The Role of Angiopoietin-like 4 in Inflammation

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**Karolinska
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The Role of Angiopoietin-like 4 in Inflammation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Terri Phua

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 TGF β -induced EMT. TAK1 deficiency also promoted Rac1-Nox1 activation, increasing overall ROS production and augmented mesenchymal markers like Snai1 and E-cadherin. H₂O₂ augmented key signaling intermediates which are involved in cellular homeostasis and increased the invasiveness of epithelial cells. In addition, H₂O₂ also desensitized cells towards TGF β 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 14-3-3 γ . In addition, 14-3-3 γ 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. *Manuscript under revision*.

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- 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 PPAR β/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*.

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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-1 β	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	Lipopolysaccharide
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- κ B	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 dysregulation 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 microbiota, 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 dysregulation 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 environment. 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-1 β [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 deadenylation [85], initiates deadenylation 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- κ B is positively regulated by TNF- α and is considered to be a prototypical pro-inflammatory signaling pathway [92]. Functionally active NF- κ B exists as either a hetero- or homo-dimer, made up from the combination of Rel A (p65), Rel B, Rel (c-Rel), NF- κ B1 (p50 and p105 precursor) and NF- κ B2 (p52 and p100 precursor) [93]. The most abundant p65/p50 heterodimer combination is usually sequestered in its inactive state by binding to cytosolic I κ B complexes [94]. Pro-inflammatory stimuli activate the I κ B kinase (IKK) complex. Consisting of 3 catalytic subunits, IKK α , IKK β and IKK γ , IKK phosphorylates I κ B (I κ B α subunit) and targets it for degradation [95] and leading to the dissociation of the I κ B-NF- κ B inhibitory complex [96]. This unmasks the nuclear localization signal on NF- κ B, 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- κ B 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- κ B, 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 phosphatase 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 ($OH^\cdot$).

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 catalases 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). Catalases are concentrated primarily in peroxisomes that are located in proximity to mitochondria and glutathione peroxidases 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- κ B [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- κ B (NF- κ B) 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 TGF β 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- κ B 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 (18206803). 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 hemopoietic 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 dimers 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 1 α (HIF-1 α) 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-1 α 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 (TGF β), 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, β 1 and β 5 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 O₂:H₂O₂ 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-1 β , TNF- α , IFN- γ increases ANGPTL4 expression. Another independent group has also shown that the acute phase protein α 1-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 TGF β , 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 mechanisms involving the regulation of Tristetraprolin (TTP) through CREB and NF- κ B 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 Transplantation

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% NH₄Cl (w/v), 0.1 mM EDTA, pH 7.2]. Cells were filtered through a 30 μ m 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 10⁷ 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 °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 THP1-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 $\pm$ 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- κ B. 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 TGF β stimulation through the canonical Smad3 signaling axis. Interestingly, **Paper II** showed that the depletion of TAK1, a non-canonical TGF β 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 β 1 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 endogenous TAK1 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 H₂O₂ levels are positively correlated to increasing tumor aggressiveness and the intensity of diffused H₂O₂ 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 TGF β -Smad3 stimulation in the tumor environment. As cells lose TAK1 expression, cells respond by increasing Rac1-Integrin β 1/3 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 H₂O₂ increase the invasiveness of epithelial cells, H₂O₂ 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 H₂O₂. Along with the reduction of Smad3 and TAK1 expression, our data show that H₂O₂-treated fibroblasts quickly become desensitized to TGF β stimulation. Similar to our earlier findings, we demonstrate that the reduction in Smad3 and TAK1 signaling further increases ROS production and p65-NF- κ B 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 TGF β 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 $\alpha 5\beta 1$ 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 14-3-3 γ -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 14-3-3 γ 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 14-3-3 γ 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 Snail1 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 14-3-3 γ :TSC2 and 14-3-3 γ :Snail complexes during EMT (**Figure 4.5f-g**). Similarly, we also observed significantly more 14-3-3 γ :TSC2 and 14-3-3 γ :Snail complexes in aggressive human tumors biopsies as compared to their benign controls. We also demonstrate that 14-3-3 γ stabilizes Snail 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: 14-3-3 γ 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 polyunsaturated 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 TGF β 1 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 log₂ 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-1 β and NF- κ B 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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Angiopoietin-like 4 Mediates Colonic Inflammation by Regulating Chemokine Transcript Stability via Tristetraprolin

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

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

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

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

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

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

To underscore the importance of CREB and NF- κ B as active transcription factors mediating TTP expression upon c $ANGPTL4$ stimulation, we inhibited the activity of CREB, NF- κ B or both using siRNAs (iCEC- C_{RELA} , iCEC- C_{CREB} and iCEC- $C_{RELA/CREB}$) and specific kinase inhibitors (IKK-2, 666-15 and IKK-2/666-15), respectively. The highly

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

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

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

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

Discussion

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

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

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

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

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

Materials and Methods

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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Author Contributions

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

Additional Information

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

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SUPPLEMENTAL INFORMATION

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

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Supplementary Table S1. Disease Activity Index (DAI) Score.

Score	Weight Loss	Stool Consistency	Visible Blood in Feces
0	0%	None	None
1	1-5%		
2	5-10%	Loose Stool	Slight Bleeding
3	10-20%		
4	>20%	Diarrhoea	Extensive Bleeding

Supplementary Table S2. Endpoint Macroscopic/Disease Severity Score.

Score	Rectal Bleeding	Rectal Prolapse	Stool Consistency	Blood
0	None	None	Normal	Normal
1	Red	Signs of prolapse	Soft	Red
2	Dark Red	Clear prolapse	Very Soft	Dark Red
3	Gross Bleeding	Extensive Prolapse	Diarrhea	Black

Supplementary Table S3. Histological Scoring for Degree of Epithelial Damage and Inflammatory Infiltration.

Score	Inflammation Severity	Crypt Damage	Ulceration
0	Rare inflammatory cells in the lamina propria	Intact crypts	absence of ulcer
1	Increased numbers of granulocytes in the lamina propria	Loss of the basal one-third	<2 foci of ulcerations
2	Confluence of inflammatory cells extending into the submucosa	Loss of the basal two-thirds	< 4 foci of ulcerations
3	Transmural extension of the inflammatory infiltrate	Loss of entire crypts	confluent or extensive ulceration
4	-	Change of epithelial surface with erosion	-
5	-	Confluent erosion	-

Supplementary Table S4. List of Differentially Expressed Genes and their respective expression values in association with Gastrointestinal Diseases.

Gene Symbol	Vehicle	SA ANGPTL4 ^{+/+}	SA ANGPTL4 ^{-/-}	DSS ANGPTL4 ^{+/+}	DSS ANGPTL4 ^{-/-}
RETNLB	-26.6188	-1.25321	4.64282	-5.39947	10.1949
SIRT6	1.58671	-1.30123	-5.54798	1.60477	-1.20498
IL17A	1.12074	-1.11832	-1.05405	-2.055	-1.7773
DRD4	1.05236	-1.5765	-1.24962	-1.32943	-1.56154
PON1	1.97514	-1.10116	-1.09437	-1.09882	-2.90767
FCER1A	1.34889	-1.07422	1.1706	-1.21276	-1.70433
NFE2L2	-2.7691	-1.35215	-1.38063	-3.7374	-1.14798
B3GNT6	-2.10196	-1.1442	-1.17655	-3.1278	-1.60115
NR5A2	-1.5738	-2.0245	-1.36879	-1.56857	-1.40889
PROC	-1.25318	-1.64026	-1.35534	-1.62156	-1.60218
TRPV1	-1.37549	-1.16311	-1.6602	-1.98386	-1.36789
CEBPE	-1.19974	-1.05284	-1.12961	-2.07957	-1.43371
SERPINE1	-1.58514	-1.41746	-1.25299	-2.10557	-1.033
CORT	-1.60273	-1.012	-1.09575	-2.01711	-1.25602
MST1	-1.75233	-1.05904	-1.10075	-1.80311	-1.07072
IL25	-1.44409	-1.04595	-1.31383	-1.68468	-1.36804
FUT7	-1.43967	-1.08146	-1.41597	-1.40863	-1.20905
AGR2	-1.31688	-1.4246	-1.33041	-1.28983	-1.07755
IL13	-1.17519	-1.06607	-1.11831	-1.40748	-1.19772
IL23A	-1.22906	-1.08807	-1.1708	-1.09771	-1.12181
HPGD	-1.02934	-1.1409	-1.88522	-1.08238	-1.73129
PDE4C	-1.16904	1.02033	-1.2904	-1.76682	-1.44068
JAK3	-1.20285	1.20139	-1.38017	-1.28041	-1.38338
CRLF2	-1.00953	1.04459	-1.32036	-1.12094	-1.16585
APOA4	-1.04016	1.30988	-1.11326	-1.42492	-1.2794
FKBP1A	-2.19355	-1.54415	-1.42833	-3.03289	1.02716
ID3	-1.38537	-1.40079	-2.20764	-3.39579	1.23223
HSF1	-1.497	-2.26297	-2.13899	-1.46727	1.03598
PTPN11	-1.51045	-1.70983	-2.33757	-1.19645	1.20427
NFKB1	-1.1295	-2.02506	-1.8885	-1.02364	1.25495
MTOR	-2.00182	-2.26367	-1.091	-1.5921	1.26378
VEGFA	-2.17882	-1.97848	-1.38317	-1.38931	2.03393
PTGS1	-1.97047	-2.0465	-1.04333	-1.08905	1.63136
MERTK	-1.85714	-1.71701	-1.57332	-1.01989	1.58254
JAK1	-1.82148	-1.4327	-1.75069	-1.1433	1.66069
RAC1	-1.93969	-1.27498	-1.0857	-1.77756	1.13242

PPP3CA	-1.71001	-1.72684	-1.39265	-1.30021	1.03945
TRAF3IP2	-1.46481	-1.51732	-1.75997	-1.591	1.1241
Map3k7	-1.54306	-1.46731	-1.6109	-1.24003	1.14156
LTB	-1.29939	-1.67018	-1.19537	-1.75935	1.02987
ITGB3	-1.4162	-1.8556	-1.17229	-1.46268	1.24162
NLRX1	-1.27339	-1.30732	-1.34163	-1.63348	1.09034
CD40	-1.41569	-1.13296	-1.38161	-1.34019	1.04531
SELE	-1.30216	-1.02828	-1.1178	-1.45899	1.0242
GCNT3	-1.7527	-1.08844	-1.54661	-1.02564	1.39333
HSP90B1	-1.45019	-1.01837	-1.28478	-1.07022	1.31761
STAP2	-1.04524	-1.82845	-1.04225	-1.01008	1.15859
TLR9	-1.11351	-1.64595	-1.14105	-1.02772	1.39376
ANXA1	-2.2405	1.02251	-2.68851	-2.00896	1.84666
S1PR4	-1.79524	1.0563	-1.00132	-1.68558	1.04522
SOD1	-1.89037	1.60346	-1.31383	-1.38129	1.06448
PROCR	-1.16328	1.14168	-1.69279	-1.25741	1.05706
IL27RA	-1.19545	1.06105	-1.13638	-1.23417	1.06253
CD44	-1.1668	1.1498	-1.14438	-1.27485	1.06489
STAT5A	-1.1369	1.22899	-1.21412	-1.11786	1.1354
NKX2-3	-1.2083	1.07732	-1.03985	-1.09584	1.34038
RAG1	-2.00338	-1.48789	2.14076	-2.07728	-1.06656
ALOX12	-1.47344	-1.67266	1.6672	-2.24655	-1.49486
CYP11B1	-1.60169	-1.54329	1.16741	-2.14192	-1.13875
SOCS1	-1.38137	-1.26633	1.28739	-2.55959	-1.19905
CALCA	-1.36846	-1.08872	1.32864	-2.02409	-1.60363
TBX21	-1.69255	-1.10917	1.44342	-1.6745	-1.27891
PLG	-1.25363	-1.04103	1.26468	-1.61668	-1.19262
IL21	-1.25166	-1.04097	1.44289	-1.79289	-1.08921
PGLYRP2	-1.29207	-1.02418	1.49765	-1.38411	-1.04748
DRD2	-1.28427	-1.33718	1.11135	-1.3641	-1.22718
BID	-1.08705	-1.3221	1.10094	-1.13724	-1.07721
PDE4A	-1.11174	-1.10246	1.09427	-1.22686	-1.06803
DRD3	-2.09993	-1.45169	1.08058	-2.07108	1.14421
ALOX5	-1.75646	-1.75883	1.21825	-1.69646	1.04325
RXRA	-1.41533	-1.99749	1.505	-1.41533	1.06201
POR	-1.56913	-2.089	1.2376	-1.03348	1.50171
CRP	-1.59623	-1.34974	1.49501	-1.73895	1.05988
BATF	-1.61161	-1.16806	1.60481	-1.7622	1.08132
TKTL1	-1.39718	-1.24981	1.60785	-1.82859	1.00825
ADORA2A	-1.57238	-1.25024	1.69087	-1.44974	1.0619
EBI3	-1.48586	-1.12135	1.48807	-1.45179	1.25108

IL2	-1.22685	-1.00257	1.75146	-1.39365	1.13582
NOD2	-1.38484	-1.44373	1.36766	-1.38311	1.07226
CDH1	-1.55877	-1.77433	1.40625	-1.20528	1.3836
NFKBID	-1.68562	-1.54046	1.52507	-1.36841	1.37383
NOS3	-1.47938	-1.53841	1.57869	-1.29937	1.28667
HTR7	-1.39511	-1.40025	1.58485	-1.22475	1.6514
RUNX3	-1.13678	-1.35953	1.43174	-1.08961	1.37892
TREX1	-1.16251	-1.00275	1.22233	-1.26651	1.11976
RASGRP4	-1.50867	-1.11397	1.06686	-1.09975	1.58407
IL1R1	-1.63231	-1.07927	1.17933	-1.192	2.81466
NFIL3	-2.60854	-2.43028	1.46341	-1.39627	3.44323
CKM	-2.21212	1.46795	1.84386	-3.88946	-1.28993
MEP1A	-1.18545	1.26504	1.62529	-1.46591	-2.99943
BSN	-1.65624	1.13292	1.29964	-1.86133	-1.05234
DRD5	-1.27351	1.0402	1.47924	-1.76156	-1.1581
MPO	-1.14963	1.24997	1.13302	-1.67043	-1.31289
IFNG	-1.0258	1.09285	1.19431	-1.12442	-1.07517
CD40LG	-1.11526	1.1636	1.47449	-1.28332	-1.16061
PGLYRP3	-1.20701	1.11443	1.59727	-1.29865	-1.11216
ESR2	-1.21616	1.0409	1.61505	-1.21272	-1.02877
CSF2	-1.06841	1.49153	1.8323	-1.14348	-1.20635
TRPM8	-1.06825	1.75878	1.14576	-1.12163	-1.39738
IL4	-1.14174	1.10666	2.22296	-1.43256	-1.19431
CYP11A1	-1.22238	1.25683	2.51989	-1.47142	-1.06899
ALB	1.19747	1.04177	1.18117	-1.06722	-1.63433
IL24	1.01303	1.5918	1.67301	-1.06248	-1.12344
MME	1.02132	1.41249	2.52821	-1.30279	-1.28318
PPARG	-1.94427	1.51837	1.24371	-1.7641	1.19287
CALCB	-1.89205	1.41566	1.70846	-2.05855	1.11461
IL12A	-1.60121	1.01815	2.12528	-1.93616	1.09617
IL22	-1.17297	1.00585	1.51543	-1.63395	1.97593
MMP7	-1.65341	1.23486	1.65503	-1.25154	1.23674
SIGIRR	-1.18122	1.05917	1.33369	-1.22699	1.08792
IL23R	-1.29396	1.13352	1.48381	-1.05412	1.35519
WAS	-1.0857	1.09616	1.59823	-1.11237	1.45503
ITGB7	-1.24647	1.10803	1.92843	-1.06249	1.23373
Ccl2	-1.66945	1.2296	2.056	-1.10835	1.90177
Ccl2	-1.66945	1.2296	2.056	-1.10835	1.90177
CR2	-1.08288	1.95694	1.57529	-1.23283	1.69063
PGLYRP4	-1.74188	1.53334	2.39859	-1.5389	1.15389
PDCD1	-1.68359	1.24356	2.84018	-1.64444	1.21547

IL10	-1.46245	1.14761	2.88133	-1.18316	1.54387
STAT6	-1.30016	-2.57358	-1.84793	1.12096	1.37862
ITGA1	-1.40833	-1.4551	-2.3596	1.1914	1.48545
IRF3	-1.06951	-1.28953	-1.75937	1.01439	1.22236
AHR	-1.04713	-1.38696	-1.58564	1.15732	1.14741
STX2	-1.08302	-1.71137	-1.69874	1.12409	1.64993
DDX58	-1.38222	-1.16978	-1.15213	1.01099	1.36929
ATG16L1	-1.06252	-1.35452	-1.10566	1.06644	1.28045
PDE4B	-1.481	-1.30477	-1.15957	1.08216	1.7176
PIK3CD	-1.19695	-1.36932	-1.21022	1.01904	1.8112
ICAM1	-1.12922	-1.41927	-1.18683	1.19976	2.0483
IL1RN	-1.10143	-1.38788	-1.03174	1.7474	1.69256
C3	-1.68126	-1.97542	-2.49761	2.31674	2.7554
TNFRSF1A	-1.09713	-1.66177	-1.80691	1.92805	2.5234
IL6ST	-1.08629	-1.31998	-1.18184	2.11966	2.62532
STAT1	-1.03316	-1.22273	-1.21251	1.90765	3.25507
CD274	-1.04028	-1.99381	-1.10074	2.18924	3.89993
PTGS2	-1.97925	-1.3019	-1.54534	1.58974	4.45818
NLRP6	1.17997	-1.78366	-2.15082	1.3403	-1.09509
RAC3	1.16945	-1.09155	-1.31408	1.03373	-1.16945
FAAH	1.31787	-1.07043	-1.47256	1.29516	-1.05414
SMAD7	1.61498	-1.29873	-1.02701	1.1959	-1.35974
PHB	2.28424	-1.07405	-1.70313	1.49525	-1.45846
TPMT	2.15391	-1.01194	-1.96843	1.72489	-1.17363
PYCARD	2.92642	-1.10603	-1.82476	3.20785	-1.3755
EDN1	1.29646	-2.51659	-2.00331	1.71405	1.48887
XBP1	1.94201	-2.65388	-1.46594	1.74532	1.12364
GPX2	2.12319	-2.67529	-1.42045	1.02146	2.02904
TRAF2	1.18797	-1.64956	-1.77769	1.10533	1.13745
GPX1	1.28542	-1.56572	-1.75153	1.40496	1.2074
ABCC4	1.2224	-1.19904	-1.42674	1.52685	1.4991
RPTOR	1.03235	-2.00154	-1.22256	1.36818	1.31137
TGFB1	1.33674	-1.74343	-1.10928	1.44106	1.70122
AKT1	2.31563	-1.78267	-1.29387	1.61879	1.05119
LGALS4	1.76501	-1.48001	-1.0853	1.62791	1.01459
STAT5B	1.62087	-1.18976	-1.4971	1.78372	1.21549
LTBR	1.90908	-1.35937	-1.27052	1.9874	1.17344
GTPBP1	2.08531	-1.053	-1.25931	2.38207	1.00968
TFF3	1.54439	-1.16569	-1.34886	2.37195	1.59218
COL1A1	1.42936	-1.89733	-1.61133	1.91599	1.92556
STAT3	1.24422	-1.49867	-1.6117	2.07328	2.01191

