The Role of Inflammation and SPARC (Secreted Protein Acidic and Rich in Cysteine) in Colorectal Tumourigenesis

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BSc (Hons)

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Abstract

Chronic inflammation of the colon is associated with an increased risk of the development of colorectal cancer (CRC). Therefore, pathways that contribute to inflammation modifying tumourigenesis in the colon are of diagnostic and therapeutic interest. This thesis analyses the contribution of inflammation to tumourigenesis in a murine model of CRC in which sporadic tumourigenesis was produced by a colonotropic mutagen, azoxymethane (AOM). Colitis was induced by ingestion of dextran sulphate sodium (DSS) and mice treated with both agents (AOM/DSS) also developed colorectal tumours.

Both sporadic (AOM) and colitis-associated tumours (AOM/DSS) were found to consist of carcinomas-in-situ and in this study these are compared for the first time in detail. The number and size of tumours were studied macroscopically, and the extent of dysplasia and tumour morphology characterised by histology. The number of proliferating cells within tumours was determined by detection of Ki-67 and the number of apoptotic cells by fragmented DNA using TUNEL. The composition of the immune cell populations infiltrating the tumours was characterised by indirect immunofluorescent antibody staining for CD4, CD8a, CD11c, F4/80, and Ly6G. Finally, a comparison of gene expression levels was performed to examine the involvement of various pathways at a global level.

The presence of chronic colitis increased the number of tumours although these were smaller than those developing in the absence of inflammation. Chronic colitis associated tumours contained fewer apoptotic stromal cells and proliferating epithelial cells but increased numbers of CD4+ T-cells, macrophages, and dendritic cells, based on CD4+, F4/80+, and CD11c+ staining, than AOM-tumours. There were also fewer CD8+ and Ly6G+ cells, suggesting a decrease in CD8+ T-cells and neutrophils compared to AOM-only tumours. The majority of the immune infiltrate in both the sporadic and colitis associated tumours consisted of CD4+ cells and F4/80+ cells, suggesting a relative preponderance of CD4+ T-cells and macrophages in both models. Through a global gene expression microarray involving about 18 000 genes, 143 genes were found to be differentially expressed between sporadic and colitis-associated tumours. Of these, the expression levels of Cd164, Csf1r, Cd44, and Mmp10 were tested by a qRT-PCR and Cd164 and Csf1r were verified as significantly increased in AOM/DSS colitis-associated
tumours. The products of *Cd164* and *Csf1r* are linked to the phosphatidylinositol 3-kinase (PI3K)-Akt-pathway, suggesting a possible role in colitis-associated tumourigenesis.

The role of secreted protein acidic and rich in cysteine (SPARC) in colitis-associated colorectal tumourigenesis was examined as earlier work by my group had found an association of SPARC with inflammation and CRC tumourigenesis. This is the first study to examine the influence of SPARC in murine models of colitis-associated colorectal tumourigenesis. For these studies, tumours were induced as before with AOM/DSS treatment of SPARC-knockout (KO) mice and compared with those in wild-type (WT) mice.

More tumours developed in the SPARC-KO mice but these were found to be of comparable size and contained similar numbers of proliferating and apoptotic cells compared to those developing in WT mice. However, the presence of SPARC was associated with fewer CD11c⁺ and F4/80⁺ cells in the tumours, suggesting that they contained lower numbers of dendritic cells and macrophages. In contrast, there were increased numbers of T lymphocytes, based on CD4⁺ and CD8a⁺ cells, in the presence of SPARC while no difference in Ly6G⁺ cell number was detected. A global gene expression microarray compared the expression levels of about 18 000 genes between WT and SPARC-KO tumours and 6 genes, in addition to SPARC, were differentially expressed. The expression levels of 4 of these genes, *Ido*, *Ifit2*, *Areg*, and *Erdr1*, were tested via multiplex qRT-PCR but no significant changes were found. While 2 genes remain to be tested, the lack of differences in gene expression between the SPARC KO and WT tumours suggests that SPARC reduces tumour initiation and alters the intra-tumoural inflammatory population by other mechanisms.

In conclusion, chronic inflammation of the colon increases the number of tumours developing, perhaps through damage to the colon and possibly involving the Pi3K-Akt pathway. Notably, inflammation-associated tumours were smaller which did not appear to be explained by the extent of apoptosis. While they contained fewer neutrophils and CD8⁺ T-cells, there was an increase in the numbers of other inflammatory populations suggesting the possibility of suppression of tumour growth by cytokines produced by immune cells. The presence or absence of the matricellular protein SPARC also impacted on the number of tumours and the composition of the tumour infiltrating cell populations. Interestingly, this occurred without substantive changes in gene expression. The chronic
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<th>Abbreviation</th>
<th>Full name</th>
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<tr>
<td>5-ASA</td>
<td>5-Aminosalicylic acid</td>
</tr>
<tr>
<td>ACF</td>
<td>Aberrant crypt foci</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AOM</td>
<td>Azoxymethane</td>
</tr>
<tr>
<td>BHQ</td>
<td>Black hole quencher</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
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<tr>
<td>BWI</td>
<td>Backwash ileitis</td>
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<tr>
<td>CAC</td>
<td>Colitis-associated colorectal cancer</td>
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<tr>
<td>CD</td>
<td>Crohn's disease</td>
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<tr>
<td>CIN</td>
<td>Chromosomal instability</td>
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<tr>
<td>Cox</td>
<td>Cyclooxygenase</td>
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<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte antigen 4</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole, dihydrochloride</td>
</tr>
<tr>
<td>DAVID</td>
<td>Database for annotation, visualization and integrated discovery</td>
</tr>
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<td>DSS</td>
<td>Dextran sodium sulphate</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>FAM</td>
<td>Fluorocein amidite</td>
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<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>GIMP</td>
<td>GNU image manipulator program</td>
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<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
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<tr>
<td>HIF</td>
<td>Hypoxia-Inducible factor 1</td>
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<td>HNPCC</td>
<td>Hereditary non-polyposis colorectal cancer</td>
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<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IKK</td>
<td>Iκβ kinase</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>K-ras</td>
<td>Kirsten -ras</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
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<td>LS</td>
<td>Lunch syndrome</td>
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<tr>
<td>M1</td>
<td>Macrophage subtype 1</td>
</tr>
<tr>
<td>M2</td>
<td>Macrophage subtype 2</td>
</tr>
<tr>
<td>MAMA</td>
<td>Monoallelic mutation analysis</td>
</tr>
<tr>
<td>MCA</td>
<td>3'-Methylcholanthrene</td>
</tr>
<tr>
<td>MLPA</td>
<td>Multiplex ligation-dependent amplification</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation response gene 88</td>
</tr>
<tr>
<td>NF-kβ</td>
<td>nuclear factor (NF)-κβ</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
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<tr>
<td>OCT</td>
<td>Optimum cutting temperature</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PD-1</td>
<td>Programmed cell death 1</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Programmed cell death ligand 1</td>
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<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PI3K-AKT</td>
<td>Phosphatidylinositol 3-kinase-AKT</td>
</tr>
<tr>
<td>PSC</td>
<td>Primary sclerosing cholangitis</td>
</tr>
<tr>
<td>PTT</td>
<td>Protein truncation test</td>
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<tr>
<td>qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator for nuclear factor κ-β ligand</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RTD</td>
<td>RealTimeDesign™</td>
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<tr>
<td>SPARC</td>
<td>Secreted protein, acidic and rich in cysteine</td>
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<tr>
<td>TAFs</td>
<td>Tumour-associated fibroblasts</td>
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<tr>
<td>TAMs</td>
<td>Tumour-associated macrophages</td>
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<tr>
<td>TE</td>
<td>tris-ethylenediaminetetraacetic acid</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>Description</td>
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<td>------------------------------------</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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</table>
List of published articles


List of conference abstracts

1. Shuyi Kristine Fu, “Comparison of the Tumour Microenvironment between Mouse Models of Colorectal Cancer” (paper presented at the annual Combined Biological Sciences Meeting, Western Australia, Australia, 2011).
2. Shuyi Kristine Fu, “Tumour Microenvironment Differs Between Murine Sporadic and Inflammation-Associated Colorectal Tumourigenesis” (poster presented at the annual meeting for the European Crohn’s and Colitis Organisation, Barcelona, Spain, 2012). (Highly commended poster)
4. Shuyi Kristine Fu, “SPARC affects colorectal tumourigenesis by altering tumour microenvironment” (Poster presented at the annual meeting for the Gastroenterological Society of Australia, Melbourne, Australia, 2013). (Awarded with Poster of Merit)

List of published abstracts

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To my family – There are simply not enough words in the world to describe my gratitude. You guys have done so much for me and now its my turn…get ready for it! 符家和黄妈妈，我爱死你们！

And last, and definitely not the least, to Amos – Thank you. ^^
Declaration

The work presented within this thesis was completed in the School of Medicine and Pharmacology at the University of Western Australia. I hereby declare that all work presented is entirely my own, unless explicitly stated otherwise. All contributions by others are formally disclosed and duly acknowledged.

__________________________
Shuyi Kristine Fu
Chapter 1

Literature review
1.1 INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related death in both men and women worldwide, and it appears to be increasing in incidence. According to the Cancer Council of Australia, the estimated annual CRC incidence in 2012 was 14,957 while the number of CRC-related deaths in 2007 was identified as 4,162 (Cancer Council of Australia, 2013). Figure 1.1 presents data from a study conducted in 2012 by the International Agency for Research on Cancer (IARC), showing that CRC mortality was highest in Central and Eastern Europe, with about 20 males and 11 females per 100,000 individuals dying annually, compared to 11 males and 9 females per 100,000 individuals in Australia. Australia and New Zealand combined had the highest incidence of CRC in the world, with 46 males and 32 females out of every 100,000 individuals affected, compared to 36 males and 24 females per 100,000 in Central and Eastern Europe (Ferlay, et al., 2013). Differences in availability of appropriate medical services within each region, lifestyle, including variability in dietary protein content of the respective diets and other causes of mortality, may contribute to the differences observed in the CRC mortality rates compared to incidence rate in each region.

Survival has been shown to be inversely related to the duration of disease but currently fewer than 40% of CRC are detected early (Australian Government Department of Health and Aging, 2013). Mortality could, thus be reduced further if the disease was detected at an earlier stage particularly before local, lymphatic or haematogenous spread. Such thinking has led to the introduction of the National Bowel Cancer Screening Program in Australia, where faecal occult blood testing is offered to all people aged 50, 55, 60 and 65 years. Individuals positive for the test are offered further investigation via colonoscopy. The aim of this program is to detect prodromal lesions or adenomatous polyps prior to the development of malignant change, and to remove them during the colonoscopic procedure. It is estimated that this program will detect an additional 12,000 new cases of CRC and could save between 300 to 500 lives each year (Australian Government Department of Health and Aging, 2013).
Improvement in disease classification for CRC would provide a more accurate prognosis for individuals, allowing for better the allocation of medical resources and implementation of the most appropriate treatments. This could be advanced by the identification of biological markers that are predictors of a pathological state. For example, high levels of the lymphangiogenic factor, vascular endothelial growth factor D (VEGF-D), may indicate aggressive tumour growth prompting the need for a more aggressive treatment approach. High levels of VEGF-D could also be an indicator of a poor long-
term prognosis (Moehler, et al., 2008). It should be noted that CRC, however, exists as multiple forms and biomarkers may differ between each disease type. Thus, a better understanding of each form of CRC might identify potentially appropriate markers.

1.2 FORMS OF COLORECTAL CANCER

CRC exists in sporadic, colitis-associated, and hereditary forms. Sporadic CRC and colitis-associated CRC (CAC) occur as a result of random non-inherited genetic mutations, unlike familial or hereditary CRC. Hereditary forms of CRC include familial adenomatous polyposis (FAP) and Lynch Syndrome (LS), and these forms of CRC are discussed further below.

1.2.1 Sporadic colorectal cancer

About 75% of CRC patients do not have a strong family history of the disease, and 64 years is the average age of detection (Amersi, et al., 2005). Around 15% of sporadic colorectal adenomas exhibit microsatellite instability (MSI) (Poulogiannis, et al., 2010), and the tumours occur on the right side of the colon. Compared to LS patients, sporadic CRC patients have a lower 5-year survival rate presumably related to the older age of presentation as opposed to more aggressive, or more advanced, disease at presentation (Ward, et al., 2001).

MSI is where mutations occur in short motifs of tandemly repeated sequences (Jensen, et al., 2009) and is caused by defects to the mismatch repair (MMR) genes; MutS protein homolog (MSH)-2, MSH3, MSH6, MutL homolog 1 (MLH1), and PMS2 postmeiotic segregation increased 2 (PMS2). These are crucial in repairing DNA mutations caused by single-base mismatches and insertion-deletion loops that occur during DNA replication. In sporadic CRC, the MSI status is regarded as high (MSI-H), where greater than 30% of microsatellite markers are mutated (Boland, et al., 2010), and hypermethylation of the MLH1 promoter is almost always the cause of MSI (Menigatti, et al., 2001).

Field defects within the colon are characterised by genetically-distinct malignancies that arise close to each other and this may explain why individuals with no family history may develop multiple primary tumours. The hypermethylation of O-6 methylguanine DNA Transferase (MGMT), a DNA-repair gene that protects cells from alkylation by removing alkyl groups from the O6-position of guanine, has also been linked to causing field defects in CRC (Shen, et al., 2005).
MSI-H polyps are frequently located in the right side of the colon and have a mucinous and poorly differentiated phenotype whilst both MSI-L and microsatellite stable (MSS) polyps have a different phenotype (Ward, et al., 2001). The phenotype between MSI-H and MSI-low (MSI-L) polyps are also different, suggesting differences in pathological pathways (Ward, et al., 2001). MGMT-promoter hypermethylation is also commonly observed in MSI-L sporadic CRCs and it is present in non-tumour tissue directly adjacent to the tumour but not in tissues more than 10 cm away from the tumour (Shen, et al., 2005).

It should be noted that around 75% of sporadic CRC cases displayed truncating somatic mutations or loss of heterozygosity (LOH) to the adenomatous polyposis coli (APC) gene. Most truncating mutations occurred within a mutation cluster region from codons 1282 to 1581, leaving 1 to 3 intact 20 amino-acid repeats. It is of interest that proximal tumours corresponded to 2 to 3 intact 20 amino-acid repeats whereas distal tumours corresponded to 1 to 2 20 amino-acid repeats, and that these were independent of MSI status (Christie, et al., 2013).

The clinicopathological features of these tumours were distinct to tumours without APC mutations (Albuquerque, et al., 2010). For example, tumours with APC mutations were associated with younger age, male gender, distal colon and rectal tumour location, well/moderate differentiation, non-mucinous histology, and MSS status (Christie, et al., 2013). In contrast, tumours with APC mutations were associated with older age, female gender, proximal tumour location, poor differentiation, mucinous histology, MSI, and weaker nuclear β-catenin staining (Albuquerque, et al., 2010).

1.2.2 Familial adenomatous polyposis

Familial Adenomatous Polyposis is an autosomal-dominant disease and is the second-most common inherited form of CRC. Its characteristic feature is the development of hundreds, to thousands, of colonic adenomas with the vast majority of the polyps (70 to 80%) occurring in the left side of the colon (Bjork, et al., 1999). The formation of polyps normally commences in the adolescent years and if left untreated, will almost invariably progress to cancer by 35 to 40 years (Bjork, et al., 1999).

Germline mutations occur in the APC gene located on chromosome 5q21 and often lead to protein truncation, which is responsible for the FAP phenotype. The APC protein functions as a scaffold protein that affects both cell-adhesion and migration and is part of
a signalling pathway complex mediated by the Wnt pathway. This pathway regulates both the phosphorylation, and degradation, of β-catenin, which is an intracellular protein that binds E-cadherin, and links it to the actin cytoskeleton and phosphorylation of β-catenin. This results in the ubiquitinylation of β-catenin and its subsequent degradation. Mutations in APC, therefore, lead to cytoplasmic accumulation of β-catenin allowing it to bind to the T-cell family of transcription factors affecting expression of genes related to cellular differentiation, proliferation, migration, and apoptosis, such as cyclin D-1 and c-Myc oncogene (Galiatsatos, et al., 2006).

Routine screening methods involve the detection of the truncated APC protein via a protein truncation test (PTT), but germline mutations have not been found in 20 to 30% of classical FAP patients (Galiatsatos, et al., 2006). Use of sensitive second-line detection methods such as monoallelic mutation analysis (MAMA) and multiplex ligation-dependent probe amplification (MLPA) are thus important. These techniques have identified large deletions in APC that result in a protein too small to be detected by PTT, thus increasing the detection rates of FAP mutations to at least 94% (Laken, et al., 1999). MAMA involves the analysis of the APC gene on both alleles, allowing investigators to determine their respective APC expression and in one study of 9 patients who were previously negative for PTT, 6 were then identified as having APC mutations by MAMA (Laken, et al., 1999). MLPA is another method that identifies large deletions using numerous probes to amplify various regions of the APC gene. This method identified 3 out of 7 PTT-negative families as containing APC mutations with another family identified to have a monoallelic expression of APC (Cao, et al., 2006). The inability to detect APC mutations in certain FAP individuals, however, raises the possibility that other genes may give rise to the FAP phenotype.

1.2.3 Lynch syndrome

Lynch syndrome, also known as hereditary non-polyposis CRC (HNPCC), accounts for 2 to 4% of all CRCs (Hampel, et al., 2005) and the criteria for the diagnosis of LS is that there should be at least 3 relatives with a LS-associated cancer with one of the cases being a first-degree relative of the other two. At least two generations must also be affected and at least one relative should be diagnosed before the age of 50 years. FAP needs to be excluded and tumours must be verified as cancers by pathological examination (Vasen, et al., 1999). The onset for LS is normally at an early age, and there is a high penetrance of cancer in genetically-affected individuals, with a lifetime risk of approximately 80%
for the development of CRC (reviewed by Chung, et al., 2003). Tumours commonly form in the right colon and the progression from adenoma to carcinoma is usually rapid with untreated adenomas progressing to carcinomas within 3 years (Vasen, 2005). The average age at diagnosis is 45 years, and patients inheriting LS also have an increased risk of extra-colonic cancers that include endometrial, renal pelvis/ureter, stomach, small bowel, brain, and sebaceous tumours with the most common extra-colonic cancer in females being endometrial (Aarnio, et al., 1995).

This disease is a result of germline mutations in genes involved in MMR, such as MSH2, MLH1, MSH6, and PMS2, and unsurprisingly the resulting tumours are commonly MSI-H (McGivern, et al., 2004). The current screening sensitivity for LS, however, is only 40 to 80% for the high-risk patients (Lynch, et al., 2003), which could be improved by screening for germline mutations in all of the above-mentioned genes. Sequencing for these mutations, however, is expensive and time-consuming and a combination of immunohistochemical analysis and genotyping for MSI has been recommended as an improvement to the current screening methods. In a study involving 1066 patients with newly diagnosed CRC regardless of age and family history, 23 patients were identified as having a mutation causing LS and 15 of these patients did not fit within the current diagnostic criteria for LS (Hampel, et al., 2005). Genotyping and immunohistochemical analysis for MSI also failed to identify 2 of 23 LS patients, which indicates a screening sensitivity of 91%. The 2 undetected LS patients raises the question as to the number of LS individuals that have been missed and the need to revise current screening methods (Hampel, et al., 2005).

1.2.4 Colitis-associated colorectal cancer

The inflammatory bowel diseases (IBDs), Crohn’s disease (CD) and ulcerative colitis (UC), are chronic relapsing inflammatory disorders of unknown aetiology that result in mucosal ulceration and macroscopic inflammation of the gastrointestinal tract (GIT). UC only affects the colon whilst CD may affect any part of the GIT, and is most likely to involve the terminal ileum and colon (Caprilli, et al., 2008). IBD increases the risk of CRC and the risk appears to be associated with the length of time since diagnosis and extent of the colon that is subjected to the chronic inflammation and how well the inflammation is kept under control (Bernstein, et al., 2001).
1.2.4.1 Ulcerative colitis

Neoplastic progression in UC is characterised by the development of epithelial dysplasia and is associated with genetic abnormalities such as the LOH for the p53 allele and DNA aneuploidy within the affected tissue (Burmer, et al., 1991). A meta-analysis of 116 studies has identified the prevalence of CRC in UC patients to be 3.7% with the cumulative risk of cancer increasing with duration of disease (Eaden, et al., 2001). The age of onset of CRC for UC patients is younger than CD patients (Choi, et al., 1994) and the CRC-risk at 10, 20 and 30 years since onset of disease could be as high as 2%, 8%, and 18%, respectively (Eaden, et al., 2001), although more recent studies suggest that the risk is not as high, with the cumulative probability of CRC in the Copenhagen county of Denmark being 0.4%, 1.1%, and 2.1% by 10, 20, and 30 years since disease onset, respectively (Winther, et al., 2004). The anti-inflammatory drug, 5-aminosalicylic acid, is used in the treatment of mild to moderate UC and for the management of remission, and has been demonstrated to protect patients against CRC by reducing its risk (Velayos, et al., 2005, Ardizzzone, et al., 2006, Velayos, et al., 2006) by up to 75% (Eaden, et al., 2000, Velayos, et al., 2005) although the literature is not unanimous on this issues with a meta-analysis of 4 observational studies indicating no effect of treatment (Nguyen, et al., 2012). Cancer risk for UC-patients is also increased with the presence of primary sclerosing cholangitis (PSC) (Shetty, et al., 1999, Mill, et al., 2013).

PSC is a disease of unknown aetiology and is associated with both CD and UC in 1 to 7% of patients (Shetty, et al., 1999, Broome, et al., 2006). PSC results in a chronic inflammatory disease of the biliary tree that is characterised by intense hepatic fibrosis and usually affects both the intra- and extrahepatic biliary ducts (Nagengast, et al., 1995, Broome, et al., 1996, Kornfeld, et al., 1997, Shetty, et al., 1999). The chronic inflammation leads to a decrease in bile drainage from the liver and a reduction of bile acid secretion into the intestinal lumen (Shetty, et al., 1999). This leads to a change in the enterohepatic circulation of bile, thereby increasing bile acid exposure to intestinal microflora and resulting in an increase in secondary bile acid production. The secondary bile acids, lithocholic and deoxycholic acids, have been implicated in the development of CRC (Nagengast, et al., 1995, Ou, et al., 2012) and may explain why patients with both UC and PSC experience higher frequencies of DNA aneuploidy and dysplasia when compared to patients suffering from UC alone (Kornfeld, et al., 1997, Shetty, et al., 1999).
1.2.4.2 Crohn’s disease

CD is an inheritable immunological disease characterised by the presence of skip lesions, which are regions of inflamed tissue separated by sections of uninvolved mucosa. Skip lesions can occur throughout the whole colon, most commonly the terminal ileum with early mucosal lesions tending to occur in Peyer’s patches (Sankey, et al., 1993). Deep linear ulceration of the intestinal mucosa, transmural inflammation, and fibrosis may result in intestinal fibrostenosis and obstruction (Barrett, et al., 2012).

CD is thought to arise from the aberrant interaction between gut contents and the innate immune system, which is exacerbated by a macrophage immunodeficiency. Macrophages extracted from CD patients exhibit a diminished cytokine response towards agonists (Smith, et al., 2009), exacerbating inflammation as a result of failing to clear foreign material/bacteria from the mucosal tissue. Over time, this can lead to the formation of granulomas, which is a feature in 50% of CD patients (Denoya, et al., 2011). Unlike UC, a meta-analysis involving 20 clinical studies has estimated the risk of CRC from CD to be mild, with a cancer prevalence of 1% amongst CD patients (Laukoetter, et al., 2011). The risk of CRC development, however, increases with the area of the intestinal wall subjected to chronic inflammation (Triantafillidis, et al., 2009). The inflammation in CD is frequently patchy and therefore less area involved than UC, thus, it is not surprising that the risk of CRC development in CD is lower than UC. A meta-analysis of population-based cohort studies confirmed that an overall increased risk of both CRC and small bowel cancer exists amongst patients with CD (Jess, et al., 2005, Laukoetter, et al., 2011). Concomitant PSC in CD patients does not appear to increase the risk of dysplasia or CRC, but again this may be secondary to the lower rates of PSC in those with CD (Braden, et al., 2012). Compared to a standard population without inherent CRC risks, CD patients, however, are still 2 to 3 times more likely to develop CRC and these occur 20 years younger than in the general population (Laukoetter, et al., 2011). The mean time from CD diagnosis to the development of cancer is around 18 years with an average age of cancer detection at 50 years of age (Laukoetter, et al., 2011).

1.2.5 Genome-wide association studies and IBD

The identification of susceptibility loci in IBD through genome-wide association (GWA) studies has increased over the past decade and four meta-analyses of GWA studies on UC (McGovern, et al., 2010, Anderson, et al., 2011, Jostins, et al., 2012), and CD (Franke, et
al., 2010, Jostins, et al., 2012), have identified new susceptibility loci. A meta-analysis of 6 UC GWAs identified 29 new loci, increasing the number of UC-associated susceptibility loci to 47, which explains about 16% of UC hereditability (Anderson, et al., 2011). A minimum of 28 susceptibility loci are shared between CD and UC, suggesting that some mechanistic pathways are shared between these diseases (McGovern, et al., 2010, Anderson, et al., 2011).

In CD, 39 new loci have been described which increases those explained by genetics from 20% to 23.2% due to the larger sample size (Barrett, et al., 2008). However, it was suggested that even if sample size were increased, the identification of possible genetic causes of CD will increase only by a fraction owing to the probability that the key genes have already been identified and that with larger patients numbers, the detection of further genes are likely to be less important in disease pathogenesis.

The single-nucleotide polymorphism (SNP) rs281379 was identified to highly correlate with a nonsense mutation in the human secretor blood group α (1, 2) fucosyltransferase gene, FUT2 (Franke, et al., 2010, McGovern, et al., 2010). Individuals who are homozygous for this SNP do not secrete blood group antigens at epithelial surfaces and have been described to having near-complete protection from symptomatic norovirus infection. It was, therefore, suggested that this SNP may be a potential link between infection and immune-mediated disease.

1.3 ANIMAL MODELS OF CRC

Studying the process of malignant change in humans is difficult due to medical treatment of the patients and the inability to trial drugs in the prodromal phases of CRC development. Animal models, on the other hand, provide the flexibility of creating a model that suits the research question of interest, with large and reproducible numbers of animals. Most importantly, it allows researchers to study tumour progression. These methods allow new insights that could lead to a better management of disease detection and treatment.

For CRC, as with many other diseases, multiple animal models exist, with the differences between each model potentially allowing the researcher to focus on one aspect of disease progression. Several types of models are available chemically induced models will be discussed below.
1.3.1 Chemically-induced models

Chemically-induced models of CRC are widely used for studying CAC and can involve the use of chemicals such as dextran sulphate sodium (DSS) and azoxymethane (AOM) and models using these two chemicals will be described below.

1.3.1.1 Dextran sulphate sodium

DSS is a non-genotoxic colonic carcinogen and is commonly used in animal models of colitis. Its exact mode of effect, however, is not presently known. There is a loss of colonic epithelial cell adhesion 3-days after DSS-administration (Kitajima, et al., 1999). In a separate study, a gradual loss of tight junction protein ZO-1 was observed over 7 days from the commencement of DSS-administration (Poritz, et al., 2007). DSS is cytotoxic but it is not known if this is a result of changes to cell adhesion and permeability, or a direct effect of DSS. These changes were observed before the onset of inflammation and it is widely believed that inflammation is a secondary effect of DSS. It should be noted that DSS is available in different molecular sizes and its size can affect the inflammatory outcome. For example, DSS with a molecular weight of 5 kD and 40 kD results in inflammation in contrast to the 500 kD variant (Kitajima, et al., 2000).

The oral administration of DSS in the drinking water of rodents can induce colitis and this is the most widely used model for investigating both acute and chronic intestinal inflammation. There are, however, strain differences to the DSS-treatment with C57Bl/6 mice being more susceptible to colitis than BALB/c mice (Melgar, et al., 2005). Also, the anatomical sites for colitis tend to vary between strains. For example, inflammation develops in the colon of C57Bl/6 mice, whereas the caecum and colon are active sites of inflammation in C3H/HeJ Bir and NOD-LtJ mice (Mahler, et al., 1998).

Chronic intestinal inflammation is associated with the induction of intestinal carcinogenesis. Long term exposure to DSS alternating with plain water over 204 days results in the development of flat-lesions, which are similar to human CAC, with a 31% incidence of dysplasia and a 25% cancer-incidence in ICR mice (Cooper, et al., 2000). Unfortunately the duration to tumour induction in this CAC model is long with a relatively low incidence and multiplicity of dysplasia and cancer.
1.3.1.2 Azoxymethane

AOM is an organotropic carcinogen and along with its metabolites, it causes DNA adducts via alkylation, forming N7-methly-guanine and O6-methly-guanine, which results in a guanine to adenine transition. AOM is injected intraperitoneally into mice, absorbed into the blood stream then transported to the liver. Cytochromes P450 then metabolises AOM to produce methylazoxymethanol, which is secreted with bile into the gut. It is further metabolised into methyldiazonium by gut microbes, and this is the final carcinogenic metabolite (Neufert, et al., 2007). These activation steps contribute to the organ specificity of AOM and its metabolites, allowing researchers to induce tumours effectively within the intestine.

The concentration of AOM is strain dependent and can vary from 10 to 15 mg/kg and colonic tumours develop over a 24-week period following 6 AOM injections, which are administered once per week. As it does not rely on the induction of inflammation for tumour growth, this model is thought to mimic the development of spontaneous CRC (Neufert, et al., 2007).

In AOM-treated rodents, aberrant crypt foci (ACF) (Vivona, et al., 1993), adenomas and adenocarcinomas develop (Vivona, et al., 1993, Maltzman, et al., 1997, Suzui, et al., 2002). Mutations to the K-ras oncogene are observed (Vivona, et al., 1993) along with β-Catenin mutations (Takahashi, et al., 1998) and the absence of full-length APC (Maltzman, et al., 1997). Changes in the expression of inducible nitric oxide synthase and cyclooxygenase 2 were also increased in AOM-induced colorectal tumours (Takahashi, et al., 2004). These changes are consistent with those found in human CRC and the AOM-induced model of CRC is widely used in the study of sporadic CRC.

1.3.1.3 The AOM and DSS model

Modifications to the DSS model to include administration of the carcinogens 1,2-dimethylhydrazine or AOM leads to the initial activation of oncogenes, which are genes that promote both cell growth and proliferation. It may also result in the inactivation of tumour suppressor genes, which are genes that act to suppress the cell cycle and, therefore, cellular proliferation through the inhibition of growth stimulation factors.

The primary aim of this combined drug model is to provide an intestinal carcinogenesis model where tumours develop over the short-term and express biological modifications similar to those found in human CRC. This could be achieved with an initial
administration of 10 mg/kg AOM, followed by a 1 week exposure to 2% DSS via consumption in the drinking water (Neufert, et al., 2007). This AOM/DSS model mimics the chronic inflammation associated with UC and CD (Tanaka, et al., 2003), and results in tumour development, which is dependent on the level of chronic inflammation. Lower levels of DSS concentration in the drinking water, therefore, decrease the numbers of tumours that develop. However, there are differences in mouse strain sensitivity to AOM/DSS administration (Suzuki, et al., 2006) and this model required further modification for C57BL/6 mice in order to overcome resistance to tumour development. The administration of 10 mg/kg of AOM was thus followed by three cycles of DSS treatment, each cycle consisting of 7 days of 1% DSS in drinking water, followed by plain water for 14 days. This protocol induces visible colonic lesions at day 20 and large tumours in 100% of mice.

