Suppressor of Cytokine Signaling-2: A Growth Hormone−Inducible Inhibitor of Intestinal Epithelial Cell Proliferation

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Background & Aims: Growth hormone (GH) and insulin-like growth factor-I (IGF-I) increase intestinal growth. GH is thought to act indirectly via IGF-I. In several models, including rats given total parenteral nutrition (TPN), IGF-I more potently stimulates mucosal growth than GH, even when GH induces similar circulating IGF-I levels. These studies test the hypothesis that GH induces a suppressor of cytokine signaling (SOCS), which inhibits intestinal epithelial cell (IEC) proliferation. *Methods*: Rats on TPN received vehicle, GH, or IGF-I. Jejunal SOCS (SOCS-1, -2, -3, and cytokine-inducible SH2-domain−containing protein [CIS]) and IGF-I messenger RNA (mRNA) were quantified. Caco-2, IEC-6 cells, and SOCS-2 null and wild-type (WT) mice were used to examine the expression and functional role of SOCS-2. *Results:* As reported previously, IGF-I, but not GH, prevented mucosal atrophy during TPN, although GH elevated plasma IGF-I and increased body weight. GH, but not IGF-I, induced jejunal SOCS-2 mRNA. SOCS-2 mRNA levels in GH and IGF-I-treated rats inversely correlated with mucosal weight. SOCS-2 is expressed in Caco-2 cells, and elevated SOCS-2 expression in postconfluent cells is associated with reduced proliferative rates. SOCS-2 overexpression in Caco-2 cells inhibited cell proliferation and promoted differentiation. In IEC-6 cells, GH induced SOCS-2 and reduced basal or IGF-I−induced proliferation. GH also reduced proliferative activity in isolated crypts from WT but not SOCS-2 null mice, and SOCS-2 null crypts showed enhanced proliferative responses to GH and IGF-I. SOCS-2 null mice have increased intestinal weight and length. *Conclusions:* SOCS-2 is a GH-inducible, novel inhibitor of intestinal epithelial cell proliferation and intestinal growth.

Growth hormone (GH) and insulin-like growth factor (IGF)-I have both been shown to exert trophic actions on the intestinal epithelium.[1–3] GH-induced increases in circulating IGF-I have traditionally been thought to mediate or contribute to GH action on in-testinal growth.[3] Examination of the literature reveals, however, that the most potent trophic effects of GH on intestine have been observed in transgenic mice with long-term GH excess.[2,3] Comparisons between GH and IGF-I transgenic mice[1,2] and in models of systemic treatment with GH or IGF-I indicate also that IGF-I has much more potent actions to increase crypt cell prolif-eration than GH.[1,2,4–9] This is somewhat surprising be-cause, in these same models, GH elevates circulating IGF-I and promotes similar or greater increases in body weight than IGF-I.[1,2,4–7,9] These findings, together with the fact that there is little evidence in the literature for direct proliferative actions of GH on isolated epithelial cells, have led us to hypothesize that GH may induce, within the intestine, a factor that limits its stimulatory effects on crypt cell proliferation or those of IGF-I. Support for the novel concept that GH may self-limit its own actions stems from recent observations that GH induces suppressors of cytokine signaling (SOCS), which act as negative feedback inhibitors of cytokine action.[10]

SOCS comprise a family of 8 related proteins: SOCS-1 through 7 and cytokine-inducible SH2-domain−con-taining protein (CIS).[10] A number of studies have shown that several SOCS family members can terminate or inhibit GH activation of the Janus kinase–signal trans-ducer and activator of transcription (STAT) path-way.[11–15] An overgrowth phenotype of mice homozygous for targeted disruption of SOCS-2 indicates a particular

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role of SOCS-2 in limiting body growth and growth of some organs.[16] The similarity in phenotype of SOCS-2 null mice and mice overexpressing GH or IGF-I, as well as increased hepatic expression of a number of GH-inducible genes in liver of SOCS-2 null mice, suggests that SOCS-2 may have a key role in regulating GH or IGF-I action on growth in vivo. However, the role of SOCS-2 or other SOCS in GH or IGF-I action in the intestine is not yet defined, and there is no direct evidence that SOCS-2 limits the mitogenic actions of GH or IGF-I. Although most current evidence suggests that SOCS interact with receptors for cytokines, which use the Janus kinase–STAT pathway,[10] yeast 2–hybrid data suggest that SOCS-2 interacts with the type 1 IGF receptor, which is a receptor tyrosine kinase mediating IGF-I action.[17] SOCS-2 is therefore an attractive candidate as a GH-induced signaling molecule that could not only limit GH action but also could modulate IGF-I action or the interactions between GH and IGF-I in regulating growth and proliferation of the intestinal epithelium. The current studies first used a rat model of total parenteral nutrition (TPN), where differences in potency of GH and IGF-I on intestinal growth have been well established.[4,5,18] We used this model to test whether GH, but not IGF-I, induces SOCS-2 or other SOCS family members and whether this correlates with effects of GH and IGF-I on mucosal mass. We then used model intestinal epithelial cell lines to examine more directly whether SOCS-2 is expressed in intestinal epithelial cells and to examine its functional effects on cell proliferation and its regulation by GH. Isolated intestinal crypts from SOCS-2 null mice and wild-type (WT) littermates were used to test whether SOCS-2 deficiency alters the mitogenic effects of GH or IGF-I on the intestinal epithelium, and intestinal mass was examined in SOCS-2 null and WT mice.

Materials and Methods

Rat TPN Model

A rat TPN model was used to compare the effects of GH and IGF-I on jejunal SOCS expression and to test whether these effects correlate with effects on mucosal growth. The TPN model permits comparisons of GH and IGF-I action under conditions of identical nutrient intake.[4] TPN experiments were performed at the University of Wisconsin-Madison and approved by the Institutional Animal Care and Use Committee and have been described in detail elsewhere.[19] Briefly, 250-g male Sprague Dawley rats (Harlan, Madison, WI) were fasted for 18 hours, anesthetized with 80 mg ketamine plus 8 mg xylazine per kg body weight, and a catheter was inserted into the superior vena cava as previously described.[4,19] The infusion of TPN solution was gradually increased from 30 mL

on the day of surgery to 50 mL on day 1 and then 60 mL on days 2–7. During the last 6 days of TPN, animals were given intravenous GH or intravenous IGF-I (human recombinant GH or IGF-I, 800 μg/day; Genentech, Inc., San Francisco, CA) or saline vehicle continuously coinfused with the TPN solution. A group of rats allowed free access to food throughout the experiment was studied in parallel with TPN groups to provide an orally fed control group. Body weight was monitored throughout the experiment. After 8 days of exclusive TPN with 6 days of concomitant growth factor treatment, animals were anesthetized and blood was collected for radioimmunoassay of total plasma IGF-I as previously described.[20] The abdomen was opened by midline incision and jejunum was dissected and flushed with ice-cold saline. Corresponding jejunal segments were collected for assay of mucosal dry mass (mg/cm) or RNA extraction as previously described.[4,5] Colon was also collected for RNA extraction.

