Growth Factors, Cytokines, Cell Cycle Molecules

Suppressor of Cytokine Signaling-2 Gene Disruption Promotes *Apc*^{Min/+} Tumorigenesis and Activator Protein-1 Activation

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Epigenetic *in vitro* and *in vivo* studies suggest that suppressor of cytokine signaling-2 (SOCS2) may normally limit tumorigenesis in the intestine; however, this theory has not been directly tested. We hypothesized that SOCS2 deficiency promotes spontaneous intestinal tumorigenesis in *Apc*^{Min/+} mice. Therefore, we quantified tumor number, size, and load in the small intestine and colon using *SOCS2*^{+/+}/*Apc*^{Min/+}, *SOCS2*^{+/-}/*Apc*^{Min/+}, and *SOCS2*^{-/-}/*Apc*^{Min/+} mice and assayed hematocrit as an indirect marker of disease severity. Biochemical and histological assays were used to assess mechanisms. Heterozygous and homozygous disruption of *SOCS2* alleles promoted 166 and 441% increases in tumor load in the small intestine, respectively, accelerated development of colon tumors, and caused severe anemia. SOCS2 deletion promoted significant increases in intestinal insulin-like growth factor-I mRNA but did not affect plasma insulin-like growth factor-I. Western blots and immunohistochemical analysis demonstrated that tumor and nontumor intestinal tissue of *SOCS2*^{-/-}/*Apc*^{Min/+} mice had increased serine 727 phosphorylation of signal transducer and activator of transcription 3 compared with *SOCS2*^{+/+}/*Apc*^{Min/+} mice. Moreover, electromobility shift assays showed that SOCS2 deletion did not alter signal transducer and activator of transcription 3 DNA binding. However, tumors and small intestine from *SOCS2*^{-/-}/*Apc*^{Min/+} showed dramatic increases in activator protein-1 (AP-1) DNA binding, and SOCS2 overexpression *in vitro* reduced levels of AP-1. These studies indicate that SOCS2 deletion promotes the spontaneous development of intestinal tumors driven by mutations in the adenomatous polyposis coli/ β -catenin pathway and activates AP-1. Therefore, reduced expression or epigenetic

silencing of SOCS2 may serve as a useful biomarker for colorectal cancer risk. (*Am J Pathol* 2010; 176:2320–2332; DOI: 10.2353/ajpath.2010.090684)

The suppressor of cytokine signaling (SOCS) family of proteins consists of SOCS1 through SOCS8 and cytokine-inducible SH2-containing protein.¹ SOCS proteins were initially discovered as negative regulators of cytokine signaling via the janus kinase (JAK) and signal transducers and activators of transcription (STAT) pathway.¹ Activated STATs form homo- and heterodimers and later translocate into the nucleus where they mediate gene transcription, proliferation, cell survival, and transformation.² Cytokine receptor-mediated JAK-STAT activation induces the expression of SOCS, which inhibits or terminates cytokine signaling by inhibiting JAK activation, competing with STATs or other signaling proteins for binding sites on cytokine receptors and by targeting signaling proteins for proteosomal degradation.¹

SOCS2 has been found to exert negative feedback effects on growth hormone (GH) signaling. *SOCS2*^{-/-} mice show enhanced body growth that is phenotypically similar to that in GH-transgenic mice and is reversed when GH is genetically inactivated, demonstrating a key role of SOCS2 in negatively regulating GH-induced body

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overgrowth.³ However, our recent studies indicate novel effects of SOCS2 deletion in the intestine whereby SOCS2^{-/-} mice show enhanced growth-promoting effects of insulin-like growth factor-I (IGF-I) as well as GH. SOCS2^{-/-} mice infused with IGF-I have significantly greater increases in the mucosal mass of the small bowel compared with wild-type littermates, establishing a role for SOCS2 in regulating the potency of action of IGF-I.⁴ These studies also show that SOCS2 deficiency enhances the proliferative and antiapoptotic actions of IGF-I in the small intestine and colon, along with the ability of IGF-I to activate STAT3. Other studies in intestinal epithelial or colon cancer cell lines have demonstrated that SOCS2 directly binds to the type I IGF-I receptor and limits its activation by IGF-I.^{4,5} Disruption of one SOCS2 allele in mice expressing a GH transgene was found to enhance transgene-induced increases in body weight and mucosal mass, to enhance colon and jejunal crypt proliferation, and to promote the formation of hyperplastic and lymphoid polyps in the colon.⁶ Although hyperplastic and lymphoid polyps are generally considered benign,⁷ this was novel *in vivo* evidence that SOCS2 may negatively regulate aberrant growth in the intestine in a model of GH and IGF-I excess. However, the role of SOCS2 in development of spontaneous precancerous adenomas in the intestine has not been established.

Limited evidence in cancers of other organs suggests that the SOCS2 gene may be epigenetically silenced by hypermethylation of CpG islands within the promoter.⁸⁻¹² In patients with melanoma, SOCS2 has been found to be silenced by methylation and its transcription is reduced in primary tumor samples.⁹ High SOCS2 expression inversely correlates with tumor grade of breast cancers and favors a good prognosis.¹³ Another study in breast carcinoma samples showed that SOCS2 protein expression is positively correlated with low-grade tumors.¹¹ Although these studies in primary human tumors and cancer cell lines suggest a potential role of SOCS2 in suppressing tumor growth, this theory has not been directly evaluated in an *in vivo* model of intestinal cancer.

The current study tested whether SOCS2 gene disruption enhances spontaneous intestinal tumor formation in *Apc*^{Min/+} mice. The *Apc*^{Min/+} mouse model is widely used to study mediators and mechanisms that govern the initiation, establishment, and progression of intestinal tumors.¹⁴ *Apc*^{Min/+} mice are heterozygous for a mutation that results in a truncated form of APC, a gene frequently mutated in human colon cancer that leads to aberrant activation of the β -catenin pathway.¹⁵ An adenomatous polyposis coli (APC)-containing complex normally degrades cytoplasmic β -catenin.¹⁶ In the absence of APC, β -catenin accumulates in the nucleus and coactivates the transcription of proliferative and prosurvival genes in conjunction with T-cell factor/lymphoid enhancer factor transcription factors.¹⁷ Depending on the genetic background, *Apc*^{Min/+} mice develop tens to hundreds of spontaneous adenomas in the small intestine and fewer adenomas in the colon, both of which typically arise due to focal loss of the remaining wild-type *Apc* allele.¹⁴ We cross-bred SOCS2^{-/-} and *Apc*^{Min/+} mice to test the hypothesis that disruption of one or both SOCS2 alleles will

increase tumor number or size in *Apc*^{Min/+} mice. Studies in our laboratory have shown that *ex vivo* treatment with IGF-I in the intestine induces STAT3 DNA binding activity, and this effect is enhanced and prolonged in SOCS2^{-/-} mice.⁴ Therefore, we postulated that loss of SOCS2 could increase activation of STAT3, which is increasingly linked to normal growth and tumorigenesis in the intestine.¹⁸ STAT3 is activated by tyrosine phosphorylation, which permits the formation of STAT3 homo- or heterodimers with other STATs as well as DNA binding activity. STAT3 is also serine-phosphorylated, and serine phosphorylation has recently been linked to activator protein-1 (AP-1) activation, cell proliferation, and transformation in cancer cell lines.¹⁹⁻²¹ Our studies demonstrate that SOCS2 gene deletion in *Apc*^{Min/+} mice enhanced serine but not tyrosine phosphorylation of STAT3, especially in tumors, and this was associated with increased AP-1 but not STAT3 binding activity. Together, these findings provide *in vivo* evidence that SOCS2 deletion promotes *Apc*^{Min/+} tumorigenesis, and this is associated with novel effects on serine phosphorylation of STAT3 and AP-1 activation.