1.4 MORPHOGENETIC PATHWAYS OF SPORADIC AND COLITIS-ASSOCIATED COLORECTAL CARCINOMAS

The natural history of progression of dysplasia to carcinoma in CAC suggests a shorter time to progression compared to the adenoma to carcinoma progression of sporadic CRC. This suggests a potential difference in the development pathway (Rhodes, et al., 2002). Also, the general morphology of sporadic CRC and CAC tumours are different. For example, the majority of sporadic tumours tend to be exophytic whilst CAC are mostly flat-lesions that grow close to the mucosa (Jaramillo, et al., 1996, Kiesslich, et al., 2003, Kukitsu, et al., 2008). It has also been observed that flat-lesions display a relatively higher grade of dysplasia (Tsuda, et al., 2002).

Tumourigenesis is associated with the accumulation of oncogene and tumour suppressor gene mutations as well as the clonal expansion of neoplastic cells. The association of mutations with tumour stage-specific genetic abnormalities has led to the development of a model for the clonal evolution of CRC by the acquisition of sequential mutations (Vogelstein, et al., 1988). A comparison can thus be made between the progression of sporadic CRCs and CACs.

1.4.1 Progression of CAC and sporadic cancer

Sporadic CRC is believed to develop from pre-existing colonic adenomas and the concept of progression from adenoma to carcinoma was strengthened when similarities between
the histology of adjacent non-invasive and invasive lesions were noted (Leslie, et al., 2002). By contrast, CAC progression is thought to follow a dysplasia to carcinoma pathway, as it was noted that carcinomas were found in regions of dysplastic tissue without the development of a discrete adenoma (Leedham, et al., 2009).

An early stage characteristic of CAC progression is the G-to-A and C-to-T transversion mutations that occur in p53 and are found in the dysplastic tissues, which later progresses to abnormal p53 expression and is related to point mutations and allelic deletions in CAC (Hussain, et al., 2000, Leedham, et al., 2009). Loss of p53 function is also strongly associated with the appearance of the flat lesion (Chang, et al., 2007). By contrast, loss of p53 occurs late in the progression of sporadic CRC as observed by gene mutation and allelic loss of p53 in carcinomas, but the presence of wild-type alleles in non-malignant adenomas (Baker, et al., 1990).

Mutations to the oncogene, Kirsten (K)-ras, may occur as well, but rarely together with the p53 mutations (Conlin, et al., 2005). K-ras mutations tend to occur earlier in the CAC progression pathway (Baker, et al., 1990) but are observed late in sporadic adenoma progression (Vogelstein, et al., 1988, Lamlum, et al., 2000). These mutations are also noted to be more common in sporadic CRC than CAC (Chaubert, et al., 1994). Mutations to K-ras in CAC are found in dysplastic tissue adjacent to cancerous lesions, but not in the cancerous lesion itself suggesting that K-ras expression may be lost with the development of the cancer (Chaubert, et al., 1994).

Tumour progression can also be associated with chromosomal gains of oncogenes and losses of the tumour suppressor genes (Greenwald, et al., 1992, Goel, et al., 2003, Cianciulli, et al., 2004). Through the use of comparative genomic hybridisation, a technique that allows for the detection of chromosomal copy number changes (Weiss, et al., 1999), the majority of CACs display a loss of heterozygosity of p53 with an average of 3 gains and 5 losses per sample compared to 3 gains and 4 losses per sample in sporadic CRC (Aust, et al., 2000). The most common alteration in CAC is the loss of chromosome arm 18q (Aust, et al., 2000), which also occurs in late stage sporadic CRC, and contains the loci for two tumour suppressor genes, deleted in pancreatic cancer locus 4, also known as a mediator of the TGF-β pathway, Smad4 (Hahn, et al., 1996, Takaku, et al., 1998, Miyaki, et al., 1999, Alazzouzi, et al., 2005), and Deleted in Colorectal Cancer (DCC) (Shibata, et al., 1996).
Both 8p loss and 8q gain, are associated with stage progression in CAC (Aust, et al., 2000) and are a common phenomenon. They are observed in many cancers with the 8p deletion associated with progression of urinary bladder (Wagner, et al., 1997), hepatocellular (Qin, et al., 1999) and prostate cancer (El Gammal, et al., 2010) while 8q gain is associated with progression of prostate (El Gammal, et al., 2010), uveal (Ehlers, et al., 2005) and ovarian cancer (Arnold, et al., 1996). Although the genes responsible for progression on both chromosomes are not known, gene mapping studies have identified several tumour suppressor genes on 8p such as Deleted in Liver Cancer 1 (Xue, et al., 2008), human β-defensin-1 (Sun, et al., 2006) with proto-oncogenes and tumour-promoting genes detected on 8q such as microRNA 151 (Ding, et al., 2010) and Development and Differentiation Enhancing Factor 1 (Ehlers, et al., 2005).

Gatekeeper mutations can also initiate the carcinogenesis pathway for each tumour, and mutations to the APC gene usually occur prior to K-ras mutations and are detected in very early sporadic adenomas (Powell, et al., 1992, Lamlum, et al., 2000). The reverse, however, it true for CAC (Leedham, et al., 2009). Biallelic mutations to the APC gene are often detected in early sporadic adenomas (Jen, et al., 1994, Lamlum, et al., 2000). As APC is widely involved in mitosis, disruption to this gene may cause CIN, which is another feature of CRC. In addition to regulating the Wnt signalling pathway, through the degradation of β-catenin, it assists in late stage mitosis by binding to, and stabilising, microtubules. Mutations to it can lead to genome instability in embryonic stem cells (Kaplan, et al., 2001). Assessment of the loss-of-heterozygosity in adenomas has shown APC truncation with nuclear localisation of β-catenin (Preston, et al., 2003). This highlights the importance of the Wnt signalling pathway in tumour progression as elevated rates of crypt fission are observed as a result of a single germ-line mutation to APC (Wasan, et al., 1998).

However, it is not the mere accumulation of mutations that is necessary for tumour progression (Figure 1.2), but the specific combination of these mutations. For example, a group of malignant adenomas can be segregated according to chromosome alterations into distinct clusters featuring mutually exclusive chromosomal aberrations such as 8q gain with 13q gain compared to 18q loss with 20q gain (Hermsen, et al., 2002).
1.4.1.1 Aberrant crypt foci

The histological sequence of normal healthy colonic mucosa through to adenocarcinoma also includes the development of aberrant crypt foci (ACF) into adenomas followed by the progression to carcinoma (Takayama, et al., 1998). ACF are early colonic abnormalities that are visible on colonoscopic examination by the use of methylene blue staining (Shpitz, et al., 1998, Takayama, et al., 1998, Kukitsu, et al., 2008). Two forms of ACF exist with each differing in their malignant potential. The first, dysplastic ACF, is believed to be a putative precursor of adenomas and thus carcinoma, while the second form, hyperplastic ACF, is thought to have minimal risk of malignancy. ACFs occur in CAC and are associated with the occurrence of dysplasia (Kukitsu, et al., 2008). Colitis-associated ACFs are distinct from sporadic CRC-ACFs under magnifying endoscopy with the ACF boundaries of individual crypts obscured, or indistinct, compared to the clear borders found in sporadic ACFs. Most colitis-ACFs are also distorted in shape in contrast to the round, or oval-shaped, sporadic ACFs (Kukitsu, et al., 2008). Colitis-ACFs are also genetically distinct to the sporadic ACFs with the colitis-ACFs negative for K-ras mutations and p16 expression unlike the sporadic ACFs (Kukitsu, et al., 2008), suggesting a different developmental pathway. As the dysplastic colitis-associated lesions are similar in genetic mutations to colitis-ACFs, it is possible that colitis-associated polyps develop from ACFs and p16 expression is detectable in ACFs with expression levels increasing with disease progression (Dai, et al., 2000).

1.4.1.2 Chromosomal instability and microsatellite instability

Chromosomal instability (CIN) is detected very early on in the adenoma-carcinoma sequence (Shih, et al., 2001) and is associated with a worse disease-free survival in stage II/III patients (Mouradov, et al., 2013). The exact cause of CIN is still unknown and is thought to be associated with genes required in mitosis such as APC (Tighe, et al., 2004) and Aurora A (Meraldi, et al., 2004, Baba, et al., 2009). It can contribute to loss-of-heterozygosity, and in CAC, CIN occurs as an early event as it is detected in nondysplastic tissue (Rabinovitch, et al., 1999, O'Sullivan, et al., 2002); it has been suggested to be a result of telomere shortening, which has an association with CAC (O'Sullivan, et al., 2002).

It should also be noted that the frequency of APC mutations do not correlate with the increase in CIN observed in adenoma-carcinoma progression and thus additional
mutations must be required for accelerating CIN development (Hermsen, et al., 2002). APC mutations are also not essential for tumour progression, as tumours can develop in the presence of wild-type APC when activating β-catenin mutations are present (Mirabelli-Primdahl, et al., 1999).

MSI occurs in both CAC and sporadic CRC and can be detected in the dysplastic colitis-associated lesions as well as in sporadic adenomas (Suzuki, et al., 1994). MSI lesions have a preponderance for the right side of the colon (Suzuki, et al., 1994) and can occur in tissues distant from the site of the cancer or dysplasia in patients with UC. MSI is noted to be an early feature of CAC with a 45% occurrence in cases of indefinite dysplasia (Fujiwara, et al., 2008), which is a histological classification that precedes the onset of early dysplasia.

It was thought that CIN and MSI are independent processes that were mutually exclusive, but recent studies have shown that both can co-exist in the same tumour and may have overlapping signalling pathways (Goel, et al., 2003, Tang, et al., 2004). It has also been demonstrated that tumours, which are both CIN+ and MSI are a biologically distinct form of CRC (Tang, et al., 2004) and this highlights the existence of at least one other mode of CRC tumourigenesis.

In chronic colitis, dysplasia develops from a field of inflammation containing shortened telomeres, DNA damage, and senescence (Risques, et al., 2011). Telomere shortening as a cause of CIN was primarily observed in patients with UC compared to patients with sporadic CRC (Konishi, et al., 2007) and appears to be more pronounced in regions surrounding high-grade dysplasia or cancer (Risques, et al., 2011).

1.4.1.3 CpG-island methylator phenotype

CpG-Island Methylator Phenotype (CIMP) is also an early event in the development of sporadic CRC, occurring in about 50% of sporadic adenomas and carcinomas (Kim, et al., 2005). These CIMP+ tumours are characterised by high levels of CpG island methylation, features that are not found as frequently in CAC (Konishi, et al., 2007). CIMP-associated methylation of MLH1, a mismatch repair protein, also accounts for most of the MSI in sporadic CRC and is associated with V-raf murine sarcoma viral oncogene homolog B (BRAF) mutations as well (Weisenberger, et al., 2006).
### 1.4.1.4 A two-step model for colorectal adenoma progression

The combination of APC and K-Ras mutations promote cell proliferation, contributing to tumour progression and enhancing their invasive potential (Janssen, et al., 2006). These mutations, however, are not sufficient in themselves to explain adenoma or cancer development and it is the activities of K-Ras, v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1), and ras-related C3 botulinum toxin substrate 1 (RAC1) that are essential for nuclear localisation of β-catenin and adenoma progression (Phelps, et al., 2009). As such, a two-step model for colorectal adenoma initiation and progression that depends on β-catenin accumulation in the nucleus has been proposed. Firstly, APC loss stabilises cytoplasmic levels of β-catenin, after which signalling occurs between the K-Ras/RAF1 and β-catenin pathway. Finally, nuclear translation occurs with the aid of RAC1 (Wu, et al., 2008, Phelps, et al., 2009).

A. Colitis-associated colorectal cancer

![Diagram of the two-step model for colitis-associated colorectal cancer]

B. Sporadic colorectal cancer

![Diagram of the two-step model for sporadic colorectal cancer]

**Figure 1.2. Molecular pathogenesis of (A) colitis-associated colorectal cancer and (B) sporadic colorectal cancer.** Both models demonstrate a stepwise development involving the accumulation of genetic mutations at specific developmental stages of the disease. Despite the difference in timing, mutations to similar genes are observed, such as APC, p53, K-ras, and the development of chromosomal instability (CIN).

### 1.4.2 Differences in colorectal cancer according to subsite

Differences between colorectal lesions in the right and left colon have been observed and include the preponderance of sporadic CRC and MSI-high tumours in the right colon
compared to CAC and CIMP+ tumours which tend to be more distally located in the left colon (Tang, et al., 2004, Weisenberger, et al., 2006, Nawa, et al., 2008). Advanced tumours are also more commonly detected in the right colon (Nawa, et al., 2008, Brenner, et al., 2010).

Other studies also indicate the occurrence of a more complex scenario, where disease severity and genetic mutations differ according to the multiple colonic subsites such as the rectum, rectosigmoid, sigmoid colon, descending colon, splenic flexure, transverse colon, hepatic flexure, ascending colon and caecum. For example, upon combining tumour databases for two prospective cohorts, it was observed that the frequency of CIMP-high, MSI-high and BRAF mutations increased in a linear manner from rectum to the ascending colon, suggesting a gradual change in the molecular characteristics in the colon (Yamauchi, et al., 2012). A separate study also demonstrated that disease severity was better characterised according to the colonic subsites with the highest proportion of stage III and IV CRCs developing in the caecum and the splenic flexure, whilst the ascending and descending colon exhibited the lowest proportions (Benedix, et al., 2011). These studies challenge the current view of left and right lesions being distinct entities but instead identify the splenic flexure as a discrete location (Iacopetta, 2002).

Not only do genetic mutations differ according to the site, the risk of developing a metachronous cancer differs. According to one study, the risk of developing a second primary cancer is increased in individuals whose first primary CRC occurred in the transverse colon and descending colon (Phipps, et al., 2013).

The presence of multiple pathways for tumour initiation and progression highlights the complexity of CRC. If tumour initiation is indeed a more superior predictor of disease severity than disease progression, then the factors that influence tumour initiation will be necessary for predicting patient prognosis.

1.5 CANCER IMMUNOEDITING

The term “cancer immunoediting” was proposed after the discovery that the immune system not only protected the host from tumours, but could also promote tumour growth via immunoselection (Dunn, et al., 2002). This observation was made after transplanting tumour cells from either immunocompetent, or immunodeficient, mice into naïve wild-type mice. It was observed that all the transplants involving immunocompetent-derived tumours cells progressed while only half of the transplants with immunodeficient-derived
tumour cells progressed. This led to the conclusion that tumours growing in mice with a immune system were less immunogenic, and were classified as “edited”, while tumours grown in immunodeficient mice were more immunogenic, and were classified as “unedited” (Shankaran, et al., 2001).

The cancer immunoediting hypothesis was postulated to have three sequential phases, namely the elimination, equilibration and escape phase (Dunn, et al., 2002).

1.5.1 Elimination

The elimination of tumours is achieved through a cooperation of both the innate and adaptive immune systems. This process is not fully understood and examples of currently known triggers for this process include interferons and alarmins (Lotfi, et al., 2007).

1.5.1.1 Interferons

Interferons (IFNs) were originally identified by their effects against viral infections (Muller, et al., 1994, Dunn, et al., 2006) and are classified based on structure as either type I or II. Type I IFNs, IFNα and IFNβ, are frequently associated with an antiviral response and recent studies have demonstrated roles for them in cancer immunosurveillance (Dunn, et al., 2006). For example, mice deficient in IFNα receptor 1, which recognises both IFNα and IFNβ, experienced a higher prostate tumour burden when compared to their wild-type counterparts and the tumour did not respond to polyinosinic-polycytidylic acid, a TLR3 activator, which induces type I IFN and IFN-inducible genes (Chin, et al., 2010). Tumour suppression, however, was observed in another model of prostate cancer and the inhibition of angiogenesis is associated with IFNα activity (Persano, et al., 2009). It also appears that IFNα and IFNβ are required to enhance CD8α⁺ dendritic cell (DC) mediated cross-priming of the adaptive response, which is essential in tumour rejection (Diamond, et al., 2011).

IFNγ is a type II IFN and is primarily expressed by natural killer (NK) cells, natural killer T cells, CD4⁺ and CD8⁺ cells (reviewed by Schoenborn, et al., 2007). It has a broad role in cancer immunosurveillance and is under-expressed in the tumours of patients with cervical cancer (Gey, et al., 2003), in the peripheral blood from patients with prostate (Tahir, et al., 2001) and CRC (Heriot, et al., 2000).

IFNγ reduces FoxP3 expression whilst inducing the expression of cytotoxic T-lymphocyte antigen 4, which leads to the in vitro inhibition of induced-T-regulatory cells
and, therefore, reduces immunosuppression (Oh, et al., 2011). IFNγ also increases major histocompatibility complex (MHC) class I molecule expression by inducing the gene transporter for antigen presentation (Tap)-2, which is involved in the transportation of MHC class I peptides into the endoplasmic reticulum for presentation to T-cells (Martini, et al., 2010).

In a mouse model of gastric cancer, IFNγ induces autophagy in gastric epithelial cells, inhibits epithelial cell apoptosis, normalises cell proliferation and reduces double cortin and CaM kinase-like-1 (DCLK-1)+ putative progenitor cell expansion, reducing inflammation and the risk for gastric cancer (Tu, et al., 2011). In a mouse xenograft model for human prostate cancer, inducing IFNγ expression was also able to suppress tumour growth by about 52%, compared to untreated animals (Zhao, et al., 2007).

1.5.1.2 Alarmins

Alarmins, also known as danger-associated molecular patterns (DAMPs), are endogenous molecules that are released by dying, stressed or injured cells to alert the adaptive immune system of infection, or other pathological processes (Bianchi, 2007, Kono, et al., 2008, Krysko, et al., 2012).

The concept of alarmins was demonstrated in vitro when resting DCs up-regulated MHC and costimulatory factors in the presence of necrotic, but not normal or apoptotic cells (Gallucci, et al., 1999). To further demonstrate the immunogenicity of necrotic cells, mice were injected with ovalbumin (OVA) mixed with either apoptotic cells, two types of necrotic cells (frozen-thawed cells and cells from damaged blood vessels), IFNα or complete Freund’s adjuvant. A week following the challenge, the T-cell response was measured and it was observed that both types of necrotic cells and IFNα were effective adjuvants for the primary anti-OVA delayed-type hypersensitivity reaction, although not as potent as complete Freund’s adjuvant. Apoptotic cells, on the other hand, did not result in the efficient priming of T-cells (Gallucci, et al., 1999). This demonstrated that necrotic cells were able to activate both the innate and adaptive immune system.

Several molecules that have been identified as alarmins include high mobility group box-1 and uric acid.
1.5.1.2.1 High mobility group box-1

High Mobility Group Box-1 (HMGB1) is a nuclear protein that binds loosely to chromatin during interphase and mitosis parts of the cell cycle, and dissociates readily into the medium when the cell membrane integrity is lost during necrosis (Degryse, et al., 2001, Scaffidi, et al., 2002). It induces cell migration, changes in cell shape and cytoskeleton reorganisation (Degryse, et al., 2001), while binding of HMGB1 to chromatin is highly dependent on cell viability which is a distinguishing feature between necrotic and apoptotic cells. It also independently triggers an inflammatory response in the absence of HMGB1 and anti-HMGB1 antibodies are noted to result in reduced inflammation in the liver (Scaffidi, et al., 2002).

1.5.1.2.2 Uric acid

Uric acid is a by-product of the purine synthesis pathway and is sequestered within the cell as a soluble chemical (Becker, 1993, Behrens, et al., 2008). Upon exposure to the extracellular environment, uric acid crystallises into bioactive monosodium urate (Bianchi, 2007, Behrens, et al., 2008). Then it can activate DCs (Kool, et al., 2011) and mediate a T-helper (Th) type 2 response (Gasse, et al., 2009, Kool, et al., 2011). It mediates bleomycin-induced inflammation of the lung (Gasse, et al., 2009); and in inflamed airways of allergen-challenged asthma patients (Kool, et al., 2011). It increases the risk for kidney disease (Obermayr, et al., 2008) and, conversely, induces tumour regression in mice (Hu, et al., 2004, Behrens, et al., 2008).

1.5.2 Equilibrium

The equilibrium stage of immunoediting occurs when variants of tumours cells that have survived the elimination stage are placed under selection pressure by the adaptive immune system. This prevents the vast majority of tumour outgrowths but this ‘Darwinian’ selection also allows for the creation of new tumour cell variants. This stage in immunoediting is thought to be the longest and may persist for years (Dunn, et al., 2002, Schreiber, et al., 2011).

The way the equilibrium phase works can be demonstrated by the observation of two renal transplant patients who succumbed to secondary melanoma 1 to 2 years after their organ transplants despite having no prior history of melanoma (MacKie, et al., 2003). Via the transplant registry it was noted that the kidneys originated from the same donor who
had died from a presumed subarachnoid haemorrhage. The donor had, however, been registered to a melanoma group following the excision of a primary melanoma, but was declared tumour-free the year before she died. One of the kidney recipients died from metastatic melanoma shortly after diagnosis, while the other survived following an induced rejection of the donated kidney. Both of the donated kidneys featured melanoma nodules. It should be noted that both individuals were immunosuppressed, which was reversed upon the diagnosis of secondary melanoma. These cases suggest that nascent melanoma cells were present in the kidneys and proliferated in the recipients in the presence of a suppressed immune system.

Equilibrium has also been demonstrated in a mouse model of chemically-induced sarcoma by 3’-methylcholanthrene (MCA) (Koebel, et al., 2007). Low doses of MCA were injected subcutaneously and mice were monitored for tumours. Animals with growing sarcomas were removed from the experiment and mice displaying small stable tumour masses were treated with either control monoclonal antibodies, or monoclonal antibodies against specific immunological components. When animals were treated with antibodies against the adaptive immune system, such as anti-CD4, anti-CD8, anti-IFNγ or anti-IL-12p40 (which induces IFNγ production), in 46% of the mice the sarcomas grew. Mice treated with monoclonal antibodies against NK cells, however, did not have tumour growth. The suggestion is that the adaptive immune system is crucial in maintaining the tumour cells at equilibrium (Koebel, et al., 2007). In order to determine if the stable tumour masses contained transformed tumour cells that could proliferate, these cells were transplanted into immunodeficient Rag2 knockout mice. This resulted in the formation of tumours, suggesting that transformed cells were prevented from proliferating by the adaptive immune response in the intact host.

Tumour cells that were maintained in equilibrium in anti-CD4 and anti-CD8-treated mice were also injected into Rag2-knockout mice and wild-type mice and tumours formed and grew in the immunodeficient animals. Between 31 and 50% of tumours from the treated animals, however, were rejected in wild-type mice, suggesting that the cells that were held in equilibrium had an unedited phenotype (Koebel, et al., 2007).

1.5.3 Escape

The escape phase occurs when tumour cell proliferation has overwhelmed the immune system and a tumour mass develops. There are several explanations as to why this occurs,
one being the induction of an immunosuppressive state through cytokines such as indoleamine 2, 3-dioxygenase (IDO), IL-10, and macrophage migration inhibitory factor (MIF), which promote tumour escape by dampening the anti-tumour response.

1.5.3.1 Indoleamine 2,3-dioxygenase

IDO is an enzyme produced by DCs and macrophages (Hwu, et al., 2000, Popov, et al., 2006) that catalyses tryptophan degradation to kynurenine and enhanced tryptophan degradation is associated with a poor prognosis (Brandacher, et al., 2006). It is over-expressed in most tumours (Uyttenhove, et al., 2003) and correlates with a poor prognosis in endometrial cancer (Ino, et al., 2008) and CRC (Brandacher, et al., 2006). Typtophan-shortage and the production of kynurenine signals T-regulatory cell generation (Fallarino, et al., 2006) but then restricts antigen-induced T-cell activation as these cells have a tryptophan-sensitive checkpoint which halts their cell-cycle in the G1 phase when tryptophan level is below 0.5-1 µM (Munn, et al., 1999). Tryptophan catabolites specifically inhibit, in a concentration-dependent manner, the proliferation of CD4⁺, CD8⁺ T-lymphocytes, and NK cells, but not B lymphocytes (Frumento, et al., 2002).

The combined effects of tryptophan depletion and tryptophan catabolites down-regulate CD3ζ (Frumento, et al., 2002, Fallarino, et al., 2006), which is a signalling molecule of the MHC class II complex (Krummel, et al., 2000). Plasmacytoid DCs have been stimulated to produce IDO, which leads to T-cell suppression in tumour-draining lymph nodes (Sharma, et al., 2007, Muller, et al., 2008). IDO induces T-regulatory (T-reg) cell expansion from CD4⁺ cells, activates resting T-reg cells (Sharma, et al., 2007, Sharma, et al., 2009) and inhibits the proliferation of NK cells and tumour-infiltrating lymphocytes (Brandacher, et al., 2006, Ino, et al., 2008, Spaggiari, et al., 2008). Its expression is induced by IFNα (Muller, et al., 2008) and IFNγ (Brandacher, et al., 2006, Muller, et al., 2008) and is under the genetic control of the tumour suppressor gene, bridging integrator 1 (Muller, et al., 2005). IDO expression is thus a potential target for pharmaceutical intervention and the competitive inhibitor, 1-methyl-tryptophan, may lead to slower tumour progression in mice compared to untreated controls (Uyttenhove, et al., 2003). As of March 2016, there are several ongoing clinical trials involving IDO inhibitor drugs and although no final results have been published, these studies will demonstrate the efficacy of these drugs in enhancing immune responses and improving patient prognosis (Reviewed by Munn, et al., 2016).
1.5.3.2 IL-10

IL-10 is produced by cells of the innate and adaptive immune system including macrophages, DCs (Jarnicki, et al., 2006), T-cells (Sharma, et al., 1999) and B-cells (Fillatreau, et al., 2002) and it inhibits the production of Th-type 1 cell cytokines following priming by antigen presenting cells. It may also reduce the capacity of DCs to present antigens (Sharma, et al., 1999, Larmonier, et al., 2007). IL-10 inhibits the production of IFNγ by lymphocytes (D'Andrea, et al., 1993), proinflammatory cytokines by polymorphonuclear leucocytes (Cassatella, et al., 1993) and decreases the number of CD4+ and CD8+ T-cells in a mouse model of lung cancer (Jarnicki, et al., 2006). It is known to down-regulate MHC class I expression and inhibit TAP-1 and 2 expression and function (Salazar-Onfray, et al., 1997).

Mice deficient in IL-10 developed colitis in specific pathogen-free conditions which progresses to dysplasia and carcinoma following colonisation with gut microbe, Enterococcus faecalis (Sellon, et al., 1998, Balish, et al., 2002). This demonstrates a role for IL-10 in maintaining homeostasis between the intestinal immune system and gut microbes, and IL-10-based therapy has been suggested reducing inflammation in IBD patients (Braat, et al., 2003).

On the other hand, transgenic mice over-expressing IL-10 had a high tumour burden compared to controls and treating them with anti-IL-10 antibodies restored anti-tumour responses (Sharma, et al., 1999), while targeting IL-10 receptor-blocking by using oligonucleotides aptamers led to the rejection of 20% of implanted tumours (Berezhnoy, et al., 2012).

Overexpression of IL-10 is associated with various cancers in humans such as ovarian cancer (Gotlieb, et al., 1992), CRC (Galizia, et al., 2002), metastatic renal cell carcinoma (Wittke, et al., 1999), and non-small-cell lung cancer (NSCLC) (Hatanaka, et al., 2000). Polymorphisms in the IL-10 promoter have also been associated with a reduction in the risk of breast cancer in humans (Langsenlehner, et al., 2005).

Taken together, these studies suggest that despite being required for maintaining gut homeostasis, the usage of IL-10 to treat IBD patients may promote tumourigenesis.
1.5.3.3 Macrophage migration inhibitory factor

MIF is secreted by the pituitary (Calandra, et al., 1994) and macrophages (Calandra, et al., 1994). It is regulated by microRNA-451 and there is an inverse relationship between MIF expression and microRNA-451 in primary gastric cancer (Bandres, et al., 2009). It impairs the release of cytolytic perforin granules from NK cells by down-regulating the NK receptor, NKG2D (Apte, et al., 1998, Krockenberger, et al., 2008), and it also plays a role in tumour-associated macrophage polarisation and promotes an immunosuppressive phenotype in mice (Yaddanapudi, et al., 2013).

Both the loss and inhibition of MIF in mice can lead to a reduction in subcutaneous and metastatic melanoma growth and progression (Yaddanapudi, et al., 2013). Heat-shock protein (HSP) 90 is a chaperone for MIF and HSP90-inhibitors destabilises MIF thereby reducing its tumour-promoting properties both in vivo and in vitro in mice with breast cancer (Schulz, et al., 2012). High levels are also found in human prostate cancer and it is a predictor of a poor prognosis (Hussain, et al., 2013).

Inhibiting MIF-function through the use of anti-MIF antibodies abrogates the in vitro MIF-dependent proliferation and survival signalling and it favours programmed cell death and suppresses in vivo growth of human prostate cancer cell xenografts in mice (Hussain, et al., 2013).

1.5.3.4 Programmed cell death-1

Programmed cell death (PD)-1 is a co-inhibitory molecule and it regulates T-cell activation, peripheral tolerance, and the prevention of bystander tissue damage during immune responses (Reviewed by Sznol, et al., 2013). Activation of the PD-1 pathway can also promote and co-stimulate proliferative responses of T-cells and the preferential expression of IL-10 by T-cells (Dong, et al., 1999).

PD-1 is expressed on activated CD4+ and CD8+ T-cells, natural killer T cells, B cells, and activated monocytes and DCs, and has two ligands, PD-ligand (L) 1 and 2 (Wang, et al., 2008). The expression of PD-L1 and not PD-L2 on DCs was required for the induction of Foxp3+ adaptive T-regulatory (T-reg) cells (Wang, et al., 2008), and tumour-associated cells expressing both PD-1 and PD-L1 can escape from an anti-tumour immune response and this has been termed adaptive immune resistance (reviewed by Sznol, et al., 2013).
PD-1 and PD-L1 expression were associated with poor patient survival in gastric cancer (Wu, et al., 2006), lung cancer (Mu, et al., 2011), hepatocellular carcinoma (Gao, et al., 2009), and PD-1 expression was associated with high-risk renal cell carcinomas in patients (Thompson, et al., 2007). As such, many studies have targeted the PD-1 and PD-L1 pathway as a form of cancer therapy.

For example, following a phase I trial of the anti-PD-1 antibody BMS-936558 in patients with treatment-refractory solid tumours, a long-term follow-up study was performed on three patients with CRC, renal cell cancer, and melanoma (Lipson, et al., 2013). The CRC patient showed a partial response to a single dose of anti-PD-1 and 3 years after receiving therapy, the patient did not show evident of disease recurrence. In the renal cell cancer patient, a pancreatic lesion and bone metastases slowly resolved and 4 years since stopping anti-PD-1 treatment, the patient remains in remission as of radiologic evaluation. On the other hand, the patient diagnosed with metastatic melanoma experienced a disease recurrence and the anti-PD-1 treatment had to be reinduced. Following treatment, CT scans showed a 40% tumour reduction. This study demonstrates that the PD-1 pathway is a potential target for tumour immunotherapy.

However, there are diseases where the expression of either PD-1 or PD-L1 are associated with better patient prognosis. For example, PD-L1 was detected in melanomas and was positively associated with improved overall survival in patients with PD-L1+ metastatic lesions (Taube, et al., 2015). Similarly, a high expression of PD-L1 was correlated with a good prognosis in CRC (Droeser, et al., 2013). In human papilloma virus-positive head and neck cancer, the expression of PD-1 on infiltrating T-cells are associated with better overall survival (Badoual, et al., 2013).

