lamins to the nuclear lamina is also thought to be important for the assembly and spatial organisation of the nuclear pore complexes (Lenz-Bohme, Wismar et al. 1997; Stuurman, Heins et al. 1998; Liu, Rolef Ben-Shahar et al. 2000) and more specifically the recruitment of nucleoporin NUP153 (Smythe, Jenkins et al. 2000), one of the key proteins which makes up the terminal ring of the nuclear pore basket [reviewed by (Bagley, Goldberg et al. 2000)].

A rapidly increasing number of proteins have also been shown to directly interact with lamins and the nuclear lamina. Examples of the most common proteins that interact at the inner nuclear membrane and with the nuclear lamina are chromatin (Heessen and Fornerod 2007), lamina-associated polypeptides (LAP's), Lamin B Receptor (LBR), Otefin, MAN1, Emerin, Nesprin, barrier-to-autointegration factor (BAF) (Schirmer and Foisner 2007), and the transcription factors retinoblastoma (Rb), sterol regulatory element binding protein (SREBP-1), Germ-cell-less (Gcl), and Oct-1 (Heessen and Fornerod 2007). Such work is providing strong evidence that the function of lamins and the nuclear lamina is much more dynamic and linked to a much wider range of physiological processes than that of the more traditional static structural role (Goldman, Gruenbaum et al. 2002).

Hence at present, the nuclear lamina as well as acting as the inner nuclear membrane scaffold has been shown to mediate chromatin organisation and condensation (Paddy, Belmont et al. 1990), nuclear assembly and disassembly (Ulitzur, Harel et al. 1992), DNA synthesis (Spann, Moir et al. 1997), transcriptional control (Mancini, Shan et al. 1994; Dreuillet, Tillit et al. 2002; Kumaran, Muralikrishna et al. 2002; Lloyd, Trembath et al. 2002) and apoptosis (Rao, Perez et al. 1996; Zhivotovsky, Gahm et al. 1997), although one would expect to see this list extend further in the coming years. The complexity of interactions occurring between the nuclear lamina, scaffolding proteins, chromatin, transcription factors, and other proteins have profound implications on not only the overall structural integrity of the cell, but also cell-cycle control, DNA replication, and regulation of gene expression (Hutchison and Worman 2004).

1.4.5.1 The Nuclear Lamina and DNA Replication

Early research has shown that cells which are depleted in functional B-type lamins are unable to support DNA replication (Newport, Wilson et al. 1990; Meier, Campbell et al. 1991). However, it was not until 1995 that evidence was published showing that lamins directly influence DNA replication (Goldberg, Jenkins et al. 1995). Research showed that upon addition of purified lamin B3 (Liii) to depleted extracts of a *Xenopus* nuclear assembly system, there was a

reinitiation of previously halted DNA replication. What is also clear is that the absence of a functional lamina leads to extreme mechanical fragility of the nuclear envelope (Newport, Wilson et al. 1990; Liu, Rolef Ben-Shahar et al. 2000) which subsequently results in nuclear deformations, such as invaginations, herniations and a greatly reduced resistance to mechanical pressure (Broers, Peeters et al. 2004)

1.4.5.2 The Nuclear Lamina and Transcriptional Regulation

It is thought that the nuclear lamina and lamins in particular influence gene transcription directly through interaction with transcriptional machinery, chromatin and transcriptional repressors. That is not to say that they always bind directly to such factors, but more often than not do so through their binding partners which associate directly with the chromatin or transcriptional components.

The main lamin-interacting proteins integral to the inner nuclear membrane are the so-called LEM domain proteins; LAP2, Emerin and MAN1. The LEM domain is a characteristic 43 amino acid sequence in the N-terminal domain of these proteins (Cohen, Lee et al. 2001) although at present there has been no conclusive evidence of direct interaction between lamins and MAN1. In his review, Foisner (Foisner 2001) nicely covers lamin interactions at the INM and interactions with peripheral chromatin.

However, in a manner seemingly independent of lamins, LAP2β interacts with chromatin via BAF (barrier to autointegration factor) and its LEM domain (Furukawa 1999). It is also capable of binding the transcription factor Gcl (germ-cell-less), a peripheral inner nuclear membrane protein, and is also able to repress the activity of the E2F-DP transcriptional complex, which is known to be under tight control by the retinoblastoma protein (pRb) (Nili, Cojocaru et al. 2001). LAP2α on the other hand does not directly integrate into the inner nuclear membrane (Berger, Theodor et al. 1996; Dechat, Gotzmann et al. 1998). Instead it is capable of binding chromatin and has also been shown to target lamins A/C to intranuclear sites during interphase where the two proteins then form tight complexes which have consequently been implicated in the structural

organisation of the nucleus (Dechat, Gotzmann et al. 1998; Dechat, Korbei et al. 2000).

Emerin is definitely one of the most interesting integral membrane protein targets of lamin A/C, despite the fact that its exact function is still unknown. It binds both lamins A/C and BAF (Lee, Haraguchi et al. 2001) which directly constitutes it a functional link with chromatin. It has also been shown more recently to bind β-catenin through its APC-like tail domain, and overexpression results in an inhibition of the β-catenin signalling pathway by preventing nuclear accumulation (Markiewicz, Tilgner et al. 2006). The localisation of Emerin to the inner nuclear membrane is also closely dependent on the availability of lamin A polymers (Vaughan, Alvarez-Reyes et al. 2001) and it is found redistributed throughout the cytoplasm, when the nuclear lamina fails to form (Gruenbaum, Lee et al. 2002).

It has been shown that the inner nuclear membrane protein MAN1 regulates the nuclear translocation of the TGF-β effecter, rSMAD. The result of this is the subsequent antagonising of the BMP signalling pathways (Osada, Ohmori et al. 2003; Pan, Estevez-Salmeron et al. 2005), which are heavily involved in many developmental pathways.

When considering the concept of cancer, it is clear that many proteins which control cell proliferation and survival are regulated by the rate at which they accumulate in the nucleus, and disruption of this balance (as in the case of constitutive β-catenin activation in colorectal carcinogenesis) leads to the initiation and development of cancer. The nuclear accumulation of pRb, rSMAD and β-catenin (which are all implicated in controlling cell proliferation) have been shown to be mediated by proteins of the inner nuclear membrane. The tumour suppressor pRb is anchored in the nucleus by lamins A/C via LAP2α (Markiewicz, Dechat et al. 2002). Under normal conditions, hypophosphorylated pRb represses the transcription of genes which typically push cells from G1 phase into S phase. Concomitantly, it also activates genes which promote cellular differentiation through negative regulation of the E2F transcriptional complexes (Chellappan, Hiebert et al. 1991; Korenjak and Brehm 2005). This occurs through tethering of pRb at the nuclear envelope in the manner mentioned earlier (Markiewicz, Dechat et al. 2002). β-catenin and rSMAD

nuclear accumulation is regulated by the presence of Emerin and MAN1 at the inner nuclear membrane (Osada, Ohmori et al. 2003; Pan, Estevez-Salmeron et al. 2005; Markiewicz, Tilgner et al. 2006), thus inhibiting the signal transduction pathways in which they are involved and preventing expression of either the Wnt or BMP target genes. Such evidence strongly suggests that there may be a mechanism by which A-type lamins are heavily implicated in tumour initiation and development.

Finally, it has also been proposed that lamins may regulate gene transcription through direct modulation of RNA synthesis. Experiments have shown that the addition of mutant lamin A lacking its globular head domain to transcriptionally active *Xenopus* nuclei causes disintegration of the nuclear lamina and specifically halted the activity of RNA polymerase II (Spann, Goldman et al. 2002).

1.4.5.3 Nucleoplasmic-Cytoplasmic interactions

The rapid response of an individual cell to stimuli, either mechanical or biochemical is crucial for cellular functioning. Within the cell, a large network of physically interconnected cellular components, which starts with the structural components of the nuclear lamina, and via cytoskeletal filaments to adhesion molecules and the extracellular matrix, constitutes an integrated matrix to elicit such responses. Aside from an obvious mechanical role, this network also has a mechanotransductional function in the response to mechanical stress. This signalling route operates not only to regulate a rapid reorganisation of structural components such as actin filaments, but also stimulates for example gene activation via NFκB and other transcription factors.

Over the last few years a novel family of spectrin-repeat proteins has been elucidated and described. Many of the members of this family are integral proteins of the inner nuclear membrane, whilst others are shown to specifically localise to the outer nuclear membrane. The family of proteins are either known as nesprins (Zhang, Skepper et al. 2001), NUANCE (NUcleus and ActiN Connecting Element) (Zhen, Libotte et al. 2002) or Synes (Apel, Lewis et al. 2000). The family of proteins is typically characterised by the giant size of some

of the alternative splice variants (it has been reported that they may have relative molecular masses in excess of 800,000 Da). They also all possess multiple clustered spectrin repeats throughout the core of the protein, amino-terminal calponin homology domains and a conserved C-terminal single pass membrane domain.

NUANCE has been shown to bind to actin and its distribution is thus influenced by the actin cytoskeleton (Zhen, Libotte et al. 2002). *In vitro*, nesprin 1α binds directly to lamins A/C and to emerin at the inner nuclear membrane (Mislow, Holaska et al. 2002b). Thus its localisation at the inner nuclear membrane is dependent on the expression of lamins A/C (Muchir, van Engelen et al. 2003). Nesprins have also been shown to self associate (Zhang, Skepper et al. 2001). In addition, emerin, which directly binds to lamins A/C *in vitro* and is subsequently localized to the inner nuclear membrane is also an actin-binding protein (Bengtsson and Wilson 2004). Thus, lamins A and C can assemble a complex of proteins at the nuclear envelope that have characteristics of cytoskeleton linker proteins (Maatta, Hutchison et al. 2004) and potentially link the actin cytoskeleton to the lamina.

1.4.5.4 The Nuclear Lamina and Apoptosis

When considering any type of cancer it is of particular interest that nuclear lamins have been postulated to be involved in the regulation of apoptosis, a key functional target of tumourigenesis. The classical morphological features of apoptosis include the appearance of highly condensed chromatin, the clustering of nuclear pore complexes and membrane blebbing. Interestingly, all of these are commonly observed in lamin-deficient nuclei (Sullivan, Escalante-Alcalde et al. 1999; Tzur, Hersh et al. 2002). At the same time, it has been shown that caspase induced cleavage of lamins is one of the earliest events associated with apoptosis (Lazebnik, Takahashi et al. 1995), and precedes DNA fragmentation (Rao, Perez et al. 1996; Takano, Takeuchi et al. 2002) and degradation of other inner nuclear membrane-associated proteins (Duband-Goulet, Courvalin et al. 1998; Buendia, Courvalin et al. 2001). It has also been shown that failure to assemble B-type lamins correctly will also directly trigger apoptosis (Steen and Collas 2001).

1.4.6 Lamins in Disease

To date, there are several different disease phenotypes associated with altered nuclear envelope function, and these are collectively referred to as laminopathies (Burke and Stewart 2002; Mounkes, Kozlov et al. 2003). Most of the laminopathies discovered up to now have been mapped to the *LMNA* locus and subsequently nuclear lamins have become a hot topic of research. The biggest reason behind this is that mutations in the *LMNA* gene can affect cardiac, muscular, skeletal, neural, or adipose tissues, or any combination of these (Hutchison, Alvarez-Reyes et al. 2001). More specifically, mutations in the *LMNA* gene are associated with at least 9 different well-characterised human diseases including the autosomal dominant (AD) form of Emery-Dreifuss muscular dystrophy (EDMD) (Bonne, Di Barletta et al. 1999), dilated cardiomyopathy type 1A (CMD-1A) with conduction system defects (Fatkin, MacRae et al. 1999), limb-girdle muscular dystrophy 1B with atrioventricular conduction disturbances (LGMD1B) (Muchir, Bonne et al. 2000), Dunnigan-type familial partial lipodystrophy (FPLD) (Shackleton, Lloyd et al. 2000), Charcot-Marie-Tooth disorder type 2B1 (CMT2B1) (De Sandre-Giovannoli, Chaouch et al. 2002), mandibuloacral dysplasia (MAD) (Novelli, Muchir et al. 2002), restrictive dermopathy (RD) (Navarro, De Sandre-Giovannoli et al. 2004), atypical Werner's syndrome (Chen, Lee et al. 2003) and Hutchison-Gilford progeria syndrome (HGPS), a premature aging disorder that affects all tissues and results in premature death (Eriksson, Brown et al. 2003; Goldman, Shumaker et al. 2004). This does not include laminopathies caused as a result of mutations in lamin associated proteins such as mutations in the *STA* gene encoding Emerin which causes the muscle wasting disease X-linked Emery-Dreifuss muscular dystrophy (Bione, Maestrini et al. 1994) and which exhibits very similar clinical features to AD-EDMD. Below I briefly outline some of the major laminopathies and conclude with the role of lamins in cancer.

1.4.6.1 Emery-Dreifuss Muscular Dystrophy (EDMD)

There are currently three diagnosable forms of Emery-Dreifuss Muscular Dystrophy, autosomal dominant (AD), autosomal recessive (AR) and X-linked (XL). Both the autosomal dominant and autosomal recessive forms are due to

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mutations in the *LMNA* gene (Bonne, Di Barletta et al. 1999; Raffaele Di Barletta, Ricci et al. 2000). As already mentioned the X-linked form is caused by a loss of function mutation in Emerin (Wulff, Ebener et al. 1997). All three forms display a very similar triad of clinical features, including early contractures of the elbows, Achilles tendons and posterior cervical muscles; progressive weakness and wasting of skeletal muscles, particularly humeral and perineal muscles; and cardiac conduction defects (cardiomyopathy) which often results in sudden heart failure (Dreifuss and Hogan 1961; Emery and Dreifuss 1966; Miller, Layzer et al. 1985).

The locus for AD-EDMD was mapped to chromosome 1q11-q23, a region which contains the *LMNA* gene. It was found that mutations in the *LMNA* gene co-segregated with the disease in five families and this became the first inherited disorder documented due to mutations in a component of the nuclear lamina (Bonne, Di Barletta et al. 1999). The exact mutation can vary between patients with mutations being found in the head, rod and tail domains (Raffaele Di Barletta, Ricci et al. 2000). Incidentally, to date, only one case of EDMD has been reported to involve both *LMNA* alleles (Raffaele Di Barletta, Ricci et al. 2000).

The X-linked form of Emery-Dreifuss Muscular Dystrophy is a recessive form but shows 100% penetrance in the third decade of life. It was first identified and reported in 1961 (Dreifuss and Hogan 1961) and then again in 1966 (Emery and Dreifuss 1966). The locus has been mapped to chromosome Xq28 (Bione, Maestrini et al. 1994), which contains the *STA* gene (also known as the *EMD* gene) encoding Emerin. So far there have been no identifiable mutation hotspots within the gene. Early research revealed five unique mutations resulting in loss of function or truncation of the protein (Bione, Maestrini et al. 1994). Later, a further six mutations were identified at novel locations along the gene (Wulff, Parrish et al. 1997). However, what they do have in common is that the vast majority are associated with the lamin A binding domain and hence disrupt lamin A – Emerin interactions (Lee, Haraguchi et al. 2001). This was also shown by evidence of a weakened interaction of mutant emerin with the nuclear lamina (Ellis, Yates et al. 1999).

1.4.6.2 Limb Girdle Muscular Dystrophy 1B (LGMD-1B)

LGMD-1B is an autosomal dominant disease which is associated with progressive muscle wasting and cardiac conduction defects (van der Kooi, Ledderhof et al. 1996). Clinical manifestations include a symmetrical weakness in proximal lower limb muscles which begins before the age of 20, progressing to upper limb muscles at about 30-40 years of age. Generally, the phenotype is considered to be somewhat milder than EDMD and patients tend to experience a reduced rate of contractures. However, in a manner similar to EDMD, cardiological abnormalities are the most life-threatening symptom. The disorder was mapped to chromosome 1q11-q21 (van der Kooi, van Meegen et al. 1997) and subsequently mutations in the LMNA gene have been identified in three LGMD-1B families (Muchir, Bonne et al. 2000).

1.4.6.3 Dilated Cardiomyopathy 1A (CMD-1A)

Dilated cardiomyopathies are somewhat different to the two diseases already discussed in that they primarily affect cardiac function and exhibit minimal skeletal muscle involvement (Olson and Keating 1996). Acute and sudden heart failure is the primary cause of death in these patients usually in the fourth decade of life. It has also been shown that mutations in the LMNA gene account for the autosomal dominant form and these mutations typically occur within the coiled-coil regions; 1A and 1B of the rod domain (Fatkin, MacRae et al. 1999; Brodsky, Muntoni et al. 2000). Interestingly it has been shown that lamin A and lamin C can be affected independently of one another, with the R571S mutation substituting an arginine residue in the lamin C specific carboxy terminus (Bonne, Mercuri et al. 2000), and the R644C mutation also substituting an arginine residue, but in the lamin A specific tail domain (Genschel, Bochow et al. 2001).

1.4.6.4 Dunnigan type – Familial Partial Lipodystrophy (FPLD)

Familial Partial Lipodystrophy is a family of diseases within which only one form (Dunnigan type) has currently been directly associated with mutations in the LMNA gene. The clinical manifestations of FPLD usually arise at the onset of

puberty and are typically characterised by abnormal distribution of subcutaneous fat. In Dunnigan type – FPLD, adipose tissue progressively disappears (lipoatrophy) from upper and lower extremities, gluteal and truncal areas and begins to accumulate on the face, neck, back and groin (Dunnigan, Cochrane et al. 1974). As a result of this, patients generally develop insulin resistance. The disease has been mapped to chromosome 1q21 and more specifically mis-sense mutations in the *LMNA* gene have been identified in five Canadian families (Cao and Hegele 2000). The mutations responsible for causing Dunnigan type – FPLD are mostly distributed on the surface of the C-terminal globular Ig domain and therefore do not appear to affect lamin structure (Hegele, Anderson et al. 2000; Speckman, Garg et al. 2000). Instead they result in nuclear lamina fragility and disorganisation, which subsequently results in the nuclear envelope herniations typically seen in lipodystrophic patients (Vigouroux, Auclair et al. 2001).

1.4.6.5 Mandibuloacral Dysplasia (MAD)

Mandibuloacral Dysplasia is an autosomal recessive disease and only homozygous patients show the classical clinical features associated with the disease. Such clinical features include; postnatal growth retardation; craniofacial malformations including a hypoplastic mandible producing severe dental crowding; skeletal abnormalities including hypoplastic clavicles; atrophy of the skin over hands and feet with mottled cutaneous pigmentation; alopecia; and partial lipodystrophy of either pattern A or B, often accompanied by extreme insulin resistance (Young, Radebaugh et al. 1971; Cutler, Kaufmann et al. 1991; Simha and Garg 2002). To date, only MAD patients which exhibit type A lipodystrophy have been found to harbour the homozygous mutation, R527H in the lamin A/C tail domain (Novelli, Muchir et al. 2002; Simha, Agarwal et al. 2003) which appears to affect normal distribution of lamins A/C at the nuclear envelope resulting in lobulations and a 'honey-comb' appearance of the nuclear lamina (Novelli, Muchir et al. 2002).

1.4.6.6 Charcot-Marie-Tooth type 2B1 (CMT2B1)

Charcot-Marie-Tooth diseases are an autosomal recessive group of disorders which typically manifest in the second decade of life and appear clinically and

genetically heterogeneous, affecting both motor and sensory nerves, and in particular nerve conduction velocity. Clinically CMT type 1 (CMT1) is a demyelinating form of the disease, whereas CMT type 2 (CMT2) of which there are two types CMT2B1 and CMT2B2, is an axonal disruption form. In separate studies on consanguineous families with CMT2B1, the disorder was directly mapped to chromosome region 1q21 which harbours the *LMNA* gene (Bouhouche, Benomar et al. 1999). A unique homozygous mutation was then later identified in the rod domain of lamin A/C in three consanguineous Algerian families (De Sandre-Giovannoli, Chaouch et al. 2002).

1.4.6.7 Hutchinson-Gilford Progeria Syndrome (HGPS)

Hutchinson-Gilford Progeria Syndrome is a rare premature ageing syndrome affecting approximately 1 in 8 million people but which can occur through both consanguineous and non-consanguineous unions suggesting both an autosomal recessive and autosomal dominant mode of inheritance. Clinical features associated with HGPS include growth retardation, abnormal dentition, loss of subcutaneous fat, alopecia, reduced bone density, incomplete sexual maturation and poor muscle development (Mounkes, Kozlov et al. 2003). Patients suffer from atherosclerosis and typically die of congestive heart failure in the first or second decade of life. The HGPS causing gene was initially mapped to chromosome 1q and then to the *LMNA* gene and a *de novo* single-base substitution affecting codon 608 (GGC → GGT) was identified as the major cause in 18 of 20 patients with classic HGPS (Eriksson, Brown et al. 2003). This substitution does not result in an amino acid change in the lamin A specific exon 11, but is responsible for partial activation of a cryptic splice site. Activation of this cryptic splice site results in a 50aa deletion at the C-terminal end of lamin A. This effectively removes the endoproteolytic cleavage site and the site for cell-cycle-dependent phosphorylation (Serine 625). The result of this is that post-translational modification of prelamin A to mature lamin A cannot occur and it is thought that the incompletely processed lamin A may act as a dominant negative mutant at the nuclear envelope (Eriksson, Brown et al. 2003).

1.4.6.8 Werner Syndrome

Werner syndrome is a very rare autosomal recessive disorder which is most recognisable for its characteristic premature aging symptoms. The syndrome clinically comprises features such as premature atherosclerosis, scleroderma-like skin changes and subcutaneous calcification (McKusick 1963). Atypical Werner Syndrome has a much more severe phenotype than the 'typical' form. Homozygous mutations in the DNA helicase-like gene *RECQL2* are typically the cause, although four atypical Werner Syndrome patients were found to express normal *RECQL2*, but were heterozygous for novel mis-sense mutations in the *LMNA* gene (Chen, Lee et al. 2003). Thus in a small subset of cases, atypical Werners Syndrome is characterised by the autosomal dominant inheritance of *LMNA* mutations which result in nuclear abnormalities, and may subsequently affect *RECQL2* function.

1.4.7 Structural and Gene Regulatory roles in laminopathies

There is still a great deal of controversy which surrounds the exact function of the nuclear lamina, especially in the case of disease. Early hypotheses stated that its functions were merely architectural although as I have discussed, there is a vastly increasing plethora of evidence that links its role to the regulation of gene transcription as well. Currently, there are two major hypotheses that attempt to explain the mechanisms responsible for A-type lamin associated diseases: nuclear fragility and global and/or specific gene expression changes (Burke and Stewart 2002; Mounkes, Kozlov et al. 2003).

A structural model in which loss of correct lamin function causes nuclear fragility is an attractive explanation for phenotypes associated with skeletal muscle and heart tissue disorders, such a Emery-Dreifuss Muscular Dystrophy, Limb-Girdle Muscular Dystrophy, and Dilated Cardiomyopathy. In such a model the nuclear lamina maintains the structural integrity of the nucleus and resists shearing stress exerted by mechanical use of the muscle which would eventually lead to damage of the nucleus and ultimately cell death (Hutchison, Alvarez-Reyes et al. 2001; Goldman, Gruenbaum et al. 2002).

However, this shear stress model fails to explain lamin-associated lipodystrophies, for example the tissue pathology of Dunnigan type – Familial Partial Lipodystrophy, a phenotype that affects the function of adipocytes or Charcot-Marie-Tooth type 2B1 which affects the function of neurons, both of which are tissues that do not experience anywhere near the same amount of repetitive mechanical stress as muscle. A more plausible model to explain the wide variety of tissue specific pathologies associated with mutations in just one protein is the gene-expression model. That is to say that A-type lamins and loss of correct function thereof must impact on other important functions of the nuclear lamina, other than, or as well as maintenance of nuclear architecture.

As I have already mentioned, lamin A has been found to directly interact with a variety of gene-regulatory factors, including SREBP1 (Lloyd, Trembath et al. 2002), MOK2 (Dreuillet, Tillit et al. 2002), pRb (Mancini, Shan et al. 1994) and BAF (Gotzmann and Foisner 1999). Using a specific example of this hypothesis, SREBP1 is a transcription factor that is known to activate genes involved in cholesterol biosynthesis, adipocyte differentiation, and lipogenesis (Kim and Spiegelman 1996). It has also been shown to directly interact with the globulin like tail of lamin A (Lloyd, Trembath et al. 2002). Thus mutations in lamin A that cause Familial Partial Lipodystrophy bind SREBP1 with approximately 25-40% less affinity (Lloyd, Trembath et al. 2002) and it therefore becomes attractive to link FPLD to changes in adipocyte function due to genes affected by altered SREBP1 function.

In addition to binding to specific gene regulatory factors, nuclear lamins and lamin A in particular can also interact directly with naked DNA (Luderus, den Blaauwen et al. 1994), histones (Taniura, Glass et al. 1995), and is thought to play a role in the organisation of heterochromatin (Kourmouli, Theodoropoulos et al. 2000). Gross disruptions in the organisation of chromatin, which is found in cells from Emery-Dreifuss Muscular Dystrophy patients, has the potential to alter global expression patterns of hundreds, or possibly thousands of genes (Ognibene, Sabatelli et al. 1999).

Recently a third model has also been proposed suggesting that malfunctions in the nuclear lamina and nuclear envelope may cause perturbations in cytoskeletal-nuclear interactions through disruption of for example the

microtubule-lamina interaction as mediated by proteins such as SUN (Sad1 / UNC-84 homology) domain proteins which transverse the nuclear envelope. Such a hypothesis postulates that the cytoskeleton and nuclear lamina are hardwired together providing a device for transducing mechanical stress sensing from the plasma membrane to the nucleus.

1.4.8 Lamins in Cancer

The expression of nuclear lamins has previously been described in various types of cancer and varies widely depending on the subtype of cancer, its aggressiveness, proliferative capacity and degree of differentiation. Cancers affecting epithelial, lymphoid and mesenchymal tissues, including basal skin cell carcinoma (BCC) (Venables, McLean et al. 2001; Tilli, Ramaekers et al. 2003), non-small cell lung cancer (Kaufmann, Mabry et al. 1991; Broers, Raymond et al. 1993), non-Hodgkin's lymphoma, acute lymphoblastic leukaemia, (Stadelmann, Khandjian et al. 1990), metastatic chondrosarcoma, rhabdomyosarcoma and leiomyosarcoma (Cance, Chaudhary et al. 1992) have been well documented in current literature. In general, the expression of A-type lamins (lamins A and C) has been correlated with a non-proliferating, differentiated state of cells and tissues (Tilli, Ramaekers et al. 2003).

At present there have been two previous studies which have investigated the differential expression and changes in lamins A/C and lamin B1 in colorectal neoplasms. Their findings however have been somewhat contradictory. Cance and co-workers (Cance, Chaudhary et al. 1992) reported that there was a constitutive expression of lamin B accompanied to a heterogeneous expression of lamins A/C in two colon adenocarcinomas, determined by immunohistochemical analysis. Moss and colleagues (Moss, Krivosheyev et al. 1999) on the other hand reported that there was a reduced nuclear immunostaining for lamins A/C in colon adenomas and adenocarcinomas, compared to normal tissue. In their investigations, Moss and co-workers evaluated a total of 17 formalin-fixed paraffin embedded tumour specimens from patients; 3 from Dukes' A patients, 3 from Dukes' B patients and 11 from Dukes' C patients. They report that in all 17 cases there was "reduced or absent" lamin A/C expression, which they classed as either 0 (no staining), 1 (weak staining) or

2 (moderate staining) on a 4 point scale (3 being classed as very strong, intense staining). They also report levels of heterogeneous non-specific cytoplasmic staining which is ultimately due to the use of their polyclonal antisera. Whilst polyconal antisera offer a cheap and more readily available method of antigen detection, they lack the homogeneity and consistency of their monoclonal counterparts, and batches vary dramatically unlike the limitless supply of a monoclonal equivalent. The heterogeneity of polyclonal antisera means they often recognise a host of antigenic epitopes both specific and non-specific which is most likely what is observed by Moss and co-workers in their paper. Interestingly, they make no mention of the use of internal controls to validate absent staining as a lack of antigen as opposed to poor staining.

Moss and co-workers were unable to support their data in further immunoblotting studies, but this may be due to the heterogeneity of tissue in samples used, which can realistically only be overcome by laser micro-dissection or enzymatic separation. They speculate that reduced lamin A/C expression is not related to Dukes' or differentiation status, but due to a very small sample size (n=17) it is not surprising that no correlation could be found. Their final conclusions suggest that a lack of lamin A/C expression in tumours may be a marker of malignancy; however they did not investigate lamin A/C expression in relation to patient outcome, disease free survival or any other clinicopathological variables (Moss, Krivosheyev et al. 1999).

In basal skin cell carcinomas, Venables (Venables, McLean et al. 2001) correlated a down-regulation of lamin A with a higher proliferation index and the loss of lamin C with slower growing tumours. However this was not supported in later work by Oguchi's team (Oguchi, Sagara et al. 2002) in which no relationship between the loss of lamin A expression and increased proliferation was observed. Instead it was shown that such a loss of lamin A was associated instead with a poor differentiation status of the tumour. This uncertainty in the link between lamin A/C expression and cellular proliferation was further highlighted by analyses of keratinocyte tumours carried out by Tilli (Tilli, Ramaekers et al. 2003). Here they showed that in basal cell carcinomas and squamous cell carcinomas, lamin A/C staining was positive accompanied by a heterogenous expression in the suprabasal and strong staining in the basal

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epidermis. However lamin A/C expression only correlated with Ki-67 staining (a proliferative index marker) in 50% of tumour cells.

On a slightly different note, Agrelo (Agrelo, Setien et al. 2005) has shown in studies that CpG Island promoter methylation of the *LMNA* gene not only correlated with loss of RNA and protein in leukaemia and lymphoma malignancies but that it is a significant predictor of poor outcome in nodal diffuse large B-cell lymphomas.

Interestingly, a class of drugs currently used to treat some forms of cancer, known as farnesyl transferase inhibitors (FTIs), has been shown reverse the phenotype of Hutchinson-Gilford Progeroid cells grown *in vitro* (Mallampalli, Huyer et al. 2005) and also in mouse model systems (Fong, Frost et al. 2006). FTIs were originally developed to block the farnesylation of *ras*, a CaaX protein that is mutated and hyperactive in a number of cancers. This is of interest since Lamin A also requires post-translational farnesylation to be incorporated into the nuclear membrane. By blocking the first step in processing of the faulty lamin in HGPS patients with FTIs, it can in part reverse some of the disease symptoms. Work such as this draws not only strong links between disease such as cancer and premature ageing syndromes, but fundamentally supports the notion that the A-type lamins can be considered as 'Guardians of the soma' (Hutchison and Worman 2004).

1.5 Thesis Aims

Changes in lamin expression and their effects on patient mortality have been reported in a small number of cancers, but as yet no large scale studies have been carried out in colorectal cancer (Cance, Chaudhary et al. 1992; Moss, Krivosheyev et al. 1999). Similarly the exact nature of how alterations in lamin expression affect the biological behaviour of tumours is not clear. Given the importance of A-type lamins and their binding partners to the regulation of growth pathways, it has been speculated that these lamins might be linked to tumour progression (Burke and Stewart 2006). The availability of large retrospective and

developing prospective archives alongside previously established *in vitro* models have allowed both questions to be addressed in this thesis.

Chapter 3 describes an initial pilot study and subsequent larger follow-up studies undertaken to immunohistochemically examine the expression pattern of lamins A/C in colorectal cancer patients. Tumour material was selected from a retrospective archive of incident colorectal cancer patients participating in the Netherlands cohort study on diet and cancer. In the pilot study, the expression pattern of lamins A/C was initially assessed in 100 incident colorectal cancer patients by immunoperoxidase staining. With strong evidence to support further investigation this was then subsequently extended to include the entire cohort of 737 patients in a similar follow-up study. Marked differences in lamin A/C expression were observed between patients of similar clinicopathological grades. These differences in expression were analysed alongside accompanying clinicopathological, biological and epidemiological data. Data shows a counter-dogma affect of A-type lamin expression in colorectal cancer. That is to say those patients with lamin A/C positive tumours are twice as likely to die as their lamin A/C negative counterparts. This lends us to conclude that tumour lamin A/C status can be considered a strong independent prognostic marker of survival in sporadic colorectal cancer.

Chapter 4 then looks at the biological question associated with the decreased mortality in lamin A/C deficient tumours of why? The starting point is the investigation into the effects of lamin A expression on the canonical Wnt signalling pathway, classically at the heart of colorectal cancer development. Data show that differential expression of lamin A in the SW480 (Dukes' B) colon cancer *in vitro* model does not directly influence β-catenin activity. Data also shows that this absence of change in relative β-catenin activity persists in the presence of either endogenous truncated or full length functional APC. Results hence suggest that the role lamin A plays in influencing colorectal cancer mortality may in this case act independently of the canonical Wnt signalling pathway.

Chapter 5 uses the same SW480 (Dukes' B) colon cancer *in vitro* model to look at global gene expression changes under conditions of differential lamin A

expression. Data mining of genome-wide microarray analysis was carried out on lamin A transfected and control colon cancer cell lines. Here I looked for alternative proteins and signalling pathways which may be affected by changes in lamin A expression. A subset of key targets was chosen and independently verified at the RNA level implicating other cellular pathways and components such as cell-cell adhesion; cytoskeletal reorganisation; protein phosphatase/kinase pathways, such as MAPK/ERK; EGF signalling pathways; BMP signalling pathways; Slit/Robo signalling pathways and more.

Finally chapter 6 looks at BMP4, a prominent protein identified by microarray data analysis which has previously been identified as having a potential role in tumour suppressor pathways as a negative regulator of tumourigenesis. Data show that the expression of lamin A significantly down-regulates BMP4 at both the RNA and protein level. A down-regulation in the level of the cell-cell adhesion protein E-cadherin (a downstream target of BMP signalling) and a concomitant up-regulation in the actin bundling protein T-Plastin is also noted at the mRNA level and such changes pertain even in the presence of exogenously applied rhBMP4. Finally I show that exogenous rhBMP4 treatment partially inhibits migratory behaviour in scratch wound assays in the SW480 GFP-Alone transfected control cells, but has no effect on the SW480 GFP-lamin A transfected cells suggesting that the presence of lamin A in these cells may abrogate BMP4 mediated growth suppression. Thus, in the absence of changes in β-catenin activity, data suggest that the detrimental effect of lamin A/C expression in patient tumours may be mediated through inhibition of BMP rather than Wnt signalling pathways.

CHAPTER 2 – Materials and Methods

2.1 Routine chemicals and materials

The majority of general routine chemicals used were purchased from one of; BDH Laboratory Supplies (VWR International Ltd, Leicestershire, England), Sigma-Aldrich (Sigma-Aldrich Company Ltd., Dorset, England) or Melford (Melford Laboratories Ltd., Suffolk, England) unless otherwise stated. Those chemicals obtained from BDH Laboratory Supplies were AnalaR® analytical grade and all other reagents were Molecular Biology grade. All plasticware for tissue culture was supplied by Greiner Bio-One Ltd, Gloucestershire, GB.

2.2 The Netherlands Cohort Study on Diet and Cancer (NLCS) – A Retrospective Archive

2.2.1 Population characteristics and background

A prospective cohort study on diet and cancer was initiated in the Netherlands in September 1986. The study design has been described in detail elsewhere (van den Brandt, Goldbohm et al. 1990). Briefly, at baseline a total of 58,279 men and 62,573 women between the ages of 55 and 69 completed a self-administered food frequency and lifestyle questionnaire. The study population originated from 204 municipal population registries throughout the Netherlands. Incident colorectal cancer cases are identified by monitoring the entire cohort for cancer occurrence through annual record linkage to the Netherlands Cancer Registry (NCR), nine regional cancer registries throughout the Netherlands and the Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA), a national database of pathology reports (van den Brandt, Schouten et al. 1990). The PALGA database was also used to identify and locate colorectal cancer tumour tissue in Dutch pathology laboratories.

In terms of patient follow-up, the first 2.3 years were excluded due to incomplete nationwide coverage of PALGA. However, from January 1989 until January

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1994, 819 incident cases with histologically confirmed colorectal cancer were identified. Colorectal cancer was classified according to site using the International Classification of Diseases (ICD) system as follows; proximal colon: [caecum through transverse colon] (ICD-0 codes 153.0, 153.1, 153.4, 153.5, 153.6); distal colon: [splenic flexure through sigmoid colon] (ICD-0 codes 153.2, 153.3, 153.7); rectosigmoid (ICD-0 code 154.0) and rectum (ICD-0 code154.1).

Patient follow-up for all incident colorectal cancer cases (excluding the first 2.3 years after commencing the trial in September 1986) was complete and subsequently obtained for the period of 1st January 1989 to 31st December 1996 (7 years).

2.2.2 Tissue Samples

Tumour material of colorectal cancer patients was collected after approval by the Ethical Review Board of Maastricht University, PALGA and the Netherlands Cancer Registry. Tissue samples from the 819 identified colorectal cancer patients were requested from 54 pathology laboratories throughout the Netherlands. Tumour tissue collection started in August 1999 and was completed in December 2001. Forty-four tumour tissue samples (5%) could not be traced at all. Of 775 traceable tissue samples, 737 (90%) contained sufficient tumour material as confirmed by a pathologist (Professor Adriaan de Bruïne, University of Maastricht, The Netherlands) for histological and molecular analysis.

All specimens had been processed according to standard Dutch Pathology guidelines. Briefly specimens were grossly dissected and immersed in 10% formalin fixative for 30 minutes at 35°C. Dehydration and embedding of tissues was fully automated and the procedure was as follows: 70% ethanol, 60 minutes at 35°C; 70% ethanol, 60 minutes at 37°C; 96% ethanol, 60 minutes at 37°C; 96% ethanol, 1 hour 30 minutes at 40°C; 100% ethanol, 1 hour 30 minutes at 40°C and 100% ethanol, 2 hours at 45°C. Samples were cleared in xylene for 15 minutes at 45°C, 15 minutes at 50°C, followed by another 30 minutes in fresh xylene at 50°C before embedding in paraffin at 60°C for two 60 minute periods

and finally for 1 hour 30 minutes. The clinical stage of the tumours was then confirmed histologically by a resident pathologist.

2.2.3 A Retrospective Lamin Study and the CREAM Database

Information about age at diagnosis, sex and family history of colorectal cancer (at baseline) was retrieved from the NLCS database. Information concerning tumour sub-localisation, Duke's stage and differentiation state of the tumour was retrieved from the NCR database.

DNA had previously been isolated for biochemical analysis (Brink, de Goeij et al. 2003) from the 737 specimens and molecular information regarding the following had been obtained: methylation status of the O^6-MGMT promoter; methylation status of the hMLH1 promoter; methylation status of the APC promoter (van Engeland, Weijenberg et al. 2003); mutational status of *Ki-ras* exon 1 (further subdivided as codon 12 or 13) (Brink, de Goeij et al. 2003; Brink, Weijenberg et al. 2004; Bongaerts, de Goeij et al. 2006); mutational status of β-catenin; mutational status of APC (Luchtenborg, Weijenberg et al. 2004; de Vogel, van Engeland et al. 2006); expression status of p53; and MSI staining status (Luchtenborg, Weijenberg et al. 2005; Luchtenborg, Weijenberg et al. 2005). These data had been entered into and stored within a new database titled the Colorectal Epidemiology and Mutation (CREAM) database, from which all of the above information was easily accessible. All 737 slides were analysed for expression of lamins A/C as described in section 2.3 and the reported results (Chapter 3) were added to the database.