Materials and Methods

Laboratory Animals

Apc^{Min/+} male mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice with targeted disruption of both SOCS2 alleles were developed as described previously and provided by Dr. Christopher Greenhalgh (Glaxo Smith Kline, Melbourne, Australia).²² To generate SOCS2 knockout mice on the *Apc*^{Min/+} background, SOCS2^{+/-} mice were cross-bred with *Apc*^{Min/+} mice, and SOCS2^{+/-}/*Apc*^{Min/+} male progeny were bred with SOCS2^{+/-} females to generate *Apc*^{Min/+} mice with SOCS2^{-/-}, SOCS2^{+/-}, or SOCS2^{+/+} genotypes. Genotyping was performed on tail DNA as described previously.^{4,23} Age-matched *Apc*^{Min/+} male and female mice with different SOCS2 genotypes were studied for number and size of tumors. Our studies emphasized female *Apc*^{Min/+} mice because male *Apc*^{Min/+} mice were primarily used as breeders as female *Apc*^{Min/+} mice exhibit complications during pregnancy.²⁴ The Institutional Animal Care and Use Committee of the University of North Carolina approved all animal studies. Study protocols were in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.²⁵

Sample Preparation and Tumor and Blood Measurements

At least six age-matched mice of each genotype were studied at 13 to 17 weeks of age. Animals were weighed and anesthetized, and blood was collected by cardiac puncture. The entire colon was dissected. The small intestine was dissected and separated into three segments roughly equal in length. Each intestinal segment was flushed with PBS supplemented with vanadate (2 mmol/L), phenylmethanesulfonyl fluoride (1 mmol/L), and phos-

phatase inhibitor cocktail (diluted 1:100, P2850, Sigma-Aldrich, St. Louis, MO). Intestinal segments were splayed open onto 3MM Whatman paper and fixed in 10% zinc-formalin (Fisher Scientific, Pittsburgh, PA) overnight at 4°C and then were dehydrated in 70% ethanol. Numbers of adenomas in the small and large intestines of each animal were counted under a Leica dissecting scope, using an in-lens micrometer to measure adenoma diameter. A portion (2–3 cm) of the proximal ileum was flash-frozen for RNA isolation. For immunohistochemical analysis, intestinal segments were rolled into a pinwheel, paraffin-embedded, and sectioned at 5 μ m. Embedding was performed by the Center for Gastrointestinal Biology and Disease Histology Core Facility. The presence and morphology of adenomas were confirmed by H&E staining and by immunohistochemical analysis for β -catenin. Because *Apc*^{Min/+} mice develop severe anemia as polypoid progresses, hematocrit was measured as an indirect marker of tumor load and disease severity²⁶; measurement was performed by the Animal Clinical Chemistry Core Facility at the University of North Carolina.

Immunohistochemical Analysis

Dewaxed and rehydrated sections underwent epitope retrieval in Reveal Decloaker (Biocare Medical, Concord, CA) and endogenous peroxidase activity was blocked with 3% H₂O₂. Sections were incubated in normal blocking serum either from a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) for rabbit antibodies or from a M.O.M. Kit (BD Transduction Laboratories, Franklin Lakes, NJ) for mouse antibodies. Sections were incubated overnight with mouse monoclonal antibody to β -catenin (BD Transduction Laboratories), rabbit anti-pS727-STAT3 (Cell Signaling, Beverly, MA), or rabbit anti-STAT3 (Cell Signaling). Bound antibody was detected with a Vector Elite Kit or biotinylated anti-mouse IgG (Vector Laboratories), followed by diaminobenzidine substrate. Tissues were counterstained with hematoxylin. Primary antibody was omitted as a negative control. Sections from both *SOCS2*^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} mice were stained on the same slide, and all slides for a particular antibody were stained in the same batch. Sections were examined on a Axio Imager A1 (Carl Zeiss, Jena, Germany), and photographs were taken using AxioCam MRc5 and associated AxioVision software (version 4.6). Comparisons were made between sections from pairs of *SOCS2*^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} mice mounted on the same slide. Brightness and contrast of photographs were selected to maximize the clarity for publication.

Plasma IGF-I Assay

IGF-I was measured by enzyme-linked immunosorbent assay, which was performed by the Center for Gastrointestinal Biology and Disease Immunotechnology Core at the University of North Carolina-Chapel Hill on plasma that was acid-ethanol extracted and cryoprecipitated to remove IGF-I binding proteins as described previously.^{6,27} A Mouse IGF-I Quantikine ELISA kit (R&D Systems, Minne-

apolis, MN) was used, according to the manufacturer's protocols, except that samples were diluted 1:500.

Quantitative RT-PCR

Total RNA was extracted from frozen ileum from at least five mice per genotype using TRIzol reagents and standard methods (Invitrogen, Carlsbad, CA). The concentration was determined by a NanoDrop system (Thermo Scientific, Wilmington, DE). Aliquots of each RNA sample were run on a 1% agarose gel to verify RNA integrity and concentration. RNA (4 μ g) was DNase-treated using the TURBO DNA-free Kit (Ambion, Foster City, CA) and transcribed into cDNA with AMV-Reverse Transcriptase (Promega, Madison, WI). A no-RT control was also generated for each sample.

To quantify IGF-I mRNA, an SYBR Green-based real-time PCR (SYBR Green JumpStart Taq ReadyMix, Sigma-Aldrich) was used with the following primers for mouse IGF-I: forward 5'-GTGTGGACCGAGGGGCTTTTACTTC-3' and reverse 5'-GCTTCAGTGGGGCACAGTACATCTC-3'. To measure c-Fos and c-Jun mRNA, TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) were used according to the manufacturer's protocol (c-Fos, Mm_00487425_m1; c-Jun, Mm_00495062_s1). Mouse hydroxymethylbilane synthase mRNA was also quantified as an invariant control using mouse SYBR Green primers (forward 5'-TGTGTTGCACGATCCTGAAAC-3' and reverse 5'-CTCCTTCCAGGTGCCTCAGAA-3') or a TaqMan Assay (Mm_01143545_m1). A standard curve was generated from dilutions (1×10^7 – 1×10^3 copies/ μ l) of a PCR product using conditions optimized for each primer or primer-probe set. DNA sequencing was performed by the University of North Carolina-Chapel Hill Genomic Analysis Facility to confirm the correct sequence of the PCR product. A no-template control was included in each run as a negative control as well as a standard to normalize the standard curve across runs. A cDNA pool generated from intestine and liver RNA from multiple mice was added to each run to control interrun variability. A melt was performed after each run to confirm that a single product was generated. Reactions were run on the Rotor-Gene 2000 (Corbett Research, Sydney, Australia) and analyzed with Rotor-Gene Software 6.0.14. Data for each sample were calculated as absolute copy number based on comparison of test sample data with a standard curve and normalized to hydroxymethylbilane synthase.

