

**IRS-1 AND SOCS2 ARE KEY REGULATORS OF INSULIN-LIKE GROWTH
FACTOR SIGNALING IN INTESTINAL CANCER**

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ABSTRACT

NICOLE M. RAMOCKI: IRS-1 and SOCS2 are key regulators of insulin-like growth factor signaling in intestinal cancer
(Under the direction of Dr. P. Kay Lund)

The growth hormone/insulin-like growth factor (GH/IGF) axis has been implicated at various stages of tumor progression in colon cancer. Insulin receptor substrate-1 (IRS-1) is a major downstream signaling molecule of insulin, IGF-I, and IGF-II and is constitutively active in various tumor types, including breast. Its role in colon cancer has not been studied *in vivo* to date. Conversely, Suppressor of Cytokine Signaling-2 (SOCS2) has been shown to limit this pathway and could thereby limit tumorigenesis in intestine. IRS-1 and SOCS2 deficient mice were crossbred with the *Apc*^{Min/+} model of intestinal polyposis to test whether IRS-1 or SOCS2 play a role in tumor formation in intestine. SOCS2 deficient mice were crossbred with GH-transgenic mice to test the hypothesis that SOCS2 normally limits GH-mediated trophic and tumorigenic actions. In mice with partial and absolute IRS-1 deficiency, intestinal crypt apoptosis levels were significantly increased, most notably in the putative stem cell region. Partial and absolute IRS-1 deficiency also lead to decreased tumor formation and decreased expression of a putative stem cell marker and β -catenin transcriptional target, Sox9, in intestine of *Apc*^{Min/+} mice. Partial SOCS2 deficiency in GH-transgenic mice lead to greater intestinal growth. SOCS2 deficiency lead to greater tumorigenesis in intestine of GH-transgenic and *Apc*^{Min/+} mice, but not using the AOM/DSS

model of inflammation-associated colon cancer. Overall, these studies suggest that IRS-1 plays a significant role in intestinal tumorigenesis and SOCS2 may serve as a crucial tumor suppressor by limiting the actions of the GH/IGF-I and IRS-1 pathway. Future studies will be aimed at determining if IRS-1, Sox9, and SOCS2 levels in human intestine can serve as useful biomarkers of colon cancer or adenoma risk.

DEDICATION

This thesis is written in dedication to my mother, Karen M. Ramocki, my very first mentor whose memory continues to inspire me.

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ABBREVIATIONS

ANOVA	analysis of variance
AOM/DSS	azoxymethane/dextran sodium sulfate
APC	adenomatous polyposis coli
Caco-2	human colon adenocarcinoma cell line
CBC	crypt base columnar
CIMP	CpG island methylator phenotype
CIS	cytokine-inducible SH2 domain-containing protein
CP	cell-permeant
CRC	colorectal cancer
DAPI	4',6-diamino-2-phenylindole
E	empty adenovirus
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
EMSA	electromobility shift assay
EMT	epithelial-mesenchymal transition
FAP	familial adenomatous polyposis
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GH	growth hormone
GHR	growth hormone receptor
GHRH	growth hormone releasing hormone
Gpr49	G-protein coupled receptor 49

GSK-3	glycogen synthase kinase-3
H&E	hematoxylin and eosin
HNPCC	hereditary nonpolyposis colon cancer
HO	homozygous
HT	heterozygous
IBD	inflammatory bowel disease
IEC	intestinal epithelial cells
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IHC	immunohistochemistry
i.p.	intraperitoneal
IR	insulin receptor
IRS-1	insulin receptor substrate-1
JAK	janus kinase
Lgr5	leucine-rich repeat-containing G protein-coupled receptor 5
LID	liver-specific IGF-I deficiency
LRC	label-retaining cell
LOH	loss of heterozygosity
LOI	loss of imprinting
MAPK	mitogen-activated protein kinase
MSI	microsatellite instability
Min	multiple intestinal neoplasia
PIRC	polyposis in the rat colon

SBS	short bowel syndrome
SEM	standard error of the mean
SOCS	supressor of cytokine signaling
Sox9	src homology-containing box
STAT	signal transducer and activator of transcription
TCF4	T-cell factor 4
TG	transgenic
TPN	total parenteral nutrition
V	vehicle
WT	wildtype

CHAPTER I

BACKGROUND AND SIGNIFICANCE

A. Structure of the normal intestine

The wall of the gastrointestinal tract consists of an epithelial cell layer closest to the lumen, the lamina propria, a submucosa comprised of fibroblasts, a layer of smooth muscle cells making up the muscularis mucosa, circular and longitudinal muscle layers, and the external serosa as shown in Figure 1.1a (132). The enteric nervous system of the gut is composed of the myenteric plexus (between the circular and longitudinal muscle layers) and the submucosal plexus (beneath the mucosal layer). The neurons within the plexi regulate smooth muscle motility and epithelial cells function (64). The smooth muscle cells that comprise the circular and longitudinal muscle layers are connected by gap junctions, allowing electrical current to spread from one cell to adjacent cells in both directions (64). The result is a coordinated contraction of the bowel wall that propels luminal contents through the digestive tract. The GI tract is richly perfused by the splanchnic circulation, which takes up nutrients and delivers nutrients, oxygen, and regulatory hormones to the gut (64).

The common bile/pancreatic duct enters the intestine at the duodenum and delivers digestive enzymes from the pancreas and bile from the liver, which are critical for digestion of carbohydrates, protein, and lipids (64). The epithelial layer secretes ions, mucus, and water, and produces brush border enzymes and transport proteins crucial for the breakdown and absorption of food. The epithelial layer also serves as a barrier, preventing bacteria and ingested toxins from entering the body. Immune cells within the bowel wall protect against toxins or infection by pathogenic bacteria in the gut. The lamina propria is composed of mesenchymal cells (fibroblasts and myofibroblasts), capillaries, immune cells, and smooth

muscle cells (132). Fibroblasts also reside in the muscularis mucosa, along with a collagen-rich extracellular matrix (132). The epithelial layer receives many of its proliferative and developmental signals from the underlying mesenchymal cells (106, 132, 139, 167).

B. The intestinal epithelium and intestinal adaptation

As shown in Figure 1.1b, the intestinal epithelium is made of a crypt-villus axis in the small intestine and a crypt-surface epithelium axis in the colon. Villi, which are finger-like luminal projections that start at the top of the crypts, greatly increase the surface area in the small intestine and allow more efficient nutrient digestion and absorption (109). The mammalian intestinal epithelium continuously renews itself every 3-5 days (84). Cells are born near the base of the crypt, where the stem cells and progenitor cells reside. Crypt cells migrate up to the villus where they differentiate into either an enterocyte, enteroendocrine, or goblet cell phenotype. Once cells reach the tip of the villus, they are sloughed off in a process called anoikis. In small intestine, a subset of cells migrate downward to the very base of the crypt to become Paneth cells, which are thought to function in innate immunity by secreting microbicidal peptides (33). Within the crypts a small but significant number of cells undergo spontaneous apoptosis (109). In order for the functional mass of the intestinal epithelium to remain constant, the rate of cell loss by anoikis and spontaneous apoptosis must closely match the rate of production of new cells by proliferation of stem cells and progenitor cells.

Maintenance of the functional mass of the epithelium is necessary for the epithelial layer to maintain its secretory, absorptive, and barrier functions. However, the intestine has a

remarkable ability to alter the rate of renewal and mass of intestinal epithelium to match physiological or pathophysiological challenge, a process termed intestinal adaptation. During oral nutrient restriction, the intestinal crypts reduce their rate of proliferation and increase apoptosis resulting in a decrease in mass of the epithelium (32, 46). This is an appropriate physiological response to the reduced need for digestion and absorption of enteral nutrients. Loss of intestinal mass due to injury, disease, or resection results in compensatory crypt and villus hyperplasia in remnant intestine that can maintain the intestine's digestive and absorptive functions despite its loss in length (36, 103). This occurs by increased crypt proliferation, increased crypt fission, and ultimately an increase in circumference of remnant intestine (36). The insulin-like growth factor (IGF) system has been shown to be a major mediator of normal and adaptive growth of the intestine (31, 55, 90, 103, 120, 137). Mice expressing an IGF-I transgene have increased intestinal mass, associated with increased villus height, crypt depth, and cell mitosis (137). IGF-I treatment in rats and smooth muscle-specific IGF-I transgenic expression in mice increase mucosal hyperplasia following small bowel resection (31, 90).

C. Crypt stem and progenitor cells

Based on stem cell characteristics in other organs such as the nervous system, it is generally believed that stem cells show slow, but long-lasting rates of cell division to give rise to shorter lived and faster cycling progenitor cells. True stem and progenitor cells should also be multipotent, having the ability to differentiate into all epithelial lineages (11, 27). At present, there is no fully defined or validated biomarker of intestinal stem cells. Current

theories indicate that a small population of multipotent stem cells reside in the crypts, which divide slowly and asymmetrically to renew the stem cell and produce a daughter progenitor cell (155). In asymmetric division, stem cells undergo selective segregation of their DNA strands during mitosis, with the mother strands retained in the stem cells while the daughter strands are donated to the newly-generated progenitor cell (155). This scenario makes teleological sense, since random DNA mutations from cell division would less likely be introduced in the conserved DNA strands in the stem cells (155). This contention is based on label-retention studies, which involve administration of multiple, high doses of ^3H thymidine or BrdU to label slowly dividing (as well as rapidly dividing) cells (27). A period of ‘washout’ spanning several weeks is then allowed so the label is diluted and undetectable in rapidly dividing cells, but is retained in long-lived and asymmetrically dividing stem cells, or ‘label retaining cells’ (LRCs) (87). Thus the LRCs retain ^3H thymidine or BrdU due to labeling of the ‘mother DNA’ and retention of this DNA in the LRC or stem cell. Early studies using autoradiography showed slow-cycling cells could be labeled radioactively, and this method could be used to identify cells that give rise to all cell types within the crypt epithelium (27). While this type of asymmetrical cell division would likely occur during homeostatic conditions, it has been proposed that stem cells can divide symmetrically into two stem cells during adaptive growth and into two daughter cells during atrophy or nutrient restriction (109). Through use of label retention and radiation to speed cellular turnover, Potten postulated that the crypt stem cells reside around positions 4-6 (151, 155), a claim that is still controversial.

Progenitor cells derived from crypt stem cells can be termed ‘transit cells.’ This is because these cells show rapid, but limited, cell division and migrate up to the villus or to the

crypt base, where they differentiate into the four terminally differentiated lineages. A major question in intestinal epithelial biology is the molecular and functional phenotype of crypt stem and progenitor cells. Many candidate stem cell markers have been proposed. Musashi and CD133 have been proposed as stem cell markers, but these biomarkers appear to label crypt stem and progenitor cells, and cells expressing these biomarkers have not been shown to be multipotent (7, 133, 136, 154). Recent data show that Lgr5, a G-protein coupled receptor with no known ligand, may represent a biomarker of multipotent crypt stem cells (7). Lgr5 immunostaining marked only an average of 3.5 cells per crypt, suggesting that this could be a marker of stem cells and, less likely, a marker of stem and transit amplifying cells (7). The Lgr5-positive cells that reside between Paneth cells have been termed crypt base columnar cells (CBCs) and lie at a site where proliferation often takes place (7). Lineage-tracing studies in which a tamoxifen-inducible Cre recombinase was knocked-in to the Lgr5 locus and then crossbred with a transgenic mouse expressing Cre-activated LacZ allowed lineage tracing of cells derived from Lgr5 positive cells. This revealed that Lgr5-positive CBCs were able to generate goblet, enterocyte, and Paneth cells (enteroendocrine cells were too rare to locate in these studies), indicating that Lgr5-positive cells are multipotent (7). Thus, Lgr5/Gpr49 may be a true intestinal stem cell marker.

At the same time these studies were being conducted, our laboratory began to turn to Sox9, or Sry-related High Mobility Group (HMG)-box DNA binding protein 9, as a potential stem cell marker. The Sox family of transcription factors are composed of 26 proteins shown to mark stem and progenitor cells in other organs and can enhance or antagonize β -catenin/Wnt signaling (186, 218). Sox9 expression requires the Wnt/ β -catenin pathway, and Sox9 has been implicated in fate determination of stem cells in other organs,

including the nervous system, pancreas, cartilage, and gonads (1, 23, 28, 104, 180). In intestine, Sox9 is normally expressed in the same regions within the crypt where proliferating cells reside, indicating Sox9 could mark intestinal stem or progenitor cells (12). When the Sox9 gene is inactivated in mice, Paneth cells failed to form and goblet cell formation was decreased (9, 125). These findings were associated with hyperplasia and dysplasia within the intestinal epithelium. These studies suggest Sox9 plays a crucial role in growth and homeostasis of the intestinal epithelium as well as differentiation of two major lineages. Furthermore, human adenocarcinoma stains positive for Sox9, indicating a potential role for Sox9 in intestinal tumorigenesis (12). Whether Sox9 is a true stem cell biomarker or plays a significant role in intestinal tumors needs to be further explored. As described in later chapters, recent data from our group indicates that Sox9 is expressed in both the CBCs (identified as the stem cells by the Clevers group) and the stem/progenitor cell region as identified by Potten's group.

D. Biological significance of crypt apoptosis

The small but significant rate of crypt apoptosis in small intestine and colon is thought to serve two potential roles. Apoptosis may remove 'excess' cells and thereby ensure that rates of cell production do not exceed rates of cell loss, preserving normal epithelial mass and crypt homeostasis. In addition, apoptosis may remove genetically-damaged crypt cells, preventing their expansion into a precancerous or cancerous lesion. Evidence in support of this concept stems from recent data in humans showing a link

between low rates of apoptosis in normal intestinal mucosa and risk of precancerous adenomatous lesions in the colon (15, 110). High proliferation rates of stem or progenitor cells may also represent a risk factor for developing colon cancer. High rates of crypt proliferation statistically increase the probability of DNA damage. Furthermore, the highest rates of apoptosis occur near the base of the small intestine or colonic crypts, where crypt stem and progenitor cells reside and apoptosis is triggered by DNA damage (72, 217). Radiation, which induces apoptosis of genetically-damaged crypt cells, is a valuable tool for signaling mediators of cell death and crypt regeneration (150).

E. Intestinal cancer

The colon and, to a lesser extent, the small intestine are sites of development of precancerous and cancerous lesions. Perpetual and rapid turnover of intestinal epithelial cells may contribute to cancer risk. As cells divide, newly synthesized DNA is susceptible to the introduction of mutations, which can lead to cancer. In the small intestine, cells that acquire genetic mutations often undergo p53-dependent apoptosis, thereby preventing rapid expansion of genetically-damaged stem cells (109). In the large intestine, however, an anti-apoptotic protein called Bcl-2 is expressed and can prevent apoptosis of genetically-damaged cells (114, 117). Therefore, the colon relies more heavily on DNA repair mechanisms to prevent aberrant growth within the epithelium (109). The colon is also subjected to a greater amount of toxins and potentially harmful microbial agents than the small intestine while lacking protection by Paneth cell secretions, a combination thought to contribute to the vastly greater cancer risk in colon compared to small intestine.

Colon cancer is the third most prevalent cancer worldwide (20). Early detection is key to colon cancer survival, since metastasis leads to poor prognosis (45). Colon cancer is curable with surgery, however metastasis occurs in at least 40% of colon cancer patients and often requires a combination of surgery, radiotherapy, and chemotherapy (45). Figure 1.2 shows the typical progression of colon cancer, with common mutations or risk factors noted at specific stages along the pathway. Mutations in genes such as *APC* lead to familial adenomatous polyposis (FAP) in which hundreds to thousands of tumors cover the colon (53). Other genetic mutations can also be inherited, leading to Hereditary Nonpolyposis Colorectal Cancer (HNPCC) (232), while mutations that are acquired and not inherited lead to sporadic colon cancer (19). Microsatellite instability (MSI) resulting from deficient function of mismatch repair genes is a hallmark of HNPCC but is also found in 15% of all sporadic cancers (41). Colon cancer is often initiated by mutations that lead to constitutive activation of oncogenes, as in the case of K-Ras, which is mutated in about 30-60% of all colon cancers (19). Conversely, silencing of tumor suppressors by hypermethylation can also lead to colon cancer, such as hypermethylation of CpG islands in the hMLH1 gene promoter, a common mismatch repair gene (48, 224). The abundance of genes affected by hypermethylation have led to a push to decode the DNA “hypermethylome” (48). Clustering of human colon cancers by CpG island methylator phenotype (CIMP) and mutation status has recently allowed colon cancer classification into separate subgroups (183). The three groups, CIMP1 (high in MSI and in BRAF mutations), CIMP2 (high in K-Ras mutations), and CIMP-negative (high in p53 mutations) subgroups show distinct trends in histology and prognosis (183).

Colonoscopy is used to detect neoplastic lesions, which are removed and biopsied

when found. While adenomas and other pedunculated polyps are typically found and removed at the time of colonoscopy, flat or sessile tumors are more difficult to detect (77). Since colon cancer is not always easily detected and colonoscopy is a highly-invasive procedure, understanding the factors underlying polyp initiation and growth is crucial for preventing cancer.

F. Obesity, insulin and colon cancer

Body mass index and waist to hip ratio both strongly correlate with colon cancer risk (16, 63). This is especially alarming because two thirds of the American population is overweight and obesity has risen from 13% to 30% in the last 40 years (63). Low physical activity, high calorie intake, and high glycemic index of foods taken in all correlate with an increased risk of colon cancer (63). Insulin resistance and colorectal neoplasia are linked, but whether these two diseases are directly linked or if they simply share similar risk factors is not known (63, 179). Insulin resistance results in high circulating levels of insulin, and this excess insulin may have proliferative or tumorigenic potential in the colon since the colon is not a typical site of insulin signaling and glucose storage (63, 179). Increased energy intake may also link to colon cancer due to an increase in triglycerides and fat metabolites, which can regulate gene transcription through PPAR γ in colonic tissue and play a role in growth and insulin sensitivity (42, 78, 170, 191). Intracellular lipids may also increase cancer risk by serving as substrates for oxidative stress, depleting available antioxidants, and leading to DNA damage (43, 63). Recent studies in our laboratory and that of Dr. Robert Sandler showed that high but within the normal range insulin increased risk of precancerous

adenomatous polyps and strongly correlated with low rates of crypt apoptosis in humans (85). Furthermore, insulin levels and obesity perturb circulating levels of insulin-like growth factors (IGFs), either directly or by affecting levels of IGF binding proteins (IGF-BPs) that modulate the bioavailability of IGFs (63). Numerous components of the IGF system of growth factors are implicated throughout the progression to colon cancer, as shown in Figure 1.2.

G. The growth hormone/insulin-like growth factor axis

Overview: Growth hormone (GH) is secreted in a pulsatile manner, with low secretion pre-puberty, a rise during puberty, and a drop at old age (101). GH releasing hormone (GHRH) is produced in the hypothalamus and stimulates the production of GH in the anterior pituitary, which is released into the circulation. Circulating GH binds the GH receptor (GHR) and stimulates the production of IGF-I primarily in the liver, although GH can act on other organs, including the intestine to increase IGF-I expression (143). Evidence suggests that Signal Transducer and Activator of Transcription 5b (STAT5b) is a key mediator of GH-induced IGF-I production in liver, since STAT5b knockout mice show decreased body growth and serum IGF-I levels and fail to increase liver IGF-I expression upon stimulation with GH (34, 198, 206, 220). GH production by the pituitary gland is limited by IGF-I via a classical negative feedback mechanism (143). The IGF system is summarized in Figure 1.3.

IGF system ligands (IGF-I, IGF-II, and insulin): Insulin is synthesized only by beta cells in the pancreas. Available evidence suggests that the IGFs are produced by

hepatocytes in liver and by mesenchymal cells (fibroblasts, myofibroblasts, smooth muscle cells) in multiple organs (65, 219). In the intestine, mesenchyme-derived IGF-I can stimulate growth of the epithelium (216). IGF-I and IGF-II are single-chain polypeptides with 70% homology in amino acid sequence (75). Both IGF-I and IGF-II contain B, A, C, and D domains as well as a carboxyl-terminal E domain that is cleaved off in the golgi prior to its trafficking (97). Proinsulin contains B, C, and A domains and, in contrast to the IGFs, the C domain is cleaved from proinsulin during post-translational processing so that insulin contains only the B and A chains linked by disulfide bonds (97). The portion of proinsulin that is cleaved off, named C-peptide, is secreted with mature insulin and is a commonly-used marker of insulin secretion (63). Since rodents express IGF-II primarily at the fetal stage, it was previously thought that IGF-II functions in fetal growth while IGF-I functions postnatally. In humans, however, IGF-I and IGF-II is produced in multiple organs throughout life with IGF-II in consistently higher abundance than IGF-I (97). Transcription of IGF-II from its first promoter site is found in adult tissue while transcription from its third and fourth promoter sites is found in fetal tissue (234).

Insulin and IGF receptors: The actions of IGF-I, IGF-II, and insulin are mediated by two receptors, the IGF-IR and the insulin receptor (IR). Activation of the IGF-IR by IGF-I or IGF-II or insulin activation of the IGF-IR or IR transduces most of the growth-promoting effects of the IGF system. The IGF-IIR (or mannose-6-phosphate receptor) specifically binds IGF-II and no other ligands and functions as a scavenger receptor, binding IGF-II and diminishing its action (211, 229).

The 60% homologous IGF-I and insulin receptors are both heterotetramers, containing 2 α extracellular subunits and 2 β transmembrane subunits linked by disulfide

bonds (211). The α subunits are mainly involved in ligand binding whereas the β subunits include a tyrosine kinase domain involved in transmitting various downstream signals. IGF-I binds the IGF-IR with at least 2 times greater affinity than IGF-II (82), which is thought to contribute to the greater role of IGF-I in adulthood compared to IGF-II, despite the overall higher levels of IGF-II. IGF-I also has 2 times greater affinity for the IGF-IR compared to the insulin receptor (IR), but physiological levels of IGF-I can activate the insulin receptor since IGF-I circulates at 10-100 times the concentration of insulin (99). Generally, circulating IGF binding proteins (IGFBPs) sequester IGF-I and prevent binding to the IR. The insulin receptor has two isoforms, an isoform that lacks exon 11 (IR-A) and an isoform that includes exon 11 (IR-B). IR-A has high affinity for IGF-II and is currently regarded as the receptor involved in proliferative effects of insulin and IGF-II, whereas the IR-B primarily binds insulin and mediates the metabolic actions of insulin on glucose and fat storage (165).

The IGF-IR and IR can heterodimerize forming hybrid receptors. The IGF-IR/IR hybrids have high affinity for IGF-I, but low affinity for insulin. This decreased response to insulin with an increase in IGF-IR/IR hybrid formation has been proposed as the mechanism behind insulin resistance in type 2 diabetes mellitus (99). Hybrids composed of the different isoforms of the IR act differently, with IGF-IR/IR-A binding IGF-I, IGF-II, and insulin, while IGF-IR/IR-B binds IGF-I, IGF-II to a lesser extent, and does not bind insulin (142).

IGF binding proteins: IGF bioavailability and action is modified by a family of six or more IGF binding proteins. The majority of IGF-I and IGF-II in the circulation is bound to an IGFBP, influencing the bioavailability of ligand (168). The majority of IGF-I and IGF-II is bound to IGFBP-3 along with an 80-kd acid-labile subunit (168). The IGFBPs increase

the half-life of circulating IGFs, sequester IGFs in the blood vessels, and control their availability to their receptors (99, 168). Some IGFBPs, however, facilitate selective transport to specific organs by allowing the ligand to cross capillary walls or directly interact with their receptors thereby augmenting receptor binding of the ligand (99, 168). Insulin does not bind IGFBPs, but can decrease the production of IGFBP-1 and IGFBP-2 (168). IGFBP proteases can hydrolyze IGFBPs and release the ligand from its binding partner, allowing it to become biologically active (231). Therefore, the IGF-IIR (which scavenges IGF-II) and IGFBPs negatively regulate the actions of the IGF system, whereas the IR, the IGF-IR, and IGFBP proteases can be regarded as positive regulators of the pathway.

H. IGF-IR and IR signaling

IGF-I or IGF-II binds to the extracellular α subunit of the IGF-IR, resulting in a conformational change and cross-autophosphorylation of the intracellular β subunits (99). This leads to further phosphorylation of tyrosine residue sites in the juxtamembrane and COOH-terminal regions of the receptor, which can then recruit downstream docking proteins such as insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) and src homology-containing and collagen-related protein (Shc) (99). These signaling molecules can then activate the PI-3 kinase and Ras/Raf/mitogen-activated protein kinase (MAPK) signaling cascades that eventually lead to the proliferative, apoptotic, and differentiative effects of IGF-I (93, 95, 173, 202). Although in cell lines IGF-I, IGF-II or insulin can activate both IRS-1 and IRS-2, findings in IRS-1 and IRS-2 knockout mice indicate a preferential role of IRS-1 in growth and a preferential role of IRS-2 in metabolism. IRS-1 knockout mice exhibit reduced body

growth and reduced growth of several organs including the intestine but do not develop type 2 diabetes (5, 145), while IRS-2 null mice show normal body and organ growth but do develop type 2 diabetes (212). In other tissues, studies indicate a preferential role of IRS-1 compared with IRS-2 in mediating the growth-promoting and anti-apoptotic actions of insulin (159, 196).

I. IGFs and IRS-1 in anti-apoptotic pathways

IGF/IGF-IR/IRS-1 link directly to numerous anti-apoptotic pathways, defined primarily in cancer cell lines. The PI3K/Akt pathway is often considered the central pathway in inhibition of apoptosis by IGF-I (93). IGF-I-induced activation of Akt promotes phosphorylation of FKHL1 and FKHR, members of the forkhead family of transcription factors, causing cytoplasmic retention (173). This reduces transcriptional activity by Forkhead proteins, putatively by recruitment of 14.3.3., which masks the nuclear localization signal and DNA binding site (93, 173). Unphosphorylated nuclear Forkhead proteins, however, can activate transcription of pro-apoptotic proteins, such as FasL, Bim, TRAIL, and TRADD (93, 173). IGF-I induces Bcl-2 and Bcl-X_L, which are anti-apoptotic if homodimerized or pro-apoptotic if heterodimerized with unphosphorylated Bad (93). IGF-I also promotes cell survival by stimulating phosphorylation of Bad via Akt/PKB, preventing it from heterodimerizing with Bcl-X_L (29, 93). IGF-I induces mTOR, which phosphorylates the cell growth-promoting 4EBP protein and S6 kinase, which is involved in ribosome biogenesis (100).

Following DNA damage in cultured fibroblasts, IGF-I opposes apoptosis by

stimulating p53 degradation (69). In myocytes, inhibition of apoptosis by IGF-I correlated with decreased p53 expression and an increase in mdm2, a RING finger ubiquitin ligase that targets p53 for degradation (98). In hepatoma, melanoma, intestinal smooth muscle cells and colon cancer cell lines, IGF-I inactivates glycogen synthase kinase-3 (GSK-3) and activates Tcf/ β -catenin pathways (37, 95, 147). IGF-I also interacts with α -catenin and E-cadherin in mediating migration and invasion in colon cancer cell lines (4). Though previously thought of as strictly an anti-apoptotic signaling molecule, ERK activation by IGF-I has been shown under various conditions to promote apoptosis, possibly by inducing cytochrome c release and caspase 3 activation or by inhibition of the pro-survival Akt pathway (236). IGF-I and IGF-II induce Cox-2 and prostaglandin E2, which are increasingly implicated in the enhanced proliferation and decreased apoptosis in intestinal adenoma and colon cancer, as well as in other systems (39, 74, 87, 148, 182). IRS-1 activation has been found in various tumor types, and its inhibition leads to a less transformed phenotype in a breast cancer cell line (24, 91). Furthermore, studies show that a G972R polymorphism in the IRS-1 gene had a significant association with increased risk of adenoma and colorectal cancer (CRC) (187), further supporting a major role of IRS-1 in cancer. Given the major role of IRS-1 in mediating the anti-apoptotic actions of insulin and IGFs, known risk factors for colon cancer, this prompted the emphasis of IRS-1 studies described here (Chapter II).

J. The role of the *APC* gene in intestine

The Wnt signaling pathway is involved in growth and development of epithelial cells, including the intestinal epithelium (131). In the absence of a Wnt signal, a multiprotein

complex comprised of adenomatous polyposis coli (APC), glycogen synthase kinase 3 β (GSK3 β), and axin binds β -catenin and targets it for destruction in the proteasome (131). When Wnt ligands are present, they bind the frizzled receptor and inhibit GSK3 β , preventing GSK3 β from binding APC and axin (131). Under these conditions, β -catenin is not degraded, accumulates in the cytoplasm, and translocates to the nucleus to serve as a co-activator of transcription once bound to the T-cell factor 4 (TCF4) transcription factor (201). Various cell cycle progression genes are transcriptionally activated under these conditions, including Cyclin D and Myc, providing favorable conditions for the intestinal epithelium to undergo neoplastic growth (76).

Mutations in the *APC* gene are an early and common event in colon cancer, occurring in approximately 85% of spontaneous colon cancers (228). Hereditary intestinal polyposis in familial adenomatous polyposis (FAP) and Gardner syndrome are also caused by *APC* gene truncations (14, 53). *Apc*^{Min/+} mice are heterozygous for an *APC* truncation mutation, but through a process called loss of heterozygosity (LOH), some cells lose their wildtype allele and become homozygous for this mutation (25). Cells that have undergone LOH have dysregulation of cellular β -catenin, leading to tumor formation (92). The *Apc*^{Min/+} mouse model is caused by a truncation in codon 850, which leads to loss of the GSK3 β binding site in the *APC* gene protein product (128). As shown in Figure 1.4, mutated *Apc* is unable to bind GSK-3 β and β -catenin is not degraded, much like in the presence of Wnt ligand (228). Thus, *Apc*^{Min/+} mice have tens to hundreds of adenomatous polyps preferentially localized, but not limited to, the small intestine. This mouse model can be used to study factors that contribute to or protect against precancerous lesions in the intestine. A Cre-lox inducible mouse model was made to more directly test the role of APC in the intestinal epithelium

(169). *APC* deletion in this mouse model resulted in perturbed differentiation, increased proliferation, and disrupted migration of intestinal epithelial cells along the crypt/villus axis, all of which are considered phenotypic hallmarks of intestinal neoplasia (169).

Recent reports indicate direct interactions between IRS-1 and β -catenin. Studies in C10 cells (a colorectal cancer cell line) show that IGF-IR binds E-cadherin and β -catenin at the membrane, forming a complex that disassembles upon IGF-IR activation (147). In the presence of IGF-I, β -catenin is released from the membrane, shows an increased half-life, and can enter the nucleus to serve as a transcriptional cofactor. Other *in vitro* studies using fibroblasts derived from mouse embryos with targeted disruption of IGF-IR, with and without stable transfection of IGF-IR, indicate that IRS-1 directly associates with β -catenin. Furthermore, IRS-1 appears to be involved in nuclear translocation of β -catenin, and IRS-1/ β -catenin complexes can bind to the promoters of growth promoting target genes of β -catenin that mediate proliferation or apoptosis (26). Given the *in vitro* evidence for an interaction between IRS-1 and β -catenin, studies in Chapter II aimed to assess if IRS-1 deficiency altered *Apc*^{Min/+} tumorigenesis *in vivo*.

K. The SOCS family of proteins

Other portions of this thesis (Chapters III and IV) focus on Suppressor of Cytokine Signaling-2 (SOCS2) as a negative regulator of the GH/IGF axis in the intestine. The family of SOCS proteins consists of SOCS 1-7 and CIS (cytokine inducible SH2-containing protein) (60). SOCS proteins are activated by various cytokines and growth factors, most commonly those that signal through the janus kinase and signal transducer and activator of transcription

(JAK/STAT) pathway (60, 61). Various SOCS family members have also been shown to bind and inhibit various cytokine and growth factor signaling receptors, including GHR as shown in Figure 1.5 (61). SOCS proteins, particularly SOCS1 and SOCS3, have been shown to bind and inactivate JAKs, which are recruited to the receptor following its activation by ligand binding. Upon activation, cytokine receptors dimerize and bind JAKs, which autophosphorylate and activate the receptor at specific tyrosine residues. These residues serve as docking sites for STATs, which become phosphorylated, dimerize, and enter the nucleus and serve as transcription factors for various target genes, including SOCS (60). By binding the receptor, SOCS can compete with STATs for binding sites on the activated cytokine receptor, thereby diminishing receptor signaling. SOCS proteins contain an N-terminus of variable homology, a central SH2 domain, and a conserved 40 amino acid motif near the C-terminus called the SOCS box. The SH2 domain binds phosphotyrosine sites on activated proteins, and the SOCS box interacts with elongins B and C, likely targeting activated proteins for destruction in the proteasome (21, 83, 233). This targeted degradation has been proposed as one of the ways SOCS proteins may terminate cytokine signaling. To date, most is known about the functions of SOCS1, SOCS2, and SOCS3. Generation of mice with targeted disruption of SOCS1 and SOCS3 genes indicates a role of SOCS1 in regulating IFN γ action and a role of SOCS3 in IL-6 signaling. Both these SOCS are therefore key negative modulators of signaling by immune cell-derived cytokines (60). Unlike SOCS1 and SOCS3, SOCS2 has been shown to play a limited role in immune function but plays a major role in growth.

L. SOCS2 as an inhibitor of the GH/IGF-I axis

SOCS2^{-/-} mice display increased body weight, similarly to GH-transgenic mice, suggesting that SOCS2 may play a role in GH signaling (61). When SOCS2^{-/-} mice were crossed with growth hormone releasing hormone receptor deficient mice (*Ghrhr*^{-/-}), GH-deficient or “*little*” mice, GH-deficiency reversed the overgrowth phenotype of the SOCS2^{-/-} (61). These SOCS2^{-/-}/*Ghrhr*^{-/-} double-mutants were also hyper-responsive to exogenous GH, and GH administration recapitulated the SOCS2^{-/-} phenotype, suggesting increased growth from SOCS2 deficiency is dependent on GH action (61). Data in yeast two-hybrid screens indicate that SOCS2 binds directly to both the activated GH and IGF-I receptors (38, 66). Recent data from our lab shows that FLAG-tagged SOCS2 associates with activated endogenous IGF-I receptor in Caco2 colon cancer cells and attenuates IGF-I induced activation of the IGF-IR and IRS-1 (120). Furthermore, in Caco2 cells, SOCS2 expression increases as cells become confluent and overexpression of SOCS2 limits proliferation and induces expression of enterocyte differentiation markers (121). In IEC-6 cells (an untransformed rat intestinal epithelial cell line that, unlike Caco-2 cells, are GH-responsive) GH treatment led to induction of SOCS2 mRNA, a decline in cellular proliferation, and also attenuated IGF-I-induced proliferation (121). Crypts isolated from SOCS2 knockout mice were more responsive to both GH and IGF-I (121). Together, these data support the novel concept that SOCS2 limits the actions of IGF-I and the IGF-IR tyrosine kinase as well as having a more traditional role in limiting the actions of the cytokine GH.

M. SOCS proteins in colon cancer

Inflammatory bowel disease (IBD) has been linked to an elevated risk of colorectal cancer, which is likely due to chronic inflammation of the intestinal mucosa (62, 77, 194). Cytokines and growth factors can help repair damage and restore the absorptive and barrier function of the intestinal epithelium, but such actions can lead to hyperplasia and predispose to dysplasia or neoplasia. GH and IGF-I are key mediators of mucosal repair during inflammation, and SOCS proteins may limit their actions to prevent an excessive wound healing response (194). SOCS proteins have been implicated in IBD, particularly SOCS3. SOCS3 was shown to be induced by GH treatment in a peptidoglycan-polysaccharide rat model of IBD and fibrosis, and SOCS3 mRNA was localized to granulomas, suggesting that SOCS3 may mediate anti-inflammatory or anti-fibrogenic roles of GH (194). Our laboratory has shown that intestinal epithelium-specific knockout of SOCS3 can increase the susceptibility to inflammation-induced intestinal tumor formation using the azoxymethane/dextran sodium-sulfate (AOM/DSS) model. Thus, SOCS3 may be crucial in promoting the beneficial effects of GH/IGF-I during mucosal repair in IBD, while limiting excessive trophic effects that could favor neoplastic growth (194). Although SOCS2 was recently shown to be required for the anti-inflammatory action of lipoxins, very little is otherwise known about the mechanisms and extent of SOCS2 activity in the immune system (105, 160). Whether SOCS2 can alter the risk of CRC by playing a similar role in the inflammatory process has yet to be fully explored.

Endogenous growth hormone is crucial for both normal intestinal growth during

development and during adaptive growth following loss of mucosal surface area (143). Recombinant GH is an FDA-approved treatment for stunted growth in GH-deficient children and for patients with short bowel syndrome (SBS), a bowel malabsorption syndrome following multiple surgeries (70). However, excess growth hormone has been implicated in various cancers, including colon cancer. Acromegals, who have GH-secreting pituitary tumors resulting in elevated GH and IGF-I levels, have increased risk of colorectal cancer, although the extent of this increased risk remains controversial (80). The role of SOCS2 in inhibiting GH/IGF-I action supports a role for SOCS2 in protecting against colon cancer by inhibiting excessive trophic effects of GH. Data showing that SOCS2 gene inactivation by hypermethylation of CpG islands within the SOCS2 promoter region correlates with multiple types of cancer support a role for SOCS2 in cancer development (30, 49, 52, 68, 190, 203). Despite an accumulating literature on SOCS2 epigenetic silencing in cancer of a number of organs, little direct evidence to-date indicates a direct role for SOCS2 in colon cancer or cancer of other organs. Thus, elucidating the role of SOCS2 in intestinal tumorigenesis was a key objective of the studies described here.

N. Hypotheses tested by the studies presented in this dissertation

- I. IRS-1 protects against irradiation-induced apoptosis in the intestinal crypt stem cell zone. Studies testing this hypothesis are described in Chapter II.
- II. IRS-1 promotes intestinal adenoma number and size in the $Apc^{Min/+}$ model of intestinal polyposis. Chapter II also describes studies testing this hypothesis.
- III. SOCS2 limits the growth-promoting actions of GH in the intestine and prevents

the formation of GH-induced aberrant lesions in intestine. These studies are described in Chapter III.

- IV. SOCS2 limits sporadic and/or inflammation-associated lesions in intestine. These studies are tested in Chapter IV.