2.2.4 Statistical Analysis

Baseline characteristics of patients (age at diagnosis, sex, family history of colorectal cancer), tumours (tumour sub-localisation, Dukes' stage and tumour differentiation) as well as tumour biology variables (gene mutation/methylation status), were compared by Student's t-test (continuous variables) and Chi-squared (χ^2) tests (categorical variables) to the study variable (lamin A/C expression). Hazard Ratios (HR) for disease and 95% confidence intervals (CI)

were calculated by conditional logistic regression. In multivariate analyses, missing values were treated as a separate category or excluded (categorical variables) or given the median-value (continuous variables). All statistical tests and corresponding p-values reported were for two-sided tests and p-values of less than 0.05 were considered statistically significant. SPSS version 12.0 (Chicago, IL, USA) was used for all statistical analyses.

2.3 Immuno-Histochemistry

2.3.1 Heat Mediated Antigen Retrieval

Formalin-fixed, paraffin-embedded tissue specimens obtained from the NLCS archive were sectioned at 4μm and incubated at 50°C for 24 hours to allow adhesion to Starfrost® adhesive microscope slides (Knittel Glaser, Braunshweig, Germany). Tissue sections were then de-paraffinised in xylene, twice for 5 minutes and rehydrated through a decreasing ethanol series: 2 x 100% ethanol for 5 minutes; 1 x 96% ethanol for 5 minutes; 1 x 70% ethanol for 5 minutes before finally being transferred to distilled water for 2 minutes.

The process of heat-mediated antigen retrieval used was adapted from a method previously described (Barker, Huls et al. 1999). Briefly, endogenous peroxidase activity was quenched by incubating slides in 3% H_2O_2 in methanol for 15 minutes. Thereafter tissue sections were immersed in 0.01 M citrate buffer, pH 6.0 (pre-heated to 90°C) for 15 minutes in a water bath at 90°C. Following this, slides were transferred to 1 x Phosphate Buffered Saline (PBS) (pre-warmed to 50°C) and allowed to cool to room temperature.

2.3.2 Three-layer peroxidase staining

Slides were mounted into Thermo Shandon Coverplates™ (Thermo Shandon Ltd. UK) and loaded into Shandon Sequenza® staining racks (Thermo Fisher Scientific Inc. US). The setup was chosen as it allows a highly organised and reliable method for immunostaining which ensures consistency and excellent

staining quality with the ability to perform large staining runs (up to 50 slides per run) with a very low chance of batch variation.

Non-specific antibody binding was blocked using 125μl/section of 5% goat serum and 2% bovine serum albumin (BSA) in PBS. Slides were washed 3 times with PBS containing 0.1% BSA (PBS/BSA). The anti-lamin A/C primary mouse monoclonal antibody (Dyer, Kill et al. 1997) was diluted 1:10 in 1 x PBS/BSA, applied (125μl/section) and incubated at 4°C for 16 hours. Unbound primary antibody was removed by washing 3 times with PBS/BSA. Biotinylated goat anti-mouse IgG secondary antibodies (used at a concentration of 1:400), diluted in 1 x PBS/BSA were used (125μl/section) and incubation was for 45 minutes at room temperature (RT). Unbound secondary antibody was removed by washing 3 x with 1 x PBS/BSA. This was followed by a 30 minute incubation at room temperature with StreptABComplex/HRP (Dako Cytomation, Glostrup, Denmark) made up according to manufacturer's guidance. 125μl/section was used, and unbound complex was removed by washing 3 x with 1x PBS/BSA after incubation. Immunological detection was carried out using 125μl activated Diaminobenzidine (DAB) chromogen (BioGenex Laboratories Inc., San Ramon, CA, US) (0.0225% H_2O_2 in 1 mg/ml filtered DAB in 1 x PBS – made up according to manufacturer's instructions). DAB was applied directly to tissue sections and colour development was monitored under a simple light microscope. Reactions were stopped by immersing slides in δH_2O. Negative controls were stained in the manner described above but substituting the lamin A/C primary antibody or biotinylated secondary for 1 x PBS/BSA.

2.3.3 Counterstaining and mounting

To visualise the nuclei of cells, sections were lightly counterstained with undiluted Mayers Haemalum for 5 seconds and rinsed in distilled water for 1 minute. The counterstain was differentiated and fixed using alkaline alcohol (3ml ammonia solution in 100ml 70% ethanol) for 30 seconds and then rinsed in distilled water for 1 minute.

Slides were dehydrated through a graded ethanol series and cleared as follows; 70% ethanol for 1 minute, 96% ethanol for 1 minute, 2 x 100% ethanol for 1 minute, 2 x xylene for 5 minutes. Specimens were then mounted and coverslipped with Entellan® (Merck kGaA, Darmstadt, Germany) and left to dry overnight.

2.3.4 Image Acquisition and Scoring

Colorectal cancer specimens were analysed using a Nikon Diaphot 300 inverted microscope (Nikon Corporation, Tokyo, Japan) equipped for bright-field microscopy with Plan 4x/0.13, 10X/0.25, 20x/0.4 and 40x/1.3 lenses. Images were taken using a Nikon DXM1200 digital camera controlled by Nikon Act-1 Version 2.20 software. Image processing was carried out using the Adobe® Photoshop® 7.0 imaging software (Adobe Systems Inc. San Jose, CA, US).

Scoring of the slides was based on a +/- system with lamin +ve being classed as 10% or more of tumour cell nuclei staining strongly positive for lamin A/C compared to an the internal control. Adjacent stromal cell nuclei were used as an internal positive control since they always stained positive. Lamin –ve was classed as less that 10% of nuclei staining strongly for lamin A/C expression compared to the internal control. The use of adjacent stromal cell nuclei as internal control demonstrated that a negative stain in tumour tissue corresponded to an absence of antigen rather than poor staining. In all studies, slides were scored blind to all database information by two independent observers, and followed by a consultatory evaluation of scoring before database information was retrieved and statistical analysis began.

2.4 Mammalian cell culture

2.4.1 Cell lines

The colon carcinoma cell line SW480 used in *in vitro* studies was originally obtained from the European Collection of Cell Cultures (ECACC), (Wiltshire, UK)

as growing cultures. The SW480 cell line is a classified as a Grade IV carcinoma cell line. The cells were originally derived from a grade 3-4 colon adenocarcinoma and were initially populated by a mixture of epithelial-like and bipolar cells. Over time the epithelial-like cells have predominated (Leibovitz, Stinson et al. 1976) and this was evident from the cultures grown. The SW480 cell line harbours a type II APC truncation which possesses both the N-terminal Nuclear Export Signal (n-NES) as well as the 5' most C-terminal NES (c-NES) (Rosin-Arbesfeld, Townsley et al. 2000) unlike cell lines harbouring Type I APC truncations whereby the 5'-most c-NES is also lost. The SW480 cell line has also been shown to strongly secrete the Carcino-Embryonic Antigen (CEA).

The parent cell line and subsequent stably-transfected clones (described in section 2.4.2) were routinely cultured in 75cm^2 plastic tissue culture flasks under the following conditions; L-15 (Leibovitz) medium with 2mM L-Glutamine (Invitrogen, UK) supplemented with 10% Foetal Bovine Serum (FBS), 100 units/ml penicillin and 100µg/ml streptomycin. Flasks were cultured in a humidified, 37°C environment in the absence of CO_2 following recommendations from the ECACC. Culture media was changed every 48-72 hours. All cells were cultured using FBS from lot 111K3397, obtained from Sigma. Cells were routinely sub-cultured between 70% and 80% confluency. Cells were detached by treatment with 0.25% trypsin in 0.5mM Ethylenediamine Tetraacetic Acid (EDTA) in PBS in a humidified environment at 37°C for 2 minutes before neutralisation with serum-containing L-15 media. The cells were routinely split 1:4 before being re-plated at a density of approximately 20-30 x 10^3 cells/cm^2

2.4.2 Transfected SW480 clones

The SW480 parent cell line had been previously stably transfected with DNA constructs encoding fusion proteins of EGFP-lamin A and EGFP (Willis, 2006). The EGFP-lamin A full length construct was a gift from Dr M. Ozumi, Institute of Physical and Chemical Research, Saitama, Japan. Single cell cloning of the stably-transfected daughter colonies was established using Geneticin® (G-418 sulphate, Invitrogen, UK) antibiotic selection and GFP-reporter stability within clones was monitored by fluorescence microscopy at monthly intervals resulting

in multiple stably transfected clones for use as an *in vitro* model. Before initial experimental work for this project was conducted, single cell cloned lines were subjected to a 2 week Geneticin® antibiotic selection (200µg/ml) followed by re-evaluation of GFP-reporter stability. Cell lines showing no loss of GFP expression were grown in Geneticin® free media for all subsequent experimental work. On a monthly basis, stable transfection was routinely re-evaluated by simple observation under fluorescence microscopy using a Zeiss Axioskop microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) equipped for epifluorescence.

2.4.3 Transient transfection using Lipofectamine™ 2000 reagent

Transfection of the SW480 cell lines using Lipofectamine™ 2000 (Invitrogen, UK) was carried out according to Invitrogen technical bulletin #116668 (2004). Briefly, cells were grown to 80% confluency in 35mm diameter 6 well plates under standard culture conditions previously described. Constructs were transfected in at required concentrations in antibiotic free media and cells were incubated for 48 hours without media change. Transfection utilising $\delta\delta H_2O$ was used as a negative control.

2.4.4 Transient Nucleofection

Transfection of the SW480 cell line was also carried out using the amaxa Nucleofector® (AAD-1001) (amaxa AG, Cologne, Germany) using the amaxa Cell Line Nucleofector® kit V (amaxa AG, Cologne, Germany) and exactly in accordance with amaxa's optimised protocol guidelines DCV-1036 (2005) for SW480 cells.

2.4.5 Dual Luciferase® Reporter Assays

Dual Luciferase Reporter Assays for β-catenin activity were carried out on the SW480 cell lines using the Dual-Luciferase® Reporter Assay System (Promega, UK) in accordance with Promega's technical manual TM040 (2003). Briefly, cells

were seeded at a density of 5×10^5 in 6 well plates 24 hours prior to transfection. Cells were then transfected using the Lipofectamine™ 2000 reagent as described in section 2.4.3. The TOPFlash and FOPFlash reporters were a gift from Ewa Markiewicz, Durham University, UK and were co-transfected at a concentration of 1µg/µl alongside the *Renilla TK* luciferase construct at a concentration of 0.1µg/µl. 24 hours post transfection, cells were lysed and the relative activity of firefly luciferase under the TOPFlash and FOPFlash promoters was measured before being quenched and measuring relative *Renilla* luciferase activity.

2.5 One-dimensional SDS-PAGE and immunoblotting

Cultured SW480 cells were harvested at 80% confluency and protein was extracted from 7×10^6 cells. Cell pellets were re-suspended in 500µl of lysis buffer [10mM Tris-HCL (pH7.4), 10 mM KCl, 3mM $MgCl_2$, 0.1% Triton X-100 and 1 x protease cocktail inhibitor] and then incubated with 0.1 units/µl DNase I on ice for 10 minutes. Cell extracts were dissolved in 500µl of 2 x sample buffer [125mM Tris-HCl (pH6.8), 2% β-mercaptoethanol and 0.25% Bromophenol Blue (w/v)], and boiled at 95°C for 3 minutes.

Protein samples were resolved using one-dimensional SDS-PAGE on a 6% or 10% poly-acrylamide resolving gel with a 5% poly-acrylamide stacking gel, both made using ProSieve® 50 acrylamide gel solution (Cambrex Bio Science Wokingham Ltd., Berkshire, UK) and containing 0.1% SDS. Samples were run at 100 volts (V) for 2 hours in tank buffer (25mM Tris, 192mM Glycine and 0.1% SDS) and transferred to nitrocellulose membrane (Protran®, grade BA85, Schleider and Schuell BioScience Inc., Keene, NH) at 30V for 16 hours at 4°C in transfer buffer (25mM Tris and 192mM Glycine, pH 9.2, plus 20% methanol and 0.1% SDS). Nitrocellulose membranes were subsequently washed once in Blot Rinse Buffer (BRB) [10mM Tris-HCL, 150mM NaCl and 1mM EDTA, pH 7.4, plus 0.1% Tween®20 (v/v)] and incubated initially in 5% blocking buffer [5% skimmed milk powder (w/v) in BRB] for 1 hour at room temperature on a shaker. After blocking, nitrocellulose membranes were washed three times in BRB for 5

minutes. Detection of proteins was accomplished using a series of specific antibodies listed in Table 2.1. β-actin was used as a control for loading.

Nitrocellulose membranes were incubated with primary antibodies diluted to their optimum working concentrations in BRB + 5% milk overnight at 4°C on a shaker and then rinsed 5 times with BRB, 15 minutes per rinse at room temperature on a shaker. The secondary antibody used was a horse-radish peroxidase (HRP)-conjugated IgG and used at a concentration of 1:2000 – 1:4000 in BRB + 5% milk. Membranes were incubated with secondary antibodies for 1 hour at room temperature with continuous agitation and then washed with BRB, 5 times for 10 minutes. Nitrocellulose membranes were exposed to ECL™ western blotting reagents 1 and 2 (1:1 v/v) (Amersham Biosciences) and following exposure, photographic film was developed using a compact X4 Automatic X-Ray Film Processor (Xograph Imaging Systems Ltd., Gloucestershire, UK). Differences in protein expression were then quantified using densitometry (see Section 2.8.1).

Table 2.1 Primary antibodies used for SDS PAGE

Antibody	Target	Type	Supplier	Dilution
Actin clone AC-40	β-actin	Mouse monoclonal	Sigma	1:1500
Anti-APC (Ab-1) (FE9)	APC	Mouse Monoclonal	CalBioChem	1:1000
Anti-BMP4	BMP4	Mouse monoclonal	R&D Systems	1:500

2.6 Semi-quantitative RT-PCR

2.6.1 Primer Design

Primers specific for the coding sequence (cds) of target genes being investigated were designed using the freely available online Primer3 web interface (http://frodo.wi.mit.edu/primer3/primer3_code.html) (Whitehead institute, Cambridge, MA. It selects suitable primers from a given DNA sequence based on a number or parameters including product size, primer length, melt temperature (Tm) and GC%. The software parameters were set to design primers between 18 and 27 bases long and select primers with a minimum Tm of

55°C, a maximum Tm of 65°C, a maximum Tm difference between sense and antisense primers of 4°C and a GC content of 45% to 70%. The use of NetPrimer online software [PREMIER Biosoft International, CA (http://www.premierbiosoft.com/Netprimer/index.html) enabled the examination of secondary structures in primer pairs, incorporating hairpin loops, self-dimers, cross-dimers, palindromes and runs/repeats, which could impinge their function or lead to non-specific products. Table 2.2 shows primer sequences designed and used within this project.

Table 2.2 Primers for semi quantitative PCR

Gene	Accession	Direction	Sequence (5'-3')	Size (bp)
APC	NM_000038	Sense	GAGGCAGAATCAGCTCCATC	958
		Antisense	CATTCCACTGCATGGTTCAC	
AREG	NM_001657	Sense	GAGCACCTGGAAGCAGTAAC	348
		Antisense	AAGAGGACCGACTCATCATTT	
β-Actin	NM_001101	Sense	GGCACCACACCTTCTACAATGAGC	834
		Antisense	CGTCATACTCCTGCTTGCTGATCC	
BMP4	D30751	Sense	AGCATGTCAGGATTAGCCGAT	400
		Antisense	ATGGAGATGGCACTCAGTTCAG	
CDH1	NM_005032	Sense	CCAAGTGCCTGCTTTTGATG	415
		Antisense	CACAATTATCAGCACCCACAC	
DUSP5	NM_004419	Sense	CTCAGGGTAGGTTCTCGGG	492
		Antisense	ATGCCAAAGTCCAAGGTCAG	
FOSL1	NM_005438	Sense	CTCATCGCAAGAGTAGCAGC	479
		Antisense	GTAAAGTGGCACCTTCTGTCA	
PLS3	NM_004360	Sense	GCATCTTCCCTCTCATACCC	307
		Antisense	GCAAACAGCTTGACAAAGCA	
ROBO1	NM_002941	Sense	CCCAGAGAAGCACAGGAACA	599
		Antisense	GGCTGATGACTGAAGCAAGAAG	

2.6.2 Total RNA Isolation

For each experiment, total RNA was isolated from three different passages of the SW480 cell line using the TRIZOL® reagent (Invitrogen, UK). The procedure was carried out exactly as described in Invitrogen's technical manual #15596-026.

Briefly, cells were expanded to approximately 70% confluency in 75cm^2 tissue culture flasks and were lysed directly on the culture surface with 2ml TRIZOL® per flask and allowed to stand for 10 minutes at room temperature. Lysates were transferred to 1.5ml RNase-free microcentrifuge tubes (Ambion Inc., Austin, TX, US) and centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase alone was immediately transferred to fresh tubes and 0.5ml RNase-free isopropanol per original 1ml of TRIZOL® was added. Samples were inverted 3 times and incubated for 10 minutes at room temperature before being incubated at -20°C overnight. Samples were then centrifuged at 12,000 x g for 10 minutes at 4°C. All supernatant was removed and the RNA precipitate was washed with 1ml RNase-free 75% ethanol per original 1ml TRIZOL® and pelleted again at 10,000 x g for 10 minutes at 4°C. All supernatant was removed and the pellets air-dried for 5-10 minutes in a laminar flow hood before being resuspended in 15-20µl nuclease-free water. Samples were then aliquoted and stored at -80°C.

2.6.3 Confirmation of quality, purity and concentration of RNA

Total RNA quality was assessed by gel electrophoresis. RNA was diluted in 6 x RNA loading buffer [6 x Tris-acetate EDTA (TAE) buffer, 8M urea, 15% ficol, 0.25% Xylene cyanol FF (w/v) and 0.25% bromophenol Blue (w/v)] and heated at 70°C for 5 minutes before adding ethidium bromide to a final concentration of 7 ng/µl. Samples were separated alongside RNA millennium™ size markers (Ambion Inc., Austin TX, US) on 1% agarose gels in 0.8x TBE buffer at 80V for 1 hour. Bands were visualised using a Gel Doc™ 2000 UV transilluminator and Quantity One™, version 4.0.3 software (Bio-Rad)

Purity was determined by measuring the ratio of absorbance in 10mM Tris-HCl (pH 7.5) at 260nm and 280nm (A_{260}/A_{280}) in a GeneQuest CE2301 Analyser (Cecil Instruments Ltd. Cambridge, UK) and the concentration was calculated by measuring the absorbance at 260nm (A_{260}) in DEPC-treated δH$_2$O.

2.6.4 Confirmation of RNA Integrity

The human gene β-actin was used to exclude the possibility that genomic DNA (gDNA) may have been co-isolated with total RNA. Primers to an 834bp fragment of β-actin (see table 2.2) were used to amplify any contaminating gDNA in 0.5μg RNA. PCR was carried out in a 25μl reaction volume comprising 1 x ReddyMix™ PCR Master Mix (ABgene, Epsom, Surrey, UK) [25 units/ml Thermoprine Plus DNA polymerase (Taq), 75mM Tris-HCl (pH 8.8 at 25°C), 20mM $(NH_4)_2SO_4$, 2.5mM $MgCl_2$, 0.01% Tween®20 (v/v) and 200 μM each dNTPs], 0.4 pmol/μl β-actin sense primer and 0.4 pmol/μl β-actin anti-sense primer. Cycling conditions were as described in section 2.6.5).

The total volume of PCR product (25μl) was electrophoresed on a 1% TAE-agarose gel with 0.3μg/ml Ethidium Bromide (EtBr). RNA samples showing evidence of gDNA contamination were treated with 0.075 units/μl RQ1 RNase-free DNase (Promega) in 1 x reaction buffer [40 mM Tris-HCl (pH 8.0), 10mM $MgSO_4$, 1mM $CaCl_2$] for 15 minutes at room temperature. The reaction was terminated by incubating the samples with 1μl DNase stop solution (20mM EGTA, pH 8.0) for 10 minutes at 65°C. RNA was then precipitated with 1/10 of the total reaction volume of Diethyl pyrocarbonate (DEPC)-treated 3M Sodium acetate (NaOAc), pH 5.2 and 3 volumes of 100% RNase free ethanol (-20°C), vortexed for 5 seconds and incubated overnight at -20°C. To recover RNA, samples were centrifuged at 12,000 x g for 10 minutes at 4°C, the supernatant removed and pellets washed with 1ml 70% RNase free alcohol (in DEPC-treated δH20). Centrifugation was repeated at 12,000 x g for 5 minutes at 4°C, the supernatant aspirated and residual ethanol allowed to evaporate for 5-10 minutes in a laminar flow hood. The resulting pellet was re-suspended in 20μl RNase free water and the quality, purity (A_{260}/A_{280}) and concentration (A_{260}) of the RNA was re-evaluated as described in section 2.6.3.

2.6.5 Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

Reverse transcription and amplification of target genes was carried out in 2 stages using Promega's Reverse Transcription system (A3500) and PCR Master Mix (M7502). For all samples, RT-PCR was completed in triplicate.

First a cDNA library was synthesised in a 40μl reaction using Promega's Reverse Transcription System in exact accordance with their technical bulletin TB099 (2006). Two -ve controls substituting RNA and the AMV-RT for δH$_2$O were used in each run. All resulting cDNA libraries were aliquoted and stored at - 20°C.

β-actin was amplified from all cDNA libraries and their accompanying AMV-RT negative controls, plus negative controls where no RNA was added to show equal amounts of cDNA had been reverse transcribed and that reverse transcription and PCR reactions were working successfully.

PCR fragments for the chosen target genes detailed in section 2.6.1 (table 2.2) were amplified from all Reverse Transcriptase reactions using A 25μl reaction containing 1 x Promega PCR Master mix [25 units/ml Taq DNA polymerase in buffer (pH 8.5), 200μM each dNTPs and 1.5 mM MgCl2], 0.4 pmol/μl sense primer. 0.4 pmol/μl antisense primer, 2μl cDNA library and Nuclease-free water. Optimisation of PCR profiles was carried out in an Eppendorf Mastercycler™ Gradient Thermal Cycler (Eppendorf UK Ltd, Cambridge, UK) using the following conditions; 94°C for 2 minutes, 26 cycles of [94°C for 45 seconds, Tm°C ± 5°C for 1 minute, 72°C for 1 minute] and finally 72°C for 5 minutes.

2.6.6 Analysis

All amplified products were run on 1% TAE-agarose gels, containing 0.3 μg/ml EtBr, at 60V for 60 minutes. Product size was compared to a 100bp DNA ladder (Promega Cat #G2101) and bands were visualised using a GelDoc™ 2000 UV transilluminator and Quantity One™ software, version 4.0.3 (Bio-Rad).

Differences in expression levels were quantified using densitometry (see section 2.8.1) and calculated as a ratio against β-actin expression.

2.6.7 RT-PCR confirmation of changes in expression of selected genes.

Primers to target genes were designed as detailed in section 2.6.1 (table 2.2). Semi-quantitative RT-PCR was set-up exactly as described in section 2.6.5 including appropriate negative controls. Experiments were carried out in triplicate for each target gene. Optimised PCR cycling conditions are detailed in section 2.6.5. Optimised Tm for each primer set is as follows: APC = 60°C; AREG = 57°C; β-Actin = 60°C; BMP4 = 57°C; CDH1 = 55°C; DUSP5 = 55°C; FOSL1 = 58°C; PLS3 = 58°C; ROBO1 = 58°C

PCR products were analysed by gel electrophoresis and subsequent densitometry as outlined in section 2.6.6. β-actin RT-PCR was performed as a positive and loading control for all samples.

2.7 Cloning

The *Escherichia coli* strain DH5α was used as the bacterial transformation recipient for the APC construct. The full length cDNA of human APC cloned into the pcDNA3.1/FLAG vector was a gift received from Kristi Neufeld [see Appendix 1 (p204) for Plasmid map]

Transformation reactions were set up using 2μl pFlag-APC and 50μl of XL1-Blue Super-competent cells (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^{q}ZΔM15* Tn*10* (Tet^r)] (Stratagene, La Jolla, CA, US), and precisely followed Stratagene's guidelines #200236 (2004)

Briefly, cells were thawed on ice before being transferred to pre-chilled reaction tubes and adding 1.7μl β-mercaptoethanol per 100μl. Upon addition of the construct, cells were incubated on ice for 30 minutes; heat shocked at 42°C for 45 seconds and cooled on ice for 2 minutes. 0.5ml preheated SOC (Super

Optimal Broth [Catabolite repression](Hanahan 1983)) nutrient broth (without antibiotics) was added and transformation reactions were incubated at 37°C for 1 hour with shaking at 225rpm. 250µl sample was then plated onto Lysogeny Broth (LB) agar plates (10g/l Triptone, 5g/L Yeast Extract, 10 g/L NaCl, pH 7.2) supplemented with 100µg/m; ampicillin and incubated at 37°C for 24 hours.

Ten colonies were selected from each transformation reaction and inoculated in LB media containing 100µg/ml ampicillin. Bacteria were incubated at 37°C with shaking (225rpm) for 16 hours. Glycerol stocks were prepared using 0.3ml culture and the rest of the cultures were used to purify plasmid DNA using Promega's Wizard® Plus Minipreps DNA Purification System (Promega, UK) as detailed in section 2.7.1.

2.7.1 DNA Purification

Plasmid DNA was purified from inoculated LB-media at 16 hours using Promega's Wizard® Plus Minipreps DNA Purification System (Promega, UK) according to their technical bulletin #117 (2002). Final elution of plasmid DNA from the spin columns was eluted in 100µl nuclease free water and stored at minus 20°C.

2.7.2 Confirmation of Plasmid

The purified DNA plasmid was checked for quantity at OD_{260}, quality was checked at OD_{260}/OD_{280} and 10µl was checked visually by electrophoresis on a 0.7% TAE-agarose gel against a known DNA standard.

Plasmid DNA was analysed by restriction digestion. 20 units of Pmel (GTTT↓AAC) were used per 1µg plasmid DNA in NEBuffer 4 supplemented with 100µg/ml BSA (New England Biolabs Inc., UK) Incubation was at 37°C for 2 hours and the products analysed by electrophoresis on 1% TAE-agarose gel. Bands of 8.4Kb (APC) and 5.5Kb (vector) were considered a positive result.

Further confirmation was undertaken by analysing extracted plasmid DNA for the presence of APC through PCR amplification using the primers detailed in table 2.2 and the methodology outlined in section 2.6.7.

2.8 Miscellaneous applications

2.8.1 Densitometry

Western Blot and RT-PCR agarose gel bands were digitally scanned in a Fujifilm Intelligent Dark Box II (Fujifilm Medical Systems, Edison, HJ) directed by Fujifilm Image Reader LAS-1000 Pro Version 2.11 software and intensities quantified in Image Gauge version 4.0 (Fujifilm).

2.9 Microarray Data Mining

Genome wide microarray analysis was carried out on the SW480 EGFP-lamin A and SW480 EGFP cell lines using the Human Genome U133 Plus 2.0 high-density oligonucleotide Affymetrix GeneChip array (Affymetrix, Santa Clara, CA, USA). Normalisation of expression ratios was carried out in accordance with the associated Affymetrix guidelines for the HG U133 GeneChip and a targeted gene list was generated by means of a series of relevance thresholds for detection *p*-value significance, expression change p-value significance and Signal Log Ratio (SLR).

Analysis of the targeted gene list and biological interpretation of results was carried out using the online NetAffx Analysis Centre from Affymetrix found at (http://www.affymetrix.com/analysis/index.affx). Genes of interest were subjected to cluster analysis by Gene Ontology including; Biological Process, Molecular Function and Cellular component. For a detailed description of the online NetAffx tools, see http://www.affymetrix.com/support./index.affx.

CHAPTER 3 – Lamin A/C status in colorectal tumours as a biomarker of patient mortality

3.1 Introduction

3.1.1 Lamin A/C expression in colorectal cancer

The differential expression of lamin A/C has already been associated with epithelial, lymphoid and mesenchymal tumours, including basal skin cell carcinoma (BCC) (Venables, McLean et al. 2001; Tilli, Ramaekers et al. 2003), non-small cell lung cancer (Kaufmann, Mabry et al. 1991; Broers, Raymond et al. 1993), non-Hodgkin's lymphoma, acute lymphoblastic leukaemia, (Stadelmann, Khandjian et al. 1990), metastatic chondrosarcoma, rhabdomyosarcoma and leiomyosarcoma (Cance, Chaudhary et al. 1992). Two groups have investigated the changes in lamin A/C expression in colorectal neoplasms. Their findings however, have been somewhat contradictory. Cance and co-workers (Cance, Chaudhary et al. 1992) reported that there was a heterogeneous expression of lamins A/C in two colon adenocarcinomas, determined by immunohistochemical analysis. Moss (Moss, Krivosheyev et al. 1999) on the other hand reported that there was a reduced nuclear immunostaining for lamins A/C in colon adenomas and adenocarcinomas, compared to normal tissue. Neither group however, has corroborated their findings in a large randomised controlled immunohistochemical study, or to date have there been any investigations of this nature looking at lamin A/C expression and disease severity or patient mortality in colorectal cancer.

Originally described as units of architecture, lamins A/C are type V intermediate filaments which form an orthogonal meshwork underlying the inner nuclear membrane. Termed the nuclear lamina, it conveys the nucleus mechanical strength (Aebi, Cohn et al. 1986), provides resistance to deformation (Liu, Rolef Ben-Shahar et al. 2000; Moir, Yoon et al. 2000b; Broers, Peeters et al. 2004) and regulates nuclear shape (Sullivan, Escalante-Alcalde et al. 1999; Schirmer, Guan et al. 2001) and size (Spann, Moir et al. 1997). Lamins also help to shape the nucleoskeleton by anchoring integral proteins of the inner nuclear

membrane, positioning nuclear pore complexes and recruiting other lamin subtypes to the nuclear lamina [reviewed by (Hutchison 2002)]. Current evidence has also implicated lamins in the control of DNA replication (Goldberg, Jenkins et al. 1995) and shown they may organise interphase chromatin and play a role in regulating transcription [reviewed by (Foisner 2001)]

3.1.2 The Netherlands Cohort Study on Diet and Cancer

The Netherlands Cohort Study (NLCS) on diet and cancer (previously described in section 2.2) is a prospective cohort study of 120,852 people. Incident colorectal cancer cases in the cohort were identified by annual record linkage to the Netherlands Cancer Registry (NCR) and PALGA and tumour material was collected after approval by the Medical Ethics Committee of Maastricht University, the NCR and PALGA. Initially tissue samples from 819 patients were requested from 54 pathology laboratories throughout the Netherlands. Incident cases included those patients developing colorectal cancer between 1989 and 1994. Tumour material from forty-four patients (~5%), could not be retrieved due to administrative inconsistencies (9 cases), unavailability of tumour blocks in the pathology archives (34 cases) or incorrect tumour classification (1 case). Of 775 available tissue samples, 737 contained sufficient tumour material for immunohistochemical and molecular analyses as determined by a senior pathologist; Professor Adriaan de Bruïne (University of Maastricht, The Netherlands). Once retrieved, the formalin-fixed, paraffin-embedded tissue blocks were sectioned at 4 μm for immunohistochemical analysis as detailed in section 2.2.2.

3.1.3 Summary

With evidence in other tumour types potentially linking lamin A/C status with patient survival (Mounkes, Kozlov et al. 2003; Agrelo, Setien et al. 2005), the pivotal role of A-type lamins in such a broad range of laminopathies, the lack of research specifically in the colorectal cancer field and the availability of access to the NLCS archive, this chapter looked to investigate whether lamin A/C may be implicated in colorectal carcinogenesis.

In this chapter two studies are described. First a pilot trial to immunohistochemically assess the expression pattern of lamins A/C in a small (n=100), randomised subset of incident colorectal cancer cases from the NLCS archive. The second is a follow-up study to more rigorously test the conclusions drawn from the initial pilot study in the complete subset (n=737) of incident colorectal cancer cases from the NLCS archive. Lamin A/C expression was determined immunohistochemically using the mouse monoclonal anti-Lamin A/C JoL2 antibody (Dyer, Kill et al. 1997), assessed and evaluated against a broad spectrum of confounding variables.

3.2 Results

3.2.1 Immunohistochemical analysis of lamin A/C expression

To determine lamin A/C expression status, slides were stained and scored for the presence or absence of lamin expression exactly as detailed in section 2.3. Lamin A/C expression was scored on +/- system with lamin positive being classed as 10% or more of tumour cell nuclei staining strongly positive for lamin A/C compared to an the internal control (figure 3.1). Adjacent stromal cell nuclei were used as an internal positive control because they always stained positive for lamins A/C. Lamin negative was classed as less that 10% of nuclei staining strongly for lamin A/C expression compared to the internal control (figure 3.2). The availability of an internal positive control demonstrated that a negative stain in tumour tissue corresponded to an absence of antigen rather than poor staining. Scoring was carried out blind by two independent observers followed by a consultatory evaluation.

3.2.2 Patient follow-up

Information on mortality from 1st January 1989 until 31st December 1996 (7 years) was retrieved through linkage with the Central Bureau for Statistics. Differences in patient, tumour and follow-up characteristics were analysed through the use of the chi-square (χ^2) tests for categorical variables and

Students *t*-tests for continuous variables. Hazard ratios (HR) (a hazard ratio is an estimate of the relative effect that a variable [i.e. lamin A/C status] exerts on an event [in this case colorectal cancer related mortality]) and corresponding 95% confidence intervals (CI) for colorectal cancer related mortality according to lamin A/C status were estimated using Cox regression analysis. Gender, age at diagnosis, family history of colorectal cancer, Dukes' stage, tumour location and differentiation, MGMT, *hMLH1*, *Ki-ras* and APC promoter methylation, *TP53* and MSI status, *Ki-ras* activating and APC truncating mutations were considered as potential confounding variables. Those variables found to have a statistically significant contribution to the multivariate model for colorectal cancer related mortality, or that influenced the risk estimates by more than 10% (age at baseline) as well as gender, were included as covariates in the analyses. All analysis was carried out in SPSS.

Figure 3.1

Positive expression of lamin A/C in colorectal tumours.

(A-H) 4μm thick sections of colorectal cancer tissue from patient specimen blocks showing strong positive JoL2 staining in tumour (T) and stromal (S) tissue. Stromal tissue was always positive acting as an internal positive control for the investigation. Tissue sections were weakly stained with Mayers Haemalum (purple) to highlight nuclei where lamin staining was negative. Positive lamin staining appears brown.

(A) Dukes' A, well differentiated tumour **(B)** Dukes' A, moderately well differentiated tumour. **(C)** Dukes' B, moderately well differentiated tumour. **(D)** Dukes' B, moderate/poorly differentiated tumour. **(E)** Dukes' C, moderately well differentiated tumour. **(F)** Dukes' C, moderate/poorly differentiated tumour. **(G)** Dukes' D, moderate/poorly differentiated tumour. **(H)** Dukes' D, moderately differentiated tumour.

In each panel, lamin A/C expression is considered positive and was scored as such for statistical analysis.

Scale bar = 30 μm
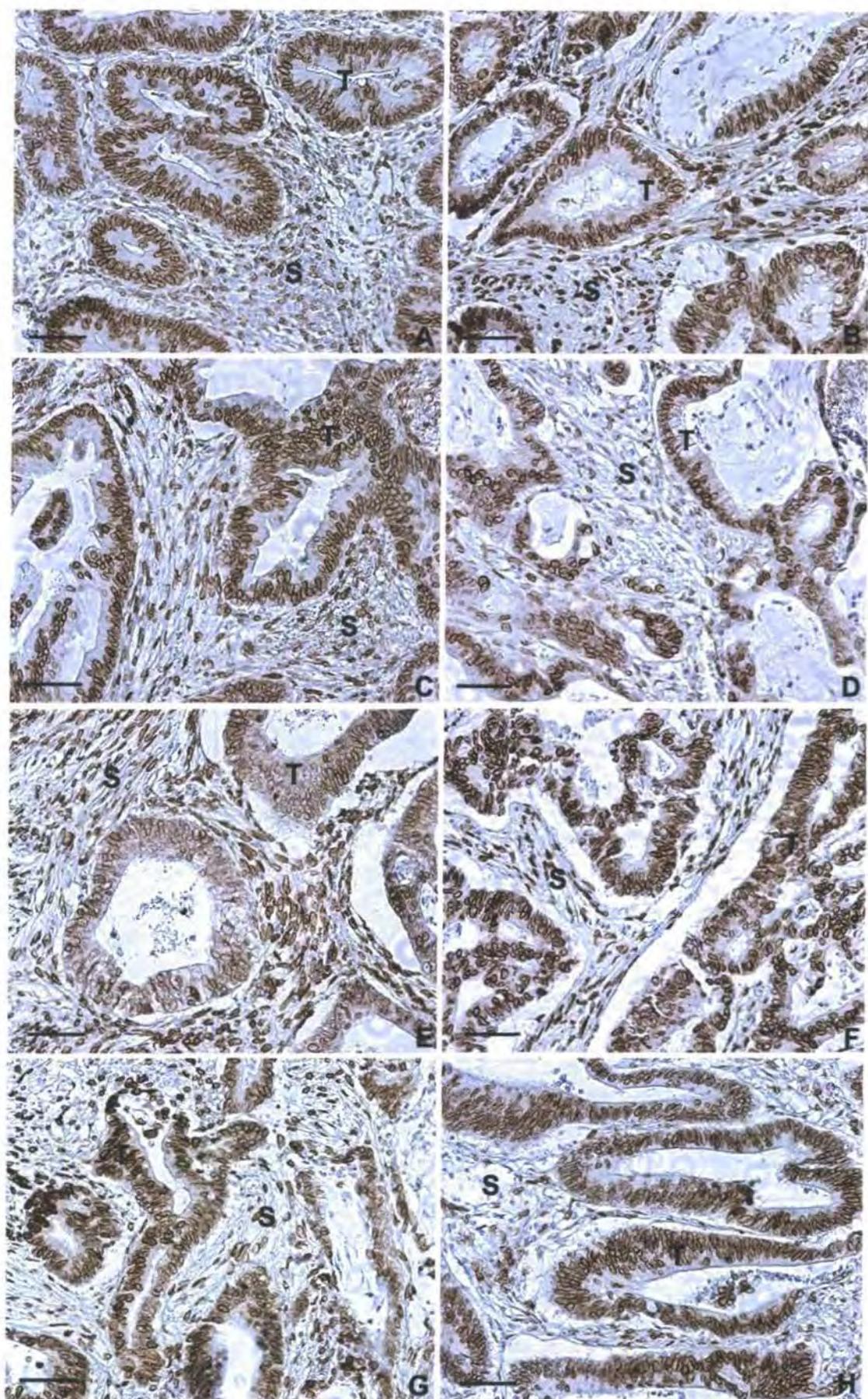
Figure 3.2

Negative expression of lamin A/C in colorectal tumours.

(A-H) 4μm thick sections of colorectal cancer tissue from patient specimen blocks showing negative JoL2 staining in tumour (T) and positive stromal (S) tissue. Stromal tissue was always positive acting as an internal positive control for the investigation and demonstrating that a negative stain corresponded to the absence of antigen rather than poor staining. Tissue sections were weakly stained with Mayers Haemalum (purple) to highlight nuclei where lamin staining was negative. Positive lamin staining appears brown.

(A-B) Dukes' A, moderately well differentiated tumour. **(C)** Dukes' B, moderately differentiated tumour. **(D)** Dukes' B, moderately well differentiated tumour. **(E)** Dukes' C, moderate/poorly differentiated tumour. **(F)** Dukes' C, moderately differentiated tumour. **(G-H)** Dukes' D, poorly differentiated tumour.

In each panel, lamin A/C expression is considered negative since less than 10% of nuclei are staining positive for lamin A/C expression and was scored as such for statistical analysis.