RNA Extraction and Northern Blot Analysis

Total RNA was isolated from jejunum and colon, and abundance of SOCS-1, -2, -3, and CIS messenger RNAs (mRNAs) was assayed by Northern blot hybridization using [^{32}P]-labeled antisense complementary RNA or complementary DNA probes and methods detailed previously.[21] Mouse SOCS-1, -2, -3, and CIS probes were provided by Dr. Douglas Hilton (Walter and Eliza Hall Institute, Melbourne, Australia).[22] Abundance of the constitutively expressed ribosomal protein PL-7 mRNA was used as a control for RNA loading. IGF-I mRNA was assayed in jejunum by ribonuclease protection assay using rat IGF-I and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (control) (Ambion, Austin, TX) complementary RNA probes as previously described.[23]

In Vitro Analyses

Caco-2 and intestinal epithelial cell (IEC)-6 cells were obtained from the American Type Culture Collection (Rockville, MD) and were used to examine the proliferative effects of GH, expression of SOCS-2 in intestinal epithelial cells, and functional effects of SOCS-2 on proliferation. Caco-2 cells are one of the few intestinal epithelial cell lines reported to be GH responsive,[24,25] and, although derived from a colon cancer, Caco-2 cells exhibit characteristics of small intestinal enterocytes in their ability to differentiate spontaneously and express sucrase-isomaltase and alkaline phosphatase. In addition, the rates of spontaneous proliferation of Caco-2 cells in serum-free or serum-containing media are known to depend largely on autocrine or serum-derived IGFs, respectively.[8,26] IEC-6 cells were used as an independent, nontransformed cell line that has not previously been tested for GH responsiveness but shows a robust proliferative response to IGF-I.[27] Both cell lines were propagated in Dulbecco modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10 mmol/L HEPES (growth medium).

For assays of GH or IGF-I action on proliferation, cells were switched to serum-free medium supplemented with 5 μg/mL

transferrin and 0.1% bovine serum albumin (Sigma, St. Louis, MO) or 5 μg/mL selenous acid (Sigma).[28] [³H]-thymidine incorporation was measured as previously described.[27,29] To study SOCS-2 expression, total RNA was isolated by using TRIzol reagent according to manufacturer's instructions (Gibco BRL). Assays of SOCS-2 mRNA in Caco-2 cells were performed as described above but used a human SOCS-2 probe provided by Dr. Richard Furlanetto (Juvenile Diabetes Research Foundation, New York, NY)[17] and a human GAPDH probe (Ambion) as a loading control. SOCS-2 and control GAPDH mRNA were measured in IEC-6 cells by reverse-transcription polymerase chain reaction (RT-PCR), using standard amplification conditions.[30] Oligomers used for RT-PCR were hSOCS-2 sense (5′GTG-GGTGACAGTGTCTGCGAGAGAC3′), hSOCS-2 antisense (5′GTCCCCGGATGGTACCGGTACACTT3′), GAPDH sense (5′CTACTGGCGCTGCCAAGGCTGT3′), and GAPDH anti-sense (5′GCCATGAGGTCCACCACCCTGTTG3′).

The functional effects of SOCS-2 over expression were studied in Caco-2 cells. Caco-2 cells were transfected with a human pBIG2i-FLAGSOCS-2 expression vector or empty vector provided by Dr. Furlanetto.[17,31] pBIG2i-FLAGSOCS-2 contains the complete coding sequence of human SOCS-2 with a FLAG epitope tag downstream from the repressed tetracycline trans-activator (rtTAN), tetracycline/doxycycline-inducible promoter, and a hygromycin resistance gene to permit selection of transfected cells. The empty vector is identical except that it lacks the inserted SOCS-2 coding sequence.[17,31] Cells were seeded in growth medium at 60%–80% confluence. After 24 hours, medium was switched to growth medium plus endotoxin-free plasmid and Effectene as per manufacturer's instructions (Qiagen Inc., Valencia, CA). Twenty-four hours after transfection, medium was changed to remove Effectene and detached cells. The remaining cells were switched to growth medium plus hygromycin (100 μg/mL) to maintain selection of transfected cells and doxycycline (1 μg/mL) to maximize SOCS-2 expression. Dependent on the experiment, functional analyses were performed between 24 hours and 15 days after initial selection with hygromycin, during which time fresh growth medium plus hygromycin and doxycycline were added every 48 hours to maintain selection of transfected cells and maximal expression of transfected plasmid. Northern hybridization for SOCS-2 mRNA and immunoprecipitation of epitope tagged SOCS-2 with an M2 Anti-FLAG antibody (Sigma Diagnostic Inc., St. Louis, MO) followed by western immunoblot with the same anti-FLAG antibody were used to verify over expression of SOCS-2.

Assays of Differentiation Markers in Caco-2 Cells

Sucrase-isomaltase mRNA was quantified by northern hybridization using a sucrase-isomaltase complementary RNA and GAPDH as control. Alkaline phosphatase activity was assayed using a kit from Sigma Diagnostic Inc. (St. Louis, MO) following manufacturer's instructions.

In Vivo Analyses: SOCS-2 Null and WT Mice

SOCS-2 null mice were derived as previously described.[16] Heterozygote male and female mice were bred to provide age-matched SOCS-2 null and WT mice for analyses. Adult (100–200-day-old) mice were anesthetized, and the entire small intestine from pylorus to ileocecal valve and colon was dissected. Length and weight of the entire small intestine and colon were recorded. To assess whether absolute SOCS-2 deficiency altered crypt proliferation, isolated colonic crypts were prepared by using a modified method described by Whitehead et al.[32] and tested for responses to GH, IGF-I, and GH plus IGF-I by using [³H]-thymidine incorporation into DNA as a readout. The lumen of dissected colon was washed sequentially in ice-cold phosphate-buffered saline (pH 7.4) plus 1.5 mmol/L dithiothreitol, then phosphate-buffered saline + 27 mmol/L Na citrate at room temperature. The colon was then incubated/washed at room temperature in 20 mL of crypt elution buffer (phosphate-buffered saline containing 3 mmol/L EDTA and 0.5 mmol/L dithiothreitol, 50 U/mL penicillin, and 50 μg/mL streptomycin). Every 15 minutes, the colon was first shaken gently and allowed to settle, and supernatants were removed and visualized under an inverted microscope. The first washes remove debris within the supernatants, and supernatants collected at 45–90 minutes contained intact crypts. Crypts were pooled into Dulbecco modified Eagle's medium plus antibiotics. Pooled crypts were gently centrifuged (200g) for 5 minutes. Supernatants were aspirated and pelleted crypts resuspended in Dulbecco modified Eagle's medium and counted by using a hemocytometer. Equal numbers of crypts from WT and SOCS-2 null mice were seeded into 24-well plates precoated with purified collagen (Vitrogen 100, 2.9 mg/mL; Celtrix Pharmaceuticals, Santa Clara, CA). After 60-minute incubation in serum-free medium, fresh medium was added with [³H]-thymidine (2 μCi/mL) plus or minus GH (10^{-9} mol/L), IGF-I (10 ng/mL), or GH and IGF-I in combination. Acid-precipitated DNA was harvested in 0.2 N sodium hydroxide, 0.1% sodium dodecyl sulfate, and counted as for cultured cells. Data were expressed as fold stimulation by treatments relative to serum-free control.