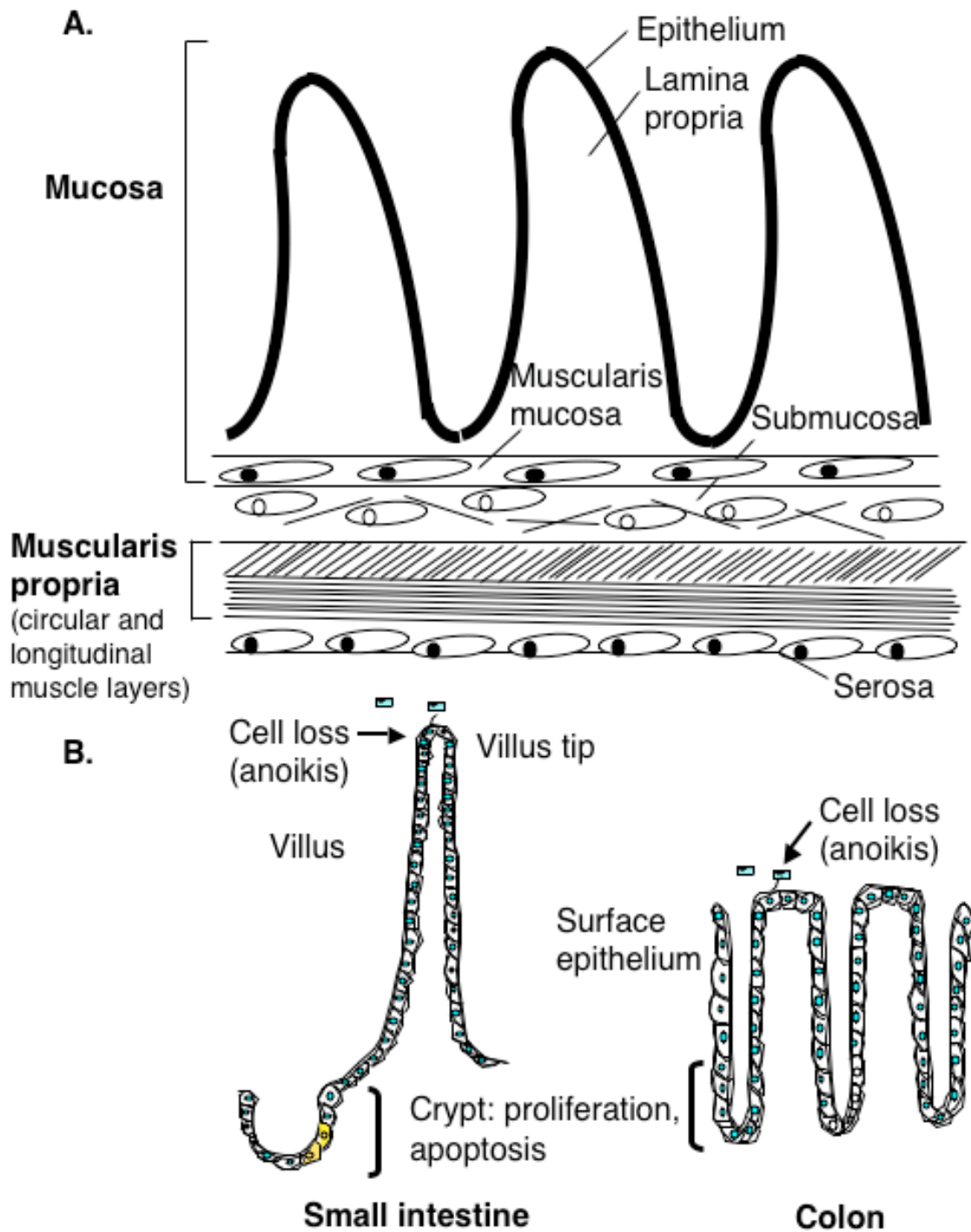


Figure 1.1. Structure of the gastrointestinal tract A. Schematic shows the layers of the gastrointestinal tract wall. B. Schematic shows the architecture of the small (left) and large (right) intestine.

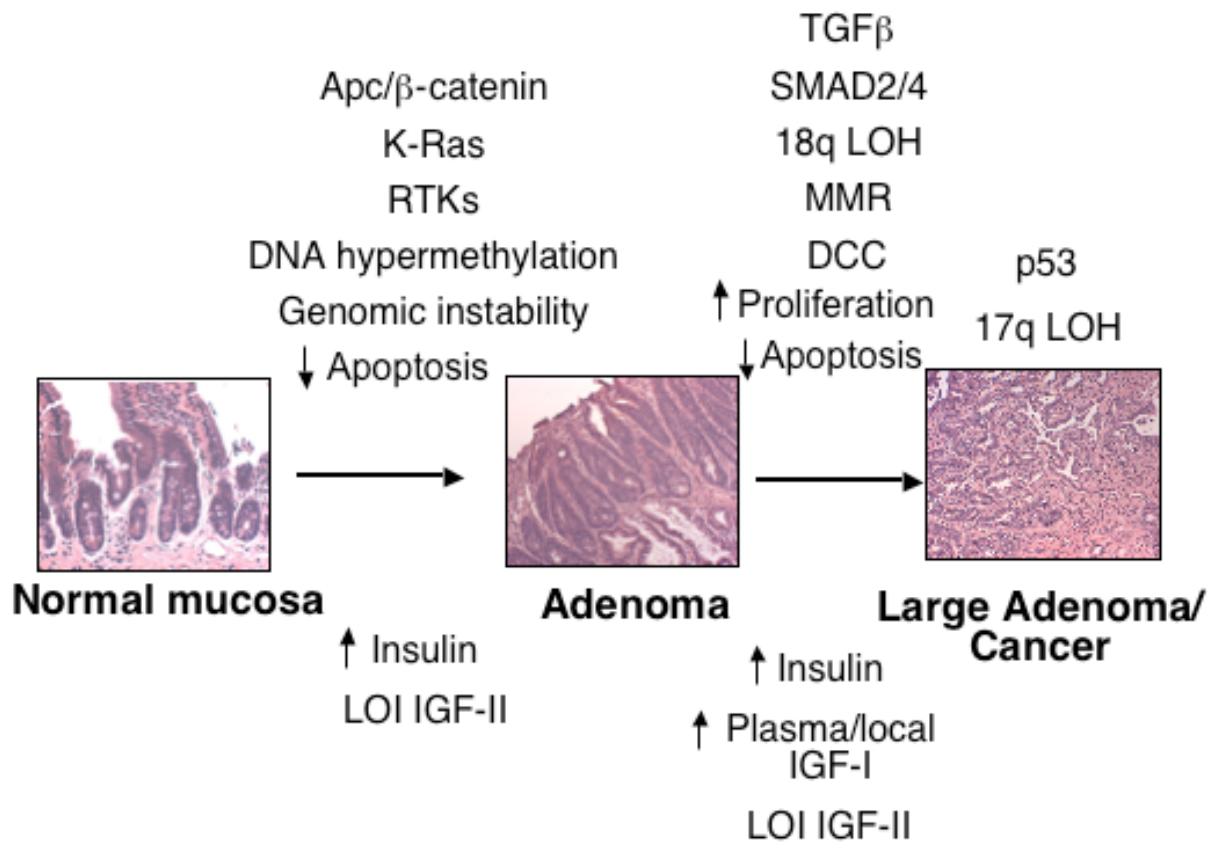


Figure 1.2. Adapted from the ‘Vogelstein model of carcinogenesis.’ Common contributing factors in colon cancer are noted at the top, with the role of the IGF system shown at the bottom.

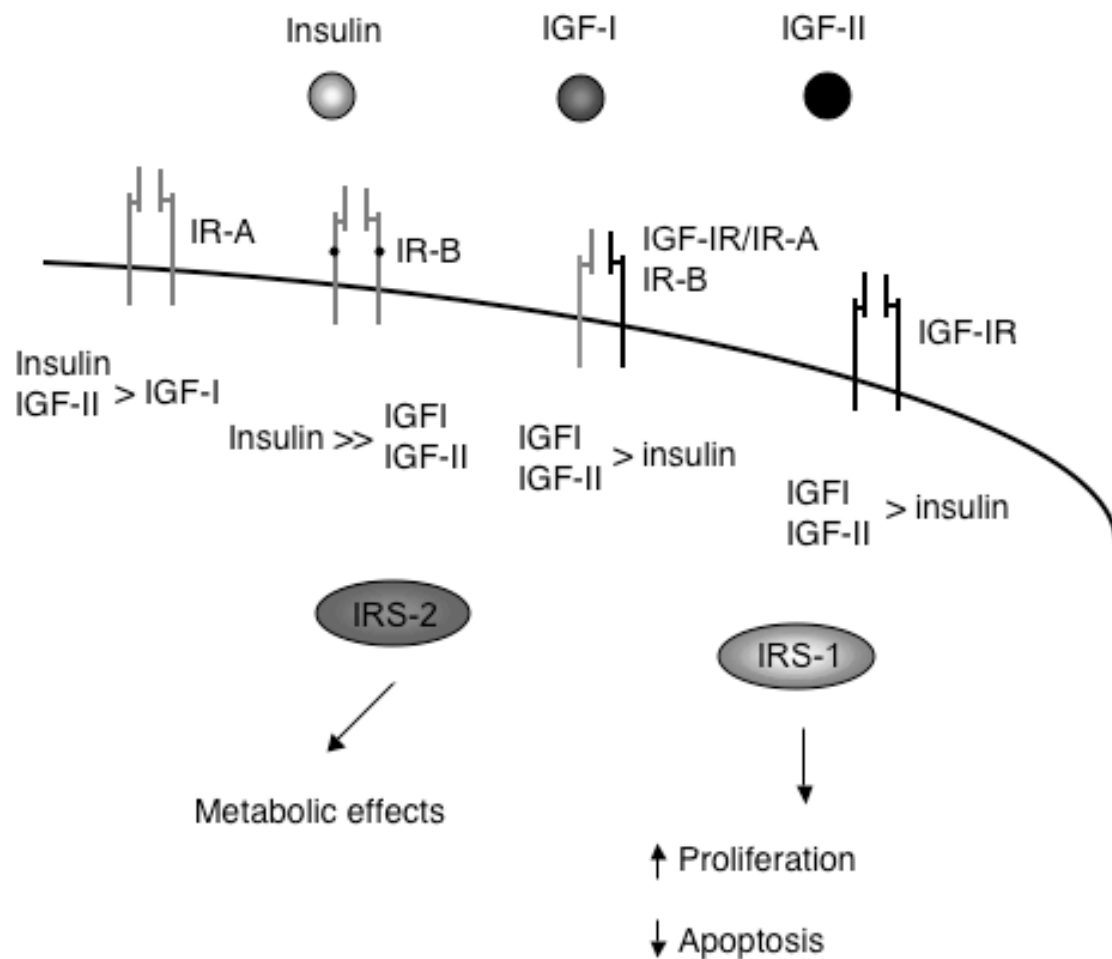


Figure 1.3. The IGF system. Schematic illustrates the IGF system, including ligands, receptors and relative binding affinities noted under each receptor.

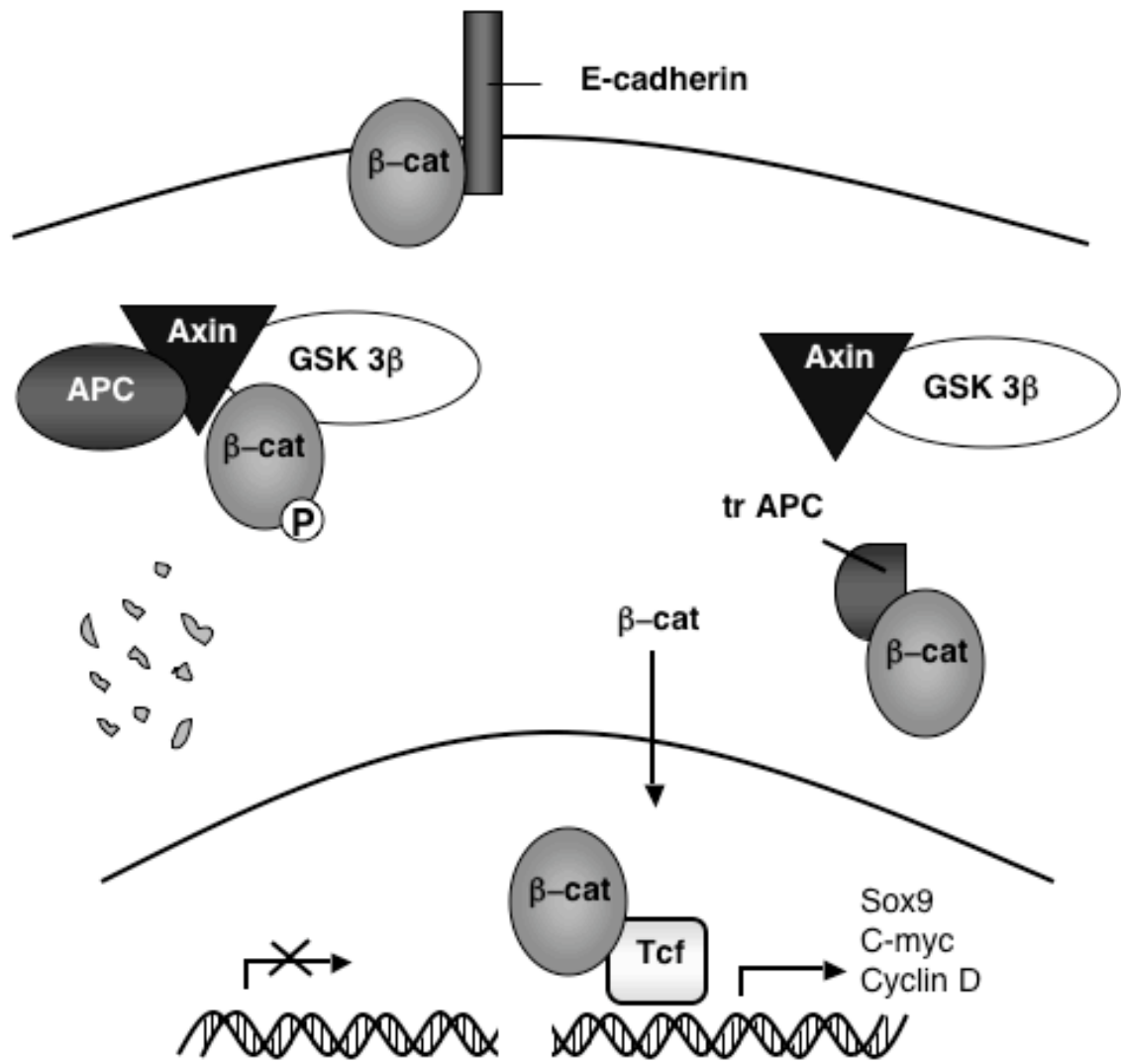


Figure 1.4. β -catenin signaling. Schematic shows the regulation of cellular β -catenin, illustrating its role at the membrane, its degradation in the cytoplasm, and its accumulation when the *Apc* gene is truncated. When β -catenin is allowed to accumulate, it translocates into the nucleus, where it functions in transcription of Wnt targets and various cell cycle progression genes, including Sox9, Myc, and Cyclin D.

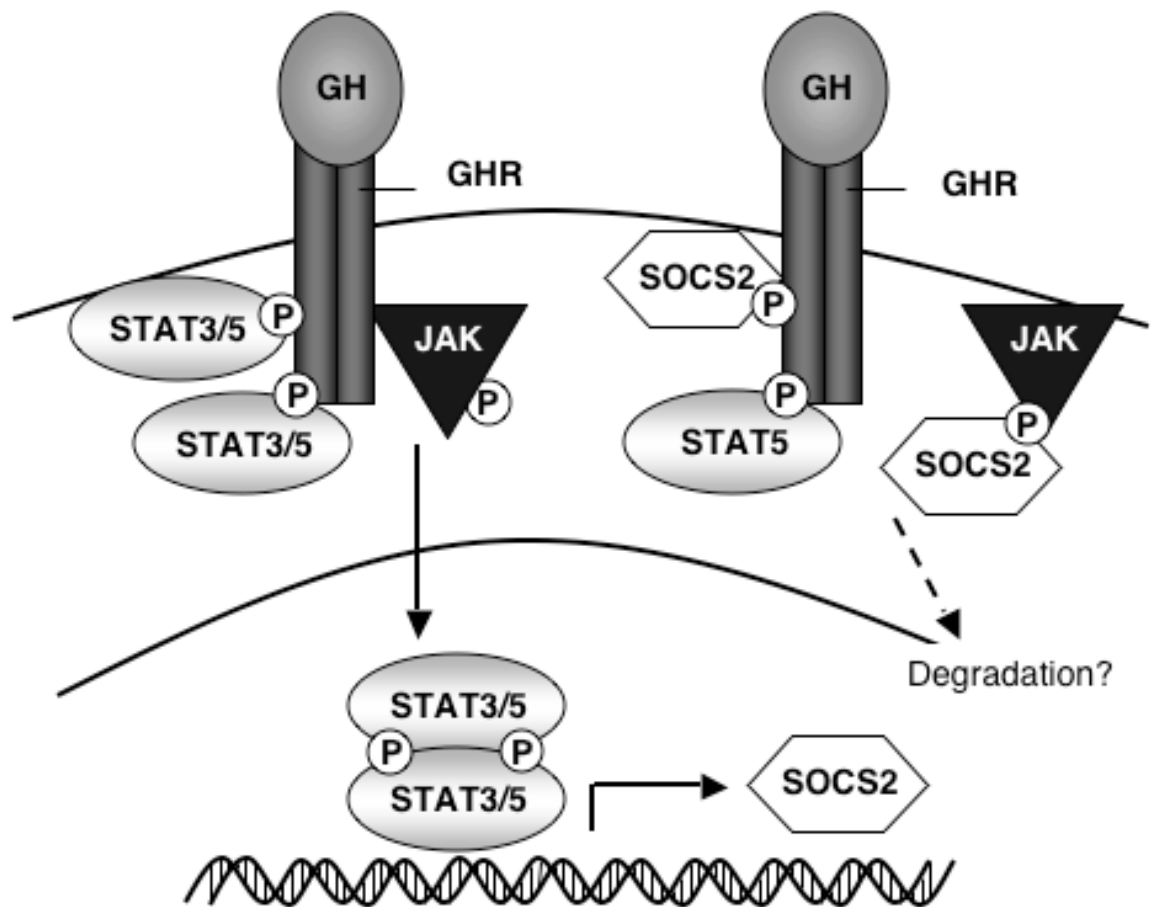


Figure 1.5. GH and SOCS2 signaling. Diagram illustrates GH signaling through the JAK/STAT pathway, leading to transcription of SOCS2, and the putative mechanisms of inhibition by SOCS2.

CHAPTER II

IRS-1 DEFICIENCY PROMOTES APOPTOSIS IN THE PUTATIVE INTESTINAL CRYPT STEM CELL REGION, LIMITS APC^{MIN/+} TUMORS, AND REGULATES SOX9

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A. Introduction

The epithelial lining of the small and large intestine is constantly renewed as a result of continuous proliferation of putative intestinal stem cells and progenitor cells within the crypts (11, 172). While the identity of intestinal stem cells is not conclusively defined, current evidence suggests that they reside near the base of the crypts (11, 172). As well as continuous proliferation, there are small but significant levels of spontaneous apoptosis in the crypts. Genotoxins such as gamma irradiation lead to major increases in crypt apoptosis, and this effect is particularly pronounced in the region of the putative crypt stem cells and progenitors (150). The radiosensitivity of intestinal stem cells is thought to protect against tumor development by reducing the probability of clonal expansion of genetically damaged stem cells (6, 150). A better understanding of the key modulators of irradiation-induced apoptosis could provide critical insight into potential cancer risk factors or point to new therapies. In animal models, insulin-like growth factors (IGFs) reduce spontaneous or irradiation-induced crypt apoptosis, with effects being especially potent in the putative crypt stem cell region (215). Human studies show that low rates of spontaneous apoptosis in normal intestinal crypts predict increased risk of precancerous adenomas in the colon (85, 110). Multiple studies have linked increased plasma or tissue IGFs to increased risk of intestinal cancer in humans (46, 56, 166, 221). Our recent studies indicate that high but within the normal range levels of insulin in humans correlate strongly with both low apoptosis in intestinal crypts and increased adenoma risk (15, 85).

This study focuses on insulin receptor substrate-1 (IRS-1), because it is a key mediator of the actions of both insulin and the IGFs and lies downstream of the insulin and

IGF-I receptors (161). Considerable evidence indicates that IRS-1 is a primary mediator of the anti-apoptotic or trophic actions of insulin and the IGFs (161, 213). IGFs and insulin can also activate IRS-2, but findings in IRS-1 and IRS-2 null mice suggest preferential roles of IRS-1 in growth and IRS-2 in metabolism. IRS-1 null mice exhibit reduced body growth and reduced growth of several organs including the intestine (145), while IRS-2 null mice are normal size but develop type 2 diabetes (5, 212).

Accumulating evidence links IRS-1 to cancer. A G972R polymorphism in the IRS-1 gene significantly increased risk of colorectal cancer (187). IRS-1 is overexpressed or constitutively active in a number of cancers and cancer cell lines, including colon cancer cell lines (24, 91, 134). Recent studies demonstrate that mice with mammary gland-specific IRS-1 overexpression develop spontaneous metastatic mammary tumors (35). Whether reduced expression of endogenous IRS-1 can protect against cancer of any organ, including colon cancer, remains unknown. The present study tested the hypothesis that reduced levels of endogenous IRS-1 promote apoptosis of genetically-damaged crypt stem or progenitor cells in the intestine and protect against spontaneous intestinal adenoma. Our studies focused on the role of IRS-1 in spontaneous intestinal tumors driven by excessive activation of β -catenin-mediated transcription, since accumulating evidence suggests that the IGF-I receptor activates β -catenin via IRS-1 dependent pathways (26, 35, 147, 222).

$Apc^{Min/+}$ mice have a truncation in *Apc* (adenomatous polyposis coli), a gene mutated in many human intestinal cancers (226). $Apc^{Min/+}$ mice spontaneously develop tens to hundreds of adenomas in small intestine and some adenomas in colon (127). Adenomas have aberrant intracellular and nuclear accumulation of β -catenin, which is normally degraded by an APC-containing complex (228). β -catenin acts in concert with Tcf/Lef transcription

factors to activate genes that regulate crypt cell proliferation and survival (76). By crossbreeding, we developed $Apc^{Min/+}$ mice with $IRS-1^{+/+}$, $IRS-1^{+/-}$, and $IRS-1^{-/-}$ genotypes to determine if partial or absolute $IRS-1$ deficiency reduces susceptibility to spontaneous intestinal tumors driven by β -catenin.

Defining IGF-IR/ $IRS-1$ regulated biomarkers of intestinal stem cells could be relevant to defining mechanisms or biomarkers for increased intestinal cancer risk due to elevated levels of IGF or insulin. The Sry-related High Mobility Group (HMG)-box DNA binding protein (Sox9) is a β -catenin/Tcf gene target implicated in fate-determination of stem cells in the pancreas, neural ectoderm, cartilage, and gonads (1, 12, 23, 28, 104, 180). In intestine, recent studies demonstrate that Sox9 localizes to the nucleus of proliferating crypt cells, particularly in regions where stem or progenitor cells reside (12). Sox9 also represses expression of intestinal differentiation markers Muc2 and Cdx2, suggesting a role for Sox9 in maintenance of an undifferentiated stem or progenitor phenotype (12). Sox9 is highly expressed in human colon cancer cell lines and in intestinal tumors (12), indicating a possible role in tumorigenesis. Studies in chondrocytes indicate that Sox9 expression is induced by IGF-I (181), but whether this occurs in other cell types or is $IRS-1$ dependent is unknown. Our studies therefore explored whether changes in the number of Sox9 positive cells or Sox9 expression levels in crypts were associated with the effects of $IRS-1$ genotype on adenoma susceptibility in the $Apc^{Min/+}$ mice, and if increased $IRS-1$ expression in intestinal epithelial cells alters Sox9 expression *in vitro*.

B. Materials and Methods

Mouse models

Mice heterozygous for targeted disruption of the IRS-1 gene (IRS-1^{+/-}) on a pure-bred C57BL/6 background were previously described (5) and provided by Dr. Ronald Kahn. IRS-1^{+/-} males and females were bred to derive sex-matched littermates with 2 (IRS-1^{+/+}), 1 (IRS-1^{+/-}), or 0 (IRS-1^{-/-}) functional IRS-1 alleles for studies of spontaneous and irradiation-induced apoptosis. Apc^{Min/+} male mice on the C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME) and crossed with female IRS-1^{+/-} mice. IRS-1^{+/-} males with the Apc^{Min/+} mutation were then crossbred with IRS-1^{+/-} females. This two-step crossbreeding yielded Apc^{Min/+} and wildtype (WT) mice with 2, 1, or 0 functional IRS-1 alleles. Genotyping was performed on tail DNA using primers described previously (145, 163). Studies in Apc^{Min/+} mice were largely confined to females since males were used primarily for breeding. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of North Carolina. Study protocols were in compliance with the Guide for the Care and Use of Laboratory Animals published by the NIH.

Irradiation and tissue collection

Mice (50-75 days old) received 5 Gy of whole body irradiation delivered at 1 Gy/min with a ¹³⁷Cs source and were killed four hours after irradiation, a time of peak irradiation-induced apoptosis (152). Non-irradiated, genotype- and sex-matched littermates were used as controls. Mice were anesthetized using sodium pentobarbital (200 µg/g body weight), and the abdomen was opened by mid-line incision. Two pieces of jejunum (each 0.5-cm long)

were fixed in 10% formalin for four hours, dehydrated in 70% ethanol, and embedded in paraffin.

Analysis of apoptosis in irradiated mice

Apoptosis was quantified in hematoxylin and eosin (H&E)-stained 4 μ m sections of jejunum as previously described (215). Briefly, morphological identification of apoptotic crypt cells was based on nuclear margination, chromatin and cytoplasmic condensation, shrinkage from neighboring cells, and the formation of apoptotic bodies due to nuclear and cytoplasmic fragmentation. Apoptosis in well-oriented crypts was recorded as previously described (149), with cells at the base designated as position 1. All scoring was performed twice by a single investigator unaware of the mouse genotype or treatment. Apoptosis was expressed as the mean number of apoptotic cells per crypt or the percent of apoptotic cells at each position from the crypt base relative to the total number of cells counted.

Tissue collection and evaluation of tumors in $Apc^{min/+}$ mice

Mice were studied at 18-20 weeks of age, when they were anesthetized and blood collected by cardiac puncture. Since $Apc^{Min/+}$ mice develop severe anemia as disease progresses (67), hematocrit was measured as an indirect marker for tumor load and disease severity. Hematocrit was assayed by the Animal Clinical Chemistry Facility in the Pathology Department, University of North Carolina at Chapel Hill. The entire small intestine and colon were dissected, and tumor load was assessed as in prior studies (163). Briefly, tumor number was counted under a Leica dissecting scope for the entire small intestine and colon, and tumor diameter was measured using an in-lens micrometer. Tumor load was calculated

by multiplying the total tumor number and mean size for small intestine and colon of each mouse. Intestinal segments were rolled into swissrolls, paraffin-embedded, and sectioned at 7 μ m. The presence and morphology of adenomas were then confirmed by H&E staining and β -catenin immunostaining. Elevated cytoplasmic and nuclear β -catenin is considered a reliable hallmark of adenomatous lesions in *Apc*^{Min/+} mice (163).

Localization of β -catenin and Sox9

β -catenin immunostaining was performed as previously described (163). A primary mouse monoclonal β -catenin antibody (BD Transduction Laboratories, #610154, Franklin Lakes, NJ) was used together with a biotinylated anti-mouse IgG from the MOM kit using a mouse on mouse blocking protocol (Vector Laboratories, Burlingame, CA). Peroxidase was visualized under brightfield using a Nikon Microphot FXA microscope. For Sox9, a rabbit polyclonal antibody (Chemicon, AB35535, Temecula, CA) was used at 1:100, followed by peroxidase or a Cy3 labeled secondary antibody at 1:100. Fluorescent images were taken under a Zeiss 510 laser-scanning confocal microscope.

Northern blot hybridization of Sox9 mRNA

Total mRNA was extracted from 2 cm of proximal ileum using TriZol reagent (Invitrogen, Carlsbad, CA) and manufacturer's instructions. Northern blot hybridization was performed as previously described (137), using a [³²P]CTP-labeled Sox9 DNA template from pCMV-SPORT6 (Open Biosystems, Huntsville, AL). Blots were reprobed for constitutively expressed 18S ribosomal RNA, and hybridization signals were visualized using a PhosphorImager (Typhoon, GE Healthcare, Piscataway, NJ) as previously described (121).

Effects of IRS-1 on Sox9 expression

Primary cultures of intestinal epithelial cells do not remain viable for sufficient times after isolation to study the role of IRS-1 in Sox9 expression. We therefore used the non-transformed intestinal epithelial cell line, IEC-6, to assess whether IRS-1 overexpression altered Sox9 expression. IEC-6 cells were grown as previously described (121). IEC-6 cells were infected with either IRS-1 adenovirus (Adex1CAIRS-1wt) (199) or an empty virus (Gene Therapy Center Virus Vector Core, University of North Carolina at Chapel Hill) at 10^8 particles/mL 18 hours prior to harvesting. Total cell lysates were prepared, and equal amounts of protein subject to SDS-PAGE as described (185). Blots were incubated in Sox9 antibody (1:1000), followed by an IRDye 800CW goat anti-rabbit secondary antibody (1:5000, #926-32211, LI-COR Biosciences, Lincoln, NE). β -actin was used as a loading control. Blots were imaged using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

Statistical analyses

Values are expressed as mean \pm SEM. ANOVA was used to compare levels of apoptosis in irradiated IRS-1^{+/+} versus IRS-1^{+/-} or IRS-1^{-/-} littermates and post hoc analyses between each genotype were performed by Fisher's protected least squares difference comparisons. Wilcoxon signed-rank test was used to compare tumor number, size, and load in IRS-1^{+/+}/Min and IRS-1^{+/-}/Min littermates. IRS-1^{-/-}/Min mice were born at lower than expected Mendelian frequency, and it was not possible to restrict comparisons to littermates. Therefore, a student's *t*-test was used to compare tumor number, size, and load in IRS-1^{-/-}

/Min mice versus age- and sex-matched IRS-1^{+/+}/Min or IRS-1^{+/-}/Min mice. Two-way ANOVA was used to determine whether there was significant effect of IRS-1 or Min genotype on Sox9 expression and if IRS-1 and Min genotype show a significant interaction. $p < 0.05$ was considered to be statistically significant.

C. Results

IRS-1 gene-dosage effects on irradiation-induced apoptosis of crypt stem cells

Gamma irradiation induces genetic damage and apoptosis in the intestinal crypts (215). Disruption of one or both IRS-1 alleles significantly increased irradiation-induced apoptosis (Figure 1a). After irradiation, IRS-1^{+/-} mice had significantly higher crypt apoptosis (2.1 ± 0.17) compared with IRS-1^{+/+} littermates (1.3 ± 0.09 $p=0.019$), and IRS-1^{-/-} mice had even higher rates of apoptosis (2.5 ± 0.30 $p=0.002$).

Prior evidence indicates that small intestinal crypt stem and progenitor cells are located primarily at cell positions 3-5 from the base of the crypts, otherwise known as the stem cell region (150, 215). This region lies immediately above Paneth cells which are located at the crypt base (cells 1 and 2 relative to the base of the crypts). Recent studies indicate that stem cells may also lie near the base of the crypts interspersed with Paneth cells (7). In IRS-1^{-/-} mice, irradiation-induced apoptosis was significantly increased at positions 2-7 from the crypt base. In IRS-1^{+/-} mice, apoptosis was increased significantly at positions 4 and 6 (Figure 1b). Thus, partial or absolute IRS-1 deficiency promotes apoptosis of genetically-damaged crypt cells, with most dramatic effects within the putative stem/progenitor cell region.

IRS-1 gene-dosage effects on tumor number in $Apc^{Min/+}$ mice

$Apc^{Min/+}$ mice with disruption of just one IRS-1 allele (IRS-1^{+/-}/Min) had a significant 24.5 ± 13.0% reduction in the number of small intestinal adenomas compared with sex-matched IRS-1^{+/+}/Min littermates (p=0.017, Figures 2 and 3). This is despite the fact that loss of one IRS-1 allele has no discernible effect on growth or size of small intestine (145). IRS-1^{-/-}/Min mice had a 55.5 ± 13.2% reduction in the number of small intestinal adenomas compared with IRS-1^{+/+}/Min mice, an effect which is greater than the 25% reduction in small intestine mass observed in IRS-1^{-/-} mice (145). In colon, IRS-1^{+/-}/Min showed a 46.9 ± 15.2% decrease, and IRS-1^{-/-}/Min a 61.9 ± 19.0% decrease in adenoma number compared with IRS-1^{+/+}/Min mice (Figure 3). There was a small but significant decrease in adenoma size in small intestine of IRS-1^{-/-}/Min versus IRS-1^{+/+}/Min, but adenoma size did not differ between IRS-1^{+/-} and IRS-1^{+/+} mice. Partial and absolute IRS-1 deficiency significantly decreased tumor load (number x size) in both small intestine and colon (Figure 3). Hematocrit is considered a useful indirect measure of tumor load in $Apc^{Min/+}$ mice (144, 209). Hematocrit was reduced in IRS-1^{+/+}/Min and IRS-1^{+/-}/Min mice compared to mice without the $Apc^{Min/+}$ mutation (Figure 4). In IRS-1^{-/-}/Min mice, hematocrit was near normal and significantly higher than in IRS-1^{+/+}/Min mice.

β-catenin accumulation is a hallmark of adenomas in $Apc^{Min/+}$ mice because loss of the second wildtype *Apc* allele leads to aberrant accumulation of β-catenin in the cytosol and nucleus. β-catenin immunostaining revealed that β-catenin was localized to the intercellular junctions of normal crypts as previously reported (163) (Figure 5). Tumor staining revealed accumulation of β-catenin in the cytoplasm and nucleus of adenomas of all genotypes

(Figure 5), confirming adenomatous phenotype.

Reductions in Sox9 positive cells and Sox9 expression in IRS-1 deficient mice

Sox9 is a reported biomarker of crypt stem or progenitor cells, is a target of β -catenin/TCF-activated transcription, and is expanded in human tumors. We therefore examined whether IRS-1 deficiency in $Apc^{Min/+}$ mice reduced the number of Sox9 positive cells or Sox9 mRNA. As shown in Figure 6a, immunofluorescence revealed that Sox9 expression was restricted to cells at the base of normal crypts. In $IRS-1^{-/-}/Min$ crypts, we observed an obvious reduction in both the number and staining intensity of Sox9 positive cells, and cell number was quantified in Figure 6b. Immunohistochemical Sox9 staining with peroxidase-labeled secondary antibodies was used to better visualize the location of Sox9 positive cells, which were found near the base of the crypts and do not appear to colocalize with granular Paneth cells at the crypt base (Figure 6c). Note the presence of Sox9 positive nuclei in cells lying between Paneth cells in $IRS-1^{+/+}/Min$ but not $IRS-1^{-/-}/Min$ (Figure 6c). This is the location of multipotent Gpr49/Lgr5 positive stem cells recently reported by the Clevers group (7). Thus, loss of IRS-1 appears to reduce the numbers of Sox9 positive crypt stem and progenitors. Evaluation of Sox9 mRNA in small intestine of $IRS-1^{+/+}$ and $IRS-1^{-/-}$ mice with or without the $Apc^{Min/+}$ mutation provided quantitative evidence that IRS-1 deficiency leads to significant reductions in Sox9 expression (Figure 7a and 7b).

Sox9 expression correlates with IRS-1 levels in cultured cells

To examine more directly if IRS-1 regulates Sox9 expression, Sox9 levels were examined in IEC-6 cells after infection with empty or IRS-1 expressing adenovirus. IEC-6

cells show constitutive Sox9 expression, and IRS-1 adenovirus significantly up-regulated Sox9 protein levels by 1.8 ± 0.1 fold (Figure 8).

D. Discussion

Our studies provide novel evidence that intestinal crypt epithelial cells show gene dosage dependence on IRS-1 for protection from irradiation-induced apoptosis. This finding is noteworthy in light of recent evidence in humans that decreased levels of crypt apoptosis are associated with increased risk of intestinal adenoma (110), that high but within the normal range insulin levels correlate with both low apoptosis and adenoma risk (85), and that IRS-1 polymorphisms predict colon cancer risk (187). IRS-1^{+/-} mice exhibit only a ~50% reduction in IRS-1 expression and loss of one IRS-1 allele has little or no effect on body or intestinal growth (145, 184). Thus, our findings that IRS-1^{+/-} mice show significant increases in irradiation-induced apoptosis, with effects most prominent in the putative stem cell region, indicate that even small variations in levels of endogenous IRS-1 significantly impact on survival of genetically-damaged stem cells.

Survival of genetically damaged stem or progenitor cells is considered a key early event in the development of intestinal tumors. Consistent with this concept, partial or absolute IRS-1 deficiency in mice carrying the Apc^{Min/+} mutation led to reduced tumor number relative to IRS-1^{+/+}/Apc^{Min/+} mice. The more dramatic effect of IRS-1 deficiency on tumor number than size suggests a predominant role of IRS-1 in initiation or survival of early stage tumors than rate of tumor growth. Our demonstration that reductions in endogenous IRS-1 limit tumor development is significant since most evidence linking IRS-1 and cancer

involves overexpression systems and, with a few exceptions, largely *in vitro* studies. Given our findings in mouse models, it could be of considerable interest to establish if the IRS-1 polymorphism linked to colon cancer risk (187) affects IRS-1 expression levels, or if patients with increased adenoma risk show high IRS-1 expression. Such studies to translate our basic science findings to the human population have been initiated in our laboratory.

Tumorigenesis mediated by the $Apc^{Min/+}$ mutation is initiated by loss of the second functional *Apc* allele, leading to cytosolic and nuclear accumulation of β -catenin and activation of β -catenin-driven oncogenes (163). Emerging evidence based in colon cancer cell lines or based on overexpression of IRS-1 in mouse embryonic fibroblasts or mammary gland *in vivo* indicate that IGF/IRS-1 pathways stabilize or activate β -catenin, and that there may be direct interactions between IRS-1 and β -catenin (26, 35, 147). Our findings that IRS-1 deficiency limits β -catenin driven intestinal tumorigenesis *in vivo* provide, to our knowledge, the first evidence that endogenous IRS-1 impacts on tumor promoting activity of β -catenin. Qualitatively, we noted that some intestinal adenomas of IRS-1^{-/-}/Min mice appeared to show less intense or obvious nuclear β -catenin immunostaining than in IRS-1^{+/-}/Min adenomas. This provides evidence that loss of endogenous IRS-1 may limit nuclear accumulation of β -catenin. Attempts to verify this by more quantitative biochemical assays proved problematic due to variability in total and nuclear β -catenin, even within genotypes.