Scale bar = 30 μm
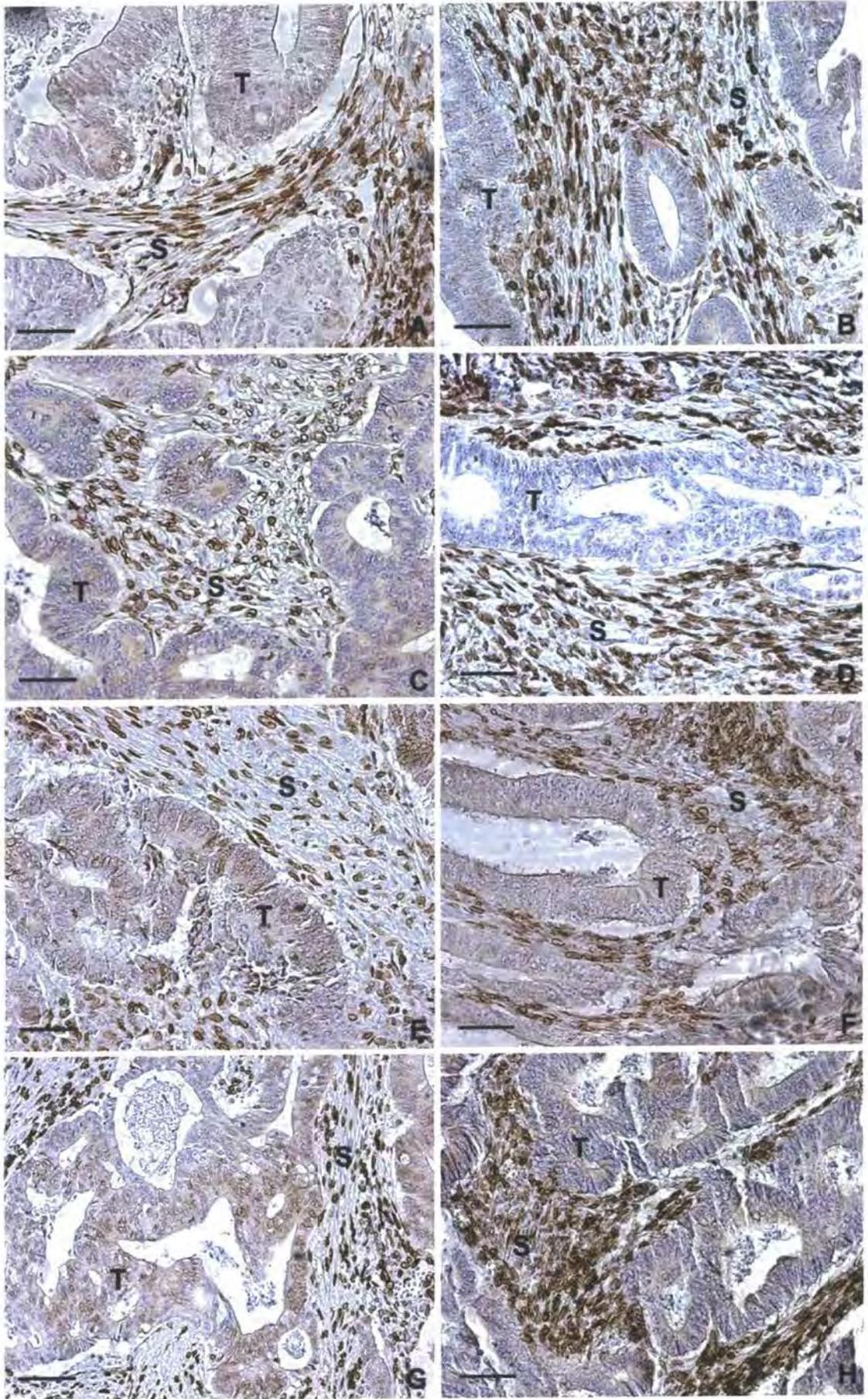
3.3 The NLCS Pilot

An initial pilot study was set up to investigate the expression pattern of lamins A/C within the NLCS archive. Of the 737 tumour specimens available, a subset of 100 incident colorectal cancer cases was randomly selected by an independent selector and immunohistochemically assessed for lamin A/C expression using the JoL2 anti-lamin A/C mouse monoclonal antibody (Dyer, Kill et al. 1997) as detailed in section 2.3. Slides were scored blind on two separate occasions and a consultatory evaluation was then carried out with a senior pathologist. A single case (1%) was lost due to poor tumour specimen leaving a total of 99 cases for statistical analyses.

During the follow-up period, from 1st January 1989 until 31st December 1996, 43 patients died, with 37 of these patients dying as a result of colorectal cancer. Information on primary cause of death as well as, primary, secondary and tertiary complications was retrieved from the PALGA database. Colorectal cancer related death was concluded if colorectal cancer was determined as either the primary cause of death or as a primary, secondary or tertiary complication, using the International Classification of Diseases system. ICD-9 codes 153.0 through 153.9; 154.0 through 154.3 and 154.8 were considered representative of colorectal cancer related death.

3.3.1 Basic analysis

Initial baseline analysis was performed on all patient and tumour characteristics in relation to colorectal cancer related mortality, as detailed in table 3.1. The study population characteristics appeared to be normal with 55% (n=55) of patients being male, 8% (n=8) of patients exhibiting family history of colorectal cancer, and Dukes' staging split as follows: Dukes' A, 23% (n=23); Dukes' B, 26% (n=26); Dukes' C, 34% (n=34); and Dukes' D 16% (n=16). Analysis detailed in table 3.1 was carried out to initially identify variables as potential confounders which may have been influencing patient survival.

Table 3.1 *Baseline population and tumour characteristics according to fate*

	No CRC related mortality (n=62)	CRC related mortality (n=37)	*p*-value
Patient characteristics			
Gender (male)	58% (n=36)	54% (n=20)	0.697
Family history of CRC (yes)	10% (n=6)	5% (n=2)	0.451
Age at diagnosis (years, mean)	68.18	67.92	0.764
(95% CI)	(67.07 - 69.29)	(66.69 - 69.15)	
Tumour characteristics			
Dukes' stage			
Dukes' A	32% (n=20)	8% (n=3)	
Dukes' B	32% (n=20)	16% (n=6)	
Dukes' C	34% (n=21)	35% (n=13)	
Dukes' D	2% (n=1)	41% (n=15)	<0.001 *
Tumour Location			
Proximal	21% (n=13)	49% (n=18)	
Distal	35% (n=22)	30% (n=11)	
Rectosigmoid	18% (n=11)	8% (n=3)	
Rectum	24% (n=15)	14% (n=5)	0.053 *
Tumour Differentiation			
Well differentiated	24% (n=15)	11% (n=4)	
Moderately differentiated	69% (n=43)	62% (n=23)	
Poorly differentiated	6% (N=4)	27% (n=10)	0.010 *
Molecular characteristics			
Ki-ras activating mutation (exon 1)	37% (n=23)	35% (n=13)	0.844
Ki-ras activating mutation (codon 12)	29% (n=18)	22% (n=8)	0.418
APC truncating mutation	44% (n=27)	49% (n=18)	0.622
Ki-ras promoter methylation	3% (n=2)	3% (n=1)	0.948
O^6-MGMT promoter methylation	45% (n=28)	30% (n=11)	0.114
APC promoter methylation	29% (n=18)	35% (n=13)	0.811
hMLH1 promoter methylation	13% (n=8)	14% (n=5)	0.712
Microsatellite Instability (Positive)	3% (n=2)	5% (n=2)	0.367
TP53 Status (Positive)	56% (n=35)	59% (n=22)	0.770
Lamin A/C status (Positive)	47% (n=29)	68% (n=25)	0.044 *
Follow-up			
Cigarette Smoker (yes)	23% (n=14)	14% (n=5)	0.268
Survival Time (years, mean)	4.76	1.59	<0.001 *
(95% CI)	(4.23 - 5.27)	(1.13 - 2.04)	

Simple chi-square (χ^2) analysis of baseline population and tumour characteristics according to fate (colorectal cancer related death or not) showed the strongest variable to be (as expected) Dukes' staging, with more patients in the later Dukes' C and D stages dying of colorectal cancer related mortality than patients in groups A and B ($p<0.001$).

Insofar as tumour location was concerned, the borderline significance ($p=0.053$) represented the increased number of proximal tumours correlating with colorectal cancer related mortality (observed 18, expected 11) and fewer rectosigmoid and rectal cancers correlating with colorectal cancer mortality. However, such observations were not in keeping with previously published work where distal tumours of the colon are typically much more closely associated with colorectal cancer related mortality and proximal tumours classically associated with a better outcome (Thibodeau, Bren et al. 1993). Although it must be said that due to the size of this study population (n=99) and also the presence of small subgroup values [n=3 and n=5 (rectosigmoid, rectum)] such a result could not be considered statistically reliable and in a larger patient population, observations may more closely follow those previously published.

There was a close correlation between tumour differentiation and colorectal cancer related mortality in the study population ($p=0.010$). Here, fewer well differentiated tumours were associated with colorectal cancer related mortality than expected (observed 4, expected 7) and more poorly differentiated tumours were associated with colorectal cancer related mortality than expected (observed 10, expected 5). This was to be expected and supported previously published work (Newland, Chapuis et al. 1981), however small subgroup numbers lead the statistical reliability of such observations to be questionable.

Unfortunately *Ki-ras* promoter methylation data was only available for 20 (20%) of patients and could not be used in analysis. For all other molecular characteristic variables, missing values were below 5% (n=5). Of all the molecular characteristics investigated, lamin A/C status showed the only statistically significant correlation ($p=0.044$) between expression status and colorectal cancer related mortality. Data showed that more lamin A/C positive tumours correlated with colorectal cancer related mortality (observed 25,

expected 20), and lamin A/C negative tumours were more closely correlated with survival (observed 33, expected 28) (p=0.044) (Table 3.1).

As would be expected, there was a significant difference in the survival time of patients between the colorectal cancer related mortality and non-colorectal cancer related mortality groups (p<0.001). The average survival for patients dying of colorectal cancer related mortality was only 1.59 years (95% CI: 1.13 – 2.04), whereas for the non-colorectal cancer related mortality group, it was 3 times longer at 4.76 years (95% CI: 4.23 – 5.27) (Table 3.1).

When the baseline characteristics were cross-tabulated with lamin A/C expression, correlations between variables could be assessed in order to identify potential confounders affecting patient survival (see table 3.2). The majority of factors investigated showed little or no correlation with lamin A/C expression, but the two key variables which cross-tabulated closely with lamin A/C status were - *Ki-ras* activating mutations in codon 12 (p=0.027) and tumour differentiation state (p=0.029). In the first instance, data showed that more lamin A/C positive tumours also harboured an activating mutation at codon 12 in the *Ki-ras* oncogene (observed 19, expected 14) than expected. In the second instance we also see that more lamin A/C positive than expected (observed 12, expected 7) and less lamin A/C negative tumours were than expected (observed 2, expected 7) were poorly differentiated.

Table 3.2 *Baseline population and tumour characteristics according to lamin A/C expression*

	Lamin A/C +ve (n=54)	Lamin A/C −ve (n=45)	p-value
Patient characteristics			
Gender (male)	50% (n=27)	64% (n=29)	0.149
Family history of CRC (yes)	6% (n=3)	11% (n=5)	0.313
Age at diagnosis (years, mean)	68.48	67.60	0.290
(95% CI)	(67.38 - 69.58)	(66.34 - 68.86)	
Tumour characteristics			
Dukes' stage			
Dukes' A	19% (n=10)	29% (n=13)	
Dukes' B	30% (n=16)	22% (n=10)	
Dukes' C	37% (n=20)	31% (n=14)	
Dukes' D	15% (n=8)	18% (n=8)	0.566
Tumour Location			
Proximal	37% (n=20)	24% (n=11)	
Distal	37% (n=20)	29% (n=13)	
Rectosigmoid	11% (n=6)	18% (n=8)	
Rectum	15% (n=8)	27% (n=12)	0.248
Tumour Differentiation			
Well differentiated	20% (n=11)	18% (n=8)	
Moderately differentiated	57% (n=31)	78% (n=35)	
Poorly differentiated	22% (n=12)	4% (n=2)	0.029 *
Molecular characteristics			
Ki-ras activating mutation (exon 1)	43% (n=23)	29% (n=13)	0.158
Ki-ras activating mutation (codon 12)	35% (n=19)	16% (n=7)	0.027 *
APC truncating mutation	41% (n=22)	51% (n=23)	0.302
Ki-ras promoter methylation	2% (n=1)	4% (n=2)	0.413
O^6-MGMT promoter methylation	44% (n=24)	33% (n=15)	0.365
APC promoter methylation	33% (n=18)	29% (n=13)	0.879
hMLH1 promoter methylation	11% (n=6)	16% (n=7)	0.205
Microsatellite Instability (Positive)	6% (n=3)	2% (n=1)	0.391
TP53 Status (Positive)	59% (n=32)	56% (n=22)	0.710
Follow-up			
Cigarette Smoker (yes)	20% (n=11)	18% (n=8)	0.744
Colorectal cancer related mortality	44% (n=25)	29% (n=12)	0.044 *
Survival Time (years, mean)	3.31	3.88	0.145
(95% CI)	(2.67 - 3.96)	(3.16 - 4.59)	

3.3.2 Extended Analysis

Since n=99 in this study, Kaplan-Meier analysis (Kaplan and Meier 1958) (also known as Product Limit Estimator Analysis) of survival was chosen instead of the more powerful multivariate statistical analysis such as Cox proportional hazards (Cox 1972). From baseline analysis, lamin A/C expression was closely correlated with colorectal cancer related patient mortality. Figure 3.3 shows the Kaplan-Meier plot for cumulative survival in relation to lamin A/C status. The graphical representation shows a clear difference in cumulative patient survival between the lamin A/C subgroups during the first 4 years of followup. Patients harbouring lamin A/C negative tumours had a much better cumulative survival compared to their lamin A/C positive counterparts. This supported data provided by simple cross-tabs analysis in table 3.1 and 3.2 and added further strength to these observations since Kaplan-Meier analysis is a much more robust statistical approach than simple chi-square (χ^2) cross-tabulation. This is because Kaplan-Meier analysis takes into account censored data, that is losses from the sample before the final outcome is observed (for instance, if a patient dies before the end of follow-up, but not as a result of colorectal cancer).

Finally, in order to test this correlation further, a binary logistic regression was used to predit the effect of lamin A/C status on colorectal canecr related mortality. Results give an odds ratio of 0.508 (95% CI: 0.219 – 0.995, p=0.047). Whilst this is on the limit of statistical significance (the 95% CI almost embrace unity and the accompanying p-value is just less than 0.05) it nonetheless supports the notion that lamin A/C status is linked to colorectal cacner mortality.

Kaplan-Meier Survival Plot
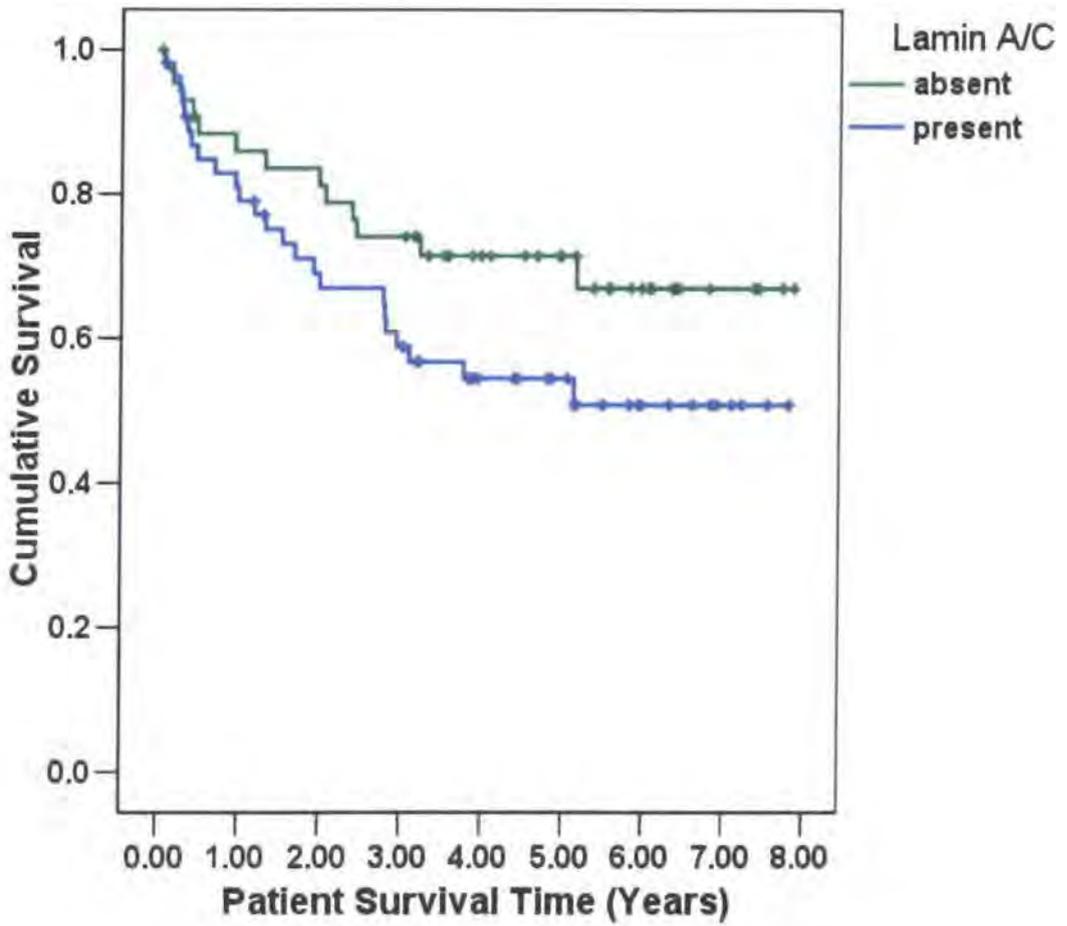
Figure 3.3

Kaplan-Meier plot of cumulative survival for colorectal cancer patients in relation to lamin A/C expression. Data show an increased cumulative survival for patients harbouring lamin A/C negative tumours over patients with lamin A/C positive tumours.

3.4 The NLCS Follow-up

3.4.1 Basic Analysis

With the data obtained from the initial study, there was a strong case to support further study in a much larger patient population, and so the original pilot study was expanded to include the remaining 637 specimens obtained from the NLCS, bringing the total number of specimens immunohistochemically profiled for lamin A/C expression to 737.

When expanding the pilot study, it was important to determine the minimal detectable risk which could be confidently detected within the projected cohort size and follow-up period in order to be sure that data was of sufficient statistical power for results to be accepted. Using standard parameters to test at the 5% significance level (i.e. $\alpha = 0.05$) and with a 90% power (i.e. $\beta = 0.1$), using 737 patients with an available 7 year follow-up, the minimal detectable risk or minimal detectable hazard ratio was calculated as 1.19 (or 0.81). Hazard ratios equal to or above 1.19 (or equal to or below 0.81 depending on the direction of comparison) would reach statistical significance. Concomitantly, in order to detect small anticipated effect sizes ($f^2 = 0.02$), with a significance of 5% and a power of 90%, the minimum population size required was 516. Thus it was safe to assume that the sample size would be large enough for data produced to be accepted as statistically significant.

Of the 737 specimens assessed, 673 (91%) were scored for lamin A/C expression. Those excluded (n=64, 9%) were due to either a poor starting specimen in which there was a total or significant lack of tumour material making scoring unreliable, or whereby there was a lack of internal control (as described in section 2.3)

The subsequent retrieval of information from the associated databases led the total number of specimens to be reduced further to 658 unique cases (89% of original total) due to duplication of patient administration codes. Incidentally, of those duplications in patient material blocks, both slides had been scored blind

for lamin A/C expression and gave the same results in all cases. A further 2 specimens were then also excluded on the grounds that they were later histologically confirmed by a senior pathologist (Professor Adriaan de Bruïne, University of Maastricht, The Netherlands) as signet ring tumours and not adenocarcinomas, which left a total of 656 patients for statistical analysis.

During the follow-up period, from 1st January 1989 until 31st December 1996, 246 patients died, with 163 (66%) of these patients dying as a result of colorectal cancer. Information on primary cause of death as well as, primary, secondary and tertiary complications was retrieved from the PALGA database. Colorectal cancer related death was concluded if colorectal cancer was determined as either the primary cause of death or as a primary, secondary or tertiary complication, using the International Classification of Diseases system. ICD-9 codes 153.0 through 153.9; 154.0 through 154.3 and 154.8 are representative of colorectal cancer related death.

Of the 656 available specimens immunohistochemically assessed, nuclear lamin A/C expression was found to be positive in 463 (70%) patients and negative in 193 (30%) patients.

Simple descriptives were carried out on baseline characteristics first to assess the study population and identify potential factors of interest for further analysis. These are reported in table 3.3 which presents population and tumour characteristics of the follow-up study according to fate. The study population was considered normal with 55% (n=365) patients being male, 10% (n=68) patients exhibiting family history of colorectal cancer and Dukes' staging being distributed as follows: Dukes' A, 25% (n=160); Dukes' B, 33% (n=215); Dukes' C, 26% n=167; and Dukes' D, 13% (n=79). 5% of patients (n=35) were not reported to have been graded and where necessary were excluded from analysis.

Table 3.3 *Baseline population and tumour characteristics according to fate*

	No CRC related mortality (n=493)	CRC related mortality (n=163)	p-value
Patient characteristics			
Gender (male)	56.1% (n=277)	53.9% (n=88)	0.625
Family history of CRC (yes)	11.7% (n=58)	12.2% (n=20)	0.407
Age at diagnosis (years, mean)	68.06	67.60	0.471
(95% CI)	(67.64 - 68.47)	(67.01 - 68.18)	
Tumour characteristics			
Dukes' stage			
Dukes A	30.2% (n=149)	6.7% (n=11)	
Dukes B	36.9% (n=182)	20.2% (n=33)	
Dukes C	22.1% (n=109)	35.5% (n=58)	
Dukes D	5.4% (n=27)	31.9% (n=52)	<0.001 *
Tumour Location			
Proximal	31.0% (n=153)	35.5% (n=58)	
Distal	33.6% (n=166)	26.9% (n=44)	
Rectosigmoid	12.1% (n=60)	9.2% (n=15)	
Rectum	21.7% (n=107)	26.9% (n=44)	0.178
Tumour Differentiation			
Well differentiated	11.1% (n=55)	7.3% (n=12)	
Moderately differentiated	64.0% (n=316)	61.3% (n=100)	
Poorly differentiated	11.7% (n=58)	17.7% (n=29)	
Undifferentiated	0.6% (n=3)	1.8% (n=3)	0.065
Molecular Characteristics			
Ki-ras activating mutation (exon 1)	30.8% (n=152)	49.6% (n=81)	0.077
Ki-ras activating mutation (codon 12)	21.7% (n=107)	37.4% (n=61)	0.132
APC truncating mutation	76.6% (n=378)	90.7% (n=148)	0.586
Ki-ras promoter methylation	2.4% (n=12)	3.6% (n=6)	0.922
O^6-MGMT promoter methylation	36.7% (n=181)	42.9% (n=70)	0.172
APC promoter methylation	32.2% (n=159)	40.4% (n=66)	0.685
hMLH1 promoter methylation	18.0% (n=89)	22.0% (n=36)	0.600
Microsatellite Instability (Positive)	8.1% (n=40)	4.9% (n=8)	0.032 *
TP53 status (Positive)	47.2% (n=233)	69.3% (n=113)	0.215
Lamin A/C status (Positive)	68.1% (n=336)	77.9% (n=127)	0.018 *
Follow-up			
Cigarette Smoker (yes)	24.9% (n=123)	24.5% (n=40)	0.237
Survival Time (years, mean)	5.01	1.59	<0.001 *
(95% CI)	(4.84 - 5.18)	(1.40 - 1.77)	

Based on a typical significance cutoff of $p \leq 0.05$, data from simple analysis carried out (detailed in table 3.3) showed that colorectal cancer related mortality was significantly higher in patients as Dukes' stage increased: Dukes' A, observed 11, expected 40; Dukes' B, observed 33, expected 53; Dukes' C, observed 58, expected 41, Dukes' D, observed 52, expected 20. This was to be expected since it is the original basis of the scoring system devised by Cuthbert Dukes in 1932 (Dukes 1932). Data did not however give strong evidence to support tumour location as a factor influencing colorectal cancer related mortality in this study group. Nor did it offer evidence to suggest that previous family history affects patient outcome supporting the notion that family history is correlated with an increased likelihood of colorectal cancer development, but not associated mortality in patients (Butterworth, Higgins et al. 2006).

Tumour differentiation state showed an indication of trend (p=0.065) and suggested that patients harbouring poorly differentiated tumours are more closely correlated with colorectal cancer related mortality than are patients with moderately or well differentiated tumours again supporting observations which have been well documented in previous work (Newland, Chapuis et al. 1981). This pattern also pertained to undifferentiated tumours, but since n=6 in this sub-group, it could not be considered statistically reliable.

There was no difference in age at diagnosis and colorectal cancer related mortality supporting the notion that outcome is not linked to age at diagnosis. As expected, the mean survival time was significantly lower for the colorectal cancer related mortality patient group. Patients in the non-colorectal cancer related mortality group survived on average 3 times longer. The average survival time of 5.01 years (95% CI: 4.84 - 5.18) for non-colorectal cancer related mortality patients appeared lower than one might have expected but this was due to the follow-up period being capped to a maximum of 7 years. This survival time is often referred to as the "patient at risk" period, i.e. the time t at risk of dying as a result of their primary colorectal tumour following surgery. Thus it can be said that in the 7 years of follow-up, the average survival time, or at risk period of patients either surviving to the end of follow-up or dying but not as a result of colorectal cancer related mortality was 5.01 years 95% CI: 4.84 - 5.18) [vs. 1.59

years (95% CI: 1.40 – 1.77) in the colorectal cancer related patient group, (p<0.001)].

When looking at molecular characteristic data in relation to colorectal cancer related mortality, there was only a single variable which reached statistical significance at baseline considering the scope of factors included. Unfortunately data for *Ki-ras* promoter methylation was only available for 102 (16%) patients (of which 18 exhibited methylation of the *Ki-ras* promoter region). This meant that the data was insufficient to support reliable conclusions in this study. For all other molecular characteristic variables, missing values were below 2% (n=13).

The two molecular characteristics which differed between the two groups were; activating mutations in the *Ki-ras* oncogene which exhibited an indication of trend (p=0.077), suggesting that patients harbouring a *Ki-ras* activating mutation in exon 1 were more closely correlated with colorectal cancer related mortality (observed 81, expected 58) than those not harbouring a *Ki-ras* mutation (observed 152, expected 175). This data follows what has previously been published in regard to the link between *Ki-ras* mutations and patient mortality [reviewed in detail by Bos (Bos 1989)]. The other was Microsatellite Instability as determined by immunohistochemical analysis. Data suggested that patients with microsatellite instable tumours were less likely to be associated with colorectal cancer related mortality following surgery (p=0.032). However, whilst this supports previously published work (Thibodeau, Bren et al. 1993), the number of microsatellite instable tumours in the general population is low (typically just less than 10%) and this was also represented in our study population (7.3%, n=48). Cigarette smoking was found to have no significant detectable effect of colorectal cancer related mortality in this study population.

The most important finding of this study was the very close correlation of lamin A/C status with colorectal cancer related mortality which strongly supported the initial data obtained from the pilot study. The data had a high statistical significance (p=0.018) and from *a priori* power calculations, we know that the sample size is big enough to support statistical credibility. Data showed that lamin A/C positive patients were more closely correlated with colorectal cancer related mortality (observed 127, expected 114) than lamin A/C negative patients

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who were less closely correlated (data shown in table 3.4, [observed 36, expected 49]).

Baseline population and tumour characteristics were then analysed according to lamin A/C expression to determine whether expression correlated with any of the previously mentioned factors. This was carried out to help to identify confounders which would need to be considered in later extended analysis.

Table 3.4 *Baseline population and tumour characteristics according to lamin A/C expression*

	Lamin A/C +ve (n=463)	Lamin A/C -ve (n=193)	p-value
Patient characteristics			
Gender (male)	54.4% (n=252)	58.5% (n=113)	0.334
Family history of CRC (yes)	10.0% (n=46)	11.4% (n=22)	0.581
Age at diagnosis (year, mean)	68.20	67.25	0.010 *
(95% CI)	(67.79-68.61)	(66.67-67.84)	
Tumour characteristics			
Dukes' stage			
Dukes A	21.1% (n=92)	36.6% (n=68)	
Dukes B	37.9% (n=165)	26.9% (n=50)	
Dukes C	26.7% (n=116)	27.4% (n=51)	
Dukes D	14.3% (n=62)	9.1% (n=17)	<0.001 *
Tumour Location			
Proximal	36.5% (n=167)	23.3% (n=44)	
Distal	31.2% (n=143)	35.4% (n=67)	
Rectosigmoid	10.3% (n=47)	14.8% (n=28)	
Rectum	22.0% (n=101)	26.5% (n=50)	0.010 *
Tumour Differentiation			
Well differentiated	11.3% (n=45)	12.9% (n=22)	
Moderately differentiated	71.9% (n=287)	75.4% (n=129)	
Poorly differentiated	16.8% (n=67)	11.7% (n=20)	0.290
Molecular Characteristics			
Ki-ras activating mutation (exon 1)	38.0% (n=176)	29.5% (n=57)	0.046 *
Ki-ras activating mutation (codon 12)	27.6% (n=128)	20.7% (n=40)	0.160
APC truncating mutation	82.% (n=381)	83.% (n=162)	0.814
Ki-ras promoter methylation	2.80% (n=13)	2.59% (n=5)	0.806
O^6-MGMT promoter methylation	38.8% (n=180)	36.7% (n=71)	0.613
APC promoter methylation	36.0% (n=167)	30.0% (n=58)	0.182
hMLH1 promoter methylation	18.% (n=85)	20.% (n=40)	0.434
Microsatellite Instability (Positive)	7.99% (n=37)	5.69% (n=11)	0.287
TP53 status (Positive)	52.6% (n=244)	52.8% (n=102)	0.842
Follow-up			
Cigarette Smoker (yes)	21.5% (n=100)	20.2% (n=39)	0.672
Colorectal cancer related mortality	27.4% (n=127)	18.6% (n=36)	0.018 *
Survival Time (years, mean)	3.73	4.49	<0.001 *
(95% CI)	(3.52-3.95)	(4.17-4.80)	

Based on a typical significance cutoff of $p \leq 0.05$, patients in the lamin A/C positive expression group were older at diagnosis by approximately 1 year [68.20 (95% CI: 67.79 – 68.61) vs. 67.25 (95% CI: 66.67 – 67.84) , $p=0.010$], had considerably higher colorectal cancer related mortality (27.4% vs. 18.6%, $p=0.018$) and exhibited a shortened survival period [3.73 (95% CI: 3.52 – 3.95) years to 4.49 (95% CI: 4.17 – 4.80) years, $p<0.001$] as compared to patients in the lamin A/C negative expression group.

Moreover, Dukes' stage and tumour location were also closely correlated with lamin A/C expression status. A higher proportion of Dukes' A tumours were found in the lamin A/C negative expression group (observed 68, expected 48) as opposed to more Dukes' D (observed 62, expected 55) tumours being present in the lamin A/C positive patient population. Interestingly, Dukes' C patients were observed as expected in both lamin A/C positive (observed 116, expected 117) and lamin A/C negative (observed 51, expected 50) ($p<0.001$). Similarly, within the lamin A/C positive expression group, more tumours were found located in the proximal colon (observed 167, expected 149) than in other regions ($p=0.010$).

As for molecular characteristics, the finding which was most significant was the association of *Ki-ras* activating mutations in exon 1 and lamin A/C status. Data suggested that fewer lamin A/C negative tumours exhibit a *Ki-ras* mutation (observed 57, expected 70) and more lamin A/C positive tumours harbour a *Ki-ras* mutation than expected (observed 176, expected 164) ($p=0.046$). The p-values for the other molecular characteristics were not statistically significant with $p \geq 0.160$ in all cases (table 3.4). Thus it could be concluded that these variables are independent of lamin A/C expression and as such this does not lead one to believe that they may be confounding factors influencing patient survival in this study. The extent of the influence of *Ki-ras* activating mutations and other molecular characteristic variables on patient outcome in relation to lamin A/C status is assessed later in this chapter.

The colorectal cancer related mortality data shown at the bottom of table 3.4 shows that more patients in the colorectal cancer related mortality group are lamin A/C positive (observed 127, expected 114) than expected and less are lamin A/C negative (observed 36, expected 49) than expected ($p=0.018$).

3.4.2 Analysis

More powerful statistical analysis using Cox regression (or proportional hazards regression)(Cox 1972) was used to investigate the effect of each variable individually and also in combination upon patient outcome. Cox regression gives much better estimates of survival probabilities and cumulative hazard than those provided by the simpler Kaplan-Meier function (as carried out in section 3.2.2). Survival probabilities and cumulative hazard ratios were calculated using unadjusted analyses based on the mortality risk for absence vs. presence of lamin A/C expression alone; stratified analyses based on the mortality risk for absence vs. presence of lamin A/C expression in patient subgroups (i.e. tumour location); and multivariate analyses based on absence vs. presence of lamin A/C expression adjusted for potential confounders.

The cumulative hazard at a time t is the risk of dying between time 0 and time t, and the survivor function at time t is the probability of surviving to that time. In this study, the cumulative hazard refers to death as a result of colorectal cancer. Coefficients or hazard ratios (HR) above 1 indicate a worse prognosis (i.e. the hazard; colorectal cancer related mortality, is more likely to occur) and a coefficient of less than 1 indicates a protective effect of the variable (against colorectal cancer mortality) with which it is associated. A coefficient of 1 indicates no effect of the variable on the likelihood of hazard occurrence.

As one would expect unadjusted survival analysis of Dukes' stage versus colorectal cancer related mortality (figure 3.4) showed a decreasing cumulative survival with increasing Dukes' stage, and this powerful analysis results in a graphical illustration of the correlation noted in table 3.3. In a similar unadjusted survival analysis of lamin A/C expression vs. colorectal cancer associated mortality (figure 3.5), the strong correlation between lack of lamin A/C expression and its associated increased cumulative survival (shown in table 3.4) is clearly seen. The relative hazard ratio (HR) is calculated at 0.59 (95%-CI: 0.41 – 0.86, p=0.006) and can be considered the key finding in this study, further supporting the pilot study and linking lamin A/C status to patient prognosis.

Cumulative Survival Function across the Dukes' Stages

Figure 3.4
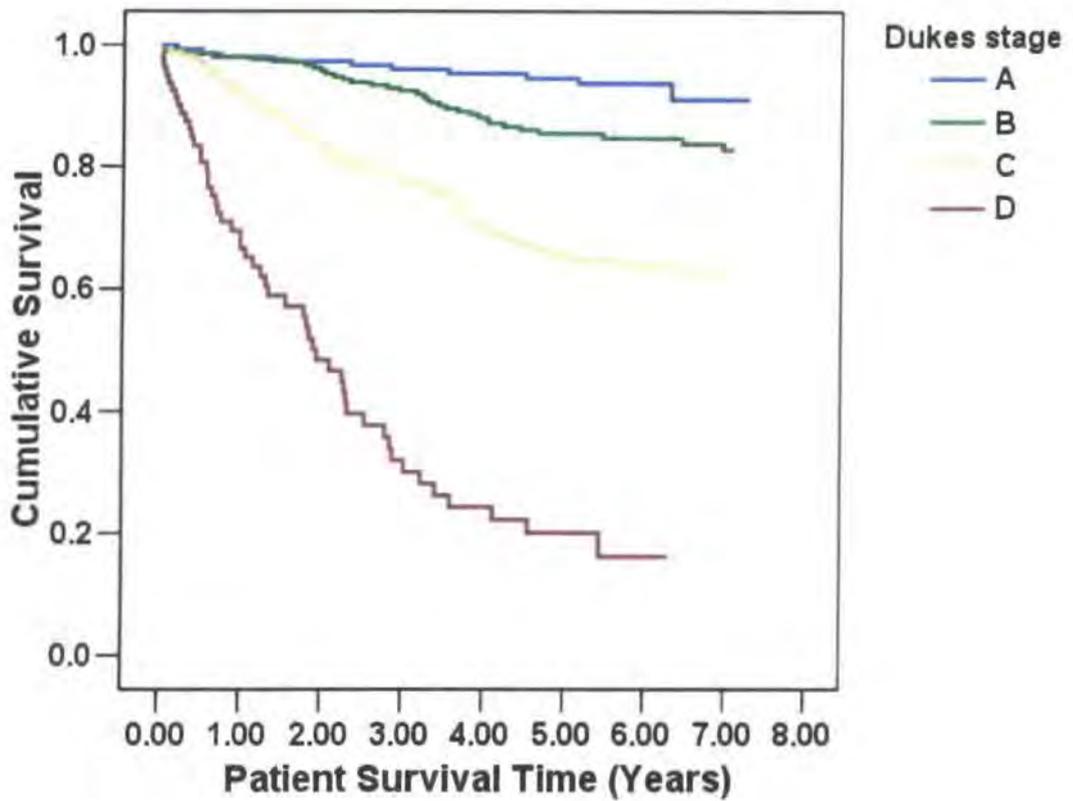
Proportional hazards regression plot of cumulative survival for colorectal cancer patients in relation to Dukes' stage.

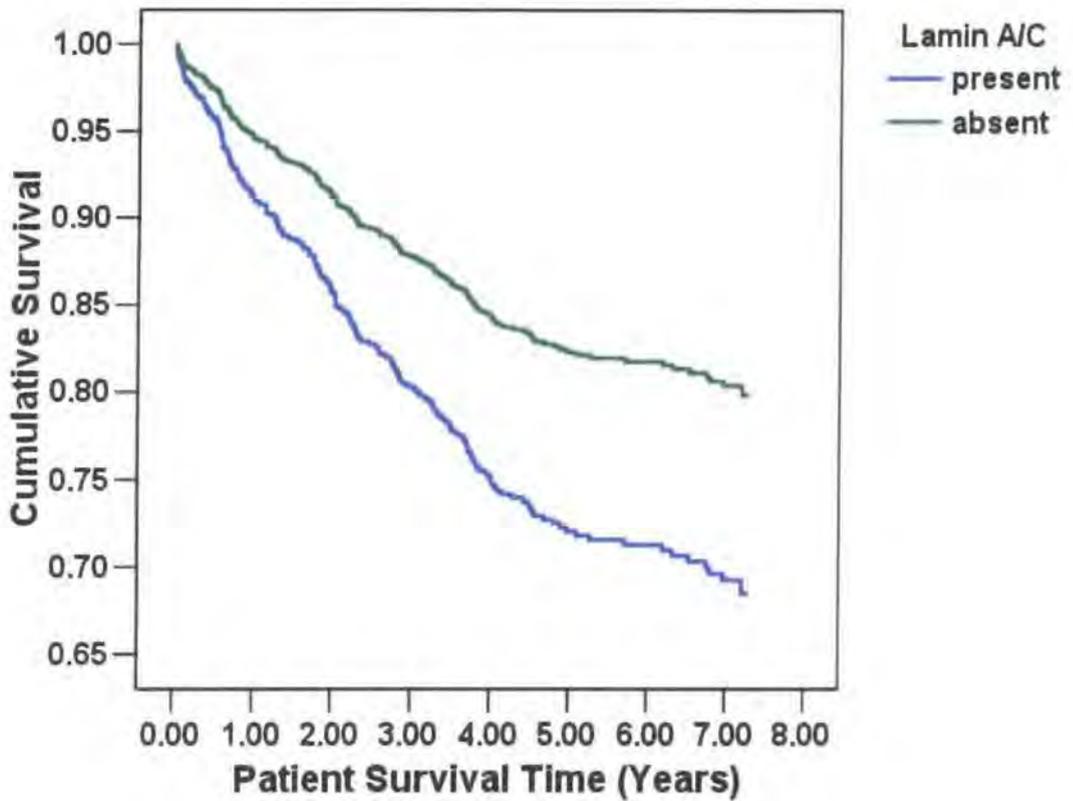**Cumulative Survival Function for Lamin Expression**

Figure 3.5

Proportional hazards regression plot of cumulative survival for unadjusted analysis in terms of differential lamin A/C expression and colorectal cancer related mortality.