This paradoxical association might be because PD-1 and PD-L1 expressions were not oncogene driven and might represent surrogate markers of endogenous anti-tumour immune response (Badoual, et al., 2013).

In addition, an anti-tumour response can be achieved by blocking immune inhibitory pathways through both PD-1 and the immune regulatory checkpoint protein, cytotoxic T-lymphocyte antigen 4 (CTLA-4). For example, the dual blocking of PD-1 and CTLA-4 combined with tumour vaccine resulted in the reversal of CD8+ tumour-infiltrating leucocyte and tumour rejection in a murine model of tumour implants (Duraiswamy, et al., 2013).
1.6 TUMOUR MICROENVIRONMENT

Tumours are heterogeneous multicellular tissues and tumours of epithelial origin consists of tumour epithelial cells, and components of the tumour stroma, such as the extracellular matrix (ECM), fibroblasts and non-structural matrix components, immune cells, and the blood and lymphatic vessel network. Its constitution resembles that of granulation tissue during wound healing and has been described as a wound that never heals (Dvorak, 1986). Tumour epithelial cells and components of the tumour stroma interact with one another, and it is the reciprocal communication that contributes to the development and phenotype of the tumour. There is also evidence that in some instances, mutations within the stroma precede those in the epithelial cells, and that the hyper-proliferation of ‘healthy’ epithelial cells encouraged by the stroma (reviewed by Tlsty, et al., 2001). However, a study on cancer-associated fibroblasts derived from breast and ovarian cancer demonstrated extremely rare loss of heterozygosity and copy number alterations, suggesting that mutations within the stroma might not promote carcinoma (Qiu, et al., 2008). The constituents of the tumour microenvironment and how they relate to tumour development will be discussed in brief below.

1.6.1 Extracellular matrix

The ECM provides structural support to solid tumours and is involved in the transmission of signals through cell adhesion receptors. It consists of various components such as matricellular proteins, fibrillar and non-fibrillar collagens. This varies between tissues, and can change through the regulation of extracellular degradation and synthesis as a response to signals during development, wound healing, and pathological processes. ECM degradation is performed by matrix metalloproteinases (MMPs) whilst synthesis is mediated by fibroblasts, which produce ECM components such as collagen and fibronectin. Brief descriptions of MMPs and fibroblasts including their roles in tumourigenesis will be provided below.

1.6.1.1 Matrix metalloproteinases

MMPs are enzymes that cleave components of the ECM and there are at least 23 MMPs, and their inhibitors are termed tissue of metalloproteinases (TIMPs). Both classes of enzymes are involved in remodelling tumour ECM (reviewed by Nagase, et al., 2006).
For example, MMPs-2 and 9, also known as type IV collagenases as they specifically degrade type IV collagens, can be inhibited by TIMP 2 and 1 respectively (Liabakk, et al., 1996; Avolio, et al., 2005). As type IV collagens are the main components of tissue basement membrane, they represent the first barrier in tumour invasion and increased expression of MMPs 2 and 9 are often associated with malignancy and worse survival rates (Liabakk, et al., 1996). MMP-2 and 9 are localised to the stromal compartments of colorectal neoplasias (Poulsom, et al., 1992), and they have been associated with increased expression with malignancy and worse survival rates (Schmalfeldt, et al., 2001, Langers, et al., 2012). MMP-2 and 9 levels correlate with levels of vascular endothelial growth factor (VEGF) A in CRC and gastric carcinoma (Zheng, et al., 2006). They appear to regulate VEGF-A expression in vivo (Belotti, et al., 2003, Hawinkels, et al., 2008), possibly by mediating its release into the microenvironment (Hawinkels, et al., 2008). In human prostate cancer, the expression of MMPs 2, 9, TIMP 1, and 2 are associated with disease progression, with increased MMPs and decreased amounts of both TIMPs reflecting advanced prostate cancer (Wood, et al., 1997).

MMP-1, also known as collagenase 1, degrades type I, II and III collagen (Ohuchi, et al., 1997). Its expression is negatively associated with poor prognosis in CRC and oesophageal cancer (Murray, et al., 1996, Murray, et al., 1998). In CRC patients, MMPs 1, 3 and TIMP-1 expression levels are higher in colorectal tumours compared to normal colorectal tissue, whilst TIMP-2 expression was greater in normal than in colorectal tumours. Total MMP activity was also higher in tumour tissues than control colon tissues (Baker, et al., 2000).

1.6.1.2 Tumour-associated fibroblasts

Fibroblasts are primarily responsible for ECM remodelling and have roles in promoting cellular proliferation, survival and apoptosis (Evan, et al., 1992, Crawford, et al., 2009). For example, stroma tissues were compared between normal, neoplastic, and cancerous prostates, and a reactive stroma, as characterised by the presence of fibroblasts and the differentiated myofibroblasts, was detected in neoplastic tissues (Tuxhorn, et al., 2002). These activated cells release ECM components and appear to contribute to the development of a cancer microenvironment.

Fibroblasts extracted from invasive human mammary ductal carcinomas appear to consist of mostly myofibroblasts, and have demonstrated a retention of pro-tumourigenic
properties following ten passages as pure cultures. This suggests that their tumourigenic properties are independent of interactions with carcinoma cells (Orimo, et al., 2005).

It is interesting to note that tumour development from admixing carcinoma cells with normal fibroblasts is quicker than carcinoma cells alone, although slower than carcinoma cells with tumour-associated fibroblasts (TAFs), suggesting that a pro-tumourigenic phenotype has been acquired. It was determined that this phenotype can be induced by activating the nuclear factor (NF)-κB pathway via IL-1β (Erez, et al., 2010).

In mice, TAFs isolated from lymphomas refractory to anti-VEGF-A treatment have been demonstrated to up-regulate platelet-derived growth factor (PDGF)-C expression, which works in concert with free VEGF-A to promote angiogenesis via enhanced endothelial cell growth (Crawford, et al., 2009). It was also demonstrated that by blocking VEGF-A and PDGF-C via antibodies, the growth of anti-VEGF-A refractory tumours can be suppressed. TAFs, therefore, play a crucial role in tumourigenesis by providing chemokines and cytokines.

### 1.6.2 Tumour infiltrating leucocytes

Leucocytes are present in early stage adenomas and are located in the stroma or within the epithelial layer within a CRC tumour. They consist mostly of T-cells and macrophages, along with DCs, neutrophils and killer cells. Associations have been made between the presence of infiltrating leucocytes and progression to dysplasia (Risques, et al., 2011), and examples of leucocytes and their role in tumourigenesis will be provided below.

#### 1.6.2.1 T-cells

T-cells are part of the cell-mediated immune response and are activated following cross-presentation of tumour antigens by antigen presenting cells. To date, sensitised T-cells are known to differentiate into helper, cytotoxic, or regulatory cells and are distinguishable by the expression of unique cell surface antigens and cytokines.

T helper (Th) cells are CD4+ cells and can be of the Th1, 2, 17, or 22 subtypes. Th1 cells typically drive the cell-mediated immune response and the production of IgG2a whilst Th2 cells induce allergy dominated IgE antibody-mediated responses (Steinman, 2007). Peripheral blood cells from renal cell carcinoma or melanoma patients with active disease were found to have a dominant Th2 phenotype while recovered patients had a balance of
Th1/Th2, and normal blood donors had a strong Th1-polarised immunity (Tatsumi, et al., 2002). This suggests that a Th2 subtype is associated with tumour development.

Th17 cells are characterised by their ability to secrete IL-17, and in an extensive study involving ovarian tumour samples, Th17 cells appear to be induced by tumour-associated macrophages (TAMs) via IL-1β secretion. Similarly, IL-1β is expressed by intestinal macrophages in response to gut microflora and polarises T-cells into Th17 cell through the IL-1 receptor (Shaw, et al., 2012). Th17 cells appear to recruit Th1-type effector cells and NK cells into the tumour microenvironment by inducing Th1-type chemokine production. IL-17 levels in tumour ascites also positively correlate with patient survival (Kryczek, et al., 2009). The activation of CD8+ cytotoxic T-cells (CTLs) requires that both CTLs and Th cells recognise antigens presented on the same antigen presenting cell (Schoenberger, et al., 1998) and the resulting antigen-specific CTLs are more effective at killing tumour cells (Celluzzi, et al., 1996).

In a study involving tumour samples from CRC patients, a panel of genes related to Th1, 2, 17 and CTL were analysed in relation to prognosis and results suggest that patients expressing high levels of Th17 genes had a worse prognosis whereas high levels of Th1 genes predicted prolonged disease-free survival. A combination of CTL, Th1 and 2 genes also improved the ability to discriminate relapse (Tosolini, et al., 2011). In addition, a study involving CRC tumour samples from patients suggested that Th17 cells might increase the metastatic ability of tumour cells and might be a strong predictor of prognosis in later stage cancer (Yoshida, et al., 2016).

Th22 cells have been characterised as CD4-positive cells that express IL-22 and not IFN-γ and IL-17, or co-express chemokine receptors 4, 6, and 10 (Page, et al., 2014, Huang, et al., 2015). They have been associated with progression and poor survival outcome in gastric cancer (Zhuang, et al., 2012), pancreatic ductal adenocarcinoma (Niccolai, et al., 2016), multiple myeloma (Di Lullo, et al., 2015), and colon cancer progression in mice (Duramad, et al., 2004). Furthermore, IL-22 inhibited apoptosis in colon cancer cell culture (Huang, et al., 2015), dexamethasone-induced apoptosis in multiple myeloid cell cultures (Di Lullo, et al., 2015), and IFN-γ-induced apoptosis in pancreatic ductal adenocarcinomas (Niccolai, et al., 2016).

T-reg cells are typically identified by the co-expression of CD4, CD25 and Foxp3, and are important in the maintenance of tolerance to self-antigens (Chen, et al., 2005). They are stimulated by the expression of TGF-β and also produce suppressor cytokines like IL-
T-reg cells also deprive the microenvironment of IL-2, which is essential to Foxp3+ CD4+ effector T-cells, resulting in the apoptosis of effector T-cells (Pandiyan, et al., 2007).

Clinically, higher numbers of Foxp3+ cells are located in metastatic lymph nodes compared to non-metastatic lymph nodes in patients with cervical cancer (Nakamura, et al., 2007). Patients with hepatocellular carcinoma have increased numbers of periphery CD4+, CD25+ T-reg cells and this level correlates with disease progression (Fu, et al., 2007). Also, more Foxp3+ cells are located in the tumour stroma of advanced cervical cancer compared to early stage cancer (Nakamura, et al., 2007). It has also been shown that high levels of CD4+ and CD25+ cells can be detected in peripheral blood from patients with malignant epithelial tumours and that these cells inhibit NK-cell-mediated cytotoxicity (Wolf, et al., 2003). The depletion of T-reg cells also promotes tumour rejection or the inhibition of tumour growth in murine models of cancer (Onizuka, et al., 1999).

1.6.2.2 Macrophages

Macrophages can differentiate into either M1 or M2 types. M1 macrophages, also known as classical macrophages, are typically stimulated by microbial products and express a pro-inflammatory and anti-tumour phenotype, whereas M2 macrophages tend to express an anti-inflammatory phenotype and encourage tumour growth by enhancing cell proliferation and angiogenesis.

Examples of the anti-tumour properties of M1 macrophages include the spontaneous rejection of metastatic mammary carcinoma (Sinha, et al., 2005), and patients with extended survival post-surgical resection of NSCLC had more M1 than M2 macrophages within tumour islets (Ohri, et al., 2009).

TAMs derive from circulating blood monocytes and are drawn to tumours along concentration gradients of the monocyte chemoattractant protein (MCP)-1 (Fujimoto, et al., 2009). These monocytes then differentiate into TAMs upon stimulation with VEGF-A and macrophage colony-stimulating factor (M-CSF). TAMs are a subtype of M2 macrophage and they secrete immunosuppressive cytokines like IL-10 and TGF-β, and also IDO, and MMPs.

Stimulation of TAMs isolated from fibrosarcomas with lipopolysaccharide (LPS) resulted in the expression an M2 phenotype characterised by high levels of IL-10 and low levels
of IL-12 (Biswas, et al., 2006). A separate in vivo study co-cultured macrophages with ovarian or breast cancer cell lines and observed activation of the c-Jun N-terminal kinase (JNK) and NF-κB pathways in tumour cells, resulting in enhanced invasiveness (Hagemann, et al., 2005). Macrophages can also stimulate angiogenesis through the production of proangiogenic factors like TNFα (Leibovich, et al., 1987) and hypoxia-inducible factor (HIF)-1α (Doedens, et al., 2010). Macrophage-derived HIF-1α also correlates with suppressed CTL responsiveness in a murine model of mammary carcinoma (Doedens, et al., 2010).

High numbers of TAMs positively correlated with the tumour stage, the depth of tumour invasion and detection of metastases in the draining lymph nodes (Kang, et al., 2010). By contrast, TAMs positively correlated with relapse-free survival in human breast cancer patients (Fujimoto, et al., 2009). These contradictory results may signal that TAMs have pleiotropic roles.

1.6.2.3 Dendritic cells

DCs function as activators of the adaptive immune response by sensitising T-cells following acquisition of their immunostimulatory abilities through maturation (Regnault, et al., 1999, Beyth, et al., 2005). DCs that mature in the presence of IL-10, a Th2 cytokine, have a reduced capacity to induce a Th1-type response (De Smedt, et al., 1997). Immature DCs have immunosuppressive activity and the injection of immature DCs inhibited CTL activity in healthy subjects, and increased the production of anti-inflammatory IL-10 by CD8⁺ CTLs (Dhodapkar, et al., 2001).

In human breast adenocarcinoma, immature DCs localised within the tumour while mature DCs were found in the peritumoural regions (Bell, et al., 1999). DC maturation can be inhibited by VEGF-A, which is produced by cancer cells and is often detected in the serum of cancer patients (Gabrilovich, et al., 1996). Heme oxygenase-1 is expressed during inflammation and also inhibited DC maturation (Chauveau, et al., 2005).

Due to the antigen-presentation abilities of mature DCs, there has been a lot of interest in immunotherapy involving sensitising DCs to tumour antigens. It has been demonstrated that efficient antigen presentation is maintained by granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-4 (Sallusto, et al., 1994). Sipuleucel-T is an immunotherapy involving DCs loaded with prostatic acid phosphatase linked to GM-CSF. In a phase I/II trial, the indicator of prostate cancer, the prostate-specific antigen (PSA),
declined in some patients by up to 50%, and disease progression slowed with increasing doses of the treatment (Small, et al., 2000). The phase III trial that led to a United States Food and Drug Administration (FDA) approval involved 512 patients with metastatic prostate cancer and sipuleucel-T achieved a 38% increase in survival at 3 years (Kantoff, et al., 2010).

1.6.2.4 Neutrophils

Neutrophils are the most abundant leucocyte in the body and they polarise into either N1 or N2 neutrophils, the former displaying anti-tumour properties and the latter promoting tumour growth. In cancer, neutrophils are often associated with disease progression and a worse prognosis. For example, in tumours from hepatocellular carcinoma patients, the quantity of peritumoural neutrophils inversely correlated with patient survival and was an independent predictor of disease relapse-free and overall survival (Kuang, et al., 2011). Similarly, a high neutrophil-to-lymphocyte ratio in human small-cell lung cancer was associated with poorer overall survival and progression-free survival (Kang, et al., 2014).

It was also reported that tumour-associated neutrophils can be recruited by IL-17-producing cells and can be activated by tumour-derived hyaluronan, in vitro (Wu, et al., 2011). Increases in the neutrophilic infiltrate is also associated with increases in MMP-9 and VEGF-A (Kuang, et al., 2011), which may promote tumour progression. A relative increase in neutrophils compared to lymphocytes is also associated with advanced stages of CRC (Satomi, et al., 1995). In addition, in a review of one hundred studies, the ratios of neutrophils to lymphocytes were measured as a predictor of overall survival in diseases with solid tumours such as pancreatic, hepatocellular, and colorectal cancer, and a high neutrophil-to-lymphocyte ratio was associated with poor survival outcome (Templeton, et al., 2014).

1.6.2.5 Natural killer cells

NK cells are large granulocytes with cytotoxic and cytokine-producing effector functions (Vivier, et al., 2008). Transplanted tumour cells progressed in the absence of NK cells, whereas the absence of T-cells led to tumour regression (Diefenbach, et al., 2001), suggesting that NK cells are essential in tumour immnosurveillance.

NK cells can exhibit cytotoxic effects via apoptosis through several effects like perforin, which creates pores in cells, granzymes which enters target cells and induces apoptosis via cleavage of caspases (Goping, et al., 2003), or the expression of TNF-related
apoptosis-inducing ligand (TRAIL) which has anti-metastatic properties in an _in vitro_ model of liver metastasis (Takeda, _et al._, 2001).

NK cells are activated by IL-2 and in a follow-up study of patients with metastatic disease, all of whom received either a combination of IL-2 and NK cells or IL-2 alone, some patients on combined therapy experienced partial to complete regression of their diseases, such as metastatic CRC and melanoma (Rosenberg, _et al._, 1987), demonstrating the anti-tumour effects of activated NK cells.

**1.6.3 Tumour vascularisation**

Angiogenesis is the process of blood vessel formation from the pre-existing vasculature via the sprouting of vascular endothelial capillaries followed by microvascular growth and fusion into a mature and functional vascular bed (Less, _et al._, 1992). This process is dependent on associations between endothelial cells, pericytes, stromal cells, the ECM, and the basement membrane.

The balance of angiogenic activators, such as VEGF-A, fibroblast growth factor (FGF), and PDGF, and inhibitors like angiostatin, thrombospondin and endostatin determines angiogenesis in steady-state conditions. In pathological conditions such as tumourigenesis, the balance is shifted towards angiogenesis and is widely accepted as the rate-limiting step in tumour growth.

The diffusion limit for oxygen through tissue is between 100 μm to 200 μm (Hoeben, _et al._, 2004), therefore, tumours that develop beyond those limits will require a vascular system. Progression beyond the limits of oxygen diffusion will result in hypoxia, stabilising HIF-1 (Minet, _et al._, 2000), which then translocates into the nucleus and assists in the transcription of _VEGF-A_ and glucose transporter 1 (Gleadle, _et al._, 1997). The products of these genes contribute to tumour progression, and hypoxia and HIF-1 are associated with metastasis and invasion of human oesophageal squamous cell carcinoma and in _in vitro_ experiments (Krishnamachary, _et al._, 2003, Pàez-Ribes, _et al._, 2009). HIF-1 expression can also occur very early in carcinogenesis (Zhong, _et al._, 1999).

A hypoxic tumour microenvironment triggers an angiogenic response pathway such as the PI3K/Akt signalling pathway, which results in the translation of HIF-1α (Mazure, _et al._, 1997), or the production of peroxisome-proliferator-activated receptor-γ co-activator-1α (PGC-1α) which then activates VEGF-A (Tang, _et al._, 2004). HIF-1α enhances VEGF-A-dependent cell motility (Tang, _et al._, 2004). HIFs can also be expressed in normoxic
conditions as a result of mutations to the tumour suppressor gene, *VHL* (Kondo, *et al*., 2002). The PDGF-BB homodimer expression increases following hypoxia and binds PDGF receptor (R)-β, leading to Akt phosphorylation and ultimately the anti-apoptotic and proliferative properties NF-κB activation (Romashkova, *et al.*, 1999, Zhang, *et al.*, 2003).

In clinical studies involving advanced cancers of the uterine cervix, levels of oxygenation were measured and it was determined that hypoxic tumours with a low apoptotic index favoured lymphatic spread and disease recurrence (Hockel, *et al.*, 1999). A second study, however, associated hypoxia with worse disease-free and overall survival (Hockel, *et al.*, 1996).

Microvessel density correlates with tumour size, depth of invasion and Dukes’ stage (Yonenaga, *et al.*, 2005). Compared to blood vasculature in healthy tissue, that of tumours are often disorganised, tortuous, and leaky, which contributes to higher interstitial fluid pressure (IFP) within a tumour compared to normal tissues (Less, *et al.*, 1992).

1.6.3.1 Treating the tumour vasculature

VEGF-A is the main growth factor for vascular endothelial cells. Alternate splicing provides four main isoforms—VEGF_{121}, VEGF_{165}, VEGF_{189}, and VEGF_{206}. These isoforms contain 121, 165, 189, and 206 amino acids respectively and can be expressed in varying degrees in diseases. For example, VEGF_{121} and VEGF_{165} are up-regulated compared to the other isoforms in breast, ovarian, lung and CRC (Cheung, *et al.*, 1998, Stimpfl, *et al.*, 2002), whilst VEGF_{189} correlates with liver metastasis and poor patient prognosis in CRC (Tokunaga, *et al.*, 1998). As VEGF-A is a driver of vascular endothelial cells proliferation, studies have experimented with VEGF-A-inhibitors as a form of cancer therapy.

The effects of two types of VEGF-A-inhibitors on tumour blood vasculature have been analysed. One inhibitor was a synthetic antibody containing the domains for VEGF-A receptor (R)-1 and 2 with the main effect of sequestering free VEGF-A. The second was an inhibitor of VEGF-A/PDGF receptor tyrosine kinases, which will inhibit VEGF-A and PDGF signalling. Treatment of tumour-bearing animals with these agents results in a reduction in endothelial cell sprouts and fenestrations, reduced blood vasculature which correlates with reduced VEGFR-2 and 3 expression on surviving endothelial cells, normalisation of pericytes, and ultimately, a reduction in tumour size (Inai, *et al.*, 2004).
The treatment of mice with anti-VEGFR 2 led to the normalisation of mammary carcinoma vasculature, which resulted in the restoration of physiological IFP. This resulted in better penetration of tumours with bovine serum albumin when compared to untreated controls, suggesting that the normalisation of vasculature may improve response to cytotoxic therapy (Tong, et al., 2004).

Bevacizumab is a humanised recombinant monoclonal antibody approved by the FDA. It binds and neutralises the isoforms of VEGF-A and is currently used as 1) second-line treatment in combination with oxaliplatin, leucovorin, and 5-fluorouracil (FOLFOX4) in metastatic CRC (Cohen, et al., 2007), 2) a single agent for the treatment of glioblastoma multiforme (Cohen, et al., 2009), 3) first-line treatment together with paclitaxel and carboplatin for advanced/metastatic recurrent non-squamous NSCLC (Cohen, et al., 2007), and 4) treatment of advanced renal cell carcinoma with interferon-α2a (Summers, et al., 2010). There are, however, serious, albeit uncommon, side-effects associated with the prolonged usage of Bevacizumab such as gastrointestinal perforation, wound-healing complications, haemorrhages, and congestive heart failure. Examples of the more common side-effects experienced by patients include nausea, diarrhoea, fatigue, hypertension and dehydration (Hapani, et al., 2009).

1.6.3.2 Tumours refractory to anti-VEGF-A therapy

Neovascularisation persists in tumours resistant to anti-VEGF-A therapy and studies have identified several mediators that contribute to this. Bone marrow mononuclear cells (BMMCs), for example, are more numerous in tumours refractory to anti-VEGF-A therapy, and when the specific depletion of Gr1+ BMMCs was coupled with anti-VEGF-A treatment, refractory tumours regressed. It was determined that CD11b*Gr1+ cells conferred refractoriness towards anti-VEGF-A therapy (Shojaei, et al., 2007, Piao, et al., 2012). This infiltration was associated with the development of a more aggressive mesenchymal phenotype in resistant glioblastoma xenografts compared to control tumours, as indicated by an increase in vimentin and smooth muscle actin staining, and a decrease in e-cadherin staining (Piao, et al., 2012). Regulation of angiogenesis in refractory tumours is also multifactorial, with the involvement of other cytokines such as granulocyte-colony stimulating factor and PDGF-C (Crawford, et al., 2009, Shojaei, et al., 2009).
1.7 SELECTIVE PRESSURE BY THE MICROENVIRONMENT

The immune system plays a crucial role in the Darwinian selection of tumour epithelial cells by eliminating vulnerable tumour cells, leaving cells that are resistant to proliferate and progress into a tumour. The question, therefore, arises as to whether it is possible to predict the likelihood of tumour progression based on selection pressure?

A multi-scale mathematical model considered several microenvironment factors including hypoxia, cell heterogeneity, motility, and proliferation rate, which all relate to the invasive potential of the tumour epithelial cell. It was predicted that harsher microenvironment conditions led to a tumour with invasive margins that were dominated by a few clones with aggressive traits. On the other hand, a milder microenvironment favoured a smooth edged tumour with no invasive margins that was composed of clones with varying degrees of aggressiveness (Anderson, et al., 2006). In the same study, this model was able to reverse the invasive tumour properties by altering microenvironment conditions, suggesting that instead of treatments like chemotherapy and anti-angiogenic therapy which aim to create a harsher environment for cells to survive in, treatments could be tailored according to a tumour’s microenvironment to target cancer microenvironment interactions instead (reviewed by Quail, et al., 2013). As such, proteins that alter the microenvironment are potential alternatives to chemotherapy and anti-angiogenic therapy. One such protein is a matrix-associated protein, secreted protein, acidic and rich in cysteine (SPARC), and will be discussed in detail below.

1.8 SECRETED PROTEIN, ACIDIC AND RICH IN CYSTEINE

SPARC, alternatively known as BM40 and osteonectin, is a calcium-binding, matricellular glycoprotein belonging to a group of matrix-associated factors that includes hevin, testican and follistatin-like proteins. SPARC has a regulatory role and it interacts with, and mediates interactions between, the cell and its environment (Iruela-Arispe, et al., 1995, Tai, et al., 2005, Said, et al., 2007).

During foetal development, bones and teeth express high levels of SPARC mRNA and protein which are expressed in osteoblasts and chondrocytes (reviewed by Alford, et al., 2006), suggesting a role of SPARC in the development of these tissues. SPARC is also involved in endothelial cell migration (Hasselaar, et al., 1992), ECM remodelling (Tremble, et al., 1993), endothelial cell shape regulation (Goldblum, et al., 1994), cellular differentiation, angiogenesis (Kupprion, et al., 1998, Barker, et al., 2005, Said, et al.,
and apoptosis (Yiu, et al., 2001, McCarty, et al., 2007). As each of these events can contribute to cancer progression, SPARC may also have a role in carcinogenesis.

SPARC knockout (KO) mice have normal embryonic development and are fertile but exhibit osteopaenia at week 17 of age (Delany, et al., 2000). At around six months of age, cataracts develop and the lens capsule may rupture (Gilmour, et al., 1998). This rupture is caused by alterations to the ECM leading to increased lens permeability, resulting in the swelling of lens fibre (Yan, et al., 2002), which is a hallmark of the osmotic cataracts sometimes found in diabetic individuals (Yan, et al., 2002). In addition, mice experience severe osteopenia which is attributed to a general reduction in osteocyte number (Delany, et al., 2000). Accelerated dermal wound closure was also observed in SPARC KO (Bradshaw, et al., 2002), which may result from the actions of VEGF-A, basic fibroblast growth factor (bFGF) and PDGF. These factors have been shown to improve wound healing (Reviewed by Slavin, 1996) and are known to be inhibited by SPARC (Chlenski, et al., 2006), hence the availability of these cytokines by the absence of SPARC may promote wound closure.

Due to the role that SPARC plays in cell migration, cell adhesion and ECM regulation, interest in a role for SPARC in the development of cancer and as a cancer therapy target has increased over recent years. Indeed, recent studies have identified a role for SPARC in the pathogenesis of various cancers. There are, however, opposing views on the matter of SPARC and cancer-free survival in CRC patients. Some studies have shown that increased levels of SPARC predict worse survival rates (Zhao, et al., 2010, Jeung, et al., 2011, Kim, et al., 2013) whilst others have detected better long-term cancer-free survival (Chew, et al., 2011). In addition, low SPARC expression as caused by promoter methylation has been associated with poor chemotherapy response (Tai, et al., 2005, Yang, et al., 2007, Cheetham, et al., 2008). It has also been suggested that the methylation status of SPARC, along with protocadherin 10 and ubiquitin thiolesterase, may be used as prognostic and predictive molecular markers for stage II CRC patients (Heitzer, et al., 2014). Demethylation of SPARC by 5-Aza reverses this (Cheetham, et al., 2008). Although the demethylation of SPARC is not specific and some studies disagree on the extent of promoter hypermethylation (Yang, et al., 2007, Cheetham, et al., 2008), they concur that increased SPARC expression is a marker for a good prognosis as low SPARC levels are associated with poor response to chemotherapy, CRC development and progression, resulting in a worse prognosis.
Therefore, the following sections will discuss the role of SPARC and tumourigenesis-related processes such as angiogenesis, inflammation, apoptosis and tumour growth, cell motility. The role of SPARC in chemosensitisation, targeted therapy and disease detection will also be discussed.

### 1.8.1 SPARC and angiogenesis

SPARC is an efficient inhibitor of angiogenic activity at various levels. Firstly, SPARC binds directly to VEGF-A, thus reducing activation of VEGF-A receptors and MAPK, hindering vascular endothelial cell growth. This has been proposed as the predominant mechanism by which SPARC inhibits VEGF-A activity on angiogenesis (Kupprion, et al., 1998). Secondly, the FS-E degradation-derived product of SPARC represents the epidermal growth factor (EGF) of the follistatin domain, and blocks the effects of bFGF in a corneal rat assay and inhibits neovascularisation in neuroblastoma cells. In addition, SPARC-degradation derived peptides rhSPARC and FS-E block bFGF-stimulated endothelial cell migration, induce apoptosis and inhibit angiogenesis in a Matrigel plug assay (Chlenski, et al., 2006). SPARC was also shown to inhibit in vitro and in vivo angiogenesis using human umbilical vein endothelial cells, possibly through the downregulation of proangiogenic molecules such as VEGF-A, FGF, PDGF, MMP-7, and MMP-9 (Zhang, et al., 2012, Gorantla, et al., 2013). These findings correspond with a reduction in the observed number of blood vessels and a higher apoptotic cell number in SPARC-transfected tumours, supporting the concept of SPARC being inhibitory for tumour growth due to its anti-angiogenic activity (Small, et al., 2000).

SPARC has also been shown to inhibit angiogenesis by inhibiting phosphorylation of extracellular signal-related kinase (ERK)/MAPK in VEGF-A signalling (Kato, et al., 2001). On the other hand, proteolysis of SPARC can result in peptide fragments containing the KGHK sequence, which has angiogenic properties (Lane, et al., 1994).

### 1.8.2 SPARC and inflammation

SPARC displays anti-inflammatory properties in relation to cancers. In ovarian cancers, SPARC ameliorates cancer-induced inflammation by attenuating lipophosphotidic acid (LPA)-induced urokinase plasminogen activator (uPA) in cancer cells (Said, et al., 2008). This reduced overall macrophage chemotaxis, and also desensitised them to the mitogenic and chemotactic effects of MCP-1. In a SPARC-KO model of bladder tumour, urothelial
cells and TAMs that were deficient in SPARC had an enhanced inflammatory phenotype compared to their SPARC-WT counterparts and had increased promoter expression of the oncogenic transcription factors, NF-κB and activator protein 1 (Said, et al., 2013). In a DSS-induced murine model of colitis, SPARC was associated with increased levels of acute and chronic inflammation endoscopically and histologically when compared to SPARC-KO mice (Ng, et al., 2013). In ovarian cancer cells, the reduction in LPA-induced uPA was also associated with tumour progression (Said, et al., 2008), with SPARC inhibiting uPA, MMP-2 and 9, thereby reducing overall ECM degradation. Reduced ECM degradation can translate into the reduced capacity for metastasis, and this is supported by studies claiming a reduction in metastasis during increased levels of SPARC expression (Koblinski, et al., 2005, Arnold, et al., 2008).