TGFB2	1.55764	-1.73728	-1.04745	2.22275	2.09301
COL1A2	1.54003	-1.29655	-1.47134	2.26325	2.42547
CFB	1.22193	-1.75355	-1.0137	2.85426	2.60359
HPGDS	1.29833	-1.29193	-1.19181	3.53669	2.42167
FAS	1.02361	-1.04475	-1.56447	2.99063	3.01426
CSF1R	2.14256	-1.35453	-1.52388	3.51295	2.35429
MYD88	2.3735	-2.78234	-1.44625	3.7431	1.91635
IL10RB	2.64618	-2.85961	-1.09453	4.06753	1.30899
KRAS	-1.0818	3.44966	-3.25919	1.50156	1.17326
TLR2	-1.23749	1.08891	-1.10471	1.14635	1.57774
DDIT3	-1.13516	1.52218	-1.08529	1.1533	1.31871
TLR1	-1.07657	1.60355	-1.16999	1.22882	1.47395
NOD1	-1.37543	1.55792	-1.02677	1.12507	1.75135
B2M	-1.01352	1.28174	-1.24845	1.89814	1.74976
HSF2	-1.03352	2.49994	-1.14628	1.53628	1.31122
TLR4	-1.05335	1.81016	-2.31818	1.5363	1.98114
CCL11	-1.58055	1.73723	-1.40988	1.38563	2.74515
CD47	-1.28137	3.82436	-1.25429	2.60584	3.05922
ATM	1.04759	1.01685	-1.99078	1.49357	1.14115
TNFAIP8	1.26517	1.04402	-1.93902	1.659	1.50925
TNFAIP3	1.29807	1.14978	-1.88753	1.63354	1.9782
SMAD3	1.02789	1.0188	-1.13196	1.40517	1.71746
F2RL1	1.02679	1.69078	-1.18163	1.69445	1.64769
ITGA4	1.61911	1.32338	-2.40836	2.68561	1.71311
AXL	1.51246	1.20566	-1.80288	2.48566	1.70236
JAK2	1.35359	1.35254	-1.61361	2.55728	1.89524
PROS1	1.43364	1.26306	-1.85696	2.73403	2.53528
IL7	1.20265	1.76105	-1.00331	2.49812	1.00466
CASP1	1.63288	2.53568	-2.15564	2.0891	1.18771
ATP11C	1.07495	2.92215	-1.58812	2.46417	1.70011
CR1L	1.65646	3.71986	-2.00429	2.71468	1.23355
CKB	2.17095	1.1382	-4.07999	3.04446	1.17899
CRBN	2.00387	1.73112	-3.1418	2.80311	1.05185
IL18	2.48296	1.24017	-2.76151	3.95895	1.49787
ENTPD1	2.49697	1.44078	-3.29715	3.97206	2.28863
LY96	1.73233	1.36609	-2.34586	4.86187	1.66274
ID2	3.41051	2.32825	-1.31236	3.82715	1.39019
VDR	-1.16707	-2.39178	1.50576	1.04406	-1.15479
FERMT1	-1.03852	-1.25715	1.15029	1.2737	-1.19526
ERN2	1.17839	-2.01899	1.18919	1.8399	-1.03618
IL22RA2	1.41036	1.35181	1.52681	1.38398	-1.23789

IFNB1	1.31756	1.01373	1.97103	1.33854	-1.14159
PIP	1.15697	1.43735	2.11715	1.03299	-1.10134
NRG4	1.1667	2.00222	2.2079	1.20857	-1.51717
AKT2	-1.4754	-2.8049	1.17837	1.06033	1.80109
CEACAM1	-1.23378	-1.40504	1.09442	1.10696	1.27983
PIK3R1	-1.20881	-1.03504	1.351	1.07032	1.20972
PLS1	-1.2801	-1.15342	1.30416	1.10937	1.55576
F10	-1.3612	-1.02499	1.34582	1.10198	1.51719
IL2RA	-1.1629	-1.20627	1.67881	1.23773	1.56122
PIK3CG	-1.00657	-1.70243	1.38037	1.5127	1.54673
CTLA4	-1.44901	-1.27595	2.04779	1.06545	1.963
DMBT1	-1.63303	-1.23155	2.16134	1.94448	3.30078
IL6	-1.2921	-1.20194	3.77616	1.23142	2.70156
TNFRSF11B	-1.20234	1.13038	1.21879	1.05709	1.29307
NR3C1	-1.21579	1.23663	1.00196	1.1518	1.34884
TLR3	-1.18448	1.20297	1.11373	1.31416	1.30573
PPP3CC	-1.33787	1.26169	1.33565	1.3808	1.33199
ALOX15	-1.03146	1.24802	1.35281	1.58157	1.11082
IL12B	-1.12705	1.0792	1.79723	1.16114	1.27572
CD80	-1.07047	1.1673	1.79182	1.16824	1.36869
ADAMTS12	-1.52372	1.08295	1.54216	1.05914	1.79677
IL13RA2	-1.02982	1.31415	2.24483	1.29808	1.95026
CXCL10	-1.23354	1.43813	1.79403	1.48444	4.08562
NLRP3	-1.19481	1.35447	2.30694	2.3255	2.83283
TYK2	1.24185	-1.353	1.06705	1.26122	1.11137
KSR1	1.14312	-1.4129	1.17775	1.55673	1.70665
NCF1	1.10163	-1.08527	1.1492	1.73512	2.03481
CD69	1.00405	-1.07282	1.57186	1.68661	2.55396
GNAI2	1.68937	-1.43281	1.13918	2.09806	1.35224
GNAI2	1.68937	-1.43281	1.13918	2.09806	1.35224
TNFRSF14	1.55123	-1.09474	1.02392	2.03333	1.78266
RELA	2.11036	-1.34066	1.52934	1.90412	1.11825
GUCY2C	2.07913	-1.53966	1.53528	2.26251	1.00212
RORC	1.70711	-1.28754	1.04349	2.94119	1.25254
FGFR2	1.28582	-1.16658	1.00974	3.01045	1.67707
ADM	1.69298	-1.10755	1.52419	2.49359	3.00715
CCR5	1.0213	-1.04346	1.30101	3.47081	3.6519
ITGB8	1.10243	1.44749	1.17829	1.18371	1.61066
SPP1	1.19356	1.26573	1.00382	2.12677	1.28588
MMP9	1.29794	1.08506	1.0841	2.15277	1.39096
PDE4D	1.53377	1.3404	1.2399	2.63356	1.15824

PPP3CB	1.55466	1.80873	1.10411	2.19592	1.04349
IL10RA	1.1248	1.07662	1.54193	1.7779	2.41555
HSD11B1	1.20162	1.1637	1.63652	1.7798	2.88012
NCF4	1.29552	1.30862	1.2629	2.04625	2.53647
STAT4	1.31313	1.04788	1.93287	2.08256	2.64075
CXCL3	1.14316	1.22465	1.99026	2.38905	2.5253
NR1H4	1.12738	1.33626	1.15467	2.67755	2.05239
S1PR1	1.38752	1.47645	1.02028	3.09947	2.22284
PTPN22	1.52082	1.18684	1.51551	3.54386	1.90429
CYLD	1.22994	3.13735	1.10372	2.35591	1.79937
MC2R	-1.18598	1.82783	4.86515	1.13151	-1.13127
RAG2	-1.06394	1.19881	4.98807	1.13033	1.0224
SLPI	-2.12001	-1.70489	1.64073	1.49557	5.57916
CXCL5	-1.23289	-1.12782	1.27556	1.44991	6.04236
TNF	-1.71711	1.07873	1.93168	1.62914	6.46805
SELP	-1.27778	1.02891	2.10372	1.9938	5.77376
CFH	-1.15477	1.50003	-2.25178	3.5769	5.69614
TREM1	-1.44136	1.15221	1.05275	4.79722	5.511
HGF	1.17141	1.34558	-1.22794	3.07991	4.4313
EREG	1.48455	1.54003	1.10689	3.71949	4.70649
Retnla	-1.00485	1.75934	-1.41336	7.45647	3.99548
CXCL2	-1.04405	1.49699	1.72401	6.70428	9.86742
CCR2	1.19437	2.44401	2.00809	7.26107	9.03643
NFKBIA	3.49968	-1.02344	1.01129	7.3258	2.67856
SOCS3	-1.8566	-1.51803	1.91034	1.81516	11.9349
LTF	-1.7211	-1.04272	1.86351	4.26719	13.3968
LBP	-1.6013	-1.37887	-1.81481	3.6804	17.7245
NOS2	-1.65396	-1.38193	1.33736	3.74231	18.9493
S100A8	-1.26127	1.06691	1.46931	6.76554	17.1899
LCN2	1.31501	-1.39367	-1.23284	18.5878	12.1224
MUC1	4.22658	1.68306	1.1663	40.5054	6.60718
IL1B	-1.22156	1.34929	1.77778	23.5381	36.4379
IGHM	60.7957	-1.16806	-1.71707	3.82298	-11.3306

Supplementary Table S5. List of Differentially Expressed Genes and their respective expression values in association with Gastrointestinal Diseases in comparison between murine and human samples.

Gene Symbol	ANGPTL4 ^{+/+} DSS	Ulcerative Colitis (non-inflamed)	ANGPTL4 ^{-/-} DSS	Ulcerative Colitis (inflamed)
CKB	2.17095	3.04446	1.17899	-1.88829
FAS	1.02361	2.99063	3.01426	-1.54032
RORC	1.70711	2.94119	1.25254	-1.51346
GUCY2C	2.07913	2.26251	1.00212	-1.44301
PIK3R1	-1.20881	1.07032	1.20972	-1.4242
EDN1	1.29646	1.71405	1.48887	-1.38378
ERN2	1.17839	1.8399	-1.03618	-1.23377
FAAH	1.31787	1.29516	-1.05414	-1.22826
EREG	1.48455	3.71949	4.70649	-1.12482
VDR	-1.16707	1.04406	-1.15479	-1.08755
ESR2	-1.21616	-1.21272	-1.02877	1.09874
ITGB3	-1.4162	-1.46268	1.24162	1.15755
JAK1	-1.82148	-1.1433	1.66069	1.17046
IL17A	1.12074	-2.055	-1.7773	1.20362
WAS	-1.0857	-1.11237	1.45503	1.22069
BID	-1.08705	-1.13724	-1.07721	1.23141
VEGFA	-2.17882	-1.38931	2.03393	1.25006
ALOX5	-1.75646	-1.69646	1.04325	1.2522
CD44	-1.1668	-1.27485	1.06489	1.25621
IL1R1	-1.63231	-1.192	2.81466	1.25621
CD40	-1.41569	-1.34019	1.04531	1.31529
ANXA1	-2.2405	-2.00896	1.84666	1.31543
HSP90B1	-1.45019	-1.07022	1.31761	1.34164
ITGB7	-1.24647	-1.06249	1.23373	1.36925
SOD1	-1.89037	-1.38129	1.06448	1.46972
MERTK	-1.85714	-1.01989	1.58254	1.50204
SOCS1	-1.38137	-2.55959	-1.19905	1.55949
PROCR	-1.16328	-1.25741	1.05706	1.59804
CCL2	-1.66945	-1.10835	1.90177	2.21867

Supplementary Table S7. Fold change of chemokine concentration in iCECs when treated with various pro- and anti-inflammatory stimuli.

	Treatments	Veh		DSS		SA		IL-1 β		TNF- α		HC		NaBu	
	Genes	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
iCEC _{Ctrl}	CXCL10	1	0	1.83	0	1.71	0	1.02	0	2.46	0	1.19	0	0.41	0
	CCL2	1	0.07	1.2	0.08	1.07	0.16	0.95	0.02	1.06	0.04	1.03	0.02	0.8	0.16
	CCL11	1	0.09	2.81	0	1.59	0.27	1.4	0.26	2.9	0	1.58	0.2	0.61	0.09
	IL-1 β	1	0.08	1.34	0.18	1.74	0.02	-	-	1.37	0.39	0.78	0.08	0.65	0.16
	IL-10	1	0.58	2.45	0.05	0.51	0.35	0.07	0.06	0.75	0.69	1.39	0.12	2.52	0.49
	IL-17	1	0.45	0.55	0.3	0.68	0.08	0.48	0.1	0.38	0.14	1.79	1.17	6.01	3.83
iCEC _{TTP}	CXCL10	1	0.28	1.55	0.62	1.7	0.96	1.59	0.58	2.65	0	1.37	0.97	1.28	0.89
	CCL2	1	0.02	1	0.02	1.11	0.04	0.95	0.1	1.22	0.12	0.88	0.16	0.91	0.14
	CCL11	1	0.22	3.33	0.56	2.31	0.62	3.1	0.79	3.33	0.56	1.6	0.07	1.37	0.9
	IL-1 β	1	0.09	1.14	0.15	1.61	0.38	-	-	2.03	0.44	0.75	0.32	0.72	0.22
	IL-10	1	0.43	3.44	0.1	1.21	0.08	0.23	0.18	4.1	0.73	1.01	0.81	6.16	0.89
	IL-17	1	0.69	1.52	0.91	1.2	0.99	0.94	0.02	2.63	0.25	0.46	0.05	8.05	0.15
iCEC _{ANGPTL4}	CXCL10	1	0.35	3.71	0.34	2.68	0.16	2.73	1.6	3.08	0.97	0.85	0.23	0.7	0.22
	CCL2	1	0.05	1.14	0.04	1.37	0.01	0.97	0.02	1.24	0.23	1.08	0.19	0.89	0.12
	CCL11	1	0.41	4.09	0	4.09	0	2.14	0.09	2.66	0.43	2.3	0.79	0.45	0.07
	IL-1 β	1	0.2	1.76	0.1	1.68	0.15	-	-	2.51	0.14	1.63	0.92	0.83	0.1
	IL-10	1	0.29	1.89	0.8	4.77	0.93	0.11	0.07	3.06	0.88	0.97	0.55	0.41	0.2
	IL-17	1	0.67	0.76	0.36	7.6	0.82	0.71	0.29	2.01	0.63	0.33	0.09	0.44	0.18

Supplementary Table S8. Sequences of human ON-TARGETplus SMARTPool siRNA targets for ANGPTL4-, CREB-, RELA- or TTP-knockdown.

Gene Symbol	Species	siRNA smartpool target sequences (5' to 3')
ANGPTL4	Human	GAUGGAGGCUGGACAGUAA
		CCACUUGGGACCAGGAUCA
		GAAAGAGGCUGCCCGAGAU
		GGCAGAAGCUUAAGAAGGG
CREB1	Human	GAGAGAGGUCCGUCUAAUG
		UAGUACAGCUGCCCAUUGG
		CAACUCCAAUUUACCAAAC
		GCCCAGCCAUCAGUUUUAUC
RELA	Human	GGAUUGAGGAGAAACGUAA
		CCCACGAGCUUGUAGGAAA
		GGCUAUAACUCGCCUAGUG
		CCACACAACUGAGCCCAUG
TTP	Human	GCAAGUAGCCAAAGCCGUU
		CCCAAUACAAGACGGAAC
		GGACAGUAAUCAAGUAAUC
		GCAUAUUUAAGGGAGGCAA

Supplementary Table S9. Sequences of real-time PCR primers used in this study.

Gene Symbol	Species	Gene ID	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
18S	Human/ Mouse	19791	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG
ANGPTL4	Human	51129	TGGTTTGGCACCTGCAGCCATTC	TGCTGCCATGGGCTGGATCAAC
ANGPTL4	Mouse	57875	CCCCACGCACCTAGACAATG	GCCTCCATCTGAAGTCATCTCA
CCL2	Human	6347	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
CCL2	Mouse	20296	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
CCL11	Human	6356	CCCCTTCAGCGACTAGAGAG	TCTTGGGGTCGGCACAGAT
CCL11	Mouse	20292	GAATCACCAACAACAGATGCAC	ATCCTGGACCCACTTCTTCTT
CREB1	Human	1385	TTAACCATGACCAATGCAGCA	TGGTATGTTTGTACGTCTCCAGA
CXCL10	Human	3627	GTGGCATTCAAGGAGTACCTC	TGATGGCCTTCGATTCTGGATT
CXCL10	Mouse	15945	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA
IL-1 β	Human	3553	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
IL-1 β	Mouse	16176	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
IL-6	Mouse	16193	TCTATACCACTTCACAAGTCGGA	GAATTGCCATTGCACAACCTTTT
IL-10	Human	3586	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG
IL-10	Mouse	16153	CTTACTGACTGGCATGAGGATCA	GCAGCTCTAGGAGCATGTGG
IL-17	Human	3605	TCCCACGAAATCCAGGATGC	GGATGTTCAAGTTGACCATCAC
IL-17	Mouse	16171	CGGAGAATTAGTCCCTGTGTTG	GAACAGTCACTTCATACTCCTGG
IFN- γ	Mouse	15978	ACAGCAAGGCGAAAAAGGATG	TGGTGGACCACTCGGATGA
pCREB/pNF- κ B Binding Site on TTP	Human	-	TGCAAGTGGAAGTCGGAG	TGGAAGTCCGGATTGCTTC
pCREB/pNF- κ B Negative Control on TTP	Human	-	CCAGACAGAGGGAAGTCAAG	GAGCATGCAGTGTGCACCAG
RELA	Human	5970	GTGGGGACTACGACCTGAATG	GGGGCACGATTGTCAAAGATG
TTP	Human	7538	GACTGAGCTATGTCGGACCTT	GAGTTCGCTCTTGATTGGGG
TTP	Mouse	22695	CCGAATCCCTCGGAGGACTT	GAGCCAAAGGTGCAAAACCA
TNF- α	Mouse	21926	CCCTCACACTCAGATCATCTTC	GCTACGACGTGGGCTACAG

Supplementary Table S10. Concentrations of kinase inhibitors in the Kinase Inhibitor Array used in this study.

Position	Inhibitor	Inhibitor Target	Concentration (nM)
A1	DMSO	DMSO	-
A2	R428	AXL	14
A3	Purvalanol B	CDK	9
A4	NVP-BGJ398	FGFR 1/2/3	10
A5	Tofacitinib	JAK3	91
A6	TAK-715	MAPK, p38 α	30
A7	PP242	mTOR	8
A8	GDC-0941	PI3K α / δ	42
A9	(R)-BI-2536	PLK	5
A10	GNF-5837	TRK	42
A11	Axitinib	c-Kit, PDGFR β , VEGFR 1/2/3	2
A12	MK-2461	c-Met, FGFR 1/2/3, PDGFR β	2.5
B1	A-674563 HCl	AKT, CDK, PKA	16
B2	ABT-737	Bcl2	40
B3	BMS387032 HCl	CDK	62
B4	PD-0173074	FGFR 1	25
B5	CC-401 HCl	JNK	50
B6	AS703026	MEK 1/2	11
B7	Rapamycin	mTOR	1
B8	IC87114	PI3K δ	500
B9	PF431396	PYK2	31
B10	GW441756	TrkA	2
B11	Bosutinib	BCR-Abl, Src	1.2
B12	Motesanib	c-Kit, PDGFR, VEGFR 1/2/3	6
C1	MK-2206 2HCl	Akt 1/2/3	65
C2	Imatinib mesylate	BCR-Abl	600
C3	PF0477736	CHK1	50
C4	Quizartinib	FLT3, FMS, cKIT, PDGFR	4.2
C5	Masatinib	c-KIT	800
C6	CI-1040	MEK 1/2	17
C7	XL388	mTOR	10
C8	PIK-75	PI3K, p110 α	6
C9	PLX4032	B-RAF	31
C10	GDC-046	TYK2	13.3
C11	Cabozantinib	c-MET, Tle2, AXL, Kit	14.3
C12	Ponatinib	BCR-Abl, VEGFR2, FGFR1, Src	6
D1	LDK378	ALK	0.2

D2	GCI1746	BTK	2
D3	CX-4945	CK2	1
D4	SB-216763	GSK-3	35
D5	AMG-47a	Lck	10
D6	Selumetinib	MEK1	50
D7	BX795	PDK1	15
D8	PIK-90	PI3K α / β / δ	60
D9	GSK429286A	ROCK	15
D10	Tivozanib	VEGFR 1/2/3, PDGFR, c-Kit	1
D11	Dasatinib	BCR-Abl, Src, c-Kit	1
D12	Regorafenib	VEGFR1/2/3, PDGFR β , KIT, RET, Raf1	46
E1	S-99	ASK	5
E2	PD173955-Analog1	c-Src	10
E3	Gefitinib	EGFR	30
E4	Mubritinib	HER2	6
E5	BMS-5	LIMK	8
E6	Crizotinib	c-MET	24
E7	GSK2606414	PERK	3.2
E8	TGX221	PI3K, p110 β	50
E9	BI-D1870	RSK	31
E10	JNJ-38158471	VEGFR2	50
E11	CHIR-258	c-KIT, FGFR 1/3, FLT3, FMS, PDGFR, VEGFR 1-4	36
E12	Sorafenib	B-RAF, VEGFR2, Raf-1	90
F1	CYC116	Aurora A/B	70
F2	Alvocidib	CDK 1/2/4/6	40
F3	Lapatinib	EGFR, HER2	11
F4	LY2784544	JAK2	3
F5	RWJ-67657	MAPK (p38MAP)	200
F6	PF04217903	c-MET	5
F7	AS-252424	PI3Ky	33
F8	YM201636 HCl	PIKfyve	33
F9	PF-04708671	S6K	160
F10	SAR131675	VEGFR3	45
F11	E7080	VEGFR 2/3	6
F12	SU-6668	VEGF, PDGFR, FGFR1	8
G1	SNS-314	Aurora A/B/C	31
G2	AT-7519 HCl	CDK 1/2/4/6/9	210
G3	PF-562271	FAK	1.5
G4	Momelotinib	JAK1/2	20
G5	SB202190	MAPK (p38MAP), p38 α / β	100
G6	Foretinib	c-MET, VEGFR	1
G7	BKM120	PI3K, p110 α / β / γ / δ	20

G8	SML-4a	Pim	21
G9	R406	Syk	41
G10	Pazopanib	c-KIT, PDGFR1/2/3, FGFR, c-Fms	146
G11	Intedanib	VEGFR1/2/3, FGFR1/2/3,PDGFR α / β	100
G12	Sunitinib malate	c-KIT, PDGFR β , VEGFR2	80
H1	Tozasertib	Aurora A	70
H2	Palbociclib Isethionate	CDK 4/6	16
H3	Brivanib	FGFR1, VEGFR2	25
H4	TG101348	JAK2	3
H5	SD169	MAPK (p38MAP)	4
H6	MPI0479605 HCl	Mps1, TTK	4
H7	GDC-0032	PI3K α / γ / δ	10
H8	Bisindoylmaleimide X HCl	PKC	15
H9	AMG-Tie2-1	TIE2	1
H10	Amuvatinib	Fit3, KIT, PDGFR α	81
H11	Linifanib	Fit1/3, PDGFR β , VEGFR2, CSF-1R	66
H12	Tandutinib	c-KIT, FLT3, PDGFR	220

Supplementary Table S11. Genotyping PCR primer sequences used in this study.

