THE ROLE OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR ALPHA (PPARα) IN THE EFFECT OF PIROXICAM ON COLON CANCER

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BSc. (Hons), MSc.

Thesis submitted to the University of Nottingham
for the degree of
Doctor of Philosophy

April 2015

Abstract

Studies with $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice were undertaken to investigate whether polyp development in the mouse gut was mediated by PPARα. Additionally, the effect of piroxicam treatment dependency on PPARα was assessed.

Results showed the number of polyps in the colon was significantly higher in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice than in $APC^{Min/+}$ mice, whilst in the small bowel the difference was not significant.

Analysis of gene expression in the colon with Affymetrix® microarrays demonstrated the largest source of variation was between tumour and normal tissue. Deletion of PPARα had little effect on gene expression in normal tissue but appeared to have more effect in tumour tissue.

Ingenuity pathway analysis of these data showed the top biological processes were growth & proliferation and colorectal cancer. Collectively, these data may indicate that deletion of PPARα exacerbates the existing $APC^{Min/+}$ mutation to promote tumorigenesis in the colon.

95 genes from Affymetrix® microarray data were selected for further analysis on Taqman® low density arrays. There was good correlation of expression levels between the two array types. Expression data of two genes proved particularly interesting; Onecut homeobox 2 (Onecut2) and Apolipoprotein B DNA dC → dU - editing enzyme, catalytic polypeptide 3 (Apobec3). Onecut2 was highly up-regulated in tumour tissue. Apobec3 was up-regulated in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice only; suggesting expression was mediated *via* PPARα.

There was a striking increase in survival accompanied by a marked reduction in small intestinal polyp numbers in mice of either genotype that received piroxicam. Taqman® low density array analysis of the same 95 genes as previously showed similar expression levels in piroxicam-treated $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice. Taken together, these data indicated that the effect of piroxicam treatment was not mediated *via* PPARα.

Declaration

The work in this thesis was performed entirely by myself (unless otherwise stated) and in no way forms part of any other thesis. The work was carried out at the Nottingham Digestive Diseases Centre (NDDC), Queens Medical Centre, University Hospital, Nottingham, and the School of Biomedical Sciences, University of Nottingham, under the joint supervision of Professor C.J. Hawkey and Dr A.J. Bennett.

Karen Marie McCartney

Publications & Presentations

Publications

- *Gut* 2011, **60** A115-A116
- *Gastroenterology* 2010 **138** (5) S502
- *Gastroenterology* 2009, **136** (5) A618-A619
- *Gastroenterology* 2009, **136** (5) A752-A753
- *Gastroenterology* 2008, **134** (4) A304

Presentations

- British Society of Gastroenterology 14/03/11 – 17/03/11 Birmingham NCC

  *Poster submitted 'Substantial chemo-preventative effect of piroxicam in Min mice independent of peroxisome proliferator activated receptor alpha'

  Awarded poster of distinction

- United European Gastroenterology Week 23/10/10 – 27/10/10 Barcelona, Spain

  Poster submitted 'Substantial chemo-preventative effect of piroxicam in Min mice independent of peroxisome proliferator activated receptor alpha'

- Digestive Diseases Week 1/05/10 – 5/05/10 New Orleans, LA

  Poster submitted 'Increased longevity in polyp-bearing mice treated with piroxicam'

- European Association of Cancer Research Symposium (EACR) 3/07/09 Nottingham

  Poster submitted 'The role of PPAR alpha in the effects of NSAIDs upon colon cancer'

Abbreviations used in this thesis

AA	Arachidonic acid
APC	Adenomatous polyposis coli
ASPA	Animals (Scientific Procedures) Act 1986
BLAST	Basic local alignment search tool
BMSU	Biomedical Science Unit
Cox	Cyclooxygenase
Cyp	Cytochrome P450
FAP	Familial adenomatous polyposis
IPA	Ingenuity pathway analysis
IPKB	Ingenuity Pathway Knowledge Base
Lox	Lipoxygenase
Min	Multiple intestinal neoplasia
NCBI	National Centre for Biotechnology Information
NSAIDs	Non-steroidal anti-inflammatory drugs
PPAR	Peroxisome proliferator activated receptor
PPRE	Peroxisome proliferator response element
RT Q PCR	Real Time Quantitative Polymerase Chain Reaction
UTR	Untranslated region

Acknowledgements

I would like to dedicate this research to my four children, Emma, Lisa, Zoe and Luke, and to the memory of their dad, Les.

I would like to thank my two supervisors, Professor Christopher Hawkey – Chris, and Dr Andrew Bennett – Andy, for their insight, support, help and encouragement during my research and personal difficulties.

I would also like to acknowledge and thank the Medical Research Council, and the School of Clinical Sciences, University of Nottingham, for funding this research.

I would like to say thank you to my children for championing their mum throughout the years of my study. Thank you also to Richard, for his unswerving support - and the odd glass or two of wine! To my dad, I would like to say – I have done it! When the going got tough, I kept going. Thank you for having faith in me.

A big thank you to all FRAME lab members especially Elke Gottschalg for her 'listening ear' and support, and Monika Owen and Nicki De Vivo, for their assistance and constructive advice in the lab.

Thank you to all of the staff in the Biomedical Support Unit for their help with my mouse studies, especially Mark Trussell and Neil Yates.

And thank you to Dr Emma King, Denise Christie and Ian Ward in the Advanced Microscopy Unit, for help and instruction with microscopes and staining techniques.

I would like to say a special thank you for the support and compassion shown to me by the late Professor Sue Watson.

Lastly, but by no means least, to my friends and colleagues in the Nottingham Digestive Diseases Centre, and especially Diane Stevenson, Jen Dumbleton and Rosemary Dainty, I would like to say thank you for being there to chat about life, music, theatre *etc. etc.* - anything but '*the PhD!*'

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1 Introduction

Colon cancer (colorectal or bowel cancer) is the third most common cancer in the United Kingdom (UK), with 22,517 male, and 17,864 female (based on 3 year average) new cases diagnosed annually between 2008-2010.

Incidences of cancer diagnoses in order of prevalence were prostate, lung and colon cancer in males, and breast, lung and colon cancer in females (Office for National Statistics, 2012).

Colon cancer was also the third most common cause of mortality in both sexes. Average annual deaths were 8,569 in males and 7,207 in females within the same time period. Lung cancer was the largest cause of mortality from cancer in both sexes, with prostate cancer in males, and breast cancer in females being the second largest causes (Office for National Statistics, 2012).

Worldwide, colon cancer is the third most common form of cancer, and the second leading cause of cancer-related mortality (Ferlay et al., 2010).

The human cost revealed by these statistics makes for sobering reading and shows how continued research into improving detection and treatment of cancer is necessary.

An earlier study by (Jackson et al., 2003) identified peroxisome proliferator activated receptor alpha (PPARα) activation as important in preventing neoplastic transformation and growth in the colon of $APC^{Min/+}$ mice.

The work described and discussed in this thesis builds on the findings of Jackson, to investigate and identify pathways for therapeutic intervention of colon cancer. The $APC^{Min/+}$ mouse model was used, both with and without a functional PPARα gene. Mice were also treated with/without the non-selective cyclooxygenase (Cox) inhibitor piroxicam.

1.1 Function, structure & histology of the small bowel & colon

The small bowel has three parts, the duodenum, jejunum and ileum. It is the major site of digestion and absorption. The major functions of the colon are reabsorption of electrolytes and water, and the elimination of undigested food and waste.

The small bowel and colon are comprised of layers of mucosa, sub-mucosa, muscularis externa and either a serosa or adventitia.

The mucosa is made up of three parts; the epithelium and basement membrane, lamina propria and muscularis mucosa.

The epithelium lines organ cavities and forms glands. The basement membrane anchors the epithelial layer to the underlying connective tissue. As epithelial layers are avascular and innervated, nutrients diffuse through the basement membrane which acts as a selectively permeable membrane.

Epithelial layers in both the small bowel and colon are comprised of densely packed simple columnar, non-ciliated cells, with very little intercellular space. The main cell types are:

Enterocytes; these cells have a life-span of 5 – 6 days and are absorptive cells. Each cell has ~ 3000 microvilli at the luminal surface. In the small bowel enterocytes absorb amino acids and monosaccharides by active transport, and monoglycerides and fatty acids by passive transport. In the colon, active transport of electrolytes and passive absorption of water occurs in these cells.

Goblet cells; these cells have a life-span of 5 – 6 days and secrete mucus. The mucus acts as a lubricant to facilitate passage of the food bolus and faecal matter. In the colon, goblet cells are more abundant in the crypts of Lieberkühn (crypts) and the number increases distally towards the rectum.

Paneth cells; these cells have a life-span of ~ 4 weeks and are only found at the base of crypts. They produce the anti-bacterial enzyme, lysozyme. Paneth cells are usually absent in the colon.

Enteroendocrine cells; these cells produce cholecystokinine (CKK), secretin and gastric inhibitory peptide (GIP – also known as glucose-dependent insulinotropic peptide). CKK stimulates pancreatic enzyme secretion and gall bladder contraction. Secretin stimulates pancreatic and biliary bicarbonate secretion. GIP induces insulin secretion (Baggio and Drucker, 2007). Enteroendocrine cells are rarely found in the colon.

Microfold (M) cells; these cells endocytosise antigens and transport them to the underlying lymphoid cells where the immune response is initiated. In the small bowel they overlie Peyers patches. Peyers patches are aggregated lymphoid nodules found in the ileum.

Undifferentiated cells; these are stem cells and are found at the base of crypts.

The lamina propria is a loose, connective tissue with numerous blood and lymphatic vessels. Gut-associated lymphatic tissue (GALT) acts as an immunological barrier to pathogens. Cells in the lamina propria include lymphocytes, plasma cells, eosinophils and macrophages. In the colon, extensive development of GALT reflects the abundance of micro-organisms.

The muscularis mucosa is a thin layer of smooth muscle separating the lamina propria from the sub-mucosa.

There are major differences in the mucosa in the small bowel and colon. In the small bowel, the mucosa is highly modified with the luminal surface covered by villi. The core of the villi is an extension of the lamina propria and is covered in simple, columnar epithelium. Crypts open onto the surface of the lumen at the base of the villi. The crypts extend downwards into the muscularis mucosae and are also covered in simple, columnar epithelium. Colonic mucosa has no villi and has numerous, straight, tubular glands that extend to the muscularis

mucosae. The glands are also covered with a simple, columnar epithelium.

The sub-mucosa is a dense, irregular connective tissue which contains blood and lymph vessels, nerve fibres and glands. In the duodenum, Brunner's glands secrete alkaline glycoproteins and bicarbonate ions which neutralise acid from the stomach and raise the pH for pancreatic enzyme activity.

The muscularis externa allows for movement that is independent of the digestive tract. It is comprised of an inner circular layer, and an outer longitudinal layer. In the colon there are three very thick bands called teniae coli; these allow segments of the colon to contract independently and facilitate mass movement of colonic contents.

The serosa or adventitia is the outermost layer which comprises of a small amount of connective tissue that contains major blood vessels and nerves (van Lommel, 2003, Seeley, 2002).

1.2 Early detection & screening for colon cancer

Several studies have shown that screening programmes for colon cancer are both beneficial (Levin et al., 2008, Hewitson et al., 2008, Atkin et al., 2010) and cost-effective (Heitman et al., 2010) in the average-risk population.

Screening methods entail either stool testing, flexible sigmoidoscopy or colonoscopy. Stool tests such as faecal immunochemical testing (FIT) and the guaiac faecal occult blood test (gFOBT) detect haemoglobin in occult blood but are less effective than sigmoidoscopy and colonoscopy in neoplastic detection. However, evidence appears to indicate these tests are more readily accepted for screening programmes (Hol et al., 2010, Quintero et al., 2012). This is likely to be due to the invasive nature of sigmoidoscopy and colonoscopy.

Colonoscopy is considered to be the optimum procedure for early detection and prevention of colon cancer. Several population-based case-control studies[1] suggest incidence and mortality due to colon cancer were substantially reduced when colonoscopy screening was routinely employed (Brenner et al., 2011b, Brenner et al., 2010, Baxter et al., 2009). Also, recent evidence appears to support the use of screening colonoscopy; several studies report that patients with no detected abnormalities on a previous colonoscopy have a substantially reduced risk of developing colon cancer (Brenner et al., 2010, Brenner et al., 2011a, Imperiale et al., 2008).

Polypectomy[2] has proven to be an effective prophylactic treatment in prevention of colon cancer. Cohort studies[3] with patients presenting with adenomas have suggested the procedure can prevent approximately 80% of colon cancers (Citarda et al., 2001, Zauber et al., 2012).

1.3 Pathogenesis of colon cancer

The development of colon cancer can be affected by both hereditary conditions which predispose to cancer, and environmental factors such as diet.

However, colonic tumours arise due to an accumulation of genetic changes including mutational activation of oncogenes and mutational inactivation of tumour suppressor genes; reviewed in (Fearon and Vogelstein, 1990, Kinzler and Vogelstein, 1996).

Figure 1-1 (page 7) shows a schematic diagram of genetic changes associated with progression of normal epithelium through to development of colon cancer and metastasis.

[1] Case-control studies investigate existing records of medical conditions (in lieu of new information). Control is obtained by comparison to people without the condition
[2] Polypectomy: Colonoscopic removal of adenomatous polyps
[3] Cohort study investigates a linked group of people exposed to a variable over time. They are compared to a similar group not exposed to the variable

Mutations in the tumour suppressor gene Adenomatous Polyposis Coli (APC) are the first event in colorectal cancer formation. K-ras is an oncogene that only requires a single genetic event for its activation. It is a GTPase involved in signal transduction. K-ras mutations are predictive of a poor response to the Epidermal Growth Factor Receptor (EGFR)-inhibiting drugs panitumumab and cetuximab that are used in the treatment of metastatic colon cancer (Dempke and Heinemann, 2010).

Mutations in DNA mismatch repair enzymes (MMR) accelerate the progression to tumour development.

p53 is also a tumour suppressor gene requiring two genetic events (one in each allele) for inactivation. Other genes that have been implicated in colorectal neoplasia are the tumour suppressor genes DCC (deleted in colorectal carcinoma), DPC4 (SMAD4 - mothers against decapentaplegic homolog 4[4]) and JV18-1 (SMAD2 - mothers against decapentaplegic homolog 2). The sequence of these events and possible other unidentified genetic changes may be accountable for the heterogeneity of characteristics seen in different cancers (Fearon and Vogelstein, 1990, Kinzler and Vogelstein, 1996). Cyclooxygenase-2 (Cox-2) mutations are not acquired during the progression to cancer. However, expression of Cox-2 progressively increases which directly correlates with colorectal adenomatous polyp size and grade of dysplasia (Sheehan et al., 2004). Therefore, inhibition of Cox-2 is a potential target for the arrest or reduction of the progression to carcinogenesis.

[4] mothers against decapentaplegic homolog 4 - The *SMAD* genes are homologs of both the *Drosophila melanogaster* gene *MAD* (*mothers against decapentaplegic)* and the *Caenorhabditis elegans* gene *sma* (*small body size*). The name is a combination of the two. A mutation in the maternal *MAD* gene represses the *decapentaplegic (dpp)* gene in the embryo. The *dpp* gene is a growth factor involved in cell-cell signalling. The *sma* gene is involved in body size regulation.

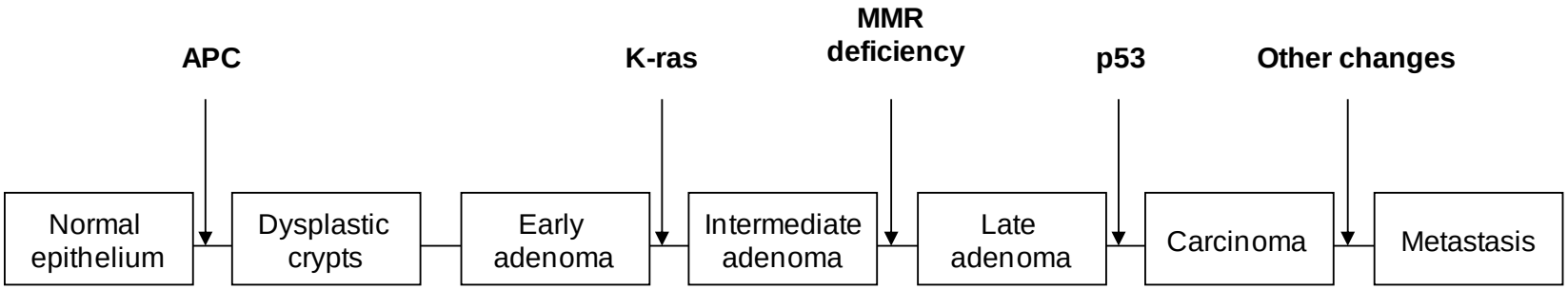


Figure 1-1 Genetic changes associated with colorectal tumorigenesis

1.4 Adenomatosis polyposis coli gene (APC gene) & the Min mouse model

The APC gene is a human gene found on chromosome 5q, and is classified as a tumour suppressor gene. The protein product of the APC gene is a large protein of approximately 310 kDa. It contains seven 20 amino acid beta-catenin (β-catenin) binding repeats. It plays a key role in the regulation of cell growth and differentiation, adhesion, signalling and apoptosis in the colon (Kinzler and Vogelstein, 1996). APC together with the protein kinases glycogen synthase kinase 3β (GSK3β) and casein kinase 1 alpha (CK1α), and the scaffolding protein axin, form the beta-catenin-destabilising complex. GSK3β within this complex phosphorylates the N-terminal serine/threonine residues of β-catenin, accelerating its rapid degradation through ubiquitylation (Polakis, 1999). If the APC gene is mutated, β-catenin is dephosphorylated by GSK3β and so accumulates in the cytoplasm, where it translocates to the nucleus to initiate transcription of Wnt target genes *via* activation of T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors (Korinek et al., 1997). A key process in polyp formation in colonic epithelium appears to require activation of the canonical Wnt pathway (Oshima et al., 1997).

Figure 1-2 is a schematic illustration of the canonical Wnt pathway.

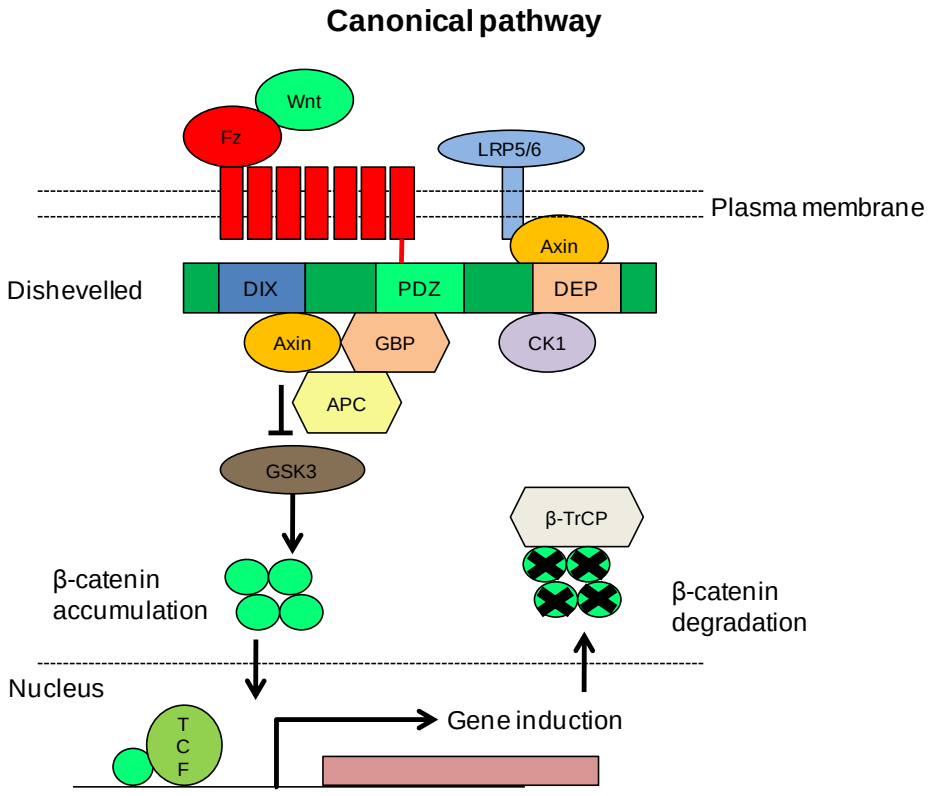


Figure 1-2 Canonical Wnt pathway

Cell signalling *via* the Frizzled (Fz) and LRP5/6 receptor complex induce stabilization of β-catenin *via* the DIX and PDZ domains of Dishevelled (Dsh) with Axin, APC, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). β-catenin translocates to the nucleus where it complexes with members of the LEF/TCF family of transcription factors to mediate transcriptional induction of target genes. β-catenin is then exported from the nucleus and degraded.

1.4.1 Min mice as a model for Familial Adenomatous Polyposis (FAP) & colon cancer

FAP is a human hereditary disease that causes numerous colonic polyps which develop into malignant adenocarcinomas if left untreated. The APC gene has been identified as a gene commonly mutated in some FAP kindreds and sporadic colon cancer (Kinzler et al., 1991).

70 – 80% of colon cancer cases in humans have APC mutations. Of these;

- 80% are sporadic
- 1 – 5% are hereditary, for example FAP and HNPCC (hereditary nonpolyposis colorectal cancer)
- ~ 20% have a family history; people are more at risk if they have 2 or more 1st or 2nd degree relatives with the disease. For

example, an APC mutation in 6% of Ashkenazi Jewish ancestry is seen in ~ 28% of Ashkenazi Jewish colon cancer patients who have a family history of the disease (Locker and Lynch, 2004).

A transversion from T to A at nucleotide 2549, that converts codon 850 from leucine (TTG) to a stop codon (TAG) in the APC gene in the Min mouse model ($APC^{Min/+}$) of this condition, predispose this animal model to the development of multiple intestinal neoplasia (Min) (Su et al., 1992). The Min mutation is found on chromosome 18 and is a fully penetrant autosomal dominant trait, transmitted by affected mice to 50% of progeny with an unbiased sex distribution (Moser et al., 1990). Heterozygote $APC^{Min/+}$ mice on a C57BL/6 background typically develop ~30 polyps throughout the intestinal tract and most die by 120 days (Moser et al., 1990).

Thus, the phenotypic and genetic similarities of the Min mutation to human FAP and sporadic colon cancer, suggest that the $APC^{Min/+}$ mouse is an excellent animal model for investigation into these conditions in humans.

Models such as the $APC^{Min/+}$ mouse provide a major resource for investigation into the pathways involved in tumorigenesis. Other mice have been genetically modified so that one or more oncogenes have a germ-line mutation, which lead to different phenotypes. Mouse models with a truncated APC gene at position 716, 1309 or 1638, and mutated Mlh1 (mutL homolog 1, colon cancer, nonpolyposis type 2) or Msh2 (mutS homolog 2, colon cancer, nonpolyposis type 1) genes and Cox-1 and Cox-2 have been engineered; reviewed by (Corpet and Pierre, 2003, Taketo and Edelmann, 2009).

Other APC mutant animal models are the PIRC (Polyposis in Rat Colon) rat and the Kyoto APC Delta (KAD) rat. The PIRC rat spontaneously develops polyps in the colon. It is heterozygous as the recessive phenotype is lethal (Amos-Landgraf et al., 2007). The KAD rat does not develop colonic tumours spontaneously, but it has enhanced

susceptibility to agents that promote their development. This rat is homozygous (Yoshimi et al., 2009).

These rats may be better models for human colon cancer as tumours occur more frequently in the colon and are more frequent in male rats, which more closely mimic the human condition.

Although the phenotypes of these rodents are not identical to the human condition, they provide a useful vehicle for the identification and manipulation of genes integral to the process of tumorigenesis and for testing of potential preventative and therapeutic agents.

1.5 Metabolism of Arachidonic Acid

The progressive up-regulation of Cox-2 during the development of cancer, has led to investigation of the mechanisms by which this could cause malignant change.

Arachidonic acid (AA) is metabolised *via* three main pathways; by cyclooxygenase (Cox), lipoxygenase (Lox) and cytochrome P-450 epoxygenase (Cyp) enzymes to produce a variety of derivative lipid signalling molecules (see Figure 1-3 and Figure 1-4a).

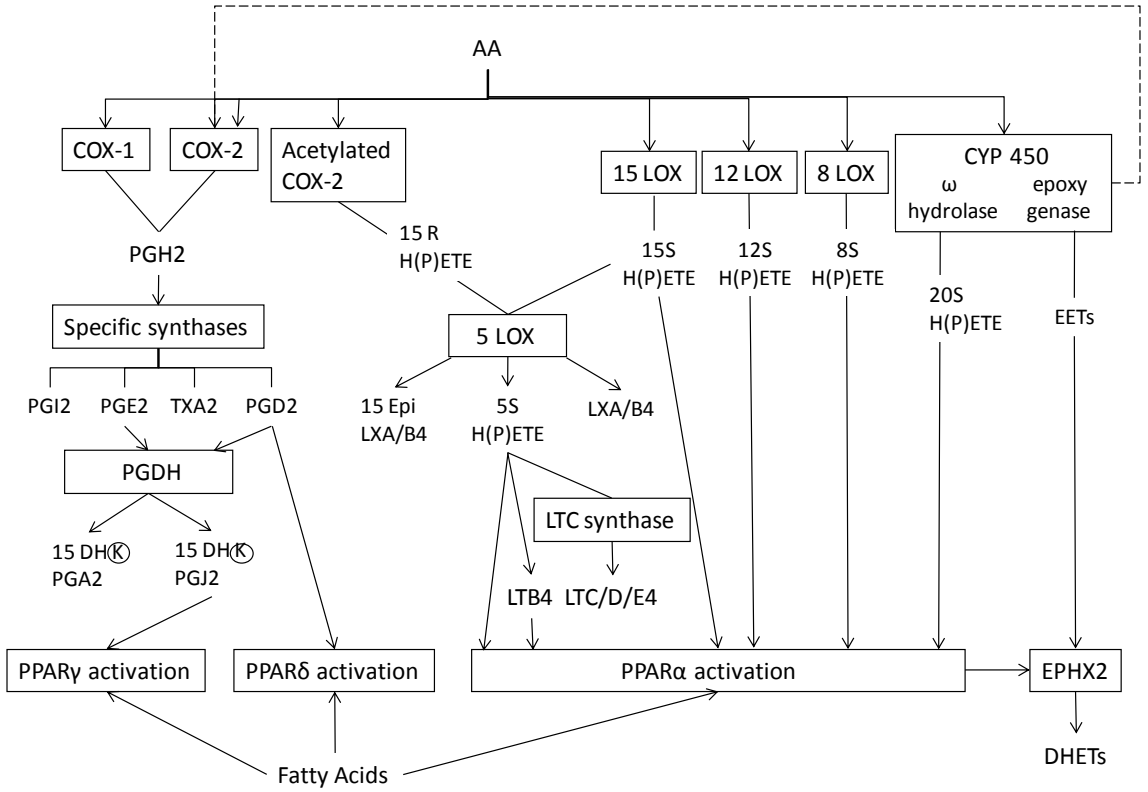


Figure 1-3 Metabolic pathways of arachidonic acid

KEY: COX Cyclooxygenase, LOX Lipoxygenase, CYP 450 Cytochrome P450, EET Epoxyeicosatrienoic acid, H(P)ETE Hydroperoxy-eicosatetraenoic acid, PGI2 Prostacyclin, PGE2 Prostaglandin E2, TXA2 Thromboxane A2, PGD2 Prostaglandin D2, 15 Epi LXA4 Aspirin-triggered 15 epimer lipoxin A4 (ATL), PGDH Prostaglandin dehydrogenase, 15 DH K PGA2 Di-hydro 15-keto prostaglandin A2, LTB4 Leukotriene B4, EPHX2 Soluble epoxide hydrolase, DHET Di-hydroxy eicosatrienoic acid, LTC synthase Leukotriene C synthase, LTB4 Leukotriene B4

Arachidonic acid (AA) is a 20-carbon omega-6 (ω-6) essential fatty acid with four double bonds (de Leval et al., 2006, Zeldin, 2001). The AA pathway metabolises a diverse variety of eicosanoids *via* three pathways that are involved in inflammation and cancer (Greene et al., 2011);

- Cyclooxygenase (Cox) pathway, produces prostanoids
- Lipoxygenase (Lox) pathway, produces hydroxyeicosatetraenoic acids (HETEs) and leukotrienes
- Cytochrome P-450 (Cyp) pathway, produces epoxyeicosatrienoic acids (EETs) and omega-hydroxylases (ω-hydroxylases)

There are two isoforms of cyclooxygenase (Cox) enzymes[5]; Cox-1, which is constitutively expressed in most tissue types, and Cox-2, expression of which is mostly induced (Needleman et al., 1976, Fu et al., 1990). Cox-2 however, is also constitutively expressed in the brain, spinal cord (Hoffmann, 2000) and kidneys (Harris et al., 1994).

Cox enzymes catalyse the irreversible, rate-limiting reaction of arachidonic acid to prostaglandin H synthase (PGH_2), which is further converted to a variety of prostanoids such as PGD_2, PGE_2, $PGF_2\alpha$, prostacyclin (PGI_2) and thromboxane A_2 (TXA_2). These bind to seven transmembrane G protein-coupled rhodopsin cell surface receptors, and also to PPAR nuclear receptors, to produce various biological effects (Cathcart et al., 2011, Ricciotti and FitzGerald, 2011).

Lipoxygenase (Lox) enzymes metabolise arachidonic acid to hydroperoxyeicosatetraenoic acids (HPETEs), which are subsequently reduced to their corresponding HETEs; 5-, 8-, 12- and 15-HETE are the predominant arachidonic acid metabolites produced by Lox enzymes (Cathcart et al., 2011).

The cytochrome P-450 (Cyp) pathway has two main branches; epoxygenases metabolise arachidonic acid (AA) to four regioisomeric epoxyeicosatrienoic acids; 5,6-EET, 8,9-EET, 11,12-EET and 14,15-

[5] Cox-3 is a splice variant of Cox-1

EET, and omega-hydroxylases (ω-hydroxylases), convert AA to hydroxyeicosatetraenoic acids (HETEs) (Panigrahy et al., 2010).

Linoleic acid is an 18-carbon ω-6 essential fatty acid and is a pre-cursor of arachidonic acid. Conversely, metabolism of linoleic acid by Lox enzymes produces hydroxy-octadecadienoic acids (HODEs); 9-HODE and 13-HODE (Cathcart et al., 2011).

Figure 1-3 shows a schematic diagram of the three arachidonic acid metabolic pathways described above. However, these enzymes also metabolise 20-carbon essential fatty acids with three or five double bonds, (dihomo-gamma-linolenic acid, DGLA and eicosapentaenoic acid, EPA, respectively) to analogous compounds (Figure 1-4b and d).

In addition, EPA and the related omega-3 (ω-3) fatty acid docosahexaenoic acid (DHA) can be converted to a group of metabolites called resolvins (Bannenberg and Serhan, 2010), as follows;

- Acetylated Cox-2 and cytochrome P450 metabolise EPA to produce E-series resolvins E1 and E2 (Figure 1-4d)
- Lipoxygenases metabolise DHA to produce D-series resolvins D1, D2, D3, D4 and aspirin-triggered resolvin D1 (Figure 1-4c)

Resolvins are generated during the resolution phase of acute inflammation and have been shown to have potent anti-inflammatory properties in animal models of inflammation, thus restoring homeostasis in affected tissue (Ji et al., 2011).

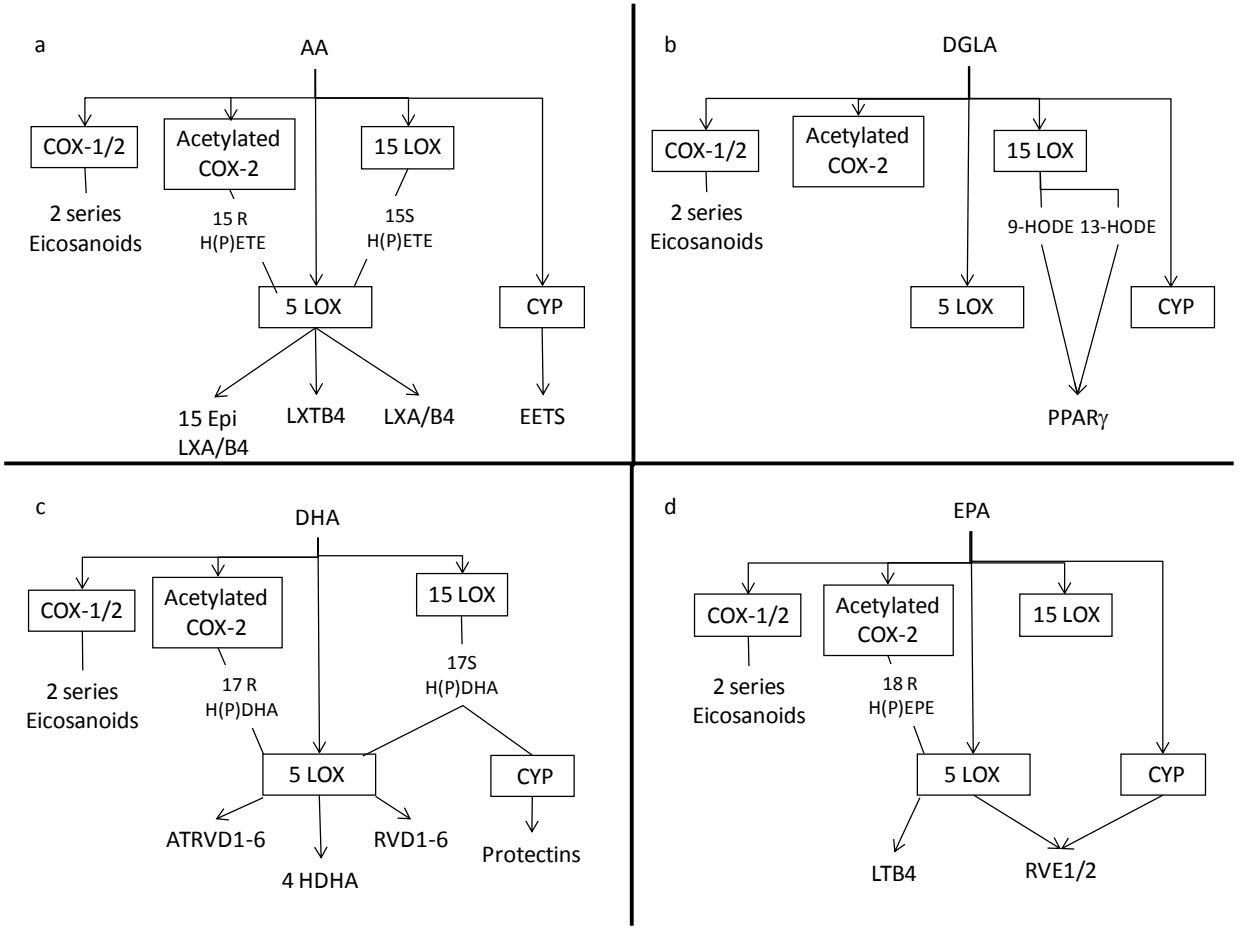


Figure 1-4 Metabolism of fatty acids

KEY: COX Cyclooxygenase, LOX Lipoxygenase, CYP 450 Cytochrome P450, H(P)ETE Hydroperoxy-eicosatetraenoic acid, 15 Epi LXA4 Aspirin-triggered 15 epimer lipoxin A4 (ATL), LTB4 Leukotriene B4, ATRVD1-6 D-series resolvins, RVE1/2 E-series resolvins, DGLA Dihomo-gamma-linolenic acid, HODE Hydroxy-octadecadienoic acid, DHA Docosahexaenoic acid, HDHA Hydroxy-docosahexaenoic acid, H(P)DHA Hydroperoxy-docosahexaenoic acid, EPA Eicosapentanoic acid

1.6 Eicosanoid pathways in colorectal cancer development & progression

1.6.1 Cyclooxygenase pathway

Cox derived prostanoids, especially prostaglandin E_2 (PGE_2), and also prostacyclin (PGI_2), thromboxane (TXA_2), prostaglandin D_2 (PGD_2) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$), are involved in colorectal cancer development and progression and mediate the pro-inflammatory and tumour-promoting effects of Cox-2 (Cathcart et al., 2011).

1.6.1.1 Prostaglandin E_2 (PGE_2)

Prostaglandins are produced by nucleated cells throughout the body. They act *via* autocrine and paracrine signalling on platelets, endothelium, uterine and mast cells to induce change in the cell.
PGE_2 is produced by the action of prostaglandin E synthases on prostaglandin H_2 (PGH_2). PGE_2 is the most dominantly expressed prostanoid in human colorectal cancer, with endogenous levels significantly higher in colon cancer mucosa than in matched normal mucosa (Mal et al., 2011).

Levels of PGE_2 are modulated by a synergy of Cox-2/prostaglandin E synthase (PGE synthase)-dependent biosynthesis and 15-hydroxy-prostaglandin dehydrogenase (15-PGDH)-dependent degradation. 15-PGDH is expressed in normal colon mucosa. However, expression is lost in tumour tissue, suggesting a regulatory role of 15-PGDH in tumour development (Backlund et al., 2005). A study by (Myung et al., 2006) demonstrated that 15-PGDH suppressed colon tumorigenesis in $APC^{Min/+}$ mice; the study showed that disruption of 15-PGDH function, with a consequential increase in PGE_2 expression, led to a large increase in the number of colon tumours.

In another study, (Hansen-Petrik et al., 2002) demonstrated that PGE_2 expression in $APC^{Min/+}$ mice protected small bowel adenomas from

NSAID-induced regression. Also, (Wang et al., 2004) found $APC^{Min/+}$ mice treated with PGE_2 had a significant increase in intestinal tumour burden compared to untreated controls. Another study with PGE_2 and azoxymethane (AOM) - treated $APC^{Min/+}$ mice recorded a significant increase in colon tumour incidence and number in these mice when compared to controls (Kawamori et al., 2003).

Microsomal prostaglandin E synthase 1 (mPGES-1) plays a critical role in the production of PGE_2, and therefore may be a factor in the effects of PGE_2 in tumorigenesis. This is supported by a study that found mPGES-1 deletion and subsequent reduction of PGE_2, suppressed intestinal tumour formation in $APC^{Min/+}$ and AOM-treated mice (Nakanishi et al., 2008). Two further studies also reinforced these findings which demonstrated that mPGES-1 deletion led to reduced colon tumour number and area in AOM-treated mice (Nakanishi et al., 2011, Sasaki et al., 2011).

Therefore, a potential target for therapeutic intervention is a novel, selective mPGES-1 inhibitor (Mbalaviele et al., 2010).

1.6.1.2 Prostacyclin (PGI_2)

Prostacyclin is produced in endothelial cells from metabolism of PGH_2 by prostacyclin synthase. It inhibits platelet activation and acts as a vasodilator.

(Mal et al., 2011) showed that the PGI_2 metabolite, 6-keto-$PGF_{1\alpha}$ was significantly reduced in colonic tumour mucosa relative to normal mucosa.

Additionally, hyper-methylation of the prostacyclin synthase promoter (PTGIS) is frequently observed in colorectal carcinogenesis (Frigola et al., 2005).

1.6.1.3 Thromboxane A_2 (TXA_2)

TXA_2 is formed in platelets from PGH_2 *via* thromboxane synthase (TXAS). It facilitates platelet aggregation and is a vasoconstrictor.
TXAS expression has been shown to be significantly higher in human colorectal cancer tissue, when compared to normal tissue (Sakai et al., 2006).

1.6.1.4 Prostaglandin D_2 (PGD_2)

Prostaglandin D_2 (PGD_2), which is also derived from PGH_2, has been shown to have anti-tumorigenic properties. A study by (Dionne et al., 2010) in human colorectal cancer demonstrated that PGD_2 promoted apoptosis *via* activation of the caspase-dependent pathway. Additionally, 15-deoxy-$\Delta^{12.14}$-PGJ_2 (15d-PGJ_2), which is a product of PGD_2 metabolism, was shown to promote colon cancer apoptosis (Koyama et al., 2010).

1.6.1.5 Other prostaglandins

Another PGH_2 product is prostaglandin $F_2\alpha$ ($PGF_2\alpha$). $PGF_2\alpha$ has been postulated as contributing to tumorigenesis by promotion of Cox-2 synthesis (Jabbour et al., 2005).

Further, expression of prostaglandin D (DP) and prostaglandin F (FP) receptors were found to be significantly reduced in colorectal tumour tissue compared to normal tissue (Gustafsson et al., 2007).

1.6.2 Lipoxygenase pathway

In human epithelial tissue, 15-Lox-1 and -2 (leucocyte 12-Lox and 8-Lox respectively, in the mouse) are usually expressed in normal tissue but not in colon cancer (Shureiqi et al., 1999, Subbarayan et al., 2005). A prospective study (cohort study) by (Shureiqi et al., 2010) demonstrated

that expression of the 15-Lox-1 metabolite 13(S)-hydroxy-octadecadienoic acid (13S-HODE) was progressively lost in the transition from normal mucosa to cancer mucosa. Expression of 5-Lox and 12-Lox however, was found to be absent in normal tissue, but often constitutively expressed in colon cancer (Ohd et al., 2003). A study by (Melstrom et al., 2008) found that 5-Lox was progressively over-expressed in human colon polyps and cancer compared to expression levels in normal mucosa. This finding was supported by another study which showed a relationship between increasing polyp size and higher tumour grade, and increased 5-Lox expression (Wasilewicz et al., 2010).

1.6.3 Cytochrome P-450 pathway

P-450-derived epoxyeicosatrienoic acids (EETs) are involved in many processes linked to cancer, including regulation of signalling pathways, gene expression, cell proliferation and inflammation (Spector et al., 2004). A recent study by (Jiang et al., 2009) showed that the P-450 epoxygenase Cyp2J2 was highly expressed in colorectal cancer; however, the small sample size in this study necessitates further investigation.

Another study demonstrated that the Cyp2J2 metabolites 8-9 and 11-12 EETs abolished the Interleukin 1 beta (Il1β) induction of NFκB (nuclear factor kappa light polypeptide gene enhancer in B-cells) *via* activation of PPARα (Wray et al., 2009).

1.7 Mechanism of action of Cyclooxygenase-2

1.7.1 Apoptosis

Several studies have shown that cyclooxygenase (Cox) derivatives, such as 15-deoxy-$\Delta^{12.14}$-PGJ_2 (15d-PGJ_2), a metabolite of PGD_2, induce apoptosis in colorectal cancer cells (Koyama et al., 2010, Shimada et al., 2002, Shin et al., 2009). Investigations into the effect of 15d-PGJ_2

on apoptosis demonstrated 15d-PGJ_2 treatment caused a decrease in expression levels of the anti-apoptotic genes, NFκB and bcl-2 (B-cell leukaemia/lymphoma 2) (Chen et al., 2002), c-Myc (cellular myelocytomatosis oncogene) (Shimada et al., 2002), and hTERT (telomerase reverse transcriptase) (Moriai et al., 2009), and an increase in expression of the proto-oncogene c-jun and DDIT3 (DNA-damage-inducible transcript 3) (Shimada et al., 2002). Also, an *in vivo* study by (Shin et al., 2009) showed treatment with 15d-PGJ_2 induced apoptosis by production of reactive oxygen species (ROS) through activation of JNK (c-Jun N-terminal kinase) and inactivation of Akt (alpha serine/threonine protein kinase).

By contrast, PGE_2 appears to have anti-apoptotic effects, with a recent study postulating this may be mediated through P-13 kinase and Wnt signalling pathways (Kaur and Sanyal, 2010).

Another study showed apoptosis in colorectal adenoma cells was suppressed by PGE_2-mediated down-regulation of Bcl2l11 (bcl2-interacting mediator of cell death) (Greenhough et al., 2010).

(Wu and Liou, 2009) showed that inhibition of another Cox derivative, PGI_2, led to induction of apoptosis *via* activation of PPAR delta (PPARδ) and increased expression of Bad protein (BCL2-associated agonist of cell death).

1.7.2 Angiogenesis

PGE_2 has also been shown to be involved in angiogenesis in colorectal cancer. PGE_2 induces expression of pro-angiogenic factors such as VEGF (vascular endothelial growth factor) and CXCL1 (chemokine C-X-C motif ligand 1) (Fukuda et al., 2003, Wang et al., 2006).

By contrast, the anti-tumour properties of 15d-PGJ_2 appear to be mediated *via* inhibition of AP-1 and VEGF or Cox-2 (Grau et al., 2006).

1.7.3 Metastasis

PGE_2 expression levels have been shown to be significantly higher in metastatic colorectal tumours, compared to non-metastatic tumours, which indicates that PGE_2 has a role in tumour metastasis (Cianchi et al., 2001). However, PGI_2 is thought to inhibit cancer cell-cell interactions to confer a protective effect against metastasis in colorectal cancer (Yoshida et al., 1999).

Platelet activation plays a crucial role in metastasis; circulating tumour cells express high levels of tissue factor (TF) and adhesion molecules, for example P-selectin ligands, which bind and activate platelets. This accelerates tumour growth and metastasis by an increase in platelet activation and blood clotting which can lead to thromboembolic disease (Gay and Felding-Habermann, 2011).

Many cancer patients present with signs of thrombosis which is more severe if tumours have metastasised. Thrombocytosis (high platelet count) indicates a poor prognosis in colon cancer. Therefore, platelets are a potential target for anti-metastatic therapy. Options for treatment include anti-thrombotic therapies such as aspirin, lipoxygenase inhibitors and cyclooxygenase inhibitors (Sierko and Wojtukiewicz, 2007).

1.7.4 Immune effects

Cox-2 and PGE_2 effects are mediated through regulatory T cells, which act to prevent anti-tumour immune responses. Adaptive and induced regulatory T cells express Cox-2 (and thus PGE_2), that suppress effector T cells. MCP-1 (monocyte chemoattractant protein-1), a chemoattractant for monocytes and macrophages, has been shown to be more highly expressed in colonic adenoma tissue than in normal tissue. MCP-1 induces Cox-2 expression, leading to the release of PGE_2 and VEGF from human macrophages (Tanaka et al., 2006).

1.7.5 Resolution of inflammation

Inflammation as a key factor in development of cancer is mediated or resolved by many metabolites of the arachidonic pathways.

Resolvins, derived from biosynthesis of omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by Cox-2 and Lox pathways (Figure 1-4c & d), are potent regulators of inflammation in colon cancer (Ji et al., 2011). Also, the cytochrome P-450 derived epoxyeicosatrienoic acids (EETs) are known to be anti-inflammatory. However, metabolism of EETs by soluble epoxide hydrolase (Ephx2), converts EETs to the pro-inflammatory dihydroxyeicosatrienoic acids (DHETs). A study by (Norwood et al., 2010) showed that inhibition of Ephx2 led to increased production of EETs with a concomitant decrease in inflammation, which suggests inhibition of Ephx2 could be a potential target for treatment of inflammation and colon cancer.

1.8 Cyclooxygenase inhibitors as cancer treatment & prevention

Chronic inflammation is a tumour promoter in almost all tissues. It is implicated in the pathogenesis of several cancers associated with the gastrointestinal tract (Coussens and Werb, 2002). Cox-2 is over-expressed in many colonic cancers and considered as having an essential role in cancer progression (Xu, 2002).

1.8.1 Aspirin

Evidence from laboratory studies suggested that aspirin use could potentially reduce cancer risk *via* inhibition of Cox-2 (Wang and Dubois, 2006, Doherty and Murray, 2009) or initiation of apoptosis (Elwood et al., 2009). Aspirin irreversibly inhibits Cox-2; the acetyl group of aspirin attaches to serine in the active site of the Cox-2 enzyme.

Subsequent randomised trials demonstrated that daily aspirin prevented colorectal polyps (Cole et al., 2009) and reduced colorectal cancer incidence and mortality, at up to 20 years follow-up (Rothwell et al., 2010, Rothwell et al., 2011).

(Thun et al., 2012) argue that these effects are mediated *via* platelets; irreversible cyclooxygenase acetylation results in prolonged functional effects since there is no capacity for re-synthesis of the Cox enzyme (platelets lack a nucleus). Thus, reversal depends on the release of fresh platelets. Consequently, platelets are susceptible to long-lasting effects of low-dose (75 – 100 mg daily) aspirin.

Platelets release a number of mediators, such as thromboxane A_2 (TXA_2), interleukin 1β (Il1β) and platelet-derived growth factor (PDGF), under the influence of Cox activity that could affect tumour growth. Therefore, inhibition of platelet activation may mediate the cancer-preventative effects of low dose aspirin.

1.8.2 Non-selective non-steroidal anti-inflammatory drugs (NSAIDs)

Two randomised clinical trials with the non-selective NSAID sulindac indicated it caused suppression of adenomatous polyps, and reduction of existing polyps in patients of Familial Adenomatous Polyposis (FAP) (Giardiello et al., 1993, Nugent et al., 1993). However, a later study of four years duration showed that sulindac did not slow the development of adenomas in FAP patients (Giardiello et al., 2002).

Also, a trial by (Ladenheim et al., 1995) with patients who had a prior history of adenomatous polyps, showed higher dose sulindac did not significantly decrease the number or size of polyps.

Although there appears to be some beneficial effects of long term use of aspirin and NSAIDs, unfortunately, there are negative side-effects. Both drugs can cause inflammation and bleeding in the stomach,

probably because of the concomitant inhibition of Cox-1, which exerts a protective effect.

1.8.3 Selective cyclooxygenase 2 (Cox-2) inhibitors

Selective Cox-2 NSAIDs were developed as a way to overcome the side-effects caused by aspirin and non-selective NSAIDs.

A study by (Bertagnolli et al., 2006) reported a reduction in adenoma incidence in patients receiving celecoxib, compared to patients receiving placebo.

Two studies that randomised patients with a previous history of colonic adenomas onto either treatment or placebo; the Prevention of Colorectal Sporadic Adenomatous Polyps (PreSAP) trial (Arber et al., 2006), which used celecoxib, and the Adenomatous Polyp Prevention on Vioxx (APPROVe) trial (Baron et al., 2006), which used rofecoxib, both demonstrated reductions in adenoma recurrence, and in advanced adenomas, compared to patients taking placebo.

Rofecoxib was subsequently found to cause serious cardiovascular problems and was withdrawn from clinical use.

1.8.4 PPARα as a target for eicosanoids

As Cox-2 metabolites of arachidonic acid (AA) such as PGE_2 are known to be pro-inflammatory and enhance tumorigenesis, and 15-Lox and CYP derived AA derivatives such as HETEs and EETs are thought to have anti-neoplastic and anti-inflammatory properties, a potential mechanism for the effects of HETEs and EETs is *via* activation of the nuclear receptor PPARα which has previously been shown to be involved in reduction of tumour burden in the $APC^{Min/+}$ mouse model of colon cancer (Jackson et al., 2003).

1.8.5 PPARs as a target for NSAIDs

Some NSAIDs directly interact with PPARs. For example, 100 μM concentrations of indomethacin, piroxicam and other NSAIDs were shown to be efficacious activators of PPARα and PPARγ to promote peroxisome proliferation and adipocyte differentiation respectively (Lehmann et al., 1997).

In another study, sulindac inhibited PPARβ/δ activation, thus preventing the binding of this PPAR/RXR heterodimer to its recognition site in DNA (He et al., 1999).

Also, (Wick et al., 2002) demonstrated inhibition of lung cancer cell growth *via* activation of PPARγ by sulindac.

NSAIDs are used as cancer chemopreventative agents. These studies have shown that NSAIDs may inhibit tumorigenesis by direct interaction with PPARs, independent of Cox enzyme activation.

1.9 Peroxisome proliferator activated receptors (PPARs)

In 1992, (Dreyer et al., 1992) identified three novel receptors in *Xenopus laevis*; they closely resembled a previously identified mammalian receptor that had been shown to induce proliferation of peroxisomes in cells when activated by a diverse class of rodent hepatocarcinogens (Issemann and Green, 1990).

These receptors were subsequently termed peroxisome proliferator activated receptors alpha, beta/delta and gamma ((PPARα, PPARβ/δ, PPARγ).

Peroxisome proliferator activated receptors (PPARs) are part of the nuclear hormone receptor superfamily of ligand-activated transcription factors which includes retinoic acid receptors (RARs), steroid hormone receptors and thyroid hormone receptors (TRs) (Laudet et al., 1992, Lemberger et al., 1996).

When a ligand binds, the PPARs form a heterodimer with the retinoid X receptor (RXR), whereupon they bind to genes with recruitment of cofactors to initiate transcription of genes involved in energy homeostasis (Wahli et al., 1995).

Figure 1-5 shows a schematic diagram of the gene transcription mechanism of PPARs.

Peroxisome proliferator response elements (PPREs) in PPAR-RXR heterodimers have an exclusive directly repeating sequence (DR-1) of two hexanucleotides, 5' AGGTCA 3' separated by one nucleotide (Lemberger et al., 1996, Juge-Aubry et al., 1997).

Several proteins function as co-factors in a ligand-dependent way to mediate the effects of PPARs to either inhibit or initiate transcription (Lemberger et al., 1996). When unliganded, the PPAR/RXR dimer associates with a co-repressor with histone deacetylase activity, for example, nuclear receptor co-repressor (NCoR) or silencing mediator for retinoid and thyroid hormone receptor (SMRT). This inhibits transcription. The opposite effect is achieved when a co-activator such as steroid receptor co-activator (SRC)-1 or PPAR binding protein (PBP) which has histone acetylase activity, associates with the ligand bound dimer to initiate transcription (Ricote and Glass, 2007, Pascual and Glass, 2006). Table 1-1 summarises transcriptional co-factors, mechanism of action and their effects in PPARs.

Table 1-1 Transcriptional co-factors of PPARs

Heterodimerized nuclear receptor	Cofactors	Mechanism of action	Effect
No ligand	***Co-repressors***	Histone deacetylase activity	Inhibition of transcription
	Nuclear receptor co-repressor (NCor)		
	Silencing mediator for retinoid and thyroid hormone receptor (SMRT)		
+ Ligand	***Co-activators***	Histone acetylase activity	Initiation of transcription
	Steroid receptor co-activator (SRC)-1		
	PPAR binding protein (PBP)		

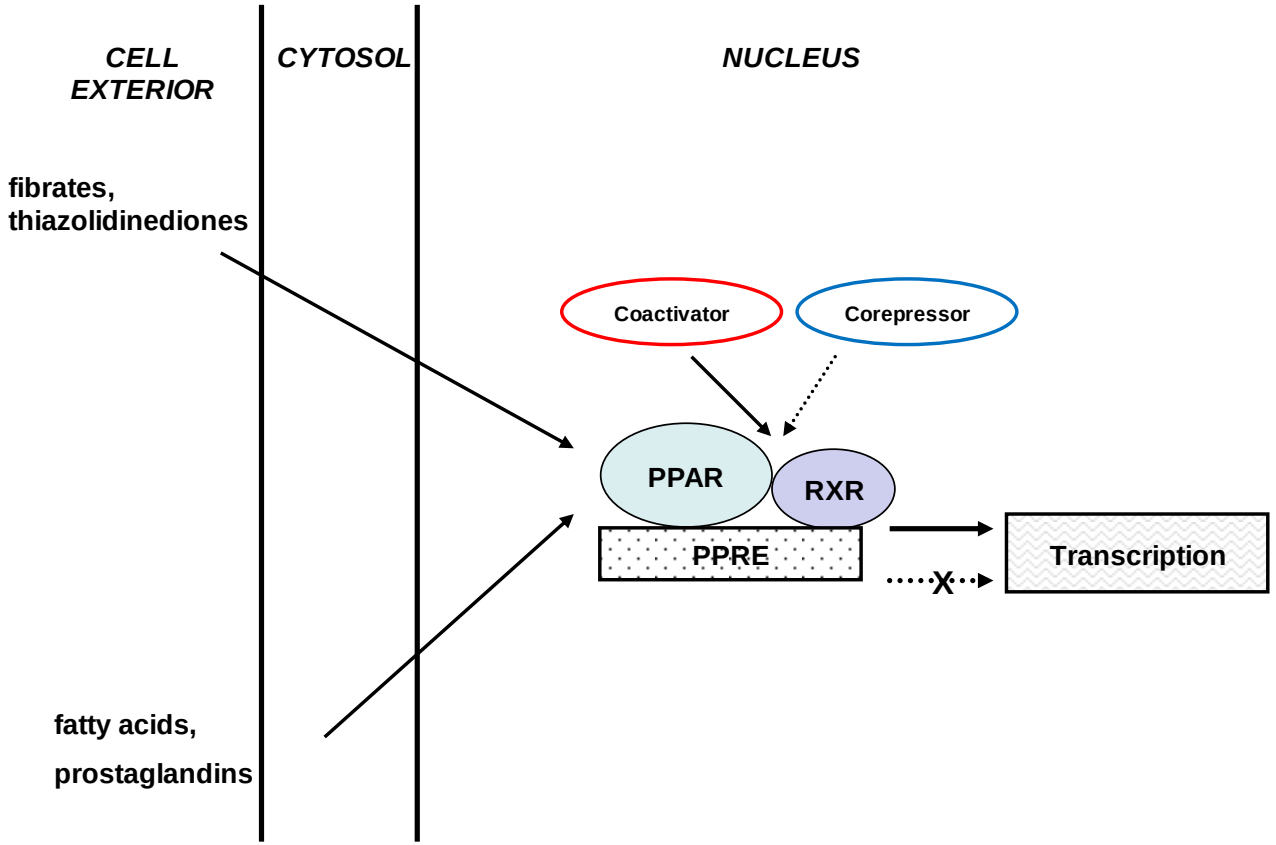


Figure 1-5 Gene transcription mechanisms of PPARs

Peroxisomes are ubiquitous organelles found in eukaryotic cells. They are rich in peroxidise, catalase and d-amino oxidase enzymes which carry out oxidative reactions such as metabolism of fatty acids and hydrogen peroxide.

There are marked species differences in response to peroxisome proliferators; rodents demonstrate high peroxisomal enzyme induction, whereas humans do not. This may be due to the lower expression of PPARα in the human liver compared to rodents, or the possible existence of an inactive PPARα splice variant in the human liver (Cattley et al., 1998).

(Ammerschlaeger et al., 2004) demonstrated that PPARα and the PPRE of target genes are important determinants for species-specificity of peroxisome proliferation.

Activation by endogenous and synthetic ligands of PPARα and PPARγ regulates the transcription of genes involved in lipid and glucose metabolism respectively. Activation of PPARβ also regulates glucose metabolism and fatty acid oxidation (Kersten et al., 2000).

The three PPAR subtypes, PPARα (NR1C1), PPARδ/β (NR1C2) and PPARγ (NR1C3), are the products of different genes (Michalik and Wahli, 1999). They are differentially expressed in various tissues (Table 1-2, (Braissant et al., 1996, Auboeuf et al., 1997, Inoue et al., 1998, Chinetti et al., 1998, Diep et al., 2000, Heneka and Landreth, 2007).

Table 1-2 Location of PPARs in the body

PPAR subtype	Expression Tissue/Organ/Cells
α	Brown adipose tissue Central nervous system (CNS) Liver Kidney **Colon** Duodenum Heart Skeletal muscle Endothelial cells Vascular smooth muscle cells (VSMCs) Monocytes/Macrophages
γ	Brown & white adipose tissue CNS Peripheral nervous system (PNS) **Colon** Retina Parts of immune system
β	Ubiquitously expressed

PPARs also share a common structure with four functional domains identified as A/B, C, D and E/F. Figure 1-6 shows a schematic representation of the functional domains of PPARs.

Briefly, the schematic shows the A/B domain contains a ligand-independent activation function 1 (AF-1) (Werman et al., 1997), which, when phosphorylated, aids the regulation of PPARα and PPARγ activation (Shalev et al., 1996, Juge-Aubry et al., 1999, Zhang et al., 1996). The C domain or DNA-binding domain (DBD) is highly conserved between the three PPAR isotypes and binds to the PPRE in the promoter region of the target gene (Kliewer et al., 1992). The D domain is a docking site for co-factors. The E/F domain or the ligand binding domain (LBD) has ligand specificity which when bound activates PPAR binding to the PPRE. This domain also contains the ligand-dependent transactivation function 2 (AF-2) which recruits PPAR co-factors (Berger and Moller, 2002).

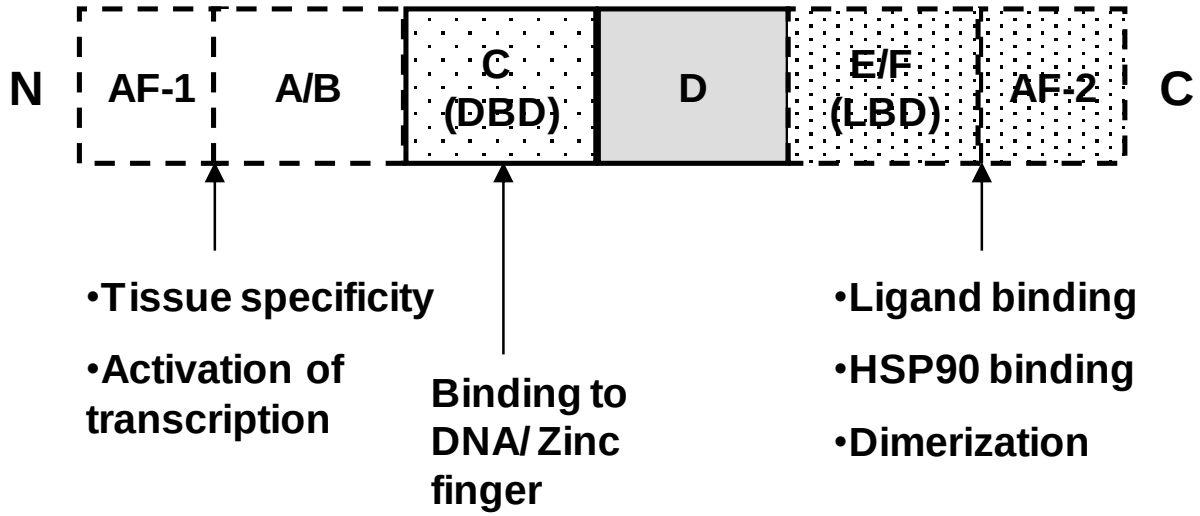


Figure 1-6 Schematic representation of the functional domains of PPARs

1.10 PPARs – Role in Cancer and Inflammation

PPARs are ligand-activated transcription factors that were originally recognised as playing a central role in glucose and lipid homeostasis. It became apparent that PPARs modulate activity of a wider range of functions including cell proliferation, differentiation, adipogenesis and inflammatory signalling. This led to consideration of their role as potential therapeutic targets in cancer pathogenesis chemo-prevention, inflammatory conditions, atherosclerosis, obesity and diabetes; reviewed in (Peters et al., 2012).

1.10.1 PPAR alpha (PPARα)

PPARα levels of expression are high in the liver (Peters et al., 2000) and also the heart, kidney, intestine, and brown adipose tissue; all of which are tissues with high oxidative capacity (Bookout et al., 2006). Consequently these tissues may be more susceptible to cancer development. Therefore, targeted activation of PPARα may be a useful strategy for cancer prevention and therapy.

Agonists of PPARα include the synthetic fibrate drugs, for example, bezafibrate, clofibrate, fenofibrate and Wy 14, 643 (Kersten et al., 2000, Willson et al., 2000, Berger and Moller, 2002). Endogenous ligands of PPARα include fatty acids and eicosanoids (Yu et al., 1995, Khan and Vanden Heuvel, 2003).

The discovery of PPARα agonist drugs now used in the treatment of chronic disorders associated with the metabolic syndrome[6] has enabled the elucidation of their mechanism of action. Fibrate drugs have been used clinically since the 1930s but their mechanism of action was only established in the 1990s. They bind and activate PPARα resulting in raised high-density lipoprotein (HDL) levels, reduced hepatic triglyceride secretion and elevated β-oxidation in the liver; effects that underpin

[6] The metabolic syndrome: A group of at least 3 risk factors that raise the probability of developing coronary heart disease, insulin resistance, diabetes and stroke. Risk factors include abdominal obesity, high triglyceride level, low HDL cholesterol, high blood pressure and high fasting blood sugar.

their use for treatment of dyslipidaemia, which is primarily associated with type 2 diabetes mellitus (Kersten et al., 2000, Willson et al., 2000, Berger and Moller, 2002).

Initially it would seem PPARα would not be an obvious target for cancer therapy and chemoprevention since the early recognition that the receptor mediated development of liver cancers in mice, induced by long-term administration of PPARα agonists such as clofibrate and bezafibrate (Reddy et al., 1980). This effect appeared to be PPARα specific in that it was not seen in PPARα null mice (Morimura et al., 2006, Shah et al., 2007). However, this effect is not evident in humans, probably as murine but not human PPARα causes down-regulation of let-7c micro RNA cluster. The let-7c miRNA destabilises Myc (myelocytomatosis oncogene) mRNA, so stability of this mitogen is increased with a concomitant reduction in let-7c. This may in turn lead to increased mitogenic signalling that causes hepatocyte proliferation (Shah et al., 2007).

Identification and activation of PPARα-dependent pathways may be useful in prevention of tumorigenesis or tumour growth. The nuclear factor–κB (NF-κB) and AP-1 signalling pathways activation of PPARα have been shown to inhibit inflammation; reviewed in (Ricote and Glass, 2007, Pascual and Glass, 2006).

NF-κB activity is controlled by the degradation of IκBα which sequesters inactive NF-κB dimers in the cytoplasm. Induction of IκBα by fibrates requires PPARα expression and the increase in IκBα results in NF-κβ nuclear deactivation, thus reducing the duration of the inflammatory response in a PPARα-dependent manner (Delerive et al., 2001).

The inhibition of AP-1-dependent activity by PPARα activators may occur by reducing the intrinsic c-Jun transactivating activity (Karin et al., 1997) and also by c-Jun inhibiting the ability of PPARs to activate PPRE-driven genes (Delerive et al., 1999). A study using the human colorectal carcinoma cell line SW620, demonstrated that the PPARα ligands LY-171883 and WY-14643, inhibited the induction of AP-1-

mediated transcriptional activation of Cox-2and vascular endothelial growth factor (VEGF) and other genes involved in inflammation and tumour progression. Thus, this established the existence of a negative cross-talk between the PPARα and AP-1-dependent signalling pathways in these cells. This effect was also found to be dependent on PPARα expression (Grau et al., 2006).

A previous study (Jackson et al., 2003) determined a functional PPARα receptor was necessary in preventing neoplastic transformation or growth in the gastrointestinal tract. This led to further investigations using a PPARα null mouse model that are reported in this thesis.

The present study concentrated on the effects of PPARα, but the two other PPAR receptors, PPAR gamma (PPARγ) and PPAR beta/delta (PPARβ/δ), have also been the subject of research into their involvement in carcinogenesis.

1.10.2 PPAR beta/delta (PPARβ/δ)

Ligand activation of PPARβ/δ inhibits or prevents metabolic syndrome, obesity, dyslipidaemia, glucose intolerance and inflammation; all of which are associated with development of cancer (Mantovani et al., 2008, Tsugane and Inoue, 2010, Wolin et al., 2010).

However, there are conflicting conclusions as to whether PPARβ/δ agonists promote or attenuate most types of cancer, although PPARβ/δ agonists have been successfully used in the treatment of non-melanoma skin cancer (Bility et al., 2010, Zhu et al., 2010).

Early attention focused on PPARβ/δ because it was up-regulated in cancer cells by the APC-beta catenin-TCF pathway and was capable of enhancing proliferation by activation of Cyclin D1 ((He et al., 1999).

There is some controversy regarding the effect of ligand activation of PPARβ/δ on colon carcinogenesis. Some evidence indicates that PPARβ/δ attenuates colorectal cancer. Two studies showed that

expression of PPARβ/δ in colon tumours from mouse and human models was lower than in normal colon epithelium (Chen et al., 2004, Marin et al., 2006).

In addition, (Marin et al., 2006) showed that specific ligand activation of PPARβ/δ induced expression of genes associated with terminal differentiation of colonocytes.

Results from a study by (Hollingshead et al., 2008) also confirmed a direct role of ligand activation of PPARβ/δ in cellular terminal differentiation, and that PPARβ/δ activation and Cox-2 inhibition act independently to attenuate colon carcinogenesis.

Conversely, results from (Gupta et al., 2004) who subjected $APC^{Min/+}$ mice to the selective PPARβ/δ agonist GW501516, showed a significant increase in the number and size of intestinal polyps.

A Phase 11 trial for dyslipidaemia using GW501516 was abandoned due to the formation of tumours in the mouse model that was used (Mackenzie and Lione, 2013).

GW501516 has been used as a performance-enhancing drug in sport. It activates PPARβ/δ and 5' AMP-activated protein kinase (AMPK). AMPK regulates uptake of glucose, β-oxidation of fatty acids and biogenesis of Glucose Transporter Type 4 (GLUT4) (Pokrywka et al., 2014).

The PPARβ/δ antagonist GSK3787 was found to inhibit PPARβ/δ-dependent activity *in vivo* and *in vitro* but with concomitant weak PPARγ agonist activity (Palkar et al., 2010). Studies using antagonists of PPARβ/δ in human cancer cells were shown to have no effect on cell proliferation (Shearer et al., 2010, Palkar et al., 2010).

The development of chemo-prevention using PPARβ/δ ligands is unlikely due to the differing effects seen in studies using PPARβ/δ agonists and antagonists.

1.10.3 PPAR gamma (PPAR γ)

Thiazolidinediones (TZDs) are used in the treatment of diabetes mellitus type 2. They bind to PPARγ to reduce insulin resistance, raise adiponectin levels, and inhibit vascular endothelial growth factor (VEGF)-induced angiogenesis. (Panigrahy et al., 2002)

Studies have shown that PPARγ agonists inhibited cell growth and increased apoptosis in cancer cell lines, as well as inhibiting tumour growth in animal models of cancer; reviewed in (Blanquicett et al., 2008).

In human chemo-prevention trials PPARγ agonists have shown modest efficacy, although PPARγ expression in colon cancer is generally associated with improved survival (Koeffler, 2003, Grommes et al., 2004).

However, clinical trials using TZDs have shown there are increased risks of negative side effects; cardiovascular effects, bone mineral density loss, bladder cancer (Piccinni et al., 2011, Erdmann et al., 2009, Grey et al., 2007, Schwartz and Sellmeyer, 2007). It is not clear whether these negative side effects are PPARγ related, related to the TZDs themselves, or off target effects. It may be possible to develop PPARγ agonists that retain chemo-preventative effects without side effects.

Ascribing a role to PPARγ in tumorigenesis is made difficult by virtue that ligands are promiscuous and have specific effects on PPARγ, but may also influence PPARα (for example, bezafibrate), or act through non-PPAR mechanisms.

Studies investigating PPARγ antagonists (for example, GW9662 and T0070907) as inhibitors of tumorigenesis have not conclusively demonstrated that the effects seen are PPARγ dependent, and may be due to independent mechanisms (Burton et al., 2008). However, the

use of PPARγ antagonists for cancer chemo-prevention remains a possibility.

1.10.4 Pan & dual-PPAR agonists

The use of pan and dual-PPAR agonists as prophylaxis or treatment of cancer is a realistic and attractive option. Studies using bezafibrate, which is a pan-PPAR agonist, have demonstrated inhibition of colon tumorigenesis in rodent (Kohno et al., 2005, Niho et al., 2003) and human cancer models (Tenenbaum et al., 2008).

Some of the effects of bezafibrate appear to be attributable to activation of PPARα (Peters et al., 2003). This is supported by findings from (Jackson et al., 2003) that showed the selective PPARα agonist, methylclophenapate (MCP) inhibited intestinal tumorigenesis.

Other effects appear to be mediated by activation of PPARγ by 15-deoxy prostaglandin J2 (15d-PGJ2), which is an endogenous PPARγ ligand (Khanim et al., 2009, Hayden et al., 2009).

Studies using high-affinity dual-PPAR agonists demonstrated development of tumours in long-term bioassays (Rubenstrunk et al., 2007). However, development of low-affinity pan-PPAR agonists for chemo-prevention treatment is supported by results of a clinical trial using bezafibrate for treatment of colon cancer in humans (Tenenbaum et al., 2008).

There are serious safety issues with pan and dual-PPAR agonist drugs and several Phase 111 clinical trials have been halted; ragaglitizar, due to formation of bladder tumours in mice, tesaglitizar, due to kidney toxicity and imiglitizar, due to liver toxicity (Fievet et al., 2006).

Saroglitizar (lipaglyn) is the first dual-PPAR (α and γ) approved drug; it was licensed for the treatment of Type 11 diabetes in June 2013 in India (Agrawal, 2014).

In summary, the use of synthetic and endogenous agonists and antagonists for chemo-preventative or chemo-therapeutic treatment of colon cancer remains a possibility.

However, further research to determine ligand-dependent and ligand-independent effects of activation of PPARs, and to reduce negative side effects is necessary.

1.11 Hypothesis

The development and progression of colon cancer is attenuated *via* activation of peroxisome activator receptor alpha (PPARα) by the non-steroidal anti-inflammatory drug (NSAID) piroxicam

1.12 Aims and Objectives

1. To investigate the role of PPARα in the $APC^{Min\ +/-}$ mouse model of colon cancer by:
   - Determining whether the reduction of tumour number observed in $APC^{Min\ +/-}$ mice is dependent on a functional PPARα locus
   - Determining whether differential gene expression observed in $APC^{Min\ +/-}$ mice is dependent on a functional PPARα locus

2. To validate levels of gene expression in the $APC^{Min\ +/-}$ mouse model of colon cancer by:
   - Determining and comparing gene expression of a sub-set of genes on 2 different platforms; Affymetrix Genechip® microarrays and Taqman® low-density arrays

3. To investigate the role of PPARα in the effect of a Cox inhibitor in the $APC^{Min\ +/-}$ mouse model of colon cancer by:

- Determining whether the reduction in tumour number observed in $APC^{Min\ +/-}$ mice is dependent on activation of PPARα by a Cox inhibitor
- Determining whether differential gene expression of the same sub-set of genes in $APC^{Min\ +/-}$ mice is dependent on activation of PPARα by a Cox inhibitor

4. To validate the $APC^{Min\ +/-}$ mouse model used in these studies by:
    - Comparison of observed tumour numbers in 2 matched cohorts of $APC^{Min\ +/-}$ mice and $APC^{Min\ +/-}$ $PPAR\alpha^{-/-}$ mice
    - Comparison of differential gene expression of the same sub-set of genes in 2 matched cohorts of $APC^{Min\ +/-}$ mice and $APC^{Min\ +/-}$ $PPAR\alpha^{-/-}$ mice

5. To investigate the role of PPARα in gene expression of Apobec3 and Onecut2 in the $APC^{Min\ +/-}$ mouse model of colon cancer by:
    - Determining gene expression of these 2 genes in 2 matched cohorts of $APC^{Min\ +/-}$ mice and $APC^{Min\ +/-}$ $PPAR\alpha^{-/-}$ mice

6. To validate levels of Apobec3 and Onecut2 gene expression in the $APC^{Min\ +/-}$ mouse model of colon cancer by:
    - Determining gene expression of these 2 genes using 3 different platforms; Affymetrix Genechip® microarrays, Taqman® low-density arrays and Real Time Quantitative PCR

7. To investigate Apobec3 isotypes in the $APC^{Min\ +/-}$ mouse model of colon cancer by:
    - Determining the sequence of the Apobec3 gene in the $APC^{Min\ +/-}$ mouse model of colon cancer using Sanger sequencing (Sanger and Coulson, 1975)

2 Materials

This chapter lists the reagents used in these studies and describes the preparation of solutions and buffers used throughout the experimental work.

2.1 Reagents

Reagent	**Supplier**
	All from Sigma-Aldrich unless otherwise stated
Acetone	
Acrylamide/bis-Acrylamide	
ACTB (beta Actin)	Applied Biosystems
Agarose	Melford
Ammonium persulfate (APS)	Promega
Boric acid	Fisher Scientific
Calcium chloride	Fisher Scientific
Chloroform	Fisher Scientific
*[1]Deoxynucleotide triphosphates (dNTPs) 400 µl	Promega
Deoxyribonuclease 1 (RNase-free) 1 U/µl	Fermentas
*[2]Diethylpyrocarbonate (DEPC)	
DNA ladder 1 kb 500 µg/ml (N32325)	New England Biolabs
DNA ladder 50 bp 100 µg/ml (N0473G)	New England Biolabs
Dimethyl sulfoxide (DMSO) D2650	
Ethanol 100% (EtOH)	BDH
Ethidium bromide 10 mg/ml	Fisher Scientific
*[3]Ethylenediamine tetraacetic acid (EDTA) E5134	
Formaldehyde F8923	
Gel loading (blue) dye 6x (B70215)	New England Biolabs
*Hin*d111 restriction enzyme	

Propan-2-ol	Fisher Chemical
N, N, N', N'-tetramethylethylenediamine (TEMED)	
Magnesium chloride	
Methylene blue	
*[4]Mouse ACTB (beta actin) 20x Taqman® endogenous control assay; FAM dye-labeled TaqMan MGB probe and two unlabeled PCR primers	
4352933E	Applied Biosystems
Phosphate buffered saline (PBS)	
Piroxicam P5654	
Proteinase K, recombinant PCR grade	Roche Diagnostics
*[5]Random hexadeoxynucleotides 20 µg	Promega
RNase OUT™Recombinant ribonuclease inhibitor 40 U/µl (10777-019)	Invitrogen
*[6]Sybr® Green 1 nucleic acid gel stain	BioWhittaker Molecular Applications (BMA)
Sodium chloride (NaCl)	Fisher Scientific
Sodium hydroxide	Fisher Scientific
TaqMan Fast universal PCR Master mix (2x) (4352042)	Applied Biosystems
Tri reagent™	
Tris (hydroxymethyl) aminomethane (Tris) base	Melford
2018 Tekland Global 18% protein rodent diet	Harlan
Rat and mouse standard diet	BEEKAY
3-Aminopropyl triethoxysilane (APES)	
Water HPLC grade	Fisher scientific

**** see 2.2 for protocols for preparation of solutions and buffers***

KITS

Affinity Script	Agilent Technologies
M-MLV Reverse Transcriptase (28025-013)	Invitrogen
Phusion® High-Fidelity DNA Polymerase	

(M0530L)	New England Biolabs
QIAquick® gel extraction kit (28704)	Qiagen
RNAse – free DNAse set (79254)	Qiagen
RNeasy mini kit (74101)	Qiagen
SuperScript® lll RT (18080-093)	Invitrogen

2.2 Protocols for preparation of working solutions & buffers

1. **Deoxynucleotide triphosphates (Promega)**

5 µl of each of dATP, dCTP, dGTP and dTTP (all at 100 mM) were added to 180 µl of water to give 10 mM concentration. 20 µl aliquots were prepared and stored at -20^{0}C.

2. **Diethylpyrocarbonate (DEPC) water (Sigma-Aldrich)**

DEPC water was prepared at 0.1% by adding 200 µl of DEPC to 200 ml of HPLC water (Fisher Scientific). This was then incubated overnight at 42^{0}C, then autoclaved.

3. **Ethylenediamine tetra-acetic acid solution (Sigma-Aldrich)**

EDTA does not go completely into solution until pH is adjusted to 8.0. To prepare a 500 ml stock solution of 0.5 M EDTA, 93.05 g of EDTA disodium salt was dissolved in 400 ml of deionized water. pH was adjusted with sodium hydroxide, and the solution topped up to a final volume of 500 ml.

4. **Mouse ACTB (beta actin) 20x Taqman® endogenous control assay; FAM dye-labeled TaqMan MGB probe and two unlabeled PCR primers (Applied Biosystems)**

To prepare a working assay, 10 µl of 20x reagent was added to 190 µl of HPLC water in an amber eppendorf tube (to protect the probe) and stored at -20^{0}C.

5. Random hexadeoxynucleotides (Promega)

The initial concentration of 500 µg/ml was diluted 1:5 to give 100 µg/ml. 20 µl (at 100 µg/ml) was added to 80 µl of water, and aliquots of 10 µl were prepared and stored at -20^{0}C.

6. Sybr® Green 1 nucleic acid gel stain (BioWhittaker Molecular Applications)

To make a working stock of Sybr Green, 1 µl of concentrated Sybr Green (10 000x in DMSO) was added to 99 µl of dimethyl sulfoxide (DMSO) in a dark glass vial and stored at room temperature.

7. Baker's formol-calcium (modified)

100 ml of 40% formaldehyde and 100 ml of 10% calcium chloride were added together. Distilled water was added to make up to 1 litre.

8. Tris-acetate-EDTA (TAE) buffer

A 50x stock solution of TAE was prepared by adding 242 g of Tris base to 750 ml of distilled water. 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) was added, and the final volume adjusted to 1 litre with distilled water.

A working solution of 1x TAE buffer was prepared by adding 20 ml of 50x stock solution to 980 ml of distilled water.

9. Tris-Borate-EDTA (TBE) buffer

A 10x stock solution of TBE buffer was prepared by adding 108 g of Tris base, 55 g of boric acid and 40 ml of 0.5 M EDTA (pH 8.0) to distilled water to make up 1 litre.

A working solution of 1x TBE buffer was prepared by adding 100 ml of 10x stock solution to 900 ml of distilled water.

3 The role of PPARα in malignant pathology of the mouse colon

3.1 Introduction

Peroxisome proliferator-activated receptor alpha (PPAR*α*) is a ligand-activated transcription factor involved in regulation of lipid uptake and metabolism, and inflammation (Desvergne and Wahli, 1999, Kersten et al., 2000).

PPARα is expressed in many tissues, particularly those that utilise fatty acid oxidation as a source of energy (Bookout et al., 2006). The receptor is activated by endogenous fatty acids and fatty acid derivatives, also synthetic agonists such as the fibrate class of drugs. Ligand-mediated activation of PPARα results in increased oxidation of fatty acids, reduction in serum lipids, improvement in insulin resistance and prevention of weight gain and adiposity (Kersten et al., 2000).

PPARα can also inhibit expression of pro-inflammatory genes in a ligand-dependent manner (ligand-dependent trans-repression) by inhibiting the activities of other transcription factors, such as members of NF-κB and AP-1 families (Pascual and Glass, 2006, Ricote and Glass, 2007).

$APC^{Min/+}$ mice were used for these studies, as this mouse has a mutated APC gene similar to the mutation observed in human familial adenomatous polyposis (FAP) and sporadic colon cancer (Moser et al., 1990).

Previous work has shown that the potent PPARα agonist, methylclophenapate (MCP) reduced both the number and size of colonic polyps in $APC^{Min/+}$ mice (Jackson et al., 2003). Therefore, the hypothesis that PPARα has a restraining effect on polyp development was investigated by comparing polyp development in $APC^{Min/+}$ mice with

those in which PPARα had been deleted ($APC^{Min/+}PPAR\alpha^{-/-}$) mice (Study 1A).

To identify changes in gene expression related to deletion of PPARα and with polyp growth in the colon in $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice, normal and polyp tissue was collected. Affymetrix® GeneChip® mouse genome 430 2.0 microarrays were used to investigate differential gene expression (Study 1B).

3.2 Aims of Study

- To investigate the effect of PPARα deletion on development of polyps in $APC^{Min/+}$ mice.
- To identify changes in gene expression associated with loss of PPARα and with polyp development in $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice.

3.3 Methods

$APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice of 28-30 days old were randomised onto study 1 (A and B) as shown in Table 3-1. Husbandry of the mice was as described in Methods 3.3.3.

Table 3-1: Study to investigate the role of PPARα in polyp development in the mouse colon

STUDY NUMBER	Genotype Number of mice on study	
	$APC^{Min/+}$	$APC^{Min/+}$ $PPAR\alpha^{-/-}$
1A	20	20
1B	5	5

Number of $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice on study 1A (polyp number) and 1B (gene expression)

All animal work was carried out at the Biomedical Science Unit (BMSU), University of Nottingham following the regulations and procedures of the Animals (Scientific Procedures) Act 1986 (ASPA). Studies 1A and

1B were managed in collaboration with Dr Tony Shonde and animal technicians at the BMSU, University of Nottingham.

3.3.1 The Min mouse model

Male PPARα$^{-/-}$ mice were a kind gift from Dr J. M. Peters, Pennsylvania State University, United States, and designated C57BL/6J PPARα$^{Tm1Gonz/Tm1Gonz}$ (Akiyama et al., 2001).

These mice (Pennsylvania male PPARα$^{-/-}$) were crossed with C57BL/6J females to produce F1 offspring. Heterozygous F1 were backcrossed with C57BL/6J mice to produce F2 offspring. This process was repeated to the F10 generation. This was to restore the C57BL/6J background as in-breeding using a limited number of breeding pairs would have led to the original background genetics of the strains being altered.[7] F10 mice were then crossed to produce homozygous wild-type or PPARα$^{-/-}$ mice. F11 mice were used for breeding to obtain F12 mice for phenotypic analysis.

Min mice (APC^{Min}/APC$^{/+}$) on a C57BL/6J background were a kind gift from Professor A. Gescher at the University of Leicester, England. (Perkins et al., 2002).

The (Leicester) Min mice were crossed with PPARα$^{-/-}$ mice to obtain APC$^{Min/+}$PPARα$^{/+}$, which were then crossed with PPARα$^{-/-}$ homozygote to obtain homozygous APC$^{Min/+}$ PPARα$^{-/-}$ on a C57BL/6J background.

The colonies of mice were managed and maintained by animal technicians at the BMSU, University of Nottingham.

3.3.2 Genotyping of mice

Ear snips from 21 day old male and female mice were used to determine the genotype of APC$^{Min/+}$ and APC$^{Min/+}$PPARα$^{-/-}$ mice.

[7] Inbreeding can lead to unwanted genetic drift. Genetic drift is a change in a populations allele frequency resulting from a random variation in the distribution of alleles from one generation to the next; it can cause some gene variants to disappear completely thereby reducing genetic variation

Genotyping was performed by Mr Declan Brady, School of Biology, University of Nottingham, England, using primers as described previously (Lee et al., 1995, Jeffery et al., 2004).

3.3.3 Husbandry of mice

Genotyped mice at 28-30 days old were introduced to the study over a period of eight months, whilst ensuring adequate breeding stock was maintained. Standard chow (Rat and mouse standard diet, BEEKAY, Humberside) and water was available *ad libitum*. Mice were subject to a light/dark cycle of 12 hours each. Date of birth (DOB) and date of death (DOD) were recorded for each mouse. Mice were weighed at the start of the study, and on a weekly basis thereafter, until sacrifice by cervical dislocation. They were terminated if they showed signs of suffering, anaemia (pale foot pads - white paw), rectal prolapse or >15% weight loss. Animal technicians in collaboration with the animal unit on-call veterinary surgeon independently monitored the mice twice daily. They advised if and when it became necessary for a mouse to be sacrificed. Reasons for sacrifice of each mouse were recorded.

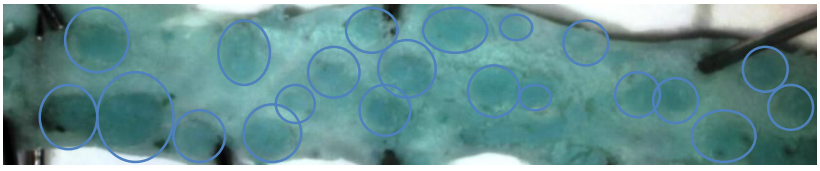
3.3.4 Quantification of mouse intestinal polyps

Whole intestines from mice on Study 1A (20 from $APC^{Min/+}$ mice, 20 from $APC^{Min/+}PPAR\alpha^{-/-}$ mice, Table 3-1) were removed immediately after sacrifice and flushed with saline. The intestines were separated into colon and small bowel, and opened lengthways with scissors. All tissue was fixed in 70% formol calcium (page 42).

50 mg of methylene blue hydrate (Sigma-Aldrich) was dissolved in 20 ml of phosphate buffered saline (PBS) to give a 0.25% methylene blue staining solution. Fixed tissue was immersed in PBS for a few minutes then briefly immersed in methylene blue stain, rinsed with water, then destained with 70% ethanol. This methodology differentially stained normal mucosa from polyp tissue.

Tissue was pinned out flat and observed through a Karl Zeiss Tessovar macroscope (x4.2 magnification). Images were acquired using a Q imaging micropublisher 5.0 RTV camera with Improvision Openlab software. Each colon and small bowel was imaged sequentially along its length. A pin placed at the edge of each 'frame' indicated where the next image was taken from (the pin could be clearly seen on the images). Images were viewed on a computer. Polyps were quantified by counting each individual polyp, up and down, left to right, on each image, then a total polyp count calculated for each piece of tissue. Extra care was taken at the longitudinal cut edges of the tissue to ensure polyps were not counted twice. Figure 3-1 shows typical images of colonic polyps and small bowel polyps.

Polyps in the mouse colon (x4.2 magnification)



Polyps in the mouse small bowel (x4.2 magnification)

Figure 3-1 Methylene blue staining of polyps in the mouse colon & small bowel

Blue circles have been added to the images to highlight the polyps

3.3.5 Processing, Cutting and Haematoxylin & Eosin staining of mouse colonic mucosa

After completion of polyp number evaluation, two mouse colons from storage in formol calcium (one representative colon from each genotype; $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$) were selected. Colons were pinned out flat onto cork boards; samples of tumour and normal tissue (at least 2 cm away from visible polyps) were excised with scissors and stored in formol saline.

For processing, samples were placed into secure cassettes and processed overnight in a Leica TP1020 tissue processor. Stations 1 and 2 (fixing stations) were not required as tissue was previously fixed in formol calcium (Table 3-2A).

After processing, the dehydrated tissue was carefully positioned centrally into a wax mould. The mould was filled with molten wax and left to set on a cold plate. A Leica RM2145 microtome was used to cut 4 µm thick sections from the wax embedded tissue samples. Sections in ribbons of 3-4 were cut and floated out into a paraffin section mounting bath (40^0C), which were then attached to an APES (3-Aminopropyl triethoxysilane) coated slide. Slides were dried on a Leica EG1120 hotplate (68^0C). Before staining the sections were rehydrated (Table 3-2B). Slides were then ready for Haematoxylin & Eosin staining (Table 3-2C).

After staining, slides were viewed on a Leica DM 4000 light microscope. Images were captured and saved using Openlab on a Q imaging Micropublisher 3.3RTV camera.

Table 3-2 Preparation of mouse colon tissue for Haematoxylin & Eosin staining

A. Tissue process steps on Leica TP1020

Leica TP1020 tissue processor sequence		
Station	**Process chemical**	**Time (hours)**
1		
2		
3	70% alcohol	1.5
4	80% alcohol	1.5
5	96% alcohol	1.5
6	100% alcohol	1
7	100% alcohol	1
8	100% alcohol	1
9	xylene	1.5
10	xylene	1.5
11	paraffin	2
12	paraffin	2

B. Rehydration steps for wax sections (pre-staining procedure)

Rehydration of wax sections	
Reagent	**Time (minutes)**
xylene	5
100% IMS	0.5
100% IMS	0.5
90% IMS	0.5
70% IMS	0.5
50% IMS	0.5
running tap water	0.5

C. Haematoxylin & Eosin stain protocol

Haematoxylin & Eosin Stain protocol	
Process	**Time**
Submerge slides in Harris Haematoxylin	5 minutes
Wash in bath of running tap water until water clears	
Dip in Acid Alcohol	10 seconds
Wash in bath of running water until no streaks on the slides	
Dip in Lithium Carbonate/Scott's tap water	10 seconds
Wash in bath of running tap water	
Check samples are blue under microscope	
Submerge slides in 1% Eosin	5 minutes
Wash in a bath of running tap water until water clears	
Dip into 50% IMS	10 seconds
Dip into 70% IMS	10 seconds
Dip into 90% IMS	10 seconds
Dip into 100% IMS	10 seconds
Dip into fresh Xylene	2 minutes
Dip into finishing Xylene	2 minutes
Add few drops of DPX to appropriately sized coverslip to mount section	

3.3.6 RNA extraction from mouse colonic mucosa

Colons from mice on Study 1B were removed immediately after sacrifice and flushed with saline. Colons were opened lengthways with scissors and laid flat for examination by eye. Samples of normal (at least 2 cm away from visible polyps) and tumour tissue were excised with scissors and separately flash frozen in liquid nitrogen, before storage at -80^0 C.

