The role of peroxisome proliferator-activated receptor β/δ in colorectal cancer

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**NANYANG
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The Role of Peroxisome Proliferator– Activated Receptor β/δ in Colorectal Cancer

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Quote:

"None of us got to where we are alone. Whether the assistance we received was obvious or subtle, acknowledging someone's help is a big part of understanding the importance of saying thank you." - Harvey Mackay.

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List of abbreviations

AOM	Azoxymethane
APC	Adenomatous polyposis coli
APC ^{min/+}	Mouse carrying a heterozygous Min (multiple intestinal neoplasia) mutation
CAC	Colitis associated cancer
CAM	Cell-cell adhesion molecules
CIN	Chromosomal instability
CRC	Colorectal Cancer
DAI	Disease activity index score
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulfate
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
FAP	Familial adenomatous polyposis
FSP-1	Fibroblast-specific protein-1
FSPCre-Pparb/d ^{-/-}	Transgenic mice harboring a stromal-specific PPAR β/δ ablation
HIF-1a	Hypoxia-induced factor 1a
HNPCC	Hereditary nonpolyposis colorectal cancer
iCEC	Immortalized colonic epithelial cell
LCM	Laser capture microdissection
LOH	Loss of heterozygosity
MHC	major histocompatibility complex
MSH2	MutS protein homolog 2
MSI	Microsatellite instability
NAC	N-acetyl cysteine
NRF2	Nuclear factor (erythroid-derived 2)-like 2
NSAID	Nonsteroidal anti-inflammatory drug
PCNA	Proliferating cell nuclear antigen
PPAR	Peroxisome proliferator activated receptor
Pparb/d ^{fl/fl}	Transgenic mice carrying a floxed PPAR β/δ exon 4
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RXR	Retinoid X receptor
TGFB	Transforming growth factor beta
TSP-1	Thrombospondin-1
VEGF-A	Vascular endothelial growth factor A

List of publications in peer-reviewed journals

1. ROS release by PPAR β / δ -null fibroblasts reduces tumor load through epithelial antioxidant response. *Oncogene*, ePub ahead of print, (2018). doi:10.1038/s41388-017-0109-8
2. Selective deletion of PPAR β / δ in fibroblasts causes dermal fibrosis by attenuated LRG1 expression. *Cell Discovery* **4**, 15, (2018). doi:10.1038/s41421-018-0014-5
3. Angiopoietin-like 4 Mediates Colonic Inflammation by Regulating Chemokine Transcript Stability via Tristetraprolin. *Scientific Reports* **7**, 44351 (2017). doi: 10.1038/srep44351.
4. Angiopoietin-like 4 Increases Pulmonary Tissue Leakiness and Damage during Influenza Pneumonia. *Cell Reports* **10**, 654–663 (2015). doi: 10.1016/j.celrep.2015.01.011

Thesis Summary

Colorectal cancer (CRC) or bowel cancer refers to the development of neoplastic growths in the rectum and colon. CRC is one of the most prevalent and lethal cancers in the world, highlighting the importance of preventive and cancer therapy research. Peroxisome proliferator-activated receptor β/δ (PPAR β/δ) belongs to the PPAR family of nuclear receptor proteins comprising of PPAR α , PPAR β/δ and PPAR γ , that act as transcription factors regulating gene expression. Due to the functions of their target genes in lipid metabolism and energy homeostasis, PPARs have been identified to play significant roles in cancer and inflammation. Of particular interest is PPAR β/δ , because unlike PPAR α and PPAR γ that exhibit tissue-specific expression, PPAR β/δ is expressed in a ubiquitous manner, opening up the study of PPAR β/δ to various tissues and diseases. The role of PPAR β/δ in CRC has been a subject of controversy with studies highlighting both pro-tumorigenic and anti-tumorigenic functions, leading to suggestions that a tissue specific approach might be required to properly dissect the role of PPAR β/δ in CRC. Studies have so far shown a pro-tumorigenic role of PPAR β/δ in the mucosal epithelium, with little known about the other cells in the tissue microenvironment. Meanwhile, available evidence in skin squamous cell carcinoma indicates that fibroblast PPAR β/δ is responsible for regulating the invasiveness, xenobiotic resistance and cellular bioenergetics of adjacent cancer cells. However, the role of fibroblast PPAR β/δ in CRC is still unclear.

To study the role of fibroblast PPAR β/δ in CRC, we used a transgenic mouse carrying a stromal-specific PPAR β/δ -deletion (FSPCre-*Pparb/d*^{-/-}), compared with *Pparb/d*^{fl/fl} littermates. Inflammatory bowel disease (IBD) is an important risk factor of colitis associated cancer of the colon. Challenging the mice to DSS-induced colitis, we showed that PPAR β/δ in fibroblasts is important for the regulation of inflammatory outcomes in the colon. The loss of fibroblast PPAR β/δ in mice resulted in resistance to DSS-induced intestinal mucosal damage and reduced disease severity. We also showed that the tissue microenvironment was altered by the deletion of fibroblast PPAR β/δ , noting presence of an anti-inflammatory cytokine milieu and an increased number of

macrophages belonging to the anti-inflammatory M2-subtype. Following this with a CRC study across 3 murine models of intestinal tumorigenesis, we found that fibroblast PPAR β/δ was also important in the tumorigenesis process. The loss of PPAR β/δ resulted in impairment of colorectal tumorigenesis in FSPC-Cre-*Pparb/d^{-/-}* mice. Using comparative gene expression analysis, we identified NRF2 signalling and oxidative processes as key differentially regulated pathways in FSPC-Cre-*Pparb/d^{-/-}* mice. Validation assays then confirmed the reduction of oxidative stress in FSPC-Cre-*Pparb/d^{-/-}* tumors compared to *Pparb/d^{fl/fl}* ones. In an *in vitro* setting, colon epithelial cell lines also experienced lower oxidative stress when co-cultured with fibroblasts with depleted PPAR β/δ expression.

In conclusion, our findings indicate that PPAR β/δ signaling from stromal sources can play a significant role in the regulation of inflammation and development of cancer in the intestine. Synthetic PPAR ligands are currently being developed as promising candidates for cancer chemotherapy and chemoprevention. Therefore, our results suggest that future studies should consider and investigate drug effects on stromal elements when designing anti-cancer therapies.

Chapter 1: Introduction

Chapter 1: Introduction

1.1 General overview

In 2012, there were 14.1 million new cancer diagnoses, 8.2 million cancer deaths and 32.6 million people living with cancer (Ferlay et al., 2013). With the rapid population growth and development worldwide, the World Health Organization (WHO) predicted that the global cancer burden in 2025 would exceed 20 million new cases (International Agency for Research on Cancer, 2014). In developed countries, the associated shift in lifestyles increases the risk of certain cancers at the population level, including female breast cancer, male prostate cancer and colorectal cancer in both sexes (International Agency for Research on Cancer, 2014). However, organ-specific cancer risk is complicated and lifestyle and hereditary factors do not fully account for increased organ-specific cancer risk (Tomasetti and Vogelstein, 2015). It has been proposed that cancer risk can be most attributed to random mutations that arise during DNA replication of stem cells and consequently, the life-time risk of developing cancer is affected by the total number of stem cell divisions in the organ. Comparing stochastic factors against lifetime cancer risk, Tomasetti and Vogelstein demonstrated a strong correlation between stem cell replication and cancer risk, with colorectal cancer types ranking highly in both lifetime risk and stemness, together with other proliferative cancers such as lung carcinoma, HPV and HCV-induced cancers (Tomasetti and Vogelstein, 2015). Thus, advancing colorectal cancer research appears to be vital considering emerging human developments.

1.2 Hallmarks of cancer

Hanahan and Weinberg published a landmark review of cancer biology in 2000, distilling the complexity of cancer to 6 basic underlying principles, namely self-sufficiency in growth signals, insensitivity to anti growth signals, evading programmed cell death, limitless replicative potential, sustained angiogenesis and tissue evasion and metastasis (Hanahan and Weinberg, 2000). The first hallmark is self-sufficiency in growth signals. Normal cells require exogenous mitogenic growth signals to transit from a quiescent cell state to an

active proliferative one, while cancer cells are able to grow without growth signals, by either producing their own signals or upregulating receptors and their pathways that are responsive to these signals. Thus, cancer cells are able to reduce their dependence on mitogenic stimulation from their tissue microenvironment. For example, more than half of studied human colon carcinomas have been shown to suffer from deregulated Ras signaling (Kinzler and Vogelstein, 1996).

The second hallmark is insensitivity to antigrowth signals. In normal cells, there are processes in place to tightly regulate cell division to prevent uncontrolled cellular growth and division, through anti-growth signals that typically interact through membrane-bound receptors on the cell. In cancer cells, these signals are rendered inactive through the downregulation or modifications of proteins that transmit these signals and thus, cancer cells are able to continue undergoing uncontrolled proliferation. In human colorectal carcinogenesis, activation of the APC/beta-catenin pathway allows intestinal enterocytes to avoid terminal differentiation (Kinzler and Vogelstein, 1996). The third hallmark is evasion of programmed cell death. Normal cells possess the ability to undergo programmed cell death or apoptosis. Apoptosis is essential in a wide variety of processes, from organismal development and tissue renewal to cellular responses towards insults and abnormalities. To develop tumors, incipient cancer cells must bypass the apoptotic machinery to avoid programmed cell death despite their abnormalities, by deregulating mechanisms that detect abnormalities or mediate apoptotic signals. One of the most common is the loss of function of the TP53 tumor suppressor gene, which acts as the guardian of the genome by sensing cellular abnormalities and in response, inducing an apoptotic circuitry. TP53 genetic mutations have been commonly reported in CRC, and thus genetic modulation of the TP53 pathway in CRC has been targeted as a potential therapy for CRC (Liu et al., 2015).

The fourth hallmark is limitless replicative potential. Normal cells are restricted to a limited number of divisions before they reach senescence or die (Hayflick, 1997). The limitation of replicative potential has been at least partially attributed to progressive telomeric shortening during DNA replication. The progressive loss of telomeric length erodes their ability to protect the ends of chromosomal DNA, leading to the hindering of end-to-end chromosomal joining

during cellular division. Malignant cells are able to maintain telomeric ends by upregulating enzymatic activity to increase telomere length (Shay and Bacchetti, 1997). However, shortened telomeres are prevalent in colorectal tumors, compared with the adjacent mucosa tissue, with suggestions that telomeric erosion is an essential early step in colorectal carcinogenesis (Bertorelle et al., 2014). The cause of this telomeric shortening has been associated with uncontrolled proliferation of tumor cells and accelerated by modifications in genes such as MSH2 and APC, which contribute to chromosomal instability (Mendez-Bermudez and Royle, 2011). While a shortened telomeric length is recognized as an early event in CRC, the correlation of telomeric length and carcinogenic progression has not been established, with study results divided on the presence of such a link (Gertler et al., 2004; Garcia-Aranda et al., 2006; Terrin et al., 2008; Rampazzo et al., 2010).

The fifth hallmark is sustained angiogenesis. A continuous supply of nutrients and oxygen is critical for cellular processes and survival. Tumor growth requires an expansive amount of resources while larger tumors must develop a sufficient vasculature to support its continued growth. Thus, the delivery of oxygen and nutrients becomes ever more important. Tumors have been shown to achieve this phenomenon by upregulating angiogenesis-promoting signals while simultaneously deregulating angiogenesis-inhibiting ones (Hanahan and Folkman, 1996). A commonly implicated angiogenesis inducer and inhibitor in cancer is vascular endothelial growth factor A (VEGF-A) and thrombospondin-1 (TSP-1) respectively. VEGF-A protein specifically acts on vascular endothelial cells to induce various effects on the vasculature, including angiogenesis, increasing vascular permeability and promoting endothelial cell growth. VEGF is a key mediator of angiogenesis in tumors, and is upregulated by oncogene expression, various growth factors and hypoxia (Carmeliet, 2005). Cancer-associated fibroblasts are the primary source of VEGF in tumors and further support tumor angiogenesis by secreting PDGF to recruit bone marrow derived cells to form endothelial and smooth muscle cells of the vessels (Tao et al., 2017). In contrast, the role of TSP-1 in tumor progression has been controversial, as both overexpression and anti-sense inhibition of TSP-1 has been shown to inhibit tumor growth, suggesting that other factors such as the level of angiogenic

stimulators and localization of TSP-1 in the tissue may be important considerations when studying TSP-1 expression (Sargiannidou et al., 2001).

The sixth and final hallmark of cancer is tissue invasion and metastasis, which refers to the ability of malignant cancer cells to dislodge from their site of origin and spread to a distant site. Invasion and metastasis represent two separate and complex processes that require multiple steps. First, malignant cells alter their expression of cell-cell adhesion molecules (CAMs) and integrins, which facilitates cellular detachment from their local site. For example, E-cadherin is an important CAM that allows adjacent epithelial cells to form adherens junctions to organize epithelial cell sheets and maintain cell quiescence. Due to its important role, downregulation and inactivation of E-cadherin is frequently observed in human carcinomas (Berx and van Roy, 2009; Cavallaro and Christofori, 2004). Furthermore, malignant cells can modify their extracellular matrix, through matrix-degrading enzymes produced by inflammatory and stromal cells recruited by the tumor (Joyce and Pollard, 2009; Kessenbrock et al., 2010; Qian and Pollard, 2010). In colon cancer, approximately 30% of patients are diagnosed with CRC with metastatic disease, highlighting its importance in the approach to CRC treatment (Riihimäki et al., 2016). The TGF- β / Smad signaling pathway has been shown to play a significant role in CRC metastasis by promoting tumor progression through the various steps of epithelial-mesenchymal transition (EMT) (Matsuzaki and Okazaki, 2006; Voorneveld et al., 2014). Likewise, the Wnt signaling pathway and its upstream components have been manipulated by tumor cells to facilitate dynamic EMT and mesenchymal-epithelial transition (MET) processes during CRC progression (Vincan and Barker, 2008).

In 2011, four additional principles were added to the list, with two emerging hallmarks namely deregulated metabolism and avoiding immune destruction; and two enabling characteristics namely, genome instability and inflammation (Hanahan and Weinberg, 2011). The first of the two new hallmarks is deregulated metabolism. Under aerobic conditions, normal cells derive their energy from the glycolytic processing of glucose, followed by oxidative phosphorylation in mitochondria. It has long been established that cancer cells exhibit altered energy metabolism, preferring to utilize glycolysis and lactic acid

fermentation to produce energy, a phenomenon also known as the “Warburg Effect” (Warburg, 1956a; 1956b). However at first glance, energy metabolism solely through glycolytic processes is rather counterintuitive for the energy-demanding cancer cell, as mitochondrial oxidative phosphorylation produces energy with much higher efficiency. Thus, the benefits of the Warburg Effect have been discussed, given special attention to rapid energy availability, the biosynthetic needs of the cell, acidification of the tumor microenvironment and modulation of redox signaling pathways (Liberti and Locasale, 2016). Hypoxia has been demonstrated to be a key player, via transcription factor hypoxia-induced factor (HIF-1a), in the energy metabolic shift during tumorigenesis. Apart from HIF-1a, it has been demonstrated that wnt signaling drives the glycolytic metabolic programming via pyruvate dehydrogenase 1 (PDK1) in colon cancer (Pate et al., 2014).

Evasion of immune destruction is the second new hallmark discussed. Immunosurveillance in a healthy host has been shown to be responsible for the detection and elimination of budding dysplastic and neoplastic cells, while defective immunosurveillance in immunocompromised individuals have been linked to the development of certain cancers (Vajdic and van Leeuwen, 2009). This phenomenon is also supported by the finding that patients with tumors bearing greater number of infiltrated cytotoxic lymphocytes have a better prognosis (Pagès et al., 2010; Fridman et al., 2011). Reduced immunosurveillance through the downregulation of MHC class I expression has been shown in colon cancer and is correlated with a poor prognosis (Watson et al., 2006; Parcesepe et al., 2016).

At the top of the 8 hallmarks of cancer, Hanahan and Weinberg also proposed that the acquisition of the multiple hallmarks of cancer occurs via two enabling characteristics namely, genome instability and tumor-promoting inflammation (Hanahan and Weinberg, 2009; 2011). The first enabling characteristic is genomic instability. The carcinogenesis process requires an accumulation of multiple gain-of-function or loss-of-functions mutations of genes that are integral to the signaling pathways that enable cancer development (Negrini et al., 2010). While the cell possesses multiple checkpoints to maintain the integrity of the genome, cancer cells often find ways to circumvent these

checkpoints to increase genetic mutation rates (Salk et al., 2010). Cancer cells may accomplish this process by inducing defects in the array of components making up the cellular DNA maintenance machinery, also known as “caretakers” of the genome (Kinzler and Vogelstein, 1997), leading to the accumulation of DNA mutations and thereby increasing the incidence of cancer (Barnes and Lindahl, 2004). Alternatively, telomeric erosion associated with old and aging cells can result in karyotypic instability that may lead to errors in chromosomal or copy number alterations that enable tumorigenesis (Artandi and DePinho, 2010). As discussed earlier, genomic instability in CRC has been classified into chromosomal instability (CIN) and microsatellite instability (MIN). The second enabling characteristic is tumor-promoting inflammation. Tumors are often infiltrated by immune cells of both the innate and adaptive immune systems, mirroring the inflammatory conditions observed during tissue injury (Egli et al., 1978). While inflammatory cells may elicit an anti-tumoral cytotoxic response, there is evidence that the tumor-associated inflammatory response may instead have the paradoxical effect of enhancing tumor development and progression. Inflammatory cells supply a large amount of secretory factors to the microenvironment including growth factors, proteases that modify the ECM, pro-survival factors, and angiogenic factors that facilitate the incipient tumor to acquire one or more hallmark capabilities (Karnoub and Weinberg, 2006; DeNardo et al., 2010; Grivennikov et al., 2010; Qian and Pollard, 2010). In the colon, the perpetual state of inflammation during inflammatory bowel diseases results in the predisposition of IBD patients to the development of colitis associated cancer (Francescone et al., 2015). Apart from the abovementioned factors, Hanahan and Weinberg (2011) have highlighted several factors that may gain prominence with further research, including epigenetic events that may promote tumorigenesis (Berdasco and Esteller, 2010), implications of miRNA expression (Garzon et al., 2010) and heterotypic interactions between cells in the tumor microenvironment.

1.3 Role of the tumor microenvironment in cancer progression and severity

An obstacle to gene-targeted anti-tumor therapies is the genetic instability of malignant cells. The tumor microenvironment has been gaining recognition as

a factor influencing tumor growth (Li et al., 2007; Chen et al., 2015) and thus represents an attractive target for anti-tumor therapies (Cairns et al., 2006; Sounni and Noel., 2013). Tumor progression is a multicellular process that implicates multi-directional communication between tumor cells and their surroundings. The tumor microenvironment refers to the cellular environment in the immediate surroundings of the tumor, containing immune, fibroblastic and endothelial cells with an extracellular matrix saturated with various growth-enhancing or suppressing signaling molecules (Witz and Levy-Nissenbaum, 2006). Studies have investigated the tumor microenvironment of breast cancer (Allinen et al., 2004) and prostate cancer (Chung et al., 2005). Studies have also showed that neutrophil-derived factors in the tumor-associated stroma are involved in the immune response in the early stage lung cancer (Eruslanov et al., 2014). Moreover in CRC, the tumor microenvironment has been thought to regulate the Wnt signaling pathway in cancer stem cells (Vermeulen et al., 2010). However, much remained to be learned about the role of microenvironmental influences in CRC progression and severity.

A chronic inflammatory microenvironment is associated with a higher cancer incidence (Grivennikov et al., 2010), providing evidence for a microenvironmental influence on tumor progression. In the liver, the link between inflammation, cirrhosis and hepatic cancer has been established, in which the inflammatory condition leads to an increased cancer incidence (Melato et al., 2006). Similarly, inflammation in the intestinal tract is thought to play a tumor-promoting role. Inflammatory bowel disease and ulcerative colitis are listed amongst some of the top risk factors for colorectal cancer (Itzkowitz and Yio, 2004). An inflammatory response leads to the production of various cytokines, growth and angiogenic signals that promote tumor growth and progression (Grivennikov et al., 2010). Conversely, immune responses can also lead to the recruitment of cells and the secretion of factors that suppress the growth of certain tumors. The immune system mounts anti-tumor responses through the production of cytotoxic factors and promotion of direct tumor cytotoxicity. The dual role of the immune system in tumorigenesis has been documented. The incidence of breast cancer was decreased, while lung, gastrointestinal, reproductive and skin cancer incidences were reported at a higher than

that predicted (Stewart et al., 1995), in a study of 25,914 female organ transplant patients who were placed on immunosuppressant treatment. Similarly, patients suffering from acquired immunodeficiency (AIDs) have been linked to higher risks of both AIDs-related cancers (eg. Kaposi's sarcoma) and non-AIDs related cancers (eg. tongue, skin, lung) (Gallagher et al., 2001). The double-edged nature of the immune system against tumorigenesis warrants a deeper investigation of the inflammatory signals at play in the tumor microenvironment.

Angiogenesis is critical for tumor growth and metastasis, constituting a rate-limiting factor for tumor development and has been suggested as a target for anti-tumor therapy (Folkman, 1971). Furthermore, the vascular endothelium has been described as a dynamic organ with important roles in regulating nutrient passage and the trafficking of immune cells in tissues (Aird, 2006). In the normal vasculature, a monolayer of endothelial cells forms tight junctions between one another, while tumor blood vessels have been shown to be abnormal morphologically with a defective endothelial layer (Warren et al., 1978). This loss of endothelial barrier function has been shown to facilitate tumor growth and progression to metastasis (Yonenaga et al., 2005; Xian et al., 2006). This vascular leakiness has also been hypothesized to disrupt the access of anti-cancer drugs to the tumor site (Jain, 2005). Leukocyte infiltration may also impact by the loss of adhesion molecule ICAM-1 expression in the tumor vasculature, thereby allowing the tumor cells to escape immune surveillance (Griffioen et al., 1996). Conversely, tumor endothelial cells may also overexpress adhesion molecules for pro-inflammatory cells which secrete angiogenic factors, thereby resulting in a positive feedback loop (Butler et al., 2010). Thus, there is an emerging appreciation of tumor endothelial cells in the regulation of the environment, with the need for further studies to ascertain its utility as a target for anti-cancer therapy (Dudley, 2012).

Fibroblastic cells are the major cell-type in the tumor microenvironment. Fibroblasts reside in connective tissues and perform multiple functions that regulates the tissue microenvironment, including the deposition of extracellular matrix (ECM), regulation of epithelial cell differentiation, modulation of immune events in the tissue and mediation of tissue homeostasis (Tomasek et al., 2002). In the tumor, cancer-associated fibroblasts (CAF) are typically found in highly

fibrotic regions, where their myriad of functions have been exploited by malignant cells to advance tumor development, and thus CAFs have been a subject of intense therapeutic research (Kalluri and Zeisberg, 2006).

CAFs have been shown to elicit pro-tumorigenic functions. Studies examining prostate and breast cancer have concluded that the presence of CAFs promotes neoplastic development in epithelial cells, a finding that has not been observed with normal fibroblasts (Olumi et al., 1999; Dumont et al., 2013). In colorectal tumors, CAFs have been shown to influence cancer stem cell clonogenicity through the secretion of HGF, leading to the activation of Wnt signaling and β -catenin-dependent transcription (Vermeulen et al., 2010). Conversely, colon cancer cells promotes TGF- β /Smad signaling in CAFs, resulting in the upregulation of MMPs, PAI-1 and TGF β -1, which perpetuates a protumorigenic feedback loop (Hawinkels et al., 2014). Apart from epithelial-mesenchymal communication, fibroblasts have also been shown to modulate immune cells through the activation of proinflammatory pathways in breast, pancreatic and lung carcinomas (Erez et al., 2010; Feig et al., 2013; Martey et al., 2004; Sharon et al., 2015). In colorectal cancer, CAFs secrete cytokines and chemokines, including IL-17A in response to chemotherapy, contributing to an immunosuppressive tumor microenvironment that leads to tumor chemoresistance (Lotti et al., 2013). With the multiplicity of roles in promoting tumor development, there has been growing research targeting CAFs for anti-cancer therapy in breast, lung and melanoma tumors (Slany et al., 2015, Ohshio et al., 2015; Togo et al., 2013).

In contrast, fibroblasts have also been shown to play a tumor-inhibiting role. Fibroblasts have been shown to inhibit tumor growth via the modulation of TGFB and HGF secretion from CAFs (Kuperwasser et al., 2004)). CAFs in pancreatic tumors have also been reported to play an anti-tumorigenic role (Özdemir et al., 2014; Rhim et al., 2014). As mentioned earlier, CAFs secrete many proinflammatory cytokines such as TGF β , TNF α , IFN γ and IL-6, leading to the recruitment and polarization of immune cells that may be involved in tumor cytotoxicity (Kalluri, 2016). Due to the duality of CAF function in different cancers, there is a need to exercise caution in targeting CAFs for anti-tumor

therapy and hence more effort should be focused on understanding the underlying mechanisms defining the role of CAFs in tumorigenesis.

Recent findings have also suggested the apparent heterogeneity of CAFs. First, a study examining oral squamous cell carcinoma, identified two distinct CAF subtypes displaying differential tumor-promoting capabilities (Costea et al., 2013). The heterogeneity has led to gene expression signature studies that offer useful prognostic information on breast cancer (Wang et al., 2013, Finak et al., 2008). Similarly, gene profiling of human colorectal cancer samples has also revealed the heterogeneity of CAFs in tumors of varying progression, suggesting that there is prognostic value for the characterization of CAFs (Herrera et al., 2013). While research has demonstrated strong evidence for the tumor-modulating functions of CAFs, the identity and source of CAFs is still debatable. As opposed to the canonical view of CAFs as an activated state of residential mesenchymal cells, some studies have proposed alternative theories, with endothelial-to-mesenchymal transition (EndMT) and epithelial-to-mesenchymal transition (EMT) touted as processes from which CAFs are derived (Zeisberg et al., 2007; Petersen et al., 2003). The multitude of processes that drive CAF formation greatly enhances the heterogeneity of different cancers.

1.3.1 FSP-1 / S100A4 as a stromal marker

Fibroblast-specific protein-1 (FSP-1), also known as S100 Calcium Binding Protein A4 (S100A4) or Malignant Transformation Suppression 1 (MTS1), is part of the S100 family of proteins characterized by an EF-hand calcium-binding motif. FSP-1 has been proposed to be a fibroblast-specific marker that was first found in cells of the mesenchymal lineage post-gastrulation (Strutz et al., 1995). A role for FSP-1 was also later described in epithelial-mesenchymal transition (EMT) and metastasis (Boye and Mælandsmo, 2010; Helfman et al., 2005). FSP-1 protein has been found to be preferentially expressed in various cell populations in the human breast tumor stroma, including fibroblasts, macrophages and activated lymphocytes, but it was not found in the tumor epithelial cell population (Cabezón et al., 2007). Thus, FSP-1 promoter driven targeted gene ablation has gained popularity in studies searching to explore tumor stroma mediated effects in carcinogenesis. In their study, Trimboli et al. adopted Fsp1 Cre-driven loxP gene tool to model tumor stroma specific

gene deletion in murine breast tumors (Trimboli et al., 2008; 2009). Cre belongs to the integrase family of site-specific recombinases and possess characteristics that enable its applications in molecular biology to manipulate the genetic material in various animal models (Buchholz et al., 1996). These characteristics include ideal enzyme efficiency at 37°C, the ability to function without additional cofactor or accessory proteins, a fixed recognition target sequence and conserved recombination activity (Buchholz et al., 1996). The Cre recombinase recognition site is the 34 bp loxP sequence. The loxP site contains two 13bp palindromic sequences separated by an 8bp non-palindromic spacer sequence. To manipulate a gene of interest, loxP sites are usually designed to flank a portion of the target gene, and hence loxP flanked genes are also referred to as "floxed" genes (fl). The directionality of the loxP sequence also determines the outcome of recombination (Nagy, 2000). The arrangement of loxP sites in the same direction results in excision of the floxed sequence, while the arrangement of the loxP sequence in opposing directions results in inversion of the floxed sequence.

Another group led by Bhowmick et al. studied the effects of FSP-1 specific ablation of the TGF- β type II receptor gene in mice, and concluded that TGF- β signaling in fibroblasts was instrumental in the modulation of the oncogenic potential of its adjacent epithelium (Bhowmick et al., 2004). Subsequently, studies have also used the FSP-1 promoter driven approach to model stromal TGF- β signaling in prostate and mammary carcinomas (Cheng et al., 2005; Li et al., 2008). Thereafter, studies have examined the effects of stromal-specific deletion of transcription factors, such as nuclear receptor ER- α and transcription factor HIF-1 α , on tumorigenesis (Chen et al., 2012; Kim et al., 2012). More recent studies have also explored the role of the transcription factor Ests2 and receptor protein Smoothed in breast and pancreatic tumor stroma respectively (Wallace et al., 2013; Liu et al., 2016). Thus, the Fsp1 Cre-driven loxP gene tool has been proven to be reliable in the study of stromal-specific deletion of a variety of genes in multiple murine cancer models. Notably, no studies have reported stromal-targeted genetic deletion and its influence in the colorectal tumor stroma. Here we propose the FSP-1 promoter-driven approach to study targeted gene deletion in murine stroma cells and study its effects on intestinal development and in the event of intestinal malignancies.

1.4 Colorectal cancer

1.4.1 Colorectal cancer overview

Colorectal cancer (CRC), also known as bowel cancer, is the development of neoplastic growth in the colon and rectum. CRC ranks as among the top three most prevalent cancers and is the fourth most lethal cancer worldwide (International Agency for Research on Cancer, 2014). CRC has been described as a lifestyle disease (Boyle and Langman, 2000), with risk factors such as diet & nutrition (Willett et al., 1990) and physical activity & obesity (Giovannucci et al., 1996). The incidence rates of CRC also differ between regions, with elevated incidences observed in emerging economies such as Japan, Singapore and countries in Eastern Europe (Hagggar and Boushey, 2009).

Various factors that are associated with cancer development play roles in tumor initiation or promotion. The tumor development model consists of 3 stages, namely initiation, promotion and progression (Hennings et al., 1993). At the initiation step of tumor development, cells acquire enabling alterations that lead to tumor development. These enabling changes often occur in the form of genetic mutations, which occurs spontaneously or is induced by chemical and physical mutagens. These enabling changes are also passed from the mutated cells to their daughter cells. During the tumor promotion stage, factors promote uncontrolled proliferation of the initiated cell and its daughter cells, leading to tumor formation. Some tumors then progress on to a metastatic state, where the primary tumor gains cellular changes that permit it to spread to secondary sites.

Almost 10% of all CRC cases are associated with a genetic predisposition in either of the two hereditary syndromes: familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) (Cancer Research UK, 2014). FAP has been ascribed to a hyperactivated Wnt signaling pathway, predominantly via the inactivation of the tumor suppressor gene adenomatous polyposis coli (Apc) on chromosome 5 of the human genome. Individuals who carry the APC mutation have a 100% chance of developing multiple colonic polyps that progress on to CRC by the time they reach the age of 40. In contrast, HNPCC has been associated with multiple genes (eg. MSH2, MLH1, PMS2,

MSH6, PMS1) which are involved in DNA repair pathways. Carriers of these HNPCC-associated mutations have an 80% chance of colon cancer development in their life-time.

Sporadic mutations account for 90% of total CRC cases. Genetic instability in CRC is typically classified into two categories; chromosomal instability (CIN) and microsatellite instability (MSI). The CIN pathway of carcinogenesis accounts for an estimated 85% of all CRC cases and is associated with chromosome number aberrations (aneuploidy) and loss of heterozygosity (LOH) events. In contrast, MSI lead to tumor development through genetic defects in mismatch-repair genes (eg. MSH2 and MLH1), thereby promoting errors in DNA replication of microsatellite regions. The uncorrected replication errors may then lead to base insertions and deletions that cause frame-shift mutations that alter the reading frame of the protein leading to alterations or loss of protein function, which may promote cancer development. Microsatellite instability is thus associated with hypermutations in cancers (Rosner et al., 2001; Pritchard et al., 2014). According to a comprehensive molecular characterization study of human CRC samples (Cancer Genome Atlas Network, 2012), the commonly deregulated pathways in were WNT signaling (92% non hypermutated tumors (nHM), 97% hypermutated tumors (HM)), TGFB signaling (27% nHM, 87% HM), PI3K signaling (50% nHM, 53% HM), RTK-RAS signaling (59% nHM, 80% HM), P53 signaling (64% nHM, 47% HM). The study results indicate that the WNT signaling pathway is the most commonly deregulated pathway regardless of the hypermutation status of the tumor.

1.4.2 Mouse models of colorectal cancer

Animal models are important for improving our understanding of colorectal carcinogenesis, with various genetic and experimental models used to study polyp and adenoma formation under defined conditions (Suman and Datta, 2012). According to a study by the Cancer Genome Atlas Network, the most commonly dysregulated pathway in colorectal tumors is the Wnt signaling pathway, with mutational modifications described in various pathway components (Cancer Genome Atlas Network, 2012). To model intestinal cancer

in an animal model, researchers typically use genetically modified mice that carry mutated Wnt signaling components, similar to the mutations reported in human FAP and sporadic colorectal cancer cases. Various models of Apc mutant mice have been developed in which there is a heterozygous mutation of the Apc gene, and tumor development occurs when the second gene undergoes a spontaneous mutation that leads to tumor development (Taketo and Edelmann, 2009). The first Apc mutant mouse model, multiple intestinal neoplasia (Apc^{min} or Apc^{Δ850}) with a truncation mutation at codon 850 of the Apc gene, is the most studied and understood mouse model of human FAP (Moser et al., 1990). Mice carrying a heterozygous Apc^{min} mutation develop multiple adenomas in both the small and large intestines, which are histologically similar to other Apc heterozygous mutant models but with variations in polyp numbers and distribution (Taketo and Edelmann, 2009).

In contrast, researchers also utilize intestinal tumorigenesis mouse models utilizing a chemically-induced method, which involves carcinogens like dimethylhydrazine (DMH) & metabolite azoxymethane (AOM), dextran sulfate sodium (DSS), 2-amino-1-methyl-6-phenylimidazol (4,5-b) pyridine (PhIP), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-Nitroso-N-methylurea (MNU) and 3,2'-dimethyl-4-aminobiphenyl (DMBA) (Karim and Huso, 2013). Among the different protocols, pro-carcinogen azoxymethane exposure followed by DSS-induced colitis is one of the most commonly used methods due to its high penetrance across the different murine genetic backgrounds (De Robertis et al., 2011). It has been reported in literature that intraperitoneal injection of AOM (10 mg/kg body weight) followed by 2% (w/v) DSS in drinking water for 1 week, induces colorectal adenocarcinomas in mice with 100% penetrance (Suzuki et al., 2005). The AOM/DSS-induced tumors have CIN, with aneuploidy and Wnt signaling activation also detected, similar to colitis-associated colorectal carcinogenesis in humans (Gerling et al., 2011).

1.4.3 Wnt signaling pathway in colorectal cancer

The canonical Wnt signaling pathway is the most frequently dysregulated pathway in colorectal cancer, according to a study by the Cancer Genome Atlas Network (Cancer Genome Atlas Network, 2012). Canonical Wnt signaling is transmitted via the transcription co-activator beta catenin (β -catenin). Without the Wnt ligand, β -catenin is targeted for degradation by a destruction complex consisting of APC, glycogen synthase kinase 3- β (GSK-3 β), Axin, and other components, via sequestration and phosphorylation. The binding of Wnt ligands to Frizzled family receptors mediate downstream signaling via Disheveled, leads to the dissociation of the complex and freeing β -catenin. This allows the accumulation of β -catenin in the cytoplasm and its translocation to the nucleus where it can induce downstream gene expression by associating with transcription factors of the LEF/TCF family (Komiya and Habas, 2008).

Growth-promoting genes, such as BMP, MYC and NOTCH, have been thought to be targets of enhanced β -catenin-LEF/TCF transcriptional activity in colorectal epithelial tumor cells, leading to the formation of benign intestinal polyps (Herbst et al., 2014). Further genetic mutations (eg. in RTK-RAS, P53, PI3K and TGFB) then work complementarily to promote development of the precancerous growth into malignant tumors (Fearon and Vogelstein, 1990). The importance of Wnt signaling has also been shown in the tumor stroma microenvironment. In human breast tumors, Wnt2 mRNA has been detected at high levels in breast CAFs but not in normal fibroblasts (Dale et al., 1996). In human colon tumors, both Wnt2 and Wnt5a mRNA are abundantly expressed in macrophages residing in the tumor stroma (Smith et al., 1999). Furthermore, Wnt signaling also appears to influence the bi-directional interaction between the tumor epithelium and its associated stroma. In phyllodes tumors of the breast, a correlation has been observed between the specific nuclear localization of β -catenin in tumor stromal cells with enhanced Wnt5a expression in the epithelium (Sawyer et al., 2002). Conversely, leukemia cells co-cultured with stroma cells also display increased β -catenin activation (Wang et al., 2007). Taken together, the studies suggest that Wnt produced in the colorectal tumor stroma may regulate signaling in tumor cells and vice versa, indicating a role for the tumor microenvironment in modulating tumor development.

1.4.4 Inflammation in colorectal cancer

Inflammation has long been associated with cancer progression, with CRC being no exception. Crohn's disease and ulcerative colitis, collectively termed inflammatory bowel disease (IBD), have been shown to be important risk factors associated with CRC development (Jess et al., 2012; Kim and Chang, 2014). More than 20% of IBD patients have been reported to develop CAC within 30 years of IBD onset, with a 50% mortality rate from CAC (Lakatos and Lakatos, 2008). Inflammation is a process that is driven and composed of a wide multitude of immune and inflammatory cells and, myriad of soluble pro-inflammatory factors such as cytokines, chemokines, lipid molecules, reactive oxygen and nitrogen species. Pathological analyses of tumor-infiltrating cells in colorectal cancer have revealed that immune cells of the innate arm, such as neutrophils, mast cells, natural killer cells (NK), dendritic cells (DC) and macrophages are frequently found in the tumors of CRC patients (Atreya and Neurath, 2008). These innate immune cells have been shown to play various roles in anti-tumor immune responses, including the suppression of tumor growth and angiogenesis, and recruitment of other cells that constitute the adaptive immune response (Deschoolmeester et al., 2011). While immune factors tend to have a promoting effect on tumor progression, immune surveillance facilitates the prevention and early elimination of aberrant development in intestinal crypts (Watson et al., 2006; Parcesepe et al., 2016). During chronic inflammation, this homeostasis is disrupted when the pro-inflammatory microenvironment curtails the immune surveillance system, resulting in the inhibition of anti-tumor mechanisms and the infiltration of pro-tumorigenic DCs and T regulatory cells (T reg) which promotes tumor growth (Rubin et al., 2012). Hence the resolution of inflammation constitutes an important step. Fibroblasts play a sizable role in the tissue repair and resolution process through the release of soluble factors such as interleukin-6 (IL-6), interleukin-8 (IL-8) and prostaglandin E2 (PGE2), which aid in the neutrophil response to halt continued aberrant proliferation (Van Linthout et al., 2014; Wilgus et al., 2013). The cyclooxygenase (COX) and lipoxygenase (LOX) pathways are implicated in the generation of PGE2 and other pro-inflammatory lipid mediators that generate a pro-tumorigenic

microenvironment (Rao et al., 2012). COX-2 is the inducible COX gene, which is found to be elevated in human CRC (Sano et al., 1995). In mice, overexpression of COX-2 promotes tumor progression in azoxymethane-induced CRC (Al-Salihi et al., 2009) while the loss of COX-2 impairs tumor formation in genetic models of colorectal cancer (Chulada et al., 2000, Oshima et al., 1996). Metabolites from the 5-LOX pathway have been implicated in the early development of CRC (Qiao et al., 1995; Avis et al., 2001). Furthermore, high 5-LOX protein expression has been reported in cancer cell lines while the expression of 5-LOX and its receptors have been negatively associated with a favorable prognosis in CRC patients (Hong et al., 1999; Ohd et al., 2003). Studies have suggested that the COX and LOX pathways interact with each other. Disruption of 5-LOX pathway components results in a compensatory increase in COX metabolites (Byrum et al., 1997; Goulet et al., 1994). It is also noteworthy that anti-inflammatory therapies have been used to prevent CRC or reduce disease severity in patients (Smalley and DuBois, 1997). Regular use of non-steroidal anti-inflammatory drugs (NSAIDs) at low doses have been reported to reduce the incidence of CRC (Thun et al., 1991; Smalley and DuBois, 1997), with studies suggesting a mechanism mediated through the inhibition of COX-2 activity.

1.4.5 Oxidative stress and reactive oxygen species in colitis and colorectal cancer

Oxidative stress refers to the imbalance between the production of free radicals in the form of reactive metabolites, oxidants or reactive oxygen species (ROS) and their elimination by cellular protective mechanisms (Sreevalsan and Safe., 2013). Several cancers, including cancers of the pancreas, lung, breast and prostate, have been associated with increased oxidative stress and ROS production (Afanas'ev, 2011). Similarly, colorectal cancer patients have also been associated with increased oxidative stress, with increased serum 8-OHdG, reduced antioxidative enzyme activity and lowered serum vitamin A and E concentrations (Chang et al., 2008). The relationship between oxidative stress and its role in tumorigenesis has since been discussed. The activity of ROS has been described in tumor initiation, in which an abundance of ROS leads to DNA

damage in the form of DNA structural alterations or base pair mutations. (Waris and Ahsan, 2006) Furthermore, ROS have been shown to regulate gene expression to promote tumorigenic processes such as cancer proliferation, cell survival, angiogenesis, invasion, metastasis and chemoresistance through the modulation of cellular signaling pathways such as NF- κ B, PI3K/Akt, heat shock proteins and the MAPK pathway (Perše, 2013). The activity of ROS has also been linked to inflammatory disorders of the gut, such as gastric ulcers and intestinal cancers (Phull et al. 1995). For example, inflammatory bowel diseases, including both CD and UC, result from inappropriate inflammatory responses to commensal gut microbiota. Increased ROS and reduced anti-oxidants in IBD leads to the pathogenesis of IBD symptoms, which are further aggravated by an activated immune response that attracts inflammatory leukocytes, thereby potentiating ROS generation (Phull et al., 1995). Hence, anti-inflammatory drug therapy has been employed to alleviate colitis and prevent colorectal cancer. NSAIDs have been shown to inhibit COX-2 activity resulting in reduced prostaglandin production that leads to the observed anti-inflammatory and anti-tumor effect in the intestines (Wang and DuBois, 2010).

While low to moderate ROS levels have been shown to have a promoting effect on cancer proliferation and development, high levels of ROS accumulate considerable oxidative stress that leads to the preferential killing of cancer cells. As such, several anti-neoplastic drugs used for cancer chemotherapy function by inducing high levels of oxidative stress (Barrera, 2012). Similarly, ionizing radiation, which is used to treat many types of cancers, are also thought to act via the generation of oxidative stress in cells, through increased ROS levels (Yoshida et al., 2012; Wang et al., 2010). The role of ROS in cancer is extremely important in the gut where it is exposed to various sources of free radicals such as the gut microbiota, dietary sources or the physical activity of the host (Perše, 2013). Thus, it becomes evident an improved understanding of oxidative stress in colorectal cancer is vital for the development of new strategies to combat the disease.

1.5 PPAR β/δ

1.5.1 PPAR β/δ overview

The peroxisome proliferator-activated receptor (PPAR) belongs to the type II subfamily of the nuclear receptor super family, together with retinoic acid receptor (RAR), retinoid X receptor (RXR) and thyroid hormone receptor. The PPAR family further comprises 3 nuclear receptor proteins, PPAR α , PPAR β/δ and PPAR γ , which act as transcription factors regulating gene expression (Mangelsdorf et al., 1995). As with type II nuclear receptors, PPARs regulate transcription in a hetero-dimeric complex, by associating with retinoid X receptors. The hetero-dimeric PPAR-RXR complex transcriptionally regulates gene expression by binding to specific DNA sequences on the target gene. These DNA sequences are termed peroxisome proliferator responsive elements (PPREs) and have a consensus sequence of AGGTCANAGGTCA with N representing any nucleotide (Kliwer et al., 1992). PPAR target genes perform a range of different functions related to lipid metabolism and energy homeostasis (Schoonjans et al., 1996).

The three subtypes, PPAR α , PPAR β/δ and PPAR γ , are evolutionarily conserved across various species, with 80% sequence identity in their DNA binding domains, but with 65% sequence identity when comparing their ligand binding domains, which thereby partially accounts for their differential activation by endogenous and exogenous ligands (Tachibana et al., 2008). There are also significant differences in the spatial distribution of the three PPAR subtypes. PPAR α expression has been detected in adipose tissue, liver, kidney, small intestine, heart, and muscle. PPAR γ expression and location differ for its splice variants. PPAR γ 2 is found mainly in the adipose tissue, which has been shown to be involved in adipocyte differentiation, lipid storage in white adipose tissue, and energy dissipation in the brown adipose tissue (Tontonoz et al., 1994). Moreover, the PPAR γ 1 and PPAR γ 3 variants are found exclusively in the digestive tract and immune cells, playing a role in the regulation of cell cycle processes and inflammation (Lehrke and Lazar, 2005). PPAR β/δ , in contrast, displays a ubiquitous expression pattern and is observed in a wide variety of cells, including cells associated with fatty acid metabolism, the epithelial lineage, and the mesenchyme and immune system (Attianese and Desvergne, 2015). Thus, the activity of PPAR β/δ has been implicated in a variety of different functions

including fatty acid oxidation, keratinocyte differentiation and wound healing (Tachibana et al., 2008).

Endogenous ligands for PPARs mainly consist of free fatty acids and eicosanoids (Desvergne and Wahli, 1999). Members of the 15-hydroxyeicosatetraenoic acid family of arachidonic acid metabolites have been shown to activate PPAR α , PPAR β/δ and PPAR γ to varying degrees (Naruhn et al., 2010). Studies have shown that prostaglandin J2 (PGJ2) specifically activates PPAR γ , and that leukotriene is the ligand for PPAR α (Devchand et al., 1996). However, the endogenous ligand specific for PPAR β/δ is still unclear, with several prostacyclins, prostaglandins and eicosanoids proposed as possible candidates (Attianese and Desvergne, 2015). On the synthetic front, compounds have been successfully developed to selectively agonize or antagonize PPAR β/δ activity. Compounds GW501516 (Pelton, 2006) and GW0742 (Sznajdman et al., 2003) have been used as PPAR β/δ agonists in animal studies while, GSK3787 has emerged as the PPAR β/δ antagonist of choice (Shearer et al., 2010). Together, the ligands, endogenous or synthetic, are important tools to aid our understanding of PPAR β/δ activities in a controlled experimental setting and maybe extended in the near future to a clinical one.

1.5.2 PPAR β/δ in inflammation and ROS

As nuclear receptors regulating metabolic signaling pathways, PPARs have been proposed as drugs to treat various components of metabolic syndrome, including dyslipidemia, hypertension, insulin resistance and inflammation (Wahli and Michalik, 2012). A chronic low-grade inflammation often associates with metabolic diseases and is characterized by an increase in inflammatory cytokines and acute phase proteins. PPAR α , PPAR β/δ and PPAR γ have all been shown to modulate inflammation in such cases (Michalik and Wahli, 2008; Bishop-Bailey and Bystrom, 2009).

With the ubiquitous expression pattern of PPAR β/δ , there has been interest in studying the role of PPAR β/δ in regulating inflammation and immunity in various tissues. In endothelial cells, ligand activation of PPAR β/δ inhibits the production of the pro-inflammatory chemokines TNF- α and C-

reactive protein (Liang et al., 2010) and through inducing the expression of endothelial adhesion molecules VCAM-1, ICAM-1 and E-selectin (Fan et al., 2008; Piqueras et al., 2009). Combining both effects results in a reduction of the immune stimulus through lowered immune cell recruitment and the blockade of monocytic attachment to vascular endothelial cells (Bishop-Bailey and Bystrom, 2009). Activation of PPAR β/δ in endothelial cells also induces 14-3-3a, superoxide dismutase-1, catalase and thioredoxin which results in an anti-oxidant effect and protects cells from oxidant stress. PPAR β/δ has also been shown to act directly on inflammatory cells. PPAR β/δ has been shown to play an anti-inflammatory role in atherosclerosis through the activation of macrophage development to the anti-inflammatory M2-subtype (Kang et al., 2008), and by lowering the levels of several inflammatory proteins, such as IL-1B, MCP-1, IL-6 and TNF-A (Lee et al., 2003; Barish et al., 2008; Takata et al., 2008).

In the skin, PPAR β/δ expression is rapidly upregulated during injury, at the epidermal wound edge, with the production of unidentified PPAR β/δ -activating ligands (Tan et al., 2001). The activation of PPAR β/δ has been shown to be vital for the suppression of keratinocyte apoptosis in the wounded tissue (Di-Poï et al., 2002), while promoting keratinocyte recruitment for wound healing (Michalik et al., 2001). Wound repair also involves a degree of epithelial-mesenchymal interaction. Fibroblast expression of PPAR β/δ is also stimulated by keratinocyte-derived IL-1 signals in the wounded tissue. Fibroblast-derived PPAR β/δ then promotes the production of secreted IL-1 receptor antagonist (sIL-Ra) that works in a negative feedback loop to limit further IL-1 stimulation, thereby resulting in reduced fibroblast-derived mitogenic factor production and ensuring homeostasis in the skin during wound healing (Chong et al., 2009). In contrast, the effect of PPAR β/δ in colonic inflammation is less understood. Studies have also been conducted in murine models of dextran sodium sulfate-induced colitis, however, disparate findings have rendered the role of PPAR β/δ in colitis inconclusive (Wahli, 2008; Peters et al., 2008). Using transgenic mice with ablated PPAR β/δ expression, the pro-inflammatory role of PPAR β/δ has been shown to be mediated through COX-2 derived PGE2 signaling (Wang and DuBois, 2014). However, another study has shown that PPAR β/δ expression in colonic mucosa protects against colitis in a ligand-independent mechanism

(Hollingshead et al., 2007). The role of mesenchymal PPAR β/δ in colitis requires further study. Thus, elucidating the role of PPAR β/δ in colitis necessitates a more detailed examination.

