

**Aspirin Effects on the Activation of c-Src-induced  
NF- $\kappa$ B-Dependent Apoptotic Mechanisms in Colorectal  
Cancer**

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## **Contents**

	<b>Page(s)</b>
<b>Contents</b>	<b>2</b>
<b>Dedications</b>	<b>3</b>
<b>Declaration</b>	<b>4</b>
<b>Acknowledgements</b>	<b>5</b>
<b>Chapter Contents</b>	<b>6</b>
<b>List of Tables and Figures</b>	<b>10</b>
<b>List of abbreviations</b>	<b>12</b>
<b>Abstract</b>	<b>16</b>
<b>Chapter 1</b>	<b>18</b>
<b>Chapter 2</b>	<b>84</b>
<b>Chapter 3</b>	<b>108</b>
<b>Chapter 4</b>	<b>147</b>
<b>Chapter 5</b>	<b>197</b>
<b>References</b>	<b>202</b>
<b>Appendix 1</b>	<b>238</b>



## **1. Dedications**

**To friends, family and loved ones who have seen me through the lowlights and highlights of this work. To those lost and found during the journey - thank you for being there with with me.**

## 2. Declaration

I declare that;

- (a) this thesis has been composed and written by myself, with advice, critique and guidance from my supervisors Dr Lesley Stark and Prof Malcolm Dunlop.
- (b) the work is my entirely own, except where otherwise stated;
  - (i) kind assistance was provided by Dr Oliver Maddocks, PhD, in helping to maintain initial short term ex-vivo cultures for optimisation, providing images for figure 4.1a and in explant C3 Damage scoring throughout chapter 4, as one of three independent scorers.
  - (ii) kind assistance was provided by Mr. Neil Waslidge in helping to maintain longer-term ex-vivo cultures for optimisation and treatments and who assisted provision of the following western blots as part of grant funded work under my supervision and direction; 4.12a, 4.14c, 4.15a, 4.16a, 4.18a, 4.18c, 4.20c. He assisted in cell counting and explant C3 Damage scoring throughout chapter 4 as one of three independent scorers.
- (c) the work has not been submitted for any other degree or professional qualification.

Richard Brady 4 October 2012

The following publications derived from the research presented in this thesis are published;

**Brady RR**, Loveridge C, Dunlop MG, Stark LA. c-Src dependency of NSAID-induced effects on NF $\kappa$ B-mediated apoptosis in colorectal cancer cells. *Carcinogenesis*. 2011;32(7):1069-77.

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## 4. Chapter Contents

<b>Chapter 1 – Introduction</b>	<b>18</b>
1.1 Colorectal cancer (CRC): incidence, treatment and detection	
1.1.1 CRC: incidence and mortality	20
1.1.2 Treatment options and outcomes	26
1.1.3 Detection and screening	30
1.2 Causation	
1.2.1 Theories of development and genetic risk factors	33
1.2.2 Inherited risk factors	42
1.2.3 Environmental risk factors	45
1.3 Prevention and chemoprevention	51
1.3.1 Folate	51
1.3.2 Calcium and Vitamin D	52
1.3.3 HRT / Oestrogens	53
1.3.4 Fibre	53
1.3.5 Statins	55
1.3.6 Other agents	55
1.3.7 Aspirin and related Non-Steroidal Anti-inflammatory Drugs	57
1.3.8 Combination approach	64
1.3.9 Conclusions	64
1.4 Putative mechanisms of aspirin's chemopreventative effects	66
1.4.1 Background	66
1.4.2 COX-2-dependent mechanisms of aspirin-induced chemoprevention	66
1.4.3 COX-2 Independent Mechanisms of aspirin-induced chemoprevention	70
1.4.4 The MAP kinase pathway	71
1.4.5 Cyclin-dependent kinases	72
1.4.6 PPAR $\gamma$ /Alternative targets	73
1.5 NF- $\kappa$ B transcription factor and its activation.	74
1.5.1 The role of the NF- $\kappa$ B transcription factor in colorectal cancer.	78

1.5.2 Aspirin effects on NF- $\kappa$ B activity in CRC	79
1.6 c-Src and the NF- $\kappa$ B pathway; relevance to CRC Chemoprevention	82
1.6.1 c-Src Structure and Function	82
1.6.2 Activation and substrate interaction with c-Src	86
1.6.3 c-Src and FAK	86
1.6.4 c-Src and the EGF Receptor Family	88
1.6.5 c-Src and apoptosis	89
1.6.6 c-Src and the NF- $\kappa$ B Pathway	90
1.6.7 c-Src and NSAIDs	90
1.7 Experimental approach	93
<b>Chapter 2 - Materials and Methods</b>	<b>94</b>
2.1 Cell Culture and methods	95
Summary of cell lines used	95
Maintenance of cell lines and media	95
Reagents (aspirin, other stimuli and inhibitors)	96
Treatment protocol	98
2.2 Generation and amplification of plasmids	98
Transformation of competent cells	88
Growth of transformed <i>E.coli</i>	98
Plasmid preparation	98
2.3 Transient transfection of adherent cells	99
2.4 <i>In-vitro</i> organ culture of human colonic mucosal explants	99
Harvesting	99
Maintenance	104
Reagents and Treatment protocols	105
2.5 Protein biology	105
Kinase assays	105
Preparation of cytoplasmic/whole cell extracts from cells/tissue	106

Protein concentration determination	107
2.6 SDS page	108
Western blot + stripping membranes	108
2.7 Annexin V –FITC assays	110
2.8 Imaging	112
Immunocytochemistry	112
Image analysis - quantification of nuclear RelA	112
2.9 Immunohistochemistry	114
Microscopy	117
Tissue damage scores	118
<b>Chapter 3 – Effects of Aspirin on c-Src, and the role of c-Src in aspirin-induced NF-<math>\kappa</math>B signaling and apoptosis in colorectal cancer cells</b>	
3.1 Introduction	120
3.2 Detection of native c-Src in CRC cells	123
3.3. Detection of aspirin–induced c-Src kinase activity	125
3.4 Aspirin induces phosphorylation of c-Src Y416 in CRC cells	127
3.5 Aspirin-induced cellular relocalisation of c-Src is consistent with c-Src activation	130
3.6 Aspirin induces Y118 phosphorylation of paxillin	135
3.7 Aspirin induces c-Src activation prior to NF- $\kappa$ B activation	137
3.8 c-Src complexes with I $\kappa$ B $\alpha$ but not RelA	139
3.9 Chemical inhibition of c-Src inhibits aspirin’s molecular effects	141
3.10 Genetic inhibition of c-Src inhibits aspirin’s molecular effects	143
3.11 Active c-Src activates NF- $\kappa$ B independently of aspirin	145
3.12 Chemical inhibitors of c-Src inhibit aspirin’s apoptotic effects	147
3.13 Genetic modulation of c-Src function mediates aspirin-induced apoptosis	147
3.14 Discussion	151

<b>Chapter 4 – <u>Study of aspirin induced effects and Wnt/notch modulation in an <i>in vitro</i> culture model of human large bowel mucosa</u></b>	<b>160</b>
4.1 Background	161
4.2 Short-term outcomes of explants are dependent on culture media	164
4.3 Longer-term outcome of explants are dependent on culture media	169
4.4 Optimisation of explant culture conditions	172
4.5 Characterisation of apoptosis and proliferation in long-term culture	173
4.6 Differentiated cell populations during long-term culture	176
4.7 Identification and characterisation of colonic stem cells in IVOC	182
4.8 Regenerative cyst characterization	184
4.9 Aspirin-mediated modulation of c-Src in colonic mucosal explants	187
4.10 Aspirin modulation of paxillin in mucosal explants	189
4.11 Aspirin-induced NF- $\kappa$ B signalling and apoptosis in human colonic mucosal explants	192
4.12 <i>Ex vivo</i> growth of colonic polyps	193
4.13 Lithium chloride activates colonic mucosal Wnt signalling	197
4.14 Inhibition of Notch signalling induces goblet cell differentiation	201
4.15 Discussion	204
<b>Chapter 5 - Discussion</b>	<b>210</b>
5.1 Elucidating the molecular mechanisms responsible for linking aspirin-induced c-Src and NF- $\kappa$ B signalling activation	210
5.2. Utility of IVOC model to future work and exploration of findings derived from optimisation.	213
5.3 Conclusion	217
<b>References</b>	<b>219</b>
<b>Appendix 1= c-Src dependency of NSAID-induced effects on NF<math>\kappa</math>B-mediated apoptosis in colorectal cancer cells.</b> Carcinogenesis. 2011; 32(7):1069-77.	256

## **5. List of Tables and Figures**

### **Chapter 1**

Table 1.1 CRC staging systems	23
Table 1.2 Stage-specific 5-year survival rates and incidence	23
Table 1.3 Histological types of CRC	24
Figure 1.1 Anatomical distribution of CRC.	26
Table 1.4 Relative survival rate (%) at 5 years for rectal /colonic cancer	26
Figure 1.2 The adenoma-carcinoma sequence	35
Table 1.5 Major genetic syndromes	45
Figure 1.3 Previous case-control trials examining aspirin/NSAIDs impact on CRC endpoints	60
Figure 1.4 The Cox pathway	70
Figure 1.5. Models of NF- $\kappa$ B Signalling	77
Figure 1.6 Structure of c-Src	86
Figure 1.7 c-Src activation and regulation: conformational opening	86

### **Chapter 2**

Figure 2.1 Reagents used	97
Figure 2.2 IVOC set-up	103
Table 2.1 Antibodies used in western blotting	111
Table 2.2 Antibodies used in immunocytochemistry	113
Table 2.3 Antibodies used in Immunohistochemistry	116
Figure 2.4 The “3C” scoring system	119

### **Chapter 3**

Figure 3.1 Detection of native c-Src in CRC cells	124
Figure 3.2 Detection of aspirin-induced c-Src kinase activity	126
Figure 3.3 Aspirin induces phosphorylation of c-Src Y416 in CRC cells	128
Figure 3.4 Aspirin-induced cellular relocalisation of c-Src is consistent with c-Src activation	131
Figure 3.5 Aspirin-induced cellular relocalisation of FAK is consistent with c-Src activation	132
Figure 3.6 Aspirin-induced cellular relocalisation of cytoskeletal elements is consistent with c-Src activation	136



Figure 3.7 Aspirin induces Y118 phosphorylation of Paxillin	136
Figure 3.8 Aspirin induces c-Src activation prior to NF- $\kappa$ B activation	138
Figure 3.9 c-Src complexes with I $\kappa$ B $\alpha$ but not RelA	140
Figure 3.10 Chemical inhibition of c-Src inhibits aspirin's molecular effects	142
Figure 3.11 Genetic inhibition of c-Src inhibits aspirin's molecular effects	144
Figure 3.12 Active c-Src activates NF- $\kappa$ B independently of aspirin	146
Figure 3.13 Chemical inhibitors of c-Src inhibit aspirin's apoptotic effects (PP2/SU6566)	148
Figure 3.14 Genetic modulation of c-Src function mediates aspirin-induced apoptosis	149
Figure 3.15 A model for aspirin/NSAID-induced modulation of NF- $\kappa$ B signalling and apoptosis	158

#### **Chapter 4**

Figure 4.1 Explant survival is dependent on culture media	166
Figure 4.2 Longer term outcome of explants are dependent on culture media	168
Figure 4.3 Optimisation of explant culture conditions	171
Figure 4.4 Characterisation of apoptosis in culture	174
Figure 4.5 Characterisation of proliferation in culture	175
Figure 4.6 Epithelial cell populations during long-term culture	177
Figure 4.7 Vil-1 positive cell populations during long-term culture	179
Figure 4.8 Secretory cells during long-term culture	180
Figure 4.9 Entero-endocrine and smooth muscle cell populations during long-term culture	181
Figure 4.10 Identification and characterisation of colonic stem cells (CSC) in IVOC	183
Figure 4.11 Regenerative cyst characterization	186
Figure 4.12 Aspirin-mediated modulation of c-Src in colonic mucosal explants	188
Figure 4.13 Aspirin-mediated modulation of c-Src aggregates in colonic mucosal explants	190
Figure 4.14 Aspirin modulation of paxillin in mucosal explants	191
Figure 4.15 Aspirin-induced NF- $\kappa$ B signalling and apoptosis in human colonic mucosal explants	194
Figure 4.16 Aspirin-induced apoptosis in human colonic mucosal explants	195
Figure 4.17 <i>Ex vivo</i> growth of colonic polyps	196
Figure 4.18 Lithium chloride activates colonic mucosal Wnt Signalling (i)	199
Figure 4.19 Lithium chloride activates colonic mucosal Wnt Signalling (ii)	200



## 6. List of abbreviations

5-FU	5-fluorouracil
8-oxo-dG	8-oxo-7,8-dihydroxy-2'-deoxyguanosine
ACF	Aberrant Crypt Foci
ACPGBI	Association of Coloproctology of Great Britain and Ireland
AMP	Adenosine monophosphate
ATP	Adenosine-5'-triphosphate
AOM	Azoxymethane
APC	Adenomatous Polyposis Coli
ATL	Aspirin-triggered lipoxin
BAX	Bcl-2-associated X protein
BMI-1	B lymphoma Mo-MLV insertion region 1 homolog
BMI	Body Mass Index
CDK	Cyclin-dependent kinases
CEA	Carcino-embryonic antigen
CIN	Chromosomal instability
CIMP	CpG Islands Methylator Phenotype
COX	Cyclo-oxygenase
CRC	Colorectal Cancer
CREB	cAMP response element-binding
CRP	C-Reactive Protein
CRT	Chemo-radiotherapy
CSC	Colonic Stem Cells
CT	Computerised Tomography
DBZ	1,2-dimethylhydrazine
<i>DCC</i>	Deleted in Colon Cancer
DNA	Deoxyribonucleic acid
DMH	Dimethylhydrazine

EBV	Epstein Barr virus
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	epithelial-mesenchymal transition
ERAS	Enhanced Recovery After Surgery
ERK	Extracellular signal-regulated kinase
FAK	Focal Adhesion Kinase
FAP	Familial Adenomatous Polyposis
FOBT	Faecal Occult Blood Testing
GFR	Growth Factor Receptor
GSK-3	Glycogen synthase kinase 3
GPCR	G protein-coupled receptors
HAT	Histone acetyl transferases
HCC	Hepato-cellular carcinoma
HIV	Human Immunodeficiency Virus
HNPCC	Hereditary non-polyposis colorectal cancer
HRT	Hormone Replacement Therapy
IGF-1	insulin-like growth factor-1
I $\kappa$ B	Inhibitor of NF $\kappa$ B
IKK	I $\kappa$ B kinase
IL-6	interleukin 6
iNOS	inducible nitric oxide synthase
IVOC	In-vitro organ culture
JNK	c-Jun N-terminal Kinase
kDa	Kilodalton
KRAS	Kirsten rat sarcoma viral oncogene homolog
LOH	Loss of heterogeneity
LPS	Lipopolysaccharide
MAPK	mitogen activated protein kinase

mGPS	modified Glasgow Prognostic Score
MIN	microsatellite instability
MMR	Mismatch Repair
mM	Millimoles
MSI	Microsatellite instability
MSI-H	MSI – High
MSK1	mitogen-and stress-activated protein kinase-1
MTHFR	Methylenetetrahydrofolate reductase
NAG-1	NSAID activated gene-1
NCI	National Cancer Institute
NEMO	NF- $\kappa$ B Essential Modifier
NF- $\kappa$ B	Nuclear Factor Kappa B
NICE	National Institute for Health and Clinical Excellence
NHS	National Health Service
NIK	NF $\kappa$ B-inducing kinase
NLR	Neutrophil:Lymphocyte Ratio
NO	Nitric Oxide
NOC	N-nitroso compounds
NLS	nuclear localisation signal
NSAIDS	Non-steroidal Anti-inflammatory Drugs
O3Fas	Omega-3 fatty acids
ODC	Ornithine decarboxylase
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3-K	Phosphatidylinositol 3-kinase
PLR	Platelet:Lymphocyte Ratio
PG	Prostaglandin
PGDH	Prostaglandin Dehydrogenase
PI	Prognostic Index

PKC	Protein kinase C
PNI	Prognostic Nutritional Index
PP2	4-amino-5-(4-chlorophenyl)- 7-( <i>t</i> -butyl) pyrazolo [3,4- <i>d</i> ]pyrimidine
PPAR $\gamma$	Peroxisome proliferator-activated receptor gamma
RCT	Randomised Controlled Trial
RER	Replication error
RHD	Rel homology domain
RR	Relative risk
RSV	Rous Sarcoma Virus
SCPRT	Short-course pre-operative radiotherapy
SFK	Src Family of tyrosine Kinases
TME	Total Mesorectal Excision
TNF- $\alpha$	Tumour necrosis factor alpha
mCRC	Metastatic CRC
RNA	Riboxynucleic acid
TGF	Tumour Growth Factor
TNF	Tumour Necrosis Factor
TS	Thymidylate synthase
UK	United Kingdom
US	United States
UV	Ultra-violet
VEGF	Vascular Endothelial Growth Factor

## 7. Abstract

Compelling evidence indicates that long-term aspirin or related non-steroidal anti-inflammatory drugs (NSAIDs) ingestion can protect against colorectal cancer (CRC). The predominant mechanism of action of NSAIDs is the induction of apoptosis and it has been shown that NSAIDs stimulate the NF- $\kappa$ B pathway as a key component of this pro-apoptotic effect. Here, I demonstrate that aspirin activates the c-Src tyrosine kinase pathway in CRC cells. This activation occurred in a time- and dose-dependent manner, preceding aspirin-mediated degradation of I $\kappa$ B $\alpha$ , nuclear/nucleolar translocation of NF- $\kappa$ B/RelA and the induction of apoptosis. Furthermore, inhibition of c-Src activity, by chemical inhibition or expression of a kinase-dead form of the protein, abrogated aspirin-mediated degradation of I $\kappa$ B $\alpha$ , nuclear translocation of RelA and apoptosis, suggesting a causal link. Expression of constitutively active c-Src mimics aspirin-induced stimulation of the NF- $\kappa$ B pathway. These data established the role of c-Src activation in the NF- $\kappa$ B and apoptotic response to aspirin in CRC cells *in vitro*.

However, difficulty exists in the translational relevance of experimental findings derived from cancer cell lines, therefore I developed and optimised an *in-vitro* organ culture (IVOC) of human *ex-vivo* colonic mucosa. This provides an experimental platform that recapitulates the complex molecular, cellular and architectural environment of the *in-vivo* human colonic mucosa. I demonstrated that following a period of degeneration, the tissue regained its original architecture and was repopulated with a cell population that was representative of the original tissue. Furthermore, I identified putative colonic stem cell (CSC) populations within the tissue and demonstrated that colonic tissue growth and differentiation can be modulated by Wnt signalling agonists and Notch signalling antagonists. Finally, I show aspirin-mediated c-Src activation, NF- $\kappa$ B signalling and apoptosis in this setting. These data provide compelling evidence that c-Src is an upstream mediator of aspirin effects on NF- $\kappa$ B signaling and apoptosis in CRC cells and have relevance to the development of future chemotherapeutic / chemopreventative agents. Furthermore, these data describe a model system providing a relevant and translational experimental platform of much utility to the future study of aspirin-induced effects, CSC regulation and dynamics in the human colonic mucosa, including further confirmation of the role of Wnt and Notch signalling pathways in cell proliferation and differentiation.

<b>Chapter 1 – Introduction</b>	<b>18</b>
1.1 Colorectal cancer (CRC): incidence, treatment and detection	
1.1.1 CRC: incidence and mortality	21
1.1.2 Treatment options and outcomes	27
1.1.3 Detection and screening	31
1.2 Causation	
1.2.1 Theories of development and genetic risk factors	34
1.2.2 Inherited risk factors	41
1.2.3 Environmental risk factors	46
1.3 Prevention and chemoprevention	52
1.3.1 Folate	52
1.3.2 Calcium and Vitamin D	53
1.3.3 HRT / Oestrogens	54
1.3.4 Fibre	54
1.3.5 Statins	56
1.3.6 Other agents	56
1.3.7 Aspirin and related Non-Steroidal Anti-inflammatory Drugs	58
1.3.8 Combination approach	65
1.3.9 Conclusions	65
1.4 Putative mechanisms of aspirin’s chemopreventative effects	67
1.4.1 Background	67
1.4.2 COX-2-dependent mechanisms of aspirin-induced chemoprevention	67
1.4.3 COX-2 Independent Mechanisms of aspirin-induced chemoprevention	71
1.4.4 The MAP kinase pathway	72
1.4.5 Cyclin-dependent kinases	73
1.4.6 PPAR $\gamma$ /Alternative targets	74
1.5 NF- $\kappa$ B transcription factor and its activation.	66
1.5.1 The role of the NF- $\kappa$ B transcription factor in colorectal cancer.	79
1.5.2 Aspirin effects on NF- $\kappa$ B activity in CRC	80



1.6 c-Src and the NF- $\kappa$ B pathway; relevance to CRC Chemoprevention	83
1.6.1 c-Src Structure and Function	83
1.6.2 Activation and substrate interaction with c-Src	87
1.6.3 c-Src and FAK	87
1.6.4 c-Src and the EGF Receptor Family	89
1.6.5 c-Src and apoptosis	89
1.6.6 c-Src and the NF- $\kappa$ B Pathway	90
1.6.7 c-Src and NSAIDs	91
1.7 Experimental approach	93

## **1.1 Colorectal cancer (CRC): incidence, treatment and detection**

### **1.1.1 CRC: incidence and mortality**

Colorectal Cancer (CRC) is a disease characterised by abnormal growth and development of a malignant neoplasm arising from the lining of the large intestine (colon or rectum) (Cooper et al 2010). In the developed world, CRC is the third most common cancer in men after lung and prostate cancer and the second most common in women after breast cancer (Parkin 2001).

Worldwide, there are 1.2 million new cases per year and over 600,000 disease-related deaths (Ferlay et al 2010), accounting for greater than 10% of the total worldwide cancer related deaths (Greenlee et al 2001). In 2008, there were 40,000 new cases of CRC in the UK and 16,000 disease-related deaths, comprising around 13% of the total cancer burden. The lifetime risk is approximately 1 in 19 in females and 1 in 14 in males (Heavey et al 2004). All age groups have demonstrated an increased incidence of CRC since 1975 but it is those in the over 60s age group that have shown the greatest increase (20%) (83% of CRCs occur within this group). Gender distribution is roughly equal, however male risk is 1.5 fold that of females (<http://info.cancerresearchuk.org/cancerstats>) (Heavey et al 2004). Hence, CRC is a major public health issue and whilst the detection and various treatment options have led to improved outcomes, the overall 5-year survival rate continues to remain around 50% (Cooper et al 2010, Gondos et al 2009).

#### *Metastatic disease*

Approximately 20–25% of patients already have metastases at the time of diagnosis and 40–50% of patients develop metastases after resection of the primary tumour (Goldberg et al 2005, Scheele et al 1990). The liver is often the first site of metastatic disease and may be the only site of spread in as many as 30–40% of patients with advanced disease. Given that tumour dissemination in this case is putatively via the portal system, surgical resection of isolated hepatic metastases from CRC may be curative in a number of cases (reviewed in Garden) (Garden et al 2006). For patients with extra-hepatic metastases or non-resectable liver metastases, systemic treatment to reduce the size and number of metastases can be undertaken. For most patients with metastatic CRC (mCRC), treatment is palliative with the aim of prolonging survival and maintaining quality of life (Folprecht 2008).

### *Pathological factors that influence prognosis*

The prognosis of patients with CRC is highly correlated with the extent of tumour penetration through the bowel wall, the presence or absence of nodal involvement and the presence or absence of distant metastases. These characteristics form the basis for all staging systems developed for this disease. Staging of the tumour is the strongest predictor of survival for patients with CRC. Some of these staging systems are described in table 1.1. The associated stage-specific 5-year survival rates are outlined in table 1.2 (Compton 2003).

The different histological types of CRC are outlined in table 1.3. More than 90% of CRC cases are adenocarcinomas, derived from the colonic mucosal epithelium (Kerr and Midgley 2010). Other histological types include, mucinous (colloid) carcinomas, which display large areas of mucus external to the cell, and signet-ring cell carcinomas, which have large amounts of mucus within the cell, pushing the nucleus to the periphery. Mucinous, signet-ring cell and small-cell carcinomas are prognostically unfavorable (Compton et al 2000, Compton 2003). Medullary carcinoma is a distinct type of non-gland-forming carcinoma, which has a favourable prognosis and a strong association with microsatellite instability (MSI), DNA mismatch repair gene (MMR) dysfunction, and the hereditary non-polyposis colon cancer (HNPCC) syndrome (Ponz de Leon and Di Gregorio 2001). Other rarer forms of colonic tumour include lymphoma or squamous cell cancers, but as these are clinically and pathologically distinct, we will not focus on these sub-types within this thesis.

Tumour grading depends on cell differentiation within the tumour and relates to the degree of glandular architecture, cellular pleomorphism, and mucosecretion of the predominant cell pattern within CRC tumours. Whilst this has been described by a variety of different grading systems, generally low grade tumours are those containing cells that are well or moderately differentiated and high grade tumours those containing cells that are poorly differentiated or undifferentiated (Compton 2003). High-grade tumours provide a poor stage-independent prognostic factor (Compton et al 2000).

Table 1.1 CRC staging systems

a

UICC/TNM	Modified Dukes'
Stage 0 Carcinoma in situ	A
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)	B
Stage III Nodal involvement, no distant metastasis 1 to 3 regional lymph nodes involved (any T, N1, M0) 4 or more regional lymph nodes involved (Any T, N2, M0)	C
Stage IV Distant metastasis (any T, any N, M1)	D

From Colorectal Cancer Pathology  
(C. C. Compton)

b

Table 1.2 Stage-specific 5-year survival rates and incidence

Dukes' Stage at diagnosis	Percentage of cases	Five-year relative survival
A	8.7%	93.2%
B	24.2%	77.0%
C	23.6%	47.7%
D	9.2%	6.6%
Unknown	34.3%	35.4%

from <http://info.cancerresearchuk.org/cancerstats>

Table 1.3 Histological types of CRC

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World Health Organization Classification of Colorectal Carcinoma

---

Adenocarcinoma  
Medullary carcinoma  
Mucinous (colloid) adenocarcinoma (<50% mucinous)  
Signet-ring cell carcinoma (>50% signet-ring cells)  
Squamous cell (epidermoid) carcinoma  
Adenosquamous carcinoma  
Small-cell (oat cell) carcinoma  
Undifferentiated carcinoma  
Other (e.g., papillary carcinoma)

---

From Compton CC 2002

Figure 1.1 (obtained from CRUK CancerStats) displays the anatomical distribution of CRC. Differing anatomical locations are associated with reported variations in survival, one of the most investigated variations is that which exists between rectal and colonic cancer (table 1.4). Right-sided CRC tumours (ascending colon and caecum) tend to have a worse prognosis compared to left sided tumours, as patients tend to present at a more advanced stage and their occurrence is proportionally greater in elderly patients (Meguid et al 2008, Suttie et al 2011). Right-sided tumours also tend to be exophytic and present with anaemia, whereas left-sided tumours tend to be more annular and can present with obstruction. Other anatomical and biochemical differences also exist between the right and left colon, as they have a different arterial supply, differing mucin production (Caldero et al 1989) and luminal content (Macfarlane et al 1989). Further molecular, microbiological and signalling differences within right and left colonic mucosa are described in chapter 4.

#### *Patient factors that influence prognosis*

Patient factors associated with poor prognosis from CRC are increasing age, BMI, smoking, patients with co-morbidities, or those requiring operative urgency. Specifically, patients presenting with bowel obstruction/bowel perforation have a particularly poor prognosis (Morris et al 2011, Tan et al 2007). Studies have also found that survival rates in the UK are significantly associated with levels of deprivation, with the most deprived in the population having the poorest outcomes (Coleman et al 2001). Racial differences in outcome have also been observed, particularly in the US. African American males experience higher CRC incidence rates and almost a 50% greater disease-related mortality, compared to white males (Edwards et al 2010). It is likely that these differences are multifactorial and reflect variations in tumor biology (increased right-sided (proximal) tumors among African Americans, genetics by race (Polite et al 2006)) or relate to other factors such as socioeconomic factors, obesity, screening rates and health care utilization (Loconte et al 2011).

#### *Molecular factors associated with prognosis*

Tumours can be distinguished by the mutational status of the distinct molecular pathways responsible for their development or continuing survival. Some of the mutations in these pathways are prognostically relevant, such as those responsible for MMR function. MMR deficiency accounts for

Figure 1.1 Distribution of CRC, Great Britain, 2006-2008

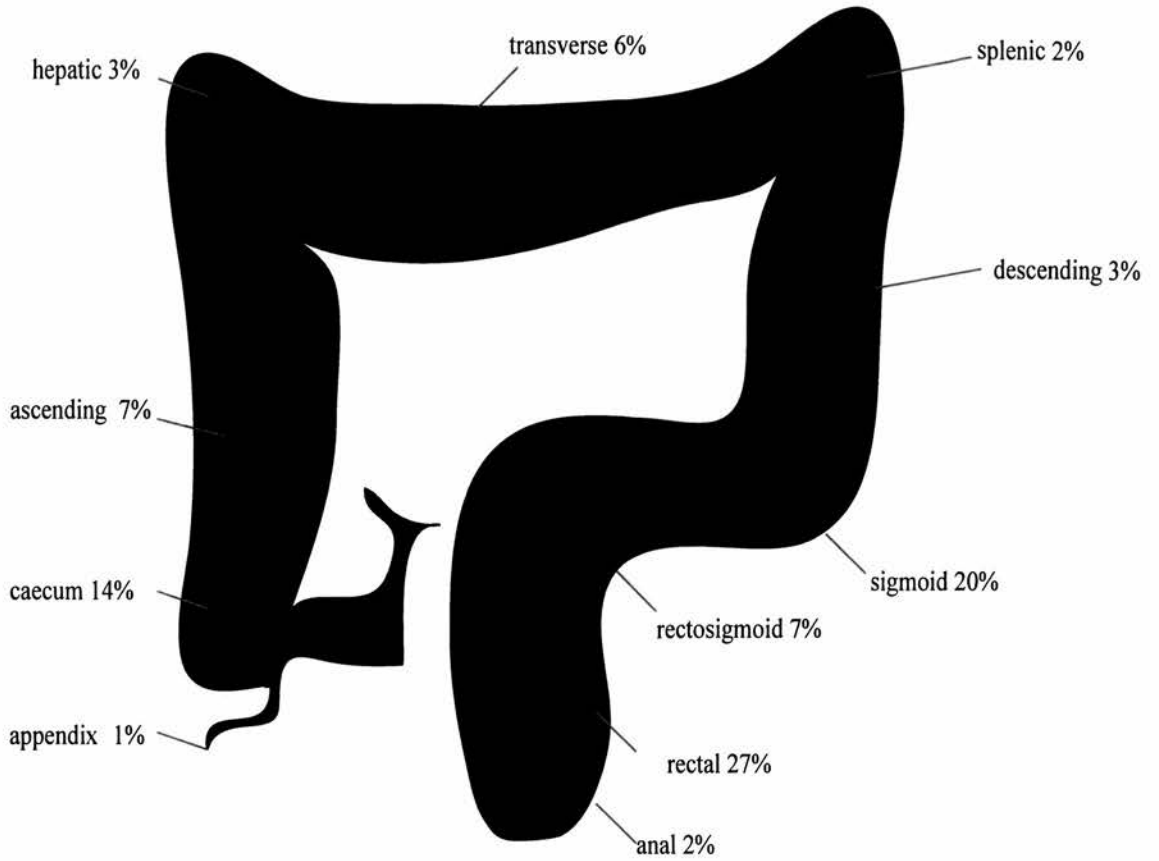


Figure modified from CRUK Cancerstats

Table 1.4 Relative survival rate (%) at 5 years for rectal /colonic cancer

	1971-1975	1991-1995
<b>Males</b>		
Colon	32.0	47.4
Rectum	30.6	45.2
<b>Females</b>		
Colon	34.8	49.5
Rectum	32.9	51.4

from McCardle MS

about 15 – 20% of sporadic CRC and also includes those patients with Lynch Syndrome (See chapter 1.2). Mutations in MMR genes cause instability of nucleotide repeats (microsatellites (MSI)). If > 30% of the microsatellite markers are altered, the tumour is defined as microsatellite-high (MSI-H). MSI-H tumours are found predominantly on the right side of the colon and have a better prognosis (Samowitz et al 2001) but are associated with a resistance to fluorouracil (FU)-based adjuvant chemotherapy (French et al 2008, Sargent et al 2010). KRAS activating mutations also engender resistance to some chemotherapeutic agents (see 1.1.2 *Chemotherapy*).

The gene, Deleted in Colon Cancer (DCC/18q), plays a role in cellular differentiation and epithelial:stromal interaction. 18q loss of heterozygosity (LOH), which is detected by PCR amplification of polymorphic MSI markers at or near 18q21, has been associated with shorter disease-free and overall survival (Wang et al 2010). However the evidence is conflicting (Carethers et al 1998).

Components of the systemic inflammatory response, C-reactive protein, albumin, white cell, neutrophil, lymphocyte and platelet counts have been studied over many years to assess their prognostic value in predicting outcome following cancer diagnosis. These have been combined to form inflammation-based prognostic scores (such as the modified Glasgow Prognostic Score (mGPS), Neutrophil Lymphocyte Ratio (NLR), Platelet Lymphocyte Ratio (PLR), Prognostic Index (PI) and Prognostic Nutritional Index (PNI)). Generally, higher inflammatory scores are associated with a reduced cancer survival (Proctor et al 2010). However, to date, many of these markers continue to remain research tools only and are excluded from treatment/diagnostic algorithms (Kerr and Midgley 2010).

### **1.1.2. Treatment options and outcomes**

#### *Surgery*

Mortality rates from CRC are progressively decreasing over time and the majority of this improvement is due to improvements in detection, surgical technique and peri-operative management (Morris et al 2010). An estimated 92% of colon cancer patients and 84% of rectal cancer patients



undergo surgical resection as the primary mode of treatment (Compton 2003). For the majority of these patients, surgery is performed with curative intent and approximately 40% will remain disease-free in the long term. In 20–30% of cases, the disease is too far advanced at initial presentation for curative intervention to be possible and many of these patients die within a few months (reviewed in the National Institute for Health and Clinical Excellence (NICE). Guidance on cancer services: improving outcomes in colorectal cancers London: NICE; 2004).

The majority of UK patients with CRC currently undergo resection by open approach but developments in surgical techniques such as laparoscopic colorectal surgery are increasingly being employed (ACPGBI *National bowel cancer audit 2010*). Whilst, there are no differences in the rates of overall survival, lymph node harvest, disease-free survival and tumour recurrence between these two approaches, patients undergoing laparoscopic resections have improved recovery times (hospital stay is 20% shorter (Abraham et al 2004) and decreased post-operative morbidity, less post-operative pain and need for analgesia (Schwenk et al 2005). Wound infection rates, incisional herniation, small bowel obstruction, blood loss and blood transfusion requirements (Schwenk et al 2005) are all reportedly reduced with laparoscopic surgery but key surgical quality indicators, such as anastomotic leakage and mortality rates, are similar to those for open procedures (Duepre et al 2003). The addition of enhanced recovery after surgery (ERAS) programmes following laparoscopic CRC surgery may further improve short-term recovery and reduce hospital stay.

### *Chemotherapy*

Adjuvant chemotherapy can help reduce the chance of recurrence of disease and significantly improve overall survival in patients with early stages of CRC undergoing surgery (Chau and Cunningham 2002). 5-fluorouracil (5-FU) has been utilized as a treatment for patients with mCRC over the past 40 years (Kohne and Folprecht 2004) but its use is associated with a significant degree of side effects (Xiong and Ajani 2004). Moreover, resistance to the drug can occur and certain molecular subtypes of tumour are prognostically relevant (MSI-H/18q LOH) (Banerjee et al 2002). Thymidylate synthase (TS) is needed for DNA synthesis in the S-phase of the cell cycle and is reported to be a target enzyme of 5-fluorouracil. Higher levels of TS putatively correlate with resistance to 5-fluorouracil, however

studies have been conflicting (Allegra et al 2003, Lenz et al 1998). Clinical studies have also reported that high TS protein expression is strongly associated with tumour recurrence (Leichman et al 1995). Furthermore, several clinical studies have reported that CRC patients with wild-type *TP53* derive a survival benefit from fluorouracil (FU)-based chemotherapy but those with mutant *TP53* do not (Iacopetta 2003).

Other chemotherapeutic agents have been developed and used to treat CRC including: capecitabine (thymidylate synthase inhibitor) and oxaliplatin (diaminocyclohexane platinum, a DNA cross-linker) (Napier and Ledermann 2000). Other drugs like CPT11 (irinotecan, a DNA topoisomerase I inhibitor) are available but like 5-FU, associated with a significant degree of toxicity. Different combinations of these drugs, such as the FOLFOX regimen (leucovorin, 5-FU and oxaliplatin), the FOLFIRI regimen (leucovorin, 5-FU and irinotecan) and the XELOX regimen (oxaliplatin and capecitabine) with or without a monoclonal antibody agent, have been shown to improve outcomes for patients with metastatic disease [Reviewed in Edwards] (Edwards et al 2012).

Bevacizumab (which targets vascular endothelial growth factor (VEGF)) was the first approved monoclonal antibody for CRC treatment in 2004 and is indicated for the first- and second-line treatment of mCRC in combination with fluoropyrimidine-based chemotherapy. Cetuximab and panitumumab, which target the epidermal growth factor receptor (EGFR), are also now in use. Activating mutations of the *KRAS* oncogene occur in 30-50% of CRC tumours and are of key relevance to treatment, as *KRAS* activating mutations give rise to tumours that are resistant to anti-EGFR antibody therapy (Kerr and Midgley 2010, Walther et al 2009). Cetuximab and panitumumab are therefore only indicated for wild-type *KRAS* mCRC as monotherapy, and cetuximab in combination with chemotherapy, in Europe and in combination with irinotecan in irinotecan-refractory wild-type *KRAS* mCRC in the US, however, despite this, 40-60% of patients with wild-type *KRAS* will still not respond to these agents (Amado et al 2008, Karapetis et al 2008, Wilson et al 2010). Mutated *BRAF* is present in 5-10% of CRC tumours and affects the tumour response to these agents, especially in those with wild-type *KRAS* i.e. wild-type *KRAS* tumours have a survival benefit with these agents, *BRAF* mutation is a marker of poor prognosis and response (Bokemeyer et al 2012).

## *Rectal Cancer*

Treatment of rectal cancer is stage specific. Surgery alone is usually undertaken for those with T1/2 disease. The role of short course preoperative radiotherapy (SCPRT) for rectal cancer has been examined in a number of large RCTs. These have found that there is little benefit in treating T1/ T2 N0 tumours and no statistically significant difference in the local recurrence rate for tumours lying from 0 to 5 cm from the anal verge. Short-course pre-operative radiotherapy (SCPRT) is therefore reserved for T3 Tumours >5 cms from anal verge (evidence is conflicting below 5cms), anterior tumours in men in which the mesorectal fascia is not breached and T1/2 tumours with grossly involved perirectal lymph nodes within fascia. Downstaging or long course chemoradiotherapy (CRT) is usually reserved for fixed, inoperable tumours or those with a high risk of breaching mesorectum and not achieving clearance through resection [reviewed in Gollins S] (Gollins 2010).

The development of local recurrence has a major influence on long-term survival after rectal cancer resection and is highly dependent on pathological clearance of the tumour at the time of resection (avoidance of a “positive resection margin”) (Nagtegaal and Quirke 2008). Significant variation in local recurrence rates of 0 to 21% among surgeons operating on rectal cancers was previously detected (McArdle et al 1990). However, the development of standardised total mesorectal excision (TME) surgery by Cecil and others (Cecil et al 2004, Moore et al 2005, Taylor et al 2011) as the surgical standard of care, was one of the most important factors in reducing local recurrence. Together with a >1mm clear resection margin, this is still the mainstay of treatment for operable tumours with no metastases and in those fit for surgery.

Perforation of the tumour during resection (Patel et al 1977, Zirngibl et al 1990) and anastomotic leakage are associated with worse survival (up to a five-fold increase in 30-day mortality) and a significant increase in the local recurrence rate (McArdle et al 2005). Therefore, surgical technique is an important factor in patient outcome after rectal resection. Recent developments such as an extra-sphincteric (prone) resection excludes the formation of a TME waist and putatively results in further reduction of recurrence rates but is associated with increased surgical morbidity (de Campos-Lobato et al 2011).

### 1.1.3 Detection and screening

The goal of screening is to reduce cancer mortality by early detection of the disease. Preventative screening is distinctly different from early detection screening, as this aims to target the disease before it becomes malignant by detecting and removing precursor lesions. CRC screening provides both detection and prevention as more than 90% of bowel cancer cases are adenocarcinomas and the majority of these are thought to arise from pre-existing adenomatous polyps (adenomas) and develop over time.

Adenomatous polyps/adenomas are growths arising from the lining of the intestine, which may or may not develop into colorectal cancer. There are three histological types of adenomas: tubular, tubulovillous and villous. Tubular adenomas are the most common and have a tube-like structure. Villous adenomas are the least common and have a 'frilly' or cauliflower-like structure. Tubulovillous adenomas are a mix between the two. Villous adenomas are the most likely to become cancerous, followed by tubulovillous adenomas (Cooper et al 2010).

Polyps are present in about a third of the European and USA populations. Indeed, approximately 5% of people have polyps by age 50, increasing to 50% for people over 70 years old (Kinzler and Vogelstein 1996). 1 in 10 of these will progress to malignancy. Flat adenomas account for about 10% of all lesions and have a greater propensity to malignant change. Adenomas that are either greater than or equal to 1 cm in diameter or have villous or tubulovillous features or severe dysplasia are called high-risk or advanced adenomas. Low-risk adenomas are less than 1 cm in diameter and are non-villous/tubulovillous without severe dysplasia (Cooper et al 2010).

The various screening options that are currently available for CRC are Faecal Occult Blood Testing (FOBT), endoscopy (Sigmoidoscopy and colonoscopy) and molecular faecal and blood markers. The rationale behind FOBT is based on the observation that traces of blood (occult blood) are released into the bowel lumen by CRC/larger polyps. The FOBT analyses stool samples for the presence of this occult blood either by guaiac-based tests (gFOBTs) (which detects the presence of any blood in the

stool, including that from raw meat), or immunochemical tests (iFOBTs), which are more specific for human blood (Bretthauer 2011).

The Scottish Bowel Screening Program biannually screens all men and women between 50 and 74 years of age. FOBTs are performed on three different bowel movements and the test is returned to a processing centre. Depending on the number of positive tests, the patient will either be retested with iFOBTs, or invited for a colonoscopy. Randomised studies have demonstrated a reduction in cancer-related mortality of around 15-33% in those undergoing routine FOBT screening (Mandel et al 1999) (Hardcastle et al 1996, Hewitson et al 2007).

However, the sensitivity of screening tests is more impressive in those undergoing more invasive screening methodologies, such as sigmoidoscopy and colonoscopy (Segnan et al 2005), with additional reductions in cancer-related mortality. A number of RCTs have reported the beneficial effect on incidence and mortality associated with distal CRC after screening with flexible sigmoidoscopy (Atkin et al 2010, Hoff et al 2009, Segnan et al 2002, Weissfeld et al 2005). In the recent large multicentre UK RCT of over 150,000 people aged 55-64 years of age, incidence of CRC in people attending for flexible sigmoidoscopy was reduced by 33% and mortality by 43%. The incidence of rectal and sigmoid cancer was reduced by 50%. In this trial, patients had all polyps under 1cm removed, and were referred for follow-up colonoscopy if there were >3 adenomas or pathology revealed >25% of the polyps were villous or had high grade dysplasia (Atkin et al 2010).

Colonoscopy has a high sensitivity for both adenomas and cancer and several countries such as Poland and Germany have introduced screening programmes. Colonoscopy is the most commonly used method for CRC screening in the USA (Hoff 2010). However, no RCT has yet reported on the effects of colonoscopy on reduction of CRC incidence or mortality.

It must also be considered that both positive results and false positives following FOBT lead to colonoscopy with associated potential for morbidity (Hewitson et al 2007). The rate of serious complications, such as bleeding and bowel perforation, due to colonoscopy is between 0.2% and 0.8%

(Hanley 2005). There is also significant psychological morbidity associated with positive test results in any screening modality, some of which can be ameliorated by better counselling and information.

Compliance with almost all CRC screening methodologies, including FOBT screening, remains consistently poor (Vernon et al 1995). Only 75% of suitable patients are likely to be compliant towards screening endoscopy, with obese patients and patients with lower educational attainment least likely to comply (Anderson et al 2011a). In a recent review of the Scottish screening programme, gFOBT uptake decreased with increasing deprivation and was higher in women than men. Furthermore, increasing deprivation was negatively associated with uptake of follow-up colonoscopy in men with a positive gFOBT. These findings may contribute to the observation that increased deprivation is associated with decreased CRC survival (Steele et al 2010).

New methods of screening in CRC are currently being developed and evaluated, including investigations of patient stool for colonocyte DNA mutations, epigenetic changes or microRNA expression and blood tests for plasma DNA mutations, epigenetic changes, heteroplasmic mitochondrial DNA mutations, plasma microRNA expression or protein and autoantibody expression [reviewed in Pawa] (Pawa et al 2011). One example, which has previously been evaluated, is screening for KRAS mutations in DNA within stools (Villa et al 1996). However, this approach is limited as these mutations occur in less than half of all CRCs and may also occur in pancreatic hyperplasia and other non-malignant conditions. Research into developing tests for multiple DNA alterations, including mutant KRAS, may offer greater potential for new non-invasive screening for CRC with high levels of sensitivity and specificity (Kopreski et al 2000, Villa et al 1996).

Given the poor patient compliance with screening, the risk of false positives, the invasiveness, unacceptability to the patient and risks associated with advanced screening methodologies; approaches to prevent the initiation of CRC have obvious attractions to assist a reduction in CRC incidence and related mortality, on a population basis. In order to develop new methods of early CRC detection and diagnosis, cancer prevention, and targeted therapies, a better understanding of the causation of CRC is required as an obvious pre-requisite.

## 1.2. Causation

Understanding of the cause and management of cancer has been pursued since ancient times. Hippocrates (BC 460-375) hypothesized that tumours were caused by an imbalance in the four humors “blood, phlegm, yellow bile, and black bile”, and nominated tumours as “cancer”, as the growths reminded him of moving crabs. Claudius Galen (AD 131-203) agreed with this “humoral” view and argued that accumulation of black bile in the breast, uterus, lips, and in hemorrhoids caused cancer. These views essentially persisted until the 18th century, truncating any moves towards innovation or developing surgical resection of tumours (as this would not resolve a humoral imbalance). However, new theories in the 18<sup>th</sup> century developed regarding the local origin of cancer, supplemented by development of additional tools such as microscopy [reviewed in Sørider] (Soreide et al 2009). During the 1920-50s, tumourigenesis was thought to be a multistep process (Foulds 1958) and latterly, as observations supported the view that CRC arose from preexisting neoplastic lesions (polyps), this evolved into the “adenoma-carcinoma- sequence” theory (Fearon and Vogelstein 1990).

The genetic aetiology of cancer became more apparent following the publication of the stepwise model (Fearon and Vogelstein 1990) and subsequent major discoveries, such as the adenomatous polyposis coli (APC) mutation, MMR deficiencies and chromosomal alterations (Powell et al 1992), (Thibodeau et al 1993). However, since then, at the very least, CRC development has demonstrated itself to be both highly complex and still not completely understood.

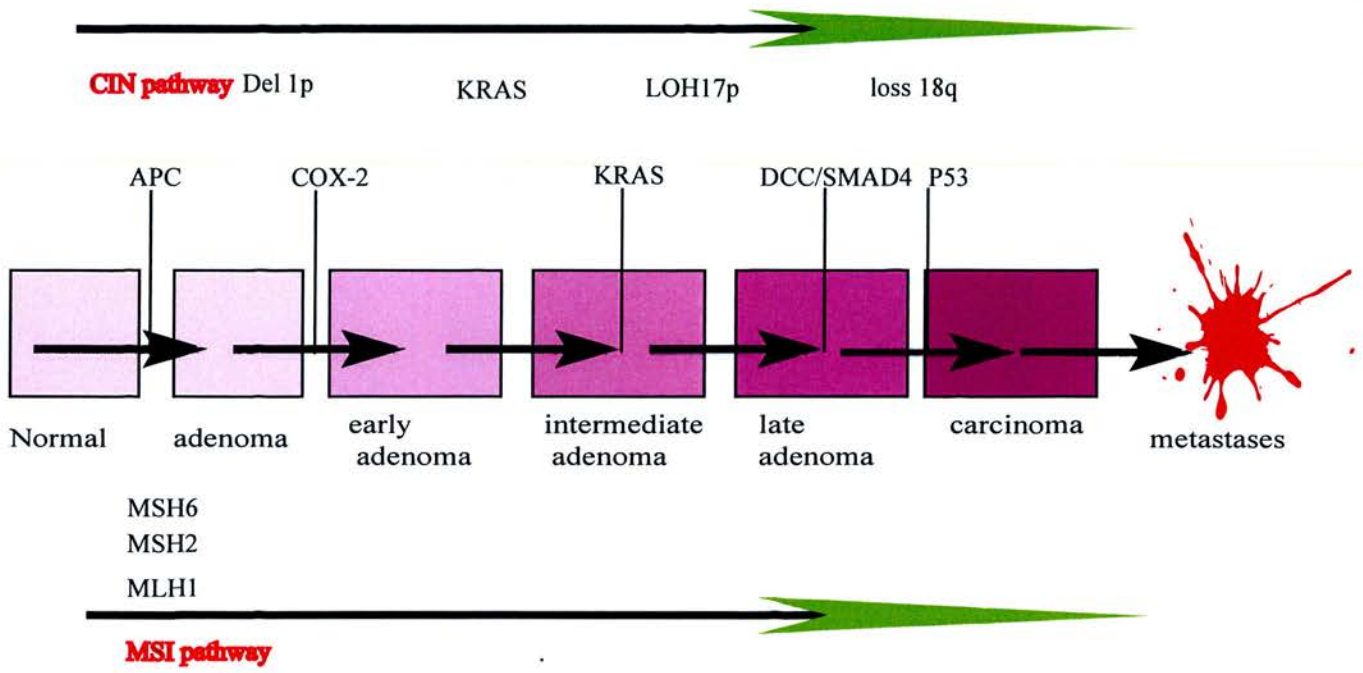
### 1.2.1 Theories of development and genetic risk factors

#### *The “adenoma-carcinoma” stepwise sequence model*

The majority (70-80%) of CRCs are sporadic in nature, putatively due to genetic mutations acquired through the accumulation of genetic “hits” over a lifetime (Knudson 1985). One theory attempting to explain this is the multi-step carcinogenesis model, which was developed by Fearon and Vogelstein in 1990, amongst others (Fearon and Vogelstein 1990). This states that tumours develop in a stepwise fashion via an adenoma-carcinoma sequence. A schematic representation is shown figure 1.2 (Janne and Mayer 2000). Normal epithelium progresses through adenoma to invasive carcinoma due to a series of accumulated mutations in oncogenes and tumour suppressor genes and histological changes



Figure 1.2 Progressive multi-step model of Colon Carcinogenesis





in which the adenomatous polyp is an intermediate step (Jung et al 2006, Kinzler and Vogelstein 1998).

The belief that CRC arises from pre-existing adenomatous polyps is supported by several lines of evidence; (a) the prevalence of adenomas correlates with that of carcinomas, (b) adenomatous tissue often accompanies cancerous tissue at resections (adenomas are found in one third of colorectal resections for cancer), (c) sporadic adenomas are also identical histologically to those from the pre-malignant adenomas of Familial Adenomatous Polyposis (FAP), (d) large adenomas are more likely to display cellular atypia and genetic abnormalities than smaller adenomas, and (e) the incidence of CRC has been shown to fall with the introduction of long term screening and polypectomy (Bond 2005).

The most common mutations in CRCs affect the Wnt signalling pathway, responsible for cell growth and differentiation. The most common genetic mutation in this pathway is in the *APC* gene (chromosome 5q). Mutations in this gene are reported as one of the earliest genetic alterations in the genesis of CRC and may be required to initiate the adenomatous process, resulting in the clonal growth of a single cell (Fodde et al 2001b). APC is a multi-functional protein that is involved in several key cellular processes, such as adhesion, migration, microtubule assembly, chromosome segregation and signal transduction (Senda et al 2005) (Fearnhead et al 2001, Fodde et al 2001a) (Polakis 1997). The majority of these functions are mediated through its interaction with  $\beta$ -catenin. APC complexes with glycogen synthase kinase 3-beta (GSK-3 $\beta$ ) and axin which binds  $\beta$ -catenin. This association allows casein kinase 1 (CK1) firstly then and GSK-3 $\beta$  subsequently, to phosphorylate  $\beta$ -catenin targeting  $\beta$ -catenin ubiquitination and degradation by proteasomes. The vast majority (95%) of *APC* germ line mutations are nonsense or frame shift mutations, which result in a truncated protein. This mutant APC is unable to bind  $\beta$ -catenin, causing  $\beta$ -catenin to accumulate in the cell and induce deregulation of growth and differentiation and subsequently, tumour development (Fearnhead et al 2002, Markowitz 2007).

Familial adenomatous polyposis (FAP) patients carry a germ line mutation of the *APC* gene. FAP is a condition that occurs in 1:7000 to 1:14,000 live births (de la Chapelle 2004, Rowley 2004) and is responsible for less than 1% of all CRC. It is characterised by the development of thousands of adenomatous polyps throughout the large bowel (de la Chapelle 2004, Rowley 2005) and presents at a much younger age in these patients (Rowley 2005). Additionally, 6% of Ashkenazi Jews hold a lysine to isoleucine amino acid substitution (I1307K or Ile1307Lys) in *APC*, which is associated with a 20% increased risk of CRC (Fodde 2002).

According to the “adenoma-carcinoma” stepwise sequence, once this initial mutation has occurred, a small polyp subsequently develops. When a cell in the polyp has acquired enough mutations and undergone further clonal expansions, it acquires the ability to invade and metastasise (Hawk and Levin 2005, Kinzler and Vogelstein 1998). Further such mutations occur in oncogenes such as *KRAS*, *RAF* and *PI3-Kinase*. Isolated activating *KRAS* mutations induce cell proliferation, leading to the development of hyperplastic lesions, but if the mutation in *KRAS* occurs after a pre-existing *APC* mutation has occurred, transformation to carcinoma is reported. As previously discussed, *KRAS* activates the EGFR pathway downstream of the EGF receptor and *KRAS* activating mutations result in a tumour phenotype that is relatively more resistant to certain anti-tumour agents, such as anti-EGFR antibodies (Kerr and Midgley 2010, Walther et al 2009).

*RAF* (proto-oncogene serine/threonine-protein kinase) is encoded by the *RAF-1* gene and functions as part of the MAPK/ERK signalling cascade responsible for accurate control of cell cycle, division, apoptosis and differentiation. Activating mutations in *RAF* genes drive dysfunction in these pathways, leading to transformation. Anti-*RAF* agents are potential targets for drug development (Sridhar et al 2005) and mutations of *RAF* are detected in about 5% of CRC (Li et al 2006).

Tumour suppressor function is also affected in the adenoma-carcinoma sequence. The p53 protein, product of the *TP53* gene (ch 17p), plays a role in regulation of cell division and inhibits the survival of cells with Wnt pathway defects. Mutations in the *TP53* gene, occurring in 40-50% of CRCs (Iacopetta 2003), putatively transform an adenoma into an invasive carcinoma. This is also similar to

the gatekeeper function of BAX, which targets cells for apoptosis, which can also be deactivated in CRC. Another protein that can be deactivated in CRC is TGF- $\beta$  or members of the downstream SMAD pathways, which have a deactivating mutation in at least half of CRC (Markowitz 2007).

However, the multi-step carcinogenesis model has a number of weaknesses. Firstly, it fails to explain the development of *de novo* CRC, which can affect between 20-40% of all tumours and where tumours evolve from the colorectal mucosa *de novo* and grow invasively into the bowel wall without passing through the adenoma stage. Secondly, Aberrant Crypt Foci (ACF) are small circumscribed areas in the colorectal mucosa with enlarged crypts, compared to surrounding normal mucosa. It is thought that accumulation of biochemical and mutational changes within the cells of some ACF can lead to the transformation of those cells into cancer. Data indicates that some ACF directly bypass the polyp stage and progress directly to carcinoma (Bedenne 1992, Takayama et al 1998).

Fearon (Fearon and Vogelstein 1990) based some of the rationale for the multi-step carcinogenesis model on the fact that many colonic tumours (from adenomas to carcinomas) were monoclonal and putatively developed from clonal expansion of a mutated cell, however, reports state some 75% of early adenomas are polyclonal and whilst, one explanation postulated is that short-range interaction between adjacent initiated crypts is responsible for polyclonality (Leedham et al 2005), further problems with this theory exist. Therefore, an alternative theory is required to explain the complex heterogeneity of primary colonic tumours and the ability of distant metastases to undergo further differentiation, when they have accumulated a level of acquired mutations presumed to prevent further differentiation. Hence the “Cancer Stem Cell Theory” has developed.

#### *The “Cancer Stem Cell Theory”*

Stem cells are defined as cells that have the ability to perpetuate themselves through self-renewal and to generate mature cells of a particular tissue through differentiation. Stem cells share many characteristics that mimic the behaviour of cancer cells, such as their ability to self-renew and avoid apoptosis. Stem cells can give rise to phenotypically heterogeneous cells that exhibit various degrees of differentiation. Hence, polyclonality could be the result of the existence of a subset of cancer cells

which produce heterogeneous progeny, replicating the behaviour of stem cells. This explains therapeutic failures achieved with molecular-biological therapeutics utilising target-based therapies which assume homogeneity of the cancer cell population within a tumour mass.

Many of the pathways utilised by stem cells to potentiate self-renewal, such as the Wnt, Shh and Notch pathways, are also aberrantly used for survival and proliferation by cancer cells in a variety of human cancers, including CRC (further description of these pathways can be found in Chapter 4). Additionally, stem cells, as they live for longer periods than normal cells, may be more prone to accumulating chance mutations. Furthermore, as stem cells already have an activated self-renewal pathway, they may require less mutations to occur in order to undergo neoplastic change, in contrast to non-stem cells which theoretically require more mutations to create an activated *de-novo* survival and proliferation signalling pathway to undergo transformation (reviewed in Reya and Clevers 2005).

However, both of these theories are yet more complicated by the observation that cancer cells can undergo an epithelial-mesenchymal transition (EMT)-like dedifferentiation. This transition involves several mechanisms and aids in invasion, with re-differentiation observed in some metastases (Brabletz et al 2005, Heslin et al 2001, Matsuyama et al 2002). The process of carcinomatous progression is therefore dynamic and genetic alterations are not irreversible. Elements in the extracellular matrix, immune cells and fibroblasts have all been postulated to play a part in this phenomenon (Fidler 2003, Heslin et al 2001, Mueller and Fusenig 2004).

#### *Polyclonicity theory*

A traditional view exists that tumours originate from a single cells which develop along the traditional monoclonal model of an established sequence of genetic events, during the multistep process of colon carcinogenesis. However, this does not explain the finding of unrelated clones in some tumour types, including CRC (Parsons et al 2008). This is supported by evidence of tumoural polyclonicity from studies in human adenomas (Thirlwell et al 2010), and rodent models of CRC (Merritt et al 1997). As such, some authors have felt that the monoclonal tumour theory, as the underlying premise in the field of oncology, could explain why some attempts at chemotherapy, based on a monoclonal population,

are ineffective (Parsons et al 2008).

The theory of polyclonicity, proposes a number of tumour developmental stages. Firstly, tumour cells, derived from a founder cell population, are affected by chromosomal instability and therefore display an increased mutation rate. This leads to cells within the tumour population possessing different genetic mutations. These mutations may or may not offer a Darwinian survival advantage against other cell populations, as the tumour cell number begins to increase. As a number of successful clones may survive, there exists a heterogenic genotype across the tumour mass. With this altered gene expression, the tumour may be composed of cells which become phenotypically heterogenous. Through further processes of clonogenic selection and poly/monoclonal expansion, the tumour may result in a heterogenous, polyclonal population of cells. Of course, if one clone is a dominant survivor, the tumour may be monoclonal, as a result of clonal selection, rather than the monoclonal theory of evolution of all cells in the tumour concurrently from a single founder (Gisselsson, 2001).

It is impossible to detect currently whether the polyclonicity theory is correct based as tumours are highly variable in themselves and the ability to determine the founder origin of a tumour and measure precisely the genetic and molecular changes which occur in a step-wise progression within a tumour's evolution are, as yet, not available (Parsons et al 2008). The situation is even more complex to determine *in-vivo* as external factors, such as changes in the tumour microenvironment may work to influence the outcome of clonal survival pressures, and clones may be dependent on each other in tumours forming a symbiotic relationship. Therefore, debate between the polyclonicity and monoclonicity theories of colorectal carcinogenesis will continue (Parsons et al., 2008).

### *Genetic Instability*

The stability of the genome in cancer cells becomes compromised because several cancer-predisposing genetic mutations also affects the function of genes that are responsible for maintaining the integrity and number of chromosomes during cell division. At least three major elements of

genetic instability are known to play a role; Chromosomal instability (CIN), microsatellite instability (MSI) and epigenetic silencing [reviewed in Søreide] (Soreide et al 2009).

#### *Chromosomal instability (CIN)*

Chromosomal instability leads to gains and losses of large segments of chromosomes (Lengauer et al 1998). Mutations in CIN-associated genes drive imbalance in the number of chromosomes per cell (aneuploidy) and an enhanced rate of loss of heterozygosity (LOH), as whole or large fractions of chromosomes are gained or lost during cell division. This chromosomal instability (CIN) phenotype, describing allelic losses, chromosomal amplifications, and translocations, accounts for 85% of sporadic CRC cases (Dunican et al 2002).

The CIN phenotype arises through mutations in many different pathways and genes, particularly those responsible for telomere maintenance, cell division, centrosome duplication and chromosome segregation (Paulovich et al 1997). Some examples are chromosome 7 amplification, deletion at *1p* (*MUTYH*) and *8p*, as well as loss of heterozygosity (LOH) of *17p* (*P53*) and *18q* (*DCC, BCL2*)

Chromosome *1p* encodes for *MUTYH* glycosylase, which is involved in oxidative DNA damage repair. Mutations in the *MUTYH* gene affect the ability of cells to correct mistakes made during DNA replication and cause an autosomal recessive form of colonic polyposis (also called *MUTYH*-associated polyposis). Polyps caused by mutated *MUTYH* do not appear until adulthood and are less numerous than those found in patients with *APC* gene mutations. *8p* comprises a metastatic susceptibility locus involved in tumour progression whose disruption increases metastatic potential involving a large number of genes, such as *TNFRSF10B* (involved in transcription of *DR5* and *TRAILR2*) (Macartney-Coxson et al 2008).

#### *Microsatellite instability (MSI)*

The microsatellite instability (MSI) phenotype is not as prevalent in sporadic CRC, accounting for 15% of such malignancies, and has been firmly linked to a faulty DNA mismatch repair system (Markowitz 2000). Nucleotide sequences (i.e. CACACACA; or [CA]<sub>4</sub>) known as “microsatellites” contribute to genetic stability and errors generated within these areas may cause frame shift mutations

leading to protein alterations and carcinogenesis. These tumours often demonstrate characteristic mutations such as those in Transforming Growth Factor beta Receptor II (*TGFβRII*) (ch 3p22), Bcl2-associated X protein (19q13.3-q13.4) and insulin-like growth factor receptor II (*IGFRII*) (Parsons et al 1995, Souza et al 1996).

Widespread MSI was discovered to be a marker in hereditary non-polyposis CRC (HNPCC), a condition accounting for approximately 3-5% of all CRC cases (de la Chapelle 2004, Rowley 2004). MSI is observed in the vast majority (>90%) of HNPCC patients and is also seen in approximately 15% of sporadic CRC (Fearhead et al 2002). HNPCC is characterised by a 70-80% risk of developing early onset CRC (Rowley 2005) and is associated with the development of other cancers, such as endometrial, stomach, ovarian, small bowel, hepatobiliary, and brain (the HNPCC syndrome) (de la Chapelle 2004, Rowley 2005). HNPCC is caused by germline mutations in DNA mismatch repair (MMR) genes, such as *hMLH1*, *hMSH2*, *hMSH6*, *PMS1* or *PMS2*, and the condition is inherited as autosomal dominant (Rowley 2004). Dysfunction of these genes essentially causes widespread genome instability, which can manifest as different numbers of simple repeated sequences, such as microsatellites, (referred to as the replication error phenotype, RER+). (Fodde et al 2001a); (Hopper 2005). A study of 48 HNPCC kindreds (Liu et al, 1996) investigated the proportion of mutations in known mismatch repair genes; 31% had mutations in the *MSH2* gene, 33% had mutations in the *MLH1* gene, 2% had mutations in the *PMS1* gene, and 4% had mutations in the *PMS2* gene. *MSH6* mutations account for 10%–20% of HNPCC patients and 0.4% of all CRC (Southey et al 2005).

Base excision repair genes also acts to prevent mutagenesis induced by 8-oxo-7,8-dihydroxy-2'-deoxyguanosine (8-oxo-dG) (Sieber et al 2003). *MUTYH* removes adenines mispaired with 8-oxo-dG or guanine and a defect in this repair mechanism also gives arise to multiple adenomas (Al-Tassan et al 2002).



### *Epigenetic silencing*

Approximately 40% of CRC tumors are characterized by epigenetic changes, such as DNA methylation (Weisenberger et al 2006). This 'Epigenetic change' affects gene function (without genetic changes) by aberrant methylation of DNA that 'silences' the gene, causing deficiency in protein expression.

CpG islands are genomic regions, typically 300-3000 base pairs in size, where there are a significant number of cytosine and guanine bases adjacent to each other in the backbone of the DNA (linked by phosphodiester bonds; thus named CpG). Hypermethylation of CpG islands in the promoter regions of tumour suppressor genes is associated with transcriptional silencing. For selected genes, epigenetic changes are tightly related to neoplastic transformation in CRC i.e. loss of the tumour suppressor gene *PTEN* located at *10q23* occurs through promoter hypermethylation in CRC with MSI-H (Goel et al 2004). Aberrant DNA methylation may be part of the age-related field defect observed in sporadic CRC. Excessive aberrant methylation through a hypermethylator phenotype termed *CpG Island Methylator Phenotype* (CIMP) contributes CRC formation (Toyota et al 1999). Non-neoplastic but epigenetically disrupted stem/progenitor cells might be a crucial target for cancer risk assessment and chemoprevention in the future (Feinberg et al 2006).

#### **1.2.2 Inherited risk factors**

Only 5-6% of cancers develop on a truly inherited genetic background (de la Chapelle 2004), however, a further 10-20% of CRCs have a "familial" clustering of cases without any detectable or known specific genetic alterations leading to increased risk (a relative risk (RR) increase of 2-3 is noted in those with a first-degree relative with CRC) (Rowley 2004). In one study, 2.3 for those with 1 first-degree relative affected, 4.3 for those with more than one first-degree relative affected, 3.9 if the relative is less than 45 years of age and 2.0 if the relative has an adenoma (Johns et al 2001). When 2 or more relatives are affected by CRC the possibility of a genetic syndrome increases substantially. These include the major genetic syndromes, described in table 1.5 (Slattery and Kerber 1994).



Hereditary conditions include those mentioned above, such as; Familial Adenomatous Polyposis (FAP) syndrome and Hereditary Non-Polyposis CRC (HNPCC; the Lynch syndrome) (Lichtenstein 2002). The lifetime gastrointestinal cancer risk associated with HNPCC is reported as around 80% for CRC (Vasen 1994). In FAP, the development of large bowel cancer is virtually certain without prophylactic surgery (Bulow 1991).

Genetic factors including high-penetrance mutations and low-penetrance polymorphisms, which determine a patient's defense and adaption when faced with exposure to carcinogens, effect susceptibility to CRC development. However, the effect of common low-penetrance risk determinants is small when in isolation, with increasing susceptibility observed only through the cumulative effect associated with multiple risk variants (Ostrowski and Wyrwicz 2009). Recently, Genome Wide Association Studies have identified that part of the heritable component of the risk of developing CRC is due to common germline genetic variants, which are of modest effect. These single nucleotide polymorphisms (SNPs), some which are associated with genes that have a putative role in CRC development and others which have yet to be characterized, have been validated in unrelated populations, and are reported to explain about 8% of heritability risk of developing CRC (Dunlop et al 2012, Peters et al 2012). However, whilst these loci are associated with development they are not associated with CRC survival (Tenesa et al 2010). A recent metanalysis review more than 600 papers naming genetic polymorphisms associations with CRC found 445 polymorphisms in 110 different genes. 16 independent variants at 13 loci (MUTYH, MTHFR, SMAD7, and common variants tagging the loci 8q24, 8q23.3, 11q23.1, 14q22.2, 1q41, 20p12.3, 20q13.33, 3q26.2, 16q22.1, and 19q13.1) were found to have the highest associations with CRC (Theodoratou et al 2012).