Samples taken from storage were homogenised in 2 ml of Tri reagent™, and extraction of RNA performed according to the manufacturer's instruction (Sigma-Aldrich). Samples were further purified using the RNeasy mini kit, incorporating the additional DNAse treatment step as outlined in the manufacturer's instruction (Qiagen). A NanoDrop® ND-1000 Spectrophotometer was used to determine the 260/280 ratio[8] and the concentration in ng/µl of all of the samples. 1 µl of each mouse RNA sample was analysed on an Agilent Bioanalyser using a RNA Nano 6000 chip. Analysis produced the RIN (RNA Integrity Number).[9] RNA was aliquoted into smaller volumes and stored at -80^0C. Table 3-3 shows sample identification numbers, genotype, tissue type and RIN.

[8] The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA. A ratio of ~2.0 signifies pure RNA. A ratio that is lower indicates the presence of protein, phenol or other contaminants that absorb at or near 280 nm.

[9] The 28S/18S rRNA (ribosomal RNA) ratio is used to determine integrity; rRNA makes up > 80% of total RNA in a ~ 2:1 ratio

Table 3-3 Identification & RNA integrity number of samples for Affymetrix® microarray

Sample identification	Genotype	Tissue type	RNA Integrity Number (RIN)
44b.3/4	$APC^{Min/+}$	normal	8.8
44b.3/4	$APC^{Min/+}$	tumour	8.5
70.3/2	$APC^{Min/+}$	normal	9.1
70.3/2	$APC^{Min/+}$	tumour	9.2
76.4/1	$APC^{Min/+}$	normal	8.4
76.4/1	$APC^{Min/+}$	tumour	9.0
76.4/3	$APC^{Min/+}$	normal	8.5
76.4/3	$APC^{Min/+}$	tumour	9.1
72.1/2	$APC^{Min/+}$	normal	6.6
72.1/2	$APC^{Min/+}$	tumour	5.3
115.4/1	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	normal	9.1
115.4/1	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	tumour	9.6
11a.3/4	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	normal	9.3
11a.3/4	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	tumour	9.4
121.1/2	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	normal	8.7
121.1/2	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	tumour	9.0
121.1/3	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	normal	8.3
121.1/3	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	tumour	8.5
121.1/5	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	normal	8.6
121.1/5	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	tumour	9.1

Nine pairs of samples, tumour and normal from four $APC^{Min/+}$ mice and five $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice were selected for further analysis (Table 3-3). Paired samples from an $APC^{Min/+}$ mouse (72.1/2) were excluded as the RIN was low indicating degradation of the samples (highlighted in red).

3.3.7 Affymetrix® microarray analysis of mouse colon normal & tumour mucosa

Mouse colon RNA samples (Study 1B, Table 3-3) were processed at the Paterson Institute, Manchester on Affymetrix® GeneChip® Mouse Genome 430.2 high-density oligonucleotide arrays (one sample per array).

The array uses 45,101 probe sets selected from sequences derived from GenBank®, dbEST and RefSeq to analyze the expression level of over 39,000 transcripts and variants from over 34,000 well characterized mouse genes (Affymetrix, 2003).

Microarray studies involve a multi-step process; these are experimental design, array design, sample preparation and labelling, hybridisation procedures and parameters, measurements, normalisation controls, data processing and analysis. It is, therefore, important to minimise variation by the use of good laboratory protocols. Microarray standard operating procedures (mSOPs) were implemented to verify that each step was accurately and consistently carried out (Forster et al., 2003). This ensured compliance of 'Minimum Information About a Microarray Experiment' (MIAME) requirements; these are necessary for publication of microarray data and ensure uniformity of standards in production and analysis of microarray data that can be readily interpreted and independently verified (Brazma et al., 2001).

The quality of data from microarrays is highly dependent on RNA quality. Mouse RNA samples for gene array were sent on dry ice at a concentration of ~ 1 µg/µl and greater than or equal to 5 µg of RNA with a 260/280 ratio of 1.9 – 2.1 and a RIN of greater than or equal to 8.0.

Figure 3-2 shows an overview of the protocol for sample preparation & analysis on Affymetrix GeneChip® arrays.

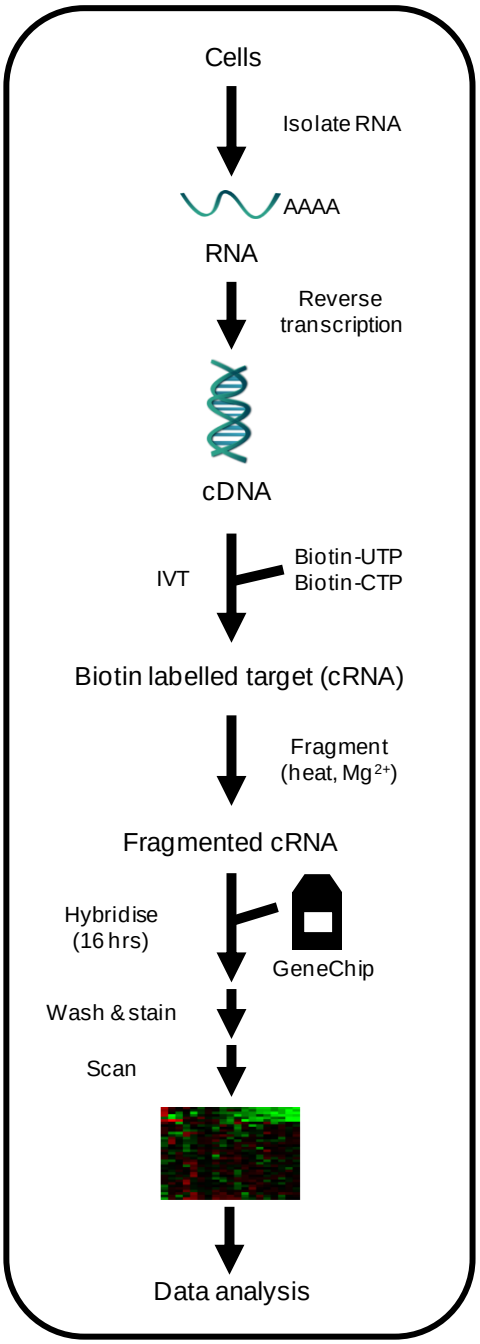


Figure 3-2 Protocol for sample preparation & analysis on Affymetrix GeneChip® arrays

RNA was reverse transcribed then labelled with Biotin-UTP and Biotin-CTP in an *in-vitro* transcription reaction. After heating with Mg2+, fragmented cRNA was hybridized onto a GeneChip before washing, staining and scanning for data analysis

Briefly, Figure 3-2 portrays how cDNA was synthesised from mouse RNA samples using reverse transcriptase and a T7 oligo-dT (deoxythymine) promoter primer. cDNA was then used in an Affymetrix 3' *in-vitro* transcription (IVT) reaction to produce amplified biotin-labelled antisense mRNA (cRNA) – the array targets.

The T7 oligo-dT primer is designed to incorporate a T7 promoter sequence during cDNA synthesis. The sequence has high affinity for the T7 RNA polymerase, and therefore, promotes a high level of expression. The primer contains an oligo-dT 24 sequence at its 3 prime (3') end for specific binding to the poly (A) tail of mRNA, and the core sequence of the T7 RNA polymerase promoter at its 5 prime (5') end.

Essentially, this means that the cDNA yield from sequences close to the 5' end of partially degraded mRNA would be significantly less than that near the poly (A) tail (Swift et al., 2000). Affymetrix arrays are designed with oligo probes that are close to the 3' end of a transcript. However, additional probe sets for reference genes including glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and actin, are designed at the 5' end of the transcript (Affymetrix, 2001). The signal intensity ratio of the 3' probe set to the 5' probe set (3'/5' ratio) is used to verify RNA integrity, efficiency of first strand cDNA synthesis, and *in vitro* transcription of cRNA (Copois et al., 2007). RNA is deemed to be degraded if the 3'/5' ratio is greater than three (Affymetrix, 2001).

After the reverse transcription step, the cDNA and a mixture of nucleotides and biotinylated nucleotide analogues were then used in a 3' IVT reaction to produce biotin labelled complementary RNA (cRNA). Antisense RNA amplification improves the sensitivity of the microarray.

Prior to hybridisation the biotinylated cRNA was heated with an endonuclease and Mg^{2+} to produce 25 – 200 base pair fragments. Fragmentation improves hybridisation to target probes to produce an enhanced signal. The cRNA fragments were then added to a hybridisation master mix before being injected into a Genechip® chamber for hybridisation for 18 hours at 45^0C.

After hybridisation the chip was processed with a series of staining and washes; the chip was stained with streptavidin-phycoerythrin, a fluorescent molecule that binds to biotin, and washed with anti-streptavidin antibody (goat) and biotinylated IgG antibody (goat).

The chips were scanned and processed using GeneChip® Operating Software (GCOS).

GCOS computed a detection call (present, absent or marginal), detection p value and average intensity value (signal) for each probe set to generate a table of 45,101 entities. Detection calls were used to determine whether a probe set was reliably detected and selected for further analysis. GCOS quality control data indicated between 55% - 60% of probe sets were detected as present in the 18 arrays.

GCOS also produced image data files (.DAT files) which provided an assessment of the quality of the scanning event; and cell intensity files (.CEL files) that were binary files derived from the .DAT files. .CEL files were the base files for further analysis using GeneSpring®.

3.3.8 Rationale for methodology of analysis of Affymetrix® microarray data

Affymetrix® microarray data were analysed to determine if there were any differences in gene expression in the colon between $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice, and between tumour and normal tissue.

GeneSpring®GX 11.0.2 software was used for the analysis. Data from each of the 18 samples were categorised into groups; that is, 4 $APC^{Min/+}$ normal samples, 4 $APC^{Min/+}$ tumour samples, 5 $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal samples, 5 $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour samples (Table 3-3).

Comparison of these data generated lists of differentially expressed genes (DEG lists), as below;

- Comparison 1: $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal
- Comparison 2: $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour
- Comparison 3: $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour
- Comparison 4: $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour

These data were further interrogated to determine whether any differences were significant in development and progression of colon cancer in the mouse colon.

DEG list data were input into Ingenuity Pathway Analysis (IPA) to produce networks of genes; these were then annotated with pathways known to be involved in tumorigenesis and PPARα; Wnt/beta catenin, ERK/MAPK, p53 (Fearon and Vogelstein, 1990) and PPARα/RXRα (Kersten et al., 2000, Jackson et al., 2003).

A sub-set of these genes were then selected for further investigation.

3.3.9 GeneSpring®GX 11.0.2 analysis of Affymetrix® microarray data

GeneSpring® is software for importing and analysing gene array data within a single platform. It supports Affymetrix® data format with guided workflows specific to GeneChip® gene expression arrays. GeneSpring® makes optimal use of Affymetrix®-specific quality control and other optimisations, for example, sample preparation, hybridisation, staining and washing.

Table 3-4 shows an overview of the guided workflow for GeneChip® mouse genome 430.2 arrays.

Table 3-4: GeneSpring® guided workflow for GeneChip Mouse Genome 430.2 arrays

PROCESS	STEP	TASK	SELECTION
Create new experiment	**1**	Load data	Import .CEL files
	2	Select technology	Affymetrix® GeneChip® Mouse Genome 430. 2
	3	Select summarization algorithm	Robust Multichip Averaging (RMA)
	4	Select baseline transformation	Baseline to median for all samples
Experiment setup	**1**	Define experiment grouping	i. Tumour or Normal ii. APC $^{Min/+}$ PPARα$^{-/-}$ or APC $^{Min/+}$
	2	Create interpretation	i. Tissue type only ii. Genotype only iii. Tissue type & Genotype
Experiment quality	**1**	Validation of biological replicates	Pivot table
Quality control (QC)	**1**	QC on samples	i. Internal controls ii. Hybridization control iii. Principle component analysis (PCA)
	2	QC on entities	Filter probesets by expression; 45.0–100.0 percentile
Analysis	**1**	Fold change >= 2	
	2	t test $p < 0.005$	Multiple testing correction not used

Overview of workflow protocol for preparation and analysis of gene array data using *GeneSpring*®

As can be seen from Table 3-4 the first process for preparation and analysis of array data was creation of a new experiment. All 18 .CEL files were imported from GCOS, and the appropriate technology selected (Affymetrix GeneChip® Mouse genome 430.2).

Robust Multichip Averaging (RMA) algorithm (Irizarry et al., 2003) was used to perform three key tasks; background correction, normalisation and probe summarisation.

Background correction was performed on each chip. The method is based on the distribution of only the perfect match (PM) values of probes; probe intensity values consist of the actual signal and a background signal comprised of non-specific binding of the fluorophore and optical noise. The background signal was corrected for by the RMA algorithm.

Normalisation was performed before analysis to ensure that apparent differences in intensities, both within and between arrays, were due to differential expression and not hybridisation or scanning variations. The RMA algorithm uses Quantile normalisation and utilises all arrays to perform normalisation on raw intensities, regardless of variance.

Briefly,

1. The intensity values were ranked in each array
2. The average of the intensity values was calculated for each probe
3. The intensity values were substituted with the calculated average
4. The ranked average intensity values were returned to the original order

This effectively caused all the distributions to be the same and corrected for array biases.

Probe summarisation estimated the actual expression value of a probe by aggregation of the expression value on the log scale with an independent noise value. Log-transformed intensity values result in a normal distribution.

Baseline transformation completed the creation of the new experiment. Baseline to median for all samples was selected; comparable to normalisation for each gene.

The next process was to set up the experiment by defining experimental groups and the interpretations to be analysed. There were four groups; $APC^{Min/+}$ normal, $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and

$APC^{Min/+}PPAR\alpha^{-/-}$ tumour. Interpretations were; the effects of tissue type only, genotype only, and tissue type & genotype (Table 3-4).

The quality of the experiment was assessed. The similarity within each group of biological replicates was examined; that is, $APC^{Min/+}$ normal group (four replicates), $APC^{Min/+}$ tumour group (four replicates), $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal group (five replicates) and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour group (five replicates).

This was done by ranking (highest to lowest) the normalised expression values of every probe for each replicate within a group. For each group, data were input into a pivot table (Excel). The pivot table presents the data to show the ranking of every probe in each sample of a group. Thus, the ranking of any probe can be compared simultaneously across all samples within a group. This allows for easy identification of an outlier sample.

Quality control (QC) of samples was performed to assess the reproducibility of gene expression measurements across replicates. QC of samples comprised of three steps. Firstly, internal controls assessed RNA sample quality by determining the 3 prime/5 prime (3'/5') ratio for GAPDH and actin for each sample. A ratio of more than three would indicate sample degradation. Secondly, hybridisation controls showed the quality of hybridisation and the washing process. Hybridisation controls were composed of a mixture of biotin-labelled cRNA transcripts of bioB, bioC, bioD and cre, at known staggered concentrations that were spiked into the hybridisation reaction. The signal intensity of the controls should increase as expected in line with the known concentrations. Lastly, Principal Component Analysis (PCA) which is suitable for large data sets such as gene expression data from microarrays, was performed. A covariance analysis was calculated to discover correlations between samples or conditions. PCA scores were visually represented on a 3D scatter plot. Samples from the same experimental condition group together on the plot.

The QC analysis of our samples led to exclusion of one $APC^{Min/+}$ tumour (70.3/2) sample from further analysis.

Quality control of entities was performed by filtering probe sets by expression. This operation removes by default the lowest 20 percentile of all intensity values. The lowest 45 percentile was removed as GCOS QC data indicated between 55% - 60% of probe sets were detected as present in the 18 arrays (3.3.7, page 51).

Comparison of groups and interpretations as defined in the experiment set up (Table 3-4) produced lists of differentially expressed genes (DEG lists). The lists from $APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal (Comparison 1), $APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2), $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3) and $APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}PPAR\alpha^{-/-}$ tumour (Comparison 4) were subject to statistical analyses; genes were excluded from the list if they did not fulfil specified criteria; that are, fold change (FC) greater than or equal to two, followed by unpaired t test, where p was less than or equal to 0.005.

Initial statistical analysis of gene lists was carried out using unpaired t test with incorporated multiple testing correction (MTC).

MTC adjusts p-values to correct for the occurrence of false positives; these occur when the null hypothesis is true, but is rejected. The null hypothesis states that there is no significant difference in gene expression between specified groups.

The false positive rate is proportional to the number of tests performed and the p-value cut-off. A microarray experiment simultaneously performs a t-test or ANOVA on all genes. A p-value of 0.05 denotes a 5% probability that the expression value of a gene in one condition is different than the expression value in the other condition, by chance alone. For example, if 10,000 genes were tested, 5% (or 500 genes) would be deemed significant by chance alone.

GeneSpring® has a choice of four types of MTC which are listed below in order of stringency; Bonferroni is the most stringent, and the Benjamini and Hochberg False Discovery Rate (FDR) the least stringent.

- Bonferroni
- Bonferroni Step-down (Holm)
- Westfall and Young Permutation
- Benjamini and Hochberg FDR

The more stringent a MTC, the fewer false positive genes are identified. Benjamini and Hochberg FDR (Benjamini and Hochberg, 1995) was selected as the MTC; the method is explained briefly below.

For example, when $p < 0.05$

1. p-values of each gene are ranked (smallest to largest)
2. Largest p-value is unchanged
3. Second largest p-value is multiplied by the total number of genes (n) and divided by its rank (n-1).

That is, corrected p-value = p-value*(n/n-1). Gene is significant if $p < 0.05$

4. Third largest p-value is multiplied as in step 3:

Corrected p-value = p-value*(n/n-2). Gene is significant if $p < 0.05$

And so on.....

Initial statistical analysis of differentially expressed gene lists (Comparisons 1-4) produced zero results. To attempt to overcome this problem, the p value stringency was changed to less than or equal to 0.05. However, this too produced zero results. Further analysis was carried out without MTC, and with FC >= 2, followed by unpaired t test, where p <= 0.005, as defined earlier (Table 3-4).

To increase the number of potential differentially expressed genes in $APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal (Comparison 1), and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2), less

stringent criteria for selection was applied. Comparison 1 and Comparison 2 were re-analysed using FC >= 1.7, p <= 0.05, also without MTC.

To ensure these data were valid and comparable as multiple testing correction (MTC) was not used in statistical analysis, differentially expressed gene (DEG) lists were cross-referenced against ranked lists of genes, as described; briefly, normalised probe intensity values[10] of pairs of groups (comparable to comparison groups 1 - 4) of $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}$ normal, $APC^{Min/+}PPAR\alpha^{-/-}$ tumour and $APC^{Min/+}$ tumour, $APC^{Min/+}$ normal and $APC^{Min/+}$ tumour, and $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour, were listed. The difference between probe intensity values (that is, the intensity value for a probe in one group minus the intensity value of the same probe in the other group, for each pair of groups) was ranked, highest to lowest. The ranking of five reference genes were determined in each list; 18s (ribosomal RNA), Actb (beta actin), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), Tfrc (transferrin receptor protein) and Pcx (pyruvate carboxylase). Reference genes were selected as expression of each was similar in all four lists. Genes were selected and listed from each of the four ranked lists if they were above a reference gene in the ranking. Genes were excluded from DEG lists if they did not appear on at least one of the ranked lists. These edited DEG lists can be viewed in Appendices (pages 311-377).

Figure 3-3 and Figure 3-4 show a flow-chart sequence of GeneSpring analysis of comparison group 1 ($APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal), comparison group 2 ($APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour), comparison group 3 ($APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour) and comparison group 4 ($APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour).

[10] The average of probe intensity values corresponds to the expression level of the hybridised gene of interest

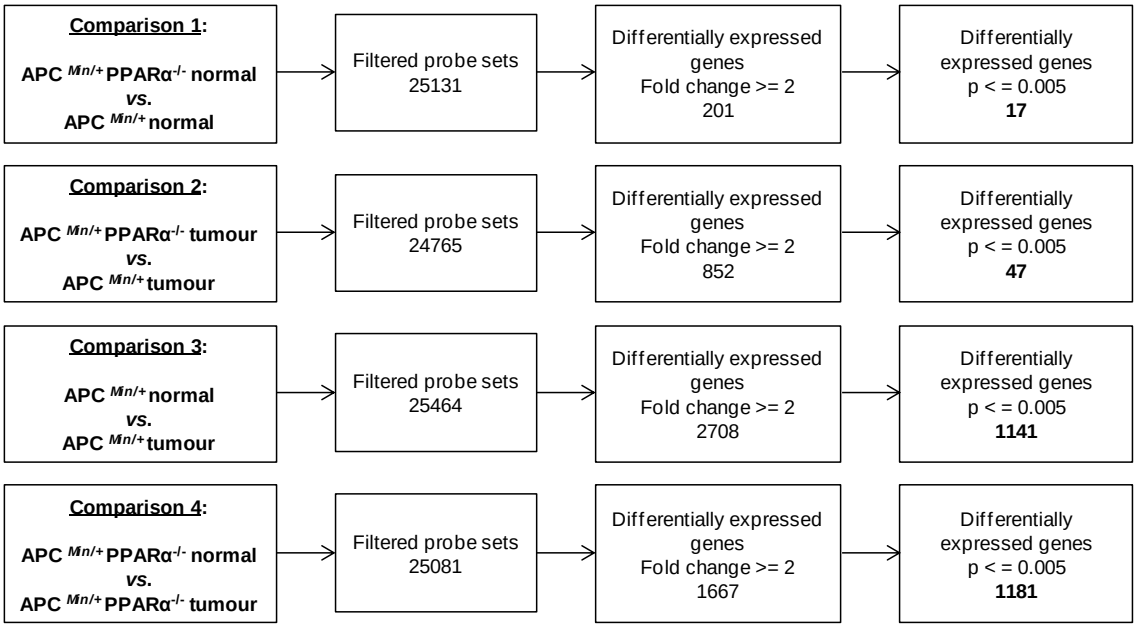


Figure 3-3: Enrichment of differentially expressed genes by GeneSpring® quality control & analysis

Numbers of differentially expressed genes were sequentially reduced by increasing the stringency of quantity control and statistical analyses

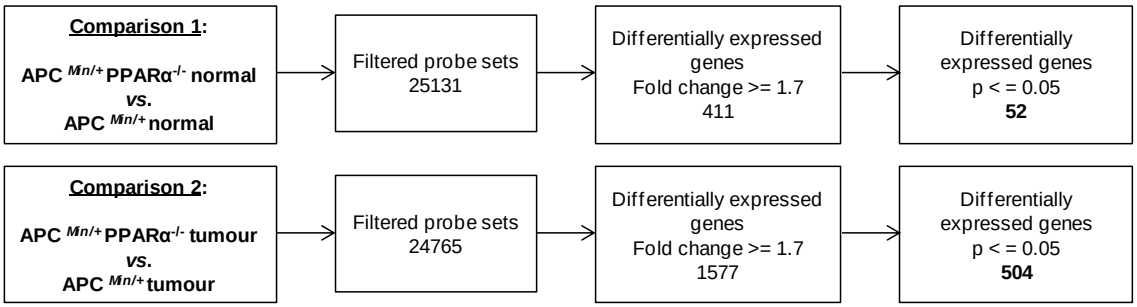


Figure 3-4: Enrichment of differentially expressed genes in Comparison 1 & Comparison 2 by GeneSpring® quality control & analysis

Numbers of differentially expressed genes in Comparison 1 and Comparison 2 were increased by decreasing the stringency of statistical analyses

3.3.10 Ingenuity® Pathway Analysis (IPA®) of Affymetrix® microarray data (Study 1B)

Ingenuity® Pathway Analysis (IPA®) is a bioinformatics tool based on the Ingenuity Pathway Knowledge Base (IPKB). The IPKB is a repository of expertly curated biological and chemical interactions, and functional annotations. It is produced from over 200,000 peer-reviewed scientific publications which are manually reviewed for accuracy.

IPA utilises IPKB to generate network maps; a biological interaction network map is a graphical representation of the molecular relationships between genes. Genes are represented as nodes, and the biological relationship between two nodes by a line. All lines are supported by at least one reference stored in the IPKB. The degree of up- (red) or down- (green) regulation is indicated by the intensity of node colour. Nodes are displayed using different shapes to represent the functional class of a gene product (see Figure 3-5). Lines describe the nature of the relationship between the nodes, that is, a solid line indicates a direct interaction and a dashed line indicates an indirect interaction between genes.

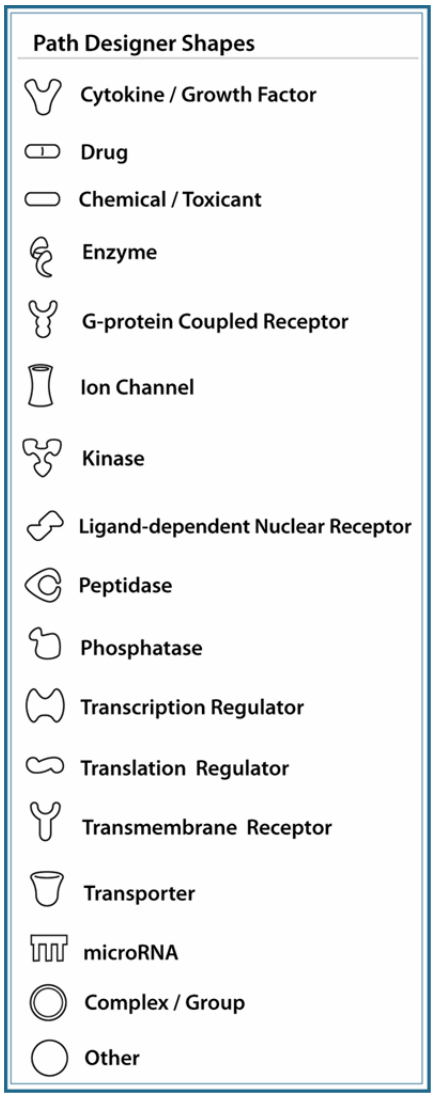


Figure 3-5 Key to symbols on Ingenuity Pathway Analysis networks

IPA was used to analyse Affymetrix® microarray data and also to aid selection of genes of interest for further investigation on Taqman® low density arrays.

Data sets comprised of the edited DEG lists (Appendices, pages 311-377) from Comparison 1 ($APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal), Comparison 2 ($APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour), Comparison 3 ($APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour) and Comparison 4 ($APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}PPAR\alpha^{-/-}$ tumour), containing gene identifiers and corresponding fold change and p values were separately uploaded into IPA. Each gene identifier was mapped to its corresponding gene in the IPKB to generate a series of biological

interaction network maps and list of biological processes, all with corresponding lists of genes, for each data set.

IPA computed a score for each network based on the input data. The score was derived from the p value and indicated the likelihood of the genes in a network being found together as a result of random chance. A score of 2 indicates that there is a 1-in-100 chance that the genes were together in a network as a result of random chance. Therefore, the higher the score the greater statistical significance that genes represented in a network were interconnected.

The IPA data of Comparison 3 ($APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour) and Comparison 2 ($APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour), were mined to extract data from the two top biological processes highlighted in these analyses; growth & proliferation and colorectal cancer. Separate networks showing the interaction of genes involved in both processes were generated.

Additionally, the growth & proliferation and colorectal cancer networks from Comparison 3 and Comparison 2 were separately annotated with;

- The Wnt/ beta Catenin signalling pathway

This pathway is known to be deregulated when the Adenomatous Polyposis Coli (APC) gene is mutated, leading to development of polyps in the colonic epithelium (Korinek et al., 1997, Oshima et al., 1997).

- The ERK/MAPK (ras) pathway

The ERK/MAPK canonical pathway is involved in regulation of gene expression. Mutations or aberrant expression of components of the pathway (for example ras and b-raf) have been revealed in human cancers (McCubrey et al., 2007).

The ERK/MAPK pathway is activated when a signalling molecule binds to the receptor on the cell membrane. This initiates a cascade of signalling events beginning with Ras (a GTPase). Ras exchanges GDP (guanosine diphosphate) for GTP (guanosine triphosphate) which

activates MAP3K. This in turn activates MAP2K, then MAPK (mitogen-activated protein kinase, also known as ERK). MAPK then activates a transcription factor, for example, Myc (Seger and Krebs, 1995).

- The p53 pathway

p53 is a tumour suppressor gene and is known as the 'guardian of the genome' (Lane, 1992). Mutation of the p53 gene can lead to loss of function, deregulated transcription of target genes and cancer (Hollstein et al., 1991).

- The PPARα pathway

A previous study (Jackson et al., 2003) suggested that PPARα ligands decreased polyp number and that PPARα expression was reduced in human tumour samples.

In addition, data from Comparison 3 and Comparison 2 were overlaid onto IPA generated canonical pathways for Wnt/ beta Catenin, ERK/MAPK (ras) p53 and PPARα to create further networks.

These data are presented in Results 3.4.5 page 77.

To validate Affymetrix® microarray data, a sub-set of 95 genes was selected for subsequent analysis with Taqman® low density arrays.

All IPA networks, with a score of ten or above were analysed for genes involved with colorectal cancer, growth & proliferation and PPARα pathways. However, as IPA is dependent on the IPKB, only networks and results based on peer-reviewed research within that database were generated. Several novel genes were significantly differentially regulated in the DEG lists (Comparison 1-4, Appendices pages 311-377), but did not appear on IPA networks. Therefore, although IPA is a useful source of information for validation of data for established pathways, such as the Wnt/ beta Catenin, ERK/MAPK (ras), p53 and PPARα pathways, it cannot be used exclusively for determination of genes of interest for further investigation.

Two novel genes of interest, Onecut2 and Apobec3 were included in the final list of genes to be validated on Taqman® low density arrays.

The National Centre for Biotechnology Information (NCBI) gene database (http://www.ncbi.nlm.nih.gov/gene) was accessed for information on nomenclature, reference sequences (RefSeqs) and pathways for each selected gene.

The 95 genes selected for verification of expression level by Taqman® low density arrays were categorised into functional groups; see Results 3.4.6 page 104, Table 3-15-Table 3-23.

3.4 Results

3.4.1 Age & Weight of mice at sacrifice: Study 1A

The mean age at sacrifice for $APC^{Min/+}$ mice was 29.49 +/- 0.93 weeks (Mean +/- Standard Error, Table 3-5). All mice in this group were sacrificed due to development of white paw (pale foot pads that indicates anaemia) except one which had rectal prolapse (Table 3-5).
$APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice mean age at sacrifice was 28.87 +/- 1.24 weeks (Table 3-6). All mice in this group were sacrificed due to white paw (Table 3-6). There was no significant difference in ages of mice at sacrifice between the two groups (Figure 3-6A).

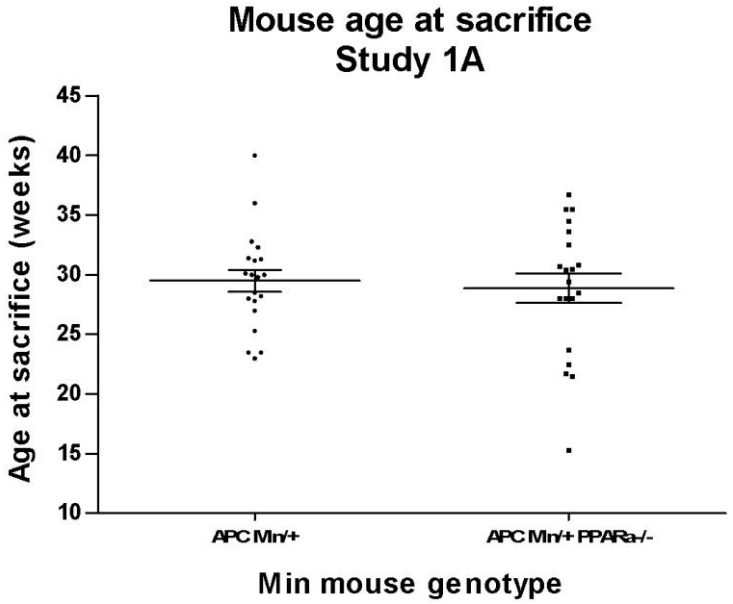
The mean weight at death of $APC^{Min/+}$ mice was 22.35 +/- 0.99 g, Table 3-5. The mean weight at death of $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice was significantly heavier 29.91 +/- 1.49 g, Table 3-6, $p = 0.0001$, Figure 3-6B.

Table 3-5: Age, weight & disposition at sacrifice of APC$^{Min/+}$ mice on study 1A

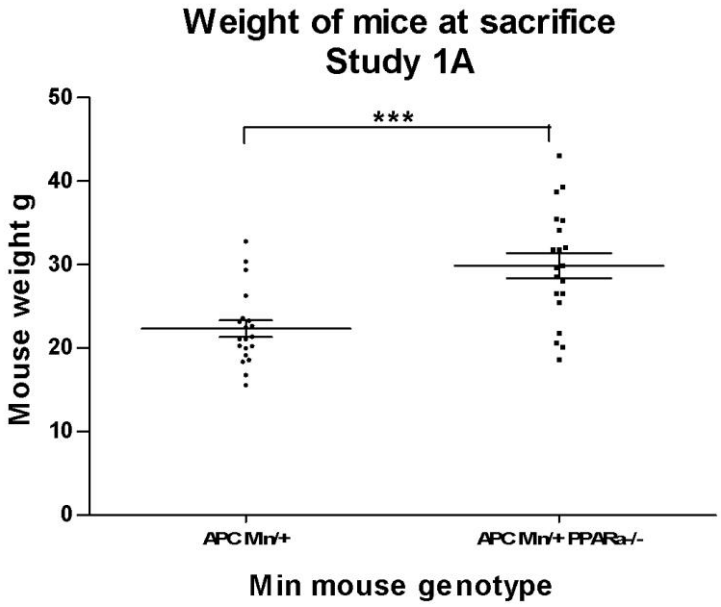
Mouse ID	Genotype	Age at death (weeks)	Weight at death (grams)	Disposition of mouse at death
35 1/1 M	APC $^{Min/+}$	28.50	21.10	white paw
36 1/4 F	APC $^{Min/+}$	31.30	23.20	white paw
31 1/3 M	APC $^{Min/+}$	31.40	20.00	white paw
29b 3/3 F	APC $^{Min/+}$	28.20	26.30	white paw
31 1/2 F	APC $^{Min/+}$	30.00	22.50	rectal prolapse
35 1/4 M	APC $^{Min/+}$	30.10	18.60	white paw
27b 4/2 M	APC $^{Min/+}$	23.00	22.70	white paw
24a 5/3 M	APC $^{Min/+}$	25.30	23.30	white paw
30 1/2 F	APC $^{Min/+}$	30.00	20.25	white paw
31 6/1 M	APC $^{Min/+}$	32.80	29.40	white paw
31 2/1 m	APC $^{Min/+}$	32.30	30.40	white paw
37 1/2 M	APC $^{Min/+}$	23.50	32.80	white paw
37 1/3 M	APC $^{Min/+}$	23.50	16.80	white paw
35 1/5M	APC $^{Min/+}$	27.00	21.10	white paw
28c. 4/1F	APC $^{Min/+}$	27.80	20.30	white paw
34 2/1 F	APC $^{Min/+}$	31.20	15.60	white paw
27b 4/1 F	APC $^{Min/+}$	29.80	18.40	white paw
24a 5/2 M	APC $^{Min/+}$	36.00	19.20	white paw
31 1/2 M	APC $^{Min/+}$	28.00	21.40	white paw
21 2/3 F	APC $^{Min/+}$	40.00	23.60	white paw
	MEAN	**29.49**	**22.35**	

Table 3-6: Age, weight & disposition at sacrifice of APC$^{Min/+}$ PPARα$^{-/-}$ mice on study 1A

Mouse ID	Genotype	Age at death (weeks)	Weight at death (grams)	Disposition of mouse at death
40 2/3 M	APC $^{Min/+}$ PPARα$^{-/-}$	15.3	20.2	white paw
37 3/2 M	APC $^{Min/+}$ PPARα$^{-/-}$	28	32.1	white paw
47b 1/3 M	APC $^{Min/+}$ PPARα$^{-/-}$	30.5	28.6	white paw
47a 1/1 M	APC $^{Min/+}$ PPARα$^{-/-}$	28.5	34.2	white paw
47b1/1 F	APC $^{Min/+}$ PPARα$^{-/-}$	32.5	35.5	white paw
40 1/4 F	APC $^{Min/+}$ PPARα$^{-/-}$	30.4	28.1	white paw
41 1/3 M	APC $^{Min/+}$ PPARα$^{-/-}$	29.4	20.7	white paw
44 1/2 F	APC $^{Min/+}$ PPARα$^{-/-}$	23.7	31.8	white paw
42 2/1 F	APC $^{Min/+}$ PPARα$^{-/-}$	21.7	39.3	white paw
40 2/2 M	APC $^{Min/+}$ PPARα$^{-/-}$	36.7	38.75	white paw
40 2/1 M	APC $^{Min/+}$ PPARα$^{-/-}$	30.7	43.1	white paw
52b 1/5 M	APC $^{Min/+}$ PPARα$^{-/-}$	21.5	18.7	white paw
47b 1/1M	APC $^{Min/+}$ PPARα$^{-/-}$	30.8	26.6	white paw
31 2/3 F	APC $^{Min/+}$ PPARα$^{-/-}$	22.5	26.55	white paw
41.1/2 M	APC $^{Min/+}$ PPARα$^{-/-}$	28	35.3	white paw
42 2/3 M	APC $^{Min/+}$ PPARα$^{-/-}$	28	29.9	white paw
37 2/2 M	APC $^{Min/+}$ PPARα$^{-/-}$	35.5	31.8	white paw
37 2/2F	APC $^{Min/+}$ PPARα$^{-/-}$	34.5	25.5	white paw
35 2/3F	APC $^{Min/+}$ PPARα$^{-/-}$	35.5	21.8	white paw
31 2/4F	APC $^{Min/+}$ PPARα$^{-/-}$	33.6	29.7	white paw
	MEAN	**28.87**	**29.91**	



A. Age at sacrifice of mice on Study 1A



B. Weight of mice at sacrifice on Study 1A

Figure 3-6 Distribution of age (A) & weight (B) of $APC^{Min/+}$ & $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice at sacrifice: Study 1A

Unpaired t test, *** p <= 0.0001

3.4.2 Number of polyps in the mouse colon

The mean number of polyps in the colon of $APC^{Min/+}$ mice was 0.60 +/- 0.17, Table 3-7. However, in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice the mean number of polyps (2.10 +/- 0.28) was significantly higher ($p < 0.0001$, Table 3-8, Figure 3-7A).

A similar profile of results was seen when age of the mice at sacrifice was considered. Mean number of polyps per week in the colon of $APC^{Min/+}$ mice was 0.019 +/- 0.006 (Table 3-7), but significantly higher in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice, 0.077 +/- 0.011 ($p < 0.0001$, Table 3-8, Figure 3-7B).

3.4.3 Number of polyps in the mouse small bowel

The mean number of polyps in the small bowel of $APC^{Min/+}$ mice was 17.80 +/- 1.23 and 19.65 +/- 1.72 in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice. There was no significant difference in number of polyps between the two genotypes (Table 3-8, Figure 3-8C).

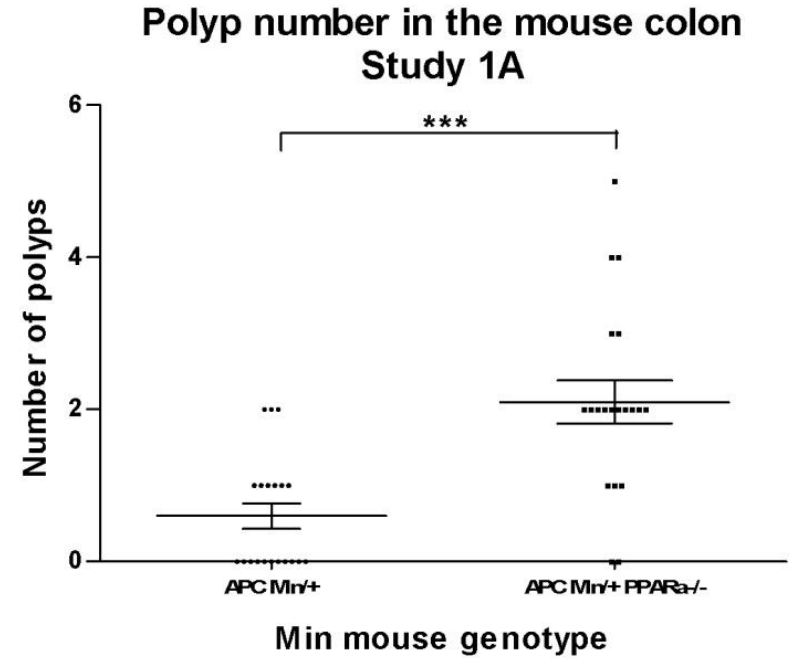
Similarly when age of mice at death was considered, there was no significant difference in mean number of polyps per week in the small bowel, (Figure 3-8D). Number of polyps per week in $APC^{Min/+}$ mice was 0.61 +/- 0.04 (Table 3-7), and in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice was 0.69 +/- 0.06 (Table 3-8).

Table 3-7 Number of polyps in the colon & small bowel of APC$^{Min/+}$ mice on study 1A

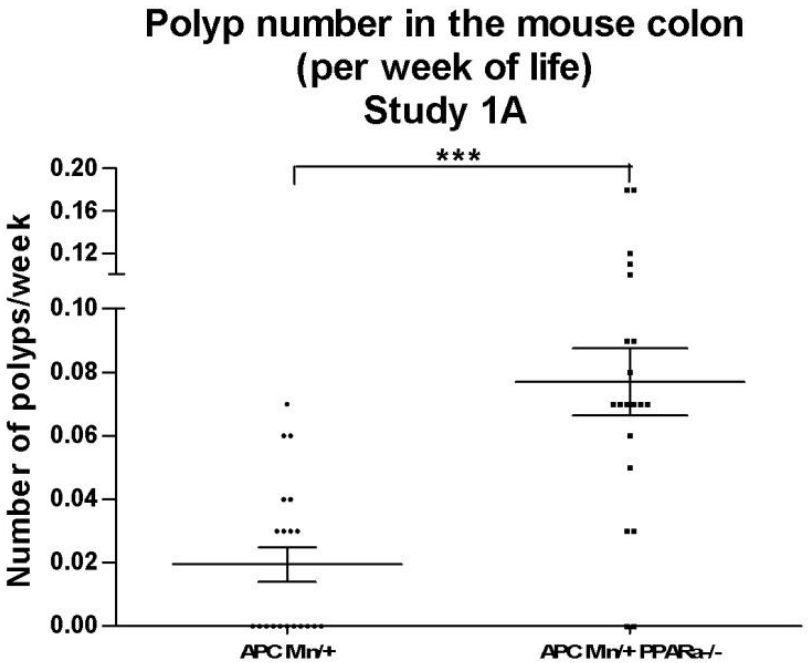
Mouse ID	Genotype	Colon polyp number	Colon polyp number/ week	Small bowel polyp number	Small bowel polyp number/ week
35 1/1 M	APC $^{Min/+}$	2	0.07	21	0.74
36 1/4 F	APC $^{Min/+}$	2	0.06	22	0.70
31 1/3 M	APC $^{Min/+}$	1	0.03	14	0.45
29b 3/3 F	APC $^{Min/+}$	0	0.00	14	0.50
31 1/2 F	APC $^{Min/+}$	1	0.03	19	0.63
35 1/4 M	APC $^{Min/+}$	0	0.00	28	0.93
27b 4/2 M	APC $^{Min/+}$	0	0.00	19	0.83
24a 5/3 M	APC $^{Min/+}$	0	0.00	11	0.43
30 1/2 F	APC $^{Min/+}$	0	0.00	13	0.43
31 6/1 M	APC $^{Min/+}$	0	0.00	6	0.18
31 2/1 m	APC $^{Min/+}$	2	0.06	18	0.56
37 1/2 M	APC $^{Min/+}$	1	0.04	15	0.64
37 1/3 M	APC $^{Min/+}$	0	0.00	14	0.60
35 1/5M	APC $^{Min/+}$	0	0.00	20	0.74
28c. 4/1F	APC $^{Min/+}$	0	0.00	24	0.86
34 2/1 F	APC $^{Min/+}$	0	0.00	19	0.61
27b 4/1 F	APC $^{Min/+}$	0	0.00	12	0.40
24a 5/2 M	APC $^{Min/+}$	1	0.03	18	0.50
31 1/2 M	APC $^{Min/+}$	1	0.04	22	0.79
21 2/3 F	APC $^{Min/+}$	1	0.03	27	0.68
	MEAN	**0.60**	**0.02**	**17.80**	**0.61**

Table 3-8 Number of polyps in the colon & small bowel of APC$^{Min/+}$ PPARα$^{-/-}$ mice on study 1A

Mouse ID	Genotype	Colon polyp number	Colon polyp number/ week	Small bowel polyp number	Small bowel polyp number/ week
40 2/3 M	APC $^{Min/+}$ PPARα$^{-/-}$	1	0.07	14	0.92
37 3/2 M	APC $^{Min/+}$ PPARα$^{-/-}$	5	0.18	20	0.71
47b 1/3 M	APC $^{Min/+}$ PPARα$^{-/-}$	2	0.07	6	0.20
47a 1/1 M	APC $^{Min/+}$ PPARα$^{-/-}$	2	0.07	22	0.77
47b1/1 F	APC $^{Min/+}$ PPARα$^{-/-}$	4	0.12	33	1.02
40 1/4 F	APC $^{Min/+}$ PPARα$^{-/-}$	2	0.07	24	0.79
41 1/3 M	APC $^{Min/+}$ PPARα$^{-/-}$	2	0.07	34	1.16
44 1/2 F	APC $^{Min/+}$ PPARα$^{-/-}$	2	0.08	20	0.84
42 2/1 F	APC $^{Min/+}$ PPARα$^{-/-}$	4	0.18	16	0.74
40 2/2 M	APC $^{Min/+}$ PPARα$^{-/-}$	2	0.05	8	0.22
40 2/1 M	APC $^{Min/+}$ PPARα$^{-/-}$	1	0.03	12	0.39
52b 1/5 M	APC $^{Min/+}$ PPARα$^{-/-}$	2	0.09	20	0.93
47b 1/1M	APC $^{Min/+}$ PPARα$^{-/-}$	3	0.10	10	0.32
31 2/3 F	APC $^{Min/+}$ PPARα$^{-/-}$	2	0.09	13	0.58
41.1/2 M	APC $^{Min/+}$ PPARα$^{-/-}$	2	0.07	19	0.68
42 2/3 M	APC $^{Min/+}$ PPARα$^{-/-}$	3	0.11	25	0.89
37 2/2 M	APC $^{Min/+}$ PPARα$^{-/-}$	0	0.00	24	0.68
37 2/2F	APC $^{Min/+}$ PPARα$^{-/-}$	1	0.03	20	0.58
35 2/3F	APC $^{Min/+}$ PPARα$^{-/-}$	2	0.06	24	0.68
31 2/4F	APC $^{Min/+}$ PPARα$^{-/-}$	0	0.00	29	0.86
	MEAN	**2.10**	**0.08**	**19.65**	**0.70**



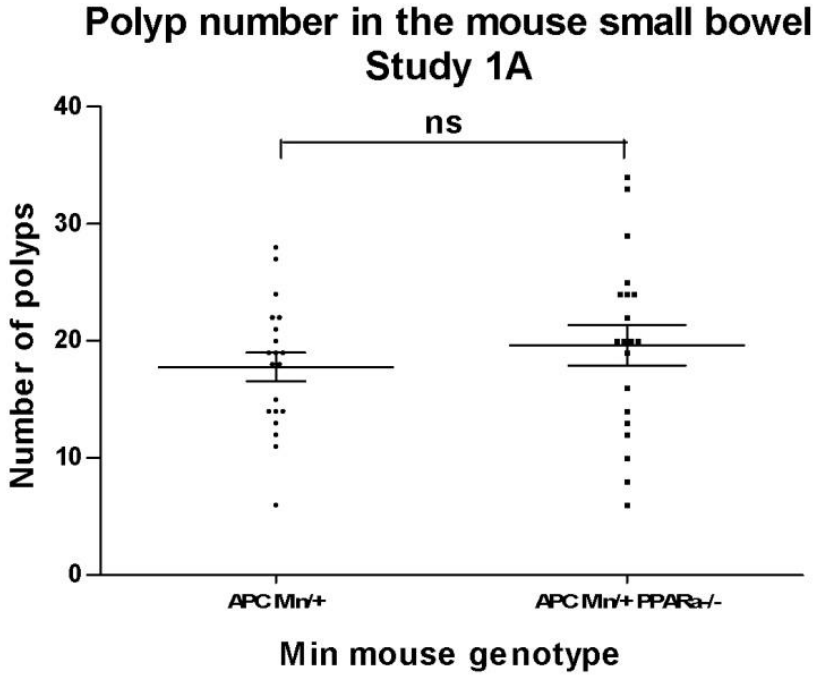
A. Polyp number in the mouse colon



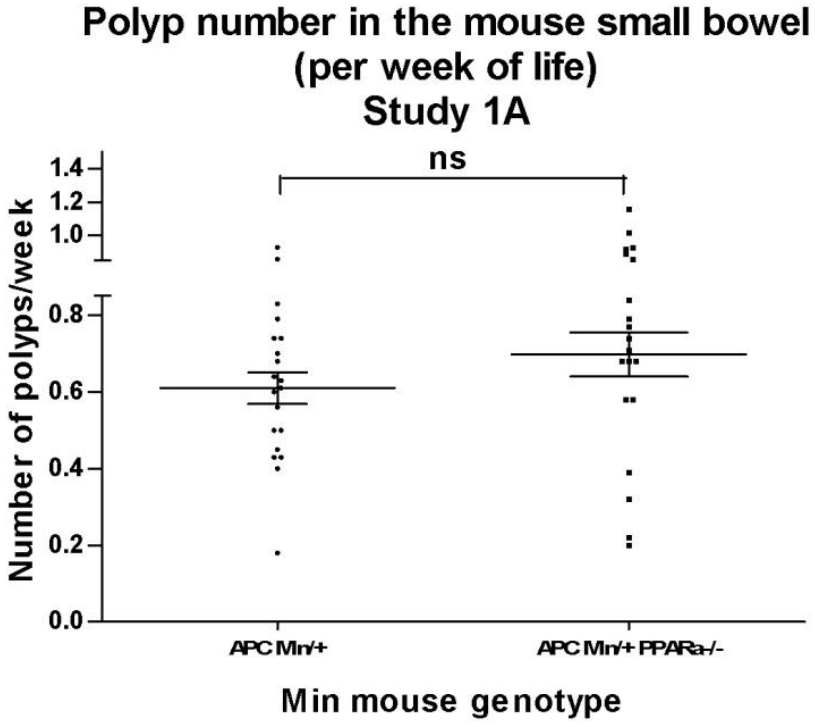
B. Polyp number per week of life in the mouse colon

Figure 3-7 Polyp number in the colon of APC$^{Min/+}$ mice and APC$^{Min/+}$ PPARα$^{-/-}$ mice

Unpaired t test, *** p < 0.0001



C. Polyp number in the mouse small bowel



D. Polyp number per week of life in the mouse small bowel

Figure 3-8 Polyp number in the small bowel of $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice

Unpaired t test, ns $p > 0.05$

3.4.4 Haematoxylin & Eosin staining of mouse colonic tissue

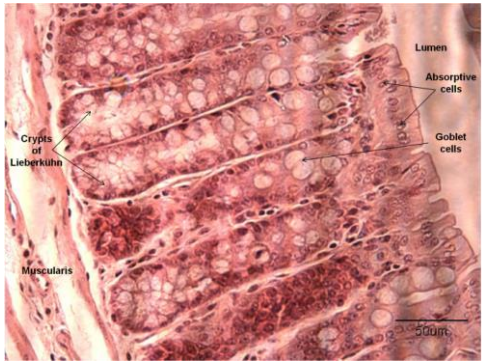
Figure 3-9 (**A – E**) shows representative examples of Methylene Blue and Haematoxylin and Eosin (H & E) stained normal, polyp and adenocarcinoma tissue from the colons of $APC^{Min/+}$ or $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice.

A is an H & E stain of normal colon tissue showing simple columnar epithelium (Wheater et al., 1990).

B shows a colonic polyp with methylene blue stain. **C** is an H & E stain of **B**. The tissue shows dysplasia indicated by cellular pleomorphism[11], an increase in nuclear/cytoplasmic ratio[12], hyperchromatism of nuclei, reduction of goblet cells and infiltration of lamina propria by lymphocytes and plasma cells (Wheater et al., 1990).

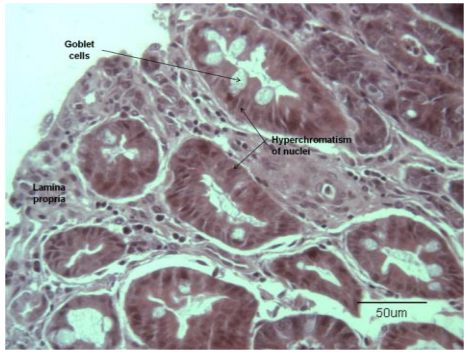
D shows an image of a colonic tumour with methylene blue stain from an $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mouse. The tumour had locally invaded the colonic wall to the serosal surface. **E** is an H & E stain of **D** showing an adenocarcinoma. Tissue is well differentiated as glandular pattern of original normal tissue is still apparent, but with hyperchromatism of nuclei and a high nuclear/cytoplasmic ratio (Wheater et al., 1990).

[11] Pleomorphism: variable size & shape of cells
[12] Increase in nuclear/cytoplasmic ratio: indicates immature cells

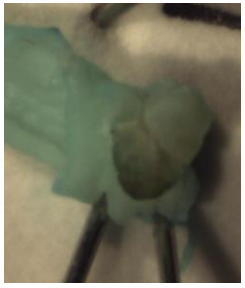


A. Haematoxylin & Eosin stain of normal colon tissue from an APC$^{Min/+}$ PPARα$^{-/-}$ mouse

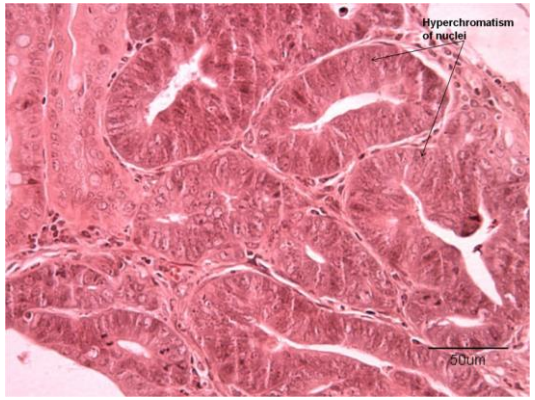
B. Methylene Blue stained colon tissue from an APC$^{Min/+}$ mouse showing a polyp (x4.2 magnification)



C. Haematoxylin & Eosin stain of colonic polyp tissue from an APC$^{Min/+}$ mouse



D. Methylene blue stained colon tissue from an APC$^{Min/+}$ PPARα$^{-/-}$ mouse showing a tumour (x4.2 magnification)



E. Haematoxylin & Eosin stain of adenocarcinoma tissue from the colon of an APC$^{Min/+}$ PPARα$^{-/-}$ mouse

Figure 3-9 Methylene blue and Haematoxylin & Eosin stains of mouse colonic tissue

3.4.5 Ingenuity® Pathway Analysis (IPA®) of Affymetrix® microarray data (Study 1B)

IPA networks of the edited DEG lists from Comparison 1 ($APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal), Comparison 2 ($APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour), Comparison 3 ($APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour) and Comparison 4 ($APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}PPAR\alpha^{-/-}$ tumour), (Appendices pages 311-377) were generated (networks not shown).

The two top scoring biological processes after IPA of both Comparison 2 and Comparison 3 were growth & proliferation and colorectal cancer. Networks of these data only were created and annotated with the Wnt/beta catenin, ERK/MAPK (ras) and p53 pathways. Additionally, canonical pathways overlaid with Comparison 2 and Comparison 3 data were produced. All of these networks are shown and discussed.

Also, IPA of Comparison 1 and Comparison 4 are reviewed.

All IPA networks (of Comparisons 1 – 4, and colorectal cancer, growth & proliferation networks of Comparison 2 and Comparison 3) were analysed for genes of interest that may be involved in colonic cancer, growth & proliferation and PPARα pathways.

Genes from networks and other novel genes were selected for further analysis on Taqman® low density arrays based on fold change, p value and function, as described previously.

3.4.5.1 Colonic tumorigenesis & the $APC^{Min/+}$ mouse model

Affymetrix® microarray analysis of colon tissue from Comparison 3 mice ($APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour) showed there were 1141 differentially regulated genes between normal and tumour tissue (Figure 3-3 and Appendices page 378).

IPA of these data showed the top biological processes affected by the differential expression of genes between normal and tumour tissue in $APC^{Min/+}$ mice, were cellular growth & proliferation (p = 1.03E-10) and colorectal cancer (p = 1.66E-07). IPA networks showing gene expression data from these two processes were produced (Figure 3-10, Figure 3-14).

Each network was also annotated with the Wnt/beta catenin, ERK/MAPK (ras) and p53 canonical pathways. Additionally, gene expression data from Comparison 3 was overlaid onto IPA generated canonical pathways to produce corresponding networks.

Table 3-9 and Table 3-10 detail expression data for genes involved in cell growth & proliferation. These data were extracted from IPA of Comparison 3 Affymetrix® microarray data.

Table 3-9 Growth & Proliferation network data for up-regulated genes(Comparison 3 $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour)

Gene Symbol	Entrez Gene Name	Fold Change	p-value
	UP-REGULATED IN TUMOURS (RED)		
Abcc4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	4.78	2.07E-04
Abcg1	ATP-binding cassette, sub-family G (WHITE), member 1	2.31	3.54E-03
Adam12	ADAM metallopeptidase domain 12	2.89	2.06E-04
Apex1	APEX nuclease (multifunctional DNA repair enzyme) 1	2.16	2.03E-03
Arntl2	aryl hydrocarbon receptor nuclear translocator-like 2	6.70	1.29E-04
Axin2	axin 2	9.42	3.94E-05
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	4.11	1.01E-03
Bmf	Bcl2 modifying factor	3.59	3.89E-03
Casp6	caspase 6, apoptosis-related cysteine peptidase	3.16	1.26E-03
Cd47	CD47 molecule	2.07	1.61E-03
Cxcl1	chemokine (C-X-C motif) ligand 2	24.79	2.42E-04
Cxcl2	chemokine (C-X-C motif) ligand 3	20.48	3.26E-03
Dbn1	drebrin 1	2.93	4.02E-04
Dusp4	dual specificity phosphatase 4	7.86	3.70E-04
Ets2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	2.48	2.05E-04
Gpc1	glypican 1	5.55	1.52E-04
Gpld1	glycosylphosphatidylinositol specific phospholipase D1	3.04	5.53E-04
Gpx1	glutathione peroxidase 1	2.02	1.45E-04
Hoxa9	homeobox A9	2.46	1.92E-03
Htra1	HtrA serine peptidase 1	4.95	4.25E-04
Hunk	hormonally up-regulated Neu-associated kinase	3.31	1.62E-04
Il11	interleukin 11	3.96	3.17E-03
Il1rl1	interleukin 1 receptor-like 1	11.33	3.54E-03
Itga6	integrin, alpha 6	2.22	1.31E-03
Jub	ajuba LIM protein	7.08	6.56E-06
Lama5	laminin, alpha 5	2.85	3.86E-04
Lcn2	lipocalin 2	22.67	3.14E-03
Mafg	v-maf musculoaponeurotic fibrosarcoma oncogene homolog G (avian)	2.97	4.97E-04
Metap2	methionyl aminopeptidase 2	2.05	4.10E-04
Mllt3	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3	3.01	5.30E-03
Mmp12	matrix metallopeptidase 12 (macrophage elastase)	18.20	5.40E-06
Mmp13	matrix metallopeptidase 13 (collagenase 3)	49.90	1.13E-05
Mmp3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	8.52	5.45E-03
Myc	v-myc myelocytomatosis viral oncogene homolog (avian)	2.40	4.30E-03
Nap1l1	nucleosome assembly protein 1-like 1	2.19	2.75E-03
Pdgfb	platelet-derived growth factor beta polypeptide	2.09	1.84E-03
Pea15a	phosphoprotein enriched in astrocytes 15	2.92	1.38E-03
Plat	plasminogen activator, tissue	14.32	2.71E-04
Prmt1	protein arginine methyltransferase 1	4.75	1.06E-03
Prox1	prospero homeobox 1	34.48	3.11E-06
Ptpre	protein tyrosine phosphatase, receptor type, E	3.44	1.23E-03
Ptprz1	protein tyrosine phosphatase, receptor-type, Z polypeptide 1	9.44	4.03E-03
Reg3b	regenerating islet-derived 3 alpha	132.99	2.29E-03
Sdc1	syndecan 1	2.04	3.02E-03
Serpine2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	3.33	4.41E-03
Sox17	SRY (sex determining region Y)-box 17	14.56	4.55E-03
Sox4	SRY (sex determining region Y)-box 4	10.95	4.19E-04
Spp1	secreted phosphoprotein 1	35.21	1.60E-04
T	T, brachyury homolog (mouse)	15.53	7.99E-04
Tcf7	transcription factor 7 (T-cell specific, HMG-box)	4.54	5.23E-03
Timp1	TIMP metallopeptidase inhibitor 1	3.82	5.29E-04
Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b	25.72	2.21E-04
Tnfrsf12a	tumor necrosis factor receptor superfamily, member 12A	4.03	5.91E-04
Tnfsf9	tumor necrosis factor (ligand) superfamily, member 9	5.42	2.58E-04
Wdr6	WD repeat domain 6	2.51	4.76E-03
Wisp1	WNT1 inducible signaling pathway protein 1	2.29	4.76E-04

Table 3-10 Growth & Proliferation network data for down-regulated genes(Comparison 3 $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour)

Gene Symbol	Entrez Gene Name	Fold Change	p-value
DOWN-REGULATED IN TUMOURS (GREEN)			
Abcb1a	ATP-binding cassette, sub-family B (MDR/TAP), member 1	-10.24	2.29E-03
Abcc3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	-4.72	1.43E-03
Abcc9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	-4.30	2.46E-03
Abcg2	ATP-binding cassette, sub-family G (WHITE), member 2	-6.87	3.89E-03
Akap12	A kinase (PRKA) anchor protein 12	-3.43	1.51E-03
Ank2	ankyrin 2, neuronal	-2.53	3.87E-03
Bin1	bridging integrator 1	-2.15	1.55E-03
Cacna2d2	calcium channel, voltage-dependent, alpha 2/delta subunit 2	-2.49	1.55E-03
Cbs	cystathionine-beta-synthase	-3.23	1.78E-03
Ccng2	cyclin G2	-2.13	2.88E-03
Cd36	CD36 molecule (thrombospondin receptor)	-5.35	5.60E-04
Cspg4	chondroitin sulfate proteoglycan 4	-2.76	6.30E-04
Cth	cystathionase (cystathionine gamma-lyase)	-5.55	4.50E-04
Ddr2	discoidin domain receptor tyrosine kinase 2	-3.04	7.76E-04
Des	desmin	-10.37	2.78E-03
Entpd5	ectonucleoside triphosphate diphosphohydrolase 5	-7.05	1.57E-03
Epas1	endothelial PAS domain protein 1	-2.29	3.65E-04
Epb4.1l3	erythrocyte membrane protein band 4.1-like 3	-2.49	1.96E-03
Esrrg	estrogen-related receptor gamma	-5.04	2.78E-04
Fbln2	fibulin 2	-2.06	5.78E-03
Fgf13	fibroblast growth factor 13	-2.82	2.97E-03
Foxo3	forkhead box O3	-2.07	5.72E-04
Fxyd1	FXYD domain containing ion transport regulator 1	-4.55	1.53E-04
Fzd5	frizzled family receptor 5	-3.10	8.61E-04
Gli2	GLI family zinc finger 2	-2.28	1.71E-03
Gstm2	glutathione S-transferase mu 1	-4.81	8.06E-04
Hhip	hedgehog interacting protein	-3.91	4.26E-04
Hlx	H2.0-like homeobox	-2.06	1.96E-03
Hnf4a	hepatocyte nuclear factor 4, alpha	-2.35	1.50E-03
Ifi30	interferon, gamma-inducible protein 30	-2.02	5.79E-04
L1cam	L1 cell adhesion molecule	-3.42	5.66E-03
Mxd1	MAX dimerization protein 1	-3.37	1.62E-03
Myh14	myosin, heavy chain 14, non-muscle	-2.17	1.50E-03
Myl9	myosin, light chain 9, regulatory	-6.94	4.31E-04
Nr1i2	nuclear receptor subfamily 1, group I, member 2	-2.03	2.02E-03
Nr3c1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	-2.24	5.22E-03
Nr3c2	nuclear receptor subfamily 3, group C, member 2	-4.48	1.97E-03
Ntn1	netrin 1	-5.71	4.21E-04
Pde3a	phosphodiesterase 3A, cGMP-inhibited	-2.65	3.97E-03
Postn	periostin, osteoblast specific factor	-2.58	3.09E-03
Sdc2	syndecan 2	-3.10	4.34E-03
Slc22a18	solute carrier family 22, member 18	-2.28	9.25E-04
Synm	synemin, intermediate filament protein	-13.41	1.28E-03
Tgfb1i1	transforming growth factor beta 1 induced transcript 1	-2.51	1.19E-04
Tgfb3	transforming growth factor, beta 3	-2.14	4.85E-03
Tgfbr3	transforming growth factor, beta receptor III	-5.61	1.94E-03
Trpm6	transient receptor potential cation channel, subfamily M, member 6	-7.92	4.12E-03
Txnip	thioredoxin interacting protein	-2.31	3.67E-03
Vip	vasoactive intestinal peptide	-20.36	5.45E-03

Figure 3-10 shows a network of differentially expressed genes associated with growth & proliferation between tumour and normal colonic tissue in the $APC^{Min/+}$ mouse. The network was annotated with the Wnt/β catenin, ERK/MAPK and p53 pathways.

Figure 3-11, Figure 3-12 and Figure 3-13 show Comparison 3 gene expression data overlaid onto IPA canonical Wnt/β catenin, ERK/MAPK and p53 pathways respectively.

Figure 3-10 and Figure 3-11 show the Wnt/β catenin pathway affected the differential regulation of several genes in tumour tissue that have previously been shown to be involved in development of cancer (Doucas et al., 2005, Zhai et al., 2002).

These genes are listed below. The figures in brackets are fold change and p value of expression of each gene in tumour tissue compared to normal tissue (Table 3-9 and Table 3-10).

- Myelocytomatosis oncogene (Myc, fold change (FC) 2.40↑, p = 4.30E-03)
- CyclinD1 (Ccnd1, FC 2.85↑, p = 2.60E-03)
- Transcription factor 4 (Tcf4, FC 4.11↑, p = 2.18E-04)
- Axin2 (FC 9.42↑, p = 3.94E-05)
- Sox4 (SRY-sex determining region Y – box 4, FC 10.95↑, p = 4.19E-04)
- Sox17 (SRY-sex determining region Y) – box 17, FC 14.56↑, p = 4.55E-03)
- Matrix metallopeptidase 7 (Mmp7, FC 78.28↑, p = 1.86E-04)
- Frizzled family receptor 5 (Fzd5, FC 3.10↓, p = 8.61E-04)

Myc is well known to be a critical mediator of neoplasia in the gastrointestinal tract when function of the APC gene is lost (Sansom et al., 2007).

Another study by (Sansom et al., 2005) demonstrated that up-regulation of Cyclin D1 was not an immediate effect of APC loss of function,

suggesting the gene may be involved in tumour progression but not initiation.

Also, an earlier study by (Roose et al., 1999) showed that mutations in the APC or β catenin genes and aberrant Wnt/β catenin signalling caused inappropriate activation of Tcf4. Thus, deregulated Tcf4 expression was shown to affect transformation of intestinal epithelial cells in mice *via* up-regulation of target genes, for example Myc.

Axin2 is known to act as a negative regulator of canonical Wnt/β catenin signalling in normal cells (Leung et al., 2002). However, a recent study has demonstrated that in tumour cells Axin2 acts as a potent tumour promoter *via* up-regulation of transcriptional repressor Snai1 which drives metastatic activity (Wu et al., 2012).

The SOX family of transcription factors has emerged as modulators of β-catenin/TCF activity. SOX proteins are able to repress or enhance Wnt transcriptional responses, and expression of several Sox genes are regulated by Wnts (Kormish et al., 2010)**.**

Several studies have shown that Sox gene expression is deregulated in human cancers. SOX proteins can be either transcriptional activators or repressors depending on cell type. Sox17 has been shown to be epigenetically silenced[13] in human colorectal cancer and appears to act as a tumour suppressor (Zhang et al., 2008)**.** However, Sox4 was shown to be over expressed in tumours and appears to act as an oncogene (Reichling et al., 2005, Andersen et al., 2009)**.**

Mmp7 is known to be up-regulated in colon cancer (Kioi et al., 2003). Elevated expression of Mmp7 has been shown to be a poor prognostic indicator in colon cancer (Koskensalo et al., 2011).

Lastly, Fzd5 is a 7-transmembrane domain protein which functions as the receptor for the Wnt5A ligand as part of the Wnt/β catenin signalling pathway (Saitoh et al., 2001)**.**

[13] Epigenetic silencing: change in gene expression caused by mechanisms other than alteration of the DNA sequence

Figure 3-10 and Figure 3-12 show Myc may also be up-regulated by ERK/MAPK signalling (Seger and Krebs, 1995, Cheng et al., 1999). As discussed earlier, mutations of components of the ERK/MAPK pathway, such as ras or b-raf can lead to deregulated activation of target genes such as Myc, and development of cancer (McCubrey et al., 2007).

v-ets erythroblastosis virus E26 oncogene homolog 2 (Ets2, FC 2.48↑, p = 2.05E-04) has also been shown to have a role in tumorigenesis in the colon. A study by (Munera et al., 2011) showed that loss of Ets2 in colonic stem cells may increase the number or sensitivity of these cells for tumour initiation. Indeed, their study demonstrated that mice with Ets2 deficient intestinal cells developed more colonic tumours in response to treatment with azoxymethane and dextran sulfate sodium (DSS). An earlier study identified intestinal stem cells as the cell of origin of intestinal tumorigenesis in mice (Barker et al., 2009).

Figure 3-10 and Figure 3-13 show Caspase 6, apoptosis-related cysteine peptidase (Casp6, FC 3.16↑, p = 1.26E-03) and Apoptotic peptidase activating factor 1 (Apaf1, FC 2.44↑, p = 3.00E-03) were transcriptional targets of the p53 pathway (Hammond et al., 2006).

Both genes have a role in apoptosis within the cell. Induction of Casp6 expression which has an important role in the execution phase of apoptosis, has been shown to lower the cell death threshold in response to activation by apoptotic signals (MacLachlan and El-Deiry, 2002).

However, data from the present study showed up-regulation of Casp6 in tumour tissue that may be explained by the findings of a study by (Lee et al., 2006) which demonstrated Casp6 was up-regulated in human colorectal and gastric carcinomas. Their study identified mutations in the Casp6 gene that could be implicated in the development of these cancers.

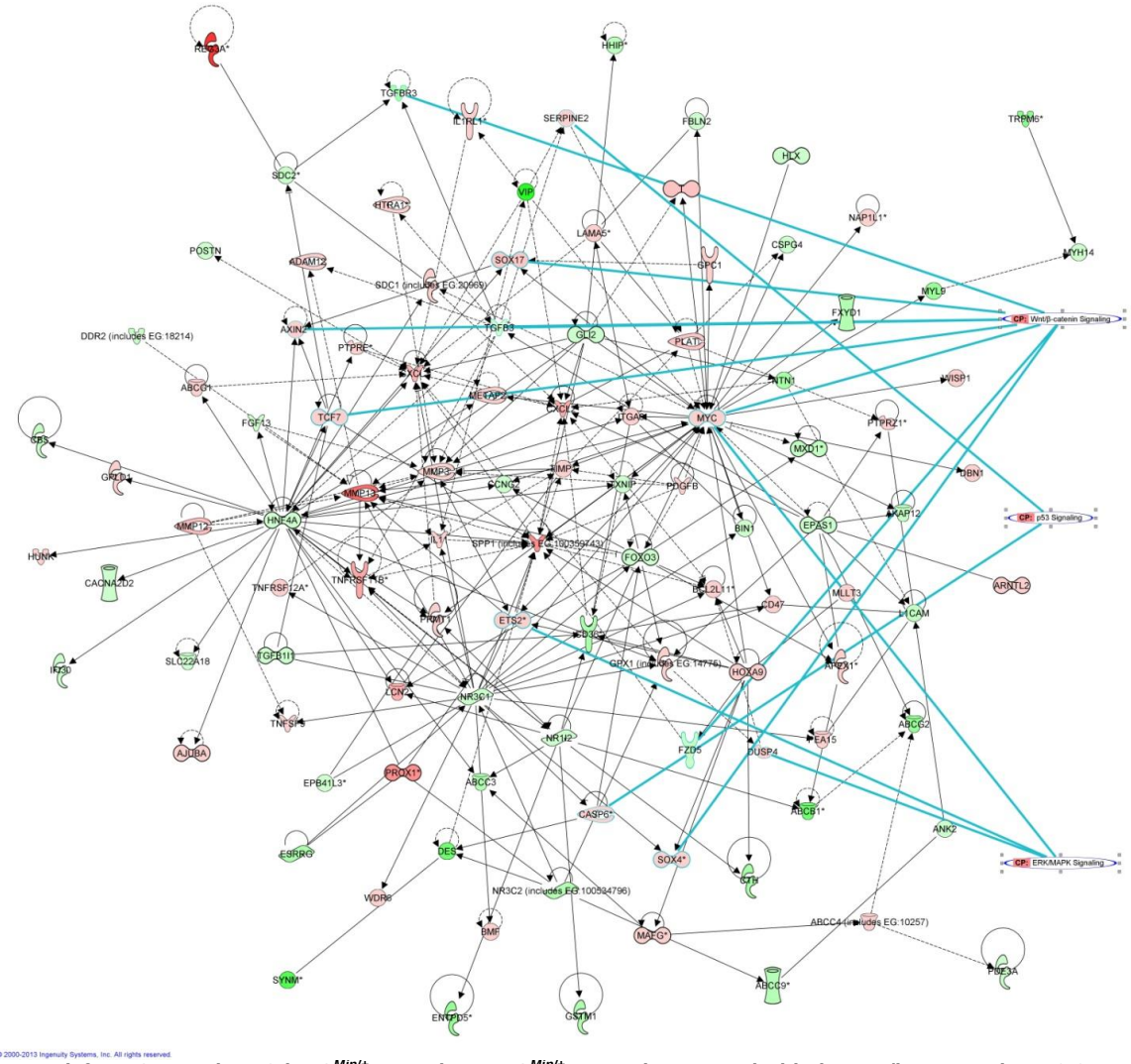


Figure 3-10 Growth & Proliferation network from Comparison 3 (APC$^{Min/+}$ normal *vs.* APC$^{Min/+}$ tumour) annotated with the Wnt/beta catenin, p53 & ERK/MAPK pathways Genes in red are up-regulated in tumours, genes in green are down-regulated in tumours. Network p value, p = 1.03E-10

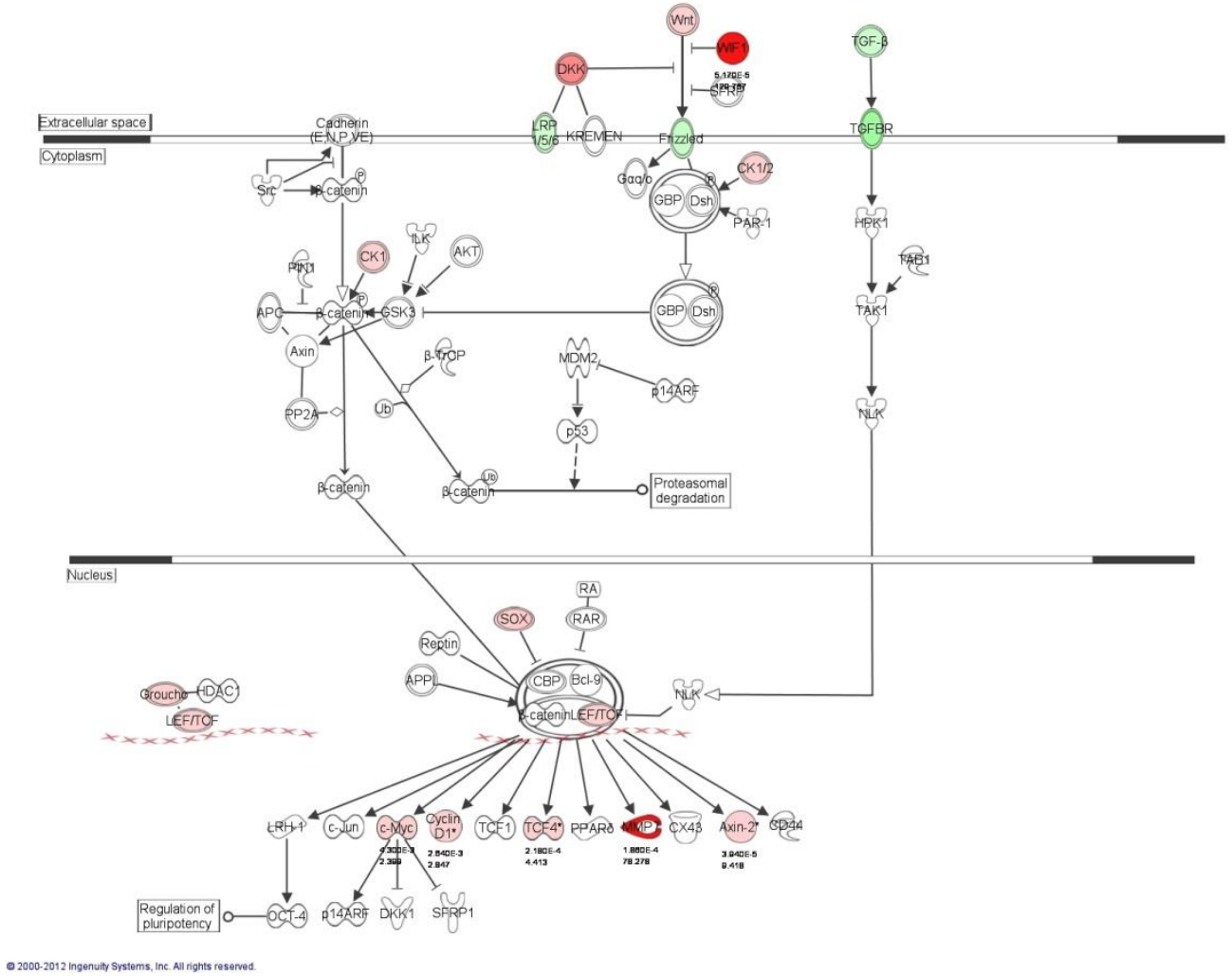


Figure 3-11 Canonical Wnt/beta catenin pathway & gene expression data from Comparison 3 ($APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour) Genes in red are up-regulated in tumours, genes in green are down-regulated in tumours.

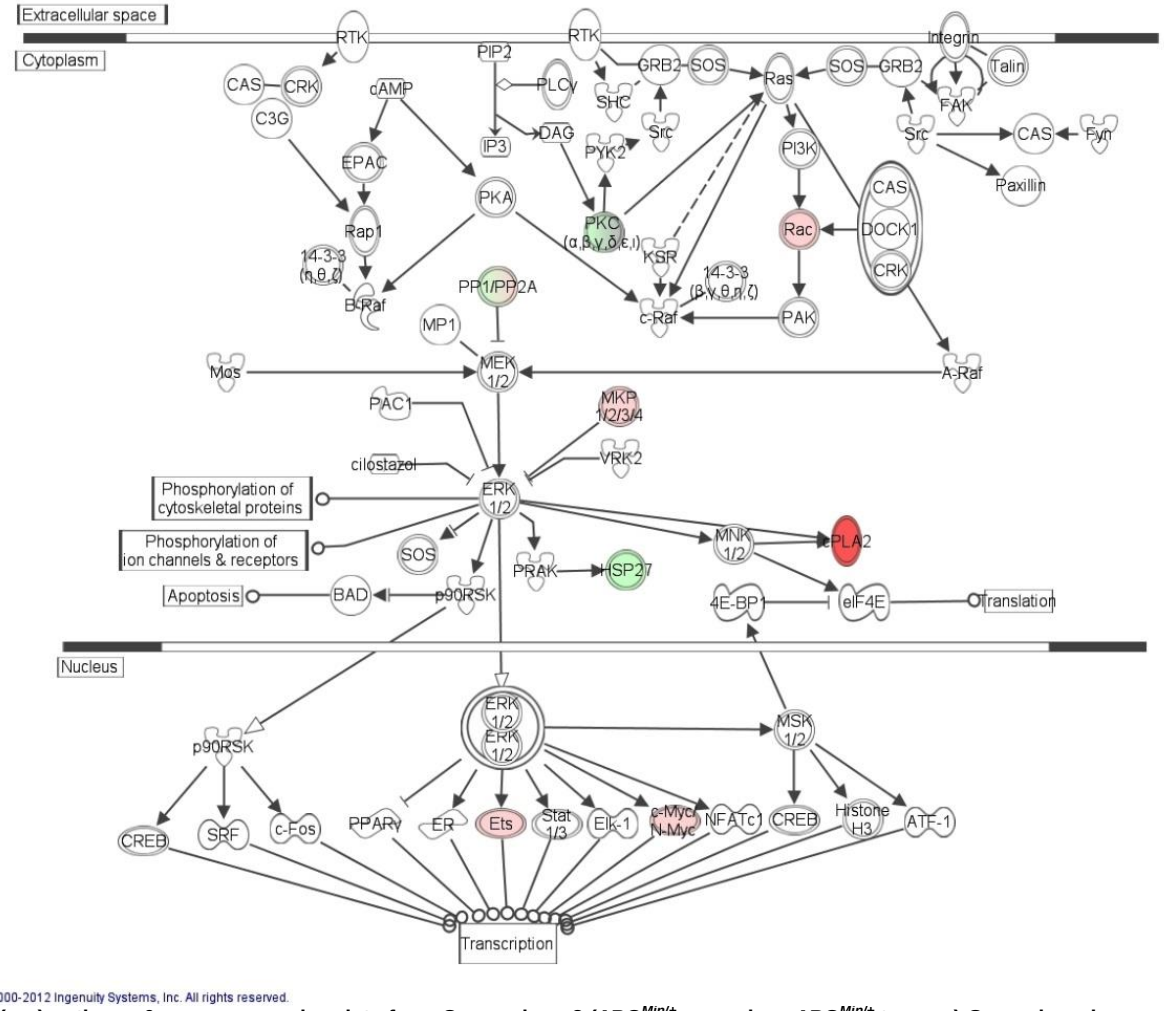


Figure 3-12 Canonical ERK/MAPK (ras) pathway & gene expression data from Comparison 3 ($APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour) Genes in red are up-regulated in tumours, genes in green are down-regulated in tumours.

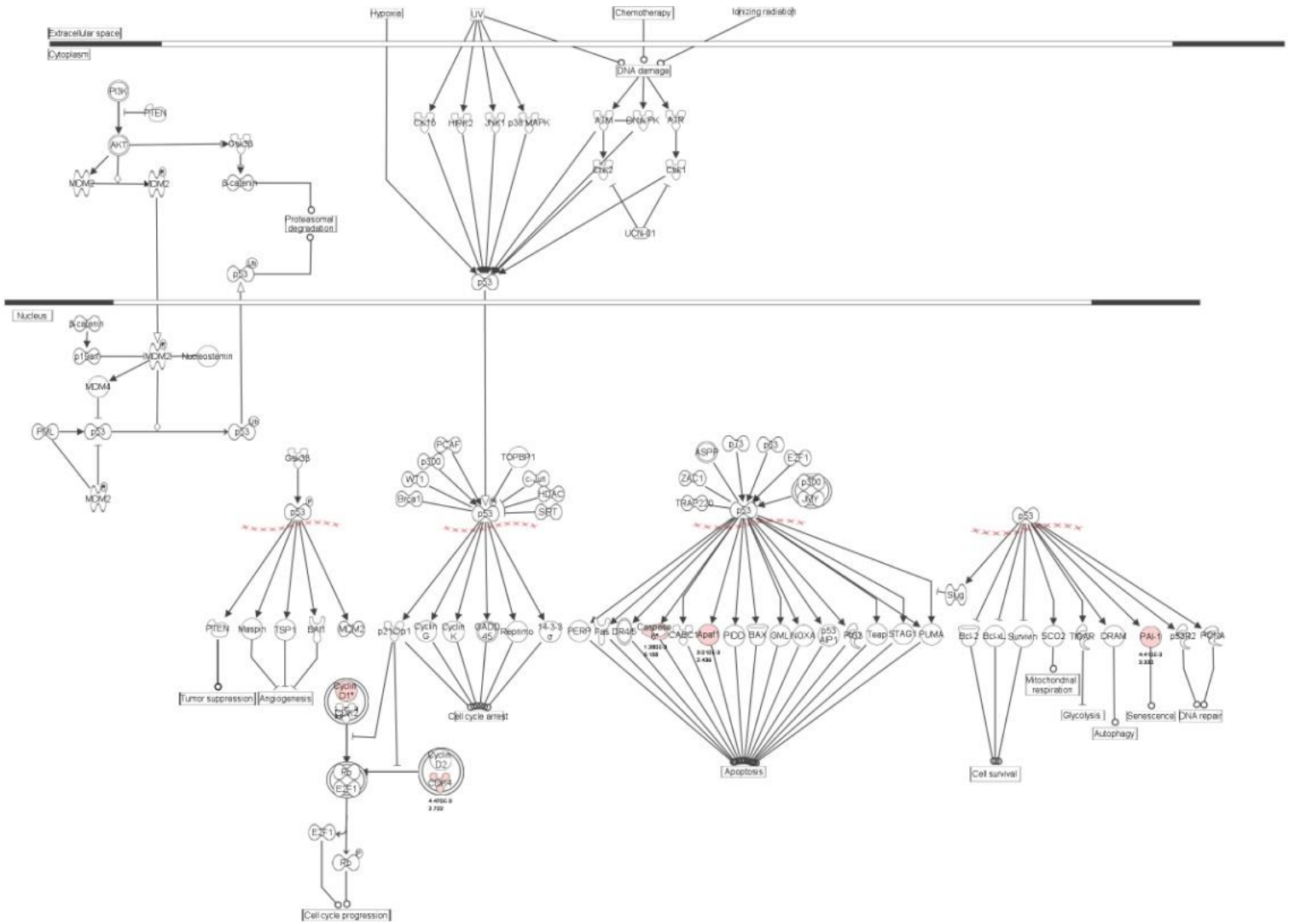


Figure 3-13 Canonical p53 pathway & gene expression data from Comparison 3 (APC$^{Min/+}$ normal *vs.* APC$^{Min/+}$ tumour) Genes in red are up-regulated in tumours, genes in green are down-regulated in tumours.

Table 3-11 details gene expression data from Comparison 3 for genes involved in colorectal cancer. These data were extracted from IPA of Comparison 3 Affymetrix® microarray data.

Figure 3-14 shows a network of differentially expressed genes associated with colorectal cancer between tumour and normal colonic tissue in the $APC^{Min/+}$ mouse. The network was annotated with the Wnt/beta catenin, ERK/MAPK and p53 pathways as before.

Figure 3-14 (see also Figure 3-11 and Figure 3-12) shows Myc (FC 2.40↑, p = 4.30E-03) was targeted by Wnt/β catenin and ERK/MAPK signalling as described previously.

Other targets of Wnt/β catenin signalling were Transforming growth factor beta 3 (Tgfβ3, FC 2.14↓, p = 4.85E-03) and Transforming growth factor receptor beta 3 (Tgfrβ3, FC 5.61↓, p = 1.94E-03), which are part of the Transforming growth factor beta (Tgfβ) signalling pathway.

The Tgfβ pathway is involved in many cellular processes including cell differentiation, cell growth, apoptosis, cellular homeostasis and other cellular functions.

A study by (Mishra et al., 2005) suggests there is an interaction between Tgfβ signalling and Wnt β catenin signalling in development of intestinal cancers. In normal intestinal epithelial cells growth inhibition is the predominant influence. Briefly, Tgfβ ligands bind to a type II serine/threonine kinase receptor, which then attach and phosphorylate a type I receptor. The type I receptor then phosphorylates a receptor-regulated SMAD gene, for example SMAD2 (JV18-1) which then bind SMAD4 (DPC4). These SMAD/SMAD4 complexes translocate to the nucleus and activate target genes including p53 and Tgfβ 11 receptor (Shi and Massague, 2003).

Mutations frequently occur in Tgfβ 11 receptors (Massague et al., 2000), SMAD2 and SMAD4 (Fearon and Vogelstein, 1990, Kinzler and Vogelstein, 1996). Therefore, this would make the Tgfβ signalling pathway vulnerable to disruption in colorectal cancer (CRC). Also,

constitutive activation of β catenin/Tcf4 complexes leading to unregulated transcription of Myc is characteristic of CRC (Doucas et al., 2005, Zhai et al., 2002).

Figure 3-14 shows that Wnt β catenin signalling affected down-regulation of components of the Tgfβ signalling pathway with concomitant up-regulation of Myc in tumour tissue.

Figure 3-14 (see also Figure 3-13) shows Cyclin-dependent kinase 4 (Cdk4, FC 2.72↑, p = 4.47E-03) was a target of p53 signalling. Cdk4 is a cell cycle regulator involved in early G1 cell cycle progression. A study by (Abedin et al., 2010) suggests deregulated Cdk4 expression plays an important role in angiogenesis during intestinal tumour formation *via* the up-regulation of E2F transcription factor 1 (E2F1[14]) target proteins Vascular endothelial growth factor b (Vegf-b) and Cyclin A (Ccna).

Figure 3-10 and Figure 3-11 show that several ATP-binding cassette (ABC) genes were differentially expressed in tumour tissue compared to normal tissue; Abcc4 (FC 4.78↑, p = 2.07E-04), Abcg1 (FC 2.31↑, p = 3.54E-03), Abcb1a (FC 10.24↓, p = 2.29E-03), Abcc3 (FC 4.72↓, p = 1.43E-03), Abcc9 (FC 4.30↓, p = 2.46E-03) and Abcg2 (FC 6.87↓, p = 3.89E-03).

The ABC superfamily of genes code for membrane proteins that play an important role in normal tissue throughout the body. They regulate the transport of substrates including fats, sugars, amino acids, phospholipids and drugs. They protect the blood-brain barrier (BBB), blood-cerobrospinal fluid (CSF) barrier and placenta from cytotoxins, and excrete toxins from the liver, gastrointestinal tract and kidneys.

The proteins all share a common domain that binds ATP; they are divided into subfamilies based on their structure and phylogenetic analysis (Dean, 2005).

[14] E2F1 transcription activator, major role in G1/S transition

Many of these proteins are important in the development of resistance to cancer therapies (multidrug resistance – MDR). Cancer cells can become resistant by increased activity of different ABC transporter proteins; for example, the protein product of Abcb1, a P-glycoprotein is a broad spectrum multidrug efflux pump. Also, the Abcc sub-group of transporters are known to be multidrug resistance proteins. Some anti-cancer therapies are poor substrates for certain transporters; multi-drug resistance is acquired by cancer cells by expression of other transporters such as Abcg2 (also known as mitoxanthrone-resistance gene) (Gottesman et al., 2002).

Detection and expression levels of ABC genes could be used as candidate markers for colorectal cancer and to inform the chemotherapy that would be most effective in treatment.

The differences in expression of the ABC genes seen in this study may imply that they have important pharmacological and physiological roles in colon cancer in the mouse.