**1.8.3 SPARC and apoptosis and tumour growth**

In an *in vitro* system using lens epithelial cells, expression levels of SPARC increased following stress and resulted in reduced apoptosis compared to SPARC-null animals. This is mediated by the activation of integrin-linked kinase as a result of an interaction between the copper-binding domain on SPARC and integrin β1 (Weaver, et al., 2008). Integrin β1 is an ECM receptor and a positive association between SPARC and integrin β1 is observed in human gastric cancer (Zhao, et al., 2010).

There has been opposing views on the relationship between SPARC and apoptosis. SPARC is positively associated with apoptosis via the function of cathepsin B in medullablastoma and neuroblastoma cell lines. Cathepsins are a family of proteases that regulated apoptosis, and under normal physiological conditions, they are localised in a lysosome and are released upon stimulation from cell damage. In these cell lines, the over-expression of SPARC leads to autophagy, followed by apoptosis (Bhoopathi, et al., 2010). The expression of SPARC also resulted in the *in vitro* apoptosis of endothelial cells (Gorantla, et al., 2013). By contrast, an *in vivo* mouse model of melanoma identified that SPARC depletion activated the mitochondrial apoptotic pathway via up-regulation of p53 and thus SPARC would also appear to be anti-apoptotic (Fenouille, et al., 2011).

**1.8.4 SPARC and cell motility**

The ability to regulate cell motility indicates a potential role of SPARC in the spread and eventual cancer metastasis. SPARC expression is associated with cell migration in a
human pancreatic carcinoma cell line, and is negatively regulated in part by tumour protein 53 induced nuclear protein 1 (TP53INP1). TP53INP1 is a pro-apoptotic target gene of p53 and it inhibits cell migration and invasion, possibly by modulating cell-ECM adhesion (Seux, et al., 2011). SPARC appears to be positively regulated by Snail, which is a zinc-finger transcription factor, and the over-expression of Snail resulted in increased SPARC-dependent invasion in vitro using lung cancer cell lines (Grant, et al., 2014).

SPARC appears to affect the migration of immune cells as SPARC-deficient mice exhibited enhanced migration of DCs from the skin to lymph nodes after induction of localised inflammation. The increased migration appeared to result from the altered microenvironment, and resulted in increased T-cell priming (Sangaletti, et al., 2005).

1.8.5 SPARC and chemosensitisation

Low doses of the chemotherapy, camptothecin, induce tumour cell senescence by inhibiting topoisomerase-1, resulting in the inhibition of DNA replication and transcription. The inhibition of DNA replication and transcription prevents the cell from replicating, and this phenomenon is known as senescence. Some tumours, however, can be resistant to camptothecin and increasing its dose would increase the risk of cytotoxicity. One solution involves sensitising tumour cells to chemotherapy, and this may be achieved by SPARC. SPARC-induced senescence in CRC in response to low doses of camptothecin is mediated by up-regulation of p53 (Chan, et al., 2010). Similarly, up-regulation of SPARC increases apoptosis in hepatocellular carcinoma cells in response to 5-fluorouracil-based chemotherapy (Atorrasagasti, et al., 2010)

1.8.6 Target molecular therapy and disease imaging

It has been suggested that altering microenvironment interactions may be beneficial as a form of treatment (Anderson, et al., 2006). Since SPARC has as a matricellular protein makes it ideal as a target for molecular therapy. SPARC-deficient tumour cells were transplanted into mice and developed tumours were chemo- and radio-therapy resistant. Sensitivity to radiotherapy, 5-fluorouracil, and irinotecan was re-established following induced expression of SPARC, resulting in regression of tumours (Tai, et al., 2005).

The observation of increased SPARC expression in tumours has led to the development of a novel imaging process, where nanoparticles specific to SPARC have been administered systemically and SPARC expression in diseased tissues analysed via
computed tomography and fluorescence mediated tomography. This method allows for the detection of lung metastasis in a murine model of prostate cancer (Thomas, et al., 2011), supporting the use of optical imaging rather than surgery for the detection of metastasis.

1.9 Aims of thesis

CRC is a major cause of morbidity and mortality in the developed world as discussed earlier (section 1.1). Those with chronic inflammation of the colon are at greater risk of developing this cancer (section 1.2.4). Therefore, identification and analysis of pathways that contribute to inflammation and modify tumourigenesis in the colon are of diagnostic and therapeutic interest. The hypothesis that inflammation impacts on colorectal tumourigenesis will be addressed by analysing the contribution of inflammation to tumourigenesis using a murine model of CRC in which tumours develop in the presence of chronic colitis. Sporadic tumourigenesis will be produced in inbred mice by AOM, a colonotrophic mutagen, and colitis by ingestion of DSS. Mice will be treated with both agents, AOM/DSS to allow development of colorectal tumours (section 1.3.1.3) during colitis. Tumours developing in these models have not been directly compared previously in detail. The first major objective will be to test whether chronic intestinal inflammation promotes tumourigenesis by analysis of the tumours to:

1. determine their pathological features (Chapter 3);
2. characterise the innate and adaptive immune cell infiltrates within the tumours (Chapter 4);
3. gain information on molecular changes that occur with chronic inflammation through a global gene expression microarray analysis (Chapter 5).

The role of the microenvironment in supporting tumour development is of great interest (reviewed in sections 1.6 and 1.7) since modification of this component could lead to new methods to treat cancer. SPARC, a matricellular protein, is a potential modifier of the microenvironment and has been implicated by my group in CRC in humans, and others previously (Section 1.8). There have been no studies of SPARC using in vivo models to analyse how it impacts on colorectal tumour development. The hypothesis that SPARC impacts on colitis-associated colorectal tumourigenesis will be addressed using SPARC-KO mice created by disruption of the SPARC gene (Norose, et al., 1998). While these have been used to examine the role of SPARC in various processes (section 1.8) this has
not been performed for colitis-associated colorectal tumourigenesis. The second major objective was to investigate the role of SPARC in colitis-associated CRC (CAC) through use of SPARC-KO mice. This will involve analysis of:

1. the pathological features of the induced tumours (Chapter 6);
2. the innate and adaptive immune cell infiltrates within the tumour (Chapter 7);
3. global gene expression by microarray analysis of tumours (Chapter 8).
Chapter 2

Materials and methods
2.1 **Materials**

All materials used for studies in this thesis were of analytical or biochemical grade unless stated otherwise.

2.1.1 **Reagents for inducing colorectal cancer and animal sacrifice**

<table>
<thead>
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<th>Supplier</th>
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<tbody>
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<td>DSS</td>
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2.1.2 **Materials and reagents for histochemistry**

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<tbody>
<tr>
<td>10% buffered paraformaldehyde</td>
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<td>Acetone</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Antibody diluent</td>
<td>DAKO, Agilent technologies</td>
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<tr>
<td>DAPI</td>
<td>Dr Jane Allan, UWA; Invitrogen, ThermoFisher</td>
</tr>
<tr>
<td>DePeX mounting medium, Gurr®</td>
<td>Merck Pty Ltd</td>
</tr>
<tr>
<td>Goat serum</td>
<td>Courtesy of Andrew Wilson, UWA; DAKO, Agilent technologies</td>
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<tr>
<td>Haematoxylin and Eosin</td>
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<tr>
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<td>SuperFrost plus glass slides</td>
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<tr>
<td>Xylene</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>ApopTag® fluorescein in situ apoptosis detection kit</td>
<td>Merck Millipore</td>
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<tr>
<td>SuperPAP pen</td>
<td>Life Technologies</td>
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</table>
**Solutions**

- 5% Goat serum
- Acetone: Methanol fixative
- PBS solution

**Formula**

- 5% (v/v) goat serum in PBS
- 1:1 (v/v) solution of acetone and methanol.
- 1 PBS tablet to 50 mL deionised water

### 2.1.2.1 Primary antibodies

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<th>Isotype</th>
<th>Format</th>
<th>Specificity</th>
<th>Supplier</th>
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### 2.1.2.2 Isotype controls

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<th>Format</th>
<th>Supplier</th>
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<tr>
<td>Rat IgG2b, κ (A95-1)</td>
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<tr>
<td>Hamster IgG1, λ1</td>
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### 2.1.2.3 Fluorescent secondary/conjugate antibodies

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<thead>
<tr>
<th>Antibody/Isotype Clone</th>
<th>Conjugate</th>
<th>Specificity</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor 594</td>
<td>None</td>
<td>Rat IgG (H+L)</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>Steptavidin</td>
<td>Biotin</td>
<td>Life Technologies</td>
</tr>
</tbody>
</table>


### 2.1.3 Reagents for mRNA and downstream applications

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose powder</td>
<td>Biochemicals</td>
</tr>
<tr>
<td>BCP</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Promega</td>
</tr>
<tr>
<td>1x First Strand buffer</td>
<td>Invitrogen, Life technologies</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>Invitrogen, Life technologies</td>
</tr>
<tr>
<td>SuperScript™ -III reverse transcriptase</td>
<td>Invitrogen, Life technologies</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>Promega</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>FastStart SYBR green master</td>
<td>Roche</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Merck</td>
</tr>
<tr>
<td>Oligo dT15</td>
<td>Promega</td>
</tr>
<tr>
<td>QIAGEN multiplex PCR kit</td>
<td>QIAGEN</td>
</tr>
<tr>
<td>RNA storage solution</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Taq PCR master mix kit</td>
<td>QIAGEN</td>
</tr>
<tr>
<td>TRI Reagent</td>
<td>Ambion</td>
</tr>
<tr>
<td>UltraPure™ Dnase/Rnase-free distilled water</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>DNA-free™ Kit</td>
<td>Ambion, Life technologies</td>
</tr>
</tbody>
</table>

### Solutions

<table>
<thead>
<tr>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% Agarose gel</td>
</tr>
<tr>
<td>3M sodium acetate, pH 5.2</td>
</tr>
<tr>
<td>70% Ethanol</td>
</tr>
<tr>
<td>75% Ethanol</td>
</tr>
<tr>
<td>TAE buffer</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Animals

The use of mice, and the animal procedures, was approved by the Animal Ethics Committee (University of Western Australia, Perth, Western Australia). FVB/NJ (animal ethics approval RA/3/100/1148) and C57BL/6J (homozygote controls and secreted protein, rich in cysteine (SPARC) knockout (KO), animal ethics arrival RA/3/100/606) female mice used in experiments were purchased from the Animal Resource Centre (Perth, Western Australia). SPARC KO mice were originally generated on the C57BL/6/129S/J background (Norose et al, 1998) and had been backcrossed ten times with C57Bl/6 mice to produce SPARC KO mice on a pure C57Bl/6 background. The resulting progenies were used in all experiments.

To confirm the genotype of wild-type (WT) and SPARC-KO mice, genomic DNA from the frozen tail tips of these mice were extracted by Ms Frances Lloyd and amplified using two sets of primers. One pair amplified a 170 base-pair region in exon 4 of the SPARC gene. The second pair amplified a segment of exon 4 along with an adjacent segment of the neomycin cassette which was used to disrupt the SPARC gene in KO mice, and is about 500 base-pair in length.

All animals were maintained by Ms Frances Lloyd and the staff of the animal facility under a specific pathogen free standard housing conditions in the animal facility operated by the University of Western Australia at Fremantle Hospital.

For animal monitoring via mini-endoscopy, mice were anaesthetised intraperitoneally with 0.01 mL/g of a xylazil and ketamine solution and were checked with a toe pinch to ensure that they are fully anaesthetised. Following the procedure, mice are returned to their cages, which were kept warm, and monitored for 1 to 2 hours after the procedure, or until they have roused. Animals were sacrificed either by cervical dislocation or cardiac puncture whilst under terminal anaesthesia induced by xylazil-ketamine.

2.2.2 AOM-only and AOM/DSS regime

Initial doses of azoxymethane (AOM) and dextran sodium sulphate (DSS) were as previously described (Neufert, et al., 2007) and modified due to variation in animal strain and with the addition of batch-to-batch variations for DSS. Concentrations of DSS were modified to ensure that the doses given were sufficient to induce a mild to moderate colitis that would lead to tumour development. As AOM causes liver damage at high
concentrations, an optimum dose was used to induce tumourigenesis but without inducing excessive liver damage.

The AOM-only animal model consisted of six weekly intraperitoneal injections of AOM at an optimised concentration of 12 mg/kg body weight for the FVB mice (Fig. 2.1a, i). Animals were sacrificed after 13 weeks from the first AOM injection.

The AOM/DSS animal model consisted of a single AOM intraperitoneal injection of 7.4 mg/kg body weight for the FVB, and 10 mg/kg for the C57Bl/6 mice, followed by two cycles of 2% w/v DSS in the drinking water for the FVB mice (Fig 2.1a, ii) or 1% w/v DSS for the C57Bl/6 mice (Fig. 2.1b, ii) mice. Each DSS cycle consisted of 7 days of the DSS in drinking water followed by 14 days of autoclaved water. The regimes were maintained by F. P. Lloyd.

**FVB Mice**

(i) **AOM-only**: 12 mg/kg body weight AOM, once a week for 6 weeks

(ii) **AOM/DSS**: 7.4 mg/kg AOM + 2% w/v DSS

Figure 2.1a. Treatment regime for the spontaneous (AOM-only) and colitis-associated (AOM/DSS) development of colorectal cancer in FVB mice.

(i) Mice received 6 weekly intraperitoneal injections of AOM (12 mg/kg) (white triangle; Δ) and were fed autoclaved water (blue box; □). Tumour development was monitored endoscopically at weeks 7, 10 and 13 (symbol = e). Mice were sacrificed following final endoscopy (black triangle; ▲). (ii) Mice received an intraperitoneal injection of AOM (7.4 mg/kg) at the start of the regime (white triangles; Δ) with the introduction of 2% w/v DSS into drinking water the next day. This continued for 7 days (pink box; □) and was replaced by autoclaved water for 14 days and the cycle repeated twice. Tumour development was monitored at week 11 and mice were sacrificed at the end of week 12.
**C57Bl/6 SPARC KO and WT mice**

(i) **AOM/DSS**: 10 mg/kg AOM + 1% w/v DSS

![Diagram](image)

**Figure 2.1b. Overview of treatment regime for colitis-associated (AOM/DSS) development of colorectal cancer in C57Bl/6 mice.**

(i) Mice initially received an intraperitoneal injection of AOM (10 mg/kg) (white triangle; ▲) with the introduction of 1% w/v DSS into drinking water the next day (pink boxes; □). This continued for 7 days and was replaced by autoclaved water for 14 days (blue boxes; ●) and the cycle repeated twice. Tumour development was monitored via endoscopy at week 10 and 11 and mice were sacrificed at the end of week 12.
2.2.3 Colonoscopy and scoring of tumour burden

Microcolonoscopy in the mice was performed on anaesthetised mice by B. Klopcic, F. P. Lloyd and S. K. Fu. A novel high-resolution miniature endoscopic system (Coloview, Karl Storz GmbH, Tuttlingen, Germany) was used to assess tumour burden by assigning a score to the frequency of tumours (table 2.1a) and the size of each tumour in relation to the diameter of the colon (table 2.1b) (Becker, et al., 2005). Tumours that were barely detectable (tumour score of 1) were viewed under magnification and only had a final score of 1 if they contained a visible blood supply.

A. Tumour Frequency

<table>
<thead>
<tr>
<th>Tumour Frequency</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1-2</td>
<td>1</td>
</tr>
<tr>
<td>3-5</td>
<td>2</td>
</tr>
<tr>
<td>6-8</td>
<td>3</td>
</tr>
<tr>
<td>9-12</td>
<td>4</td>
</tr>
<tr>
<td>≥13</td>
<td>5</td>
</tr>
</tbody>
</table>

B. Tumour Size

<table>
<thead>
<tr>
<th>Size in relation to area of field of view</th>
<th>Diagrammatic representation of tumour size</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Just detectable</td>
<td><img src="image" alt="Diagram" /></td>
<td>1</td>
</tr>
<tr>
<td>1/8</td>
<td><img src="image" alt="Diagram" /></td>
<td>2</td>
</tr>
<tr>
<td>1/4</td>
<td><img src="image" alt="Diagram" /></td>
<td>3</td>
</tr>
<tr>
<td>1/2</td>
<td><img src="image" alt="Diagram" /></td>
<td>4</td>
</tr>
<tr>
<td>≥1/2</td>
<td><img src="image" alt="Diagram" /></td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2.1A Scoring of tumour frequency. The total number of tumours visible during endoscopy was allocated a score ranging from 0 to 5. The lowest score of 0 was allocated to a tumour frequency of 0, while the highest tumour score of 5 was allocated to a tumour frequency that was equal to or more than 13.

Table 2.1B. Scoring of tumours size in relation to colon diameter. Tumours were scored according to size in relation to the field of view. Tumours given a score of 1 were just detectable and had a distinct blood supply. Tumours that occupied one-eighth, one-quarter, half and more than half of the field of view area were scored as 2, 3, 4 and 5 respectively.
2.2.4 Digital imaging and analysis

All microscopic images were viewed using an inverted microscope (Nikon Eclipse, TE2000-U, Nikon, Tokyo, Japan) while macroscopic images were viewed using a zoom stereomicroscope (Nikon SMZ800, Nikon, Tokyo, Japan). Both microscopes were attached to a relay lens (Nikon 0.7x DXM relay lens, Nikon, Tokyo, Japan) and images were captured using the Nikon elements Basic Research software (Nikon, Tokyo, Japan).

Where required, images were analysed and/or merged using an image editing software (GNU Image Manipulation Program (GIMP), version 2.6, United States of America) and composite images were created using a panoramic image stitcher (Microsoft Image Composite Editor (ICE), version 1.4.4, Microsoft Corporation, Washington, United States of America).

2.2.5 Microscopic assessment of tumours

All tumours were assessed microscopically by Dr. Cynthia Forrest (School of Pathology and Laboratory Medicine, University of Western Australia) a specialist gastrointestinal histopathologist. Tumours were classified as either pedunculated or sessile, whilst the grade of dysplasia was assigned as either low grade, low grade with focal high grade, or high grade. The extent of tumour cell or gland proliferation was also assessed using a scoring system that graded the proportion of colorectal tumour crypts, also known as glands, relative to tumour stroma. This was devised with the assistance of Dr. Cynthia Forrest. Both glands and stroma were graded from 1-3. A score of 1 indicated a mild or low presence of the feature, a score of 2 with moderate presence, while a score of 3 with severe or marked presence of the specific feature (table 2.2). As an indicator of dysplasia, the gland to stroma ratio informs the investigator of the extent of tumour cell proliferation and allows for the statistical comparison of gland complexity between tumour treatment groups.

<table>
<thead>
<tr>
<th>Polyp type</th>
<th>Pedunculated or sessile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysplasia</td>
<td>Low grade, low grade with focal high grade, or high grade</td>
</tr>
<tr>
<td>Gland:stroma ratio</td>
<td>Gland</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Stroma</td>
<td>Mild</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.2. Tumour assessment system
2.2.6 Reassessing tumour burden post-mortem

For the reassessment of tumour burden, the distal 5 cm of each colon was excised and placed next to a scale. The colons were then dissected longitudinally to expose the tumours. The colons were photographed using a stereomicroscope and composite images were created for each animal using Microsoft ICE. The projected areas of each tumour were then digitally measured using GIMP. The projected area of a tumour is defined as the area of a three-dimensional surface as projected onto a two-dimensional plane. The number of visible tumours was also noted for each animal.

2.2.7 Tissue selection

Colonic tumours and the surrounding non-tumour tissue were placed in DNase and RNase-free tubes, flash frozen in either optimum cutting temperature (OCT) compound or in pyrogen and then stored at -20°C and -80°C respectively until later use.

2.2.8 Tissue sectioning

Cryosections 7 µm thick were cut on a cryomicrotome (Cryocut 1800, Reichert-Jung, Ney York, USA), courtesy use by the department of Histopathology, Fremantle Hospital. Sections were mounted onto adhesive SuperFrost plus glass slides (Menzel-Gläser, Braunschweig, Germany) and allowed to air dry at room temperature for 1 hour prior to storage at -20°C or being fixed in 10% buffered formalin prior to staining with haematoxylin and eosin.

2.2.9 Haematoxylin and eosin

Formalin-fixed cryosections were stained regressively using the Leica staining system (Linear stainer, Leica ST4040, Leica Microsystems, Australia). The staining protocol consisted of immersing slides for 30 seconds in each station in sequence as follows: four xylene stations, two stations containing absolute alcohol, one station containing 95% alcohol, one station containing 70% alcohol, one distilled water wash station, six haematoxylin stations, two distilled water wash stations, one 0.25% acid alcohol wash, one distilled water wash station, one Scott’s tap water substitute station, one distilled water wash, one eosin station, two 95% alcohol stations, two absolute alcohol stations, and a final xylene station. Slides were then coverslipped using DePeX with the Tissue-Tek® Glas™ Coverslipper (Olympus Australia, Australia) (courtesy use by the
2.2.10 Immunofluorescence

For the detection of immune cells, antibodies were used against the following cell-surface antigens: CD4 and CD8α (T-cells), CD11c (dendritic cells), F4/80 (macrophages), and Ly6G (neutrophils). Proliferative cells were detected via the detection of the nuclear protein Ki-67. Information on the antibodies used is presented in Tables 2.3a, b and c, and representative images of the isotype controls are presented in Figure 2.2.

Frozen sections were fixed for two minutes in ice-cold solution containing acetone and methanol at 50% v/v each. Slides were then removed from the fixative and allowed to completely air dry before rehydrating in a phosphate buffered saline (PBS)-filled coplin jar with shaking for ten minutes. Excess moisture was removed from the slides and around the section using lint-free tissues. Hydrophobic barriers were then drawn around each tissue sample using a SuperPAP pen (Life Technologies, California, United States of America). Protein blocking was achieved by incubating the sections with 5% v/v goat serum (courtesy of Andrew Wilson of the Animal Facility at the University of Western Australia, Perth, Western Australia; DAKO, Agilent technologies) at room temperature for 30 minutes in a humidified chamber. Excess goat serum was tipped off followed by an overnight incubation in a humidified chamber with the primary antibody diluted with antibody diluent (DAKO, Denmark) at 4°C. Either an isotype control or an antibody diluent were used as negative controls. Slides were then washed twice in PBS for 5 minutes each before incubation with the appropriate secondary fluorescent antibody for 1 hr in the dark (Table 2.3c). Sections were then washed for 10 minutes in a PBS solution containing 25 pg/µL 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Gift from Associate Professor Jane E. Allan, University of Western Australia, Perth, Australia). Slides were then air-dried prior to mounting and coverslipping with ProLong® Gold antifade reagent (Life Technology, California, United States of America). Sections were stored in a light-proof box for a day at 4°C before assessment and photographing and then subsequently stored at -20°C.
<table>
<thead>
<tr>
<th>Antibody Clone</th>
<th>Isotype</th>
<th>Format</th>
<th>Specificity</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 (GK 1.5)</td>
<td>Rat (LEW) IgG2b, κ</td>
<td>Biotinylated</td>
<td>Mouse CTL clone V4</td>
<td>1:200</td>
</tr>
<tr>
<td>CD8a (53-6.7)</td>
<td>Rat (LOU) IgG2a, κ</td>
<td>Biotinylated</td>
<td>CD8a</td>
<td>1:200</td>
</tr>
<tr>
<td>CD11c (HL3)</td>
<td>Armenian Hamster IgG1, λ2</td>
<td>Biotinylated</td>
<td>Integrin α-χ chain</td>
<td>1:200</td>
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<tr>
<td>F4/80 (Cl:A3-1)</td>
<td>Rat IgG2b</td>
<td>Biotinylated</td>
<td>F4/80 antigen</td>
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<tr>
<td>Ly-6G (1A8)</td>
<td>Rat IgG2a, κ</td>
<td>Biotinylated</td>
<td>Ly-6G</td>
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<tr>
<td>Ki-67 (TEC-3)</td>
<td>Rat IgG2a</td>
<td>Purified</td>
<td>Ki-67</td>
<td>1:200</td>
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</table>

Table 2.3A. Primary antibodies directed against cell surface markers

<table>
<thead>
<tr>
<th>Antibody/Isotype Clone</th>
<th>Format</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat IgG2a, κ (R35-95)</td>
<td>Biotinylated</td>
<td>1:200</td>
</tr>
<tr>
<td>Rat IgG2b, κ (A95-1)</td>
<td>Biotinylated</td>
<td>1:200</td>
</tr>
<tr>
<td>Hamster IgG1, λ1</td>
<td>Biotinylated</td>
<td>1:200</td>
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</tbody>
</table>

Table 2.3B. Isotype controls

<table>
<thead>
<tr>
<th>Antibody/Isotype Clone</th>
<th>Conjugate</th>
<th>Specificity</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor 594</td>
<td>None</td>
<td>Rat IgG (H+L)</td>
<td>1:200</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>Streptavidin</td>
<td>Biotin</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Table 2.3C. Fluorescent secondary antibodies/conjugates
Figure 2.2. Isotype controls. Immunofluorescent staining of Hamster IgG1, λ1, Rat IgG2a, κ, and Rat IgG2b, κ co-stained with a nuclear dye (blue) in non-tumour tissue and colorectal polyps. Scale bar represents 100 µm.
2.2.11 Terminal deoxynucleotidyl transferase dUTP nick end-labelling

For the detection of apoptosis within the colonic tumours, the terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) was performed using the ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Merck Millipore, Massachusetts, United States of America). Manufacturer’s recommendations were followed and are described below.

Tissue sections were fixed in 1% paraformaldehyde in PBS, pH 7.4 for 10 minutes before washing in two changes of PBS at 5 minutes each. Sections were then post-fixed for 5 minutes in an ice-cold solution consisting of 2 parts ethanol and 1 part acetic acid. Sections were washed in PBS solution twice for 5 minutes per wash before the application of the equilibrium buffer. Sections were then incubated for up to an hour before the buffer was gently tapped off and then incubated with working strength terminal deoxynucleotidyl transferase (TdT) enzyme at 0.3 Units/µL. Sections were then incubated for 1 hour in a humidified chamber at 37°C and the reaction was stopped by agitating sections in a jar containing the stop/wash buffer followed by an incubation at room temperature for 10 minutes. Sections were washed in three changes of PBS prior to the application of working strength anti-digoxigenin-fluorescein antibody at 13 µL/cm². Sections were incubated in the dark at room temperature for 30 minutes before washing in 3 changes of PBS for 2 minutes per wash. A fourth wash was performed containing 25 pg/µL DAPI in PBS before the slides were removed and briefly air-dried before mounting and coverslipping in ProLong® Gold anti-fade reagent (Life Technology, California, United States of America). Sections were then stored in a light-proof box for a day at 4°C prior to assessment and photographing and were then subsequently stored at -20°C.

2.2.12 Cell counting

Whole tumours were visualised and photographed at 200x magnification. Composite images of whole tumours were made by stitching individual images using Microsoft ICE, version 1.4.4 (Microsoft Corporation, Washington, United States of America). The number of positively-stained cells within each tumour with a visible DAPI-stained nucleus were counted by eye. Tumour crypts and stroma were differentiated based on tissue histology.
2.2.13 RNA extraction and analysis

2.2.13.1 RNA extraction

Tissue samples were homogenised in 10-20 volumes of ice-cold TRI Reagent® Solution (Life technologies, California, United States of America) using the TissueLyser (Qiagen, Hilden, Germany) at 50Hz for 90 seconds. The homogenates were incubated for 5 minutes at room temperature before being centrifuged at 13300 g for 10 minutes at 4°C. The resulting supernatants were transferred to a low-adhesion 1.5 mL tube and 100 μL of bromo-chloropropane per 1 mL of TRI Reagent® Solution (Life Technologies, California, United States of America) was added to each sample for nucleic acid precipitation. Samples were mixed thoroughly for 15 seconds prior to incubation at room temperature for 15 minutes. The solutions were centrifuged at 13300 g for 15 minutes at 4°C and the aqueous phases were transferred to nuclease-free tubes. 500 μL of isopropanol was added to each sample and mixed using a vortex for 10 seconds to precipitate the RNA. The solutions were centrifuged at 13300 g for 8 minutes at room temperature and the resulting supernatants discarded. 1 mL of 75% ethanol was added to each of the resulting pellets and centrifuged at 7500 g for 5 minutes. The ethanol was aspirated and the RNA pellets were air dried for 3 minutes before dissolving them overnight at 4°C in 20 μL of RNA Storage Solution (Life Technologies, California, United States of America).

2.2.13.2 Eliminating contaminating DNA

To 20μL of dissolved RNA, 2 Units of the rDNase I enzyme and 3 μL of DNase I Buffer (Life Technologies, California, USA) was added and the mixture was left to incubate at 37°C for 30 minutes. The reaction was terminated by the addition of 3 μL of DNase Inactivation Reagent at room temperature for 2 minutes. Tubes were centrifuged at 13300 g for 90 seconds. The resulting supernatant was collected and realiquoted into several tubes for storage at -80°C.
2.2.13.3 Quantification and quality assessment of RNA

RNA samples were quantified by low volume NanoDrop spectrophotometry (Thermo Scientific, Massachusetts, United States of America). RNA concentrations were calculated by the following formula:

\[ A_{260} \times 40 = \mu\text{g of RNA/\muL} \]

The purity of the RNA samples was assessed by spectrophotometric readings at \(A_{260}, A_{280}\) and \(A_{230}\). “Pure” RNA samples are considered to have an \(A_{260}:A_{280}\) ratio between 1.8 to 2.1 with a similar \(A_{260}:A_{230}\) ratio. Contamination with protein, or phenol, will result in the ratio being appreciably lower. Phenol contamination can be determined by the assessment of the spectral pattern of each sample. A typical spectral pattern for nucleic acids show a trough at 230 nm and a peak at around 260 nm, while samples with phenol contamination from TRI Reagent will show an additional peak at around 230 nm. Samples that are contaminated were purified via re-precipitation. Resulting RNA has an \(A_{260}:A_{280}\) ratio of between 1.8-2.0.

RNA degradation was determined via 2.0% agarose gel electrophoresis containing ethidium bromide at 90 mAmp for 30 minutes. Bands were visualised using VersaDoc Model 4000 Imaging System (Bio-Rad Laboratories, California, United States of America) with non-degraded RNA producing sharp 28S and 18S bands, representing the 28s and 18s ribosomal RNA respectively, and degraded RNA producing a smear. Degraded samples were discarded and only intact RNA was used in this thesis.

2.2.13.4 RNA re-precipitation

RNA was re-precipitated by the addition of 1 volume of 3M sodium acetate and 3 volumes of absolute ethanol and left to incubate at -20°C for 30 minutes. Precipitated RNA was pelleted via centrifugation at 13300 g for 30 minutes. Supernatants were aspirated while carefully avoiding the resulting RNA pellet. Residual salt was removed by washing the RNA pellet in 1 mL of 75% ethanol via centrifugation at 13300 g for 5 minutes. The supernatants were then discarded and the RNA pellets left to dry at ambient temperature for 5 minutes or until the pellet became translucent. The RNA pellet was dissolved in 20 μL of RNA Storage Solution (Life Technologies, Ambion, California, USA) at 4°C overnight before re-quantitation and reassessment of RNA quality.
2.2.13.5 Global genes expression microarray

A MouseRef-8 v2.0 Expression BeadChip kit (Illumina, Inc, California, USA) was used to analyse global gene expression of about 25,600 genes between 2 groups of 4 mice, with one tumour per mouse, and were performed as a service at the Australian Genome Research Facility (Victoria, Australia). Of interest, there were two probes that detected SPARC in the microarray, namely ILMN_3136561 and ILMN_3059326. The microarray was performed on one chip, and RNA samples used were not degraded and had an $A_{260}/A_{280}$ ratio of 1.8-2.0.

