

**ROLE OF SUPPRESSOR OF CYTOKINE SIGNALING 3
IN COLORECTAL CANCER**

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ABSTRACT

**KATHRYN ELIZABETH HAMILTON: Role of suppressor of cytokine
signaling 3 in colorectal cancer
(Under the direction of Dr. P. Kay Lund)**

Patients with inflammatory bowel diseases (IBD) have an increased lifetime risk of developing colorectal cancer (CRC). Suppressors of cytokine signaling (SOCS) are intracellular proteins that provide negative feedback on pro-inflammatory cytokine signaling. SOCS3 silencing in intestinal epithelial cells has previously been shown to promote tumorigenesis in the azoxymethane/dextran sodium sulfate (AOM/DSS) mouse model of inflammation-associated CRC. Mechanisms associated with this effect were increased activation of signal transducer and activator of transcription 3 (STAT3) and NF κ B, and increased expression of TNF α receptor 2 (TNFR2). TNFR2 is increased in IBD and CRC, but how this receptor is regulated remains undefined. Studies in this dissertation tested the hypothesis that TNFR2 is induced by STAT3 and/or NF κ B pathways, that SOCS3 limits TNFR2 expression, and that SOCS3 is a tumor suppressor in both sporadic and inflammation-associated

cancer. Colon cancer cell lines were treated with IL-6 and TNF α in the presence of STAT3 or NF κ B inhibitors. STAT3 inhibition dramatically decreased cytokine-induction of TNFR2, implicating STAT3 as a critical mediator of TNFR2 induction. SOCS3 limited cytokine-induction of TNFR2, as well as STAT3 binding to consensus sequences within the TNFR2 promoter. SOCS3 also limited TNFR2-mediated proliferation and anchorage-independent growth of colon cancer cells. Together these findings support the concept that SOCS3 exerts a tumor suppressor role in part by limiting the growth-promoting abilities of TNFR2. To test the whether low SOCS3 expression predicts risk of early stage CRC, biopsies of normal mucosa from colonoscopy patients with and without adenomas were assayed for SOCS3 mRNA. No significant difference in SOCS3 mRNA was observed in normal mucosa of patients with and without adenoma. Thus SOCS3 silencing in normal mucosa does not predict adenoma risk. To test whether SOCS3 normally limits tumorigenesis in sporadic CRC, mice with IEC-SOCS3 silencing were subjected to the AOM model of spontaneous, non-inflammatory CRC. Mice with SOCS3 silencing exhibited a 75% increase in colon tumor incidence. Collectively, these data indicate that SOCS3 normally modulates multiple pro-tumorigenic pathways that contribute to both inflammation-associated and sporadic CRC.

DEDICATION

This dissertation was written in dedication to my parents Arthur and Sheila Hamilton, and to my husband Jeremy Sadler.

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LIST OF ABBREVIATIONS

ACF	aberrant crypt foci
AOM	azoxymethane
AP-1	activator protein 1
APC	adenomatous polyposis coli
BRAF	B-Raf proto-oncogene serine/threonine-protein kinase
CKI	casein kinase I
CRC	colorectal cancer
DSS	dextran sodium sulfate
EGF	epidermal growth factor
ESS	extended SH2 domain
FAP	familial adenomatous polyposis
FGF	fibroblast growth factor
G-CSF	granulocyte colony-stimulating factor
GI	gastrointestinal
GP130	glycoprotein 130
GSK3 β	glycogen synthase kinase 3 beta
HCC	hepatocellular carcinoma
HIC1	hyper-methylated in cancer 1
HNPCC	hereditary nonpolyposis colorectal cancer
HPP1	hyperplastic polyposis protein gene
IBD	inflammatory bowel disease

ICAM	intercellular adhesion molecule
IEC	intestinal epithelial cell
IFN γ	interferon gamma
IGF1	insulin-like growth factor 1
IL	interleukin
IRF-1	interferon regulatory factor 1
IKK β	inhibitor of nuclear factor kappa-B kinase subunit beta
JAK	Janus kinase
KGF	keratinocyte growth factor
KIR	kinase inhibitory region
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LEF	lymphoid enhancer factor
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MIN	multiple intestinal neoplasia
MGMT	O ⁶ -methylguanine-DNA methyltransferase gene
MLCK	myosin light chain kinase
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)
MMR	mismatch repair
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)
NF κ B	nuclear factor kappa B
OSM	oncostatin M
PEST	Pro/Glu/Ser/Thr-rich

PI3KCA	phosphoinositide-3-kinase, catalytic, alpha polypeptide
SFRP2	secreted frizzled-related protein gene 2
SH2	src-homolgy 2
SOCS	suppressor of cytokine signaling
STAT	signal transducers and activators of transcription
TACE	TNF- converting enzyme
TAK1	TGF β activated kinase 1
TCF	T-cell factor
TGFBR2	transforming growth factor beta receptor 2
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TP53	tumor protein 53
TRAF	TNF receptor associated factor
TYR	tyrosine
VC	villin-Cre

CHAPTER I

BACKGROUND AND INTRODUCTION

A. Anatomy of the intestine

The small intestine and colon are a continuous tube that primarily functions to digest and absorb nutrients while maintaining a protective barrier between the external environment and the underlying tissue. The mucosa interfaces directly with the lumen of the intestine. The mucosa comprises an epithelial layer directly facing the lumen, the lamina propria, and the muscularis mucosa. The lamina propria contains mesenchymal cells (fibroblasts, myofibroblasts, smooth muscle), as well as capillaries and nerve endings. In the small intestine, particularly ileum, lymphoid tissue within the lamina propria is found in Peyer's patches. Peyer's patches are surrounded by M-cells that transport antigens from the epithelium to the lymphoid tissue in order to elicit an immune response (1). In the colon, aggregates of lymphoid tissue within lamina propria are organized as lymphoid follicles (2). Immediately below the mucosa resides the loose connective tissue of the submucosa, which contains primarily support tissue, mesenchymal cells and connective tissue, and houses larger blood vessels. Enveloping the submucosal layer, the muscularis externa is innervated by neurons of the myenteric plexus, which aid in the coordination of resident smooth muscle layers to produce peristaltic movement of luminal contents through the intestine. In most regions of the small and large intestine, the muscularis externa is composed of an inner, circular smooth muscle layer and an outer, longitudinal layer. The outer-most intestinal layer is the serosa, which includes connective tissue and a layer of mesothelium (squamous epithelial cells) Figure 1.1) (3).

The mucosal layer of the small intestine consists of macroscopic folds of Kerckring, and then is further organized into microscopic villi and crypts of Lieberkühn. The villi protrude into the lumen and are surrounded by the glandular crypts. The villi are absent in the colon, which instead consists of surface epithelium and crypts (4). The macroscopic organization of the small intestine and colon function to provide maximal surface area of these organs, with the small intestine having a surface area of roughly 200 m², and the colon 25 m² (4). The surface area of the small intestine facilitates its absorption of roughly 6.5 liters of dietary, salivary, gastric, pancreatic, and biliary fluid per day, with the colon absorbing another 2 liters per day (4).

B. Renewal and homeostasis of the intestinal epithelium

The intestinal epithelium is constantly and rapidly renewed. This process depends on production of new cells within the crypts, which balances constant loss of cells from the surface of the villi or colon. Current views indicate that this renewal depends on proliferation of long-lived stem cells, which reside in the crypts that give rise to faster proliferating and shorter-lived progenitor or transit amplifying cells (5-7). Stem cells are thought to reside at or near the base of the crypts in small intestine, and available evidence suggests that stem cells reside in the mid-crypt of the ascending colon, and at the base of the crypt in the descending colon (Figure 1.2) (8). Stem cells undergo asymmetrical division to maintain the stem cell and produce a new daughter cell (8). Symmetrical division to produce two stem cells occurs much less frequently under normal

physiological conditions (9). Daughter cells continue to proliferate up the crypt axis within the transit-amplifying region and then differentiate into one of the four major epithelial lineages (Figure 1.2) (7, 10, 11).

The subsets of intestinal epithelial cells (IEC) are absorptive (enterocytes or colonocytes), or secretory (goblet, enteroendocrine, and Paneth cells). The distribution of these cell types varies along the length of the small intestine and colon based on the functional requirement of each segment. Enterocytes predominate the small intestine where dietary nutrients are absorbed. These cells have increased absorptive capabilities due to the presence of apical microvilli and are the most abundant of the IEC types. Goblet cells are interspersed among the enterocytes and increase in number from the duodenum to the colon. These cells secrete mucin to form a mucus layer, which contributes to the epithelial barrier and facilitates the movement of stool towards the rectum. Paneth cells are found at the crypt base in the small intestine and secrete anti-microbial proteins. Enteroendocrine cells comprise less than 1% of the intestinal epithelium and represent a very diverse group of cells, which produce multiple gastrointestinal hormones (12, 13). Even though enteroendocrine cells represent less than 1% of gut epithelial mass, they represent the largest endocrine organ of the human body. Enteroendocrine cell types and hormones secreted vary along the length of small intestine and colon. Functionally, hormones regulate a wide variety of activities ranging from appetite and motility, to gut, pancreas, and liver secretions.

The high turnover rate of the epithelium requires a carefully coordinated balance of proliferation in the lower portion of the crypt, and differentiation and cell shedding on the villus or surface epithelium. This coordination and a balance between cell production and cell loss maintains the integrity of the mucosal lining. Once at the villus tip or surface epithelium, cells lose their attachment to the underlying basement membrane and undergo the apoptotic process of anoikis (14). The balance of cell proliferation and shedding is achieved through sophisticated cross-talk between the mucosal layer, immune and mesenchymal cells, and the commensal microflora (15, 16).

The Wnt/ β -catenin pathway is a major proliferative pathway in the intestinal crypts. When Wnt signals are absent, β -catenin is degraded by the proteasome through direct binding to a complex of adenomatous polyposis coli (APC), glycogen synthase kinase 3 β (GSK3 β), and casein kinase I (CKI). In the presence of Wnt signaling, β -catenin is liberated from the destruction complex and is translocated to the nucleus, where it acts as a transcriptional co-activator of the T cell factor (TCF)/lymphocyte enhancer factor (LEF) family of transcription factors. Downstream targets of Wnt signaling promote cell proliferation, and various studies have lead to the model of a signaling gradient in which Wnt signaling is highest in the proliferative zone of the crypt (Figure 1.2) (17). Wnt activation is also a major component of intestinal tumorigenesis, which will be discussed later.

C. Epithelial-immune interactions and physiological inflammation

The epithelial barrier functions to protect underlying tissue from harmful agents present in the lumen. Insult to the epithelium, including pathogens and toxins, can compromise the integrity of the epithelial barrier and expose the lamina propria to antigens that elicit an acute inflammatory response. This is marked by neutrophil infiltration, macrophage activation, and induction of pro-inflammatory cytokines. Mild damage, limited to superficial injury to the epithelium, can be resolved within hours due to rapid epithelial restitution. During this process, cells neighboring the wound flatten and migrate into the damaged area and form new cell:cell junctions to close the wound. During more extensive or deeper injury, or chronic insult, restoration of the epithelial barrier requires hyper-proliferation of crypt epithelial cells to regenerate the crypts and injured epithelium (18, 19).

Aside from the ability to rapidly proliferate to restore the integrity of the intestinal barrier, the intestine is intrinsically “primed” to handle environmental challenge due to the presence of resident pools of immune cells within Peyer’s patches (small intestine) and lymphoid follicles (colon). Lymphocytes also reside within the epithelial and lamina propria layers (15, 20). The presence of these immune cells in normal intestine and the fact that these cells can exhibit a controlled inflammatory response has been termed “physiological inflammation” of the intestine. This term encompasses the fact that immune cells play a role in maintaining normal intestinal homeostasis (20). Indeed, resident immune cells (specifically gamma delta T-cells, a subset of intra-epithelial lymphocytes) are thought to aid in IEC turnover under normal conditions (21) and promote repair of

the epithelium (22). Cross-talk between IECs and resident immune cells occurs through direct cell:cell interactions, as well as through chemokine and cytokine signaling (23-25) Through these same interactions, IECs can recruit leukocytes to sites of injury, where neutrophils and macrophages can phagocytose and destroy invading microbes or antigens and secrete pro-proliferative factors, including epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), and keratinocyte growth factor (KGF) to promote epithelial healing (26-30).

D. Tumor necrosis factor α (TNF α) and its receptors

In addition to growth factors, cytokines are secreted by immune cells and activate proliferative and/or anti-apoptotic signaling pathways within IECs to promote regeneration and repair. TNF α is synthesized as a 26kd membrane-bound precursor that forms biologically active trimers and can be cleaved into its soluble form by TNF-converting enzyme (TACE, also known as ADAM-17). It is secreted by a variety of immune cells, including macrophages, neutrophils, T and B cells (31-35). Low levels of TNF α mRNA transcripts have also been detected in the Paneth cells of the normal intestine (36). Other studies have shown that low levels of TNF α can be produced by cultured human IEC and the rat cell line IEC-6 in response to lipopolysaccharide (LPS) stimulation (37). These studies showed that autocrine production of TNF α lead to growth restriction of these cells, and higher levels of exogenous TNF α -induced caspase-dependent apoptosis (37-39). In non-transformed mouse colonic epithelial cells, low doses of TNF α (0.1 and 1ng/mL) stimulated proliferation, while high doses (100 and

1000ng/mL) inhibited proliferation (40). TNF α has also been shown to decrease barrier function of the mucosal lining by altering tight junctions through down-regulation of expression of ZO-1, an integral component of the tight junction (41, 42).

TNF α signals through two receptors: TNFR1 (p55TNFR) and TNFR2 (p75TNFR). Early studies in mice showed that TNFR1 mediates cellular toxicity and TNFR2 mediates proliferation (43). However, other studies indicate that the two receptors may cooperate in certain contexts through ligand passing (44). TNFR1 contains an intracellular death domain that binds to several caspase-recruiting proteins and mediates stress-induced apoptosis (45, 46). Signaling through TNFR1 has also been shown to activate the transcription factors NF κ B and c-Jun (47). TNFR1^{-/-} mice have accelerated skin wound healing after injury due to reduced immune infiltration, and exhibit decreased liver and lung metastases in cancer cell transplantation models (48-50). Recent studies have also shown that TNFR1^{-/-} mice have decreased tumor incidence in the azoxymethane/dextran sodium sulfate (AOM/DSS) tumor model associated with a significant decrease in neutrophil and macrophage recruitment to the underlying lamina propria and submucosa (51). The same study showed that treatment with anti-TNF α monoclonal antibodies decreased tumor load in wild-type mice in the AOM/DSS model (51). These findings suggest that TNFR1 is a critical mediator of immune cell recruitment in the context of inflammation-associated colorectal cancer, and may contribute to increased local TNF α signaling.

TNFR2 does not have an intracellular death domain, but can activate NF κ B through the adaptor protein TNF receptor-associated factor 2 (TRAF2) (52). Like TNFR1, TNFR2 can activate NF κ B and AP-1, but in some settings activates other transcription factors, including STAT3 (53, 54). While TNFR1 is ubiquitously expressed in most tissues, TNFR2 expression is induced in the context of multiple cytokines, including IFN γ , or a combination of IL-6, TNF α , and IL-1 β (55, 56). TNFR2 is required for TNF α -mediated myosin light chain kinase (MLCK)-dependent barrier dysfunction *in vitro* (55) and is up-regulated in the epithelium of mice and humans with inflammatory bowel diseases and colorectal cancer (56-58). Studies described in CHAPTER II of this dissertation assessed the mechanisms by which cytokines induce TNFR2.

E. Interleukin-6 (IL-6)

Interleukin-6 (IL-6) is a cytokine that is induced in some settings by other cytokines via mechanisms that involve NF κ B (59-61). IL-6 activates a receptor heterodimer composed of IL-6R α and gp130, a common subunit of multiple receptors that share some signaling mechanisms with IL-6 (62, 63). Ligand-induced activation of IL-6R α /gp130 leads to activation and phosphorylation of Janus kinases (JAKs). JAKs in turn activate signal transducers and activators of transcription (STATs) by recruiting them to the cytokine receptor/gp130 subunit and mediating phosphorylation of key tyrosine residues (64). Tyrosine phosphorylation of STATs leads to their dimerization and translocation to the nucleus where they bind to consensus STAT binding sites in DNA to mediate

transcriptional regulation of target genes (64). The IL-6R α /gp130 potently activates STAT3, one of a family of STAT proteins. A major focus of this dissertation is on the regulation of IL-6/STAT3 signaling by suppressor of cytokine signaling 3 (SOCS3) (65-67).

F. Suppressors of cytokine signaling (*portions of this section are excerpted from (68)*).

Suppressors of cytokine signaling (SOCS) were discovered in the late 1990's as a family of proteins that exert negative feedback on cytokine receptor signaling through the JAK-STAT pathway (69-71). Initial characterization of SOCS found that they comprise a family of eight structurally related proteins (SOCS1-7 and CIS) that have major roles to limit the extent of cytokine signaling in the immune system (72-74). SOCS have since been implicated in a variety of other cell types, including IECs.

SOCS proteins share a similar structural organization with a COOH-terminal SOCS box, an SH2 domain, and an NH₂-terminal domain. The SOCS box is an approximately 40-residue motif showing strong homology across all SOCS family members. The SOCS box is essential for interactions with elonginBC and the E3 ubiquitin ligase scaffold cullin5, which targets SOCS-bound proteins for poly-ubiquitination and proteasomal degradation (75-78). The SH2 domain of SOCS proteins is critical for interactions between SOCS and phospho-tyrosine residues of target signaling proteins (77). Additionally, SOCS1

and SOCS3 contain kinase-inhibitory regions (KIR) that can specifically target JAK activity (79, 80).

Consistent with the discovery of SOCS proteins as cytokine-inducible, negative feedback inhibitors of cytokine receptor JAK-STAT signaling, a wealth of evidence suggests that the expression of one or more SOCS mRNAs and proteins is induced by activation of particular cytokine receptors. The SOCS proteins then exert their negative feedback effects through multiple mechanisms (Figure 1.3) depending on the particular SOCS protein (77, 81). These include:

- a) Direct inhibition of activated JAK, which has been demonstrated for SOCS1 and SOCS3 and relies on the presence of a KIR in these SOCS.
- b) Binding of SOCS to the cytokine receptor cytoplasmic domain via SH2 domain interactions and subsequent inhibition of JAK activity.
- c) Binding to SH2 domains of cytokine receptors and competitive inhibition of STAT binding to the cytokine receptor SH2 domains.
- d) Recruitment of the E3 ubiquitin ligase complex to the SOCS box motif leading to ubiquitination and degradation of receptors/signaling molecules associated with SOCS.

Mice with targeted deletion of genes encoding particular SOCS proteins exhibit various phenotypes, including multi-organ inflammatory response (SOCS1), gigantism, altered growth hormone and insulin-like growth factor- 1 signaling (SOCS2), and embryonic lethality (SOCS3) (77, 82-85). Because SOCS3^{-/-} mice are not viable, cell-specific deletion models have been used to identify its role in regulating IL-6 signaling in specific cell types (86, 87).

G. Suppressor of cytokine signaling 3

SOCS3 is an inhibitor of cytokines and receptors that share the gp130 receptor subunit as a signaling molecule, including interleukin 6 (IL-6), leukemia inhibitory factor (LIF), oncostatin M (OSM), granulocyte colony-stimulating factor (G-CSF), interleukin 11 (IL-11), interleukin 23 (IL-23), and leptin (86-94).

Mutational studies and identification of the crystal structure of murine SOCS3 have lead to insights about the structure and function of specific SOCS3 protein domains (Figure 1.4A). Initial reports showed that SOCS3 binds JAK2 through its SH2 domain, and that the KIR is required for functional inhibition of JAK2 (79). The SH2 domain is also responsible for SOCS3 binding to the gp130 receptor, which is facilitated by an extended SH2, or ESS domain (95).

As mentioned earlier, the C-terminal SOCS-box binds elongin BC and cullin5 to promote proteasomal degradation of SOCS3-bound proteins (75-78). In addition, the SOCS-box also permits ubiquitination and degradation of SOCS3 itself, providing a mechanism that regulates SOCS3 protein turnover (96).

SOCS3 turnover can also be regulated through its PEST (Pro/Glu/Ser/Thr-rich) domain, though this domain is not required for the STAT-inhibitory effects of SOCS3 (Figure 1.4A) (97). Inherent to SOCS3 function as a negative regulator of cytokine signaling are the characteristics that SOCS3 mRNA and protein are tightly controlled such that cytokine induction of SOCS3 mRNA is typically rapid and transient over the course of 30 minutes to 3 hours. In addition, the intrinsic structural determinants of the SOCS3 protein, the SOCS box and PEST sequence, allow for rapid regulation of SOCS3 at the protein level.

Available evidence suggests that the SOCS3 negative feedback loop has a particular role in attenuating STAT3 signaling, which mediates transcription of genes that elicit a multitude of context-specific, cellular effects (Figure 1.4B). In addition to genes involved in a pro-inflammatory response mediated by multiple cytokines (IL-6R, IL-11, IL-4R, IRF-1), STAT3 transcriptional targets include genes that mediate increased proliferation (c-Myc, cyclin D1), decreased apoptosis (survivin, Bcl-2, Bcl-XL), and angiogenesis (ADM, EPAS1) (98). SOCS3 has been implicated in various chronic, inflammatory diseases of the gastrointestinal tract, including inflammatory bowel diseases (IBD) and hepatitis (66, 99, 100). Very recent studies have explored the role of SOCS3 in cancer. Mounting evidence suggests that aberrant STAT3-activation resulting from SOCS3 silencing may be a key mechanism to promote tumorigenesis during gastrointestinal inflammation. Indeed, SOCS3 is known to be silenced by promoter hyper-methylation in lung, liver, and gastrointestinal cancers (101-105). Table 1.1 summarizes available information about SOCS3 methylation in human gastrointestinal cancers.