More direct evidence for effects of IRS-1 on β -catenin transcription pathways stems from findings that expression of Sox9, a newly recognized β -catenin/Tcf target, is reduced in crypts of IRS-1 deficient $Apc^{Min/+}$ mice *in vivo* and is induced in an IEC cell line by IRS-1 overexpression. These findings also provide novel evidence linking IRS-1 to Sox9. Sox9 is emerging as a potentially important biomarker of crypt stem or progenitor cells and belongs

to a family of proteins known to maintain stem cells in an undifferentiated state (12). Recent studies show that inactivating the Sox9 gene at a developmental timepoint prior to enterocyte differentiation ablates Paneth cells and, to a lesser extent, goblet cells (9, 125). These findings suggest that Sox9 may play a functional role in stem or progenitor cell potency. Sox9 is also highly expressed in cells within human colon adenocarcinoma (12). Thus, our evidence that IRS-1 regulates Sox9 expression has important ramifications for how IGF/insulin signaling may regulate stem cell survival and cancer risk. The function of Sox9 in cancer is unknown, but one speculation is that Sox9 may mark ‘cancer-associated stem cells.’ Thus, our findings indicate that the link between IRS-1 regulation of Sox9 and genetically-damaged or cancer-associated stem cells will be an interesting avenue of future investigation. Furthermore, examining if Sox9 expression correlates with apoptosis, adenoma risk, or alterations in insulin/IGFs and IRS-1 that predict adenoma risk in humans may provide new insights into the etiology of early stage intestinal cancer.

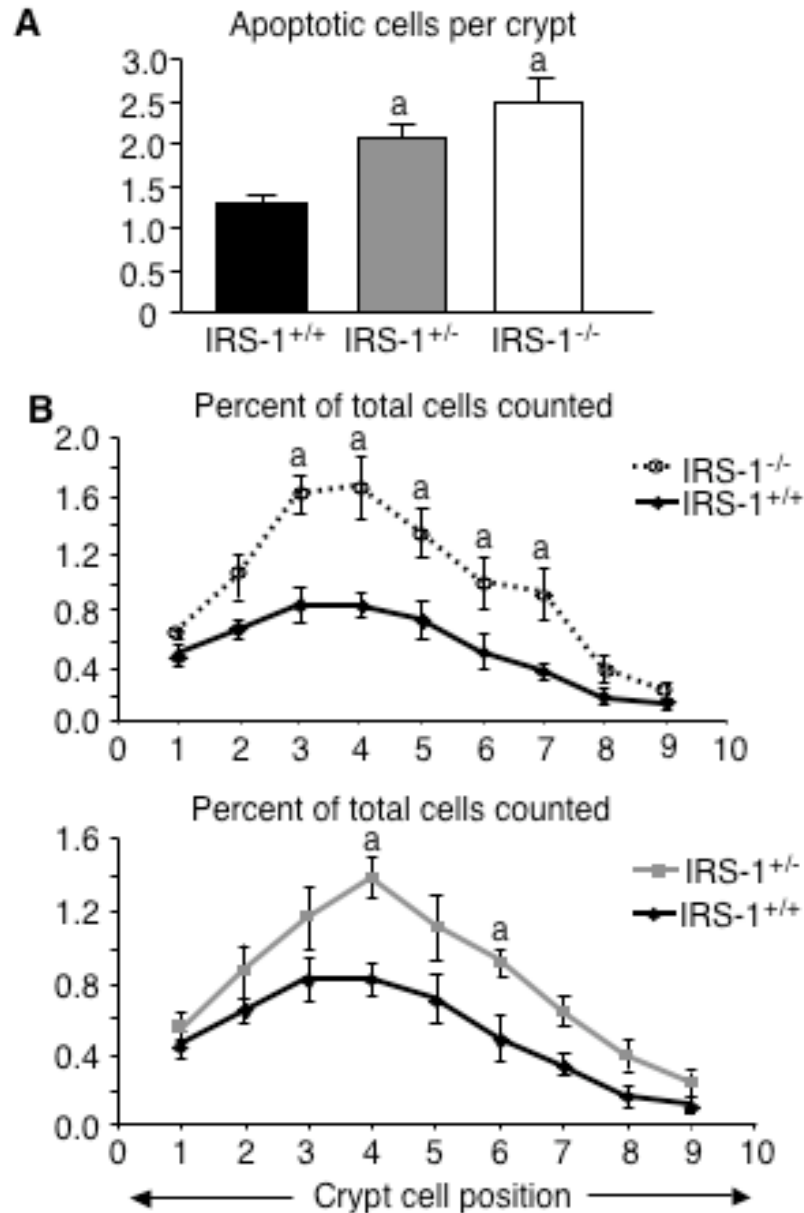


Figure 2.1 Irradiation-induced apoptosis in jejunal crypts of IRS-1^{+/+}, IRS-1^{+/-}, and IRS-1^{-/-} mice. A. Histogram shows the mean number of apoptotic cells per crypt. ^a=*p*<0.05 vs. IRS-1^{+/+}. N=5 for each genotype. B. Graph shows the percent apoptotic cells at each cell position along the jejunal crypt for irradiated IRS-1^{+/+}, IRS-1^{+/-}, and IRS-1^{-/-} mice. Data are expressed as percent apoptotic cells per total cells counted at each location among ≥40 crypts for 5 animals of each genotype. The increase in apoptosis in IRS-1^{+/-} and IRS-1^{-/-} vs. IRS-1^{+/+} is most prominent in the putative stem or progenitor cell region. ^a=*p*<0.05 vs. IRS-1^{+/+}.

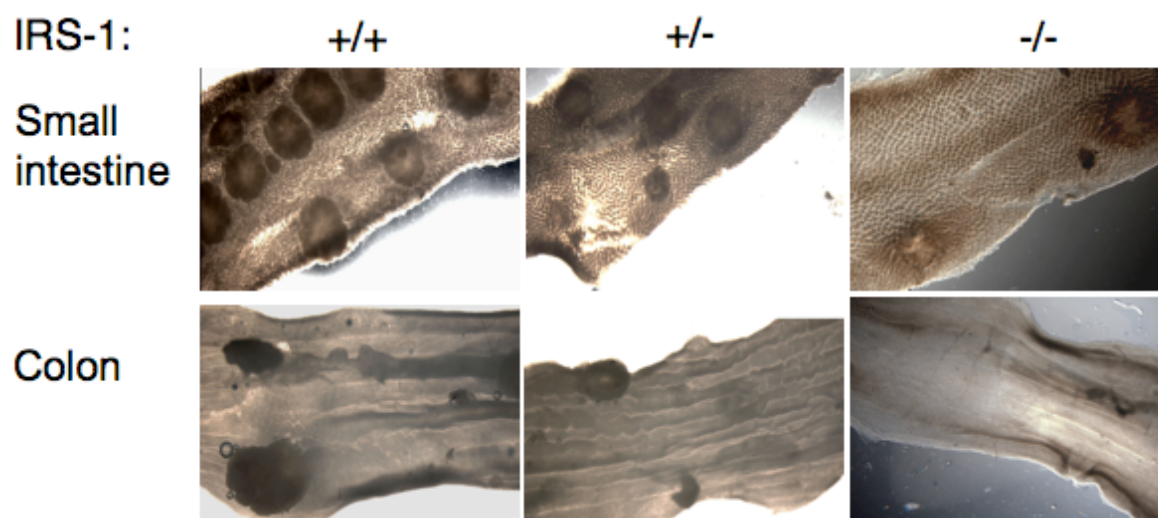


Figure 2.2 Representative images of adenomas in small and large intestine of $Apc^{Min/+}$ mice. Adenomas in small intestine (top) and colon (bottom) of $Apc^{Min/+}$ mice that IRS-1^{+/+}, IRS-1^{+/-}, and IRS-1^{-/-} genotypes are shown. Photographs were taken at 12x under a dissecting scope with white light optics underneath the specimen. Note the decrease in adenomas in IRS-1 deficient mice.

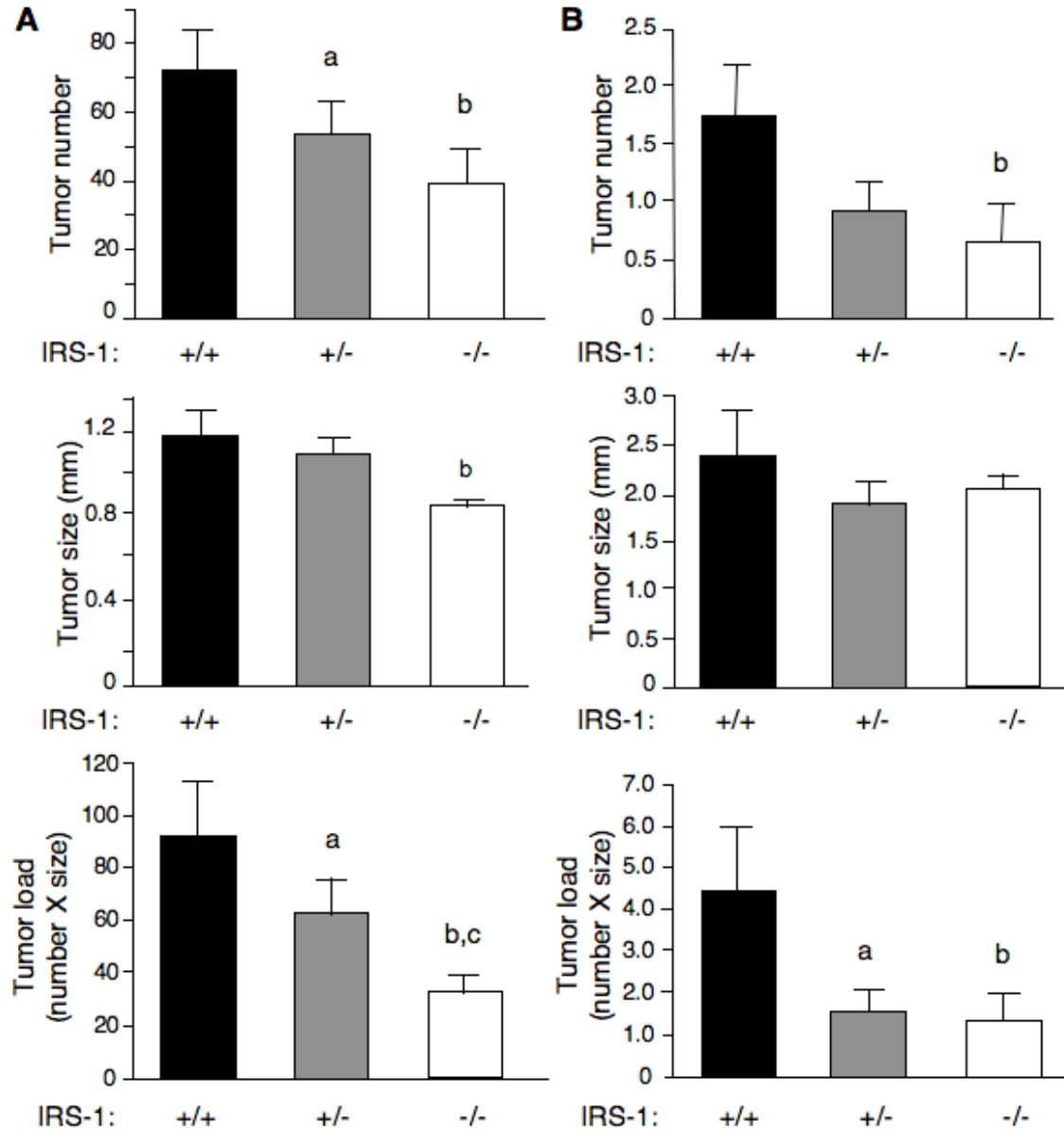


Figure 2.3 Effects of IRS-1 gene disruption on tumors in $Apc^{Min/+}$ mice. Histograms show mean tumor number, size, and load in small intestine (A) and colon (B). $N=10$ for $IRS-1^{+/+}/Min$ and $IRS-1^{+/-}/Min$ littermates, $n=3$ for $IRS-1^{-/-}/Min$ age-matched with $IRS-1^{+/+}/Min$ or $IRS-1^{+/-}/Min$ mice. $a=p<0.05$ for $IRS-1^{+/-}/Min$ vs. $IRS-1^{+/+}/Min$ littermates; b and $c=p<0.05$ for $IRS-1^{-/-}/Min$ vs. age-matched $IRS-1^{+/+}/Min$ or $IRS-1^{+/-}/Min$ mice, respectively.

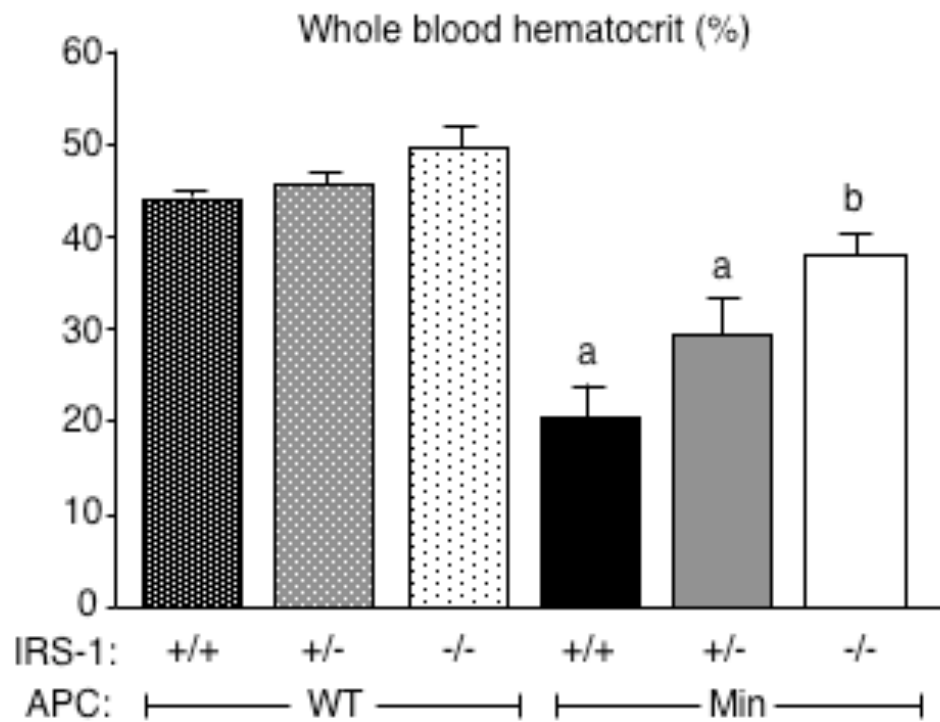


Figure 2.4 Effects of IRS-1 gene disruption on hematocrit in WT or $Apc^{Min/+}$ mice.

Histogram of whole blood hematocrit from IRS-1^{+/+}, IRS-1^{+/-}, and IRS-1^{-/-} mice with and without the Min mutation as indicated. All hematocrit values were at or near normal range in mice lacking the Min mutation (32.8 - 48.0% hematocrit). IRS-1^{+/+}/Min and IRS-1^{+/-}/Min mice had significantly decreased percent hematocrit levels, while IRS-1^{-/-}/Min mice were within the normal range. a=p<0.05 vs. all non-Min genotypes; b=p<0.05 vs. IRS-1^{+/+}/Min.

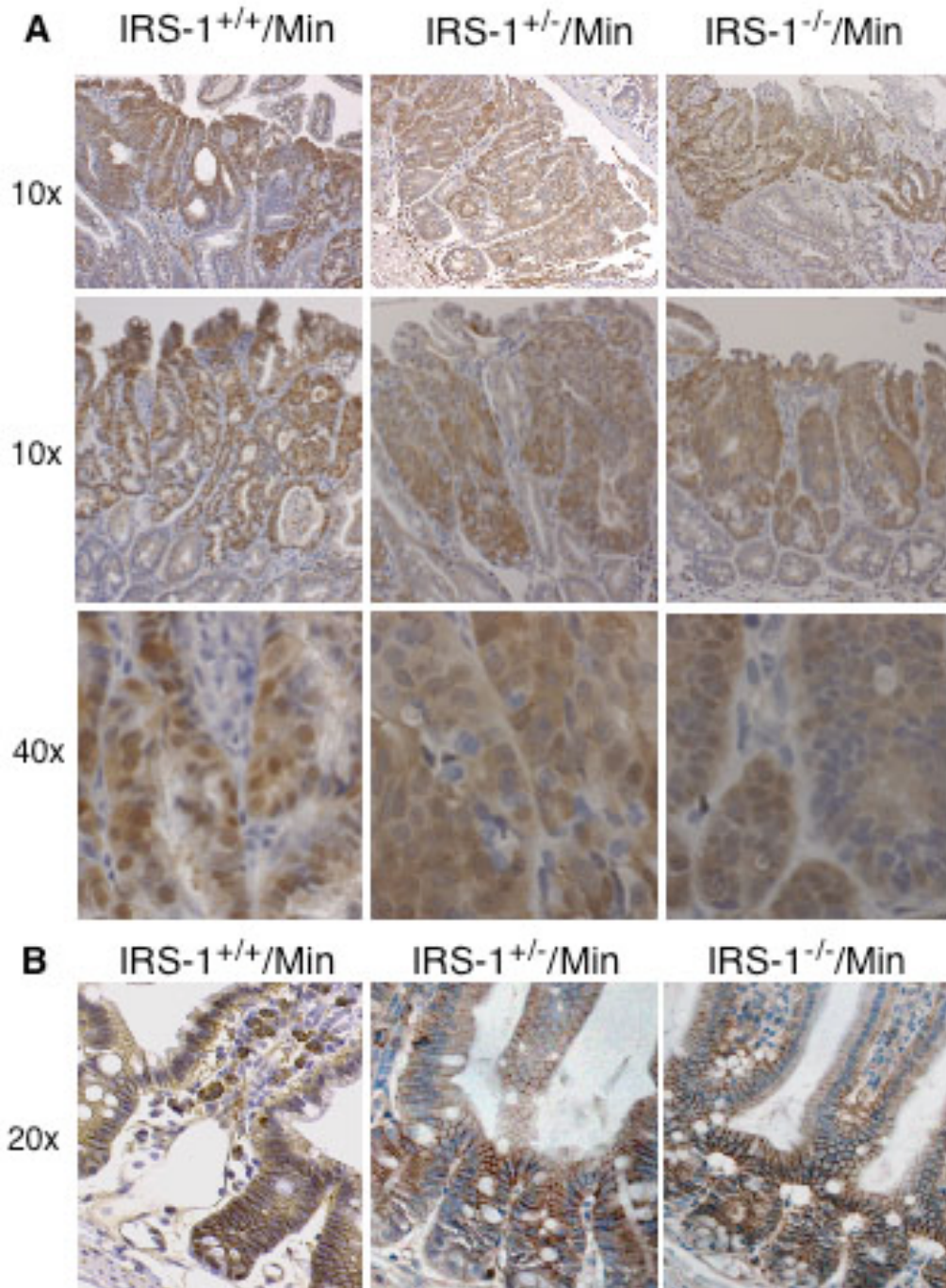


Figure 2.5 β -catenin immunostaining of adenomas and normal mucosa. A. Images show small intestinal adenomas (10x and 40x magnification) in IRS-1^{+/+}/Min, IRS-1^{+/-}/Min, and IRS-1^{-/-}/Min small intestine. Note the accumulated nuclear and cytoplasmic β -catenin in adenomas from all three genotypes. B. In contrast, the normal mucosa (20x magnification) shows prominent localization of β -catenin specifically at the lateral membrane.

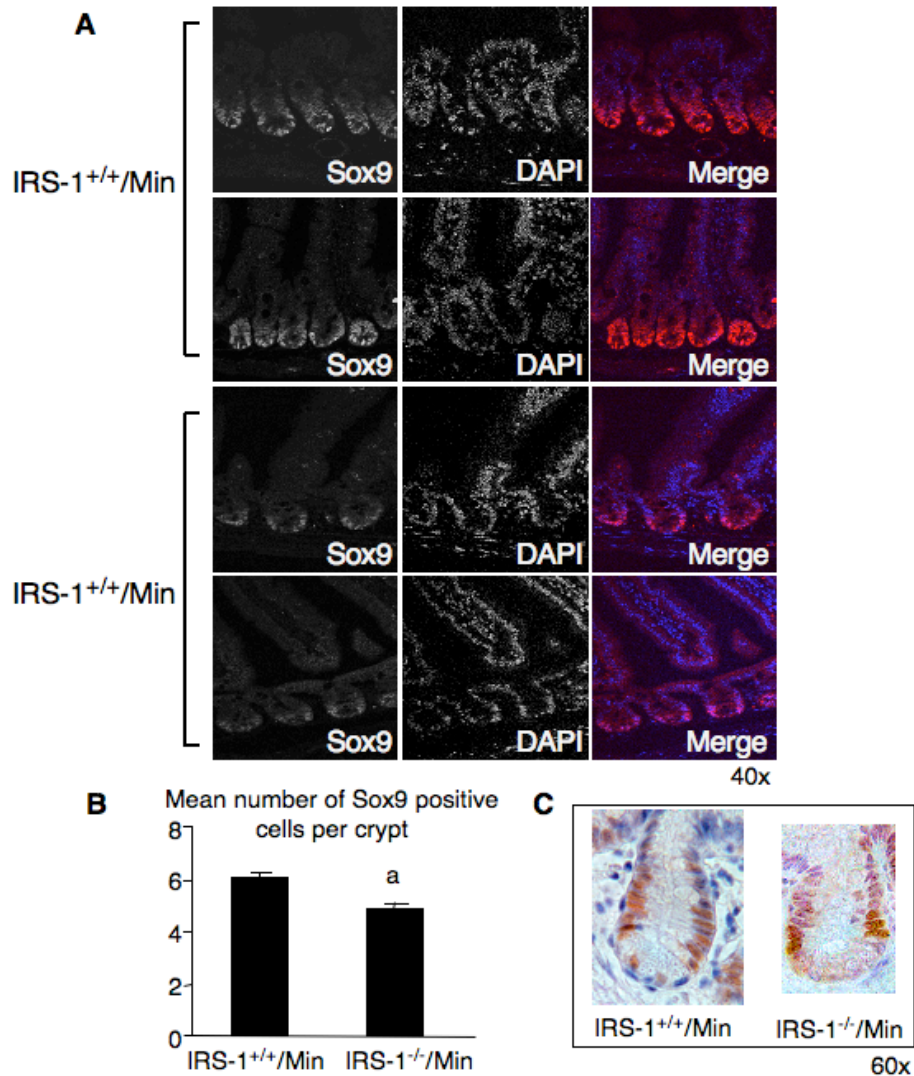


Figure 2.6 Decreased Sox9 expression with IRS-1 deficiency. A. Confocal images showing Sox9, 4',6-diamino-2-phenylindole (DAPI), and merged immunofluorescence in IRS-1^{+/+}/Min and IRS-1^{-/-}/Min small intestine (40x). Representative images are from 2 animals per genotype. B. Histogram shows the mean number of Sox9 positively stained cells for each side of the crypt in IRS-1^{+/+}/Min vs. IRS-1^{-/-}/Min jejunum. ^a $p < 0.05$ vs. IRS-1^{+/+}/Min. C. Sox9 immunohistochemistry of crypts (60x), showing that cells with obvious Sox9 positive nuclei appear distinct from the Paneth cells. Note the nuclear Sox9 in cells intercalating between the Paneth cells in IRS-1^{+/+}/Min, but not IRS-1^{-/-}/Min.

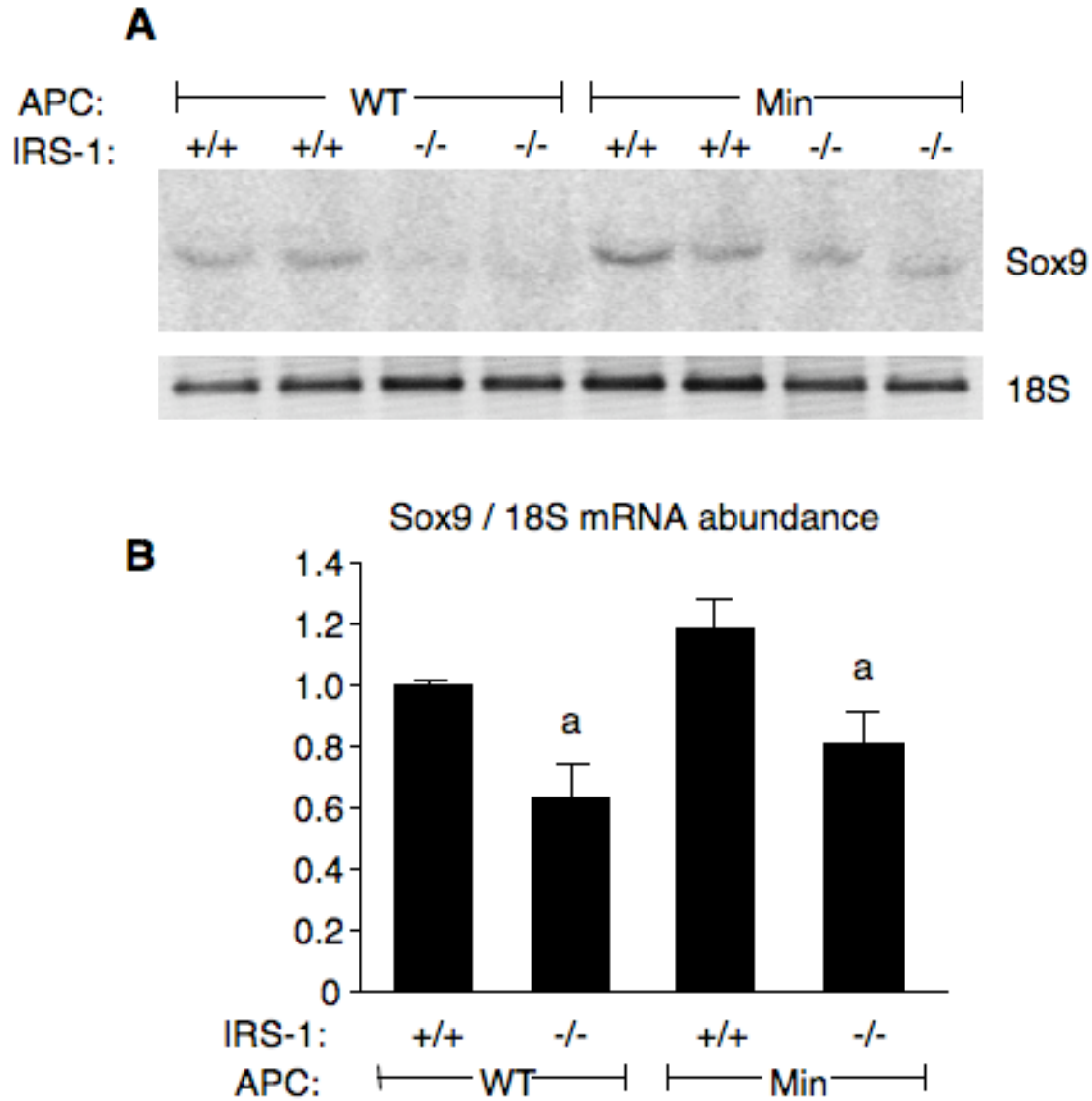


Figure 2.7 Sox9 mRNA expression. A. Representative northern blot of Sox9 and 18S RNAs in ileum of IRS-1^{+/+}, IRS-1^{+/-}, and IRS-1^{-/-} with and without the Apc^{Min/+} mutation. B. Histograms show mean relative abundance of Sox9 mRNA normalized to the 18S loading control. a=p<0.05 IRS-1^{-/-} vs. IRS-1^{+/+} mice. n≥4 for each genotype.

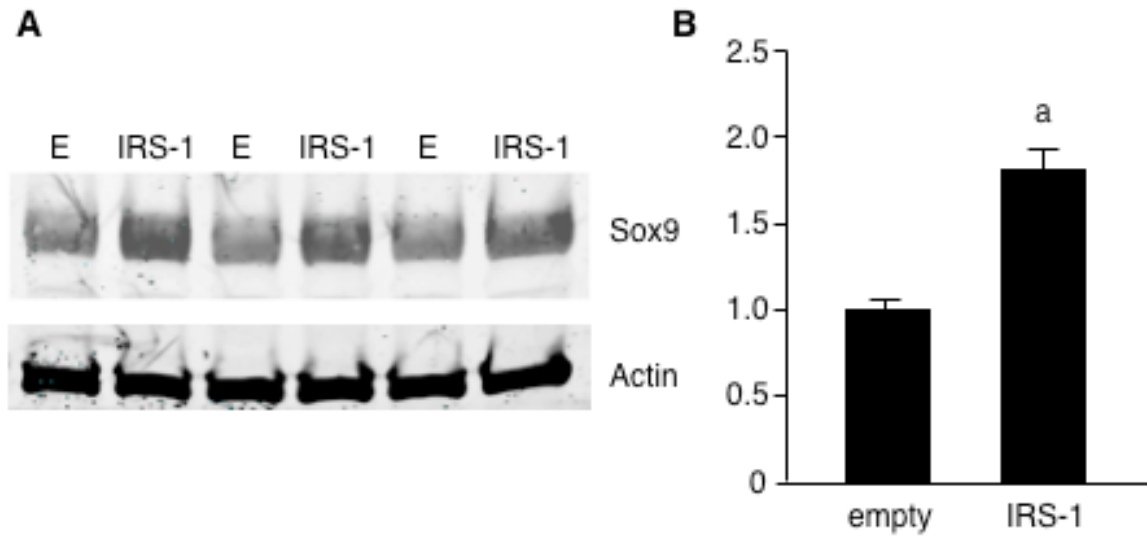


Figure 2.8 Sox9 protein levels are regulated by IRS-1 and IGF-I. A. Western immunoblot for Sox9 or β -actin in IEC-6 cells infected with empty virus (E) or IRS-1 adenovirus. B. Histogram shows Sox9 protein abundance for empty vs. IRS-1 infected IEC-6 cells normalized to actin levels. ^a $p < 0.05$ vs. empty control, $n = 6$ for each adenovirus.

CHAPTER III

HAPLOTYPE INSUFFICIENCY FOR SOCS2 ENHANCES GROWTH AND PROMOTES POLYP FORMATION IN INTESTINE OF GH-TRANSGENIC MICE

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A. Introduction

Even though growth hormone (GH) is FDA approved as treatment for patients with short bowel syndrome, the ability of exogenous GH to promote adaptive growth of the intestine is controversial (135, 174). Several studies in patients with short-bowel syndrome (SBS) treated with GH have reported variable results (175, 177, 178). In animal studies, enterotrophic actions of exogenous GH in intestine have primarily been observed in GH deficient models such as hypophysectomized rats infused with GH (192, 230). or in models of GH excess such as GH transgenic mice (GH-TG) (200). Studies in intact rats receiving total parenteral nutrition (TPN) identified postreceptor resistance to exogenous GH in the jejunal mucosa (32). In TPN-fed rats, GH was shown to induce expression of suppressor of cytokine signaling-2 (SOCS2) in intestine, and SOCS2 mRNA levels negatively correlated with intestinal mass (121). *In vitro* studies demonstrated that SOCS2 overexpression inhibits proliferation of intestinal epithelial cell lines (121). The current studies aimed to directly assess if SOCS2 normally limits the trophic actions of GH on intestine *in vivo*. Mice with germ-line transmission of a mouse metallothionein-driven bovine GH gene (113). which we have previously characterized for small intestinal growth (200). were crossbred with mice heterozygous for targeted disruption of the SOCS2 allele (118) (118). Our aim was to test the effects of SOCS2 deficiency on GH-transgene mediated growth of small intestine or colon. Since SOCS2 was shown previously to induce sucrase expression in Caco2 cells (121), we also examined sucrase mRNA to assess if SOCS2 deficiency affected sucrase expression *in vivo*.

SOCS2 belongs to a family of eight structurally-related proteins, SOCS-1 to -7 and CIS (cytokine inducible SH2-domain containing protein). These proteins contain an N-terminal region of variable length and amino acid composition, a central SH2 domain, and a conserved 40 amino acid motif on the C-terminus referred to as the SOCS box (96). SOCS are induced by cytokines and bind cytokine receptors or JAK kinases, acting as negative feedback inhibitors to limit cytokine action (60). One mechanism of SOCS action is to limit the duration or magnitude of cytokine-dependent STAT activation (60). In addition, the SOCS box motif interacts with elongins B and C, which are members of the proteasome-degradation complex (233) and SOCS may therefore limit the duration of cytokine signaling by targeting activated signaling complexes for proteolytic degradation (60, 233). The phenotype of SOCS2 null mice indicated a particular role of SOCS2 to limit the trophic actions of GH. Mice homozygous for SOCS2 gene disruption are 1.3 to 1.5 times the size of their SOCS2^{+/+} littermates (118). The increase in weight becomes evident around 42 days of age, a time slightly later than the onset of a body overgrowth phenotype observed in GH-TG mice (113, 118, 200). The increase in body weight in SOCS2 null mice is associated with an increase in long bone length and a proportionate increase in the size of several organs (118). SOCS2 null mice have normal circulating levels of IGF-I but show characteristics of deregulated GH action, including increased local IGF-I production in some but not all organs studied (118). SOCS2 null mice also show decreased production of major GH-regulated urinary protein in liver, and increased collagen deposition in the dermis (118). Recent studies demonstrated enhanced body and organ growth responses to exogenous GH in GH-deficient SOCS2 null mice, providing direct evidence that SOCS2 limits GH action in vivo

(61). The effect of SOCS2 on GH-induced intestinal growth has, however, not been analyzed previously.

Identifying novel mechanisms that limit the trophic effects of GH or its downstream effector IGF-I in the intestine is of interest, because considerable evidence implicates these factors in intestinal neoplasia (57). Patients with acromegaly, due to GH secreting pituitary adenomas, have dramatically increased circulating levels of GH and IGF-I (50) and have increased risk of developing precancerous polyps and colorectal cancer compared to normal individuals (158, 193, 208). The GH-TG mice used in these studies are a mouse model of acromegaly (94, 138). SOCS2 gene silencing by hypermethylation is found in various cancer types (52, 108, 190, 214). indicating that SOCS2 may function as an endogenous inhibitor of the tumor-promoting actions of GH. The present study therefore assessed the effect of partial deletion of SOCS2 on growth of small intestine and colon and examined the intestine for abnormal lesions and signs of dysplasia. Results from these studies demonstrate that haplotype insufficiency for SOCS2, associated with only a 40% reduction in normal SOCS2 expression levels, enhances the trophic actions of GH in small intestine. SOCS2 deficiency also promotes the development of lymphoid and hyperplastic polyps in colon, and adenomas in the duodenum of GH-TG mice.

B. Materials and methods

Animal Care and Genotyping

Derivation of mice with targeted disruption of one or both SOCS2 alleles was previously described (118). Mice homozygous for SOCS2 gene disruption (SOCS2 null

mice) on a C57BL/6 background were provided by Drs. Douglas Hilton and Christopher Greenhalgh (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia) (118). Derivation of transgenic mice (GH-TG) that constitutively overexpress a bovine GH-transgene comprising the entire bovine GH gene linked to a mouse metallothionein 1 promoter, was previously described (113). Hemizygous GH-TG mice on a C57BL/SJL background were originally provided by Drs. Richard Palmiter (University of Washington, Seattle WA) and Ralph Brinster (University of Pennsylvania Veterinary School, Philadelphia, PA) (113). Mice homozygous for SOCS2 gene disruption ($SOCS2^{-/-}$) and GH-TG mice were crossbred to generate $SOCS2^{-/-}$ /GH-TG ($SOCS2^{-/-}$ /TG) and $SOCS2^{-/-}$ /WT ($SOCS2^{-/-}$ /WT). A second round of crossbreeding ($SOCS2^{-/-}$ /TG x $SOCS2^{-/-}$ /WT) aimed to generate transgenic (TG) and wild-type (WT) mice with 0 ($SOCS2^{-/-}$ /WT and $SOCS2^{-/-}$ /TG), 1 ($SOCS2^{+/-}$ /WT and $SOCS2^{+/-}$ /TG) or 2 ($SOCS2^{+/+}$ /WT and $SOCS2^{+/+}$ /TG) functional SOCS2 alleles. Genotyping for the WT or disrupted SOCS2 allele was performed on tail DNA by PCR with primers specific for the WT SOCS2 gene (sense: 5'-CGAGCTCAGTCAAACAGGTAGG-3'; antisense: 5'-GCTTTCAGATGTAGGGTGCTTCC-3') or for β -galactosidase present in the disrupted allele (sense: 5'-GCAGACGATGGTCAGGATATCC-3'; antisense: 5'-GGATCGACAGATTTGATCCAGC-3'). Genotyping for the presence of the GH-transgene was performed using primers specific for the bovine GH-transgene (sense: 5'-TTGACACAAACATGCGCAGT-3'; antisense: 5'-GCACTTCATGACCCTCAGGTAC-3'). All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Body and intestine growth

Adult female mice (100-120 days) were studied to assess the effects of partial SOCS2 deficiency on body and intestinal growth. We focused on females because cross-breeding of SOCS2 null and GH-TG mice yielded greater numbers of female littermate pairs with appropriate genotypes for comparisons, and male SOCS2^{+/-}/TG mice were used for breeding purposes. Body weights were monitored weekly starting at day 21 (weaning). A subcutaneous injection of BrdU (200mg/kg, Sigma Diagnostic Inc, St. Louis, MO) was administered 90 minutes prior to sacrifice. At sacrifice, mice were anesthetized with sodium pentobarbital (200µg/g, Abbot Laboratories, Chicago, IL) and entire small intestine and colon were removed and wet weight and length assessed. Corresponding segments of jejunum were quick frozen in liquid nitrogen and stored at -80°C for future analyses. Distal segments of jejunum (0.5cm) were fixed in 4% formalin and paraffin-embedded for morphometric measurements. Whole colon was placed on filter paper for support and opened longitudinally prior to fixing in 4% formalin. Fixed colon was later analyzed under a Leica MZ 16 FA dissecting scope by a single observer blinded to the genotype of the samples to screen for and quantify abnormal lesions. Colon was later paraffin embedded in 'swiss-roll fashion' for further histological examination. Since benign polyps were found in the colon of 100-120 day old mice, follow-up studies were performed on older mice (200-350 days of age) to establish if the colon polyps increased in number, progressed to precancerous or cancerous lesions, or if lesions developed in small intestine. In the follow-up studies, small intestine and colon were collected, fixed, and examined under a dissecting scope to visualize and quantify abnormal lesions.

Small intestine mass; villus height and crypt depth

Frozen jejunum segments were thawed on ice and a longitudinal cut was made along the entire segment. The segments were gently opened and scraped with a cold microscope slide resulting in a mucosal fraction and a submucosal/muscularis fraction (216). The wet weight of the mucosal fraction per unit length of jejunum was measured to assess the effects of partial SOCS2 deficiency on mucosal mass (mg/cm).

Paraffin embedded samples of jejunum were sectioned at a thickness of 4 μm , placed on positively charged slides and stained with hemotoxylin and eosin (H&E). Crypt depth and villus height were measured in stained sections of jejunum by a single blinded observer. Measurements were performed using light microscopy and computer assisted morphometry as described by Williams et al., 2002 (216). Six to ten well-oriented villi and a similar number of crypts were measured per segment.