CRC may also develop on a background of colonic inflammation, such as in long-standing ulcerative colitis, with cumulative probabilities for cancer development of 2% by 10 years, 8% by 20 years, and 18% at 30 years (Ullman and Itzkowitz 2011). It is reported there is a similar step-wise progression of genetic mutations from inflamed colitic mucosa, through dysplasia to cancer development. For instance, loss of p53 function is an important step in progression of colitis-associated cancer and allelic deletion of *p53* is observed in approximately 50%–85% of colitis-associated tumors (Burmer et

al 1992). If there is a clear family history of CRC i.e. those who have two or more first- or second-degree relatives (or both) with CRC – then the risk is even higher (Lynch and de la Chapelle 2003). Other settings with an increased risk include a personal history of CRC or adenomas and a personal history of ovarian, endometrial, or breast cancer.

Familial Adenomatous Polyposis (FAP)	1:7000 autosomal dominance, mutations of the <i>APC</i> gene ; 5q21, >100 adenomatous polyps in the colon and rectum, other extra-colonic features (Gardner syndrome) may include upper gastrointestinal tract polyps, congenital hypertrophy of retinal pigment epithelium, osteomas and epidermoid cysts, supernumerary teeth, desmoid formation, and other malignant changes such as thyroid tumours, small bowel cancer, hepatoblastoma, and brain tumors, particularly medulloblastoma. 90% CRC risk by age of 45 years.
Attenuated FAP	Missense mutations in APC, Fewer 30-100 polyps, CRC develops later, similar extra-colonic manifestations, 69% CRC risk by 80 years
Lynch	Amsterdam criteria = 3+ cases of CRC over 2+ generations, with at least 1 diagnosed < 50, and no evidence of FAP. 25% test positive for an informative <i>MSH2</i> or <i>MLH1</i> mutation. MSI-H tumours. Familial clustering with early age at onset; high risk of second primary tumour; preferentially right colon; improved clinical outcome; extracolonic sites= endometrium, ovaries, other GI tract, uroepithelium, brain, and sebaceous tumor. 40%-80% CRC risk by 75 years;
MYH-associated polyposis	Develop fewer adenomas and at a later age compared to FAP. Adenomas, serrated adenomas, and hyperplastic polyps can be seen in MAP patients. CRCs tend to be right-sided and synchronous; carry a better prognosis than sporadic CRC. Extracolonic cancers have been reported including gastric, small intestinal, endometrial, liver, ovarian, bladder, and thyroid and skin cancers including melanoma, squamous epithelial, and basal cell carcinomas. 35-53% CRC lifetime risk,
Peutz-Jeghers	Early-onset autosomal dominant ; germline mutations in the <i>STK11</i> gene at chromosome 19p13.3 melanocytic macules on the lips, and the perioral and buccal regions, and multiple gastrointestinal polyps, both hamartomatous and adenomatousAutosomal recessive 39% CRC risk by 70 years
Juvenile Polyposis	Genetically heterogeneous, rare, childhood-onset, autosomal dominant germline mutations in <i>SMAD4</i> , 18q21 (15-20%) and <i>BMPRIA</i> ; 10q22 (20-40%); presents as hamartomatous polyposis throughout the GI tract and diarrhea, GI tract hemorrhage, and protein-losing enteropathy.17-68%CRC risk by 60 years
CHEK-2	Rare subset of families with hereditary breast and colon cancer; mutation in the <i>CHEK2</i> gene.

Fig 1. 5 Major Genetic Syndromes

### **1.2.3 Environmental risk factors**

CRC is also considered to be an environmental disease, as the majority of CRCs are 'sporadic' with no apparent genetic predisposition. "Environmental" is defined broadly to include a wide range of often ill-defined cultural, social, and lifestyle factors. As such, modifiable causes may be identified which would allow a large proportion of cases to theoretically be prevented (Boyle and Langman 2000, Johnson and Lund 2007). Evidence of environmental risk comes from large scale epidemiological studies, twin cohorts or studies showing that CRC cases increase in migrant populations that move from a low incidence to a high incidence countries (Johnson and Lund 2007). The major environmental factor thought to influence CRC risk is diet.

#### *Dietary factors*

It has been proposed that changes in food habits could reduce CRC in the western world by up to 70% (Michaud et al 2005, Willett 2001). However, the dietary constituents that contribute to the development of CRC have not yet been fully defined, primarily because of methodological and practical difficulties in undertaking dietary studies. Results from case-control studies often fail to replicate data from cohort studies and vice versa.

Fish oils, especially rich in polyunsaturated omega-3 fatty acids (O3Fas) have also been shown to reduce the risk of bowel cancer (Cho et al 2011). A multitude of other studies are currently investigating the effects of Vitamin (especially C and E), nutrient and mineral supplementation in reducing CRC risk (see below).

A number of factors identified to contribute to CRC risk are listed below:

#### ***Fruit and vegetables***

The EPIC study recruited 520,000 healthy people from 10 European countries and tracked their diet and health over the years (Norat et al 2005). The findings from this study demonstrated that for every two portions of vegetables and fruit consumed per day there was a 2.5 percent lower risk of cancer.

However, in a metaanalysis of seven studies evaluating fruit and vegetables intake and CRC risk there was a weak but statistically significant reduction in risk (RR=0.99), per 100 g/d taken. However, when taken individually, there was no significant association between fruit and vegetable intake and colon cancer risk or rectal cancer risk. Therefore, when viewed against the strong effect of confounding lifestyle issues, the evidence is not convincing that fruit and vegetable intake is related to reductions in CRC risk (Norat et al 2010).

### ***Saturated fats***

The dietary factor which has the strongest association with CRC risk is saturated animal fats [reviewed in Martinez] (Martinez 2005). CRC rates are high in populations with high total fat intake and low in populations consuming less fat. Furthermore, populations migrating from low fat intake to high fat intake areas significantly increase their CRC risk (Key et al 2002). Several theories exist as to the mechanism of this association, including that dietary intake of saturated fats results in increased bile acid and cholesterol in the colonic lumen, which favours the development of a bacterial flora capable of degrading bile salts to potentially carcinogenic *N*-nitroso compounds (Gill and Rowland 2002, Giovannucci and Willett 1994, Mason 2002).

### ***Red meat and N-nitroso compounds (NOC)***

High meat consumption has also been implicated in the development of CRC (Larsson and Wolk 2006, Santarelli et al 2008, Santarelli et al 2010). A recent meta-analysis of 18 published, prospective trials, (Larsson and Wolk 2006), found an RR of 1.28 for CRC risk in high meat intake versus low meat intake populations. This association was stronger for rectal cancer than colon cancer (Larsson and Wolk 2006). The EPIC study, (Norat et al 2005) the largest ever prospective cohort study to investigate the association between diet and cancer, involving 520,000 participants in 10 European countries, also demonstrated a significant association between CRC risk and consumption of red *and* processed meats. However, red meat alone (in the absence of processed meat) was not found to be significantly associated, despite the very large numbers of study subjects. Based on the association between meat consumption and disease risk, the UK Committee on Medical Aspects of Food and Nutrition Policy (Truswell 2002) has advised that people who are consuming high levels of red meat

—more than 14 portions a week (140-g cooked weight per day)—should consider a reduction. The World Cancer Research Fund recommend individuals “limit intake of red meat to less than 80g daily”.

N-nitroso compounds (NOCs), including nitrosamines and nitrosamides, have previously been implicated in gut carcinogenesis in humans. This is of interest as nitrosamines have been detected in processed foods with added nitrates or nitrites (such as salt-preserved fish and meat, and in foods processed by smoking or direct-fire drying), which may explain the association between processed food consumption and CRC risk. NOCs are produced from nitrogen-containing compounds by the nitrosation of amines, amides and urea, following the ingestion of high protein/ high red meat diets. (Bingham et al 1996, Bingham et al 2002, Santarelli et al 2010). Furthermore, NOCs are alkylating agents with the potential to cause intrinsic DNA damage in the colorectal epithelium. The most common *KRAS* mutations found in CRC are characteristic of alkylating agents, namely G-A transitions at the 3' G of a GG pair at codon 12 or 13. Indeed, there have been reports showing an excess prevalence of *KRAS* mutations in CRCs from high dietary meat consumers (Lewin et al 2006, Santarelli et al 2008)

Other hypotheses to explain why processed meat intake is linked to cancer risk are that cooking meat at a high temperature forms carcinogenic heterocyclic amines and polycyclic aromatic hydrocarbons and that the heme iron in red meat can promote carcinogenesis because it is a pro-oxidant (Lund et al 1999), which increases cell proliferation in the mucosa, through lipoperoxidation and/or cytotoxicity of fecal water [reviewed in Santarelli] (Santarelli et al 2008).

### ***Physical activity***

A substantial body of epidemiological evidence supports an association of a sedentary lifestyle with an increased risk of CRC (Martinez 2005). Friedenreich *et al.* studied physical activity and risk of colon and rectal cancers amongst more than 400 000 individuals concluding that 2 hours of moderate physical activity per day or 1 hour of more vigorous activity was associated with a risk reduction for colon cancer of 20–25% overall and of around 35% for right-sided colon cancer (Friedenreich et al 2006). This is supported by the findings of earlier studies that have demonstrated a 30%-50%

reduction in CRC incidence among individuals with the highest levels of physical activity compared to those with low levels of activity (Ahmed 2004, Giovannucci 2003, Lao and Brenner 2004).

In the dose-response meta-analyses of 3 studies, the AICR/WCRF report found total physical activity (expressed as increase of 5 METs-hour/day) associated with a significant decreased RR of CRC (0.97) and colon cancer (0.92), but not with rectal cancer (1.02). Interestingly, further analysis revealed a statistically significant RR reduction was observed for proximal colon cancer (0.91) and not for distal colon cancer (1.01) (Norat et al 2010).

A high body mass index (BMI) as a result of positive energy balance, is also associated with an increased risk of CRC (BMI>30 = 2 x CRC risk) [reviewed in (Ahmed 2004, Gatof and Ahnen 2002, Gunter and Leitzmann 2006); (Martinez 2005)]. This effect increases significantly with age and is stronger for men than for women. A larger waist size was associated with increased risk of CRC in both sexes, and this effect was independent of BMI (Moore et al 2004). In another larger trial, body weight and BMI were significantly associated with CRC risk in men but not in women. Risk of colon cancer was associated with waist circumference and waist: hip ratio in men and in pre-menopausal women, but not for post-menopausal women using hormone replacement therapy. The association was not significant for rectal cancer (Pischon et al 2006, Pischon et al 2008). Several studies have found increased adenoma occurrence in those patients who are obese (Kim et al 2009). In a recent meta-analysis, obesity (BMI $\geq$ 30) or overweight ( $25 \leq$  BMI  $\leq$ 29.9) was examined as a risk factor for colorectal adenoma (CRA). Adenoma occurrence risk in those with BMI  $\geq$  22 was 1.42,  $\geq$  25 was 1.81 and  $\geq$  30 was 1.47 (Omata et al 2013).

Overall, a statistically significant increased RR risk of CRC was found with increasing waist circumference. The RR per inch increase for colorectal, colon and rectal cancer were 1.03 (95%CI=1.02-1.04), 1.05 (95%CI=1.03-1.06) and 1.03 (95%CI=1.01-1.04), respectively, with the association between waist circumference and colon cancer stronger in men than women. Even with adjustment for BMI - the results indicated that abdominal obesity might be a CRC risk factor independent of BMI (Norat et al 2010).

A recent metanalysis demonstrated that diabetes, a condition increased in obese people, is associated with both CRC incidence and mortality (Jiang et al 2011b). High concentrations of circulating insulin, as occurs in obesity, has a mitogenic effect on colonocytes and induces activation of signalling pathways such as protein kinase C (PKC) and mitogen activated protein kinase (MAPK) (Campos et al 2005); (Gunter and Leitzmann 2006). They also increase the proliferative effect and availability of insulin-like growth factor-1 (IGF-1) (Campos et al 2005, Giovannucci 2003). Another mechanism to consider is the production of endocrines ('adipokines' such as leptin and adiponectin), and cytokines (such as tumour necrosis factor alpha (TNF- $\alpha$ ) and interleukin 6 (IL-6)) (Rondinone 2005), by adipose tissue. These factors, released by excessive amounts of adipose tissue, have been shown to induce increased expression of inducible nitric oxide synthase (iNOS) and COX-2 and activate NF- $\kappa$ B transcription factor signaling within colonic mucosa (John et al 2006), suppressing apoptosis, which is a prerequisite for tumourigenesis.

### *Alcohol*

Case-control and prospective studies suggest an association between high alcohol intake and increased risk of CRC development, but reports have been conflicting (Heavey et al 2004); (Ahmed 2004, Martinez 2005). In a recent study, in comparison with non-drinkers with no family history, the RR for colon cancer was 2.8 for individuals who consumed  $\geq 30$  g/d and who had a family history of CRC (Cho et al 2011). In a pooled analysis of primary data from 8 cohort studies in 5 countries, increased risk for CRC was limited to persons with an alcohol intake of greater than 30 g/day (approximately 2 drinks/day). Therefore, regular alcohol intake was correlated with a modest relative elevation in CRC rate, mainly at the highest levels of alcohol intake (Cho et al 2005).

Breakdown products of alcohol are known to inactivate protective elements, such as folate (Giovannucci and Willett 1994, Giovannucci 2003) (Martinez 2005). As folate is implicated in key

cellular processes such as DNA synthesis and repair, reduced folate levels, which occurs as a result of excessive alcohol intake, could underlie the a 2-5 fold elevation in CRC risk among individuals with high intakes of alcohol and low intakes of folate (Giovannucci 2003). Additionally, reports suggest alcohol stimulates cell proliferation in the rectum and can induce a MSI phenotype (Heavey et al 2004).

### ***Smoking***

Smokers have increased risk of both adenomatous polyps (Giovannucci 2002) and hyperplastic polyps (Martinez et al 2011), but evidence of the association between smoking and CRC is conflicting (Ahmed 2004, Martinez et al 2011, Martinez 2005). Some studies have found CRC risk is 2-4 times higher in smokers (Ahmed 2004, Giovannucci 2002). Compared to non-smokers, female smokers had an increased risk of advanced adenomas with an exposure of 10 or more pack-years while men had an increased risk with smoking over 30 pack-years. An increased association with smoking was observed for proximal advanced adenomas and large hyperplastic polyps in women (Anderson et al 2011b).

Molecularly, cigarette smoking increases concentrations of carcinogens, such as polycyclic aromatic hydrocarbons, nitrosamines and aromatic amines (Ahmed 2004).



### **1.3 Prevention and chemoprevention**

Given that conventional treatment is expensive and associated with significant morbidity and mortality, and that early detection and prompt surgery in the early stages of colon cancer offers the best chances of a long-term cure, there has been a great deal of focus on developing primary, secondary and tertiary prevention strategies to combat the disease. Primary prevention is concerned with aetiological factors, such as lifestyle and dietary modification, to eliminate potential mutagens (Krishnan et al 1997). Secondary prevention attempts to reduce incidence by identifying and treating pre-malignant conditions in subjects at risk (Rigas and Williams 2002). In tertiary prevention or chemoprevention, the use of natural or laboratory-made substances is employed to prevent a disease such as cancer (Cooper et al 2010), the aim is either to prevent the initiation of carcinogenesis in healthy individuals or to prevent recurrence, invasion and metastasis in people diagnosed with symptomatic disease (Vainio and Miller 2003). Although dietary and lifestyle intervention can have a considerable impact on reducing the risk of developing CRC, it is very difficult to devise and implement such prevention strategies on a large scale due to lack of compliance.

Chemoprevention therefore represents an attractive, alternative approach to reducing the mortality from CRC and essentially involves the long-term use of chemical agents that can delay, prevent or reverse the development of adenomas and interfere with the multi-step progression from adenoma to carcinoma (Arber and Levin 2005, Benamouzig et al 2005). The challenge for those attempting chemoprevention in CRC is to develop safe and effective drugs which are easy to administer and have a low cost (Thun et al 2002). Several agents have been postulated as having a chemopreventative effect on the development of premalignant lesions, polyps or cancers in human studies (Hong and Sporn 1997). Here I will concentrate on a few of the more extensively studied candidates.

#### **1.3.1 Folate**

Folic Acid is important in DNA synthesis and in control of cellular division and differentiation. Studies of patients who have polymorphisms in the genes which control folate processing i.e. Methylene tetrahydrofolate reductase (MTHFR), report a reduced relative risk of CRC compared to the unaffected population (Ma et al 1997, Slattery et al 1999). Evidence for the chemopreventative effect

of folate is mixed. In a pooled analysis of 13 prospective studies of folate supplementation and CRC risk, including 725,134 participants, a 2% risk reduction (95% CI 0-3%) was estimated for every 100 µg/day increase in total folate intake, concluding that higher folate intake is modestly associated with reduced risk of colon cancer (Kim et al 2010). However, in a combined metanalysis of RCTs of folic acid supplementation (0.5 or 1.0 mg/day) in patients with an adenoma history, after 3.5 years of folic acid use, there was no clear decrease or increase in the occurrence of new adenomas (Figueiredo et al 2011). Furthermore, there is evidence that excessive folate supplementation can actually transform normal bowel mucosa. It has been reported that once a malignant lesion has developed, there is a direct inhibitory effect on its growth by folate deficiency and the reported opposite effect with folate supplementation (Kim 2007).

### **1.3.2 Calcium and vitamin D**

Calcium binds to bile salts and fatty acids (Janne and Mayer 2000). Vitamin D levels (1,25 dihydroxyvitamin D, 1,25(OH)D), are related to solar ray exposure and demonstrated an inverse relationship with solar radiation and CRC incidence in human populations (Emerson and Weiss 1992). Both calcium and vitamin D have an antiproliferative effect on colorectal epithelial proliferation, promoting cellular differentiation and cell death (apoptosis) (Lamprecht and Lipkin 2003). Calcium putatively also reduces CRC tumour initiation in animal models of intestinal carcinogenesis (Pence 1993).

Epidemiological studies suggest a reduction in adenomas in persons who take calcium supplementation (1200–2000 mg/day) (18% reduction in the risk of adenoma recurrence after 3–4 years) and a non-significant reduction in the risk of advanced adenomas (RR 0.77) (Baron et al 1999, Martinez et al 2002). However, a metanalysis of 3 RCTs of calcium with or without another agent versus placebo in individuals with a history of adenomas showed a reduced RR for adenoma recurrence (RR = 0.80 [95% CI, 0.69–0.94], P = 0.006) for those receiving calcium 1200 to 2000 mg/d, but no effect was seen in advanced adenoma (RR = 0.77 [95% CI, 0.501.17], P = NS).

However, evidence from trials is mixed when observing CRC risk (Martinez and Willett 1998). Studies assessing calcium (1000–1500 mg/day) plus vitamin D (400–1100 IU/day) in populations with no increased baseline risk of CRC have reported no protective effect on the relative risk of CRC (Lappe et al 2007, Wactawski-Wende et al 2006). Of note, differences in vitamin D levels (sun exposure) amongst participants could account for discrepancies found in studies reporting on calcium, given its confounding effect and relevance in the regulation of calcium metabolism, control and uptake (Holt et al 2002).

### **1.3.3 Hormone Replacement therapy (HRT) / Oestrogens**

The correlation between HRT and CRC has been widely researched and the majority of case-control and cohort studies, to date, have demonstrated that women who use HRT postmenopausally have an approximately 20-40% decreased risk of CRC (Hawk et al 2004, La Vecchia et al 2005, Raju and Cruz-Correa 2006). In the cancer prevention study II, CRC mortality was reduced in those who took HRT for greater than 10 years (Calle et al 1995). Oestrogens decrease the synthesis of secondary bile acids, decrease response to Insulin-like growth factor and have direct regulatory effects on epithelial proliferation (Janne and Mayer 2000), providing a putative mechanism by which HRT may exert its effect. It is also possible that HRT confers protection through biological mechanisms involving vitamin D (via UV and other sources) and/or components of the vitamin D signaling pathway (Gallagher et al 1980).

### **1.3.4 Fibre**

Burkitt first proposed that dietary fibre reduces the risk of CRC in the 1970s whilst observing the low rates of CRC among rural Africans (Burkitt 1971). A large number of animal and epidemiological studies examining fibre intake and CRC risk have been performed; the majority show a protective effect [reviewed in (Ahmed 2004, Howe et al 1992, Martinez 2005, Trock et al 1990)]. However, increasing the daily amount of fibre does not seem to increase the dose effect (Alberts et al 2000). Fibre is a complex carbohydrate and several mechanisms have been postulated to explain the protective effect, including increased stool bulk and dilution of carcinogens in the colonic lumen (limiting exposure of colonic epithelium to carcinogens) (Lao and Brenner 2004, Lipkin et al 1999).

Fibre may also act as a substrate for bacteria in the gut, resulting in an increase in concentration of short chain fatty acids, such as butyrate (Campos et al 2005). Butyrate has diverse and apparently paradoxical effects on cellular proliferation, apoptosis and differentiation and may be either be pro-neoplastic or anti-neoplastic, depending upon factors such as the level of exposure, availability of other metabolic substrate and the intracellular milieu (Sengupta et al 2006). However, butyrate has been shown to be anti-carcinogenic and can induce apoptosis in colon adenoma and colon cell lines (Key et al 2002). Furthermore, butyrate is an important fuel for regeneration of the colonic epithelium and may also have protective effects by lowering the pH in the colonic lumen (Heavey et al 2004).

Whole grains are a major source of dietary fibre and contain germ, endosperm, and bran, in contrast with refined grains that contain only the endosperm. The germ and bran contain numerous nutrients, which are removed during the refining process. In addition, whole grains are a major source of several vitamins, minerals, and phytochemicals, which have anticancer properties and could influence the risk of CRC by several potential mechanisms (Slavin et al 1999).

To clarify the association between dietary fibre and whole grain intake and risk of CRC, Aune et al. recently carried out a systematic review and meta-analysis of all published prospective studies. The RR of developing CRC with 10 g daily total dietary fibre (16 studies) was 0.90, for fruit fibre was 0.93, for vegetable fibre was 0.98, for legume fibre was 0.62, and for cereal fibre was 0.90. The summary RR for an increment of three servings daily of whole grains was 0.83 (Aune et al 2011).

In a metanalysis of previous studies within the AICR/WCRF report, the RR per 3x60g of wholegrain servings /day of intake was 0.79 for CRC, 0.84 for colon cancer (n=3) and 0.72 for rectal cancer (n=2). Although the heterogeneity of studies was high due to diverse protocols and dosages. (Norat et al 2010).

### 1.3.5 Statins

Statins are small-molecule inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and are used as cholesterol-lowering drugs. In addition to their use in the treatment of lipid disorders, studies have suggested they may have chemopreventative potential against CRC (Agarwal et al 1999, Cho et al 2008). However, large-scale epidemiological studies evaluating the preventative effects of statins against CRC are inconsistent (Coogan et al 2007). This is partially as data relied on retrospective self-reporting by study participants and there was an element of non-compliance. In addition, few studies control for confounding variables such as concurrent NSAID/aspirin use, exercise, vegetable consumption, hypercholesterolaemia, and family history of CRC (Poynter et al 2005). Unfortunately, even in clinical studies specifically set-up to measure the clinical effect of statin use, CRC case numbers are often small and thus fail to demonstrate any risk reduction with the agents (Shepherd et al 1995, Strandberg et al 2004).

### 1.3.6 Other agents

Oxidation of molecules can produce free radicals (molecules with an unpaired electron), which are highly reactive and cause cellular damage. Antioxidants which prevent oxidation, thereby reducing the number of free radicals, such as micronutrients (vitamins A, C, D, E, folic acid and B6; selenium; and carotenoids and flavonoids), have been shown to have a protective effect against CRC (Gatof and Ahnen 2002, Heavey et al 2004, Lin et al 1998, Martinez 2005). However, the results of clinical trials with vitamin supplementation have been disappointing (Giovannucci et al 1998). The Physician Health Study and the  $\alpha$ -tocopherol  $\beta$ -carotene cancer prevention study both demonstrated no encouraging chemopreventative effects and a further RCT with a combination of  $\beta$ -carotene, vitamin C or E, also demonstrated no difference in colonic carcinogenesis endpoints after one year (Giacosa et al 1997). Reported side effects of antioxidant have included pruritus (vitamins A, C, E), epistaxis (vitamin E), risk of haemorrhagic stroke (vitamin E), alopecia and dermatitis (selenium), and yellowing of the skin and belching (beta-carotene) (Cooper et al 2010). Other reviews have reported a possible association between  $\beta$ -carotene and vitamin A and an increased risk of upper GI cancer (Bjelakovic et al 2007). Additional agents such as Ursodiol, a modulator of Bile acid composition (Earnest et al 1994), e-flornithine, an inhibitor of cellular proliferation by altering polyamine

metabolism (Meyskens and Gerner 1999) and Oltipraz, an inducer of mutagen detoxification enzyme, glutathione S-transferase (Helzlsouer and Kensler 1993) have been reported in isolated studies to reduce the risk of CRC in animal models, but evidence has yet to be verified and therefore the weight given to such findings requires appropriate mitigation.

### 1.3.7 Aspirin and related Non-Steroidal Anti-inflammatory Drugs (NSAIDS)

Aspirin belongs to a family of compounds called the salicylates. Aspirin is absorbed primarily in the upper GI tract following first order kinetics and has an absorption half-life of 5-16 minutes. It is mainly unchanged in the lumen of the GI tract but once absorbed is hydrolysed by carboxylesterases in the gut wall and liver, so that only 68% of the dose reaches the systemic circulation as acetylsalicylic acid. Salicylates are excreted via the kidney and broken down into salicylic acid. (reviewed in Hare et al 2003). The serum half-life of Aspirin is 20 mins, Salicylic acid is the principal metabolite of aspirin which has a serum half-life of 2-30 hours dependant on concentration (Needs et al 1985).

Salicylic acid is also present in dietary fruits and vegetables, with some herbs and spices containing particularly good sources of dietary salicylates (Venema et al 1996). A normal diet provides 0–6 mg of salicylates daily, and no measurable aspirin (Janssen et al 1996).

Aspirin and related compounds (of which there are greater than 20) are the most widely studied agents in the chemoprevention of CRC (Janne and Mayer 2000). They have a major role in the primary and secondary prevention of myocardial infarction, thromboembolic disease and stroke (Sanmuganathan et al 2001) and it was through analysis of larger trials of the above conditions that a protective effect against CRC was first noted. Compelling evidence from a number of experimental, animal, human and population studies indicates that these agents have a CRC chemopreventative effect (Dube et al 2007, Levy 1997, Thun et al 1991).

#### *Effects on aberrant crypt foci development*

Case control and animal studies demonstrate a reduction in both Aberrant Crypt Foci (ACF) formation and the incidence of early adenomas (DuBois et al 1996, Reddy et al 1993). In one particular study, the total number of ACF were significantly reduced in colon carcinoma resections in patients who received preoperative aspirin for greater than one year (Shpitz et al 2006). The incidence of ACF is also noted to be decreased in individuals treated with sulindac (Kuno et al 2002).

### *Effects on the development of colorectal lesions in high-risk patients*

Several studies of NSAIDs in FAP (sulindac, celecoxib or tiracoxib) have demonstrated that NSAIDs reduce the number and size of polyps in FAP patients (polyp regression) (Guldenschuh et al 2001, Makela and Laitinen 1994, Rigau et al 1991, Waddell and Loughry 1983, Winde et al 1997). The metabolite of sulindac, sulindac sulfone (exisulind), has also been shown to have a protective effect against the development of rectal adenomas in FAP (Stoner et al 1999).

The CAPP1 study (Colorectal Adenoma Prevention Programme 1) studied daily resistant starch or daily aspirin (600 mg as 2 tablets) in 206 FAP patients. After a median of 17 months, the primary endpoint of polyp risk, reported that polyp numbers in the rectum and sigmoid colon was not significantly reduced in either treatment group but there was a trend toward reduced polyp number and diameter of the largest polyp detected in the aspirin arm. This was later supplemented by evidence from subgroup analyses of patients who continued on the study for more than one year, which found a significant reduction in the size of the largest polyp (Burns J et al 2011, Burns et al 2013).

In CAPP2, 1009 Lynch syndrome gene carriers were recruited and after a mean of 29 months, there was no evidence that aspirin 600mg/d influenced development of colonic neoplasia after 2 years. However, the design included double blind follow-up for at least 10 years, and after a mean of 55.7 months, 48 recruits developed CRC. The HR for CRC with aspirin was 0.63. The evidence was sufficient to recommend aspirin to all Lynch syndrome gene carriers (Burn J et al 2011). This was also the first time that a trial had demonstrated a beneficial effect of aspirin on the incidence of CRC or death.

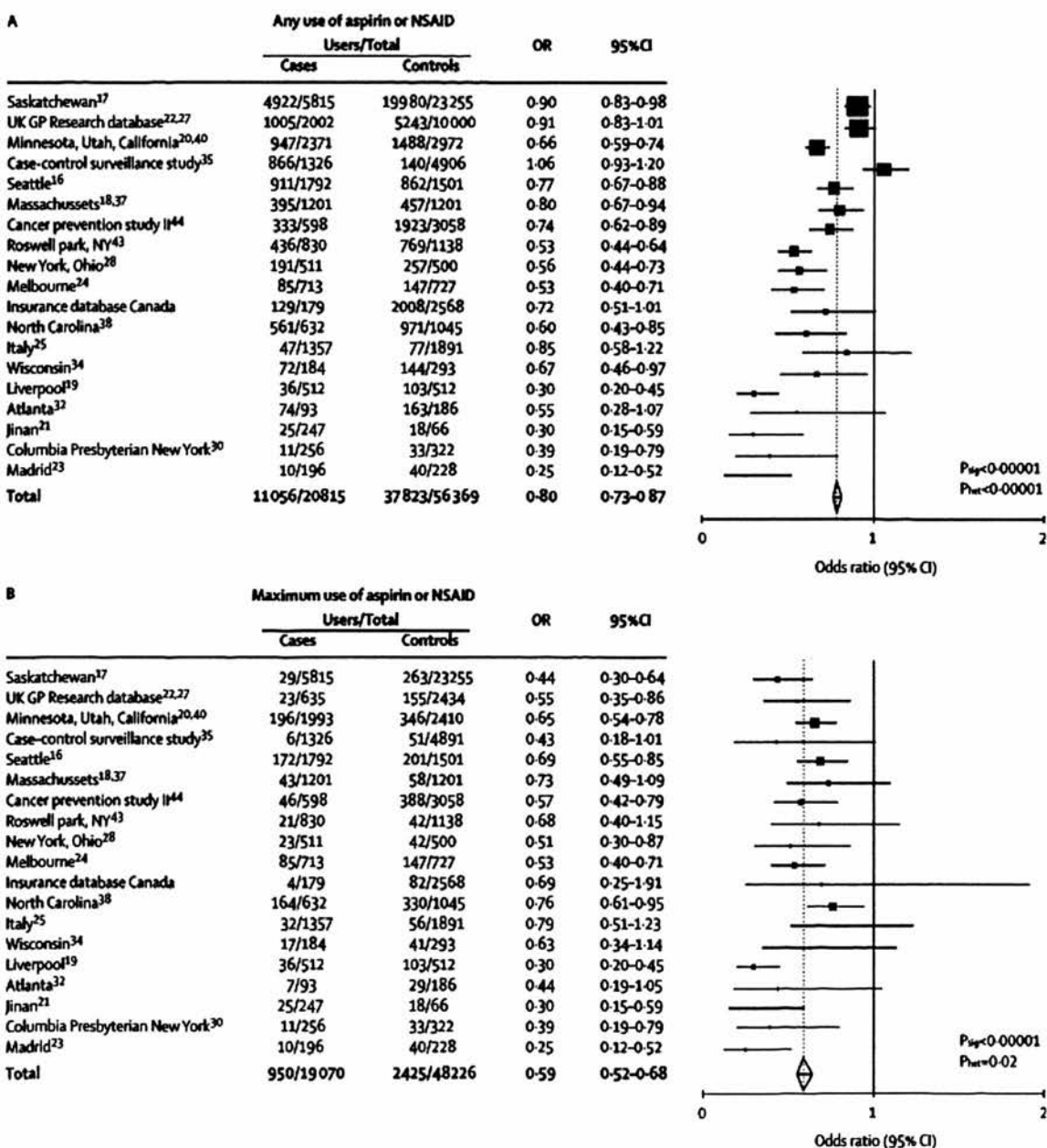
### *Effects on adenoma occurrence in persons of “normal” risk*

Epidemiological studies demonstrate persons who regularly take aspirin or related non-aspirin NSAIDs have a 40-50% lower risk of developing colorectal adenomas and tumours. Some studies even suggest NSAIDs reduce CRC-related deaths (Asano et al 2004, Giovannucci et al 1998, Janne and Mayer 2000) [reviewed in (Benamouzig et al 2005, Brown and DuBois 2005, Imperiale 2003)



(Giovannucci 1999, Shiff and Rigas 1997)]. Flossman et al. (Flossmann and Rothwell 2007) retrospectively analysed CRC incidence data from two previous prospective British trials involving aspirin randomisation, the British Doctors Aspirin Trial and the UK-TIA Aspirin Trial, originally designed to examine association with myocardial infarction and Transient Ischaemic Attacks (TIAs), respectively. The maximum chemopreventative effect occurred after 5 years, or more, duration of aspirin intake and at a dosage of greater than 300mg per day (lesser dosages gave more inconsistent results), with a latency period until the chemopreventative effect became significant of over 10 years.

Figure 1. 3 Previous case control trials examining aspirin and related NSAIDs impact on CRC endpoints (A) any use of aspirin or NSAID and (B) maximum reported use of aspirin or NSAID (Flossmann and Rothwell).



Metanalysis of four RCTs of aspirin in the general population, (two low dose (100–325 mg every other day) and two higher dose (300–1500 mg/day), with a combined follow-up to 10-23 years, demonstrated no effect on CRC risk over the first 10 years of follow-up (RR 1.01). However, analysis of the higher dose studies demonstrated a significant reduction in CRC incidence over the full 23-year follow-up period (RR 0.74). An even greater reduction was observed when analysing years 10–19 only (RR 0.61) (Cook et al 2005, Cooper et al 2010). Only one trial to date has demonstrated an increased risk of CRC following Aspirin or NSAID administration (Paganini-Hill et al 1989). However, this questionnaire study had considerable flaws including compliance weakness, recall bias and the reliance on analysis of both male and female subpopulations combined, to present a significant increase statistically.

#### *Intermediate risk - secondary prevention*

Previous RCTs report that aspirin (81–325 mg/day) imparts a significantly reduced risk (RR = 0.65-0.96) of adenoma recurrence in patients previously diagnosed with CRC or adenoma, within 3 years of follow-up (Baron et al 2003, Benamouzig et al 2003, Cole et al 2009, Sandler et al 2003). Metanalysis of these studies demonstrates an overall statistically significant 21% reduction in the relative risk of adenoma recurrence (Cooper et al 2010).

#### *Interventional studies*

Two recent epidemiological studies also suggest regular aspirin use after CRC diagnosis improves outcomes and that aspirin could have a role as an adjuvant therapy in cancer (Chan et al 2009) (Holmes et al 2010). The benefit of aspirin was greatest in those whose tumours overexpressed COX-2. Furthermore, those who had taken aspirin before diagnosis did not appear to benefit (Rothwell et al 2010). A small trial of only 66 patients evaluated 1200 mg of aspirin daily compared with placebo as adjuvant treatment for Duke's B2 and C CRC demonstrated a non-significant reduction in HR for survival 0.65 (95% CI: 0.02–18.06) (Lipton et al 1982). In addition, the recent Capp2 study examining the role of aspirin in reducing the development of CRC in high-risk groups has already been discussed and has led to the recommendation of aspirin for chemopreventative use in MMR

carriers (Burn et al 2011).

Recently, Rothwell et al., (Rothwell and Algra 2012) published a systematic review of all case-control and cohort studies over a 61 year period that reported associations between aspirin use and risk or outcome of any cancer. This included more than 77,000 patients in 51 studies. Meta-analyses demonstrated that aspirin was associated with a reduced risk of CRC (0.62) and the 20-year risk of death due to CRC. Regular use of aspirin was also associated with a reduced risk in the development of distant metastasis but not with any reduction in regional spread.

Almost concurrently, Rothwell et al. (Rothwell et al 2012) also published an additional meta-analysis of five large UK randomised trials of daily aspirin ( $\geq 75$  mg daily) versus control for the prevention of vascular events, specifically looking at the frequency of distant metastasis in aspirin treated versus aspirin-naive patients who developed cancer during trials of daily aspirin versus control. Aspirin reduced the risk of developing distant metastasis in all cancers, but with greater effect in adenocarcinomas. This effect was present at initial diagnosis (HR 0.69) and on subsequent follow-up in patients without metastasis initially (HR 0.45)(particularly in patients with CRC - HR 0.26). There was also a reduction in death due to cancer in patients who developed adenocarcinoma, especially in those without metastasis at diagnosis (HR 0.50).

#### *Dose effect*

The epidemiological data and RCTs assessing primary prevention support the notion that the anti-cancer effects of aspirin are seen when higher, daily dosages of aspirin are used over longer follow-up (Chan et al 2005, Chan et al 2008). At higher doses, aspirin is a more potent inhibitor of COX-2 but interestingly, in the majority of cohort studies, the chemopreventative dose for aspirin is at a dose of aspirin much higher than that required to prevent the Cox activity (Frantz and O'Neill 1995). Some exceptions have been reported (Thrombosis Prevention Trial, Swedish Aspirin Low Dose Trial) (Rothwell et al 2010). Baron et al. detected the maximum RR effect with adenomas at 81mg OD dosage (RR 0.81 CI 0.69-0.96) compared to the 325mg OD dosage (RR 0.96 CI 0.81-1.13) (Baron

2003). A recent case-control Scottish study has also demonstrated preventative effects at levels as low as 75mg Aspirin daily (Din et al 2010).

Of note, in the previously discussed paper looking at the frequency of distant metastasis in aspirin treated versus aspirin-naive patients who developed cancer during vascular event prevention RCTs (Rothwell et al 2012), low-dose formulations of aspirin (designed to inhibit platelets but to have little systemic bioavailability) were as effective as higher doses. Previous reports have also found that whilst aspirin reduced the 20-year risk of CRC (HR= 0.7) and that benefit increased with scheduled duration of treatment, such as allocation to aspirin of 5 years or longer, there was no increase in benefit at doses of aspirin greater than 75 mg daily (Rothwell et al 2010).

There are a number of ongoing studies due to report in the next few years which aim to elucidate the relationship of aspirin dose and its effect on adenoma formation, CRC recurrence and CRC survival. These include The Systematic Evaluation of Aspirin and Fish Oil polyp prevention (SEAFOOD) (evaluating adenoma number in high risk individuals; EPA and 300mg aspirin/d), the Japan Colorectal Aspirin Polyps Prevention (J-CAPP) study (new CRC in those who have had previously undergone resection for CRC; 100mg aspirin/d) and Aspirin for Dukes C and High Risk Dukes B Colon Cancer Trial (ASCOLT) trial (evaluating disease free survival in those with previous Duke's C or high risk Duke's B CRC) (reviewed in Chan et al 2012). The future CAPP3 study will report on findings made in previous CAPP studies and aims to recruit 3000 high risk gene carriers into a dose inferiority study to test the relative chemopreventative benefits of 100mg, 300 or 600mg daily doses (Burn et al 2013)

#### *Animal studies*

In addition to epidemiological studies, the anti-neoplastic properties of aspirin and related NSAIDs have been consistently reported in animal (mainly rodent) models of CRC. In carcinogen-induced (DMH (1,2-dimethylhydrazine) or AOM (azoxymethane)) CRC models it has been shown that animals have reduced of adenomas following NSAID administration (Fukutake et al 1998, Haanen 2001, Pollard and Luckert 1981, Reddy et al 1993, Shiff and Rigas 1997). In the Min (APC<sup>+/-</sup>) mouse (which has an autosomal, dominant heterozygous mutation of the APC gene leading to spontaneous

intestinal neoplasia) aspirin has also been shown to reduce adenoma formation (Barnes and Lee 1998, Chiu et al 1997, Giovannucci 1999, Jacoby et al 1996). NSAIDs (Goldman et al 1998, Williams et al 2000) also suppress CRC cell growth in the nude mouse xenograft model (where cultured CRC cells are xenografted onto the flanks of nude (athymic) mice) (Gupta and Dubois 2001).

Aspirin has previously demonstrated a CRC cell-specific ability to both reduce the viability of CRC cancer cells *in vitro* and induce apoptosis, in comparison to its effects on cells originating from other tissues (Din et al 2004), (Piazza et al 1995). Recent evidence from CRC cell lines also suggests the increasing importance of aspirin's effects on inducing cell cycle arrest as a predominant mechanism in aspirin-induced apoptosis (Thoms et al 2007a). Further details of these mechanisms will be explored in chapter 1.4.

#### *Side Effects*

Long-term aspirin use is consistently associated with a statistically significant elevated risk of gastrointestinal bleeding (RR 1.6- 2.5) and increased risk of adverse gastrointestinal symptoms, such as nausea and dyspepsia (OR, 1.7) (Dube et al 2007). The side effects of NSAIDs are associated with inhibition of Cox-1. Therefore, selective Cox-2 inhibitors were developed. It was found that the incidence of perforation, ulceration or bleeding was significantly lower, but not negligible, with rofecoxib (Cox-2 specific inhibitor) than with traditional NSAIDs in a metaanalysis of patients taking these medications for arthritis (cumulative incidence 1.6% vs. 3.1%,  $p < 0.001$ ) (Watson 2004). Based on these data, the agents were licensed in the US for use in conditions associated with high numbers of adenomatous polyps. However, in 2004, after analysis of a RCT of the Cox-2 inhibitor, rofecoxib in patients with adenomas, there was still a higher level of perforations, ulcer obstructions or GI bleeding episodes in this group compared to placebo treatment (relative risk, 3.8) (Lanas et al 2007). Furthermore, the use of these agents was associated with significant cardiovascular thrombotic events (Bresalier 2005). This safety profile was such that Rofecoxib (Vioxx) was withdrawn from recommended use (Rostrom 2007) (Topol, 2004; Samoha and Arber, 2005). Similar findings were reported from evaluation of a trial of celecoxib, which also demonstrated significant cardiovascular side effects, especially in high-dose groups (Solomon et al., 2005).

The mechanism linking the use of COX-2 inhibitors to an increased incidence of thrombotic vascular events has not been precisely elucidated. However one theory is that Prostaglandin I<sub>2</sub>, derived from COX-2, has previously been shown to be the predominant cyclooxygenase product in endothelium, inhibiting platelet aggregation, causing vasodilatation, and preventing the proliferation of vascular smooth-muscle cells *in vitro*. *In vitro* these effects contrast with those of thromboxane A<sub>2</sub>, the major COX-1 product of platelets, which causes platelet aggregation, vasoconstriction, and vascular proliferation. Whereas aspirin and traditional NSAIDs inhibit both thromboxane A<sub>2</sub> and prostaglandin I<sub>2</sub>, the coxibs leave thromboxane A<sub>2</sub> generation unaffected, but suppress formation of prostaglandin I<sub>2</sub> potentially predisposing patients to myocardial infarction or thrombotic stroke (Fitzgerald 2004).

### **1.3.8 Combination Approach**

In addition to the above individual potential agents, authors have suggested that two or more agents in combination may provide a better chemopreventative action than one recommended agent alone. In a trial in the APC<sup>Min/+</sup> mice model of intestinal polyposis, the use of the EGFR kinase inhibitor, EKI-785, in addition to a NSAID (Sulindac) caused a 50% regression of adenomas compared to untreated controls (Torrance et al 2000) and further studies have postulated the benefits of COX inhibitors in addition to P13-Kinase, PPAR $\gamma$  inhibitors or 15-PGDH inhibitors (Markowitz 2007). Another example utilizes the polyamine synthesis pathway. Arginine, a precursor of ornithine, induces polyamine synthesis via ornithine decarboxylase (ODC). Polyamines enhance colonic tumourigenesis while inhibition of ODC inhibits carcinogenesis. Combination of an inhibitor of ODC with sulindac, which inhibits polyamine biosynthesis, was found to have strong chemopreventative effects on sporadic colorectal adenomas (Meyskens et al 2008).

### **1.3.9 Conclusions**

The agents described above are in no way exhaustive and many ongoing trials continue to search for a reliable and safe means of chemopreventative therapy in CRC. The importance in understanding the mechanisms of action of those agents that have demonstrated most promise cannot be underestimated, as it is through this understanding that safer, more effective agents may be designed. Hence, whilst

aspirin and related NSAIDs are some of the best characterised agents, they are also some of the most toxic and urgent priority must be given to research aimed at understanding more about intracellular events stimulated and modified by aspirin administration.

## **1.4 Putative mechanisms of aspirin's chemopreventative effects**

### **1.4.1 Background**

Tissue mass is critically affected by rates of cell proliferation and cell death (by apoptosis), and the colonic mucosa in both normal and pathological states is not an exception. Exploration of aspirin's molecular effects on colonic mucosa would therefore aid in the development of more effective and safer chemopreventative agents.

### **1.4.2 COX-2-dependent mechanisms of aspirin induced chemoprevention**

One of the most notable effects of aspirin and the majority of NSAIDs is their ability to inhibit activity of cellular COX (cyclo-oxygenase) enzymes. A basic schematic of the pathway is seen in figure 1.3 but briefly, these enzymes provide the rate-limiting step in the synthesis of eicosanoids, such as prostaglandins E<sub>2</sub> and D<sub>2</sub>, from arachidonic acid. Arachidonic acid is released from membrane phospholipids by phospholipase A<sub>2</sub> and converted by COX-1 or COX-2 into prostaglandin (PG) G<sub>2</sub> and then to PGH<sub>2</sub> by peroxidase catalytic activity. Production of PGs (PGE<sub>2</sub>, PGD<sub>2</sub>, PGI<sub>2</sub>, PGF<sub>2</sub>α and thromboxane A<sub>2</sub>) from PGH<sub>2</sub>, the precursor of all PGs, is catalysed by a specific isomerase (Brown and DuBois 2005). PGE<sub>2</sub> levels are elevated in colorectal tumours and adenomas compared to normal adjacent mucosa (Backlund et al 2005) and it has been suggested that these elevated levels of prostaglandins accelerate the growth and invasion of cancer (Easty and Easty 1976). Indeed, it has been demonstrated that certain oncogenes induce prostaglandin synthesis (Levine 1981) and addition of prostaglandins to CRC cell lines promotes tumour growth, resistance to apoptosis, secretion of pro-angiogenic molecules and promotion of cell proliferation (Oshima and Taketo 2002, Sakamoto 1998, Seno et al 2002, Sonoshita et al 2001) (Cha and DuBois 2007). Furthermore, 15-PGDH (15-Prostaglandin Dehydrogenase), a potent suppressor of the prostaglandin pathway, inhibits tumour growth in murine models of CRC, is abolished in the majority of colon cancers (Markowitz 2007).

COX-1 and COX-2 are homodimers of similar molecular weight, approximately 70 (576 amino acids) and 72 kDa (581 amino acids) respectively. Both contain three high mannose oligosaccharides, a



fourth oligosaccharide, present only in COX-2, regulates its degradation. Each subunit of the dimer consists of the epidermal growth factor domain, the membrane binding domain and the catalytic domain comprising the bulk of the protein (containing a cyclooxygenase and peroxidase active sites). COX-1 is coded for by *Ptgs-1*, whose mRNA is relatively stable unlike the COX-2 gene *Ptgs-2*, which is activated by a wide variety of stimuli, and produces a relatively unstable mRNA (Smith et al. 2000). COX-1 provides prostaglandins involved in homeostasis (including gastric cytoprotection and haemostasis), whereas COX-2 plays a role during inflammation and tumorigenesis.

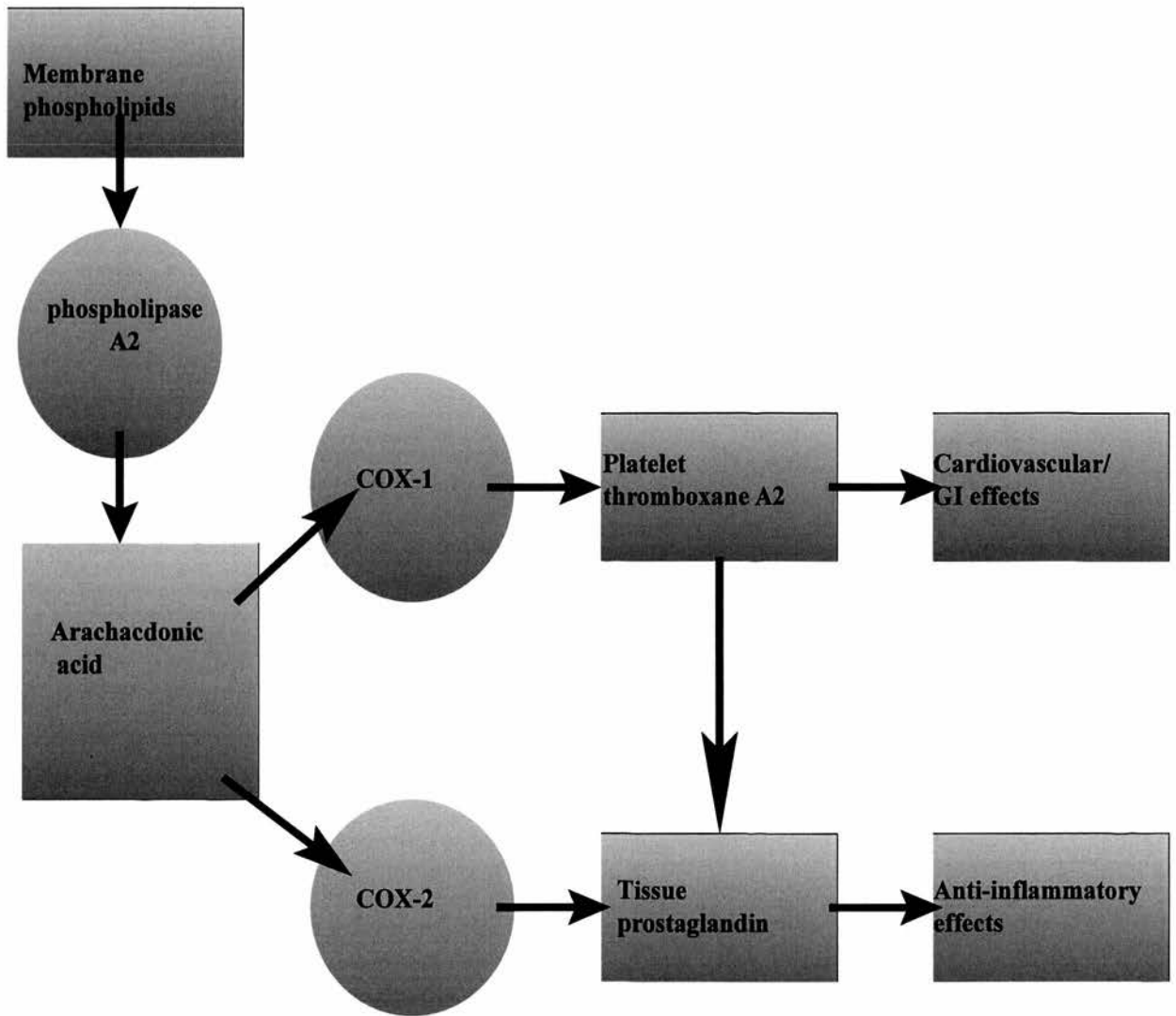
Aspirin is an approximately 150- to 200-fold more potent inhibitor of the constitutive enzyme COX-1 than the inducible isoform COX-2. This explains the different dosage requirements of aspirin as an antithrombotic (COX-1) and an anti-inflammatory drug (COX-2), respectively (Schrör K. et al. 1997). Aspirin is the only clinically used NSAID that covalently modifies the COX protein. All other NSAIDs act non-covalently and most can be classified as either rapidly reversible, competitive inhibitors or slow, tight binding inhibitors. The kinetics and mechanism of inhibitor binding were recently reviewed in detail (Rouzer and Marnett 2009). The substitution of isoleucine at position 523 in COX-1 with valine in COX-2, allows for selective inhibition via binding of drug molecules, such as the coxibs. Because COX-2 is usually specific to inflamed tissue, there is much less gastric irritation associated with COX-2 inhibitors, with a decreased risk of peptic ulceration. (Blobaum and Marnett 2007).

COX-1 is a constitutively and widely expressed “house-keeping” enzyme which supplies tissues with the prostaglandins required to maintain physiological function i.e. renal blood flow and cytoprotection of the gastric mucosa. COX-1 is expressed in platelets and gastric mucosa and inhibition of this enzyme, following NSAID administration, potentially leads to ulceration and bleeding (Patrignani 2000). COX-2 is an inducible form of the COX isoform, which is upregulated in injury/inflammation and serves to sensitise nociceptors and increase inflammatory mediators (Backlund et al 2005, Brown and DuBois 2005, Gupta and Dubois 2001, Janne and Mayer 2000). It is induced by a wide variety of agents including cytokines, mutagens and growth factors and increases new blood vessel growth and survival in tumours by, in some cases, decreasing apoptosis (Tsuji and DuBois 1995). Elevated levels of prostaglandins in CRC are attributed to increased COX-2 activity. In fact, multiple

lines of evidence indicate that this enzyme acts as a tumour promoter in this and other cancers. For instance, COX-2 mRNA and protein are found to be upregulated in over 90% of colorectal cancers and 40% of adenomas compared to normal mucosa (Fujita et al 1998, Patrignani 2000, Sano et al 1995). There is increased COX-2 expression found in adenomas from FAP patients (Giardiello et al 1998) and in CRCs from inducible cancer animal models (Williams and DuBois 1996), (Boolbol et al 1996). Both gene disruption and pharmacological inhibition of COX-2 have been shown to reduce the number and size of polyps in APC<sup>Δ716(+/-)</sup> knockout mice (Oshima et al 1996) and inhibition of COX-2 leads to increased levels of arachidonic acid, which in turn stimulates apoptosis (Chan et al 1998, Janne and Mayer 2000). Furthermore, in COX-1- or COX-2-deficient APC deficient mice, the tumourigenic effect of the APC mutation is decreased by 80% (Chulada et al 2000).

Given the evidence that COX-2 contributes to colorectal carcinogenesis, it is not surprising that the anti-tumour activity of NSAIDs has been attributed to their ability to inhibit these enzymes. Aspirin, unlike other NSAIDs (Mitchell et al 1993), inhibits both COX-1 and COX-2 activity by irreversibly acetylating the proteins (Taketo 1998, Vane 1996), although there is preferential inhibition of COX-1 (Simmons et al 2004). Acetylation of COX-2 by aspirin switches COX-2 induced promotion of prostaglandins (PGE<sub>2</sub>) to promotion of the antitumourigenic 15-epi-lipoxin-A<sub>4</sub> (LXA<sub>4</sub>) or 'aspirin-triggered lipoxin (ATL). ATL inhibits proliferation of carcinoma cells (Claria et al 1996) and is induced at low, antiplatelet doses of the agent (Morris et al 2009). In addition to irreversible acetylation of COX-2, it has been suggested that NSAIDs modulate COX-2 activity through induction of the NSAID activated gene-1 (NAG-1). Expression of this gene is induced by NSAIDs and this expression correlates with reduced levels of COX-2 activity and increased apoptosis. Furthermore, over expression of NAG-1 mediates apoptosis of colorectal cancer cells while anti-sense NAG-1 causes attenuation of NSAID induced apoptosis and suppression of xenografted tumour growth in nude mice (Baek et al 2010, Eling et al 2006). High expression of COX-2 in human CRC tumour tissue is related to low expression of NAG-1, suggesting a reciprocal relationship (Iguchi et al 2009). It is suggested that increased COX-2 expression in CRC tumours suppresses NAG-1 but that induction of NAG-1 by aspirin and non-aspirin NSAIDs might constitute a possible chemopreventative mechanism.

Figure 1.4 The Cyclo-oxygenase pathway



Evidence from recent clinical trials supports the notion of the existence of a subpopulation of colon tumours or polyps which express higher levels of COX-2 are more responsive to the anti-tumour effects of aspirin/NSAIDs (Chan et al 2007). Earlier case control studies also suggested that NSAIDs reduce the relative risk of adenomatous polyps, especially in those cases showing high COX-2 mRNA levels (Haile et al 2005). However, there is also considerable evidence that NSAIDs have COX independent mechanisms of action.

### **1.4.3 COX-2 independent mechanisms of aspirin induced chemoprevention**

There remains intense debate over whether a COX-independent mechanism is responsible for the anti-cancer effects of aspirin and related NSAIDs (Levy 1997, Rigas and Williams 2002). Evidence that COX-2 independent pathways may be responsible comes from several sources. Important discoveries have been made in CRC cell lines that do not express COX-2 or NSAIDs that have no known anti-COX 2 activities. Smith et al. (Smith et al 2000a), investigated the action of aspirin, indomethacin and NS-398 (a COX-2 specific NSAID) in a panel of COX-2 expressing and non-expressing CRC cell lines, found that whilst NS-398 had no effect on the COX-2 negative cell lines, indomethacin induced cell cycle arrest and apoptosis in both COX-2 positive and negative cell lines, irrespective of their COX-2 status. Hanif et al., demonstrated that aspirin exhibits a dose-dependent inhibition of growth in CRC cell lines irrespective of their COX-2 expression profile (Hanif et al 1996). Piazza et al., reported dose-related induction of apoptosis in sulindac treated colon cancer cell lines, independent of COX inhibition (Piazza et al 1997b). Furthermore, mouse embryo fibroblasts that do not express either COX isoforms show similar sensitivity to high dose NSAID treatment (Zhang et al 1999).

In animal models of colonic carcinogenesis, many COX-2 inhibitors continue to demonstrate an anti-tumour effect despite possessing no anti-COX activity (Kawamori et al 1998, Oshima and Taketo 2002, Yoshimi et al 1999). The R-enantiomer of Flurbiprofen retained chemopreventative activity in the APC Min Mouse Model of intestinal polyposis despite no known anti-COX activity (Wechter et al

1997) and sulindac sulphone (a metabolite of sulindac which does not inhibit COX activity) inhibited AZO induced colonic cancer in rats (Piazza et al 1997a).

In epidemiological studies, data suggests that the chemopreventative action of aspirin is observed at doses higher than that required to reduce E2 and F2 prostaglandins synthesis in the intestinal mucosa (Ricchi et al 2003, Tegeder et al 2001). These data suggest that important anti-tumour cell mechanisms are induced at higher dosages (>100 fold higher) than the amount required to inhibit prostaglandin production (Kralj et al 2001, Smith et al 2000b)(Chiu et al 1997, Qiao et al 1998a)(Ricchi et al 1997).

Taken together, these data suggest that NSAIDs may have COX-independent mechanisms of action against colorectal cancer cells. Several alternative pathways have been suggested as putative targets for the anti-tumour mechanism of these agents (Elwood et al 2009, Jankowski and Anderson 2004). The most studied of these pathways will be discussed in detail below.

#### **1.4.4 The MAP kinase pathway**

The MAPK/ERK stress response pathway includes ERK-1, -2, p42, p44 MAP Kinase, c-Jun N-terminal Kinase (JNK) and p38 Map kinase. These pathways regulate inflammation, proliferation and cell survival. MAPKs are activated by MAPK kinases (MAPKKs or MAP2Ks). MAPKKs are in turn activated by a family of serine-threonine kinases called MAPKK kinases (MAPKKKs, MAP3Ks or MEKKs) (Saklatvala 2004). Activation of MAPKs occurs in times of cell stress with the eventual activation of p38, leading to resultant changes in cell cycle progression and apoptosis (Thoms et al 2007b) Classically, activation of MAP kinases is associated with cell survival and proliferation and even transformation (Mansour et al 1994), (Xia et al 1995) however, their importance to the induction of apoptosis and cell cycle arrest have now also been noted, and importantly, depend on the cellular context (Thoms et al 2007b) (Stanciu et al 2000).

Sodium salicylate inhibits JNK and ERK activity in human fibroblasts and other cellular contexts (Pillinger et al 1998, Rice et al 2001, Schwenger et al 1998, Wang and Baldwin 1998). However,

individual MAP kinase family members respond to aspirin and related-NSAIDs with variation depending on cellular context. For example, Sodium Salicylate can activate ERK 1, 2 and p38 MAPK and increased apoptosis (Schwenger et al 1998). Salicylates also induce apoptosis in eosinophils via activation of c-JNK and p38 MAPK (Wong et al 2000). In human colon cancer cells, treatment with NS-398, a NSAID, caused sustained activation of ERK, associated with apoptosis (Elder et al 2002) and chemical inhibition of these kinases blocks the effects of the salicylates-induced apoptosis (Lee et al 2003).

The host laboratory have previously reported that a very early response to aspirin in colorectal cancer cells is activation of the p38 MAP kinase and that this activation is critical for the pro-apoptotic effect of the agent. They demonstrated that upon aspirin-mediated activation, p38 induces degradation of Cyclin D1, inhibition of CDK 4, activation of the NF- $\kappa$ B pathway, repression of NF $\kappa$ B transcription and induction of apoptosis, with inhibitors of MAPK blocking these effects (Thoms et al 2007a) (see below for more details). These data suggest that MAP kinase signalling could be a primary target of aspirin and related NSAIDs.

#### **1.4.5 Cyclin-dependent kinases (CDKs)**

Cell cycle progression is promoted by cyclin-dependent kinases (CDKs) which complex with cyclins, driving the cell forward through the cell cycle (Schwartz and Shah 2005). Progression mediated by cyclin/CDK complexes is monitored at several positions, known as cell cycle check points (Collins and Garrett 2005). The activity of the CDK/cyclin complex is further negatively regulated by a number of CDK inhibitors (CDKIs), including p21 (<sup>waf1/cip1</sup>) and p27 (<sup>kip1</sup>) (Vermeulen et al 2003a).

A range of NSAIDs have been reported to induce cell cycle arrest by altering of CDK expression particularly within G<sub>0</sub>/G<sub>1</sub> phases (Qiao et al 1998b) (Maier et al 2004, Shiff et al 1995, Shiff et al 1996). NSAIDs also up-regulate CDKIs, such as p27<sup>kip1</sup> and p21<sup>waf1/cip1</sup> (Jung et al 2005, Marra et al 2000, Ricchi et al 2003, Yang et al 2001). As mentioned above, the host laboratory has previously demonstrated that inhibition of CDK4 can mimic aspirin effects on the NF- $\kappa$ B pathway and mediate

apoptosis. It was shown that this effect was independent of the effects of the agent on the cell cycle (Thoms et al 2007b).

#### **1.4.6 PPAR $\gamma$ /Alternative targets**

PPAR $\gamma$  is a ligand-dependent transcription factor that is highly expressed in breast and CRC. PPARs are members of the nuclear hormone superfamily, including steroid hormones, thyroid hormone and retinoid nuclear hormone receptors (He et al 1999). PPAR $\gamma$  agonists induce differentiation and apoptosis in CRC cell lines (Vandoros et al 2006) and some CRC are associated with a PPAR $\gamma$  loss of function mutation (Tegeeder et al 2001). Indomethacin and other NSAIDs directly bind to PPAR $\gamma$  (He et al 1999, Jaradat et al 2001, Lehmann et al 1997), thus suggesting a putative direct molecular target for NSAIDs. However, genetic ablation of PPAR $\gamma$  in CRC cells does not alter their sensitivity to sulindac-induced apoptosis, (Park et al 2001). Other targets which have been postulated as regulating NSAID-induced effects in colon cancer are Ribosomal S6 Kinases, STAT-1, catabolism of polyamines, caspase induction and many others (Tegeeder et al 2001)(Din 2012).

Whilst the above alternative pathways have been highlighted as potential targets for the anti-tumour mechanism of NSAIDs, studies from the host laboratory have identified activation of the NF $\kappa$ B transcription factor as critical in this regard. Therefore, the remainder of this chapter will be focused on this mechanism as this is of direct relevance to the work outlined in this thesis.

### **1.5. NF- $\kappa$ B transcription factor and its activation**

The NF- $\kappa$ B (Nuclear Factor Kappa B) family of transcription factors comprises of NF $\kappa$ B1 (p105 precursor cleaved to p50), NF $\kappa$ B2 (p100 precursor cleaved to p52), RelA, C-Rel and RelB (Gilmore and Herscovitch 2006). These NF- $\kappa$ B proteins form a variety of homo and heterodimers that bind to target DNA sequences and regulate a large spectrum of genes including immunoregulatory and inflammatory genes; anti-apoptotic genes; genes that positively regulate cell proliferation; and genes that encode negative feedback regulators of NF- $\kappa$ B itself (Karin et al 2002); (Campbell et al 2006). Each member of the NF $\kappa$ B transcription factor family contains an amino-terminal region of approximately 300 bases long called the Rel homology domain (RHD). This domain is responsible for the DNA binding potential of the proteins, protein dimerisation and nuclear localisation. In addition to their Rel homology domain, RelA, C-Rel and RelB have a C terminal transactivation domain, which is not observed in p50 or p52. The dimerisation domain has an area called the nuclear localisation signal (NLS) and an additional binding site for NF- $\kappa$ B inhibitors.

NF- $\kappa$ B proteins are found ubiquitously in all human cells but are normally retained in the cytoplasm by their inhibitory proteins, Inhibitors of NF $\kappa$ B (I $\kappa$ Bs). The I $\kappa$ B family of proteins includes I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\epsilon$ , I $\kappa$ B $\gamma$ , Bcl-3, p100 and p105 (Ravi and Bedi 2004) and are characterised by their multiple ankyrin repeats. The ankyrin repeats allow I $\kappa$ Bs to bind NF- $\kappa$ B, which are characterised by multiple ankyrin repeats to provide protein-protein interaction sites with NF- $\kappa$ B (Ghosh and Karin 2002), which masks the NLS of NF- $\kappa$ B, thus retaining the protein in the cytoplasm, by inhibiting the NLS of NF- $\kappa$ B (Thanos and Maniatis 1995); (Stancovski and Baltimore 1997). Most stimuli activating the NF- $\kappa$ B transcription factor do so via induced phosphorylation of the I $\kappa$ B $\alpha$  protein on serine/threonine residues (32/36). The I $\kappa$ B proteins are then targeted for polyubiquitination and eventual proteosomal destruction. This unmaskes the NLS of NF- $\kappa$ B allowing it to translocate to the nucleus and activate target genes (Karin and Ben-Neriah 2000, Perkins 2007). The kinetics of I $\kappa$ B degradation and rate at which NF- $\kappa$ B translocates to the nucleus depends on the nature of the stimuli and the cellular context, but can have a direct effect on the range of target genes induced. Activation of NF $\kappa$ B is generally transient as upon induction, it controls transcription of its inhibitor proteins (I $\kappa$ B, p105 (NF- $\kappa$ B1) and



p100 (NF- $\kappa$ B2)) (Karin et al 2002). Once transcribed, I $\kappa$ B is translated in the cytoplasm, translocates to the nucleus and transports NF $\kappa$ B back into the cytoplasm, thus terminating the signal.