Oxidative stress-mediated signaling mechanisms are involved in mediating inflammation and tissue injury (Mittal et al., 2014). PPARs regulate downstream processes, including fatty acid oxidation and lipid metabolism, although few studies have examined if the increased lipid oxidation leads to increased oxidative stress. Liver is a key metabolic organ for lipid metabolism. PPAR-pan activation using the agonist GW625019, leads to increased hepatic oxidative stress and pro-inflammatory eicosanoids in rats (Ament et al., 2016). The role of PPAR in ROS signaling in the brain and cardiovascular system has also been further discussed (Aleshin and Reiser, 2013; Kim, 2013). PPAR α has been implicated in fasting-mediated oxidative stress (Abdelmegeed et al., 2009) while PPAR γ is known to mediate oxidative stress by interacting with various pathways, including the wnt/ β -cat, erythroid 2-related factor 2 (NRF2) and forkhead box proteins O (FOXO) pathways (Polvani et al., 2012). Despite detailed studies conducted for both PPAR α and PPAR γ , our understanding of PPAR β/δ in oxidative stress modulation remains to be improved. Our previous study has shown that treatment of diabetic wounds with PPAR β/δ agonist GW501516 is mediated by redox modulation. Another PPAR β/δ agonist, GW0742, has been shown to mediate anti-oxidant effects in the heart, resulting in protective effects on the vasculature of the cardiac endothelium (Zarzuelo et al., 2013) and inhibition of cardiac myocyte hypertrophy (Galatou et al., 2014).

1.5.3 PPAR β/δ in CRC

PPARs, with their role as transcription factors modulating gene expression, regulate an array of biological functions and thus have been shown to play an important role in many diseases, including cancer. PPARs have been implicated in cancers of the brain, lung, gut, liver, pancreas and urinary system (Youssef and Badr, 2011; Tachibana et al., 2008). Studies of PPAR α in cancer have largely revolved around its role in liver carcinogenesis. Prolonged PPAR α agonist treatment has been shown to promote liver cancer in rodents (Reddy et

al., 1980), whereas PPAR α -null mice exhibit resistance to the hepatocarcinogenic effects of PPAR α agonist treatment (Peters et al., 1997). However, this effect was not observed in humans, owing to the differences in PPAR α expression levels, roles of PPAR α in proliferative and apoptotic pathways, and inter-species differences in the ability of PPAR α to suppress miRNA let-7C which in turn mediates the downstream regulation of cellular proliferation (Tachibana et al., 2008). In contrast, PPAR γ , is expressed in a variety of cancers, including colonic, breast, esophageal, lung and the prostate (Tachibana et al., 2008). PPAR γ has been shown to inhibit tumorigenesis through cell cycle regulation and apoptosis induction (Theocharis et al., 2004, Grommes et al., 2004). It plays an anti-inflammatory role by inhibiting the production of proinflammatory signaling proteins, and thus has been implicated in the risk for cancers associated with chronic inflammation (Peters et al., 2012). For example, PPAR γ expression in intestinal epithelial cells and macrophages has been shown to inhibit inflammatory pathways thereby leading to reduced colon carcinogenesis (Adachi et al., 2006, Shah et al., 2007). While PPAR γ and PPAR α have relatively established roles in regulating carcinogenesis, the role of PPAR β/δ in colorectal cancer has been one of much controversy, with both pro- and anti-tumorigenic functions reported in literature.

On the one hand, studies have suggested that PPAR β/δ is an enabler of colorectal tumorigenesis through enhancing cell proliferation. First, mRNA expression of PPAR β/δ is elevated in human and rat colorectal carcinomas (Gupta et al., 2000). NSAIDs have been used in therapy for CRC. A study showed that NSAIDs work by disrupting the DNA binding of PPAR β/δ /RXR heterodimers, and that ectopic expression of PPAR β/δ , in a colorectal cancer cell line, results in resistance to sulindac-induced apoptosis (He et al., 1999). The same study also proposed that PPAR β/δ is a downstream target of the canonical Wnt signaling pathway, involving APC/ β -catenin / T cell factor-4 (TCF-4) (He et al., 1999). This finding was echoed by studies of DMBA-induced gastric tumors in mice where increased β -catenin levels were reported to be involved in the promotion of PPAR β/δ -mediated gastric tumorigenesis (Pollock et al., 2010). Furthermore, high-fat diet-induced PPAR β/δ activation leads to increased β -catenin activity and expression of β -catenin target genes in intestinal stem cells,

leading to increased self-renewal potential and enabling oncogenic transformation (Beyaz et al., 2016). In HCT116 intestinal carcinoma cells, the cooperation of β -catenin and PPAR β/δ has been demonstrated to activate VEGF transcription, where PPAR β/δ activation has been shown to regulate β -catenin-mediated chromatin looping (Hwang et al., 2012). Although studies have hypothesized that APC/ β -catenin signaling regulates PPAR β/δ activity, changes in PPAR β/δ protein expression was not observed in multiple colon cancer cell lines with mutations in APC or β -catenin, and intestinal polyps of Apc^{min/+} mice (Foreman et al., 2009). This suggests that the regulation of PPAR β/δ transcriptional activity by APC/ β -catenin, if any, occurs without changes to PPAR β/δ expression. Thus, further studies will be required to investigate the molecular mechanism in which APC/ β -catenin regulates PPAR β/δ activity. Other evidences, including treatment with the PPAR β/δ agonist GW501516 showed an increased number and size of intestinal polyps in Apc^{min} mice (Gupta et al., 2004), while transgenic PPAR β/δ ^{-/-} mice, on a Apc^{min} background, demonstrated reduced formation of intestinal adenomas and were resistant to the tumorigenic effect of GW501516 agonist treatment (Wang et al., 2006).

However, other studies have also reported that ligand-activation of PPAR β/δ results in terminal differentiation and growth inhibition of colorectal tumors. The pattern of PPAR β/δ expression in the tumor has also been debated, and Marin et al. reported a decrease in PPAR β/δ expression in both Apc^{min} and azoxymethane (AOM)-treated wild-type mouse polyps (Marin et al., 2006). A contradiction also exists in a study conducted by Hollingshead et al. who showed that GW501516 and GW0742, two highly specific PPAR β/δ ligands, did not produce an increase in cellular proliferation in several human colon cancer cell lines (HT-29, HCT116 and LS-174T) or in Apc^{min} mouse intestines (Hollingshead et al., 2007). Moreover, further studies on PPAR β/δ ^{-/-}Apc^{min} and AOM-treated PPAR β/δ ^{-/-} mice have reported an increase in colon polyp formation (Reed et al., 2004; Harman et al., 2004). The effect was attributed to the induction of colonocyte differentiation and apoptosis enhancement via the action of PPAR β/δ (Marin et al., 2006).

The reason for the apparently disparate findings concerning the role of PPAR β/δ in colorectal cancer has not yet been fully identified. The different

strategies used for the genetic ablation of the PPAR β/δ gene were identified as a potential factor (Wang et al., 2006). A popular strategy involves targeting exon 4 of the PPAR β/δ gene, which encodes the DNA binding domain, resulting in the disruption of transcription factor activity of PPAR β/δ (Nadra et al., 2006). Another strategy involves targeted ablation of the last exon, exon 8, of the PPAR β/δ gene and is thought to result in the production of a hypomorphic PPAR β/δ variant (Marin et al., 2006). While targeting of exon 4 abrogates the transcriptional activity of PPAR β/δ , the hypomorphic PPAR β/δ variant produced by the deletion of exon 8 may retain some part of its transcription factor function. Evidently, PPAR β/δ exon 4 deletion results in high rates of embryonic lethality and abnormal placental development, but this was not observed with the PPAR β/δ exon 8 deletion (Barak et al., 2002).

To pursue the intricacies underlying the role of PPAR β/δ in colorectal cancer, Zuo et al. employed a tissue-specific approach, generating transgenic mice with an intestinal epithelium-specific disruption of PPAR β/δ exon 4 (Zuo et al., 2009). The intestinal epithelium-specific loss of PPAR β/δ resulted in suppressed colonic tumorigenesis in mice, supporting the pro-tumorigenic role of PPAR β/δ . A subsequent study then demonstrated that ectopic expression of PPAR β/δ in the intestinal epithelium markedly increased colon tumor size and numbers in both Apc^{min} and AOM-treated models of murine colorectal cancer (Zuo et al., 2014). A study has also indicated the prognostic significance of PPAR β/δ expression in vascular endothelial cells in colorectal tumors, highlighting the important role played by supporting cells in tumorigenesis (Zhou et al., 2014). Although the role of the tumor stroma is gaining traction in recent studies, the influence of stromal PPAR β/δ in colorectal tumorigenesis has not been adequately addressed in the literature. To emphasize the role of stromal PPAR β/δ , PPAR β/δ signaling has been shown to participate in reverse crosstalk with TGF β signaling, a commonly dysregulated pathway in colorectal tumors (Stockert et al. 2011). Thus, PPAR β/δ signaling derived from the colorectal stroma and its effect on tumor development, present an interesting and yet unexplored territory in colorectal cancer research.

1.5.4 PPAR β/δ in fibroblasts

Fibroblastic cells have a major influence on the tissue microenvironment, secreting factors that regulates surrounding cells and shape the extracellular matrix (Tomasek et al., 2002). In the skin, PPAR β/δ is the abundant subtype found in fibroblasts and has been shown to play a role in modulating its neighboring cells via epithelial-mesenchymal interactions (Di-Poi et al., 2002). In a skin wounding model, ligand activation of PPAR β/δ in rat skin fibroblasts also increased fibroblast proliferation and improved wound repair via the upregulation of Ski protein (Li et al., 2012). Furthermore, fibroblast derived PPAR β/δ signals have been shown to exert a paracrine influence on oxidative homeostasis in the adjacent epithelium (Wang et al., 2015). Similarly, treatment with the PPAR β/δ agonist GW501516 reduced corneal stromal fibrosis by deregulating myofibroblast transdifferentiation, leading to reduced aberrant ECM deposition (Gu et al., 2014). A role for PPAR β/δ in the tumor stroma has also been previously discussed, citing possible roles of stromal PPAR β/δ in regulating the proliferation and differentiation of tumor endothelial cells leading to angiogenesis (Müller et al., 2008; Müller-Brüsselbach et al., 2007, Abdollahi et al., 2007). However, the role of PPAR β/δ in the colorectal fibroblastic populations has not been well studied. Thus, we investigated this gap in research through the generation and characterization of a transgenic mouse line carrying a fibroblast-specific protein (FSP)-1-promoter driven deletion of PPAR β/δ expression.

Figure 1 Schematic of PPAR β/δ function in colonic epithelial cell, macrophage, endothelial cell and fibroblast

1.7 Aims / Objectives of this study

Despite more than a decade of research, how PPAR β/δ plays a role in colorectal cancer development remains to be answered. Given that current literature emphasizes the role of PPAR β/δ in a classical transgenic knock-out system or one that targets tumor epithelial cells, we hypothesize that improving our understanding of the role of the tumor stroma in colorectal cancer may resolve much of the controversy. Notably, the role of PPAR β/δ in fibroblasts of the stroma in developing intestinal carcinomas remains a topic that requires further study. To ascertain whether *FSP1Cre-Pparb/d^{-/-}* intestines carry the stromal-specific deletion of PPAR β/δ , we will first validate transcript and protein

levels of PPAR β/δ in the intestines and examine whether they are histologically different from wild type mice. As inflammatory bowel diseases constitute an important risk factor for colitis-associated cancer in the intestines, we will assess whether the FSPCre-*Pparb*/*d*^{-/-} behave any differently from wild type intestines during DSS-induced colitis. To perform a holistic assessment of the *in vivo* role of stromal PPAR β/δ during colorectal carcinogenesis, we will study three different models of colorectal cancer in mice carrying a stromal-specific mutation of the PPAR β/δ gene, to represent human FAP and IBD. To understand the mechanism underlying any observed effects, we will perform microarray comparative gene expression analysis. A variety of cells have been shown to influence the tissue microenvironment, and thus to observe direct epithelial-mesenchymal interaction, we will perform validation assays on an *in vitro* platform comprising intestinal epithelial cell lines with fibroblast cells.

Chapter 2: Materials and Methods

Chapter 2: Materials & Methods

2.1 Materials

2.1.1 Antibodies

Table 1: Parameters used for protein and flow cytometric analysis.

Target Molecule	Primary Antibody Dilution	Secondary Antibody Dilution
PPAR β/δ	1:10000 (Santa Cruz Biotechnology; #sc-1987)	1:20000 (LI-COR Biosciences, #926-68074)
NRF2	1:5000 (Santa Cruz Biotechnology; #sc-722)	1:10000 (LI-COR Biosciences, #925-68071)
β -Tubulin	1:10000 (Santa Cruz Biotechnology; #sc-9104)	1: 20000 (LI-COR Biosciences, #925-68071)
PCNA	1:5000 (Santa Cruz Biotechnology; #sc-56)	1:10000 (LI-COR Biosciences, #925-68070)
EpCAM-PE	1:100 (Miltenyi Biotec; #130-117-863)	n/a
LGR5-Biotin	1:100 (Miltenyi Biotec; #130-110-123)	1:200 (Streptavidin-APC; Miltenyi Biotec, #130-106-791)
CD11b-APC	1:100 (Miltenyi Biotec; #130-109-286)	n/a
Ly6G-PE	1:100 (Miltenyi Biotec; #130-107-975)	n/a
CD3-FITC	1:100 (Miltenyi Biotec; #130-109-878)	n/a
F/480-FITC	1:100 (Miltenyi Biotec; #130-102-988)	n/a
MHC Class II (I-ab)-APC	1:100 (Miltenyi Biotec; #130-108-004)	n/a
CD206-PE	1:100 (Miltenyi Biotec; ##141705)	n/a

2.1.2 Primer sequences

Table 2: PCR primer sequences

Genotyping	Primer	Sequences
APC ^{min/+}	Forward primer 1 (Apcwt)	5'-GCCATCCCTTCACGTTAG-3'
	Forward primer 2 (Apcmin)	5'-TTCTGAGAAAGACAGAAGTTA-3'
	Common reverse primer (ApcR)	5'-TTCCACTTTGGCATAAGGC-3'
PPAR β/δ floxed	Forward primer (Pbx10)	5'-GCAGCTGCTCAGCTGCCTGC-3'
	Reverse Primer 1 (Ab008)	5'-GGACCCCGTAGTGGAAGCCCGAG-3'
	Reverse Primer 2 (Ab021)	5'-ATGCCGAGTGCCAGGCACTTCTG-3'
Fsp1Cre	Forward primer (fspcreF)	5'-GCGGTCTGGCAGTAAAACTATC-3'
	Reverse primer (fspcreR)	5'-GTGAAACAGCATTGCTGTCACTT-3'
Real-time PCR	Primer	Sequences
PPAR β/δ	Forward	5'- TGCAGATGGGCTGTGATGGG-3'
	Reverse	5'-CTCGAGCTTCATGCGGATTGTC-3'
Mouse catalase	Forward	5'- CCCCTATTGCCGTTTCGATTCT-3'
	Reverse	5'- TTCAGGTGAGTCTGTGGGTTT-3'
	Forward	5'-CCTAGTTCTCCGCTGCTGGG-3'

Mouse nuclear factor erythroid-derived factor-2 (mNRF2)	Reverse	5'-CCATGTCCTGCTGGGACTGTA-3'
Mouse superoxide dismutase 1	Forward	5'-ATGGCGATGAAAGCGGTGT-3'
	Reverse	5'-CCTTGTGTATTGTCCCCATACTG-3'
Mouse superoxide dismutase 2	Forward	5'-CAGACCTGCCTTACGACTATGG-3'
	Reverse	5'-CTCGGTGGCGTTGGATTGTT-3'
Mouse superoxide dismutase 3	Forward	5'-ACCGGCTTGGTTCTCTTCC-3'
	Reverse	5'-CTCCATCGGGTTGTAGTGCG-3'
Mouse glutathione peroxidase 1	Forward	5'-AATGTCGCGTCTCTCTGAGG-3'
	Reverse	5'-TCCGAACTGATTGCACGGG-3'
Mouse glutathione peroxidase 2	Forward	5'-GAGCTGCAATGTCGCTTTCC-3'
	Reverse	5'-TGGGTAAGACTAAAGGTGGG -3'
Mouse glutathione peroxidase 3	Forward	5'-TTTGTGCCTAATTTCAGCTCTT-3'
	Reverse	5'-GTCCATCTTGACGTTGCTGA-3'
Mouse glutathione peroxidase 4	Forward	5'-GATGGAGCCCBTTCCTGAACC-3'
	Reverse	5'-CCCTGTACTTATCCAGGCAGA-3'
Mouse peroxiredoxin 1	Forward	5'-AATGC AAAAATTGGGTATCCTGC-3'
	Reverse	5'-CCGTGGGACACACAAAAGTAAA-3'
Mouse peroxiredoxin 2	Forward	5'-GGTAACGCGCAAATCGGAAAG-3'
	Reverse	5'-TCCAGTGGGTAGAAAAAGAGGT-3'

Mouse peroxiredoxin 3	Forward	5'- GTCTCGACGACTTTAAGGGAAAA- 3'
	Reverse	5'- ACTGAAACTGCAACTACTTCACA- 3'
Mouse peroxiredoxin 4	Forward	5'- CTCAAAGTACTGACTATCGTGG- 3'
	Reverse	5'- CGATCCCCAAAAGCGATGATTTC- 3'
Human catalase	Forward	5'- TCATCAGGGATCCCATATTGTT- 3'
	Reverse	5'- CCTTCAGATGTGTCTGAGGATTT- 3'
Human nuclear factor erythroid- derived factor-2	Forward	5'-ACACGGTCCACAGCTCATC-3'
	Reverse	5'-TGCCTCCAAAGTATGTCAATCA- 3'
Human superoxide dismutase 1	Forward	5'-TCATCAATTTTCGAGCAGAAGG- 3'
	Reverse	5'-GCAGGCCTTCAGTCAGTCC-3'
Human superoxide dismutase 2	Forward	5'-AAGTACCAGGAGGCGTTGG-3'
	Reverse	5'-TGAAGTTCAGTGCAGGCTGA-3'
Human superoxide dismutase 3	Forward	5'-GGTGCAGCTCTCTTTTCAGG-3'
	Reverse	5'- AACACAGTGGCGCCAGCATGGCC- 3'
Human glutathione peroxidase 1	Forward	5'-TCTGTTGCTCGTAGCTGCTG-3'
	Reverse	5'-CTATGGATCACCGGGATTTTG- 3'
Human glutathione peroxidase 2	Forward	5'-CTGGTGGTCCTTGGCTTC-3'
	Reverse	5'-TGTTTCAGGATCTCCTCATTCTG- 3'

Human glutathione peroxidase 3	Forward	5'- GGGGACAAGAGAAGTCGAAGA-3'
	Reverse	5'-TGTTCAGGATCTCCTCATTCTG- 3'
Human glutathione peroxidase 4	Forward	5'-TTTCCGTGTAACCAGTTCG-3'
	Reverse	5'-TGCCTCCAAAGTATGTCAATCA- 3'
Human peroxiredoxin 1	Forward	5'- AATGCAAAAATTGGGTATCCTGC- 3'
	Reverse	5'- CCGTGGGACACACAAAAGTAAA- 3'
Human peroxiredoxin 2	Forward	5'-GGTAACGCGCAAATCGGAAAG- 3'
	Reverse	5'- TCCAGTGGGTAGAAAAAGAGGT- 3'
Human peroxiredoxin 3	Forward	5'- GTCTCGACGACTTTAAGGGAAAA- 3'
	Reverse	5'- ACTGAAACTGCAACTACTTCACA- 3'
Human peroxiredoxin 4	Forward	5'- CTCAAAGTACTGACTATCGTGG- 3'
	Reverse	5'- CGATCCCCAAAAGCGATGATTTC- 3'
Human NADPH oxidase 1	Forward	5'-AAGGATCCTCCGGTTTTACC-3'
	Reverse	5'-TTTGGATGGGTGCATAACAA-3'
Human NADPH oxidase 3	Forward	5'-ACGGGTCGGATTGTTCGAGG-3'
	Reverse	5'-ACGGTCGGATTGTTCGAGG-3'
	Forward	5'-TGCCTGCTCATTTGGCTGT-3'

Human NADPH oxidase 4	Reverse	5'- CCGGCACATAGGTAAAAGGATG- 3'
18S Ribosomal RNA (18S rRNA)	Forward	5'-GTAACCCGTTGAACCCCATT-3'
	Reverse	5'-CCATCCAATCGGTAGTAGCG-3'
60S Ribosomal protein L27 (RPL27)	Forward	5'-GCTGGTTATCGGGAGTTGG-3'
	Reverse	5'-ACTGGCCTGGTGTCTAGAG-3'
siRNA	Primer	Sequences
PPAR β/δ siRNA (J-003435-06)	Antisense	5'-GACCUGGCCCUAUUCAUUG-3'
PPAR β/δ siRNA (J-003435-07)	Antisense	5'-UAUCAUUGAGCCUAAGUUU-3'
PPAR β/δ siRNA (J-003435-08)	Antisense	5'-GAGCGCAGCUGCAAGAUUC-3'
PPAR β/δ siRNA (J-003435-09)	Antisense	5'-CCACUACGGUGUUCAUGCA-3'

2.2 Mice

2.2.1 Transgenic mouse models

Generation of FSPCre-*Pparb/d*^{-/-} mice

FspCre transgenic mice were a kind gift from Prof Gustavo Leone (Ohio State University, USA). FspCre transgenic mice express the Cre recombinase protein under the control of Fsp1 promoter, resulting in stromal-specific expression of Cre recombinase as reported in several organs, such as the mammary gland, prostate and the forestomach (Trimboli et al., 2008; 2009).

The *Pparb/d*^{*fl/fl*} transgenic mice were derived by Prof Walter Wahli's laboratory (University of Lausanne, Switzerland). *Pparb/d*^{*fl/fl*} transgenic mice

carry alleles of the PPAR β/δ gene with a loxP-flanked exon 4, which encodes for the DNA-binding domain (Iglesias et al., 2012).

FSPCre-*Pparb/d*^{-/-} mice, whose exon 4 only of PPAR β/δ gene was deleted in stroma cells, were then generated in-house by crossing *Pparb/d*^{fl/fl} and FspCre mice over several generations.

Generation of mice on APC^{min/+} x FSPCre-*Pparb/d*^{-/-} background

The *Apc* multiple intestinal neoplasia (APC^{min/+} or *Apc* ^{Δ 850}) mouse carries the *Apc* gene allele with a truncation mutation of codon 850 (Moser et al., 1990). APC^{min/+} mice were obtained from The Jackson Laboratory (The Jackson Laboratory, USA) and maintained in-house as heterozygous min/+ mice.

APC^{min/+} mice were crossed with FSPCre-*Pparb/d*^{-/-} mice over several generations to generate progenies with mixed APC^{min/+} and FSPCre-*Pparb/d*^{-/-} backgrounds.

2.2.2 Mouse PCR genotyping

To perform mouse genotyping, small ear biopsies (<0.5 cm) were taken from 3-week old mice and lysed for DNA using the KAPA Express Extract kit (KapaBiosystems, USA). Briefly, DNA extraction was performed at 50 μ L volume, in PCR-grade water, 10X KAPA Express Extract buffer and 1U/ μ L KAPA Express Extract enzyme. The manufacturer's lysis protocol was used, with a lysis step of 75 °C for 10 min followed by an enzyme activation step of 95 °C for 5 min. Thereafter, polymerase chain reaction (PCR) was performed using the 2X KAPA2G Fast Genotyping Mix (KapaBiosystems, USA) with a final primer concentration of 0.5 μ M and 1 μ L of extracted mouse template DNA. Primer sequences can be found in Table 2. The manufacturer's KAPA2G Robust PCR Protocol was used, with an initial denaturation step (95 °C for 3 min), followed by 35 cycles of Denaturation (95 °C for 15 sec), Annealing (60 °C for 15 sec) and Extension (72 °C for 30 sec), ending with a final extension step (72 °C for 1 min).

2.2.3 Mouse treatment: Azoxymethane / Dextran sulfate sodium (AOM/DSS)

For chemical induction of colonic tumor formation, 6 to 8-week old mice were first injected with azoxymethane (AOM; 10 mg/kg bodyweight; Sigma-Aldrich Co. LLC, USA) dissolved in sterile 0.9% saline i.p.. One week after AOM injection, mice were fed with 2% (w/v) dextran sulfate sodium (DSS; MP Biomedicals LLC, USA) dissolved in drinking water for 7 days *ad libitum*. After the 1-week DSS treatment, mice were returned to normal drinking water until sacrifice. For anti-oxidant treatment, mice were exposed to 400 μ L of 40 mM N-acetylcysteine (NAC, Sigma-Aldrich Co. LLC, Missouri, USA) intra-gastrically for 5 consecutive days before the administration of DSS treatment. Clinical symptoms were recorded as detailed in Table 3 below (adapted from Maxwell et al., 2009).

Table 3: Disease Activity Index (DAI) scoring matrix

DAI Score	Stool Consistency	Presence of bleeding	Mouse appearance
0	Normal	None	Normal
1	Moist or sticky	Visible blood traces in stool or around anus	Ruffled fur or altered gait
2	Soft	Gross bleeding	Lethargic or moribund
3	Diarrhea		

2.2.4 Animal handling and husbandry

All procedures were performed according to the Responsible Care and Use of Laboratory Animals (RCULA) guidelines and approved by the Institutional Animal Care and Use Committees (IACUC) in Nanyang Technological University (NTU), Singapore (Reference number: ARF-SBS/NIE-A0217) and SingHealth, Singapore (Reference number: IACUC

#2013/SHS/866). Mice were maintained at the designated animal facility under a 12-h dark-light cycle at $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and given standard mouse chow and water ad libitum.

2.3 Cell Culture

2.3.1 Cell culture maintenance

Human colon adenocarcinoma cell lines (HCT116; ATCC No. CCL-247), human immortalized skin-derived fibroblasts (BJ1; ATCC No. CRL-2522) and human colon myofibroblasts (CCD18Co, ATCC No. CRL-1459) were procured from American Type Culture Collection (ATCC). These cell lines were cultured in DMEM (HyClone, USA) supplemented with 10% FBS (HyClone, USA). SV40-immortalized human colon epithelial cells were obtained from Applied Biological Materials Canada (iCECs; T0570; ABM, Canada) and cultured in Prigrow III Medium (ABM Canada) with collagen-coating, according to manufacturer's protocol. Culture media was changed every three days. Cells were seeded to a density of 5×10^5 cells/ mL media and grown to 70 - 80% confluence, detached with 0.25% trypsin-EDTA, and then re-plated in fresh culture flasks.

2.3.2 HCT116 treatment with conditioned media from explanted fibroblasts

HCT116 cells were cultured in conditioned media (CM) from explanted fibroblasts cultures of FSPC*Cre-Pparb*^{d^{-/-}} or WT from APC^{+/+} background. Briefly, conditioned media from explanted fibroblasts were explanted from 3 mm mouse colonic tissues and cultured on a petridish with 10% FBS-DMEM. Tissue explants were removed and attached fibroblasts were cultured for 48 hours in serum-free DMEM before the CM were harvested. CM of explanted fibroblasts from FSPC*Cre-Pparb*^{d^{-/-}} and *Pparb*^{d^{fl/fl}} mice were denoted as CM- FSPC*Cre-Pparb*^{d^{-/-}} and CM-WT, respectively. HCT116 cells were seeded at a density of 2×10^4 cells/ mL in 24-well transwell plate (Corning, USA) and incubated overnight. Subsequently, HCT116 cells were cultured for 24 hours of either CM- FSPC*Cre-Pparb*^{d^{-/-}} or CM-WT, denoted as HCT116^{CM-FSPC*Cre-Pparb*^{d^{-/-}}} and HCT116^{CM-WT}. Untreated HCT116 in serum-free DMEM, denoted as HCT116^{Untreated}, acted as a control.

2.3.3 Silencing of PPAR β/δ expression in fibroblasts by siRNA

Transient suppression of PPAR β/δ expression in fibroblasts was performed in 24-well culture plates using 2.5 μ L of 5 μ M ON-TARGETplus SMARTpool PPAR β/δ (Dharmacon, USA; J-003435-06, J-003435-07, J-003435-08, J-003435-09) and transfected using 0.5 μ L of DharmaFECT1 reagent diluted in 49.5 μ L of serum-free media (Dharmacon, USA), as per manufacturer's instructions. The sequences of PPAR β/δ siRNAs are listed in Table 2. BJ1 and CCD18co were seeded with an equal seeding density of 2×10^4 cells/mL and incubated overnight. Subsequently, cell cultures were treated with siRNA at least 24 hours before co-culture with HCT116. The siRNA-mediated knockdown of PPAR β/δ (siPPAR β/δ) in BJ1 and CCD18co were designated as BJ1-siPPAR β/δ and CCD18co-siPPAR β/δ , respectively. The DharmaFECT1 vehicle-treated BJ1 and CCD18co cells were used as controls and denoted as BJ1-CTRL and CCD18co-CTRL, respectively.

2.3.4 Transwell co-culture

HCT116 (bottom chamber of transwell plate) were co-cultured with either BJ1 or CCD18co in the top chamber of a transwell 0.4 μ m pore size membrane insert (Corning, USA). HCT116 cells were seeded with a cell density of 2×10^4 cells/mL in a 24-well transwell plate and incubated overnight. Subsequently HCT116 cells were co-cultured for 48 hours in 1 mL serum-free DMEM with either BJ1-siPPAR β/δ or CCD18co-siPPAR β/δ , denoted as HCT116^{BJ1-siPPAR β/δ} and HCT116^{BJ1-siPPAR β/δ} , respectively. HCT116 cells treated with BJ-CTRL or CCD18co-CTRL were denoted as HCT116^{BJ1-CTRL} or HCT116^{CCD18co-CTRL}, respectively.

2.3.5 Treatment with H₂O₂ and N-acetyl cysteine (NAC)

We examined the effect of direct H₂O₂ stimulation and subsequent sequestering by NAC. HCT116 and ICEC cells were seeded with a cell density of 2×10^4 cells/mL in a 24-well transwell plate and allowed to attach overnight. The cells were treated with 1 μ M H₂O₂ with or without 5mM NAC (Sigma-Aldrich Co. LLC, USA) in 1mL of appropriate culture media.

2.4 Histology

2.4.1 Tissue preparation and embedding

Biopsies were first fixed for 16 h in 4% paraformaldehyde dissolved in PBS (Sigma-Aldrich Co. LLC, USA) at 4°C. Tissues were either processed for cryo-sectioning by snap-freeze in tissue freezing medium (Leica Microsystems, USA), or for paraffin-sectioning by embedding in paraffin wax (Leica Microsystems, USA). Tissues were sectioned to 8µm and 5µm thickness for cryopreserved and paraffin-embedded tissues respectively. The tissue sections were processed for subsequent histological or immunofluorescence staining.

2.4.2 Hematoxylin and eosin (HE) staining

Tissue sections were stained in Hematoxylin (Merck Millipore, USA) for 5 min, rinsed in 0.1% HCl solution then allowed to develop under running tap water for 5 min, followed by counter-staining with 0.5% eosin solution (Merck Millipore, USA) for 3 min. The sections were rinsed for 10 sec in tap water. Stained tissue sections were imaged using Nikon Eclipse 90i bright-field microscope using a Plan Fluor, 10x/0.30 objective and Qcapture Pro software version 5.0.1.26 (QImaging, Canada).

2.4.3 Laser capture microdissection (LCM) for FFPE tissue collection

For LCM, tissue sections were mounted onto MembraneSlide 1.0 PEN (Carl Zeiss, Germany) and histologically stained accordingly. Tissues were dissected using the PALM MicroBeam laser capture microdissection system (Carl Zeiss, Germany) and captured on CapSure Macro LCM Caps (Life Technologies, USA). After tissue collection, the LCM caps were capped onto 0.5 mL microfuge tubes, resuspended in the appropriate lysis buffer and processed for RNA extraction.

2.4.4 Antigen retrieval

Antigen retrieval of FFPE tissues was performed in sodium citrate buffer (10 mM, pH 6.0) using the 2100 Retriever (Aptum Biologics Ltd., U.K.). Briefly, glass slides containing tissue sections were placed in slide chambers containing 100 mL of buffer. Slide chambers are arranged in the 2100 Retriever body and secured before the initialization of antigen retrieval cycle. The antigen retrieval heating cycle runs for approximately 20 min and the machine is allowed to cool and depressurize for another 2 hours before the sample slides are retrieved.

2.4.5 Immuno-histocompatibility (IHC) staining

Tissue sections were first allowed to equilibrate at room temperature for 5 min and then subsequently blocked in 1% Bovine Serum Albumin (BSA; Sigma-Aldrich Co. LLC, USA) and probed with suitable antibodies as listed in Table 1. After each antibody incubation period, sections were washed 3X 5 min in PBS at RT.

For fluorescent staining, stained sections were mounted with Vectashield mounting media with DAPI (Vector Laboratories, USA) and imaged with LSM 710 confocal microscope (Carl Zeiss, Germany) using the Plan-Apochromat 20x/0.8 NA objective. Captured images were processed and analyzed in Zen 2012 Lite Edition software (Carl Zeiss, Germany).

For colorimetric staining, sections were further processed using Vectastain ABC HRP kit (Vector Laboratories, USA) and developed using DAB Peroxidase (HRP) Substrate Kit (Vector Laboratories, USA). Stained sections were imaged using Nikon Eclipse 90i bright-field microscope using a Plan Fluor, 10x/0.30 objective and Qcapture Pro software version 5.0.1.26 (QImaging, Canada).

2.4.5 Proliferation and Cell death assay

Colon tissues were fixed with 2% paraformaldehyde in 1X PBS for 2 h, washed twice with 1X PBS and embedded in Tissue-Tek OCT compound medium (Sakura Leica) overnight at 4 °C. Cryosections, 8- μ m thickness, of mouse colon tissue were generated using cryostat (Leica Biosystems, Germany) and analyzed for proliferation and cell death.

To identify apoptotic cells, in situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) 4',6-diamidino-2-phenylindole (DAPI) assay was performed using TUNEL cell death detection kit (Roche, Germany), as per manufacturer's protocol. Briefly, sections were permeabilized using a labeling solution (0.1% Triton X-100 in 10 mM Tris-HCL at pH 7.4 - 8.0) and enzymatic solution (10X diluted) and incubated at 37 °C for 1 h in the dark. Fluorescence images were then taken using LSM 710 confocal laser scanning microscope (Carl Zeiss, Germany) with Plan-Apochromat 10x/0.8 NA objective and analyzed on

Zen 2012 Lite Edition software (Carl Zeiss, Germany). Percentage of TUNEL-positive cells per microscopic field was quantified using ImageJ software.

To identify proliferating cells in the colon tissue sections, proliferating cell nuclear antigen (PCNA) staining was performed. Briefly, heat-antigen retrieval was performed in 10 mM sodium citrate buffer (pH, 6.0) for 30 min at 95 °C and allowed to cool for 30 min at room temperature. Endogenous peroxidase activity was then quenched by 30 min incubation with 3% H₂O₂ in distilled water. Sections were rinsed in 1X PBS for 5 min and incubated in blocking solution (3% neonatal goat serum in 0.05% Triton X) for 1 h and 30 min at room temperature. Following washing and blocking, the sections were probed with antibodies as described in Table 4.

Table 4: *Conditions for immunohistochemistry analysis*

Target Molecule	Primary Antibody	Secondary Antibody Dilution
PCNA	1:100 in blocking solution	1:200 in blocking solution
	15 hours	1 hour
	4 °C	60 °C

After secondary antibody incubation, sections were incubated with avidin–biotin complex (ABC) H₂O₂ reagent (Vector Laboratories, USA) for 45 min. Sections were washed three times for 5 min in 1X PBS after each antibody incubation period and avidin-biotin staining. Sections were counterstained with 2% hematoxylin (Merck Millipore, USA), dehydrated, mounted and imaged under bright-field (10x and 20x objectives) using EVOS XL Core microscope (Thermo Scientific, USA). Quantification of PCNA positive cells was performed using the Cell Counter Module in ImageJ software version 1.48 (National Institutes of Health, USA).

2.5 mRNA analyses

2.5.1 RNA isolation and cDNA amplification from microdissected FFPE tissue

Total RNA was extracted from microdissected FFPE tissue using the Ambion RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Life Technologies, USA), according to manufacturer's protocol. Briefly, tissues were resuspended in the kit digestion buffer and digested in RecoverALL Protease. Samples are transferred to an isolation additive/ethanol mixture and passed through a filter cartridge. Samples are washed 3X in the filter cartridge using provided wash buffers. On-cartridge nuclease digestion was then performed to remove unwanted DNA. Samples were washed another 3X with wash buffers from elution in nuclease-free water. The purity and quantity of the collected RNA were measured using Nanodrop ND1000 (Thermo scientific, USA).

The collected RNA was reverse-transcribed to cDNA in a 2-step synthesis and amplification reaction using the Full Spectrum Complete Transcriptome RNA Amplification kit (System Biosciences, USA), as per manufacturer's protocol. Briefly, approximately 100 ng of starting RNA was mixed with 2 μ L Universal Primer Mixer to a final volume of 5 μ L and incubated at 70°C for 4 min then 25°C for 5 min. Next, first-strand cDNA synthesis was performed using the kit's 2X master mix and incubated accordingly (42°C for 50 min, then 95°C for 5 min). Second strand cDNA synthesis and amplification was then performed in a PCR master mix containing 2 μ L Full spectrum PCR primer and polymerase. The second strand cDNA synthesis was performed using the suggested PCR protocol (95°C for 4 min, 68°C for 5 min, 58°C for 1 min, 68°C for 1.5 min, 13 cycles of 95°C 25 sec followed with 68°C for 2 min, and finally 68°C 2.5 min, then hold at 15°C).

2.5.2 RNA isolation and cDNA synthesis from intestinal tissue

Samples were lyzed using TRIzol Reagent (Invitrogen, USA) and total RNA was purified by e.Z.N.A Total RNA Kit (Omega Bio-Tek, USA). Briefly, 1 cm mouse intestinal biopsies were lyzed and homogenized in 1 mL TRIzol Reagent. Samples were incubated for 5 min to allow complete dissociation of the nucleoproteins complex before adding 0.2 mL of chloroform allowed to incubate for another 2 min. Sample mix was then centrifuged for 15 min at 12000G at 4°C. The aqueous phase containing the RNA is transferred into mini-columns

provided in the kit. Samples are digested on-column with DNase I, before washing 3X in provided wash buffers and elution in 40 µL of DEPC water. The purity and quantity of total RNA were then recorded using Nanodrop ND1000 (Thermo scientific, USA). Reverse transcription (RT) was performed using the qScript cDNA SuperMix (Quanta Biosciences, USA) on the iCycler thermal PCR System (Bio-Rad laboratories, USA).

2.5.3 Quantitative Real-time PCR (qRT-PCR)

Amplified cDNA samples were processed for qRT-PCR with KAPA SYBR FAST qPCR master mix (Kapa Biosystems, USA) and analyzed using C1000 Thermal Cycler with CFX96 Real-Time System module (Bio-Rad Laboratories, USA). Signal detection was accomplished with KAPA SYBR FAST qPCR master mix (Kapa Biosystems, USA). The primers and their respective sequences are found in Table 2.

2.6 Genomic DNA analysis

2.6.1 DNA isolation and tissue-specific genotyping using cryo-embedded tissue

Collected tissues were washed 3X in PBS and lysed for DNA using the KAPA Express Extract kit (Kapa Biosystems, USA) and the manufacturer's lysis protocol. Using the extracted DNA and appropriate genotyping primers, polymerase chain reaction (PCR) was performed with 2X KAPA2G Fast Genotyping Mix (Kapa Biosystems, USA) and the manufacturer's KAPA2G Robust PCR Protocol.

2.7 Flow cytometry analyses

2.7.1 Animal Tissue dissociation and staining

Fresh tissue biopsies were dissected from mice following end of treatment. Tissues were then resuspended in DMEM containing 1 mg/ml collagenase-3 and 40 U/mL DNase I and homogenized into single-cell suspensions using the gentleMACS Dissociator (Miltenyi, Biotec, USA).

Homogenates were then passed through a 70 µm nylon cell strainer and washed in PBS. For the blocking step, the cells were resuspended in PBS containing 3% FBS. Cells were then stained with the respective fluorophore-conjugated antibodies on ice, washed and resuspended in PBS. Flow cytometry was performed using Accuri C6 Flow Cytometer (BD Biosciences, USA) and analyses were completed on FlowJo v10.0.7 (FlowJo LLC, USA).

2.7.2 *In vitro* cell staining

Treated cells were detached with 0.25% Trypsin-EDTA and washed in PBS. Cell suspensions were blocked in PBS containing 3% FBS, then stained with the respective fluorophore-conjugated antibodies on ice. The stained cells were washed and resuspended in PBS for flow cytometric analyses in Accuri C6 Flow Cytometer (BD Biosciences, USA). Data analyses were performed on FlowJo v10.0.7 (FlowJo LLC, USA).

2.8 Protein extraction and western blot analysis

Samples were lysed in mammalian protein extraction reagent (M-PER; Thermo Fisher Scientific Inc., USA) supplemented with complete protease inhibitor mix (Roche Applied Science, Germany). For subcellular compartment protein extraction, samples were lysed using Bio Basic's membrane, nuclear and cytoplasmic protein extraction kit (Bio Basic Inc, Canada; #BSP002).

Protein extracts were normalized for equal concentration and resolved by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using Mini-PROTEAN II electrophoresis cell (Bio-Rad Laboratories, USA). Then, proteins were electroblotted to polyvinylidene difluoride (PVDF) membranes using Mini Trans-Blot Module (Bio-Rad Laboratories, USA).

Membranes were blocked in Rockland's Blocking Buffer (Rockland Immunochemicals, USA) for fluorescent-based western blotting for 1 h and incubated with primary antibodies overnight (~ 17 h) at 4°C. Subsequently, membranes were incubated with infrared-conjugated secondary antibodies (LI-COR Biosciences, USA) for 1 h at room temperature. Membranes were then visualized using ODYSSEY CLx Imager (LI-COR Biosciences, USA). Protein

bands were quantified using ImageJ software version 1.48 (National Institutes of Health, USA). Antibodies and conditions used are listed in Table 1.

2.9 Microarray analyses

2.9.1 Microarray preparation and hybridization

Total RNA was extracted from LCM-collected tissues using the Ambion RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Life Technologies, USA). The quantity and purity of the isolated RNA was measured using Nanodrop ND1000 (Thermo scientific, USA). The RNA samples were normalized for concentration and processed for array hybridization using the GeneChip® WT Pico Kit (Affymetrix, USA) and GeneAtlas® Hybridization, Wash, and Stain Kit (Affymetrix, USA). Briefly, cDNA synthesis and fragmentation was performed using GeneChip® WT Pico Kit (Affymetrix, USA). The fragmented cDNA was labelled and prepared for hybridization using GeneAtlas® Hybridization, Wash, and Stain Kit (Affymetrix, USA). The final hybridization mixes were loaded into Affymetrix Genechip Mouse Gene 1.0 ST Array gene chips (Affymetrix, USA) and incubated at 45°C for 18 h. After the hybridization mix was removed from the chips, the chips were washed at a GCOS fluidics station (Affymetrix, USA). Gene chips were loaded with 100 µL array holding buffer for scanning.

2.9.2 Gene expression analysis using Partek Genomics Suite

Array expression files (.CEL) were normalized by RMA and analyzed in the Partek Genomics Suite v6.6 (Partek Inc., USA). FSPCre-*Pparb/d*^{-/-} and *Pparb*^{fl/fl} samples were compared using multi-way ANOVA analysis in the Partek Genomics Suite.

2.9.3 Pathway analysis using Ingenuity Pathway Analysis

Gene expression data were filtered for fold-change and uploaded to Ingenuity Pathway Analysis software (Ingenuity Systems Inc., USA) for gene ontology (GO) analysis of gene subsets. The microarray protocols and data have been deposited in NCBI's Gene Expression Omnibus (GEO) database accession number GSE97473 (GSE97472 normal, GS97339 - tumor).

2.10 Reactive oxygen species analyses

2.10.1 Intracellular ROS reading using flow cytometry

To assess oxidative changes, flow cytometric analyses were performed for measurement of H₂O₂ levels. Production of H₂O₂ in cultured cells was measured using 5-&-6-carboxy-2', 7'dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; ThermoFisher Scientific, USA). Briefly, cells were incubated in 10 µM CM-H₂DCFDA at 37°C for 30 min. The oxidation of CM-H₂DCFDA to dichlorofluorescein (DCF) by H₂O₂ is representative of cellular ROS generated. Production of H₂O₂ in dissociated intestinal tissue cells was measured using CellROX Green oxidative stress detection kit (Thermo Fisher Scientific, USA), as per the manufacturer's protocol. Briefly, cells were incubated in 5 µM CellROX Green reagent for 30 min at 37°C, followed by 3X washing in PBS and fixation in 3.7% formaldehyde.

Flow cytometric readings were performed using Accuri C6F flow cytometer (BD Biosciences, USA) and analyzed with Accuri Cflow software (BD Biosciences, USA). A minimum of 5000 events was collected for each condition and all experiments were performed at least in triplicates. FlowJo software (Tree Star, USA) was used for downstream data analyses.

2.10.2 Whole animal tissue using AmplexRed H₂O₂ assay

Fresh mouse colon tissues were analysed for H₂O₂ levels using Amplex Red hydrogen peroxidase (H₂O₂) assay kit (Life Technologies, USA) as described by the manufacturer's protocol. Briefly, samples were homogenised and supernatants incubated with appropriate reagents in a 96-well microplate (Corning, USA), at 37 °C for 1 h. Fluorescence intensities of resorunfin, from oxidation of Amplex Red, were measured using Infinite M200 plate reader (Tecan, Germany) (571 nm Ex and 585 nm Em). A calibration curve was prepared using H₂O₂ in assay buffer (0 to 20 µM) and used to calculate H₂O₂ concentration of samples.

2.11 Cytokine detection by multiplex microbead immunoassay

Fresh colon biopsies were dissected and lysed in mammalian protein extraction reagent (M-PER; Thermo Fisher Scientific Inc., USA) supplemented with complete protease inhibitor mix (Roche Applied Science, Germany). Samples were sent to a multiplex immunoassay service (Discovery Assay; Eve Technologies, Canada). The multiplex immunoassays were completed on a mouse cytokine kit (EMD Millipore, USA) and readings were recorded on the BioPlex 200 system (Bio-Rad Laboratories, Inc., USA). The immunoassay included 31 cytokines, namely Eotaxin, G-CSF, GM-CSF, IFN γ , IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1 α , MIP-1 β , MIP-2, RANTES, TNF α , VEGF. The sensitivities of the assay ranged from 0.1 to 33.3 pg/mL.

2.12 Statistical analyses

Statistical analyses were performed using two-tailed non-parametric tests, either Mann-Whitney test (for 2 variables) or Kruskal-Wallis test (for > 2 variables). Statistical tests were performed using GraphPad Prism software (version 5; GraphPad Software Inc, USA), with p values of <0.05 considered significant. (*P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant.)

Chapter 3: Results

Chapter 3: Results:

3.1 Verification of PPAR β / δ deletion in colon stroma and the effect of stromal PPAR β / δ ablation on colitis severity

3.1.1 Background information

With the increasing prominence of microenvironmental effects driving cellular processes, there is a need to better understand the drivers that enable such effects. Consequently, as the most abundant cell type in the microenvironment, fibroblasts have emerged as potential targets in therapies (Hanley et al., 2018; Li et al., 2016; Zhou et al., 2015). Fibroblasts have been implicated in a multitude of roles in regulating the tissue microenvironment, including extracellular matrix (ECM) deposition, regulating epithelial cell differentiation, immune modulation in the tissue and regulating tissue homeostasis (Tomasek et al., 2002). During tumor development, tumor epithelial cells make use of fibroblasts and their various functions to facilitate tumor development, and thus research has focused on targeting cancer-associated fibroblasts as a cancer therapy (Kalluri and Zeisberg, 2006).

The use of cellular markers allows for the characterization and targeting of specific cellular populations in vivo. While fibroblasts express a variety of markers, fibroblast-specific protein-1 (FSP-1) is the most effective as a selective marker of fibroblasts (Strutz et al., 1995). While FSP-1 was originally identified as fibroblast cell marker that was first detected in mesenchymal cells post-gastrulation (Strutz et al., 1995), later studies discovered that FSP-1 also plays a role in epithelial-mesenchymal transition (EMT) and subsequently metastasis (Boye and Mælandsmo, 2010; Helfman et al., 2005). FSP-1 has also been found to be expressed preferentially by cells in the tumor stroma, but not in tumor epithelial cells of human breast cancer (Cabezón et al., 2007).

Technological advancements in science have allowed scientists to create cell-type specific gene expression ablation in mouse models. To characterize the functions of fibroblast cell proteins in vivo, researchers have employed FSP1-promoter-driven targeted gene modifications in mice. Trimboli et al. first adopted

Fsp1 Cre-driven loxP gene deletion, in their study of tumor stroma-specific effects in a murine breast cancer model (Trimboli et al., 2008; 2009). Subsequently, using the same technique, studies of the stroma population employed the ablation of various genes in fibroblasts (Bhowmick et al., 2004; Cheng et al., 2005; Li et al., 2008). Thus, Fsp 1 Cre-driven loxP gene deletion presents a way to study stromal-specific deletions in cancer. To study the role of fibroblast PPAR β/δ in a murine model, we have used Cre-lox technology (Maddison and Clark, 2005). To perform targeted gene deletion of PPAR β/δ , loxP sites were engineered to flank the exon 4 sequence in the PPAR β/δ gene in the same direction (Sng et al., 2018). Here we propose a FSP-1 promoter-driven approach to study the effects of stroma cell-derived PPAR β/δ signals on normal and malignant intestines. We generated transgenic mice whose exon 4 only of PPAR β/δ gene was selectively deleted in fibroblasts by crossing *Pparb/d^{fl/fl}* with Fsp1 Cre mice, herein called FSPCre-*Pparb/d^{-/-}* mice.

