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THE ROLE OF ANGIOPOIETIN-LIKE 4 IN INFLAMMATION

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The Role of Angiopoietin-like 4 in Inflammation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Academic Dissertation

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"It is not the most intellectual of the species that survives; it is not the strongest that survives; but the species that survives is the one that is able best to adapt and adjust to the changing environment in which it finds itself."

Leon C. Megginson.

ABSTRACT

Inflammation is a homeostatically fundamental feature of the host defence surveillance system made up of an army of sentinel cells that guard against foreign intrusion. At the onslaught of an attack, primary immune responders (macrophages and neutrophils) secrete a plethora of cytokines and chemokines that attract secondary responders (T and B cells) to the site of inflammation, to eliminate the intrusion and encourage tissue healing. The acute inflammatory stimulation is rapidly attenuated following tissue repair to prevent hypercytokinemia. However, the dysregulation of inflammatory stimulation can also lead to a chronic, erroneous attack on the host immune system, leading to autoimmune diseases and in some cases, cancer initiation. The chronic inflammation theory established by Rudolf Virchow in 1845 is perhaps the first that accurately described the inflammation-induced cancer phenomenon. Since the advancement of technologies, independent researchers have brought forth increasing novel evidence to support the role of inflammation in tumorigenesis. In the compilation of this thesis, we will explore the multi-faceted role of Angiopoietin-like 4 (ANGPTL4) in the regulation of the chemokine landscape during both acute and chronic inflammation.

In Part I, we shall investigate the role of ANGPTL4 in attenuating acute inflammation through the intermediate tristetraprolin (TTP). We show that ANGPTL4 deficiency resulted in an exacerbated inflammatory response in mice subjected to pro-inflammatory stimulation. Confirming the current paradigm, we also reveal that significant microbial divergence was only visible during inflammation and not at steady states. Using immortalized colonic epithelial cells, we connected ANGPTL4 and TTP in the inflammatory signaling axis, and show that ANGPTL4 is able to regulate the acute inflammatory landscape through both TTP-dependent and independent signaling pathways.

In Parts II and III, we demonstrate that ANGPTL4 regulates oxidative stress levels in the tumor and tumor microenvironment during chronic inflammation, and induces the epithelial-mesenchymal transition (EMT) process. Capitalizing on numerous cultures like A5RT3, MKN78 and HSC5, we first establish that TAK1 protected cells against TGFβ-induced EMT. TAK1 deficiency also promoted Rac1-Nox1 activation, increasing overall ROS production and augmented mesenchymal markers like Snai1 and E-cadherin. H_2O_2 augmented key signaling intermediates which are involved in cellular homeostasis and increased the invasiveness of epithelial cells. In addition, H_2O_2 also desensitized cells towards TGFβ stimulation by decreasing Smad3 and TAK1 expression. To underscore the medical relevance of ANGPTL4 in tumorigenesis, we reveal that ANGPTL4 drives EMT by elevating cellular energy flux through 14-3-3γ. In addition, 14-3-3γ promotes the transcription of E-cadherin by stabilizing the binding of Snai1 to its promoter during EMT.

These findings illustrate the importance of ANGPTL4 in regulating the inflammatory landscape during both acute and chronic inflammation, and in tumorigenesis, expanding its role outside of metabolism, insulin resistance, angiogenesis and wound healing.

LIST OF PUBLICATIONS

This thesis is based on two publications and one manuscript:

I. **Phua T**, Sng MK, Tan EHP, Chee DSL, Li Y, Wee JWK, Teo Z, Chan JSK, Lim MMK, Tan CK, Zhu P, Arulampalam V, Tan NS. Angiopoietin-like 4 mediates Colonic Inflammation by Regulating Chemokine Transcript Stability via Tristetraprolin. Sci Rep. 2017; 7:44351.

II. Lam CR, Tan C, Teo Z, Tay CY, **Phua T**, Wu YL, Cai PQ, Tan LP, Chen X, Zhu P, Tan NS. Loss of TAK1 increases cell traction force in a ROS-dependent manner to drive epithelial-mesenchymal transition of cancer cells. Cell Death Dis. 2013; 4:e848.

III. Teo Z, Sng MK, Chan JSK, Li Y, Li L, Lim MMK, **Phua T**, Zhu P, Tan NS. Metabolic Reprogramming by Angiopoietin-like 4 is Required for Epithelial-Mesenchymal Transition. M*anuscript under revision.*

Other related publications and/or manuscripts are as follows:

IV. Chan JSK , Tan MJ, Sng MK, Teo Z, **Phua T**, Choo CC, Li L, Zhu P, Tan NS. Cancer-associated fibroblasts enact field cancerization by promoting extratumoral oxidative stress. Cell Death Dis. 2017;8:e2562.

V. Sng MK, Chan JSK, Teo Z, **Phua T**, Tan EHP, Tan CK, Chen JP, Tong BMK, Tnay YL, Chiba S, Wang XM, Wahli W, Tan NS. Fibroblast PPARβ/δ deficiency causes dermal fibrosis by attenuated LRG1 expression. *Submitted Manuscript.*

VI. Al-Asmakh M, Anuar F, **Phua T**, Yip SFM, Kundu P, Zadjali F, Rafter J, Hibberd ML, Fundele R, Tan NS, Parini P, Hedin L, Pettersson S. Commensal microbiota supports placenta development and maternal metabolism. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

18S	18S Ribosomal RNA
AKT (PKB)	Protein kinase B
AMPs	Antimicrobial peptides
Ang	Angiopoietin
ANGPTL4	Angiopoietin-like 4
AREs	Adenosine-Uridine Rich Elements
cANGPTL4	c terminus of ANGPTL4
CECs	Colon Epithelial Cells
CCL11	Chemokine (C-C motif) ligand 11
CCL2	Chemokine (C-C motif) ligand 2
CD68	Cluster of differentiation 68
cFos	FBJ murine osteosarcoma viral oncogene homolog
ChIP	Chromatin immunoprecipitation
CREB	cAMP response element-binding protein
CRP	C-Reactive Protein
CXCL10	Chemokine (C-X-C motif) ligand 10
CXCL3	Chemokine (C-X-C motif) ligand 3
CXCL5	Chemokine (C-X-C motif) ligand 5
DMSO	Dimethyl sulfoxide
DSS	Dextran sulfate sodium salt
ELK1	ETS domain-containing protein
ERK	Extracellular signal-regulated kinases
fANGPTL4	full length fragment of ANGPTL4
FIAF	Fasting-inducing adipose factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GF	Germ-free
GI tract	Gastrointestinal tract
h-cANGPTL4 Ab	Human cANGPTL4 antibody
HDL	High density lipoprotein
HMP	Human Microbiome Project
HRARP	Hepatic Fibrinogen/ Angiopoietin-related Protein
IBD	Inflammatory bowel disease

iCECs	Immortalized Human Colon Epithelial Cells
IECs	Intestinal Epithelial Cells
IFN-γ	Interferon Gamma
IGFR	Insulin-like Growth Factor
IgG	Immunoglobulin G
IL-10	Interleukin 10
IL-17	Interleukin 17
IL-1β	Interleukin 1, beta
IL-23p19	Interleukin 23, alpha subunit p19
IL-6	Interleukin 6
IRS	Insulin Receptor Substrate
LCFA	Long chain fatty acids
LDL	Low density lipoprotein
LPL	Lipoprotein Lipase
LPS	Lipopolysaccaharide
Ly6G	Lymphocyte antigen 6 complex, locus G
MAMP	Microbe-associated Molecular Patterns
Muc2	Mucin-2 protein
n	Sample size
NaBu	Sodium butyrate
nANGPTL4	n terminus of ANGPTL4
NEFA	Non-esterified Fatty Acid
NF-κB	NuclearFactor kappa-light-chain-enhancer of activated B cells
NGS	Normal goat serum
OA	Oleic acid
PBS	Phosphate buffered saline
pc-Fos	Phosphorylated FBJ murine osteosarcoma viral oncogene homolog
PCR	Polymerase chain reaction
pCREB	Phosphorylated cAMP response element-binding protein
pELK1	Phosphorylated ETS domain-containing proteinhomolog
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR response element

PRR	Pattern Recognition Receptors
Re-ChIP	Re-chromatin immunoprecipitation
Rel Exp	Relative expression
rh-ANGPTL4	Recombinant human c-ANGPTL4
S6K	S6 Kinase
SA	Stearic acid
SCFA	Short chain fatty acids
SEM	Standard error of mean
SIRT1	Sirtuin 1
STAT3	Signal Transducer and Activator of Transcription 3
TBP	TATA box binding protein
TBS	Tris buffered saline
TBST	Tris buffered saline with tween 20
TLR4	Toll-like receptor 4
TNF-α	Tumor Necrosis Factor Alpha
TNFR	Tumor necrosis factor receptor
TTP	Tristetraprolin
Veh	Vehicle control
VLDL	Very low density lipoprotein
WHO	World health organization
ZFP36	Zinc finger protein 36
β-tubulin	Beta tubulin

1 INTRODUCTION

1.1 THE GASTROINTESTINAL TRACT: FUNCTION AND DEFENCE

An estimated 10^{14} microorganisms make up the human flora, comprising of more than 10 000 bacterial species altogether [1, 2]. Hence, it is critical that we distinguish and recognize both specific microbes, metabolites and genes in the collection of microbiome that confer health benefits to its host [3, 4]. To date, the exact composition that makes up the microbiota of a healthy individual remains unknown. Scientists estimate that about 300 – 1000 microbial species make up the microflora in the entire gastrointestinal tract. In addition, the lower gastrointestinal tract including both the small and large intestine was found to contain the largest reservoir of intestinal microflora. Microbes residing along the human gastrointestinal tract exhibit habitat-specific traits. To date, studies suggest that more than 99% of the total microbiota in the gastrointestinal tract consists of anaerobic bacteria, while only those residing in the caecum are believed to be predominantly aerobic [5]. For instance, microbial species representing *Bacteroides, Clostridium, Fusobacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus*, and *Bifidobacterium* are amongst the most common microbial genera present in the lower gastrointestinal tract of a normal healthy individual [5-8].

Intestinal epithelial cells (IECs) lining the gastrointestinal tract play an important role as a physical barrier to prevent the invasion of bacteria to the host lamina propria. Hence, the presence of a dynamic and viable intestinal immune system as the first line of defence, allows for a balanced homeostasis between the host and the microbiota community, and in turn, the disregulation of the system, leads to diseases. Although the morphology of the small intestine and the colon differs, the host innate immune approach consists of 2 main strategies: to keep the integrity of the gastrointestinal wall through the continuous production of mucus; and the secretion of antimicrobial peptides (AMPs) by underlying immune and epithelial cells in the lamina propria and submucosa (**Figure 1.1**).

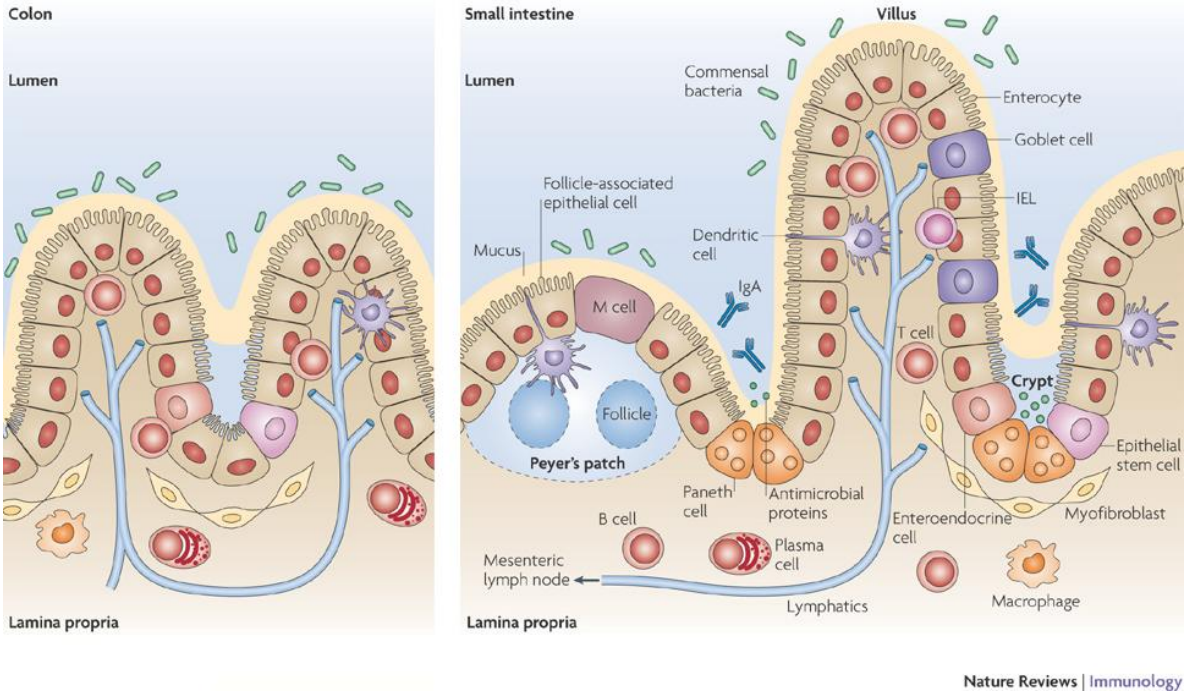

Figure 1.1 The immune landscape in the gastrointestinal tract. A monolayer of intestinal epithelial cells (IECs) acts as a barrier that partitions the host cells in the lamina propria and the resident microflora in the intestinal lumen. IECs in the colon (colonocytes) and the small intestine (enterocytes) play roles in absorption of water and nutrients respectively. Enteroendocrine and Paneth cells secrete enteric hormones and antimicrobial peptides respectively. Located in proximity with the Paneth cells, epithelial stem cells reside in the crypt regions divide to give daughter cells, which later differentiate into mature IECs as they move towards the luminal surface. Other immune cells like T cells, B cells, dendritic cells and resident macrophages also reside under the lamina propria located close to the IECs. Peyer's patches consisting of lymphatic tissues are also found throughout the ileum, and are primarily responsible for monitoring intestinal bacterial load. Modified and reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol. 10(2):131-144. Copyright 2010.

In the colon, goblet cells are the specialized IECs that keep the resident community of microbiota at bay. Goblet cells residing amongst colonocytes mainly produce mucin-2 protein, which are the building blocks for the mucus matrix [9]. The colon is coated with a double layer of mucus – an inner, stratified layer of mucus in proximity with the epithelial apical surface, as well as an outer, non-attached mucus layer that is in contact with the lumen. Mucin glycans found in the matrix of the outer mucus layer also act as a nutrient supply for resident microbiota. In turn, by-products of microbial fermentation like short chain fatty acids (SCFAs) are absorbed by colonocytes as additional energy source for the host. Because the colon is home to more than 10^{12} bacteria per gram of faeces, it becomes of utmost importance that the mucus barrier remains intact to limit events of direct contact between host colonocytes and the gut microbiota. In contrast, the small intestinal mucus layer has been found to be discontinuous because enterocytes are primarily responsible for nutrient absorption and must be in contact with the luminal space [9]. To avert possible assaults,

Paneth cells located close to the crypt region of the small intestine secrete AMPs like defensins and lectins to form a biochemical barrier in place of the lack of a continuous mucus barrier [10, 11] while other host plasma cells secrete immunoglobins (i.e. IgA) [12]. Although once considered to be commensal, the prokaryotic resident microbiota can trigger an inflammatory response and poses severe health risks to its host once the gastrointestinal barrier is breached. Microbes that are capable of degrading mucin-2 and its related glycans can weaken the mucus matrix and hence, reside and grow in proximity with the host epithelial cells.

1.1.1.1 The Benefits and Hindrance of Host-Microbiota Interactions in the GI tract

In humans, only about 85% of all dietary carbohydrates are absorbed in the proximal small intestine [13, 14]. Indigestible carbohydrates, short chain fatty acids (SCFAs), and polysaccharides representing about 25% of the total ingested energy enter the distal small intestine and colon [15, 16]. Without the fermentation action of colonic microbes, this form of energy would have remained inaccessible to the host. The fermentation process of colonic microbes causes the breakdown of large carbohydrates and dietary fibers to give SCFAs like acetate, propionate and butyrate [17], which can then bind to G-protein coupled receptors (GPRs) to regulate a variety of downstream processes [18, 19], or be directly utilized as a source of energy by the host (**Figure 1.2**).

Numerous studies have implicated a continuous interplay between the microbiota residing in the gastrointestinal tract and various host functions. A stable gut microbial landscape resembles a form of defence system because it reduces the risks of pathogenic invasion through competitive exclusion [1]. Commensal microbes have been reported to be involved in the normal development of the gut and also control systemic functions including development, immunity and metabolism in the host [6, 20]. In addition to secreting digestive enzymes, gut microbes are essential for the production of SCFAs through fermentation of dietary fibres [21]. SCFAs like acetate, propionate and butyrate also play an integral component of intestinal energy balance and defence [22] (**Figure 1.3**).