Table 3-11 Colorectal Cancer network data (Comparison 3 $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour)

Gene Symbol	Entrez Gene Name	Fold Change	p-value
UP-REGULATED IN TUMOURS (RED)			
CDK4	cyclin-dependent kinase 4	2.72	4.47E-03
GPX1	glutathione peroxidase 1	2.02	1.45E-04
HDAC2	histone deacetylase 2	2.12	2.96E-03
HOXA9	homeobox A9	2.46	1.92E-03
ITGA6	integrin, alpha 6	2.22	1.31E-03
KRT18	keratin 18	2.25	8.48E-04
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	2.40	4.30E-03
PATZ1	POZ (BTB) and AT hook containing zinc finger 1	2.77	1.19E-03
SDC1	syndecan 1	2.04	3.02E-03
TUBB5	tubulin, beta class I	2.81	1.05E-03
WISP1	WNT1 inducible signaling pathway protein 1	2.29	4.76E-04
ZC3H12C	zinc finger CCCH-type containing 12C	2.22	5.08E-03
Gene Symbol	**Entrez Gene Name**	**Fold Change**	**p-value**
NO SIGNIFICANT CHANGE IN EXPRESSION (NO COLOUR)			
ARID5B	AT rich interactive domain 5B (MRF1-like)	2.70	0.11
EIF1AD	eukaryotic translation initiation factor 1A domain containing	1.18	0.24
HNRNPA2B1	heterogeneous nuclear ribonucleoprotein A2/B1	1.36	0.37
KIAA1704	RIKEN cDNA 1200011I18 gene	1.14	0.36
PAQR7	progestin and adipoQ receptor family member VII	1.00	0.99
PSME4	proteasome (prosome, macropain) activator subunit 4	1.19	0.43
RNF6	ring finger protein (C3H2C3 type) 6	1.11	0.68
SERINC2	serine incorporator 2	1.43	0.25
STAT1	signal transducer and activator of transcription 1	1.47	0.65
TGFB	transforming growth factor, beta	1.07	0.74
UBC	ubiquitin C	1.18	0.63
ZFP161	zinc finger protein 161	1.08	0.78
CROT	carnitine O-octanoyltransferase	-1.21	0.78
MLC1	myosin, light polypeptide 1	-1.22	0.28
progesterone	progesterone	-1.03	0.93
SLC26A1	solute carrier family 26 (sulfate transporter), member 1	-2.14	0.47
TLR4	toll-like receptor 4	-1.01	0.66
UST	uronyl-2-sulfotransferase	-1.24	0.31

Gene Symbol	Entrez Gene Name	Fold Change	p-value
DOWN-REGULATED IN TUMOURS (GREEN)			
ABCB1A	ATP-binding cassette, sub-family B (MDR/TAP), member 1	-10.24	2.29E-03
ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	-4.72	1.43E-03
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	-6.87	3.89E-03
ACTA2	actin, alpha 2, smooth muscle, aorta	-3.88	2.10E-04
CACNB2	calcium channel, voltage-dependent, beta 2 subunit	-2.26	3.54E-03
CAR2	carbonic anhydrase II	-2.81	2.24E-03
CD36	CD36 molecule (thrombospondin receptor)	-5.35	5.60E-04
CLCN2	chloride channel, voltage-sensitive 2	-2.85	6.10E-04
CLIC5	chloride intracellular channel 5	-6.87	5.21E-03
CRYL1	crystallin, lambda 1	-3.78	9.53E-04
DES	desmin	-10.37	2.78E-03
DPYD	dihydropyrimidine dehydrogenase	-11.78	1.33E-03
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	-4.50	7.11E-04
EPAS1	endothelial PAS domain protein 1	-2.29	3.65E-04
EPB4.113	erythrocyte membrane protein band 4.1-like 3	-2.49	1.96E-03
GLI2	GLI family zinc finger 2	-2.28	1.71E-03
GREM2	gremlin 2	-9.75	5.37E-04
GSTM2	glutathione S-transferase mu 1	-4.81	8.06E-04
GSTT1	glutathione S-transferase theta 1	-4.50	1.35E-05
GUCY2C	guanylate cyclase 2C (heat stable enterotoxin receptor)	-2.49	1.82E-03
HHIP	hedgehog interacting protein	-3.91	4.26E-04
HNF4A	hepatocyte nuclear factor 4, alpha	-2.35	1.50E-03
LPAR1	lysophosphatidic acid receptor 1	-2.35	5.05E-03
MEIS1	Meis homeobox 1	-3.72	2.46E-04
MGLL	monoglyceride lipase	-6.03	3.44E-03
MYLK	myosin light chain kinase	-6.59	3.44E-04
NDRG4	NDRG family member 4	-2.56	3.41E-03
PLD1	phospholipase D1, phosphatidylcholine-specific	-2.25	1.65E-03
PLSCR4	phospholipid scramblase 4	-2.04	1.43E-03
PPARa	peroxisome proliferator-activated receptor alpha	-3.94	5.57E-03
QPCT	glutaminyl-peptide cyclotransferase	-3.08	2.31E-03
SELENBP1	selenium binding protein 1	-16.93	2.47E-03
SGK2	serum/glucocorticoid regulated kinase 2	-8.09	5.39E-03
SLC26A2	solute carrier family 26 (sulfate transporter), member 2	-7.37	4.69E-03
STMN2	stathmin-like 2	-3.39	3.38E-03
SYNE1	spectrin repeat containing, nuclear envelope 1	-2.06	2.35E-03
SYNM	synemin, intermediate filament protein	-13.41	1.28E-03
TGFB3	transforming growth factor, beta 3	-2.14	4.85E-03
TGFBR3	transforming growth factor, beta receptor III	-5.61	1.94E-03
VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor	-7.48	2.50E-03

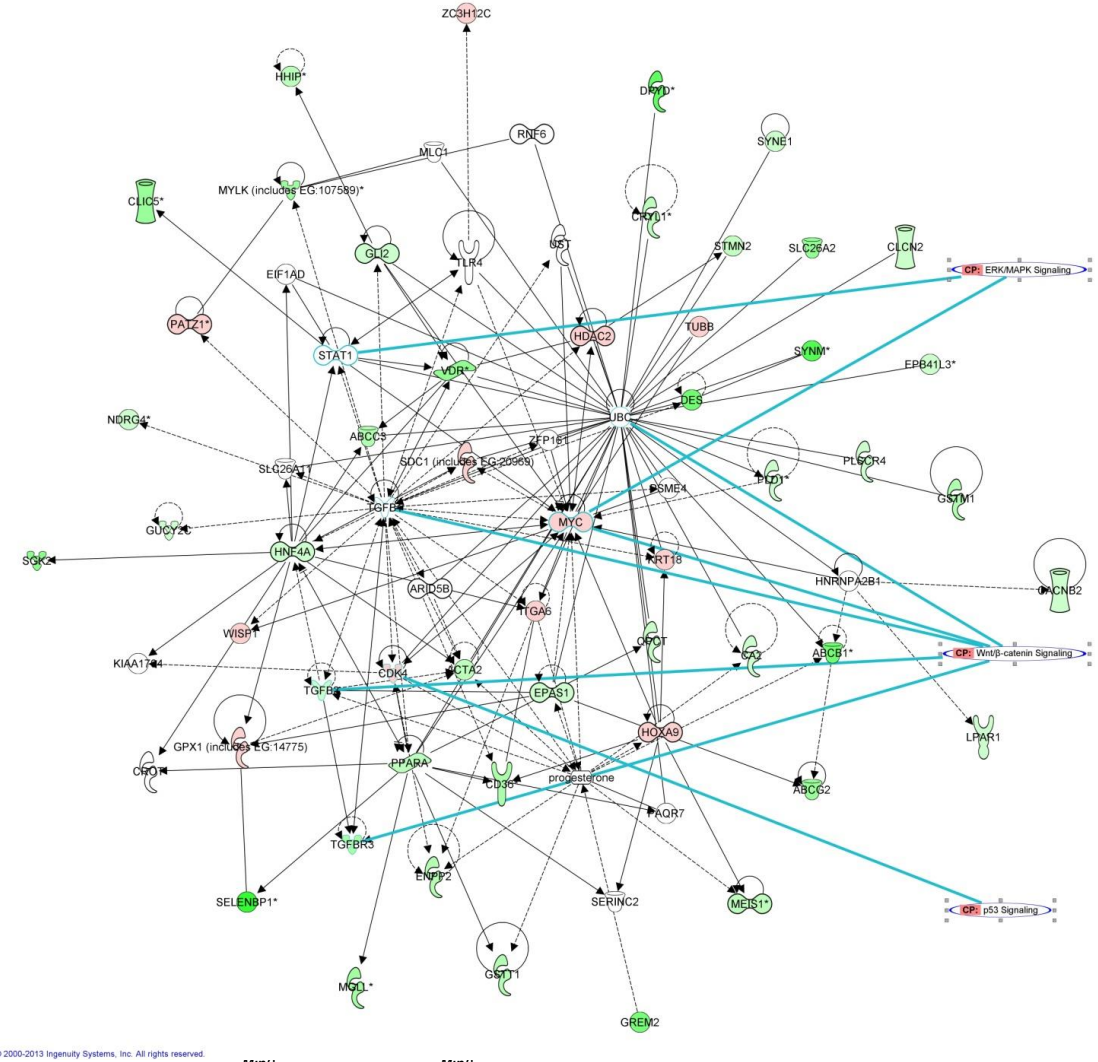


Figure 3-14 Colorectal Cancer network of Comparison 3 ($APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour) annotated with the ERK/MAPK, Wnt/beta catenin & p53 pathways Genes in red are up-regulated in tumours, genes in green are down-regulated in tumours. Network p value, p = 1.66E-07

3.4.5.2 The effect of PPARα deletion in the APC$^{Min/+}$ mouse model; tumorigenesis & gene expression in the colon

Affymetrix® microarray analysis of Comparison 2 (APC$^{Min/+}$PPARα$^{-/-}$ tumour *vs.* APC$^{Min/+}$ tumour) tumour tissue from the colons of mice found 504 genes were differentially regulated between APC$^{Min/+}$PPARα$^{-/-}$ mice and APC$^{Min/+}$ mice (Figure 3-4 and Appendices page 378).
IPA analysis showed the top biological processes affected by the differential expression of genes between tumour tissue in APC$^{Min/+}$PPARα$^{-/-}$ mice and APC$^{Min/+}$ mice, were also cellular growth & proliferation (p = 3.14E-10) and colorectal cancer (p = 5.67E-04). Networks showing gene expression data from these two processes were generated (Figure 3-15 and Figure 3-18).

Each network was also annotated with the Wnt/beta catenin and PPARα canonical pathways. Additionally, gene expression data from Comparison 2 was overlaid onto IPA generated canonical pathways to produce corresponding networks.

Table 3-12 details gene expression data for cell growth & proliferation of genes from Comparison 2 (APC$^{Min/+}$PPARα$^{-/-}$ tumour *vs.* APC$^{Min/+}$ tumour). These data were extracted from IPA of Comparison 2 Affymetrix® microarray data.

Figure 3-15 shows a network of differentially expressed genes associated with growth & proliferation between tumours in APC$^{Min/+}$PPARα$^{-/-}$ mice and APC$^{Min/+}$ mice. The network was annotated with the Wnt/β catenin and PPARα pathways.

Figure 3-15 and Figure 3-16 show genes targeted by Wnt/β catenin signalling;

- Transforming growth factor beta receptor 3 (Tgfβr3, FC 2.57↑, p = 0.03)
- Gap protein alpha 1/Connexin 43 (Gjα1/CX43, FC 2.48↑, p = 0.02)

- Secreted frizzled-related protein 1 (Sfrp1, FC 6.15↑, p = 0.05)

Tgfβr3 and the associated signalling pathway were described previously. Data from the present study showed that in $APC^{Min/+}$ mice (Comparison 3), Tgfβr3 was down-regulated in tumour tissue compared to normal tissue.

However, Tgfβr3 was up-regulated in tumour tissue in $APC^{Min/+}PPAR\alpha^{-/-}$ mice compared to tumour tissue in $APC^{Min/+}$ mice (Comparison 2). Figure 3-15 shows Tgfβr3 was also a target of PPARα/RXRα signalling. Taken together, these data may infer PPARα exerts an inhibitory effect in expression of Tgfβr3.

Gjα1 functions in control of cell growth and differentiation, and has been shown to be down-regulated in human cancers (Mesnil et al., 2005). A recent study by (Sirnes et al., 2012) found that Gjα1 was down-regulated in colon cancer cell lines and colorectal carcinomas. Further, their study demonstrated that Gjα1 co-localised with β-catenin to down-regulate the Wnt signalling pathway and increase apoptosis. Therefore, they concluded that Gjα1 functions as a colorectal cancer tumour suppressor protein.

A recent study has presented evidence to suggest that Sfrp1 may suppress tumour formation by functioning as a mediator of senescence *via* inhibition of Wnt/β catenin signalling and activation of the retinoblastoma (Rb) pathway. In addition, mutations in the Sfrp1 gene, as have previously been identified in human cancers (Bovolenta et al., 2008), may impair the senescence-inducing activity of Sfrp1 (Elzi et al., 2012).

Figure 3-15 and Figure 3-17 show Interleukin 6 (Il6, FC 7.36↑, p = 7.34E-03) was targeted by PPARα/RXRα signalling.

Il6 has been shown to be an important tumour promoting cytokine. Briefly, Il6 is secreted by cells of the innate or adaptive immune system and then binds to soluble Il6 receptor (sIl6r). This complex interacts with gp130 on tumour cells to induce activation of Janus kinases (JAKs) and

phosphorylation of signal transducer and activator transcription 3 (STAT3). Phosphorylated STAT3 then translocates to the nucleus to induce transcription of target genes to promote proliferation, cell growth and inhibition of apoptosis (Waldner et al., 2012).

Il6 expression has been demonstrated to be associated with tumour stage, size, metastasis and prognosis in patients with colorectal cancer (Knupfer and Preiss, 2010).

Table 3-12 Growth & Proliferation network data (Comparison 2 APC$^{Min/+}$PPARα$^{-/-}$ tumour *vs.* APC$^{Min/+}$ tumour)

Gene Symbol	Entrez Gene Name	Fold Change	p value
UP-REGULATED IN APC$^{Min/+}$ PPARα$^{-/-}$ TUMOURS (RED)			
CCL11	chemokine (C-C motif) ligand 11	3.58	8.54E-03
CCL13	chemokine (C-C motif) ligand 13	4.07	1.05E-02
CNTN1	contactin 1	4.66	5.42E-03
COL14A1	collagen, type XIV, alpha 1	3.74	3.03E-02
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	2.43	1.41E-02
Cxcl12	chemokine (C-X-C motif) ligand 12	2.47	1.43E-02
DCN	decorin	1.77	8.12E-03
EDNRA	endothelin receptor type A	2.22	4.91E-02
ELN	elastin	1.93	4.56E-02
GHR	growth hormone receptor	2.23	2.68E-03
GJA1	gap junction protein, alpha 1, 43kDa	2.48	1.50E-02
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	1.78	4.45E-02
IL13RA1	interleukin 13 receptor, alpha 1	1.90	4.60E-03
IL13RA2	interleukin 13 receptor, alpha 2	1.90	4.79E-02
IL6	interleukin 6 (interferon, beta 2)	7.36	7.34E-03
ITGAM	integrin, alpha M (complement component 3 receptor 3 subunit)	2.40	2.86E-02
ITGAV	integrin, alpha V	1.77	1.34E-02
LILRB3	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	2.57	6.41E-03
LOX	lysyl oxidase	1.75	2.10E-02
LUM	lumican	9.47	2.30E-02
OSMR	oncostatin M receptor	2.50	1.58E-02
PLAUR	plasminogen activator, urokinase receptor	2.44	1.13E-03
PYCARD	PYD and CARD domain containing	2.03	3.67E-02
RECK	reversion-inducing-cysteine-rich protein with kazal motifs	1.91	1.40E-02
S1PR3	sphingosine-1-phosphate receptor 3	2.21	4.88E-02
SOCS2	suppressor of cytokine signaling 2	1.87	2.61E-02
TGFBR3	transforming growth factor, beta receptor III	2.57	3.34E-02
TNC (includes EG:116640)	tenascin C	2.23	4.68E-02
VCAN	versican	2.48	5.25E-03
Gene Symbol	**Entrez Gene Name**	**Fold Change**	**p value**
DOWN-REGULATED IN APC$^{Min/+}$ PPARα$^{-/-}$ TUMOURS (GREEN)			
CISH	cytokine inducible SH2-containing protein	-1.84	9.69E-03
EDN3	endothelin 3	-1.96	3.15E-02
GPNMB	glycoprotein (transmembrane) nmb	-6.69	8.33E-05
IRAK4	interleukin-1 receptor-associated kinase 4	-3.90	9.91E-04
MMP14	matrix metallopeptidase 14 (membrane-inserted)	-2.20	3.46E-02
TBX3	T-box 3	-2.95	3.15E-02

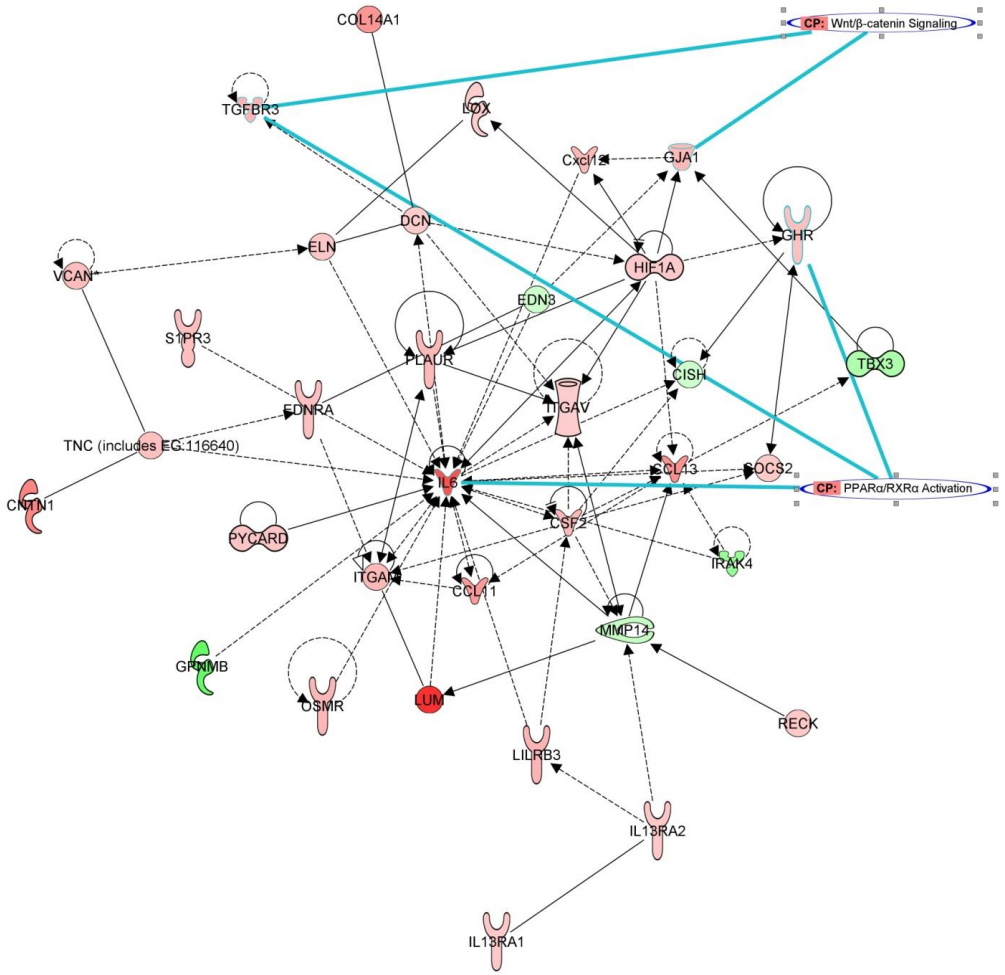


Figure 3-15 Growth & Proliferation network of Comparison 2 (APC$^{Min/+}$PPARα$^{-/-}$ tumour *vs.* APC$^{Min/+}$ tumour) annotated with the Wnt/beta catenin & PPARα/RXRα pathways Network p value, p = 3.14E-10

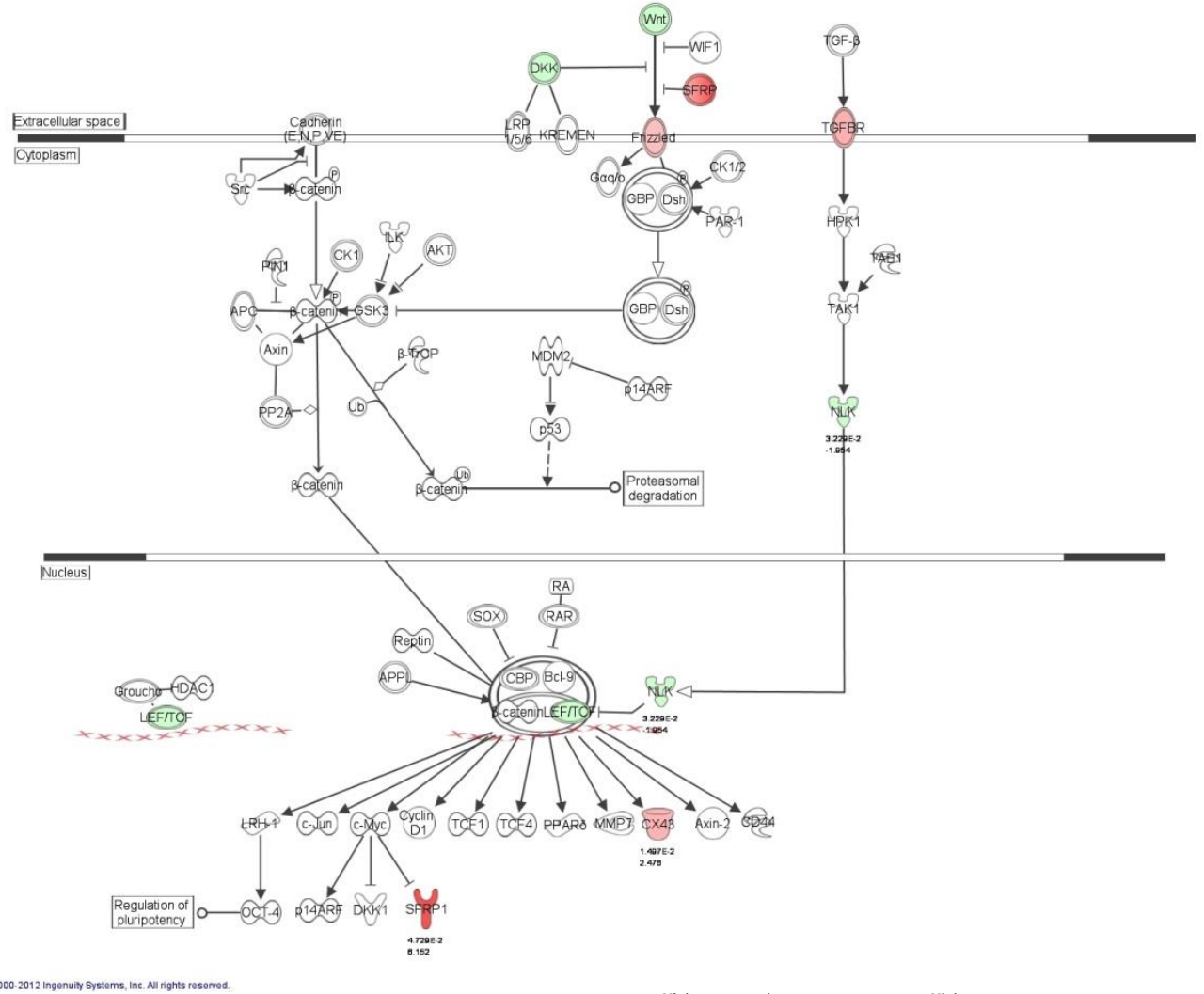


Figure 3-16 Canonical Wnt/beta catenin pathway & gene expression data from Comparison 2 ($APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour)

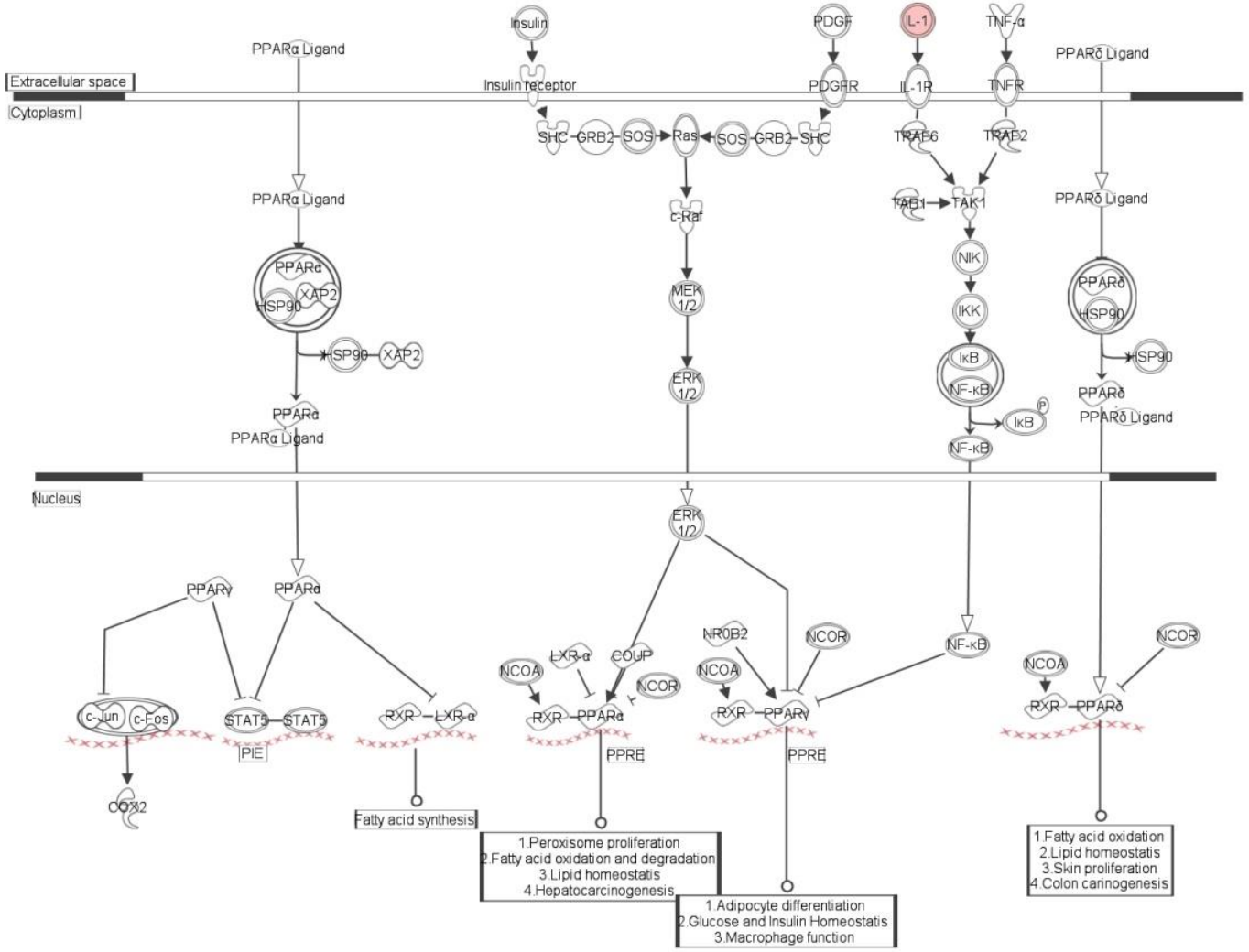


Figure 3-17 Canonical PPAR signalling & gene expression data from Comparison 2 (APC$^{Min/+}$PPARα$^{-/-}$ tumour *vs.* APC$^{Min/+}$ tumour

Table 3-13 details gene expression data for colorectal cancer of genes from Comparison 2 ($APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour). These data were extracted from IPA of Comparison 2 Affymetrix® microarray data.

Figure 3-18 shows a network of differentially expressed genes associated with colorectal cancer between tumours in $APC^{Min/+}PPAR\alpha^{-/-}$ mice and $APC^{Min/+}$ mice. The network was annotated with the Wnt/beta catenin and PPARα pathways as before.

Figure 3-18 (see also Figure 3-16) show Wnt/β catenin signalling targeted Sfrp1 (FC 6.15↑, p = 0.05) and Gjα1 (FC 2.48, p = 0.02) as explained before.

Also, PPARα/RXRα signalling (see Figure 3-17) targeted Il6 (FC 7.36↑, p = 7.34E-03) as described previously.

Table 3-13 Colorectal Cancer network data (Comparison 2 $APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour)

Gene Symbol	Entrez Gene Name	Fold change	p-value
UP-REGULATED IN $APC^{Min/+}$ $PPAR\alpha^{-/-}$ TUMOURS (RED)			
ANGPTL4	angiopoietin-like 4	2.16	3.55E-02
CNNM4	cyclin M4	2.07	4.03E-02
EPB41L3	erythrocyte membrane protein band 4.1-like 3	1.76	1.04E-02
GJA1	gap junction protein, alpha 1, 43kDa	2.48	1.50E-02
GSTM1	glutathione S-transferase mu 1	1.77	1.62E-02
HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	2.17	7.58E-03
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	1.78	4.45E-02
IGFBP5	insulin-like growth factor binding protein 5	2.48	4.58E-02
IL6	interleukin 6 (interferon, beta 2)	7.36	7.34E-03
KLF6	Kruppel-like factor 6	1.98	1.88E-02
NDRG4	NDRG family member 4	1.91	4.02E-02
NRP1 (includes EG:18186)	neuropilin 1	1.79	4.43E-02
OSMR	oncostatin M receptor	2.50	1.58E-02
P4HA1	prolyl 4-hydroxylase, alpha polypeptide I	2.17	1.84E-02
PLAUR	plasminogen activator, urokinase receptor	2.44	1.13E-03
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	1.97	1.31E-02
SFRP1	secreted frizzled-related protein 1	6.15	4.73E-02
TBC1D4	TBC1 domain family, member 4	2.03	4.87E-02
TFF3	trefoil factor 3 (intestinal)	2.15	3.46E-02
TNC (includes EG:116640)	tenascin C	2.23	4.68E-02
Gene Symbol	**Entrez Gene Name**	**Fold change**	**p-value**
NO SIGNIFICANT CHANGE IN EXPRESSION (NO COLOUR)			
B3GNT2	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2	1.18	> 0.05
GALE	UDP-galactose-4-epimerase	1.24	0.07
IL17RC	interleukin 17 receptor C	1.04	0.63
KIAA1522	C77080	1.32	> 0.05
POU2F1	POU class 2 homeobox 1	1.00	0.97
SNX10	sorting nexin 10	1.64	> 0.05
TMEM9B	TMEM9 domain family, member B	1.32	0.13
UBC	ubiquitin C	1.20	0.27
GINS1	GINS complex subunit 1 (Psf1 homolog)	-1.29	0.09
KLK8	kallikrein-related peptidase 8	-1.02	0.84
MARCH7	membrane-associated ring finger (C3HC4) 7, E3 ubiquitin protein ligase	-1.08	0.41
SLC39A14	solute carrier family 39 (zinc transporter), member 14	-1.13	0.43
SMOX	spermine oxidase	-1.18	> 0.05
TBC1D9	TBC1 domain family, member 9 (with GRAM domain)	-1.07	0.03
TTYH1	tweety homolog 1 (Drosophila)	-1.42	0.51

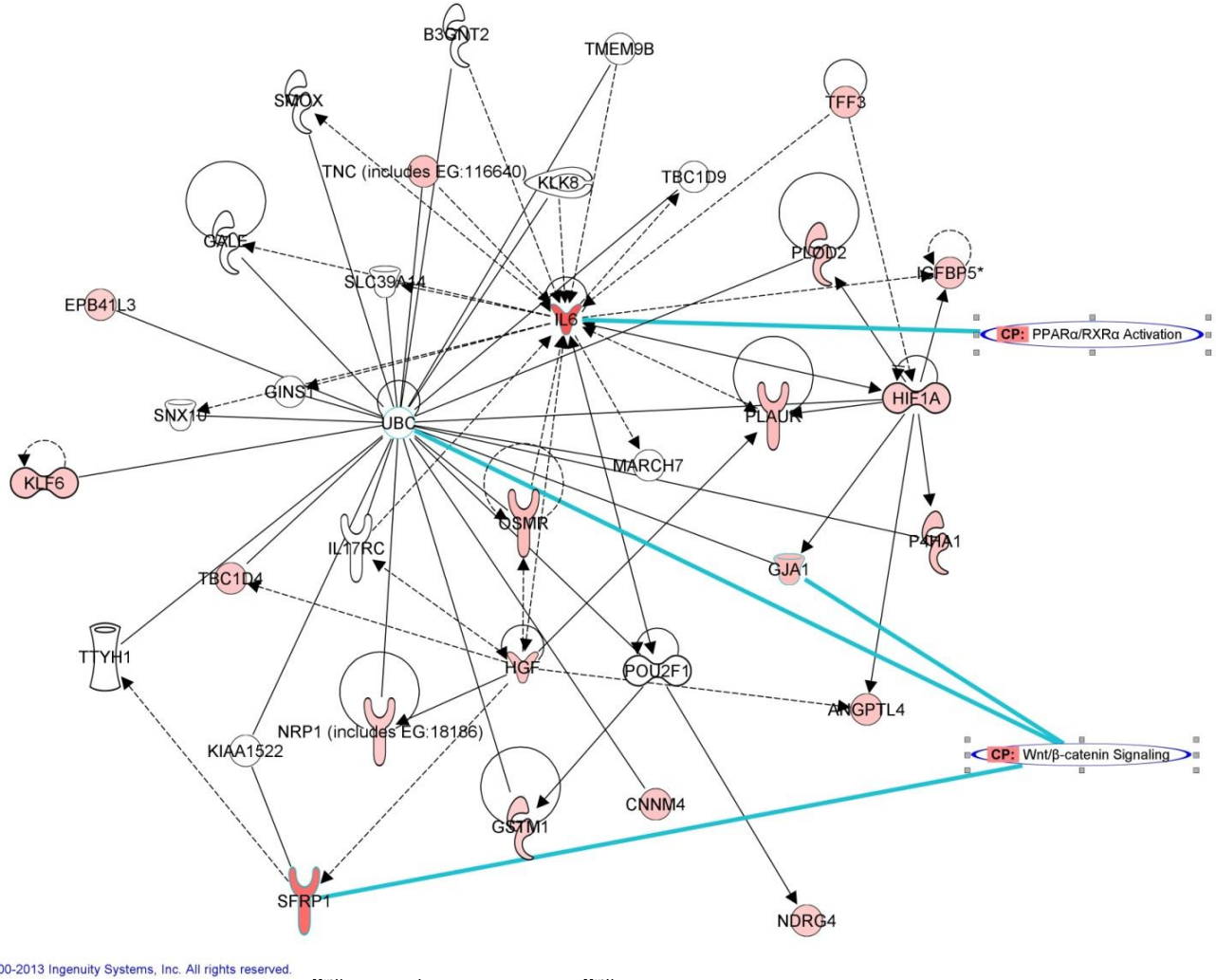


Figure 3-18 Colorectal Cancer network of Comparison 2 (APC$^{Min/+}$PPARα$^{-/-}$ tumour *vs.* APC$^{Min/+}$ tumour) annotated with the Wnt/beta catenin & PPARα/RXRα pathways Network p value, p = 5.67E-04

3.4.5.3 Ingenuity® Pathway Analysis (IPA®) of Comparison 1 & Comparison 4

An overview of results for IPA of Comparison 1 ($APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal) and Comparison 4 ($APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}PPAR\alpha^{-/-}$ tumour) are below.

- Comparison 1 ($APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal)

Affymetrix® microarray analysis of normal colonic tissue found only 52 genes were differentially expressed between $APC^{Min/+}PPAR\alpha^{-/-}$ mice and $APC^{Min/+}$ mice (Figure 3-4 and Appendices page 378). IPA of these data showed the top biological function was lipid metabolism (p = 6.10E-03). However, the IPA network showed no genes were highlighted when annotated with the PPARα/RXRα and Wnt/β catenin signalling pathways.

Therefore, these data taken together indicate that deletion of PPARα had little effect on gene expression in normal tissue of the colon.

- Comparison 4 ($APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}PPAR\alpha^{-/-}$ tumour)

Affymetrix® microarray analysis of colon tissue from $APC^{Min/+}PPAR\alpha^{-/-}$ mice found there were 1181 differentially regulated genes between normal and tumour tissue in these mice (Figure 3-3 and Appendices page 378).

IPA analysis of these data indicated the two top biological processes affected by the differential expression of genes between normal and tumour tissue in $APC^{Min/+}PPAR\alpha^{-/-}$ mice, were cancer (p = 1.61E-21) and cellular growth & proliferation (p = 1.47E-14).

Expression of several genes was significantly up-regulated in tumour tissue. Genes included Gjα1 (FC 2.12↑, p = 7.28E-04), Mmp7 (FC 63.93↑, p = 1.26E-08) and Myc (FC 2.40↑, p = 1.55E-06). Also, two genes associated with the Wnt/β Catenin signalling pathway were

differentially-regulated in tumour tissue including Myc and Tgfβr (FC 2.5↓, p = 6.65E-04). Expression of these genes in tumour tissue has been discussed previously.

In summary, IPA results and analysis of Comparison 3 ($APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour) growth & proliferation and colorectal cancer networks, and canonical pathways associated with tumorigenesis; Wnt/beta catenin, ERK/MAPK and p53, clearly demonstrated the differential expression of genes and activation of pathways involved in these processes. IPA highlighted many genes that have previously been shown to be involved in tumorigenesis in the colon. In addition, the high number of differentially expressed genes in tumour tissue compared to normal tissue is as would be expected in the $APC^{Min/+}$ mouse model used in these studies.

Also, IPA results and analysis of Comparison 2 ($APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour) growth & proliferation and colorectal cancer networks, the Wnt/β catenin and PPARα/RXRα pathways, showed there were far fewer differentially expressed genes between $APC^{Min/+}PPAR\alpha^{-/-}$ tumour tissue and $APC^{Min/+}$ tumour tissue (504), than between $APC^{Min/+}$ normal tissue and $APC^{Min/+}$ tumour tissue (1141).

Therefore, genes highlighted on the IPA networks may indicate that any differential expression is due to the effect of PPARα. A key regulatory gene was demonstrated to be Il6.

3.4.6 Genes selected for analysis & verification on Taqman® low density arrays

Table 3-14 lists the reference genes chosen for normalisation of the 95 genes selected for validation by Taqman® low density arrays.

Expression data from all 18 arrays (samples) for each of the selected reference genes is shown. The antilog2 of intensity values were calculated which were used to determine the mean and standard deviation for each reference gene for all 18 samples. These data

confirmed that the expression level for each reference gene in all samples were within three standard deviations of the mean (three-sigma rule[15], (Ruan, 2005). Therefore, this showed expression of each reference gene was invariant across each array.

The final list of genes selected for analysis and verification on Taqman® low density arrays are presented in Table 3-15 to Table 3-23.

[15] The three-sigma rule (empirical rule) states that for a normal distribution, nearly all values lie within 3 standard deviations of the mean.

Table 3-14 Reference gene selection for normalisation of Taqman® low density arrays

Gene Symbol	Gene Name	Function
		Process
Actb	actin, beta	Cytoskeletal protein
		Cell structure
Hmbs	hydroxymethylbilane synthase	Hydrolase, Deaminase
		Coenzyme and prosthetic group metabolism
Hprt1	hypoxanthine guanine phosphoribosyl transferase 1	Transferase, Glycosyltransferase
		Nucleoside, nucleotide and nucleic acid metabolism
Ppia	peptidylprolyl isomerase A	Isomerase
		Protein metabolism and modification

Reference genes for Taqman® low density arrays

Samples	Genotype	Tissue type	Expression data of reference genes (intensity values from Affymetrix® microarrays)				(antilog2) of expression data			
			Actb	Hmbs	Hprt1	Ppia	Actb	Hmbs	Hprt1	Ppia
1	APC $^{Min/+}$	normal	-0.07	-0.14	0.02	-0.25	0.96	0.91	1.01	0.84
2	APC $^{Min/+}$	normal	0.09	0.08	0.08	0.03	1.07	1.06	1.06	1.02
3	APC $^{Min/+}$	normal	0.02	-0.08	-0.02	0.02	1.02	0.94	0.99	1.01
4	APC $^{Min/+}$	normal	-0.02	0.12	-0.30	-0.02	0.98	1.08	0.81	0.99
5	APC $^{Min/+}$	tumour	-0.05	-0.27	0.14	-0.05	0.97	0.83	1.10	0.97
6	APC $^{Min/+}$	tumour	0.03	0.25	-0.04	-0.04	1.02	1.19	0.97	0.97
7	APC $^{Min/+}$	tumour	0.02	-0.12	0.04	0.09	1.01	0.92	1.03	1.07
8	APC $^{Min/+}$	tumour	-0.02	0.12	-0.25	0.04	0.99	1.09	0.84	1.03
9	APC $^{Min/+}$ PPARα $^{-/-}$	normal	-0.04	0.06	0.21	-0.04	0.97	1.04	1.16	0.97
10	APC $^{Min/+}$ PPARα $^{-/-}$	normal	0.00	-0.03	-0.07	0.00	1.00	0.98	0.95	1.00
11	APC $^{Min/+}$ PPARα $^{-/-}$	normal	-0.04	-0.07	0.00	-0.04	0.97	0.95	1.00	0.97
12	APC $^{Min/+}$ PPARα $^{-/-}$	normal	0.00	0.04	-0.18	0.00	1.00	1.03	0.88	1.00
13	APC $^{Min/+}$ PPARα $^{-/-}$	normal	0.08	0.00	0.20	0.08	1.06	1.00	1.15	1.06
14	APC $^{Min/+}$ PPARα $^{-/-}$	tumour	0.05	0.00	0.03	-0.08	1.04	1.00	1.02	0.95
15	APC $^{Min/+}$ PPARα $^{-/-}$	tumour	0.01	0.10	0.07	-0.01	1.01	1.08	1.05	0.99
16	APC $^{Min/+}$ PPARα $^{-/-}$	tumour	-0.12	-0.02	-0.02	0.02	0.92	0.98	0.99	1.01
17	APC $^{Min/+}$ PPARα $^{-/-}$	tumour	0.00	0.00	0.00	0.06	1.00	1.00	1.00	1.04
18	APC $^{Min/+}$ PPARα $^{-/-}$	tumour	-0.07	0.00	-0.22	0.00	0.95	1.00	0.86	1.00
						Mean	1.00	1.00	0.99	0.99
						Standard Deviation (SD)	0.04	0.08	0.10	0.05
						Upper limit (Mean + 3SD)	1.11	1.25	1.28	1.14
						Lower limit (Mean - 3SD)	0.89	0.76	0.70	0.84

Affymetrix® microarray expression data of selected reference genes

Table 3-15 Genes implicated in fatty acid & lipid metabolism

Gene Symbol	Gene Name	Function Process	Comparison group	Regulation	fold change	p value
Acot2	acyl-CoA thioesterase 2	Hydrolase, Esterase, Enzyme Lipid, fatty acid and steroid metabolism	1	down	1.8	3.40E-02
Ptgs1	prostaglandin-endoperoxide synthase 1	Oxidoreductase activity, Enzyme Lipid, fatty acid and steroid metabolism	1	-	1	5.00E-02
Angptl4	angiopoietin-like 4	Enzyme inhibitor activity, Receptor binding Negative regulation of apoptosis. Fatty acid & lipid metabolism	2	up	2.2	4.00E-02
Bdh1	3-hydroxybutyrate dehydrogenase, type 1	Oxidoreductase, Dehydrogenase, Enzyme Lipid, fatty acid and steroid metabolism	2	up	2.3	5.00E-02
Cyp2b10	cytochrome P450, family 2, subfamily b, polypeptide 10	Oxidoreductase, Oxygenase, Enzyme Fatty acid and lipid metabolism	2	down	4.9	4.00E-02
Decr1	2,4-dienoyl CoA reductase 1, mitochondrial	Oxidoreductase, Enzyme Lipid, fatty acid and steroid metabolism, Fatty acid beta-oxidation	2	up	3.2	7.81E-04
Pla2g2a	phospholipase A2, group IIA (platelets, synovial fluid)	Hydrolase, Lipase, Enzyme Lipid, fatty acid and steroid metabolism,Phospholipid metabolism,Signal transduction	2	down	2.2	5.40E-04
Alox12	arachidonate 12-lipoxygenase	Oxidoreductase, Oxygenase, Enzyme Lipid, fatty acid and steroid metabolism	3	down	23.8	2.04E-05
Alox15	arachidonate 15-lipoxygenase	Oxidoreductase, Oxygenase, Enzyme Lipid, fatty acid and steroid metabolism	3	down	2.8	4.00E-02
Cyp11a1	cytochrome P450, family 11, subfamily a, polypeptide 1	Oxidoreductase, Oxygenase, Enzyme Lipid, fatty acid and steroid metabolism, Electron transport	3	down	3.7	1.00E-03
Cyp2c55	cytochrome P450, family 2, subfamily c, polypeptide 55	Oxidoreductase, Oxygenase, Enzyme Fatty acid and lipid metabolism	3	up	42.5	2.00E-02
Ech1	enoyl coenzyme A hydratase 1, peroxisomal	Lyase, Hydratase, Enzyme Lipid, fatty acid and steroid metabolism, Fatty acid beta-oxidation	3	up	2.5	2.00E-03
Echdc2	enoyl Coenzyme A hydratase domain containing 2	Catalytic activity, Lyase activity, Enzyme Lipid & fatty acid metabolism	3	down	3.9	4.00E-03
Hadha	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit	3-hydroxyacyl-CoA dehydrogenase activity, Enzyme Carbohydrate metabolism,Lipid, fatty acid and steroid metabolism, Fatty acid beta-oxidation, Vitamin metabolism	3	up	2	4.00E-03
Lrp1	low density lipoprotein receptor-related protein 1	Transmembrane receptor Lipid, fatty acid and steroid metabolism	3	up	2.1	2.00E-03
Ptgis	prostaglandin I2 (prostacyclin) synthase	Oxidoreductase, Isomerase, Enzyme Lipid, fatty acid and steroid metabolism, Electron transport	3	up	3.2	4.00E-03
Alox5ap	arachidonate 5-lipoxygenase activating protein	Enzyme binding Lipid, fatty acid and steroid metabolism	4	down	1.7	2.00E-02
Ptgs2	prostaglandin-endoperoxide synthase 2	Oxidoreductase activity, Enzyme Lipid, fatty acid and steroid metabolism	4	down	9.2	3.17E-04
Steap4	STEAP family member 4	Ion binding, oxidoreductase Oxidation reduction, homeostasis, ion transport. Fatty acid & lipid metabolism	4	down	2.9	7.36E-04

Comparison group refers to;
$APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal (Comparison 1)
$APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)
$APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)
$APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}PPAR\alpha^{-/-}$ tumour (Comparison 4).
p value and fold change values are from Affymetrix microarray expression data

Table 3-16 Genes implicated in signal transduction

Gene Symbol	Gene Name	Function / Process	Comparison group	Regulation	fold change	p value
Apcdd1	adenomatosis polyposis coli down-regulated 1	Wnt-protein binding Wnt receptor signalling pathway	2	down	8.5	3.00E-02
Ereg	epiregulin	Growth factor Angiogenesis, cytokine-mediated signaling pathway	2	up	2.9	4.00E-02
Ghr	growth hormone receptor	Transmembrane receptor Signal transduction	2	up	2.2	3.00E-03
Il6	interleukin 6	Signaling molecule, Cytokine Signal transduction, Cytokine and chemokine mediated signaling pathway,Intracellular signaling cascade,MAPKKK cascade, JNK cascade, JAK-STAT cascade, Apoptosis	2	up	7.4	7.00E-03
Sfrp1	secreted frizzled-related protein 1	Signaling molecule, Transmembrane receptor Signal transduction	2	up	6.2	5.00E-02
Gulp1	GULP, engulfment adaptor PTB domain containing 1	Signaling molecule, Protein receptor Signal transduction, Receptor protein tyrosine kinase signaling pathway,Intracellular signaling cascade,MAPKKK cascade, Apoptosis	3	up	2.5	7.12E-04
Inhba	inhibin beta-A	Signaling molecule, Cytokine, Growth factor Signal transduction	3	down	6.4	5.17E-06
Krt18	keratin 18	Protein binding Apoptosis. Signal transduction	3	down	2.3	8.48E-04
Lama5	laminin, alpha 5	Extracellular matrix linker protein Signal transduction,Cell communication,Cell adhesion-mediated signaling	3	down	2.9	3.86E-04
Ptger3	prostaglandin E receptor 3 (subtype EP3)	G-protein coupled receptor Signal transduction, G-protein mediated signaling, Apoptosis	3	up	5.6	8.00E-03
Rhoj	ras homolog gene family, member J	Select regulatory molecule, Enzyme Signal transduction,Cell surface receptor mediated signal transduction,G-protein mediated signaling	3	down	2.9	2.00E-03
Il1b	interleukin 1 beta	Signaling molecule, Cytokine Signal transduction, Cytokine and chemokine mediated signaling pathway	4	down	11.3	1.11E-06
Lama1	laminin, alpha 1	Protein binding Cell adhesion, Regulation of cell migration. Signal transduction	4	down	4	9.35E-06
Ppbp	pro-platelet basic protein	Chemokine, Cytokine Signal transduction, Cytokine and chemokine mediated signaling pathway	4	down	6.4	2.67E-04
Tnf	tumor necrosis factor	Signaling molecule, Cytokine Signal transduction, Cytokine and chemokine mediated signaling pathway, Apoptosis	4	down	2	1.42E-04

Comparison group refers to;
$APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal (Comparison 1)
$APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)
$APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)
$APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}PPAR\alpha^{-/-}$ tumour (Comparison 4).
p value and fold change values are from Affymetrix microarray expression data

Table 3-17 Genes implicated in transcription

Gene Symbol	Gene Name	Function / Process	Comparison group	Regulation	fold change	p value
Cbx7	chromobox homolog 7	Transcription factor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription,mRNA transcription regulation	1	down	2.1	4.00E-03
Ang	angiogenin, ribonuclease, RNase A family, 5	Select regulatory molecule, Enzyme Nucleoside, nucleotide and nucleic acid metabolism, Angiogenesis, Transcription	2	down	47.8	3.00E-02
Chd8	chromodomain helicase DNA binding protein 8	Transcription factor, Enzyme Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription	2	down	2.5	5.00E-03
Id4	inhibitor of DNA binding 4	Transcription factor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription,mRNA transcription regulation	2	up	2.9	8.00E-03
Foxc2	forkhead box C2	Transcription factor Carbohydrate metabolism,Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription, Signal transduction, Embryogenesis, Neurogenesis, Cell cycle, Cell proliferation and differentiation	3	down	4.2	1.30E-04
Gucy2c	guanylate cyclase 2c	Receptor, Kinase Transcription	3	up	2.5	2.00E-03
Klf4	Kruppel-like factor 4 (gut)	Transcription factor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription,mRNA transcription regulation	3	up	3.2	3.00E-03
Meis1	Meis homeobox 1	Transcription factor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription, Oncogenesis, Cell proliferation and differentiation	3	up	3.7	2.46E-04
Arnt2	aryl hydrocarbon receptor nuclear translocator 2	Transcription factor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription,mRNA transcription regulation	4	down	5.6	1.36E-06
Baz1a	bromodomain adjacent to zinc finger domain 1A	Transcription factor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription,mRNA transcription regulation	4	down	2.1	1.70E-06
Crem	cAMP responsive element modulator	Transcription factor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription	4	down	2.4	1.66E-04
Jun	Jun oncogene	Transcription factor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription, Signal transduction, JNK cascade,Oncogenesis, Cell cycle, Cell proliferation and differentiation	4	down	2.2	4.00E-03
Phf17	PHD finger protein 17	Transcription factor Transcription	1	up	2.3	2.00E-03
Tcf12	transcription factor 12	Transcription factor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription	2	down	2	4.00E-02
Onecut2	one cut domain, family member 2	Transcription factor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription,mRNA transcription regulation	3	down	41.7	3.95E-04
Pou2af1	POU domain, class 2, associating factor 1	Transcription factor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription	3	up	4.8	5.00E-02
Rbms1	RNA binding motif, single stranded interacting protein 1	Nucleic acid binding Transcription	3	down	2.1	3.00E-03
Myc	myelocytomatosis oncogene	Transcription factor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription, Oncogenesis, Cell cycle, Apoptosis	4	down	2.4	1.55E-06
Nfkbie	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	Transcription factor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription, Signal transduction, NF-kappaB cascade	4	down	2.2	2.76E-04
Nr1d1	nuclear receptor subfamily 1, group D	Ligand dependent nuclear receptor receptor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription	4	up	2.1	3.56E-04
Rarb	retinoic acid receptor, beta	Ligand dependent nuclear receptor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription, Oncogenesis	4	down	5.5	1.85E-04
Rorc	RAR-related orphan receptor gamma	Ligand dependent nuclear receptor Nucleoside, nucleotide and nucleic acid metabolism,mRNA transcription,mRNA transcription regulation	4	up	2.7	1.94E-05

Comparison group refers to;
$APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal (Comparison 1)
$APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)
$APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)
$APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}PPAR\alpha^{-/-}$ tumour (Comparison 4).
p value and fold change values are from Affymetrix microarray expression data

Table 3-18 Genes implicated in the cell cycle

Gene Symbol	Gene Name	Function / Process	Comparison group	Regulation	fold change	p value
Apobec3	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3	Cytidine deaminase, Enzyme Innate inhibitor of retroviral replication. Cell cycle	1	down	1.7	4.10E-03
Bin1	bridging integrator 1	Membrane traffic protein Oncogenesis,Tumor suppressor, Cell cycle, Cell proliferation and differentiation	3	up	2.2	1.00E-03
Ccnd1	cyclin D1	Select regulatory molecule Cell cycle, Cell proliferation and differentiation	3	down	2.9	2.00E-03
Cdkn1c	cyclin-dependent kinase inhibitor 1C (P57)	Select regulatory molecule Oncogenesis,Cell cycle, Cell proliferation and differentiation	4	down	3.1	4.00E-03

Table 3-19: Genes implicated in apoptosis

Gene Symbol	Gene Name	Function / Process	Comparison group	Regulation	fold change	p value
Bik	BCL2-interacting killer	Protein binding Apoptosis	2	down	2.4	6.34E-04
Apaf1	apoptotic peptidase activating factor 1	Select regulatory molecule Intracellular protein traffic, Apoptosis	3	down	2.4	3.00E-03
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	Protein binding Oncogenesis, Apoptosis	3	down	4.1	1.00E-03
Bmf	BCL2 modifying factor	Protein binding Apoptosis	3	down	3.6	4.00E-03
Endod1	endonuclease domain containing 1	Endonuclease, Hydrolase, Nuclease, Enzym;e Apoptosis	3	up	2.4	4.00E-03
Khdc1a	KH domain containing 1A	RNA binding Apoptosis	3	down	20.4	1.89E-04
Sbk1	SH3-binding kinase 1	Kinase, Transferase, Nucleotide binding Phosphorylation. Apoptosis	4	down	2.2	1.47E-04

Table 3-20: Genes implicated in protein transport

Gene Symbol	Gene Name	Function / Process	Comparison group	Regulation	fold change	p value
Abcc4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	Nucleotide binding, ATP binding, Transporter Protein transport	3	down	4.8	2.10E-04
Alb	albumin	Transfer/carrier protein, Transporter Protein transport	3	down	20.9	2.90E-04
Ap1s3	adaptor-related protein complex AP-1, sigma 3	Membrane traffic protein, Transporter Intracellular protein traffic, Transport	3	up	1.9	3.00E-02
Crip1	cysteine-rich protein 1 (intestinal)	Ion binding Protein transport	4	up	2.4	1.55E-04

Comparison group refers to;
$APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal (Comparison 1)
$APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)
$APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)
$APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}PPAR\alpha^{-/-}$ tumour (Comparison 4).
p value and fold change values are from Affymetrix microarray expression data

Table 3-21 Genes implicated in protein metabolism & modification

Gene Symbol	Gene Name	Function / Process	Comparison group	Regulation	fold change	p value
Irak4	interleukin-1 receptor-associated kinase 4	Protein kinase receptor / Protein metabolism and modification	1	down	2.7	1.56E-05
Ptprg	protein tyrosine phosphatase, receptor type, G	Phosphatase / Protein metabolism and modification	1	down	1.8	4.00E-03
Casp6	caspase 6	Protease, Peptidase / Protein metabolism and modification, Apoptosis	2	down	2.6	3.00E-02
Htra1	HtrA serine peptidase 1	Protease, Peptidase / Protein metabolism and modification,Proteolysis,Signal transduction	3	down	4.9	4.25E-04
Mep1b	meprin 1 beta	Protease, Peptidase / Protein metabolism and modification,Proteolysis	3	up	5.9	2.00E-03
Mmp2	matrix metallopeptidase 2	Protease, Peptidase / Protein metabolism and modification,Proteolysis, Oncogenesis, Angiogenesis	3	up	1.7	8.00E-03
Mmp7	matrix metallopeptidase 7	Protease, Peptidase / Protein metabolism and modification, Oncogenesis	3	down	78.3	1.86E-04
Pdk2	pyruvate dehydrogenase kinase, isoenzyme 2	Kinase / Protein metabolism and modification,Protein modification	3	up	2.8	2.00E-03
Plat	plasminogen activator, tissue	Protease, Peptidase / Protein metabolism and modification,Proteolysis	3	down	14.3	2.71E-04
Timp1	tissue inhibitor of metalloproteinase 1	Select regulatory molecule / Protein metabolism and modification	3	down	3.8	5.29E-04
Akt3	thymoma viral proto-oncogene 3	Kinase / Protein metabolism and modification, Oncogenesis, Cell cycle,Apoptosis,Cell proliferation and differentiation	4	up	2.1	4.00E-03
B4galt6	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6	Transferase, Glycosyltransferase, Enzyme / Protein metabolism and modification,Protein modification,Protein glycosylation	4	down	2.1	7.27E-05
Cdk4	cyclin-dependent kinase 4	Kinase / Protein metabolism and modification, Oncogenesis,Cell cycle, Cell proliferation and differentiation	4	down	2.2	5.16E-04

Table 3-22 Genes implicated in cell adhesion

Gene Symbol	Gene Name	Function / Process	Comparison group	Regulation	fold change	p value
Ctgf	connective tissue growth factor	Signaling molecule, Growth factor / Cell adhesion	3	down	2.1	5.00E-02
Itga6	integrin alpha 6	Cell adhesion molecule / Cell adhesion	3	down	2.2	1.00E-03
Sell	selectin, lymphocyte	Cell adhesion molecule / Cell adhesion	4	down	1.7	5.00E-03

Table 3-23 Genes involved in various cellular functions

Gene Symbol	Gene Name	Function / Process	Comparison group	Regulation	fold change	p value
Nisch	nischarin	Select regulatory molecule, Transmembrane / Cell structure and motility	2	down	2.3	3.00E-03
Aoc3	amine oxidase, copper containing 3	Oxidoreductase, Oxidase, Enzyme / Amino acid metabolism	3	up	6.5	1.31E-05
Cbr1	carbonyl reductase 1	Oxidoreductase, Reductase, Enzyme / Toxin metabolism	3	up	2.9	1.96E-04
Cryab	crystallin, alpha B	Structural protein / Cellular structure	4	up	2.9	9.72E-04

Comparison group refers to;
$APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal (Comparison 1)
$APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)
$APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)
$APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}PPAR\alpha^{-/-}$ tumour (Comparison 4).
p value and fold change values are from Affymetrix microarray expression data

3.5 Discussion

This study determined what effect PPARα may have on polyp burden and expression of genes in the colon of $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice. Age and disposition at death of these mice were also compared.

At the conclusion of the mouse study there was no significant difference in age at sacrifice between $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice (Figure 3-6A). All of the mice except one $APC^{Min/+}$ mouse (rectal prolapse) were sacrificed due to white paw (Table 3-5, Table 3-6). However, $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice were significantly heavier than $APC^{Min/+}$ mice at sacrifice ($p < 0.0001$, Figure 3-6B). This corresponds with the role of PPARα in lipid and fatty acid metabolism as $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice do not have a functional PPARα gene (Desvergne and Wahli, 1999, Kersten et al., 2000). However, it must also be noted that food consumption was not monitored. Mice were fed *ad libitum*. Therefore, it may be that $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice consumed more feed than $APC^{Min/+}$ mice.

The mean polyp numbers (lowest number counted and highest number counted in brackets) of $APC^{Min/+}$ mice in this study at death were 0.6 (0.0, 2.0) in the colon, and 17.8 (6.0, 28.0) in the small bowel (Table 3-7). These numbers were lower than those in an earlier study by (Jackson et al., 2003), using the same mouse model, where numbers were 6.5 (4.1, 10.0) and 24.0 (15.8, 30.3) in the colon and small bowel respectively. The polyp numbers in these two studies were considerably fewer than the first Leicester University study using this mouse model; 2.5 (0.0, 5) in the colon and 120 (70, 170) in the small bowel (Perkins et al., 2002).

In other studies that have used the $APC^{Min/+}$ mouse model, polyp numbers were highly variable. However, this is likely to be due to differing experimental protocols. These other studies differed in their

duration, the agent and dose used in treatment and are reviewed in (Corpet and Pierre, 2003).

The reduction in polyp numbers seen in the present studies may be due to the in-breeding of the mouse colony over many generations, which has afforded a survival advantage to mice with fewer polyps. This may have perpetuated the decline of the phenotypic effects of the Min mutation, evident in fewer polyp numbers.

At sacrifice, both mouse genotypes had a similar disposition and number of polyps in the small bowel and were of similar age. However, in the colon there were more polyps in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice than in $APC^{Min/+}$ mice which may suggest that PPARα has a role in preventing initiation or growth of tumours in the colon.

Microarray technology has facilitated the analysis of whole transcriptomes, leading to recognition and understanding of the functional roles that PPARs play in gene expression. Affymetrix® microarrays were used in this study to evaluate gene expression levels in normal and tumour tissue from the colons of $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice.

A critique of the method used for tissue collection, preparation and subsequent RNA extraction, could be that as tissue is a heterogeneous mix of cell types (see 1.1, page 2), it was not known which cell types, or proportion of these, were present in each tissue sample, and hence, which genes were expressed in the different cells. However, the method for tissue collection and preparation was consistent. Also, RNA samples were rigorously checked for integrity before analysis. In addition, after analysis, Affymetrix® quality control systems assessed groups of samples for any outliers.

If future studies required cell types and numbers to be known, tissue would be mixed with a dissociation enzyme such as collagenase, centrifuged, then re-suspended in culture medium for subsequent analysis *via* fluorescence – activated cell sorting (FACs).

Affymetrix® microarray results from the present study showed that the largest source of variation in gene expression was between tumour and normal samples.

Ingenuity® Pathway Analysis (IPA®) of Comparison 3 ($APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour) showed growth & proliferation and colorectal cancer were the two top scoring biological processes. Myc, CyclinD1, Tcf4, Axin2 and Mmp7 were significantly up-regulated in tumour tissue; this was shown in the IPA® networks of these functions and the Wnt/beta catenin, ERK/MAPK (ras) and p53 canonical pathways; all of which are known pathways linked to tumorigenesis (Fearon and Vogelstein, 1990, Kinzler and Vogelstein, 1996).

Similarly, IPA® of Comparison 4 ($APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}PPAR\alpha^{-/-}$ tumour) showed growth & proliferation and colorectal cancer were the two top scoring biological processes. Myc and Mmp7 were again shown to be significantly up-regulated in tumour tissue.

Other microarray studies have demonstrated a similar profile of gene expression involved in colonic tumorigenesis to that shown in our study; (Paoni et al., 2003) used Affymetrix® Mouse Genome – U74Av2 (MG-U74Av2)[16] GeneChip® arrays to analyse gene expression in tumour tissue compared to normal tissue in the colons of $APC^{Min/+}$ mice. Many differentially expressed genes were shown to be involved in pathways that regulate cell growth and proliferation. In agreement with findings from the present study, significantly up-regulated genes included Casp6 (Caspase 6) and Sox17 (SRY-box containing gene 17).

A study by (Williams et al., 2003) used microarrays to investigate gene expression in human tumour and normal colon tissue. A sub-set of genes from the arrays were selected based on expression levels, their association with aberrant cellular growth and to assess their importance in the pathogenesis of colorectal cancer. Real time quantitative PCR was used to validate expression levels of the selected genes in the

[16] Affymetrix® MG-U74Av2 GeneChip® array is the predecessor of the Affymetrix® MG-430.2 GeneChip® arrays used in this study

human tissue samples. Expression of the same genes were also determined in HCT116 cells (Brattain et al., 1981) and found to be comparable to levels in human tumour tissue. Genes that were found to be differentially-regulated in the present study and also highlighted in the (Williams et al., 2003) study included down-regulated Tnfsf10 (tumour necrosis factor (ligand) superfamily member 10) and Myc which is well documented for its role in cell growth and proliferation was up-regulated.

The differentially Expressed Gene (DEG) lists; Comparison 1 ($APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal) and Comparison 2 ($APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour)**,** Appendices pages 311-377, list the PPARα dependent genes that were identified on Affymetrix® microarrays after filtering by statistical analysis for fold change and p value (as defined previously 3.3.9, page 56).

These DEG lists were compared to PPARα target genes identified in previous studies (Mandard et al., 2004, Rakhshandehroo et al., 2010). Twelve genes on the DEG lists had been previously identified; interleukin 6 (Il6), acyl-CoA thioesterase 2 (Acot2), 2,4-dienoyl CoA reductase 1 (Decr1), 3-hydroxybutyrate dehydrogenase, type 1 (Bdh1), angiopoietin-like 4 (Angptl4), epoxide hydrolase 2 (Ephx2), acyl-CoA synthetase short-chain family member 2 (Acss2), fatty acid binding protein 5 (Fabp5), thioredoxin interacting protein (TXNIP), phosphoenolpyruvate carboxykinase 1 (PCK1), interleukin 1 receptor antagonist (Il1rn) and chemokine (C-C motif) ligand 2 (Ccl2).

Only two genes from the (Mandard et al., 2004, Rakhshandehroo et al., 2010) studies; Fabp5 and Acot2, were highlighted on Comparison 1 ($APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal) DEG list. As discussed earlier (Results 3.4.5.3, page 103) this DEG list identified only 52 genes differentially expressed between normal colon tissue in $APC^{Min/+}PPAR\alpha^{-/-}$ mice and $APC^{Min/+}$ mice. Therefore, these data show that in general, deletion of PPARα had little effect on gene expression in normal tissue of the colon.

However, as discussed previously (Results 3.4.5.2, page 93), Ingenuity® Pathway Analysis (IPA®) of Comparison 2 ($APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour) highlighted many more differentially expressed genes between tumour colon tissue in $APC^{Min/+}PPAR\alpha^{-/-}$ mice and $APC^{Min/+}$ mice; including ten that had been identified in previous studies by (Mandard et al., 2004, Rakhshandehroo et al., 2010). IPA® networks showing genes involved in growth & proliferation (Figure 3-15) and colorectal cancer (Figure 3-18) highlighted interleukin 6 (Il6) as a key regulatory gene involved in these processes.

Previous microarray analyses of gene expression in the liver and skeletal muscle have confirmed PPARα is involved in many regulatory pathways; lipid metabolism, cell death, signal transduction, cell cycle and DNA repair. However, differences in gene expression profiles and levels could be explained by how fatty acids are metabolised in different tissues. Fatty acid metabolism provides a major source of energy for the body. The colon metabolises short chain fatty acids from dietary fibre, whereas long chain fatty acids are firstly metabolised in the liver before being transported to other tissues in the blood.

A study by (Rakhshandehroo et al., 2007) investigated gene expression in the liver of Sv129 mice (Lee et al., 1995) and Sv129 $PPAR\alpha^{-/-}$ mice. Results from this study demonstrated a major role for PPARα in hepatic gene expression, liver homeostasis and regulation of lipogenesis.

A study by (Finck et al., 2005) demonstrated the role of PPARα in skeletal muscle using transgenic MCK PPARα (muscle-specific creatine kinase promoter PPARα) mice. These mice over-express PPARα in skeletal muscle. PPARα was shown to cause increased muscle fatty acid oxidation, decreased AMP-activated protein kinase activity and glucose uptake *via* transcriptional repression of GLUT4 (Slc2a4, solute carrier family 2 (facilitated glucose transporter), member 4). Thus, their study established a potential link between PPARα, obesity, level of glucose intolerance and insulin resistance.

A potential mechanism for the effects seen in the present study could be as explained in Figure 1-3 (page 12). Briefly, arachidonic acid (AA) is converted to prostaglandins by cyclooxygenase enzymes (Cox) and to epoxyeicosatrienoic acids (EETs) by cytochrome P450 enzymes (CYPs). EETs are regulated and degraded by soluble epoxide hydrolase (Ephx2), which is activated by PPARα. Increased production of EETs or inhibition of Ephx2 leads to repression of Cox2 induction (Inceoglu et al., 2008). EETs are pro-angiogenic, however, when degraded by Ephx2, there is a reduction in tumour angiogenesis and growth (Pozzi et al., 2007).

The study by (Pozzi et al., 2007) also investigated whether the anti-angiogenic effects of Wyeth-14643 (Wy-14643), a PPARα-selective ligand, were associated with altered Cyp2c expression. Their study using mice injected with tumour cells, and treated with Wy-14643, recorded marked reductions in tumour growth and vascularization, with concomitant reductions in epoxygenase metabolites, 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET, hepatic EET biosynthesis and Cyp2c29, -2c38, -2c40 and -2c44 epoxygenase transcripts. Similar effects were seen when primary cultures of mouse lung endothelial cells were treated with Wy-14643. Cell proliferation was reduced in the cells when treated with Wy-14643. However, the effects were reversed on addition of synthetic 5,6-, 8,9-, 11,12- and 14,15- EETs. None of these effects were seen in cells obtained from PPARα$^{-/-}$ mice, which confirmed PPARα as having an involvement in cell proliferation and angiogenesis *via* its relationship with Cyp2c epoxygenase expression and EET biosynthesis (Pozzi et al., 2007).

Another study by (Pozzi et al., 2010) used a mouse xenograft model of tumorigenesis, to show that activation of murine Cyp2c44 epoxygenase by PPARα produced a reduction in tumour volume, mass and vascularisation. Cyp2c44 is expressed in endothelial cells (Pozzi et al., 2007) and generates 11,12- and 14,15-EET as its major products (Pozzi et al., 2010). Their study also showed that CYP2C9, a previously identified human catalytic homologue of murine Cyp2c44 (Zeldin et al.,

1995) is an endothelial epoxygenase expressed in the vasculature of several human tumours (clear cell renal carcinoma, metastatic melanoma, lung adenoma), and was confirmed as a regulatory target of human PPARα (Pozzi et al., 2010).

In conclusion, the studies discussed here show that PPARα is instrumental in regulation of many biological pathways within different tissues in the body. Results from the present study clearly demonstrate that deletion of PPARα led to differential expression of genes involved in biological functions linked with cancer and a concomitant increase in tumour burden in the colon. Therefore, these data and the studies discussed here support the hypothesis that PPARα has a role in the pathophysiology of cancer and further endorses the possibility that ligands of PPARα could be viable candidates for cancer prevention and treatment.

To validate and verify Affymetrix® microarray results, 95 significantly differentially expressed genes were selected from Ingenuity® Pathway Analysis networks, selected canonical signalling pathways and novel genes of interest from differentially expressed gene (DEG) lists (Table 3-14 - Table 3-23).

Chapter 4 describes how these genes were assayed on Taqman® low density arrays to determine expression levels. Also, expression levels for each gene as determined on Affymetrix® microarrays and Taqman® low density arrays were compared.

4 Gene quantitation with Taqman® low density arrays: Study 1B

4.1 Introduction

Microarray based assays allow the global changes in gene expression caused by disease or defined experimental conditions to be measured. In Chapter 3, Affymetrix® microarray analysis and results of gene expression for the whole transcriptome in colons of $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice were presented and discussed. These data identified many differentially expressed genes in $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice, and in tumour and normal tissue, which may be involved in PPARα signalling and tumorigenesis.

Therefore, Taqman® low density arrays were used to determine and verify gene expression of a selected sub-set of these genes. Taqman® low density arrays are microfluidic cards that function as miniaturised reaction wells for PCR of target genes. Each well of the array contains a Taqman® Gene Expression Assay that detects the real-time amplification of a target gene. Relative levels of gene expression are determined from the fluorescence data that is generated during PCR cycling.

The principle of the Taqman probe relies on the 5' – 3' nuclease activity of the enzyme *Taq*-polymerase and fluorophore based detection. TaqMan probes are oligonucleotide probes with a fluorophore/reporter (6-carboxy fluorescein, FAM) at the 5' end, and a quencher (tetramethylrhodamine, TAMRA) at the 3' end. The probe anneals within the DNA region between forward and reverse specific primers. *Taq*-polymerase extends the primer and synthesises the nascent strand (3' – 5' of the complementary strand). The 5' – 3' nuclease activity of the enzyme degrades the probe annealed to the template. This releases the fluorophore from the close proximity of the quencher allowing fluorescence (see Figure 4-1). The level of detected

fluorescence is directly proportional to the amount of template DNA (Holland et al., 1991).

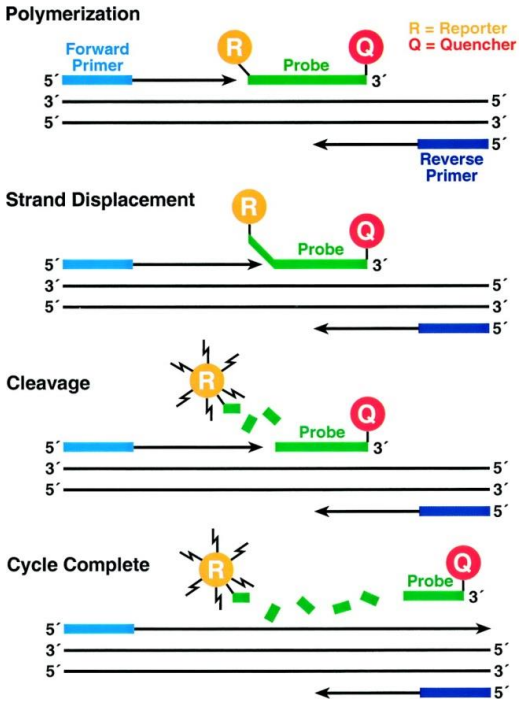


Figure 4-1 Schematic of the principle of a Taqman probe

Figure from (Yuan et al., 2000)

Genes to be assayed and endogenous control genes are preloaded onto one card which facilitates a high throughput of samples. Taqman® low density arrays are very economical with reagents and samples. They are also very easy to use; samples to be assayed are simply mixed with master mix and loaded into fill reservoirs using a micropipette. Once the array is sealed and centrifuged, it is ready to run. The whole assay is automated with decreased assay time and without loss of fidelity. This contrasts with the assay of samples on individual arrays, which would be very time consuming and expensive.

Analysis of Taqman® low density array results using SDS RQ Manager Version 1.2 (Applied Biosystems) allows up to ten assays at once to be reviewed. This means array to array reproducibility and stringency of reference genes can be checked across all arrays simultaneously.

The main disadvantage of Taqman® low density arrays is cost. The requirement for the sophisticated and expensive 7900HT system and software, and peripheral equipment for preparation, assay and analysis of arrays, means initial set-up costs are very high.

Applied Biosystems Taqman® low density arrays were used to analyse and verify gene expression levels of 95 selected genes that had previously been determined by Affymetrix® microarrays (Chapter 3).

4.2 Aims of study

- To determine gene expression of selected genes in the colon of $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice using TaqMan® low density arrays.
- To compare and validate the expression level of selected genes on Taqman® low density arrays against expression level of the same genes on Affymetrix® microarrays.

4.3 Methods

The customised Taqman® low density arrays used in the present study were 384-well microfluidic cards that enabled 384 simultaneous Real-Time PCR reactions to be performed. Four duplicated cDNA samples were run in parallel against 96 (including one mandatory control gene, 18s) pre-loaded Taqman® gene expression assay targets. Figure 4-2 shows an image of a Taqman® low density array.

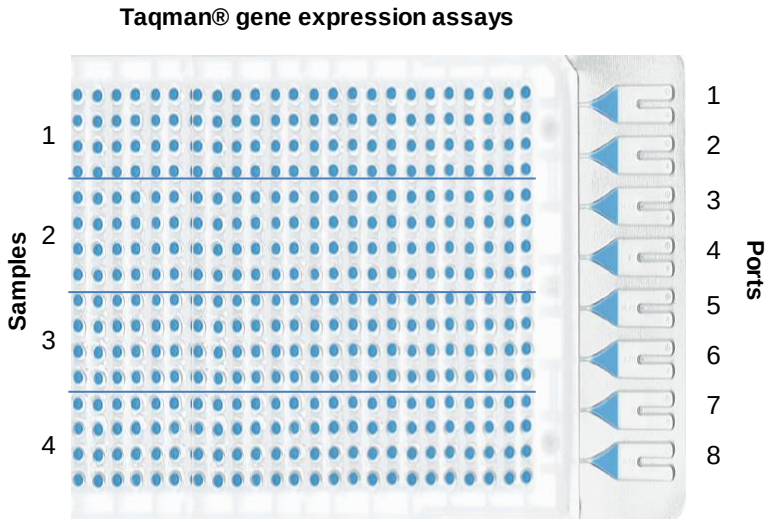


Figure 4-2 Taqman® low density array

(appliedbiosystems.com, 2012)

Taqman® low density arrays can be customised by selecting genes of interest to fit research goals and criteria dictated by the user; genes for the arrays were selected based on function and statistical relevance following GeneSpring® and Ingenuity® Pathway analysis of Affymetrix® microarray data (Table 3-15-Table 3-23).

TaqMan® low density arrays perform relative quantitation (RQ) of gene targets using the comparative C_T ($\Delta\Delta C_T$) method[17] (as opposed to an absolute quantitation method that uses a standard curve) Applied Biosystems 7900HT fast real-time PCR system with sequence detection system (SDS) software v2.1 was used to detect RQ of gene expression from fluorescence data generated during the real-time amplification of gene targets. SDS also alerts the user to any errors or faults within the run, array or samples.

4.3.1 Preparation & assay of Taqman® low density arrays

Aliquots of RNA samples extracted from colon tissue of mice on Study 1B (3.3.6, page 50, Table 3-3) were removed from storage at -80^{0}C.

[17] Comparative C_T method: is an approximation method that assumes the amplification efficiencies of target and reference genes are equal. Expression of a target gene is normalised to a reference gene. Expression is then compared to expression of that target in a nominated reference sample

RNA concentration was assessed on a NanoDrop® ND-1000 Spectrophotometer. Two samples were found to have a very low concentration and there was insufficient quantity to prepare cDNA (44b.3/4 $APC^{Min/+}$ normal & 115.4/1 $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour). The remaining 16 samples were diluted to 250 ng/µl and cDNA prepared, in duplicate using 500 ng of RNA with random primers (Promega, page 41) and SuperScript® lll RT (Invitrogen™). The manufacturer's protocol was followed with some modification; reagent and sample volumes were scaled-up to produce a cDNA reaction volume of 30 µl instead of 20 µl.

Four samples at a time were prepared for a TaqMan® low density array by combining the duplicate cDNA reactions and adding 50 µl of RNase-free water (to give 110 µl). An equal volume of TaqMan® Fast universal PCR master mix (Applied Biosystems) was added. Samples were thoroughly mixed by vortexing, and centrifuged to remove any bubbles.

A TaqMan® low density array equilibrated to room temperature was carefully removed from protective packaging. 100 µl of the first sample was loaded into the first fill reservoir on the array using a micropipette. Another 100 µl of the same sample was loaded into the adjacent fill reservoir. The process was repeated for the other three samples to complete the loading of the array.

The array was centrifuged on a Sorvall® centrifuge with custom-made buckets and array holders for 2 x 1 minute. The array was checked to ensure the fill reservoirs had correctly distributed the cDNA samples to the reaction wells. The array was then sealed and fill reservoirs were removed using scissors.

All TaqMan® low density arrays are shipped with an Array Information CD, which contains an SDS setup file. The SDS software on the 7900HT system used this file to configure the plate document grid and setup table. The plate document was saved as a plate document template for use with further assays with identical assay configurations.

The completed sealed array was loaded into the 7900HT instrument and the run commenced.

A total of four arrays were completed to assay all 16 samples.

4.3.2 Interpretation & Analysis of TaqMan® low density array data

All four arrays (16 sample results) were analysed simultaneously using SDS RQ Manager software. Average Ct (cross threshold)[18] values of each target gene (average calculated from Ct value from each sample across all 4 arrays) were copied into an Excel spreadsheet.

Consistency of average Ct value of each selected reference gene (Table 3-14) and 18s were checked for outliers before further analysis (Table 4-1). To justify their use for normalisation the expression level of reference genes must not alter across arrays. Outliers were defined as an average Ct value not within three standard deviations of the mean (three-sigma rule[19]), (Ruan, 2005). 18s was not used for normalisation as one sample average Ct value did not fulfil the stringency of the statistical testing (Table 4-1, shown in red).

[18] The threshold value is a defined level of fluorescence set by the user. The point at which an amplification plot crosses the threshold is known as the Ct (cross threshold) value; the lower the Ct value for a sample the greater the starting amount of DNA in the sample.

[19] The three-sigma rule (empirical rule) states that for a normal distribution, nearly all values lie within 3 standard deviations of the mean.

Table 4-1 Expression (average Ct value) of reference genes in Study 1B samples

Sample identification	Genotype	Tissue type	Reference Genes Average Ct values				
			18s	Actb	Hmbs	Hprt1	Ppia
70.3/2	$APC^{Min/+}$	normal	16.001	16.484	25.980	23.852	21.837
76.4/1	$APC^{Min/+}$	normal	12.638	16.722	24.934	23.908	20.425
76.4/3	$APC^{Min/+}$	normal	12.530	16.513	25.250	23.635	20.266
44b.3/4	$APC^{Min/+}$	tumour	12.735	17.358	25.305	23.999	20.706
70.3/2	$APC^{Min/+}$	tumour	12.790	16.474	24.471	23.300	20.197
76.4/1	$APC^{Min/+}$	tumour	11.499	16.499	24.331	23.395	19.074
76.4/3	$APC^{Min/+}$	tumour	12.564	16.938	25.028	23.761	19.976
115.4/1	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	normal	11.829	16.712	24.617	23.360	19.699
11a.3/4	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	normal	12.038	15.895	24.704	23.262	19.948
121.1/2	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	normal	11.263	16.431	24.710	23.684	19.894
121.1/3	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	normal	11.103	15.953	24.596	23.257	19.663
121.1/5	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	normal	11.561	16.927	24.878	23.489	20.156
11a.3/4	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	tumour	11.464	15.973	24.484	23.215	19.194
121.1/2	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	tumour	12.723	16.315	24.767	23.643	20.100
121.1/3	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	tumour	12.687	17.391	25.159	24.044	20.089
121.1/5	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	tumour	11.964	16.972	24.934	23.929	19.941
Mean			12.337	16.597	24.884	23.608	20.073
Standard Deviation (SD)			1.139	0.451	0.407	0.288	0.624
Upper limit (Mean + 3SD)			15.755	17.951	26.104	24.471	21.946
Lower limit (Mean - 3SD)			8.919	15.244	23.664	22.746	18.199

The geometric mean[20] of the remaining four reference genes was calculated and used for normalisation of all average Ct values of target genes.

The mean relative quantity (RQ) of gene expression in $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples to expression in $APC^{Min/+}$ normal sample 76.4/1 was calculated from normalised average Ct values of Study 1B TaqMan® low density array results. SPSS 16.0.0.247 was used to perform univariate analysis of variance on the same data. Genes were determined as differentially expressed due to; the effect of PPARα status, or the effect of tissue, or the effect of an interaction between PPARα status and tissue, where $p <= 0.05$.

[20] Geometric mean: normalises ranges of numbers to give an average that indicates a central tendency or typical value. Numbers are multiplied together, then the *n*th root (where *n* is the count of numbers in the range) is taken of the resultant product.

These data were presented in groups (as defined previously, Table 3-15-Table 3-23) in Table 4-2-Table 4-10. Mean RQ data were presented as clustered bar graphs in similar groups (Figure 4-3-Figure 4-11) using a $\log_{10}$ scale for genes where RQ was greater than or equal to two (fold change increase >= 2) or less than or equal to 0.5 (fold change decrease >=2).

4.3.3 Ingenuity® Pathway Analysis (IPA®) of Taqman® low density array data

Data from Taqman® low density arrays were analysed on IPA to generate networks showing genes influenced by PPARα and tissue type.

Fold change (relative quantity of $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal to $APC^{Min/+}$ normal) and corresponding p values of significantly expressed genes (p <= 0.05) listed in Table 4-2-Table 4-10 were input into IPA to show the effect of PPARα status on gene expression. The generated network was annotated with the PPARα pathway.

Similarly, fold change (relative quantity of $APC^{Min/+}$ tumour to $APC^{Min/+}$ normal) and corresponding p values (p <= 0.009) from Table 4-2-Table 4-10 were input into IPA to show significantly expressed genes due to the effect of tissue. The generated network was annotated with the Wnt/beta catenin, p53 and ERK/MAPK signalling pathways.

4.3.4 Comparison of Gene Expression in Affymetrix® Microarrays & TaqMan® Low Density Arrays

To ascertain whether the two methodologies used to analyse the same sample set (Study 1B) produced similar results, gene expression results of the selected 95 genes (Table 3-15-Table 3-23) from Affymetrix® microarrays and Taqman® low density arrays were compared.
Relative quantity (RQ) to $APC^{Min/+}$ normal sample 76.4/1 was calculated for each selected gene from Affymetrix® microarray and Taqman® low

density array data. These data were presented on scatter plots and clustered bar charts.

4.4 Results

4.4.1 Gene expression on Taqman® low density arrays

For simplicity all table and figure headings have been abbreviated as follows; $APC^{Min/+}$ abbreviated to Min, and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ abbreviated to $PPAR\alpha^{-/-}$.

- **Fatty acid & lipid metabolism**

Table 4-2 and Figure 4-3 show expression data for genes implicated in fatty acid and lipid metabolism.

Hadha (hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit) expression was reduced in $APC^{Min/+}$ tumour samples only with a fold change reduction of 2.2 ($p = 0.01$).

Expression of Ptgs1 (prostaglandin-endoperoxide synthase 1) was reduced 2.5 fold (p is ns) in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples.

Echdc2 (enoyl Coenzyme A hydratase domain containing 2) and Steap4 (STEAP family member 4) expression increased in tumours with fold change increases of 4.2 and 3.0 in Echdc2 ($p = 0.012$) and 4.7 and 5.8 in Steap4 ($p = 0.002$) in $APC^{Min/+}$ tumour and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples respectively.

Expression of Alox15 (arachidonate 15-lipoxygenase) and Ptgs2 (prostaglandin-endoperoxide synthase 2) also increased with fold change increases of 4.3 and 2.0 in Alox15 (p is ns), and 5.1 and 6.5 in Ptgs2 ($p = 0.001$) in tumour samples as before.

Large increases in expression of Alox12 (arachidonate 12-lipoxygenase), Cyp2b10 (cytochrome P450, family 2, subfamily b,

polypeptide 10), Cyp11a1 (cytochrome P450, family 11, subfamily a, polypeptide 1) and Pla2g2a (phospholipase A2, group IIA) in tumours was observed, with fold change increases in expression of 36.3 and 29.7 in Alox12 ($p = 0.001$), 97.3 and 37.6 in Cyp2b10 ($p = 0.01$), 17.0 and 17.8 in Cyp11a1 ($p < 0.0001$), and 37.1 and 17.2 in Pla2g2a ($p < 0.0001$) in respective tumour samples as previously.

Expression of Angptl4 and Cyp2c55 was reduced in both normal and tumour $APC^{Min/+}PPAR\alpha^{-/-}$ samples; fold change reductions of 7.3 and 3.4 in Angptl4 (p is ns), and 2.0 and 10.7 in Cyp2c55 (p is ns) were recorded in $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples respectively.

There was significant interaction between PPARα status and the effects of tissue in the expression of Pla2g2a ($p = 0.002$).

Table 4-2 Genes implicated in fatty acid & lipid metabolism

Gene	Mean Relative Quantity of gene expression to Min normal			p value		
	Min tumour	PPARα-/- normal	PPARα-/- tumour	Effect of PPARα	Effect of tissue	Interaction between PPARα & tissue
Acot2	0.65	0.80	0.54	ns	0.027	ns
Alox12	36.27	1.54	29.74	ns	0.001	ns
Alox15	4.30	1.35	2.02	ns	ns	ns
Alox5ap	1.70	1.04	1.78	ns	0.002	ns
Angptl4	0.39	0.14	0.29	ns	ns	ns
Bdh1	0.40	0.62	0.35	ns	0.014	ns
Cyp11a1	17.03	1.68	17.78	ns	<0.0001	ns
Cyp2b10	97.33	0.91	37.63	ns	0.01	ns
Cyp2c55	0.04	0.50	0.09	ns	ns	ns
Decr1	0.41	0.70	0.45	ns	<0.0001	ns
Ech1	0.45	0.63	0.30	0.024	0.001	ns
Echdc2	4.24	0.78	3.00	ns	0.012	ns
Hadha	0.45	0.88	0.63	ns	0.01	ns
Lrp1	0.59	1.13	0.78	ns	0.001	ns
Pla2g2a	37.14	1.13	17.23	0.002	<0.0001	0.002
Ptgis	0.11	1.41	0.15	ns	0.01	ns
Ptgs1	0.55	0.53	0.40	ns	ns	ns
Ptgs2	5.15	0.97	6.50	ns	0.001	ns
Steap4	4.74	1.74	5.84	ns	0.002	ns

Mean relative quantity (RQ) of gene expression in $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples to expression in $APC^{Min/+}$ normal sample 76.4/1. Univariate analysis of variance of $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ groups was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

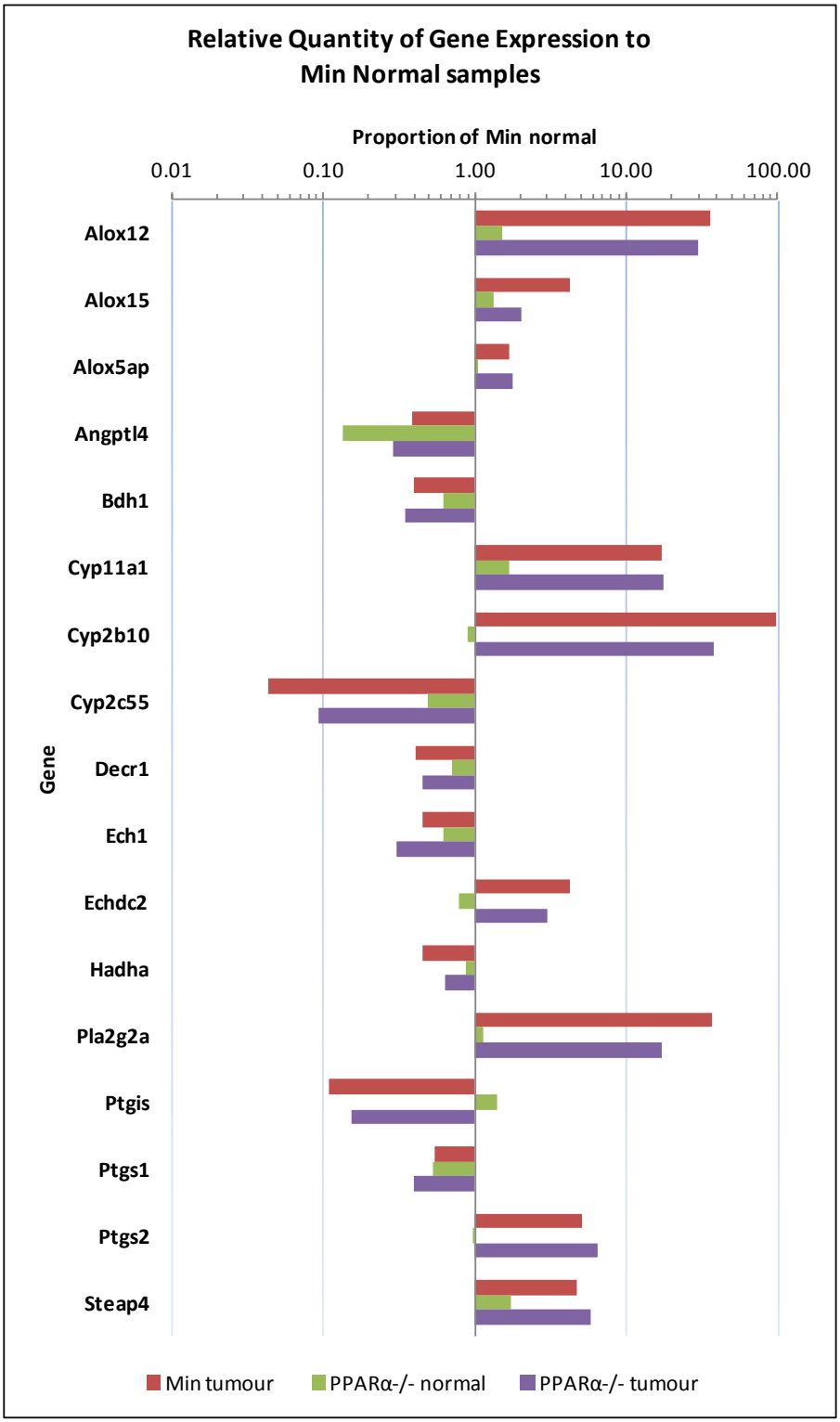


Figure 4-3 Genes implicated in fatty acid & lipid metabolism

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour groups to expression in $APC^{Min/+}$ normal, where RQ was >= 2 or <= to 0.5

- **Signal transduction**

Table 4-3 and Figure 4-4 show expression data for genes implicated in signal transduction.