Statistical Analyses

Values are expressed as mean ± SEM. One-way analysis of variance followed by Fisher protected least significant differences multiple t test was used to test for differences between groups.

Results

Effects of GH and IGF-I in TPN-Fed Rats

Body weight, plasma IGF-I, and mucosal mass data for some of the treatment groups have been reported previously for the animals used in this study.[19] These prior data are shown in Table 1 to show key points relevant to the new analyses, which tested for correlations

Table 1. Body Weight Gain, Jejunal Mucosa Dry Weight, Serum IGF-I, and Jejunal IGF-I mRNA Abundance

	Body weight gain (g/8d)	Jejunal mucosa dry wt (mg/cm)	Serum IGF-I (µg/L)	Jejunal IGF-I mRNA[a] (arbitrary units)
Oral	40 ± 3[b]	7.9 ± 0.6[b]	377 ± 14[b]	4.3 ± 2.6
TPN	12 ± 3	3.9 ± 0.3	304 ± 9	2.0 ± 0.6
TPN + GH	34 ± 1[b]	4.1 ± 0.3	435 ± 15[b]	3.4 ± 2.2
TPN + IGF-I	31 ± 3[b]	7.7 ± 0.1[b,c]	694 ± 21[b,c]	2.7 ± 0.8

NOTE. Values are means ± SE. n = 5/group.
[a]IGF-I mRNA abundance normalized to GAPDH control mRNA.
[b]$P < 0.01$ vs. TPN control.
[c]$P < 0.01$ vs. TPN + GH.

between mass of jejunal mucosa and jejunal SOCS-2 mRNA. As reported previously in these animals[19] and previous studies with similar experimental design,[4,5] TPN-fed rats showed reductions in body weight gain, plasma IGF-I, and mass of jejunal mucosa relative to orally fed animals (Table 1). Administration of IGF-I to TPN-fed rats restored body weight gain, plasma IGF-I, and mucosal mass toward values observed in orally fed controls. In contrast, administration of GH restored body weight gain and plasma IGF-I but not the mass of jejunal mucosa (Table 1). Levels of plasma IGF-I achieved in TPN-fed rats given GH were not as high as in IGF-I-infused rats. It is important to note, however, that body weight gain achieved in GH- and IGF-I–infused rats was identical. In previous, similarly designed studies, even when plasma IGF-I levels did not differ significantly between GH- and IGF-I–treated TPN-fed rats, GH still did not increase jejunal mucosal mass, despite similar efficacy as IGF-I in restoring body weight gain.[4,5] Jejunal mucosal IGF-I mRNA was assayed in the present study, and, consistent with previous findings,[33] there was a nonsignificant trend for reduced IGF-I mRNA in TPN-fed rats relative to orally fed rats, and neither GH nor IGF-I significantly affected jejunal IGF-I mRNA (Table 1).

Effects of GH and IGF-I on SOCS mRNA in TPN-Fed Rats

SOCS-2 and CIS mRNA were expressed at low levels in the intestine of orally fed or TPN-fed rats, and there was no significant effect of TPN itself on the abundance of SOCS-2 or CIS mRNA (Figure 1). SOCS-2 mRNA was significantly induced in the jejunum by GH but not by IGF-I (Figure 1). Regression analysis in the GH and IGF-I treated groups showed an inverse correlation ($R = -0.63$; $P < 0.05$) between SOCS-2 mRNA and mucosal mass, indicating a significant association between high level SOCS-2 expression in GH-treated rats and the lack of observed enterotrophic action of GH. CIS expression was more variable than SOCS-2 and was

not significantly affected by TPN or TPN plus GH or IGF-I infusion (Figure 1). SOCS-1 and SOCS-3 mRNA were expressed at barely detectable levels in the jejunum of TPN-fed rats, and, as shown in Figure 1A, these mRNA were not discernibly induced by GH or IGF-I. Recently, we have found that, in colon, as in jejunum, IGF-I, but not GH, significantly increased crypt depth (E. Dahly, Ph.D. thesis) in TPN-fed rats. We therefore assessed SOCS-2 expression in colon and found that GH increased SOCS-2 mRNA 2.2 ± 0.75-fold relative to TPN alone ($P < 0.05$), whereas IGF-I was without effect (1.0 ± 0.33-fold relative to TPN, $P = 0.98$). We attempted to localize the sites of SOCS-2 mRNA expression by in situ hybridization histochemistry, but this yielded signals that were not conclusively different from
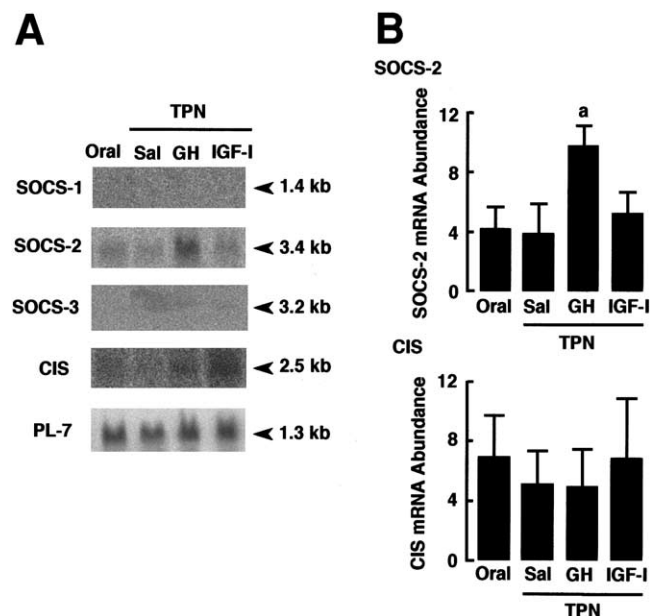

Figure 1. Jejunal SOCS mRNA expression in rats on TPN given GH, IGF-I, or saline vehicle (Sal). (*A*) Representative autoradiograms of northern blots probed for SOCS-1, SOCS-2, SOCS-3, CIS, and PL-7 control mRNAs. (*B*) Histograms of SOCS-2 or CIS mRNA normalized to PL-7 mRNA (means ± SEM; n = 5 per group; a = $P < 0.05$ vs. other groups).

background, even when probes were double labeled with [^{35}S]-UTP and [^{35}S]-CTP to increase sensitivity (data not shown). Immunohistochemistry with available SOCS-2 antibodies also did not detect SOCS-2 protein. We attribute this to the low abundance of SOCS-2 mRNA and protein and note that there is currently, to our knowledge, only 1 article localizing SOCS-2 mRNA, and this was performed in developing brain.[34] We could find no report on cellular localization of SOCS-2 protein in any tissue in vivo, suggesting that suitable antibodies and/or sufficiently sensitive methods are yet to be developed. We therefore turned to in vitro systems to test specifically whether SOCS-2 is expressed in intestinal epithelial cells and to examine its functional effects on cell proliferation.