RNA Extraction and Northern Blot Analysis

Total RNA was isolated from jejunum by the guanidine thiocyanate-caesium chloride method. Abundance of SOCS2, IGF-I and sucrase-isomaltase mRNAs was assayed by northern blot hybridization using [^{32}P]-labeled antisense cRNA or cDNA probes and methods detailed previously (171). The mouse SOCS2 probe was provided by Dr. Douglas Hilton (Walter and Eliza Hall Institute, Melbourne, Australia), and the sucrase-isomaltase probe was provided by Dr. Susan Henning (Baylor University, TX). Blots were re-probed for the constitutively expressed GAPDH mRNA (Ambion, Austin, TX) to control for RNA loading. Blots were scanned on a phosphorimager and abundance of specific mRNAs was quantified using Image Quant software for Macintosh. Abundance of each mRNA examined was

normalized to the abundance of GAPDH mRNA.

Plasma IGF-I analysis

Plasma IGF-I levels were analyzed using the DSL-10-2900 ACTIVE mouse/rat IGF-I enzyme immunoassay (EIA) kit (Diagnostic Systems Laboratories, Inc., Webster, TX) following the manufacturer's instructions. Prior to assay, all samples were acid-ethanol extracted to remove IGF binding proteins (IGFBPs).

Immunohistochemistry

Crypt proliferation was assessed by immunohistochemistry (IHC) using a BrdU immunostaining kit (Zymed BrdU Staining Kit, San Francisco, CA) to label cells in S phase of the cell cycle based on incorporation of BrdU into DNA. Coded sections were scored under a light microscope to assess the number of BrdU-positive cells per crypt. Number of cells per crypt were counted so that data could be expressed as the fraction of total cells per crypt labeled with BrdU.

Colon histology and immunostaining

Examination of the colon revealed polypoid lesions in colon of SOCS2^{+/-}/TG and, to a lesser extent, SOCS2^{+/+}/TG mice. Swissroll embedded sections were therefore examined to assess histology. Histology revealed lymphoid polyps in colon of SOCS2^{+/-}/TG mice. IHC was therefore performed to assess the immune cell types present within the lesions. Formalin-fixed, paraffin-embedded colon was sectioned (4 μ m) and placed on positively charged slides. Analysis for the presence of specific lymphocyte markers used the following

antibodies, CD45R/B220 (B cell marker, BD Biosciences, San Jose, CA) and MAC-3 (macrophage marker, BD Biosciences, San Jose, CA) and CD-3 (T cell marker, Dako, Carpinteria, CA). In brief, sections were deparaffinized in xylene, rehydrated in graded ethanols (95% 4 min, 70% 3 min) to distilled water and rinsed in 0.05M Tris buffer (pH 7.6). This was followed by methanol, hydrogen peroxide block (30% hydrogen peroxide 1:10 methanol, 10 min) and a rinse in water and Tris buffer (0.05M TRIS buffer, pH 7.6, 3min). Blocking was then performed with 2% fish gelatin (Sigma, St. Louis, MO) in Tris prior to incubation with primary antibody (1:25 in 2% Fish Gelatin/Tris buffer, overnight, humid chamber, 4°C for 18-24 hours). The following day, sections were washed in Tris (3x2 min) and incubated in secondary antibody (biotinylated mouse anti-rat IgG 1/2 α , BD Biosciences, San Jose, CA, 1:100, room temperature, 90 min). Slides were incubated in avidin-biotin complex (Vectastain, Burlingame, CA) for 75 min and labeling visualized using DAB. β -catenin staining of tumors was performed to determine if tumors exhibited cytoplasmic or nuclear β -catenin, a hallmark of early-stage precancerous lesions in mouse and human. β -catenin staining was performed as described in Chapter 2, using a mouse primary antibody (610154, BD Transduction Laboratories, Franklin Lakes, NJ) and a mouse-on-mouse blocking kit (BMK-2202, Vector Laboratories, Burlingame, CA). Slides were counterstained in hematoxylin, dehydrated in ethanol, coverslipped and analyzed with a light microscope.

Effects of SOCS2 deletion on STAT5 activation by GH ex vivo

Corresponding segments of small intestine (~8 cm) were isolated from SOCS2 null and WT mice. The segments were flushed with 1X PBS to remove luminal contents. One cm segments were incubated at room temperature with serum-free medium with or without

GH (10^{-7} M). After 30 - 90 minutes at room temperature, segments were dounce-homogenized in 1 ml ice-cold 1X TBS for nuclear protein extraction. Nuclei and nuclear proteins were extracted using standard methods (188). Electromobility shift assays (EMSA) were performed as previously described (188) on 30 μ g of protein using double-stranded oligomers corresponding to a consensus STAT5 binding sequence (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Analysis

Values are expressed as mean \pm standard error of the mean (SEM). Absolute values were analyzed by two-way analysis of variance (ANOVA) to test for main effects of the GH transgene, or SOCS2 gene disruption or an interaction between GH transgene and SOCS2 gene disruption. Pair-wise comparisons were performed using Tukey's test. Statistical significance was set at $p < 0.05$.

C. Results

Effects of partial SOCS2 deletion on body growth

Theoretically, crossbreeding of SOCS2^{+/-}/TG and SOCS2^{+/-}/WT should yield transgenic (TG) and wild-type (WT) mice with 0 (SOCS2^{-/-}/WT and SOCS2^{-/-}/TG), 1 (SOCS2^{+/-}/WT and SOCS2^{+/-}/TG) or 2 (SOCS2^{+/+}/WT and SOCS2^{+/+}/TG) functional SOCS2 alleles. Surprisingly, no SOCS2^{-/-}/mice that were also GH-TG were obtained over the duration of the initial intestinal growth study. To date, only one SOCS2^{-/-}/mouse that is also GH-TG has been generated. The cross did, however, generate appropriate numbers of

SOCS2^{+/-} and SOCS2^{+/+} mice that were GH-TG. Since partial SOCS2 deficiency represents what may be considered small and more physiological reductions in SOCS2 expression, our analyses focused on SOCS2^{+/+}/WT, SOCS2^{+/-}/TG, SOCS2^{+/-}/WT and SOCS2^{+/-}/TG to assess if haplotype insufficiency for SOCS2 altered intestinal growth induced by the GH-transgene. Figure 1 shows growth curves for SOCS2^{+/+}/WT, SOCS2^{+/-}/WT, SOCS2^{+/-}/TG and SOCS2^{+/-}/TG based on body weights measured weekly from day 21. Consistent with previous reports (118), mice with one disrupted SOCS2 allele but otherwise WT (SOCS2^{+/-}/WT), showed no significant increase in body weight compared with their WT littermates (SOCS2^{+/+}/WT). Consistent with prior reports, SOCS2^{+/-} mice expressing the GH transgene showed an increase in body weight relative to SOCS2^{+/+}/WT, which was apparent immediately after weaning (21-28 days) and then continued to be significantly larger than SOCS2^{+/+}/WT littermates throughout the entire study period. GH-TG mice lacking one copy of SOCS2 (SOCS2^{+/-}/TG) did not show a significant difference in body weight compared to SOCS2^{+/+}/TG littermates at time points immediately after weaning. The SOCS2^{+/-}/TG mice did, however, demonstrate a significant increase in body weight compared to SOCS2^{+/+}/TG beginning at 49 days of age and continued to be significantly larger throughout the rest of the study period (Figure 3.1).

Loss of one copy of SOCS2 in GH-TG mice leads to increased growth in jejunum.

Effects of partial SOCS2 deficiency on body growth were compared with effects on intestinal growth. Adult mice (100-120 days old) were studied, because prior studies had revealed a greater effect of SOCS2 deletion on intestinal growth in adult compared to young

mice. As shown in Table 3.1, compared to their SOCS2^{+/+}/WT littermates, adult SOCS2^{+/-}/TG mice showed significant increases in body, small intestine and colon weight. Evaluation of SOCS2 expression revealed a significant approximately 50% reduction in SOCS2 mRNA in SOCS2^{+/-}/WT and SOCS2^{+/-}/TG compared with SOCS2^{+/+}/WT and SOCS2^{+/+}/TG (Figure 3.2). Interestingly, neither SOCS2^{+/-}/TG nor SOCS2^{+/+}/TG showed an increase in SOCS2 expression relative to littermates lacking the GH transgene. The SOCS2^{+/-}/TG mice showed a significantly greater transgene-induced increase in body and small intestine wet weight compared with SOCS2^{+/+}/TG mice having both intact SOCS2 alleles. Disruption of one SOCS2 allele led to a greater effect of transgene on body and small intestine weight compared with effects in SOCS2^{+/+}/TG mice. There was also a trend for a greater increase in colon weight in SOCS2^{+/-}/TG vs. SOCS2^{+/+}/TG, although this increase did not reach statistical significance (Table 3.1).

Partial SOCS2 deficiency results in increased mucosal growth and crypt cell proliferation in jejunum of GH-TG mice

The effects of partial SOCS2 deficiency on jejunal growth were assessed, because prior studies demonstrated increased growth of jejunum in GH-TG (200). Jejunal mucosal mass did not differ significantly in SOCS2^{+/+}/WT and SOCS2^{+/-}/WT but was increased in SOCS2^{+/+}/TG vs. SOCS2^{+/-}/WT mice, and an even greater increase was observed in SOCS2^{+/-}/TG mice than in SOCS2^{+/+}/TG (Figure 3.3A). Morphometry revealed no significant difference in villus height and crypt depth compared with SOCS2^{+/+}/WT mice

(Figures 3.3B and 3.3C). Only the SOCS2^{+/-}/TG mice showed a statistically significant increase in villus height and crypt depth in SOCS2^{+/-}/WT compared to SOCS2^{+/+}/WT or SOCS2^{+/-}/WT (p<0.001) and these parameters were significantly greater in SOCS2^{+/-}/TG than SOCS2^{+/+}/TG (p<0.001) (Figures 3.3B and 3.3C).

Previous studies in adult GH-TG mice did not identify a difference in crypt cell mitoses compared to WT littermates (200). Consistent with these results no significant change in percentage of BrdU positive cells per crypt were observed in SOCS2^{+/-}/TG mice compared to their SOCS2^{+/+}/WT littermates. Disruption of one SOCS2 allele in GH-TG mice caused a small but significant increase in BrdU labeling, indicating that SOCS2 normally limits the proliferative effects of GH on intestinal epithelial cells (Figure 3.4).

Increased local but not circulating IGF-I in GH-transgenic mice lacking one copy of SOCS2

To determine if more pronounced GH-transgene induced increases in jejunal growth in SOCS2^{+/-}/TG vs. SOCS2^{+/+}/TG mice were associated with an increase in circulating or locally expressed IGF-I, plasma IGF-I levels and jejunal IGF-I mRNAs were measured. As shown in Figure 5A, the SOCS2^{+/-}/WT mice showed no significant difference in plasma IGF-I compared with SOCS2^{+/+}/WT mice. The SOCS2^{+/+}/TG mice showed the predicted increase in plasma IGF-I compared with SOCS2^{+/+}/WT mice. The SOCS2^{+/-}/TG mice showed virtually identical plasma IGF-I levels as SOCS2^{+/+}/TG mice, indicating that enhanced jejunal growth was not associated with more pronounced increases in plasma IGF-I (Figure 3.5A). Figure 3.5B shows data on local levels of IGF-I mRNA expression in

jejunum. Jejunal IGF-I mRNA levels did not differ significantly between SOCS2^{+/+}/WT, SOCS2^{+/-}/WT or SOCS2^{+/-}/TG. However, SOCS2^{+/-}/TG mice showed a significant increase in local IGF-I expression (Figure 3.5B). These results indicate that the increased trophic effects observed in intestine of SOCS2^{+/-}/TG mice could be driven at least in part by an increase in locally expressed IGF-I, which would be expected to exert paracrine effects on growth.

Decreased sucrase-isomaltase expression in jejunum of GH-TG mice lacking one copy of SOCS2

Previous studies in Caco-2 cells have shown that SOCS2 overexpression results in increased sucrase-isomaltase expression and alkaline phosphatase activity (121), suggesting that SOCS2 may promote enterocyte differentiation. To determine the effects of partial SOCS2 deficiency on differentiation, sucrase-isomaltase mRNA was measured in jejunum. SOCS2^{+/-}/WT mice showed no significant change in sucrase mRNA expression compared to SOCS2^{+/+}/WT, indicating that reduced SOCS2 expression alone has no effect on expression of this differentiation marker in the intestine of WT mice. While SOCS2^{+/+}/TG sucrase mRNA levels were not significantly different from SOCS2^{+/-}/WT, SOCS2^{+/-}/TG mice had significantly decreased sucrase-isomaltase mRNA expression (Figure 3.6). These results suggest that reduced SOCS2 expression in conjunction with GH excess results in diminished expression of a key marker of enterocyte differentiation.

Formation of aberrant lesions in colon of GH-TG mice lacking one copy of SOCS2

Acromegaly is associated with an increased risk of colorectal cancer (158, 193). Because the GH-TG mice used in these studies are a model of acromegaly, the colon was analyzed for the presence of aberrant lesions. No significant lesions were observed in SOCS2^{+/+}/WT and SOCS2^{+/-}/WT mice. A small number of possible aberrant lesions were identified under the dissecting microscope in colon of SOCS2^{+/+}/TG mice but could not be histologically-verified as polyps. The colon of SOCS2^{+/-}/TG mice contained a large number of polyp-like lesions, which were primarily localized to the proximal colon (Figure 3.7).

Further histological examination demonstrated the presence of lymphoid polyps as well as increased presence of hyperplastic mucosa or hyperplastic polyps in colon of SOCS2^{+/-}/TG mice. To better characterize the lymphoid aggregates, immunohistochemistry was performed using specific markers for T cells (CD-3), B cells (CD-45R/B220) and macrophages (MAC-3) were performed. The lymphoid aggregates showed no significant staining for Mac-3 or CD-3 (data not shown), while there was intense staining for CD45R/B220, indicating that these aggregates are composed primarily of B cells (Figure 3.8). Lymphoid polyps also stained positively for BrdU, indicating ongoing B cell proliferation. Increased BrdU was also consistently observed in hyperplastic mucosa adjacent to polypoid lesions as shown in examples in Figure 3.8.

Increased STAT5 activation by GH in intestine of SOCS2 null mice

The effect of SOCS2 deficiency on GH signaling was addressed in small intestine of SOCS2 null and WT mice treated with exogenous GH. Activation of STAT5, a major downstream mediator of GH signaling that has been linked to leukemia (22). was assessed by

EMSA. In response to GH treatment, the intestine of SOCS2 null mice showed enhanced binding of nuclear proteins to a STAT5 DNA binding sequence compared with WT control (Figure 3.9).

Effects of aging on the development of aberrant lesions in small intestine and colon

Older, 200 day old SOCS2^{+/+}/TG, SOCS2^{+/-}/TG, and one SOCS2^{-/-}/TG mouse born across all litters were examined for aberrant lesions in the colon and small intestine. In colon, we found that polyps did not progress to a precancerous phenotype and with age, the difference in polyp number between SOCS2^{+/+}/TG and SOCS2^{+/-}/TG was attenuated (Figure 3.10). One SOCS2^{-/-}/TG had a large number of colonic polyps. Interestingly, in aged but not younger mice, we observed duodenal lesions. Histology confirmed adenomatous polyps in SOCS2^{+/-}/TG, and the one SOCS2^{-/-}/TG animal had a duodenal adenocarcinoma (Figure 3.11). β -catenin staining revealed largely membrane-associated β -catenin in duodenal adenomas but obvious nuclear β -catenin in the duodenal adenocarcinoma.

D. Discussion

These studies identified a novel effect of haplotype insufficiency for SOCS2 in enhancing the actions of excess GH in the intestine. The results from these studies are relevant because GH is used in an increasing number of children to correct growth delay (102, 116) and is also used clinically in patients with long-term TPN due to SBS (40). Results from the current studies suggest that the intestinal response to GH therapy may be affected by even small changes in the levels of expressed SOCS2.

In this study, we aimed to examine the effects of disruption of one or both SOCS2 alleles on GH-transgene dependent intestinal growth, because the GH transgenic mice represent a model of acromegaly, and we were interested in whether SOCS2 status impacts on normal or aberrant intestinal growth resulting from GH excess. Other than a single animal, we were unable to generate multiple GH-TG mice homozygous for SOCS2 gene disruption. This suggests that homozygous deletion of both SOCS2 genes and expression of the GH-TG is incompatible with embryonic survival. This was unexpected because the phenotypes of both the SOCS2 null and the GH-TG mice do not become evident until post-weaning. When SOCS2 null mice were crossed with GH-transgenics overexpressing an ovine transgene, this also did not yield mice both homozygous for SOCS2 gene disruption and positive for the transgene (Greenhalgh, personal communication). The mechanism by which the GH-TG compromises survival of SOCS2 null embryos is unknown. Although body and organ phenotypes have been studied in GH-TG post-natally, there is no information about prenatal effects of GH-TG expression. However, recent studies suggest that both GH and SOCS2 play important roles in neurogenesis during development of the embryonic brain (157, 197). It may be that the transgene-derived growth hormone affects embryonic brain development and, when combined with SOCS2 deficiency, has detrimental effects on the development of the central nervous system and compromises viability. This is speculative at present and will require further analysis. Nonetheless, the fact that combined GH overexpression and absolute SOCS2 deficiency appears compromise embryonic viability provides new, albeit preliminary and indirect, evidence that there may be interactions between GH and SOCS2 during embryonic development.

Studies in WT or GH-TG mice lacking one copy of the SOCS2 gene revealed

significant effects of modest (40%) reductions in SOCS2 expression on body growth and intestinal responses to GH excess. This supports a concept that small and what may be considered physiological variations in SOCS2 expression may profoundly impact the effects of GH on body or intestinal growth in clinical settings of GH therapy or situations of GH excess such as acromegaly. It is noteworthy that dual roles of SOCS2 as both an inhibitor and enhancer of GH action have been suggested. The potential enhancer role was based on small increases in body weight and weight of some organs in mice with widespread expression of supraphysiological levels of SOCS2 due to a ubiquitin promoter-driven SOCS2 transgene, and findings that over-expressed SOCS2 bound to the GH receptor (59). Potential mechanisms suggested for these effects of SOCS2 overexpression were that supraphysiological levels of ectopically expressed SOCS2 may have a dominant negative effect to perturb the actions of endogenous SOCS2, or that over-expressed SOCS2 may perturb the actions of other SOCS family members that normally repress GH action (59). Since intestine was not examined in the mice that over-express SOCS2, it could be of interest to generate cross-breeds of the GH-TG and SOCS2 overexpressing mice to assess how high levels of SOCS2 impact on phenotypic effects of GH excess in the intestine. However, the current studies demonstrating that small reductions in SOCS2 expression in SOCS2-HT enhance GH action strengthen the argument that endogenous SOCS2 generally serves as a negative modulator of GH action in the intestine.

In small intestine, disruption of one SOCS2 allele alone had no significant effect on growth or markers of differentiation. However, haplotype insufficiency for SOCS2 amplified the trophic effects of excess GH on jejunal mucosa based on major increases in jejunal mass, crypt depth and villus height. The enhanced trophic effect of GH was also

associated with decreased expression of sucrase-isomaltase, a marker of terminal differentiation of enterocytes. Thus partial SOCS2 deficiency appears to favor a less mature epithelium. These results are consistent with previous observations in Caco-2 cells where SOCS2 overexpression resulted in increased sucrase-isomaltase expression and alkaline phosphatase activity (121). It is unlikely that SOCS2 is essential for enterocyte differentiation since the SOCS2 null mice do not display the dramatic phenotypes of impaired enterocyte differentiation observed in mice with deletion in other genes such as Cdx-2 (10). Nonetheless, our prior in vitro findings and the current in vivo findings indicate that signaling pathways regulated by SOCS2 may interact with transcription pathways mediating sucrase-isomaltase expression. In this regard it is of interest to note that the sucrase-isomaltase gene contains several potential STAT binding sites upstream of Cdx-2 binding sites known to induce sucrase expression (121).

Partial SOCS2 deficiency in GH-TG mice resulted in increased crypt cell proliferation and increased local IGF-I mRNA expression in jejunum. Because IGF-I has well-established mitogenic effects on intestinal epithelial cells (137), the increase in crypt proliferation may be mediated by the increase in locally expressed IGF-I. This is also supported by findings from our lab that an IGF transgene targeted to mesenchymal cells had paracrine effects to increase growth of small intestinal epithelium (216). The increased local IGF-I expression or trophic effects of the GH-transgene observed in the intestine of SOCS2^{+/-}/TG mice also may be due to enhanced activation of early downstream mediators of GHR signaling known to be negatively regulated by SOCS2. A likely candidate is increased activation of signal-transducer and activator of transcription-5b (STAT5b). The growth phenotype observed in the SOCS2 null mice has been shown to require STAT5b (59).

STAT5b has also been shown to be essential for GH-induced IGF-I gene expression in liver (34). Consistent with these prior observations, we observed enhanced STAT5 activation in intestine of SOCS2 null mice in response to short-term treatment with exogenous GH.

The presence of polyp-like lesions in the colon of GH-TG mice lacking one copy of SOCS2 provides novel evidence for a role of SOCS2 in limiting the development of aberrant intestinal lesions during GH excess. Although hyperplastic polyps are not typically considered pre-cancerous lesions, prior studies have demonstrated increased frequency of hyperplastic polyps in acromegalic patients who are also more susceptible to colorectal cancer (111). The presence of lymphoid polyps is also of interest because recent studies have demonstrated an increased incidence of mucosal lymphoid polyps in patients with colorectal cancer (130). A few cases of non-Hodgkin's lymphoma have been reported in patients with acromegaly (2). The lymphoid polyps observed in the SOCS2^{+/-}/TG colon were primarily composed of B cells. One study reported that induction of SOCS2 in B cell lymphoma cells by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a potent immunosuppressor, renders these cells less responsive to mitogenic stimulation (17). Further studies assessing the role of SOCS2 in B cell pathogenesis may therefore be of interest. Since the mice used in these studies were relatively young, we went on to determine if the colonic lesions observed in SOCS2^{+/-}/TG mice progress to a more neoplastic phenotype in older animals. The colonic lesions looked similar regardless of age, both grossly and histologically. However, duodenal adenomas were found in older mice, and the one SOCS2^{-/-}/TG developed duodenal adenocarcinoma. Our evidence that SOCS2 may promote tumorigenesis in the intestine is consistent with emerging evidence in tumors of other organs, for example SOCS2 expression has been shown to be down-regulated in pulmonary adenocarcinoma (214) and SOCS2

hypermethylation has been observed in ovarian carcinoma cells and in patients with endometrial cancer (52, 190). Our studies support a role for SOCS2 as a tumor suppressor and indicate that further analyses of the role of SOCS2 in tumor development and growth are warranted.

GH is known to play an important role in post-natal growth. This is highlighted in the GH-TG mice, which begin to show a significant increase in body weight compared to WT littermates at points immediately following weaning (200). In the present studies, we show that the increase in body weight shown by these transgenic mice is further enhanced by small reductions in SOCS2 expression due to disruption of one SOCS2 allele. These results are consistent with recent studies, which showed enhanced trophic effects of exogenous GH in GH-deficient mice lacking both copies of SOCS2 (61). However, the fact that haplotype insufficiency for SOCS2 enhances the growth response to GH excess supports a concept that small variations in SOCS2 expression may impact on body and intestinal phenotype in acromegaly. A novel finding of our studies is that the increase in body growth observed in the SOCS2^{+/-}/TG mice did not manifest until the mice were 49 days of age. This indicates that small reductions in SOCS2 play an important role in limiting the trophic actions of GH in adult mice, but have no effect at earlier stages in life. This is consistent with prior observations in SOCS2 null mice, where no significant effect on body weight was observed until the mice were 42-55 days of age (118). Given the current interest in the effects of the GH-IGF-I axis in aging and longevity (8, 71, 189), future studies assessing the effects of SOCS2 on aging and longevity may be of interest.

In conclusion, our findings in GH-TG mice with partial SOCS2 deficiency provide new evidence that endogenous SOCS2 normally limits intestinal growth and promotes

enterocyte differentiation during GH excess in adult animals and that normal SOCS2 expression limits neoplasia in intestine during GH excess. Together these studies provide new evidence that SOCS2 directly impacts on the actions of GH on intestine and suggest that the efficacy of GH therapy to promote intestinal growth in patients with SBS or intestinal abnormalities in acromegalic patients may be dependent on SOCS2 status.

Table 3.1 Deletion of 1 copy of SOCS2 in GH-TG mice leads to increases in both body and small intestine weight.

Genotype	Body Weight (g)	Small Int. Weight (g)	Colon Weight (g)
SOCS2 ^{+/+} /WT	22.1 ± 0.2	1.05 ± 0.02	0.24 ± 0.01
SOCS2 ^{+/-} /WT	26.1 ± 1.2	1.14 ± 0.02	0.24 ± 0.01
SOCS2 ^{+/+} /TG	43.1 ± 1.3 ^a	1.78 ± 0.06 ^a	0.43 ± 0.03 ^a
SOCS2 ^{+/-} /TG	54.4 ± 1.8 ^{a,b}	2.33 ± 0.06 ^{a,b}	0.49 ± 0.04 ^a

Values are mean ± SEM. n=7 littermate pairs; a=p<0.05 for TG vs. littermate control of the same SOCS2 genotype; b=p<0.05 for SOCS2^{+/-}/TG vs. SOCS2^{+/+}/TG.

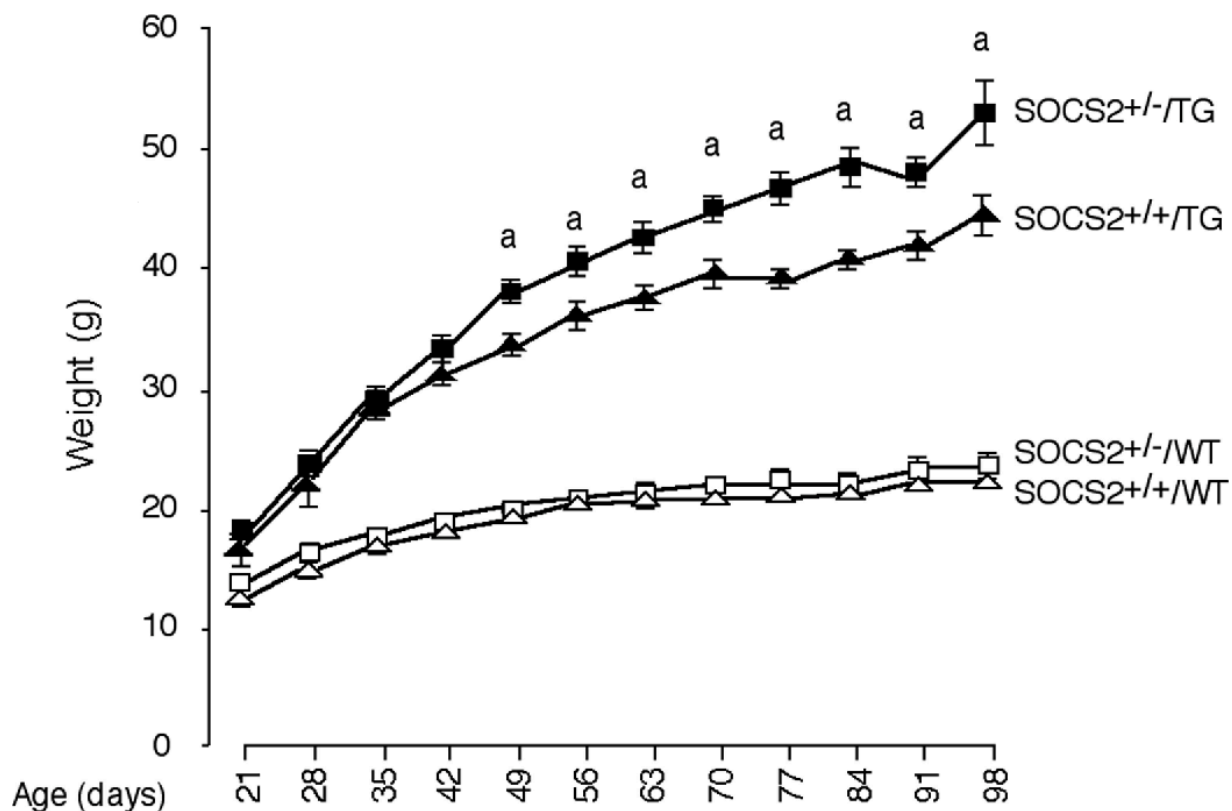


Figure 3.1 Growth Curves. Growth curves for female SOCS2^{+/-}/WT (open triangles), SOCS2^{+/-}/WT (open squares), SOCS2^{+/-}/TG (solid triangles), SOCS2^{+/-}/TG (solid squares). Mouse body weights were measured weekly and each point represents mean \pm SEM; n=6-10 per time point; * SOCS2^{+/-}/TG and SOCS2^{+/-}/TG differed from SOCS2^{+/-}/WT and SOCS2^{+/-}/WT at all time points analyzed. a = p<0.05 for SOCS2^{+/-}/TG vs. SOCS2^{+/-}/TG.

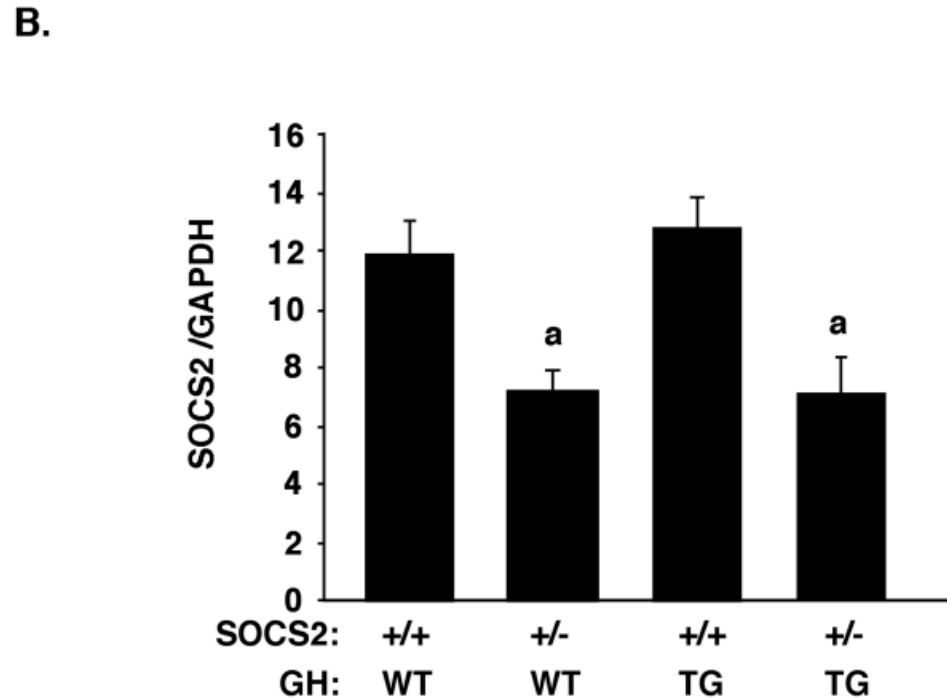
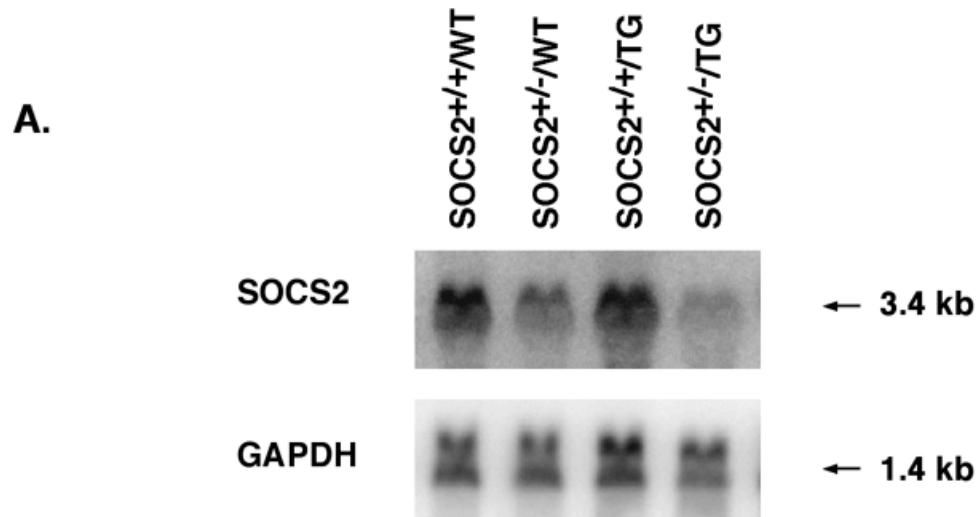


Figure 3.2 Reduced SOCS2 expression in jejunum of GH-TG mice lacking 1 copy of SOCS2. A. Representative autoradiograms of northern blots probed for SOCS2 and GAPDH control mRNAs in jejunum. B. Histograms show mean \pm SEM of SOCS2 mRNA normalized to GAPDH mRNA; n = 4; a = p < 0.05 vs. other genotypes.

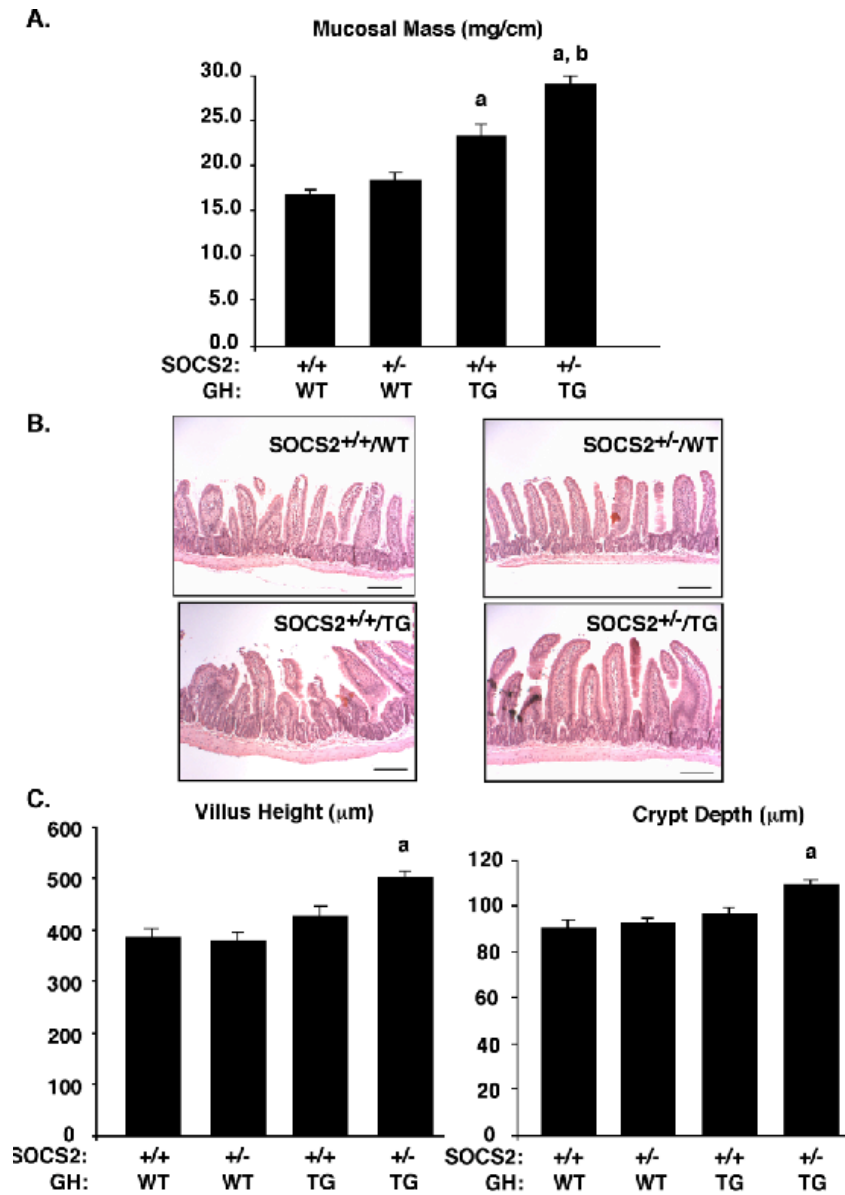
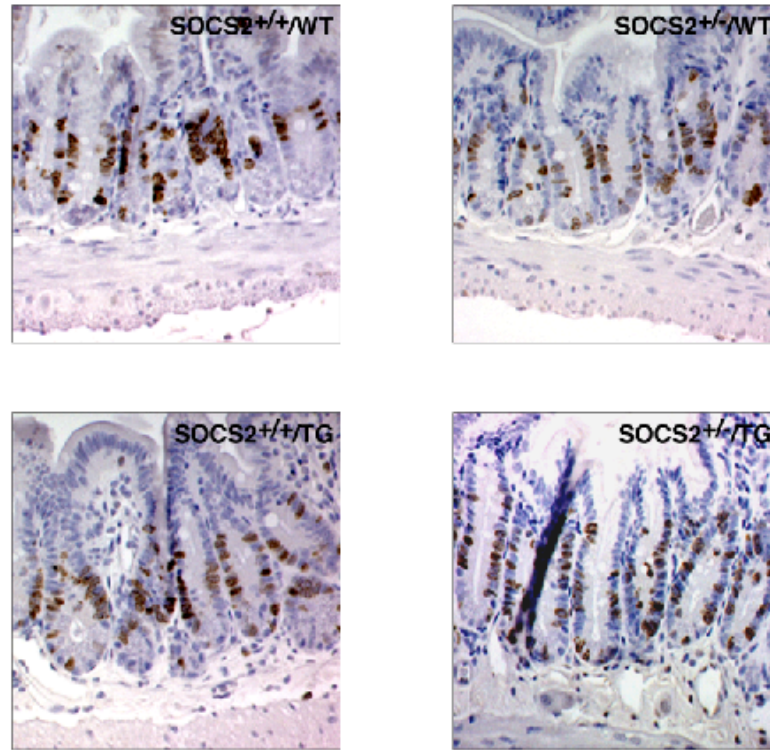


Figure 3.3 Effects of partial SOCS2 deficiency on morphometric measures in jejunum of GH-TG mice. A. Histograms show wet mass of jejunal mucosa for SOCS2^{+/+}/WT, SOCS2^{+/-}/WT, SOCS2^{+/+}/TG, and SOCS2^{+/-}/TG mice. Values are means \pm SEM; n=7; a= p<0.05 for TG vs. WT control of the same SOCS2 genotype, b=p<0.05 for SOCS2^{+/-}/TG vs. SOCS2^{+/+}/TG. B. Representative 4X bright field images of H&E stained jejunum. C. Histograms show mean \pm SEM of villus height and crypt depth compared to control of same SOCS2 genotype; n=6-7; a=p<0.05 vs. other genotypes.