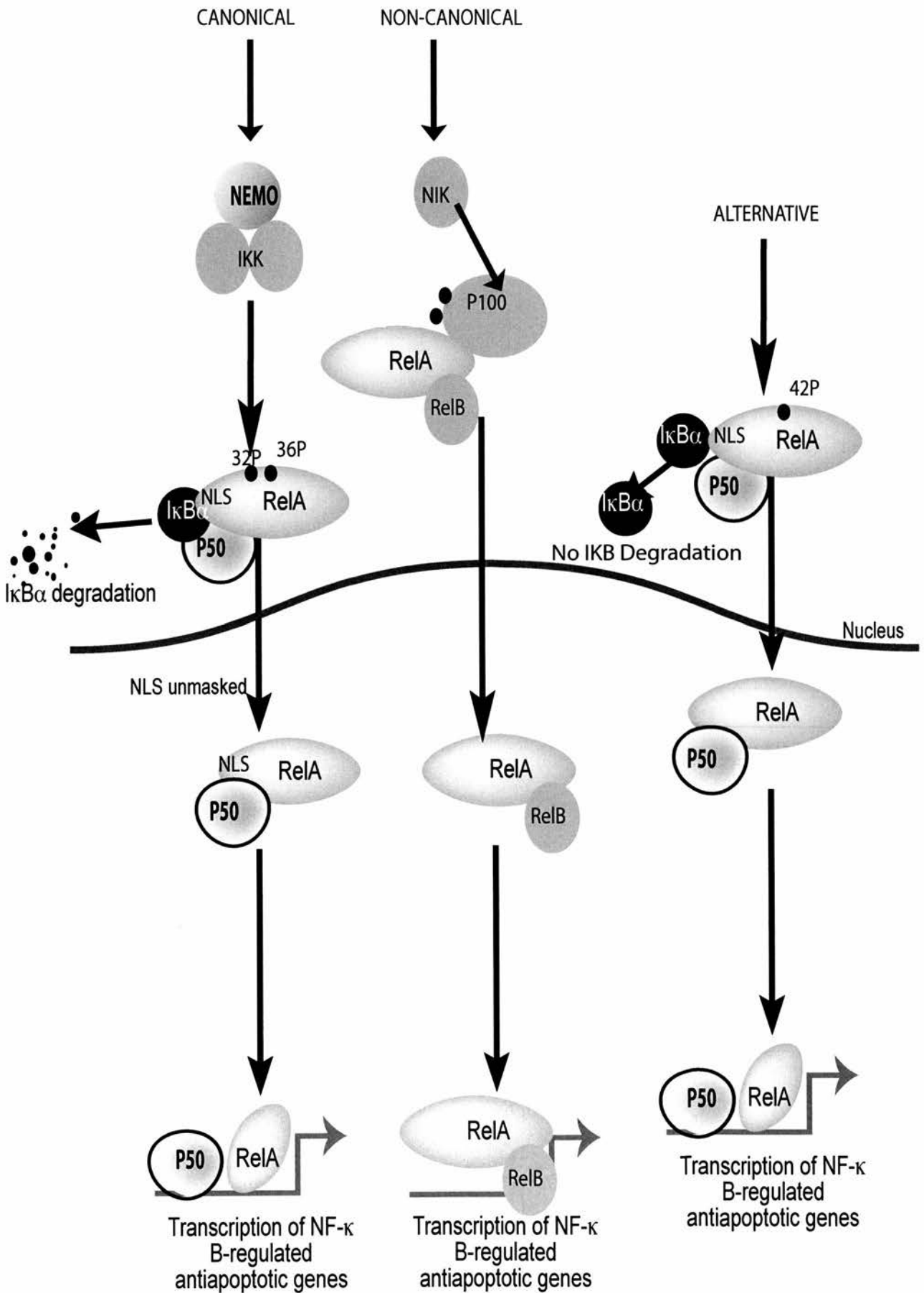
*Activation of NF- $\kappa$ B (see Fig 1.5)*

A large spectrum of environmental and cellular stimuli activate NF- $\kappa$ B signalling through a number of different upstream pathways. The most defined of these pathways is the classical or canonical pathway. When the canonical pathway is stimulated, a 700-900-kDa-protein complex, known as the I-Kappa B kinase (IKK) complex, phosphorylates I $\kappa$ B $\alpha$  at two critical serine (Ser) residues, Ser32 and which unmasks the NLS on RelA, allowing nuclear translocation of NF- $\kappa$ B. The IKK complex comprises IKK $\alpha$ , IKK $\beta$  and the non-catalytic regulatory scaffolding protein NEMO (IKK $\gamma$ ) (NF- $\kappa$ B Essential Modifier) (Karin and Ben-Neriah 2000). IKK $\beta$ /NEMO and IKK $\alpha$  have distinct signal transmission and disparate regulation. The proteins are phosphorylated at conserved serine residues within their activation loop by one or more of a large number of putative upstream kinases. Proteins proposed to act as IKK kinases include Caspase-8 (Skrzydowska et al 2005),  $\beta$ -catenin (Lamberti et al 2001), Cyclin D1 (Kwak et al 2005), HIF-2 $\alpha$  (Bracken et al 2005), NIK (Ling et al 1998, Regnier et al 1997), and c-Src (Huang et al 2003a), in diverse cellular environments and following various stimuli (reviewed in Scheidereit) (Scheidereit 2006).

The non-canonical or p100 processing pathway is activated by a limited number of stimuli and selectively requires IKK $\alpha$ . It is characterised by NIK (NF $\kappa$ B-inducing kinase)-dependent processing of the p100 precursor to p52 (Beinke et al 2004, Hayden and Ghosh 2004). Several physiological inducers of the non-canonical pathway are well known, including lymphotoxin A, BAFF (B-cell activating factor)), and CD40. These activate NIK/ IKK1 inducing p100 phosphorylation, ubiquitination and 26S proteasome proteolytic processing to yield p52. Activation is important for lymphoid organ development and the adaptive immune responses (Delhalle et al 2004).

The non-canonical pathway cross-stimulates the canonical pathway and the activation of both cascades are linked. However, the non-canonical pathway has slower activation kinetics, requiring hours to initiate a response following specific stimuli, compared to that of the canonical pathway, which is stimulated within seconds to minutes. (Reviewed in (Perkins 2007).

### 1.5 Models for NFκB signalling



An additional alternative activation pathway exists which has been shown to play a role in NF- $\kappa$ B activation following atypical agents such as UVB/UVC light, radiation, hypoxia, and certain chemotherapeutic agents (etoposides and anthracyclines (daunorubicin and doxorubicin)). In this response, both IKK-dependent and independent mechanisms of activation have been suggested (Campbell and Perkins 2004, Perkins 2004) (Campbell et al 2006, Perkins and Gilmore 2006). This pathway does not depend on utilising the IKK kinase function to phosphorylate the I $\kappa$ B protein. In some cases direct phosphorylation of alternative serine residues (UVC) (Kato et al 2003) or the use of the IKK complex as a scaffold structure only, occurs (Campbell and Perkins 2004, Perkins 2004)

Reports suggest that yet another route to activation of NF $\kappa$ B involving I $\kappa$ B $\alpha$  tyrosine 42 phosphorylation. This type of phosphorylation has been reported in response to pervanadate, (Fan et al 2003, Imbert et al 1996), redox stress (Schoonbroodt et al 2000), silica (Kang et al 2000, Kang et al 2006) and in some contexts, TNF $\alpha$  (Abu-Amer et al 1998). These routes of activation occur with slower kinetics and may or may not be associated with I $\kappa$ B $\alpha$  degradation (Campbell and Perkins 2004, Campbell et al 2006, Perkins 2007). The tyrosine kinase candidates proposed to be responsible for this response are p56<sup>lck</sup> (Imbert et al 1996, Livolsi et al 2001, Mahabeleshwar and Kundu 2003); c-Abl (Kawai et al 2002); Syk (Takada et al 2003) and c-Src (Abu-Amer et al 1998); (Fan et al 2003, Kang et al 2006).

RelA is known to possess a number of critical phosphorylation sites and can be directly targeted by a number of kinases. Phosphorylation affects several functions of RelA, including DNA binding and transactivation potential (Chen and Greene 2004). For example, phosphorylation of RelA at Ser276 by protein kinase A (PKA<sub>C</sub>) occurs in response to LPS, increasing RelA binding to cAMP response element-binding (CREB) protein (CBP)/p300 in the nucleus, resulting in increased acetylation and transactivation (Zhong et al 2002). Interleukin-1 (IL1) or TNF $\alpha$  induced activation of Protein kinase C- $\zeta$  (PKC $\zeta$ ) triggers phosphorylation of S311. TNF $\alpha$  and IL1 also induce phosphorylation of serine 529 through stimulation of casein kinase II (CKII). TNF $\alpha$  stimulates mitogen-and stress-activated protein kinase-1 (MSK1), resulting in enhanced NF- $\kappa$ B transcriptional activity (Vermeulen et al 2003b). S536 phosphorylation occurs following activation of IKK and the RelA c-terminus is also a

target of GSK-3 $\beta$  and PI 3-kinase (Chen and Greene 2004, Schmitz et al 2004, Schwabe and Brenner 2002). In summary, activation of the NF $\kappa$ B pathway is highly complex, involves a number of distinct upstream kinases and downstream co-regulators and is highly dependent upon the stimuli and the cellular context.

RelA is also regulated by reversible acetylation. For example, RelA can be acetylated on lysines 122 and 123 by p300/CREB-binding protein (CBP)-associated factor (PCAF) and p300/CBP respectively, which decreases the transcriptional activity of RelA by reducing the binding of RelA to the  $\kappa$ B enhancer. Conversely, acetylation of K221 by p300/CBP increases transcriptional activity and DNA-binding affinity of RelA for the  $\kappa$ B enhancer (Quivy and Van Lint 2004, Schmitz et al 2004)]. Histone acetyl transferases (HATs) acetylate histone tails, allowing access of transcription factors to promoters. NF- $\kappa$ B transcription depends upon coactivators, such as CBP/p300, p300/CBP-associated factor (PCAF) or steroid receptor coactivator-1 (SRC-1), which possess histone acetyl transferase (HAT) activity (Gerritsen et al 1997, Perkins et al 1997, Sheppard et al 1999, Zhong et al 2002) (Quivy and Van Lint 2004).

### **1.5.1 The role of the NF- $\kappa$ B transcription factor in CRC.**

Although NF- $\kappa$ B is involved in the regulation of both anti-apoptotic and pro-apoptotic genes, it is generally thought to have anti-apoptotic properties and contribute to carcinogenesis. This notion is based on a number of findings. Firstly, aberrant NF- $\kappa$ B activity is observed in numerous tumour cell types, including leukaemia, lymphoma, myeloma, breast and colon (Basseres and Baldwin 2006, Bharti and Aggarwal 2002, Kucharczak et al 2003).

In resected CRCs, NF $\kappa$ B is constitutively activated in the majority of tumour tissue, but not normal mucosa and activation correlates with tumour progression from adenoma to carcinoma and with tumour stage and invasion (Kojima et al 2004). In the majority of CRC cell lines, NF- $\kappa$ B is constitutively active, and has been postulated to regulate anti-apoptotic mechanisms, conferring resistance to radiotherapy and chemotherapy (Basseres and Baldwin 2006). However, whilst some cell

lines have increased promotion of survival as a result of NF- $\kappa$ B activation (Barger et al 1995), suppression of NF- $\kappa$ B signalling inhibits proliferation, causes cell cycle arrest, and leads to apoptosis. Genes that promote growth (c-Myc, p53, and COX-2) and inhibit apoptosis (BCL-XL and BCL-2) of CRC are regulated by NF $\kappa$ B. Furthermore, activation of NF $\kappa$ B confers resistance to radio and chemotherapy in CRC (Bharti and Aggarwal 2002).

Thus, the NF- $\kappa$ B signaling pathway is a strong potential candidate for the anti-tumour effects of NSAIDs in the colon (LaCasse et al 1998).

### **1.5.2 Aspirin effects on NF- $\kappa$ B activity in CRC**

Kopp and Ghosh were the first to report the inhibitory action of sodium salicylate and aspirin on NF- $\kappa$ B activity (Kopp and Ghosh 1994). They found that these agents inhibited cytokine-mediated degradation of I $\kappa$ B and nuclear translocation of NF- $\kappa$ B and concluded that inhibition of NF- $\kappa$ B signalling is a critical mechanism for the anti-inflammatory effects of the agents. Following these initial studies, it was suggested that salicylate-induced inhibition of NF $\kappa$ B nuclear translocation might confer sensitivity of cancer cells to apoptosis-inducing treatments or stress and there followed suggestions that salicylate-induced inhibition of NF- $\kappa$ B nuclear translocation might confer sensitivity of cancer cells to apoptosis-inducing treatments or stress (McDade et al 1999). Yin *et al.* (Yin et al 1998), subsequently reported that sodium salicylate and aspirin inhibited stimulus (TNF $\alpha$ , NIK, TAX or MEKK1) -induced activation of NF- $\kappa$ B by directly binding IKK $\beta$ , thus reducing its ability to bind ATP. They also found that sulindac and its derivatives inhibited IKK activity in colorectal cancer cells and suggested that this mechanism account for the anti-tumour properties of the agents (Yamamoto, 1999).

Following these initial studies there have been a wealth of papers suggesting that NSAIDs inhibit tumour cell growth by inhibiting activation of the NF $\kappa$ B pathway. However, in the majority of these studies, cells are pre-treated with aspirin or related NSAIDs for a short period prior to stimulation with a powerful cytokine such as TNF or IL-1. These experimental conditions are unlike those used to demonstrate the induction of apoptosis by NSAIDs, where colorectal cancer cell lines are treated with

NSAIDs for a prolonged period in the absence of any additional cytokines. Under these conditions, aspirin and related NSAIDs have been found to activate the NF- $\kappa$ B pathway. For example, Niederberger et al., found that celecoxib mediates activation of NF- $\kappa$ B through a direct interaction (Niederberger et al 2001). Cho et al. (Cho et al 2005), also demonstrated in some settings that diclofenac induced degradation of I $\kappa$ B $\alpha$ , supported by similar findings from Smartt et al., who reported the COX-2-selective inhibitor, NS-398 caused degradation of I $\kappa$ B $\alpha$  (Smartt et al 2003).

Studies from the host laboratory have confirmed the importance of activation the NF- $\kappa$ B pathway to the anti-tumour activity of NSAIDs in CRC. They demonstrated that aspirin (Stark et al 2007), sulindac, sulindac sulphone and indomethacin (Loveridge et al 2008) activate the NF- $\kappa$ B pathway by phosphorylation of I $\kappa$ B $\alpha$  at serine residues 32/36 causing degradation of the protein complex. Inhibiting nuclear translocation of NF- $\kappa$ B using a super-repressor I $\kappa$ B $\alpha$  (blocked from phosphorylation and degradation by mutagenesis), effectively blocked aspirin-induced cell death confirming that aspirin-induced apoptosis is due, at least in part, to nuclear translocation of NF- $\kappa$ B (Stark 2001). In subsequent studies they have shown that this NF $\kappa$ B response to NSAIDs is cell type dependent, as it was not observed in a panel of non-CRC cell lines (Din, 2004). However, it was found that the response was independent of basal I $\kappa$ B $\alpha$ , RelA and COX-2 expression levels and APC, beta-catenin, p53 or DNA MMR gene (specifically HMLH1) mutational status (Din FV 2005). These *in vitro* data have been supported by data from advanced models of colon cancer, including xenografted HT29 tumours in mice, APC $\pm$  mice adenomas (Stark 2007) and patient rectal biopsy explants (Stark LA 2001). These *In vivo* studies suggested that NF $\kappa$ B-mediated apoptosis in response to aspirin is much more accentuated in neoplastic tissue compared to normal mucosa (Stark LA 2007).

Following aspirin-induced RelA translocation to the nucleus, it localises in the subcellular/ nuclear compartment of the nucleolus (Stark and Dunlop 2005). This response was also noted in common with other pro-apoptotic known stimuli of NF $\kappa$ B, including UVC, serum withdrawal and radiation (Stark and Dunlop 2005). Nucleolar localisation of RelA following aspirin treatment is paralleled by increased apoptosis and a reduction in NF- $\kappa$ B driven transcription (Stark and Dunlop 2005), (Loveridge et al 2008). The host laboratory utilised site-directed mutagenesis to create a deletion

mutant of RelA (deletion of amino acids 27-30) which interfered with the nucleolar localisation signal (NoLS) and prevented its targeting to the nucleolus (Stark and Dunlop 2005). This had the effect of abrogating aspirin and NSAID-induced repression of NF $\kappa$ B-driven transcription and the induction of apoptosis (Loveridge et al 2008, Stark and Dunlop 2005). These data demonstrated the importance of nucleolar accumulation of RelA in NSAID-mediated apoptosis.

Recent work from the host laboratory (see above) has shown that activation of the p38 MAPK pathway is implicated in the NF- $\kappa$ B response to aspirin (Thoms et al 2007a). As mentioned above, it was shown that aspirin stimulates the p38 MAP kinase pathway within minutes of exposure and that this activation causes the phosphorylation and proteosomal degradation of cyclinD1. It was shown that specific inhibition of p38 signalling using chemical inhibitors or siRNA knock-down attenuated aspirin-mediated degradation of I $\kappa$ B, nuclear/nucleolar translocation of RelA and apoptosis in SW480 CRC cells, while mimicking cyclinD1 degradation, using a CDK4 inhibitor mimicked aspirin effects on NF $\kappa$ B signalling and apoptosis.

In summary, there is compelling evidence that aspirin induces Serine 32/36 phosphorylation and proteosomal degradation of I $\kappa$ B $\alpha$ , translocation of the RelA transcription factor to the nucleus then the nucleolus, causing repression of NF $\kappa$ B-driven transcription. There is also compelling evidence that this pathway is critical for the apoptotic effects of the agents in CRC cell lines. The data is further supported by experiments in other models of CRC cancer. However, the exact upstream mechanisms by which aspirin stimulates the NF $\kappa$ B pathway remain unclear. To achieve more targeted chemotherapeutic strategies, elucidating these upstream mechanisms is of vital importance.

## 1.6 c-Src and the NF- $\kappa$ B pathway; relevance to CRC Chemoprevention

As described above, aspirin induces a time dependent NF- $\kappa$ B signalling response in CRC cells *in vitro* and in colorectal neoplasia *in vivo*. However, this response has a significantly delayed kinetics occurring hours, rather than minutes after administration. Hence, it is therefore likely that aspirin acts through intermediate upstream kinases to induce the NF- $\kappa$ B response. One upstream candidate kinase is the proto-oncogene product, c-Src.

c-Src is upregulated and activated in CRC progression and metastases and c-Src activation is well-reported to induce NF- $\kappa$ B signaling in response to a wide variety of inflammatory mediators, bacterial stimuli and other chemical stimulants (Chang et al 2004, Fan et al 2003, Funakoshi-Tago et al 2005, Jalal and Kone 2006, Kang et al 2005, Lin et al 2006, Liu et al 2001, Lluís et al 2007, Mendez-Samperio et al 2007). The remainder of this thesis will therefore describe the rationale and experimental exploration undertaken to detect and understand the role of c-Src in the aspirin induced NF- $\kappa$ B signaling and apoptotic response in CRC.

### 1.6.1 c-Src structure and function

c-Src is the human cellular equivalent of the viral oncogene v-Src. v-Src was discovered by Peyton Rous in the late 20th century in a series of experiments describing the infective agent causing transmissible soft tissue sarcomas in chickens (named RSV (**R**ous **S**arcoma **V**irus)). The Src Family of tyrosine **K**inases (SFKs) have multiple cellular functions of importance to cancer, including control of proliferation, survival, migration and adhesion (Frame 2002, Schwartzberg 1998, Thomas and Brugge 1997). The SFK members include Src, Lyn, Fyn, Lck, Hck, Fgr, Blk and Yes and whilst some of the SFK members are broadly expressed (i.e. Src, Fyn, Yes) others have a more specialised tissue expression (i.e. Hck, Blk, Lck) (Thomas and Brugge 1997).

c-Src signaling affects cell growth and differentiation in the intestine, where it is thought to play a role in villin-induced regulation of cell migration (Mathew et al 2008). Previous studies have demonstrated that c-Src activity is elevated in colonic polyps, particularly malignant polyps and those greater than



2-cm that contain a villous structure and severe dysplasia (Cartwright et al 1990). Additionally, c-Src activity and expression is elevated in malignant and severely dysplastic epithelia in ulcerative colitis, and is 6-10-fold higher in mildly dysplastic than in nondysplastic epithelia. Thus, c-Src activity is elevated in premalignant UC epithelia and in polyps that are at greatest risk for developing cancer. The data suggests that activation of the c-Src proto-oncogene may be an early event in the genesis of CRC (Cartwright et al 1994).

Over-expression or activation of c-Src is believed to contribute to the progression of breast and CRC (Biscardi et al 2000). The activity of c-Src is increased in >80% of CRCs (Bolen et al 1987, Cartwright et al 1989, Talamonti et al 1993) and further increases are seen in metastases relative to primary tumours (Mao et al 1997, Termuhlen et al 1993). Furthermore, c-Src activity increases through progressive stages of the disease and is predictive of poor clinical prognosis (Maurer et al 2007). However, whilst c-Src activating mutations have been reported in some primary colon cancers (Irby and Yeatman 2000), other studies did not show correlation with CRC and c-Src mutations, thus activating mutations are rare. More common are those mutations that lead to increased expression rather than activity (Biscardi et al 2000).

c-Src is regulated by a complex series of phosphorylation and de-phosphorylation (see figure 1.5). c-Src dephosphorylating (inducing c-Src activation) proteins such as PTP $\alpha$ , SHP-1 and PTP1B have been detected at elevated levels in some cancers, including epidermal and breast. Conversely, proteins such as Csk and Chk that inactivate c-Src by phosphorylation of Tyr527 have been detected at reduced levels in other cancers and are thereby considered to have a tumour-suppressing ability. The complex mechanisms behind the regulation and activation of its tyrosine phosphorylation and kinase abilities is outlined in figure 1.4/1.5. An intricate auto-regulation has evolved amongst SFKs which allows for tight control of these functions. (Reviewed by (Boggon and Eck 2004, Frame 2002).

*Structure and activation (see figures 1.4 and 1.5)*

The structure of c-Src includes; a *SH4 n-Terminus*, a membrane targeting region, a *Unique Domain* (this varies amongst SFKs) and *SH1, 2 and 3 (Src Homology) Domains*. The conserved residue within

the *SH2 Domain* at Arginine 175 is critical to the phospho-tyrosine recognition process. In the auto-inhibited form, the SH2 domain is configured to bind to the phosphorylated Y537 on the c-terminal tail of c-Src and prevent any protein-protein interactions with c-Src substrates. However, it also contains a non-catalytic domain of about 100 residues which is functionally important in substrate protein recognition (Pawson 1995). This domain has two pockets, one that recognises the phosphorylated tyrosine and one that binds to one or more hydrophobic residues at the c-terminal of the target protein. The former is conserved and the latter has variety across the Src family members and accounts for differing substrates and functions of family members and their interactions downstream. The *SH3 (Src Homology 3)* domain in the auto-inhibited form binds to the linker region of the Type 2 helix between the Kinase and the SH2 domain. This domain again modulates protein-protein interactions with substrates, specifically, it contains a recognition site for proline-rich sequences.

c-Src kinase activity can be controlled by phosphorylation of the c-terminal Y527 or through protein-protein interactions in the *SH1 (Src Homology 1) domain*. Phosphorylation of the Y416 residue within the SH1 domain causes intra-protein conformational displacement and “opening up” of the kinase domain. When there is phosphorylation of c-terminal Y527 (phosphorylated by CSK (C-Src Kinase) or de-phosphorylated by SHP-1), inactivation occurs through the previously described interaction of SH2 with Y527. Direct protein/protein interactions are also important and both platelet-derived growth factor (PDGF) and focal adhesion kinase (FAK) are able to bind to the SH2 domain, causing c-Src to open up independently into the active form. The auto-inhibited c-Src is therefore a precariously set “mousetrap” (Boggon and Eck 2004) and small perturbations can be sufficient to spring the kinase into active conformation allowing the substrate access to its kinase site.

Figure 1.6 Src structure

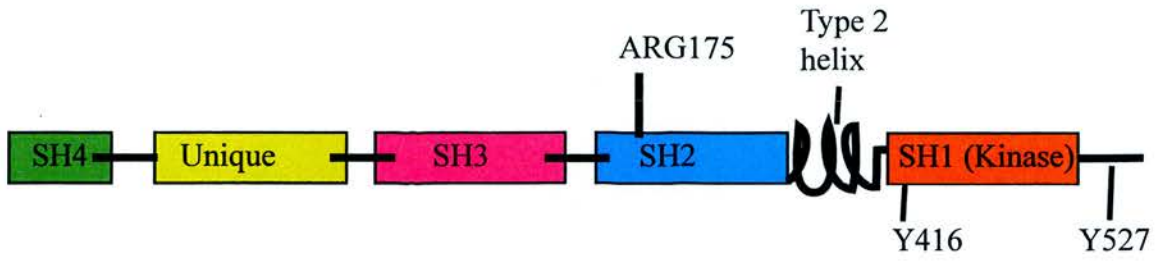
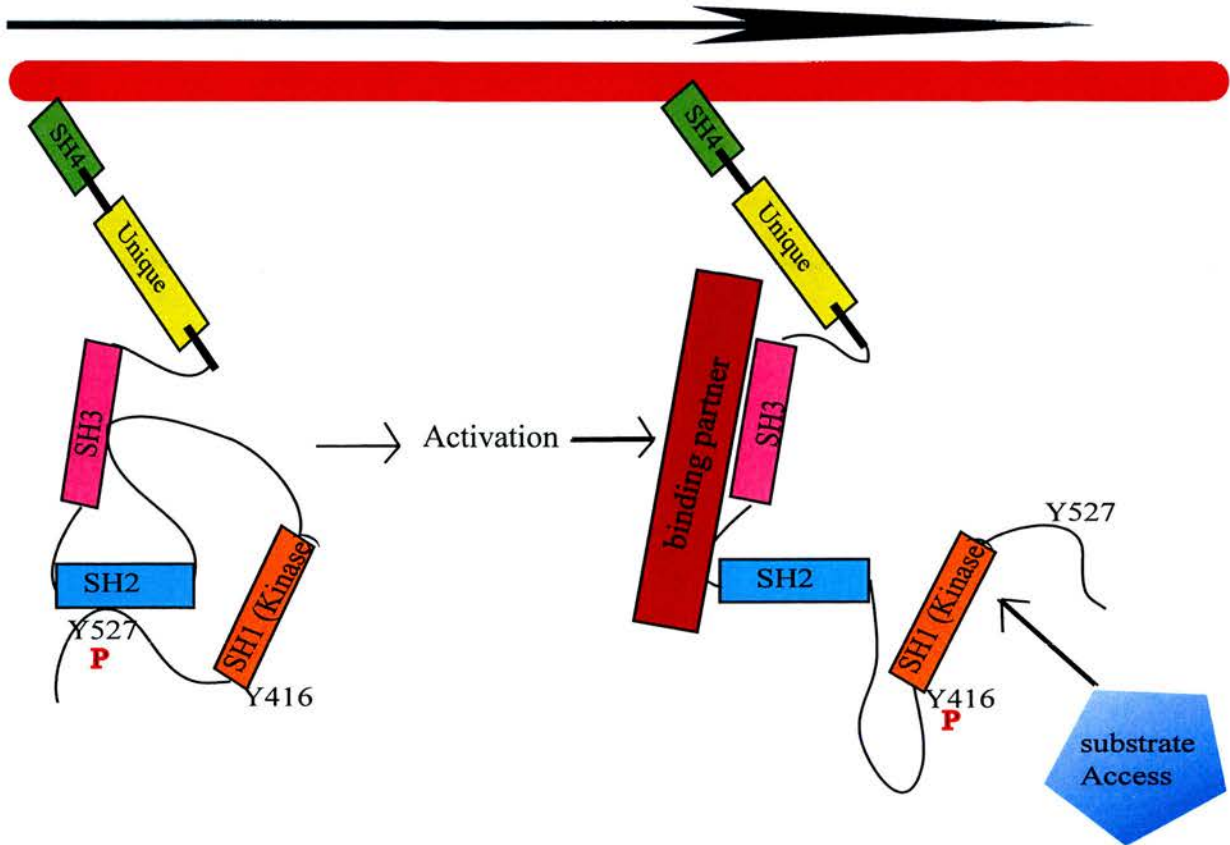


figure 1.7 c-Src activation and regulation: conformational opening

Protein tyrosine phosphatases, reduced CSK activity  
displacement of intramolecular interactions  
mutational activation (mutation or deletion of Y527)



(Modified from Frame MC)

### 1.6.2 Activation and substrate interaction with c-Src

c-Src is associated with the cellular membrane, the nuclear membrane and mitochondria. At the cell membrane, it is closely related to membrane receptors, especially EGFR and VGF receptors. Activation occurs by a number of molecular interactions with other kinases, such as via SH2 domain interaction with the tyrosine-phosphorylated receptor of PDGF (Kypta et al 1990), SH3/SH2 interaction with FAK (Thomas et al 1998) or activation of SH3, as in Hck's interaction with the HIV protein, Nef (Moarefi et al 1997). The ability of Src to be "turned on by touch" (Boggon and Eck 2004) is important for maintaining specificity and fidelity in initiating signalling cascades. Engagement of the c-Src SH3 and SH2 domains, with molecular ligands such as FAK, p130 (CAS) and Paxillin (proteins found in focal adhesions complexes), results in high affinity bonds with c-Src and activation the kinase by inducing conformational changes (Thomas et al 1998, Walter et al 1999).

Active dephosphorylation of Y527 forms an important mechanism for c-Src activation. PTP $\alpha$  is a transmembrane phosphatase, and its over expression has been shown to activate c-Src *in vivo* and can induce cellular transformation (Zheng et al 2000). PTP1B (SHP-2), a kinase that plays an important role in integrin signalling, has also been suggested as another possible candidate to activate c-Src via dephosphorylation of Y527 (von Wichert et al 2003). A list of some of the more investigated candidate substrates for c-Src are discussed in the next sections, with particular focus on NF- $\kappa$ B.

### 1.6.3 c-Src and FAK

FAK was first identified as a c-Src associated protein in v-Src transformed cells (Kanner et al 1991). Amongst the first clues that c-Src was involved in FAK/ integrin signalling was the localisation of temperature-sensitive v-Src to focal adhesions. The SH3 domain was fundamental to this localisation (Kaplan et al 1994). In contrast, the localisation of non-activated c-Src is primarily perinuclear and in cellular endosomes (Kaplan et al 1994, Schaller et al 1999, Timpson et al 2001). FAK contains phosphotyrosines and proline rich regions that bind to the SH2 and SH3 domains, respectively.

Following phosphorylation at FAK Y397, there is association with c-Src, resulting in increased protein tyrosine kinase activity of c-Src (Schlaepfer et al 1994). FAK tyrosine 397 residue is therefore

vital for initial recruitment of c-Src and following this, c-Src continues to phosphorylate FAK on 5 further tyrosine residues 406, 577, 576, 861 and 925. Whilst a degree of FAK phosphorylation is known to occur in the absence of c-Src, in c-Src negative cells FAK largely remains unphosphorylated. Authors suggest it is possible that the c-Src SH2 domain may stabilise the phosphorylation of FAK via direct interaction with the phosphorylated protein, thus protecting the FAK phosphorylated residues from cellular phosphatases (Brunton et al 2005). However, tyrosine 397 also serves as a binding site for PI3K, Grb7 and Phospholipase C gamma and therefore some competition of association between substrates may form part of cellular control in downstream signalling (Chen and Guan 1994). PI3K is a known regulator of apoptosis downstream of Growth Factor Receptor (GFR) signalling and has also been shown to bind to FAK 397. As Y397 is a c-Src/PI3K binding site, it is possible that c-Src plays a role in mediating this process (Howe and Juliano 2000).

c-Src dependent phosphorylation of FAK promotes detachment at the trailing edge of a migrating cell (Brunton et al 2005). Phosphorylation of FAK Y925 is dependent on c-Src kinase function and lack of phosphorylation at this site is associated with an inability of the cell to extend and retract protrusions associated with cell adhesions (Liu et al 2002). c-Src induced phosphorylation of FAK is linked with proteolytic cleavage of FAK by Calpain (Carragher et al 2001), which is required for both focal adhesion turnover and migration. c-Src located at peripheral cell adhesions therefore plays a key role in cell motility (Fincham et al 1995, Kaplan et al 1994).

FAK and c-Src interaction seems to play a key role in apoptosis avoidance and persists as long as cells are in contact with the extracellular matrix (Defilippi et al 1993). As part of a signal transmitted through FAK, c-Src and PI3 Kinase function to impair mitochondrial translocation of Bax and thus blocks apoptosis when cells are adherent (Gilmore et al 2000). c-Src associates directly with the p85 subunit of PI3 Kinase (Fincham et al 1995) and this association is sufficient to activate AKT, which is critical in inducing anoikis resistance (Windham et al 2002). Detachment of normal epithelial cells from the extra-cellular matrix leads to FAK dephosphorylation and dissociation of the FAK: Src

complex (Frisch and Francis 1994). This is followed by rapid Caspase 3-mediated, degradation of FAK, cessation of PI3 Kinase-mediated survival signals and anoikis (Weiner et al 1993).

#### **1.6.4 c-Src and the EGF Receptor Family**

Members of the EGFR family regulate differentiation, proliferation, survival, motility, angiogenesis and cancer initiation and progression (Holbro and Hynes 2004). c-Src physically associates with such receptors when they are activated (Maa et al 1995), and becomes transiently activated itself, phosphorylating downstream targets (Osheroov and Levitzki 1994). One target of c-Src activation is EGFR itself, which can be further phosphorylated on multiple residues, most notably Y845 (Biscardi et al 2000).

Phosphorylation of EGFR Y845 activates two signals; one that promotes EGF-induced cell proliferation through STAT 5B and another that enhances cell survival through upregulation of COX-2. Given that the phosphorylation of this residue is c-Src and not EGFR activation dependent, it allows for the possibility that cascades downstream of EGFR can be activated by stimuli that activate other receptors, which in turn activate c-Src. EGFR transactivation in response to integrins and GPCRs activation requires c-Src. Other stimulants of these pathways include clathrins and dynamin, proteins involved in the internalisation of multiple types of membrane receptors (including EGFR) and importantly regulate intracellular adhesions in colonocytes (Ishizawar and Parsons 2004) (see chapter 4).

### 1.6.5 c-Src and apoptosis

Activation of c-Src is primarily regarded as inhibiting apoptosis. For example, it inactivates the Caspase 8-APAF-apoptotic pathway (De Toni et al 2007), and blocks TNF $\alpha$  and NSAID induced apoptosis (Bernardi et al 2006, Park et al 2004). Reports also suggest reduced rates of apoptosis are observed in cells with high or activated c-Src (Irby and Yeatman 2000). v-Src (viral truncated form of cellular c-Src), the constitutively active form of c-Src, activates several kinases that can inhibit cell death, including ERK and PI3K (Johnson et al 2000). In rat intestinal epithelial cells, v-Src expression is sufficient to induce anoikis resistance via BCL-XL expression (Coll et al 2002). Additionally, it has been reported that TGF $\alpha$  inhibits anoikis through a c-Src mediated pathway (Fodde et al 2001a).

However, the consequences of increased cellular c-Src activity appears to be pleiotropic, and mimics the behaviour of the NF- $\kappa$ B pathway, i.e. the response of c-Src to either stimulate survival or apoptosis may be both cell environmental and stimulant specific. For example, c-Src induced c-Myc expression in other cell environments suggests a likely pro-apoptotic signal (Aziz et al 1999) and v-Src transformed cells are associated with loss of actin reorganisation and reduced cell-extracellular matrix adhesion, leading to cell rounding and cell detachment. (Fincham et al , Jones et al). In cells partially transformed by c-Src over-expression, sensitivity to apoptotic stress is increased (Zhong et al 2003). In CRCs with high innate c-Src activity, a PI3 Kinase inhibitor sensitises cells to anoikis and whenever the functions of Ras and PI3 kinase are inhibited, v-Src transformed Rat fibroblasts undergo apoptosis (Windham et al). This suggests v-Src generates a pro-apoptotic signal that under normal circumstances is counterbalanced by survival signalling through Ras and PI3 Kinase and is only revealed when the activities of these proteins are inhibited. v-Src expression can also down-regulate the CDK inhibitor p27, suggesting that deregulation of the cell cycle machinery by Src may be involved in the apoptotic process (Fincham et al 1995, Frisch and Francis 1994, Zhong et al 2002). Further details of c-Src and its relevance to apoptosis are described in the discussion of chapter 3.



### **1.6.6 c-Src and the NF- $\kappa$ B Pathway**

Many stimuli of c-Src-dependent pathways are classically also associated with NF- $\kappa$ B activation, and suggest a linkage/co-operation between these cellular pathways. c-Src is one of the many known upstream activators of NF- $\kappa$ B and members of the NF- $\kappa$ B family are well known as substrates of c-Src. What emerges is a complex picture of c-Src's multiplicity in relation to activation of the NF- $\kappa$ B transcription pathway and a multitude of cellular environments in which NF- $\kappa$ B signalling is dependent on upstream c-Src activation. It would be obviously advantageous, given the dysregulation of the NF- $\kappa$ B pathway and the upregulation of c-Src in many human cancers, to further investigate this linkage in the setting of CRC. These reports describe a multitude of cellular environments in which NF- $\kappa$ B signalling is dependent on upstream c-Src activation. It would obviously be advantageous, given the dysregulation of the NF- $\kappa$ B pathway and c-Src in many cancers, to further investigate this linkage. A further discussion of the crossover between the NF- $\kappa$ B and c-Src pathways can be found in the introduction of chapter 3.

### **1.6.7 c-Src and NSAIDs**

Initial evidence suggesting NSAIDs target c-Src was observed in the search for early c-Src chemical inhibitors. Ramdas et al. tested 43 compounds from 5 different chemical families, including NSAIDs, to evaluate their effect on the tyrosine kinase activity of c-Src (Ramdas and Budde). NSAIDs were demonstrated to confer inhibition of c-Src kinase activity. Many studies have examined the importance of c-Src in platelet responses, specifically in response to aspirin (one of the best known anti-platelet agents). Propylgallate is a platelet agonist that causes platelet aggregation, protein tyrosine phosphorylation and platelet factor 3 activities. It induces phosphorylation of c-Src in this process, but this phosphorylation is inhibited by aspirin, thus putatively accounting for some of the anti-platelet actions of the agent (Rao et al).

Several reports have suggested NSAIDs interferes with c-Src dependent molecular responses. For instance, treatment of bronchial cell lines with IL-8, which normally induces STAT through a c-Src dependent process, was inhibited after pre-treatment with NSAIDs (Perez-G et al). Additionally, angiotensin II and PDGF induced COX-2 production in cardiac fibroblasts, another process also



involving c-Src activation, is inhibited, by pre-treatment with salicylate (5-20mMol) (Wang and Brecher). Lu et al., examined the transformation of chicken embryonic fibroblasts to neoplastic cells following infection with v-Src. The authors reported that, in common with other studies, transfection of the cells with v-Src induced an independent apoptotic signal causing 11-23% of cells to undergo apoptosis. However, when they added diclofenac, a well-known NSAID, the level of apoptosis approached 100%. They concluded “NSAID-induced apoptosis” in neoplastic cells, “proceeds through a c-Src/Myc-dependent pathway” (Lu et al 1997).

Previous work from the host laboratory investigated effects of NSAIDs on c-Src and NF- $\kappa$ B signalling pathways in CRC. As part of her PhD project, Dr Loveridge performed a number of experiments with direct relevance to the rationale behind our onward investigation. In time-course experiments, CRC cells treated with sulindac, sulindac sulfone and indomethacin, demonstrate increased phosphorylation of c-Src Tyr 416 (signalling activation of the kinase) at 1-2 hours after NSAID administration. NSAIDs induced c-Src activation prior to degradation of I $\kappa$ B and nucleolar translocation of RelA suggesting a possible causal link. Chemical inhibition of c-Src activity (utilising PP2) blocked NSAID-mediated nuclear/nucleolar localisation of RelA. It was also found that cells expressing a kinase dead form of c-Src were resistant to NSAID-mediated apoptosis. These earlier data suggested NSAIDs activate c-Src in CRC cells and that this activation may be involved in the NF- $\kappa$ B and pro-apoptotic response to the agents. However, the effect of the most commonly used NSAID, aspirin, was not examined in these studies.

Together these data represent a compelling basis in which to further explore the role of c-Src in aspirin-related molecular effects in CRC. c-Src represents a dynamic kinase which is upregulated in CRC, is prognostically relevant and the regulation and control of which is reasonably well characterised. This kinase interacts with a diverse range of pathways of theoretical importance in carcinogenesis, and has reported roles in survival, migration and apoptotic signalling. Critically, this kinase is also known to function as an upstream agonist of NF- $\kappa$ B signalling activation in a variety of cellular settings and transmits molecular signals to the NF- $\kappa$ B signalling pathway through a variety of interactions with NF- $\kappa$ B members.

Additionally, c-Src plays a key role in the control of apoptosis and is known to be modulated by NSAIDs directly. Furthermore, preliminary data from the host laboratory has suggested evidence of a dependency between c-Src activation and NF- $\kappa$ B signalling in response to NSAIDs treatment of CRC cell lines. Taken together, these data provide compelling rationale for further studying the role of c-Src in the NF $\kappa$ B and apoptotic response to aspirin in colorectal cancer.

## 1.7 Experimental Approach

Initial work concentrated on characterising the response of the c-Src signalling pathway to aspirin in CRC cell lines. I utilised c-Src kinase assays and phospho-specific antibodies against known markers of c-Src activation to detect any agent-induced change to the activity or levels of c-Src. Furthermore, I evaluated other surrogate markers of c-Src activity, such as detecting the phosphorylation of substrate tyrosine residues and changes to cell adhesion dynamics. I attempted to characterise the kinetics of the aspirin-induced c-Src response, and its relationship to the known kinetics of aspirin-induced effects on the NF- $\kappa$ B pathway in CRC cell lines. I also attempted to detect physical molecular interaction between c-Src and NF- $\kappa$ B following aspirin exposure.

In order to evaluate if there is any dependency between c-Src activation and NF $\kappa$ B activation, I utilised various experimental tools, such as chemical and genetic interference with c-Src activity. Finally, given the importance of NF- $\kappa$ B signalling in aspirin induced apoptosis, and the known role of c-Src in other settings, I evaluate the c-Src dependency of the aspirin-induced pro-apoptotic response using the molecular and genetic tools above (chapter 3).

Initial experimentation above involves the use of CRC cell lines, including the SW480 CRC cell line, which is very well characterised in the host laboratory and in which a wealth of data has been generated indicating the cellular responses to aspirin. However, I also developed and studied a complex organ culture model that goes some way to re-capitulate the human situation *in vivo*.

Optimisation of an *in vitro* organ culture model of human *ex vivo* colonic mucosa with comparison and evaluation of previous culture methodologies was undertaken. Early investigation within the model was undertaken in order to decipher the molecular mechanisms controlling growth and differentiation. This has begun to allow me to explore the relevance of *in vitro* findings in the *in vivo* setting. Specifically, I evaluate the effects of aspirin, its influence on c-Src and NF- $\kappa$ B signalling pathways and apoptosis (chapter 4).

<b>Chapter 2 - Materials and Methods</b>	<b>94</b>
2.1 Cell Culture and methods	95
Summary of cell lines used	95
Maintenance of cell lines and media	95
Reagents (aspirin, other stimuli and inhibitors)	96
Treatment protocol	98
2.2 Generation and amplification of plasmids	98
Transformation of competent cells	88
Growth of transformed <i>E.coli</i>	98
Plasmid preparation	98
2.3 Transient transfection of adherent cells	99
2.4 <i>In-vitro</i> organ culture of human colonic mucosal explants	99
Harvesting	99
Maintenance	104
Reagents and Treatment protocols	105
2.5 Protein biology	105
Kinase assays	105
Preparation of cytoplasmic/whole cell extracts from cells/tissue	106
Protein concentration determination	107
2.6 SDS page	108
Western blot + stripping membranes	108
2.7 Annexin V –FITC assays	110
2.8 Imaging	112
Immunocytochemistry	112
Image analysis - quantification of nuclear RelA	112
2.9 Immunohistochemistry	114
Microscopy	117
Tissue damage scores	118

## **2.1 Cell Culture and Methods**

### **Summary of cell lines used**

SW480 cells were obtained from the American Type Culture Collection/European Collection of Animal Cell Cultures (ATCC/ECACC; number CCL-228). They are an adherent human CRC cell line that are derived from a Dukes' stage B colon adenocarcinoma from a male Caucasian aged 50 years. This cancer cell line expresses the oncogenes WT BRAF, WT PTEN, APC Q1338, WT beta-catenin, myc, ras and p53, but does not express COX and is also known to express low levels of c-Src. However, the aspirin-induced pro-apoptotic effect of interest (involving non-cox dependent pathways) has been well characterized in this cell line previously in the host laboratory and low level expression of c-Src was felt to offer an ideal environment in which to perform over-expression studies (further detailed in chapter 3.14). SW480 cells constitutively expressing a kinase-dead mutant form of c-Src (SW480-SrcKD) and equivalent control cells (SW480-pBpuro) were obtained as a kind gift from Prof. M. Frame (Cancer Research UK Beatson Laboratories, Glasgow). HT29 cells were also obtained from the American Type Culture Collection/European Collection of Cell Cultures (ATCC/ECACC; number HTB-38). They are an adherent human CRC cell line derived from a Dukes' stage B adenocarcinoma from a female Caucasian aged 42 years expressing constitutively high levels of c-Src and COX.

### **Maintenance of cell lines and media**

SW480 cells were maintained in L-15 medium (Gibco BRL), SW480-pBpuro and SW480-SrcKD cells were maintained in RPMI medium and HT29 cells were maintained in DMEM. All media were supplemented with 10% foetal calf serum (FCS) and 1% penicillin and streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. All cell lines were grown in 25 cm<sup>2</sup> or 75 cm<sup>2</sup> flasks (Greiner Bio-one) until a confluent monolayer was formed.

Cells were passaged or seeded for experimentation at a 1:3 → 1:5 dilution by washing in phosphate buffered saline (PBS) [137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 10 mM sodium phosphate dibasic (anhydrous) (Na<sub>2</sub>HPO<sub>4</sub>), 2 mM potassium phosphate monobasic (KH<sub>2</sub>PO<sub>4</sub>), pH 7.4], followed by a 5 minute (min) incubation with Trypsin/Versene (T/V)\* (1:1).

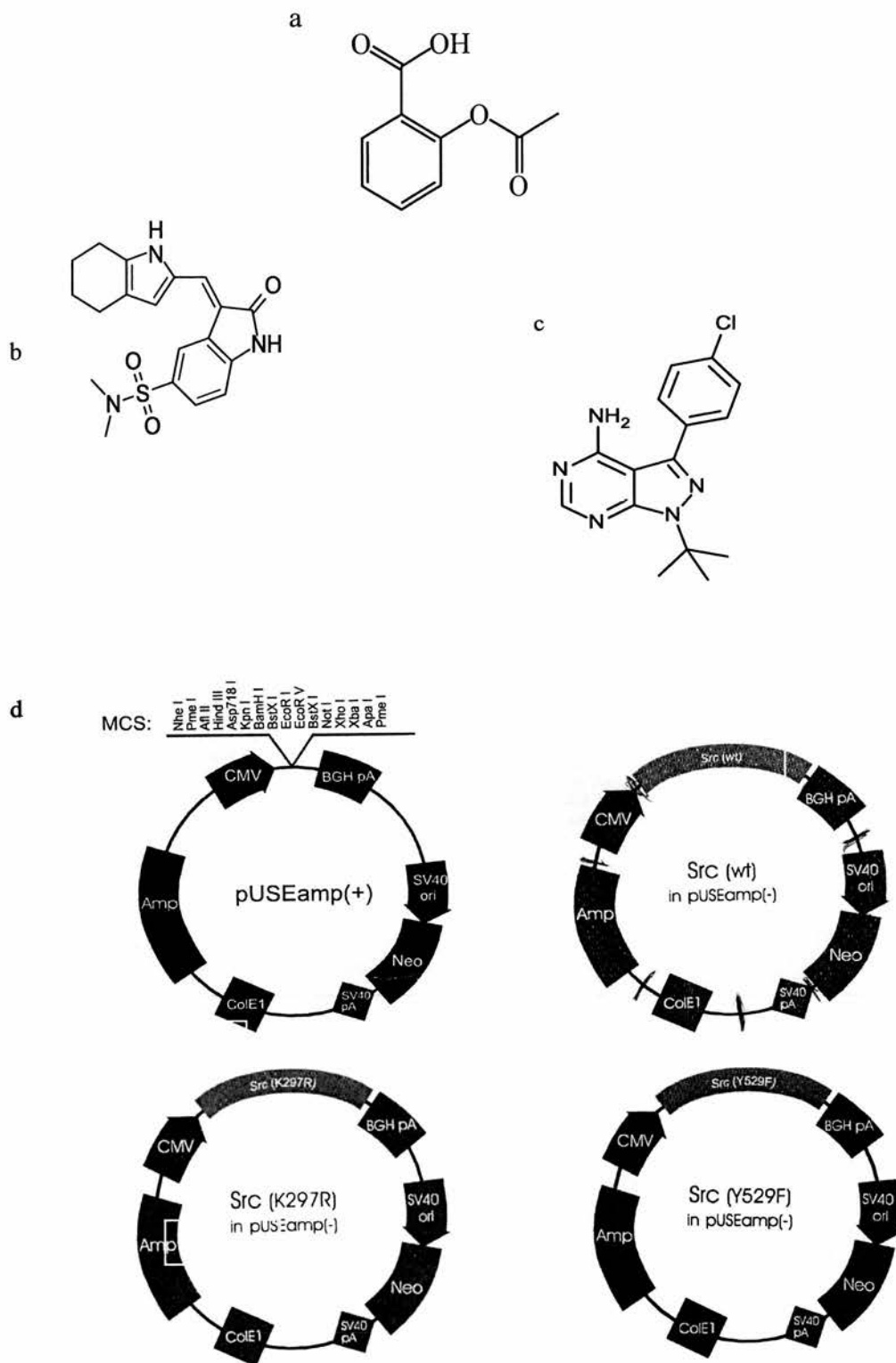
To freeze cell lines, 1 ml of appropriate cell culture media was added to detached cells prior to centrifugation at 200 x g for 5 minutes at room temperature. The media was then discarded and the cell pellet resuspended in 1 ml freezing media [10% dimethylsulfoxide (DMSO) in FCS] before being transferred to a cryotube. Cells were subsequently incubated on ice for 1 hour (hr) then stored in liquid nitrogen. To retrieve frozen cells, aliquots were rapidly thawed at 37°C, cells collected by centrifugation at 200 x g for 5 min, washed in culture medium then seeded into 25 cm<sup>2</sup> tissue culture flasks.

The modified cell lines were raised in RPMI (10%FCS, 1%Pen-strep, 0.75ug/ml puramycin (selection agent). The cells were defrosted at 37°C in a water bath then transferred to tubes with 10mls of above medium. The cells were spun at 1000rpm x 5mins and the supernatant was removed to exclude the storage solution. The cells were then re-suspended in 5mls of medium and transferred to a small flask for growth. On day 2 the medium was changed following successful appearance of growth and on day 2 the cells were transferred to a larger flask containing the above medium.

#### **Reagents (aspirin, other stimuli and inhibitors)**

Stock solutions of aspirin (Sigma) (0.5 M, pH 7.0), were prepared by solubilising the reagents in distilled water (dH<sub>2</sub>O), using NaOH to adjust the pH as required. All drugs were filter sterilized using a 0.2 micron filter (Schleicher and Schuell Microscience) prior to use in cell culture (See figure 2.1a for chemical structure of aspirin).

Stock solutions of PP2 (10µM)(4-Amino-5-(4-Chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) and the c-Src inhibitor SU6656 (0.5 µM) (2-oxo-3-(4,5,6,7-tetrahydro-1 H-indol-2-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide) were obtained as solid from Calbiochem and dissolved in DMSO prior to administration to cells at the concentration required (See figure 2.1b-2.1c for chemical structures of inhibitors). Epithelial growth factor (EGF) (Invitrogen, Carlsbad, CA) was obtained, dissolved in DMSO, and added to the cells as required (20ng/ml, 0.5hrs).



**Figure 2.1 Structure of reagents**

Chemical structures of 1. aspirin, 2. Su6656, 3. PP2.

4. plasmids used in transfection.

### **Treatment protocol**

The cells were plated at a density of  $0.2 \times 10^5/\text{cm}^2$  and were treated in 0.5% serum when they were 60% to 80% confluent. To study the effects of inhibitors on the response to aspirin, cells were seeded and grown as above. Once 60-80% confluent, cells were pre-treated with the required inhibitor in low serum medium for 2 hr prior to continuous treatment with aspirin as specified.

### **2.2 Generation and amplification of plasmids**

The following plasmids were utilised for transformation;

- Src cDNA (Constitutively activated) in pUSEamp (Y529F mutation) (Millipore)
- Src cDNA (kinase defective) in pUSEamp (K297R mutation) (Millipore)
- Src cDNA (Wild type) (Millipore)
- pUSEamp vector only (Millipore)

The plasmids used in these studies are summarised in figure 2.1d.

### **Transformation of competent cells**

In order to generate a sufficient quantity of plasmid, vectors were amplified in *E. coli*. Each of the plasmids used carried the gene for ampicillin, allowing selection of bacteria that were successfully transformed. A single transformed bacterial clone could then be grown in bulk and the required plasmid subsequently extracted. Initially, the vectors were transformed into *E. coli* that had been made chemically competent for transformation. The bacteria used were Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen Life Technologies) and the method as per the manufacturer's instructions. Briefly, competent *E. coli* cells were thawed on ice prior to transformation by the gentle addition of 10-15 ng of ligated plasmid DNA to approximately  $5 \times 10^6$  bacteria and incubating cells on ice for 30 min. Following heat shock at 42°C for 45 seconds, cells were incubated on ice for 2 minutes and 900 µl SOC medium (Invitrogen Life Technologies) [2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM magnesium chloride (MgCl<sub>2</sub>), 10 mM magnesium sulphate (MgSO<sub>4</sub>), 20 mM glucose] was added prior to incubation at 37°C for 60 min with vigorous shaking (225 rpm). An aliquot (100 µl) of transformed bacteria was plated onto L-agar (Luria Bertani Broth (L-broth)\* [0.1% tryptone, 0.05% yeast extract, 171 mM NaCl] containing 0.15% agar) plates containing the



appropriate antibiotic [L-agar-Amp\* (L-agar containing ampicillin (100 µg/ml)) and the plates were then incubated at 37°C overnight to allow colony formation.

### **Growth of transformed *E.coli***

Following transformations, a single transformed *E. coli* colony was picked from the plate and grown in 2 ml L-broth containing the appropriate antibiotics [ampicillin (100 µg/ml)] overnight at 37°C with vigorous shaking (225 rpm). An aliquot (500 µl) of this culture was used to inoculate 500 mls of L-broth, containing ampicillin, which in turn was grown overnight to allow large scale amplification of the plasmid in *E. coli*. Transformed *E. coli* colonies were stored on L-agar plates at 4°C for up to one month or at -70°C in L-broth containing 50% glycerol for longer-term storage. Strains were re-isolated from glycerol stocks by spreading some of the frozen cells using a sterile loop onto an L-agar plate containing the appropriate antibiotic and then incubating the plate overnight at 37°C.

### **Plasmid preparation**

Plasmids were harvested from bacterial cells using a purification kit (Qiagen) and reagents provided by the manufacturer according to their instructions. Briefly, cells were collected by centrifugation at 6,000 x g for 15 min at 4°C. The cell pellet was then resuspended in 10 ml chilled buffer P1 [10 mM ethylenediaminetetraacetate (EDTA), 50 mM Tris-(hydroxymethyl)-methylamine (Tris)-HCl (pH 8.0), 100µg/ml ribonuclease A (RNase A)] and subsequently lysed with 10 ml buffer P2 [200mM NaOH, 1% sodium dodecyl sulphate (SDS)] to allow disruption of the bacterial cell wall. This alkaline lysate was neutralised with 10 ml chilled buffer P3 [3.0 M potassium acetate (C<sub>2</sub>H<sub>3</sub>KO<sub>2</sub>) (pH 5.5)] and then incubated on ice for 20 min. Cell debris was removed by centrifugation at 20,000 x g for 30 min. The soluble fraction (including the plasmid DNA) was applied to a pre-equilibrated QIAGEN-tip 500, which contains a specific Anion-Exchange resin. The plasmid DNA adhered to the resin whilst the remaining debris, such as RNA, protein and metabolites, was removed by washing the tip with 60 ml buffer QC [1.0 M NaCl, 50 mM 4-morpholinepropanesulfonic acid (MOPS) (pH 7.0), 15% isopropanol]. The plasmid DNA was detached from the resin by the addition of buffer QF [1.25M NaCl, 50mM Tris-HCl (pH 8.5), 15% isopropanol], precipitated with isopropanol and immediately pelleted by centrifugation at 15,000 x g for 30 min at 4°C. The DNA was then washed with 70%

EtOH, re-pelleted by centrifugation at 15,000 x g for 10 min at 4°C and allowed to air-dry before being dissolved in 300 µl TE buffer [10mM Tris-HCl (pH 8.0), 1mM EDTA] and stored at -20°C.

Plasmid DNA was quantified by optical densitometry on a UV spectrophotometer (GeneQuant Pro, Biochrom Ltd.). 1 µl DNA was diluted with 99 µl dH<sub>2</sub>O and placed in quartz cuvettes. DNA quantities were calculated automatically from the measured absorbency at 260 nm ( $A^{260}$ ) and 280 nm ( $A^{280}$ ), taking into account the dilution factor of 100. An  $A^{260}/A^{280}$  ratio of 1.8 was taken as optimum purity of DNA.

### **2.3 Transient transfection of adherent cells**

Cells were grown in 25 cm<sup>2</sup> flasks or on sterile cover slips in the wells of 6 well plates until 50-60% confluent prior to transfection. Transient transfection of cells with plasmid DNA was carried out using a Lipofectamine<sup>TM</sup> protocol (Invitrogen Life Technologies) as per the manufacturer's instructions. Briefly, for each transfection, 20 µl lipofectamine was added to 500 µl optimem (Gibco BRL) and separately 500 µl optimem was added to the 8µg plasmid DNA. Each solution was incubated separately for 5 minutes then combined at room temperature and incubated for a further 20 minutes. Cells were washed twice with 2 ml optimem before 3 ml of serum free media was added. 1ml of cDNA / lipofectamine /optimem mix was then added. Cells were then incubated for 24 hrs at 37°C. After this incubation, the cells were washed with low serum medium and grown for a further 24 hours before being harvested or treated .

### **2.4 In vitro organ culture of human colonic mucosal explants**

#### **Harvesting**

Ethical approval and permissions were obtained from the Lothian Research Ethics Committee and the NHS Research and Development office, Lothian University Hospitals Division (LUHD) (06/F1101/16 & 01/MREC0/5). Patients were identified from those undergoing colonic resection for colorectal cancer or polyps through prior consultation with the patient's colorectal surgical consultant (see acknowledgements). Operative resection was performed at the Western General Hospital, Edinburgh, Scotland and IVOC (*In Vitro* Organ Culture) was maintained on campus at the Medical

Research Council's Human Genetics Unit (MRC HGU), Western General Hospital, Edinburgh, Scotland.

Prior to surgical resection, patients were identified, fully informed and provided written consent. An anonymous code was generated by the study investigators and used to label the sample from the time of resection. Matched patient details were stored securely but blinded from MRC HGU investigators by the study data guardians at the ECMC.

At the time of colonic resection, the resected colonic specimen was immediately transferred to the investigators. The colon was kept at room temperature throughout the harvesting process. It was then transported within minutes from the operating theatre to the Department of Pathology, Western General Hospital. Before and after the tissue dissection process all surfaces and equipment were decontaminated with vircon and universal precautions were used to prevent cross-contamination and risk of infection.

In the case of retrieving normal colonic mucosa, the colon was sectioned longitudinally to expose the colonic mucosa. Pathological areas were noted and prognostically/diagnostically important areas were excluded from the harvesting process. The colonic lumen was initially gently rinsed with water to remove superficial debris. A 5x 5 cm section of non-pathogenic colonic mucosa was separated by sharp dissection with a scalpel from the underlying muscularis layers.

In the case of colonic polyps, the pathologist examined the colon and provided non-pathologically/diagnostically relevant polyps, by dissecting the polyp by sharp dissection from the underlying muscularis layer. In the case of CRC, a section of non-prognostically/diagnostically relevant tumour was separated from the tumour mass by the pathologist using sharp dissection.

At the time of retrieval, all colonic specimens were then rinsed briefly x 3 in sterile water and then immediately transferred to a universal container containing pre-warmed transport culture media (see

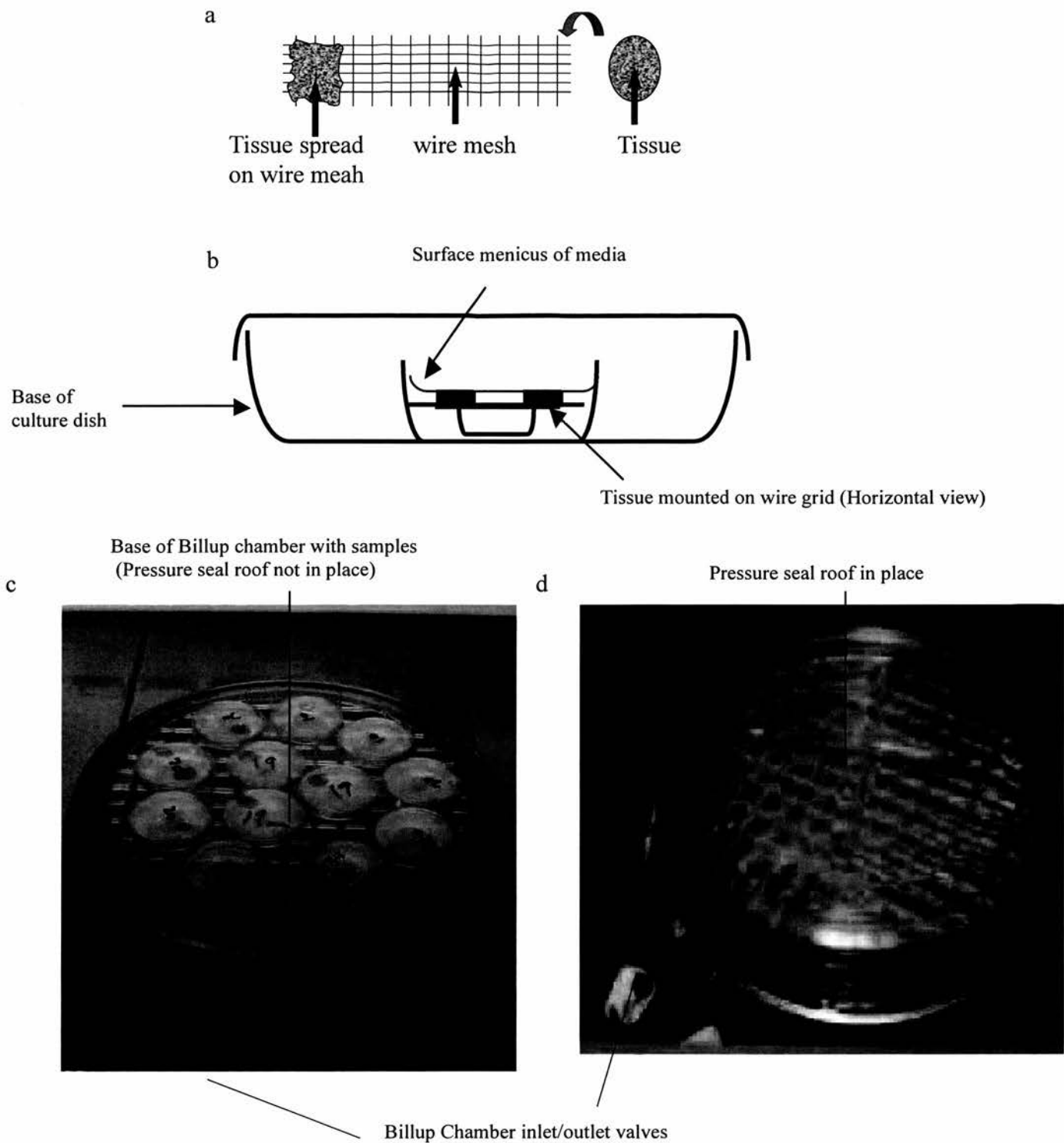
below) at 37°C. The residual non-harvested colonic specimen was placed in formalin and entered the normal clinical and diagnostic standard of care protocols for resected colonic specimens.

The universal container was placed on ice within a sealed, marked biohazard box and transferred to the IGMM within 10 minutes by trained investigators or study nurses. There, the sample was transferred directly to Containment Level 2 facilities where awaiting investigators took the process further. The sample details (tissue type, location of colon, time since resection, anonymous identifier) were recorded on arrival.

Under CL2 conditions, the sample was removed from the universal tube. Under sterile conditions, using a scalpel, explants were then cut into smaller sections of roughly 0.5 cm x 1 cm in size. Explants were then gently drawn across and mounted on sterilised porous plain weave stainless steel wire gauze (0.224mm diameter pores) (United Wire, Edinburgh) and placed submerged within culture dishes containing pre-warmed media at 37°C (BD Organ culture dishes 60 x 15 mm, [www.Scientificlabs.co.uk](http://www.Scientificlabs.co.uk)). The explant culture was then loosely covered and placed with others in a sealed modular incubator container (Billups-Rothenberg, Del Mar, CA). The container was purged with 95% oxygen/5% CO<sub>2</sub> (BOC) for 2 minutes. The sealed container was then incubated at 37°C and gently horizontally rotated at 5 cycles per minute (see figure 2.2 for details of in-vitro culture set-up).

The gas and media phase were changed daily. At the time of explant harvest, explants for use in histology were rinsed briefly in PBS and then placed initially in 4% Formaldehyde solution (Sigma) overnight prior to transfer to 70% alcohol for longer term storage until later fixation in paraffin blocks. Explants for use in protein analysis were taken from culture, briefly rinsed in PBS and then placed immediately in dry-ice prior to storage at -80°C.

Waste material and contaminated materials/ consumables are placed in Virkon™ solution overnight and then marked for disposal for incineration the next day in accordance with strict protocols for biohazard waste disposal within the unit.



**Figure 2.2 IVOC set-up**

- a. Stretching out mucosa on metal grid - view from above.
- b. Side view of explant set-up.
- c. View of multiple explants within billup chamber (central explants were infected).
- d. View of sealed billup chamber with roof apparatus in place.

and recorded in the register. All material not used or superfluous to demand must be returned to storage or disposed of with disposal recorded in the register.

### **Maintenance**

#### **Medium**

Non-supplemented (serum-free) RPMI media (Gibco), modified CMRL media (Gibco) (Glucose 15 mM, methionine 7 micromolar, tricine buffer 20Mm, hydrocortisone hemisuccinate 1.5 micromolar, (retinyl acetate 1 microgram/ml, glutamine 3 mM, 5% Bovine Serum Albumin, 1.5% DMSO, Penicillin 100 units/ml, streptomycin 100 units/ml, gentamicin 50 micrograms/ml, amphotericin 0.25micrograms/ml) (Autrup 1980) or modified Waymouth's media (Gibco MB752/1) (Foetal Calf serum 10% (Gibco), ascorbic acid 300ug/ml (Sigma A4544), Hydrocortisone-21-sodium succinate 3.0 microg/ml (Autogen Bioclear cat no K3520), ferrous sulphate 4.5µg/ml, penicillin/ streptomycin 100 units/ ml (Gibco), antimycotic/antibiotic mix 1/100 (Sigma)) (Senior et al 1982) were used to culture colonic mucosal explants. Explants were transported to the laboratory in 50 mls of their respective long-term culture media. When added, lithium chloride (LiCl<sub>2</sub>)(0.5mM) was added as a supplement to Modified Waymouth's media on a daily basis throughout the culture process and timelines (Li et al 2011). Dibenzazepine (DBZ) (10µM) (Calbiochem) was added once daily for 5 days prior to harvest (Ootani et al 2009). DMSO was added as a carrier control in these experiments.

#### **Medium changes**

On a daily basis, inside a class II microbiological safety cabinet (msc), fresh, labelled organ culture dishes containing 3mL pre-warmed medium was prepared. Any additional supplements (e.g. drug treatments) were added at this stage using sterile filter tips and all stocks removed from msc to reduce risk of cross contamination.

The Billups chamber was gently removed from heated shaker/incubator and placed in msc. Pressure was slowly released using inlet/outlet clips and the metal securing clip was opened. Culture dishes were removed individually and place on a sterilised metal tray inside the msc. Any samples to be 'harvested' were set aside. Using fine forceps, pre-sterilised in 15ml Barrycide and rinsed in 2 x

50mL sterile PBS (Lab services), gauzes were transferred to the appropriately-labelled fresh dishes. After completion, the samples were placed inside the Billups container and resealed. The container was purged with 100% oxygen (BOC Cat No 299031 AV-PC 95% oxygen 5 % CO<sub>2</sub>) for 20 secs the chamber was transferred to the incubator shaker as above. Samples for harvest were either transferred to PBS/formaldehyde 4% or -80 °C for storage.

### **Reagents and treatment protocols**

Lithium chloride (LiCl<sub>2</sub>) (Sigma) (0.5mM) was added as a supplement to Modified Waymouth's media on a daily basis throughout the culture process timelines (Li et al., 2011a). Dibenzazepine (DBZ) (10µM) (Calbiochem) was added once daily for 5 days prior to harvest (Ootani et al., 2009). DMSO was added as a carrier control in these experiments. Aspirin 0.5M stock was prepared as before and added at the concentration specified.