Nuclear Extracts and Immunoblotting

In a subset of *SOCS2*^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} mice, tumors were finely dissected under a dissecting scope from the middle third of the small intestine (~12 cm in length), and tissue was immediately used for extraction of nuclear proteins. Size-matched biopsy samples of grossly normal, tumor-free intestinal mucosa were also dissected for comparison. Tumor or nontumor samples were homogenized using a Dounce homogenizer in 1 ml of ice-cold 1 $\times$ Tris-buffered saline supplemented

with vanadate (2 mmol/L) and phenylmethanesulfonyl fluoride (1 mmol/L). Nuclear proteins were extracted as described previously.²⁸ Concentration was determined by a Bradford assay using Coomassie Plus reagent (Pierce Chemicals, Rockford, IL) and confirmed by examining samples on an SDS-polyacrylamide gel using the NuPAGE gel system (Invitrogen). Gels were visualized and analyzed using the LI-COR Odyssey Infrared Imaging System (version 3.0, LI-COR Biosciences, Lincoln, NE).

Western Immunoblotting

Equal amounts of protein (30 μ g) were size-fractionated on a 4 to 12% TEO-Cl SDS-polyacrylamide gel (PAGEgel, Fisher Scientific) and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) using a NuPAGE gel system. After blocking in Blocker Casein in PBS (Pierce), blots were incubated with primary antibodies against phospho-serine 727 (pS727)-STAT3, phospho-tyrosine 705 (pY705)-STAT3 (Cell Signaling), total STAT3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or histone H1 (Santa Cruz Biotechnology) for 16 hours at 4°C. Blots were washed in PBS containing 0.1% Tween and incubated with secondary antibodies conjugated to DyLight800 for ~1 hour at room temperature. Immunoreactive proteins were visualized and analyzed using the LI-COR Odyssey Infrared Imaging system. Densitometry was performed on visualized bands, and results were normalized to the total protein gel.

Electromobility Shift and Supershift Assays

DNA-protein binding reactions were performed in a 20- μ l total volume containing 100 mmol/L NaCl, binding buffer [20% glycerol, 1 mmol/L $MgCl_2$, 1 mmol/L EDTA, 0.5 mmol/L dithiothreitol, and 0.2 ng of poly(dI:dC)], 20 to 30 μ g of nuclear protein extract, and 100,000 to 400,000 cpm of a ^{32}P -labeled double-stranded oligomer corresponding to a consensus STAT3 binding site (STAT3-RE, Santa Cruz Biotechnology) or consensus AP-1 binding site (AP-1-RE, Promega). Binding was performed at room temperature for 20 minutes. For cold competition, an excess of unlabeled oligomer that was specific to either AP-1 or STAT3 or unrelated oligomer specific to the T-cell factor (TCF) DNA response element (S: 5'-GGTAAGATCAAAGGG 3', Integrated DNA Technologies Inc., Coralville, IA).²⁹ Samples were then loaded onto nondenaturing 4% polyacrylamide gels and electrophoresed in $0.5 \times$ TBE ($1 \times = 89$ mmol/L Tris borate, pH 8.0, and 2 mmol/L EDTA) for 2 to 3 hours. After electrophoresis, gels were dried on 3MM Whatman paper and exposed to PhosphorImager screens. Screens were viewed on the Typhoon 6400 scanner (GE Healthcare, Piscataway, NJ). The intensity of the shifted DNA-STAT3 and DNA-AP-1 complexes was quantified using Image-Quant Software v1.2 and is expressed as a percentage of values in nontumor samples for SOCS2^{+/+}/Apc^{Min/+} mice, which was set as 100%.

Supershift assays were performed to establish the composition of AP-1 binding activity according to meth-

ods published previously.²⁸ Antibodies used for Supershift assays were obtained from Santa Cruz Biotechnologies. Each supershift reaction included equal amounts of nuclear extract and 6 μ g of one of the following antibodies: rabbit antibodies specific for c-Jun, JunD, or c-Fos or mouse antibody specific for phospho-c-Jun (p-c-Jun, phosphorylated at serine 63). Nuclear extracts were incubated for 30 minutes at room temperature followed by the addition of γ - ^{32}P -labeled AP-1 oligomer and further incubated for 20 minutes. Samples were analyzed on 4% nondenaturing polyacrylamide gels as described above for electromobility shift assay (EMSA). Intensities of supershifted complexes were quantified using Image-Quant Software v1.2.

SOCS2 Overexpression in IEC-6 Cells

IEC-6 cells infected with SOCS2-expressing adenovirus or empty virus were used as a simple *in vitro* system to confirm the *in vivo* findings that SOCS2 affects AP-1 binding activity. IEC-6 cells are a nontransformed intestinal epithelial cell line previously shown to exhibit robust AP-1 activation in response to combined IGF-I and epidermal growth factor (EGF).³⁰ IEC-6 cells at 80% confluence were infected with either FLAG-tagged SOCS2 (Ad-SOCS2) or empty control adenovirus (Ad-Empty) in 10-cm² dishes as described previously.⁴ In brief, growth medium plus fetal bovine serum was removed, and serum-free medium (SFM) containing the appropriate virus at a multiplicity of 100 viral particles/cell was added. Twenty-four hours after infection, fresh SFM or SFM plus recombinant human (rh) IGF-I (20 ng/ml, Genentech, South San Francisco, CA) and rhEGF (5 ng/ml, Sigma-Aldrich) was added for 0, 15, and 30 minutes. Cells were scraped, and nuclear extracts were prepared as described above. AP-1 binding activity was assessed by EMSA and supershift assay as described above. Data were quantified as a percentage of the values in Ad-Empty infected cells in SFM alone.

Statistical Analyses

Values for average adenoma number, size, and tumor load and hematocrit are expressed as means $\pm$ SEM. Analysis of variance was used to compare data derived from Apc^{Min/+} mice, which have SOCS2^{+/+}, SOCS2^{+/-}, or SOCS2^{-/-} genotypes to determine whether there was a significant effect of SOCS2 deletion. Plasma IGF-I and IGF-I, c-Jun, and c-Fos mRNA data were compared between SOCS2^{+/+}/Apc^{Min/+} and SOCS2^{-/-}/Apc^{Min/+} mice by Student's *t*-test. Densitometry data for STAT3 and AP-1 DNA binding in tumor versus nontumor tissue of SOCS2^{+/+}/Apc^{Min/+} and SOCS2^{-/-}/Apc^{Min/+} mice were analyzed by analysis of variance. Pairwise comparisons were performed using Fisher's exact test. Densitometry results for AP-1 DNA binding in *in vitro* studies were compared between Ad-SOCS2 and Ad-Empty infected cells using Student's *t*-test. A value of *P* < 0.05 was considered statistically significant. Statistical tests were performed using Statview 4.1.

Results

Disruption of Both SOCS2 Alleles Modestly Affects Body Weight

SOCS2^{-/-} mice have a body overgrowth phenotype.³ We determined whether SOCS2^{-/-} mice retained this phenotype when cross-bred onto the *Apc*^{Min/+} line. SOCS2^{+/-}/*Apc*^{Min/+} mice had a final body weight of 21.4 ± 0.4 g at sacrifice. Loss of one SOCS2 allele had no significant effect on body weight (21.8 ± 0.3 g). However, SOCS2^{-/-}/*Apc*^{Min/+} mice had body weights of 27.8 ± 2.6 g, representing a significant 29.6% increase in final body weight (*P* < 0.05).