SOCS-2 Is Expressed in Intestinal Epithelial Cells and SOCS-2 Expression Is Increased in Postconfluent Cells

We first characterized endogenous SOCS-2 expression in Caco-2 cells, which show a characteristic log phase proliferation when subconfluent, followed by slower rates of proliferation postconfluence.[8,26] As shown in Figure 2, SOCS-2 is expressed in Caco-2 cells but at low abundance in subconfluent cells (2–5 days after plating) and at significantly higher levels in confluent and postconfluent cells (10–15 days after plating), which show reduced proliferative rates (Figure 2).

SOCS-2 Overexpression in Caco-2 Cells Reduces Proliferation and Promotes Differentiation

Transfection-mediated SOCS-2 over expression was used to test directly whether SOCS-2 inhibits proliferation of Caco-2 cells. We initially identified conditions that minimized endogenous SOCS-2 expression to avoid complications because of the observed density-dependent changes in endogenous SOCS-2. As shown in Figure 3A, endogenous SOCS-2 mRNA was significantly down-regulated in cells grown in medium plus serum compared with serum-free medium. In subsequent experiments to test the functional effects of SOCS-2, cells were maintained in serum after transfection to minimize endogenous SOCS-2 and switched to serum-free medium 4–16 hours before experiments. We also verified that transfection, followed by incubation in serum-containing medium plus hygromycin to select transfected cells and doxycycline to induce expression of transfected plasmid, yielded overexpression of SOCS-2 mRNA and FLAG-epitope-tagged SOCS-2 protein in cells
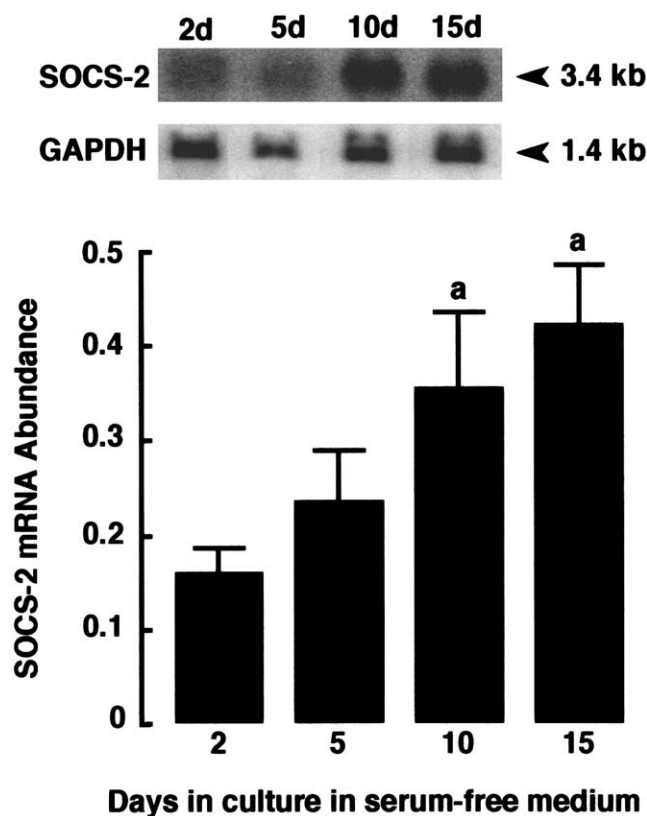

Figure 2. Density-dependent changes in expression of SOCS-2 in Caco-2 cells. Autoradiograms are of SOCS-2 and control GAPDH mRNAs in subconfluent (2–5 days after plating) and confluent (10–15 days after plating) Caco-2 cells grown in serum-free medium. Histograms are of SOCS-2 mRNA abundance normalized to control GAPDH mRNA. Data are means ± SEM (n = 5 or 6 at each time point; a = $P < 0.05$ vs. values at day 2).

transfected with SOCS-2 expression vector compared with empty vector (Figure 3B).

DNA synthesis and cell morphology were monitored in Caco-2 cells transfected with empty vector or SOCS-2 expression vector over 4–13 days after plating. As reported in the literature for Caco-2 cells,[8,26] empty vector transfected cells showed a typical log phase growth from days 4 to 8, when cells were at low density, and then plateau phase growth at higher densities (Figure 4A). SOCS-2 transfected cells showed similar levels of DNA synthesis at 4 days after plating (immediately after the initial hygromycin selection of transfectants), but, by day 6, DNA synthesis was significantly reduced relative to empty vector transfected cells. After this time point, SOCS-2-transfected cells failed to exhibit the typical log phase growth and showed earlier plateau phase growth than empty vector transfected cells. Morphologic examination verified similar numbers of surviving empty vector or SOCS-2 transfectants at day 4, immediately after initial hygromycin selection, but, by day 13, empty
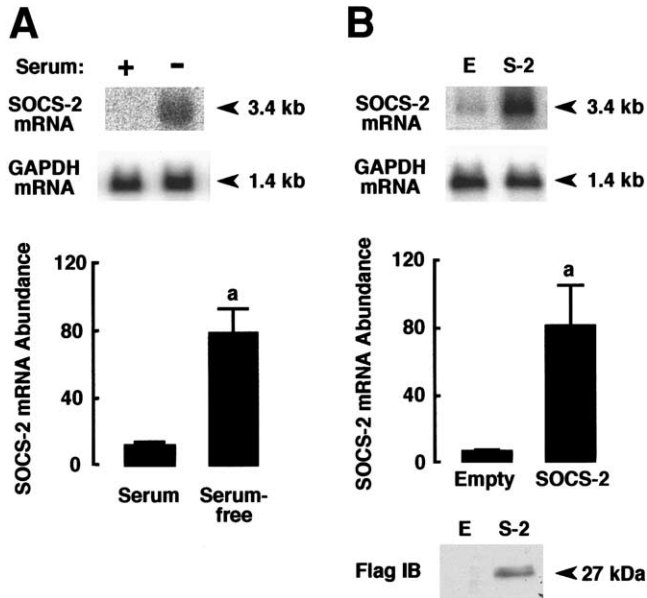
Figure 3. SOCS-2 expression in Caco-2 cells grown in serum or serum-free medium or after transfection with SOCS-2 vector. (*A*) SOCS-2 mRNA abundance in subconfluent Caco-2 cells grown in medium with or without serum (serum-free). Autoradiograms show representative examples of cells at 10 days after plating. Histograms show mean SOCS-2 mRNA abundance normalized to control GAPDH mRNA in cells cultured for 5–10 days in serum-free or serum-containing medium (n = 3 per group; a = $P < 0.05$ in cells grown in serum-free vs. serum-containing medium). (*B*) Increased SOCS-2 mRNA and protein in Caco-2 cells transfected with SOCS-2 (S-2) expression vector vs. empty vector (E). Autoradiograms show SOCS-2 mRNA (*top*) and SOCS-2 protein (immunoprecipitated with antibody to the FLAG epitope) in cells at 5 days after transfection. *Histograms* show mean SOCS-2 mRNA abundance at 5–10 days after transfection.

vector transfected cells formed larger colonies than SOCS-2-transfected cells (Figure 4*B*). Detached or dead cells per plate were collected and counted daily over the entire study period and did not differ significantly in empty vector transfected cells (2750 ± 281) and SOCS-2 transfected cells (3237 ± 314, $P = 0.27$). Thus, SOCS-2 overexpression appeared to reduce cell proliferation rather than cell survival. SOCS-2–transfected cells showed increased expression of sucrase-isomaltase mRNA and elevated alkaline phosphatase activity (Figure 5), indicating that, in addition to exerting antiproliferative actions, SOCS-2 overexpression promotes differentiated phenotype.