Gene	Species	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
ANGPTL4 ^{+/+}	Mouse	GCAAGATCCAGCAATTGTTCCAG	ACCCTTGATGAGAGCCTAGTGAG
ANGPTL4 ^{-/-}	Mouse	GCAAGATCCAGCAATTGTTCCAG	GGTGCCCAAGGACCTGAAATGA

Supplementary Figures and Legends

Supplementary Fig. S1. Parameters of colon samples from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice given Veh, DSS and SA at 8-day post-treatment.

(a) Percentage change in body weight of ANGPTL4^{+/+} and ANGPTL4^{-/-} mice given Veh, DSS or SA over an 8-day treatment. (b) Macroscopic (*left panel*) and histological (*right panel*) scoring for mice at the endpoint; scoring criteria can be found in Supplementary Tables 2-3 respectively. Quantitative measurements of (c) colon length, (d) muscularis thickness and (e) villus length at the 8-day post-treatment experimental endpoint. (f) Immunofluorescence staining for Ly6G (neutrophils; red) and CD68 (macrophages; red) and nuclei (blue) in colon samples from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice following the indicated treatments. (g) TUNEL staining (*left panel*) and immunoblot analysis of cleaved caspase 3 (*right panel*) and for apoptotic cells in colon sections from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice following the indicated treatment. Scale bar = 100 μ m. (h) Quantitative measurements of plasma blood glucose levels in ANGPTL4^{+/+} and ANGPTL4^{-/-} mice after Veh, DSS and SA treatments for 8 days. For the Veh, DSS or SA groups, n=20 mice were used in each treatments. The Mann–Whitney U test was used. *p < 0.05, **p < 0.01, ***p < 0.001.

Supplementary Fig. S2. Chemokine expression levels in ANGPTL4^{+/+} and ANGPTL4^{-/-} mice.

(a) Relative concentration of the indicated chemokines in colon tissues from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice treated with Veh, DSS or SA. (b) Relative expression (*upper panel*) and immunoblot analysis (*lower panel*) of ANGPTL4, TTP and HuR levels in ANGPTL4^{+/+} littermates for DSS and SA treatments with respect to Veh. (c) Relative mRNA and protein levels of TTP in colon tissues from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice. (d) Relative mRNA expression of the indicated chemokines in iCECs treated with DSS, SA, IL-1 β , TNF- α , HC or NaBu. Relative mRNA (*left panel*) and protein (*right panel*) levels accounting for levels of (e) ANGPTL4 and (f) TTP following siRNA knockdown as determined by real-time PCR and immunoblot analysis, respectively. (g) Heatmap showing log-transformed protein concentration of indicated chemokine mRNA transcripts in iCEC_{Ctrl}, iCEC_{TTP} and iCEC_{ANGPTL4} following stimulation with DSS, SA, IL-1 β , TNF- α , HC or NaBu. (h) Decay curves of ANGPTL4 and TTP in iCEC_{ANGPTL4}, iCEC_{TTP} or iCEC_{Ctrl}, following actinomycin D treatment after NaBu stimulation. (i) Transwell migration assay set-up. siRNA knockdown was performed on iCECs seeded in wells while THP1-derived macrophages were cultured on inserts. The macrophages were later introduced to iCECs and further stimulated with either pro- or anti-inflammatory stimuli. The Mann–Whitney U test was used. *p < 0.05, **p < 0.01, ***p < 0.001.

Supplementary Fig. S3. Expression of TTP transcript in iCECs treated with various kinase inhibitors.

(a) Relative mRNA (*left panel*) and protein (*right panel*) levels accounting for TTP expression in iCECs treated with rh-cANGPTL4 and rh-nANGPTL4. (b) A schematic illustration of the rationale behind the kinase inhibitor array, which led up to mapping the IPA pathway in Fig. 4a. When treated with rh-cANGPTL4, TTP expression was increased in iCECs (*left panel*). Kinase inhibitors (XX_n) that negated this up-regulation of TTP in the presence of rh-cANGPTL4 meant that these kinases were directly responsible for regulating TTP expression (*middle panel*). On the other hand, the addition of kinase inhibitors that brought about an increase in TTP expression in the presence of rh-cANGPTL4 (*right panel*) suggested that ANGPTL4 works through other unique pathways to up-regulate the expression of TTP, independent of the target kinase. (c) Relative mRNA level of TTP after treatment with various kinase inhibitors from a SYNLibrary 95 array. Relative mRNA (*left panel*) and protein (*right panel*) levels accounting for the levels of (d) RELA and CREB, (e) CREB and (f) RELA following siRNA knockdown as determined by real-time PCR and immunoblot analysis, respectively. (g) Relative expression of TTP in iCECs after the depletion of CREB and/or NF- κ B using siRNA knockdown (iCEC_{Ctrl}, iCEC_{RELA}, iCEC_{CREB} and iCEC_{RELA/CREB}) and CREB and/or NF- κ B inhibitors (Veh iCEC, IKK-2 iCEC, 666-15 iCEC and IKK-2 /666-15 iCEC). (h) Immunoblot analysis of pCREB, CREB, and pNF- κ B for nuclear (*upper panel*) and cytoplasmic fractions (*lower panel*) isolated from iCECs (iCEC_{Ctrl}, iCEC_{RELA}, iCEC_{CREB}, iCEC_{RELA/CREB}, Veh iCEC, IKK-2 iCEC, 666-15 iCEC and IKK-2 /666-15 iCEC) stimulated with or without rh-cANGPTL4. Mann–Whitney U test was used. Three independent experiments (n = 3) were performed (means $\pm$ s.e.m.); *p < 0.05, **p < 0.01, ***p < 0.001.

Supplementary Fig. S4. Schematics of the microarray analysis.

Schematics illustrating the flow of the microarray analysis. Both murine and human samples were subjected to ANOVA in Partek Genomics Suite software to evaluate and identify changes in gene transcriptomic profiles. Subsequently, datasets were analyzed using Ingenuity Pathway Analysis to identify top hits in canonical pathways and biological processes.

Loss of TAK1 Increases Cell Traction Force in a ROS-dependent Manner to Drive Epithelial-Mesenchymal Transition of Cancer Cells

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Loss of TAK1 increases cell traction force in a ROS-dependent manner to drive epithelial–mesenchymal transition of cancer cells

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Epithelial–mesenchymal transition (EMT) is a crucial step in tumor progression, and the TGF β –SMAD signaling pathway as an inducer of EMT in many tumor types is well recognized. However, the role of non-canonical TGF β –TAK1 signaling in EMT remains unclear. Herein, we show that TAK1 deficiency drives metastatic skin squamous cell carcinoma earlier into EMT that is conditional on the elevated cellular ROS level. The expression of TAK1 is consistently reduced in invasive squamous cell carcinoma biopsies. Tumors derived from TAK1-deficient cells also exhibited pronounced invasive morphology. TAK1-deficient cancer cells adopt a more mesenchymal morphology characterized by higher number of focal adhesions, increase surface expression of integrin $\alpha 5 \beta 1$ and active Rac1. Notably, these mutant cells exert an increased cell traction force, an early cellular response during TGF $\beta 1$ -induced EMT. The mRNA level of ZEB1 and SNAIL, transcription factors associated with mesenchymal phenotype is also upregulated in TAK1-deficient cancer cells compared with control cancer cells. We further show that TAK1 modulates Rac1 and RhoA GTPases activities via a redox-dependent downregulation of RhoA by Rac1, which involves the oxidative modification of low-molecular weight protein tyrosine phosphatase. Importantly, the treatment of TAK1-deficient cancer cells with Y27632, a selective inhibitor of Rho-associated protein kinase and antioxidant *N*-acetylcysteine augment and hinders EMT, respectively. Our findings suggest that a dysregulated balance in the activation of TGF β –TAK1 and TGF β –SMAD pathways is pivotal for TGF $\beta 1$ -induced EMT. Thus, TAK1 deficiency in metastatic cancer cells increases integrin:Rac-induced ROS, which negatively regulated Rho by LMW-PTP to accelerate EMT.

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Subject Category: Cancer

Metastasis accounts for more than 90% of cancer-related deaths.¹ Metastasis is a multi-stage process involving cancer cell motility, intravasation, transit in the blood or lymph, extravasation and growth at a new site.¹ Conceivably, the various stages consist of regulatory networks with many opportunities for cancer therapy. The potential for new therapeutics avenues and their clinical impact to reduce cancer mortality rate underscores the immense importance to identify novel target modulators of these regulatory networks during metastasis.

Numerous evidences showed metastasis of epithelial cancer involve the conversion of primary cancerous epithelial cells to a migratory form, that is, undergoes epithelial–mesenchymal transition (EMT) for their dissemination to secondary tumor sites.² It is a process whereby cobblestone-shaped epithelial cell colonies acquire distinct mesenchymal traits such as the loss of E-cadherin and the upregulation of N-cadherin to weaken cell–cell junctional adhesion, augmenting cell dissociation.² EMT also exhibit

extensive cytoskeletal remodeling, whereby actin stress fibers are formed in conjunction with an increased establishment of integrin-containing focal contacts, effectively enhancing cell attachment to the extracellular matrix. Cancer cells undergoing EMT are also enriched with mesenchymal type intermediate filaments (i.e. desmin, vimentin). The expression of mesenchymal features and suppression of epithelial traits are induced by early EMT-associated transcriptional factors such as SNAIL and ZEB1. Studies suggested that this mesenchymal transformation enhances the ability of cancer cells to intravasate and disseminate. Thus, the identification of mechanistic drivers of EMT would provide crucial insights and therapeutic targets to halt cancer spreading.

There are many evidences that clearly support a tumor-promoting role of inflammation in the development and progression of epithelial cancer.³ Data from various animal models clearly emphasize that the tumor-promoting micro-environment is indispensable for tumor formation and progression. Although EMT culminates from an integrated

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Keywords: reactive oxygen species; cell traction force; epithelial–mesenchymal transition

Abbreviations: A5RT3_{CTRL}, control- knockdown A5RT3 cancer cells; A5RT3_{TAK1}, TAK1-knockdown A5RT3 cancer cells; CAFs, cancer-associated fibroblasts; CTF, cell traction force; EMT, epithelial–mesenchymal transition; NF κ B, nuclear factor kappa B; PLA, Proximity ligation assay; PNS, perilesional normal skin; qPCR, quantitative PCR; ROS, reactive oxygen species; SCC, squamous cell carcinomas; TGF β , transforming growth factor- β ; TAK1, TGF β activated kinase 1

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cascade of proinflammatory cytokines, much more remains to be understood about the intracellular signaling pathways that were essential for EMT. Of the numerous cytokines produced at the tumor microenvironment, transforming growth factor- β (TGF β) has the broadest effects, influencing many aspects of tumorigenesis.⁴ TGF β has a leading role in eliciting inflammation, serving as a chemotactic factor to recruit immune cells. TGF β triggers EMT in tumor cells through the canonical Smad signaling.^{5,6} TGF β also signals through the non-canonical TGF β activated kinase 1 (TAK1) cascade, which is a pivotal proinflammatory signaling mediator. However, the role of TGF β -mediated TAK1 signaling in EMT is unclear. A hallmark of inflammation is an increase in reactive oxygen species (ROS), which also has a protumorigenic role.^{7,8} TAK1 has been shown to modulate ROS level in normal and tumor cells. The ablation of TAK1 causes accumulation of ROS, resulting in epithelial cell death and inflammation.^{9,10} However, the effect of ROS on EMT is not well understood. In this study, we investigated the role of TAK1 in TGF β -induced EMT of tumor cells. We found that TAK1 deficiency in tumor cells accelerated EMT in ROS-dependent manner, thus providing a previously unexplored mechanistic regulation of EMT by TAK1.

Results

Reduced expression of TAK1 in invasive human squamous cell carcinomas (SCC). To assess the clinical relevance of TAK1, we examined the expression of TAK1 from 15 clinically graded SCCs. Tumors were formerly graded by in-house pathologist for their invasiveness, and were grouped into benign or invasive group. We observed reduced mRNA and protein levels of TAK1 in invasive SCCs when compared with its perilesional normal skin (PNS), which served as cognate-paired control. No difference was observed between benign SCCs and its corresponding PNS (Figures 1a and b). This observation suggests reduced TAK1 expression is associated with an invasive prognosis.

TAK1-deficient A5RT3 cells form skin tumors with invasive morphology. Next we set out to investigate the *in vivo* effect of TAK1 deficiency on tumor invasiveness by using SSC orthotopic nude mice model. In the first instance, we suppressed TAK1 expression in human SCC A5RT3 cell line using lentiviral-mediated RNAi (designated herein A5RT3_{TAK1}). A5RT3 cells transduced with a scrambled sequence served as the control A5RT3_{CTRL}. The efficiency of TAK1-knockdown was verified with immunoblotting and quantitative PCR (qPCR) (Supplementary Figure S1A). Immunoblot analysis of A5RT3_{TAK1} cells further revealed that diminished TAK1 corresponded with a reduced downstream phosphorylation of nuclear factor kappa B (NF κ B) upon treatment with interleukin-1 (IL-1)⁴ (Supplementary Figure S1B). The activation of other stress response pathways like ERK and JNK were likewise also found to be suppressed in A5RT3_{TAK1} (Supplementary Figure S1C). A5RT3_{CTRL} and A5RT3_{TAK1} cells showed no significant difference in their rate of proliferation (Supplementary Figure S1D). A5RT3_{TAK1} and A5RT3_{CTRL} were injected subcutaneously into nude mice to induce tumor formation.

A5RT3_{TAK1}- and A5RT3_{CTRL}-derived tumors were harvested and sections were immunostained for laminin-332, a recognized marker of tumor invasiveness.^{11–13} Laminin-332 immunostaining in A5RT3_{CTRL}-derived tumors appeared as demarcated boundaries characteristic of basement membrane type architecture, whereas the corresponding staining in A5RT3_{TAK1}-derived tumors was disorganized and interspersed among the tumor epithelial cells, indicative of enhanced invasiveness. This spatial disparity in laminin-332 immunostaining also corresponded with an increased laminin-332 protein (Figure 1d). Gelatin zymography also revealed increased MMP-9 activity, another marker of tumor invasiveness, in A5RT3_{TAK1}-tumor. These observations indicate that the TAK deficiency is associated with enhanced invasiveness (Figure 1d).

TAK1 deficiency promotes TGF β -1 induced EMT in A5RT3 cells. Reduced TAK1 expression is a common attribute of invasive human SCCs. In addition, TAK1-deficient tumors exhibited more prominent invasive features. The TGF β 1–SMAD signaling pathway is central to EMT induction. However, the role of TGF β 1–TAK1 signaling in EMT remains unknown. A5RT3_{TAK1} colonies treated with TGF β 1 showed earlier separation at cell–cell borders at 48 h post treatment (Figure 1e). Immunofluorescence staining revealed a decreased E-cadherin and a corresponding increased N-cadherin staining (Figure 1f). The mRNA levels of EMT markers measured by qPCR revealed an EMT phenotype as early as 24 h after exposure in A5RT3_{TAK1} treated with TGF β compared with cognate controls (Figure 1g). The protein expression of EMT markers vimentin, fibronectin, Snail 1 and Snail 2 was also altered, indicating an earlier switch to an EMT phenotype by A5RT3_{TAK1} cells (Figure 1h). To further assess the potential role of TAK1 in EMT, we examined TGF β 1-induced EMT in three other tumor lines, HSC-5, I14 and MKN28, whose endogenous TAK1 was suppressed by siRNA. HSC-5 was established from a clinically derived human squamous cell carcinoma.¹⁴ I14 cell line is a Ras-transformed HaCat cell line that is not metastatic.¹⁵ MKN28 is a well-differentiated stomach adenocarcinoma cell line.¹⁶ Importantly, we also observed similar augmentation of TGF β 1-induced EMT in these TAK1-knockdown cells. TAK1-deficient cells were observed to adopt mesenchymal-like cell–cell separation upon TGF β 1 induction (Supplementary Figure S1E) and corresponded to the expression of mesenchymal markers (Supplementary Figure S1F). These observations support a role of TAK1 deficiency in promoting mesenchymal transition. We also found that TGF β -induced EMT phenotype was prevented in A5RT3_{TAK1} transfected with an expression vector for TAK1 pCDNA–TAK1 (Supplementary Figure S2). The TAK1 mRNA sequence transcribed from pCDNA–TAK1 contains silent mutations that are not targeted by the siRNA produced in A5RT3_{TAK1}.