Inflammation is mostly positively associated with cancer progression, with CRC being no exception. Crohn's disease and ulcerative colitis, which are collectively termed inflammatory bowel disease (IBD), have been shown to be important risk factors associated with CRC development (Jess et al., 2012; Kim and Chang, 2014). PPAR β/δ is thought to stimulate a variety of cells and soluble factors that contribute to the pro-tumorigenic stimulation of inflammatory pathways in the colon (Wang et al., 2014; Peters et al., 2008; Foreman et al., 2009). Studies have detailed the role of PPAR β/δ in colonic epithelial cells towards CRC development and colitis severity (Zuo et al., 2009; 2014). Although there is growing awareness of the role of stromal components during tumorigenesis, the precise mechanisms and roles of stromal PPAR β/δ in colorectal tumorigenesis and inflammation have not been fully explored. In this chapter, we sought to explore this aspect using our novel FSPCre-*Pparb/d^{-/-}* transgenic mouse model.

Well-established animal models for ulcerative colitis and inflammatory bowel syndrome have used DSS to induce colitis in mice (Chassaing et al., 2014). Dextran sodium sulfate (DSS) is a water soluble chemical colitogen that is used to induce colitis in mice. DSS is a negatively charged sulfated polysaccharide and is available in a variety of molecular weights ranging from 5

to 1400 kDa, with severe colitis resulting from the administration of 40-50kDa DSS in drinking water (Okayasu et al., 1990). To examine whether stromal PPAR β/δ ablation affects experimental-induced colitis in mice, we employed DSS to induce intestinal inflammation in our experimental murine model.

3.1.2 Results

3.1.2.1 PPAR β/δ expression is ablated in stromal cells of the colon

We have previously generated a mouse carrying a FSP1-promoter driven deletion of PPAR β/δ exon 4, which encodes for the DNA-binding domain (Sng et al., 2018). To confirm that this deletion is also found in the colon, we extracted the different colonic tissue layers using microscopy-directed laser capture microdissection (LCM) (Figure 3.1A). The extracted tissues were genotyped for the PPAR β/δ exonic deletion (Figure 3.1B). Our genotyping results confirmed that the PPAR β/δ deletion is only found in the lamina propria layer of the colon and is absent in the mucosal villi and smooth muscular layers. This result agrees with our observations for explanted colon fibroblasts (Figure 3.1C). We observed a reduction in the PPAR β/δ mRNA transcript and protein levels in the FSPCre-*Pparb/d*^{-/-} mice compared with their *Pparb/d*^{fl/fl} littermates (Figure 3.1D-E).

Figure 3.1 PPAR β/δ is deleted in the stromal tissue of FSPCre-*Pparb/d*^{-/-} mice.

(A) Depicts representative H&E stained images of colons before and after laser capture microdissection of colonic layers, mucosal, lamina propria and muscularis. Scale: 500

μm. **(B)** Schematic diagram depicting the relative position of PCR genotyping primers (red, blue, green) for floxed and deleted PPARβ/δ exon 4 alleles, at 400 bp and 490 bp respectively (top). DNA gel image showing genotyping results indicated tissue layers (colonic submucosa and lamina propria layer (L.p.); mucosal villi (Vil.); smooth muscle (Mus.) layers). **(C)** Representative images showing the outgrowth of fibroblasts from colonic explants of *Pparb/d^{fl/fl}* and *FSPCre-Pparb/d^{-/-}* mice after 48 h and 96 h. Scale bar: 500μm. **(D-E)** Relative fold change in expression of PPARβ/δ mRNA (D) and PPARβ/δ protein (E) in migrated colonic fibroblasts. β-tubulin that served as housekeeping protein was from the same samples. Data are represented as mean ± S.E.M. from at least four independent experiments (n=4).

3.1.2.2 Ablation of PPARβ/δ expression in stromal cells results in greater gut mucosal thickness in mice and increased proliferation of colonic epithelial cells.

After confirming the presence of the PPARβ/δ deletion in the colon of *FSPCre-Pparb/d^{-/-}* mice, we wanted to investigate if the deletion causes any gross morphological changes in the gut of the transgenic mice. Hence, we measured the thickness of the villus, muscularis interna and muscularis externa layers of the stomach, small intestines and large intestines (Figure 3.2A). In all the measured tissues, we found that the mucosal layer of *FSPCre-Pparb/d^{-/-}* mice was significantly thicker than its *Pparb/d^{fl/fl}* counterparts (Figure 3.2B-G). In addition, we also observed thicker muscularis layers in the stomach, jejunum, ileum and colon. We hypothesized that this increased mucosal layer thickness might be attributed to increased proliferation in colonic epithelial cells. Thus, we performed flow cytometry to investigate the number of Lgr5+ crypt stem cells in the intestines (Figure 3.3A-C). We found a significantly higher number of Lgr5+ cell in *FSPCre-Pparb/d^{-/-}* colons but not in the ileum (Figure 3.3C). Using PCNA staining to identify proliferative cells in the colon, we also found that *FSPCre-Pparb/d^{-/-}* colons contained more PCNA+ cells in the mucosal layers, compared with their *Pparb/d^{fl/fl}* littermates (Figure 3.3D-E). We did not observe any differences in total leukocyte numbers (CD45+) in the colons (Figure 3.3F-G).

Figure 3.2 Thickening of intestinal epithelial layer is prominent in FSPCre-*Pparb/d*^{-/-} mice.

(A) Representative images of the stomach, small intestines and large intestines from *Pparb/d*^{fl/fl} and FSPCre-*Pparb/d*^{-/-} mice. Scale: 100 μ m. (B-D) Bar graphs depicting layer thickness of epithelial and muscularis layer of *Pparb/d*^{fl/fl} (n = 6) and FSPCre-*Pparb/d*^{-/-} (n = 6) mouse gut, namely (B) Fore stomach, (C) Glandular stomach, (D) Duodenum, (E) Jejunum, (F) Ileum and (G) Colon. Values are mean $\pm$ S.E.M.

Figure 3.3 FSPCre-*Pparb*/*d*^{-/-} colons have increased LGR5 and PCNA staining in the colons

(A - B) FACS analysis of LGR5 fluorescence stained in epithelial cells (EpCAM+) of (A) Ileum and (B) Colons from FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice. (C) Bar graph depicting the percentage of LGR5+ cells in ileum and colons of FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice (n=4) as reported in (A-B). Values are mean $\pm$ S.E.M. (D) Representative IHC images of colon tissues from FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice stained with Anti-PCNA antibody and counterstained with hematoxylin. Scale: 100 μ m. (E) Bar graph depicting the percentage of PCNA+ cells in the colonic epithelial mucosa of FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice (n=10) as reported in (D). Values are mean $\pm$ S.E.M. (F) FACS analysis of CD45+ cells in colons from FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice. (G) Bar graph depicting the percentage of leukocytes (CD45+) in colons of FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice as reported in (F). Values are mean $\pm$ S.E.M. (n=6).

*3.1.2.3 The FSPCre-*Pparb*/*d*^{-/-} background confers protection against experimentally-induced colitis*

While we observed obvious morphological differences between the FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} intestines, we were also interested in investigating whether the increased proliferation in the intestinal mucosa translated to any significant differences during colitis challenge by DSS treatment. We challenged the mice to induce colitis by feeding them 2% DSS in their drinking water for 5 days (Figure 3.4A). During the course of treatment, we scored the mice for changes in gait, stool consistency and presence of bleeding as stated in Chapter 2 (Figure 3.4B). We observed that the FSPCre-*Pparb*/*d*^{-/-} background had a lower disease activity index (DAI) compared with *Pparb*/*d*^{fl/fl}, suggesting a protective effect from stromal PPAR β/δ deletion (Figure 3.4C). While DSS treatment caused a slight reduction in mouse weights, we did not observe a difference in weight between the two genotypes during the course of treatment (Figure 3.4D).

Figure 3.4 FSPCre-*Pparb/d^{-/-}* mice have lower severity scores during DSS-treatment (A) Schematic of DSS-induced colitis treatment programme. (B) Representative image of rectal bleeding observed during DSS-treatment. (C) Mean DAI score of DSS-treated mice over 1 week (n=5). (D) Mean mouse weight of DSS-treated mice over the span of 1 week (n=5). Data are represented as mean $\pm$ S.E.M.

To perform histological analyses, we dissected the mice for colon biopsies (Figure 3.5A). As expected, the mice suffered colon shortening after DSS treatment (Chassaing et al., 2014), but we did not observe any differences between genotypes (Figure 3.5B). Microscopically, we observed no significant changes in the villus number and crypt depth in the FSPCre-*Pparb/d^{-/-}* mice, while the *Pparb/d^{fl/fl}* rodents suffered severe villus loss and crypt damage (Figure 3.5C-D). Colons were stained with TUNEL to observe apoptotic cells (Figure 3.5E). In agreement with the observed reduction of villus damage in FSPCre-*Pparb/d^{-/-}* mice, there were also fewer apoptotic cells compared with *Pparb/d^{fl/fl}* colons, indicative of a protective effect against apoptosis during DSS treatment (Figure 3.5F).

Figure 3.5 Mice with the FSPCre-*Pparb/d^{-/-}* background are more resistant to DSS-induced villus damage

(A) Representative images of colons from vehicle- and DSS-treated *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{-/-}* mice. (B) Dot plot illustrating the length of colons from *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{-/-}* mice after vehicle or DSS treatment. (C) Representative H&E stained images of ileum and colon tissues from FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice treated with vehicle or DSS. Scale: 100 μ m. (D) Bar graph depicting the mean villus number of mouse ileum recorded (n=8). Data are represented as mean $\pm$ S.E.M. (E) Representative images of colons from vehicle- and DSS-treated *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{-/-}* mice stained with TUNEL apoptotic stain (green) and counterstained with DAPI (blue). Scale: 100 μ m. (F) Bar graph illustrating the percentage of apoptotic cells in the colons of vehicle- and DSS-treated *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{-/-}* mice. Data are represented as mean $\pm$ S.E.M.

3.1.2.4 FSPCre-Pparb/d^{-/-} colons exhibit a more anti-inflammatory cellular and cytokine profile compared with their Pparb/d^{fl/fl} littermates

During inflammation, immune cells infiltrate the site of tissue damage, secreting various factors that recruit other inflammatory cells and initiate processes to clear up the damage site. To examine any changes in the soluble factors present in the tissue microenvironment, we performed a multiplex cytokine ELISA array of 31 common cytokines involved in inflammatory processes (Table 5). Comparatively, the FSPCre-*Pparb/d^{-/-}* colons had a milieu of increased levels of anti-inflammatory cytokines and reduced levels of pro-inflammatory signals, supporting the observation of an anti-inflammatory profile in the tissue microenvironment of FSPCre-*Pparb/d^{-/-}* mice. Next, we investigated if the difference in the cytokine profile was due to changes in the inflammatory cell make-up in the tissue. Thus, we performed flow cytometry analyses of mouse colon biopsies. In our analyses, we did not observe any differences between the percentage of infiltrating monocytic (CD11b+) cells in colons (Fig. 3.6A & B). However, the multiplex immunoassay analysis highlighted differences in several cytokines, suggesting potential differences in the relative abundances of neutrophils, T-cells and macrophages between FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* colons, and prompting our further investigation into cell type specific analysis. Although we did not find any significant differences in the percentage of infiltrating neutrophils (Ly6G+CD11b+) and T lymphocytes (CD3+) between FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* colons (Figure 3.6C-D), we noted that the vehicle-treated FSPCre-*Pparb/d^{-/-}* colons had a higher percentage of infiltrated macrophages (F4/80+CD11b+) than *Pparb/d^{fl/fl}*, which was abrogated by the DSS treatment (Figure 3.6E-F). We further characterized macrophage subtypes and found that vehicle-treated FSPCre-*Pparb/d^{-/-}* colons had more anti-inflammatory M2 macrophages, but more M1 macrophages were observed upon DSS treatment (Figure 3.6G-H). The lower M1:M2 ratio in FSPCre-*Pparb/d^{-/-}* colons is in agreement with the reduced inflammatory cytokine landscape.

Table 5: Cytokine levels in colon extracts of FSPCre-Pparb/d^{-/-} mice vs Pparb/d^{fl/fl} controls

Cytokine in colon extracts (pg /mg protein $\pm$ s.e.m.)		
Cytokine	<i>Pparb/d^{fl/fl}</i>	<i>FSP-Pparb/d^{-/-}</i>
Eotaxin	1.345 $\pm$ 0.195	1.361 $\pm$ 0.273
G-CSF	0.03 $\pm$ 0.01	0.026 $\pm$ 0.005
GM-CSF	0.013 $\pm$ 0.005	0.003 $\pm$ 0.003
IFNγ	0.004 $\pm$ 0.002	0.002 $\pm$ 0.002
IL-1a	0.248 $\pm$ 0.036	0.346 $\pm$ 0.112
IL-1B	0.022 $\pm$ 0.007	0.035 $\pm$ 0.009
IL-2	0.048 $\pm$ 0.008	0.05 $\pm$ 0.016
IL-3	0.001 $\pm$ 0.001	0.002 $\pm$ 0.001
IL-4	0.001 $\pm$ 0.001	0.002 $\pm$ 0.001
IL-5	0.023 $\pm$ 0.005	0.01 $\pm$ 0.004
IL-6	0.006 $\pm$ 0.002	0.01 $\pm$ 0.001
IL-7	0.073 $\pm$ 0.007	0.051 $\pm$ 0.01
IL-9	0.124 $\pm$ 0.013	0.279 $\pm$ 0.083
IL-10	0.018 $\pm$ 0.002	0.044 $\pm$ 0.013
IL-12 (p40)	0.012 $\pm$ 0.005	0.01 $\pm$ 0.003
IL-12 (p70)	0.027 $\pm$ 0.007	0.012 $\pm$ 0.006
IL-13	0.004 $\pm$ 0.001	0.002 $\pm$ 0.001
IL-15	0.196 $\pm$ 0.035	0.22 $\pm$ 0.076
IL-17	0.01 $\pm$ 0.003	0.007 $\pm$ 0.002
IP-10	0.443 $\pm$ 0.07	0.115 $\pm$ 0.026
KC	0.104 $\pm$ 0.034	0.21 $\pm$ 0.047
LIF	0.019 $\pm$ 0.001	0.029 $\pm$ 0.003
LIX	0.028 $\pm$ 0.022	0.006 $\pm$ 0.006
MCP-1	0.09 $\pm$ 0.019	0.074 $\pm$ 0.017
M-CSF	0.015 $\pm$ 0.004	0.036 $\pm$ 0.017
MIG	2.282 $\pm$ 1.16	0.694 $\pm$ 0.415
MIP-1a	0.203 $\pm$ 0.022	0.034 $\pm$ 0.013
MIP-1B	0.368 $\pm$ 0.127	0.051 $\pm$ 0.032
MIP-2	0.399 $\pm$ 0.055	0.63 $\pm$ 0.177
RANTES	1.883 $\pm$ 0.659	0.116 $\pm$ 0.045
TNFa	0.02 $\pm$ 0.004	0.012 $\pm$ 0.002
VEGF	0.047 $\pm$ 0.006	0.136 $\pm$ 0.042

Highlighted **Bold** - indicates difference above 2-fold.

Figure 3.6 Macrophage, Neutrophil and T-cell profiles of FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} colons.

(A) FACS analyses of CD11b fluorescence of Vehicle control and DSS-treated colons from FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice. Values are mean $\pm$ S.E.M. (n=4). (B-D) Bar graph depicting the percentage of (B) monocytic cells (CD11b+), (C) neutrophils (CD11b+ Ly6G+) and (D) T cells (CD3+) in vehicle control and DSS-treated colons of FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice (n=4) as reported in (A). (E) FACS analyses of F/480 fluorescence of Vehicle control and DSS-treated colons from FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice. Values are mean $\pm$ S.E.M. (n=4). (F) Bar graph depicting the percentage of macrophages (F/480+) in vehicle control and DSS-treated colons of FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice (n=4) as reported in (E). (G) Representative FACS analyses of macrophage subtypes M1 (MHCII+) and M2 (MHCII- CD206+) of Vehicle control colons from FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice. Values are mean $\pm$ S.E.M. (n=4). (H) Bar graph depicting the M1/M2 ratio of macrophagea in vehicle control and DSS-treated colons of FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice (n=4) as reported in (G).

3.1.3 Summary

There has been a growing interest in the role of the tissue microenvironment in tumor development. While research has shown various roles for PPAR β/δ in pro-tumorigenic and anti-tumorigenic functions in the gut epithelium, little work has been done in terms of stromal PPAR β/δ functions. Hence, we sought to address this issue using our transgenic mice carrying a stromal-specific ablation of PPAR β/δ , FSPCre-*Pparb/d*^{-/-}. We examined whether the intestinal stromal cells carried the PPAR β/δ exon 4 deletion by DNA genotyping, RNA transcript expression analysis and western analysis. Our results confirmed that PPAR β/δ was reduced in the stromal cells and confirms the validity of the model for studying the role of intestinal stromal PPAR β/δ . With the model validated, we sought to discover whether the genetic ablation caused any physiological changes in gross mouse intestinal morphology. To obtain quantifiable parameters, we measured the thickness of the villus and muscularis layers of the different sections of the mouse gut, comparing FSPCre-*Pparb/d*^{-/-} mice to their *Pparb/d*^{fl/fl} littermates. In general, across all of the measured tissues we noticed that the FSPCre-*Pparb/d*^{-/-} gut had thicker villus mucosal layers than the *Pparb/d*^{fl/fl} littermates. To investigate if this phenotype was due to the increased proliferative ability of intestinal epithelial cells in the FSPCre-*Pparb/d*^{-/-} gut, we examined two different markers of proliferative capacity, namely the number of intestinal stem cells and the presence of PCNA staining in the intestinal mucosa. Our flow cytometric analyses indicate an increased number of LGR5+ stem cells in the FSPCre-*Pparb/d*^{-/-} colon, indicative of an enhanced proliferative and self-renewal capacity in the FSPCre-*Pparb/d*^{-/-} colons. This observation was supported by an increase in PCNA IHC staining in FSPCre-*Pparb/d*^{-/-} colon mucosa.

Inflammatory bowel disorders have important implications for colorectal cancer risk. Experimentally-induced colitis by DSS is commonly used in rodents to model human inflammatory bowel disorders. We challenged our FSPCre-*Pparb/d*^{-/-} mice to a 1-week 2% DSS regime, to investigate if the mice responded differently to experimentally-induced colitis. In general, the FSPCre-*Pparb/d*^{-/-} mice were more resistant to the DSS treatment, displaying fewer colitis-induced physiological effects and little or no change in the intestinal mucosal

morphology. Moreover, their *Pparb/d^{fl/fl}* littermates had visible changes in gait and physiology, together with extensive intestinal mucosal damage. We also noted fewer apoptotic cells in the FSPCre-*Pparb/d^{-/-}* colons after DSS-treatment, compared to their *Pparb/d^{fl/fl}* counterparts, again indicating the general retention of normal intestinal physiology post DSS-treatment. During inflammation, inflammatory cells infiltrate the tissue site, releasing a cytokine storm and resulting in much of the observed tissue damage (Matricon et al., 2010; Clark, 2007). To assess whether the FSPCre-*Pparb/d^{-/-}* background resulted in any changes in cytokine involvement, we performed a multiplex ELISA array of 31 common cytokines involved in inflammation and flow cytometric analyses of FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* colons. We found that the colons with the FSPCre-*Pparb/d^{-/-}* background contained higher concentrations of anti-inflammatory cytokines and reduced pro-inflammatory signals, suggesting an anti-inflammatory profile in the tissue microenvironment of FSPCre-*Pparb/d^{-/-}* mice. Notably, we detected significant changes in the cytokines responsible for T-lymphocyte (RANTES, MIG), macrophage (GM-CSF, MIP-1a, MIP-1b) and neutrophil (LIX) recruitment and response. To further examine this finding, we performed flow cytometric analyses to determine whether the observed cytokine profile was reflected in changes in T-lymphocyte, macrophage or neutrophil infiltration during DSS-induced colitis. As expected, immune cell infiltration was increased in the DSS-treated mice due to inflammation. However, we did not notice any significant differences in the monocyte, neutrophil or T-lymphocyte infiltration between FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice, in either vehicle or DSS-treatments. While there were no significant differences in FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* macrophage infiltration during DSS treatment, and there were more macrophages in FSPCre-*Pparb/d^{-/-}* colons with vehicle treatment. Deeper analysis of macrophage subtypes then revealed that the increased macrophages in FSPCre-*Pparb/d^{-/-}* colons correspond to the anti-inflammatory M2 subtype.

In conclusion, we presented our transgenic FSPCre-*Pparb/d^{-/-}* mouse model for stromal-specific deletion of PPAR β/δ expression. This deletion was present in the intestinal tissues, resulting in mucosal villus layer thickening, possibly due to increased epithelial cell proliferation, highlighting the role of

stromal PPAR β/δ in regulating intestinal cell proliferation. The loss of stromal PPAR β/δ expression also conferred FSPCre-*Pparb/d*^{-/-} transgenic mice with resistance against DSS-induced deleterious effects in the intestinal mucosa. A more detailed analysis of the cytokine and immune cell profiles of the transgenic mice showed that the FSPCre-*Pparb/d*^{-/-} background resulted in an anti-inflammatory phenotype in the gut epithelium. Taken together, using our transgenic mouse carrying a stromal PPAR β/δ deletion, we have demonstrated that PPAR β/δ exhibits roles in the regulation of mucosal epithelial cell proliferation and the modulation of inflammatory signals in the tissue microenvironment.

3.2 Effect of stromal PPAR β/δ ablation on colorectal cancer development

3.2.1 Background information

We generated a novel transgenic mouse harboring a stromal-specific PPAR β/δ ablation (FSPCre-*Pparb/d*^{-/-}). These transgenic mice had a thicker gut mucosa, possibly due to increased proliferative activity of the intestinal stem cells. Mice with the FSPCre-*Pparb/d*^{-/-} background are also protected against colitis-mediated intestinal damage caused by DSS-treatment, compared with their *Pparb/d*^{fl/fl} littermates. A closer examination of the immune cell populations and cytokine profiles, revealed an anti-inflammatory phenotype of the FSPCre-*Pparb/d*^{-/-} colons prior to DSS treatment, possibly contributing to the reduction in colitis-induced mucosal damage. As inflammation is usually associated with pro-tumorigenic outcomes in CRC, an anti-inflammatory phenotype suggests that the FSPCre-*Pparb/d*^{-/-} background may confer a degree of protection against colorectal tumorigenesis (Colins et al., 1987.; Gilat et al., 1988). There has been supporting evidence showing that the deletion of PPAR β/δ in CRC mouse models results in fewer intestinal polyps while conversely, treatment with the PPAR β/δ agonist GW501516 increased polyp numbers and size (Gupta et al., 2004; Wang et al., 2006). However, PPAR β/δ has also been shown to exert an anti-tumorigenic effect, proposed to be through the induction of colonocyte differentiation and enhancement of apoptosis (Reed et al., 2004; Harman et al., 2004; Marin et al., 2006). While the classical transgenic ablation mouse model has been studied in detail for PPAR β/δ , a stroma-specific deletion model of PPAR β/δ allows the understanding of the contribution of stroma PPAR β/δ in colorectal tumorigenesis. In this study, we attempted to tackle this problem using our mouse model carrying a fibroblast-specific protein (FSP)-1-promoter specific deletion of PPAR β/δ expression.

Several mouse models have been developed to model human colorectal cancer in transgenic mice (Karim and Huso., 2013). Two main models have been commonly used for CRC studies, namely APC^{min/+} mice to model human familial adenomatous polyposis (FAP) and AOM/DSS treatment to model colitis-associated CRC. APC^{min/+} mice harbor a Min (multiple intestinal neoplasia) mutant allele for the murine adenomatous polyposis coli (Apc) locus. Mice on

the APC^{min/+} background are predisposed to adenomatous polyp formation in the intestine, similarly to humans with germline mutations in the APC gene (Moser et al., 1990). To mimic human colitis-associated CRC (CAC) in a mouse model, the AOM/DSS treatment model was developed (Tanaka et al., 2003; Tanaka, 2012). Tanaka et al. found that a single intraperitoneal injection of the colonic genotoxic carcinogen AOM (10 mg/kg body weight) and followed by treatment with 2% colitis-inducing agent DSS in the drinking water was sufficient to produce colorectal adenomas and adenocarcinomas within 4 weeks of AOM administration (Tanaka et al., 2003). Studies have also reported that the combination of the APC^{min/+} model with DSS treatment results in tumor growth 4 weeks after DSS treatment (Tanaka, 2006). To assess the role of stromal PPAR β/δ in colorectal carcinogenesis, we studied APC^{min/+} mice and, AOM/DSS and APC/DSS treatments to model CRC *in vivo*.

3.2.2 Results

3.2.2.1 *The FSPCre-Pparb/d^{-/-} background confers increased disease survival and reduced symptoms associated with disease severity in murine CRC.*

To obtain APC^{min/+} mice on the FSPCre-Pparb/d^{-/-} background, APC^{min/+} mice from The Jackson's laboratory were crossed with Pparb/d^{fl/fl} and then backcrossed with FSPCre-Pparb/d^{-/-} over several generations (Figure 3.7A). To model CAC, the mice were injected with AOM (i.p. 10 mg / kg bw) and then fed with 2% DSS in drinking water for a week (Figure 3.7B). As a combinatorial treatment, we fed APC^{min/+} mice 2% DSS in the drinking water for 1 week (Figure 3.7C). Across all 3 models, we found that mice on the FSPCre-Pparb/d^{-/-} background were less prone to CRC complications and had a longer survival compared with their Pparb/d^{fl/fl} counterparts. To assess the well-being of the DSS-treated mice, we scored their fecal consistency, presence of fecal bleeding and physical gait as a disease activity index (DAI) score. While there was little or no difference between FSPCre-Pparb/d^{-/-} and Pparb/d^{fl/fl} mice during DSS treatment, mice on a FSPCre-Pparb/d^{-/-} background maintained a lower DAI score after returning to normal drinking water, across both AOM/DSS and APC/DSS treatments (Figure 3.7D and E). Across all three tested models of CRC, the FSPCre-Pparb/d^{-/-} background confers a survival advantage compared with Pparb/d^{fl/fl} mice (Figure 3.7F-H).

Figure 3.7 Treatment course, disease severity graphs and survival plots of CRC disease.

(A) Left: Breeding plan depicting the crossing of $APC^{min/+}$ mice to $Pparb/d^{fl/fl}$ genetic background, and subsequently to mice carrying the Fsp1 promoter-driven Cre recombinase to produce $APC^{min/+}$ mice carrying the stromal-specific PPAR β/δ deletion ($APC^{min/+}$ FSPCre- $Pparb/d^{-/-}$). Right: Representative genotyping results for the transgenic mice. (B) Schematic of AOM/DSS treatment. Mice of 6 to 8 weeks old were injected with 10 mg/kg AOM (i.p.) at the start of the experiment and 1 week after, mice were subjected to 2% DSS in drinking water for 1 week. Mice were then returned to normal drinking water until the end of the experiment. (C) Schematic of DSS treatment for $APC^{min/+}$ mice (APC/DSS). Mice were given 2% DSS in drinking water for 1 week and then returned to normal drinking water until the end of the experiment. (D-E) Mean DAI score of DSS-treated mice over 5 weeks. (D) AOM/DSS treatment of FSPCre- $Pparb/d^{-/-}$ mice (n=13) compared with $Pparb/d^{fl/fl}$ littermates (n=18) (E) DSS treatment of $APC^{min/+}$ FSPCre- $Pparb/d^{-/-}$ mice (n=14) compared with $APC^{min/+}$ $Pparb/d^{fl/fl}$ littermates (n=13). Data are represented as mean $\pm$ S.E.M. (F-H) Percentage survival charts of (F) $APC^{min/+}$ FSPCre- $Pparb/d^{-/-}$ (n=14) and $APC^{min/+}$ $Pparb/d^{fl/fl}$ mice (n=15) over 25 weeks, (G) FSPCre- $Pparb/d^{-/-}$ mice (n=13) and $Pparb/d^{fl/fl}$ treated with AOM/DSS, or (H) $APC^{min/+}$ FSPCre- $Pparb/d^{-/-}$ (n=13) and $APC^{min/+}$ $Pparb/d^{fl/fl}$ mice (n=14) treated with DSS.

3.2.2.2 Mice on a *FSPCre-Pparb/d^{-/-}* background have decreased CRC

tumorigenesis

Mice on the *FSPCre-Pparb/d^{-/-}* background were shown to be protected from disease outcomes associated with CRC, namely longer survival and less disease burden. Hence, we were also interested to determine if the observed reduction in disease burden and survival was due to reduced tumorigenesis in the *FSPCre-Pparb/d^{-/-}* mice. Mouse colon biopsies were dissected longitudinally and observed for tumors under a dissecting microscope (Figure 3.8A). To perform tumor size comparisons, we classified the tumors by diameter as small (<2mm), intermediate (2-4mm) and large (>4mm). Across all three models, we found that *FSPCre-Pparb/d^{-/-}* mice had a significantly fewer tumors in total and significantly fewer large tumors (Figure 3.8B-G). Additionally, in 25-week old *APC^{min/+}* mice, fewer tumors of the intermediate size category was also recorded in *FSPCre-Pparb/d^{-/-}* mice (Figure 3.8C). The reduction of tumor size in *FSPCre-Pparb/d^{-/-}* colons was also apparent microscopically (Figure 3.8H).

Figure 3.8 FSPCre-*Pparb*/*d*^{-/-} mice display reduced colon tumor numbers and size in murine CRC models.

(A) Representative images of *Pparb*/*d*^{fl/fl} and FSPCre-*Pparb*/*d*^{-/-} colons from mice undergoing the respective treatments: Untreated control, APC^{min/+} background (25 weeks old), AOM/DSS treatment and APC/DSS treatment. (B, D, F) Bar graphs depicting total colonic tumor numbers per mouse for (B) APC^{min/+} background (25 weeks old), (D) AOM/DSS treatment and (F) APC/DSS treatment. Values are mean ± S.E.M. (C, E, G) Box-and-whisker plot showing distribution of tumor number and size in mice of (C) APC^{min/+} background (25 weeks old), (E) AOM/DSS treatment and (G) APC/DSS treatment. (H) Representative H&E stained images of tumors and adjacent colon tissues from APC^{min/+} FSPCre-*Pparb*/*d*^{-/-} and APC^{min/+} *Pparb*/*d*^{fl/fl} mice. Scale: 200 μm.

*3.2.2.3 Comparative gene expression analyses reveal perturbed oxidative processes in FSPCre-*Pparb*/*d*^{-/-} normal and malignant mucosal tissues*

To identify the mechanisms underlying the observed reduction in colon tumors in FSPCre-*Pparb*/*d*^{-/-} mice, we performed comparative gene expression analyses of epithelial and mucosal layers from normal intestines and tumors. We collected tissue from intestinal normal and malignant sections using laser capture microdissection (LCM) (Figure 3.9A). Samples were processed for microarray chip analyses as per the manufacturer's protocol, raw chip intensities were processed in Partek Genomic Suite (PGS) and all gene ontological (GO) and pathway analyses were performed using ingenuity pathway analysis (IPA). GO analyses identified mitochondrial dysfunction and oxidative phosphorylation as commonly enriched ontological pathways in FSPCre-*Pparb*/*d*^{-/-} mice across all tissues (Figure 3.9B-D). To identify a master gene regulating the perturbed pathways, we performed unguided hierarchical cluster analysis of upstream gene regulators and pathways of differentially expressed genes in normal, tumor adjacent and tumor epithelial tissues from FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice. Our analyses suggested the activation of the NRF2-mediated stress response in normal, tumor adjacent and tumor epithelia of FSPCre-*Pparb*/*d*^{-/-} colons (Figure 3.9E & F). Our validation assay showed that the expression of NRF2 was elevated in FSPCre-*Pparb*/*d*^{-/-} colons, concomitant with an increase in antioxidant enzyme expression (Figure 3.10A) The expression of NRF2

protein was also elevated in FSPC*Cre-Pparb/d^{-/-}* colons as shown by western blot analysis and IHC staining (Figure 3.10B-C). To confirm our gene and protein expression results, we performed analyses of the oxidant levels of FSPC*Cre-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* colons (Figure 3.10D). We observed reduced intracellular H₂O₂ in the colonic tissue of APC^{+/+} and early APC^{min/+} mice on the FSPC*Cre-Pparb/d^{-/-}* background (Figure 3.10D). In older APC^{min/+} mice with tumor burden, this difference was no longer observed (Figure 3.10D).

Figure 3.9 Microarray analyses of laser microdissected colonic epithelial and tumoral tissues.

(A) Representative H&E stained images of intestines before and after laser capture microdissection of tumor epithelium and adjacent intestinal mucosa from *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{-/-}* mice. Scale: 200 μ m. (B-D) Gene Ontological Pathway Analysis of differentially expressed genes from (B) normal colon mucosa, (C) normal ileum mucosa, and (D) intestinal tumor epithelium. The top 5 GO analysis were in descending order as determined by their p-value. Pathway analyses were conducted using Ingenuity Pathway Analysis (IPA), comparing FSPCre-*Pparb/d^{-/-}* (KO) against *Pparb/d^{fl/fl}* (WT) samples (n=3; Fold change cut-off $\geq \pm 1.3$; p<0.05). * Oxidative Phosphorylation and Mitochondrial Dysfunction were two common pathways placed in the top 3 most significant pathways across all tissues. (E-F) Unguided hierarchical clustering by (D) Upstream regulator and (E) Pathway analysis of differentially expressed genes in normal, tumor adjacent and tumor epithelium tissues from FSPCre-*Pparb/d^{-/-}* (KO) and *Pparb/d^{fl/fl}* (WT) mice. Our analysis also flagged NRF2-mediated oxidative stress response as an upregulated pathway in FSPCre-*Pparb/d^{-/-}* colonic epithelium. *NFE2L2 (Nuclear factor erythroid 2-related factor 2) is a synonym of NRF2 (Nuclear factor erythroid 2-related factor 2).

Figure 3.10 Increased NRF2 expression and reduced intracellular H₂O₂ in FSPCre-*Pparb/d^{-/-}* colons.

(A) Relative fold change in mRNA levels of 13 genes associated with oxidative stress response in *APC^{min/+}FSPCre-Pparb/d^{-/-}* and *APC^{min/+}Pparb/d^{fl/fl}* colons. Reverse transcription real-time quantitative PCR (RT-qPCR) were performed with ribosomal RNA L27 used as housekeeping gene. Values are mean $\pm$ S.E.M. (n=4). * $P < 0.05$. (B) Fold change in NRF2 protein in colons from FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice.

Representative immunoblot and graph for NRF2 protein are shown. PCNA that served as housekeeping protein were from the same samples. Data are represented as mean $\pm$ S.E.M. from at least three independent experiments. **(C)** Representative images of immunohistochemical staining for NRF2 in FSPC*Cre-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* colons, counterstained with hematoxylin for visualization of tissue morphology. Black arrows indicate representative NRF2-positive regions. Scale bar: 50 μ m. **(D)** Intracellular H₂O₂ levels in colons from FSPC*Cre-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice as determined by Amplex Red H₂O₂ assay (571 nm ex and 585 nm em). A calibration curve was prepared (0 to 20 μ M) and used to calculate H₂O₂ concentration of samples. The concentration of H₂O₂ was normalized to total protein. Data are represented as mean $\pm$ S.E.M. from at least four independent experiments. **(E-G)** Bar graph depicting the percentage of (E) monocytic cells (CD11b+), (F) neutrophils (CD11b+ Ly6G+) and (G) T cells (CD3+) in normal APC^{+/+} colons and APC^{min/+} colon tumors of FSPC*Cre-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice (n=5). **(E)** FACS analyses of F/480 fluorescence of Vehicle control and DSS-treated colons from FSPC*Cre-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice. Values are mean $\pm$ S.E.M. (n=4). **(H)** Bar graph depicting the percentage of macrophages (F/480+) in normal APC^{+/+} colons and APC^{min/+} colon tumors of FSPC*Cre-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice (n=5). **(I)** Bar graph depicting the M1/M2 ratio of macrophages in normal APC^{+/+} colons and APC^{min/+} colon tumors of FSPC*Cre-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice (n=5).

As elaborated in the colitis experiments of Chapter 3, we found that FSPC*Cre-Pparb/d^{-/-}* colons had a less inflamed phenotype, with more anti-inflammatory and less inflammatory cytokines, and an increased number of anti-inflammatory macrophages in the unchallenged FSPC*Cre-Pparb/d^{-/-}* colon, as compared to their *Pparb/d^{fl/fl}* littermates. In our current pathway analyses of comparative gene data, we also discovered an enrichment of processes related to immune cell activity in FSPC*Cre-Pparb/d^{-/-}* colons, compared with their *Pparb/d^{fl/fl}* littermates (Figure 3.9F). We observed a reduction in infiltrated monocytic cells, neutrophils, T-cells and macrophages in FSPC*Cre-Pparb/d^{-/-}* tumors, corresponding to the reduction in inflammation previously observed during DSS-treatment (Figure 3.10E-H). However, no significant difference in the M1/M2 ratio of macrophages was observed (Figure 3.10I).

3.2.2.4 Recapitulation of the oxidative stress phenotype in intestinal cell lines cultivated together with PPAR β/δ ablated fibroblast cell lines or with conditioned medium from FSPCre-Pparb/d^{-/-} explanted colon fibroblasts.

In our animal experiments, FSPCre-Pparb/d^{-/-} colonic mucosa were shown to have lower oxidative stress and higher expression of antioxidant genes. To establish if the oxidative stress phenotype could be reproduced in vitro, we cultured the intestinal cell lines HCT116 and HT29 with conditioned medium from the explanted colonic fibroblast explants. We observed a significant increase in anti-oxidant gene expression in HCT116 and HT29 cells cultured in FSPCre-Pparb/d^{-/-} conditioned medium (Figure 3.11A and C). We also observed a corresponding decrease in intracellular oxidative stress in HCT116 cells cultured in FSPCre-Pparb/d^{-/-} conditioned medium (Figure 3.11B). We observed greater extracellular secretion of H₂O₂ from FSPCre-Pparb/d^{-/-} colonic fibroblast explants (Figure 3.11D), without significant intracellular H₂O₂ buildup in the fibroblasts. We confirmed that the increased H₂O₂ secretion from explanted fibroblasts from FSPCre-Pparb/d^{-/-} colons was also reflected in the decreased gene expression of antioxidant genes (Figure 3.11G). However, we did not observe any difference in intracellular H₂O₂ in the explanted fibroblasts (Figure 3.11E and F).

Figure 3.11 Increased antioxidant expression and reduced intracellular oxidative stress in intestinal epithelial cells lines treated with conditioned medium from FSPCre-*Pparb*/*d*^{-/-}

(A, C) Relative fold change in mRNA levels of 13 genes associated with oxidative stress response in (A) HCT116 and (C) HT29 cells cultured in conditioned medium from FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} explanted fibroblasts. Reverse transcription real-time quantitative PCR (RT-qPCR) were performed with 18S rRNA used as housekeeping gene. Values are mean $\pm$ S.E.M. (n=4). * P<0.05, ** P<0.01, ***P<0.001. (B) Intracellular oxidative stress levels in HCT116 cells cultured in condition medium of explanted fibroblasts from FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice, determined by CM-H2DCFDA assay. Values are mean $\pm$ S.E.M. (n=4). (D) Extracellular H₂O₂ levels of explanted fibroblasts from *Pparb*/*d*^{fl/fl} and FSPCre-*Pparb*/*d*^{-/-} colons detected by Amplex Red Assay. Values are mean $\pm$ S.E.M. (n=6). (E, F) Intracellular H₂O₂ levels of explanted fibroblasts from *Pparb*/*d*^{fl/fl} and FSPCre-*Pparb*/*d*^{-/-} colons detected by CellROX. Cells were first positively gated for fibroblast marker (CD140a or PDGRA) and the CellROX fluorescence readings were taken from the CD140a+ gated cells. (E) Representative histogram of CellRox fluorescence. (F) Bargraph plotting the percentage of fibroblasts with positive CellRox staining. Values are mean $\pm$ S.E.M. (n=4). (G) Relative fold change in mRNA levels of 13 genes associated with oxidative stress response in explanted fibroblasts from *Pparb*/*d*^{fl/fl} and FSPCre-*Pparb*/*d*^{-/-} colons. Reverse transcription real-time quantitative PCR (RT-qPCR) were performed with 18S rRNA as a housekeeping gene. Data are represented as mean $\pm$ S.E.M. from 4 independent experiments. * P<0.05, ** P<0.01.

To further confirm our findings in fibroblastic cell lines, we performed targeted siRNA depletion of PPAR β/δ (siPPAR β/δ) in BJ-1 fibroblast and CCD-18co colonic myofibroblast cell lines (Figure 3.12A and D). We co-cultured HCT116 intestinal cell line with fibroblastic cell lines pre-treated with siPPAR β/δ or a scrambled control. The results showed that of our conditioned medium treatment led to increased anti-oxidant gene expression in HCT116 cells cultured with BJ-1 or CCD-18co cells treated with siPPAR β/δ , coupled with a corresponding decrease in intracellular oxidative stress (Figure 3.12B, C, E, F). Chronic low exposure to H₂O₂ signalling has been reported to affect epithelial cell proliferation (Chan et al., 2017), and thus we were interested to determining whether PPAR β/δ depletion in fibroblastic cell lines exerted a similar proliferative effect on HCT116 intestinal cell lines. We observed an increase in

cells in the G2/M or S phases among HCT116 cells co-cultured with PPAR β/δ -depleted fibroblast cell lines compared with their cognate controls (Figure 3.12G).

Figure 3.12 siRNA directed depletion of PPAR β/δ in fibroblast cell lines results in increased antioxidant gene expression and decreased oxidative stress in co-cultured intestinal epithelial cells.

(A, D) Relative fold change in PPAR β/δ mRNA expression in (A) BJ1 fibroblasts and (D) CCD-18co myofibroblasts, whose endogenous PPAR β/δ was suppressed by siRNA at 72 h post-transfection. Cells transfected with Scrambled siRNA served as cognate controls. 18S rRNA served as housekeeping gene. Data are represented as mean $\pm$ S.E.M. from 4 independent experiments. * $P < 0.05$, ** $P < 0.01$. (B, E) Relative fold change in mRNA levels of 13 genes associated with oxidative stress response in HCT116 co-cultured with BJ1 (B) and CCD18Co cells (E) whose endogenous PPAR β/δ was

suppressed by siRNA. Cells transfected with siRNA of PPAR β/δ and Scrambled were denoted by superscript. Data are represented as mean $\pm$ S.E.M. from 4 independent experiments. * P<0.05, ** P<0.01. **(C, F)** Intracellular oxidative stress levels in HCT116 co-cultured with BJ1 * P<0.05 (C) and CCD18Co cells (F) whose endogenous PPAR β/δ was suppressed by siRNA, determined by CM-H2DCFDA assay. Cells transfected with siRNA of PPAR β/δ and Scrambled were denoted by superscript. Values are mean $\pm$ S.E.M. (n=4). **(H)** Cellular proliferation assay by BrDU-FITC and PI staining of HCT116 cells co-cultured with normal BJ fibroblasts (BJ-1^{siScrambled}) and CCD-18co myofibroblasts (CCD18Co^{siScrambled}) or ablated of PPAR β/δ expression (BJ-1^{siPPAR β/δ} and CCD18Co^{siPPAR β}). Values in bold denote percentage of proliferating cells in S phase and G2/M phase from cells in cell cycle arrest at G1/0 phase.

3.2.2.5 NAC-treatment reduces colon tumor numbers and size in DSS-treated

APC^{min/+} mice and attenuates the H₂O₂-mediated increase in anti-oxidant gene expression

We established a reduction of oxidative stress in the colonic epithelium of FSPCre-*Pparb/d*^{-/-} mice and a recapitulation of this phenotype *in vitro* with intestinal epithelial cell lines co-cultured with fibroblastic cells depleted of PPAR β/δ expression. In human CRC patients, antioxidant treatment has been shown to be useful for the control and prevention of colorectal cancer (Gorrini et al., 2013). Thus, we wondered whether anti-oxidant treatment with N-acetyl cysteine (NAC) would enhance the anti-tumorigenic effect of our FSPCre-*Pparb/d*^{-/-} transgenic mice. We were also interested in determining whether these findings could be repeated *in vitro* with a non-transformed colonic epithelial cell line. First, we repeated the conditioned medium incubation experiment using a normal immortalized colonic epithelial cell line (iCEC) to validate the upregulation of NRF2 and antioxidant genes incubated with FSPCre-*Pparb/d*^{-/-} fibroblast conditioned medium. To confirm that NAC could exert a direct antioxidant effect, we treated the intestinal epithelial cell lines iCEC and HCT116 with H₂O₂ as an oxidative stress challenge, and observed intracellular oxidative stress and anti-oxidant response gene expression as stress markers (Figure 3.13A, C and D). The addition of NAC abrogated the effect of the H₂O₂ induced stress response.

To observe this effect in a CRC mouse model, we pre-treated APC^{min/+} mice with NAC for 5 consecutive days prior to the commencement of DSS treatment. NAC has been reported to reduce tumor growth in CRC mouse models (Seril et al., 2007). We observed a similar effect in our experiments, with increased survival, a reduced disease severity score and decreased tumor development in NAC-treated APC^{min/+} mice (Table 6; Figure 3.13E-I). However, NAC-treated APC^{min/+} mice on FSPCre-*Pparb*/*d*^{-/-} background did not show a decrease in tumor numbers, suggesting that further reduction of oxidative stress through anti-oxidant supplementation had a minimal impact on tumor development (Table 6; Figure 3.13E-I). Paradoxically, we also observed that NAC treatment prolonged the survival of *Pparb*/*d*^{fl/fl} mice but decreased that of FSPCre-*Pparb*/*d*^{-/-} mice.

Figure 3.13 NAC treatment abrogates the effect of reduced tumorigenesis conferred by ablated fibroblast PPARβ/δ expression

(A-C) Relative fold change in mRNA levels of 13 genes associated with oxidative stress response in (A) HCT116 treated with NAC and H₂O₂, and (B, C) ICEC (B) cultured in conditioned medium of fibroblasts from *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{-/-}* or (C) treated with NAC and H₂O₂. Data are represented as mean ± S.E.M. from 4 independent experiments. * P<0.05, ** P<0.01, n.d. expression below assay thresholds. (D) Intracellular oxidative stress levels in ICEC cells either cultured in condition medium of fibroblasts from *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{-/-}* or treated with H₂O₂ or NAC. Values are mean ± S.E.M. (n=4). * P<0.05, ** P<0.01, *** P<0.001 (E) Mean DAI score of NAC/DSS-treated APC^{min/+}FSPCre-*Pparb/d^{-/-}* mice (n=5) and APC^{min/+}*Pparb/d^{fl/fl}*

littermates (n=5) over 5 weeks. Data are represented as mean $\pm$ S.E.M. ** P<0.01 **(F)** Percentage survival of APC^{min/+}FSPCre-*Pparb/d^{-/-}* and APC^{min/+}*Pparb/d^{fl/fl}* mice (n=5) that were pretreated with NAC and then DSS (N). **(G)** Bar graph depicting total colonic tumor numbers per mouse, in NAC/DSS-treated APC^{min/+} FSPCre-*Pparb/d^{-/-}* (n=5) and APC^{min/+}*Pparb/d^{fl/fl}* mice (n=5). Values are mean $\pm$ S.E.M. ** P<0.01 **(H)** Box-and-whisker plot showing distribution of tumor number and size in APC^{min/+}FSPCre-*Pparb/d^{-/-}* and APC^{min/+}*Pparb/d^{fl/fl}* mice that were pretreated with NAC and then DSS. * P<0.05, ** P<0.01 **(I)** Representative images of colons from NAC/DSS-treated *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{-/-}* mice in APC^{min/+} background at indicated weeks after treatment. Scale: 1 cm.

Table 6: Summary of murine CRC experiments

	AOM/DSS Treatment		APC ^{min/+} (25 week old)		APC/DSS Treatment		APC/DSS Treatment with NAC	
	<i>Pparb/d^{fl/fl}</i>	<i>FSPCre-Pparb/d^{-/-}</i>	<i>Pparb/d^{fl/fl}</i>	<i>FSPCre-Pparb/d^{-/-}</i>	<i>Pparb/d^{fl/fl}</i>	<i>FSPCre-Pparb/d^{-/-}</i>	<i>Pparb/d^{fl/fl}</i>	<i>FSPCre-Pparb/d^{-/-}</i>
Mouse genotype								
Sample size (n)	18	13	14	15	13	14	5	5
End Point								
Survival (%)	77.78	100.00	50.00	80.00	53.85	71.43	80.00	40.00
Total number of tumors	13.17 ± 2.1	9.2 ± 2.43	4 ± 0.89	2.8 ± 0.54	18.43 ± 3.99	11.8 ± 2.68	3 ± 0.41	10 ± 0.5
<u>Tumors by Size (mm)</u>								
< 0.02	1.4 ± 0.37	1.17 ± 0.37	0.8 ± 0.34	1.33 ± 0.31	8.29 ± 3.31	2 ± 0.54	1.5 ± 0.2	6.5 ± 0.75
0.02 - 0.04	7.17 ± 1.18	6.2 ± 1.7	3.25 ± 1.02	0.57 ± 0.26	6.29 ± 2.65	4.1 ± 1.27	1 ± 0.26	3 ± 0.82
> 0.04	7.17 ± 1.18	6.2 ± 1.7	1.2 ± 0.16	0.29 ± 0.16	4.5 ± 1.39	0.75 ± 0.43	0.48 ± 0.13	0.48 ± 0.13

3.2.3 Summary

We confirmed the presence of the PPAR β/δ exon4 deletion in the intestinal stroma of our transgenic mouse line harboring the FSPCre-*Pparb/d*^{-/-} transgene. The deletion conferred increased intestinal mucosal proliferation, resistance to colitis-induced intestinal mucosal damage and a reduction in inflammatory signals in the intestinal tissue microenvironment. Chronic inflammatory signals have been shown to promote the development of CRC (Gilat et al., 1988), while PPAR β/δ has been shown to regulate both chronic inflammation and colorectal cancer (Wang and Dubois et al., 2010). Thus, we investigated whether the FSPCre-*Pparb/d*^{-/-} transgenic deletion could also result in changes in colorectal tumorigenic development. To model CRC in transgenic mice, we employed 3 commonly used models, namely the APC^{min/+} mouse model for human FAP, AOM/DSS for colitis-associated CRC and a combinatorial model of APC/DSS. We found that across all treatments, the FSPCre-*Pparb/d*^{-/-} background conferred increased survivability over the course of treatment, indicative of reduced disease burden. To assess the well-being of the mice undergoing DSS-treatment in quantitative terms, we graded the physiological traits of mice during and after DSS-treatment, according to a scoring matrix. We found that FSPCre-*Pparb/d*^{-/-} mice had reduced severity scores, indicative of a better disease prognosis during and after DSS treatment. At the end of the treatment course, we also observed a reduced number and size of colon tumors in the FSPCre-*Pparb/d*^{-/-} mice across all 3 treatments, demonstrating the anti-tumorigenic effect of FSPCre-*Pparb/d*^{-/-} background.