Expression of Ptger3 (prostaglandin E receptor 3, subtype EP3) was reduced in tumours with fold change decreases of 6.1 in $APC^{Min/+}$ tumour samples and 5.5 in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples ($p < 0.0001$). Expression of Gulp1 (GULP, engulfment adaptor PTB domain containing 1) was reduced in $APC^{Min/+}$ tumour samples only with a fold change reduction of 2.8 (p is not significant, ns). However, expression of Ereg (epiregulin), Krt18 (keratin 18), Lama1 (laminin, alpha 1), Lama5 (laminin, alpha 5), Rhoj (ras homolog gene family, member J) and Tnf (tumour necrosis factor) were increased in tumours. Fold change increases in expression were 2.7 and 3.8 in Ereg ($p = 0.007$), 2.2 and 2.0 in Krt18 ($p < 0.0001$), 5.1 and 6.0 in Lama1 ($p < 0.0001$), 3.7 and 4.4 in Lama5 ($p = 0.001$), 3.2 and 2.3 in Rhoj ($p = 0.001$), and 3.7 and 2.5 in Tnf ($p = 0.002$) in $APC^{Min/+}$ tumour and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples respectively.

Large increases in expression in tumours were observed in Apcdd1 (adenomatosis polyposis coli down-regulated 1), Il1b (interleukin 1 beta), Il6 (interleukin 6), Inhba (inhibin beta-A) and Ppbp (pro-platelet basic protein). Fold change increases of 18.0 and 10.0 in Apcdd1 ($p = 0.007$), 6.7 and 15.9 in Il1b ($p = 0.001$), 7.9 and 19.9 in Il6 ($p < 0.0001$), 12.2 and 20.5 in Inhba ($p < 0.0001$)) and 10.2 and 7.8 in Ppbp ($p = 0.003$) were recorded in $APC^{Min/+}$ tumour and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples respectively. Expression of Tnf was reduced 2.7 fold in $APC^{Min/+}PPAR\alpha^{-/-}$ normal samples and increased 2.5 fold in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples (p is ns). There was also a fold change decrease of 2.4 in Apcdd1 expression in $APC^{Min/+}PPAR\alpha^{-/-}$ normal samples, whilst in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples there was a fold change increase of 10.0 (p is ns). There was significant interaction between PPARα status and tissue type in the expression of Il6 ($p = 0.022$).

Table 4-3 Genes implicated in signal transduction

Gene	Mean Relative Quantity of gene expression to Min normal			p value		
	Min tumour	PPARα-/- normal	PPARα-/- tumour	Effect of PPARα	Effect of tissue	Interaction between PPARα & tissue
Apcdd1	17.95	0.42	9.96	ns	0.007	ns
Ereg	2.65	1.24	3.83	ns	0.007	ns
Ghr	0.69	0.85	0.67	ns	0.012	ns
Gulp1	0.36	0.77	0.68	ns	ns	ns
Il1b	6.75	0.74	15.86	ns	0.001	ns
Il6	7.88	1.40	19.92	0.016	<0.0001	0.022
Inhba	12.17	2.08	20.51	ns	<0.0001	ns
Krt18	2.23	1.25	1.99	ns	<0.0001	ns
Lama1	5.07	0.85	6.01	ns	<0.0001	ns
Lama5	3.68	1.07	4.43	ns	0.001	ns
Ppbp	10.21	1.10	7.77	ns	0.003	ns
Ptger3	0.16	0.71	0.18	ns	<0.0001	ns
Rhoj	3.17	1.05	2.26	ns	0.001	ns
Sfrp1	0.98	1.25	1.45	ns	ns	ns
Tnf	3.71	0.37	2.47	ns	0.002	ns

Mean relative quantity (RQ) of gene expression in $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples to expression in $APC^{Min/+}$ normal sample 76.4/1. Univariate analysis of variance of $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ groups was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

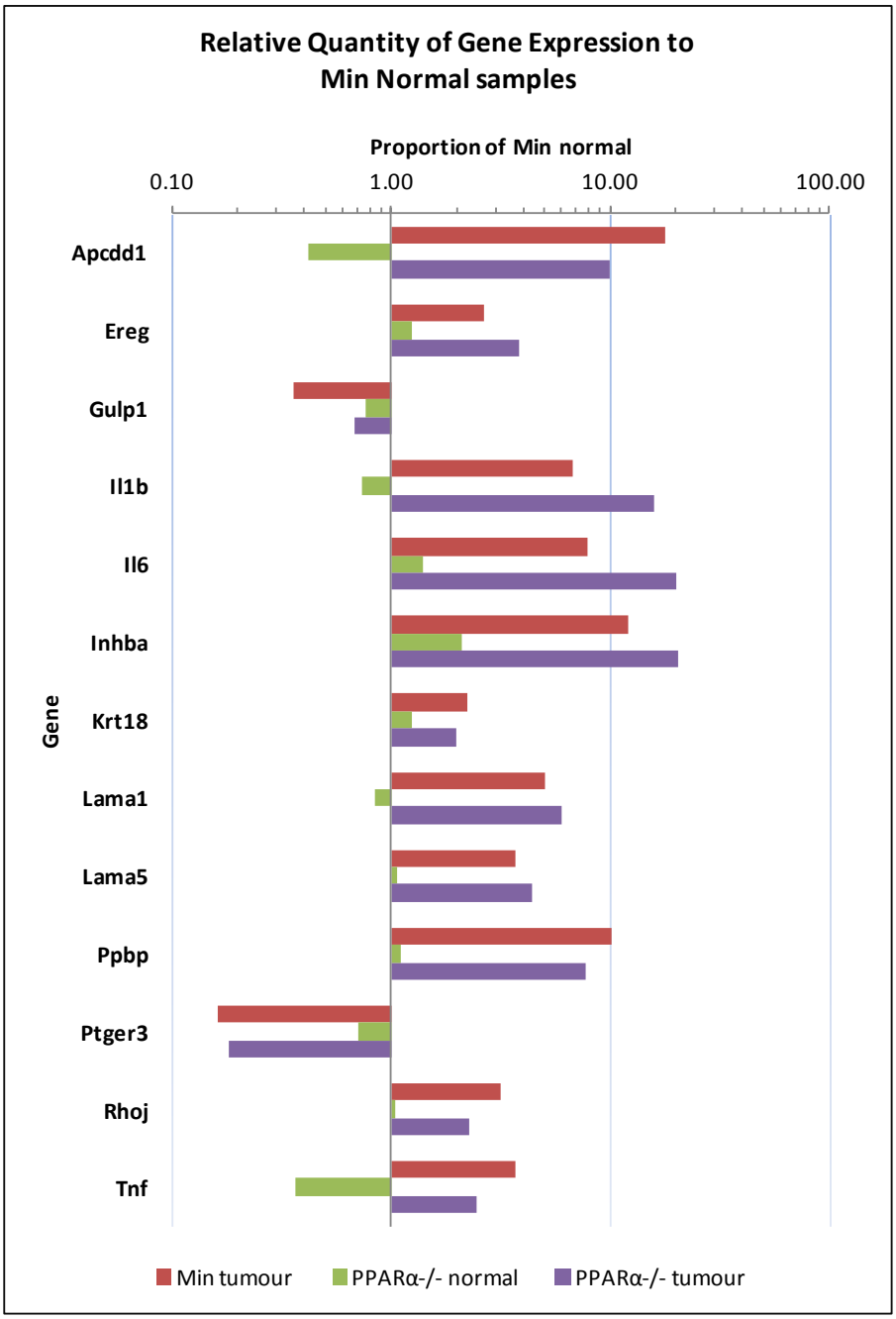


Figure 4-4 Genes implicated in signal transduction

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour groups to expression in $APC^{Min/+}$ normal, where RQ was >= 2 or <= to 0.5

- **Transcription**

Table 4-4 and Figure 4-5 show expression data for genes implicated in transcription.

Expression decreased in Gucy2c (guanylate cyclase 2c), Meis1 (Meis homeobox 1), Nr1d1 (nuclear receptor subfamily 1, group D), Id4 (inhibitor of DNA binding 4), Klf4 (Kruppel-like factor 4, gut) and Rorc (RAR-related orphan receptor gammaRAR-related orphan receptor gamma); fold change decreases of 2.5 and 2.9 in expression of Gucy2c ($p < 0.0001$), 2.2 and 3.5 in Meis1 ($p = 0.029$), 3.4 and 3.5 in Nr1d1 ($p = 0.001$), 4.1 and 4.5 in Id4 ($p = 0.001$), 4.1 and 6.1 in Klf4 ($p < 0.0001$), and 5.4 and 7.1 in Rorc ($p < 0.0001$) were recorded in $APC^{Min/+}$ tumour and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples respectively.

Expression of Ang (angiogenin), Cbx7 (chromobox homolog 7) and Pou2af1 (POU domain, class 2, associating factor 1) decreased in $APC^{Min/+}PPARa^{-/-}$ tumour samples only with fold change reductions of 4.5 in Ang (p is ns), 2.2 in Cbx7 (p is ns) and 2.5 in Pou2af1 (p is ns) respectively.

Expression of Myc (myelocytomatosis oncogene), Rbms1 (RNA binding motif, single stranded interacting protein 1), Arnt2 (aryl hydrocarbon receptor nuclear translocator 2), Foxc2 (forkhead box C2) and Rarb (retinoic acid receptor, beta) increased in tumours. Fold change increases of 2.4 and 2.3 in Myc ($p < 0.0001$), 2.1 and 2.4 in Rbms1 ($p = 0.009$), 3.5 and 6.8 in Arnt2 ($p < 0.0001$), 5.3 and 2.8 in Foxc2 ($p = 0.006$), and 3.4 and 3.5 in Rarb ($p = 0.019$) were observed in $APC^{Min/+}$ tumour and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples respectively.

A massive increase of Onecut2 expression ($p < 0.0001$) in tumours was seen with fold change increases of 145.8 in $APC^{Min/+}$ tumour and 196.8 in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples.

Expression of Tcf12 (transcription factor 12) increased in $APC^{Min/+}$ tumour samples only with a fold change increase of 2.5 ($p = 0.003$). Expression of Arnt2 was reduced 2.1 fold in $APC^{Min/+}PPAR\alpha^{-/-}$ normal

samples but increased 6.8 fold in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples (p is ns).

A similar pattern was observed for expression of Rarb; expression in $APC^{Min/+}PPAR\alpha^{-/-}$ normal samples decreased 2.3 fold, whereas expression increased 3.5 fold in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples (p is ns).

Table 4-4 Genes implicated in transcription

Gene	Mean Relative Quantity of gene expression to Min normal			p value		
	Min tumour	PPARα-/- normal	PPARα-/- tumour	Effect of PPARα	Effect of tissue	Interaction between PPARα & tissue
Ang	0.78	0.61	0.22	0.014	ns	ns
Arnt2	3.49	0.48	6.75	ns	<0.0001	ns
Baz1a	1.37	0.76	1.46	ns	0.007	ns
Cbx7	1.45	0.61	0.45	0.002	ns	ns
Chd8	1.01	0.85	0.82	ns	ns	ns
Crem	1.65	1.03	1.02	ns	ns	ns
Foxc2	5.27	0.65	2.83	ns	0.006	ns
Gucy2c	0.40	0.70	0.35	ns	<0.0001	ns
Id4	0.24	1.20	0.22	ns	0.001	ns
Jun	1.49	0.61	1.36	ns	0.004	ns
Klf4	0.24	0.64	0.16	0.024	<0.0001	ns
Meis1	0.45	1.28	0.29	ns	0.029	ns
Myc	2.44	0.77	2.30	ns	<0.0001	ns
Nfkbie	1.61	0.90	1.80	ns	0.014	ns
Nr1d1	0.30	0.73	0.28	ns	0.001	ns
Onecut2	**145.82**	**1.01**	**196.83**	ns	**<0.0001**	ns
Phf17	0.58	0.88	0.56	ns	0.01	ns
Pou2af1	0.56	0.52	0.40	ns	ns	ns
Rarb	3.35	0.44	3.50	ns	0.019	ns
Rbms1	2.14	1.54	2.44	ns	0.009	ns
Rorc	0.19	0.55	0.14	0.021	<0.0001	ns
Tcf12	2.48	0.76	1.48	ns	0.003	ns

Mean relative quantity (RQ) of gene expression in $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples to expression in $APC^{Min/+}$ normal sample 76.4/1. Univariate analysis of variance of $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ groups was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

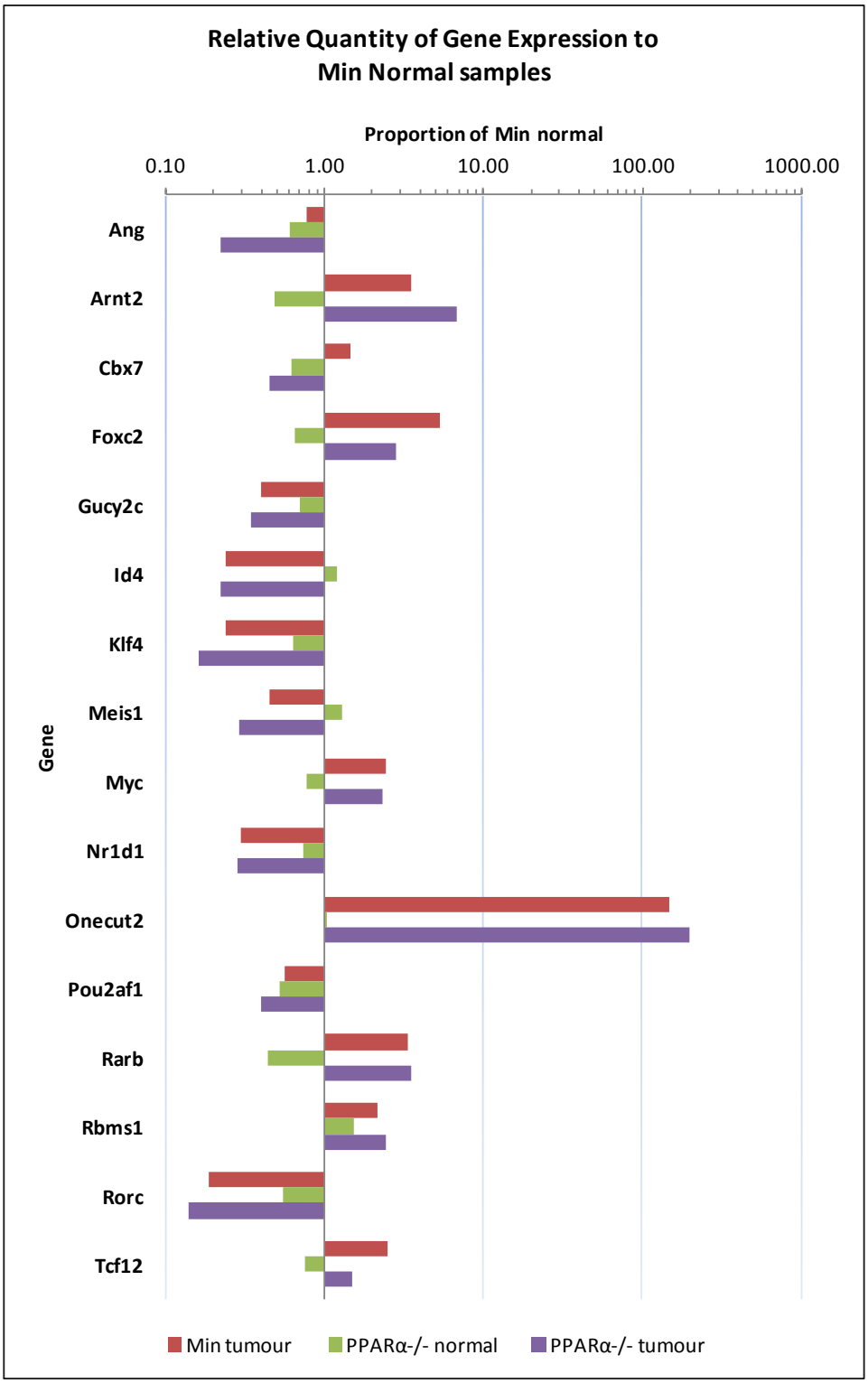


Figure 4-5 Genes implicated in transcription

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour groups to expression in $APC^{Min/+}$ normal, where RQ was >= 2 or <= to 0.5

- **Cell cycle**

Table 4-5 and Figure 4-6 show expression data for genes implicated in the cell cycle.

Expression of Bin1 (bridging integrator 1) decreased in tumours ($p = 0.003$) with fold change decreases of 2.4 in $APC^{Min/+}$ tumour samples and 7.7 in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples.

However, expression of Apobec3 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3), Ccnd1 (cyclin D1) and Cdkn1c (cyclin-dependent kinase inhibitor 1C, P57) increased in tumours. Fold change increases of 2.1 and 128.7 in Apobec3 ($p = 0.035$), 3.0 and 2.7 in Ccnd1 ($p < 0.0001$), and 2.2 and 2.1 in Cdkn1c ($p = 0.001$) were observed in $APC^{Min/+}$ tumour and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour groups respectively.

Expression of Cdkn1c decreased 2.1 fold in $APC^{Min/+}PPAR\alpha^{-/-}$ normal samples and increased 2.1 fold in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples (p is not significant).

There was a very large increase in expression of Apobec3 ($p < 0.0001$) in $APC^{Min/+}PPAR\alpha^{-/-}$ normal samples and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples with fold change increases of 77.9 and 128.7 respectively.

There was also significant interaction between PPARα status and tissue type in the level of expression of Apobec3 ($p = 0.042$).

Table 4-5 Genes implicated in the cell cycle

Gene	Mean Relative Quantity of gene expression to Min normal			p value		
	Min tumour	PPARα-/- normal	PPARα-/- tumour	Effect of PPARα	Effect of tissue	Interaction between PPARα & tissue
Apobec3	2.07	77.89	128.68	<0.0001	0.035	0.042
Bin1	0.42	0.87	0.13	ns	0.003	ns
Ccnd1	2.97	1.22	2.65	ns	<0.0001	ns
Cdkn1c	2.22	0.47	2.08	ns	0.001	ns

Mean relative quantity (RQ) of gene expression in $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples to expression in $APC^{Min/+}$ normal sample 76.4/1. Univariate analysis of variance of $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ groups was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

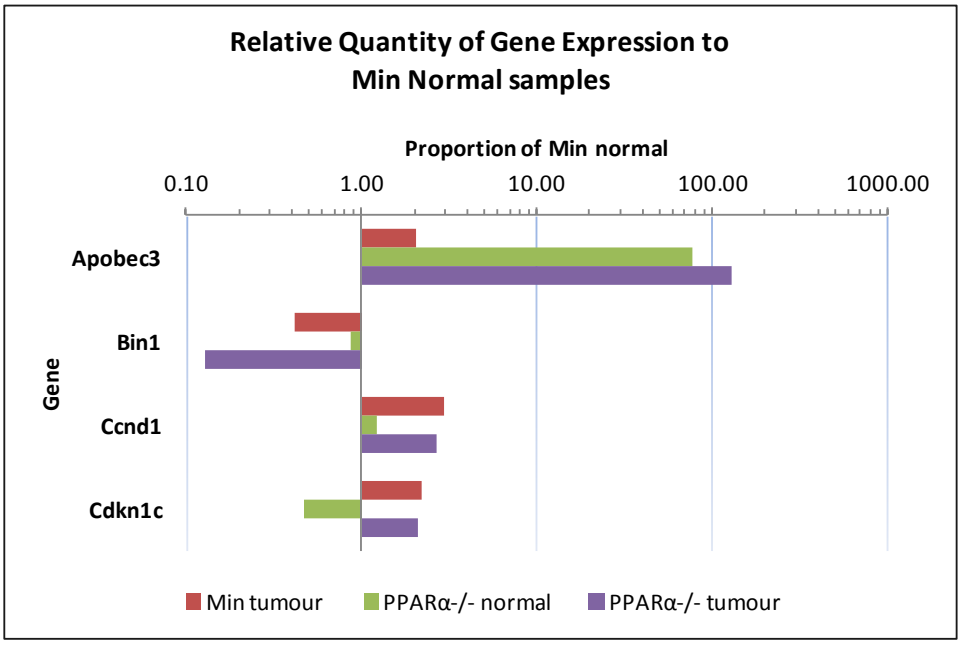


Figure 4-6 Genes implicated in the cell cycle

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour groups to expression in $APC^{Min/+}$ normal, where RQ was >= 2 or <= to 0.5

- **Apoptosis**

Table 4-6 and Figure 4-7 show expression data for genes implicated in apoptosis.

Expression of Endod1 (endonuclease domain containing 1) decreased in tumours ($p < 0.0001$) with fold change decreases of 3.2 in $APC^{Min/+}$ tumour samples and 4.0 in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples.

Expression of Bcl2l11 (BCL2-like 11, apoptosis facilitator) and Bmf (BCL2 modifying factor) increased in tumours. Bcl2l11 ($p < 0.0001$) and Bmf ($p = 0.005$) had fold change increases of 2.8 and 3.2, and 4.1 and 3.6, in $APC^{Min/+}$ tumour and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour groups respectively. Khdc1a (KH domain containing 1A) had decreased expression in $APC^{Min/+}PPAR\alpha^{-/-}$ samples. Fold change decreases of 4.0 in $APC^{Min/+}PPAR\alpha^{-/-}$ normal and 3.4 in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples were observed ($p = 0.005$). Bik (BCL2-interacting killer) had a 2.2 fold decrease in expression in $APC^{Min/+}PPAR\alpha^{-/-}$ normal samples only ($p = 0.001$)

Table 4-6 Genes implicated in apoptosis

Gene	Mean Relative Quantity of gene expression to Min normal			p value		
	Min tumour	PPARα-/- normal	PPARα-/- tumour	Effect of PPARα	Effect of tissue	Interaction between PPARα & tissue
Apaf1	1.89	0.86	1.51	ns	<0.0001	ns
Bcl2l11	2.80	0.88	3.20	ns	<0.0001	ns
Bik	1.82	0.46	0.72	0.001	0.016	ns
Bmf	4.13	1.08	3.60	ns	0.005	ns
Endod1	0.31	0.81	0.25	ns	<0.0001	ns
Khdc1a	1.91	0.25	0.29	0.005	ns	ns
Sbk1	1.85	0.98	1.70	ns	0.011	ns

Mean relative quantity (RQ) of gene expression in $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples to expression in $APC^{Min/+}$ normal sample 76.4/1. Univariate analysis of variance of $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ groups was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

Figure 4-7 Genes implicated in apoptosis

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour groups to expression in $APC^{Min/+}$ normal, where RQ was >= 2 or <= to 0.5

- **Protein transport**

Table 4-7 and Figure 4-8 show expression data for genes implicated in protein transport.

Expression of Ap1s3 (adaptor-related protein complex AP-1, sigma 3) and Crip1 (cysteine-rich protein 1, intestinal) decreased in tumours. Fold change decreases of 2.2 and 3.0 in Ap1s3 ($p < 0.0001$) and 2.2 and 2.3 in Crip1 ($p = 0.001$) in $APC^{Min/+}$ tumour samples and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples respectively, were recorded. Large increases in Alb (albumin) expression in tumours were observed with fold change increases of 28.0 in $APC^{Min/+}$ tumour samples and 7.7 in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples ($p = 0.003$).

Table 4-7 Genes implicated in protein transport

Gene	Mean Relative Quantity of gene expression to Min normal			p value		
	Min tumour	PPARα-/- normal	PPARα-/- tumour	Effect of PPARα	Effect of tissue	Interaction between PPARα & tissue
Abcc4	1.49	0.92	1.22	ns	ns	ns
Alb	28.02	1.17	7.65	0.046	0.003	0.043
Ap1s3	0.46	1.01	0.34	ns	<0.0001	ns
Crip1	0.46	1.39	0.44	ns	0.001	ns

Mean relative quantity (RQ) of gene expression in $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples to expression in $APC^{Min/+}$ normal sample 76.4/1. Univariate analysis of variance of $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ groups was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

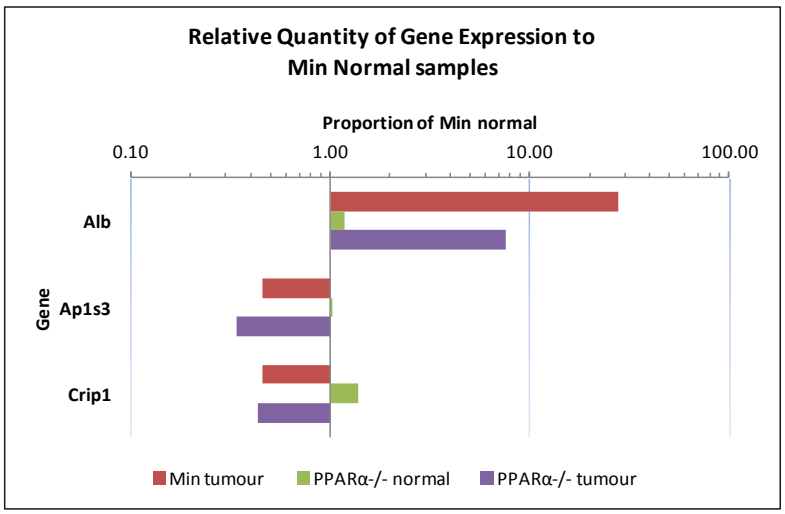


Figure 4-8 Genes implicated in protein transport

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour groups to expression in $APC^{Min/+}$ normal, where RQ was >= 2 or <= to 0.5

- **Protein metabolism & modification**

Table 4-8 and Figure 4-9 show expression data for genes implicated in protein metabolism and modification.

Expression of Pdk2 (pyruvate dehydrogenase kinase, isoenzyme 2) and Mep1b (meprin 1 beta) decreased in tumours with fold change decreases of 3.7 and 3.9 in Pdk2 ($p < 0.0001$), and 5.5 and 2.8 in Mep1b ($p = 0.003$) in $APC^{Min/+}$ tumour and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples respectively.

Expression of Akt3 (thymoma viral proto-oncogene 3) and Irak4 (interleukin-1 receptor-associated kinase 4) decreased in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples only with fold change decreases of 2.8 in Akt3 ($p = 0.042$) and 2.2 in Irak4 ($p = 0.021$).

Expression of Casp6 (caspase 6), Cdk4 (cyclin-dependent kinase 4), Htra1 (HtrA serine peptidase 1), Timp1 (tissue inhibitor of metalloproteinase 1), Mmp7 (matrix metallopeptidase 7) and Plat (plasminogen activator, tissue) increased in tumours. Fold change increases were 2.9 and 2.5 in Casp6 ($p = 0.001$), 2.3 and 2.1 in Cdk4 ($p < 0.0001$), 4.6 and 2.6 in Htra1 ($p = 0.014$), and 4.9 and 5.1 in Timp1 ($p = 0.019$), and 21.0 and 20.8 in Plat ($p = 0.001$) in $APC^{Min/+}$ tumour and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples respectively.

There was a large increase in expression of Mmp7 ($p < 0.0001$); fold change increases of 185.4 in $APC^{Min/+}$ tumour samples and 146.4 in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples were observed. Timp1 expression increased 2.4 fold in $APC^{Min/+}PPAR\alpha^{-/-}$ normal samples and 5.1 fold in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples (p is ns). However, expression of Irak4 decreased approximately 2-fold in both $APC^{Min/+}PPAR\alpha^{-/-}$ normal and tumour samples ($p < 0.0001$).

Table 4-8 Genes implicated in protein metabolism & modification

Gene	Mean Relative Quantity of gene expression to Min normal			p value		
	Min tumour	PPARα-/- normal	PPARα-/- tumour	Effect of PPARα	Effect of tissue	Interaction between PPARα & tissue
Akt3	0.55	0.99	0.36	ns	0.042	ns
B4galt6	1.45	0.74	1.65	ns	0.001	ns
Casp6	2.85	0.90	2.47	ns	0.001	ns
Cdk4	2.29	0.90	2.14	ns	<0.0001	ns
Htra1	4.56	0.98	2.61	ns	0.014	ns
Irak4	0.78	0.54	0.46	<0.0001	0.021	ns
Mep1b	0.18	0.94	0.35	ns	0.003	ns
Mmp2	1.57	1.18	1.58	ns	ns	ns
Mmp7	185.40	1.38	146.44	ns	<0.0001	ns
Pdk2	0.27	0.72	0.26	ns	<0.0001	ns
Plat	21.02	1.66	20.81	ns	0.001	ns
Ptprg	1.27	0.69	0.89	0.01	ns	ns
Timp1	4.89	2.38	5.06	ns	0.019	ns

Mean relative quantity (RQ) of gene expression in $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples to expression in $APC^{Min/+}$ normal sample 76.4/1. Univariate analysis of variance of $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ groups was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

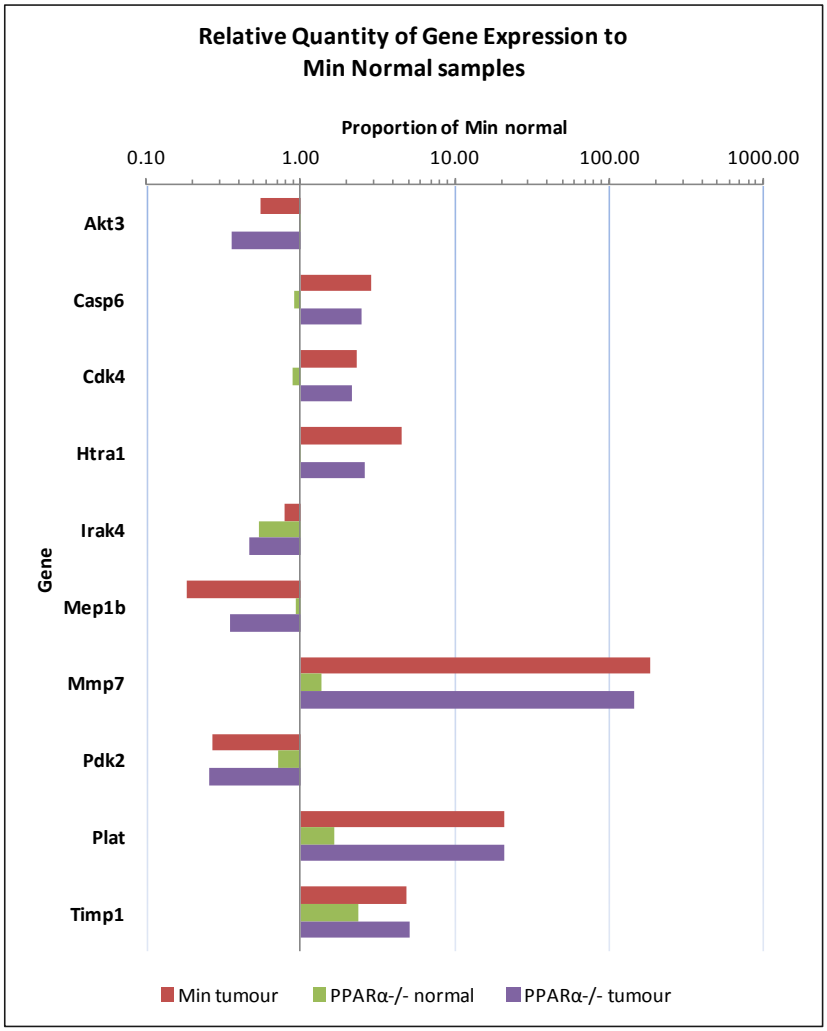


Figure 4-9 Genes implicated in protein metabolism & modification

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour groups to expression in $APC^{Min/+}$ normal, where RQ was >= 2 or <= to 0.5

- **Cell adhesion**

Table 4-9 and Figure 4-10 show expression data for genes implicated in cell adhesion.

Expression of Sell (selectin) decreased 2.9 fold in $APC^{Min/+}$ tumour samples and approximately 2-fold in $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples (p is ns). Expression of Itga6 (integrin alpha 6) increased 2.1 fold in $APC^{Min/+}$ tumour samples only (p = 0.001). Sell expression also decreased in $APC^{Min/+}PPAR\alpha^{-/-}$ normal samples with a fold change decrease of 4.2 (p is ns).

Table 4-9 Genes implicated in cell adhesion

Gene	Mean Relative Quantity of gene expression to Min normal			p value		
	Min tumour	PPARα-/- normal	PPARα-/- tumour	Effect of PPARα	Effect of tissue	Interaction between PPARα & tissue
Ctgf	1.29	1.10	1.13	ns	ns	ns
Itga6	2.09	1.23	1.83	ns	0.001	ns
Sell	0.34	0.24	0.53	ns	ns	ns

Mean relative quantity (RQ) of gene expression in $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples to expression in $APC^{Min/+}$ normal sample 76.4/1. Univariate analysis of variance of $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ groups was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

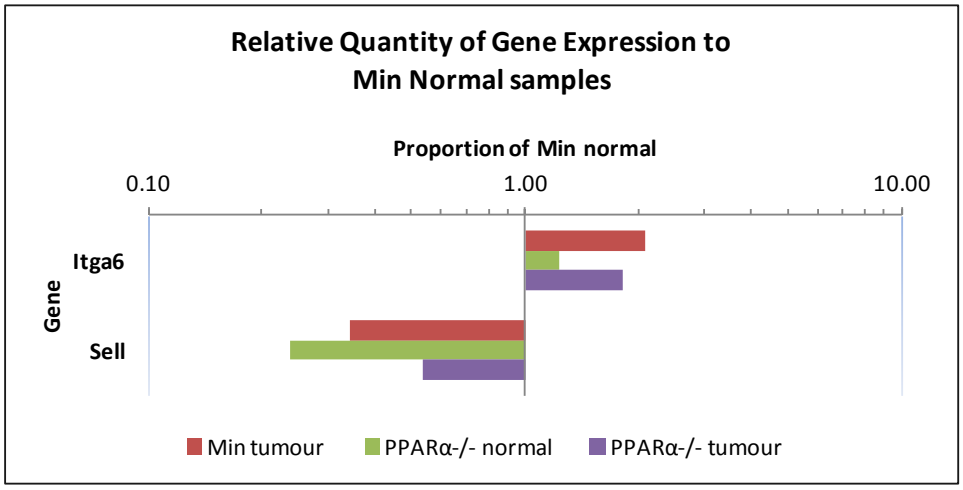


Figure 4-10 Genes implicated in cell adhesion

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour groups to expression in $APC^{Min/+}$ normal, where RQ was >= 2 or <= to 0.5

- **Various cellular functions**

Table 4-10 and Figure 4-11 show expression of genes implicated in toxin metabolism, amino acid metabolism and cellular structure.

Expression of Aoc3 (amine oxidase, copper containing 3), Cbr1 (carbonyl reductase 1) and Cryab (crystallin, alpha B) decreased in tumours with fold change decreases of 3.4 and 2.5 in Aoc3 ($p = 0.013$), 3.5 and 3.6 in Cbr1 ($p < 0.0001$), and 3.2 and 3.3 in Cryab ($p < 0.0001$) in $APC^{Min/+}$ tumour and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples respectively.

Expression of Nisch (nischarin) was slightly elevated in tumour samples ($p = 0.009$).

Expression of genes in this group did not appear to be affected by PPARα.

Table 4-10 Genes involved in various cellular functions

Gene	Mean Relative Quantity of gene expression to Min normal			p value		
	Min tumour	PPARα-/- normal	PPARα-/- tumour	Effect of PPARα	Effect of tissue	Interaction between PPARα & tissue
Aoc3	0.29	1.12	0.40	ns	0.013	ns
Cbr1	0.29	0.72	0.28	ns	<0.0001	ns
Cryab	0.31	0.95	0.30	ns	<0.0001	ns
Nisch	1.24	0.86	1.26	ns	0.009	ns

Mean relative quantity (RQ) of gene expression in $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour samples to expression in $APC^{Min/+}$ normal sample 76.4/1. Univariate analysis of variance of $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ groups was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

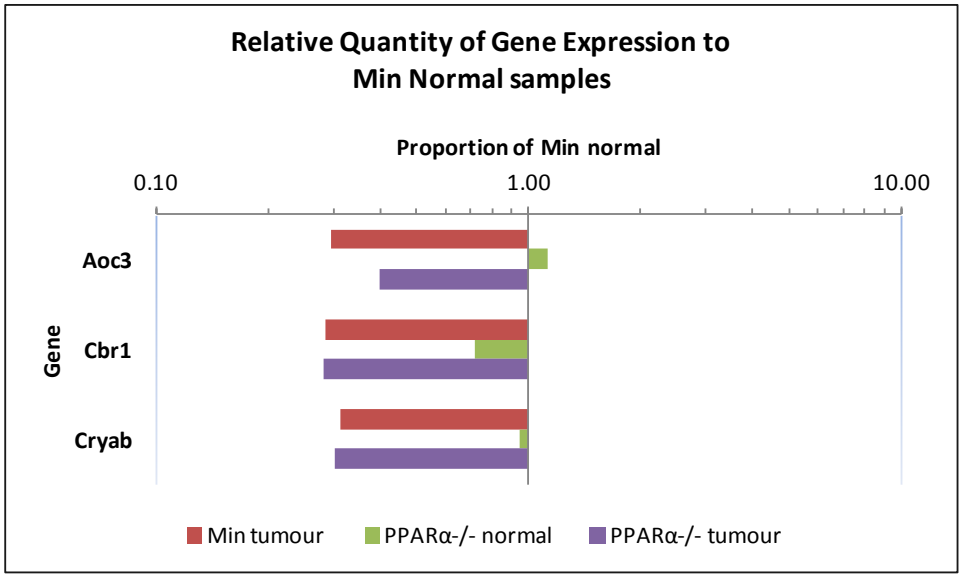


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Figure 4-11 Genes involved in various cellular functions

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour groups to expression in $APC^{Min/+}$ normal, where RQ was >= 2 or <= to 0.5

4.4.2 Ingenuity® Pathway Analysis (IPA®) networks of Taqman® low density array data

Figure 4-12 and Figure 4-13 show IPA networks of Taqman® low density array data for genes where expression may be influenced by PPARα and tissue type respectively.

Figure 4-12 was annotated with the PPARα signalling pathway and shows Il6 (interleukin 6) was a central regulatory gene in this network. This corresponds with IPA networks of Affymetrix® microarray data (Figure 3-15 and Figure 3-18) which indicate a similar regulatory role for Il6.

Figure 4-13 was annotated with the Wnt/beta catenin, p53 and ERK/MAPK signalling pathways. These pathways targeted similar genes to IPA networks of Affymetrix® microarray data as shown previously.

The Wnt/β catenin and ERK/MAPK signalling pathways were associated with Myc (myelocytomatosis oncogene) gene expression as previously demonstrated. Similarly, p53 signalling targeted Casp6 (caspase 6) as shown before (Figure 3-10 and Figure 3-14).

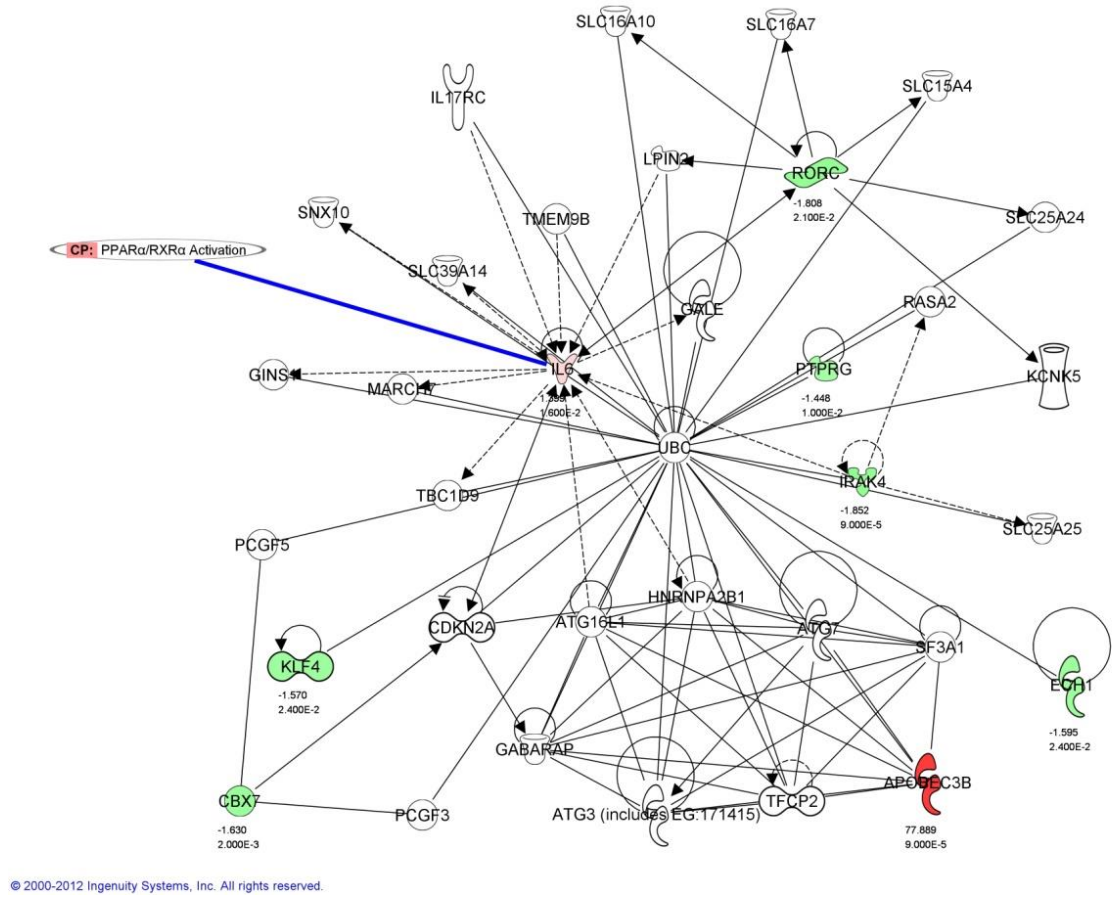

Figure 4-12 IPA network of significantly expressed genes (p <= 0.05) affected by PPARα

Network is annotated with the PPARα/RXRα pathway. Network p value, p = 1.09E-05

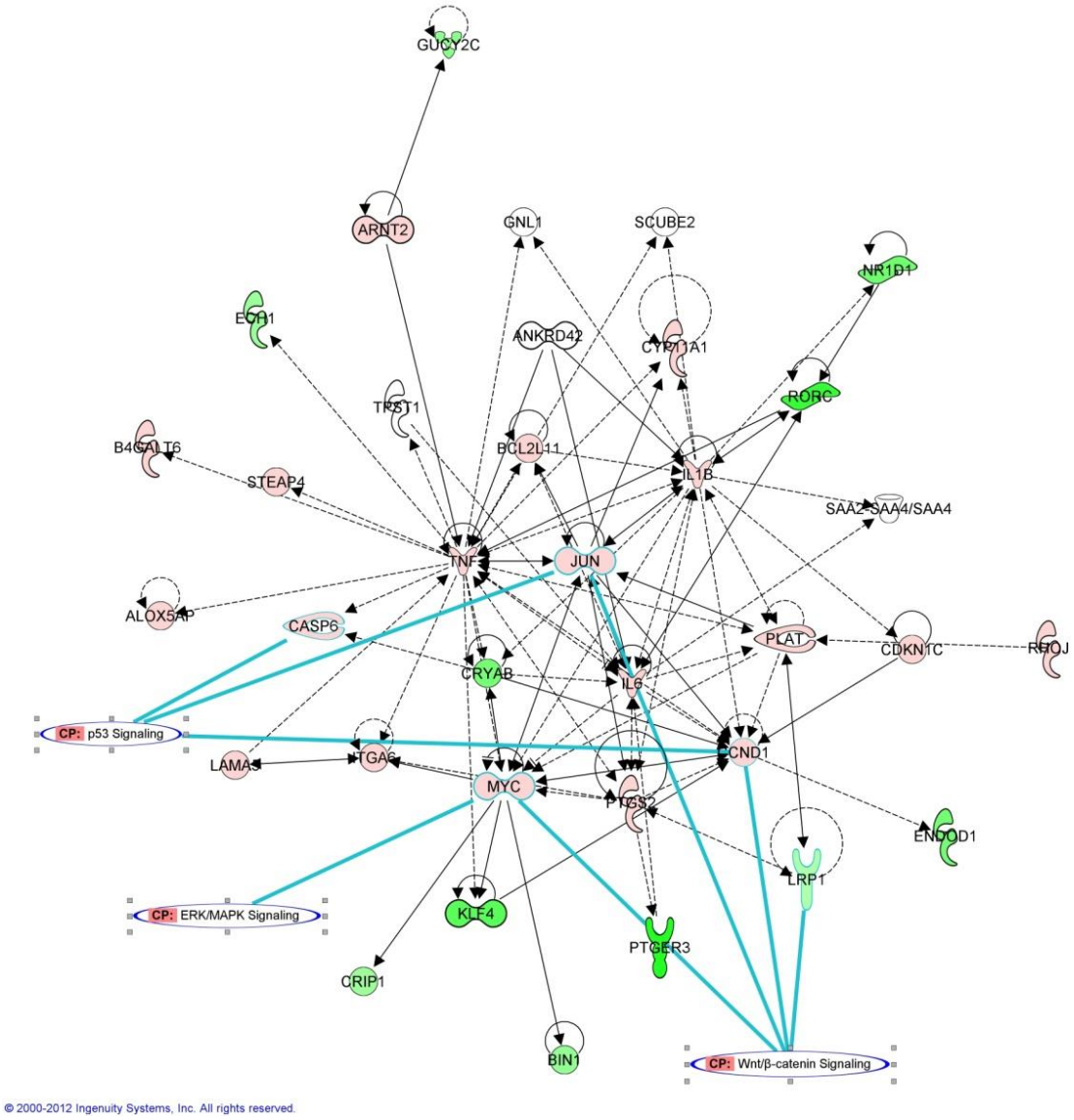

Figure 4-13 IPA network of significantly expressed genes (p <= 0.009) affected by tissue type

Network is annotated with the Wnt/beta – catenin, p53 and ERK/MAPK signalling pathways. Network p value, p = 4.42E-09

4.4.3 Comparison of relative quantity of gene expression in Taqman® low density arrays & Affymetrix® microarrays

Pairs of Relative Quantity (RQ) values from Taqman® low density arrays *vs.* Affymetrix® Microarrays (Table 4-11a-c) were compared on scatter plots and clustered bar charts.

$APC^{Min/+}$ tumour *vs.* $APC^{Min/+}$ tumour was shown in Figure 4-14 and Figure 4-17.

$APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal was shown in Figure 4-15 and Figure 4-18.

$APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour was shown in Figure 4-16 and Figure 4-19.

Table 4-11 Comparison of relative quantity (to APC$^{Min/+}$ normal) in Taqman® low density arrays & Affymetrix® microarrays (a)

Taqman arrays Relative Quantity to APC$^{Min/+}$ normal (76.4/1)				Microarrays Relative Quantity to APC$^{Min/+}$ normal (76.4/1)			
GENE	Min tumour	PPARα$^{-/-}$ normal	PPARα$^{-/-}$ tumour	GENE	Min tumour	PPARα$^{-/-}$ normal	PPARα$^{-/-}$ tumour
Abcc4	1.49	0.92	1.22	Abcc4	3.74	0.75	3.07
Acot2	0.65	0.80	0.54	Acot2	0.70	0.68	0.71
Akt3	0.55	0.99	0.36	Akt3	0.69	1.23	0.81
Alb	28.02	1.17	7.65	Alb	19.19	1.23	7.04
Alox12	36.27	1.54	29.74	Alox12	17.05	0.98	8.74
Alox15	4.30	1.35	2.02	Alox15	2.78	1.33	1.69
Alox5ap	1.70	1.04	1.78	Alox5ap	1.45	1.16	2.00
Ang	0.78	0.61	0.22	Ang	0.79	0.26	0.17
Angptl4	0.39	0.14	0.29	Angptl4	1.22	0.91	1.59
Aoc3	0.29	1.12	0.40	Aoc3	0.19	0.71	0.24
Ap1s3	0.46	1.01	0.34	Ap1s3	0.54	0.79	0.48
Apaf1	1.89	0.86	1.51	Apaf1	2.20	1.30	2.25
Apcdd1	17.95	0.42	9.96	Apcdd1	22.48	0.92	7.51
Apobec3	**2.07**	**77.89**	**128.68**	**Apobec3**	**3.19**	**0.57**	**0.91**
Arnt2	3.49	0.48	6.75	Arnt2	1.52	1.00	2.51
B4galt6	1.45	0.74	1.65	B4galt6	1.58	0.87	1.73
Baz1a	1.37	0.76	1.46	Baz1a	1.82	1.01	2.09
Bcl2l11	2.80	0.88	3.20	Bcl2l11	2.72	0.97	2.59
Bdh1	0.40	0.62	0.35	Bdh1	0.36	0.88	0.39
Bik	1.82	0.46	0.72	Bik	1.19	0.66	0.73
Bin1	0.42	0.87	0.13	Bin1	0.82	0.99	0.84
Bmf	4.13	1.08	3.60	Bmf	1.59	0.94	1.45
Casp6	2.85	0.90	2.47	Casp6	2.60	0.83	2.09
Cbr1	0.29	0.72	0.28	Cbr1	0.35	0.78	0.35
Cbx7	1.45	0.61	0.45	Cbx7	0.86	0.68	0.60
Ccnd1	2.97	1.22	2.65	Ccnd1	2.79	1.22	2.44
Cdk4	2.29	0.90	2.14	Cdk4	2.64	1.06	2.20
Cdkn1c	2.22	0.47	2.08	Cdkn1c	2.55	0.57	1.76
Chd8	1.01	0.85	0.82	Chd8	1.03	0.89	0.93
Crem	1.65	1.03	1.02	Crem	1.75	1.04	1.97
Crip1	0.46	1.39	0.44	Crip1	0.57	1.26	0.53
Cryab	0.31	0.95	0.30	Cryab	0.57	1.09	0.58
Ctgf	1.29	1.10	1.13	Ctgf	1.78	0.96	1.69
Cyp11a1	17.03	1.68	17.78	Cyp11a1	2.18	1.01	2.59
Cyp2b10	97.33	0.91	37.63	Cyp2b10	3.43	0.80	1.59
Cyp2c55	0.04	0.50	0.09	Cyp2c55	0.04	0.30	0.10
Decr1	0.41	0.70	0.45	Decr1	0.54	0.94	0.66
Ech1	0.45	0.63	0.30	Ech1	0.43	0.80	0.48
Echdc2	4.24	0.78	3.00	Echdc2	3.26	0.92	2.37

Relative quantity of gene expression in APC$^{Min/+}$ tumour, APC$^{Min/+}$ PPARα$^{-/-}$ normal and APC$^{Min/+}$ PPARα$^{-/-}$ tumour groups to APC$^{Min/+}$ normal; Affymetrix® microarrays and Taqman® low density arrays

Comparison of relative quantity (to $APC^{Min/+}$ normal) in Taqman® low density arrays & Affymetrix® microarrays (b)

Taqman arrays Relative Quantity to $APC^{Min/+}$ normal (76.4/1)				Microarrays Relative Quantity to $APC^{Min/+}$ normal (76.4/1)			
GENE	Min tumour	$PPAR\alpha^{-/-}$ normal	$PPAR\alpha^{-/-}$ tumour	GENE	Min tumour	$PPAR\alpha^{-/-}$ normal	$PPAR\alpha^{-/-}$ tumour
Endod1	0.31	0.81	0.25	Endod1	0.45	0.99	0.59
Ereg	2.65	1.24	3.83	Ereg	2.79	1.33	6.08
Foxc2	5.27	0.65	2.83	Foxc2	3.97	0.94	2.27
Ghr	0.69	0.85	0.67	Ghr	0.68	0.87	0.87
Gucy2c	0.40	0.70	0.35	Gucy2c	0.45	0.80	0.49
Gulp1	0.36	0.77	0.68	Gulp1	0.54	0.91	0.83
Hadha	0.45	0.88	0.63	Hadha	0.53	0.84	0.60
Htra1	4.56	0.98	2.61	Htra1	4.01	0.96	3.14
Id4	0.24	1.20	0.22	Id4	0.28	1.20	0.35
Il1b	6.75	0.74	15.86	Il1b	5.71	1.09	12.77
Il6	7.88	1.40	19.92	Il6	3.95	0.96	17.16
Inhba	12.17	2.08	20.51	Inhba	3.47	1.38	5.12
Irak4	0.78	0.54	0.46	Irak4	0.95	0.53	0.50
Itga6	2.09	1.23	1.83	Itga6	1.84	1.19	1.83
Jun	1.49	0.61	1.36	Jun	1.95	0.79	1.71
Khdc1a	1.91	0.25	0.29	Khdc1a	16.15	1.07	3.85
Klf4	0.24	0.64	0.16	Klf4	0.35	1.00	0.33
Krt18	2.23	1.25	1.99	Krt18	2.11	1.20	2.07
Lama1	5.07	0.85	6.01	Lama1	3.31	1.10	4.39
Lama5	3.68	1.07	4.43	Lama5	2.19	1.02	1.74
Lrp1	0.59	1.13	0.78	Lrp1	0.59	0.94	0.69
Meis1	0.45	1.28	0.29	Meis1	0.50	0.90	0.53
Mep1b	0.18	0.94	0.35	Mep1b	0.23	0.76	0.33
Mmp2	1.57	1.18	1.58	Mmp2	1.74	1.10	1.80
Mmp7	185.40	1.38	146.44	Mmp7	77.85	0.95	62.22
Myc	2.44	0.77	2.30	Myc	2.55	1.07	2.56
Nfkbie	1.61	0.90	1.80	Nfkbie	1.81	0.95	1.90
Nisch	1.24	0.86	1.26	Nisch	1.36	0.82	1.05
Nr1d1	0.30	0.73	0.28	Nr1d1	0.55	0.92	0.45
Onecut2	**145.82**	**1.01**	**196.83**	**Onecut2**	**30.66**	**1.02**	**44.91**
Pdk2	0.27	0.72	0.26	Pdk2	0.42	0.97	0.32
Phf17	0.58	0.88	0.56	Phf17	0.76	1.30	1.10
Pla2g2a	37.14	1.13	17.23	Pla2g2a	45.93	1.71	23.99
Plat	21.02	1.66	20.81	Plat	6.43	1.07	8.11
Pou2af1	0.56	0.52	0.40	Pou2af1	0.41	0.60	0.75
Ppbp	10.21	1.10	7.77	Ppbp	5.95	0.99	6.44
Ptger3	0.16	0.71	0.18	Ptger3	0.58	1.13	0.63
Ptgis	0.11	1.41	0.15	Ptgis	0.61	1.02	0.65
Ptgs1	0.55	0.53	0.40	Ptgs1	0.59	0.63	0.47
Ptgs2	5.15	0.97	6.50	Ptgs2	3.84	1.03	8.85
Ptprg	1.27	0.69	0.89	Ptprg	1.56	0.73	0.99
Rarb	3.35	0.44	3.50	Rarb	2.44	0.63	3.56

Relative quantity of gene expression in $APC^{Min/+}$ tumour, $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour groups to $APC^{Min/+}$ normal; Affymetrix® microarrays and Taqman® low density arrays

Comparison of relative quantity (to APC$^{Min/+}$ normal) in Taqman® low density arrays & Affymetrix® microarrays (c)

Taqman arrays Relative Quantity to APC$^{Min/+}$ normal (76.4/1)				Microarrays Relative Quantity to APC$^{Min/+}$ normal (76.4/1)			
GENE	Min tumour	PPARα$^{-/-}$ normal	PPARα$^{-/-}$ tumour	GENE	Min tumour	PPARα$^{-/-}$ normal	PPARα$^{-/-}$ tumour
Rbms1	2.14	1.54	2.44	Rbms1	1.89	1.09	1.95
Rhoj	3.17	1.05	2.26	Rhoj	2.68	1.05	2.22
Rorc	0.19	0.55	0.14	Rorc	0.45	0.93	0.43
Sbk1	1.85	0.98	1.70	Sbk1	2.13	0.94	1.82
Sell	0.34	0.24	0.53	Sell	1.00	0.66	1.33
Sfrp1	0.98	1.25	1.45	Sfrp1	1.22	1.55	2.27
Steap4	4.74	1.74	5.84	Steap4	3.19	1.30	4.61
Tcf12	2.48	0.76	1.48	Tcf12	2.02	0.94	1.69
Timp1	4.89	2.38	5.06	Timp1	4.18	1.58	5.50
Tnf	3.71	0.37	2.47	Tnf	1.98	0.87	1.75

Relative quantity of gene expression in APC$^{Min/+}$ tumour, APC$^{Min/+}$ PPARα$^{-/-}$ normal and APC$^{Min/+}$ PPARα$^{-/-}$ tumour groups to APC$^{Min/+}$ normal; Affymetrix® microarrays and Taqman® low density arrays

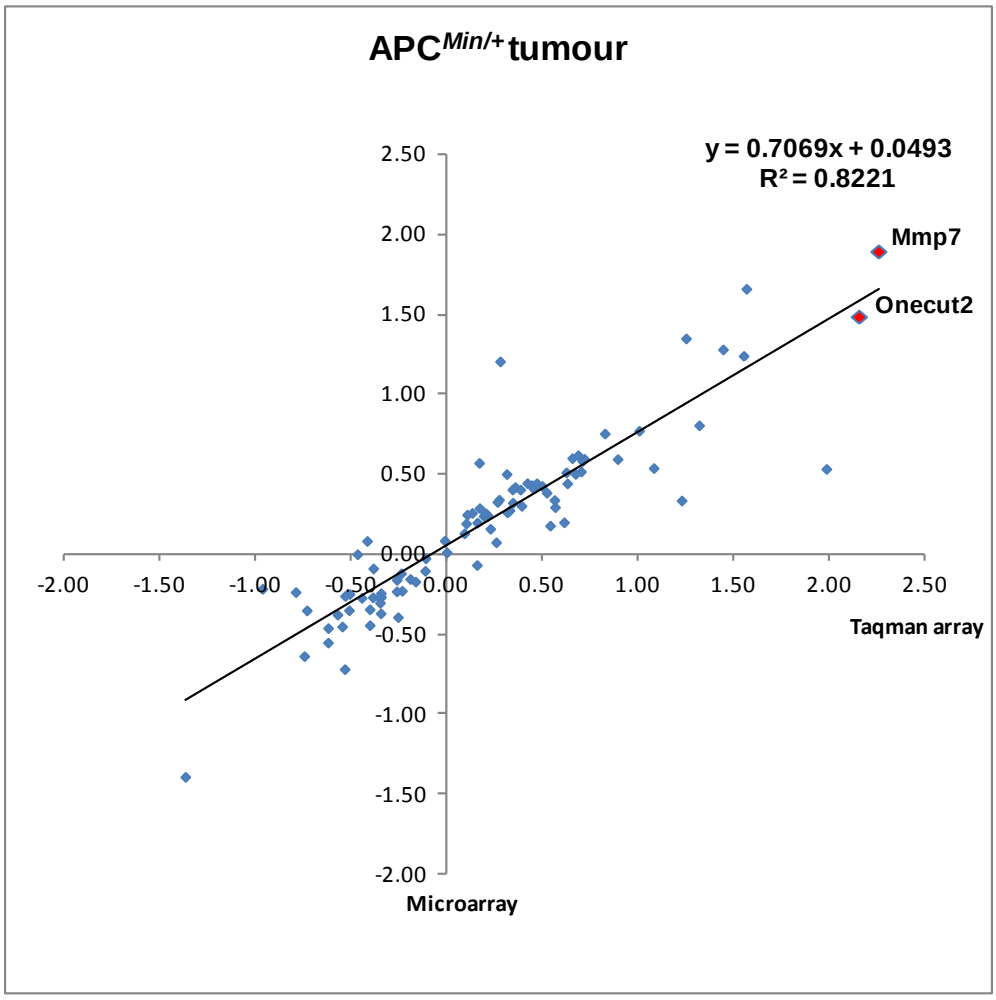

Figure 4-14 Differential expression of genes in tumour *vs.* normal tissue taken from APC$^{Min/+}$ mice as assessed by Taqman® low density arrays & Affymetrix® microarrays

Relative Quantity (RQ) of gene expression in tumour tissue compared to normal tissue in APC$^{Min/+}$ mice assessed by Taqman® low density arrays and Affymetrix® microarrays represented on a $\log_{10}$ scatter plot

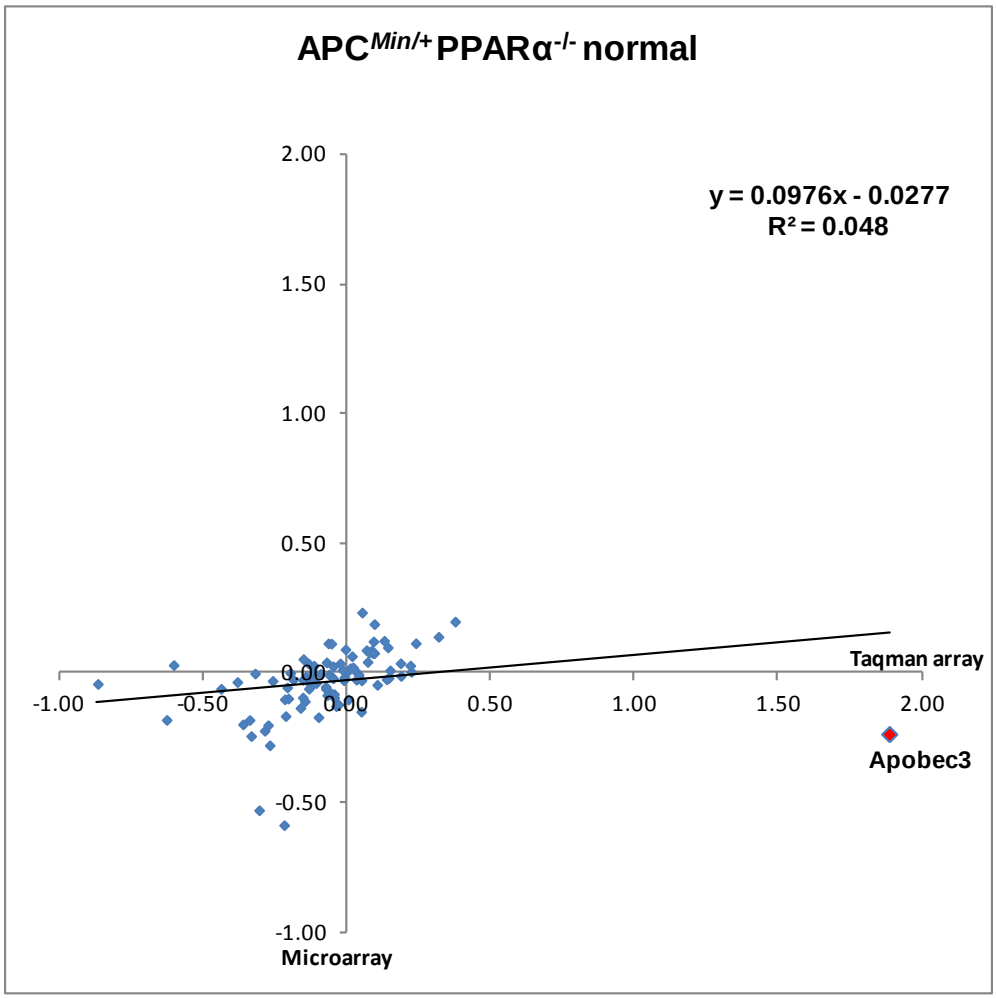

Figure 4-15 Differential expression of genes in normal tissue taken from APC$^{Min/+}$ PPARα$^{-/-}$ mice *vs.* normal tissue taken from APC$^{Min/+}$ mice as assessed by Taqman® low density arrays & Affymetrix® microarrays

Relative Quantity (RQ) of gene expression in normal tissue of APC$^{Min/+}$ PPARα$^{-/-}$ mice compared to normal tissue in APC$^{Min/+}$ mice assessed by Taqman® low density arrays and Affymetrix® microarrays represented on a $\log_{10}$ scatter plot

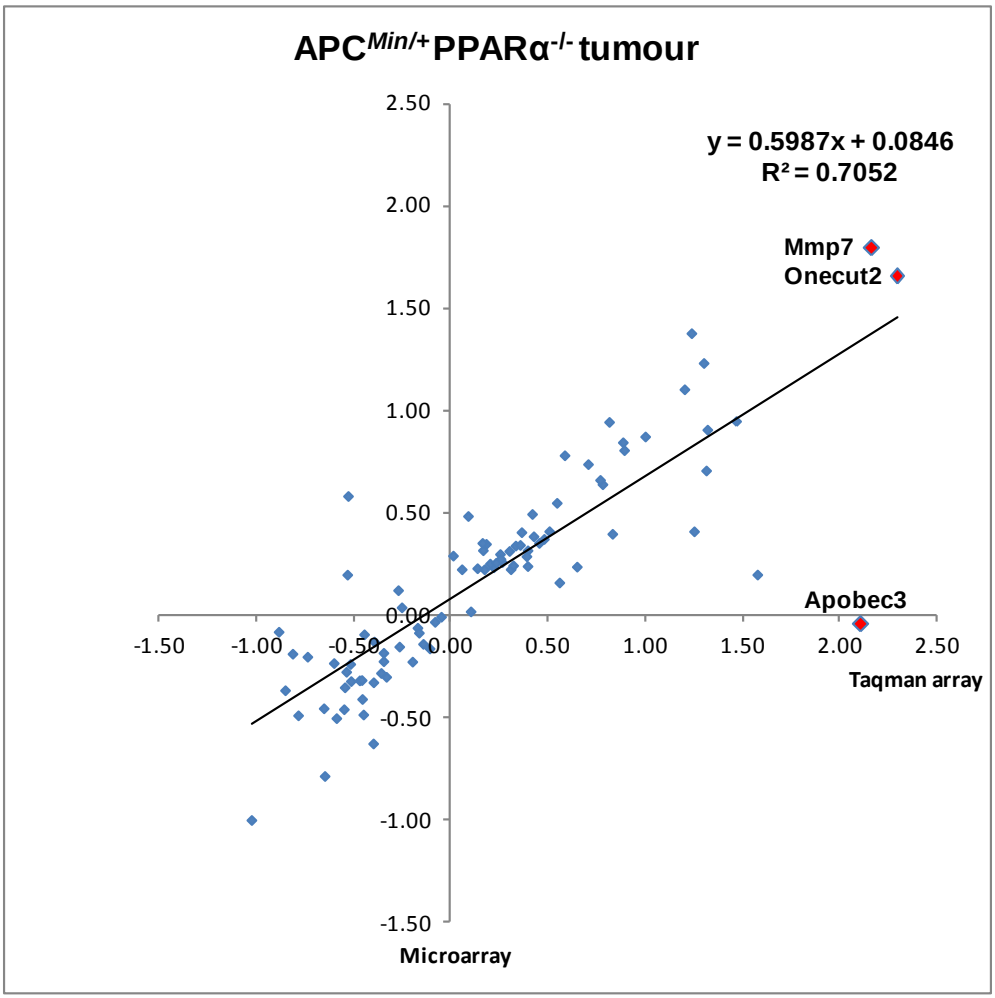

Figure 4-16 Differential expression of genes in tumour tissue taken from APC$^{Min/+}$ PPARα$^{-/-}$ mice *vs.* normal tissue taken from APC$^{Min/+}$ mice as assessed by Taqman® low density arrays & Affymetrix® microarrays

Relative Quantity (RQ) of gene expression in tumour tissue of APC$^{Min/+}$ PPARα$^{-/-}$ mice compared to normal tissue in APC$^{Min/+}$ mice assessed by Taqman® low density arrays and Affymetrix® microarrays represented on a $\log_{10}$ scatter plot

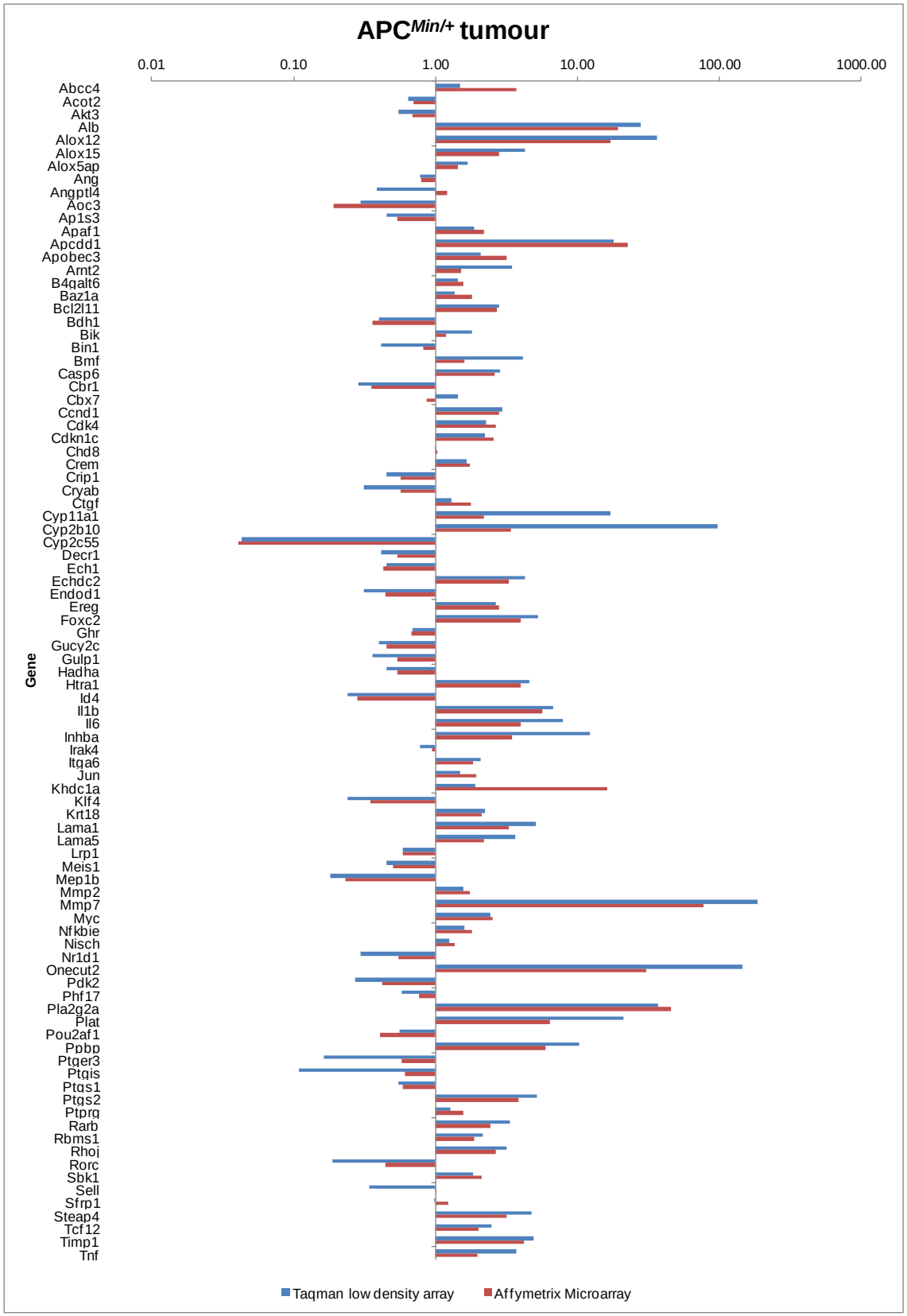

Figure 4-17 Differential expression of genes in tumour *vs.* normal tissue taken from APC$^{Min/+}$ mice as assessed by Taqman® low density arrays & Affymetrix® microarrays

Relative quantity (RQ) of gene expression in tumour tissue compared to normal tissue in APC$^{Min/+}$ mice assessed by Taqman® low density array (blue bars) and Affymetrix® microarray (red bars) represented on $\log_{10}$ scale bar chart

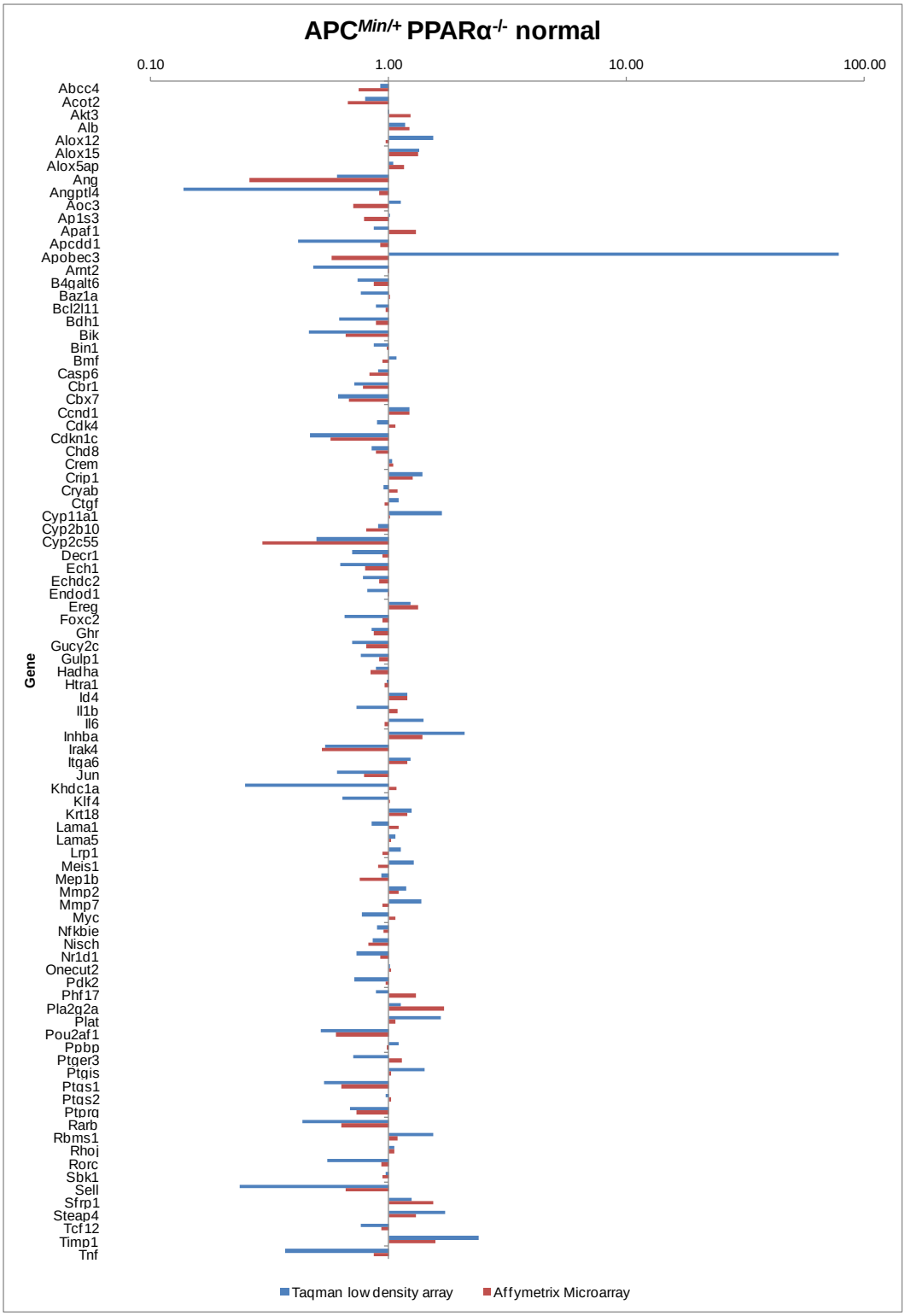

Figure 4-18 Differential expression of genes in normal tissue taken from APC$^{Min/+}$ PPARα$^{-/-}$ mice *vs.* normal tissue taken from APC$^{Min/+}$ mice as assessed by Taqman® low density arrays & Affymetrix® microarrays

Relative Quantity (RQ) of gene expression in normal tissue of APC$^{Min/+}$ PPARα$^{-/-}$ mice compared to normal tissue in APC$^{Min/+}$ mice assessed by Taqman® low density array (blue bars) and Affymetrix® microarrays (red bars) represented on a $\log_{10}$ scatter plot

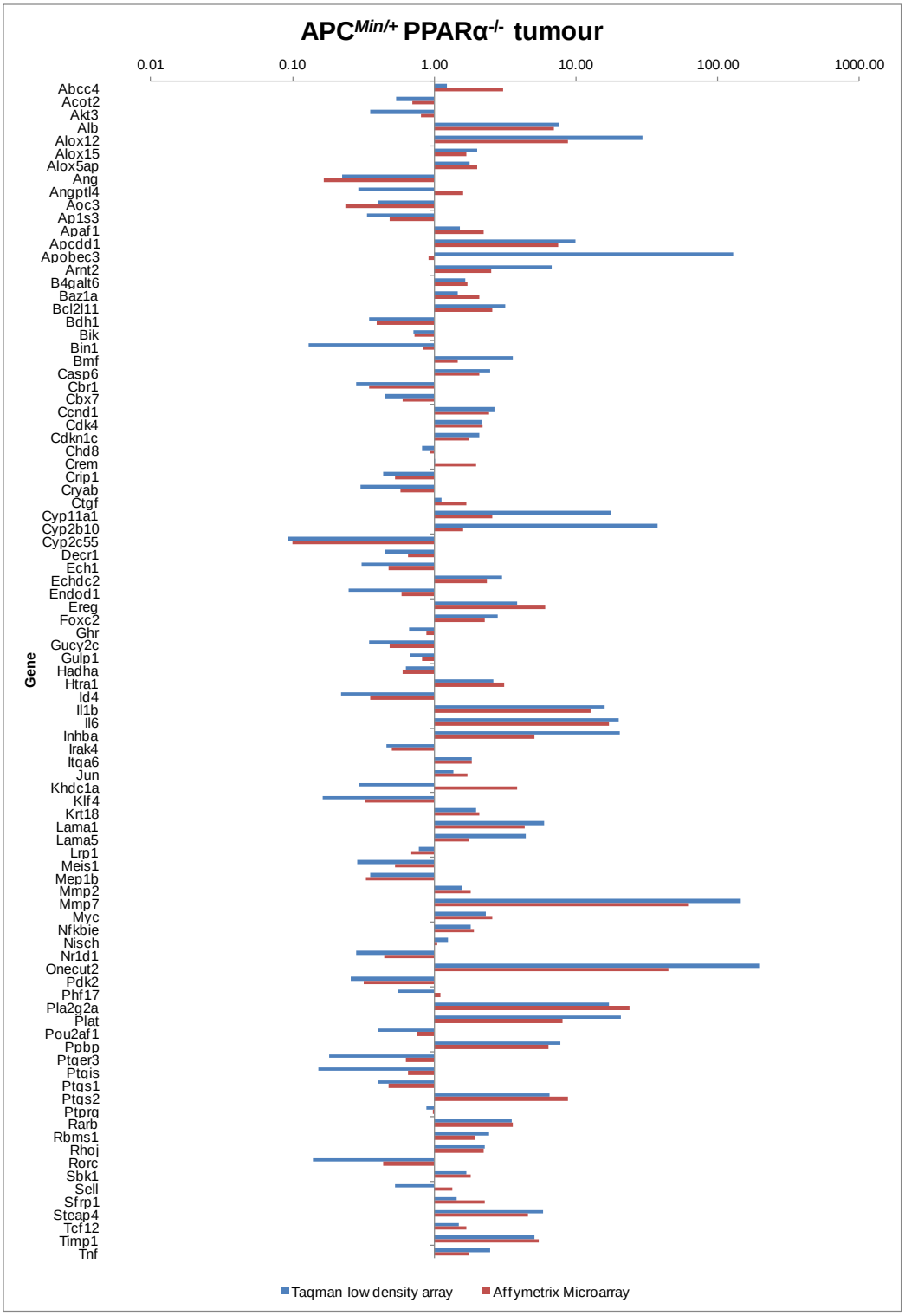

Figure 4-19 Differential expression of genes in tumour tissue taken from APC$^{Min/+}$ PPARα$^{-/-}$ mice *vs.* normal tissue taken from APC$^{Min/+}$ mice as assessed by Taqman® low density arrays & Affymetrix® microarrays

Relative Quantity (RQ) of gene expression in tumour tissue of APC$^{Min/+}$ PPARα$^{-/-}$ mice compared to normal tissue in APC$^{Min/+}$ mice assessed by Taqman® low density array (blue bars) and Affymetrix® microarrays (red bars) represented on a log$_{10}$ scatter plot

4.5 Discussion

This study used Taqman® low density arrays to determine the expression level of 95 genes of interest from normal and tumour tissue from the colons of $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice.

Also, results were compared to see whether Taqman® low density arrays would produce similar gene expression levels of these genes to levels previously determined on Affymetrix® microarrays.

The findings of Taqman® low density arrays were in agreement with data from Affymetrix® microarrays, in that the largest source of differential gene expression was between tumour and normal samples.

Two genes, Onecut2 (one cut domain, family member 2) and Mmp7 (matrix metallopeptidase 7) were highly up-regulated in tumour tissue.

There has been much research into Mmp7 and it is known to be up-regulated in colon cancer (Kioi et al., 2003). Mmp7 has been suggested as a potential target for colon cancer therapy, as high Mmp7 expression in tumours has been shown to be associated with a poor prognosis (Koskensalo et al., 2011).

By contrast, there has been very little research into the role and function of Onecut2 in the intestine. One study by (Dusing et al., 2010) suggested Onecut2 may have a regulatory role in the expression of genes related to transport and lipid metabolism in the mouse intestine. Results from the present study showed a large increase in Onecut2 expression in colonic tumours ($p < 0.0001$).

To validate these findings further analyses into Onecut2 expression levels were carried out; these are described and discussed in Chapter 6.

In the absence of malignant change there were very few examples of genes differentially regulated by PPARα genotype (Figure 4-15, Figure

4-18). However, in tumour tissue, differences in the expression of PPARα dependent genes were evident (Figure 4-16, Figure 4-19).

The lack of gene induction in normal tissue in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice meant there were few large excursions from expression levels seen in normal tissue from $APC^{Min/+}$ mice. Apobec3 was a notable exception. Consequently, correlations between repeated measurements of gene expression in normal tissue were weak.

Where malignant change produced observed differences in gene expression from normal mucosa in $APC^{Min/+}$ mice (Figure 4-14, Figure 4-17) and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice (Figure 4-16, Figure 4-19), correlation was generally good.

These data show colon gene expression results from the same cohort of mice, using two alternative analysis techniques were comparable. Overall these results show there was good correlation (R^2 = x to y[21]) between experiments employing Affymetrix® microarrays, and those employing Taqman® low density arrays.

There was strong correlation between Affymetrix® microarrays and Taqman® low density arrays in $APC^{Min/+}$ tumour and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour groups (Figure 4-14, Figure 4-16). However, gene expression in Taqman® low density arrays was consistently higher compared to Affymetrix® microarrays.

Apobec3 showed highly induced expression in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice compared to $APC^{Min/+}$ mice in Taqman® low density arrays ($p < 0.0001$). However, in Affymetrix® microarrays Apobec3 expression was lower in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice compared to $APC^{Min/+}$ mice. This discrepancy is investigated in Chapter 6, and may be explained by the sequences used for amplification by the two different methodologies which appear to have targeted different parts of the gene, or different isotypes of the gene.

[21] R^2 = x to y; this is the relationship between the 2 $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal groups, 2 $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour groups and 2 $APC^{Min/+}$ tumour groups. The R^2 value measures how successful the 'fit' is in explaining the variation of the data. When R^2 is 1.0 all points lie exactly on the line with no scatter.

There was good correlation of expression of Onecut2 in both methodologies, which confirmed the gene as being highly expressed in tumours.

The focus of the present studies was to determine gene expression levels; protein levels of expression were not assessed. However, transcription data is useful for identifying potential candidates for follow-up work at protein level. mRNA levels give an indication of the presence of a protein.

As there are many processes between transcription and translation; the variety of translated proteins means correlation of a particular protein level with its related mRNA can be highly variable (Vogel and Marcotte, 2012).

Future work will determine protein levels of two genes initially, Apobec3 and Onecut2 in the mouse, and also in human colonic tissue using immunoblotting and immunohistochemistry techniques.

In summary, gene expression results from Taqman® low density arrays showed that overall the differential expression of selected genes of interest corresponded to their functional process and role in tumorigenesis. Two genes which were highly up-regulated, namely Apobec3 and Onecut2, had greatly increased expression in PPARα samples and tumour samples respectively and are discussed in depth in Chapter 6.

These data also demonstrated that analysis of the same sample set on Affymetrix® microarrays and Taqman® low density arrays showed comparable gene expression levels.

Ingenuity® pathway analysis networks of Affymetrix® microarray data and Taqman® low density array data showed similar genes were highlighted when annotated with PPARα, Wnt/beta – catenin, ERK/MAPK and p53 signalling pathways. Il6, Myc and Casp6 were three genes that featured on both sets of networks.

However, Onecut2 was not displayed on the IPA networks of Taqman® low density array data. This may be due to the selective nature of the data input. Or, that there is no current research in the Ingenuity Pathway Knowledge Base (IPKB) for interactions between Onecut2 and the other genes on the networks.

The present studies with $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice have shown PPARα may have a role in preventing initiation or growth of tumours in the mouse intestine. Additionally, previous work has demonstrated that the PPARα ligand methylclophenapate (MCP) reduced the number of polyps in the mouse colon and small bowel (Jackson et al., 2003).

As discussed in Chapter 1, it is known that non-steroidal anti-inflammatory drugs (NSAIDs) reduce the number and incidence of colonic polyps. Therefore, to investigate whether NSAIDs act through a functional PPARα gene to reduce intestinal polyp number and incidence $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice were treated with the non-selective Cox-inhibitor piroxicam. Polyp burden and expression of the same 95 genes as selected for Chapter 4 were analysed.

5 The role of PPARα in the effects of piroxicam on the APC$^{Min/+}$ mouse colon & small bowel

5.1 Introduction

As discussed in Chapter 1 there is considerable evidence that aspirin, non-selective NSAIDs (non-steroidal anti-inflammatory drugs) and selective Cox-2 inhibitors can reduce development of colonic polyps and are associated with a reduced incidence of colorectal cancer.

Results from Chapter 3 and Chapter 4 showed that PPARα status has an effect on tumorigenesis. Subsequently, this study was instigated to determine whether the effects produced by treatment with an NSAID act *via* a functional PPARα pathway.

Piroxicam was selected for this study as the duration of the study necessitated the use of a drug that could be tolerated over a long period of time. Piroxicam is a non-selective Cox inhibitor with a good safety record in mice compared to other NSAIDs such as indomethacin which has a high rate of toxicity and ileal perforation (Trujillo et al., 1994). Also, piroxicam has been successfully used in previous mouse studies; (Jacoby and al., 1996) used piroxicam at 50, 100 and 200 ppm, (Jacoby et al., 2000) used the drug at 12, 25 and 50 ppm, and (Ritland and Gendler, 1999) used it at 200 ppm. The present study dosed mice with piroxicam at 100 ppm in their feed. This dose is comparable to that used in previous studies and has been shown to be efficacious. The study by (Jacoby et al., 2000) measured thromboxane B_2 levels in plasma after different doses of piroxicam were given to mice. Their study demonstrated an increase in serum levels of piroxicam with increasing dose. There was also a significant dose-related reduction in plasma thromboxane levels.

The possible role of PPARα in the action of piroxicam treatment on the mouse gut was therefore investigated.

5.2 Aim of study

- To investigate the effect of piroxicam on polyp formation in $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice.
- To investigate the effect of piroxicam on gene expression in $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice.
- To determine whether the effects of piroxicam are mediated *via* activation of PPARα.

5.3 Methods

A study to determine the effect of piroxicam on polyp formation and gene expression in the colon and small bowel of $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice was established. Mice were allowed food and water *ad libitum* with half of the mice dosed with piroxicam at 100 ppm (parts per million) mixed in their feed. Mice were sacrificed at one year old if they were still on the study at that time.

Polyp number at death in the colon and small bowel of $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice from control and piroxicam-treated mice were compared (Study 2A). Expression of genes in the colon from similarly treated $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice were analysed using Taqman® low density arrays (Study 2B).

Table 5-1 shows the numbers of mice on study 2A and 2B.

Table 5-1 Number of $APC^{Min/+}$ mice & $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice on Study 2A & Study 2B

Genotype	Number of mice on study			
	$APC^{Min/+}$		$APC^{Min/+}$ $PPAR\alpha^{-/-}$	
Feed regime	**control**	**piroxicam 100 ppm**	**control**	**piroxicam 100 ppm**
STUDY 2A	18	18	18	18
STUDY 2B	5	5	5	5

Animal work using an APC$^{Min/+}$ mouse model (3.3.1, page 45) was carried out at the Biomedical Science Unit (BMSU), University of Nottingham following Animals (Scientific Procedures) Act 1986 (ASPA) protocols and guidelines as before (3.3, page 44). Genotyping was performed as previously (3.3.2, page 45).

Four groups of mice (aged 28-30 days) were allocated onto one year (maximum) studies (Table 5-1). Mice were dosed with piroxicam or control mixed in their feed (5.3.1, page 167). Food and water were available *ad libitum.* Husbandry of mice was as before (3.3.3, page 46). Mice were sacrificed by cervical dislocation at the end of the study or sooner if they showed signs of suffering, anaemia, rectal prolapse or >15% weight loss as previously. Mesenteric fat[22] was collected from all mice after sacrifice (5.3.2, page 168); as PPARα is known to be involved in lipid metabolism, it would be expected that there would be more mesenteric fat in APC$^{Min/+}$PPARα$^{-/-}$ mice (as this mouse model does not have a functional PPARα gene).

Two mice on the studies were sacrificed prematurely; one APC$^{Min/+}$ control mouse was found to have hydrocephalus, and an APC$^{Min/+}$PPARα$^{-/-}$ piroxicam-treated mouse was found to have a red, sore eye which was not due to the Min mutation (assessment was made by the on-call veterinary surgeon). Two other mice, an APC$^{Min/+}$ control mouse and an APC$^{Min/+}$PPARα$^{-/-}$ control mouse, were found dead in their cages.

5.3.1 Preparation of mouse feed with piroxicam

To prepare mouse feed with piroxicam at 100 parts per million (ppm) 200 mg of piroxicam was dissolved in 4 ml of chloroform. 1.2 ml of this was added to 300 ml of acetone. The resultant solution was then thoroughly mixed with 600 g of ground mouse feed and left to dry in a fume hood. Control feed was prepared by mixing 300 ml of acetone with 600 g of ground mouse feed and left to dry in a fume hood.

[22] Mesenteric fat – fat in the folds of the peritoneum (serous membrane encasing the intestines)

5.3.2 Mesenteric fat collection

Mesenteric fat was collected from mice on Study 2A (polyp number) and Study 2B (gene expression). After sacrifice all visible mesenteric fat surrounding the intestines was removed with scissors and forceps and placed on a Petri dish.

The fat was weighed (fat mass calculated by subtraction of the mass of the Petri dish) and the mass recorded. The fat was then disposed of as appropriate to tissue disposal protocols within the animal unit.

5.4 Evaluation of polyp number in the mouse colon & small bowel: Study 2A

Whole intestines from mice on Study 2A were removed immediately after sacrifice and separated into small bowel and colon. Tissue was processed for quantification of mouse intestinal polyps as described previously (3.3.4, page 46).