SOCS2 Gene Disruption Dramatically Increases *Apc*^{Min/+} Tumorigenesis

Small Intestine

Figure 1A shows representative images of tumors in small intestine of *Apc*^{Min/+} mice with SOCS2^{+/+}, SOCS2^{+/-}, or SOCS2^{-/-} genotypes. Quantitative data for tumor number, size, and total tumor burden (number × size) are shown in Figure 1 for the entire small intestine (Figure 1B) and different regions of the small intestine (Figure 1C). Compared with SOCS2^{+/+}/*Apc*^{Min/+} mice, SOCS2^{-/-}/*Apc*^{Min/+} mice showed significant and dramatic increases in total tumor number and tumor size, together representing a 441% increase in overall tumor burden in the small intestine. *Apc*^{Min/+} mice with disruption of one SOCS2 allele showed an intermediate phenotype with a significant increase in tumor number and tumor size (Figure 1B). This result suggests that SOCS2 has a gene-dosage effect on *Apc*^{Min/+} tumorigenesis. Examination of different regions of the small intestine revealed that SOCS2^{-/-}/*Apc*^{Min/+} mice showed significant increases in tumor number, size, and load in all segments (Figure 1C). Although a trend for increased tumor number and size was observed in all small bowel regions of SOCS2^{+/-}/*Apc*^{Min/+} mice, this increase was statistically significant only for tumor number in the most distal small bowel segment.

Colon

SOCS2 gene disruption in *Apc*^{Min/+} mice also had a dramatic effect on tumor number and size in colon. By 13 to 17 weeks of age, 100% of SOCS2^{-/-}/*Apc*^{Min/+} mice (six of six) and 71% of SOCS2^{+/-}/*Apc*^{Min/+} (five of seven) mice had at least one colon tumor, whereas only one of six SOCS2^{+/+}/*Apc*^{Min/+} mice studied (17%) had an observable colon tumor. Figure 2A shows representative images of colon tumors, and Figure 2B shows quantitative data for tumor number and size. These analyses revealed significant increases in tumor number, size, and burden in colon of SOCS2^{-/-}/*Apc*^{Min/+} mice compared with those in SOCS2^{+/+}/*Apc*^{Min/+}. As was seen with the small intestine, SOCS2^{+/-}/*Apc*^{Min/+} showed an intermediate phenotype.

Figure 1. SOCS2 gene disruption in *Apc*^{Min/+} mice increases tumor number and size in the small intestine. **A:** Representative gross images of adenomas in small intestine of SOCS2^{+/+}/*Apc*^{Min/+}, SOCS2^{+/-}/*Apc*^{Min/+}, and SOCS2^{-/-}/*Apc*^{Min/+} mice taken under a dissecting scope with white light optics underneath the specimen. Original magnification, × 12.3. **B:** Histograms show means ± SE for tumor number (top), size (middle), and load (number × size) (bottom) in the entire small intestine. **C:** Means ± SE for tumor number, size, and load in different regions of the small intestine. SI-1 is the first third of the small intestine that contains the duodenum and is most proximal, whereas SI-3 contains the ileum and is most distal. **P* < 0.05 versus SOCS2^{+/+}/*Apc*^{Min/+} mice; ***P* < 0.05 versus SOCS2^{+/-}/*Apc*^{Min/+}.

We used hematocrit as an indirect measure of tumor burden. Consistent with increases in tumor burden, we found that SOCS2^{+/+}/*Apc*^{Min/+} mice had hematocrit within normal range (34.2 ± 5.9%), SOCS2^{-/-}/*Apc*^{Min/+} mice had significant reductions in hematocrit (22.4 ± 5.3%, *P* < 0.05), and SOCS2^{+/-}/*Apc*^{Min/+} mice had an intermediate hematocrit (29.5 ± 7.7%). Further studies compared SOCS2^{+/+}/*Apc*^{Min/+} and SOCS2^{-/-}/*Apc*^{Min/+} mice, in which the most dramatic changes in tumor number and size were observed.

β-Catenin Immunostaining

Tumor development in *Apc*^{Min/+} mice is driven by focal loss of heterozygosity of the *Apc* gene.¹⁴ This leads to translocation of β-catenin from the lateral membranes of the epithelium to the nucleus where it promotes gene transcription. In the normal small intestine of both SOCS2^{+/+}/*Apc*^{Min/+} and SOCS2^{-/-}/*Apc*^{Min/+} mice,

Figure 2. SOCS2 gene disruption in *Apc*^{Min/+} mice increases tumor number and size in the colon. **A:** Representative gross images of colon adenomas of female SOCS2^{+/+}/Apc^{Min/+}, SOCS2^{+/-}/Apc^{Min/+}, and SOCS2^{-/-}/Apc^{Min/+} mice at 13 to 17 weeks of age taken under a dissecting scope. Original magnification, × 12.3. Colon tumors from female mice are shown because they demonstrated the greatest increases in tumor size compared with age-matched female SOCS2^{+/+}/Apc^{Min/+}. At this time, 17% of age- and sex-matched SOCS2^{+/+}/Apc^{Min/+} mice had colon tumors, whereas 71% of SOCS2^{+/-}/Apc^{Min/+} and 100% of SOCS2^{-/-}/Apc^{Min/+} mice had tumors. The small lesions visible in SOCS2^{+/+}/Apc^{Min/+} colon were determined to be lymphoid aggregates. **B:** Histograms show means ± SE for tumor number (**top**), tumor size (middle), and tumor load (**bottom**) in colon. *P < 0.05 versus SOCS2^{+/+}/Apc^{Min/+} mice by analysis of variance.

β -catenin was detected at the lateral and basement membranes of epithelial cells along the villi and in cell membranes and some nuclei in the crypts (Figure 3A). Adenomas in the small intestine of both SOCS2^{+/+}/Apc^{Min/+} and SOCS2^{-/-}/Apc^{Min/+} mice showed positive nuclear and cytoplasmic staining for β -catenin in epithelial-like cells of adenomas, but no obvious differences in staining intensity or nuclear localization were observed (Figure 3B). Colon adenomas in SOCS2^{-/-}/Apc^{Min/+} mice were positive for nuclear and cytoplasmic β -catenin, indicating activated β -catenin signaling (Figure 3C). Because so few SOCS2^{+/+}/Apc^{Min/+} mice develop colon tumors, β -catenin was not analyzed. The data confirm that increased nuclear β -catenin occurs in adenomas of both SOCS2^{+/+}/Apc^{Min/+} and SOCS2^{-/-}/Apc^{Min/+} mice but provide no evidence that loss of SOCS2 enhances nuclear β -catenin accumulation.

SOCS2^{-/-}/Apc^{Min/+} Mice Show Increased IGF-I Expression in the Small Intestine but No Elevation in Plasma IGF-I

We used real-time PCR to assess whether the enhanced tumorigenesis in the small intestine of SOCS2^{-/-}/Apc^{Min/+} mice was associated with increases in local IGF-I mRNA. Figure 4A shows that, compared with the liver, which is the major target of GH-induced IGF-I expression, the small intestine expresses relatively low amounts of IGF-I mRNA. As shown in Figure 4B, SOCS2^{-/-}/Apc^{Min/+} mice had small but statistically significant 1.5 fold increases in IGF-I mRNA in the ileum. In contrast, plasma IGF-I levels did not differ significantly in SOCS2^{+/+}/Apc^{Min/+} and SOCS2^{+/-}/Apc^{Min/+} mice (Figure 4C). These data indicate that disruption of both SOCS2 alleles in Apc^{Min/+} mice promotes increased local intestinal IGF-I expression, without affecting circulating IGF-I.