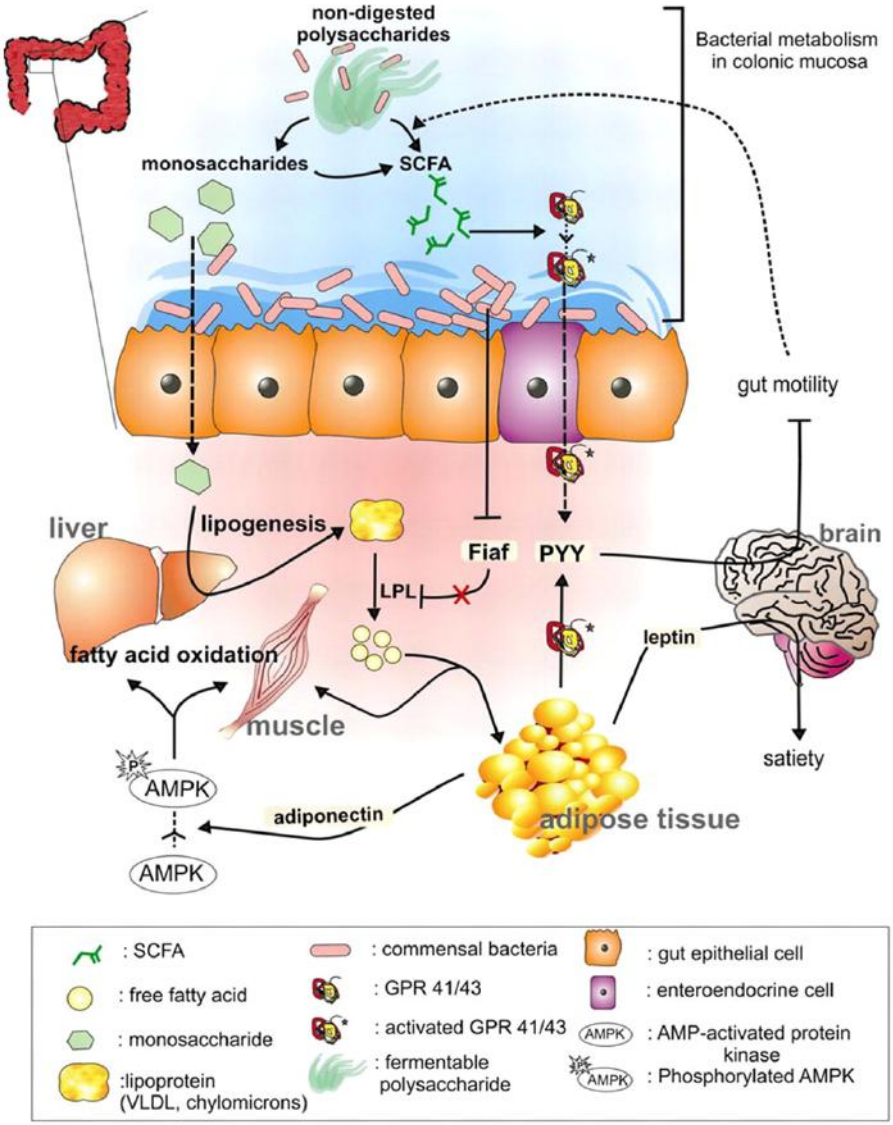

Figure 1.2 Regulation of intestinal microflora on host energy metabolism. If not for intestinal micrbiota, about 30% worth of the total dietary energy cannot be harvested because the host digestive system cannot break down complex polysaccharides and carbohydrates. The fermentation process by intestinal microbiota gives rise to SCFAs which can be utilized by the host. SCFAs positively regulate GPR 41/43 receptors to increase fermentation. The regulation of ANGPTL4 (FIAF) in intestinal epithelial cells also mediates LPL activity, which alters fat deposition and accumulation in surrounding adipocytes. Reprinted from Nutr Clin Pract., Rosa Krajmalnik-Brown et al, Effects of gut microbes on nutrient absorption and energy regulation, 2012 Apr; 27(2): 201–214, Copyright 2012, with permission from SAGE Publications.

Different members of SCFAs have also been reported to contribute to unique host homeostatic processes that might be both tissue and species specific. The binding affinities between SCFAs are also unique to specific GPRs. For example, it was previously demonstrated that GPR109A is highly selectively for only butyrate [23], while GPR43 binds acetate and propionate over butyrate and GPR41 binds propionate and butyrate over acetate [24, 25]. Perhaps of all multivariate roles of SCFAs, the anti-inflammatory action of butyrate has most widely been associated with its selectivity and specificity as a potent, non-

competitive histone deacetylase (HDAC) inhibitor [26]. Butyrate has been reported to mediate the crosstalk between the gut microbiota and host diseases like colitis [27], type II diabetes and obesity [28] through epigenetic regulation and also energy homeostasis through GPR41 [29]. On the other hand, acetate and propionate has been reported to regulate other processes like adipose-specific insulin signaling [30, 31] via GPR43. In a recent study conducted by Ang A *et al.*, acetate was demonstrated to decrease the expression of inflammatory cytokines by increasing p38 phosphorylation and repressing the phosphorylation of AKT and ERK2 in human monocytes [32]. Interestingly, only an increase in p38 and not AKT or ERK2 phosphorylation was observed when mouse monocytes were treated with acetate. Although more studies are needed to show if this acetate/GPR43/41 signaling cascade is unique to monocytes or if it also applies to other cell types or host processes, this monocyte acetate-dependent inflammatory response has been demonstrated to be species-specific.

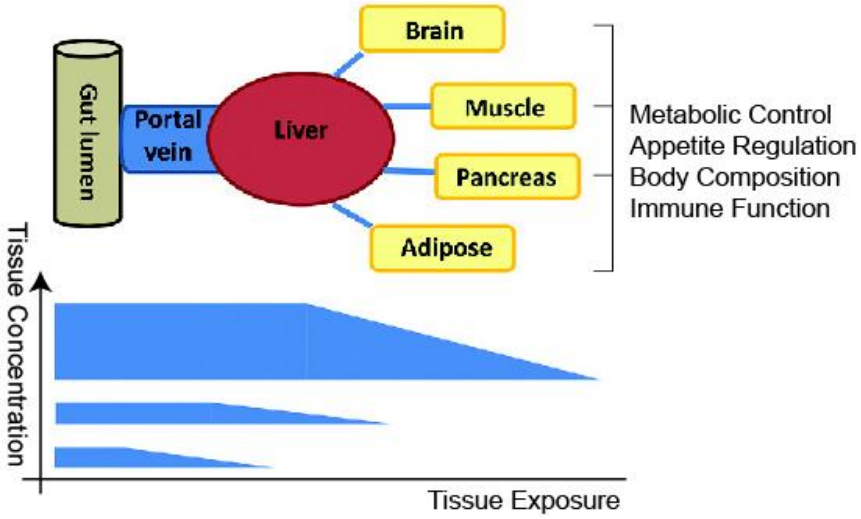

Figure 1.3 The impact and distribution of short chain fatty acids (SCFAs) on host homeostatic processes. The lumen of the gastrointestinal tract is one of the primary sites of SCFA production. As nutrients are absorbed by the epithelial cells and channeled away into the circulation, the concentration of SCFAs reduces. SCFAs are believed to play important roles in regulating host metabolism and immunity. Modified from Gut Microbes, Douglas J. Morrison and Tom Preston. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism, 2016; 7(3): 189-200. Copyright The Author(s); permitting unrestricted use, distribution and reproduction.

Microbes residing in the colonic mucosa can regulate the levels of epithelial ANGPTL4 [33, 34], while others suggest that ANGPTL4 is a downstream target of GPR 41/43 activation [22, 35]. The regulation of ANGPTL4 alters lipoprotein lipase (LPL) activity in the gastrointestinal tract, directly affecting lipogenesis and the amount of fat deposition and accumulation in circulation, as well as in other storage organs like adipocytes, liver and skeletal muscles. Adipocytes also secrete hormones like adiponectin and leptin in response to changes in SCFA levels. Leptin regulates satiety and appetite in the arcuate nucleus region of the hippocampus [36, 37] (opposed by ghrelin) while adiponectin activates AMP to AMPK to

stimulate cellular fatty acid oxidation [38, 39]. Activated by GPR41/43, the secretion of peptide YY (PYY) also reduces peristalsis and gut motility, allowing for increased nutrient absorption [40, 41]. Current studies underscored a significant role for commensal gut microbes in energy regulation, the unwanted enrichment of a subset of microbes can lead to excess energy harvest and storage [42, 43] that contribute to the development of various facets of the metabolic syndrome [44, 45]. Current metabolomics findings revealed that obesity is associated with an enrichment of microbes belonging to *Actinobacteria* and *Firmicutes*, while decreased fat storage (leanness) has been correlated to the enrichment of *Bacteroidetes* population [43]. Indeed, genetically obese (*ob/ob*) mice on normal chow diet demonstrated an increase in *Firmicutes:Bacteroidetes* population ratios [46]. However, the cause and effect remains to be determined; it is unclear if the state of obesity selects for *Firmicutes* population or if a stable population of *Firmicutes* in the gastrointestinal tract results in inevitable obesity [47].

Despite great mutualistic benefits between microbiota in the gastrointestinal tract and its hosts, it is important to note that these microbes can also become opportunistic pathogens (**Figure 1.4**). Some famous examples of bacterial-induced inflammation of the gastrointestinal tract (gastroenteritis) include inflammatory bowel disease (IBD). Ulcerative Colitis and Crohn's Disease are well characterized IBDs whereby the commensal gut microbiota in the gastrointestinal tract invade host epithelial cells, causing inflammation predominantly in the ileum and/or the colon which might potentially progress to give colorectal cancer [48]. On the other hand, cholera involves the colonization of *Vibrio cholera* and infection to the small intestine [49]. A seemingly mild infection by *Helicobacter pylori* can bring about ulcer formation in the stomach and duodenum. However, *H. pylori* also secrete a cocktail of enzymes and proteases which further injures epithelial cells, leading to the development of gastric cancer [50].

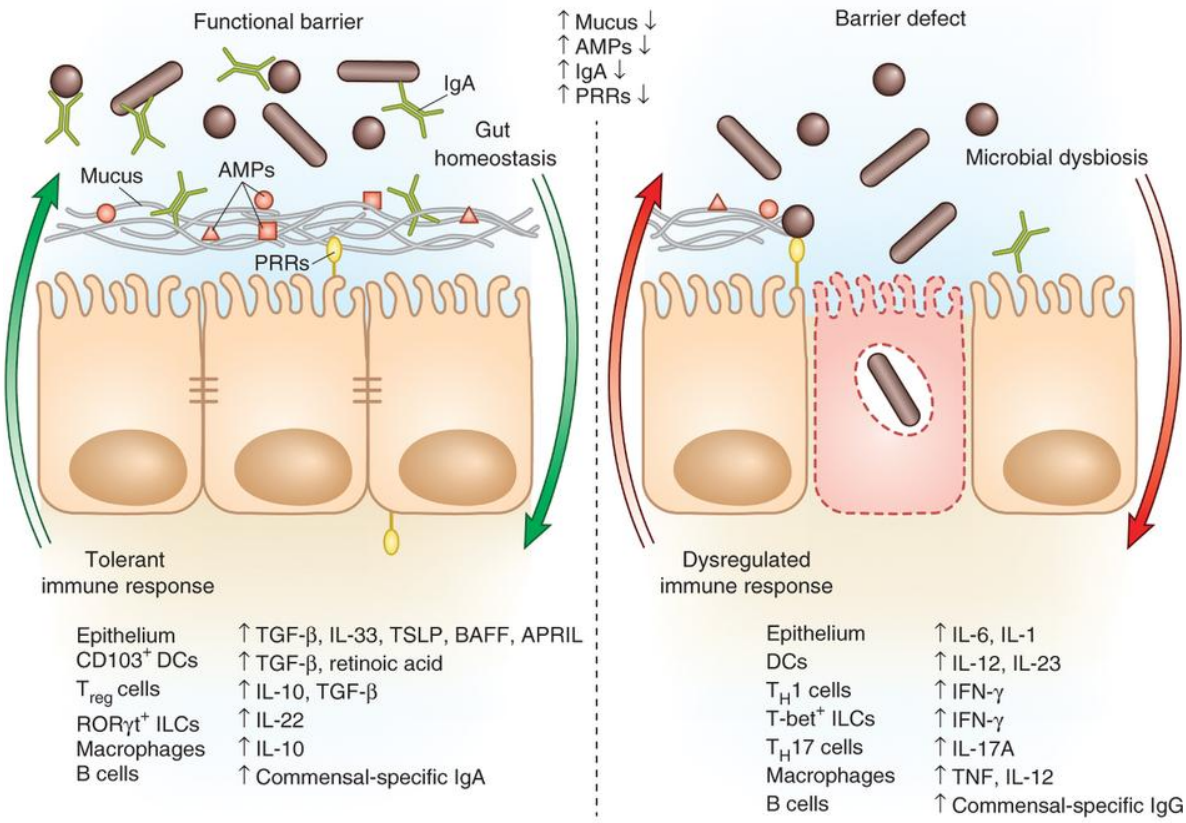

Figure 1.4 An intact and continuous epithelial barrier supports healthy gastrointestinal homeostasis. In a healthy gut environment, cells lining the gastrointestinal wall continuously secrete mucus and anti-microbial peptides (AMPs). Not only does the mucus layer form a physical barrier to keep gut microbiota at bay, it acts as a matrix layer that also allows for a unique habitat for the selection and colonization of commensal gut bacteria. Anti-microbial peptides are secreted by a variety of immune cells located further away in the submucosa and the lamina propria, creating a tolerant immune environment made up of a unique balance of both pro- and anti- inflammatory chemokines. However, a disregulation of mucus or AMP production disrupts healthy gut homeostasis. The lack of a physical compartmentalization of the host from the plethora of gut microbes allows the invasion of opportunistic pathogens. In response to the onslaught, immune cells secrete large amounts of pro-inflammatory chemokines to launch an immune attack against invading pathogens, causing a dysregulated immune envioronment. Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology. Brown EM et al. The role of the immune system in governing host-microbe interactions in the intestine. Nat Immunol. 14(7): 660-667. Copyright 2013.

IECs and a subset of immune cells express protein recognition receptors (PRRs) which aid in monitoring the bacterial load and maintaining healthy intestinal homeostasis [51]. The major PRRs are the Toll-like receptors (TLRs), as well as the nuclear oligomerization domain-like receptors (NOD-like receptors or NLRs). PRRs recognise microbe-associated molecular patterns (MAMPs), which consists of a wide variety of bacterial-related components like peptidoglycans, lipopolysaccharides and microbial RNA/DNA [52].

1.1.1.2 Effects of Development and Lifestyle Adaptations on Microbiota Variability

Research suggests changes in the landscape of microbiota in the gastrointestinal tract are also simultaneously observed over the course of development. Currently, 2 distinct milestones during human development – namely, birth and weaning, have observed significant change in the composition of the microbiota in the gastrointestinal tract. While the gastrointestinal tract of the foetus within the womb is thought to be almost sterile, newborns acquire microflora immediately following birth through contact with the mother and also from the surrounding environment. At the outset of birth, infants are believed to first be colonized by facultative anaerobes such as *Enterococcus, Streptococcus, Staphylococcus* and *Enterobacter* spp, and thus, creating a reducing environment conducive for the subsequent colonization of strict anaerobic microbes like *Bacteroides* and *Clostridium* spp [53, 54]. Although the intestinal microbiota ecosystem is believed to be stabilized up to three years after birth, reports suggest that the various methods of childbirth contributes significantly to the rate of gut microbiota colonization [55, 56]. During weaning, facultative anaerobes has been observed to give way to obligate anaerobes [2]. As such, the dominant mode of energy synthesis in the gut microbiota switches from aerobic respiration to anaerobic fermentation. Although commensal gut microbiota has been identified to be unique between males and females, little was known about its function on host development. Recent research shows that alterations in the composition of gut microbiota modify the levels of circulating testosterone in males and females to a different extent. This implies that an active crosstalk is present between gut microbiota and host development even in adults [57]. This results in the production of a different landscape of metabolites, which in turn is believed to affect host metabolism.

Consensus accredits the composition of microbiota in the gastrointestinal tract to be predominantly dependent on the lifestyle of the host. For instance, the use of antibiotics during treatment has been observed to alter the microbial ecosystem along the gastrointestinal tract [58]. Recent studies have shown that the reduction of commensal bacteria in the gastrointestinal tract through excessive use of antibiotics in early life increases the risks of developing inflammatory bowel disease and asthma [59]. Strikingly, it has come to attention that the diet could potentially also modify the components of the flora in the gastrointestinal tract. While diet containing high amounts of carbohydrates select for *Prevotella* species of microbes, diet with high amounts of proteins and fats will favor *Bacteroides* species. Since the gut microbiota is impermanent and fluid, a change in diet is likely to modify the ecosystem of microbes living in the gastrointestinal tract [60].