To understand the effects of stromal PPAR β/δ deletion on intestinal epithelial cells, we performed comparative gene expression analyses of normal, tumor adjacent and tumor epithelium of intestines. Our gene ontological and pathway analysis revealed an enrichment of oxidative stress pathways with activation of NRF2 as a possible upstream regulator of these processes. We first validated our gene expression findings by qPCR, IHC staining and western analysis of NRF2. Consequently, we also detected reduced oxidative stress in FSPCre-*Pparb/d*^{-/-} colons of APC^{+/+} and early APC^{min/+} mice. Given the complexities of an *in vivo* model in understanding the effects of stromal PPAR β/δ ablation on epithelial cells, we cultured the human intestinal epithelial cell lines

HCT116, HT29 and iCEC to investigate intercellular interactions with PPAR β/δ -ablated stromal cells. Colonic fibroblast explants were derived from FSPC*Cre-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice, and cultured for conditioned media. Our results indicated that the culture of intestinal cells with FSPC*Cre-Pparb/d^{-/-}* fibroblast conditioned medium induced a significant increase in antioxidant gene expression, with a concomitant reduction in intracellular oxidative stress, very similar to our observations for the FSPC*Cre-Pparb/d^{-/-}* mouse colons. A similar observation was also noted when we co-cultured intestinal epithelial cell lines with stromal cell lines, BJ-1 fibroblasts and CCD-18co myofibroblasts transiently depleted of PPAR β/δ expression. A reduction of intracellular oxidative stress was detected in intestinal epithelial cells, together with increased anti-oxidant gene expression. We found the PPAR β/δ deletion in FSPC*Cre-Pparb/d^{-/-}* fibroblasts resulted in an increase in extracellular H₂O₂ secretion into its microenvironment. Treatment with exogenous H₂O₂ also recapitulated the NRF2 antioxidant response and was attenuated in the presence of antioxidant N-acetyl cysteine. Chronic low levels of H₂O₂ have been reported to exert a proliferative stimulus on epithelial cell growth (Chan et al., 2017). We observed a similar proliferative effect with increased proportion of intestinal cells in G2/M or S phase when co-cultured with PPAR β/δ -depleted fibroblast cell lines compared with their cognate controls.

To examine whether the anti-oxidant treatment yield any changes in tumorigenesis in FSPC*Cre-Pparb/d^{-/-}* mice, we fed mice N-acetyl cysteine (NAC) for 5 days prior to the start of the DSS regime in the APC/DSS treatment protocol. NAC has been shown to exert potent anti-inflammatory and antioxidant activities and reduces tumor development in mice and humans (Seril et al., 2002; Uraz et al., 2013; Estensen et al., 1999). With NAC treatment, we observed a decrease in tumor development in APC^{min/+} mice, but we did not observe any changes in tumorigenesis for APC^{min/+} mice on a FSPC*Cre-Pparb/d^{-/-}* background while paradoxically observed shorter median survival of APC^{min/+} mice on a FSPC*Cre-Pparb/d^{-/-}* background.

In conclusion, we have shown that the FSPC*Cre-Pparb/d^{-/-}* background confers resistance to the development of colorectal tumors in mice. Comparative gene expression analysis and subsequent functional validation assays in vivo

suggest that oxidative processes may mediate the delayed tumorigenesis phenotype in FSPCre-*Pparb/d^{-/-}* mice. We then replicated the observation *in vitro*, again showing a reduction of oxidative stress in intestinal epithelial cells cultured with PPAR β/δ -ablated stromal cells or with conditioned medium from FSPCre-*Pparb/d^{-/-}* fibroblasts. Finally, antioxidant treatment prior to DSS treatment reduces tumorigenesis in APC^{min/+} mice but not in APC^{min/+} mice on the FSPCre-*Pparb/d^{-/-}* background, indicating that the deletion of PPAR β/δ in stromal cells may exert its tumorigenesis-reducing effect by modulating oxidative stress in the associated intestinal epithelium.

Chapter 4: Discussion & Future studies

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4.1 Colon cancer and PPAR β/δ in perspective

In our study, we were interested to elucidate the role of PPAR β/δ in colorectal cancer carcinogenesis. Colorectal cancer ranks among the top 5 cancers in both prevalence and mortality rates, highlighting the importance and urgency of advancing colorectal cancer research (Tomasetti and Vogelstein, 2015). A variety of risk factors affect CRC, including age, personal history of inflammatory bowel diseases (IBD), familial history of CRC, hereditary genetic conditions, dietary practices, obesity, cigarette smoking and alcohol consumption (Haggar and Boushey, 2009). Genetic predisposition of CRC occurs in either of two hereditary syndromes, Familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) (Cancer Research UK, 2014). FAP is associated with hyperactivated Wnt signaling program, most commonly via the inactivation of the tumor suppressor APC gene. Inflammatory bowel diseases, namely Crohn's disease and ulcerative colitis, have been demonstrated as important risk factors associated with progression to CRC (Jess et al., 2012; Kim and Chang, 2014). Experimental models have been developed to model FAP and IBD in a transgenic mouse lines, namely the APC^{min/+} and AOM/DSS treatment, facilitating CRC research in an animal model (Taketo and Edelmann, 2009; Suzuki et al., 2005).

PPAR β/δ is part of a 3-member PPAR family of nuclear receptor proteins that hetero-dimerizes with RXR to bind to specific peroxisome proliferator responsive elements (PPREs) to function as transcription factors that regulate gene expression (Mangelsdorf et al., 1995). Various studies have implicated all three PPARs in the regulation of inflammation and cancer (Michalik and Wahli, 2008; Bishop-Bailey and Bystrom, 2009; Youssef and Badr, 2011; Tachibana et al., 2008) While PPAR γ and PPAR α display tissue-specific expression, PPAR β/δ has a ubiquitous expression pattern and is found in a wide variety of cell types, allowing its study in diverse tissue types and diseases. However, studies on PPAR β/δ in colorectal cancer has been largely complicated by experimental design, leading to varied conclusions on PPAR β/δ 's role in colon cancer and suggesting that a tissue specific approach may be need to investigate the cell-

specific role of PPAR β/δ in different cells of the colon (Wang et al., 2014; Harman et al. 2004).

The cellular roles and expressional patterns of PPAR β/δ differ between cell types. Although both epithelial and endothelial PPAR β/δ levels have been associated with the severity of colorectal cancer in humans, an inverse relationship between endothelial and epithelial PPAR β/δ levels was established for patient prognostic predictions (Zhou et al., 2014). Vascular endothelial PPAR β/δ has been shown to exert an inhibitory effect on the production of pro-inflammatory chemokines (Liang et al., 2010). On the other hand, mucosal epithelial PPAR β/δ has been shown to exert a pro-tumorigenic role when ectopically expressed, while intestinal epithelium-specific loss of PPAR β/δ resulted in suppressed colonic tumorigenesis in mice (Zuo et al., 2009; 2014). While there is a growing realization of the role of tumor stroma, there is very little literature addressing the role of stromal PPAR β/δ in regards to colorectal tumorigenesis. Thus, the role of stromal PPAR β/δ in orchestrating intestinal tumorigenesis represents a sizable gap in research.

4.2 Effect of stromal PPAR β/δ deletion in the intestines during colitis and colorectal cancer

In this study, we sought to explore this issue, using a transgenic mouse line carrying a stromal cell-specific deletion of PPAR β/δ expression, to better understand the role that stromal-derived PPAR β/δ plays in colitis and tumorigenesis. We observed mucosal thickening, associated with increased proliferative markers in epithelial cell types. We also noted an increase in intestinal stem cells in FSPC*Cre-Pparb/d^{-/-}* mice. Our result further illustrated the role of PPAR β/δ in intestine, where PPAR β/δ was previously found to regulate intestinal cell differentiation by downregulating Indian hedgehog (Ihh) in Paneth cells (Varnat et al., 2006). The gut mucosa was found to be significantly thicker in FSPC*Cre-Pparb/d^{-/-}* than *Pparb/d^{fl/fl}*, we think that a stronger proliferative capacity in the intestinal epithelial cells may have contributed to the mucosal

thickening phenotype. The increase in villus lengths has been thought to increase the effective surface area for nutrient absorption in the intestines (Goodlad and Wright, 1990). Future studies can explore if the changes in villus lengths also result in metabolic changes in FSPCre-*Pparb*/*d*^{-/-} mice, such as nutritional absorption or enzyme secretion. We have also noted thicker smooth muscle layers in the stomach, colon and parts of the small intestines. Multiple studies have reported the dysregulation of colonic smooth muscle development and physiology in irritable bowel syndrome (Shi et al., 2005; Zhang et al., 2010; Li et al., 2013). Further investigations can assess functional changes in the intestinal smooth muscle layer, such as contractility, and how they impact intestinal ailments. An altered intestinal physiology and functions have been associated with chronic heart failure in human patients (Sandek et al., 2007; 2008). Heart failure patients present with abnormalities in intestinal permeability, resulting in inappropriate permeability to bacterial endotoxins and reduced intestinal absorption of nutrients such as fats, proteins and iron (Sandek et al., 2009; Arutyunov et al., 2008; King et al., 1996). Increase in plasma levels of bacterial endotoxins, such as lipopolysaccharides, results in the activation of pro-inflammatory signaling pathways such as TNF- α , which has been linked as an inducer of ventricular remodeling, and leading to heart failure (Bozkurt et al., 1998; Sivasubramanian et al., 2001). Given the significant changes in intestinal physiology, further investigations can examine the cardiac physiology of FSPCre-*Pparb*/*d*^{-/-} mice.

As colonic inflammation in IBD poses an important risk factor for CRC, we challenged the FSPCre-*Pparb*/*d*^{-/-} mice with DSS-induced colitis. The FSPCre-*Pparb*/*d*^{-/-} mice displayed greater resistance to DSS-induced physiological effects and intestinal mucosal damage, compared with *Pparb*/*d*^{fl/fl} mice. Our results are consistent with findings in classical PPAR β / δ knockout mice and intestinal-epithelial specific knockout mice, highlighting the pro-inflammatory role played by PPAR β / δ in overall colon physiology (Wang et al., 2006; Zuo et al., 2009). For FSPCre-*Pparb*/*d*^{-/-} mice, we observed an anti-inflammatory effect at the homeostatic baseline, with a greater number of macrophages of the anti-inflammatory M2 subtype and a cytokine milieu representative of an anti-inflammatory tissue microenvironment, providing a

plausible explanation for the reduction of colitis-induced damage in FSPCre-*Pparb/d^{-/-}* mice. Intestinal fibroblasts are known to exert a paracrine regulation of the inflammatory secretory responses of intestinal epithelial cells (Berschneider and Powell, 1992). However, PPAR β/δ has also been shown to be essential for the regulation of macrophage polarization to the M2-subtype in adipose tissue and liver (Kang et al., 2008; Odegaard et al., 2008). Furthermore, activation of PPAR β/δ in adipocytes also results in the inhibition of cytokines following lipopolysaccharide-induction (Rodriguez-Calvo et al., 2008), demonstrating the importance of PPAR β/δ as a regulator of inflammatory cells and signals in the tissue microenvironment. Studies have suggested that PPAR β/δ signaling may be involved in the modulation of T-cell mediated inflammatory response by regulating of T-cell differentiation (Dunn et al., 2010; Kanakasabai et al., 2010). Wang and colleagues have also showed a reduction in total T-lymphocyte (CD3+) and T helper cell (CD4+) cell infiltration in DSS-treated PPAR β/δ -null mice (Wang et al., 2014). While we did not observe a significant change in total T-lymphocyte numbers between FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice, further examination should be performed studying on T-lymphocyte subtypes to establish if the FSPCre-*Pparb/d^{-/-}* background also results in the preferential differentiation of T-lymphocytes. Future studies should be conducted to examine the precise mechanism responsible for the colonic anti-inflammatory phenotype in FSPCre-*Pparb/d^{-/-}* mice.

Available research provides some clues to the pathways that may be perturbed in FSPCre-*Pparb/d^{-/-}* colons. The activation of PPAR β/δ in transgenic mice results in inflammatory skin disease psoriasis through a STAT3-dependent mechanism (Romanowska et al., 2010). PPAR β/δ regulation of cytokine production in adipocytes is mediated through NF-KB and erk1/2-dependent pathways (Rodriguez-Calvo et al., 2008). Moreover, proteomic profiling of fibroblasts from PPAR β/δ -null mice provides some useful insight about the molecular changes that occur in PPAR β/δ -null fibroblasts, in which the authors highlighted the significant increase in cellular retinol binding protein 1 (CRBP1) and reduction of calcium intracellular channel-4 (CLIC4) (Adamkiewicz et al., 2007). CRBP1 negatively regulates the AKT signaling pathway, which is activated by PPAR β/δ signaling via PDK1 and PTEN transcription (Di-Poi et al.,

2002). Thus the loss of PPAR β/δ may reduce AKT signaling both by reducing activation and increasing CRBP1-mediated inhibition. AKT signaling has been shown to be necessary for the acute inflammatory reaction, through the regulation of vascular permeability, thereby inducing edema and restricting leukocyte extravasation and intravasation (Di Lorenzo et al., 2009). In contrast, CLIC4 has been shown to be involved in the modulation of cellular proliferation and morphogenesis (Tung et al., 2009; Song et al., 2016). In a similar manner, PPAR β/δ has been shown to be anti-proliferative and differentiation-promoting in several cell types (Tan et al., 2012; Nadra et al., 2006; Varnat et al., 2006). Future studies can investigate the role of stromal PPAR β/δ in conjunction with CLIC4 and CRBP1 to further evaluate the anti-inflammatory and increased proliferation phenotype that we have observed in FSPC*Cre-Pparb/d^{-/-}* mice.

Following our investigations in the colitis experiment, we assessed whether CRC was affected in FSPC*Cre-Pparb/d^{-/-}* mice. We observed reduced colorectal tumorigenesis in mice carrying the FSPC*Cre-Pparb/d^{-/-}* transgene, which was consistent across the three murine CRC models investigated, indicating a robustness of the phenotype. This finding is in agreement to previous reported experimental outcomes on transgenic mice carrying a classical transgenic PPAR β/δ ablation (Wang et al., 2006). Furthermore, the role of PPAR β/δ in the intestinal epithelium has been thought to be pro-tumorigenic, with increased tumorigenesis with ectopic PPAR β/δ expression and deletion of PPAR β/δ expression resulting in reduced tumorigenesis (Zuo et al., 2009; 2014). Our results also echoed findings of a recent study on lung metastasis in PPAR β/δ -null mice, where the authors observed a reduction in tumor size and number from cancer cells injected into *Pparb/d^{+/-}* mice, indicative of the importance of the PPAR β/δ signaling pathways in the supporting tissue microenvironment for tumor growth and development (Zuo et al., 2017). We then sought to understand the effect of PPAR β/δ -null fibroblasts on the gene expression profile of their adjacent intestinal and tumor epithelial cells, through comparative gene expression analysis. We observed an enrichment of genes in oxidative stress pathways in the gene ontological analysis and identified NRF2 activation as a plausible gene candidate for the upstream regulator of the altered oxidative processes. NRF2 is well-recognized as the master regulator of the antioxidant

response to oxidant damage, and it plays an important role in the homeostasis and structural integrity of mitochondria (Sporn and Liby, 2012; Holmström and Finkel, 2014). To validate this finding, we performed qPCR, IHC staining and western blot analysis, which confirmed the upregulation of protein and mRNA expression of NRF2 and associated genes in FSPCre-*Pparb/d*^{-/-} colons. Consequently, we also noted reduced oxidative stress levels in FSPCre-*Pparb/d*^{-/-} colons, in agreement with the gene expressional data. In the literature, NRF2-null mice have been demonstrated to be more susceptible to colorectal tumorigenesis, as compared to wild type mice (Khor et al., 2008). PPAR-dependent signaling pathways have shown to be important for NRF2-mediated oxidative regulation (Reddy and Standiford, 2010), as supported by evidence showing a functional PPAR response element in several anti-oxidant genes (Cho et al., 2010; Park et al., 2004; Wang et al., 2015). Furthermore, in cardiomyocytes, PPARβ/δ exerts an antioxidant effect by inhibiting PI3K/Akt signaling to suppress ROS generation induced by AngII; however, its role in redox regulation in the gut remains poorly understood (Kim et al., 2011; 2012; Liu et al., 2011). Thus it is possible that the reduction in colon tumors in FSPCre-*Pparb/d*^{-/-} mice is, at least in part, due to the activation of NRF2 and associated oxidative processes driven by PPARβ/δ deletion in associated stromal cells. However, the oxidative stress phenotype was not observed in late stage APC^{min/+} colons, suggesting exacerbated oxidative stress plays a more critical role during tumor initiation. At the late stage, complex changes to cellular and soluble signals in the tumor microenvironment contributed to tumor growth (Reuter et al., 2010; Voest et al., 1992).

However, our animal model is not without its limitations. A limitation to our model is the gene deletion strategy which ablates the exon containing the DNA-binding domain of PPARβ/δ (Iglesias et al., 2012). It has been proposed that other gene deletion strategies may affect the different phenotypic severity or outcomes owing to differences in vulnerabilities to oxidative stress (Zuo et al., 2009). Another limitation was that only one genetic background was used in our murine study. Strain differences have been shown to influence the susceptibility of mice to colonic inflammation and colorectal tumor development (Suzuki et al., 2005b). The use of FSP-1 promoter to perform selective gene deletion in

fibroblasts also has its criticisms, with studies showing that other cell types may also express FSP-1. Indeed, FSP-1 expression was reported in the breast tumor stroma, expressed in tumor-associated fibroblasts, macrophages and lymphocytes (Cabezón et al., 2007). FSP-1 expression was also found in an inflammatory subtype of hepatic tissue macrophages and in a population of CD11b+ cells in infarcted hearts (Österreicher et al., 2010; Kong et al., 2013). We had also previously detected the deleted PPAR β/δ allele in a population of CD45+ cells in the spleen (Sng et al., 2018). However, the achievement of pure fibroblast-specific gene deletion is severely limited by existing technology. Nevertheless, the FSP1-Cre approach, when complemented with supporting *in vitro* studies, represents a feasible and commonly used method to study gene deletion in fibroblasts. Further work to more clearly define the origin, fate, and function of FSP-1-positive populations in the colon will be required moving forward.

In an effort to reduce the complexities of multiple cell-cell interactions present in an *in vivo* model (Luz-Crawford et al., 2016), we proceeded to study specifically the interaction between fibroblastic and intestinal epithelial cells in a controlled *in vitro* setting. Similar to our *in vivo* findings, we observed an increase in antioxidant gene expression and a reduced oxidative stress phenotype in our culture of intestinal epithelial cell lines treated with conditioned medium from PPAR β/δ -null fibroblasts or co-cultured with fibroblastic cell lines transiently ablated of PPAR β/δ mRNA expression. Detailed investigations reveal that PPAR β/δ -null explanted fibroblasts secreted an increased amount of H₂O₂ in the tissue microenvironment. Wounded fibroblasts have been shown to transcriptionally regulate the expression of the antioxidant enzymes catalase and gpx1 in a PPAR β/δ -dependent manner, resulting in a reduction of ROS in the wound microenvironment (Wang et al., 2015). Furthermore, fibroblasts in cancerous tissue were previously demonstrated to enact field cancerization by increasing H₂O₂ secretion (Chan et al., 2017). Indeed, the NRF2 antioxidant response was recapitulated when intestinal epithelial cell lines were treated with exogenous H₂O₂ and attenuated with the addition of the antioxidant NAC. Similar to the *in vivo* findings of greater proliferative markers in FSPCre-*Pparb/d*^{-/-} murine intestinal epithelial cells, we also noted an increased

proliferation of intestinal epithelial cells when co-cultured with PPAR β/δ -depleted fibroblast cell lines, compared with their cognate controls. Studies have shown that PPAR β/δ is a promoter of cellular differentiation, thus leading to reduced differentiation in colon cancer cells (Yang et al., 2010; Kim et al., 2006). Thus, PPAR β/δ signaling in the stroma appears to play a role in regulating the growth and cellular stress levels of adjacent intestinal epithelial cells, thereby regulating tumorigenesis.

In our *in vivo* APC/DSS model, we also found that NAC pre-treatment reduced the tumor number and size of APC^{min/+} mice, suggesting that ROS may be important in early tumor development, in agreement with the known effects of NAC on CRC (Seril et al., 2002). Although we observed significant changes in *Pparb/d^{fl/fl}* mice, NAC pre-treatment did not reduce the number of tumors in APC^{min/+} FSPCre-*Pparb/d^{-/-}* mice, indicating that a further reduction of epithelial ROS with NAC treatment might not yield a palpable impact on tumor development. Interestingly, while the median survival rate of APC^{min/+} mice increased with NAC pretreatment, we observed, in contrast, a reduction of the survival rate of APC^{min/+} FSPCre-*Pparb/d^{-/-}* mice. While we did not explore this phenotype in detail, we speculate that further reduction of oxidative stress by NAC treatment might have disrupted essential ROS cellular functions during homeostasis. Reactive oxygen species (ROS) at non-toxic concentrations have been known to mediate crucial cellular functions through intracellular signaling pathways, where further destabilization of the intracellular redox state with NAC treatment could have adverse effects on normal cellular functions and animal physiology (Gamaley and Klyubin, 1999; Di Meo et al., 2016). Thus, antioxidant therapies should not aim to remove oxidants entirely, but instead limit oxidants below the threshold of triggering signaling pathways that support inflammation and tumor growth (Rhee, 2006; Valko et al., 2007).

In our *in vitro* model, we only briefly examined the direct epithelial-mesenchymal interaction aspect of stromal PPAR β/δ signaling and its effect on oxidative stress. PPAR β/δ signaling has also been shown to be important in the development and recruitment of various immune cell types that are involved in inflammation (Barish et al., 2008; Paterniti et al., 2012; Kang et al., 2008). We observed an enrichment of leukocyte recruitment and inflammatory pathways in

the comparative gene expression analysis and changes in the cytokine profile of FSPC*Cre-Pparb/d^{-/-}* colons. Due to the limited scope of this study, we did not fully explore the immune cellular changes associated with the FSPC*Cre-Pparb/d^{-/-}* genetic background. Activation of PPAR β/δ with the agonists GW501516 and L165041 modulates T helper type 1 and Th 17 mediated response by blocking the production of IFN- γ and IL-17 (Kanakasabai et al., 2010). Inflammatory cells release a myriad of soluble factors that can drastically modify the tissue microenvironment, and thus PPAR β/δ may also influence the redox state of the tissue through the modulation of immune cells and inflammatory mechanisms. Future studies can be designed to explore the mechanisms by which stromal PPAR β/δ modulates redox activity through immune cell regulation.

Inflammatory bowel diseases (IBDs), due to disrupted homeostatic processes in the host immune system, results in the manifestation of cutaneous disorders (Huang et al., 2012). As the predominant PPAR subtype in dermal fibroblasts, fibroblast PPAR β/δ expression has been shown to be important in the control of keratinocyte proliferation during wound healing (Di-Poï et al., 2002). Furthermore, PPAR β/δ activation results in inflammatory skin disease psoriasis in transgenic mice (Romanowska et al., 2010). We previously showed that FSPC*Cre-Pparb/d^{-/-}* mice displayed morphological characteristics reminiscent of morphea scleroderma and gene expressional analysis indicated that the FSPC*Cre-Pparb/d^{-/-}* dermis and epidermis have a similar nuclear receptor transcriptomic profile with fibroblasts from scleroderma patients (Sng et al., 2018). An inflammatory tissue environment promotes the development of skin cancer through the inhibition of natural anti-tumor immunity (Balkwill and Mantovani, 2001; Tang and Wang, 2016). Unsurprisingly, PPAR β/δ has also shown to be implicated as a regulator of UV-induced skin tumorigeneis in mice (Montagner et al., 2014). Like in the colon, skin carcinogenesis protocols have also been developed for the cancer investigations in mice (Abel et al., 2009). Thus, skin cancer represents a potential direction of future studies for the FSPC*Cre-Pparb/d^{-/-}* transgenic mice.

Figure 4.1 A graphic illustration showing outcomes of FSPCre-Pparb/d^{-/-} intestinal epithelium during normal conditions, inflammation and cancer

FSPCre-*Pparb/d*^{-/-} stomach and intestines have a thicker mucosa layer than *Pparb/d*^{fl/fl} gut tissues, while cytokine multiplex ELISA assay showed an anti-inflammatory microenvironment in FSPCre-*Pparb/d*^{-/-} intestines. During DSS-induced colitis, the anti-inflammatory microenvironment in FSPCre-*Pparb/d*^{-/-} intestines translated to reduced inflammation-induced tissue damage, compared to *Pparb/d*^{fl/fl}. In CRC model, FSPCre-*Pparb/d*^{-/-} mice harbored significantly fewer tumors. Microarray analyses and confirmation assays highlighted the importance of NRF2-mediated stress response in the FSPCre-*Pparb/d*^{-/-} phenotype.

4.3 Closing remarks

We have modeled the fibroblast-specific deletion of PPARβ/δ gene in a transgenic murine model, the FSPCre-*Pparb/d*^{-/-}. The deletion of fibroblast PPARβ/δ increased proliferation of intestinal villi, resulting in thicker intestinal mucosa layers in FSPCre-*Pparb/d*^{-/-} mice. Our study then showed the importance of fibroblast PPARβ/δ in colitis and tumorigenesis. Backed by gene expression analyses, together with *in vivo* and *in vitro* experiments to validate, we showed

that PPAR β/δ depletion in fibroblasts results in the modification of the antioxidant response and thus oxidative status of the adjacent epithelium.

The precise role of PPAR β/δ in CRC is met with much controversy as studies have shown that PPAR β/δ has both an anti-tumorigenic (Wang et al., 2014) and pro-tumorigenic role in the development of CRC (Harman et al., 2004). While our current results have implicated a pro-tumorigenic role for PPAR β/δ through the modulation of ROS, it is conceivable that PPAR β/δ has dual roles in tumorigenesis, much like ROS during the different stages of tumorigenesis. Oxidative stress has been seen as a double-edged sword of sorts, linked as a promoter of tumorigenesis (Chan et al., 2017), while the modulation of oxidative stress as an anti-cancer therapeutic agent has also been discussed (Gorrini et al., 2013). The temporal and dose-dependent basis of oxidative stress on tumor formation and development may help to explain the duality of the effect of PPAR β/δ on tumorigenesis. As discussed, further investigations are needed to examine the role of ROS on tumorigenesis and normal cellular functions in FSPC*Cre-Pparb/d^{+/-}* mice in the various tissue types, particularly in the skin.

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END

ROS release by *PPAR* β/δ -null fibroblasts reduces tumor load through epithelial antioxidant response

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Abstract

Tumor stroma has an active role in the initiation, growth, and propagation of many tumor types by secreting growth factors and modulating redox status of the microenvironment. Although *PPAR* β/δ in fibroblasts was shown to modulate oxidative stress in the wound microenvironment, there has been no evidence of a similar effect in the tumor stroma. Here, we present evidence of oxidative stress modulation by intestinal stromal *PPAR* β/δ , using a *FSPCre-Pparb/d*^{-/-} mouse model and validated it with immortalized cell lines. The *FSPCre-Pparb/d*^{-/-} mice developed fewer intestinal polyps and survived longer when compared with *Pparb/d*^{fl/fl} mice. The pre-treatment of *FSPCre-Pparb/d*^{-/-} and *Pparb/d*^{fl/fl} with antioxidant *N*-acetyl-cysteine prior DSS-induced tumorigenesis resulted in lower tumor load. Gene expression analyses implicated an altered oxidative stress processes. Indeed, the *FSPCre-Pparb/d*^{-/-} intestinal tumors have reduced oxidative stress than *Pparb/d*^{fl/fl} tumors. Similarly, the colorectal cancer cells and human colon epithelial cells also experienced lower oxidative stress when co-cultured with fibroblasts depleted of *PPAR* β/δ expression. Therefore, our results establish a role for fibroblast *PPAR* β/δ in epithelial–mesenchymal communication for ROS homeostasis.

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Introduction

Tumor initiation, growth, and propagation are frequently accompanied by desmoplasia and acquisition of a reactive stroma, mainly comprising leukocytes, fibroblasts, and endothelial cells [1]. The dynamic and reciprocal relationship between epithelial and mesenchymal compartments of the tumor dictates almost every aspect of cancer progression, even governing the efficacy of therapy and influencing the risk of disease relapse [2]. This epithelial–mesenchymal communication has been usually ascribed to a complex signaling network of growth factors and cytokines. Reactive oxygen species (ROS) have also been identified as potent modifiers of signaling pathways and consequently cell behavior. The balance between production of ROS and their elimination by antioxidants is important for tissue homeostasis [3, 4]. Microenvironmental oxidative stress conjures protective antioxidant response from tissues to regain homeostasis. However, ROS imbalance or the loss of homeostatic control in a continued oxidative microenvironment can either result in cell death caused by oxidative catastrophe or contribute to carcinogenesis, which are tissue- and context-dependent [5–7]. Indeed, ROS are by-products of aberrant cancer cell metabolism and

mitochondrial defects. However, little is known about the effect of tumor modulating ROS signals by fibroblasts, the most prominent cell type in tumor microenvironment.

Recent study showed that a deficiency in fibroblast peroxisome proliferator-activated receptor β/δ (PPAR β/δ) increased oxidative stress in wound microenvironment to delay keratinocyte migration [8]. PPAR β/δ has also been implicated in the pathogenesis of colorectal cancer (CRC). However, its role remains controversial as both pro- and anti-tumorigenic roles were reported from studies using conventional PPAR β/δ -knockout mice [9, 10]. On one hand, PPAR β/δ has been shown to exert potent pro-tumorigenic effects. Early study suggested that nonsteroidal anti-inflammatory drugs (NSAIDs) reduced tumorigenesis through inhibition of PPAR β/δ [11]. PPAR β/δ has been shown to promote UV-induced skin tumorigenesis by upregulating Src expression and activity thereby enhancing the downstream EGFR/Erk1/2 pathway [12]. PPAR β/δ also enhanced CRC progression by regulating Glut1 and SLC10A5 expression, promoting cancer cell metabolic programming [13]. PPAR β/δ also promoted angiogenesis by stimulating the expression of VEGF in CRC and bladder cancer [14–16]. In addition, PPAR β/δ promoted a pro-tumorigenic inflammatory environment through the increased production of COX-2-derived PGE₂ [16]. On the other hand, PPAR β/δ has also been reported to exert potent anti-inflammatory functions through interfering with the functions of transcription factors such as NF κ B, AP1, STAT3 [17]. In melanoma, PPAR β/δ exhibited inhibitory effects on tumorigenicity via the modulation of inflammatory and apoptotic mechanisms [18]. In apparent discrepancies with earlier studies, several studies showed that NSAIDs increased PPAR β/δ expression in human CRC cell lines, suggesting that elevated PPAR β/δ level, rather than decreased level, contributed to the chemopreventive effects of NSAIDs. Using a tissue-specific approach to delineate the problem, subsequent studies using an intestinal epithelia-specific deletion and transgene overexpression of PPAR β/δ in mice supported a pro-tumorigenic role for epithelial PPAR β/δ [19, 20]. Recent work on skin squamous cell carcinoma showed that the expression of PPAR β/δ was reduced in cancer-associated fibroblasts [21]. Further investigation revealed that PPAR β/δ in cancer-associated fibroblasts reduced the invasiveness, xenobiotic resistance and cellular bioenergetics of the adjacent epithelial cancer, at least, in part via the secretion of growth factors and cytokines [21]. However, the role of fibroblast PPAR β/δ in the initiation and progression of CRC remains unclear.

Our understanding on the role of fibroblasts in pathologic conditions is hampered by the absence of specific markers. Fibroblast-specific protein (FSP)1 (or S100A-4) has been suggested as a fibroblast-specific marker in normal and fibrotic tissues. The intestinal mesenchyme comprises of a

heterogeneous population of stromal cells of varying origins [22]. FSP1-directed deletion occurs in fibroblast sub-population that had an active FSP1 promoter activity. Although FSP1 has been identified as a fibroblast marker and used for fibroblast depletion [23, 24], the complete coverage of the entire fibroblast population by a single marker has proven to be tricky. Although FSP1 is only expressed in the mesenchymal lineage and commonly used for stromal-specific gene deletion strategy, it is lowly expressed if at all, in matured fibroblasts and myofibroblasts. Despite these limitations, FSP1 reporter mice and FSP1-Cre-driven gene deletion are considered reliable strategies to investigate fibroblast biology. Transgenic mice with green fluorescent protein expression driven by the FSP1 promoter have been used to map cell fate in fibroblast populations, and that FSP1-Cre animals have served as important tools to dissect the effects of fibroblast-specific gene deletion in fibrotic and neoplastic conditions. FSP1-directed deletion of genes, like Smoothed, PTEN, TGF receptor type II, and BMP receptor 2 in the fibroblasts have been used to study their roles in different tumor types such as acinar–ductal metaplasia, prostate, breast, and squamous cell carcinoma of the forestomach [25–28].

Drawing on the parallels between wound healing and cancer, we investigated the role of fibroblast PPAR β/δ in CRC. We generated a mouse whose PPAR β/δ gene was deleted in the fibroblasts (FSPCre-*Pparb/d*^{-/-}) by crossing a floxed PPAR β/δ mouse (*Pparb/d*^{fl/fl}) [29] with transgenic mice with a Cre transgene under the control of FSP1 promoter (FSP1-Cre) [30]. The FSPCre-*Pparb/d*^{-/-} mice developed fewer CRC when compared to their *Pparb/d*^{fl/fl} littermates, regardless of genetic-, or chemical-induced carcinogenesis. Microarray gene expression analyses revealed an altered oxidative processes in adjacent intestinal or tumor epithelium of FSPCre-*Pparb/d*^{-/-} mice. We recapitulated the reduced oxidative stress phenotype in two human intestinal cancer cell lines (HCT116 and HT29) when co-cultured with colonic fibroblasts from FSPCre-*Pparb/d*^{-/-} mice or fibroblasts whose endogenous PPAR β/δ were suppressed by siRNA. Taken together, our results indicate that PPAR β/δ expression in fibroblast modulates oxidative responses in the adjacent epithelium, and oxidative stress may be an initiating factor in CRC.

Results and discussion

PPAR β/δ expression is ablated in colonic fibroblasts of FSPCre-*Pparb/d*^{-/-} mice

We generated a transgenic mouse line harboring a fibroblast-specific deletion of PPAR β/δ exon 4 allele (FSPCre-*Pparb/d*^{-/-}) (Sng et al., in revision). To study

fibroblast PPAR β / δ ablation in the gastrointestinal tract, we examined the deletion PPAR β / δ exon 4 in the different colonic tissue layers of the FSPCre-*Pparb/d*^{-/-} transgenic mice by dissecting the mucosa villi, stroma lamina propria and muscularis layers with a laser dissecting microscope (Fig. 1a; Supplementary Fig. 1A–D). Subsequent PCR and DNA gel analysis confirmed the deletion of exon 4 of PPAR β / δ gene has occurred in the intestinal fibroblasts, which were enriched in the locality of the stroma lamina propria region (Fig. 1b). To examine PPAR β / δ protein expression in colonic stroma, we enriched for colonic fibroblasts from FSPCre-*Pparb/d*^{-/-} and their *Pparb/d*^{fl/fl} littermates by the fibroblast explant method. Our analysis of explanted FSPCre-*Pparb/d*^{-/-} fibroblasts showed a 60 and 80% decrease in PPAR β / δ protein and mRNA levels, respectively, when compared with *Pparb/d*^{fl/fl} littermates (Fig. 1c, d). The observed residual PPAR β / δ expression may be contributed by two technical difficulties. As mentioned above, FSP1 promoter is active in a subpopulation of fibroblasts. Although, the explant method is a reliable method to enrich and culture primary fibroblasts *in vitro*, it is also inevitable that other cell types such as endothelial cells and inflammatory immune cells from the stroma might have contaminated the culture [22, 31]. Importantly, our observation mirrored that in other studies where FSP1 promoter-driven gene deletion was targeted to the stroma of breast and pancreatic tissues [26, 30]. Taken together, we have established that the FSPCre-*Pparb/d*^{-/-} colonic mesenchyme harbors perturbed PPAR β / δ expression.

Loss in fibroblast PPAR β / δ expression delays intestinal tumorigenesis

To investigate the effects of fibroblast PPAR β / δ expression on intestinal tumorigenesis, we employed three commonly used murine models, chemical (azoxymethane, AOM, and dextran sulfate sodium, DSS), genetic (APC^{min/+}), and combined (APC^{min/+} with DSS) approaches to induce colon tumors in FSPCre-*Pparb/d*^{-/-} mice.

Ulcerative colitis and inflammatory bowel syndrome are common risk factors associated with CRC development [32, 33]. Well-established animal models for ulcerative colitis and inflammatory bowel syndrome have used DSS to induce colitis in mice, thus DSS was used to induce intestinal inflammation in two of the murine models. We investigated whether there were differences in the inflammatory processes that lead to tumor initiation. In DSS-induced colitis, the FSPCre-*Pparb/d*^{-/-} mice yielded a lower disease activity index (DAI) (Fig. 2a and Supplementary Table 2), reduced DSS-induced morphological aberrations and loss of the mucosa layer (Fig. 2b, c) when compared with wild-type littermates. No significant difference in colon lengths was observed (Fig. 2d). Consistent

with the lower disease severity, we detected a reduction of pro-inflammatory cytokines and an increase of anti-inflammatory cytokines in FSPCre-*Pparb/d*^{-/-} when compared with *Pparb/d*^{fl/fl} mice as determined by multiplex immunoassay (Supplementary Table 1). These observations suggest that a deficiency in fibroblast PPAR β / δ confers some protection against colitis, possibly by modulating an inflammatory response.

Next, we looked at chemical induction of colon tumors using AOM/DSS treatment. In similar fashion to our colitis experiment, the FSPCre-*Pparb/d*^{-/-} mice demonstrated better survival and DAI over the course of AOM/DSS treatment, compared to the wild-type (*Pparb/d*^{fl/fl}) mice (Fig. 2e, f). The FSPCre-*Pparb/d*^{-/-} mice also produced 50% fewer and smaller tumors when compared with *Pparb/d*^{fl/fl} littermates (Fig. 2g–j). The results showed that the FSPCre-*Pparb/d*^{-/-} genetic background confers a protection against CRC in mice, suggesting that fibroblast PPAR β / δ is important in the promotion of tumor development.

To study intestinal tumor formation in a spontaneous CRC murine model, we crossed FSPCre-*Pparb/d*^{-/-} mice to an APC^{min/+} background (Supplementary Figure 1E–M). APC^{min/+} mice were either allowed to spontaneously develop tumors or stimulated chemically by a 1-week DSS treatment course. First, we noted a lower DAI in APC^{min/+} transgenic mice on the FSPCre-*Pparb/d*^{-/-} background (Supplementary Figure 1E & F). In both models, we also noted that mice deficient in fibroblast PPAR β / δ were more resistant to the disease burden of tumor development (Supplementary Figure 1G) as suggested by higher survival rates (Supplementary Figure 1H & K). The mice deficient in fibroblast PPAR β / δ also developed fewer and smaller tumors compared to cognate wild-type *Pparb/d*^{fl/fl} littermates (Supplementary Figure 1G, I–J & L–M). Thus, regardless of the oncogenic stimuli, either with AOM/DSS, APC^{min} or combined, our results indicate that reduced PPAR β / δ expression in fibroblasts delays CRC development.

Fibroblast PPAR β / δ alters NRF2-mediated response in FSPCre-*Pparb/d*^{-/-} intestinal epithelium

To gain mechanistic insight into how PPAR β / δ expression in fibroblasts mediates changes in its adjacent epithelial cells, we performed a comparative gene expression analysis of epithelial and mucosal layers from normal intestines and tumors from FSPCre-*Pparb/d*^{-/-} and APC^{min/+}FSPCre-*Pparb/d*^{-/-}. Ingenuity pathway analyses revealed that genes involved in oxidative phosphorylation and mitochondrial dysfunction were enriched in FSPCre-*Pparb/d*^{-/-} mice (Fig. 3a–c). Interestingly, our upstream regulator analyses of normal tumor adjacent and tumor epithelia revealed

Fig. 1 *PPAR β/δ* expression is ablated in colonic fibroblasts of FSPCre-*Pparb/d*^{-/-} mice. **a** Representative H&E-stained images of colons before and after laser capture microdissection of colonic layers, mucosal, lamina propria, and muscularis. FSPCre-*Pparb/d*^{-/-} mice were generated in-house by crossing *Pparb/d*^{fl/fl} and FspCre mice progenies, and backcrossed with *Pparb/d*^{fl/fl} for at least six generations. Scale: 500 μ m **(b)** Schematic diagram depicting the relative position of PCR genotyping primers for floxed and deleted *Pparb/d* exon 4 alleles, at 400 bp (red), and 490 bp (blue), respectively (top). Sample DNA were processed as instructed in the KAPA Express Extract kit and 2 $\times$ KAPA2G Fast Genotyping Mix. DNA gel image showing genotyping results indicated tissue layers colonic submucosa and lamina propria layer (L.p.); mucosal villi (Vil.); smooth muscle (Mus.) layers. **c** Representative images showing the outgrowth of fibroblasts from colonic explants from *Pparb/d*^{fl/fl} and FSPCre-*Pparb/d*^{-/-} mice. Fresh colons were dissected longitudinally and washed in ice cold phosphate

buffered solution (PBS) containing 30% antimycotic/antibiotic solution. Specimens were minced to 1–2 mm² pieces and incubated in fresh culture media for 72 h to allow colonic fibroblasts to migrate out of the explants. Migrated cells were washed and cultured for another 48 h before protein and RNA analyses. Scale bar: 500 μ m. **c–d** Relative fold change in expression of *PPAR β/δ* mRNA **c** and *PPAR β/δ* protein **d** in migrated colonic fibroblasts. Total RNA was extracted from cells using TRIzol Reagent with E.Z.N.A Total RNA Kit according to manufacturer's protocol. Reverse transcription real-time quantitative PCR (RT-qPCR) were performed with 18S rRNA as a housekeeping gene. Samples were homogenized and lysed in mammalian protein extraction reagent (M-PER) supplemented with complete protease inhibitor mix. Far-infrared immunoblotting was performed. β -tubulin that served as housekeeping protein was from the same samples. Data are represented as mean $\pm$ S.E.M. from at least four independent experiments

enhanced NRF2-mediated stress responses owing to the deletion of fibroblast *PPAR β/δ* (Fig. 3d, e). NRF2 is a master regulator of antioxidant responses through binding to the antioxidant response elements in the gene's promoter region. It upregulates the expression of numerous antioxidant proteins to protect the cell from oxidative damages [34]. Available experimental evidence also clearly indicates that NRF2 is an important player in the maintenance of mitochondrial homeostasis and structural integrity (Holmstrom, Kostov et al. 2016). This role becomes particularly critical under conditions of oxidative, electrophilic, and inflammatory stress which influences the overall health and survival of the organism. The loss of NRF2 in mouse has been associated with increased susceptibility to CRC development [35], underscoring NRF2 as a potential chemopreventive target [36]. PPARs have been implicated as

one of the downstream mediators of NRF2 [37]. For example, functional PPAR response element has been identified in the glutathione S-transferase [38, 39], glutathione peroxidase 1, and catalase genes [8]. *PPAR β/δ* has also been shown to exert antioxidant effect on cardiomyocytes by inhibiting PI3K/Akt signaling pathway to suppress ROS generation induced by AngII, however very little is known about its role in redox regulation in the gut [40–42]. Therefore, we winnowed our attention on NRF2 and associated antioxidant genes in the colon of FSPCre-*Pparb/d*^{-/-} mice.

We observed an increase in NRF2 expression, concomitant with elevated expression of antioxidant enzymes in intact FSPCre-*Pparb/d*^{-/-} colons (Fig. 3f). We also found higher expression of antioxidant (e.g., glutathione peroxidase and catalase) genes in the FSPCre-*Pparb/d*^{-/-}

compared with *Pparb/d^{fl/fl}* intestines (Fig. 3g). This was supported by increased NRF2 staining in the intestinal villi, as well as increased nuclear NRF2 protein in FSPCre-

Pparb/d^{fl/fl} colons (Fig. 3h-i). Indeed, Therefore, our data provides evidence that NRF2 and its downstream oxidative stress signaling pathways were elevated in FSPCre-*Pparb/d*

◀ **Fig. 2** Loss in fibroblast PPAR β/δ expression retards intestinal tumorigenesis. **a** Mean disease activity index (DAI) score of DSS-treated mice over 7 days. Litter-matched mice were fed with 2% DSS in drinking water ad libitum and observed at days 3, 5, and 7. DAI score was computed as detailed in Supplementary Materials and Methods. Values are mean $\pm$ S.E.M. ($n=5$ for each genotype per condition). **b** Representative H&E stained images of mouse colons and ileums from *Pparb/d^{fl/fl}* and FSPC*re-Pparb/d^{-/-}* in vehicle- and DSS-treated conditions. Scale: 100 μ m. **c** Mean villus number measurement of mouse ilea in Vehicle and DSS-treated mice. H&E stained images of mouse ilea ($n=8$) were used for the recording of villus number in ImageJ software version 1.45 s. **d** Length of colons from *Pparb/d^{fl/fl}* and FSPC*re-Pparb/d^{-/-}* mice after treatment with vehicle or DSS. Each data point represents one mouse. **e** Percentage survival of mice after AOM/DSS treatment for 10 weeks. Mice were injected with 10 mg/kg AOM. After 1 week, they were given 2% DSS in drinking water ad libitum. Litter-matched FSPC*re-Pparb/d^{-/-}* mice ($n=13$) and *Pparb/d^{fl/fl}* mice ($n=18$) were used. **f** Mean DAI score of AOM/DSS-treated FSPC*re-Pparb/d^{-/-}* mice ($n=13$) and *Pparb/d^{fl/fl}* littermates ($n=18$). DAI score was computed as detailed in Supplementary Materials and Methods. Values are mean $\pm$ S.E.M. **g** Representative images of colons from vehicle- and AOM/DSS-treated *Pparb/d^{fl/fl}* and FSPC*re-Pparb/d^{-/-}* mice. Colons were dissected longitudinally from the distal to proximal end (right to left) and inspected for tumor number and size using an upright dissecting microscope. Scale: 1 cm. **h** Bar graph depicting total colonic tumor numbers per mouse in AOM/DSS-treated FSPC*re-Pparb/d^{-/-}* mouse colons ($n=6$) and cognate *Pparb/d^{fl/fl}* colons ($n=6$). Values are mean $\pm$ S.E.M. **i** Box-and-whisker plot showing distribution of tumor number and size in AOM/DSS-treated *Pparb/d^{fl/fl}* and FSPC*re-Pparb/d^{-/-}* mice. The tumors were categorized into three groups by tumor diameter (small < 2 mm; middle, 2–4 mm, large > 4 mm). Fewer large tumors were observed in FSPC*re-Pparb/d^{-/-}* colons than *Pparb/d^{fl/fl}* colons. Values are mean $\pm$ S.E.M. ($n=6$ for each genetic background). **j** Representative H&E-stained images of tumors and adjacent colon tissues from FSPC*re-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice. Scale: 200 μ m

$^{-/-}$ colons and may be a major contributing factor for the reduced CRC in these mutant mice.

PPAR β/δ deficiency in fibroblasts lowers oxidative stress in HCT116, HT29, and iCEC cells

As shown earlier in the inflammatory model, FSPC*re-Pparb/d^{-/-}* colons had a less-inflamed phenotype, with more anti-inflammatory cytokines and less-inflammatory cytokines, compared with their *Pparb/d^{fl/fl}* littermates. Inflammatory immune cells release a myriad cytokines, growth factors, and ROS to create an inflammatory microenvironment that is conducive for tumor development [43, 44]. Thus, we questioned if infiltrating immune cells could contribute to the difference in the tumor load between FSPC*re-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice. We did not observe differences in the number of CD11b⁺ immune cell numbers between two genotypes (Fig. 4a, b). Although the total number of infiltrating CD11b⁺ immune cells was similar, multiplex immunoassay analysis (Supplementary Table 1) suggested potential differences in the relative

abundance of neutrophils, T-cells and macrophages between FSPC*re-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* colons. We did not find any significant difference in the number of infiltrating neutrophils (Ly6G⁺CD11b⁺) and T lymphocytes (CD3⁺CD11b⁺) between the FSPC*re-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* colons as determined by FACS analysis (Supplementary Fig. 2A & B). We noted that the vehicle-treated FSPC*re-Pparb/d^{-/-}* colons have a higher infiltrated macrophage (F4/80⁺Cd11b⁺) than *Pparb/d^{fl/fl}*, which was no longer distinguishable upon DSS stimulation (Supplementary Fig. 2C). Further characterization of macrophage subtypes revealed that vehicle-treated FSPC*re-Pparb/d^{-/-}* colons have more of the anti-inflammatory M2 macrophages, but higher M1 macrophages upon DSS treatment (Supplementary Fig. 2D). The lower M1:M2 ratio in FSPC*re-Pparb/d^{-/-}* colon was consistent with the reduced inflammatory cytokine landscape. Clinical and experimental evidence have indicated that inflammatory milieu and elevated ROS contribute to cancer initiation [45, 46]. ROS production is usually associated with the activation and functions of M1 rather than M2 macrophages. Together, they suggest that immune cells have minor role in tumor initiation of FSPC*re-Pparb/d^{-/-}* mice.