H. Colorectal cancer

Within the literature, colorectal cancer (CRC) can be broadly classified as sporadic (spontaneously arising) or inflammation-associated (typically CRC that occurs in a setting of ongoing IBD) (106). Work by Vogelstein has provided a multi-step sequence of CRC development, which involves multiple mutations or 'hits' within normal colon epithelial cells ultimately permitting the development of

invasive adenocarcinoma (107). These 'hits' can result from a combination of genetic and lifestyle-related factors (108). The CRC sequence involves an initial change in function of crypt epithelial cells such that a genetically aberrant cell evades normal apoptotic mechanisms that remove damaged cells. In addition, this cell proliferates to clonally expand the aberrant cell and fails to migrate to the colonic surface epithelium where it could be removed by the normal process of anoikis.

The earliest aberrant lesions detectable are 'aberrant crypt foci,' or ACF, which have atypical morphology and hyper-proliferation (109). Not all ACFs have the potential to become precancerous and can be targeted by immune-surveillance (109). However, if ACFs survive and acquire additional mutations, they can expand to become precancerous, adenomatous lesions. Mutations that favor establishment and growth of adenomas, and survival to become large adenomas, include loss of tumor suppressors and mutations in oncogenes (107). Ultimately, late adenomas can transform to adenocarcinomas that grow and invade submucosal layers and can metastasize to lymph nodes and distant organs. In sporadic CRC, early mutations in APC and β -catenin are common in ACF and adenoma; adenoma and late adenomas acquire additional mutations in tumor suppressor genes such as *TGFBR2* (*transforming growth factor beta receptor 2*) and *TP53*, and oncogenes such as *KRAS*, *BRAF*, and *PI3KCA* (107, 110). Mutations in cell-cycle arrest and DNA repair genes commonly occur later in the sequence, within late adenoma or adenocarcinoma.

CRC can be an inherited condition that arises in individuals with either familial adenomatous polyposis (FAP) or hereditary non-polyposis colorectal cancer (HNPCC). FAP is caused by an autosomal dominant mutation in the *APC* gene (111, 112). Clinically, FAP patients may present with several hundred adenomatous polyps in the colon during the mid-teens to early 20's and develop CRC in the early 40's if the colon is not removed. HNPCC is caused by mutations in mismatch repair (MMR) genes, including *MLH* and *MSH2*, and is also inherited in an autosomal-dominant fashion (110). These familial cancers account for ~20% of the total CRC cases.

Current evidence suggests that the sequence of IBD-associated cancer may differ from sporadic CRC in etiology and sequence of progression (Figure 1.5). IBD-associated cancer involves earlier mutations in p53, while *APC* or β -catenin mutations occur later in the sequence of dysplasia to colitis-associated cancer (Figure 1.5) (106). Furthermore, IBD-associated cancer can involve flat versus polypoid lesions. In IBD, the setting of chronic inflammation is thought to create a permissive tumor microenvironment characterized by DNA-damaging reactive oxygen and nitrogen species (ROS and RNS), along with induction of anti-apoptotic and growth-promoting cytokine signaling (106, 113, 114). Patients with IBD have an increased risk of CRC over a lifetime because of cumulative damage that results from repeated cycles of chronic inflammation and injury and healing (106). Chronic inflammation of the intestine is associated with persistent infiltration of immune cells into the mucosa, including neutrophils, macrophages,

and activated T-cells, which produce pro-inflammatory cytokines including IL-6, TNF α and IL-1 β , among others.

With this knowledge in mind, research in the Lund laboratory first addressed the role of SOCS3 in inflammation-associated colon cancer (115). Recent concepts suggest that inflammatory changes, milder in nature than IBD, may contribute to the risk of sporadic CRC, or at least of subset of CRC. Therefore other chapters assess whether there is an association between SOCS3 and sporadic precancerous adenoma in humans or mouse models. Mouse models relevant to this dissertation are summarized in Table 1.2.

I. SOCS3 in colorectal cancer

After initial discovery, a majority of studies focused on anti-inflammatory roles of SOCS3 in immune cells (116). Our laboratory hypothesized that because of its anti-inflammatory roles and negative regulation of STAT3, SOCS3 expressed in IEC may have a tumor suppressor role. As an initial test of this hypothesis, Rigby *et al.* used Cre-recombinase (Cre) technology to generate a mouse model with specific disruption of both SOCS3 alleles in IEC (115). This was accomplished by crossing mice expressing a villin-Cre transgene with mice homozygous for LoxP modifications of the SOCS3 genes, and comparing these animals with littermates carrying LoxP modified, but intact SOCS3 alleles, or wild-type mice carrying the villin-Cre transgene.

The IEC-SOCS3 ^{Δ/Δ} mice had no obvious phenotype in the basal state and so they were given two challenges. In one series of experiments they were given

a single cycle of 3% DSS for 5 days and were studied at the end of DSS and for 3-7 days following DSS. DSS treatment causes acute inflammation, crypt loss, and mucosal injury, followed by a period of IEC hyper-proliferation, crypt regeneration and healing. At the end of DSS treatment, the IEC-SOCS3^{ΔΔ} mice showed similar inflammation and crypt loss as mice with intact SOCS3 genes. However, during the healing and regeneration period, IEC-SOCS3^{ΔΔ} showed dramatically enhanced crypt proliferation and crypt hyperplasia (115). This was the first *in vivo* demonstration that SOCS3 expressed in IEC normally restrained crypt proliferation.

The AOM/DSS model was then used to assess if the loss of SOCS3 in IEC affected inflammation-associated colon cancer. AOM/DSS combines a chemical mutagen with multiple cycles of DSS to model chronic, reactivating inflammation. Mice with IEC-SOCS3 deletion had a four-fold increase in tumor load compared to controls, which reflected an increase in tumor number and tumor size (115). SOCS3 over-expression decreased proliferation of colon cancer cells lines, further supporting the role of SOCS3 as a suppressor of tumor cell growth (115). Colon and tumor tissue from AOM/DSS treated IEC-SOCS3^{ΔΔ} mice were assessed for signaling pathways affected by IEC-SOCS3 deletion. As anticipated, loss of SOCS3 was associated with an increase in activated STAT3 (115). In addition, these tumors showed an increase in NFκB activation.

The increase in NFκB activation in IEC-SOCS3 deletion mutants was a critical finding, as a key role of NFκB had been demonstrated in the AOM/DSS model of inflammation-associated CRC. This was accomplished by development

of mice with IEC- or macrophage-specific IKK β deletion. IKK β phosphorylates I κ B, which is bound in the cytoplasm to NF κ B sub-unit dimers (p65, c-Rel, and p50), targeting it for proteasomal degradation. This permits NF κ B translocation to the nucleus, and transcription of multiple pro-inflammatory genes (117). Mice lacking IEC-IKK β (and thus decreased NF κ B activation) exhibited a decrease in incidence of AOM/DSS induced colon tumors associated with enhanced apoptosis, and this was irrespective of DSS-induced inflammation. Mice lacking macrophage-IKK β also had a modest decrease in tumor incidence, but the most striking effect was the decrease in tumor size due to decreased expression of pro-inflammatory cytokines (117). This led to the conclusion that IEC-NF κ B is essential for tumor initiation, while macrophage-NF κ B promotes tumor growth through the up-regulation of paracrine factors.

Other studies have shown that constitutive activation of NF κ B promotes proliferation and prevents apoptosis in colon cancer cells (118). Additionally, activation of NF κ B protects colon tumor cells from irradiation, thus promoting tumor survival (118). IL-6 activates NF κ B in Caco-2 cells to induce ICAM, which can be reversed by over-expression of SOCS3 (65). SOCS3 has been shown to promote degradation of TRAF6 and TAK1, which are both downstream signaling molecules of NF κ B (119). Data from our laboratory were, to our knowledge, the first to demonstrate that IEC-SOCS3 normally limits NF κ B activation *in vivo* (115).

These data provide preclinical evidence that SOCS3 normally acts as a tumor suppressor, and limits activation of STAT3, as well as NF κ B. As discussed

below, increasing evidence points to STAT3 as a key mediator of CRC. Thus, identification of SOCS3 as an intrinsic inhibitor of both NFκB and STAT3 pathways suggests that SOCS3 may represent a biomarker of CRC risk and potential target for therapies that might mimic SOCS3.

J. IL-6/STAT3 in sporadic and inflammation-associated colorectal cancer

A number of recent studies have highlighted the role of the IL-6/STAT3 pathway in both sporadic and inflammation-associated CRC in mouse models and humans. This evidence, summarized below, underscores the importance of exploring the role of SOCS3 in sporadic neoplasia, as is addressed in CHAPTERS III and IV.

Studies in the AOM/DSS model of colitis-associated cancer and the APC^{Min/+} model of spontaneous tumors showed that loss of IL-6 leads to decreased tumor load and this was associated with decreased tyrosine-phosphorylation of STAT3 (120, 121). Prior studies in mice lacking macrophage- $\text{IKK}\beta$ in the AOM/DSS model showed that myeloid production of IL-6 is a critical component in tumor initiation (117). Consistent with these findings, wild-type mice had increased IL-6 expression in infiltrating immune cells in adenomas of AOM/DSS-treated mice, with weaker IL-6 expression in the IEC (121). These studies suggest that IL-6 derived from immune cells may have paracrine actions to stimulate STAT3 activation in IEC and to promote tumor growth. Other studies suggest that IECs themselves can produce IL-6, and may therefore promote IL-6 activation of STAT3 in an autocrine fashion (122, 123).

Mice with a Y757F mutation in the gp130 subunit of the IL-6 receptor (gp130^{Y757F} mice), which leads to constitutive gp130 activation, have enhanced ligand-dependent STAT3 activation due to the inability of SOCS3 to bind and inhibit phosphorylation of the gp130 subunit and therefore inhibit STAT3 activation (124). These mice exhibit enhanced tumor incidence and size in the AOM/DSS model, and this is associated with increased IEC proliferation and also increased expression of the cell-cycle progression genes cyclin D1, cyclin B1, c-Myc and cdc2 (125). These same mice showed an increase in tumor incidence in the APC^{Min/+} model as well (125). Interestingly, deletion of either IL-6 or IL-11 in gp130^{Y757F} mice did not affect the IEC hyper-proliferation seen in gp130^{Y757F} mice. However, deletion of both IL-6 and IL-11 in the gp130^{Y757F} mutant led to a significant decrease in proliferation as measured by BrdU incorporation (125). This suggests functional redundancy between IL-6 and IL-11 with respect to regulation of proliferation of colon epithelial cells.

Consistent with findings in the gp130^{Y757F} mice, mouse models lacking IEC-STAT3 (Stat3^{ΔIEC}) are highly resistant to development of AOM/DSS-mediated tumors (121). This is despite the fact that Stat3^{ΔIEC} mice had more severe DSS-induced colitis than control mice, implicating a protective role of STAT3 in inflammation. This could reflect the inability of Stat3^{ΔIEC} mice to induce SOCS3, which would provide negative feedback of other pro-inflammatory pathways, such as NFκB (115). However, further studies must be done to look at the specific role of SOCS3 in Stat3^{ΔIEC} mice under these conditions.

Similar to the effect seen in the AOM/DSS model, Stat3^{ΔIEC} mice crossed with APC^{Min/+} mice exhibited a decrease in tumor number compared to wild-type APC^{Min/+} mice (126). Interestingly, despite this affect, aged Stat3^{ΔIEC}/APC^{Min/+} mice exhibited increased tumor size and a higher percentage of invasive carcinoma and lymph node metastases than their wild-type counterparts, with earlier death in mice lacking IEC-STAT3 (126). Intriguingly, the invasive regions of Stat3^{ΔIEC}/APC^{Min/+} tumors showed a decrease in phosphorylation of STAT3 on the key tyrosine 705 residue, compared to the main tumor mass, indicating that factors leading to the enhanced invasive phenotype may be independent of Tyr-705-phosphorylation of STAT3. However, this would be inconsistent with findings in human CRC that Tyr-705-phosphorylation of STAT3 is significantly correlated with tumor invasion (127). These studies collectively identify a seemingly paradoxical role of STAT3 as both a promoter of tumor initiation, yet an inhibitor of tumor progression. This possibility to date has been addressed in only the APC^{Min/+} model of spontaneous tumors and not in the colitis-associated AOM/DSS model. Further studies must be done to determine the underlying mechanism of the phenotypic switch of STAT3 from oncogene in early stages of sporadic CRC to anti-oncogene in later stage tumor progression.

Recent evidence in humans revealed that IL-6 and STAT3 are increased, and SOCS3 is decreased, in tumors from both ulcerative colitis-associated and sporadic CRC. Moreover, low SOCS3 expression was attributed to DNA hypermethylation in inflammation-associated tumors (128). These same studies also indicated that while SOCS3 silencing occurs in tumors, its expression is not

decreased in pre-cancerous, inflamed tissue. This is consistent with our recent report in which SOCS3 expression did not differ significantly in the normal colonic mucosa of patients with adenoma versus adenoma-free patients (129). Also, logistic regression did not provide evidence that SOCS3 in normal mucosa had an independent predictive value for adenoma risk (129). These studies are described in detail in CHAPTER III. Together, the available information in humans suggests that loss of SOCS3 does not occur in normal mucosa, but its loss in adenoma may predispose to survival and continued progression of these lesions.

Collectively, the studies in mice and humans indicate that de-regulated IL-6/STAT3 signaling promotes intestinal tumorigenesis. SOCS3 functions as a tumor suppressor in the intestine and negatively regulates both STAT3 and NFκB, two key signaling pathways that appear integral to development of inflammation-associated CRC. The aforementioned mouse studies of the IL-6/STAT3/SOCS3 pathway in intestinal tumorigenesis are summarized in Table 1.3. As well as a role in CRC, accumulating evidence suggests a role of STAT3 and SOCS3 in cancers of other regions of the GI tract as summarized below.

K. SOCS3 in esophageal cancer

To date, few studies have analyzed the potential role of SOCS in esophageal cancer. However, activated STAT3 is detected in the nuclei of dysplastic Barrett's esophagus tissues (130). In addition, bile acids and low pH induce the IL-6/STAT3 pathway in Seg-1 esophageal adenocarcinoma cells

(130). Consistent with these findings, analysis of human tissue in various stages of Barrett's adenocarcinoma revealed that SOCS3, and to a lesser extent SOCS1, is down-regulated by promoter hyper-methylation (131). This emerging evidence suggests a potential tumor suppressor role for SOCS3 in the pathogenesis of Barrett's adenocarcinoma, but further studies, such as tissue-specific SOCS3 silencing using the squamous esophageal-specific ED-L2 promoter (132) would be useful to confirm this hypothesis.

L. SOCS3 in stomach cancer

Considerable evidence supports tumor-suppressor roles of SOCS3 in the stomach. A study of RGM-1 rat gastric mucosal cells showed that infection with *H. pylori* induced SOCS3 expression, while SOCS1 was not expressed and SOCS2 expression remained unchanged (133). This is intriguing as *H. pylori* infection is a factor associated with increased risk of gastric cancer. T3b (non-classical major histocompatibility class 1 molecule) promoter-Cre recombinase mediating silencing of SOCS3 in the gastric epithelium and small and large intestines, resulted in spontaneous hyperplastic lesions in the stomach as early as three weeks of age, and gastric adenocarcinoma by 12-15 weeks (134). These effects of SOCS3 deletion were associated with increased nuclear STAT3, as well as nuclear accumulation of β -catenin in the gastric epithelium (134).

The gp130^{Y757F} mouse expresses mutant a gp130 receptor, which is unable to bind SOCS3. These mice have enhanced STAT3 and STAT1 activation and developed spontaneous hyperplastic lesions in the stomach by 4

weeks of age (135). These effects were associated with dysregulated SHP2, Erk, and AP-1 transcription (135). Recent studies in the gp130^{Y757F} mouse have highlighted IL-11, perhaps more so than IL-6, as pro-proliferative factors in the development of gastritis-associated gastric tumorigenesis (136). This is interesting because other inflammation-associated cancers in other regions of the GI tract appear to be mediated predominantly through IL-6 signaling. Importantly IL-11 can activate both STAT3 and STAT1, and inhibition of either of these STATs in the gp130^{Y757F} mouse decreased gastric tumors and IL-11 signaling (137).

M. SOCS3 in liver and gall bladder cancers

The nitrosodiethylamine (DEN) model of hepatocellular carcinoma (HCC) has been used by two independent groups to define the role of hepatocyte-SOCS3. Collectively, these studies showed that mice lacking hepatocyte-SOCS3 had an increase in liver tumor incidence, number, and size when compared to mice with floxed, but intact SOCS3 alleles. SOCS3 silencing led to increased cellular proliferation, increased STAT3 (and its target genes Bcl-XL, Bcl-2, c-Myc, cyclin D1), increased VEGF, increased ERK activation, and increased circulating IL-6 (105, 138). In a concanavalin-A model of hepatitis, mice lacking liver-SOCS3 had increased STAT3 and NFκB activation, with associated decreases in apoptosis (105). These findings strongly support the role of SOCS3 as a tumor suppressor in inflammation-associated carcinogenesis of the liver.

Several studies have shown that hepatitis B and C are major risk factors for the development of HCC in humans (139, 140). In a study of hepatitis C (HCV) patients, SOCS3 expression was found to be significantly elevated in non-HCC-regions compared to HCC-regions, as well as compared to patients without HCV. Indeed, in non-HCC areas, HCV patients had significantly higher levels of local IL-6 and IFN γ expression and associated increases in STAT3 and STAT1. These data suggest that HCV patients exhibit a local inflammatory environment in which SOCS3 may become silenced, thus permitting tumor formation (105). Paradoxically, a different study of human HCC showed that SOCS3 expression was low in only a subset of HCC areas studied, and that high SOCS3 correlated with vascular invasion, an indicator of poor prognosis (141). A separate protein microarray analysis of HCC and normal liver tissues showed that both SOCS3 and STAT3 were differentially up-regulated, and this was confirmed by western blot (142). These data suggest a disconnect in the STAT3/SOCS3 feedback loop in liver that promotes HCC, whereby available SOCS3 is insufficient to attenuate STAT3 activation.

Few studies have examined the role of SOCS proteins in gall bladder cancers. In a study looking at SOCS3 in human cholangiocarcinoma tissues and cell lines, there was an inverse correlation between tyrosine-phosphorylated STAT3 and SOCS3 protein levels, and SOCS3 was highly methylated in tumor compared to non-tumor tissue and in two cholangiocarcinoma cell lines (102, 143). These data suggest that SOCS3 silencing leads to prolonged STAT3 activation and subsequent tumorigenesis in the biliary tract.

N. Hypotheses tested by the studies presented in this dissertation

The aim of the studies in this dissertation was to contribute to the growing body of knowledge of SOCS3 as a tumor suppressor in inflammation-associated and sporadic CRC. *In vitro* studies assessed mechanisms of SOCS3 tumor suppression in the context of cytokines, while translational studies evaluated the potential of low SOCS3 expression as a biomarker of adenoma risk. Finally, preliminary studies in mice addressed the role of SOCS3 silencing in sporadic CRC. These studies are based on the following hypotheses:

- I. Basal or cytokine-induced TNFR2 expression is mediated by STAT3 and suppressed by SOCS3. SOCS3 limits TNFR2-mediated growth of colon cancer cell lines. These studies are described in Chapter II.
- II. Low SOCS3 expression in biopsies of normal colonic epithelium correlates with adenoma risk. These studies are described in Chapter III.
- III. SOCS3 deletion in intestinal epithelial cells promotes tumor formation in the azoxymethane (AOM) mouse model of sporadic CRC. These studies are described in Chapter IV.