## **2.5 Protein biology**

### **Kinase assays**

In the KineActive ELISA method, a cellular extract is added to the wells of an antibody-capture plate. This is followed by addition of a kinase-specific antibody, which binds to the plate and captures the kinase of interest (c-Src). After the unbound proteins are washed away, a biotinylated, unphosphorylated peptide substrate is added in the presence of ATP. The immobilized kinase phosphorylates this substrate peptide, which is subsequently also captured by the addition of a second antibody that is specific for the phosphorylated form of the substrate peptide. Addition of streptavidin-HRP and subsequent developing solution provides a quantitative colorimetric readout that is directly proportional to the activity of the kinase

Detection of the kinase activity was performed using the KineActive Src kit (Invitrogen) manufacturer's instructions. Briefly, cells were treated as above. At harvest, cells were washed with 8 mls ice cold PBS x 2. Cells were scraped into 3 mls ice cold PBS then centrifuged at 1000 rpm x 10mins at 4°C. 3x volume of Complete Lysis Buffer was added with additional 1ul DDT + 10 ul

Protease inhibitor/ ml of Lysis buffer was also added. The mixture was mixed with a pipette several times and incubated for 30 mins. Following this, the mix was again centrifuged for 20 mins at 14,000 rpm at 4 degrees and supernatant was collected and stored at -80°C with a Bradford assay performed to detect protein concentration.

Assay strips were prewashed with 3 x 200ul of wash buffer, dried and the diluted capture antibody was added. The protein sample (20µg) was added and incubated for 1.5 hours at room temperature on a shaking platform (60 rpm). The kinase mix was prepared; including 1:500 antibody, 1:500 peptide substrate and 5x ATP buffer 1:5 in dH<sub>2</sub>O. The plates were washed after incubation with 5 x 200 ul of wash buffer. 100ul of kinase reaction mix was added to each well and the mixture was further incubated for another 1.5 hours at 30°C with agitation. After incubation the wells were washed x 5 with 200ul of wash. 100ul of Streptavidin: HRP antibody solution was added and plates incubated for 30 mins at room temperature with gentle agitation. One final round of washing 5x200ul of wash buffer was performed before developing the colour. 100ul of developing solution was added and the mix incubated for 10 minutes (protected from sunlight) until the colour was moderate to dark blue. 100ul of the Stop solution was added turning the solution yellow and the absorbance was read within 5 minutes on a spectrometer at 450 nM with optimal reference wavelength of 655 nm.

#### **Preparation of cytoplasmic extracts from cells**

Treated cells were washed with 2ml PBS prior to harvesting by scraping the cells into 5ml PBS. Cells were subsequently pelleted by centrifugation at 1000 rpm for 2 min and then lysed for five min at 4°C in approximately 3 X cell volume of lysis buffer [50 mM NaCl, 10 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) (pH 8.0), 500 mM sucrose, 1mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.2% triton X-100] containing Complete™ protease inhibitor cocktail (Roche Applied Science) at 1:1250 dilution, 1 mM pepstatin A (Sigma) and 100 mM Pefabloc SC (Roche Applied Science). Phosphatase inhibitors [100 µM sodium fluoride (NaF), 10 mM β-glycerophosphate, 10 mM Na<sub>3</sub>VO<sub>4</sub>] were also added where appropriate, that is for the analysis of phosphorylated proteins. The mixture was then further centrifuged at 6000 rpm for 20mins and the



supernatant was transferred to fresh eppendorfs and the protein concentration of the samples was measured with storage subsequently at  $-20^{\circ}\text{C}$ .

#### **Preparation of whole cell extracts from cells**

Treated cells were washed with 5ml PBS prior to harvesting, which was then discarded. Then cells were scraped into 1 ml PBS and placed on ice. Cells were subsequently pelleted by centrifugation at 13,000rpm for 15 secs. The supernatant was then removed and cells were incubated for 30 minutes on ice following resuspension in approximately 3 X cell volume of lysis buffer [100uL/mL Cell Lysis Buffer x10 Cell Signalling #9803, 40uL /mL Protease Inhibitor Complete Roche # 11697498001 x25 in dH<sub>2</sub>O, 1uL /mL Pepstatin A Santa Cruz sc-45036, 20ul / mL (10 mM) Sodium orthovanadate (Sigma s-6508) 500mM stock, 10uL / mL (10 mM)  $\beta$ -glycerophosphate (Sigma g-6251), 1 uL/mL (100  $\mu\text{M}$ ) Sodium Fluoride (Sigma s-7920), 10uL/mL PEFA Bloc SC, AEBSF (4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride 100mg/ml stock in H<sub>2</sub>O (Sigma 76307). 10 uls PMSF was added for every 1 ml of buffer. The mixture was vortexed occasionally throughout the incubation. Cells were then pelleted at 13,000 rpm for 5mins at  $4^{\circ}\text{C}$ . The supernatant was transferred to fresh eppendorfs and the protein concentration of the samples was measured with storage subsequently at  $-20^{\circ}\text{C}$ .

#### **Preparation of whole cell extracts from tissue**

Tissue was removed from  $-80^{\circ}\text{C}$  freezer and samples were cut to size on dry ice. The samples were rinsed with ice cold PBS and diced into pieces using a sterile scalpel. The samples underwent 3 cycles of freeze-thawing and were homogenised by use of a mini homogeniser. The homogenate was then centrifuged at 13,000rpm 5 min at  $4^{\circ}\text{C}$ . The supernatant was then collected and stored on ice.

#### **Protein concentration determination**

Bradford Reagent (Bio-RAD) was diluted 1:5 with dH<sub>2</sub>O and filtered using Whatman filter paper (27.0 cm) prior to use. 1  $\mu\text{l}$  protein sample was added to 200  $\mu\text{l}$  Bradford Reagent in the wells of a 96 well plate. A concentration curve was included in each experiment using BSA standards (0.2  $\mu\text{g}/\mu\text{l}$ , 0.5  $\mu\text{g}/\mu\text{l}$ , 0.8  $\mu\text{g}/\mu\text{l}$ , 1.0  $\mu\text{g}/\mu\text{l}$ , 1.5  $\mu\text{g}/\mu\text{l}$  and 2.0  $\mu\text{g}/\mu\text{l}$ ). The standards were loaded in duplicate and

samples loaded in triplicate. Following a 10 min incubation at room temperature, the absorbance was measured at 595 nm ( $A^{595}$ ) using an ELISA (enzyme-linked immunosorbent assay) plate reader. The protein concentration was calculated from the  $A^{595}$  automatically by the plate reader, taking into account the standards. The average of the 3 readings for each sample was determined and subsequently used.

## **2.6 SDS Polyacrylamide Gel Electrophoresis (PAGE)**

A tenth of the volume of Sample Buffer [20% glycerol, 2% SDS, 0.25% bromophenol blue, 1 X Stacking Buffer [500 mM Tris, 0.4% SDS, pH 6.8], 5%  $\beta$ -mercaptoethanol] was added to protein samples (10-30  $\mu$ g) prior to boiling at 95°C for 5 min and then placing on ice. The Protean II mini-gel apparatus (Bio-RAD) and a two-tier gel system, comprising a lower resolving gel and upper stacking gel, were used to resolve protein extracts by denaturing SDS PAGE. c-Src is 62 KDa, therefore, an 8% polyacrylamide was used.

*Resolving Gel;* 4.02ml-distilled H<sub>2</sub>O, 1.48 ml 40% Acrylamide, 1.9 mls, 4x Resolving Buffer, 112 ul 10% APS and 5 ul of TEMED)

*Stacking gels* were; 1.081mls of Distilled H<sub>2</sub>O, 0.444 ml of 4X Stacking Gel, 0.218 mls of 40% Acrylamide, 28ul of 10% APS, 5ul of TEMED). 10 $\mu$ l of Kaleidoscope pre-stained molecular weight standards (Bio-RAD) were loaded in a single well of each gel and run in parallel with protein samples to aid the identification of proteins of interest. Gels were electrophoresed in 1 X Running Buffer [25 mM Tris, 0.2 M glycine, 0.1% SDS] at 160 volts for approximately 1 hr.

## **Western blot**

Antibodies used for Western blot analysis and details of the host species they were raised in, dilutions, incubation time and manufacturer are summarised in Table 2.1. Western blot analysis was carried out using standard procedures. Briefly, protein extracts were resolved on SDS PAGE gels as described in section 2.7. Proteins were then transferred to Hybond PVDF membrane (Bio-RAD) using a semi-dry

blotter (Bio-RAD) with 1 X semi-dry transfer buffer [47 mM Tris, 40 mM glycine, 0.037% SDS, 100 mM methanol]. Gels were included in a stack which included 3x Whatman blotting paper 7x9cm layers and 1xHybond PVDF membrane 7x9cm, 1x gel then 3xWhatman blotting paper 7x9cm. Gels were run at 12 volts for 45 minutes as per manufacturers instructions. The membrane was then blocked in TBS (1 L Distilled H<sub>2</sub>O, 2.42g Tris base, 8.77g NaCl pH 7.4) containing 0.1% TWEEN<sup>®</sup> 20 (Sigma) (TBST) plus 5 % dried milk (TBSTM) for 1 hr at room temperature or overnight at 4°C prior to the addition of primary antibody for 2 hr at room temperature or overnight at 4°C (See table 2.1). The membrane was subsequently washed with TBST (3 X 10 min) before being incubated with the appropriate secondary antibody conjugated to horseradish peroxidase for 30 min at room temperature (See table 2.8). The membrane was then washed with TBST (3 X 10 min) before detection of specifically bound antibody by chemiluminescence. ECL reagents (Santa Cruz Biotechnology) were mixed 1:1, applied to the membrane for 1 minute, excess removed, then the membrane covered with cling film and exposed to Hyperfilm (Amersham Biosciences) for the time as appropriate for the antibody signal to be captured. Films were subsequently developed using an SRX-101A X-ray processor (Konica Minolta, Medical + Graphic Inc.).

### **Stripping Membranes**

Membranes were stripped in a fume hood with 50 ml strip buffer [62.4 mM Tris, 2 % SDS, pH 6.7], which was pre-warmed in a glass dish to 60°C using a heat block. One or two membranes were placed into the buffer and 400 µl β-mercaptoethanol added and gently mixed prior to heating at 60°C for a further 30-45 min. The strip solution was then discarded and membranes washed with PBST (3 X 10 min) before being re-blocked in PBSTM for 1 hr or overnight at 4°C. Membranes were then re-probed with primary antibody as above.

Whilst it is possible to quantify western blot output by densitometry methods, the output from this assay is by definitely qualitative in nature. Recent reviews of densitometry have heavily criticized this methodology for inaccuracy, openness to bias and lack of uniform protocols (Gassman 2009). Different densitometry procedures applied to the identical Western blots have also revealed wide-ranging results with poor correlation (Gassman 2009).

Given the known weaknesses regarding this methodology, in addition to the published nature of the majority of western blot data presented within this thesis, without densitometry, it was not felt that this methodology would provide robust or additional strength to the cumulative data presented.

### **2.7 Annexin V-FITC assays**

Staining for cell surface phosphatidyleserine was used as a marker for apoptosis and was carried out using an AnnexinV-FITC apoptosis detection kit (Calbiochem), as per the manufacturer's instructions. Briefly, the media from a flask of treated adherent cells was transferred to a 15 ml conical tube and placed on ice. Adherent cells were then washed with 2 mls PBS (which was added to the 15ml conical tube) prior to incubation with 1ml T/V (per 5cm<sup>2</sup> flask) at 37°C for 5 minutes and until cells appeared to be detached. Cells were then released from flasks with firm tapping and subsequently resuspended in the media that was initially removed. The number of cells/ml was determined by haemocytometric counts then cells were resuspended at a concentration of approximately 1 x 10<sup>6</sup> cells/ml. 0.2 ml cell suspension (1 x 10<sup>6</sup> cells) was then transferred to a 1.5 ml eppendorf along with 20 µl media binding reagent (Calbiochem) and 0.5 µl Annexin V-FITC. Cells were incubated for 15 min at room temperature in the dark, pelleted by centrifugation at 2000rpm/1,000 x g for 5 min at room temperature and then resuspended in 0.2 ml cold 1 X binding buffer (made by diluting 5 X binding buffer (Calbiochem) with dH<sub>2</sub>O). 10ul cell solution was placed onto a glass slide and 5ul of Vectashield-DAPI was placed onto a coverslip. The coverslip was placed over the cell solution. The percentage of cells undergoing apoptosis in the population was determined using fluorescent microscopy. The total number of cells per field of view was determined using DAPI to identify viable cell nuclei then the number of apoptotic cells quantified using the FITC channel. For all cases, at least 200 cells from a minimum of 10 independent fields of view were analysed.

Table 2. 1 – Antibodies used for western blot analysis.

Antibody	Host Species and Antibody Type	Dilution	Incubation Time	Manufacturer
c-Src (Y416)	Rabbit (Monoclonal)	1:1000 in TBSTM	2 hour (RT) or 16 hrs at 4 °C	Cell-signaling Technology, Inc
Anti-c-Src	Mouse (IgG) (Monoclonal)	1 : 500 in PBSTM (PBS + 0.1% TWEEN*20 (Sigma) + 5% dried milk)	16 hrs at 4 °C	Upstate Cell Signaling Solutions
Paxillin (Y118)	Rabbit (Monoclonal)	1:1000 in TBSTM	2 hour (RT) or 16 hrs at 4 °C	Invitrogen
Paxillin	Mouse (Monoclonal)	1:1000 in PBSTM	1hour (RT)	BD Pharmingen
Anti-IkB $\alpha$	Sheep (Polyclonal)	1 : 4000 in PBSTM	1 hr at room temperature (RT)	Gift from Ron Hay
Anti-Actin	Mouse (IgM) (Monoclonal)	1 : 15,000 in PBSTM	1 hr at RT	Calbiochem
Anti-Mouse IgG (HRP Conjugate)	Donkey (Polyclonal)	1 : 1000 in PBSTM	30 min at RT	Amersham Biosciences
Anti-Mouse IgM (HRP Conjugate)	Goat (Polyclonal)	1 : 1000 in PBSTM	30 min at RT	Calbiochem
Anti-Sheep IgG (HRP Conjugate)	Donkey (Polyclonal)	1: 1000 in PBSTM	30 min at RT	Jackson Immunoresearch
Anti-Rabbit IgG (HRP Conjugate)	Donkey (Polyclonal)	1 : 1000 in PBSTM	30 min at RT	Amersham biosciences

## **2.8 Imaging**

### **Immunocytochemistry**

Antibodies used for immunocytochemistry and details of the host species in which they were raised, dilutions, incubation time and manufacturer are summarised in Table 2.2. Cells were grown on sterilised coverslips in 6 well plates until 60-80% confluent then treated as specified. After treatment, cells were washed briefly with TBS then fixed in 1 : 1 methanol : acetone at -20°C for 20min. Cells were subsequently washed with TBST (2 X 10 min) prior to incubation with 10% donkey serum (Sigma) for 30 min to block non-specific binding. After blocking, cells were incubated with primary antibody diluted in 10% donkey serum for 1 hr and washed with TBST (2 X 10 min) prior to incubation with secondary antibody diluted in 1.5% donkey serum for 30 min. Cells were washed with TBS (2 X 10 min) then coverslips mounted in Vectastain (Vector Laboratories) containing DAPI (4',6'-diamido-2-phenylindole) (1 µg/ml) (Sigma) to stain DNA.

### **Quantification of nuclear RelA**

In order to quantify the induced nuclear translocation of RelA, individual exposure channels for each immunocytochemistry image field were captured and stored using identical exposures throughout image collection and across treatment arms of the experiment. Later image analysis created nuclear outline masks in DAPI channel images, which were subsequently transposed onto the matched FITC channel exposures for that field of view. The mean intensity of nuclear RelA staining within previously defined DAPI masks were then quantified in at least 3 separate experiments, in least 15 random fields per experiment.

**Table 2.2 Antibodies used in immunocytochemistry**

Antibody	Host Species and Antibody Type	Dilution	Manufacturer
c-Src (Y416)	Rabbit (Monoclonal)	1:1000 in TBSTM	Cellsignaling Technology, Inc
Antic-Src	Mouse (IgG) (Monoclonal)	1 : 500 in PBSTM (PBS + 0.1% TWEEN 20 (Sigma) + 5% dried milk)	Upstate Cell Signaling Solutions
Antc-Src	Rabbit (IgG)	1:100	Cellsignaling Technology, Inc
Paxillin (Y118)	Rabbit (Monoclonal)	1:1000 in TBSTM	Invitrogen
Paxillin	Mouse (Monoclonal)	1:1000 in PBSTM	BD Pharmingen
AntiFAK alexa-flour 555	Mouse (Monoclonal)	1:100	Upstate Cell Signaling Solutions
Antic-Yes	Mouse (Monoclonal)	1:100	Biosource
Antic-Fyn	Mouse (Monoclonal)	1:100	Biosource
AntiBeta-Tubullin	Mouse Monoclonal	1:1000	Sigma
Antimouse IgG (XRd Conjugate)	Donkey (Polyclonal)	1 : 100	Jackson Immunoresearch
Antirabbit IgG (FITC conjugate)	Donkey (Polyclonal)	1 : 100	Jackson Immunoresearch
Antirabbit IgG (TxRd conjugate)	Donkey (Polyclonal)	1 : 100	Jackson Immunoresearch
Antigoat IgG (TxRd conjugate)	Donkey (Polyclonal)	1 : 100	Jackson Immunoresearch

## **2.9 Immunohistochemistry**

4 micrometer widths sections were cut from paraffin embedded tissue and fixed on glass slides. For fluorescence immunohistochemistry, slides were heated at 60 °C for 45 minutes then submerged twice for 5 minutes in Xylene. Samples were then rehydrated through 2 sequential steps of 100% ethanol and then 70 % ethanol, each for 5 minutes. Samples were rinsed with deionised water x 2 for 5 minutes each, prior to microwave induced boiling in citrate buffer for 25 minutes. After cooling samples to room temperature for 1 hour, samples were washed again in deionised water and placed in sequenza clips. Samples were permeabilised in 1% Triton-x 100 in PBS for 20 minutes. Following 2 further 10 minute washes in PBS, the samples were then placed in protein block overnight at 4 °C (10% Donkey Serum (Sigma), 5% Bovine Serum Albumin (Sigma, St Louis, MO) in PBS with 1 % Tween).

Antibodies used for immunocytochemistry and details of the host species in which they were raised, dilutions, incubation time and manufacturer are summarised in Table 2.3. Primary antibodies were added in blocking solution at the concentration specified for the time specified followed by 3x 5 minute washes with T/PBS containing 1% Tween. The relevant fluorescent secondary antibodies were then added at the concentration specified. Following incubation, protected from light, the samples were washed 3 times in T/PBS 1% tween for 5 minutes each and then air dried. Cover slips were applied to the samples after 10 microlitres of DAPI:Vectashield (Vector Lab Inc., Burlingame, CA) had been added. Samples were stored at 4 °C until ready to be viewed by immuno-fluorescence microscopy.

Primary antibodies included Ki67 (1:500) (DakoCytomation, clone MIB-1), PCNA (1:100) (Santa Cruz, clone C-20), Active Caspase-3 (1:300)(BD Pharmingen, clone C92-605), TUNEL “In Situ cell death detection” kit (concentration as per manufacturers instructions) (Roche) , mouse anti- $\beta$ -catenin (1:250)( BD Transduction Labs ), rabbit anti-chromogranin A (1:50) (Autogen Bioclear Cat no ABJ1411), Mouse anti-Vil-1 (1:100)(Lifespan Biosciences), mouse anti-Muc-2 (1:100) (Genetex Inc), mouse FITC-conjugate, anti- $\alpha$ -smooth muscle actin (Sigma), rabbit anti-LGR5 (1:100) (MBL International Corporation, Woburn, WA), mouse anti-BMI-1 (1:100)(Abcam ab14389-25) and mouse



anti-CD133 (Prominin 1)(#SC-130127; Santa-Cruz Biotechnology Inc). Secondary antibodies used were anti-rabbit FITC (5:1000) and anti-mouse Texas-red (5:1000) (Jackson Laboratories).

**-Table 2.3 Antibodies used in immunohistochemistry**

Antibody	Host Species and Antibody Type	Dilution	Manufacturer
Anti-RelA	Rabbit (Polyclonal)	1 : 200	Santa Cruz Biotechnology
Anti-c-Src (Y416)	Rabbit (Monoclonal)	1:200	Cell-signaling Technology, Inc
Anti-c-Src	Mouse (IgG) (Monoclonal)	1 : 200	Upstate Cell Signaling Solutions
Anti-Paxillin (Y118)	Rabbit (Monoclonal)	1:200	Invitrogen, Camarillo, Ca
Anti-Paxillin	Mouse (Monoclonal)	1:200	BD Pharmingen
Anti-Hu villin 1	Mouse	1:100	Lifespan biosciences
Anti- $\beta$ -catenin	Mouse	1:250	BD Transduction labs
Anti-Muc-2	Mouse	1:100	Genetex Inc.
Anti-Chromogranin A	Rabbit	1:50	Autogen Bioclear
Anti-alpha -smooth muscle actin	Mouse	1:500	Sigma
Anti-BMI-1	Mouse	1:100	Abcam
Anti-Lgr5	Rabbit	1:100	MBL
Anti-PCNA	Mouse	1:250	Santa cruz
Anti-active caspase - 3	Rabbit	1:250	BD Pharmingen
TUNEL In situ cell death kit	--	As per kit	Roche
Anti-Ki67	Mouse	1:250	Dako cytotation
Anti-mouse IgG (FITC Conjugate)	Donkey (Polyclonal)	1 : 100	Jackson Immunoresearch
Anti-mouse IgG (TxRd Conjugate)	Donkey (Polyclonal)	1 : 100	Jackson Immunoresearch
Anti-rabbit IgG (FITC conjugate)	Donkey (Polyclonal)	1 : 100	Jackson Immunoresearch
Anti-rabbit IgG (TxRd conjugate)	Donkey (Polyclonal)	1 : 100	Jackson Immunoresearch

## **Microscopy**

For brightfield colour imaging using a monochrome camera, the imaging system comprises a Coolsnap HQ CCD camera (Photometrics Ltd, Tucson, AZ) and Zeiss Axioplan II fluorescence microscope with Plan-neofluoro objectives (Carl Zeiss, Welwyn Garden City, UK). Colour additive filters (Andover Corporation, Salem, NH) installed in a motorised filter wheel (Ludl Electronic Products, Hawthorne, NY) were used sequentially to collect red, green and blue images that were then superimposed to form a colour image. Image capture and analysis were performed using in-house scripts written for IPLab Spectrum (Scanalytics Corp, Fairfax, VA). White adjustment was performed once per session on a representative slide using a non-tissue section of the slide. For fluorescence imaging, the imaging system comprises a Coolsnap HQ CCD camera (Photometrics Ltd, Tucson, AZ), Zeiss Axioplan II fluorescence microscope with Plan-neofluar objectives, a 100W Hg source (Carl Zeiss, Welwyn Garden City, UK) and Chroma #83000 triple band pass filter set (Chroma Technology Corp., Rockingham, VT) with the excitation filters installed in a motorised filter wheel (Prior Scientific Instruments, Cambridge, UK). Image capture and analysis were performed using in-house scripts written for IPLab Spectrum (Scanalytics Corp, Fairfax, VA).

### Tissue damage scores


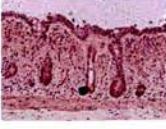



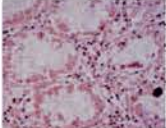
The “C<sub>3</sub> tissue damage scale” was developed to describe ex-vivo mucosal colonic histological changes within the IVOC system. Further examples of scoring and an explanation of the system can be found in figure 2.4. The score describes 3 main histological qualities noted within explant samples (a) *Crypt density* (0= high crypt density /inter-crypt regions narrower than the crypts), 1= Medium crypt density (inter-crypt regions the same width as crypts), 2= Low crypt density (inter-crypt regions larger than the crypts), 3= no obvious crypt structures present (b) *Epithelial Continuity (surface and crypts)* (0= continuous superficial epithelial layers, 1= minor surface epithelium breaks, 2= major discontinuity of surface epithelium) plus (0= continuous epithelial layers within crypts, 1= Some crypts have epithelium breaks, 2= major discontinuity or absence of epithelium in crypts) or alternatively, 5= little or no continuous epithelium in the field) (c) *Composition of crypt cell population* (0= goblet cells in all crypts throughout, 1= few goblet cells in some crypts, 2= No goblet cells apparent but epithelial cells present, 3= no epithelium present). All scores represented were derived as an average from 3 trained and blinded participants. Scores ranged from 0 to 11 with a mean difference in participant’s scores across experiments of 0.72 (SD = 0.47). R-squared values ranged from 0.81-0.86. Initial preparation to evaluate the strength of relationship between rater’s scores was undertaken. Further analysis was undertaken in order to evaluate levels of agreement between independent blinded reviewers of the base data (i.e. sample of 200 explant histology pictures) to ensure that scoring was similar across reviewers. As this was, in the first instance, categorical data and 3 reviewers were involved, a Light’s kappa analysis was undertaken as this computes Light's Kappa as an index of inter-rater agreement between more than 2 raters on categorical data (Conger 1980). The results of this analysis, corresponding to the agreement between raters, with squared weights was 0.898. This is judged as a “very good” strength of agreement according to Altman’s scale (Altman, 1991).

Mann-whitney U testing has been performed throughout to compare C3 Damage scores, following statistician advice. Chi-Squared testing was undertaken to compare outcomes for the categorical data of explant survival failure/success. Significance was set at 0.05.

a

<b>Crypt density</b>	<p>0=High crypt density /inter-crypt regions narrower than the crypts</p> <p>1= Medium crypt density (inter-crypt regions the same width as crypts)</p> <p>2= Low crypt density (inter-crypt regions larger than the crypts)</p> <p>3= No obvious crypt structures present</p>	
<b>Epithelial Continuity</b>	<i>Surface</i>	<p>0= Continuous superficial epithelial layers</p> <p>1= Minor surface epithelium breaks</p> <p>2= Major discontinuity of surface epithelium</p>
	<i>Crypt</i>	<p>0= Continuous epithelial layers within crypts</p> <p>1= Some crypts have epithelium breaks</p> <p>2= Major discontinuity or absence of epithelium in crypts</p>
	<i>Both</i>	<p>5= Little or no continuous epithelium in the entire field</p>
<b>Composition of crypt population</b>	<p>0= Goblet cells in all crypts throughout</p> <p>1= Few goblet cells in some crypts</p> <p>2= No goblet cells apparent but epithelial cells present</p> <p>3= No epithelium present</p>	

b

	Crypt Density	Epithelial Continuity		Composition of Crypt Population	C3 score
		Surface	Crypt		
	0	0	0	0	0
	1	0	0	1	1
	1	0	0	2	3
	2	0	2	1	5
	2	1	2	2	7
	3	-	5	-	11

**Figure 2.4. The C3 scoring scale**

a. Tabular representation of the C3 score. b. Some examples of the scoring in practice

**Chapter 3 – Effects of Aspirin on c-Src, and the role of c-Src in aspirin-induced NF- $\kappa$ B signaling and apoptosis in colorectal cancer cells**

3.1 Introduction	120
3.2 Detection of native c-Src in CRC cells	123
3.3. Detection of aspirin-induced c-Src kinase activity	125
3.4 Aspirin induces phosphorylation of c-Src Y416 in CRC cells	127
3.5 Aspirin-induced cellular relocalisation of c-Src is consistent with c-Src activation	130
3.6 Aspirin induces Y118 phosphorylation of paxillin	135
3.7 Aspirin induces c-Src activation prior to NF- $\kappa$ B activation	137
3.8 c-Src complexes with I $\kappa$ B $\alpha$ but not RelA	139
3.9 Chemical inhibition of c-Src inhibits aspirin's molecular effects	141
3.10 Genetic inhibition of c-Src inhibits aspirin's molecular effects	143
3.11 Active c-Src activates NF- $\kappa$ B independently of aspirin	145
3.12 Chemical inhibitors of c-Src inhibit aspirin's apoptotic effects	147
3.13 Genetic modulation of c-Src function mediates aspirin-induced apoptosis	147
3.14 Discussion	151

### 3.1 Introduction

Previously, the host laboratory has established that pharmacologically relevant concentrations of aspirin induce a concentration and time dependent molecular response characterized by degradation of I $\kappa$ B $\alpha$  and nuclear translocation of NF- $\kappa$ B in colorectal cancer cells *in vitro* and in colorectal neoplasia *in vivo*, and that this NF- $\kappa$ B response is essential for the pro-apoptotic effects of the agent (Stark et al 2001, Stark et al 2007). However, the aspirin induced response differs from the canonical pathway described above in that, firstly, there is a significant delay in the kinetics observed between aspirin administration and the activation of the NF- $\kappa$ B pathway. Secondly, the agent induces a sustained reduction in cytoplasmic I $\kappa$ B $\alpha$ , initially caused by a serine 32/36 phosphorylation-dependent, proteasome-mediated degradation of the protein (Din et al 2004, Stark et al 2001, Stark et al 2007). Finally, following aspirin treatment, unbound RelA translocates to the nucleus and is subsequently sequestered in the nucleolar compartment of the nucleus. These key molecular events result in a paradoxical response characterised by initial activation of the NF- $\kappa$ B pathway but the subsequent RelA nucleolar sequestration is paralleled by a decrease in basal levels of NF- $\kappa$ B transcriptional activity. RelA is therefore causally involved in the induction of apoptosis stark (Stark and Dunlop 2005).

As discussed, in contrast to the *in vitro* kinetics of TNF- $\alpha$ , the NF- $\kappa$ B response to NSAIDs is delayed, occurring hours rather than minutes after administration. Hence, it is likely that aspirin, rather than targeting I $\kappa$ B $\alpha$  directly, acts through the induction of intermediate upstream kinase signalling pathways. Previously, the p38 MAP Kinase was identified as a potential candidate as it is activated within minutes of aspirin administration and inhibition of p38 attenuates the aspirin induced NF- $\kappa$ B response (Thoms et al 2007a). However, due to the delay between p38 MAP Kinase activation and the NF- $\kappa$ B response, it is unlikely that there is direct interaction between p38 MAP Kinase and the NF- $\kappa$ B pathway members and that further intermediate candidate kinases are involved.



One such candidate kinase is the proto-oncogene product, c-Src. c-Src is upregulated and activated in CRC progression and metastases and c-Src activation is well-reported to induce NF- $\kappa$ B signaling in response to a wide variety of inflammatory mediators, bacterial stimuli and other chemical stimulants (Abu-Amer et al 1998, Huang et al 2003a) (Chang et al 2004, Fan et al 2003, Funakoshi-Tago et al 2005, Jalal and Kone 2006, Kang et al 2005, Lin et al 2006, Liu et al 2001, Lluís et al 2007, Mendez-Samperio et al 2007).

The mechanisms suggested for c-Src activation of NF- $\kappa$ B are diverse and have been described in many settings. In some reports, c-Src is involved in the direct phosphorylation of I $\kappa$ B $\alpha$  tyrosine residues (Y42) in response to stimulants such as pervanadate, hypoxia or VEGF, which result in nuclear translocation of NF- $\kappa$ B in the absence of I $\kappa$ B $\alpha$  degradation (Elder et al 2002, Fan et al 2003, Grosjean et al 2006, Imbert et al 1996, Koong et al 1994). Alternatively, c-Src-induced NF- $\kappa$ B activation is also known to occur more indirectly, through induced stimulation of intermediate kinase pathways, such as Protein Kinase C Alpha (PKC $\alpha$ ) and the I $\kappa$ B $\alpha$  kinase (IKK) signaling pathways. These activation pathways classically induce NF- $\kappa$ B signaling by I $\kappa$ B $\alpha$  serine 32/36 residue phosphorylation and subsequent proteasomal-mediated I $\kappa$ B $\alpha$  degradation (Huang et al 2003a, Mendez-Samperio et al 2007) (Lin et al 2006). Furthermore, reports also describe a mechanism by which c-Src acts as a scaffold for the IKK complex itself and contributes to signal-mediated degradation of I $\kappa$ B $\alpha$  in a manner that does not require the c-Src tyrosine kinase domain or function (Chang et al 2004, Funakoshi-Tago et al 2005).

Critically, in support of the notion that c-Src acts as an intermediary in aspirin/NSAID activation of the NF- $\kappa$ B pathway, NSAIDs and aspirin are also reported to effect the activity of c-Src in a variety of cellular contexts (Perez-G et al 2002, Ramdas and Budde 1998, Wang and Brecher 2001). Wang et al. have reported that salicylates alter the phosphorylation state of c-Src by inducing a modification of the intra-cellular calcium/redox state. Kunte et al., (Kunte et al 2008) reported that the NSAID, sulindac sulfide, modifies c-Src activity in CRC cells while Casanova et al. reported that another NSAID, Celecoxib, modified c-Src activity in acute myeloid leukaemia cell lines, together with the induction of a subsequent pro-apoptotic response (Casanova et al 2008).



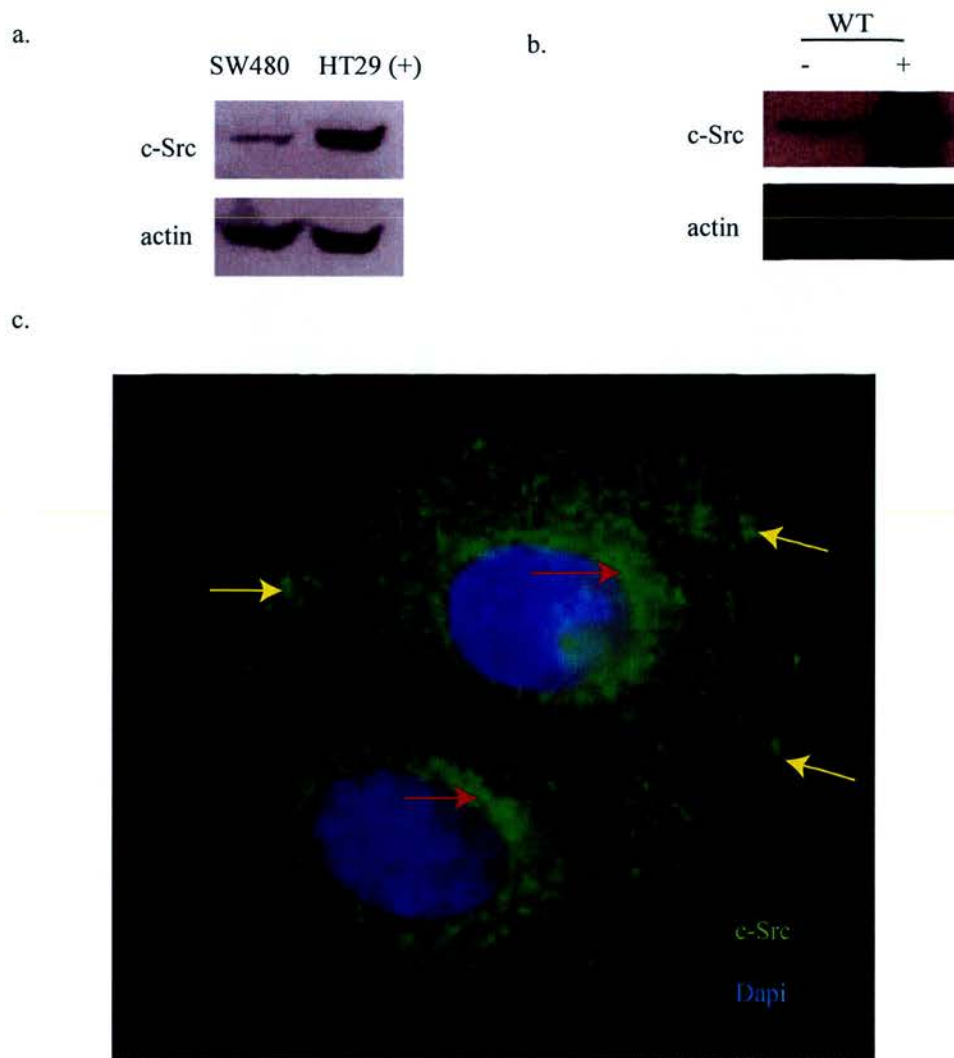
In summary, c-Src plays a critical role in CRC initiation and progression and NSAIDs are known to modulate the activity of kinase. Therefore, there is a compelling case to further investigate the role of c-Src in the aspirin-induced NF- $\kappa$ B response in CRC cells.

### **3.2 Detection of native c-Src in CRC cells**

SW480 colorectal cancer cells are a well-characterized experimental system in which to dissect the molecular responses to aspirin (Din et al 2004, Stark et al 2001, Stark and Dunlop 2005, Thoms et al 2007a). As such, this was a logical choice as an available model in which to pursue my line of investigation. However, whilst activation of c-Src is a consistent early finding in CRC (Bolen et al 1987, Cartwright et al 1989, Talamonti et al 1993, Termuhlen et al 1993), the protein expression levels amongst different CRC cell lines have been reported as being highly variable (Dehm et al 2001).

Previous reports have demonstrated that CRC cell lines such as SW480, SW620, HCT-116, HCT-15 and DLD-1 have low levels of c-Src mRNA, intermediate levels of c-Src protein expression and low levels of c-Src kinase activity, compared to CRC cell lines such as HT29, WiDr, COLO201 and COLO205, which display high levels of *SRC* transcriptional activation (Dehm et al 2001). Furthermore, even those cells which express very low levels of c-Src expression have been previously documented to exhibit high levels of phosphorylated / activated c-Src (Serrels et al 2006). Therefore, given these previous reports, my first objective was to confirm that c-Src could be detected in SW480 cells.

Using extracts from the SW480 cell line and the HT29 CRC cell line (a CRC cell line which is known to constitutively express higher levels of c-Src, utilised as a positive control), I found that native c-Src was detectable by western blot analysis in SW480 whole cell extracts (figure 3.1a). However, in keeping with previous reports, c-Src levels were reduced in SW480 cells compared to HT29 cells. To confirm the specificity of the antibody to active and native protein, I utilised extracts from SW480



**Figure 3.1. Detection of native c-Src in CRC cells**

a+b. Anti-c-Src western blot analysis was performed on whole cell extracts from a. SW480 cells and HT29 cells (positive control) or b. SW480 cells transfected with a wild-type c-Src expression vector (WT). Actin was used as a protein loading control (n=3) c. Anti-c-Src immunocytochemistry was performed on SW480 cells. DAPI was used as a nuclear marker. c-Src localises to a peri-nuclear rim (red arrow) and to aggregates at the cell peripheries (yellow arrow) (n=5).

cells that had been transiently transfected with an expression vector to overexpress the wild-type form of c-Src (figure 3.1b).

Immunocytochemistry confirmed the expression of c-Src within the cell cytoplasm and primarily around the peri-nuclear envelope however, some peripheral aggregates were also noted at the cell membrane. (figure 3.1c). This is consistent with the known c-Src cellular location in CRC cell lines and invasive tumours (Kaplan et al 1992, Reynolds et al 1989).

### **3.3. Detection of aspirin-induced c-Src kinase activity**

Having established that SW480 cells expressed c-Src, I then utilised this cell line to determine the effect of aspirin on the tyrosine kinase activity of c-Src. Initially, I aimed to measure c-Src activity using a commercially available kinase assay specific for c-Src activity (“c-Src kineactive” kit ®). In the KineActive ELISA method, a cellular extract is added to the wells of an antibody-capture plate. This is followed by addition of a c-Src-specific antibody, which binds to the plate and captures c-Src from the extract. After the unbound proteins are washed away, a biotinylated, unphosphorylated peptide substrate is added in the presence of ATP. The immobilized c-Src phosphorylates this substrate peptide, which is subsequently also captured by the addition of a second antibody that is specific for the phosphorylated form of the substrate peptide. Addition of streptavidin-HRP and subsequent developing solution provides a quantitative colorimetric readout that is directly proportional to the activity of the kinase (see methods). Cytoplasmic extracts of SW480 cells were utilised in time course experiments with aspirin.

Results initially indicated that aspirin induced c-Src tyrosine kinase activity in SW480 CRC cells, and that this activation peaked 4 hours after treatment (Figure 3.2). Unfortunately, shortly after optimisation of this assay, the manufacturer withdrew the kit from the market for financial reasons. Further assessment of dose or inhibitor responses utilising this assay could therefore not be

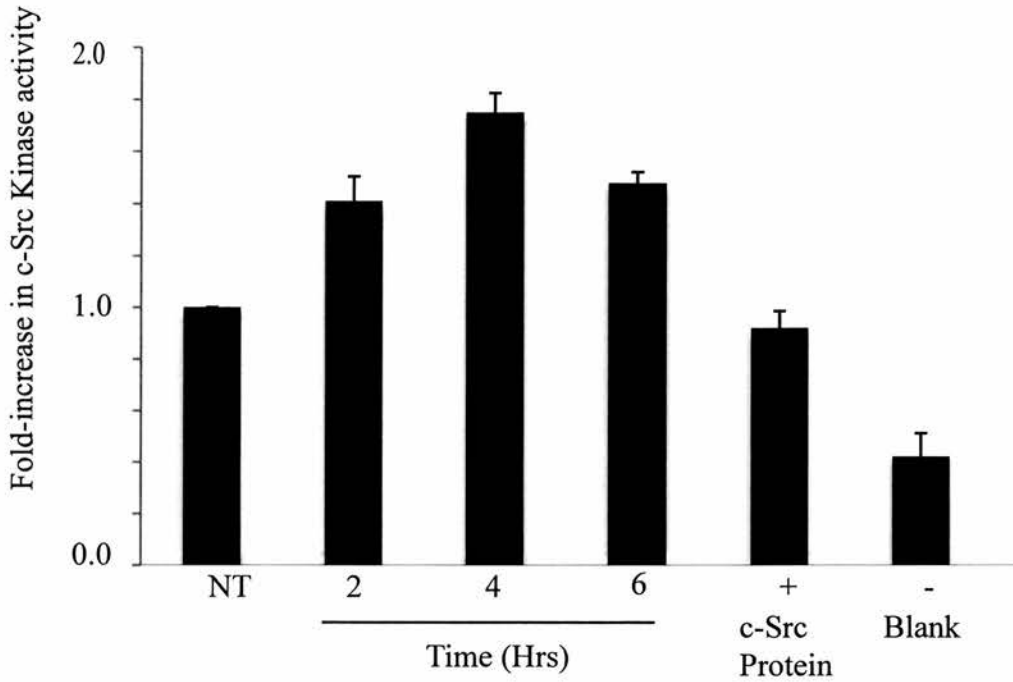


Figure 3.2 Detection of aspirin-induced c-Src kinase activity

SW480 cells were untreated (NT) or aspirin treated (5mM) for the times specified. Cell extracts were processed by c-Src kinase assay kit and fluorometric output (equivalent to kinase activity) was recorded. Values are presented as a fold increase compared to base-line SW480 activity (NT). Fluorometric output increased following aspirin treatment, peaking at 4 hours following administration. Fluorometric outputs were normalised to untreated SW480 c-Src kinase activity baseline levels. Recombinant Src protein (+) and lysis buffer (-) were used as positive and negative controls. Data are the average of 4 independent experiments (+/- standard error).

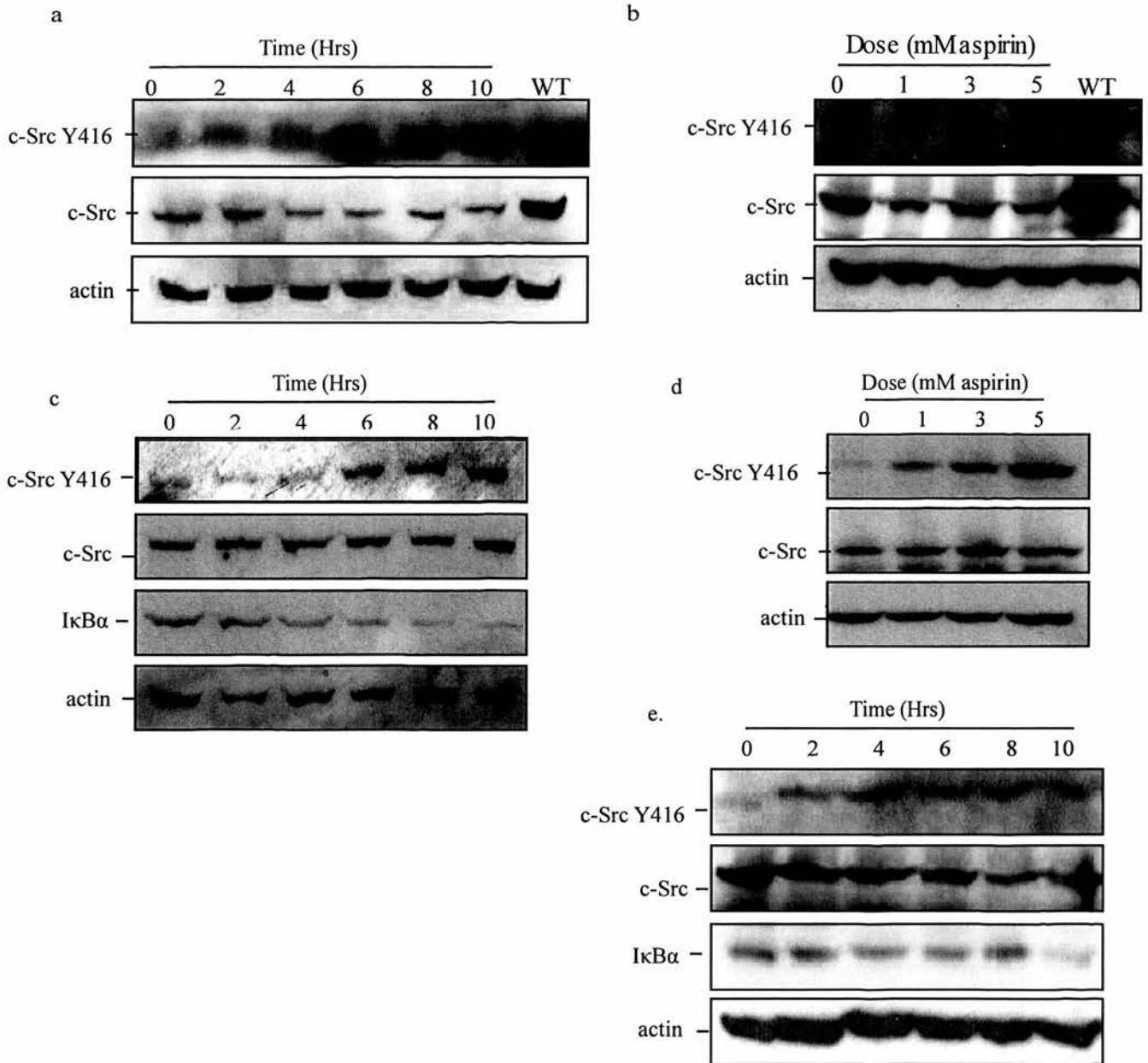
undertaken. Further attempts to obtain the kit or elements of the kit by alternative means were not successful. I therefore sought to detect c-Src kinase activation by alternative means.

### **3.4 Aspirin induces phosphorylation of c-Src Y416 in CRC cells**

Intermolecular auto-phosphorylation of the Y416 residue on the activation loop of c-Src induces internal protein displacement and conformational opening of the kinase domain, permitting access of substrates and signifying activation of the c-Src tyrosine kinase (Brown and Cooper 1996). Phosphorylation of this residue is widely used as a surrogate marker of c-Src activation and increased c-Src tyrosine kinase activity (Roskoski, Jr. 2005)(Frame 2002). A further detailed description of the mechanism of c-Src regulation and function are presented in chapter 1.6 (figure 1.6).

Given the difficulties experienced with the c-Src kinase assays, I next examined phosphorylation of the c-Src Y416 residue as a surrogate marker for activation of the kinase. A commercially available phospho-specific antibody that detects phosphorylation of the Y416 residue on c-Src was obtained to assess cellular c-Src activation status and specifically to characterize the effect of aspirin.

Time-course studies indicated Y416 phosphorylation increased 4-6 hours following aspirin (5mM) administration (figure 3.3a). Furthermore, the kinetics of this activation were in keeping with the kinetics of those observed previously when using the “c-Src kineactive” kit ®. The c-Src (phospho-Y416) activation signal corresponded to a protein size of 60 kDa and extracts from cells transiently transfected to over-express the wild-type form c-Src protein were used as a control for phospho-protein specificity. Following stripping, membranes were subsequently reprobed with an antibody to detect native c-Src. This revealed that the previous Y416 antibody detection signal also matched native c-Src in regards to molecular weight. Notably, in contrast to the increase observed with activated c-Src, the level of native c-Src protein expression remained unchanged in response to aspirin administration.



**Figure 3.3 Aspirin induces phosphorylation of c-Src Y416 in CRC cells**

SW480 cells (a, b), HT29 cells (e) or SW480 cells which were transiently transfected with a wild type c-Src expression vector (c, d) were aspirin treated (5mM) for the times specified (a, c, e) or 0-5mM, 4hrs (b, d). (a-e) Western blot analysis was performed on whole cell extracts using antibodies to phosphorylated c-Src (Y416) and IκBα. Blots were stripped and reprobbed for endogenous c-Src. Lysates from wild-type transfected cells were used as positive controls. SW480 cells transfected with a wild type c-Src expression vector (WT) were used as a positive control. Actin was used as a control for protein loading. Aspirin induces a dose- and time-related increase in phosphorylated c-Src (Y416) and time-dependent decrease in levels of IκBα (n>3).

Next, the dose-dependency of aspirin-induced c-Src activity was examined. SW480 cells were treated with 0-5mM aspirin for 4 hours, then whole cell extracts were analysed for activation of c-Src using Y416 phosphorylation as a marker of activation. I found activation of c-Src occurred in a dose dependent manner. Subsequent probing demonstrated that levels of native c-Src remained unchanged irrespective of the dose of aspirin administered (figure 3.3b).

Given the relatively low levels of c-Src expression in SW480 cells, the strength of the antibody signal obtained was weak and small changes in protein phosphorylation were difficult to detect despite using high total protein concentrations. In order to maximise antibody signal detection, dose and time-course experiments were replicated in SW480 cells that had been transiently transfected to over-express the wild-type form of c-Src. In this setting, a time and dose -dependent increase in phosphor-Y416 c-Src levels was again observed, confirming data observed from the endogenous protein setting (figures 3.3c and 3.3d).

The commercial antibody used in these experiments can cross-react with c-Src Y416 residues and with other SFK members. However, the c-Src over-expression experiments confirmed that c-Src is indeed activated after aspirin administration. These data also indicate that the level of c-Src expression within CRC cell lines is not a relevant factor in whether a cell has a c-Src response to aspirin, as the response was observed in both high (transiently transfected) and low (native cells) c-Src expression environments.

To determine the generality of the c-Src response to aspirin with regard to CRC cells, the response was assessed in HT29 cells, which as discussed previously, expresses high levels of native c-Src. HT29 cells were treated in time course experiments. Aspirin induced a time-dependent increase in c-Src Y416 phosphorylation, consistent with results from SW480 cells (figure 3.3e). These data demonstrate that the aspirin induced response is of relevance to CRC cell lines more generally.

### 3.5 Aspirin-induced cellular localisation of c-Src is consistent with c-Src activation

In previous reports, native c-Src localises primarily to the peri-nuclear envelope (Reynolds et al 1989). However, c-Src activation is associated with remodelling of the actin cytoskeleton and a profound change in its normal cellular location, characterised by rapid translocation from the perinuclear area to peripheral focal adhesions/cell membrane. This “trafficking” putatively occurs along actin fibres facilitating relocation of c-Src to membrane protrusions/lamellipodia (Fincham et al 1995).

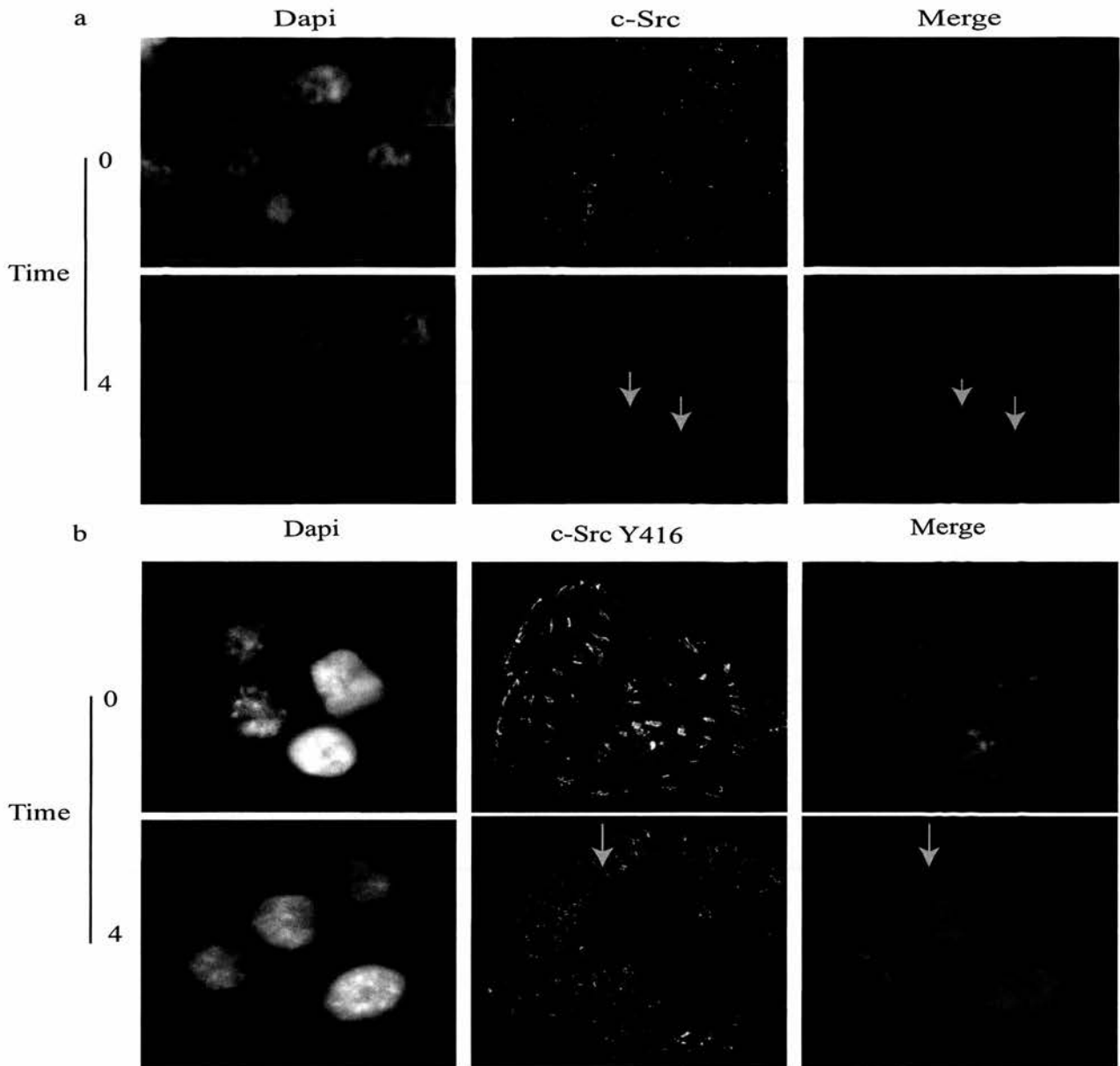
I therefore used immunocytochemistry to determine the location of native and active c-Src within the cell before and after aspirin treatment. Following aspirin treatment, the native c-Src signal was of increased intensity at the cell periphery, approximating to the location of the cell membrane (Sakai et al 1998)(Figure 3.4a).

These studies were repeated using antibodies to detect the phospho-Y416 c-Src signal. Prior to aspirin treatment phospho-Y416 was found in relatively low levels around the perinuclear area. However, there were also numerous phospho-Y416 staining aggregates localising at the cell membrane (Figure 3.4b). These aggregates were reminiscent of the appearance and location of focal adhesions. Therefore, co-staining for activated c-Src and anti-FAK (Focal Adhesion Kinase) was undertaken.

I found that peripheral aggregates contained a central dense FAK-rich core surrounded by active an intensely staining phospho-Y416 ring (Figure 3.5a and figure 3.5b). These appearances were consistent with the known characteristics, appearance and molecular structure of focal adhesions/focal complexes (Worth and Parsons 2008). The localisation of activated c-Src within focal adhesions is well described (Howell and Cooper 1994). Activated c-Src genetic mutants are known to associate in podosomes, which are areas of the cell in which there are components of focal adhesions (Kaplan et al 1994).

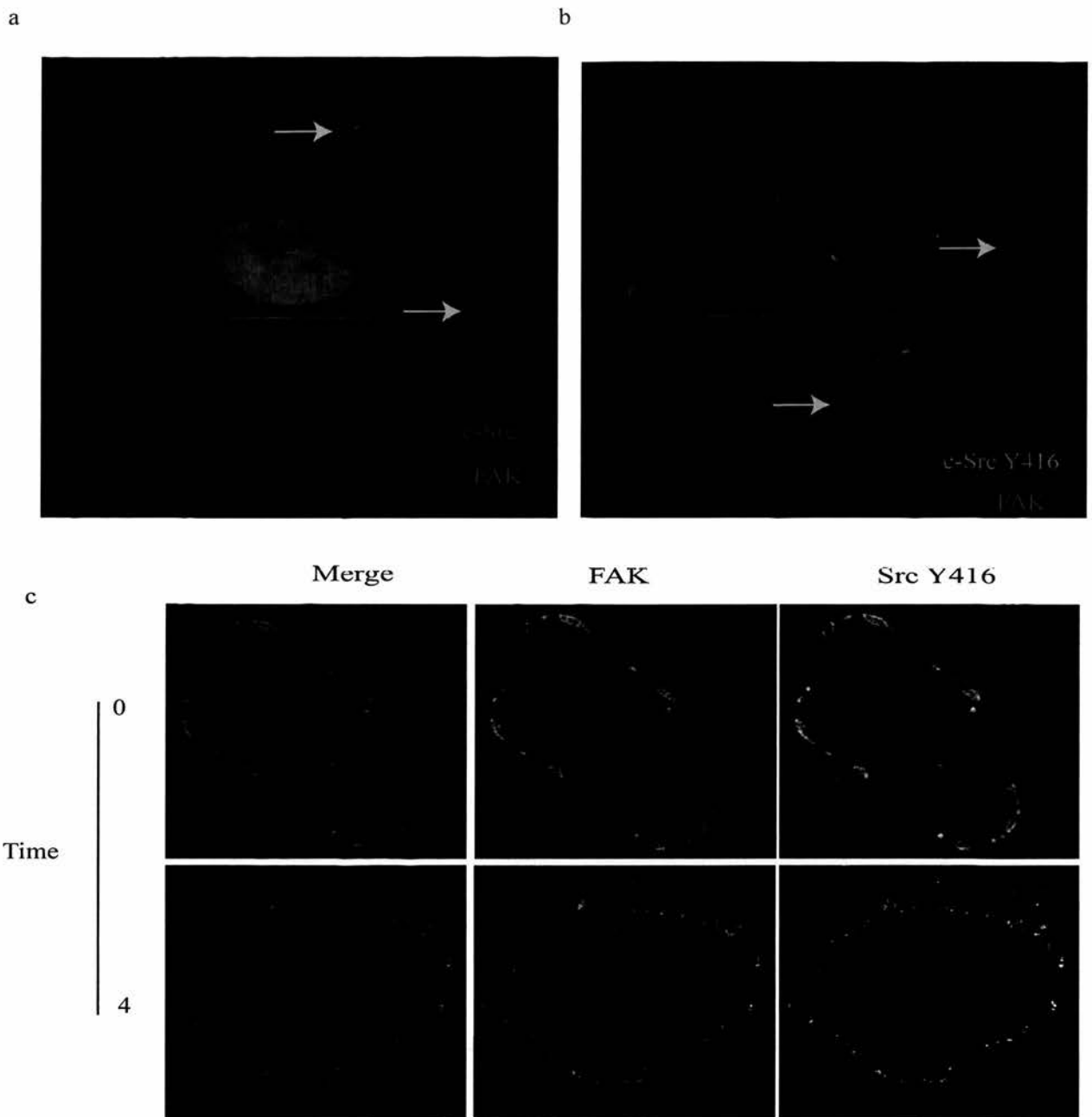
In contrast to the tightly-structured appearance, I found that aspirin caused a dramatic breakdown in focal adhesions. This breakdown, from larger active c-Src aggregates to smaller c-Src foci, occurred





**Figure 3.4 Aspirin-induced cellular localisation of c-Src is consistent with c-Src activation**

Immunocytochemistry utilising antibodies against endogenous c-Src (a) phosphorylated c-Src (Y416) (b) was performed on SW480 cells at baseline or 4 hrs after treatment with aspirin (5mM). a. Aspirin induces migration of endogenous c-Src to the cell membrane (n=5), Yellow arrows denote increased staining intensity at the cell membrane b. Focal aggregates of densely staining c-Src Y416 are observed at the cell periphery prior to aspirin treatment. In response to aspirin levels of phosphorylated c-Src (Y416) were increased and distributed diffusely (n=5).



**Figure 3.5 Aspirin-induced cellular relocalisation of FAK is consistent with c-Src activation**

Immunocytochemistry utilising antibodies against endogenous c-Src (a) phosphorylated c-Src (Y416) (b,c) or FAK (a, b, c) was performed on SW480 cells at baseline (a,b) or 4 hrs (c) after treatment with aspirin (5mM) a. Anti-c-Src and anti-FAK immunocytochemistry was performed on untreated cells. Endogenous c-Src localises primarily to the peri-nuclear region but a smaller proportion localises closely to membrane aggregates that are FAK rich (yellow arrows). DAPI was used as a nuclear marker. (n=3) b. Phospho-c-Src Y416 localises primarily to membrane aggregates that have a FAK rich core. DAPI was used as a nuclear marker. (n=5) c. Larger FAK dense aggregates undergo dissolution after aspirin treatment and become more numerous (n=3).

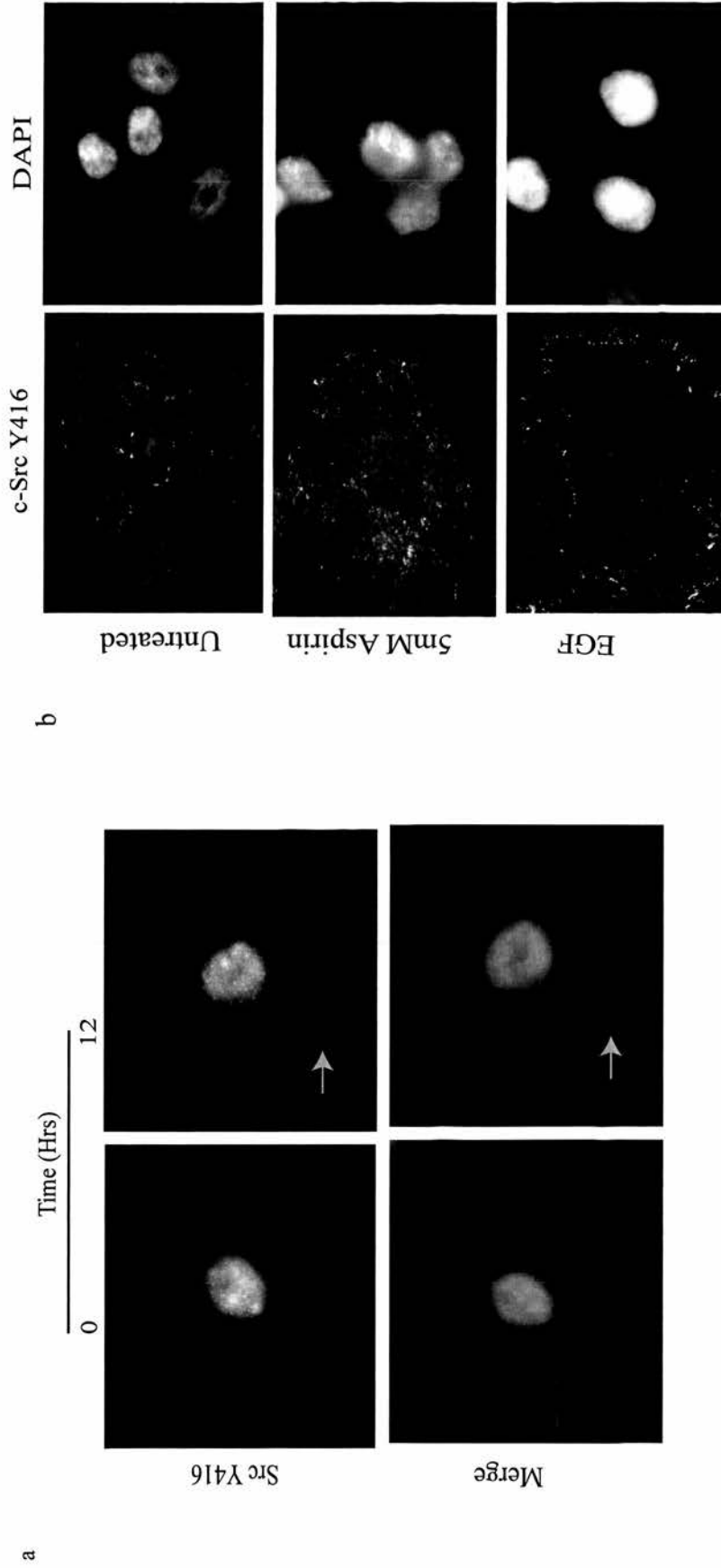
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simultaneously with the increase in c-Src activation observed using western blot analysis. It also correlated with the appearance of membrane ruffles and lamellipodia. The small c-Src foci appeared to align along the edges of these membrane protrusions and remain co-localised with smaller FAK aggregates. There was a time-dependent re-localisation of activated c-Src staining from dense focal adhesion-like structures, to smaller, more numerous aggregates at the cell periphery in areas which resembled the appearance of membrane ruffles and lamellipodia-like protrusions (figure 3.5c).

Whilst c-Src is known to play a central role in focal adhesion assembly, deregulated c-Src activity also has a destructive effect on these structures and on cell-matrix adhesion (Volberg et al 2001). Previous studies report that in addition to inducing disassembly of focal adhesions and relocation of the protein to membrane ruffles/podia, activation of c-Src also induces rearrangement of certain cytoskeletal elements (Westhoff et al 2004).

Eukaryotic cells contain three main kinds of cytoskeletal filaments; microfilaments, intermediate filaments, and microtubules. These cytoskeletal elements interact extensively and intimately with the cellular membranes (Doherty and McMahon 2008). Microtubules are highly dynamic and comprise of polymers of  $\alpha$  and  $\beta$ -tubulin. To examine aspirin effects on the cytoskeleton in relation to the activation of c-Src, we performed immunocytochemical analysis with antibodies to the major cytoskeletal component  $\beta$ -tubulin. I found that aspirin treatment induced significant changes in  $\beta$ -tubulin staining and structure, with loss and deterioration of staining of peripheral fibres, disordered fibre structures and less numerous fibres overall. This occurred following the reduction in the number and intensity of c-Src phospho-Y416 aggregates and dissolution/disassembly of FAK staining membrane bodies (figure 3.5f).

The changes observed in Src/FAK localisation and membrane structure were similar to those observed for the known activator of c-Src, Epidermal Growth Factor (EGF) (Figure 3.6a). EGF is known to cause an increase in c-Src kinase activity, relocalisation of c-Src to the cell periphery and phosphorylation of FAK in the carcinoma cells (Brunton et al 1997) (Figure 3.6b).



**Figure 3.6 Aspirin-induced cellular relocalisation of cytoskeletal elements is consistent with c-Src activation**

Immunocytochemistry utilising antibodies against phosphorylated c-Src (Y416) (a, b), or tubulin (a) was performed on SW480 cells 4 hrs (b) or 12hrs (a) after treatment with 5mM aspirin or 30mins after treatment with EGF (b). DAPI was used as a nuclear marker (b). The  $\beta$ -Tubulin cytoskeletal structure is changed after aspirin treatment, particularly in peripheral areas of the cell, with fewer fibres, and deterioration in signal strength. This is particularly evident in aggregates of increased phospho-c-Src (416) staining at the periphery (yellow arrows) (n=3). b. After aspirin, the membrane distribution of phospho-c-Src Y416 is diffusely increased, similar to the pattern observed after treatment with the known c-Src stimulant, EGF (n=5).