Previous study reported that PPAR β/δ in wound fibroblasts transcriptionally regulates the expression of H₂O₂-detoxifying enzymes, glutathione peroxidase 1 and catalase, culminating in reduced ROS in the wound microenvironment [8]. Indeed, we detected elevated extracellular H₂O₂ in the conditioned medium of PPAR β/δ -deficient fibroblasts compared to their wild-type counterparts (Fig. 4c). No significant difference in intracellular H₂O₂ levels in the fibroblasts (Fig. 4d, e). Explanted FSPC*re-Pparb/d^{-/-}* fibroblasts also have overall reduction in the expression antioxidant genes (8 of 12 genes), suggesting a more oxidative phenotype that contributed to the elevated H₂O₂ production and secretion into the microenvironment (Fig. 4f). Numerous studies have showed that the activation of cytoprotective NRF2-mediated stress responsive genes in response to elevated oxidative stress, like extracellular H₂O₂. Thus, to strengthen our observation that the novel epithelial-mesenchymal communication in oxidative stress homeostasis was dysregulated in FSPC*re-Pparb/d^{-/-}*, as well as a direct effect of fibroblast PPAR β/δ on the oxidative status of adjacent epithelia, we measured ROS in two different human intestinal cancer cell lines (HCT116 and HT29) co-cultured with either PPAR β/δ -deficient fibroblasts or in conditioned media from FSPC*re-Pparb/d^{-/-}* and *Pparb/d^{-/-}* colonic fibroblasts (Fig. 4g–o). Consistent with our gene expression data, we detected higher expression of NRF2 and associated antioxidant enzymes in HCT116 and HT29 cultured in conditioned media from FSPC*re-Pparb/d^{-/-}* than in *PPARb/d^{fl/fl}* colonic fibroblasts (Fig. 4g, h). This is coupled with reduced ROS levels in

HCT116 cells cultured in conditioned media from FSPCre-Pparb/d^{-/-} colonic fibroblasts (Fig. 4i). In support, we also detected higher NRF2 expression, elevated mRNA levels of

antioxidant enzymes and reduced ROS levels in HCT116 cells when co-cultured with siPPAR β / δ -knockdown BJ-1 fibroblasts (BJ-1^{siPPAR β / δ}) (Fig. 4 j-l) or CCD18Co

◀ **Fig. 3** Fibroblast PPAR β/δ alters NRF2 and associated oxidative processes in FSPC*Cre-Pparb/d^{-/-}* intestinal epithelium. **a–c** Gene Ontological Pathway Analysis of differentially expressed genes from colon mucosa (**a**), ileum mucosa (**b**), and intestinal tumor epithelium (**c**). Total RNA was isolated from LCM tissues using the Ambion RecoverAll Total Nucleic Acid Isolation Kit for FFPE and processed for array hybridization using the GeneChip WT Pico Kit and GeneChip Mouse Gene 1.0 ST Array gene chips. The data were normalized and analyzed using the Partek Genomics Suite v6.6. Multi-way ANOVA analysis was used to compare FSPC*Cre-Pparb/d^{-/-}* (KO) against *Pparb/d^{fl/fl}* (WT). Gene ontology (GO) analysis of gene subsets was performed using the Ingenuity Pathway Analysis software. The top five GO analysis were in descending order as determined by their *p*-value. Pathway analyses were conducted using Ingenuity Pathway Analysis (IPA), comparing FSPC*Cre-Pparb/d^{-/-}* (KO) against *Pparb/d^{fl/fl}* (WT) samples (*n* = 3; Fold change cutoff $\geq \pm 1.3$; *p* < 0.05). * Oxidative Phosphorylation and Mitochondrial Dysfunction were two common pathways placed in the top three most significant pathways across all tissues. **d, e** Unguided hierarchical clustering by upstream regulator (**d**) and pathway analysis (**e**) of differentially expressed genes in normal, tumor adjacent and tumor epithelium tissues from FSPC*Cre-Pparb/d^{-/-}* (KO) and *Pparb/d^{fl/fl}* (WT) mice. Our analysis also flagged NRF2-mediated oxidative stress response as an upregulated pathway in FSPC*Cre-Pparb/d^{-/-}* colonic epithelium. *NFE2L2 (Nuclear factor erythroid 2-related factor 2) is a synonym of NRF2 (Nuclear factor erythroid 2-related factor 2). **f** Intracellular H₂O₂ levels in colons from FSPC*Cre-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice as determined by Amplex Red H₂O₂ assay. Samples were homogenized and supernatants incubated with appropriate reagents according to manufacturer's instructions. Oxidation of Amplex Red results in the production of fluorescent resorunfin (571 nm ex and 585 nm em). A calibration curve was prepared (0–20 μ M) and used to calculate H₂O₂ concentration of samples. The concentration of H₂O₂ was normalized to total protein. Data are represented as mean $\pm$ S.E.M. from at least four independent experiments. **g** Relative fold change in mRNA levels of 13 genes associated with oxidative stress response in APC^{min/+}FSPC*Cre-Pparb/d^{-/-}* and APC^{min/+}*Pparb/d^{fl/fl}* colons. Total RNA was extracted, and RT-qPCR was performed as described in legends of Fig. 1d, e. Ribosomal RNA L27 used as housekeeping gene. Values are mean $\pm$ S.E.M. (*n* = 4). **p* < 0.05. **h** Representative images of immunohistochemical staining for NRF2 (black arrows) in FSPC*Cre-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* colons. Sections were counterstained with hematoxylin for visualization of tissue morphology. The number of NRF2-positive cells was recorded using ImageJ software version 1.45 s. Black arrows indicate representative NRF2-positive regions. Scale bar: 50 μ m. **i** Fold change in NRF2 protein in colons from FSPC*Cre-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice. Representative immunoblot and graph for NRF2 protein are shown. Samples were processed for western blot as described in legends of Fig. 1d, e. PCNA that served as housekeeping protein were from the same samples. Data are represented as mean $\pm$ S.E.M. from at least three independent experiments

myofibroblasts (CCD18Co^{siPPAR β/δ}) (Fig. 4m–o) compared with cognate controls (BJ-1^{siScrambled} and CCDCo^{siScrambled}). We similarly observed an NRF2 antioxidant response in immortalized non-tumorigenic human colon epithelial cells cultured in either conditioned media from FSPC*Cre-Pparb/d^{-/-}* or the presence of exogenous H₂O₂ (Supplementary Figure 2 E–G). This response was attenuated in the presence of antioxidant N-acetyl cysteine (NAC). Chronic or repeated exposure to low level of H₂O₂, in combination with

growth factors, has been shown to exert transformative and growth-promoting effects on epithelial cells [47]. We observed an increase in HCT116 cells at either G2/M or S phase when was co-cultured with PPAR β/δ -knockdown fibroblast cell lines compared with cognate controls (Supplementary Figure 2H).

Our findings suggest that an elevated NRF2-related response in the colon epithelia was associated with reduced CRC. Thus, we explore the effect of NAC pre-treatment on CRC initiation in DSS-treated APC^{min/+}*Pparb/d^{fl/fl}* and APC^{min/+}FSPC*Cre-Pparb/d^{-/-}* mice (NAC/DSS treatment). APC^{min/+}*Pparb/d^{fl/fl}* mice developed more and larger CRCs than APC^{min/+}FSPC*Cre-Pparb/d^{-/-}* (Supplementary Figure 1L–M). The pre-treatment with NAC reduced the number of tumors in APC^{min/+}*Pparb/d^{fl/fl}* by 87% when compared no NAC pre-treatment (Supplementary Figure 1N–P), suggesting that ROS have an important role in early tumor development. There was no difference in the number of tumors between NAC pre-treated or untreated APC^{min/+}FSPC*Cre-Pparb/d^{-/-}* mice (Supplementary 1L & O), suggesting that further reduction in epithelial ROS has minimal impact on tumor development. Paradoxically, we observed a reduced median survival rate of the APC^{min/+}FSPC*Cre-Pparb/d^{-/-}* mice pre-treated with NAC (Fig. 1n). Although the precise reason for this observation is unclear, we speculate that additional reduction in epithelial ROS might have disrupted critical ROS-mediated cellular functions necessary for homeostasis in APC^{min/+}FSPC*Cre-Pparb/d^{-/-}* mice, which already have elevated NRF2 antioxidant response. Studies from the past two decades have established that ROS serve as signaling molecules to regulate biological and physiological processes [48]. Clearly, future experiments will be necessary to understand the effect of antioxidant treatment on normal cellular functions in FSPC*Cre-Pparb/d^{-/-}* mice, as well as the optimal antioxidant treatment regime to reduce CRC in wild-type mice.

We showed a major role for stromal PPAR β/δ in ROS production that consequently conjures an NRF2-mediated antioxidant response, reducing ROS levels in the adjacent intestinal epithelial cancer cells. NRF2-mediated response also results in the activation of various signaling pathways that facilitates tumor cell senescence [49, 50]. Collectively, we propose that FSPC*Cre-Pparb/d^{-/-}* colonic epithelium are subjected to a constant dose of ROS, extracellular H₂O₂ from their neighboring stroma, which triggers NRF2-mediated signaling that suppresses tumor development.

Conclusion

Our data have shown that PPAR β/δ ablation in fibroblasts results in reduced tumorigenesis across three different mice models of CRC. Microarray gene expression and gene

ontology analyses, together with *in vivo* and *in vitro* experiments indicate that PPARβ/δ depletion in fibroblasts alters the antioxidant responses and thus the oxidative status

of the adjacent epithelium. We propose that PPARβ/δ deficiency in fibroblasts increases extracellular H₂O₂, triggering an NRF2-mediated antioxidant response in the

Fig. 4 PPAR β/δ deficiency in fibroblasts lowers oxidative stress in colon cell lines. **a, b** FACS analysis of CD11b⁺ cell numbers in colons from FSPCre-*Pparb/d*^{-/-} and *Pparb/d*^{fl/fl} mice treated with vehicle or DSS (**a**) and APC^{min/+}FSPCre-*Pparb/d*^{-/-} and APC^{min/+}*Pparb/d*^{fl/fl} colons (**b**). Fresh tissue biopsies were dissociated using the gentleMACS Dissociator according to manufacturer's instruction. Homogenates were strained, washed, and processed for staining with fluorophore-conjugated antibodies on ice. Flow cytometry was performed using Accuri C6 Flow Cytometer and analyzed on FlowJo v10.0.7. Values are mean $\pm$ S.E.M. ($n = 6$). **c** Extracellular H₂O₂ levels of explanted fibroblasts from *Pparb/d*^{fl/fl} and FSPCre-*Pparb/d*^{-/-} colons detected by Amplex Red Assay as described in legend of Fig. 3f. Values are mean $\pm$ S.E.M. ($n = 6$). **d, e** Intracellular H₂O₂ levels of explanted fibroblasts from *Pparb/d*^{fl/fl} and FSPCre-*Pparb/d*^{-/-} colons detected by CellROX. Cells were first positively gated for fibroblast marker (CD140a or PDGRA) and the CellROX fluorescence readings were taken from the CD140a⁺ gated cells. Values are mean $\pm$ S.E.M. ($n = 4$). **f** Relative fold change in mRNA levels of 13 genes associated with oxidative stress response in explanted fibroblasts from *Pparb/d*^{fl/fl} and FSPCre-*Pparb/d*^{-/-} colons. Total RNA was extracted, and RT-qPCR was performed as described in legends of Fig. 1d, e. were Ribosomal 18S rRNA served as a housekeeping gene. Data are represented as mean $\pm$ S.E.M. from 4 independent experiments. * $p < 0.05$, ** $p < 0.01$. **g, h, k, n** Relative fold change in mRNA levels of 13 genes associated with oxidative stress response in HCT116 (**g, k, n**) and HT29 cells (**h**) cultured in conditioned medium of fibroblasts from *Pparb/d*^{fl/fl} and FSPCre-*Pparb/d*^{-/-} (**g, h**), or co-cultured with BJ-1 (**k**) and CCD18Co cells (**n**) whose endogenous PPAR β/δ was suppressed by siRNA. Cells transfected with siRNA of PPAR β/δ and Scrambled were denoted by superscript. Data are represented as mean $\pm$ S.E.M. from four independent experiments. * $p < 0.05$, ** $p < 0.01$. **i, l, o** Intracellular oxidative stress levels in HCT116 cells (**i, l, o**) either cultured in condition medium of fibroblasts from *Pparb/d*^{fl/fl} and FSPCre-*Pparb/d*^{-/-} (**i**) or co-cultured with BJ-1 (**l**) and CCD18Co cells (**o**) whose endogenous PPAR β/δ was suppressed by siRNA. Cells transfected with Scrambled siRNA served as cognate controls. Intracellular ROS level of HCT116 cells cultured in conditioned medium from or co-cultured with PPAR β/δ -deficient fibroblasts as determined by CM-H₂DCFDA assay. Values are mean $\pm$ S.E.M. ($n = 4$). **j, m** Relative fold change in PPAR β/δ mRNA expression in BJ-1 fibroblasts (**j**) and CCD18co myofibroblasts (**m**) whose endogenous PPAR β/δ was suppressed by siRNA at 48 and 72 h post transfection. Cells transfected with Scrambled siRNA served as cognate controls. 18S rRNA served as housekeeping gene. Data are represented as mean $\pm$ S.E.M. from four independent experiments. * $P < 0.05$, ** $P < 0.01$

adjacent epithelia. The elevated expression of NRF2-dependent proteins is critical for eliminating carcinogens to maintain cellular redox homeostasis. Consequently, FSPCre-*Pparb/d*^{-/-} mice have reduced colonic polyp formation.

Nuclear receptor PPAR β/δ has been implicated in CRC, although it remained controversial as studies have shown supporting evidence for PPAR β/δ playing an anti-tumorigenic [16] and pro-tumorigenic roles in CRC [51]. It is conceivable that PPAR β/δ has dual roles in tumorigenesis, much like TGF β -1 and ROS. By regulating cell growth, death, and immortalization, TGF β signaling pathways exert tumor suppressor effects in normal cells and early carcinomas. But as tumors progress, these protective

and cytostatic effects of TGF β are often lost, switching to promote cancer progression, invasion, and tumor metastasis. Similarly, chronic oxidative stress has been shown to promote tumorigenesis [47, 52], whereas the modulation of oxidative stress as an anticancer therapeutic agent has also been discussed [53]. With the temporal and dose-dependent basis of oxidative stress on tumor formation and development, this may explain the dual effect of PPAR β/δ on tumorigenesis.

Limitations of our study include that one genetic background of mouse and our deletion strategy consists of the deletion of exons coding for the DNA-binding domain of PPAR β/δ . Different mouse strains may exhibit different susceptibility to carcinogen or tolerance to oxidative stress. It is also conceivable that other gene deletion strategies may result in different phenotypic severity or outcomes owing to differences in vulnerabilities to the oxidative stress. FSP1 is a key marker of a specific subset of macrophages in the liver during fibrosis and injury [54], although no report has described confounding issues in other organs. Nevertheless, to the best of our knowledge, this is the first study to examine the role of fibroblast PPAR β/δ in CRC, which reveals novel epithelial-mesenchymal communication in ROS homeostasis. Our results illustrate that PPAR β/δ modulation of oxidative stress has potential medical applicability.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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ROS release by *PPAR* β / δ -null fibroblasts reduces tumor load through epithelial antioxidant response.

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Supplementary Information

Supplementary Materials and Methods

Generation of FSPCre-Pparb/d^{-/-} mice

Fsp1Cre transgenic mice were from Jackson Laboratory, Maine, USA (stock number 012641). The FspCre transgenic mice express Cre recombinase under the control of Fsp1 promoter. The *Pparb/d^{fl/fl}* transgenic mice were obtained from Prof Walter Wahli (University of Lausanne, Lausanne, Switzerland). The transgenic mice carry alleles of the PPAR β/δ gene where the exon 4, which encodes the DNA-binding domain, was flanked by loxP sites ⁴. FSPCre-*Pparb/d^{-/-}* mice were generated in-house by crossing *Pparb/d^{fl/fl}* and FspCre mice progenies, and backcrossed with *Pparb/d^{fl/fl}* in C57BL/6 background for at least 6 generations.

Generation of mice on APC^{min} x FSPCre-Pparb/d^{-/-} background

APC^{min/+} mice were purchased from The Jackson Laboratory (The Jackson Laboratory, Maine, USA) and maintained as heterozygous min/+ mice. APC^{min/+} mice were crossed with FSPCre-*Pparb/d^{-/-}* mice to generate mice with mixed APC^{min/+} and FSPCre-*Pparb/d^{-/-}* backgrounds.

Mouse genotyping

Small ear biopsies (< 0.5 cm) were taken from 3-week old mice and harvested for DNA as instructed in the KAPA Express Extract kit (Kapa Biosystems, Massachusetts, USA). PCR was performed with 2X KAPA2G Fast Genotyping Mix (Kapa Biosystems, Massachusetts, USA) with appropriate primers (Primer list attached).

Chemical-induced murine colorectal cancer and colitis

To chemically induce colonic tumors, mice were intraperitoneally injected with 10 mg/kg body weight azoxymethane (AOM; Sigma-Aldrich Co. LLC, Missouri, USA) dissolved in sterile 0.9 % saline. One week after injection, mice were fed 2% (w/v) dextran sulfate sodium (DSS; MP Biomedicals LLC, California, USA) dissolved in drinking water for 7 days *ad libitum*. To induce colitis, APC^{min} mice were given 2% (w/v) DSS. Mice were then returned to normal drinking water until sacrifice. For anti-oxidant treatment, mice were administered with 400 µL of 40 mM N-acetylcysteine (NAC, Sigma-Aldrich Co. LLC, Missouri, USA) intra-gastrically for 5 consecutive days prior to DSS treatment. Mice were maintained at the facility under a 12-h dark-light cycle at 23°C ± 2°C and given standard mouse chow and water *ad libitum*. The DAI of treated mice were determined by combining scores of stool consistency, gross bleeding and gait changes (Supp. Table 2) ⁷. Power analysis was used to estimate sample size. Double blinded randomization was used. All protocols were approved by the Institutional Animal Care and Use Committees (IACUC) in Nanyang Technological University (NTU), Singapore (Reference number: ARF-SBS/NIE-A0217) and SingHealth, Singapore (Reference number: IACUC #2013/SHS/866).

Colonic fibroblast explant

Colons were dissected longitudinally and washed for 30 min in ice cold phosphate buffered solution (PBS) containing 30% antimycotic/antibiotic solution (AA) (Thermo Fisher Scientific Inc., Massachusetts, USA). Specimens were further dissected to 1-2 mm² pieces and placed into empty culture plates for 10-15 min to promote attachment. Next, DMEM-10 medium supplemented with 10% FBS and 10% AA was added. The explants were incubated in a humidified incubator at 37°C and 5% CO₂. Colonic fibroblasts migrate out of the

explants after 72 h of explant incubation. Colonic fibroblasts were cultured for another 48 h before harvest.

Cell Culture

Human colon adenocarcinoma cell lines (HCT116; ATCC No. CCL-247), human immortalized skin-derived fibroblasts (BJ1; ATCC No. CRL-2522) and human colon myofibroblasts (CCD18Co, ATCC No. CRL-1459) were obtained from American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% FBS (HyClone, Utah, USA). SV40-immortalized human colon epithelial cells were procured from Applied Biological Materials Canada (iCECs; T0570; ABM, Vancouver, Canada) and cultured in Prigrow III Medium (ABM Canada) in collagen-coated flasks. The siRNA knockdown against PPAR β/δ was accomplished using Dharmacon ON-TARGETplus siRNA according to the manufacturer's protocol (Thermo Fisher Scientific Inc., Massachusetts, USA). Colon epithelial cells were seeded in 24-well transwell plates (Corning Inc, New York, USA) and were either incubated with conditioned media of explanted fibroblasts Pparb/d^{fl/fl} and FSP-Pparb/d^{-/-} mice for 24 hours, co-cultured with BJ1 or CCD18co in the top chamber of a transwell 0.4 μ m pore size membrane insert for 48 hours, or treated with 1 μ M H₂O₂ with or without 5mM NAC (Sigma-Aldrich Co. LLC), Missouri, USA.

Laser capture microdissection for FFPE tissue collection

Tissue sections were mounted onto MembraneSlide 1.0 PEN (Carl Zeiss AG, Oberkochen, Germany) and histologically stained. Laser capture microdissection was performed using the PALM MicroBeam system (Carl Zeiss AG, Oberkochen, Germany) as previously described ^{1, 2}.

Real-time qPCR

Total RNA was extracted from cells or tissues using TRIzol Reagent (Invitrogen, California, USA) followed by E.Z.N.A Total RNA Kit (Omega Bio-Tek, Georgia, USA). The purity and quantity of total RNA were recorded using Nanodrop ND1000 (Thermo Fisher Scientific Inc., Massachusetts, USA). Reverse transcription and real-time quantitative PCR (qPCR) were performed as previously described^{5, 6}. The primers and their respective sequences are found in the attached primer list.

Microarray Analysis

LCM-collected tissues were processed for total RNA using the Ambion RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Life Technologies, California, USA). The quantity of the collected RNA was determined using Bioanalyzer and processed for array hybridization using the GeneChip® WT Pico Kit (Affymetrix, California, USA)⁸. Microarray and data analysis were performed by Denova Science Pte Ltd (Singapore). The GeneChip® Mouse Gene 1.0 ST Array gene chips (Affymetrix, California, USA) were used. The data were normalized and analyzed using the Partek Genomics Suite v6.6 (Partek Inc., Missouri, USA). FSPCre-*Pparb*/*d*^{-/-} and *Pparb*^{fl/fl} samples were compared using multi-way ANOVA analysis in the Partek Genomics Suite. Gene ontology (GO) analysis of gene subsets was performed using the Ingenuity Pathway Analysis software (Ingenuity Systems Inc., California, USA). The microarray protocols and data have been deposited in NCBI's Gene Expression Omnibus (GEO) database accession number (GSE97473).

Flow cytometry

Fresh tissue biopsies were dissociated using the gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Homogenates were strained, washed and processed for

staining with the respective fluorophore-conjugated antibodies on ice, washed and resuspended in PBS. Flow cytometry was performed using Accuri C6 Flow Cytometer (BD Biosciences, New Jersey, USA) and analyses were completed on FlowJo v10.0.7 (FlowJo LLC, Oregon, USA).

Production of H₂O₂ in cultured cells was measured using 5-&-6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; Sigma-Aldrich Co. LLC, Missouri, USA), as per the manufacturer's protocol. The oxidation of CM-H₂DCFDA to dichlorofluorescein (DCF) by H₂O₂ is representative of cellular ROS generated. Production of H₂O₂ in dissociated intestinal tissue was measured using CellROX Green oxidative stress detection kit (Thermo Fisher Scientific Inc., Massachusetts, USA), as per the manufacturer's protocol.

Protein extraction and western blot.

Fresh tissues were homogenized and lysed in mammalian protein extraction reagent (M-PER; Thermo Fisher Scientific Inc., Massachusetts, USA) supplemented with complete protease inhibitor mix (Roche Applied Science, Penzberg, Germany). Proteins from different subcellular compartment were extracted using Bio Basic's membrane, nuclear and cytoplasmic protein extraction kit (#BSP002; Bio Basic Inc, Ontario, Canada). Far-infrared immunoblotting was performed as previously described³. Antibodies used were PPAR β/δ (#sc-1987), NRF2 (#sc-722), PCNA (#sc-56) and β -Tubulin (#sc-9104) from Santa Cruz Biotechnology Inc., Texas, USA.

Cytokine detection by multiplex microbead immunoassay

Colon biopsies were processed and lysed in mammalian protein extraction reagent (M-PER; Thermo Fisher Scientific Inc., Massachusetts, USA) supplemented with complete protease inhibitor mix (Roche Applied Science, Penzberg, Germany). Samples were processed using a multiplex immunoassay service (Discovery Assay; Eve Technologies, Alberta Canada). The multiplex immunoassay was performed using the Milliplex mouse cytokine kit (EMD Millipore, Massachusetts, USA) and reading were obtained using the BioPlex 200 system (Bio-Rad Laboratories, Inc., California, USA). The assay examined 31 cytokines, namely Eotaxin, G-CSF, GM-CSF, IFN γ , IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1 α , MIP-1 β , MIP-2, RANTES, TNF α , VEGF. The sensitivities of the assay ranged from 0.1 to 33.3 pg/mL.

Statistical analysis

Statistical analysis was performed using two-tailed non-parametric tests, either Mann-Whitney test (for 2 variables) or Kruskal-Wallis test (for > 2 variables). All statistical tests were performed using GraphPad Prism software (version 5; GraphPad Software Inc, California, USA), with p values of <0.05 considered significant. (*P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant.)

Supplementary Figure Legends

Suppl Figure 1.

(A - D) Relative fold change in mRNA levels of Vilin (A, C) and vimentin (B, D) in Colon (A, B) and Ileum (C, D) in laser microdissected mesenchyme (mes), and mucosal villi (villi) samples, compared to whole undissected intestine (whole). The results show the enrichment of villin expressing tissue in the villi samples and vimentin expressing tissue in the mesenchyme samples.

(E, F) Mean DAI score of DSS-treated mice over 5 weeks. FSPCre-*Pparb*/*d*^{-/-} mice (n=14) displayed a lower DAI after withdrawal of DSS at week 2 when compared with *Pparb*/*d*^{fl/fl} littermates (n=13) (E). Pretreatment with NAC before DSS results in FSPCre-*Pparb*/*d*^{-/-} mice (n=5) and *Pparb*/*d*^{fl/fl} littermates (n=5) (F). Data are represented as mean ± S.E.M.

(G) Representative images of colons from vehicle-, DSS- or NAC/DSS-treated *Pparb*/*d*^{fl/fl} and FSPCre-*Pparb*/*d*^{-/-} mice in APC^{min/+} background at indicated weeks after treatment. APC^{min/+} mouse spontaneously develops tumors after 25 weeks. Colons from APC^{min/+}FSPCre-*Pparb*/*d*^{-/-} mice showed fewer tumors than APC^{min/+}*Pparb*/*d*^{fl/fl} mice. Pretreatment with NAC before DSS greatly reduces tumor formation in *Pparb*/*d*^{fl/fl}. Scale: 1 cm

(H, K, N) Percentage survival of APC^{min/+}FSPCre-*Pparb*/*d*^{-/-} and APC^{min/+}*Pparb*/*d*^{fl/fl} mice (n=13-14) that were either developed tumors spontaneously for 25 weeks (H), given DSS treatment for 5 weeks (K) or pretreated with NAC and then DSS (N). In untreated and DSS-treated models, APC^{min/+}FSPCre-*Pparb*/*d*^{-/-} mice maintained higher survivability than APC^{min/+}*Pparb*/*d*^{fl/fl} (n=13-15). Pretreatment with NAC before DSS challenge improved the survival for APC^{min/+}*Pparb*/*d*^{fl/fl} mice and poorer survival for APC^{min/+}FSPCre-*Pparb*/*d*^{-/-} mice.

(I, L, O) Bar graph depicting total colonic tumor numbers per mouse. In untreated and DSS-treated models, colons of APC^{min/+} FSPCre-*Pparb*/*d*^{-/-} mice (n=14-15) showed 50% fewer tumors than cognate APC^{min/+} *Pparb*/*d*^{fl/fl} colons (n=13-14). NAC pretreatment of APC^{min/+} FSPCre-*Pparb*/*d*^{-/-} mice (n=5) and cognate APC^{min/+} *Pparb*/*d*^{fl/fl} colons (n=5). Values are mean ± S.E.M.

(J, M, P) Box-and-whisker plot showing distribution of tumor number and size in APC^{min/+} FSPCre-*Pparb*/*d*^{-/-} and APC^{min/+} *Pparb*/*d*^{fl/fl} mice that either spontaneously developed tumors (J), were treated with DSS (M) or were pretreated with NAC and then DSS.

Suppl Figure 2.

(A - D) FACS analysis of immune cells in colons from FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice treated with vehicle or DSS, gated for neutrophils (A), T-lymphocytes (B) and Macrophages (C, D). As expected, DSS treatment resulted in an increase immune cell numbers compared with vehicle treatment. No significant difference in infiltrated neutrophils and T-lymphocytes was observed between the two genotypes (A, B), but FSPCre-*Pparb*/*d*^{-/-} colons showed higher macrophage numbers in the vehicle treated control (C). The macrophages were found to be the anti-inflammatory M2 subtype, agreeing with our earlier observations that FSPCre-*Pparb*/*d*^{-/-} colons contained more anti-inflammatory cytokines. Values are mean ± S.E.M. (n=4).

(E, F) Relative fold change in mRNA levels of 13 genes associated with oxidative stress response in ICEC (E, F) cultured in conditioned medium of fibroblasts from *Pparb*/*d*^{fl/fl} and FSPCre-*Pparb*/*d*^{-/-} (E) or treated with NAC and H₂O₂ (F). The expression of epithelial NRF2 and associated anti-oxidant genes were elevated in colon cancer cells cultured in conditioned medium from PPARβ/δ -deficient fibroblasts. Treatment of cells with H₂O₂ resulted in increased expression of antioxidant genes over vehicle-treated controls. Data are represented

as mean $\pm$ S.E.M. from 4 independent experiments. * $P < 0.05$, ** $P < 0.01$, n.d. expression below assay thresholds.

(G) Intracellular oxidative stress levels in ICEC cells either cultured in condition medium of fibroblasts from *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{-/-}* or treated with H₂O₂ or NAC. Cells with no treatment served as cognate controls. Similar to prior results in HCT116, we observed a significant reduction in intracellular ROS level of ICEC cells cultured in conditioned medium from or co-cultured with PPAR β/δ -deficient fibroblasts as determined by CM-H2DCFDA assay. While H₂O₂-treated cells were significantly differed from untreated and NAC-treated ICECs, NAC-treatment did not produce a significant reduction in intracellular stress. Values are mean $\pm$ S.E.M. (n=4).

(H) Cellular proliferation assay by BrDU-FITC and PI staining of HCT116 cells co-cultured with normal BJ fibroblasts (BJ-1^{siScrambled}) and CCD-18co myofibroblasts (CCD18Co^{siScrambled}) or ablated of PPAR β/δ expression (BJ-1^{siPPAR β/δ} and CCD18Co^{siPPAR β}). Values in bold denote percentage of proliferating cells in S phase and G2/M phase from cells in cell cycle arrest at G1/0 phase.

Suppl Fig. 1

Suppl Fig. 2

Suppl. Table 1: Cytokine levels in colon extracts of *FSP-Pparb/d^{-/-}* mice vs *Pparb/d^{fl/fl}*

Cytokine	<i>Pparb/d^{fl/fl}</i>	<i>FSP-Pparb/d^{-/-}</i>
Eotaxin	1.345 ± 0.195	1.361 ± 0.273
G-CSF	0.03 ± 0.01	0.026 ± 0.005
GM-CSF	0.013 ± 0.005	0.003 ± 0.003
IFNγ	0.004 ± 0.002	0.002 ± 0.002
IL-1a	0.248 ± 0.036	0.346 ± 0.112
IL-1B	0.022 ± 0.007	0.035 ± 0.009
IL-2	0.048 ± 0.008	0.05 ± 0.016
IL-3	0.001 ± 0.001	0.002 ± 0.001
IL-4	0.001 ± 0.001	0.002 ± 0.001
IL-5	0.023 ± 0.005	0.01 ± 0.004
IL-6	0.006 ± 0.002	0.01 ± 0.001
IL-7	0.073 ± 0.007	0.051 ± 0.01
IL-9	0.124 ± 0.013	0.279 ± 0.083
IL-10	0.018 ± 0.002	0.044 ± 0.013
IL-12 (p40)	0.012 ± 0.005	0.01 ± 0.003
IL-12 (p70)	0.027 ± 0.007	0.012 ± 0.006
IL-13	0.004 ± 0.001	0.002 ± 0.001
IL-15	0.196 ± 0.035	0.22 ± 0.076
IL-17	0.01 ± 0.003	0.007 ± 0.002
IP-10	0.443 ± 0.07	0.115 ± 0.026
KC	0.104 ± 0.034	0.21 ± 0.047
LIF	0.019 ± 0.001	0.029 ± 0.003
LIX	0.028 ± 0.022	0.006 ± 0.006
MCP-1	0.09 ± 0.019	0.074 ± 0.017
M-CSF	0.015 ± 0.004	0.036 ± 0.017
MIG	2.282 ± 1.16	0.694 ± 0.415
MIP-1a	0.203 ± 0.022	0.034 ± 0.013
MIP-1B	0.368 ± 0.127	0.051 ± 0.032
MIP-2	0.399 ± 0.055	0.63 ± 0.177
RANTES	1.883 ± 0.659	0.116 ± 0.045
TNFA	0.02 ± 0.004	0.012 ± 0.002
VEGF	0.047 ± 0.006	0.136 ± 0.042

Highlighted **Bold** - indicates difference above 2-fold.
Cytokine in colon extracts (pg /mg protein s.e.m.)

Suppl Table 2: Disease activity index (DAI) scoring matrix for DSS treatment

DAI Score	Stool Consistency	Presence of bleeding	Mouse appearance
0	Normal	None	Normal
1	Moist or sticky	Visible blood traces in stool or around anus	Ruffled fur or altered gait
2	Soft	Gross bleeding	Lethargic or moribund
3	Diarrhea		

Primer List

List of genotyping primers

APC ^{min/+}	
Forward primer 1 (Ap ^{cwt})	5'-GCCATCCCTTCACGTTAG-3'
Forward primer 2 (Ap ^{cmin})	5'-TTCTGAGAAAGACAGAAGTTA-3'
Common reverse primer (Ap ^{cR})	5'-TTCCACTTTGGCATAAGGC-3'
PPAR β/δ floxed	
Forward primer (Pb ^{x10})	5'-GCAGCTGCTCAGCTGCCTGC-3'
Reverse Primer 1 (Ab008)	5'-GGACCCCGTAGTGGAAGCCCGAG-3'
Reverse Primer 2 (Ab021)	5'-ATGCCGAGTGCCAGGCACTTCTG-3'
Fsp1Cre	
Forward primer (fsp ^{creF})	5'-GCGGTCTGGCAGTAAAACTATC-3'
Reverse primer (fsp ^{creR})	5'-GTGAAACAGCATTGCTGTCACTT-3'

List of mouse qPCR primers

PPAR β/δ	
Forward	5'- TGCAGATGGGCTGTGATGGG-3'
Reverse	5'- CTCGAGCTTCATGCGGATTGTC-3'
Mouse catalase	
Forward	5'- CCCCTATTGCCGTTTCGATTCT-3'
Reverse	5'- TTCAGGTGAGTCTGTGGGTTT-3'
Mouse nuclear factor erythroid-derived factor-2 (mNRF2)	
Forward	5'-CCTAGTTCTCCGCTGCTGGG-3'
Reverse	5'-CCATGTCCTGCTGGGACTGTA-3'
Mouse superoxide dismutase 1	
Forward	5'-ATGGCGATGAAAGCGGTGT-3'
Reverse	5'-CCTTGTGTATTGTCCCCATACTG-3'
Mouse superoxide dismutase 2	
Forward	5'-CAGACCTGCCTTACGACTATGG-3'
Reverse	5'-CTCGGTGGCGTTGGATTGTT-3'
Mouse superoxide dismutase 3	
Forward	5'-ACCGGCTTG GTTCTCTTCC-3'
Reverse	5'-CTCCATCGGGTTGTAGTGCG-3'
Mouse glutathione peroxidase 1	
Forward	5'-AATGTCGCGTCTCTCTGAGG-3'
Reverse	5'-TCCGAAGTATTGCACGGG-3'
Mouse glutathione peroxidase 2	
Forward	5'-GAGCTGCAATGTCGCTTTCC-3'
Reverse	5'-TGGGTAAGACTAAAGGTGGG -3'
Mouse glutathione peroxidase 3	
Forward	5'-TTTGTGCCTAATTTCCAGCTCTT-3'

Reverse	5'-GTCCATCTTGACGTTGCTGA-3'
Mouse glutathione peroxidase 4	
Forward	5'-GATGGAGCCCBTTCCTGAACC-3'
Reverse	5'-CCCTGTACTTATCCAGGCAGA-3'

List of human qPCR Primers

Human catalase	
Forward	5'- TCATCAGGGATCCCATATTGTT-3'
Reverse	5'- CCTTCAGATGTGTCTGAGGATTT-3'
Human nuclear factor erythroid-derived factor-2	
Forward	5'-ACACGGTCCACAGCTCATC-3'
Reverse	5'-TGCCTCCAAAGTATGTCAATCA-3'
Human superoxide dismutase 1	
Forward	5'-TCATCAATTTTCGAGCAGAAGG-3'
Reverse	5'-GCAGGCCTTCAGTCAGTCC-3'
Human superoxide dismutase 2	
Forward	5'-AAGTACCAGGAGGCGTTGG-3'
Reverse	5'-TGAACTTCAGTGCAGGCTGA-3'
Human superoxide dismutase 3	
Forward	5'-GGTGCAGCTCTCTTTTCAGG-3'
Reverse	5'-AACACAGTGGCGCCAGCATGGCC-3'
Human glutathione peroxidase 1	
Forward	5'-TCTGTTGCTCGTAGCTGCTG-3'
Reverse	5'-CTATGGATCACCGGGATTTTG-3'
Human glutathione peroxidase 2	
Forward	5'-CTGGTGGTCCTTGGCTTC-3'
Reverse	5'-TG TTCAGGATCTCCTCATTCTG-3'
Human glutathione peroxidase 3	
Forward	5'-GGGGACAAGAGAAGTCGAAGA-3'
Reverse	5'-TG TTCAGGATCTCCTCATTCTG-3'
Human glutathione peroxidase 4	
Forward	5'-TTTCCGTGTAACCAGTTCG-3'
Reverse	5'-TGCCTCCAAAGTATGTCAATCA-3'
Human peroxiredoxin 1	
Forward	5'-AATGCAAAAATTGGGTATCCTGC-3'
Reverse	5'-CCGTGGGACACACAAAAGTAAA-3'
Human peroxiredoxin 2	
Forward	5'-GGTAACGCGCAAATCGGAAAG-3'
Reverse	5'-TCCAGTGGGTAGAAAAAGAGGT-3'
Human peroxiredoxin 3	
Forward	5'-GTCTCGACGACTTTAAGGGAAAA-3'
Reverse	5'-ACTGAAACTGCAACTACTTCACA-3'
Human peroxiredoxin 4	

Forward	5'-CTCAAAGTACTGACTATCGTGG-3'
Reverse	5'-CGATCCCCAAAAGCGATGATTC-3'
Human NADPH oxidase 1	
Forward	5'-AAGGATCCTCCGGTTTTACC-3'
Reverse	5'-TTTGGATGGGTGCATAACAA-3'
Human NADPH oxidase 3	
Forward	5'-ACGGGTCGGATTGTTCGAGG-3'
Reverse	5'-ACGGTCGGATTGTTCGAGG-3'
Human NADPH oxidase 4	
Forward	5'-TGCCTGCTCATTTGGCTGT-3'
Reverse	5'-CCGGCACATAGGTAAAAGGATG-3'

List of mouse and human reference gene qPCR primers

18S Ribosomal RNA (18S rRNA)

Forward	5'-GTAACCCGTTGAACCCCAT-3'
Reverse	5'-CCATCCAATCGGTAGTAGCG-3'

60S Ribosomal protein L27 (RPL27)

Forward	5'-GCTGGTTATCGGGAGTTGG-3'
Reverse	5'-ACTGGCCTGGTGTCTAGAG-3'

List of siRNA sequences used in the study

PPAR β/δ siRNA (J-003435-06)	5'-GACCUGGCCCCUAUUCAUUG-3'
PPAR β/δ siRNA (J-003435-07)	5'-UAUCAUUGAGCCUAAGUUU-3'
PPAR β/δ siRNA (J-003435-08)	5'-GAGCGCAGCUGCAAGAUUC-3'
PPAR β/δ siRNA (J-003435-09)	5'-CCACUACGGUGUUC AUGCA-3'

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ARTICLE

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Selective deletion of PPAR β/δ in fibroblasts causes dermal fibrosis by attenuated LRG1 expression

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Abstract

Connective tissue diseases of the skin are characterized by excessive collagen deposition in the skin and internal organs. Fibroblasts play a pivotal role in the clinical presentation of these conditions. Nuclear receptor peroxisome-proliferator activated receptors (PPARs) are therapeutic targets for dermal fibrosis, but the contribution of the different PPAR subtypes are poorly understood. Particularly, the role of fibroblast PPAR β/δ in dermal fibrosis has not been elucidated. Thus, we generated a mouse strain with selective deletion of PPAR β/δ in the fibroblast (FSPCre-*Pparb*/*d*^{-/-}) and interrogated its epidermal and dermal transcriptome profiles. We uncovered a downregulated gene, leucine-rich alpha-2-glycoprotein-1 (*Lrg1*), of previously unknown function in skin development and architecture. Our findings suggest that the regulation of *Lrg1* by PPAR β/δ in fibroblasts is an important signaling conduit integrating PPAR β/δ and TGF β 1-signaling networks in skin health and disease. Thus, the FSPCre-*Pparb*/*d*^{-/-} mouse model could serve as a novel tool in the current gunnery of animal models to better understand dermal fibrosis.

Introduction

Connective skin diseases are commonly characterized by excessive deposition of collagen in the skin and internal organs^{1, 2}. The classical hallmark of collagen overproduction is caused by myofibroblasts that are undergoing a dysregulated balance of signaling mediators, including cytokines and growth factors, resulting in dermal fibrosis that is observed in such diseases³. A prototypical and well-studied example of a connective skin disease is scleroderma (SSc). To date, treatments for these conditions exist only to provide symptomatic reliefs and helps to prevent further complications⁴. The precise etiology for the various clinical manifestations, however,

remains unclear. Many animal models have been developed to better understand the disease pathogenesis^{5, 6}. Although each animal model could not completely recapitulate all aspects of connective tissue diseases, these models collectively have advanced our understanding of the different aspects of these conditions. Importantly, they have underscored that connective tissue diseases, and the underlying dermal fibrosis and overproduction of collagen, are multigenic and involves communications among key cell types, such as keratinocytes, fibroblasts, and immune cells⁵⁻⁷.

Nuclear receptors (NRs) represent one of the largest classes of transcription factors that are also known to be druggable targets from the clinical perspective⁸⁻¹⁰. A variety of skin diseases is currently treated with FDA-approved drugs that target NRs, such as glucocorticoids (cortisone) and vitamin D (calcitriol)¹¹. There is a total of 48 NRs in humans, yet only a handful of them have well-defined roles in dermal fibrosis and collagen

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overproduction, and thus are unexplored therapeutic targets for these conditions¹². The discovery of peroxisome-proliferator activated receptors (PPARs) has

attracted attention due to their crucial role in lipid homeostasis and tissue repair^{8, 11}. There are three PPAR subtypes: PPAR α , β/δ , and γ . A recent study showed that

Fig. 1 (See legend on next page.)

(see figure on previous page)

Fig. 1 *Pparb/d* ablation in fibroblasts enhances epidermal inflammation. **a** PCR analysis of mouse fibroblast DNA. The non-deleted floxed allele is represented by the 2.2-kb band. The *Pparb/d*-deleted allele is represented by the 600-bp band. n.c., negative control. **b, c** Relative PPAR α , PPAR β/δ , and PPAR γ mRNA (**b**) and protein (**c**) levels in fibroblasts of FSPCre-*Pparb/d*^{fl/fl} and *Pparb/d*^{fl/fl} mice. Representative immunoblots are shown. 18S rRNA served as housekeeping gene. β -tubulin that served as housekeeping protein was from the same samples. Values are mean $\pm$ S.D. ($n = 5$). **d** Body weight of male and female mutant mice over 6 weeks post birth. Values represent mean $\pm$ S.D. ($n = 35$ for male and $n = 40$ for female mice). **e** Representative hematoxylin and eosin (H&E) stained skin sections from FSPCre-*Pparb/d*^{fl/fl} and *Pparb/d*^{fl/fl} mice at weeks 1, 4, and 8 of age. Scale bar = 50 μ m. ($n = 12$). **f** Epidermis and dermis thickness in both genotypes at weeks 1, 4, and 8. Values represent mean $\pm$ S.D. ($n = 31$). **g** Relative protein expression of epidermal markers for proliferation (PCNA) and differentiation (TGase1) in FSPCre-*Pparb/d*^{fl/fl} and *Pparb/d*^{fl/fl} mice at age week 4. Representative immunoblots were shown. Coomassie stained of blots showed equal loading and transfer. β -tubulin that served as housekeeping protein was from the same samples. Values represent mean $\pm$ S.D. ($n = 5$). **h** Barrier function test. Representative images of skins from FSPCre-*Pparb/d*^{fl/fl} and *Pparb/d*^{fl/fl} mice topically treated with Lucifer yellow dye. Nuclei were stained with Hoechst dye (blue). Scale bar = 50 μ m. **i, j** Graphs showing the epidermal thickness (**i**) and the number of PCNA-positive keratinocytes (**j**) on dorsal skin treated with either vehicle (Veh) or 6.5 nmol of 12-O-tetradecanoylphorbol-13-acetate (TPA) for 24 h. Values represent mean $\pm$ S.D. ($n = 8$). **k** Representative H&E stained section of TPA-treated skins from FSPCre-*Pparb/d*^{fl/fl} and *Pparb/d*^{fl/fl} mice. Intra-epidermal microabscesses were observed in skins from FSPCre-*Pparb/d*^{fl/fl} mice. Scale bar = 200 μ m. Graph showing the relative protein expression of neutrophil marker Ly-6C/Ly-6G in Veh-treated and TPA-treated skin of FSPCre-*Pparb/d*^{fl/fl} compared to *Pparb/d*^{fl/fl} mice. Representative immunoblots are shown. Coomassie stained of blots show equal loading and transfer. Values represent mean $\pm$ S.D. ($n = 5$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. not significant

pan PPAR agonist IVA337 was effective in the prevention and treatment of experimental skin fibrosis¹³. The study pointed to a role for PPAR in dermal fibrosis, however, the contribution of various PPAR subtypes remains unclear. The skin of whole-body PPAR γ heterozygous and fibroblast-specific PPAR γ -deficient mice did not exhibit any significant alterations in skin thickness or matrix accumulation. Nonetheless, the deletion of PPAR γ resulted in enhanced susceptibility to bleomycin-induced skin fibrosis and specific agonists of PPAR γ have been shown to alleviate the extent of the development of cutaneous sclerosis^{14, 15}. As the most dominant PPAR subtype in the skin, the role of epidermal PPAR β/δ in skin physiology is well-documented^{16–19}. Sclerodermic keratinocytes can trigger an inflammatory response from the fibroblasts via secreted IL-1 α ^{20, 21}. Interestingly, fibroblast PPAR β/δ has been shown to curb excessive epidermal proliferation during wound healing via the production of secreted IL-1 receptor antagonist²². Several studies have suggested that cellular redox state may play a significant role in the progression of scleroderma fibrosis^{23, 24}. Fibroblasts obtained from the skin of patients with SSc showed increase in reactive oxygen species production when compared to normal fibroblasts²⁵. Incidentally, Wang et al. underscored a novel role of fibroblast PPAR β/δ in the modulation of oxidative stress in a diabetic wound microenvironment²⁶. These findings suggested a possible role for fibroblast PPAR β/δ in dermal fibrosis. However, whole-body PPAR β/δ knockout mice do not exhibit any dermal fibrotic phenotype^{8, 27–29}, and this may be because of a tissue-specific role for fibroblast PPAR β/δ .

To understand the role of PPAR β/δ in fibrotic skin diseases, we generated a fibroblast-selective PPAR β/δ deleted mouse (FSPCre-*Pparb/d*^{-/-}). FSPCre-*Pparb/d*^{-/-} mice exhibited profound morphological changes to the skin architecture at an early age, pronounced wound

contraction and have altered gene expression profile that resembles fibroproliferative diseases.

Results

PPAR β/δ -deficient fibroblasts enhance epidermal hyperplasia and inflammation

We generated a mouse whose exons 4–5 of PPAR β/δ gene were deleted in the fibroblasts by crossing *Pparb/d*^{fl/fl} with FSP1-Cre mice, herein called FSPCre-*Pparb/d*^{-/-}. The excision of *Pparb/d*^{fl/fl} was identified by the amplification of a 600-bp DNA fragment in dermal fibroblasts isolated from FSPCre-*Pparb/d*^{-/-} and FSPCre-*Pparb/d*^{+/-} mice (Fig. 1a). Intact floxed *Pparb/d*^{fl/fl} appeared as a 2.2-kb band (Fig. 1a). Multiplex PCR of CD45⁺, CD31⁺, and MHC⁺ revealed differential deletion of PPAR β/δ allele in these isolated cells. No deleted PPAR β/δ allele was observed in either CD31⁺ or MHC⁺ cells, whereas PPAR β/δ was deleted in CD45⁺ cells (Fig. S1a). PPAR β/δ mRNA and protein were not detected in fibroblasts from FSPCre-*Pparb/d*^{-/-} mice (Fig. 1b, c). The relative expression of PPAR α and PPAR γ in FSPCre-*Pparb/d*^{-/-} fibroblasts was not altered when compared to that in *Pparb/d*^{fl/fl} fibroblast (Fig. 1b, c). This was similarly observed in human dermal fibroblasts whose endogenous PPAR β/δ was suppressed by siRNA (Fig. S1b). The deletion did not affect the number of pups per litter or gender ratio (Fig. S1c). The body weight of male offspring was significantly lower than that of their wildtype counterparts, but this difference was not observed in female offspring (Fig. 1d, S1d). Thus, the FSPCre-*Pparb/d*^{-/-} mouse is viable and PPAR β/δ expression in fibroblasts is not critical for embryonic development.