Table 1.1. SOCS3 methylation in gastrointestinal cancers

Cancer	SOCS3 Methylation Pattern	Tissues Studied	Citations
Barrett's adenocarcinoma	SOCS3 promoter hyper-methylated in 74% of tissues studied	Human Barrett's adenocarcinoma (n=19), intraepithelial neoplasia (n=56), and precursor (n=30) or normal tissue (n= 20)	(131)
Colorectal adenocarcinoma	SOCS3 is hyper-methylated in ulcerative colitis-associated colorectal cancer	Human tissue, methylation in tumor versus non-tumor tissue (n=4)	(103)
Hepatocellular carcinoma (HCC)	SOCS3 hyper-methylation in HCC subclass with poor survival	Human HCC tissues with surrounding normal tissue (n=80), n=55 for cirrhosis-associated, n=58 for poor survival (<3 years) group	(144)
	SOCS3 is hyper-methylated in HCC cell lines and in 33.3% of primary HCC tumors; aberrant methylation of SOCS1 in 67% of human HCC tumors; concomitant methylation of SOCS1 and SOCS3 in 28% of human HCC tumors	HuH2, Hap3B, HT17 HCC cell lines; primary HCC tumors (n=18)	(104)
Cholangiocarcinoma	SOCS3 hyper-methylated in cholangiocarcinoma tissues and cell lines	Human cholangiocarcinoma cell lines Mz-ChA-1 and CCLP1, resected liver tissue with intrahepatic cholangiocarcinoma (n=26)	(102, 143)

Table 1.2. Mouse models of intestinal cancer discussed in this dissertation

Mouse Model	Mechanisms	Tumor Phenotype	Citations
Adenomatous polyposis coli-multiple intestinal neoplasia (APC ^{Min/+})	Heterozygous mutation in <i>APC</i> gene, loss of wild-type copy of <i>APC</i> gene leads to β -catenin activation and TCF/LEF-mediated cell cycle progression	Hundreds of small intestinal polyps, small number of colon tumors	(145-148)
Azoxymethane (AOM)	Mutations in <i>APC</i> and <i>Ctnnb1</i> genes leads to β -catenin activation and TCF/LEF-mediated cell cycle progression	Colon tumor development*	(149-153)
AOM/dextran sodium sulfate (AOM/DSS)	β -catenin activation and TCF/LEF-mediated cell cycle progression, elevated COX-2 and iNOS expression in tumor lesions	Colon tumor development*	(154, 155)

* Strain-dependent differences in tumor phenotype

Table 1.3. IL-6/STAT3/SOCS3 pathway in mouse models of intestinal cancer

	Mutation	Mouse Model	Tumor Phenotype	Mechanisms	Citations
IL-6	IL-6 ^{-/-}	AOM/ DSS*	↓ tumor load	↓ proliferation ↓ phospho-STAT3 ↓ cyclin-D1 ↓ COX-2 ↓ MMP9 ↓ Hsp70 ↓ Bcl-X _L	(121)
	IL-6 ^{-/-}	APC ^{Min/+} **	↓ tumor load	↓ phospho- to total STAT3 ratio	(120)
STAT3	gp130 ^{Y757F} †	AOM/ DSS	↑ tumor incidence and size	↑ proliferation ↑ cyclin D1 ↑ c-Myc ↑ cyclin B1 ↑ cdc2	(125)
	gp130 ^{Y757F}	APC ^{Min/+}	↑ tumor incidence	Unspecified	Unpublish ed data noted in (125)
	IEC- STAT3 ^{ΔΔ}	AOM/ DSS	↓ tumor load	↓ tumor multiplicity ↓ tumor size ↓ proliferation ↑ apoptosis	(121, 125)
	IEC- STAT3 ^{ΔΔ}	APC ^{Min/+}	↓ tumor number, but ↑ tumor size in aged mice	↑ IEC proliferation, ↑ nuclear β- catenin ↑ cyclin D1 ↓ ceacam1	(126)
SOCS3	IEC- SOCS3 ^{ΔΔ}	AOM/ DSS	↑ tumor load	↑ IEC proliferation ↑ IL-6/STAT3 and TNFα/NFκB activation	(115)
	IEC- SOCS3 ^{ΔΔ}	AOM ^{††}	↑ tumor incidence	Not yet determined	Chapter IV

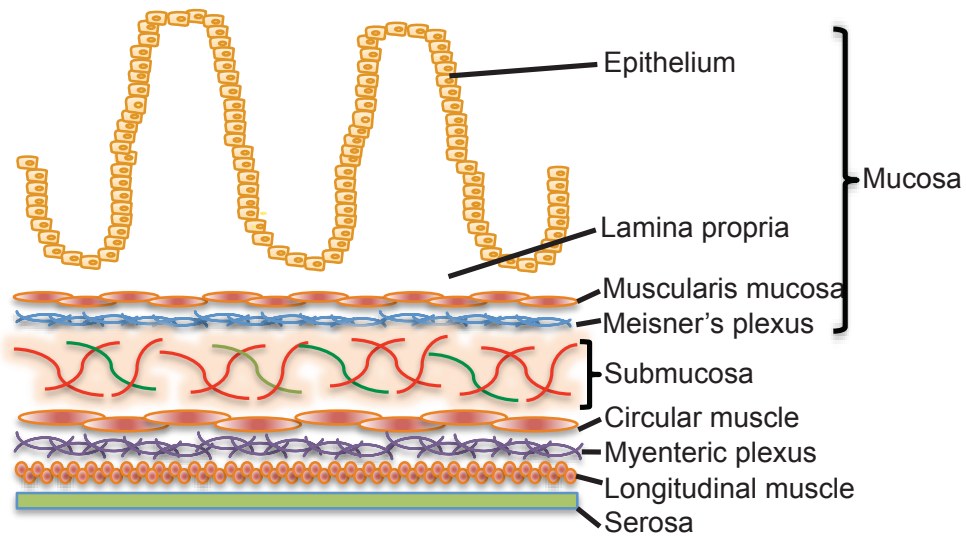
* Azoxymethane/dextran sodium sulfate model (inflammation-associated CRC)

** Genetic model of spontaneous CRC

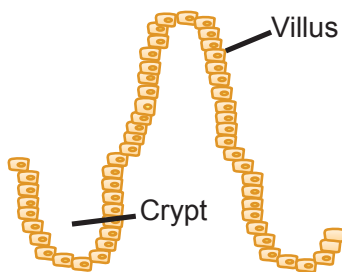
† Causes STAT3 hyper-activation

†† Chemically-induced model of spontaneous CRC

A Intestinal Layers



B Small intestine



C Colon

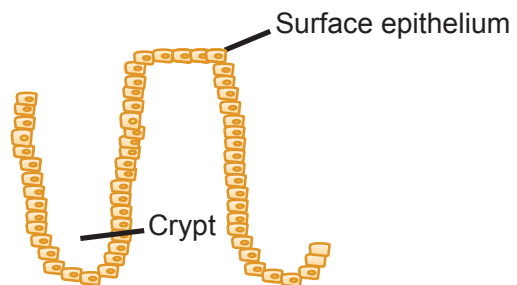
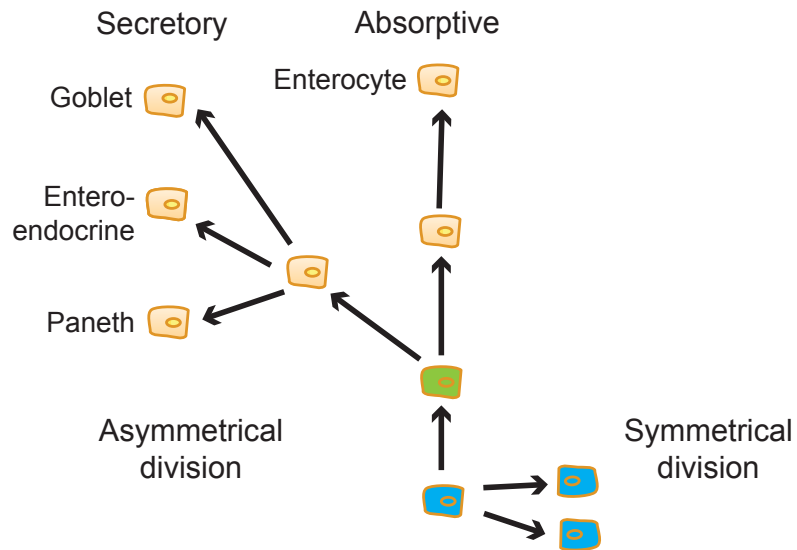


Figure 1.1. Schematic of intestinal layers and organization of small intestine and colon epithelium. (A) The intestine is comprised of multiple layers. (B & C) The small intestine is organized into crypts and villi, while the colon is comprised of crypts and surface epithelium.

A Intestinal stem cell division



B Colonic crypt zones

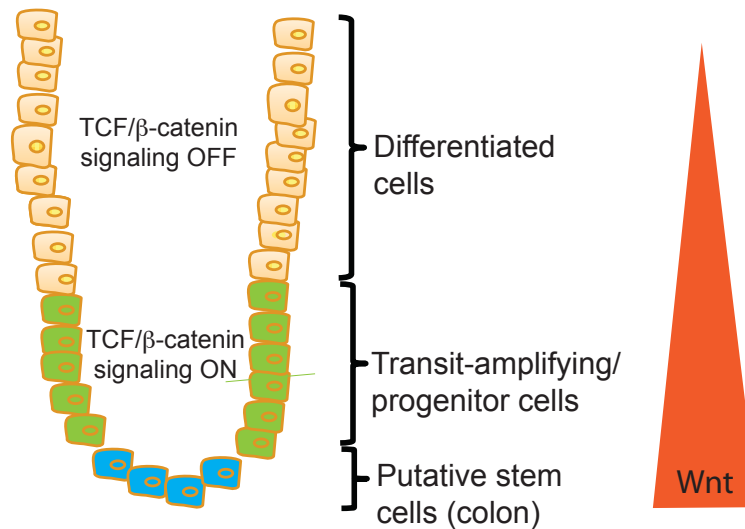
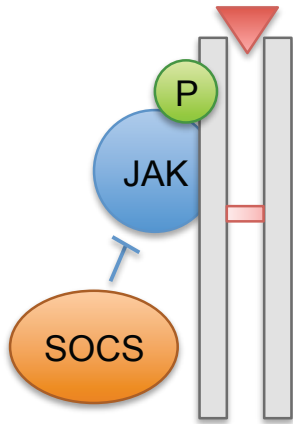
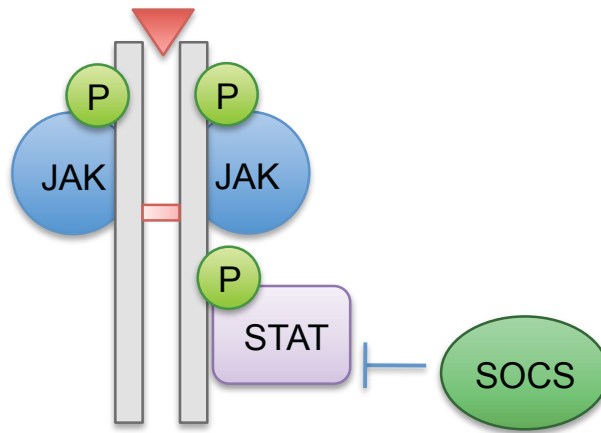


Figure 1.2. Schematic of intestinal stem cell division and location of stem cell, proliferative, and differentiation zones in the crypt. (A) Stem cells undergo asymmetric division to produce cells of all four intestinal epithelial lineages. Symmetric division occurs infrequently and can facilitate production of two stem cells. (B) Stem cells are thought to reside at the base of the crypt in the descending colon (and in the mid-crypt in ascending colon). After stem cell division, daughter cells proliferate in the transit-amplifying zone, and differentiate as they migrate towards the surface epithelium. The presence of Wnt signaling promotes proliferation in the stem cell and transit-amplifying zones, while Wnt signaling is thought to be “turned off” in the differentiation zone (5-7).

A JAK inhibition



B Inhibition of STAT binding to cytokine receptor



C Degradation of signaling complexes

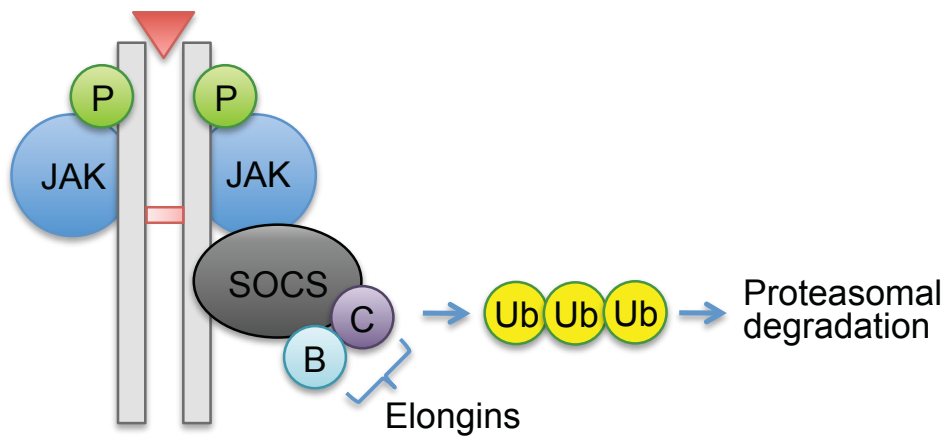
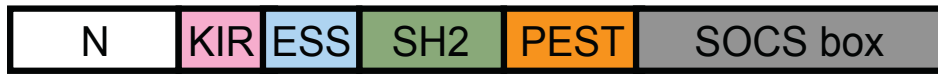


Figure 1.3. General actions of SOCS proteins. (A) SOCS proteins inhibit JAK through direct binding or through binding to JAK docking sites on cytokine receptors. (B) SOCS proteins compete with STAT proteins for binding sites on cytokine receptors. (C) SOCS proteins target their binding proteins for proteasomal degradation through interactions with E3 ubiquitin ligases.

A SOCS3



B

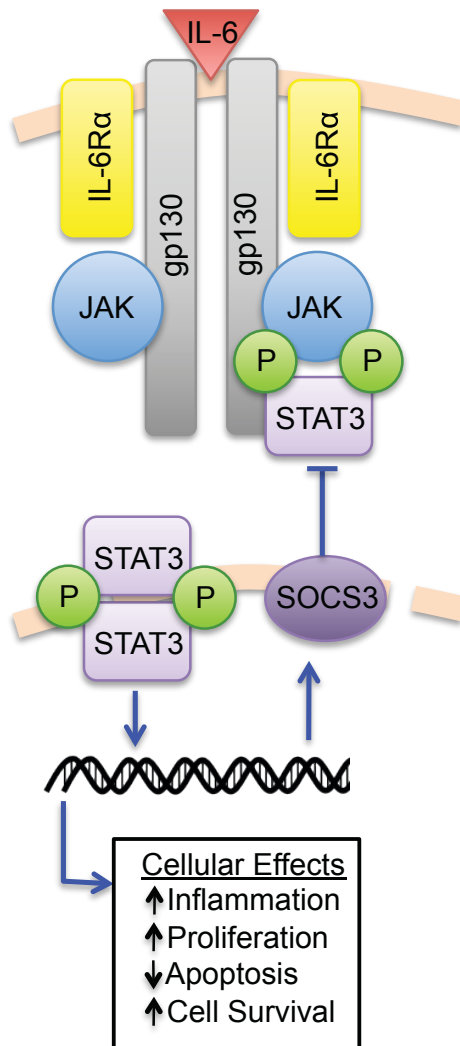


Figure 1.4. SOCS3 protein domains and general pathway. (A) SOCS3 binds other proteins through its SH2 and ESS (extended SH2) domains, and inhibits JAK phosphorylation through its kinase inhibitory region (KIR). Alternatively, SOCS3 can promote degradation of its binding partners through SOCS-box dependent binding to E3 ubiquitin ligases. SOCS3 protein turnover is mediated through the PEST (Pro/Glu/Ser/Thr-rich) domain. (B) Upon IL-6 binding to its receptor, phosphorylated JAK binds to and phosphorylates STAT3, which then dimerizes and translocates to the nucleus. SOCS3 is induced by and subsequently inhibits STAT3 and the various cellular effects of STAT3 activation.

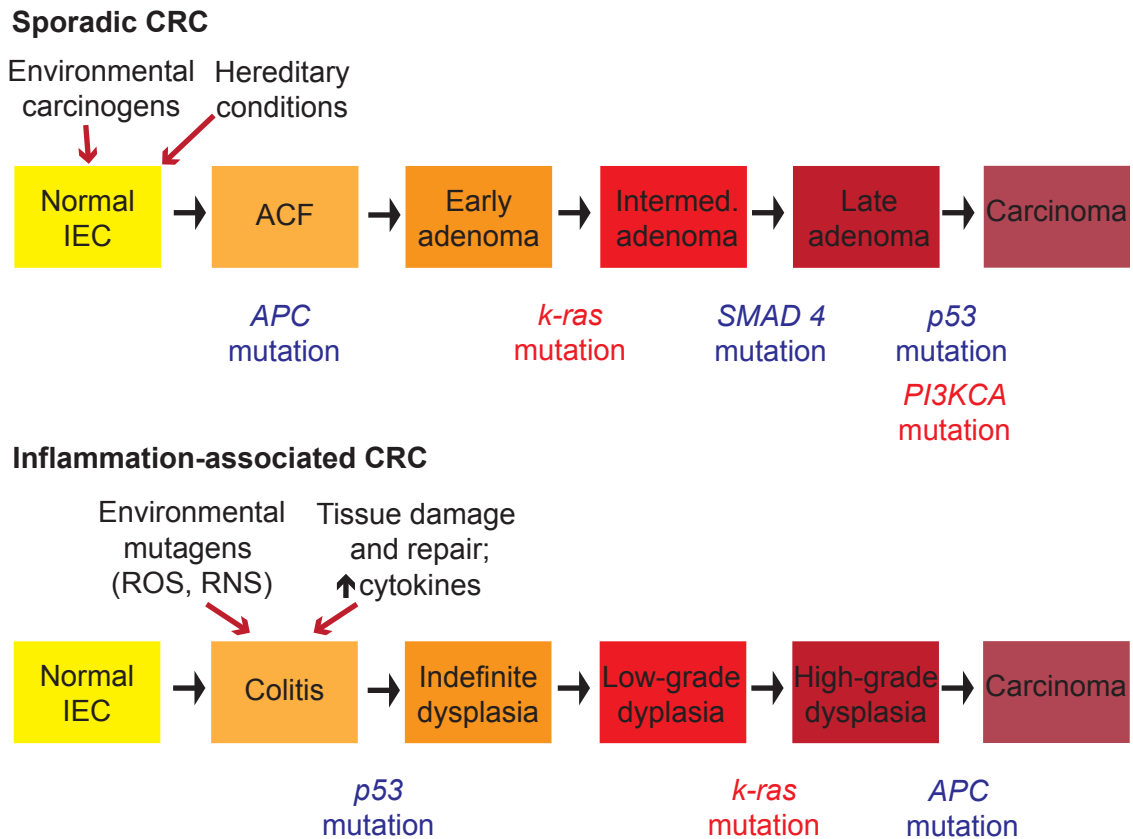


Figure 1.5. Initiating events and common mutations in progression of sporadic and inflammation-associated CRC. Both cancer types share common mutations, but differ in the point of progression in which such mutations occur. Adapted from (106, 107, 110, 156, 157)

CHAPTER II

CYTOKINE-INDUCTION OF TUMOR NECROSIS FACTOR RECEPTOR 2 (TNFR2) IN COLON CANCER CELLS IS MEDIATED BY STAT3 AND SUPPRESSED BY SOCS3

A. Introduction

Patients with inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis have an increased lifetime risk of developing inflammation-associated colorectal cancer (CRC) (158-160). Chronic increases in proliferation of intestinal epithelial cells (IEC) driven by pro-inflammatory factors have been shown to promote tumorigenesis. The IL-6/STAT3 (121, 125, 161-164) and TNF α /NF κ B (117, 165, 166) pathways are both major mediators of inflammation-associated CRC and recent studies show that a TNF α neutralizing antibody decreases intestinal tumor formation in mice (51, 57, 121).

TNF α signals through two receptors: TNFR1 and TNFR2. TNFR1 exerts pro-apoptotic functions due to its intracellular death domain (167). TNFR2, which lacks a death domain, promotes proliferation of IEC and colon cancer cells (56, 168). This supports a concept that TNFR2 may mediate pro-tumorigenic effects of TNF α . The role of TNFR2 in inflammation-associated cancer is a topic of increasing interest, as recent studies indicate that TNFR2 is up-regulated in IBD and in the azoxymethane/dextran sodium sulfate (AOM/DSS) model of inflammation-associated cancer (56-58). *In vitro* studies have shown that TNFR2 is induced in colon cancer cells treated with both TNF α and IL-6, but neither cytokine alone (56). Other studies have demonstrated TNFR2 induction by IFN γ (55). These findings suggest that the STAT pathways activated by IL-6 or IFN γ and/or NF κ B pathways typically activated by TNF α may interact to induce TNFR2 expression. In support of this possibility, the human TNFR2 promoter contains two consensus STAT binding sites and two consensus NF κ B binding sites (169).

Suppressors of cytokine signaling (SOCS) proteins limit cytokine signaling (170). IEC-specific SOCS3 gene deletion increased tumor load in the AOM/DSS model of colitis-associated CRC (115). This effect was associated with enhanced activation of both STAT3 and NF κ B (115). *In vitro*, SOCS3 over-expression reduced proliferation of colon cancer cell lines and inhibited both IL-6-induced STAT3 activation and TNF α -induced NF κ B activation (115). SOCS3 genes are silenced by promoter hyper-methylation in various human cancers, including CRC (171-174). Together these data provide strong evidence that SOCS3 normally acts as a suppressor of inflammation-associated colorectal cancer. The current study tested the hypothesis that SOCS3 limits the expression and growth-promoting actions of TNFR2 in colon cancer cells. We show that SOCS3 over-expression decreases TNFR2 expression, as well as the ability of STAT3 to bind to the TNFR2 promoter. We also demonstrate that TNFR2 over-expression increases (and TNFR2 silencing decreases) proliferation. TNFR2 over-expression has modest but significant effects to increase anchorage-independent growth of colon cancer cells. Together, these findings provide to our knowledge the first direct evidence that increased TNFR2 expression promotes tumor growth. SOCS3 over-expression dramatically reduces proliferation and anchorage-independent growth of colon cancer cells. This provides direct evidence that SOCS3 is a potent suppressor of colon cancer cell growth and may act in part by limiting TNFR2.

B. Materials and Methods

Cell culture and cytokine treatments

SW480 and COLO205 cells were used in this study because both express low levels of endogenous SOCS3. SW480 cells were used for the majority of experiments because our prior studies demonstrated that these cells are responsive to both IL-6 and TNF α , which robustly activate STAT3 and NF κ B, respectively (115). COLO205 cells were used as an independent cell line to confirm cytokine induction of TNFR2. Since COLO205 cells grow well in soft agar they were also used to address effects of TNFR2 and SOCS3 on anchorage-independent growth. SW480 and COLO205 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in RPMI 1640 media (Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, 50U/mL penicillin, and 50mg/mL streptomycin. Given prior findings that both IL-6 and TNF α were required to induce TNFR2 (56), cells were treated with recombinant human IL-6 and/or TNF α (Peprotech, Rocky Hill, NJ) at 50ng/mL in serum-free medium. Cells were harvested at various times after cytokine treatment for evaluation of TNFR2 mRNA and protein, or STAT3 and NF κ B binding to consensus regions in the TNFR2 promoter.