Caco-2 cells did not prove useful to study whether SOCS-2 alters the mitogenic actions of GH or IGF-I because the cells proved unresponsive to exogenous GH or IGF-I. We attribute this to their documented high-level secretion of IGF-II, which exerts autocrine mitogenic effects via the same type 1 IGF receptor as IGF-I.[8,26] We therefore turned to IEC-6 cells and isolated crypts from SOCS-2 null and WT mice as alternate systems.

GH Inhibits Spontaneous or IGF-I–Induced Proliferation of IEC-6 Cells and Induces SOCS-2

We examined GH action in IEC-6 cells as an independent measure of the direct proliferative actions of GH in a nontransformed intestinal epithelial cell line. As shown in Figure 6*A*, GH reduced basal DNA synthesis in IEC-6 cells. RT-PCR showed that levels of SOCS-2 mRNA were very low in untreated cells but were induced by GH (Figure 6*B*). Because IEC-6 cells show robust proliferative responses to IGF-I, we tested whether coincubation with GH affected this response. As shown in Figure 7, IGF-I alone potently stimulated
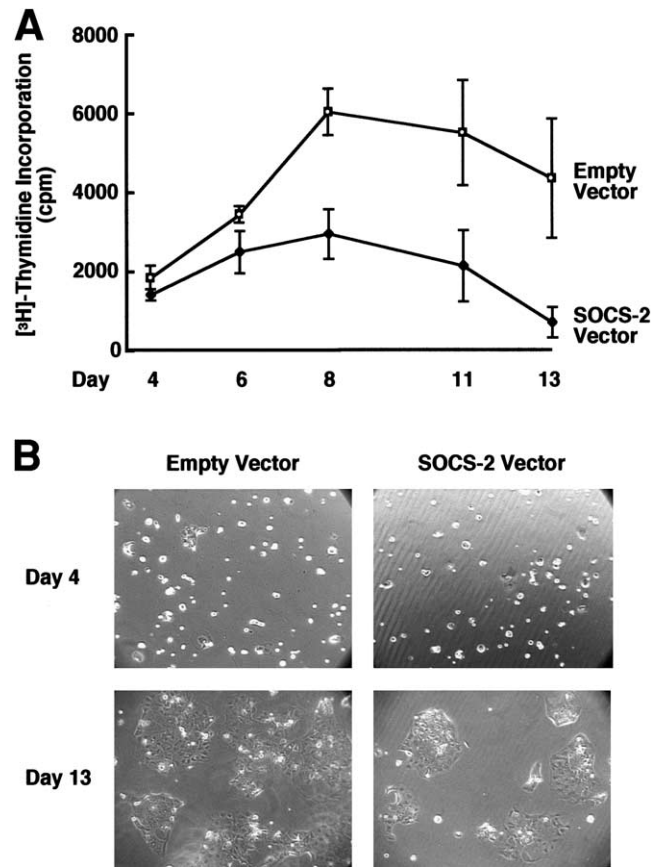

Figure 4. Effects of SOCS-2 overexpression on basal proliferation in serum. (*A*) Growth curves in SOCS-2 vector transfected vs. empty vector transfected Caco-2 cells at different times after transfection. *Line graph* shows cell proliferation (means ± SEM) measured as incorporation of [³H]-thymidine into DNA (n = 4 per time point). Cells were maintained in serum up to the 24 hours prior to study and switched to serum-free medium plus thymidine for 24 hours. SOCS-2 transfected cells showed significantly lower DNA synthesis ($P < 0.05$) at each time point assessed except day 4. (*B*) Morphology of Caco-2 cells transfected with SOCS-2 vector or empty vector. Phase-contrast pictures of Caco-2 cells transiently transfected with SOCS-2 vector or empty vector control taken at day 4 and day 13. Note the similar numbers of cells at day 4 and the smaller colonies in SOCS-2 transfected cells at day 13.
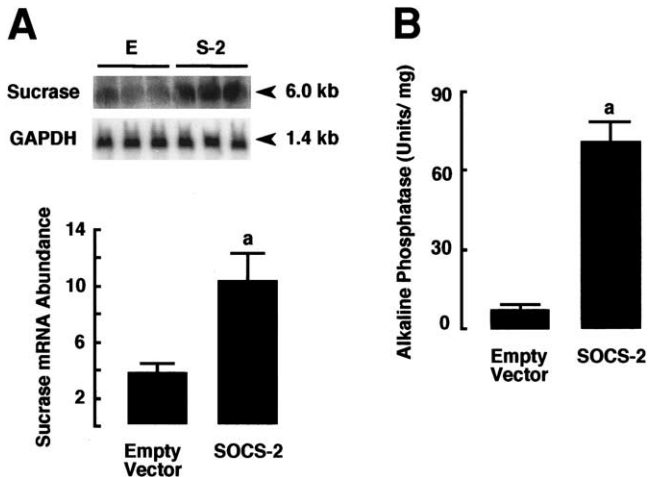
Figure 5. SOCS-2 overexpression promotes Caco-2 cell differentiation. (*A*) Autoradiograms of sucrase-isomaltase mRNA in Caco-2 cells transfected with empty vector (E) or SOCS-2 expression plasmid (S-2). Histograms show means ± SEM of sucrase mRNA abundance (a = $P < 0.05$ vs. empty vector control at day 7 after transfection). (*B*) Shows alkaline phosphatase activity in SOCS-2 vs. empty vector transfected cells. *Histograms* represent means ± SEM at day 7 after transfection.

DNA synthesis in IEC-6 cells, but this effect was attenuated by coincubation with GH. The attenuation of basal and IGF-I–induced DNA synthesis in IEC-6 cells by GH, coincident with induction of SOCS-2, supports our hypothesis that SOCS-2 serves as a GH-inducible factor, which limits the mitogenic actions of GH or IGF-I in intestinal epithelial cells.