A.



B.

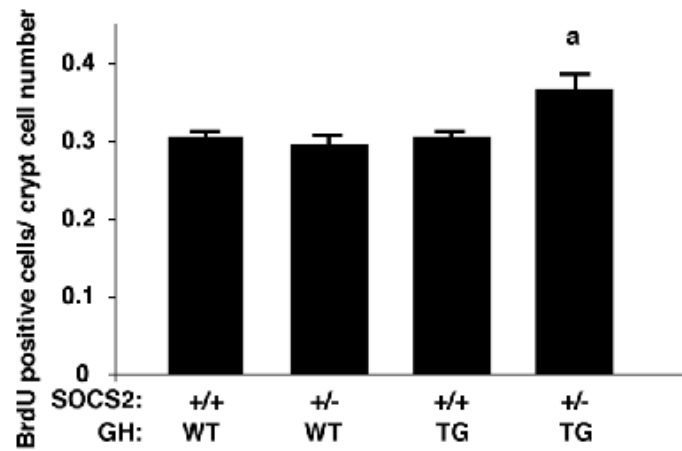


Figure 3.4 BrdU incorporation in jejunal crypt cells.

A. Representative 15X bright field microphotographs of BrdU immunostained cells in jejunal crypts. B. Histograms show mean number of BrdU positive cells expressed as a fraction of mean number of cells per crypt total. Values are means \pm EM; $n \geq 6$; $a = p < 0.05$ vs. other genotypes.

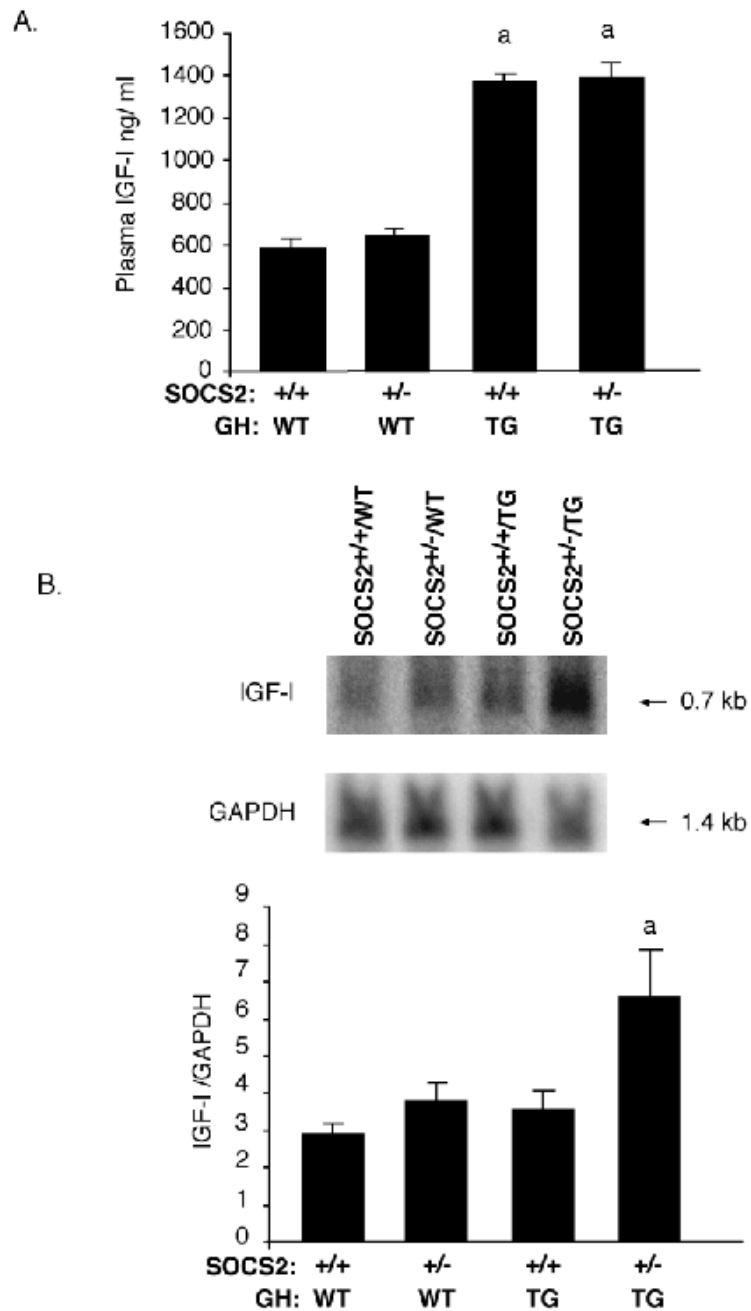


Figure 3.5 Plasma and jejunal IGF-I expression. A. Histograms show mean \pm SEM of plasma IGF-I levels; $n = 7$; $a = p < 0.05$ vs. control of same SOCS2 genotype. B. Top: Representative autoradiograms of northern blots probed for IGF-I and GAPDH mRNAs in jejunum. Bottom: Histograms show mean \pm SEM of IGF-I mRNA normalized to GAPDH mRNA; $n = 6$; $a = p < 0.05$ vs. other genotypes.

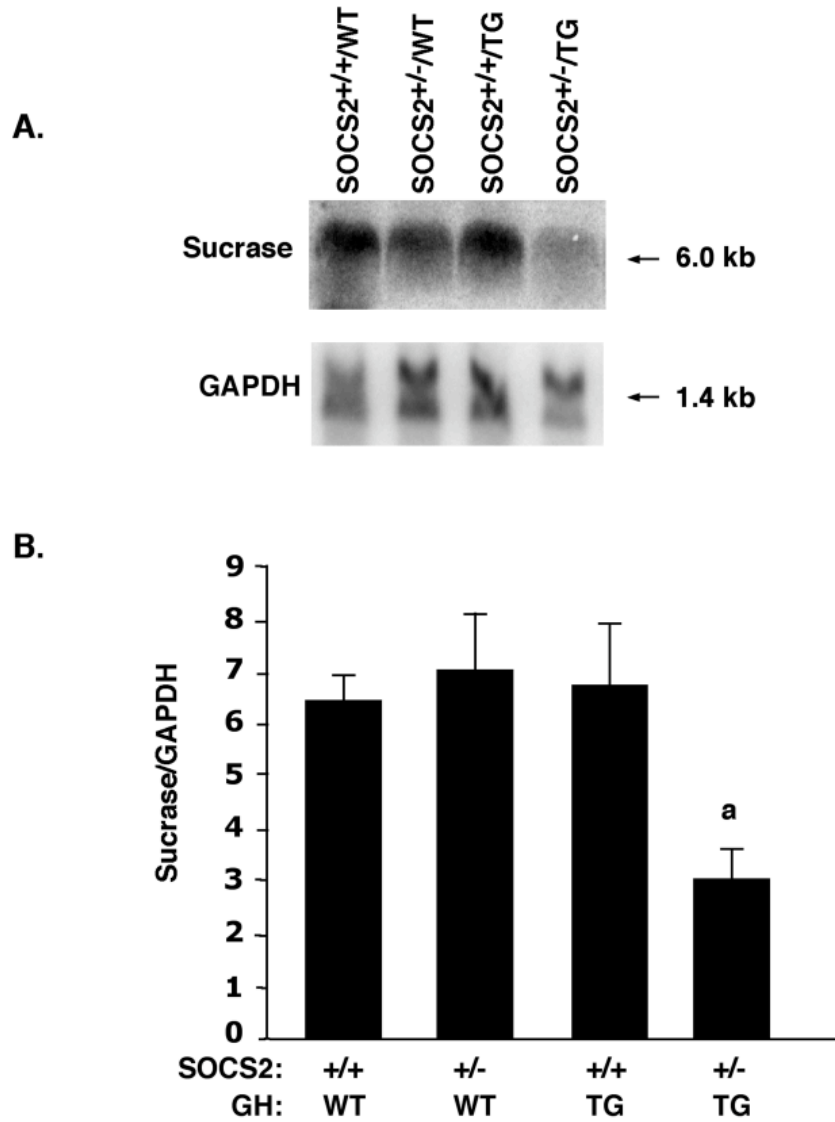
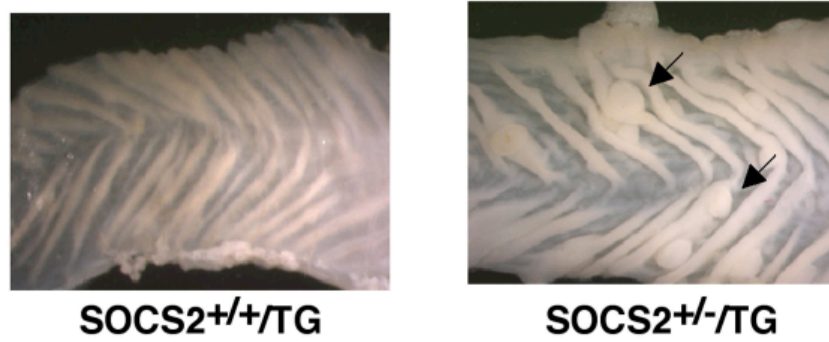


Figure 3.6 Reduced sucrase-isomaltase mRNA in GH-TG mice with partial SOCS2 deficiency. A. Representative autoradiograms of northern blots probed for sucrase-isomaltase and control GAPDH mRNAs in jejunum. B. Histograms show mean \pm SEM of sucrase-isomaltase mRNA normalized to GAPDH mRNA; $n=6$; $a=p<0.05$ vs. other genotypes.

A.



B.

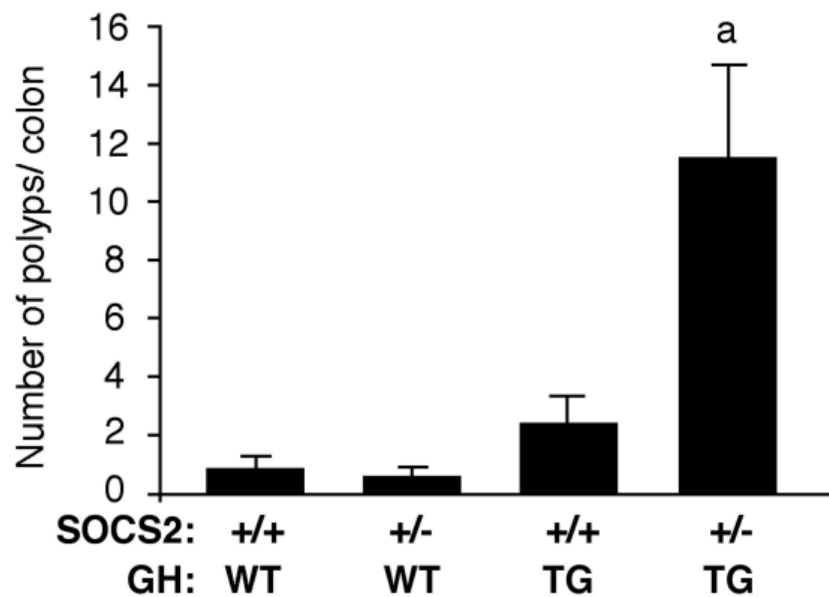


Figure 3.7 Formation of colonic polyp-like lesions in GH-TG mice with partial SOCS2 deficiency. A. Representative 8X microphotographs of colon from SOCS2^{+/+}/TG and SOCS2^{+/-}/TG mice as visualized under a dissecting scope. B. Histograms show mean number of polyp-like lesions present in colon. Values are mean \pm SEM; n=6-7; a= p<0.05 vs. SOCS2^{+/+}/TG and other genotypes.

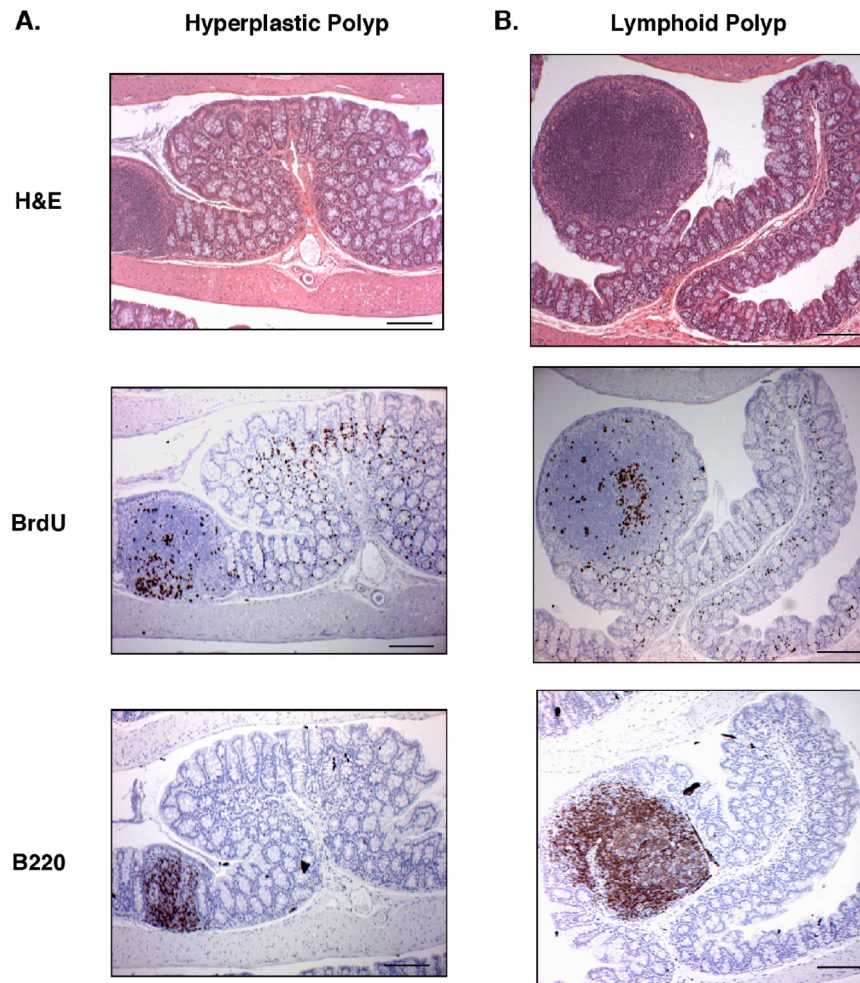


Figure 3.8 Histology of colonic polyp-like lesions in HT-TG. A. Top: Representative 10X bright field microphotograph of H&E stained hyperplastic polyp. Middle: Serial section labeled with BrdU. Bottom: Immunohistochemistry shows presence of B-cells in a nearby lymphoid aggregate but not within the hyperplastic polyp. B. Top: Representative 10X bright field microphotograph of H&E stained lymphoid aggregate. Middle: BrdU labeled serial section showing the presence of proliferating cells within the immune aggregate. Bottom: Immunohistochemistry showing the presence of CD45R/B220 positive cells within the lymphoid polyp.

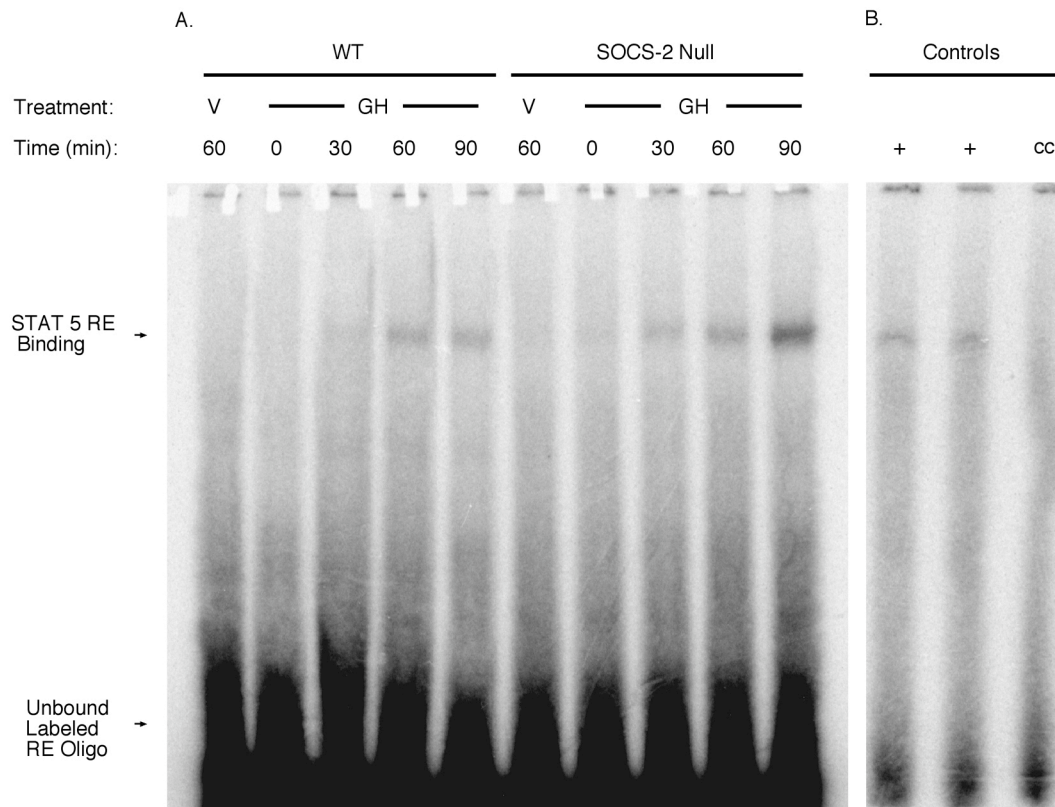


Figure 3.9 Increased STAT5 activation in intestine of SOCS2 null mice treated with GH. A. Representative autoradiograms of EMSA for binding of nuclear proteins to a ^{32}P -labeled STAT5 consensus sequence in small intestine of WT and SOCS2 null mice treated with GH or vehicle (V) for the indicated times at 0, 30, 60 and 90 min treatment with GH. B. Shows STAT5-RE binding activity in positive control jejunal extracts and competition with excess unlabeled STAT5-RE.

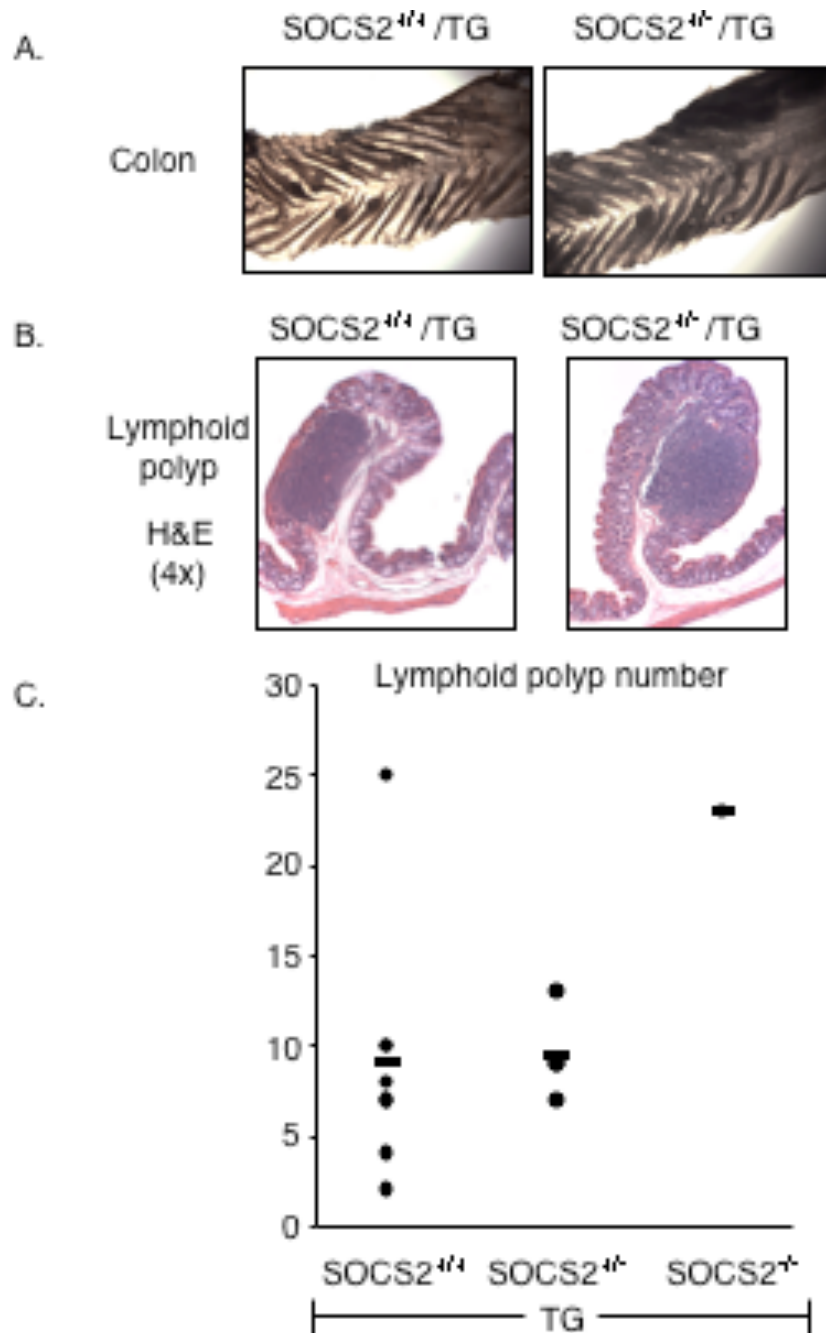


Figure 3.10. Colonic lymphoid polyps did not show signs of progression. A. Representative images show colonic lymphoid polyps in $SOCS2^{+/+}/TG$ mice and in $SOCS2^{+/-}/TG$ mice. B. Representative images of H&E (4x) staining of lymphoid polyps, showing similar histology to polyps found in younger mice. C. Dot plot shows the number of lymphoid polyps in $SOCS2^{+/+}/TG$, $SOCS2^{+/-}/TG$, and the one $SOCS2^{-/-}/TG$ mice, with no clear difference between genotypes.

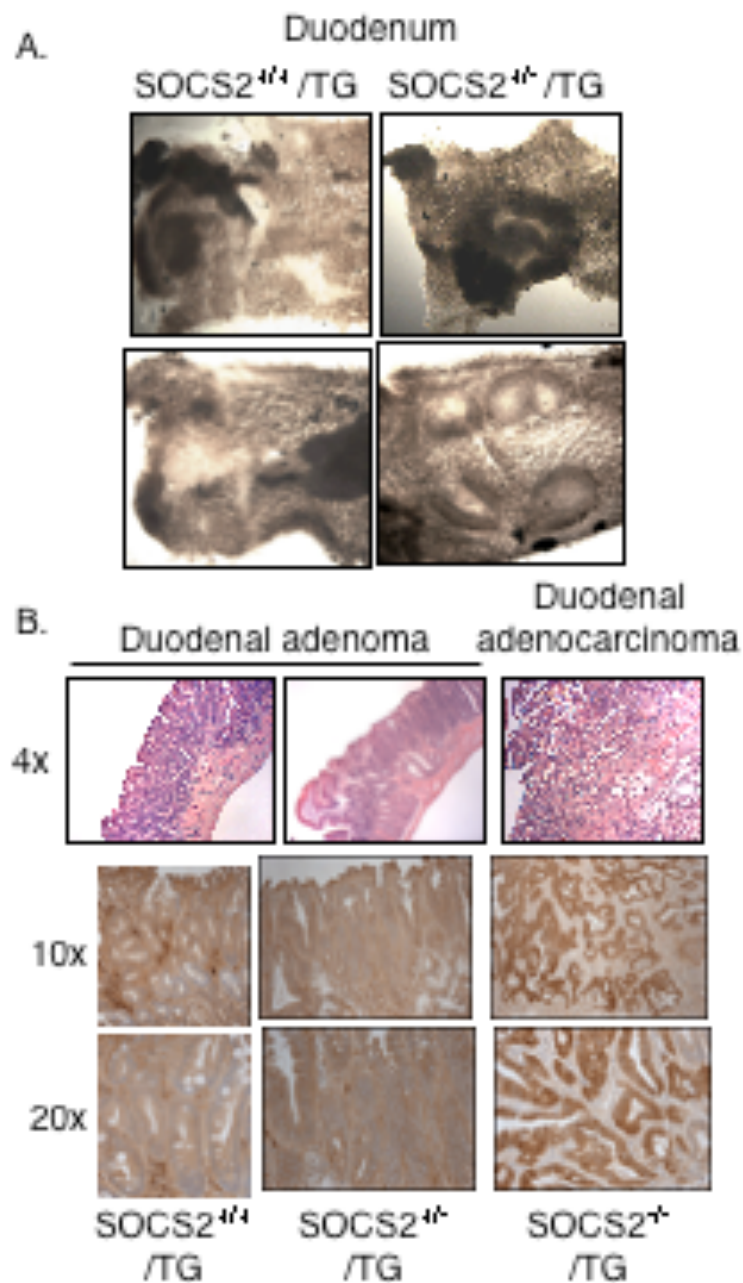


Figure 3.11. Duodenal neoplastic lesions in partial or absolute SOCS2 deficient mice expressing the GH-transgene. A. Representative images show duodenal adenomatous lesions in $SOCS2^{+/-}/TG$ mice and not in $SOCS2^{+/+}/TG$ mice. B. Representative images of H&E (4x) and β -catenin staining (10x or 20x) of duodenal neoplastic lesions. Note little nuclear β -catenin staining in duodenal adenomas, while the adenocarcinoma found in the one $SOCS2^{-/-}/TG$ mouse that was generated had β -catenin positive nuclei.

CHAPTER IV

SOCS2 DEFICIENCY ENHANCES SPORADIC INTESTINAL TUMOR DEVELOPMENT, BUT HAS A LIMITED ROLE IN INFLAMMATION-MEDIATED INTESTINAL TUMORS

A. Introduction

Suppressor of Cytokine Signaling 2 (SOCS2) has been shown to inhibit the growth hormone/insulin-like growth factor (GH/IGF) axis. Mice with disruption of one or both SOCS2 alleles exhibit a body overgrowth phenotype similar to the phenotype observed in GH transgenic and IGF-I transgenic mice (113, 118, 140). By crossbreeding SOCS2^{-/-} mice and mice with a point mutation in the gene encoding Growth Hormone Releasing Hormone (*Ghrh*^{-/-}), the body overgrowth phenotype in SOCS2^{-/-} mice was reversed (61). Prior studies from our laboratory suggested that SOCS2 could limit intestinal growth induced by GH or GH-induced IGF-I (121). This was based on observations that IGF-I but not GH prevented atrophy of the intestinal epithelium during total parenteral nutrition (TPN), and this effect correlated with the induction of SOCS2 by GH but not IGF-I (121). This was intriguing because GH still increased body growth and circulating IGF-I. Since the elevated IGF-I was unable to elicit intestinal growth, this raised the possibility that SOCS2 limited IGF-I action on intestine. The ability of SOCS2 to limit IGF-I/IGF-IR action would be quite novel, since this would suggest that SOCS2 may limit the actions of the IGF-IR tyrosine kinase, as well as its role in the action of GH.

IGF-I has beneficial effects in cases of mucosal atrophy or bowel surgery, yet excess IGF-I levels can lead to colon cancer. Circulating IGF-I levels and IGF binding proteins that modulate the bioavailability of IGF-I impact on risk of colon cancer in humans (56, 126, 141). Using liver-specific IGF-I deficient (LID) mice that have 25% the serum IGF-I levels of wildtype mice, it was found that the presence of IGF-I increases formation, growth, and metastasis of intestinal tumors (47, 223). Therefore, factors that permit the beneficial trophic

actions of IGF-I while limiting its excessive activation may help prevent colon cancer. In support of a role of SOCS2 in limiting IGF-I action, SOCS2 was shown to directly bind IGF-IR and inhibit its activation, along with activation of insulin receptor substrate-1 (IRS-1), in the Caco2 colon carcinoma cell line (120). IRS-1 has been linked to intestinal cancer risk in human epidemiology studies (187) and mouse studies (see Chapter II), suggesting that SOCS2 may protect against colon cancer by inhibiting IRS-1 as well. Previous studies from our laboratory using IGF-I infused SOCS2^{-/-} and SOCS2^{+/+} mice provided direct evidence that SOCS2 deficiency enhanced the proliferative, trophic, and anti-apoptotic actions of IGF-I (120). SOCS2 deficiency also augmented the growth-promoting actions of epidermal growth factor (EGF) on intestine (120), another growth factor linked to colon cancer in humans and mouse mutant studies (51, 115, 124, 163). Furthermore, increased GH secretion in acromegaly correlates with increased risk of colorectal cancer in humans (79, 112), and mice with disruption of one SOCS2 allele that express a GH transgene have increased benign colonic lesions and duodenal adenomas (see Chapter 3). This suggests that during GH excess, also associated with elevated IGF-I, reduced levels of SOCS2 may promote aberrant lesions in the colon. We therefore postulate that SOCS2 may normally protect against cancer by inhibiting the tumorigenic actions of numerous growth factors, including GH, IGF-I, IRS-1, and EGF.

SOCS2 has been linked to cancer susceptibility, particularly in epigenetic studies. A role of SOCS2 in tumor formation is supported by data showing that SOCS2 gene inactivation by hypermethylation of CpG islands within the SOCS2 promoter region correlates with various types of cancer, including prostate, breast, ovarian, and endometrial cancer (30, 49, 52, 68, 190, 203). Other SOCS proteins have been implicated in cancerous

growth as well. SOCS1 is hypermethylated in breast cancer and in glioblastoma cells (235). SOCS3 expression was increased in glioblastoma cells (235) and both SOCS1 and SOCS3 were hypermethylated in Barrett's adenocarcinoma (195). Our laboratory recently demonstrated that epithelial-specific SOCS3 deletion leads to greater tumor load using the azoxymethane/dextran sodium sulfate (AOM/DSS) mouse model of inflammation-induced intestinal cancer (162). This indicates a role for SOCS3 in normally limiting inflammatory bowel disease-related colon cancer. Thus, SOCS3 may have a particular role in protecting against intestinal tumors during inflammation. Despite the evidence that SOCS3 limits inflammation-mediated colon cancer and correlations between SOCS2 levels and colon cancer, the role of SOCS2 in intestinal cancer is not defined.

The current study tested whether SOCS2 plays a role in sporadic intestinal tumor formation in genetically-susceptible mice. We crossbred SOCS2 knockout mice with the *Apc^{Min/+}* model of spontaneous polyposis in intestine and hypothesized that SOCS2 deficiency would lead to greater or larger tumors in this model. Since our laboratory has linked SOCS3 to inflammation-associated tumors using the AOM/DSS model, we also treated SOCS2^{-/-} mice with AOM/DSS to help distinguish the role of SOCS2 in spontaneous versus inflammation-associated cancer. We hypothesized that while SOCS3 deficiency promotes inflammation-mediated colon cancer, SOCS2 deficiency would preferentially affect sporadic, rather than inflammation-mediated, tumorigenesis in normal intestine. SOCS proteins are negative regulators of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, inhibiting signaling by either inactivating JAKs or blocking receptor binding sites for STATs (60, 160). STAT5b is necessary for GH-stimulated IGF-I production, and the body overgrowth phenotype of SOCS2^{-/-} mice is

dependent on STAT5b (34, 59). We therefore postulated that SOCS2 may inhibit intestinal tumorigenesis through inhibition of STAT5 or its homologues STAT1 and STAT3. This hypothesis is consistent with data showing that GH infusion increases STAT5 transcriptional binding activity in intestine, and IGF-I infusion increases STAT3 transcriptional binding activity in mice (119, 120). STAT activity is linked to cancer in other organs (18) but, to our knowledge, STAT activation has not previously been reported in $Apc^{Min/+}$ or β -catenin-driven tumors.

B. Materials and methods

Laboratory animals

$Apc^{Min/+}$ male mice were purchased from Jackson lab, and mice with targeted disruption of the SOCS2 gene were developed as previously described and provided by Dr. Christopher Greenhalgh (118). To generate SOCS2 knockout mice on the $Apc^{Min/+}$ background, $SOCS2^{+/-}$ were crossbred with $Apc^{Min/+}$ mice, and $SOCS2^{+/-}/Apc^{Min/+}$ mice were selected among their progeny. $SOCS2^{+/-}/Apc^{Min/+}$ mice were bred with $SOCS2^{+/-}$ to generate $Apc^{Min/+}$ mice that were $SOCS2^{-/-}$, $SOCS2^{+/-}$ and $SOCS2^{+/+}$. Genotyping was performed on tail DNA using oligomers from a previous publication (119, 163). Males and females across litters were studied for tumor load in the $Apc^{Min/+}$ model to maximize the number of mice per genotype, since studies included $SOCS2^{+/+}$, $SOCS2^{+/-}$, and $SOCS2^{-/-}$. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of North Carolina. Study protocols were in compliance with the Guide for the Care and Use of Laboratory Animals published by the NIH.

Electromobility shift assay (EMSA) for STAT binding in $Apc^{Min/+}$ tumors versus normal tissue

We first compared STAT DNA binding activity in tumors versus normal tissue of $Apc^{Min/+}$ mice to assess if STAT activation accompanied tumor development. Segments of intestine were collected from small intestine and were flushed with ice-cold PBS to removed luminal contents. Tumors were dissected out from remaining tissue under a dissecting scope and both tumor tissue and normal surrounding intestinal tissue were dounced homogenized in 1ml ice-cold 1x Tris-buffered saline for nuclear protein extraction using methods described previously (188). EMSAs were performed as established (188) using 30 μ g protein using double-stranded oligomers corresponding to a consensus STAT1, 3, or 5 binding sequence (Santa Cruz Biotechnology, Santa Cruz, CA).

Tumor scoring in $SOCS2^{+/+}$ and $SOCS2^{-/-}$ mice with the $Apc^{Min/+}$ mutation

At the time of sacrifice, mice were anesthetized and blood was collected by cardiac puncture for hematocrit. The small intestine was separated into 3 pieces and the colon kept intact. Each intestinal region was flushed with PBS, splayed open onto 3MM Whatman paper and fixed in 10% formalin overnight at 4° C. Adenoma number for small and large intestine of each animal was counted under a dissecting scope, using an in-lens micrometer to measure adenoma diameter. Intestinal sections were rolled into a swissroll, paraffin-embedded, and sectioned at 7 μ m. The presence and morphology of adenomas were confirmed by H&E- and β -catenin-staining of swissrolls. Since $Apc^{Min/+}$ mice are known to get severe anemia as disease progresses, hematocrit was measured as an indirect marker for

tumor load and disease severity.

Immunohistochemistry

Dewaxed and rehydrated swissroll sections underwent epitope retrieval in 10mM citrate buffer and were blocked of endogenous peroxidase activity with 3% H₂O₂. Normal blocking serum was used from a Vectastain ABC kit (Vector laboratories, Burlingame, CA) or from a MOM kit for mouse antibodies (BD Transduction laboratories, #610154), followed by incubation with antibodies for CD3 (T cell marker; Dako, Carpinteria, CA), CD45/B220 (B cell marker; BD Biosciences, San Jose, CA), F4/80 (macrophage marker; Abcam #6640, Cambridge, MA), or β -catenin (BD Transduction Laboratories, #610154, Franklin Lakes, NJ). Bound antibody was detected with a Vector Elite kit (Vector laboratories, #PK-61601) or biotinylated anti-mouse IgG from the MOM kit, followed by DAB substrate. Tissues were counterstained in hematoxylin. Primary antibody was omitted as a negative control. When possible, tissues known to highly express or lack proteins of interest were used as positive and negative controls, respectively.

AOM/DSS treatment

Sex- and age-matched SOCS2^{+/+} and SOCS2^{-/-} pairs were given a single intraperitoneal (i.p.) injection of 10 mg/Kg AOM. Seven days later, mice were administered 2.5% DSS for 5 days (TDB Consultancy, Sweden), followed by 14 days recovery on water. This cycle was repeated three times, and mice were monitored for blood in their stool using a Hemocult Sensa kit (Beckman Coulter, Fullerton, CA) to confirm that treated mice were undergoing intestinal inflammation and neoplastic growth. Mice were sacrificed and studied

60 or 80 days following AOM injection.

Statistical analyses

Values for average adenoma number and size were expressed as mean \pm SEM. Comparisons between SOCS2^{+/+}/Min, SOCS2^{+/-}/Min, and SOCS2^{-/-}/Min were not confined to littermate or sex-matched pairs due to the heterogeneity of genotypes derived in each litter. Therefore, ANOVA was also used in Apc^{Min/+} mice to determine if there was a significant interaction between SOCS2 genotype and tumor number, size, or load, as well as percent hematocrit. In AOM/DSS studies, ANOVA was used to determine if there was a significant interaction between tumor number or percent hematocrit and SOCS2 status. $P < 0.05$ was considered to be statistically significant.

C. Results

Increased STAT transcription factor binding in tumor versus normal intestine

Tissue from intestinal tumors and normal tissue of Apc^{Min/+} mice were compared for STAT1, STAT3, and STAT5 promoter binding by electromobility shift assay. As shown in Figure 4.1, preliminary data suggests that tumor tissue may have enhanced binding of nuclear proteins to STAT1, 3, and 5 promoter binding sequences compared to normal tissue. Although additional experiments are required to confirm this finding, this supports the concept that SOCS2, a major regulator of STAT activation, may impact on Apc^{Min/+} tumors.

Increased adenoma in SOCS2 deficient $Apc^{Min/+}$ mice

In small intestine, tumor number significantly increased by over 2-fold in $SOCS2^{-/-}/Min$ and by 1.4-fold in $SOCS2^{+/-}/Min$ compared to $SOCS2^{+/+}/Min$ mice (shown in Figure 4.2 and quantified in Figure 4.3). Tumor number in colon increased 2.8-fold in $SOCS2^{-/-}/Min$ and 1.6-fold in $SOCS2^{+/-}/Min$ compared to $SOCS2^{+/+}/Min$. Tumor size was not significantly affected in small intestine or colon. Tumor number and size were multiplied for small intestine and colon to calculate tumor load, which was increased in all genotypes compared to $SOCS2^{+/+}/Min$ in both small intestine and colon. Hematocrit was measured as a secondary measure of tumor load, indicating significant anemia in $SOCS2^{-/-}/Min$ mice, but not in $SOCS2^{+/+}/Min$ or $SOCS2^{+/-}/Min$ mice (Figure 4.4).

$Apc^{Min/+}$ tumors are not comprised of immune cells

Adenomas in the $Apc^{Min/+}$ model of both $SOCS2^{+/+}$ and $SOCS2^{-/-}$ mice were stained for various immune cell markers, including CD45/B220 (B cells), CD3 (T cells), and F4/80 (macrophages) as shown in Figure 4.5. While Peyer's patches stained positively for B and T cells as expected, $Apc^{Min/+}$ tumors had little or no detectable CD45/B220 or CD3 cell staining, indicating neither $SOCS2^{+/+}$ nor $SOCS2^{-/-}$ adenomas had an upregulation of B or T cells. F4/80, a macrophage marker, was detected in tumors with no obvious difference between staining in the tumors of $SOCS2^{+/+}$ and $SOCS2^{-/-}$ mouse intestine.