To understand the relationship between SMAD and TAK1 in TGF β 1-induced EMT, we assessed the relevance of SMAD3 in TGF β 1-induced EMT of A5RT3_{TAK1} cells, which was previously unclear. A5RT3_{TAK1} cells were transiently transfected with siRNA targeting SMAD3 (Figure 2). Loss of SMAD3 in A5RT3_{TAK1} cells inhibited TGF β 1-induced EMT

Figure 1 TAK1 deficiency promotes TGFβ1 induced EMT in A5RT3 cells. **(a and b)** Relative mRNA **(a)** and protein **(b)** expression of TAK1 in human squamous cell carcinomas (SCC) biopsies and their cognate perilesional normal skin (PNS). Biopsies #1–9 and #10–15 are benign and invasive grades SCC samples, respectively. Biopsies #5–7 and #10–14 were used for western blot analysis. For qPCR, data points from the same individual are linked by colored lines. **(c)** Immunofluorescence staining of laminin-332 (green) in cryosections of A5RT3_{CTRL}- and A5RT3_{TAK1}-derived tumors. Nuclei were counterstained with DAPI (blue). **(d)** Expression of invasive markers laminin-332 and MMP-9 in A5RT3_{CTRL}- and A5RT3_{TAK1}-derived tumors as determined by immunoblot and gelatin zymography, respectively. Values below each band represent the mean fold differences ($n = 3$) in expression level when compared with A5RT3_{CTRL}-derived tumor. **(e)** Phase-contrast microscopy images of A5RT3_{CTRL} and A5RT3_{TAK1} cell colonies 48 h after TGFβ1 treatment. Scale bar, 100 μ m. **(f)** Immunostaining of E-cadherin and N-cadherin (green) in A5RT3_{CTRL} and A5RT3_{TAK1} cells under the indicated treatment. Nuclei were counterstained with DAPI (blue). Scale bar, 100 μ m. **(g)** qPCR analysis of EMT markers in A5RT3_{CTRL} and A5RT3_{TAK1} 24 h after TGFβ1 treatment. Samples were normalized with reference gene, L27. **(h)** Representative western blots of EMT markers in A5RT3_{CTRL} and A5RT3_{TAK1} 48 h after TGFβ1 treatment. The densitometry values as indicated below respective lanes were normalized with respect to the control. For western blot, β-tubulin was used as a loading control. For qPCR, all samples were normalized with housekeeping gene L27 gene. Data represent means $\pm$ S.D.; $n = 3$

(Figure 2a). The expression of mesenchymal markers was downregulated whereas epithelial markers were upregulated in SMAD3-deficient A5RT3_{TAK1} cells (Figures 2b and c). We observed that EMT also occurred in A5RT3_{CTRL} cell colonies

after prolonged (i.e. 72 h) treatment with TGFβ1 as compared to 48 h for A5RT3_{TAK1} (Supplementary Figure S3A). Similarly, SMAD3 deficiency in A5RT3_{CTRL} cells inhibited TGFβ1-induced EMT (Supplementary Figures S3B & S3C).

Figure 2 TGFβ1-induced EMT is effected through SMAD signaling. (a) Phase-contrast images of SMAD3 siRNA-transfected A5RT3_{TAK1} and respective control cells with 48 h of TGFβ1 (10 ng/ml) treatment. Scale bar, 100 μm. (b) qPCR analysis of EMT markers in SMAD3 siRNA-transfected A5RT3_{TAK1} cells with and without TGFβ1 treatment. (c) Immunoblot analysis of SMAD3 siRNA-transfected A5RT3_{TAK1} cells for their knockdown and EMT markers upon TGFβ1 treatment. Representative blots were shown with respective densitometry values indicated below lanes, normalized with respect to the control. For western blot, β-tubulin was used as a loading control. For qPCR, all samples were normalized with housekeeping gene L27 gene. Data represent means ± S.D.; *n* = 3

The phosphorylation of SMAD3 was also evaluated in the SMAD3-knockdown cells by immunoblotting (Supplementary Figure S3D). Altogether, our observations indicates that TAK1 limits TGFβ driven pro-EMT signals mediated by SMAD3 pathway, and suggests a signaling balance between the canonical SMAD3 and non-canonical TAK1 in regulating EMT.

TAK1 deficiency increases the expression of surface integrin β1 and β3. Our above observations suggest that TAK1 deficiency in A5RT3 facilitated TGFβ1-induced EMT. EMT involves the transition of the epithelial cancer cells from predominantly cell–cell adhesion to a cell–ECM interaction. Integrins are major cell surface receptors that enable bidirectional communication between the cells with the ECM are thus well apt to influence cellular behavior involved in EMT including migration and adhesion.^{17,18} Thus, we examined the surface expression of integrins β1, β3, β4, β5 and β7 on A5RT3_{TAK1} (Figure 3a). We found that A5RT3_{TAK1} had significantly increased surface levels of β1 and β3 integrins (Figure 3a). We further conducted staining of α5β1 integrin and found that it is also significantly elevated in a manner reminiscent of integrin β1 (Figure 3b). Proximity

ligation assay (PLA) also confirmed higher level of surface α5β1 integrin in A5RT3_{TAK1} than A5RT3_{CTRL} (Figure 3c, upper panel). PLA assay was conducted without Triton-X permeabilization to detect only to surface integrin levels. PLA signals for integrin α5β1 were predominantly situated along the cell–cell boundary (Figure 3c, upper panel). PLA assay performed using antibody against active integrin β1 and configuration-specific monoclonal anti-Rac1-GTP antibody further confirmed that the increase expression of surface integrin was associated with an elevated Rac1 activation, suggesting a remodeling of the cytoskeleton (Figure 3c, lower panel).

Loss of TAK1 increases cell traction force in A5RT3 during EMT. During EMT, integrins are recruited to distinct contact points to form focal adhesions accompanied by cytoskeletal remodeling of cortical actin network to filamentous stress fibers. Interestingly, we already observed more focal adhesions and better developed stress fiber formation in A5RT3_{TAK1} than A5RT3_{CTRL}, before TGFβ1 treatment (Figure 4a). These differences in stress fiber formation correlated with vinculin-positive focal adhesion staining at their cell junctions of A5RT3_{TAK1} (Figure 4a). The vinculin

Figure 3 Expression of surface integrin in A5RT3_{CTRL} and A5RT3_{TAK1}. **(a and b)** FACS analysis of A5RT3_{CTRL} and A5RT3_{TAK1} cells immunostained with antibodies against indicated surface integrin β subunits **(a)** and with integrin $\alpha 5\beta 1$ **(b)**. The distribution of tumor cells with regard to their extent of integrin staining was presented in histogram plots with the identity of the stained integrin subunit indicated (top left). Cells stained with only control IgG served as the negative controls. Each image is representative of at least three different experiments. Values shown indicate mean fluorescence intensity. **(c)** PLA assay of $\alpha 5\beta 1$ integrin expression (upper panel) and active integrin $\beta 1$:active Rac1 (lower panel) in A5RT3_{CTRL} and A5RT3_{TAK1} cells. Representative images were shown with mean number of PLA spots per nucleus $\pm$ S.D. indicated; $n = 3$

Figure 4 TAK1 deficiency increases cell traction force in A-5RT3 facilitating EMT. (a) Vinculin immunostaining (green) and phalloidin F-actin (red) staining were conducted of A5RT3_{CTRL} and A5RT3_{TAK1} cell colonies with and without TGFβ1 treatment for 48 h with representative images shown. Scale bar, 100 μm. (b and c) Cell traction force profiling (b) and mean measured cell traction stress (c) of A5RT3_{CTRL} and A5RT3_{TAK1} with and without TGFβ1 (10 ng/ml) treatment for 24 h. Color scale bar denotes traction stress (kPa). Scale bar, 10 μm. Values (mean ± S.D.) of three independent measurements. (d) A5RT3_{CTRL} and A5RT3_{TAK1} cells were seeded on a transwell membrane of pore size and evaluated for their migration through the pore with and without TGFβ1 treatment for 48 h. Migrated cells were stained with crystal violet and destained with a fixed volume of 0.5% Triton-X solution. Absorbance at 595 nm of the solutions was measured to quantify transwell migration. Graph displays mean absorbance values ± S.D.; *n* = 3

staining also displayed differences in spatial distribution observed in PLA staining for integrin $\alpha 5 \beta 1$. As expected, the treatment with TGFβ1 increased stress fiber in both cell lines despite their different cell–cell dispersion. Differences in stress fibers and focal adhesion contact formation in cells are known to effect differential cell traction force (CTF) generation. These force differences represent the early detectable changes in cell responses, preceding the appearance of visible morphological changes. Thus, we measured the CTF generated in A5RT3_{TAK1} and A5RT3_{CTRL} using CTF microscopy. Cells were cultured on fibronectin-treated compliant PDMS substrate (10 kPa) coated with a monolayer of fluorescence beads. Traction force is determined by measuring the displacement of the fluorescence beads in the *x-y* plane because of the contraction exerted by the cell. Notably, A5RT3_{TAK1} generated a considerably different CTF profile from that of control A5RT3_{CTRL} cells. Although traction field was observed to be concentrated along the periphery of the A5RT3_{CTRL} cell colony, intra-colony traction can only be detected in the A5RT3_{TAK1} group (Figure 4b). As the CTF exerted is also relative to the cell colony size and shape, measured CTF values are divided over the area covered by the cell colonies to derive traction stress values for comparison between cell colonies of differing sizes.

Untreated A5RT3_{CTRL} cells displayed a mean traction stress value of 93.4 ± 14.8 Pa, whereas that of A5RT3_{TAK1} displayed a mean CTF of 170.7 ± 20.2 Pa (Figure 4c), indicating higher contractility in TAK1-deficient cancer cells. TGFβ1 induction resulted in significant CTF increments for both A5RT3_{CTRL} and A5RT3_{TAK1} as reflected in their elevated traction stress values of 143.96 ± 31.7 Pa and 461.4 ± 113.22 Pa, respectively (Figure 4c). Notably, TGFβ1-induced A5RT3_{CTRL} cell colonies also adopted a CTF profile pattern comparable with that of untreated A5RT3_{TAK1}. In transwell invasion assay, we found that A5RT3_{TAK1} significantly migrated faster through an 8-μm pore transwell with TGFβ1 as the chemotactic factor (Figure 4d). Our results indicate that TAK1 deficiency in A5RT3 promoted the expression of $\beta 1$ and $\beta 3$ integrins, and altered stress fiber formation. This cytoskeletal remodeling corresponded with an increased CTF profile and metastatic potential in A5RT3_{TAK1} cells, suggesting an inhibitory role of endogenous TAK1 during TGFβ1-induced EMT.

Elevated ROS levels in TAK1-deficient tumor cells accelerate EMT. TAK1 deficiency was reported to upregulate ROS level in human keratinocytes and in cancer cells.^{9,19} Integrin-Rac1 signaling mediates multiple pathways

that control actin cytoskeletal changes, transcriptional activity and ROS production.^{20,21} First, we examine if the increased active Rac1 in A5RT3_{TAK1} (Figure 3c) was associated with a higher recruitment of Rac1-dependent Nox1 for ROS generation. PLA assay using antibodies against active Rac1 and Nox1 revealed higher number of PLA signals in A5RT3_{TAK1} than A5RT3_{CTRL}, indicating an elevated Rac1-dependent activation of Nox1 (Figure 5a). Next, we evaluated intracellular ROS production in A5RT3_{TAK1} and A5RT3_{CTRL} using fluorescence dye CM-H2DCFDA followed by FACS analysis. We found that A5RT3_{TAK1} cells have increased ROS level, which was further potentiated by TGF β 1 induction. In fact, the ROS level in untreated A5RT3_{TAK1} was comparable with TGF- β 1-treated A5RT3_{CTRL} (Figure 5b). The difference in ROS level between A5RT3_{CTRL} and A5RT3_{TAK1} upon TGF β 1 treatment was also verified by fluorescence microscopy. Interestingly, we observed that A5RT3_{TAK1} cells that have separated from the tumor colony were more intensely stained with CM-H2DCFDA compared with A5RT3_{CTRL} (Figure 5c). As ROS upregulation in A5RT3_{TAK1} correlated with the mesenchymal phenotype induced by TGF β 1, it was conceivable that the observed elevated oxidative stress may be mechanistically involved in EMT. Antioxidant *N*-acetylcysteine (NAC) addition was utilized to quench ROS. First, we determine the optimal concentration of NAC (30 μ M–30 mM) for our experiments. At NAC concentrations of 3–30 mM, annexin V/propidium iodide staining of A5RT3_{TAK1} revealed increased percentage of apoptotic cells (Supplementary Figure S4A). A corresponding CM-H2DCFDA staining showed that the ROS level was increased in cells treated with high 30 mM NAC concentration, most likely due to cell death. The treatment with 300 μ M–3 mM of NAC did not lower the ROS further than that obtained by 100 μ M of NAC. At 30 μ M of NAC, the decrease in ROS was less effective (Figure 4b). Thus, we determine the optimal NAC concentration to be 100 μ M, which lowers the ROS level without substantially increasing cell death. We quenched the ROS in the cancer cells with NAC and observed that the treatment hindered the cell–cell separation characteristic of TGF β 1-induced EMT in A5RT3_{TAK1} (Figure 5d). The NAC-treated cells were significantly diminished of their EMT traits (Figures 5e and f). NAC treatment neither altered the phenotype of A5RT3_{CTRL} cells (Supplementary Figure S4C) nor elicited any off-target effects as verified by immunoblotting of various EMT markers (Supplementary Figure S4D).

Inhibition of Rho accelerated TGF β 1-induced EMT in A5RT3_{TAK1}. Our above observations on increased CTF of untreated A5RT3_{TAK1} corresponded with the increased surface integrin expression, focal adhesion and stress fiber formation. RhoA and Rac1 have key roles in the formation of stress fiber, focal adhesion, cytoskeleton organization and cellular migration. We first examined their activities in A5RT3_{CTRL} and A5RT3_{TAK1} with and without TGF β 1 induction. In both TGF β 1-treated and -untreated groups, the activities of Rac1 and RhoA were elevated and reduced, respectively, in A5RT3_{TAK1} compared with A5RT3_{CTRL} (Figure 6a). We observed that NAC treatment increased RhoA activity, but has not significantly affected the level of

active Rac1 (Figure 6a). This is consistent with our earlier observation that increased surface expression of integrin β 1 activated Rac1, which subsequently engaged Nox1 for ROS generation. It also suggested that the activity of RhoA was redox-dependent. To further understand the relationship between Rac1 and ROS, we transfected A5RT3_{CTRL} and A5RT3_{TAK1} with expression vectors containing constitutively active (G12V) and dominant-negative Rac mutant (T17N), respectively. FACS analysis revealed that A5RT3_{CTRL}(G12V) cells have increased ROS content compared with control vector transfected A5RT3_{CTRL} (Figure 6b). Conversely, the oxidative stress level of A5RT3_{TAK1}(T17N) was significantly decreased compared with A5RT3_{TAK1} (Figure 6b). In contrast to their cognate parent cell lines, A5RT3_{CTRL}(G12V) and A5RT3_{TAK1}(T17N) showed increased and reduced TGF β 1-induced EMT, respectively (Figure 6c).

To understand how ROS can modulate the activity of RhoA, we employed the sensitive cysteine-targeted biotin switch method to label oxidatively modified proteins. We showed that the oxidation of low-molecular weight protein tyrosine phosphatase (LMW-PTP), a potential downstream target of ROS was significantly increased in A5RT3_{TAK1}. LMW-PTP oxidation was, however, undetectable in NAC-treated A5RT3_{TAK1} cells (Figure 6a) regardless of TGF β 1 treatment. Oxidatively modified LMW-PTP has been shown to inhibit RhoA activity.²⁰ To understand the role of RhoA in TGF β 1-induced EMT, we treated A5RT3_{TAK1} with Y27632, a selective inhibitor of Rho-associated protein kinase, the downstream mediator of RhoA. The inhibition of Rho augmented EMT in A5RT3_{TAK1} cells detected within 24 h post-TGF β 1 treatment (Figure 6d). The Y27632-treated cells had decreased epithelial marker expression and increased expression of mesenchymal markers (Figure 6e). To further confirm the role of RhoA in attenuating EMT, we transfected A5RT3_{TAK1} cells with RhoA siRNA and observed an increase EMT-like dispersion (Figure 6d). These RhoA-knockdown cells also exhibited changes in the expression of EMT markers (Figure 6e). The inhibition of ROCK and the suppression of RhoA by siRNA in A5RT3_{CTRL} also stimulated the expression of EMT markers, albeit at a reduced level (Supplementary Figure S5). Altogether, our observations showed that TAK1 deficiency increased integrin:Rac1-induced ROS, which negatively regulated RhoA by LMW-PTP to accelerate EMT.

Discussion

Chronic inflammation has been linked to various steps involved in tumorigenesis, including cellular transformation, promotion, invasion and metastasis.^{22,23} Among the numerous cytokines involved in inflammation and tumorigenesis, TGF β 1 has the broadest effect, influencing cell growth, cancer invasion and spreading.^{24,25} Cellular response to TGF β 1 is cell-type specific and context-dependent. EMT is a crucial step in tumor progression, and the TGF β -SMAD signaling pathway as an inducer of EMT in many tumor types is well-recognized.^{26,27} However, the role of TGF β -TAK1 signaling in EMT remains unclear. Earlier studies showed that TAK1 through the NF κ B signaling pathway triggered increased EMT and invasiveness in tumor.²⁸ Recent studies also

implicated ROS-mediated oxidative stress to promote tumorigenesis²⁹. Herein, we found that squamous cell carcinoma progressed earlier into EMT that was conditional on elevated cellular ROS level resulting from TAK1 deficiency (Figure 7). Notably, the expression of TAK1 was consistently reduced in invasive SCCs, but not in benign SCCs, when compared with their cognate perilesional normal tissues. Furthermore, tumors derived from TAK1-deficient cells expressed elevated levels of MMP-9 and laminin-332, the latter was spatially dispersed in the epithelial tumor region. Our investigation further showed that TAK1-deficient cancer cells exhibited an increased cell traction force, one of the earliest cellular response during EMT.^{30,31} This cellular response was associated with an increase in surface integrin $\alpha 5\beta 1$, a well-known mesenchymal marker in cancer.³² We found increased active integrin and active Rac1 interactions in TAK1-deficient cancer cells. The elevated active Rac1 mediated the recruitment of Rac-dependent Nox1 and higher ROS production. Rac1-mediated ROS production resulted in the downregulation of Rho activity in TAK1-deficient cancer cells. The redox-dependent decrease in Rho activity was required for Rac1-induced formation of integrin-mediated cell spreading during TGF β 1-induced EMT. Conversely, the suppression of Rac1 hindered the progression of EMT by TAK1-deficient cancer cells. The redox-dependent downregulation of RhoA involves the inhibition of LMW-PTP.²⁰ Thus, TAK1 modulates Rac1 and Rho GTPases activities, which are critical regulators of actin cytoskeleton remodeling during EMT.

Numerous studies have revealed a biphasic role of epithelial TGF β 1 signaling in tumorigenesis, but our molecular understanding for this phenomenon remains unclear. In the early stages of cancer, TGF β 1 has tumor suppressive effects, whereas in the advanced stages of cancer, TGF β 1 can promote the growth of tumor cells, as well as induce EMT, which results in metastasis.^{25,33} The role of TGF β –TAK1 in tumorigenesis is only emerging. It was reported that in mouse skin tumor, the deletion of *TAK1* gene increases cellular apoptosis due to elevated ROS level.¹⁹ In fact, TAK1-deficient cancer cells displayed increased propensity to undergo EMT upon TGF β 1 exposure. Thus, our findings together with that of others suggest that TGF β –TAK1 signaling also exhibited a biphasic role during tumor progression. In early development of tumor, TAK1 promotes tumor growth by reining ROS production and ROS-mediated apoptosis. In advance tumor stage, TAK1 discourages EMT and thus delays metastasis. Although we observed a similar increase ROS in our TAK1-deficient cancer cells, in contrast to the mouse study,¹⁹ no significant difference in proliferation or apoptosis were observed. This discrepancy may be due the influence of other

surrounding cell types, such as cancer-associated fibroblasts (CAFs). The development and progression of neoplastic epithelia can be understood to arise from the deregulation of this dynamic interaction between normal epithelial and stromal fibroblasts.^{34,35} Recent studies of TGF β signaling in stromal fibroblasts showed significant paracrine influence on the behavior of adjacent epithelial cell. Mice with a fibroblast-specific deletion of TGF β receptor type II developed intraepithelial neoplasia in prostate and invasive squamous cell carcinoma of the forestomach.^{36,37} Clearly, a better understanding the role of TGF β –TAK and TGF β –SMAD signalings in CAFs, and their influence on epithelial cell behaviors requires our future attention. In summary, we showed that TAK1 deficiency increases CTF by a ROS-dependent manner to drive EMT in metastatic A5RT3 cells. Our findings suggest that a dysregulated balance in the activation of TGF β –TAK1 and TGF β –SMAD pathways is pivotal for TGF β 1-induced EMT.