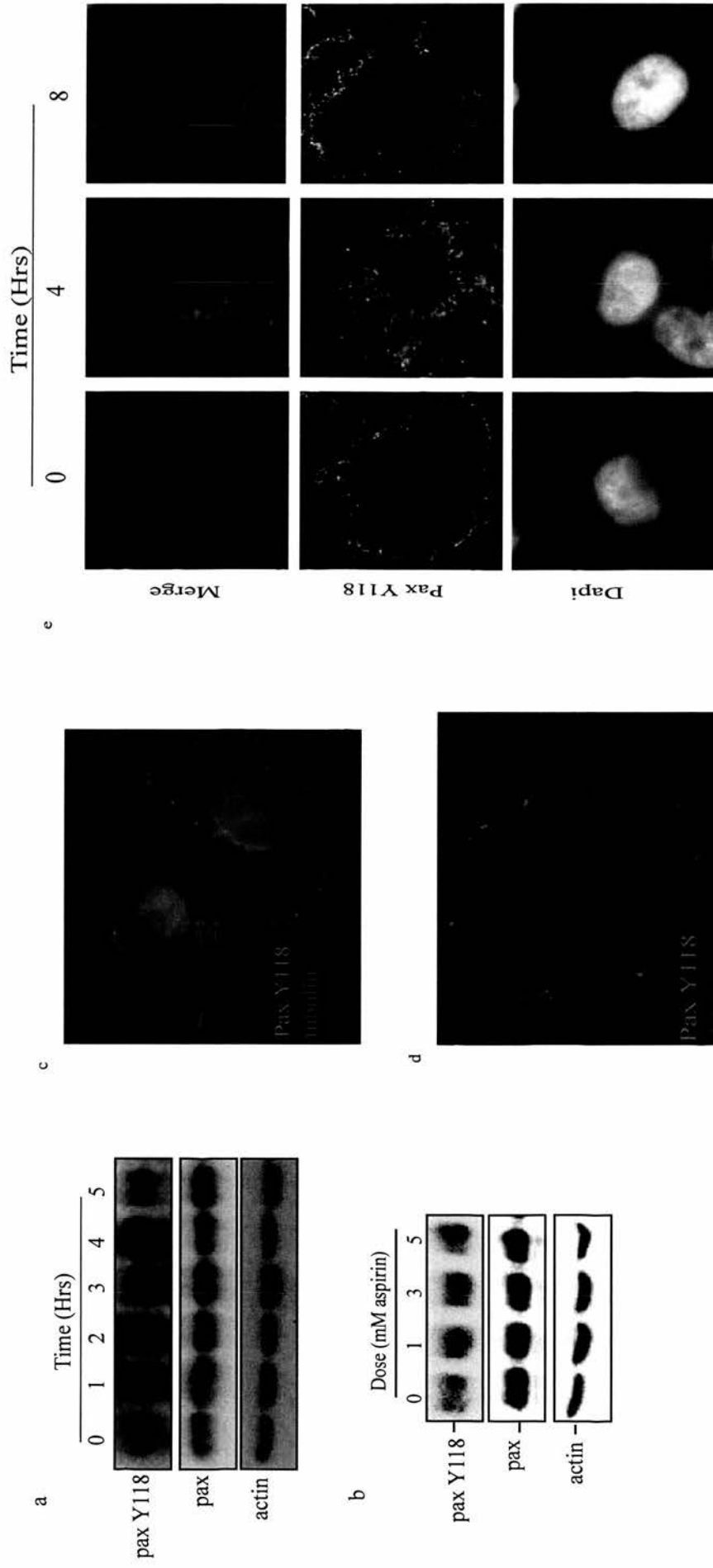
Taken together, these data confirm that aspirin activates c-Src in CRC cells. They also reveal the interesting observation that aspirin causes early changes to the cell cytoskeletal and membrane structures.

### **3.6 Aspirin induces Y118 phosphorylation of paxillin**

Paxillin is a multi-domain scaffold protein that localizes to the intracellular surface of sites of cell adhesion. It was first identified as a c-Src substrate when it was identified as the 68-kDa protein that exhibited increased tyrosine phosphorylation following the transformation of chick embryonic fibroblasts with the v-Src-expressing Rous sarcoma virus (Glenney and Zokas 1989). As one of the earlier identified members of the focal adhesion “proteome”, it has a number of widely recognised c-Src phosphorylation sites and is therefore of key interest, both in relation to its potential role in focal adhesions and also its use as a surrogate marker of c-Src activity (Serrels et al 2006). In addition to its structural role linking the ECM with the actin cytoskeleton (Brown and Turner 2004), it is also involved in many other cellular signalling functions, including survival signalling (Deakin and Turner 2008).

To further analyse aspirin effects on the c-Src pathway, I next utilised phospho-specific antibodies raised against paxillin phosphorylated at tyrosine residue 118, a known c-Src specific site (Sachdev et al 2009). In keeping with data showing activation of c-Src in response to aspirin, western blot analysis demonstrated a time-dependent increase in phosphorylated Y118 paxillin. Furthermore, this response occurred in parallel with the kinetics of the Y416 phosphorylation of c-Src noted previously (figure 3.7a). Similarly, the level of phospho-Y118 paxillin signal also increased concurrently with dose of aspirin administration (figure 3.7b).

Next, immunocytochemistry was used to examine the localisation of phosphorylated paxillin in relation to FAK and Y416 c-Src. Prior to aspirin treatment, the cellular distribution of phosphorylated Y118 paxillin in relation to  $\beta$ -tubulin and FAK was similar to that observed with c-Src Y416 (figure



**Figure 3.7 Aspirin induces Y118 phosphorylation of Paxillin**

SW480 cells were aspirin treated (5mM for the times specified) (a, c) or 0-5mM at 4hrs (b). Western blot analysis was performed on whole cell extracts using antibodies to phosphorylated paxillin (Y118). Blots were stripped and reprobed for native paxillin. Actin was used as a control for protein loading. Aspirin induces a dose- and time-related increase in phosphorylated paxillin (Y118). (N=5) c. Anti-paxillin (Y118) and anti- $\beta$ -tubulin immunocytochemistry was performed on SW480 cells. Paxillin (Y118) is expressed primarily in aggregates at the cell periphery and at the extremities of  $\beta$ -tubulin fibres. (N=3) d. Anti-phospho-paxillin (Y118) and FAK immunocytochemistry was performed. Phospho-paxillin (Y118) localises primarily to membrane aggregates that have a FAK-dense core in a similar to pattern to that observed with phospho-c-Src (Y416) distribution. DAPI was used as a nuclear marker. (N=3) e. Immunocytochemistry utilizing antibodies against phosphorylated paxillin (Y118) was performed on SW480 cells 4 and 8 hrs after treatment with 5mM aspirin. DAPI was used as a nuclear marker. Prior to aspirin treatment, phosphorylated paxillin (Y118) is located in aggregates at the cell membrane. Aspirin induces dissolution of these aggregates and an increase in diffuse phosphorylated paxillin (Y118) staining intensity at the cell membrane (N=5).

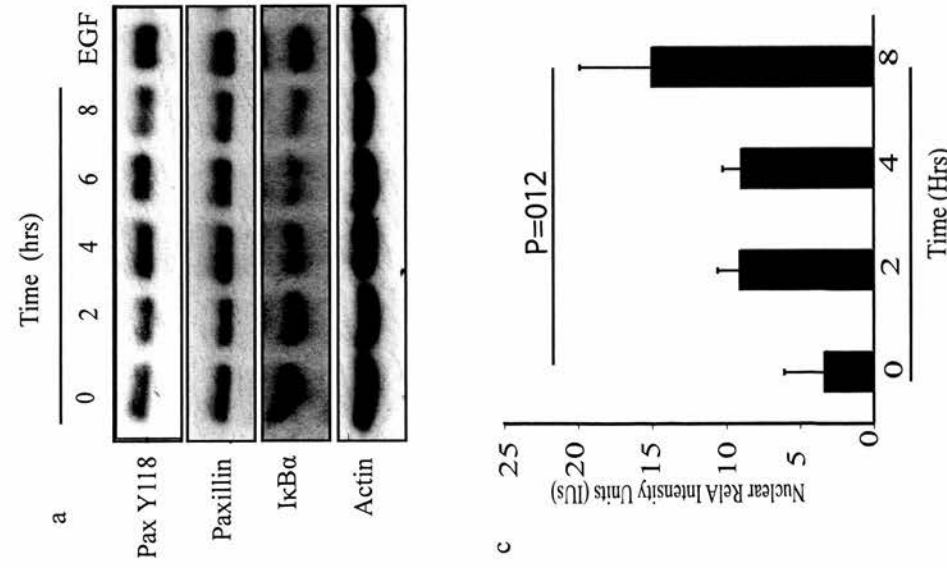
3.7c and 3.7d). However, following aspirin treatment, there was an increase in the intensity of the antibody signal at the cell membrane in concert with dissolution of FAK rich aggregates. Furthermore, there was a redistribution of Y118 phosphorylation along the cell membrane similar to that observed with c-Src (figure 3.7e). These findings suggest that aspirin induces phosphorylation of paxillin Y118 and that this may lie downstream of the effects of the agent on c-Src. The data also suggest that the phosphorylation of paxillin Y118 could therefore have potential utility as a surrogate marker of cellular c-Src-mediated tyrosine phosphorylation. However, whether paxillin Y118 phosphorylation occurs as a result of c-Src activity, or as a result of an unknown alternative kinase or is indeed a reflection of a more global effect of aspirin, in which there is tyrosine residue phosphorylation on many different cellular proteins, was at this point undetermined.

### **3.7 Aspirin induces c-Src activation prior to NF- $\kappa$ B activation**

Having established that aspirin activated the c-Src pathway, I next wished to determine whether this was linked to the effects of aspirin on activation of NF- $\kappa$ B signalling in CRC cells. To begin to establish if there was a relationship, I firstly examined the kinetics of the response.

Aspirin-induces I $\kappa$ B $\alpha$  degradation before the nucleoplasmic accumulation of RelA (Stark et al 2001). In order to further clarify the link between c-Src and the effects on the NF- $\kappa$ B pathway, western blot analysis was performed on cytoplasmic extracts to analyse aspirin effects on cytoplasmic levels of I $\kappa$ B $\alpha$  (as a marker for activation of the NF- $\kappa$ B pathway) in relation to activation of c-Src and phosphorylation of paxillin in time-course studies. Figure 3.8a demonstrates that aspirin-induced activation of c-Src and phosphorylation of paxillin precedes the effects of the agent on degradation of I $\kappa$ B $\alpha$  (see also figure 3.4c and 3.7a). This response was also confirmed in the HT29 CRC cell line (Figure 3.3e).

Upon I $\kappa$ B $\alpha$  degradation, RelA relocates to the nucleoplasm of the cell. Therefore, I next used immunocytochemical analysis to confirm the kinetics of aspirin-mediated stimulation of the NF- $\kappa$ B



**Figure 3.8 Aspirin induces c-Src activation prior to NF-κB activation**

SW480 cells were aspirin treated (5mM) for the times specified. **a.** Western blot analysis was performed on whole cell extracts using antibodies to phosphorylated paxillin (Y118) or IκBα. Blots were stripped and reprobed for native paxillin. Lysates from EGF treated SW480 cells were used as positive controls. Actin was used as a control for protein loading. Aspirin induces a time-dependent increase in phosphorylated paxillin (Y118) prior to time-dependent decrease in levels of IκBα. **b+c.** Anti-RelA immunocytochemistry was used to examine the location of RelA in response to aspirin. The subcellular localization of RelA (FITC) and of the nucleolar marker fibrillarin (Texas red) was then assessed by immunocytochemical staining. The merged panel is a pseudo-colour image generated by combining the FITC (green), Texas red (red), and DAPI (blue) channels. Nuclear localisation of RelA is observed prior to nucleolar translocation of the protein. **b/c.** Quantification was used to determine nuclear levels of RelA. Nuclear DAPI exposure image outlines which were transposed onto RelA exposure images. Comparative nuclear RelA staining intensity was then quantified by computer image analysis software. The nucleolar localisation of RelA was examined using anti-RelA immunocytochemistry. Representative fields of view (x63 objective) are shown. Aspirin induced increased RelA nuclear/nucleolar location at time-points subsequent to c-Src/paxillin phosphorylation and IκBα degradation (N>5).



pathway. Quantification of nuclear levels of RelA demonstrated that following aspirin administration, there was a trend for increased levels of nuclear RelA protein at 8 hours after administration of the agent. This time-point was subsequent to the observed kinetics of both aspirin-induced c-Src Y416, paxillin Y118 phosphorylation and aspirin-induced I $\kappa$ B $\alpha$  degradation (Figure 3.8a).

I next quantified the levels of nuclear RelA and confirmed that nucleolar localisation of RelA occurred at 10 hours following administration of the agent. This time-point confirmed aspirin-induced nucleolar targeting of RelA occurred subsequent to c-Src pathway activation (Figure 3.8c). Taken together, these lines of evidence suggest that aspirin activates c-Src upstream of the effects of the agent on NF-KB signalling.

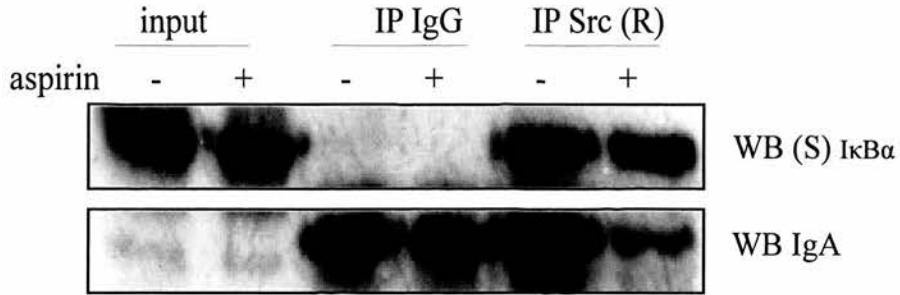
### **3.8 c-Src complexes with I $\kappa$ B $\alpha$ but not RelA**

Several studies have described a functional role for c-Src in a protein complex involved in initiating the degradation of I $\kappa$ B $\alpha$  (Abu-Amer et al 1998, Fan et al 2003, Jalal and Kone 2006, Kang et al 2005, Kang et al 2006, Lluís et al 2007). Therefore, in view of my results suggesting that aspirin activated c-Src prior to effects of the agent in the NF-KB pathway, I wished to determine if a direct physical interaction between c-Src and members of the NF-KB family occurs in CRC cells in response to aspirin treatment.

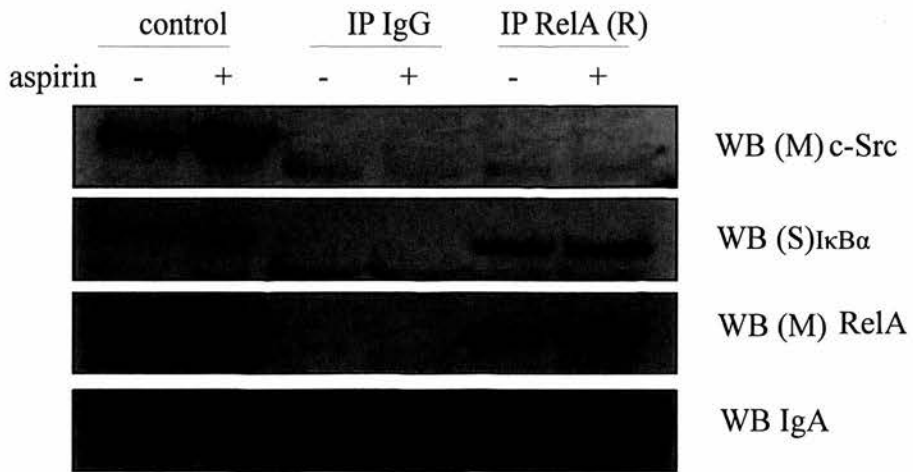
Immunoprecipitation experiments were undertaken to detect the presence of RelA/c-Src or I $\kappa$ B $\alpha$ /c-Src protein complexes in SW480 cytoplasmic cell extracts, before and after 4 hours treatment with 5mM aspirin. I found that c-Src was physically associated in immunoprecipitation complexes with I $\kappa$ B $\alpha$  before and after treatment with aspirin, but it was not found to physically precipitate in complexes with RelA (Figures 3.9a and 3.9b).

However, despite multiple attempts at replicate experiments, it was not possible to reliably report whether this interaction was quantitatively increased or decreased after aspirin treatment.

a



b



### Figure 3.9 c-Src complexes with IκBα but not RelA

SW480 cells were treated with 5mM aspirin for 0 and 4 hours and then immunoprecipitation (IP) experiments were performed with antibodies to c-Src (a) or RelA (b). a. IκBα is present in complexes with c-Src before and after aspirin treatment. Input control lanes, Rabbit (R) IgG and light chain signals are given for negative and loading controls. (n=3) b. RelA complexes with IκBα before and after aspirin treatment but not with c-Src. Input control lanes, Rabbit IgG and light chain signals are given for negative and loading controls (n=3).

R=Rabbit, M=Mouse, S=Sheep.

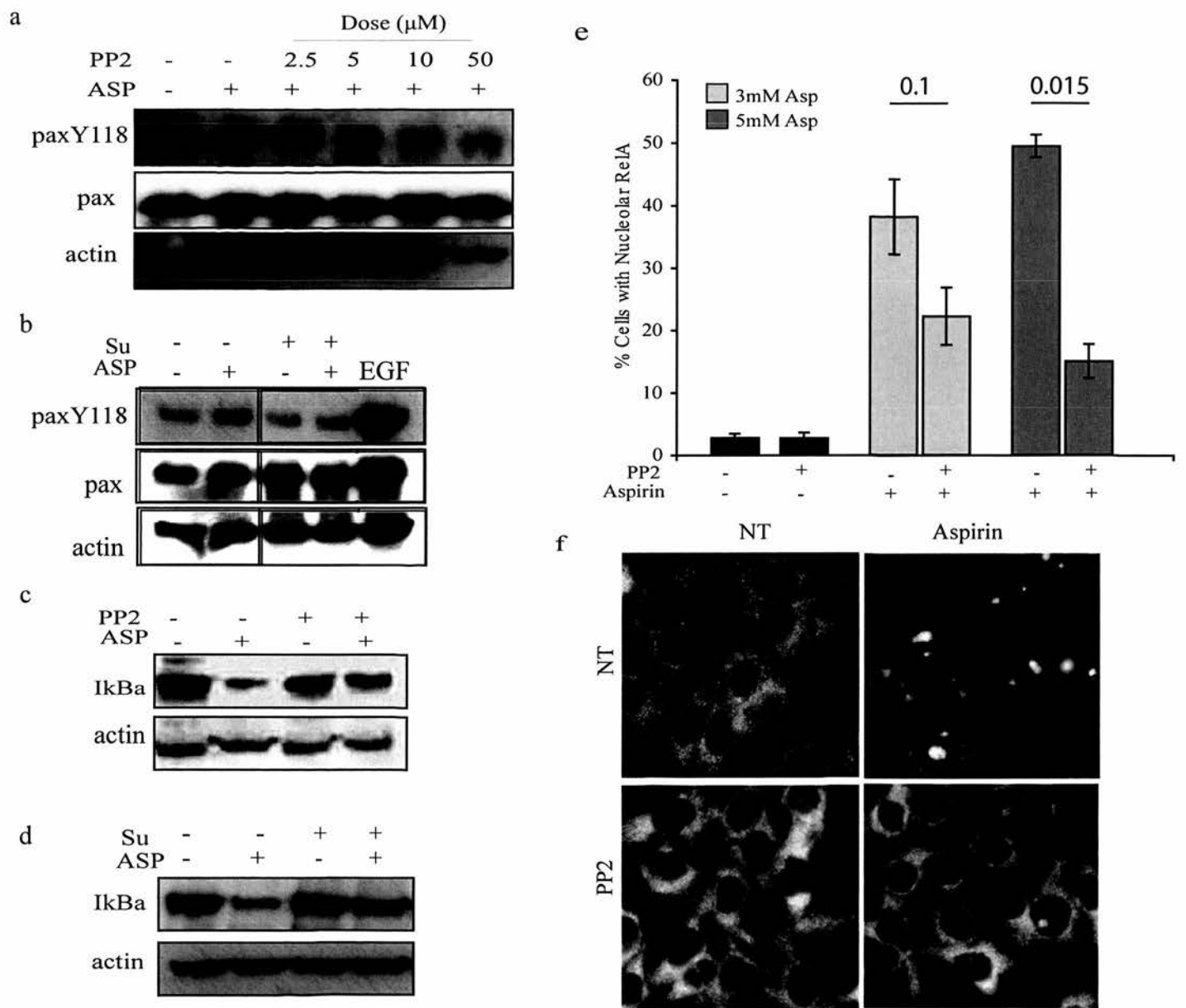
Additionally, as discussed previously, in some reports, c-Src is involved in the direct phosphorylation of I $\kappa$ B $\alpha$  tyrosine residues (Y42) in response to a range of stimulants (Elder et al 2002, Fan et al 2003, Grosjean et al 2006, Imbert et al 1996, Koong et al 1994). Attempts to assess the Y42 phosphorylation status of I $\kappa$ B $\alpha$  with antibodies raised against the phosphorylated form of I $\kappa$ B $\alpha$  Y42 were unfortunately not found to yield interpretable results due to technical difficulties with the specific antibody.

### **3.9 Chemical inhibition of c-Src inhibits aspirin's molecular effects**

Having established that aspirin activates the c-Src pathway upstream of effects on the NF- $\kappa$ B pathway, I further explored this relationship using chemical inhibitors of c-Src. PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-*d*] pyrimidine), is a widely used (but relatively non-specific) inhibitor of SFKs (Hanke et al 1996, Nam et al 2002). SU6656 (2-oxo-3-(4,5,6,7-tetrahydro-1H-indol-2-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide) is a more specific SFK inhibitor, with a high affinity to block c-Src. Modelling of SU6656 in the ATP binding pockets of c-Src suggests it functions as a competitive inhibitor (Blake et al 2000). These chemical inhibitors affect the kinase domain function of c-Src but not the Y418 phosphorylation status (Blake et al 2000). Therefore, I utilised phosphorylation of paxillin Y118 as a surrogate readout of the effective inhibitory dose of PP2/SU6656 on c-Src activity. For PP2, a dose of 10  $\mu$ M was found to be effective, in keeping with the published therapeutic window of this agent (Lu et al 2008) (Figure 3.10a). Similarly, 0.5  $\mu$ M SU6656 was demonstrated as the most effective inhibitory dose on aspirin-induced phosphorylation of paxillin Y118 (Blake et al 2000) (figure 3.10b).

Inhibition of aspirin-induced phosphorylation of paxillin Y118, by PP2/SU6656 pre-treatment, suggested that the aspirin-induced phosphorylation of paxillin Y118 was c-Src dependent, occurred downstream of aspirin-induced c-Src tyrosine kinase activity and was not a result of a non-specific global phosphorylation of tyrosine residues of many cellular proteins by aspirin.

Having established the c-Src inhibitors blocked aspirin-induced increases in the kinase activity of c-Src, I next examined their effect on the NF- $\kappa$ B response to aspirin. Firstly, western blot analysis



### Figure 3.10 Chemical inhibition of c-Src inhibits aspirin's molecular effects

SW480 cells were untreated (-) or pre-treated for 2 hrs with PP2 50 $\mu\text{M}$  (+) (a) or 10  $\mu\text{M}$  (c,e,f), 2hr) or SU6656 (Su) (0.5  $\mu\text{M}$ ) (b, d) then either untreated (-) or aspirin (Asp) treated (+) (5mM), 4hrs (a, b), 8 hrs (c+d) or 16 hrs (e, f) (a, b, c, d) Western blot analysis was performed on cell extracts using antibodies to phosphorylated paxillin (Y118) (a, b) or I $\kappa$ B $\alpha$  (c, d). Blots were stripped and reprobbed for native paxillin. An antibody to actin was used as a control for protein loading.

a+b. Aspirin induces an increase in phosphorylated paxillin (Y118) which was abrogated by pre-treatment with PP2 (a) or SU6656 (b) (n=3) c+d. Aspirin-induced I $\kappa$ B $\alpha$  degradation was abrogated by chemical inhibitors of c-Src. (n=3) e. The nucleolar localisation of RelA was examined using anti-RelA immunocytochemistry as above. At least 200 cells in at least 5 random fields of view were analysed and the proportion showing RelA in the nucleolus determined. Data are the average of 3 independent experiments (+/- standard error) (n=5). g. Representative fields of view (x63 objective). Aspirin-induced RelA nucleolar location is abrogated by c-Src chemical inhibition over a range of aspirin dosages.

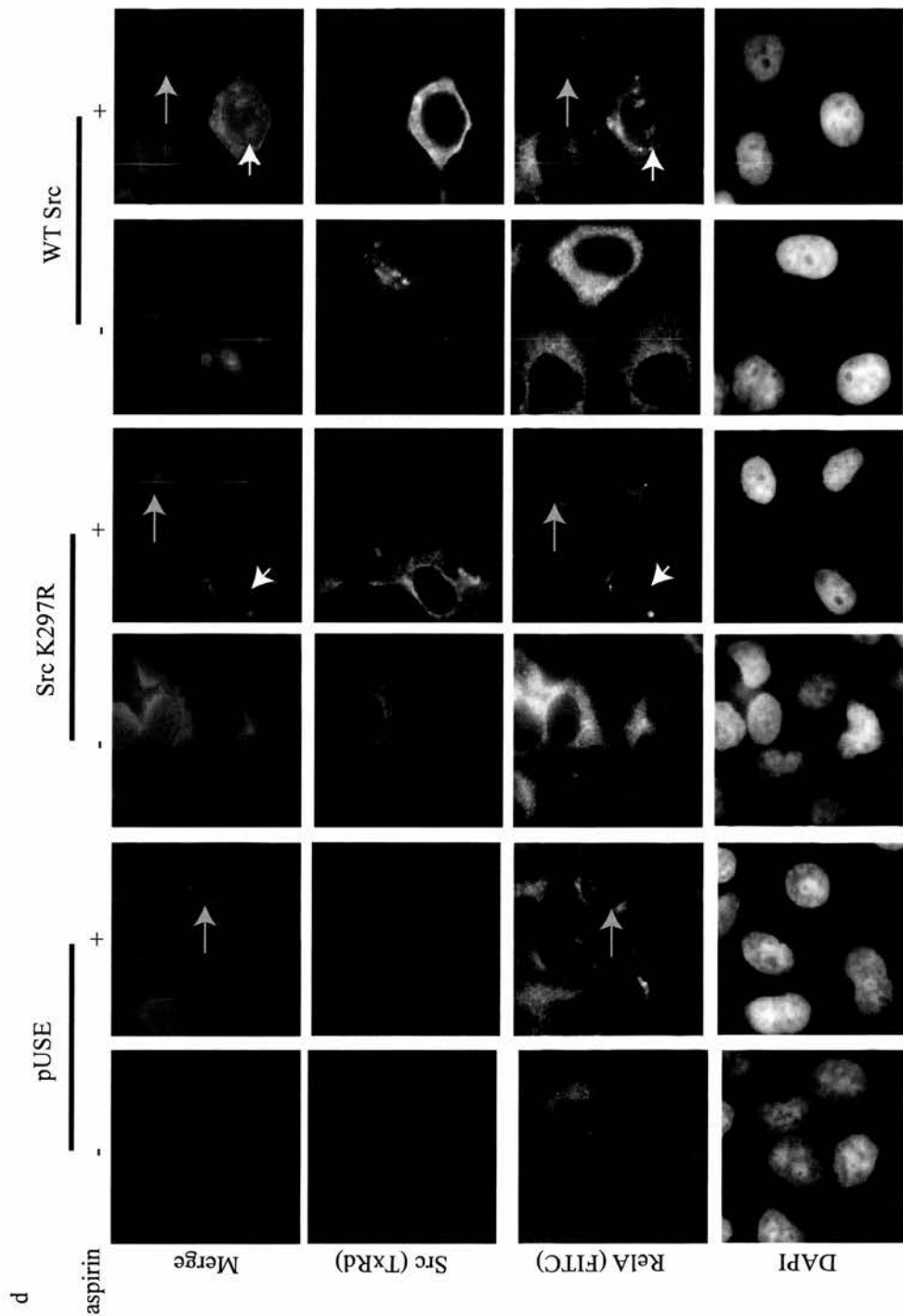
indicated that both SU6566 and PP2 blocked aspirin-mediated degradation of  $\text{I}\kappa\text{B}\alpha$  at a similar dose to that which blocked aspirin-induced phosphorylation of Y118 paxillin (Figures 3.10c and 3.10d). Next, using immunocytochemistry with antibodies directed against RelA and the nucleolar protein fibrillarin, I found that PP2 pre-treatment also inhibited aspirin-induced RelA nucleolar sequestration at 16 hours. This inhibition was observed over a range of aspirin dosages (figures 3.10e and 3.10f). The above evidence suggests that c-Src plays a key role in transmitting an activation signal stimulated by aspirin to induce Y118 phosphorylation of paxillin, degradation of  $\text{I}\kappa\text{B}\alpha$  and nuclear/nucleolar localisation of RelA.

### **3.10 Genetic inhibition of c-Src inhibits aspirin's molecular effects**

In common with many competitive ATP inhibitors, both PP2 and SU6656 have “off target” non-specific inhibitory actions/side effects affecting other SFK members or other protein tyrosine kinases. Therefore, further dissection of the relationship between c-Src and NF- $\kappa\text{B}$  signalling responses to aspirin was undertaken utilising genetic methods.

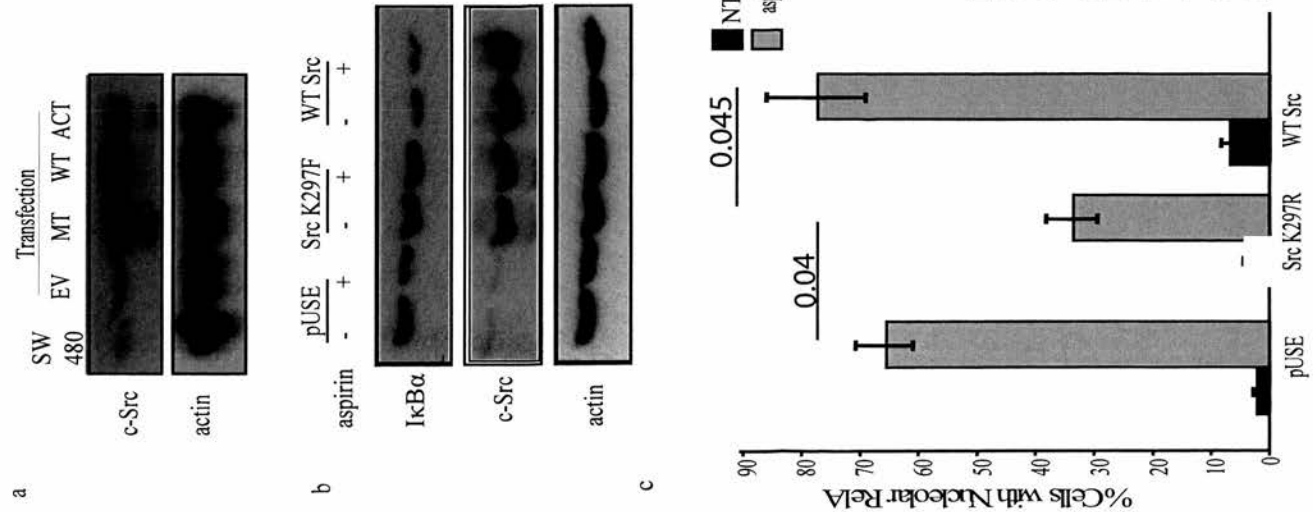
Firstly, to further investigate the role of c-Src in the NF- $\kappa\text{B}$  response to aspirin, I utilised vectors that over-expressed either a wild-type form of c-Src or a kinase-dead equivalent. Empty control pUSEamp vector was used as a control (Figure 3.11a). SW480 CRC cells were transiently transfected with the vectors then western blot analysis was used to analyse aspirin effects on cytoplasmic levels of  $\text{I}\kappa\text{B}\alpha$ . Consistent with the results obtained from c-Src chemical inhibitory studies,  $\text{I}\kappa\text{B}\alpha$  degradation after aspirin treatment was inhibited in cells expressing the kinase-dead form of c-Src but was maintained in those over-expressing wild-type protein or control vector (figure 3.11b). Of note, wild type c-Src cells expressed constitutively lower levels of  $\text{I}\kappa\text{B}\alpha$  at base line.

Furthermore, immunocytochemistry with antibodies to c-Src (to identify transfected cells) and anti-RelA indicated that the increase in the number of cells showing nuclear RelA localization in cells expressing the control vector or wild-type protein, was abrogated in cells expressing the mutant inactive c-Src. Quantification of the percentage of cells with nucleolar RelA confirmed this



**Figure 3.11 Genetic inhibition of c-Src inhibits aspirin's molecular effects**

SW480 cells were transiently transfected with an empty pUSEamp vector, kinase-inactive c-Src expression vector (pUSE Src K297R), wild-type c-Src, or constitutively active c-Src (pUSE Src Y529F). 48 hrs after transfection, cells were untreated (-) or treated with 5mM aspirin (+) for 8 hrs (b) or 16 hrs (c). a. In order to confirm transfection efficiency, anti-c-Src western blot analysis was performed on cytoplasmic cell extracts as above. Actin was used as a control for protein loading (n=3). b. anti-IkB $\alpha$  western blot analysis was performed. Blots were stripped and reprobed for endogenous paxillin. Actin was used as a control for protein loading (n=3). c. Immunocytochemistry was performed on transfected cells and the percentage of cells showing nucleolar RelA quantified as above. Data are the mean of three independent experiments  $\pm$  SE. d. Anti-RelA/c-Src immunocytochemistry was used to examine effects of expression of the c-Src proteins on aspirin-induced nucleolar translocation of RelA. Aspirin induced nuclear translocation of RelA in non-transfected cells (yellow arrows). However, those cells over-expressing pUSE Src K297R demonstrated reduced RelA translocation in response to aspirin, compared to the response in non-expressing cells or cells expressing wild-type protein (white arrows = transfected cells)(n=6).



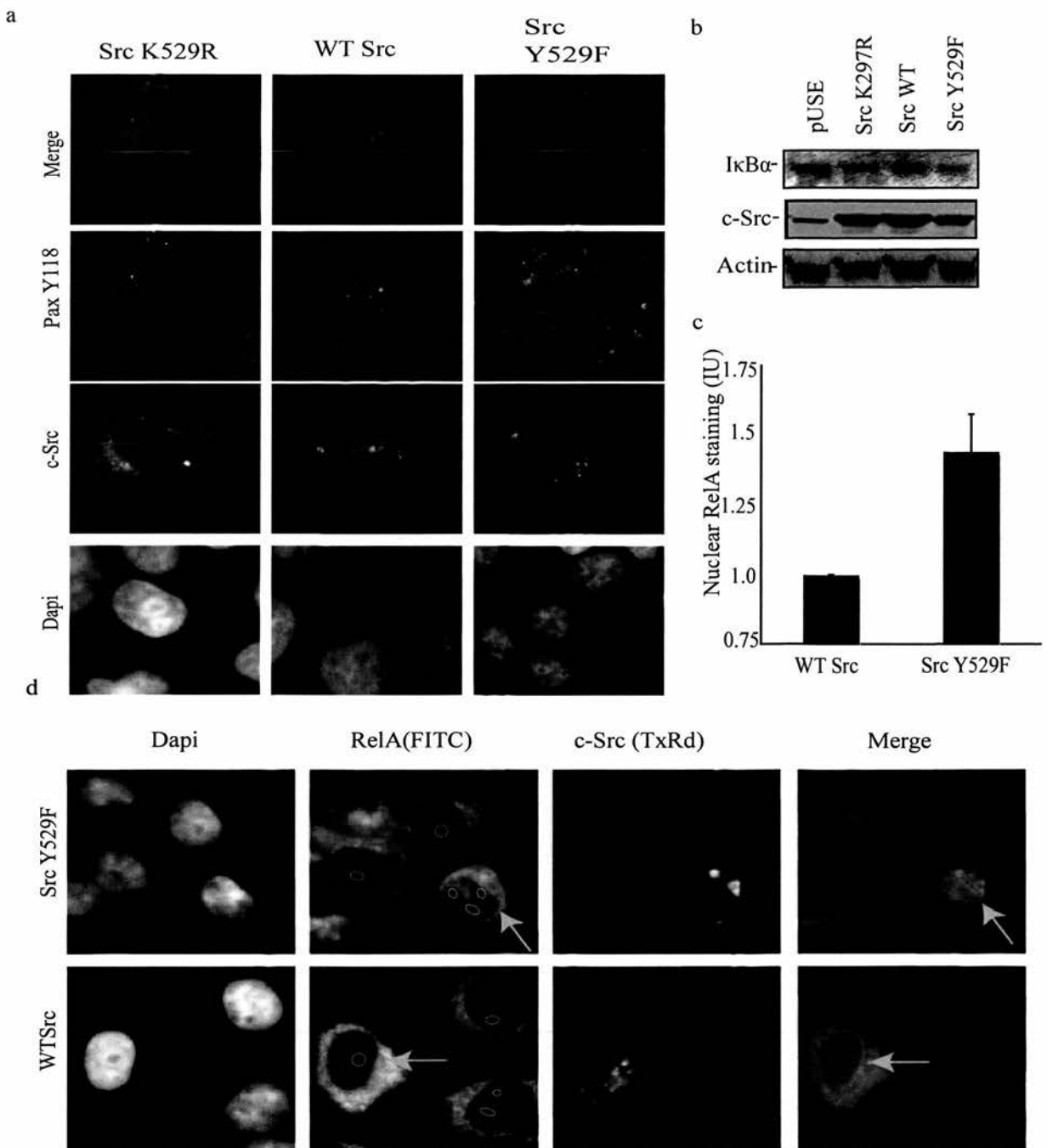
observation (figure 3.11c and 3.11d). These results confirmed that c-Src kinase function played a key role in aspirin-induced degradation of I $\kappa$ B $\alpha$  and the RelA response to aspirin.

### **3.11 Active c-Src activates NF- $\kappa$ B independently of aspirin**

Whilst the above results suggested a role for c-Src kinase function in the aspirin-induced NF- $\kappa$ B response, I also aimed to determine whether this response was specific to aspirin induced stimulation of c-Src. Utilising cells which were transiently transfected with a vector to over-express a constitutively active form of c-Src, c-Src (Y529F) effects on NF- $\kappa$ B signalling were assessed.

Firstly, I confirmed that the active mutant was effective by performing immunocytochemistry for Y118 phosphorylated paxillin. Active c-Src over-expression induced widespread phosphorylation of paxillin Y118 within the cell, producing a dramatically different appearance in contrast to those cells expressing the wild type protein or the kinase-dead c-Src mutant, K297R (Figure 3.12a). The membrane distribution was similar in nature to that observed following EGF treatment but of much greater intensity. These data confirmed the activity of the mutant. On examination of NF- $\kappa$ B signalling, I found that over-expression of the active form of c-Src mimicked aspirin-induced molecular effects by inducing degradation of I $\kappa$ B $\alpha$  in SW480 cells after 24 hours (figure 3.12b). Those cell populations which expressed the empty pUSEamp vector, or those expressing the wild type form of c-Src, did not demonstrate an independent effect on I $\kappa$ B $\alpha$  levels. Immunocytochemical analysis demonstrated that expression of active c-Src induced an increase in nuclear RelA. Computerised quantification of images confirmed that whilst wild-type c-Src transfected cells did not independently induce RelA nuclear translocation, activated c-Src (Y529F) induced high levels of nuclear RelA within transfected cells (figure 3.12c). Whilst expression of active c-Src induced nuclear RelA, further analysis utilising the latter endpoint of RelA nucleolar targeting was undertaken. Examining individual c-Src(Y529F) expressing cells demonstrated that c-Src (Y529F) did not appear to induce RelA nucleolar localisation (figure 3.12d). As this is highly related to the pro-apoptotic response to aspirin, there may be a requirement for an, as yet unknown, additional aspirin-induced co-factor and/or other cellular response within the nucleus that targets RelA to the nucleolus.





**Figure 3.12 Active c-Src activates NF-κB independently of aspirin**

SW480 cells were transiently transfected with an empty pUSEamp vector, kinase-inactive c-Src expression vector (Src K297R), wild-type c-Src, or constitutively active c-Src (Src Y529F) and harvested forty eight hours after transfection a. Anti-paxillin (Y118) immunocytochemistry. Phosphorylated paxillin Y118 is highly expressed, primarily at the cell periphery in cells expressing Src Y529F. This activation was in a pattern similar to that observed with aspirin and EGF stimulation. pUSE Src K297R-and wild-type c-Src expressing cells demonstrated much-reduced paxillin Y118 expression levels. DAPI was used as a nuclear marker. (n=3) b. Anti-IκBα western blot analysis was performed. Src Y529F induced a decrease in the cytoplasmic levels of IκBα that was not observed in the Src K297R, wild-type c-Src or pUSEamp vector transfected cells (n=4) c. Anti-RelA/anti-c-Src immunocytochemistry was used to examine effects of expression vectors on RelA localization (yellow arrows = transfected cells, yellow circles = nucleoli; areas devoid of DAPI surrounded by a hyperchromatic ring). Src Y529F induced RelA nuclear but not nucleolar localisation. (n=4) d. The nuclear localisation of RelA in c-Src transfected cells was quantified, using anti-RelA immunocytochemistry, as described in Figure 3.7. Src Y529F induced RelA nuclear localisation (n=4).



### **3.12 Chemical inhibitors of c-Src inhibit aspirin's apoptotic effects (PP2/SU6566)**

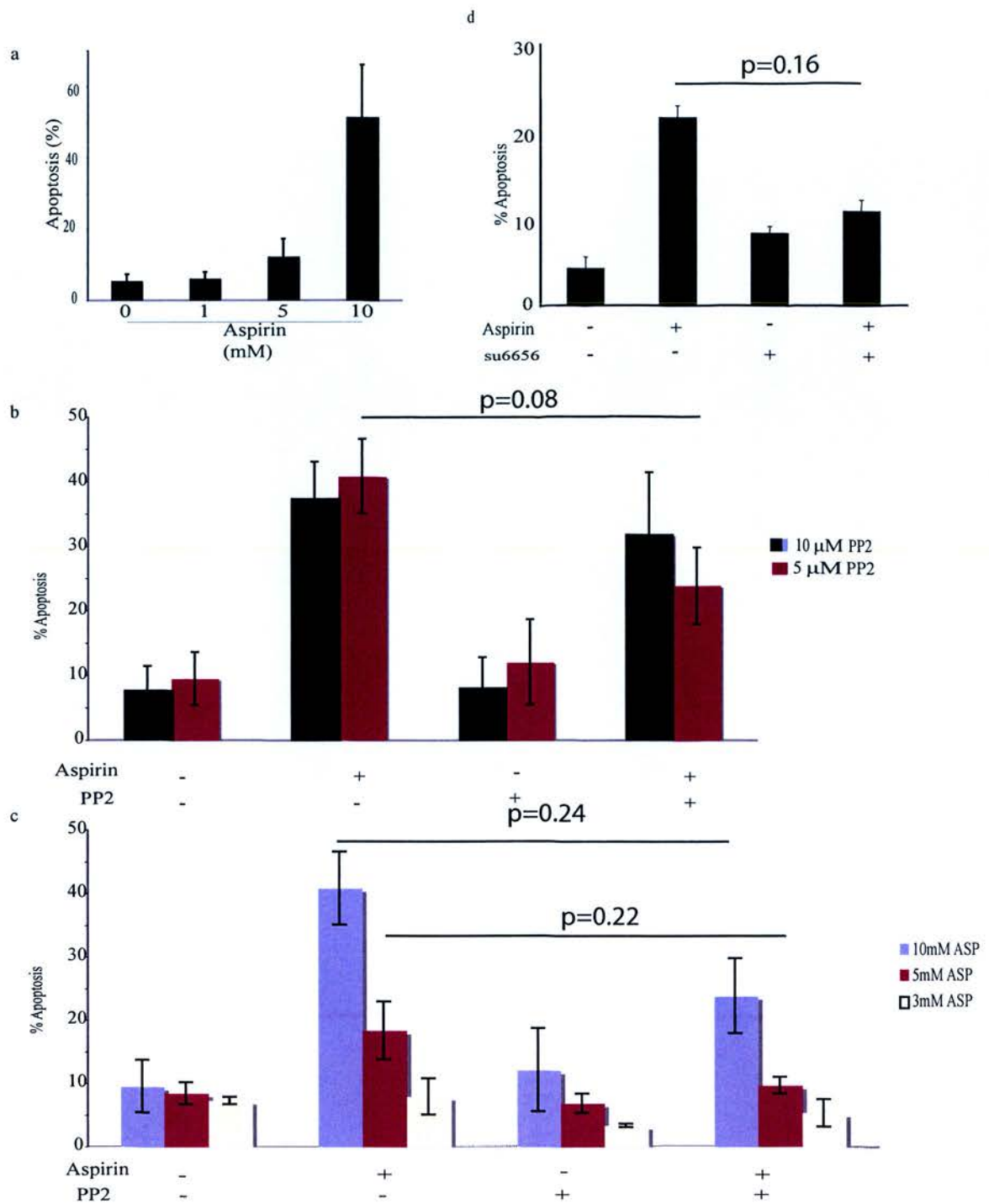
As NF- $\kappa$ B signalling is a key element in the aspirin-induced pro-apoptotic effect in CRC cells, and experimentation to date has suggested a c-Src dependency in aspirin-mediated NF- $\kappa$ B activation, further investigation was required to explore the role of c-Src role in the aspirin-induced pro-apoptotic response.

Previous studies have demonstrated that nuclear fragmentation and classical apoptotic bodies are rarely seen in conjunction with aspirin-induced apoptosis in SW480 CRC cells (Castano et al 1999, Stark et al 2001). However, aspirin does induce an increase in the percentage of cells showing externalization of phosphatidylserine (PS), a marker for apoptosis detected by FITC-AnnexinV binding (Stark et al 2001). Apoptosis in the following studies was therefore detected using an AnnexinV apoptosis assay kit which has been well characterised in the host laboratory.

Firstly, experiments were performed to confirm a pro-apoptotic response to aspirin in SW480 cells (figure 3.13a). In order to assess the role of c-Src in the apoptotic response to aspirin, SW480 cells were pre-treated with c-Src chemical inhibitors, prior to aspirin administration. PP2 pre-treatment was partially effective in inhibiting aspirin-induced apoptosis over a range of aspirin doses. In addition, the inhibitory effect of PP2 was of a similar dose to that which had inhibited aspirin-induced paxillin Y118 phosphorylation, I $\kappa$ B $\alpha$  degradation and RelA nucleolar localisation (figures 3.13b and 3.13c). Further confirmatory studies utilising the alternative c-Src chemical inhibitor, SU6566, also demonstrated an inhibitory effect on apoptosis following aspirin at a dose which also previously demonstrated an inhibitory effect on aspirin-induced paxillin Y118 phosphorylation, I $\kappa$ B $\alpha$  degradation and RelA nucleolar targeting (figure 3.13d).

### **3.13 Genetic modulation of c-Src function mediates aspirin-induced apoptosis**

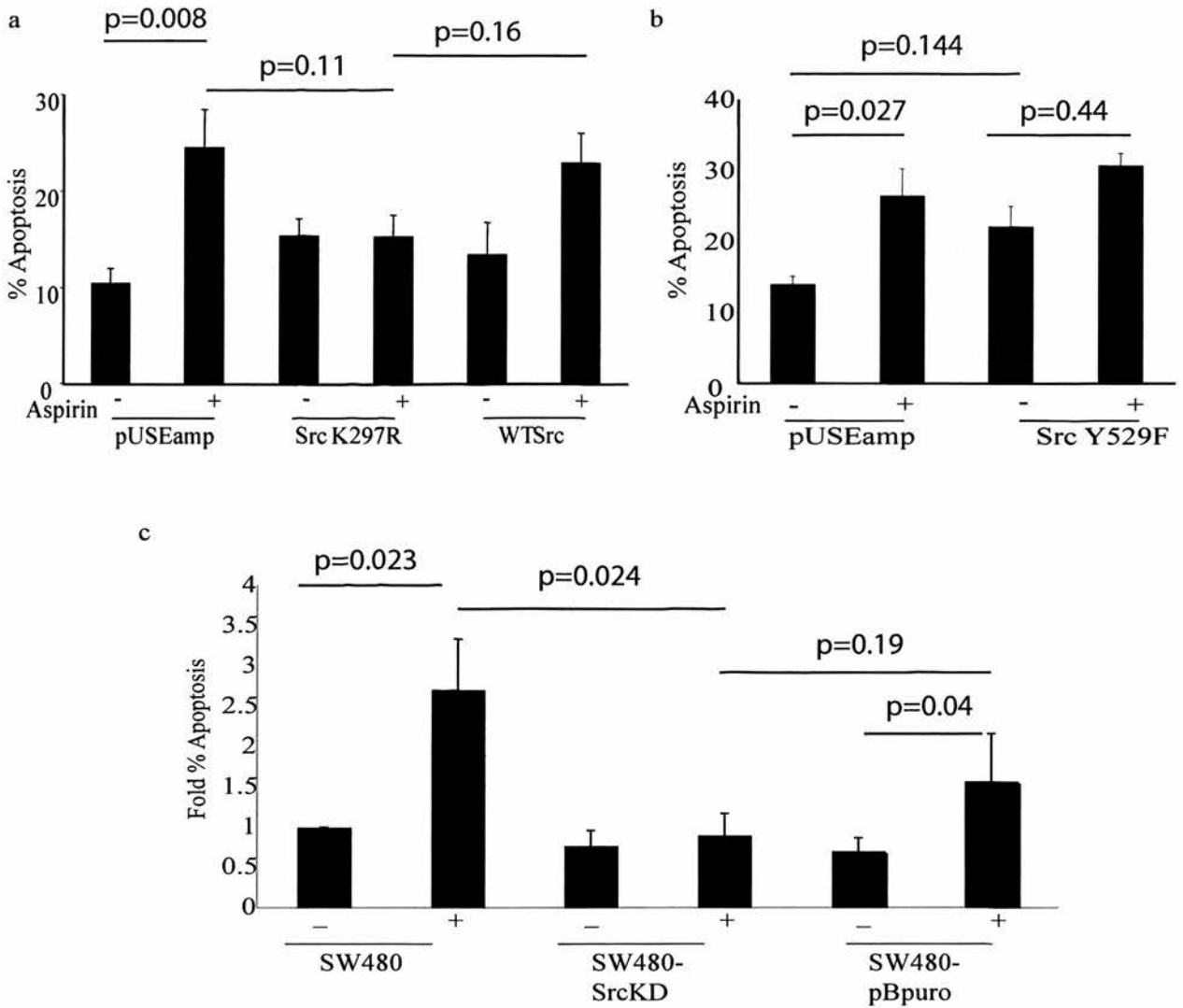
In keeping with the above data, I found that expression of kinase-dead c-Src also partially inhibited aspirin-mediated apoptosis, compared to empty vector or wild-type protein expression. However, there was also a slight increase in baseline apoptosis in cell populations expressing Src K297R, suggesting over expression of an inactive c-Src mutant protein could be pro-apoptotic (figure 3.14a).



**Figure 3.13 Chemical inhibitors of c-Src inhibit aspirin's apoptotic effects (PP2/SU6566)**

SW480 cells were either untreated (-) or pre-treated with chemical inhibitors PP2 (b, c) or SU6656 (d) prior to aspirin (0(-), 5 (+) mM, 16hrs (a-d) or dose stated). The percentage of cells in the population undergoing apoptosis was determined using AnnexinV-FITC apoptosis assays. Data presented is the minimum of 3 independent experiments (+/- standard error).

a. Aspirin induces a dose-related pro-apoptotic trend in SW480 cells (n=4) b. PP2 reduced aspirin-induced apoptosis over a range of PP2 dosages (n=5). c. SW480 cells were either untreated (-) or pre-treated with PP2 (10  $\mu$ m) for 2hrs prior to treatment with aspirin (5 or 10 mM). PP2 partially inhibited aspirin induced apoptosis over a range of aspirin dosages. (n=4) d. SW480 cells were either untreated (control) or pre-treated with SU6656 for 2hrs prior to treatment with aspirin (0(-), 5(+) mM; 16hrs). SU6656 reduced aspirin induced apoptosis (n=4).



**Figure 3.14 Genetic modulation of c-Src function mediates aspirin-induced apoptosis**  
 SW480 cells were transiently transfected with c-Src expression plasmids as described in figure 3.10, (a, b) or SW480-SrcKD or SW480-pBpuro cells (c) were treated with aspirin (0 (-) 16hrs (+)). The percentage of cells in the population undergoing apoptosis was determined using AnnexinV-FITC apoptosis assays. Data presented is the minimum of 3 independent experiments (+/- standard error). a. Src K297R expression partially inhibited the aspirin induced pro-apoptotic response (n=8). b. Src Y529F expression induced a trend towards a pro-apoptotic response independent of aspirin effects (n=3) c. SW480-SrcKD cells were resistant to the aspirin-induced apoptotic response whilst the response was preserved in SW480-pBpuro cells (n=6).

The observation that over-expression of wild type c-Src does not affect the magnitude of the aspirin-induced apoptotic response but that expression of an inactive form of the protein does inhibit this response again confirms that the level of c-Src expression in CRC cells was less relevant to aspirin-induced apoptosis than the functionality and level of c-Src activation.

Fig 3.14 demonstrates that there is a *trend* towards increased mean apoptosis in cells which were transfected with Y529F active c-Src construct, compared to cells transfected with the empty vector. However, this trend was not statistically significant. Aspirin induced an apoptotic response in both those cells transfected with an empty vector and in those Y529F transfected cell populations, with a *partial trend* towards a higher response in those cells transfected with the active c-Src construct and treated with aspirin. Therefore, I found that this mutant produced a pro-apoptotic effect that increased baseline levels of apoptosis, mimicking the pro-apoptotic effects of aspirin treatment. This effect was increased further when aspirin was administered (figure 3.14b). Taken together, the above data provides potential evidence that activation of c-Src can induce NF- $\kappa$ B signaling and function as a pro-apoptotic stimulus in CRC cells.

As there is variation in transfection efficiency with transient transfections and concerns over the potential for the confounding effect of non-transfected cell sub-populations, I investigated the use of stably transfected SW480 cell engineered to express a kinase dead form of c-Src (SW480-Src KD), (a gift from Professor Margaret Frame, Edinburgh Cancer Centre). Control cell were those stably transfected with the pBpuro empty vector alone (SW480-pBpuro). To determine whether kinase-dead c-Src also blocked the apoptotic effects of aspirin in this system, Annexin V apoptosis assays were performed on SW480, SW480-SrcKD and SW480-pBpuro cells. Figure 3.14c demonstrates aspirin-induced apoptosis was abrogated in SW480-SrcKD cells, compared to the control SW480-pBpuro cell line. However, the aspirin-induced apoptotic response was also blunted in the control cell lines transfected with the empty SW480-pBpuro vector. There are a number of possible explanations for this, including that the inhibitory effect in these cells may not be c-Src specific, may relate to contamination of the cell populations, or as a consequence of the vector transfection process or of the specific vectors used.

In further attempts to characterise these cell lines, the aspirin-induced RelA nucleolar targeting response was assessed. Contrary to expectations, neither SW480-SrcKD nor SW480-pBpuro KD cell lines demonstrated an aspirin-induced NF- $\kappa$ B response. Therefore, further experimental work in these cell lines was abandoned in lieu of their further characterisation.

### 3.14 Discussion

The present studies were undertaken to determine the upstream mechanisms by which aspirin stimulates the NF- $\kappa$ B pathway and mediates apoptosis with specific emphasis on the role of the c-Src tyrosine kinase. The work presented here provides new insights into the anti-tumour activity of the agent. Firstly, I demonstrate that aspirin induces phosphorylation of c-Src and activates c-Src tyrosine kinase activity. Secondly, I show that the NF- $\kappa$ B response to aspirin is mediated through c-Src and finally, demonstrate that the apoptotic effects of aspirin are dependent upon the activation status of the c-Src tyrosine kinase. These findings could have implications for the development of novel chemopreventative agents and specifically, those that modulate c-Src kinase activity.

The evidence presented here suggests that aspirin induces c-Src activity in SW480 CRC cells, as determined by Y418 phosphorylation of the protein and phosphorylation of the recognised c-Src substrate, paxillin Y118. However, these data are in contrast to those of Kunte *et al.*, (Kunte et al 2008) who reported that sulindac sulfide activates the negative regulator of c-Src, CSK, to inhibit c-Src activity in HT29 CRC cells. This apparent difference may in fact relate more to the dose and type of NSAID used in his study and the particularly prolonged duration of exposure to the agent (72hrs). Casanova *et al.*, (Casanova et al 2008) also reported that the NSAID, Celecoxib, inhibits c-Src activity in acute myeloid leukaemia cell lines. However, interestingly, in 4 out of the 5 cell lines presented within his report, there was actually an apparent transient increase in c-Src Y418 phosphorylation at 4 hours following agent administration. Furthermore, this appeared to occur concurrently with the proteolytic cleavage of Poly (ADP-ribose) polymerase (PARP) and activation of caspases. It is likely that aspirin and NSAID effects on c-Src activity are related to the cellular context, chemical class, timing and concentration used. Notably, these differential effects are consistent with the variation in aspirin/NSAID-induced effects observed on NF- $\kappa$ B pathway signalling in different cell environments and treatment conditions. For example, following TNF $\alpha$  pre-treatment of CRC cells, certain NSAIDs inhibit activation of NF- $\kappa$ B signalling however, in the absence of TNF $\alpha$  pre-treatment, the converse molecular response is observed (Stark et al 2001, Yamamoto et al 1999).

Inhibiting c-Src activity, using chemical inhibitors or a kinase-dead mutant, reduced aspirin-mediated degradation of I $\kappa$ B $\alpha$  and nuclear translocation of RelA, suggesting a relationship between these two events. A variety of putative mechanisms for c-Src-mediated activation of the NF- $\kappa$ B signalling pathway have been reported. Fan (Fan et al 2003) describes an IKK-independent pathway in which c-Src directly interacts with I $\kappa$ B $\alpha$  and directly phosphorylates the protein on tyrosine residue 42. They suggested that this phosphorylation event leads to nuclear translocation of NF- $\kappa$ B in the absence of I $\kappa$ B $\alpha$  degradation. This response follows a c-Src physical interaction with I $\kappa$ B $\alpha$ . Other authors have suggested that c-Src-induced tyrosine phosphorylation of I $\kappa$ B $\alpha$  may potentially play a role in controlling an alternative activation pathway concerning NF- $\kappa$ B (Lluis et al 2007). Kang et al., (Kang et al 2005, Kang et al 2006) also suggested a physical interaction between c-Src and I $\kappa$ B $\alpha$  is required for LPS- and pervanadate-induced NF- $\kappa$ B activation. They also demonstrated that chemical and genetic inhibitors of c-Src blocked this interaction. Kang et al., (Kang et al 2006) also described how silica-induced NF- $\kappa$ B activation required tyrosine phosphorylation of I $\kappa$ B $\alpha$  in macrophages. An effect again blocked by c-Src chemical inhibitors.

These reports contrast with my results that indicate that c-Src plays a key role in aspirin-induced degradation of I $\kappa$ B $\alpha$ . Interestingly, c-Src-mediated NF- $\kappa$ B activation has also been shown to involve stimulation of intermediate kinase pathways, such as Protein Kinase C Alpha (PKC $\alpha$ ) and I $\kappa$ B $\alpha$  kinase (IKK) signaling pathways. For example, IL-1 induced activation of c-Src has been linked to sustained Ser177/181 phosphorylation of I $\kappa$ B $\alpha$  Kinase (IKK $\beta$ ), which in turn phosphorylates I $\kappa$ B $\alpha$  at Ser 32/36, leading to its polyubiquitination and proteosomal degradation. The Ser/Thr phosphatase PP2A, dephosphorylates Ser 177/181 IKK $\beta$  following IL-1 stimulation, thus allowing re-accumulation of I $\kappa$ B $\alpha$ . Interestingly, PP2A is a known substrate of c-Src. c-Src induces Tyr307 phosphorylation of PP2A, which subsequently inhibits the activity of the phosphatase, causing sustained I $\kappa$ B $\alpha$  degradation and therefore NF- $\kappa$ B activation (Barisic et al 2008). Sustained I $\kappa$ B $\alpha$  degradation is a notable feature of aspirin-induced NF- $\kappa$ B responses and, in the context of previous studies evaluating aspirin's chemopreventative potential in colorectal cancer cells, it has also been found to inhibit PP2A (Bos et al 2006). These putative mechanisms classically induce NF- $\kappa$ B signalling by I $\kappa$ B $\alpha$  serine



32/36 residue phosphorylation and subsequent proteosomal-mediated I $\kappa$ B $\alpha$  degradation (Mendez-Samperio et al 2007) (Huang et al 2003a) (Lin et al 2006).

c-Src has also been described to be an integral part of the IKK complex. In work looking at the NF $\kappa$ B-dependent COX-2 and ICAM-1 promoter activity, Huang et al. (Huang et al 2003b)) reported that TNF $\alpha$  activated a c-Src dependent pathway, through activating NIK, which resulted in the physical association of c-Src with IKK, phosphorylation of IKK tyrosine residues 188 and 199, and NF- $\kappa$ B activation. Chemical and genetic inhibition abrogated this association and blocked the activation of downstream NF- $\kappa$ B activity. Similarly, Lin et al. (Lin et al 2006) reported that Thrombin induced a physical complex of PKC, c-Src and IKK $\alpha/\beta$  and that PKC chemical inhibition or over-expression of a dominant negative c-Src blocked I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B signalling induced by thrombin. It will now be of extreme interest to identify the pathway that links c-Src to I $\kappa$ B $\alpha$  degradation in response to aspirin and NSAIDs. One potential mechanism may involve NF- $\kappa$ B activation following aspirin-induced c-Src-mediated effects on the cytoskeleton.

CRC chemopreventative drugs are known to modify integrin-mediated signaling pathways, including those responsible for motility and survival (Weyant et al 2000). c-Src has a key role in focal adhesion turnover, and its role in invasion and motility is one of the best characterised (Serrels et al 2006). Upon integrin engagement (as previously discussed in chapter 1.6.3), Focal Adhesion Kinase (FAK) becomes phosphorylated at Y397, creating a high-affinity binding site for c-Src, which then further phosphorylates FAK on other specific residues. The resultant c-Src/FAK complex phosphorylates a number of other proteins, including Grb2 and other focal adhesion proteins, such as paxillin on Y118 and talin, causing the recruitment of other signalling proteins to the FAK complex. NSAIDs, such as sulindac sulfide, have been shown to cause rearrangement of the actin cytoskeleton and the attendant loss of focal adhesion plaques (Weyant et al 2000). These findings suggest that aspirin may stimulate factors upstream of c-Src, potentially involving the extra-cellular matrix, through pathways that are mediated by integrins and FAK. Further identification of these responsive elements would be advantageous to fully characterizing if a specific receptor or molecular element exists which is responsible for the aspirin-induced effect.



Previous reports have suggested that c-Src substrates, including MEK/ERK and p38 are involved in the molecular mechanisms underlying aspirin/NSAID-induced cell cycle arrest and apoptosis (Elder et al 2002, Thoms et al 2007b). As discussed above, the host laboratory previously demonstrated that p38 is activated in response to aspirin (Thoms et al 2007a) and that this effect is upstream of effects on the NF- $\kappa$ B pathway. Since p38 is a known target of c-Src, it is possible that c-Src effects a response on the NF- $\kappa$ B pathway through p38 signalling. Bernardi et al. reported that activation of c-Src is required for the anti-proliferative effects of indomethacin in C6 Glioma cells and that chemical inhibition of c-Src prevented NSAID-induced ERK activation and the pro-apoptotic response (Bernardi et al 2006). Determining the pathways that link c-Src, p38 and NF- $\kappa$ B is an area where further dissection of the molecular mechanisms by which c-Src activates or interacts with I $\kappa$ B $\alpha$  requires much further work.

Whilst increased c-Src activity has previously been associated with CRC progression and metastasis (Mao et al 1997, Talamonti et al 1993, Termuhlen et al 1993), here we show that activating endogenous c-Src using aspirin, or expressing a constitutively active form of the protein, induces apoptosis in CRC cells. These findings are in keeping with those of Welman et al., who showed that expression of active c-Src had growth inhibitory effects against HCT116 and SW480 CRC cells (Welman et al 2006). In early reports in chicken embryoblasts, cells which expressed the constitutively active form of c-Src induce a pro-apoptotic response and that this effect is further amplified by the addition of aspirin/NSAIDs (Lu et al. 1997). Using an *in situ* approach in *Drosophila*, it has also been reported that the cellular outcome of altering Src signaling is dependent upon the degree of activation. Low levels of activation are associated with epithelial cell survival and proliferation, whilst high levels of activation mediated apoptosis (Vidal et al 2007). Cells which over-express c-Src, such as neoplastic colonic cells, can become sensitized to apoptotic stress in particular cellular environments (Vidal et al 2007, Zhong et al 2002). Indeed, serum starvation and RAS/PI3K inhibition favour pro-apoptotic signaling in cells which over-express activated c-Src (Webb et al 2000). This is of interest, as in my studies, treatment took place in low-serum media.

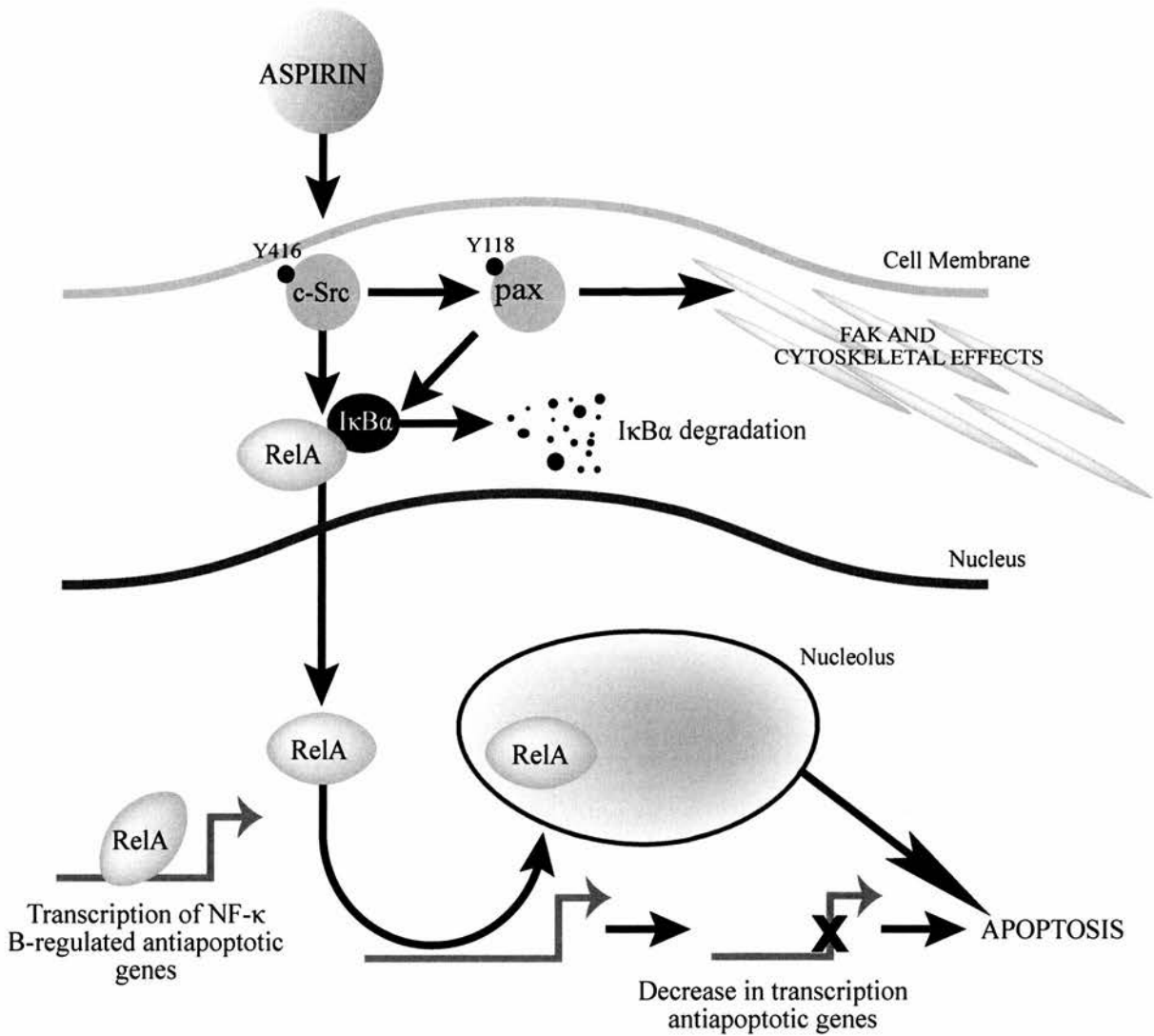
Cells which were transiently transfected to over-express active c-Src had activated NF- $\kappa$ B signalling, and a pro-apoptotic response, but in contrast to aspirin treatment, RelA nucleolar localization was not observed. This suggests that whilst c-Src may be causally involved in mediating stimulation of the NF- $\kappa$ B activation, there is a requirement for a further aspirin-induced co-factor to induce RelA nucleolar localization. Although the nature of this cofactor is unknown at present, putative candidates have been previously suggested, including NFBP, nucleophosmin/B23 and p14<sup>ARF</sup> (Stark and Dunlop 2005). Work on the potential role of these candidates continues in other research streams within the host laboratory.