No significant difference in AOM/DSS tumorigenesis in SOCS2 deficient mice

Mice that had both SOCS2 alleles deleted ($SOCS2^{-/-}$) and mice with both alleles

intact (SOCS2^{+/+}) mice were given AOM injections as a mutagen, followed by DSS to induce colitis. All mice treated with AOM/DSS had bloody stool and grossly visible hyperplasia and inflammation (Figure 4.6a and b). There was no significant difference between tumor number in the colon (Figure 4.7a) or whole-blood percent hematocrit (Figure 4.7b) between SOCS2^{+/+} and SOCS2^{-/-} mice. Tumors were confirmed histologically by H&E and nuclear β -catenin staining, showing similar histology. Nuclear β -catenin staining was observed in AOM/DSS-induced tumors in both SOCS2^{+/+} and SOCS2^{-/-} mice (Figure 4.6c).

D. Discussion

We explored the potential role of SOCS2 in β -catenin-mediated and inflammation-associated intestinal tumor formation. SOCS2 deficiency increases susceptibility to β -catenin-mediated intestinal tumors, leading to an increase in tumor number in small intestine and colon. Therefore, studies examining the effect of SOCS2 levels on β -catenin transcriptional targets are currently being performed in the laboratory. Despite evidence that SOCS3 is involved in inflammation-associated cancer, SOCS2 deficiency did not lead to greater tumor load in the AOM/DSS model. Furthermore, tumors in the Apc^{Min/+} mice were not rich in B cells or T cells, suggesting that the immune system plays little role in the increased tumor load with SOCS2 deficiency. The intriguing concept that SOCS3 plays a role in limiting inflammation-associated cancer and SOCS2 in sporadic, β -catenin-mediated cancer suggests that studies of the roles of SOCS2 or SOCS3 silencing in sporadic versus inflammation-associated cancer in humans will be of interest.

Since immune cell types commonly found in inflammatory tumors were not

upregulated in the $Apc^{Min/+}$ tumors, we will explore possible mediators of SOCS2 tumorigenic action beyond the pro-inflammatory cytokines typically linked to SOCS2 signaling. We hypothesize that SOCS2 deficiency may promote tumor development by enhancing STAT signaling. STAT3 is constitutively expressed in colon cancer (88). Constitutive activation of STAT3 and STAT5 is associated with cell transformation, tumor formation, and growth in breast cancer, leukemia, and lymphoma tumors and cell lines (18, 54, 123). Furthermore, STAT5b is activated in response to GH (89), and data from our lab shows that GH-induced STAT5 promoter binding is enhanced in SOCS2 deficient mice (119). Our preliminary studies show that STAT1, 3, and 5 promoter binding is increased in $Apc^{Min/+}$ tumors compared to normal surrounding tissue, suggesting that STAT signaling is activated during tumor formation in this model. STAT binding had not previously been examined in this model and such studies were needed prior to studies examining the effect of SOCS2 status on STAT binding activity. We are currently testing whether SOCS2 deficiency impacts on STAT promoter binding in tumors and normal tissue from the $Apc^{Min/+}$ mice.

Additionally, IGF-I and EGF are two main tumor-promoting signaling molecules that are inhibited by SOCS. SOCS2 may normally protect the intestinal epithelium against cancer by inhibiting IGF and/or EGF signaling. SOCS2 deficient mice have augmented intestinal growth in response to both IGF-I and EGF infusion (120). IGF-I is the main mediator of GH action, and we have shown that GH excess can lead to intestinal lesions in SOCS2 deficient mice (see Chapter 3). We have also examined SOCS2 deficient mice expressing an IGF-I transgene and observed preliminary data for numerous pre-cancerous colonic lesions in this model similar to those in the GH transgenics discussed in Chapter 3 (data not shown). These

studies were not pursued due to difficulties in breeding this mouse line and therefore sample size for these data are limited. EGFR deficiency can protect against adenoma development in *Apc^{Min/+}* mice (163), and the EGFR has been found to be overexpressed in cancer cell lines (89). SOCS2 may inhibit EGF signaling by its inhibition of GH action, since GH can transactivate the EGFR (227). SOCS2 could also inhibit EGF activity by inhibiting STAT5b, since EGF has been linked to STAT5b activation (89). Future studies will therefore aim at testing if adenomas formed in SOCS2 deficient mice have increased IGF-I expression, elevated IGF-IR or EGFR activation, or elevated STAT activation.

If IGF-I, EGF, and STATs are all implicated in tumor development in SOCS2 deficient mice, it is possible that SOCS2 may serve as an attractive therapeutic target itself, by simultaneously inhibiting the tumorigenic actions of all three pathways. Cell-permeant (CP) forms of other SOCS have been developed and so far CP-SOCS3 has been shown to limit acute inflammation in liver (81). CP-SOCS2 mimetics could therefore be developed and tested for therapeutic potential in intestinal cancer. Since SOCS2 is normally produced in the body, SOCS2-based therapies, alone or in conjunction with lower-dose chemotherapy, may serve as an attractive cancer therapy in the future.

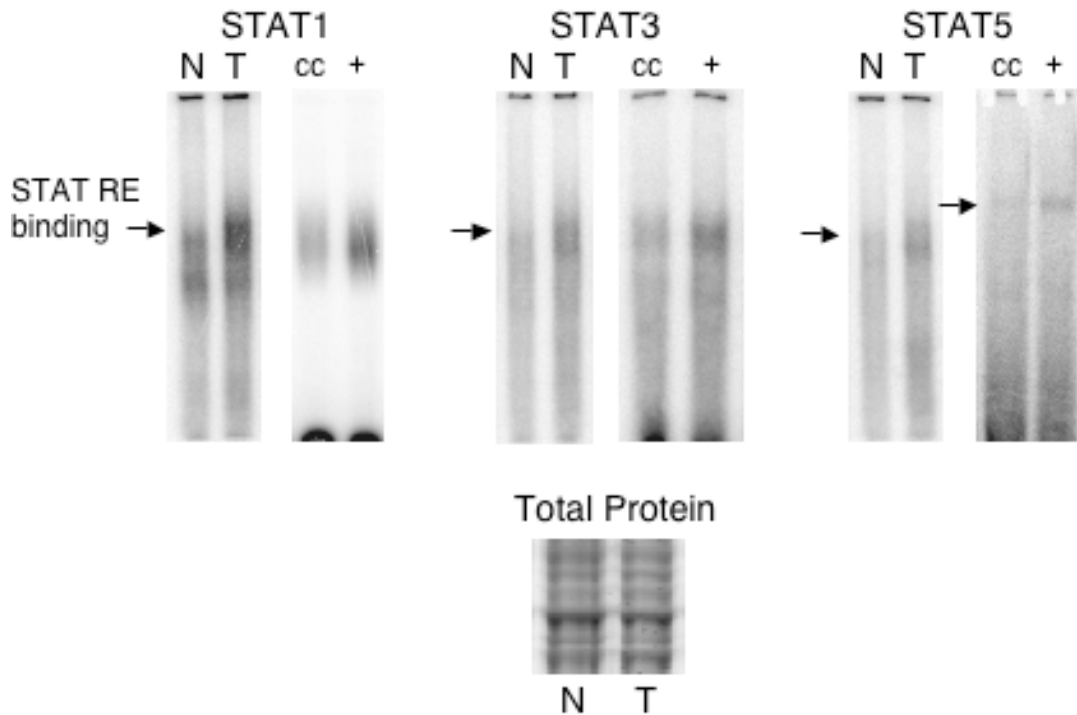


Figure 4.1. Increased STAT1, 3, and 5 transcription factor binding in tumor compared to normal intestine. Electromobility shift assay for STAT1, STAT3, and STAT5 binding in tumors compared to normal tissue from $Apc^{Min/+}$ mouse small intestine. Cold competitor (cc) and a positive control (+) are shown for each response element. Total protein is shown as a loading control. Note the increased STAT1, 3, and 5 binding activity in tumor compared to normal tissue.

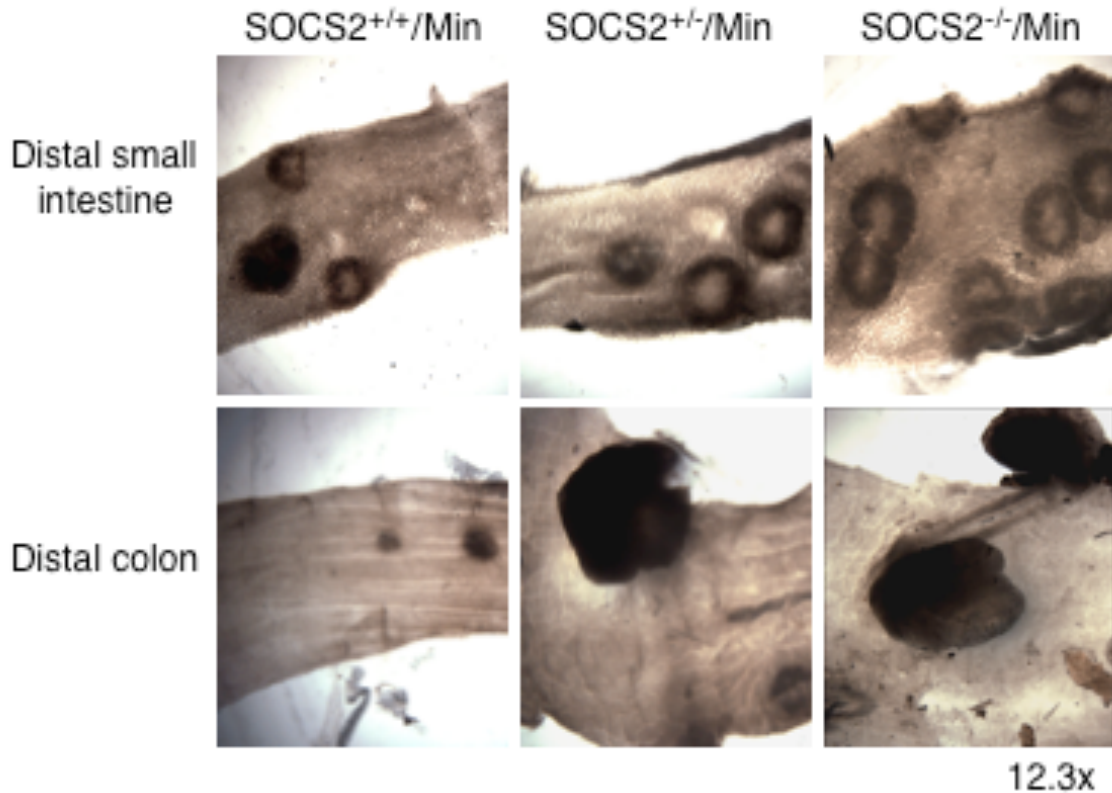


Figure 4.2. Representative images of adenomas in small and large intestine of *Apc*^{Min/+} mice. Adenomas in small intestine (top) and colon (bottom) of *Apc*^{Min/+} that are SOCS2^{+/+}, SOCS2^{+/-}, and SOCS2^{-/-} genotypes are shown. Photographs were taken at 12.3x under a dissecting scope with white light optics underneath the specimen. Note the increase in adenomas in SOCS2 deficient mice.

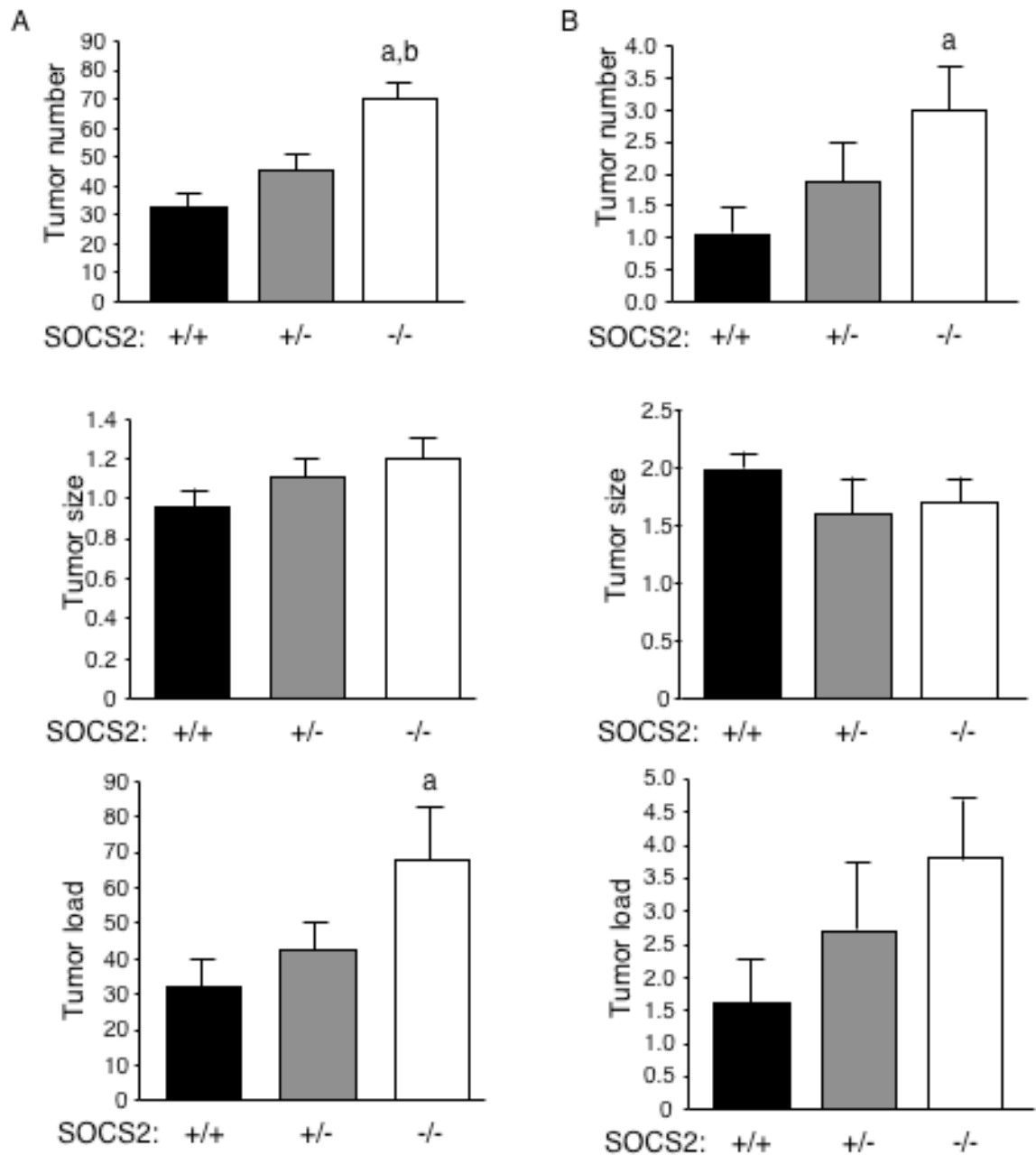


Figure 4.3. Effects of SOCS2 gene disruption on tumors in *Apc*^{Min/+} mice. Histograms show mean tumor number, size, and load in small intestine (a) and colon (b). $N \geq 9$ for *Apc*^{Min/+} mice that were SOCS2^{+/+}, SOCS2^{+/-}, and SOCS2^{-/-}. A= $p < 0.05$ for SOCS2^{-/-}/Min vs. SOCS2^{+/+}/Min and b= $p < 0.05$ for SOCS2^{+/-}/Min vs. SOCS2^{+/+}/Min mice.

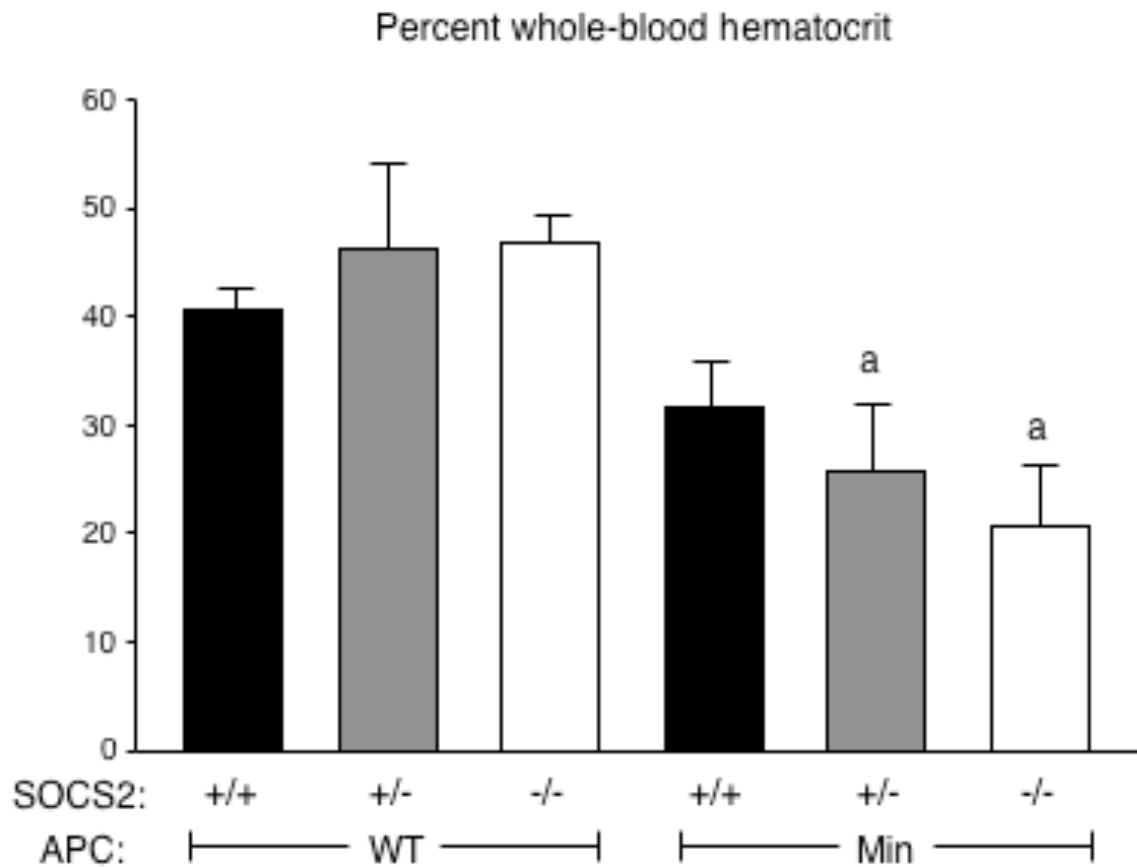


Figure 4.4. Effects of SOCS2 gene disruption on hematocrit in WT or $Apc^{Min/+}$ mice. Histogram of whole blood hematocrit from SOCS2^{+/+}, SOCS2^{+/-}, and SOCS2^{-/-} mice with and without the $Apc^{Min/+}$ mutation as indicated. All hematocrit values were at or near normal range in mice lacking the Min mutation as well as in SOCS2^{+/+}/Min mice (32.8-48.0% hematocrit). SOCS2^{+/-}/Min and SOCS2^{-/-}/Min mice had significantly decreased percent hematocrit levels. a= $p < 0.05$ vs. all non-Min genotypes.

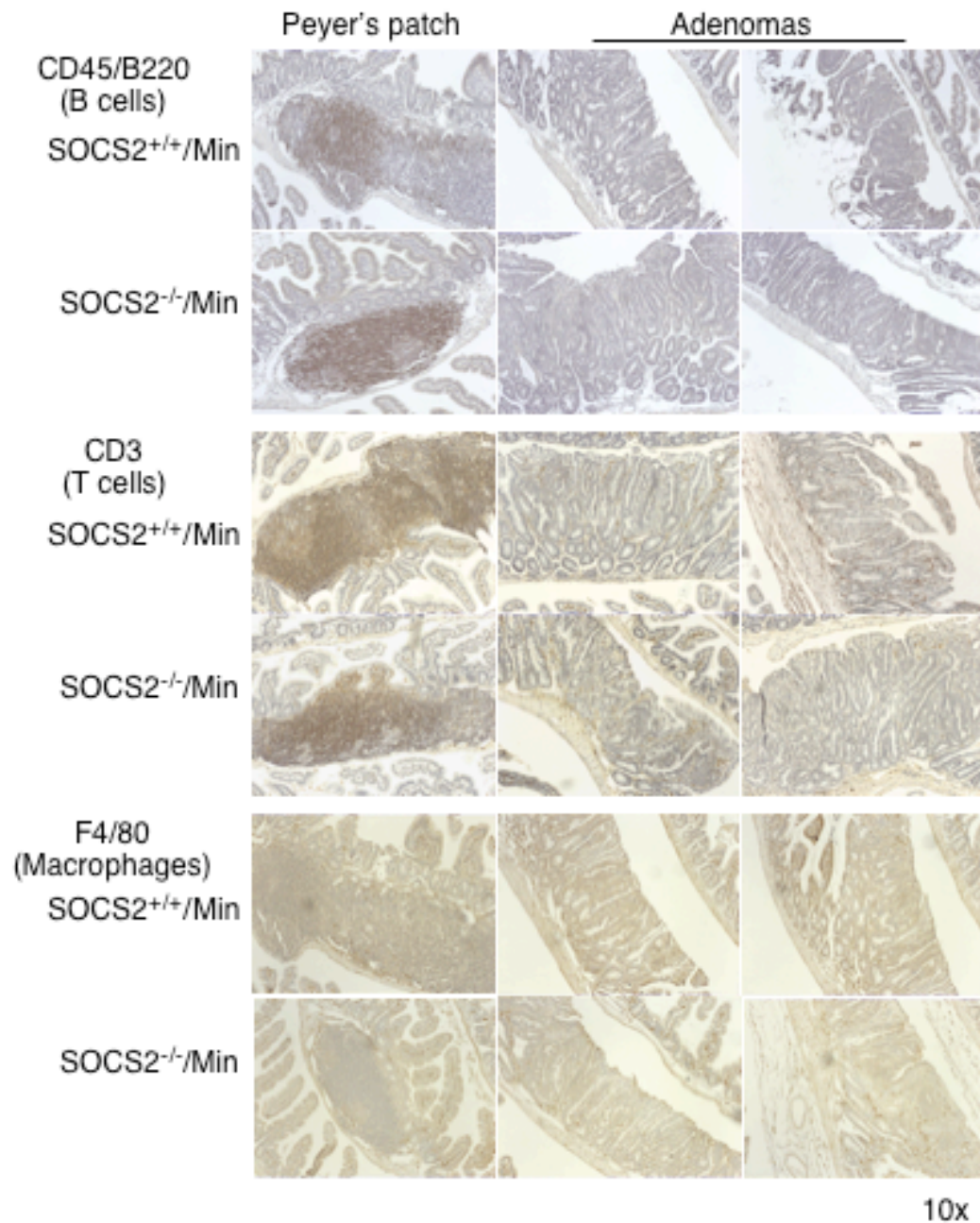


Figure 4.5. Immune cell staining in Peyer's patches and adenomas. SOCS2^{+/+}/Min and SOCS2^{-/-}/Min mouse Peyer's patches and intestinal adenomas stained for B cells with CD45/B220 (A), T cells with CD3 (B), and macrophages with F4/80 antibody (C) are shown at 10x magnification. Note no obvious difference in staining between adenomas of SOCS2^{+/+}/Min and SOCS2^{-/-}/Min mice.

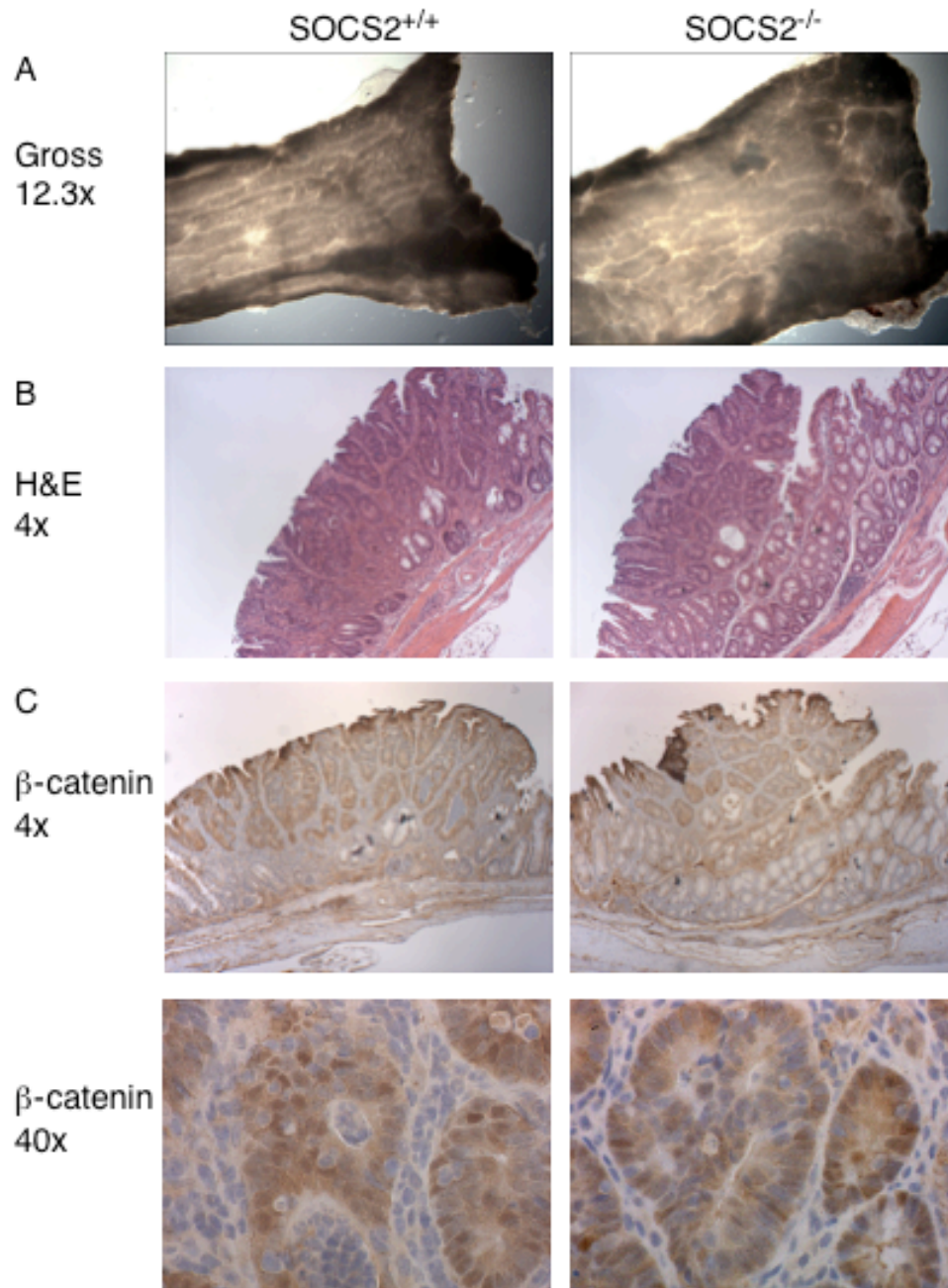


Figure 4.6. Gross and histological images from SOCS2^{+/+} and SOCS2^{-/-} mice treated with AOM/DSS. Representative images show gross morphology (A), H&E (B), and β-catenin at 4x and 40x magnification (C) in SOCS2^{+/+} and SOCS2^{-/-} mice treated with AOM/DSS. Note no noticeable change in tumor number or morphology between SOCS2^{+/+} and SOCS2^{-/-} mouse intestine.

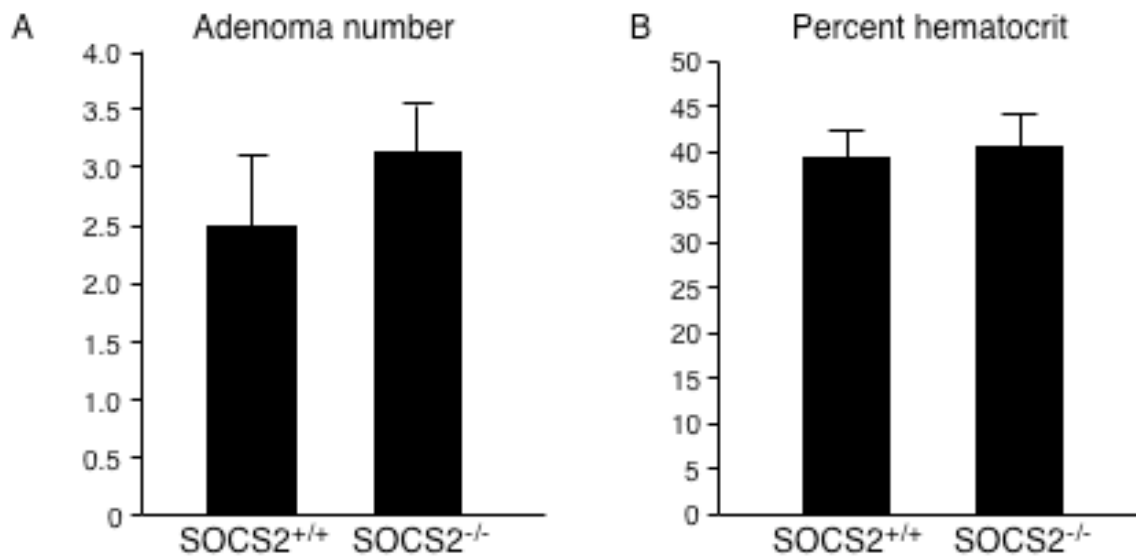


Figure 4.7. No significant change in adenoma number and percent hematocrit in $SOCS2^{+/+}$ compared to $SOCS2^{-/-}$ mice treated with AOM/DSS. Histogram shows mean adenoma number (A) and percent hematocrit (B) for $SOCS2^{+/+}$ compared to $SOCS2^{-/-}$, with no significant change found.

CHAPTER V

GENERAL DISCUSSION

A. Targeting IRS-1 in intestinal tumors

Colon cancer prevalence is most dramatically increasing in developed countries, suggesting that environmental factors likely play a role in its development (56). A strong correlation has been observed between colon cancer and obesity, a major health issue throughout the world (58, 63). Obesity has reached epidemic proportions and is most prevalent in areas that eat a Westernized diet high in fat, processed foods, and refined grains (63, 179). Therefore, numerous studies have been aimed at identifying specific dietary and lifestyle factors related to obesity that predispose to colon cancer, with very few studies providing any substantial links. Dietary factors like fiber, meat, and fat intake have all been tested for their colon cancer preventative properties, with controversial results or little correlation between intake and disease susceptibility (164). While the cancer promoting or protective effects of specific nutrients remain elusive, it has been shown that elevated insulin levels in obese and diabetic individuals are linked to an increased risk of colon cancer (85). Furthermore, high circulating levels of related peptides IGF-I and IGF-II have both been linked to colon cancer (210). Insulin and IGFs all exert their growth-promoting actions predominantly through IRS-1. Therefore, we conducted studies testing if IRS-1 levels could affect crypt apoptosis and tumor formation in mouse intestine.

Chapter II reports our findings that IRS-1 deficiency promotes irradiation-induced apoptosis of potential crypt stem or progenitor cells, a process that most likely protects against clonal expansion of genetically-damaged cells to neoplastic lesions (152, 155). Since our findings suggested a role for IRS-1 in intestinal cancer, IRS-1 deficient mice were crossbred with the *Apc^{Min/+}* model of intestinal polyposis. In these studies, it was found that

even partial IRS-1 deficiency in mice heterozygous for IRS-1 gene disruption protects against β -catenin-mediated tumors. IRS-1 deficiency also correlated with a decrease in protein and mRNA levels of Sox9, a potential stem cell marker known to be involved in colon cancer (12). These experiments indicate that further study of IRS-1 as a biomarker of colon cancer risk or a therapeutic target to prevent or treat intestinal cancer would be of interest. Studies exploring the use of IGF-IR tyrosine kinase inhibitors are already underway in numerous laboratories (73, 205, 229). Inhibiting the IGF-IR, however, could be ineffective since this would not disrupt insulin signaling. Inhibiting insulin signaling may be vital in cancer prevention, since high insulin levels correlate with intestinal adenoma formation, and insulin stimulates breast cancer cells to proliferate (85, 229). IGF-II activation of the insulin receptor has also been shown in a breast cancer cell line (229). While potentially protective against colon cancer, inhibiting both the IGF-IR and the insulin receptor could have detrimental effects on glucose homeostasis (229). By targeting IRS-1, the anti-apoptotic or trophic actions of IGF-I, IGF-II, and insulin (all of which are linked to colon cancer) could be inhibited, while leaving IRS-2 signaling intact. This is favorable because available evidence indicates that trophic and anti-apoptotic effects in intestine are mainly mediated by IRS-1 while IRS-2 is important in maintaining glucose homeostasis and can compensate for the metabolic side effects of loss-of-function of IRS-1 (5).

B. IRS-1/ β -catenin interactions

Once we established that IRS-1 deficiency protects against intestinal adenoma in *Apc^{Min/+}* mice, we wanted to examine the mechanism by which IRS-1 levels modulate

adenoma susceptibility. Original proposed mechanistic studies were based on potential interactions between IRS-1 and β -catenin. Various studies in cell lines indicate that IRS-1 and β -catenin are bound at the membrane, until they translocate to the nucleus upon IGF-I stimulation and activate various cell cycle progression genes downstream of β -catenin (26, 147, 222). This interaction at the membrane may play an important role in cell-cell adhesion, since β -catenin is part of the adherens junction, a dynamic membrane complex comprised of E-cadherin, α -catenin, and actin (44, 225). Further support for a role of IRS-1 in cellular adhesion derives from a study in a neuroblastoma cell line showing that IRS-1 binds β 1-integrins, and IRS-1 protein degradation disrupts this interaction causing the cell to undergo anoikis (86). Not only is a role for IRS-1 in adhesion likely important for cell survival and apoptosis, but it has been shown that adhesion and cytoskeletal components play a key role in tumor metastasis. Studies show in breast cancer metastasis that epithelial cells acquire a more mesenchymal cell phenotype, losing E-cadherin-mediated adhesion at the membrane and expressing mesenchymal cell markers, termed epithelial mesenchymal transition (EMT) (107). This mesenchymal-like cell type is favorable for migration into the blood stream and invading distant organs, where it reverts back to its original epithelial phenotype (107). Whether IRS-1 plays a role in cytoskeletal organization in this EMT process remains unknown.

Although *in vivo* IRS-1/ β -catenin interactions were theoretically an interesting avenue to pursue, preliminary studies proved quite difficult. In immunoprecipitation experiments, β -catenin was found to bind Protein A and G agarose beads in controls that had no IRS-1 or β -catenin antibody added. IRS-1 is not abundant enough in intestinal tissue for immunostaining for colocalization studies. IRS-1 adenovirus overexpression and siRNA

could be an easier potential means of studying IRS-1/ β -catenin interactions, but such studies would simply support already published *in vitro* data and these less novel experiments were not a priority during the studies described here. One study that could be pursued in the laboratory, which has not yet been performed due to a lack of IRS-1^{-/-}/Min mice, is to immunoprecipitate E-cadherin in IRS-1^{+/+} versus IRS-1^{-/-} mouse intestine or isolated crypt protein extracts and immunoblot for β -catenin. This would help determine whether IRS-1 levels play a major role in the stability of the adherens junction, which could be a potential mechanism whereby IRS-1 promotes intestinal tumor formation. Since such studies were dependent on the generation of IRS-1^{-/-}/Min mice, we went on to examine levels of Sox9 in our tumor model, since Sox9 is a key transcriptional target of β -catenin.

C. The intestinal stem cell

Our studies described in Chapter II show that IRS-1 deficiency promotes irradiation-induced apoptosis within the putative stem cell area, a region that is labeled by Sox9 staining. However, much controversy surrounds studies aimed at proving where exactly the intestinal stem cells reside. Label-retaining studies or studies by Potten on susceptibility of stem cells to radiation have been the major method for finding potential stem cells in the past and have indicated that the stem cells reside around positions 3-5 in the crypt (27, 153, 155). Quite recent data claim that cells that express Lgr5, a G-protein coupled receptor (Gpr49) with no known ligand, can give rise to all intestinal cell types in lineage-tracing studies (7). Lgr5/Gpr49 expression is confined to crypt base columnar cells that intercalate between Paneth cells (7). This study introduced a new crypt cell numbering system, whereby the

bottom of the crypt is referred to as 0 and intercalating crypt base columnar cells are numbered 1', 2', and so on, depending on their position. Comparisons of putative stem cell position between these and Potten's studies are made difficult by this new numbering system along with the general heterogeneity of crypt architecture. Therefore, whether Lgr5 marks the same cells that express Sox9 is not yet known. In staining shown in Chapter II, it appears that Sox9 is expressed in crypt cell positions 3-5 and in the intercalating cells between the Paneth cells at the base of the crypt.

Dr. Scott Magness, a collaborator of our laboratory, has made Sox9/eGFP mice that show high and low expressing Sox9/eGFP cells in the intestinal crypts. Low Sox9/GFP cells are small cells at the base of the crypts, intercalated with Paneth cells, virtually identical to the location of Lgr5/Gpr49 'stem cells' reported by Barker et al., 2007 (7). Dr. Magness' lab is currently FACS sorting the high versus low expressing cells for use in microarray studies. Such studies will be important to determine if certain levels of Sox9 correlate with elevated expression of genes associated with stem cells or commitment to particular differentiation pathways as well as cancer. It is possible that low levels of Sox9 keep cells proliferative and undifferentiated as a stem or progenitor cell, while high levels of Sox9 promote differentiation of Paneth and/or goblet cells. Experiments in the lab will compare Lgr5 and Sox9 expression patterns in both WT and IRS-1 deficient intestine, and the effects of IGF-I on survival of Lgr5 and Sox9 positive cells. Because current data suggest that Sox9 labels a greater number of cells within the crypt than Lgr5, it is likely that Lgr5 labels a subset of Sox9 positive cells. An exciting area to pursue in the future will be to differentiate between cells that express both Lgr5 and Sox9 versus only Sox9 to test if Lgr5/Sox9-positive cells show more stem cell and Sox9 only-positive cells show more progenitor cell characteristics.

Even if Lgr5 proves to be a true stem cell marker and Sox9 does not, it will still be imperative to determine the physiological function of Sox9 in normal intestine and in tumors, since it is highly expressed in proliferative crypt compartments and tumors.