2.2.13.6 Analysis of microarray data

Raw signal intensity values of samples were analysed by the Australia Genome Research Facility (Victoria, Australia). Differentially expressed genes were determined as genes with a fold-change of more than 1.5, which was regarded significantly different with a p-value of $\leq 0.05$. The entire expression profile of samples were visualised in a principal component analysis plot which displays intra and inter-variation.

Differentially expressed genes were then analysed according to gene function, ontology, pathways and statistical analysis tools using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang, et al., 2008, Huang da, et al., 2009).

2.2.13.7 Quantitative real-time polymerase chain reaction target information

Differentially regulated genes identified by analysis of whole-genome microarray required confirmation. Cd44, colony stimulating factor 1-receptor (Csf1r), metalloproteinase 10 (Mmp10), Cd164 were selected from a list of differentially expressed genes between sporadic and colitis-associated colorectal polyps in FVB mice (Table 2.4).

Similarly, amphiregulin (Areg), erythroid differentiation regulator-1 (Erdr1), indoleamine-pyrrole 2,3-dioxygenase (Ido), and interferon-induced protein with tetracopeptide repeats 2 (Ifit2) were selected from a list of differentially expressed genes between WT and SPARC-KO colitis-associated colorectal polyps in C57Bl/6 mice (Table 2.5).
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Accession Number</th>
<th>Amplicon Length (nt)</th>
<th>In silico specificity screen</th>
<th>Location of primers by exon or intron</th>
<th>Targeted Splice Variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd44</td>
<td>NM_001037859.2</td>
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<td>BLAST</td>
<td>Exon 14</td>
<td>All</td>
</tr>
<tr>
<td>Csf1r</td>
<td>NM_001037859.2</td>
<td>69</td>
<td>BLAST</td>
<td>Exon 22</td>
<td>None</td>
</tr>
<tr>
<td>Mmp10</td>
<td>NM_019471.2</td>
<td>78</td>
<td>BLAST</td>
<td>Exon 1</td>
<td>None</td>
</tr>
<tr>
<td>Cd164</td>
<td>NM_016898.2</td>
<td>75</td>
<td>BLAST</td>
<td>F: Spanning intron 4 R: Exon 5</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 2.4. Information of targets to be confirmed via multiplex and standard (Cd164) quantitative real-time polymerase chain reaction in colonic polyps induced in FVB WT mice.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Accession Number</th>
<th>Amplicon Length (nt)</th>
<th>In silico specificity screen</th>
<th>Location of primer by exon or intron</th>
<th>Targeted Splice Variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Areg</td>
<td>NM_009704.3</td>
<td>87</td>
<td>BLAST</td>
<td>F: Exon 4 R: Exon 5</td>
<td>None</td>
</tr>
<tr>
<td>Erdr1</td>
<td>NM_133362.2</td>
<td>127</td>
<td>BLAST</td>
<td>Exon 1</td>
<td>None</td>
</tr>
<tr>
<td>Ido1</td>
<td>NM_008324.1</td>
<td>78</td>
<td>BLAST</td>
<td>Exon 7</td>
<td>None</td>
</tr>
<tr>
<td>Ifit2</td>
<td>NM_008332.3</td>
<td>100</td>
<td>BLAST</td>
<td>Exon 3</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 2.5. Information of targets to be confirmed multiplex quantitative real-time polymerase chain reaction in colonic polyps induced in SPARC KO and WT mice.

2.2.13.8 Selection of reference genes

Reference genes are required for determining relative gene expression levels and the reference gene chosen for any assay was dependent on the type of samples that were used for validation in quantitative real-time polymerase chain reaction (qPCR).

Six potential reference genes, centrin-2 (Cetn2), β-glucuronidase (GusB), hypoxanthine-guanine phosphoribosyltransferase (Hprt), phosphomannomutase 1 (Pmm1), polymerase (RNA) II (DNA-directed) polypeptide A (Polr2a), proteasome subunit, beta type, 6 (Psmb6) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were selected following a literature search for suitable candidates and an optimisation experiment was performed in triplicates using mRNA extracted from AOM-only and AOM/DSS colonic tumours. The expression level of a reference gene must be stable across all test samples and this was determined using a normalisation algorithm (Andersen, et al., 2004). The gene that displayed the most stable expression level across all samples was selected for relative gene-expression analysis. Hprt was selected as the most stable reference gene and is described below.
### Table 2.6: Information of reference gene to be used in relative quantitative expression analysis.

<table>
<thead>
<tr>
<th>Acession Number</th>
<th>Amplicon Length (nt)</th>
<th>In silico specificity screen</th>
<th>Location of primers by exon or intron</th>
<th>Targeted Splice Variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_013556.2</td>
<td>87</td>
<td>BLAST</td>
<td>Exon 9</td>
<td>None</td>
</tr>
</tbody>
</table>

2.2.13.9 Reverse transcription polymerase chain reaction

First-strand cDNA synthesis was performed using 2 ug of total RNA, 0.44 mM of deoxynucleotide triphosphates and 33 ng/uL of oligo dT15 per sample. This mixture was then heated at 65°C for 5 minutes to denature the RNA template and primer. After the initial denaturation, the solution was cooled to 4°C for 4 minutes to allow for the annealing of primers to the RNA template. Whilst cooling, a mixture containing 1x first-strand buffer, 4 mM of dithiothreitol (DTT), 100 Units of Superscript-III reverse transcriptase (Life Technologies, California, United States of America) and 10 Units of RNAse inhibitor (Promega, Wisconsin, United States of America) were added to each sample. Samples were mixed gently and centrifuged before reverse transcription at 50°C for 1 hr. The reactions were inactivated at 70°C for 15 minutes before diluting with 18 uL of tris-ethylenediaminetetraacetic acid (TE) buffer. Aliquots were made and stored at -20°C.

2.2.13.10 Primer design

For multiplex quantitative-polymerase chain reaction, appropriated sequences for primers and their respective dual-labelled Black Hole Quencher® (BHQ®) probes were determined using RealTimeDesign™ (Biosearch Technologies, California, United States of America). RealTimeDesign™ received gene sequences according to the specified accession number and confirmed primer specificity by linking directly to NCBI. As several primer pairs will be present together in the sample during multiplex qPCR, the sequence for each primer must only anneal to the target gene. The expression levels for *Cd164* will be analysed via standard qPCR as suitable primers for a multiplex qPCR were not available (table 2.4).

To determine the expression level of genes via multiplex qPCR, fluorophores were assigned according to the gene’s expression level as determined by gene expression microarray analysis, for example, the dimmest fluorophore was assigned to the gene with...
the highest expression level and the brightest fluorophore was assigned to the gene with the lowest expression level. Also, only one fluorophore was selected per optical channel as recommended for the Rotor-Gene Q (Qiagen, Hilden, Germany). The sequences and associated modifications are listed in the Table 2.7.

Primers for *Cd164* were designed using the Universal ProbeLibrary Assay Design Centre (version 2.49, Roche Diagnostics, Basel, Switzerland) under the following conditions, 1) the primers were between 18 to 27 nucleotides in length; 2) the primers melting temperature (Tm) were between 59°C and 61°C; 3) the PCR product size ranged from 70 to 1000 nucleotides (nt) in length with the optimal annealing temperature being 60°C, and 4) the difference in the Tm between forward and reverse primer was 3°C.

2.2.13.11 Quantitative real-time polymerase chain reaction

To quantify the gene expression of *Cd164*, a qPCR was performed using the FastStart SYBR Green Master (Roche, Basel, Switzerland). For each reaction, 0.25 uM of primers and 30 ng/uL of cDNA were used with a 1x solution of the FastStart SYBR Green Master. Each reaction was mixed gently before being heated to 94°C for 3 minutes to activate the FastStart Taq DNA polymerase. Samples then underwent 35 cycles of amplification with each cycle consisting of a 30 sec denaturation step at 94°C, an annealing stage at 60°C for 30 seconds and a primer extension phase at 72°C for 30 seconds. Acquisition of fluorescence intensity was performed after every extension phase onto the green channel of the Rotor-Gene Q (Qiagen, Hilden, Germany). After 35 cycles, a final elongation step occurred at 71°C. To confirm that *Cd164* was amplified in each sample, a melt-curve was generated end of all amplification cycles by measuring total fluorescence at the end of each 1°C increase in temperature from 72°C to 95°C. Peaks appearing at similar melting temperatures confirm the amplification of the same product in every sample.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Oligonucleotide Sequences (5’ – 3’)*</th>
<th>Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hprt*</td>
<td>F: CGAGTCTGAAGCTCTCGATTTCCT</td>
<td>Probe: 5’ CAL Fluor Orange 560, 3’ BHQ1</td>
</tr>
<tr>
<td></td>
<td>R: CAGCCAACACTGCTGAAACATG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P: CAGCATCTAAGAGGTTTTTGCTCAGTGG</td>
<td></td>
</tr>
<tr>
<td>Cd164</td>
<td>F: AGTGCCAACAAATTTACTACAGC</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>R: GATGTCACAACCTGAGGGAGTAGGG</td>
<td></td>
</tr>
<tr>
<td>Erdr1</td>
<td>F: GGGCGTGAATGGGAAGTCTCAAC</td>
<td>Probe: 5’ CAL Fluor Orange 560, 3’ BHQ1</td>
</tr>
<tr>
<td></td>
<td>R: GGGCATGGCAGAGACATC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P: CTCTGTTCAGCGTCGGCGG</td>
<td></td>
</tr>
<tr>
<td>Ifit2</td>
<td>F: GGGTGAGTCAGAAGGGTCACAT</td>
<td>Probe: 5’ CAL Fluor Orange 560, 3’ BHQ1</td>
</tr>
<tr>
<td></td>
<td>R: AGGCCAGAGTAGGGGAGACATC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P: TGCAAGCAGTAACTTGCAAGCGGC</td>
<td></td>
</tr>
<tr>
<td>Areg</td>
<td>F: GGCATCGCCATCGTTATCAC</td>
<td>Probe: 5’ Quasar 705, 3’ BHQ2</td>
</tr>
<tr>
<td></td>
<td>R: TCGAAGCTCTCTCTTITTCTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P: TGGAACGATATCTCCAGGGAAATATGAAGGA</td>
<td></td>
</tr>
<tr>
<td>Ido1</td>
<td>F: CAGTGCACTAGAGCAGTGCAAGAC</td>
<td>Probe: 5’ Quasar 705, 3’ BHQ2</td>
</tr>
<tr>
<td></td>
<td>R: TTGGCTTTTCTCCAGACTG7GA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P: AAAGCATTTGAAAAGGCACTGCACGA</td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>F: GCTCTGTCGAAGCTCTCCTCATGA</td>
<td>Probe: 5’ CAL Fluor Red 610, 3’ BHQ2</td>
</tr>
<tr>
<td></td>
<td>R: CACCAGCTTGGAAGCAAGTGTGA</td>
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</tr>
<tr>
<td></td>
<td>P: CCCCCTGATGCAGGGGAGG</td>
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</tr>
<tr>
<td>Csf1R</td>
<td>F: GCCACACCTCTTGACCTTCA</td>
<td>Probe: 5’ FAM, 3’ BHQ1</td>
</tr>
<tr>
<td></td>
<td>R: AGCTGCGCCTCAGTACAAG</td>
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</tr>
<tr>
<td></td>
<td>P: TCTGTCTATAGTCCCCGCTCATCC</td>
<td></td>
</tr>
<tr>
<td>Mmp10</td>
<td>F: CCGAGGATCATCAGTGTCGA</td>
<td>Probe: 5’ Quasar 705, 3’ BHQ2</td>
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<td></td>
<td>R: AGCAGCCAGCTGTTTGCTCTTC</td>
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</tr>
<tr>
<td></td>
<td>P: TGACACCCATGCGCAGGACGG</td>
<td></td>
</tr>
</tbody>
</table>

*Probe for Hprt was excluded for the singleplex analysis of Cd164

Table 2.7. Nucleotide sequences and modifications for primers (F: forward, R: reverse, P: probe)

2.2.13.12 Multiplex quantitative real-time polymerase chain reaction

For the simultaneous amplification and analysis of multiple genes within each sample, a 10-fold serial dilution standard curve with 12 points was created for each gene product and the reference gene, and tumour samples were analysed in duplicate using the rotor-gene multiplex PCR kit (Qiagen, Hilden, Germany) in accordance to manufacturer’s recommendations.

Each 1x reaction contained 0.5 µM of gene-specific primers (forward and reverse) and 0.2 µM of the conjugated probe. Samples were gently mixed before aliquoting into PCR-clean reaction tubes and 300 ng of cDNA from each sample was added. An initial
incubation step at 95°C for 5 minutes was required to activate the HotStarTaq Plus DNA Polymerase before entering the 35 cycling phases, each phase containing a denaturation step at 95°C for 15 seconds followed by an annealing and extension step at 60°C for 15 seconds. An automatic gain optimisation was performed at the first extension step and fluorescence intensities were acquired on appropriate optical channels at the end of all amplification cycles. The identity of amplicons was confirmed at the completion of all cycles via agarose gel electrophoresis as described in section 1.1.12.3.

2.2.14 Statistical analysis

A loglinear regression analysis was used to explore relationships between the different treatments and the number of tumours and immunofluorescent cells. The data in sections 4.2.1 and 7.2.3 were analysed via an analysis of variance (ANOVA) to test for association between the different types of tumour-immune cells. All other data were analysed using an independent-sample T-test. Where applicable, normality of the data was checked and normalised where necessary. Probability values less than or equal to 0.05 were regarded as significantly different. All analyses were performed using IBM SPSS 21. Scatter plots were graphed using the Companion to Applied Regression library (Fox, et al., 2011) in the statistical program, R (R Core Team, 2015).
Chapter 3

Comparison of sporadic and colitis-associated colorectal tumour pathology in FVB mice
3.1 **Introduction**

As discussed in Chapter 1, sporadic CRC and CAC in humans both develop from precancerous dysplastic mucosa, but result in lesions with differing features. Sporadic colorectal tumours result from dysplasia located in one or two foci within the colon and generally develop into well-differentiated circumscribed polyps. In contrast, CAC tumours can develop simultaneously in multiple regions due to the multifocal nature of the dysplasia and frequently result in poorly differentiated, flat, aggressive cancers (Delaunoit, *et al.*, 2006, Ullman, *et al.*, 2011). These differences may be due to differences in the initiation and progression pathways of the two tumour types but what these are is presently unknown.

A common mouse model for sporadic tumourigenesis involves the administration of AOM, an alkylating chemical that functions as a colon-specific carcinogen (Chumanevich, *et al.*, 2010, Cui, *et al.*, 2010). On the other hand, colitis-associated tumourigenesis required the combination of AOM and oral DSS which induces chronic intestinal inflammation and tumourigenesis (Rosenberg, *et al.*, 2009, Cui, *et al.*, 2010). Both models result in the development of multiple tumours and to date, no studies have compared these tumour models with each other. For example, it is not known if there are differences in the tumour burden and histopathology between tumours from the AOM-only and the combined AOM/DSS model. As chronic colitis increases the risk of CRC in humans (Rubin, *et al.*, 2013), it might be predicted that the AOM/DSS model will produce a relatively higher tumour burden than the AOM-only model.

To address whether there are differences between tumours that develop in the presence of chronic inflammation it is important to first define the morphological and histological features of each type and to also examine the relative quantity of proliferative and apoptotic tumour cells between these. In this chapter, FVB mice treated with AOM or AOM/DSS (as described in section 2.2.2) and were assessed for tumour growth (as described in section 2.2.3), microscopic features of their tumours (as described in section 2.2.5) and the extent of cellular proliferation and apoptosis (as described in sections 2.2.10 and 2.2.11, respectively) were measured in each.
3.2 Results

3.2.1 Macroscopic examination of AOM and AOM/DSS tumours

In both the sporadic model (AOM-only), and the CAC model (AOM/DSS), the mice developed colonic polyps of varying sizes that were generally semi-pedunculated and located within the distal colon (Figure 3.1).

There were 1.5 times more tumours in AOM/DSS-treated mice (n=14 mice, 284 tumours) compared to AOM-only (n=22 mice, 300 tumours) (p<0.0001, SE=0.083) when using loglinear regression analysis to compare the number of tumours generated in each model.

Using an independent T-test, AOM-only tumours (n=13 mice, 174 tumours; SEM=0.36) were significantly larger than AOM/DSS tumours (n=9 mice, 140 tumours; SEM=0.40) by 1.4-fold (3.7 mm$^2$ vs 2.8 mm$^2$, p=0.03, SEM=0.5) (Figure 3.2).

Figure 3.1. Representative images of polyps, stained post-mortem with methylene blue, induced via AOM-only (A) and a combination of AOM and DSS (B) contained within the distal colon. Dotted circles represent examples of measured projected tumour areas. Each division on the scale is 0.1 mm.
3.2.2 Microscopic examination of AOM and AOM/DSS tumours

Histological differences between the tumours generated from the AOM-only and AOM/DSS models were assessed using haematoxylin and eosin (H&E)-stained cryosections by Dr Cynthia Forrest, a specialist gastrointestinal histopathologist (PathWest, Western Australia), who was blinded to the treatments used. Colorectal polyps were classified by assessing crypt architecture and the degree of dysplasia within polyps.

In general, polyps that developed in both models were a mixture of both sessile and semi-pedunculated lesions with all displaying either low or high-grade dysplasia. About 85% of lesions in both AOM and AOM/DSS-treated mice were polyps with high-grade dysplasia (AOM: 22 high-grade tumours/34 tumours, 8 mice, AOM/DSS: 22 high-grade tumours/ 26 tumours, 7 mice), as shown by crowding of epithelial cells, the increased density of crypts featuring cribriform structure, and loss of cell nuclei polarity (Figure 3.3). There was no association between the type of tumour induction and grade of dysplasia. It should be noted that a range of tumour sizes were sampled and it was observed that the crypt architecture was complex with crypt branching occurring
throughout the length of the crypts (Figure 3.3 B, arrows), even in early lesions. There was no association between the treatment groups and the grade of dysplasia or the type of lesion that developed.

Also, there was only one incidence of intra-mucosal and sub-mucosal tumour invasion, both of which developed in a single tumour from the AOM-only model. In cancer, a stromal reaction is the development of tissue stroma in response to invasion and Figure 3.4 demonstrates intramucosal invasion with stromal reaction (circle with dotted line) against an area in the same tissue without stromal reaction (Figure 3.4, circle with solid line).

Submucosal invasion developed in a single sessile polyp from an AOM-only treated mouse where the tumour epithelial cells invaded into the submucosa (Figure 3.5, arrows) with surface ulceration also observed with pink fibrin exudate (Figure 3.5, box). No visible signs of micrometastasis were observed in the liver of mice from either model (data not shown).

Figure 3.3. Representative images of tumours stained using H&E. (A) AOM-only (B) AOM/DSS. Arrows denote regions of crypt branching. Magnification is 200x. Scale bar represents 100 µm.
Despite the absence of differences in tumour dysplasia between the AOM-only and AOM/DSS tumours, the tumour epithelium to stroma ratio, which indicates crypt complexity, was significantly greater in the AOM/DSS compared to AOM polyps (p=0.04) (Figure 3.6).

![Image](image1.png)

**Figure 3.4.** Representative image of intramucosal invasion displaying stromal reaction. H&E section of a colonic polyp featuring intramucosal invasion (dotted line) and neighbouring dysplastic tissue without intramucosal invasion (circle). Magnification is 400x.

![Image](image2.png)

**Figure 3.5.** Submucosal invasion in a sessile lesion from an AOM-only mouse. H&E staining of a flat colorectal lesion with tumour epithelial cells invading the muscularis mucosa (arrows) with surface ulceration (box). Magnification is 200x.
Cell proliferation and apoptosis

Tumours can only develop if the proliferation of tumour cells exceeds the number of those dying. Cells positive for Ki-67, as a marker of cell division, and fragmented DNA, as a marker of apoptosis, were assessed to determine if chronic inflammation alters proliferation and apoptosis respectively within colonic polyps. All Ki-67+ cells were counted in each tumour as described in section 2.2.12. Based on the location of positive cells, both proliferative and apoptotic cells were mostly located within the crypts and tumour stroma respectively (Figure 3.7). There were 1.3-times more proliferative crypt cells (AOM-only: 6 tumours, 6 mice; AOM/DSS: 7 tumours, 5 mice; p<0.05) and 1.9-times more apoptotic stromal cells (AOM-only: 14 tumours, 8 mice; AOM/DSS: 16 tumours, 6 mice; p<0.05) in AOM-only induced polyps compared to AOM/DSS polyps (Figure 3.8).
Figure 3.7. Immunofluorescent staining of Ki-67 (red) and TUNEL (green) -positive cells co-stained with a nuclear-stain (blue) in AOM-only and AOM/DSS colorectal polyps. C – Crypt, S – Stroma. Scale bar represents 100 µm.

Figure 3.8. Scatterplot of the number of positively stained cells in AOM (blue) and AOM/DSS (red) tumours against the area (mm²) of sample for Ki-67 and TUNEL.
3.3 Discussion

Tumourigenesis is a multistep process that includes initiation (transformation of a normal cell into a neoplastic cell), promotion (expansion of neoplastic cell populations) and progression (continued growth of the neoplastic mass). In this chapter, both AOM and AOM/DSS caused lesions in the distal colon that were usually semi-pedunculated polyps. The occurrence of lesions in the distal colons in the AOM-only and AOM/DSS models may be an outcome of AOM activation and in the AOM/DSS model, DSS as well. As AOM relies on colonic microflora to exert its mutagenicity, mutagens are produced in the colon with the resulting neoplastic colonic cells giving rise to colonic polyps. On the other hand, DSS disrupts the integrity of the intestinal epithelial cell layer and in DSS-treated rats, the distal colon featured the most severe lesions and inflammation when compared to untreated controls (Vetuschi, et al., 2002). Therefore, due to the main activity of both AOM and DSS in the colon, it is not surprising that AOM, or a combination of AOM and DSS, led to the development of multiple colonic polyps.

In addition, the development of chronic inflammation in the AOM/DSS model was associated with greater numbers of tumours when compared with AOM treatment alone, suggesting that tumour initiation occurred more frequently in the time frame studied when there was chronic inflammation. Thus, it may be hypothesised that a greater number of cells may have been initiated and transformed into dysplastic cells through DSS-induced colitis in AOM/DSS treated mice.

Despite differences in tumour numbers, the polyps that developed in both the AOM-only and AOM/DSS model are histologically similar. As AOM injections increases the chance of an APC mutation which is associated with adenoma formation, as demonstrated in sporadic CRC and APC^{Min/+} mice (Yamada, et al., 2002), the similarities in histology might be a result of the AOM injections received by mice in both models.

Mutations to APC and other tumour suppressor genes and/or oncogenes increase the chance of tumour development, hence the relatively early development of tumours in the AOM/DSS protocol (day 40; data not shown) compared to a 204-day DSS-only protocol (Cooper, et al., 2000).

In the model described here, AOM/DSS polyps were found to contain fewer proliferative and apoptotic cells than AOM-only polyps. Although oxidative and nitrosative stress from inflammation can increase both cellular proliferation and apoptosis (Lee, et al., 2006), cells experiencing a high mutational rate, such as one caused by AOM, can also...
experience increased apoptosis (Kujoth, et al., 2005). Therefore, these results suggest that the effect of oxidative and nitrosative stress caused by DSS-induced colitis might not exceed the mutation rate in AOM-only tumour cells.

In humans, the dysplastic lesions of sporadic and CAC are often different with sporadic CRC developing from adenomatous polyps which are commonly found in the distal colon, whereas CAC lesions may be polypoid to flat, and may be localised, diffused, or multifocal in both the proximal and distal colon (Connell, et al., 1994, Goldstone, et al., 2012). However, there are currently no direct comparisons of proliferation and apoptosis between human CRC and CAC. Regardless, differences in tumour morphology between the murine models and CRC may reflect some key differences between human and murine bowels.

3.4 Conclusion

The data presented here demonstrate that the tumour promoting effects of chronic inflammation may not differ to those that arise from multiple exposures to a colonotropic carcinogen. AOM-only lesions, however, were generally larger and had a higher number of proliferating cells. Nevertheless, chronic inflammation increased the number of tumours which may be an outcome of an immunosuppressed microenvironment with decreased immunosurveillance that favours the expansion of neoplastic cells. Further analysis of the inflammatory cells and their potential role is investigated in the next chapter.
Chapter 4

Infiltrating cells of the innate and adaptive immune system in sporadic and colitis-associated murine colorectal tumours
4.1 Introduction

As described in Chapter 3, there were more tumours in AOM/DSS-treated mice than AOM-treated mice possibly owing to reduced immunosurveillance. Tumours arise from mutated epithelial cells they develop within a complex microenvironment that consists of a diverse range of immune cells (Pages, et al., 2005, Lewis, et al., 2006, Strauss, et al., 2007, Murdoch, et al., 2008, Salama, et al., 2009).

As described in Chapter 1, section 1.6, Tumour Associated Macrophages (TAMs) are a specific macrophage subtype of the innate immune response (Macatonia, et al., 1993), which may promote tumour growth (reviewed by Erreni, et al., 2011, Balkwill, et al., 2012, Fan, et al., 2014) and are positively associated with tumour cell apoptosis (Sugita, et al., 2002). Dendritic Cells (DCs) are another member of the innate immune response, the presence of which has been demonstrated to increase with CRC stage (Nagorsen, et al., 2007). An elevated number of neutrophils, another innate immune cell, combined with a low number of lymphocytes (also known as the Neutrophil to Lymphocyte Ratio (NLR) predicted worse overall survival in human CRC, human small-cell lung, pancreatic, and hepatocellular cancer (Hung, et al., 2011, Kang, et al., 2014, Templeton, et al., 2014). High densities of the adaptive CD8+ cytotoxic T-cells within the primary tumour of CRC patients were associated with significant protection against tumour recurrence (Camus, et al., 2009) while an increased in CD8+ cells was associated with prolonged survival in glioblastoma patients (Kniecik, et al., 2013). Also, patients with a low CD4+ to CD8+ ratio had a significantly higher 5-year survival (Diederichsen, et al., 2003).

In mice, TAMs promoted the development of CRC in a N-methyl-N-nitrosourea and H. pylori model of CRC (Liu, et al., 2011). In a murine model of ovarian cancer, tumour-infiltrating DCs promoted tumour development (Conejo-Garcia, et al., 2004). Similarly, in a murine model of pancreatic cancer, tumour-infiltrating neutrophils promoted early tumour development (Nozawa, et al., 2006). Another study using Lewis lung carcinoma and mesothelioma cancer cell lines linked tumour-associated neutrophils with tumour progression (Mishalian, et al., 2013). Also, CD4+ and CD8+ T-cells modulated tumour growth and were predominant tumour-infiltrating cells in an AOM/DSS model of CRC (Becker, et al., 2004), and high co-expression of CD4+ and CD8+ T-cells along with the high expression of DCs in the tumour stroma of patients with NSCLC correlated with improved disease-specific survival (Hald, et al., 2013)
With innate and adaptive immune cells playing a significant role in tumour progression and disease outcome, the studies in this chapter aim to determine if the differences identified between the two mouse models of tumourigenesis in Chapter 3 were accompanied by changes in the cellular composition of tumour-associated immune cells. In this chapter, tumours described in Chapter 3 were stained for the presence of F4/80 (macrophages), CD11c (dendritic cells), Ly6g (neutrophils), and CD4 and CD8 (T-cells) (section 2.2.10).

4.2 Results

4.2.1 Relative proportions of tumour associated immune cells

As tumour-associated immune cells are related with tumour progression, it was of interest to determine the relative quantity of each of the immune cell types in both AOM-only and AOM/DSS tumour models by immunofluorescence.

Comparisons were made between all the inflammatory cell types within AOM-only and AOM/DSS tumours using an ANOVA, and F4/80+ and CD4+ cells were the predominant tumour immune cells. AOM-only tumours contained 1.3-times more F4/80+ than CD4+ cells (p<0.05), and contained 17.6, 25.6, and 18.4-times more F4/80+ than CD11c+, Ly6G+, and CD8a+ cells, respectively (p<0.00005). There were also 13.3, 19.4, and 14-times more CD4+ than CD11c+, Ly6G+, and CD8a+ cells, respectively (p<0.0005) (Figure 4.1A).