1.1.1.3 Gnotobiotic Animals

With the advancement of technology, many groups embarked on independent journeys to study the symbiotic relationship between commensal microbiota and its host. As such, independent groups tried to create the first gnotobiotic model. When the use of antibiotics did

not successfully eradicate the entire profile of microbes, scientists utilized new technologies that kept animals germ free.

Presumably pioneered by James Reyniers in 1920 [61], germ-free (GF) animals are often used as tools for proof-of-concept trials and were popular in fields like microbiology [43]. However, scientists soon realize that because GF animals had under-developed immune systems, GF mice were more susceptible to pathogenic infections when compared with specific-pathogen-free (SPF) mice that were raised in normal conditions [62]. As interests to understand the plasticity of commensal microbiota grew, attention was drawn to the lower gastrointestinal tract because the ileum and colon harbors the highest density of microbes [5-8]. To date, evidence suggests that neonatal microbial exposure is crucial in the development of the brain [63] and immune system [64], as well as the membrane barriers of the testis [65] and the blood brain barrier [66]. In addition, recent data indicates that the intestinal microflora might play roles in energy harvest [33, 67], and in modulating neuronal signaling cascades that govern behavior and motor control [68].

In recent years, the increasing interests of characterizing GF mice also highlight the importance of PRRs- microbiota associations. GF mice exhibited an under-developed immune system [69]. GF mice lacked immune-associated structures like lymphoid follicles and Peyer's patches in the small intestine [70]. They exhibited an immature and under-developed gastrointestinal tract lacking proper mucus formation [71, 72]. In addition, the removal of an important downstream target of TLR signalling, MyD88, saw a decreased response towards inflammatory signals like IL-18 and IL-1β [73], and reduced intestinal lymphocytes [74]. PRR-MAMP complexes also support and boost the production of glycoproteins in the mucus matrix, hence maintaining the integrity of the mucosal barrier [75].

1.2 INFLAMMATION

Inflammation is a fundamental, homeostatic role of the host defence system. It usually categorised by the cause and mechanism, and can be further divided into the severity (intensity) or eventual outcome. The early surveillance system is made up of an army of immune sentinel cells that alert the host of the intrusion of foreign particles in the body during acute inflammation events. Primary immune cell responders secrete a variety of cytokines and chemokines that attract other specialized immune cells like macrophages, neutrophils, T- and B-cells to combat and eliminate the intrusion – mechanical or microbial – and to encourage healing of surrounding tissues. As the wound repair, the inflammation is gradually resolved. Thus, a timed and balanced secretion of both anti- and pro-inflammatory cytokines allow for the rapid initiation and attenuation of inflammatory responses. Chronic inflammation occurs when the inflammation signals continue to persist even after the removal of foreign particles. In these less well-defined events, the sustained secretion of pro-inflammatory cytokines and chemokines continue to attract immune cells to the presumed site-of-injury, culminating in tissue-damaging cytokine storm, or hypercytokinemia.

In retrospect, the inflammatory bowel disease (IBD) is one of the well-documented microbial-associated, chronic inflammatory events in the gastrointestinal tract. The epithelial cells that line the gastrointestinal tract serve as a physical barrier that separates the host from its surrounding microbiota, nonetheless, they are in close proximity with environmental and dietary carcinogens. It was reported that the increase activation of $CD8^+$ cells coupled with the elevation in ROS production, or KRAS and TP53 mutations aggravate chronic IBD-related inflammation, supporting a close link to colorectal cancer initiation and progression [76-79]. Recent studies demonstrate that microbial infections also pose significant cancer risks [80]. For instance, *Helicobacter pylori* (gram negative) and *Streptococcus bovis* (gram positive) are bacteria that have been associated with gastric and colon cancers. Some viruses have also been shown to cause host DNA mutations by inserting its DNA or RNA into the host cell in attempts for replication. For example, the Epstein-Barr virus (belonging to the family of herpes virus) has been documented to increase the risks to developing lymphoma while certain strains of the human papilloma group of viruses have been linked to the development of cervical cancer. Hepatitis B/C viruses are also correlated to increased risks of developing liver cancer and the human immunodeficiency virus causes the acquired immune-deficiency syndrome (AIDS), which does not cause cancer directly but weakens the immune system of the infected, ultimately elevating the risk of developing other types of cancers or diseases.

1.2.1 Tristetraprolin (TTP) in Acute Inflammation

The role of TTP in acute inflammation was first identified when the embryonically non-lethal mouse deficient of TTP developed progressive manifestations autoimmune, inflammatory responses like erosive arthritis, dermatitis, cachexia and eventually high titers of antinuclear antibodies [81]. Subsequently, it was demonstrated that TTP is involved in the formation of a negative feedback loop responsible of modulating the production of TNF-α by destabilizing its mRNA transcripts [82]. Further studies showed that the removal of the TTP-binding region in the 3' UTR of TNF-α was sufficient to cause inflammatory arthritis [83]. TTP binds to the AREs located at the 3' UTR of TNF-α [84] and initiate deadenlyation [85], initiates deadenlyation to destabilize mRNA transcripts and target them for degradation [82, 86]. Subsequent findings have identified that TTP also targets other pro-inflammatory and cell cycle proteins [87] like CCL2 [88], c-myc [89], IFN-γ [90] and iNOS [91].

The transcription factor NF-κB is positively regulated by TNF-α and is considered to be a prototypical pro-inflammatory signaling pathway [92]. Functionally active NF-κB exists as either a hetero- or homo-dimer, made up from the combination of Rel A (p65), Rel B, Rel (c-Rel), NF-κB1 (p50 and p105 precursor) and NF-κB2 (p52 and p100 precursor) [93]. The most abundant p65/p50 heterodimer combination is usually sequestered in its inactive state by binding to cytosolic IκB complexes [94]. Pro-inflammatory stimuli activate the IκB kinase (IKK) complex. Consisting of 3 catalytic subunits, IKKα, IKKβ and IKKγ, IKK phosphorylates IκB (IκBα subunit) and targets it for degradation [95] and leading to the dissociation of the IκB-NF-κB inhibitory complex [96]. This unmasks the nuclear locatlization signal on NF-κB, allowing its nuclear translocation to regulate the transcription of a plethora of pro- and anti-inflammatory target genes engaged in immune modulation [97]. Observations from independent groups indicate that cytosolic TTP is able to bind and prevent the nuclear import of the NF-κB p65 subunit [98, 99], providing an alternative function of TTP exclusive of its mRNA destabilization activity. Put together, these evidences are consistent with previous postulations [82] that suggest that TTP might have further inhibitory effects on the transcription of a subset of inflammatory-related genes.

1.2.1.1 Expression and Regulation of TTP

Tristetraprolin (TTP), also known as zinc finger protein 36 homolog (ZFP36), Nup475 or GOS24, belongs to a family of proteins containing the CCCH (CX8CX5CX3H) tandem repeats. TTP was first identified by two independent groups in 1990 using the adipocyte-like, mouse 3T3-L1 cells [100, 101]. TTP has two exons and one intron. Currently, there are four known isoforms of TTP: ZFP36, ZFP36-like 1 (ZFP36L1), ZFP36-like 2 (ZFP36L2) and ZFP36-like 3 (ZFP36L3). Most mammals express three of four TTP isoforms (ZFP36, ZFP36L1 and ZFP36L2) while ZFP36L3 is expressed only in the placenta of rodents [102].

The mouse TTP gene is located on chromosome 7 encoding for a 319-amino acid long protein while the human TTP gene is located on chromosome 19, translating into a 326-

amino acid long homologue. TTP proteins exist in low levels in non-stimulated cells, predominantly in the nucleus in its unphosphorylated state. Upon stimulation, TTP translocates into the cytoplasm and bind the Adenosine-Uridine rich elements (AU-rich elements, AREs) located at the 3'UTR of target mRNAs [103] (**Figure 1.5**). The formation of translationally repressed mRNA (translational repression complex) gives rise to two primary forms of mRNA degradation: 3' to 5' decay through exosome complexes or 5' to 3' decay through processing bodies.

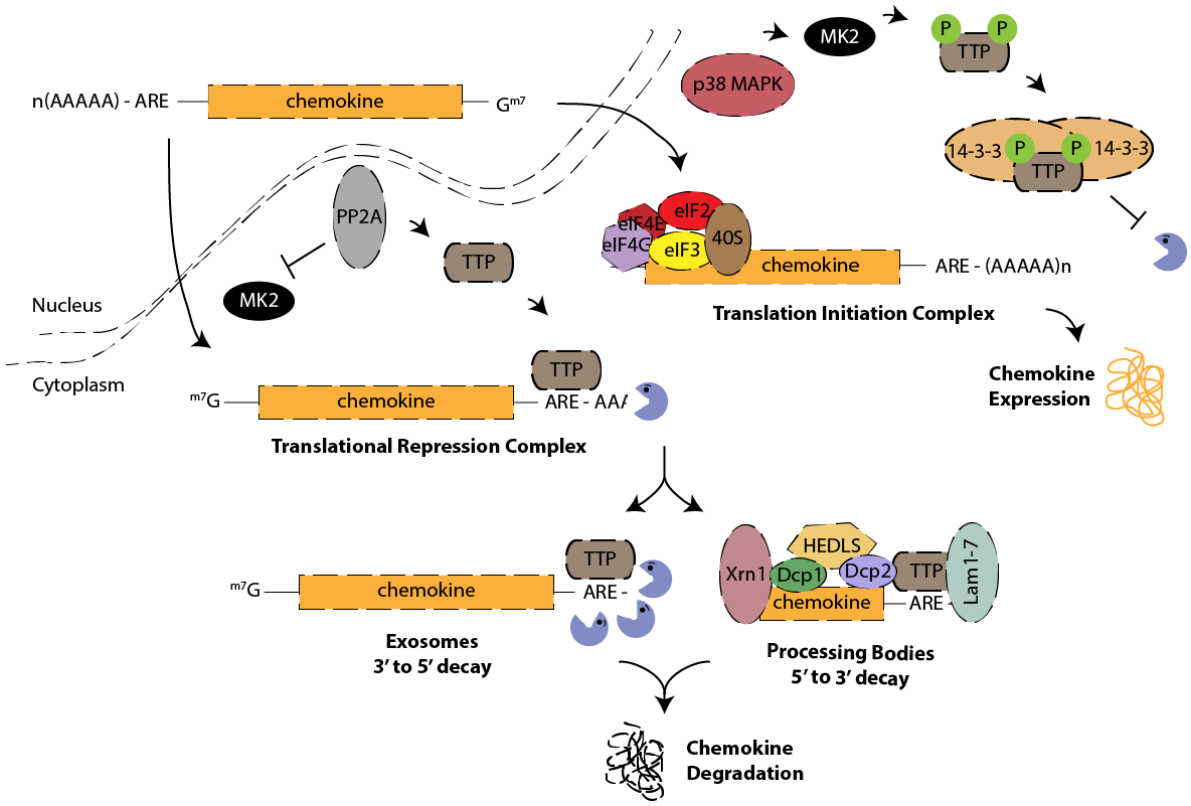

Figure 1.5 Regulation of chemokines by Tristetraprolin. The activity of TTP is an outcome of a balance between PP2A and MK2. p38 MAPK-MK2 dependent signaling causes the phosphorylation of TTP and recruits 14-3-3 complex, sequestering the activity of TTP in the cytosol. On the contrary, PP2A inhibits the action of MK2, resulting in the unphosphorylation of TTP. This allows TTP to recognize and bind the AREs of target mRNAs. This process first triggers deadenylation and the formation of translational repression complexes and the eventual degradation of target chemokines. mRNA degradation happens predominantly through the exosomes (3' to 5' decay) and the processing bodies (p-bodies; 5' to 3' decay).

Gathering work on TTP revealed that transcription factors and other molecules like SP1, AP2, NF-κB, EGR1, TPE1 and ELK1 can transcriptionally regulate the expression of TTP [104, 105]. TTP was initially identified as an acute-response gene because of its rapid induction towards stimuli like insulin, serum as well as other known mitotic agents like TGF-α and p38 MAPK [87, 100, 106-108].

Several serine residues have been found to be phosphorylated in the active form of TTP. Subsequently, TTP has been reported to be stimulated by p38 MAPK and later

phosphorylated by the mitogen-activated protein kinase-activated protein kinase 2 (MK2) [109] at S60 and S186 in human and S52 and S178 in mouse [87]. However, whether phosphorylated TTP displayed altered ARE-binding capability remains unclear [109]. Interestingly, phosphorylated TTP recruits 14-3-3 adaptor proteins, which reduces the degradation activity of TTP and allowing for downstream chemokine stabilization [110, 111]. The protein phosphotase 2A (PP2A) has been identified to be antagonistic towards MK2 activity, dephosphorylating TTP and causing the degradation of mRNA [110, 112].

1.2.1.2 mRNA Transcript Stability and its Impact on Immunosuppression

The balance between target mRNA destabilization and stabilization depends on the ratio of the different cellular regulators present in its environment. In contrast to TTP, the human antigen R (HuR) protein, competes for binding at the ARE of the 3' UTR region of target mRNA transcripts, stabilizing and putting a stop to mRNA degradation [113, 114]. Human HuR is encoded for by the ELAVL1 gene, is thus, a positive regulator of mRNA stabilization and gene expression [115]. Although much remains to be investigated, it was reported that HuR plays imperative roles in the transcriptional regulation of mRNA transcripts involved in microRNA regulation [116], immune response [117, 118], cancer [119, 120], as well as virus replication [121]. Recently, it was reported that HuR is detrimental in positively regulating the translation of TNF precursors and protein expression into functional TNF-α [122].

The dysregulation or overexpression of pro-inflammatory chemokines and cytokines, i.e. a chemokine storm more commonly known as hypercytokinemia, leads to erroneous damage on the host tissue [123]. Immunosuppressive drugs have been used to rein the hyperactivation of the host immune system. Immunosuppressive drugs are classified into 5 categories: cytostatics, antibodies, anti-immunophilins, glucocorticoids, and other drugs that target active components of the innate immune system. Cytostatics affect the proliferation of B and T cells and largely inhibit cell division [124]. Antibodies are sometimes used as quick-response agents to quench the immune response [125, 126]. The use of drugs against immunophilins are targeted at the inhibition of prolyl isomerise to prevent the interconversion between the cis and trans position of peptidyl-prolyl groups [127]. Glucocorticoids are arguably the most extensively used immunosuppressant to date [128]. Glucocorticoids inhibit a wide assortment of interleukins [129] and chemokines [128, 130], as well as decreasing eicosanoid and cyclooxygenase (COX-1/2) synthesis and expression [131, 132]. Interestingly, glucocorticoids have been observed to positively regulate MKP-1 (also recognised as DUSP1) [133-135], which has shown to increase the activity of TTP-dependent destabilization of pro-inflammatory cytokines [136-138].

1.2.2 Oxidative Stress in Chronic Inflammation and Cancer

The rapid development and advancement of technology over the last century fueled novel research techniques that supported Rudolf Virchow's 'chronic irritation theory'. In 1845, Virchow coined the term leukemia when he reported significant elevation of white blood

cells in patients. Subsequently, he demonstrated that normal cells can become neoplastic to give rise to cancers [139], and described the cancer initiation and propagation as a result of tissues under sources of severe irritation that spread through a liquid medium. Virchow also described that cancers were intrinsically related to white blood cells which were the original cause of this irritation, an observable fact we now relate to as tumor-associated macrophages during inflammation [140-142]. In subsequent breakthrough findings, *Katsusaburo Yamagiwa et al* demonstrated the role of chemical carcinogens in cancer [143] while *Francis Peyton Rous et al* established the existence of cancer-causing viruses [144]. Scientists then discovered a cluster of genes that regulated cellular growth (proto-oncogenes that are mutated to give oncogenes) as well as the cellular DNA repair mechanism (tumor suppressor genes). Researchers also hypothesized the two-hit hypothesis (also known as the Knudson hypothesis), which suggests that multiple hits were required to induce cancer. In this respect, the term field cancerization becomes an extension of the two-hit hypothesis because an earlier hit increases the predisposition of the cells in the cancer vicinity to developing a larger tumor.

Numerous mechanisms by which chemical carcinogens caused tumorigenesis have been proposed [145, 146]. One common underlying theme is the production of free radicals, such as reactive oxygen (ROS) and reactive nitrogen species (RNS) [147]. Because of their highly reactive nature, the balance between the production of ROS and their elimination by antioxidants is important for tissue homeostasis. It is now well-established that low concentrations of these reactive species served as signalling mediators [148, 149]. 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. Environmental factors like heightened background radiation and pollutant levels in cities, or increase intake of drugs and xenobiotics also increases the exposure to exogenous ROS/RNS. Endogenous sources of ROS/RNS are generated by essential biological processes such as respiration and inflammation. During respiration, free electrons are transported from one membrane-bound donor protein to its successive recipients arranged in progressively lower energy levels along the electron transport chain to create a proton gradient during mitochondrial respiration and ATP synthesis. Under normal homeostatic conditions, oxygen is completely reduced to water at the end of the respiratory cycle. However, incomplete reduction of molecular oxygen leads to the formation of the superoxide radical (O_2^-), a regular precursor of most other ROS. In its protonated form, hydroperoxyl (HO_2^-) has been shown to inactivate enzymes and accelerate lipid peroxidation [150, 151]. The dismutation of O_2^- gives rise to hydrogen peroxide (H_2O_2), and may further be partially reduced to give the hydroxyl radical (OH^-).