Materials and Methods

Materials. Antibodies were purchased against integrin $\alpha 5\beta 1$ (Millipore, Billerica, MA, USA), TAK1, NF κ Bp65, phospho NF κ Bp65, E-cadherin, SMAD3, phospho-SMAD3, ERK, phospho-ERK, JNK, phospho-JNK (Cell Signaling, Danvers, MA, USA), Rac1, RhoA (Cytoskeleton Inc), activated RhoA, activated Rac1 (NewEast Biosciences, King of Prussia, PA, USA), vimentin, snail2 (Millipore), laminin-332 (AB14509, Abcam, Cambridge, MA, USA), N-cadherin, β -tubulin, snail1, fibronectin, Nox1 and secondary HRP-conjugated antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) for immunoblotting or immunostaining. Transfection reagent ExGen 500 was purchased from Fermentas. Double-promoter lentivirus-based siRNA vector and pFIV packaging kit were acquired from System Biosciences (Mountain View, CA, USA). Pooled siRNA and transfection reagent were purchased from Dharmacon (Pittsburgh, PA, USA). All restriction enzymes and DNA/RNA-modifying enzymes were obtained from Fermentas. TGF β 1 and IL-1 were purchased from Peprotech (Rehovot, Israel). Otherwise, chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Reverse-transcription and real-time PCR (qPCR). Total RNA was isolated and reverse transcribed as previously described.⁹ The cDNA were used for qPCR using SYBR-Green Master mix (KAPABiosystem, Woburn, MA, USA). Melt curve analysis was included to assure that only one PCR product was formed. Expression was normalized to the control gene ribosomal L27, which did not change under any of the experimental conditions studied. Average fold change was derived from three independent experiments and two-tailed student *t*-tests were performed to evaluate statistical significance of expressional changes. The sequences of primers are in Supplementary Table S1.

Tumorigenicity assay. Primary tumor formation was monitored over a period of 2 months after subcutaneous injection of 2×10^5 tumor cells resuspended in 100μ l of DMEM medium containing Matrigel (5 mg/ml) into the interscapular region of 6-week-old BALB/c athymic nude mice. Animals were killed at tumor size of 10 mm diameter. Excised tumors were fixed in 4% paraformaldehyde overnight followed by overnight equilibration in 30% sucrose/PBS for cryoprotection before embedding and freezing in OCT medium (Tissue-tek, Alphen aan den Rijn, The Netherlands). Frozen tumor tissues were sectioned and immunostained as previously described.³⁸

Figure 5 Role of elevated ROS during TGF β 1-induced EMT in A5RT3_{TAK1}. (a) PLA assay of active Rac1 and Nox1 in A5RT3_{CTRL} and A5RT3_{TAK1} cell. Representative images were shown with mean number of PLA spots per nucleus $\pm$ S.D. indicated; *n* = 3. (b) A5RT3_{CTRL} and A5RT3_{TAK1} with or without 48 h of TGF β 1 (10 ng/ml) treatment were stained with DCF and analyzed with flow cytometry. Antioxidant NAC-treated cells (100 μ M) served as a negative control. Image shown is representative of three different experiments. Values shown indicate mean fluorescence intensity. (c) Phase-contrast images showing DCF staining of A5RT3_{CTRL} and A5RT3_{TAK1} after 48 h of TGF β 1 (10 ng/ml) treatment. Scale bar, 100 μ m. (d) Phase-contrast images A5RT3_{TAK1} cells subjected to indicated treatments. NAC (100 μ M) was used to quench ROS. Scale bar, 100 μ m. (e) qPCR analysis of EMT markers in TGF β 1 induced A5RT3_{TAK1} with NAC treatment. Samples were normalized with reference gene, L27. (f) Representative blots of EMT markers in TGF β 1 induced A5RT3_{TAK1} with NAC treatment were shown with densitometry values indicated below respective lanes. Samples were normalized with tubulin as a loading control. Data represent means $\pm$ S.D.; *n* = 3

Immunostaining and proximity ligation assay. Cells or tissue sections were fixed with 4% paraformaldehyde for 15 min, blocked and permeabilized in 3% BSA with 0.05% Triton-X for 1 h. Cells or tissue sections were incubated with either anti-vinculin (1:100), or monoclonal anti-E-cadherin (1:25), or anti-N-cadherin (1:25) or in anti-laminin-332 (1:50) in 0.3% BSA overnight at 4 °C. Secondary goat anti-rabbit Alexa Fluor 488 antibodies (1:200) in 0.2% BSA were added with incubation for 1 h at room temperature in the dark. Cells were then incubated with 1:50 dilution of Alexa Fluor 594 phalloidin (Molecular Probes, Eugene, OR, USA) in 2% BSA for 2 h and counterstained with DAPI (Vectashield, Burlingame, CA, USA). Between each step, slides were washed thrice with PBS. Immunofluorescent and phase-contrast images were taken using Nikon 80i Eclipse microscope equipped with a Nikon DS-Si1 camera, using a (20x/0.5) DIC objective and the NIS element D3.0 software with constant exposure and gain. Respective PLA assays were carried out as per manufacturer protocol (Olink Bioscience, St. Louis, MO, USA). Images were taken using Carl Zeiss (Thornwood, NY,

USA) confocal microscope LSM 710meta using a Plan-Apochromat $\times 63/1.4$ oil DIC objective, and ZEN 2009 LE software with constant exposure and gain. The resulting fluorescent spots were quantified using Duolink ImageTool (Olink Bioscience).

Flow cytometry. Cells were seeded and grown on 75 cm² culture flask (Corning, Tewksbury, MA, USA) until 50% confluence. Cells were then washed with PBS and incubated with 25 mM EDTA/PBS for 20 min. EDTA suspended cells were then spun down at 250 $\times$ g and fixed with resuspension in 4% paraformaldehyde for 20 min at room temperature. Fixed cells were spun down at 700 r.p.m. and washed with PBS twice through resuspension. Washed cells were then treated with anti- $\beta 1$, $\beta 3$, $\beta 4$, $\beta 5$, $\beta 7$, $\alpha 5\beta 1$ integrin antibodies, respectively at concentration (1:25) in 10% BSA and incubated at room temperature for 1 h. Cells were washed twice with PBS resuspension before incubation in secondary antibody conjugated with Alexa Fluor 488 at concentration (1:100) in 10% BSA at room temperature in the dark for 1 h. Stained cells were analyzed with Becton Dickinson (BD, Franklin Lakes, NJ, USA) LSRII flow cytometer. Analysis of flow cytometry results were performed with Flowjo software (Ashland, OR, USA). For ROS detection, cells were incubated with 10 μ M CM-H2DCFDA (Invitrogen, Carlsbad, CA, USA) for 30 min at 37 °C prior to trypsinization and analysis by flow cytometry (LSRII from BD Biosciences). Results were analyzed by the Flowjo software. ROS was quenched with the addition of *N*-acetyl-L-cysteine (NAC) to a final concentration of 100 μ M. CM-H2DCFDA-labeled cells were also imaged with the Nikon 80i Eclipse microscope equipped with a Nikon DS-Si1 camera, using a ($\times 20/0.5$) DIC objective and the NIS element D3.0 software with constant exposure and gain.

Western blot analysis and detection of oxidized proteins. Cells (5×10^5) were lysed in 100 μ L lysis buffer (50 mM Na₂HPO₄, 250 mM NaCl, 1% Triton-X and 0.1% SDS). Equal amount of proteins were resolved by SDS-PAGE and transferred onto PVDF membrane (Millipore). Immunoblotting for β -tubulin was used to ensure equal loading of total proteins. The primary antibodies were used at dilutions of 1:4000 (TAK1), 1:2000 (NF κ Bp65), 1:2000 (phospho NF κ Bp65), 1:1000 (SMAD 3), 1:2000 (Fibronectin), 1:1000 (Vimentin) and 1:5000 (β -tubulin). Secondary antibodies were used at 1:5000 (anti-rabbit IgG), 1:10000 (anti-mouse IgG) and 1:5000 (anti-goat IgG). All incubations were performed in 1 $\times$ TBS, pH 7.5, with 0.1% Tween-20 and either 5% fat-free milk (β -tubulin) or 5% bovine serum albumin (TAK1). Washing was performed with the same buffer without milk or BSA. The procedure to identify oxidized proteins (i.e. LMW-PTP) with biotin-NM was performed as earlier described.³⁹ Densitometry measurements of bands were performed with ImageJ software. The first band in each blot was given an arbitrary density of 1, and relative densities of the remaining bands were determined. Densitometry results of all individual bands are divided by those from tubulin and the ratio is presented as values below individual lanes.

Cell culture and transient transfection assay. Human embryonic kidney (HEK293-T) and human skin squamous cell carcinoma (A5RT3) cells were routinely maintained in DMEM and FBS (Hyclone, Pittsburgh, PA, USA). Transfection of A5RT3_{CTRL} and A5RT3_{TAK1} cells was carried out as per manufacturer's protocol (Fugene, Madison, WI, USA) with either plasmids expressing constitutive-active Rac1 G12V or dominant-negative Rac T17N.

Cell traction force (CTF) measurements. To measure cell contraction forces, cells were cultured on PDMS substrate embedded with fluorescent beads. Briefly, glass coverslips were cleaned by use of oxygen plasma before it was placed in a vacuum desiccator containing a small drop of 1H,1H,2H,2H-perfluorooctyltrichlorosilane for 30 min. Following which, the surface-treated coverslips were removed and 100 μ L of diluted yellow-green FluoSpheres microspheres (0.2 μ m, carboxylate-modified, 0.1 vol%,

Figure 7 Proposed mechanism by which TAK1 inhibits TGF β 1-induced EMT. TAK1 likely counters the well-established TGF β 1-SMAD3 induction of EMT with the suppression of integrin expression and hence Rac1 activation. Rac activity otherwise promotes ROS production via the recruitment of Nox1, which oxidizes LMW-PTP and correspondingly inhibit RhoA activity, promoting EMT. Elevated ROS also leads to increased CTF, a characteristic of EMT progression

Figure 6 Redox regulation of RhoA by Rac1 facilitates TGF β 1 induced EMT. (a) RhoA and Rac1 activities and LMW-PTP oxidation were compared with immunoblotting as respectively indicated. 10 ng/ml TGF β 1 and 100 μ M of antioxidant NAC were used respectively for treatment duration of 48 h. (b) FACS analysis of tumor cells transfected with or without constitutive-active (G12V) and dominant-negative Rac1 (T17N). Antioxidant NAC-treated cells (100 μ M) served as a negative control. Values shown indicate mean fluorescence intensity. (c) Representative images of E-cadherin (green/488 nm) immunostaining of tumor cells with respectively indicated treatments and transfection. Cell nucleus is stained with DAPI (blue). Scale bar, 100 μ m. (d) Phase-contrast images of A5RT3_{TAK1} cells with and without TGF β 1 induction were inhibited with Y27632 (10 μ M) for 24 h or transfected with siRNA for RhoA. Representative phase-contrast images of treated cells. Scale bar, 100 μ m. (e) Representative blots of EMT markers in TGF β 1-induced A5RT3_{CTRL} and A5RT3_{TAK1} treated with ROCK Y27632 or transfected with pooled siRNA for RhoA. Values shown with densitometry values indicated below respective lanes. Samples were normalized with tubulin as a loading control. Data represent means $\pm$ S.D.; *n* = 3

Molecular Probes) was added to the coverslips with the treated surface facing upwards. This step forms a monolayer of fluorescent beads on the coverslips. Thin film of PDMS were generated by placing 15 μ l of PDMS mixture (before curing, 1:60 w/w of cross-linker to base ratio) on the fluorescent microspheres coated glass coverslips. One layer of Scotch tapes was applied as a spacer before putting the other bare glass coverslip on the top of fluorescent microspheres coated glass coverslips. The PDMS droplet was flattened under the weight of the upper coverslip. The entire assembly was left undisturbed for 5 min and then placed in 37 °C incubator overnight before carefully peeling off the upper bonded PDMS thin film with fluorescent microspheres on its top. Only samples with well-dispersed beads were used in the study. Substrates were UV sterilized for 30 min. As PDMS surface does not allow cells to adhere, the substrate was coated by incubation with human plasma derived fibronectin solution (50 μ g/ml in 0.02 M acetic acid for thin coating) overnight to promote cell attachment. Coated substrates were rinsed with PBS, and stored in the dark at 4 °C prior to culture. 1×10^3 cells/inserts were seeded for 48 h under cell culture conditions. Cells were subsequently treated with 10 ng/ml TGF β 1 for 24 h before traction force measurement. All experiments were triplicated.

Calculation of CTFs was based on the elastic moduli of PA substrate and fluorescent beads displacements as previously described.⁴⁰ Spatial displacement of the beads was tracked by taking images of the fluorescent beads before and after cell trypsinization. The cell traction mapping was based on the DIC image of cells on PA substrate and the corresponding traction stress mapping in pseudocolor, which indicated regions of traction stresses (dark blue to light pink according to stresses from low to high). Briefly, the traction force at discrete point f_i , located at the

position (x_i, y_i) was calculated based on the following formulation: $\vec{u}_t(x, y) = \sum_{i=1}^n \vec{G}(x - x_i, y - y_i) \vec{f}_i$, where $\vec{G}$ denoted the Greens' tensor and $\vec{u}_t$ denoted the experimental displacements of fluorescent beads at position (x_i, y_i) .

The overall force of the cell (F) is an integral of the traction field magnitude over the area, $F = \iint \sqrt{T_x^2(x, y) + T_y^2(x, y)} dx dy$, where $T(x, y) = [T_x(x, y) + T_y(x, y)]$ is the continuous field of traction vectors defined at any spatial position (x, y) within the cell.

Transwell invasion assay. 5×10^4 cells were dispensed into the top chamber of transwell migration chambers (8 μ m Millicell, Billerica, MA, USA; Hanging Cell Culture Inserts) loaded on a 6-well plate and allowed to attach overnight in cell culturing conditions. One thousand microliters of complete medium (supplemented with 10 ng/ml TGF- β 1) was added to each of the underlying wells. For control wells, no TGF- β 1 was supplemented. This construct was then incubated for 48 h. Following incubation, the inserts were washed with PBS and fixed with 1% glutaraldehyde for 10 min. The inserts were rinsed with PBS and subsequently stained in 0.1% crystal violet for 25 min. Cells were thoroughly rinsed with PBS after crystal violet staining. Cotton buds were used to swipe the upper surface of the transwell membrane, ensuring thorough removal of unmigrated cells. The inserts were then immersed in a 500 μ l of 0.5% Triton-X solution dispensed in a clean 6-well plate and incubated overnight in the dark. The 6-well plate was sealed with parafilm tape to prevent evaporation. Absorbance of the 500 μ l volume of 0.5% Triton-X at 595 nm was then measured using Nanodrop. The absorbance difference for the test and control wells were used as an index for the comparison of cellular migration.

Conflict of Interest

The authors declare no conflict of interest.

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SUPPLEMENTAL INFORMATION

Loss of TAK1 increases cell traction force in a ROS-dependent manner to drive epithelial-mesenchymal transition of cancer cells.

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Running title: TAK1 modulates epithelial-mesenchymal transition.

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Figure S1

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Supplementary Figure Legends

Figure S1. Comparison of cellular proliferation and death in A5RT3_{CTRL} and A5RT3_{TAK1} cells.

(A) The expression levels of TAK1 mRNA and protein in A5RT3_{CTRL} and A5RT3_{TAK1} cells.

(B-C) Immunoblot analysis of TAK1 downstream signaling mediator, NF κ B p65 subunit (B) and ERK and JNK (C) in cells treated with cytokine IL-1 at 20 ng/ml prior to analysis. β -tubulin immunoblotting showed equal loading and transfer. Values below each band represents mean fold change compared with control.

(D) FACS analysis of proliferation rate in A5RT3_{CTRL} and A5RT3_{TAK1} cells as determined by BrdU labelling. Results were gated to show percentage of cell population in the various mitotic phases (ie. G1, S and G2/M). Quadrant percentages are indicated in the respective dot plots.

(E) Phase contrast microscopic images of TAK1-knockdown HSC-5, II4 and MKN28 treated with TGF β 1 (10 ng/ml) for 48 h. Scale bar: 100 μ m.

(F) Representative immunoblots of indicated EMT markers of cell samples mentioned in (E). Values below each band represents mean fold change compared with control.

For immunoblotting, β -tubulin serves as loading control.

Figure S2. TAK1 overexpression in A5RT3_{TAK1} rescued TGF β 1-induced EMT.

(A) Phase contrast images of A5RT3_{TAK1} transfected with expression vector containing TAK1 cDNA and treated with TGF β 1 (10 ng/ml) for 48 h. Scale bar: 100 μ m.

(B) qPCR analysis of EMT markers in TAK1-transfected A5RT3_{TAK1} cells with and without TGF β 1 treatment. Samples were normalized with reference gene, L27.

(C) Immunoblot analysis of TAK1-transfected A5RT3_{TAK1} cells for the overexpression of TAK1 and EMT phenotype upon TGFβ1 treatment. Representative blots were shown with respective densitometry values indicated below lanes, normalised with respect to the control. Samples were normalized with β-tubulin as a loading control.

Data represent means± S.D; n=3.

Figure S3. TGFβ1-induced EMT is effected through SMAD signaling.

(A) Phase contrast images of SMAD3 siRNA transfected A5RT3_{CTRL} cells with 72 h of TGFβ1 (10 ng/ml) treatment. Scale bar: 100μm.

(B) qPCR analysis of EMT markers in SMAD3 siRNA transfected A5RT3_{CTRL} cells with and without TGFβ1 treatment. Samples were normalized with reference gene, L27.

(C) Immunoblot analysis of SMAD3 siRNA transfected A5RT3_{CTRL} cells for their knockdown and EMT markers upon TGFβ1 treatment. Representative blots were shown with respective densitometry values indicated below lanes, normalised with respect to the control. Samples were normalized with β-tubulin as a loading control.

(D) Immunoblot analysis of phosphorylated SMAD3 in SMAD3-knockdown A5RT3_{CTRL} cells. Values below each band represents mean fold change compared with control. For immuoblotting, β-tubulin serves as loading control.

Data represent means± S.D; n=3.

Figure S4. Determination of optimal NAC concentration.

(A-B) Apoptosis assay by annexin V/PI staining (A) and CM-H2DCFDA staining (B) of A5RT3_{TAK1} cells treated with indicated concentrations of NAC as determined by FACS. Numbers indicate percentages of cells in the respective quadrants.

(C) Phase contrast microscopy images of A5RT3_{CTRL} cells after 48 h of 100 μ M NAC treatment.

(D) Representative immunoblots indicated EMT markers in A5RT3_{CTRL} cells treated NAC.

Values below each band represents mean fold change compared with control. For immunoblotting, β -tubulin serves as loading control.

Data represent means $\pm$ S.D; n=3.

Figure S5. Effect of different concentration of Y27632 in ROCK activity.

(A) Representative immunoblots of phosphorylated ROCK in A5RT3_{CTRL} treated with indicated concentration of ROCK inhibitor Y27632. Values below each band represents mean fold change compared with control. β -tubulin serves as loading control.

(B) Relative protein expression of RhoA in siRNA RhoA-knockdown A5RT3 cells treated with or without TGF- β 1.

Data represent means $\pm$ S.D; n=3.

Supplemental Table 1- qPCR Primer sequences used in this study.