HR 0.59 (0.41 – 0.86) p=0.006

The long term survival of Duke's D patients is known to be extremely low, approximately 3% (as discussed in section 1.1.3) and figure 3.4 shows this poor cumulative survival. Thus with this in mind, figure 3.6 shows the stratification of lamin A/C influence on colorectal cancer associated mortality across the Dukes' stages. The apparent protective effect of lamin A/C negative expression in tumours pertains to Dukes' A through C, but appears to be absent in Dukes' D (figure 3.6); Dukes' A: HR 0.10 (0.01 – 0.82) p=0.032, Dukes' B: HR 0.60 (0.25 – 1.45) p=0.254, Dukes' C: HR 0.59 (0.33 – 1.07) p=0.083, Dukes' D: HR 1.01 (0.71 – 1.93) p=0.385. However, the only statistically significant data is for Dukes' A where $p < 0.05$.

To further test the extent of the Dukes' D stage influence on unadjusted survival analysis (figure 3.5) further unplanned exploratory stratified analysis was performed. Figure 3.7A shows the difference in cumulative survival between pre-metastatic (Dukes' stage A through C) and metastatic forms of the disease. Analysis showed that the effect of Dukes' D on the crude unadjusted hazard ratio was indeed present although its influence was considered relatively small. The relative lamin A/C negative vs. lamin A/C positive hazard ratio for Dukes' A, B and C decreased from 0.59 (95%-CI: 0.41 – 0.86), p=0.006 to 0.53 (95%-CI: 0.33 – 0.85) p=0.008 (figure 3.7B) when Dukes' D data was excluded. Since Dukes' D is an advanced post-operative clinical stage of the Dukes' system where distant metastasis has already occurred and will ultimately cause death, it seems logical that the lamin A/C status of the primary tumour bears little or no significant influence on survival based on the parameters of this study.

Further survival analysis showed that decreased colorectal cancer related mortality was associated with patient tumour location in the following manner; rectosigmoid junction, distal colon, proximal colon, rectum (figure 3.8). Whilst this was not statistically significant in simple baseline analysis of patient outcome (table 3.3, χ^2 test p-value = 0.178), tumour location was shown to be correlated to lamin A/C expression (table 3.4, χ^2 test p-value = 0.010). Stratified analysis of tumour location with lamin A/C expression shows that the protective effect observed by lamin A/C negativity in tumours was highest in the rectosigmoid junction (HR 0.22 (95%-CI: 0.05 – 0.96) p=0.044), followed by the rectum (HR 0.58 (95%-CI: 0.29 – 1.14) p=0.113), then the distal colon (HR 0.63 (95%-CI:

0.32 – 1.24) *p*=0.177) with the protective effect being almost non-existent in the proximal colon (HR 0.96 (95%-CI: 0.51 – 1.82) *p*=0.909) (figure 3.9). However, since there appeared to be no statistically significant effect on patient outcome in relation to tumour location and due to non significant *p*-values (*p*>0.05), the only data which could be considered of any significance, would be that from the rectosigmoid junction where the protective effect of lamin A/C expression appeared to be at its greatest, and the *p*-value fell just within the confines of statistically significant. That said, when undertaking stratified analysis such as this, it is important to bear in mind that the power of such analysis decreases. Within the rectosigmoid junction substrata, n=75 which makes it the smallest of the four substratum, and as such the number of patients is insufficient to count such observations as statistically reliable in this case.
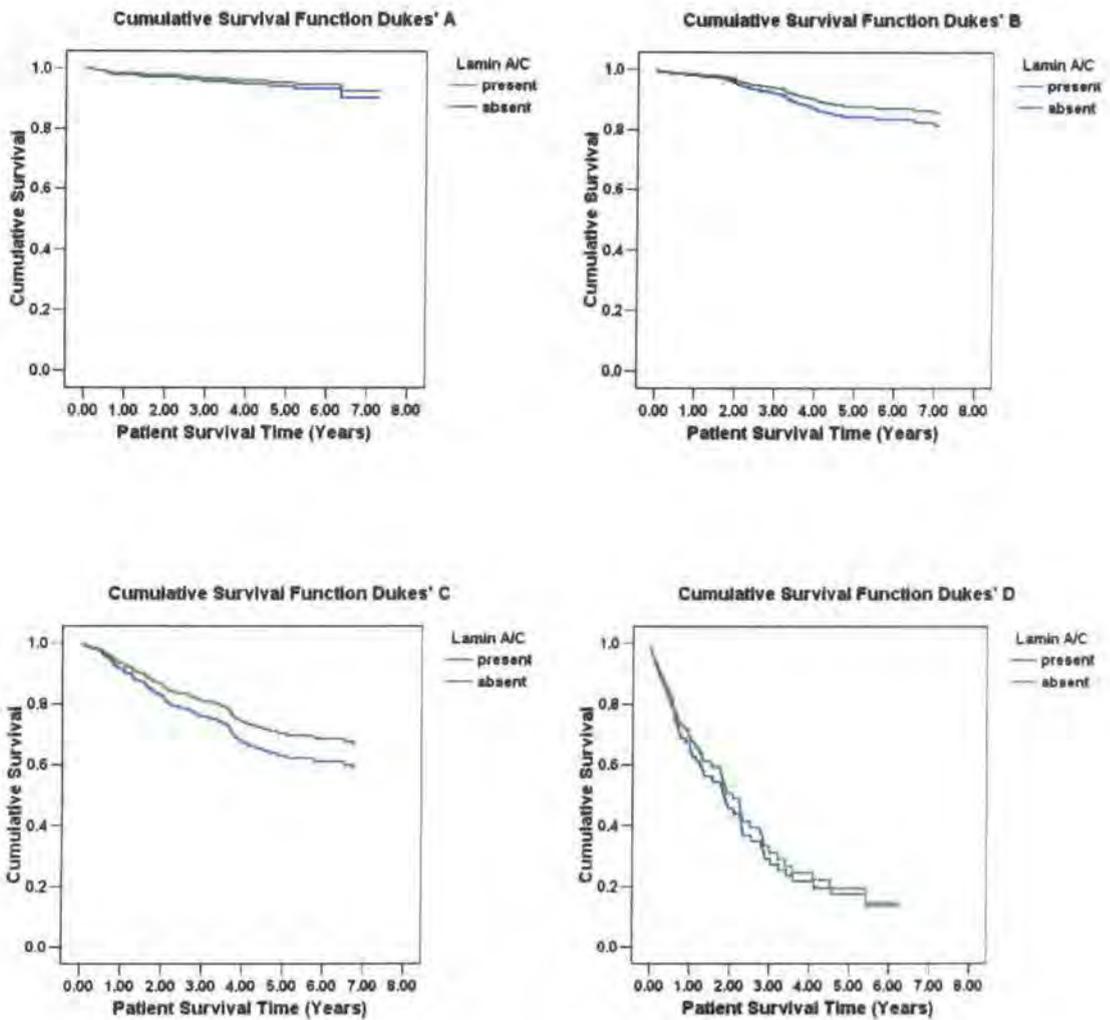
Figure 3.6

Proportional hazards regression plots of survival analysis for differential lamin A/C expression and colorectal cancer related mortality stratified for Dukes' stage. Data shows an increased cumulative survival for lamin A/C negative patients across Dukes' stages A to C, but a loss of this protective effect within the Dukes' D strata.

Dukes' A: HR 0.10 (95% CI: 0.01 – 0.82) p=0.032 (n = 160)

Dukes' B: HR 0.60 (95% CI: 0.25 – 1.45) p=0.254 (n = 215)

Dukes' C: HR 0.59 (95% CI: 0.33 – 1.07) p=0.083 (n = 167)

Dukes' D: HR 1.01 (95% CI: 0.71 – 1.93) p=0.385 (n = 79)

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Figure 3.7

(A) Proportional hazards regression plot of survival analysis for colorectal cancer related mortality stratified for Dukes' stage A, B and C vs. D. Data highlights the significant difference in survival rate between pre-metastatic (Dukes' A, B and C) and metastatic (Dukes' D) tumours.

(B) Proportional hazards regression plot of cumulative survival for differential lamin A/C expression and colorectal cancer related mortality for Dukes' stages A through C only. Data show a small decrease in hazard ratio when Dukes' D patients are excluded (see figure 3.5).

3.7B HR 0.53 (95%-CI: 0.33 - 0.85) *p*=0.008

Cumulative Survival Function according to Dukes' Stage
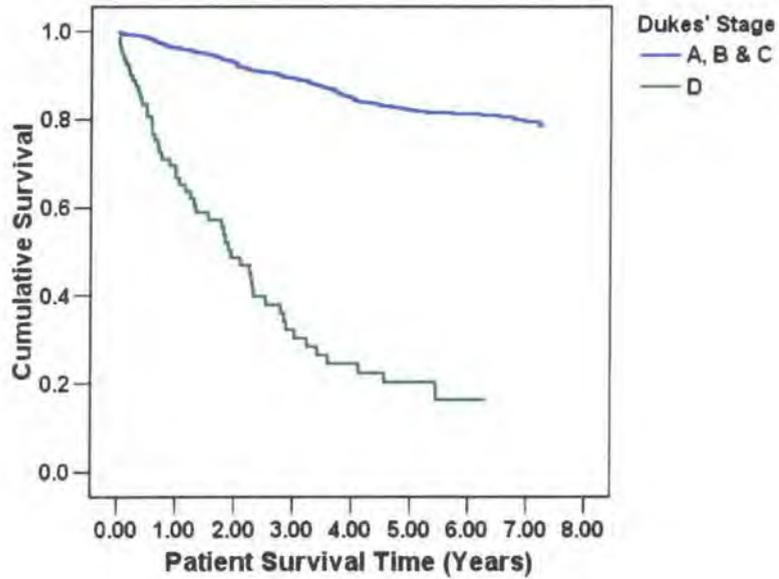
A

Cumulative Survival Function: Dukes' A, B & C

B

117
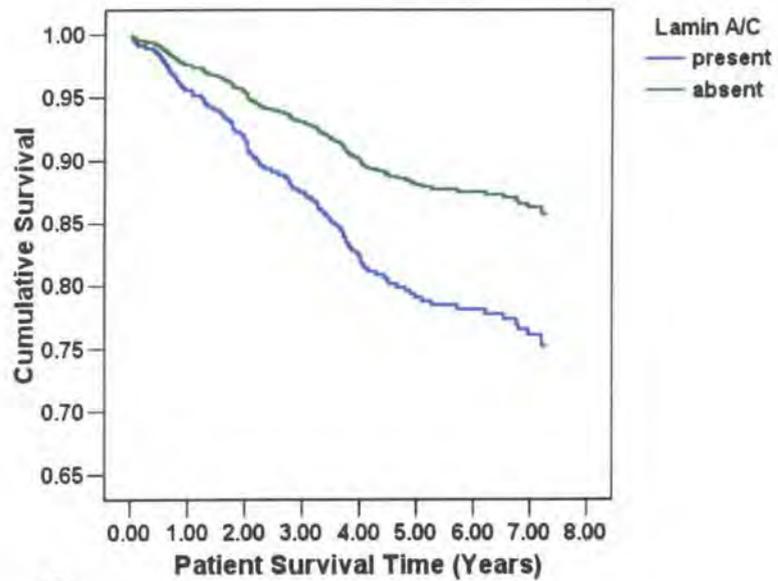
Cumulative Survival Function according to location
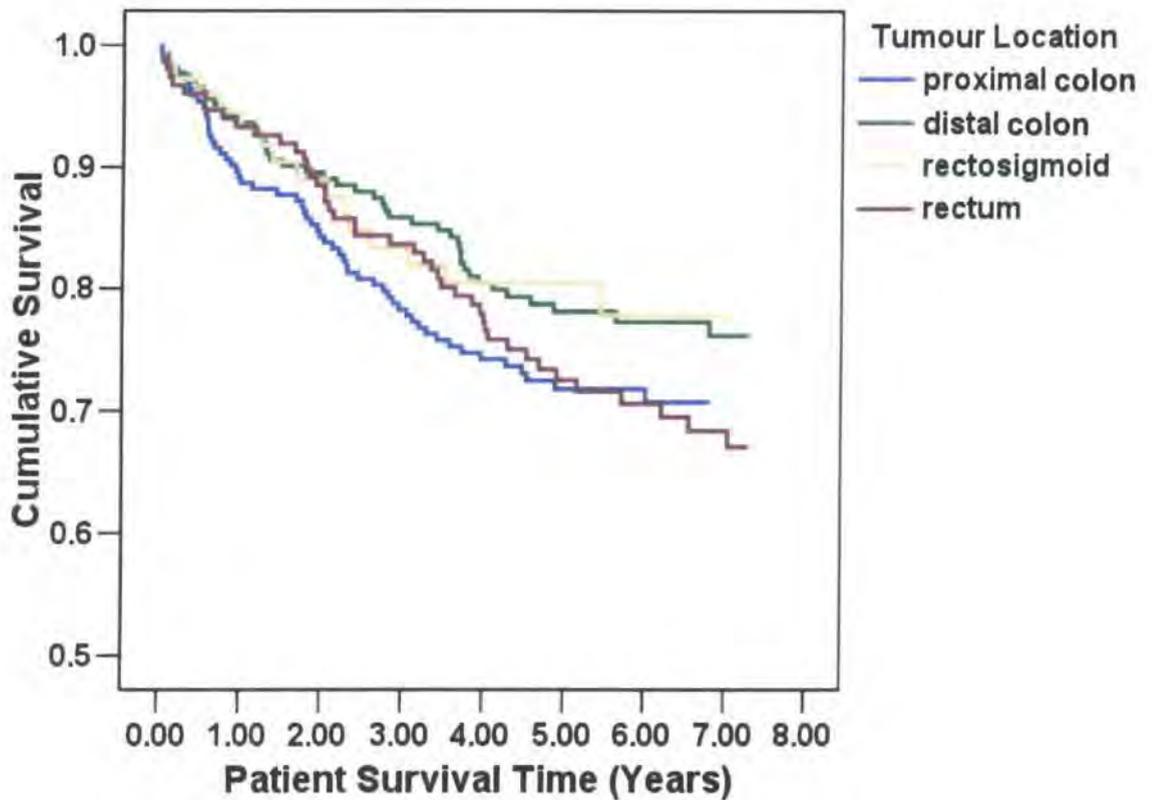
Figure 3.8

Proportional hazards regression plot of cumulative survival for colorectal cancer patients in relation to tumour location. Data shows highest cumulative survival is observed in patients with rectosigmoid tumours and the lowest in patients with rectal tumours.
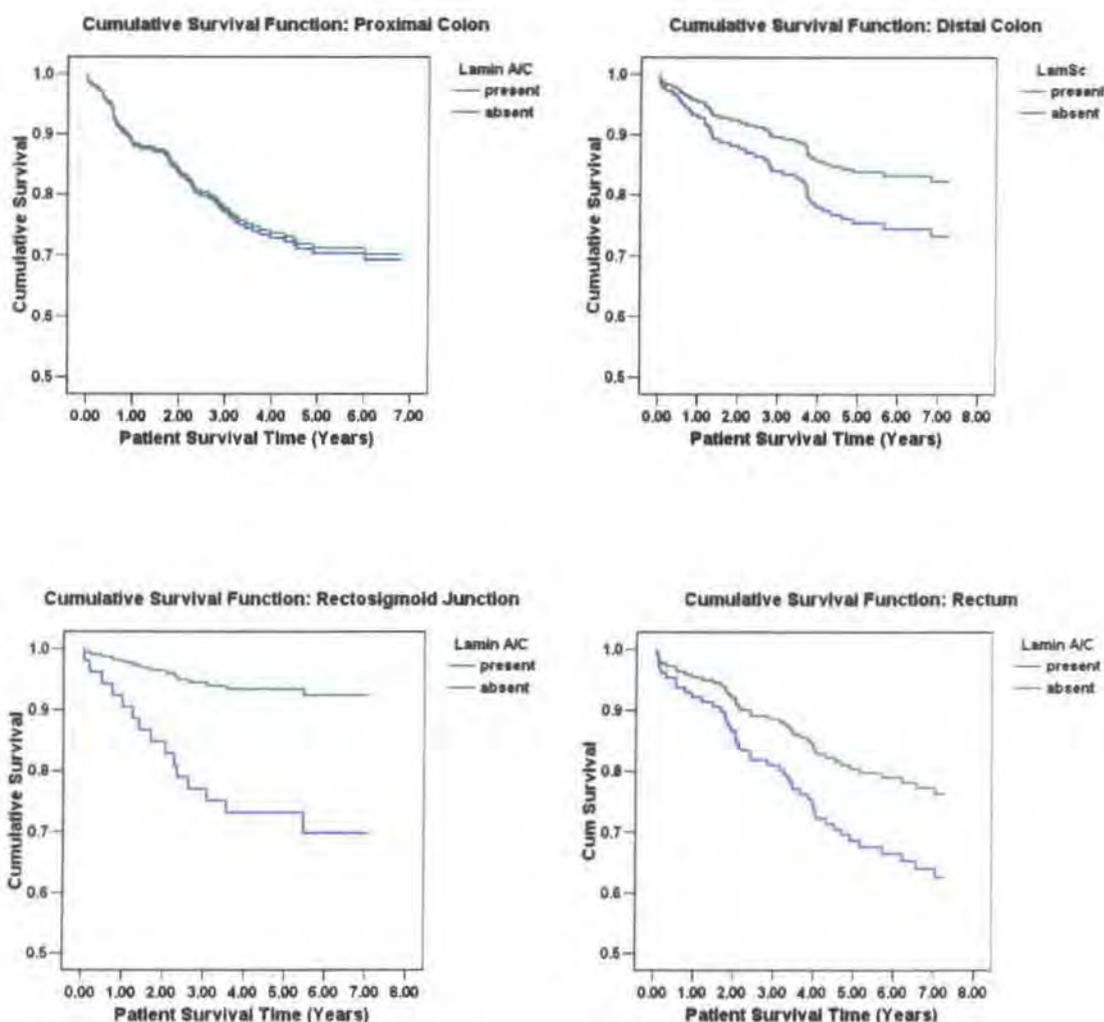
Figure 3.9

Proportional hazards regression plots of survival analysis for differential lamin A/C expression and colorectal cancer related mortality stratified for tumour location. Data shows an increased cumulative survival for lamin A/C negative patients as we move from distal to proximal locations.

Proximal Colon: HR 0.96 (95% CI: 0.51 – 1.82) *p*=0.909

Distal Colon: HR 0.63 (95% CI: 0.32 – 1.24) *p*=0.177

Rectosigmoid Junction: HR 0.22 (95% CI: 0.05 – 0.96) *p*=0.044

Rectum: HR 0.58 (95% CI: 0.29 – 1.14) *p*=0.113

Table 3.5 combines an overview of the stratified analyses of lamin A/C across various factors graphically represented, alongside other analyses which have not been shown graphically but were considered interesting from baseline analysis.

Table 3.5 *Stratified analysis of potential confounders for patient outcome*

Stratification		n	HR	p-value	95% CI Lower	95% CI Upper
Unadjusted		656	0.59	0.006	0.41	0.86
Gender	Male	365	0.58	0.019	0.37	0.92
	Female	291	0.43	0.004	0.24	0.77
Dukes' Stage	A	160	0.10	0.032	0.01	0.82
	B	215	0.60	0.254	0.25	1.45
	C	167	0.59	0.083	0.33	1.07
	D	79	1.01	0.385	0.71	1.93
(Premetastatic)	A→C	542	0.53	0.008	0.33	0.85
Tumour Location	Proximal	211	0.96	0.909	0.51	1.82
	Distal	210	0.63	0.177	0.32	1.24
	Rectosigmoid	75	0.22	0.044	0.05	0.96
	Rectum	151	0.58	0.113	0.29	1.14
Tumour Differentiation	Well	67	0.29	0.100	0.06	1.27
	Moderate	416	0.67	0.064	0.44	1.02
	Poorly	87	0.34	0.041	0.12	0.96
	Undifferentiated	6	1.42	0.775	0.13	2.43
Family history	Yes	68	0.54	0.001	0.38	0.78
	No	588	0.20	0.123	0.03	1.56
Ki-ras activating mutation (exon 1)	No	423	0.64	0.015	0.42	1.05
	Yes	233	0.55	0.025	0.29	1.01
Microsatellite Instability	No	608	0.54	0.475	0.16	1.43
	Yes	48	0.58	0.060	0.39	0.84
Cigarette Smoker	No	517	0.49	0.030	0.209	0.718
	Yes	139	0.68	0.145	0.406	1.159

The stratified analysis data detailed in table 3.5 looked to compare the protective effect of lamin A/C negative tumours on survival across patient subgroups. A change in hazard ratio of more than 10% ($\pm$ 0.06) with a corresponding statistically significant p-value was considered for further analysis.

Stratified analysis for gender showed that in females the protective effect of lamin A/C negative expression in tumours increased from a HR 0.59 to a HR 0.43 (p=0.004) suggesting that women benefited more from tumours being lamin

A/C negative. Data also suggested that the protective effect was marginally increased (HR 0.59 to HR 0.54, p=0.001, n=68) in patients with a family history of colorectal cancer.

Simple baseline analysis of microsatellite instability and patient outcome had suggested a close correlation between the two. However, just as in baseline analysis of microsatellite instability and lamin A/C expression, the stratified analysis showed that there was no reliably detectable evidence to correlate the two directly. That is to say that whilst microsatellite instability as an independent variable may exert a protective effect on patient survival, it had no influence on lamin A/C status. Nor did the correlation between lamin A/C status and colorectal cancer related mortality alter with differential microsatellite instability status.

What was supported from baseline analysis was the correlation between *Ki-ras* activating mutations in exon 1 and lamin A/C expression. The stratified analysis indicated that the protective effect noted in lamin A/C negative tumours was marginally increased (HR 0.59 to HR 0.55, p=0.015) in tumours harbouring a *Ki-ras* exon 1 activating mutation and decreased in patients not harbouring a *Ki-ras* exon 1 activating mutation (HR 0.59 to HR 0.64, p=0.015). Such data called for further multivariate analysis in order to determine the extent (if any) of interaction between the two variables in influencing patient mortality and is discussed later.

Another interesting finding was some clear evidence to suggest that the protective effect of a lamin A/C negative status in tumours was most beneficial in individuals that did not smoke. This is interesting considering that in simple univariate baseline analysis (table 3.3 and table 3.4) there appeared to be no correlation between cigarette smokers and colorectal cancer related mortality, or cigarette smokers and lamin A/C status. Data suggested that the protective effect is increased (HR 0.59 to HR 0.49, p=0.03) in non-smokers.

Multivariate analysis involved observation and analysis of lamin A/C expression as well as another variable at the same time to look for correlations and to test the independence of an observed effect by one variable alone. Figure 3.5 gives an unadjusted hazard ratio of 0.59 for lamin A/C negative tumours vs. lamin A/C positive tumours. Multivariate analysis was carried out and tabulated in table 3.6

showing results from the analyses and highlighting significant changes in the unadjusted lamin A/C hazard ratio in relation to each of the separate covariates.

Table 3.6 *Multivariate analysis of lamin A/C expression with potential confounders*

Multivariate adjustment	HR	p-value	95% CI Lower	Upper
Unadjusted lamin A/C	0.593	0.006	0.409	0.858
Adjusted for;				
Gender	0.593	0.006	0.409	0.859
Family History	0.595	0.006	0.410	0.861
Age at Diagnosis	0.588	0.005	0.405	0.852
Dukes' stage	0.673	0.039	0.462	0.980
Tumour Location	0.609	0.007	0.419	0.884
Tumour differentiation	0.641	0.013	0.436	0.941
Ki-ras activating mutation (exon 1)	0.603	0.007	0.411	0.883
Ki-ras activating mutation (codon 12)	0.602	0.007	0.410	0.881
APC truncating mutation	0.583	0.006	0.398	0.851
Ki-ras promoter methylation	0.592	0.006	0.409	0.857
O^6-MGMT promoter methylation	0.593	0.006	0.409	0.858
APC promoter methylation	0.589	0.005	0.406	0.853
hMLH1 promoter Methylation	0.594	0.006	0.410	0.860
Microsatellite Instability	0.561	0.005	0.379	0.827
TP53 expression	0.591	0.006	0.404	0.864
Cigarette smoker	0.590	0.006	0.407	0.854

Hazard ratios listed in table 3.6 show the relative effect of lamin A/C expression on colorectal cancer related mortality when the influence of the covariate is removed. Multivariate analysis is an extremely powerful statistical technique, much more so than univariate and stratified analyses and uses much more stringent parameters to avoid false positives. Typically one would not undertake such analysis on confounders which show little or no effect in univariate or stratified analysis. However, all factors investigated in univariate and stratified analyses have been included for completeness.

As an example, when we carry out multivariate analysis for gender, we observed no change in the unadjusted hazard ratio (0.59) which indicated that any effect on survival related to patient gender does not influence the protective effect of lamin A/C negative status in tumours. It is worth noting that the stratified analysis which suggested that a lamin A/C negative status was more protective in women

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is still valid, since multivariate analysis removes the influence of the protective effect of gender from the observed protective effect of lamin A/C status. Typically changes of less than 10% (0.06) in the hazard ratio were not considered to be statistically significant.

With the above in mind, when multivariate analysis adjusting for Dukes' staging was carried out, the relative protective effect of lamin A/C negative tumour status was diminished (HR 0.59 to HR 0.67, p=0.039). This was expected since Dukes' stage has an undeniably large effect on patient survival. It is worth noting that none of the other molecular characteristics investigated exhibited any significant influence (on an individual basis) on the effect of lamin A/C status on colorectal cancer related mortality in patients. Similarly where stratified analysis seemed to suggest that non-smokers benefited from the protective effect of lamin A/C negative tumours, this was not evident in multivariate analysis. This was also true for *Ki-ras* activating mutations and microsatellite instability. Such observations are to be expected, since where there was little or no effect shown in univariate analysis of a variable on colorectal cancer related mortality, the removal of this 'non-existent' effect would not affect the observed influence of lamin A/C status on survival.

3.5 Discussion

The availability of access to the NLCS allowed the investigation of lamin A/C expression in a large scale, controlled study. Using an initial pilot study of 100 patients allowed the feasibility of a much larger study to be tested beforehand. Since some of the tumour specimens had been stored for anything up to 20 years before they were used it was important to ascertain whether it would be possible to achieve reproducible results in a study of this design. Following a successful optimisation of previously established protocols (Shi, Key et al. 1991; Igarashi, Sugimura et al. 1994; Kawai, Serizawa et al. 1994; Taylor, Shi et al. 1996; Barker, Huls et al. 1999), the pilot study showed that A-type lamin epitopes can be successfully re-exposed in formalin-fixed, paraffin-embedded tissue sections. With a successful protocol in place which was closely related to those currently used in standard pathology laboratories the full archive could be

accessed and data obtained accepted as both reliable and reproducible. This would have further implications in the role of clinical diagnostic testing.

In short, data shows that patients harbouring lamin A/C positive tumours are almost twice as likely to die than patients of the same clinicopathological grade who harbour a lamin A/C positive tumour [HR 0.59 (95%-CI: 0.41 – 0.86) p=0.006]. When this effect is stratified for the clinicopathological Dukes' staging, it is seen to be at its greatest in the earliest (Dukes' A) stage [HR 0.10 (95%-CI: 0.01 – 0.82) p=0.032 (n = 160)], and almost non-existent in the latest (Dukes' D) stage [HR 1.01 (95%-CI: 0.71 – 1.93) p=0.220 (n = 79)]. Thus, when Duke's D patients are excluded from analysis (since greater that 90% die of colorectal cancer related mortality due to the presence of distant metastases), we see an improvement in the hazard ratio of lamin A/C negative tumours from 0.59 to 0.53 [(95%-CI: 0.33 - 0.85) p=0.008].The inverse of this analysis gives a hazard ratio of is 1.88 [(95%-CI: 1.17 – 3.03) p=0.008] showing that patients with lamin A/C positive tumours are almost twice as likely to die from colorectal cancer related mortality than their clinicopathologically similar lamin A/C negative counterparts across Dukes' stages A to C.

When we undertake multivariate analysis to remove the effect of Dukes' stage on survival from the observed protective effect of lamin A/C negative status in tumours, we see as would be expected, a decrease in the hazard ratio of HR 0.59 to a HR 0.67 [(95%-CI: 0.46 – 0.98) p=0.039]. That is to say, that once any effect on patient survival which is correlated with a particular Dukes' stage is removed, we still see a clear yet diminished protective effect of lamin A/C negative status in tumours. It is important to remember here though that Dukes' stage does not influence the tumour, but that the tumour influences the Dukes' stage, i.e. the tumour is of a particular Dukes' stage due to its behaviour.

However, since the 95% confidence intervals almost embrace unity and the significance is markedly decreased, it suggests that whilst lamin A/C when used in collaboration with the clinicopathological Dukes' staging system is a strong predictor of patient mortality, when used as an independent biomarker it is unable to offer a prognosis equal to or better than Dukes' alone. As has been previously discussed in chapter 1, there is an urgent need to identify prognostic

markers that can be used alongside these clinicopathological staging systems to highlight intermediate stage cancer patients (Dukes' B & C) that suffer tumour recurrence which cannot be detected by the clinicopathological staging systems alone. Thus one would seek to use lamin A/C expression in a clinical setting as a prognositic biomarker alongside the clinicopathological Dukes' staging system to predict high risk intermediate stage patients likely to experience such relapse. Thus lamin A/C status could be used as one of a panel of biomarkers to guide the administration of adjuvant therapies in intermediate stage colorectal cancer.

When stratified for various subgroups, we see that the protective effect is most notable in the following categories; female patients, rectosigmoid tumours, absence of *Ki-ras* activating mutations, Dukes' A tumours and non-smokers.

When we look more closely at lamin A/C expression across the clnicopathological Dukes' staging system, we note that the apparent protective effect of lamin A/C negative expression in tumours pertains to Dukes' A through C; HR 0.53 (95% CI: 0.33 – 0.85) p=0.008. However, within a particular Dukes' stage alone, we only see a statistically significant contribution to the Dukes' A strata patients; Dukes' A: HR 0.10 (0.01 – 0.82) p=0.032, Dukes' B: HR 0.60 (0.25 – 1.45) p=0.254, Dukes' C: HR 0.59 (0.33 – 1.07) p=0.083, Dukes' D: HR 1.01 (0.71 – 1.93) p=0.385. This suggests that independently of Dukes' staging, lamin A/C expression status could only be used as a prognostic marker of mortality in early stage colorectal adenocarcinoma (before invasion of the *muscularis mucosae* occurs). That said, one could speculate that since lamin A/C negative tumours proffer a statistically significant better prognosis in Dukes' A, which also pertains to Dukes' B and C (albeit non-significantly) that lamin A/C expression may be indicative of more aggressive tumour phenotype and hence earmark patients harbouring these tumours as high risk.

When we look at the case of smoking however, we know that colorectal cancer is a multiple step progressive disease which develops over many years. As such it is impossible to tell when ex-smokers quit or when current smokers may have started in relation to the initiation and subsequent progression of the disease. Hence this single variable itself is not accurate enough to implicate it reliably in this study without retrieving further detailed information such as; cigarette

smoking status (never vs. ever and never vs. former or current), frequency (number of cigarettes per day), duration (years), age at first exposure (years), and time since cessation (years). Such information is available within the NLCS archive and could be retrieved for further analysis but was not in this study.

In regard to other stratified variables, data for *Ki-ras* activating mutations in exon 1 is intersting. It has been documented in the international, collaborative RASCAL study that colorectal cancer patients who harbour *Ki-ras* activating mutations exhibit a poorer survival than those who do not due to an associated more aggressive tumour phenotype within these tumours (Andreyev, Norman et al. 1998; Andreyev, Norman et al. 2001). Data shows that there is an increase in the lamin A/C negative HR [0.59 to 0.64 (p=0.015)] for tumours not harbouring *Ki-ras* activating mutations and a decrease in the lamin A/C negative HR [0.59 to 0.55 (p=0.025)] for tumours that harbour a *Ki-ras* activating mutation. This would suggest that tumours harbouring a *Ki-ras* mutation, a lamin A/C negative status may actually allow the tumour to become even more aggressive, thus resulting in a poorer patient outcome than their lamin A/C positive counterparts. Such an observation however calls for further in depth analysis before reliable conclusions can be drawn.

Where we observed an effect of microsatellite instability on patient survival (table 3.3), this effect appears to have little or no influence on the protective effect of lamin A/C status when we consider it as a covariate. However, this observation is limited in that n=48 for MSI positive patients and so there are insufficient numbers in both the lamin A/C negative (n=37) and lamin A/C positive (n=11) groups for statistical reliability.

To date there have been no large scale studies of this type for colorectal cancer, and previous work has reported ambiguous correlations of lamin A/C expression in colorectal adenocarcinomas. Thus findings contained here within break new ground in implicating A-type lamins in colorectal carcinogenesis and most importantly linking them to patient mortality. These results are extremely interesting in the light of work published in other cancer types (BCC, SCC, lung and lymphoid) where loss of lamin A/C expression is correlated with a poorer survival in patients (Venables, McLean et al. 2001; Agrelo, Setien et al. 2005). In

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previous work, the loss of A-type lamin expression is correlated with an increase in aggressiveness of the cancer due to a loss in the regulation of proliferation.

Moreover this work contradicts work by Moss and co-workers (Moss, Krivosheyev et al. 1999) who showed that in all colorectal adenocarcinomas they studied (n=17), there was reduced or absent lamin A/C expression. However, as has been previously discussed, their investigations were limited to a very small number of patients as opposed to the 656 described in this study. The use of a highly sensitive monoclonal anti-lamin A/C antibody and direct comparison with an internal control in every sample used in this study compared to the polycolonal antisera and lack of internal controls used by Moss and co-workers lends greater credibility to the immunohistochemical staining obtained. Most importantly though, the size of the study population and available followup means that for the first time, lamin A/C expression in colorectal tumours could be directly correlated with patient mortality.

The data described in this chapter suggests that positive lamin A/C expression results in a poorer patient survival, therefore suggesting that the loss of lamin A/C expression may be detrimental to the aggressiveness of the tumour in this instance. In previous work in *in vitro* colorectal cancer cell lines, Willis (Willis 2005) has suggested that there is a gradual loss of A-type lamin expression in pre-metastatic colorectal tumours, with returning strong expression in the metastases associated with these tumours. Unfortunately, such findings could not be corroborated in immunohistochemical studies and may serve to highlight the inherent difficulties associated with extrapolating *in vitro* models to *in vivo* situations of this type.

Work carried out concomitantly to this study by Casañs, Cox *et al.* (unpublished data), has focussed on full length colonic crypt profiling and revealed both interesting and relevant data. Results have shown lamin A/C to be a putative marker of adult stem cells located at the base of the colonic crypts. With this in mind, it could shed light onto the apparent protective effect of lamin A/C negative status in tumours. Casañs, Cox *et al* have shown that stem cells residing at the base of colonic crypts immunohistochemically stain strongly positive for lamin A/C using the same JoL2 anti-lamin A/C antibody (Dyer, Kill et al. 1997).

Meanwhile, cells located adjacent to this putative stem cell zone, in the transient amplifying zone are clearly negative for lamin A/C expression. Based on this, the observation that lamin A/C positive tumours result in a poorer cumulative survival in patients could be attributed to differences in a stem-cell like phenotype of the tumour cells. That is to say that lamin A/C negative tumours may possess a phenotype more characteristic of the transient amplifying cells, whereas those lamin A/C positive tumour possess a phenotype more characteristic of the colonic stem cells, thus resulting in a potentially more aggressive phenotype, although the exact causal mechanism remains unclear.

This hypothesis has also been tested further in *in vitro* models by Willis, Cox *et al* (unpublished data). We have shown that changes in lamin A/C expression in the SW480 Dukes' B cell line result in concomitant changes in both actin bundling proteins and cell adhesion molecules at both the RNA and protein level. Such data has led to the conclusion that loss of lamin A/C expression in these cells results in a less motile, more adherent cellular phenotype than the lamin A/C positive counterparts. Such data begins to answer the biological question as to why lamin A/C positive tumours proffer a poorer patient prognosis than their clinicopathologically similar lamin A/C negative counterparts.

It has long been known that whilst clinicopathological staging of solid tumours is the most reliable method for predicting patient outcome and determining patient treatment regimens, it fails to discriminate the innate biological characteristics and behaviour of the these tumours. As such, patients in clinicopathologically similar groups exhibiting markedly different outcomes and also responding in very different manners to adjuvant therapies. The call for promising measurable biomarkers which can be used in conjunction with current staging systems is becoming greater in order to allow more accurate predictions of patient prognosis in these situations where clinical staging alone may be problematic and potentially misleading (Mandrekar, Grothey et al. 2005; O'Connell, Atha et al. 2005; He 2006; Natarajan and FitzGerald 2007).

Data shown in this chapter (figure 3.1 and figure 3.2) serve to highlight this problem clearly. Figure 3.1C and 3.2D are clinicopathologically identical, Dukes' B, moderately well differentiated tumours. Standard classification would not

distinguish the two tumours as different and subsequent recommended treatment regimens would almost certainly be the same for both patients. It is obvious however, that there must be an innate biological difference between these two tumours based on the markedly differential lamin A/C expression patterns. Studies such as this go a long way to justifying the call for biomarker panels to complement current clinicopathological staging systems.

Since we observe no correlation between the clinicopathological status of tumours, i.e. tumour differentiation state and there also appears to be little correlation with other molecular variables investigated, the notion of using lamin A/C as a complementary prognostic marker to the clinicopathological Dukes' stagin system to identify high risk patients for is very promising. Just as Moss and co-workers concluded in theri paper in 1999 (Moss, Krivosheyev et al. 1999) that altered lamin expression may be a biomarker of malignancy in the gastrointestinal tract, it is safe to say that evidence presented herein allows lamin A/C to be considered a sensitive and also independent biomarker of a high-risk colorectal cancer population and as such could be used in conjunction with other markers in order to establish a reliable biomarker panel to guide the therapeutic strategy of colorectal cancer patients.

3.5.1 Relevant Information:

International Patent No: *PCT/GB2007/002316* University of Durham. (2007) *Determining a Prognosis of Colorectal Cancer.*

***Appendix II (p205)* – CRT Licensing Opportunity:** *Lamin A/C: Prognostic marker in colorectal cancer.* Cancer Research Technologies, London, UK.

CHAPTER 4 – Differential lamin A expression does not affect β-catenin activity in the SW480 colorectal cancer cell line

4.1 Background

4.1.1 Wnt signalling and its implication in colorectal cancer

The proto-oncogenic and tumour suppressor effects of Wnt genes was first discovered 25 years ago (Nusse and Varmus 1982) and quickly led to the intense investigation into the role of these genes in human cancer. The later discovery of wingless (*Wg*) the *Drosophila* homolog of wnt-1, then led to the elucidation and assembling of a signalling pathway which was subsequently found to contain many cancer causing genes (Cabrera, Alonso et al. 1987; Rijsewijk, Schuermann et al. 1987).

The normal regulation of cell growth and survival can be subverted by a variety of genetic defects that alter transcriptional programs responsible for controlling cell number. The canonical Wnt signalling pathway is an outstanding example of this. The Wnt ligands, of which there are approximately 20 members in vertebrates, are secreted glycoproteins that are loosely categorised according to their ability to promote neoplastic transformation [for review, see (Wodarz and Nusse 1998)] .