In normal cells, oxidative stress conjures protective antioxidant response from tissues to regain homeostasis. Antioxidant mechanisms involving enzymatic and non-enzymatic pathways are in place to prevent ROS accumulation. Notably, antioxidant molecules like vitamins A and E, glutathione, bilirubin and co-enzyme Q assist in quenching ROS in the body. Enzymes belonging to the family of superoxide dismutases (SODs) or catalyses can be site-specific and help catalyze the reaction of superoxides to the less harmful hydrogen

peroxides and oxygen. SODs contain different cationic metallic reactive centres and are located in the cytoplasm (SOD1), mitochondria (SOD2) and even in the extracellular matrix (SOD3). Catalyses are concentrated primarily in peroxisomes that are located in proximity to mitochondria and glutathione peroxidises rely on its reactive sulphur centre to reduce ROS.

Perturbations to the balance of antioxidant and oxidants, resulting in accumulation of ROS/RNS causes oxidative stress, and have been documented to cause breakages in the DNA double helixes, mismatches in the complementary DNA base-pairings and also base transversions [152]. Many studies also show that the build-up of ROS promotes disease pathogenesis by acting as a precursor to fuel chronic inflammation and malignancy. Metabolically active organs like the brain and the liver are also highly susceptible to ROS accumulation. ROS elevation in the brain has been reported to increase risks of Alzheimer's disease [153, 154] while chronic hepatitis and liver fibrosis have been attributed to the accumulation of ROS in the liver [155, 156]. ROS has been reported to cause mutations in critical cell cycle proteins like checkpoint kinases 1/2 (Chk1 and Chk2) and ataxia telangiectasia (ATM), as well as induce G2-M phase cell cycle arrest [157]. Others found that the increase in ROS [158, 159] in the hypoxic tumor environment stimulated the subsequent augmentation of TNF-α, COX-2 [160], NF-κB [161], STAT3 [162] and JNK/p38 MAPK [163] activity, which brought about further increase in IL-6, TNF-α and BCL-X_L levels, hence promoting tumor cell survival, migration and metastasis.

1.2.3 TAK1 Expression and Regulation in Chronic Inflammation and Cancer

The transcription factors nuclear factor-κB (NF-κB) and activating protein-1 (AP-1) are critical regulators of stress responses, immunity, inflammation and cancer. A large variety of cellular stimuli utilize these signaling pathways through a common upstream kinase transforming growth factor-β-activated kinase 1 (TAK1). TAK1 belongs to the family of mitogen-activated protein kinase kinase kinase (MAPKKK) class of threonine/serine kinases and is encoded for by the *MAP3K7* gene on chromosome 6. TAK1 has been found to complex with both Tab1 and Tab2 proteins in the cytosol to facilitate its downstream signaling. The association with Tab1 is believed to activate the TAK1 kinase activity [164] while Tab2 assists in the binding with the TRAF6 complex [165]. TAK1 deficiency in mouse resulted in embryonically lethal phenotypes because of the inability to undergo important processes like angiogenesis and vascularization during development [166].

TAK1 has been well characterized in recent literature in playing pivotal roles in development and innate inflammation and has been found to respond to a plethora of inflammatory stimulation. Bacterial LPS, inflammatory cytokines and TLRs have been reported to stimulate TAK1 activity [167-171]. Although non-canonical, it was also reported that TGFβ can stimulate TAK1 activity through Smad3 independent pathways [172]. TAK1 was also found to phosphorylate other downstream MAP kinases, culminating in the activation of transcription factors like p38 MAPK, NF-κB and AP-1 that regulates pro-inflammatory genes

[173-176]. Importantly, evidence have showed that TAK1 deficiency decreases cJun activation and promotes ROS production and inflammation in the skin [177]. Others demonstrate that TAK1 phosphorylates MAPKs [178] and increases TNF-α signaling [179, 180], resulting in the increased production of COX-2 and ROS.

1.3 ANGIOPOIETINS AND ANGIOPOIETIN-LIKE PROTEINS

There are 4 members that make up the angiopoietin family of proteins (Ang). While pro-angiogenic Ang1 was found to bind its cognate receptor Tie1 and Tie2 to promote angiogenesis and downstream vessel remodelling [181], it was later reported that Ang2 antagonises this effect by competitive binding to Tie2 [182]. Angiopoietins were first identified to be mediators of blood vessels formation during embryonic and postnatal development, and also for the assemblage of the vascular endothelial wall [183, 184].

The Ang family of proteins share structural and sequence homology to the Angiopoietin-like family of proteins (ANGPTL) because both Ang and ANGPTL family of proteins are characterized by a coiled-coil N-terminal domain with a fibrinogen-like C-terminal domain [185-187]. ANGPTL represents a family of 8 proteins. But unlike their cousins, ANGPTL do not bind Tie proteins to mediate downstream responses. To date, ANGPTL remain as orphan ligands with little knowledge on their direct binding partners. Thus far, studies have observed that ANGPTL2, ANGPTL3, ANGPTL4, ANGPTL5, ANGPTL7 are involved in hemapoietic stem cell maintenance and regulation while ANGPTL3, ANGPTL4, ANGPTL6 and ANGPTL8 have been reported to mediate glucose and lipid metabolism [188-190]. Recent studies have also detailed the multifaceted role of ANGPTL in inflammation and cancer progression and metastasis [191-193].

1.3.1 Angiopoietin-like 4 (ANGPTL4)

ANGPTL4 is perhaps one of the most important members of the ANGPTLs because it mediates a broad variety of processes. Studies show that ANGPTL4 is involved in a wide assortment of pathways ranging from energy homeostasis, wound repair, inflammation and cancer [194-197]. Emerging evidence indicates that ANGPTL4 alters endothelial barrier integrity and is involved in cancer cell migration and metastasis [198]. Lately, the dysregulation of ANGPTL4 has also been attributed to complications associated with the metabolic syndrome.

1.3.1.1 Expression and Regulation of ANGPTL4

ANGPTL4 is a secretory adipokine previously known as fasting induced adipose factor (FIAF), hepatic fibrinogen/angiopoietin-related protein (HRARP) and peroxisome proliferator-activated receptor gamma angiopoietin-related protein (PGAR). The ANGPTL4 protein is encoded for by the ANGPTL4 gene, a 11.09 kb gene located on human chromosome 19, and translated into a 406-amino acid long protein. On the other hand, the mouse ANGPTL4 gene is 6.6 kb on chromosome 17 encoding for a 410-amino acid ANGPTL4 protein. The molecular weight of full length ANGPTL4 (fANGPTL4) in both mice and humans remains contentious, ranging between 45 to 65 kDa due to post-transcriptional modifications such as glycosylation (**Figure 1.6**).

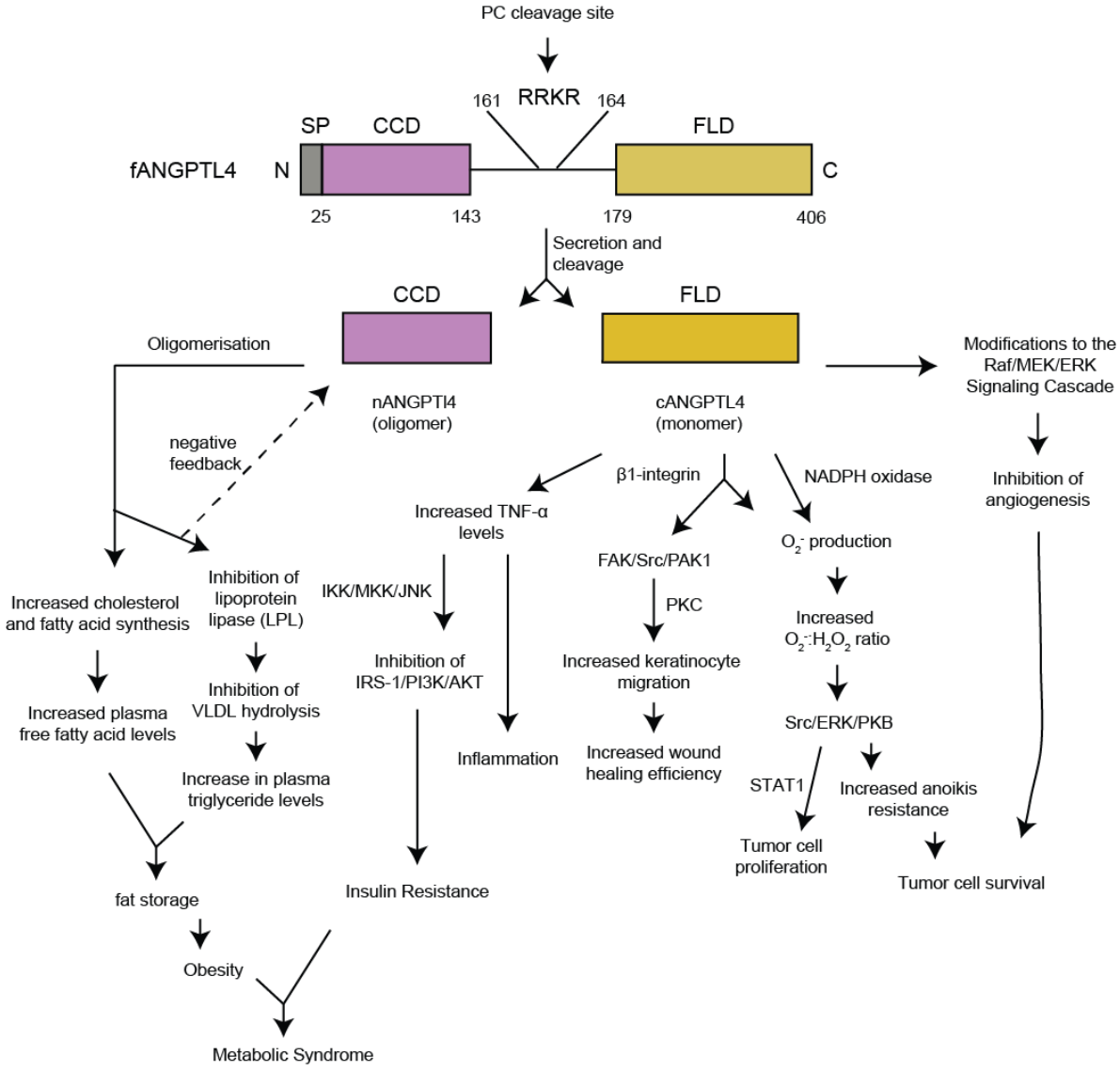

Figure 1.6 Regulation and function of Angiopoietin-like 4 (ANGPTL4). Proprotein convertases like furin have been reported to recognize a conserved sequence –RRKR- of non-functional full length ANGPTL4 (fANGPTL4) to give an N-terminal coiled-coil fragment (nANGPTL4, 26 kDa) as well as a C-terminal fibrinogen-like fragment (cANGPTL4, 35-47 kDa). Functional nANGPTL4 oligomerizes into dimmers or tetramers, and has been found to inhibit lipoprotein lipase (LPL), primarily controlling downstream fat metabolism and storage. On the other hand, functional cANGPTL4 exists as monomers. Recent findings direct the function of cANGPTL4 to play regulatory roles in inflammation, as well as tumor cell survival and proliferation. Modified and reprinted from Mol Cancer Res, 2012, 10, 677-688, Tan MJ et al, Emerging Roles of Angiopoietin-like 4 in Human Cancer, with permission from AACR.

Once secreted, fANGPTL4 is cleaved by proprotein convertases at the -RRXR- consensus region to give a 25 kDa N-terminus coiled-coil fragment (nANGPTL4) and a 25-35 kDa C-terminus fibrinogen-like fragment (cANGPTL4). To date, the proteolytic cleavage mechanism of fANGPTL4 remains to be elucidated. *In vitro* studies have shown that proprotein convertases like furin and paired basic amino acid-cleaving enzyme 4 can cleave fANGPTL4 at its consensus site [199]. However, little is known if these proprotein convertases are indeed responsible for the *in vivo* processing of secreted fANGPTL4, or if the

expression of the different classes of proprotein convertases correlates to the presence of different ANGPTL4 fragments. Unlike cANGPTL4 which exists as a monomer, disulfide bond formation at the highly conserved 76 and 80 residues of the N-terminus coiled-coil domain allows fANGPTL4 to oligomerize and exist either as a tetramer or a dimer [200]. Likewise, the nANGPTL4 fragment also oligomerizes after cleavage and regulates lipid metabolism via its interaction with lipoprotein lipase.

During hypoxia, the expression of hypoxia-inducing factor 1α (HIF-1α) is up-regulated, which mediated downstream ANGPTL4 expression [201, 202]. ANGPTL4 is also a direct target gene of the nuclear receptor PPAR (peroxisome proliferator-activated receptor) and functional PPAR response element (PPRE) resided in the intron 3 of the ANGPTL4 gene [203]. HIF-1α and PPAR were also found to synergistically up-regulate the expression of ANGPTL4 [204]. Other reports indicated that a variety of growth factors and stimuli like the toll-like receptors (TLRs), transforming growth factor beta (TGFβ), glucocorticoid receptor (GR), the metabolites from gut microbes and even the circadian rhythm signals ARNT2/SIM1 also regulate the expression of ANGPTL4 [188, 205-210].

fANGPTL4 is readily detected in hepatocytes, adipocytes and macrophages, as well as a lower level in other tissues such as the skin, kidneys and intestines [188] while cANGPTL4 and nANGPTL4 fragments are detected in the liver [203, 211]. The nANGPTL4 associates with lipoproteins [212] and also inhibits the activity of lipoprotein lipases (LPL). This interaction is dependent on the oligomerization of nANGPTL4 fragments in order to effectively inhibit LPL and participate in lipid metabolism [213] (**Figure 1.7**). Hence, the function of nANGPTL4 hinges on its ability to form active three-dimensional structures which are functional. In contrast, the cANGPTL4 fragment exists as a monomer. cANGPTL4 associates with specific extracellular matrix proteins, β1 and β5 integrin molecules [214]. In wound healing, cANGPTL4 facilitates keratinocyte migration through the FAK/Src/PAK-1 cascade [215]. The cANGPTL4 also suppresses angiogenesis through the inhibition of the Raf/MEK/ERK pathway [216]. Furthermore, cANGPTL4 also increases the O_2^-:H_2O_2 ratio, promoting tumor cell survival and proliferation [197]. Put together, these evidences suggest that the expression of specific ANGPTL4 fragments is tissue/organ dependent, and that the various ANGPTL4 fragments play distinct cellular functions.

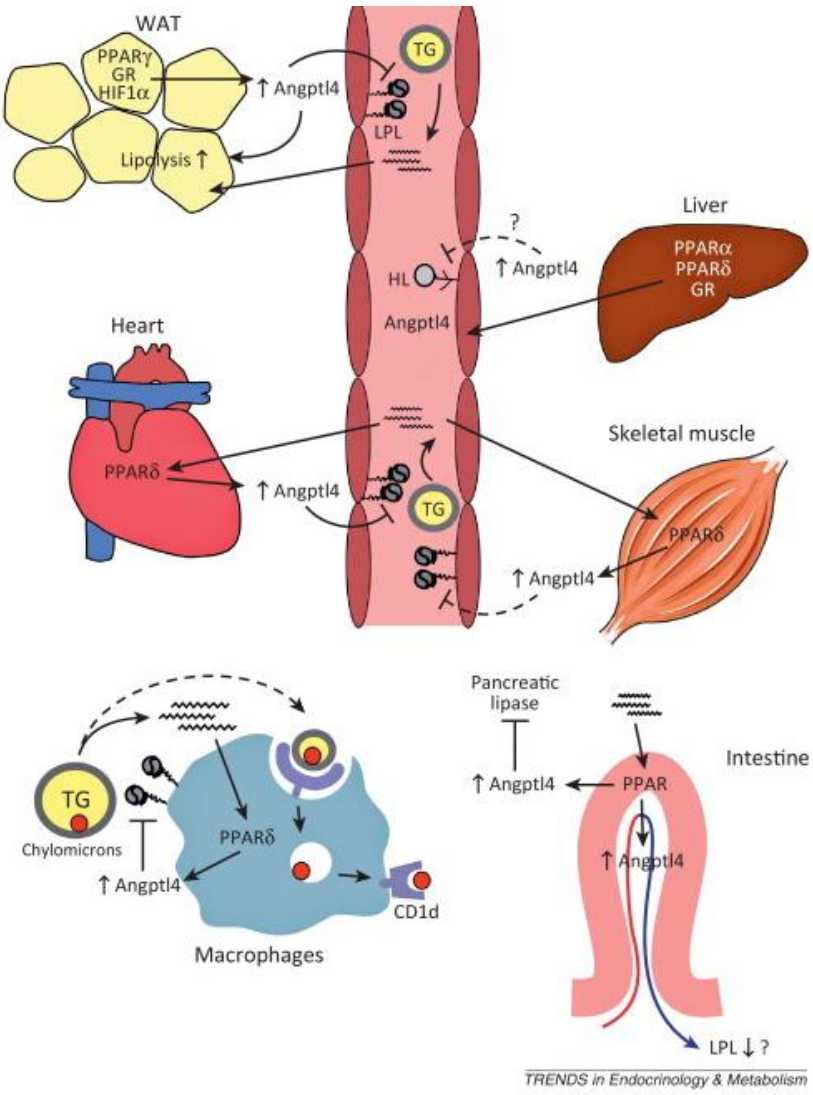

Figure 1.7 The paracrine effects of Angiopoietin-like 4 (ANGPTL4) in lipid metabolism. ANGPTL4 is chiefly expressed in the liver, white adipose tissue (WAT), skeletal muscle but is recently reported to be also expressed in macrophages, heart as well as enteroendocrine cells in the intestine. The function of ANGPTL4 in lipid metabolism is tissue specific, and is primarily under the regulation of peroxisome proliferator-activated receptors (PPARs) and glucocorticoid receptors (GRs). In WAT, increased ANGPTL4 stimulation inhibits lipoprotein lipases and increases lipolysis of accumulated triglycerides. Because the liver does not produce LPL, ANGPTL4 is secreted into the circulation and targeting peripheral LPL activity. In the muscle, heart and in macrophages and intestinal enteroendocrine cells, positive stimulation by fatty acids increases ANGPTL4 levels, bringing about an inhibition of local LPL activity. Reprinted from Trends in Endocrinology & Metabolism, 25/3, Dijk W et al, Regulation of lipoprotein lipase by Angptl4, Pages No. 146–155, Copyright 2014, with permission from Elsevier.