SOCS2 Gene Disruption Promotes Serine Phosphorylation but Not Tyrosine Phosphorylation of STAT3 in Apc^{Min/+} Mice

In normal intestine, SOCS2 gene disruption has previously been linked to enhanced IGF-I-induced STAT3 DNA binding activity.⁴ Therefore, we examined whether grossly normal mucosa (nontumor) or dissected tumors of SOCS2^{+/+}/Apc^{Min/+} or SOCS2^{-/-}/Apc^{Min/+} mice showed altered levels of nuclear tyrosine-phosphorylated STAT3 (pY705), which is integral to STAT3 DNA binding activity, and compared this with levels of serine-phosphorylated STAT3 (pS727). Surprisingly, levels of pY705-STAT3 did not differ in tumors or nontumor tissue from SOCS2^{-/-}/Apc^{Min/+} versus SOCS2^{+/+}/Apc^{Min/+} mice (Figure 5). However, the immunoreactive pY705-STAT3 in SOCS2^{-/-}/Apc^{Min/+} tumors was an obvious doublet, most likely due to concurrent serine phosphorylation. Western immunoblot of the same samples revealed that pS727-STAT3 was upregulated in nontumor tissue from SOCS2^{-/-}/Apc^{Min/+} mice and especially tumors of SOCS2^{-/-}/Apc^{Min/+} mice, showing an overall 2.5 ± 0.45-fold increase in pS727-STAT3 (P < 0.05) (Figure 5). When analyzed across multiple blots, total STAT3 did not significantly differ in tumor and nontumor tissues between SOCS2^{+/+}/Apc^{Min/+} and SOCS2^{-/-}/Apc^{Min/+} mice (Figure 5).

To confirm increased serine phosphorylation of STAT3 in the tumors of SOCS2^{-/-}/Apc^{Min/+} mice, we performed immunohistochemical analysis on small intestine and colon. In SOCS2^{+/+}/Apc^{Min/+} mice, pS727-STAT3 immunoreactivity was barely detectable except at the base of the crypts (Figure 6A). Immunostaining revealed dramatic increases in pS727-STAT3 in the nucleus and cytoplasm of crypt and villus epithelial cells in the normal small intestine of SOCS2^{-/-}/Apc^{Min/+} mice (Figure 6A). Serine-phosphorylated-STAT3 was detectable in the adenomas of SOCS2^{+/+}/Apc^{Min/+} mice. However, small intestine adenomas of SOCS2^{-/-}/Apc^{Min/+} mice showed dramatic increases in pS727-STAT3 in the cytoplasm and nucleus (Figure 6B). Intense staining of pS727-STAT3 was also

Figure 3. Tumors of *SOCS2*^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} mice are positive for nuclear β -catenin. Representative histological images. **A:** Grossly normal small intestine of *SOCS2*^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} mice immunostained for β -catenin. Original magnification: $\times 20$ (top); $\times 40$ (bottom). **B:** β -Catenin-positive adenomas in the small intestine of *SOCS2*^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} mice. Original magnification: $\times 5$ (top); $\times 40$ (bottom). **C:** Adenoma in the colon of a *SOCS2*^{-/-}/*Apc*^{Min/+} mouse positive for β -catenin immunostaining. Original magnification: $\times 5$ (top); $\times 40$ (bottom). Scale bar = 200 μ m unless otherwise noted.

detected in colon adenomas from *SOCS2*^{-/-}/*Apc*^{Min/+} (Figure 6C). In addition, we examined total STAT3 by immunohistochemistry and detected strong staining throughout the epithelium in the normal intestine that did not differ between *SOCS2*^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} (Figure 6D). Compared with nontumor tissue, tumors from both *SOCS2*^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} mice showed stronger cytosolic and nuclear STAT3 immunostaining but, in contrast to pS727-STAT3 immunostaining, there was no dramatic difference in total STAT3 in *SOCS2*^{-/-}/*Apc*^{Min/+} versus *SOCS2*^{+/+}/*Apc*^{Min/+} tumors (Figures 6, E and F). These studies suggest that deletion of *SOCS2* genes promotes serine 727 phosphorylation of STAT3 in intestinal epithelium and that this is further enhanced in small intestine and colon tumors.

Figure 4. Homozygous *SOCS2* gene disruption increases local intestinal IGF-I expression, but not plasma IGF-I. **A** and **B:** Real-time PCR was performed on RNA extracted from wild-type liver and small intestine (ileum) or entire ileum from *SOCS2*^{+/+} or *SOCS2*^{-/-} mice carrying the *Apc*^{Min/+} mutation. Absolute copy number was calculated from C_t values for either IGF-I or hydroxymethylbilane synthase using a standard curve. Histograms show the relative expression (means $\pm$ SE) of three runs for IGF-I/hydroxymethylbilane synthase. **A:** Histogram shows fold change of IGF-I expression in the liver relative to the small intestine. $n \geq 3$. Note that expression in the liver is ~ 45 -fold higher compared with that the small intestine. $^*P < 0.05$ versus *SOCS2*^{+/+}/wild-type. **B:** Histograms show IGF-I expression in ileal RNA from *SOCS2*^{-/-}/*Apc*^{Min/+} mice relative to *SOCS2*^{+/+}/*Apc*^{Min/+} mice analyzed in the same run. $^*P < 0.05$ versus *SOCS2*^{+/+}/*Apc*^{Min/+}. **C:** Enzyme-linked immunosorbent assay for plasma IGF-I levels (means $\pm$ SE) in *SOCS2*^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} mice. Binding proteins were removed before detection of IGF-I. Samples were run in duplicate, $n > 4$.

SOCS2 Deficiency Enhances AP-1 but Not STAT3 DNA Binding

Because serine phosphorylation of STAT3 was increased in the tumors of *SOCS2*^{-/-}/*Apc*^{Min/+} mice, we tested whether this increase was associated with changes in STAT3 DNA binding activity. STAT3 DNA binding activity was evaluated in nuclear extracts from pooled tumors and matched biopsies of grossly normal intestine from

Figure 5. Tumors of *SOCS2*^{-/-}/*Apc*^{Min/+} mice show increased serine phosphorylation of STAT3. Western blots on nuclear extracts from either normal intestine or a pool of tumors from the small intestine of *SOCS2*^{+/+}/*Apc*^{Min/+} or *SOCS2*^{-/-}/*Apc*^{Min/+} mice. Duplicate blots were immunoblotted for pY705-STAT3 or pS727-STAT3. Blots were then reprobbed for histone H1 (H1), a nuclear protein used as a loading control, or total STAT3. Shown are reprobes for H1 on the pY705 blot and total STAT3 on the pS727 blot. Protein stain of nuclear proteins is shown in the bottom panel to demonstrate equal loading. A ~ 86 kDa band was detected by both the pY and pS-STAT3 antibodies, although a doublet was apparent in the tumor samples with the pY705 antibody, most likely due to serine phosphorylation of STAT3. Overall, no difference in pY705-STAT3 was detected between *SOCS2*^{+/+}/*Apc*^{Min/+} (100 $\pm$ 9%) and *SOCS2*^{-/-}/*Apc*^{Min/+} (108 $\pm$ 10%) (values normalized to the H1 control). *SOCS2*^{-/-}/*Apc*^{Min/+} mice had a significant increase in serine phosphorylation (normalized to total STAT3) compared with *SOCS2*^{+/+}/*Apc*^{Min/+} mice (258 $\pm$ 45% versus 100 $\pm$ 23%, $P < 0.05$). Although total STAT3 appeared elevated in tumors of *SOCS2*^{-/-}/*Apc*^{Min/+} on this representative blot, across multiple blots and independent samples ($n = 5$), there was no significant difference in total STAT3 between *SOCS2*^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} mice (tumor, 1.13 $\pm$ 0.06 versus 1.15 $\pm$ 0.06; nontumor, 1.00 $\pm$ 0.05 versus 1.00 $\pm$ 0.08).