SOCS-2 Deficiency Increases Responsiveness of Isolated Crypts to GH and IGF-I and Promotes Increased Intestinal Growth

Attempts to manipulate SOCS-2 expression in IEC-6 cells by transfection-based approaches proved problematic because transfection even with an empty vector transformed the cells, making them unresponsive to IGF-I. Isolated crypts from SOCS-2 null and WT mice were therefore used to test whether the antiproliferative effects of GH observed in IEC-6 cells occurred in normal intestinal epithelium and whether SOCS-2 is required for this effect. As in IEC-6 cells, GH reduced DNA synthesis in WT crypts, and this effect was abolished in crypts from SOCS-2 null mice (Figure 8*A*). IGF-I alone did not significantly affect DNA synthesis in WT crypts but tended to increase DNA synthesis in SOCS-2 null crypts. GH and IGF-I in combination significantly increased DNA synthesis in crypts from SOCS-2 null but not WT mice, and the

response to GH plus IGF-I in SOCS-2 null crypts was significantly greater than the response to GH alone (Figure 8*A*). These findings directly support the concept that SOCS-2 limits the proliferative effects of GH or GH in combination with IGF-I in the intestinal epithelium. Increased weight and length of the small intestine and colon in SOCS-2 null mice relative to age-matched WT littermates (Figure 9) also show that SOCS-2 limits intestinal growth in vivo. Increases in weight (41.1% ± 8.3% colon, 27.6% ± 3.9% small intestine) but not length (19.9% ± 2.8% colon, 12.7% ± 1.1% small intestine) were comparable with increases in body weight (30.1% ± 3.2%).
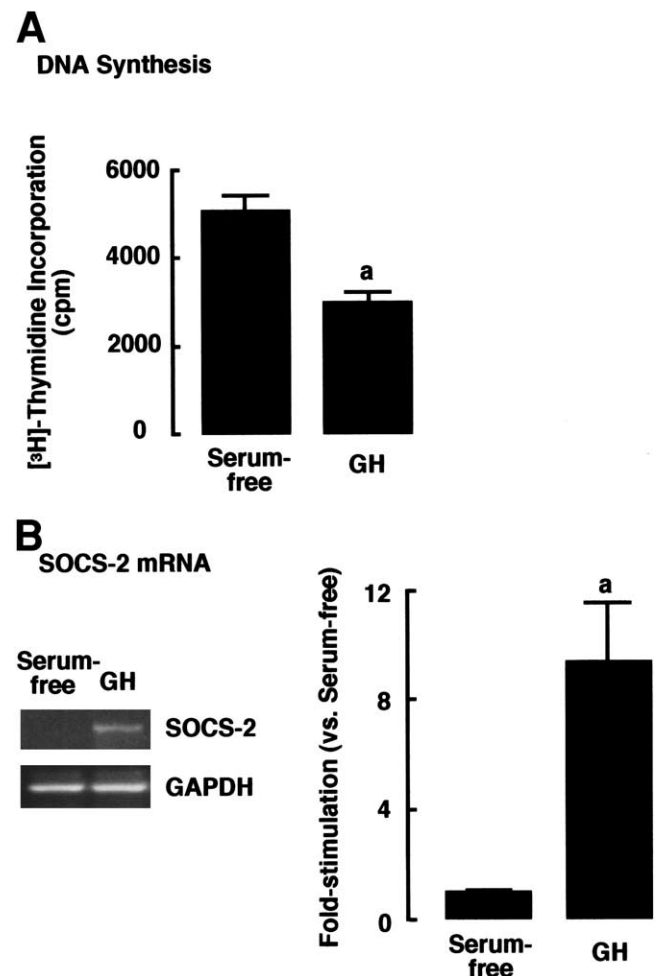

Figure 6. GH inhibits DNA synthesis in IEC-6 cells and induces SOCS-2. (*A*) *Histograms* show [³H]-thymidine incorporation into DNA in subconfluent IEC-6 cells grown on 24-well plates in serum-free medium alone or plus GH (2×10^{-8} mol/L) for 24 hours. Values are means ± SEM (n = 4 per group; a = $P < 0.05$ vs. serum-free control). Data were replicated in 2 independent experiments. (*B*) RT-PCR of SOCS-2 and GAPDH mRNA in serum-free or GH-treated IEC-6 cells. Representative gel is shown at *left* after 24-hour GH treatment. *Right* shows histograms of mean SOCS-2 mRNA abundance normalized to control GAPDH mRNA (n = 4; a = $P < 0.05$ vs. serum-free controls, showing significant induction of SOCS-2 mRNA by GH).
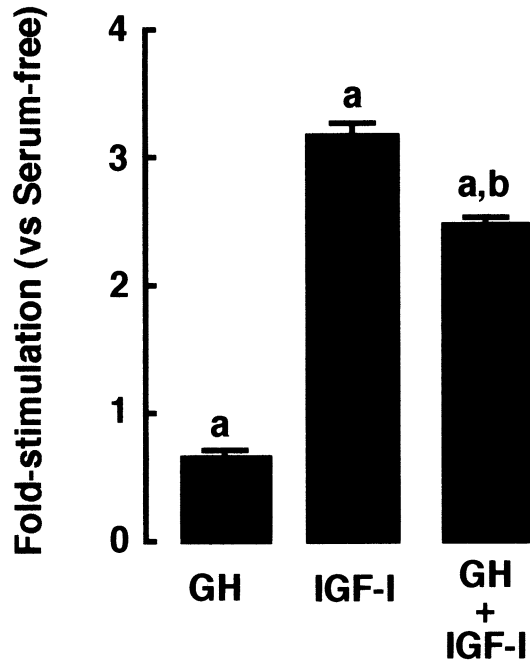
Figure 7. GH attenuates spontaneous and IGF-I–mediated proliferation of IEC-6 cells. DNA synthesis in cells treated with IGF-I (10 ng/mL) or GH (2×10^{-8} mol/L) alone or in combination for 24 hours. *Histograms* show means $\pm$ SEM of [^{3}H]-thymidine incorporation into DNA expressed as fold-stimulation relative to serum-free control (n = 4, a = $P < 0.05$ vs. no treatment control; b = $P < 0.05$ vs. GH or IGF-I alone).