To assess whether the FSPCre-*Pparb/d*^{-/-} mouse exhibited post-natal alterations, we performed histomorphometric analysis of the skin. The FSPCre-*Pparb/d*^{-/-} mice exhibited a thicker epidermis, dermis, and

Fig. 2 Increased collagen deposition in dermis of FSPCre-Pparb/d^{-/-} mice. **a** Representative Van Gieson's stained sections of skins from FSPCre-Pparb/d^{-/-} and Pparb/d^{fl/fl} mice at weeks 1, 4, and 8. Scale bar = 50 μm. **b** Hydroxyproline content in FSPCre-Pparb/d^{-/-} and Pparb/d^{fl/fl} mice at week 4. Hydroxyproline content was normalized to total protein concentration. Values represent mean ± S.D. (n = 5). **c** Mean number and cross-sectional area of collagen fibrils in the dermis of the FSPCre-Pparb/d^{-/-} and Pparb/d^{fl/fl} mice at week 4. Values represent mean ± S.D. (n = 5). **d** Representative images of immunofluorescence staining for vimentin (green) and α-SMA (red) in dermis of both genotypes. Sections were counterstained with DAPI for nuclei (blue). The dotted line represents the epidermis-dermis junction. Scale bar = 50 μm. **e** Relative protein expression of vimentin (vim) and α-SMA in skin tissues from FSPCre-Pparb/d^{-/-} and Pparb/d^{fl/fl} mice. Representative immunoblots for vimentin and α-SMA are shown. β-tubulin served as housekeeping protein and was from the same samples. Values represent mean ± S.D. (n = 5). **f** Wound closure rate of full-thickness excisional wounds on FSPCre-Pparb/d^{-/-} and Pparb/d^{fl/fl} mice over a period of 18 days. Values are mean ± S.D. (Mann-Whitney U test; n = 7 per time point). **g** Rate of wound contraction determined as the distance between the first hair follicles on the wound edges. A 8-mm full-thickness excisional wound was created on dorsal backs of mice. Wound biopsies were harvested at the indicated days post-wounding and stained with H&E. Values are mean ± S.D. (Mann-Whitney U test; n = 10 per time point). *P < 0.05; **P < 0.01; ***P < 0.001; n.s. not significant

hypodermis (Fig. 1e, f, S1e). Immunohistochemical staining and western blot analysis revealed an elevated expression of PCNA (proliferation marker) as well as

transglutaminase type 1 (TGase1; differentiation marker), suggesting an increase in the number of keratinocytes that proceeded to differentiate (Fig. 1g, S1f–g). We did not

detect differences in the penetration of Lucifer Yellow dye into the epidermis of FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice (Fig. 1h), suggesting that the skin barrier function was unaffected. Although, FSP1 has been reported to be a key marker of a specific subset of macrophages in the liver during fibrosis and injury³⁰, the FSPCre-*Pparb/d^{-/-}* mice neither exhibited hepatomegaly nor had increased collagen deposition in the liver or gastrointestinal tract (Fig. S1h–i).

Previous studies showed that global PPAR β/δ knockout mice had an enhanced epidermal hyperplasia response when topically treated with 12-O-Tetradecanoylphorbol-13-acetate (TPA) compared to their cognate wild-type littermates^{27, 28}. We questioned the role of fibroblast PPAR β/δ in this acute TPA response, thus FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice were treated with TPA. We observed an enhanced epidermal hyperplasia with more PCNA-positive proliferating keratinocytes in FSPCre-*Pparb/d^{-/-}* mice compared to *Pparb/d^{fl/fl}* mice in response to topical TPA treatment (Fig. 1i, j, S1j–k). FSPCre-*Pparb/d^{-/-}* mice also exhibited intra-epidermal microabscesses with greater infiltration of Ly-6C/Ly-6G-positive neutrophils (Fig. 1k, S1l). Thus, PPAR β/δ ablation in fibroblasts led to an aggravated inflammatory response of the adjacent epidermis to TPA.

FSPCre-*Pparb/d^{-/-}* mice exhibit dermal thickening and faster dermal wound healing

Dermal thickening was the most obvious phenotypic difference between FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice. There was more collagen deposition in the FSPCre-*Pparb/d^{-/-}* dermis compared with *Pparb/d^{fl/fl}* as indicated by Van Gieson's stain and hydroxyproline measurement (Fig. 2a, b). We also stained the tissues using PicroSirius Red, which is intended for use in the histological visualization of Type I and III collagen in red and the cytoplasm in yellow. When viewed under polarized light microscope, a red fluorescence indicates the presence of Type 1 collagen (Fig. S2a). Consistently, we observed a thicker dermis and more collagen in the FSPCre-*Pparb/d^{-/-}* compared with *Pparb/d^{fl/fl}* mice (Fig. S2a). Focused ion beam scanning electron microscopy of the dermis region revealed a larger mean cross-sectional area but fewer collagen fibrils in FSPCre-*Pparb/d^{-/-}* mice compared to *Pparb/d^{fl/fl}* (Fig. 2c, S2b). We also detected more α -smooth muscle actin (α -SMA)-positive myofibroblasts, but not vimentin-positive fibroblasts in FSPCre-*Pparb/d^{-/-}* mice compared to *Pparb/d^{fl/fl}* mice (Fig. 2d, e). These findings suggest that PPAR β/δ deficiency in fibroblasts promotes the activation of fibroblasts to myofibroblasts, leading to increased dermal collagen deposition. To study how this phenotype affects dermal wound healing, we performed full-thickness excisional wounding assays. FSPCre-*Pparb/d^{-/-}* mice exhibited more rapid wound

closure than the *Pparb/d^{fl/fl}* mice (Fig. 2f, S2c), which was associated with a significantly higher rate of wound contraction (Fig. 2g, S2d). These observations suggest that the higher number of myofibroblasts and increased collagen deposition with thicker collagen fibers contributed to the rapid wound contraction in FSPCre-*Pparb/d^{-/-}* mice.

FSPCre-*Pparb/d^{-/-}* mice exhibit a pro-fibrotic gene expression profile

The above observations suggest that FSPCre-*Pparb/d^{-/-}* mice exhibited certain localized SSc-like hallmarks, including excessive collagen deposition leading to thickening of the dermis and greater number of myofibroblasts. Previous studies also concluded that localized SSc involves autoimmune abnormalities with elevated levels of anti-nucleosome antibodies^{31, 32}. We detected higher levels of anti-nucleosome antibodies in 69% of our FSPCre-*Pparb/d^{-/-}* mice, with a higher frequency in female mice than male mice (Fig. S2e). To further strengthen our findings, we performed comparative microarray gene expression analysis of skin biopsies from SSc patients, other mouse models of SSc and FSPCre-*Pparb/d^{-/-}* mice.

An intrinsic SSc gene set comprising 995 genes was previously identified by Milano et al (GSE9285)³³. We examined the expression of these genes in the tight skin (Tsk) 1 or 2 heterozygous mutant mouse model (GSE71999), the bleomycin-induced fibrosis mouse model (GSE71999), the sclerodermatous graft versus host disease (SGVHD) mouse model (GSE24410), and our FSPCre-*Pparb/d^{-/-}* mice (Fig. S3). Unguided hierarchical clustering of samples revealed that the mouse models of SSc mainly represented the fibroproliferative subtypes of human SSc. Both fibroproliferative 1 and 2 subtypes of human SSc share a common biological program of proliferation^{33–35}. FSPCre-*Pparb/d^{-/-}* mice samples branched off from the same node as fibroproliferative 2 subtype of human SSc samples, indicating that the gene expression profiles of the two sample groups are highly similar (Fig. S3).

A subset of 113 genes involved in cell:cell signaling and interaction, connective tissue development and function, and inflammation was downregulated in fibroproliferative human SSc compared with normal human skin (Fig. 3a). On the one hand, only our FSPCre-*Pparb/d^{-/-}* mice recapitulated this molecular signature of the diseased tissue, whereas these 113 genes were mostly upregulated in the Tsk1/2, SGVHD and bleomycin-induced mouse models of SSc. On the other hand, a subset of 110 genes that was upregulated in human SSc samples was better represented by Tsk1/2, SGVHD and bleomycin-induced mouse models of SSc than FSPCre-*Pparb/d^{-/-}* mice (Fig. 3b). However, we noted that the gene ontologies (GO) of deregulated genes in all mouse models of SSc,

including our FSPCre-*Pparb*/*d*^{-/-} mice, highlight connective tissue abnormalities and inflammation as critical processes in the development of SSc. Because our FSPCre-*Pparb*/*d*^{-/-} mice uniquely recapitulated the cell:cell signaling aspect of fibroproliferative 2 subtype of human SSc, we separated the epidermis and the dermis of FSPCre-*Pparb*/*d*^{-/-} mice skin to analyze their compartment-specific gene expression by microarray. Genes that were differentially regulated by >1.5-

fold in the epidermis of FSPCre-*Pparb*/*d*^{-/-} mice enriched the GO of lipid metabolism, molecular transport, small molecule biochemistry, cell death and survival, and cellular movement, ranked according to their *P*-values (a lower *P*-value indicates a stronger association to the indicated GO) (Fig. 4a). A total of 80 unique gene transcripts were responsible for the top three enriched GOs in the epidermis. Among these 80 genes, 85% of them (68 genes: 37 upregulated, 31 downregulated) were lipid-

related. 40 of these 68 genes (~60%) had overlapping functions in inflammation, and 25 of these 40 inflammation-related genes (~63%) were involved in TGFβ1 signaling (Fig. 4a, S4a). In the dermis, 58 genes that were differentially regulated by >1.5-fold, enriched the GOs of connective tissue development, tissue development, cellular growth and proliferation, immune cell trafficking and inflammatory response. 49 of the 58 genes (~85%) were migration/proliferation-related, 35 genes (~60%) were connective tissue-related and 29 genes (50%) were involved in TGFβ1 signaling (Fig. 4b, S4b).

The findings from microarray analysis of the two skin compartments suggest that TGFβ1 signaling is a major paracrine axis that is altered by the deletion of PPARβ/δ in fibroblasts. The microarray data is consistent with the increased immune cell infiltration within the skin of FSPCre-Pparb/d^{-/-} mice compared with their wild-type counterparts. This is also consistent with our current observation of epidermal hyperproliferation in the skin of FSPCre-Pparb/d^{-/-} mice. These observations suggest that the deletion of PPARβ/δ in fibroblasts creates a hotbed for facilitated immune cell entry, which is in part responsible for thickening of the skin.

Fibroblast PPARβ/δ regulates *Lrg1* to modulate TGFβ1 signaling

The interrogation of our microarray datasets suggested that dysregulated TGFβ1 signaling plays a central role in the profibrotic phenotype of FSPCre-Pparb/d^{-/-} mice (Fig. S4). Indeed, FSPCre-Pparb/d^{-/-} mice exhibited an enhanced activation of TGFβRII and SMAD3 via phosphorylation compared to Pparb/d^{fl/fl} mice (Fig. 5a). Of interest is the diminished expression of LRG1 in the dermis of FSPCre-Pparb/d^{-/-} mice (Fig. 5b, S4b). LRG1 modulates TGFβ1 signaling in a tissue-specific manner and has been implicated to play a role in vascularization^{36, 37}. Thus, the role of LRG1 is aligned with the dysfunctional microvascularization and dysregulated TGFβ1 signaling observed in SSc patients. PPARβ/δ agonist GW501516 (GW) increased LRG1 mRNA and protein levels, which were attenuated either by PPARβ/δ antagonist GSK0660 (GSK) or an inverse agonist compound 10h (10h) in normal primary human fibroblasts (Fig. 5c, d). In silico analysis of the intragenic regulatory region of mouse and human *Lrg1* promoters revealed putative PPAR response elements (PPREs) (Fig. S5). Chromatin immunoprecipitation of primary mouse and

Fig. 5 (See legend on next page.)

(see figure on previous page)

Fig. 5 Fibroblast PPAR β/δ modulates TGF β 1 signaling via LRG1. **a, b** Relative expression levels of phosphorylated TGF β RII and SMAD3 (**a**) and LRG1 proteins in dermis and epidermis (**b**) from FSPC*Cre-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice. Representative immunoblots for phosphorylated, total TGF β RII and SMAD3, as well as LRG1 were shown. β -tubulin served as housekeeping protein was from the same samples. Values are mean $\pm$ S.D. ($n = 5$). **c, d** Relative LRG1 mRNA (**c**) and protein (**d**) levels in human fibroblasts treated with GW501516 (GW), a specific PPAR β/δ agonist, and in combination with GSK0660 (GSK), a PPAR β/δ antagonist, and compound **10h** (**10h**), a PPAR β/δ inverse agonist. TBP, TATA-Box binding protein, served as housekeeping gene for qPCR. Representative immunoblots are shown. β -tubulin that served as housekeeping protein was from the same samples. Values are mean $\pm$ S.D. ($n = 5$). **e** Chromatin immunoprecipitation (ChIP) using PPAR β/δ antibodies or pre-immune Ig (p.i.) in human and mouse fibroblasts that were stimulated with either vehicle (Veh) or GW. Enrichment of either a PPRE-containing fragment from the endogenous *Lrg1* promoter or a control DNA fragment (2 kb upstream of the PPRE) was evaluated by PCR. Aliquots of the extract were also analyzed before immunoprecipitation (input). **f, g** Relative ALOX5AP protein level in skins from FSPC*Cre-Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice (**f**) and in human keratinocytes treated with either TGF β 1 or recombinant LRG1 proteins (**g**). Representative immunoblots for ALOX5AP were shown. β -tubulin served as housekeeping protein was from the same samples. Values represent mean $\pm$ S.D. ($n = 5$). **h, i** Relative LRG1 mRNA (**h**) and protein (**i**) levels in human fibroblasts transfected with either siRNA against LRG1 or scrambled siRNA control. 18 S rRNA served as housekeeping gene for qPCR. Representative immunoblot for LRG1 is shown. β -tubulin that served as housekeeping protein was from the same samples. Values are mean $\pm$ S.D. ($n = 3$). **j, k** Relative expression of TGFBR2, SMAD3 mRNAs (**j**) and α -SMA protein (**k**) in control, LRG1-knockdown human fibroblasts, and LRG1-knockdown human fibroblasts treated with an increasing dose of recombinant LRG1 protein. 18S rRNA served as housekeeping gene for qPCR. Representative immunoblot for α -SMA was shown. β -tubulin that served as housekeeping protein was from the same samples. Values are mean $\pm$ S.D. ($n = 3$). **l, m** Relative protein levels of α -SMA, SMAD3, and phosphorylated SMAD3 (p-SMAD3) in PPAR β/δ - (siPPARb/d) (**l**), LRG1- (siLRG1) knockdown fibroblasts (**m**) and control fibroblasts transfected with scrambled siRNA (siScrambled). p-SMAD3 was normalized to total SMAD3 protein. β -tubulin that served as housekeeping protein was from the same samples. Values are mean $\pm$ S.D. ($n = 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. not significant

human fibroblasts showed that PPAR β/δ is recruited to the PPRE of *Lrg1* (Fig. 5e). These experiments identified *Lrg1* as a direct target of PPAR β/δ in fibroblasts.

Microarray analyses revealed that the expression of genes related to lipid mediators of inflammation was upregulated in FSPC*Cre-Pparb/d^{-/-}* epidermis (Fig. 4a). One of the most upregulated genes was arachidonate 5-lipoxygenase-activating protein (*Alox5ap*), which mediates inflammatory responses towards external stimuli³⁸ (Fig. S4). The FSPC*Cre-Pparb/d^{-/-}* mice expressed higher levels of ALOX5AP mRNA and protein in the epidermis than *Pparb/d^{fl/fl}* mice (Fig. 5f). Recombinant LRG1 decreased ALOX5AP expression in a dose-dependent manner in primary human keratinocytes (Fig. 5g). These results also suggest that the expression of ALOX5AP in the epidermis was regulated, in part, by paracrine production of LRG1 by the fibroblasts. We conclude that the upregulation of ALOX5AP in the epidermis of FSPC*Cre-Pparb/d^{-/-}* mice may contribute to the increased susceptibility of the epidermis to inflammatory insults.

The contractile and secretory α -SMA-positive myofibroblasts contribute to tissue repair during wound healing, but organ function can be severely impaired when contraction and collagen secretion become excessive, such as in fibrosis.^{3, 39} We studied the TGF β 1-mediated α -SMA expression in LRG1-deficient fibroblasts (Fig. 5h, i). LRG1 deficiency led to an increase in the mRNA levels of TGF β RII and SMAD3, whereas treatment of LRG1-deficient fibroblasts with recombinant LRG1 reduced TGF β RII and SMAD3 expression (Fig. 5j). The increased expression of α -SMA observed in LRG1-deficient fibroblasts was indicative of enhanced fibroblast activation (Fig. 5k), consistent with the higher number of α -SMA-

positive fibroblasts in FSPC*Cre-Pparb/d^{-/-}* mice. The exposure to increasing doses of recombinant LRG1 protein decreased the expression of α -SMA in a dose-dependent manner (Fig. 5k). To examine if recombinant LRG1 protein could rescue the fibrotic phenotype of PPAR β/δ -deficient fibroblasts, we treated control (siScrambled) and PPAR β/δ -knockdown (siPPARb/d) human fibroblasts with TGF β 1 in the presence or absence of recombinant LRG1 protein (Fig. 5l). TGF β 1 treatment resulted in higher expression of phosphorylated SMAD3 (p-SMAD3) and α -SMA in siPPARb/d fibroblasts when compared with siScrambled fibroblasts (Fig. 5l). Importantly, this increase expression of p-SMAD3 and α -SMA was attenuated in the presence of recombinant LRG1. Conversely, we treated siScrambled-knockdown and LRG1-knockdown (siLRG1) human fibroblasts with TGF β 1 in the presence or absence of PPAR β/δ agonist GW501516 (GW) (Fig. 5m). Similar to observation in Fig. 5k, reduced LRG1 resulted in higher p-SMAD3 and α -SMA, i.e., greater fibroblast activation in siLRG1 fibroblasts when compared with control. The co-treatment with GW did not significantly attenuate the increase (Fig. 5m). Thus, LRG1 acted downstream of PPAR β/δ and was able to rescue the fibrotic phenotype of PPAR β/δ -deficient fibroblasts.

These observations suggest a role of fibroblast PPAR β/δ in the homotypic regulation of TGF β 1 signaling, via increased LRG1 production, in fibroblast activation and collagen production, which contribute to the fibrotic phenotype of FSPC*Cre-Pparb/d^{-/-}* mice. Taken together, our results indicate that in fibroblasts, PPAR β/δ transcriptionally upregulates *Lrg1* that in turn acts in an

Fig. 6 PPARβ/δ and LRG1 reduce bleomycin-induced dermal fibrosis. **a** Representative H&E stained sections of FSPCre-*Pparb*^{d/d} and *Pparb*^{d/f} mice skin treated with imiquimod cream 4% daily for 4 days compared to vehicle-treated (Veh) cognate mice. Scale bar = 50 μm. **b** Graph showing the dermal thickness of Veh-treated and imiquimod-treated skins from indicated mice. Values represent mean ± S.D. (n = 5). **c** Representative H&E stained sections of vehicle-treated and GW-treated skins from bleomycin-induced SSc. Scale bar = 150 μm. **d** Dermal thickness of vehicle-treated and GW-treated skins from bleomycin-induced SSc. The dermal thickness of untreated FSPCre-*Pparb*^{d/d} and *Pparb*^{d/f} mice skin was included for comparison purpose. Values represent mean ± S.D. (n = 5). **e** Representative images of immunofluorescence staining against cytokeratin 10 (CK10) in bleomycin-injected mouse skin that was treated with either vehicle or the PPARβ/δ agonist, GW. Scale bar = 50 μm. **f** Hydroxyproline content in vehicle-treated and GW-treated skins from bleomycin-induced sclerodermic mice. Hydroxyproline content was normalized to total protein concentration. Values represent mean ± S.D. (n = 5). **g** Correlation plot of PPARβ/δ and LRG1 proteins in fibroblasts from normal patients (n = 9) and from scleroderma patients (n = 28). Representative immunoblots for PPARβ/δ and Lrg1 are in Supplementary Figure S5b. **h, i** Relative expression of α-SMA protein in normal and sclerodermic fibroblasts treated with either PPARβ/δ agonist (GW), antagonist (GSK), and inverse agonist (10h) (**h**) or treated with increasing doses of recombinant LRG1 protein (**i**). Representative immunoblots for α-SMA are shown. β-tubulin that served as housekeeping protein was from the same samples. Values are mean ± S.D. (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001; n.s. = not significant

autocrine and paracrine manner to modulate tissue-specific TGF β 1 effects.

Ligand-activated PPAR β / δ reduces the fibrotic effect in SSc

Although the best treatment for localized SSc is uncertain and current treatments often fail to help, benefits have been reported following topical 5% imiquimod cream (Aldara®) treatment^{40–43}. Imiquimod is an immune response modifier. It induces interferon and subsequently inhibits TGF β 1 and was reported to attenuate SSc symptoms in patients⁴⁰. FSPCre-*Pparb/d*^{-/-} mice topically treated with imiquimod exhibited a thinning of the thickened dermis (Fig. 6a). The resultant dermal thickness was no longer significantly different from *Pparb/d*^{fl/fl} (Fig. 6b). To evaluate the potential of PPAR β / δ as a therapeutic target for dermal fibrosis, we examined the effect of the PPAR β / δ agonist GW on the widely used bleomycin-induced mouse model of dermal fibrosis. Dermal fibrosis was induced in C3H/He wildtype mice through daily subcutaneous injection of bleomycin for a period of 28 days. We concurrently co-injected them with either dimethyl sulfoxide (DMSO) (vehicle) or GW. Histological analysis revealed that the dermis of the mice co-treated with GW were thinner than that of mice co-treated with vehicle (Fig. 6c, d). The epidermis of mice co-treated with GW showed increased differentiation of the epidermal layer by PPAR β / δ activation, as evidenced by an increased CK10 staining (Fig. 6e). Hydroxyproline assay also showed a lower level of collagen in the dermis of GW-treated mice (Fig. 6f).

To further underscore the clinical relevance of PPAR β / δ , we examined the effect of GW on human dermal fibroblasts that were explanted from 28 SSc patients. Scleroderma fibroblasts expressed lower levels of PPAR β / δ mRNA and protein than normal fibroblasts (Fig. 6g, h). The protein level of LRG1 was lower in SSc fibroblasts than in normal fibroblasts isolated from healthy volunteers (Fig. 6h). GW-activated PPAR β / δ reduced the expression of α -SMA in SSc fibroblasts, while the co-treatment with either GSK or compound 10h (PPAR β / δ inverse agonist) increased the expression of α -SMA (Fig. 6i). Recombinant LRG1 similarly reduced the expression of α -SMA in SSc fibroblasts (Fig. 6j), indicating that PPAR β / δ exerted its effect through LRG1, a direct downstream target of PPAR β / δ . This observation suggested that the activation of PPAR β / δ could reduce fibrosis, underscoring its therapeutic potential.

Discussion

Connective skin diseases are characterized by dermal fibrosis and excessive collagen deposition in the skin and internal organs. Fibroblasts are the main producers of collagen in the extracellular matrix, and thus the pivotal cell type involved in the clinical presentation of

connective skin diseases, including SSc. Yet, the transcriptional networks within the fibroblasts that contribute to dermal fibrosis are not well-studied. We showed here that fibroblast PPAR β / δ is not critical for embryonic development in mice. However, the epidermis of FSPCre-*Pparb/d*^{-/-} mice had an aggravated response to TPA, suggesting that PPAR β / δ ablation in the fibroblasts rendered the mutant mice more susceptible to developing inflammatory skin disorders. Notably, FSPCre-*Pparb/d*^{-/-} mice exhibited pronounced dermal thickening associated with excessive collagen production, a greater number of myofibroblasts and Ly6-positive immune cells, which somewhat represents a fibrosing skin disorder phenotype. These observations were also supported by our comparative gene analyses, which showed that the expression of the respective genes for collagen production and immune cells activation were correspondingly altered.

The FSPCre-*Pparb/d*^{-/-} mice exhibited dermal fibrosis. The skin of FSPCre-*Pparb/d*^{-/-} mice was characterized by increased collagen synthesis associated with increased activation of fibroblasts to myofibroblasts. However, FSPCre-*Pparb/d*^{-/-} livers exhibited neither hepatomegaly, which manifests with systemic SSc-associated primary biliary cirrhosis⁴⁴, nor increased collagen deposition in the intestinal wall, which could cause bowel hypomotility⁴⁵. These indicate that the role of PPAR β / δ in fibroblasts is likely to be tissue/organ-specific. We detected dysregulated TGF β 1 signaling in the FSPCre-*Pparb/d*^{-/-} mice, which has been implicated in the etiology of dermal fibrosis⁴⁶. Dermal fibrotic symptoms may be further aggravated by an enhanced epidermal inflammatory response in these mutant mice. In clinical settings, activated fibroblasts give rise to an immune-inflammatory environment with abnormal repair of damaged vessels and fibrosis^{47, 48} and ROS production^{49–52}. PPAR β / δ -deficient fibroblasts contribute to greater microenvironment oxidative stress due to reduced expression of antioxidant genes such as catalase and glutathione peroxidase 1²⁶. Microarray analyses of FSPCre-*Pparb/d*^{-/-} skin revealed upregulation of numerous genes that would culminate an increased ROS production in the PPAR β / δ -ablated dermis, including CCL2 and CCL5. Notably, CCL2 and CCL5 upregulation was found to precede skin and lung fibrosis in a mouse model that recapitulates the pathology of SSc⁵³. Finally, we also identified gene networks in FSPCre-*Pparb/d*^{-/-} mice that closely resemble fibroproliferative 2 subtype of human SSc. Thus, the fibroblast PPAR β / δ deficiency causes dermal fibrosis. Unlike FSPCre-*Pparb/d*^{-/-} mice, fibroblast-specific PPAR γ -deficient skin did not exhibit any manifestations of dermal fibrosis or collagen overproduction, suggesting that fibroblast PPAR β / δ and PPAR γ play different roles in skin physiology¹⁴. FSPCre-*Pparb/d*^{-/-} exhibited epidermal hyperplasia which is

characteristic of psoriasis, another type of connective tissue diseases. Several studies have also implicated a role for PPAR β/δ in psoriasis^{54–58}. However, the relative contribution of epidermal and dermal PPAR β/δ in the etiology and pathophysiology of psoriasis remains unclear. This study is still ongoing in the lab, and is beyond the scope of this work.

Our current study revealed that TGF β 1 signaling has an overarching influence on skin architecture in FSPCre-*Pparb/d*^{-/-} mice. We identified *Lrg1* as a direct downstream target of PPAR β/δ that contributes to the observed phenotype. Thus far, LRG1 had been reported to augment endothelial TGF β 1 signaling and promote angiogenesis³⁶. LRG1 is expressed in various human tissues, including the breast, skin, and intestine, but little is known about its role in these tissues. Our study shows that fibroblast-secreted LRG1 acts in a paracrine manner to influence the epidermal TGF β 1 response to inflammatory challenges. The underlying mechanism involved transcriptional upregulation of LRG1 by fibroblast PPAR β/δ , which modulated homotypic and heterotypic TGF β 1 signaling. Limitations of our study include that one genetic background of mouse and our deletion strategy consists of the deletion of exons coding for the DNA-binding domain of PPAR β/δ . It is also conceivable that other gene deletion strategies may result in different phenotypic severity or outcomes⁸. In addition to fibroblasts, FSP1 driven gene deletion may affect other cell types, although in vitro experiments were performed to strengthen the conclusion. Nevertheless, to the best of our knowledge, this is the first study to examine the role of fibroblast PPAR β/δ in skin, which reveals novel epithelial–mesenchymal communication.

Current treatment of SSc is directed toward managing complications and providing symptomatic relief. Although several regimens have shown benefit in case series, few controlled trials have been performed and data suggest wide variation in the approach to treatment⁵⁹. For example, topical 5% imiquimod cream has been reported for the treatment of localized SSc⁴⁰. We showed that FSPCre-*Pparb/d*^{-/-} mice were responsive to topical treatment with imiquimod cream. The dermis of these mice became thinner, resulting in a thickness similar to that of their wildtype littermates. We also showed that ligand-activated PPAR β/δ reduced dermal fibrosis using the bleomycin-induced mouse model of dermal fibrosis. In the 28 SSc patients analyzed, fibroblasts consistently had reduced level of PPAR β/δ and LRG1 compared to healthy subjects. In addition, PPAR β/δ activation, or LRG1 treatment, could reduce α -SMA levels in these profibrotic fibroblasts. In conclusion, we identified an important signaling conduit that integrates PPAR β/δ and TGF β 1 signaling networks via LRG1 to be key players in fibrosis.

Materials and methods

Cell culture

Human keratinocytes (Lonza, USA) were routinely cultured in keratinocyte-SFM culture media (Invitrogen, USA). Fibroblasts were maintained in FibroGROTM Medium (Millipore, USA). All cultures were maintained in a humidified incubator at 37 °C with 5% CO₂. Normal human dermal and sclerodermic fibroblasts were procured from Asterand Bioscience, UK and Proteogenex, USA. Scleroderma patients were classified accordingly to American College of Rheumatology criteria⁶⁰. SSc patients presented limited cutaneous SSc with skin thickening was limited to hands and face. SSc patients have Raynaud's phenomenon for >5 years, minimal joint pain and presented telangiectasias. The male:female ratio was approximately 2:7. The balanced median age of the normal ($n = 9$) and SSc ($n = 28$) donors were 42.8 ± 3.1 and 49.1 ± 2.5 years old, respectively. Due to the role of PPAR β/δ in energy homeostasis, patients with diabetes, obese patients (BMI > 30) or patients with hyperlipemia were excluded. Skin fibroblasts were isolated from skin biopsy of the forearm using the explant method⁶¹ and flash frozen at the third passages for subsequent analysis.

Antibodies and chemicals

Primary antibodies used in this study: monoclonal anti- α -SMA, PCNA, GAPDH, polyclonal anti-PPAR β/δ , β -tubulin, TGase1, collagen type I and ALOX5AP (Santa Cruz, USA); polyclonal anti-Ly6C/Ly-6G (BioLegend, USA); polyclonal anti-vimentin and LRG1 (Abcam, USA). IRDye[®] 680LT conjugated secondary antibodies were from Li-Cor (Biosciences, USA); DyLight[®] conjugated secondary antibodies were from Vector Laboratories, USA. GW501516 (PPAR β/δ agonist; GW), GSK0660 (PPAR β/δ antagonist; GSK) and compound **10h** (PPAR β/δ inverse agonist; **10h**) were synthesized in-house.

Generation of fibroblast-selective PPAR β/δ -knockout mice

The *Pparb/d*^{fl/fl} mouse was provided by Prof Walter Wahli (University of Lausanne, Switzerland). The *Pparb/d*^{fl/fl} mouse carries alleles of the PPAR β/δ gene where the exon 4, which encodes the DNA-binding domain, was flanked by loxP sites^{62, 63}. The FSPCre transgenic mouse expresses Cre recombinase under the control of the FSP1 promoter⁶⁴. FSPCre mice were always maintained as hemizygotes. FSPCre and *Pparb/d*^{fl/fl} mice were independently backcrossed to C57BL/6J wild-type mice for at least six generations. The FSPCre-*Pparb/d*^{+/-} mouse was generated in-house by crossing *Pparb/d*^{fl/fl} and FSPCre mice. FSPCre-*Pparb/d*^{+/-} progenies were bred with *Pparb/d*^{fl/fl} mice to obtain FSPCre-*Pparb/d*^{-/-} mice and the wild-type control mice. To sustain this genotype, we bred FSPCre-*Pparb/d*^{-/-} progenies with *Pparb/d*^{fl/fl} mice. Mice were housed in a specific-pathogen free facility with

a 12 h/12 h light/dark cycle. Food and water were provided *ad libitum*.

Mice in the telogen phase of their hair cycle were anaesthetized and depilated on their dorsal back before being subjected to a biopsy punch (~8 mm). The skin biopsy was then treated with 3.8% ammonium thiocyanate (Sigma, USA) in 1 × PBS for 30 min at 37 °C. Subsequently, the epidermis was mechanically separated from the dermis with a pair of forceps. The dermis was then curetted with a 30-gauge needle. The dermis was washed with 1 × PBS before being subjected to the genotyping procedure, real-time qPCR assay and Western blot. Mice genotyping were performed using the Mouse Genotyping Kit HotStart as described by manufacturer (KAPA Biosystems, USA). Genotyping primers are provided in Table S1.

Animal experiments were carried out in accordance to the guidelines of the University Institutional Animal Care and Use Committee (IACUC, ARF-SBS/NIE-A0216AZ, A0321, and A0322), Singapore.

Real-time qPCR and Western blot

RNA extraction, reverse transcription, qPCR and western blot were performed as previously described⁶⁵. Total RNA was extracted using TRIzol® as described by manufacturer (Invitrogen, USA). Reverse transcription was performed using the iScriptTM Reverse Transcription Supermix for RT-qPCR (Bio-Rad, USA) and qPCR done using KAPA SYBR® FAST Universal qPCR Kit (KAPA Biosystems, USA). Primer sequences are provided in Table S1. Total protein was extracted using M-PERTM Mammalian Protein Extraction Reagent (ThermoFisher Scientific). Infrared western blot was done as previously described⁶⁶. Protein bands were analyzed using the Odyssey CLx scanner and Image Studio V2.1 (LI-COR Biosciences, USA).

Histological, immunohistochemical, and immunofluorescence staining

Tissues were processed, sectioned and stained as previously described⁶⁵. PicroSirius Red staining was performed as recommended by the manufacturer (ab150681, Abcam, Cambridge, United Kingdom). Colorimetric images were captured using Axio Scan.Z1 with Plan Apochromat 20×/0.8 objective (Carl Zeiss, Germany). Fluorescence images were captured using the LSM 710 confocal microscope with the Zeiss EC Plan-NEOFLUAR 20×/0.5 NA objective. Analyses were performed with ZEN 2012 Light Edition software (Carl Zeiss, Germany).

Lucifer yellow permeability assay

Lucifer yellow dye (1 mg/mL; Sigma-Aldrich, USA) was topically applied on the epidermis for 1 h. Excess Lucifer yellow was washed away with 1 × PBS, and the skin

tissues were cryofrozen with OCT tissue freezing medium (Leica, USA). Tissues were cryosectioned at 8 µm and mounted with Hoechst 33,342 dye (Life Technologies, USA). Images were taken using the LSM 710 confocal microscope with the Zeiss EC Plan-NEOFLUAR 20×/0.5 NA objective. Analyses were performed with ZEN 2012 Light Edition software (Carl Zeiss, Germany).

Hydroxyproline assay

Hydroxyproline content in tissues was determined as previously described⁶⁷. Trans-4-hydroxy-L-proline (0–300 µg/mL) was used to generate a standard graph. Absorbance was measured at 550 nm using a SpectraMax M2e Multi-Mode Microplate Reader and SoftMax Pro Microplate Data Acquisition & Analysis Software (Molecular Devices, USA). The value of unknown hydroxyproline was determined from the standard graph. The weight of each respective wound biopsy was used for normalization.

12-O-tetradecanoylphorbol 13-acetate (TPA)-induced skin inflammation

Mice of ~4 weeks of age were shaven on their dorsal skin and topically treated with 6.5 nmol of TPA (Sigma-Aldrich, USA) in acetone for 24 h. The vehicle-treated and TPA-treated skins were then harvested and processed accordingly for histological studies and protein analysis.

Wounding experiment

Mice of ~8 weeks of age were shaved and full-thickness excisional wounds were inflicted on the dorsal skin of mice as previous described⁶⁸. Wound surface areas and contraction rates were measured over the indicated duration.

Imiquimod topical application

Mice of ~8 weeks of age were shaved and subsequently imiquimod cream 4% (Selleck Chemicals, USA) was applied topically daily for a period of 4 days. Skin tissues were then harvested and processed for histological analysis.

Bleomycin-induced dermal fibrosis

C3H/He mice of 4 weeks of age were subcutaneously injected with 10 µg of bleomycin sulfate (Selleck Chemicals, USA) together with either DMSO (*n* = 5) or 0.25 mg of GW (*n* = 5) daily for a period 28 days⁶⁹. Skin tissues were then harvested and processed accordingly for histological studies.

Gene expression microarray

cDNA synthesis was performed using the Applause WT-Amp ST System (NuGEN Technologies, USA). The resulting cDNA was purified with MinElute Reaction

Cleanup Kit (Qiagen, USA), followed by fragmentation and labeling using the Encore Biotin Module (NuGEN Technologies, USA). The fragmented and labeled cDNA were mixed with a hybridization master mix (Affymetrix, USA) in accordance to the manufacturer's protocol, and 90 μ L of the resultant mixture were injected into the GeneChip® Mouse Gene 1.0 ST Array gene chips (Affymetrix, USA). The scanned data was collected for analyses using the Partek Genomics Suite v6.6 (Partek Inc., USA). Differences in gene expression between FSPCre-*Pparb*/*d*^{-/-} and wild-type samples were identified using the ANOVA function of the Partek Genomics Suite. Microarray datasets from FSPCre-*Pparb*/*d*^{-/-} mice were subsequently hierarchically clustered with microarray datasets from tight skin (Tsk) 1 or 2 heterozygous mutant mice (GSE71999), bleomycin-induced fibrosis mice (GSE71999), sclerodermatous graft versus host disease (SGVHD) mice (GSE24410), and human samples of SSc (PMC2481301, GSE9285), downloaded from NCBI Gene Expression Omnibus (GEO) database. We focused our analysis on an intrinsic SSc gene set comprising 995 genes that was previously reported³³ (GSE9285). Gene ontology (GO) analysis of gene subsets was performed using the Ingenuity Pathway Analysis software (Ingenuity Systems Inc., USA). The microarray protocols and data have been deposited in NCBI's Gene Expression Omnibus (GEO) database accession number GSE71419.

Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed as previously described⁶⁵ except that anti-PPAR β / δ antibodies was used (see above). ChIP primer sequences are listed in Table S1.

Transfection and luciferase assay

TGF β 1 responsive luciferase reporter assay in keratinocytes was performed as previously described⁷⁰. Luciferase assay was performed using the Dual Luciferase® Reporter Assay System and the Glomax 20/20 Luminometer (Promega, USA). The ON-TARGETplus SMARTpool siRNA were used for knockdown studies (Lrg1, Cat # L-056177-01; PPAR β / δ , Cat #L-042751-01; Dharmacon). Cells were transfected using DharmaFect 1 as recommended by manufacturer. As control, the ON-TARGETplus non-targeting control pool (D-001810-10; Dharmacon) was used.

Anti-nucleosome antibody (ANA) detection

Mice of 4–6 weeks of age were studied for their ANA levels. ANA levels in mice plasma samples were measured by using the anti nucleosome IgG Antibody (AnuA, IgG) BioAssay™ ELISA Kit (United States Biological, USA). Readings were performed on the Infinite® M200 Pro machine (Tecan, Switzerland) with the Magellan™ v7.0 software (Tecan, Switzerland).

Statistical analysis

Statistical analyses were performed using two-tailed Mann-Whitney U test using SPSS software (IBM Corporation, USA). A *P*-value of <0.05 was considered significant.

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Author contributions

M.K.S. and N.S.T. designed, performed, and organized most of the experiments; analyzed and interpreted the data; and co-wrote the manuscript, which was edited and read by all authors. M.K.S., J.S.K.C., and E.H.P.T. performed microarray analyses. J.S.K.C., Z.T., T.P., and N.J.N.K. assisted in performing experiments and interpretation of data. P.Z. performed picosirus red staining and imaging. J.W. K.W. and E.H.P.T. performed the cell sorting, FACS analysis and multiplex genotyping. J.P.C., C.K.T., and W.W. designed and executed experiments with mouse livers and gastrointestinal tract. B.M.K.T., Y.L.T., and S.C. synthesized and provided the various ligands of PPAR β / δ . X.W. provided insights on LRG1-related experiments and M.P. worked on normal and scleroderma fibroblasts experiments. W.W. provided the floxed PPAR β / δ mice. N.S.T., M.K.S., W.W., and X.W. are involved scientific discussion. N.S.T., M.K.S., and J.S.K.C. co-wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Selective deletion of PPAR β/δ in fibroblasts causes dermal fibrosis by attenuated LRG1 expression.

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Supplementary Figure Legends

Figure S1. FSPCre-*Pparb/d^{-/-}* mice are viable and have thicker epidermis and dermis.

(a) Representative scatterplots and gel runs of CD45⁺ (left panel), CD31⁺ and MHC⁺ positive cells (right panel) from indicated tissues of FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice. The diagram showed the relative position of the multiplex genotyping primers, resulting in a larger deleted PPARβ/δ allele compared to floxed PPARβ/δ allele type.

(b) Relative PPARα, PPARβ/δ, and PPARγ mRNA and protein levels in human fibroblasts transfected with either control (siScrambled) or PPARβ/δ (siPPARβ/δ) siRNAs. Representative immunoblots are shown. 18S rRNA served as housekeeping gene. β-tubulin that served as housekeeping protein was from the same samples. Values are mean ± S.D. (n=3).

(c) Average number of male versus female pups in each litter. n.s., not significant.

(d) Body length of male and female mutant mice over 6-week post birth. Values represent mean ± S.D. (n=35 for male and n=40 for female mice).

(e) Thickness of the hypodermis and skeletal muscle for both genotypes at weeks 1, 4, and 8. Values represent mean ± S.D. (n=10).

(f-g) Representative sections of colorimetric staining of PCNA (d) and TGase1 (e) in the skin tissues of FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice. Scale bar = 50 μm.

(h) Representative macroscopic images of liver from FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice.

(i) Representative immunoblots of type I collagen and β-actin from gastrointestinal tract and liver of FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice.

(j-l) Representative sections of H&E (j), immunohistochemical staining of PCNA (k) and Ly-6C/Ly-6G (l) for vehicle- (Veh) and TPA-treated skins of FSPCre-*Pparb/d^{-/-}* and *Pparb/d^{fl/fl}* mice. Scale bar = 50 μm. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; n.s., not significant.

Figure S2. FSPCre-*Pparb/d^{-/-}* mice show greater wound contraction.

- (a) Representative PicroSiruis Red staining against collagen in the skins of FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice at 1 and 8 weeks of age (n=3 for each age). Scale bar = 50 μ m.
- (b) Representative transmission electron microscopy images of dermis of FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice. Scale bars = 0.2 μ m (inset) and 1 μ m.
- (c) Macroscopic images of wounds from FSPCre-*Pparb*/*d*^{-/-} and *Pparb*/*d*^{fl/fl} mice at indicated day post-injury.
- (d) Representative images of wound contraction at indicated day post-injury. Wound contraction is determined using the distance between the first hair follicles at the wound edges. Red arrows indicate wound edges. Scale bar = 500 μ m.
- (e) Frequency of male and female FSPCre-*Pparb*/*d*^{-/-} mice that were positive for anti-nucleosome antibodies in their plasma. Seven male and 22 female mice were studied.

Figure S3. Microarray analyses of an intrinsic scleroderma gene set comprising 995 genes in mouse models of scleroderma and human SSc samples. Microarray datasets from FSPCre-*Pparb*/*d*^{-/-} mice were hierarchically clustered with microarray datasets from Tsk1/2 heterozygous mutant mice, bleomycin-induced fibrosis mice, SGVHD mice, and human SSc. Each mouse model or human disease cluster is represented by a unique color. Microarray datasets for other mouse models of scleroderma and human SSc were downloaded from the NCBI GEO database. The white box represents a subset of 110 genes that are upregulated in human fibroproliferative SSc and well-represented by Tsk1/2 heterozygous mutant mice, bleomycin-induced fibrosis mice and SGVHD mice. The black box represents a subset of 113 genes that are downregulated in human fibroproliferative SSc and well-represented by FSPCre-*Pparb*/*d*^{-/-} mice. Bar charts represent the gene ontologies (GO) associated with the 995 gene set, ranked by $-\log(\text{p-value})$.

Figure S4. Gene ontology (GO) analyses of epidermis and dermis.

(a) GO of genes altered in the epidermis of FSPCre-*Pparb/d*^{-/-} mice. Genes that were altered were highly associated with lipid metabolism (80 genes). Out of these 80 genes, approximately 60% (40 genes) were associated with inflammatory responses. One of the most up-regulated genes was arachidonate 5-lipoxygenase activating protein (*Alox5ap*; circled in red), with an up-regulation of approximately 4.6-fold.

(b) GO of genes altered in the dermis of FSPCre-*Pparb/d*^{-/-} mice. Altered genes were highly associated with cellular function and maintenance, which could alter the behavior and function of fibroblasts in the dermis. Leucine-rich a-2-glycoprotein 1 (*Lrg1*) was one of the most down-regulated genes detected upon fibroblast-specific deletion of *Pparb/d*. This down-regulation was approximately 4-fold.

Figure S5. *Lrg1* is a novel direct target of PPARβ/δ.

(a) Schematic diagram showing the relative position of PPRE sequences in mouse and human *Lrg1* (red boxes) and their respective NUBIScan scores.

(b) Representative immunoblots of PPARβ/δ and *Lrg1* from fibroblasts of normal (n = 9) and sclerodermic patients (n = 28). GAPDH served as housekeeping protein and was from the same samples. Densitometric values were used for the correlation plot.

Supplementary Table

Supplementary Table S1. Primer sequences used in this study (5' to 3').

Mouse Genotyping	
PPAR β/δ Exons 4/5 Forward	GCA GCT GCT CAG CTG CCT GC
PPAR β/δ Exon 4 Reverse	CCG CCT CTC GCC ATC CTT TCA GA
PPAR β/δ Exon 5 Reverse	CAG GTG CTG GGC GCC CTC TGA CAG AAT
FSP1Cre Control Forward	CTA GGC CAC AGA ATT GAA AGA TC
FSP1Cre Control Reverse	GTA GGT GGA AAT TCT AGC ATC ATC C
FSP1Cre Transgene Forward	GCG GTC TGG CAG TAA AAA CTA TC
FSP1Cre Transgene Reverse	GTG AAA CAG CAT TGC TGT CAC TT
Real-time qPCR	
Mouse PPAR α Forward	TCG GCG AAC TAT TCG GCT G
Mouse PPAR α Reverse	GCA CTT GTG AAA ACG GCA GT
Mouse PPAR β/δ Forward	TTG AGC CCA AGT TCG AGT TTG
Mouse PPAR β/δ Reverse	CGG TCT CCA CAC AGA ATG ATG
Mouse PPAR γ Forward	TGT GGG GAT AAA GCA TCA GGC
Mouse PPAR γ Reverse	CCG GCA GTT AAG ATC ACA CCT AT
Human Lrg1 Forward	CAG ACA GCG ACC AAA AAG C
Human Lrg1 Reverse	GGA ACA CCT GGC AGT CTT TG
Human Tgfbr2 Forward	CGT GGA GTC GTT CAA GCA GAC G
Human Tgfbr2 Reverse	CCG CAC CTT GGA ACC AAA TGG
Human Smad3 Forward	GAA CGT CAA CAC CAA GTG CAT
Human Smad3 Reverse	ACG CAG ACC TCG TCC TTC T
Human Coll1A2 Forward	GGT CTC GGT GGG AAC TTT G
Human Coll1A2 Reverse	CCA GGT GGG CCT CTA GGT
Rpl27 Forward	CGC AAA GCT GTC ATC GTG

Rpl27 Reverse	GTC ACT TTG CGG GGG TAG
18S Forward	GTA ACC CGT TGA ACC CCA TT
18S Reverse	CCA TCC AAT CGG TAG TAG CG
TBP Forward	GCT GGT TAT CGG GAG TTG G
TBP Reverse	ACT GGC CTG GTG TCC TAG AG
Chromatin immunoprecipitation (ChIP)	
Human Lrg1 Forward	CCA GGA ATA GTG CCT TGC AAA C
Human Lrg1 Reverse	CCA GTC CAG GCA GGT ATA AGG C
Human Lrg1 Negative Forward	CTC CTG GGT GCA AGC AAT TCT C
Human Lrg1 Negative Reverse	GGG ATT ACA GGC GTG AGC CA
Mouse Lrg1 Forward	ACC TGC TTG GTT CCT TGT GGA AGA C
Mouse Lrg1 Reverse	CAA ACT CAG TCC CTG GAT CCT GGT G
Mouse Lrg1 Negative Forward	TGT GCG TGT GTG CGA CTC AG
Mouse Lrg1 Negative Reverse	CTT CGA CCA CCA GCT GAC CAT

Synthesis of PPAR β/δ ligands

^1H NMR (400 MHz) spectra were recorded on a Bruker Avance 400 spectrometer in CDCl_3 [with $(\text{CH}_3)_4\text{Si}$ ($\delta = 0.00$ ppm) as an internal standard]. ^{13}C NMR (100 MHz) spectra were recorded on a Bruker Avance 400 spectrometer in CDCl_3 [with residual CHCl_3 ($\delta = 77.00$ ppm) as an internal standard]. The following abbreviations are used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, sept = septet, br s = broad singlet, m = multiplet. High-resolution mass spectra were obtained with a Finnigan MAT 95 XP mass spectrometer (Thermo Electron Corporation). Flash column chromatography was performed by using Merck silica gel 60 with distilled solvents.

Synthesis of GW501516 (GW)

GW501516 (IUPAC: {4-[(4-methyl-2-[4-(trifluoromethyl)phenyl]-1,3-thiazol-5-yl)methyl)sulfanyl]-2-methylphenoxy}acetic acid) was prepared according to reported synthetic route as shown in Scheme 1 (Ham and Kang, 2005).

Scheme 1

Step 1: To a solution of 4-iodo-2-methylphenol (2.46 g, 10.5 mmol) in THF (80 mL) was added isopropylmagnesium chloride (5.3 mL, 2.0 M solution in THF, 10.5 mmol) at 0°C under a N_2

atmosphere (Edgar and Falling, 1990). The mixture was stirred for 10 min at 0 °C, and then further cooled to –78 °C before *tert*-butyllithium (12.4 mL, 1.7 M solution in pentane, 21.0 mmol) was added dropwise and the reaction mixture was stirred for 30 min at –78 °C. A suspension of sulfur (337 mg, 10.5 mmol) in anhydrous THF (10 mL) was then dropped into the reaction mixture and then warmed to room temperature and left to be stirred for 30 min. Finally, the reaction mixture was cooled to 0 °C and a solution of 5-(chloromethyl)-4-methyl-2-(4-(trifluoromethyl)phenyl)thiazole (3.06 g, 10.5 mmol) in anhydrous THF (10 mL) was added dropwise, and then the reaction mixture was stirred at room temperature for 1 h (Sznaidman et al., 2003). The reaction was quenched with saturated aqueous NH₄Cl and then acidified with aqueous 1 M HCl. The mixture was extracted thrice with ethyl acetate and the combined organic extract was washed with brine and dried over anhydrous MgSO₄, filtered, and evaporated under reduced pressure to give the crude product. The crude compound was purified by silica-gel flash column chromatography (*n*-hexane/ethyl acetate=75:25) to obtain 2-methyl-4-(((4-methyl-2-(4-(trifluoromethyl)phenyl)thiazol-5-yl)methyl)thio)phenol (**A1**) (3.13 g, 7.9 mmol, 75%) as a white solid.