Similarly, AOM/DSS tumours contained 1.5-times more F4/80+ than CD4+ cells (p<0.05), and contained 20.1, 31.3, and 9-times more F4/80+ than CD11c+, Ly6G+, and CD8a+ cells, respectively (p<0.00005). There were also 13.5, 21, and 6.1-times more CD4+ than CD11c+, Ly6G+, and CD8a+ cells, respectively (p<0.0005) (Figure 4.1B).
Figure 4.1. Average number of inflammatory cells per mm$^2$ of (A) AOM-only tumours and (B) AOM/DSS tumours. N=number of tumours (number of mice). *: p-value < 0.05, #: p-value < 0.00005. Columns represent average and bars represent ± SEM.
4.2.2 Inflammatory cell infiltration

4.2.2.1 Tumour-adjacent mucosa

To ascertain if induced-colitis alters immune cell composition in colonic tissues adjacent to tumours, the phenotype and number of immune cells were compared between tumour-adjacent mucosae from a sporadic model (AOM-only) and an inflammatory model (AOM/DSS) of CRC. Cells were stained for CD11c, F4/80, and Ly6G antigens respectively by immunohistochemistry as described in Chapter 2. Adaptive CD4+ and CD8a+ T-cells were also quantified (Figure 4.2). A log-linear regression analysis was used and the numbers of each cell type were compared between AOM and AOM/DSS tumour-adjacent tissues whilst controlling for tissue area. As presented in table 4.1, AOM/DSS tumours have significantly more F4/80+, Ly6G+, CD4+, and CD8a+ cells than AOM-only tumours, and no significant difference in the number of CD11c+ cells between AOM and AOM/DSS tumour-adjacent mucosae.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Number of tumours (number of mice)</th>
<th>Relative fold-change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AOM</td>
<td>AOM/DSS</td>
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</tr>
<tr>
<td>F4/80</td>
<td>8 (3)</td>
<td>6 (3)</td>
<td>AOM &lt; AOM/DSS by 1.2-fold</td>
</tr>
<tr>
<td>Ly6G</td>
<td>8 (6)</td>
<td>6 (5)</td>
<td>AOM &lt; AOM/DSS by 10-fold</td>
</tr>
<tr>
<td>CD11c</td>
<td>7 (4)</td>
<td>7 (4)</td>
<td>AOM ≈ AOM/DSS</td>
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<tr>
<td>CD4</td>
<td>6 (6)</td>
<td>4 (5)</td>
<td>AOM &lt; AOM/DSS by 3-fold</td>
</tr>
<tr>
<td>CD8a</td>
<td>5 (3)</td>
<td>5 (3)</td>
<td>AOM &lt; AOM/DSS by 2.4-fold</td>
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</tbody>
</table>

Table 4.1 The fold-difference in F4/80+, Ly6g+, CD11c+, CD4+, and CD8+ cells between AOM and AOM/DSS non-tumour colonic mucosa. AOM/DSS tumour-adjacent mucosae have significantly more F4/80+, Ly6G+, CD4+, and CD8a+ cells than AOM-only tumour-adjacent mucosae. No significant difference was detected in the number of CD11c+ cells between AOM and AOM/DSS tumour-adjacent mucosae. P-values < 0.05 are considered significant.
Figure 4.2. Immunofluorescent staining of F4/80, Ly6G, CD4 and CD8a (red)-positive cells co-stained with a nuclear dye (blue) in AOM-only and AOM/DSS colorectal polyps. C – Crypt, S – Stroma. Magnification 200x. Scale bar represents 100 µm.
Figure 4.3. Scatterplot of the number of positively stained F4/80, Ly6G, CD11c, CD4, and CD8a cells against the area of tissue sample (mm²) in non-tumour colonic mucosa of AOM (blue) and AOM/DSS (red)-treated mice.
4.2.2.2 Infiltration of tumours

To determine if DSS-induced chronic colitis alters the composition of the inflammatory cells within tumours, the presence and location of the innate immune cells, DCs, macrophages, and neutrophils, were quantified in tumours induced with and without DSS. Cells were stained for CD11c, F4/80, and Ly6G antigens was determined by immunohistochemistry (Figure 4.3) as described in Chapter 2. Adaptive CD4+ and CD8+ T-cells were also quantified (Figure 4.4). A loglinear regression analysed the number of each cell type between DSS and AOM/DSS tumours and tissue area. As displayed in table 4.2, AOM/DSS tumours have significantly more F4/80+, CD11c+, and CD4+ cells, and significantly fewer Ly6G+ and CD8α+ cells when compared to AOM-only tumours.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Number of samples (number of mice)</th>
<th>Relative fold-change</th>
<th>p-value</th>
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<tr>
<td></td>
<td>AOM</td>
<td>AOM/DSS</td>
<td></td>
</tr>
<tr>
<td>F4/80</td>
<td>5 (3)</td>
<td>3 (3)</td>
<td>AOM&lt;AOM/DSS by 1.6-fold</td>
</tr>
<tr>
<td>CD11c</td>
<td>13 (6)</td>
<td>12 (5)</td>
<td>AOM&lt;AOM/DSS by 1.2-fold</td>
</tr>
<tr>
<td>CD4</td>
<td>6 (4)</td>
<td>5 (4)</td>
<td>AOM&lt;AOM/DSS by 2.1-fold</td>
</tr>
<tr>
<td>Ly6G</td>
<td>13 (6)</td>
<td>15 (5)</td>
<td>AOM&gt;AOM/DSS by 1.1-fold</td>
</tr>
<tr>
<td>CD8α</td>
<td>6 (3)</td>
<td>6 (3)</td>
<td>AOM&gt;AOM/DSS by 1.2-fold</td>
</tr>
</tbody>
</table>

Table 4.2 The fold-difference in F4/80+, CD4+, CD11c+, Ly6G+, and CD8α+ cells between AOM and AOM/DSS tumours. AOM/DSS tumours contained significantly more F4/80+, CD11c+, and CD4+ cells, whereas AOM-only tumours contained more Ly6G+ and CD8α cells. P-values<0.05 are considered significant.
Figure 4.4. Immunofluorescent staining of CD11c (green), F4/80, and Ly6G (red)-positive cells co-stained with a nuclear dye (blue) in AOM-only and AOM/DSS colorectal polyps. C – Crypt, S – Stroma. Magnification 200x. Scale bar represents 100 µm.
Figure 4.5. Immunofluorescent staining of CD4 and CD8a (red)-positive cells co-stained with a nuclear dye (blue) in AOM-only and AOM/DSS colorectal polyps. C – Crypt, S – Stroma. Magnification 100x.
Figure 4.6. Scatterplot of the number of positively stained F4/80, Ly6G, CD11c, CD4, and CD8a cells against the area of tissue sample (mm\(^2\)) in the colonic tumours of AOM (blue) and AOM/DSS (red)-treated mice.
4.3 Discussion

Tumourigenesis-promoting immune cells

It is of interest to determine the predominant immune cell types within each tumour model as the balance of tumour-associated immune cells affects tumourigenesis. In both the AOM-only and AOM/DSS tumours, there were more CD4+ and F4/80+ cells compared to other immune cell markers, suggesting that these cell types are important in tumourigenesis regardless of treatment.

In addition, the association between T-cells and macrophages has been linked with tumourigenesis. For example, in a genetic mouse model of breast cancer, CD4+ and CD8+ T-cells increased in number with cancer development and CD4+ T-cells promoted metastases by working together with and activating M2 macrophages (DeNardo, et al., 2009). In another mouse model of murine breast cancer, infiltration of CD4+ and F4/80+ cells increased with cancer progression, which is similar to breast cancer development in humans (DeNardo, et al., 2009). As AOM/DSS tumours contained more CD4+ and F4/80+ cells, it may be assumed that invasion and metastasis might occur earlier due to chronic inflammation (reviewed by Solinas, et al., 2010).

In Chapter 3, the AOM/DSS model generated more tumours when compared to the AOM-only model. This may be explained in part by reduced CD8a+ T-cell numbers in AOM/DSS tumours, which may result in a reduced cytotoxic response towards tumour cells. Adjacent non-tumour AOM/DSS tissues, however, contained higher CD8a+ cells than AOM-only tissues and whether this discrepancy is a result of tumour formation or the method by which tumours are induced is unknown.

CD4+ and CD11c+ cells are increased whilst CD8a+ and Ly6G+ cells are decreased in AOM/DSS tumours compared to AOM-only tumours

In this model, non-tumour AOM/DSS colonic mucosa contained more inflammatory cells in general than AOM-only non-tumour mucosa, with the exception of CD11c+ cells, which were present in similar numbers in both models. AOM/DSS-induced tumours, on the other hand, contained a higher density of CD11c+ cells and fewer CD8a+ and Ly6G+ cells. This demonstrates that the chronic inflammatory microenvironment in AOM/DSS tumours increased CD4+, CD11c+ cells recruitment whilst reducing CD8a+ and Ly6G+ cell recruitment when compared to AOM-only tumours.
This might be explained in part by the role mature DCs have in the expansion of CD25+ CD4+ T-cell and in the absence of specific antigen, DCs require pro-inflammatory IL-2 for CD25+ CD4+ T-cell expansion (Yamazaki, et al., 2003). This might account for AOM/DSS tumours containing more CD4+ T-cells and DCs. This positive association between DCs and T-cells may be a result of increased CD4+ T-reg cell proliferation caused by DC-secreted TGF- β (Yamagiwa, et al., 2001, Ghiringhelli, et al., 2005). It should be noted, however, that CD4+ T-cells, CD8+ T-cells, DCs, and tumour epithelial cells also express TGF-β in an AOM/DSS mouse model of CRC (Becker, et al., 2004), which may promote T-reg cell proliferation, resulting in an increased CD4+ cell density in AOM/DSS tumours as shown in this chapter.

AOM-only tumours contained more Ly6G+ cells than AOM/DSS tumours. The role of neutrophils in immune regulation may explain the reduced presence of CD4+ cells and CD11c+ cells in sporadic CRC tumours. Depletion of neutrophils in mice that were injected with adjuvant resulted in an increase in CD4+ cells, DCs, and macrophages, whilst a decrease in CD8a+ cells was observed (Yang, et al., 2010). Similarly, depleting CD4+ cells resulted in reduced neutrophil infiltration (Zwacka, et al., 1997). This suggests that neutrophils and CD4+ cells regulate each other.

**CD8+ cells are increased in AOM-only tumours compared to AOM/DSS tumours and relate to increased apoptosis**

Assessing CD8+ cell densities in tumours is relevant to patient prognosis. For example, tumours from CRC patients who had relapsed contained low CD8+ cell densities regardless of the initial tumour stage (Mlecnik, et al., 2011). In another study involving stage I and II CRC patients, a combined assessment of CD8+ T-cells and CD45RO+ memory T-cells predicted patient prognosis, with higher intra-tumour T-cell densities predicting disease-free and overall survival (Pages, et al., 2009). Also, in human hepatocellular carcinoma, the number of intratumoural neutrophils positively correlated with CD8+ T-cells and was a poor prognostic factor for the disease (Li, et al., 2011). However, similar findings in mice CACs have not been observed. Regardless, the above-mentioned studies might imply a worse prognosis for AOM-only mice, as AOM-only tumours contained more CD8+ cells than AOM/DSS tumours.

High number tumour infiltrating leucocytes also correlate with increased apoptosis (Michael-Robinson, et al., 2001), which may explain the significantly higher number of TUNEL+ cells in AOM-only tumours as described in Chapter 3. In addition, apoptotic
tumour cells increase CD8+ T-cell cross-presentation, resulting in host anti-tumour responses and the reduction in tumour sizes in a mouse transplant model of mesothelioma (Nowak, et al., 2003). It should be noted, however, that even though AOM-only tumours contained more CD8+ and apoptotic cells, a decrease in tumour size was not detected. In fact, AOM-only tumours were significantly larger. This discrepancy may be due to the differences in animal models as mesothelioma tumours developed until they reached 100mm², whereas tumours that developed in the AOM-only model were smaller.

4.4 Conclusion

Although colorectal tumours in both AOM-only and AOM/DSS mice were predominantly occupied by CD4+ and F4/80+ cells, the chronic inflammation in the AOM/DSS model was associated with reduced infiltration of CD8+ cells and Ly6G+ cells, and increased infiltration of CD4+, CD11c+, and F4/80+ cells. This demonstrates that chronic inflammation alters the microenvironment of a tumour by influencing immune cell infiltration.
Chapter 5

Molecular differences between murine models of sporadic and colitis-associated colorectal tumours
5.1 Introduction

In chapters 3 and 4, the AOM-only and AOM/DSS models of CRC were shown to be different with more tumours induced in the AOM/DSS model. The AOM-only tumours had more proliferation of epithelial cells present in the crypts and this was also observed in stromal cells. The tumours were also larger in AOM-only compared with the AOM/DSS tumours. Both the AOM-only and AOM/DSS tumours contained higher numbers of CD4+ and F4/80+ cells than surrounding tissue but overall, AOM/DSS tumours contained more CD4+ and F4/80+ cells than AOM-only tumours suggesting a greater presence of immune cells.

In addition to the differences mentioned above, chronic inflammation might impact on other tumourigenic processes such as angiogenesis (Kim, et al., 2003, Numasaki, et al., 2003), transition of cell phenotypes (reviewed by Orlichenko, et al., 2008), and structural changes to the ECM (reviewed by Kass, et al., 2007). These changes are also preceded, and accompanied, by changes in the expression levels of a multitude of genes (Thomas, et al., 1998, Glickman, et al., 2001, Yu, et al., 2004, Orlichenko, et al., 2008). Therefore, to detect gene expression changes on a global level, a technique such as a gene expression microarray is required.

The use of gene expression microarrays makes the monumental task of assessing the changes in gene expression more manageable through the concurrent analyses of thousands of genes. For example, gene expression microarray analysis on stage I and III lung adenocarcinomas identified 50 genes that impacted on patient survival. This group included genes that had not been previously associated with patient survival and thus the technique has the potential to identify high-risk patients during early-stage lung adenocarcinoma (Beer, et al., 2002).

In the studies presented in this chapter gene expression microarrays, as described in section 2.2.13, were used to identify differentially expressed genes between the AOM-only and AOM/DSS tumours. This assesses whether or not they were of genes likely to provide a biological rationale underlying the differences presented in chapter 3 and 4 between tumours of the two models of tumourigenesis in FVB mice.
5.2 Results

5.2.1 Differentially expressed genes

RNA expression levels of a total of 18,138 genes were quantified in 4 AOM-only and 4 AOM/DSS tumours of similar size (section 2.2.13.5). A principal analysis component plot (section 2.2.13.6) demonstrates that the gene expression profiles of AOM-only and AOM/DSS tumours are distinct to each other. Although sample A from the AOM-only tumour group did not cluster with the other samples in its group, it will be included in the analysis as it is distinct to the tumours in the AOM/DSS group. A total of 143 genes were found to be differentially expressed with at least 1.5-fold difference in gene expression between the treatment groups with a p<0.05. Of these 143 genes, 56 genes were relatively over-expressed in AOM-only tumours compared to AOM-DSS tumours, and the remaining 87 genes were over-expressed in AOM/DSS tumours relative to AOM-only tumours. These genes are presented in Tables 5.1 and 5.2 respectively.

5.2.2 Enriched biological processes between AOM and AOM/DSS colorectal tumours

Differentially expressed genes were analysed using DAVID, an online classification system for gene ontology studies (Huang da, et al., 2009, 2009). The absence of chronic inflammation in AOM-only tumours was associated with increased blood vessel development through the up-regulation of Vegfa, Plcd3, Lox, Cyr61, and Ctgf (p=0.007), the up-regulation of the extracellular matrix components by increasing Tn-c, Vegfa, Mmp10, Mmp13, Lox, Timp1, Ctgf (p=0.0003), and an increase in growth factor activity by up-regulating of Vegfa, Inha, Pdgfb, Il11, Gdf15 (p=0.00009) (Table 5.3). The biological processes that appeared to be significantly enriched in AOM/DSS tumours when compared to AOM-only tumours was that of the positive regulation of adaptive immune response (p=0.009), as denoted by the relative over-expression of Ido, Tnfsf13b, and Tap2 (Table 5.3).
Figure 5.1. Principal component analysis plot displaying the gene profile of AOM-only (samples A, B, C, and D; blue) and AOM/DSS (samples E, F, G, and H; red) tumours with principal component 2 plotted against principal component 1.
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<th>p-value</th>
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<td>Matrix metalloproteinase 13</td>
<td>5.2</td>
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<td>Reg3b</td>
<td>Regenerating islet-derived 3 beta</td>
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<td>8.1E-04</td>
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<td>Hba-a1</td>
<td>Hemoglobin alpha, adult chain 1</td>
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<td>Il11</td>
<td>Interleukin 11</td>
<td>3.5</td>
<td>8.1E-03</td>
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<td>Mmp10</td>
<td>Matrix metalloproteinase 10</td>
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<td>Dnajc6</td>
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<td>Thrombospondin 2</td>
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<td>G1 to S phase transition 2</td>
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<td>Vdtr</td>
<td>Very low density lipoprotein receptor</td>
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<td>Syt12</td>
<td>Synaptotagmin-like 2</td>
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Table 5.1. Genes that were relatively over-expressed in AOM-only tumours compared to AOM/DSS tumours.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
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<th>p-value</th>
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<td>4.3E-02</td>
</tr>
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<td>Tissue inhibitor of metalloproteinase 1</td>
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<td>3.4E-03</td>
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<td>Uck2</td>
<td>Uridine-cytidine kinase 2</td>
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<td>6.3E-03</td>
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<td>Uridine-cytidine kinase 2</td>
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<td>5.0E-04</td>
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<td>Dual specificity phosphatase 4</td>
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<td>Vegfa</td>
<td>Vascular endothelial growth factor A</td>
<td>1.5</td>
<td>1.7E-02</td>
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Table 5.1 continued. Genes that were relatively over-expressed in AOM-only tumours compared to AOM/DSS tumours.

<table>
<thead>
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<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold-Change</th>
<th>p-value</th>
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<td>Indoleamine-pyrrole 2,3 dioxygenase</td>
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<td>4.3E-02</td>
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<td>Pnliprp1</td>
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<td>Expressed sequence AI747448</td>
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<td>2.4E-02</td>
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<tr>
<td>Cldn6</td>
<td>Claudin 6</td>
<td>2.2</td>
<td>3.3E-02</td>
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<tr>
<td>Mt3</td>
<td>Metallothionein 3</td>
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<td>3.0E-02</td>
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<tr>
<td>Prf1</td>
<td>Perforin 1</td>
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<td>4.7E-02</td>
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<tr>
<td>Dmbt1</td>
<td>Deleted in malignant brain tumors 1</td>
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<td>3.1E-02</td>
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<tr>
<td>AA467197</td>
<td>Expressed sequence AA467197</td>
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<td>2.7E-03</td>
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<td>Aapo</td>
<td>Apolipoprotein E</td>
<td>2</td>
<td>4.8E-02</td>
</tr>
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<td>Atp12a</td>
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<td>7.3E-03</td>
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<td>Cd177</td>
<td>CD177 antigen</td>
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<td>Gbp1</td>
<td>Guanylate binding protein 1</td>
<td>2</td>
<td>4.5E-02</td>
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<tr>
<td>Tac1</td>
<td>Tachykinin 1</td>
<td>2</td>
<td>1.9E-02</td>
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<tr>
<td>Usp18</td>
<td>Ubiquitin specific peptidase 18</td>
<td>2</td>
<td>3.1E-02</td>
</tr>
</tbody>
</table>

Table 5.2. Genes that were over-expressed in AOM/DSS tumours relative to AOM-only tumours.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold-Change</th>
<th>p-value</th>
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<tbody>
<tr>
<td>2600011E07Rik</td>
<td>RIKEN cdna 2600011E07 gene</td>
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<td>3.6E-02</td>
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<td>A1987692</td>
<td>Expressed sequence A1987692</td>
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<tr>
<td>Irx5</td>
<td>Iroquois related homeobox 5</td>
<td>1.9</td>
<td>4.0E-02</td>
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<tr>
<td>Krt20</td>
<td>Keratin 20</td>
<td>1.9</td>
<td>1.1E-02</td>
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<tr>
<td>Mt4</td>
<td>Metallothionein 4</td>
<td>1.9</td>
<td>2.3E-03</td>
</tr>
<tr>
<td>Ogn</td>
<td>Osteoglycin</td>
<td>1.9</td>
<td>2.5E-02</td>
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<tr>
<td>Smpdl3a</td>
<td>Sphingomyelin phosphodiesterase, acid-like 3A</td>
<td>1.9</td>
<td>6.6E-03</td>
</tr>
<tr>
<td>Trim40</td>
<td>Tripartite motif-containing 4</td>
<td>1.9</td>
<td>1.0E-02</td>
</tr>
<tr>
<td>2510009E07Rik</td>
<td>RIKEN cdna 2510009E07 gene</td>
<td>1.7</td>
<td>9.4E-03</td>
</tr>
<tr>
<td>Aim1</td>
<td>Absent in melanoma 1</td>
<td>1.7</td>
<td>8.8E-03</td>
</tr>
<tr>
<td>BC040758</td>
<td>Cdna sequence BC040758</td>
<td>1.7</td>
<td>1.3E-02</td>
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<tr>
<td>Bglap-rs1</td>
<td>Bone gamma-carboxyglutamate protein, related</td>
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<td>Mep1b</td>
<td>Meprin 1 beta</td>
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<td>2.2E-02</td>
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<tr>
<td>Saa1</td>
<td>Serum amyloid A 1</td>
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<td>Tmc4</td>
<td>Transmembrane channel-like gene family 4</td>
<td>1.7</td>
<td>1.3E-03</td>
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<tr>
<td>Xdh</td>
<td>Xanthine dehydrogenase</td>
<td>1.7</td>
<td>2.3E-03</td>
</tr>
<tr>
<td>Casp4</td>
<td>Caspase 4, apoptosis-related cysteine peptidase</td>
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<td>2.1E-02</td>
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<tr>
<td>Ceacam10</td>
<td>Carcinoembryonic antigen-related cell adhesion</td>
<td>1.6</td>
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<td>Dkk3</td>
<td>Dickkopf homolog 3</td>
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<td>Leng9</td>
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<td>LOC100038882</td>
<td>PREDICTED: Mus musculus hypothetical protein</td>
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<td>LOC547343</td>
<td>Similar to H-2 class I histocompatibility antigen, L-D alpha chain precursor</td>
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<td>Man2a1</td>
<td>Mannosidase 2, alpha 1</td>
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<td>1.0E-03</td>
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<td>Mast cell protease 6</td>
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<td>8.0E-03</td>
</tr>
<tr>
<td>Plac8</td>
<td>Placenta-specific 8</td>
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<td>1.2E-02</td>
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<tr>
<td>Samhd1</td>
<td>SAM domain and HD domain, 1</td>
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<td>5.9E-04</td>
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<td>Sepp1</td>
<td>Selenoprotein P, plasma, 1</td>
<td>1.6</td>
<td>4.2E-02</td>
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<tr>
<td>Slc46a3</td>
<td>Solute carrier family 46, member 3</td>
<td>1.6</td>
<td>5.2E-04</td>
</tr>
<tr>
<td>Smap2</td>
<td>Stromal membrane-associated gtpase-activating protein 2</td>
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<td>2.5E-02</td>
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<tr>
<td>Tap2</td>
<td>Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)</td>
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<td>3.2E-02</td>
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<tr>
<td>Tnfsf13b</td>
<td>Tumor necrosis factor (ligand) superfamily, member 13b</td>
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<tr>
<td>Capn13</td>
<td>Calpain 13</td>
<td>1.5</td>
<td>9.5E-03</td>
</tr>
<tr>
<td>Cideb</td>
<td>Cell death-inducing DNA fragmentation factor, alpha subunit-like effector B</td>
<td>1.5</td>
<td>1.1E-02</td>
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</tbody>
</table>

Table 5.2 continued. Genes that were over-expressed in AOM/DSS tumours relative to AOM-only tumours.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold-Change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CtsS</td>
<td>Cathepsin S</td>
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<td>1.0E-03</td>
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<tr>
<td>Cyp2d34</td>
<td>Cytochrome P450, family 2, subfamily d, polypeptide 34</td>
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<td>8.6E-04</td>
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<tr>
<td>D4Bwg0951e</td>
<td>DNA segment, Chr 4, Brigham &amp; Women's Genetics 0951 expressed</td>
<td>1.5</td>
<td>2.5E-02</td>
</tr>
<tr>
<td>Dcxr</td>
<td>Dicarboxyl L-xylulose reductase</td>
<td>1.5</td>
<td>1.0E-02</td>
</tr>
<tr>
<td>Gsdmdc1</td>
<td>Gasdermin domain containing 1</td>
<td>1.5</td>
<td>4.7E-03</td>
</tr>
<tr>
<td>Gstm5</td>
<td>Glutathione S-transferase, mu 5</td>
<td>1.5</td>
<td>4.4E-02</td>
</tr>
<tr>
<td>Hist1h1c</td>
<td>Histone cluster 1, h1c</td>
<td>1.5</td>
<td>3.4E-02</td>
</tr>
<tr>
<td>Lamp2</td>
<td>Lysosomal-associated membrane protein 2</td>
<td>1.5</td>
<td>1.9E-02</td>
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<tr>
<td>Mocos</td>
<td>Molybdenum cofactor sulfurase</td>
<td>1.5</td>
<td>1.7E-02</td>
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<tr>
<td>Nr1h4</td>
<td>Nuclear receptor subfamily 1, group H, member 4</td>
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<td>1.1E-02</td>
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<td>Ogt</td>
<td>O-linked N-acetylglucosamine transferase</td>
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<td>7.6E-03</td>
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<td>Pglyrp1</td>
<td>Peptidoglycan recognition protein 1</td>
<td>1.5</td>
<td>2.9E-02</td>
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<td>Pip4k2a</td>
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<td>4.6E-02</td>
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<td>Scn2b</td>
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<td>1.6E-02</td>
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<td>Scl39a4</td>
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<td>8.2E-03</td>
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<tr>
<td>Scl6a8</td>
<td>Solute carrier family 6 (neurotransmitter transporter, creatine), member 8</td>
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<td>5.4E-04</td>
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<td>Stac</td>
<td>Src homology three (SH3) and cysteine rich domain (Stac)</td>
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<td>4.5E-02</td>
</tr>
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<td>Tmem50b</td>
<td>Transmembrane protein 50B</td>
<td>1.5</td>
<td>1.2E-03</td>
</tr>
<tr>
<td>Tnfrsf19</td>
<td>Tumor necrosis factor receptor superfamily, member 19</td>
<td>1.5</td>
<td>1.9E-02</td>
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Table 5.2 continued. Genes that were over-expressed in AOM/DSS tumours relative to AOM-only tumours

<table>
<thead>
<tr>
<th>Relative expression</th>
<th>Annotation clusters (ontology ID), p-values, genes</th>
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<tr>
<td><strong>Overexpressed in AOM-only tumours</strong></td>
<td>Blood vessel development (GO:0001568), p-value=0.007</td>
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<tr>
<td></td>
<td>Vegfa, Plcd3, Lox, Cyr61, Ctgf</td>
</tr>
<tr>
<td></td>
<td>Growth factor activity (GO:0008083), p-value=0.00009</td>
</tr>
<tr>
<td></td>
<td>Vegfa, Inhba, Pdgfb, Il11, Gdf15, Ctgf</td>
</tr>
<tr>
<td></td>
<td>Extracellular matrix (GO:0031012), p-value=0.0003</td>
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<tr>
<td></td>
<td>Tnc, Vegfa, Mmp10, Mmp13, Lox, Timp1, Ctgf</td>
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<tr>
<td><strong>Overexpressed in AOM/DSS tumours</strong></td>
<td>Positive regulation of the adaptive immune response (GO:0002821), p-value=0.009</td>
</tr>
<tr>
<td></td>
<td>Ido, Tnfsf13b, Tap2</td>
</tr>
<tr>
<td></td>
<td>Defence response (GO:0006952), p-value=0.01</td>
</tr>
<tr>
<td></td>
<td>Pglyrp1, Ido, Samhd1, C2, Pnliprp2, Tac1, Saa1</td>
</tr>
</tbody>
</table>

Table 5.3. Biological processes and cellular components that are significantly enriched between AOM-only and AOM/DSS tumours.
5.2.3 Quantification of selected differentially expressed genes

Mmp10, CD44, and Csf1r were selected due to their implications in cancer. For example, MMP10 is over-expressed in human lung carcinomas and promotes invasion in head and neck cancer cells (Gill, et al., 2004, Deraz, et al., 2011). Similarly, CD44 positively correlates with cancer progression while inhibiting its expression attenuates the activation of regulators of adhesion, migration, and invasion (Wielenga, et al., 1993, Zaytseva, et al., 2012). Finally, the blocking of Csf1r-signalling can stop tumour-infiltrating myeloid cells and can improve the efficacy of radiotherapy in prostate cancer cells (Xu, et al., 2013).

CD164, which did not satisfy the statistical threshold as it had a fold-change of 1.2 (over-expression in AOM/DSS tumours), was selected due to its known roles in metastasis of human colorectal and prostate cancer (Matsui, et al., 2000, Havens, et al., 2006), and proliferation and mobility in human cancer cell lines (Tang, et al., 2012). CD164 expression levels were confirmed by qPCR as described in Chapter 2, section 2.2.13.11.

To validate the genes of greatest interest, 4 differentially expressed genes were selected and the expression levels were quantified in 7 colorectal tumours from AOM/DSS and AOM-only each, different to the tumours used in the gene expression microarray experiment, by either a standard or multiplex quantitative polymerase chain reaction (qPCR) as described in Chapter 2, section 2.2.13.11 and 2.2.13.12, respectively. These genes were Mmp10, Csf1r, Cd44, and Cd164. The expression levels of Mmp10, Csf1r, Cd44 were determined via multiplex qPCR while the expression level of Cd164 was confirmed via a standard qPCR. Of these 4 genes, only Cd164 and Csf1r were confirmed to be statistically different via multiplex qPCR and both were significantly over-expressed in CAC tumours. Table 5.4 displays the selected genes along with their relative fold-change to AOM-only tumours and respective p-values.
Table 5.4. Relative fold-change to AOM-only tumours and p-values of MMP10, CD164, CSf1r, and CD44. The blue row indicates a gene selected from the list of over-expressed genes in AOM-only tumours and rows in red are genes selected from genes that are over-expressed in AOM/DSS tumours.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Relative fold-change to AOM-only tumours</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mmp10</td>
<td>Matrix metalloproteinase 10</td>
<td>-1.6</td>
<td>0.19</td>
</tr>
<tr>
<td>Cd164</td>
<td>CD164 antigen</td>
<td>3.3</td>
<td>0.006</td>
</tr>
<tr>
<td>Csfr1</td>
<td>Colony stimulating factor 1 receptor</td>
<td>1.6</td>
<td>0.04</td>
</tr>
<tr>
<td>Cd44</td>
<td>CD44 antigen</td>
<td>-1.1</td>
<td>0.13</td>
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</table>

5.3 Discussion

In chapters 3 and 4, clinicopathological and immunological differences were described between CRC and CAC tumours, highlighting the impact of chronic inflammation on tumourigenesis. In this chapter, these changes were characterised at the gene expression level using a cDNA microarray. Of the 18 138 genes investigated, 56 genes were over-expressed and 87 genes that were under-expressed in CRC tumours relative to CAC tumours. Gene ontology analysis identified an increase in angiogenesis in AOM-only tumours when compared to AOM/DSS tumours, with the up-regulation of pro-angiogenic genes such as VEGFA, Lox, Cyr61, and Ctgf. These genes have been described in scientific literature as being expressed in CRC. VEGFA expression is associated with progression, invasion, and metastasis in humans (Ishigami, et al., 1998, George, et al., 2001). LOX expression in CRC patient samples is clinically correlated with VEGF-A expression and blood vessel formation (Baker, et al., 2013) and is essential for hypoxia-induced metastasis in CRC patients (Erler, et al., 2006). CTGF expression is significantly associated with early stage CRC tumours in patients (Ladwa, et al., 2011), indicating a possible role in early stage tumour development.

As tumour development and progression is dependent on angiogenesis, the increased expression of pro-angiogenic LOX in AOM-only tumours suggests increased angiogenesis compared to AOM/DSS tumours, resulting in enhanced tumour growth.
This concurs with the findings in Chapter 3 with AOM-only tumours having larger projected area than AOM/DSS tumours.

Another gene cluster that was identified as increased in AOM-only tumours when compared to AOM/DSS tumours is the ECM, due to increased expression of Tnc, Mmp-10, Mmp-13, and Timp1. TNC encodes the extracellular matrix protein tenascin-C, which has been associated with distant metastasis in breast cancer cells (Oskarsson, et al., 2011) and is increased in colorectal tumour stroma when compared to non-neoplastic colonic tissue (Hanamura, et al., 1997). MMPs are proteolytic enzymes that digest ECM proteins and TIMPs are the main inhibitors of MMPs. Together, these proteins regulate the degradation of the ECM. A number of MMPs and TIMPs have been implicated in CRC, with MMP-10, MMP-13, and TIMP-1 being some of them. For example, MMP-13 is associated with poor prognosis in CRC and is over-expressed in CRC with liver metastasis (Leeman, et al., 2002, Yamada, et al., 2010). Protein levels of TIMP-1 are increased in human CRC tissues and elevated mRNA levels correlate with lymph node and distant metastasis (Lu, et al., 1991, Zeng, et al., 1995). MMP-10 expression, on the other hand, is frequently associated with enterocyte migration during intestinal wound healing (Vaalamo, et al., 1998, Salmela, et al., 2004). Increases in the gene expression levels of these genes in AOM-only tumours suggests that disease progression in AOM-only tumours may be greater when compared to AOM/DSS tumours. In addition, Gbp1 was relatively under-expressed in AOM-only tumours. The under-expression of GBP1 in colorectal tumours was identified as a marker of tumour aggressiveness and was associated with lymphatic invasion, histologic type, tumour stage, lymph node status, and metastasis (Cancer Genome Atlas, 2012), suggesting that AOM-only tumours might be more aggressive than AOM/DSS tumours. Taken together, these gene expression profile which may explain the increase in AOM-only tumour projected areas when compared to AOM/DSS tumours in Chapter 3.