To help elucidate if Sox9 or Lgr5 are true stem cell markers, future studies will also determine if Lgr5 or Sox9 expressing cells are capable of regenerating the entire crypt following irradiation. When the intestine is subjected to high dose irradiation, our lab has observed that the majority of crypt cells are lost. After irradiation, small foci of proliferating cells (microcolonies) regenerate crypts and these colonies are believed to arise from clonal expansion of single surviving stem cells. Preliminary studies in our laboratory using Sox9/eGFP mice given high-dose irradiation indicate that microcolonies express the Sox9/eGFP reporter, but Lgr5 expression has not yet been examined. Future studies will confirm this finding and determine whether these Sox9 positive cells are capable of reversing the irradiation-induced mucosal atrophy over time. Our laboratory is currently studying the timecourse by which these cellular foci are formed and when the crypts begin to regenerate. Once the timecourse is established, our lab will examine various characteristics of the cells within the foci, such as Sox9, IRS-1, and Lgr5 expression levels and if IGF-I promotes regeneration by increasing the number of Sox9/eGFP cells or microcolonies. Dr. Magness and Dr. Lund are also developing Sox4/eGFP reporter mice since Sox4 labels a more restricted population of cells in the crypt than Sox9 (Magness, personal communication), and the laboratory is also obtaining the Lgr5/LacZ reporter mouse used by Barker, et al., 2007 (7). These mice will be used in the future to more definitively address the role of the IGF/IRS-1 system in stem cell survival and in tumor-associated stem cells.

D. SOCS2 as a modifier of GH action

GH is an FDA-approved therapy for short bowel syndrome (SBS), a malnutrition and malabsorption syndrome that results from repeated or massive bowel resection. Patients with SBS often require TPN feeding for extended time periods, if not for the patient's entire lifetime. GH stimulates growth of the remainder of the bowel, both in length and mucosal thickness of the gut, leading to improved nutrient absorption that is vital to further intestinal growth and repair (143). GH is also abused in competitive sports due to its stimulatory actions in lean muscle mass growth. Although GH has beneficial effects, excess levels of GH or its downstream mediator IGF-I are linked to increased risk of intestinal neoplasia. Increased GH secretion in acromegaly correlates with increased risk of colorectal cancer in humans (79, 112). Therefore, understanding the mechanisms whereby GH can promote mucosal growth and repair during atrophy or pathological settings, while not becoming excessive and promoting cancerous growth, are vital.

Studies of SOCS2 in our lab have stemmed from findings in TPN-fed rats given GH or IGF-I infusion. TPN fed rats showed severe mucosal atrophy, which was almost completely reversed by IGF-I infusion (121, 146). However, rats given GH infusion, in which circulating IGF-I levels were similar to those in IGF-I infused rats, did not show mucosal regrowth. Screening for mRNA levels of the SOCS family of proteins showed that SOCS2 was expressed following GH treatment, but not IGF-I treatment. These studies led to our later proven hypothesis that SOCS2 is a GH-inducible inhibitor of its own actions (121). The more potent effects of IGF-I infusion suggest that IGF-I may serve as a more effective therapy for SBS than GH. However, despite promising intestinal adaptive growth effects of

IGF-I in animal models, IGF-I therapy has numerous unwanted side effects preventing it from becoming an approved therapy, including generalized edema, arthralgia, myalgia, hypotension, and intracranial hypertension (122, 156, 204). GH is most likely a safer therapy due to its induction of SOCS2, which could provide the balance between the adaptive growth actions of GH while limiting excess growth that could lead to neoplasia.

As described in Chapter III, GH transgenic expression in SOCS2^{+/-} mice increased overall body size as well as numerous growth parameters in the intestinal mucosa, including villus height, crypt depth, increased proliferation, decreased apoptosis and decreased differentiation. One potential mechanism of GH action in intestine of SOCS2 deficient mice is increased transcriptional activity of STATs. Our data show increased STAT5 activity in response to GH, which is further increased with SOCS2 deficiency. Overall, these studies suggest that SOCS2 expression levels in humans may help determine the efficacy of GH therapy in SBS or growth delay. Responses to GH infusion in SBS patients have been variable (207). We have samples from SBS patients given GH, and future studies will test if levels of SOCS2 predict responses to therapy.

E. Mechanisms of SOCS2 action in intestinal cancer

In Chapter III, SOCS2 deficient mice expressing a GH transgene had increased hyperplastic and lymphoid lesions. These lesion types are generally accepted as benign and not pre-cancerous in humans, but their potential to become cancerous in mice is unknown. These mice were aged to determine if these colonic polyps could progress to a more pre-cancerous or cancerous phenotype and to determine if varying SOCS2 levels could further

impact on the growth-promoting actions of GH excess over time. These lesions did not progress to a pre-cancerous phenotype, nor did the tumor multiplicity in SOCS2 deficient compared to WT mice with GH excess further diverge with aging. Despite limited sample size, one interesting observation was the formation of numerous adenomatous lesions in SOCS2^{+/-}/GH-TG mice and an invasive adenocarcinoma in the one generated SOCS2^{-/-}/GH-TG mouse. This suggests that during GH excess, reduced levels of SOCS2 promote aberrant lesions in intestine.

To test the potential role of SOCS2 alone in intestinal tumorigenesis, we crossbred SOCS2 deficient mice with the Apc^{Min/+} tumor model. Our data in Chapter IV shows that SOCS2 deficiency increases adenoma in Apc^{Min/+} mice and are consistent with epidemiological data found in humans that SOCS2 silencing by epigenetic hypermethylation is linked to various cancer types, including endometrial and ovarian (52, 190). We used the Apc^{Min/+} mouse model since it is a model of pre-cancerous adenoma, and the protective effects we observed of SOCS2 in this model support a role of SOCS2 specifically in early adenoma formation. We also tested if SOCS2 deficiency could increase susceptibility to inflammation-associated colon tumors using the AOM/DSS model, since intestinal epithelial-specific knockout of SOCS3 leads to an increase in inflammation-associated tumorigenesis (162). There was no significant difference in tumors in SOCS2^{-/-} or SOCS2^{+/-} tumors using this model, suggesting that the role of SOCS2 in inflammation-associated neoplasia is limited.

Future studies in the laboratory will be aimed at determining the mechanism by which SOCS2 deficiency leads to increased tumors. Tumors in the SOCS2/Apc^{Min/+} and GH/SOCS2 models will be assessed for phosphoEGFR, phosphoIGF-IR, and signaling

molecules downstream of β -catenin-mediated transcription by immunohistochemistry, Western immunoblotting, or quantitative RT-PCR. An interesting experiment would be to crossbreed IRS-1 deficient mice with SOCS2 deficient mice to determine if IRS-1 deficiency reverses the increased tumor phenotype in SOCS2 deficient mice, since SOCS2 has been shown to inhibit IRS-1 activation (120). Because IRS-1^{-/-} mice are born at a low frequency and breeding would be complicated by a triple-crossbreeding strategy (IRS-1 knockout, SOCS2 knockout, and Apc^{Min/+}), such studies might be limited to IRS-1^{+/-} rather than IRS-1^{-/-}. IRS-1^{+/-} mice may have sufficient IRS-1 levels for SOCS2 deficiency to promote tumors via the IGF/IRS pathway. Thus, simpler studies could be performed by treating SOCS2^{-/-}/Min mice with IGF-IR kinase inhibitors like VP-AEW541 or PPP, which are commercially available (129, 176). Ideally, these studies would be performed using a cell-permeant IRS-1 inhibitor, to most closely mimic the effect in IRS-1^{-/-} mice, however IRS-1 inhibitors have not yet been generated. Since SOCS2 may protect against cancer by inhibiting the EGF pathway as well, SOCS2^{-/-} mice could be treated with EGFR inhibitors, such as the commercially-available mouse-human chimeric EGFR monoclonal antibody Cetuximab (13), to determine if inhibiting this pathway could reverse the effect of disrupting SOCS2 signaling.

Increasing evidence suggests that SOCS2 may serve as an endogenous inhibitor of the trophic actions of IRS-1 and manipulating SOCS2 levels may serve as a safer and more effective mode of cancer prevention therapy than introducing drugs that are not normally produced in the body. Our studies show that SOCS2 deficiency leads to greater susceptibility to intestinal tumors, providing further support for its normal role as a tumor

suppressor. Since SOCS2 has been linked to several tumor-promoting signaling pathways, including GH, IGF-I, IRS-1, EGF, and STATs, SOCS2 may have greater efficacy in preventing or treating colon cancer than one inhibitor alone. Furthermore, SOCS2 mimetics may be useful in combination with other non-specific modes of treatment, such as chemotherapy. The major limitation of chemotherapeutic treatment of cancer are the side-effects that occur, including nausea, diarrhea, and malabsorption due to the toxic effects on the intestinal epithelium. In combination with SOCS2 mimetics, chemotherapy may be effective at lower doses, limiting harmful side effects. Our laboratory is interested in testing cell-permeant SOCS2 mimetics (CP-SOCS2) for prevention or treatment of neoplastic growth. CP-SOCS2 mimetics in cell lines will likely be crucial in determining the pathways that SOCS2 modulates, by testing for activated IGF-I, IRS-1, EGF, and STAT transcriptional activity. In the future, we also wish to test the efficacy of CP-SOCS2 in the $Apc^{Min/+}$ model of polyposis.

F. Relevance of the $Apc^{Min/+}$ model

We chose the $Apc^{Min/+}$ model of intestinal polyposis to study the role of IRS-1 and SOCS2 in intestinal adenoma since chemical carcinogen-induced intestinal tumors have a long latency and low penetrance, and tumor xenografts in nude mice lack the tumor microenvironment typical of a tumor that is formed *in situ* (3). However, one potential caveat of our studies is that the $Apc^{Min/+}$ mouse model develops polyposis mainly in the small intestine, while humans develop *APC*-mediated polyposis in the colon. While this difference between humans and mice may limit the human relevance of our findings, it is also possible

that because the mouse small intestine and human colon are both particularly susceptible to β -catenin mediated tumors, the mouse small intestine may have crucial genetic similarities to human colon that make it useful for studying colon cancer. Since this is difficult to test, performing additional studies in PIRC (polyposis in the rat colon) rats that get colon tumors may be of considerable interest. PIRC rats have a knockout allele of the *Apc* gene and mimic human colon cancer, showing the ability to progress to invasive adenocarcinoma with age and displaying sexual dimorphism, with tumor prevalence moderately higher in males compared to females (3). This rat model could be ideal for longitudinal studies of the effect of IRS-1 and SOCS2 inhibitors or mimetics over time, since they have proven useful in MicroCT and endoscopy studies (3).

G. Identifying cancer pathways through tumor microarrays

Microarray studies performed in the Threadgill laboratory at UNC-CH suggest that tumors that form in *Apc*^{Min/+} mice despite an EGFR inactivation mutation (EGFR^{wa2}) have elevated IGFBP5 and IGF-IR expression (communication with Dr. Ming Yu and Dr. David Threadgill). Experiments testing whether tumors that form in *Apc*^{Min/+} mice despite abrogated IGF system signaling (such as in IRS-1 deficient mice) have enhanced EGF signaling will be crucial to determine if IGF-I and EGF signal, at least partially, through independent tumor-promoting pathways. Studies in our laboratory will test whether treating IRS-1 deficient mice with the *Apc*^{Min/+} mutation with an EGFR inhibitor decreases tumor formation to a greater extent than IRS-1 deficiency or EGFR inhibition alone. If tumors still form despite inhibiting both the IRS-1 and EGFR pathways, our lab will perform microarrays

on the remaining tumors to identify any novel genes contributing to the tumor phenotype. Since SOCS2 may serve as a potent endogenous inhibitor of IGF-I and EGF signaling simultaneously, it would be desirable to examine $Apc^{Min/+}$ mice treated with CP-SOCS2 for tumors. Any tumors arising despite inhibition of pro-tumorigenic pathways by SOCS2 action could be examined by microarray to help explore how such tumors can “escape” inhibition of regulatory pathways and elucidate the pathways in which SOCS2 may be uninvolved or only weakly involved.

H. Biomarkers for colon cancer screening

The current studies have important implications for prevention and treatment of colon cancer. IRS-1 levels may determine susceptibility to colon cancer and could potentially serve as a biomarker in screening for genetic factors that increase one's risk of developing the disease. Identifying relative colon cancer susceptibility could help health care professionals focus their screening on ‘high risk’ groups, leading to earlier diagnosis and saving on health care costs. By screening for mutations or polymorphisms of genes involved in colon cancer, dietary, lifestyle, and screening recommendations could be tailored to an individual's relative risk.

Our laboratory is involved in the North Carolina colon cancer screening study led by Dr. Robert Sandler, in which patients with no prior history of colon cancer undergo routine colonoscopy. Patients are classified as a case if an adenoma was found or as a control if not, while patients in which cancer was detected are excluded. During colonoscopy, normal mucosa is biopsied to determine a “field effect” of certain lifestyle or genetic factors in

susceptibility to low apoptosis and adenoma formation in cases versus controls and blood is collected to assay circulating hormone and lipid profiles. These studies have already shown a significant link between less frequent NSAID use, low levels of apoptosis, high but within the normal range of insulin, and high IGFBP3 in adenoma (85, 110).

A human polymorphism in IRS-1 (G972R) is associated with increased risk of colon cancer (187), but the physiological effect of this polymorphism is not known. This polymorphism is found in a portion of the IRS-1 coding sequence that is not conserved between humans and mice. Direct test of the effect of this polymorphism would be limited to human cell lines, in which one could explore differences in response to IGF-I, proliferation, apoptosis, and anchorage-independent growth in cells with and without the polymorphism. However, such studies would have to be conducted in human colon cancer cell lines, since normal epithelial cell lines are not available. Future studies in our laboratory will therefore determine if the G972R polymorphism in IRS-1 is linked to an increase in local IRS-1 production in human colon biopsy samples. We postulate that the G972R IRS-1 polymorphism leads to increased colon cancer risk by increasing the levels or activity of IRS-1. Levels of Sox9 will also be assessed to determine if Sox9 levels in the intestinal mucosa are indicative of adenoma susceptibility, since Sox9 levels mimicked IRS-1 status in our mouse studies. In addition, SOCS2 levels in mucosal biopsies will be assessed to determine if elevated SOCS2 levels correlate with decreased adenoma development. We will also explore whether SOCS2 silencing by methylation of CpG islands within the SOCS2 promoter correlates with intestinal adenoma risk in humans undergoing routine colonoscopy. These studies are aimed at correlating early pre-cancerous adenoma with SOCS2 methylation in patients with no previous history of colon cancer to help determine the molecular

mechanisms whereby individuals develop the very earliest stages of intestinal neoplasia.

I. Summary and working model

Data provided in this body of work are summarized in our current working model of IRS-1 and SOCS2 action in intestinal tumorigenesis, as shown in Figure 5.1. In our proposed working model, IGF-I, IGF-II, and insulin are produced locally in intestine or are released in the circulation and act in an endocrine manner by binding the insulin receptor or the IGF-IR. IRS-1 lies downstream of all three ligands and docks to the activated receptor and becomes phosphorylated. IRS-1 promotes proliferation and prevents apoptosis of intestinal epithelial cells. Our studies in Chapter II show that the presence of IRS-1 protects against apoptosis of potential progenitor/stem cells and promotes intestinal polyposis. The exact mechanism of IRS-1 action remains unknown, although studies described here show that Sox9 levels and Sox9 expressing cells are reduced by IRS-1 deficiency, suggesting that Sox9 could be a tumor-promoting transcription factor downstream of IRS-1. Other potential candidates that need further exploration are association of IRS-1 with β -catenin and E-cadherin, as well as activation of β -catenin transcriptional targets such as Myc and Cyclin D. SOCS2 has been shown to inhibit GH (Chapter III) and IGF-I/IRS-1 (120) action, thereby leading to growth inhibition and early differentiation. SOCS2 deficiency leads to greater tumor number in intestine, indicating that SOCS2 normally inhibits tumorigenesis. In addition to GH and IGF/IRS inhibition, SOCS2 may protect against intestinal tumors by inhibiting EGF or STAT signaling, both of which have been shown to be major pathways in the pathogenesis of colon cancer (18, 115). A key aim of future studies in the laboratory will be to determine the

molecular mechanisms whereby SOCS2 protects against intestinal tumorigenesis.

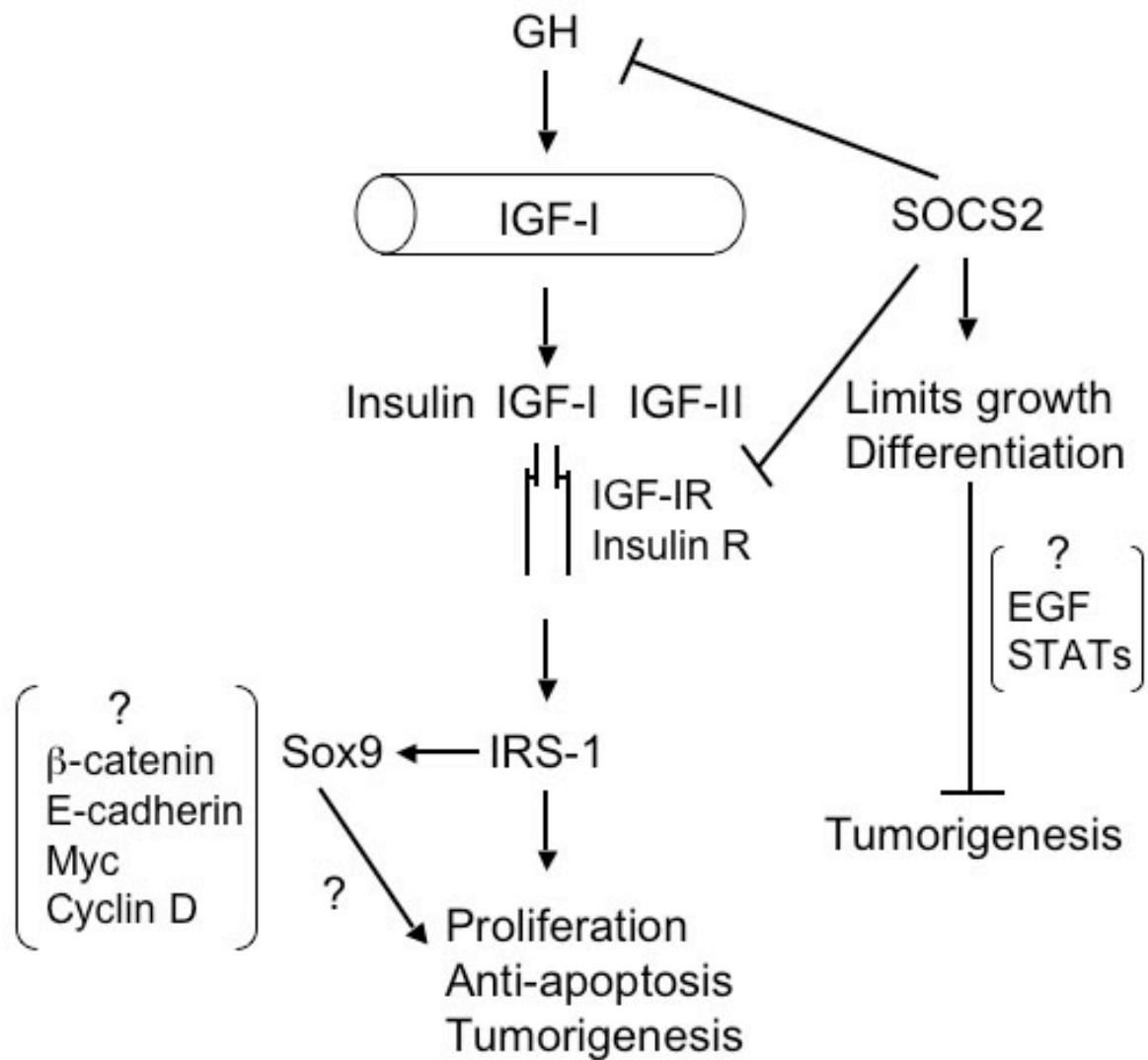


Figure 5.1. Hypothetical model of IGF/IRS and SOCS2 action in intestinal growth and tumorigenesis. Future directions will be aimed at understanding mechanisms of IRS-1 and Sox9 action in normal and aberrant intestinal growth as well as SOCS2 action in tumorigenesis.

CHAPTER VI

APPENDIX

A. List of publications

The list below provides citations of the publications to which Nicole M. Ramocki has contributed. Percent contribution by Nicole M. Ramocki is noted in parentheses.

1. Ramocki NM, Wilkins HR, Magness ST, Lee GH, Scull BP, McNaughton KK, Lund PK. Insulin receptor substrate-1 (IRS-1) inhibits apoptosis in the crypt stem cell region and promotes intestinal adenoma in mice. *Endocrinology* 2008 Jan;149(1):261-7. (90%)
2. Zhang H, Morgan D, Cecil G, Burkholder A, Ramocki N, Scull B, Lund PK. Biochromoendoscopy: Molecular imaging with capsule endoscopy for detection of polypoid lesions in the GI tract. *Gastrointestinal Endoscopy*. Accepted. (15%)
3. Michaylira CZ, Simmons JG, Ramocki NM, Scull BP, McNaughton KK, Fuller CR, Lund PK. Suppressor of cytokine signaling-2 limits intestinal growth and enterotrophic actions of IGF-I *in vivo*. *Am J Physiol Gastrointest Liver Physiol*. 2006 Sep;291(3): G472-81. (15%)
4. Michaylira CZ, Ramocki NM, Simmons JG, Tanner CK, McNaughton KK, Woosley JT, Greenhalgh CJ, Lund PK. Haplotype insufficiency for suppressor of cytokine signaling-2 enhances intestinal growth and promotes polyp formation in growth hormone-transgenic mice. *Endocrinology*. 2006 Apr;147(4): 1632-41. (20%)

B. References

1. Akiyama Ddagger H, Kim Ddagger JE, Nakashima K, Balmes G, Iwai N, Deng JM, Zhang Z, Martin JF, Behringer RR, Nakamura T, and de Crombrughe B. Osteochondroprogenitor cells are derived from Sox9 expressing precursors. *Proc Natl Acad Sci U S A* 102: 14665-14670, 2005.
2. Alves RH, Vaisman M, Brasil RR, and Gadelha MR. Acromegaly and non-Hodgkin's lymphoma. *Endocr Pract* 4: 279-281, 1998.
3. Amos-Landgraf JM, Kwong LN, Kendzierski CM, Reichelderfer M, Torrealba J, Weichert J, Haag JD, Chen KS, Waller JL, Gould MN, and Dove WF. A target-selected Apc-mutant rat kindred enhances the modeling of familial human colon cancer. *Proc Natl Acad Sci U S A* 104: 4036-4041, 2007.
4. Andre F, Janssens B, Bruyneel E, van Roy F, Gespach C, Mareel M, and Bracke M. Alpha-catenin is required for IGF-I-induced cellular migration but not invasion in human colonic cancer cells. *Oncogene* 23: 1177-1186, 2004.
5. Araki E, Lipes MA, Patti ME, Bruning JC, Haag B, 3rd, Johnson RS, and Kahn CR. Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature* 372: 186-190, 1994.
6. Bach SP, Renehan AG, and Potten CS. Stem cells: the intestinal stem cell as a paradigm. *Carcinogenesis* 21: 469-476, 2000.
7. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, and Clevers H. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449: 1003-1007, 2007.
8. Bartke A. Minireview: role of the growth hormone/insulin-like growth factor system in mammalian aging. *Endocrinology* 146: 3718-3723, 2005.
9. Bastide P, Darido C, Pannequin J, Kist R, Robine S, Marty-Double C, Bibeau F, Scherer G, Joubert D, Hollande F, Blache P, and Jay P. Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. *J Cell Biol* 178: 635-648, 2007.
10. Beck F, Chawengsaksophak K, Luckett J, Giblett S, Tucci J, Brown J, Poulsom R, Jeffery R, and Wright NA. A study of regional gut endoderm potency by analysis of Cdx2 null mutant chimaeric mice. *Dev Biol* 255: 399-406, 2003.
11. Bjerknes M, and Cheng H. Gastrointestinal stem cells. II. Intestinal stem cells. *Am J Physiol Gastrointest Liver Physiol* 289: G381-387, 2005.

12. Blache P, van de Wetering M, Duluc I, Domon C, Berta P, Freund JN, Clevers H, and Jay P. SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. *J Cell Biol* 166: 37-47, 2004.
13. Blick SK, and Scott LJ. Cetuximab : a review of its use in squamous cell carcinoma of the head and neck and metastatic colorectal cancer. *Drugs* 67: 2585-2607, 2007.
14. Bodmer WF. Cancer genetics: colorectal cancer as a model. *J Hum Genet* 51: 391-396, 2006.
15. Bogazzi F, Russo D, Locci MT, Chifenti B, Ultimieri F, Raggi F, Cosci C, Sardella C, Costa A, Gasperi M, Bartalena L, and Martino E. Apoptosis is reduced in the colonic mucosa of patients with acromegaly. *Clin Endocrinol (Oxf)* 63: 683-688, 2005.
16. Boutron-Ruault MC, Senesse P, Meance S, Belghiti C, and Faivre J. Energy intake, body mass index, physical activity, and the colorectal adenoma-carcinoma sequence. *Nutr Cancer* 39: 50-57, 2001.
17. Boverhof DR, Tam E, Harney AS, Crawford RB, Kaminski NE, and Zacharewski TR. 2,3,7,8-Tetrachlorodibenzo-p-dioxin induces suppressor of cytokine signaling 2 in murine B cells. *Mol Pharmacol* 66: 1662-1670, 2004.
18. Bowman T, Garcia R, Turkson J, and Jove R. STATs in oncogenesis. *Oncogene* 19: 2474-2488, 2000.
19. Brink M, de Goeij AF, Weijenberg MP, Roemen GM, Lentjes MH, Pachen MM, Smits KM, de Bruine AP, Goldbohm RA, and van den Brandt PA. K-ras oncogene mutations in sporadic colorectal cancer in The Netherlands Cohort Study. *Carcinogenesis* 24: 703-710, 2003.
20. Buchanan FG, Holla V, Katkuri S, Matta P, and DuBois RN. Targeting cyclooxygenase-2 and the epidermal growth factor receptor for the prevention and treatment of intestinal cancer. *Cancer Res* 67: 9380-9388, 2007.
21. Bullock AN, Debreczeni JE, Edwards AM, Sundstrom M, and Knapp S. Crystal structure of the SOCS2-elongin C-elongin B complex defines a prototypical SOCS box ubiquitin ligase. *Proc Natl Acad Sci U S A* 103: 7637-7642, 2006.
22. Calo V, Migliavacca M, Bazan V, Macaluso M, Buscemi M, Gebbia N, and Russo A. STAT proteins: from normal control of cellular events to tumorigenesis. *J Cell Physiol* 197: 157-168, 2003.
23. Chaboissier MC, Kobayashi A, Vidal VI, Lutzkendorf S, van de Kant HJ,

Wegner M, de Rooij DG, Behringer RR, and Schedl A. Functional analysis of Sox8 and Sox9 during sex determination in the mouse. *Development* 131: 1891-1901, 2004.

24. Chang Q, Li Y, White MF, Fletcher JA, and Xiao S. Constitutive activation of insulin receptor substrate 1 is a frequent event in human tumors: therapeutic implications. *Cancer Res* 62: 6035-6038, 2002.

25. Chang SC, Lin JK, Lin TC, and Liang WY. Loss of heterozygosity: an independent prognostic factor of colorectal cancer. *World J Gastroenterol* 11: 778-784, 2005.

26. Chen J, Wu A, Sun H, Drakas R, Garofalo C, Cascio S, Surmacz E, and Baserga R. Functional significance of type 1 insulin-like growth factor-mediated nuclear translocation of the insulin receptor substrate-1 and beta-catenin. *J Biol Chem* 280: 29912-29920, 2005.

27. Cheng H, and Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. *Am J Anat* 141: 537-561, 1974.

28. Cheung M, and Briscoe J. Neural crest development is regulated by the transcription factor Sox9. *Development* 130: 5681-5693, 2003.

29. Chrysis D, Calikoglu AS, Ye P, and D'Ercole AJ. Insulin-like growth factor-I overexpression attenuates cerebellar apoptosis by altering the expression of Bcl family proteins in a developmentally specific manner. *J Neurosci* 21: 1481-1489, 2001.

30. Cooper JC, Boustead JN, and Yu CL. Characterization of STAT5B phosphorylation correlating with expression of cytokine-inducible SH2-containing protein (CIS). *Cell Signal* 18: 851-860, 2006.

31. Dahly EM, Guo Z, and Ney DM. IGF-I augments resection-induced mucosal hyperplasia by altering enterocyte kinetics. *Am J Physiol Regul Integr Comp Physiol* 285: R800-808, 2003.

32. Dahly EM, Miller ME, Lund PK, and Ney DM. Postreceptor resistance to exogenous growth hormone exists in the jejunal mucosa of parenterally fed rats. *J Nutr* 134: 530-537, 2004.

33. Dann SM, and Eckmann L. Innate immune defenses in the intestinal tract. *Curr Opin Gastroenterol* 23: 115-120, 2007.

34. Davey HW, Xie T, McLachlan MJ, Wilkins RJ, Waxman DJ, and Grattan DR. STAT5b is required for GH-induced liver IGF-I gene expression. *Endocrinology* 142: 3836-3841, 2001.

35. Dearth RK, Cui X, Kim HJ, Kuitse I, Lawrence NA, Zhang X, Divisova J, Britton OL, Mohsin S, Allred DC, Hadsell DL, and Lee AV. Mammary Tumorigenesis and Metastasis Caused by Overexpression of Insulin Receptor Substrate 1 (IRS-1) or IRS-2. *Mol Cell Biol* 26: 9302-9314, 2006.
36. Dekaney CM, Fong JJ, Rigby RJ, Lund PK, Henning SJ, and Helmrath MA. Expansion of intestinal stem cells associated with long-term adaptation following ileocecal resection in mice. *Am J Physiol Gastrointest Liver Physiol* 293: G1013-1022, 2007.
37. Desbois-Mouthon C, Cadoret A, Blivet-Van Eggelpoel MJ, Bertrand F, Cherqui G, Perret C, and Capeau J. Insulin and IGF-1 stimulate the beta-catenin pathway through two signalling cascades involving GSK-3 β inhibition and Ras activation. *Oncogene* 20: 252-259, 2001.
38. Dey BR, Spence SL, Nissley P, and Furlanetto RW. Interaction of human suppressor of cytokine signaling (SOCS)-2 with the insulin-like growth factor-I receptor. *J Biol Chem* 273: 24095-24101, 1998.
39. Di Popolo A, Memoli A, Apicella A, Tuccillo C, di Palma A, Ricchi P, Acquaviva AM, and Zarrilli R. IGF-II/IGF-I receptor pathway up-regulates COX-2 mRNA expression and PGE2 synthesis in Caco-2 human colon carcinoma cells. *Oncogene* 19: 5517-5524, 2000.
40. DiBaise JK, Young RJ, and Vanderhoof JA. Intestinal rehabilitation and the short bowel syndrome: part 1. *Am J Gastroenterol* 99: 1386-1395, 2004.
41. Dionigi G, Bianchi V, Villa F, Rovera F, Boni L, Annoni M, Castano P, and Dionigi R. Differences between familial and sporadic forms of colorectal cancer with DNA microsatellite instability. *Surg Oncol* 2007.
42. Doney AS, Fischer B, Leese G, Morris AD, and Palmer CN. Cardiovascular risk in type 2 diabetes is associated with variation at the PPARG locus: a Go-DARTS study. *Arterioscler Thromb Vasc Biol* 24: 2403-2407, 2004.
43. Draper HH, and Bettger WJ. Role of nutrients in the cause and prevention of oxygen radical pathology. *Adv Exp Med Biol* 366: 269-289, 1994.
44. Drees F, Pokutta S, Yamada S, Nelson WJ, and Weis WI. Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell* 123: 903-915, 2005.
45. Durai R, Yang W, Gupta S, Seifalian AM, and Winslet MC. The role of the insulin-like growth factor system in colorectal cancer: review of current knowledge. *Int J Colorectal Dis* 20: 203-220, 2005.

46. Duran B. The effects of long-term total parenteral nutrition on gut mucosal immunity in children with short bowel syndrome: a systematic review. *BMC Nurs* 4: 2, 2005.
47. Ealey KN, Xuan W, Lu S, and Archer MC. Colon carcinogenesis in liver-specific IGF-I-deficient (LID) mice. *Int J Cancer* 122: 472-476, 2007.
48. Esteller M. Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum Mol Genet* 16 Spec No 1: R50-59, 2007.
49. Farabegoli F, Ceccarelli C, Santini D, and Taffurelli M. Suppressor of cytokine signalling 2 (SOCS-2) expression in breast carcinoma. *J Clin Pathol* 58: 1046-1050, 2005.
50. Ferone D, Resmini E, Bocca L, Giusti M, Barreca A, and Minuto F. Current diagnostic guidelines for biochemical diagnosis of acromegaly. *Minerva Endocrinol* 29: 207-223, 2004.
51. Fichera A, Little N, Jagadeeswaran S, Dougherty U, Sehdev A, Mustafi R, Cerda S, Yuan W, Khare S, Tretiakova M, Gong C, Talerico M, Cohen G, Joseph L, Hart J, Turner JR, and Bissonnette M. Epidermal growth factor receptor signaling is required for microadenoma formation in the mouse azoxymethane model of colonic carcinogenesis. *Cancer Res* 67: 827-835, 2007.
52. Fiegl H, Gatringer C, Widschwendter A, Schneitter A, Ramoni A, Sarlay D, Gaugg I, Goebel G, Muller HM, Mueller-Holzner E, Marth C, and Widschwendter M. Methylated DNA collected by tampons--a new tool to detect endometrial cancer. *Cancer Epidemiol Biomarkers Prev* 13: 882-888, 2004.
53. Galiatsatos P, and Foulkes WD. Familial adenomatous polyposis. *Am J Gastroenterol* 101: 385-398, 2006.
54. Garcia R, Bowman TL, Niu G, Yu H, Minton S, Muro-Cacho CA, Cox CE, Falcone R, Fairclough R, Parsons S, Laudano A, Gazit A, Levitzki A, Kraker A, and Jove R. Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. *Oncogene* 20: 2499-2513, 2001.
55. Gillingham MB, Kritsch KR, Murali SG, Lund PK, and Ney DM. Resection upregulates the IGF-I system of parenterally fed rats with jejunocolic anastomosis. *Am J Physiol Gastrointest Liver Physiol* 281: G1158-1168, 2001.
56. Giovannucci E. Insulin, insulin-like growth factors and colon cancer: a review of the evidence. *J Nutr* 131: 3109S-3120S, 2001.
57. Giovannucci E. Nutrition, insulin, insulin-like growth factors and cancer. *Horm*

Metab Res 35: 694-704, 2003.

58. Giovannucci E, and Michaud D. The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas. *Gastroenterology* 132: 2208-2225, 2007.

59. Greenhalgh CJ, Bertolino P, Asa SL, Metcalf D, Corbin JE, Adams TE, Davey HW, Nicola NA, Hilton DJ, and Alexander WS. Growth enhancement in suppressor of cytokine signaling 2 (SOCS-2)-deficient mice is dependent on signal transducer and activator of transcription 5b (STAT5b). *Mol Endocrinol* 16: 1394-1406, 2002.

60. Greenhalgh CJ, Miller ME, Hilton DJ, and Lund PK. Suppressors of cytokine signaling: Relevance to gastrointestinal function and disease. *Gastroenterology* 123: 2064-2081, 2002.

61. Greenhalgh CJ, Rico-Bautista E, Lorentzon M, Thaus AL, Morgan PO, Willson TA, Zervoudakis P, Metcalf D, Street I, Nicola NA, Nash AD, Fabri LJ, Norstedt G, Ohlsson C, Flores-Morales A, Alexander WS, and Hilton DJ. SOCS2 negatively regulates growth hormone action in vitro and in vivo. *J Clin Invest* 115: 397-406, 2005.

62. Greenstein AJ. Cancer in inflammatory bowel disease. *Mt Sinai J Med* 67: 227-240, 2000.

63. Gunter MJ, and Leitzmann MF. Obesity and colorectal cancer: epidemiology, mechanisms and candidate genes. *J Nutr Biochem* 17: 145-156, 2006.

64. Guyton AC, and Hall JE. *Textbook of Medical Physiology*. Philadelphia: Saunders, 1998.

65. Han VK, Snouweart J, Towle AC, Lund PK, and Lauder JM. Cellular localization of tyrosine hydroxylase mRNA and its regulation in the rat adrenal medulla and brain by in situ hybridization with an oligodeoxyribonucleotide probe. *J Neurosci Res* 17: 11-18, 1987.

66. Hansen JA, Lindberg K, Hilton DJ, Nielsen JH, and Billestrup N. Mechanism of inhibition of growth hormone receptor signaling by suppressor of cytokine signaling proteins. *Mol Endocrinol* 13: 1832-1843, 1999.

67. Hassan AB, and Howell JA. Insulin-like growth factor II supply modifies growth of intestinal adenoma in Apc(Min/+) mice. *Cancer Res* 60: 1070-1076, 2000.

68. Hendriksen PJ, Dits NF, Kokame K, Veldhoven A, van Weerden WM, Bangma CH, Trapman J, and Jenster G. Evolution of the androgen receptor pathway during progression of prostate cancer. *Cancer Res* 66: 5012-5020, 2006.

69. Heron-Milhavet L, and LeRoith D. Insulin-like growth factor I induces MDM2-

dependent degradation of p53 via the p38 MAPK pathway in response to DNA damage. *J Biol Chem* 277: 15600-15606, 2002.

70. Hintz RL. Growth hormone treatment of idiopathic short stature: clinical studies. *Growth Horm IGF Res* 15 Suppl A: S6-8, 2005.

71. Holzenberger M. The GH/IGF-I axis and longevity. *Eur J Endocrinol* 151 Suppl 1: S23-27, 2004.

72. Hong MY, Turner ND, Carroll RJ, Chapkin RS, and Lupton JR. Differential response to DNA damage may explain different cancer susceptibility between small and large intestine. *Exp Biol Med (Maywood)* 230: 464-471, 2005.

73. Hopfner M, Baradari V, Huether A, Schofl C, and Scherubl H. The insulin-like growth factor receptor 1 is a promising target for novel treatment approaches in neuroendocrine gastrointestinal tumours. *Endocr Relat Cancer* 13: 135-149, 2006.

74. Houchen CW, Stenson WF, and Cohn SM. Disruption of cyclooxygenase-1 gene results in an impaired response to radiation injury. *Am J Physiol Gastrointest Liver Physiol* 279: G858-865, 2000.

75. Howarth GS. Insulin-like growth factor-I and the gastrointestinal system: therapeutic indications and safety implications. *J Nutr* 133: 2109-2112, 2003.

76. Hu R, Khor TO, Shen G, Jeong WS, Hebbar V, Chen C, Xu C, Reddy B, Chada K, and Kong AN. Cancer chemoprevention of intestinal polyposis in ApcMin/+ mice by sulforaphane, a natural product derived from cruciferous vegetable. *Carcinogenesis* 2006.