TAK1 qPCR primer 1	GTTCCGTGTAAGGGCTTTGA
TAK1 qPCR primer 2	GAGCAGCTGCCACTTACCTTTACA
E-cadherin qPCR primer 1	GCCGAGAGCTACACGTTCA
E-cadherin qPCR primer 2	GACCGGTGCAATCTTCAA
N-cadherin qPCR primer 1	TGCGGTACAGTGTAAGTGGG
N-cadherin qPCR primer 2	GAAACCGGGCTATCTGCTCG
Vimentin qPCR primer 1	GTTTCCCCTAAACCGCTAGG
Vimentin qPCR primer 2	AGCGAGAGTGGCAGAGGA
SNAIL qPCR primer 1	TCGGAAGCCTAACTACAGCGA
SNAIL qPCR primer 2	AGATGAGCATTGGCAGCGAG
L27 qPCR primer 1	TGATGGCACCTCAGATCGC
L27 qPCR primer 2	AGAGTACCTTGTGGGCATTAGG

Reprogramming of Cellular Bioenergetics Primes Cancer Cells for Epithelial-Mesenchymal Transition

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Reprogramming of cellular Bioenergetics Primes cancer cells for Epithelial-Mesenchymal Transition

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Summary

Background: Epithelial-mesenchymal transition (EMT) empowering cancer cells with enhanced invasive capabilities. Although molecular and cellular changes are well-studied during EMT, little is known about bioenergetics changes during EMT and the specific molecular players that reprogram cancer cell bioenergetics to drive EMT.

Methods: Three *in vitro* EMT model, a novel dual-inducible EMT mouse model and clinical patient's samples were used to characterize bioenergetics changes during EMT. Cellular levels of ATP, ADP and AMP were measured using reverse-phase HPLC and used to determine cancer cells' energy charge status after indicated treatment. Comparative microarray analysis and gene knock-down assays were used to identify critical players that reprogrammed cancer cell metabolism during EMT. The mechanism was elucidated using a combination of proximity ligation assay, immunofluorescence, immunoblots and protein and chromatin immunoprecipitation.

Results: We found that EMT is an energy-demanding process fueled by glucose metabolism-derived ATP. We identified angiopoietin-like 4 (ANGPTL4) as an important driver of cancer cell metabolism within a larger EMT gene program. ANGPTL4-knockdown suppresses energy charge elevation, delaying EMT and reduces cancer metastasis to the lung and liver. Unbiased kinase inhibitor screens revealed that ANGPTL4 differentially regulates the expression of 14-3-3 adaptor proteins to synchronize metabolic shift necessary for EMT.

Conclusions: Our study shows that metabolic reprogramming is necessary to prime cancer cells for EMT competency. We identified a novel ANGPTL4:14-3-3 signaling axis which coordinates energy demand and stability of critical EMT proteins necessary to drive EMT and promote metastasis. These findings offer new therapeutic opportunities for metastasis with important clinical implications.

Introduction

Metastasis, the spread of cancer cells to distant tissues and/or organs, is a complex and multi-step process, making it a challenging therapeutic target (1). Metastasis accounts for more than 90% of cancer mortality (2). Initiation of the metastatic process resembles the epithelial-mesenchymal transition (EMT) that occurs during embryogenesis and wound healing (3-5). During EMT in cancer, epithelial cells lose their cell polarity and cell-cell adhesions are weakened as they adopt a mesenchymal phenotype. This change is accompanied by cytoskeletal remodeling, ultimately allowing the cells to gain motility and the ability to invade distant organs (5). These energy-demanding processes during cancer EMT suggest that changes in cellular metabolism may be required to drive the EMT event.

Metabolic reprogramming has been recognized as one of the 10 hallmarks of cancer (6). Our current understanding of cancer metabolism is based primarily on metabolic status comparison between primary tumors and normal healthy cells. Cancer cells derive a substantial amount of ATP from aerobic glycolysis (i.e., the Warburg effect) instead of oxidative phosphorylation to support their anabolic growth and proliferation (7). Cancer cells also exploit glutaminolytic flux, amino acid and lipid metabolism, mitochondrial biogenesis, the pentose phosphate pathway, and macromolecular biosynthesis to progress malignancy (8,9). Recent studies have suggested that oncogenes and tumor suppressors function as critical modulators of metabolic reprogramming to support tumor progression (10,11). Despite the significance of EMT in metastasis, little is known about the changes in cellular bioenergetics that occur during this process and whether such metabolic reprogramming is a functional prerequisite.

The exponential growth of the primary tumor exposes cancer cells to various microenvironmental stresses, including hypoxic, acidic, and inflammatory milieus (3,4). Interestingly, though these constraints can kill cancer cells, they also exert selective pressures

on cells to exploit adverse microenvironments by modifying their cellular behavior and selecting for a highly invasive phenotype that facilitates metastasis (12). Microenvironmental stimuli initiate the EMT process by controlling the expression and function of a myriad of molecular players, such as Snail, ZEB-1, Wnt ligands and matricellular proteins which are pivotal for EMT (3,13,14). However, majority of these players are unable to initiate EMT alone, suggesting that there are unique molecular drivers capable of driving the EMT event (15-17). Interestingly, hypoxia and TGF- β , which are known initiators of EMT, can also modulate cancer cell metabolism during tumorigenesis (12,18). Hence, whether these microenvironmental signals can influence specific molecular players to reprogram cancer cell bioenergetics and drive EMT remains unknown.

Here, we show how EMT is coordinated with cancer cell metabolism. Using *in vitro*, *in vivo* EMT models, stage-specific human tumor biopsies, comparative microarray analysis, and energy charge measurements, we showed that reprogramming of cancer cells bioenergetics is required for EMT. We further identified angiopoietin-like 4 (ANGPTL4) as a key player in cellular bioenergetics and elucidated the mechanism by which it coordinates the energy demands required for EMT.

Results

Altered cancer cell bioenergetics during EMT

To examine whether EMT utilizes cellular energy, we carried out a series of molecular and biochemical analyses to examine changes in cellular bioenergetics during EMT. First, we established three *in vitro* EMT models using the polarized gastric carcinoma cell line MKN74. In two models, EMT was initiated by either hypoxia (1% O₂, Figure 1A) or TGF- β (Supplementary Figure 1A, available online) to mimic the microenvironment of stress-induced EMT. The third model utilized the transgenic MKN74 cell line harboring a Snai1-ER transgene (MKN74_{Snai1ER}) that allows direct initiation of EMT by 4-hydroxytamoxifen (4-OHT) (Figure 1B; Supplementary Figure 1B, available online). Upon exposure to either microenvironmental stress or 4-OHT, the epithelial colonies of MKN74 cells scattered and adopted a mesenchymal-like phenotype, with diminished E-cadherin staining bordering the epithelial cells, after 48 h (Figure 1A; Supplementary Figure 1A, available online) and 96 h (Figure 1B), respectively. qPCR and immunoblotting for critical EMT genes showed downregulation of epithelial genes (CDH1; DDR1; ErBb3), with a concomitant increase in mesenchymal gene expression (Snai1; ZEB-1; Figure 1, A and B; Supplementary Figure 1A, available online). Similar EMT phenotype was also observed in MCF7, II4, HSC, and HepG2 cancer cell lines (Supplementary Figure 4D-G, available online).

Cancer cells are known to exploit aerobic glycolysis to metabolize glucose to lactate instead of pyruvate, resulting in the acidification of the extracellular environment (7). We observed increased glucose uptake and extracellular acidification rate in MKN74_{CTRL} and MKN74_{Snai1ER} cells during EMT, suggesting that glycolysis was augmented (Figure 1, C and D). *In vivo* 2-deoxyglucose (2-DG) imaging of our inducible EMT xenograft mice, transplanted with MKN74_{Snai1ER} cells, showed similar elevation in glucose uptake in 4-OHT-

treated tumors compared to vehicle-treated tumors (Figure 1E). To further confirm that cellular metabolism was altered during cancer EMT, we investigated the energy charge status of the cells which is indicative of the cellular metabolic activity (19-21). We observed an increased energy charge in MKN74_{CTRL} and MKN74_{Snai1ER} cells during EMT progression, which mirrored the increased glycolytic activity (Figure 1F). This increase in energy charge during EMT was also observed in four different cancer cell lines and our *in vivo* xenograft tumors (Figure 1, G and H). Importantly, clinical samples showed significant correlations between the energy charge status and tumor grades, with the higher grade metastatic tumors exhibiting a higher energy charge (Figure 1I; Supplementary Table 3). These observations suggest that cancer cells undergo metabolic reprogramming during EMT and the increased cellular bioenergetics could fuel the aggression of the metastatic cancer cells.

Metabolic reprogramming enhanced metastatic capability of cancer cells

Cancer EMT is often accompanied by energy-intensive processes like cytoskeletal remodeling and enhanced drug resistance, ultimately transforming these cancer cells into malignant forms with superior survivability and invasiveness (22-25). The observations above prompted us to examine whether alteration of cancer cellular bioenergetics can influence malignant behavior. The PI3K/AKT and AMPK pathways are two major regulatory nodes for cellular bioenergetics (26). Therefore, dominant-negative and constitutive forms of AKT and AMPK were introduced into MKN74_{CTRL} and MKN74_{Snai1ER} cells (Supplementary Figure 2A, available online). Constitutive active AKT (caAKT) and AMPK (caAMPK) increased energy charge status of both MKN74_{CTRL} and MKN74_{Snai1ER} cells (Figure 2A-D). Conversely, dominant-negative AKT (dnAKT) and AMPK (dnAMPK) resulted in a decrease in the energy charge status of these cells (Figure 2A-D). In the absence of EMT-inducing signals, the colony morphology of MKN74 cells harboring caAKT (MKN74_{caAKT}), dnAKT (MKN74_{dnAKT}), caAMPK (MKN74_{caAMPK}) and dnAMPK (MKN74_{dnAMPK}) resembled that of

MKN74_{CTRL} cells. Interestingly, upon exposure to hypoxia or 4-OHT, MKN74_{caAKT} and MKN74_{caAMPK} cells exhibited a more pronounced EMT phenotype, as early as 24 h after hypoxia exposure, compared to 48 h in cognate MKN74_{CTRL} cells (Figure 2, E and F). The dominant-negative forms of AKT and AMPK suppressed energy charge elevation, delaying EMT progression (Figure 2A-F; Supplementary Figure 2B, available online). These observations suggest that the consolidation of cellular bioenergetics may be a pre-requisite to prime the cancer cells for EMT.

To gain further insights into the biological significance of altered cancer cellular bioenergetics during EMT, we created an inducible EMT xenograft mouse model using MKN74_{Snai1ER} cells. Histological analysis of tumor sections revealed a compact vehicle-treated control tumors with clear encapsulation of the tumor mass, defined by strong distinct E-cadherin and basement membrane laminin 332 staining within the tumors (Figure 2G; Supplementary Figure 2C, available online). Conversely, 4-OHT-treated tumors appeared dispersed with diminished E-cadherin staining at the cell-cell borders and clear disruption of basement membrane laminin 332 compared to vehicle-treated tumors, indicative of an aggressive tumor phenotype (Figure 2G; Supplementary Figure 2C, available online). Indeed, elevated activities of pro-invasive matrix metalloproteinases (MMPs)-2 and -9 were detected in 4-OHT-treated tumors and these mice harbored significantly more lung metastases than the vehicle control mice (Figure 2, H and I). Likewise, we also observed improved xenobiotic resistance of cancer cells during EMT. One of the most important resistance mechanisms is the active efflux of anti-cancer drugs via the ATP-binding cassette (ABC) transporter family. Drug efflux was enhanced during EMT and the exposure of MKN74_{CTRL} and MKN74_{Snai1ER} cells to chemotherapeutic drug, cisplatin, during EMT resulted in a significant reduction in cellular apoptosis (Figure 2, J and K). The ABC transporters expression was also elevated during EMT as revealed by focus array analysis (Supplementary Figure 2D, available online).

This was further confirmed by immunoblot analysis of multidrug resistant receptor 1 (MDR1), a major ABC transporter in tumor (Figure 2, L and M).

Altogether, our findings indicated that the increased cellular bioenergetics during EMT could fuel malignant transformation, conferring enhanced invasive and chemoresistant capabilities to the cancer cells.

ANGPTL4 regulates cellular bioenergetics during EMT

EMT induction signals could influence various molecular players to modify cancer cells behaviors and drive EMT. To identify driver genes involved in metabolic reprogramming during EMT from differentially expressed genes associated with cellular metabolism, we performed knockdown experiments followed by microarray analysis. We observed an unanimous enrichment of metabolic regulatory genes, particularly in glucose, fatty acid, autophagy, amino acid, and ROS metabolism, as early as 24 h after EMT across the three *in vitro* EMT models (Figure 3, A and B). qPCR and immunoblot analysis of selected metabolic genes and ROS production measurements further confirmed that these processes were enhanced during EMT, which could contribute to the elevated energy charge of the cancer cells during EMT (Figure 3, C and D; Supplementary Figure 3B, available online). Interestingly, we found elevated angiopoietin-like 4 (ANGPTL4) expression in several gene clusters across all EMT models. ANGPTL4 protein levels, specifically the C-terminal fibrinogen-like form (cANGPTL4), were elevated during EMT in both *in vitro* models and *in vivo* xenograft tumors (Figure 3, E and F; Supplementary Figure 3C, available online). Furthermore, clinical analysis revealed that metastatic cancers expressed higher ANGPTL4 expression, which suggested important roles for ANGPTL4 in regulating cellular bioenergetics during EMT (Figure 3G).

ANGPTL4 has well-defined roles in peripheral metabolism, however, its roles in cellular metabolism remains unknown. The suppression of ANGPTL4 resulted in significant

alteration of >50% of genes identified in our microarray analysis (Figure 3B). Treatment with either neutralizing antibodies (α -cANGPTL4) or siRNA against ANGPTL4 (siANGPTL4) diminished glucose uptake and energy charge elevation in MKN74_{CTRL}, MKN74_{Snai1ER} and 4 other cancer lines during EMT. Conversely, the addition of exogenous rh-cANGPTL4 alone was able to rescue glucose uptake and energy charge status (Figure 3H-K; Supplementary Figure 3C-F, available online). To further understand the role(s) of ANGPTL4 in controlling cancer cells' bioenergetics, we created a dual-inducible EMT xenograft mouse model. A doxycycline (Dox)-inducible shANGPTL4 expression plasmid was introduced into MKN74_{Snai1ER} cells to generate the dual-inducible MKN74_{Snai1ER:shANGPTL4} cell line prior to xenograft (Supplementary Figure 3G, available online). *In vivo* xenograft tumors exhibited a similar elevation in glucose uptake and energy charge status (~1.5-fold) during EMT progression, and this elevation was diminished by Dox-diet-induced ANGPTL4 depletion (Figure 3L; Supplementary Figure 3H, available online). These changes in the cellular energy charge corresponded with the intracellular ATP levels, lending further credence to the importance of ANGPTL4 in regulating cellular bioenergetics during EMT (Supplementary Figure 3I-K, available online).

Metabolic reprogramming by ANGPTL4 augments EMT

Our observations above prompted us to investigate whether metabolic reprogramming by ANGPTL4 could influence the malignant transformation of cancer cells during EMT. The depletion of ANGPTL4 delayed EMT, with the cells retaining their epithelial-like morphology characterized by clear E-cadherin staining and reduced nuclear accumulation of Snai2 (Figure 4, A and B; Supplementary Figure 4, A and H available online). qPCR and immunoblot analysis revealed a delay in the expression of critical EMT genes (Figure 4, A and B; Supplementary Figure 4B, D-G, available online). Interestingly, MKN74 cells treated with recombinant human cANGPTL4 (rh-cANGPTL4) resulted in the repression of epithelial

markers and significant elevation of mesenchymal markers (Figure 4C; Supplementary Figure 4C, available online).

The depletion of ANGPTL4 in our dual-inducible EMT xenograft model rescued E-cadherin and laminin 332 expression in the tumors compared to the 4-OHT-treated tumors (Supplementary Figure 4I, available online). Pro-invasive MMPs activities and secondary metastases in the lungs were also significantly decreased (Figure 4, D and E). Furthermore, immunofluorescence analysis of benign and metastatic mouse skin tumor sections revealed that metastatic tumors expressed high levels of ANGPTL4 that is correlated to diminished E-cadherin expression (Figure 4F). Finally, the depletion of ANGPTL4 was able to sensitize MKN74_{CTRL} and MKN74_{Snai1ER} cells to xenobiotics and suppressed MDR1 expression during EMT (Figure 4G-I). These observations suggest that ANGPTL4 can augment malignant transformation by controlling cellular bioenergetics to drive EMT.

ANGPTL4 regulates specific 14-3-3 expression

The 14-3-3 protein family consists of adaptor proteins that play critical roles in numerous important cellular functions via binding to specific phosphorylation signals on target proteins (27). During wound healing, keratinocytes adopt a migratory mesenchymal phenotype highly reminiscent of cancer EMT (28). We demonstrated that ANGPTL4 can influence the expression of specific members of the 14-3-3 protein family during wound healing. Thus, we examined the expression of all seven 14-3-3 protein isotypes during cancer EMT. In response to EMT-inducing signals, MKN74_{CTRL} and MKN74_{Snai1ER} cells exhibited increased expression of 14-3-3 γ , η , and τ and decreased expression of 14-3-3 σ , whereas the remaining 14-3-3 isotypes were unchanged (Figure 5, A and B). Suppression of ANGPTL4 resulted in a significant decrease in the expression of 14-3-3 γ compared to 14-3-3 η and τ , whereas the exposure of MKN74_{CTRL} cells to rh-cANGPTL4 enhanced 14-3-3 γ expression (Figure 5, A and C). Notably, tumor biopsies from patients exhibited similar alterations in 14-

3-3 γ expression as in four other cancer cell lines during EMT (Figures 5I; Supplementary Figure 5B-E, available online). These observations suggest that ANGPTL4 may exert its influence through these 14-3-3 isotypes to reprogram cancer cell bioenergetics and drive EMT.

Although a multitude of studies demonstrated the importance of 14-3-3 in various signaling pathway, how specific 14-3-3 expression are regulated remains unclear. Exposure of MKN74_{CTRL} cells to rh-cANGPTL4 increased the expression of 14-3-3 γ , η , and τ mRNA within 6 hours, while 14-3-3 σ expression was suppressed (Supplementary Figure 5A, available online). To obtain insight into the signaling pathways involved in ANGPTL4-mediated expression of 14-3-3 isotypes, we performed an unbiased kinase inhibitor screen to identify key signaling mediators whose inhibition would suppress ANGPTL4-mediated up-regulation of 14-3-3 expression. qPCR followed by Ingenuity Pathway Analysis showed that the majority of involved kinases converged on the PI3K/AKT and MAPK signaling cascades, implicating several transcription factors known to be involved in cancer progression (Figure 5D). We further showed that MKN74 cells exposed to hypoxia, 4-OHT and exogenous rh-cANGPTL4 displayed increased phospho-activation of AKT, whereas the depletion of ANGPTL4 opposed this effect during hypoxia-induced EMT (Figure 5E-G). Analysis of four different cancer cell lines, xenograft EMT tumors and tumor biopsies from patients revealed similar phospho-activation of AKT dependent on ANGPTL4 (Figure 5I; Supplementary Figure 5B-E, available online). Importantly, immunoblot analysis of the identified transcription factors showed that three of these transcription factors, namely CREB, cFOS, and Stat3, were phospho-activated during EMT (Figure 5J). These observations confirm that ANGPTL4 can activate specific transcription factors via the PI3K/AKT pathway to differentially regulate specific 14-3-3 isotypes.

The ANGPTL4:14-3-3 γ axis coordinates energy demand during EMT

To further decipher how ANGPTL4:14-3-3 γ axis control cellular metabolism during EMT, we interrogated the ANnotation and Integrated Analysis of the 14-3-3 interactome (ANIA) database to find key signaling mediators of cancer cell metabolism and pivotal transcription factors in EMT that could interact with 14-3-3 (29). Remarkably, our analysis identified tuberlin sclerosis complex (TSC2) and Snai1 as highly ranked interaction partners for 14-3-3. TSC2 is an important gatekeeper of cellular metabolism that integrates extracellular signals to direct anabolic or catabolic activities (30,31). Depending on the TSC2 phosphorylation sites, 14-3-3 can bind and inhibits TSC2 activities. In our experiment, inhibitory TSC2 phosphorylation at Ser939 (TSC2_{S939}) was increased during EMT (Supplementary Figure 6A-C, available online). Co-immunoprecipitation and proximity ligation assay (PLA) revealed increased 14-3-3 γ :TSC_{S939} complex formation, indicating that TSC2 was suppressed during EMT (Figure 6A-D; Supplementary Figure 6E-F and 7A-C, available online). Conversely, ANGPTL4 deficiency diminished 14-3-3 γ :TSC_{S939} complex formation (Figure 6A-D; Supplementary Figure 6E-F, available online). These observations revealed a novel ANGPTL4:14-3-3 signaling axis that exerts significant influences to control cellular bioenergetics during EMT.