Semi-quantitative real time PCR analyses

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Reverse-transcription was performed using AMV-RT (Promega, Madison, WI). PCR and

analyses were completed on the Rotorgene 2000 (Qiagen) using Invitrogen (Carlsbad, CA) Platinum qPCR Supermix-UDG and the following Taqman primer-probe sets (Applied Biosystems, Carlsbad, CA): human TNFR2 Hs00961755_m1, human IL-6 (Hs00985639_m1), human ICAM-1 (positive control as NFκB-induced gene). Hydroxymethylbilane synthase (human HMBS) Hs00609297_m1 was used as an invariant control. Non-reverse transcribed (no RT) samples were used as negative controls. Gene expression was calculated using the $R = 2^{-[\Delta\Delta Ct]}$ method, where changes in Ct values for the genes of interest were normalized to HMBS. In all cases, gene expression for particular treatment groups was expressed as fold change versus mean values for no treatment control. Real time PCR reactions were performed in triplicate and replicated in at least three independent experiments.

ELISA for TNFR2

Soluble TNFR2 levels in cell supernatants were measured using Quantikine ELISA system (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. Samples were normalized to total protein as measured by BCA protein assay (Pierce, Rockford, IL).

Flow cytometric analysis of surface TNFR2 expression

Cell surface expression of TNFR2 was assessed using flow cytometry as previously described (56). SW480 cells (1×10^5 cells per condition) were trypsinized, washed with serum-free RPMI 1640 media (Gibco) and incubated

with IL-6 and TNF α at 50ng/mL for 10 hours at 37°C with rotation in 15mL conical tubes. Cells were then resuspended in wash buffer (phosphate-buffered saline supplemented with 1% bovine serum albumin and 1mg/mL DNase (Roche, Palo Alto, CA), and Fc-blocked with 1 μ g human IgG (R&D Systems) for 15 minutes. Cells were then incubated with fluorescein-conjugated anti-TNFR2 (R&D Systems) or isotype control (BD Pharmingen, Franklin Lakes, NJ) for 45 minutes at 4°C. Following antibody incubation, cells were washed and resuspended in 2% paraformaldehyde. Flow cytometric analysis of surface TNFR2 was then performed using a CyAn flow cytometer (Beckman-Coulter-Dako, Brea, CA). Effect of cytokine treatment on TNFR2 surface expression was measured based on fluorescein intensity.

STAT3 and NF κ B inhibition

To test if cytokine-induced TNFR2 mRNA induction requires STAT3 and/or NF κ B activation, SW480 cells were treated with the STAT3 inhibitor Cucurbitacin I (Tocris, Ellisville, MO) at 20 μ M, or I κ B kinase inhibitor Bay 11-7082 at 5 μ M. Bay 11-7082 was kindly provided by Dr. Albert Baldwin (University of North Carolina, Chapel Hill, NC). Inhibitor doses were based on maximum effective doses used in prior studies (175, 176). SW480 cells were seeded in complete media, grown for 24 hours, and treated with inhibitors and IL-6, TNF α , or both cytokines at 50ng/mL for 10 hours prior to mRNA extraction. No treatment controls were treated with vehicle (dimethyl sulfoxide (DMSO)). ICAM-1, which was previously shown to be regulated by cytokine-induction of NF κ B

(65), was measured by Taqman qRT-PCR to confirm effectiveness of Bay 11-7082.

Chromatin immunoprecipitation (ChIP) to assess STAT3 and NF κ B binding to the TNFR2 promoter

For ChIP, SW480 cells were serum-deprived overnight followed by treatment with IL-6, TNF α , or both cytokines (50ng/mL) for 5-60 minutes. After treatment, cells were cross-linked with 1% formaldehyde for 10 minutes. Subsequent steps were performed as specified in the ChIP-IT Express (Active Motif, Carlsbad, CA) user manual. Briefly, cross-linked cells were lysed and sonicated, followed by overnight immunoprecipitation with anti-STAT3 (SC-483x) and anti-p65 NF κ B (SC-372) (Santa Cruz Biotechnology, Santa Cruz, CA), or Negative Control IgG antibody (Active Motif). Eluted, reverse cross-linked protein:DNA complexes were treated with proteinase K for 1 hour, followed by column purification (QIAquick PCR Purification Kit, Qiagen) and then PCR with primers specific to STAT3 and NF κ B-binding elements within the TNFR2 promoter. Oligomers to amplify these binding sites were purchased from Sigma and sequences are shown in Table 2.1. Densitometry was performed to quantify the PCR amplified transcription factor binding sites.

Western blot for activated STAT3 in nuclear extracts

Western blots were performed on nuclear extracts from SW480 cells to determine whether IL-6 combined with TNF α enhances STAT3 activation and

nuclear STAT3. Nuclear extracts were prepared as previously described (177). Briefly, SW480 cells were grown to confluence, serum-deprived overnight, and treated with IL-6 and TNF α (50 ng/mL) for 30 minutes. Cells were then pelleted in lysis buffer containing 10mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 2 μ g/mL aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Nuclei were obtained by adding 10% NP40 and centrifuging for 5 minutes at 15,000 x g. Pellets were then resuspended in buffer containing 20 mM Hepes pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF to release nuclear proteins. The nuclear suspension was centrifuged at 15,000 x g for 5 minutes, and supernatants containing nuclear extracts were then subjected to immunoprecipitation and immunoblot with the following antibodies: anti-phosphotyrosine STAT3: rabbit polyclonal pTyr705 (#9131, Cell Signaling, Danvers, MA); anti-STAT3 (total): rabbit polyclonal SC-7179 (Santa Cruz Biotechnologies). Coomassie-stained protein gels verified equivalent amounts of nuclear protein in samples used for immunoprecipitation.

Constitutive activation of STAT3

Constitutively-active STAT3 (CA-STAT3) adenovirus was kindly provided by Dr. Christian Jobin. This vector is constitutively activated due to C661A and C663N mutations and has been functionally characterized in prior studies (54, 178). SW480 cells were treated with CA-STAT3 at 50 MOI or with IL-6 and TNF α for 10 hours prior to mRNA extraction for evaluation of TNFR2 expression.

SOCS3 and TNFR expression constructs

Cells treated with SOCS3 and/or TNFR2 expression constructs were used to evaluate effects of SOCS3 on cytokine-induced TNFR2 mRNA, proliferation, and anchorage-independent growth. Plasmid pBIG2i expressing human SOCS3 or empty vector were kindly provided by Drs. Richard Furlanetto and Peter Nissley and used to generate adenovirus expressing human SOCS3 as previously described (115). Adenoviruses were used at a multiplicity of infection (MOI) of 100. Cells were treated with adenovirus for 24-48 hours in complete media and switched to serum-free media overnight prior to cytokine stimulation. Adenovirus-mediated over-expression of SOCS3 was confirmed by Northern blot and qRT-PCR (data not shown).

Retroviral expression vector pQCXIP containing c-myc-tagged human TNFR2 was kindly provided by Dr. Daniella Männel (University of Regensburg, Regensburg, Germany). Empty vector pQCXIP was obtained from BD Biosciences Clontech (Mountain View, CA). HEK293 cells were co-transfected with retroviral vectors and packaging vector as previously described (179) using jetPei (Polyplus Transfection, West Chester, PA) according to manufacturers instructions. Media containing TNFR2 retrovirus was collected from transfected HEK293 cells and used to treat SW480 or COLO205 cells for 24-48 hours. TNFR2 over-expression was confirmed using western immunoblot (data not shown).

TNFR2 silencing

SW480 or COLO205 cells were grown to approximately 50-70% confluence in RPMI 1640 medium (Gibco, with 10% FBS, plus antibiotics). Cells were trypsinized and counted. 1×10^6 cells of each cell line were then transfected using nucleofector technology according to manufacturers instructions (Kit V for SW480, Kit T for COLO205, Lonza, Conshohocken, PA) with 100 pmoles of each of 3 different siRNAs: oligonucleotides encoding TNFR1 and TNFR2, or scrambled control (Applied Biosystems). Each transfected cell culture was divided into 2 parts: one was seeded for subsequent RNA isolation; the other into 24-well culture plates for a tritiated-thymidine incorporation/proliferation assay. RNA was isolated after 24 hour incubation in serum-containing medium. Cells in the 24-well plate were incubated for 24 hours in serum-containing medium, then switched to serum-free medium containing tritiated-thymidine (2 uCi/mL, PerkinElmer, Waltham, MA). After 24 hours, labeled cells were washed once with 1XPBS, fixed in 10% trichloroacetic acid for 10 minutes, harvested in 0.2N NaOH/0.1% SDS, collected and counted by liquid scintillation counting (Packard model 1600).

Analysis of cell proliferation and anchorage-independent growth

Assays of [^3H]thymidine incorporation into DNA were used as a measure of cell proliferation and were performed as previously described (180). SW480 cells were plated in 24-well plates at a density of 1×10^4 cells per well and treated with SOCS3 and/or TNFR2 expression constructs for 24 hours. Medium

was then supplemented with 2 $\mu\text{Ci/mL}$ [^3H]thymidine overnight and thymidine incorporation was measured using scintillation counting. Values are expressed as fold change compared to empty vector control.

COLO205 cells, which show robust colony formation in soft agar, were used to test the effects of SOCS3 or TNFR2 on anchorage-independent growth. Cells were treated with empty vector, SOCS3, TNFR2 or both SOCS3 and TNFR2 expression constructs. COLO205 cells were trypsinized and suspended in complete culture media supplemented with 0.3% agar followed by plating in 6-well culture dishes coated with 5% agar. Cells were treated with expression constructs at Days 1, 7, and 14. At day 21, viable cells were stained with 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) for 4 hours and colonies quantified using NIH ImageJ (181).

Statistics

Values are expressed as mean \pm standard error (S.E.). Comparisons between cell treatments were analyzed using one-way analysis of variance followed by post-hoc, pair-wise comparisons using Fisher's PLSD. A p -value of <0.05 was considered statistically significant for all experiments.

C. Results

IL-6 and TNF α induce TNFR2 in SW480 and COLO205 cells

Prior studies in colon cancer cells indicate that TNFR2 expression is increased by combined IL-6 or TNF α treatment, with only modest effects due to

either cytokine alone (56). Figure 2.1A and B confirm increased TNFR2 mRNA levels upon combined treatment with IL-6 and TNF α in SW480 and COLO205 cells. Evaluation of TNFR2 protein by ELISA and flow cytometry (Figure 2.1C, D, and E) verifies these data. Surprisingly, SW480 cells treated with TNF α alone exhibit a similar magnitude of induction as treatment with both cytokines. We therefore tested if TNF α -treated cells had increased IL-6 mRNA levels, indicating that these cells may be exhibiting autocrine IL-6 stimulation, an emerging concept seen in other cancers (123, 182, 183). Figure 2.1F demonstrates that TNF α treatment leads to induction of IL-6 mRNA in SW480 cells, which suggests that TNF-induced IL-6 may contribute to the induction of TNFR2 by TNF α to similar levels as observed in cells treated with both IL-6 and TNF α .

STAT3 inhibitors more potently inhibit TNFR2 expression than NF κ B inhibitors

To functionally assess the role of STAT3 or NF κ B in regulating TNFR2 expression, we examined the effects of a STAT3 (cucurbitacin) or NF κ B (Bay 11-7082) inhibitors on basal and cytokine-induced TNFR2 mRNA. Based on published and pilot studies, we used maximally effective doses of 20 μ M cucurbitacin or 5 μ M Bay 11-7082. Cucurbitacin significantly decreased both basal- and cytokine-induced TNFR2 mRNA (Figure 2.2A). Bay 11-7082 treatment had no significant effect on basal or IL-6 mediated TNFR2 mRNA levels, but decreased TNF α -induced TNFR2, and modestly although non-significantly decreased TNFR2 mRNA in cells treated with both IL-6 and TNF α .

Importantly the inhibitory effects of cucurbitacin combined with Bay 11-7082 on basal or cytokine-induced TNFR2 did not differ significantly from STAT3 inhibitor alone, indicating a primary role for STAT3 in mediating basal or cytokine-induced TNFR2 expression (Figure 2.2A). Since it has been established that cytokines induce ICAM-1 through NFκB-dependent mechanisms, we verified the efficacy of Bay 11-7082 by showing that IL-6 and TNFα induced a 4.4 ± 0.6 -fold increase in ICAM-1 mRNA, and treatment with Bay 11-7082 potently inhibited this effect (Figure 2.2B). Thus, the modest effects of NFκB inhibitor versus STAT3 inhibitor on TNFR2 expression were not due to a lack of effective Bay 11-7082 dosing. Together these findings suggest a predominant role of STAT3 in mediating cytokine-induced TNFR2 expression.

To confirm a role for STAT3 in TNFR2 expression, we treated SW480 cells with a constitutively-active STAT3 (CA-STAT3) adenovirus. Expression of CA-STAT3 in the absence of cytokine treatment induced TNFR2 mRNA levels to the same degree as observed with combined IL-6 and TNFα treatment (Figure 2.2C). Together, the data with STAT3 inhibitor and constitutively activated STAT3 suggest that STAT3 is necessary and sufficient for basal and IL-6/TNFα-induced TNFR2 expression.

IL-6 and TNFα induce STAT3, but not NFκB binding to the TNFR2 promoter

Given that our prior data suggested a predominant role of STAT3 versus NFκB in mediating increased TNFR2 expression in response to IL-6 and TNFα, we used ChIP to more directly assess the effect of cytokines on binding of

STAT3 and NF κ B to putative binding elements in the TNFR2 promoter (Figure 2.3A). Using antibodies specific to STAT3 or NF κ B, we performed ChIP assays followed by PCR amplification of the putative STAT3 and NF κ B binding sites. Combined IL-6 and TNF α treatment induced binding of STAT3 to both of the putative STAT binding sites, with maximal binding at 30 and 60 minutes for -364 and -1578 sites, respectively (Figure 2.3B). Densitometry revealed that IL-6 and TNF α induced 2.0 ± 0.4 -fold and 3.8 ± 1.6 -fold and increases in STAT3 binding to the -364 and -1578 sites, respectively. There was a small degree of basal NF κ B binding, but surprisingly, this was not enhanced by IL-6 and TNF α treatment.

To delineate the individual contribution of IL-6 and TNF α on STAT3 binding to the TNFR2 promoter, we treated cells with either cytokine alone or in combination and performed ChIP for STAT3 as described above. IL-6 and TNF α alone modestly induced STAT3 binding to the -1578 STAT binding site, while both cytokines combined induced dramatic increases in STAT3 binding to this -1578 element (Figure 2.3C). Putative STAT3 binding site -364 differed in that IL-6 alone, but not TNF α induced STAT3 binding and combined cytokines had similar effects as IL-6 alone. Thus, the cooperative effects of IL-6 and TNF α to activate STAT3 appear selective for the -1578 STAT3 binding site.

Because combined IL-6 and TNF α led to a dramatic increase in STAT3 binding to the -1578 STAT3 binding site in the TNFR2 promoter, we assessed whether the two cytokines in combination enhanced tyrosine phosphorylation of STAT3 relative to IL-6 or TNF α alone. Western blot on nuclear extracts revealed

that IL-6 increased phosphorylated and total STAT3 in the nucleus, whereas TNF α alone has no effect (Figure 2.3D). Both cytokines together did not dramatically augment tyrosine phosphorylation of STAT3 or total nuclear STAT3. Thus, enhanced tyrosine phosphorylation of STAT3 does not appear to account for the combinatorial effects of IL-6 and TNF α on STAT3 binding to the -1578 STAT3 binding site. It is noteworthy that the treatment time points used to examine TNF α effects on STAT3 binding by CHIP and STAT3 tyrosine phosphorylation are much shorter than the times (10 hours) needed for TNF α to induce IL-6 mRNA.

SOCS3 inhibits cytokine-induced TNFR2 expression and STAT3 binding to the -1578 STAT3 site

Negative regulation of STAT3 by SOCS3 is well established (66, 86, 87). To test whether SOCS3 inhibits TNFR2 expression, we treated SW480 cells with SOCS3 adenovirus or empty vector control and examined TNFR2 mRNA. As anticipated, SOCS3 over-expression significantly inhibited cytokine-induced TNFR2 (Figure 2.4A). ChIP assay also revealed that SOCS3 over-expression dramatically inhibited cytokine-induced STAT3 binding to the -1578 site, but had variable and non-significant effects on STAT3 binding to the -364 site (Figure 2.4B).

TNFR2 over-expression increases proliferation

Prior *in vivo* studies suggest that TNFR2 null mice show reduced crypt proliferation during intestinal inflammation (56). To directly test the effects of TNFR2 on cancer cell proliferation, and whether SOCS3 can inhibit this effect, we over-expressed TNFR2 and/or SOCS3 in SW480 cells and measured [³H]thymidine incorporation into DNA. TNFR2 increased [³H]thymidine incorporation into DNA, and SOCS3 markedly inhibited basal and TNFR2-induced proliferation of SW480 cells (Figure 2.5A). To assess whether endogenous TNFR2 mediates colon cancer cell proliferation, we silenced TNFR2 using siRNA (Figure 2.5B). TNFR2 expression was decreased 45% using TNFR2-targeted siRNA, and TNFR1 mRNA was unaffected. A control TNFR1 siRNA had no effect on TNFR2 mRNA but inhibited TNFR1 by 80%. Western blots verified that TNFR2 protein levels were decreased by 40% with TNFR2-specific siRNA (data not shown). TNFR2 silencing modestly, but significantly decreased [³H]thymidine incorporation (Figure 2.5C). Together, these results demonstrate that TNFR2 directly promotes proliferation of SW480 cells.

SOCS3 over-expression limits TNFR2-mediated anchorage-independent growth

The ability of cancer cell lines to exhibit anchorage-independent growth in soft agar is indicative of phenotypic transformation towards unregulated growth. We used COLO205 cells to test the effect of TNFR2 and SOCS3 on anchorage-independent growth. We first confirmed that SOCS3 over-expression limits TNFR2 expression in these cells (Figure 2.6A). Next, COLO205 cells were plated

in soft agar and treated with empty vector, TNFR2, and/or SOCS3 expression constructs. Cells over-expressing TNFR2 showed a small, but significant increase in colony formation when compared to empty vector controls. SOCS3 over-expression dramatically decreased (>70%) colony formation compared to empty vector control and SOCS3 also dramatically decreased colony formation in cells over-expressing TNFR2 (Figure 2.6B & C).

D. Discussion

The etiology of inflammation-associated CRC is based strongly on the model that chronically up-regulated cytokines drive excessive proliferation of intestinal epithelial cells, tumor initiation and progression. TNFR2 has recently emerged as a pro-proliferative factor that is up-regulated in IBD and in the AOM/DSS model of IBD-associated cancer (56, 57). Mechanisms regulating TNFR2 expression in IBD or CRC are not fully defined, although prior studies suggest that combined effects of IL-6 and TNF α promote TNFR2 expression (56). The current study provides novel evidence that IL-6 and TNF α act predominantly through STAT3 to induce TNFR2 in CRC (Figure 2.7). We also demonstrate that SOCS3 inhibits cytokine induction of TNFR2 and STAT3 binding to the TNFR2 promoter, and can limit the ability of TNFR2 to promote proliferation or anchorage-independent growth of colon cancer cells.

Mizoguchi and colleagues provided the first evidence for up-regulation of TNFR2 during intestinal inflammation by demonstrating that TNFR2 was increased during acute DSS-colitis, and this was preceded by IL-6/STAT3

activation. They also demonstrated that TNFR2 disruption led to decreased IEC proliferation in the T-cell receptor α (TCR α) null model of colitis (56).

Furthermore, TCR α mice with disruption of both IL-6 alleles showed reduced colitis severity and decreased TNFR2 expression compared to TCR α mice with intact IL-6 (56). While these studies suggested an association between IL-6/STAT3 and TNFR2, the ability of STAT3 to directly regulate TNFR2 expression has not been tested. Prior *in vitro* studies indicated that both IL-6 and TNF α are required to induce TNFR2 in CRC cells, potentially reflecting a physiological micro-environment of induction of multiple cytokines, as found in IBD or IBD-associated CRC. The current study confirmed induction of TNFR2 mRNA and protein by combined IL-6 and TNF α in two different colon cancer cell lines and provides novel and direct evidence for predominant role of STAT3 in TNFR2 induction. We also provide evidence that TNF α induces IL-6 in SW480 cells suggesting that autocrine effects of TNF α -induced IL-6 contribute to the ability of TNF α and IL-6 to cooperatively stimulate TNFR2 expression.