Step 2: To a solution of **A1** (2.87 g, 7.24 mmol) in DMSO/water (54 mL/ 6 mL) was added K₂CO₃ (1.49, 10.9 mmol) and ethyl bromoacetate (0.96 mL, 8.69 mmol) at room temperature. The reaction mixture was warmed to 50 °C and then vigorously stirred for 2 h. After the reaction was completed, the mixture was poured into water (100 mL) and extracted thrice with ethyl acetate. The combined organic extract was washed twice with water, dried over anhydrous MgSO₄, filtered, and evaporated under reduced pressure to give the crude product. The crude product was purified by silica-gel flash column chromatography (*n*-hexane/ethyl acetate=80:20) to obtain ethyl 2-(2-methyl-4-(((4-methyl-2-(4-(trifluoromethyl)phenyl)thiazol-5-yl)methyl)thio)phenoxy)acetate (**A2**) (3.26 g, 6.77 mmol, 94%) as a pale yellow solid. NMR analysis was in agreement with the literature data.

Step 3: A 3N aqueous solution of NaOH (3.40 mL, 10.2 mmol) was dropped into a solution of **A2** (3.26 g, 6.77 mmol) in ethanol (135 mL) and left to be stirred at room temperature for 30 min. Next, the reaction mixture was acidified with 1N HCl to pH 2–3 and ethanol was removed under reduced pressure. The residue was diluted with water and extracted thrice with ethyl acetate. The combined organic extract was then washed with brine, dried over anhydrous MgSO₄, filtered, and evaporated under reduced pressure to give the crude product. The crude compound was purified by silica-gel flash column chromatography (AcOH/ethyl acetate=1:99) to obtain GW501516 as a white solid (2.50 g, 5.51 mmol, 81%).

4-[(4-methyl-2-[4-(trifluoromethyl)phenyl]-1,3-thiazol-5-yl)methyl)sulfanyl]-2-methylphenoxy}acetic acid (GW501516)

¹H NMR (400 MHz, CDCl₃) δ 2.15 (s, 3H), 2.23 (s, 3H), 4.10 (s, 2H), 4.67 (s, 2H), 6.62 (d, 1H, *J* = 8.4 Hz), 6.82 (br s, 1H), 7.11 (dd, 1H, *J* = 8.4, 2.0 Hz), 7.22 (d, 1H, *J* = 1.6 Hz), 7.66 (d, 2H, *J* = 8.4 Hz), 7.95 (d, 2H, *J* = 8.4 Hz). ¹³C NMR (100 MHz, CDCl₃) δ 14.5, 16.1, 32.4, 65.1, 111.5, 123.9 (q, *J* = 271.0 Hz), 125.3, 125.9 (q, *J* = 4.0 Hz), 126.5, 128.4, 130.9, 131.6 (q, *J* = 33.0 Hz), 132.3, 136.3, 136.4, 151.2, 156.2, 163.7, 172.5.

Synthesis of GSK0660 (GSK)

GSK0660 (IUPAC: methyl 3-([2-(methoxy)-4-phenyl]amino)sulfonyl)-2-thiophenecarboxylate) was synthesized using the synthetic route as shown in Scheme 2.

Scheme 2

Step 1: To a solution of 2-methoxy-4-nitroaniline (1.75 g, 10.4 mmol), NaH (1.46g, 60 % in mineral oil w/w, 36.4 mmol) in THF (100 ml) was added di-*tert*-butyl dicarbonate (7.49 g, 34.3 mmol). The mixture was stirred at room temperature under a N₂ atmosphere for 4 h to form a slurry solution. The reaction was quenched carefully by slow addition of water, and extracted thrice by ethyl acetate. The combined organic extract was washed with brine, dried over anhydrous MgSO₄, filtered, and evaporated under reduced pressure to give the crude product. The crude product was purified by silica-gel flash column chromatography (*n*-hexane/ethyl acetate=80:20) to obtain the *N*-protected 2-methoxy-4-nitroaniline (**B1**) (3.49, 9.47 mmol, 91%) as a pale yellow solid.

¹H NMR (400 MHz, CDCl₃) δ 1.41 (s, 18H), 3.94 (s, 3H), 7.27 (d, 1H, *J* = 8.5 Hz), 7.78 (d, 1H, *J* = 2.4 Hz), 7.86 (dd, 1H, *J* = 8.5, 2.4 Hz). ¹³C (100 MHz, CDCl₃) δ 27.7, 56.1, 83.0, 106.3, 115.6, 129.5, 134.5, 147.7, 150.5, 154.8. ESI HRMS: Found: *m/z* 369.1663. Calcd for C₁₇H₂₅N₂O₇: (M+H)⁺ 369.1662.

Step 2: To a solution of iodobenzene (0.4 ml, 3.57 mmol) in THF (8 ml) cooled at $-20\text{ }^{\circ}\text{C}$ was added isopropylmagnesium chloride (1.8 ml, 2.0 M solution, 3.60 mmol) dropwise (Sapountzis and Knochel, 2002). The mixture was stirred at $-20\text{ }^{\circ}\text{C}$ under a N_2 atmosphere for 30 mins to allow I/Mg exchange. **B1** (553 mg, 1.5 mmol) was added and the mixture was left to be stirred at $-20\text{ }^{\circ}\text{C}$ for 2 h. Ethanol (2 mL) was then added to the reaction mixture, followed by addition of FeCl_2 (0.38 g, 3 mmol), NaBH_4 (50 mg, 1.5 mmol) was added and the mixture was allowed to stir at room temperature under a N_2 atmosphere for 2 h. The reaction was finally quenched by water, and extracted thrice by diethyl ether. The combined organic extract was washed with brine, dried over anhydrous MgSO_4 , filtered, and evaporated under reduced pressure to give the crude product. The crude product was purified by silica-gel flash column chromatography (*n*-hexane/ethyl acetate=70:30) to obtain the *N*-protected di-phenyl aniline (**B2**) (354 mg, 0.855 mmol, 57 %) as a pale brown solid.

^1H NMR (400 MHz, CDCl_3) δ 1.43 (s, 18H), 3.76 (s, 3H), 5.73 (br s, 1H), 6.59-6.62 (m, 2H), 6.93-6.97 (m, 2H), 7.08-7.10 (m, 2H), 7.28-7.30 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 28.0, 55.5, 82.1, 101.1, 109.2, 118.2, 121.2, 121.5, 129.4, 129.5, 143.0, 144.0, 152.4, 155.3. ESI HRMS: Found: m/z 415.2236. Calcd for $\text{C}_{23}\text{H}_{31}\text{N}_2\text{O}_5$: $(\text{M}+\text{H})^+$ 415.2233.

Step 3: To a stirred solution of acetyl chloride (41 μL , 0.579 mmol) in methanol (1 ml) was added a solution of **B2** (60 mg, 0.145 mmol) in methanol (0.4 ml) at $0\text{ }^{\circ}\text{C}$ under a N_2 atmosphere. The mixture was then allowed to warm to room temperature before refluxed for 1 h. The reaction was then allowed to cool to room temperature and quenched by addition of saturated aqueous NaHCO_3 , and extracted thrice with ethyl acetate. The combined organic extract was washed with brine, dried over anhydrous MgSO_4 , filtered, and evaporated under

reduced pressure to give the crude product. The crude product was purified by silica-gel flash column chromatography (*n*-hexane/ethyl acetate=70:30) to obtain 3-methoxy-*N*-phenylbenzene-1,4-diamine (**B3**) in (24 mg, 0.113 mmol, 78%) as a dark purple solid.

¹H NMR (400 MHz, CDCl₃) δ 3.79 (s, 3H), 6.60 (dd, 1H, *J* = 2.4, 8.0 Hz), 6.65-6.68 (m, 2H), 6.79 (tt, 1H, *J* = 1.1, 7.3 Hz), 6.84-6.88 (m, 2H), 7.16-7.24 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 55.7, 106.0, 114.8, 115.3, 115.7, 119.2, 129.5, 132.0, 134.1, 146.1, 148.1. ESI HRMS: Found: *m/z* 215.1186. Calcd for C₁₃H₁₅N₂O: (M+H)⁺ 215.1184.

Step 4: To a solution of **B3** (21 mg, 0.100 mmol) in dichloromethane (1 ml) was added pyridine (13 μL, 0.16 mmol) and methyl 3-(chlorosulfonyl)thiophene-2-carboxylate (36 mg, 0.15 mmol) at room temperature under an inert atmosphere and left to be stirred for 3 h. The reaction was quenched by addition of water and extracted thrice with ethyl acetate. The combined organic extract was washed with brine, dried over anhydrous MgSO₄, filtered, and evaporated under reduced pressure to give the crude product. The crude product was purified by silica-gel flash column chromatography (*n*-hexane/ethyl acetate=60:40) to obtain GSK0660 (38 mg, 0.091 mmol, 91%) as an orange solid.

Methyl 3-(*N*-(2-methoxy-4-(phenylamino)phenyl)sulfamoyl)-thiophene-2-carboxylate (GSK0660) (Toth et al., 2012)

¹H NMR (400 MHz, CDCl₃) δ 3.52 (s, 3H), 4.01 (s, 3H), 5.65 (br s, 1H), 6.45 (d, 1H, *J* = 2.4 Hz), 6.58 (dd, 1H, *J* = 2.4, 8.8 Hz), 6.94 (t, 1H, *J* = 7.2 Hz), 6.92-7.02 (m, 2H), 7.23-7.27 (m, 2H), 7.38-7.44 (m, 3H), 8.27 (br s, 1H). ¹³C NMR (100 MHz, CDCl₃) δ 53.3, 55.6, 100.6, 109.9, 118.1, 118.4, 121.6, 126.9, 129.6, 129.7, 131.6, 131.8, 142.6, 142.8, 145.4, 152.8, 160.9. ESI HRMS: Found: *m/z* 419.0731. Calcd for C₁₉H₁₉N₂O₅S₂: (M+H)⁺ 419.0735.

Synthesis of compound 10h (10h)

Compound 10h (IUPAC: methyl 3-(N-(4-(isopentylamino)-2-methoxyphenyl)sulfamoyl)-thiophene-2-carboxylate) was prepared according to reported synthetic route as shown in Scheme 3 (Toth et al., 2012).

Scheme 3

Step 1: To a solution of 4-fluoro-2-methoxy-1-nitrobenzene (471 mg, 2.75 mmol) in anhydrous acetonitrile (2 ml) was added 3-methylbutan-1-amine (0.56 mL, 4.82 mmol) and the reaction mixture was heated to 120 °C under microwave irradiation in a sealed 10 mL reaction vessel for 2 h. The solvent was evaporated under reduced pressure, the residue was purified by silica-gel flash column chromatography (*n*-hexane/ethyl acetate=80:20) to obtain *N*-isopentyl-3-methoxy-4-nitroaniline (**C1**) (513 mg, 2.16 mmol, 78%) as a yellow solid. NMR analysis is in agreement with the literature data.

Step 2: To a solution of **C1** (460 mg, 1.93 mmol) in EtOAc (4 ml) was added Pd/C (63 mg, 10% Pd in carbon w/w%) under a N₂ atmosphere. The atmosphere was then removed *in vacuo* and replaced by hydrogen via balloon (1 atm). The reaction mixture was then left to be stirred at room temperature in the dark for 12 h. The catalyst was filtered off through a pad of celite and the filtrate was concentrated under reduced pressure to give the light sensitive aniline **C2**, which was directly used in the next step without further purification.

Step 3: Aniline **C2** was dissolved in anhydrous pyridine (2 mL) under a N₂ atmosphere. *N,N*-dimethylpyridin-4-amine (DMAP) (118 mg, 0.966 mmol) and methyl 3-(chlorosulfonyl)thiophene-2-carboxylate (558 mg, 2.32 mmol) were added and the reaction mixture was stirred for 48 h in the dark. The reaction was quenched by addition of water, and extracted thrice by dichloromethane. The combined organic extract was washed with brine, dried over anhydrous MgSO₄, filtered, and evaporated under reduced pressure to give the crude product. The crude product was purified by silica-gel flash column chromatography (*n*-

hexane/ethyl acetate=60:40) to obtain compound 10h (390 mg, 0.945 mmol, 49% in two steps) as a yellow solid.

Methyl 3-(*N*-(4-(isopentylamino)-2-methoxyphenyl)sulfamoyl)-thiophene-2-carboxylate (10h)

^1H NMR (400 MHz, CDCl_3) δ 0.92 (d, 6H, $J = 6.8$ Hz), 1.45 (q, 2H, $J = 7.3$ Hz), 1.67 (sept, 1H, $J = 6.8$ Hz), 3.04 (t, 2H, $J = 7.4$ Hz), 3.48 (s, 3H), 3.57 (br s, 1H), 3.99 (s, 3H), 5.92 (d, 1H, $J = 2.4$ Hz), 6.11 (dd, 1H, $J = 8.6, 2.4$ Hz), 7.27 (d, 1H, $J = 8.6$ Hz), 7.36 (s, 2H), 8.10 (br s, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 22.5, 25.9, 38.4, 42.1, 52.9, 55.1, 95.6, 104.4, 114.2, 128.1, 129.1, 131.4, 131.5, 145.6, 148.2, 153.4, 160.7.

¹H NMR Spectra of GW501516

¹³C NMR Spectra of GW501516

¹H NMR Spectra of GSK 0660

¹³C NMR Spectra of GSK 0660

¹H NMR Spectra of 10h

¹³C NMR Spectra of 10h

995 gene signature for human SSC

110 genes upregulated in fibroproliferative SSC

113 genes downregulated in fibroproliferative SSC

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Angiopoietin-like 4 Mediates Colonic Inflammation by Regulating Chemokine Transcript Stability via Tristetraprolin

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Many gastrointestinal diseases exhibit a protracted and aggravated inflammatory response that can lead to hypercytokinaemia, culminating in extensive tissue damage. Recently, angiopoietin-like 4 (ANGPTL4) has been implicated in many inflammation-associated diseases. However, how ANGPTL4 regulates colonic inflammation remains unclear. Herein, we show that ANGPTL4 deficiency in mice (ANGPTL4^{-/-}) exacerbated colonic inflammation induced by dextran sulfate sodium (DSS) or stearic acid. Microbiota was similar between the two genotypes prior DSS challenge. A microarray gene expression profile of the colon from DSS-treated ANGPTL4^{-/-} mice was enriched for genes involved in leukocyte migration and infiltration, and showed a close association to inflamed ulcerative colitis (UC), whereas the profile from ANGPTL4^{+/+} littermates resembled that of non-inflamed UC biopsies. Bone marrow transplantation demonstrates the intrinsic role of colonic ANGPTL4 in regulating leukocyte infiltration during DSS-induced inflammation. Using immortalized human colon epithelial cells, we revealed that the ANGPTL4-mediated upregulation of tristetraprolin expression operates through CREB and NF- κ B transcription factors, which in turn, regulates the stability of chemokines. Together, our findings suggest that ANGPTL4 protects against acute colonic inflammation and that its absence exacerbates the severity of inflammation. Our findings emphasize the importance of ANGPTL4 as a novel target for therapy in regulating and attenuating inflammation.

An aggravated inflammatory response is a common feature of many gastrointestinal disorders, such as inflammatory bowel diseases, enteritis, and colitis. Many of these conditions are caused by changes in dietary fat intake, the ingestion of bacteria-contaminated food and water, and certain chemicals. These insults trigger an inflammatory response by inducing the recruitment of macrophages to the site of inflammation to combat pathogens, neutralize harmful immunogens and promote tissue repair¹. However, a protracted inflammatory response can cause tissue damage and lead to hypercytokinaemia, which is a potentially fatal immune reaction. Immune cell infiltration into the site of damage is highly regulated by chemotactic factors, such as macrophage inflammatory protein 1 α and chemokine (C-C motif) ligand 2 (CCL2)^{2,3}. As the initial cellular barrier that encounters luminal insults, intestinal and colonic epithelia play important roles in the early recruitment of inflammatory cells to the mucosa. Epithelial cells are a major source of chemoattractants, and epithelial chemokine production has been proposed as a key target of future therapies for gastrointestinal disorders⁴. However, much remains to be understood about the mechanisms that regulate the levels of these chemokines in the gastrointestinal and colonic tracts.

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Angiopoietin-like 4 (ANGPTL4) is a matricellular protein that has been implicated in many inflammation-associated diseases⁵. Native full-length ANGPTL4 (fANGPTL4) is proteolytically cleaved into two functionally distinct isoforms: the N-terminal domain (nANGPTL4) inhibits lipoprotein lipase (LPL) and directly regulates energy homeostasis, while the C-terminal domain (cANGPTL4) has been implicated in various processes such as cancer metastasis, skin wound and pulmonary inflammation^{6–8}. Diabetic wounds show low endogenous cANGPTL4 levels and have been associated with an elevated F4/80⁺ macrophage population at the wound site. The infiltration of F4/80⁺ macrophages was reduced upon treatment of diabetic wounds with recombinant cANGPTL4 when compared with saline⁹. ANGPTL4 can also protect against the severe pro-inflammatory effects of saturated fat by inhibiting fatty acid uptake by mesenteric lymph node macrophages¹⁰. Similarly, ANGPTL4 confers protective effects against the development of atherosclerosis¹¹, which has been associated with atherogenesis and macrophage polarization¹². ANGPTL4 has also been identified as an angiogenic mediator in arthritis¹³. ANGPTL4 has been observed to exacerbate influenza-associated inflammation through IL-6–Stat3 signaling in the lung¹⁴. Furthermore, serum ANGPTL4 was associated with the C-reactive protein level in type II diabetic patients, suggesting that ANGPTL4 may be involved in the progression of inflammation during metabolic syndrome¹⁵. Thus, ANGPTL4 may exert both anti- and pro-inflammatory effects in a context-dependent manner. Despite numerous reports of the role of ANGPTL4 in inflammation, the mechanisms whereby ANGPTL4 modulates inflammation in various diseases remain largely unclear.

Herein, we describe an anti-inflammatory role for colonic ANGPTL4 in dextran sulfate sodium salt (DSS)-induced colitis and dietary stearic acid (SA) intake *in vitro* and *in vivo*. We showed that the microbiota was similar between ANGPTL4^{+/+} and ANGPTL4^{-/-} mice at steady states, but with perturbation such as DSS treatment some differences in microbiota community become accentuated. Bone marrow transplantation and microarray analysis confirmed the intrinsic role of colonic ANGPTL4 in regulating leukocyte infiltration during DSS-induced inflammation, and thus the colonic inflammatory landscape. The underlying mechanism involves the regulation of tristetraprolin (TTP or ZFP36), an mRNA-binding protein that is involved in chemokine destabilization, by ANGPTL4 via activation of CREB and NF- κ B transcription factors.

Results

ANGPTL4 reduces DSS- and saturated fat-induced colonic inflammation. We first characterized the intestinal and colonic tract of unchallenged ANGPTL4-knockout (ANGPTL4^{-/-}) and wild-type (ANGPTL4^{+/+}) mice. There was no significant difference in body weight, colon length, disease activity index (DAI), endpoint macroscopic scores or histological scores between the genotypes (Fig. 1a and Supplementary Fig. S1a,c). Detailed examination revealed that ANGPTL4^{-/-} mice exhibited an increased muscularis thickness and shorter colonic villus length than ANGPTL4^{+/+} littermates (Fig. 1b, Supplementary Fig. S1d,e). To gain insights into the role of ANGPTL4 in acute colonic inflammation, we challenged ANGPTL4^{-/-} and ANGPTL4^{+/+} mice to either 5% DSS or 15% (w/w) stearic acid (SA) for 8 days.

ANGPTL4^{-/-} mice receiving DSS lost significantly more weight compared with ANGPTL4^{+/+} mice from day 6 onwards (Supplementary Fig. S1a). DSS-treatment resulted in a significant increase in both the DAI and endpoint macroscopic score (Fig. 1c and Supplementary Fig. S1b) as well as a reduction in the ANGPTL4^{+/+} colonic villus length, whereas there was no significant reduction observed in ANGPTL4^{-/-} mice compared with their cognate controls (Fig. 1b and Supplementary Fig. S1e). Histological analysis of the DSS-challenged ANGPTL4^{-/-} colon sections revealed a severe loss of epithelial integrity and a massive infiltration of immune cells into the lamina propria when compared with cognate ANGPTL4^{+/+} colon (Fig. 1b). Indeed, we observed a higher number of Ly6G⁺ neutrophils and CD68⁺ macrophages in the colons of DSS-treated ANGPTL4^{-/-} mice (Fig. 1d and Supplementary Fig. S1f). FACS analysis also confirmed the higher number of Ly6G⁺, CD11b⁺ and F4/80⁺ cells in ANGPTL4^{-/-} mice compared to ANGPTL4^{+/+} mice (Fig. 1e,f). Such differences were not attributed to an increase in apoptotic cells between the genotypes, as indicated by TUNEL-positive cells and the expression of cleaved caspase 3 (Supplementary Fig. S1g). Consistent with the elevated inflammation status, we also detected higher levels of TNF- α , IL-6 and IFN- γ mRNA transcripts (Fig. 1g).

As expected, stearic acid induced a more subdued inflammatory response compared to the DSS challenge (Fig. 1g). No significant differences in weight gain, blood glucose levels, DAI, endpoint macroscopic scores or histological scores were observed between ANGPTL4^{-/-} and ANGPTL4^{+/+} mice on the SA diet compared with mice fed a normal chow diet (Fig. 1c and Supplementary Fig. S1a–c,h). ANGPTL4^{+/+} mice on an SA diet exhibited a longer colon, a thicker muscularis wall and a shorter colonic villus length at the endpoint compared with mice fed a standard diet (Fig. 1a,b and Supplementary Fig. S1c,d). Immunofluorescence staining showed a similar elevated number of Ly6G⁺ neutrophils and CD68⁺ macrophages in ANGPTL4^{-/-} colon (Fig. 1d and Supplementary Fig. S1f). FACS analysis also detected an elevated number of Ly6G⁺, CD11b⁺ and F4/80⁺ cells in ANGPTL4^{-/-} mice (Fig. 1e,f). Together, our data demonstrated that the absence of ANGPTL4 enriched for a subset of the immune cell population not limited to neutrophils, macrophages and eosinophils during inflammation. Altogether, our observations are consistent with a model whereby ANGPTL4 attenuates colonic inflammation in response to inflammatory stimuli.

ANGPTL4 deficiency does not influence colonic commensal microbiota at steady state. Several reports have suggested that dysbiosis aggravates host inflammatory and metabolic diseases^{16–19}. Studies also suggested that a subset of microbes could regulate ANGPTL4 expression in the gut in response to changes in energy demands^{20–22}. To examine if the exacerbated colonic inflammatory response in ANGPTL4^{-/-} mice could be attributed to microbe composition, we performed 16S metagenomics sequencing using the V4 region to identify microbe abundance between ANGPTL4^{+/+} and ANGPTL4^{-/-} littermates before and after DSS-induced inflammation. Redundancy analysis showed that genotype and inflammation amounted to 41.2% of the total variation observed between microbial compositions in the colon. Genotype could only explain for 10.7% of the differences

Figure 1. ANGPTL4 attenuates colonic inflammation. (a–e) Images of colon samples (a) Representative colon sections stained with hematoxylin and eosin (H&E) (b). (c) Disease activity index (DAI) of mice assessed every alternate day over the treatment regimes. Scoring criteria can be found in Supplementary Table S1. (d) Percentage of Ly6G⁺ and CD68⁺ cells per field of view. Total DAPI-stained nuclei were taken as the total cell number. Microscopic views from 5 different sections were numerated. (e) Representative FACS histograms for Ly6G expression (neutrophils) from ANGPTL4^{+/+} and ANGPTL4^{-/-} littermates for the indicated treatments. (f) Representative FACS analysis of the colons from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice double stained for F4/80 (macrophages) and CD11b (macrophages, monocytes, granulocytes, NK cells, dendritic cells) for each of the treatments. (g) Relative mRNA levels of pro-inflammatory cytokines (TNF- α , IL-6 and IFN- γ) from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice at the endpoint. For the Veh, DSS and SA groups, n = 20 mice were used for each treatment. The Mann–Whitney U test was used. *p < 0.05; **p < 0.01.

Figure 2. ANGPTL4 deficiency does not influence commensal microbe composition. Pie charts showing the commensal microbe landscape at steady state (*upper panel*) and after DSS treatment (*lower panel*) of age- and gender-matched $ANGPTL4^{+/+}$ and $ANGPTL4^{-/-}$ littermates. Values in the charts denote mean percentage of abundance. $n = 6$ mice per group.

observed, while inflammation amounted to at least 31.1%. The steady state phylogenetic microbe composition was predominated by Firmicutes and Bacteroidetes, which did not differ much between both $ANGPTL4^{+/+}$ and $ANGPTL4^{-/-}$ mice before DSS treatment (Fig. 2). As expected, DSS-induced inflammation caused a sharp decrease in Bacteroidetes and a concomitant increase in Firmicutes and Proteobacteria. We found that Firmicutes were more abundant in inflamed $ANGPTL4^{+/+}$ mice, while Proteobacteria was more prevalent in $ANGPTL4^{-/-}$ mice (Fig. 2). Collectively, our data showed that microbe composition was similar between $ANGPTL4^{+/+}$ and $ANGPTL4^{-/-}$ mice at steady state, but with perturbation such as DSS treatment, some differences in the microbiota community become accentuated.

Intrinsic role of colonic ANGPTL4 in leukocytes infiltration during DSS-induced inflammation.

To further define the role of epithelial-derived ANGPTL4, we performed bone marrow transplantation (BMT) experiment. We transferred bone marrow from $ANGPTL4^{+/+}$ and $ANGPTL4^{-/-}$ donors to γ -irradiated wild-type $ANGPTL4^{+/+}$ (WT IR) recipient mice (BMT ($ANGPTL4^{+/+}$) or BMT ($ANGPTL4^{-/-}$); Fig. 3a,b). $CD4^{+}$ and $CD8^{+}$ cells were depleted from bone marrow inoculum before BMT to limit possible graft-versus-host-disease (GVHD)-related mortality. The bone marrow of WT IR mice appeared necrotic with a significant reduction in the number of bone marrow cells. The spleen was smaller in size and has reduced $CD45^{+}$ splenic cells (Fig. 3c,d). Hematopoietic reconstitution was observed through the repopulation of bone marrow cells in the femur and $CD45^{+}$ cells in the spleen at 4-week post BMT (Fig. 3c,d). Genotype PCR confirmed the successful reconstitution of the respective donor cells in WT IR mice (Fig. 3e). No significant difference in classical clinical scorings was observed between BMT ($ANGPTL4^{+/+}$) or BMT ($ANGPTL4^{-/-}$) chimeras on vehicle treatment (Fig. 3f). Histological analysis also revealed little difference between BMT ($ANGPTL4^{+/+}$) or BMT ($ANGPTL4^{-/-}$) chimeras (Fig. 3g). As expected, DSS-induced inflammation caused an erosion of the epithelial lining with increased infiltration of immune cells into the lamina propria (Fig. 3g). Notably, there was no significant difference recorded in the DAI, endpoint macroscopic score or histological score between BMT ($ANGPTL4^{+/+}$) and BMT ($ANGPTL4^{-/-}$) chimeras after DSS treatment (Fig. 3f,g and Supplementary Tables S1–3). FACS analysis also showed no significant difference in the number of $CD11b^{+}$ and $F4/80^{+}$ cells in the inflamed colons of BMT ($ANGPTL4^{+/+}$) and BMT ($ANGPTL4^{-/-}$) chimeras (Fig. 3h). Taken together, our data demonstrates the intrinsic role of epithelial-derived ANGPTL4 in regulating colonic leukocyte infiltration during DSS-induced inflammation, and thus the colonic inflammatory landscape.

ANGPTL4 deficiency alters colonic inflammatory gene expression. Our observations suggest that ANGPTL4 deficiency increased the susceptibility to inflammation induced by SA and DSS, albeit with differential severities. To strengthen our findings, we performed a comparative microarray gene expression analysis using colon tissues from $ANGPTL4^{-/-}$ and $ANGPTL4^{+/+}$ mice challenged with either DSS or SA. Using Ingenuity Pathway Analysis (IPA), we identified 250 differentially expressed genes associated with “gastrointestinal diseases”. As expected, the gene expression profiles of DSS-challenged colon samples showed a more aggravated

Figure 3. Intrinsic role of colonic ANGPTL4 in leukocytes infiltration during DSS. (a,b) Graphical illustrations depicting the experimental setup for bone marrow transplantation (BMT). CD4⁺ and CD8⁺ depleted bone marrow cells from age- and gender-matched ANGPTL4^{+/+} or ANGPTL4^{-/-} donors were transplanted into γ -irradiated ANGPTL4^{+/+} wild-type recipient (WT IR). Chimera BMT ANGPTL4^{+/+} and BMT ANGPTL4^{-/-} denote WT IR transplanted with bone marrow cells from ANGPTL4^{+/+} and ANGPTL4^{-/-} donor, respectively. Graph showed the number of CD45⁺ cells from spleen of indicated mice (Mean $\pm$ S.D.; n = 6 mice per group). (c,d) Representative

images of the femur (c) and spleen (d) from indicated mice (WT and WT IR) and chimeras (BMT ANGPTL4^{+/+} and BMT ANGPTL4^{-/-}). Inserts in (c) showed the bone marrow cell pellet before and after red blood cell lysis. Graph in (c; *left panel*) showed the number of bone marrow cells from femur of indicated mice (Mean $\pm$ S.D.; n = 6 mice per group). (e) Representative agarose gel image of genotype PCR products from CD45⁺ splenic cells from indicated mice. (f) Graphs showing the disease activity index (DAI; *left panel*), macroscopic score (*middle panel*) and histological score (*right panel*) of indicated mice treated with vehicle or 2% DSS. Individual scoring criteria can be found in Supplementary Tables S1–S3. (g) Representative hematoxylin and eosin (H&E) images of colon section from vehicle and DSS treated mice post BMT. (h) Representative FACS analysis for CD11b⁺ and F4/80⁺ cells from colons of BMT ANGPTL4^{+/+} and BMT ANGPTL4^{-/-} chimeras treated with vehicle or DSS. n = 6 per group. The Mann-Whitney U test was used. *p < 0.05; **p < 0.01 and ***p < 0.001. n.s. denotes not significant.

response when compared with the SA-challenged colon samples. Consistent with the above observations, the heat map also indicated that ANGPTL4 deficiency resulted in more severe inflammation in ANGPTL4^{-/-} mice when compared with ANGPTL4^{+/+} mice (Fig. 4a,b and Supplementary Table S4). Further studies of the 2 islands of dissimilarly regulated genes between either challenge groups or genotypes revealed that 52% of the transcripts were related to TNF- α signaling, whereas 27% and 31% were linked to the IL-10 and IL-6 signaling cascades, respectively (Fig. 4c). Gene ontology maps suggested that these genes were primarily involved in leukocyte migration and infiltration (Fig. 4d). The top diseases identified from previous studies listed in the IPA database include colitis, inflammation of the intestine, diabetes mellitus and gastrointestinal tract cancers and tumors (Fig. 4d). Next, a comparative gene expression analysis was performed between DSS-challenged ANGPTL4^{-/-} and ANGPTL4^{+/+} mice and human ulcerative colitis (UC) colonoscopy samples. The colonic mucosal samples from UC patient biopsies were classified as “inflamed” or “non-inflamed”, according to whether the samples exhibited signs of inflammation during collection. Unsupervised hierarchical clustering revealed that the gene expression profile of DSS-challenged ANGPTL4^{+/+} mice was more closely associated with the non-inflamed UC samples, while the profile of ANGPTL4^{-/-} mice was clustered more closely with the inflamed UC samples (Fig. 4e and Supplementary Table S5). Further analysis revealed that the UC samples showed the highest number of up-regulated genes, followed by the DSS-challenged ANGPTL4^{-/-} mice; the DSS-challenged ANGPTL4^{+/+} mice showed the lowest number of up-regulated genes (Fig. 4f). Our analysis also identified a cluster of distinctively regulated genes across both the mouse and human samples. Among these genes, 29%, 27%, and 23%, were involved in the IL-4, IL-1 β and IL-6 signaling cascades, respectively (Fig. 4g). Gene ontology analysis revealed that the top molecular and cellular functions included cellular movement, cellular development, cellular function and maintenance, and cellular proliferation, as well as cell death and survival (Fig. 4h). These transcripts have also been found to be involved in myeloid cell homeostasis, immune responses, and leukocyte homeostasis, activation and migration (Fig. 4h). Together, the histological analysis and gene expression profiling indicated that ANGPTL4^{-/-} mice were more susceptible to colonic inflammation, implicating a role for ANGPTL4 in immune cell infiltration.

ANGPTL4 deficiency increases the infiltration of immune cells. ANGPTL4 deficiency exacerbated colonic inflammation upon DSS and SA challenge. Guided by histological differences and gene expression changes between colon samples from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice, we next investigated the chemokine expression profile during inflammation. Consistent with the microarray findings, we detected an overall elevated expression of pro-inflammatory chemokines and a reduced expression of anti-inflammatory cytokines IL-10 and IL-17 in ANGPTL4^{-/-} mice compared with their ANGPTL4^{+/+} littermates (Fig. 5a). The highest levels of these chemokines were also consistent with greater infiltration of inflammatory cells into the colons of ANGPTL4^{-/-} mice (Fig. 1d–f and Supplementary Fig. 1f). The protein concentrations of CCL2, CCL11 and CXCL10 were also found to be elevated in ANGPTL4^{-/-} littermates in both the DSS- and SA- treated groups (Supplementary Fig. S2a). Furthermore, we observe a decreasing trend, albeit not statistically significant, in the protein levels of IL-1 β , IL-10 and IL-17 between ANGPTL4^{+/+} and ANGPTL4^{-/-} littermates among different treatments.

To date, little is known about the mechanisms by which ANGPTL4 exerts such effects on the immune and chemokine landscape. Recent publications suggest that ANGPTL4 may modulate the inflammatory response in a variety of disease models^{9–11,14,15,23}. Our microarray analysis and a review of the literature directed us to two potential intermediary proteins that respond quickly during acute inflammation and acts broadly to attenuate inflammation^{24,25}. Human antigen R (HuR) and tristetraprolin (TTP) are mRNA binding proteins well-characterized for their opposite roles in binding mRNA at AU-rich regions located at the 3′-UTR of target transcripts, including chemokines, to modify the inflammatory landscape at the onset of inflammation. HuR is highly selective in its interaction with its cognate mRNA binding partners and has been described as an active participant in promoting mRNA stability^{26,27}. In contrast, TTP promotes mRNA degradation by destabilizing target transcripts^{28–30}. The acute inflammation elicited by DSS and SA treatments significantly suppressed both ANGPTL4 and TTP expression in ANGPTL4^{+/+} littermates (Supplementary Fig. 2b). However, we detected no significant change in HuR expression in both pro-inflammatory treatments. Analysis of our focused gene expression array revealed that the expression of TTP was altered, as was the expression of genes involved in immune cell infiltration and responses. The reduced mRNA and protein levels of TTP in ANGPTL4^{+/+} and ANGPTL4^{-/-} littermates were confirmed by real-time PCR and western blot analyses (Supplementary Fig. 2c). Together, our data suggests the involvement of TTP in the regulation of local colonic inflammation.

To further understand the role that colonic epithelial cells play in regulating chemokine expression, we treated human colon epithelial cells (iCECs) with a panel of pro-inflammatory (DSS, SA, IL-1 β or TNF- α) or

Figure 4. ANGPTL4 deficiency alters colonic inflammatory gene expression. (a,e) Using Partek Genomic Suite software, we generated a microarray heat map showing changes in gene expression among colon tissues from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice fed with Veh, DSS or SA (a) and among colons from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice treated with DSS, inflamed and non-inflamed colon biopsies from ulcerative colitis patients (GSE9452) (e). Transcripts were estimated using a log₂ transformation and subjected to unbiased ANOVA to detect differentially expressed genes between samples. Only genes with a fold change < -1.2 or > 1.2 were of significance. The genes were then hierarchical clustered based on significance to generate heatmaps. For (a), among the 434 transcripts associated with “gastrointestinal diseases” identified with the IPA database,

63% were found to be commonly regulated across the three treatment groups. For (e), 32% of the transcripts associated with “gastrointestinal diseases” were found to be shared among the groups, with the gene profile from DSS-induced inflammation in ANGPTL4^{-/-} mice most closely associated with the inflamed UC biopsies. (b,f) A larger number of up-regulated genes was correlated with an increasing severity of colonic inflammation in the microarray analysis. (c) Gene ontology analysis of gene clusters (black boxes in (a)) indicated that the major pathways included IL-6-, IL-10- and TNF-related pathways. (d) IPA analysis ranked colitis, inflammation of the intestine and inflammatory bowel disease as the most closely associated gastrointestinal diseases, with leukocyte migration and infiltration as the predominant functions. (g) Among the genes that were commonly regulated between human and mouse colon samples (black box), most genes encode for components of the IL-1 β , IL-6 and IL-4 pathways. (h) The IPA database ranked cellular movement, development, function, growth and proliferation as the top molecular processes involved, along with immune processes such as leukocyte activation, myeloid cell homeostasis and immune responses being the most significant biological functions involved. The numbers in the bar graphs in (d,h) represent the p-values of the process in the GO analysis, with the smallest p-value being the most significant. An in-depth overview is described in Supplementary Fig. S4.

anti-inflammatory (HC or NaBu) stimuli. Notably, ANGPTL4 and TTP levels decreased when iCECs were treated with DSS, SA, IL-1 β and TNF- α (Fig. 5b). Exposure to such pro-inflammatory stimuli increased the levels of CCL2, CCL11, CXCL10 and IL-1 β mRNA transcripts and decreased the levels of IL-10 and IL-17 mRNA transcripts (Supplementary Fig. 2d). Conversely, anti-inflammatory HC and NaBu increased both ANGPTL4 and TTP levels, prompting a decreased expression of CCL2, CCL11, CXCL10 and IL-1 β concomitant with an increase in IL-10 and IL-17 (Fig. 5b and Supplementary Fig. 2d). Next, we examined the role of ANGPTL4 and TTP in mediating the abundance and stability of chemokines. iCECs were subjected to transient ANGPTL4 (iCEC_{ANGPTL4}) or TTP knockdowns (iCEC_{TTP}) and subsequently exposed to pro- or anti-inflammatory stimulation (Fig. 5c and Supplementary Fig. S2e–g, Tables S6 and 7). As a control, scrambled siRNA was used (iCEC_{Ctrl}). Pro-inflammatory stimuli (DSS, SA, IL-1 β and TNF- α) increased CCL2, CCL11, CXCL10 and IL-1 β expression but decreased IL-10 and IL-17 in iCEC_{Ctrl}. Conversely, HC and NaBu stimulation in iCEC_{Ctrl} suppressed CCL2, CCL11, CXCL10 and IL-1 β but heightened IL-10 and IL-17. The absence of ANGPTL4 (iCEC_{ANGPTL4}) raised the basal levels of CCL2, CCL11, CXCL10, IL-1 β , IL-10 and IL-17 expression independent of the nature of stimulation. Interestingly, the CCL11, IL-10 and IL-17 levels surged independent of stimulation when TTP was ablated (iCEC_{TTP}). Further investigation into chemokine stability demonstrated that the half-lives of CCL11, IL-10 and IL-17 mRNA transcripts, but not CCL2, IL-1 β or CXCL10, were prolonged in iCEC_{ANGPTL4} and iCEC_{TTP} (Fig. 5d). The decreased half-life of TTP in iCEC_{ANGPTL4}, and not vice versa, also confirmed that ANGPTL4 positively regulates downstream TTP (Supplementary Fig. S2g). In an effort to understand immune cell infiltration, THP1-derived macrophages were co-cultured with iCECs (iCEC_{ANGPTL4}, iCEC_{TTP} and iCEC_{Ctrl}) in the presence of various treatments, and macrophage transwell migration was investigated. Consistent with the above results, we found that deficiency in ANGPTL4 and TTP was sufficient to increase chemotactic signals from epithelial cells and accelerate the migration of macrophages (Fig. 5e and Supplementary Fig. S2i). Taken together, our results demonstrate that ANGPTL4 is an important regulator that modulates the chemokine landscape and immune cell infiltration during inflammation. Our data also highlights the role of ANGPTL4 in attenuating inflammation simultaneously through TTP-dependent and independent pathways, although the mechanism for the latter remains to be elucidated.

ANGPTL4 up-regulates TTP via CREB and NF- κ B. Full-length ANGPTL4 is proteolytically cleaved into N-terminal coiled-coil (nANGPTL4) and C-terminal fibrinogen-like (cANGPTL4) fragments³¹. To understand which fragment of ANGPTL4 is responsible for regulating TTP expression, we treated iCECs with recombinant (rh) cANGPTL4 and rh-nANGPTL4. This investigation showed that cANGPTL4 was potent in significantly up-regulating TTP expression (Supplementary Fig. 3a). Next, we sought to elucidate the mechanism whereby ANGPTL4 regulates TTP expression. We subjected rh-cANGPTL4-stimulated iCECs to a kinase inhibitor array screen to identify key signaling pathways involved in the cANGPTL4-mediated up-regulation of TTP (Supplementary Fig. 3b). TTP expression was increased when iCECs were treated with rh-cANGPTL4 (Supplementary Fig. 3b, *left panel*). Hence, we reasoned that kinase inhibitors that negated or inhibited the rh-cANGPTL4-mediated up-regulation of TTP mRNA transcripts indicated the involvement of those specific kinases in the selected signaling cascades (*middle panel*). Conversely, kinase inhibitors that did not attenuate the cANGPTL4-mediated up-regulation of TTP would indicate that those kinases were not involved in ANGPTL4-mediated signaling (*right panel*). Our results showed that inhibitors against CDK, JNK, MEK1/2, mTOR, PI3K δ , TYK2, BTK, CK2, GSK3, PDK1 and Aurora A/B/C targeted key signaling regulators of cANGPTL4-mediated TTP transcription (Supplementary Fig. 3c). To avoid bias, all kinases that prevented the up-regulation of TTP expression under the stimulation of rh-cANGPTL4 were used to study how their availability impacted the overall level of inflammatory signaling. Using IPA, we further established that these signaling mediators resulted in the activation of downstream transcription factors, such as AP1 (cFos-cJun complex), CREB and NF- κ B (Fig. 6a). Immunoblot analysis revealed that rh-cANGPTL4 increased the phosphorylation of CREB (pCREB) and NF- κ B (pNF- κ B) but not phosphorylated cFos (pcFos) (Fig. 6b). *In silico* analysis identified putative CREB (+455 to +453 bp), AP1 (+388 to +395 bp) and NF- κ B (+528 to +537 bp) transcription binding sites in the promoter of TTP (Fig. 6c).

To underscore the importance of CREB and NF- κ B as active transcription factors mediating TTP expression upon cANGPTL4 stimulation, we inhibited the activity of CREB, NF- κ B or both using siRNAs (iCEC_{RELA}, iCEC_{CREB} and iCEC_{RELA/CREB}) and specific kinase inhibitors (IKK-2, 666-15 and IKK-2/666-15), respectively. The highly

Figure 5. ANGPTL4 deficiency increases the infiltration of immune cells. (a) Relative mRNA levels of indicated chemokines in colon tissues from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice treated with Veh, DSS or SA. (b) Relative expression (left panel) and immunoblot analysis (right panel) of ANGPTL4 and TTP levels in iCECs when stimulated with DSS, SA, IL-1 β , TNF- α , HC or NaBu. (c) Heatmap displaying log-transformed relative expression levels of indicated chemokine mRNA transcripts in iCEC_{Ctrl}, iCEC_{TTP} and iCEC_{ANGPTL4} when stimulated with DSS, SA, IL-1 β , TNF- α , HC or NaBu. (d) Decay curves of IL-1 β , IL-10, IL-17, CCL2, CCL11 and CXCL10 in iCEC_{ANGPTL4}, iCEC_{TTP} or iCEC_{Ctrl} following actinomycin D treatment after NaBu stimulation. (e) Transwell migration assay measuring the percentage change in relative fluorescence intensity of THP1 migration in response to chemokines secreted by iCEC_{Ctrl}, iCEC_{TTP} and iCEC_{ANGPTL4} (bottom panel). Five independent experiments (n = 5) were performed in triplicate. The Mann–Whitney U test was used. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 6. ANGPTL4 up-regulates TTP expression via CREB and NF- κ B. (a) IPA identified CREB, NF- κ B and AP-1 as potential transcription factors that regulate the expression of TTP when stimulated with rh-cANGPTL4. IPA-assisted pathways were mapped following experimental screens using a kinase inhibitor array. (b) Immunoblot analysis revealed an increase in the phosphorylation of CREB (pCREB) and NF- κ B p65 (pNF- κ B) but not cFos (pcFos) after rh-cANGPTL4 stimulation. (c) A schematic illustration of the promoter of the human *TTP* gene. The putative transcription factors binding sites were determined *in silico* online with the

Jaspar database. The relative location of the ChIP primers was indicated. **(d)** Chromatin Immunoprecipitation (ChIP) and re-ChIP using pCREB or pNF- κ B/p65 and coactivator p300 antibodies in iCECs that were stimulated with rh-ANGPTL4. **(e)** Relative expression and immunoblot analysis of TTP for iCECs repressed of CREB and/or NF- κ B activity using siRNA (iCEC_{Ctrl}, iCEC_{RELA}, iCEC_{CREB} and iCEC_{RELA/CREB}) and specific kinase inhibitors (Veh, IKK-2, 666-15 and IKK-2/666-15) in the absence and presence of rh-cANGPTL4. **(f)** Densitometry analysis showing the relative expression of pCREB and pNF- κ Bp65 in nuclear and cytoplasmic fractions of iCECs as treated in **(e)**, normalized against Histone H3 and GAPDH for nuclear and cytoplasmic fractions, respectively, for Supplementary Fig. 3h. The Mann–Whitney U test was used. Three independent experiments (n = 3) were performed.

potent CREB inhibitor (666-15) hinders the interaction of KID-KIX, which is essential for CREB-dependent gene transcription³², while the IKK-2 inhibitor IV (IKK-2) selectively targets IKK-2 and retards downstream p65NF- κ B signaling. In both instances, the lack of either CREB or NF- κ B was sufficient to reduce TTP expression in iCECs even in the presence of rh-cANGPTL4 (Fig. 6e and Supplementary Fig. S3g). We also observed reduced pCREB and pNF- κ B levels in nuclear and cytoplasmic fractions of iCECs during cANGPTL4-stimulation, suggesting that the nuclear translocation of pCREB and pNF- κ B was severely compromised (Fig. 6f and Supplementary Fig. 3h). In conclusion, our data suggests that the CREB and NF- κ B transcription factors play irreplaceable roles in the ANGPTL4-mediated, TTP-dependent signaling axis.

Discussion

The epithelial cells that line the gastrointestinal tract serve as a physiological barrier that prevents the invasion of pathogens and a selective conduit of luminal signals to the host. When harmful immunogens and pathogens are detected, epithelial cells produce chemoattractants to initiate the infiltration of immune cells into the mucosa. Our findings highlight the importance of colonic epithelial cell-secreted ANGPTL4 as a prospective regulator that alters the chemokine landscape in the colon to influence downstream inflammation. We showed that ANGPTL4-deficient mice were more susceptible to acute DSS and SA exposure. 16S metagenomic sequencing indicated little difference in microbe composition between ANGPTL4^{+/+} and ANGPTL4^{-/-} mice that could account for the exacerbated inflammatory response in ANGPTL4^{-/-} mice. Bone marrow transplant study emphasized the intrinsic role of epithelial-derived ANGPTL4 in regulating the inflammatory landscape in the colon. Using human colonic epithelial cells, we further showed that ANGPTL4 regulated the expression of TTP, an mRNA destabilizing agent, via the activation of CREB and NF- κ B. The absence of ANGPTL4 or TTP prolonged the mRNA half-life of a specific subset of chemokines.

An exuberant and protracted inflammatory response contributes to the development of many gastrointestinal disorders. Infiltrating macrophages are the major cellular components of this inflammatory response. As the initial cellular barrier, the colonic epithelium plays an important role in the recruitment of inflammatory cells to the mucosa. We showed that ANGPTL4-knockout mice fed DSS or SA exhibited a greater colonic inflammatory response, which was associated with greater infiltration of immune cells when compared with wild-type mice fed similar treatments. Similarly, pro-inflammatory stimuli such as DSS, SA, IL-1 β and TNF- α suppressed the expression of ANGPTL4 and TTP in iCECs, whereas anti-inflammatory stimuli such as HC and NaBu increased its expression. In support of these findings, previous studies showed that anti-inflammatory glucocorticoid treatment boosted TTP expression in lymphocytes³³, while the synthetic glucocorticoid dexamethasone increased TTP levels in A549 cells³⁴ as well as ANGPTL4 levels in hepatocytes³⁵ and adipocytes³⁶. Although the exact pathways remain to be elucidated, dietary NaBu was also found to stimulate ANGPTL4 expression via a PPAR-independent pathway³⁷. HC has also been demonstrated to up-regulate IL-10 and IL-17 levels in natural killer cells to improve pneumonia survival rates³⁸, although no detailed mechanism was reported. Interestingly, our findings cement the importance of ANGPTL4 in regulating the general inflammatory landscape in the gastrointestinal tract. Although other ANGPTL4-dependent but TTP-independent signaling mechanisms remain to be identified, we demonstrated that ANGPTL4 modifies the availability of a subset of chemokine signals that alter the ease of immune cell infiltration through a TTP-dependent signaling circuit.