In vitro co-immunoprecipitation analysis and PLA of Snai1 and 14-3-3 revealed a significant increase in 14-3-3 γ :Snai1 complex formation during hypoxia-induced EMT. This interaction was diminished in the absence of ANGPTL4, while Snai1:14-3-3 η/τ complex formation remained unchanged during EMT (Figure 6E-H; Supplementary Figure 6G-H, available online). Similarly, PLA analysis revealed significantly more 14-3-3 γ :Snai1 complexes in 4-OHT-treated tumors than in vehicle-treated tumors from mice fed a normal diet (Figure 6G). Tumor sections from 4-OHT-treated mice fed a Dox diet exhibited fewer 14-3-3 γ :Snai1 interactions than mice fed a normal diet (Figure 6G). PLA analysis of tumor biopsies from patients further confirmed that higher grade, more aggressive tumors harbored

significantly more 14-3-3 γ :Snail complexes than their benign counterparts (Figure 6H; Supplementary Figure 7D-F, available online). The interaction of 14-3-3 with Snail was proposed to stabilize Snail binding on the E-cadherin promoter to facilitate transcriptional repression during EMT (32). We showed that the reduction in 14-3-3 γ :Snail complex in MKN74 Δ ANGPTL4 cells resulted in decreased Snail occupancy of the E-cadherin promoter during hypoxia-induced EMT, indicating that the repression of E-cadherin gene expression was impaired. Furthermore, EMT was delayed in the absence of ANGPTL4, highlighting the importance of 14-3-3 γ :Snail complexes during malignancy (Figure 6I).

Taken together, our data revealed a previously unknown mechanism where the ANGPTL4:14-3-3 γ signaling axis coordinates energy demand and stability of critical EMT proteins necessary to drive EMT (Figure 6J).

Discussion

The EMT is the first step in metastasis initiation and confers invasive characteristics to cancer cells (4). The chronic exposure of cancer cells to microenvironmental stresses, such as hypoxia and inflammation, allows the selection of EMT-competent cells in cancer (33). Incidentally, these stresses also modify cancer cell metabolism to sustain their growth potential (26). However, whether such metabolic reprogramming is a prerequisite for EMT remains unclear. Using a combination of *in vitro*, *in vivo* and clinical models, we found that reprogramming and consolidation of cellular bioenergetics are required to prime cancer cells for EMT. Furthermore, the elevated cellular bioenergetics can fuel malignant transformation and enhanced cancer metastasis. Conceivably, there may be molecular drivers that modulate metabolic reprogramming and prime these cancer cells for EMT competency. Microarray analysis of three EMT models revealed ANGPTL4 as an important molecular driver of metabolic changes during EMT. We went on to demonstrate that ANGPTL4 exert its influence through a novel ANGPTL4:14-3-3 signaling axis to coordinate energy demand and stability of critical EMT proteins necessary to drive EMT.

Metastasis requires the cooperative effects of multiple signaling events and biological progresses (3,33). While the role of cellular metabolism is well-known during cancer cell proliferation, its role during malignant progression remains unclear. During metastasis initiation, the primary cancer cells undergo major alterations in cell morphology and functions to gain a pro-invasive and chemoresistance phenotype. We demonstrated that during EMT, the cancer cells increase glucose uptake and aerobic glycolysis to reprogram their cellular bioenergetics, which may be necessary to fuel the acquisition of these malignant traits. The active remodelling of the actin cytoskeleton requires ATP binding and hydrolysis to confer invasive abilities to the cancer cells (22,23). Similarly, the enhanced chemoresistance involved active efflux of various chemotherapeutic compounds from cancer cells (24). Thus, it

is conceivable that metabolic reprogramming can prime the cancer cells for EMT. Interestingly, when we enhanced the cancer cellular biogenesis by constitutively activating PI3K/AKT and AMPK pathways, these cancer cells showed accelerated transition into mesenchymal forms. Importantly, the suppression of cellular bioenergetics hindered EMT progression, suggesting that the alteration of cellular bioenergetics during EMT primes these cancer cells for increased mobility and xenobiotic-resistance. Moreover, the loss of E-cadherin has been shown to augment glycolysis to favor EMT. Thus, our study revealed that the consolidation of cellular bioenergetics is essential for metastasis initiation.

Cancer cells are known to express higher ANGPTL4 levels compared to their normal counterparts. As the tumor progresses, the chronic exposure of the cancer cells to microenvironmental signals can trigger further elevation in ANGPTL4 expression. This suggests that ANGPTL4 could play significant roles during malignant progression (34-37). Many studies have linked ANGPTL4 to cancer metastasis, from enhancement of metastatic cells motility, angiogenesis to the disruption of vascular integrity to facilitate distal dissemination. Although ANGPTL4 has well-established roles in peripheral metabolism, its role in cellular metabolism is unknown. By demonstrating ANGPTL4 as a molecular driver of metabolic changes during EMT, we provide a novel insight for its function in pathological metabolism. By suppressing ANGPTL4, we are able to reconfigure cancer cell metabolism and delay EMT. *In vitro* and *in vivo* EMT models showed that cancer cells deficient in ANGPTL4 were unable to rewire aerobic glycolysis to increase cellular energy charge during EMT, resulting in reduced metastasis. These suggest that ANGPTL4 can consolidate the cellular bioenergetics of cancer cells and prime them to an “excited” state for EMT. Notably, all of these changes were also detected in clinical tumor biopsies and strongly correlated with the stage and aggressiveness of the tumors, further underscoring the clinical relevance of our

findings. It will be interesting to explore if cancer cells within tumors have energetic “threshold” for EMT competency.

A key mechanistic insight is that ANGPTL4 regulates the expression of specific isoforms of 14-3-3 and contributes to an important new signaling axis that coordinates multiple biological processes for metastasis. Interestingly, the functions of many mediators in autophagy, glycolysis, survival, and cell cycle, among others, are known to be regulated by the 14-3-3 protein family (27,38). The expression of 14-3-3 protein was also aberrantly up-regulated in numerous tumor types (39-41). However, due to the diverse influences 14-3-3 proteins has over critical cellular pathways, the development of inhibitors targeting 14-3-3 remains a challenge (39-41). In contrast, ANGPTL4 is a secreted extracellular protein that can be easily targeted by either small molecules or antibodies. Furthermore, the elevated expression of ANGPTL4 and its multi-faceted roles in cancer makes it an attractive therapeutic target. Therefore, through the inhibition of ANGPTL4, we may be able to apply a multi-prong approach to inhibit tumor progression and EMT, keeping the tumors in a low-grade status, potentially increasing therapeutic efficacy of cancer treatments.

Our study shows that metabolic reprogramming in cancer cells is a pivotal event in the progression of EMT. Our findings identified the involvement of the ANGPTL4:14-3-3 signaling axis that coordinates multiple biological processes for metastasis and revealed a previously unknown role for ANGPTL4 as a molecular driver of cellular metabolism in the EMT to promote metastasis with important clinical implications.

Experimental Procedures

Antibodies. Antibodies for E-cadherin, pan-AKT, p-AKT (S473), AMPK, p-AMPK (T172), TSC2, p-TSC2, CREB, p-CREB, cFOS, p-cFOS, ELK1, p-ELK1, STAT3, p-STAT3 and various 14-3-3 isotypes (Cell Signaling, USA); N-cadherin and Snai2 (Millipore, USA); β -tubulin, Snai1, HIF-1 α and 14-3-3 σ (Santa Cruz, USA); Vimentin, laminin 332 and mouse monoclonal anti-human cANGPTL4 mAb11F6C4 (Abcam, USA); pan-cytokeratin and IRdye 680-conjugated secondary antibodies (Thermo Scientific, USA); Alexa Fluor 488-conjugated secondary antibodies and Alexa Fluor 594-conjugated phalloidin (Molecular Probes, USA). Rabbit polyclonal anti-human cANGPTL4 were produced in-house.

Cell culture. MKN74 human gastric carcinoma (ATCC, USA) were cultured in RPMI-1640 supplemented with 10% FBS in a humidified atmosphere of 5% CO₂ at 37 °C. All other cell lines were cultured in DMEM with 10% FBS. All ANGPTL4 knock-down cells were generated as per manufacturer's protocol (Thermo Scientific, USA) with DharmaFECT transfection reagent and Smartpool siRNA against ANGPTL4. AKT activity was measured using the AKT activity kit (Cell Signaling, USA).

Human tumor biopsies. Fresh frozen breast, colon, gastric and head and neck tumor biopsies (Proteogenex, USA) were used for protein, energy charge and immunofluorescence analysis.

Retrovirus transduction. MKN74_{Snai1ER} cells were generated using retroviral transduction as previously described (42). Briefly, HEK 293T cells were transfected with pCL-10A1 retrovirus packaging vector (Imgenex, USA) and pWZL-Snai1-ER (Addgene, USA).

Harvested pseudoviruses were used to infect MKN74 cells. Following transduction, cells were selected with 5 µg/mL of blasticidin hydrochloride (Sigma Aldrich, USA).

Generation of dual-inducible MKN74_{Snai1ER/shANGPTL4} cell line. Doxycycline-inducible pSingle-tTS-shRNA vector carrying either shANGPTL4 or scrambled shRNA was introduced into MKN74_{Snai1ER} cells using Fugene HD reagent as per manufacturer protocol (Promega, USA). MKN74_{Snai1ER/shANGPTL4} and MKN74_{Snai1ER/shscrambled} cells were selected with 1 mg/mL of G418 (Sigma Aldrich, USA). The efficiency of knockdown was verified by qPCR and immunoblotting after treatment with various concentrations of doxycycline (up to 10 µg/mL).

***In vitro* induction of EMT.** Cells were seeded at a density of 5.25×10^2 cell/cm² to allow the formation of individual colonies before indicated treatments. Hypoxia treatments were performed in hypoxic chamber (Stem cell Technology, USA) purged with 5% CO₂ and 95% N₂ to obtain O₂ concentration at 1% as determined by an O₂ sensor. For TGF-β-induced EMT, cells were treated with 10 ng/mL of TGF-β for 2 days. For Snail-mediated EMT, MKN74_{Snai1ER/shANGPTL4} and MKN74_{Snai1ER} cells were exposed to 20 ng/mL of 4-hydroxytamoxifen (4-OHT) for 4 days.

***In vivo* induction of EMT.** Six-week-old BALB/c athymic female nude mice (20-22 g) were injected subcutaneously with either 1×10^6 MKN74_{Snai1ER/shANGPTL4} or MKN74_{Snai1ER/shscrambled} cells resuspended in matrigel. A week after xenograft was established, each xenograft group was randomly divided into four subgroups. Two subgroups received intraperitoneal injection of 4-OHT (4 mg/kg) and were fed on either normal chow diet or Dox-diet (625 mg/kg, Harlan Laboratories, USA). The other two subgroups received vehicle and similarly fed on the two different diets. Injection was done twice weekly using sunflower seed oil as carrier for 4-

OHT. After 8 weeks, the mice were sacrificed. The tumors and lungs were harvested for further analysis. Animal experiments were carried out in accordance to the guidelines of the University Institutional Animal Care and Use Committee (IACUC, ARF-SBS/NIE-A0250AZ), Singapore.

Chromatin-immunoprecipitation (ChIP) and Re-ChIP. ChIP experiments were carried out as previously described with slight modifications (43). Briefly, chromatin was cross-linked using 0.5% formaldehyde for 10 min at 37°C and sonicated in SDS lysis buffer (1% SDS, 10mM EDTA, and 50mM Tris-HCl, pH 8.1) to obtain DNA fragments of 200-500 bp. 10% of the supernatant was retained as input, while the remaining amount was immunoprecipitated using anti-Snail antibody (Santa Cruz, USA) and immunocomplexes were affinity precipitated by Protein A/G Sepharose (Santa Cruz, USA). Re-ChIP was performed by subsequent probing with anti-14-3-3 γ antibody (Thermo Scientific, USA). DNA fragments were reverse cross-linked at 65 °C for 6 h. The ChIP primer sequences are listed in Supplementary Table S1.

Energy charge determination. The cellular levels of AMP, ADP and ATP were determined by HPLC. Nucleotides were extracted as previously described (44). Nucleotides standards (Sigma Aldrich, USA) were prepared in milli-Q water and resolved on a Shimadzu LC-20AD series Quaternary Gradient HPLC system with PDA detector using a Hypersil ODS 150 mm x 4.6 mm I.D. C18 reverse-phase column with 3 μ m particle size and 5 μ m Hypersil ODS C18 guard column (Thermo Scientific, USA). Separation of the nucleotides were carried out with a mobile phase containing 60 mmol/L of KH₂PO₄, 0.45 mmol/L of tetrabutylammonium bromide (TBAB) and 1.26 mol/L of acetonitrile. The pH of the mobile phase was adjusted to 3.20 using concentrated phosphoric acid. Retention times of the individual nucleotides were

matched to that of the respective nucleotide standards. Areas under the peaks for the respective nucleotides were tabulated and energy charge was calculated using the formula:
Energy Charge = $[ATP] + \frac{1}{2}[ADP] / [ATP] + [ADP] + [AMP]$.

Kinase inhibitor array. MKN74 cells were treated with 95 different kinase inhibitors (SYN-2103; Synkinase, Australia) in the absence and presence of rh-cANGPTL4 (10 µg/mL) for 6 h. RNA was isolated and reverse transcribed using the iScript Reverse Transcription Supermix. qPCR was done using the KAPATM SYBR qPCR Universal Master Mix (KAPABiosystems).

Statistical analysis. Statistical analyses were performed using two-tailed Mann-Whitney test with SPSS software. All statistical tests were two-sided. p value of ≤ 0.05 is considered significant.

Accession numbers. Microarray data have been submitted to the GEO database under accession number GSE 71280

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Contribution: ZT performed experiments, analyzed the results and wrote the article; MKS, JSKC, YL, MMKL, LL, TP and PZ performed experiments and contributed to discussion. NST analyzed the results, contributed to discussion, reviewed and edited the article.

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Figure Legends

Figure 1. Altered cancer cell bioenergetics during EMT. (A, B, left panel) Immunofluorescence staining of E-cadherin in hypoxia- (A) or 4-OHT-treated MKN74_{Snai1ER} cells (B) at the indicated time intervals. Cells were counterstained with DAPI (blue) for nuclei and phalloidin (red) for actin cytoskeleton. Scale bar = 40 μ m. (A, B, middle and right panels) Relative expression (middle) and immunodetection (right) of EMT markers in hypoxia-exposed MKN74 cells (A) or 4-OHT-treated MKN74_{Snai1ER} cells (B) at the indicated time intervals. Data (mean $\pm$ SD) from three independent experiments. Loading controls are from the same sample. * $P<0.05$, ** $P<0.01$. (C) FACS analysis of fluorescent glucose analog, 2-NBDG, uptake in MKN74_{CTRL} and MKN74_{Snai1ER} cells after the indicated treatments. (D) Glycolysis rate in MKN74_{CTRL} cells after the indicated treatments. (E) *In vivo* 2-DG fluorescence imaging of mice bearing tumors that received the indicated treatments. (F-I) Energy charge measurement by HPLC in MKN74_{CTRL}, MKN74_{Snai1ER}, MCF7, HepG2, H4, and HSC cells (F, G), xenograft tumors (H) and tumor biopsies from patients (I) after the indicated treatments. veh: vehicle

Figure 2. Metabolic reprogramming enhanced metastatic capability of cancer cells. (A-D) Energy charge measurement by HPLC in MKN74_{Snai1ER} (A, C) or MKN74_{CTRL} (B, D) cells harboring the respective modification after the indicated treatments. * $P<0.05$; ** $P<0.01$. (E, F) Immunofluorescence staining of E-cadherin in MKN74 cells with the indicated modification under normoxic or hypoxic conditions. Nuclei were counterstained with DAPI (blue). Scale bar = 50 μ m. (G) Representative immunofluorescence images of E-cadherin and laminin 332 from MKN74_{Snai1ER} tumor sections exposed to the indicated treatments. Scale bar = 40 μ m (top) or 100 μ m (bottom). (H) Gelatin zymography and quantification of MMP activities in the indicated tumors. (I) Representative macroscopic images of lungs and the relative expression of human TBP from the indicated lungs. Black

arrows in **(I)** indicate micrometastases (**n=5**). **(J, K)** FACS analysis of fluorescent dye efflux **(J)** and Annexin/PI **(K)** staining in MKN74_{CTRL} and MKN74_{Snai1ER} cells after the indicated treatments. **(L, M)** Immunodetection of MDR1 in MKN74_{CTRL}, MKN74_{Snai1ER} cells **(L)** and tumor biopsies from patients **(M)** after indicated treatment.

Figure 3. ANGPTL4 regulates cellular bioenergetics during EMT. **(A, left panel)** Top five most represented biological processes in hypoxia (**top**), TGF- β (**middle**), and 4-OHT (**bottom**) induced EMT models. **(A, right panel)** Top five most enriched metabolic processes. **(B)** Heat map representation of commonly regulated genes across the three EMT models (control vs. ANGPTL4-knockdown) over the indicated time points. Green: down-regulated; Red: up-regulated; White box: groups of genes that are differentially regulated in ANGPTL4-knockdown cells compared to control cells. **(C)** Relative expression of selected genes involved in the four enriched metabolic processes. **(D)** Total ROS content of MKN74 or MKN74_{Snai1ER} cells during EMT based on fluorescence measurements. Loading controls are from the same sample. **(E-G)** Immunodetection of ANGPTL4 in MKN74_{CTRL}, MKN74_{Snai1ER} cells **(E)**, xenograft tumors **(F)** and tumor biopsies from patients **(G)** after indicated treatment. Loading controls are from the same sample. **(H-L)** Energy charge measurement by HPLC in MKN74_{CTRL}, MKN74_{Snai1ER}, MCF7, HepG2, II4, and HSC cells **(H-K)** and xenograft tumors **(L)** after the indicated treatments. veh: vehicle; Ab: α -cANGPTL4; Kd: ANGPTL4-knockdown.

Figure 4. Metabolic reprogramming by ANGPTL4 augments EMT. **(A-C, top panel)** Immunofluorescence staining of E-cadherin in hypoxia- **(A)** or rh-cANGPTL4- **(C)** treated MKN74 cells or 4-OHT-treated MKN74_{Snai1ER} cells **(B)** in the presence of α -cANGPTL4 at the indicated time intervals. Cells were counterstained with DAPI (**blue**) for nuclei and phalloidin (**red**) for actin cytoskeleton. Scale bar = 40 μ m. **(A-C, bottom panels)** Immunodetection of EMT markers in hypoxia-exposed MKN74 or MKN74 Δ ANGPTL4 cells

(A), rh-cANGPTL4-treated MKN74 cells (C), or 4-OHT-treated MKN74_{Snai1ER} cells (B) exposed to α -cANGPTL4 at the indicated time intervals. Data (mean $\pm$ SD) from three independent experiments. * $P<0.05$, ** $P<0.01$. Loading controls are from the same sample. (D) Gelatin zymography and quantification of MMP activities in the indicated tumors. (E) Representative macroscopic images of lungs and the relative expression of human TBP from the indicated lungs. Black arrows in (E) indicate micrometastases (n=5). (F) Immunofluorescence staining of cANGPTL4 and E-cadherin in benign and metastatic skin tumors. Scale bar = 100 μ m. (G, H) FACS analysis of fluorescent dye efflux MKN74_{CTRL}, MKN74 Δ ANGPTL4 (G) and MKN74_{Snai1ER:shANGPTL4} (H) cells after the indicated treatments. (I) Immunodetection of MDR1 in MKN74_{CTRL}, MKN74 Δ ANGPTL4 and MKN74_{Snai1ER:shANGPTL4} cells after indicated treatment.