Conversely, genes encoding growth factors, such as Il-11, were up-regulated in AOM/DSS tumours compared to AOM-only tumours. IL-11 has been implicated in the regulation of tumour progression and is highly expressed in human colorectal adenocarcinoma (Yamazumi, et al., 2006). IL-11 transgenic mice and human umbilical vascular endothelial cells treated with IL-11 experienced increased levels of an anti-apoptotic protein, survivin (Mahboubi, et al., 2001). Chapter 3, however, described an increased in apoptotic epithelial cells in AOM-only tumours, suggesting that there are other factors involved in regulating apoptosis in this model.
In contrast to the AOM-only tumours, the positive regulation of the adaptive immune response and defence response were over-represented in AOM/DSS tumours, thus reflecting the effects of DSS-induced chronic inflammation. For example, the relative over-expression of Ido and SAA suggests a role for these genes in colitis-associated CRC. As described in Chapter 1, IDO is an enzyme that is associated with poor prognosis in CRC and is expressed at the invasive border of CRC tumours (Brandacher, et al., 2006, Ferdinande, et al., 2012). SAA expression increased with CRC malignancy with invaded colon cancer cells expressing the highest protein levels of SAA (Gutfeld, et al., 2006). In addition, a subcutaneous injection of recombinant SAA into mice recruited polymorphonuclear cells and monocytes to the site of injection, suggesting that SAA functions as a chemo-attractant (Badolato, et al., 1994). These findings support the findings described in Chapter 4, such as the increased quantities of CD4+, CD11c+, and F4/80+ cells.

**Gene validation via qPCR**

Of the 4 genes chosen for confirmation via multiplex qPCR, only C164 and Csf1r were significantly different and are over-expressed in AOM/DSS compared to AOM-only tumours. The mRNA level of CD164 was lower in human CRCs compared to non-tumour colonic mucosa, and CD164 mRNA expression in non-tumour mucosa is positively associated lymphatic vessel tumour invasion (Matsui, et al., 2000). The soluble CD164 to transmembrane CD164 ratio inversely correlates with venous and remote metastasis (Matsui, et al., 2000). Conversely, CD164 positively correlates with high-grade human ovarian tumours (Huang, et al., 2013) and in a mouse xenograft model of ovarian cancer. CD164 over-expression can drive tumour formation. Knockdown of CD164 in an ovarian cancer model resulted in reduced tumourigenicity and enhanced mouse survival (Huang, et al., 2013). Therefore, the increase in C164 expression in AOM/DSS tumours implies that chronic inflammation increases tumourigenicity when compared to the AOM-only model.

CSF1 is the ligand for CSF1R, alternatively known as macrophage-colony stimulating factor (M-CSF), and is involved in the differentiation of macrophages from monocytes, and the production of Langerhan cells and osteoclasts (Dai, et al., 2002). Binding of CSF1 to CSF1R initiates a phosphorylation cascade resulting in increases in gene transcription and protein translation, which induces cytoskeleton remodelling via multiple signalling pathways, resulting in increased survival, proliferation, and differentiation of target cells.
CSF1 also promotes cell proliferation as the stimulation of bone marrow-derived macrophages with CSF1 promotes entry into the S-phase of the cell cycle (Tushinski and Stanley, 1985). As Cs1fr is over-expressed in AOM/DSS tumours when compared to AOM-only tumours, one might expect an increase in Ki-67$^+$ cells in AOM/DSS tumours. However, this was not the case as shown in Chapter 3, with AOM/DSS tumours containing fewer Ki-67$^+$ cells when compared to AOM-only tumours.

5.4 Conclusion

When compared to a model of sporadic CRC, chronic inflammation resulted in unique gene expression changes, which are associated with increased tumourigenesis, as suggested by the increase in Cd164 and Csf1r in AOM/DSS tumours. As described in Chapter 1, section 1.9, SPARC is a potential modifier of inflammation and to determine if SPARC impacts on colitis-induced tumourigenesis, the following chapter assessed changes to tumour morphology, histology, proliferative and apoptotic tumour cells between SPARC-KO and WT mice.
Chapter 6

Clinicopathological comparison of colitis-induced colorectal tumours in SPARC-knockout and wild-type mice.
6.1 Introduction

As tumour cells interact with components of their microenvironment during tumourigenesis, it is of interest to identify what modulates these interactions as a possible means to both inhibit tumour formation and also tumour spread. As described in section 1.8, secreted protein acidic and rich in cysteine (SPARC) is a protein that may play a role. SPARC is positively correlated with the tumour stages (T) of 2, 3, and 4 when compared to T1 gastric cancer, when considering the depth of the gastric wall invasion (Wang, et al., 2004). However, SPARC expression did not correlate with gastric tumour location, size, morphology type, or histology. In a DSS-induced murine model of colitis, SPARC expression increased the level of acute and chronic inflammation endoscopically and histologically when compared to SPARC-KO mice (Ng, et al., 2013), while in a spontaneous mouse model of CRC, it was suggested that SPARC promotes intestinal tumourigenesis (Sansom, et al., 2007). Conversely, it was also noted in a lymphoma cell transplant model that tumours grew more rapidly without SPARC compared to their WT counterparts (Brekken, et al., 2003).

In addition, SPARC has been shown to inhibit cell proliferation while inducing apoptosis in ovarian cancer cells (Yiu, et al., 2001) and others have shown this in a variety of cancer types for apoptosis (Ilic, et al., 1998, Yiu, et al., 2001, Shi, et al., 2004, Shi, et al., 2007, Tang, et al., 2007, Weaver, et al., 2008) and proliferation (Schiemann, et al., 2003, Capper, et al., 2010). However, in contrast another group demonstrated that SPARC did not affect the proliferation, or apoptosis, of tumour cells in a murine subcutaneous syngeneic tumour transplant model (Brekken, et al., 2003).

In light of the findings described in chapters 3, 4, 5, and the findings in the SPARC literature, this chapter aimed to determine if SPARC modifies colitis-induced colorectal tumourigenesis. SPARC-KO were generated from C57BL/6 mice (Norose, et al., 1998). This model is widely referenced (Francki, et al., 1999, Bradshaw, et al., 2003, Brekken, et al., 2003, Puolakkainen, et al., 2004, Ng, et al., 2013) and has been used in our lab (Klopcic, et al., 2008, Ng, et al., 2013). To generate CAC, SPARC-KO and WT mice received an intraperitoneal injection of AOM to mimic tumour initiation, followed by oral DSS to simulate chronic colitis. Induced tumours were then compared for differences in (i) morphological and histological features as described in section 2.2.5, (ii) the number of tumours that developed and tumour burden assessed post-mortem as described in
section 2.2.4 and 2.2.6, respectively, and (iii) the quantity of proliferative and apoptotic
tumour cells as described in section 2.2.10 and 2.2.11, respectively.

6.2 Results

6.2.1 Macroscopic examination of SPARC-KO and WT tumours

Both SPARC-KO and WT mice developed pedunculated colorectal lesions of various
sizes distal to the caecum following treatment (Figure 6.1). The effect of SPARC on
colorectal tumour initiation and growth were also determined. Differences in tumour
initiation were assessed by analysing the number of tumours in SPARC-KO and WT mice
via loglinear regression analysis as described in section 2.2.13, and 1.3-times more
tumours developed in KO mice (n=184 tumours, 16 mice) compared to WT mice (n=136
tumours, 15 mice) (p=0.02, SE=0.11). Using an independent sample T-test, differences
between WT and SPARC-KO projected tumour sizes were interpreted as being indicative
of real differences in tumour growth; however, there was no evidence for differences in
tumour growth between WT and SPARC-KO mice (5.2 mm² vs 4.4 mm², respectively, p
=0.08, SEM=0.5) (Figure 6.2).

![Figure 6.1. Representative images of polyps in WT and SPARC KO mice, stained post-mortem with methylene blue. Dashed circles represent examples of measured projected tumour areas. Distance between each major division on the scale is 1 cm.](image)
To determine if SPARC impacts on tumour histology, tumours were assessed in a blinded manner by a histopathologist (Dr Cynthia Forrest, PathWest). There were no observable histological differences between the SPARC-KO and WT tumours with most tumours containing focal high-grade dysplasia (Figure 6.3). A tumour epithelium to stroma ratio was also determined to assess the complexity of the crypts within the tumours and no significant differences were found between the SPARC groups via independent-sample T-test (KO: n=25 tumours, 18 mice, tumour epithelium:stroma=1.92; WT: n=20 tumours, 17 mice, tumour epithelium:stroma=1.90; p=0.92, SEM=0.19) (Figure 6.4).

**6.2.2 Microscopic examination of SPARC-KO and WT tumours**

Figure 6.2. The average projected area of tumours in the colons of WT and SPARC-KO mice. Average projected area ± SEM in mm², independent sample T-test (WT: n=15 mice, 136 tumours; KO: n=16 mice, 184 tumours). Whiskers represent lower and upper quartiles of projected tumour areas.
Figure 6.3. Representative histological images of WT and SPARC-KO tumours. Top panel: WT tumours. Bottom panel: SPARC-KO tumours. Magnification is 40x. Scale bar represents 500 µm.

Figure 6.4 Average tumour epithelium to stroma ratio of tumours in the colons of WT and SPARC-KO. Average tumour projected area ± SEM in mm² (WT:20 tumours, 17 mice; SPARC-KO:25 tumours, 18 mice)
6.2.3 Cell proliferation and apoptosis

Multiple studies have demonstrated a role for SPARC in tumour progression; for example, SPARC has been positively associated with gastric cancer progression (Wang, et al., 2004, Zhao, et al., 2010) and there are increases in SPARC expression between stage I and II/III/IV in CRC (Chew, et al., 2011).

As tumour development relies on a balance between proliferating and dying cells, the number of proliferative and apoptotic cells were assessed through the visualisation of antigen Ki-67 and fragmented DNA within the tumour as described in 2.2.12. No statistical differences, however, were found via loglinear regression analysis between Ki-67 staining and fragmented DNA between the WT and SPARC-KO tumours (Ki67 – WT:7 tumours, 7 mice; KO: 9 tumours, 9 mice; p=0.16, and TUNEL – WT: 9 tumours, 9 mice; KO: 10 tumours, 9 mice; 0.28, respectively) (Figure 6.5).

![Figure 6.5](image)

Figure 6.5. Immunofluorescent staining of Ki-67 (red) and TUNEL (green) -positive cells co-stained with a nuclear-stain (blue) in AOM-only and AOM/DSS colorectal polyps. C – Crypt, S – Stroma. Scale bar represents 100 µm.
Discussion

In this chapter the data do not appear to demonstrate an association of SPARC expression with dysplasia, tumour size, and cellular proliferation. However, the data suggest that SPARC reduces tumour initiation in the AOM/DSS mouse model of CRC with significantly fewer tumours developing. Despite a trend, no association of SPARC expression with apoptosis of colorectal epithelium could be confirmed in inflammation-mediated colonic tumourigenesis. Therefore, the following paragraphs will discuss these findings in relation to current scientific literature.

In the model studied here SPARC reduces the initiation of early-stage tumours in the AOM/DSS treated mice when compared with SPARC-KO mice. In other studies, the colons of SPARC-KO mice resulted in lower numbers of infiltrating macrophages and neutrophils following DSS-treatment (Ng, et al., 2013). Furthermore, SPARC-KO mice were unable to mount an immune response following inject of footpads with lipopolysaccharide (Rempel, et al., 2007). These studies suggest a less effective immunosurveillance in SPARC-KO mice, which may explain the increase in colonic tumours when compared to WT mice in the present study.

When compared to wild-type mice, syngeneic transplant tumours from SPARC-KO mice had altered ECM constituent deposition of collagen and laminin-1, resulting in smaller

Figure 6.6. Scatterplot of the number of positively stained cells in SPARC-KO (blue) and WT (red) tumours against the area (mm²) of sample for Ki-67 and TUNEL.
collagen bundles with fewer cross-links (Brekken, et al., 2003). These mice also had more rapid tumour growth (Brekken, et al., 2003). Similarly, subcutaneously injected pancreatic adenocarcinoma cells in SPARC-KO mice developed into larger tumours when compared to their WT counterparts (Puolakkainen, et al., 2004). However, there was no difference in tumour sizes when comparing to SPARC-KO tumours to WT-tumours in this chapter.

In addition to modulating tumour growth, SPARC alters cell proliferation and apoptosis. Although no difference in proliferative and apoptotic cells were detected between WT and SPARC-KO colonic tumours, as previously suggested by mouse models of Lewis lung cancer and melanoma (Brekken, et al., 2003, Prada, et al., 2007), many studies have observed SPARC to alter cell proliferation and apoptosis. For example, the over-expression of SPARC in CRC cell lines increased the activation of apoptosis through interaction with pro-caspase 8 (Tang, et al., 2007). Cellular senescence was also induced in irinotecan-resistant CRC cells after exposure to either exogenous or endogenous SPARC (Chan, et al., 2010). Also, knocking down SPARC expression in ovarian cancer cells suppressed proliferation and induced apoptosis (Chen, et al., 2012).

6.4 Conclusion
The data presented suggest that SPARC may alter tumourigenesis through its involvement in ECM modulation. However, it is not known if immune cell infiltration is impacted upon in this model of CAC. Therefore, the following chapter aims to investigate the presence of inflammatory cells in SPARC-KO and WT CAC tumours to determine differences in immune cell infiltration caused by SPARC.
Chapter 7

Differences in tumour-associated immune cells associated with loss of SPARC in a colorectal cancer model.
7.1 Introduction

Studies described in the previous chapter examined the role of SPARC on tumour initiation and growth, apoptosis and proliferation in a colorectal tumour model and showed that SPARC is associated with reduced tumour number. Cancer progression is dependent on complex interactions between the tumour and its microenvironment, which also includes tumour-associated inflammatory cells. Therefore, it is of interest to determine if SPARC alters the presence of tumour-associated immune cells in the AOM/DSS model of CAC.

SPARC was found to reduce cancer-associated inflammation in an in vitro model of ovarian cancer (Said, et al., 2007, Said, et al., 2008). However, in various models of inflammation, SPARC can either increase inflammation, as demonstrated in the angiotensin II murine model of renal inflammation (Socha, et al., 2007), or decrease inflammation, as seen in two murine models of bleomycin-induced lung injury (Savani, et al., 2000, Sangaletti, et al., 2011). In addition, Lewis lung carcinoma tumours that developed in SPARC-KO mice contained fewer infiltrating macrophages (Brekken, et al., 2003). The involvement of SPARC its effect on individual inflammatory cells needs to be clarified.

As shown by studies conducted in our lab, there was almost no presence of Ly6G+ cells, and a minimal presence of CD68+ cells in both untreated SPARC-KO and WT colons (Ng, et al., 2013). Additionally, the induction of chronic colitis via DSS treatment in SPARC-KO and WT mice resulted in WT mice containing higher levels of Ly6g+, CD68+ and F4/80+, and CD11b+ cells (Ng, et al., 2013). In another study, SPARC-KO mice contained more epidermal Langerhans cells, which are DCs located in the skin within the draining lymph nodes of antigen-sensitised ears compared to their WT-counterparts which resulted in enhanced T-cell priming (Sangaletti, et al., 2005). Neutrophils also accumulate in greater numbers in SPARC-KO lungs of a bleomycin-injured mouse model when compared to their WT equivalents and this is also seen in SPARC-KO mice following acute chemical peritonitis (Savani, et al., 2000). In the same study, an intraperitoneal injection of bleomycin, however, did not result in differences in infiltrating neutrophils between WT and SPARC-KO mice suggesting that the involvement of SPARC in pulmonary injury is different and may rely on a variety of factors such as the site of injury.
In light of the demonstrated impact of SPARC on inflammation this chapter aims to determine the effect of SPARC on the cellular composition of immune cell infiltrates in an attempt to explain the clinicopathological differences previously identified in the previous chapter. Tumours will be stained for following antigens via immunofluorescence as described in section 2.2.10: F4/80, CD11c, Ly6g, CD4, and CD8a.

7.2 Results

7.2.1 Innate and adaptive immune cells in tumour-adjacent tissues

To ascertain if SPARC alters the immune cell composition in colonic tissues adjacent to tumours, immune cells were compared between tumour-adjacent tissues between SPARCK-KO and WT animals using immunohistochemistry to identify cells positive for antigens CD11c (dendritic cells), F4/80 (macrophages), and Ly6G (neutrophils). Adaptive CD4+ and CD8+ T-cells were also quantified. The number of each cell-type present within the tissues was assessed according to the treatment and tissue area and a loglinear regression analysis was used to define any associations.

As presented in table 7.1, there were significantly more numbers of F4/80+, CD11c+, and CD4+ cells, and significantly fewer CD8a+ cells in SPARC KO tumour-adjacent tissues than in the WT counterparts. No significant differences were detected in the number of Ly6G+ cells (Figure 7.1).
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Number of tumours (number of mice)</th>
<th>Relative fold-change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4/80</td>
<td>SPARC-KO 6 (6) VS WT 4 (4)</td>
<td>SPARC-KO&gt;WT by 1.9-fold</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>CD11c</td>
<td>SPARC-KO 10 (10) VS WT 7 (7)</td>
<td>SPARC-KO&gt;WT by 1.5-fold</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>CD4</td>
<td>SPARC-KO 5 (5) VS WT 3 (3)</td>
<td>SPARC-KO&gt;WT by 2.8</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>CD8a</td>
<td>SPARC-KO 9 (8) VS WT 6 (6)</td>
<td>SPARC-KO&lt;WT by 1.5-fold</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Ly6G</td>
<td>SPARC-KO 6 (6) VS WT 5 (5)</td>
<td>SPARC-KO≈WT</td>
<td>p&gt;0.05</td>
</tr>
</tbody>
</table>

Table 7.1 The fold-difference in F4/80⁺, CD11c⁺, CD4⁺, CD8⁺, and Ly6G⁺, cells between SPARC-KO and WT non-tumour colonic mucosa. SPARC-KO tumours have significantly more numbers F4/80⁺, CD11c⁺, and CD4⁺, and fewer numbers of CD8a⁺ cells than WT tumour-adjacent tissues. No significant difference was detected in the number of Ly6G⁺ cells between AOM and AOM/DSS tumours. P-values<0.05 are considered significant.
Figure 7.1. Immunofluorescent staining of CD11c (green), F4/80, CD4, and CD8a (red) co-stained with a nuclear dye (blue) in WT and SPARC KO tumour-adjacent tissues. C – Crypt, S – Stroma. Magnification 200x. Scale bar represents 100 µm.
Figure 7.2. Scatterplot of the number of positively stained F4/80, Ly6G, CD11c, CD4, and CD8a cells against the area of tissue sample (mm²) in tumour-adjacent tissues of WT (red) and SPARC-KO (blue) mice.
7.2.2 *Innate and adaptive immune cells within tumours*

To determine if SPARC alters the composition of tumour inflammatory cells, the presence of innate and adaptive cells were determined via the detection of the following antigens – CD11c (DCs), F4/80 (macrophages), Ly6G (neutrophils), CD4 and CD8a (T-cells). A regression analysis was performed to determine a relationship between the number of antigen-positive cells and SPARC status along with tissue size.

As presented in table 7.2, SPARC-KO tumours contained significantly more numbers of cells positive for F4/80 or CD11c, and significantly fewer cells positive for CD8a or CD4 when compared to WT tumours. No significant differences were detected in the number of Ly6G+ cells between SPARC-KO tumours and WT tumours. (Figure 7.2).

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Number of tumours (number of mice)</th>
<th>Relative fold-change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4/80</td>
<td>SPARC-KO 9 (9)  WT 6 (6)</td>
<td>SPARC-KO&gt;WT by 1.3-fold</td>
<td>p&lt;0.0005</td>
</tr>
<tr>
<td>CD11c</td>
<td>SPARC-KO 11 (11)  WT 11 (11)</td>
<td>SPARC-KO&gt;WT by 1.1-fold</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td>CD8a</td>
<td>SPARC-KO 12 (12)  WT 7 (7)</td>
<td>SPARC-KO&lt;WT by 1.5-fold</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td>CD4</td>
<td>SPARC-KO 9 (9)  WT 6 (6)</td>
<td>SPARC-KO&lt;WT by 1.2-fold</td>
<td>p&lt;0.0005</td>
</tr>
<tr>
<td>Ly6G</td>
<td>SPARC-KO 10 (8)  WT 6 (6)</td>
<td>SPARC-KO≈WT</td>
<td>p&gt;0.05</td>
</tr>
</tbody>
</table>

Table 7.2 The fold-difference in F4/80+, CD11c+, CD4+, CD8+, and Ly6g+, cells between SPARC-KO and WT colonic tumours. SPARC-KO tumours have significantly more numbers F4/80+ or CD11c+ cells, and fewer CD4+ or CD8a+ cells than WT colonic tumours. No significant difference was detected in the number of Ly6G+ cells between AOM and AOM/DSS tumours. P-values<0.05 are considered significant.
Figure 7.3. Immunofluorescent staining of CD11c (green), F4/80, Ly6G, CD4, and CD8a (red) co-stained with a nuclear dye (blue) in WT and SPARC KO colonic polyps. C – Crypt, S – Stroma. Magnification 200x. Scale bar represents 100 µm.
Figure 7.4. Scatterplot of the number of positively stained F4/80, Ly6G, CD11c, CD4, and CD8a cells against the area of tissue sample (mm$^2$) in tumour tissues of WT (red) and SPARC-KO (blue) mice.
7.2.3 Overall trend of immune cells within WT and SPARC KO tumours

To determine the main inflammatory cell component within tumours, the numbers of each antigen-positive cell count were analysed using an ANOVA. In general, there were significantly more CD4\(^+\), CD11c\(^+\) and F4/80\(^+\) cells than CD8a\(^+\) cells in both WT and SPARC KO tumours (Figures 6.3A and B). There were also significantly more F4/80\(^+\) cells than CD11c\(^+\) cells in WT tumours by 1.4-fold (p=0.01) (Figure 7.3A) whereas in SPARC KO tumours, this difference was not significant (p=0.8) (Figure 7.3B).

The ratio between CD4\(^+\) to CD8\(^+\) cells, and T-cells to antigen presenting cells, have been associated with disease prognosis (Diederichsen, et al., 2003, Shah, et al., 2011) and the activation of T-reg cells (Denning, et al., 2011), respectively. In this study, the ratio between CD4\(^+\) and CD8\(^+\) in WT and SPARC-KO tumours was 6:1 and 10:1, respectively. The ratios for CD4\(^+\) to F4/80\(^+\) and CD4\(^+\) to CD11c\(^+\) cells in WT were 1:1.4 and 1:1, respectively; and in SPARC-KO tumours, both ratios were 1:2.
Figure 7.5. Bar chart and table displaying the average number of F4/80, CD11c, Ly6G, CD8a, and CD4+ cells per mm² in (A) WT and (B) SPARC-KO tumours. #: p-value < 0.00005. Columns represent average cell numbers and bars represent ±2SEM. N= number of tumours (number of mice).
7.3 Discussion

The assessment of innate and adaptive immune cell markers in colitis-associated tumours that developed in WT and SPARC-KO mice following the AOM/DSS determined that SPARC facilitated the infiltration of CD4\(^+\) and CD8\(^a\)\(^+\) cells whilst inhibiting the infiltration of CD11c\(^+\) and F4/80\(^+\) cells. These differences might be explained by the effect of SPARC on the ECM, and chemoattractants. Furthermore, the composition of tumour-associated immune cells might impact on tumourigenesis.

As demonstrated in our lab, the collagen fibrils that constitute intestinal ECM in SPARC-KO mice are significantly smaller in diameter when compared to WT mice (Ng, paper in submission). As both dye and radioactive tracer penetration increased through the lens capsule of SPARC-KO eyes compared to WT lens (Yan, et al., 2002), the positive association between collagen pore size and fluid permeability (O'Brien, et al., 2007) suggests that collagen pore size would be increased in the absence of SPARC. This may support the finding of more infiltrating CD11c\(^+\) and F4/80\(^+\) cells, but it does not explain why there is a reduction in infiltrating CD4\(^+\) and CD8a\(^+\) cells and no difference detected in the infiltrating Ly6G\(^+\) cells. This suggests that pore size alone is insufficient to explain the differences in immune cell infiltration.

SPARC may alter immune cell infiltration by altering the expression of chemoattractants and adhesion molecules. In a murine model of acute DSS-induced colitis, SPARC-KO colons expressed lower levels of the T-cell chemoattractant, monokine induced by gamma interferon (MIG) (Aging, 2013). SPARC also reduced the expression of macrophage chemoattractant protein (MCP)-1 which down-regulates macrophage recruitment (Said, et al., 2008). This may explain why there were more tumour-associated F4/80\(^+\) cells in SPARC-KO mice.

In addition to analysing the impact of SPARC on immune cell infiltration, it is important to analyse the composition of immune cells within a tumour. Immune cells co-exist and cooperate with each other to influence tumourigenesis. For example, in the colons of healthy mice, F4/80\(^+\) lamina propria macrophages were able to induce FoxP3-expressing T-reg cells in a T-cell to macrophage cell ratio of 1:1 (Denning, et al., 2011). On the other hand, lamina propria DCs were less efficient at inducing T-reg cell formation, requiring a T-cell to DC ratio of at least 1:5 (Denning, et al., 2011). In this chapter, the ratios of both CD4\(^+\)/F4/80\(^+\) and CD4\(^+\)/CD11c\(^+\) cells in SPARC-KO tumours were each approximately 1:1, suggesting that there may be T-reg cells amongst the CD4\(^+\) cells. WT
colonic tumours had a T-cell to antigen presenting cell ratio of less than 1:1, suggesting a less efficient induction of T-reg cells. In support of this concept, the colons of SPARC-KO mice with DSS-induced colitis contained significantly more T-reg cells when compared to their WT counterparts (Aging, 2013). T-reg cells are commonly associated with a better prognosis in humans (Salama, et al., 2009, Correale, et al., 2010, Frey, et al., 2010, Chew, et al., 2011), however, the data here imply that SPARC might indicate worse prognosis. SPARC-KO mice have altered haematopoiesis, which may be the cause of an altered immune cell infiltration compared with humans.

A low CD4 to CD8 ratio is associated with better disease prognosis in cervical and CRC patients (Diederichsen, et al., 2003, Shah, et al., 2011). This chapter demonstrates that colonic tumours from WT mice contained fewer CD4+ and more CD8a+ cells compared to SPARC-KO mice, suggesting a lower CD4 to CD8 ratio in WT tumours. Therefore, SPARC may be associated with better survival in this model, as demonstrated in stage II CRC patients (Chew, et al., 2011).

Although the data in this chapter present dual roles for SPARC in CRC survival, it must be noted that (i) the tumours analysed in this chapter are very early-stage tumours, and the dynamics of SPARC in tumourigenesis may change as disease progresses, and (ii) the altered haematopoiesis in SPARC-KO mice may confound tumour immune cell infiltration in relation to SPARC.

7.4 Conclusion

The differences in the numbers of tumour-associated immune cells might be a combined result of a more permeable SPARC-deficient ECM with altered haematopoiesis due to the onset of osteopaenia. This suggests that the role of SPARC in this model of colorectal tumourigenesis might not reflect the role SPARC in human cancer. This is because patients tend to express varying levels of SPARC instead of an absence of SPARC, as displayed by SPARC-KO mice. Despite this, colorectal tumours from WT and SPARC-KO mice displayed different tumour-associated immune cells and as described in Chapter 6, WT mice developed fewer tumours.

Tumourigenesis is a process that consists of multiple processes which might explain the differences mentioned above, such as growth factor regulation, cell migration, and angiogenesis. Therefore, the studies described in the next chapter aim to determine pro-tumourigenic pathways that are altered by SPARC via global gene expression microarray.
Chapter 8

Molecular differences between WT and SPARC-knockout colorectal tumours
8.1 Introduction

The previous two chapters described the changes observed in CAC tumours that were caused by SPARC, such as reduced tumour initiation, a reduction in CD11c+, F4/80+, CD4+ cells, and an increase in CD8a+ cells. However, tumourigenesis is complex and involves other processes such as cell migration, growth factor expression, angiogenesis and ECM formation. Therefore, changes in these processes might be observed in SPARC-KO tumours.

SPARC promotes intestinal epithelial cell migration in an APCMin/+ mouse model of spontaneous CRC (Sansom, et al., 2007). SPARC also increases apoptosis in colonic mucosal cells following AOM-treatment (Aoi, et al., 2013). In addition, SPARC induces the expression of collagen type I and regulates TGFβ1 signalling in murine mesangial cells (Francki, et al., 1999, Francki, et al., 2004), and enhances β-catenin signalling in mice (Nie, et al., 2009). Also, SPARC inhibits angiogenesis and impairs tumour growth (Chlenski, et al., 2006).

As SPARC is involved in a myriad of processes, a technique such as a global gene expression microarray is suitable for detecting changes across these pathways. Therefore, it was hypothesised that SPARC influences that expression of genes associated with tumour initiation, immune cell, and the processes mentioned above. To achieve this, the mRNA of WT and SPARC-KO colitis-induced tumours were extracted as described in Chapter 2, section 2.2.13.1, and the expression level of genes were determined as described in Chapter 2, section 2.2.13.5. In addition, differentially expressed genes were identified analysed via gene ontology as described in section 2.2.13.6, and the expression level of these genes were confirmed via multiplex qPCR, as described in section 2.2.13.12. The outcome of these studies will be described in this chapter.
8.2 Results

8.2.1 Differentially expressed genes between wild-type and SPARC knockout colonic tumours

Of 18,138 genes, only 7 were differentially expressed between SPARC-KO and WT tumours (four tumours per group, one tumour per mouse). Five of these were relatively over-expressed in WT tumours (Table 8.1) and 2 were relatively over-expressed in SPARC KO tumours (Table 8.2).

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold-Change</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sparc</td>
<td>Secreted protein acidic and rich in cysteine</td>
<td>10.2</td>
<td>0.0003</td>
</tr>
<tr>
<td>Indo</td>
<td>Indoleamine-pyrrole 2,3 dioxygenase</td>
<td>4.1</td>
<td>0.03</td>
</tr>
<tr>
<td>Ifit2</td>
<td>Interferon-induced protein with tetratricopeptide repeats 2</td>
<td>1.9</td>
<td>0.03</td>
</tr>
<tr>
<td>Areg</td>
<td>Amphiregulin</td>
<td>1.9</td>
<td>0.04</td>
</tr>
<tr>
<td>Hoxa7</td>
<td>Homeo box A7</td>
<td>1.6</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 8.1. Genes that are relatively over-expressed in wild-type colonic tumours compared to SPARC-knockout colonic tumours with their respective fold-change and p-values.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold-Change</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erdr1</td>
<td>Erythroid differentiation regulator 1</td>
<td>1.9</td>
<td>0.04</td>
</tr>
<tr>
<td>Hamp2</td>
<td>Hepcidin antimicrobial peptide 2</td>
<td>1.7</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 8.2. Genes that are relatively over-expressed in SPARC-knockout colonic tumours compared to wild-type colonic tumours with their respective fold-change and p-values.
8.2.2 Genes selected for confirmation via multiplex qPCR and their respective fold-change and p-values.