Tissue was pinned out flat and observed through a Karl Zeiss Tessovar macroscope (x4.2 magnification). Images were acquired using a Q imaging micropublisher 5.0 RTV camera with Improvison Openlab programme as described previously. Saved images were analysed for polyp number, polyp size and area using Aperio ImageScope® software.

5.5 Gene expression in the mouse colon: Study 2B

Colons from mice on Study 2B were removed immediately after sacrifice. Paired samples of normal and tumour mucosa, (paired samples each from $APC^{Min/+}$ control, $APC^{Min/+}$ piroxicam, $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ piroxicam groups) were identified and collected for RNA extraction as before (3.3.6, page 50).
From the twenty mice on the study, seventeen pairs of normal and tumour mucosa samples were collected. Tumour tissue was not apparent in three of the colons collected. From the seventeen pairs of

samples, twelve pairs (twenty four samples) were selected for analysis on Taqman® low density arrays based on RNA integrity number (RIN), concentration and 260/280 ratio (to give n = 3 in each group).

Twenty four RNA samples were prepared and analysed on Taqman® arrays (six arrays, four samples per array) as previously (4.3.1, page 122). Taqman® arrays were designed with identical gene assays as before (Table 3-14 - Table 3-23).

All six arrays (24 sample results) were analysed simultaneously using SDS RQ Manager software as previously (4.3.2, page 124). Average Ct (cross threshold) values of each target gene (average calculated from Ct value from each sample across all six arrays) were copied into an Excel spreadsheet.

The expression level of reference genes must not alter across arrays. Therefore, average Ct values of each selected reference gene (Table 3-14) and 18s were checked for outliers before further analysis (Table 5-2).

As previously, outliers were defined as an average Ct value not within three standard deviations of the mean (three-sigma rule[23]), (Ruan, 2005). All five reference genes were used in analysis. One sample (123.35, highlighted in red) had outlying average Ct values for four of the reference genes. However, as the values were consistently higher in each case, the sample was not excluded from analysis.

The geometric mean[24] of all five reference genes was calculated and used for normalisation of all average Ct values of target genes.

[23] The three-sigma rule (empirical rule) states that for a normal distribution, nearly all values lie within 3 standard deviations of the mean.

[24] Geometric mean: normalises ranges of numbers to give an average that indicates a central tendency or typical value. Numbers are multiplied together, then the *n*th root (where *n* is the count of numbers in the range) is taken of the resultant product.

Table 5-2 Expression (average Ct value) of reference genes in Study 2B samples

Sample identification	Genotype	Group	Tissue type	Reference Genes Average Ct values				
				18s Ct	Actb Ct	Hmbs Ct	Hprt1 Ct	Ppia Ct
121.46	APC $^{Min/+}$	Control	Normal	13.80	17.52	26.32	24.63	21.38
119.33	APC $^{Min/+}$	Control	Normal	13.26	16.98	25.97	24.72	20.93
122.37	APC$^{Min/+}$	Control	Normal	13.86	17.49	26.24	24.65	21.39
119.33	APC$^{Min/+}$	Control	Tumour	13.37	17.27	26.90	25.11	22.60
119.34	APC$^{Min/+}$	Control	Tumour	13.52	18.34	26.50	25.15	21.88
122.37	APC$^{Min/+}$	Control	Tumour	13.67	16.55	25.98	24.23	21.00
113.22b	APC$^{Min/+}$ PPARα$^{-/-}$	Control	Normal	13.84	16.80	26.06	24.71	21.93
113.23	APC$^{Min/+}$ PPARα$^{-/-}$	Control	Normal	12.54	16.63	25.08	24.21	20.17
113.23b	APC$^{Min/+}$ PPARα$^{-/-}$	Control	Normal	12.91	17.31	25.91	24.58	20.87
113.22b	APC$^{Min/+}$ PPARα$^{-/-}$	Control	Tumour	14.49	17.54	26.60	25.18	21.65
113.22	APC$^{Min/+}$ PPARα$^{-/-}$	Control	Tumour	12.66	17.36	26.39	25.00	21.21
113.23b	APC$^{Min/+}$ PPARα$^{-/-}$	Control	Tumour	13.94	17.97	26.76	25.94	21.66
123.35	APC$^{Min/+}$	Piroxicam	Normal	13.38	20.18	29.64	28.03	24.81
123.36	APC$^{Min/+}$	Piroxicam	Normal	12.90	18.94	27.18	26.19	22.73
114.44	APC$^{Min/+}$	Piroxicam	Normal	14.02	18.57	26.67	25.78	21.79
113.44	APC$^{Min/+}$	Piroxicam	Tumour	12.92	18.32	26.51	25.34	21.46
113.45	APC$^{Min/+}$	Piroxicam	Tumour	12.58	17.90	26.15	24.90	21.34
114.44	APC$^{Min/+}$	Piroxicam	Tumour	13.37	17.52	26.54	25.10	21.91
119.17	APC$^{Min/+}$ PPARα$^{-/-}$	Piroxicam	Normal	12.13	17.73	25.59	24.48	21.04
120.15	APC$^{Min/+}$ PPARα$^{-/-}$	Piroxicam	Normal	11.30	17.64	25.55	24.38	20.78
112.33	APC$^{Min/+}$ PPARα$^{-/-}$	Piroxicam	Normal	12.67	17.84	26.21	25.07	21.80
119.17	APC$^{Min/+}$ PPARα$^{-/-}$	Piroxicam	Tumour	13.75	17.71	25.77	24.28	20.55
120.15	APC$^{Min/+}$ PPARα$^{-/-}$	Piroxicam	Tumour	12.99	17.97	25.94	24.53	20.76
120.17	APC$^{Min/+}$ PPARα$^{-/-}$	Piroxicam	Tumour	12.68	17.96	25.96	24.28	20.54
Mean				**13.19**	**17.75**	**26.35**	**25.02**	**21.51**
Standard Deviation (SD)				**0.71**	**0.78**	**0.84**	**0.84**	**0.94**
Upper limit (Mean + 3SD)				**15.31**	**20.08**	**28.88**	**27.52**	**24.33**
Lower limit (Mean - 3SD)				**11.07**	**15.42**	**23.82**	**22.51**	**18.68**

5.5.1 The effect of piroxicam on gene expression

To determine effect of piroxicam on gene expression, relative quantity (RQ) of gene expression in the piroxicam-treated mouse groups of $APC^{Min/+}$ normal, $APC^{Min/+}$ tumour, $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour groups to expression in the corresponding control groups were calculated from normalised average Ct values (to sample 121.46 $APC^{Min/+}$ normal control).

Mean RQ was determined for each group.

Normalised average Ct values of all groups were analysed using SPSS 16.0.0.247 to calculate univariate of analysis of variance for effects of piroxicam only, piroxicam with PPARα effects, and piroxicam with tissue type effects.

5.6 Validation of Taqman® low density array data: Correlation between Study 1B & control groups of Study 2B

The reliability and reproducibility of Taqman® low density arrays was assessed by comparing expression of selected genes as determined in Study 1B (Chapter 4) to the gene expression of similar genes in Study 2B.

5.7 Results

5.7.1 Survival of mice

The mean age at sacrifice for $APC^{Min/+}$ control mice was 23.7 +/- 0.7 weeks (mean +/- Standard Error). This was significantly older than $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice which had a mean age at sacrifice of 21.0 +/- 1.1 weeks ($p = 0.043$, Figure 5-1A, Table 5-3, Table 5-4). All mice in control groups were sacrificed due to white paw (Table 5-3, Table 5-4).

The mean age at sacrifice of mice treated with piroxicam was much higher than mean age at sacrifice of control mice. The mean age of piroxicam-treated $APC^{Min/+}$ mice at sacrifice was 52.3 +/- 0.5 weeks compared to 23.7 +/- 0.7 weeks in control mice ($p < 0.0001$, Figure 5-1A, Table 5-5). $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice treated with piroxicam had a mean age at sacrifice of 46.5 +/- 2.1 weeks, whereas control mice had a mean age of 21.0 +/-1.1 weeks at sacrifice ($p < 0.0001$, Figure 5-1A, Table 5-6).

Piroxicam-treated $APC^{Min/+}$ mice were significantly older at sacrifice than piroxicam-treated $APC^{Min/+}$ $PPAR\alpha^{-/-}$mice ($p = 0.019$, Figure 5-1A). 72% of $APC^{Min/+}$ mice and 61% of $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice were sacrificed due to achieving the experimental cut off age of one year. The remainder of the mice in these groups were sacrificed earlier due to white paw or rectal prolapse (Table 5-5, Table 5-6).

Table 5-3 Age, weight & disposition of $APC^{Min/+}$ control group mice at sacrifice

Mouse	Group	Genotype	Age at death (weeks)	Weight at start (grams)	Weight at death (grams)	Disposition at death
97.3/5	control	$APC^{Min/+}$	24.4	16.5	26.3	white paw
104.6/8	control	$APC^{Min/+}$	17.7	14.3	20.0	white paw
101.6/6	control	$APC^{Min/+}$	26.1	20.0	23.2	white paw
104.5/7	control	$APC^{Min/+}$	31.6	15.7	24.5	white paw
116.1/3	control	$APC^{Min/+}$	24.0	21.1	27.1	white paw
122.1/7	control	$APC^{Min/+}$	24.9	14.5	18.7	white paw
122.1/1	control	$APC^{Min/+}$	23.9	20.0	31.6	white paw
122.1/3	control	$APC^{Min/+}$	22.9	17.5	26.0	white paw
122.1/5	control	$APC^{Min/+}$	18.7	17.4	27.8	white paw
122.1/6	control	$APC^{Min/+}$	23.9	18.2	33.4	white paw
123.1/3	control	$APC^{Min/+}$	24.9	14.0	25.0	white paw
114.3/4	control	$APC^{Min/+}$	21.9	17.0	21.2	white paw
114.3/5	control	$APC^{Min/+}$	20.0	17.2	20.6	white paw
114.3/6	control	$APC^{Min/+}$	26.1	17.4	23.4	white paw
122.3/9	control	$APC^{Min/+}$	23.1	18.2	20.9	white paw
119.3/1	control	$APC^{Min/+}$	26.1	22.1	26.0	white paw
120.3/6	control	$APC^{Min/+}$	22.0	17.2	22.6	white paw
121.4/7	control	$APC^{Min/+}$	25.1	17.5	17.4	white paw
		MEAN	**23.7**	**17.5**	**24.2**	

Table 5-4 Age, weight & disposition of $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control group mice at sacrifice

Mouse	Group	Genotype	Age at death (weeks)	Weight at start (grams)	Weight at death (grams)	Disposition at death
76b.4/2	control	$APC^{Min/+}PPAR\alpha^{-/-}$	16.4	19.5	27.7	white paw
108.1/5	control	$APC^{Min/+}PPAR\alpha^{-/-}$	18.3	20.4	21.7	white paw
102.1/6	control	$APC^{Min/+}PPAR\alpha^{-/-}$	23.4	18.3	21.8	white paw
102.1/8	control	$APC^{Min/+}PPAR\alpha^{-/-}$	25.0	20.6	26.1	white paw
101.1/3	control	$APC^{Min/+}PPAR\alpha^{-/-}$	31.1	24.2	37.8	white paw
103.2/7	control	$APC^{Min/+}PPAR\alpha^{-/-}$	31.3	19.5	23.1	white paw
108.1/6	control	$APC^{Min/+}PPAR\alpha^{-/-}$	21.4	17.4	20.5	white paw
104.3/4	control	$APC^{Min/+}PPAR\alpha^{-/-}$	19.1	21.5	26.9	white paw
104.3/5	control	$APC^{Min/+}PPAR\alpha^{-/-}$	19.3	24.7	29.4	white paw
107.1/3	control	$APC^{Min/+}PPAR\alpha^{-/-}$	17.4	21.6	24.8	white paw
107.1/9	control	$APC^{Min/+}PPAR\alpha^{-/-}$	17.3	16.7	19.9	white paw
115.1/2	control	$APC^{Min/+}PPAR\alpha^{-/-}$	15.4	21.1	27.8	white paw
107.3/1	control	$APC^{Min/+}PPAR\alpha^{-/-}$	18.9	21.8	21.9	white paw
116.1/1	control	$APC^{Min/+}PPAR\alpha^{-/-}$	22.9	22.4	27.6	white paw
116.1/3	control	$APC^{Min/+}PPAR\alpha^{-/-}$	25.1	20.4	22.7	white paw
118.2/9	control	$APC^{Min/+}PPAR\alpha^{-/-}$	17.4	17.9	20.0	white paw
118.2/3	control	$APC^{Min/+}PPAR\alpha^{-/-}$	18.7	20.7	24.0	white paw
118.2/7	control	$APC^{Min/+}PPAR\alpha^{-/-}$	18.7	21.7	23.2	white paw
		MEAN	**21.0**	**20.6**	**24.8**	

Table 5-5 Age, weight & disposition of $APC^{Min/+}$ piroxicam group mice at sacrifice

Mouse	Group	Genotype	Age at death (weeks)	Weight at start (grams)	Weight at death (grams)	Disposition at death
98b.4/1	piroxicam	$APC^{Min/+}$	55.1	13.9	33.4	culled due to age
98b.4/2	piroxicam	$APC^{Min/+}$	55.1	11.0	31.0	culled due to age
98b.4/5	piroxicam	$APC^{Min/+}$	55.1	14.3	30.2	culled due to age
98b.4/6	piroxicam	$APC^{Min/+}$	46.0	13.3	30.6	white paw
104.5/1	piroxicam	$APC^{Min/+}$	52.9	22.2	37.4	culled due to age
104.5/2	piroxicam	$APC^{Min/+}$	52.9	24.1	35.6	culled due to age
104.5/3	piroxicam	$APC^{Min/+}$	52.9	22.3	32.5	culled due to age
104.5/8	piroxicam	$APC^{Min/+}$	52.9	22.3	32.9	culled due to age
101.6/2	piroxicam	$APC^{Min/+}$	51.6	23.9	32.8	white paw
101.6/3	piroxicam	$APC^{Min/+}$	51.6	25.3	32.1	white paw
115.2/3	piroxicam	$APC^{Min/+}$	52.1	13.1	27.3	culled due to age
115.2/6	piroxicam	$APC^{Min/+}$	52.1	13.6	28.3	rectal prolapse
118.2/5	piroxicam	$APC^{Min/+}$	50.9	14.0	26.3	rectal prolapse
120.1/1	piroxicam	$APC^{Min/+}$	52.1	23.2	24.2	culled due to age
120.1/2	piroxicam	$APC^{Min/+}$	52.1	19.9	36.3	culled due to age
120.1/3	piroxicam	$APC^{Min/+}$	52.1	20.0	37.6	culled due to age
113.4/1	piroxicam	$APC^{Min/+}$	52.0	18.7	30.0	culled due to age
113.4/2	piroxicam	$APC^{Min/+}$	52.0	20.1	32.7	culled due to age
		MEAN	**52.3**	**18.6**	**31.7**	

Table 5-6 Age, weight & disposition of $APC^{Min/+}$ $PPAR\alpha^{-/-}$ piroxicam group mice at sacrifice

Mouse	Group	Genotype	Age at death (weeks)	Weight at start (grams)	Weight at death (grams)	Disposition at death
102.1/3	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	23.3	22.7	30.0	white paw
101.1/5	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	52.4	17.6	25.6	culled due to age
101.1/6	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	52.4	18.4	25.7	culled due to age
101.1/7	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	52.4	18.7	24.0	culled due to age
94.3/2	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	52.0	23.6	42.3	culled due to age
98.3/2	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	41.0	16.0	25.6	rectal prolapse
98.3/3	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	41.0	14.7	24.7	rectal prolapse
108.1/1	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	52.1	21.7	29.8	culled due to age
108.1/2	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	34.3	21.8	32.2	rectal prolapse
108.1/3	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	52.1	19.8	33.0	culled due to age
102.4/2	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	52.1	21.1	47.4	culled due to age
102.4/3	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	52.1	19.3	30.0	culled due to age
104.3/6	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	46.9	18.5	27.9	rectal prolapse
115.1/7	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	52.0	17.9	35.1	culled due to age
115.1/8	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	52.0	16.6	36.9	culled due to age
115.1/9	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	52.0	18.3	26.7	culled due to age
107.3/2	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	31.3	23.4	40.5	rectal prolapse
116.2/2	piroxicam	$APC^{Min/+}PPAR\alpha^{-/-}$	45.0	21.8	27.2	rectal prolapse
		MEAN	**46.5**	**19.6**	**31.4**	

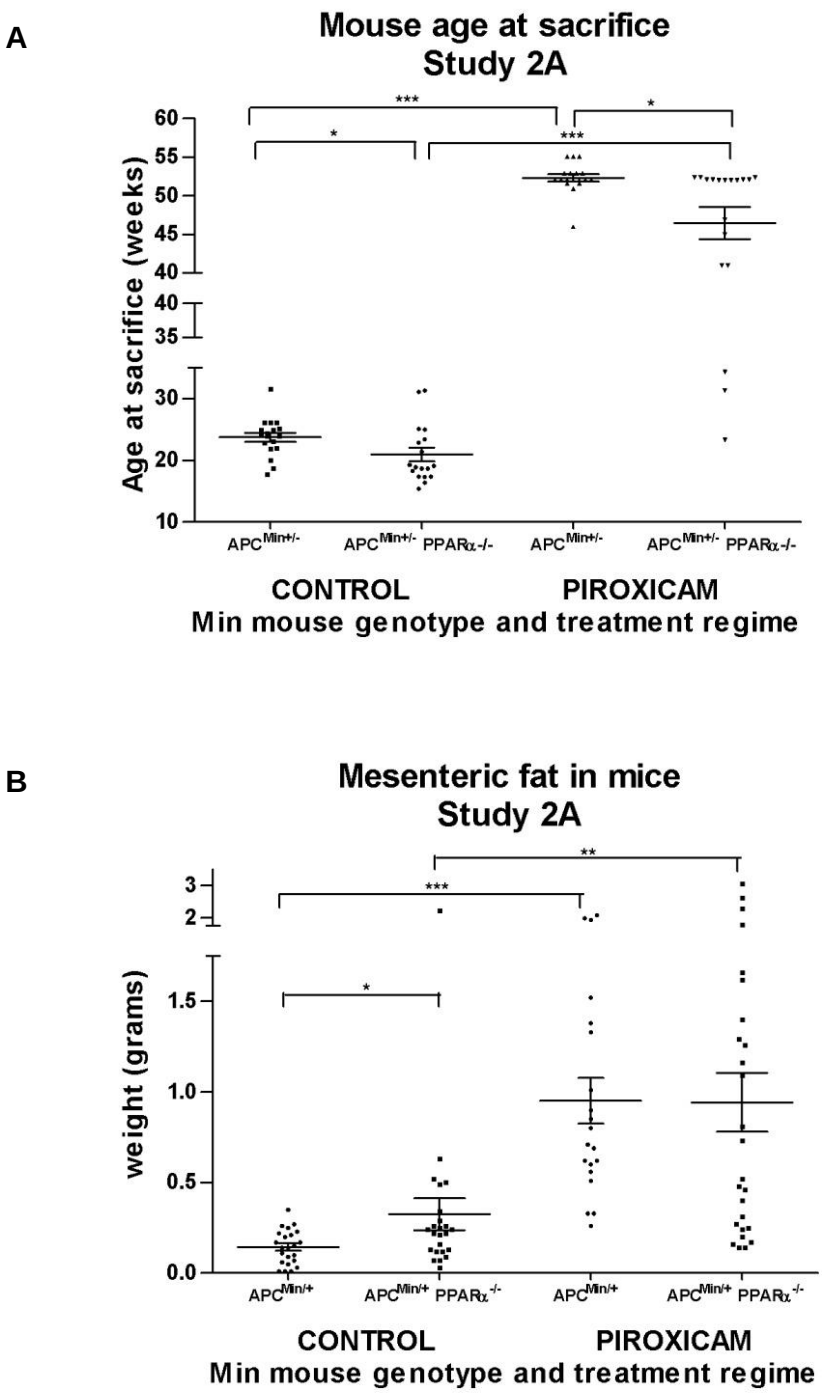

Figure 5-1 Distribution of age & mesenteric fat in APC$^{Min/+}$ & APC$^{Min/+}$ PPARα$^{-/-}$ mice after treatment with piroxicam or control diet

Unpaired t test, * p <= 0.05, ** p <= 0.001, *** p <= 0.0001

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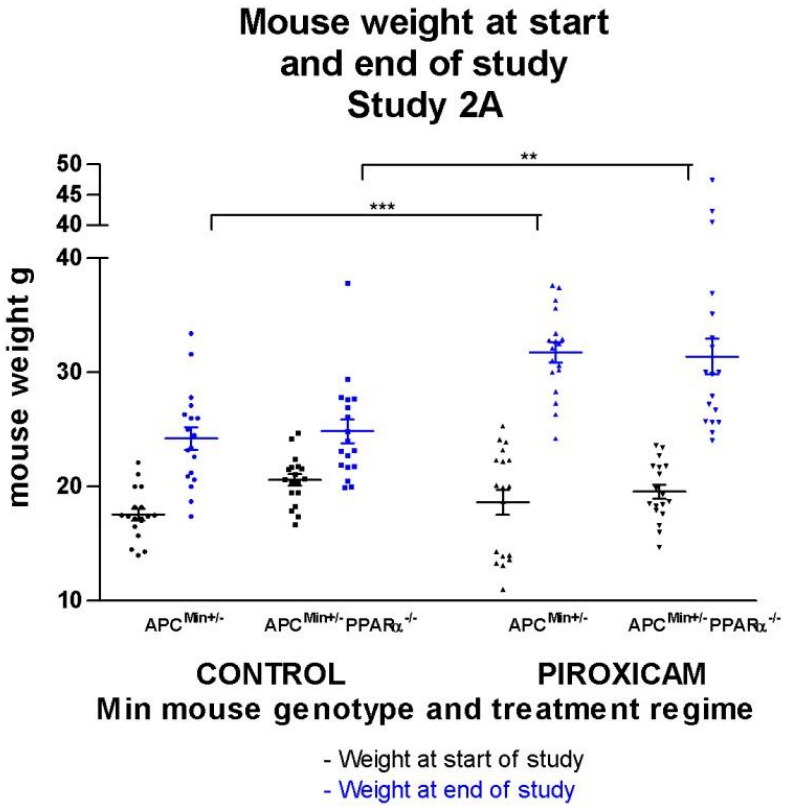

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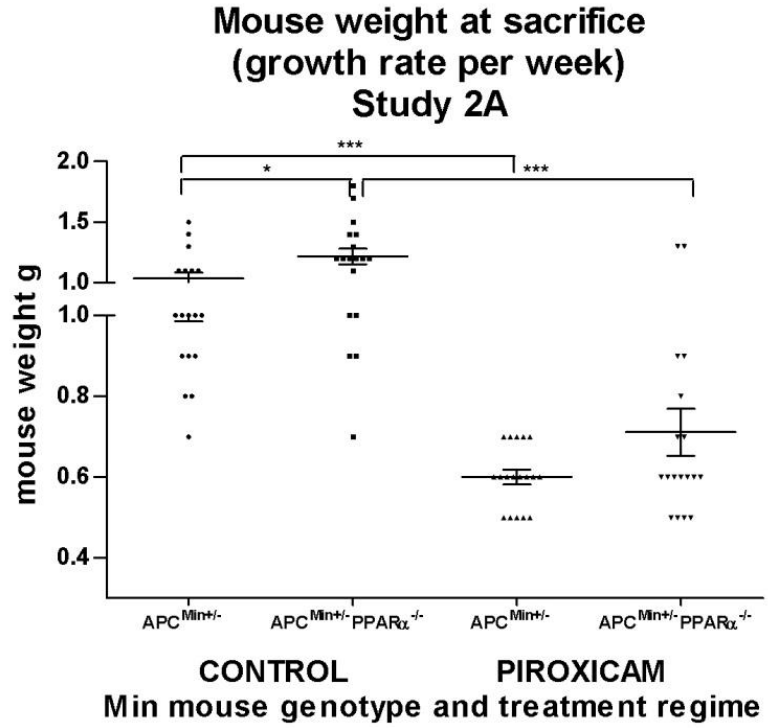


Figure 5-2 Distribution of weight in APC$^{Min/+}$ & APC$^{Min/+}$ PPARα$^{-/-}$ mice after treatment with piroxicam or control diet

Unpaired t test, * p <= 0.05, ** p <= 0.001, *** p <= 0.0001

5.7.2 Weight of mice

The mean weight of $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice was more than $APC^{Min/+}$ mice at the beginning of the study (20.1 +/- 0.4 grams in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice compared to 18.1 +/- 0.6 grams in $APC^{Min/+}$ mice, p = 0.0083, Figure 5-1C, Table 5-3-Table 5-6).

However, at time of sacrifice, there was no significant difference in mean weight between $APC^{Min/+}$ control mice or $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice. The mean weight of $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice was 24.8 +/- 1.0 grams and in $APC^{Min/+}$ control mice was 24.2 +/- 1.0 grams (p was not significant, Figure 5-1C, Table 5-3, Table 5-4).

Mice treated with piroxicam were significantly heavier at sacrifice than control mice at sacrifice. Mean weight at sacrifice in piroxicam-treated $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice was 31.4 +/- 1.6 grams (p = 0.0014, Figure 5-1C, Table 5-6). Similarly, the mean weight at sacrifice in piroxicam-treated $APC^{Min/+}$ mice was 31.7 +/- 0.9 grams (p < 0.0001, Figure 5-1C, Table 5-5).

There was no significant difference in weight between piroxicam-treated $APC^{Min/+}$ mice and piroxicam-treated $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice at time of sacrifice.

When age at sacrifice was considered, growth rate (weight per week of life, wt/wk) in control mice was significantly higher in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice (1.21 +/- 0.065 grams) than $APC^{Min/+}$ mice (1.033 +/- 0.048 grams, p = 0.028, Figure 5-1D).

Mice receiving piroxicam treatment had a significantly reduced growth rate (wt/wk) to 0.711 +/- 0.058 grams in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice (p < 0.0001) and 0.600 +/- 0.018 grams in $APC^{Min/+}$ mice (p < 0.0001). However, there was no significant difference in weight at sacrifice (wt/wk) between $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice after piroxicam treatment (Figure 5-1D).

5.7.3 Mesenteric fat in mice (Study 2A and Study 2B)

Mesenteric fat mass readings from study 2A and 2B were amalgamated for analysis. However, mesenteric fat was not collected at sacrifice for all mice which is the reason for the difference in sample numbers in this analysis*.

(* Sacrifice of mice and tissue/fat collection was performed by me. However, at times when I was absent due to holiday or illness, mice were sacrificed and **gut tissue only** was collected by BMSU technical staff).

At the end of the study, mean mesenteric fat mass in $APC^{Min/+}$ control mice (n = 18) was 0.12 +/- 0.02 grams, whereas in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice (n = 19) mean mesenteric fat mass was significantly higher at 0.25 +/- 0.04 grams (p = 0.004, Figure 5-1B).

However, the mean mesenteric fat mass in $APC^{Min/+}$ mice receiving piroxicam (n = 20) was 0.95 +/- 0.13 grams, which was a large increase compared to the corresponding control group, $APC^{Min/+}$ control mice, 0.12 +/- 0.02 grams ($p < 0.0001$).

Similarly, the mean mesenteric fat mass in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice receiving piroxicam (n = 22) was 0.99 +/- 0.18 grams and also increased compared to the corresponding control group, $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice, 0.25 +/- 0.04 grams (p = 0.0003, Figure 5-1B). The difference in mean mesenteric fat mass between piroxicam-treated $APC^{Min/+}$ mice and piroxicam-treated $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice was not significant (p = 0.42).

5.7.4 Number of polyps in the mouse colon: Study 2A

There was no significant difference in mean polyp number in the mouse colon of $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice, whether they had received piroxicam treatment or not. The mean number of polyps in control groups were 1.50 +/- 0.28 in $APC^{Min/+}$ mice and 1.22 +/- 0.26 in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice, and in piroxicam groups, 1.17 +/- 0.29 in

$APC^{Min/+}$ mice and 1.22 +/- 0.32 in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice (Figure 5-3A, Table 5-7-Table 5-10).

When age at sacrifice was considered (mean number of polyps per week of life, mean number polyps/week), piroxicam-treated mice had significantly reduced mean polyp number compared to control mice.

$APC^{Min/+}$ mice had 0.06 +/- 0.011 mean number polyps/week in control mice compared to 0.02 +/- 0.006 in piroxicam-treated mice ($p = 0.0042$, Figure 5-3B, Table 5-7-Table 5-10). Also, $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice had 0.06 +/- 0.014 mean number polyps/week in control mice compared to 0.03 +/- 0.006 in piroxicam-treated mice ($p = 0.028$, Figure 5-3B, Table 5-7-Table 5-10). However, there was no significant difference in mean number of polyps/week between $APC^{Min/+}$ control mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice, or piroxicam-treated $APC^{Min/+}$ mice and piroxicam-treated $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice.

Table 5-7 Polyp number & Area in the colon of $APC^{Min/+}$ control group mice at sacrifice

Mouse	Group	Genotype	Polyp number	Polyp number/week of life	Total area (mm^2)	Total area/ week of life (mm^2)	Average polyp size ($mm^{2)}$	Average polyp size/ week of life (mm^2)
97.3/5	control	$APC^{Min/+}$	2	0.08	1.97	0.08	0.99	0.04
101.6/6	control	$APC^{Min/+}$	2	0.08	1.78	0.07	0.89	0.03
104.5/7	control	$APC^{Min/+}$	4	0.13	4.39	0.14	1.10	0.03
104.6/8	control	$APC^{Min/+}$	1	0.06	0.66	0.04	0.66	0.04
114.3/4	control	$APC^{Min/+}$	1	0.05	1.10	0.05	1.10	0.05
114.3/5	control	$APC^{Min/+}$	2	0.10	0.46	0.02	0.23	0.01
114.3/6	control	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
116.1/3	control	$APC^{Min/+}$	4	0.17	2.62	0.11	0.66	0.03
119.3/1	control	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
120.3/6	control	$APC^{Min/+}$	2	0.09	4.17	0.19	2.08	0.09
121.4/7	control	$APC^{Min/+}$	2	0.08	13.43	0.53	6.71	0.27
122.1/1	control	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
122.1/3	control	$APC^{Min/+}$	1	0.04	0.69	0.03	0.69	0.03
122.1/5	control	$APC^{Min/+}$	1	0.05	0.66	0.04	0.66	0.04
122.1/6	control	$APC^{Min/+}$	2	0.08	7.28	0.30	3.64	0.15
122.1/7	control	$APC^{Min/+}$	2	0.08	0.35	0.01	0.17	0.01
122.3/9	control	$APC^{Min/+}$	1	0.04	0.37	0.02	0.37	0.02
123.1/3	control	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
		MEAN	**2**	**0.06**	**2.22**	**0.09**	**1.11**	**0.05**

Table 5-8 Polyp number & Area in the colon of $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control group mice at sacrifice

Mouse	Group	Genotype	Polyp number	Polyp number/week of life	Total area (mm^2)	Total area/ week of life (mm^2)	Average polyp size ($mm^{2)}$	Average polyp size/ week of life (mm^2)
79.4/2	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	0	0.00	0.00	0.00	0.00	0.00
101.1/3	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	0	0.00	0.00	0.00	0.00	0.00
102.1/6	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	1	0.04	14.35	0.61	14.35	0.61
102.1/8	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	0	0.00	0.00	0.00	0.00	0.00
103.2/7	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	2	0.06	0.52	0.02	0.26	0.01
104.3/4	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	1	0.05	0.93	0.05	0.93	0.05
104.3/5	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	1	0.05	1.36	0.07	1.36	0.07
107.1/3	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	1	0.06	5.40	0.31	5.40	0.31
107.1/9	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	3	0.17	5.09	0.29	1.70	0.10
107.3/1	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	2	0.11	5.76	0.30	2.88	0.15
108.1/5	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	1	0.05	1.52	0.08	1.52	0.08
108.1/6	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	0	0.00	0.00	0.00	0.00	0.00
115.1/2	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	1	0.06	5.57	0.36	5.57	0.36
116.1/3	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	2	0.08	0.41	0.02	0.20	0.01
116.1/1	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	2	0.09	10.41	0.45	5.21	0.23
118.2/3	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	1	0.05	14.29	0.76	14.29	0.76
118.2/7	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	4	0.21	9.92	0.53	2.48	0.13
118.2/9	control	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	0	0.00	0.00	0.00	0.00	0.00
		MEAN	**1**	**0.06**	**4.20**	**0.21**	**3.12**	**0.16**

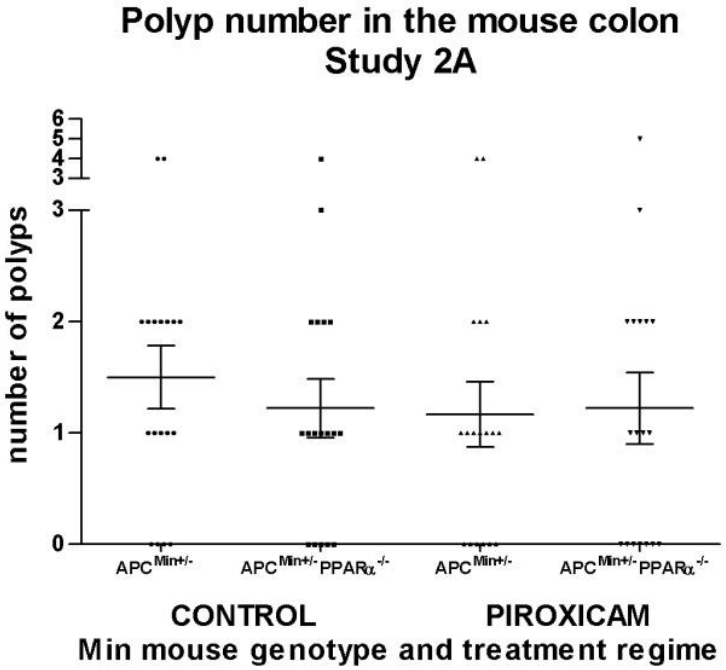
Table 5-9 Polyp number & Area in the colon of $APC^{Min/+}$ piroxicam group mice at sacrifice

Mouse	Group	Genotype	Polyp number	Polyp number/week of life	Total area (mm^2)	Total area/ week of life (mm^2)	Average polyp size ($mm^{2)}$	Average polyp size/ week of life (mm^2)
98b.4/1	piroxicam	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
98b.4/2	piroxicam	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
98b.4/5	piroxicam	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
98b.4/6	piroxicam	$APC^{Min/+}$	1	0.02	3.82	0.08	3.82	0.08
101.6/2	piroxicam	$APC^{Min/+}$	4	0.08	9.01	0.17	2.25	0.04
101.6/3	piroxicam	$APC^{Min/+}$	1	0.02	0.74	0.01	0.74	0.01
104.5/3	piroxicam	$APC^{Min/+}$	2	0.04	9.28	0.18	4.64	0.09
104.5/1	piroxicam	$APC^{Min/+}$	1	0.02	3.23	0.06	3.23	0.06
104.5/2	piroxicam	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
104.5/8	piroxicam	$APC^{Min/+}$	2	0.04	7.38	0.14	3.69	0.07
113.4/1	piroxicam	$APC^{Min/+}$	1	0.02	1.99	0.04	1.99	0.04
113.4/2	piroxicam	$APC^{Min/+}$	1	0.02	8.34	0.16	8.34	0.16
115.2/3	piroxicam	$APC^{Min/+}$	1	0.02	1.56	0.03	1.56	0.03
115.2/6	piroxicam	$APC^{Min/+}$	1	0.02	0.96	0.02	0.96	0.02
118.2/5	piroxicam	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
120.1/1	piroxicam	$APC^{Min/+}$	2	0.04	24.05	0.46	12.03	0.23
120.1/2	piroxicam	$APC^{Min/+}$	4	0.08	30.07	0.58	7.52	0.14
120.1/3	piroxicam	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
		MEAN	**1**	**0.02**	**5.58**	**0.11**	**2.82**	**0.05**

Table 5-10 Polyp number & Area in the colon of APCMin/+ PPARα-/- piroxicam group mice at sacrifice

Mouse	Group	Genotype	Polyp number	Polyp number/week of life	Total area (mm^2)	Total area/ week of life (mm^2)	Average polyp size ($mm^{2)}$	Average polyp size/ week of life (mm^2)
94.3/2	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	0	0.00	0.00	0.00	0.00	0.00
98.3/2	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	1	0.02	7.95	0.19	7.95	0.19
98.3/3	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	1	0.02	10.00	0.24	10.00	0.24
101.1/5	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	5	0.10	40.75	0.78	8.15	0.16
101.1/6	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	3	0.06	13.44	0.26	4.48	0.09
101.1/7	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	2	0.04	11.68	0.22	5.84	0.11
102.1/3	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	0	0.00	0.00	0.00	0.00	0.00
102.4/2	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	2	0.04	3.34	0.06	1.67	0.03
102.4/3	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	0	0.00	0.00	0.00	0.00	0.00
104.3/6	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	0	0.00	0.00	0.00	0.00	0.00
107.3/2	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	0	0.00	0.00	0.00	0.00	0.00
108.1/1	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	1	0.02	1.94	0.04	1.94	0.04
108.1/2	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	2	0.06	17.06	0.50	8.53	0.25
108.1/3	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	2	0.04	13.00	0.25	6.50	0.12
115.1/7	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	1	0.02	0.82	0.02	0.82	0.02
115.1/8	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	0	0.00	0.00	0.00	0.00	0.00
115.1/9	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	2	0.04	1.25	0.02	0.63	0.01
116.2/2	piroxicam	$APC^{Min/+}$ $PPAR\alpha^{-/-}$	0	0.00	0.00	0.00	0.00	0.00
		MEAN	**1**	**0.03**	**6.73**	**0.14**	**3.14**	**0.07**

A



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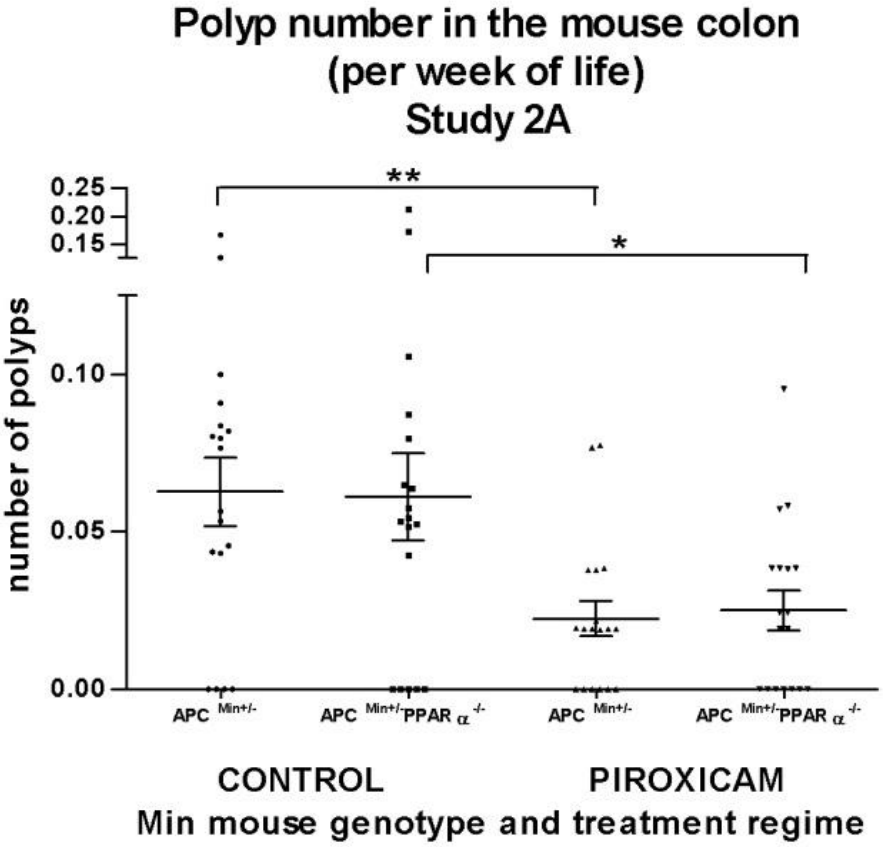
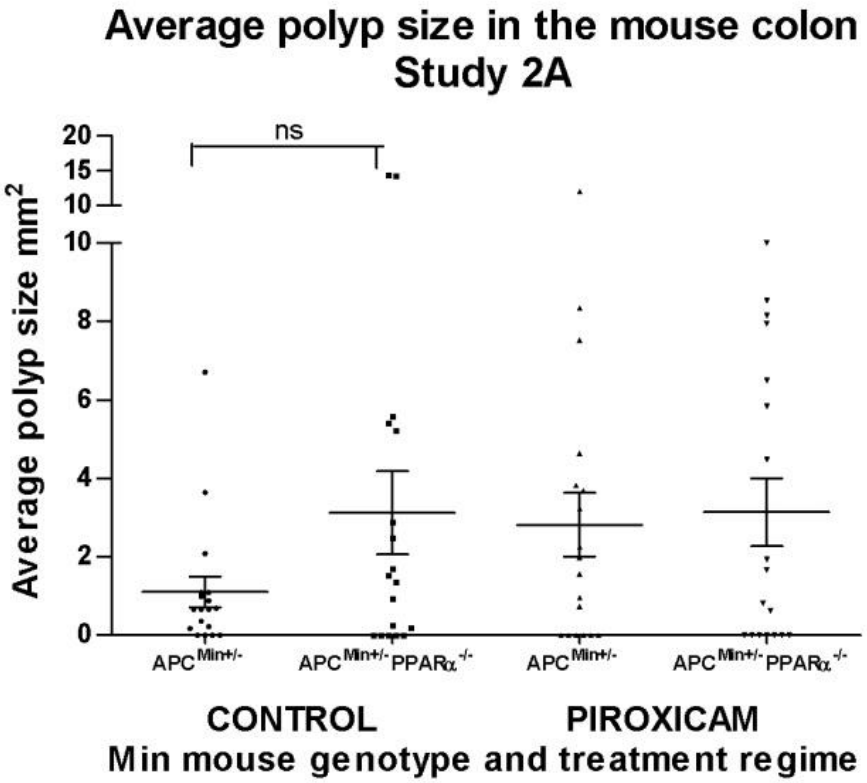


Figure 5-3 Polyp number in the colon of APC$^{Min/+}$ & APC$^{Min/+}$ PPARα$^{-/-}$ mice after treatment with piroxicam or control diet

Unpaired t test, * p <= 0.05, ** p <= 0.001

C



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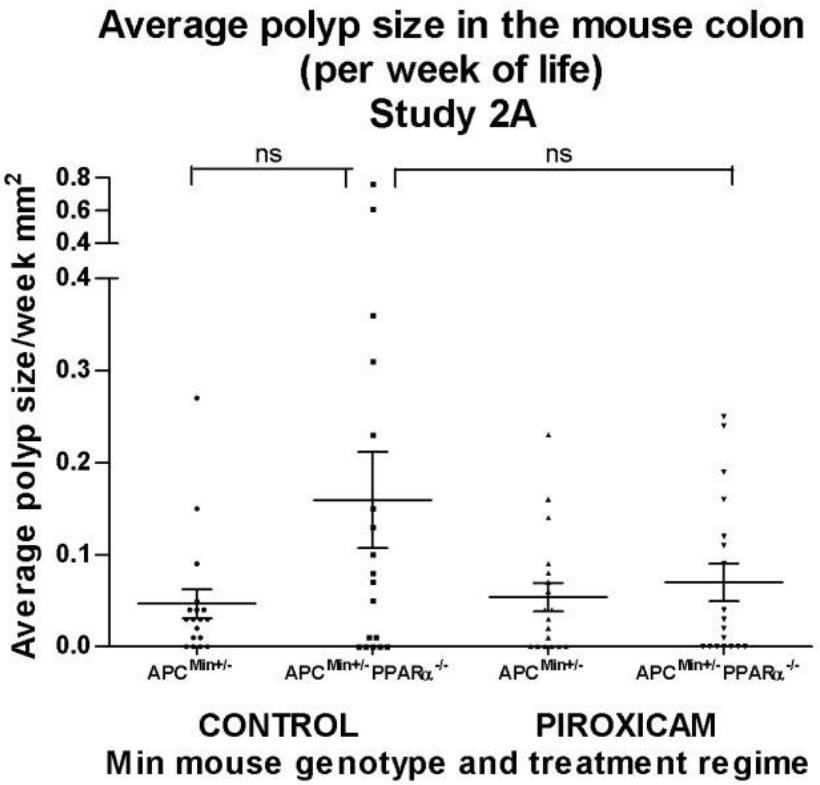
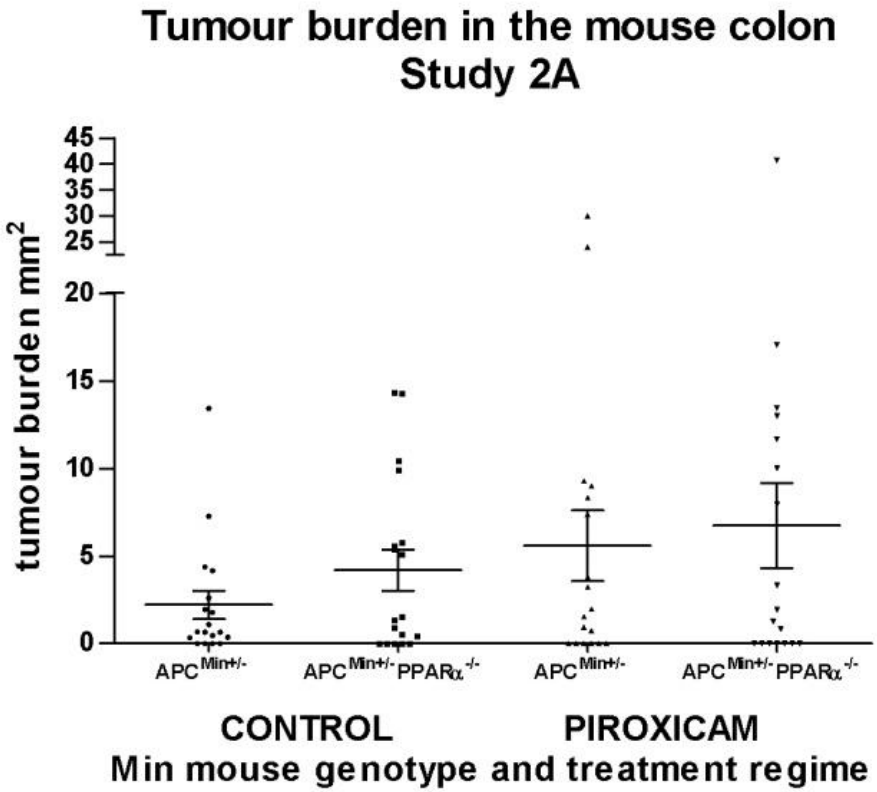


Figure 5-4 Polyp size in the colon of APC$^{Min/+}$ & APC$^{Min/+}$ PPARα$^{-/-}$ mice after treatment with piroxicam or control diet

Unpaired t test, ns not significant

E



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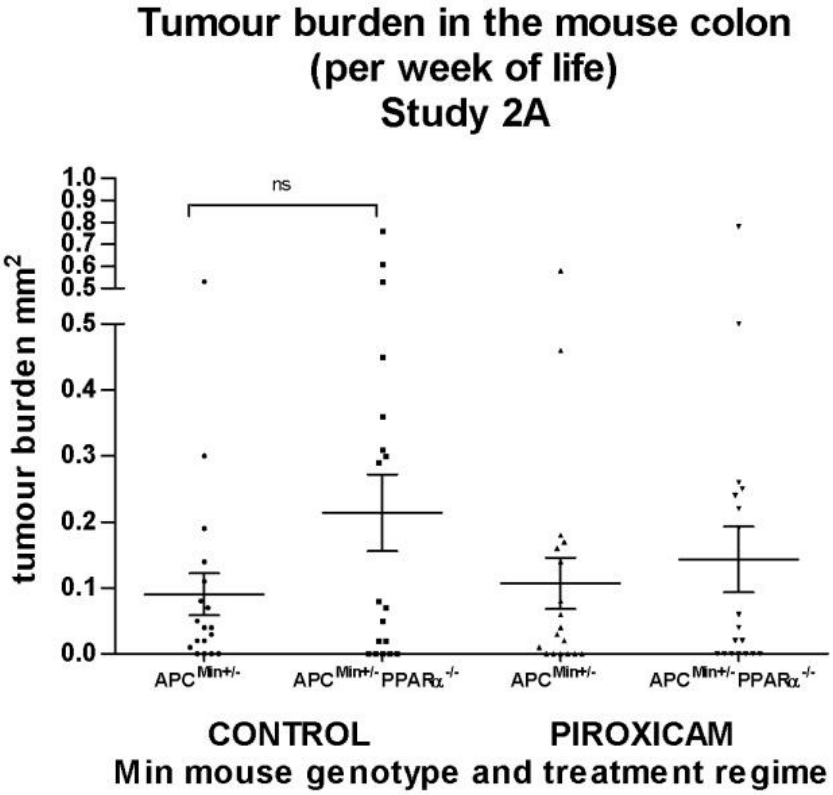


Figure 5-5 Tumour burden in the colon of APC$^{Min/+}$ & APC$^{Min/+}$ PPARα$^{-/-}$ mice after treatment with piroxicam or control diet

Unpaired t test, ns not significant

5.7.5 Average polyp size in the mouse colon (tumour burden/number of polyps): Study 2A

Mean average polyp size was not significantly different in $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice or whether they had received piroxicam treatment or not (Figure 5-3C, Table 5-7-Table 5-10).

Mean average polyp size was larger in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice (3.12 +/- 1.06 mm^2) than in $APC^{Min/+}$ control mice (1.11 +/- 0.39 mm^2) but the difference was not significant (Figure 5-3C, Table 5-7-Table 5-10). Also, piroxicam-treated $APC^{Min/+}$ mice had larger mean average polyp size than $APC^{Min/+}$ control mice but the increase was not significant (2.82 +/- 0.81 mm^2).

However, the mean average polyp size in piroxicam-treated $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice (3.14 +/- 0.86 mm^2) was very similar to mean average polyp size in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice (Figure 5-3C, Table 5-7-Table 5-10).

Similarly, when age at sacrifice was considered (mean average polyp size per week of life), there was no significant difference between $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice or whether they had received piroxicam or not (Figure 5-3D, Table 5-7-Table 5-10).

Mean average polyp size/week was larger in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice (0.16 +/- 0.05 mm^2) than in $APC^{Min/+}$ control mice (0.05 +/- 0.02 mm^2) but the difference was not significant (Figure 5-3D, Table 5-7-Table 5-10).

Also, piroxicam-treated $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice had a lower mean average polyp size/week (0.07 +/- 0.02 mm^2) than $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice (p was not significant).

However, mean average polyp size/week in piroxicam-treated $APC^{Min/+}$ mice (0.05 +/- 0.02 mm^2) was identical in $APC^{Min/+}$ control mice (Figure 5-3D, Table 5-7-Table 5-10).

5.7.6 Tumour burden (total area) in the mouse colon: Study 2A

At sacrifice there was no significant difference in mean tumour burden in the mouse colon between $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice, or whether the mice were in a control group or piroxicam-treated group. Mean tumour burden in $APC^{Min/+}$ control mice was 2.22 +/- 0.80 mm^2 and in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice it was 4.19 +/- 1.18 mm^2. In piroxicam-treated mice, mean tumour burden was 5.58 +/- 2.02 mm^2 in $APC^{Min/+}$ mice and 6.74 +/- 2.43 mm^2 in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice (Figure 5-3E, Table 5-7-Table 5-10).

The same profile of results were seen when age at sacrifice was considered (mean tumour burden per week of life, mean tumour burden/week). There was no significant difference in mean tumour burden/week between $APC^{Min/+}$ mice and $APC^{Min/+}PPAR\alpha^{-/-}$ mice, or whether the mice were in a control group or piroxicam-treated group.

Mean tumour burden/week in control mice was 0.09 +/- 0.03 mm^2 in $APC^{Min/+}$ mice and 0.21 +/- 0.06 mm^2 in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice. In piroxicam-treated mice, tumour burden/week was 0.11 +/= 0.04 mm^2 in $APC^{Min/+}$ mice and 0.14 +/- 0.05 mm^2 in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice (Figure 5-3F, Table 5-7-Table 5-10).

5.7.7 Number of polyps in the mouse small bowel: Study 2A

The mean number of polyps in the mouse small bowel between control groups was not significantly different; 7.39 +/- 1.61 in $APC^{Min/+}$ mice and 14.28 +/- 3.62 in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice, Figure 5-6A, Table 5-11-Table 5-14). Also, there was no significant difference in the mean number of polyps in piroxicam-treated mice. However, mice treated with piroxicam had significantly fewer mean polyp numbers than in control mice; 2.11 +/- 0.57 in $APC^{Min/+}$ mice ($p = 0.0055$) and 1.50 +/- 0.41 in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice ($p = 0.0006$, Figure 5-6A, Table 5-11-Table 5-14).

The mean polyp number per week of life (mean polyp number/week) was also reduced in mice treated with piroxicam compared to control mice. The mean number of polyps/week in piroxicam-treated $APC^{Min/+}$ mice was 0.04 +/- 0.01 and 0.30 +/- 0.06 in $APC^{Min/+}$ control mice ($p = 0.0003$, Figure 5-6B, Table 5-11-Table 5-14). Similarly, in piroxicam-treated $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice, the mean number of polyps/week was 0.04 +/- 0.01 and 0.71 +/- 0.19 in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice ($p = 0.0003$, Figure 5-6B, Table 5-11-Table 5-14). There was no significant difference in the mean number of polyps/week between $APC^{Min/+}$ control mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice, or $APC^{Min/+}$ piroxicam-treated mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ piroxicam-treated mice.

Table 5-11 Polyp number & Area in the small bowel of $APC^{Min/+}$ control group mice at sacrifice

Mouse	Group	Genotype	Polyp number	Polyp number/week of life	Total area (mm2)	Total area/week of life ($mm^{2)}$	Average polyp size ($mm^{2)}$	Average polyp size/week of life ($mm^{2)}$
104.5/7	control	$APC^{Min/+}$	23	0.73	39.92	1.26	1.74	0.05
122.1/5	control	$APC^{Min/+}$	12	0.64	15.42	0.82	1.28	0.07
114.3/4	control	$APC^{Min/+}$	10	0.46	8.58	0.39	0.86	0.04
122.1/7	control	$APC^{Min/+}$	11	0.44	13.59	0.55	1.24	0.05
116.1/3	control	$APC^{Min/+}$	11	0.46	13.04	0.54	1.19	0.05
122.1/6	control	$APC^{Min/+}$	8	0.33	19.69	0.82	2.46	0.10
97.3/5	control	$APC^{Min/+}$	10	0.41	18.07	0.74	1.81	0.07
104.6/8	control	$APC^{Min/+}$	2	0.11	2.55	0.14	1.28	0.07
101.6/6	control	$APC^{Min/+}$	22	0.84	72.99	2.80	3.32	0.13
123.1/3	control	$APC^{Min/+}$	4	0.16	3.30	0.13	0.82	0.03
114.3/5	control	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
122.1/3	control	$APC^{Min/+}$	5	0.22	15.34	0.67	3.07	0.13
120.3/6	control	$APC^{Min/+}$	1	0.05	0.32	0.01	0.32	0.01
122.1/1	control	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
114.3/6	control	$APC^{Min/+}$	6	0.23	53.57	2.05	8.93	0.34
119.3/1	control	$APC^{Min/+}$	2	0.08	29.11	1.12	14.56	0.56
121.4/7	control	$APC^{Min/+}$	5	0.20	30.34	1.21	6.07	0.24
122.3/9	control	$APC^{Min/+}$	1	0.04	13.23	0.57	13.23	0.57
		MEAN	**7**	**0.30**	**19.39**	**0.77**	**3.45**	**0.14**

Table 5-12 Polyp number & Area in the small bowel of $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control group mice at sacrifice

Mouse	Group	Genotype	Polyp number	Polyp number/week of life	Total area (mm2)	Total area/week of life ($mm^{2)}$	Average polyp size ($mm^{2)}$	Average polyp size/week of life ($mm^{2)}$
116.1/3	control	$APC^{Min/+}$ PPARα-/-	3	0.13	16.21	0.68	5.40	0.23
101.1/3	control	$APC^{Min/+}$ PPARα-/-	11	0.35	53.07	1.71	4.82	0.16
103.2/7	control	$APC^{Min/+}$ PPARα-/-	15	0.48	21.86	0.70	1.46	0.05
107.1/3	control	$APC^{Min/+}$ PPARα-/-	34	1.95	140.18	8.06	4.12	0.24
108.1/5	control	$APC^{Min/+}$ PPARα-/-	10	0.55	34.04	1.86	3.40	0.19
79b.4/2	control	$APC^{Min/+}$ PPARα-/-	19	1.16	77.50	4.73	4.08	0.25
104.3/5	control	$APC^{Min/+}$ PPARα-/-	16	0.83	45.10	2.34	2.82	0.15
107.1/9	control	$APC^{Min/+}$ PPARα-/-	51	2.95	152.65	8.82	2.99	0.17
102.1/6	control	$APC^{Min/+}$ PPARα-/-	6	0.26	15.10	0.65	2.52	0.11
104.3/4	control	$APC^{Min/+}$ PPARα-/-	7	0.37	17.88	0.94	2.55	0.13
107.3/1	control	$APC^{Min/+}$ PPARα-/-	11	0.58	16.01	0.85	1.46	0.08
118.2/3	control	$APC^{Min/+}$ PPARα-/-	0	0.00	0.00	0.00	0.00	0.00
102.1/8	control	$APC^{Min/+}$ PPARα-/-	48	1.92	219.55	8.78	4.57	0.18
108.1/6	control	$APC^{Min/+}$ PPARα-/-	1	0.05	0.30	0.01	0.30	0.01
116.1/1	control	$APC^{Min/+}$ PPARα-/-	16	0.70	83.00	3.62	5.19	0.23
118.2/7	control	$APC^{Min/+}$ PPARα-/-	0	0.00	0.00	0.00	0.00	0.00
115.1/2	control	$APC^{Min/+}$ PPARα-/-	0	0.00	0.00	0.00	0.00	0.00
118.2/9	control	$APC^{Min/+}$ PPARα-/-	9	0.52	37.96	2.18	4.22	0.24
		MEAN	**14.3**	**0.71**	**51.69**	**2.55**	**2.77**	**0.13**

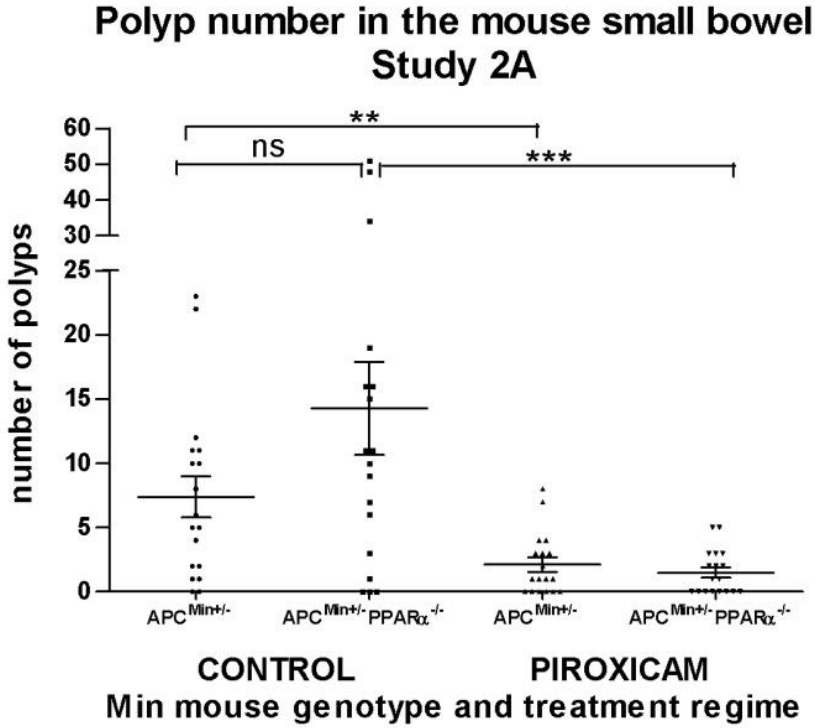
Table 5-13 Polyp number & Area in the small bowel of $APC^{Min/+}$ piroxicam group mice at sacrifice

Mouse	Group	Genotype	Polyp number	Polyp number/week of life	Total area (mm2)	Total area/week of life ($mm^{2)}$	Average polyp size ($mm^{2)}$	Average polyp size/week of life ($mm^{2)}$
98b.4/2	piroxicam	$APC^{Min/+}$	8	0.15	10.47	0.19	1.31	0.02
98b.4/5	piroxicam	$APC^{Min/+}$	1	0.02	0.37	0.01	0.37	0.01
115.2/3	piroxicam	$APC^{Min/+}$	4	0.08	9.28	0.18	2.32	0.04
104.5/2	piroxicam	$APC^{Min/+}$	4	0.08	3.36	0.06	0.84	0.02
118.2/5	piroxicam	$APC^{Min/+}$	3	0.06	6.68	0.13	2.23	0.04
104.5/3	piroxicam	$APC^{Min/+}$	2	0.04	2.60	0.05	1.30	0.02
104.5/1	piroxicam	$APC^{Min/+}$	3	0.06	1.08	0.02	0.36	0.01
98b.4/6	piroxicam	$APC^{Min/+}$	3	0.07	1.84	0.04	0.61	0.01
101.6/3	piroxicam	$APC^{Min/+}$	7	0.14	3.71	0.07	0.53	0.01
113.4/1	piroxicam	$APC^{Min/+}$	1	0.02	0.54	0.01	0.54	0.01
113.4/2	piroxicam	$APC^{Min/+}$	1	0.02	0.33	0.01	0.33	0.01
104.5/8	piroxicam	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
115.2/6	piroxicam	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
101.6/2	piroxicam	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
120.1/1	piroxicam	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
120.1/2	piroxicam	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
120.1/3	piroxicam	$APC^{Min/+}$	1	0.02	0.50	0.01	0.50	0.01
98b.4/1	piroxicam	$APC^{Min/+}$	0	0.00	0.00	0.00	0.00	0.00
		MEAN	**2.1**	**0.04**	**2.26**	**0.04**	**0.62**	**0.01**

Table 5-14 Polyp number & Area in the small bowel of $APC^{Min/+}$ $PPAR\alpha^{-/-}$ piroxicam group mice at sacrifice

Mouse	Group	Genotype	Polyp number	Polyp number/week of life	Total area (mm2)	Total area/week of life ($mm^{2)}$	Average polyp size ($mm^{2)}$	Average polyp size/week of life ($mm^{2)}$
98.3/3	piroxicam	$APC^{Min/+}$ PPARα-/-	2	0.05	0.79	0.02	0.40	0.01
102.4/2	piroxicam	$APC^{Min/+}$ PPARα-/-	2	0.04	2.26	0.04	1.13	0.02
102.4/3	piroxicam	$APC^{Min/+}$ PPARα-/-	3	0.06	1.62	0.03	0.54	0.01
101.1/5	piroxicam	$APC^{Min/+}$ PPARα-/-	5	0.10	4.34	0.08	0.87	0.02
108.1/2	piroxicam	$APC^{Min/+}$ PPARα-/-	3	0.09	2.22	0.06	0.74	0.02
104.3/6	piroxicam	$APC^{Min/+}$ PPARα-/-	5	0.11	4.32	0.09	0.86	0.02
107.3/2	piroxicam	$APC^{Min/+}$ PPARα-/-	3	0.10	1.72	0.06	0.57	0.02
101.1/7	piroxicam	$APC^{Min/+}$ PPARα-/-	1	0.02	0.31	0.01	0.31	0.01
108.1/3	piroxicam	$APC^{Min/+}$ PPARα-/-	1	0.02	0.58	0.01	0.58	0.01
102.1/3	piroxicam	$APC^{Min/+}$ PPARα-/-	2	0.09	0.73	0.03	0.37	0.02
116.2/2	piroxicam	$APC^{Min/+}$ PPARα-/-	0	0.00	0.00	0.00	0.00	0.00
108.1/1	piroxicam	$APC^{Min/+}$ PPARα-/-	0	0.00	0.00	0.00	0.00	0.00
94.3/2	piroxicam	$APC^{Min/+}$ PPARα-/-	0	0.00	0.00	0.00	0.00	0.00
115.1/7	piroxicam	$APC^{Min/+}$ PPARα-/-	0	0.00	0.00	0.00	0.00	0.00
101.1/6	piroxicam	$APC^{Min/+}$ PPARα-/-	0	0.00	0.00	0.00	0.00	0.00
98.3/2	piroxicam	$APC^{Min/+}$ PPARα-/-	0	0.00	0.00	0.00	0.00	0.00
115.1/8	piroxicam	$APC^{Min/+}$ PPARα-/-	0	0.00	0.00	0.00	0.00	0.00
115.1/9	piroxicam	$APC^{Min/+}$ PPARα-/-	0	0.00	0.00	0.00	0.00	0.00
		MEAN	**1.5**	**0.04**	**1.05**	**0.02**	**0.35**	**0.01**

A



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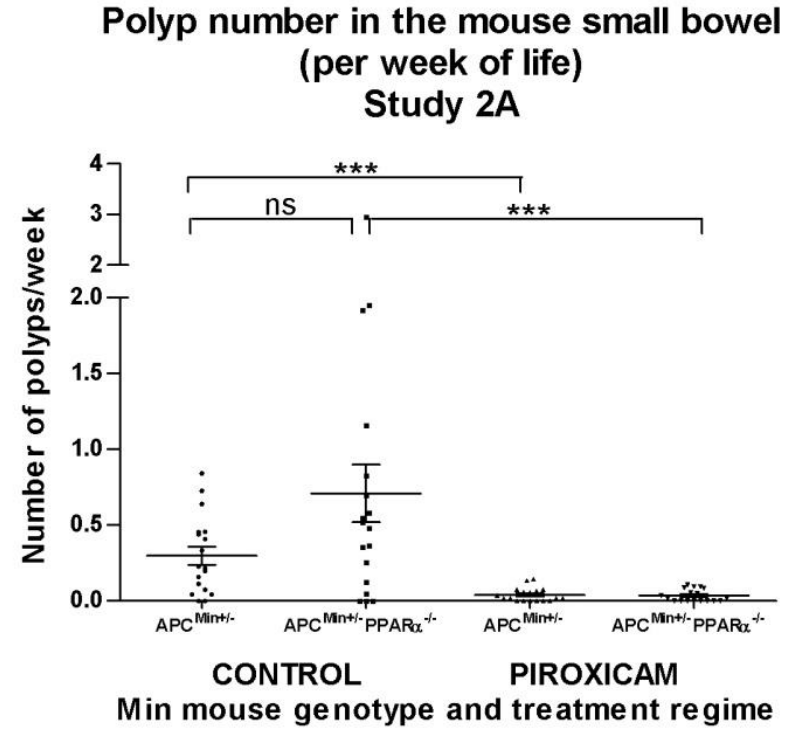
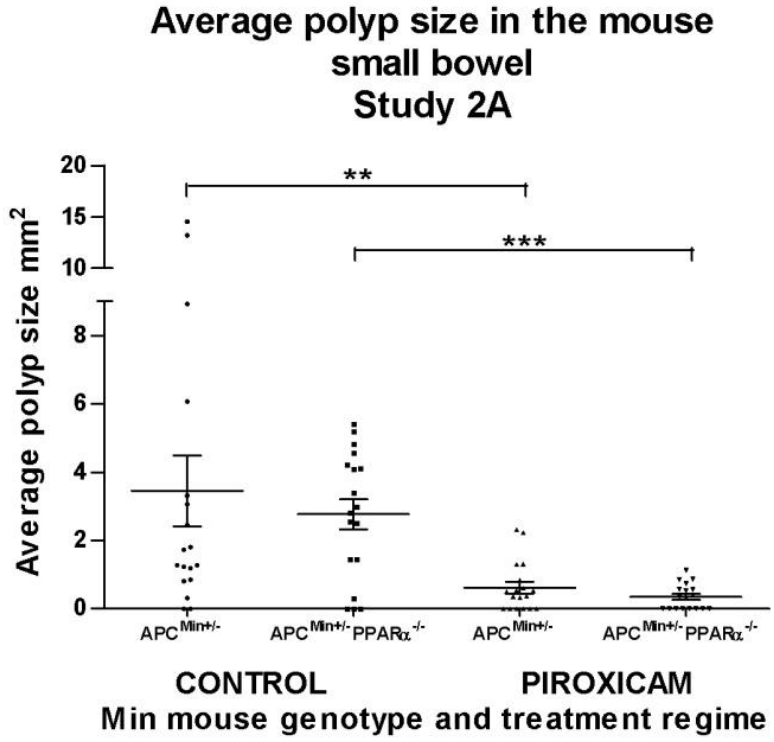


Figure 5-6 Polyp number in the small bowel of APC$^{Min/+}$ & APC$^{Min/+}$ PPARα$^{-/-}$ mice after treatment with piroxicam or control diet

Unpaired t test, ** p <= 0.001, *** p <= 0.0001, ns not significant

C



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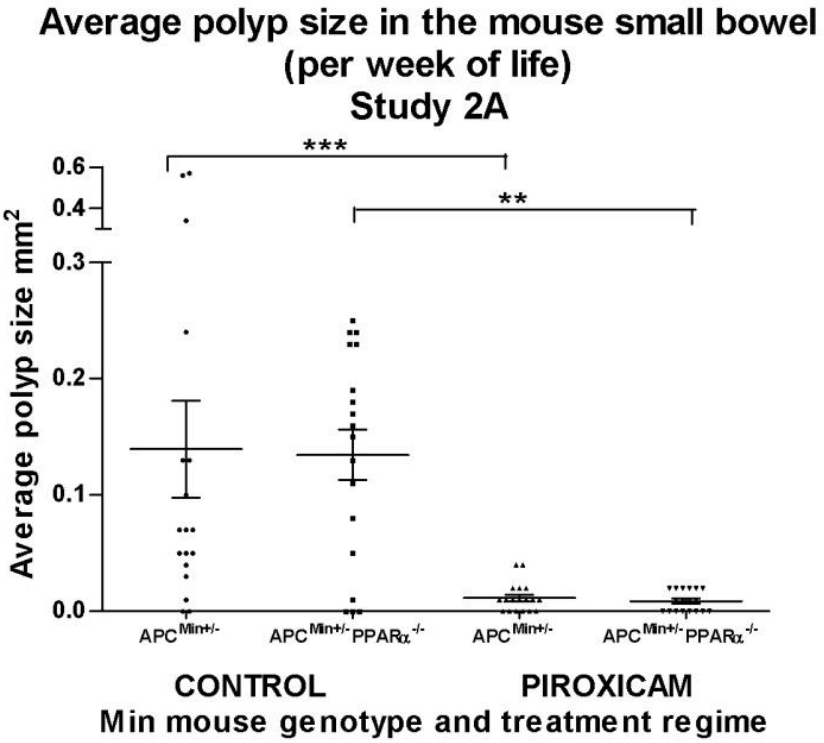


Figure 5-7 Polyp size in the small bowel of APC$^{Min/+}$ & APC$^{Min/+}$ PPARα$^{-/-}$ mice after treatment with piroxicam or control diet

Unpaired t test, ** p <= 0.001, *** p <= 0.0001

5.7.8 Average polyp size in the mouse small bowel (tumour burden/number of polyps): Study 2A

Piroxicam-treated $APC^{Min/+}$ mice had a significantly reduced mean average polyp size of 0.62 +/- 0.17 mm^2 compared to 3.45 +/- 1.04 mm^2 in $APC^{Min/+}$ control mice (p = 0.004, Figure 5-6C, Table 5-11-Table 5-14). Similarly, the mean average polyp size of $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice was 2.77 +/- 0.44 mm^2 which was far larger than in piroxicam-treated mice. Piroxicam-treated $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice had a mean average polyp size of 0.35 +/- 0.09 mm^2 (p < 0.0001, Figure 5-6C, Table 5-11-Table 5-14). The mean average polyp size did not differ significantly between control groups or between piroxicam-treated groups.

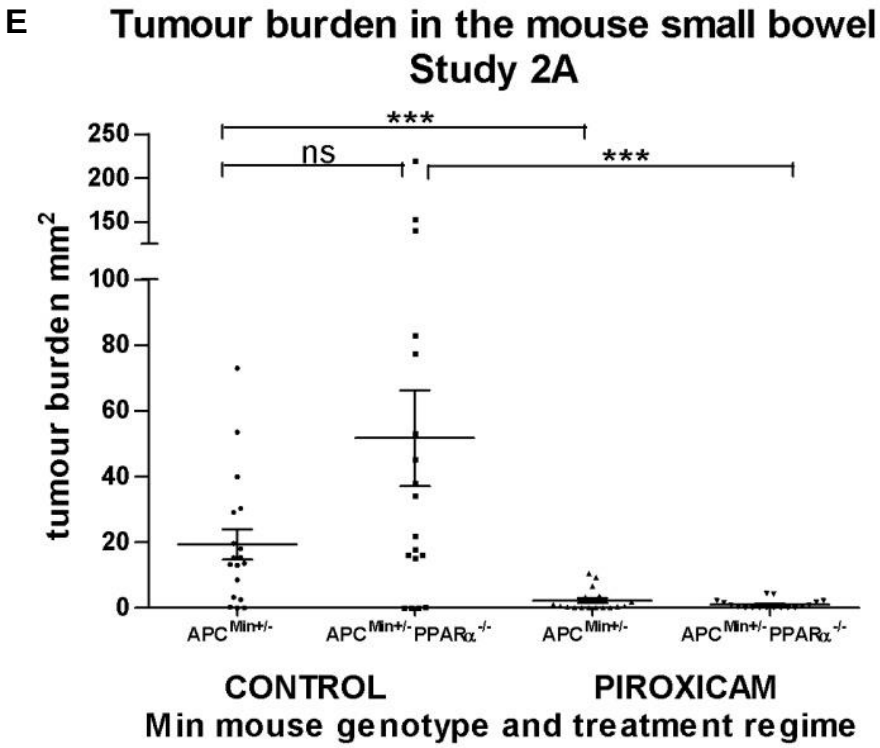
The same profile of results were seen when age at sacrifice was taken into consideration (mean average polyp size per week of life). Piroxicam-treated $APC^{Min/+}$ mice had a mean average polyp size of 0.01 +/- 0.003 mm^2 compared to 0.14 +/- 0.04 mm^2 in $APC^{Min/+}$ control mice (p = 0.0001, Figure 5-6D, Table 5-11-Table 5-14). Also, mean average polyp size/week in piroxicam-treated $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice was 0.009 +/- 0.002 mm^2 and 0.13 +/- 0.02 mm^2 in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice (p = 0.0003, Figure 5-6D, Table 5-11-Table 5-14). There was no significant difference in mean average polyp size/week between control groups or between piroxicam groups.

5.7.9 Tumour burden (total area) in the mouse small bowel: Study 2A

Piroxicam treatment significantly reduced tumour burden. The mean tumour burden in $APC^{Min/+}$ control mice was 19.39 +/- 4.64 mm^2 and 2.26 +/- 0.78 mm^2 in $APC^{Min/+}$ piroxicam-treated mice (p = 0.0007, Figure 5-6E, Table 5-11-Table 5-14). Similarly in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice mean tumour burden was 51.69 +/- 14.57 mm^2 and 1.05 +/- 0.34 mm^2 in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ piroxicam-treated mice (p = 0.0004,

Figure 5-6E, Table 5-11-Table 5-14). However, there was no significant difference in mean tumour burden between control groups or between piroxicam-treated groups.

Similar results were seen when age at sacrifice was considered (mean tumour burden per week of life). The mean tumour burden/week in $APC^{Min/+}$ control mice was 0.77 +/- 0.17 mm^2 and 0.04 +/- 0.01 mm^2 in $APC^{Min/+}$ piroxicam-treated mice (p = 0.0001, Figure 5-6F, Table 5-11-Table 5-14). $APC^{Min/+}$ $PPAR\alpha^{-/-}$ control mice had a mean tumour burden/week of 2.55 +/- 0.72 mm^2, whereas in piroxicam-treated $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice it was 0.02 +/- 0.01 mm^2 (p = 0.0003, Figure 5-6F, Table 5-11-Table 5-14). There was no significant difference in mean tumour burden/week between control groups or between piroxicam-treated groups.



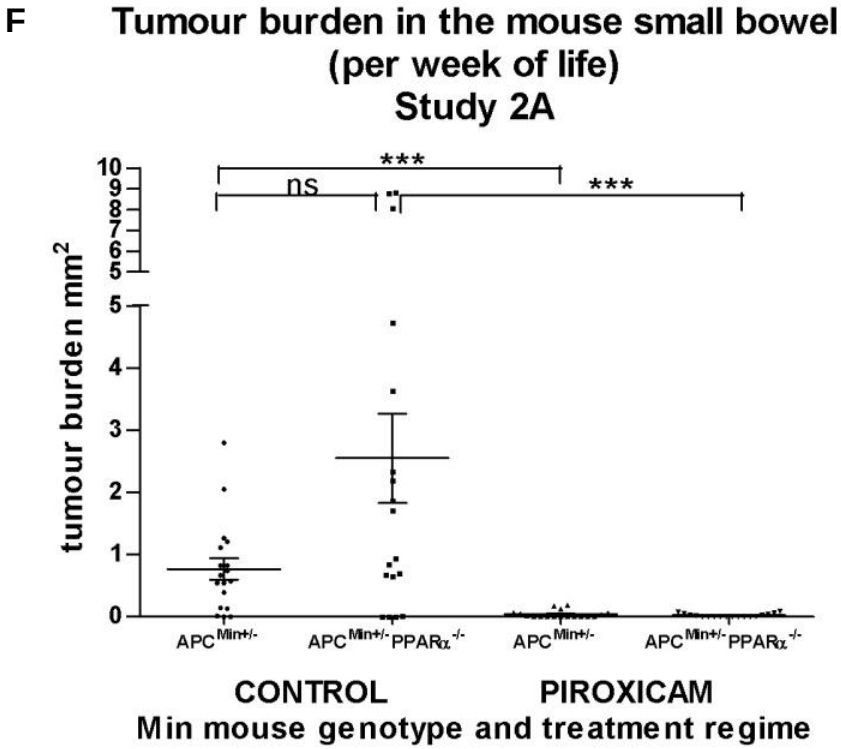


Figure 5-8 Tumour burden in the small bowel of APC$^{Min/+}$ & APC$^{Min/+}$ PPARα$^{-/-}$ mice after treatment with piroxicam or control diet

Unpaired t test, *** p <= 0.0001, ns not significant

In summary, these data indicated that the effect of piroxicam on polyp number, total tumour burden and average polyp size in the colon and small bowel was not mediated *via* PPARα.

$APC^{Min+/-}$ (control and piroxicam-treated) mice were significantly older than $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ mice at sacrifice. Also, $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ mice weighed more than $APC^{Min+/-}$ mice, but only at the beginning of the study. All control and piroxicam-treated mice increased in weight during the study, but there was no significant difference in weight at sacrifice between $APC^{Min+/-}$ mice and $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ mice. Conversely, mesenteric fat was greater in $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ mice than $APC^{Min+/-}$ mice at sacrifice, but only in control groups and not piroxicam-treated groups.

5.7.10 The effect of piroxicam on gene expression in the mouse colon

The mean relative quantity (RQ) of gene expression in $APC^{Min/+}$ normal, $APC^{Min/+}$ tumour, $APC^{Min/+}PPAR\alpha^{-/-}$ normal and $APC^{Min/+}PPAR\alpha^{-/-}$ tumour piroxicam-treated groups to gene expression in their corresponding control groups were calculated from normalised average Ct values of Study 2B TaqMan® low density array results. Mean RQ data were presented as clustered bar graphs in groups as before (Figure 5-9-Figure 5-17) using a $\log_{10}$ scale for genes where RQ was greater than or equal to two (fold change increase >= 2) or less than or equal to 0.5 (fold change decrease >=2).

SPSS 16.0.0.247 was used to perform univariate analysis of variance on the same data. Genes were determined as differentially expressed due to; the effect of piroxicam treatment, or the effect of an interaction between piroxicam treatment and PPARα status, or the effect of an interaction between piroxicam treatment and tissue type, where $p <= 0.05$. These data were presented in groups as defined previously (Table 5-15-Table 5-23).

For simplicity all table and figure headings have been abbreviated as follows; $APC^{Min/+}$ abbreviated to Min, and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ abbreviated to $PPAR\alpha^{-/-}$.

5.7.10.1 Fatty acid & lipid metabolism

Table 5-15 and Figure 5-9 show expression data for the effect of piroxicam on genes implicated in fatty acid and lipid metabolism.

These data showed that in the piroxicam-treated $PPAR\alpha^{-/-}$ normal group, expression of Cyp2c55 (cytochrome P450, family 2, subfamily c, polypeptide 55) was slightly increased (1.75-fold), whereas in the piroxicam-treated Min normal group, expression of Cyp2c55 was decreased 3.70-fold. This may imply a PPARα mediated effect with piroxicam treatment ($p = 0.049$).

However, in tumour tissue expression of Cyp2c55 in the piroxicam-treated Min and $PPAR\alpha^{-/-}$ groups was down-regulated 3.85-fold and 20-fold respectively ($p = 0.04$).

Piroxicam treatment of the Min normal group significantly increased expression of Alox12 (arachidonate 12-lipoxygenase) 2.11-fold ($p = 0.044$). Cyp2c55 and Cyp2b10 (cytochrome P450, family 2, subfamily b, polypeptide 10) were significantly down-regulated; Cyp2c55 expression was reduced 3.70-fold ($p = 0.036$) and Cyp2b10 expression reduced 10-fold ($p = 0.038$) in the piroxicam-treated Min normal group.

The effect of an interaction between piroxicam and tissue type was apparent in five genes, including Cyp2c55; expression of Alox12 and Cyp2b10 were up-regulated in piroxicam-treated Min tumour and $PPAR\alpha^{-/-}$ tumour groups. Expression of Alox12 increased 5.80-fold and 2.81-fold ($p = 0.047$), and Cyp2b10 increased 2.83-fold and 6.41-fold ($p = 0.022$), in Min tumour and $PPAR\alpha^{-/-}$ tumour groups respectively.

Expression of Lrp1 (low density lipoprotein receptor-related protein 1) and Ptgis (prostaglandin I2 (prostacyclin) synthase) were down-

regulated in the piroxicam-treated PPARα$^{-/-}$ tumour group only. Lrp1 expression was reduced 2.5-fold (p = 0.008), and Ptgis expression was reduced 2.17-fold (p = 0.017).

Table 5-15 Effect of piroxicam on genes implicated in fatty acid & lipid metabolism

Gene	Mean Relative Quantity of gene expression to corresponding control group				p value		
	Min normal piroxicam	Min tumour piroxicam	PPARα$^{-/-}$ normal piroxicam	PPARα$^{-/-}$ tumour piroxicam	Effect of piroxicam only	Interaction between piroxicam & PPARa	Interaction between piroxicam & tissue
Acot2	0.74	0.26	1.62	0.53	ns	ns	ns
Alox 12	2.11	5.80	0.87	2.81	0.044	ns	0.047
Alox 15	0.42	2.38	1.23	1.16	ns	ns	ns
Alox 5ap	1.47	1.14	0.65	1.11	ns	ns	ns
Angptl4	0.19	0.35	2.85	0.54	ns	ns	ns
Bdh1	0.75	0.50	0.90	0.56	0.003	ns	ns
Cyp11a1	0.93	2.52	1.61	1.24	ns	ns	ns
Cyp2b10	0.10	2.83	1.80	6.41	0.038	ns	0.022
Cyp2c55	0.27	0.26	1.75	0.05	0.036	0.049	0.04
Decr1	0.67	0.75	0.84	0.59	0.007	ns	ns
Ech1	0.74	0.51	1.08	0.48	0.009	ns	ns
Echdc2	1.17	1.28	1.06	1.18	ns	ns	ns
Hadha	0.51	0.56	0.82	0.73	0.005	ns	ns
Lrp1	0.93	0.56	1.29	0.40	0.034	ns	0.008
Pla2g2a	0.77	1.23	0.44	1.05	ns	ns	ns
Ptgis	1.57	0.62	1.29	0.46	ns	ns	0.017
Ptgs1	1.61	0.51	0.88	0.86	ns	ns	ns
Ptgs2	2.35	2.16	0.86	1.14	ns	ns	ns
Steap4	0.63	1.54	2.23	2.52	0.034	ns	ns

Mean relative quantity (RQ) of gene expression in APC$^{Min/+}$ normal, APC$^{Min/+}$ tumour, APC$^{Min/+}$PPARα$^{-/-}$ normal and APC$^{Min/+}$PPARα$^{-/-}$ tumour samples to expression in corresponding control groups. Univariate analysis of variance was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

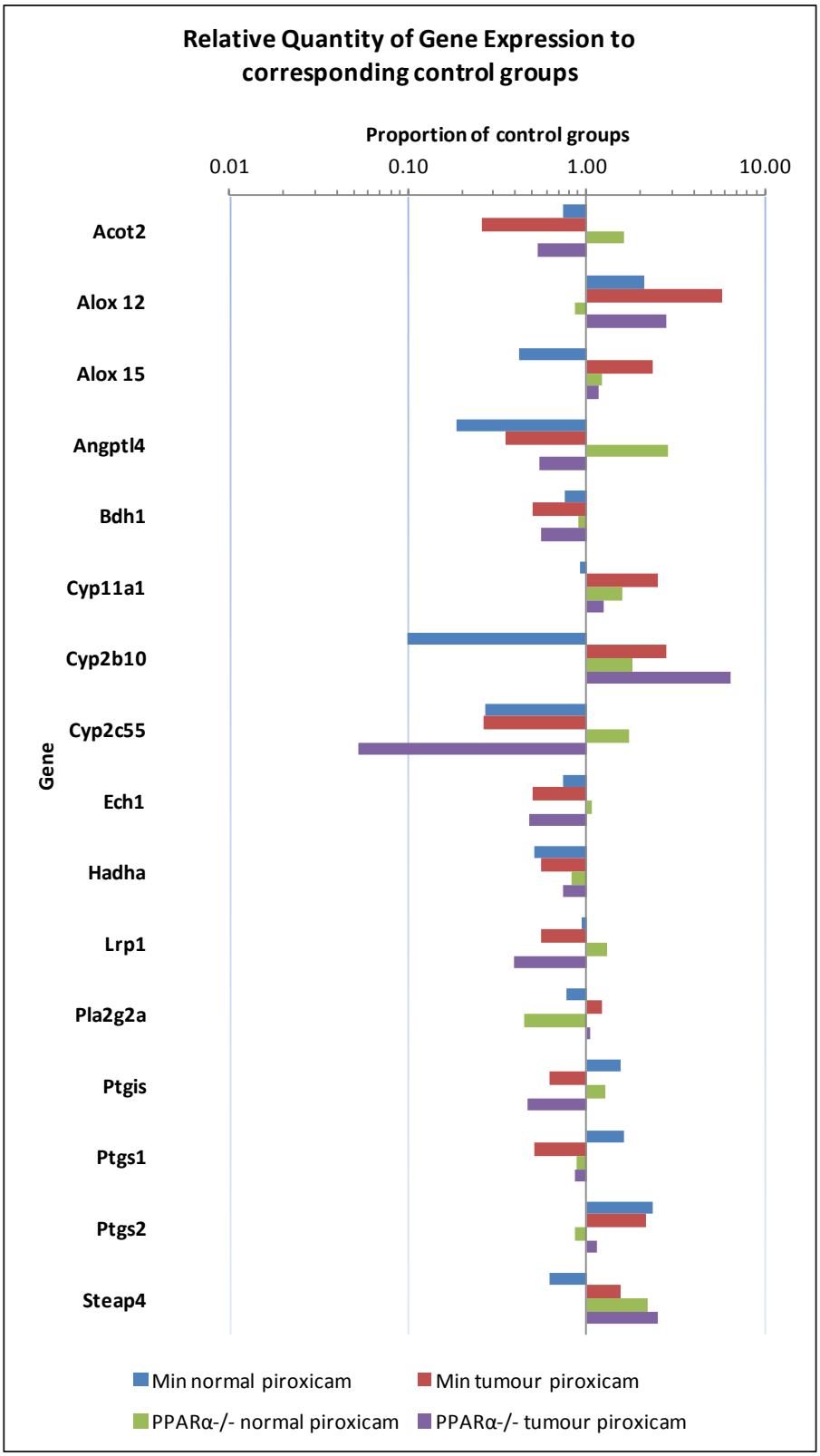


Figure 5-9 Effect of piroxicam on genes implicated in fatty acid & lipid metabolism

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of piroxicam groups to control groups, where RQ was >= 2 or <= to 0.5

5.7.10.2 Signal transduction

Table 5-16 and Figure 5-10 show expression data for the effect of piroxicam on genes implicated in signal transduction.

These data show expression of genes in this group were not altered by piroxicam *via* a PPARα pathway.

However, expression of Lama1 (laminin, alpha 1) was up-regulated 2.29-fold ($p = 0.004$) and Rhoj (ras homolog gene family, member J) was up-regulated 2.14-fold ($p = 0.031$) in the piroxicam-treated Min normal group.

Also, a significant interaction between piroxicam and tissue type was seen in Apcdd1 (adenomatosis polyposis coli down-regulated 1) and Lama1.

Expression of Apcdd1 was increased 2.90-fold and 2.68-fold ($p = 0.022$) in piroxicam-treated Min tumour and PPARα$^{-/-}$ tumour groups respectively.

Similarly, Lama1 was up-regulated 2.22-fold and 2.29-fold ($p = 0.004$) in tumour groups as before.

Table 5-16 Effect of piroxicam on genes implicated in signal transduction

Gene	Mean Relative Quantity of gene expression to corresponding control group				p value		
	Min normal piroxicam	Min tumour piroxicam	PPARα$^{-/-}$ normal piroxicam	PPARα$^{-/-}$ tumour piroxicam	Effect of piroxicam only	Interaction between piroxicam & PPARa	Interaction between piroxicam & tissue
Apcdd1	1.14	2.90	1.54	2.68	0.014	ns	0.022
Ereg	1.53	1.53	0.87	0.70	ns	ns	ns
Ghr	0.71	0.70	1.11	0.82	ns	ns	ns
Gulp1	1.83	0.57	1.56	0.66	ns	ns	0.0001
Il1b	1.20	4.48	0.72	1.79	ns	ns	ns
Il6	0.24	4.10	1.38	0.21	ns	ns	ns
Inhba	2.53	3.96	1.14	1.34	ns	ns	ns
Krt18	0.83	1.06	0.70	1.16	ns	ns	ns
Lama 1	2.29	2.22	0.61	2.29	0.004	ns	0.004
Lama 5	1.64	1.35	0.81	1.85	ns	ns	ns
Ppbp	0.34	1.68	0.67	1.17	ns	ns	ns
Ptger3	0.69	0.67	1.04	0.87	ns	ns	ns
Rhoj	2.14	1.13	1.23	1.51	0.031	ns	ns
Sfrp1	1.91	1.12	1.16	1.01	ns	ns	ns
Tnf	1.18	1.22	1.59	1.67	ns	ns	ns

Mean relative quantity (RQ) of gene expression in APC$^{Min/+}$ normal, APC$^{Min/+}$ tumour, APC$^{Min/+}$PPARα$^{-/-}$ normal and APC$^{Min/+}$PPARα$^{-/-}$ tumour samples to expression in corresponding control groups. Univariate analysis of variance was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

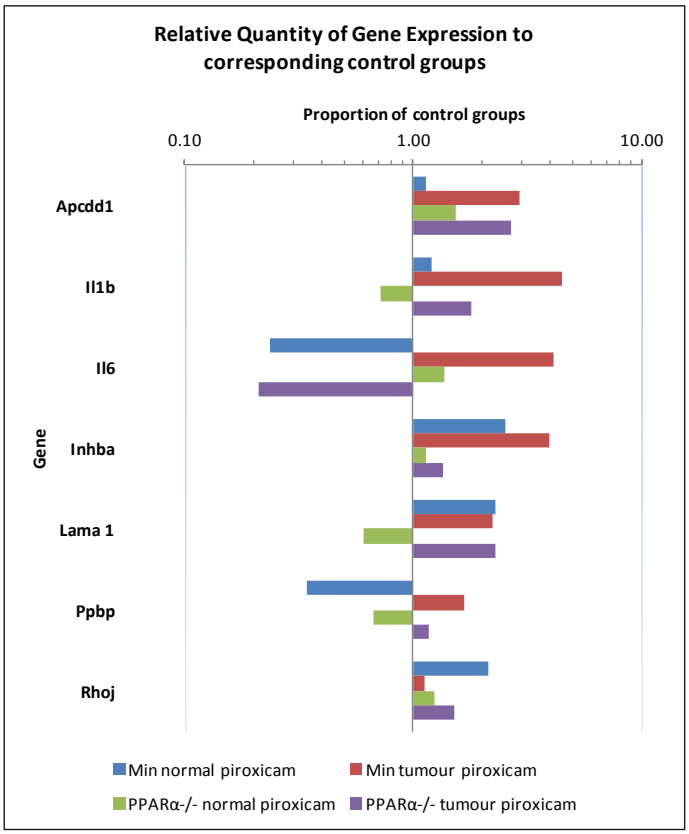


Figure 5-10 Effect of piroxicam on genes implicated in signal transduction

Gene expression represented on $\log_{10}$ scale clustered bar graph as relative quantity (RQ) of piroxicam groups to control groups, where RQ was >= 2 or <= to 0.5

5.7.10.3 Transcription

Table 5-15 and Figure 5-9 show expression data for the effect of piroxicam on genes implicated in transcription.