Figure 6. Increased pS727-STAT3 but not total STAT3 immunostaining in *SOCS2*^{-/-}/*Apc*^{Min/+} mice. Representative images of tissue sections from *SOCS2*^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} mice immunostained for pS727-STAT3 (**A–C**) or total STAT3 (**D–F**). **A–C:** Immunostaining for pS727-STAT3. **A:** Normal small intestine from *SOCS2*^{+/+}/*Apc*^{Min/+} or *SOCS2*^{-/-}/*Apc*^{Min/+} mice. Original magnification: ×20 (**top**); ×40 (**bottom**). **B:** Small intestine adenoma from *SOCS2*^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} mice. Original magnification: ×10 (**top**); ×40 (**bottom**). **C:** Colon adenoma from *SOCS2*^{-/-}/*Apc*^{Min/+} mouse. Original magnification: ×5 (**top**); ×40 (**bottom**). **D–F:** Immunostaining for total STAT3. **D:** Jejunum. Original magnification, ×20. **E:** Small intestine adenoma from *SOCS2*^{+/+}/*Apc*^{Min/+} or *SOCS2*^{-/-}/*Apc*^{Min/+} mice. Original magnification: ×10. **F:** Colon adenoma from *SOCS2*^{-/-}/*Apc*^{Min/+} mouse. Original magnification: ×5. Scale bars = 50 μm unless otherwise noted.

SOCS2^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} mice. As shown in Figure 7A, tumor or nontumor samples from *SOCS2*^{+/+} and *SOCS2*^{-/-} carrying the *Apc*^{Min/+} mutation had multiple STAT3 DNA binding complexes. Tumors from both *SOCS2*^{+/+}/*Apc*^{Min/+} and *SOCS2*^{-/-}/*Apc*^{Min/+} mice showed a trend for increased STAT3 DNA binding activity relative to nontumor tissue, but this achieved statistical significance only when tumor and nontumor tissues of *SOCS2*^{-/-}/*Apc*^{Min/+} mice were compared (Figure 7B). Surprisingly, however, there was no difference in STAT3 DNA binding activity in tumor or nontumor tissues of *SOCS2*^{-/-}/*Apc*^{Min/+} mice compared with those of *SOCS2*^{+/+}/*Apc*^{Min/+} mice (Figure 7B). Thus, loss of *SOCS2* did not significantly increase overall STAT3 binding activity as might have been expected.

Because phosphorylation of serine 727 of STAT3 has been linked to AP-1 activation in tumor cell lines, we analyzed whether *SOCS2* deletion affected nuclear AP-1 DNA binding. This analysis revealed enhanced AP-1 binding activity in nontumor tissues and particularly in

tumor tissue of *SOCS2*^{-/-}/*Apc*^{Min/+} compared with those of *SOCS2*^{+/+}/*Apc*^{Min/+} (Figure 8A). The results of three independent experiments were quantified using densitometric volume analysis and demonstrated significant increases in AP-1 DNA binding activity in the tumors of the *SOCS2*^{-/-}/*Apc*^{Min/+} mice compared with both tumor and nontumor tissue from *SOCS2*^{+/+}/*Apc*^{Min/+} mice (Figure 8B).

To verify the specificity of enhanced AP-1 DNA binding activity in *SOCS2*^{-/-}/*Apc*^{Min/+} mice, we performed supershift analysis for c-Jun, JunD, and phosphorylated c-Jun and c-Fos, as well as competition with unlabeled AP-1 oligomer versus an unrelated oligomer (TCF consensus sequence). As shown in Figure 8C, AP-1 DNA binding complexes in tumors from *SOCS2*^{-/-}/*Apc*^{Min/+} mice showed supershifted complexes with JunD, p-c-Jun, and c-Jun antibodies and showed specific competition with AP-1 but not TCF oligomers. Antibodies to c-Fos did not elicit a supershifted complex or major shift inhibition (ie, a reduction in the intensity of the DNA-

Figure 7. *SOCS2* gene disruption does not increase nuclear STAT3 DNA binding activity in *Apc^{Min/+}* mice. **A:** Autoradiogram of EMSA for nuclear binding activity to a STAT3 DNA binding sequence in either tumors or grossly normal small intestine of *SOCS2^{+/+}/Apc^{Min/+}* and *SOCS2^{-/-}/Apc^{Min/+}* mice. cc, inhibition of DNA-protein binding complexes by an excess of unlabeled oligomer. Both panels are from the same blot. Multiple DNA-protein binding complexes were detected with the STAT3 response element. WT, wild-type; cc, cold competition. **B:** Densitometric analysis of STAT3 DNA binding complexes. Results show means $\pm$ SE for intensity of DNA-protein complexes from three independent experiments expressed as a percentage of the *SOCS2^{+/+}/Apc^{Min/+}* nontumor samples. * $P < 0.05$ versus nontumor tissue from *SOCS2^{-/-}/Apc^{Min/+}* mice.

protein complex). Thus, the enhanced AP-1 DNA binding activity in tumors from *SOCS2^{-/-}/Apc^{Min/+}* mice contains primarily p-c-Jun, JunD, and c-Jun. Supershifted complexes were lower in intensity in nontumor samples, yet qualitatively similar in composition (Supplemental Figure 1, see <http://ajp.amjpathol.org>).

To assess whether the enhanced AP-1 DNA binding activity reflected increased expression of c-Jun or c-Fos, we quantified mRNA using quantitative real-time PCR. This revealed no significant difference in c-Fos or c-Jun between *SOCS2^{-/-}/Apc^{Min/+}* (c-Fos, 1.20 ± 0.45 ; c-Jun, 1.09 ± 0.10) versus *SOCS2^{+/+}/Apc^{Min/+}* (c-Fos, 1.0 ± 0.3 ; c-Jun 1.0 ± 0.1).

SOCS2 Overexpression Reduced AP-1 DNA Binding in IEC-6 Cells

Previous studies from our laboratory have demonstrated that IGF-I and EGF act synergistically to promote proliferation of IEC-6 cells and induce AP-1 transcriptional activity greater than that of either alone.³⁰ We therefore used this *in vitro* system to directly test whether *SOCS2* affects AP-1. As shown in Figure 9, IGF-I and EGF induced AP-1 binding activity in IEC-6 cells and *SOCS2* overexpression significantly attenuated growth factor-induced AP-1 DNA binding (Figure 9, A and B). This finding complements the *in vivo* data in *SOCS2^{-/-}/Apc^{Min/+}* mice, providing additional evidence that *SOCS2* affects AP-1 transcriptional activity in intestinal epithelial cells.

Figure 8. *SOCS2* gene disruption promotes increased nuclear AP-1 DNA binding activity in *Apc^{Min/+}* mice. **A:** Autoradiogram of EMSA for nuclear binding activity to an AP-1 DNA binding sequence in either tumors or grossly normal small intestine of *SOCS2^{+/+}/Apc^{Min/+}* and *SOCS2^{-/-}/Apc^{Min/+}* mice. cc, inhibition of shifted complexes by an excess of unlabeled oligomer. **B:** Densitometric analysis of AP-1 DNA binding complex. Results show means $\pm$ SE for intensity of AP-1 DNA binding complexes expressed as a percentage of the *SOCS2^{+/+}/Apc^{Min/+}* nontumor samples run on the same gel ($n = 3$ independent experiments). * $P < 0.05$ versus *SOCS2^{+/+}/Apc^{Min/+}* nontumor tissue; ** $P < 0.05$ versus *SOCS2^{+/+}/Apc^{Min/+}* tumor tissue by analysis of variance. **C:** Autoradiograms of supershift (SS) assays and cold competition (cc) specificity controls on pooled tumors from *SOCS2^{-/-}/Apc^{Min/+}* mice. **Arrowheads**, supershifted complexes. **Left panel**, supershifted complexes for JunD, p-c-Jun, and c-Jun. **Middle panel**, image intensity was increased to visualize c-Jun supershifted complex. **Right panel**, cold competition with excess of unlabeled AP-1 oligomer and no inhibition of AP-1 binding with an unrelated oligomer corresponding to the TCF binding site. No Ab, no antibody.