Confluent iCECs mimic an intact gastrointestinal mucosal layer and express low basal levels of ANGPTL4 and TTP. Many previous studies have utilized cancerous lines, such as HT-29, Caco-2 and HCT-116 cells, as surrogates for an *in vitro* gastrointestinal model^{39,40}. Potentially, this ANGPTL4-dependent mechanism may be impaired or altered in cancerous lines, as the expression of ANGPTL4 is known to be elevated in many types of cancer⁷. We further established that iCECs secreted ANGPTL4, which acted in an autocrine manner to induce the expression of TTP. We previously showed that secreted ANGPTL4 bound and activated integrin-mediated pathways⁴¹. Indeed, TTP is an important factor that contributes to mediating, modulating and attenuating inflammatory responses. Using the combination of an unbiased kinase inhibitor screen assay, along with ChIP and re-ChIP experiments, our current work unveiled multiple signaling conduits whereby ANGPTL4 stimulates TTP expression, resulting in CREB and NF- κ B activation. Interestingly, BTK and Aurora inhibitors were identified to significantly inhibit the ANGPTL4-dependent up-regulation of TTP, suggesting that they exert dominant roles in this signaling cascade. Lending support for a role for Aurora in NF- κ B activation, Katsha A *et al.* also reported that Aurora kinase A promoted and sustained inflammation through NF- κ B, leading to tumor formation⁴². Again, the importance of Aurora in the ANGPTL4-mediated up-regulation of TTP may be compromised when studied in cancer cell lines. Our study also emphasizes a potential role for Aurora kinases in regulating inflammation. Similarly, other studies have shown that cancer cells that express low levels of TTP are correlated with a genetic signature of low expression of CREB-related target genes⁴³. Numerous studies have also shown that TTP binds to AU-rich sequences at the 3'-UTRs of specific mRNA transcripts such as TNF- α ⁴⁴, IFN- γ ⁴⁵, IL-10 and IL-17⁴⁶. In

agreement with these previous reports, we showed that the mRNA half-lives of specific inflammatory chemokines were prolonged in the absence of ANGPTL4 and TTP.

The pathogenesis of inflammatory bowel disease in humans is complex and has a multifactorial and diverse aetiology⁴⁷. Dysbiosis has been associated with inflammation and impaired mucosal immune function in intestine of humans^{48–50}. Our 16S sequencing revealed little difference in microbe composition between two genotypes that can account for the exacerbated colonic inflammatory response in ANGPTL4^{-/-} mice. In this respect, the extent to which ANGPTL4 expression defines the colonic microbiota profiles remains to be delineated. It is also conceivable that mechanisms other than ANGPTL4-TTP-mediated chemokine mRNA stability might be involved. Our microarray analyses showed that *Lrg1* and *Gpx2* expression was up-regulated by 6- and 4-fold, respectively, in the colons of ANGPTL4^{-/-} mice compared with wild-type counterparts. *Lrg1* is proposed to be a possible biomarker for the diagnosis of ulcerative colitis⁵¹. Both *Lrg1* and *Gpx2* proteins are up-regulated during the acute stages of pediatric appendicitis⁵² and colitis⁵³, respectively. These data suggest that additional ANGPTL4-dependent mechanisms may contribute to the severity of colitis in ANGPTL4^{-/-} mice. Furthermore, ANGPTL4 has been implicated in many inflammation-associated pathologies. Although not explored in the present study, the mechanism whereby ANGPTL4 may be targeted to influence cell infiltration via TTP-mediated chemokine mRNA stability could be applicable to these pathologies.

Materials and Methods

Antibodies and Reagents. Antibodies against c-Fos (#2250), phospho-c-Fos (Ser32; #5348), Elk-1 (#9182), phospho-Elk-1 (Ser383; #9186), CREB (#48H2) and phospho-CREB (Ser133; #9198) were from Cell Signaling (USA). Antibodies against Histone H3 (05-499), NF- κ B/p65 (MAB3026) and phospho-NF- κ B/p65 (Ser276; AB3375) were from Merck Millipore, USA. Antibodies against Ly-6G (#127602 and #127614), CD68 (#137002) were from Biolegend, USA. FACS antibodies (APC-/FITC-conjugated) against CD11b, CD45, F4/80 were from Miltenyi Biotec, USA. Anti-CD4- and anti-CD8a- conjugated microbeads (#130-049-201, #130-049-401) was purchased from Miltenyi Biotec, USA. Anti-TTP (sc-14030, Santa Cruz, USA), active/cleaved Caspase 3 (NB100-56113, Novus Biological, USA),

ChIP-grade antibodies against NF- κ B/p65 (ab7970) and CREB (ab31387) were from Abcam, UK and p300 (RW128) from Upstate Biotechnology, USA. IRDye[®] 680 LT goat anti-rabbit IgG, goat anti-mouse IgG and donkey anti-goat IgG were purchased from Li-Cor Biosciences, USA. Dextran sulfate sodium salt (DSS; 36 000 to 50 000M.Wt) was from Affymetrix, USA (#14489). Recombinant human cANGPTL4 (rh-cANGPTL4) and anti-ANGPTL4 antibodies were produced in-house as previously described²⁴. Recombinant human nANGPTL4 (rh-nANGPTL4; 8249-AN, RnD Systems), IL-1 β (ab9617, Abcam), TNF- α (#717904, BioLegend) and hydrocortisone (CAS 50-23-7, Calbiochem). All other chemicals were purchased from Sigma-Aldrich.

Animals. ANGPTL4^{+/-} C57BL/6J mice were acquired from the Mutant Mouse Regional Resource Center (MMRRC) and were generated by Genentech. The mice were crossed to produce ANGPTL4^{+/+} and ANGPTL4^{-/-} offspring. DNA from mouse ear clippings was isolated using the KAPA Express Extract reactions and genotyped using Mouse Genotyping HotStart in accordance with the manufacturer's recommendations (KAPA Biosystems, USA).

The PCR products were visualized on 2% agarose gels stained with SYBR Safe (ThermoFisher Scientific, USA). In the DSS treatment study, age-matched ANGPTL4^{+/+} and ANGPTL4^{-/-} males (n = 20) were treated with 5% DSS in the drinking water for 8 days; the DSS was changed every 2 days. In the SA study, mice (n = 20) were fed with a 15% SA:85% ground chow (w/w) diet for 8 days. The disease activity index (DAI) was assessed every alternate day, while macroscopic and histological scorings were performed at the experimental endpoint. The scoring criteria are available in Supplementary Tables S1–3. Protein and RNA samples were collected by scraping colonic epithelial cells. Animal experiments were approved by and carried out in accordance with the guidelines of Nanyang Technological University's Institutional Animal Care and Use Committee (NTU, IACUC, ARF SBS/NIE-A0321 and ARF SBS/NIE-A0324), Singapore.

Cell Culture. SV40-immortalized human colon epithelial cells (iCECs; T0570; ABM Canada) were cultured in Prigrow III Medium (ABM Canada) in collagen-coated flasks and maintained at 5% CO₂ at 37 °C. Confluent iCECs were stimulated with either 1 μ g/mL DSS, 500 μ M SA, 10 ng/mL IL-1 β , 10 ng/mL TNF- α , 0.4 μ g/mL HC or 2 mM NaBu for 6 h prior to harvesting. The siRNA knockdown against ANGPTL4, CREB, RELA or TTP was accomplished using Dharmacon ON-TARGETplus siRNA (Supplementary Table S8) according to the manufacturer's protocol (Thermo Scientific, USA). Inhibition of the transcription factors CREB and IKK were carried out using 0.1 μ M CREB inhibitor (666-15; #5661; Tocris Bioscience, UK) and/or 1 μ M IKK-2 inhibitor IV (IKK-2; 40-1481; Merck Millipore) respectively. iCEC_{ANGPTL4}, iCEC_{TTP} and iCEC_{Ctrl} were pre-treated with 2 mM NaBu for 2 h, followed by 10 μ g/mL actinomycin D for half-life measurements. *In vitro* experiments were carried out in triplicate.

RNA Extraction and Real-time Quantitative PCR. RNA was extracted using TRIzol (Invitrogen, USA) and was reverse transcribed using the iScript[™] Reverse Transcription Supermix according to the manufacturer's recommendations (Bio-Rad, USA). Quantitative PCR (qPCR) was conducted as previously described⁵⁴. Primer sequences are presented in Supplementary Table S9. The relative expression levels of the respective mRNAs described in this manuscript were normalized against those of the housekeeping ribosomal 18S RNA.

Microarray. RNA was transcribed into cDNA using an Applause WT-Amp ST System in accordance with the manufacturer's recommendations (NuGEN Technologies, USA). cDNA was purified using a MinElute Reaction Cleanup Kit (Qiagen, USA), fragmented and labeled using an Encore Biotin Module kit (NuGEN Technologies,

USA). Labeled cDNA molecules were hybridized using the Affymetrix hybridization master mix and injected onto GeneChip Mouse Gene 1.0 ST Array gene chips (Affymetrix, USA) for scanning. Partek Genomic Suite (Partek Inc., USA) and Ingenuity Pathway Analysis (IPA) software were used to perform analyses, an overview of which is available in Supplementary Fig. S4. The human colonic mucosal samples used for the comparative analyses in Fig. 3e–h were published by Olsen *et al.* under the GEO accession number GSE9452⁵⁵. The microarray data (accession number GSE78500) have been deposited in the GEO database.

Protein Extraction and Western Blot Analysis. Proteins were extracted in ice-cold lysis buffer (20 mM $\text{Na}_2\text{H}_2\text{PO}_4$, 250 mM NaCl, 1% Triton-X, 0.1% SDS, 1 mM PMSF, and 200 μM sodium orthovanadate) as previously described⁴⁴. Equal amounts of proteins were resolved using SDS-PAGE gels and electro-transferred to low-fluorescence PVDF membranes (IPFL00010; Merck Millipore, USA). The cytoplasmic and nuclear fractions were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (#78833; Thermo Scientific). Briefly, all protein samples for western blot analysis performed in this manuscript were first normalized using the NanoDrop 3300 Spectrophotometer (Thermo Scientific) prior to the ensuing downstream analyses. All loading controls for immunoblot analyses were obtained from the same sample. Membranes were blocked with 0.5 $\times$ Odyssey Blocking Buffer (LI-COR Biotechnology, USA) and probed with the respective primary antibodies overnight at 4 °C in 1 $\times$ Blocking Buffer with 0.1% Tween-20. Membranes were washed thrice in TBST (50 mM Tris HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 5 min each and incubated for 1 h with the respective secondary antibodies in 1 $\times$ Blocking Buffer at room temperature. Each membrane was washed thrice in TBST and then air-dried. Images were analyzed using a CLx scanner and Image Studio V2.1 (LI-COR Biosciences, USA). All membranes were first probed for target proteins before being re-probed for GAPDH housekeeping protein. Densitometry analyses were processed using ImageJ (NIH, USA). Densitometry values are presented under each blot, with values in bold representing significance ($p < 0.05$), or in graphs (for Fig. 4e and Supplementary Fig. 3h). The LEGENDplex Multi-Analyte Flow Assay Kit was used to measure the protein concentrations of targets (CCL2, CCL11, CXCL10, IL-1 β , IL-10 and IL-17) for both mouse and humans and was customized through Biolegend, USA and performed in accordance with the manufacturer's recommendations.

Tissue Processing, H&E, Immunofluorescence and TUNEL Staining. Tissues were harvested and fixed in 4% paraformaldehyde overnight at 4 °C and subsequently were dehydrated and embedded in paraffin as previously described⁵⁶. Tissues were rehydrated and stained with hematoxylin and eosin (H&E) according to the manufacturer's protocol (Sigma–Aldrich, USA). Slides were mounted with Fluka Eukitt quick-hardening mounting medium (Sigma–Aldrich, USA). Images were obtained using a Zeiss Axiovert 200M microscope with a Zeiss FLUAR 10x/0.5 NA objective and PALMRobo V4.3 software (Carl Zeiss, Germany).

The TUNEL assay was performed in accordance with the manufacturer's protocol (Roche). For immunofluorescence analyses, tissue sections were rehydrated and incubated in 5% normal goat serum (NGS) for 1 h and then incubated with the primary antibodies in 3% NGS overnight at 4 °C. Subsequently, sections were washed and then incubated with respective secondary Alexa Fluor 594-conjugated antibodies (Invitrogen) for 1 hour at room temperature. Slides were then mounted with Hoechst 33342 dye (Life Technologies, USA). Images were obtained using a LSM 710 confocal microscope with a Zeiss EC Plan-NEOFLUAR 20x/0.5 NA objective and were analyzed using ZEN 2012 Blue Edition software (Carl Zeiss, Germany).

Tissue Dissociation and Flow Cytometry. Mouse colon tissues from various treatment groups were first homogenized into single-cell suspensions using the gentleMACS Dissociator (Miltenyi Biotec, USA) in DMEM containing 1 mg/mL collagenase-3 and 40 units/mL DNase 1. Homogenates were filtered through a 70 μm nylon cell strainer, washed and finally resuspended in PBS blocking buffer containing 3% FBS. The resultant suspension was then incubated with the respective FITC-/APC- conjugated antibodies on ice. Samples were washed twice and resuspended in PBS, and were then subjected to FACS analysis on the Accuri C6 Flow Cytometer (BD Biosciences, USA). All FACS analyses were performed for 5000 events.

Transwell Migration Assay. THP1 monocytes were differentiated in 100 ng/mL TPA complete medium for 48 h and allowed to recover for 24 h on transwell inserts. Respective siRNA knockdowns were performed on iCECs (iCEC_{ANGPTL4}, iCEC_{TTP} and iCEC_{Ctrl}). Inserts containing differentiated THP1 cells were then introduced to the transfected iCECs and exposed to various pro- and anti-inflammatory stimuli for 10 h (Fig. 3e). Inserts were then washed with PBS twice and fixed in 1% glutaraldehyde for 10 min, rinsed with PBS and stained with SYTO 60 (Thermo Fisher, USA) for 30 min. Cotton buds were used to remove all un migrated cells trapped in the upper chamber of the inserts. Inserts were rinsed again in PBS before the quantification of fluorescence using the CLx scanner and Image Studio V2.1 (LI-COR Biosciences, USA). Relative fluorescence as a readout for cellular migration was calculated by normalizing the intensities between test wells (THP1 and iCECs) and control wells (THP1 without iCECs).

Chromatin Immunoprecipitation (ChIP) and Re-ChIP. ChIP and re-ChIP experiments were performed as described previously⁵⁷. In addition, samples were subjected to washes with increased stringencies: twice each with the low (1% Triton X-100, 0.1% SDS, 2 nM EDTA, 20 mM Tris-HCl, 150 mM NaCl at pH 8.1) and high salt (1% Triton X-100, 0.1% SDS, 2 nM EDTA, 20 mM Tris-HCl, 500 mM NaCl at pH 8.1) wash buffers. Primer sequences are presented in Supplementary Table S9.

Kinase Inhibitor Array. Immortalized human colon cells were subjected to treatment with 95 kinase inhibitors (SYN-2103; SYNkinase, Australia) in the presence or absence of rh-c-ANGPTL4 (8 $\mu\text{g}/\text{mL}$) for 6 h (Supplementary Table S10). RNA was then isolated and reverse transcribed as described above.

16S Metagenomics Sequencing. Post-weaned, age- and gender-matched ANGPTL4^{-/-} or ANGPTL4^{+/-} mice were used. Fresh fecal pellets were collected before and after DSS treatment. Bacterial genomic DNA was isolated from feces using the FASTDNA spin kit (#116570200, MP Biomedicals, USA). 16S sequencing of the 16S V4 variable region was performed by SeqMatic LLC, USA using the Illumina MiSeq sequencing platform.

Bone Marrow Transplant Model. BMT was performed using bone marrow cells isolated from donor mice (ANGPTL4^{-/-} or ANGPTL4^{+/-}) and transplanted into recipient γ -irradiated ANGPTL4^{+/-} wild-type mice (WT IR; n = 6 per group). Recipient WT mice were kept on acidified water (pH 3.0) over the course of the BMT. WT mice were medicated with sulfamethoxazole (40 mg/kg) and trimethoprim (8 mg/kg) in oral suspension for one week prior to and after γ -irradiation (9.5 Gy) using the Biobeam 8000 (Gamma-Service Medical GmbH, Germany). Red blood cells from donor bone marrow cells were lysed (0.89% NH₄Cl, 0.1 mM EDTA, pH 7.2). Residual bone marrow cells were washed with PBS and filtered through 30 μ M nylon cell strainer before depleting CD4⁺ and CD8⁺ cell populations using the QuadroMACS kit (#130-091-051, Miltenyi Biotec, USA). Approximately 10⁷ CD4⁺ and CD8⁺ depleted bone marrow cells were introduced to WT IR mice via retro-orbital injection. Chimera mice [BMT (ANGPTL4^{+/-}) and BMT (ANGPTL4^{-/-})] were allowed to recover for 4-weeks before 2% DSS treatment for 8 days. Bone marrow reconstitution was determined using genotype PCR (Supplementary Table S11). PCR products were visualized on 2% agarose gels tinted with SYBR Safe (ThermoFisher Scientific, USA). Cell count was numerated using the automated ADAM-MC cell counter (NanoEntek, USA). Cells from the spleen and colon were analyzed for immune cell infiltration using FACS (BD LSRFortessa X-20).

Statistical Analysis. Statistical analyses were performed using two-tailed Mann-Whitney U tests. P-values < 0.05 denote statistically significant differences between means; *p < 0.05; **p < 0.01 and ***p < 0.001. Values are expressed as the means $\pm$ standard error.

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Author Contributions

N.S.T. and T.P. designed, organized and interpreted the data, and wrote this manuscript. T.P., M.K.S., E.H.P.T., D.S.L.C., Y.L., J.W.K.W., J.S.K.C. and M.M.K.L. performed the experiments. Z.T., C.K.T., P.C.Z. and V.A. assisted in data analyses. N.S.T. supervised the entire study.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

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Angiopoietin-like 4 Increases Pulmonary Tissue Leakiness and Damage during Influenza Pneumonia

Graphical Abstract

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In Brief

Li et al. show that influenza infection stimulates the expression of ANGPTL4 through a STAT3-mediated mechanism. Host ANGPTL4 enhances pulmonary tissue leakiness and exacerbates inflammation-induced lung damage. ANGPTL4 deficiency improves pulmonary tissue integrity and accelerates lung tissue recovery.

Highlights

- ANGPTL4 is upregulated by a STAT3-mediated mechanism during influenza pneumonia
- ANGPTL4-deficient mice show reduced lung damage and accelerated lung recovery
- Antibodies targeting ANGPTL4 reduce pulmonary tissue leakiness and damage
- ANGPTL4 is a potential biomarker for respiratory infection and pneumonia

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Angiopoietin-like 4 Increases Pulmonary Tissue Leakiness and Damage during Influenza Pneumonia

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SUMMARY

Excessive host inflammatory responses negatively impact disease outcomes in respiratory infection. Host-pathogen interactions during the infective phase of influenza are well studied, but little is known about the host's response during the repair stage. Here, we show that influenza infection stimulated the expression of angiopoietin-like 4 (ANGPTL4) via a direct IL6-STAT3-mediated mechanism. ANGPTL4 enhanced pulmonary tissue leakiness and exacerbated inflammation-induced lung damage. Treatment of infected mice with neutralizing anti-ANGPTL4 antibodies significantly accelerated lung recovery and improved lung tissue integrity. ANGPTL4-deficient mice also showed reduced lung damage and recovered faster from influenza infection when compared to their wild-type counterparts. Retrospective examination of human lung biopsy specimens from infection-induced pneumonia with tissue damage showed elevated expression of ANGPTL4 when compared to normal lung samples. These observations underscore the important role that ANGPTL4 plays in lung infection and damage and may facilitate future therapeutic strategies for the treatment of influenza pneumonia.

INTRODUCTION

The occurrence of annual epidemics and random global pandemics of influenza exerts a large public health burden worldwide (Mizgerd, 2006; Armstrong et al., 1999). However, designing effective vaccines and treatment options has proven challenging in view of the rapid evolution of the virus. While many aspects of host-pathogen interactions during the course of an influenza infection have been studied, there is less informa-

tion on the host response during the repair stage of an infection (Mizgerd, 2008). A better understanding of the host response during the pulmonary repair phase may facilitate innovative treatment strategies. Host-specific biomarkers, indicative of the severity of lung tissue damage, could be exploited to delineate opportunities for therapeutic intervention.

Host immune responses are extremely important for containing influenza infections (Julkunen et al., 2000). Through the combined action of innate and adaptive immune responses, the infectious pathogen becomes inactivated and cleared from the body, repair processes start to resolve the tissue damage, and long-term immunity is ultimately established. However, excessive and prolonged inflammation may be detrimental to the host and contribute to the greater morbidity and mortality associated with influenza-induced inflammatory injury (Akaike et al., 1996; Monsalvo, 2010; Nicholls and Peiris, 2005; Buchweitz et al., 2007). Exaggerated inflammatory responses in the lung parenchyma can destroy alveoli, induce excessive edema, precipitate hypoxia, and cause pulmonary impairment (Narasaraju et al., 2011). Studies have documented that inflammatory injury to the lungs represents a major factor for the fatalities associated with pandemic H1N1-2009, highly pathogenic avian influenza viruses, and severe acute respiratory syndrome (SARS) coronavirus (Monsalvo, 2010; Nicholls and Peiris, 2005). Although inflammatory processes represent important therapeutic targets, anti-inflammatory therapies may also inhibit critical immune functions that mediate pathogen clearance, and they run the risk of enhancing pathogen replication and secondary infection (Uchida and Toyoda, 2011; Snelgrove et al., 2006; Aldridge et al., 2009; Ballinger and Standiford, 2010). An ideal treatment regimen should minimize the tissue damage caused by inflammation and facilitate recovery without interfering with the host's antiviral and antibacterial responses.

Angiopoietin-like 4 (ANGPTL4) belongs to a family of angiogenic-regulating, secreted proteins that bear a high similarity to members of the angiopoietin (ANG) family. However, ANGPTL4 does not bind to ANG receptor TIE1/2, indicating that ANGPTL4 exerts its distinct functions via a different mechanism from ANG proteins (Zhu et al., 2012; Grootaert et al., 2012).

Native full-length ANGPTL4 (fANGPTL4) contains a secretory signal peptide, an N-terminal coiled-coil structure, and a C-terminal fibrinogen-like domain. ANGPTL4 undergoes proteolytic processing by proprotein convertases at the linker region, thereby releasing the N-terminal region (nANGPTL4) and the monomeric C-terminal portion (cANGPTL4) (Zhu et al., 2012; Grootaert et al., 2012). The nANGPTL4 assembles into oligomeric structures, which is important for its function as a lipoprotein lipase inhibitor (Lei et al., 2011; Dijk and Kersten, 2014). The cANGPTL4 interacts with integrin β 1/5, vascular endothelial (VE)-cadherin, or claudin-5 to trigger intracellular pathways that aid wound healing and support tumor growth and metastasis (Goh et al., 2010a, 2010b; Huang et al., 2011; Zhu et al., 2011). The expression of ANGPTL4 is elevated by numerous stimuli that are also involved in influenza pneumonia, including glucocorticoids, transforming growth factor β , and hypoxia-inducible factor 1- α (HIF1- α) (Zhu et al., 2012; Grootaert et al., 2012). Furthermore, ANGPTL4 compromises the integrity of endothelial vascular junction by integrin signaling and disruption of intercellular VE-cadherin and claudin-5 cluster (Huang et al., 2011).

Interestingly, pulmonary edema due to vascular leakiness is a component of the fully developed viral lesion in the mouse (Harford et al., 1950). However, to our knowledge, the role of ANGPTL4 has not been studied in detail in influenza pneumonia, and study on this host response factor may open door to future intervention strategies. Thus in this study, we elucidate the role of host response protein ANGPTL4 during influenza pneumonia.

RESULTS

ANGPTL4 Expression Is Elevated in Influenza Virus-Infected Lungs

To investigate if ANGPTL4 is involved in the host response to influenza, we examined ANGPTL4 expression in mice infected with the PR8 influenza A H1N1 virus that is related to the 1918 pandemic influenza virus. The infection was performed via intratracheal inoculation with a nonlethal PR8 viral challenge that was sufficient to cause serious pulmonary damage. Viral replication was detected on bronchial structures at 3 days postinfection (dpi) and peaked at 5 dpi, as indicated by the presence of NS1 viral protein (Figure 1A). Virus was also detected in alveolar type II epithelial cells throughout the lungs by coimmunofluorescence staining with surfactant protein C (Figure 1B). At day 7, the viral protein expression began to decrease, and it became undetectable at 9 dpi (Figure 1A). The viral replication profile during the disease progression was further confirmed by qPCR of viral nucleoprotein RNA from infected lungs (Figure 1C). In addition, the expression of interferon- γ (IFN- γ) and interleukin-6 (IL-6), cytokines critical for innate and adaptive immunity against viral infections, peaked at 5 dpi (Figure 1C).

H&E staining of infected lung sections revealed that bronchial cells were damaged at 5 dpi, corresponding to the peak of viral load (Figure S1A). We observed extensive lung tissue damage that was marked by large regions of pulmonary hemorrhage and infiltration of host immune cells at 13 dpi (Figure S1A). Inflammatory cells, such as macrophages and neutrophils, infiltrated the alveolar spaces of infected lungs, causing tissue damage and bleeding (Figure S1B). Together with damaged tissue

debris, the infiltrated cells formed dense cell clusters and filled up the alveolar spaces. In our model, the mice recovered from the infection, which allowed us to investigate the events that occurred during the recovery phase. Indeed, overall recovery of tissue integrity was observed at 19 dpi (Figure S1A).

Next, we determined the kinetics of ANGPTL4 mRNA and protein levels in the infected lungs by qPCR and immunoblot analyses, respectively. ANGPTL4 mRNA was significantly upregulated at day 5 and remained elevated until 9 dpi. Thereafter, its expression decreased, reaching the basal level by 17 dpi (Figure 1D). Elevated levels of ANGPTL4 protein, specifically cANGPTL4, were detected in lung tissue homogenates at day 7 and remained elevated until 19 dpi, compared to day 0 controls (Figure 1E). We also examined the spatiotemporal expression of cANGPTL4 during influenza infection by immunofluorescence staining (Figure 1F). At day 0, cANGPTL4 protein was restricted to tubular structures such as the blood vessels and bronchioles (Figure 1F). At 5 and 13 dpi, corresponding to the peaks of ANGPTL4 mRNA and protein respectively, we observed stronger cANGPTL4 staining within the inflamed regions infiltrated by immune cells (Figure 1F). To eliminate the possible interference from autofluorescence, we provided technical controls for the staining using isotype immunoglobulin G (IgG) control and Alexa Fluor 647 fluorescence antibody. Both the blood vessels and infiltrated alveolar space showed positive staining with anti-cANGPTL4 mouse monoclonal antibody as the primary antibody and anti-mouse Alexa Fluor 647 as the secondary antibody. In contrast, no staining of the blood vessel and infiltrated alveolar space at the corresponding regions in neighboring slide was observed when control IgG and anti-mouse Alexa Fluor 647 antibody were used (Figure S1C). The result confirmed the ANGPTL4 signal was specific. To confirm that the elevated cANGPTL4 expression was not virus-strain specific, we examined its expression in the lungs of mice challenged with a sublethal dose of a mouse-adapted H3N2 strain. H3N2 infection of BALB/c mice exhibited increased levels of cANGPTL4 as well as full-length ANGPTL4. The cANGPTL4 protein peaked at 10 dpi, while the expression of native ANGPTL4 protein remained elevated at 15 dpi during the recovery of lung tissues (Figures S1D and S1E). This confirmed that ANGPTL4 upregulation was not restricted to a specific strain of influenza virus or mouse. Notably, different strains of virus and mouse showed distinct ANGPTL4 mRNA and protein expression profiles.

ANGPTL4 Expression Is Regulated by a STAT3-Mediated Mechanism

As the ANGPTL4 mRNA expression profile mirrored the pattern of viral replication, we asked if viral infection could influence the expression of ANGPTL4. Dual immunofluorescence staining for NS1 viral protein and cANGPTL4 revealed that infected alveolar type II epithelial cells displayed cANGPTL4 staining, whereas the uninfected alveolar type II epithelial cells within the same lung did not exhibit cANGPTL4 staining (Figure 2A). Although Clara cells were also stained positive for ANGPTL4 (Figure S2A), we did not detect any significant ANGPTL4 mRNA level in Clara cells isolated by laser capture microdissection (Figure S2B), suggesting that the ANGPTL4 staining most likely derived from secretion from heterotypic cell types. Next,

Figure 1. Elevated cANGPTL4 mRNA and Protein Expression during Influenza Virus-Induced Pneumonia

C57BL/6J mice were infected with the PR8 virus, and lungs were harvested at the indicated days postinfection (dpi).

(A) Representative immunofluorescence staining for viral protein NS1 (green) and cell nuclei (blue) of influenza-infected lungs. Scale bar, 50 μ m.

(B) Representative dual immunofluorescence staining of alveolar epithelial type II cells (SPC, red) and viral protein NS1 (green) showing viral infection in the alveolar space is limited to alveolar epithelial type II cells. Scale bar, 60 μ m.

(C) Relative mRNA expression of viral NP mRNA (means $\pm$ SEM, $n = 5$) and protein levels (means $\pm$ SEM, $n = 4$) of cytokine interleukin-6 (IL-6, blue) and interferon- γ (IFN- γ , red) in BALF as determined by Bioplex as described in [Experimental Procedures](#).

(D and E) Relative expression of ANGPTL4 mRNA (D) and protein (E) in lungs harvested at the indicated dpi. mRNA expression was normalized to that of the housekeeping gene ribosomal protein L27, which did not change under any of the studied experimental conditions (means $\pm$ SEM, $n = 5$). β -Tubulin served as a loading and transfer control for immunoblotting.

(F) Representative H&E images and immunofluorescence-stained images of ANGPTL4 (green) counterstained with DAPI (blue) of infected lung sections. Scale bar, 1,000 μ m.

All the staining pictures shown in this figure are representative images from 15 mice for each time point. See also [Figure S1](#).

we examined ANGPTL4 expression during influenza infection of mice with a lethal dose of PR8 virus (1,000 plaque-forming units [pfu]). We detected an earlier upregulation of ANGPTL4 mRNA and protein at 3 dpi, while the expression of native ANGPTL4 and cANGPTL4 remained elevated until the mice were euthanized at 7 dpi (Figure 2B).

We next sought to elucidate the underlying mechanism of this upregulation. HIF1- α has been shown to increase the expression of ANGPTL4 in endothelial cells (Zhu et al., 2012; Grootaert et al., 2012). To investigate if HIF1- α could be responsible for the expression of ANGPTL4 mRNA, we examined the HIF1- α protein expression profile in our animal model. The peak HIF1- α protein expression was detected at 13 dpi, which did not coincide with the peak mRNA expression of ANGPTL4 (Figure S2C), suggesting HIF1- α was unlikely to be a major regulator of ANGPTL4 at the early stages of infection. However, we cannot exclude that HIF1- α may maintain or sustain ANGPTL4 expression at later stages of infection. Interrogation of GEO data sets revealed that STAT3 deficiency in pulmonary alveolar type II epithelial cells was related to lower ANGPTL4 levels (Xu et al., 2007). Interestingly, viral infection triggered host responses through IFN- γ and IL-6 pathways, which activate STAT, and displayed overlapping profiles to the ANGPTL4 mRNA expression pattern (Figure 1C). In silico analysis of the promoter of the mouse *ANGPTL4* gene also revealed a putative STAT-binding site (Figure 2C). Thus, we performed chromatin immunoprecipitation (ChIP) using phospho-STAT3 (pSTAT3) antibody on the *ANGPTL4* promoter in uninfected and infected lung tissues. The sequences spanning the STAT-binding site were enriched in the immunoprecipitates obtained from the virus-infected lung tissues compared to uninfected tissues (Figure 2D). pSTAT3 ChIP experiments followed by re-ChIP with p300, a STAT3 coactivator, further confirmed the existence of direct regulation of ANGPTL4 via a STAT3-mediated mechanism (Figure 2D). To further strengthen our in vivo findings, we suppressed endogenous STAT3 expression by small interfering RNA (siRNA) and examined the expression of ANGPTL4 in a human small airway epithelial cell culture exposed to IL-6. We observed a significant increase in ANGPTL4 mRNA in response to an IL-6 challenge, which was abrogated when endogenous STAT3 was knocked down by ON-TARGETplus siRNA (Figure S2D). We confirmed that the STAT3 protein was also reduced by western blot analysis (Figure S2D).

The fANGPTL4 undergoes proteolytic processing to release cANGPTL4. Furin proprotein convertase is known to cleave fANGPTL4 (Lei et al., 2011). To determine whether furin-like activity is present and consistent with the peak of cANGPTL4 protein expression in influenza-infected lungs, we measured furin activity in the bronchoalveolar lavage fluid (BALF) of mice that were sublethally infected with PR8 virus. Furin activity

in the BALF started to increase at 5 dpi, coinciding with the peak in virus titer (Figure 2E). Importantly, the furin activity profile overlapped significantly with that of cANGPTL4 protein (Figure 2E). To strengthen the role of furin in ANGPTL4 cleavage, we performed similar experiments in the presence or absence of a furin inhibitor. Furin inhibitor was added to BALF taken from 11 dpi, a time point when the furin activity was elevated. Recombinant fANGPTL4 protein with FLAG tag at the C terminus was used as exogenous substrate. We detected a reduced cANGPTL4:fANGPTL4 ratio in BALF containing furin inhibitor when compared to BALF alone (Figure S2E), confirming that furin was a major contributor of post-translational cleavage of fANGPTL4 in our animal model. Taken together, our data demonstrated that ANGPTL4 mRNA was upregulated via a STAT3-dependent pathway during influenza pneumonia. The concomitant increase in furin activity subsequently cleaved fANGPTL4 to generate cANGPTL4, which peaked at 13 dpi, corresponding to extensive lung injury marked by large regions of pulmonary hemorrhage and infiltration of host immune cells.

Immunoneutralization of cANGPTL4 Significantly Reduces Tissue Leakiness to Accelerate Lung Recovery

To understand the role of cANGPTL4 in influenza pathogenesis, we investigated the in vivo effect of a neutralizing cANGPTL4 monoclonal antibody (mAb; clone 3F4F5) on the host response to influenza viral pneumonia. We employed two treatment strategies based on the stage of disease progression (Figure 3A). Virus-induced inflammation caused severe lung damage, which was observed until 13 dpi. Thereafter, tissue regeneration began to restore lung structural integrity and function. Thus, we defined the period before 13 dpi as the “damage window,” and days after 13 dpi as the “recovery window.” Daily intravenous injections of anti-ANGPTL4 mAb (10 mg/kg body weight) were administered for 5 days starting either at 6 dpi during the damage window or day 13 during the recovery period (Figure 3A). Negative control groups included mock-infected mice that received either isotype-matched control mouse IgG or anti-cANGPTL4 mAb alone. Mice infected with influenza virus and treated with control mouse IgG served as another control group. Lung tissues were harvested 24 hr after the last injection.

The anti-cANGPTL4 mAb treatment during the “damage window” did not significantly alleviate the early inflammation-induced lung damage (Figure S3A). In contrast, anti-cANGPTL4 mAb treatment during the recovery stage resulted in reduced lung damage and a significant improvement in tissue recovery compared to control groups (Figure 3B). The alveolar spaces showed a remarkable increase of noninfiltrated areas with reduced pulmonary bleeding and accelerated regeneration of alveolar type I epithelial cells (Figure 3C). To pursue this further,

(C) Schematic diagram showing the relative position of a putative STAT-binding site in the mouse *ANGPTL4* gene promoter.

(D) ChIP assay was conducted using preimmune IgG or antibodies against pSTAT3 (top panel) and re-ChIP with anti-p300 (bottom panel) in infected (I) and mock-infected (N) lungs. The specific region spanning STAT3 binding site of *ANGPTL4* gene was amplified using appropriate primers. A control region served as a negative control.

(E) Bar graph shows furin activity in the BALF extracts at the indicated time points after PR8 infection (means $\pm$ SEM, $n = 10$). Expression profile of viral NP mRNA (dark blue), ANGPTL4 mRNA (red), and protein (light blue) were plotted.

See also Figure S2.

Figure 3. Immunoneutralization of cANGPTL4 Improves Lung Tissue Recovery after Influenza Infection

(A) Schematic diagram showing administration protocols during “damage” (yellow arrows) and “recovery” (blue arrows) windows. Graph shows a summary of various events of mouse influenza infection model plotted based on earlier observations.

(legend continued on next page)

we examined the *in vivo* integrity of the pulmonary vasculature by *in vivo* imaging of the mice receiving anti-cANGPTL4 mAb during the recovery period (Figure 3E). Mice were injected intravenously with IRDye 800CW PEG contrast agent (50 nmol/kg body weight) 24 hr after the last mAb injection. The presence of infrared (IR) signal indicates the accumulation of IRDye 800CW PEG in tissue due to increased leakiness of the local tissue. As expected, infected mice treated with control IgG showed a very high IR signal in the lung compared to mock-infected mice (Figure 3E). Infected mice treated with anti-cANGPTL4 mAb showed a significantly subdued IR signal in the lungs compared with control IgG treatment, indicating that immunoneutralization of cANGPTL4 markedly abrogated tissue leakiness. *Ex vivo* imaging of resected mouse lungs confirmed the reduced accumulation of IRDye 800CW PEG agent following anti-cANGPTL4 mAb therapy. To more precisely define the cell types responsible for the increased tissue leakiness, we examined the cell-cell junction of human small airway epithelial cells and microvascular endothelial cells after treatment with recombinant cANGPTL4 by immunofluorescence staining with tight junction protein zonula occludens-1 (ZO-1). The cANGPTL4 protein perturbed the cell-cell boundary of primary human endothelial and epithelial cells (Figure 3D), indicating that cANGPTL4 exacerbated pulmonary bleeding and edema and could accelerate the infiltration of immune cells inside the lung tissue to aggravate tissue damage.

To further strengthen our above observation, we examined the effect of influenza infection on wild-type (ANGPTL4^{+/+}) and ANGPTL4 mutant mice (knockout ANGPTL4^{-/-} and heterozygous ANGPTL4^{+/-}). We observed lung damage in ANGPTL4^{-/-} at 11 dpi, albeit at reduced severity when compared to influenza-infected ANGPTL4^{+/+} (Figure S3B). Consistent with above observation with neutralizing anti-cANGPTL4, infected ANGPTL4^{-/-} mice showed rapid lung tissue recovery at 18 dpi compared with ANGPTL4^{+/+} (Figure 3F) and accelerated regeneration of alveolar type I epithelial cells (Figure 3G). We also studied the effect of ANGPTL4 gene dosage on lung tissue damage and recovery using ANGPTL4^{+/-} mice. Similar to cANGPTL4 antibody-treated ANGPTL4^{+/+} mice, significant improvement in tissue recovery was observed in PR8-infected ANGPTL4^{+/-} mice when compared with ANGPTL4^{+/+} mice at 18 dpi, but no significant difference in tissue damage was observed at 11 dpi (Figures S3C and S3D). We also confirmed the observations by immunoblotting of podoplanin (PDPN) as a marker of lung damage as well as scoring of lung tissue damage by trained pathologist (Figures S3E and S3F).

To explore the biological impact of anti-cANGPTL4 mAb therapy on infected lungs, we performed microarray gene expression analyses of lungs of mice treated with either anti-cANGPTL4 antibody or control IgG from 13 to 17 dpi. Data analyses revealed that mAb therapy resulted in differences in numerous major physiological activities, including angiogenesis, lung tissue development, inflammatory responses, and extracellular matrix and endopeptidase activities (Figures 4A and S4). Genes involved in the development of lung alveoli, respiratory tubes, and blood vessels were identified in the anti-cANGPTL4 mAb-treated infected mice, indicating earlier tissue regeneration. The mAb treatment also dampened the inflammation-related tissue damage, consistent with the pathological phenotype. Taken together, our findings indicate that the functional neutralization of cANGPTL4 during the tissue recovery stage promoted lung tissue recovery and was associated with improved tissue integrity.

cANGPTL4 Expression Is Enhanced in Human Lung Biopsy Specimens of Patients with Infection-Induced Pneumonia

To underscore the clinical relevance of ANGPTL4 in lung infection and lung damage, we performed a retrospective immunofluorescence examination of ANGPTL4 expression in 40 archived human lung biopsy specimens from patients either without pneumonia or with pneumonia induced by various infections as approved by the institutional review board of the National University Hospital (reference number 2012/00661). The staining and microscopic imaging techniques were performed simultaneously under the same conditions, allowing the signal intensity to serve as a semiquantitative measure of the cANGPTL4 expression level. In the ten pneumonia samples that were symptomatic or microbe positive, we found brightly stained structures (Figure 4B; Table S2). These structures included the thickened layers around tubular structures as well as intensively stained structures in the damaged alveolar regions that appeared to be collapsed membrane-like structures (Figure 4B; Table S2). These structures were abundant in regions with dense infiltration or collapsed alveolar spaces. The nonpneumonia samples showed either very weak staining or staining restricted to a thin layer along the tubular structures, consistent with our observations in healthy mouse lung tissues (Figures 4B and 1F; Table S2). The obstructive pneumonia sample that was not caused by infection did not show positive staining of cANGPTL4, which is consistent with our proposed mechanism whereby

(B) Representative H&E images of lung sections from mice either mock-infected or PR8-infected and treated with control isotype IgG or anti-cANGPTL4 monoclonal antibody. Images show alveolar space, bronchiole, and pulmonary edema. Scale bar, 100 μ m.

(C) Representative immunofluorescence staining of PDPN (red) as marker of alveolar type I epithelial cells and nuclei (blue) show tissue regeneration in the lungs. Scale bar, 50 μ m. Representative images from five mice for each group.

(D) Representative immunofluorescence staining of ZO-1 (green) of confluent human microvascular endothelial cell and human small airway epithelial cell cultures treated with either vehicle PBS or recombinant cANGPTL4 protein (6 μ g/mL). Scale bar, 40 μ m, $n = 3$. Dotted scale bar, 10 μ m.

(E) Infrared imaging of lungs *in vivo* (bottom panel) and *ex vivo* (top panel) using PEG-800 contrast agent to reveal pulmonary leakiness. Mice were treated with antibodies from 13 to 17 dpi, and the imaging was performed at 18 dpi. Scale bar, 1 cm. Representative images of three mice for each group were shown. Intensity of fluorescence signal from the lungs was measured *ex vivo* by Li-Cor MousePOD (means $\pm$ SEM, $n = 3$).

(F) H&E images of lungs from ANGPTL4^{-/-} and ANGPTL4^{+/+} mice infected with sublethal PR8 at the indicated dpi. Scale bar, 100 μ m.

(G) Immunofluorescence staining of PDPN (red) and nuclei (blue) showed tissue regeneration in the lungs. Scale bar, 50 μ m. Representative images from five mice for each group are shown.

See also Figure S3.

Figure 4. ANGPTL4 Deficiency Reduces Infection-Associated Pulmonary Damage

(A) Heatmaps show gene expression profiles of influenza-infected lungs left untreated or treated with anti-cANGPTL4 antibody (I versus A). Genes are clustered according to their biological functions. The color spectrum from green to red depicts the fold change in LOG scale. See also Figure S4.

(B) Representative immunofluorescence images of cANGPTL4 expression in human lung biopsy specimens of patients with or without pneumonia. Scale bar, 100 μ m. See also Table S2 for a complete list of patient information and immunofluorescence staining observations.

coincided with the inflammation phase of influenza infection, localized to lung regions with elevated immune cell infiltration and tissue damage. Similarly, our analysis of human clinical infection-associated pneumonia samples showed higher levels of ANGPTL4 compared to uncomplicated human lung sections. Notably, influenza infection of ANGPTL4-knockout mice and immunoneutralization of ANGPTL4 in wild-type mice showed significantly improved pulmonary tissue integrity and accelerated recovery from inflammation-induced tissue damage.

Recent high-throughput RNA sequencing of formalin-fixed, paraffin-embedded autopsy lung tissue samples from the 1918 and 2009 influenza pandemics revealed that ANGPTL4 mRNA was one of the most significantly up-regulated genes in both samples (Xiao et al., 2013). Our interrogation of microarray data from influenza-infected mouse lungs also consistently detected elevated ANGPTL4 expression (Pommerenke et al., 2012). These observations underscore the importance of ANGPTL4 in

an infection-induced STAT pathway contributes to ANGPTL4 upregulation. These findings suggest that cANGPTL4 may be a potential biomarker for respiratory infection and pneumonia. Clearly, further validation with a larger patient cohort that includes human virally induced ARDS cases and other bodily fluids such as blood and sputum will be necessary.

DISCUSSION

One of the hallmarks of influenza pneumonia is the aggravated inflammatory host response accompanied by pulmonary edema and associated acute lung injury (Mizgerd, 2008). In this study, we showed that influenza infection elevated the expression of the host protein ANGPTL4 via direct transcriptional regulation by STAT3. The spatiotemporal expression of ANGPTL4

response to pneumonia. However, its role in infected lungs remained unclear. From our influenza mouse model, we observed that the ANGPTL4 mRNA expression profile mirrored the pattern of viral replication. Probing further, we showed that IL-6-activated STAT3 directly regulated the expression of ANGPTL4. This is consistent with microarray analysis showing that pulmonary alveolar type II epithelial cells deficient in STAT3 have lower ANGPTL4 mRNA levels compared to their wild-type counterparts (Xu et al., 2007).

Furin or furin-like proprotein convertases (PCs) play multiple roles in host response to influenza infection. Toll-like receptor 7 (TLR7) triggers antiviral immune responses by recognizing viral single-stranded RNA in endosomes. hTLR7 is proteolytically processed by furin-like PCs, and the C-terminal fragment of hTLR7 selectively accumulates in endocytic compartments.

TLR7 processing was required for its functional response to TLR7 agonists such as R837 or influenza virus (Hipp et al., 2013). Interestingly, we observed that the peak expression of cANGPTL4 protein at 13 dpi was likely attributed to the increase in activity of furin PCs that preceded and overlapped with cANGPTL4 protein expression. Recent work showed that two novel peptidomimetic furin inhibitors inhibit hemagglutinin cleavage and viral propagation of a highly pathogenic avian H7N1 influenza virus strain in vitro (Becker et al., 2012). The in vivo effect of such furin inhibitors, which conceivably prevent the proteolytic cleavage of fANGPTL4 to release cANGPTL4, on lung tissue recovery remains to be explored.

Previous work has shown that cANGPTL4 can affect the paracellular permeability of blood vessels in cancer (Huang et al., 2011; Guo et al., 2014). In vivo and ex vivo imaging of infected mice treated with neutralizing anti-cANGPTL4 mAb revealed diminished pulmonary tissue leakiness compared to isotype control IgG treatment. The direct consequence was reduced pulmonary edema and immune cell-infiltrated lung regions. The overall lung tissue integrity was improved and the alveolar space appeared to be well recovered for normal function as supported by microarray analysis. Administration of anti-cANGPTL4 mAb during the damage window, when cANGPTL4 protein levels were low, showed no observable difference compared to control. Thus, targeting cANGPTL4 to modulate tissue leakiness along with its collateral benefits is a promising approach toward the development of therapy for influenza treatment, specifically lung recovery. Our finding that host protein ANGPTL4 participates in pulmonary leakiness and lung injury responses during influenza pneumonia is an important step toward a better understanding of influenza pathogenesis and how it can be manipulated to reduce the burden of pneumonia.

EXPERIMENTAL PROCEDURES

Mice, Viruses, and Infections

Female 8- to 12-week-old C57BL/6J mice and BALB/c mice were purchased from the Biological Resource Centre, Biopolis, Singapore. Wild-type mice and mice heterozygous for ANGPTL4 (C57/B6 background) were obtained from the Mutant Mouse Regional Resource Center (MMRRC), an NIH-funded strain repository, and were donated to the MMRRC by Genentech. ANGPTL4 KO mice were identified using RT-PCR quantification of ANGPTL4 expression compared to wild-type mice. Influenza H1N1 virus A/PR/8/34 strain (PR8) was purchased from the American Type Culture Collection. PR8 was propagated in embryonated eggs at 37°C for 72 hr, and the allantoic fluid was harvested as a viral stock. Virus titers were determined by the plaque assay via infection of Madin-Darby canine kidney (MDCK) cells. Mice were housed in BSL2 facilities and infected with a sublethal dose of PR8 (30 pfu in 75 μ l of PBS [pH 7.4] per mouse) or with a lethal dose (1,000 pfu) by intratracheal inhalation under anesthesia. Mice were anesthetized using a ketamine/xylazine cocktail. When fully anesthetized, the mouse was held up and 30 pfu of PR8 virus in 75 μ l of PBS was introduced into the back of its mouth to be breathed into the trachea, with its tongue held tightly to prevent swallowing of the liquid pipetted in. Lungs were harvested from anesthetized mice at indicated time points and stored at -80°C until further use. Female 8- to 12-week-old BALB/c mice were infected with a sublethal dose of mouse-adapted influenza A/Aichi/2/68 H3N2 virus (1,500 pfu). Female 8- to 12-week-old ANGPTL4^{-/-} mice were infected also using 30 pfu of PR8 virus for comparison with ANGPTL4^{+/+} mice. Lung tissues were harvested for further analysis at 11 and 18 dpi. All animal protocols were approved by the respective institutional animal care and use committees

at National University of Singapore (050/11) and Nanyang Technological University (ARF SBS/NIE-A0200AZ).

Antibody Treatment of Mice

Anti-cANGPTL4 mAb (clone 3F4F5) was produced using hybridoma as described previously (Goh et al., 2010a, 2010b; Zhu et al., 2011). Mice were intraperitoneally injected daily with the antibody in 200 μ l saline at a dose of 10 mg/kg body weight on 6–10 dpi (harvested on day 11) or on 13–17 dpi (harvested on day 18). More details on the experimental procedures can be found in Supplemental Experimental Procedures.

In Vivo Imaging of Mice

Mice were mock infected with heat-inactivated influenza virus, infected with influenza virus and injected with control IgG, or infected with influenza virus and treated with anti-cANGPTL4 antibody on 13–17 dpi as described above. At the day of lung harvesting, mice were injected through the tail vein with IRDye 800CW PEG contrast agent (Li-Cor 926-50401) and subjected to in vivo imaging under anesthesia, using the Li-Cor MousePOD in vivo imaging facility. The IR fluorescent-tagged PEG800 was used to detect tissue leakiness when the tissue exhibited abnormally high paracellular permeability. Following imaging, the lungs of the mice were harvested and reimaged, and quantification of fluorescence signal was done according to the manufacturer's instructions.

ACCESSION NUMBERS

Microarray data have been submitted to the GEO database under accession number GSE58647.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.01.011>.

AUTHOR CONTRIBUTIONS

L.L., V.T.K.C., and N.S.T. conceived and designed the experiments. L.L., H.C.C., E.H.P.T., C.C.C., S.Y.N., K.W.K., and Z.T. performed the experiments. L.L., J.E.S., H.W.C., V.T.K.C., and N.S.T. analyzed the data. L.L. and N.S.T. wrote the manuscript. V.T.K.C., M.L.B., and N.S.T. supervised the work. J.E.S., M.L.B., V.T.K.C., and N.S.T. helped edit the paper.

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