Here, I employed the SW480 cell line as an experimental model, as it has been extensively studied in relevant experiments in the host laboratory and the NF- $\kappa$ B and apoptotic response to aspirin within this system have been well characterised. Furthermore, it was also felt that such a CRC cell line, with low levels of c-Src expression, would allow over-expression studies to be carried out without the confounding effects of high levels of endogenous protein. Research from this laboratory has demonstrated that the aspirin-induced pro-apoptotic and NF- $\kappa$ B effects occur in a wide variety of CRC cell-lines, irrespective of the known levels of high and low c-Src expression (Din et al 2004). Through modification of the genetic expression levels of c-Src within SW480 cells and the use of alternative cell lines, data presented here confirmed that the level of c-Src protein expression was not relevant to the aspirin-induced response, rather the cellular activation status of the kinase.

It is clear that the mechanisms by which aspirin acts against CRC cells are complex. The diversity of biological systems in which c-Src has been implicated suggests that there is unlikely to be a single c-Src substrate target protein or pathway involved. The work presented here proposes a model for aspirin/NSAID-induced modulation of NF- $\kappa$ B signalling and apoptosis. In the absence of aspirin/NSAIDs, NF- $\kappa$ B signalling drives the transcription of anti-apoptotic genes. Following treatment with aspirin/NSAIDs, c-Src is activated, leading to phosphorylation of paxillin, focal adhesion disassembly, tubulin reorganisation, and stimulation of intermediate kinase pathways resulting in downstream degradation of the NF- $\kappa$ B cytoplasmic inhibitor, I $\kappa$ B $\alpha$ . Subsequently,

RelA/NF- $\kappa$ B complexes undertake translocation into the nucleus/nucleolus. Nucleolar localization of RelA is causally involved in the repression of NF- $\kappa$ B driven transcription of anti-apoptotic genes leading to cellular apoptosis (figure 3.15). These data therefore indicate that activation of c-Src is a key component of the pro-apoptotic effects of aspirin in CRC and invokes molecular mechanisms through stimulation of the NF- $\kappa$ B pathway.

These findings have relevance to the future development of chemopreventative agents by shedding further light on the complex nature of the cellular and molecular effects of aspirin on CRC. I next set out to translate these, and other, aspirin-related molecular findings derived from *in vitro* experimental work into a human *in vivo* model system.



**Figure 3.15 A model for aspirin/NSAID-induced modulation of NF-κB signalling and apoptosis.** In the absence of aspirin/NSAIDs, constitutive NF-κB signalling drives the transcription of anti-apoptotic genes. Following treatment with aspirin/NSAIDs, c-Src induces phosphorylation of paxillin (Y118), FAK aggregate dissolution and changes in β-tubulin cytoskeletal structure. It also stimulates intermediate kinase pathways resulting in downstream degradation of the NF-κB cytoplasmic inhibitor, IκBα. Subsequently, RelA/NF-κB complexes translocate into the nucleus/nucleolus. Nucleolar localization of RelA is causally involved in the repression of NF-κB-driven transcription of anti-apoptotic genes, leading to cellular apoptosis.

## **Chapter 4 – Study of aspirin induced effects and Wnt/notch modulation in an *in vitro* culture**

<b><u>model of human large bowel mucosa</u></b>	<b>160</b>
4.1 Background	160
4.2 Short-term outcomes of explants are dependent on culture media	164
4.3 Longer-term outcome of explants are dependent on culture media	169
4.4 Optimisation of explant culture conditions	172
4.5 Characterisation of apoptosis and proliferation in long-term culture	173
4.6 Differentiated cell populations during long-term culture	176
4.7 Identification and characterisation of colonic stem cells in IVOC	182
4.8 Regenerative cyst characterization	184
4.9 Aspirin-mediated modulation of c-Src in colonic mucosal explants	187
4.10 Aspirin modulation of paxillin in mucosal explants	189
4.11 Aspirin-induced NF- $\kappa$ B signalling and apoptosis in human colonic mucosal explants	192
4.12 <i>Ex vivo</i> growth of colonic polyps	193
4.13 Lithium chloride activates colonic mucosal Wnt signalling	197
4.14 Inhibition of Notch signalling induces goblet cell differentiation	201
4.15 Discussion	204

#### **4.1 Background**

The human intestine is a self-renewing organ, with one of the highest cell turnover rates in the body. It is composed of stem cells, transit amplifying cells and terminally differentiated cells (including absorptive enterocytes, mucus secreting goblet cells, hormone secreting entero-endocrine cells and paneth cells) (Gregorieff and Clevers 2005, Wilson and Radtke 2006). The data described thus far, was elucidated using *in vitro* culture of homogenous CRC cell lines. However, cell culture techniques fail to accurately recapitulate the colonic architectural complexity, the diversity of the differentiated colonocyte populations or reproduce the important regulatory role played by stroma/epithelial interactions (Bjerknes and Cheng 2006). Therefore, more representative models are required so that the *in vivo* relevance of data obtained *in vitro* can be explored.

There have been many attempts to produce experimental model systems that mimic normal intestinal growth and differentiation. Previously, some of the most widely used systems to explore homeostasis in the intestinal epithelium include animal models. The drosophila mid-gut demonstrates usefulness in providing a platform for dissection of basic molecular processes (Jiang and Edgar 2011, Jiang et al 2011a, O'Keefe et al 2011, Shaw et al 2010) but fundamentally, experimental results cannot directly translate findings with relevance to the human *in vivo* environment. Additionally, even more advanced models often exhibit significant behavioural differences to the responses reported from research in the human *in vivo* setting (Menke et al 2010, Schuller et al 2007). For example, mouse tumour models clearly demonstrate fundamental differences between mouse and human gut. Humans carrying APC deletions are susceptible to colon cancer, whereas APC-deficient mice suffer mainly from small intestinal tumours (Moser et al 1995). Human and animal *in vivo* studies are also restricted by important ethical considerations surrounding the toxicity and side-effect profiles of potential chemical agents (such as inhibitors or agonists) required to dissect the molecular pathways involved in colonocyte responses to agents and to explore regulation of mucosal homeostasis.

Organotypic assays are occasionally utilised as they are more biologically relevant than simple monolayer cell cultures (Honer zu Bentrup et al 2006, Kalabis et al 2008, Ootani et al 2009, Sato et al

2009). However, these systems are labour-intensive and can be technically challenging. Therefore, whilst a variety of experimental models continue to generate a wealth of valuable information, there remains a requirement to translate findings into a more representative human context in order to discover what is of genuine biological relevance.

Long-term *in vitro* organ culture (IVOC) of human *ex vivo* colonic mucosa could provide a more representative model. One of the first descriptions of successful organ culture of human intestinal mucosa was described by Browning and Trier in 1969 (Browning and Trier 1969). Prior to this, experimentation had been limited by the observation of marked mucosal epithelial cell necrosis and disintegration beyond 2-3 hrs *in vitro*. In Browning and Trier's experiments, small bowel mucosa was biopsied from human volunteers and placed on small metal grids within supportive media, with prophylactic antibiotics, in a high oxygen environment. It was found that explants were maintained to reasonable architectural integrity within this culture system, for up to 24 hours after biopsy.

Interest in this technique increased, and by the late seventies a series of papers described longer term intestinal mucosal survival within *in vitro* organ culture systems. Mak and Chang (Mak and Chang 1978) reported rabbit intestinal mucosa culture for 3 days, Autrup et al. (Autrup et al 1978b) initially reported rat colonic mucosa maintained in culture for 4 weeks and subsequently human colonic mucosal culture for up to 22 days in CMRL media (Autrup et al 1978a). Furthermore, Shamsuddin et al., (Shamsuddin et al 1978) reported the maintenance of rat colonic epithelium within *in vitro* culture for up to 9 weeks. Critically, in these early studies, a delayed but progressive and permanent deterioration in the quality of the explant epithelium was noted, starting from relatively early in the biopsy period. In fact, studies often reported only a simple monolayer of epithelium was actually maintained beyond the early post-biopsy days in culture. Thus, undermining the strength of this model to accurately represent the nature of the human colonic *in vivo* environment.

In a seminal paper in 1982, Senior et al. reported their experience with long-term *in vitro* organ culture of human mucosa in Weymouth's media (Senior et al 1982). This group were the first to describe "remarkable regenerative changes" within explants. They noted "regenerating crypt cells

exhibiting intense proliferative activity” within explants after prolonged *in vitro* culture. This proliferation led to repopulation of explants with epithelium and goblet cells and the reappearance of complex architectural features such as cysts and crypts, producing an explant exhibiting features consistent with baseline *in vivo* mucosal architecture. These results have been supported by findings from other centres (Bareiss et al 2008, Schiff and Moore 1980). However, despite these initial findings and the obvious potential of this model for testing of agents and investigation of the intestinal regeneration system, reported description of the use of this model system have been relatively rare over the last few decades (Schuller et al 2007).

The presence of actively proliferating cells and the reported reappearance of a diverse differentiated colonocyte population, suggests that host colonic stem cells (CSCs) may remain present and active in this culture system. These cells are defined by their ability for self renewal and the generation of diverse cell lineages (Wagers and Weissman 2004). The recent identification of putative CSC markers such as Lgr-5, BMI-1, musashi-1 and aldehyde-dehydrogenase 1(ALDH-1) (Barker et al 2007, Haegebarth and Clevers 2009, Huang et al 2009, Klonisch et al 2008, van der Flier and Clevers 2009, Vermeulen et al 2008), together with the increasing recognition of the role of CSCs in the aetiology of human colonic disease, such as CRC (Clarke and Becker 2006, Hill et al 2009), have stimulated much interest in further characterising CSC regulation and control, differentiation and proliferation within the human colonic mucosa.

Here, I set out to optimise an *in vitro* culture of human *ex vivo* colonic mucosa in order to provide a more representative model system for testing aspirin-induced molecular responses. I also aimed to evaluate the presence and dynamics CSC populations within this system.

Furthermore, as discussed in chapter 1, and following the data obtained in chapter 3, epidemiological and experimental studies have provided strong evidence that aspirin and related NSAIDs have growth inhibitory effects against CRC. This includes a wealth of evidence derived from studies focusing on cellular and molecular changes induced by aspirin/NSAIDs within animal and human colonic mucosal tissue.



This laboratory has previously investigated agent-induced changes to NF- $\kappa$ B signalling in colonic mucosa. It was shown that short-term treatment with aspirin induced I $\kappa$ B $\alpha$  degradation prior to RelA nuclear localisation in colorectal mucosal tissue and tumours from patients undergoing colorectal resection (Stark et al 2001). Aspirin treatment studies were subsequently repeated in two animal models, including a model of HT-29 xenograft tumours and in adenomas from APC<sup>Min/+</sup> mice. In these studies, aspirin mediated a time-dependent increase in phosphorylated I $\kappa$ B $\alpha$ , a parallel decrease in cytoplasmic levels of the protein and was associated with a time-dependent increase in nuclear RelA. Aspirin-induced NF- $\kappa$ B signalling in this setting was associated with apoptosis *in vivo*, in benign neoplasm and malignant cells (Stark et al 2007). Critically, the aspirin-induced molecular response observed in these animal studies was restricted to pre-cancerous or carcinomatous tissue, as a similar response was absent from relevant normal colonic mucosa. This is similar to that reported in studies examining NO-indomethacin/NO-aspirin induced suppression of AOM-induced  $\beta$ -catenin expression, in which effects were present in colon adenocarcinoma but not in neighbouring mucosa (Rao et al 2006). Given that some of the above studies suggest that aspirin induces a targeted effect, limited to pre-cancerous/hyperproliferative colonic tissue *in vivo*, further development of the IVOC system to attempt to maintain polyps or hyperproliferative tissue would provide a highly relevant experimental model.

## **Results**

### **4.2 Short-term culture of explants is dependent on culture media**

Initial *in vitro* organ culture (IVOC) studies followed experimental protocols described by Moorghen et al., (Moorghen et al 1996), who reported on the successful long term culture of human colorectal mucosa obtained from patients undergoing surgical resections. In that report, colorectal explants, maintained in serum-free RPMI media, underwent moderate degeneration within the first 24 hours but epithelial proliferation, and regeneration of the cryptal architecture, was observed from 5- 21 days. Of note, that report contrasts to those of other authors, who described poor tissue survival of colonic explants grown in serum free medias (Dame et al 2010). In order to investigate this culture system, I initially tested the use of non-supplemented RPMI media to culture colonic explants.

Patients, undergoing CRC resection were recruited into optimisation and treatment categories and provided colorectal tissue, from which epithelial mucosal explants were obtained. Further details of the recruitment, preparation and culture set-up are described within chapter 2, but briefly, upon collection, biopsies of normal colorectal mucosa were divided into multiple pieces then plated on steel wire gauze and bathed within medium-filled plates. Tissue was harvested at 0, 8 hours and 24 hours following plating. Tissue was fixed in formalin; a citrate buffer retrieval system with microwaving was used for antigen retrieval. Tissue architecture was analysed initially by H&E staining. Immunohistochemistry utilizing antibodies directed against known colonic epithelial cell proliferation markers (PCNA and Ki67) was also undertaken. These antibodies have been reported by previous authors to be reliable in the setting of formalin-fixed samples and a citrate/microwave based antigen retrieval (Cattoretti et al 1992, Holt et al 1997). Additional markers of early (activated caspases-3) and late (Tunel assay) apoptosis were also utilized to evaluate the presence of apoptosis within the cultured explants.

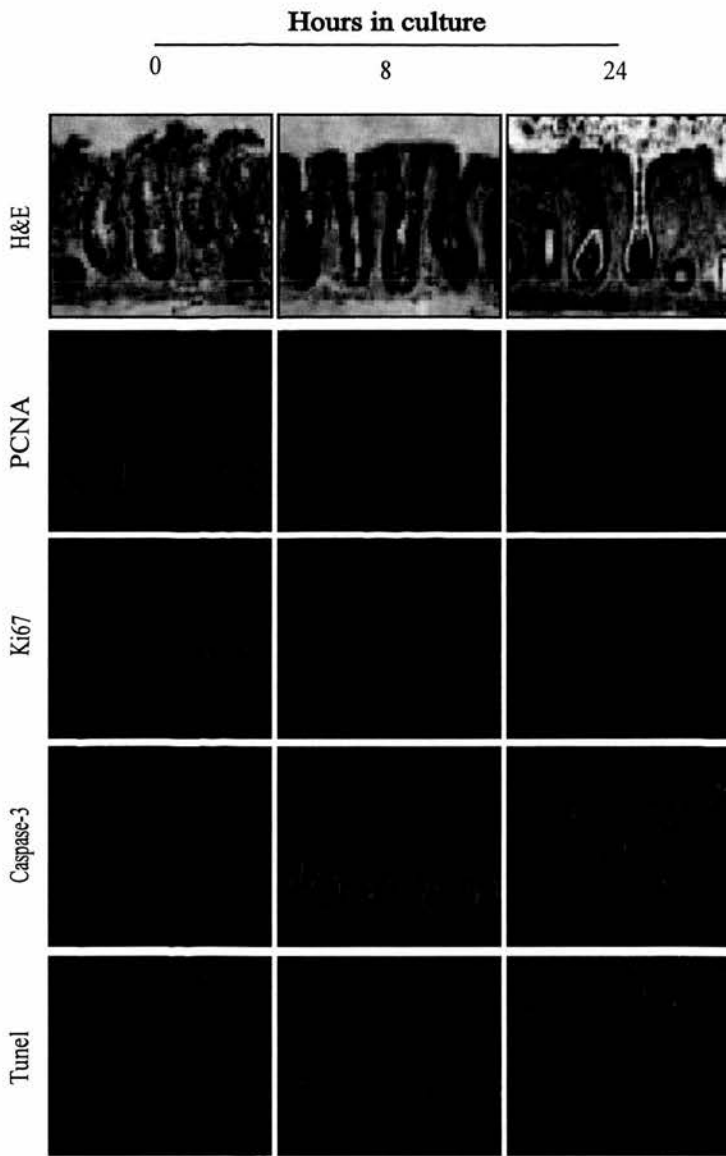
In the normal colonic mucosal tissue architecture, crypts are aligned parallel to the crypt bases. Crypts are straight and narrow and mostly unbranched, separated by a thin rim of lamina propria, composed of loose, areolar connective tissue. The distance between the crypts and the internal diameter of the

crypts are constant (although slight variation in intercryptal spacing and occasional crypt branching may occur in some normal biopsies). Crypts are composed of absorptive (tall columnar), goblet and endocrine cells, with the ratio of tall columnar cells to goblet cells usually 4:1. The proliferative zone in the base of the crypt is composed of low cuboidal CSCs. The muscularis mucosae is a thin smooth muscle layer (inner circular and outer longitudinal layer) and the submucosa is composed of loose connective tissue with collagen and elastic fibres. Meissner's plexus of autonomic nerve fibres with ganglion cells are present within this.

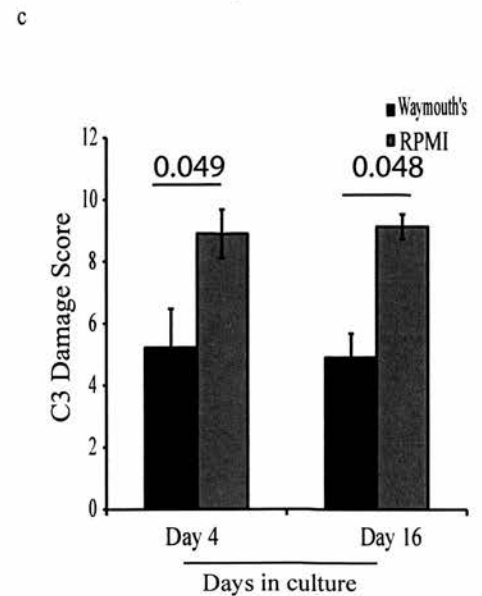
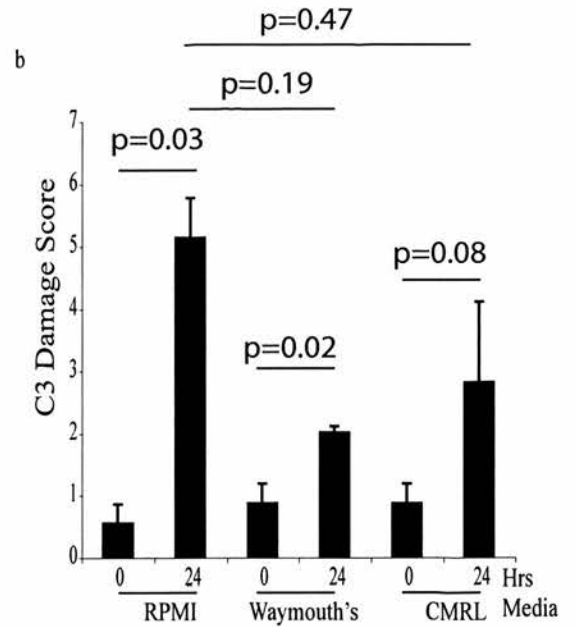
Initial observations demonstrated that at time 0, the tissue was very similar in appearance to the normal expected appearance of colonic mucosa described above. However, by 8-24 hours following retrieval, colonic tissue underwent progressive rapid architectural deterioration, with epithelial layer detachment, marked apoptosis and decreased proliferation (figure 4.1a). Histological examination confirmed that this initial architectural disruption and degradation of the epithelium was progressive and did not resolve in samples maintained in RPMI for 16 days of culture (figure 4.1c).

To determine whether this loss of architecture was associated with changes in cell proliferation, I utilized immunohistochemistry with antibodies to PCNA and Ki67. Figure 4.1a demonstrates that at time 0, Ki67 and PCNA staining was as expected, predominantly at the crypt base. However, the number of cells staining for both markers and the overall intensity of this stain within the explants, decreased at 8 hours and 24 hours. The use of secondary antibody only controls confirmed the specificity of both of these antibodies (data not shown).

To examine the presence of apoptosis within the explants cultured in RPMI, I initially utilized immunohistochemistry with antibodies to active caspases-3. This antibody only detects the cleaved (and therefore active) form of the protein. I found that there were few positive cells at the tops of crypts at time 0, in keeping with the expectation that this compartment contains crypt epithelial cells which undergo apoptosis and are shed. However, the number of positive cells increased dramatically after 8 and 24 hours. Furthermore, positive cells were also found at the base of the crypts. These data were confirmed utilizing TUNEL assays. This assay detects apoptotic cells on the basis of DNA



Pictures (OM)



#### 4.1 Explant survival is dependent on culture media

Explants were cultured in either RPMI, modified CMRL or modified Waymouth's media & harvested at the time points shown. Immunohistochemical analysis was performed on formalin-fixed tissue using antibodies to proliferation markers PCNA, Ki67 and the apoptosis marker active caspases-3. Explants undergo a decrease in proliferation and an increase in apoptosis when grown in RPMI medium. (n=3) b. C3 damage scale scoring of explants after 24 hours by 3 independent and blinded observers (see chapter 2.9 for further description of the C3 damage scale). (n=4) c. C3 damage scale scoring of explants after 4 and 16 days in culture in RPMI compared to modified Waymouth's media (graph represents mean +/- SE) (n=3).

fragmentation. Again I found that there were a small number of apoptotic cells at the top of the crypt at time 0 and that this number increased substantially from 8 to 24 hrs.

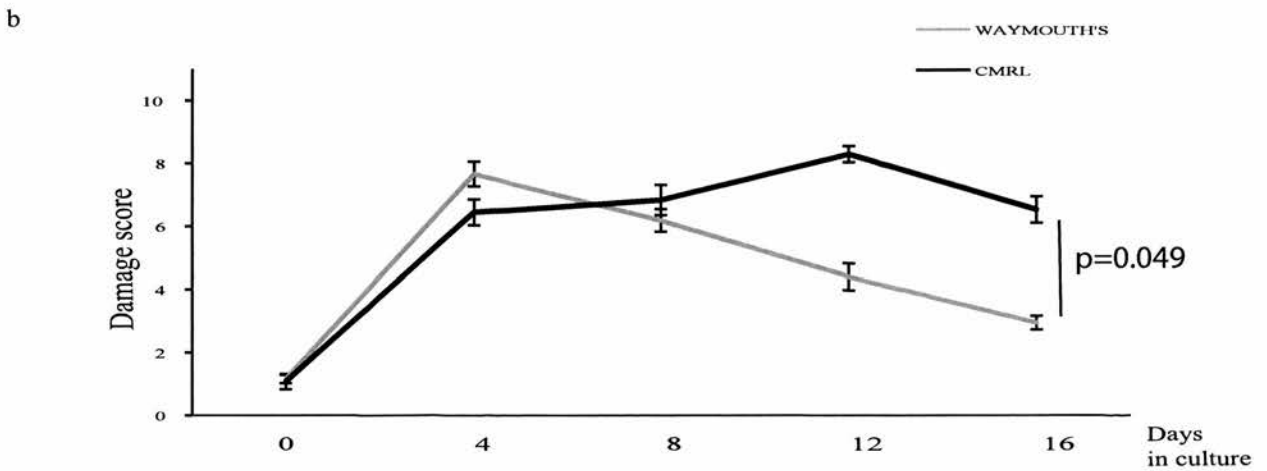
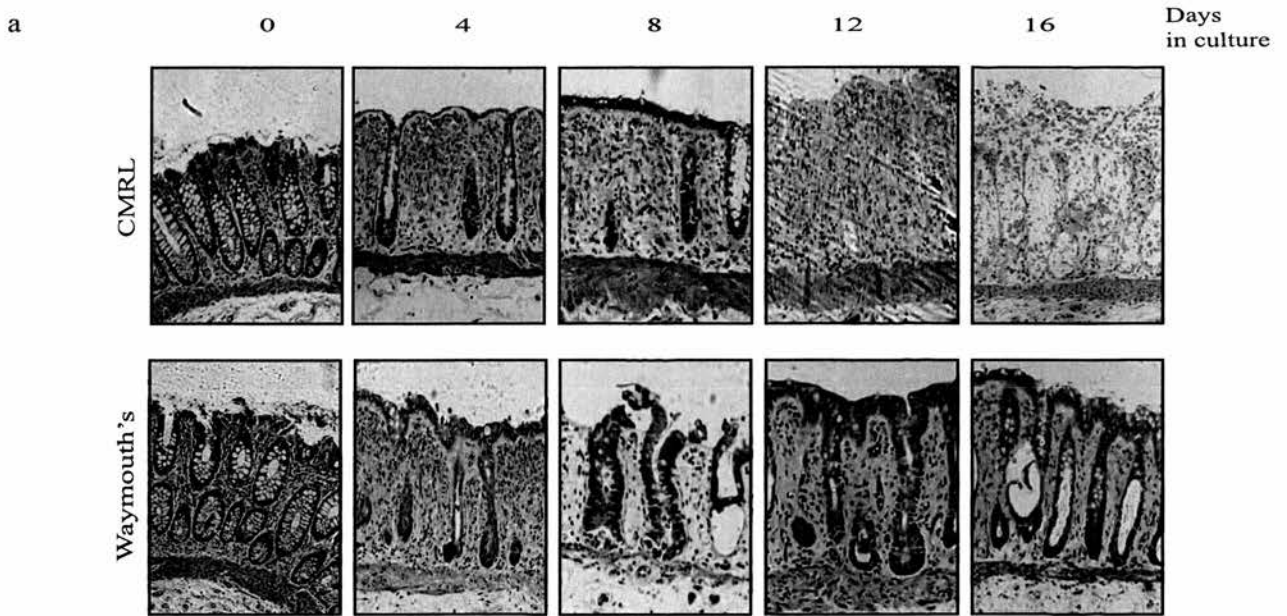
Other authors have reported more success with the use of alternative culture medias, such as modified Waymouth's or CMRL (Autrup et al 1978a, Schiff and Moore 1980, Senior et al 1982). Therefore, I next tested short term growth of explants within these alternative media.

In order to aid in the comparison of growth conditions, the "C<sub>3</sub>" scoring system was devised. This system is further described in more detail in chapter 2. Examination of H+E stained sections, and subsequent C<sub>3</sub> scoring, suggested that there was less tissue damage in these alternative media after 24 hrs (figure 4.1b). When longer-term outcomes after 16 days of *in vitro* culture were compared with RPMI media, there was significantly less tissue damage observed in explants maintained in Waymouth's media (figure 4.1c).

#### **4.3 Longer-term outcome of explants are dependent on culture media**

Given the improvement in tissue quality observed from short-term culture in these alternative media, direct comparison of cultures maintained in RPMI, Waymouth's, or CMRL were performed to provide a more a detailed histological assessment of explants in long-term culture utilizing these alternative medias.

Explants cultured in Waymouth's media showed significantly improved survival over 16 days compared to RPMI. When Waymouth's was compared to CMRL, I found that there was a gradual loss of goblet cells, loss of surface epithelial continuity, epithelial detachment and shortening of the crypt height from day 1 to day 8. However, critically, there was a subsequent divergence of explant outcomes, dependant on the media (figure 4.2a). After day 8 in culture, explants maintained in CMRL medium underwent progressive degradation until day 16, similar in nature to the response described by Autrup et al. (Autrup et al 1978a). However, after 8 days of modified Waymouth's medium, there was the development of epithelial-lined cystic formations within the stromal layer, surface epithelial repopulation and the reappearance of complex colonic crypt architecture, containing secretory cells.



#### 4.2 Longer-term outcome of explants are dependent on culture media

Explants were cultured in either modified CMRL or modified Waymouth's media and harvested at the time points shown. H+E staining of formalin-fixed tissue was used to analyse tissue damage as above a. Histological explant structure demonstrated by H&E staining of formalin-fixed samples b. C3 damage scale scoring of explants at the time points shown (n=3).

When histological sections were scored for C<sub>3</sub> damage as previously described, it was clear that cultures underwent initial crisis in both media but at 8 days in culture, samples plated in Waymoth's media began to recover, showing demonstrably less damage than samples grown in CMRL by day 12. This response mimics that previously described by Senior et al., and other groups (Moorghen et al 1996, Schiff and Moore 1980, Senior et al 1982) (figure 4.2b).

An attempt was made to grow biopsies beyond 16 days, however, I found that beyond 20 days in culture, mucosal explants underwent progressive deterioration and loss of architectural maturity and epithelium, irrespective of the culture constituent media. This was in keeping with previous reports citing a gradual reduction in the regenerative capacity of *ex vivo* colonic tissue in prolonged *in vitro* culture (Bareiss et al 2008) (data not shown).

These observations suggested that whilst a decline in explant tissue architectural quality occurred in the early stages of culture within all media, media-specific factors induced the development of complex architectural structures and cellular repopulation of explants at latter stages. In light of these findings, focus was placed on optimizing the culture and conditions using Waymouth's media and further characterising the cellular processes involved in the observed "regenerative changes" (Senior et al 1982).

#### **4.4 Optimisation of explant culture conditions**

##### ***Oxygenation***

Previous studies reported that the oxygen content of the gas phase is an important variable in successful organ culture of colonic tissue. That is, explants maintained in normal air have a reportedly worst outcome, than those maintained in high oxygen concentrations (Schiff and Moore 1980). Autrup et al., postulated that this was due to reduced tissue lactate, reduced acid production and therefore reduced tissue damage (Schiff and Moore 1980). However, the toxic effects of high oxygen concentrations on cell culture are also well described (Wright and Shay 2006).

To determine whether culturing oxygen concentration affected growth of colonic tissue in our hands, I initially compared the histology of explants grown in high oxygen phase culture setting (78% O<sub>2</sub>) compared with explants maintained in a low oxygen setting (38% O<sub>2</sub>). There was no significant difference in explant outcomes between the two oxygen concentrations (figure 4.3a).

### *Antimicrobials*

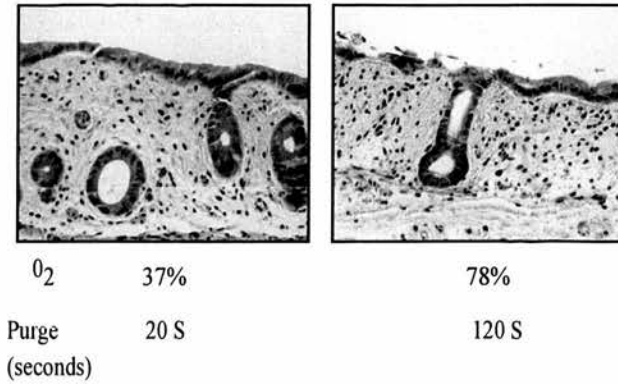
In the initial stages of optimisation, 65 patients were recruited and entered tissue into the various optimisation and treatment arms. I found that a proportion of explants became infected with bacteria. This was detected by visual cloudiness, abnormal colour change of the media (pH sensitive), abnormal growths on explants, or light microscopy showing bacterial growth. Furthermore, subclinical detection of infections was also confirmed by analysing subsequent histological slides (figure 4.3b). As explants were being cultured in media with added prophylactic antibiotics, the development of infection within this setting caused suspicion that multidrug resistance (MDR) bacteria may be involved. MDR bacteria are known to be commonly present within the NHS clinical environment and therefore explants obtained from patients from such environments have the theoretical potential of hosting bacteria that are resistant to the prophylactic antibiotics in laboratory culture use.

In order to provide antibiotic sensitivities of infecting bacterial species (to detect the presence of MDR), bacteriological culture of a selection of infected cultures and sensitivity testing of the resultant bacterial growth was undertaken through an arrangement with the local NHS microbiology laboratory (Dr Pota Kalima, Department of Laboratory Medicine, Western General Hospital, LUHT, Edinburgh).

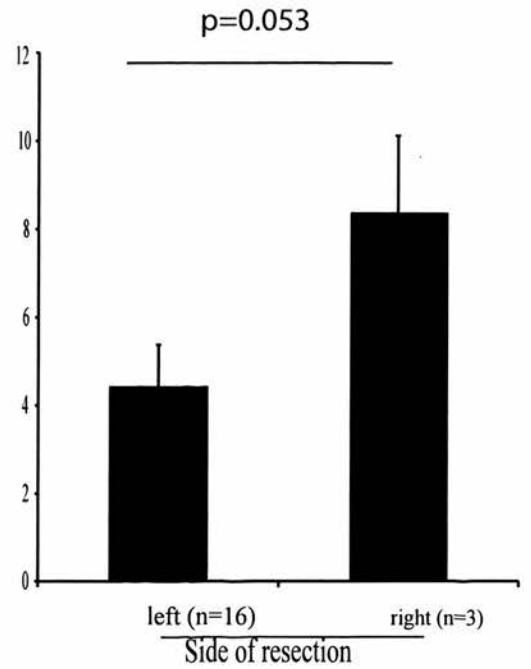
Due to labour and costs involved in this process, analysis was limited to 7 explants. 500ul of media fluid was obtained from 4 infected explants (RB19, RB21, RB23, RB24) and 3 apparently healthy explants (RB17, RB20, RB24). Media was obtained by sterile methods and transported in sterile tubes at room temperature to the bacteriology laboratory within 24 h. Similar to the process involved in the normal standard of care for patient samples, swabs were dipped in the media and were inoculated onto two Blood agar plates (Columbia Agar with Horse Blood; Oxoid Ltd, Basingstoke, UK). These were



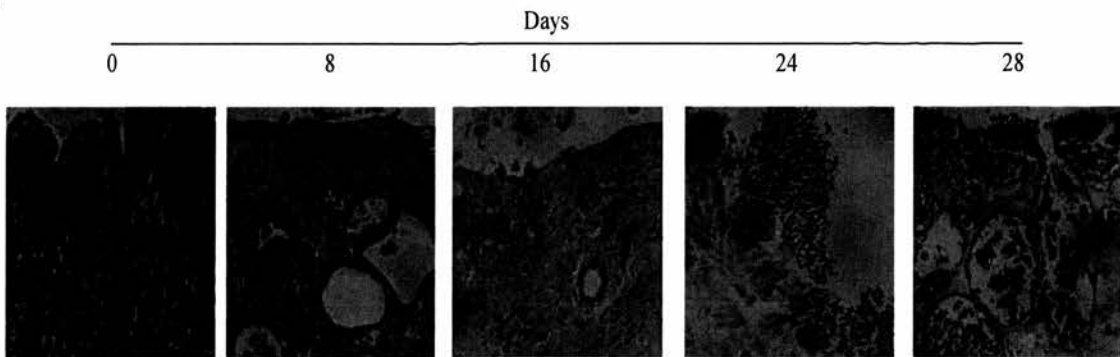
a



c



b



#### 4.3 Optimisation of explant culture conditions

- a. Explants were grown in either low (37% O<sub>2</sub>) or high (78% O<sub>2</sub>) oxygen conditions then harvested after 16 days in culture. All results are representative of at a minimum of 3 separate experiments
- b. H&E staining of formalin-fixed samples demonstrating appearance of progressive bacterial infection of explants in prolonged culture. c. Decreased damage scores were observed in long term culture of explants from left colon. C3 damage scoring (determined as in figure 4.2) were compared for damage scoring of right- (n=3) and left- (n=16) sided sourced explants in Waymouths media at 16 days. All measures of damage scale determined by 3 independent and blinded observers.

incubated, one aerobically and the other anaerobically, at 37°C for 48 h. All plates were inspected daily for visible growth and any microorganisms present were identified using standard laboratory procedures. Antibiotic sensitivities were assessed using standard antibiotic growth inhibitory discs.

Four of the seven (57%) explants demonstrated bacterial growth. None of the apparently healthy explants exhibited obvious bacterial growth except for RB24. However, this sample demonstrated no growth at day 4, but yeasts and anaerobes were identified by 24 days. This later growth was assumed to be the result of potential cross-contamination within the Billroth chamber from another infected sample. Of the four samples provided from infected cultures, RB19 and RB23 demonstrated infection with yeast, RB19, RB21 and RB23 demonstrated infection with anaerobes (all sensitive to metronidazole) and RB21 and RB22 demonstrated infection with *Escherichia coli* (all resistant to amoxicillin but sensitive to gentamicin).

In an attempt to increase infection-free yields, in addition to penicillin/streptomycin, which was routinely added to culture media, a commercial antimycotic/antibiotic mix containing amphotericin B and gentamicin was included in the subsequent culture media (see methods section 2.2).

#### ***Anatomical harvest site***

Analysis of data gathered through the recruitment process suggested that there was an association between anatomical site of colonic resection and successful, non-infected long-term growth of explants. It appeared that colonic explants derived from right-sided resections (right hemicolectomies (77%)) had a trend towards increased incidence of failure due to infection, compared to that from left-hand sided resections (left hemicolectomies (36%), anterior resections (48%)). Additionally, there was an association between C<sub>3</sub> tissue damage scores at day 16 and the anatomical site of the tissue source. Right-sided resections exhibited greater explant C<sub>3</sub> tissue damage scores compared to left-hand sided resections ( $p < 0.06$ ) (figure 4.3b). Therefore, a decision was made to focus subsequent recruitment of mucosa from patients undergoing left sided colonic resections.

### *Success of the model system*

In total, initial optimisation experiments utilised tissue from 38 individual patients, which were all maintained in culture for 16 days. Successful explant outcome was defined as the appearance of organised mucosal epithelial structures (crypts), with epithelialisation of the surface of explants at 16 days, in the absence of infection. Using this standard, the initial overall success rate from these experiments was 28.9%.

However, following optimisation of the protocol (which included the use of Waymouth's media, the inclusion of antimycotic/antibiotic, stable oxygenation conditions and a preference for utilising only tissue derived from left sided colonic sources, the success rate was markedly improved (8/13 cultures = 62% success). This success rate is higher than the 33% previously observed using IVOC of human colonic mucosa (Autrup 1980).

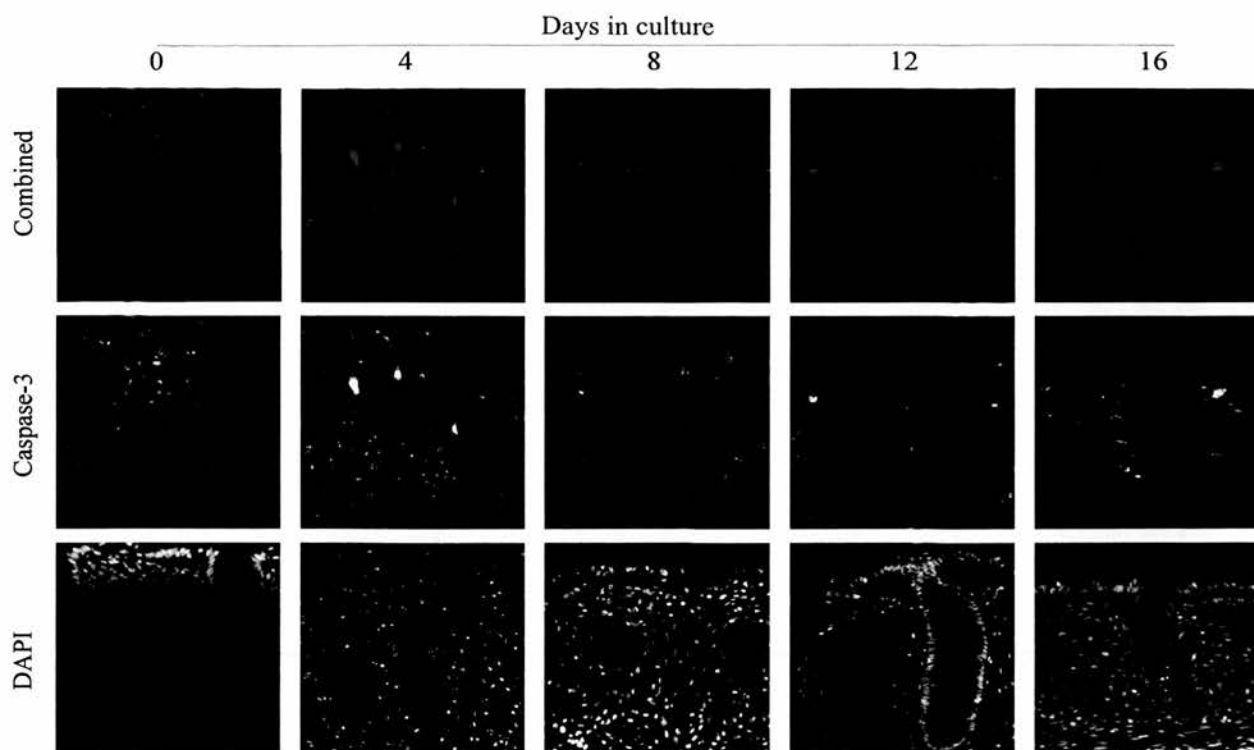
### **4.5 Characterisation of apoptosis and proliferation rates in long-term culture**

Given the above histological findings, a more detailed characterization of the role of proliferation and apoptosis during prolonged IVOC culture was undertaken.

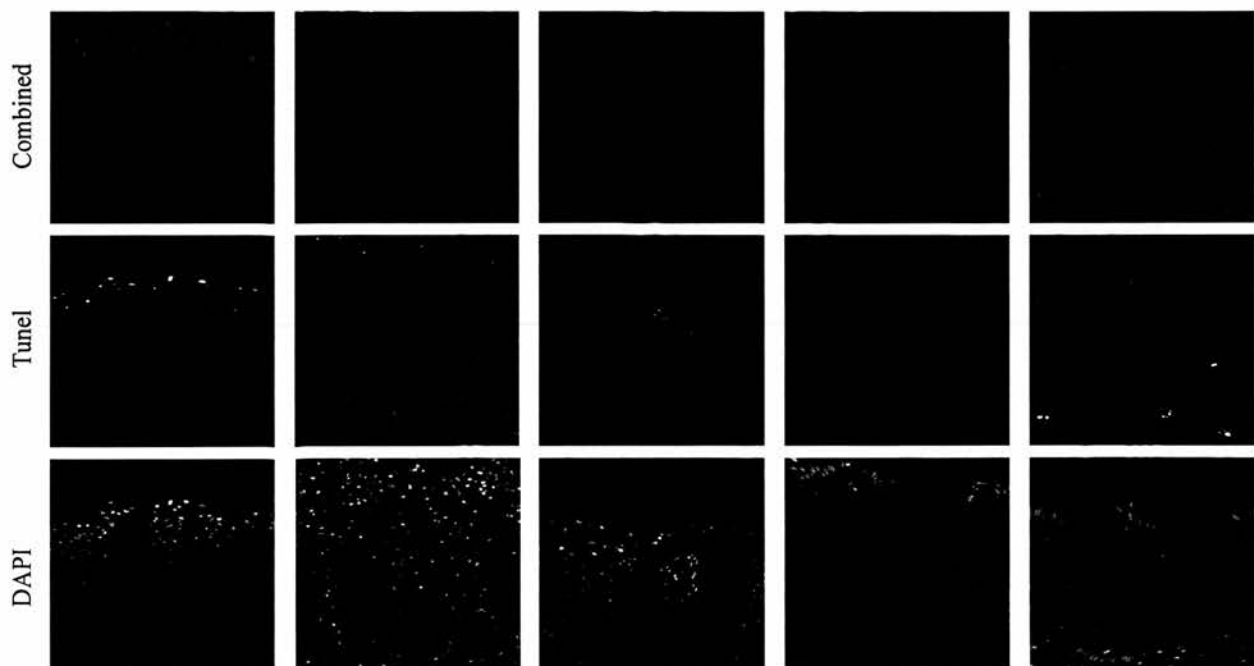
Firstly, using similar techniques described previously involving immunohistochemical markers of early (active caspase-3) and late (Tunel assay) apoptosis, I examined explants for apoptosis. This was increased in samples obtained between days 4 and 8 but subsequently there was a decrease in the levels of apoptotic markers, returning to a level relative to baseline by day 16 (Figure 4.4a and 4.4b). During the initial stages, apoptotic marker expression was confined to the explant epithelial layer, particularly the crypt lumen and detached epithelial cells. There was little apoptotic marker expression within the stroma. Following the initial phase, apoptotic marker expression reduced to return to baseline levels for the remainder of the culture process.

To quantify explant proliferation, immunohistochemical markers of cell proliferation (Ki67 and PCNA) were also analyzed. Proliferative markers at baseline were expressed in the lower third of the

a

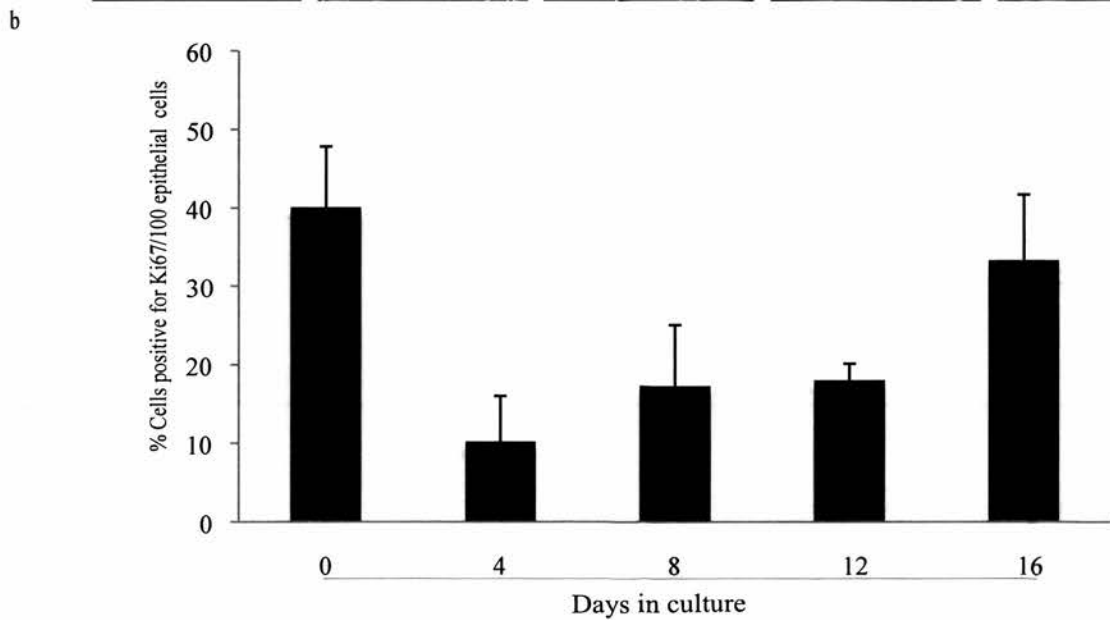
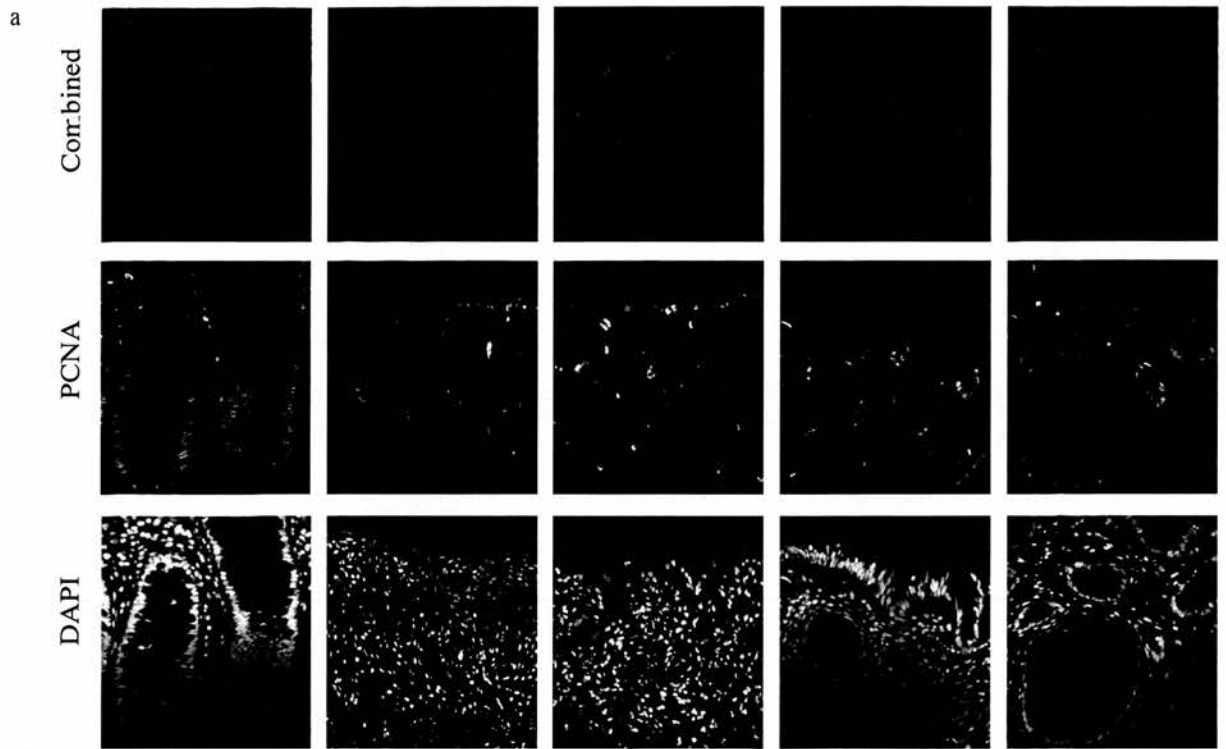


b



#### 4.4 Characterisation of apoptosis in long-term culture

Explants were harvested at 4 day intervals for the 16 days. To assess apoptosis, immunohistochemical analysis was performed on formalin-fixed tissue, with antibodies to the apoptosis markers, Tunel and active caspases-3. Images were captured at x20. Representative immunocytochemistry images of explants. All results are representative of at a minimum of 3 separate experiments.



#### 4.5 Characterisation of proliferation in long-term culture

Explants were harvested at 4 day intervals for 16 days. To assess proliferation immunohistochemical analysis was performed on formalin-fixed tissue, with antibodies to the proliferation markers, Ki67 and PCNA. Images were captured at x20. a Representative PCNA immunocytochemistry images of explants. b. The number of Ki67 positive cells were counted in a minimum of 5 separate fields. Graph represents mean of 3 independent experiments +/- standard error at the time points shown.

colonic crypt. During the initial apoptotic phase, there were few actively proliferating cells apparent, however subsequently, the number of cells expressing proliferative markers increased within surface epithelium and focally within early epithelial cystic structures developing within the stroma. PCNA expression was strongest in the lower third of crypts and within these early epithelial cystic structures within the explant stroma. As the crypt architecture developed, PCNA expression was observed throughout the full length of the crypts (Figure 4.5a). The proportion of Ki67 positive epithelial cells was evaluated and confirmed that proliferation decreased in the earlier stages of culture, after which there was a period increased proliferation between days 8 and 12, returning to baseline levels by day 16 (figure 4.5b).

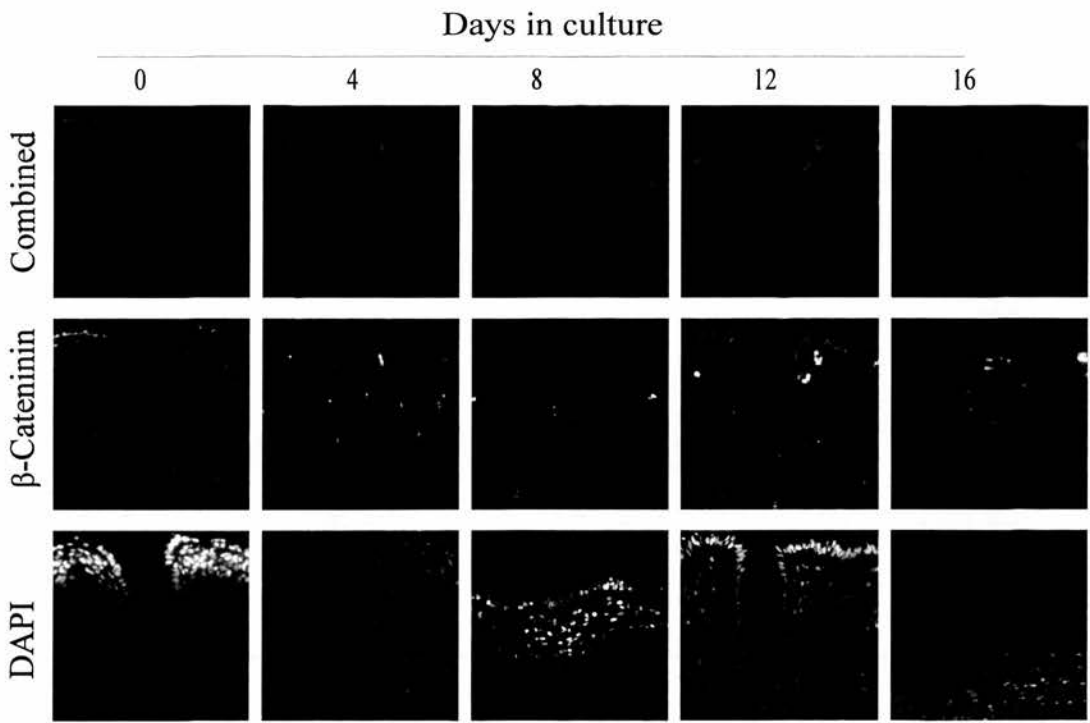
Taken together these data suggest a biphasic process. Firstly, characterised by rapid deterioration in explant architecture within the first 24 hours after retrieval and a subsequent apoptotic phase lasting until days 4-8. During this, cells became detached from the crypts, crypt width was reduced, crypt height was shortened and there was a loss in secretory/goblet cell numbers. This phase was followed by progressive repopulation of the explant, and the eventual reappearance of goblet cells within maturing colonic crypts.

#### **4.6 Differentiated cell population dynamics in long-term culture**

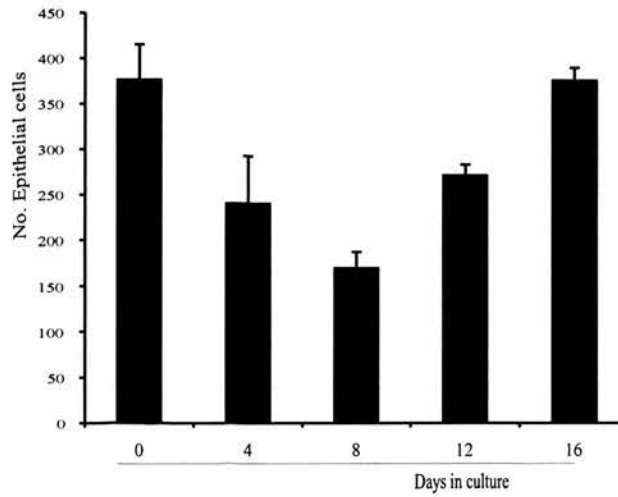
Given the subsequent proliferation observed in longer-term culture, cell type-specific immunohistochemical analysis was undertaken to further characterize the type of cells that repopulated explants and to assess the diversity of the populations which resulted from this process.

The epithelial cell marker,  $\beta$ -catenin, was employed to determine changes in epithelial cell number (figure 4.6a).  $\beta$ -catenin staining, and quantification of the number of positive cells, confirmed that whilst epithelial cell counts reduced during the apoptotic phase of culture, this was followed by a subsequent rapid increase in the epithelial cell population at later culture time-points (Figure. 4.6b). The previously noted cystic and cryptal structures within the stroma were lined with epithelial cells.

a



b



#### 4.6 Epithelial cell populations during long term culture

Paraffin embedded formalin-fixed explants were harvested at 4-day intervals for 16 days.

At the times shown, immunohistochemistry was used to identify cells of different lineages

a.  $\beta$ -catenin used to identify epithelial cells, b. The number of  $\beta$ -catenin positive staining cells reduces before returning to baseline appearance at day 16. These cells were counted in a minimum of 5 separate fields. Graph is representative of average number of cells +/- standard error (3 separate experiments).

Differentiated colonocytes with absorptive/sampling function, have a brush border of microvilli to increase the cell surface area. This is highly positive for the marker protein Vil-1. Within the initial apoptotic phase at days 4 to 8, enterocytes positive for this microvilli marker became discontinuous, accompanied by a reduction in overall enterocyte numbers. However, during days 8 to 12, positively staining cells were present both within epithelial-lined cystic structures and in increasing numbers on the explant surface. By the latter stages of explant culture (day 16), the pattern of Vil-1 positively staining cells mirrored that observed within samples at day 0 (Fig. 4.7a).

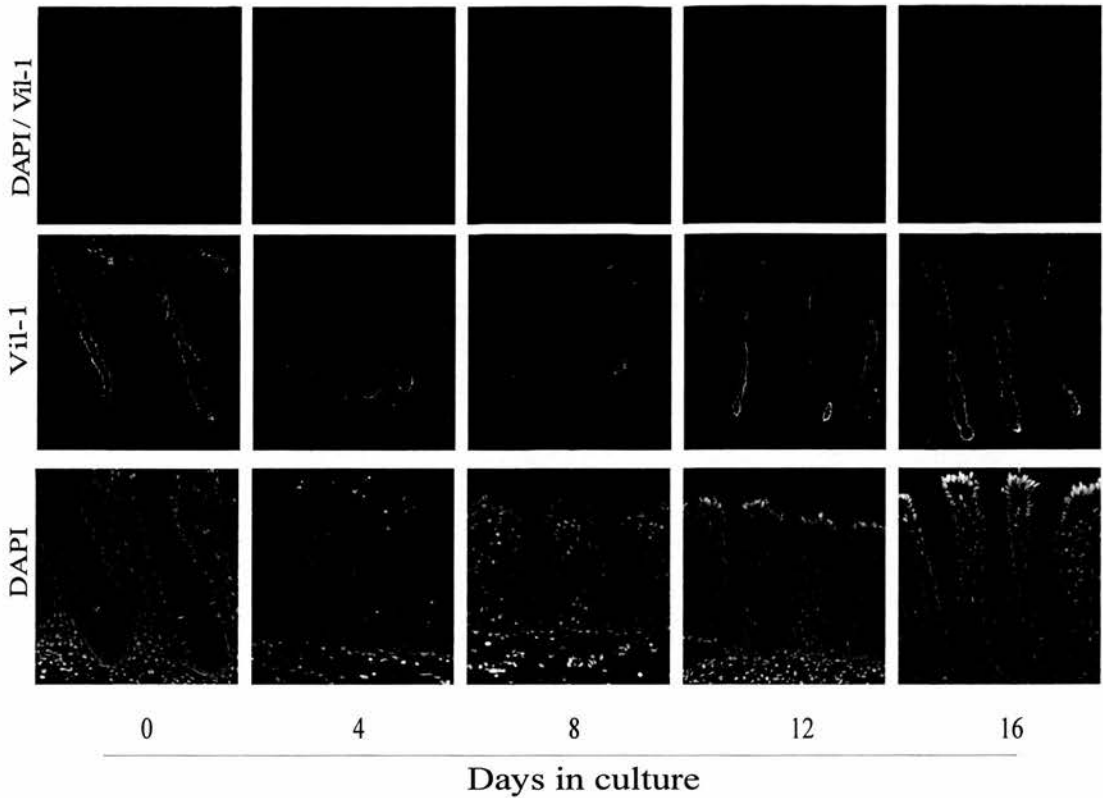
Muc-2 is a marker of Mucin, produced by colonocytes of the secretory/goblet cell lineage. Immunohistological analysis demonstrated that the proportion of goblet cells rapidly decreased in initial stages of culture, with subsequent increases during the later proliferation phase. The goblet cell proportion of the total epithelial cell population rebounded after initial decline. Cell counts demonstrated that at day 16, the proportion of Muc-2 positively staining cells within epithelium, reflected that observed at day 0 (Fig. 4.8a and 4.8b).

There are a small number of entero-endocrine cells within colonic crypts which perform paracrine and exocrine functions. There were typically 1-2 entero-endocrine cells (positively staining for the marker Chromogranin A) per crypt at day 0. Whilst the presence of positively-staining cells could not be detected at culture midpoints (days 4-8), Chromogranin A-staining cells were subsequently observed in small numbers at day 16 (figure 4.9a).

Whilst immunohistochemistry using the paneth cell marker lysozyme was performed, no positive staining was detected. However, given that paneth cells are usually only present in the small bowel, caecum and proximal colon, and that the presence of paneth cells more distally in the colon is usually only associated with metaplastic change in chronic infection, together with our preference for left-sided resection samples latterly, this was perhaps not surprising.

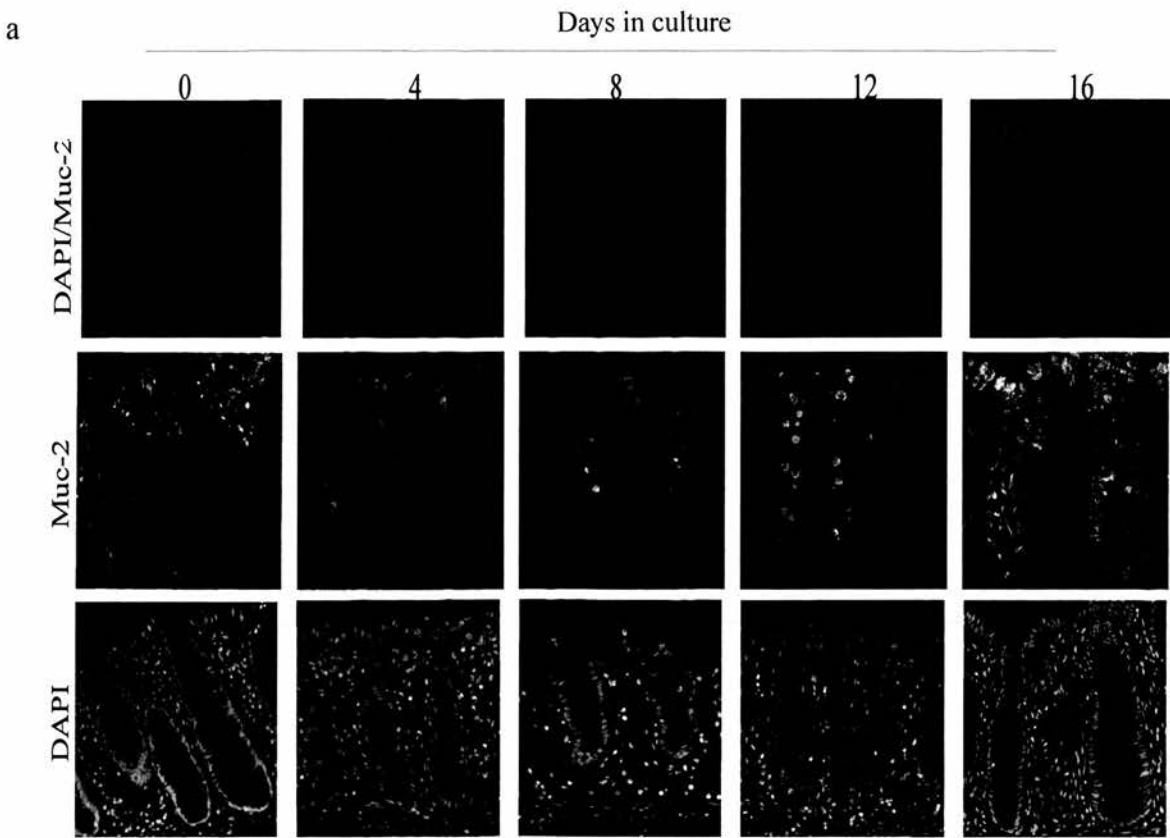


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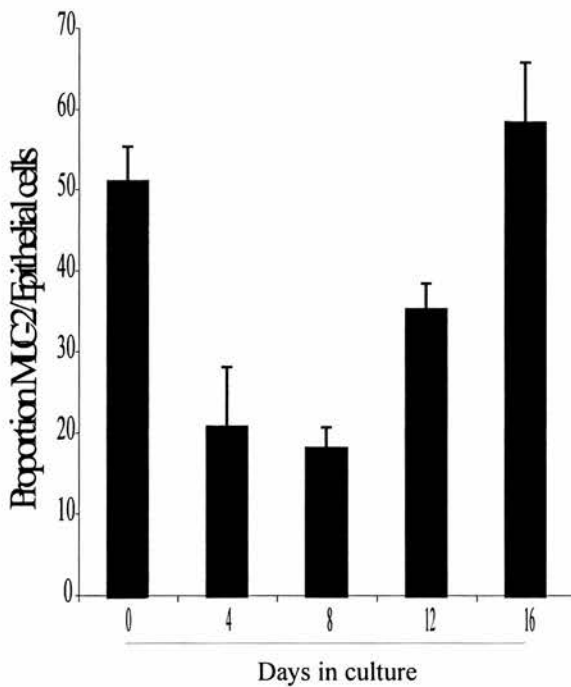


#### 4.7 Vil-1 positive cell populations during long term culture

Paraffin embedded formalin-fixed explants were harvested at 4-day intervals for 16 days. At the times shown, immunohistochemistry was used to identify cells which were positive for the epithelial marker Vil-1a. Representative micrographs are shown of Vil-1 positive cells. All results are representative of at a minimum of 3 separate experiments



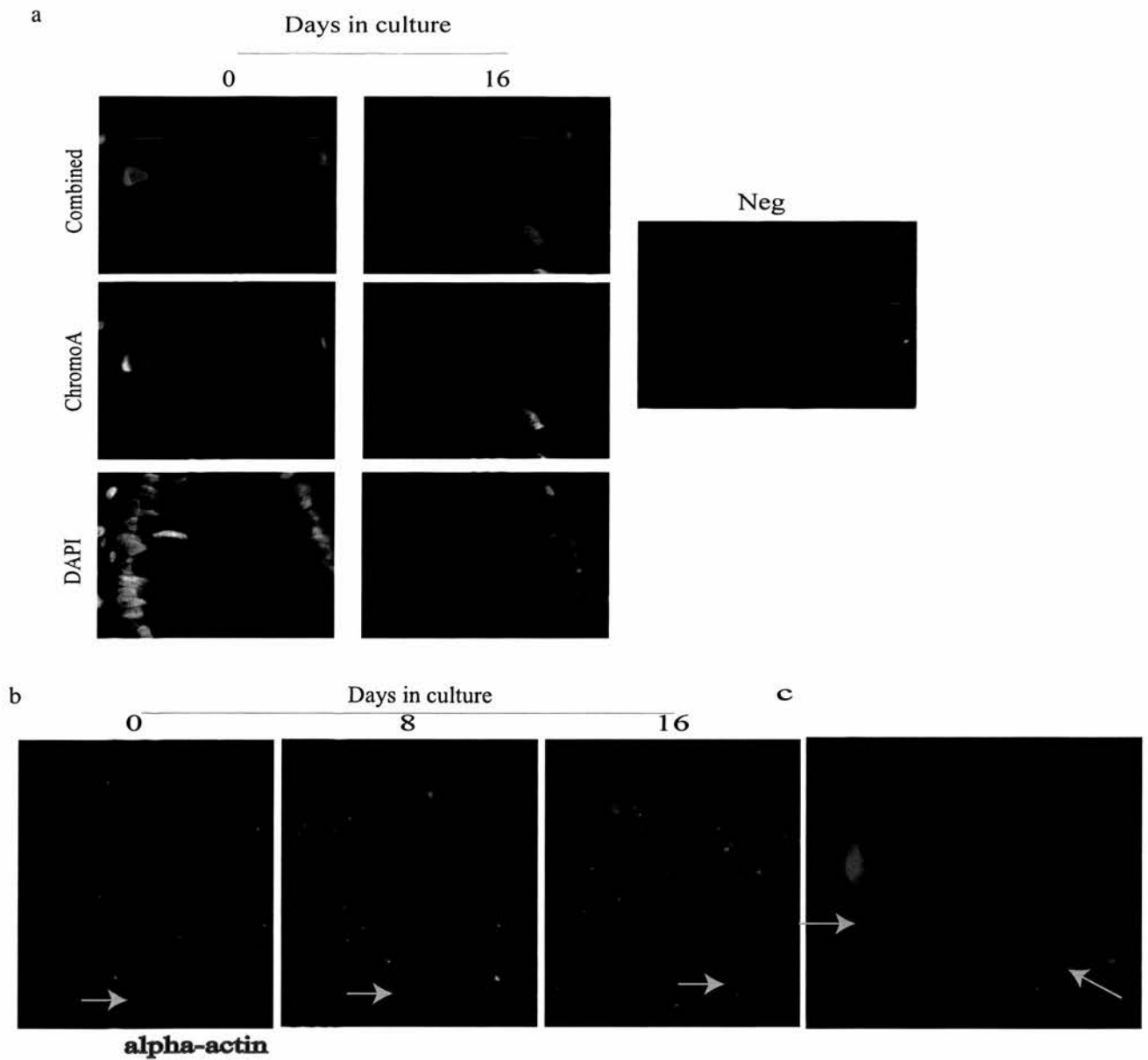
b



#### 4.8 Secretory cell populations during long term culture

Explants were harvested at 4-day intervals for 16 days and then paraffin-fixed for immunohistochemistry to identify cells of different lineages

a. Representative micrographs are shown of Muc-2 positive cells, b. The number of Muc-2 staining cells reduces before returning to baseline appearance at day 16. Muc-2-staining cells were counted and compared with number of epithelial cells in 5 separate fields. Graph is representative of average number of cells +/- standard error (3 separate experiments). All results are representative of at a minimum of 3 separate experiments. There is rapid initial decrease in the number of Muc-2 positive cells, before increasing at later stages in culture. The number of Muc-2-positive staining cells reduces before returning to baseline appearance at day 16.