Up till now, there is no known protein crystal structure of fANGPTL4, cANGPTL4 or nANGPTL4. Current knowledge of the different roles of ANGPTL4 fragments were elucidated via a combination of biochemical analysis and ANGPTL4-knockout animal model. ANGPTL4 has been implicated to play a role in tumorigenesis, however its effect remained controversial. Although the precise reason for the controversy is unclear, it is conceivable that different ANGPTL4 fragments may be involved, as these fragments have

been reported to stimulate different signaling cascades Thus, more effort is required to elucidate the structures and functions of the different ANGPTL4 fragments.

1.3.1.2 ANGPTL4 and Metabolism

The oligomerized nANGPTL4 inhibits LPL and regulates lipid metabolism (**Figure 1.8**). LPLs are found at the luminal surface of capillary endothelial cells and are regulated by hormones like insulin, glucagon and adrenaline. LPL homodimers hydrolyses plasma triglycerides found in VLDL into intermediate-density lipoproteins (IDL) and eventually into free fatty acids (FFA), allowing the subsequent uptake of fatty acids into the liver, adipocytes and macrophages for storage purposes. ANGPTL4 mediates the rate of plasma triglyceride clearance. fANGPTL4 and nANGPTL4 oligomers cause the dissociation and subsequent inactivation of LPL dimers, resulting in the inhibition of triglyceride hydrolysis. Furthermore, it was observed that ANGPTL4 further exacerbates the inhibition of triglyceride hydrolysis by targeting LPLs for proteolysis by proprotein convertases [199]. However, studies also showed that increasing circulating ANGPTL4 further increases plasma FFA levels through increasing cholesterol and fatty acid synthesis [211, 217], suggesting a negative feedback mechanism on plasma LPL inhibition. The over-expression of ANGPTL4 was also demonstrated to increase levels of nonesterified fatty acids (NEFA) in adipocytes [212] and cause hypertriglyceridemia in mice [212, 218].

Apart from lipid metabolism, ANGPTL4 was recently found to be involved in glucose metabolism. ANGPTL4 has been shown to reverse glucose insensitivity in diabetic mice. ANGPTL4 lowers hyperglycemia primarily through augmenting insulin-dependent inhibition of gluconeogenesis and impeding glucose synthesis in the liver [219]. In adipocytes, insulin down-regulates ANGPTL4 levels through the PI3K/FOXO1 signalling cascade [220].

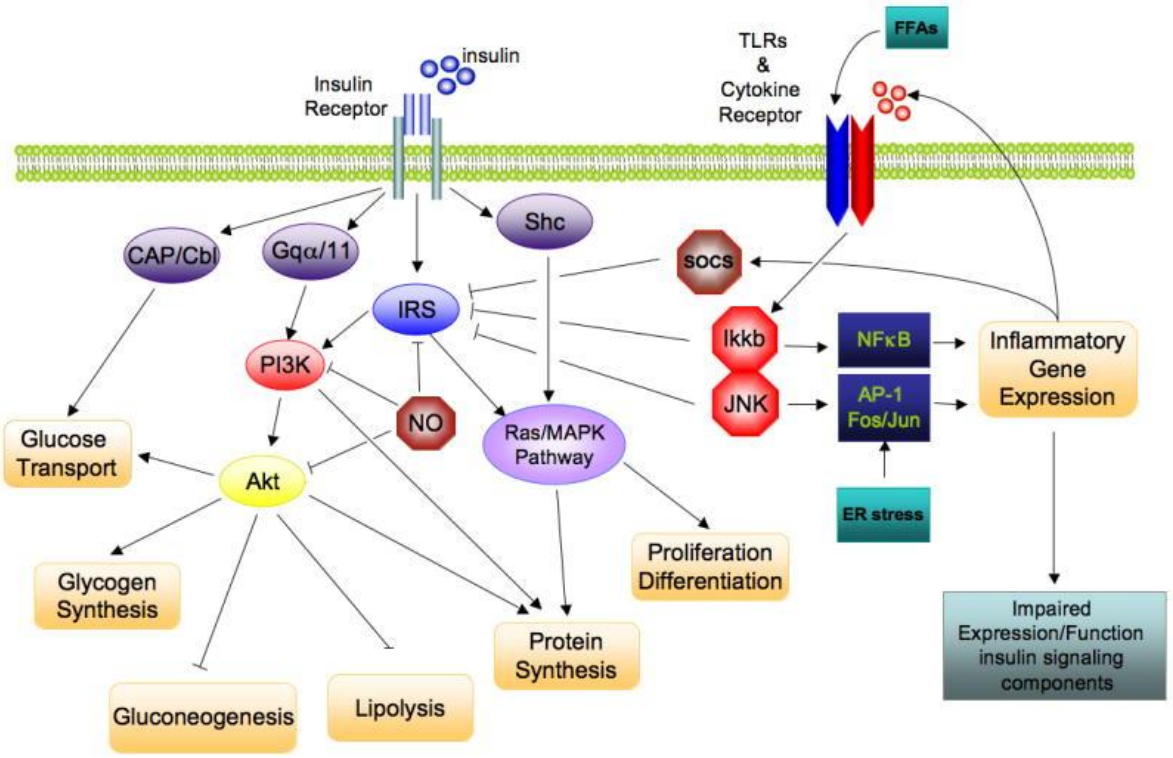

Figure 1.8 Inflammatory pathways implicated in insulin and FFA stimulation. Insulin binds cell surface insulin receptors to increase glucose uptake into cells predominantly through the phosphorylation of IRS, leading to the activation of the PI3K/AKT and Ras/MAPK signaling cascades to support growth and development. The abundance of FFA in the system hyperactivates TLRs and other chemokine receptors, causing an increase in phosphorylation of downstream inflammatory mediators like IKKB and JNK. Signaling through the NFκB and AP1 (Jun/Fos) pathway increases, leading to an increase but impaired expression of inflammatory components. The activation of IKKB and JNK serine kinases also inhibits the signaling ability of IRS, which further impairs conventional insulin signaling. In turn, other inflammatory signals like NO and SOCS also impairs the activity of IRS signaling, resulting in an overall increase in the production of inflammatory mediators and a decrease in normal glucose uptake and metabolism. Reprinted from FEBS Letters, 582/1, De Luca C et al, Inflammation and Insulin Resistance, Pages No. 97–105, Copyright 2008, with permission from Elsevier.

1.3.1.3 ANGPTL4 and Inflammation

The dysregulation of ANGPTL4 impacts lipid and glucose metabolism, leading to obesity-induced inflammation and the unwanted accumulation of fat and energy storage [221, 222]. The increase in FFA and SFA was found to positively regulate pro-inflammatory cytokine production and insulin resistance, the latter directly involved in mediating cellular glucose uptake [223, 224]. FFA and SFA also hyperactivates Toll-like receptor 4 (TLR4), resulting in the activation of an assortment of immune responses. In turn, this triggers the synthesis and secretion of increased levels of pro-inflammatory cytokines like TNF-α and IL-6 [225]. Augmented pro-inflammatory signals further activate downstream S6K/IRS/PI3K/AKT signalling cascade in a positive feedback manner, causing the eventual insulin resistance and development of type II diabetes.

Numerous studies have implicated a role for ANGPTL4 in inflammation, where ANGPTL4 plays either anti- or pro-inflammatory roles in a context- or tissue-dependent manner. ANGPTL4 prevents foam cell formation by inhibiting fatty acid uptake into mesenteric lymph node macrophages, thus protecting against severe pro-inflammatory effects of saturated fatty acids [226]. ANGPTL4 also protects against atherosclerosis development [227] and acts as an angiogenic mediator in arthritis [227, 228]. ANGPTL4 was observed to exacerbate influenza and inflammation through independent IL-6-STAT3 and SIRT1-NFkB mediated pathways in the lungs [196, 229]. Interestingly, serum ANGPTL4 was correlated to C reactive protein (CRP) levels in type II diabetic patients, suggesting that ANGPTL4 might be involved in the inflammatory progression during the metabolic syndrome [230]. In the context of infection-associated inflammation, bacterial lipopolysaccharide (LPS) was found to be able to up-regulate ANGPTL4 expression in the mouse serum within 8 hours upon LPS stimulation and was subsequently labelled as an acute phase protein [231]. Furthermore, it was also shown that stimulating 3T3-L1 adipocytes with inflammatory chemokines like IL-1β, TNF-α, IFN-γ increases ANGPTL4 expression. Another independent group has also shown that the acute phase protein α1-antitrypsin (A1AT) increases ANGPTL4 expression through ERK1/2 signaling cascade [232]. Albeit the numerous reports of the role of ANGPTL4 in inflammation, the pathways by which ANGPTL4 modulates inflammation are largely unknown and remains unclear.

1.3.1.4 ANGPTL4 and Cancer

We consider the immune system as our friend; it protects us by fighting infections while keeping us healthy. But there is a darker side to the immune system. Often, when it comes to cancer, we find that the immune system can turn traitor and actually promote cancer development. The presence of leukocytes in tumors has been noted for many decades and provides the first clue that inflammation is linked to cancer. Yet it is only within the last few years that we have obtained clear evidence that inflammation plays a critical role in cancer development, and we are just beginning to understand the molecular mechanisms of how this happens. Indeed, chronic infections, obesity, smoking, alcohol consumption, environmental pollutants and high fat diets are now recognized as major risk factors for most common types of cancer; and, importantly, all these risk factors are linked to cancer through inflammation.

In an inflammatory and oxidative milieu, cells begin to accumulate damages to their DNA that facilitates the accumulation of pro-oncogenic mutations. Furthermore, the incessant secretion of ROS, MMPS, cytokines, chemokines and other soluble pro-inflammatory factors like CSF-1 and IL-6 by tumor-associated macrophages (TAMs) amongst other inflammatory infiltrates, potentiates angiogenesis and tumor progression [233]. TAMs also secrete TGFβ, TNF-α and IL-10 [234, 235], and attenuating cytotoxic T cell activation [236]. Coupled onto sustained cell proliferation, reduced DNA repair and the subversion of apoptosis, cells in the chronic inflammatory environment develop neoplastic phenotypes and progress into cancer [237, 238].

A role for ANGPTL4 in regulating inflammation has been implicated (see above, chapter *1.3.1.3*). Conceivably, ANGPTL4 has a well-described role in carcinogenesis. ANGPTL4 is both a pro-angiogenic and anti-apoptotic factor that promotes endothelial vascularisation [209, 228, 239, 240]. Recently, we found that ANGPTL4 facilitates cell migration [215] and also regulates intracellular oxidative O_2^-:H_2O_2 ratios in tumors [197], In addition, ANGPTL4 has also been implicated in cancer progression and metastasis. Independent groups have also reported that ANGPTL4 levels were increased in gastric cancer cells [241] and renal epithelial tumor cells [242, 243]. *Nakayama et al* and *Shibata et al* proposed that ANGPTL4 play significant roles in both venous and lymphovascular invasion [241, 244], suggesting that ANGPTL4 affects tumor migration and metastasis. Others identified ANGPTL4 as a marker of breast-lung metastasis [207] and oral squamous cell carcinoma [245]. These findings suggest that ANGPTL4 is a potential regulator in cancer progression and tumor vascularisation. Even though ANGPTL4 has been identified to play vital roles in regulating both inflammation and tumorigenesis, the exact roles by which ANGPTL4 regulates cancer through inflammatory pathways remain to be elucidated. Therefore, it is of utmost importance to understand the relationship between the ANGPTL4-dependent, inflammation-tumorigenesis signalling axis.

2 AIMS

We aim to explore various ANGPTL4-dependent intermediates that regulate the inflammatory landscape. Using independent models, we further delineate the respective signaling cascades that primarily govern both the acute and chronic phases of inflammation in the inflammation-cancer signaling axis.

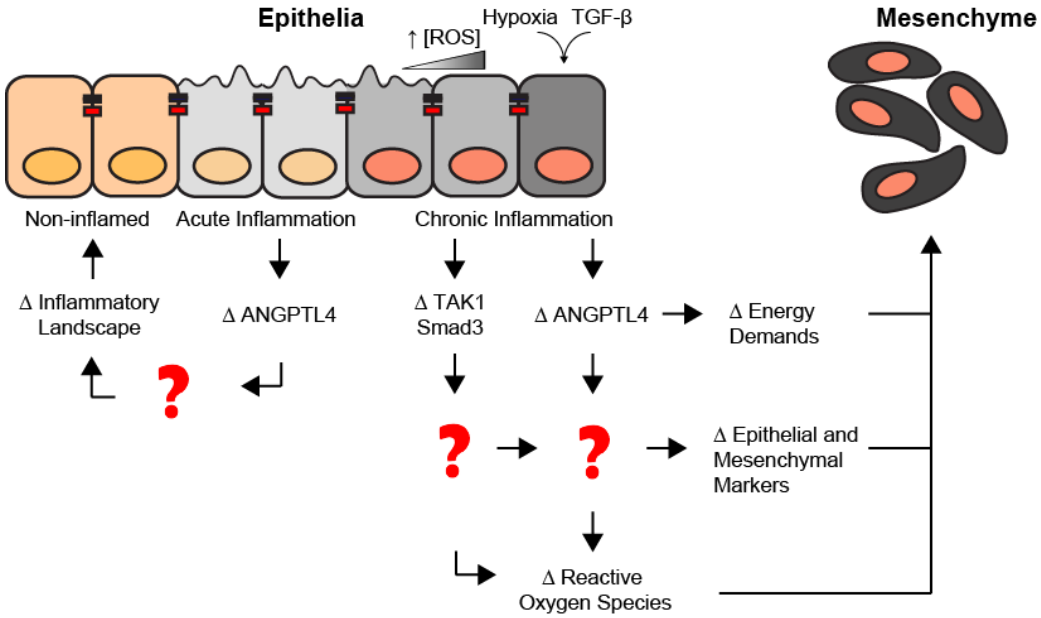

Figure 2.1 A graphical summary of ANGPTL4-dependent signaling cascades during acute and chronic inflammation which promotes epithelial-to-mesenchymal transition.

In **PART I** of this thesis, we demonstrate that the importance of ANGPTL4 in regulating the acute colonic inflammatory environment.

I. An anti-inflammatory role for colonic ANGPTL4 in dextran sulfate sodium salt (DSS)-induced colitis and dietary stearic acid (SA) intake.
II. The role of the commensal gastrointestinal microbiota in inflammation.
III. The intrinsic role of colonic ANGPTL4 in regulating leukocyte infiltration during DSS-induced inflammation, and thus, the colonic inflammatory landscape.
IV. The underlying mechansims involving the regulation of Tristetraprolin (TTP) through CREB and NF-κB transcription factors.

In **PARTS II and III** of this thesis, we aim to understand the role of TAK1 and ANGPTL4 in regulating ROS production during the chronic inflammatory state in the tumor microenvironment.

V. The role of TAK1 and ANGPTL4 in mediating the pro-inflammatory ROS landscape in the early, pre-tumor microenvironment.
VI. To understand the role of ROS in promoting field cancerization.
VII. To delineate possible signalling downstream of ANGPTL4 during chronic inflammation that promotes tumor cell migration and the epithelial-mesenchymal transition (EMT) process.

3 METHODOLOGY

3.1 METHODOLOGICAL HIGHLIGHTS

Detailed experimental methodologies can be obtained in the individual papers and manuscripts. Therefore, this section will describe methodological highlights utilized in this thesis.