The expression levels of Areg, Indo, Ifit2, and Erdr1 were quantified via multiplex qPCR as described in Chapter 2. However, no significant differences were detected in the expression level of these genes between 4 WT and 4 SPARC KO tumours, each from a different mouse (Table 8.3).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Fold-change</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indo</td>
<td>Indoleamine-pyrrole 2,3 dioxygenase</td>
<td>1.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Areg</td>
<td>Amphiregulin</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>Ifit2</td>
<td>Interferon-induced protein with tetratricopeptide repeats 2</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>Erdr1</td>
<td>Erythroid differentiation regulator 1</td>
<td>1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Table 8.3. Fold-change and p-values for Areg, Indo, Ifit2, and Erdr1 between WT and SPARC KO colonic tumours.

8.3 Discussion

8.3.1 AOM/DSS-treated wild-type and SPARC- knockout colons

Of the 18 138 genes, only Ido, Ifit2, Areg, Hoxa7, Erdr1, and Hamp2 were differentially expressed between WT and SPARC-KO tumours. However, no significant differences were detected via qRT-PCR in the expression levels of Indo, Ifit2, Areg, and Erdr1. It should be noted that these tumours were obtained from the same animals that were studied in chapters 6 and 7, which displayed differences in tumour burden and tumour-associated immune cells. Therefore, it is surprising that no differences in the expression of genes were detected. For example, of the six genes that were differentially expressed, we might expect differences in Indo, Ifit2, and Areg due to their roles in cancer. In addition, given that studies in Chapter 6 suggested that SPARC alters tumour initiation, differences in the gene expression of initiation-associated genes might be expected.

INDO is an immunosuppressive enzyme, which is over-expressed in carcinomas including prostatic, bladder, ovarian, colorectal, endometrial and small-cell lung (Uyttenhove, et al., 2003) and correlates with a poor prognosis in endometrial cancer (Ino,
et al., 2008) and CRC (Brandacher, et al., 2006). INDO is positively associated with metastasis in human metastatic pancreatic ductal carcinoma and CRC (Witkiewicz, et al., 2008, Ferdinande, et al., 2012). Plasmacytoid DCs have been stimulated to produce Indo, which leads to T-cell suppression in tumour-draining lymph nodes (Sharma, et al., 2007, Muller, et al., 2008). Indo induces T-reg cell expansion from CD4+ cells, activates resting T-reg cells (Sharma, et al., 2007, Sharma, et al., 2009) and inhibits the proliferation of NK cells and tumour-infiltrating lymphocytes (Brandacher, et al., 2006, Ino, et al., 2008, Spaggiari, et al., 2008).

Low IFIT2 expression is associated with distant metastasis and a poor prognosis in oral squamous cell carcinoma patients (Lai, et al., 2013). In vitro inhibition of IFIT2 induced endothelial-mesenchymal transition, migration, and invasion in oral squamous cell carcinoma cells (Lai, et al., 2013). IFIT2 induces apoptosis in transfected HeLa cell cultures (Stawowczyk, et al., 2011) and is an important mediator in lipopolysaccharide-induced septic shock (Siegfried, et al., 2013). It also enhances the secretion of IL-6 and TNF-α by bone marrow-derived macrophages (Siegfried, et al., 2013) and is involved in antiviral defences (Cho, et al., 2013).

AREG is part of the epidermal growth factor family and is a Th2 cytokine (Zaiss, et al., 2006) and it is an independent prognostic factor of liver metastasis from CRC (Yamada, et al., 2008). Patients with higher AREG expression levels predict a better control of metastatic CRC with cetuximab and a longer progression-free survival (Khambata-Ford, et al., 2007). It mediates prostaglandin E2 (PGE2)-induced cell proliferation in a CRC cell line (Shao, et al., 2003). AREG also promotes the expression of CD147, known alternatively as extracellular matrix metalloproteinase inducer (EMMPRIN). Basigin, the murine version of EMMPRIN, stimulates the production of MMP-2 and 9 in fibroblasts (Li, et al., 2001).

There are several genetic events that are associated with the early phases of colorectal tumourigenesis. For example, mutations are observed in p53, K-ras, and APC (Baker, et al., 1990, Hussain, et al., 2000, Lamllum, et al., 2000). Therefore, it is interesting to note that none of the differentially expressed genes identified are associated to these gene mutations.

The absence of changes in gene expression as confirmed by multiplex qPCR suggests that differences in tumour initiation-associated genes may present as gene mutations, and not as gene expression, in these carcinomas in situ. However, it is not unusual for PCR results
to conflict with gene expression microarray data. The overall accuracy of differential expression decreases for low-level expression and microarrays may have poor sensitivity at detecting small fold-changes, such as 2-fold changes (Wang, *et al.*, 2006), which was the case for Ifit2, Areg, and Erdr2. Ido, on the other hand, had a differential fold-change of 4.1. One study detected a 13 to 16% non-concordance between gene expression microarray and qRT-PCR results, hence, the validation of results via qRT-PCR is strongly recommended (Dallas, *et al.*, 2005). The lack of differences in gene expression can also be explained by negative post-transcriptional regulation mediated by microRNAs, which are non-coding RNAs of 21-22 nucleotides (Lai, 2002). For example, the expression of microRNA-224, which targets apoptosis inhibitor-5, is up-regulated in hepatocellular carcinoma patients, resulting in a reduction in apoptosis inhibitor-5 expression (Wang, *et al.*, 2008).

It should be noted that the difference in expression of SPARC was a mere 10-fold difference and this can be attributed to the binding location of the probes. The probe ILMN_3136561 binds to exon 10, whilst ILMN_3059326 binds exon 2, which are not disrupted in the generation of SPARC-KO mice (Norose, *et al.*, 1998). Therefore, these probes might not detect the true expression levels of SPARC in KO mice.

### 8.4 Conclusion

Despite being associated with altered tumour initiation and immune cell infiltration, SPARC expression does not appear to be associated with gene expression changes. This is unusual, as one would expect gene expression to reflect the initiation of fewer tumours, fewer tumour-associated CD11c⁺, F4/80⁺, and CD4⁺ cells and more tumour-associated CD8a⁺ cells (chapters 6 and 7). Analysing tumours for gene mutations and the presence of micro RNAs might support macroscopic and immunological observations.
Chapter 9

General discussion and conclusion
9.1 General introduction

It is hypothesised that inflammation increases the risk of cancer. For example, there is evidence that prostatitis promotes tumour initiation in the prostate (Dennis, et al., 2002, Kwon, et al., 2014). Similarly, Helicobacter pylori-induced gastritis is associated with gastric cancer risk (Ohata, et al., 2004). In addition, the inflammatory bowel diseases, CD and UC, increase the risk of CRC (Lutgens, et al., 2008). It is important to note that the human gut is susceptible to inflammation caused by a variety of factors such as intestinal pathogens, gut commensal flora, and food antigens such as wheat gluten (reviewed by Macdonald, et al., 2005). However, the mechanisms behind cancer-inducing inflammation are unknown. Therefore, models such as the murine AOM-only model of sporadic CRC and the murine AOM/DSS model of colitis-associated CRC, as described in this thesis, are essential in understanding the effect of chronic inflammation on colorectal tumourigenesis. In addition, as there is evidence that SPARC alters inflammation (Savani, et al., 2000, Said, et al., 2008, Sangaletti, et al., 2011), there is interest in SPARC as a modulator of colitis-associated CRC.

The features of cancer, such as cellular proliferation and apoptosis, tumour dysplasia, and tumour-associated inflammation were compared between a sporadic and inflammatory model of CRC to determine the impact inflammation has on tumourigenesis. Also, to determine the extent by which chronic colitis modifies tumourigenesis, gene expression levels were compared globally between sporadic and colitis-induced tumours. In addition, studies on inflammation-induced CRC in WT and SPARC-KO mice to determine if SPARC alters colitis-induce CRC. These findings will be discussed in the sections below.

9.1.1 Chronic inflammation on tumour initiation and growth

Scientific literature has suggested that a chronically inflamed tumour microenvironment impacts on tumourigenesis. In addition, as discussed in Chapter 1, DNA mutations are also associated with CRC and as AOM-treated mice received higher doses of the colonotrophic carcinogen than AOM/DSS-treated mice, we might expect more AOM-only tumours due to increased DNA mutations. However, this was not the case. In this thesis, tumour incidence was increased while tumour size was decreased in AOM/DSS mice compared to AOM-only mice which might be explained by the following, (i) nitrosative damage and oxidative stress, (ii) the positive association between chronic inflammation and the Iκβ kinase (IKK) complex, and (iii) the increase in tumour-infiltrating
lymphocytes (TILs) as described in Chapter 4. These factors will be discussed in the following paragraphs.

Nitrosative DNA damage occurs during DSS-induced inflammation as indicated by increased levels of nitrotyrosine, an indicator of nitric oxide (NO) production (Suzuki, et al., 2006), and inducible nitric-oxide synthase, an enzyme that synthesises NO (Shaked, et al., 2012). In addition to causing DNA damage, NO stabilises hypoxia-inducible factor (HIF)-1α, which promotes angiogenesis and tumour development (Riano, et al., 2011, Chowdhury, et al., 2012).

Another explanation for the increase in tumour incidence in AOM/DSS mice is the positive association between chronic inflammation and the IKK complex. IKK activates the transcription factor, nuclear factor (NF)-κβ, by phosphorylating NF-κβ bound Iκβs, thus targeting them for ubiquitin-dependent degradation. Freed NF-κβ dimers are then able to translocate into the nucleus, resulting in the transcription of pro-inflammatory genes (Li, et al., 1999). One study investigated the role of IKKβ in CAC through the tissue-specific deletion of IKKβ in enterocytes and macrophages in an AOM/DSS mouse model of CAC and demonstrated that IKKβ was associated with increased colitis-associated tumour incidence and reduced enterocyte apoptosis (Greten, et al., 2004). This mechanism might explain the reduction in apoptotic cells seen in the experiments reported in Chapter 3, and supports the observation of more AOM/DSS tumours due to higher numbers of F4/80+ cells than AOM-only tumours as reported in Chapter 4. In addition, the activation of NF-κB stimulates cellular proliferation and inhibits apoptosis (Li, et al., 1999) with the balance between these processes being essential for tumour progression. AOM-only tumours were found to be larger than those from AOM/DSS animals, which correlates with the finding that AOM-only polyps contain a greater number of proliferating cells than AOM/DSS polyps. However, it should be noted that there were also 1.9-times fewer apoptotic cells in AOM/DSS polyps than AOM-only polyps, supporting an anti-apoptotic function of NF-κβ as a product of chronic inflammation.

Another possible explanation for a reduction in tumour size in the AOM/DSS model might be the increase in TILs in AOM/DSS tumours. TGF-β production in TILs suppressed colonic tumour growth via IL-6 production and subsequent IL-6 signal transduction (Becker, et al., 2004). As demonstrated in Chapter 3, AOM/DSS tumours are smaller than AOM-only tumours, which supports the notion that TILs in AOM/DSS tumours might be producing TGF-β.
The AOM-only and AOM/DSS models allow certain aspects of tumourigenesis to be studied, such as the impact of inflammation on tumour growth, tumour cell death and proliferation, and immune cell infiltration as demonstrated in this thesis. Therefore, detailed studies in these areas and a comparison between both animal models will shed light on the involvement of inflammation on tumourigenesis.

9.1.2 Genetic differences caused by chronic inflammation

As described in Chapter 5, a comparison in the global gene expression levels between the sporadic and chronic colitis models of CRC allowed for the identification of differentially expressed genes. These genes might be associated with differences observed between AOM-only and AOM/DSS tumours as discussed in the sections above. Of these genes, *Cd164* and *Csf1r* were confirmed to be over-expressed in AOM/DSS tumours. Therefore, a discussion of these genes and the mechanics that link them to the observations made in chapters 3 and 4 will be presented in the following two sections.

9.1.2.1 Inflammation-induced tissue damage and tumourigenesis

Understanding the link between the increase in *Cd164* gene expression and chronic inflammation, as shown in Chapter 5, might hint at the pathogenic pathways involved in the AOM/DSS model of colitis-induced colorectal tumourigenesis. As DSS is a tissue irritant causes tissue damage, the expression of the alarmin, high-mobility group box 1 (HMGB1), which is a nuclear protein that is released by necrotic and severely stressed cells such as in DSS-induced colitis (Maeda, *et al.*, 2007), might explain the increase in *Cd164* and *Csf1r* in AOM/DSS tumours. Evidence for this will be discussed below.

CD164 expression is induced by stromal cell-derived factor (SDF)-1 (Sun, *et al.*, 2007), also known as CXCL12, which is a chemokine that is chemotactic to neutrophils. However, there is no association between CXCL12 mRNA expression and colonic inflammation in UC (Katsuta, *et al.*, 2000). On the other hand, there is an association between the alarmin, HMGB1, and CXCL12. HMGB1 forms a complex with CXCL12 thus protecting it from degradation (Campana, *et al.*, 2009) which might promote *Cd164* expression as described in Chapter 5. Also, it triggers signalling via CXCR4, the CXCL12 receptor (Molyneaux, *et al.*, 2003) promotes the recruitment of mononuclear cells into mice muscles injured via the creation of sterile air pouches (Schiraldi, *et al.*, 2012).
Therefore, this mechanism might explain the increase in F4/80$^+$ cells in AOM/DSS tumours as described in Chapter 4.

Furthermore, the inhibition of HMGB1 by either dipotassium glycyrrhizate or ethyl pyruvate can reduce murine colitis (Dave, et al., 2009, Vitali, et al., 2013), while a neutralising anti-HMGB1 antibody reduces both tumour incidence and size in an APCMin/+ DSS mouse model of CAC (Maeda, et al., 2007). Therefore, the increased \textit{Cd164} gene expression in AOM/DSS tumours might be a result of HMGB1-induced CXCL12 expression. HMGB1 is also detectable in human specimens. Stool samples from children suffering from IBD contain significantly higher levels of HMGB1 when compared to controls (Vitali, et al., 2011).

These studies suggest that tissue damage resulting from DSS-induced chronic inflammation might initiate AOM/DSS tumourigenesis, resulting in the differences observed between AOM/DSS and AOM-only tumours.

9.1.2.2 PI3K pathway and colitis-induced tumourigenesis

As mentioned in the previous section, the increase in \textit{Cd164} expression might be associated with the expression of both CXCL12 and CXCR4. As both CXCL12 and CXCR4 are associated with the phosphatidylinositol 3-kinase (PI3K)-Akt-pathway (Zheng, et al., 2007, Ping, et al., 2011), the PI3K-Akt pathway might be associated with the observations made in chapters 3, 4, and 5, and will be discussed in the following paragraphs.

\textit{In vitro} studies have revealed that CD164 and the chemokine, CXCR4, co-immunoprecipitate after CXCL12 stimulation in the presence of fibronectin, and that silencing or inhibiting CD164 significantly inhibits cell migration (Forde, et al., 2007). Also, silencing CD164 attenuates phosphorylated-protein kinase B (p-Akt) and p-protein kinase C-ζ (p-PKC-ζ), but not p-p42/44 MAPK signalling through CXCR4 (Forde, et al., 2007). On the other hand, over-expressing CD164 in human ovarian surface epithelial cells has been shown to increase chemokine CXCR4, 7, and CXCL12 expression, which induced the PI3K-Akt-pathway (Huang, et al., 2013). Both CXCR4 and CXCL12 were noted, however, to be down-regulated in primary CRC and CRC metastases compared to normal colonic tissues (Brand, et al., 2005).

In addition, IBD patients and mice with DSS-induced colitis express higher levels of CXCR4 compared to controls, with CXCR4 expression on peripheral T-cells from IBD
patients also being significantly higher and positively correlating with disease progression (Mikami, et al., 2008). Also, both CXCL12 and CXCR4 are over-expressed in DSS-induced murine colitis and CXCR4 antagonists reduce inflammation in the same mice (Mikami, et al., 2008). This suggests that CXCL12 and CXCR4 may play a role in CAC tumourigenesis, and that blocking either CXCL12, or CXCR4, might reduce inflammation in IBD patients and, hence, the risk of cancer.

Interestingly, the PI3K-Akt pathway is also involved in CSF1R signalling, another gene that was confirmed to be significantly over-expressed in AOM/DSS tumours. CSF1 is the ligand for CSF1R, alternatively known as macrophage-colony stimulating factor (M-CSF), and is involved in the differentiation of macrophages from monocytes, and the production of Langerhan cells and osteoclasts (Dai, et al., 2002). Binding of CSF1 to CSF1R initiates a phosphorylation cascade resulting in increases in gene transcription and protein translation, which induces cytoskeleton remodelling via multiple signalling pathways, resulting in increased survival, proliferation and differentiation of target cells. CSF1 also promotes cell proliferation as the stimulation of bone marrow-derived macrophages with CSF1 promotes entry into the S-phase of the cell cycle (Tushinski, et al., 1985). Therefore, the increase of Csf1r in AOM/DSS mice confers a higher chance of increased cellular proliferation. However, this was not observed in AOM/DSS tumours, suggesting that Csf1r signalling was not activated.

The terminal differentiation of macrophages involves the coordination of Akt, and activation of extracellular receptor kinases (ERK) 1 and 2, which are controlled by CSF1 and CSF1R (Jacquel, et al., 2009). The activation of the PI3K-Akt pathway activates caspases that drive the monocyte to macrophage differentiation process by cleaving specific proteins and regulating gene transcription (Jacquel, et al., 2009). PI3K-Akt-induced differentiation of monocytes to macrophages may explain why, in the work reported in Chapter 4, CAC tumours contained greater numbers of F4/80+ macrophages, when compared to CRC tumours.

The activation of the PI3K/Akt pathway in target cells by both CD164 and CSF1R could result in increased cell survival by reducing cell death (Brunet, et al., 1999). Conversely, the in vitro inhibition of PI3K/Akt signalling-induced apoptosis (Francois, et al., 2005, Greenhough, et al., 2007, Lue, et al., 2007), may explain the reduction in the number of apoptotic cells in CAC tumours when compared to CRC tumours described in Chapter 3.
Taking together the results from chapters 3, 4, and 5, and the studies mentioned above, PI3K-Akt signalling pathway might be activated in the AOM/DSS model of colorectal tumourigenesis. As the PI3K-Akt signalling pathway is activated in cancer (reviewed by Chang, et al., 2003, reviewed by Fresno Vara, et al., 2004, reviewed by Wong, et al., 2010), the AOM/DSS model represents a good tool for exploring the outcome of activating and blocking PI3K/Akt signalling in colorectal cancer.

9.1.3 Inflammation in AOM-only tumours

It is of interest to note that AOM-only lesions described in this thesis also contained immune cells. The efficacy of anti-inflammatory drugs in treating sporadic polyps in the human colons suggests that these sporadic lesions might contain an inflammatory microenvironment. Therefore, inflammation might be important in sporadic colorectal tumourigenesis and several examples will be described below.

For example, the administration of curcumin, an anti-inflammatory agent, in AOM-only mice prior to, during, and after tumour initiation, and also during late-stage tumour development, reduced the overall numbers of tumours compared to untreated AOM-only mice (Kawamori, et al., 1999). Sulindac, a non-steroidal anti-inflammatory drug (NSAID) that inhibits prostaglandin synthesis, is well known to induce regression of sporadic colorectal adenomatous polyps (Matsuhashi, et al., 1997). A 20-year follow-up of five randomised trials also suggested that low doses of aspirin reduced the long-term incidence and mortality secondary to CRC (Rothwell, et al., 2010). Low oral doses of difluoromethylorinthine combined with aspirin also reduced the recurrence of colorectal adenomas in a placebo-controlled, double-blinded study (Meyskens, et al., 2008).

9.1.4 SPARC and CAC

Despite differences in colitis-induced tumours between SPARC-KO and WT mice (chapters 6 and 7), namely the reduction in tumour numbers, fewer tumour-associated dendritic cells and macrophages in WT mice when compared to SPARC-KO mice, some studies suggests that the SPARC-KO model might develop contradictory results. As SPARC is crucial in the development of bone, SPARC-KO mice develop extramedullary haematopoiesis as a result of severe osteopaenia in SPARC-KO mice (Visnjic, et al., 2004) which may alter tumourigenesis (Lee, et al., 2003). Therefore, the use of SPARC-KO mice obscures the exact role of SPARC on colitis-associated tumourigenesis. However,
as SPARC might be associated with metastasis, and calcium-related cell motility and CRC risk, it remains clinically relevant and some examples will be discussed below.

Inflammatory cell-derived SPARC appears to be necessary for metastases to occur in mice and macrophage-derived SPARC induces metastasis (Sangaletti, et al., 2008). Alternatively activated macrophages endocytose SPARC for internal degradation via stabilin-1, highlighting a self-regulatory role of maintaining homeostatic levels of SPARC (Kzhyshkowska, et al., 2006a, Kzhyshkowska, et al., 2006b). Stabilin-1 is a scavenger receptor, which is expressed by tissue macrophages and different subtypes of endothelial cells during chronic inflammation and tumourigenesis, and is also involved in clearing apoptotic cells (reviewed by Kzhyshkowska, et al., 2006, Schledzewski, et al., 2006, Park, et al., 2009). It is, therefore, possible that a dysfunctional stabilin-1-mediated clearance of SPARC promotes metastasis and represents a candidate for predicting prognosis and therapy.

As SPARC is a calcium-binding protein, the absence of SPARC may alter extracellular and intracellular calcium levels. For example, intracellular levels of calcium regulate neutrophil motility by binding to integrins αv and β3 and inhibiting motility on vitronectin (Hendey, et al., 1996). However, no differences in the number of neutrophils were detected between the WT and SPARC-KO tumours, suggesting that intratumoural calcium levels were not significantly affected. There is also contradictory evidence regarding the benefit of calcium and/or vitamin D supplementation on reducing CRC risk in patients. Moderate amounts of calcium were associated with reduced distal colon cancer risk in male and female health professionals in two randomised trials with follow-up durations of 10 to 20 years (Wu, et al., 2002). A four-year population-based, double-blind, randomised placebo-controlled study on post-menopausal women also associated a reduction in CRC risk with calcium and vitamin D supplementation (Lappe, et al., 2007). On the other hand, a six-year randomised-controlled trial involving individuals over the age of 70 years, and a four-year randomised, blinded and placebo-controlled study involving post-menopausal women, reported no association between calcium and/or vitamin D supplementation and a reduced risk of CRC (Wactawski-Wende, et al., 2006, Avenell, et al., 2012). Thus, determining how SPARC impacts on calcium levels might explain the association of SPARC and patient prognosis.
Regardless of the need for a better SPARC model, SPARC remains relevant in cancer biology and the following section will discuss methods to obtain a more appropriate SPARC model.

9.1.5 Future directions

To follow-up on the findings described in this thesis, the following paragraphs will provide suggestions that will further our understanding of inflammation and colorectal tumourigenesis. For example, (i) the use of small animal imaging to improve the study of tumour development, (ii) the use of additional immune cell-specific markers and detection via flow cytometry, (iii) the quantification of proteins encoded by differentially expressed genes identified in chapters 5 and 8, (iv) the study of SPARC-associated proteins and their association with colorectal tumourigenesis, (v) the improvement of the SPARC-KO model, and lastly, (vi) the use of an alternate murine model of CAC which develop tumours that closely resemble the histology of human CAC. These suggestions will be discussed in detail in the following paragraphs.

Small-animal molecular imaging methods provide a non-invasive method for detecting very early stage tumours in vivo, and can accurately determine the rate of proliferation, apoptosis, and development. In vivo imaging using fluorescent probes detect microscopic tumours under 300 μm in diameter from fluorescent signals that are emitted from tumour cells (Weissleder, et al., 1999). By using the mini-endoscopy system, biopsies of tumours of varying sizes could also be used to sequence genomic DNA and to identify mutations to common oncogenes such as p53, K-ras, and APC to confirm if early, and late, stage mutations in human CRC and CAC are reflected in the FVB and C57Bl/6 mice. In addition, immunohistochemistry could be used to identify β-catenin in tumour biopsies as mutations to APC are associated with either increased protein levels or nuclear localisation of β-catenin (Munemitsu, et al., 1995, Alman, et al., 1997, Kariola, et al., 2005).

In chapters 4 and 7, only one immune cell marker was used to identify the innate and adaptive immune cells, which limited the ability to fully characterise the tumour-associated immune cells. To identify them more accurately, the co-localisation of multiple specific antigens would be required, and this is something to be considered for future work. T_{17} cells are of particular interest as they promote intestinal inflammation, and can be identified by the co-expression of IL-23R, CCR6, and RORγt (Annunziato, et al., 2007, Kleinschek, et al., 2009, Leppkes, et al., 2009).
Flow cytometry allows the simultaneous analysis of multiple antigens per sample, making this technique suitable for analysing the immune cell population within tumours. The tumours that develop in either the AOM, or AOM/DSS, models in both C57Bl/6 and FVB mice, however, were too small and did not contain sufficient immune cells for single-sample analysis. The use of multiple antigens, or labels, could also be performed using immunofluorescence, and such studies have reported success with up to triple and quadruple labelling (Reddy, et al., 2001, Lindl, et al., 2007). For example, the concurrent staining of immune cells for F4/80, CD11c, and CD206 can used to differentiate M1 from M2 macrophages (Nishimura, et al., 2009) and can determine which sub-type of macrophages promote tumourigenesis. In addition, flow cytometry can be used to detect intracellular cytokines of tumour-associated immune cells (Duramad, et al., 2004). This is important as the cytokine profiles might indicate the T-helper cell lymphocyte profile, and has been associated with cancer prognosis and prediction of treatment efficacy (reviewed by Fridman, et al., 2011, Yoshida, et al., 2016). Furthermore, flow cytometry can also be used to detect intracellular and membrane-bound chemokines, which have been implicated in CRC progression and prognosis (Murakami, et al., 2013, Rupertus, et al., 2014). It should be noted that an advantage of immunofluorescence over flow cytometry is the ability to identify the cellular location of immune cells. This is important as the cellular location of immune cells has been associated with clinical outcome in human CRC (Galon, et al., 2006).

To follow-up on the gene-microarray results, the expression level of proteins that are encoded by the genes identified in chapters 5 and 8, including the non-significantly different genes, could be quantified. mRNA and protein levels may not correlate and differences might estimate contributions of post-transcriptional and post-translational regulation of a gene (Anderson, et al., 1997, Chen, et al., 2002, Greenbaum, et al., 2003). In addition, gene expression analyses on individual tumour components, such as the tumour stroma, tumour epithelial cells, and tumour-associated immune cells, might inform on the specific role of SPARC on different tumour components in relation to studies conducted in chapters 3, 4, 6, and 7.

2010), and also predicts a poor prognosis in SATB2-negative CRC (Nodin, et al., 2012). Also, SATB1 regulates the expression of SPARC (Li, et al., 2007). Therefore, it is of interest to quantify expression levels of SATB1, 2, and SPARC to determine if there are correlations with colorectal tumorigenesis in mouse models.

As evident from the studies described in chapters 6 and 7, mice devoid of SPARC developed colorectal tumours that contain an immune cell population that is different to WT mice. Despite changes in the site of haematopoiesis, the role of SPARC in ECM organisation may also impact on the infiltration of immune cells into tumours. It is thus of interest to determine if the ECM is altered between WT and SPARC-KO tumours and to determine if these changes are associated with the expression levels of genes identified through microarray gene expression (Chapter 8.2.3), namely Indo, Areg, Ifit2, Hoxa7, Erdr1, and Hamp2. In addition, the association of SPARC and genes that were not identified through microarray gene expression, such as MCP-1, which is associated with immune cell migration (Said, et al., 2008), should be confirmed.

SNP haplotypes in the SPARC 3’ untranslatable region are associated with idiopathic osteoporosis in Caucasian men (Delany, et al., 2008), and it is of interest to determine if these individuals have altered bone marrow haematopoiesis, and whether this affects cancer prognosis.

To assess the difference in extramedullary haematopoiesis between WT and SPARC-KO animals, CD34, CD133, and CD117 could be used to identify haematopoietic progenitor and precursor cells in the liver, spleen, mesenteric lymph nodes, and thymus (Krause, et al., 1994, Kaplan, et al., 2005). Likewise, these tissues could be labelled with antibodies that recognise a panel of antigens, which typically characterise the subpopulations of immune cells. For example, F4/80, CD11c, and CD206 could be used to differentiate M1 from M2 macrophages (Luo, et al., 2006, Nishimura, et al., 2009).

To improve on the SPARC-KO model, the use of a conditional knockout system such as the Cre-lox system might reduce the potential confounding effect of extramedullary haematopoiesis on SPARC-associated tumourigenesis. The benefit of using the Cre-lox system is that the conditional expression of Cre recombinase allows a Cre-lox deletion to occur in a specific organ, such as the intestine, as demonstrated by the creation of mice expressing a β-catenin mutation solely in the intestine. Using this method, exon 3 of the β-catenin gene was deleted in the intestine and when mice heterozygous for this mutation were examined, polyposis was present in the small intestine and not the caecum or colon,
although nascent microadenomas were found in the colonic mucosa (Harada, et al., 1999). In addition, the Cre-lox method results in stable gene inactivation in the intestine in adult mice (Hayashi, et al., 2002).

SPARC expression can be down-regulated in cells using microRNA29a (Zhu, et al., 2012). MicroRNAs are non-coding RNAs of 21-22 nucleotides that mediate negative post-transcriptional regulation (Lai, 2002). Also, short-interfering RNAs (siRNA), which are double-stranded RNAs of 20-25 nucleotides in length, can be used to reduce SPARC expression. siRNAs suppress gene expression by guiding nucleases in a sequence-specific manner (Baulcombe, 2006), and in our lab, the dose-dependent silencing of SPARC expression using siRNA was demonstrated in murine intestinal fibroblasts.

As inflammation is important in sporadic colorectal tumourigenesis, it is of interest to test the effect of SPARC on AOM-only colorectal tumourigenesis. Interestingly, SPARC has been implicated in therapy-refractory CRCs and it has been shown that upregulating SPARC expression re-sensitises tumours to chemotherapy (Tai, et al., 2005). Therefore, further work on the role of SPARC in CRC mice might improve current knowledge of SPARC in CRC pathology.

Regarding clinically-relevant animal models, a DSS-only model develops tumours that resemble human CAC lesions more closely than the AOM/DSS model (Cooper, et al., 2000). However, the total duration of only 204 days makes it a challenging model to adopt. An alternate DSS/AOM model was trialled, but only one round of DSS was tested before an AOM injection, which did not result in polyp formation, possibly due to the lack of chronic colitis (Tanaka, et al., 2003). An alternative is to induce chronic colitis with three cycles of DSS before the intraperitoneal injection of AOM, and to maintain mice on cycles of DSS to establish a chronically inflamed microenvironment during tumour development. These lesions can then be compared to the AOM/DSS model to assess the impact of a different timing for AOM. It should be noted that different doses of AOM in the AOM-only and AOM/DSS models might impact on observations. To assess these differences, a comparison should be made between an AOM-only model using a higher dose and one using a lower dose to study the impact of AOM on tumour growth, tumour immunology, and genetic mutations.
9.1.6 Conclusion

The animal models studied in this thesis demonstrate the importance of inflammation in colorectal tumourigenesis and, potentially, the involvement of the PI3K-Akt signalling pathway in CAC. These studies support the role of inflammation in the promotion of cancer. The different composition of AOM tumour-associated immune cells compared to colitis-associated tumours demonstrates the complexity of the role of inflammation in tumourigenesis, reinforcing the need for continual research using appropriate disease models.

The decreased number of initiated tumours and the altered composition of tumour-associated immune cells caused by SPARC demonstrate a possible role in colitis-associated colorectal tumourigenesis. In addition, the possibility of SPARC having regulatory roles outside that of gene expression suggest that other pathways will need to be analysed to understand the influence of SPARC on tumour development.
Chapter 10

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