Discussion

Although trophic actions of GH on the intestine have been noted in animal models of chronic GH excess[2] or with GH treatment of GH-deficient animals,[35,36] GH does not prevent or reverse the atrophy of the intestinal mucosa associated with restriction of oral nutrient and TPN in rats.[4] This is despite the fact that GH treatment increases circulating IGF-I and causes similar increases in body weight as observed with IGF-I treatment, which does potently stimulate mucosal growth and crypt cell proliferation in the TPN model.[4] The differential responsiveness of the intestinal mucosa to GH and IGF-I during TPN was confirmed in the present study and was shown to correlate with induction of SOCS-2 in the intestine by GH and not IGF-I. These findings support a hypothesis that, during TPN, SOCS-2 limits the actions of GH and/or GH-induced circulating IGF-I on intestinal epithelial cell proliferation and mucosal growth. In other tissues such as liver, GH has been shown to induce CIS, SOCS-1, and SOCS-3 as well as SOCS-2.[22,37,38] Our study provides new evidence that, in intestine, SOCS-2 is specifically or preferentially induced by GH. We cannot exclude the possibility that other SOCS are induced in intestine at other times after GH, although preliminary analyses at earlier time points in-

dicate that GH induces SOCS-2 but has no effect on CIS, SOCS-1, or SOCS-3.[39]

Although we have used the TPN model to compare GH and IGF-I action, we note that a majority of studies testing GH in other models, including orally fed rodents[40,41] and resection models,[7,9,42] report that GH has little or no effect on crypt proliferation, despite increasing body weight and plasma IGF-I. One study of 90% small bowel resection and very high-dose GH[43] and another of 70% resection, TPN, and intermediate-dose GH report positive effects on crypt proliferation.[44] It would be of interest to establish whether variable growth responses in these models correlate with SOCS-2 expression. Studies of GH in humans with short-bowel syndrome also indicate anabolic effects,[45–47] but variable
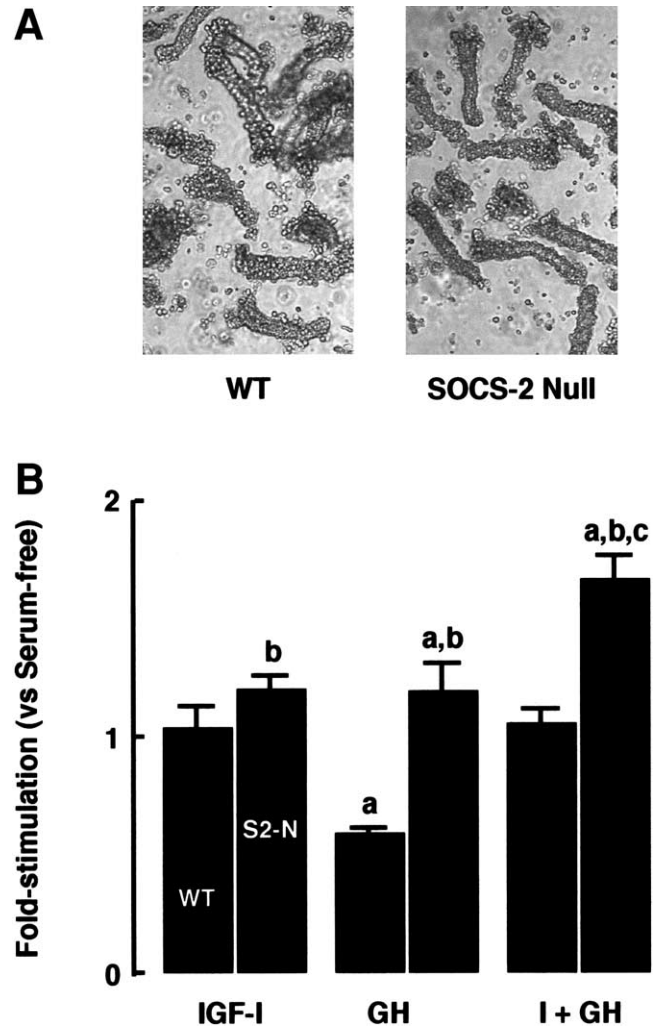

Figure 8. Mitogenic effects of GH and IGF-I in isolated crypts from WT and SOCS-2 null mice. (*A*) Shows representative photomicrographs of isolated crypts. (*B*) Shows [^{3}H]-thymidine incorporation (means $\pm$ SEM) in WT and SOCS-2 null (S2-N) crypts treated with IGF-I, GH, or IGF-I plus GH (a = $P < 0.05$ vs. no treatment, b = $P < 0.05$ vs. WT, and c = $P < 0.05$ vs. GH alone).
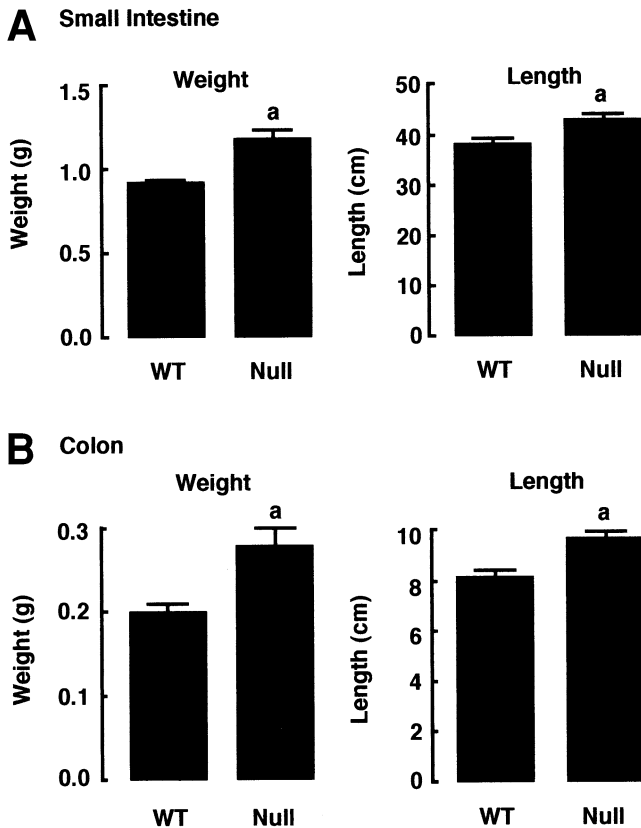
A **Small Intestine**

B **Colon**

Figure 9. SOCS-2 deficiency increases length and weight of the small intestine and colon. *Histograms* show mean weight and length of entire small intestine and colon for SOCS-2 null mice and age- and sex-matched WT mice (n = 6, a = *P* < 0.05 vs. WT).

effects on intestinal growth or nutrient uptake have been reported.[48–50] The variable responses may reflect dose of GH or coadministered nutritional interventions. However, our current studies suggest that SOCS-2 may play a critical role in the outcome of GH treatment. Evaluation of SOCS expression in intestine in other rodent models and in GH-treated patients would therefore be of significant future interest. Crypt proliferation was induced in GH transgenic mice vs. WT during recovery from colitis induced by dextran sulfate,[51] although crypt proliferation is not increased in untreated GH transgenics.[2] This points to distinct mechanisms of action of GH in normal and inflamed intestine.