Supershift analysis demonstrated that the AP-1 complex in untreated or IGF/EGF-treated IEC-6 cells contained c-Jun, JunD, and p-c-Jun, as observed in *SOCS2^{-/-}/Apc^{Min/+}* tumors. Growth factor treatment increased the intensity of the p-c-Jun supershifted complex (Figure 9C). Supershifted complexes were uniformly lower in intensity in *SOCS2*-overexpressing cells (Figure 9C), but the relative percentage of AP-1 binding represented by JunD, phosphorylated c-Jun, and c-Jun was similar between empty vector and *SOCS2*-overexpressing cells (Figure 9C). This result indicates that *SOCS2* down-regulates all

Figure 9. Overexpression of *SOCS2* reduces nuclear AP-1 DNA binding activity in IEC-6 cells. **A:** Representative autoradiogram of EMSA with radiolabeled AP-1 oligomer on nuclear extracts from IEC-6 cells infected with either Ad-Empty or Ad-SOCS2. Cells were treated with SFM alone (0) or combined rhIGF-I (20 ng/ml) and rhEGF (5 ng/ml) (I + E) for 15 or 30 minutes. cc, cold competition; S, unlabeled AP-1 oligomer; NS, unrelated TCF oligomer. **B:** Densitometric analysis of AP-1 DNA binding complex. Results show means $\pm$ SE for intensity of AP-1 DNA binding complex expressed as a percentage of the Ad-Empty in SFM alone ($n = 3$ independent experiments). * $P < 0.05$ versus Ad-Empty; ** $P < 0.05$ versus no treatment. **C:** Autoradiogram of supershift (SS) assays on extracts from Ad-Empty or Ad-SOCS2-infected IEC-6 cells treated with SFM (left panel) or IGF-I and EGF for 30 minutes (right panel). Arrowheads, supershifted complexes with JunD, p-c-Jun, and c-Jun antibodies relative to the no antibody (no Ab) control. Numbers at the bottom indicate the percentage of JunD, p-c-Jun, or c-Jun in the AP-1 DNA protein complex (assessed as intensity supershifted band/intensity shifted complex in the no antibody control $\times$ 100) in each treatment group. Note that although the intensity of supershifted complexes is lower in Ad-SOCS2 versus Ad-Empty cells, the relative proportions of JunD, p-c-Jun, or c-Jun in AP-1 complexes are similar. Thus, SOCS2 overexpression reduces all Jun isoforms within the AP-1 complexes.

Jun isoforms within AP-1 DNA binding complexes to an approximately similar extent.

Discussion

Our studies show that disruption of both *SOCS2* alleles in *Apc*^{Min/+} mice dramatically increases tumor number and tumor size in the small intestine and colon. We found these increases to be associated with increased local intestinal IGF-I expression. Serine phosphorylation of STAT3 was increased in the tumors of *SOCS2*^{-/-}/*Apc*^{Min/+} mice and, to a lesser extent, in grossly normal intestine of *SOCS2*^{-/-}/*Apc*^{Min/+} mice. Neither tyrosine phosphorylation of STAT3 nor STAT3 DNA binding activity was enhanced by *SOCS2* gene deletion. The protumorigenic effects of *SOCS2* gene disruption were, however, associated with enhanced AP-1 DNA binding activity in the tumors of *SOCS2*^{-/-}/*Apc*^{Min/+} mice, when the AP-1 complex contained c-Jun, phosphorylated c-Jun, and JunD. Overexpression of *SOCS2* in IEC-6 cells reduced AP-1 DNA binding, with an approximately equal reduction in c-Jun, phosphorylated c-Jun, and JunD.

The increases in tumor number in *SOCS2*^{-/-}/*Apc*^{Min/+} mice occurred across all regions of the small intestine, with maximal effects in the most distal regions. Effects of *SOCS2* deletion on tumor size were less dramatic, but represent a significant finding because most modifiers of *Apc*^{Min/+} tumorigenesis alter tumor number but not size.³¹ Our findings, therefore, suggest that loss of *SOCS2* affects the rate of tumor growth as well as the number of initiated tumors or their survival and are strongly supported by data in the colon of *SOCS2*^{-/-}/*Apc*^{Min/+} mice. Very few *SOCS2*^{+/-}/*Apc*^{Min/+} mice developed colon tumors at the time studied. In contrast, 100% of *SOCS2*^{-/-}/*Apc*^{Min/+} mice and 71% of *SOCS2*^{+/-}/*Apc*^{Min/+} mice developed colon tu-

mors by 13 to 17 weeks, and tumor size was increased in colon as observed in small intestine. The current findings in the *Apc*^{Min/+} model are consistent with our prior findings that loss of one *SOCS2* allele in GH-transgenic mice promotes benign colon polyps.⁶ However, to our knowledge, this current study is the first to demonstrate that deletion of one or both *SOCS2* alleles promotes spontaneous precancerous lesions that are driven by aberrant β -catenin activation, a pathway that is dysregulated in many human intestinal tumors. This finding suggests that epigenetic *SOCS2* silencing may be relevant to colon cancer risk in humans.

A number of studies have shown that *SOCS2* genes are epigenetically inactivated by hypermethylation of CpG islands within the promoter region of biopsy samples from primary human tumors or cancer cell lines from a number of organs, including prostate, melanoma, breast, ovary, and endometrium, and this inactivation correlates with reduced *SOCS2* expression.⁸⁻¹² Other *SOCS* genes, including *SOCS1* and *SOCS3*, have also been shown to be hypermethylated in Barrett's adenocarcinoma.³² *SOCS1* is hypermethylated in breast cancer biopsy samples and in glioblastoma cells.^{33,34} *SOCS3* is also methylated in glioblastoma cells, and hypermethylation of *SOCS3* is associated with an unfavorable clinical outcome.³³ However, relatively few studies have shown a functional role for *SOCS* in tumor development or growth *in vivo*. Mice with *SOCS3* deleted specifically in liver parenchymal cells exhibited increased tumor development when treated with the carcinogen diethylnitrosamine.³⁵ Deletion of *SOCS3* specifically in intestinal epithelial cells led to greater tumor load in the azoxymethane/dextran sodium sulfate mouse model of inflammation-induced colon cancer.³⁶ This finding indicates a role for *SOCS3* in normally limiting inflammation-associated colon cancer.

Our current data in $SOCS2^{-/-}/Apc^{Min/+}$ mice indicate that endogenous SOCS2 normally limits the development of tumors that derive from dysregulated β -catenin, a pathway that is integral to the development of many human colon cancers. To date, the relative roles of SOCS2 compared with those of SOCS1 or SOCS3 in tumor development have not been formally compared in the same animal model of intestinal tumorigenesis. In this regard, preliminary findings in the azoxymethane/dextran sodium sulfate model provided no evidence that SOCS2 gene disruption affects tumor number or load ($SOCS2^{+/+}$, 2.2 ± 0.6 ; $SOCS2^{-/-}$, 2.9 ± 0.4 ; $n = 5$) in this model of inflammation-associated colon cancer. Although more study is needed, this finding suggests that SOCS2 and SOCS3 should be compared as potentially useful biomarkers of the risk of human colon tumors driven by two distinct and major initiating factors: activation of β -catenin or inflammation.