These data showed that in the piroxicam-treated PPARα$^{-/-}$ normal group, expression of Rorc (RAR-related orphan receptor gamma) was slightly decreased (1.75-fold), whereas in the piroxicam-treated Min normal group expression of Rorc was decreased 6.25-fold. This may imply a PPARα mediated effect with piroxicam treatment ($p = 0.016$).

Also, expression of Rorc in the piroxicam-treated Min and PPARα$^{-/-}$ tumour groups was downregulated 4.17-fold and 2.38-fold respectively, but this effect was not significant.

The effect of piroxicam treatment alone was significant in the expression of two genes; Nfkbie (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon) was up-regulated 2.15-fold ($p = 0.001$) and Rorc was down-regulated 6.25-fold ($p = 0.0001$) in the piroxicam-treated Min normal group.

Expression of Gucy2c (guanylate cyclase 2c) was significantly decreased 2.5-fold ($p = 0.003$) in the piroxicam-treated PPARα$^{-/-}$ tumour group only.

Table 5-17 Effect of piroxicam on genes implicated in transcription

Gene	Mean Relative Quantity of gene expression to corresponding control group				p value		
	Min normal piroxicam	Min tumour piroxicam	PPARα$^{-/-}$ normal piroxicam	PPARα$^{-/-}$ tumour piroxicam	Effect of piroxicam only	Interaction between piroxicam & PPARa	Interaction between piroxicam & tissue
Ang	0.89	0.52	0.88	0.23	0.029	ns	ns
Arnt2	1.82	1.42	2.05	1.17	ns	ns	ns
Baz1a	0.96	1.15	0.63	1.12	ns	ns	0.037
Cbx7	1.22	0.53	1.46	0.80	ns	ns	0.044
Chd8	0.88	0.84	0.90	1.11	ns	ns	ns
Crem	1.01	0.76	0.85	0.91	ns	ns	ns
Foxc2	33.31	1.30	0.41	3.62	ns	ns	ns
Gucy2c	1.20	0.52	1.13	0.40	ns	ns	0.003
Id4	2.18	0.59	1.29	0.53	ns	ns	0.001
Jun	0.80	0.87	0.52	1.34	ns	ns	ns
Klf4	0.59	0.38	0.46	0.64	0.0001	ns	ns
Meis 1	1.88	0.50	1.67	0.62	ns	ns	ns
Myc	1.14	1.62	0.83	1.48	ns	ns	ns
Nfkbie	2.15	1.54	1.45	1.44	0.001	ns	ns
Nr1d1	0.59	0.37	0.53	0.28	0.0001	ns	ns
Onecut2	1.95	3.98	0.41	1.21	ns	ns	ns
Phf17	0.80	0.63	0.89	0.87	0.006	ns	ns
Pou2af1	1.34	3.14	2.80	0.66	ns	ns	ns
Rarb	1.73	1.13	1.20	0.98	ns	ns	ns
Rbms1	0.98	0.99	0.97	1.28	ns	ns	ns
Rorc	0.16	0.24	0.57	0.42	0.0001	0.016	ns
Tcf12	0.70	0.87	0.99	1.07	ns	ns	ns

Mean relative quantity (RQ) of gene expression in APC$^{Min/+}$ normal, APC$^{Min/+}$ tumour, APC$^{Min/+}$PPARα$^{-/-}$ normal and APC$^{Min/+}$PPARα$^{-/-}$ tumour samples to expression in corresponding control groups. Univariate analysis of variance was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

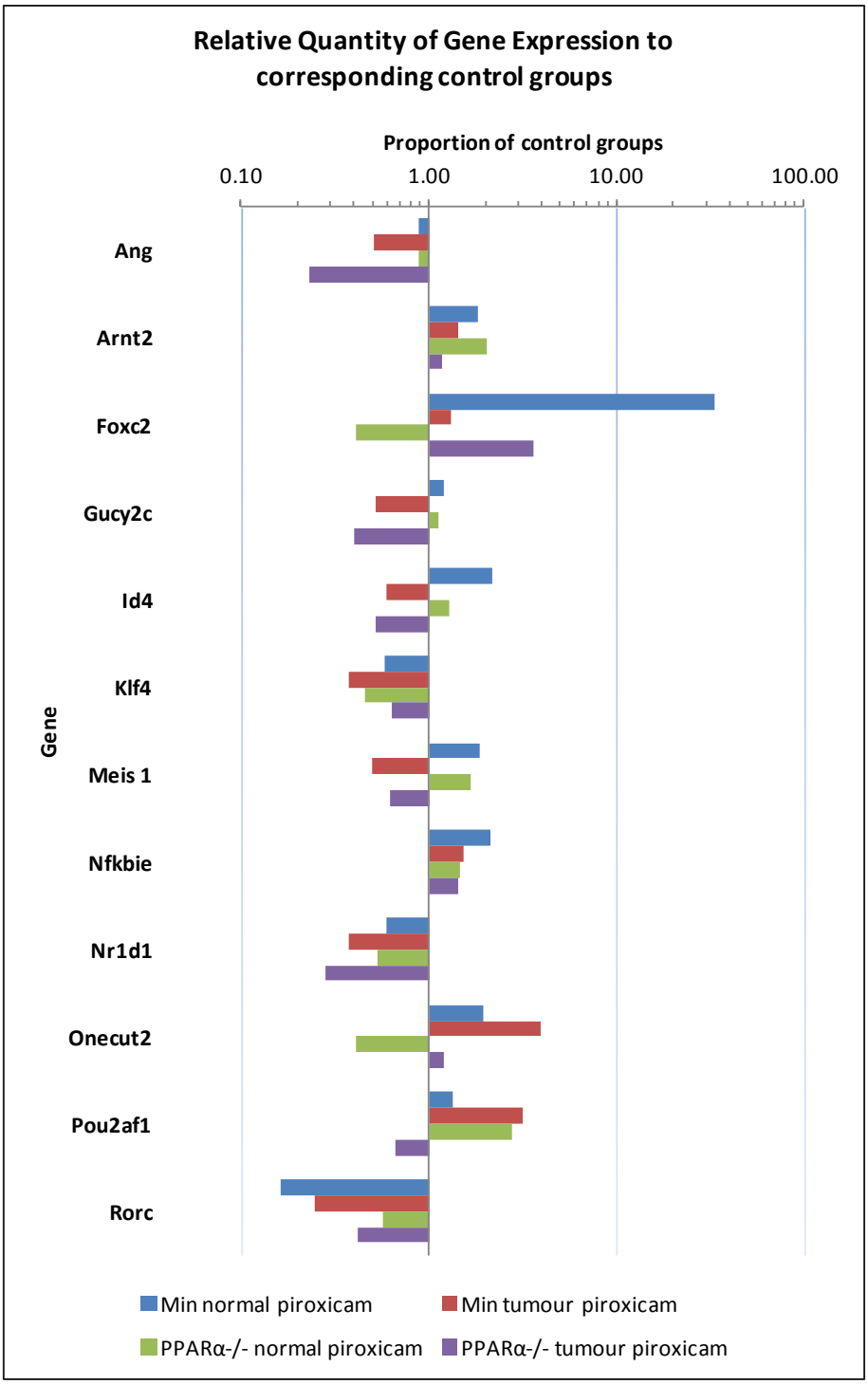


Figure 5-11 Effect of piroxicam on genes implicated in transcription

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of piroxicam groups to control groups, where RQ was >= 2 or <= to 0.5

5.7.10.4 Cell cycle

Table 5-18 and Figure 5-12 show expression data for the effect of piroxicam on genes implicated in the cell cycle.

These data show piroxicam treatment did not significantly effect the expression of genes in this group.

Table 5-18 Effect of piroxicam on genes implicated in the cell cycle

Gene	Mean Relative Quantity of gene expression to corresponding control group				p value		
	Min normal piroxicam	Min tumour piroxicam	PPARα$^{-/-}$ normal piroxicam	PPARα$^{-/-}$ tumour piroxicam	Effect of piroxicam only	Interaction between piroxicam & PPARa	Interaction between piroxicam & tissue
Apobec3	2.91	3.15	0.89	1.37	ns	ns	ns
Bin 1	0.82	0.43	1.94	0.33	ns	ns	ns
Ccnd1	1.56	1.51	1.01	1.18	0.039	ns	ns
Cdkn1c	0.97	0.61	1.24	0.86	ns	ns	ns

Mean relative quantity (RQ) of gene expression in APC$^{Min/+}$ normal, APC$^{Min/+}$ tumour, APC$^{Min/+}$PPARα$^{-/-}$ normal and APC$^{Min/+}$PPARα$^{-/-}$ tumour samples to expression in corresponding control groups. Univariate analysis of variance was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

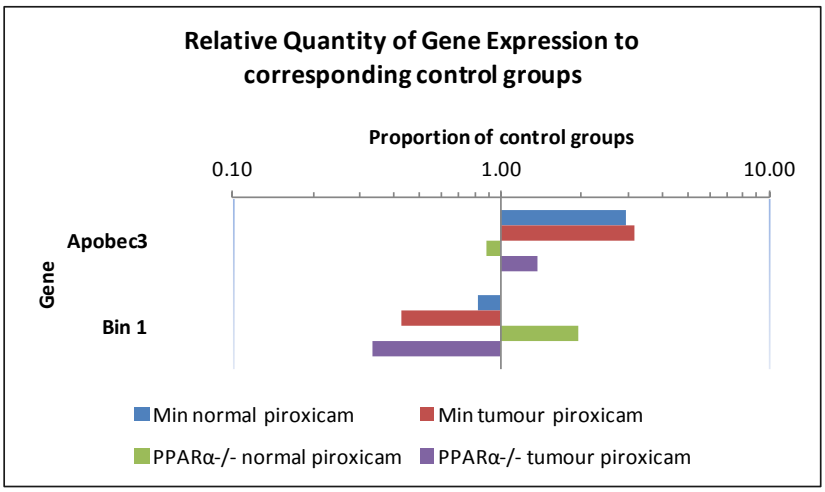


Figure 5-12 Effect of piroxicam on genes implicated in the cell cycle

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of piroxicam groups to control groups, where RQ was >= 2 or <= to 0.5

5.7.10.5 Apoptosis

Table 5-19 and Figure 5-13 show expression data for the effect of piroxicam on genes implicated in apoptosis.

These data showed that in the piroxicam-treated PPARα$^{-/-}$ normal group, expression of Endod1 (endonuclease domain containing 1) was slightly decreased (1.64-fold), whereas in the piroxicam-treated Min normal group expression was decreased 2.94-fold. This may imply a PPARα mediated effect with piroxicam treatment ($p = 0.002$).

The effect of piroxicam treatment only was significant in reduction of Endod1 expression in the Min normal group (2.94-fold, $p = 0.0001$).

Bmf (BCL2 modifying factor) expression was significantly increased 2.52-fold in the PPARα$^{-/-}$ tumour group only ($p = 0.01$).

Table 5-19 Effect of piroxicam on genes implicated in apoptosis

Gene	Mean Relative Quantity of gene expression to corresponding control group				p value		
	Min normal piroxicam	Min tumour piroxicam	PPARα$^{-/-}$ normal piroxicam	PPARα$^{-/-}$ tumour piroxicam	Effect of piroxicam only	Interaction between piroxicam & PPARa	Interaction between piroxicam & tissue
Apaf1	1.29	0.97	0.92	1.28	ns	ns	ns
Bcl2l11	0.87	1.44	1.20	1.56	ns	ns	ns
Bik	1.38	0.80	1.32	1.47	ns	ns	ns
Bmf	0.94	1.12	1.42	2.52	0.002	0.004	0.01
Endod 1	0.34	0.38	0.61	0.49	0.0001	0.002	ns
Khdc1a	0.06	0.61	1.94	0.12	ns	ns	ns
Sbk1	0.69	0.85	1.01	1.10	ns	ns	ns

Mean relative quantity (RQ) of gene expression in APC$^{Min/+}$ normal, APC$^{Min/+}$ tumour, APC$^{Min/+}$PPARα$^{-/-}$ normal and APC$^{Min/+}$PPARα$^{-/-}$ tumour samples to expression in corresponding control groups. Univariate analysis of variance was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

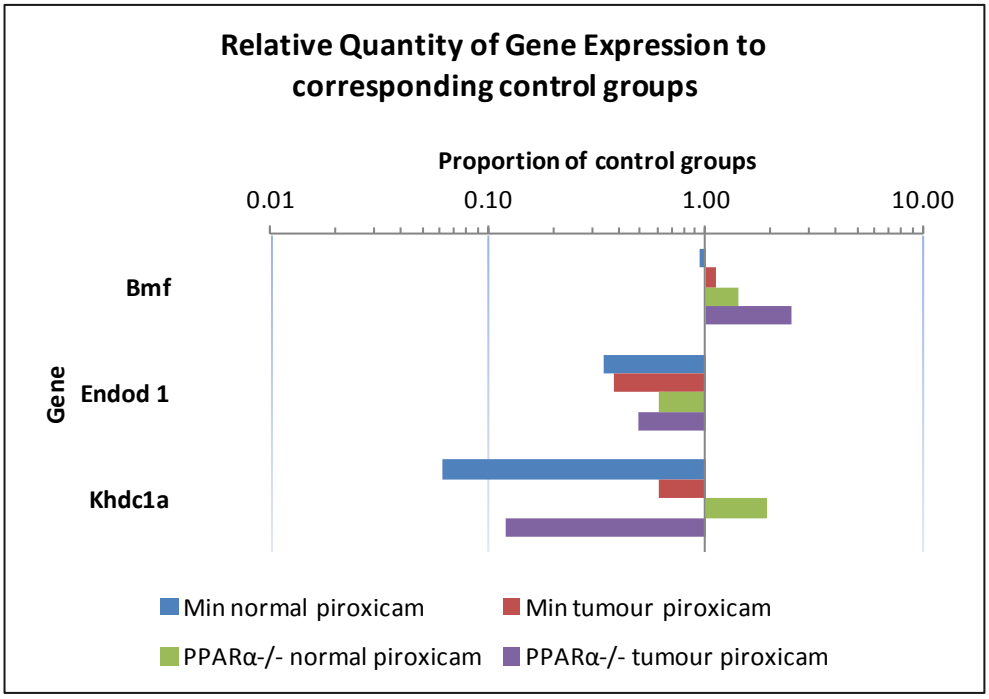


Figure 5-13 Effect of piroxicam on genes implicated in apoptosis

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of piroxicam groups to control groups, where RQ was >= 2 or <= to 0.5

5.7.10.6 Protein transport

Table 5-20 and Figure 5-14 show expression data for the effect of piroxicam on genes implicated in protein transport.

These data showed that in the piroxicam-treated PPARα$^{-/-}$ normal group, expression of Ap1s3 (adaptor-related protein complex AP-1, sigma 3) was slightly decreased (1.72-fold), whereas in the piroxicam-treated Min normal group expression was decreased 3.13-fold.

This difference was highly significant ($p = 0.0001$) and may imply a PPARα-mediated effect with piroxicam treatment.

Ap1s3 expression was also significant with just piroxicam treatment ($p = 0.0001$) and in the piroxicam-treated Min tumour group (2.70-fold, $p = 0.033$).

Table 5-20 Effect of piroxicam on genes implicated in protein transport

Gene	Mean Relative Quantity of gene expression to corresponding control group				p value		
	Min normal piroxicam	Min tumour piroxicam	PPARα$^{-/-}$ normal piroxicam	PPARα$^{-/-}$ tumour piroxicam	Effect of piroxicam only	Interaction between piroxicam & PPARa	Interaction between piroxicam & tissue
Abcc4	1.40	1.23	1.04	1.53	ns	ns	ns
Alb	0.60	3.05	1.30	0.19	ns	ns	ns
Ap1s3	0.32	0.37	0.58	1.16	0.0001	0.0001	0.033
Crip 1	0.90	0.91	0.80	0.34	0.02	ns	ns

Mean relative quantity (RQ) of gene expression in APC$^{Min/+}$ normal, APC$^{Min/+}$ tumour, APC$^{Min/+}$PPARα$^{-/-}$ normal and APC$^{Min/+}$PPARα$^{-/-}$ tumour samples to expression in corresponding control groups. Univariate analysis of variance was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

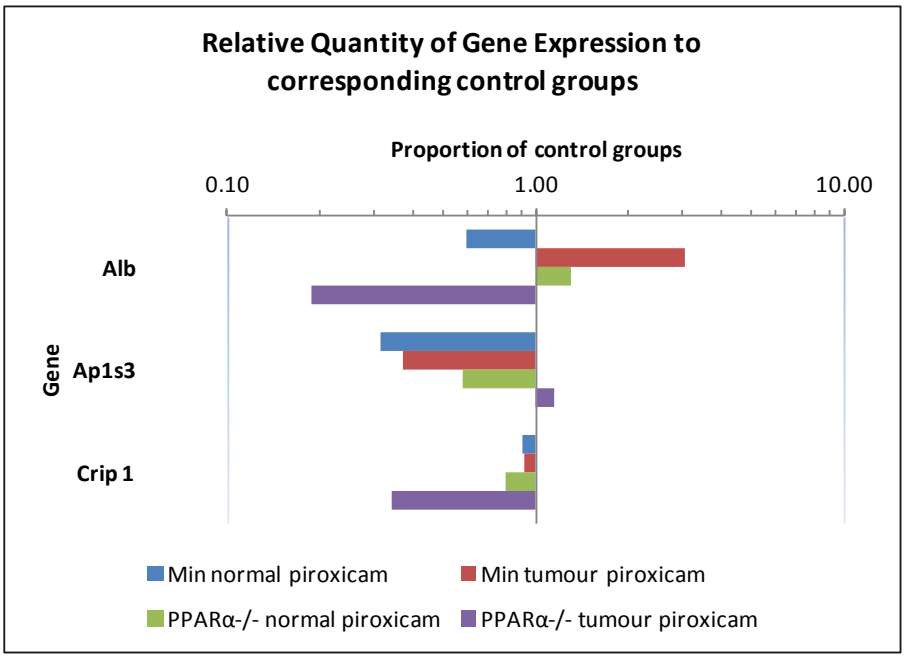


Figure 5-14 Effect of piroxicam on genes implicated in protein transport

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of piroxicam groups to control groups, where RQ was >= 2 or <= to 0.5

5.7.10.7 Protein metabolism & modification

Table 5-21 and Figure 5-15 show expression data for the effect of piroxicam on genes implicated in protein metabolism & modification.

These data showed there was no significant difference in expression of genes in this group *via* PPARα-mediated piroxicam treatment.

Expression of Htra1 (HtrA serine peptidase 1) was up-regulated 2.07-fold in the piroxicam-treated Min normal group ($p = 0.0001$).

Htra1 expression was also increased in the piroxicam-treated Min tumour (2.29-fold) and PPARα tumour (2.43-fold) groups ($p = 0.01$).

Table 5-21 Effect of piroxicam on genes implicated in protein metabolism & modification

Gene	Mean Relative Quantity of gene expression to corresponding control group				p value		
	Min normal piroxicam	Min tumour piroxicam	PPARα$^{-/-}$ normal piroxicam	PPARα$^{-/-}$ tumour piroxicam	Effect of piroxicam only	Interaction between piroxicam & PPARa	Interaction between piroxicam & tissue
Akt3	1.71	0.62	1.10	1.42	ns	ns	ns
B4galt6	1.26	0.98	0.84	0.80	ns	ns	ns
Casp 6	0.65	0.98	1.10	1.59	ns	0.037	ns
Cdk4	1.33	1.65	1.02	1.41	0.03	ns	ns
Htra1	2.07	2.29	1.23	2.43	0.0001	ns	0.01
Irak 4	0.80	0.82	0.72	0.69	0.001	ns	ns
Mep1b	1.02	0.97	0.84	0.13	0.038	0.041	ns
Mmp2	1.48	0.97	1.31	1.10	ns	ns	ns
Mmp7	0.58	1.36	1.00	1.46	ns	ns	ns
Pdk2	0.56	0.48	0.83	0.50	0.001	ns	ns
Plat	1.54	1.53	1.17	1.31	ns	ns	ns
Ptprg	0.66	1.00	1.00	1.17	ns	ns	0.046
Timp 1	0.83	4.40	0.70	0.97	ns	ns	ns

Mean relative quantity (RQ) of gene expression in APC$^{Min/+}$ normal, APC$^{Min/+}$ tumour, APC$^{Min/+}$PPARα$^{-/-}$ normal and APC$^{Min/+}$PPARα$^{-/-}$ tumour samples to expression in corresponding control groups. Univariate analysis of variance was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

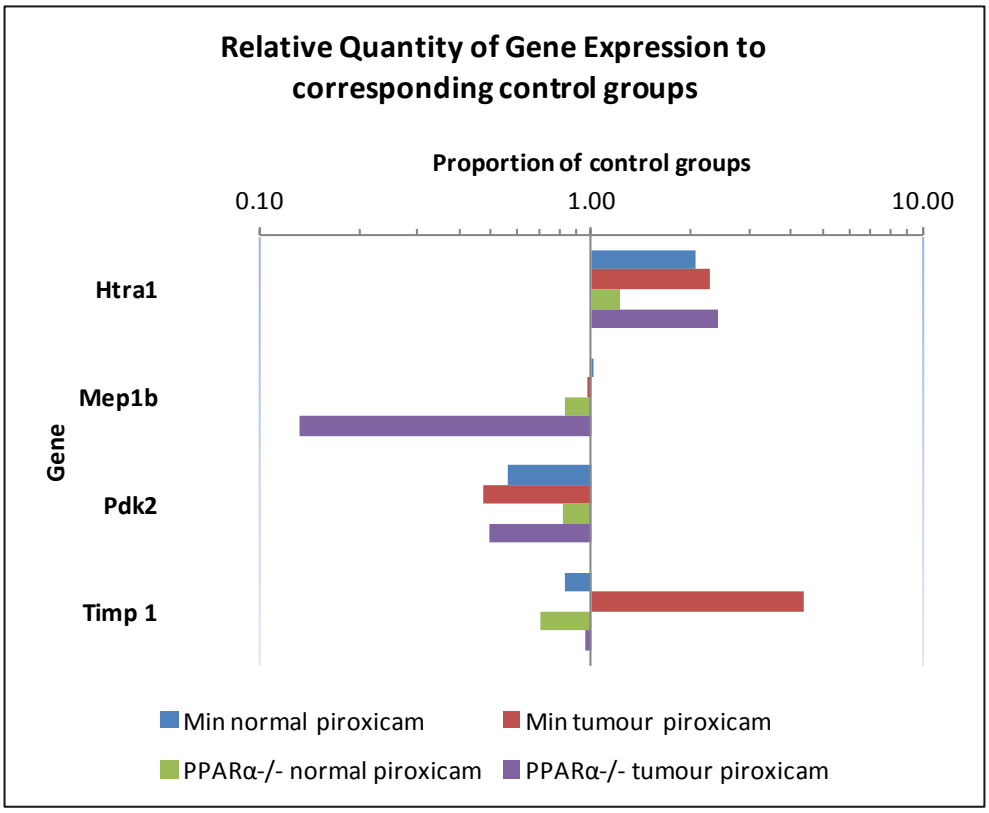


Figure 5-15 Effect of piroxicam on genes implicated in protein metabolism & modification

Gene expression represented on $\log_{10}$ scale clustered bar graph as relative quantity (RQ) of piroxicam groups to control groups, where RQ was >= 2 or <= to 0.5

5.7.10.8 Cell adhesion

Table 5-22 and Figure 5-16 show expression data for the effect of piroxicam on genes implicated in protein metabolism & modification.

These data show piroxicam treatment did not significantly affect the expression of genes in this group.

Table 5-22 Effect of piroxicam on genes implicated in cell adhesion

Gene	Mean Relative Quantity of gene expression to corresponding control group				p value		
	Min normal piroxicam	Min tumour piroxicam	PPARα$^{-/-}$ normal piroxicam	PPARα$^{-/-}$ tumour piroxicam	Effect of piroxicam only	Interaction between piroxicam & PPARa	Interaction between piroxicam & tissue
Ctgf	1.67	1.15	0.66	1.03	ns	ns	ns
Itga6	1.29	1.06	0.96	0.95	ns	ns	ns
Sell	1.96	5.81	1.70	1.58	ns	ns	ns

Mean relative quantity (RQ) of gene expression in APC$^{Min/+}$ normal, APC$^{Min/+}$ tumour, APC$^{Min/+}$PPARα$^{-/-}$ normal and APC$^{Min/+}$PPARα$^{-/-}$ tumour samples to expression in corresponding control groups. Univariate analysis of variance was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

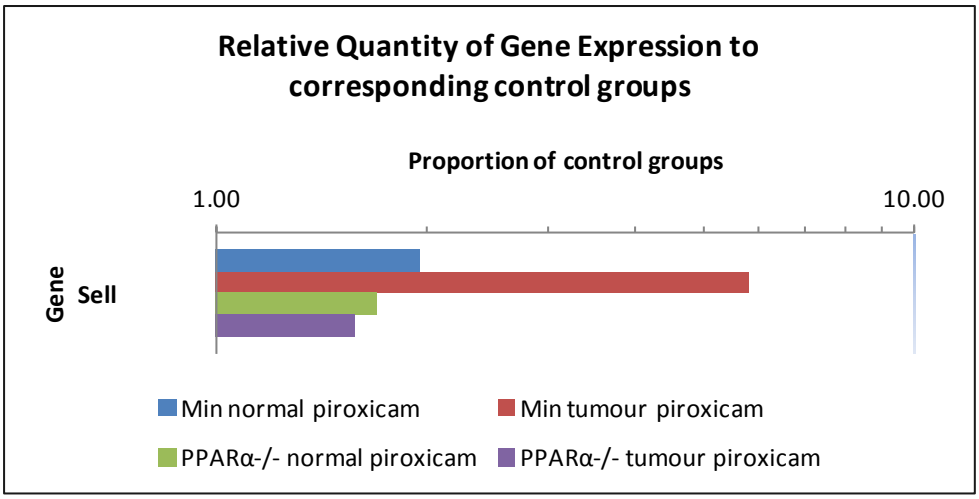


Figure 5-16 Effect of piroxicam on genes implicated in cell adhesion

Gene expression represented on log_{10} scale clustered bar graph as relative quantity (RQ) of piroxicam groups to control groups, where RQ was >= 2 or <= to 0.5

5.7.10.9 Various cellular functions

Table 5-23 and Figure 5-17 show expression data for the effect of piroxicam on genes implicated in toxin metabolism, amino acid metabolism and cellular structure.

These data showed piroxicam treatment was not mediated *via* PPARα in the differential expression of genes in this group.

The effect of just piroxicam treatment decreased expression of Cbr1 (carbonyl reductase 1) by 2.04-fold ($p = 0.001$), and increased expression of Cryab (crystallin, alpha B) by 3.85-fold ($p = 0.045$).

Aoc3 (amine oxidase, copper containing 3) expression decreased in the piroxicam-treated PPARα tumour group (2.13-fold, $p = 0.0001$).

Table 5-23 Effect of piroxicam on genes implicated in various cellular functions

Gene	Mean Relative Quantity of gene expression to corresponding control group				p value		
	Min normal piroxicam	Min tumour piroxicam	PPARα$^{-/-}$ normal piroxicam	PPARα$^{-/-}$ tumour piroxicam	Effect of piroxicam only	Interaction between piroxicam & PPARa	Interaction between piroxicam & tissue
Aoc3	3.12	0.59	2.29	0.47	ns	ns	0.0001
Cbr1	0.49	0.38	1.26	0.78	0.001	0.0001	ns
Cryab	3.85	1.03	0.93	1.01	0.045	0.035	0.049
Nisch	1.49	0.94	0.96	1.13	ns	ns	ns

Mean relative quantity (RQ) of gene expression in APC$^{Min/+}$ normal, APC$^{Min/+}$ tumour, APC$^{Min/+}$PPARα$^{-/-}$ normal and APC$^{Min/+}$PPARα$^{-/-}$ tumour samples to expression in corresponding control groups. Univariate analysis of variance was used to determine differentially expressed genes with p <= 0.05 (ns - not significant)

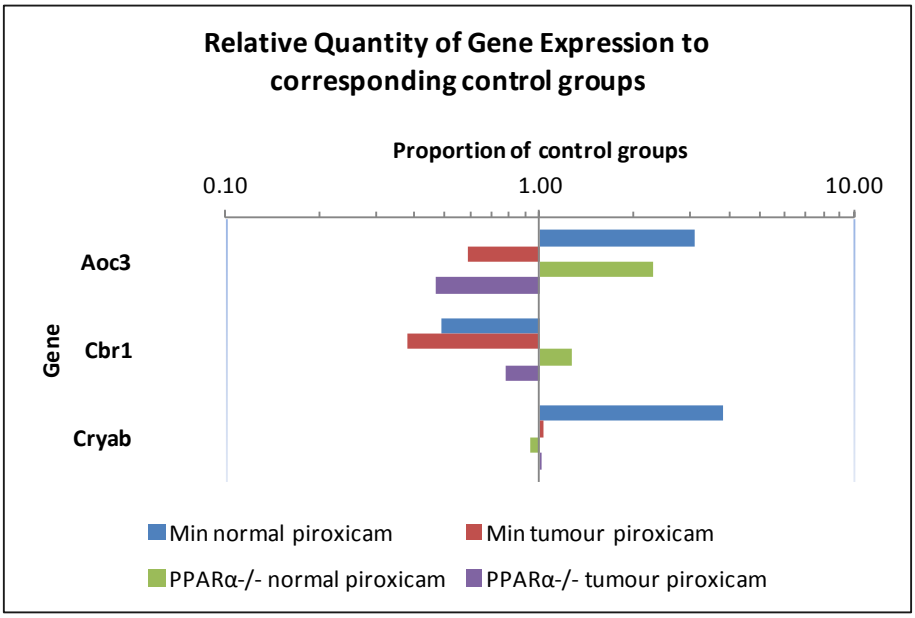


Figure 5-17 Effect of piroxicam on genes implicated in various cellular functions

Gene expression represented on $\log_{10}$ scale clustered bar graph as relative quantity (RQ) of piroxicam groups to control groups, where RQ was >= 2 or <= to 0.5

5.7.11 Validation of Taqman® low density array data: Correlation between Study 1B & control groups of Study 2B

Table 5-24 shows the relative quantities (RQ) of genes to $APC^{Min/+}$ normal groups. The three left hand columns show RQ to $APC^{Min/+}$ normal in Study 1B, and the three right hand columns the RQ to $APC^{Min/+}$ normal control group in Study 2B.

As discussed previously, expression of Apobec3 was greatly increased in $APC^{Min/+}$ PPARα$^{-/-}$ mice, and expression of Onecut2 was highly up-regulated in tumour tissue. These two genes and corresponding data are highlighted in red in Table 5-24.

Table 5-24 Relative Quantities of gene expression in APC$^{Min/+}$ tumour, APC$^{Min/+}$ PPARα$^{-/-}$ normal & APC$^{Min/+}$ PPARα$^{-/-}$ tumour groups (Study 1B & Study 2B control groups only) to corresponding APC$^{Min/+}$ normal groups (1)

Gene	Relative Quantity to APC$^{Min/+}$ normal (Study 1B)			Relative Quantity to APC$^{Min/+}$ normal control (Study 2B)		
	Min tumour	PPARα$^{-/-}$ normal	PPARα$^{-/-}$ tumour	Min tumour control	PPARα$^{-/-}$ normal control	PPARα$^{-/-}$ tumour control
Abcc4	1.5	0.9	1.2	0.9	0.8	1.2
Acot2	0.6	0.8	0.5	1.9	0.5	0.8
Akt3	0.6	1.0	0.4	1.0	1.0	0.6
Alb	28.0	1.2	7.6	1.8	0.7	5.8
Alox12	36.3	1.5	29.7	9.9	2.3	33.0
Alox15	4.3	1.3	2.0	1.5	0.3	7.6
Alox5ap	1.7	1.0	1.8	1.5	1.8	2.2
Ang	0.8	0.6	0.2	1.1	0.9	0.8
Angptl4	0.4	0.1	0.3	1.8	0.2	0.8
Aoc3	0.3	1.1	0.4	2.2	1.0	1.0
Ap1s3	0.5	1.0	0.3	0.8	0.5	0.3
Apaf1	1.9	0.9	1.5	1.2	1.0	1.6
Apcdd1	18.0	0.4	10.0	2.9	1.0	7.3
Apobec3	**2.1**	**77.9**	**128.7**	**0.8**	**190.6**	**251.9**
Arnt2	3.5	0.5	6.8	2.5	1.1	7.1
B4galt6	1.5	0.7	1.7	1.4	1.2	2.2
Baz1a	1.4	0.8	1.5	1.2	1.2	1.6
Bcl2l11	2.8	0.9	3.2	0.9	0.7	1.8
Bdh1	0.4	0.6	0.3	1.5	1.1	1.0
Bik	1.8	0.5	0.7	1.1	0.4	0.5
Bin1	0.4	0.9	0.1	0.3	0.2	0.2
Bmf	4.1	1.1	3.6	1.2	0.8	1.5
Casp6	2.9	0.9	2.5	1.2	1.0	1.4
Cbr1	0.3	0.7	0.3	1.1	0.7	0.5
Cbx7	1.4	0.6	0.4	0.9	0.3	0.4
Ccnd1	3.0	1.2	2.7	1.2	1.2	2.3
Cdk4	2.3	0.9	2.1	0.8	1.0	1.6
Cdkn1c	2.2	0.5	2.1	1.7	0.8	2.5
Chd8	1.0	0.8	0.8	0.9	0.9	0.9
Crem	1.7	1.0	1.0	1.0	0.9	0.9
Crip1	0.5	1.4	0.4	0.9	1.2	1.3
Cryab	0.3	0.9	0.3	0.7	1.3	0.6
Ctgf	1.3	1.1	1.1	0.4	0.8	0.6
Cyp11a1	17.0	1.7	17.8	0.6	0.3	3.4
Cyp2b10	97.3	0.9	37.6	2.8	0.1	1.8
Cyp2c55	0.0	0.5	0.1	2.8	0.9	0.8
Decr1	0.4	0.7	0.4	0.9	1.0	0.9
Ech1	0.5	0.6	0.3	1.2	0.8	0.8
Echdc2	4.2	0.8	3.0	1.2	0.9	1.9
Endod1	0.3	0.8	0.2	0.9	1.0	0.4
Ereg	2.7	1.2	3.8	2.8	1.7	8.1
Foxc2	5.3	0.7	2.8	1.6	1.1	1.9
Ghr	0.7	0.8	0.7	0.9	0.7	0.7
Gucy2c	0.4	0.7	0.3	1.3	0.9	0.9
Gulp1	0.4	0.8	0.7	1.3	1.0	0.8
Hadha	0.4	0.9	0.6	1.2	0.7	0.6
Htra1	4.6	1.0	2.6	1.8	1.8	3.6
Id4	0.2	1.2	0.2	0.9	1.2	1.1
Il1b	6.7	0.7	15.9	6.0	2.7	30.0
Il6	7.9	1.4	19.9	3.7	1.2	33.1
Inhba	12.2	2.1	20.5	3.9	1.9	22.6
Irak4	0.8	0.5	0.5	1.1	0.6	0.7
Itga6	2.1	1.2	1.8	1.1	1.0	1.6
Jun	1.5	0.6	1.4	1.4	0.9	1.3
Khdc1a	1.9	0.3	0.3	0.1	0.2	0.8
Klf4	0.2	0.6	0.2	1.1	1.0	0.5
Krt18	2.2	1.2	2.0	1.0	1.4	1.5
Lama1	5.1	0.9	6.0	3.3	3.1	7.4
Lama5	3.7	1.1	4.4	1.1	1.0	1.5
Lrp1	0.6	1.1	0.8	1.3	0.8	1.1
Meis1	0.5	1.3	0.3	1.0	0.7	0.7
Mep1b	0.2	0.9	0.4	2.0	2.0	3.7
Mmp2	1.6	1.2	1.6	1.9	1.6	2.5
Mmp7	185.4	1.4	146.4	14.1	1.1	46.0
Myc	2.4	0.8	2.3	0.9	0.9	1.3

Relative Quantities of gene expression in APC$^{Min/+}$ tumour, APC$^{Min/+}$ PPARα$^{-/-}$ normal & APC$^{Min/+}$ PPARα$^{-/-}$ tumour groups (Study 1B & Study 2B control groups only) to corresponding APC$^{Min/+}$ normal groups (2)

Gene	Relative Quantity to APC$^{Min/+}$ normal (Study 1B)			Relative Quantity to APC$^{Min/+}$ normal control (Study 2B)		
	Min tumour	PPARα$^{-/-}$ normal	PPARα$^{-/-}$ tumour	Min tumour control	PPARα$^{-/-}$ normal control	PPARα$^{-/-}$ tumour control
Nfkbie	1.6	0.9	1.8	1.6	1.3	2.8
Nisch	1.2	0.9	1.3	1.0	1.1	1.1
Nr1d1	0.3	0.7	0.3	1.2	1.1	0.7
Onecut2	**145.8**	**1.0**	**196.8**	**25.7**	**2.1**	**184.9**
Pdk2	0.3	0.7	0.3	1.1	0.7	0.6
Phf17	0.6	0.9	0.6	0.8	0.8	0.6
Pla2g2a	37.1	1.1	17.2	4.3	0.8	11.6
Plat	21.0	1.7	20.8	3.0	1.0	9.0
Pou2af1	0.6	0.5	0.4	1.1	0.8	2.7
Ppbp	10.2	1.1	7.8	2.3	0.9	6.7
Ptger3	0.2	0.7	0.2	0.4	0.6	0.3
Ptgis	0.1	1.4	0.2	0.9	0.8	0.8
Ptgs1	0.6	0.5	0.4	1.2	1.1	0.7
Ptgs2	5.1	1.0	6.5	1.9	1.4	5.3
Ptprg	1.3	0.7	0.9	1.0	0.9	1.1
Rarb	3.4	0.4	3.5	1.8	0.7	3.7
Rbms1	2.1	1.5	2.4	1.0	0.8	1.2
Rhoj	3.2	1.1	2.3	1.4	0.9	1.6
Rorc	0.2	0.6	0.1	0.6	0.4	0.3
Sbk1	1.9	1.0	1.7	1.0	0.8	1.3
Sell	0.3	0.2	0.5	1.2	0.8	4.2
Sfrp1	1.0	1.2	1.4	0.7	1.2	1.1
Steap4	4.7	1.7	5.8	1.4	0.5	1.9
Tcf12	2.5	0.8	1.5	1.0	0.8	1.1
Timp1	4.9	2.4	5.1	1.5	1.3	5.3
Tnf	3.7	0.4	2.5	2.0	0.7	2.8

Data from Table 5-24 were plotted onto scatter plots (Figure 5-18, Figure 5-19, Figure 5-20) and bar graphs (Figure 5-21,

Figure 5-22, Figure 5-23) as matched paired groups; that is APC$^{Min/+}$ tumour, APC$^{Min/+}$ PPARα$^{-/-}$ normal and APC$^{Min/+}$ PPARα$^{-/-}$ tumour groups from Study 1B and the corresponding untreated (control) groups from Study 2B.

Figure 5-18 is a scatter plot showing the correlation between APC$^{Min/+}$ tumour groups. The highly expressed gene Onecut2 is highlighted in red. These data were also represented on a bar graph (Figure 5-21) which shows good correlation between APC$^{Min/+}$ tumour groups from study 1B and 2B.

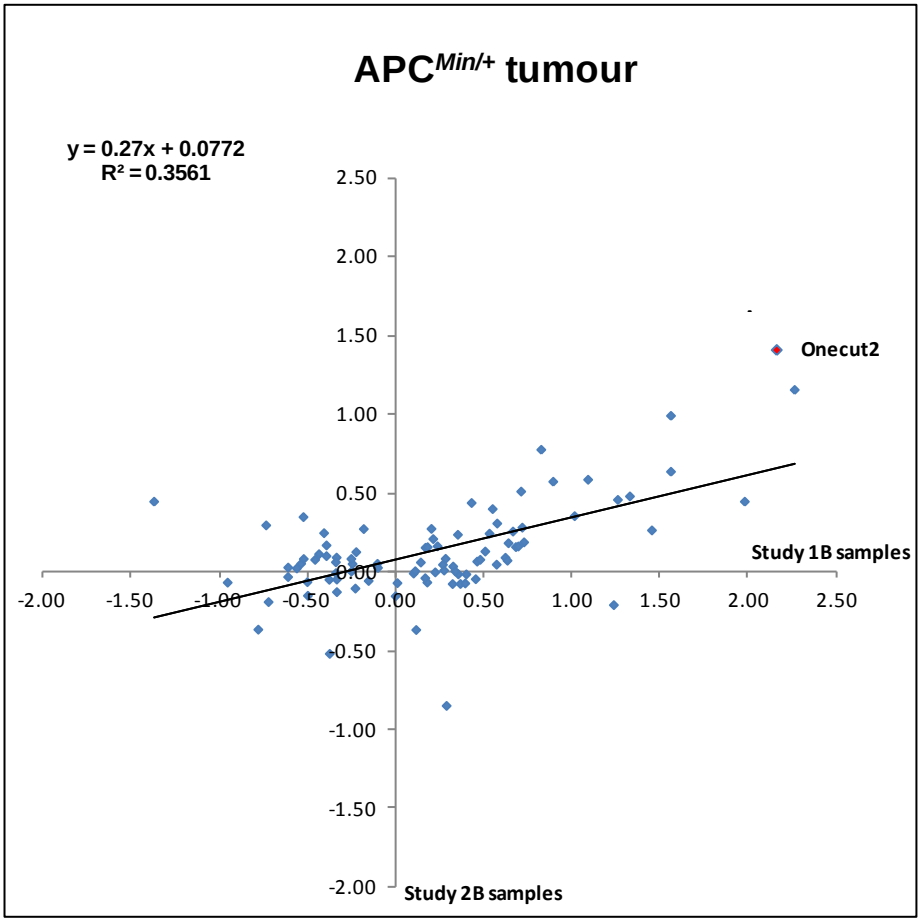


Figure 5-18 Correlation between expression of genes in APC$^{Min/+}$ tumour groups of Study 1B & Study 2B

Comparison of relative quantity (log_{10}) of gene expression in APC$^{Min/+}$ tumour group to APC$^{Min/+}$ normal (Study 1B) *vs.* RQ (log_{10}) of gene expression in APC$^{Min/+}$ tumour group to APC$^{Min/+}$ normal (Study 2B control groups) represented on scatter plot

Figure 5-19 is a scatter plot showing the correlation between APC$^{Min/+}$ PPARα$^{-/-}$ normal groups. Apobec3 is highlighted in red. The corresponding bar graph (

Figure 5-22) shows expression of genes was highly comparable in APC$^{Min/+}$ PPARα$^{-/-}$ normal groups of study 1B and 2B.

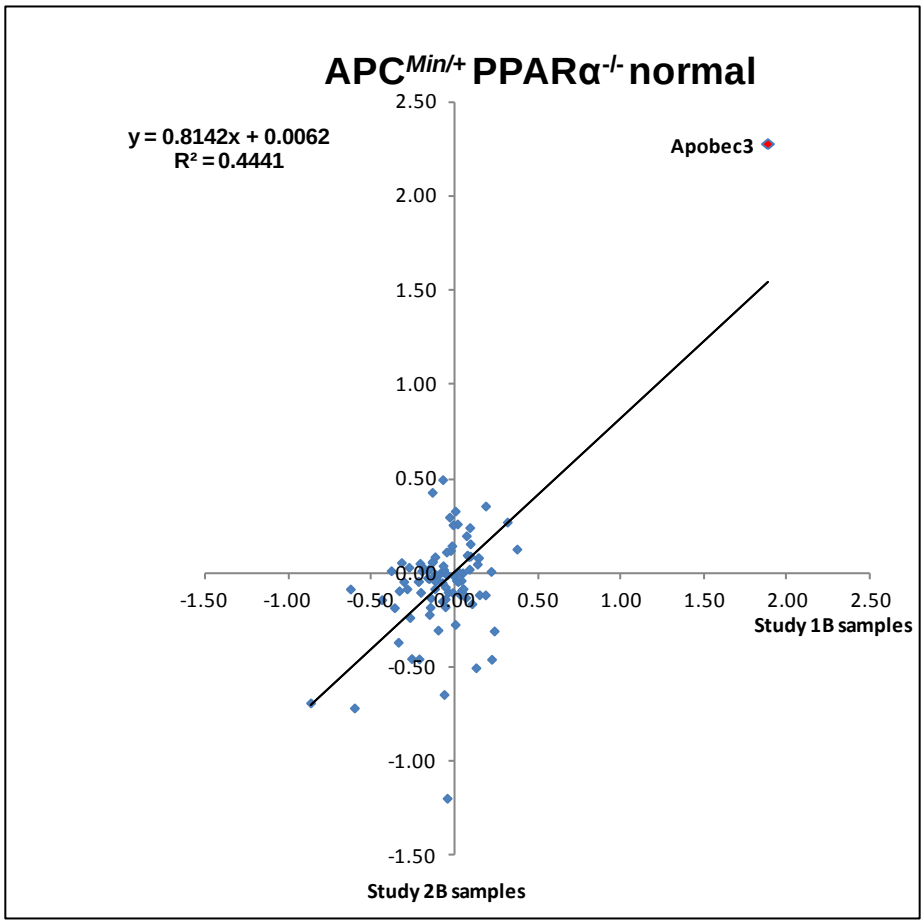


Figure 5-19 Correlation between expression of genes in APC$^{Min/+}$ PPARα$^{-/-}$ normal groups of Study 1B & Study 2B

Comparison of relative quantity (log_{10}) of gene expression in APC$^{Min/+}$ PPARα$^{-/-}$ normal group to APC$^{Min/+}$ normal (Study 1B) *vs.* RQ (log_{10}) of gene expression in APC$^{Min/+}$ PPARα$^{-/-}$ normal group to APC$^{Min/+}$ normal (Study 2B control groups) represented on scatter plot

Figure 5-20 shows a scatter plot that represents the correlation between $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour groups. Apobec3 and Onecut2 are highlighted in red. Figure 5-23 shows that genes have very similar expression levels in each of $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour groups from Study 1B and 2B.

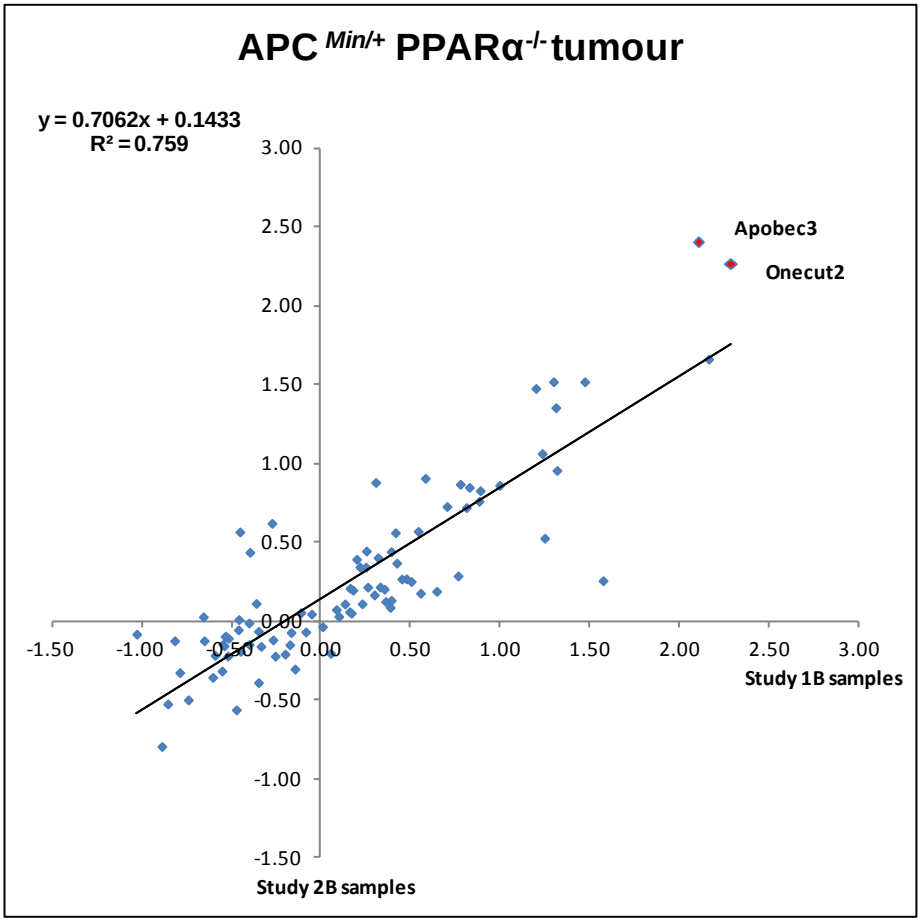


Figure 5-20 Correlation between expression of genes in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour groups of Study 1B & Study 2B

Comparison of relative quantity (log_{10}) of gene expression in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour group to $APC^{Min/+}$ normal (Study 1B) *vs.* RQ (log_{10}) of gene expression in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour group to $APC^{Min/+}$ normal (Study 2B control groups) represented on scatter plot

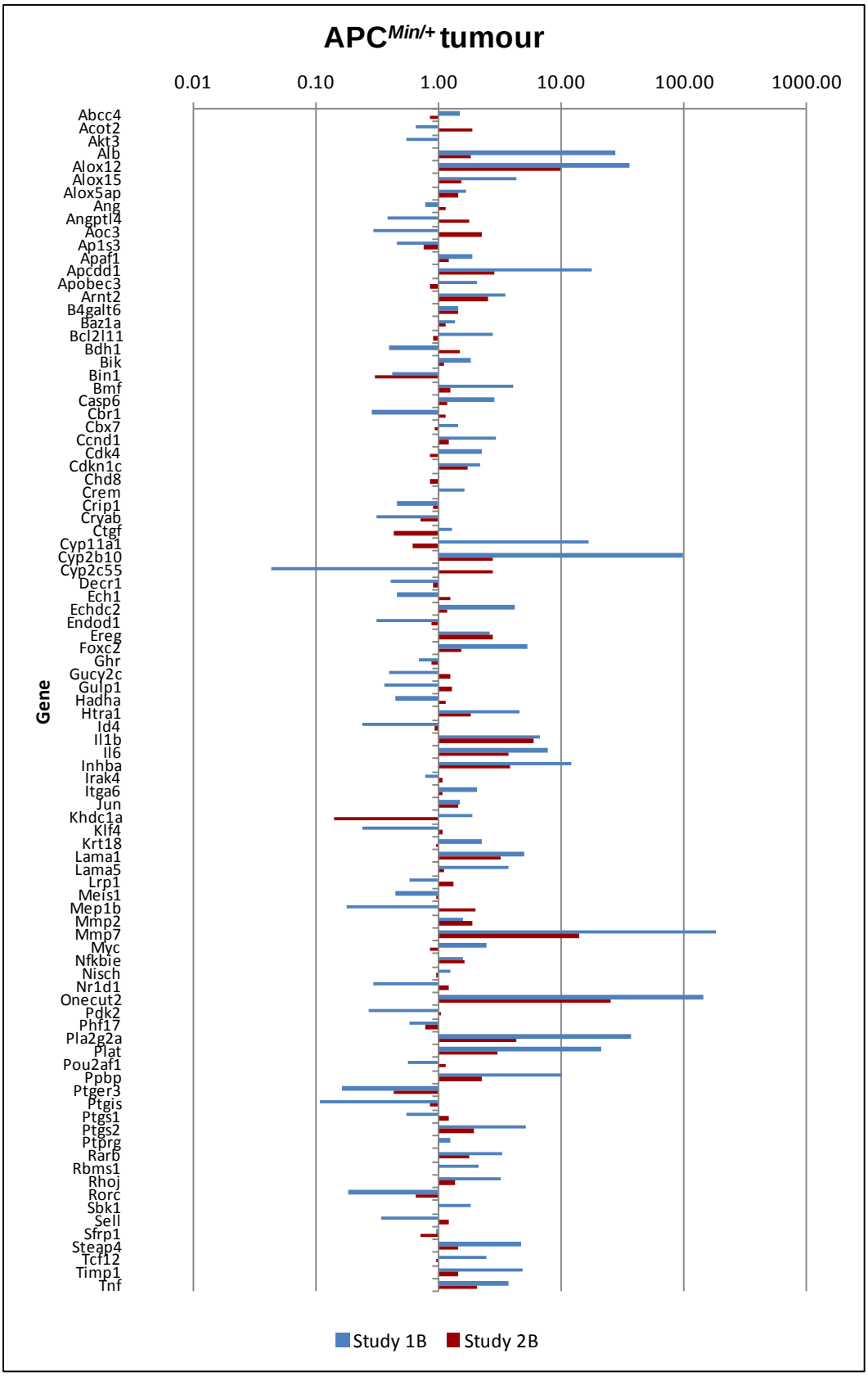


Figure 5-21 Comparative expression of genes in APC$^{Min/+}$ tumour groups of Study 1B & Study 2B

Relative quantity (RQ) of gene expression in APC$^{Min/+}$ tumour group to APC$^{Min/+}$ normal (Study 1B, blue bars) and RQ of gene expression in APC$^{Min/+}$ tumour group to APC$^{Min/+}$ normal (Study 2B control groups, red bars) represented on log_{10} scale bar chart

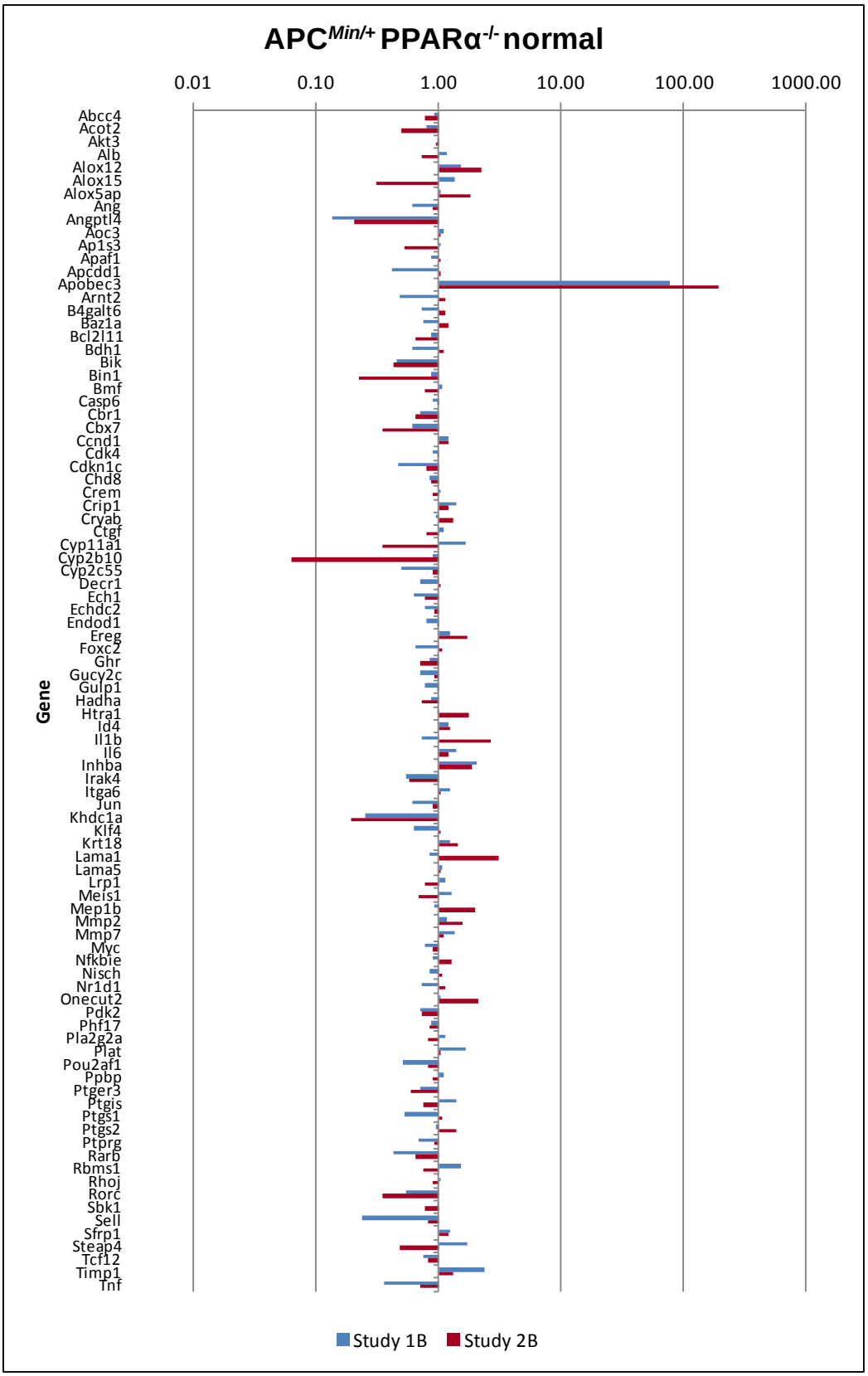


Figure 5-22 Comparative expression of genes in APC$^{Min/+}$ PPARα$^{-/-}$ normal groups of Study 1B & Study 2B

Relative quantity (RQ) of gene expression in APC$^{Min/+}$ PPARα$^{-/-}$ normal group to APC$^{Min/+}$ normal (Study 1B, blue bars) and RQ of gene expression in APC$^{Min/+}$ PPARα$^{-/-}$ normal group to APC$^{Min/+}$ normal (Study 2B control groups, red bars) represented on log_{10} scale bar chart

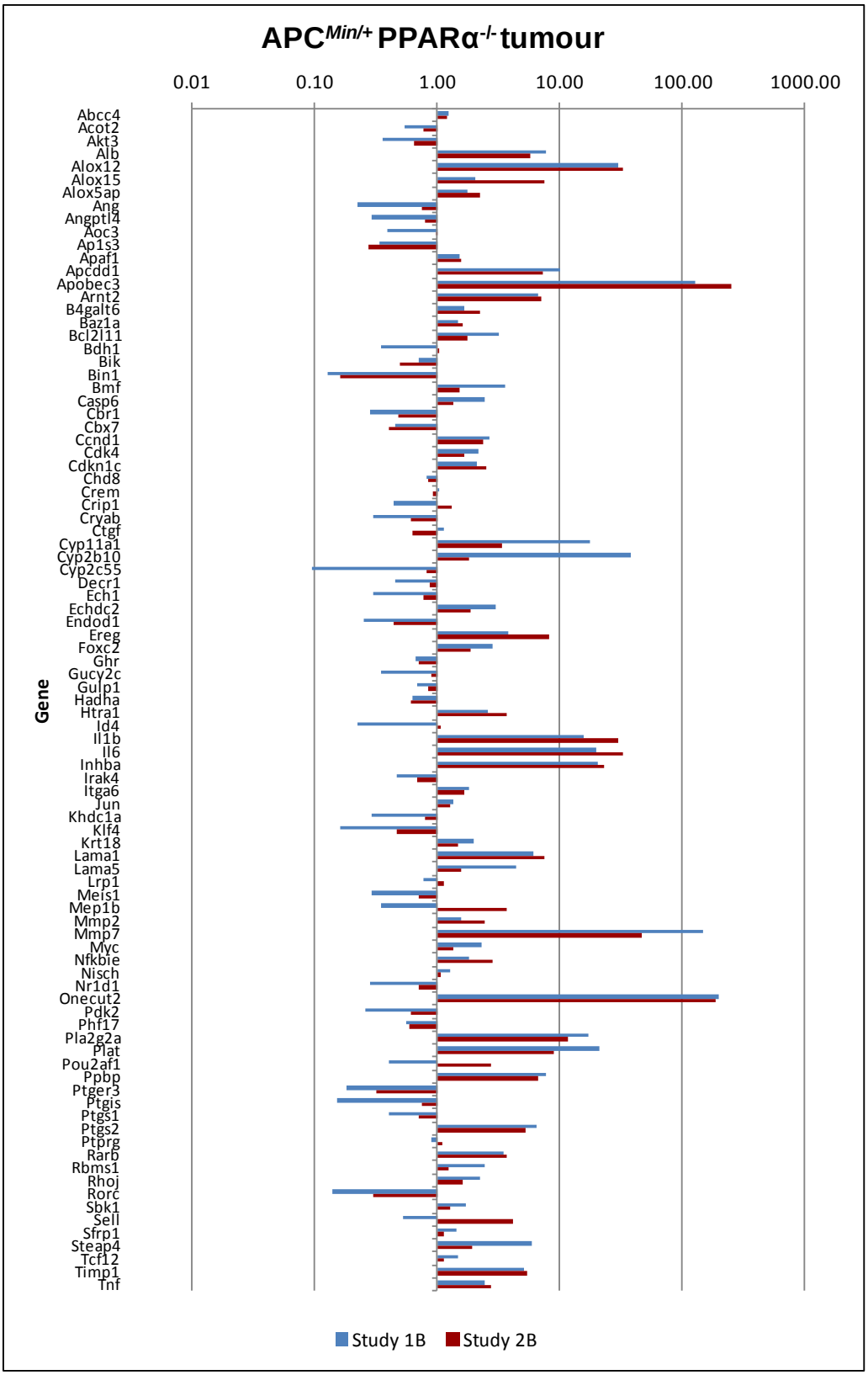


Figure 5-23 Comparative expression of genes in APC$^{Min/+}$ PPARα$^{-/-}$ tumour groups of Study 1B & Study 2B

Relative quantity (RQ) of gene expression in APC$^{Min/+}$ PPARα$^{-/-}$ tumour group to APC$^{Min/+}$ normal (Study 1B, blue bars) and RQ of gene expression in APC$^{Min/+}$ PPARα$^{-/-}$ tumour group to APC$^{Min/+}$ normal (Study 2B control groups, red bars) represented on $\log_{10}$ scale bar chart

5.8 Discussion

5.8.1 Are the effects of piroxicam mediated *via* PPARα?

Results from the analyses of polyp burden and gene expression in the mouse intestine of $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice have shown that PPARα may have a role in preventing development and growth of polyps in the mouse intestine. Also, it has previously been demonstrated that the PPARα ligand methylclophenapate (MCP) reduced the number of polyps in the mouse colon and small bowel (Jackson et al., 2003).

The present study investigated the long-term effects of piroxicam (a non-selective Cox inhibitor) on survival, polyp formation and gene expression in the colon and small bowel of $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice, and whether these effects were mediated *via* PPARα.

Results of mean age at sacrifice showed $APC^{Min+/-}$ control mice were significantly older than $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ control mice ($p = 0.043$) which could imply that PPARα has a role in life expectancy; perhaps due to downstream effects of PPARα activation such as lipid metabolism, fatty acid transport and oxidation (Erol, 2007).

Also, mice dosed with piroxicam lived considerably longer than control mice, and to a greater extent in $APC^{Min+/-}$ mice than $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ mice ($p = 0.019$). This effect was also reported in a previous study investigating the effects of piroxicam on intestinal polyps in $APC^{Min/+}$ mice (Ritland and Gendler, 1999).

In the colon, polyp number, polyp size and total tumour burden did not alter significantly between all groups. However, when corrected for age, polyp number, but not polyp size or total polyp burden was significantly reduced in all piroxicam-treated mice. This would imply that the polyps are larger although fewer in number.

Also, in the small bowel, polyp number, polyp size and total tumour burden were reduced significantly with piroxicam treatment in both $APC^{Min+/-}$ mice and $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ mice. However, there was no significant difference in the number of polyps, polyp size or total tumour burden between control groups or between piroxicam-treated groups. Similar results were seen when age at sacrifice (per week) was considered.

A study by (Jacoby et al., 1996) reported similar findings to those in the present study. Their study showed piroxicam treatment at 100 ppm for six weeks significantly reduced polyp numbers in the small bowel from 16.7 +/- 2.7 in control mice to 3.2 +/- 0.4 ($p < 0.0001$). Also, polyp number in the colon increased from 0.6 +/- 0.3 in control mice to 1.2 +/- 0.5 (p was not significant).

Similarly, (Ritland and Gendler, 1999) demonstrated a 94.4% reduction (at 100 days old) and 95.7% reduction (at 200 days old) in intestinal polyp numbers in $APC^{Min/+}$ mice given piroxicam at 200 ppm. Their study also reported that the distribution of tumours remaining after treatment with piroxicam implied that colonic tumours were relatively resistant to chemo-suppression.

The weight of $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ mice was greater than $APC^{Min+/-}$ mice at the start of the present study ($p = 0.0083$), but there was no significant difference in weight in control groups at the end of the study.

Results showed mice in piroxicam-fed groups were significantly heavier at sacrifice than mice in control groups. However, they were also significantly older, so an increase in weight would be expected. Also, there was no significant difference in the weight of piroxicam-fed $APC^{Min+/-}$ mice and piroxicam-fed $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ mice at sacrifice.

Growth rate (defined as weight per week of life) in both $APC^{Min+/-}$ mice and $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ mice was significantly reduced with piroxicam treatment. However, as piroxicam-fed mice were significantly older than non-treated mice, factoring in age skewed the actual growth rate.

Also, there was no significant difference in growth rate between control groups or between piroxicam-fed groups.

The mesenteric fat in $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ control mice was significantly higher than that in $APC^{Min+/-}$ control mice at sacrifice ($p = 0.004$). Also, piroxicam-fed mice had significantly increased mesenteric fat at sacrifice compared to control mice. However, this is likely to be due to increased survival in piroxicam-treated mice. There was no significant difference in mesenteric fat between mice on piroxicam diet.

The results of the present study and the studies discussed here may suggest that in the colon, piroxicam inhibits tumorigenesis and nascent polyp development, but not polyp enlargement and carcinogenesis. However, the dramatic reduction in the number of polyps in the small bowel may have led to increased survival in the piroxicam-treated mice and therefore, more time for pre-existing colonic polyps to increase in size.

Analysis of gene expression in the colon of piroxicam-treated $APC^{Min+/-}$ and $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ mice using Taqman® low density arrays demonstrated similar gene expression levels in each genotype.

These data indicated that piroxicam did not act through a PPARα pathway to significantly affect expression levels of the genes that were investigated.

However, Cyp2b10 (fold change decrease of 10.0, $p = 0.038$) and Cryab (fold change increase of 3.85, $p = 0.045$) demonstrated differential expression due to the effect of piroxicam treatment only.

In summary, these data indicate that the effect of piroxicam on polyp number, polyp size and total tumour burden in the colon and small bowel of $APC^{Min+/-}$ and $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ mice was not mediated *via* PPARα.

In addition, Taqman® low density array analysis of selected gene expression in the colon of piroxicam-treated mice indicated that

piroxicam does not act through a PPARα pathway to significantly affect expression levels.

Taken together, these data suggest that the PPARα and cyclooxygenase pathways may act independently to influence polyp development in the colon and small bowel Therefore, a potential prophylactic and treatment therapy for intestinal cancers could be the simultaneous targeting of both pathways with an NSAID, such as piroxicam and a PPARα ligand, such as the fibrate group of drugs.

5.8.2 Comparison of Taqman® low density array data of Study 1B & untreated groups of Study 2B

Gene expression results from Study 2B (untreated groups only) were compared to Study 1B results to ascertain whether Taqman® low density arrays would produce comparable results from two matched studies.

Figure 5-18 and Figure 5-21 show a comparison of gene expression results in the $APC^{Min+/-}$ tumour group from Study 1B and the untreated $APC^{Min+/-}$ tumour group from Study 2B. The pattern of gene expression in both groups was similar, although expression in Study 1B was consistently higher. Expression of Onecut2 (one cut domain, family member 2) was highly up-regulated in tumour tissue in both studies.

Figure 5-19 and

Figure 5-22 show a comparison of gene expression results in the $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ normal group from Study 1B and the untreated $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ normal group from Study 2B.

There was very little gene induction in the $APC^{Min+/-}PPAR\alpha^{-/-}$ normal groups which meant expression levels were comparable to levels in normal tissue from $APC^{Min/+}$ mice (See Table 5-24). Apobec3 (Apolipoprotein B, catalytic polypeptide 3) was an exception.

Expression of Apobec3 was significantly increased in both $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ normal groups.

Figure 5-20 and Figure 5-23 show a comparison of gene expression results in the $APC^{Min+/-}PPAR\alpha^{-/-}$ tumour group from Study 1B and the untreated $APC^{Min+/-}PPAR\alpha^{-/-}$ tumour group from Study 2B. There were similar gene expression levels between both groups. Apobec3 and Onecut2 were both highly up-regulated in both studies.

In summary, these data show gene expression levels of similar genes from two separate matched studies were comparable. The results demonstrated good correlation of Taqman® low density array analysis from the two studies.

Apobec3 and Onecut2 have been referred to in previous chapters and were confirmed as highly up-regulated in $PPAR\alpha^{-/-}$ samples and tumour samples respectively. These two genes were selected for further investigation. The findings and results are discussed in depth in Chapter 6.

6 Investigation of the role of Apobec3 & Onecut2 in tumorigenesis in the colon

6.1 Introduction

Apolipoprotein B DNA dC → dU - editing enzyme, catalytic polypeptide 3 (Apobec3) and Onecut homeobox 2 (Onecut2) were selected for further analysis as the large changes in expression levels of these genes in the colon in relation to genotype and malignancy respectively were the most striking novel results from Affymetrix® microarrays and Taqman® low density arrays.

As reported in previous chapters, analysis of the expression level of Apobec3 using Affymetrix® microarrays showed a decrease in $APC^{Min+/-}$ $PPAR\alpha^{-/-}$ samples compared to $APC^{Min+/-}$ samples.

Conversely, Taqman® low density array expression results showed a large increase in $APC^{Min+/-}PPAR\alpha^{-/-}$ samples compared to $APC^{Min+/-}$ samples.

These discrepancies may be explained by methodological differences that it is important to resolve because the size of the effects may indicate that direct or indirect regulation of Apobec3 is an important function of PPARα- dependent control.

Further investigations into these findings are described and discussed in this chapter.

Expression of Onecut2 was consistently high in colonic tumour tissue in both Affymetrix® microarrays and Taqman® low density arrays, suggesting that the product of this gene may play an important role in carcinogenesis.

To validate these data real time quantitative PCR (RT Q PCR) was carried out.

6.1.1 Apobec3

Organisms are subject to assault by a vast range of pathogens, and have evolved a range of protective measures. The adaptive immune responses by antibodies and T cells play a key role in protection from infection. However, the innate immune response is an immediate response, affording the organism protection from opportunistic or zoonotic pathogens (Tosi, 2005).

A vital part of the innate immune response involves detection and clearance of foreign DNA that would cause disruption to the stability of host genomes (Ishii and Akira, 2006, Stetson and Medzhitov, 2006). Apobec3 proteins play a major part in this. Apobec3 proteins restrict foreign gene transfer by deamination[25] of cytidines to uridines, which are then acted on by uracil DNA glycosylase (UNG), leading to degradation of the DNA (Stenglein et al., 2010).

Apobec3 was named because of significant homology to Apobec1, Apobec2 and activation-induced deaminase (AID). All members of these families of genes share a conserved cytidine deaminase active site (CDA), (Jarmuz et al., 2002). Apobec1 edits a single C residue to U on the mRNA encoding Apolipoprotein B (ApoB), introducing a premature stop codon and production of a truncated protein with a different biological function (Wedekind et al., 2003). The role of Apobec2 has not been determined. AID functions in activated B cells and randomly edits single-stranded DNA dC residues to dU on the immunoglobulin locus to cause immunoglobulin gene diversification (Bransteitter et al., 2003).

The human genome encodes seven Apobec3 proteins (A3A, A3B, A3C, A3DE, A3F, A3G and A3H) that are encoded on a single gene cluster on chromosome 22. All are cytidine deaminases with specificity for single-stranded DNA (Jarmuz et al., 2002). Mice only encode a single Apobec3 protein (Mariani et al., 2003). Analysis of the human genome

[25] Hydrolysis reaction of cytosine → uracil with the release of ammonia

suggests the human Apobec3 gene cluster developed after infection by historical exogenous retroviruses that were able to circumvent Apobec3–mediated inhibition of viral replication. Therefore, exogenous retroviruses may be the cause of selective pressure that promoted the amplification and diversification of the human Apobec3 gene (Sawyer et al., 2004, Zhang and Webb, 2004).

A study by (Nik-Zainal et al., 2012) explored patterns of mutations in the genomes of breast cancers to assemble 'mutational signatures of the underlying processes' (Nik-Zainal et al., 2012). Their study identified localised hypermutation (kataegis). Areas of kataegis differ between cancers. However, these areas are generally found with somatic base substitutions, usually cytosine at TpC dinucleotides which may suggest Apobec family cytidine deaminase genes play a role in carcinogenesis.

Another study by (Roberts et al., 2012) also identified mutation clusters in yeast, and human cancer genomes of multiple myelomas, prostate cancers and head and neck squamous cell carcinomas (HNSCCs). These were shown to have arisen from simultaneous mutations of cytosines or guanines in long single-stranded DNA. Interestingly, the clusters were found to be enriched with a motif targeted by Apobec family cytidine deaminases.

A study by (Ding et al., 2011) suggests human Apobec3G is one of several genes that play an important role in mediating colorectal cancer hepatic metastasis. Their study showed Apobec3G was highly expressed in human hepatic metastatic and primary colorectal tumours. Further, the study proposed a novel mechanism for the promotion of hepatic metastasis was *via* Apobec3G directed inhibition of miR-29-mediated suppression of Mmp2, although the mechanism for this remains to be elucidated.

Our data showed some evidence that expression of Apobec3 in the mouse colon may be mediated by PPARα. Therefore, to investigate these findings further analyses were performed.

6.1.2 Onecut2

Onecut genes are transcription factors that have two conserved domains involved in DNA recognition and binding; a cut domain and a homeodomain. The cut domain encodes a DNA-binding motif that can bind independently, or with the homeodomain. The homeodomain binds DNA in a sequence-specific manner in the promoter region of target genes, in complex with other transcription factors.

The mammalian Onecut (OC) family is comprised of OC-1, OC-2 and OC-3. OC-1 was originally designated HNF-6, and is expressed in the liver, pancreas, brain, spleen and testis, but not the small intestine (Lannoy et al., 1998, Lemaigre et al., 1996). OC-2 and OC-3 were discovered by homology to OC-1 (Jacquemin et al., 2003, Vanhorenbeeck et al., 2002). OC-2 is expressed in the liver, pancreas and brain, and crypt and villous epithelium of the small intestine, whilst OC-3 is expressed in the stomach and brain, and enteroendocrine cells of the small intestine (Maier et al., 2006, Vanhorenbeeck et al., 2007).

OC genes are important regulators of pancreas and liver development (Beaudry et al., 2006, Margagliotti et al., 2007). However, there is very little research into the function and role of these genes in the intestine.

A study by (Dusing et al., 2010) used knockout mouse models of OC-2 and OC-3, to show that ablation of OC-2 led to a failure to thrive and increased mortality in post-natal development in these mice compared to wild-type mice. OC-3 ablation had no effect on growth and weight in the mice.

The same study used Affymetrix® microarrays to assess the effect of OC-2 on gene expression in the small intestine in mice. (Dusing et al., 2010) showed that a large percentage of differentially expressed genes were involved in transport and lipid metabolism, which may indicate a regulatory role for OC-2 in expression of these genes in the small intestine.

The novelty of our data which showed a large increase in Onecut2 expression in tumour tissue in the colon led to further investigation of this gene. Real time quantitative PCR was used to validate and confirm expression levels previously determined by Affymetrix® microarrays and Taqman® low density arrays.

6.2 Aims

- To ascertain the basis of the difference in expression levels of Apobec3 in Affymetrix® microarrays and Taqman® low density arrays.
- To confirm the DNA sequence of *Mus musculus* Apobec3.
- To determine and validate expression levels of Apobec3 and Onecut2 in the mouse colon using Real Time Quantitative PCR.

6.3 Methods

Methods for determination of Apobec3 and Onecut2 expression levels in the mouse colon are explained in previous chapters;

Chapter 3 describes the method used for Affymetrix® microarrays (Methods 3.3.7, page 51), Chapter 4 (Methods 4.3.2, page 124) and Chapter 5 (Methods 5.5.1, page 171) describe the method used for Taqman® low density arrays.

Expression levels of Onecut2 in all groups were comparable in Affymetrix® microarrays and Taqman® low density arrays.

However, expression of Apobec3 in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal and tumour groups greatly increased in Taqman® low density arrays but not Affymetrix® microarrays.

To ascertain the reason for the discrepancy further investigations were carried out.

6.3.1 Investigation of differences in Apobec3 gene expression on Affymetrix® microarrays & Taqman® low density arrays

Investigation of the Apobec3 gene reference sequences (mRNA) from the National Centre for Biotechnology Information (NCBI) show that in the mouse there are two variants; NM_001160415.1, variant 1 which encodes the longer isoform 1 and NM_030255.3, variant 2 which lacks an alternate in-frame exon in the coding region, compared to variant 1. The encoded isoform 2 is shorter than isoform 1 as the sequence corresponding to exon 5 in isoform 1 is absent (See Table 6-1 and Figure 6-1).

Table 6-1 Exon base numbers within Apobec3 genomic & mRNA sequences

Apobec3						
Exon	Variant 1			Variant 2		
	DNA base numbers	mRNA base numbers	Number of bases in exon	DNA base numbers	mRNA base numbers	Number of bases in exon
1	1-162	1-162	162	1-162	1-162	162
2	3004-3151	163-310	148	3004-3151	163-310	148
3	5288-5564	311-587	277	5288-5564	311-587	277
4	5704-5818	588-702	115	5704-5818	588-702	115
5	6613-6711	703-801	99			
6	13037-13193	802-958	157	13037-13193	703-859	157
7	13934-14195	959-1220	262	13934-14195	860-1121	262
8	14444-14556	1221-1333	113	14444-14556	1122-1234	113
9	14897-16022	1334-2459	1126	14897-16022	1235-2360	1126

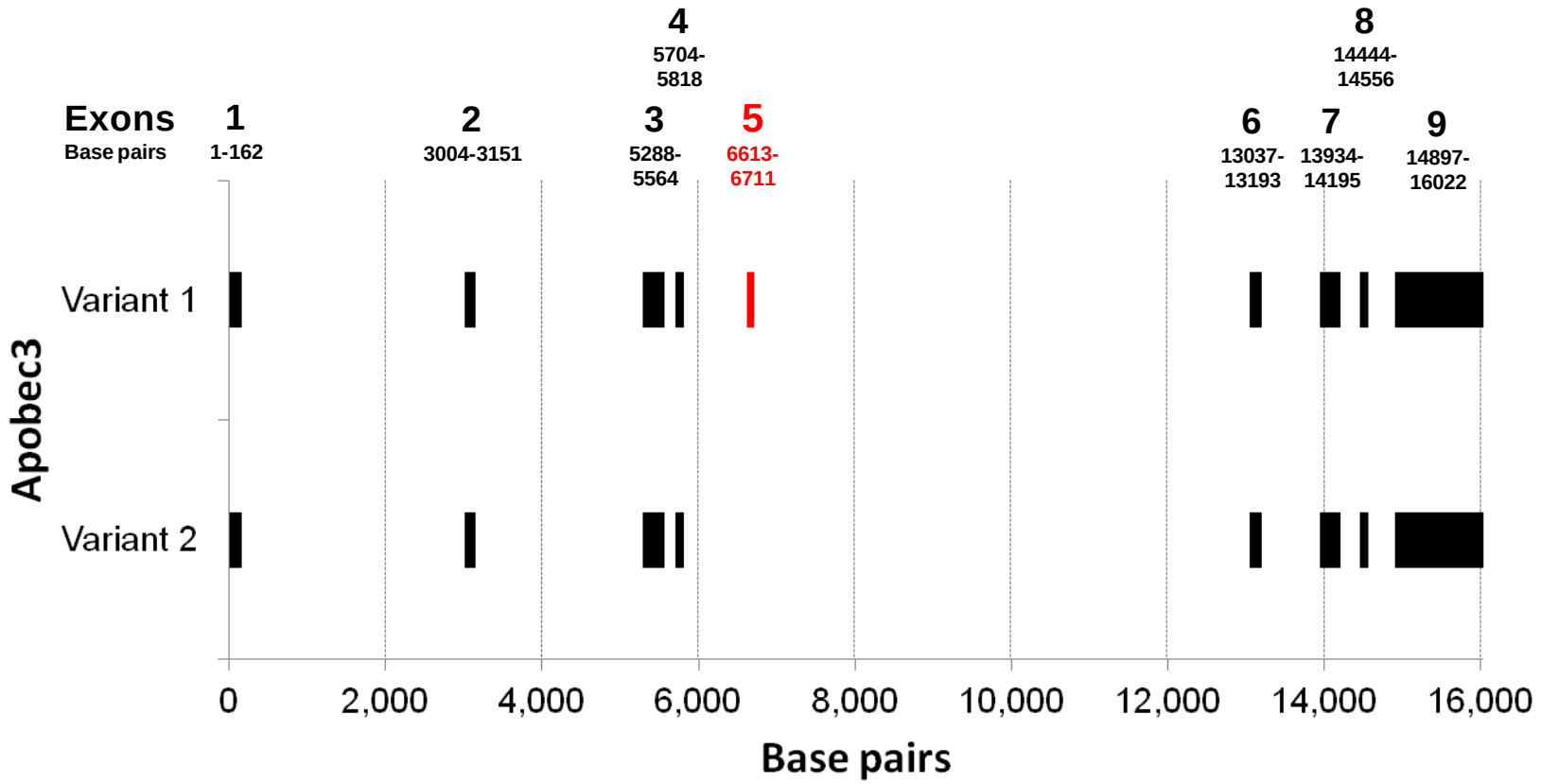


Figure 6-1 Exons of Apobec3 variant 1 & variant 2 in genomic DNA (to scale)

The differences in expression could be explained by the sequences used for detection by the two different methodologies which may have targeted different parts or different isoforms of the gene.

Therefore, to determine what may have produced these differences, the target sequences of the Apobec3 assays used in Affymetrix® microarrays and Taqman® low density arrays were investigated and further analysis performed.

Information about the Apobec3 assay used in the Affymetrix® microarrays was accessed from the Affymetrix® web site (http://www.affymetrix.com/). The code for the Apobec3 assay was identified as *1417470_at*. The sequence of the *1417470_at* assay was aligned against the sequence for Apobec3 variant 1 and then also variant 2 using nucleotide BLAST (Basic Local Alignment Search Tool).

The results of BLAST confirmed the sequence of Affymetrix® assay *1417470_at* matched Apobec3 variant 1 bases 1359 – 1825 (Figure 6-2) and variant 2 bases 1260 – 1726 (Figure 6-3), both within exon 9 of the Apobec3 gene.

Apobec3 Variant 1 (NM_001160415.1)

Exon 1	1 aaggtggggc ctgcattcac ttggcccggg aggtcagttt cacttctggg ggtcttccat
1-162	61 agcctgctca cagaaaatgc aaccccagcg cctggggccc agagctggga tgggaccatt
Exon 2	121 ctgtctggga tgcagccatc gcaaatgcta ttcaccgatc agaaacctga tatctcaaga
163-310	181 aacattcaag ttccacttta agaacctagg ctatgccaaa ggccggaaag ataccttctt
	241 gtgctatgaa gtgactagaa aggactgcga ttcacccgtc tcccttcacc atggggtctt
Exon 3	301 taagaacaag gacaacatcc acgctgaaat ctgcttttta tactggttcc atgacaaagt
311-587	361 actgaaagtg ctgtctccga gagaagagtt caagatcacc tggtatatgt cctggagccc
	421 ctgtttcgaa tgtgcagagc agatagtaag gttcctggct acacaccaca acctgagcct
	481 ggacatcttc agctcccgcc tctacaacgt acaggaccca gaaacccagc agaatctttg
Exon 4	541 caggctggtt caggaaggag cccaggtggc tgccatggac ctatacgaat ttaaaaagtg
588-702	601 ttggaagaag tttgtggaca atggtggcag gcgattcagg ccttggaaaa gactgcttac
Exon 5	661 aaattttaga taccaggatt ctaagcttca ggagattctg agaccttgct acatctcggt
703-801	721 cccttccagc tcttcatcca ctctgtcaaa tatctgtcta acaaaaggtc tcccagagac
Exon 6	781 gaggttctgg gtggagggca ggcgaatgga cccgctaagt gaagaggaat tttactcgca
802-958	841 gttttacaac caacgagtca agcatctctg ctactaccac cgcatgaagc cctatctatg
Exon 7	901 ctaccagctg gagcagttca atggccaagc gccactcaaa ggctgcctgc taagcgagaa
959-1220	961 aggcaaacag catgcagaaa tcctcttcct tgataagatt cggtccatgg agctgagcca
	1021 agtgacaatc acctgctacc tcacctggag cccctgccca aactgtgcct ggcaactggc
	1081 ggcattcaaa agggatcgtc cagatctaat tctgcatatc tacacctccc gcctgtattt
	1141 ccactggaag aggcccttcc agaaggggct gtgttctctg tggcaatcag ggatcctggt
Exon 8	1201 ggacgtcatg gacctccaac agtttactga ctgctggaca aactttgtga acccgaaaag
1221-1333	1261 gccgttttgg ccatggaaag gattggagat aatcagcagg cgcacacaaa ggcggctccg
Exon 9	1321 caggatcaag gagtcctggg gtctgcaaga tttggtgaat gactttggaa acctacagct
1334-2459	1381 tggacccccg atgtcttgag aggcaagaag agattcaaga aggtcttttg gtgacccccc
	1441 cacccaaccc caagtctagg agacctttttg ttctcctgtt tgtttcccct tttgttttat
	1501 cttttgttgt tttgctttgt tttgaagaca gagtctcact gggtagcttg ctactctgga
	1561 actcactact agactaagct ggccttaaac tctaaaatcc acctgccagt gccttctgag
	1621 agccaggctt aaggtgtgcg ctgcccactc ccagccttaa cccactgtgg cttttccttc
	1681 ctctttcttt tattatcttt ttatctcccc tcacctccc gccatcaata ggtacttaat
	1741 tttgtacttg aaatttttaa gttgggccag gcatggtgga gcagcgtgcc tctaatcgca
	1801 ggcaggagga tttccacgag cttgaggcta gcctgatcta catagtgggc tccaggacag
	1861 ccagaactac acagagaccc tgtctcaaaa ataaatttag atagataaat acataaataa
	1921 atggaagaag tcaaagaaag aaagacaaga ttaagttgtg catgattggg tcatatgtgt
	1981 ttgaggcagg aggattgcca catcctgggc tatacaatga gaccctgtct caaacaaaac
	2041 aaaacaaaac acaccaaaag aacagttttt aaatccccaa acctgaattc ttttctaata
	2101 aagtggacaa ccaggcaggc cccctcaccc atcagagtgt gaacatcaag gaagggagcc
	2161 agcaaactgg ctcacaggtg agcaagctga ctgcacctga agatcagagt tcagatccca
	2221 gcacccacct cctgtagctc acaattgatc taactctagc tccagggtac ctggtgccct
	2281 ctcctggact ccatggcacc ccatatatat gtatgtgtgt ttatgtatgt ataatgtgta
	2341 cattatatat gcatacatgc aatatataat ttatatgtgt gtgtatatat atatataatg
	2401 tatatattat atatctccct gtagttgcaa cccaacccag ttccctctac atagggtgt

KEY TO COLOURS

Exon junctions

Target sequence of Taqman low density array *Mm00518791_m1*

Target sequence of Affymetrix probe *1417470_at*

Figure 6-2 mRNA sequence of Apobec3 variant 1 (NM_001160415.1)

Apobec3 Variant 2 (NM_030255.3)

Exon 1	1 aaggtggggc ctgcattcac ttggcccggg aggtcagttt cacttctggg ggtcttccat
1-162	61 agcctgctca cagaaaatgc aaccccagcg cctggggccc agagctggga tgggaccatt
Exon 2	121 ctgtctggga tgcagccatc gcaaatgcta ttcaccgatc agaaacctga tatctcaaga
163-310	181 aacattcaag ttccacttta agaacctagg ctatgccaaa ggccggaaag ataccttctt
	241 gtgctatgaa gtgactagaa aggactgcga ttcacccgtc tccttcacc atggggtctt
Exon 3	301 taagaacaag gacaacatcc acgctgaaat ctgcttttta tactggttcc atgacaaagt
311-587	361 actgaaagtg ctgtctccga gagaagagtt caagatcacc tggtatatgt cctggagccc
	421 ctgtttcgaa tgtgcagagc agatagtaag gttcctggct acacaccaca acctgagcct
	481 ggacatcttc agctcccgcc tctacaacgt acaggaccca gaaacccagc agaatctttg
Exon 4	541 caggctggtt caggaaggag cccaggtggc tgccatggac ctatacgaat ttaaaaagtg
588-702	601 ttggaagaag tttgtggaca atggtggcag gcgattcagg ccttggaaaa gactgcttac
Exon 6	661 aaattttaga taccaggatt ctaagcttca ggagattctg aggcgaatgg acccgctaag
703-801	721 tgaagaggaa ttttactcgc agttttacaa ccaacgagtc aagcatctct gctactacca
	781 ccgcatgaag ccctatctat gctaccagct ggagcagttc aatggccaag cgccactcaa
Exon 7	841 aggctgcctg ctaagcgaga aaggcaaaca gcatgcagaa atcctcttcc ttgataagat
959-	901 tcggtccatg gagctgagcc aagtgacaat cacctgctac ctcacctgga gcccctgccc
1220	961 aaactgtgcc tggcaactgg cggcattcaa aagggatcgt ccagatctaa ttctgcatat
	1021 ctacacctcc cgcctgtatt tccactggaa gaggcccttc cagaaggggc tgtgttctct
Exon 8	1081 gtggcaatca gggatcctgg tggacgtcat ggacctccca cagtttactg actgctggac
1221-1333	1141 aaactttgtg aacccgaaaa ggccgttttg gccatggaaa ggattggaga taatcagcag
Exon 9	1201 gcgcacacaa aggcggctcc gcaggatcaa ggagtcctgg ggtctgcaag atttggtgaa
1334-2459	1261 tgactttgga aacctacagc ttggacccccc gatgtcttga gaggcaagaa gagattcaag
	1321 aaggtctttt ggtgaccccc ccacccaacc ccaagtctag gagacctttt gttctcctgt
	1381 ttgtttcccc ttttgtttta tcttttgttg ttttgctttg ttttgaagac agagtctcac
	1441 tgggtagctt gctactctgg aactcactac tagactaagc tggccttaaa ctctaaaatc
	1501 cacctgccag tgccttctga gagccaggct taaggtgtgc gctgcccact cccagcctta
	1561 acccactgtg gcttttcctt cctctttctt ttattatctt tttatctccc ctcaccctcc
	1621 cgccatcaat aggtacttaa ttttgtactt gaaattttta agttgggcca ggcatggtgg
	1681 agcagcgtgc ctctaatcgc aggcaggagg atttccacga gcttgaggct agcctgatct
	1741 acatagtggg ctccaggaca gccagaacta cacagagacc ctgtctcaaa aataaattta
	1801 gatagataaa tacataaata aatggaagaa gtcaaagaaa gaaagacaag attaagttgt
	1861 gcatgattgg gtcatatgtg tttgaggcag gaggattgcc acatcctggg ctatacaatg
	1921 agaccctgtc tcaaacaaaa caaaacaaaa cacaccaaaa gaacagtttt taaatcccca
	1981 aacctgaatt cttttctaat aaagtggaca accaggcagg ccccctcacc catcagagtg
	2041 tgaacatcaa ggaagggagc cagcaaactg gctcacaggt gagcaagctg actgcacctg
	2101 aagatcagag ttcagatccc agcacccacc tcctgtagct cacaattgat ctaactctag
	2161 ctccagggta cctggtgccc tctcctggac tccatggcac cccatatata tgtatgtgtg
	2221 tttatgtatg tataatgtgt acattatata tgcatacatg caatatataa tttatatgtg
	2281 tgtgtatata tatatataat gtatatatta tatatctccc tgtagttgca acccaaccca
	2341 gttccctcta catagggtgt

KEY TO COLOURS

Exon junctions

Target sequence of Taqman low density array *Mm00518791_m1*

Target sequence of Affymetrix probe *1417470_at*

Figure 6-3 mRNA sequence of Apobec3 variant 2 (NM_030255.3)

Applied Biosystems web site (http://bioinfo.appliedbiosystems.com/) was accessed to gain information on assay identification number *Mm00518791_m1*, the Apobec3 assay used on Taqman® low density arrays. The assay information identified the amplicon size (129 bases) and sequence.