The TNFR2 promoter contains two putative STAT binding sequences and NF κ B binding sequences (169). Since TNF α typically activates NF κ B and IL-6 typically activates STAT3, we hypothesized that IL-6 and TNF α induction of TNFR2 would be mediated by activation of both of these transcription factors. We provide several independent pieces of evidence to indicate that STAT3, rather than NF κ B, is the predominant mediator of TNFR2 induction by IL-6 and TNF α . Specifically, a STAT3 inhibitor reduces basal TNFR2 expression and completely reverses the induction of TNFR2 by IL-6 and TNF α . In contrast, NF κ B inhibitor

had no effect on basal TNFR2 expression and only modestly and non-significantly reduced induction of TNFR2 by combined IL-6 and TNF α . This was despite data verifying that the NF κ B inhibitor potently and completely reverse cytokine induction of ICAM-1 mRNA, whose expression is known to be dependent on NF κ B. Importantly combined STAT3 and NF κ B inhibitors did not reduce basal or cytokine-induced TNFR2 expression compared with STAT3 inhibitor alone and constitutively activated STAT3 was able to induce TNFR2 to a similar extent as IL-6 and TNF α . Together these findings indicate a predominant role of STAT3 in mediating TNFR2 induction and demonstrate that STAT3 activation alone is sufficient to mimic cytokine effects on TNFR2 expression. ChIP assays also confirmed that IL-6 and TNF α induced STAT3 binding to two putative STAT3 binding sites, but had no effect on NF κ B binding. This was in spite of the fact that TNF α is known to induce phosphorylation of NF κ B in this same cell system (115). It is also notable that combined IL-6 and TNF α more potently induced STAT3 binding to the -1578 binding site in the TNFR2 promoter than the -364 site. Interestingly, the -1578 STAT3 binding site also showed dramatic cooperative effects of IL-6 and TNF α to induce STAT3 binding while alone only modestly induced STAT3 binding to this site.

Collectively, these observations provide compelling evidence that IL-6 and TNF α interact to promote maximal STAT3 binding to the TNFR2 promoter and TNFR2 induction, and that this cooperative effect appears to occur primarily at the -1578 STAT binding site. While we cannot rule out the possibility that NF κ B binds to other regions in the TNFR2 gene, observations that IL-6 and TNF α did

not induce NF κ B binding to consensus NF κ B sites and the minimal effects of NF κ B inhibitor on TNFR2 expression support a novel mechanism of TNF α and IL-6 interaction to induce TNFR2 by predominant effects on STAT3.

A number of recent studies implicate TNFR2 as a mediator of colitis-associated cancer. TNFR2 has been shown to increase proliferation and migration of colon cancer cell lines and is up-regulated in mouse models and patients with inflammatory bowel diseases. Additionally, disruption of TNFR2 genes decreased proliferation of crypt epithelial cells (56, 168). Recent studies in the AOM/DSS model of inflammation-associated CRC revealed that TNFR2 is preferentially up-regulated over TNFR1 and that treatment with the anti-TNF α mAb MP6-XT22 reduced the number and size of tumors, although colitis severity was unchanged (57). In a separate study, anti-TNF α antibodies given at late stages of the AOM/DSS model reduced tumor load (121). To our knowledge, a direct effect of TNFR2 on CRC proliferation or transformed phenotype has not been demonstrated. Our current study used TNFR2 over-expression in two different colon cancer cell lines to demonstrate that TNFR2 directly enhances proliferation and anchorage independent growth of colon cancer cells. This strongly supports a direct effect of TNFR2 on colon cancer cell proliferation and supports the concept that TNFR2 is a key pro-tumorigenic factor.

SOCS3 silencing has been implicated in risk of lung, liver, and squamous cell cancers (101, 104, 105, 184, 185). Our previous study showed that IEC-specific deletion of SOCS3 led to an increase in tumor load in the AOM/DSS model, supporting the hypothesis that SOCS3 may act as a suppressor of colitis-

associated cancer (115). This same study revealed that loss of IEC-SOCS3 resulted in enhanced activation of both STAT3 and NF κ B. We report here that SOCS3 over-expression limits TNFR2 expression in colon cancer cell lines and limits STAT3 binding to the TNFR2 promoter. Importantly, SOCS3 limits basal and TNFR2-induced proliferation of colon cancer cells and dramatically decreases anchorage-independent growth. Together, these findings support a direct role of SOCS3 as an inhibitor of colon cancer cell growth and indicate that loss of SOCS3 may promote colon tumors at least in part by promoting increases in TNFR2 expression.

Anti-TNF α therapies are widely used in the treatment of human IBD (186-190). However, the effect of anti-TNF α therapy on risk of colitis-associated cancer is not well defined. These studies are seemingly difficult to perform due to the incidence of bowel resection in patients with IBD. However, reports of using anti-TNF α therapies in renal cell carcinoma showed promising results to prevent tumor progression in established disease (236). Anti-TNF α would reduce activation of both TNFR1 and TNFR2 by TNF α . Our findings that TNFR2 directly promotes CRC growth and that TNF α plays a role in TNFR2 induction suggest that TNFR2 may provide a useful biomarker of effects of anti-TNF α on risk of colitis-associated cancer, or represent a specific target to decrease colon cancer risk in IBD. Furthermore, the fact that STAT3 is a mediator of TNFR2 induction by combined TNF α and IL-6 adds to the growing evidence for STAT3 as a key mediator of colitis-associated cancer (121, 125).

Table 2.1. Oligomers used in chromatin immunoprecipitation assays

TNFR2 Promoter Site	Oligomers	Product Size (bp)
STAT3 (-1578)	F-5'-CTGCAGTGAGCTATGGGTGA-3' R-5'-GAGGGTGTGGCTGGTATGAC-3'	223
STAT3 (-364)	F-5'-CTGCAGTGAGCTATGGGTGA-3' R-5'-GGGTGAGGCACTAATTTGGA-3'	172
NFκB (-1890)	F-5'-TTGAATTCGTTCCCAGGATG-3' R-5'-CTAGTTGTCCCCCACACACC-3'	171
NFκB (-1517)	F-5'-AAGGCTCTGTGGGTCATACCAG-3' R-5'-GGCTGCCTGAAGAGGTACAG-3'	228

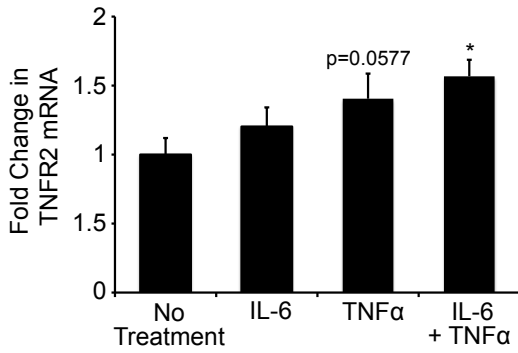
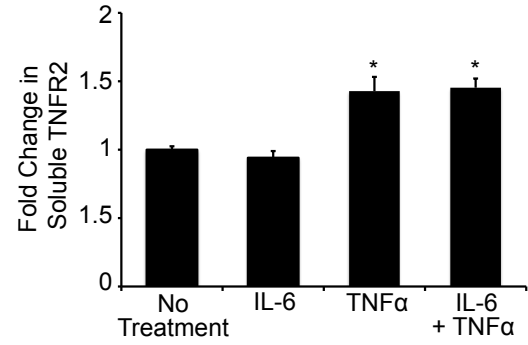
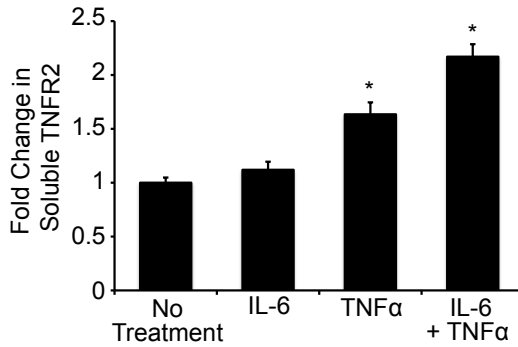
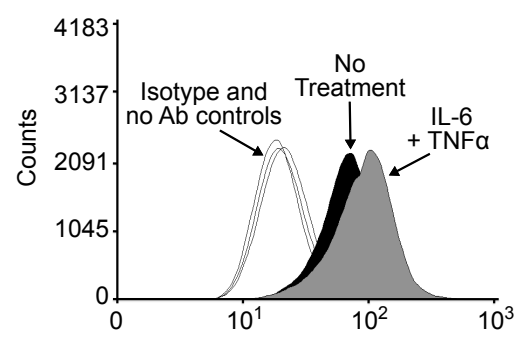
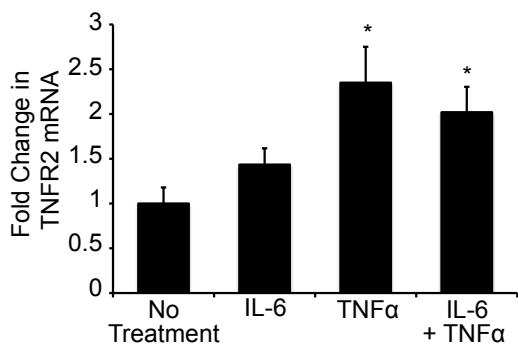
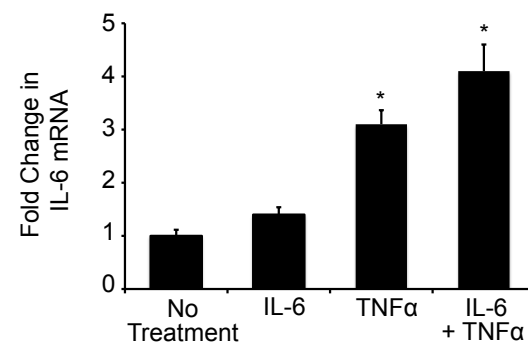
A COLO205**D SW480****B COLO205****E SW480****C SW480****F SW480**

Figure 2.1. Induction of TNFR2 mRNA and protein by IL-6 and TNF α . (A and C) Histograms show levels of TNFR2 mRNA in COLO205 and SW480 cells treated 50ng/mL IL-6 plus TNF α for 10 hours. TNFR2 mRNA was normalized to HMBS and all values are expressed as fold change (mean \pm SE) versus mean levels in untreated controls. TNFR2 mRNA was significantly increased by IL-6 and TNF α treatment in both cell lines, and by TNF α in SW480. (B and D) COLO205 and SW480 cells were treated with 50ng/mL IL-6 plus TNF α for 10 hours followed by ELISA on cell supernatants to measure TNFR2 protein levels. Samples were normalized to total protein. Consistent with findings for TNFR2 mRNA, protein levels of soluble TNFR2 were significantly increased with IL-6 and TNF α treatment. (* $p \leq 0.05$ compared to no treatment). (E) Representative figure showing cell surface expression of TNFR2 using flow cytometry. Treatment with IL-6 and TNF α increased TNFR2 surface expression. (F) Induction of IL-6 mRNA in SW480 cells treated with TNF α , or IL-6 combined with TNF α for 10 hours. IL-6 mRNA was normalized to HMBS and values are expressed as fold change (mean \pm SE) versus mean levels in untreated controls.

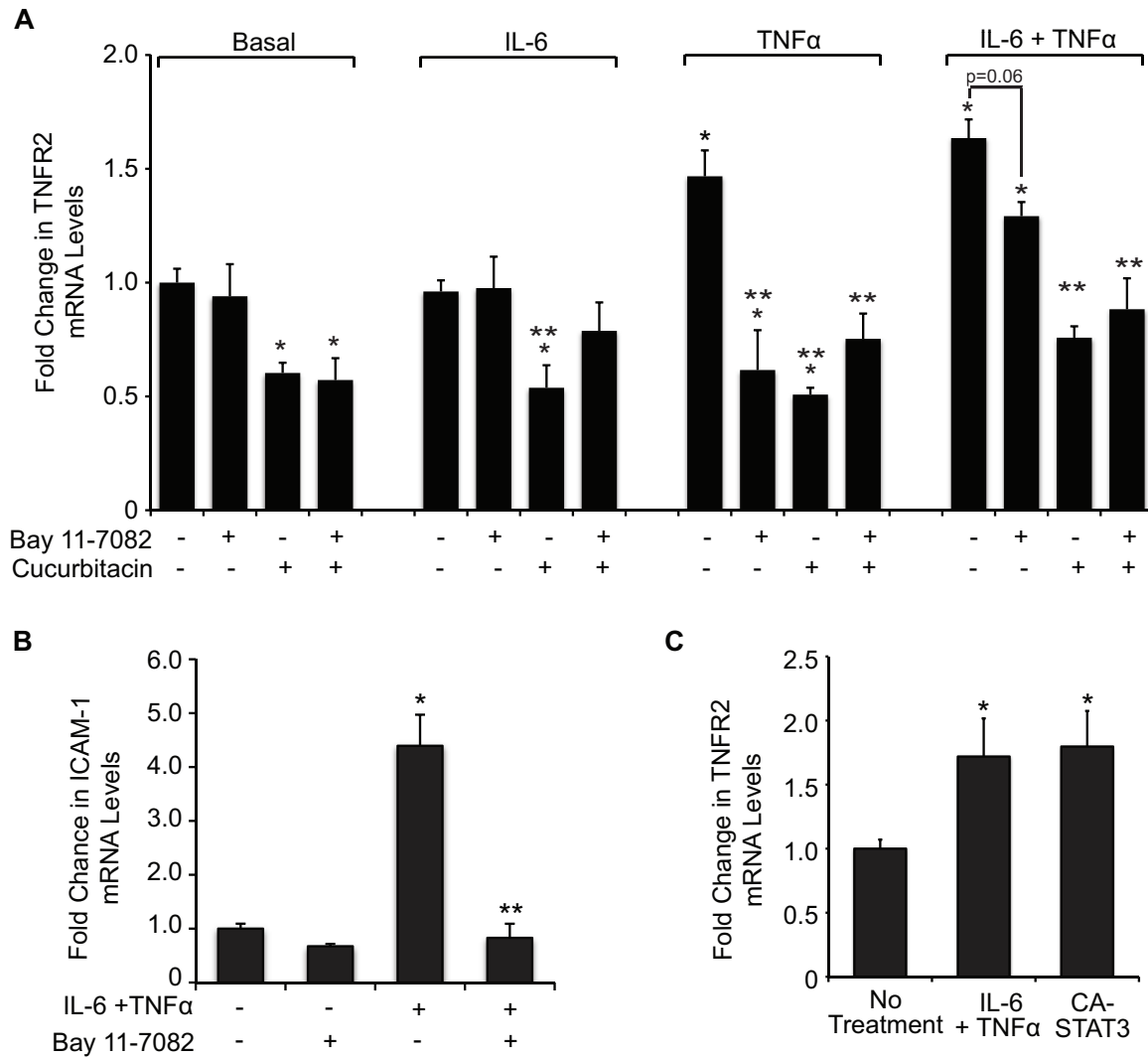


Figure 2.2. Regulation of TNFR2 by STAT3 Inhibition or Constitutive STAT3 Activation. (A) Histogram shows levels of TNFR2 mRNA in SW480 cells treated with vehicle or IL-6, TNF α , or both cytokines in the absence (-) or presence (+) of 20 μ M of the STAT3 inhibitor cucurbitacin or 5 μ M of the NF κ B inhibitor Bay 11-7082. Note the dramatic inhibitory effect of cucurbitacin on both basal and cytokine-induced TNFR2 compared with Bay 11-7082. (* $p \leq 0.05$ compared to vehicle, no cytokine; ** $p \leq 0.05$ compared to vehicle, cytokine-treated cells). (B) ICAM-1 mRNA was measured in SW480 cells treated with IL-6 and TNF α in the presence or absence of Bay 11-7082. Note the complete inhibition of cytokine-induced ICAM-1 mRNA by Bay 11-7082. (* $p \leq 0.05$ compared to vehicle; ** $p \leq 0.05$ compared to cytokine-treated cells). (C) SW480 cells were treated with adenovirus to over-express constitutively-active STAT3 (CA-STAT3) for 10 hours followed by mRNA collection. Expression of CA-STAT3 significantly increased TNFR2 mRNA levels to the same level as that found with IL-6 and TNF α treatment. (* $p \leq 0.05$ compared to empty vector).

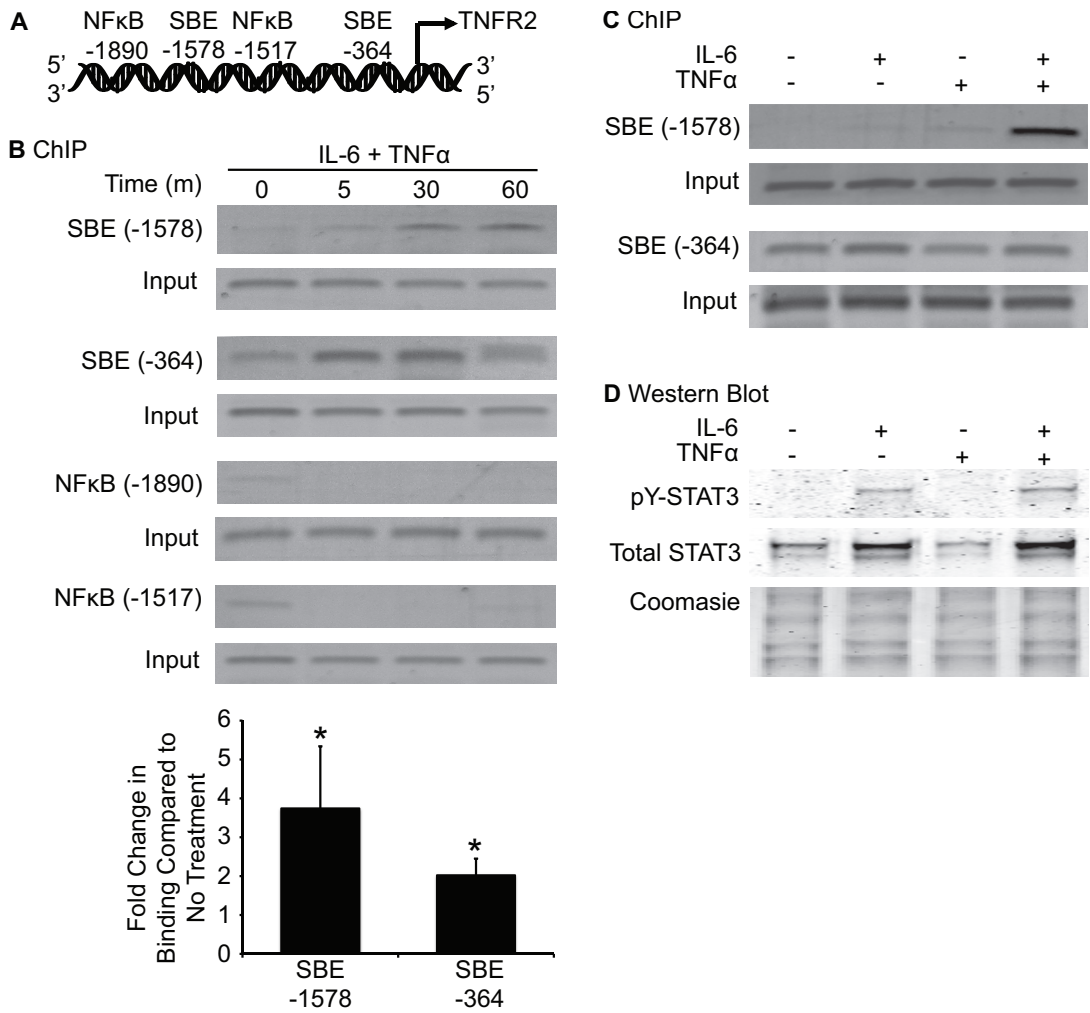


Figure 2.3. IL-6 and TNF α induce STAT3 binding to the TNFR2 promoter. (A) Schematic shows the locations of two putative STAT3 binding sites and two putative NFkB binding sites in the human TNFR2 promoter. (B) PCR products from ChIP assays of STAT3 or NFkB binding to consensus STAT3 or NFkB sites in untreated cells or cells treated with IL-6 and TNF α for the indicated times. IL-6 and TNF α treatment lead to a time-dependent increase in STAT3 binding at both consensus sites. Cytokine treatment has no effect on NFkB binding. Densitometric analysis revealed a 3.75 ± 1.62 -fold and 2.02 ± 0.43 -fold increase in overall STAT3 binding at -1578 and -364 sites, respectively. (* $p \leq 0.05$ compared to no treatment). (C) PCR products from ChIP assays in SW480 cells. IL-6 or TNF α alone or in combination for 30 minutes had an additive effect on STAT3 consensus binding for -1578, but this effect is not seen for the -364 site. (D) Western immunoblots on nuclear extracts from SW480 cells treated with IL-6, TNF α , or both cytokines. Upper panels show immunoblots for tyrosine-phosphorylated (pY) and total STAT3. IL-6 treatment induced pY-STAT3 and increased total nuclear STAT3, while TNF α alone had no detectable effect, and IL-6 and TNF α combined gave a similar effect as IL-6 alone. Equal protein loading was confirmed with Coomassie-stained protein gels on nuclear extracts.

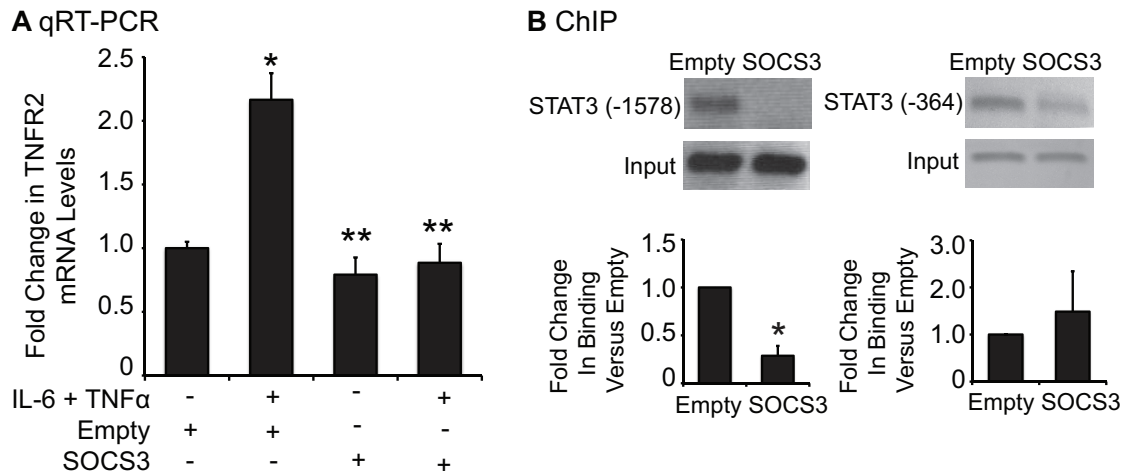


Figure 2.4. SOCS3 over-expression decreases TNFR2 expression and STAT3 binding to TNFR2 promoter. (A) Histogram shows levels of TNFR2 mRNA in SW480 cells in the absence (-) or presence (+) of IL-6 and TNF α and/or SOCS3 adenovirus. Cytokine treatment significantly increased TNFR2 mRNA, and SOCS3 over-expression attenuated this effect. (* $p \leq 0.05$ compared to empty vector; ** $p \leq 0.05$ compared to cytokine treatment). (B) Upper panel shows PCR products from ChIP assays of SW480 cells stimulated with IL-6 and TNF α for 30-60 minutes. Histograms indicate fold change in STAT3 binding with SOCS3 over-expression compared to empty vector. SOCS3 decreased STAT3 binding to the TNFR2 promoter at the -1578 site and had variable effects on binding at the -364 site. (* $p \leq 0.05$ compared to empty vector).