#### 4.9 Entero-endocrine and smooth muscle cell populations during long term culture

Paraffin embedded formalin-fixed explants were harvested at 4-day intervals for 16 days. At the times shown immunohistochemistry was used to identify a. chromogranin--positive and, b. Smooth muscle  $\alpha$ -actin-positive cells.

a. Representative micrographs are shown of chromogranin-A positive cells at baseline and day 16 in culture with secondary antibody only control (neg).

b. Deterioration in the size and quality of the  $\alpha$ -actin positive cells within the smooth muscle layer (yellow arrows) and decreased peri-cryptal myofibroblasts over time during prolonged culture (magnification x20). c. High power image (x63) of crypt base demonstrating crypt surrounding myofibroblasts at time 0 (yellow arrows).

All results are representative of at a minimum of 3 separate experiments

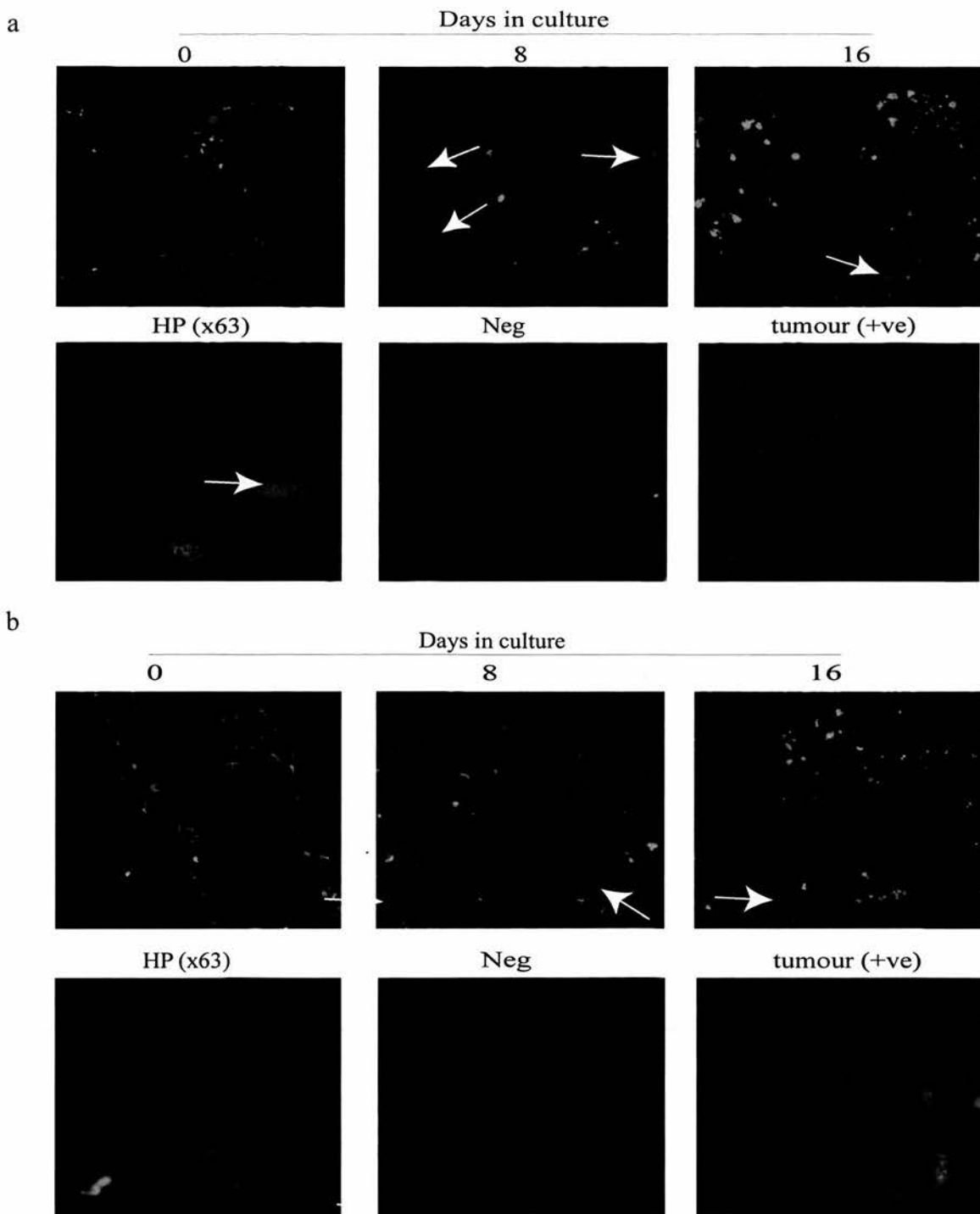
In conclusion, I found that after prolonged culture, explants exhibited a diversity of cell population, resembling the populations observed at the time of initiation of culture. This suggested the IVOC model provided a representative platform of actively proliferating human colonic mucosa at day 16.

#### **4.7 Identification and characterisation of colonic stem cells in IVOC**

The presence of actively proliferating cells and the reappearance of diverse differentiated colonocyte populations within explants suggested the presence and activity of colonic stem cells (CSC). In the small bowel, it is thought that around 6 CSCs exist either as Crypt Base Columnar cells which over-express the proliferation marker Ki67 (Barker et al 2007), and are interspersed with basal Paneth cells, (Bjerknes and Cheng 2005, Sato et al 2009) or reside in the +4 position immediately above basal Paneth cells (Bjerknes and Cheng 2005). However, less is known about the location and regulation of large bowel CSCs, particularly in human tissue.

To examine for the presence of CSCs and to investigate their role in long-term culture of colonic explants, I next used immunohistochemistry with the putative stem cell markers Lgr-5 and BMI-1. Lgr-5 is a target of active Wnt signalling and has recently been identified as a putative CSC marker (van der Flier and Clevers 2009). Its expression is restricted to the base of the crypt, in particular, the crypt basal columnar (CBC) cells (Haegebarth and Clevers 2009). This is particularly interesting as, described above, positive staining for cell proliferation markers Ki67 and PCNA during days 8-12 of *in vitro* culture localised to the lower third of the early colonic crypts (figure 4.5c), akin to the location of the putative colonic stem cell compartment (Barker et al 2008). Indeed, I found that whilst there were few positively staining Lgr5<sup>+</sup> cells at baseline and throughout the early apoptotic phase, by day 8 there was an increase in positively staining cells localising to the epithelium of early crypt-like structures. By day 16, Lgr5<sup>+</sup> cells were more frequently located at the base of cryptal structure (Figure 4.10a).

BMI-1 is involved in the self-renewal of neuronal, haematological and leukemic cells but has also been suggested as a putative marker of intestinal stem cells *in vivo* (Sangiorgi and Capecchi 2008). The distribution of cells expressing BMI-1 is reduced whilst progressing distally within the



**Figure 4.10 Identification and characterisation of colonic stem cells (CSC) in IVOC**  
 Paraffin embedded formalin-fixed explants were harvested at 4-day intervals for 16 days. Fluorescent immunohistochemistry was used to identify CSC markers a. Lgr-5 and b. BMI-1. a, b. Immunohistochemistry images of CSC markers during IVOC (white arrows =positively staining cells) and high power images. Upper panel images were captured at x20, the lower high panel high power at x63. Positive (tumour) and negative (Neg=secondary antibody only) control micrographs are also shown. All results are representative of at a minimum of 3 separate experiments

gastrointestinal tract (Sangiorgi and Capecchi 2008). BMI-1 positive cells were found to localise to nuclei in the lower third of colonic crypts at day 0. However, by day 8, aggregates of positively staining BMI-1 cells were found within early cryptal structures. By day 16, levels of BMI-1 staining within the crypt cell nuclei was notably increased, with positive cells populating more than half of the lower portion of the regenerating crypt, akin to the pattern of Ki67/PCNA distribution described previously (figure 4.10b).

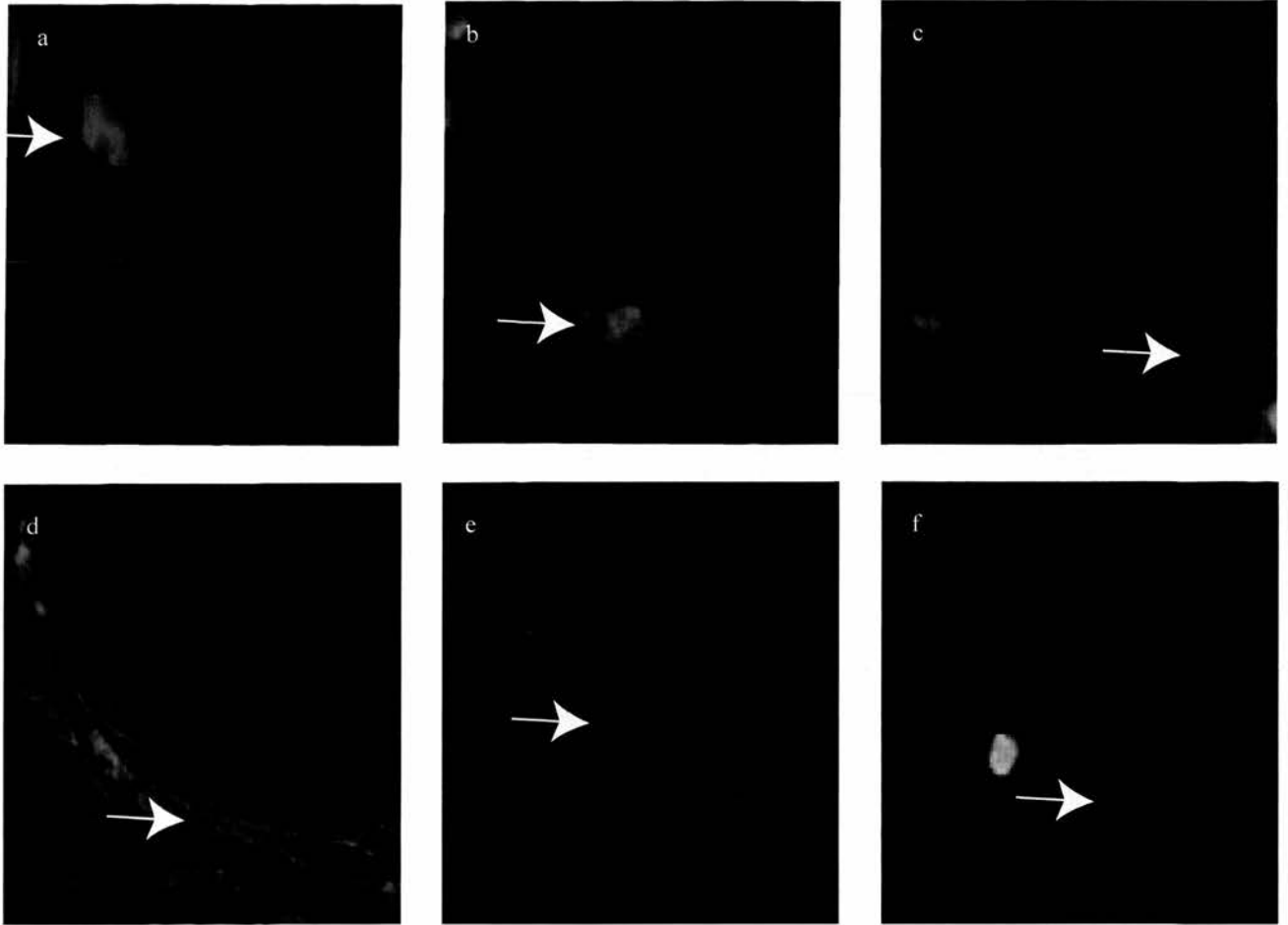
Previous reports have suggested that stromal elements can produce inhibitory factors that prevent stem-cell proliferation and modulate differentiation within the colonic crypt (Vermeulen et al 2010). Many of these factors have been identified to originate from the smooth muscle layer/myofibroblasts that surround the lower 3<sup>rd</sup> of crypts. In order to observe if physical changes within this muscle layer occurred in prolonged culture, immunohistochemistry was performed to detect  $\alpha$ -actin, a marker expressed by smooth muscle cells. Prolonged culture of explants resulted in marked degeneration in cell numbers and size of this smooth muscle layer in all explants (Figure 4.9b).

#### **4.8 Regenerative cyst characterization**

Further examination of the nature of the cystic structures within the regenerating colonic mucosal tissue in long-term culture was undertaken, as they represented a potential precursor of the later development of more architecturally-advanced colonic crypt structures. The epithelial walls of the cysts were composed of a thin layer of epithelium ( $\beta$ -catenin, Vil-1). This demonstrated both high levels of cells which were positive for proliferative markers (Ki67, PCNA) and the stem cell marker (BMI-1) and were also composed of both types of differentiated epithelial cell (Vil-1, Muc-2) (Figure 4.11).

Lgr-5 staining of crypts was undertaken but there were no detectable positive staining cells. This may be due to a. the crypts being lined by highly proliferative cells (as demonstrated by ki-67, PCNA and BMI-1 staining) but not stem cells or b, the cross-sectioning of the cysts sample missing individual stem cells positive for the Lgr-5 marker. The experiments were repeated several times but no obvious Lgr-5 positively staining cells could be detected.

Having conducted various optimisation phases and characterised this model, I now focused on assessing the effects of aspirin on the model in the supra-cellular context of the epithelium as an organ.



**Figure 4.11 Regenerative cyst characterization**

Micrographs of immunohistological staining of stromal epithelial cysts from samples at day 12 in culture stained for markers of proliferation (a. Ki67, b. PCNA, c. BMI-1) and differentiation (d. beta-catenin, e. Muc-2, f. Vil-1)(n=3).

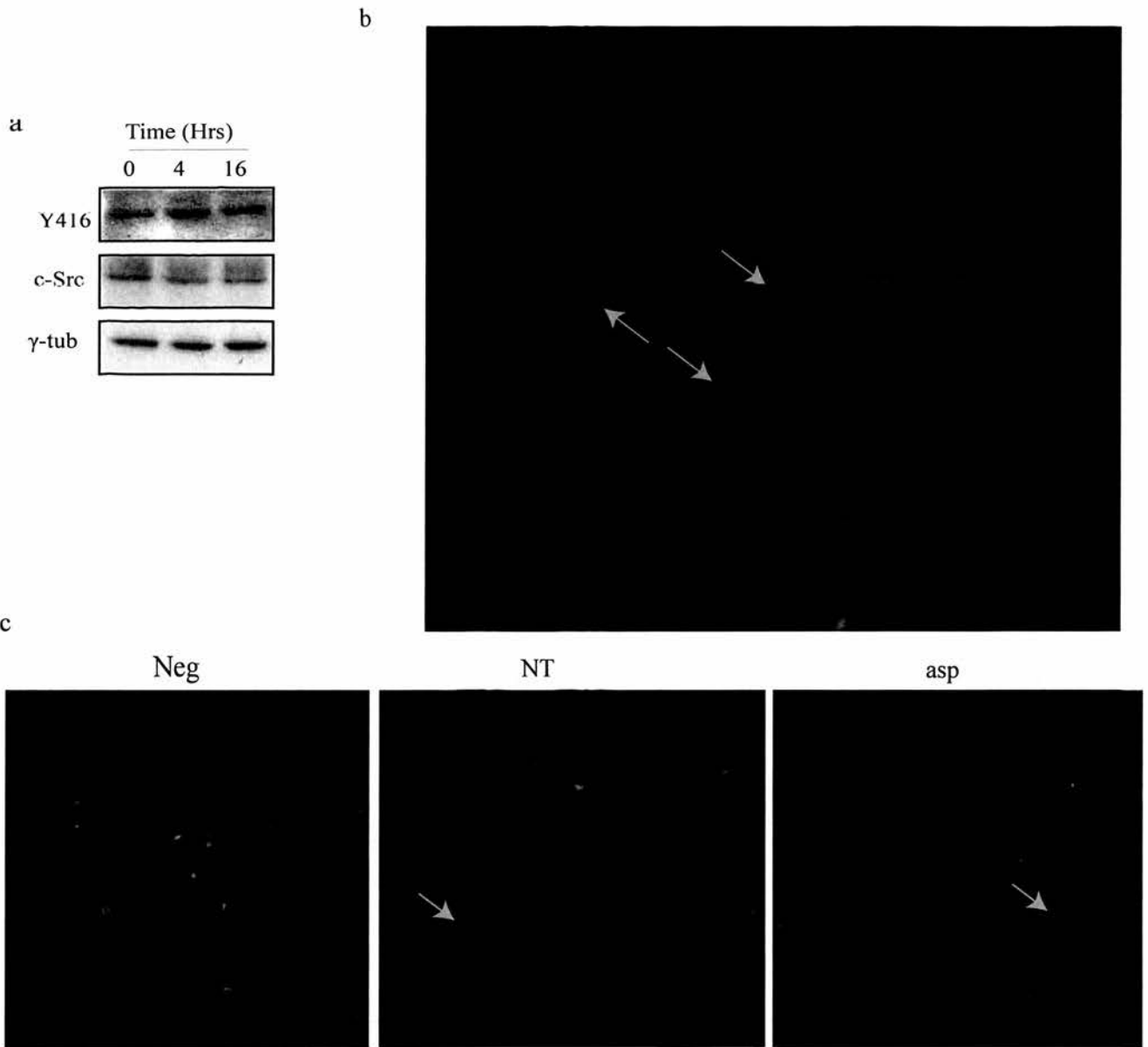


#### **4.9 Aspirin-mediated modulation of c-Src in colonic mucosal explants**

As presented in chapter 3, aspirin induces phosphorylation of c-Src in CRC cell lines and this phosphorylation plays a key role in the apoptotic effects of the agent. To determine whether aspirin had a similar effect on colonic mucosa *in vivo*, I utilized the above protocols developed for growing human colonic mucosa as explant cultures.

The first aim was to evaluate whether c-Src could be detected in IVOC tissue. Western blot analysis was initially undertaken to optimise the detection of c-Src within human mucosal tissue. Previous CRC cell line findings indicate that aspirin activates c-Src in a time-dependent manner through a mechanism involving phosphorylation of the c-Src Y416 residue. Western blot analysis was initially utilized to examine the effects of aspirin on day 16 cultures in time-course studies. Similar to CRC cell lines, I found that in 3 of 4 cultures treated, aspirin induced an increased in c-Src Y416 phosphorylation within 4 hours of treatment. There was a return to baseline levels 16 hours after treatment. This was consistent with the previous kinetics of the transient c-Src activation observed in CRC cell lines. In contrast to phosphorylated c-Src Y416, re-probing of the membranes revealed no change in the levels of native protein (figure 4.12a).

Immunocytochemistry in CRC cell lines revealed aspirin induced changes in c-Src cellular location and activation in response to aspirin (see chapter 3). Therefore, I next performed immunocytochemistry on aspirin-treated explants. Unfortunately, despite extensive optimization attempts, detection of native c-Src by immunocytochemistry was not successful, in that the antibody gave a diffuse, non-specific staining pattern. However, I was able to specifically detect activated c-Src (Y416 phosphorylated). I found that at time 0, phospho-c-Src Y416 was located primarily at the luminal surface of enterocytes, lining the upper levels of colonic crypts. High power magnification demonstrated that the signal for phospho-c-Src Y416 was highest at the plasma membrane, and focally in aggregates along the membrane surface of these enterocytes. In keeping with a role for c-Src in cell adhesion structures, this signal was found to be most highly concentrated in aggregates at the junction with neighbouring cells (figure 4.12b).



#### 4.12 Aspirin-mediated modulation of c-Src in colonic mucosal explants

16 day explants were either untreated (0) or treated with 5mM aspirin for the time points stated a. Western blot analysis on whole cell extracts from aspirin-treated day 16 explants. Extracts were probed for phospho-c-Src Y416 and native c-Src.  $\gamma$ -tubulin was used as a protein loading control (n=3) b. Anti-phospho-c-Src Y416 immunohistochemistry was performed on time 0 explants. DAPI was used as a nuclear marker. Activated c-Src expression localises to the plasma membrane of epithelial cells lining upper third of colonic crypts (yellow arrow). c. Anti-phospho-c-Src Y416 immunohistochemistry was performed on aspirin-treated explants. DAPI was used as a nuclear marker. Activated c-Src expression is increased in the epithelium of regenerating crypts but this is less pronounced in surface epithelium (yellow arrow). Secondary antibody only negative control is also represented (Neg) (all data minimum of 3 experiments).

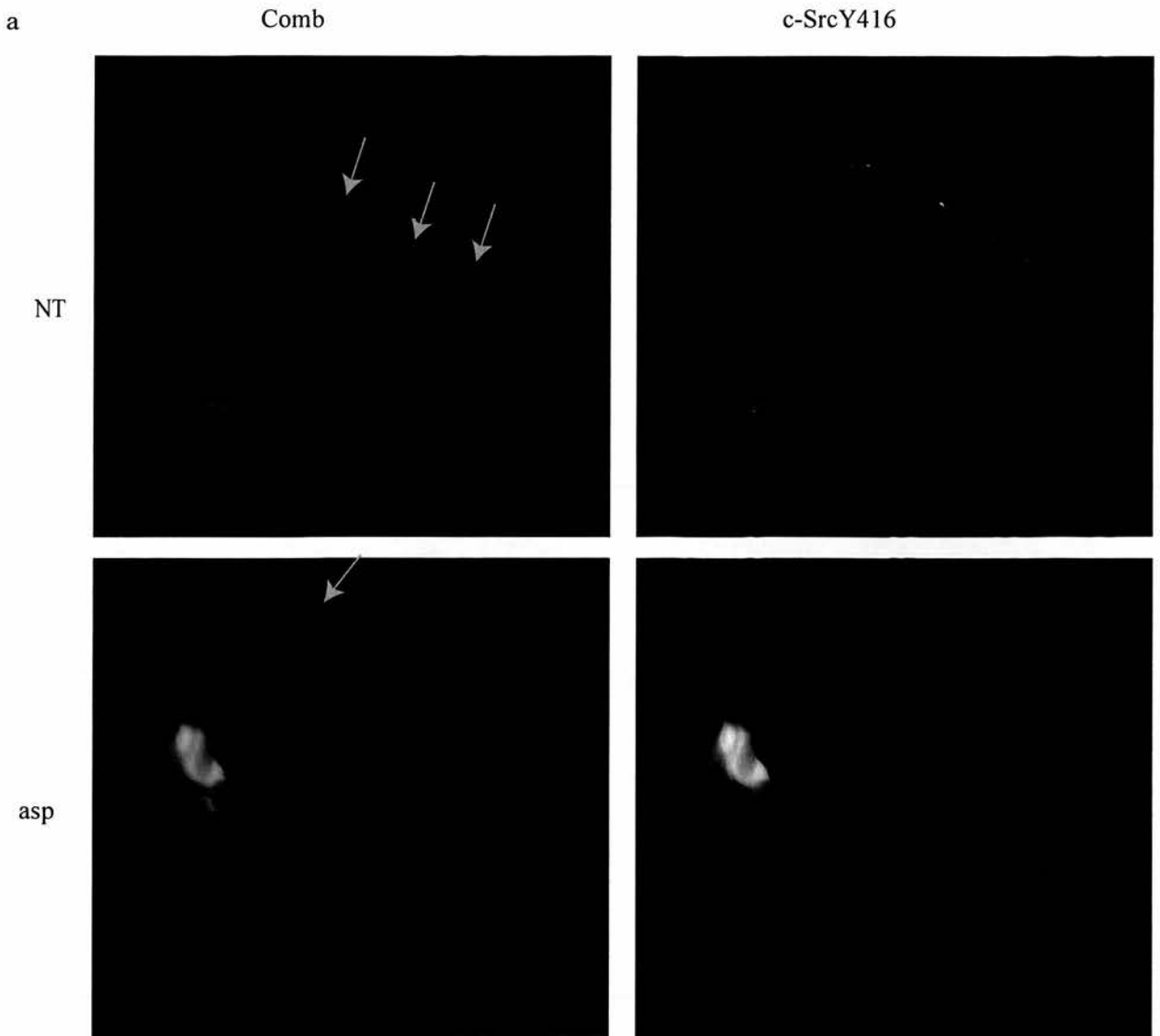
Interestingly, enterocytes within regenerating crypts expressed higher levels of activated c-Src on their cell surface compared to baseline levels (figure 4.12c). c-Src activation is highly associated with CRC progression and analysis of hyperproliferative and premalignant colonic epithelium within the AOM-rat model found that prior to the development of neoplastic lesions, the tumor suppressor gene C-terminal Src kinase (Csk) (a negative regulator of c-Src) was down-regulated, with a concomitant increase in c-Src activity (Kunte et al 2008). Increased expression within the explant may therefore be a reflection of underlying epithelial hyperproliferation within regenerating crypts.

Colonocytes on the explant surface demonstrated a change in the pattern of c-Src membrane aggregates following aspirin administration (figure 4.13a). Namely, both the size and density of membrane focal aggregates decreased. This pattern had similarity to the aspirin-induced effect on FAK/phospho-c-Src Y416 aggregates in CRC cell lines (chapter 3.5). Attempts to further characterise these membrane aggregates were unsuccessful as, despite optimisation attempts, it was not possible to detect a positive signal in immunohistochemistry of explant tissue utilising the currently available FAK antibody.

#### **4.10 Aspirin modulation of paxillin in mucosal explants**

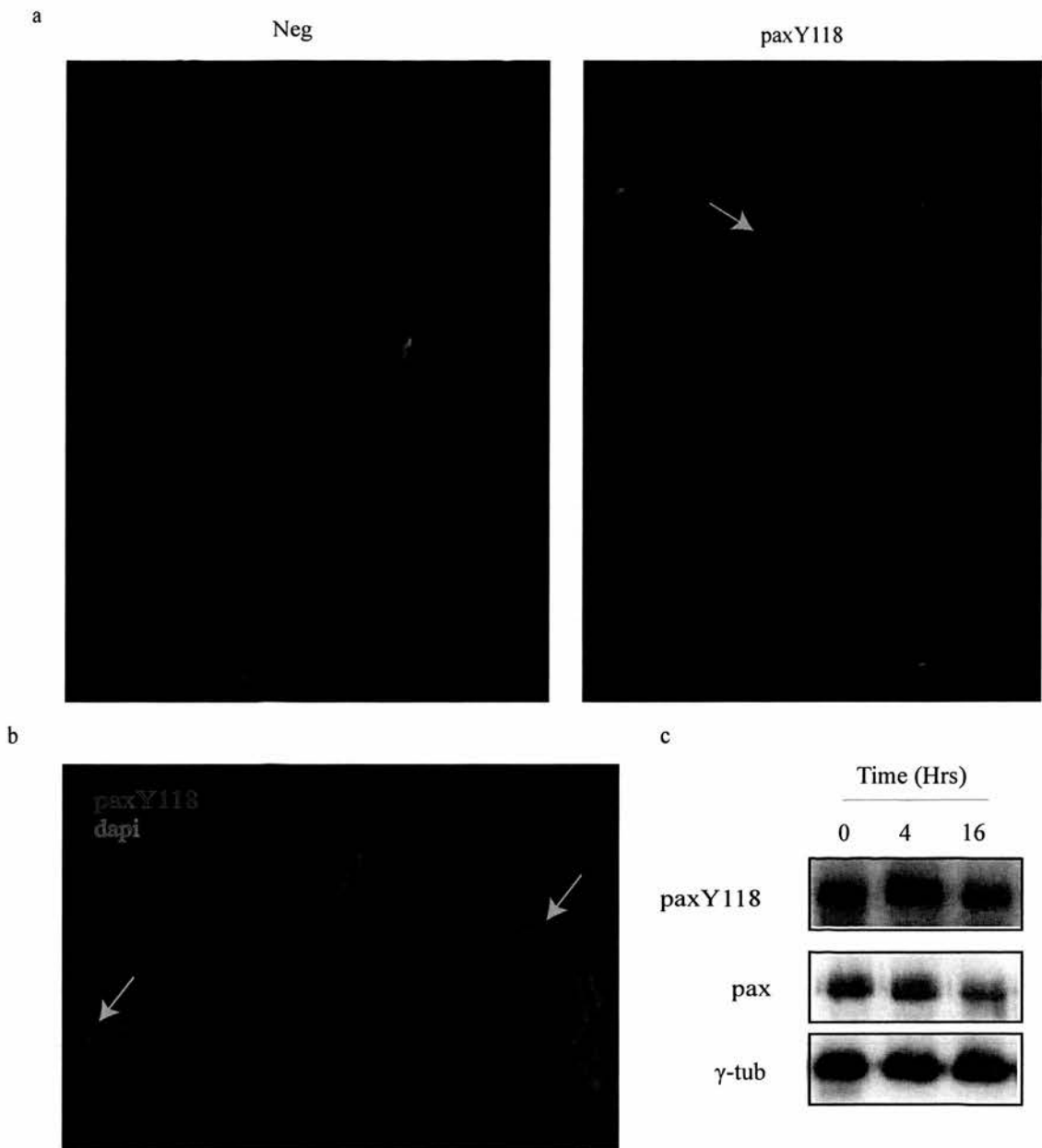
Given that aspirin induced phosphorylation of c-Src Y416 and changes to its membrane location, further confirmation of c-Src activation within explants was undertaken. I had previously demonstrated that phosphorylation of paxillin at Y118 occurs in response to aspirin and that this was dependent on c-Src kinase activity. Therefore, I next examined paxillin phosphorylation in mucosal explants.

Immunohistochemical analysis of time 0 explants demonstrated the location of phosphorylated paxillin Y118 mimicked that of activated c-Src in human colonic mucosa in that the paxillin Y118 signal localized to the luminal surface of enterocytes in the upper levels of the crypt (figure 4.14a and 4.14b).



**Figure 4.13 Aspirin-mediated modulation of c-Src aggregates in colonic mucosal explants**

16 day explants were either untreated (NT) or treated with 5mM aspirin for 4 hours  
 a. Anti-phospho-c-Src Y416 immunohistochemistry performed on aspirin-treated explants. DAPI was used as a nuclear marker. Focal aggregates of phospho-c-Src Y416 undergo dissolution following aspirin (yellow arrow). Findings consistent in 3/4 experiments.



#### 4.14 Aspirin modulation of paxillin in mucosal explants

Explants were either untreated (NT) (a, b) or treated with 5mM aspirin for the time points stated (c). a.b. Anti-phospho-paxillin Y118 immunohistochemistry was performed on explants. DAPI was used as a nuclear marker. Phosphorylated paxillin Y118 signal localises to the plasma membrane of epithelial cells lining the upper third of colonic crypts (yellow arrow) (n=7) c. Western blot analysis performed on whole cell extracts from aspirin-treated day 16 explants (n=3). Extracts were probed for phospho-paxillin Y118, native paxillin and  $\gamma$ -tubulin was used as a protein loading control.

Western blot analysis of extracts from aspirin-treated explants demonstrated increased phosphorylation of paxillin Y118 at 4 hours following administration of the agent, concurrent with the above kinetics of the aspirin-induced phosphorylation of c-Src Y416 and consistent with the response observed in CRC cell lines. However, 16 hours following treatment, the levels of phosphorylated paxillin Y118 decreased, in parallel with the overall level of the native protein (figure 4.14c). The reasons for this, when set against the findings for activated c-Src at 16 hours, are unclear at present.

These findings support the earlier data obtained from CRC cancer cell lines, that aspirin induces phosphorylation/activation of c-Src and phosphorylation of paxillin Y118 within hours in human *ex vivo* colonic mucosa.

#### **4.11 Aspirin-induced NF- $\kappa$ B Signalling and apoptosis in human colonic mucosal explants**

Previous findings suggested the c-Src-dependent apoptotic effect of aspirin occurred through modulation of the NF- $\kappa$ B signalling pathway (see chapter 3). Therefore, I next assessed the effects of aspirin on NF- $\kappa$ B signalling and apoptosis within the colonic mucosal explants.

Firstly, I examined aspirin effects on cytoplasmic levels of I $\kappa$ B $\alpha$ . Western Blot analysis of I $\kappa$ B $\alpha$  levels in aspirin-treated IVOC tissue extracts found that these decreased 16 hrs after treatment, subsequent to phosphorylation of paxillin (figure 4.15a). Next, I examined nuclear levels of RelA using immunohistochemistry (Stark et al 2001). In aspirin treated samples, increased RelA nuclear staining of cells was noted along the uppermost mucosal surface of the crypts compared to controls (figure 4.15b). However, due to the nature of the staining, the complexity of the plane of tissue dissection for mounting and variability of the nuclei, quantification of this effect was not possible.

As NF- $\kappa$ B activation is a key element of the pro-apoptotic effect of aspirin in CRC cells, experiments to detect aspirin-induced apoptosis, using a known marker of apoptosis, such as activated caspase-3, was undertaken. Aspirin induced a time-dependent increase in activated caspase-3 at 16 hours after administration (Figure 4.16a). Furthermore, the increased detection of the apoptotic marker occurred

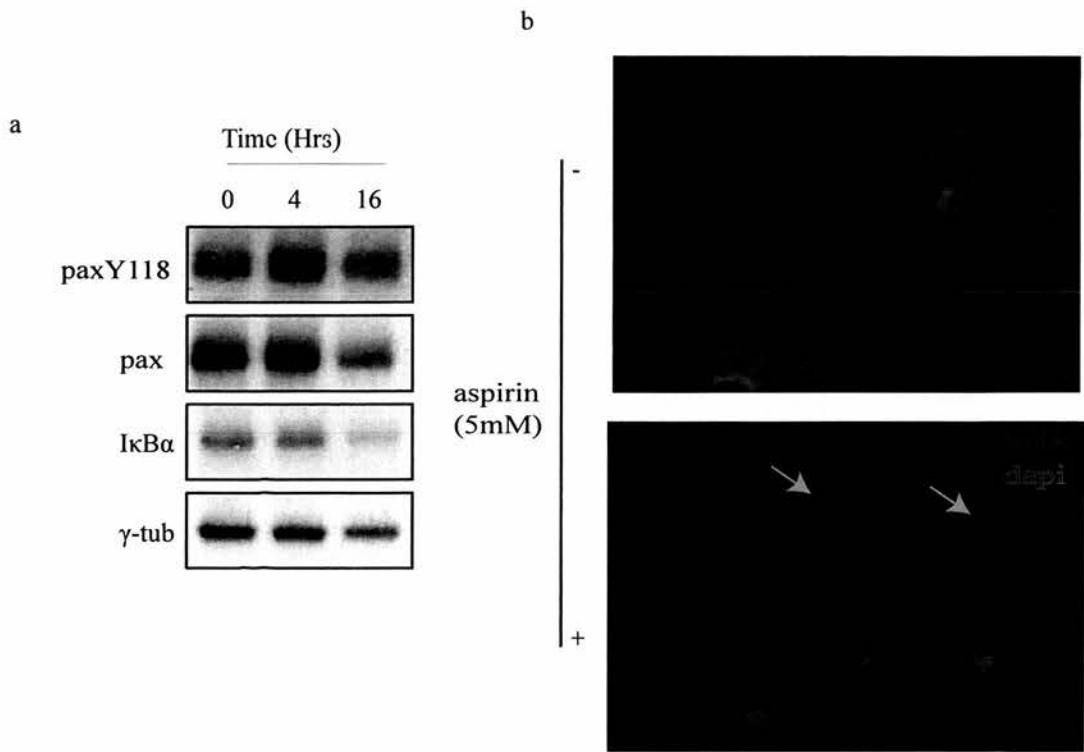
subsequent to aspirin-induced phosphorylation of paxillin Y118 and concurrently with detection of aspirin-induced I $\kappa$ B $\alpha$  degradation (figure 4.15a).

Previous studies from this laboratory have demonstrated that nuclear fragmentation and classical apoptotic bodies are rarely seen in conjunction with aspirin induced-apoptosis in SW480 CRC cells (Stark et al 2001). However, histological assessment of aspirin-treated 16-day explants demonstrated that aspirin induced apoptotic bodies, breaks in epithelium and higher tissue damage scores (figure 4.16b and 4.16c).

#### **4.12 Ex vivo growth of colonic adenomatous polyps**

Aspirin appears to prevent the initiation rather than the progression of CRC. Therefore, in order to further extend the relevance of these studies for testing aspirin and anti-cancer agents, *ex vivo* culture of colorectal adenoma tissue was also undertaken. 3 patients were recruited for colorectal polyp culture optimisation protocols. *Ex vivo* culture of colorectal adenomas from these 3 patients was attempted. However, I found that polyps underwent massive apoptosis and tissue destruction both macroscopically and histologically without subsequent regeneration. Explants were characterised by detachment of the surface epithelium and disintegration of any remaining epithelium (figure 4.17a).

It became apparent that the growth conditions for these neoplastic lesions were dissimilar to normal mucosa and would require considerable optimisation. Unfortunately, as outlined in the methodology section, the material obtained from adenomas was also of insufficient quantity to allow testing of varying growth conditions for a single lesion.



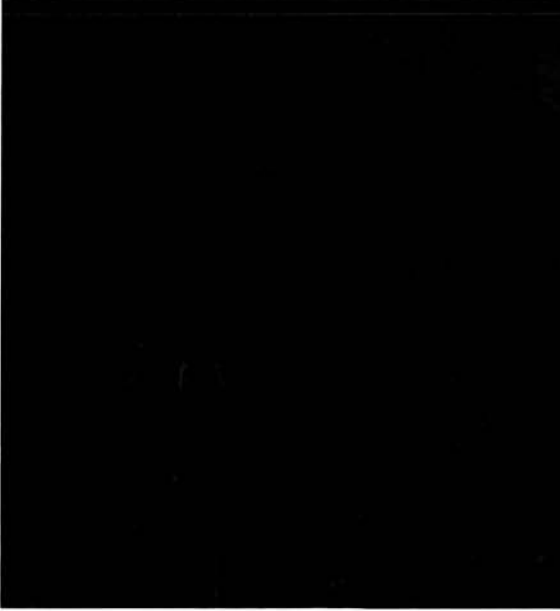
#### 4.15 Aspirin-induced NF- $\kappa$ B Signalling in human colonic mucosal explants

Explants were either untreated (0) or treated with 5mM aspirin for the time points stated (a), or 4 hrs (b) a. Western blot analysis on whole cell extracts from aspirin-treated day 16 explants. Extracts were probed for phospho-paxillin Y118, native paxillin, I $\kappa$ B $\alpha$  and  $\gamma$ -tubulin was used as a protein loading control. Results consistent in 3/4 experiments. b. Anti-RelA immunohistochemistry was performed on explants. DAPI was used as a nuclear marker. Aspirin induced RelA translocation to the nucleus in surface cells (yellow arrow)(n=3).

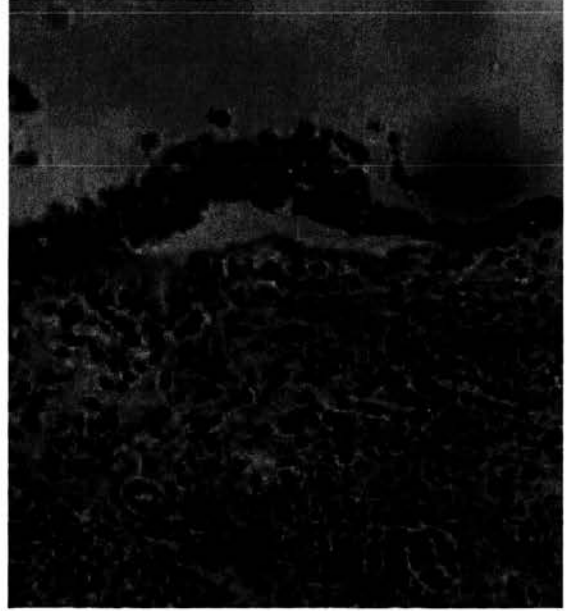




a



day 0



day 4

#### **4.17 Ex vivo growth of colonic polyps**

a. Histological explant structure demonstrated by H&E staining of formalin-fixed samples at day 0 and day 4. (results are representative of 3 independent experiments).

#### **4.13 Lithium chloride activates colonic mucosal Wnt signalling**

Given the failure to maintain polyps in long-term IVOC, in order to further replicate the hyperproliferative state at which chemoprevention is most relevant, I next attempted to induce explants to mimic the hyper-proliferative state found in colonic polyps and tumours.

Wnt signalling is known to be the most important pathway for intestinal epithelial cell proliferation and plays a critical role in CRC tumorigenesis. Briefly, in the canonical Wnt signalling pathway, stimulation of the frizzled receptor leads to higher levels of cytosolic  $\beta$ -catenin initially, subsequent  $\beta$ -catenin nuclear transportation and the stimulation of Wnt-target genes (including Lgr-5 and other genes involved in cellular proliferation) (van der Flier and Clevers 2009).

Lithium treatment mimics the effects of canonical Wnt signalling by inhibiting glycogen synthase kinase-3 $\beta$  (GSK- $\beta$ ), leading to reduced GSK3-mediated phosphorylation of  $\beta$ -catenin (including Ser 552), thus preventing its subsequent degradation by the ubiquitin proteasome system. Accumulated cytosolic  $\beta$ -catenin then localises to the nucleus and promotes the expression of genes regulated by Lef-Tcf transcription factor (Hedgepeth et al 1997, Klein and Melton 1996, Li et al 2011). These include c-Myc and cyclin-D1 (Brabletz et al 2009, Kolligs et al 2002), genes that promote proliferation and survival (Sinha et al 2005). Lithium also affects the inhibitory BMP pathway, inhibiting Smad 1,5 and 8 phosphorylation (Li et al 2011), thereby inducing stem cell derived epithelial proliferation.

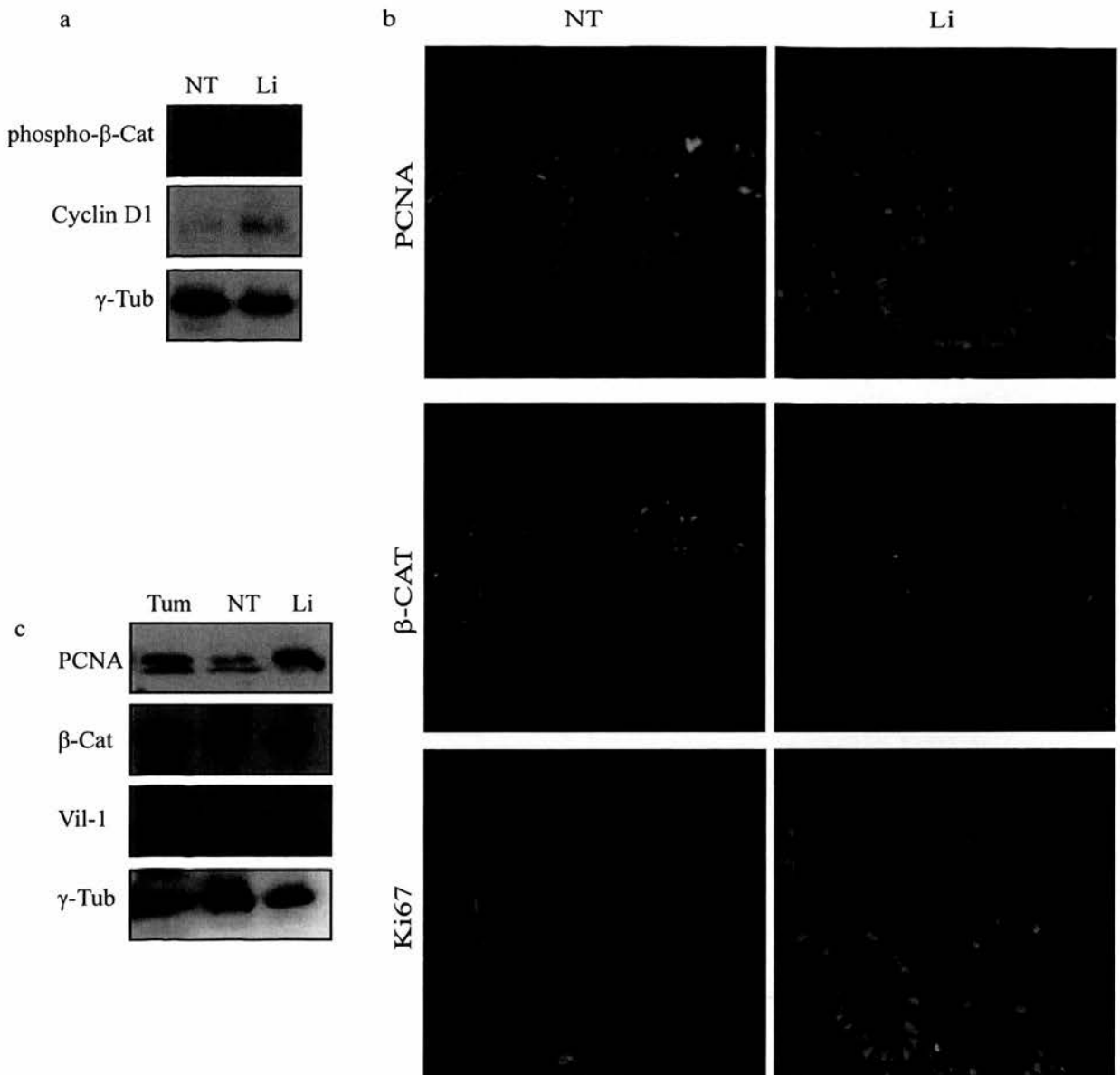
As lithium chloride modulates major pathways responsible for stem cell activation, I set out to determine whether this agent could induce proliferation of human CRC mucosa. Firstly, we optimised the use of lithium chloride in CRC cell lines. Lithium chloride was found to induce a dose-related increase in expression of c-Myc within CRC cell lines (supplementary data 7.1). Therefore, experimentation next turned to focus to the effects of daily supplementation of lithium chloride within the IVOC model system.

$\beta$ -catenin is itself a direct target of active Wnt signalling. Immunohistochemistry (fig 4.18b) and western blot analysis (figure 4.18a) indicated that levels of this protein also increased in mucosal explants when exposed to lithium chloride. Phosphorylation of  $\beta$ -catenin on Ser 552 residue is a marker of Wnt activity and results in  $\beta$ -catenin nuclear accumulation with subsequent Wnt-dependent transcription of Lef–Tcf dependent genes (Fang et al 2007). Lithium chloride addition also resulted in increased levels of phosphorylation of  $\beta$ -catenin Ser 552 within explants.

Lithium supplementation also increased expression of the downstream Wnt-dependent gene, cyclin D1 (Kolligs et al 2002) (Figure 4.18a). As Cyclin D1 is a marker of proliferation and cell cycle progression, these data would suggest lithium chloride stimulates proliferation of explants. In keeping with these data, immunohistochemistry demonstrated that lithium chloride increased levels of the proliferation markers PCNA (figure 4.18b and 4.19a) and Ki67 (figure 4.18b and 4.19b). Furthermore, western blot analysis confirmed a dramatic increase in PCNA expression in explants grown in the presence of lithium chloride, compared to no lithium chloride controls (figure 4.18c).

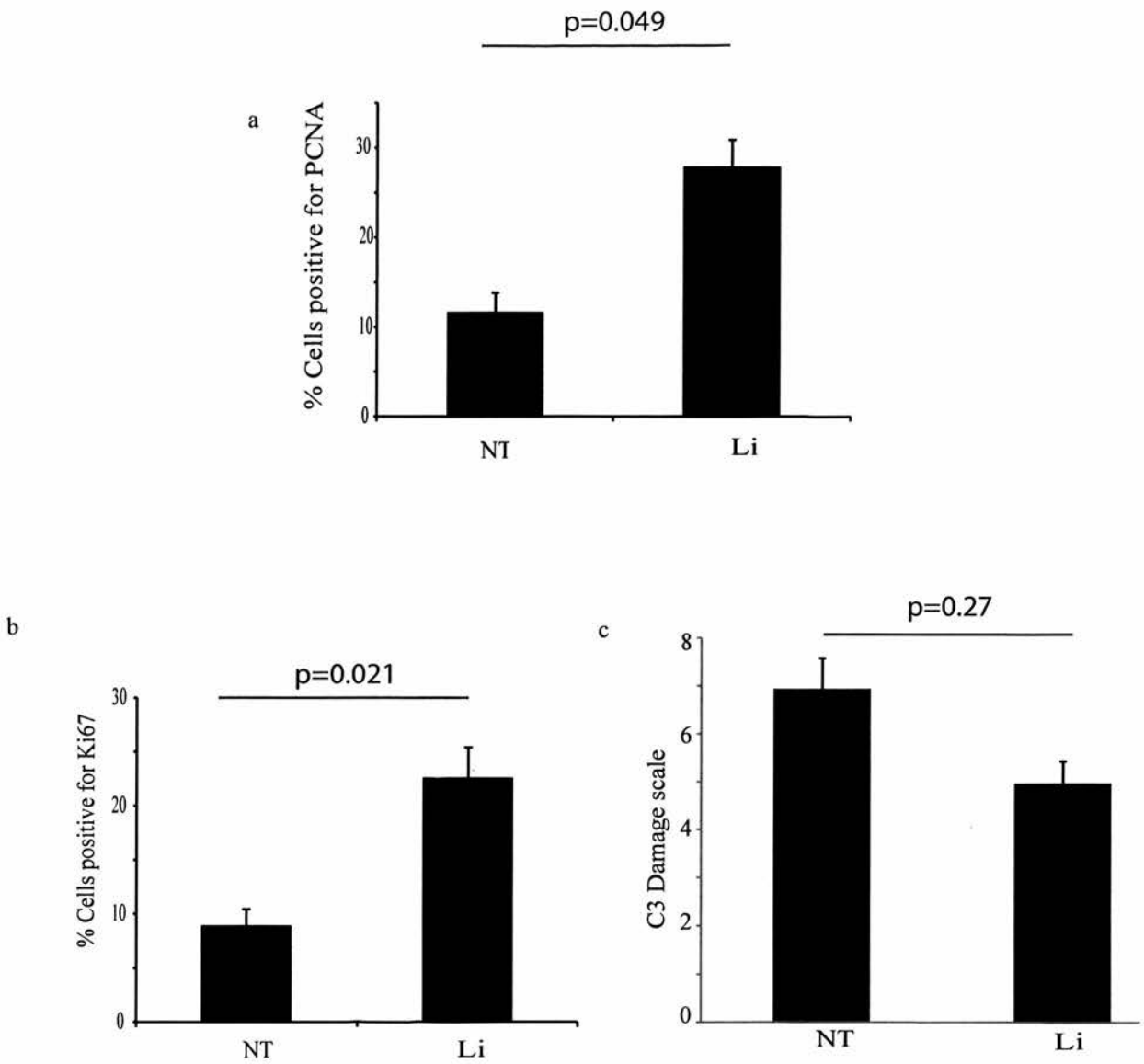
In addition, lithium chloride also increased expression of the differentiated enterocyte marker, Vil-1 (Figure 4.18c). Taken together, these data suggest that this agent caused increased proliferation and differentiation of colonic epithelial cells. Indeed, when H&E sections were scored, it was found that cultures grown in the presence of lithium chloride had reduced damage scores compared to controls (figure 4.19c).

Thus, the induction of Wnt stimulation by daily lithium supplementation induces an apparent hyperproliferative state within the explant that was characterised by Wnt stimulation, increased cell cycle progression, proliferation, and an increasing population of differentiated epithelial cells within explants.



#### 4.18 Lithium chloride activates colonic mucosal Wnt Signalling (i)

Explants were cultured for 16 days in modified Weymouth's media and were either untreated (NT), or treated daily with 0.5 mM Lithium chloride (Li). a. Addition of Lithium results in increased detection of phosphorylated β-catenin and increased expression of the Wnt-dependent gene, cyclin D1. γ-tubulin is used as a loading control. (n=3) b. Representative images from explants demonstrate staining for β-catenin, PCNA and Ki67 (n=5). c. Lithium supplementation induces increased expression of PCNA, β-catenin and Vil-1. Colorectal tumour tissue (Tum) is used as a positive control. γ-tubulin is used as a loading control (n=3).



**4.19 Lithium chloride activates colonic mucosal Wnt Signalling (ii)**

Explants were cultured for 16 days in modified Weymouth's media and were either untreated (NT), or treated daily with 0.5 mM Lithium chloride (Li) a, b. The percentage of positively staining cells for proliferative markers (a. PCNA, b. Ki67) were counted in a minimum of 5 separate fields +/- standard error (n=3). c. C3 damage scale scoring of explants as determined by 3 independent and blinded observers (n=3)(+/- Standard error)

#### **4.14 Inhibition of Notch signalling induces goblet cell differentiation**

The above data suggested that proliferation within explants could be influenced by the addition of external Wnt stimulants. Given that control of proliferation could be influenced, I further investigated whether differentiation within explants could also be modified.

Notch signalling maintains crypt progenitor cells in an undifferentiated state, and it controls progenitor cell differentiation between secretory and absorptive cell fates (Wilson and Radtke 2006). The activation of Notch signalling is critically dependent on the transmembrane protease complex  $\gamma$ -secretase (Baron 2003, De Strooper et al 1999, Mumm et al 2000). Following Notch receptor activation, canonical Notch signalling leads to Hes1 upregulation and Atoh1/Hath1/Math1 down-regulation (Zheng et al 2011). The Atoh1 transcription factor induces differentiation towards a secretory cell lineage. As Notch signalling down-regulates Atoh1, it is implicated in maintaining colonocytes in a progenitor state, inhibiting goblet cell differentiation (reviewed in (Katoh and Katoh 2007)).

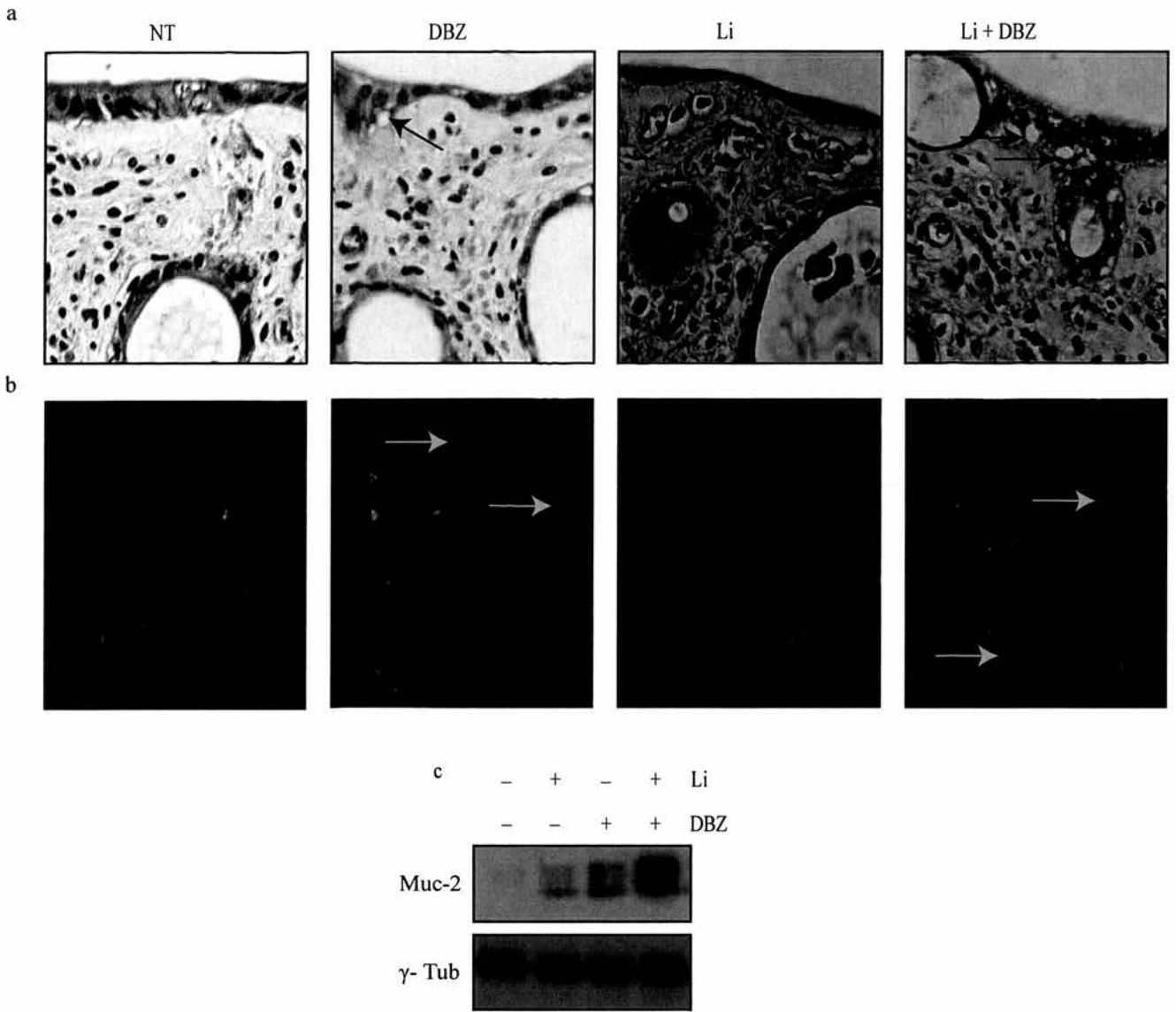
Previous studies have demonstrated that genetic disruption of Notch signalling in the intestinal crypt compartment, or Notch inhibition by chemical antagonists, induces colonic epithelium to differentiate towards a secretory/goblet cell phenotype (resulting in increased Muc-2 expression) (Coant et al 2010, Okamoto et al 2009). Indeed, in a recent human preclinical study of  $\gamma$ -secretase inhibitors as a trial treatment for Alzheimer's disease (one setting in which their use is being evaluated), addition of the inhibitor induced a side effect characterised by the appearance of goblet cells within the intestine, mirroring the effects of deliberate genetic disruption of Notch signaling, reported from some animal studies (Lundkvist and Naslund 2007).

In order to assess whether it was possible to influence the cell fate decisions of progenitor cells within the IVOC model, the  $\gamma$ -secretase inhibitor, Dibenazepine (DBZ) was added to IVOC culture media on a daily basis for 5 days between days 10-15 of culture. Previous reports have suggested that differentiation of colonic epithelium to a secretory/goblet cell phenotype (muc-2 expression) after  $\gamma$  -

secretase inhibition is most efficacious in those cells that are actively proliferating (Menke et al 2010). Given that the above results demonstrated that lithium chloride increased proliferation within the explants, I therefore also assessed the effect of the concurrent addition of these two agents on explant differentiation.

At day 16, treatment with DBZ alone induced a moderate induction of the secretory cell phenotype, however the most pronounced effect occurred in those explants that had been concurrently treated with lithium chloride supplementation. Using immunohistochemistry, there was an increase in the number of cells that stained positive for Muc-2 expression (figure 4.20a) and following western blot analysis with antibodies against Muc-2, there was a large increase in the of levels of Muc-2 protein expression within the tissue extracts after DBZ and lithium chloride treatment (figure 4.20b).





#### 4.20 Inhibition of Notch signalling induces goblet cell differentiation

Explants were cultured for 16 days in modified Weymouth's media and were either untreated (NT), or treated daily with 0.5 mM Lithium chloride (Li) supplementation or with daily doses of Dibenazepine (DBZ) for 5 days before harvesting. a. H&E histology demonstrated the induction of a secretory cell phenotype in DBZ treated explants. b. Anti-Muc-2 immunocytochemistry demonstrated increased expression of Muc-2 in Li- and DBZ-treated explants. DAPI was used as a nuclear marker. c. Anti-Muc-2 western blot analysis of protein extracts from Li/DBZ treated explants.  $\gamma$ -tubulin was used as a protein loading control. Results are representative of 2/3 independent experiments.

#### **4.15 Discussion**

This research phase established a number of important findings. Firstly, I made substantial progress in establishing the optimal conditions for long-term *in vitro* organ culture of human *ex vivo* colonic mucosa. Through this, I observed a biphasic process of apoptosis and proliferation during long-term culture. Subsequently, proliferation within this model resulted in a diverse, differentiated cell population organised in epithelial crypt-like structures, thus mimicking the histological features of the human *in vivo* colonic mucosa in architectural complexity and population heterogeneity. Cells positive for the putative colonic stem markers, Lgr-5 and BMI-1, were also identified for the first reported time in a colonic IVOC culture model of this type, suggesting a potential underlying mechanism for the proliferative and regenerative response observed.

Secondly, I tested a range of agents (lithium chloride, DBZ and aspirin) within an IVOC model of human *ex vivo* colonic mucosa. I induced a hyperproliferative effect in the epithelium of explants, by stimulating Wnt signalling and influenced cell differentiation by Notch inhibition. Importantly, the experimental outputs following aspirin treatment provide considerable support for the human biological relevance of findings derived from previous experimentation in CRC cell lines, specifically in regards to the aspirin-induced c-Src and NF- $\kappa$ B dynamics.

This study is the first to report the detection both Lgr-5- and BMI-1-positively staining cells in human colonic mucosa within culture. The pattern of staining suggest, in concordance with previous reports, that these populations of cells are distinct. Focal staining of Lgr-5 cells at the base of crypts was consistent with their location in previous studies (Barker et al 2007). However, the non-specific staining pattern of BMI-1 in repopulating crypts, mirroring that of the proliferation markers Ki67 and PCNA, suggested that whilst BMI-1 is a sensitive marker of proliferating cells, it lacks the specificity to accurately detect a putative small number of CSC within crypts. Previous studies have suggested an apparent lack of BMI-1 expression in colon and distal small bowel, which suggest the existence of a BMI-1-negative intestinal CSC population. It remains to be seen if BMI-1 is expressed in a stem cell

population only, or provides a non-specific marker for highly proliferative cells, including those positive for Lgr-5 expression (Haegebarth and Clevers 2009).

There are two main strategies used to identify stem cells. These rely on confirmation of the characteristics of self-renewal and multi-potency *in vitro* and *in vivo*. Stem cells can be identified by marker expression and then *in vitro* culture and/or transplantation into animal models is performed. Alternatively, cells are genetically marked *in situ* and the cell and its progeny can then be traced over time. This human mucosal model provides an ideal future platform on which to confirm the characteristics of multi-potency by potentially allowing the identification of marked stem cell populations over time or alternatively observation of the dynasty of animal stem cells which are genetically marked and transplanted into such a model (Bareiss et al 2008). The lack of an immune response within the model also allows flexibility in avoiding the host/graft responses potentially present in other more advanced models.

Embryonic cryptal “spheroids”/ residual epithelial formations (Astrup et al 1978a, Astrup 1980) and cyst-like structures (Bareiss et al 2008) have been described elsewhere in regenerating explants and may comprise a functional stem cell compartment, augmented by potential stem cells (Astrup et al 1978a). Here, cystic structures contained cells of all lineages but importantly were highly proliferative, staining heavily for both proliferation markers and BMI-1. The Intestinal Stem Cell Niche (ISCN) is notable for myofibroblasts adjacent to the crypt base which are believed to secrete paracrine signals regulating the neighbouring ISCN (Ootani et al 2009, Vermeulen et al 2010). Previous reports have suggested that these stromal elements can produce inhibitory factors, such as BMP, which prevent stem-cell proliferation and modulate differentiation within the colonic crypt (Kosinski et al 2007, Powell et al 2011). There was progressive deterioration and reduction in the alpha-actin staining smooth muscle layer and muscle fibres surrounding the base of crypts. Potentially, this degradation could reduce previous inhibitory signals, allowing uninhibited stem cell activity, and thus the development of mature architectural structures such as crypts.

Studies using the drosophila midgut suggest that as intestinal epithelial cells encounter damaging stress (e.g. bacterial infection) they are able to signal to stem cells to increase proliferation to replace damaged cells (Jiang et al 2011a, Shaw et al 2010). The simple structure of the fly gut means that signalling from epithelial cells to stem cells, which are adjacent, is potentially very straightforward. Whether this type of signalling exists in the human intestine and how the signal may be transduced from surface epithelial cells to stem cells near the base of crypts, remains to be elucidated. IVOC of human colonic tissue is an ideal model to answer this question due to the presence of the diverse cell populations found *in vivo*, particularly the stromal cells which may play a role in relaying signals from the surface epithelium to crypt cells. Such signalling could also explain the selective nature of the apoptosis seen during short-term culture in RPMI media.

The intestinal epithelium is subject to continuous renewal. This includes progenitor proliferation, directional migration of epithelial cells from the crypt region and, ultimately, cell death (Gregorieff and Clevers 2005, Wilson and Radtke 2006). The IVOC model undergoes relatively controlled regeneration of surface epithelium following a biphasic process of apoptosis and proliferation. The maintenance of such intestinal homeostasis depends on regulation of various cell processes such as differentiation, migration and proliferation, which are tightly controlled by a small number of highly conserved signalling pathways (Kanwar et al 2010).

Extracellular Wnt signals are absolutely required within the stem cell compartment/niche in order to drive and regulate this cell proliferation (Ootani et al 2009), and studies have observed the rapid ablation of proliferation and secondary loss of differentiation in the setting of Wnt inhibition (Ootani et al 2009, Yen and Wright 2006). Here, daily pulses of lithium chloride (for 16 days) stimulated endogenous Wnt signalling, demonstrated by increased phosphorylation (activation) of  $\beta$ -catenin and the expression of the Wnt transcriptional targets, cyclin D1 and c-Myc. Lithium-treated explants demonstrated improved cryptal maturity, proliferation, and an increased differentiated epithelial cell population. These data are highly significant, as they demonstrate that a hyperproliferative state can be induced within explants of normal colonic mucosa, mimicking the very early stages of colorectal

carcinogenesis. This model is therefore relevant for future testing of the efficacy of chemopreventative agents.

Colonic mucosal epithelium is tightly controlled to ensure maintenance of a monolayer-type organization and adequate barrier function, however, NSAIDs have been shown to disrupt intestinal integrity and long-term treatment leads to inflammation of the colonic mucosa (Bjarnason et al 1986). Specifically, NSAIDs have a deleterious structural effect on the tight junctions that are integral to the barrier function of the colon (Mima et al 2008). TJs contain the transmembrane proteins occludin and claudin, which are connected to the cytoskeleton via zonula occludens (Anderson and Van Itallie 1995) (Mima et al 2008). NSAIDs are known to reduce the transcription of claudin (Mima et al 2008) and previous analysis of the cumulative effects of celecoxib-induced changes in the gene expression within rectal mucosa from HNPCC patients indicates substantial changes to gene expression of adhesion-related molecules.

Analysis of the cumulative effects of celecoxib-induced changes in the gene expression within rectal mucosa indicates substantial changes occur in the expression of proteins associated with cell adhesion. Forty of these genes encode proteins that are involved in focal contacts and in fact previous authors have suggested that the pro-apoptotic effect of celecoxib was related to changes in focal adhesion (Glebov et al 2006). Of interest, both c-Src and FAK play a key role in focal contact turnover, adhesion and NF- $\kappa$ B signalling (see chapter 3). c-Src localises at the TJ and the tyrosine kinase activity of c-Src has been found to be involved in the disruption of TJ in human intestinal epithelium (Basuroy et al 2003). Inhibition of c-Src (by chemical or genetic methods) has been previously reported to prevent normal tight junction opening in response to selected stimuli (Basuroy et al 2003, Kevil et al 2001). Given that aspirin induced activation of c-Src, phosphorylation of paxillin Y118 and the disassembly of membrane aggregates in this model, findings here could suggest that activation of c-Src in colorectal mucosal tissue occurs concomitantly to junctional disassembly and may be involved in the pro-apoptotic effects of these agents, but further research focusing on the constituency of the c-Src-rich membrane aggregates and component proteins of TJ would be required.

Surgical procedures on the large intestine are common and offer considerable opportunities for harvesting surplus fresh tissue from human subjects without ethical problems in principle— provided the perceived obstacles finding collaborative surgeons can be overcome (Dame et al 2010). Therefore, this model potentially provides an available, reliable and representative colonic mucosal model in which to examine molecular mechanisms of relevance to aspirin's chemopreventative effect. However, further exploration could improve explant quality and survival rates.