The amplicon sequence and Apobec3 variant 1 (NM_001160415.1) sequence were input into NCBI and a nucleotide BLAST of the two sequences was performed. BLAST was also performed for the amplicon sequence and Apobec3 variant 2. This confirmed the amplicon sequence of *Mm00518791_m1* as a match to Apobec3 variant 1 bases 740-868, crossing exons 5–6 (Figure 6-2). Also, Apobec3 variant 2 bases 701-769 (60 bases of the amplicon did not match with variant 2) (Figure 6-3). This showed that assay *Mm00518791_m1* could only amplify variant 1 of Apobec3 as variant 2 lacks exon 5.

In summary, these data demonstrate that Affymetrix® microarrays targeted Apobec3 variant 1 or variant 2 and Taqman® low density arrays targeted Apobec3 variant 1 only.

6.3.2 Polymerase Chain Reaction (PCR) of the Apobec3 gene

PCR of Apobec3 was performed in $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ samples with three sets of primers targeting specific exons of the gene. Primers to amplify exon 1 – exon 9 (Primer pair A), to amplify exon 1 – exon 5 (Primer pair B) and to amplify exon 5 to exon 9 (Primer pair C), were designed using NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/. Sequences of each primer pair are below.

Primer pair A: exon 1- exon 9

Sequences (5' → 3')

Forward primer GGCCCAGAGCTGGGATGGGA (bases 96 – 115 →)

Reverse primer AGAGGGAACTGGGTTGGGTTGC (bases 2448 – 2427 ←)

Primer pair B: exon 1 – exon 5

Sequences (5' → 3')

Forward primer GGCCCAGAGCTGGGATGGGA (bases 96 – 115 →)

Reverse primer GCCCTCCACCCAGAACCTCGT (bases 799 – 779 ←)

Primer pair C: exon 5 – exon 9

Sequences (5' → 3')

Forward primer TGGGTGGAGGGCAGGCGAAT (bases 788 – 807 →)

Reverse primer AGAGGGAACTGGGTTGGGTTGC (bases 2448 – 2427 ←)

Primers were synthesised by Eurofins MWG Operon. They were supplied in a lyophilized state and were reconstituted in water following manufacturer's instructions to give a stock concentration of 100 pmol/µl. Aliquots of 10 pmol/µl were prepared. Stock and aliquots were all stored at -20^0C until required.

Figure 6-4 is a schematic figure of Apobec3 variant 1. The position of primer pairs A, B and C are shown.

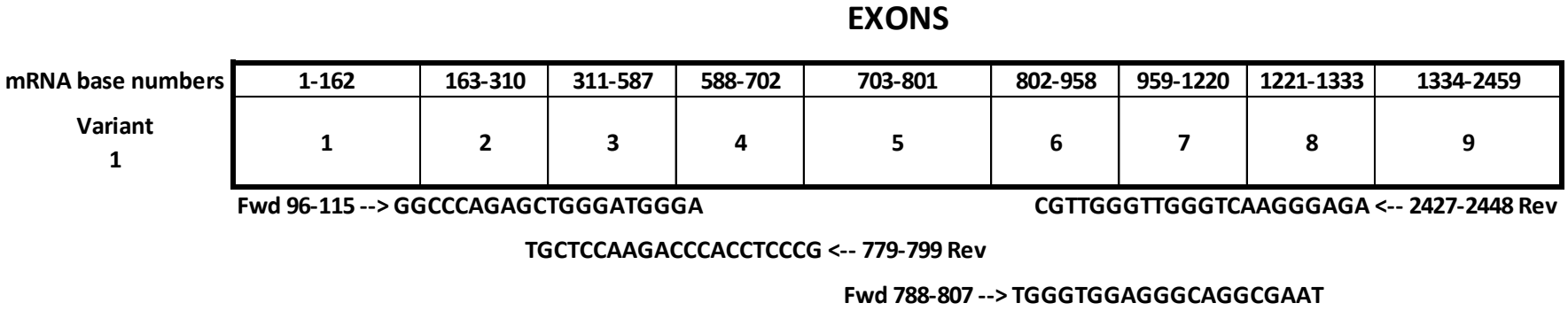


Figure 6-4 Schematic of Apobec3 variant 1 showing primer pairs A, B and C

Primer pair A target sequence exon 1 to exon 9
(Forward primer bases 96 – 115 → Reverse primer bases 2448 – 2427 ←)
Primer pair B target sequence exon 1 to exon 5
(Forward primer bases 96 – 115 →Reverse primer bases 799 – 779 ←)
Primer pair C target sequence exon 5 to exon 9
(Forward primer bases 788 – 807 →Reverse primer bases 2448 – 2427 ←

- **Preparation of cDNA**

Two APC$^{Min/+}$ normal (76.4/3 & 70.3/2) and two APC$^{Min/+}$ PPARα$^{-/-}$ normal (121.1/3 & 121.1/2) RNA samples from Study 1B were selected from storage at -80^{0}C.

The concentration and integrity of the RNA were assessed on a NanoDrop® ND-1000 Spectrophotometer and then diluted to 250 ng/µl. cDNA was prepared with random primers (page 42) and 500 ng of RNA using Affinity Script (Agilent Technologies) following the manufacturer's protocol.

- **PCR of cDNA**

cDNA samples were prepared for PCR cycling using Phusion® High-Fidelity DNA polymerase (New England Biolabs) as per the manufacturer's protocol but modified with the addition of DMSO at 3% of the final volume.

For a 50 µl reaction the following reagents were added to a PCR tube; DMSO 1.5 µl, 5x Phusion HF buffer 10 µl, dNTPs (prepared as on page 41) 1 µl, water 29 µl, forward primer A 2.5 µl, reverse primer A 2.5 µl, template cDNA (undiluted) 3 µl and polymerase 0.5 µl.

50 ul reactions were similarly prepared using forward primer B and reverse primer B, and forward primer C and reverse primer C.

- **Controls**

Two negative controls were used;

A non-template control (NTC) was used to detect the presence of contaminating nucleic acids. Water was added to a PCR reaction instead of template cDNA.

A no enzyme control (NEC) was used to detect genomic DNA contamination. The reverse transcriptase enzyme was omitted from cDNA preparation of the RNA samples. The samples (templates) were then used in PCR reactions.

One endogenous positive control was used;

Primers to detect the *Mus musculus* Rplp0 (ribosomal protein, large P0) gene were used. The primers were added to separate PCR reactions to amplify a second target on the cDNA samples (templates).

All of the PCR reactions were then run on a Bio-Rad T100 thermocycler as shown in Table 6-2.

Table 6-2 Cycling protocol for PCR on Bio-Rad T100 thermocycler

Times and temperatures				
Initial denaturation	PCR (35 Cycles)		Final extension	Hold
	Melt	Anneal/Extend		
30 seconds at 98^0C	30 seconds at 98^0C	2 minutes at 68^0C	10 minutes at 72^0C	4^0C

- **Agarose gel of PCR products**

Agarose gels were prepared with the addition of ethidium bromide to visualise the PCR products. The amplicon size of PCR with primer pairs A and C was 2353 and 1661 base pairs respectively. 0.8% agarose gels were prepared to detect these PCR products. However, PCR with primer pair B produced a smaller amplicon size of 704 base pairs so 1.5% agarose gels were prepared.

Preparation of agarose gels is described below.

To prepare 0.8% gel; 0.4 g agarose was added to 50 ml of 1x TAE buffer (page 42) in a conical flask. This was heated to boiling in a microwave oven and allowed to cool before the addition of 6 µl of ethidium bromide. The gel was cast and allowed to set.

To prepare 1.5% gel; 0.75 g of agarose was added to 50 ml of 1x TAE buffer (page 42) and prepared as above.

PCR products and DNA ladders (New England Biolabs) were prepared;

To prepare PCR products, 4 µl of loading buffer was added to 16 µl of PCR product (or water for non-template control).

1 kilobase (kb) ladders were prepared by adding 1 µl of ladder to 4 µl of loading buffer and 15 µl of water.

50 base pair (bp) ladders were prepared by adding 2 µl of ladder to 4 µl of loading buffer and 14 µl of water.

0.8% gels were loaded with 1 kb markers, controls and PCR products (from primer pairs A and C), and run at 100 V for 50 minutes.

1.5% gels were loaded with 50 bp markers, controls and PCR products (from primer pair B), and run at 100 V for 50 minutes.

Bands of DNA corresponding to the expected amplicon sizes from primer pairs A (2353 bp) and C (1661 bp) were not seen on the agarose gels. However, primer pair B amplified a sequence of ~ 704 base pairs as expected which was highly expressed in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal samples (Figure 6-5). These samples were investigated further.

6.3.3 Sequencing of Apobec3 PCR product (primer pair B)

A 1.5% agarose gel was prepared as before, but without addition of ethidium bromide.

PCR products (from amplification of two $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal, 121.1/3 & 121.1/2 cDNA samples with Primer pair B as before 6.3.2, page 239), non-template control (NTC) and a 50 base pair (bp) ladder were prepared as previously, but with addition of 2 µl Sybr® Green 1 nucleic acid gel stain to each tube (page 42). The tubes were incubated at room temperature for 5 minutes before addition of loading buffer.

The agarose gel was loaded with 50 bp markers, PCR products and NTC, and run at 100 V for 50 minutes.

The gel was viewed using a Dark Reader® (Clare Chemical Research). DNA bands corresponding to 704 base pairs were excised from the gel with a clean, sharp scalpel. The bands were placed into separate pre-weighed Eppendorf tubes, and the weights of the bands calculated.

A QIA gel extraction kit (Qiagen) was used to extract and clean the DNA from the agarose, following the manufacturers' protocol.

Sequencing of DNA samples was carried out by Source Bioscience Life Sciences, Nottingham, UK (www.lifesciences.sourcebioscience.com).

6.3.4 Real Time Quantitative Polymerase Chain Reaction (RT Q PCR) of Apobec3 & Onecut2

The expression levels of Apobec3 and Onecut2 as determined by Affymetrix® microarrays and Taqman® low density arrays were validated by RT Q PCR.

Primer Express® software v3.0 (Applied Biosystems) was used to design primers and probes for Apobec3 and Onecut2. Two sets of primers and probes were designed for Apobec3. The first set was designed to cross the boundary of exon 5 and exon 6, to amplify a target sequence in Apobec3 variant 1 only. The second set was designed to cross the boundary of exon 2 and exon 3, to amplify target sequences in both Apobec3 variants 1 and 2. One set of primers and probe was designed for Onecut2.

Primers and probes were synthesised by Eurofins MWG Operon.

Apobec3 sequences (specific to variant 1 only)

Sequences (5' → 3')

Forward primer TCTCCCAGAGACGAGGTTCTG

Reverse primer TTGGTTGTAAAACTGCGAGTAAAATT

Probe TGGAGGGCAGGCGA (5'-FAM, 3'-TAM)

Apobec3 sequences (variant 1 and 2)

Sequences (5' → 3')

Forward primer GGAAAGATACCTTCTTGTGCTATGAA

Reverse primer CGTGGATGTTGTCCTTGTTCTTAA

Probe	TGACTAGAAAGGACTGCGATTCACCCGTC (5'-FAM, 3'-TAM)

Onecut2 sequences

Sequences (5' → 3')	
Forward primer	CCAGCGCATGTCTGCCTTA
Reverse primer	TTGTTCCTGTCTTTGTTTGGTTCTT
Probe	CCTGGCAGCATGCAAACGCAAA (5'-FAM, 3'-TAM)

Primers and probes were supplied in a lyophilised state. Primers were reconstituted in water, and probes in the supplied dilution buffer (10mM Tris-HCl; pH 8; 1mM EDTA), following manufacturer's instructions to give a stock concentration of 100 pmol/µl. Aliquots at 10 pmol/µl were prepared. Stock and aliquots were all stored at -20^{0}C until required.

Actin, beta (ACTB, prepared as on page 41) was used as the reference gene for normalisation of all samples.

Previously prepared aliquots of undiluted RNA samples from Study 1B (3.3.6, page 50) and Study 2B (5.5, page 168) were removed from storage at -80^{0}C; integrity and concentration were assessed on a NanoDrop® ND-1000 spectrophotometer. RNA was then diluted with DEPC water (prepared as on page 41) to a concentration of 250 ng/µl. cDNA was synthesised using 500 ng of RNA and random primers (page 42) with an M-MLV Reverse Transcriptase kit (Invitrogen) following manufacturers protocol.

5 µl from each newly prepared cDNA sample were pooled for preparation of standards. The remainder of each cDNA sample was diluted 1:16 with water. Aliquots of the pooled samples (8 µl) and diluted cDNA samples (20 µl) were prepared. All were stored at -20^{0}C until required.

To prepare solutions for running a standard curve (sufficient to run two plates), an aliquot of pooled samples was defrosted and prepared as below:

STANDARD 1: Pooled samples (8 µl aliquot) + 32 µl water → defined as **NEAT**

STANDARD 2: 20 µl of NEAT + 20 µl water → **1/2**

STANDARD 3: 20 µl of 1/2 + 20 µl water → **1/4**

STANDARD 4: 20 µl of 1/4 + 20 µl water → **1/8**

STANDARD 5: 20 µl of 1/8 + 20 µl water → **1/16**

A standard curve was run on every plate. Each standard (1 - 5) was run in triplicate.

Master mixes were prepared for each plate;

Preparation of master mix for RT Q PCR with ACTB (reference gene), per well;

6.5 µl* TaqMan Fast universal PCR Master mix, 2x (Applied Biosystems), 2.25 µl* water and 1.25 µl* ACTB (primers and probe supplied in one tube, see 2.2, page 41), were added to an Eppendorf tube, then briefly mixed and centrifuged (*multiplied by number of wells to be used on the assay[26]).

Preparation of master mix for RT Q PCR with target gene primers and probes, per well;

6.5 µl* TaqMan Fast universal PCR Master mix (2x), 2.5 µl* water, 0.375 µl* forward primer, 0.375 µl* reverse primer and 0.25 µl* probe were added to an Eppendorf tube, then briefly mixed and centrifuged (*multiplied by number of wells to be used on the assay).

Individual 96-well plates were used to assay diluted (1:16) cDNA samples from Study 1B and Study 2B, standards and a non-template control with;

[26] Number of wells per assay = standards (3 x 5) + non template control (1 x 3) + samples n (n x 3) + 10% to allow for error

- ACTB
- Apobec3 variant 1 primers and probe
- Apobec3 variant 1 and 2 primers and probe
- Onecut2 primers and probe

10 µl of master mix (prepared as above) was pipetted into each well of a plate. 3 µl of either a standard, diluted cDNA sample or non-template control (water) were added to appropriate wells in triplicate. Plates were sealed, spun on a MPS 1000 mini plate spinner (Labnet International, Inc.) then loaded onto a StepOne Plus™ Real-Time PCR system and run as below (Table 6-3).

Table 6-3 Cycling protocol for StepOne™ Plus Real-Time PCR system

Times and Temperatures		
Initial Step	**PCR (40 Cycles)**	
Activation	**Melt**	**Anneal/Extend**
HOLD	CYCLE	
10 minutes at 95^{o}C	15 seconds at 95^{o}C	60 seconds at 60^{o}C

At the end of each cycle protocol, all data were saved and exported into an Excel spreadsheet for analysis. Fold change values of Apobec3 and Onecut2 expression in all samples relative to APC$^{Min/+}$ normal/untreated samples were calculated from normalised Ct values (relative quantity (RQ).

Also, SPSS 16.0.0.247 was used to calculate univariate analysis of variance of normalised Ct values.

6.4 Results

Results of Affymetrix® microarrays for Apobec3 and Onecut2 gene expression can be seen in Chapter 3 (Results 3.4.5, page 77). Taqman® low density array results can be seen in Chapter 4 (Results 4.4, page 127) and Chapter 5 (Results 5.7.10, page 195).

6.4.1 Polymerase Chain Reaction (PCR) analysis of the Apobec3 gene

Products of PCR of $APC^{Min/+}$ normal and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal samples with primer pairs A, B and C (Methods 6.3.2, page 239) were analysed on agarose gels.

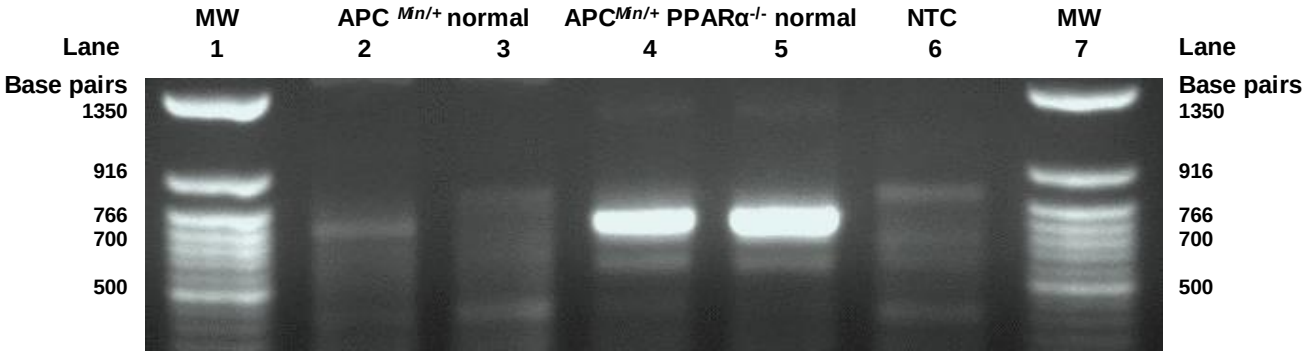
As stated previously, bands of DNA corresponding to the expected *amplicon sizes from primer pairs A and C were not seen on the agarose* gels. Therefore, no further analyses were performed using these primer pairs.

However, primer pair B (see below) amplified a sequence of ~ 704 base pairs which was highly expressed in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal samples (See Figure 6-5).

(Primer pair B: Forward primer GGCCCAGAGCTGGGATGGGA, bases 96 – 115 →

Reverse primer GCCCTCCACCCAGAACCTCGT, bases 799 – 779 ←)

The PCR products of the two $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal samples were re-run on a 1.5% agarose gel with the addition of Sybr® Green 1 nucleic acid gel stain. Bands corresponding to 704 base pairs were excised and prepared for sequencing as previously described (Methods 6.3.3, page 244).



1.5% agarose gel of Apobec3 PCR products (primer pair B)
Lanes 1 & 7, MW molecular weight marker (DNA ladder 50 base pairs, New England Biolabs
Lanes 2 & 3, APC$^{Min/+}$ normal
Lanes 4 & 5, APC$^{Min/+}$ PPARα$^{-/-}$ normal
Lane 6, NTC (non-template control)

Figure 6-5 Agarose gel of primer pair B amplicon

6.4.2 Sequence analysis of Apobec3 gene (primer pair B amplicon)

The amplicon sequences for each APC$^{Min/+}$ PPARα$^{-/-}$ normal sample (forward and complement) recorded by Source Biosciences were input into National Centre for Biotechnology Information (NCBI) BLAST (Basic Local Alignment Search Tool) and aligned against the sequence for Apobec3 variant 1 (See Figure 6-6) and variant 2 (See Figure 6-7), see also Table 6-4 below.

Table 6-4 Alignment of base numbers of Primer pair B amplicon of APC$^{Min/+}$ PPARα$^{-/-}$ normal samples against Apobec3 variant 1 & variant 2

APC$^{Min/+}$ PPARα$^{-/-}$ sample number	Sequence	ALIGNMENT	
		Apobec3 variant 1 base numbers	Apobec3 variant 2 base numbers
121.1/2	forward	139 - 800	139 - 702
	complement	754 - 96	702 - 96
121.1/3	forward	139 - 800	139 - 702
	complement	754 - 106	702 - 106

Primer pair B amplicon sequences (forward and complement) of APC$^{Min/+}$ PPARα$^{-/-}$ normal samples were aligned against the mRNA sequences of Apobec3 variant 1 and variant 2

The results from sequencing show that the forward primer sequences aligned with Apobec3 variant 1 from exon 1 to exon 5. Also, both reverse primer sequences aligned with Apobec3 variant 1 from exon 5 to exon 1 (Figure 6-6).

It can be seen in Table 6-4 that the sequence results did align with Apobec3 variant 2 but only to base number 702. This is the end of exon 4 as Apobec3 variant 2 does not have exon 5.

Therefore, the results of PCR with primer pair B and subsequent sequencing data, may indicate that Apobec3 variant 1 is highly expressed in the colon of APC$^{Min/+}$ PPARα$^{-/-}$ mice.

A possible reason for the lack of bands on the agarose gel after PCR with primer pairs A and C is a large, predicted 3'UTR (untranslated region) after the protein coding region. The sequence of the reverse

primer was within this region. In addition, the sequence of the Affymetrix® probe was also found to be in this region. This provides an explanation for both the lack of amplification with primer pairs A and C, and the difference observed between Taqman® low density array and Affymetrix® microarray data.

Future work will see primers re-designed to amplify the coding region. The gene will then be sequenced.

Apobec3 Variant 1 (NM_001160415.1)

Exon 1
1-162
Exon 2
163-310
Exon 3
311-587
Exon 4
588-702
Exon 5
703-801
Exon 6
802-958

```
   1 aaggtggggc ctgcattcac ttggcccggg aggtcagttt cacttctggg ggtcttccat
  61 agcctgctca cagaaaatgc aaccccagcg cctgggggccc agagctggga tggga ccatt
 121 ctgtctggga tgcagccatc gcaaatgcta ttcaccgatc agaaacctga tatctcaaga
 181 aacattcaag ttccacttta agaacctagg ctatgccaaa ggccggaaag ataccttctt
 241 gtgctatgaa gtgactagaa aggactgcga ttcacccgtc tcccttcacc atggggtctt
 301 taagaacaag gacaacatcc acgctgaaat ctgcttttta tactggttcc atgacaaagt
 361 actgaaagtg ctgtctccga gagaagagtt caagatcacc tggtatatgt cctggagccc
 421 ctgtttcgaa tgtgcagagc agatagtaag gttcctggct acacaccaca acctgagcct
 481 ggacatcttc agctcccgcc tctacaacgt acaggaccca gaaacccagc agaatctttg
 541 caggctggtt caggaaggag cccaggtggc tgccatggac ctatacgaat ttaaaaagtg
 601 ttggaagaag tttgtggaca atggtggcag gcgattcagg ccttggaaaa gactgcttac
 661 aaattttaga taccaggatt ctaagcttca ggagattctg agaccttgct acatctcggt
 721 cccttccagc tcttcatcca ctctgtcaaa tatctgtcta acaaaaggtc tcccagagac
 781 gaggttctgg gtggagggca gg
```

KEY TO COLOURS

Exon junctions

Forward primer B amplicon from APC$^{Min/+}$ PPARα$^{-/-}$ samples (bases 139 – 800)

Reverse primer B amplicon from APC$^{Min/+}$ PPARα$^{-/-}$ samples (bases 96 – 754)

Forward primer

Reverse primer

Figure 6-6 mRNA sequence of Apobec3 variant 1 (exons 1 – 5)

Primer pair B amplicon sequences (forward and complement) of APC$^{Min/+}$ PPARα$^{-/-}$ normal samples were aligned against the sequence for Apobec3 variant 1

Apobec3 Variant 2 (NM_030255.3)

Exon 1
1-162
Exon 2
163-310
Exon 3
311-587
Exon 4
588-702

```
   1 aaggtggggc ctgcattcac ttggcccggg aggtcagttt cacttctggg ggtcttccat
  61 agcctgctca cagaaaatgc aaccccagcg cctggggccc agagctggga tgggaccatt
 121 ctgtctggga tgcagccatc gcaaatgcta ttcaccgatc agaaacctga tatctcaaga
 181 aacattcaag ttccacttta agaacctagg ctatgccaaa ggccggaaag ataccttctt
 241 gtgctatgaa gtgactagaa aggactgcga ttcacccgtc tcccttcacc atggggtctt
 301 taagaacaag gacaacatcc acgctgaaat ctgcttttta tactggttcc atgacaaagt
 361 actgaaagtg ctgtctccga gagaagagtt caagatcacc tggtatatgt cctggagccc
 421 ctgtttcgaa tgtgcagagc agatagtaag gttcctggct acacaccaca acctgagcct
 481 ggacatcttc agctcccgcc tctacaacgt acaggaccca gaaacccagc agaatctttg
 541 caggctggtt caggaaggag cccaggtggc tgccatggac ctatacgaat ttaaaaagtg
 601 ttggaagaag tttgtggaca atggtggcag gcgattcagg ccttggaaaa gactgcttac
 661 aaattttaga taccaggatt ctaagcttca ggagattctg ag
```

KEY TO COLOURS

Exon junctions

Forward primer B amplicon from APC$^{Min/+}$ PPARα$^{-/-}$ samples (bases 139 - 702)

Reverse primer B amplicon from APC$^{Min/+}$ PPARα$^{-/-}$ samples (bases 96 - 702)

Figure 6-7 mRNA sequence of Apobec3 variant 2 (exons 1 – 4)

Primer pair B amplicon sequences (forward and complement) of APC$^{Min/+}$ PPARα$^{-/-}$ normal samples were aligned against the sequence for Apobec3 variant 2

6.4.3 Alignment of Apobec3 sequences in *Mus musculus* & *Homo sapiens*

Protein sequences of mouse Apobec3 were aligned with protein sequences of human Apobec3 to determine whether there was any similarity. Protein sequences, rather than DNA sequences were used, due to the inherent variability of the genetic code. Clustal X version 2[27] was used to perform the alignments (Larkin et al., 2007).

The gi numbers of the sequences used for alignment are listed below (aa → amino acids).

Sequence 1: gi|199581417_Apobec3_Mus_musculus	429 aa
Sequence 2: gi|218511520_APOBEC3H_Homo_sapiens	200 aa
Sequence 3: gi|38303911_APOBEC3G_Homo_sapiens	79 aa
Sequence 4: gi|22907044_APOBEC3F_isoform_a_Homo_sapiens	373 aa
Sequence 5: gi|54873619_APOBEC3F_isoform_b_Homo_sapiens	101 aa
Sequence 6: gi|187608816_APOBEC3D_Homo_sapiens	386 aa
Sequence 7: gi|22907039_APOBEC3C_Homo_sapiens	190 aa
Sequence 8: gi|31753108_APOBEC3B_Homo_sapiens	251 aa
Sequence 9: gi|219520199_APOBEC3A_Homo_sapiens	181 aa

An average quality score for each aligned sequence was generated; a high score indicated the sequences were well conserved, whereas a low score indicated low conservation.

Table 6-5 shows the average quality scores for alignments of each human Apobec3 protein sequence against mouse Apobec3 protein sequence.

[27] Clustal X2 is a programme for multiple alignments of nucleic acid and protein sequences

Table 6-5 Alignment quality analysis of Apobec3 genes in *Mus musculus* & *Homo sapiens*

Alignment with *Mus musculus* Apobec3	
	Average quality score %
Homo sapiens APOBEC3H	43
Homo sapiens APOBEC3G	41
Homo sapiens APOBEC3F isoform a	34
Homo sapiens APOBEC3F isoform b	33
Homo sapiens APOBEC3D	34
Homo sapiens APOBEC3C	47
Homo sapiens APOBEC3B	37
Homo sapiens APOBEC3A	39

These data show the sequences of *H. Sapiens* Apobec3C, H and G were most similar to the sequence of *M. musculus* Apobec3.

Other average quality scores indicated the degree of similarity between each human Apobec3 protein;

Sequences (Apobec3H: Apobec3G) Aligned. Score: 27
Sequences (Apobec3H:Apobec3Fa) Aligned. Score: 33
Sequences (Apobec3H:Apobec3Fb) Aligned. Score: 24
Sequences (Apobec3H:Apobec3D) Aligned. Score: 33
Sequences (Apobec3H:Apobec3C) Aligned. Score: 34
Sequences (Apobec3H:Apobec3B) Aligned. Score: 31
Sequences (Apobec3H:Apobec3A) Aligned. Score: 34
Sequences (Apobec3G: Apobec3Fa) Aligned. Score: 82
Sequences (Apobec3G: Apobec3Fb) Aligned. Score: 78
Sequences (Apobec3G: Apobec3D) Aligned. Score: 53
Sequences (Apobec3G: Apobec3C) Aligned. Score: 34
Sequences (Apobec3G: Apobec3B) Aligned. Score: 53
Sequences (Apobec3G: Apobec3A) Aligned. Score: 24
Sequences (Apobec3Fa:Apobec3Fb) Aligned. Score: 74
Sequences (Apobec3Fa: Apobec3D) Aligned. Score: 79
Sequences (Apobec3Fa: Apobec3C) Aligned. Score: 78
Sequences (Apobec3Fa: Apobec3B) Aligned. Score: 68
Sequences (Apobec3Fa: Apobec3A) Aligned. Score: 43
Sequences (Apobec3Fb: Apobec3D) Aligned. Score: 45
Sequences (Apobec3Fb: Apobec3C) Aligned. Score: 30
Sequences (Apobec3Fb: Apobec3B) Aligned. Score: 44
Sequences (Apobec3Fb: Apobec3A) Aligned. Score: 27
Sequences (Apobec3D: Apobec3C) Aligned. Score: 78
Sequences (Apobec3D: Apobec3B) Aligned. Score: 70

Sequences (Apobec3D: Apobec3A) Aligned. Score:	45
Sequences (Apobec3C: Apobec3B) Aligned. Score:	53
Sequences (Apobec3C: Apobec3A) Aligned. Score:	38
Sequences (Apobec3B: Apobec3A) Aligned. Score:	38

Sequence alignment data were used to generate a phylogenetic tree with TreeView[28](Page, 1996). See Figure 6-8 below. This represents the inferred evolutionary relationship between human Apobec3 and mouse Apobec3.

The tree shows there were three branches from a common ancestral node (far left of figure). The node at each subsequent branch represents the common ancestor of further descendants. Branch lengths give an estimate of time elapsed (left to right).

Figure 6-8 shows Apobec3G was closely related to Apobec3F isoform a and b. In addition, it can be seen Apobec3F isoform a was associated to Apobec3C and Apobec3B. Furthermore, Figure 6-8 was also supported by the average quality scores of the sequence alignments, which demonstrated high scores for these alignments.

[28] TreeView is a phylogenetic tree visualisation software

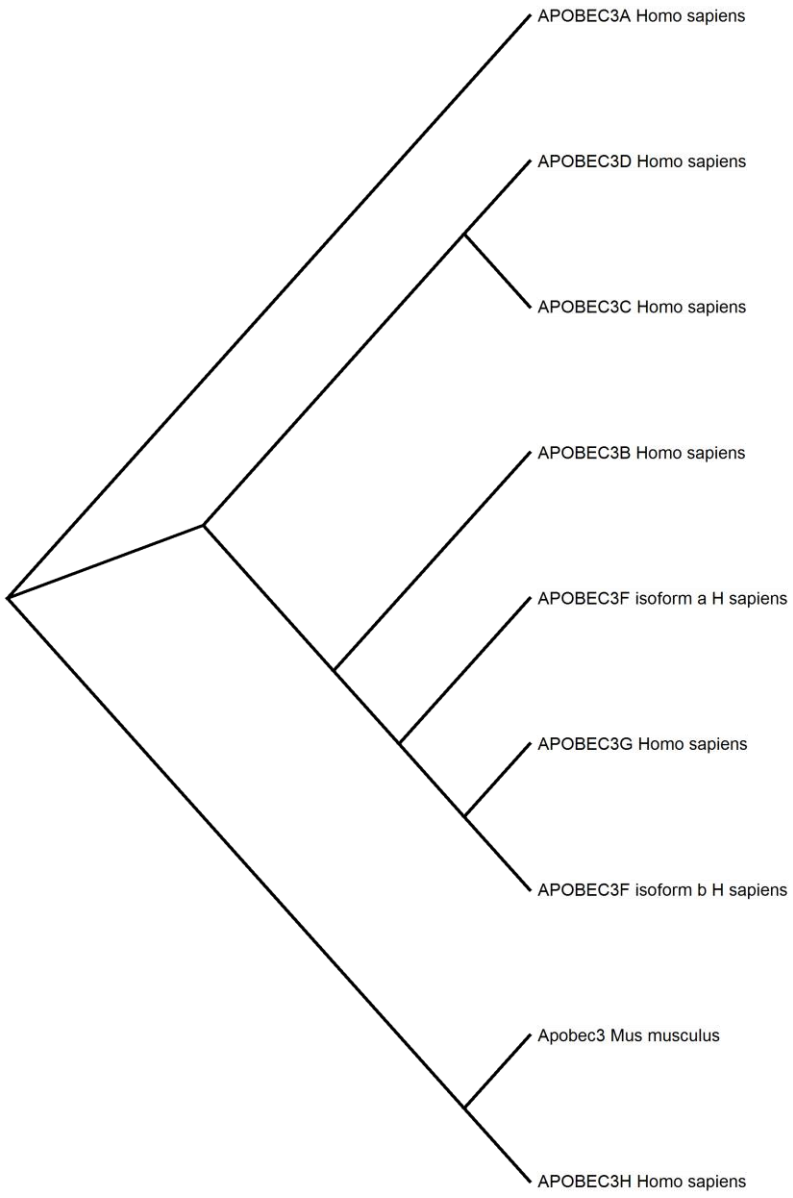


Figure 6-8 Phylogenetic relationship between Apobec3 proteins of *Mus musculus* & *Homo sapiens*

The rooted tree was generated using Clustal X2 programme with the neighbour joining (NJ)[29] method (Saitou and Nei, 1987) and visualised by TreeView.

[29] NJ – algorithm that calculates the distance between each pair of sequences within the multiple alignment

6.4.4 Validation of gene expression of Apobec3 & Onecut2 using Real Time Quantitative PCR

Study 1B: Investigation of the role of PPARα on gene expression in the mouse colon

The relative quantity (RQ) of Apobec3 and Onecut2 expression in all Study 1B samples relative to $APC^{Min/+}$ normal samples were calculated from normalised Ct values.

Also, p values to show the statistical significance of the effect of PPARα, tissue and the interaction of PPARα and tissue were calculated (Table 6-6).

These data were in agreement with earlier investigations of Study 1B samples using Affymetrix® microarrays and Taqman® low density arrays.

Amplification of Apobec3 with primers and probe specific to Apobec3 variant 1 showed expression in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ samples was significantly highly up-regulated compared to $APC^{Min/+}$ samples (Table 6-6).

This verified the findings of Taqman® low density arrays which showed high expression in the same samples.

Similarly, when Apobec3 was amplified with variant 1/2 primers and probe, expression levels were comparable to those seen in Affymetrix® microarrays. That is, expression in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ samples was significantly down-regulated compared to $APC^{Min/+}$ samples (Table 6-6).

Expression of Onecut2 was confirmed as being significantly highly up-regulated in tumour tissue compared to normal tissue (Table 6-6).

Table 6-6 Real Time Quantitative PCR of Apobec3 & Onecut2: Study 1B

Gene	Mean relative quantity of gene expression to $APC^{Min/+}$ normal +/- standard error			p value		
	Min tumour	$PPAR\alpha^{-/-}$ normal	$PPAR\alpha^{-/-}$ tumour	Effect of PPARα	Effect of tissue	Interaction between PPARα & tissue
Apobec3 (primers & probe specific to variant 1)	3.1 +/- 0.02	52.6 +/- 0.22	65.4 +/- 0.20	< 0.0001	not significant	not significant
Apobec3 (primers & probe for variant 1 & variant 2)	2.9 +/- 0.37	0.5 +/- 0.05	0.8 +/- 0.08	< 0.0001	0.001	0.006
Onecut2	167.5 +/- 1.93	1.1 +/- 0.01	141.5 +/- 0.65	not significant	0.012	not significant

Study 2B: Investigation of the role of PPARα in the effects of piroxicam on gene expression in the colon

RT Q PCR results were used to calculate the relative quantity (RQ) of Apobec3 and Onecut2 expression in all Study 2B samples relative to $APC^{Min/+}$ normal untreated samples from normalised Ct values. Similarly, p values to show the statistical significance of the effect of PPARα only, tissue only and the interaction of PPARα and tissue were calculated (Table 6-7).

Also, the effect of piroxicam treatment on RQ of Apobec3 and Onecut2 expression in all samples relative to $APC^{Min/+}$ normal untreated samples were calculated from normalised Ct values. p values to show the statistical significance of the effect of piroxicam treatment only, the interaction of PPARα and piroxicam treatment, and the interaction of tissue and piroxicam treatment were calculated (Table 6-8).

Real time quantitative PCR (RT Q PCR) of Apobec3 and Onecut2 in Study 2B untreated samples (Table 6-7) showed comparable results to the earlier Taqman® low density array analysis of the same samples (Chapter 5).

Expression of Apobec3 variant 1 showed significantly high up-regulation in $APC^{Min/+}$ $PPARα^{-/-}$ samples compared to $APC^{Min/+}$ samples (Table 6-7).

Similarly, Apobec3 variant 1/2 expression levels were comparable to those seen in Affymetrix® microarrays. That is, expression in $APC^{Min/+}$ $PPARα^{-/-}$ samples was significantly down-regulated compared to $APC^{Min/+}$ samples (Table 6-7).

Expression of Onecut2 was confirmed as being significantly highly up-regulated in tumour tissue compared to normal tissue (Table 6-7).

Also, these data demonstrated a similar expression pattern of Apobec3 and Onecut2 to that seen in RT Q PCR analysis of the same genes in Study 1B samples (Table 6-6). This confirmed that expression of

Apobec3 and Onecut2 in the two sets of samples (Study 1B and Study 2B untreated samples) were comparable.

However, RT Q PCR analysis of these genes in Study 2B piroxicam-treated samples (Table 6-8) produced differing results to those seen in Taqman® low density array analysis of the same samples.

These data indicate that expression levels of Apobec3 variant 1 and Onecut2 were significantly increased with piroxicam treatment in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ samples and in tumour samples respectively (Table 6-8).

This contradicts earlier results produced by Taqman® low density arrays which found the effect of piroxicam treatment on Apobec3 expression was not significantly affected by PPARα status (Table 5-18). Similarly, Taqman® low density array results showed piroxicam treatment did not significantly affect Onecut2 expression in tumour samples (Table 5-17).

Table 6-7 Real Time Quantitative PCR of Apobec3 & Onecut2: Study 2B (untreated samples)

Gene	Mean relative quantity of gene expression to $APC^{Min/+}$ normal (untreated) +/- standard error			p value		
	Min tumour (untreated)	$PPAR\alpha^{-/-}$ normal (untreated)	$PPAR\alpha^{-/-}$ tumour (untreated)	Effect of PPARα	Effect of tissue	Interaction between PPARα & tissue
Apobec3 (primers & probe specific to variant 1)	0.8 +/- 0.003	77.2 +/- 0.06	76.2 +/- 0.32	< 0.0001	0.013	0.012
Apobec3 (primers & probe for variant 1 & variant 2)	0.6 +/- 0.15	0.3 +/- 0.02	0.3 +/- 0.08	0.011	not significant	not significant
Onecut2	23.0 +/- 0.14	3.3 +/- 0.01	107.0 +/- 0.64	0.004	< 0.0001	0.005

Table 6-8 Real Time Quantitative PCR of Apobec3 & Onecut2: Study 2B (piroxicam-treated samples)

Gene	Mean relative quantity of gene expression to $APC^{Min/+}$ normal (untreated) +/- standard error				p value		
	Min normal (piroxicam treated)	Min tumour (piroxicam treated)	$PPAR\alpha^{-/-}$ normal (piroxicam treated)	$PPAR\alpha^{-/-}$ tumour (piroxicam treated)	Effect of treatment	Interaction between PPARα & treatment	Interaction between tissue & treatment
Apobec3 (primers & probe specific to variant 1)	3.5 +/- 0.01	3.2 +/- 0.02	86.3 +/- 0.29	155.2 +/- 0.21	0.001	0.003	0.011
Apobec3 (primers & probe for variant 1 & variant 2)	1.1 +/- 0.32	1.5 +/- 0.71	0.4 +/- 0.04	1.0 +/- 0.07	0.03	not significant	not significant
Onecut2	2.7 +/- 0.007	113.7 +/- 0.55	1.7 +/- 0.003	343.7 +/- 0.41	0.003	not significant	0.003

6.5 Discussion

Earlier chapters analysed Apobec3 expression in the mouse colon using Affymetrix® microarrays and Taqman® low density arrays. The two arrays produced differing results; a decrease in Apobec3 expression was recorded in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ samples on Affymetrix® microarrays. However, a large increase in Apobec3 expression in the same samples was seen with Taqman® low density arrays. These data indicated that PPARα may be involved in the regulation of Apobec3. Therefore, the gene was analysed further.

A reason for the discrepancy in array results may be due to different sequences of the probes on the two arrays. Further investigation revealed that there are two isoforms of Apobec3; variant 1 and variant 2. The sequences of the probes did indeed show that Affymetrix® microarrays amplified variant 1 or variant 2, whereas Taqman® low density arrays only amplified variant 1 of the gene. These results were verified by real time quantitative PCR which showed comparable expression levels of Apobec3 to the levels determined on Affymetrix® microarrays (variant 1 or 2) and Taqman® low density arrays (variant 1 only).

Therefore, these data raised two questions:

Why do changes in expression of Apobec3 with PPARα deletion as detected by Affymetrix® microarrays and Taqman® low density arrays go in opposite directions?

How does PPARα affect the expression of Apobec3?

A possible answer to the first question may be the existence of mutation clusters which feature many somatic substitutions of cytosine, as demonstrated by other studies (Roberts et al., 2012, Nik-Zainal et al., 2012). These studies strongly support the idea that the Apobec family of cytidine deaminases is instrumental in development of a cancer genome.

Therefore, analysis of the colon cancer genome in mice and humans could potentially identify mutation clusters, cytidine deaminase motifs and miRNA sequences.

A potential answer to the second question could be regulation of miRNAs by PPARα that control expression of Apobec3. As discussed earlier, a study by (Ding et al., 2011) suggested existence of a novel pathway involving Apobec3 and miRNA-29 in promotion of hepatic metastasis.

PCR was carried out to verify the DNA sequence of Apobec3 variant 1 and variant 2. However, PCR with primer pairs A and C did not amplify the targeted sequences; primer pair A to amplify exon 1 – exon 9, and primer pair C to amplify exon 5 – exon 9. As discussed previously, this is possibly due to the existence of a large predicted 3' UTR at the end of the protein coding region. However, exon 1 - exon 5 (primer pair B) of the gene was sequenced which established that variant 1 of Apobec3 was highly up-regulated in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ samples. Future work will be to design new primers to amplify and sequence Apobec3.

It would also be interesting to establish which isoform of Apobec3 is present in the colon; that is whether Apobec3 variant 1 is the only isoform or whether there is a combination of both isoforms.

Alignment of National Centre of Biotechnology Information (NCBI) *Mus musculus* Apobec3 protein sequences against *Homo sapiens* Apobec3 protein sequences revealed significant similarity between *Mus musculus* Apobec3 and *Homo sapiens* Apobec3C, Apobec3H and Apobec3G. It would be interesting to determine if there is any functional significance in the similarities, and also whether PPARα is implicated in regulation of this gene in humans.

Results of Real time quantitative PCR (RT Q PCR) of Apobec3 and Onecut2 in Study 1B and Study 2B (untreated samples) showed similar results for each gene in each study. Samples in both studies demonstrated significant down-regulation of Apobec3 variant 1/2 in

$APC^{Min/+}$ $PPAR\alpha^{-/-}$ samples, and up-regulation of Onecut2 in tumour samples. These data showed that two different sample sets from the same mouse models and under similar experimental conditions produced comparable data.

These RT Q PCR results also verified Affymetrix® microarray data of Apobec3 variant 1/2 expression and Onecut2 in Study 1B samples, which showed significant down-regulation in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ samples and tumour samples respectively.

RT Q PCR of piroxicam study (Study 2B) untreated samples demonstrated that expression of Apobec3 variant 1 was significantly up-regulated in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ samples (Table 6-7). In addition, these data showed piroxicam treatment significantly up-regulated Apobec3 variant 1 in similar samples (Table 6-8).

Similarly, RT Q PCR of Onecut2 in Study 2B untreated and piroxicam-treated samples showed it was significantly up-regulated in tumours (Table 6-7, Table 6-8).

These data show Onecut2 was highly up-regulated in tumour tissue of the colon. This may indicate an important role for Onecut2 in tumorigenesis in the colon. It would be interesting to investigate how silencing of the gene with miRNAs affects tumorigenesis. Additionally, establishing the downstream targets of Onecut2 activation could potentially lead to identification of new targets for colon cancer therapy.

This body of work has highlighted some interesting avenues for future study. In particular, does PPARα have a role in the regulation of Apobec3 expression in humans? Or, do miRNAs feature in the control of expression levels?

These possibilities for further research are discussed in detail in Chapter 7.

7 Discussion & Future Work

7.1 Summary of findings of Study 1 and Study 2

The present studies with $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice were undertaken to support and provide confirmation of previous research by (Jackson et al., 2003) which suggested a PPARα-mediated effect in polyp development in the mouse gut.

The $APC^{Min/+}$ mouse model was selected for these studies as it has a mutated Adenomatous Polyposis Coli (APC) gene similar to the mutation observed in human Familial Adenomatous Polyposis (FAP) and sporadic colon cancer (Moser et al., 1990).

Study 1 of this research used two groups of $APC^{Min/+}$ mice, one group with deletion of PPARα ($APC^{Min/+}$ $PPAR\alpha^{-/-}$) to investigate the effect of PPARα on survival and weight, polyp development and gene expression in the colon.

Results of this study showed there was no difference in the ages of $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice at sacrifice, but $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice were significantly heavier ($p < 0.0001$). As discussed previously this could be because of the role of PPARα in lipid and fatty acid metabolism (Desvergne and Wahli, 1999, Kersten et al., 2000). However, as food intake was not monitored, it may be that $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice consumed more feed.

The number of polyps in the colon was significantly higher in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice than in $APC^{Min/+}$ mice ($p < 0.0001$). Similarly, when age at sacrifice was taken into account (number of polyps per week) the same result was seen ($p < 0.0001$).

However, the difference in number of polyps in the small bowel between $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice and $APC^{Min/+}$ mice was not significant. A similar result was seen when age at sacrifice was considered.

Polyp number in the small bowel in these mice was high and probably led to anaemia (visible sign is pale foot pads - white paw), necessitating sacrifice of the mice. Polyp number in the colon was comparatively much lower. However, the higher number of polyps in the colon of $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice than $APC^{Min/+}$ mice may suggest that PPARα has a role in preventing initiation or growth of tumours in the colon.

Analysis of gene expression in the colon with Affymetrix® microarrays demonstrated that the largest source of variation was between tumour and normal tissue.

There were far fewer differentially expressed genes between $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice. Deletion of PPARα had little effect on gene expression in normal tissue but appeared to have more effect in tumour tissue. Therefore, this may indicate that deletion of PPARα exacerbates the existing $APC^{Min/+}$ mutation to promote tumorigenesis.

Differentially expressed gene data from Affymetrix® microarray analysis (Comparison groups 1 – 4) were input into Ingenuity® Pathway Analysis (IPA). IPA showed the two top-scoring biological processes were growth and proliferation (p = 1.03E-10), and colorectal cancer (p = 1.66E-07); 95 genes were selected for further analysis. Selection of genes was based on molecular function; and fold change and p value as previously assessed on Affymetrix® microarrays.

The 95 genes were analysed on Taqman® low density arrays. Expression data of two genes proved particularly interesting; Onecut homeobox 2 (Onecut2) and Apolipoprotein B DNA dC → dU - editing enzyme, catalytic polypeptide 3 (Apobec3).

Affymetrix® microarrays and Taqman® low density arrays showed that Onecut2 was highly up-regulated in tumour tissue in the colon. This was a novel finding as this gene had previously been shown to be expressed in the small bowel but not the colon (Maier et al., 2006, Vanhorenbeeck et al., 2007). Also, a recent study demonstrated a

potential regulatory role of Onecut2 in the small bowel (Dusing et al., 2010).

Real time quantitative PCR (RT Q PCR) of Onecut2 was performed which validated the previous expression levels shown on Affymetrix® microarrays and Taqman® low density arrays.

Therefore, these data may indicate a role for Onecut2 in tumorigenesis in the colon.

Results from Affymetrix® microarrays and Taqman® low density arrays for expression of Apobec3 were conflicting. However, both sets of results suggested that expression of Apobec3 may be mediated *via* PPARα. Affymetrix® microarray results showed expression of the gene was down-regulated in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ samples. Whereas, Taqman® low density array results showed the gene was highly up-regulated in similar samples.

The discrepancy of the results led to investigation of the Apobec3 gene and the sequences of the probes used on each array type. Investigations showed that there are two isotypes of Apobec3 in the mouse; variant 1 and variant 2. Variant 2 is shorter than variant 1 as it lacks an alternate in-frame exon in the coding region compared to variant 1 (exon 5). It was demonstrated that the probe sequence of Apobec3 on Affymetrix® microarrays could target either isotype. However, the probe sequence on Taqman® low density arrays matched sequence on exon 5 of the Apobec3 gene and therefore could only target variant 1.

To validate these data RT Q PCR of Apobec3 with primers and probes designed to amplify variant 1 and variant 2, and variant 1 only was performed. Amplification with variant 1/2 primers and probe produced comparable levels of expression to levels shown in Affymetrix® microarrays. Similarly, variant 1 only primers and probe produced expression levels comparable to results of Taqman® low density arrays.

How PPARα may mediate expression levels of Apobec3, and why amplification of different sequences (exons) on the gene should produce such differing results could be due to regulation by miRNAs (Ding et al., 2011) and/or the existence of mutation clusters (Roberts et al., 2012, Nik-Zainal et al., 2012), as discussed earlier. Future work to address these questions is suggested later in this chapter.

Study 2 assessed the long-term effect of piroxicam treatment on survival and weight, polyp development and gene expression in the colon. Also, whether these effects were mediated *via* PPARα was investigated. Mouse models as previously were used and were dosed with piroxicam at 100 parts per million (ppm) in their diet or diet only.

Results from this study demonstrated a striking increase in survival in all mice dosed with piroxicam. However, $APC^{Min/+}$ mice lived significantly longer than $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice ($p = 0.019$). Additionally, all piroxicam-treated mice were significantly heavier than non-treated mice. However, these mice were significantly older so this result would be expected.

The number of polyps, polyp size and total tumour burden were not significantly reduced with piroxicam treatment in the colon. However, when age at sacrifice was considered (per week), polyp number but not polyp size or total tumour burden was significantly reduced with piroxicam treatment. This may indicate that colonic polyps although fewer in number may be larger.

The effect of piroxicam treatment on polyp number, polyp size and total tumour burden in the small bowel was dramatic. All were significantly reduced in all piroxicam-treated mice, with similar effects seen when age at sacrifice was considered.

The significant reduction in small bowel polyps with piroxicam treatment is likely to be the reason for the increase in survival in piroxicam-treated mice. The increase in life-span appears to have also had an impact on polyp development in the colon. These data may indicate that the effect

of piroxicam treatment in the colon may inhibit development of nascent polyps but not existing polyp growth.

Taqman® low density array analysis of selected genes (as previously) in piroxicam-treated mice showed similar expression levels in $APC^{Min/+}$ mice and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice.

The results of polyp analysis and gene expression taken in combination indicated that the effect of piroxicam treatment was not mediated *via* PPARα.

Apobec3 and Onecut2 were previously identified as particular genes of interest. Therefore, to validate the findings of the piroxicam treatment study (Study 2), RT Q PCR was carried out as before.

Conversely, results from RT Q PCR indicated a significant PPARα mediated effect of piroxicam treatment in Apobec3 variant 1 expression ($p = 0.003$). Similarly, expression of Onecut2 was significantly higher in tumour tissue with piroxicam treatment ($p = 0.003$).

In summary, these studies have demonstrated that deletion of PPARα produced a phenotype that made development of tumorigenesis in the colon more likely.

In addition, expression of Apobec3 and Onecut2 were shown to be highly up-regulated in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ samples and tumour samples respectively. This may point to a role for these genes in tumorigenesis. However, the mechanisms for these effects remain to be elucidated.

Also, these results indicate the effect of piroxicam treatment was not mediated *via* PPARα. However, subsequent analysis of Apobec3 variant 1 expression with RT Q PCR did point to a PPARα mediated effect. Similarly, RT Q PCR of Onecut2 showed expression was up-regulated in tumour tissue with piroxicam treatment.

It would be interesting to investigate these discrepancies further. Future works to examine possible reasons for these, along with other suggestions to advance this research are discussed.

7.2 Future work

Analysis of Study 1 gene expression data of the mouse colon revealed a list of possible PPARα-controlled genes, but few of these matched those identified in earlier studies (Mandard et al., 2004, Rakhshandehroo et al., 2010). Potentially, this could be due to the discovery of previously unrecognised PPARα-controlled genes, or perhaps pleiotropic effects independent of PPARα activation.

To further investigate these possibilities, a short-term (7 day) study treating $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice with the potent PPARα agonists' methylclophenapate (MCP) and fenofibrate will be carried out.

Also, Study 2 gene expression data of the mouse colon indicated that the effects of piroxicam treatment were not mediated *via* PPARα. However, these data also indicated that changes in expression of Apobec3 may be mediated *via* PPARα, and conversely, expression of this gene was increased with piroxicam treatment. Therefore, to address these inconsistencies another short-term (7 day) study will be carried out. $APC^{Min/+}$ and $APC^{Min/+}$ $PPAR\alpha^{-/-}$ mice will be treated with piroxicam at 100ppm as previously. In addition, the effects of piroxicam treatment at 300ppm will be assessed.

Mouse intestines from both short-term studies will be harvested immediately after sacrifice; however, due to the short term nature of the study, polyps or tumours would be unlikely to have developed. Therefore, instead of collecting separate tumour and normal tissue as previously, epithelial cells will be extracted from the colon and the small bowel as described (Bartnik et al., 1980, Mahida et al., 1997). Gene expression of selected genes in the mouse colon and small bowel will be determined using Taqman® low density arrays as before.

In addition to analysis of the short term effects of the PPARα agonists MCP and fenofibrate, and piroxicam on gene expression in the colon and small bowel, other potential opportunities for further research have been presented during the duration of this research. Results from the

present studies indicated that two genes, namely Apobec3 and Onecut2 may have an important role in tumorigenesis in the colon.

As discussed previously, the mechanism for regulation of Apobec3 expression may occur *via* miRNA mediated control of PPARα activation. In addition, localised mutation of cytosine bases by cytidine deaminases such as Apobec3 may have led to the development of a tumour phenotype. Therefore, it could be informative to explore the mouse and human colon cancer genomes for evidence of localised mutations and cytidine deaminase motifs.

Also, characterisation of the mouse and human transcriptomes using high-throughput sequencing (HTS) methods (RNA-Seq) could accurately predict the functional properties of RNAs and how these functions are altered in disease states (Blencowe et al., 2009).

Other methods for transcriptomics include a probabilistic inference method, for example, Emu, to identify areas of mutational processes; mutations in cancer genomes can be explained by a few mutational processes such as kataegis C > G and C > T in a TpCpX context (Fischer et al., 2013). Also, the ENCODE (Encyclopaedia of DNA Elements) project defines functional DNA elements in the human genome and includes information on RNA transcripts, transcriptional regulator binding sites and chromatin states (Kellis et al., 2014). The use of 3 approaches, that is, gene expression, genomic coverage (Lander et al., 2001) and evolutionary conservation (Johnsson et al., 2014) could provide complementary information to determine genomic function in human biology and disease.

In addition, further studies using a mouse model of Apobec3 deletion to investigate the effect of Apobec3 on gene expression in the colon and small bowel could be instigated.

As was discussed previously, the design of new primers to amplify and sequence the Apobec3 gene could lead to establishing the reasons for the apparent differences in expression in $APC^{Min/+}$ mice and $APC^{Min/+}$

PPARα$^{-/-}$ mice. Also, it would be interesting to determine the isoform/s of Apobec3 present in the mouse colon.

Analysis of the *Mus musculus* Apobec3 gene sequence against *Homo sapien* Apobec3 sequences demonstrated significant alignments. This solicits the question as to whether there is any functional significance in these similarities.

Therefore, gene expression studies using human colonic cell lines and/or tissue could be instigated to investigate;

1. Does Apobec3 have a role in tumorigenesis in the human colon?
2. Is PPARα implicated in the regulation of Apobec3 in the human colon?

In addition, the protein level of Apobec3 could be detected using specific antibodies with immunohistochemistry and/or protein immunoblots.

The present studies also defined Onecut2 as a novel gene that was highly up-regulated in tumour tissue in the colon, indicating the gene may be involved in tumorigenesis. Further analysis of the Affymetrix® microarray data and Ingenuity® Pathway Analysis (IPA) may reveal downstream targets of Onecut2 that are involved in development of cancer. Also, interrogation of the mouse and human transcriptome may highlight miRNAs involved in regulation of Onecut2.

Additionally, further research using human cell based and tissue based studies to investigate Onecut2 gene and protein expression are proposed.

As with Apobec3, a further study using a mouse model with Onecut2 deletion is suggested. A study by (Dusing et al., 2010) showed Onecut2 was highly expressed in the duodenum of postnatal wild-type mice but not in mice where Onecut2 was deleted. Their study showed Onecut2 is implicated in the temporal regulation and altered expression levels of many genes related to transport and metabolism in the epithelial

membrane, and also adenosine deaminase (ADA[30]). Mice with Onecut2 deletion have a significantly reduced size and weight, and increased mortality compared to wild type mice. Nevertheless, they have a similar life-span and fertility to wild-type mice after weaning (Dusing et al., 2010).

Analysis of polyp data and Taqman® low density array data from Study 2 (piroxicam study) indicated that the effects of piroxicam on polyp development and gene expression in the mouse gut were not mediated by PPARα. However, subsequent analysis with RT Q PCR indicated Apobec3 and Onecut2 expression may be affected by PPARα. Therefore, to investigate these anomalous results, the piroxicam study could be replicated using cell based or tissue based human studies.

In summary, this research has confirmed the role of PPARα in development of a tumour phenotype in $APC^{Min/+}$ mice. In addition, it appears that the effect of piroxicam may not be mediated *via* PPARα. However, the mechanisms that produce the effects that were seen remain to be elucidated.

Apobec3 and Onecut2 were shown to be significantly up-regulated in $APC^{Min/+}$ $PPAR\alpha^{-/-}$ samples and tumour samples respectively, indicating they may have an important role in the development of tumorigenesis in the mouse colon. Consequently, further investigations into the role and function of these two genes in humans are warranted and recommended.

PPARα is expressed in many tissues in the body (Table 1-2), therefore, there is the real possibility that the results and findings from this study may have implications in the development and progression of other cancers.

The rapid increase in available resources for interrogation of the human transcriptome could lead to identification of areas of mutational processes and RNA transcriptional regulation. For example, areas of

[30] ADA is ubiquitous in mammal cells and is a key enzyme in purine metabolism

kataegis are found with cytosine base substitutions at TpC dinucleotides suggesting Apobec3 plays a role in this. The results and findings of the work described here may imply that PPARα plays an important role in the regulation of Apobec3. Therefore, it is possible that Apobec3 or other members of the cytosine deaminase family have a similar role in other parts of the body.

In addition, Onecut2 is also known to be expressed in areas of the body other than the colon (6.1.2 page 232). Demonstration that Onecut2 was highly expressed in tumour tissue in the colon was a novel finding in this work. Therefore, it is feasible that Onecut2 could be implicated in tumorigenesis in other cancers.

Piroxicam, the NSAID used in this study, showed dramatic reductions in polyp growth in the mouse intestines. However, the Committee for Medicinal Products for Human Use (CHMP, June 2007) has recommended restrictions on its use in humans due to increased risk of gastro-intestinal side effects and serious skin reactions.

The current recommendations for first-line treatment of colon cancer are surgery and/or folinic acid plus fluorouracil (FOLFOX); NICE guidelines [CG 131], December 2014,

http://www.nice.org.uk/guidance/cg131/chapter/introduction

The use of aspirin as prophylaxis for colon cancer is not currently recommended.

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Appendices

1. Differentially Expressed Gene (DEG) Lists

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal (Comparison 1)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Ang	down	9.8	3.31E-02
Rpgrip1	down	8.8	2.24E-02
Ang	down	7.3	2.87E-02
D130051D11Rik	down	3.4	5.51E-05
Igh /// Ighg1	down	3.1	3.90E-02
Irak4	down	2.7	1.56E-05
Igh	down	2.5	3.94E-02
Ppara	down	2.4	4.60E-03
Serhl	down	2.4	1.44E-03
Erdr1	down	2.2	4.99E-02
EG633640	down	2.2	2.94E-02
Cbx7	down	2.1	3.83E-03
Fabp5	down	2.0	4.94E-02
Tmem117	down	1.9	6.60E-03
Ppara	down	1.9	3.19E-02
Fabp5	down	1.9	4.07E-02
Serhl	down	1.9	3.02E-03
Acot2	down	1.8	3.38E-02
Higd1a	down	1.8	4.87E-02
C920006O11Rik	down	1.7	3.60E-02
Ptprg	down	1.7	3.63E-03
Slc38a1	down	1.7	1.60E-03
Satb1	down	1.7	4.80E-02
Apobec3	down	1.7	4.09E-03
Mmrn1	up	4.8	1.39E-02
EG665955	up	4.7	1.18E-03
Supt16h	up	2.4	9.82E-04
Phf17	up	2.3	1.77E-03
Uba6	up	2.3	9.81E-05
Cntn1	up	2.3	4.23E-02
Cntn1	up	2.1	1.05E-02
LOC433762	up	2.0	1.41E-02
Oasl1	up	1.9	1.44E-02
Celsr1	up	1.8	1.93E-02
Hspa8	up	1.8	3.66E-02
Ifi27l1	up	1.8	4.37E-02
Vcl	up	1.7	3.91E-02

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Ang	down	47.8	3.36E-02
Ang4	down	31.0	2.20E-02
Rpgrip1	down	17.1	4.26E-02
Ang	down	14.5	1.61E-02
Khdc1a	down	11.2	9.40E-03
D130051D11Rik	down	9.9	3.23E-05
Apcdd1	down	8.5	3.02E-02
Gpnmb	down	6.7	8.33E-05
Cbx6	down	5.7	4.27E-04
Cyp2b10	down	5.1	4.87E-02
Cyp2b10	down	4.9	3.77E-02
Cbx6	down	4.8	8.45E-04
Irx5	down	4.7	2.62E-02
Apobec3	down	4.7	1.65E-04
Ang4	down	4.5	3.97E-02
Greb1	down	4.4	7.72E-03
Alb	down	4.2	4.23E-02
Ang	down	3.9	1.62E-03
Slc7a8	down	3.9	3.72E-02
Irak4	down	3.9	9.91E-04
Higd1c	down	3.5	3.46E-02
Pik3ip1	down	3.3	8.35E-03
Syngr1	down	3.3	6.65E-03
Adh6a	down	3.3	3.57E-02
Serhl	down	3.2	1.89E-03
Hoxa11	down	3.2	4.64E-02
Entpd3	down	3.1	2.54E-03
Lef1	down	3.1	3.58E-02
1700097N02Rik	down	3.0	3.42E-02
Npb	down	3.0	5.13E-03
Tbx3	down	2.9	3.15E-02
Col23a1	down	2.9	3.94E-02
Tmed6	down	2.9	4.57E-02
Prkg2	down	2.9	2.34E-02
Sorbs2	down	2.8	4.97E-02
Zc3h6	down	2.8	4.00E-02
4833428M15Rik	down	2.7	4.82E-02
Slc38a1	down	2.7	2.94E-04
Tesc	down	2.6	3.06E-02
Casp6	down	2.6	2.73E-02

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
1810005K13Rik	down	2.6	3.27E-02
Ptprg	down	2.6	3.22E-02
Bik	down	2.6	1.29E-03
Greb1	down	2.6	3.28E-03
Fam89a	down	2.6	2.20E-02
Prei4	down	2.6	2.28E-02
1110034G24Rik	down	2.6	3.28E-02
Fgfrl1	down	2.6	9.66E-03
Chd8	down	2.5	4.77E-03
Tesc	down	2.5	9.35E-03
Ldhb	down	2.5	7.99E-03
1700097N02Rik	down	2.5	7.68E-03
Snhg11	down	2.5	3.50E-02
Bik	down	2.4	6.34E-04
Rabl4	down	2.4	2.64E-04
Fam69b	down	2.4	3.22E-02
Sp6	down	2.4	4.19E-02
C1qtnf6	down	2.4	1.61E-02
Prei4	down	2.4	3.00E-02
Wdr19	down	2.4	2.77E-02
Malat1	down	2.4	2.55E-02
Zfp783	down	2.4	1.10E-02
Atg9b	down	2.3	2.61E-02
Sbsn	down	2.3	4.04E-02
Nisch	down	2.3	3.19E-03
Ldhb	down	2.3	3.38E-02
Srd5a3	down	2.2	9.59E-03
2810410L24Rik	down	2.2	1.12E-02
Ldhb	down	2.2	2.13E-02
2310034G01Rik	down	2.2	4.06E-02
Mmp14	down	2.2	3.46E-02
Limch1	down	2.2	3.19E-02
Limch1	down	2.2	1.35E-02
Patz1	down	2.2	1.09E-02
1110059G02Rik	down	2.2	4.43E-02
Usp20	down	2.2	3.83E-02
4933429F08Rik	down	2.2	3.06E-02
Pvrl4	down	2.2	4.50E-02
Pla2g2a	down	2.2	5.40E-04
Trav12n-2	down	2.1	2.87E-02

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
E130012A19Rik	down	2.1	7.06E-03
BC057079	down	2.1	3.70E-02
Rabl4	down	2.1	7.90E-04
Comt1	down	2.1	1.12E-02
Prei4	down	2.1	1.96E-02
1700012B15Rik	down	2.1	4.51E-03
Svopl	down	2.1	4.17E-02
Pvrl4	down	2.1	3.47E-02
Plekhg5	down	2.1	2.32E-02
Hsf2bp	down	2.1	4.05E-02
D630039A03Rik	down	2.1	3.24E-02
1110059G02Rik	down	2.1	1.81E-02
Afap1l1	down	2.0	1.38E-02
Fgfrl1	down	2.0	7.75E-03
Odz4	down	2.0	4.03E-02
9530067D14Rik	down	2.0	4.26E-02
2310047M10Rik	down	2.0	2.87E-02
Fam109a	down	2.0	2.86E-02
Tcf12	down	2.0	3.75E-02
Pcbp4	down	2.0	1.09E-02
Sbsn	down	2.0	2.48E-02
Txnrd3	down	2.0	3.38E-02
1500012F01Rik	down	2.0	7.78E-04
Mafg	down	2.0	4.60E-02
Il17rd	down	2.0	2.77E-02
2810408A11Rik	down	2.0	1.72E-02
Pacsin3	down	2.0	2.27E-02
BC031353	down	2.0	9.51E-03
Edn3	down	2.0	3.15E-02
Prei4	down	2.0	3.38E-02
Nlk	down	2.0	3.23E-02
Ppp4r1l	down	1.9	3.86E-02
Serhl	down	1.9	1.07E-03
9330133O14Rik	down	1.9	2.11E-02
Klc3	down	1.9	4.94E-02
Tnfrsf19	down	1.9	6.74E-03
Hpn	down	1.9	2.22E-02
Slc20a2	down	1.9	9.52E-04
Ppp4r1l	down	1.9	4.69E-02
B930041F14Rik	down	1.9	1.23E-02

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Wbscr27	down	1.9	7.52E-04
Slc35f2	down	1.9	1.91E-02
Wnt5b	down	1.9	3.19E-02
Dkk2	down	1.9	5.60E-03
Nisch	down	1.9	4.35E-03
Pacsin3	down	1.9	3.82E-02
Pnp1 /// Pnp2	down	1.9	2.97E-03
C79946	down	1.9	5.80E-03
Zbtb20	down	1.9	4.05E-02
B4galnt4	down	1.9	2.15E-02
Klhl8	down	1.9	3.92E-02
Pik3r3	down	1.9	2.99E-02
Slc41a1	down	1.9	4.80E-02
9030601B04Rik	down	1.9	1.11E-03
1700052K11Rik	down	1.8	1.18E-02
Cish	down	1.8	9.69E-03
Plekha1	down	1.8	4.72E-02
BC062109	down	1.8	3.29E-02
3110045A19Rik	down	1.8	2.12E-02
Matr3	down	1.8	1.66E-02
Lass4	down	1.8	3.97E-02
Slc48a1	down	1.8	2.02E-03
A130086G11Rik	down	1.8	1.25E-02
Hunk	down	1.8	2.42E-02
Fem1c	down	1.8	2.79E-02
Fam43a	down	1.8	3.28E-02
Ptprg	down	1.8	3.12E-02
6720401G13Rik	down	1.8	2.82E-02
Six4	down	1.8	9.71E-03
Ptprg	down	1.8	1.09E-02
Zfp560	down	1.8	2.89E-02
Neu1	down	1.8	1.46E-02
2310005E10Rik	down	1.7	6.74E-03
Clps	down	1.7	4.42E-02
Hexdc	down	1.7	1.11E-02
Bckdha	down	1.7	9.94E-03
C730025P13Rik	down	1.7	4.72E-03
Haghl	down	1.7	3.59E-02
Zfp688	down	1.7	2.24E-02
6720475J19Rik	down	1.7	3.99E-02

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Cep78	down	1.7	1.84E-02
C230037E05Rik	down	1.7	3.15E-02
BC031353	down	1.7	1.02E-02
Zfp783	down	1.7	5.62E-03
Dlx3	down	1.7	2.82E-02
Mblac2	down	1.7	2.31E-03
Ccdc85b	down	1.7	2.59E-02
Haus4	down	1.7	1.27E-02
D130062J10Rik	down	1.7	3.08E-03
Gadd45g	down	1.7	8.81E-03
Trim45	down	1.7	4.80E-02
Ces6	up	15.1	3.30E-02
Ighg	up	11.3	2.71E-02
Snca	up	9.8	1.25E-03
Lum	up	9.5	2.30E-02
Igh-3 /// Ighg	up	9.1	3.91E-02
Hsd17b13	up	7.8	2.43E-02
Il6	up	7.4	7.34E-03
Vip	up	6.8	2.19E-02
Sfrp1	up	6.2	4.73E-02
Mmrn1	up	6.0	1.05E-02
2010001M09Rik	up	5.7	2.36E-02
Aldob	up	5.3	2.90E-02
Scin	up	4.9	2.93E-02
Slc39a5	up	4.8	2.17E-02
Atp10d	up	4.7	4.06E-04
Cntn1	up	4.7	5.42E-03
Selp	up	4.5	1.87E-03
Igk-V19-14	up	4.4	3.05E-02
Phf17	up	4.4	7.09E-04
Pck1	up	4.2	8.17E-03
Ccl2	up	4.1	1.05E-02
Col14a1	up	3.7	3.03E-02
Slc5a8	up	3.7	1.86E-02
Chgb	up	3.7	4.70E-02
Ncf4	up	3.6	1.11E-02
Il11	up	3.6	2.13E-02
Hspa1a	up	3.6	1.08E-02
Ccl11	up	3.6	8.54E-03
Rsad2	up	3.4	9.42E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Aspn	up	3.4	1.07E-02
Flrt3	up	3.4	2.32E-02
B3galt5	up	3.4	3.30E-02
Rsad2	up	3.3	8.07E-03
Sprr1a	up	3.3	4.99E-02
Ccl7	up	3.3	8.39E-03
Ccl21a	up	3.3	2.73E-02
Cd59a	up	3.3	7.22E-03
Flrt3	up	3.2	2.42E-02
Decr1	up	3.2	7.81E-04
Abcb1b	up	3.1	9.68E-04
Paqr5	up	3.1	3.07E-02
Gstm3	up	3.1	3.90E-02
Abcc9	up	3.0	1.71E-02
Arnt2	up	3.0	3.04E-02
Clca2	up	3.0	3.96E-02
Supt16h	up	3.0	1.03E-02
Atp2c2	up	3.0	1.32E-02
Pdzd3	up	2.9	1.71E-02
Arg2	up	2.9	2.85E-02
Prkce	up	2.9	1.93E-02
Ereg	up	2.9	3.99E-02
Ephx2	up	2.9	4.83E-02
Id4	up	2.9	8.21E-03
Cyp7b1	up	2.8	1.84E-02
Frzb	up	2.8	1.62E-03
Grem2	up	2.8	4.57E-02
Obfc2a	up	2.8	1.82E-02
Lgi2	up	2.8	1.24E-02
Gucy1a3	up	2.8	2.54E-02
Epas1	up	2.7	3.83E-02
Fam107b	up	2.7	1.05E-02
Igj	up	2.6	3.86E-02
Hspa1b	up	2.6	4.03E-02
Chl1	up	2.6	3.90E-03
Slc6a14	up	2.6	4.27E-02
1810014B01Rik	up	2.6	3.84E-02
Tgfbr3	up	2.6	3.34E-02
Pira2	up	2.6	6.41E-03
Clca1	up	2.6	4.80E-02

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
9930013L23Rik	up	2.6	1.35E-02
Hyou1	up	2.5	2.72E-03
Uba6	up	2.5	2.37E-05
Cxcl3	up	2.5	9.32E-03
Trpa1	up	2.5	4.47E-02
Osmr	up	2.5	1.58E-02
Igfbp5	up	2.5	4.58E-02
Hspa1b	up	2.5	4.69E-02
Vcan	up	2.5	5.25E-03
Gja1	up	2.5	1.50E-02
Ap1s2	up	2.5	1.49E-03
Cxcl12	up	2.5	1.43E-02
Lrrk2	up	2.5	4.43E-04
Hspa1b	up	2.5	4.35E-02
D730039F16Rik	up	2.4	3.02E-02
Plaur	up	2.4	1.13E-03
Csf2	up	2.4	1.41E-02
Slpi	up	2.4	4.21E-03
Jam2	up	2.4	3.93E-03
Rsad2	up	2.4	4.58E-02
Golph3l	up	2.4	7.18E-03
Itgam	up	2.4	2.86E-02
Tcf23	up	2.4	9.69E-04
Tgoln1 /// Tgoln2	up	2.3	2.18E-02
Bdh1	up	2.3	4.52E-02
Dnajc3	up	2.3	3.23E-03
2610018G03Rik	up	2.3	1.65E-02
Ndn	up	2.3	4.69E-02
Vcan	up	2.3	1.63E-02
Fam55b	up	2.3	1.57E-02
Gja4	up	2.2	2.52E-02
Tgoln1	up	2.2	4.40E-02
Slc40a1	up	2.2	2.86E-02
Dpt	up	2.2	2.97E-02
Tspan2	up	2.2	2.65E-02
Aldh1a3	up	2.2	4.64E-02
Prkce	up	2.2	6.13E-03
Nr3c1	up	2.2	2.45E-02
Sod3	up	2.2	4.97E-02
Ghr	up	2.2	2.68E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Tnc	up	2.2	4.68E-02
Gucy1b3	up	2.2	3.59E-02
Tgoln1	up	2.2	3.56E-02
Ednra	up	2.2	4.91E-02
Tmtc3	up	2.2	7.97E-03
S1pr3	up	2.2	4.88E-02
Etnk1	up	2.2	6.46E-03
Col4a5	up	2.2	2.95E-02
2010109K11Rik	up	2.2	2.13E-02
Tmem47	up	2.2	2.19E-02
Hgf	up	2.2	7.58E-03
Pck1	up	2.2	5.39E-03
P4ha1	up	2.2	1.84E-02
Angptl4	up	2.2	3.55E-02
Hs3st1	up	2.2	2.69E-02
Tff3	up	2.2	3.46E-02
Aspn	up	2.1	4.59E-02
Atoh1	up	2.1	8.25E-03
Slc35a1	up	2.1	4.32E-02
Dmp1	up	2.1	4.88E-02
Sema6d	up	2.1	1.66E-02
Mef2c	up	2.1	8.20E-03
Bnc2	up	2.1	4.75E-02
P2ry14	up	2.1	4.96E-02
Cnnm4	up	2.1	4.03E-02
Ddr2	up	2.1	1.35E-02
Crispld2	up	2.1	2.86E-02
Slc16a9	up	2.1	1.22E-02
Igfbp5	up	2.0	2.83E-02
Rab2b	up	2.0	1.77E-02
Adamts4	up	2.0	3.33E-02
Sertad4	up	2.0	4.79E-02
Fzd5	up	2.0	3.43E-02
Tbc1d4	up	2.0	4.87E-02
Pycard	up	2.0	3.67E-02
Adamts4	up	2.0	2.46E-02
Zfp9	up	2.0	8.34E-03
Ercc6l	up	2.0	1.81E-02
Fgf7	up	2.0	2.42E-02
Ehd2	up	2.0	3.23E-02

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Gfpt1	up	2.0	1.88E-02
Rabif	up	2.0	4.17E-02
Sord	up	2.0	1.22E-02
Tm6sf1	up	2.0	1.29E-02
Darc	up	2.0	4.69E-03
Tmem195	up	2.0	1.45E-02
5830444B04Rik	up	2.0	7.38E-03
Klf6	up	2.0	1.88E-02
Slco2b1	up	2.0	1.46E-02
Txnrd1	up	2.0	3.51E-02
Plod2	up	2.0	1.31E-02
Fgf7	up	2.0	1.63E-02
Btbd3	up	2.0	4.44E-02
Fam107b	up	2.0	2.27E-02
St6gal1	up	2.0	4.05E-03
Icam2	up	2.0	3.76E-02
Naalad2	up	2.0	4.28E-02
Atp8a1	up	2.0	3.36E-03
Dock8	up	2.0	3.01E-02
Armcx3	up	1.9	2.92E-02
Il1rn	up	1.9	3.40E-02
2610018G03Rik	up	1.9	4.37E-02
Ikzf2	up	1.9	4.51E-02
Mapre2	up	1.9	1.02E-02
Eln	up	1.9	4.56E-02
Cd59a	up	1.9	1.24E-02
Atf2	up	1.9	1.52E-02
Acss2	up	1.9	2.78E-02
Sec24d	up	1.9	4.81E-02
Efnb2	up	1.9	1.45E-02
AW987390	up	1.9	3.35E-02
Ndrg4	up	1.9	4.02E-02
Reck	up	1.9	1.40E-02
Lgals7	up	1.9	1.88E-02
Il13ra1	up	1.9	4.60E-03
Vhl	up	1.9	1.85E-02
Il13ra2	up	1.9	4.79E-02
Ifi27l2b	up	1.9	4.67E-02
Ifi27l1	up	1.9	1.66E-02
Rgmb	up	1.9	4.73E-02

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Lats1	up	1.9	3.42E-02
Uba6	up	1.9	2.29E-04
Mapre2	up	1.9	3.82E-02
Socs2	up	1.9	2.61E-02
Cd72	up	1.9	2.80E-02
E030049G20Rik	up	1.9	9.81E-03
Pmm2	up	1.9	4.43E-02
Rnf141	up	1.8	4.15E-03
Chpt1	up	1.8	2.63E-02
Fam107b	up	1.8	2.86E-02
Golph3l	up	1.8	2.06E-02
Crispld2	up	1.8	2.96E-02
Ap1s2	up	1.8	1.96E-02
Rbp7	up	1.8	9.64E-03
Hnrnpc	up	1.8	1.98E-03
Piga	up	1.8	4.63E-02
St8sia6	up	1.8	2.60E-02
Mier1	up	1.8	3.10E-02
Yy1	up	1.8	1.09E-02
Etnk1	up	1.8	5.37E-03
Gucy1b3	up	1.8	3.88E-02
Dcbld1	up	1.8	9.85E-03
Lgals12	up	1.8	1.49E-02
Nrp1	up	1.8	4.43E-02
Ifi27l1	up	1.8	3.11E-02
Hif1a	up	1.8	4.45E-02
Rgs4	up	1.8	6.71E-03
Trim25	up	1.8	1.15E-02
Slc35a3	up	1.8	3.51E-02
Antxr2	up	1.8	4.16E-02
Gstm2	up	1.8	1.62E-02
4732429D16Rik	up	1.8	3.26E-02
Itgav	up	1.8	1.34E-02
Gigyf2	up	1.8	2.80E-02
Dcn	up	1.8	8.12E-03
Nup54	up	1.8	2.38E-02
1110032E23Rik	up	1.8	2.16E-03
Mmp10	up	1.8	1.93E-02
Epb4.1l3	up	1.8	1.04E-02
Txnip	up	1.8	3.03E-02

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour (Comparison 2)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Ckmt1	up	1.8	3.97E-02
Lgals12	up	1.7	1.31E-02
Lox	up	1.7	2.10E-02
Rp2h	up	1.7	1.83E-02
Dgkh	up	1.7	4.80E-02
Rnf141	up	1.7	2.79E-02
Far1	up	1.7	4.72E-02
Mmrn2	up	1.7	3.29E-02
Tmem106a	up	1.7	2.86E-02
Pcolce	up	1.7	3.89E-02
Sema6d	up	1.7	4.25E-02
Canx	up	1.7	5.75E-03
Phlda2	up	1.7	2.01E-02
Armcx2	up	1.7	2.88E-02
Atp8a1	up	1.7	8.01E-03
AU040320	up	1.7	4.31E-02
2810417H13Rik	up	1.7	1.06E-02
Gfpt1	up	1.7	4.56E-02
Vps35	up	1.7	4.91E-02
Lin7c	up	1.7	5.64E-03
Endod1	up	1.7	3.61E-02
Cpd	up	1.7	5.08E-03
Prkx	up	1.7	3.64E-02
Bmper	up	1.7	3.41E-02
Casd1	up	1.7	1.99E-02
Pja1	up	1.7	3.64E-02

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Reg3b	down	133.0	2.29E-03
Wif1	down	129.8	5.17E-05
Defa5	down	117.3	3.36E-03
Defa24	down	102.9	2.71E-03
Reg3b	down	102.7	1.55E-03
Mmp7	down	78.3	1.86E-04
Itln1	down	60.0	1.36E-04
Mt4	down	55.3	1.09E-03
Apcdd1	down	51.5	1.83E-04
Mmp13	down	49.9	1.13E-05
Pla2g2a	down	48.5	1.07E-03
Apcdd1	down	46.7	6.64E-04
Expi	down	45.3	3.70E-03
Cxcl5	down	44.3	4.82E-04
Arg1	down	43.7	5.16E-07
Onecut2	down	41.7	3.95E-04
Slc38a4	down	40.6	7.13E-05
Mmp10	down	38.3	1.78E-06
Adcy8	down	37.4	5.43E-06
Onecut2	down	36.7	2.07E-04
Apcdd1	down	36.7	3.45E-04
Spp1	down	35.2	1.60E-04
Prox1	down	34.5	3.11E-06
Prox1	down	32.0	6.43E-07
Dkk2	down	32.0	5.36E-07
Reg3g	down	30.9	2.73E-03
Gm106	down	30.5	3.01E-04
Notum	down	29.8	4.05E-06
Apcdd1	down	29.4	2.93E-04
Nfe2l3	down	29.3	6.65E-05
Onecut2	down	28.3	5.66E-04
Spock2	down	28.3	6.79E-06
Prox1	down	27.3	1.09E-06
Cfi	down	27.3	6.54E-04
Tnfrsf11b	down	25.7	2.21E-04
Nebl	down	25.2	9.97E-04
Slc38a4	down	25.0	7.62E-04
Cxcl1	down	24.8	2.42E-04
Slc30a2	down	24.8	1.92E-05
Tacstd2	down	24.3	6.89E-05

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Alox12	down	23.8	2.04E-05
Pnliprp1	down	23.3	6.55E-04
Serpinb11	down	23.1	1.31E-03
Al747448	down	23.0	8.31E-10
Pnliprp2	down	22.9	6.23E-04
Lcn2	down	22.7	3.14E-03
Myl7	down	21.6	3.70E-05
Alb	down	21.0	2.88E-04
Cxcl2	down	20.5	3.26E-03
Khdc1a	down	20.4	1.89E-04
Pnliprp2	down	20.2	6.11E-04
Hp	down	18.6	5.06E-04
Gm106	down	18.3	3.44E-04
Mmp12	down	18.2	5.40E-06
Sox21	down	18.2	2.72E-04
Nkd1	down	18.2	7.64E-05
Nkd1	down	17.8	2.98E-06
Fam84a	down	17.7	8.08E-04
Lrg1	down	16.8	2.39E-03
A730054J21Rik	down	16.4	1.83E-04
Prr18	down	15.5	4.75E-05
Pdgfrl	down	15.5	1.22E-06
T	down	15.5	7.99E-04
Avil	down	15.3	1.78E-04
Krt23	down	14.7	1.05E-03
Kif26b	down	14.7	7.20E-05
Rnase1	down	14.6	4.24E-05
Sox17	down	14.6	4.55E-03
Plat	down	14.3	2.71E-04
Apcdd1	down	13.1	5.94E-04
Isl1	down	12.4	1.72E-03
Tnfrsf19	down	12.4	1.77E-05
Robo1	down	12.3	5.75E-04
Tnfrsf11b	down	11.8	1.74E-04
Spock2	down	11.8	1.48E-05
Cubn	down	11.7	1.14E-03
Cxcl3	down	11.6	3.19E-05
Il1rl1	down	11.3	3.54E-03
BC007180	down	11.1	4.76E-06
Rnf180	down	11.0	1.62E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Tex15	down	11.0	1.49E-04
Sox4	down	10.9	4.19E-04
Bex1	down	10.8	5.84E-04
Atg9b	down	10.6	5.22E-04
Cubn	down	10.5	1.12E-03
Marcksl1	down	10.3	3.48E-05
Lgr5	down	9.9	1.23E-03
Ptprz1	down	9.4	4.03E-03
Axin2	down	9.4	3.94E-05
Gprin2	down	9.4	7.54E-06
2900084O13Rik	down	9.3	8.03E-04
Fam89a	down	9.2	1.71E-07
Ptpro	down	9.2	2.87E-04
9930013L23Rik	down	9.2	9.86E-05
Isl1	down	9.2	8.34E-04
Adam8	down	9.1	2.19E-06
Prr18	down	9.1	1.28E-05
Sox4	down	9.1	1.12E-04
Odz4	down	9.1	2.59E-03
Marcksl1	down	9.1	5.56E-05
8030425K09Rik	down	9.0	1.92E-05
Slco5a1	down	8.8	5.42E-04
Foxq1	down	8.8	2.01E-03
Sox4	down	8.6	1.26E-04
2700046A07Rik	down	8.6	1.09E-03
Gata5	down	8.6	6.83E-06
Mt3	down	8.5	7.75E-05
Sp5	down	8.2	1.46E-05
Stra6	down	8.2	1.59E-04
Tbx3	down	8.1	4.45E-03
Sox4	down	8.0	1.44E-05
Odz4	down	8.0	6.30E-04
5730457N03Rik	down	8.0	2.71E-03
Tmem173	down	7.9	1.23E-04
Wnt10a	down	7.9	3.61E-04
Irx5	down	7.9	8.81E-04
Dusp4	down	7.9	3.70E-04
Gsdma	down	7.7	3.29E-05
Tead2	down	7.7	7.52E-05
Asprv1	down	7.6	1.69E-06

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Ptpro	down	7.4	8.19E-04
Plxnb1	down	7.4	1.66E-04
Cxcl1	down	7.4	6.40E-04
Rnf43	down	7.3	2.72E-04
Ttc9	down	7.2	5.02E-04
LOC100047138	down	7.2	4.63E-04
Grhl3	down	7.2	6.68E-05
6720475J19Rik	down	7.1	1.16E-04
Ecscr	down	7.1	7.01E-05
Il1rl1	down	7.1	5.42E-04
Il23a	down	7.1	3.61E-03
Jub	down	7.1	6.56E-06
Krt36	down	6.9	3.72E-03
Odz4	down	6.8	4.15E-04
Vash2	down	6.8	1.66E-06
Sox4	down	6.7	2.56E-05
BC037703	down	6.7	3.78E-03
Arntl2	down	6.7	1.29E-04
Neto2	down	6.6	2.24E-03
EG664949	down	6.6	2.43E-04
Rem2	down	6.6	2.18E-04
Cldn4	down	6.6	1.21E-03
Padi4	down	6.5	1.30E-03
Dio2	down	6.4	1.93E-04
Arl4c	down	6.4	1.39E-04
Inhba	down	6.4	5.17E-06
LOC100047138	down	6.3	5.68E-04
Basp1	down	6.2	2.79E-03
Scn2b	down	6.1	4.72E-03
Marcksl1	down	6.1	1.15E-04
Fam62c	down	6.0	2.15E-03
Mtap	down	6.0	1.42E-04
Cited1	down	6.0	1.66E-04
Mtap	down	5.9	2.38E-04
100042016	down	5.9	1.74E-04
Igfbp4	down	5.9	1.48E-03
Neurl1a	down	5.9	8.65E-04
Bmp7	down	5.8	3.41E-03
Phlda1	down	5.8	7.45E-04
Nfe2l3	down	5.8	6.87E-04