Signalling is initiated by the secreted Wnt proteins, which bind to a class of seven-pass transmembrane receptors encoded by the frizzled genes (Bhanot, Brink et al. 1996; Yang-Snyder, Miller et al. 1996; He, Saint-Jeannet et al. 1997). Activation of the frizzled receptor leads to the phosphorylation of the dishevelled (Dsh) protein which, through its association with axin prevents glycogen synthase kinase 3β (GSK3β) from phosphorylating β-catenin (Itoh, Krupnik et al. 1998; Kishida, Koyama et al. 1999; Lee, Ishimoto et al. 1999; Peters, McKay et al. 1999; Smalley, Sara et al. 1999). Unphosphorylated β-catenin then escapes recognition by β-transducin repeat containing protein (β-TrCP), a component of

an E3 ubiquitin ligase and is able to translocate to the nucleus of the cell where it engages transcription factors such as Tcf and Lef (Behrens, von Kries et al. 1996; Molenaar, van de Wetering et al. 1996; Hart, Concordet et al. 1999). Additional components in the pathway include casein kinases I and II, both of which have been proposed to phosphorylate dishevelled (Dsh) (Willert, Brink et al. 1997; Peters, McKay et al. 1999; Sakanaka, Leong et al. 1999) (see figure 1.7).

Although Lef/Tcfs bind directly to DNA through their high-mobility group (HMG) domains, they are incapable of independently activating gene transcription (Eastman and Grosschedl 1999; Roose and Clevers 1999). This has best been illustrated for Lef, which through its binding to the cofactor ALY, makes indirect contacts with a second transcription factor AML (Bruhn, Munnerlyn et al. 1997). The Tcfs however do not contain the ALY binding site and like Lefs they cannot independently activate genes. However, target genes are transcribed on co-activation with β-catenin suggesting that β-catenin supplies the additional cofactors required for transcriptional activation (Molenaar, van de Wetering et al. 1996).

The simple interpretation is that the Tcf/Lef-β-catenin complex comprises a bipartite positive acting transcription factor in the canonical Wnt signalling pathway. This interpretation agrees well with developmental studies in which the manipulation of Lef/Tcf function results in phenotypes consistent with the genetic manipulation of Wnt/β-catenin signalling (Behrens, von Kries et al. 1996; Huber, Korn et al. 1996; Brunner, Peter et al. 1997; van de Wetering, Cavallo et al. 1997). Thus the manifestation of cancer by aberrant Wnt signalling is known to result from inappropriate gene activation mediated by stabilised β-catenin.

We now know that at least three regulatory genes in the Wnt signalling pathway are mutated in primary human cancers and several others promote experimental cancers in rodents. The primary cause in colorectal cancer is mutations affecting the Adenomatous Polyposis Coli (APC) gene (as discussed in section 1.2). Other mutations include those in axin, GSK3β and β-catenin itself. Nonetheless, in all of these cases the common denominator is the constitutive activation of gene transcription by β-catenin.

Recent evidence has been published which also implicates Emerin (a lamin A/C type II integral membrane protein target) in the regulation of β-catenin activity. This regulation is mediated through the APC-like tail domain of Emerin. When over-expressed in HEK293 cells, β-catenin is restricted to the cytoplasm and activity is inhibited. Expression of dominant negative mutant Emerin (lacking its APC-like tail domain) results in constitutive β-catenin activity and enhanced nuclear accumulation (Markiewicz, Tilgner et al. 2006). Such evidence has suggested that inner nuclear membrane proteins such as Emerin can influence cell signalling pathways by restricting access of transcription co-activators to the nucleus (Markiewicz, Tilgner et al. 2006). Whilst such data holds true for the HEK293 and human fibroblasts, to date it has not been investigated in colorectal cancer or the gastrointestinal epithelia.

4.1.2 The Adenomatous Polyposis Coli (APC) protein

The tumour suppressing APC gene is located on chromosome, 5q21 and encodes a large protein (312kDa) that is involved in many cellular processes . Central to APC's tumour suppressive function is its down-regulatory capacity of β-catenin, a key component of the Wnt signalling transduction pathway. This pathway plays an important role in both embryonic development and tumourigenesis. In the central region of APC, seven motifs of 20 amino acids have been identified, which not only bind β-catenin but also facilitate down-regulation of β-catenin through interactions with axin and GSK3β . The spectrum of APC mutations is large, with inactivation usually mediated through truncation of the protein removing the central and N-terminal parts of the APC gene. The result is complete or partial loss of function allowing β-catenin to accumulate in the cell and translocate to the nucleus where it serves as a co-activator of the Tcf/Lef proteins and activates transcription of Wnt target genes .

Most sporadic colorectal cancers have an APC truncation in one allele and loss of heterozygosity in the other allele (Kinzler and Vogelstein 1996; Rowan, Lamlum et al. 2000). The APC mutations associated with cancer are consistently truncated before amino acid 1638 and hence lack the axin binding domains (Smits, Kielman et al. 1999). The APC protein is pivotal in controlling β-catenin

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activity in colonic epithelia and disruption of this tight regulation results in colorectal neoplasia. Thus it is also important in this chapter to assess the effect (if any) of differential lamin A expression on β-catenin activity in a system where the functional regulation of β-catenin by APC is in place.

The SW480 colon cancer cell line has previously been transfected with full length functional APC constructs and shown to partially restore β-catenin regulation in these cells (Neufeld, Zhang et al. 2000). Using the same pFlag-APC construct, the SW480-GFP and SW480-GFP-lamin A cell lines were transfected to restore APC function and to enable the potential effects of differential lamin A expression on β-catenin activity to be evaluated.

4.1.3 Selection of colorectal cancer cell lines

Colorectal carcinoma is known to develop over many years through a series of pathologically and histologically distinct stages. By explanting tissue from individual stages, cell lines can be created representing each order of malignancy and enabling *in vitro* studies of the molecular characteristics concomitant with disease progression. Previous work has already established a model system of cell lines with which the nature of A-type lamin expression in respect to colorectal cancer development could be investigated (Willis 2005).

ECACC is a European cell repository of cell lines, particularly those derived from tumours. From this source, the SW480 colorectal cancer cell line – established from a Broders' grade IV (Broders 1925) (Dukes' B) adenocarcinoma of the colon in a 50 year old white Caucasian male (Leibovitz, Stinson et al. 1976) was obtained. The SW480 colorectal cancer cell line produces carcinoembryonic antigen (CEA), typical of colorectal adenocarcinomas and harbours a type I truncating mutation in the APC protein. That is to say that the truncated protein still retains its n-terminal Nuclear Export Signal (n-NES) but lack the c-terminal Nuclear Export signals (c-NESs). It has also been shown to be naturally deficient in lamin A (Willis 2005) and as such the cell line had been previously stably transfected with EGFP-lamin A and EGFP constructs (as described in section

2.4.2) providing an *in vitro* model system whereby the effects of differential lamin A expression can be evaluated.

4.1.4 Summary

Evidence from chapter 3 has shown a protective effect of lamin A/C negative status in patient tumours. In this chapter I address the hypothesis that differential lamin A expression in an *in vitro* colorectal cancer cell line affects the activity of the transcriptional co-activator β-catenin either directly or via its binding partners. The effects of differential lamin A expression on β-catenin activity are investigated in the presence of both the non-functional truncated and full length functional forms of the APC protein. Data show that the canonical Wnt signalling pathway is not directly implicated as the mediator of differential prognosis in the lamin A/C positive vs. negative tumour patients.

4.2 Results

4.2.1 Lamin A expression does not affect β-catenin activity

Dual luciferase TOPFlash reporter assays (Korinek, Barker et al. 1997) were carried out to determine the effect of differential lamin A expression on the relative activity of β-catenin in the SW480 colon cancer cell line. These assays are used widely to measure the transcriptional activity of TCF and β-catenin [pTOPFlash contains multimerised Tcf-binding sites linked to a luciferase reporter; (Korinek, Barker et al. 1997)].

Transfection of the SW480 clones was very successful, and their TOPFlash activity as a ratio of firefly luciferase:*Renilla* values were determined. The SW480-GFP and SW480-GFP-Lamin A clones were also transfected with pFOPFlash (whose Tcf-binding sites are mutated). All FOPFlash values were exceedingly low, confirming the specificity of the Tcf/β-catenin input. Triplicate data did not show any significant difference in relative TOPFlash mediated β-catenin activity between the SW480-GFP-lamin A or SW480-GFP lines (Figure

4.1A) Δ = 7.3%, *t* = 1.0454, *df* = 4, *p* = 0.3548. Concomitantly to this, there was no statistically significant differences in relative β-catenin activity for untransfected and FOPFlash transfected clones; Δ = 5.0%, *t* = 0.2999, *df* = 4, *p* = 0.7792 and Δ = 29.5%, *t* = 2.6835, *df* = 4, *p* = 0.0550 retrospectively.

Figure 4.1B shows whole cell extract immunoblotted with the anti-lamin A/C JoL2 mouse monoclonal antibody (Dyer, Kill et al. 1997). Data shows a clear expression of both GFP-lamin A and stabilised endogenous lamin A in the GFP-lamin A transfected cell line vs. control.

Figure 4.1

(A) Relative β-catenin activity as measured by Dual luciferase TOPFlash reporter assays. Measurements are arbitrary firefly luciferase luminosity relative to *Renilla* luminosity. Data shows no significant difference in relative TOPFlash luciferase activity between the SW480-GFP and SW480-GFP-lamin A clones.

TOPFlash Δ = 7.3% t = 1.0454, df = 4, p = 0.3548.
FOPFlash; Δ = 29.5%, t = 2.6835, df = 4, p = 0.0550.
Untransfected; Δ = 5.0%, t = 0.2999, df = 4, p = 0.7792.

(B) Whole cell extract from SW480-GFP (lane 1) and SW480-GFP-lamin A (lane 2) resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with mAb JoL2 – anti-lamin A/C (Dyer, Kill et al. 1997). Equal loading was confirmed by co-blotting for β-actin. Data shows an absence of lamin A in the SW480-GFP cells, alongside the presence of GFP-lamin A and stabilised endogenous lamin A in the SW480-GFP-lamin A cells.

Molecular markers are in kDa.

(A)

SW480 β-Catenin Reporter Assays

Legend:
- ■ Untransfected
- ▦ TOPFlash
- ☐ FOPFlash

x-axis: SW480-GFP, SW480-GFP-lamin A
y-axis: Relative luciferase activity

(B)

Lane 1, Lane 2

Molecular weight markers: 120, 99, 53, 38

Bands:
- ← GFP-lamin A
- ← lamin A
- ← lamin C
- ← β-actin
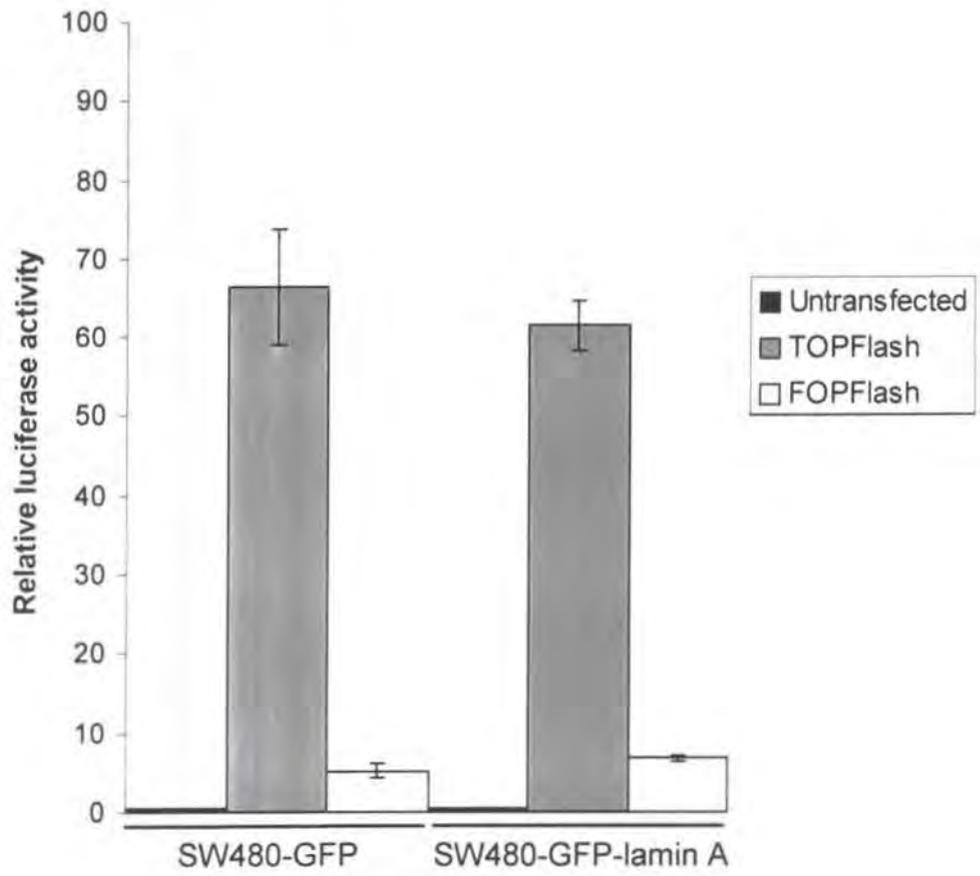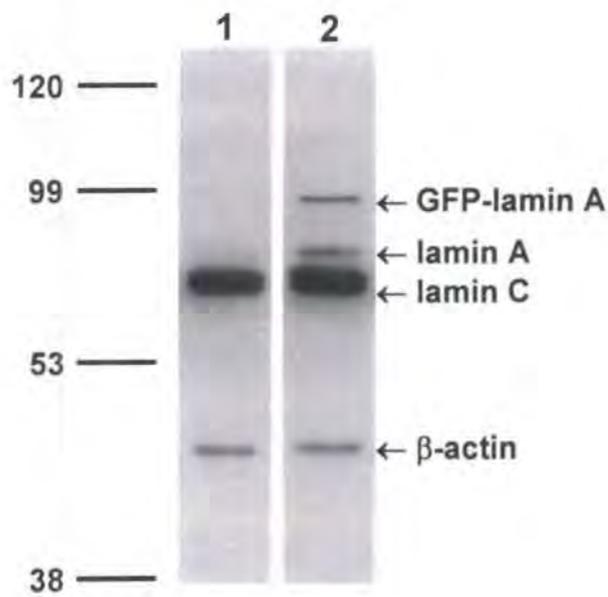
4.2.2 pFlag-APC transfection of the SW480 clones

Both the SW480-GFP and SW480-GFP-Lamin A clones were successfully transfected with the full length pFlag-APC construct. Following successful transformation of the *Escherichia coli* strain DH5α, the pFlag-APC construct was extracted and purified. After extraction and purification, gel electrophoresis yielded a single band of approximately 14,000 bp when run against known DNA standards (figure 4.2A). PCR analysis on the purified product using the APC primers detailed in table 2.2 and subsequent gel electrophoresis yielded the expected fragment size of roughly 950 bp indicating the plasmid was intact and also contained the full-length APC (figure 4.2B).

Following successful transfection of the pFlag-APC construct into the SW480 clones, APC expression was determined at both the RNA and protein level. Confirmation at the RNA level was via RT-PCR of whole cell RNA extracts using primers designed specifically for the n-terminal region of the APC gene as detailed in table 2.2, (figure 4.3B). This region is lost by the type I truncating mutation associated with the SW480 colon cancer cell line and served to highlight expression of the transfected full length APC construct. β-actin levels were also monitored to ensure equal amounts of cDNA had been reverse transcribed and that the reverse transcription system and PCR reactions were working successfully (figure 4.3A). Data show a clear band visible at approximately 950 bp in the pFlag-APC transfected SW480-GFP-lamin A clone and an absence of this band in the untransfected SW480-GFP-lamin A clone indicating successful expression of the pFlag-APC construct at the RNA level (figure 4.3B).

Following a successful confirmation at the RNA level, functional expression of the pFlag-APC construct was evaluated by one-dimensional SDS-PAGE and immunoblotting of whole cell extracts as detailed in section 2.5 (figure 4.4). Data show equal β-actin loading (A) and equal expression levels of the endogenous truncated APC protein, as well as a lower expression of the pFlag-APC construct (*) containing full length APC, in the pFlag-APC transfected SW480-GFP-lamin A clone (B).
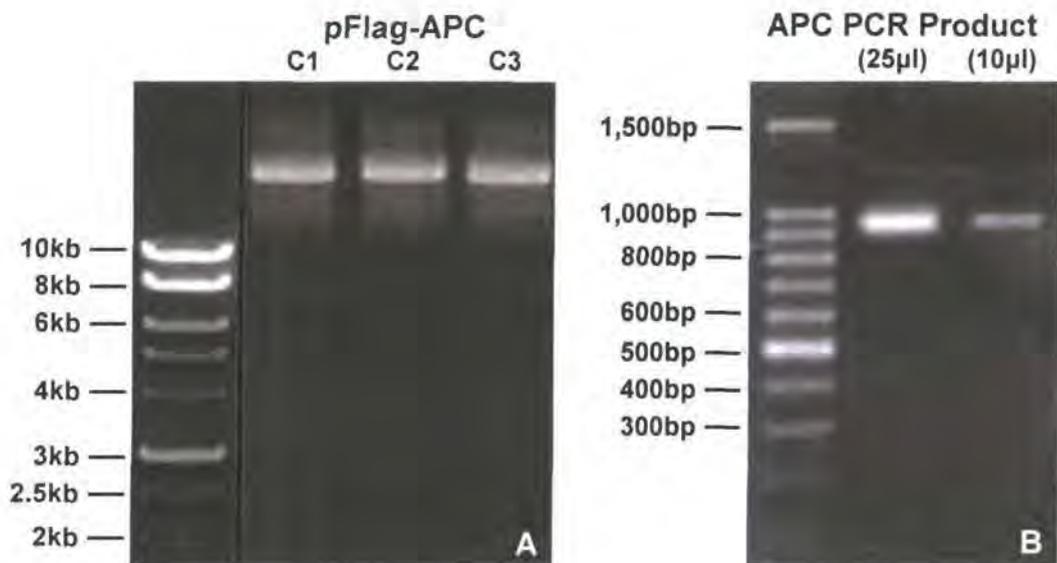
Figure 4.2 Gel electrophoresis of pFlag-APC construct extracted and purified from *E.coli* DH5α.

(A) Plasmid extraction and purification of three bacterial colonies with bands showing at approximately 14,000 bp representative of the full length pFlag-APC construct.

(B) PCR of extracted and purified plasmid DNA for n-terminal APC fragment. Bands at approximately 950bp indicate the presence of the 958bp cloned n-terminal APC fragment.
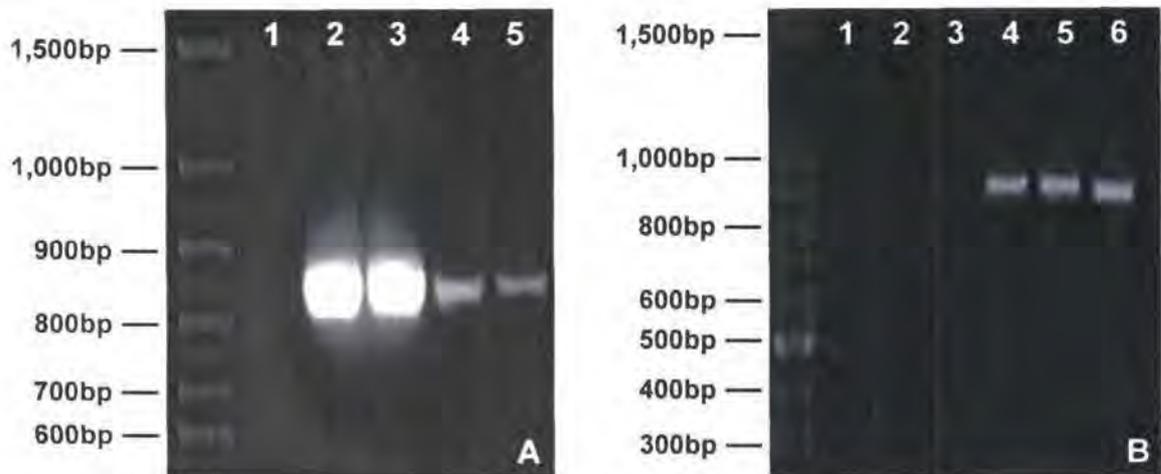
Figure 4.3

(A) β-actin RT-PCR. Lane 1, negative control; Lane 2, SW480-GFP (10μl loading); Lane 3, SW480-GFP-lamin A (10μl loading); Lane 4, SW480-GFP (1μl loading); Lane 5, SW480-GFP-lamin A (1μl loading). Data shows that β-actin levels are consistent between clones.

(B) APC RT-PCR. Lane 1-3, Untransfected SW480-GFP-lamin A; Lanes 4-6, SW480-GFP-Lamin A; pFlag-APC. Data shows no band in any of the 3 untransfected SW480-GFP-lamin A lines (lanes 1-3) indicating the absence of the pFlag-APC construct. Meanwhile a clear band is observed in all 3 of the SW480-GFP-Lamin A; pFlag-APC lines (lanes 4-6) representative of successful pFlag-APC transfection, at approximately equal efficiencies.

Figure 4.4 Western Blot analysis of pFlag-APC and β-actin expression.

(A) Whole cell extracts from SW480-GFP-lamin A (lane1) and SW480-GFP-Lamin A; pFlag-APC (lane 2) resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with anti-β-actin which was used as a loading control.

(B) Whole cell extracts from SW480-GFP-lamin A (lane1) and SW480-GFP-lamin A; pFlag-APC (lane 2) resolved on a 5% SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with anti-APC antibody (Ab-1).

Immunoblots show an equal loading as revealed by β-actin staining (A). Blots also show a comparable level of endogenous truncated APC (~180kDa) (B) and a clear band in SW480-GFP-Lamin A pFlag-APC transfected line (B, lane 2) at approximately 320kDa indicating a positive expression of the construct at the protein level.

Molecular markers are in kDa
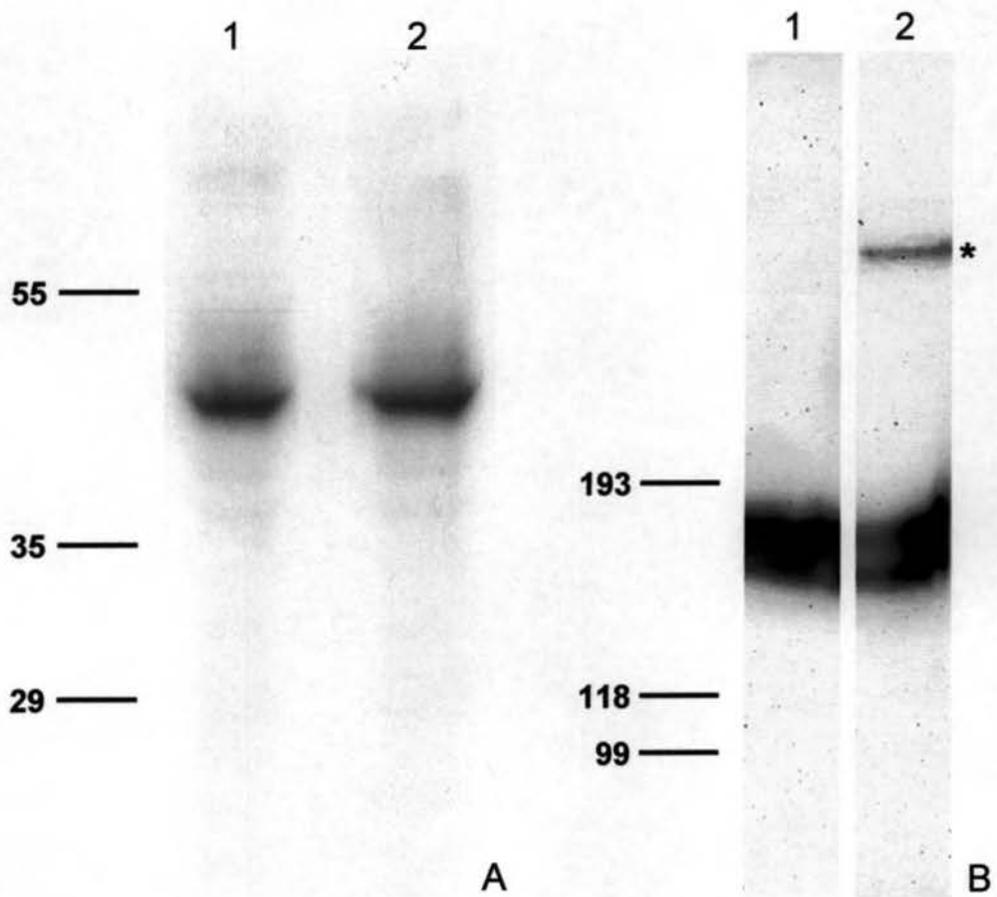
4.2.3 In the presence of full length functional APC, lamin A does not affect β-catenin activity

Following successful transfection of the SW480-GFP and SW480-GFP-Lamin A cell lines with the pFlag-APC construct, dual luciferase TOPFlash reporter assays were carried out again to assess the influence of lamin A expression on β-catenin activity in the presence of the fully functional APC protein.

Data from reporter assays (figure 4.5) show that there is still no effect on β-catenin activity in either the presence or absence of GFP-lamin A with full length functional APC. Data shows a difference in relative TOPFlash luciferase activity between the SW480-GFP;pFlag-APC and SW480-GFP-lamin A;pFlag-APC clones of; Δ = 7.5%, t = 0.9180, df = 4, p = 0.4106. This is comparable to the 7.3% change observed in figure 4.1. β-catenin reporter activity is however repressed by approximately 40% upon transfection with pFlag-APC as would be expected due to the partial reinstating of its regulation (figure 4.6).

SW480 (pFlag-APC) β-Catenin Reporter Assays
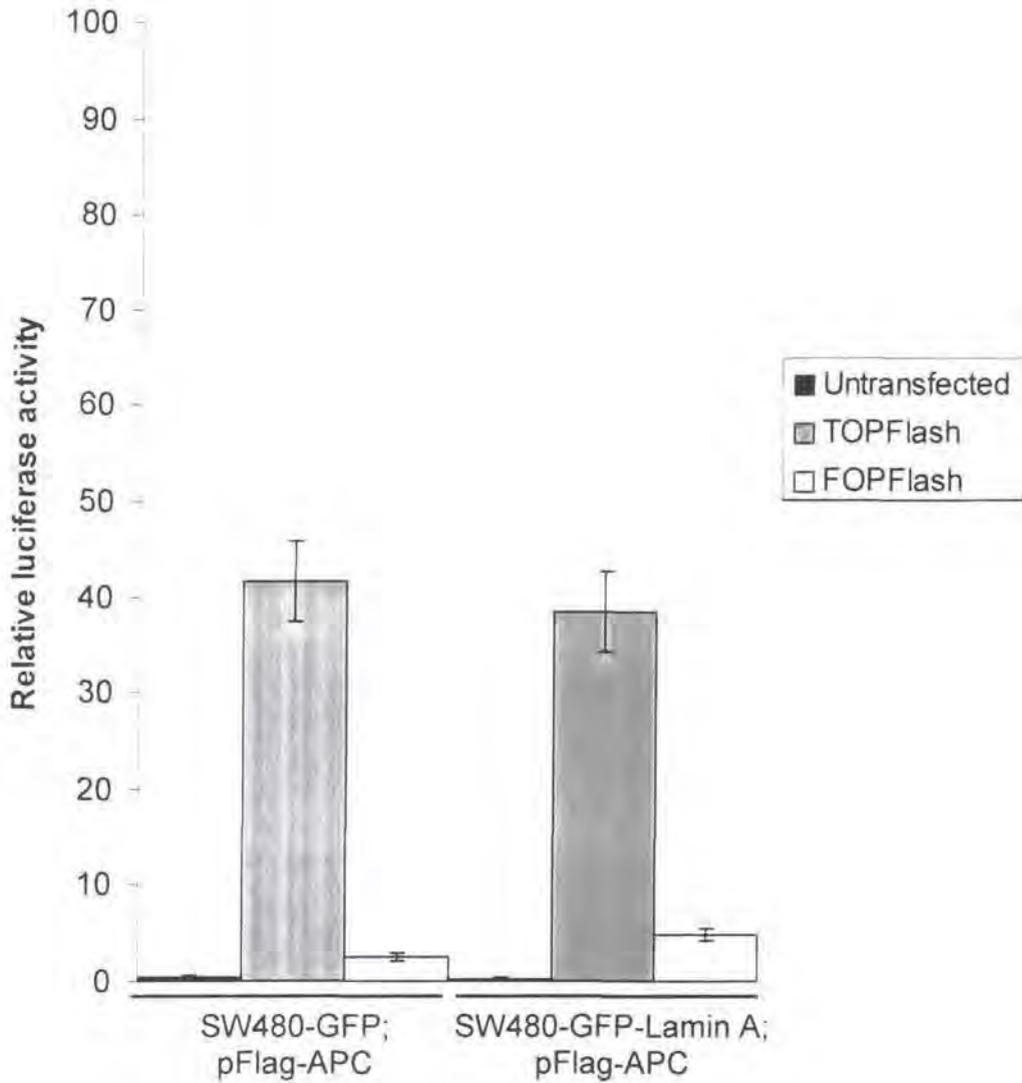
Figure 4.5

Relative β-catenin activity as measured by dual luciferase TOPFlash reporter assays. Measurements are arbitrary firefly luciferase luminosity relative to *Renilla* luminosity. Data shows no significant difference in relative TOPFlash firefly luciferase activity between the SW480-GFP; pFlag-APC and SW480-GFP-lamin A, pFlag-APC transfected clones; $\Delta = 7.5\%$, $t = 0.9180$, $df = 4$, $p = 0.4106$.

SW480 (pFlag-APC) β-Catenin Reporter Assays
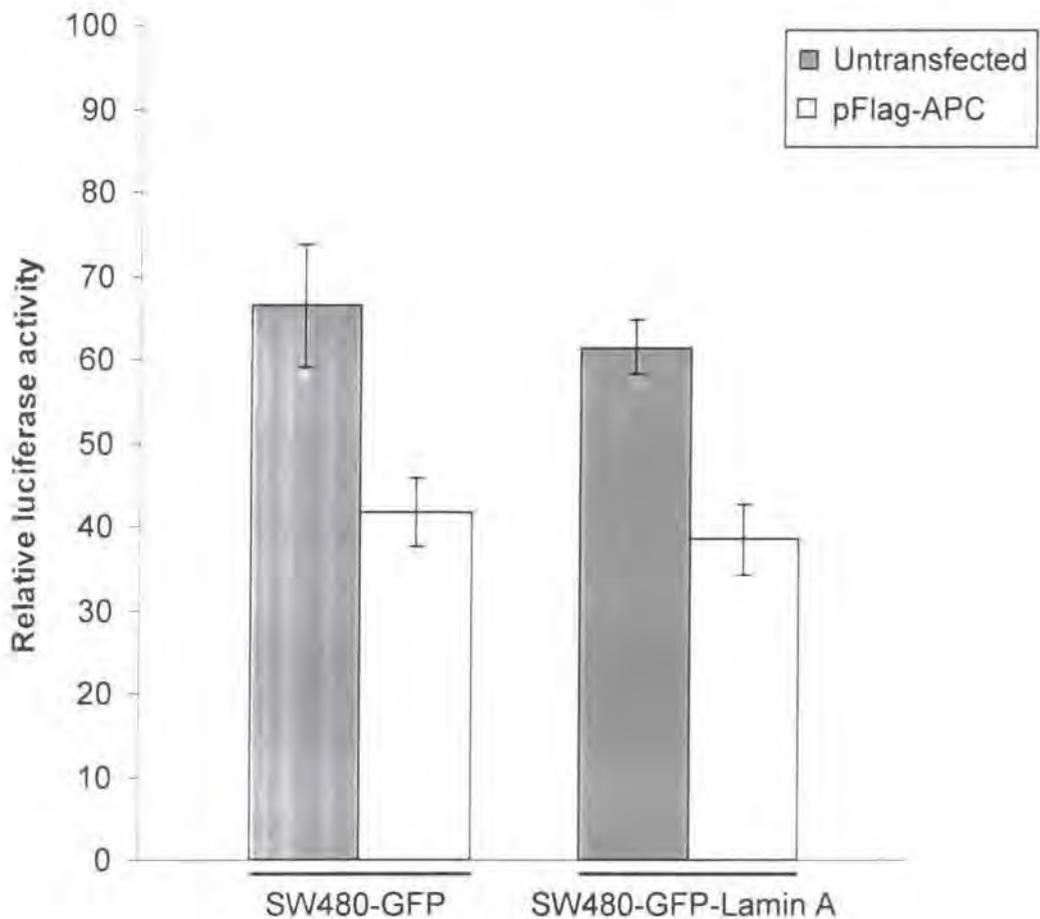
Figure 4.6

Relative β-catenin activity as measured by dual luciferase TOPFlash reporter assays. Measurements are arbitrary firefly luciferase luminosity relative to *Renilla* luminosity. Data shows no significant difference in relative TOPFlash luciferase activity between the SW480-GFP and SW480-GFP-Lamin A, untransfected and also pFlag-APC transfected clones. Data does show a down-regulation in β-catenin activity following successful pFlag-APC transfection of approximately 40% in both cases.

4.3 Discussion

The TOPFlash assay was chosen to measure β-catenin activity since it is highly quantitative and reproducible and also measures the most relevant function of APC in tumour suppression (i.e. its ability to reduce Tcf/β-catenin mediated transcription). Furthermore, it provides a direct read-out of β-catenin activity that is highly specific, unlike measuring endogenous target genes whose expression levels reflect inputs from Tcf and other signalling pathways at the same time.

The hypothesis behind this chapter's experiments was to determine whether the observed protective effects of lamin A/C negativity in colorectal tumours, which was established in chapter 3 was mediated through alterations in the canonical Wnt signalling pathway. Primary experiments showed that the relative activity of β-catenin was not affected by the presence or absence of GFP-lamin A in the SW480 colon cancer cell line. As such this would lead one to believe that the Wnt target genes which are under the control of β-catenin mediated transcription are not altered by the presence or absence of lamin A expression in these cells.

However, such conclusions were limited by the fact that β-catenin activity is no longer being correctly regulated in light of the APC type I truncating mutation present in the SW480 cell lines. In order to address this, full length functional APC was also transfected into the cells and shown to be expressed at both the RNA and protein level. Dual luciferase TOPFlash reporter assays were then undertaken but again showed no changes in β-catenin reporter activity with differential lamin A expression. This further supports the null hypothesis that the protective effect lamin A/C absence in colorectal tumours is not mediated through the canonical Wnt signalling pathway.

As already mentioned, the type II integral membrane protein Emerin has been shown to play a role in the regulation of β-catenin activity through its APC-like tail domain (Markiewicz, Tilgner et al. 2006). This work has shown that the presence of Emerin at the nuclear envelope down-regulates β-catenin activity by excluding it from the nucleus in the HEK293 cell line and human fibroblasts. In addition to this, it was shown several years ago that the lamin A is required for the correct

localisation of Emerin to the inner nuclear membrane and in the event of a failure of nuclear envelope assembly, Emerin is redistributed throughout the cytoplasm (Vaughan, Alvarez-Reyes et al. 2001). With this in mind it would seem logical to assume that in the case of colorectal tumours where lamin A/C is completely absent from the nuclear envelope that there would be a redistribution of Emerin from the nuclear envelope to the cytoplasm. Concomitant to this one would also expect to see an increase in the activity of β-catenin in the light of this previous work. This could then be extrapolated further to proffer a poorer prognosis in patients due to still further increased β-catenin activity in lamin A/C negative tumour cells.

What we actually see is the reverse of this and a better prognosis in patients with lamin A/C negative tumours, contradicting the mechanism proposed above. However it must be said that whilst these observations were true for both the HEK293 cells and human fibroblasts, similar experiments have not been done in colorectal epithelia or colorectal cancer cell lines. Since the behaviour of these cells is so closely regulated by the Wnt signalling pathway, it may be feasible that Emerin does not play a role in regulating β-catenin activity in these cells, or if it does, its influence is not to detected by the dual luciferase TOPFlash reporter assays. In terms of experimental design, if the effect of Emerin on β-catenin activity is small enough not to be detected then it could not account for the differences observed in survival between clinicopathologically similar patients with lamin A/C positive and lamin A/C negative tumours.

Other work which has been carried out in the same SW480-GFP and SW480-GFP-lamin A cell lines has shown the mRNA expression of c-*MYC*, a key Wnt target whose expression is directly regulated by β-catenin activity and responsible for controlling cell proliferation (van de Wetering, Sancho et al. 2002) remains unchanged with differential lamin A expression (Willis 2005). Consistent with this, cell proliferation indices as measured by flow cytometry were also shown not to change (Willis 2005) also suggesting β-catenin activity remains unchanged with differential lamin A expression.

A better prognosis but concomitant lack of change in β-catenin activity between lamin A/C positive and lamin A/C negative tumours, indicates that the molecular

mechanisms behind the observed differences in patient survival must be a result of alterations in a pathway or pathways other than the canonical Wnt signalling pathway. Combined with data from chapter 3 which excludes other key tumourigenic factors such as *Ki-ras* mutations and *TP53* status, it would be logical to look for alternative regulatory pathways which may be implicated in mediating this apparent protective effect.

CHAPTER 5 – Genome-wide DNA Microarray analysis

5.1 Background and Introduction

5.1.1 The call for genome-wide array studies

Traditionally tumours have been categorised on the basis of clinicopathological and histological features (Cancerbackup 2008). However, the information gained from this type of microscopic examination of simple staining patterns does not reveal the underlying molecular events which are involved in tumourigenesis and progression leading up to that time. In order to obtain a deeper understanding of the biology of tumours, genome-wide DNA microarray is becoming more widely used. This is a powerful technique which can uncover gene expression signatures associated with different genotypes of clinicopathologically similar tumours.

The multi-step process of tumourigenesis starts with a genetic or epigenetic alteration in a cell giving it a growth advantage. As aberrant cells propagate, genetic instability is accumulated and abnormal characteristics are gained. Cancer cells are typically self sufficient in growth signals, insensitive to apoptotic stimuli and have an increased telomerase activity enabling a larger number of cell divisions (Hanahan and Weinberg 2000). As a tumour grows it often further gains the ability to infiltrate through surrounding tissue and induce angiogenesis to supply itself with additional required nutrients and oxygen. The tumour will often ultimately spread to other organs distant from the primary tumour, and 90% of all cancer deaths are as a result of this secondary tumour metastasis (Sporn 1997). Colorectal cancer is no exception to this (Fearon and Vogelstein 1990). Research carried out over the last decade has implicated numerous genes in the initiation and progression of colorectal cancer as well as highlighting the role of epigenetic changes as a result of DNA promoter methylation in this progression (Jones and Laird 1999; Herman and Baylin 2003). For example CpG island promoter hypermethylation of the *LMNA* gene in leukaemia and lymphoma has been correlated with the abrogation of lamin A expression at the level of

transcription and subsequently associated with poor survival in diffuse large B-cell lymphomas (Agrelo, Setien et al. 2005).

To investigate all possible genetic aberrations in any form of cancer using traditional techniques is inconceivable hence the development of these high throughput expression microarray technologies. Such advancements have revolutionised our understanding of the cancer genome since they can evaluate changes in expression of thousands of genes at once (Clarke, te Poele et al. 2001; Stremmel, Wein et al. 2002; Basik, Mousses et al. 2003). DNA microarrays have enabled the identification of novel diagnostic and prognostic indicators which would not have been discovered otherwise (Dhanasekaran, Barrette et al. 2001; Agrawal, Chen et al. 2002; Korkola, Estep et al. 2003) as well as enabling the classification of tumours according to their individual genetic signature (Golub, Slonim et al. 1999; Perou, Sorlie et al. 2000; Fuller, Wang et al. 2002; Korkola, Houldsworth et al. 2005) and subsequent genome-wide monitoring of responses to chemotherapeutic agents (Marton, DeRisi et al. 1998; Scherf, Ross et al. 2000).