3.1.1 Bone Marrow Tranplantation

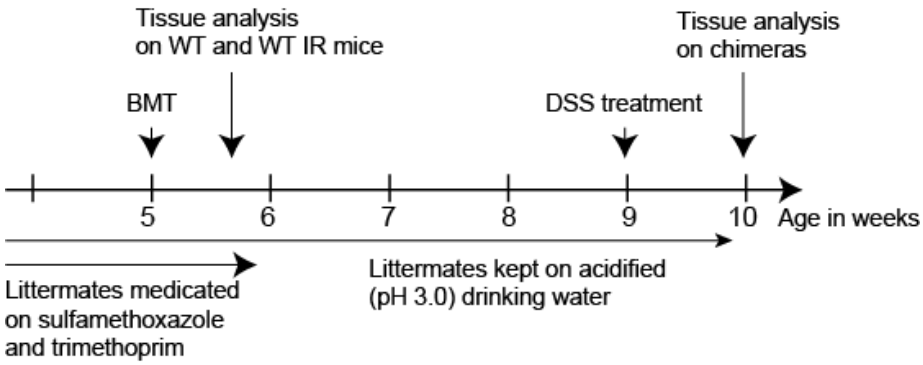

Figure 3.1 Experimental Timeline for the Bone Marrow Transplant.

To exclude the cell-autonomous (intrinsic) effects of ANGPTL4 originating from non-colonic epithelial cells, we further performed a bone marrow transplant using the bone marrow from donor ANGPTL4$^{-/-}$ or ANGPTL4$^{+/+}$ mice to recipient ANGPTL4$^{+/+}$ mice (**Figure. 3.1**). Recipient mice were kept on acidified water (pH 3.0) over the course of the entire experiment. BMT (ANGPTL4$^{-/-}$) and BMT (ANGPTL4$^{+/+}$) chimeras were medicated with trimethoprim (8 mg/kg) and sulfamethoxazole (40 mg/kg) in oral suspension (Allpets Asia, Singapore) for one week before and after lethal γ-irradiation (9.5 Gy), using the Biobeam 8000 (Gamma-Service Medical GmbH).

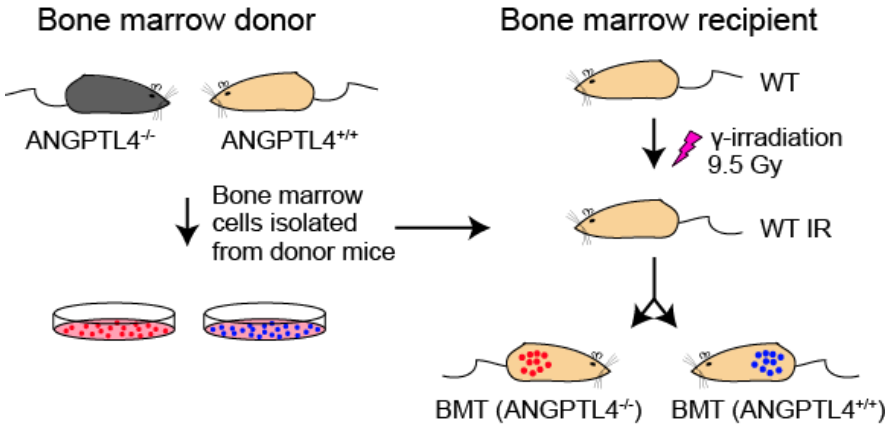

Figure 3.2 Bone Marrow Transplant Procedures.

Donor bone marrow cells from the humerus, tibia and femur of donor mice were isolated in serum-free DMEM. Cells were first washed with PBS followed by red blood cells lysis [0.89% NH_4Cl (w/v), 0.1 mM EDTA, pH 7.2]. Cells were filtered through a 30 µm nylon cell

strainer before depleting mature $CD4^+$ and $CD8^+$ donor immune cell populations (QuadroMACS kit with anti-CD4- and anti-CD8a-conjugated microbeads, #130-049-201, #130-049-401 and #130-091-051; from Miltenyi Biotec; in accordance to manufacturer's recommendations; degassed, ice cold PBS containing 0.5% BSA, 2 mM EDTA at pH 7.2). Cells were resuspended in serum-free DMEM and approximately 10^7 bone marrow cells (in 100 µL total injected volume) were introduced into WT IR recipient mice via retro-orbital injection (**Figure 3.2**). BMT ($ANGPTL4^{-/-}$) and BMT ($ANGPTL4^{+/+}$) chimeras were allowed to recover for 4 weeks before the subsequent 8-day 2% DSS treatment.

Hematopoietic reconstitution was evaluated using genotype PCR. At experimental endpoint, cell counts were performed for the whole femur and spleen tissues using the ADAM-MC cell counter (NanoEntek, USA). Splenic and colonic cells were analyzed for F4/80 and CD11b expression using FACS (BD LSRFortessa X-20) to determine the extent of immune cell infiltration.

3.1.2 16S Metagenomics Sequencing

Age-matched, post-weaned $ANGPTL4^{-/-}$ and $ANGPTL4^{+/+}$ males were co-housed for the entire experiment. Fresh fecal samples were collected from both $ANGPTL4^{-/-}$ and $ANGPTL4^{+/+}$ littermates before and after 8-day 5% DSS treatment, and stored at -80 ^{o}C. Bacterial genomic DNA was isolated using the FASTDNA spin kit for feces in accordance to manufacturer's recommendations (MP Biomedicals, USA; #116570200). 16S metagenomics sequencing was performed by SeqMatic, USA, using primers targeting the 16S V4 region by means of the high throughput NGS Illumina MiSeq platform.

3.1.3 Transwell Migration Assay

THP1 monocytes were initially differentiated into macrophages by incubating cells in DMEM supplemented with 10% FBS and 100 ng/mL TPA for 48 h. Cells are left to recover for 24 h before seeding onto transwell inserts. Concurrently, respective siRNA knockdowns were performed on iCECs ($iCEC_{Ctrl}$, $iCEC_{ANGPTL4}$ and $iCEC_{TTP}$) that were pre-seeded onto wells. Cells on both the inserts and wells are kept apart until the start of the transwell migration experiment as shown in **Figure 3.3**.

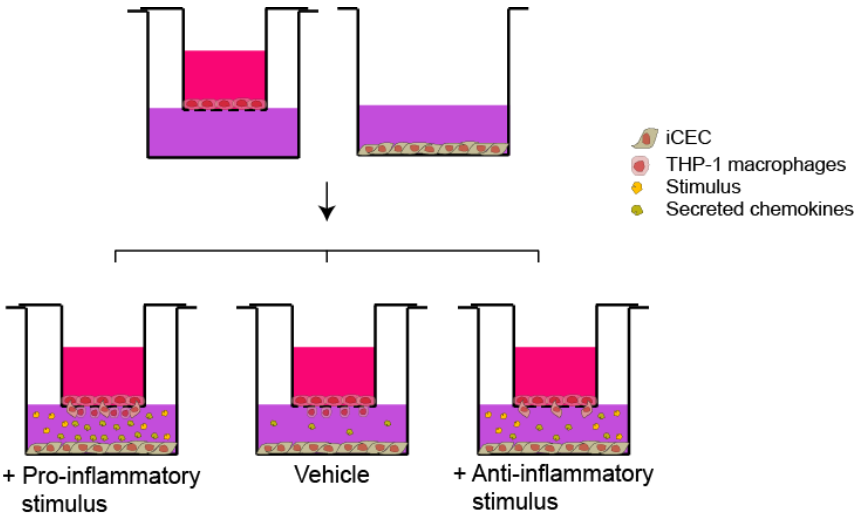

Figure 3.3 A graphical illustration of the transwell migration assay set up.

Inserts containing differentiated THPI-derived monocytes were then introduced to the respective transfected iCECs and exposed to various pro- or anti-inflammatory stimuli for 10 h. Subsequently, inserts were then removed and rinsed twice with PBS and fixed in 1% glutaraldehyde for 10 min. Inserts were rinsed again and stained with SYTO 60 (Thermo Fisher, USA) for 30 min. Consequently, cotton buds were used to scrape away all non-migrated THP1 cells trapped within the upper chamber of each insert and rinsed for the last time in PBS. Relative fluorescence was quantified using the CLx scanner and Image Studio V2.1 (LI-COR Biosciences, USA).

3.1.4 Statistical Analysis

Statistical analyses were performed using 2-tailed Mann Whitney U-test using Graphpad Prism 5 and Microsoft Excel software. P values are expressed as means ± standard error; and $p < 0.05$ represent statistically significant differences, where * denotes $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

4 RESULTS AND DISCUSSION

4.1 ANGIOPOIETIN-LIKE 4 IN ACUTE INFLAMMATION

Recent studies report that Angiopoietin-like 4 (ANGPTL4) plays a multi-faceted role in the regulation of host inflammatory response. ANGPTL4 has been observed to play angiogenic roles in mediating arthritis [227, 228] and atherosclerosis development [227]. ANGPTL4 was found to exhibit anti-inflammatory functions in preventing macrophages from absorbing fatty acids, hence preventing foam cell formation [226]. However, others attribute ANGPTL4 to exacerbating inflammation. Serum C-reactive protein (CRP) levels have been correlated to ANGPTL4 expression in patients with type II diabetes [230]. LPS was detailed to positively up-regulate ANGPTL4 expression [231]. To date, ANGPTL4 has been shown to exert both anti- and pro- inflammatory responses through a wide array of stimuli. However, little is known about how ANGPTL4 regulates inflammation.

In **Part I** of this thesis, we sought to outline possible mechanisms that could explain the anti-inflammatory role of ANGPTL4 (**Figure 4.1**). Using both dietary C18 saturated fatty acid (stearic acid; SA) and dextran sodium salt (DSS) as separate models to induce acute inflammation in mice, we show that mice exhibited an exacerbated inflammatory response in the absence of ANGPTL4. Confirming the current paradigm, 16S metagenomics sequencing showed little differences in the microbiota communities between the genotypes at steady state but show divergence during DSS-induced inflammation [246-248]. Both microarray analysis and bone marrow transplantation experiments further affirm the importance of epithelial-derived ANGPTL4 in regulating the local inflammatory landscape. Subsequently, we demonstrated an increased stability in a subset of chemokines that were previously found to be regulated by an mRNA destabilizing protein, TTP. To reconfirm our *in vivo* findings, we subjected SV40-immortalized human colonic epithelial cells (iCECs) to pro- and anti-inflammatory treatments. We observe that ANGPTL4 and TTP expression decreases with pro-inflammatory treatments and increases with anti-inflammatory stimulation. In summary **Paper I** demonstrated that ANGPTL4 is able to regulate the inflammatory landscape through TTP-dependent and TTP–independent pathways.

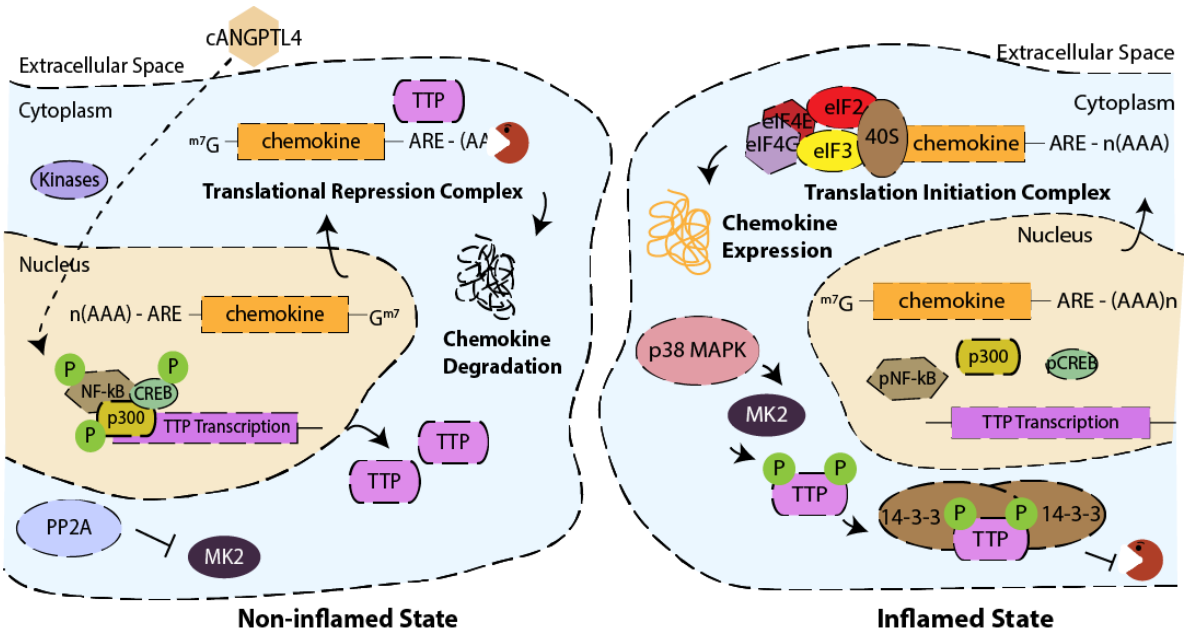

Figure 4.1 A graphical summary of the TTP-dependent ANGPTL4 signaling axis. cANGPTL4 mediates colon epithelial cell homeostasis by regulating TTP expression. At basal state, cANGPTL4 stimulation maintains TTP expression through the phosphorylation of transcription factors CREB and NF-κB. TTP binds at the AU-rich regions of the chemokine 3' UTR, causing the degradation of target chemokine mRNA transcripts. During inflammation, positive p38MAPK-MK2 signaling causes the phosphorylation of TTP, allowing the 14-3-3 complex to sequester and inactivate TTP. Chemokine transcription ensues, bringing about increased amounts of chemotactic signals to aid the infiltration of immune cells and to remove the source of inflammation.

In a similar genome-wide study aimed at identifying the differences in the gene expression between UC and Crohn's disease (CD), Wu *et al* found that even though UC and CD resulted to similar clinical IBD characteristics, each disease exhibited a unique and non-overlapping pattern of genomic expression (GSE6731) [249]. Using their study cohort of CD, we conducted a comparative microarray gene expression analysis of CD patients against our murine ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ colon samples (**Figure 4.2**).

Unlike UC, the gene expression and hierarchical clustering profile was very different between human CD and murine samples (**Figure 4.2a**). Gene ontology analysis demonstrates that top molecular and cellular functions include cellular movement, cellular growth and proliferation, as well as cellular function and maintenance (**Figure 4.2b**). Further inquisition identified a region of differentially expressed inflammatory genes (black box demarcated in Figure 4.2a), but primary canonical pathways that were affected were not confined to the gastrointestinal tract (**Figure 4.2c**). One conceivable explanation to this difference is the varying aetiology and manifestation of UC and CD even though they belonged to the category of IBD. UC is limited to the colon, while CD has been reported to mostly affect the ileum and the colon, as well as other regions of the gastrointestinal tract. Recent correlation studies also suggest that CD might also be hereditary, and that genetic factors (mutations in genes like *NOD2*, *IRGM*, *ATG16L1*) are also believed to be contributing to the development of CD [250, 251].

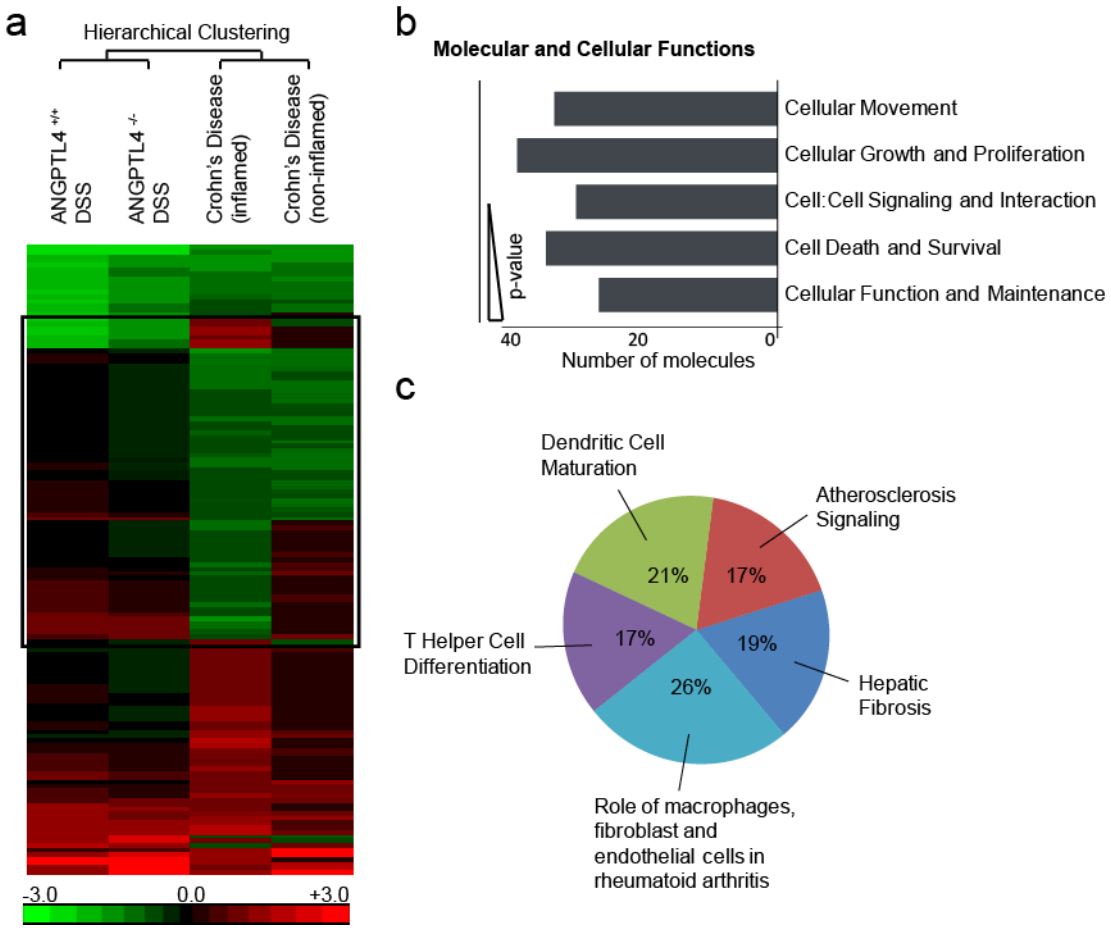

Figure 4.2 Comparative microarray gene expression analysis between colonic samples from DSS-challenged mice and human Crohn's disease. (**a**) Microarray heat map showing changes in gene expression between murine ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ mice treated with DSS, and inflamed and non-inflamed Crohn's disease (CD) biopsies (GSE6731). (**b**) The IPA database ranks genes responsible for regulating cellular movement, cellular growth and proliferation, as well as cellular maintenance amongst the most significantly varied between murine and human samples. (**c**) Inflammatory genes that were found to be differentially expressed (as demarcated by the black box in figure 4.2a), were primarily responsible for the regulation of inflammatory processes not limited to the gastrointestinal tract.