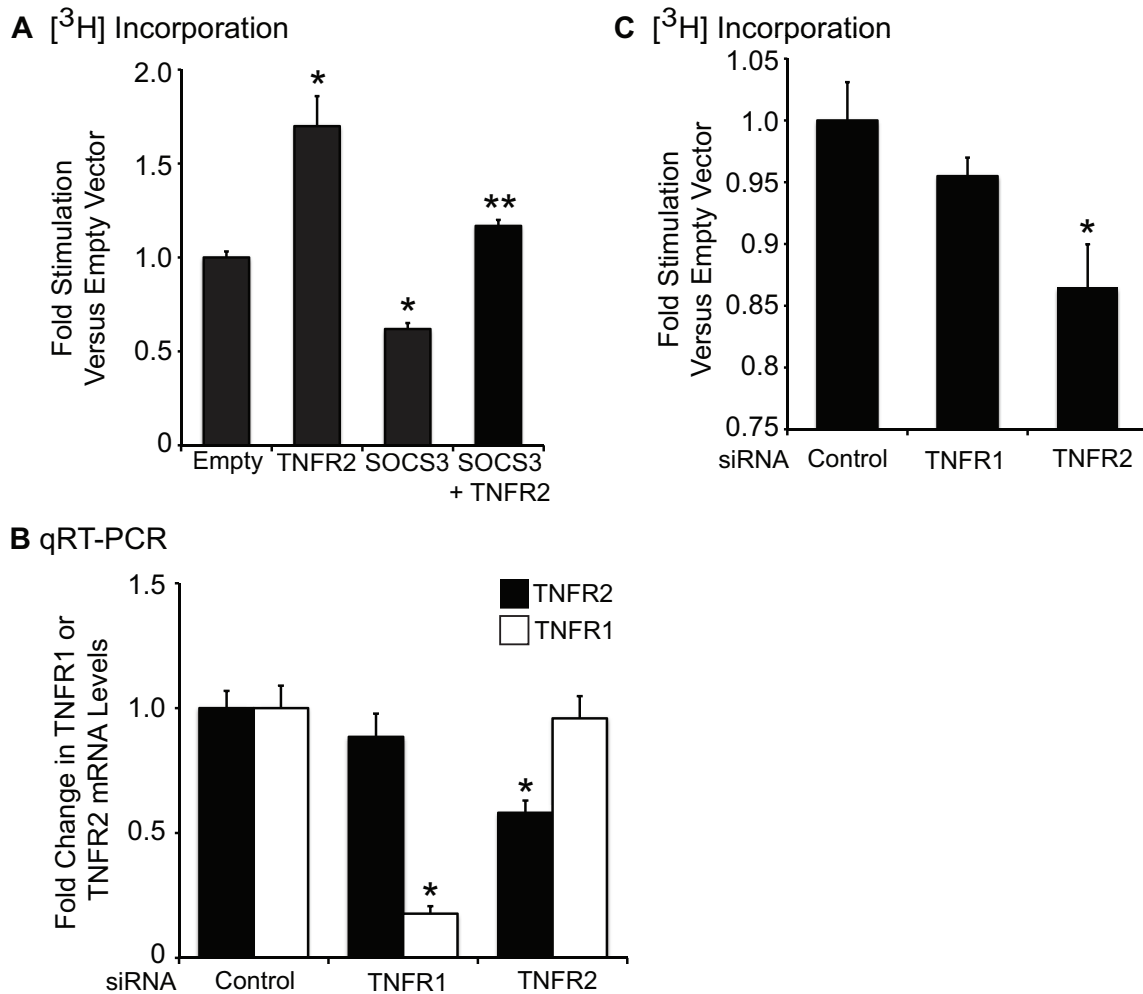


Figure 2.5. SOCS3 over-expression limits TNFR2-mediated [3H] incorporation. (A) Histogram of [3H] incorporation into DNA as a measure of SW480 cell proliferation after 24-hour over-expression of TNFR2, SOCS3 or both. TNFR2 over-expression increased cell proliferation, and SOCS3 over-expression dramatically limited this effect. (*p ≤ 0.05 compared to empty vector; **p ≤ 0.05 compared to TNFR2-treated cells). (B) TNFR2 mRNA levels are decreased by 45% using siRNA specific to TNFR2, while TNFR1 expression is not effected. (*p ≤ 0.05 compared to control siRNA). (C) Histogram of [3H] incorporation with TNFR2 siRNA treatment. Knock-down of endogenous TNFR2 using siRNA lead to a modest, but significant decrease in [3H] incorporation into DNA. (*p ≤ 0.05 compared to control siRNA).

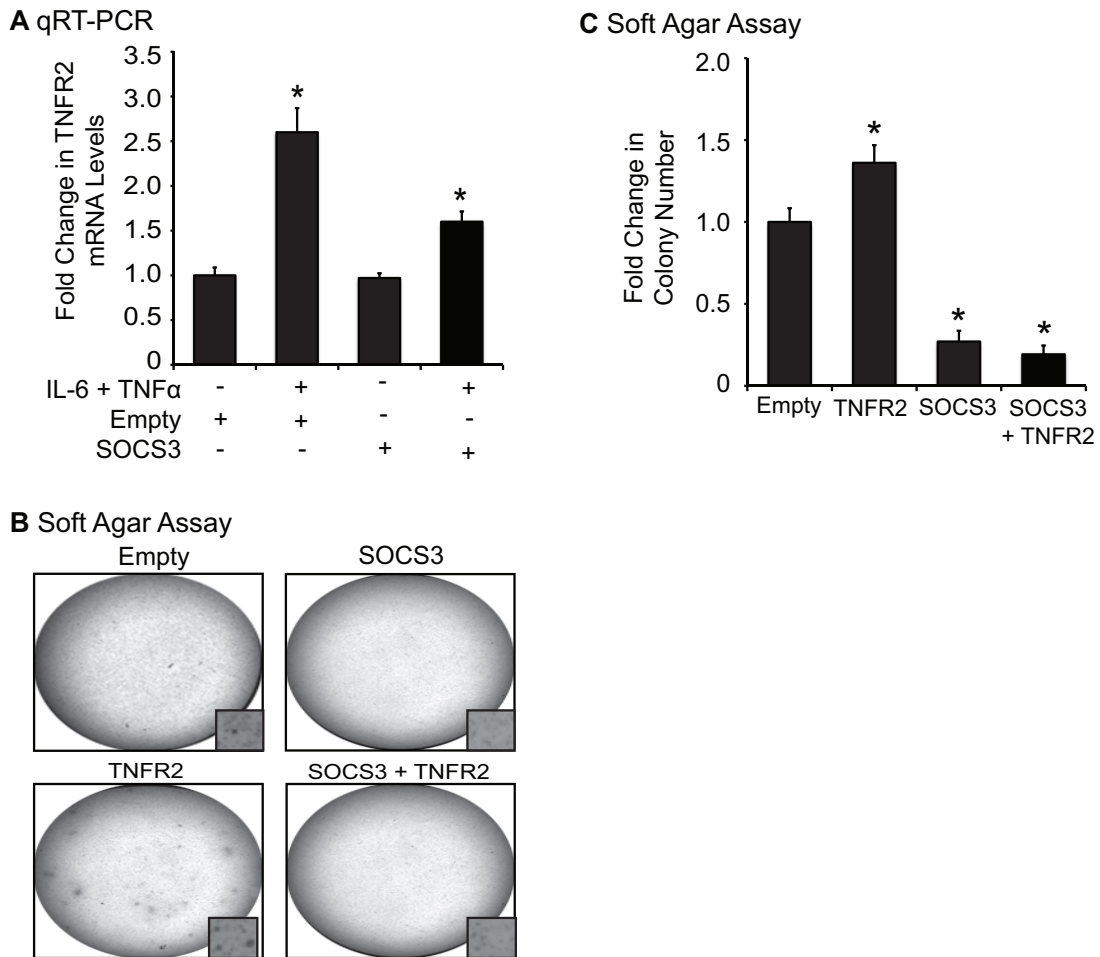


Figure 2.6. SOCS3 over-expression limits anchorage-independent growth of COLO205 cells. (A) Histogram shows levels of TNFR2 mRNA in COLO205 cells in the absence (-) or presence (+) of IL-6 and TNF α and/or SOCS3 adenovirus. SOCS3 over-expression limits IL-6 and TNF α induction of TNFR2 in COLO205 cells. (* $p \leq 0.05$ compared to empty vector). (B) Representative photographs of individual wells from a 6-well plate containing COLO205 cells grown in 0.3% soft agar and over-expressing TNFR2 and/or SOCS3. Images are representative of at least three total experiments. (C) Colonies were stained with MTT and quantified using NIH ImageJ. Cells treated with TNFR2 retrovirus exhibited a modest, but significant increase in colony number, and treatment with SOCS3, or SOCS3 combined with TNFR2, caused a dramatic, >70% decrease in colony number. (* $p \leq 0.05$ compared to empty vector).

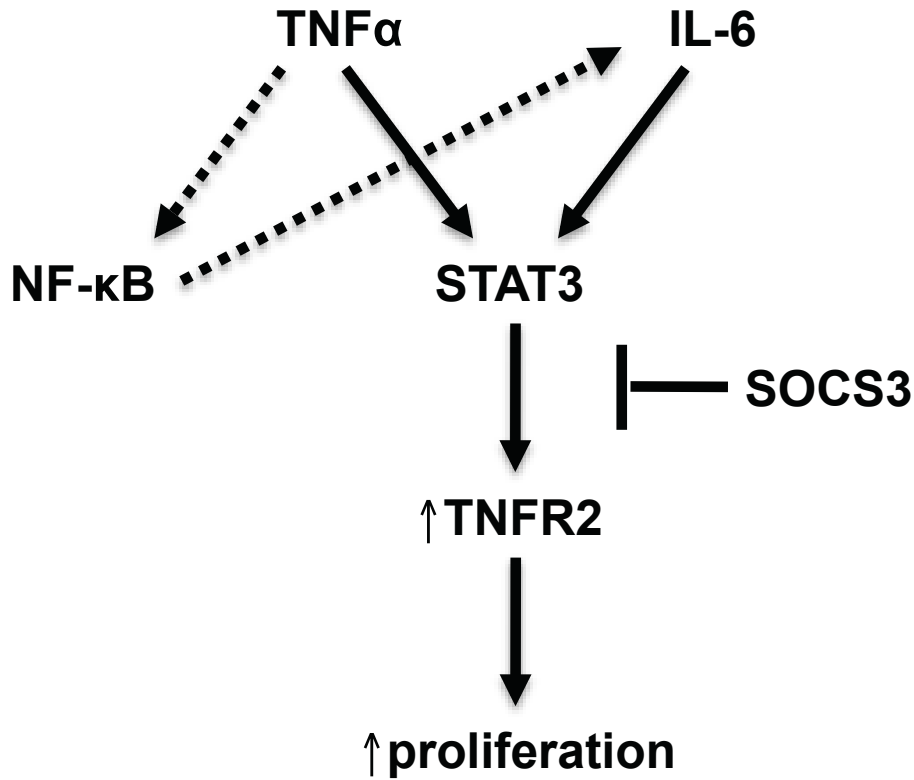


Figure 2.7. Schematic of STAT3-mediated induction of TNFR2 expression by IL-6 and TNF α . IL-6 and TNF α induce STAT3 binding to the -1578 site of the TNFR2 promoter to induce TNFR2 expression. In SW480 cells, TNF α can induce IL-6 expression, which may then promote autocrine actions of IL-6 to cooperatively up-regulate TNFR2 expression with TNF α . SOCS3 limits TNFR2 expression and the proliferative effects of TNFR2 in CRC cells.

CHAPTER III

SOCS3 IS NOT AN INDEPENDENT BIOMARKER OF ADENOMA RISK

The contents of the following chapter were originally published by the open access publisher Biomed Central (66).

A. Introduction

Recent evidence in mice and humans suggest that the anti-inflammatory protein Suppressor of cytokine signaling 3 (SOCS3) may act as a tumor suppressor in the colon (115, 128). Specific silencing of SOCS3 expression in intestinal epithelial cells (IEC) increased tumor load in the azoxymethane/dextran sodium sulfate (AOM/DSS) mouse model of inflammation-associated CRC (115). Furthermore, SOCS3 expression is low or silenced by promoter hypermethylation in other cancers, including lung, liver, and squamous cell carcinoma (101, 104, 185).

Prior studies from our group demonstrated that increased systemic levels of pro-inflammatory cytokines IL-6 and TNF α correlate with risk of colorectal adenoma (191). SOCS3 has been shown to limit the actions of both of these cytokines as well as their downstream targets STAT3 and NF κ B, which are frequently activated in humans and mouse models of CRC (57, 65, 66, 86, 125, 128, 192). Based on these studies, we investigated SOCS3 mRNA expression in the normal mucosa of patients undergoing routine colonoscopy screening to determine if low SOCS3 expression predisposes to adenoma and could thus be considered an early biomarker of CRC risk.

B. Methods

Study population and data collection

All eligible subjects provided written, informed consent. Consenting participants were enrolled in Diet and Health Study IV at University of North

Carolina Hospitals as previously described (193). Briefly, subjects undergoing routine colonoscopy provided rectal biopsies and blood samples for the study. Subjects also consented to a follow-up interview to collect diet and lifestyle information. High quality RNA from 322 subjects (93 with adenoma, 229 without adenoma, with complete information on plasma IL-6 and TNF α , age, race, sex, waist hip ratio (WHR), family history and use of non-steroidal anti-inflammatory drug (NSAIDs)) was assayed for SOCS3. Patients in the adenoma group were defined as having one or more adenomas by the study pathologist based on standard criteria. The study was approved by the University of North Carolina School of Medicine Institutional Review Board.

RNA extraction and real-time qRT-PCR

RNA from four pooled, normal colon biopsies per subject was extracted using Qiagen's RNeasy kit (Valencia, CA) and reverse transcribed with AMV-Reverse Transcriptase (Promega, Madison, WI) as previously described (193).

SOCS3 mRNA abundance was determined using the ABI Prism 7900HT (Applied Biosystems, Foster City, CA) and Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA). Human SOCS3 (NM_003955.3) was quantified using Applied Biosystem primer/probe set targeting exon 2. The housekeeping gene hydroxymethylbilane synthase (HMBS, NM_000190.3) was chosen as a low-abundance, invariant control. Standard curves for each primer/probe set were generated using gel-isolated, sequence-confirmed PCR products. Cycling included initial denaturation at 95°C for 5 minutes followed by 45 cycles of 95°C

denaturation for 15 seconds and 60°C annealing for 45 seconds. Threshold cycles analysis was performed using Applied Biosystem SDS v2.2.2 software and values are expressed as copy number relative to HMBS. All PCR runs included standards and inter-run calibrator controls (pooled sample cDNA), as well as non-reverse transcribed (no-RT) and water controls. Samples were run in triplicate.

Statistical analysis

Means and standard errors were generated for continuous variables, and frequencies and percentages were generated for categorical variables. T-tests and Mann-Whitney tests were used to compare cases with controls on continuous variables. SOCS3 values were log-transformed to normalize the distribution. Chi-Square tests were used to compare cases and controls on categorical variables. Logistic regression was used to test for an association between case/control status and SOCS3. Levels of SOCS3 were categorized into tertiles based on control values. Age, race, sex, WHR, NSAIDS, IL-6, TNF α , and family history were assessed as potential confounders of SOCS3-adenoma association. Each variable was put into a model separately with SOCS3, and if one of the two dummy variables for SOCS3 changed by at least 15% compared to when only SOCS3 was in the model, then that co-variable passed the first stage for being a confounder. All such variables were then entered into a model with SOCS3 and a backwards, stepwise regression was done with the SOCS3 variable being forced into the model. Only age and sex met these criteria for

confounding factors and thus they were included in the final model.

C. Results

Descriptive characteristics of the population in the SOCS3-adenoma study are shown in Table 3.1. Consistent with results from our prior reports for the study population, subjects with adenomas were older, more likely to be male, had higher waist-hip ratios and increased plasma IL-6 (191, 193). There was no difference in median SOCS3 expression between individuals with or without adenomas.

To determine if low SOCS3 expression was associated with having an adenoma, odds ratios and 95% confidence intervals were generated using logistic regression analysis (Table 3.2). There was no difference in odds ratios in subjects in the lower tertiles of SOCS3 values, indicating that low levels of local SOCS3 expression were not associated with adenoma risk.

D. Discussion

Identifying local factors that predispose patients to early, pre-cancerous lesions may make it possible to stratify screening based on risk, but risk factors that occur early in CRC development are not well defined. Our group has shown that patients with adenoma have reduced apoptosis in their normal mucosa, demonstrating a field effect that predisposes the individual to a higher risk of developing precancerous adenomas (194, 195). We hypothesized that SOCS3

might be a promising biomarker of colorectal neoplasia risk, but contrary to expectation the study was negative.

Patients with inflammatory bowel diseases (Crohn's disease or ulcerative colitis) have an increased risk of developing CRC, which is associated with the degree and duration of intestinal inflammation (reviewed in (196)). In addition, recent data suggest that individuals with chronic, low levels of inflammation (such as increased circulating IL-6 and TNF α) have increased odds of having adenoma and thus increased CRC risk (191). SOCS3 is an anti-inflammatory protein that limits IL-6 induction of STAT3, as well as TNF α induction of NF κ B. IEC-specific silencing of SOCS3 leads to a dramatic increase in tumor load in a mouse model of inflammation-associated CRC (115). Furthermore, recent studies show that STAT3 activation is increased, and SOCS3 is silenced in tumors of patients with ulcerative colitis-associated and sporadic CRC (128, 163). The current study tested the hypothesis that patients with adenoma may have lower SOCS3 in the normal mucosa than patients without adenoma, thus contributing to a permissive environment for aberrant growth. However, our study found that low or silenced SOCS3 expression does not occur in the normal mucosa of patients with colorectal adenoma.

One potential limitation of the study is possible effect of standard bowel preparation on SOCS3 expression. While we have not directly tested this for SOCS3, similar studies of other genes have shown that there is no significant change in mucosal gene expression in patients using bisacodyl or polyethylene glycol bowel preparations (197). Another possible limitation is that SOCS3 mRNA

levels do not reflect changes in SOCS3 phosphorylation, which targets SOCS3 for proteasomal degradation (198). However, other studies show that SOCS3 is silenced by DNA hyper-methylation in CRC tumors (128), indicating that evaluating changes in gene expression is an appropriate measure of SOCS3 in this study. Finally, while our results could indicate that SOCS3 is more important in the underlying pathogenesis of inflammation-associated rather than sporadic CRC, recent studies comparing SOCS3 expression in both ulcerative colitis-associated and sporadic tumors found that SOCS3 was decreased and there was no significant difference between the two groups for SOCS3 (128). Taken together these studies suggest that SOCS3 silencing occurs later in the progression from adenoma to adenocarcinoma, and is not an independent, early biomarker of CRC risk in the normal mucosa.

Table 3.1. Descriptive characteristics of study participants

Variable	Case (n=93)	Control (n=229)	p-value *
Age median (1 st , 3 rd quartile)**	58 (51, 56)	55 (50, 63)	0.03
Race- White N (%)†	73 (78)	177 (77)	0.88
Sex- Male N (%)	51 (55)	85 (37)	0.004
WHR median (1 st , 3 rd quartile)	0.922 (0.854, 1.000)	0.888 (0.804, 0.979)	0.04
Family History of CRC- N (%)	16 (20)	31 (15)	0.37
Plasma IL-6 median (1 st , 3 rd quartile)	0.21 (0.00, 0.56)	0.07 (0.00, 0.41)	0.01
Plasma TNF α median (1 st , 3 rd quartile)	1.93 (1.44, 2.74)	1.75 (1.30, 2.44)	0.14
Plasma CRP median (1 st , 3 rd quartile)	3,959 (1,934, 14,278)	7,239 (2,294, 16,728)	0.14
Tissue SOCS3 median (1 st , 3 rd quartile)	3.09 (2.33, 3.77)	3.09 (2.35, 3.80)	0.77

* p-values are from Fishers Exact Test for categorical variables and Mann-Whitney test to compare medians for continuous variables.