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Dyrk3	down	5.8	1.94E-04
Limch1	down	5.8	5.82E-04
Limch1	down	5.7	1.66E-03
Evl	down	5.6	2.75E-03
2810404F17Rik	down	5.6	1.67E-03
Pcdhb9	down	5.6	1.06E-03
Gpc1	down	5.6	1.52E-04
Tmem173	down	5.5	3.38E-04
Btbd11	down	5.4	2.22E-03
Tnfsf9	down	5.4	2.58E-04
Smox	down	5.4	7.07E-04
Gata5	down	5.4	1.64E-05
Chrnb1	down	5.4	1.07E-04
6720418B01Rik	down	5.4	6.78E-04
Pmepa1	down	5.3	1.57E-04
Rem2	down	5.3	9.11E-04
Tnfrsf19	down	5.3	1.12E-04
Vangl2	down	5.3	1.58E-04
Evx1	down	5.3	1.50E-04
Tubb2b	down	5.3	1.95E-03
Axin2	down	5.2	2.52E-04
Tubb2a-ps2	down	5.2	3.39E-03
Prei4	down	5.1	1.02E-03
Pgm2l1	down	5.1	2.24E-04
Mex3a	down	5.0	8.87E-06
Ascl2	down	5.0	1.99E-03
Htra1	down	5.0	4.25E-04
Cd244	down	4.9	3.58E-03
Slc16a10	down	4.9	1.13E-03
Rgs12	down	4.9	1.07E-04
Pgm2l1	down	4.8	1.02E-03
Tbx3	down	4.8	4.38E-03
Htra1	down	4.8	1.06E-03
Abcc4	down	4.8	2.07E-04
Ephb6	down	4.8	3.16E-03
Prmt1	down	4.7	1.06E-03
Scn2b	down	4.7	3.96E-04
Macc1	down	4.7	4.33E-03
Ramp3	down	4.7	1.00E-03
Elavl2	down	4.7	1.02E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Cdh13	down	4.6	5.15E-04
Ctxn1	down	4.6	4.04E-03
Znrf3	down	4.5	7.41E-04
Ascl2	down	4.5	1.81E-03
Wnt5a	down	4.5	4.51E-04
Rnf32	down	4.5	7.41E-04
Nav2	down	4.5	7.39E-04
Sox21	down	4.4	6.66E-05
Tcf4	down	4.4	2.18E-04
Mtap	down	4.4	1.73E-03
Wnt6	down	4.4	2.28E-03
Wnt6	down	4.4	4.38E-04
Tmeff1	down	4.4	3.80E-03
Mapk4	down	4.4	3.37E-03
6720475J19Rik	down	4.3	5.69E-05
Pvrl4	down	4.3	4.28E-04
Tcf4	down	4.3	1.19E-04
Slc7a2	down	4.3	1.05E-03
Slc18a1	down	4.2	4.57E-03
2810037O22Rik	down	4.2	3.59E-04
Foxc2	down	4.2	1.30E-04
BC057079	down	4.2	2.07E-04
Slc18a1	down	4.2	2.25E-03
Entpd3	down	4.2	1.04E-03
Ifitm3	down	4.1	5.84E-04
2700094K13Rik	down	4.1	3.77E-04
Btbd11	down	4.1	1.08E-03
Bcl2l11	down	4.1	1.01E-03
Nav2	down	4.1	1.30E-03
Tcf4	down	4.1	9.48E-05
Mex3d	down	4.1	3.00E-04
Lef1	down	4.1	4.91E-05
Tnfrsf12a	down	4.0	5.91E-04
Ptgr1	down	4.0	1.97E-03
Pmepa1	down	4.0	8.14E-04
Pla1a	down	4.0	1.88E-03
Sema3f	down	4.0	2.89E-04
Nr4a2	down	4.0	4.75E-03
Il11	down	4.0	3.17E-03
Echdc2	down	3.9	3.62E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Tgif2	down	3.9	6.77E-04
Mex3b	down	3.9	2.95E-04
Amot	down	3.9	8.15E-04
Igfbp4	down	3.9	1.88E-03
Prss12	down	3.9	4.77E-03
Cdh13	down	3.8	9.53E-04
Rdh10	down	3.8	2.29E-03
Wnt5a	down	3.8	3.04E-03
Mmp14	down	3.8	3.82E-04
Timp1	down	3.8	5.29E-04
Krt7	down	3.8	4.84E-05
Nuak1	down	3.8	2.07E-03
Ascl2	down	3.8	6.04E-04
Tcf4	down	3.8	2.99E-03
Elk3	down	3.8	8.55E-04
Slc44a2	down	3.8	4.41E-04
Tcf4	down	3.7	1.71E-03
Cyp11a1	down	3.7	1.42E-03
Snx10	down	3.7	1.09E-03
Smox	down	3.7	2.92E-04
Lef1	down	3.7	1.82E-03
Slc41a1	down	3.7	1.85E-03
Cd244	down	3.7	2.13E-03
Rnf183	down	3.6	3.25E-05
Csnk1e	down	3.6	5.39E-04
Nav2	down	3.6	2.80E-04
Bcl2l11	down	3.6	2.90E-03
Bmf	down	3.6	3.89E-03
Arl4c	down	3.6	2.03E-04
B4galnt4	down	3.6	4.11E-03
Marcksl1	down	3.6	4.17E-05
E030016H06Rik	down	3.5	2.59E-03
A330049M08Rik	down	3.5	2.04E-03
Mmp14	down	3.5	8.50E-04
Igfbp4	down	3.5	1.41E-03
Higd1c	down	3.5	3.43E-03
Nanos1	down	3.5	1.47E-04
Lamp2	down	3.5	5.80E-04
ENSMUSG00000073000	down	3.5	1.82E-03
2700081O15Rik	down	3.5	4.76E-04

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Ptpre	down	3.4	1.23E-03
9530048O09Rik	down	3.4	4.76E-04
Bcl2l11	down	3.4	1.34E-03
Avpi1	down	3.4	4.30E-04
Nr4a2	down	3.4	4.06E-03
Camta1	down	3.4	2.02E-03
Adamts12	down	3.4	2.73E-03
4921525O09Rik	down	3.4	3.46E-04
Klhl8	down	3.4	3.20E-04
D17H6S56E-5	down	3.3	3.18E-03
Serpine2	down	3.3	4.41E-03
Hebp2	down	3.3	9.04E-04
Prss22	down	3.3	1.73E-04
Rbms3	down	3.3	4.83E-03
Tiam1	down	3.3	2.62E-04
Hunk	down	3.3	1.62E-04
Col18a1	down	3.3	4.71E-03
Map4k4	down	3.3	3.96E-04
Vim	down	3.3	2.93E-03
Igfbp4	down	3.3	1.38E-03
Il17rd	down	3.3	6.71E-04
4732423E21Rik	down	3.3	4.59E-03
Spns2	down	3.3	2.76E-04
Krt6a	down	3.2	2.68E-04
Ly6e	down	3.2	1.35E-03
Tmem184c	down	3.2	4.38E-03
1700017B05Rik	down	3.2	1.01E-03
Vangl2	down	3.2	1.28E-04
Sh3pxd2b	down	3.2	7.41E-04
Fgfrl1	down	3.2	4.40E-03
4631416L12Rik	down	3.2	7.99E-04
Ptpre	down	3.2	2.16E-04
Lamp2	down	3.2	1.66E-04
Casp6	down	3.2	1.26E-03
Col18a1	down	3.1	2.81E-03
Prei4	down	3.1	1.42E-03
Psapl1	down	3.1	1.17E-03
Zfp518b	down	3.1	3.32E-05
Csgalnact1	down	3.1	1.16E-03
Sema4c	down	3.1	2.03E-04

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Slc16a3	down	3.1	3.70E-04
Apobec3	down	3.1	1.25E-03
Bcl2l11	down	3.1	3.34E-03
Vill	down	3.0	2.24E-04
Csnk1e	down	3.0	3.48E-04
Nme4	down	3.0	1.45E-04
Gpld1	down	3.0	5.53E-04
Tnfrsf12a	down	3.0	3.77E-03
Elk3	down	3.0	5.07E-04
Gusb	down	3.0	2.78E-04
Rhoj	down	3.0	1.79E-03
Ddit4l	down	3.0	1.49E-03
4732490B19Rik	down	3.0	7.15E-04
Mafg	down	3.0	4.97E-04
Pdlim4	down	3.0	2.99E-03
Vim	down	3.0	2.30E-03
Fam60a	down	3.0	4.55E-05
Pcdhb21	down	3.0	1.21E-03
Csrnp2	down	2.9	1.32E-04
D15Wsu126e	down	2.9	1.48E-03
Tnik	down	2.9	3.94E-03
Gpx2	down	2.9	7.76E-04
Map4k4	down	2.9	6.41E-04
Rcc2	down	2.9	8.86E-04
Dbn1	down	2.9	4.02E-04
Pea15a	down	2.9	1.38E-03
Stx1a	down	2.9	6.55E-04
Sepn1	down	2.9	4.63E-03
D17H6S56E-5	down	2.9	1.24E-03
Map4k4	down	2.9	1.35E-03
Adam12	down	2.9	2.06E-04
Mtap	down	2.9	1.18E-03
Rac3	down	2.9	1.32E-03
Usp22	down	2.9	1.28E-04
Nlk	down	2.9	1.20E-03
Lama5	down	2.8	3.86E-04
Ccnd1	down	2.8	2.64E-03
Phldb2	down	2.8	4.33E-03
Phlda3	down	2.8	1.85E-03
Rgs12	down	2.8	2.22E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Mex3a	down	2.8	4.85E-05
Tubb5	down	2.8	1.05E-03
Rtn4rl1	down	2.8	8.39E-04
Klc3	down	2.8	2.92E-05
Zbtb12	down	2.8	1.99E-05
Dpysl3	down	2.8	1.97E-03
Patz1	down	2.8	1.19E-03
Sh3kbp1	down	2.8	4.71E-03
Rbm24	down	2.8	1.12E-03
Pvrl4	down	2.7	4.17E-04
Prkd1	down	2.7	3.46E-03
4930426D05Rik	down	2.7	2.98E-04
Ddah2	down	2.7	1.06E-04
Sox6	down	2.7	4.17E-03
Cdk4	down	2.7	4.47E-03
Smarcc1	down	2.7	1.69E-04
Slc39a6	down	2.7	3.79E-03
Cdk4	down	2.7	3.89E-03
Cdk4	down	2.7	3.81E-03
Mafg	down	2.7	8.36E-04
Tcfe2a	down	2.7	1.26E-03
Slc25a30	down	2.7	2.73E-04
Sh3kbp1	down	2.7	3.16E-03
2310008H09Rik	down	2.7	1.70E-04
Abhd12	down	2.7	3.59E-03
S100a11	down	2.7	2.97E-04
Iffo2	down	2.7	5.89E-04
D730005E14Rik	down	2.7	1.59E-03
Hopx	down	2.7	2.21E-03
Nhp2	down	2.6	6.09E-05
Mpzl1	down	2.6	4.08E-03
Klhl8	down	2.6	3.21E-04
Elk3	down	2.6	2.50E-04
Stx1a	down	2.6	4.25E-04
Cbx6	down	2.6	1.20E-03
Cep72	down	2.6	1.91E-04
Cep78	down	2.6	3.12E-04
Hopx	down	2.6	9.34E-04
Pacsin3	down	2.6	1.45E-03
Bcam	down	2.6	1.55E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Pxdn	down	2.6	4.11E-03
Ckap4	down	2.6	4.59E-03
Efna4	down	2.6	1.79E-03
Runx1	down	2.6	3.61E-03
Sbk1	down	2.6	1.04E-03
Plcd3	down	2.6	6.97E-06
Nhp2	down	2.6	1.27E-05
Psapl1	down	2.6	2.91E-04
Armc10	down	2.6	1.94E-04
Hist3h2ba	down	2.6	3.90E-03
Zdhhc15	down	2.5	4.67E-03
Ifitm2	down	2.5	3.30E-03
Ezh2	down	2.5	2.95E-03
Aspscr1	down	2.5	3.21E-06
Slc25a30	down	2.5	7.66E-04
Plscr3	down	2.5	2.12E-04
Wdr6	down	2.5	4.76E-03
Iffo2	down	2.5	6.21E-04
2010109K09Rik	down	2.5	2.63E-03
9430064K01Rik	down	2.5	6.53E-04
Smarcc1	down	2.5	1.33E-03
Lgi2	down	2.5	4.48E-03
Zfp703	down	2.5	4.61E-03
Ets2	down	2.5	2.05E-04
Dlg5	down	2.5	3.33E-05
1700017B05Rik	down	2.5	2.49E-03
Bok	down	2.5	2.47E-03
Ppp1r14b	down	2.5	4.26E-03
Sypl	down	2.5	3.48E-03
Nme1	down	2.5	8.85E-05
Hoxa9	down	2.5	1.92E-03
Zfp451	down	2.5	1.63E-03
Dpysl3	down	2.4	1.27E-03
9430081I23Rik	down	2.4	4.74E-03
C79946	down	2.4	6.10E-04
Fmnl2	down	2.4	2.89E-03
Zfp90	down	2.4	1.06E-03
Apaf1	down	2.4	3.01E-03
Vasn	down	2.4	3.23E-03
Arhgdig	down	2.4	3.69E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Prdx2	down	2.4	2.21E-04
Smoc1	down	2.4	3.76E-03
Rnf122	down	2.4	3.25E-03
5730601F06Rik	down	2.4	2.48E-04
2610301F02Rik	down	2.4	7.34E-04
Cbx2	down	2.4	1.35E-03
Myc	down	2.4	4.30E-03
Vangl2	down	2.4	2.24E-05
Epb4.1l5	down	2.4	9.98E-04
Neto2	down	2.4	1.32E-03
Lysmd2	down	2.4	4.98E-03
Hmgb3	down	2.4	1.84E-05
Lama5	down	2.4	2.63E-04
Slco5a1	down	2.4	1.09E-03
Cbx6	down	2.4	1.74E-04
Usp11	down	2.4	1.49E-03
Ypel1	down	2.4	8.28E-04
Pcdhb16	down	2.4	4.05E-03
Skap2	down	2.4	4.64E-04
6720460F02Rik	down	2.4	2.05E-03
Runx1	down	2.4	2.72E-03
Map4k4	down	2.4	7.22E-04
Slc25a30	down	2.3	1.97E-03
Rtn4	down	2.3	2.80E-03
6820402I19Rik	down	2.3	2.45E-03
Slc44a2	down	2.3	2.34E-04
Abcg1	down	2.3	3.54E-03
Cdca7	down	2.3	2.61E-03
E430016P22Rik	down	2.3	5.28E-04
Cdc42ep1	down	2.3	1.34E-03
Wisp1	down	2.3	4.76E-04
Qtrt1	down	2.3	7.26E-04
Tulp3	down	2.3	6.71E-05
Rasal2	down	2.3	1.41E-03
Pabpc1	down	2.3	1.49E-03
Pabpn1	down	2.3	1.58E-03
Chic2	down	2.3	1.38E-03
Neurl1a	down	2.3	2.63E-03
Krt18	down	2.3	8.48E-04
Lif	down	2.3	4.55E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Fhl2	down	2.2	2.63E-03
Rab11fip5	down	2.2	6.88E-04
Rnmt	down	2.2	3.54E-03
5430404G13Rik	down	2.2	3.23E-03
Tbcel	down	2.2	4.76E-03
Gtf2i	down	2.2	2.78E-03
Ptplb	down	2.2	4.53E-03
Rere	down	2.2	2.96E-03
Notch1	down	2.2	2.72E-05
Cnn3	down	2.2	6.52E-04
Vgll4	down	2.2	1.91E-03
Rpl13a	down	2.2	1.77E-03
Etv6	down	2.2	2.77E-03
Mmp14	down	2.2	4.52E-04
Itga6	down	2.2	1.31E-03
Nop56	down	2.2	2.73E-03
Rnf122	down	2.2	2.11E-03
D930015E06Rik	down	2.2	4.73E-04
Sbk1	down	2.2	2.44E-03
Stmn1	down	2.2	3.14E-03
Nap1l1	down	2.2	2.75E-03
8030447M02Rik	down	2.2	1.65E-03
Tmod3	down	2.2	2.22E-03
100042016	down	2.2	2.06E-03
Nap1l1	down	2.2	1.88E-04
Cpsf6	down	2.2	2.59E-03
Patz1	down	2.2	1.11E-03
5730601F06Rik	down	2.2	3.11E-03
Traf4	down	2.2	1.42E-03
Skp1a	down	2.2	1.07E-03
Abhd12	down	2.2	1.67E-03
Apex1	down	2.2	2.03E-03
Cnn3	down	2.2	2.28E-03
Cd276	down	2.2	2.24E-04
Gng10	down	2.2	4.63E-04
Ext1	down	2.1	4.08E-03
Ets2	down	2.1	2.75E-04
Nhsl1	down	2.1	4.94E-05
Gar1	down	2.1	2.42E-04
Nap1l1	down	2.1	4.61E-04

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
2700023E23Rik	down	2.1	2.14E-03
Spnb3	down	2.1	4.58E-03
Rtn4	down	2.1	3.73E-03
Nap1l1	down	2.1	1.20E-03
2610201A13Rik	down	2.1	3.25E-04
1190003J15Rik	down	2.1	7.33E-04
Rbms1	down	2.1	2.96E-03
Sh3tc2	down	2.1	1.52E-03
Gm22	down	2.1	1.56E-03
Napepld	down	2.1	2.73E-03
Hdac2	down	2.1	2.96E-03
Hoxa10	down	2.1	3.23E-04
Mex3c	down	2.1	3.43E-03
Etv6	down	2.1	5.03E-06
Steap2	down	2.1	4.12E-03
Syngr1	down	2.1	9.66E-04
Ikbkap	down	2.1	9.42E-04
Pdgfb	down	2.1	1.84E-03
Clcn3	down	2.1	1.75E-04
Ift81	down	2.1	2.35E-03
Cnn3	down	2.1	4.91E-03
Hnrnpa1	down	2.1	3.14E-03
Mettl11a	down	2.1	2.39E-05
Hmgn1	down	2.1	1.58E-04
Ptk7	down	2.1	1.95E-04
Cd47	down	2.1	1.61E-03
Ccdc125	down	2.1	3.79E-03
Fam40a	down	2.1	3.02E-04
Cnn3	down	2.1	1.90E-03
Fam129b	down	2.1	2.86E-04
Zfand5	down	2.1	5.97E-04
F830028O17Rik	down	2.1	4.52E-03
Metap2	down	2.1	4.10E-04
9530048O09Rik	down	2.1	1.53E-03
2610024B07Rik	down	2.0	3.40E-03
2810026P18Rik	down	2.0	4.44E-03
Tbcel	down	2.0	2.84E-03
Sdc1	down	2.0	3.02E-03
Ppl	down	2.0	2.39E-03
Zfp282	down	2.0	8.79E-04

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Atxn10	down	2.0	1.92E-03
Nup155	down	2.0	2.62E-03
Hmgn1	down	2.0	2.12E-04
Apex1	down	2.0	4.83E-03
Nkd1	down	2.0	9.28E-04
Gpx1	down	2.0	1.45E-04
Rnmt	down	2.0	4.74E-04
Slc7a5	down	2.0	2.83E-03
Nap1l1	down	2.0	7.81E-04
Cul7	down	2.0	2.09E-03
2310008H09Rik	down	2.0	1.60E-03
Fam115a	down	2.0	1.42E-03
Xpot	down	2.0	4.61E-04
Ltv1	down	2.0	1.19E-03
Atxn10	down	2.0	1.19E-03
Fam40a	down	2.0	1.16E-03
Fbf1	down	2.0	8.53E-04
Aqp8	up	33.6	3.59E-03
Ces6	up	22.2	8.29E-04
Cnn1	up	19.0	4.17E-03
Sult1a1	up	18.7	1.47E-03
Selenbp1	up	16.9	2.47E-03
Selenbp1	up	16.8	2.11E-03
Maob	up	16.2	7.68E-04
Aadac	up	15.6	1.03E-03
Myh11	up	15.0	1.70E-03
Aldh1a7	up	15.0	3.67E-03
Ces2	up	14.7	2.28E-03
Synpo2	up	14.1	1.94E-03
Scin	up	13.8	9.69E-04
Synm	up	13.4	1.28E-03
Akr1c14	up	12.4	1.59E-03
Abca8a	up	12.4	4.74E-04
Pck1	up	12.3	1.15E-03
Ndn	up	12.3	4.84E-05
Cbr3	up	11.9	2.30E-03
Dpyd	up	11.8	1.33E-03
Ugt1a1	up	11.4	2.80E-03
Pgm5	up	11.4	3.00E-04
Actg2	up	11.3	1.21E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Usp2	up	10.8	7.61E-04
Ndn	up	10.7	1.26E-04
Ndn	up	10.7	9.81E-05
Sult1c2	up	10.6	3.57E-03
Acer1	up	10.4	1.74E-03
Des	up	10.4	2.78E-03
Abcb1a	up	10.2	2.29E-03
Hsd3b2	up	9.9	3.33E-03
Mep1a	up	9.8	4.51E-03
Grem2	up	9.7	5.37E-04
Foxp2	up	9.7	3.19E-03
Abcb1a	up	9.6	2.11E-03
Car3	up	9.5	1.11E-03
Slc26a3	up	9.2	4.06E-04
Tpm2	up	9.0	1.18E-03
Slc5a8	up	8.9	2.74E-03
Itih5	up	8.5	3.94E-03
Synm	up	8.5	1.39E-03
Aldob	up	8.2	2.02E-03
Slc16a5	up	8.1	3.05E-03
Rims1	up	8.1	1.75E-03
Trpm6	up	7.9	4.12E-03
Gstm3	up	7.9	2.85E-03
Ndn	up	7.8	9.31E-05
Fa2h	up	7.8	2.18E-03
Apob	up	7.8	2.99E-03
Gucy1a3	up	7.7	3.23E-05
Otc	up	7.7	2.11E-04
0610005C13Rik	up	7.7	1.64E-03
Ugt1a1	up	7.6	4.21E-03
Tpm2	up	7.6	1.23E-03
Tmprss8	up	7.6	4.65E-03
Tagln	up	7.5	1.30E-03
Id4	up	7.5	4.66E-03
Vdr	up	7.5	2.50E-03
Slc26a2	up	7.4	4.69E-03
Pdlim3	up	7.4	5.11E-04
Sspn	up	7.2	7.47E-05
Cyp2s1	up	7.2	2.20E-03
100042999 /// Sprr2a	up	7.1	4.80E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Entpd5	up	7.0	1.57E-03
Foxp2	up	7.0	2.96E-03
Mamdc2	up	7.0	4.82E-03
Myl9	up	6.9	4.31E-04
Abcg2	up	6.9	3.89E-03
Ugt1a1	up	6.7	2.43E-03
Entpd5	up	6.6	2.67E-03
Mylk	up	6.6	3.44E-04
Vdr	up	6.6	2.73E-03
Fgfbp1	up	6.5	6.14E-04
Aoc3	up	6.5	1.31E-05
Ccl28	up	6.4	4.97E-03
Acer1	up	6.3	1.68E-03
Tnxb	up	6.2	7.77E-05
Kcnd3	up	6.2	1.40E-03
Pdlim3	up	6.1	1.37E-03
Mgll	up	6.0	3.44E-03
Mgll	up	6.0	3.12E-03
Mep1b	up	6.0	1.93E-03
Es22	up	5.9	3.53E-03
Slc24a3	up	5.8	2.22E-04
Slc13a2	up	5.8	4.96E-03
Kcnf1	up	5.8	1.71E-03
Dhrs11	up	5.7	2.91E-04
Ntn1	up	5.7	4.21E-04
Ogn	up	5.7	6.18E-04
Tgfbr3	up	5.6	1.94E-03
Maoa	up	5.6	1.47E-03
Ccl28	up	5.6	4.08E-03
Cth	up	5.5	4.50E-04
Clic5	up	5.5	4.39E-03
LOC73899	up	5.5	2.78E-03
Rims1	up	5.5	1.46E-03
Clybl	up	5.4	3.09E-04
9530008L14Rik	up	5.4	3.21E-03
Entpd5	up	5.4	1.90E-03
Cd36	up	5.3	5.60E-04
Slco2b1	up	5.3	2.04E-03
Ahcyl2	up	5.3	8.91E-04
Myo15b	up	5.3	4.78E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Dpyd	up	5.3	1.14E-03
Enpep	up	5.3	2.30E-03
Sparcl1	up	5.2	8.01E-04
Dpt	up	5.2	8.40E-04
B130021B11Rik	up	5.1	2.59E-03
Scn7a	up	5.1	1.01E-03
Gstm3	up	5.1	1.62E-03
Prkaa2	up	5.1	3.13E-03
Scn7a	up	5.1	2.21E-03
Cxcl12	up	5.1	2.93E-04
Ppp1r12b	up	5.1	9.00E-05
Esrrg	up	5.0	2.78E-04
Entpd5	up	5.0	2.92E-03
Fmo1	up	5.0	2.68E-03
Tns1	up	5.0	9.25E-04
Bche	up	5.0	4.92E-03
Cyp2d22	up	5.0	3.22E-03
Sdpr	up	4.9	3.34E-03
B3galnt1	up	4.9	2.57E-04
Dio1	up	4.9	1.04E-03
Gpr160	up	4.8	3.67E-03
Tspan2	up	4.8	1.60E-03
Maoa	up	4.8	7.54E-04
Iyd	up	4.8	1.45E-03
Gstm2	up	4.8	8.06E-04
Pkdcc	up	4.8	1.32E-03
Smtn	up	4.8	4.96E-04
Pcdh24	up	4.8	2.95E-03
Myom1	up	4.7	4.71E-03
Abcc3	up	4.7	1.43E-03
Gm967	up	4.7	3.58E-03
Cpe	up	4.7	3.83E-03
Mfap5	up	4.7	3.08E-04
Mylk	up	4.7	1.90E-04
Pck1	up	4.6	4.28E-03
Sorbs1	up	4.6	2.65E-03
Entpd5	up	4.6	2.63E-03
Tns1	up	4.6	7.34E-04
Sdpr	up	4.6	2.41E-03
2610027H17Rik	up	4.6	2.96E-04

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Adcy5	up	4.6	3.25E-04
Fxyd1	up	4.6	1.53E-04
Bdh1	up	4.5	6.98E-04
Enpp2	up	4.5	7.11E-04
Gstt1	up	4.5	1.35E-05
Nr3c2	up	4.5	1.97E-03
Wscd2	up	4.5	1.73E-04
Speg	up	4.5	9.12E-04
0610040B09Rik	up	4.5	1.34E-03
Cox7a1	up	4.5	1.24E-03
Synpo2	up	4.4	4.81E-03
Prkaa2	up	4.4	2.57E-03
LOC100046086	up	4.4	7.01E-04
2010003H20Rik	up	4.4	2.04E-03
Glod5	up	4.4	1.25E-03
Sorbs1	up	4.4	1.64E-03
Sdpr	up	4.4	1.75E-03
Sorbs1	up	4.3	6.12E-04
Rbpms2	up	4.3	6.88E-04
Lhfpl2	up	4.3	1.21E-03
Slc25a34	up	4.3	3.23E-04
Pcp4	up	4.3	1.88E-03
Abcc9	up	4.3	2.46E-03
Espn	up	4.3	3.16E-03
Slco2a1	up	4.2	3.40E-04
EG240055	up	4.2	1.23E-03
2010012P19Rik	up	4.2	2.29E-03
Ugdh	up	4.2	4.08E-03
Lims2	up	4.2	1.66E-04
Gstm1	up	4.2	8.29E-04
Lhfpl2	up	4.2	9.44E-04
Gm967	up	4.1	1.28E-04
Ssbp2	up	4.1	3.34E-03
Myo15b	up	4.0	2.27E-03
Chrdl1	up	4.0	3.13E-04
Retsat	up	3.9	1.44E-04
Bnc2	up	3.9	8.74E-04
Hhip	up	3.9	4.26E-04
Acta2	up	3.9	2.10E-04
Gstm1	up	3.9	1.33E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Hhip	up	3.8	4.32E-04
Slco2a1	up	3.8	1.53E-03
Kcnk5	up	3.8	1.03E-03
Tns1	up	3.8	2.97E-03
Foxd2	up	3.8	4.64E-03
Cideb	up	3.8	4.59E-05
Cryl1	up	3.8	9.53E-04
LOC552901	up	3.7	2.85E-03
Chrdl1	up	3.7	4.90E-04
Meis1	up	3.7	2.46E-04
Dst	up	3.7	1.50E-03
Lmod1	up	3.7	3.26E-03
5730409N24Rik	up	3.7	3.67E-03
Pdzd3	up	3.7	9.36E-04
Cryl1	up	3.7	1.11E-03
Rspo3	up	3.7	1.09E-03
Trpm6	up	3.7	4.99E-03
Pycard	up	3.7	8.22E-04
Synpo2	up	3.6	8.06E-04
Calb2	up	3.6	3.71E-03
Ppfia3	up	3.6	3.68E-03
Mylk	up	3.6	6.14E-04
Hlf	up	3.6	2.72E-03
Prkg1	up	3.5	5.10E-04
Metrnl	up	3.5	1.21E-03
Mab21l2	up	3.5	2.61E-04
Slc4a4	up	3.5	4.24E-03
Sorbs1	up	3.5	3.00E-05
Ppargc1b	up	3.5	9.63E-04
Prkaa2	up	3.4	1.29E-03
Hhip	up	3.4	9.00E-04
Akap12	up	3.4	1.51E-03
Dgkh	up	3.4	6.93E-04
Scg5	up	3.4	2.87E-03
Rgs7bp	up	3.4	8.45E-04
Stmn2	up	3.4	3.38E-03
9130016M20Rik	up	3.4	3.93E-03
Mxd1	up	3.4	1.62E-03
Hspb7	up	3.4	4.09E-03
2310044G17Rik	up	3.3	1.41E-04

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Reck	up	3.3	8.70E-04
Cds1	up	3.3	3.22E-03
Me2	up	3.3	2.15E-03
Slc9a3r1	up	3.3	7.41E-04
Ssbp2	up	3.3	3.33E-03
Tmem117	up	3.3	3.21E-03
2010001M06Rik	up	3.3	9.96E-04
Mertk	up	3.2	1.38E-04
Cd36	up	3.2	1.56E-03
Klf4	up	3.2	3.12E-03
4931406C07Rik	up	3.2	3.71E-03
Cbs	up	3.2	1.78E-03
Sntg2	up	3.2	2.22E-03
Ptgis	up	3.2	3.64E-03
Myocd	up	3.2	4.06E-03
Slc9a3r1	up	3.2	1.45E-03
Ctdspl	up	3.2	6.35E-04
Sord	up	3.2	1.67E-03
Pkia	up	3.2	1.42E-03
Entpd8	up	3.2	2.26E-04
Slc9a3r1	up	3.2	1.42E-03
Popdc2	up	3.1	1.79E-03
Iqgap2	up	3.1	1.73E-03
AI605517	up	3.1	2.66E-03
Etv1	up	3.1	1.43E-03
Mupcdh	up	3.1	2.47E-03
Pcdh21	up	3.1	5.85E-04
Slc25a20	up	3.1	1.64E-03
Zfp385b	up	3.1	2.88E-03
Stk10	up	3.1	1.23E-03
Retsat	up	3.1	2.77E-04
Fzd5	up	3.1	8.61E-04
9130409J20Rik	up	3.1	2.85E-03
Fam13a	up	3.1	3.12E-03
Sdc2	up	3.1	4.34E-03
Chrdl1	up	3.1	8.05E-04
Itm2a	up	3.1	9.13E-04
Mustn1	up	3.1	2.72E-03
Qpct	up	3.1	2.31E-03
Mapre2	up	3.1	1.02E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Prkce	up	3.1	3.38E-03
Sdc2	up	3.1	4.81E-03
Tmem171	up	3.0	4.83E-04
Acad11 /// Nphp3	up	3.0	1.50E-05
Ddr2	up	3.0	7.76E-04
Cmbl	up	3.0	6.18E-04
Itga7	up	3.0	2.43E-03
Abcc9	up	3.0	1.97E-03
Tlr1	up	3.0	1.45E-03
Mpdz	up	3.0	8.40E-04
Fam13a	up	3.0	1.40E-03
Fahd1	up	3.0	1.97E-03
Aldh1b1	up	3.0	2.10E-03
Dst	up	3.0	1.40E-03
Cisd1	up	3.0	9.61E-04
Acss1	up	3.0	6.18E-04
Gstm1	up	3.0	9.41E-04
Cbr1	up	3.0	1.96E-04
Mrvi1	up	3.0	2.48E-03
Synpo	up	3.0	1.07E-03
Gstt3	up	2.9	5.87E-05
Tmlhe	up	2.9	1.17E-04
4931406C07Rik	up	2.9	4.21E-03
Clrn3	up	2.9	1.22E-03
Mxd1	up	2.9	1.82E-03
Ndn	up	2.9	1.54E-04
Arhgap26	up	2.9	1.06E-05
1810014F10Rik	up	2.9	6.19E-04
Gulp1	up	2.9	3.12E-03
2310044G17Rik	up	2.9	4.38E-03
Sgce	up	2.9	4.45E-04
Clcn2	up	2.9	6.10E-04
9130004J05Rik	up	2.8	2.06E-03
Acad11 /// Nphp3	up	2.8	3.68E-05
Gulp1	up	2.8	7.12E-04
Cds1	up	2.8	1.43E-03
Ppp1r3c	up	2.8	1.73E-04
Sh3gl2	up	2.8	1.42E-03
Kcne4	up	2.8	1.68E-03
Il17rc	up	2.8	2.58E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Fgf13	up	2.8	2.97E-03
Car2	up	2.8	2.24E-03
Meis1	up	2.8	3.31E-03
LOC100036521	up	2.8	3.57E-04
Trim31	up	2.8	8.67E-05
Filip1	up	2.8	2.48E-03
Slc30a1	up	2.8	2.91E-03
3110049J23Rik	up	2.8	3.06E-03
Pdk2	up	2.8	1.54E-03
Plcd1	up	2.8	2.14E-03
Cspg4	up	2.8	6.30E-04
100041434	up	2.8	2.46E-03
Sepp1	up	2.8	1.77E-03
Tspan2	up	2.7	1.90E-03
Heph	up	2.7	3.34E-04
Anks4b	up	2.7	9.35E-04
Sh3gl2	up	2.7	1.40E-03
Galm	up	2.7	4.30E-03
1110032E23Rik	up	2.7	9.18E-04
Tspan1	up	2.7	1.58E-03
2010003K11Rik	up	2.7	3.05E-03
Meis2	up	2.7	1.18E-03
Tmlhe	up	2.7	4.56E-04
Fam26e	up	2.7	1.77E-03
Satb2	up	2.7	6.86E-04
Tmlhe	up	2.7	7.00E-04
Pde3a	up	2.6	3.97E-03
Mtm1	up	2.6	4.10E-03
Frat2	up	2.6	9.27E-04
4930539E08Rik	up	2.6	4.28E-03
Rfk	up	2.6	3.56E-03
Prkg1	up	2.6	4.01E-03
Naprt1	up	2.6	3.54E-04
Rgs7bp	up	2.6	7.05E-04
Nedd4l	up	2.6	4.12E-04
Pcca	up	2.6	3.76E-04
Postn	up	2.6	3.09E-03
Msra	up	2.6	2.56E-03
1110032E23Rik	up	2.6	2.83E-03
Echdc3	up	2.6	8.77E-04

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Fbxo25	up	2.6	8.10E-04
Ppp1r14d	up	2.6	5.53E-04
Uap1	up	2.6	1.69E-03
Ndrg4	up	2.6	3.41E-03
Mapre2	up	2.5	3.85E-03
Ank2	up	2.5	3.87E-03
Neurod1	up	2.5	1.80E-03
Pfkfb4	up	2.5	1.21E-03
Lclat1	up	2.5	2.19E-03
Mapk12	up	2.5	6.25E-04
Ush1c	up	2.5	2.67E-03
Tgfb1i1	up	2.5	1.19E-04
Gucy2c	up	2.5	1.82E-03
Cacna2d2	up	2.5	1.55E-03
Epb4.1l3	up	2.5	1.96E-03
B130021B11Rik	up	2.5	3.94E-03
Mkrn1	up	2.5	2.64E-03
Kcnk5	up	2.5	5.15E-04
Ech1	up	2.5	1.64E-03
Uap1	up	2.5	1.95E-03
Mfsd8	up	2.5	3.66E-03
Hdgfrp3	up	2.5	4.64E-03
Hdgfrp3	up	2.5	4.25E-03
Epb4.1l3	up	2.4	3.02E-04
Pank3	up	2.4	1.62E-03
Bdh1	up	2.4	3.38E-04
Galm	up	2.4	1.70E-03
Erlin1	up	2.4	9.70E-04
2310044G17Rik	up	2.4	3.75E-03
Tmem106a	up	2.4	3.50E-03
Pttg1ip	up	2.4	4.55E-03
Fibin	up	2.4	2.71E-03
Endod1	up	2.4	4.21E-03
Mapre2	up	2.4	2.32E-03
5033414D02Rik	up	2.4	2.22E-03
Brpf3	up	2.4	2.04E-03
Tesk2	up	2.4	2.30E-03
9030425E11Rik	up	2.4	2.12E-03
Plcd1	up	2.4	2.84E-04
Clmn	up	2.4	2.16E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Myo1a	up	2.4	3.36E-03
Ankhd1 /// Eif4ebp3	up	2.4	4.73E-03
Tspyl4	up	2.4	6.73E-04
9030425L15Rik	up	2.4	1.41E-03
Ipmk	up	2.4	9.54E-04
Hnf4a	up	2.3	1.50E-03
B3gnt2	up	2.3	2.14E-04
AI317158	up	2.3	3.19E-03
Fam110c	up	2.3	9.66E-04
Txnip	up	2.3	3.67E-03
Plcl2	up	2.3	9.80E-04
Fam161a	up	2.3	2.38E-03
Plce1	up	2.3	2.02E-03
Jph2	up	2.3	2.59E-03
Itpk1	up	2.3	1.27E-03
Tspan3	up	2.3	3.25E-04
Cryl1	up	2.3	2.37E-03
Epas1	up	2.3	3.65E-04
Cpt1a	up	2.3	2.46E-03
Gli2	up	2.3	1.71E-03
Slc22a18	up	2.3	9.25E-04
Entpd2	up	2.3	9.86E-04
Srr	up	2.3	1.20E-03
Abcc9	up	2.3	3.78E-03
Suclg2	up	2.3	1.41E-03
Ndrg4	up	2.3	4.90E-03
Gng3	up	2.3	3.38E-03
Cacnb2	up	2.3	3.54E-03
1700040L02Rik	up	2.3	3.07E-03
Pld1	up	2.3	1.65E-03
Sri	up	2.3	2.13E-03
Suclg2	up	2.2	8.51E-04
Nudt19	up	2.2	6.33E-04
1300010F03Rik	up	2.2	1.19E-05
1300010F03Rik	up	2.2	4.70E-04
B3gnt2	up	2.2	9.75E-05
Rasgef1b	up	2.2	2.39E-03
Slc2a4	up	2.2	4.45E-03
Selm	up	2.2	4.91E-03
Plekha8	up	2.2	3.70E-03

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Slc2a4	up	2.2	5.68E-04
Prkca	up	2.2	3.95E-03
Tmem82	up	2.2	1.95E-03
Slc44a4	up	2.2	4.29E-04
Pld1	up	2.2	5.80E-04
Hadh	up	2.2	6.83E-05
Nsg2	up	2.2	1.50E-03
Serhl	up	2.2	1.64E-03
Wfdc1	up	2.2	2.62E-03
Myh14	up	2.2	1.50E-03
Slc22a23	up	2.2	4.88E-03
D4Bwg0951e	up	2.2	2.96E-03
Fmo2	up	2.2	3.21E-03
Ccdc68	up	2.2	3.41E-03
Bin1	up	2.2	1.55E-03
Dopey2	up	2.1	7.34E-04
Tgfb3	up	2.1	4.85E-03
Aldh1a2	up	2.1	2.72E-03
Ccng2	up	2.1	2.88E-03
Fnbp1	up	2.1	7.69E-04
Plekhg6	up	2.1	2.89E-03
Nedd4l	up	2.1	3.19E-03
Tep1	up	2.1	2.05E-05
Gdnf	up	2.1	3.28E-03
Chchd10	up	2.1	8.94E-04
Flna	up	2.1	1.82E-03
Zzef1	up	2.1	2.37E-03
Ints6	up	2.1	2.01E-03
Tst	up	2.1	2.22E-03
Smpdl3a	up	2.1	3.75E-03
Foxo3	up	2.1	5.72E-04
Runx1t1	up	2.1	1.07E-03
Oxsm	up	2.1	4.53E-04
Lrp1	up	2.1	2.25E-03
Syne1	up	2.1	2.35E-03
Pdss1	up	2.1	3.86E-03
Arl4d	up	2.1	1.37E-04
Hlx	up	2.1	1.96E-03
Fam110c	up	2.0	3.27E-04
Klc4	up	2.0	9.26E-04

Differentially Expressed Genes $APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour (Comparison 3)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Plscr4	up	2.0	1.43E-03
Gstt2	up	2.0	3.01E-03
Nr1i2	up	2.0	2.02E-03
Man1a	up	2.0	5.91E-04
Ifi30	up	2.0	5.79E-04
Abhd6	up	2.0	1.87E-03
Pttg1ip	up	2.0	4.89E-03
Hadha	up	2.0	3.91E-03
Atp6v0a2	up	2.0	3.67E-04
Hadh	up	2.0	6.05E-04
Acadm	up	2.0	3.20E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Reg3g	down	115.9	6.04E-06
Reg3b	down	114.8	9.68E-05
Reg3b	down	107.0	9.62E-05
Mmp7	down	63.9	1.26E-08
Onecut2	down	54.1	7.44E-08
Wif1	down	49.9	5.04E-05
Defa24	down	47.6	1.59E-05
Onecut2	down	44.1	1.03E-07
Cfi	down	42.5	7.40E-07
Slc38a4	down	41.2	1.13E-05
Mmp13	down	40.7	1.10E-05
Lcn2	down	39.9	6.71E-07
S100a9	down	39.4	4.48E-05
S100a8	down	38.1	5.06E-05
Defa5	down	37.4	1.25E-03
Mmp10	down	35.8	2.28E-05
Onecut2	down	35.7	2.56E-07
Spp1	down	35.4	6.17E-06
Arg1	down	34.1	1.04E-05
Tnfrsf11b	down	34.0	8.88E-06
Cxcl2	down	31.0	3.86E-06
Cxcl3	down	30.1	1.19E-07
Cxcl5	down	30.1	2.86E-05
Cxcl1	down	27.5	5.62E-05
Gm106	down	25.3	9.96E-06
Dkk2	down	24.9	7.56E-09
Adcy8	down	21.6	2.37E-06
Itln1	down	20.3	1.13E-03
Mmp12	down	19.8	1.08E-07
Slc38a4	down	19.0	1.78E-05
Il6	down	18.3	4.36E-05
Prox1	down	18.3	1.10E-05
Expi	down	17.8	5.33E-04
1190003M12Rik	down	17.4	7.54E-04
Lrg1	down	16.9	1.83E-05
Nfe2l3	down	16.8	3.68E-06
Saa3	down	16.2	7.18E-04
Lgals2	down	16.1	8.04E-04
Prox1	down	15.1	1.30E-05
Pnliprp1	down	15.0	1.29E-04

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Hp	down	15.0	4.07E-06
Apcdd1	down	14.4	3.45E-04
Gm106	down	14.0	1.44E-05
Prkg2	down	13.8	3.00E-03
Pla2g2a	down	13.7	7.89E-06
Plat	down	13.7	1.60E-04
Apcdd1	down	13.4	1.31E-03
Il1rl1	down	13.4	9.47E-04
T	down	13.1	1.86E-06
Slc30a2	down	12.9	6.56E-05
Notum	down	12.3	8.59E-05
Prox1	down	12.3	2.41E-05
Sox17	down	12.2	1.95E-03
9930013L23Rik	down	12.2	2.09E-04
Apcdd1	down	12.2	1.61E-03
Myl7	down	12.1	3.20E-04
Clu	down	11.9	4.24E-05
AI747448	down	11.6	2.94E-03
Ttc9	down	11.5	7.26E-08
Il1b	down	11.3	1.11E-06
BC037703	down	10.9	1.29E-05
Spock2	down	10.8	3.39E-03
Clu	down	10.8	5.59E-05
Krt23	down	10.7	1.18E-05
Cxcl1	down	10.3	2.88E-05
Clec4e	down	10.3	2.22E-07
Tnfrsf11b	down	10.2	6.37E-06
Clu	down	10.2	3.96E-05
A730054J21Rik	down	9.4	6.86E-05
Clu	down	9.3	3.67E-05
Cp	down	9.3	7.77E-08
Il11	down	9.3	6.43E-04
Ptgs2	down	9.2	3.17E-04
Robo1	down	9.2	2.60E-04
Prkg2	down	9.2	1.71E-03
Nkd1	down	9.2	1.95E-04
Nkd1	down	8.8	2.87E-05
Prr18	down	8.8	1.11E-05
1190003M12Rik	down	8.7	2.23E-03
Il1rl1	down	8.7	2.48E-05

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Tnfrsf19	down	8.6	1.35E-07
Gata5	down	8.3	3.47E-06
Ptgs2	down	8.2	4.09E-04
Marcksl1	down	7.9	8.54E-04
Cp	down	7.9	1.67E-07
Cp	down	7.7	7.18E-07
Avil	down	7.6	3.69E-06
Dio2	down	7.5	6.97E-04
Sox21	down	7.4	8.19E-04
Mmp9	down	7.4	8.25E-07
Pcdhb9	down	7.4	1.49E-05
Pdgfrl	down	7.3	3.41E-04
Cp	down	7.3	8.55E-08
Lgr5	down	7.2	3.48E-07
Inhbb	down	7.2	6.09E-06
Kif26b	down	6.7	2.72E-04
Mmp9	down	6.7	4.56E-06
Marcksl1	down	6.6	1.56E-03
Sox4	down	6.6	1.24E-04
Npnt	down	6.5	7.88E-04
Dusp4	down	6.5	1.58E-04
Sp5	down	6.5	7.76E-06
Ptpro	down	6.4	3.14E-05
Ppbp	down	6.4	2.67E-04
Tmem173	down	6.3	6.23E-05
Otop2	down	6.3	6.77E-07
Sox4	down	6.3	1.08E-04
Foxq1	down	6.3	2.69E-04
Ier3	down	6.3	2.01E-05
Isl1	down	6.2	1.09E-03
Neto2	down	6.1	5.01E-06
Fam84a	down	6.0	6.29E-05
Adamts12	down	5.9	1.34E-08
Ptpro	down	5.9	4.55E-05
Padi4	down	5.9	1.37E-04
Lgals2	down	5.8	4.80E-03
Clec4d	down	5.8	3.69E-04
Bex1	down	5.8	1.59E-04
Adamts4	down	5.8	2.53E-04
Adam8	down	5.8	8.99E-06

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
F5	down	5.8	1.69E-04
Cp	down	5.7	1.29E-05
Sox4	down	5.7	6.72E-06
Npnt	down	5.7	2.62E-04
Sox4	down	5.6	2.13E-04
Socs3	down	5.6	1.32E-05
Alb	down	5.6	7.57E-04
Arnt2	down	5.6	1.36E-06
5830408B19Rik	down	5.6	4.36E-03
A330049M08Rik	down	5.5	3.68E-06
Rarb	down	5.5	1.85E-04
Mtap	down	5.5	1.21E-04
Samd5	down	5.5	4.57E-03
Phlda1	down	5.5	6.46E-05
Selp	down	5.5	5.80E-04
Basp1	down	5.4	8.12E-05
H19	down	5.4	2.83E-04
Serpine2	down	5.4	1.80E-03
Il23a	down	5.4	6.47E-04
Arntl2	down	5.3	1.95E-06
Mtap	down	5.3	1.28E-04
Prkg2	down	5.2	8.16E-04
Inhba	down	5.2	3.05E-04
Tubb2b	down	5.1	1.06E-06
Cyp11a1	down	5.0	1.51E-05
Sema7a	down	5.0	8.38E-05
Rnase1	down	5.0	2.86E-03
Marcksl1	down	5.0	7.28E-04
Tbx3	down	5.0	1.19E-03
Jub	down	5.0	2.57E-04
Atg9b	down	5.0	7.69E-06
Odz4	down	5.0	3.61E-05
Evl	down	5.0	6.10E-04
8030425K09Rik	down	5.0	1.92E-04
Tbx3	down	5.0	2.24E-03
Cxcl14	down	5.0	3.26E-03
Arl4c	down	5.0	1.12E-05
6720475J19Rik	down	4.9	1.05E-06
Sox4	down	4.9	8.13E-05
Socs3	down	4.9	3.45E-05

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Igfbp5	down	4.9	2.33E-03
Ltbp2	down	4.8	4.80E-04
Tead2	down	4.8	2.86E-04
Rnf43	down	4.7	2.05E-04
Prss22	down	4.6	4.21E-06
Cldn4	down	4.6	8.47E-04
Stra6	down	4.6	2.55E-03
Odz4	down	4.6	4.94E-04
Ifitm1	down	4.6	1.70E-05
Gata5	down	4.5	1.81E-05
Ereg	down	4.5	2.36E-03
Ecscr	down	4.5	1.26E-04
Cd244	down	4.5	2.50E-04
Lox	down	4.4	7.16E-04
Mcpt2	down	4.4	4.33E-03
Pion	down	4.4	1.30E-04
Plaur	down	4.4	1.82E-04
Gp49a /// Lilrb4	down	4.3	1.46E-04
Pmepa1	down	4.3	3.31E-03
Fam89a	down	4.3	5.16E-04
Limch1	down	4.3	3.28E-05
Elk3	down	4.3	1.14E-04
Lyz1	down	4.3	2.47E-06
Isl1	down	4.3	1.30E-03
Lox	down	4.2	8.23E-04
Etv5	down	4.2	8.87E-04
Igfbp4	down	4.2	5.75E-04
Smoc2	down	4.2	6.96E-04
Prr18	down	4.2	2.35E-04
Bmp7	down	4.2	2.33E-04
Vash2	down	4.2	7.00E-05
Adcy1	down	4.2	2.72E-03
Smox	down	4.2	9.53E-05
Igf1	down	4.1	3.15E-03
Nfe2l3	down	4.1	1.31E-03
Abca1	down	4.1	5.33E-06
Wnt5a	down	4.1	9.37E-04
Steap4	down	4.1	1.73E-03
Vangl2	down	4.1	8.01E-04
Igfbp4	down	4.1	1.39E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Igf1	down	4.1	1.33E-03
Dyrk3	down	4.1	7.07E-07
Rhox5	down	4.1	1.20E-03
Abcc4	down	4.1	3.76E-04
Slco5a1	down	4.1	6.21E-04
Lama1	down	4.0	9.35E-06
Axin2	down	4.0	2.04E-03
Pgm2l1	down	4.0	1.94E-03
Fam55c	down	4.0	1.08E-03
Marcksl1	down	4.0	3.74E-04
Stc1	down	3.9	4.32E-05
Wnt5a	down	3.9	4.03E-04
Pitpnc1	down	3.9	5.85E-07
Cited1	down	3.9	2.07E-04
Tmem173	down	3.8	1.50E-04
A130040M12Rik	down	3.8	1.88E-03
D0H4S114	down	3.8	1.88E-03
Smoc2	down	3.8	1.36E-03
Rem2	down	3.8	1.15E-03
Pla1a	down	3.8	9.43E-05
Tcf7	down	3.7	1.27E-04
Hdc	down	3.7	4.17E-06
Ccl2	down	3.7	2.96E-04
Fgfrl1	down	3.7	3.88E-03
Pgm2l1	down	3.7	1.52E-03
Ly6d	down	3.7	2.85E-03
Chrnb1	down	3.7	1.18E-03
Limd2	down	3.6	1.40E-04
Mfsd2	down	3.6	1.26E-04
Fpr2	down	3.6	1.10E-03
A330021E22Rik	down	3.6	2.56E-05
Clec4n	down	3.6	3.80E-05
Stx11	down	3.6	9.49E-06
Pthlh	down	3.5	1.86E-03
Socs3	down	3.5	5.34E-05
Fam167a	down	3.5	6.53E-05
Wisp1	down	3.5	1.29E-03
Pla2g12a	down	3.5	4.39E-05
Odz4	down	3.5	6.49E-04
Plxnb1	down	3.5	1.18E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Bmf	down	3.5	2.45E-03
Etv5	down	3.5	8.60E-04
Adamts4	down	3.5	9.14E-05
Ramp3	down	3.5	5.66E-04
Lyz1	down	3.5	3.45E-06
Sulf1	down	3.5	4.76E-03
Abca1	down	3.4	7.65E-05
Cd44	down	3.4	6.06E-04
6720475J19Rik	down	3.4	9.65E-06
Cldn2	down	3.4	3.52E-04
Thbs2	down	3.4	3.69E-03
Lect2	down	3.4	1.59E-03
Igf1	down	3.4	5.34E-04
Trpa1	down	3.4	1.72E-03
Htra1	down	3.3	4.90E-03
Bmp7	down	3.3	7.72E-04
Tnfsf9	down	3.3	1.89E-04
Rdh10	down	3.3	6.76E-05
Btc	down	3.3	5.13E-04
Clec7a	down	3.3	3.81E-03
Prkd1	down	3.3	4.90E-03
Smox	down	3.3	1.38E-04
Map4k4	down	3.3	5.02E-04
Slc15a3	down	3.3	1.25E-04
Csgalnact1	down	3.2	1.54E-03
Runx1	down	3.2	2.05E-04
Igfbp4	down	3.2	4.34E-04
Abca1	down	3.2	4.64E-05
Tanc2	down	3.2	7.78E-04
Arl4c	down	3.2	3.68E-07
9430081I23Rik	down	3.2	6.49E-04
Cd44	down	3.2	5.16E-05
Sox6	down	3.2	1.39E-03
Mex3d	down	3.2	6.23E-04
Htra1	down	3.2	3.04E-03
Bcl2l11	down	3.2	3.22E-04
Sirpb1	down	3.2	7.61E-04
Stx1a	down	3.1	1.48E-06
Col18a1	down	3.1	5.37E-04
Mycl1	down	3.1	1.45E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Cdkn1c	down	3.1	3.84E-03
2700094K13Rik	down	3.1	6.46E-05
Col18a1	down	3.1	4.27E-04
9530048O09Rik	down	3.1	1.98E-05
Cldn2	down	3.1	1.52E-03
Gpm6b	down	3.1	1.76E-04
Slc7a11	down	3.1	6.44E-05
Igfbp4	down	3.1	5.58E-04
Pmepa1	down	3.1	2.66E-03
Sulf2	down	3.1	5.84E-04
Igf1	down	3.1	1.60E-03
B4galnt4	down	3.1	5.16E-04
Snx10	down	3.1	1.55E-04
Gls2	down	3.1	1.49E-04
Foxq1	down	3.1	9.34E-04
Myo1b	down	3.1	4.27E-06
Mtap	down	3.1	1.04E-03
Macc1	down	3.1	3.23E-03
Myo1b	down	3.0	1.55E-05
Bcl2l11	down	3.0	8.17E-05
Pcdhb16	down	3.0	1.30E-03
C630043F03Rik	down	3.0	4.20E-05
Mmp14	down	3.0	1.64E-03
D0H4S114	down	3.0	3.38E-03
Pcdhb17	down	3.0	1.30E-03
Igfbp4	down	3.0	5.99E-04
Sh3pxd2b	down	3.0	6.46E-04
Il1r2	down	3.0	3.83E-06
Lmo2	down	3.0	3.33E-04
Fscn1	down	3.0	2.52E-03
Myo1b	down	3.0	1.84E-05
Tcf4	down	3.0	7.42E-04
Scara3	down	3.0	2.00E-03
Zfpm1	down	3.0	3.27E-05
Slc18a1	down	3.0	3.94E-03
Gpc1	down	3.0	3.69E-03
Shf	down	3.0	3.58E-04
Palmd	down	3.0	2.12E-03
4732490B19Rik	down	2.9	3.48E-06
Elk3	down	2.9	6.65E-05

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Steap4	down	2.9	7.36E-04
Tmtc4	down	2.9	6.98E-06
Cyr61	down	2.9	2.28E-03
Cd44	down	2.9	1.72E-04
Cdh13	down	2.9	8.55E-04
Phgdh	down	2.9	2.67E-06
Mex3a	down	2.9	1.02E-03
Tgif1	down	2.9	5.76E-05
Sox21	down	2.9	2.48E-03
Etv5	down	2.9	1.59E-03
Nav2	down	2.9	2.82E-03
Runx1	down	2.9	4.10E-04
Tanc2	down	2.9	1.06E-03
Chst11	down	2.9	3.03E-03
Sulf2	down	2.9	2.10E-03
Icam1	down	2.8	1.69E-04
Cyba	down	2.8	5.97E-06
Tmtc4	down	2.8	1.15E-05
Clec4n	down	2.8	1.54E-04
Tesc	down	2.8	3.53E-03
Cyr61	down	2.8	2.02E-03
Vill	down	2.8	1.26E-04
Rbms3	down	2.8	1.33E-03
Bcl2a1a	down	2.8	9.67E-04
Mmp14	down	2.8	1.42E-03
Ifitm3	down	2.8	7.28E-04
Gp1bb	down	2.8	3.52E-03
Elk3	down	2.8	3.36E-04
Casp6	down	2.8	7.51E-05
Tspan12	down	2.8	1.72E-05
Ddit4	down	2.8	2.10E-05
Cadm1	down	2.8	2.54E-03
Tcf4	down	2.8	2.95E-03
Ptgr1	down	2.8	1.53E-04
Znrf3	down	2.8	7.44E-04
Cd244	down	2.8	4.26E-04
Sema3e	down	2.8	2.59E-03
Tbx3	down	2.8	1.20E-03
Tcf4	down	2.8	2.76E-03
Pxdn	down	2.8	3.42E-05

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Tnfaip2	down	2.7	1.25E-03
Csf2rb	down	2.7	1.08E-04
Tmem184c	down	2.7	2.31E-04
Map4k4	down	2.7	9.40E-05
Dock4	down	2.7	2.42E-05
Plek	down	2.7	3.07E-04
Lamp2	down	2.7	2.34E-05
Fam60a	down	2.7	6.73E-06
Rnf183	down	2.7	6.63E-04
Fam125b	down	2.7	4.21E-05
Tcf4	down	2.7	2.79E-03
Tcf4	down	2.7	3.28E-03
Mpzl1	down	2.7	4.68E-05
Ppic	down	2.7	1.62E-03
Map4k4	down	2.7	7.86E-04
Stx1a	down	2.7	5.61E-06
Bcl2l11	down	2.6	4.12E-04
Rgs12	down	2.6	1.35E-03
Cxcr4	down	2.6	1.76E-03
Crem	down	2.6	1.86E-04
Slc41a1	down	2.6	1.01E-04
Steap1	down	2.6	5.81E-04
Rps6ka6	down	2.6	1.86E-03
Rnf32	down	2.6	9.12E-04
Echdc2	down	2.6	4.60E-03
Fam62c	down	2.6	1.86E-03
Zfp647	down	2.6	8.96E-05
Phgdh	down	2.6	6.43E-07
Fmnl2	down	2.6	1.37E-05
Pion	down	2.6	2.59E-04
Srgn	down	2.6	3.54E-05
Afap1l1	down	2.6	2.14E-03
Ascl2	down	2.6	3.25E-03
Rbms3	down	2.6	2.24E-03
Irg1	down	2.6	5.80E-05
Sema3f	down	2.6	1.18E-03
2610028L16Rik	down	2.6	3.12E-06
D17H6S56E-5	down	2.6	2.31E-03
Col5a2	down	2.6	3.97E-03
Angptl4	down	2.6	2.18E-04

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Myo1b	down	2.5	3.56E-04
Slc39a6	down	2.5	3.51E-03
Nrg1	down	2.5	1.57E-04
Mtap	down	2.5	1.13E-03
Chsy1	down	2.5	4.55E-05
Fam65b	down	2.5	1.97E-04
Nbea	down	2.5	3.55E-04
Otop3	down	2.5	1.26E-03
Myo1b	down	2.5	2.62E-05
Myo1b	down	2.5	1.49E-05
Igfbp4	down	2.5	3.73E-03
8430436O14Rik	down	2.5	6.27E-04
4631416L12Rik	down	2.5	7.12E-05
Phgdh	down	2.5	7.18E-06
Hebp2	down	2.5	1.25E-04
Csf2rb	down	2.5	2.90E-04
Btc	down	2.5	2.20E-03
Prkd1	down	2.5	3.27E-03
Spns2	down	2.5	6.34E-04
Hmgcll1	down	2.5	1.19E-05
Cdh13	down	2.5	3.22E-03
Tiam1	down	2.5	1.61E-06
Amot	down	2.5	3.04E-04
Cadm1	down	2.5	2.84E-03
6430514M23Rik	down	2.5	2.04E-07
Plek	down	2.5	2.87E-04
Dock11	down	2.5	1.63E-04
Csf2rb2	down	2.5	4.61E-04
Al467606	down	2.5	1.37E-03
Slc39a10	down	2.4	3.52E-03
Prex2	down	2.4	5.24E-04
9530048O09Rik	down	2.4	4.06E-05
Ptpre	down	2.4	2.28E-04
Nfil3	down	2.4	1.62E-03
Cadm1	down	2.4	4.38E-03
Csnk1e	down	2.4	4.37E-04
Csnk1e	down	2.4	7.44E-04
D17H6S56E-5	down	2.4	2.27E-03
Sema3e	down	2.4	6.37E-04
Gusb	down	2.4	8.41E-05

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
AI120166	down	2.4	7.65E-04
Vangl2	down	2.4	5.13E-04
Smoc1	down	2.4	5.39E-05
Lamp2	down	2.4	1.78E-04
Zfp703	down	2.4	2.64E-03
Sox6	down	2.4	1.27E-03
Kif12	down	2.4	2.56E-03
Mpzl1	down	2.4	2.92E-05
5430404G13Rik	down	2.4	4.99E-03
Myc	down	2.4	1.55E-06
Tgif2	down	2.4	8.33E-04
Pfkfb3	down	2.4	2.93E-04
Cep55	down	2.4	7.73E-06
Tesc	down	2.4	2.18E-03
Map4k4	down	2.4	1.28E-04
Steap2	down	2.4	2.15E-03
Odz3	down	2.4	4.23E-03
Slc44a2	down	2.4	1.38E-03
Tnfaip8l1	down	2.4	7.57E-05
Lamp2	down	2.4	3.69E-05
Ptpre	down	2.4	6.23E-04
Crem	down	2.4	1.66E-04
Gusb	down	2.3	2.85E-05
Slc39a10	down	2.3	3.72E-03
Hunk	down	2.3	1.73E-04
Spon1	down	2.3	2.64E-05
Zfp518b	down	2.3	1.34E-03
2310008H09Rik	down	2.3	1.47E-04
Acot1	down	2.3	2.29E-03
Fgfrl1	down	2.3	2.02E-03
Prei4	down	2.3	1.19E-03
Nme4	down	2.3	8.31E-04
Ttc9	down	2.3	6.30E-04
Cks1b	down	2.3	9.34E-07
Gm98	down	2.3	2.61E-04
2310016C08Rik	down	2.3	3.77E-04
Tnfrsf19	down	2.3	8.40E-06
Sap30	down	2.3	1.84E-04
Grasp	down	2.3	9.85E-04
Col1a1	down	2.3	3.22E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
C630043F03Rik	down	2.3	2.02E-03
Slc16a3	down	2.3	3.46E-03
Slc19a2	down	2.3	1.86E-05
Arid5b	down	2.3	1.77E-03
Pea15a	down	2.3	3.01E-03
Cxcl16	down	2.3	2.08E-04
D16Ertd472e	down	2.3	4.33E-04
Itgav	down	2.3	2.04E-03
2210408K08Rik	down	2.3	1.64E-04
Runx1	down	2.3	4.64E-06
Bcl11a	down	2.3	1.76E-04
Ms4a6d	down	2.3	3.28E-03
Cks1b	down	2.3	1.17E-05
Enc1	down	2.3	3.02E-04
Steap2	down	2.3	1.83E-03
Rtn4	down	2.3	1.97E-05
Bok	down	2.3	1.05E-04
Pla2g7	down	2.3	2.40E-04
Tcf12	down	2.3	1.07E-04
Prmt1	down	2.2	1.45E-03
Myef2	down	2.2	1.26E-07
Chic2	down	2.2	3.38E-04
Csf3r	down	2.2	1.56E-03
Bcl2l11	down	2.2	8.52E-04
5430407P10Rik	down	2.2	1.23E-03
Ascl2	down	2.2	3.69E-03
Nfkbie	down	2.2	2.76E-04
Psat1	down	2.2	4.37E-03
Enc1	down	2.2	3.61E-04
Odz3	down	2.2	2.20E-03
Usp22	down	2.2	3.82E-04
Csrnp2	down	2.2	8.71E-04
BC057079	down	2.2	1.36E-03
Pcdhb21	down	2.2	5.47E-04
Sox9	down	2.2	1.54E-03
Pctk2	down	2.2	7.74E-05
Patz1	down	2.2	9.90E-04
Lyz2	down	2.2	2.74E-03
Casp6	down	2.2	5.05E-04
Lrp8	down	2.2	1.39E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Runx2	down	2.2	1.92E-04
Slain1	down	2.2	4.10E-03
Dctd	down	2.2	1.99E-03
Cd24a	down	2.2	2.37E-03
Armc10	down	2.2	1.46E-05
Sirpb1	down	2.2	2.28E-03
Sema4c	down	2.2	1.61E-03
Nap1l1	down	2.2	1.52E-04
Jun	down	2.2	4.32E-03
Sbk1	down	2.2	1.47E-04
Avpi1	down	2.2	3.91E-03
Cdk4	down	2.2	5.16E-04
Klhl8	down	2.2	1.65E-03
Wdr6	down	2.2	7.30E-04
Nap1l1	down	2.2	2.18E-05
Gja1	down	2.2	6.58E-04
Habp2	down	2.2	3.08E-04
Nlk	down	2.1	4.40E-03
1700017B05Rik	down	2.1	2.97E-04
Slc19a2	down	2.1	1.65E-04
6720460F02Rik	down	2.1	2.27E-04
Sox9	down	2.1	1.65E-03
8430436O14Rik	down	2.1	3.54E-03
Gch1	down	2.1	4.12E-03
Zfp451	down	2.1	2.14E-04
Arhgdig	down	2.1	4.37E-03
Bub1	down	2.1	8.44E-05
Samsn1	down	2.1	1.52E-03
Mafg	down	2.1	8.31E-04
Cd300lf /// LOC10004711	down	2.1	6.36E-05
Chic2	down	2.1	1.03E-03
Lamp2	down	2.1	5.55E-05
Baz1a	down	2.1	1.71E-06
Rtn4	down	2.1	6.32E-06
2210010C17Rik	down	2.1	2.09E-04
Pcgf6	down	2.1	2.37E-05
Usp22	down	2.1	1.95E-04
Gja1	down	2.1	3.91E-04
Depdc1a	down	2.1	3.81E-04
AI467606	down	2.1	1.45E-04

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Vav2	down	2.1	4.48E-04
Mirhg1	down	2.1	1.93E-05
Ifitm2	down	2.1	8.17E-04
B4galt6	down	2.1	1.81E-03
2310008H09Rik	down	2.1	3.16E-05
Gch1	down	2.1	1.75E-03
Pira2	down	2.1	1.73E-03
Angpt4	down	2.1	3.85E-03
Gja1	down	2.1	7.28E-04
1110008L16Rik	down	2.1	2.13E-04
Myef2	down	2.1	2.45E-06
Tubb5	down	2.1	5.76E-04
Lef1	down	2.1	6.62E-04
Srpx2	down	2.1	5.15E-04
Lama5	down	2.1	2.07E-03
Lysmd2	down	2.1	1.37E-03
Rps9	down	2.1	1.04E-05
Cebpb	down	2.1	3.14E-03
B4galt6	down	2.1	7.27E-05
2610201A13Rik	down	2.1	2.95E-03
Bmp1	down	2.1	4.64E-03
Pdgfc	down	2.1	1.80E-03
Slc25a30	down	2.1	7.55E-04
Nap1l1	down	2.1	9.66E-05
1700017B05Rik	down	2.1	3.53E-03
Hnrnpa1	down	2.1	2.98E-04
2010109K09Rik	down	2.1	7.67E-05
Niacr1	down	2.1	1.03E-05
Cdc42ep1	down	2.1	8.33E-04
Zdhhc15	down	2.1	7.34E-04
Psip1	down	2.1	6.97E-04
Fabp5	down	2.1	9.26E-04
Ikbkap	down	2.1	3.32E-04
D16Ertd472e	down	2.1	1.11E-03
Lrp8	down	2.1	4.73E-04
Ttc26	down	2.1	3.88E-04
Sema3e	down	2.0	2.49E-05
Sypl	down	2.0	3.69E-06
Bcl11a	down	2.0	8.43E-04
Fxyd3	down	2.0	1.26E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
D930015E06Rik	down	2.0	4.38E-03
Tle1	down	2.0	6.86E-04
Hoxa9	down	2.0	1.69E-03
B930098A02Rik	down	2.0	2.11E-03
Snhg6	down	2.0	1.44E-04
Tnf	down	2.0	1.42E-04
Steap1	down	2.0	7.50E-04
Slc25a30	down	2.0	9.96E-04
Ttc8	down	2.0	3.57E-03
Spg20	down	2.0	5.62E-04
Kif23	down	2.0	5.01E-04
Slc7a6	down	2.0	7.53E-04
Baz1a	down	2.0	2.67E-06
Ncl	down	2.0	4.66E-06
Fabp5	down	2.0	1.82E-03
Trem1	down	2.0	3.83E-04
Tcf12	down	2.0	1.76E-05
1110001D15Rik	down	2.0	3.71E-03
Mafg	down	2.0	2.85E-03
Ccr1	down	2.0	1.53E-03
Pitpnc1	down	2.0	2.79E-04
Gas2l3	down	2.0	6.02E-04
Itgav	down	2.0	1.29E-04
2310008H09Rik	down	2.0	8.34E-05
D1Ertd564e	down	2.0	3.83E-04
Ets1	down	2.0	6.12E-05
Trit1	down	2.0	7.48E-04
Hdc	down	2.0	1.69E-04
Zfand5	down	2.0	6.36E-04
Hey1	down	2.0	3.93E-03
Bace1	down	2.0	2.67E-03
Aqp8	up	19.7	2.09E-04
Slc26a3	up	16.8	1.38E-05
Slc26a3	up	15.7	3.58E-05
Car4	up	13.7	5.86E-06
Car4	up	12.8	6.49E-06
Slc26a3	up	12.7	2.86E-05
Synm	up	12.4	3.94E-05
Sult1a1	up	11.5	3.82E-04
Sycn	up	11.0	1.16E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Cnn1	up	10.7	1.56E-04
Sycn	up	10.4	9.23E-04
Maob	up	9.2	1.94E-04
Des	up	8.8	1.08E-04
Synpo2	up	8.8	1.06E-04
Myh11	up	8.8	1.47E-05
Synm	up	8.6	1.31E-05
Rims1	up	8.1	6.79E-07
Trpm6	up	7.9	3.71E-04
Eno3	up	7.9	3.26E-03
Pgm5	up	7.5	3.35E-05
Selenbp1	up	7.5	2.09E-04
Clic5	up	7.2	1.61E-04
Slc26a3	up	7.1	2.55E-04
Selenbp1	up	7.1	2.27E-04
Actg2	up	7.0	8.24E-05
Mamdc2	up	6.9	4.61E-06
Abat	up	6.9	3.90E-05
Clic5	up	6.6	2.14E-04
Abca8a	up	6.5	1.93E-05
Vip	up	6.5	7.38E-05
Tpm2	up	6.3	1.61E-05
Clic5	up	6.0	2.29E-04
Usp2	up	5.9	5.57E-04
Pdlim3	up	5.9	1.05E-05
Scg2	up	5.9	7.68E-05
Rims1	up	5.8	3.87E-07
Foxp2	up	5.8	2.84E-05
Itih5	up	5.8	1.95E-04
Itih5	up	5.8	6.90E-05
Tnni1	up	5.6	3.59E-03
Acta2	up	5.6	6.06E-04
Tppp3	up	5.5	4.67E-04
Ndn	up	5.4	1.30E-04
Pdlim3	up	5.4	6.51E-06
Akr1c14	up	5.4	1.24E-05
Acer1	up	5.4	8.87E-05
Aadac	up	5.3	4.04E-03
Fgf13	up	5.3	2.68E-06
Gcg	up	5.2	7.34E-04

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Clic5	up	5.2	2.26E-04
Ndn	up	5.2	9.37E-05
Tpm2	up	5.2	1.28E-05
Itih5	up	5.2	1.50E-04
Esrrg	up	5.1	1.41E-04
Myl9	up	5.1	4.70E-05
Kcnf1	up	5.1	9.70E-06
Hao3	up	5.0	5.64E-04
Tagln	up	5.0	1.09E-04
Foxp2	up	5.0	2.17E-05
Sh3bgr	up	5.0	2.68E-03
Ppp1r12b	up	4.9	4.24E-06
Tppp	up	4.9	3.86E-05
Scin	up	4.9	1.01E-03
Foxp2	up	4.8	3.74E-05
Ndn	up	4.7	1.86E-04
Sgk2	up	4.7	1.34E-03
Synpo2	up	4.7	3.29E-04
Cpe	up	4.6	2.94E-06
Wscd2	up	4.6	2.49E-06
Sspn	up	4.6	2.21E-05
Art3	up	4.6	9.31E-05
Tns1	up	4.6	7.64E-05
Trpm6	up	4.6	8.86E-04
Smtn	up	4.5	4.59E-05
Myom1	up	4.4	7.73E-06
Kcnd3	up	4.4	3.56E-06
Tnxb	up	4.3	9.35E-04
Iyd	up	4.3	6.02E-04
Cyp2d34	up	4.3	7.85E-04
Id4	up	4.2	8.24E-04
Mylk	up	4.2	1.37E-05
Slc26a2	up	4.2	3.08E-06
Tns1	up	4.1	1.54E-04
Hspb6	up	4.1	1.35E-04
Cox7a1	up	4.1	3.64E-04
Cyp2s1	up	4.0	1.86E-04
Acer1	up	4.0	9.44E-05
Slc20a1	up	4.0	4.79E-03
Pck1	up	3.9	1.03E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Tns1	up	3.9	3.06E-04
Hspb7	up	3.9	6.03E-04
Fmo2	up	3.9	1.68E-04
Sparcl1	up	3.8	2.47E-05
Sdpr	up	3.8	6.11E-06
Cyp2d22	up	3.8	1.44E-04
Pcp4l1	up	3.8	9.94E-04
Pck1	up	3.8	9.80E-04
Slc24a3	up	3.8	1.77E-05
Vdr	up	3.7	6.59E-05
2010110P09Rik	up	3.7	2.17E-04
Pcx	up	3.7	5.73E-04
Sdpr	up	3.7	6.74E-05
Slc13a2	up	3.6	5.14E-04
Ndn	up	3.6	1.34E-04
Sdpr	up	3.6	3.02E-05
Fmo1	up	3.6	4.81E-03
Pcx	up	3.6	2.69E-04
Klf4	up	3.6	2.25E-06
Grem2	up	3.6	2.04E-04
Slc20a1	up	3.6	1.81E-03
Adcy5	up	3.6	3.75E-06
Prdx6	up	3.6	1.16E-04
Gal3st2	up	3.6	2.49E-03
Lipg	up	3.6	5.97E-04
Chrdl1	up	3.5	7.50E-07
Fmo2	up	3.5	2.44E-04
Htr4	up	3.5	9.10E-04
Fgfbp1	up	3.5	1.02E-03
Synpo2	up	3.5	2.40E-03
Pcp4	up	3.5	4.84E-03
Lims2	up	3.5	1.32E-05
Slc16a5	up	3.5	3.36E-03
2010003H20Rik	up	3.5	1.62E-03
Rasd2	up	3.5	5.29E-05
Stmn2	up	3.5	4.49E-05
Ogn	up	3.5	6.64E-04
Sprr2a	up	3.5	6.84E-04
Rbpms2	up	3.4	2.06E-05
Dhrs11	up	3.4	7.35E-04

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Vdr	up	3.4	1.35E-04
Mylk	up	3.4	1.65E-05
Tcfcp2l1	up	3.4	7.60E-08
Fmo2	up	3.4	2.85E-04
Fxyd1	up	3.4	7.36E-05
Dbp	up	3.3	3.00E-03
Myo15b	up	3.3	2.16E-03
Hpgd	up	3.3	9.95E-05
Chrdl1	up	3.3	1.35E-06
Sult1c2	up	3.3	3.11E-03
Lipg	up	3.3	8.63E-04
Gstm3	up	3.3	4.16E-03
Enpp2	up	3.3	4.26E-04
Gstm2	up	3.3	1.98E-07
Usp2	up	3.2	2.16E-03
Id4	up	3.2	5.29E-04
Ptgis	up	3.2	2.54E-03
Slc6a4	up	3.2	3.36E-03
Ogn	up	3.2	5.21E-04
Ccl28	up	3.2	9.22E-04
Mylk	up	3.2	1.57E-05
0610005C13Rik	up	3.2	3.00E-04
Ppp1r14a	up	3.1	1.29E-04
B130021B11Rik	up	3.1	8.38E-07
Kcnk5	up	3.1	5.04E-05
Matn2	up	3.1	4.91E-03
Ccl28	up	3.1	1.12E-03
Sorbs1	up	3.1	1.09E-05
Scn7a	up	3.1	2.46E-06
Sorbs1	up	3.1	9.83E-05
Tspan2	up	3.1	9.76E-05
Maoa	up	3.1	3.75E-03
Lmod1	up	3.1	3.34E-05
Lhfpl2	up	3.1	2.45E-04
Fcgbp	up	3.1	1.34E-03
Abcg2	up	3.0	2.49E-03
Hpgd	up	3.0	2.47E-03
Lhfpl2	up	3.0	7.68E-04
Hlf	up	3.0	2.78E-04
Aoc3	up	3.0	1.10E-04

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
2010003K11Rik	up	3.0	2.83E-04
Sult1b1	up	3.0	6.17E-04
Qpct	up	3.0	9.33E-07
5730409N24Rik	up	3.0	1.65E-06
0610040B09Rik	up	3.0	2.71E-03
Scn7a	up	2.9	1.87E-06
Cryab	up	2.9	9.72E-04
Me2	up	2.9	4.63E-03
Hsd17b2	up	2.9	3.30E-03
Pdk2	up	2.9	1.26E-03
Scnn1a	up	2.9	1.29E-05
Ntn1	up	2.9	1.37E-04
Mrvi1	up	2.9	1.71E-03
Lrig3	up	2.9	5.49E-05
4930539E08Rik	up	2.9	7.60E-04
Slc25a34	up	2.9	1.03E-03
Sorbs1	up	2.9	3.17E-05
Ahcyl2	up	2.9	2.79E-03
Syn2	up	2.9	1.02E-03
Chgb	up	2.9	1.09E-03
Zbtb16	up	2.9	4.39E-03
S3-12	up	2.9	5.36E-04
Pcx	up	2.8	2.04E-05
Sntg2	up	2.8	3.25E-05
Phlppl	up	2.8	2.72E-04
Acta2	up	2.8	1.31E-04
Cryab	up	2.8	8.18E-04
Car2	up	2.8	2.61E-04
Foxp2	up	2.8	6.78E-05
Pdzk1ip1	up	2.8	2.71E-03
Sorbs1	up	2.8	1.30E-04
Gm967	up	2.8	2.43E-03
Gucy1a3	up	2.8	7.65E-04
C630028N24Rik	up	2.8	8.30E-04
Uchl1	up	2.8	1.27E-04
Itga8	up	2.8	4.62E-03
Gstm1	up	2.8	1.74E-05
Rorc	up	2.7	1.94E-05
Cyp2d22	up	2.7	1.67E-04
Slco2b1	up	2.7	1.28E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Chga	up	2.7	1.03E-03
Slc16a1	up	2.7	5.39E-04
Nr3c2	up	2.7	3.63E-05
Sema4g	up	2.7	1.23E-04
Slco2a1	up	2.7	3.97E-04
Il17rc	up	2.7	2.89E-04
Slc4a4	up	2.7	1.82E-03
Trnp1	up	2.7	1.77E-04
Pln	up	2.7	3.77E-03
Filip1	up	2.7	7.56E-05
Myh11	up	2.7	5.02E-04
Klf4	up	2.7	3.40E-06
9530009M10Rik	up	2.7	2.03E-05
5033414D02Rik	up	2.7	6.27E-04
Gstm1	up	2.7	2.29E-05
Calb2	up	2.7	6.74E-05
Gstm3	up	2.7	2.72E-03
Lrig3	up	2.7	5.20E-04
Prkg1	up	2.6	2.51E-05
Cav1	up	2.6	4.30E-03
Fam129a	up	2.6	2.29E-03
Al844685	up	2.6	8.16E-04
Fam129a	up	2.6	1.21E-03
Meis1	up	2.6	1.67E-04
Rasgef1b	up	2.6	1.91E-04
Cryl1	up	2.6	1.38E-04
2310044G17Rik	up	2.6	1.08E-04
Gstt1	up	2.6	1.22E-04
Ppp1r3c	up	2.6	4.78E-04
Prph	up	2.6	2.85E-06
Pcdh24	up	2.6	4.36E-03
Cryl1	up	2.6	1.36E-04
Mxd1	up	2.6	4.88E-06
Chpt1	up	2.6	3.57E-03
Lrig3	up	2.6	3.20E-05
Ssbp2	up	2.5	5.14E-04
Fez1	up	2.5	6.15E-04
Kcnk5	up	2.5	2.61E-04
Gm967	up	2.5	4.61E-03
Bche	up	2.5	2.01E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Tgfbr3	up	2.5	6.65E-04
Cspg4	up	2.5	4.78E-03
Ndrg2	up	2.5	4.37E-04
Abcc3	up	2.5	2.47E-03
2610027H17Rik	up	2.5	3.49E-04
Gsn	up	2.5	3.87E-03
Slco2a1	up	2.5	1.70E-04
Gng4	up	2.5	3.23E-03
Pln	up	2.5	2.30E-03
Clcn2	up	2.5	2.16E-04
Tcf3	up	2.5	4.45E-03
Al451617	up	2.5	3.55E-03
Rgs7bp	up	2.5	5.20E-05
Clybl	up	2.5	2.24E-03
Plbd1	up	2.5	4.38E-03
Prdx6	up	2.5	4.96E-05
Atp1a1	up	2.5	4.33E-04
Jph2	up	2.5	2.47E-05
Synpo2	up	2.4	2.74E-03
Tsc22d3	up	2.4	1.22E-04
Myocd	up	2.4	9.41E-05
Tsc22d3	up	2.4	5.90E-05
Tpm2	up	2.4	1.35E-04
Rab11fip4	up	2.4	1.31E-05
Gsn	up	2.4	3.66E-03
Fahd1	up	2.4	2.72E-04
Tspan2	up	2.4	2.83E-05
Per2	up	2.4	1.23E-04
Hdgfrp3	up	2.4	4.77E-05
Crip1	up	2.4	1.55E-04
Tesk2	up	2.4	2.57E-04
Gstm1	up	2.4	8.61E-05
Dtna	up	2.4	2.34E-04
Cbr2	up	2.4	2.38E-04
Rhbdl2	up	2.4	5.21E-04
Sult2b1	up	2.4	1.02E-03
Zfp467	up	2.4	8.01E-05
Lass4	up	2.4	3.05E-03
Acss2	up	2.4	4.05E-04
Meis2	up	2.4	1.35E-04

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Cnnm4	up	2.4	2.58E-04
Cds1	up	2.4	1.35E-03
Cabc1	up	2.4	3.73E-05
Mxd1	up	2.4	1.57E-04
Gsdmc2 /// Gsdmc4	up	2.4	9.16E-05
Syt1	up	2.4	9.90E-05
Myo15b	up	2.4	2.34E-03
Rhbdl2	up	2.4	2.59E-03
Ppargc1b	up	2.4	2.39E-03
Pyroxd2	up	2.4	9.94E-04
Scg3	up	2.3	6.81E-05
Tspan1	up	2.3	1.72E-05
Acpp	up	2.3	3.18E-03
Fam26e	up	2.3	1.17E-04
Hsd11b2	up	2.3	9.35E-04
Hhip	up	2.3	4.16E-04
Zfp385b	up	2.3	2.50E-04
Styk1	up	2.3	9.88E-05
Slc6a14	up	2.3	4.89E-03
2010001M06Rik	up	2.3	2.39E-03
Kazald1	up	2.3	1.34E-06
Plp1	up	2.3	2.11E-05
Bdh1	up	2.3	3.59E-03
Pmp22	up	2.3	8.27E-04
Dgkh	up	2.3	5.22E-04
9130404H23Rik	up	2.3	2.71E-03
Entpd8	up	2.3	1.12E-04
Casq2	up	2.3	3.88E-04
Cideb	up	2.3	2.16E-03
Stmn3	up	2.3	6.23E-05
Plscr4	up	2.3	1.95E-06
Chpt1	up	2.3	3.92E-04
Fbxo32	up	2.3	2.44E-04
Popdc2	up	2.3	2.77E-04
Mustn1	up	2.3	1.41E-03
Stk10	up	2.3	3.54E-04
Flnc	up	2.3	4.53E-03
Ctdspl	up	2.3	2.78E-05
Agpat4	up	2.3	5.68E-05
Cntn1	up	2.2	1.78E-03

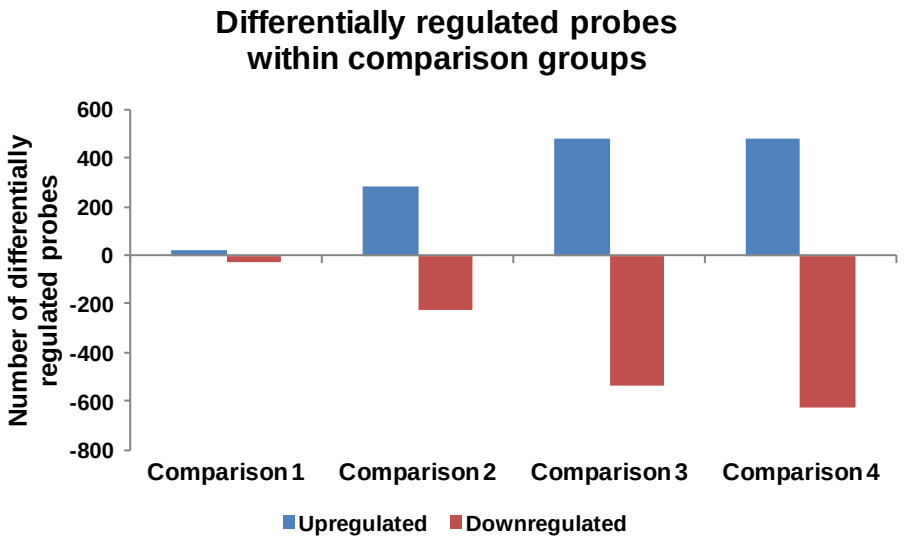
Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Slc2a4	up	2.2	8.37E-04
Stim1	up	2.2	7.83E-04
Zan	up	2.2	5.22E-05
Mupcdh	up	2.2	5.49E-04
Hspb7	up	2.2	3.27E-03
Gfpt1	up	2.2	1.70E-03
Mertk	up	2.2	6.74E-04
Hhip	up	2.2	2.43E-05
Ssbp2	up	2.2	1.34E-04
Tpcn1	up	2.2	5.84E-05
Gstt3	up	2.2	1.91E-04
Asb2	up	2.2	1.71E-04
Muc3	up	2.2	2.45E-06
Per2	up	2.2	8.13E-04
Atp1a1	up	2.2	1.36E-04
Hk1	up	2.2	5.58E-05
Fam161a	up	2.2	7.32E-07
Flnc	up	2.2	3.62E-03
Nsg2	up	2.2	6.04E-04
Chpt1	up	2.2	1.06E-03
Tcf21	up	2.2	1.46E-03
Frat2	up	2.2	1.78E-04
Tgfb1i1	up	2.2	1.89E-03
Synpo	up	2.2	6.79E-06
Rhbdl2	up	2.2	4.84E-03
Klhl23	up	2.2	7.97E-06
Agpat4	up	2.2	6.05E-05
Clrn3	up	2.2	1.33E-03
Cbr1	up	2.2	1.02E-03
Cacna2d2	up	2.2	3.59E-05
Ush1c	up	2.2	7.18E-05
9130009M17Rik	up	2.2	7.15E-07
Ryr3	up	2.2	3.55E-05
5730414M22Rik	up	2.2	1.70E-03
Marveld1	up	2.2	7.19E-04
Gsdmc	up	2.2	4.99E-03
Tlr1	up	2.2	3.67E-03
Ceacam20	up	2.2	2.59E-03
Adam23	up	2.2	2.20E-04
Anks4b	up	2.2	1.86E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Nkd2	up	2.2	1.53E-05
Ckm	up	2.2	1.87E-04
Ank1	up	2.2	2.96E-04
Fbxo32	up	2.2	1.78E-04
S1pr3	up	2.1	2.50E-03
Sdc2	up	2.1	5.41E-05
Kank2	up	2.1	4.57E-04
Ggt6	up	2.1	1.20E-05
Ccdc32	up	2.1	3.20E-04
Plekhb1	up	2.1	1.79E-05
Per3	up	2.1	7.91E-05
Hlf	up	2.1	1.94E-04
Vipr1	up	2.1	9.88E-06
Enpp2	up	2.1	9.41E-05
2310044G17Rik	up	2.1	6.02E-05
Tmem171	up	2.1	1.84E-03
Nos1	up	2.1	5.20E-04
2310044G17Rik	up	2.1	7.31E-05
2900046F13Rik	up	2.1	9.66E-04
Nr1d1	up	2.1	3.56E-04
Cisd1	up	2.1	3.03E-04
Bcas1	up	2.1	2.23E-03
Meg3	up	2.1	4.88E-03
Ankhd1 /// Eif4ebp3	up	2.1	5.23E-04
Kcnmb1	up	2.1	3.27E-04
Fgfr3	up	2.1	4.06E-04
Prkce	up	2.1	5.85E-05
Rgs7bp	up	2.1	3.78E-05
Aldh2	up	2.1	2.11E-04
Slc39a5	up	2.1	3.75E-03
Usp53	up	2.1	6.01E-04
Reln	up	2.1	9.01E-04
Meis2	up	2.1	2.83E-03
D730039F16Rik	up	2.1	1.74E-05
Zfp467	up	2.1	2.33E-05
Satb2	up	2.1	1.41E-04
D730039F16Rik	up	2.1	1.50E-04
2810432L12Rik	up	2.1	1.94E-03
9030425E11Rik	up	2.1	1.35E-04
Zfp467	up	2.1	1.73E-05

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Tspan2	up	2.1	7.95E-05
Cys1	up	2.1	1.03E-04
Cacnb2	up	2.1	1.15E-04
Arl4d	up	2.1	6.27E-07
Akt3	up	2.1	4.54E-03
Hhip	up	2.1	1.71E-03
3110049J23Rik	up	2.1	2.26E-03
Rab27a	up	2.1	2.06E-03
Tgoln1	up	2.1	4.32E-03
Per3	up	2.1	3.22E-05
Slc25a12	up	2.1	1.79E-03
1810014F10Rik	up	2.1	4.53E-03
Ppp1r14d	up	2.1	2.08E-03
Ssbp2	up	2.1	1.19E-03
Mpdz	up	2.1	8.94E-04
Atoh1	up	2.1	9.67E-04
Gnao1	up	2.1	3.07E-05
Klf9	up	2.1	1.36E-04
Ndn	up	2.1	1.04E-05
Ccbl2	up	2.1	2.24E-03
Slc2a13	up	2.1	1.97E-03
Sdr42e1	up	2.1	4.04E-03
Cdx1	up	2.0	1.43E-04
Sema3a	up	2.0	4.84E-04
Akap12	up	2.0	4.61E-05
Myo1a	up	2.0	9.96E-04
Tgoln1	up	2.0	1.61E-03
Garnl4	up	2.0	4.24E-05
Iqgap2	up	2.0	2.36E-03
Il17re	up	2.0	5.11E-05
Ttll7	up	2.0	6.91E-04
Prkcb	up	2.0	2.70E-03
Slc4a4	up	2.0	3.67E-03
Rab3b	up	2.0	3.86E-04
Acad11 /// Nphp3	up	2.0	3.98E-03
Ipmk	up	2.0	4.78E-03
Etv1	up	2.0	2.37E-05
Cd109	up	2.0	3.60E-03
Reck	up	2.0	4.20E-05
Marveld1	up	2.0	1.22E-03

Differentially Expressed Genes $APC^{Min/+}$ $PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ $PPAR\alpha^{-/-}$ tumour (Comparison 4)			
Gene Symbol	**Regulation**	**FCAbsolute**	**p-value**
Sdc2	up	2.0	4.14E-05
Hk1	up	2.0	8.11E-05
Nlrp9b	up	2.0	9.35E-05
Hhip	up	2.0	2.14E-04
Depdc6	up	2.0	1.30E-05
Galm	up	2.0	4.86E-03
Golph3l	up	2.0	2.79E-03
Cryl1	up	2.0	1.39E-04
Usp53	up	2.0	1.19E-04
Cnnm4	up	2.0	1.95E-05
Slc2a4	up	2.0	4.30E-06
Sdc2	up	2.0	2.38E-04
Ddx26b	up	2.0	9.48E-05

2. Number of differentially regulated probe sets in Comparison groups 1 – 4



Comparison 1 ($APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}$ normal)
Comparison 2 ($APC^{Min/+}PPAR\alpha^{-/-}$ tumour *vs.* $APC^{Min/+}$ tumour)
Comparison 3 ($APC^{Min/+}$ normal *vs.* $APC^{Min/+}$ tumour)
Comparison 4 ($APC^{Min/+}PPAR\alpha^{-/-}$ normal *vs.* $APC^{Min/+}PPAR\alpha^{-/-}$ tumour)

Criteria for selection of differentially regulated probes in Comparison 1 & Comparison 2 was fold change >= 1.7, p <= 0.05
Criteria for selection of differentially regulated probes in Comparison 3 & Comparison 4 was fold change >= 2, p < = 0.005