4.2 TAK1 DEFICIENCY PROMOTES PRO-INFLAMMATORY ROS LANDSCAPE FOR FIELD CANCERIZATION

As a classical theory that connects inflammation and cancer, the sustained state of chronic inflammation and irritation has frequently been correlated to drive spontaneous tumor development [238]. Epithelial cells in the chronic inflammatory environment are exposed to ROS and are at risk of accumulating unwanted DNA mutations that could further impede tumor suppressor mechanisms in place to prevent tumor formation. Under typical circumstances, the accumulation of ROS-dependent DNA damage or genomic instability would result in senescence or apoptosis via the p53 pathway [252]. However, most tumor cells are incapable of responding to the accumulation of oxidative stress levels because of defective anti-oxidant signaling pathways [253], which further leads to tumor malignancy.

In **Part II** of this thesis, we capitalized on a variety of tumorigenic cultures like A5RT3, MKN78, HSC-5 and the non-metastatic Hacat to show that epithelial to mesenchymal transition (EMT) is favored with TGFβ stimulation through the canonical Smad3 signaling axis. Interestingly, **Paper II** showed that the depletion of TAK1, a non-canonical TGFβ signaling partner, further promotes cytoskeleton remodeling and EMT. Similar to other groups, we validate a sustained production of ROS in TAK1-ablated cells [254, 255]. We further demonstrate an increase in Rac1-Nox1 activation and a corresponding decrease in RhoA activity in TAK1-depleted cells, which further elevates ROS levels in the microenvironment. An increase in Rac1-Integrin β1 interaction and an augmented expression of mesenchymal markers like vimentin, N-cadherin, fibronectin and Snai1/2 also hint at the initiation of cell migration. An increase in cell traction force was also recorded for cells depleted of TAK1. Put together, our data suggests a regulatory role of endogenousTAK1 in Rac-RhoA activation in limiting ROS production, as well as cytoskeleton remodeling and cell migration (**Figure 4.4**).

Follow-up investigations in a related publication reveal that H_2O_2 levels are positively correlated to increasing tumor aggressiveness and the intensity of diffused H_2O_2 throughout the tumor epithelial and stromal regions is amplified with increased malignancy [256]. Since the early days of cancer treatments, surgeons and oncologists alike have reported a rising trend of patients developing recurrent second primary malignancies even after the successful removal of the index tumors. As such, *Slaughter et al* first coined the term ‘field cancerization’ in 1953 when they first demonstrated histological changes in the epithelium that resided beyond the tumor boundary [257]. The heterogeneous population of cancer-associated fibroblasts (CAFs) which reside in the adjacent stroma is also believed to sustain tumor proliferation and metastasis. However, due to the complexity of defining the region beyond the tumor that possesses malignant transformations, research contributions towards field cancerization remains in its infancy. To date, it was noted that almost 30% of all cancer-related deaths are attributed to second primary malignancies and this remains an important finding in cancer treatment regimens and research.

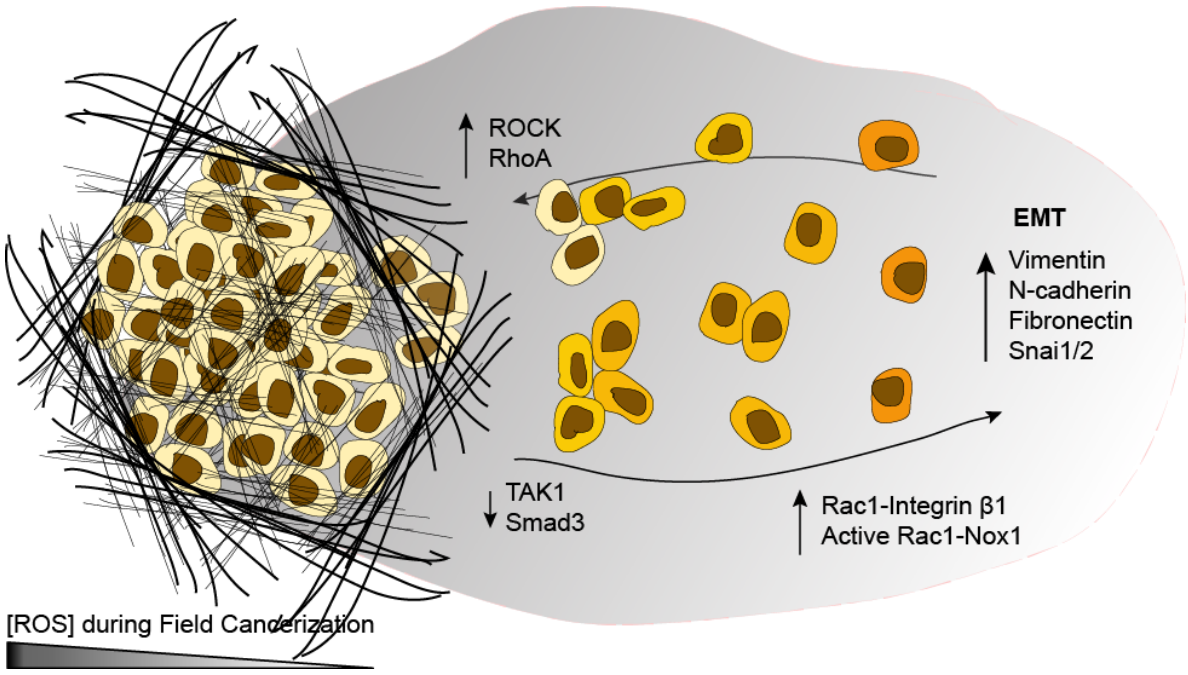

Figure 4.4 A graphical summary of the TAK1-dependent signaling cascade which promotes epithelial-to-mesenchymal transition. TAK1 activity in benign cells protects against the TGFβ-Smad3 stimulation in the tumor environment. As cells lose TAK1 expression, cells respond by increasing Rac1-Integrin β1/3 interactions, promoting increased activity of Rac1-Nox1 complex formation. This association increases the production of reactive oxygen species (ROS), which further fuels the up-regulation of inflammatory cytokines, resulting in the formation of a chronic inflammatory landscape in the tumor microenvironment. Elevated ROS levels also suppress the expression of epithelial marker E-cadherin but raise the levels of mesenchymal markers like vimentin and N-cadherin. In this respect, undergo cytoskeleton remodeling, allowing for the increase the number of focal adhesions and stress fibers to facilitate migration.

Experimental findings reveal that not only does H_2O_2 increase the invasiveness of epithelial cells, H_2O_2 also disrupts cellular homeostasis by oxidizing key signaling intermediates like PTEN, Src, JNK and EGFR. We show that fibroblasts incubated with the conditioned media of CAFs experience a similar oxidative stress with those treated with exogenous H_2O_2. Along with the reduction of Smad3 and TAK1 expression, our data show that H_2O_2–treated fibroblasts quickly become desensitized to TGFβ stimulation. Similar to our earlier findings, we demonstrate that the reduction in Smad3 and TAK1 signaling further increases ROS production and p65-NF-κB activity in fibroblasts, elevating the overall oxidative stress while promoting a pro- inflammatory microenvironment. These combined conditions not only cause cells to develop resistance to TGFβ signaling, it also primes the conversion of fibroblasts to become CAFs. Interestingly, we found that CAFs express low levels of Gpx1, rendering them incapable of detoxifying ROS and hence, allowing for the accumulation of oxidative stress. In all, our data is in agreement that ROS-induced chronic inflammation is sufficient to give a pro-inflammatory and pro-tumor microenvironment which promotes field cancerization in the epithelium.

4.3 ANGPTL4 PROMOTES TUMOR CELL MIGRATION AND EMT

In complex diseases like cancer, both the host immune system and the tumor (tumor cells and cells that make up the tumor microenvironment) play crucial roles in maintaining the dynamics that keep the cancer viable.

Huang et al show that tumor cells express high levels of ANGPTL4 and that cANGPTL4 interacts with junction proteins like claudin-5, VE-cadherin and integrin α5β1 to disrupt endothelial continuity and vascular integrity [198]. *Kubo et al* also reported that cancer cells exposed to hypoxic environments increased ANGPTL4 expression [258] while *Tanaka et al* found that ANGPTL4 increases the metastatic potential of tumorigenic cells [245]. *Zhu et al* demonstrated that ANGPTL4 levels correlated to increase ERK-AKT signaling in cancer, as well as increased E-cadherin and decreased vimentin expression [259], a feature that is characteristic of the EMT process.

To understand the medical relevance of ANGPTL4, we first characterized the expression of cANGPTL4 in human tumor biopsies. We observed that cANGPTL4 is elevated with increasing stages of cancer in a stage-dependent manner (**Figure 4.5a**). In a submitted manuscript (**Paper III**), we also demonstrated that ANGPTL4 drives the EMT process by elevating cellular energy flux through 14-3-3γ-dependent signaling cascades. We also found that ANGPTL4 phospho-activates AKT and AMPK in cancer cells during EMT and might be a possible modulator of cellular bioenergetics. Using polarized gastric carcinomas MKN74 cells, we show that the induction of EMT accompanied by an increase in Glut 1 expression occurs within 48 hours post hypoxia exposure. Interestingly, this same hypoxia exposure delayed EMT in cells with impaired ANGPTL4 signaling (treated with neutralizing cANGPTL4 antibody; α-cANGPTL4). However, stimulation with recombinant cANGPTL4 in normoxic conditions caused cells to lose their epithelial morphology and E-cadherin expression within 48 hours and was sufficient to drive cells into EMT (**Figure 4.5b-c**).

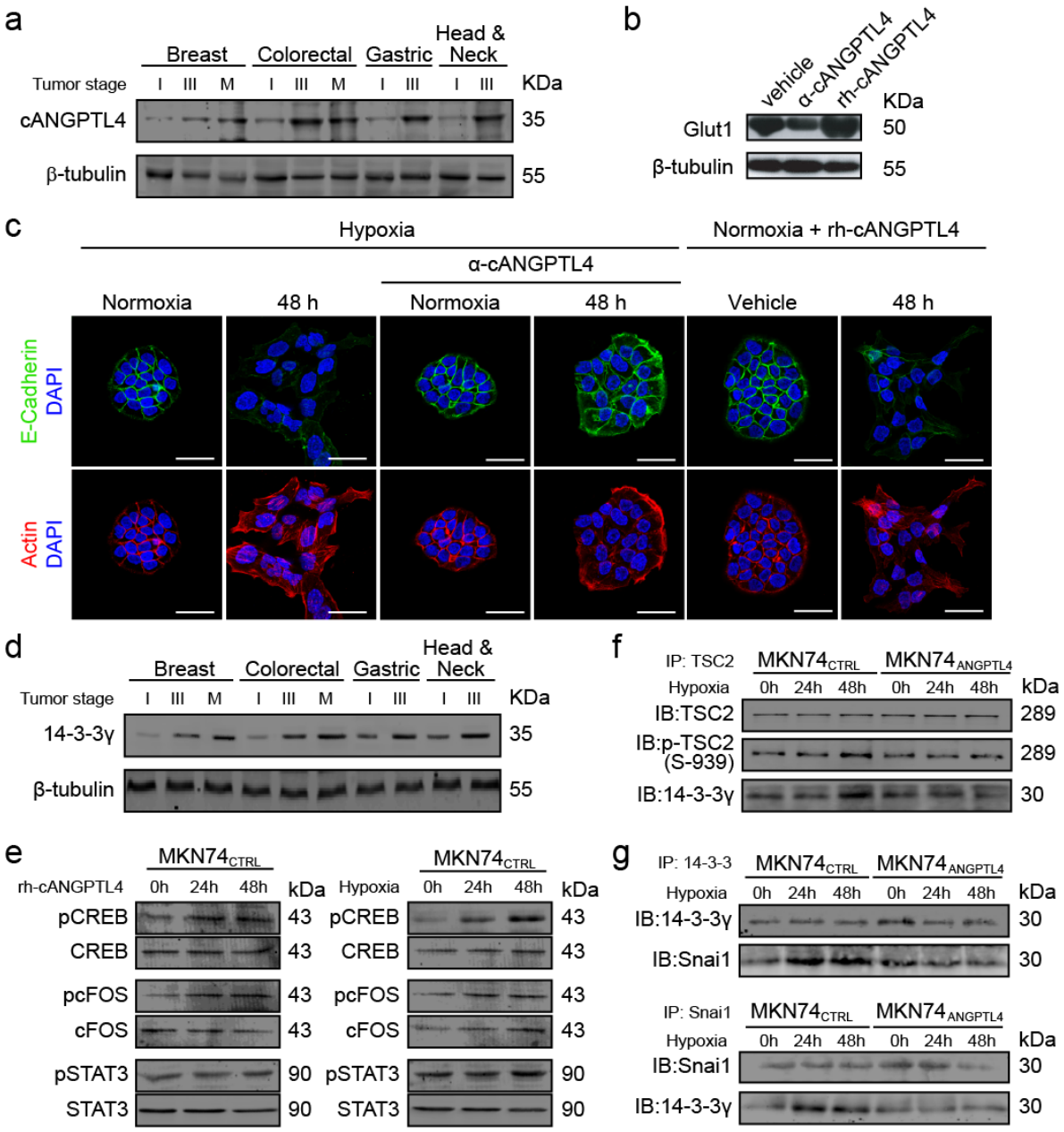

Figure 4.5 ANGPTL4 promotes cytoskeletal remodeling during epithelial to mesenchymal transition (EMT) and alters cancer cell bioenergetics. (**a-b, d**) Representative immunoblot analysis of (a) cANGPTL4 and (d) 14-3-3γ in human samples (purchased from Proteogenex, USA), of breast, colorectal, gastric and head and neck cancer samples with varying tumor stages and (b) Glut 1 in α-cANGPTL4- and rh-cANGPTL4-treated MKN74 cells. (**c**) Immunofluorescence staining of the epithelial marker E-cadherin (in green) and phalloidin for the actin cytoskeleton (in red) in MKN74 gastric carcinoma cells, counterstained with DAPI (in blue). MKN74 cells were treated with rh-cANGPTL4 in normoxia (*right panel*), in hypoxia (*middle panel*) and in hypoxia with human cANGPTL4 antibody (α-cANGPTL4; *left panel*) for 48 hours. A sharp decrease in E-cadherin expression and a remodeling of the actin cytoskeleton was observed at 48 hours with both rh-cANGPTL4 and hypoxia treatment. On the other hand, treatment with α-cANGPTL4 antibody rescued this loss and promoted EMT induction, suggesting that ANGPTL4 plays crucial roles in cytoskeletal remodeling during EMT. (**e**) Representative immunoblot analysis showing CREB/pCREB, cFOS/pcFOS and STAT3/pSTAT3 expression in rh-cANGPTL4- and hypoxia-stimulated MKN74. Immunoblot analysis showing the expression of indicated proteins after co-immunoprecipitation of (**f**) TSC2, (**g**) 14-3-3γ or Snai1 during hypoxia-induced EMT in $MKN74_{Ctrl}$ and $MKN74_{ANGPTL4}$ cells.