Following optimisation, greater than 60% of left sided resection explants were maintained to day 16. Whilst better, more targeted, antibiotic prophylaxis may have negated some bacterial infections and improved culture yield, the anatomical site of origin of explant was found to be an important determinant in culture success. Whilst conflicting reports exist (Wang et al 2005), previous authors have reported differences in the bacterial species which dominate at different colonic sites, i.e. *Lactobacilli spp.* favouring the sigmoid/rectum and *Eubacterium rectale* and *faecalibacterium prausnitzii* more dominant in the ascending and descending colon (Ahmed et al 2007). Site-specific bacterial influences could therefore account for some differences in the success/infection rates experienced. Additionally, known colonic site-specific gene expression differences, particularly in developmental gene expression (Noble et al 2008) or stem cell distribution (Sangiorgi and Capecchi 2008) may play an unknown role in the differences observed in tissue damage scores observed in regenerating right- versus left-sided colonic explants.

As a result of early optimisation and explant survival data, I focused on recruitment of patients who underwent left-handed resection. Therefore, studies described here are not designed to detect a differential colonic regional response to aspirin. The wider implications of aspirin-induced side effects on other areas of the GI tract are, however, of fundamental importance in considering the viability/design of any future chemopreventative agent and in the interpretation of data derived from any colonic region-specific study. Of major relevance to this project, recent research has also demonstrated a divergent response to sulindac in different regions of the colon. Sulindac administration reduced azoxymethane-induced distal colon tumours in all mice but in the proximal colon, sulindac induced inflammatory lesions on the mucosal folds, which further developed into

adenocarcinoma in some mice with a distinct profile of pro- and anti-inflammatory factors including upregulation of hypoxia inducible factor 1 $\alpha$  and macrophage inflammatory protein 2 (Mladenova et al 2011).

In conclusion, explants of colonic epithelium can be manipulated in culture to represent early stages of carcinogenesis and may provide an ideal model for studying the molecular mechanisms of CRC initiation. Furthermore, aspirin-induced effects observed previously within *in vitro* CRC cell lines, specifically effects on c-Src/NF- $\kappa$ B signalling and apoptosis, were also demonstrated to be biologically relevant in the IVOC model of *ex vivo* human colonic mucosal. The confirmation of the molecular mechanisms involved in aspirin's cancer-preventative role has obvious implications for the design of future, safer, more effective cancer preventative agents. In addition, this process has created a highly representative human tissue model, which has much value for use in future studies focused on the confirmation of previous laboratory-based molecular findings and in future drug development/evaluation.

## Chapter 5 –Discussion

Collectively, the data presented in this thesis shows aspirin activates c-Src in CRC cells and in human colonic epithelium. I present evidence that c-Src plays a key role in aspirin-induced activation of the NF- $\kappa$ B pathway, nucleolar sequestration of RelA and apoptosis. Furthermore, I have optimised an *in vitro* organ culture model of human *ex vivo* colonic mucosa, through which I have identified cells expressing CSC markers and modified growth and differentiation in colonic explants in long-term culture. Using this model, I confirmed that aspirin's effects on the c-Src and NF- $\kappa$ B pathways in previous CRC cell line experiments were relevant in human colonic mucosa. These findings shed further light on the mechanisms behind aspirin's chemopreventative effects in CRC but also highlight areas of further research and exploration. The implications of the data presented in this thesis, in terms of the direction of future research, will be the subject of this discussion.

### 5.1 Elucidating the molecular mechanisms responsible for linking aspirin-induced c-Src and NF- $\kappa$ B signalling activation

Whilst data presented in this thesis demonstrate that c-Src was activated following aspirin administration at a time point prior to NF- $\kappa$ B activation, the precise mechanism underpinning both aspirin-induced c-Src activation and c-Src-induced stimulation of NF- $\kappa$ B signalling is still not fully understood.

Previously, in chapter 3, I demonstrated that aspirin effects on the NF- $\kappa$ B pathway and apoptosis *in vitro* were dependent upon c-Src and in chapter 4, I demonstrated the human *in vivo* relevance of these data by showing the agent activates c-Src in association with I $\kappa$ B $\alpha$  degradation and nuclear translocation of RelA in explants of normal colonic mucosa. The experimental data suggested that the mechanism behind aspirin induced c-Src mediated activation of NF- $\kappa$ B involves a) I $\kappa$ B $\alpha$  degradation, b) a physical association of c-Src and I $\kappa$ B $\alpha$  and, c) activation through delayed kinetics.



Future work is required to further elucidate this mechanisms involved in the activation of these pathways. Co-immunoprecipitation studies were unable to definitively demonstrate if a physical interaction of c-Src with NF- $\kappa$ B was modified in CRC cells by aspirin treatment. However, optimisation of these studies, perhaps in cells which over-express c-Src may help to provide such data. Direct phosphorylation of I $\kappa$ B $\alpha$  on Y42 by c-Src is one potential mechanism for c-Src mediated NF- $\kappa$ B pathway activation and is reported in other settings (Abu-Amer et al 1998, Fan et al 2003). However experimental difficulties prevented the detection of whether aspirin treatment induces I $\kappa$ B $\alpha$  Y42 phosphorylation. This mechanism is responsible for the liberation of RelA from the cytoplasmic inhibitor and subsequent RelA nuclear/nucleolar translocation and is therefore critical to the understanding of the potential kinase pathways involved in the agent-induced response and in detecting future chemopreventative targets. Using immunoprecipitation to isolate cellular I $\kappa$ B $\alpha$ , prior to further western blotting analysis with the phospho-specific Y42 antibody may yield better more specific results.

Given the delay between the activation of c-Src and I $\kappa$ B $\alpha$  degradation, it is likely that c-Src may activate intermediate kinases, such as PI3-kinase, (which interacts with c-Src and associates with tyrosine phosphorylated I $\kappa$ B $\alpha$ ) (Haefner et al 1995, Kang et al 2003) or IKK. The use of IKK/ PI3-kinase inhibitors, IKK tyrosine residue genetic mutants (c-Src phosphorylates tyrosine residues on the activation ring of IKK) or IKK:c-Src/ PI3-kinase: c-Src immunoprecipitation experiments to dissect this response could also raise future candidates of future potential pursuit as chemopreventative targets.

The mechanism of c-Src activation involves phosphorylation of Y418 and occurs some hours after aspirin administration. Whilst, the regulation of c-Src kinase activity is highly complex, further dissection of this activation may yet yield information about upstream targets of aspirin. Both Csk and protein tyrosine phosphatases regulate phosphorylation of c-Src at tyrosine 527 and previous reports of their modification by other NSAIDs already exist (Kunte et al 2008, Playford and Schaller 2004). Therefore, the potential for future experimentation utilising assays for the activity of Csk and/or

protein tyrosine phosphatases may therefore be an intriguing option to identify if these proteins are implicated in the activation of c-Src by aspirin.

Aspirin-induced activation of c-Src is also associated with other cellular and molecular effects, including modification of adhesion/membrane dynamics and paxillin Y118 phosphorylation. FAK is both a known effector of c-Src (Frame 2002), and substrate of c-Src kinase activity. Whether FAK itself is directly targeted by aspirin, or activates c-Src as a consequence of upstream integrin signalling, would be of great interest. Exploration of the role of FAK in this response, utilising FAK chemical inhibitors, or site directed mutagenesis of FAK (such as at the key c-Src substrate site, FAK Y925) could be undertaken to assess the effect of FAK inhibition on aspirin-induced apoptosis and the NF- $\kappa$ B signalling response.

Another possible mechanism by which aspirin may translate c-Src activation to induce a NF- $\kappa$ B signalling response is through MAPK signalling, a known substrate of c-Src activity and involved in aspirin effects on NF- $\kappa$ B and apoptosis (Thoms et al 2007b). Exploration of the role of the p38 MAPK in the aspirin-mediated effects of c-Src may shed further light on the intermediate pathways involved in the transmission of the aspirin-induced pro-apoptotic effect. Evaluating the effect of MAPK inhibition on the NF- $\kappa$ B and apoptotic response to activated c-Src would be of interest to further elucidating the role of MAPK in this response.

Importantly, it is also important to consider if the active agent behind these molecular effects *in-vivo* are specific to aspirin or its' metabolite salicylilate or NSAIDs generally. As already discussed, the host lab has previously demonstrated c-Src and NF- $\kappa$ B activation following NSAIDs (Brady et al 2010) and inhibition of the NSAID pro-apoptotic effect with the use of c-Src Inhibitors in CRC cell lines. Testing the response of NSAIDs within the ex-vivo model would therefore be of great interest.

There is also the consideration of dosage. In a study of 10 subjects given 40.5 mg aspirin, the mean peak plasma salicylic acid concentration was 11.8  $\mu$ mol/l, but there was a large inter-individual

variability in the salicylic acid concentrations after the same dose of aspirin. (Ruffin et al., 1997) Higher serum concentrations of salicylic acid were present in vegetarians than in non-vegetarians, and there was overlap in the serum concentrations between vegetarians and those taking aspirin (75 mg daily). The median serum concentration of salicylic acid in a group of vegetarians not taking salicylate drugs was 107 nmol/l, with the highest concentration being 2468 nmol/l (Blacklock et al 2001 )

The study here used supra-pharmacological doses of aspirin, as this has been well characterized before within the host laboratory, allowing for reasonable logistical analysis of cells (i.e. effects measurable within realistic time-points). However, further analysis of serum and tissue salicylate levels would be useful in further evaluation of the ex-vivo model. The host lab have previously demonstrated that aspirin tumour concentration in xenografted models was of the order of >0.5mM after a dose of 40mg/kg in rats (Stark et al 2005).

Of note, sodium salicylate and aspirin at pharmacological concentrations inhibit Cox-2 transcription in endothelial cells and fibroblasts. The salicylic acid concentration which inhibited Cox-2 transcription by 50% is estimated to be 5000 nmol/l, although lower concentrations (100 nmol/l) appear to also have some effect (Xu et al., 1999). The anti-colon cancer effect of aspirin could therefore be contributed by suppression of Cox-2 expression by salicylate rather than inhibition of activity. Here, we focused on non-Cox pathways within this thesis and the host cell line SW480 was Cox negative, but the relationship of c-Src activity, its' expression and expression of Cox in other cell line models and within the ex-vivo model, at pharmacological dosages of aspirin, would be of relevance to further explaining the Cox-dependent and independent nature of the chemopreventative effect of aspirin and in other settings.

## **5.2. Utility of IVOC model to future work and exploration of findings derived from optimisation.**

The results of optimisation experiments utilising the IVOC model of colonic mucosa demonstrated that the strongest predictors of explant survival were a) media-dependent and b) location-dependent.

Further work is however required to evaluate the effect of individual constituents of media and additives that were used. Due to time constraints, this could not be performed within the timeframe of this project, however, evaluating the specific growth factors and chemical constituents of Weymouth' media which are responsible for the divergent biphasic response may provide some key findings to the underlying control of cell growth and differentiation within human colonic mucosa. Further variables such as temperature, patient specific variables (sex, age, co-morbidities; such as diabetes etc.), anaesthetic time or agents, time delay from harvest to culture, frequency of media changes, pressure conditions, size of explant and volume of media are areas where further information may assist in providing further improved conditions to improved explant culture outcomes.

The unpredicted divergent survival patterns that were obtained from explants from right and left colon harvest sites adds to a compelling weight of evidence that colonic tissue from these two anatomical locations behave and respond in different ways. As discussed previously, differences in luminal bacteria, response to NSAIDs, genetic expression patterns, CRC site-specific mortality and morbidity outcomes, genetic signatures of site-specific CRCs and racial differences in incidence have all been previously reported. This leads to the suggestion of whether there exists right- and left-sided CRC disease phenotypes. Exploration of the response to individual chemopreventative or chemotherapeutic agents with a focus on anatomical location may yield further evidence of this effect. The concept of tailored therapeutics, geared towards the molecular signatures of cancers, is well described in relation to chemotherapy and KRAS/BRAF/MMR status, however, whether location-based tailoring for chemopreventatives/chemotherapeutics is also required, is an area which may benefit from further evaluation.

The focus of this thesis has been the exploration of the molecular effects of aspirin treatment on c-Src signalling in colorectal epithelial cells, as a means to further explain aspirin's chemopreventative effect. However, whilst there are many potential molecular targets, understanding this response is even more complex when one also considers the other potential factors which putatively effect tumour growth and metastases. The factors which regulate tumour development and growth are not limited to the epithelium. This regulation involves key interactions between epithelial cells, activated stromal

cells, components of the extracellular matrix, platelets and the vascular supply. Of key relevance is aspirin's widely known property as an anti-platelet agent and the potential effect of aspirin on the surrounding stroma.

The anti-thrombotic action of aspirin is due to inhibition of platelet cyclooxygenase (COX) causing irreversible inhibition of platelet-dependent thromboxane formation (Shror et al. 1997). Complex interactions between tumour cells and circulating platelets play an important role in cancer growth and dissemination. Platelets facilitate cancer-related coagulation by providing a procoagulant surface, which can shroud tumor cells from immune responses, and facilitate dissemination of tumour cells when vascular invasion has occurred. Platelets are also stores of proangiogenic and anti-angiogenic proteins that can enhance angiogenesis and therefore tumour growth. Platelets also facilitate tumour cell invasion and induce endothelial cell proliferation. Additional platelet-related proteins and metabolites which facilitate proteolysis and tissue remodelling also enhance tumor growth and metastasis. For instance, inhibiting platelet receptors, such as GPIb/IX/V, GPIIb/IIIa and GPVI attenuates metastases (reviewed in Bambace and Holmes 2011). Aspirin's antiplatelet actions could therefore have a highly relevant role in the CRC chemopreventative effect.

Aspirin also has mesenchymal targets and has been demonstrated to induce both growth inhibition/apoptosis of mesenchymal stem cells through a mechanism which involves the WNT/beta-catenin pathway (Wang Y, Deng L) and also has important effects on the angiogenic response, a key stromal effect that is required to generate a sufficient blood supply for a solid tumor to grow. For instance, it is known that tumour cells secrete growth factors such as vascular endothelial growth factor (VEGF), which stimulates nearby vascular cells to form new blood vessels. Cox-2 inhibitors modulate angiogenesis indirectly by reducing expression of angiogenic factors, such as VEGF in the tumour micro-environment (Borthwick 2006). COX-1 and COX-2 over-expression is frequent in large and high-grade dysplasia adenomas and it is deep stromal but not epithelial expression of COX-2 in some studies which is a more important predictor of recurrence of such adenomas (Benamouzig et al 2010)

These non-epithelial targets remain important to explore, as the microenvironment remains the key incubator of tumour mass and potentially these effects are actually more stable to establish and are more predictable than the responses of highly disorganized mutating tumour cells (Borthwick G). The *Ex-vivo* model therefore provides a useful setting in which to observe mesenchymal effects and, as the stroma layer is maintained, possibly provides a more attractive and representative model than simple cell culture or 3d crypt culture models. Observing the aspirin induced stromal effects in such a setting has obvious interest to this field of future scientific exploration.”

Previous reports have documented that Notch inhibition within the setting of clinical trials confers a differentiation pressure towards a secretory cell lineage within the *in vivo* patient colonic mucosa. Given that I also found that Notch signalling antagonists induced colonocytes to differentiate towards a secretory/Mucin-2 expressing cell lineage in explants of normal colonic mucosa, the replication of this response in the IVOC setting adds evidence to the representative nature of the model to the *in vivo* situation.

Whilst the majority of CRCs are moderately well-differentiated and possess few Mucin-secreting goblet cells, 10%-20% of CRCs overproduce Mucin, with a characteristic set of clinical/pathologic features and distinct molecular genetic attributes (Skrzydowska et al 2005) (Okudaira et al 2010) (Kim et al 2005). Mucin-related genes (*HATH1* and *SOX2*) are also key components of the Notch signalling pathway and implicated in the pathogenesis of such cancers (Park et al 2008). Forced expression of *HATH1* in CRC cells results in the up-regulation of Muc-2 expression (Park et al 2006) and 80% of this subset of CRCs demonstrate increased expression of Muc-2 (Okudaira et al 2010). This model therefore provides a potential experimental platform in which to explore molecular mechanisms responsible for growth and differentiation generally and more specifically, the underlying mucinous/signet ring cell CRC pathogenesis. Future research could also be extended to assess the cell-specific response to aspirin in this setting and the implication of chemoprevention in this subset of CRCs.

I experienced significant problems with attempting prolonged culture of human polyps. The difficulties with polyps include variability of the samples, the lack of availability and the small quantity of tissue available for experimentation and analytical purposes. Here, I observed an inducible hyperproliferative state within explants by the daily addition of a small molecule Wnt agonist. There is considerable future merit in evaluating aspirin-induced c-Src and NF- $\kappa$ B pathway responses within the setting of induced hyper-proliferative mucosa in order to evaluate if the agent induced response changes within this setting. Importantly, it is perhaps within hyperproliferative mucosa that exploration and definition of the precise mechanisms underlying the chemopreventative effect of aspirin is most relevant and as such, this model may provide a means at which to adequately explore this setting.

The successful optimisation of the *ex vivo* model therefore provides considerable value beyond the original aims (to provide a platform for the investigation of chemopreventatives). In just one such instance, the work undertaken is already being used in the Colon Cancer Genetics Group to study the function of a number of signalling pathways that have been identified as contributing to colorectal cancer susceptibility, through ongoing large scale genetic studies. To date the CCGG have identified 14 new genetic loci contributing to colorectal cancer aetiology, many in TGF/BMP signalling pathways and the model is now being used to explore the mechanisms by which these genetic variants lead to cancer.

### **5.3 Conclusion**

Taken as a whole, the work presented has explored the mechanism whereby aspirin induces apoptosis in CRC cells and human mucosa. These data suggest aspirin-induces phosphorylation of c-Src Y418, activating c-Src kinase function, upstream of NF- $\kappa$ B signalling. The aspirin-induced NF- $\kappa$ B signalling response involving I $\kappa$ B $\alpha$  degradation, RelA nuclear/ nucleolar translocation, and resultant aspirin-induced apoptosis have demonstrated c-Src-dependency. The upstream mechanisms responsible for aspirin-induced c-Src activation and the mechanistic link between c-Src activation and the NF- $\kappa$ B signalling response require further exploration.

Utilising this model system, exploration of the aspirin induced c-Src and NF- $\kappa$ B signalling response confirmed that findings derived from *in vitro* CRC cell lines were relevant in human colonic mucosa. The findings presented here, in combination with previously published work from the host laboratory, has progressed the understanding of the signal transduction involved in effecting aspirin-induced apoptosis in CRC cells and human colonic mucosa. Despite treatments, in view of the high mortality associated with CRC, it is of vital importance to understand these mechanisms, as this could allow the rational development of novel therapeutic agents. Hence work aimed at identifying targets for novel chemopreventative agents, and developing models to expedite the development of such agents, has much to commend it.



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## Appendix 1=

c-Src dependency of NSAID-induced effects on NF{ $\kappa$ }B-mediated apoptosis in colorectal cancer cells. *Carcinogenesis*. 2011; 32(7):1069-77.



## c-Src dependency of NSAID-induced effects on NF- $\kappa$ B-mediated apoptosis in colorectal cancer cells

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**Long-term aspirin or related non-steroidal anti-inflammatory drugs (NSAIDs) ingestion can protect against colorectal cancer (CRC). NSAIDs have a pro-apoptotic activity and we have shown that stimulation of the nuclear factor-kappaB (NF- $\kappa$ B) pathway is a key component of this pro-apoptotic effect. However, the upstream pathways have yet to be fully elucidated. Here, we demonstrate that aspirin activates the c-Src tyrosine kinase pathway in CRC cells. We show that c-Src activation occurs in a time- and dose-dependent manner, preceding aspirin-mediated degradation of I $\kappa$ B $\alpha$ , nuclear/nucleolar translocation of NF- $\kappa$ B/RelA and induction of apoptosis. Furthermore, inhibition of c-Src activity, by chemical inhibition or expression of a kinase dead form of the protein abrogates aspirin-mediated degradation of I $\kappa$ B $\alpha$ , nuclear translocation of RelA and apoptosis, suggesting a causal link. Expression of constitutively active c-Src mimics aspirin-induced stimulation of the NF- $\kappa$ B pathway. The NSAIDs sulindac, sulindac sulphone and indomethacin all similarly activate a c-Src-dependent NF- $\kappa$ B and apoptotic response. These data provide compelling evidence that c-Src is an upstream mediator of aspirin/NSAID effects on NF- $\kappa$ B signalling and apoptosis in CRC cells and have relevance to the development of future chemotherapeutic/chemopreventative agents.**

### Introduction

In the developed world, colorectal cancer (CRC) is the third most common cancer in men after lung and prostate cancer and the second most common in women after breast cancer (1). Worldwide, it accounts for 1.2 million new cases each year and 608 000 deaths, representing a major public health concern (2). Despite continuing research, the overall 5 years survival remains ~50% as patients often present with advanced or metastatic disease (3). Hence, attention is focusing on earlier detection and prevention as the most promising approaches for combating this disease.

Epidemiological, case-control and randomized controlled trials indicate that aspirin and related non-steroidal anti-inflammatory drugs (NSAIDs) have considerable potential as chemopreventative agents against CRC (4–6). We have recently shown protective effects within a year and at low-dose aspirin (7). However, the chemopreventative effect imparted by these agents is not complete. Furthermore, even at low doses, side effect profiles argue against their adoption for CRC prevention for average risk individuals in the population (5,6). However, understanding the molecular mechanisms underlying the observed chemopreventative properties of NSAIDs could provide the basis for rational design of safer, more targeted, agents.

Induction of apoptosis through modulation of NF- $\kappa$ B signalling has been shown to be a key component of the antitumour activity of NSAIDs against CRC (8,9). NF- $\kappa$ B is an ubiquitously expressed inducible-transcription factor that plays a critical role in inflammatory and stress responses, proliferation, differentiation and apoptosis (10).

**Abbreviations:** CRC, colorectal cancer; FCS, fetal calf serum; NSAID, non-steroidal anti-inflammatory drug; NF- $\kappa$ B, nuclear factor-kappaB.

It is generally found as a p50/RelA (p65) heterodimer, bound in an inactive state in the cytoplasm by the inhibitor protein, I $\kappa$ B $\alpha$  nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha. Following cellular stimulation, I $\kappa$ B $\alpha$  is phosphorylated then undergoes poly-ubiquitination and proteasomal degradation, allowing NF- $\kappa$ B to translocate to the nucleus and regulate expression of target genes, many of which regulate cell growth and apoptosis (10,11).

Under normal circumstances, activation of the NF- $\kappa$ B pathway is transient and tightly controlled. However, several lines of evidence indicate that in CRC, NF- $\kappa$ B is constitutively activated and that this activation plays a significant role in the carcinogenic process, regulating the expression of a number of genes implicated in CRC progression (Cyclin D1, Bcl-x1), angiogenesis (VEGF, IL-8, COX2) and metastasis (MMP9) (12). A number of chemopreventative agents and other inhibitors have been shown to induce suppression of NF- $\kappa$ B signalling, inhibition of proliferation, cell cycle arrest and apoptosis of CRC cells (12,13).

We have previously reported that aspirin and NSAIDs induce degradation of I $\kappa$ B $\alpha$  and nuclear translocation of NF- $\kappa$ B in colorectal neoplasia both *in vitro* and *in vivo* and that the resulting stimulation of the NF- $\kappa$ B pathway in these conditions is responsible for repression of NF- $\kappa$ B-driven transcription and critical for the pro-apoptotic effects of these agents in CRC cells (9,14–16). In more recent studies, attention has focused on identifying upstream regulators of this response and we have identified that activation of p38 is an important upstream mediator of the NF- $\kappa$ B response to aspirin (17). However, the regulation of this response is highly complex and there remain aspects of these upstream mechanisms that require further definition.

One kinase of particular interest is the 60 kDa non-receptor, membrane-associated proto-oncogene product, c-Src, a protein tyrosine kinase. c-Src is over-expressed in >80% of colon carcinomas and is associated with CRC progression, invasion, metastasis and poor survival (18–20). Crucially, it is known to modulate NF- $\kappa$ B signalling in response to a wide variety of stimuli; either directly, through tyrosine phosphorylation of I $\kappa$ B $\alpha$  (Y42) (21–24) or indirectly, through stimulation of intermediate kinase pathways, such as Protein Kinase C Alpha and I $\kappa$ B kinase (25–31). Furthermore, NSAIDs have been shown to modulate cellular c-Src tyrosine kinase activity in response to a broad range of stimulants, in a variety of cellular environments (31–34).

We set out to test the hypothesis that the aspirin/NSAIDs-induced NF- $\kappa$ B response observed in CRC cells is mediated via c-Src activity and that c-Src has an important role in pro-apoptotic response induced by these agents.

### Experimental procedures

#### Tissue culture

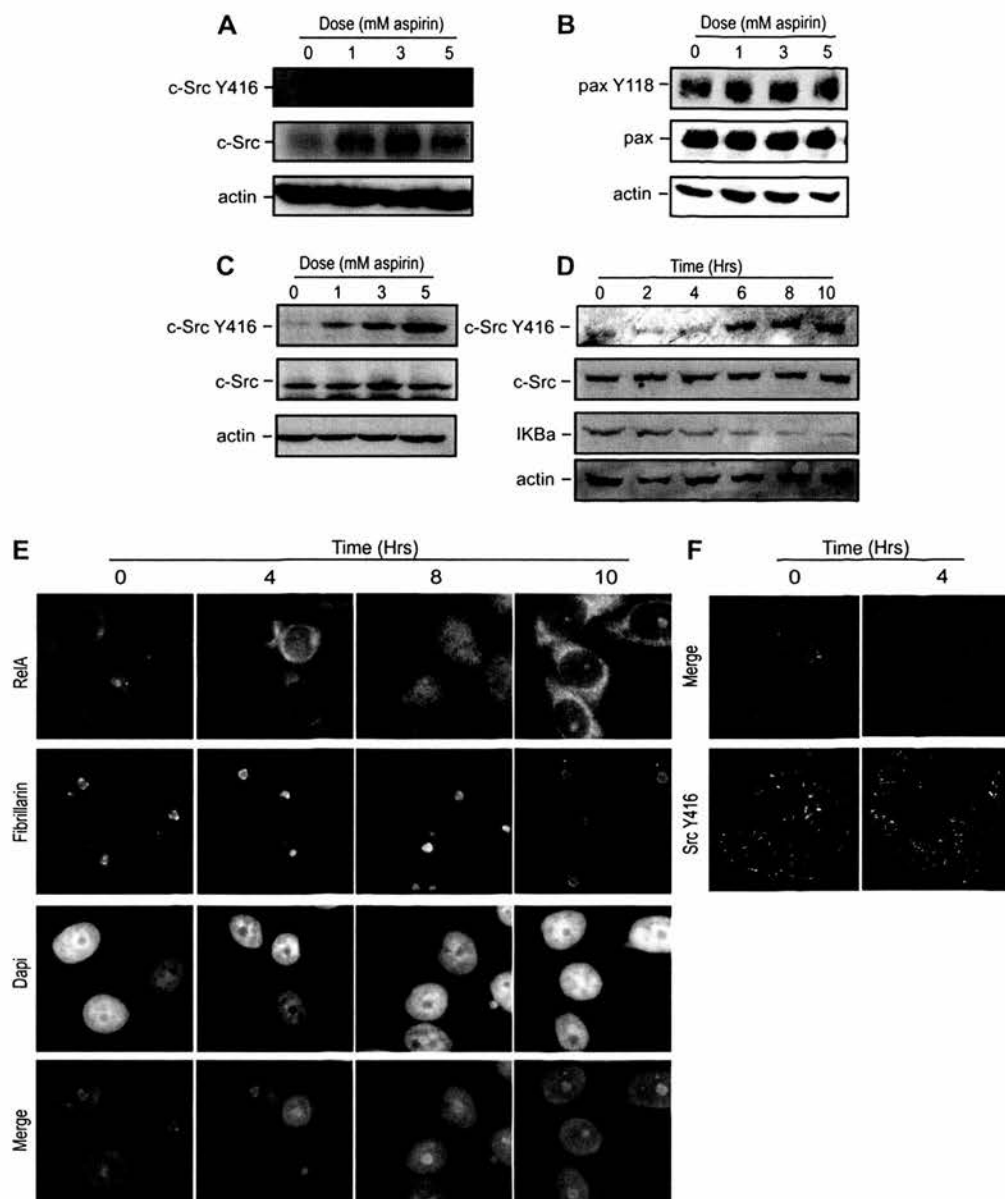
Culture of SW480 CRC cells has been described previously (14). SW480 cells stably transfected with a kinase dead form of c-Src (SW480-SrcKD) were obtained (kind gift from Prof. M.Frame, Edinburgh Cancer Research centre) along with control cells stably transfected with the pBpuro empty vector alone (SW480-pBpuro) and were maintained in RPMI medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS) and 1% penicillin and streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were plated at a density of 0.2 × 10<sup>5</sup>/cm<sup>2</sup> and treated in 0.5% serum at 60–80% confluence. Aspirin (Sigma, St Louis, MO), sulindac (Sigma, Gillingham, UK), sulindac sulphone (MP Biomedicals, Solon, OH) or indomethacin (Sigma) were added at the concentrations specified as described previously (16). Aspirin (Sigma) was dissolved in water using 10 N NaOH and the pH was then adjusted to 7.0 as described previously (17). For chemical inhibition of c-Src, the inhibitors PP2 (10 μM)

amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) SU6656 (0.5  $\mu$ M) (2-oxo-3-(4,5,6,7-tetrahydro-1H-indol-2-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide) were obtained from Calbiochem, Nottingham, UK), were dissolved in dimethyl sulfoxide and added to the cells in L-15 (Gibco BRL) with 10% FCS, 1% penicillin/streptomycin.

#### Western blot analysis

Following treatment, whole-cell extracts were prepared using a commercially available lysis buffer according to the manufacturer's instructions (Cell Signaling Technology, Hertfordshire, UK), and western blots were performed as described previously (35). The membranes were probed with the following primary antibodies: rabbit phosphorylated c-Src

(Y416) (#2101; Cell-signaling Technology), rabbit phosphorylated paxillin (Y118) (Invitrogen, Camarillo, CA), mouse c-Src (Upstate Biotechnology, Lake Placid, NY), mouse paxillin (BD Pharmingen, Oxford, UK), sheep polyclonal anti-I $\kappa$ B $\alpha$  (a gift from Professor Ron Hay, Dundee) and mouse actin (Clone AC-15; Sigma) was utilized as a control for protein loading. The horseradish peroxidase-conjugated secondary antibodies used were anti-rabbit IgG (Cell Signaling Technology and Amersham Biosciences, Buckinghamshire, UK), anti-sheep and anti-mouse IgM (Calbiochem). To examine the levels of non-phosphorylated proteins, membranes were stripped by heating at 60°C for 45 min in buffer [60 mmol/l Tris-HCl (pH 6.7), with 2% sodium dodecyl sulfate and 0.8%  $\beta$ -mercaptoethanol] and blocked before reprobing using a native antibody.



**1.** Aspirin-induced activation of the c-Src pathway in CRC precedes NF- $\kappa$ B activation. SW480 cells (A, B, E and F) or those which were transiently transfected with a wild-type c-Src expression vector (C and D) were cultured and treated with aspirin [0–5 mM; 4 h (A, B, C and F) or 5 mM (D and E)] for the specified times. (A–D) Western blot analysis was performed on whole cell extracts using antibodies to phosphorylated Src (Y416), paxillin (Y118) or I $\kappa$ B $\alpha$ . Blots were stripped and reprobed for endogenous c-Src or paxillin. Actin was used as a control for protein loading. (E) Anti-RelA and fibrillarlin (a nucleolar marker) immunocytochemistry was performed on cells treated with aspirin (5 mM) for the specified times. 4',6-Diamidino-2-phenylindole (DAPI) was used as a nuclear marker. All data is representative of at least three independent experiments. Aspirin (5 mM) induces RelA translocation from the cytoplasm to the nucleus at 8 h, and further localization within nucleolar bodies at 10 h (fibrillarlin-stained bodies). (F) Immunocytochemistry utilizing antibodies against phosphorylated Src (Y416) was performed on SW480 cells 0–4 h after treatment with 5 mM aspirin. DAPI was used as a nuclear marker.



### Transfections

SW480 CRC cells were transiently transfected with empty pUSEamp vector or a vector that expresses wild-type c-Src, inactive c-Src (pUSE Src K297R) or constitutively active c-Src (pUSE Src Y529F) (all obtained from Upstate Biotechnology). Cells were transfected using a Lipofectamine protocol [as described by manufacturers (Gibco BRL)] grown for 24 h in low serum (0.5% FCS) medium [as described previously, Stark *et al.* (9)] then treated with aspirin at various concentrations. Western blot and immunocytochemistry were subsequently performed to confirm adequate transfection efficacy with this protocol.

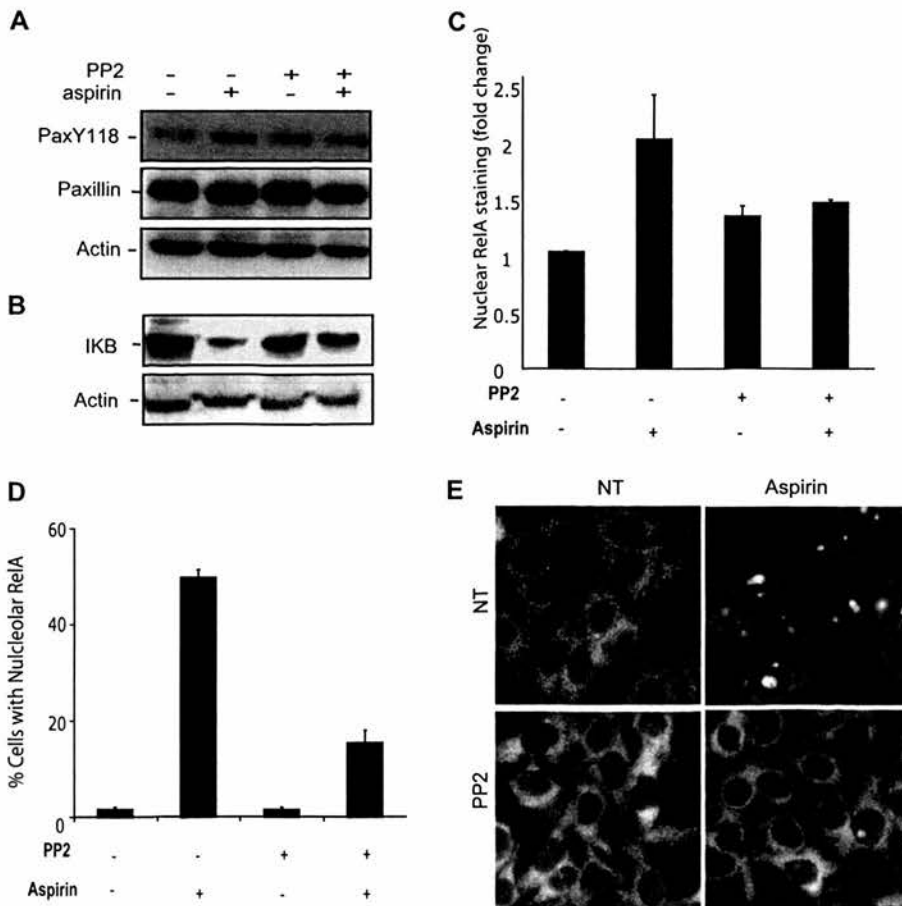
### Immunocytochemistry

For fluorescence imaging, the imaging system comprises a Coolsnap HQ CCD camera (Photometrics Ltd, Tucson, AZ) Zeiss Axioplan II fluorescence microscope with Plan-neofluar objectives, a 100 W Hg source (Carl Zeiss, Welwyn Garden City, UK) and Chroma #83000 triple band pass filter set (Chroma Technology Corp., Rockingham, VT) with the excitation filters installed in a motorized filter wheel (Prior Scientific Instruments, Cambridge, UK). Image capture and analysis were performed using in-house scripts written for IPLab Spectrum (Scanalytics Corp, Fairfax, VA). The primary antibodies used at 1:100 were as follows: NF- $\kappa$ B p65 (RelA; C-20), rabbit

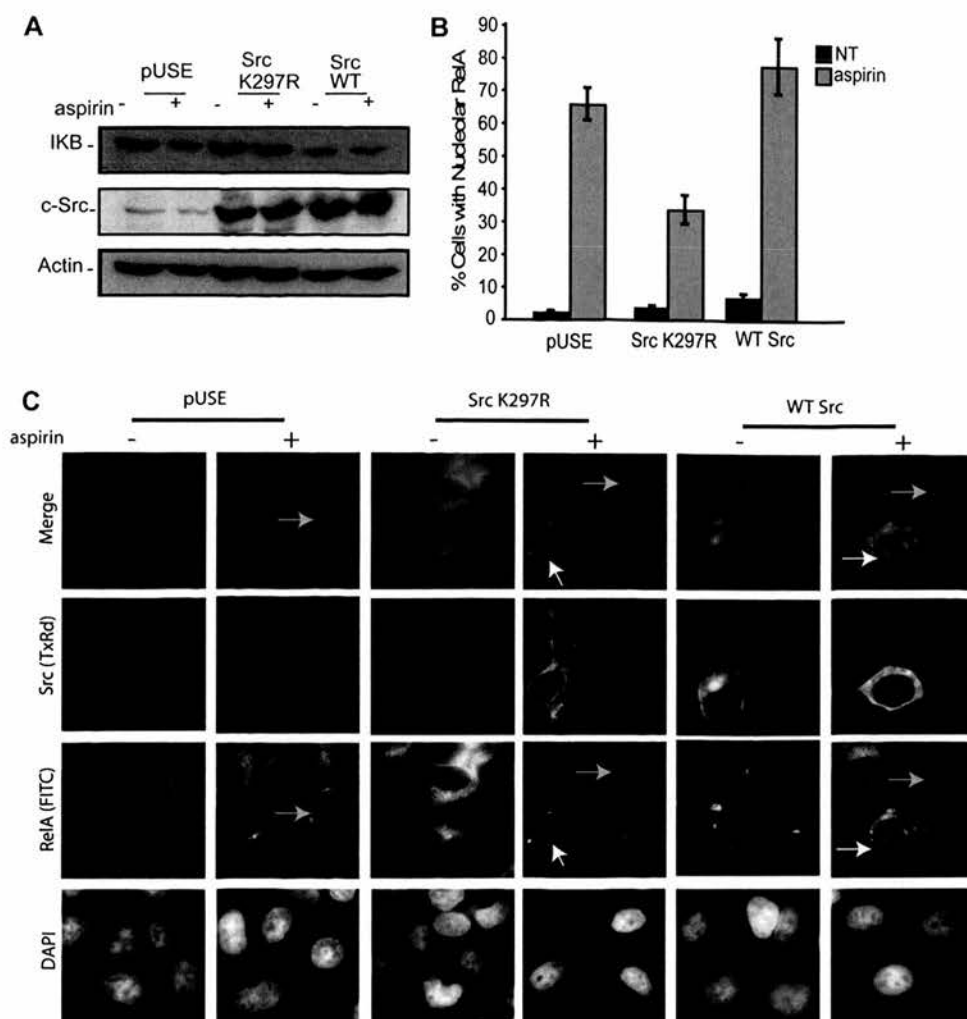
phosphorylated c-Src (Y416) (#2101; Cell Signaling Technology), c-Src (Upstate Biotechnology), C23 (Santa Cruz Biotechnology, Santa Cruz, CA) and fibrillarin (AFB01; Cytoskeleton, Denver, CO). The fluorescein isothiocyanate- and Texas red-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at a dilution of 1:200, and the cells were mounted using Vectashield (Vector Laboratories, Peterborough, UK) containing 1  $\mu$ g/ml 4',6-diamidino-2-phenylindole. In order to quantify the induced nuclear translocation of RelA, individual exposure channels for each immunocytochemistry image field were captured and stored using identical exposures throughout image collection and across treatment arms of the experiment. Later, image analysis created nuclear outline masks in 4',6-diamidino-2-phenylindole channel images, which were subsequently transposed onto the matched fluorescein isothiocyanate channel exposures for that field of view. The mean intensity of nuclear RelA staining within previously defined 4',6-diamidino-2-phenylindole masks were then quantified in at least three separate experiments, in at least 15 random fields per experiment.

### Apoptosis assays

Staining for cell surface phosphatidylserine residues was conducted using an Annexin V-fluorescein isothiocyanate apoptosis detection kit



**Fig. 2.** Chemical inhibition of c-Src activity blocks aspirin-mediated stimulation of the NF- $\kappa$ B pathway (A–E) SW480 cells were either untreated (–) or pre-treated with PP2 for 2 h prior to treatment with aspirin [4 h (A), 8 h (B and C) or 16 h (D and E); 0 (–)/5 mM (+)]. (A and B) Western blot analysis using antibodies to phosphorylated paxillin (Y118), total paxillin or I $\kappa$ B $\alpha$  were performed on cell extracts. Anti- $\beta$ -actin was used as a loading control. (C) SW480 nuclear 4',6-diamidino-2-phenylindole exposure image outlines were transposed onto RelA exposure images. Comparative nuclear RelA staining intensity was then quantified by computer image analysis software. (D–E) The nucleolar localization of RelA was examined using anti-RelA immunocytochemistry as above. (D) For each experiment, the percentage of cells showing nucleolar RelA (as depicted by areas devoid of DNA/4',6-diamidino-2-phenylindole staining) in at least 100 cells from a minimum of five fields of view was determined. Data are the average of three independent experiments ( $\pm$  standard error). (E) Representative fields of view from (D) ( $\times 63$  objective).



**3.** Expression of kinase-inactive c-Src inhibits the NF- $\kappa$ B signalling response to aspirin. (A–C) SW480 cells were transiently transfected with an empty pUSE vector, wild-type c-Src or kinase-inactive c-Src expression vector (pUSE Src K297R). Forty-eight hours after transfection, cells were untreated (–) or treated with 5 mM aspirin (+) for 8 h (A) or 16 h (C). (A) Anti-I $\kappa$ B $\alpha$  western blot analysis was performed on cytoplasmic cell extracts as above. Membranes were probed and reprobed for c-Src to confirm expression of c-Src plasmids. Actin was used as a loading control. (B) Anti-RelA immunocytochemistry was performed on transfected cells and the percentage of cells showing nucleolar RelA quantified as above. Data are the mean of three independent experiments  $\pm$  standard error. (C) Anti-RelA/anti-c-Src immunocytochemistry was used to examine effects of expression vectors on aspirin-induced nucleolar RelA localization. Aspirin induced nuclear translocation of RelA in non-transfected cells (white arrows). However, within cells over-expressing Src from transfected plasmids (yellow arrows), those over-expressing pUSE Src K297R demonstrated reduced RelA translocation in response to aspirin, compared to the response in non-expressing cells. However, cells over-expressing wild-type c-Src displayed a similar aspirin-induced RelA nuclear translocation to that observed in non-expressing cells.

Genzyme Research Products, La Jolla, CA) according to the manufacturer's instructions. The fraction of apoptotic cells was determined by fluorescent microscopy as described previously (35).

**Results**

*Aspirin induces c-Src activity in CRC cells*

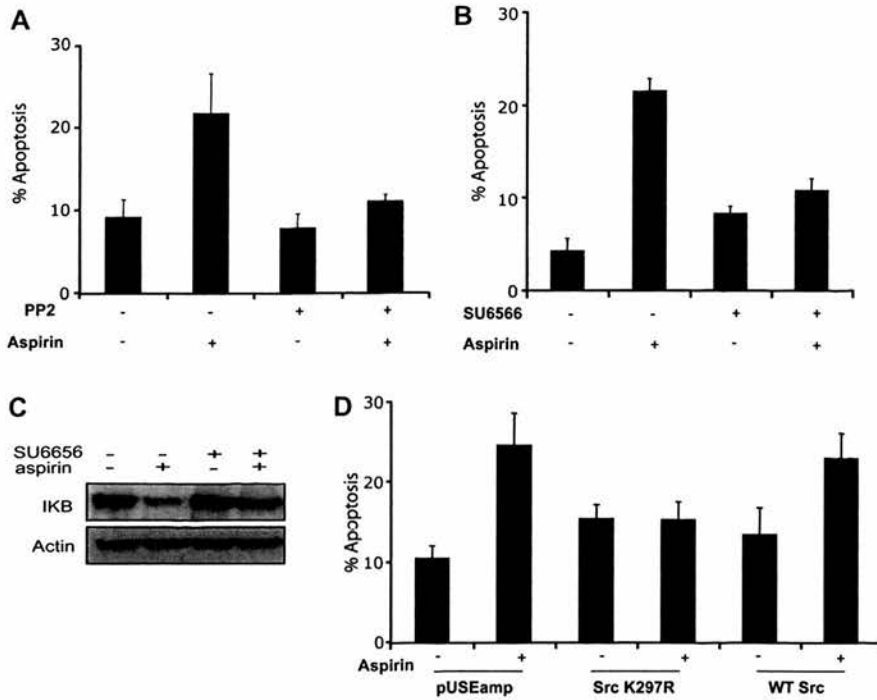
We initially investigated the effects of aspirin on c-Src activity. Phosphorylation of c-Src on tyrosine 416 triggers its kinase activity by inducing a conformational change that exposes the kinase domain to potential substrates (36). Using this phosphorylation event as a marker for activation, we found that aspirin induces a dose-dependent increase in endogenous c-Src activity in SW480 CRC cells (Figure 1A). c-Src activation was further confirmed by demonstration of phosphorylation of the c-Src substrate, paxillin at Y118 (Figure 1B) (37). SW480 cells constitutively express low levels of endogenous c-Src, and so we created these experiments in SW480 cells in which we over-expressed wild-type c-Src. These experiments further confirmed that aspirin induces c-Src activation in CRC cells through dose-dependent phos-

phorylation at Y416 of c-Src as a key member of Src Family Kinases (Figure 1C).

*Aspirin effects on c-Src activity precede stimulation of the NF- $\kappa$ B pathway*

Having established that aspirin activates the c-Src kinase pathway, we next examined the temporal relationship between c-Src activation and aspirin-induced stimulation of NF- $\kappa$ B signalling, to begin to determine whether these two events are causally linked. Using western blot analysis, we found that aspirin (5 mM)-induced c-Src Y416 phosphorylation within 6 h of treatment, concurrent with the observed kinetics of aspirin-induced I $\kappa$ B $\alpha$  degradation (Figure 1D).

Our previous work demonstrated that upon aspirin/NSAID-mediated degradation of I $\kappa$ B $\alpha$ , the RelA component of NF- $\kappa$ B translocates from the cytoplasm to the nucleoplasm and then to the nucleolus. We also showed that this was required for the agent's pro-apoptotic effect (9,35). We next used immunocytochemical analysis to examine the kinetics of nuclear/nucleolar localization of RelA in response to aspirin. Fibrillarlin was used as a nucleolar marker. We



**Fig. 4.** Variation in c-Src activity modulates aspirin-induced apoptosis. (A, C and D) SW480 cells were either pre-treated with chemical inhibitors PP2, SU6656 or transiently transfected with c-Src expression plasmids as described in Figure 3, prior to aspirin [0 (-), 5 mM (+); 16 h] treatment. The percentage of cells in the population undergoing apoptosis was determined using Annexin V-fluorescein isothiocyanate apoptosis assays. Data presented is the minimum of three independent experiments ( $\pm$  standard error). (B) SW480 cells were either untreated (-) or pre-treated with SU6656 for 2 h prior to treatment with aspirin [0 (-), 5 mM (+); 8 h]. Western blot analysis using antibodies to I $\kappa$ B $\alpha$  were performed on cell extracts. Anti- $\beta$ -actin was used as a loading control.

found that aspirin-induced nucleolar translocation of RelA occurred subsequent to c-Src Y416 phosphorylation (Figure 1E). Immunocytochemistry with antibodies to phosphorylated Y416 c-Src confirmed that aspirin induced an increase in active form of the protein and that this preceded effects of the agent on translocation of RelA (Figure 1F). Taken together, these data suggest that c-Src activation may lie upstream of stimulation of the NF- $\kappa$ B pathway in response to aspirin.

#### Inhibiting c-Src activity blocks aspirin-mediated stimulation of the NF- $\kappa$ B pathway

To further investigate the potential for the c-Src dependency of aspirin-induced NF- $\kappa$ B activation, we used the Src family kinase inhibitor, PP2 (38). As this inhibitor does not block c-Src autophosphorylation at Y416, we used phosphorylation of paxillin at Y118 as an indicator of c-Src kinase activity. We found that pretreatment of SW480 cells with PP2-abrogated aspirin-mediated phosphorylation of paxillin (Figure 2A). This supports the notion that paxillin phosphorylation in response to aspirin lies downstream of c-Src.

We next determined whether PP2 influenced the effect of aspirin on NF- $\kappa$ B signalling. Indeed, we observed that aspirin-induced degradation of I $\kappa$ B $\alpha$  was prevented in the presence of the inhibitor (Figure 2B). Furthermore, we observed that both the aspirin-induced cytoplasmic to nuclear translocation of RelA (Figure 2C) and subsequent aspirin-induced nucleolar localization of RelA were abrogated in those cells pretreated with the inhibitor (Figure 2D and E).

To further confirm the association between aspirin effects on c-Src and on the NF- $\kappa$ B pathway, we utilized a kinase dead c-Src mutant, Src K297R. We initially examined the effects of expressing this mutant on I $\kappa$ B $\alpha$  levels. We found that aspirin mediated a reduction in cytoplasmic I $\kappa$ B $\alpha$  in those cells expressing vector alone and wild-type Src. However, this reduction was completely blocked in cells expressing Src K297R. We also found that over-expression of WT Src-induced some degree of degradation of I $\kappa$ B $\alpha$ , which was further enhanced in the presence of aspirin, suggesting low level NF- $\kappa$ B stimulation in the presence of WT Src over-expression alone (Figure 3A). Similarly, immunocytochemical analysis confirmed that, following aspirin treat-

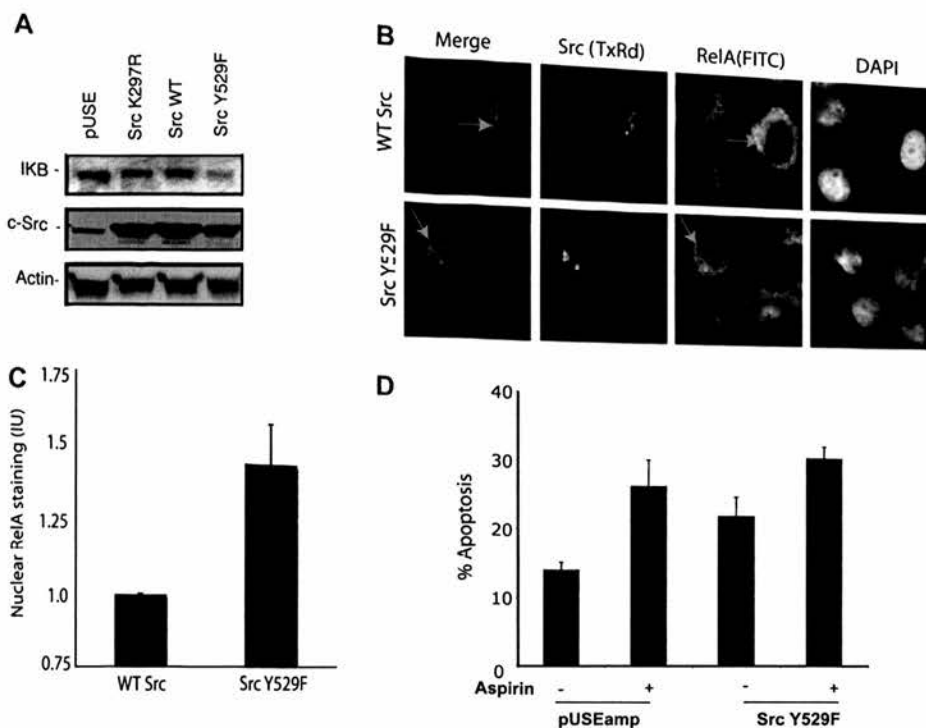
ment, the number of cells demonstrating nucleolar RelA accumulation increased in cells transfected with vector or expressing wild-type Src but that this response was inhibited in cells expressing mutant protein (Figure 3B and C).

#### Inhibiting c-src activity blocks aspirin-mediated apoptosis

Having established a role for c-Src in mediating aspirin effects on the NF- $\kappa$ B pathway, we next investigated whether c-Src is also involved in the apoptotic effects of the agent, as might be expected from our previous studies (35). Using Annexin V apoptosis assays, we found that blocking c-Src activation, using PP2, abrogated aspirin-mediated apoptosis of CRC cells (Figure 4A). A further c-Src inhibitor, Su6656 (39) similarly blocked aspirin-induced degradation of I $\kappa$ B $\alpha$  and abrogated aspirin-induced apoptosis in CRC cells (Figure 4B and C). c-Src dependency of aspirin-induced apoptosis was further supported by demonstration that over-expression of a kinase-dead form of c-Src also inhibited aspirin-mediated apoptosis of CRC cells (Figure 4D).

#### c-Src mutant (Y529F) mimics the molecular effects of aspirin in CRC cells

To further establish the role of c-Src activation in stimulation of the NF- $\kappa$ B pathway in CRC cells, we expressed a constitutively active c-Src mutant (Y529F) in SW480 cells. We found that this mimicked the effects of aspirin, causing similar degradation of I $\kappa$ B $\alpha$  (Figure 5A) and nuclear translocation of RelA (Figure 5B and C). However, expression of c-Src Y529F did not induce nucleolar translocation of RelA, consistent with our previous studies, which indicated that nucleoplasmic to nucleolar translocation of RelA requires an additional aspirin-induced cofactor. Interestingly, we also found that expression of c-Src (Y529F) increased baseline levels of apoptosis in SW480 cells and enhanced the apoptotic effects of aspirin (Figure 5D). This suggests that active Src mutant may induce apoptosis by a pathway distinct from that utilized by aspirin. Taken together, these data provide compelling evidence that activation of c-Src can induce NF- $\kappa$ B signalling and function as a pro-apoptotic stimulus in CRC cells.



**Figure 5.** The active c-Src mutant (Y529F) induces the molecular effects of aspirin in CRC cells. (A–D) SW480 cells were transiently transfected with an empty pUSEamp vector, kinase-inactive c-Src expression vector (pUSE Src K297R), wild-type c-Src or constitutively active c-Src (pUSE Src Y529F) and harvested 48 h after transfection (A–C) or treatment with aspirin [0 (–), 5 mM (+); 16 h] (D). (A) Anti-IκBα western blot analysis was performed as described in Figure 3. (B) Anti-RelA/anti-c-Src immunocytochemistry was used to examine effects of expression vectors on RelA localization [yellow arrows = transfected cells, yellow circles = nucleoli (areas devoid of DAPI surrounded by a hyperchromatic ring)]. (C) The nuclear localization of RelA in c-Src-transfected cells was quantified using anti-RelA immunocytochemistry, as described in Figure 2(D). The percentage of cells in the population undergoing apoptosis was determined using Annexin V fluorescein isothiocyanate apoptosis assays. Data presented is the minimum of three independent experiments (± standard error).

#### Evidence that activation of c-Src and NF-κB signalling is a class effect of NSAIDs

We have previously reported that non-aspirin NSAIDs induce apoptosis in SW480 CRC cell lines through stimulation of the NF-κB pathway and nuclear translocation of RelA (16). Therefore, we next examined the requirement for c-Src in the NF-κB response to these related agents. Using western blot analysis, we found that sulindac, sulindac sulphone and indomethacin-induced c-Src activation (Y418 phosphorylation) in CRC cells (Figure 6A–C). Activation was transient, in keeping with the transient effects of these agents on degradation of IκBα. Importantly, c-Src activation was detectable at time points at which we previously showed that these agents induce degradation of IκBα (16).

These data suggest an association between activation of c-Src by non-aspirin NSAIDs and stimulation of the NF-κB pathway was further examined by immunocytochemical analysis of RelA in CRC cells treated with non-aspirin NSAIDs in the presence of the chemical Src inhibitor, PP2. We demonstrate that chemical inhibition of c-Src blocked cytoplasmic to nuclear/nucleolar translocation of RelA in response to all the NSAIDs (Figure 6D), indicating that c-Src is required for activation of the NF-κB pathway by these agents.

To determine whether c-Src is required for the apoptotic effect of non-aspirin NSAIDs, we utilized CRC cells that constitutively express a kinase dead form of the protein SW480-SrcKD (kind gift from Prof. James Frame, Edinburgh Cancer Research Centre). Annexin V apoptosis assays indicated that sulindac, sulindac sulphone and indomethacin induced a significant increase in apoptosis in cells stably transfected with the control vector (pBPuro). They also indicated that the apoptotic effects of these agents were significantly abrogated in cells expressing the kinase dead c-Src (Figure 6E). Taken together, these data provide compelling evidence that c-Src is required for the NF-κB mediated apoptotic response to NSAIDs as a class (Figure 6F).

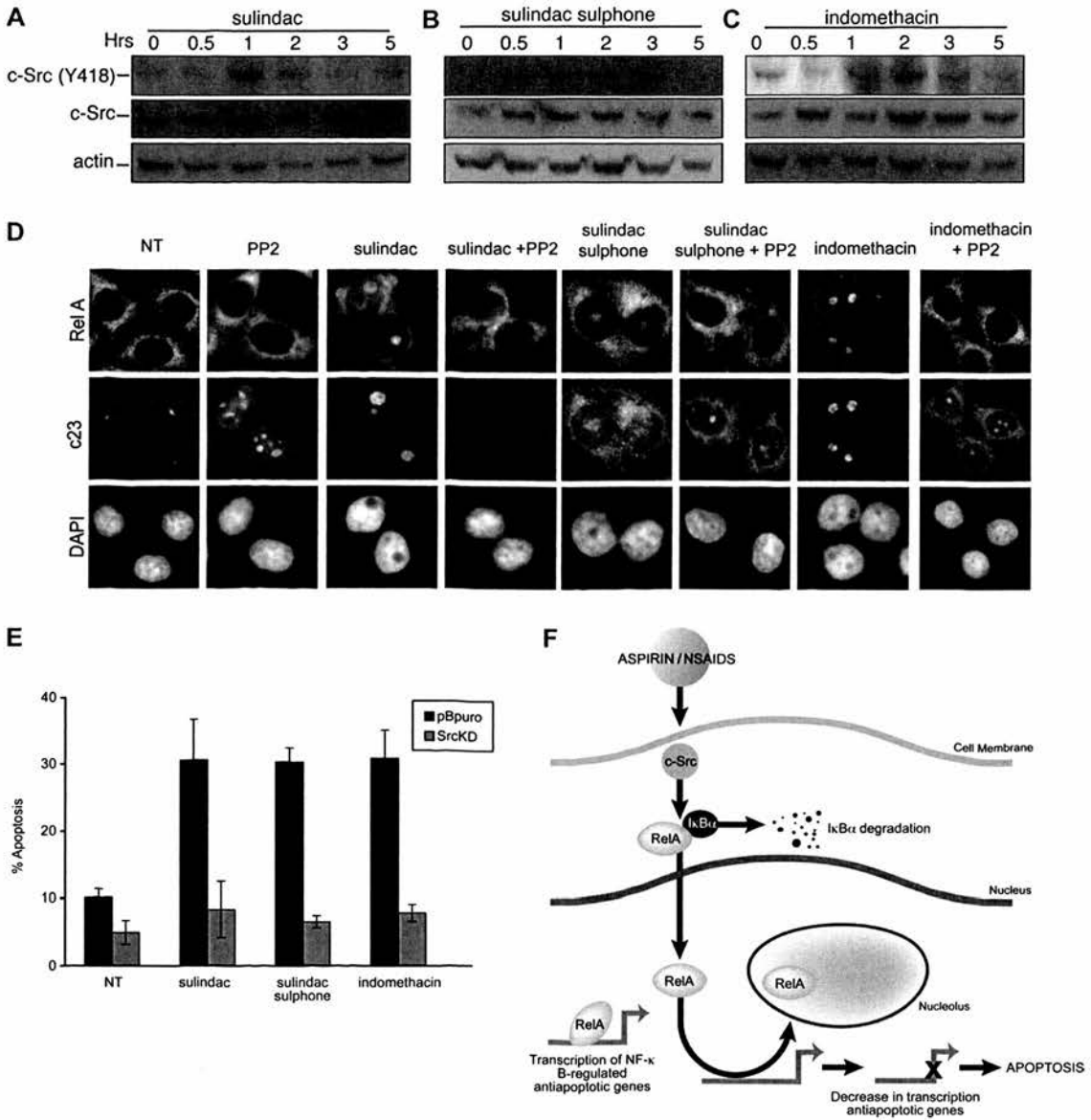
#### Discussion

The present study was undertaken to determine the upstream mechanisms by which aspirin and NSAIDs stimulate the NF-κB pathway and pro-apoptotic response and specifically, the role of the c-Src tyrosine kinase. The work presented provides two important new insights into the antitumour activity of the agent. Firstly, under the experimental conditions used here, we show that the apoptotic effects of aspirin are dependent upon activation of the c-Src tyrosine kinase. Secondly, we provide compelling evidence that the NF-κB response to NSAIDs is mediated through c-Src. These findings have important implications for the development of novel chemopreventative agents and specifically, those that modulate c-Src kinase activity.

We demonstrate here that aspirin and other NSAIDs (sulindac, sulindac sulphone and indomethacin) activate c-Src in SW480 CRC cells, as determined by Y416 phosphorylation of the protein and phosphorylation of the c-Src target site, paxillin Y118. These data are in contrast to those of Kunte *et al.* (40) who recently reported that sulindac sulfide activates the negative regulator of c-Src, csk, to inhibit c-Src activity in HT29 CRC cells. However, these apparent differences may be related to both the dose and type of NSAID used and the time of exposure to the agent, which in their study was prolonged (72 h). Casanova *et al.* (41) have also reported that the NSAID, Celecoxib, inhibits c-Src activity in acute myeloid leukaemia cell lines. However, interestingly, in four of the five cell lines presented within the report, there was an apparent transient increase in the marker for c-Src Y416 phosphorylation at 4 h following agent administration. This occurred concurrently with proteolytic cleavage of Poly (ADP-ribose) polymerase and activation of caspases and mirrored the kinetics of c-Src activation observed here.

In addition, a number of other studies have also demonstrated that pretreatment with NSAIDs modulates stimulant-induced cellular c-Src activation (31,34). It is therefore probable that the effects of





**Fig. 6.** Activation of c-Src and consequent effects on NF- $\kappa$ B signalling are effects of NSAIDs as a class. SW480 cells were cultured as described previously and then treated for 0–5 h (A–C) or 16 h (C–D) with sulindac (500  $\mu$ M), sulindac sulphone (500  $\mu$ M) or indomethacin (250  $\mu$ M). (A–C) Western blot analysis was performed on cytoplasmic extracts using antibodies to phosphorylated Src (Y416). Blots were stripped and reprobed for endogenous c-Src. Actin was used loading control. (D) Anti-RelA immunocytochemistry was performed on SW480 cells untreated (NT) or pre-treated (2 h) with the c-Src inhibitor PP2 (3.5  $\mu$ M) and then treated with sulindac, sulindac sulphone or indomethacin for 16 h. Representative micrographs ( $\times 63$ ) illustrating localization of Rel A and the nucleolar protein, C23, are shown. Chemical inhibition of c-Src inhibits NSAID-induced targeting of Rel A to the nucleolus. (E) SW480-pBpuro and SW480-SrcKD cells were treated for 16 h with sulindac, sulindac sulphone and indomethacin. The percentage of cells in the population undergoing apoptosis was determined using Annexin V-fluorescein isothiocyanate apoptosis assays. Data presented is the minimum of three independent experiments ( $\pm$  standard error). (F) A model for aspirin/NSAID-induced modulation of NF- $\kappa$ B signalling and apoptosis. In the absence of aspirin/NSAIDs, inducible NF- $\kappa$ B signalling drives the transcription of anti-apoptotic genes. Following treatment with aspirin/NSAIDs, c-Src is activated leading to stimulation of intermediate kinase pathways resulting in downstream degradation of the NF- $\kappa$ B cytoplasmic inhibitor, I $\kappa$ B $\alpha$ . Subsequently, RelA/NF- $\kappa$ B complexes undertake translocation into the nucleus/nucleolus. Nucleolar localization of RelA is causally involved in the repression of NF- $\kappa$ B driven transcription of anti-apoptotic genes leading to cellular apoptosis.

aspirin and related NSAIDs on c-Src activity are dependent upon the cellular context, chemical class, timing and concentration of the NSAID used. These differential effects are consistent with the variation in NSAID-induced effects observed on NF- $\kappa$ B pathway signalling in different cell environment settings and treatment conditions. For example, following tumor necrosis factor  $\alpha$  pretreatment of CRC cells, certain NSAIDs inhibit activation of NF- $\kappa$ B signalling, however, in the absence of tumor necrosis factor  $\alpha$  pretreatment, the converse molecular response is observed (8,9).

We found that blocking c-Src activity, using chemical inhibitors or a kinase dead mutant, blocked NSAID-mediated degradation of I $\kappa$ B $\alpha$  and nuclear translocation of RelA. Previous reports describe

a number of mechanisms by which c-Src can activate the NF- $\kappa$ B pathway (26–30). In some reports, phosphorylation of I $\kappa$ B $\alpha$  on tyrosine 42 (Y42) in response to pervanadate, hypoxia or Vascular endothelial growth factor stimulation, results in nuclear translocation of NF- $\kappa$ B in the absence of I $\kappa$ B $\alpha$  degradation (23,42–44). However, these reports contrast with our reported model that aspirin/NSAID-induced effects on the NF- $\kappa$ B pathway are associated with degradation of I $\kappa$ B $\alpha$  (9,16). In keeping with our observations, it is important to emphasise that c-Src-induced NF- $\kappa$ B activation has also been shown to occur more indirectly, through induced stimulation of intermediate kinase pathways, such as PKC $\alpha$  and I $\kappa$ B kinase. These activation pathways classically

duce NF- $\kappa$ B signalling by I $\kappa$ B $\alpha$  serine 32/36 residue phosphorylation and subsequent proteasomal-mediated I $\kappa$ B $\alpha$  degradation (28,29). Additionally, have been reported toc-Src has also been shown to form part of the I $\kappa$ B kinase complex and contribute to proteasomal-mediated degradation of I $\kappa$ B $\alpha$  in a manner that does not require its kinase domain (25,27).

Molecular mechanisms underlying aspirin/NSAID-induced cell cycle arrest and apoptosis induction in CRC have been reported to involve phosphorylation of known downstream substrates of c-Src, including p38 and mitogen-activated protein kinase/extracellular signal-regulated kinase pathways (17,45). We have previously demonstrated that p38 is activated in response to aspirin (17) and that this effect is upstream of effects on the NF- $\kappa$ B pathway. Since p38 is a known target of c-Src, it is possible that c-Src effects a response on the NF- $\kappa$ B pathway through p38 signalling. Bernardi *et al.* (46) recently reported that activation of c-Src is required for the anti-proliferative effects of indomethacin in C6 glioma cells and that chemical inhibition of c-Src-prevented NSAID-induced ERK activation and the pro-apoptotic response. Determining the pathways that c-Src, p38 and NF- $\kappa$ B and specifically, the further dissection of the molecular mechanism by which c-Src activates or interacts with I $\kappa$ B, is the focus of our ongoing work.

It is clear that the mechanisms by which NSAIDs act against CRC are complex. While increased c-Src activity is associated with CRC progression and metastasis (18–20), here, we show that activation of endogenous c-Src using aspirin, or expressing a constitutively active form of the protein, induces apoptosis in CRC cells. The findings presented in this study are in keeping with those of Welman *et al.* (20) who showed that expression of active c-Src had growth inhibitory effects against HCT116 and SW480 CRC cells. Using an *in situ* approach in *Drosophila*, it has also been reported that the cellular mechanism of altering Src signalling is dependent upon the degree of activation. Low levels of activation are associated with epithelial cell survival and proliferation, while high levels of activation mediate apoptosis (48). Importantly, cells which over-express c-Src, such as preneoplastic colonic cells, can become sensitized to apoptotic stress in particular cellular environments (48,49). Indeed, serum starvation and mTOR/SPI3K inhibition favour pro-apoptotic signalling in cells which over-express activated c-Src (50) and of relevance here, treatment conditions took place in the setting of a low-serum media culture environment. We have also observed, consistent with previous reports, that cells which express the constitutively active form of c-Src induce a pro-apoptotic response and that this effect is further amplified by the inhibition of aspirin/NSAIDs (51).

The work presented here indicates that activation of c-Src is a key component of the pro-apoptotic effects of NSAIDs in CRC cancer and uncovers molecular mechanisms through stimulation of the NF- $\kappa$ B pathway. These findings have considerable relevance to the future development of chemopreventative agents by shedding new light on the complex nature of the cellular and molecular effects of aspirin on CRC.

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