** Standard error (se); Waist Hip Ratio (WHR); Suppressor of Cytokine Signaling 3 (SOCS3)

† Percentages within columns

Table 3.2. Adjusted association between local SOCS3 levels and adenoma

Tissue SOCS3	Case / Control*	OR (95% CI) †
Tertile 3 (36.4-180.7)‡	28 / 69	1.0 (Reference)
Tertile 2 (13.8-36.4)	27 / 72	0.9 (0.5, 1.7)
Tertile 1 (0.6-13.8)	29 / 69	1.0 (0.5, 1.8)

* Adjusted for age and sex

† Odds ratio (OR) and 95% confidence interval (CI); odds of having colorectal adenomas

‡ Tertile cut-offs based on distribution of SOCS3 among control subjects; tertile 3 was used as reference

CHAPTER IV

DELETION OF SOCS3 GENES IN THE INTESTINAL EPITHELIUM INCREASES TUMOR INCIDENCE IN THE AZOXYMETHANE (AOM) MODEL OF SPORADIC COLORECTAL CANCER

A. Introduction

Suppressor of cytokine signaling 3 (SOCS3) is commonly known as an inhibitor of the IL-6/STAT3 pathway, and it is silenced in tumors of several cancers, including colorectal cancer (CRC) (66, 79, 86, 104, 174, 185, 199). In the azoxymethane/dextran sodium sulfate (AOM/DSS) model of inflammation-associated CRC, mice with intestinal epithelial cell (IEC)-SOCS3 deletion had a four-fold increase in tumor burden (accounting for increased tumor number and size) compared to control mice (115). STAT3 and NF κ B activation were enhanced in IEC-SOCS3 $^{\Delta/\Delta}$ mice, and *in vitro* studies showed that SOCS3 overexpression reduced IL-6 or TNF α -mediated activation of both pathways (115). While these studies support the hypothesis that SOCS3 normally acts as a tumor suppressor in inflammation-associated CRC, it is unclear whether SOCS3 prevents tumorigenesis in sporadic CRC.

Sporadic CRC is characterized by genomic instability associated with mutations or loss function of tumor suppressor genes, including APC. Loss of APC function leads to cytoplasmic and nuclear accumulation of β -catenin, which activates TCF-regulated growth regulatory genes (148). The APC $^{Min/+}$ mouse model of spontaneous CRC was developed as a pre-clinical model to study the consequence of such mutations. These mice have enhanced β -catenin activation and develop tens to hundreds of adenomas in the small intestine and multiple colon adenomas in the absence of overt intestinal inflammation. Few studies have explored inflammatory pathways in β -catenin driven tumorigenesis in the intestine, but recent evidence suggests that STAT3 and NF κ B pathways may

play a role. Kawada *et al* showed a correlation between poor CRC prognosis and nuclear staining of both STAT3 and β -catenin as compared with patients without co-localized staining (164). This same study demonstrated that dominant-negative STAT3 caused β -catenin to move out of the nucleus in colon cancer cell lines (164). A similar study in human esophageal squamous cell carcinomas showed that STAT3 staining correlated with nuclear β -catenin accumulation, and that TCF4 binding enhanced STAT3 expression and transcriptional activity, indicating that STAT3 is a downstream target of β -catenin/TCF4 (200).

Studies in transgenic mice over-expressing IEC-progastin (Fabp-PG), which exhibit enhanced intestinal proliferation and β -catenin accumulation, showed that treatment with the NF κ B inhibitor NEMO (NF κ B essential modulator) peptide reduced proliferation and β -catenin levels in the proximal colon (201). This suggests that NF κ B activation may be upstream of β -catenin-mediated proliferation in the intestine. Other studies in colon cancer cell lines showed that β -catenin over-expression caused a reduction in NF κ B activation, and that expression of its downstream target Fas was inversely correlated with β -catenin in colon and breast cancer tissues (202). Furthermore, β -catenin and the NF κ B subunits p50 and p65 are thought to directly interact, likely in complex with GSK-3 β , as inhibition of GSK-3 β suppressed NF κ B activation in colon cancer cell lines (203). More studies must be performed to fully understand the interaction between NF κ B and β -catenin in the context of CRC.

Based on these prior studies and our findings that SOCS3 negatively regulates both STAT3 and NF κ B in colitis-associated cancer, the goal of the

present study was to determine if SOCS3 plays a role in sporadic tumorigenesis, possibly through its ability to limit STAT3 and NFκB activation. Our laboratory has recently shown that another SOCS family member, SOCS2, normally acts as a tumor suppressor in the APC^{Min/+} mouse model (204). However, SOCS2 is primarily linked to negative regulators of the growth hormone/insulin-like growth factor 1 axis rather than pro-inflammatory signaling. In the present study we used the AOM model of sporadic tumorigenesis in order to provide a direct comparison to our prior studies with IEC-SOCS3^{Δ/Δ} mice using AOM/DSS (115, 149). AOM causes sporadic colon tumors and this is most commonly linked to APC or β-catenin mutations (149, 205). Here we show preliminary evidence that AOM-treated IEC-SOCS3^{Δ/Δ} mice have increased tumor incidence compared to wild-type mice. Ongoing studies will determine specific mechanisms affected by loss of SOCS3 in this model. We will also utilize fluorescence molecular tomography to visualize tumors in ProSense[®] 680- treated mice, in order to detect flat or otherwise difficult to detect lesions.

B. Methods

Generation of study mice

All mice are on the inbred C57BL6 background and are maintained in conventional, but specific-pathogen free (SPF) conditions. Mice with villin-Cre excised SOCS3 alleles have been derived using the villin-promoter-Cre system that selectively disrupts the SOCS3 gene and ablates SOCS3 expression in IEC (115). Study mice are denoted as IEC-SOCS3^{Δ/Δ} and compared to age-matched,

wild-type mice . For mouse imaging with molecular probes, GI tract auto fluorescence was eliminated by maintaining animals on liquid diet (Nestle Nutrition) (Nutren 1.0 Fiber: dH₂O=1:1) for 4 days before the imaging. The diet was prepared fresh everyday and served *ad libitum* in mouse feeding bottles (Bio-serv, Frenchtown, NJ) suspended from the cage walls with special holders (Bio-serv, Frenchtown, NJ). Water was supplied during the liquid diet feeding. These studies were approved by the Institutional Animal Care and Use Committee of the University of North Carolina.

Azoxymethane model and sample collection

Littermate pairs were treated with a modified protocol azoxymethane (AOM) as previously described (206). Briefly, 8-10 week-old study and control mice were given an intraperitoneal injection of 10 mg/kg AOM once per week, for four consecutive weeks. Animals were harvested 80-90 days after initial AOM injection, depending on visualization of colon tumors using colonoscopy. Colon samples were collected and fixed in 10% zinc-buffered formalin for subsequent immunohistochemical staining.

Murine colonoscopy

Animals were screened by colonoscopy to follow tumor development. Colonic tumors were visualized *in vivo* starting at 12 weeks after initial AOM injection using the "Coloview System" (Karl Storz Veterinary Endoscopy) as previous described (207). Mice were fasted overnight prior to the procedure and

anesthetized with 1.5% to 2% isoflurane prior to visualization. The colonoscopic procedures were digitally recorded on an AIDA Compaq PC.

Molecular imaging methodology

A commercially available cathepsin-activatable NIR imaging probe, ProSense[®] 680 (VisEn Medical, Inc., Bedford, MA), was given via intravenous injection to all imaged animals as indicated. The dose used was 2nmol/150 μ L in 1 X PBS (phosphate buffered saline) which is the recommended for adult mice. Both *in vivo* and *ex vivo* imaging were performed 24 hours after injection of the probe.

***In vivo* tissue imaging**

All planar and tomographic optical imaging studies were performed using a fluorescence molecular tomography system (FMT 2500[™] LX) (VisEn Medical Inc). For *in vivo* imaging, mice were anaesthetized, positioned in the imaging cassette, and placed into the imaging chamber, where they were maintained on inhaled isoflurane anaesthesia, as previously described (208). A NIR laser diode trans-illuminated each mouse (i.e. passed light through the body of the animal), with signal detection occurring via a thermoelectrically cooled CCD camera placed on the opposite side of the imaged animal. Appropriate optical filters allowed collection of both fluorescence and excitation datasets, and the fluorescence datasets were normalized to the laser excitation data. The entire image acquisition sequence took approximately 3-5 min per mouse. FMT 2500[™]

LX 2D fluorescence reflectance imaging (FRI) was also routinely performed prior to each tomographic imaging session using built-in LED front illuminators and collection of single camera images. These whole body FRI images were represented as controls for tomographic datasets. FMT has been effectively used for non-invasive detection of tumors in other organs (209, 210) using the 680 nm laser channel.

The fluorochrome quantification within the GI region of each mouse was determined using 3D region of interest (ROI) analysis. Briefly, images were displayed as rotatable reconstructed three-dimensional datasets, allowing views in transverse, sagittal and coronal planes. The target 3D region was defined by ROI placement in all three viewing planes to enclose appropriate regions of fluorescence within the imaging dataset. A threshold was applied to all animals equal to 30% of the mean GI fluorescence of the control mice. Fluorochrome concentration in the target tissue was then automatically calculated from the reconstructed images using FMT's TrueQuant Imaging Software (Ver. 2.0.0.19) using pre-acquired calibrations for the specific probe. Data are expressed as absolute pmol fluorescence per region.

***Ex Vivo* tissue imaging**

To confirm the fluorescent signal detected by in vivo imaging originates from distal colon; mice were dissected under isoflurane immediately after live imaging. Colon tissues were flushed with ice cold 1 X PBS and imaged *ex vivo*

by FRI and FMT. Relative fluorescence unit intensity was calculated based on FRI image and the total amount of fluorescence was calculated as described.

Signaling pathway analyses

Activation of STAT3 and NF κ B-p65 were evaluated by western immunoblotting for tyrosine phosphorylated (pY) and total protein. Activation of these mediators were quantified as the ratio of phosphorylated proteins to total protein. β -actin were used as loading control. Immunohistochemistry (IHC) provided qualitative/semi-quantitative data to establish if major differences in activation of particular mediators are specific to IEC, regions of dysplasia, or tumors. IHC tested for cytoplasmic and nuclear accumulation of β -catenin. Additional studies used gene microarray to define if STAT3 and/or NF κ B (and their downstream targets) are up-regulated in this model in the absence of IEC-SOCS3.

Statistics

Data are expressed as mean \pm standard error and will be analyzed by Student's *t*-test or ANOVA for an effect of IEC-SOCS3 $^{\Delta/\Delta}$. Post-hoc, pair-wise comparisons compared individual groups. Statistical significance was set at $p < 0.05$.

C. Results

To date we have examined four IEC-SOCS3^{Δ/Δ} mice and four age and sex-matched wild-type controls. Three out of four IEC-SOCS3^{Δ/Δ} mice had visible tumors on colonoscopy and verified by visual inspection under dissecting microscope (Figure 4.1A, E). None of the wild-type mice had visible tumors at the same time point. IEC-SOCS3^{Δ/Δ} mice had decreased total body weight prior to and after AOM treatment (Figure 4.1B), despite the increase in colon size in IEC-SOCS3^{Δ/Δ} mice (Figure 4.1C). *In vivo* colonoscopy of study mice allowed for tumor surveillance prior to mouse necropsy. A representative image from colonoscopy on IEC-SOCS3^{Δ/Δ} mice is shown in Figure 4.1D. Imaging with a dissecting microscope was used for visual confirmation of large tumors (Figure 4.1E). Figure 4.2A and B represent proof-of-principle *ex vivo* and *in vivo* experiments in which AOM-induced tumors are imaged in KK/HIJ mice injected with ProSense 680.

D. Discussion

Pathophysiological inflammation has emerged as a considerable driving force in intestinal tumorigenesis, highlighting the potential role of anti-inflammatory therapeutics in colorectal cancer (CRC) prevention. Until recently, sporadic and colitis-associated CRC have been characterized by distinct etiologies and genetic events occurring in the progression from pre-cancerous lesions to carcinoma. Patients with Crohn's disease and ulcerative colitis who exhibit dramatic intestinal inflammation have an increased lifetime risk of developing CRC. New evidence suggests that other conditions in which patients

exhibit low, but chronic levels of circulating cytokines such as IL-6, TNF α , and CRP, including patients with obesity, have a higher risk of developing CRC (191). Further evidence for a role of inflammatory pathways in sporadic CRC is that the use of non-steroidal anti-inflammatory drugs (NSAIDs) or non-NSAID COX-2 inhibitors has been shown to be protective against the development of sporadic CRC. However, the prophylactic use of NSAIDs use remains controversial due to gastrointestinal or cardiovascular side-effects (194, 211-214). Together these studies suggest that inflammation-associated and sporadic CRC represent a continuum of disease risk relative to levels of inflammation or activation of inflammatory pathways.

Initial studies exploring inflammatory factors such as STAT3 and NF κ B as oncogenes were performed in the AOM/DSS model (117, 121, 125). These studies confirmed that both STAT3 and NF κ B facilitate tumorigenesis in the context of inflammation. Recent studies in APC^{Min/+} mice showed that loss of IEC-STAT3 led to a decrease in tumor load, providing the first direct *in vivo* evidence of a functional role for STAT3 in sporadic tumorigenesis (126). Our findings suggest that SOCS3, an intrinsic modulator of inflammation, may normally prevent tumors in the AOM model of sporadic CRC, which is typically driven by APC/ β -catenin mutations and is not associated with grossly evident inflammation. The finding that IEC-SOCS3 ^{Δ/Δ} mice had decreased body weight compared to controls is consistent with findings in the T3b-SOCS3 knockout mouse of spontaneous gastric tumorigenesis (134). Studies in this model showed that T3b-SOCS3 knock-out mice had enlarged stomachs, which is also consistent with our

findings that IEC-SOCS3^{Δ/Δ} mice had increased colon size compared to wild-type. The decreased body weight observed in T3b-SOCS3 mice was attributed to decreased feeding, however it is unclear if decreased feeding is the reason for lower body weight in our studies (134).

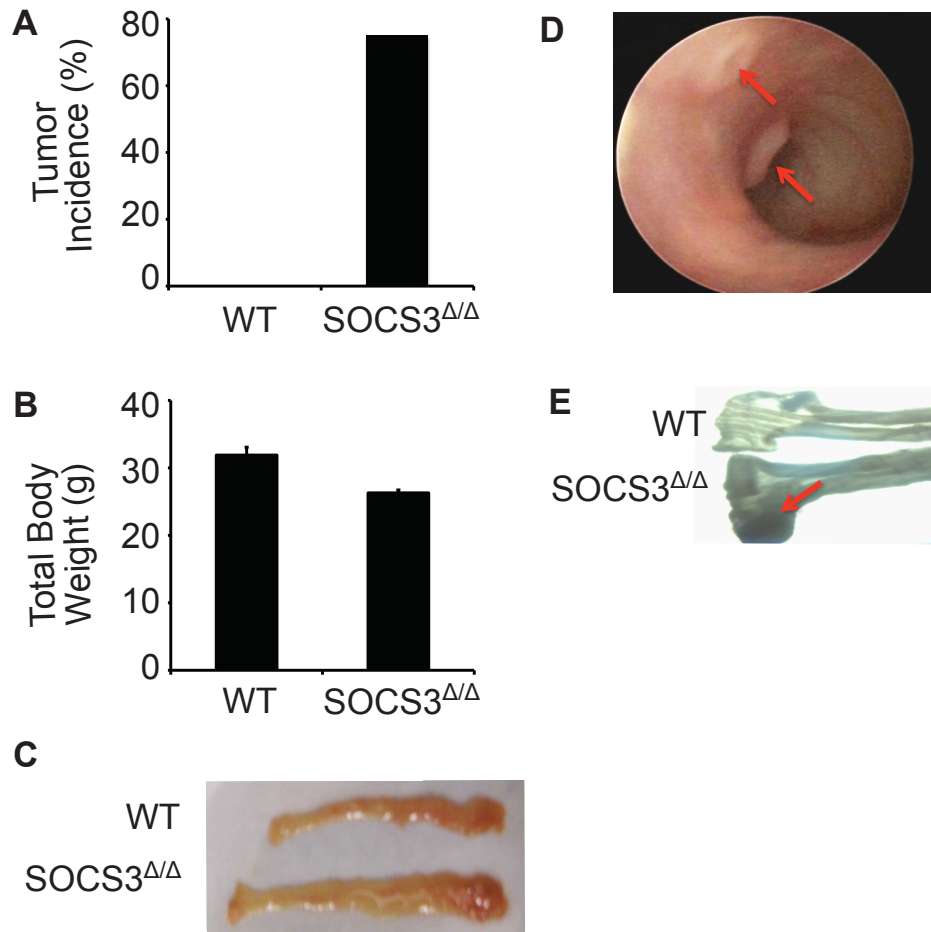


Figure 4.1 Mice with IEC-SOCS3 silencing have increased tumor incidence in the AOM model. (A) Mice lacking IEC-SOCS3 (SOCS3^{Δ/Δ}) have a 75% increase in colon tumor incidence compared to wild-type mice. (B) SOCS3^{Δ/Δ} mice have decreased body weight compared to wild-type mice. (C) SOCS3^{Δ/Δ} mice have increased colon size compared to wild-type mice. (D) Image taken from colonoscopy of SOCS3^{Δ/Δ} mouse. Red arrows indicate tumor lesions. (E) Image of colon tumor from SOCS3^{Δ/Δ} mouse under dissecting scope.

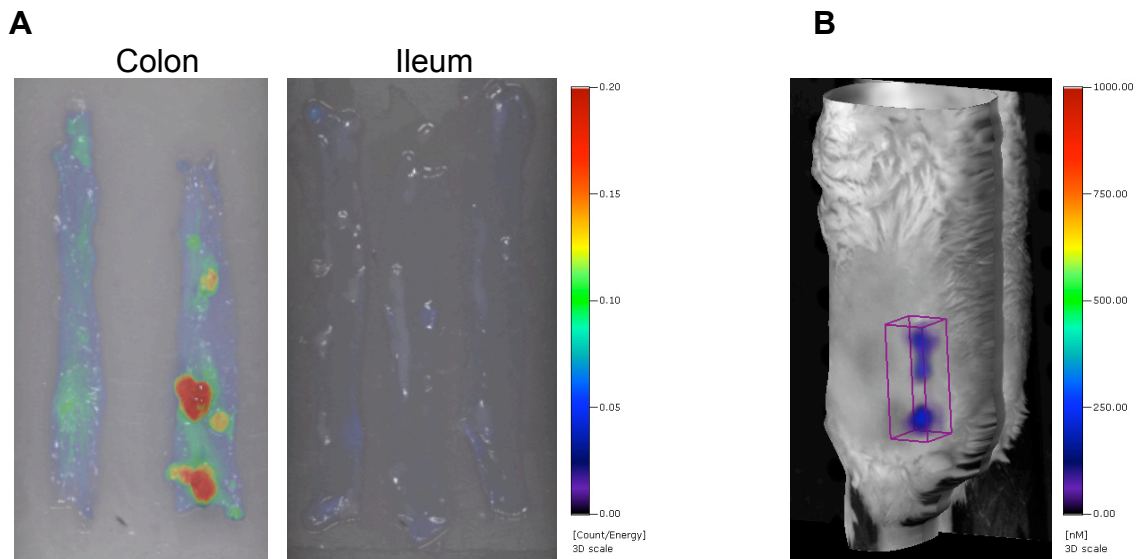


Figure 4.2 Imaging of AOM-injected KK/HIJ mice with ProSense 680. 2-3 month old KK/HIJ background mice received AOM injections once a week for four weeks to induce colonic tumors. Mice were imaged at 20 weeks after first AOM injection. ProSense 680 was injected 24 hours before imaging. (A) *Ex vivo* imaging of tumors in distal colon. (B) *In vivo* detection of colon tumors with ProSense 680.

CHAPTER V

GENERAL DISCUSSION

A. Cellular traits in colorectal cancer

In 2000, Hanahan and Weinberg described the six cellular traits that define most, if not all, human cancers. These include self-sufficient growth, evasion of apoptosis, insusceptibility to growth inhibitory signals, angiogenesis, and limitless replication (immortalization) (215). The potential contribution of inflammation to cancer development was observed as early as 1863, when Rudolph Virchow described the presence of immune cells within tumor tissues (216). However, studies within the past several decades have elucidated the involvement of inflammation in cancer at the cellular and molecular levels. Indeed, cancer-related inflammation (CRI), as it refers to the inflammatory tumor microenvironment as well as inflammation-associated genetic instability, has recently been proposed as the seventh hallmark of cancer (157). This concept is important to long-held views that sporadic and inflammation-associated cancer have different etiologies. Indeed a growing consensus is that they may, at least in some instances, reflect a continuum.

B. Convergent STAT3 and NFκB pathways in colorectal cancer

STAT3 and NFκB are transcription factors that can be activated by pro-inflammatory mediators and have both been implicated in the pathogenesis of inflammation-associated CRC. Emerging data suggest that these pathways may also contribute to sporadic CRC. Activation of NFκB leads to IL-6 production (117, 120, 121, 217, 218). IL-6 can elicit paracrine effects on tumor cells when produced by cancer-associated immune cells, or can exert autocrine effects

when produced by IEC or cancer epithelial cells (60, 121, 123, 219). IL-6 has been shown to stimulate proliferation of some, but not all, cancer cell lines (121, 220-222). Findings in this dissertation suggest that the outcome of IL-6 action in CRC may depend on interactions with other cytokines and induction of proliferative or pro-tumorigenic receptors such as TNFR2. IL-6 produced by immune cells in the lamina propria has also been shown to protect normal and pre-neoplastic IEC cells from apoptosis (121).