14-3-3 belongs to a family of highly conserved adaptor proteins and has been previously reported in regulating cell cycle proteins Chk1 [260], ATM and CDC25 [261], and also acute phase TTP during inflammation (Paper 1). In addition, we previously found that ANGPTL4 augmented 14-3-3 expression during wound healing [195, 215], suggesting that ANGPTL4 could modulate 14-3-3 during EMT. In addition to EMT-induced MKN74, we found that tumors at higher stages express elevated levels of 14-3-3γ but not other 14-3-3 isotypes (**Figure 4.5d**). Using a combination of the unbiased kinase inhibitor arrays and *in silico* analysis using the IPA software, we report that ANGPTL4 regulates 14-3-3γ transcription through transcription factors like CREB, cFOS and STAT3 (**Figure 4.5e**). The ANnotation and Integrated Analysis of the 14-3-3 interactome (ANIA) database also identified TSC2 and Snai1 as likely interaction partners of the 14-3-3 complex. To strengthen our findings, proximity ligation assays and co-immunoprecipitation experiments reveal an increase in the formation of 14-3-3γ:TSC2 and 14-3-3γ:Snai1complexes during EMT (**Figure 4.5f-g**). Similarly, we also observed significantly more 14-3-3γ:TSC2 and 14-3-3γ:Snai1complexes in aggressive human tumors biopsies as compared to their benign controls. We also demonstrate that 14-3-3γ stabilizes Snai1 binding to the E-cadherin promoter region during EMT, allowing for the transcription of the E-cadherin. Put together, our findings show that ANGPTL4 exert control over the EMT process by stabilizing key EMT proteins and meeting the increased cellular energy demands through AKT-dependent pathways. More importantly, our data underscores the significance of the ANGPTL4: 14-3-3γ signaling axis during EMT.

Interestingly, *Wang et al*, *Griseri et al* and *Young et al* recently discussed about the importance of proteins like TTP and HuR that can regulate cytokine stability play important roles in inflammation-related cancers and also in cancer progression [262-264]. In this respect, the dysregulation in cytokine stability after the initial inflammatory phase allows for a persistent feedback loop of increased inflammatory signals (chronic inflammation) and generation of ROS around the developing tumor. Since there is a need for increased nutrient supply to support the growing tumor, genes that promote angiogenesis should also be highly expressed in the vicinity.

To date, independent groups have brought forth convincing evidence to suggest that various cancers over-express immune checkpoint proteins as a method to evade immuno-surveillance [265]. Evolutionarily, the binding of PD-1 from B and T cells to its ligands PD-L1/2 negatively regulates T cell activation, muting out immune responses to prevent protracted immune reactions and tissue injury. It was found that immune cells in the pro-inflammatory tumor microenvironment do not mount any anti-cancer response because of the increased PD-1/PD-L1 signaling, further lowering $CD8^+$ T cell survival, proliferation and cytokine production [266, 267].

5 CONCLUDING PERSPECTIVES

The advancement of Science and Research is both rapid and brisk. In a short span of time, seemingly new findings can pave the way to become novel applications aimed at increasing the efficiency of diagnostics. Hence, the need to keep abreast of new findings is crucial to pilot new experiments in directions that pioneer novel work. In this thesis, we explored possible regulatory pathways of ANGPTL4 in both acute and chronic inflammation, and also examined its role in promoting EMT and tumor metastasis.

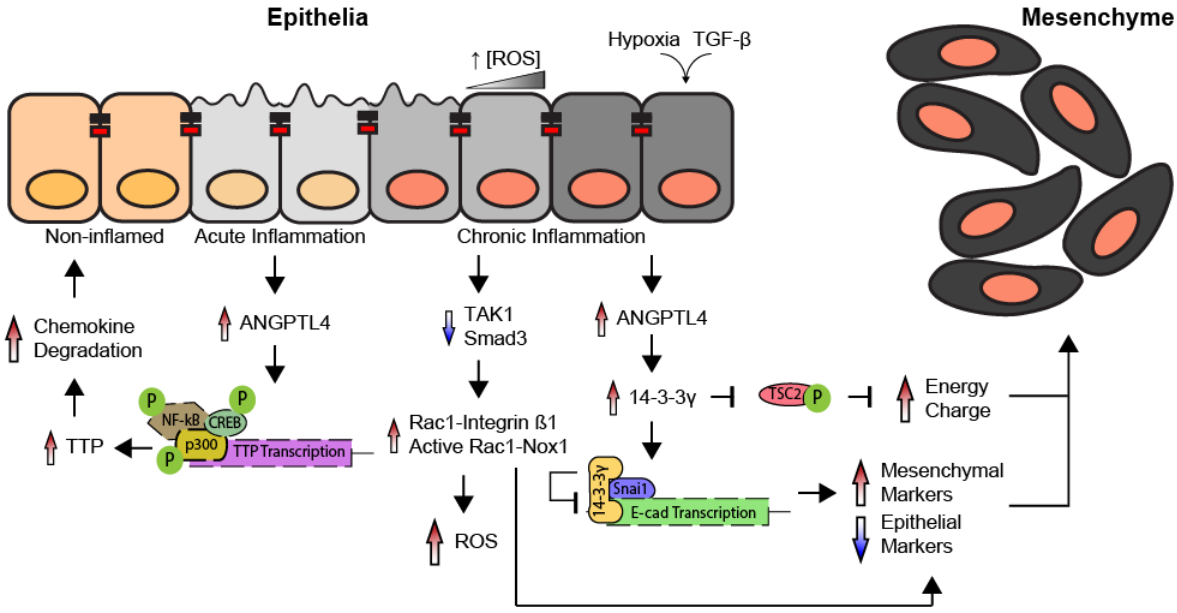

Figure 5.1 A graphical illustration showing ANGPTL4-dependent regulatory pathways during both acute and chronic inflammation. The induction of ANGPTL4 expression during acute inflammation initiates immune cell infiltration to the site of injury. Subsequently, ANGPTL4 stimulates TTP transcription, a RNA-binding intermediate which destabilizes cytokines. This quenches the local pro-inflammatory landscape and returns the tissue to its non-inflamed state. During chronic inflammation, a tightly regulated shift in ANGPTL4 and TAK1/Smad3 signaling disrupts cellular homeostasis. Cells desensitize towards the steady state of inflammation and accumulate increasing amounts of reactive oxygen species. The increase in expression of mesenchymal:epithelial markers promotes cytoskeleton remodeling and causes cells to adopt a more mesenchymal phenotype. Augmented 14-3-3γ also inhibits TSC2, accommodating for the increase in cellular energy demands during EMT.

We began by exploring the value of ANGPTL4 in regulating the immune landscape in both acute and chronic inflammatory states, leading up to cancer formation, EMT and metastasis (**Figure 5.1**). For the reason that functional ANGPTL4 exists in two forms with non-overlapping roles (dimeric or tetrameric nANGPTL4 is primarily involved in energy metabolism while monomeric cANGPTL4 has been reported to mediate angiogenesis and inflammation), ANGPTL4 has been implicated to be an ideal environmental sensor. As such, the dysregulation of ANGPTL4 has been described in literature to influence the outcome of a variety of disease models varying from cancer metastasis, atherosclerosis, metabolic syndrome and acute inflammation in association to type II diabetes.

Aberrant energy metabolism leading up to the accumulation of excess fat in the body through nutritional and hormonal imbalance have also been under scrutiny as a cause for the rising global obesity trends. Recent findings suggest apart from conferring an immunological advantage, the gastrointestinal microbial community might be responsible to modulate host energy metabolism and disease susceptibility. Metabolomics has also spearheaded efforts in identifying the plethora of microbial metabolites and their effects on diseases and health [268]. In addition, nANGPTL4 mediates both glucose and lipid metabolism, suggesting that signaling crosstalk between the host and its commensal microbial community through ANGPTL4 remains to be investigated.

Similar to the dysregulation of the inflammatory landscape during acute colonic inflammation, dietary fat like stearic acid could pose eminent health issues not limiting to inflammatory responses. Generally, common fat found in diet can be classified into saturated, unsaturated and trans fat categories. Using correlation studies, *Corwin et al* reported that saturated fat intake was inversely associated with bone mineral density [269] while *Martínez-Ramírez et al* demonstrated that increased polyunstaturated fat intake increases the predisposition of osteoporotic fractures in elderly patients [270]. However, the complexity involved in categorizing the type of dietary fat intake in human subjects complicates data collection and might explain for the lack of literature.

As a pilot study, we intend to delineate possible differences between different fat types and its effects on bone homeostasis. We explored the effects of the various C18 fat molecules: stearic acid (SA; saturated), oleic acid (OA; non-saturated) and elaidic acid (EA; trans). Using different sources of fat as a model to understand how the excess intake of fat can affect other bone homeostasis, we briefly challenged age-matched mice to 15% fat: 85% grounded chow (w/w) for 8 days and recorded for any morphological alterations on the tibia. Scanning electron micrographs reveal that the tibia of fat-challenged mice exhibited a range of depressions and grooves on the bone surface, while the bone matrix formed irregular pits when compared to mice on normal diet (**Figure 5.2a**). Preliminary findings reveal that connective tissue disorders, organismal injury and abnormalities as well as skeletal and muscular disorders are amongst the top disease functions mapped for fat challenge. Interestingly, we observed a brief increase in TNF and TGFβ1 expression in both EA and SA but not OA challenge (**Figure 5.2b**), suggesting that both an increase in trans and saturated fat intake might attribute to the chronic inflammatory phenotype associated to high fat diet observed in several population-based correlative studies. We also observed that the most significant disease associated to an increase in fat diet relates to bone homeostasis: osteoporosis, abnormal bone density and size, as well as skeletal and connective tissue morphology.

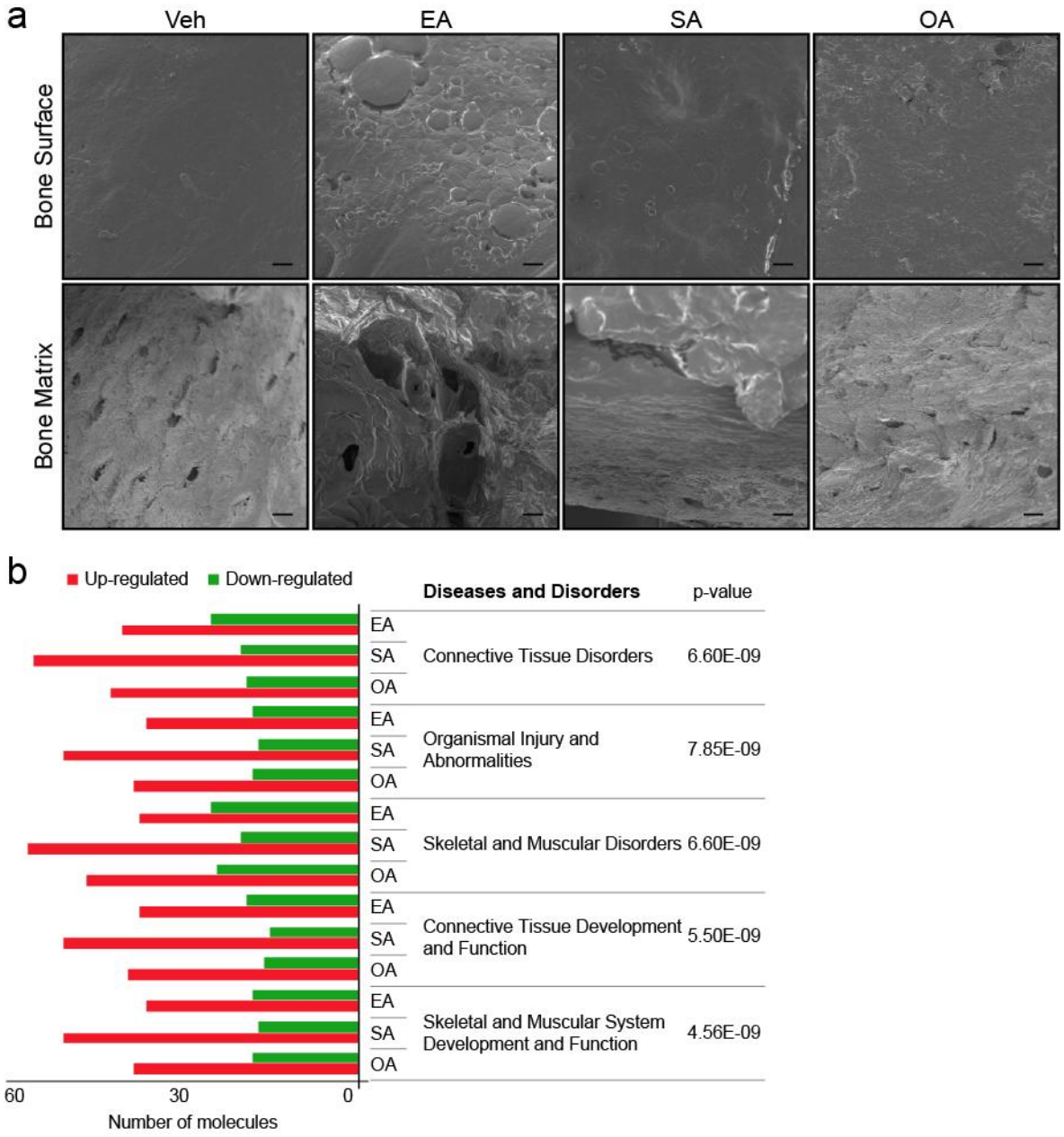

Figure 5.2 Dietary long chain fatty acid and bone homeostasis. (**a**) Scanning electron micrographs showing the surface (upper panel) and matrix of mouse tibia, isolated from age-matched mice challenged with EA, SA or OA. (**b**)Microarray gene expression analysis of colon samples of mice challenged with EA, SA or OA versus Veh. Transcripts were estimated using a $\log_2$ transformation and later subjected to unbiased ANOVA to identify gene clusters that are differentially expressed between challenge groups. Only genes that recorded fold changes <-1.2 or >1.2 were of significance. Connective tissue disorders, organismal injury and abnormalities and skeletal and muscular disorders ranks top spots in correlation to diseases commonly associated with changes in gene expression, with corresponding up-regulated or down-regulated genes in red or green. Scale bar = 10 µm.

In this respect, authors of population-based cohort studies once reasoned that bone mineral density could be a more appropriate predictor of mortality compared to other prominent markers like cholesterol and blood pressure [271, 272]. Although purely epidemiological, it was found that menopausal women were less likely to lose significant amount of bone mass and develop osteoporosis if they possessed a higher body fat mass [273, 274]. Osteoporosis is

a disease characterized by decreased bone density, mass and strength, causing skeletal fragility. Since the density of bone mass depended on a fine balance between bone formation by osteoblasts and bone resorption by osteoclasts, this observation suggests a potential homeostatic loop between the bone-adipose axis. To date, *Gomez-Ambrosi et al* suggested an active glucose homeostasis crosstalk through proteins like osteocalcin and osteopontin that are secreted by bone cells that could impact adipocyte signaling by augmenting circulating adiponectin and insulin [275, 276]. However, intimate signaling pathways within the bone-adipose axis remains to be elucidated.

Quite recently, *Shafik et al* and *Li et al* reported that ANGPTL4 exerts its effects through IL-1β and NF-κB in breast cancer [277], as well as through IL-6-Stat3 signaling in influenza [229]. However, the identities of other regulatory proteins involved in the ANGPTL4-dependent signaling axis remains to be elucidated. On the other hand, independent groups have previously characterized TTP for its role in regulating mRNA transcript stability of a subset of cytokines, and so, have extensively demonstrated that TTP acts as a molecular switch to allow cells to respond quickly to external stimuli during acute inflammation. Despite the wealth of accumulated literature generated by independent laboratories, there has not been much known between environmental sensor proteins like ANGPTL4 and an intermediate, fast-response switch like TTP that has been implicated in the plethora of diseases.

TAMs in close proximity of the pro-inflammatory tumor microenvironment are believed to play key roles in incubating the growing tumor [278, 279]. The plasticity of macrophages allows them to be further polarized into pro-inflammatory M1 or anti-inflammatory M2 states as a response to environmental stimuli [280, 281]. However, it was reported that TAMs predominantly adopt M2 polarized states [282, 283], promoting a muted immune response essential for immune-suppression around the tumor site. Interestingly, *Schumann et al* demonstrated that TAMs express high levels of ANGPTL4 [284] while *Schmid et al* suggested that patients with elevated C-reactive protein (CRP) levels had a worsened prognosis [285]. *Wu et al*, *Waschki et al* and *Tjeerdema et al* later found that ANGPTL4 share a close relationship with CRP during chronic obstructive pulmonary disease [286, 287] and type II diabetes [230]. *Hersoug et al* also reported that gut microbial-derived LPS increases chylomicron transport in the lymph circulation, promoting fat absorption and also macrophage M2 to M1 polarization [288]. However, the relationship between ANGPTL4 and macrophage polarization involved in tumor proliferation, migration and the EMT process remains largely unclear.

In conclusion, novel findings brought forth in this thesis describes the multi-faceted role of ANGPTL4, drawing links between the external environment, commensal microbiota, host metabolic and immune systems that determine the parameters for health or illness.

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