Figure 5. ANGPTL4 regulates specific 14-3-3 expression. (A-C) Immunodetection of various 14-3-3 isoforms in MKN74_{CTRL}, MKN74 Δ ANGPTL4, and MKN74_{Snai1ER} cells under hypoxia- (A) and 4-OHT-induced (B) EMT or in the presence of rh-cANGPTL4 (C) at the indicated time intervals. (D) Analysis map of kinases (solid line) whose inhibition attenuates ANGPTL4-induced 14-3-3 expression and possible downstream transcriptional regulators (dotted line). (E-J) Immunodetection of the indicated proteins in hypoxia-treated MKN74_{CTRL} or MKN74 Δ ANGPTL4 cells (E, J), 4-OHT-treated MKN74_{Snai1ER} cells (F), rh-cANGPTL4-treated MKN74_{CTRL} cells (G, J), xenograft tumors (H), and tumor biopsies from patients (I) at the indicated time points. Loading controls are from the same sample.

Figure 6. The ANGPTL4:14-3-3 γ axis coordinates energy demand during EMT. (A, E) Immunodetection of indicated 14-3-3 isoforms with TSC2_{S939} (A) or Snai1 (E) after immunoprecipitation with Snai1, TSC2, or pan 14-3-3 antibodies. (B-D and F-H) Proximity ligation assay (PLA) quantification of 14-3-3 γ :TSC2_{S939} and 14-3-3 γ :Snai1 interactions in

MKN74_{CTRL} and MKN74_{ΔANGPTL4} cells (**B, F**), xenograft tumors (**C, G**), and tumor biopsies from patients (**D, H**) after the indicated treatments. Data (mean±SD) from three independent experiments. * $P<0.05$, ** $P<0.01$. (**I**) Representative ChIP results using anti-Snai1 (**top panel**) followed by re-ChIP with anti-14-3-3 γ (**bottom panel**) in MKN74_{CTRL} and MKN74_{ΔANGPTL4} cells under normoxic or hypoxic conditions. Pre-immune serum (IgG) served as control. (**J**) Schematic diagram depicting the coordination of metabolic reprogramming by the ANGPTL4:14-3-3 axis during EMT.

SUPPLEMENTAL INFORMATION

Reprogramming of cellular Bioenergetics Primes cancer cells for Epithelial-Mesenchymal Transition

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Supplementary Figure Legends

Figure S1, related to Figure 1. (A, C) Immunofluorescence staining of E-cadherin in TGF- β -treated MKN74 cells (A) or Snai2 in hypoxia-treated MKN74 cells (C) at indicated time intervals. Cells were counterstained with DAPI (blue) for nuclei and phalloidin (red) for actin cytoskeleton. Scale bar = 40 μ m. (A, right panel) Immunodetection and relative gene expression of EMT markers in MKN74 cells exposed to TGF- β at indicated time intervals. Data (mean $\pm$ SD) from three independent experiments. * $P<0.05$, ** $P<0.01$. (B) Immunodetection and relative gene expression of Snai1 in MKN74_{CTRL} or MKN74_{Snai1ER} cells. (D) Cellular ATP measurement in MKN74_{CTRL} and MKN74_{Snai1ER} cells based on bioluminescence after the indicated treatments. The ATP concentration was determined from a standard curve using pure ATP. For immunoblots, loading controls are from the same sample.

Figure S2, related to Figures 2. (A) Immunodetection of dominant-negative (dn) or constitutively active (ca) AKT and AMPK in MKN74 cells after transfection (n=3). (B) Immunodetection of E-cadherin and Snai1 in hypoxia-treated MKN74 cells harboring the respective modifications. (C) Representative macroscopic and H&E images of tumors from MKN74_{Snai1ER} tumor sections exposed to the indicated treatments. Scale bar = 40 μ m (D) Focus array of critical ABC transporters' genes involved in drug resistance across the three EMT models over the indicated time points. Green: down-regulated; Red: up-regulated. For immunoblots, loading controls are from the same sample.

Figure S3, related to Figures 3. (A, C, G) Relative gene expression of ANGPTL4 in MKN74_{CTRL}, MKN74 Δ ANGPTL4, MKN74_{Snai1ER} or MKN74_{Snai1ER:shANGPTL4} cells after indicated treatments. (B) Immunodetection of selected proteins involved in the autophagy in MKN74_{CTRL}, MKN74 Δ ANGPTL4 and MKN74_{Snai1ER} cells. (D, F) FACS analysis of fluorescent glucose analog, 2-NBDG, uptake in MKN74_{CTRL}, MKN74 Δ ANGPTL4, MKN74_{Snai1ER}, or

MKN74_{Snai1ER:ΔANGPTL4} cells after the indicated treatments. **(E)** Glycolysis rate in MKN74_{CTRL} and MKN74_{ΔANGPTL4} cells after the indicated treatments. **(H)** *In vivo* 2-DG fluorescence imaging of mice bearing tumors that received the indicated treatments. **(I-K)** Cellular ATP measurement in MKN74_{CTRL}, MKN74_{ΔANGPTL4}, MKN74_{Snai1ER}, or MKN74_{Snai1ER:ΔANGPTL4} cells based on bioluminescence after the indicated treatments. The ATP concentration was determined from a standard curve using pure ATP. veh: vehicle; Ab: α -cANGPTL4.

Figure S4, related to Figure 4. (A, H) Immunofluorescence staining of E-cadherin in TGF- β - treated MKN74 cells **(A)** or Snai2 in hypoxia-treated MKN74 cells in the presence of antibodies against cANGPTL4 and rh-cANGPTL4-treated MKN74 cells **(H)** at the indicated time intervals. Cells were counterstained with DAPI **(blue)** for nuclei and phalloidin **(red)** for actin cytoskeleton. Scale bar = 40 μ m. **(B, C)** Relative expression of EMT markers in hypoxia-exposed MKN74, 4-OHT-treated MKN74_{Snai1ER} cells **(B)** or rh-cANGPTL4-treated MKN74 cells **(C)** exposed to α -cANGPTL4 at the indicated time intervals. Data (mean $\pm$ SD) from three independent experiments. * $P<0.05$, ** $P<0.01$. **(D-G)** Immunodetection of indicated proteins in hypoxia-treated, wild-type or ANGPTL4-knockdown MCF7 **(D)**, HSC **(E)**, H12 **(F)** and HepG2 cells **(G)**. Loading controls are from the same sample. **(I)** Representative H&E images and immunofluorescence images of E-cadherin and laminin 332 from MKN74_{Snai1ER:shANGPTL4} tumor sections exposed to the indicated treatments. Scale bar = 40 μ m (top) or 100 μ m (bottom). For immunoblots, loading controls are from the same sample.

Figure S5, related to Figure 5. (A) Relative expression of various 14-3-3 isoforms in MKN74 cells in the presence of rh-cANGPTL4 after 6 hours. Data (mean $\pm$ SD) from three independent experiments. * $P<0.05$, ** $P<0.01$. **(B-E)** Immunodetection of indicated

proteins in hypoxia-treated MCF7 (**B**), II4 (**C**), HSC (**D**) and HepG2 cells (**E**) at the indicated time points with or without ANGPTL4. Loading controls are from the same sample.

Figure S6, related to Figure 6. (A-D) Immunodetection of the indicated proteins in hypoxia-treated MKN74_{CTRL} or MKN74_{ΔANGPTL4} cells (**A**), 4-OHT-treated MKN74_{Snail^{IER}} cells (**B**), rh-cANGPTL4-treated MKN74_{CTRL} cells (**C**), and after immunoprecipitation with Snail antibody (**D**) at the indicated time points. Loading controls are from the same sample. **(E-H)** Proximity ligation assay (PLA) of 14-3-3γ:TSC2_{S939} (**E, G**) and 14-3-3γ:Snail (**F, H**) interactions in MKN74_{CTRL} and MKN74_{ΔANGPTL4} cells or xenograft tumor after the indicated treatments. PLA signals are in red. MKN74 and xenograft tumor cells were counterstained with DAPI (**blue**) for nuclei and alexa-488 phalloidin (**green**) for actin cytoskeleton. Scale bar = 40 μm (**E, G**) or 50 μm (**F, H**). Data (mean±SD) from three independent experiments. * $P<0.05$, ** $P<0.01$.

Figure S7, related to Figure 7. (A-F) Proximity ligation assay (PLA) of 14-3-3γ:TSC2_{S939} (**A-C**) and 14-3-3γ:Snail (**D-F**) interactions in patient biopsies with the indicated tumor grades. PLA signals are in red. Tumor cells were counterstained with DAPI (**blue**) for nuclei and pan-cytokeratin (**green**) to differentiate epithelial and mesenchymal origin. Scale bar = 50 μm. Data (mean±SD) from three independent experiments. * $P<0.05$, ** $P<0.01$.

Supplementary Experimental Procedures

Retrovirus transduction. MKN74_{Snai1ER} cells were generated using retroviral transduction as previously described (Mani et al., 2008). Briefly, HEK 293T cells were transfected with pCL-10A1 retrovirus packaging vector (Imgenex, USA) and pWZL-Snai1-ER (Addgene, USA). Harvested pseudoviruses were used to infect MKN74 cells. Following transduction, cells were selected with 5 µg/mL of blasticidin hydrochloride (Sigma Aldrich, USA).

Generation of dual-inducible MKN74_{Snai1ER/shANGPTL4} cell line. Doxycycline-inducible pSingle-tTS-shRNA vector carrying either shANGPTL4 or scrambled shRNA was introduced into MKN74_{Snai1ER} cells using Fugene HD reagent as per manufacturer protocol (Promega, USA). MKN74_{Snai1ER/shANGPTL4} and MKN74_{Snai1ER/shscrambled} cells were selected with 1 mg/mL of G418 (Sigma Aldrich, USA). The efficiency of knockdown was verified by qPCR and immunoblotting after treatment with various concentrations of doxycycline (up to 10 µg/mL).

Expression and purification of recombinant cANGPTL4 proteins. The expression and purification of recombinant cANGPTL4 were performed as previously described (Goh et al., 2010).

Microarray analysis. Cells were seeded at a density of 5.25×10^2 cell/cm² to allow the formation of individual colonies before indicated treatments. Hypoxia treatments were performed in hypoxic chamber (Stem cell Technology, USA) purged with 5% CO₂ and 95% N₂ to obtain O₂ concentration at 1% as determined by an O₂ sensor. For TGF-β-induced EMT, cells were treated with 10 ng/mL of TGF-β for 2 days. For Snai1-mediated EMT, MKN74_{Snai1ER/shANGPTL4} and MKN74_{Snai1ER} cells were exposed to 20 ng/mL of 4-hydroxytamoxifen (4-OHT) for 4 days. RNA was extracted from the respective treatments with Trizol following the manufacturer's protocol. Further sample processing of the RNA

was carried out using Applause® WT-Amp ST System (NuGEN), and microarray experiments were performed on GeneChip® Human Gene 1.0 ST arrays according to the manufacturer's instructions.

Immunofluorescence staining. EMT was identified by immunofluorescence staining for E-cadherin and Snai2. MKN74 cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton-X 100 for 10 min and blocked with 3% normal goat serum (NGS) containing 0.05% Triton-X 100 in a humidified chamber for 1 h at room temperature. Following three washes in PBS, cells were incubated overnight at 4 °C with either anti-human-E-cadherin (1:200) or Snai1 (1:100) antibodies in 3% NGS. After three washes, cells were incubated for 1 h at room temperature with Alexa488-secondary antibodies (1:250) and counterstained with Alexa594-phalloidin for F-actin and DAPI for nuclei. Immunostainings performed without primary antibodies served as negative controls. Images were acquired using Zeiss LSM710 confocal microscope with a Plan-APOCHROMAT 40x/1.4 oil objective and ZEN2009 software.

Proximity Ligation Assay (PLA) and analysis. MKN74_{CTRL} or MKN74_{ΔANGPTL4} (5×10^3 cells) were subjected to either normoxia or hypoxia for 2 days at 37 °C. At indicated time, MKN74 cells were fixed, permeabilized and blocked similar to immunofluorescence staining. Next, MKN74 cells were incubated with monoclonal mouse-anti human 14-3-3 γ antibody (1:200) and monoclonal rabbit anti-human Snai1 antibody (1:200) in 3% NGS overnight at 4 °C. PLA was carried out as per manufacturer's protocol (Olink Bioscience, USA). Images were taken using Carl Zeiss confocal microscope LSM710 using a Plan-APOCHROMAT 63x/1.4 oil DIC objective, and ZEN2012 LE software with constant exposure and gain. Number of protein interaction was quantified using the "BlobFinder" (Allalou and Wählby, 2009).

Immunoprecipitation and immunoblot. For immunoprecipitation, at the indicated time points after treatments, cells were lysed using IP lysis buffer (25mM Tris-HCl pH7.4, 150mM NaCl, 1% NP-40, 1mM EDTA). The lysate was then incubated with indicated antibodies overnight at 4 °C with constant rotation. Following that, the antibodies were affinity precipitated using protein A/G beads (Santa Cruz, USA). Proteins were released by boiling for 10 min in Lamelli's buffer. Protein extracts were resolved using 12 % SDS-polyacrylamide gel electrophoresis and electrotransferred (25 mM Tris, 192 mM glycine, 20% methanol) onto a low fluorescence PVDF membrane for immunoblot analysis. Membranes were stripped and re-probed for other proteins as described (Yeung and Stanley, 2009). Protein bands were detected using Odyssey CLx Infrared Imaging system (LI-COR, USA).

Energy charge determination. Cellular levels of AMP, ADP and ATP were determined by HPLC. A Shimadzu LC-20AD series Quaternary Gradient HPLC system with PDA detector using a Hypersil ODS 150 mm x 4.6 mm I.D. C18 reverse-phase column with 3 µm particle size and 5 µm Hypersil ODS C18 guard column (Thermo Scientific, USA) was used. Nucleotides were extracted as previously described (44). Nucleotides standards were from Sigma Aldrich, USA. Separation of the nucleotides were carried out with a mobile phase containing 60 mmol/L of KH₂PO₄, 0.45 mmol/L of tetrabutylammonium bromide and 1.26 mol/L of acetonitrile. The pH of the mobile phase was adjusted to 3.20 using concentrated phosphoric acid. Retention times of the individual nucleotides were matched to that of the respective nucleotide standards. Areas under the peaks for the respective nucleotides were tabulated and energy charge was calculated using the formula: Energy Charge = $[ATP] + \frac{1}{2}[ADP] / [ATP] + [ADP] + [AMP]$.

ATP quantification. ATP level in the cancer cells were quantified using the ATP determination assay kit (Invitrogen, USA) as per manufacturer protocols. Briefly, ATP was released from the cells using somatic cell lysis buffer (Sigma Aldrich, USA) after treatment for indicated time period. ATP levels were assayed and the luminescence readings were taken using a GloMax 20/20 luminometer (Promega, USA). Luminescence readings were plotted against an ATP standard curve to obtain the actual ATP concentration followed by normalization to the protein concentration.

Glucose uptake assay. Briefly, cells were incubated with 10 μ M of 2-NBDG for 12 h. The cells were then harvested and subjected to FACS analysis using BD Accuri C6 flow cytometer (BD Biosciences, USA). Data was analysed using FlowJo and plotted based on mean intensity.

Metabolic analysis. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in real-time using a XF96 flux analyzer (Seahorse Bioscience, USA). Cells were seeded on 96-well microplates. All measurements were normalized against total protein content from cell lysates.

Drug efflux assay. The activities of the multidrug resistance proteins MDR1, MRP and BCRP were measured using eFluxx-ID Green Multidrug Resistance Assay Kit (ENZ-51029-K100, Enzo Lifesciences, Farmingdale, NY) according to the manufacturer's instructions. Briefly, single cell suspensions were harvested and equal numbers of cells (2×10^5 /condition) were resuspended in full media containing either DMSO or specific inhibitors (Verapamil: MDR1 inhibitor; MK-571: MRP1 inhibitor; Novobiocin: BCRP inhibitor; 200 μ m) and incubated for 10 min at 37°C. EFLUXX-ID[®] Green Detection Reagent was then added and incubated at 37°C for 40 min before FACS analysis using BD Accuri C6 flow cytometer (BD Biosciences, USA). Data was analysed using FlowJo and plotted based on mean intensity.

Kinase inhibitor array. MKN74 were treated with 95 different kinase inhibitors (SYN-2103; Synkinase, Australia) in the absence and presence of rh-cANGPTL4 (10 µg/mL) for 6 h. RNA was isolated and reverse-transcribed using the iScript Reverse Transcription Supermix. qPCR was done using the KAPATM SYBR qPCR Universal Master Mix (KAPABiosystems).

Table S1. ChIP Primer sequences.

	Forward (5' -> 3')	Reverse (5' -> 3')
Snail-binding region	TAGAGGGTCACCGCGTCTAT	GGCTGGAGTCTGAACTGACTTC
Negative Control	TCTAGGAGCAGCAGGAAGGGAG	GTCCCTTTCATCTCCTGGCAAG

Table S2. Real-time PCR Primers

Genes	Forward Sequence (5' to 3')	Reverse Sequence (3' to 5')
ANGPTL4	TCCAACGCCACCCACTTAC	TGAAGTCATCTCACAGTTGACCA
CDH1	GCCGAGAGCTACACGTTCA	GACCGGTGCAATCTTCAAA
DDR1	CACTCCGCTCCCTGTGTC	AGAGGTGCGACTGGAACAA
ERBB3	CTGATCACCGGCCTCAAT	GGAAGACATTGAGCTTCTCTGG
Snail	GCCTTCAACTGCAAATACTGC	CTTCTTGACATCTGAGTGGGTC
TBP	GCTGGTTATCGGGAGTTGG	ACTGGCCTGGTGTCTAGAG
ZEB1	GCCAACAGACCAGACAGTGTT	TCTTGCCCTTCCTTTTCCTG
14-3-3α/β	GGCAAAGAGTACCGTGAGAAG	CTGGTTGTGTAGCATTGGGAATA
14-3-3γ	AGCCACTGTCGAATGAGGAAC	CTGCTCAATGCTACTGATGACC
14-3-3ϵ	GATTCGGGAATATCGGCAAATGG	GCTGGAATGAGGTGTTTGTCC
14-3-3η	TGGCTGATGGAAACGAAAAGAA	CCTCTGCTAAGTAGCGGTAGT
14-3-3σ	TGACGACAAGAAGCGCATCAT	GTAGTGGAAGACGGAAAAGTTCA
14-3-3τ	AGGGTCATCTCTAGCATCGAG	CCACTTTCTCCCGATAGTCCTT
14-3-3ζ	CCTGCATGAAGTCTGTAAGTCTGAG	GACCTACGGGCTCCTACAACA

Table S3. Human tumor biopsies details

Sample Source	Sex	Age	Diagnosis	Histological diagnosis	Grade	TNM	Stage
Breast	F	51	cancer	infiltrating ductal carcinoma	G2	T1cN0M0	I
Breast	F	62	cancer	infiltrating lobular carcinoma	G2	T2N3aM0	IIIC
Breast to ovary	F	59	metastases	infiltrating ductal carcinoma	N/A	recurrent	
Colon	M	66	cancer	adenocarcinoma	G1	T2N0M0	I
Colon	M	80	cancer	adenocarcinoma	G1	T3N1M0	IIIB
Colon to lung	F	42	metastases	adenocarcinoma	N/A	recurrent	
Stomach	M	64	cancer	adenocarcinoma	G2	T2N0M0	IB
Stomach	M	58	cancer	adenocarcinoma	G3	T3N2M0	IIIA
Tongue	M	64	cancer	squamous cell carcinoma	G2	T2N1M0	III
Tongue	M	58	cancer	squamous cell carcinoma	G1	T2N0M0	II

Supplemental References

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