One of the major downstream pathways induced by IL-6 is the STAT3 pathway, which is hyper-activated in a variety of cancers including CRC (174). TNF α , which potently activates NF κ B in colon cancer cells, is also increased in the tumor microenvironment (157). Since both STAT3 and NF κ B can serve as mitogens, this has led to a recent concept of an NF κ B/IL-6/STAT3 signaling cascade, in which STAT3 and NF κ B activation lead to the perpetual activation of oncogenes, such as c-Myc, Cyclin D1 and Bcl-2, amongst several others. This mechanism, coupled with the presence of mutated DNA repair genes and silencing of tumor suppressors, contribute to genomic instability and proliferation of tumor cells (121, 157). Defining intrinsic molecules that control both STAT3 and NF κ B pathways is of considerable importance to understanding mechanisms of CRC and for the development of new therapies. This dissertation focused on SOCS3 and its role in regulating TNFR2.

C. Mechanisms of TNFR2 induction by IL-6 and TNF α

At the outset of the current studies, it was known that TNFR2 is receptor that promotes proliferation of IEC, is up-regulated during intestinal inflammation, and is induced by a combination of pro-inflammatory cytokines such as IL-6 and TNF α (56). Interest in TNFR2 in promoting colon cancer cell growth is increasing (57). Our study in CHAPTER II addressed the molecular mechanisms by which IL-6 and TNF α induce TNFR2 (223). Our study shows that STAT3 has a key role in TNFR2 induction, and provides direct evidence that TNFR2 over-expression induces proliferation and modestly promotes anchorage-independent growth of colon cancer cells. It has been shown in other systems that TNF α activates NF κ B via TNFR2 (54, 57, 224). Thus, STAT3-mediated induction of TNFR2 could provide a novel link between STAT3 and NF κ B pathways, which would be expected to amplify activation of both pathways. The activation of TNFR2 by STAT3 adds to growing evidence that STAT3 inhibitors should be explored as therapy for CRC.

D. SOCS3 limits STAT3-mediated TNFR2 expression and actions, and provides a novel link between the STAT3 and NF κ B pathways in inflammation-associated colorectal cancer

Prior work by our laboratory demonstrated that loss of SOCS3 in IECs led to an increase in tumor load in the AOM/DSS model of inflammation-associated CRC (115). This work also reported *in vivo* evidence that loss of IEC-SOCS3 led to up-regulation of both STAT3 and NF κ B pathways. Chapter II of this dissertation reports that loss of IEC-SOCS3 leads to up-regulation of TNFR2

staining. Collectively, our studies support a concept in which SOCS3 limits CRC-promoting pathways, directly and indirectly:

- 1) SOCS3 normally provides negative feedback on IL-6/STAT3-mediated growth (66, 86, 87, 96, 225).
- 2) Through its ability to limit STAT3 activation, SOCS3 decreases cytokine-induction of the pro-tumorigenic TNFR2, and limits proliferation and anchorage-independent growth (CHAPTER II). The ability of SOCS3 to limit TNFR2 expression may be one way in which NF κ B activation is normally attenuated.

Our working model demonstrates the potential interactions between the STAT3 and NF κ B pathways in CRC, and how SOCS3 normally limits these pathways to prevent tumorigenesis (Figure 5.1).

E. STAT3 activation and SOCS3 silencing may represent a common pathway between inflammation-associated and sporadic colorectal cancer

STAT3 and β -catenin/Wnt pathways share common downstream targets c-Myc and cyclin D1 (226-229). This supports a theory that STAT3 may have roles in the β -catenin/Wnt-driven sporadic CRC, or that β -catenin/Wnt may impact on STAT3 (Figure 5.2). CRC patients with nuclear co-localization of STAT3 and β -catenin have poorer prognoses (164). STAT3 staining positively correlated with nuclear β -catenin accumulation in human esophageal squamous cell carcinomas (200). APC^{Min/+} mice, in which β -catenin is constitutively active, exhibit decreased tumor incidence when crossed with IEC-STAT3 ^{Δ/Δ} mice (126).

Mice with hyper-active STAT3 (gp130^{Y757F} mice) display activating mutations in β -catenin within colon tumors in the AOM/DSS model (125).

Preliminary gene microarray data from our lab demonstrated that SOCS3 expression is decreased in tumors of APC^{Min/+} mice compared to non-tumor tissue, indicating that low SOCS3 expression may promote spontaneous tumorigenesis in this model. In this thesis work, we attempted to test this hypothesis, by generating mice with IEC-SOCS3 silencing on the APC^{Min/+} background. However, after sixteen months of breeding, we were unable to obtain study mice with IEC-SOCS3 deletion and the APC^{Min/+} mutation. Mice with global SOCS3 gene disruption die at embryonic day 11-13 due to placental defects (85), so it is therefore feasible that the combination of IEC-SOCS3 gene disruption with the alterations in Wnt/ β -catenin pathway found in APC^{Min/+} mice might lead to embryonic lethality. To determine if this were the case, we would need to employ a series of timed pregnancies to identify at which point the pups were dying. However, we opted to assess the role of SOCS3 silencing in a different model of sporadic CRC mediated by multiple injections of AOM.

The AOM model results in spontaneous colon tumors that have frequent mutations in APC or β -catenin (205, 230). Our studies in CHAPTER IV provide preliminary data that mice with IEC-SOCS3 silencing have a 75% increase in tumor incidence in the AOM model. To our knowledge, this is the first evidence that SOCS3 is a tumor suppressor in a model of sporadic CRC. Follow-up studies must be performed to confirm these observations and assess the levels of STAT3 and NF κ B activation in tumors from these mice, as well as define

expression of downstream targets affected by SOCS3, including TNFR2 expression. The study in CHAPTER IV used colonoscopy to monitor tumor development in the AOM model. This provides a useful approach to biopsy tissues over the course of tumor progression to define early and late targets of SOCS3. Our laboratory is currently taking this approach in mice with IEC-SOCS3 deletion and controls in both AOM and AOM/DSS models so that common or different targets of SOCS3 in inflammation-associated versus sporadic CRC may be identified.

F. Anti-TNF α therapy in cancer

In studies of isolated APC^{Min/+}-IEC co-cultured with intra-epithelial lymphocytes isolated from wild-type C57BL6 mice, TNF α production was dramatically increased in the culture supernatants. In addition, co-culturing APC^{Min/+}-IEC with intra-epithelial lymphocytes led to a dramatic increase in TNFR2 expression, whereas IEC isolated from wild-type mice did not exhibit this increase. These studies indicate that loss of APC in IEC promotes TNF α -induced increases in TNFR2 (231), and provides further evidence for looking at anti-TNF α therapies in sporadic CRC.

Anti-TNF α drugs were developed in the late 1990's and are currently utilized for a number of diseases including Crohn's disease and rheumatoid arthritis (232, 233). There are currently three types of FDA-approved anti-TNF α agents: infliximab (chimeric monoclonal antibody); adalimumab (recombinant human monoclonal antibody); and etanercept (TNFR2:Fc fusion protein) (234).

All three agents neutralize the effects of soluble and membrane-bound TNF α , but may also have additional cellular effects (234). Studies in mice have shown that infliximab treatment reduces immune cell recruitment and pro-inflammatory cytokine production, while inducing apoptosis in macrophages residing in the lamina propria in mouse models of Crohn's disease (235).

Recent pre-clinical studies in mouse models of inflammation-associated CRC showed that treatment with the anti-TNF α monoclonal antibody MP6-XT22 decreased tumor load associated with enhanced NF κ B activation (57). Another study showed that anti-TNF α treatment in established, late-stage inflammation-associated CRC lead to decreased tumor load as well as decreased local IL-6 expression, supporting the NF κ B/IL-6/STAT3 signaling cascade hypothesis (121). At present, it is not known if anti-TNF α affects sporadic CRC. One way to test if inflammatory pathways linked to TNF α may contribute to spontaneous CRC would be to treat APC^{Min/+} or AOM-treated mice with anti-TNF α drugs and assess tumor onset, size, and number as well as expression of IL-6 and TNF α and activation of STAT3 and NF κ B.

To date, anti-TNF α drugs have not been used clinically with the specific goal of prevention or treatment of CRC. Recent phase II clinical trials in patients with renal cell carcinoma (RCC) showed that anti-TNF α prevented increases in tumor number, albeit in a small cohort of patients. In this study, RCC patients in two separate groups were given 5mg/kg (Group 1) or 10mg/kg (Group 2) infliximab at time 0, 2, and 6 weeks, then every 8 (Group 1) or 4 weeks (Group 2) until further disease progression was observed. Group 1 exhibited 16% partial

response (PR, defined as a decrease in tumor size or extent), and 16% had stable disease (SD, no new tumors), with median duration of disease response to treatment at 7.7 months (ranging from 5 to 40 months) (236). Group 2 had 61% of patients reach SD, with median response of 6.2 months (ranging from 3.5 to 24 months) (236). Together these results suggest that anti-TNF α drugs may be useful in preventing disease progression, especially at higher doses, though there was no significant change in tumor size (236). There is also no evidence as to why patients eventually did reach a point of disease progression, indicating that while anti-TNF α therapy shows promise for cancer treatment, future studies must be done to improve its efficacy and potential for longer-term treatment.

Based on studies in other cancers and pre-clinical studies in CRC, evaluating the role of anti-TNF α drugs in CRC should be of utmost importance because these therapeutics are already FDA-approved, and could therefore benefit patients sooner. IBD-associated CRC is an obvious first starting point since Crohn's disease patients are already being treated with anti-TNF α .

However, it is important to keep in mind that immune surveillance has a protective role in cancer, even though inflammation may promote cancer (237, 238). Anti-TNF α drugs can have considerable side effects, including severe infection and risk of developing hematological malignancies, and may have adverse effects in cancer patients who have already received immune-suppressing drugs (239). Thus, drugs targeted at specific inhibition of TNFR2 would be advantageous, however there are currently no such drugs available. Alternatively, TNFR2 expression could be used as a specific cell-surface marker

in targeted therapy approaches. Based on recent studies, including those presented in this dissertation, new therapies aimed at mimicking or restoring the function of the tumor suppressor SOCS3 may also be beneficial for the prevention or treatment of CRC. There is considerable merit in development of drugs that mimic intrinsic tumor suppressors as they may have fewer adverse effects than current cancer therapies.

G. Potential therapeutic strategies for SOCS3 in colorectal cancer (*portions of this section are excerpted from (68)*).

To date, the mechanisms regulating SOCS3 promoter hyper-methylation are unknown. One study demonstrated that IL-6 regulates the transcription factor Fli-1, which is required for expression and activity of DNA methyltransferase enzyme 1 (dnmt-1) (240). Additional studies showed that IL-6 directly contributes to aberrant methylation of the tumor suppressor p53 by dnmt-1 (241). IL-6-mediated hyper-methylation of SOCS3 could be a mechanism in which continuous, unregulated IL-6 signaling potentiates a permissive tumor environment where inflammatory signaling is left unchecked by SOCS3. The prevalence of SOCS3 hyper-methylation in GI cancers (CHAPTER I) highlights the importance of developing epigenetic therapies to reverse the silencing of tumor suppressor genes such as SOCS3. In cholangiocarcinoma cell lines that exhibit SOCS3 promoter methylation, treatment with the demethylating agent 5-aza-2'-deoxycytidine (DAC) reversed SOCS3 silencing and decreased STAT3 activation, providing exciting pre-clinical data on the prospect of using DAC to

restore SOCS3 expression in cancer cells (102). One problem with drugs such as DAC is that they are non-specific and so more specific therapies are desirable.

In addition to epigenetic therapies, small molecule SOCS agonists may be useful therapies to restore SOCS actions in cancer. JAK inhibitors are currently being tested for a variety of myeloproliferative disorders, but may have broader implications for other cancers where aberrant cytokine or STAT signaling occurs, such as those GI cancers where one or more SOCS proteins are silenced (242). The SOCS1 mimetic Tkip (tyrosine kinase inhibitor protein) binds to the autophosphorylation site of JAK2 and inhibits STAT3 activation in prostate cancer cells (243). However, this compound has not yet been tested in CRC.

A cell-permeant, recombinant mouse SOCS3 protein (CP-SOCS3) was generated by attaching the 12 amino acid hydrophobic signaling sequence of fibroblast growth factor (FGF) to facilitate membrane translocation. Mice under staphylococcal enterotoxin B or lipopolysaccharide challenge that were given CP-SOCS3 were protected from the increased IL-6 and TNF α levels seen in control mice, providing promising pre-clinical data for intracellular SOCS3 protein therapies (244). However, no additional studies using CP-SOCS3 have been reported thus far. Our laboratory has attempted to test the CP-SOCS3 construct with little success. Small molecule, permeant SOCS3 mimetics could be alternative approaches and these proteins may not be as susceptible to proteasomal degradation as endogenous SOCS3 (245).

H. SOCS3 as a biomarker for colorectal cancer

Both SOCS3 and STAT3 are up-regulated in IBD tissues from patients and mouse models of IBD (66, 128). It is thought that the high levels of SOCS3 seen concurrent with hyper-activated STAT3 in chronic inflammation reflects the inability of SOCS3 to overcome STAT3 activation levels under these conditions, due in large part to constitutive IL-6 induction of STAT3 (66). In contrast, SOCS3 is silenced in CRC, although this has been shown in only a few patients (128). We therefore sought to evaluate if SOCS3 expression is decreased in the normal mucosa of adenoma patients to determine if low SOCS3 may promote CRC risk. Studies in CHAPTER IV describe our findings that low SOCS3 expression does not correlate with patients who have adenoma(s) as we had hypothesized, indicating that SOCS3 silencing likely does not occur prior to the adenoma stage in CRC progression (129). However, it is not known if SOCS3 silencing occurs during the adenoma stage, and may be a possibility considering STAT3 hyper-activation is seen in this stage in CRC (127). Future studies looking at SOCS3 expression or hyper-methylation in adenoma versus normal tissue are needed to determine if SOCS3 silencing occurs prior to or as a result of malignant transformation in the colon.

Another possible approach that has yet to be explored is whether or not SOCS3 hyper-methylation can be detected in DNA from fecal samples. The ability to detect DNA methylation from fecal samples is a novel screening tool that could circumvent the clinical hurdles of low sensitivity of fecal occult blood

tests and the non-compliance with colonoscopy screening (246). Studies looking at other genes known to be hyper-methylated in CRC, including *HIC1* (hyper-methylated in cancer 1), *vimentin*, *SFRP2* (secreted frizzled-related protein gene 2), *HPP1* (hyperplastic polyposis protein gene), and *MGMT* (O^6 -methylguanine-DNA methyltransferase gene), amongst others, have been able to detect aberrant methylation of DNA in stool from patients who were diagnosed at various stages in CRC progression, including hyperplastic polyps (246-248). While it is not likely that any single gene will hold the key to early detection by this method, it will be important for SOCS3 to be included as panels of hyper-methylated genes are developed.

I. Summary

The studies in this dissertation promote the concept that SOCS3 is a tumor suppressor in CRC and reveals mechanisms underlying this role. STAT3 and NF κ B are two key pathways that are up-regulated in inflammation-associated CRC, and emerging studies suggest these same pathways may be involved in sporadic CRC as well. Through its ability to directly limit STAT3 activation, SOCS3 decreases cytokine-induction of the proliferative TNF α receptor, TNFR2, in colon cancer cells. These studies not only highlight the possibility of utilizing FDA-approved anti-TNF α agents for CRC treatment, but also support the premise that specific TNFR2 antagonists or SOCS3 mimetics should be developed as novel therapeutic approaches. Such mimetics could be useful in the treatment of both inflammation-associated and sporadic CRC, as

our preliminary findings in the AOM model of spontaneous tumorigenesis showed that mice with IEC-SOCS3 silencing had a 75% increase in tumor incidence. Finally, while we have found that evaluating SOCS3 expression in the normal colonic mucosa is not an independent biomarker of adenoma risk in humans, future studies will determine if SOCS3 is indeed silenced in pre-cancerous adenomas, which could contribute a new biomarker for early stage CRC and be a useful biomarker in newly emerging non-invasive technologies for assessing CRC risk.

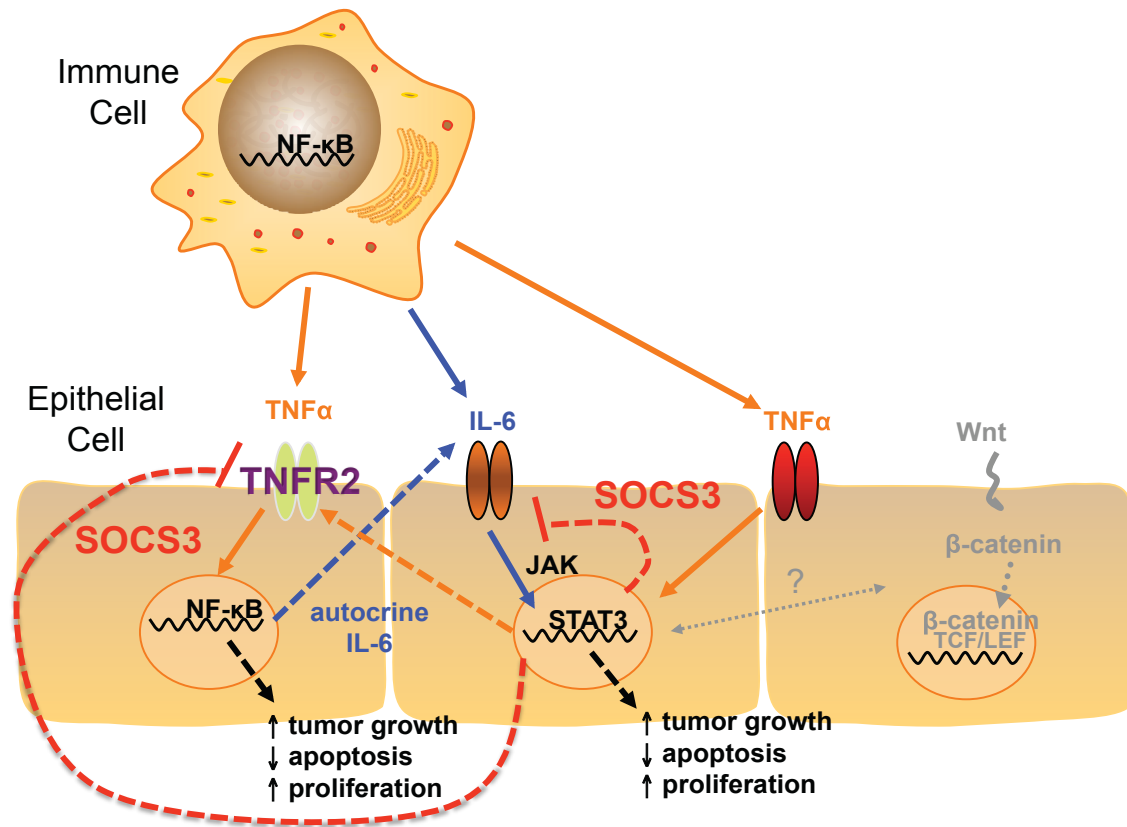


Figure 5.1. Model of NF κ B/IL-6/STAT3 signaling cascade in epithelial cells and the inhibitory effects of SOCS3. NF κ B activation leads to IL-6 up-regulation, which in turn promotes STAT3 signaling in IEC. IL-6 is also produced by local immune cells, along with TNF α , which leads to up-regulation of STAT3-mediated TNFR2 expression, which can in turn promote TNF α activation of NF κ B. SOCS3 elicits anti-tumor activity by inhibiting the JAK/STAT3 signaling cascade and its downstream targets, including TNFR2, which can in turn lead to decreased NF κ B activation. Dashed lines indicate gene transcription, solid lines indicate stimulation or activation.

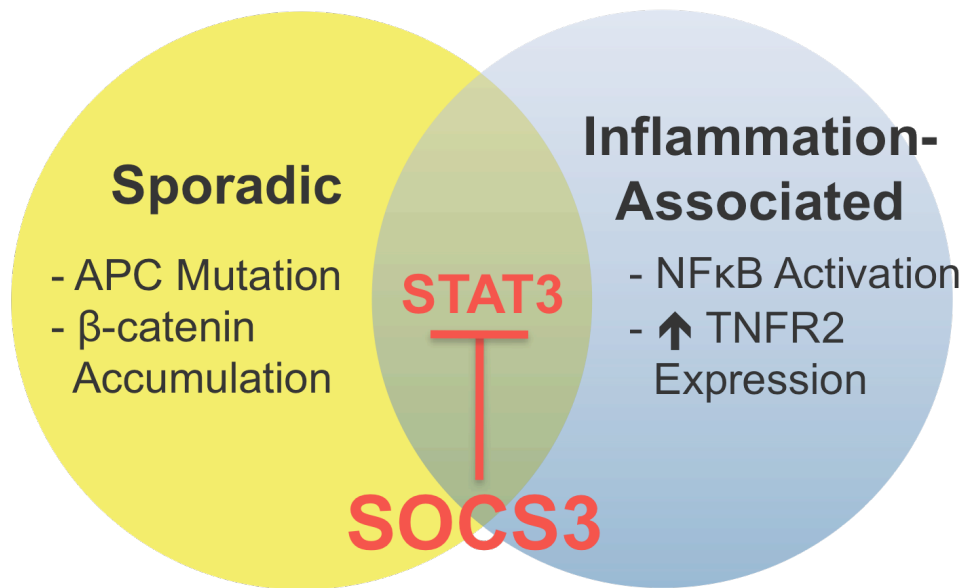


Figure 5.2. STAT3, and its endogenous repressor SOCS3 represent a common pathway in the pathogenesis of sporadic and inflammation-associated CRC. Emerging studies suggest that STAT3 is hyper-activated in tumors of both types of CRC, but the role for SOCS3 in sporadic CRC has yet to be defined. Our preliminary data shows a 75% increase in tumor incidence in mice with loss of IEC-SOCS3 in the AOM model of spontaneous CRC.

CHAPTER VI

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