77. Itzkowitz SH, and Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. *Am J Physiol Gastrointest Liver Physiol* 287: G7-17, 2004.

78. Jansson EA, Are A, Greicius G, Kuo IC, Kelly D, Arulampalam V, and Pettersson S. The Wnt/beta-catenin signaling pathway targets PPARgamma activity in colon cancer cells. *Proc Natl Acad Sci U S A* 102: 1460-1465, 2005.

79. Jenkins PJ. Acromegaly and cancer. *Horm Res* 62 Suppl 1: 108-115, 2004.

80. Jenkins PJ, Mukherjee A, and Shalet SM. Does growth hormone cause cancer? *Clin Endocrinol (Oxf)* 64: 115-121, 2006.

81. Jo D, Liu D, Yao S, Collins RD, and Hawiger J. Intracellular protein therapy with SOCS3 inhibits inflammation and apoptosis. *Nat Med* 11: 892-898, 2005.

82. Jones JI, and Clemmons DR. Insulin-like growth factors and their binding

proteins: biological actions. *Endocr Rev* 16: 3-34, 1995.

83. Kamura T, Sato S, Haque D, Liu L, Kaelin WG, Jr., Conaway RC, and Conaway JW. The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. *Genes Dev* 12: 3872-3881, 1998.

84. Kapoor A, Li HJ, and Leiter AB. Intestinal development: the many faces of Wnt signaling. *Gastroenterology* 133: 710-712, 2007.

85. Keku TO, Lund PK, Galanko J, Simmons JG, Woosley JT, and Sandler RS. Insulin resistance, apoptosis, and colorectal adenoma risk. *Cancer Epidemiol Biomarkers Prev* 14: 2076-2081, 2005.

86. Kim B, van Golen CM, and Feldman EL. Insulin-like growth factor I induces preferential degradation of insulin receptor substrate-2 through the phosphatidylinositol 3-kinase pathway in human neuroblastoma cells. *Endocrinology* 146: 5350-5357, 2005.

87. Kim HJ, and Kim TY. IGF-II-mediated COX-2 gene expression in human keratinocytes through extracellular signal-regulated kinase pathway. *J Invest Dermatol* 123: 547-555, 2004.

88. Klampfer L. The role of signal transducers and activators of transcription in colon cancer. *Front Biosci* 13: 2888-2899, 2008.

89. Kloth MT, Catling AD, and Silva CM. Novel activation of STAT5b in response to epidermal growth factor. *J Biol Chem* 277: 8693-8701, 2002.

90. Knott AW, Juno RJ, Jarboe MD, Profitt SA, Erwin CR, Smith EP, Fagin JA, and Warner BW. Smooth muscle overexpression of IGF-I induces a novel adaptive response to small bowel resection. *Am J Physiol Gastrointest Liver Physiol* 287: G562-570, 2004.

91. Koda M, Sulkowska M, Kanczuga-Koda L, and Sulkowski S. Expression of insulin receptor substrate 1 in primary breast cancer and lymph node metastases. *J Clin Pathol* 58: 645-649, 2005.

92. Kongkanunt R, Bubb VJ, Sansom OJ, Wyllie AH, Harrison DJ, and Clarke AR. Dysregulated expression of beta-catenin marks early neoplastic change in Apc mutant mice, but not all lesions arising in Msh2 deficient mice. *Oncogene* 18: 7219-7225, 1999.

93. Kooijman R. Regulation of apoptosis by insulin-like growth factor (IGF)-I. *Cytokine Growth Factor Rev* 2006.

94. Kovacs M, Kineman RD, Schally AV, Zarandi M, Groot K, and Frohman LA. Effects of antagonists of growth hormone-releasing hormone (GHRH) on GH and insulin-like growth factor I levels in transgenic mice overexpressing the human GHRH gene, an animal model of acromegaly. *Endocrinology* 138: 4536-4542, 1997.
95. Kuemmerle JF. Endogenous IGF-I protects human intestinal smooth muscle cells from apoptosis by regulation of GSK-3 beta activity. *Am J Physiol Gastrointest Liver Physiol* 288: G101-110, 2005.
96. Larsen L, and Ropke C. Suppressors of cytokine signalling: SOCS. *Apmis* 110: 833-844, 2002.
97. Le Roith D. The insulin-like growth factor system. *Exp Diabetes Res* 4: 205-212, 2003.
98. Leri A, Liu Y, Claudio PP, Kajstura J, Wang X, Wang S, Kang P, Malhotra A, and Anversa P. Insulin-like growth factor-1 induces Mdm2 and down-regulates p53, attenuating the myocyte renin-angiotensin system and stretch-mediated apoptosis. *Am J Pathol* 154: 567-580, 1999.
99. LeRoith D, and Roberts CT, Jr. The insulin-like growth factor system and cancer. *Cancer Lett* 195: 127-137, 2003.
100. Levine AJ, Feng Z, Mak TW, You H, and Jin S. Coordination and communication between the p53 and IGF-1-AKT-TOR signal transduction pathways. *Genes Dev* 20: 267-275, 2006.
101. Lieman HJ, Adel TE, Forst C, von Hagen S, and Santoro N. Effects of aging and estradiol supplementation on GH axis dynamics in women. *J Clin Endocrinol Metab* 86: 3918-3923, 2001.
102. Lopez-Siguero JP, Garcia-Garcia E, Carralero I, and Martinez-Aedo MJ. Adult height in children with idiopathic short stature treated with growth hormone. *J Pediatr Endocrinol Metab* 13: 1595-1602, 2000.
103. Lund PK. Molecular basis of intestinal adaptation: the role of the insulin-like growth factor system. *Ann N Y Acad Sci* 859: 18-36, 1998.
104. Lynn FC, Smith SB, Wilson ME, Yang KY, Nekrep N, and German MS. Sox9 coordinates a transcriptional network in pancreatic progenitor cells. *Proc Natl Acad Sci U S A* 2007.
105. Machado FS, Johndrow JE, Esper L, Dias A, Bafica A, Serhan CN, and Aliberti J. Anti-inflammatory actions of lipoxin A4 and aspirin-triggered lipoxin are SOCS-2 dependent. *Nat Med* 12: 330-334, 2006.

106. Madison BB, Braunstein K, Kuizon E, Portman K, Qiao XT, and Gumucio DL. Epithelial hedgehog signals pattern the intestinal crypt-villus axis. *Development* 132: 279-289, 2005.
107. Mani SA, Yang J, Brooks M, Schwaninger G, Zhou A, Miura N, Kutok JL, Hartwell K, Richardson AL, and Weinberg RA. Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. *Proc Natl Acad Sci U S A* 104: 10069-10074, 2007.
108. Marini A, Mirmohammadsadegh A, Nambiar S, Gustrau A, Ruzicka T, and Hengge UR. Epigenetic Inactivation of Tumor Suppressor Genes in Serum of Patients with Cutaneous Melanoma. *J Invest Dermatol* 2005.
109. Marshman E, Booth C, and Potten CS. The intestinal epithelial stem cell. *Bioessays* 24: 91-98, 2002.
110. Martin C, Connelly A, Keku TO, Mountcastle SB, Galanko J, Woosley JT, Schliebe B, Lund PK, and Sandler RS. Nonsteroidal anti-inflammatory drugs, apoptosis, and colorectal adenomas. *Gastroenterology* 123: 1770-1777, 2002.
111. Martino A, Cammarota G, Cianci R, Bianchi A, Sacco E, Tilaro L, Marzetti E, Certo M, Pirozzi G, Fedeli P, Pandolfi F, Pontecorvi A, Gasbarrini G, and De Marinis L. High prevalence of hyperplastic colonic polyps in acromegalic subjects. *Dig Dis Sci* 49: 662-666, 2004.
112. Matano Y, Okada T, Suzuki A, Yoneda T, Takeda Y, and Mabuchi H. Risk of colorectal neoplasm in patients with acromegaly and its relationship with serum growth hormone levels. *Am J Gastroenterol* 100: 1154-1160, 2005.
113. Mathews LS, Hammer RE, Behringer RR, D'Ercole AJ, Bell GI, Brinster RL, and Palmiter RD. Growth enhancement of transgenic mice expressing human insulin-like growth factor I. *Endocrinology* 123: 2827-2833, 1988.
114. McEntee MF, Chiu CH, and Whelan J. Relationship of beta-catenin and Bcl-2 expression to sulindac-induced regression of intestinal tumors in Min mice. *Carcinogenesis* 20: 635-640, 1999.
115. Mendelsohn J, and Baselga J. The EGF receptor family as targets for cancer therapy. *Oncogene* 19: 6550-6565, 2000.
116. Mericq MV, Eggers M, Avila A, Cutler GB, Jr., and Cassorla F. Near final height in pubertal growth hormone (GH)-deficient patients treated with GH alone or in combination with luteinizing hormone-releasing hormone analog: results of a prospective, randomized trial. *J Clin Endocrinol Metab* 85: 569-573, 2000.
117. Merritt AJ, Potten CS, Watson AJ, Loh DY, Nakayama K, and Hickman JA.

Differential expression of bcl-2 in intestinal epithelia. Correlation with attenuation of apoptosis in colonic crypts and the incidence of colonic neoplasia. *J Cell Sci* 108 (Pt 6): 2261-2271, 1995.

118. Metcalf D, Greenhalgh CJ, Viney E, Willson TA, Starr R, Nicola NA, Hilton DJ, and Alexander WS. Gigantism in mice lacking suppressor of cytokine signalling-2. *Nature* 405: 1069-1073, 2000.

119. Michaylira CZ, Ramocki NM, Simmons JG, Tanner CK, McNaughton KK, Woosley JT, Greenhalgh CJ, and Lund PK. Haplotype insufficiency for suppressor of cytokine signaling-2 enhances intestinal growth and promotes polyp formation in growth hormone-transgenic mice. *Endocrinology* 147: 1632-1641, 2006.

120. Michaylira CZ, Simmons JG, Ramocki NM, Scull BP, McNaughton KK, Fuller CR, and Lund PK. Suppressor of cytokine signaling-2 limits intestinal growth and enterotrophic actions of IGF-I in vivo. *Am J Physiol Gastrointest Liver Physiol* 2006.

121. Miller ME, Michaylira CZ, Simmons JG, Ney DM, Dahly EM, Heath JK, and Lund PK. Suppressor of cytokine signaling-2: a growth hormone-inducible inhibitor of intestinal epithelial cell proliferation. *Gastroenterology* 127: 570-581, 2004.

122. Mohamed-Ali V, and Pinkney J. Therapeutic potential of insulin-like growth factor-1 in patients with diabetes mellitus. *Treat Endocrinol* 1: 399-410, 2002.

123. Mora LB, Buettner R, Seigne J, Diaz J, Ahmad N, Garcia R, Bowman T, Falcone R, Fairclough R, Cantor A, Muro-Cacho C, Livingston S, Karras J, Pow-Sang J, and Jove R. Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. *Cancer Res* 62: 6659-6666, 2002.

124. Moran AE, Hunt DH, Javid SH, Redston M, Carothers AM, and Bertagnolli MM. Apc deficiency is associated with increased Egfr activity in the intestinal enterocytes and adenomas of C57BL/6J-Min/+ mice. *J Biol Chem* 279: 43261-43272, 2004.

125. Mori-Akiyama Y, van den Born M, van Es JH, Hamilton SR, Adams HP, Zhang J, Clevers H, and de Crombrughe B. SOX9 is required for the differentiation of paneth cells in the intestinal epithelium. *Gastroenterology* 133: 539-546, 2007.

126. Moschos SJ, and Mantzoros CS. The role of the IGF system in cancer: from basic to clinical studies and clinical applications. *Oncology* 63: 317-332, 2002.

127. Moser AR, Hegge LF, and Cardiff RD. Genetic background affects susceptibility to mammary hyperplasias and carcinomas in Apc(min)/+ mice. *Cancer Res* 61: 3480-3485, 2001.

128. Moser AR, Luongo C, Gould KA, McNeley MK, Shoemaker AR, and Dove WF. ApcMin: a mouse model for intestinal and mammary tumorigenesis. *Eur J Cancer* 31A: 1061-1064, 1995.
129. Mulvihill MJ, Ji QS, Coate HR, Cooke A, Dong H, Feng L, Foreman K, Rosenfeld-Franklin M, Honda A, Mak G, Mulvihill KM, Nigro AI, O'Connor M, Pirrit C, Steinig AG, Siu K, Stolz KM, Sun Y, Tavares PA, Yao Y, and Gibson NW. Novel 2-phenylquinolin-7-yl-derived imidazo[1,5-a]pyrazines as potent insulin-like growth factor-I receptor (IGF-IR) inhibitors. *Bioorg Med Chem* 2007.
130. Nascimbeni R, Di Fabio F, Di Betta E, Mariani P, Fisogni S, and Villanacci V. Morphology of colorectal lymphoid aggregates in cancer, diverticular and inflammatory bowel diseases. *Mod Pathol* 18: 681-685, 2005.
131. Nateri AS, Spencer-Dene B, and Behrens A. Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. *Nature* 437: 281-285, 2005.
132. Ney DM. Insulin-like Growth Factors in Relation to Gastrointestinal Diseases and Parenteral Nutrition. In: *IGF and Nutrition in Health and Disease*, edited by Houston MS, Holly JMP, and Feldman EL. Totowa, NJ: Humana Press, 2004, p. 271-289.
133. Nishimura S, Wakabayashi N, Toyoda K, Kashima K, and Mitsufuji S. Expression of Musashi-1 in human normal colon crypt cells: a possible stem cell marker of human colon epithelium. *Dig Dis Sci* 48: 1523-1529, 2003.
134. Nishiyama M, and Wands JR. Cloning and increased expression of an insulin receptor substrate-1-like gene in human hepatocellular carcinoma. *Biochem Biophys Res Commun* 183: 280-285, 1992.
135. Nucci AM, Finegold DN, Yaworski JA, Kowalski L, and Barksdale EM, Jr. Results of growth trophic therapy in children with short bowel syndrome. *J Pediatr Surg* 39: 335-339; discussion 335-339, 2004.
136. O'Brien CA, Pollett A, Gallinger S, and Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 445: 106-110, 2007.
137. Ohneda K, Ulshen MH, Fuller CR, D'Ercole AJ, and Lund PK. Enhanced growth of small bowel in transgenic mice expressing human insulin-like growth factor I. *Gastroenterology* 112: 444-454, 1997.
138. Olsson B, Bohlooly YM, Fitzgerald SM, Frick F, Ljungberg A, Ahren B, Tornell J, Bergstrom G, and Oscarsson J. Bovine growth hormone transgenic mice are resistant to diet-induced obesity but develop hyperphagia, dyslipidemia, and diabetes on a high-fat diet. *Endocrinology* 146: 920-930, 2005.

139. Ormestad M, Astorga J, Landgren H, Wang T, Johansson BR, Miura N, and Carlsson P. Foxf1 and Foxf2 control murine gut development by limiting mesenchymal Wnt signaling and promoting extracellular matrix production. *Development* 133: 833-843, 2006.
140. Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, and Evans RM. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature* 300: 611-615, 1982.
141. Palmqvist R, Hallmans G, Rinaldi S, Biessy C, Stenling R, Riboli E, and Kaaks R. Plasma insulin-like growth factor 1, insulin-like growth factor binding protein 3, and risk of colorectal cancer: a prospective study in northern Sweden. *Gut* 50: 642-646, 2002.
142. Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R, and Belfiore A. Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *J Biol Chem* 277: 39684-39695, 2002.
143. Pereira PM, and Bines JE. New growth factor therapies aimed at improving intestinal adaptation in short bowel syndrome. *J Gastroenterol Hepatol* 21: 932-940, 2006.
144. Perkins S, Verschoyle RD, Hill K, Parveen I, Threadgill MD, Sharma RA, Williams ML, Steward WP, and Gescher AJ. Chemopreventive efficacy and pharmacokinetics of curcumin in the min/+ mouse, a model of familial adenomatous polyposis. *Cancer Epidemiol Biomarkers Prev* 11: 535-540, 2002.
145. Pete G, Fuller CR, Oldham JM, Smith DR, D'Ercole AJ, Kahn CR, and Lund PK. Postnatal growth responses to insulin-like growth factor I in insulin receptor substrate-1-deficient mice. *Endocrinology* 140: 5478-5487, 1999.
146. Peterson CA, Carey HV, Hinton PL, Lo HC, and Ney DM. GH elevates serum IGF-I levels but does not alter mucosal atrophy in parenterally fed rats. *Am J Physiol* 272: G1100-1108, 1997.
147. Playford MP, Bicknell D, Bodmer WF, and Macaulay VM. Insulin-like growth factor 1 regulates the location, stability, and transcriptional activity of beta-catenin. *Proc Natl Acad Sci U S A* 97: 12103-12108, 2000.
148. Pold M, Krysan K, Pold A, Dohadwala M, Heuze-Vourc'h N, Mao JT, Riedl KL, Sharma S, and Dubinett SM. Cyclooxygenase-2 modulates the insulin-like growth factor axis in non-small-cell lung cancer. *Cancer Res* 64: 6549-6555, 2004.

149. Potten CS. Protection of the small intestinal clonogenic stem cells from radiation-induced damage by pretreatment with interleukin 11 also increases murine survival time. *Stem Cells* 14: 452-459, 1996.
150. Potten CS. Radiation, the ideal cytotoxic agent for studying the cell biology of tissues such as the small intestine. *Radiat Res* 161: 123-136, 2004.
151. Potten CS. Stem cells in gastrointestinal epithelium: numbers, characteristics and death. *Philos Trans R Soc Lond B Biol Sci* 353: 821-830, 1998.
152. Potten CS. The significance of spontaneous and induced apoptosis in the gastrointestinal tract of mice. *Cancer Metastasis Rev* 11: 179-195, 1992.
153. Potten CS, Booth C, and Pritchard DM. The intestinal epithelial stem cell: the mucosal governor. *Int J Exp Pathol* 78: 219-243, 1997.
154. Potten CS, Booth C, Tudor GL, Booth D, Brady G, Hurley P, Ashton G, Clarke R, Sakakibara S, and Okano H. Identification of a putative intestinal stem cell and early lineage marker; musashi-1. *Differentiation* 71: 28-41, 2003.
155. Potten CS, Owen G, and Booth D. Intestinal stem cells protect their genome by selective segregation of template DNA strands. *J Cell Sci* 115: 2381-2388, 2002.
156. Ranke MB. Insulin-like growth factor-I treatment of growth disorders, diabetes mellitus and insulin resistance. *Trends Endocrinol Metab* 16: 190-197, 2005.
157. Ransome MI, Goldshmit Y, Bartlett PF, Waters MJ, and Turnley AM. Comparative analysis of CNS populations in knockout mice with altered growth hormone responsiveness. *Eur J Neurosci* 19: 2069-2079, 2004.
158. Renehan AG, O'Connell J, O'Halloran D, Shanahan F, Potten CS, O'Dwyer ST, and Shalet SM. Acromegaly and colorectal cancer: a comprehensive review of epidemiology, biological mechanisms, and clinical implications. *Horm Metab Res* 35: 712-725, 2003.
159. Renstrom F, Buren J, Svensson M, and Eriksson JW. Insulin resistance induced by high glucose and high insulin precedes insulin receptor substrate 1 protein depletion in human adipocytes. *Metabolism* 56: 190-198, 2007.
160. Rico-Bautista E, Flores-Morales A, and Fernandez-Perez L. Suppressor of cytokine signaling (SOCS) 2, a protein with multiple functions. *Cytokine Growth Factor Rev* 17: 431-439, 2006.
161. Riedemann J, and Macaulay VM. IGF1R signalling and its inhibition. *Endocr Relat Cancer* 13 Suppl 1: S33-43, 2006.

162. Rigby RJ SJ, Fuller CR, Lund PK. Intestinal epithelial specific deletion of suppressor of cytokine signaling 3 (SOCS3) increases inflammation induced hyperproliferation. *Gastroenterology* 128: 699, 2005.
163. Roberts RB, Min L, Washington MK, Olsen SJ, Settle SH, Coffey RJ, and Threadgill DW. Importance of epidermal growth factor receptor signaling in establishment of adenomas and maintenance of carcinomas during intestinal tumorigenesis. *Proc Natl Acad Sci U S A* 99: 1521-1526, 2002.
164. Robertson DJ, Sandler RS, Haile R, Tosteson TD, Greenberg ER, Grau M, and Baron JA. Fat, fiber, meat and the risk of colorectal adenomas. *Am J Gastroenterol* 100: 2789-2795, 2005.
165. Rother KI, and Accili D. Role of insulin receptors and IGF receptors in growth and development. *Pediatr Nephrol* 14: 558-561, 2000.
166. Sakatani T, Kaneda A, Iacobuzio-Donahue CA, Carter MG, de Boom Witzel S, Okano H, Ko MS, Ohlsson R, Longo DL, and Feinberg AP. Loss of imprinting of Igf2 alters intestinal maturation and tumorigenesis in mice. *Science* 307: 1976-1978, 2005.
167. Sala FG, Curtis JL, Veltmaat JM, Del Moral PM, Le LT, Fairbanks TJ, Warburton D, Ford H, Wang K, Burns RC, and Bellusci S. Fibroblast growth factor 10 is required for survival and proliferation but not differentiation of intestinal epithelial progenitor cells during murine colon development. *Dev Biol* 299: 373-385, 2006.
168. Sandhu MS, Dunger DB, and Giovannucci EL. Insulin, insulin-like growth factor-I (IGF-I), IGF binding proteins, their biologic interactions, and colorectal cancer. *J Natl Cancer Inst* 94: 972-980, 2002.
169. Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, Battle E, Simon-Assmann P, Clevers H, Nathke IS, Clarke AR, and Winton DJ. Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. *Genes Dev* 18: 1385-1390, 2004.
170. Sarraf P, Mueller E, Smith WM, Wright HM, Kum JB, Aaltonen LA, de la Chapelle A, Spiegelman BM, and Eng C. Loss-of-function mutations in PPAR gamma associated with human colon cancer. *Mol Cell* 3: 799-804, 1999.
171. Savendahl L, Underwood LE, Haldeman KM, Ulshen MH, and Lund PK. Fasting prevents experimental murine colitis produced by dextran sulfate sodium and decreases interleukin-1 beta and insulin-like growth factor I messenger ribonucleic acid. *Endocrinology* 138: 734-740., 1997.
172. Schier S, and Wright NA. Stem cell relationships and the origin of gastrointestinal cancer. *Oncology* 69 Suppl 1: 9-13, 2005.

173. Schwab TS, Madison BB, Grauman AR, and Feldman EL. Insulin-like growth factor-I induces the phosphorylation and nuclear exclusion of forkhead transcription factors in human neuroblastoma cells. *Apoptosis* 10: 831-840, 2005.
174. Scolapio JS. Current update of short-bowel syndrome. *Curr Opin Gastroenterol* 20: 143-145, 2004.
175. Scolapio JS. Effect of growth hormone, glutamine, and diet on body composition in short bowel syndrome: a randomized, controlled study. *JPEN J Parenter Enteral Nutr* 23: 309-312; discussion 312-303., 1999.
176. Scotlandi K, Manara MC, Nicoletti G, Lollini PL, Lukas S, Benini S, Croci S, Perdichizzi S, Zambelli D, Serra M, Garcia-Echeverria C, Hofmann F, and Picci P. Antitumor activity of the insulin-like growth factor-I receptor kinase inhibitor NVP-AEW541 in musculoskeletal tumors. *Cancer Res* 65: 3868-3876, 2005.
177. Seguy D, Vahedi K, Kapel N, Souberbielle JC, and Messing B. Low-dose growth hormone in adult home parenteral nutrition-dependent short bowel syndrome patients: a positive study. *Gastroenterology* 124: 293-302., 2003.
178. Seguy D, Vahedi, K., Crenn, P., Morin, M.C., Beliah, M., Souberbielle, J.C., Postel-Vinay, M.C., Gober, J.G., Messing, B. Growth hormone benefit in very short bowel patients: A randomized controlled trial. In: *American Gastroenterological Association* 1999, p. G2530.
179. Seidell JC. Obesity, insulin resistance and diabetes--a worldwide epidemic. *Br J Nutr* 83 Suppl 1: S5-8, 2000.
180. Seymour PA, Freude KK, Tran MN, Mayes EE, Jensen J, Kist R, Scherer G, and Sander M. SOX9 is required for maintenance of the pancreatic progenitor cell pool. *Proc Natl Acad Sci U S A* 104: 1865-1870, 2007.
181. Shakibaei M, Seifarth C, John T, Rahmanzadeh M, and Mobasheri A. Igf-I extends the chondrogenic potential of human articular chondrocytes in vitro: Molecular association between Sox9 and Erk1/2. *Biochem Pharmacol* 72: 1382-1395, 2006.
182. Shao J, Sheng H, Inoue H, Morrow JD, and DuBois RN. Regulation of constitutive cyclooxygenase-2 expression in colon carcinoma cells. *J Biol Chem* 275: 33951-33956, 2000.
183. Shen L, Kondo Y, Guo Y, Zhang J, Zhang L, Ahmed S, Shu J, Chen X, Waterland RA, and Issa JP. Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. *PLoS Genet* 3: 2023-2036, 2007.
184. Shirakami A, Toyonaga T, Tsuruzoe K, Shirotani T, Matsumoto K, Yoshizato K, Kawashima J, Hirashima Y, Miyamura N, Kahn CR, and Araki E. Heterozygous

knockout of the IRS-1 gene in mice enhances obesity-linked insulin resistance: a possible model for the development of type 2 diabetes. *J Endocrinol* 174: 309-319, 2002.

185. Simmons JG, Pucilowska JB, Keku TO, and Lund PK. IGF-I and TGF-beta1 have distinct effects on phenotype and proliferation of intestinal fibroblasts. *Am J Physiol Gastrointest Liver Physiol* 283: G809-818, 2002.

186. Sinner D, Kordich JJ, Spence JR, Opoka R, Rankin S, Lin SC, Jonatan D, Zorn AM, and Wells JM. Sox17 and Sox4 Differentially Regulate {beta}-Catenin/T-Cell Factor Activity and Proliferation of Colon Carcinoma Cells. *Mol Cell Biol* 27: 7802-7815, 2007.

187. Slattery ML, Samowitz W, Curtin K, Ma KN, Hoffman M, Caan B, and Neuhausen S. Associations among IRS1, IRS2, IGF1, and IGFBP3 genetic polymorphisms and colorectal cancer. *Cancer Epidemiol Biomarkers Prev* 13: 1206-1214, 2004.

188. Smith DR, Hoyt EC, Gallagher M, Schwabe RF, and Lund PK. Effect of age and cognitive status on basal level AP-1 activity in rat hippocampus. *Neurobiol Aging* 22: 773-786, 2001.

189. Sonntag WE, Carter CS, Ikeno Y, Ekenstedt K, Carlson CS, Loeser RF, Chakrabarty S, Lee S, Bennett C, Ingram R, Moore T, and Ramsey M. Adult-onset growth hormone and insulin-like growth factor I deficiency reduces neoplastic disease, modifies age-related pathology, and increases life span. *Endocrinology* 146: 2920-2932, 2005.

190. Sutherland KD, Lindeman GJ, Choong DY, Wittlin S, Brentzell L, Phillips W, Campbell IG, and Visvader JE. Differential hypermethylation of SOCS genes in ovarian and breast carcinomas. *Oncogene* 23: 7726-7733, 2004.

191. Tanaka T, Kohno H, Yoshitani S, Takashima S, Okumura A, Murakami A, and Hosokawa M. Ligands for peroxisome proliferator-activated receptors alpha and gamma inhibit chemically induced colitis and formation of aberrant crypt foci in rats. *Cancer Res* 61: 2424-2428, 2001.

192. Taylor B, Murphy GM, and Dowling RH. Pituitary hormones and the small bowel: effect of hypophysectomy on intestinal adaptation to small bowel resection in the rat. *Eur J Clin Invest* 9: 115-127, 1979.

193. Terzolo M, Reimondo G, Gasperi M, Cozzi R, Pivonello R, Vitale G, Scillitani A, Attanasio R, Cecconi E, Daffara F, Gaia E, Martino E, Lombardi G, Angeli A, and Colao A. Colonoscopic Screening and Follow-up in Patients with Acromegaly: A Multicentre Study in Italy. *J Clin Endocrinol Metab* 2004.

194. Theiss AL, Fruchtmann S, and Lund PK. Growth factors in inflammatory bowel

disease: the actions and interactions of growth hormone and insulin-like growth factor-I. *Inflamm Bowel Dis* 10: 871-880, 2004.

195. Tischoff I, Hengge UR, Vieth M, Ell C, Stolte M, Weber A, Schmidt WE, and Tannapfel A. Methylation of SOCS-3 and SOCS-1 in the carcinogenesis of Barrett's adenocarcinoma. *Gut* 56: 1047-1053, 2007.

196. Tseng YH, Kriauciunas KM, Kokkotou E, and Kahn CR. Differential roles of insulin receptor substrates in brown adipocyte differentiation. *Mol Cell Biol* 24: 1918-1929, 2004.

197. Turnley AM, Faux CH, Rietze RL, Coonan JR, and Bartlett PF. Suppressor of cytokine signaling 2 regulates neuronal differentiation by inhibiting growth hormone signaling. *Nat Neurosci* 5: 1155-1162, 2002.

198. Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, Ram PA, Waxman DJ, and Davey HW. Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc Natl Acad Sci U S A* 94: 7239-7244, 1997.

199. Ueki K, Yamauchi T, Tamemoto H, Tobe K, Yamamoto-Honda R, Kaburagi Y, Akanuma Y, Yazaki Y, Aizawa S, Nagai R, and Kadowaki T. Restored insulin-sensitivity in IRS-1-deficient mice treated by adenovirus-mediated gene therapy. *J Clin Invest* 105: 1437-1445, 2000.

200. Ulshen MH, Dowling RH, Fuller CR, Zimmermann EM, and Lund PK. Enhanced growth of small bowel in transgenic mice overexpressing bovine growth hormone. *Gastroenterology* 104: 973-980, 1993.

201. Van der Flier LG, Sabates-Bellver J, Oving I, Haegebarth A, De Palo M, Anti M, Van Gijn ME, Suijkerbuijk S, Van de Wetering M, Marra G, and Clevers H. The Intestinal Wnt/TCF Signature. *Gastroenterology* 132: 628-632, 2007.

202. van Golen CM, Schwab TS, Kim B, Soules ME, Su Oh S, Fung K, van Golen KL, and Feldman EL. Insulin-like growth factor-I receptor expression regulates neuroblastoma metastasis to bone. *Cancer Res* 66: 6570-6578, 2006.

203. Varma RR, Hector SM, Clark K, Greco WR, Hawthorn L, and Pendyala L. Gene expression profiling of a clonal isolate of oxaliplatin-resistant ovarian carcinoma cell line A2780/C10. *Oncol Rep* 14: 925-932, 2005.

204. Vos PE, Koppeschaar HP, de Vries WR, and Wokke JH. Insulin-like growth factor-I: clinical studies. *Drugs Today (Barc)* 34: 79-90, 1998.

205. Wang Y, Hailey J, Williams D, Lipari P, Malkowski M, Wang X, Xie L, Li G, Saha D, Ling WL, Cannon-Carlson S, Greenberg R, Ramos RA, Shields R, Presta L, Brams P, Bishop WR, and Pachter JA. Inhibition of insulin-like growth factor-I

receptor (IGF-IR) signaling and tumor cell growth by a fully human neutralizing anti-IGF-IR antibody. *Mol Cancer Ther* 4: 1214-1221, 2005.

206. Wang Y, and Jiang H. Identification of a distal STAT5-binding DNA region that may mediate growth hormone regulation of insulin-like growth factor-I gene expression. *J Biol Chem* 280: 10955-10963, 2005.

207. Washizawa N, Gu LH, Gu L, Openo KP, Jones DP, and Ziegler TR. Comparative effects of glucagon-like peptide-2 (GLP-2), growth hormone (GH), and keratinocyte growth factor (KGF) on markers of gut adaptation after massive small bowel resection in rats. *JPEN J Parenter Enteral Nutr* 28: 399-409, 2004.

208. Webb SM, Casanueva F, and Wass JA. Oncological complications of excess GH in acromegaly. *Pituitary* 5: 21-25, 2002.

209. Wechter WJ, Murray ED, Jr., Kantoci D, Quiggle DD, Leipold DD, Gibson KM, and McCracken JD. Treatment and survival study in the C57BL/6J-APC(Min)/+(Min) mouse with R-flurbiprofen. *Life Sci* 66: 745-753, 2000.

210. Wei EK, Ma J, Pollak MN, Rifai N, Fuchs CS, Hankinson SE, and Giovannucci E. A prospective study of C-peptide, insulin-like growth factor-I, insulin-like growth factor binding protein-1, and the risk of colorectal cancer in women. *Cancer Epidemiol Biomarkers Prev* 14: 850-855, 2005.

211. Werner H, Hernandez-Sanchez C, Karnieli E, and Leroith D. The regulation of IGF-I receptor gene expression. *Int J Biochem Cell Biol* 27: 987-994, 1995.

212. White MF. Insulin signaling in health and disease. *Science* 302: 1710-1711, 2003.

213. White MF. IRS proteins and the common path to diabetes. *Am J Physiol Endocrinol Metab* 283: E413-422, 2002.

214. Wikman H, Kettunen E, Seppanen JK, Karjalainen A, Hollmen J, Anttila S, and Knuutila S. Identification of differentially expressed genes in pulmonary adenocarcinoma by using cDNA array. *Oncogene* 21: 5804-5813, 2002.

215. Wilkins HR, Ohneda K, Keku TO, D'Ercole AJ, Fuller CR, Williams KL, and Lund PK. Reduction of spontaneous and irradiation-induced apoptosis in small intestine of IGF-I transgenic mice. *Am J Physiol Gastrointest Liver Physiol* 283: G457-464, 2002.

216. Williams KL, Fuller CR, Fagin J, and Lund PK. Mesenchymal IGF-I overexpression: paracrine effects in the intestine, distinct from endocrine actions. *Am J Physiol Gastrointest Liver Physiol* 283: G875-885, 2002.

217. Wilson JW, Pritchard DM, Hickman JA, and Potten CS. Radiation-induced p53

and p21WAF-1/CIP1 expression in the murine intestinal epithelium: apoptosis and cell cycle arrest. *Am J Pathol* 153: 899-909, 1998.

218. Wilson M, and Koopman P. Matching SOX: partner proteins and co-factors of the SOX family of transcriptional regulators. *Curr Opin Genet Dev* 12: 441-446, 2002.

219. Winesett DE, Ulshen MH, Hoyt EC, Mohapatra NK, Fuller CR, and Lund PK. Regulation and localization of the insulin-like growth factor system in small bowel during altered nutrient status. *Am J Physiol* 268: G631-640, 1995.

220. Woelfle J, and Rotwein P. In vivo regulation of growth hormone-stimulated gene transcription by STAT5b. *Am J Physiol Endocrinol Metab* 286: E393-401, 2004.

221. Woodson K, Flood A, Green L, Tangrea JA, Hanson J, Cash B, Schatzkin A, and Schoenfeld P. Loss of insulin-like growth factor-II imprinting and the presence of screen-detected colorectal adenomas in women. *J Natl Cancer Inst* 96: 407-410, 2004.

222. Wu A, Chen J, and Baserga R. Nuclear insulin receptor substrate-1 activates promoters of cell cycle progression genes. *Oncogene* 2007.

223. Wu Y, Yakar S, Zhao L, Hennighausen L, and LeRoith D. Circulating insulin-like growth factor-I levels regulate colon cancer growth and metastasis. *Cancer Res* 62: 1030-1035, 2002.

224. Xu XL, Yu J, Zhang HY, Sun MH, Gu J, Du X, Shi DR, Wang P, Yang ZH, and Zhu JD. Methylation profile of the promoter CpG islands of 31 genes that may contribute to colorectal carcinogenesis. *World J Gastroenterol* 10: 3441-3454, 2004.

225. Yamada S, Pokutta S, Drees F, Weis WI, and Nelson WJ. Deconstructing the cadherin-catenin-actin complex. *Cell* 123: 889-901, 2005.

226. Yamada Y, and Mori H. Pre-cancerous lesions for colorectal cancers in rodents: a new concept. *Carcinogenesis* 24: 1015-1019, 2003.

227. Yamauchi T, Ueki K, Tobe K, Tamemoto H, Sekine N, Wada M, Honjo M, Takahashi M, Takahashi T, Hirai H, Tsushima T, Akanuma Y, Fujita T, Komuro I, Yazaki Y, and Kadowaki T. Growth hormone-induced tyrosine phosphorylation of EGF receptor as an essential element leading to MAP kinase activation and gene expression. *Endocr J* 45 Suppl: S27-31, 1998.

228. Yang J, Zhang W, Evans PM, Chen X, He X, and Liu C. Adenomatous polyposis coli (APC) differentially regulates beta-catenin phosphorylation and ubiquitination in colon cancer cells. *J Biol Chem* 281: 17751-17757, 2006.

229. Yee D. Targeting insulin-like growth factor pathways. *Br J Cancer* 94: 465-468, 2006.

230. Yeh KY, and Moog F. Hormonal influences on the growth and enzymic differentiation of the small intestine of the hypophysectomized rat. *Growth* 42: 495-504, 1978.
231. Yu H, and Rohan T. Role of the insulin-like growth factor family in cancer development and progression. *J Natl Cancer Inst* 92: 1472-1489, 2000.
232. Zecevic M, Amos CI, Gu X, Campos IM, Jones JS, Lynch PM, Rodriguez-Bigas MA, and Frazier ML. IGF1 gene polymorphism and risk for hereditary nonpolyposis colorectal cancer. *J Natl Cancer Inst* 98: 139-143, 2006.
233. Zhang JG, Farley A, Nicholson SE, Willson TA, Zugaro LM, Simpson RJ, Moritz RL, Cary D, Richardson R, Hausmann G, Kile BJ, Kent SB, Alexander WS, Metcalf D, Hilton DJ, Nicola NA, and Baca M. The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. *Proc Natl Acad Sci U S A* 96: 2071-2076, 1999.
234. Zhang L, Kashanchi F, Zhan Q, Zhan S, Brady JN, Fornace AJ, Seth P, and Helman LJ. Regulation of insulin-like growth factor II P3 promotor by p53: a potential mechanism for tumorigenesis. *Cancer Res* 56: 1367-1373, 1996.
235. Zhou H, Miki R, Eeva M, Fike FM, Seligson D, Yang L, Yoshimura A, Teitell MA, Jamieson CA, and Cacalano NA. Reciprocal regulation of SOCS 1 and SOCS3 enhances resistance to ionizing radiation in glioblastoma multiforme. *Clin Cancer Res* 13: 2344-2353, 2007.
236. Zhuang S, and Schnellmann RG. A Death-Promoting Role for Extracellular Signal-Regulated Kinase. *J Pharmacol Exp Ther* 2006.