It is well established that IGF-I stimulates proliferation of multiple cell lines derived from the intestinal epithelium (reviewed in Lund[3]), but such information for GH is scarce. One study reported proliferative actions of GH in Caco-2[24] cells, but we could not replicate this observation in the current studies. Cells in culture commonly lose GH responsiveness, especially with increasing passage number.[3] Although the reasons for this are unknown, our inability to replicate the previously observed effects of GH in Caco-2 cells, even when utilizing similar

culture conditions, may reflect increasing passage number of available Caco-2 cells. Another report in confluent, differentiated T84 colon cancer cells indicates responsiveness to GH in terms of regulation of chloride secretion and is consistent with the ability of GH to regulate some aspects of differentiated enterocyte function in vivo.[4] Although the focus of this study was on trophic actions of GH, the T84 cell line may prove useful for future studies of the relationship between SOCS-2 and GH action on differentiation in intestinal epithelial cells. In the nontransformed IEC-6 cell line and in isolated crypts from WT animals, GH exerts antiproliferative actions. GH unresponsive Caco-2 cells spontaneously express SOCS-2, GH induces SOCS-2 in IEC-6 cells, and SOCS-2 deficiency abrogates the antiproliferative effects of GH in isolated crypts. This, together with findings that SOCS-2 overexpression in Caco-2 cells limits spontaneous proliferation, provides strong evidence that SOCS-2 limits proliferation of intestinal epithelial cells and that SOCS-2 mediates or contributes to the less potent mitogenic effect of GH than IGF-I. Inhibitory effects of GH on proliferation of IEC-6 cells and isolated crypts counter the traditionally held view of GH as a growth-promoting hormone. However, it is important to note that, in other systems, GH has been shown to antagonize the actions of the insulin receptor, which is structurally related to the type 1 IGF receptor,[52] and this effect has recently been linked to SOCS-1.[53] The concept that GH may restrain IGF-I action or attenuate the duration or magnitude of IGF-I action is fundamentally important to delineating the relative roles GH vs. IGF-I in growth of intestine and other organs. Observations that GH induces SOCS-2 in colon of TPN-fed animals, as well as small intestine, and that SOCS-2 inhibited proliferation of Caco-2 colon cancer cells indicates that additional studies of the possible role of SOCS-2 in colorectal cancer or the effects of the GH–IGF-I axis on colorectal cancer[54] are warranted. In this regard, it is of interest that recent studies show that SOCS-2 expression is down-regulated in pulmonary adenocarcinoma.[55]

SOCS generally serve as feedback inhibitors, which limit the duration or magnitude of cytokine or growth factor action rather than preventing their biologic effects.[10] In this regard, we propose that SOCS-2 attenuates the magnitude or duration of crypt cell proliferation and mucosal growth in response to GH or GH-induced IGF-I rather than completely preventing IGF-I action. In the IEC-6 cells, GH coincubation attenuated but did not prevent IGF-I action on IEC-6 cells. The intestine is not totally unresponsive to GH during TPN. In the same TPN model as utilized here GH does, for example,

normalize some aspects of differentiated function of enterocytes such as glucose transport,[4] as well as inducing SOCS-2 as shown here.

Intriguingly, in Caco-2 cells, forced SOCS-2 overexpression enhanced expression of markers of enterocyte differentiation, sucrase, and alkaline phosphatase. This is consistent with enhanced sucrase activity in GH transgenic mice[2] but not IGF-I transgenic mice.[1] A recent report indicates that GH induction of SOCS-2 plays a role in differentiation of neurons from progenitors within the brain.[56] This, together with our current findings in intestinal epithelial cells, suggests that SOCS-2 may have a key role in dictating the balance between proliferation and differentiation of multiple cell types.

At present, the mechanisms by which SOCS-2 attenuates proliferation of intestinal epithelial cells and promotes differentiation are not defined. In other systems, it is well established that SOCS limit STAT activation by cytokines.[10] In Caco-2 cells, proliferation is known to be associated with high-level STAT-3 activation and confluence and differentiation with reduced STAT-3 activation.[57] It is therefore tempting to speculate that the increases in SOCS-2 expression observed here in confluent Caco-2 cells mediates STAT-3 inactivation at confluence. Forced SOCS-2 overexpression also may induce sucrase or alkaline phosphatase by inhibiting STAT-3. Future studies will be required to test this possibility, but it is noteworthy that a blast search revealed that the sucrase-isomaltase promoter contains 7 STAT-like binding sites within 3000 bases of the transcription start site, and several lie in proximity to caudal-type homeobox (Cdx) and GATA binding sites implicated in sucrase regulation.[58,59]

SOCS-2 null mice exhibit increases in body weight, which is first observed at times associated with rapid postnatal increases in growth known to be dependent on GH and IGF-I.[16] The body growth phenotype of SOCS-2 null mice is intermediate between that observed in transgenic mice with GH or IGF-I excess, and SOCS-2 null mice show increased expression of some but not all GH-regulated genes in liver.[16] Additionally, a high-growth phenotype observed in the high-growth mouse model was found to be the result of loss of the SOCS-2 gene.[60] These phenotypes support the concept that SOCS-2 expression in vivo normally exerts a suppressive effect on growth mediated by the GH–IGF-I axis and are consistent with the new in vivo and in vitro data reported here, which show a suppressive role of SOCS-2 on epithelial cell proliferation and intestinal growth. In SOCS-2 null mice, circulating IGF-I was normal, although some organs exhibited increases in IGF-I expression.[16] Interestingly, this was shown in only a few of a number of organs exhibiting overgrowth in the mice. SOCS-2 may therefore inhibit IGF-I induction by GH in a cell- or tissue-specific manner. During TPN, this may be the case because GH treatment increased serum IGF-I but did not elevate local IGF-I expression in the intestine in the present or previous studies.[4,5] Our current findings show increased weight and length of small intestine and colon in null mice vs. WT, providing in vivo evidence that SOCS-2 normally limits intestinal growth. Colonies of SOCS-2 null mice are currently being established in our laboratory to analyze intestinal phenotype in more detail, including analysis of enterocyte differentiation during development and studies of intestinal responsiveness to GH and IGF-I.

Isolated crypts potentially provide very useful systems to study ex vivo actions of GH and IGF-I in a simple culture system with a nontransformed phenotype, which retains some of the cell contacts and architectural features of crypts in vivo. Comparisons of SOCS-2 null and WT crypts here provided evidence for effects of SOCS-2 to limit the proliferative actions of GH and IGF-I in combination. It was, however, somewhat surprising that IGF-I alone did not have mitogenic actions in the isolated crypt preparations. Variations in isolation and culture conditions may promote optimal IGF-I responsiveness or preservation of IGF-I receptor expression. Ongoing studies are evaluating levels of expression of IGF-I receptor and key signaling molecules under different isolation and culture conditions to identify strategies that will maximize the usefulness of this system for future studies of GH and IGF-I action and interactions.

In conclusion, these studies show that SOCS-2 is induced in response to GH treatment in intestine in vivo and in intestinal epithelial cells in vitro. Evidence in 2 cell lines and isolated crypts from SOCS-2 null and WT mice indicates that SOCS-2 limits epithelial cell proliferation. These data indicate that SOCS-2 mediates or contributes to the reduced potency of GH vs. IGF-I as a mediator of growth of the mucosal epithelium. Indeed, our findings in IEC-6 cells and isolated crypts support a new mechanism of GH:IGF-I interaction, whereby direct actions of GH on epithelial cells are to limit basal proliferation or mitogenic effects of IGF-I. Our data suggest that SOCS-2 mediates or is required for these effects of GH.

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