5.1.2 DNA Microarray Analysis

The core objective in microarray data analysis is identifying genes whose transcript levels have been altered between different conditions. Usually this is presented as a ranking list of genes according to a statistical significance score. In the early days of microarray technology, articles were published using fold change as the single parameter to make a decision if a gene had changed or not (Sgroi, Teng et al. 1999; Rihn, Mohr et al. 2000; Carninci, Kasukawa et al. 2005). Considering the large number of genes and the small number of replicates the number of false positives produced was considerable. This is especially the case when we consider transcripts with high natural variation which will lead to high fold change values. Similarly a few of the large number of non-differentially expressed genes can result in deviating intensities in some experiments due to noise and thus again be falsely assigned as differentially expressed. That said, even with stringent controls and replicates, the main issue in microarray studies is how to retrieve valuable information from the enormous amount of generated

data. Since the field is relatively new, methods are continuously being developed to meet the demands of biological researchers. The main processes in the data analysis are the extraction of spot signal intensities, filtering of data, normalisation of data, assessment of differential expression, clustering and classification. Even this is not the whole story with the most important stage being the placing into context of other sources of information, such as biological databases, clinical features and other microarray experiments. There are several reviews which nicely summarise the complexity of these procedures (Brazma, Hingamp et al. 2001; Quackenbush 2006).

In 2001, the Minimum Information About a Microarray Experiment (MIAME) was introduced by the Microarray and Gene Expression Data (MGED) society (Brazma, Hingamp et al. 2001) in an effort to standardise the manipulation and presentation of microarray data. These standards have now been adopted by the microarray community and are the basis of the majority of array databases.

5.1.2.1 Clustering of data

Nowadays, extraction of spot signal intensities, filtering of data, normalisation of data and assessment of differential expression is an automated part of genechip array software provided by many companies such as Affymetrix. What are then produced are lists of data from which researchers can begin their analysis. Whereas previously scientists would look for highly up- or down-regulated genes, the preferred approach now is that of cluster analysis, which is performed to look for expression changes in groups of closely related or similar genes.

Clustering is performed to divide the massive amounts of gene expression data into groups based on similarity. In its simplest form this can be accomplished with one of two different strategies with each being suited for a different purpose. The first is known as unsupervised clustering and is used for exploratory analysis such as that undertaken later in this chapter. The second is supervised clustering which is often used to create a diagnostic device based on previously established gene expression signatures (Meyer and Ginsburg 2002; Spang 2003; Wiese, Auer et al. 2007).

Unsupervised clustering is a way of obtaining a more comprehensible representation of the data set. With reduction techniques, such as principal component analysis (PCA), singular value decomposition (SVD) and multidimensional scaling, it is possible to visualise the data in two (and more recently three dimensional) space so that the distance relationships can be explored by visual inspection. Clustering aims to show relationships between genes or groups of genes and the biological samples which might be of biological relevance.

Among the most common methods for exploratory grouping or clustering are hierarchical clustering, self organising maps (SOM) (Kohonen 1999; Toronen, Kolehmainen et al. 1999) and K-means clustering (Hartigan 1973). Hierarchical clustering is an agglomerative method which produces a dendrogram with a bottom-up structure. To start with, all data points are treated as separate clusters. The dendrogram is then formed by subsequently assigning the two closest clusters together to form new clusters. Distance between clusters can be calculated by using the minimum, maximum or the average distance between samples in two different clusters. For K-means and SOM, the number of clusters to be formed is predetermined by the user. In K-means clustering, the samples are first randomly assigned to one of the K clusters. The centre of each cluster is obtained by calculating the mean or median of all incorporated expression profiles. The samples are then reassigned to the closest cluster. New cluster locations are calculated from the new cluster members, followed by another reassigning procedure. This procedure is repeated until there are no more changes. The SOM algorithm is similar to K-means assigning samples to the closest cluster in an iterative process. The difference is that the samples in this case are mapped onto a two dimensional grid. To determine optimal numbers of clusters the ratio between cluster diameter and distance between clusters can be used.

Supervised clustering on the other hand is performed to create a tool which can be used for discrimination of new data. Development of the classifier is based on prior information of how for instance tumour types are connected to appearance of the gene expression profiles. Supervised clustering techniques thus need a

dataset with information of the sample labels, i.e. if a tumour is malignant or not. The aim is to create a classifier which can classify new unlabelled samples and genes based on the microarray data. These can be divided into machine learning algorithms such as support vector machines (SVM), ANN and K-nearest neighbours (KNN), and statistical linear discriminate analysis. SVM was first introduced by Vapnik in 1995 (Vapnik 1995) and applied to microarray data in 2000 (Brown, Grundy et al. 2000; Furey, Cristianini et al. 2000). The algorithm calculates a separating hyperplane with maximised margins to the data points. SVM has been much used in supervised clustering of genome-wide expression data compared to the other methods mainly due to its capability of providing good generality despite the small sample sizes typical for microarray experiments (Furey, Cristianini et al. 2000). A weakness for clustering methods based on a molecular signature of thousands of features rather than a small set is that they are difficult to interpret in a biological context and not applicable to lower throughput techniques. Combining these however with feature selection methods makes them more useful and also reduces risk of over-fitting. Filtering and pruning are examples of strategies for feature selection. Filtering is based on the input data by filtering features based on a specific criterion. Selection can be performed based on a threshold or a number of top genes on a ranking or by evaluating the performance.

5.1.2.2 Extraction of Biological Information

Data mining microarray analysis often gives rise to long lists of potentially differentially expressed genes or clusters of genes and poses the problem of how to interpret these from a biological point of view. A number of tools have been developed to deal with this and the choice of which to use depends on the questions being asked.

Identification of metabolic pathways is an extremely important part of gene expression profiling in order to acquire a mechanistic understanding of the disease in question. In order to establish the biological reasons behind why some genes or clusters of genes appear to be differentially expressed, one tries to find the biological features which are overrepresented in these groups. There

are several examples of biological annotations which can be used for this purpose which include; Gene Ontology terms, Swissprot key words and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways (Ashburner, Ball et al. 2000; Kanehisa, Goto et al. 2004). Currently the biggest effort in this field is the Kyoto Encyclopaedia of Genes and Genomes (KEGG) which consists of graphical representations of almost all currently known biochemical pathways (Ogata, Goto et al. 1999).

It may also be of interest to find out more about the function of the genes and how they are related to other biological pathways and processes. For this purpose, the Gene Ontology (GO) Consortium has constructed a vocabulary to describe genes and gene product attributes for biological processes, cellular components and molecular functions (Ashburner, Ball et al. 2000). There are several pieces of commercial software available which enable comprehensible visualisation of gene expression levels in the different pathways such as PathwayStudio (Nikitin, Egorov et al. 2003) (Ariadne Genomics, MD, USA) and GeneSpring (Agilent Technologies, USA) alongside other freely available utilities such as Pathway Assist and GenMAPP (Dahlquist, Salomonis et al. 2002). A number of open access online tools are also available, which can calculate overrepresentation statistics for all GO terms with respect to a given data set; so called gene class testing (GCT) (Allison, Cui et al. 2006), e.g. EASE (Dennis, Sherman et al. 2003), MAPPfinder (Doniger, Salomonis et al. 2003), Onto-Express (Draghici, Khatri et al. 2003) and until recently the NetAffx Analysis centre provided by Affymetrix.

One of the biggest reasons for genes being concordantly expressed is down to regulation by the same transcription factors in a particular signalling pathway. In eukaryotes combinations of transcription factors form *cis* regulatory modules in order for a gene to be expressed. Thus global changes in apparently unrelated genes can be clustered on this basis of co-occurrence of transcription factors in conserved promoter regions and then evaluated with software such as CRÈME, JASPAR and cisRED (Sharan, Ben-Hur et al. 2004; Robertson, Croce et al. 2006; Vlieghe, Sandelin et al. 2006)

5.1.3 Validation of array data

Although microarray technology and data analysis is becoming more standardised and thus more reliable, artefacts can still be introduced at any time during an experiment and it is therefore necessary to validate the results. Once a number of target genes have been identified by microarray analysis, it is desirable to confirm RNA expression levels by an independent method. The most common method is quantitative RT-PCR (Rajeevan, Ranamukhaarachchi et al. 2001; Rajeevan, Vernon et al. 2001) because of its speed and relative cheapness. Northern blot has also been reported to give consistent results when compared to microarray (Taniguchi, Miura et al. 2001).

In addition to validating transcript levels it is also important to measure levels of the corresponding proteins as we know that they are not always correlated. This can be done by various antibody based methods e.g. Western blot, immunohistochemistry, immunofluorescence or immunohistochemically via tissue microarrays (Kallioniemi, Wagner et al. 2001; Chuaqui, Bonner et al. 2002; Mousses, Kallioniemi et al. 2002). These methods are however dependent on antibody availability, which can pose a problem in the case of unknown transcripts. However with the Human Proteome Atlas (http://www.proteinatlas.org/) well on the way, this should soon be solved.

5.1.4 Summary

Data from chapter 3 has shown a protective effect of lamin A/C negative status in tumours of colorectal cancer patients. Furthermore, data from chapter 4 has shown that the canonical Wnt signalling pathway classically at the heart of colorectal adenocarcinoma initiation and progression is not influenced by differential lamin A expression in our *in vitro* model and hence it can be concluded that it is not directly implicated in the observed protective effect of lamin A/C negative status in tumours.

With no correlations being drawn from chapter 3 to link differential lamin expression with other oncogenic mutations in proteins such as *TP53* and *Ki-ras*

either, this suggests that the observed protective effect is mediated through one or more other developmental pathways which have yet to be investigated. Thus, with the availability of the same SW480 colon cancer cell line model system used in chapter 4, an opportunity to conduct a screening genome-wide Affymetrix DNA Microarray analysis was accepted to explore members of other potential signalling pathways which may be influenced by differential lamin A expression and thus be implicated in mediating these observed differences in patient survival.

5.2 Results

5.2.1 DNA Microarray dataset

An opportunity was taken to carry out a qualitative Genome-wide DNA microarray pair-wise screening on the SW480-GFP-lamin A and SW480-GFP cell lines using the Human Genome U133 Plus 2.0 high-density oligonucleotide Affymetrix GeneChip array (Affymetrix, Santa Clara, CA, USA). Extraction of spot signal intensity, filtering and normalisation of data was undertaken in accordance with the associated Affymetrix guidelines for the HG-U133 Plus 2.0 GeneChip using the Affymetrix GeneChip Operating Software (GCOS).

The use of a simple 2 fold change cut-off to define differentially expressed genes was avoided as this is considered inadequate for elucidating pathway changes (Miller, Galecki et al. 2001; Hsiao, Worrall et al. 2004) as it does not take into account variance and offers no associated level of confidence (Budhraja, Spitznagel et al. 2003; Hsiao, Worrall et al. 2004). Instead, a preliminary gene list was generated by means of a series of relevance thresholds for signal detection level, signal detection p-value significance, expression change p-value significance and Signal Log Ratio (SLR) between the SW480-GFP and SW480-GFP-lamin A lines so as to avoid missing potentially important, yet subtle changes that would be missed using the simpler fold change cut-off method. Thanks go to Dr Heiko Peters at Newcastle University for provision of this preliminary gene list.

At baseline the resulting gene list showed changes in expression were observed in a total of 7,577 genes between the SW480-GFP control and SW480-GFP-lamin A cell lines. 3,320 (43.8%) of these genes showed either up-regulation or moderate up-regulation in expression in the SW480-GFP-lamin A cell line and 4,257 (56.2%) showed down-regulation or moderate down-regulation in expression as compared to the SW480-GFP-Alone control (figure 5.1B).

Figure 5.1.

Changes in gene expression at baseline (before analysis) between the SW480-GFP-lamin A and SW480-GFP-Alone control cells.

(A) Heat map showing the number of up-regulated genes (green) and down-regulated genes (red) in the SW480 GFP-lamin A transfected cell line compared to the SW480 GFP-Alone transfected control cell line. Colour intensity is representative of the degree of up or down-regulation, black or dark squares indicate little or no change.

(B) Comparative analysis showing a higher proportion of down-regulated genes vs. up-regulated genes in the GFP-lamin A transfected cell line.
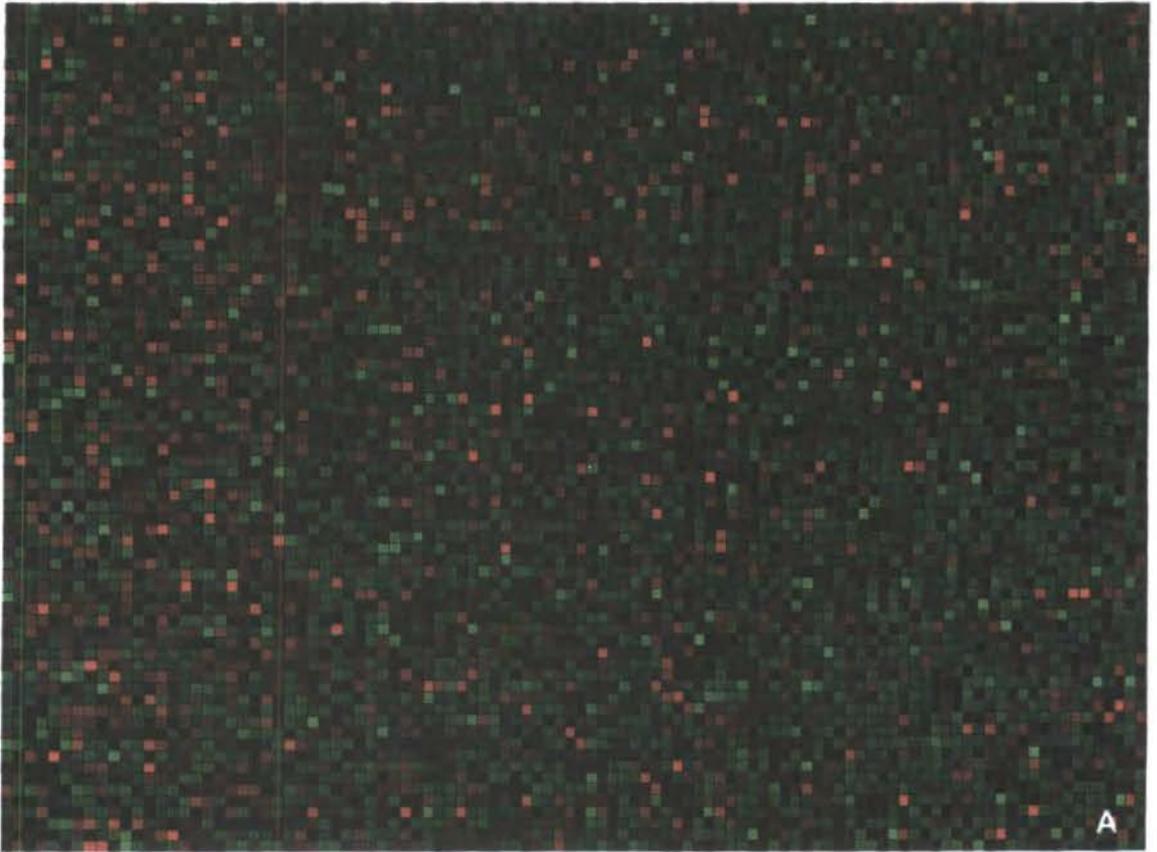
Baseline Changes in Gene Expression
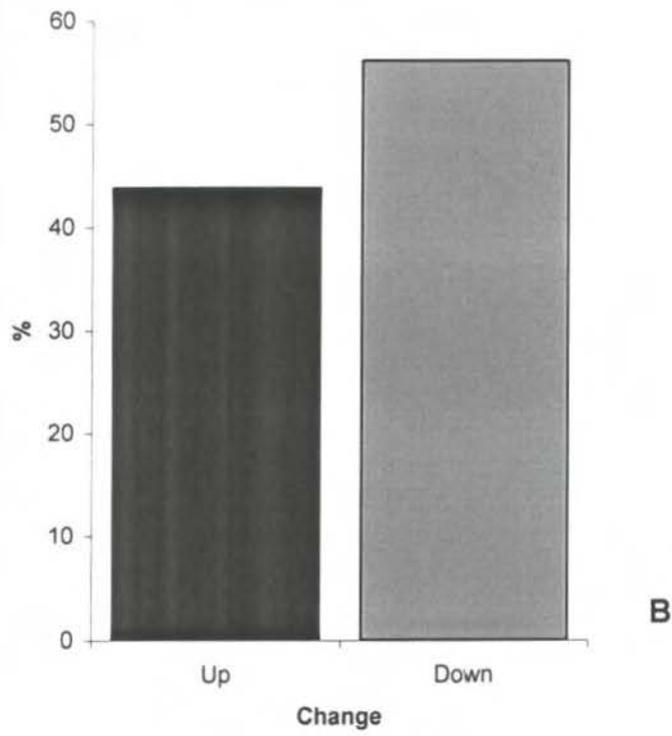
The gene list was imported into the then freely available online NetAffx Analysis centre (https://www.affymetrix.com/analysis/netaffx/index.affx). The tools available in the NetAffx analysis centre are designed specifically for use with Affymetrix GeneChip catalogue arrays.

Expression batch queries were carried out on all 7,577 genes to retrieve information for the complete probe list ready for further analysis. Information retrieved included but was not limited to the following;

- Gene Ontology biological process classifications
- Gene Ontology molecular function classifications
- Gene Ontology cellular component classifications
- GenMAPP pathway details
- Ortholog Targets
- Entrez Annotations and grades
- OMIM , Ensembl, UniGene, and RefSeq entries
- SwissProt and InterPro descriptions
- Chromosomal Locations
- GenBank descriptions
- As well as Affymetrix own descriptives

For the purposes of this chapter, only the first three, Gene Ontology biological process, molecular function and cellular component were used for cluster analysis, the rest were used in a purely qualitative manner.

5.2.2 Cluster Analysis

Unsupervised hierarchical cluster analysis (as described in section 5.1.2.1) was carried out on the assembled gene list using the Gene Ontology Browser (GO Browser) provided in the Affymetrix online NetAffx Analysis Centre [(https://www.affymetrix.com/analysis/netaffx/) – which has now unfortunately been retired and replaced with the independent AMIGO GO Browser (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) and EBI GO Browser (http://ep.ebi.ac.uk/EP/GO/)].

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By definition, an ontology is a controlled vocabulary used to define an object or concept. In the case of microarray data, gene ontologies have been designed which encompass a structure of defined terms to describe genes by one of three different processes; biological process, molecular function or cellular component. It is important to note here that gene products can fall into more than one category within an ontological branch. Such cases are observed when a gene falls into both a general and specific description which is classically associated with cluster analysis.

Initial parameters for Gene Ontology mining were established before the process was run. Such parameters included the ontology branch to be investigated (i.e. biological process, molecular function or cellular component); the probe count threshold, i.e. the minimum number of clustering genes within a single node or cluster (also know as the ontological definition); and clustering significance, i.e. the chi-square (χ^2) value of significance of association for the genes clustering in a particular node, typically expressed as a p-value and set at $p \le 0.05$. Once these had been set, cluster analysis was carried out and resulted in a gene ontology map, or gene ontology graph as shown in figure 5.2.

The gene ontology graph shown in figure 5.2 is a simple example of one constructed for the Biological Processes ontology branch with a minimum probe threshold of 5. It is organised as such that as we move from left to right, we go from very general descriptions of biological process (such as cell metabolism) to very detailed description of biological process (such negative regulation of mitosis). The ontology graph is colour-coded such that the branches appearing red or purple are those exhibiting high chi-square (χ^2) significance. That is to say that several if not all genes clustering within these nodes are closely related based on their ontological description and represent nodes or clusters which immediate attention can be focussed on. Numbers shown in brackets detail how many clustering genes are present in each node and this generally decreases as we move from left to right as nodes become more specific.
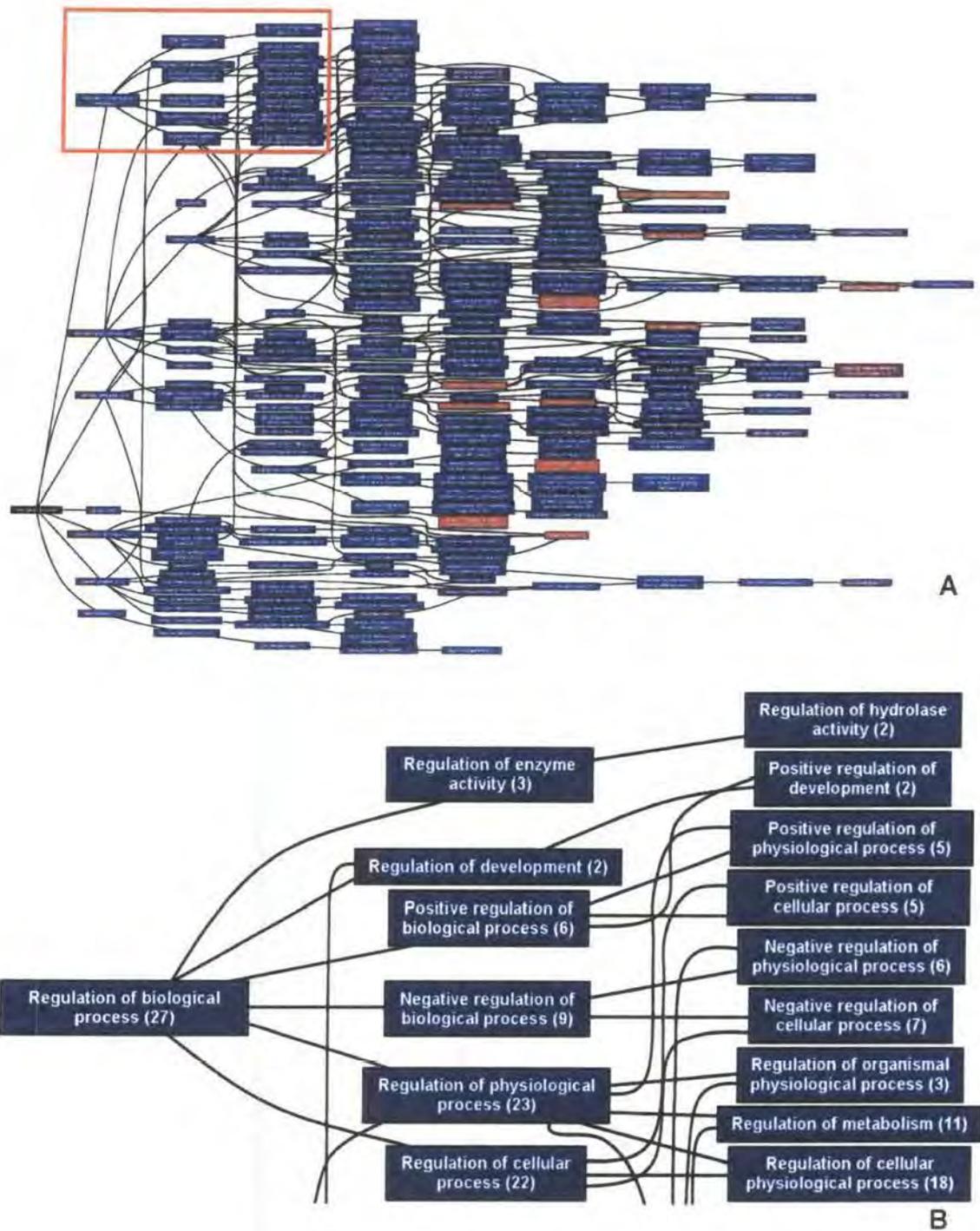
Figure 5.2

Gene Ontology map clustered by Biological Process for genes differentially expressed between the SW480-GFP and SW480-GFP-lamin A cell lines. Ontological definitions move from broad to specific as we move from left to right. Nodes shown in red or purple indicate tightly clustering significant genes.

Gene Ontology cluster analysis facilitates the identification of Gene Ontology terms which are enriched in a given gene list with a corresponding pre-determined significance based on biological, physiological, and functional descriptions of the gene products. What results is a list of enriched and unique gene ontology terms and a targeted list of genes which can be further evaluated. Put into the context of this study, it allowed the generation of a list of biological processes (such as signalling pathways), molecular functions (such as cell adhesion) and cellular compartments (i.e. golgi apparatus) which exhibited differential gene expression between the SW480-GFP-Alone and SW480-GFP-lamin A cell lines.

Large scale data sets such as that shown in figure 5.2 are typical of Gene Ontology analysis when starting with large gene lists. Whilst it does not provide useful direct information from the outset, it establishes a clustering platform in which more detailed exploration can occur. Initial isolation of those nodes or clusters which are shown to have strong chi-square (χ^2) significance allowed them to be looked at in more detail and in particular allowed the inspection of individual node members. Such information allowed details on up- and down-regulation of particular genes within the same node or cluster to be tabulated together. It also allowed individual genes which cluster in more than one node to be highlighted more easily.

Gene ontology graphs were constructed for all three gene ontologies; biological process, molecular function and cellular component and nodes of particular interest within each ontology were extracted. Clustering according to biological process was the most successful, closely followed by molecular function. Since I was looking for genes which may be implicated in mediating the protective effect of lamin A/C status in tumours, the cellular component ontology was explored, but not in any great detail.

When looking at the clustered data, the aim was to focus initial attention on novel pathways not yet investigated. Since the starting gene list was over 7,577 genes, clustering for biological process and molecular function resulted in a huge number of genes and nodes being isolated (some 2,000 genes and 200 nodes). Inspection on an individual gene-by-gene or node-by-node basis would have

been an inefficient use of time. Instead in order to identify what could be considered as significant genes and nodes in the pathways highlighted, a filter was applied (as described in section 5.1.2.1). The initial filter was simple, yet stringent and was designed to isolate nodes where 3 or more of its clustering genes underwent a 4 fold change in expression between the SW480-GFP and SW480-GFP-lamin A cell lines. This quickly reduced the list of target genes to a much more manageable size for visual inspection. The same analysis using a 2-fold cut-off was also later used, but yielded little extra useful information.

To summarise, to this point, the original 54,000+ lines of data produced from the microarray were initially analysed to reduce this to 7,577 genes showing statistically significant detectable spot intensities alongside statistically significant changes in gene expression between the SW480-GFP and SW480-GFP-lamin A cell lines. These 7,577 genes were then clustered based on Gene Ontology terms for biological, physiological, and functional descriptions of the gene products with cluster threshold counts and chi-square (χ^2) significance value cut-offs further reducing the target list of genes to approximately 2,000.

The final step was to apply a filter which selected for ontological nodes containing 3 or more genes where there was a 4-fold change in expression between the SW480-GFP and SW480-GFP-lamin A cell lines. The fold change filter reduced the list further to 133 genes and the node clustering filter reduced the number of nodes to approximately 40. In the interest of highlighting potential signalling pathways from the biological processes ontology branch, only ontological nodes representing cellular signalling pathways were extracted along with their clustering genes and are shown in table 5.1. Since the number of nodes within the molecular function ontology branch was much smaller, the gene ontology terms classically associated with cancer were selected and are detailed in table 5.2.

Table 5.1 Targeted ontological gene list (biological process) for genes showing a 4 fold change (Fc) in expression

Biological Processes		Clustering genes (4 fold change)		Fc
Inflammatory response signalling		Fibronectin 1	FN1	4.3 x Up
		Serum amyloid A1	SAA1	5.3 x Up
		Serum amyloid A2	SAA2	8.6 x Up
TGFβ signalling		Serpin peptidase inhibitor, clade E, member 1	SERPINE1	4.9 x Up
	*	Bone morphogenetic protein 4	BMP4	7.5 x Down
		Transforming growth factor, β-induced	TGFβI	4.9 x Up
		Latent transforming growth factor β-binding protein 2	LTβP2	4.3 x Up
G Protein signalling		A kinase (PRKA) anchor protein (gravin) 12	AKAP12	8.0 x Up
		G protein-coupled receptor 115	GPR155	4.0 x Down
		GNAS complex locus	GNAS	4.0 x Down
		Guanine nucleotide binding protein (G protein), α inhibiter 1	GNAI1	4.0 x Down
		Guanine nucleotide binding protein (G protein), γ 11	GNG11	7.0 x Up
Wnt signalling	*	Dual Specificity Phosphatase 5	DUSP5	4.6 x Up
	*	FOS-like antigen 1	FOSL1	4.3 x Up
		Platelet-activating factor acetylhydrolase isoform 1b	PAFAH1B1	4.1 x Down
BMP signalling	*	Bone morphogenetic protein 4	BMP4	7.5 x Down
		SMAD family member 6	SMAD6	4.0 x Down
		Activin membrane-bound homolog	AMB	4.0 x Up
Insulin receptor signalling		Insulin-like growth factor 2 (somatomedin A)	IGF2	8.6 x Down
		Synaptosomal-associated protein	SNAP25	9.8 x Down
		Phosphoinositide-3-kinase, regulatory subunit 1 (p85α)	PIK3R1	4.0 x Down
Small GTPase mediated signalling		RaP2 interacting protein 8	RPIP8	7.0 x Down
		Ras and Rab interactor 2	RIN2	4.0 x Down
		Ras homolog enriched in brain	RHEB	4.0 x Down

Table 5.2 Targeted ontological gene list (molecular function) for genes showing a 4 fold change (Fc) in expression

Molecular Function	Clustering genes (4 fold change)		Fc
Cell Adhesion	Collagen, type XIII, α1	COL13A1	4.0 x Up
	Fibronectin 1	FN1	4.3 x Up
	Cadherin 1, type 1, E-cadherin (epithelial)	CDH1	4.6 x Down
	* Roundabout, axon guidance receptor, homolog 1 (Drosophila)	ROBO1	7.0 x Up
	Serum amyloid A1	SAA1	5.3 x Up
	Serum amyloid A2	SAA2	8.6 x Up
Angiogenesis	* Bone morphogenetic protein 4	BMP4	7.5 x Down
	Serpin peptidase inhibitor, clade E, member 1	SERPINE1	4.9 x Up
	Nucleolin	NCL	4.0 x Up
	* Roundabout, axon guidance receptor, homolog 1 (Drosophila)	ROBO1	7.0 x Up
Cell differentiation	* Bone morphogenetic protein 4	BMP4	7.5 x Down
	Doublecortin and CaM kinase-like 1	DCAMKL1	5.3 x Up
	Achaete-scute complex homolog 2 (Drosophila)	ASCL2	4.0 x Down
	* Roundabout, axon guidance receptor, homolog 1 (Drosophila)	ROBO1	7.0 x Up
Apoptosis	Nuclear protein 1	NUPR1	4.0 x Up
	Tumour necrosis factor receptor superfamily, member 11b	TNFRSF11B	4.3 x Down
	Brain-derived neurotrophic factor	BDNF	4.6 x Down
Regulation of transcription	* FOS-like antigen 1	FOSL1	4.3 x Up
	Paired related homeobox 1	PRRX1	4.0 x Up
	Zinc finger protein 33A	ZNF33A	4.0 x Down
	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	MAFB	6.1 x Down
	Achaete-scute complex homolog 2 (Drosophila)	ASCL2	4.0 x Down
	Pinin, desmosome associated protein	PNN	4.3 x Down
Cell proliferation	* Amphiregulin (schwannoma-derived growth factor)#	AREG	4.0 x Up
	Epithelial membrane protein 1	EMP1	4.3 x Up
	Interleukin 6 receptor	IL6R	4.3 x Up

The amphiregulin precursor is Colorectum cell-derived growth factor)(CRDGF)

When looking at the molecular function ontologies, the keys terms which arose as significant are all those typically associated with cancer, cancer progression and ultimately patient survival such as cell adhesion, angiogenesis, cell differentiation, apoptosis, cell proliferation and regulation of transcription. On the other hand when looking at biological processes and in particular at the signalling pathways which were overrepresented, we see the canonical Wnt, TGFβ and BMP signalling pathways which are classically associated with tumour initiation and progression. Other pathways involved in Inflammatory response, G-protein, small GTPase and Insulin receptor signalling are also present indicating the complexity of signalling pathway disruption which often occurs in cancers.

5.2.3 Semi-Quantitative confirmation of gene expression

Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) (as detailed in section 2.6) was carried out to confirm the expression levels of chosen transcripts highlighted from the Microarray. A subset of 5 genes was selected from table 5.1 and table 5.2 and whole cell RNA extracts were evaluated for expression of these genes. The genes chosen were Amphiregulin (AREG), Bone morphogenic protein 4 (BMP4), Dual specificity Phosphatase 5 (DUSP5), FOS-Like Antigen 1 (FOSL1) and Roundabout 1 (ROBO1). The decisions behind gene selection are discussed in section 5.3.

Quantification was carried out in three separate passages of the two cell lines to evaluate RNA transcript expression using the primers shown in table 2.2. RT-PCR reactions were carried out and products were resolved on 1% agarose gel (figure 5.3). Densitometric analysis assessment of the gels was carried out (figure 5.4) and confirmed either the up- or down-regulation of mRNA transcripts.

Figure 5.3

Expression of AREG, BMP4, DUSP5, FOSL1 and ROBO1 in GFP and GFP-lamin A transfected SW480 colon carcinoma cells. Microarray data identified significant changes in the level of these 5 transcripts which was confirmed by semi-quantitative RT-PCR. Primers were designed to amplify Amphiregulin **(A)**, Bone morphogenic protein 4 **(B)**, Dual Specificity Phosphatase 5 **(C)**, FOS-Like Antigen 1 **(D)** and Roundabout Homolog 1 **(E)**. Equal loading of starting material was verified by monitoring the β-actin levels.
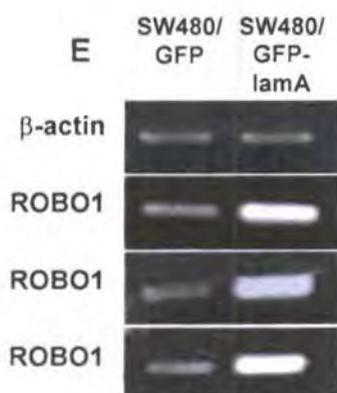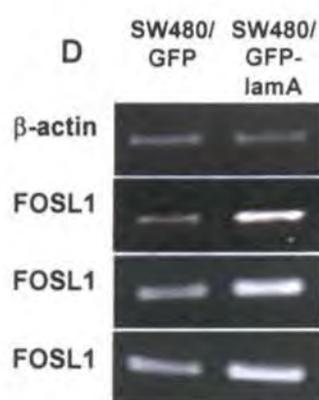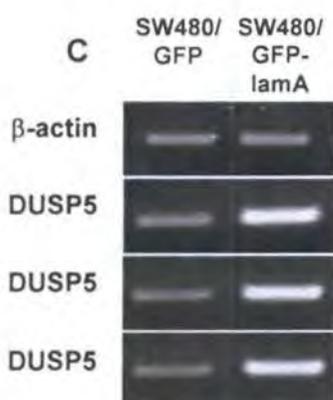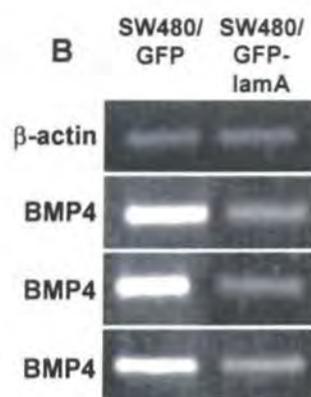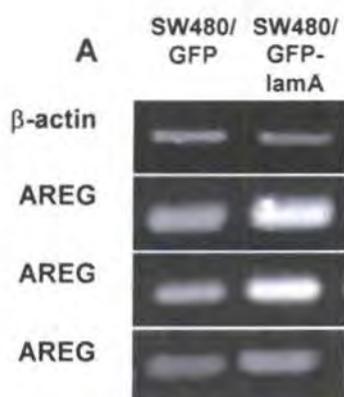
Figure 5.4 Densitometric analysis of Semi-quantitative RT-PCR

(A) Densitometric analysis of Amphiregulin (AREG) mRNA expression in SW480-GFP and SW480-GFP-lamin A cell lines. Data show an up-regulation in amphiregulin expression of the SW480-GFP-lamin A transfected cell line;
Δ = +1.84, t = 4.3558, df = 4 and p-value 0.012 ('two-tailed' Students t-test).

(B) Densitometric analysis of Bone morphogenic protein 4 (BMP4) mRNA expression in SW480-GFP and SW480-GFP-lamin A cell lines. Data show a down-regulation of BMP4 expression in the SW480-GFP-lamin A cell line;
Δ = -5.89, t = 5.9319, df = 4 and p-value 0.004 ('two-tailed' Students t-test).

(C) Densitometric analysis of Dual Specificity Phosphatase 5 (DUSP5) mRNA expression in SW480-GFP and SW480-GFP-lamin A cell lines. Data show an up-regulation of DUSP5 expression in the SW480-GFP-lamin A transfected cell line;
Δ = +3.71, t = 15.9216, df = 4 and p-value <0.001 ('two-tailed' Students t-test).

(D) Densitometric analysis of FOS-Like Antigen 1 (FOSL1) mRNA expression in SW480-GFP and SW480-GFP-lamin A cell lines. Data show an up-regulation of FOSL1 expression in the SW480-GFP-lamin A transfected cell line;
Δ = +2.66, t = 3.4669, df = 4 and p-value 0.026 ('two-tailed' Students t-test).

(E) Densitometric analysis of Roundabout homolog 1 (ROBO1) mRNA expression in SW480-GFP and SW480-GFP-lamin A cell lines. Data show an up-regulation of ROBO1 expression in the SW480-GFP-lamin A transfected cell line;
Δ = +9.22, t = 17.6643, df = 4 and p-value <0.001 ('two-tailed' Students t-test).