Our findings suggest some novel regulatory mechanisms by which SOCS2 deletion may promote $Apc^{Min/+}$ -mediated intestinal tumorigenesis. We initially assessed local IGF-I because SOCS2 gene deletion was shown to increase local IGF-I gene expression in some tissues²² and to increase GH-stimulated, local IGF-I expression in the intestine.^{4,6} Our findings demonstrate modest up-regulation of local IGF-I expression in $SOCS2^{-/-}/Apc^{Min/+}$ intestine, which may contribute to enhanced tumor load. IGF-I signaling has been reported to enhance stability, nuclear localization, and transcriptional activation of β -catenin.^{37,38} This enhancement is thought to occur through insulin receptor substrate-1, which is an immediate downstream mediator of IGF-I receptor signaling.^{37,38} However, observations that tumors from $SOCS2^{+/+}/Apc^{Min/+}$ and $SOCS2^{-/-}/Apc^{Min/+}$ mice show comparable increases in β -catenin accumulation argue against a major effect of SOCS2 deletion or accompanying increases in local IGF-I on β -catenin activation or accumulation, at least at the stage of tumor development examined. We note that increased local IGF-I expression in the intestine of $SOCS2^{-/-}/Apc^{Min/+}$ was not accompanied by an elevation in plasma IGF-I. This finding consistent with prior findings in $SOCS2^{-/-}/Apc^{Min/+}$ mice^{3,6} and suggests that SOCS2 gene disruption has a preferential impact on local intestinal IGF-I expression rather than on circulating IGF-I levels.

We examined the STAT3 pathway because of our prior findings that $SOCS2^{-/-}$ mice show enhanced IGF-I-induced STAT3 activation in normal intestine.⁴ STAT3 activation is frequently associated with colon cancer in a setting of chronic inflammation.^{39–41} Recent studies demonstrated that specific STAT3 deletion in intestinal epithelial cells reduces tumor multiplicity in $Apc^{Min/+}$ mice.⁴² Phosphorylation of STAT3 at tyrosine residue 705 is required for dimerization, nuclear translocation, and DNA binding.^{20,43} Unexpectedly, we found similar levels of tyrosine-phosphorylated STAT3 in normal small intestine tissue and tumors from $SOCS2^{-/-}/Apc^{Min/+}$ compared with those in $SOCS2^{+/+}/Apc^{Min/+}$ mice, and our studies revealed an increase in serine 727-phosphorylated STAT3 in $SOCS2^{-/-}/Apc^{Min/+}$ mice. Serine-phosphorylated STAT3 localized to epithelial cells in normal intestine and to tumors in $SOCS2^{-/-}/Apc^{Min/+}$ mice. The increased pS727-STAT3 in $SOCS2^{-/-}/Apc^{Min/+}$ com-

pared with that in $SOCS2^{+/+}/Apc^{Min/+}$ mice observed by Western immunoblot was independently confirmed by immunohistochemistry. This result is intriguing because a series of recent articles has demonstrated upregulation of pS727-STAT3 in mammary gland tumors⁴⁴ and in a number of tumor cell lines including ras-transformed fibroblasts, bladder and lung tumor cell lines,²¹ and medullary thyroid tumor cell lines.¹⁹ Studies using prostate cancer cell lines have shown that a phosphomimetic mutation of serine727 to a glutamine promoted anchorage-independent growth, invasion, and, in xenograft models, increased tumor numbers.⁴⁵ These effects were reversed by an un-phosphorylatable STAT3 mutant.⁴⁵ In another study, mutation of serine727 in STAT3 to an alanine decreased postnatal growth and survival in mice lacking one functional STAT3 allele.⁴⁶ This decrease correlated with reduced circulating GH and IGF-I.⁴⁶ Together, these studies provide evidence that serine phosphorylation of STAT3 may regulate normal and neoplastic growth. Our current findings add novel evidence that loss of SOCS2 preferentially enhances pS727-STAT3 in the intestine *in vivo*, and this is associated with dramatically increased intestinal tumor development and growth in animals carrying an inactivating Apc mutation.

The role of pS727-STAT3 in DNA binding and transcriptional activation of STAT3 is not fully defined. Prior studies have linked pS727-STAT3 to reduced tyrosine phosphorylation and transcriptional activation of STAT3.^{47,48} Other reports indicate that serine phosphorylation of STAT3 does not affect DNA binding.⁴⁹ More recent studies indicate that there are context-, cytokine- and growth factor-dependent differences in serine phosphorylation versus tyrosine phosphorylation of STAT3.^{19,20,50–52} Importantly, a recent report in medullary thyroid carcinoma cell lines demonstrated that pS727-STAT3 is essential to transcriptional activation of AP-1 DNA binding activity.¹⁹ This finding is consistent with the current observations that nuclear AP-1 DNA binding activity is dramatically upregulated in tumors from $SOCS2^{-/-}/Apc^{Min/+}$ mice. Our findings that $SOCS2^{-/-}/Apc^{Min/+}$ mice show enhanced AP-1 DNA binding activity but no detectable increase in c-Fos or c-Jun mRNA indicate that loss of SOCS2 promotes activation of AP-1 rather than increased synthesis of AP-1 components. Furthermore, supershift data indicate that the AP-1 binding complex in $SOCS2^{-/-}/Apc^{Min/+}$ mice contains primarily c-Jun and particularly phosphorylated c-Jun and JunD. These results were complemented with *in vitro* studies in IEC-6 cells, demonstrating that adenoviral overexpression of SOCS2 reduced basal and growth factor-stimulated AP-1 DNA binding activity with approximately equal inhibitory effects on c-Jun, phosphorylated c-Jun, and JunD. This finding is intriguing because recent studies suggest that Jun proteins are strongly expressed in tumors of mice with specific activation of β -catenin in intestinal epithelium.⁵³ Activation of c-Jun N-terminal kinase, which mediates c-Jun phosphorylation at serine 63, was also shown to activate TCF4/ β -catenin gene targets and increase crypt proliferation.⁵⁴ These studies provide indirect evidence to support a concept that the enhanced AP-1 activity observed here in $SOCS2^{-/-}/Apc^{Min/+}$ may contribute to the increased tumorigenesis. Additional

studies will be required to fully delineate the mechanisms by which loss of SOCS2 expression promotes serine phosphorylation of STAT3 and AP-1 DNA binding activity, their possible causal connection, and their functional roles in enhanced tumorigenesis in *SOCS2*^{-/-}/*Apc*^{Min/+} mice. However, our findings provide novel evidence that SOCS2 normally serves as a suppressor of *Apc*^{Min/+} tumorigenesis in intestine and acts on pathways other than tyrosine phosphorylation of STAT3.

In summary, the results from our study suggest that SOCS2 gene deletion promotes tumor initiation and progression in the *Apc*^{Min/+} model of spontaneous intestinal cancer driven by β -catenin activation and that this is associated with local increases in IGF-I, serine phosphorylation of STAT3, and AP-1 activation but not increased tyrosine phosphorylation of STAT3. These studies provide novel evidence that SOCS2 normally limits tumor growth in both the small intestine and colon and suggest that reduced expression or epigenetic silencing of SOCS2 and activation of pS727-STAT3 or AP-1 may be potential biomarkers for intestinal tumor development and growth in a setting of APC mutation or β -catenin activation.

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