A AREG mRNA Expression

B BMP4 mRNA Expression
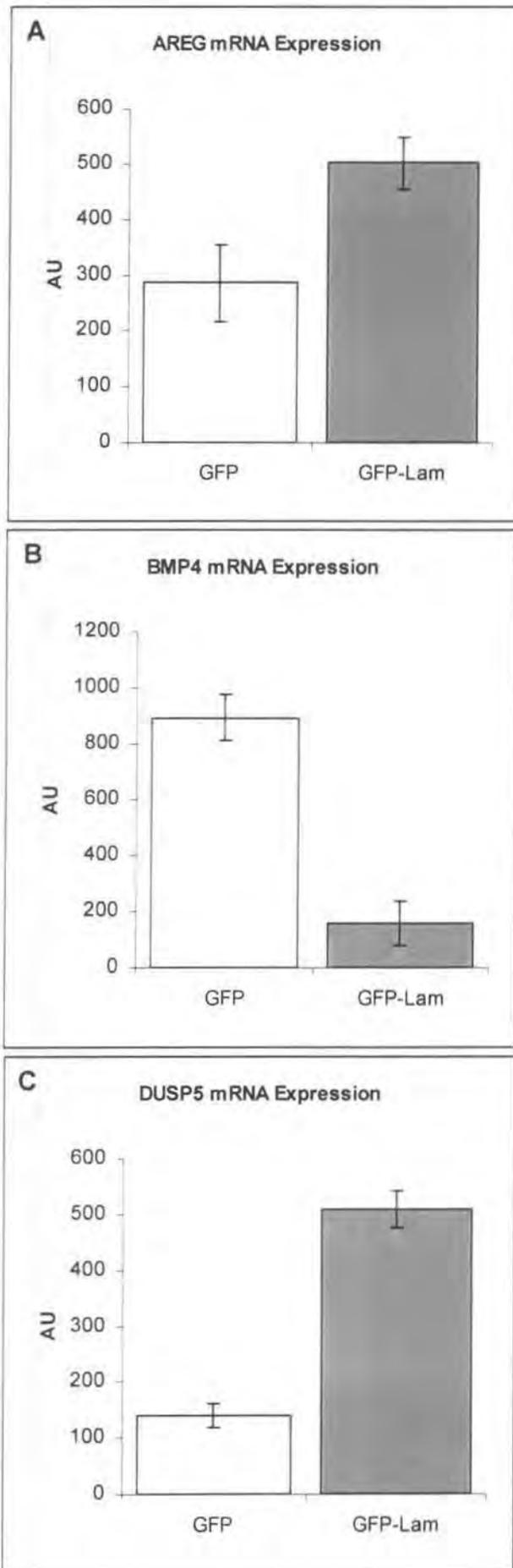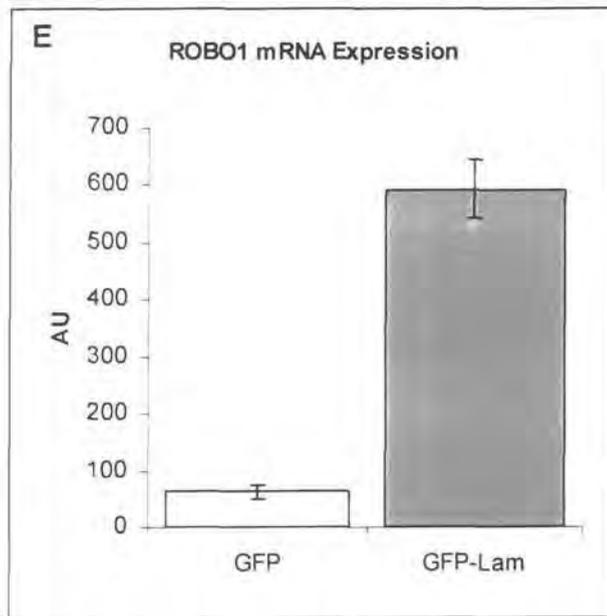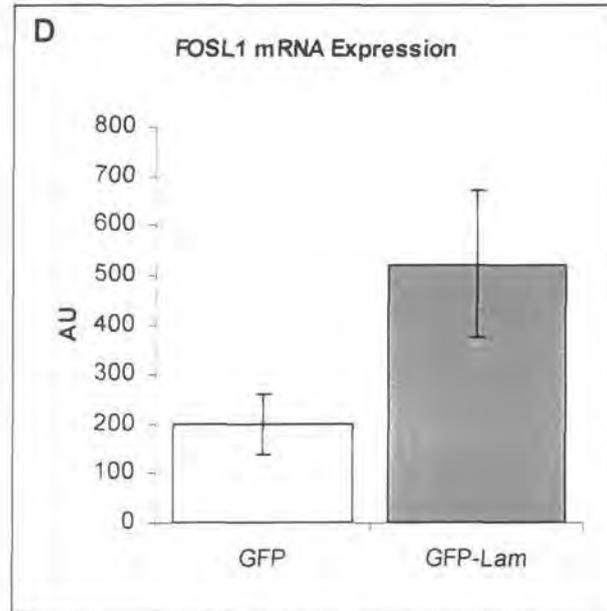
C DUSP5 mRNA Expression

Figure 5.4 A - C

Figure 5.4 D & E

Data show that the level of all 5 genes evaluated by semi-quantitative RT-PCR is in keeping with data obtained from the microarray. We see a 1.84 fold increase in AREG expression in the SW480-GFP-lamin A cell line which is less that the observed 4 fold up-regulation shown in Microarray analysis, but still supports the notion of an up-regulation of expression in the presence of lamin A. There is a 5.89 fold down-regulation in the expression of BMP4 expression in the SW480-GFP-lamin A transfected cell line which is in keeping with the 7.5 fold down-regulation observed in the microarray. There is a 3.71 fold up-regulation in DUSP5 expression in the GFP-lamin A transfected SW480 cell line, again in keeping, albeit slightly lower than the 4.6 fold up-regulation noted in the microarray. The expression of FOSL1 is 3.4 fold up-regulated in the SW480-GFP-lamin A cell line according to densitometric studies reflecting the 4.3 fold up-regulation noted in the microarray. Finally there is a 9.22 fold up-regulation in the expression of ROBO1 in the SW480-GFP-lamin A cell line which is actually higher than the observed 7.0 fold up-regulation noted in the microarray. These results strongly support data provided by the genome-wide microarray.

5.3 Discussion

It is well known in cancers that down-regulation of cell-cell adhesion molecules is associated with tumour metastases; up-regulation of angiogenic factors and cell proliferation genes alongside a down-regulation of apoptotic factors is associated with tumour growth (Yu, May et al. 2004; Pedraza-Farina 2006); loss of cellular differentiation is associated with poorer patient survival (Rindi, D'Adda et al. 2007), and loss of transcriptional regulation is associated with malignant tumour progression (Gonciarz, Pierzchalski et al. 2004; de Lau, Barker et al. 2007). Thus it is not surprising that these clusters were highlighted in the SW480-GFP lamin A transfected cell line which, if the *in vivo* observation of poor prognosis in patients with lamin A/C positive tumours (Chapter 3) were applied to the *in vitro* model system, should exhibit poorer prognosis.

The genome-wide microarray analysis work carried out within this chapter was in essence a qualitative screening exercise aimed at highlighting potential candidates for further experimental investigation. This is due to the lack of biological replicates undertaken, but the independent validation through the use

of RT-PCR allowed a much greater degree of reliability to be assigned to the data obtained. The selection of the five genes which were subsequently confirmed from the microarray was based on current literature at the time and is discussed shortly. The experiment was successful with expression data for all genes subsequently investigated correlating closely with data obtained from the microarray, and so qualitative data obtained from the data-mining exercise could reliably be used as the basis for further experiments.

ROBO1 is an integral member of the Slit/ROBO pathway. Whilst this pathway did not significantly cluster on its own, the presence of ROBO1 in the cell adhesion and angiogenesis molecular function groups draws light to it. Results show that there is a significant up-regulation of ROBO1 expression in SW480-GFP-lamin A transfected cells over the GFP control.

A review of the literature reveals it is a member of the immunoglobulin superfamily and was originally shown to play a major role in the process of axonal guidance in neurogenesis (Grone, Doebler et al. 2006). More recently however it has been implicated in tumour angiogenesis via the Slit2 ligand (Hohenester 2008). Similar work has also shown ROBO1 to be a novel hepatocellular carcinoma antigen and work is currently being undertaken to assess its potential as a therapeutic and diagnostic target (Ito, Funahashi et al. 2006). Ito et al. have shown a high over-expression in hepatocellular carcinoma and a much lower expression in surrounding normal tissue. Work then went on to show that treatment with the monoclonal mouse antibody mAb B2318C was capable of inducing complement-dependent cytotoxicity in the ROBO1 expressing hepatocellular carcinoma cells.

This work has been further supported by another group who showed that in a human oral squamous cell carcinoma line, a ROBO1 over-expressing cell line, treatment with the mouse monoclonal antibody mAb R5 lead to decreased proliferation and increased apoptosis as mediated by fas and fasL (Ma, Wang et al. 2006)

The third study which is more closely linked to this work was by Grone et al. (Grone, Doebler et al. 2006). Their work was carried out in colorectal cancer

tissue samples and they showed by microarray analysis and real-time PCR that ROBO1 is significantly up-regulated in cancerous tissue vs. normal in just fewer than 80% of patients at the mRNA level. It is interesting to note here that data shown in chapter 3 reports 70% of patients harbour lamin A/C positive tumours. If we assume that data from our SW480 colon cancer *in vitro* model can be extrapolated to an *in vivo* setting, then one would expect to see an up-regulation of ROBO1 expression in 70% of patients in the NLCS study, which would correlate closely with that observed by Grone *et al*.

Dual Specificity Phosphatase 5 (DUSP5) is a nuclear, heat shock and growth factor inducible (Ishibashi, Bottaro et al. 1994; Kwak and Dixon 1995) VH-1-like enzyme which hydrolyses substrates phosphorylated on both the tyrosine and serine/threonine residues. DUSP5 was shown by microarray to be up-regulated by 4.6 fold in the SW480-GFP-lamin A transfected cell line as opposed to the SW480-GFP control.

Among other roles DUSP5 is involved in mediating responses to oxidative stress (Foltz, Ryu et al. 2006) and in the ERK/MAPK pathway acting as a negative regulator of ERK signalling (Mandl, Slack et al. 2005) thus regulating MAPK signal propagation. The Ras/extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase signalling pathway mediates a diverse array of distinct intracellular responses to extracellular stimuli (Nishida and Gotoh 1993; Robinson and Cobb 1997; Chang and Karin 2001). These include cell proliferation, differentiation, transformation, and survival, and these responses are mediated via ERK-dependent phosphorylation. As a result, this pathway is often up regulated in human tumours and as such currently represents an attractive target for the development of anticancer drugs.

The precursor to Amphiregulin (AREG) is the Colorectum cell-derived growth factor (CRDGF). Microarray data showed an up regulation of Amphiregulin expression of 4 fold which was confirmed by semi-quantitative RT-PCR as closer to 2 fold. Nonetheless, Amphiregulin expression has been shown to be up-regulated in a number of tumours, including colorectal cancer and is an EGF family ligand for the ERBB family receptors. It has also been characterised as an

autocrine growth factor and as a potent target gene of the Wnt/β-catenin signalling pathway.

Picihard et al. (Picihard, Berthois et al. 2006) have shown that Amphiregulin is strongly involved in colorectal carcinoma cell adhesion, growth and differentiation and its involvement is mediated through the ERB1/ERK1,2 and ERB1/FAK pathways which are themselves directly involved in pRb regulation. Concomitantly, Shao et al. (Shao, Lee et al. 2003) have shown that Amphiregulin exerts a strong mitogenic effect on the human colon carcinoma LS-174 cell line and partially mediates a Prostaglandin E2 – induced growth stimulation in these cells. Prostaglandin E2, a major product of cyclo-oxygenase enzymes is closely implicated in colorectal carcinogenesis and has been shown to stimulate the growth of human colorectal carcinoma cells.

Thus, in keeping with this previously published data, it would make sense to observe an up-regulation of Amphiregulin expression in the SW480-GFP-lamin A transfected cell line which one would postulate to be more aggressive based on conclusions drawn from chapter 3. Thus, the lack of lamin A/C expression in tumours may be mediating an increased survival through the down-regulation of growth stimulatory factors such as amphiregulin.

FOS-Like antigen 1 (FOSL1) expression was up-regulated in the SW480-GFP-lamin A cell line by approximately 4 fold and this was confirmed by semi-quantitative RT-PCR. FOSL1 is a member of the FOS gene family which consists of 4 leucine zipper proteins that can dimerise the proteins of the JUN family, thereby forming the transcription factor complex AP-1. To this end the FOS genes have been implicated as regulators of proliferation, differentiation and transformation.

Previous work has shown that the over expression of FOSL1 enhances the motility and invasion of breast and colorectal cancer cells, but inhibits the tumourigenicity of cervical carcinoma cell lines (Milde-Langosch 2005). Research concomitant to this by Zhang et al. (Zhang, Hart et al. 2005), has shown that in 75 human colorectal neoplasms the level of FOSL1 was markedly elevated when compared to normal non-neoplastic colorectal epithelial cells.

They also postulate that the up-regulation is an early event in human colorectal tumourigenesis.

Again, data produced here would be in keeping with previously published work insofar that the presence of lamin A expression and concomitant up regulation of FOSL1 expression could correlate with a poorer prognosis in patients by increasing the motility and invasiveness of lamin A/C positive tumours through increased transcription of AP-1 target genes.

Bone morphogenic protein 4 (BMP4) is a member of the transforming growth factor-β (TGF-β) superfamily, which utilise BMP receptors and intracellular SMADs to transduce their signals in order to regulate cell differentiation, proliferation and apoptosis. Currently the contribution of BMPs generally to the development of epithelial-derived colorectal cancers remains largely unknown since its effects have been more widely investigated in tissues of mesenchymal origin. However with a 7.5 fold down-regulation in BMP4 expression reported in the microarray and subsequent 5.9 fold down regulation as confirmed by semi-quantitative RT-PCR, BMP4 would certainly be a target for further investigation.

Work by Nishanian et al. (Nishanian, Kim et al. 2004) has previously shown the tumour suppressive properties of BMP4. They showed a successful elimination of tumourigenic potential from the undifferentiated human cancer cell line NTERA2 by exogenous treatment with BMP4. They also went on to show that over expression of BMP4 can induce a variety of Wnt signalling molecules and that this activation of the Wnt pathway by BMP4 has functional consequences.

In lung cancer, an up-regulation of BMP4 signalling has also been shown to induce senescence and modulate the oncogenic phenotype of A549 lung adenocarcinoma cells (Buckley, Shi et al. 2004). In gastric cancer, data has indicated that BMP4 mRNA is under expressed in poorly differentiated tumours classically associated with poorer survival (Katoh and Terada 1996). Lastly, Piccirillo et al. have shown that BMP4 will inhibit the tumourigenic potential of human brain tumour-initiating (human glioblastoma) cells (Piccirillo, Reynolds et al. 2006).

Thus it seems that BMP4 is heavily implicated in tumourigenesis as a negative regulator of proliferation and activator of apoptotic programs, as well as an important mediator of cell cycle profiles. The current hypothesis is that the effects of BMP4 are mediated at least in part by activation of the canonical Wnt signalling pathway (Nishanian, Kim et al. 2004). The exact mechanism behind this proposed theory is still unclear but activation would not necessarily involve direct alteration of the activity of β-catenin but would instead be mediated through interaction of transcriptional co-activators in promoter regions containing both SMAD and Tcf binding sequences

Thus the significant down-regulation of BMP4 expression in GFP-lamin A transfected SW480 cells would suggest that BMP4 and its associated signalling pathway(s) may be responsible for mediating the observed difference in patient survival between lamin A/C negative and lamin A/C positive patient groups.

In short this chapter serves to highlight the genome-wide changes which occur as a result of changes in a single protein. The observed change in over 7,500 genes between the SW480-GFP and SW480-GFP-lamin A cell lines indicates that the function of lamin A irrefutably extends beyond that of structural. We know from chapter 3 that patients with lamin A/C positive tumours exhibit a much poorer survival than their clinicopathologically similar lamin A/C negative counterparts. Evidence from chapter 4 has shown that this differential lamin expression does not appear to directly influence β-catenin activity in the Wnt signalling pathway. Instead, data from this chapter suggests that these differences are perhaps mediated though other pathways such as the TGFβ-BMP signalling pathway, through the alteration of endogenous expression of growth-stimulatory factors such as Amphiregulin, or by potentially increasing the level of specific transcription factors such as AP-1 instead.

CHAPTER 6 – Lamin A down-regulates BMP4 and abrogates BMP4-mediated growth suppression in the SW480 colorectal cancer cell line

6.1 Introduction

6.1.1 The bone morphogenic protein signalling pathway

Bone morphogenic proteins are members of the Transforming Growth Factor-β (TGF-β) superfamily which have been shown to regulate proliferation, differentiation, chemotaxis and apoptosis in various cell types including mesenchymal cells, epithelial cells, haematopoietic cells and neuronal cells (Leivonen and Kahari 2007). Whilst their participation in the mesenchymal development of most tissues and organs is well known, their role in epithelial growth regulation is currently not well understood. As part of the TGF-β superfamily, BMPs utilise a similar signalling cascade to that of TGF-β. The BMP ligands use serine/threonine kinase receptors type IA (BMPRIA), type IB (BMPRIB) and type II (BMPRII) to transmit their signals into the cell (Koenig, Cook et al. 1994; ten Dijke, Yamashita et al. 1994; Gilboa, Nohe et al. 2000). The BMP ligands bind either cooperatively to preformed receptor complexes or first to a BMPRI receptor that then recruits BMPRII (Kirsch, Sebald et al. 2000). Additionally BMPs can bind to activin type II (ACVRII) and type IIB (ACVRIIB) receptors (Liu, Ventura et al. 1995; Rosenzweig, Imamura et al. 1995; Yamashita, ten Dijke et al. 1995). It has been shown that BMP7 preferentially binds to ACVRII and ACVRI but also has an affinity for BMPRII, BMPRIA and BMPRIB, whereas BMP2 and BMP4 appear to only bind to BMPRII and BMPRI (Macias-Silva, Hoodless et al. 1998; Greenwald, Groppe et al. 2003). Biologically active BMP4 is a disulphide-linked homodimer of the carboxyl-terminal 116 amino acid residues, which contains the characteristic seven conserved cysteine residues involved in the formation of the cysteine knot and the single interchain di-sulphide bond. Cellular responses to BMP4 have been shown to be mediated by the formation of hetero-oligomeric complexes of the type I and type II serine/threonine kinase receptors. Upon ligand binding, BMPRII phosphorylates

BMPRI which in turn phosphorylates intracellular SMAD1, SMAD5 or SMAD8 at the COOH terminals (Kretzschmar, Liu et al. 1997). It is thought that the phosphorylated (p)SMADs then associate with SMAD4, and the complex translocates to the nucleus to regulate the expression of various genes controlling cell proliferation, cell differentiation and apoptosis (Balemans and Van Hul 2002).

BMPs and their receptors have already been linked to the pathogenesis of some solid tumours. BMPRIA, BMPRII, and BMP2 mRNA levels have been found to be up-regulated in pancreatic cancers, 55% of which also have biallelic loss of SMAD4 (Moskaluk, Hruban et al. 1997; Zhang, Zhou et al. 1997). BMP2 is also thought to promote pancreatic cancer progression by enhancing the growth of pancreatic cancer cells (Kleeff, Maruyama et al. 1999; Dicuonzo, Angeletti et al. 2001). mRNAs for BMP receptors and BMP ligands have also been shown to be greater in cancer cells with less metastatic potential than in cells with higher metastatic potential (Arnold, Tims et al. 1999). However, BMP2 inhibits the proliferation of breast cancer cell lines that express both SMAD1 and SMAD4 (CAMA-1, MCF7, MDA-MB-231, T-47D and ZR-75-1) and up-regulates the cyclin-dependent kinase inhibitor p21^{WAF1} in these cells (Pouliot and Labrie 2002). BMP2 has also been shown to inhibit the proliferation of and cause cell cycle arrest in the G_1 phase of the MKN74 gastric cells (Wen, Miyake et al. 2004). Furthermore, the growth inhibitory effects of BMP2 have been seen in the androgen-sensitive prostate cancer cell line LNCAP, but no effect has been seen in the androgen-insensitive PC-3 and DU145 cell lines (Brubaker, Corey et al. 2004).

Recently Hardwick et al. (Hardwick, Van Den Brink et al. 2004) found that BMP2 inhibits normal colonic epithelial growth by promoting apoptosis and differentiation and inhibiting proliferation. They also found that BMP2, BMPRIA, BMPRIB, BMPRII pSMAD1, and pSMAD4 are expressed predominantly in mature colonocytes at the epithelial surface in normal adult human and mouse colon tissue samples and expression is significantly lower and/or absent at the base of the colonic crypts (Hardwick, Van Den Brink et al. 2004). Such work has been complemented by Kosinski (Kosinski, Li et al. 2007) who has shown high levels of expression of BMP antagonists at the base of colonic crypts.

Colorectal cancer develops as a result of uncontrolled cellular proliferation and dysregulation of cell death mechanisms. As such the inactivation of TGF-β superfamily signalling appears to play a key role in this and such inactivation of TGF-β signalling is seen in ~80% of all colorectal cancers (Grady, Rajput et al. 1998). On the other hand, inactivation of activin signalling via mutations in ACVRII, another TGF-β superfamily receptor, occurs in the majority of colon tumours with microsatellite instability (MSI) (Hempen, Zhang et al. 2003; Jung, Doctolero et al. 2004). Previous work has shown that BMP signalling mechanisms are in essence intact in human colon cancer specimens and also in several colorectal cancer cell lines and exogenous activation of these signalling pathways is moderately growth suppressive (Beck, Jung et al. 2006)

6.1.2 SW480 Colorectal cancer cell line

Review of the literature reveals that the SW480 colon cancer cell line used as the *in vitro* experimental model is Chapter 4 and 5 is microsatellite stable and SMAD4 null (Goyette, Cho et al. 1992; Hahn, Schutte et al. 1996). It has been shown that treatment with exogenous rhBMP2 and rhBMP7 does not lead to an increase in transcriptional activity of BMP target genes as determined by the BMP-specific SMAD-induced Luciferase reporter BRE-Luc (Beck, Jung et al. 2006). That said however, whilst transcriptional activity of target genes was unchanged by exogenous rhBMP treatment, data did show that treatment of the SW480 colon cancer cell line with rhBMP2 and rhBMP7 showed significant growth suppression even in the absence of SMAD4 as determined by direct cell counting (Beck, Jung et al. 2006). This was then taken further to show that treatment with exogenous rhBMP2 and to a lesser extent rhBMP7 significantly decreased scratch wound closure in the SW480 cell line such that wound closure was almost inhibited upon rhBMP treatment. (Beck, Jung et al. 2006)

Previous work by Willis, Cox *et al.* (unpublished data) has shown that there is a concomitant up-regulation of the actin-bundling protein T-plastin and down-regulation of the cell adhesion protein E-cadherin [a key regulator of adhesive properties in epithelial cells (Masciari, Larsson et al. 2007)] in the SW480 GFP-

Lamin A transfected cell line. It has previously been shown that E-cadherin is a down-stream target of the BMP signalling pathways and is also implemented in other solid tumours including breast and pancreatic (Zeisberg, Shah et al. 2005; von der Hardt, Bakkers et al. 2007; Yang, Du et al. 2007)

6.2 Results

Based on data from the chapter 5 microarray BMP4 appeared to be a good target to investigate as one of the potential mechanisms mediating the observed survival differences in patients harbouring lamin A/C positive and negative tumours.

In order to confirm data obtained from the transcriptomic analysis of chapter 5, work initially set out to assess the pattern of BMP4 expression at the protein level in both the SW480-GFP and SW480-GFP-lamin A cell lines. Whole cell extracts from 3 passages of both cell lines were taken and resolved on a 12% SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with the Anti-human BMP4 mouse monoclonal antibody (R&D Systems Inc., MN, USA). The antibody is stated to have no cross-reactivity with BMP-2, -3, -5, -6, and -7 in Western Blot analyses (R&D Systems Inc., MN, USA). Equal loading was confirmed by blotting for β-actin (figure 6.1B). Data showed a significant down-regulation of BMP4 in the presence of lamin A in the SW480-GFP-lamin A cells (figure 6.1A). This down-regulation was determined by densitometry to be 6.67 fold (p=0.0012) (figure 6.2) correlating with the observed down-regulation of BMP4 expression at the mRNA level.

6.2.1 Exogenous rhBMP4 does not alter CDH1 or PLS3 transcript levels

In order to assess the effect of exogenous Recombinant Human (rh)BMP4 treatment on the SW480-GFP-lamin A cell line, 100ng/ml of recombinant human BMP4 (R&D Systems Inc.) was added to media of cells. Whole cell extracts were then taken at t = 0hr, 2hr, 4hr, 6hr, 12hr, 18hr, 24hr and 48hr following 12hr pulsed rhBMP4 treatment. RNA was extracted and RT-PCR was run (as detailed

in section 2.6) to evaluate the transcript levels of E-cadherin (CDH1) and T-plastin (PLS3) based on previous work by Willis, Cox *et al.* (unpublished data).

E-Cadherin is a calcium-dependent cell adhesion molecule whose intact function is crucial for the establishment and maintenance of epithelial tissue polarity and structural integrity. Thus it is generally known that loss of E-cadherin expression is associated with the development of more diffuse and aggressive colorectal and other solid tumours (Becker, Atkinson et al. 1994; Oda, Kanai et al. 1994; Yoshiura, Kanai et al. 1995). Plastins are a family of actin bundling proteins which are involved in organising the actin cytoskeleton and are expressed in a wide range of tissues (Lin, Park et al. 1993; Delanote, Vandekerckhove et al. 2005). Enhanced expression of T-plastin has been associated with both drug and radiation resistant cancer cells. Of particular note is the significantly increased expression of T-plastin reported in cisplatin-resistant human cancers (Delanote, Vandekerckhove et al. 2005) and it follows that drug-resistant tumours are often associated with a much more aggressive phenotype.

Work carried out in duplicate shows an up-regulation in T-plastin mRNA levels in the SW480-GFP-lamin A transfected cell line and a concomitant down-regulation of E-cadherin vs. the SW480-GFP-Alone cell line at *t* = 0 (untreated). However, there was no detectable differences in the expression of these RNAs following treatment with 100ng/ml exogenous rhBMP4 at 12hr intervals (figure 6.3), suggesting that exogenous rhBMP4 treatment does not affect the transcriptional activity of these genes in this instance.

Figure 6.1 Western Blot analysis of BMP4 expression.

(A) Whole cell extracts from SW480-GFP (lane1) and SW480-GFP-Lamin A (lane 2) resolved on a 12% SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with anti-human BMP4.

(B) Whole cell extracts from SW480-GFP (lane1) and SW480-GFP-lamin A (lane 2) resolved on a 12% SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with anti-β-actin.

Immunoblots show an equal loading as revealed by β-actin staining (B). Blots also show a down regulation of BMP4 protein expression in the SW480-GFP-lamin A transfected cell line.

Molecular markers are in kDa.

Densitometric Analysis of BMP4 expression
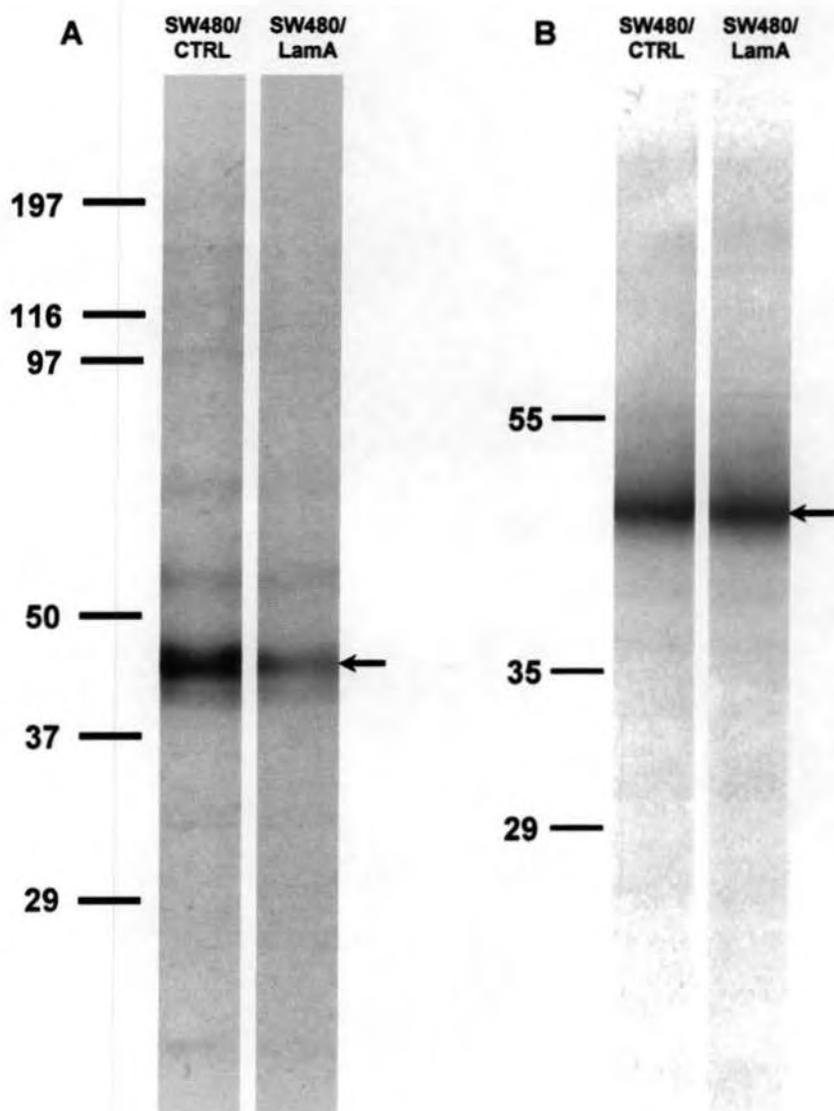
Figure 6.2

Densitometric analysis of Bone morphogenic protein 4 (BMP4) protein expressions in SW480-GFP and SW480-GFP-lamin A cell lines. Data show a down-regulation of BMP4 expression in the SW480-GFP-lamin A transfected cell line;

Δ = -6.67, t = 8.2187, df = 4 and p-value 0.0012 ('two-tailed' Students t-test)

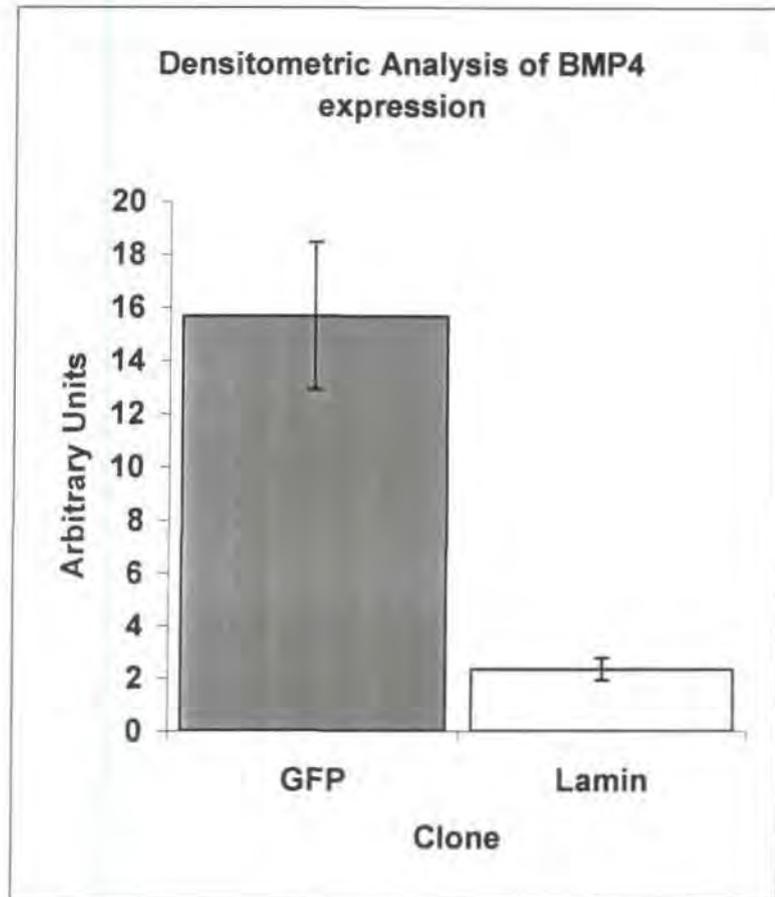**Figure 6.3** T-Plastin and E-cadherin mRNA transcript levels do not change following rhBMP4 treatment in the SW480-GFP-Lamin A cell line

Two step RT-PCR was performed on 0.1µg total RNA isolated from whole cell extracts taken at t = 0hr, 2hr, 4hr, 6hr, 12hr, 18hr, 24hr and 48hr. Expression of E-cadherin (CDH1) and T-Plastin (PLS3) was investigated using specific primers as detailed in table 2.2

L = Ladder
1 = SW480-GFP-Alone Control, t=0hr
2 = SW480-GFP-Lamin A + 100ng/ml rhBMP4, t=0hr
3 = SW480-GFP-Lamin A + 100ng/ml rhBMP4, t=2hr
4 = SW480-GFP-Lamin A + 100ng/ml rhBMP4, t=4hr
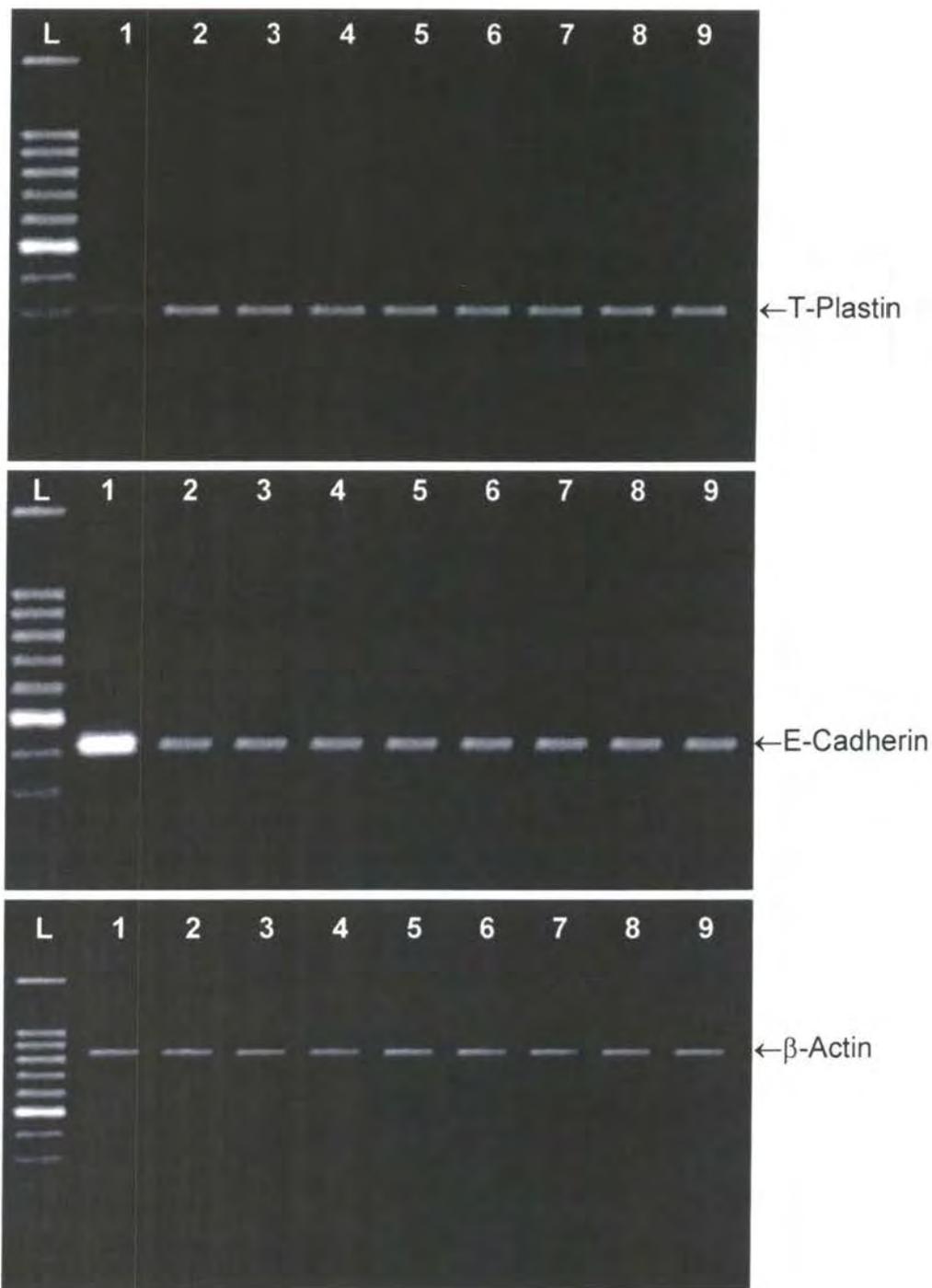5 = SW480-GFP-Lamin A + 100ng/ml rhBMP4, t=6hr
6 = SW480-GFP-Lamin A + 100ng/ml rhBMP4, t=12hr
7 = SW480-GFP-Lamin A + 100ng/ml rhBMP4, t=18hr
8 = SW480-GFP-Lamin A + 100ng/ml rhBMP4, t=24hr
9 = SW480-GFP-Lamin A + 100ng/ml rhBMP4, t=48hr

Equal loading of RNA in each sample was verified by monitoring the transcriptional level of β-actin.

←T-Plastin

←E-Cadherin

←β-Actin

6.2.2 Exogenous rhBMP4 partially decreases wound closure in SW480 control but not lamin A transfected cells

Despite a lack of change in mRNA expression of both T-plastin and E-cadherin, the effects of exogenous rhBMP4 treatment on cells was tested at a functional level by means of a simple wounding assay similar to that carried out by Beck *et al.* (Beck, Jung et al. 2006).

Briefly, cells were plated in six-well plates and grown to 95% confluency. A wound was then created in the shape of a cross with a p10 plastic pipette tip. Following wounding cells were washed twice with pre-warmed media and fresh media applied with and without 100ng/ml rhBMP4. Wounds were then photographed using a Nikon Diaphot 300 inverted microscope (Nikon corporation, Tokyo, Japan) at *t* = 0hr, 2hr, 4hr, 6hr, 12hr, 18hr, 24hr and 48hr. Media was replaced every 12hr, with or without fresh rhBMP4.

Results show that GFP-lamin A transfected cells are capable of closing scratch wounds at a rate approximately 5 times faster that the GFP-Alone control (figure 6.4 and figure 6.5A). However application of 100ng/ml exogenous rhBMP4 did not affect closure time of scratch wounds in the GFP-lamin A transfected cell line (figure 6.4 and figure 6.5B), but did significantly slow wound closure by approximately 2 fold in the GFP-Alone transfected cell line (figure 6.4 and figure 6.5C).

Figure 6.4 Expression of lamin A causes increased cell motility in SW480 colon cancer cells.

Three wounds more than 10mm in length and of equal thickness were made in 95% confluent cultures of SW480/cntl or SW480/lamA cells with a 10µl disposable Eppendorf tip. Cultures were then either left untreated or media was supplemented with 100ng/ml recombinant human BMP4 and pulsed with the same dose every 12hr thereafter. Phase contrast images were taken at t = 0hr, 2hr, 4hr, 6hr, 12hr, 18hr, 24hr and 48hr from identical regions.

Results show that the untreated SW480-GFP-Lamin A cell line is capable of closing the scratch wound within 18 hours whereas the wound remains unclosed after 48 hours in the SW480-GFP-Alone control cell line. Results also show that treatment with 100ng/ml recombinant human BMP4 has no observable effect on the SW480-GFP-Lamin A cell line but partially inhibits wound closure in the SW480-GFP-Alone control cell line.

Scale bar = 200µm
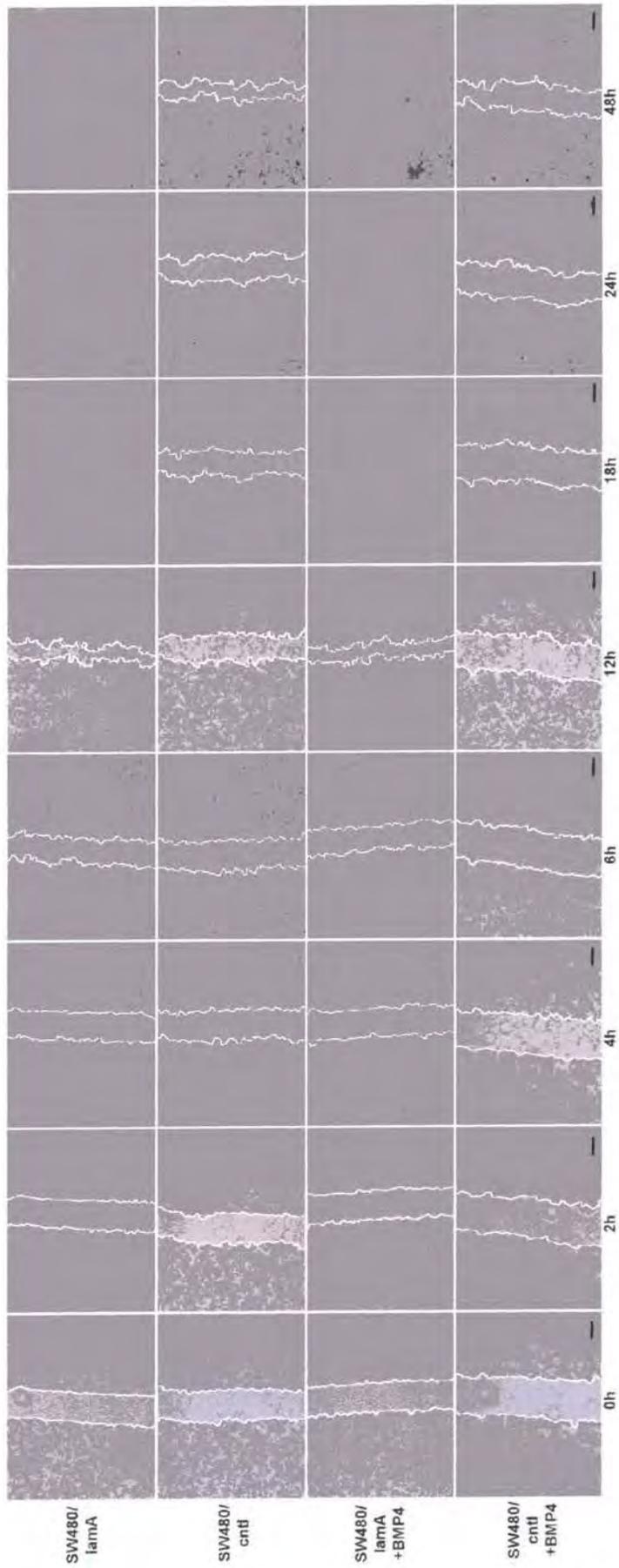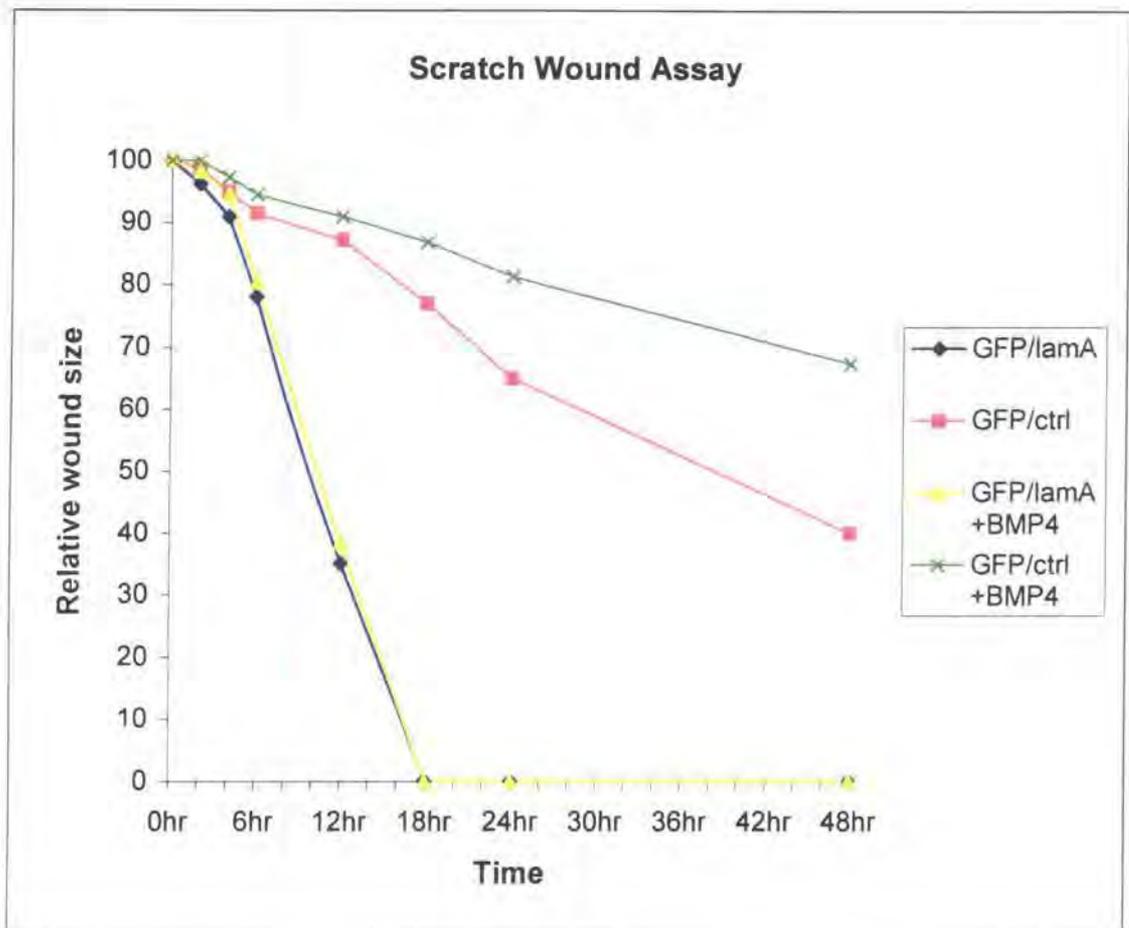
Figure 6.5 Relative wound size of SW480-GFP-lamin A and SW480-GFP-Alone control cells with or without exogenous rhBMP4 treatment over time.

The wound size relative to the starting wound size was measured using Zeiss LSM Image Browser software, version 3.1 at each time point in three independent experiments and expressed as a % of starting wound size. Both rhBMP4 treated and untreated SW480-GFP-lamin A cells closed the wound within 18 hours showing no difference in speed of closure between treatments. Wound closure was however approximately 5 times faster than the SW480-GFP-Alone untreated cell line. Despite no observable differences in the SW480-GFP-Lamin A cell line, rhBMP4 treatment did significantly suppress wound closure in the SW480-GFP-alone control cells by approximately 2 fold, with the wound reaching 40% of its starting size in untreated cells vs. ~70% in rhBMP4-treated cells at t = 48hr.

6.3 Discussion

BMPs are known to play a role in tissue development but until recently little work has been done examining the significance of BMP signalling in colorectal cancer. Work by several groups has however previously shown BMP signalling to affect normal epithelial cell growth (Pouliot and Labrie 2002; Brubaker, Corey et al. 2004; Hardwick, Van Den Brink et al. 2004). TGF-β and activin receptor ligands from the same superfamily as BMPs are known to be growth suppressors and have been shown to be inactivated in a subset of colon cancers. The loss of TGFβRI, TGFβRII and ACVR2 cell surface receptors have been shown to confer resistance of cancer cells to TGF-β induced growth suppression and as such have been postulated as candidate tumour suppressor genes in gastrointestinal cancers. (Markowitz and Roberts 1996; Grady, Rajput et al. 1998; Hempen, Zhang et al. 2003; Jung, Doctolero et al. 2004).

Data from chapter 5 has shown that there is a down-regulation of BMP4 mRNA in the GFP-lamin A transfected SW480 cell line vs. the GFP transfected control. Data from this chapter has also shown that this down-regulation subsequently results in a functional down regulation of BMP4 at the protein level by approximately 6.5 fold.

Previous work utilising the same microarray data as that used in Chapter 5 (Willis, Cox et al., unpublished data) has shown that transfection of SW480 with GFP-lamin A results in the down-regulation in expression of E-cadherin, a down-stream target of BMP signalling and an important cell adhesion molecule and a simultaneous up-regulation in T-plastin whose primary function is as a mediator of actin bundling. Data from transcriptomic analysis in this chapter supports this previous work by showing an up-regulation of T-plastin and down-regulation of E-cadherin at the mRNA level in the SW480-GFP-Lamin A transfected cell line (figure 6.3). Data also shows that upon treatment with exogenous rhBMP4 there are no detectable changes in these transcript expression levels which would follow work from Beck et al. (Beck, Jung et al. 2006) who have shown that treatment of the SW480 cell line with exogenous BMP2 and BMP7 does not alter the transcriptional activity of BMP target genes.

Concomitant to this (Willis, Cox *et al.*, unpublished data) and independent validation in this chapter has shown that the SW480-GFP-lamin A cell line also exhibits a much faster wound closure in wounding assays with the SW480-GFP-lamin A cell line closing a wound within 18 hours and the SW480-GFP transfected control failing to close the wound at 48 hours. This data supports similar work by Beck *et al.* where the SW480 parent cell line fail to close identically created wounds at 48 hours.

E-Cadherin is a calcium-dependent cell adhesion molecule whose intact function is crucial for the establishment and maintenance of epithelial tissue polarity and structural integrity. Thus it is generally known that loss of E-cadherin expression is associated with the development of more diffuse and aggressive colorectal and other solid tumours (Becker, Atkinson et al. 1994; Oda, Kanai et al. 1994; Yoshiura, Kanai et al. 1995). Work by Wen and colleagues has shown that rhBMP2 treatment significantly increases the levels of E-Cadherin at the protein level in OUMS-37 gastrointestinal tumour cells partially rescuing their aggressive phenotype (Wen, Miyake et al. 2004). Plastins are a family of actin bundling proteins which are involved in organising the actin cytoskeleton and are expressed in a wide range of tissues (Lin, Park et al. 1993; Delanote, Vandekerckhove et al. 2005). Enhanced expression of T-plastin has been associated with both drug and radiation resistant cancer cells. Of particular note is the significantly increased expression of T-plastin reported in cisplatin-resistant human cancers (Delanote, Vandekerckhove et al. 2005) and it follows that drug-resistant tumours are often associated with a much more aggressive phenotype.

Several recent findings suggest the involvement of BMP signalling in the tumourigenesis of several organs including the gastrointestinal tract. First the anti-proliferative activity of rhBMP2 in vitro has been implicated in several cancer cells, such as prostrate, gastric and colon (Ide, Yoshida et al. 1997; Hardwick, Van Den Brink et al. 2004; Horvath, Henshall et al. 2004; Wen, Miyake et al. 2004). Second, loss of BMP2 expression has been reported in several cancers, including prostrate and colon (Hardwick, Van Den Brink et al. 2004; Horvath, Henshall et al. 2004). Third, the close similarity of BMP2 and BMP4 and their shared receptors suggests a tight correlation in signalling mechanism. Thus, any

molecular alterations in the BMP signalling cascade may contribute to enhanced tumourigenesis.

Hence one could postulate that in this situation, the expression of lamin A/C in colorectal adenocarcinomas results in the down regulation of BMP4 expression as well as the abrogation of BMP4 mediated growth suppression and therefore directly influences colorectal carcinogenesis. However, what cannot be concluded from these studies is whether the two are mutually dependent and in this case, data would even suggest that they are in fact independent of one another. Thus the down-regulation of BMP4 at the protein level is not the mechanism of action by which BMP4 mediated growth suppression is occurring. This is not unusual as it has been shown in other cancers that the intra- or extra-cellular localisation of the same protein exhibit markedly different roles (Erler, Bennewith et al. 2006). Nonetheless, such evidence provided in this chapter does link expression of lamin A/C to colorectal tumour malignancy through loss of the anti-proliferative and growth suppressive effects of BMP4 mediated signalling, although the exact mechanism(s) by which this is occurring remain unknown.

At such a point one could postulate a mechanism by which that above data can be drawn together. The up-regulation of lamin A expression in colorectal tumours has been shown to be associated with a poor mortality in patients. Using *in vitro* models it has been shown that lamin A expression results in a significant down-regulation at both the RNA and protein level of BMP4. Similarly, we see a down-regulation of the same magnitude in E-cadherin which is known to be a downstream target of BMP signalling (Zeisberg, Shah et al. 2005; von der Hardt, Bakkers et al. 2007; Yang, Du et al. 2007) and is an important cell adhesion molecule. We also observe an increase in expression of the actin bundling protein T-plastin and work by Willis (Willis, Cox *et al.* unpublished data) has shown that expression levels of these two proteins are mutually dependent.

It has previously been shown that activation of BMP signalling suppresses Wnt signalling (Nishanian, Kim et al. 2004), through the merger of these signalling pathways via PTEN and this interplay between Wnt and BMP signalling is critically important in normal vertebrate development (He, Zhang et al. 2004;

Huang and Klein 2004). However, data from chapter 4 would suggest that such suppression is not occurring by direct alteration in β-catenin activity as measured by dual luciferase reporter assays, nor does previous work by Willis (Willis 2005) suggest that Wnt target genes such as c-Myc are altered by lamin A expression in the SW480 colon carcinoma cell line.

Data herein has shown that transfection of GFP-lamin A into the SW480 cell line also results in a more motile and less adherent cellular phenotype as determined by scratch wounding assays (figure 6.4). Similar assays carried out on BMP treated SW480 parent cells (Beck, Jung et al. 2006) has shown that the presence of exogenous BMPs almost completely inhibits would closure in this cell line and such work would suggest that treating the SW480 colon cancer cell line with exogenous BMPs can therefore exert SMAD4 independent growth effects by an as-yet-uncharacterised pathway.

However data here show that exogenous treatment of the SW480-GFP-lamin A transfected cell line with high levels of rhBMP4 does not alter mRNA levels of the BMP target gene CDH1 (E-cadherin) nor PLS3 (T-plastin) in either the short or long term. Concomitant to this, treatment fails to show any observable effect on wound closure as reported in previous similar experiments suggesting that lamin A expression in the SW480 cell line may abrogate BMP4 mediated responses in these cells, whereas in the SW480-GFP-Alone cell line wound closure is observed to be partially inhibited by exogenous BMP4 treatment.

Data obtained in chapter 4 has shown that β-catenin activity remains unchanged between the SW480-GFP-lamin A and SW480-GFP control transfected cell lines discounting the direct involvement of the canonical Wnt signalling pathway as the central mediator in the observed differences in cellular behaviour. Thus one could propose that the differences in patient survival between lamin A/C negative tumour patients and lamin A/C positive tumour patients is not mediated through the canonical Wnt signalling pathway. Instead it is perhaps mediated through the TGF-β signalling pathway via the bone morphogenic proteins resulting in a less adherent, more motile cellular phenotype in the lamin A/C positive cells through abrogation of BMP mediated responses. This difference in phenotype would go part way to explain the higher colorectal cancer related mortality in lamin A/C

positive tumour patients by means of potentially conferring a more invasive and aggressive phenotype to those tumours.

The mechanism by which abrogation of BMP/TGFβ signalling may be occurring could be through disruption of receptor expression and/or the signalling cascade. It would be interesting to assess the expression and localisation of BMP4 receptors BMPRII and BMPRI (which are BMP4 specific) in both the lamin A and control transfected cell lines at both the mRNA and protein levels.

Similarly, the expression of lamin A/C and in particular its many binding partners may be involved in the regulation of nuclear translocation of the TGFβ SMAD effectors thus antagonising BMP signalling pathways directly in this manner. It has recently been shown that a complex of MAN1 and A-type lamins has been shown to interact with the receptor regulated SMAD (rSMAD) and to antagonise TGF-β signalling by inhibiting rSMAD at the inner nuclear membrane (Lin, Morrison et al. 2005; Van Berlo, Voncken et al. 2005). Thus in lamin A/C negative tumours, the absence of nuclear lamin A/C expression, would allow nuclear translocation of TGFβ-SMAD signals and mediation of their associated growth suppressive properties.

Alternatively, another mechanism by which lamin A/C expression may regulate BMP signalling is via the upregulation of other pathways such as the ERK/MAPK pathway. It has been shown that up-regulation of this pathway leads to the phosphorylation of intracellular SMADs at their mid-linker regions (Kretzschmar, Liu et al. 1997) by activated ERK kinases (Beck, Jung et al. 2007) resulting in the inhibition of nuclear accumulation of BMP-activated SMAD1. Thus the negative regulation of BMP signalling by lamin A/C via this mechanism would be a means by which inhibition of BMP growth suppression could occur in the absence of mutation in BMP signalling components.

The mechanisms discussed above are purely speculative but what is true, is that evidence presented within this chapter may for the first time implement A-type lamin expression as a negative regulator of TGFβ/BMP signalling. More work is definitely needed to elucidate the exact mechanism by which this may be occurring, but it is intriguing to speculate that restoration of functional TGFβ/BMP

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signalling could contribute to new therapeutic strategies for colorectal carcinoma in the future.

CHAPTER 7 – General Discussion

7.1 Background to project

The implications of non-functional lamin A/C are borne out in the tissue-specific disorders such as autosomal inherited Emery-Dreifuss Muscular Dystrophy, Dilated Cardiomyopathy type-1A, Dunnigan type-Familial Partial Lipodystrophy, Mandibuloacral Dysplasia and Charcot-Marie-Tooth type-2B1 (Bonne, Di Barletta et al. 1999; Fatkin, MacRae et al. 1999; Cao and Hegele 2000; Raffaele Di Barletta, Ricci et al. 2000; De Sandre-Giovannoli, Chaouch et al. 2002; Novelli, Muchir et al. 2002). Mutations in the lamin A binding region of Emerin also give rise to an X-linked form of Emery-Dreifuss Muscular Dystrophy with similar clinical features to Autosomal Dominant Emery-Dreifuss Muscular Dystrophy (Bione, Maestrini et al. 1994; Lee, Haraguchi et al. 2001). Therefore a role for A-type lamins and their binding partners in tissue-specific diseases is well established. Considering that such disorders are not associated with the development of any malignancy, it is very interesting that altered expression and distribution of A-type lamins has also been reported in a growing number of neoplasms affecting epithelial, mesenchymal and lymphoid lineages, to the extent that the importance of A-type lamins and their binding partners in the regulation of growth pathways may be closely linked to tumour progression

The expression of nuclear lamins has been studied most extensively in lung cancer (Kaufmann, Mabry et al. 1991; Broers, Raymond et al. 1993) and keratinocytic tumours of the skin (Venables, McLean et al. 2001; Oguchi, Sagara et al. 2002; Tilli, Ramaekers et al. 2003), but work has also focussed on acute lymphoblastic leukaemia, non-Hodgkin's lymphoma (Stadelmann, Khandjian et al. 1990) and colorectal cancer (Cance, Chaudhary et al. 1992; Moss, Krivosheyev et al. 1999). In general these investigations have reported down-regulation of lamin A/C in association with increased proliferation and dedifferentiation in tumours.

The aforementioned studies provided the first indications that lamins may be important in the development and progression of cancer. However they failed to

link changes in expression to patient prognosis or directly to tumour progression and to date immunohistochemical investigations into A-type lamin expression in colorectal cancer have produced highly contradictory results (Cance, Chaudhary et al. 1992; Moss, Krivosheyev et al. 1999).

Colorectal cancer is the third most commonly diagnosed malignancy and the second most important cause of cancer mortality within the general population in the UK (Toms 2004). While methods for early detection have been developed, and in the case of Faecal Occult Blood (FOB) testing, are currently being rolled out in a nationwide program across the UK (Alexander and Weller 2003), the majority of treatment is curative rather than preventative. Clinicians still rely on the Dukes' staging and TNM classifications of colorectal tumours (Dukes 1932; AJCC 2002) in order to determine patient treatment regimens and predict prognosis. Although one cannot deny the contribution made by pathologists such as Cuthbert Dukes in improving treatment of the disease through the establishment of a universal staging system, the current morphological and pathological staging criteria are simply unable to provide detailed predictions of individual patient outcome [discussed by (Johnston 2004)]. Many biomarkers of tumour advancement and prognosis have been proposed, but a definitive marker or genetic signature has yet to be demonstrated. Given the intriguing data currently being expounded on nuclear lamin expression in epithelial tumours of the lung and skin, it is perhaps surprising that detailed investigations into the expression and regulation of lamins in a malignancy as common as colorectal cancer have not be undertaken so far. This thesis has aimed to go some way in rectifying this.

7.2 Differential Expression of lamin A/C in colorectal cancer is linked to patient prognosis

The expression of A-type lamins (lamins A and C) was evaluated both in a preliminary pilot and subsequent larger retrospective tissue archive originating from the Netherlands. Formalin fixed, paraffin embedded tumour specimens from 656 incident colorectal cancer patients taking part in the Netherlands Cohort Study on Diet and Cancer were immunohistochemically assessed using the anti-

lamin A/C JoL2, mouse monoclonal antibody (Dyer, Kill et al. 1997). Interestingly, differential lamin A/C expression was noted in patients of identical clinicopathological staging and appeared to have no correlation with standard clinical or pathological features of the tumours. Of the 656 patients assessed, 463 (70%) patients exhibited positive lamin A/C staining in the nuclei of tumour cells whilst the remaining 193 (30%) stained negative for nuclear lamin A/C expression in tumour nuclei. The availability of an internal control in terms of adjacent stromal tissue which always stained positive confirmed that a negative stain was as a result of an absence of lamin A/C and not poor staining. Within the follow-up period, 246 patients died with 163 of these patients dying as a result of CRC. Of those patients who died of colorectal cancer related causes within the study period (seven years) 127 (78%) scored positive for lamins A/C while 36 (22%) scored negative.

Statistical analysis carried out on data obtained in conjunction with patient, tumour and other molecular data extracted from the NLCS database showed that expression of lamin A/C in colorectal adenocarcinomas is more closely correlated with colorectal cancer related mortality following surgery than an absence of expression. Cox Hazard regression analysis indicates that this pertains to a Hazard ratio of 0.59 (95% CI: 0.41 – 0.86, p=0.006) for lamin A/C negative tumours or 1.89 (95% CI: 1.16 – 2.97, p=0.006) for lamin positive tumours indicating that patients with lamin A/C positive tumours are almost twice as likely to die from colorectal cancer related mortality than their clinicopathologically similar lamin A/C negative counterparts.

Further statistical analysis showed that the protective affect of lamin A/C negative status in tumours is independent of all other investigated patient, tumour and molecular characteristics supporting the notion that lamin A/C status could be used as an independent prognostic biomarker in colorectal cancer patients.

The finding that expression of lamin A/C in colorectal cancer tissue is correlated with a two fold increase in colorectal cancer related mortality, for the first time directly links these proteins to progression of a common disease. Previous studies have described altered expression of A-type lamins in a range of

cancers, including cancers of the skin, lung, lymphatics and soft tissue. However none of these studies was able to link either absence or presence of A-type lamins to tumour progression, although at least one study suggested that loss of expression of lamins A/C was correlated with enhanced proliferation rates in tumours. The other problems with these previous studies is that a limited number of samples were available for analysis and consequently study sizes fell below the threshold for reliable statistical analysis and made use of batch dependent polyclonal antisera (Cance, Chaudhary et al. 1992; Moss, Krivosheyev et al. 1999). In contrast, the findings presented in this thesis are based on a sufficiently large enough cohort to offer complete confidence in the significance levels reported and made use of the highly sensitive mouse monoclonal JoL2 anti-lamin A/C antibody.

7.3 Differential lamin A expression does not affect β-catenin activity in the SW480 colorectal cancer cell line

In order to address the question as to why there is a difference in survival between patients harbouring lamin A/C negative and lamin A/C positive tumours, I first looked to the canonical Wnt signalling pathway. Wnt signalling is an extremely important regulator of developmental pathways and has been shown to be implicated in over 90% of sporadic colorectal cancer cases (Miyoshi, Nagase et al. 1992; Powell, Zilz et al. 1992; Kinzler and Vogelstein 1996) through constitutive activation of the signalling pathway. With this in mind investigations were carried out to determine whether further changes in the Wnt signalling pathway were observed between lamin A positive and negative tumours using the naturally lamin A null SW480 colorectal cancer cell line. Results showed that there was no difference in β-catenin activity as investigated by Dual luciferase Tcf reporter assays. Upon stable transfection with full length pFlag-APC there was a decrease in β-catenin mediated transcriptional activity in both the GFP-lamin A transfected and GFP-alone control transfected cell lines. There was however no differences noted between cell lines thus rejecting the hypothesis that differential lamin A expression was exerting any significant influence on the canonical Wnt signalling pathway.

7.4 Differential lamin A expression results in multiple genome wide changes

Data from chapter 4 showed that the canonical Wnt signalling pathway, classically at the heart of colorectal adenocarcinoma initiation and progression is not influenced by differential lamin A expression. Thus it would suggest that the canonical Wnt signalling pathway is not centrally implicated in the protective effects mediated by lamin A/C negative status in patient tumours. Similarly with no correlations being drawn from chapter 3 to link differential lamin expression in tumours with other oncogenic mutations in proteins such as *TP53* and *Ki-ras*, it suggests that the observed protective effect is mediated through one or more other developmental pathways which had yet to be investigated. Hence a genome-wide Affymetrix DNA Microarray analysis of the SW480 colorectal cancer cell line was embarked upon to explore members of other potential signalling pathways which may have been influenced by differential lamin A expression and thus may be implicated in mediating these observed differences in patient survival.

Simple microarray data mining based on international ontological definitions highlighted a series of genes implicated in other cellular pathways and components such as cell-cell adhesion; cytoskeletal reorganisation; protein phosphatase/kinase pathways, such as MAPK/ERK; EGF signalling pathways; BMP signalling pathways; Slit/Robo signalling pathways and more. A subset of 5 genes selected was independently confirmed using RT-PCR providing a platform for further investigation.

7.5 Differential lamin A expression may abrogate BMP4-mediated signalling in the SW480 colorectal cancer cell line

The expression of the GFP-lamin A fusion protein in the SW480 colorectal cancer cell line was shown in chapter 5 to dramatically down-regulate the expression of BMP4 at the mRNA level (by approximately 7 fold) and this was independently confirmed using RT-PCR to be approximately 6.5 fold. This down-

regulation was then shown to pertain to a functional down-regulation at the protein level (chapter 6) of approximately 6.5 fold, potentially implicating lamin A expression in BMP signalling pathways.

Work carried out by Willis, Cox *et al.* (unpublished data) has shown that in the same GFP-lamin A transfected SW480 cell line there is a 5 fold up-regulation in expression of the actin bundling protein T-Plastin and a concomitant 7 fold down-regulation in the expression of the cell adhesion molecule E-cadherin. Their work also showed that this down-regulation pertains to the functional protein level in terms of E-cadherin, although unfortunately reliable T-Plastin antibodies are currently unavailable. As such, scratch wound assays performed on the GFP-Alone and GFP-lamin A transfected cell lines show a remarkable difference in wound closure time (chapter 6), with the GFP-lamin A transfected SW480 cells closing the wound roughly 5 times faster within 18 hours and remaining unclosed after 48 hours in the SW480-GFP-Alone cells. Thus a hypothesis can be put forward that the synergistic down-regulation in the cell-cell adhesion protein E-cadherin, and up-regulation in the actin-bundling protein T-plastin leads to a less adherent, more motile cellular phenotype which is functionally observed in scratch wound assays.

Work has also been carried out in the SW480 parent cell line by Beck (Beck, Jung et al. 2006) whereby using a similar wounding assay they show that wound closure time is greatly increased to a point of almost total inhibition upon treatment with exogenous BMP2 or BMP7. However, using the BMP-specific SMAD-induced Luciferase reporter (BRE-Luc) they also show that whilst there appears to be growth suppressive properties of exogenous BMP treatment, the transcriptional activity of BMP target genes appear to remain unchanged. Data from chapter 6 supports such work since E-cadherin is known to be a down stream target of BMP signalling, yet we observe no changes in CDH1 mRNA expression levels upon treatment with high levels of exogenous BMP4 in the SW480-GFP-lamin A cell line, nor do we see changes in the mRNA expression levels of PLS3.

In line with Beck's study, treatment with exogenous recombinant BMP4 does partially inhibit wound closure in the SW480-GFP-Alone control cell line by just

under 2 fold, but does not appear to affect wound closure time in the SW480 GFP-lamin A transfected cell line. Such data would suggest that the expression of lamin A seems to abrogate BMP mediated growth suppressive responses and as such may be implicated as a central mediator in the protective effects observed by a lamin negative status in colorectal tumours. The exact mechanism by which this is facilitated remains unclear, but for the first time, this evidence may implicate A-type lamins in the regulation of TGFβ/BMP signalling pathways.

7.6 Final Conclusions and Future Work

In summary I have shown that the expression of lamin A/C in a large retrospective archive of incident colorectal cancer cases is closely linked to patient mortality and as such a lamin A/C positive status in tumours is linked to a 2 fold increase in patient mortality. I have then moved on to show that differential expression of lamin A/C in a colorectal cancer *in vitro* model cell line does not influence β-catenin activity in the case of either truncated or full-length APC and as such determine that the protective effects observed due to a lamin A/C negative status of tumours must be mediated by other mechanisms. Through the use of a genome-wide microarray, I then identify a series of other cellular pathways which may be implicated instead and move to investigate the effects of BMP4 signalling as the central mediator, showing the presence of lamin A results in a more motile, less adherent cellular phenotype by potentially abrogating the growth suppressive responses of BMP signalling

Future work should concentrate on investigating BMP signalling further in our lamin A *in vitro* models with the recent acquisition of the full length BMP4 construct (Wyeth, UK). By stably transfecting the BMP4 gene into the SW480-GFP-alone and GFP-lamin A transfected cells lines under an inducible promoter, I could test whether overexpression of BMP4 at the transcriptional level is capable of overcoming the effects of lamin A expression. Continuing this work, the use of BMP-inducible reporters such as the BMP-specific SMAD-induced Luciferase reporter (BRE-Luc) used by Beck *et al.* could be used to monitor the transcriptional activity of BMP4 target genes under the above conditions. The reverse experiment would be to knockdown the expression of BMP4 in the

SW480-GFP-alone cell line in order to assess whether it adopted phenotypic characteristics more closely resembling that of the GFP-lamin A cell line, for example, decreases in scratch wound closure time. These experiments would then shed light on whether or not the protective effects of lamin A expression are primarily mediated through BMP signalling.

Elucidating the exact mechanism by which lamin A is affecting BMP signalling is of central importance. Evaluation of BMP receptor expression and also of intracellular SMAD expression and phosphorylation status would also be of particular interest to ascertain whether or not lamin A transfected cells possess the capability to respond to BMP signals in terms of receptors, and if so whether the nuclear translocation of such signals is intact and functional in lamin A expressing cells.

Further work into other potential gene targets identified from the genome-wide microarray would also be pertinent in order to find secondary markers which can be taken back to the NLCS and screened in order to create a bank of biomarkers which can be used as a prognostic tool. Similarly, it may be interesting to look at other available archives such as the Western Australian tissue array bank which also contains over several thousand specimens alongside similar detailed information for patients as that obtained from the NLCS. With such a large repository of tissue samples available, it would allow individual substrata to be investigated, for example colon vs. rectal, in order to determine the full extent of A-type lamins role in colorectal cancer. It would also allow investigations to be conducted subdividing patients into those who did and did not receive adjuvant therapies and such a trial would allow one to determine whether differential lamin A/C expression also influences the response of tumours to these adjuvant therapies.

It would also be of interest to explore whether or not the expression of Apoptotic markers correlated with lamin A/C status in order to determine whether lamin A/C negative tumour cells are more likely to undergo programmed cell death thus conferring the apparent protective effect observed *in vivo*.

In terms of re-probing the microarray database, one would like to perform further cluster analysis based on conserved promoter regions and *cis*-regulatory modules in order to determine whether the absence of lamin A/C may be directly (or through its binding partners) affecting gene transcription, such as would be the case of the AP-1 transcription factor complex mentioned in section 5.3. Software such as that mentioned in 5.1.2.2 would facilitate such an investigation.

In conclusion, one would postulate from evidence provided herein that expression of nuclear lamin A in colorectal adenocarcinomas could confer a more stem-cell like characteristic to tumour cells which is mediated through multiple genome wide changes including suppression of BMP-mediated and other developmental signalling responses, activation of endogenous growth-stimulatory factors and loss of regulation of transcription factor complexes resulting in a more motile, less adherent phenotype of the cells. Such changes in cellular phenotype would then manifest themselves clinically in terms of poorer patient prognosis and as such, based on this work lamin A expression may be an important, independent prognostic marker for colorectal cancer in the future.

Appendix I

pFlag-APC Plasmid map
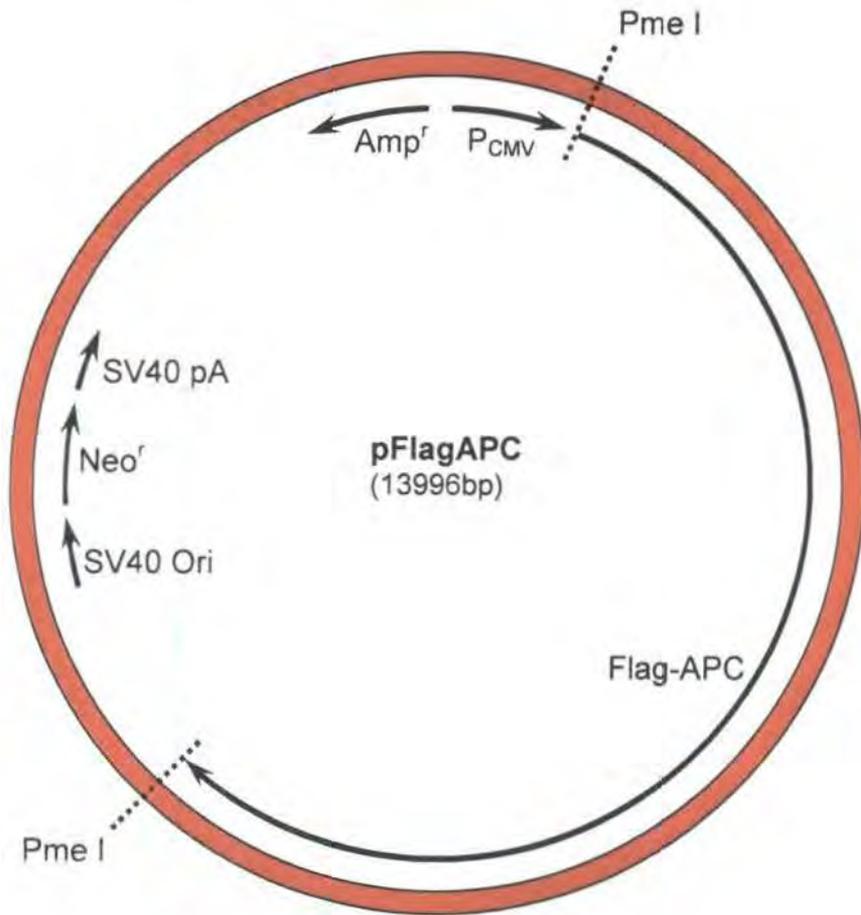
Appendix II

Cancer Research Technologies licensing opportunity:

Lamin A/C: Prognostic marker in colorectal cancer

CRT Licensing Opportunity

Lamin A/C: Prognostic marker in colorectal cancer

· Urgent unmet medical need for improved stratification of CRC patients
· Lamin A/C is an independent prognostic marker for relapse or progression of early CRC
· Validated by immunohistochemistry in 656 independent patient samples
· Expression of Lamin A/C correlates with an increased risk of colorectal cancer death

DIAGNOSTICS

Introduction

Studies in the laboratory of Professor Chris Hutchison at the University of Durham have demonstrated that the presence of the proteins lamin A/C in early stage colorectal cancer (CRC) sensitively predicts increased risk of tumour recurrence and likelihood of cancer-related death. As such, lamin A/C is a biomarker useful for guiding therapeutic strategy and disease follow-up

Background

Colorectal cancer is the third most common form of cancer, and accounts for the greatest proportion of cancer deaths in the Western world. Currently, colorectal cancers are graded pathologically by the Dukes scale (A – D) according to the size, invasiveness and spread of the tumour. The long-term outlook for Dukes C and D patients is poor, although adjuvant chemotherapy has been shown to reduce the rate of recurrence after surgery by 40-50% and overall deaths by 16%. In contrast, greater than 85% of Dukes A patients are treated successfully by surgery alone

The effects of adjuvant chemotherapy for the Dukes B subset of patients have been harder to prove, though small survival benefits have been demonstrated. As such the majority of Dukes B cases are currently treated by surgical resection alone, though greater than 30% of these suffer tumour recurrence and cancer-related death. There is an urgent medical need to develop diagnostic tests that can identify this high-risk group of Dukes B patients that may

benefit from a more aggressive treatment regime after diagnosis

The Technology

The Lamin A/C gene encodes a number of intermediate type filament proteins, the A-type lamins, which are major components of the nuclear lamina. The inventors have found that positive nuclear expression of lamin A/C in colorectal tumours is significantly correlated with poor prognosis. The inventors have observed that cells expressing lamin A become significantly more motile and less adherent than control cultures and expression of lamin A affects the expression profile of a number of genes known to be associated with tumour progression

Proprietary antibodies were used to determine nuclear lamin A/C expression by double blind scoring of IHC staining of >650 randomly-selected independent CRC patient samples from the Netherlands Cohort Study on Diet and Cancer. The samples were derived from a large number of centres and associated with 5-year follow-up data. Cox hazard ratio scoring indicated that patients expressing lamin A/C are twice as likely to suffer CRC-related death compared to patients lacking the biomarker (p=0.001)
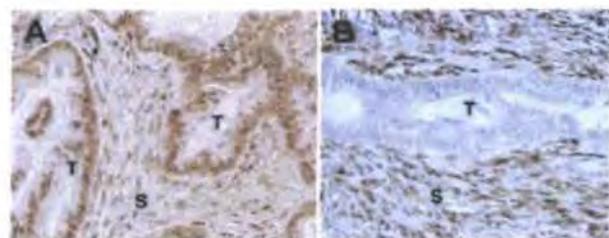
CRT Licensing Opportunity

Figure 1 Staining of CRC tissue with anti-lamin A/C monoclonal antibody JoL2. JoL2 was either strongly positive (A) or negative (B) in the tumour (T). Surrounding stromal tissue (S) was always positive giving an internal positive control and demonstrating that a negative stain corresponds to absence of the antigen rather than poor staining.

Notably, based upon previous studies on this cohort, lamin A/C was the only highly significant prognostic indicator amongst other known potential indicators also assessed (including APC promoter methylation, APC truncations, lack of p53 expression and Ras activating mutations). Of 515 samples assessed in the Dukes A, B and C patient groups, the presence of lamin A/C predicted CRC-related death with 81% sensitivity.

Taken together, these data indicate that lamin A/C is a highly sensitive independent marker of a high-risk CRC population that could be used in conjunction with other markers to establish a biomarker panel to guide therapeutic strategy for CRC patients.

Intellectual Property

CRT holds the rights to a US provisional patent application which is available for licensing together with antibody clones JoL2 and JoL4 and associated data. CRT is seeking partners for exclusive licensing or collaborative development.
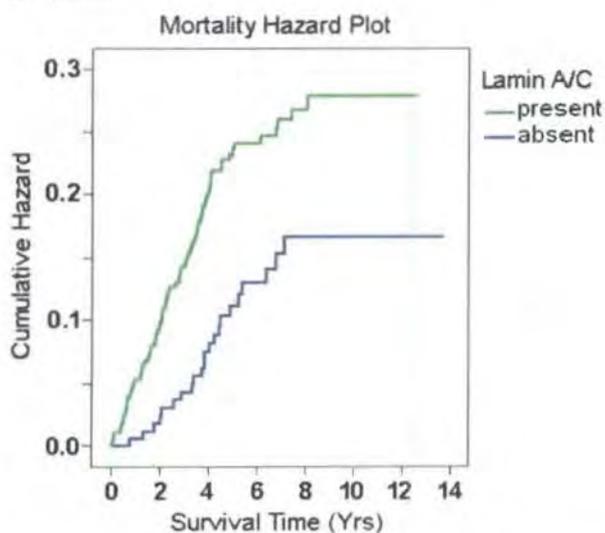
Contact: *Laura Fletcher lfletcher@CancerTechnology.com*

Figure 2 Kaplan-Meier plot of cumulative hazard for colorectal cancer patients in relation to lamin A/C expression.

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