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Interleukin-6 production induced by leptin treatment promotes cell proliferation in an Apc ($^{Min/+}$) colon epithelial cell line

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Increased visceral adipose tissue results in elevated plasma leptin, which are associated with increased risk of a number of obesity-related cancers. However, research is contradictory regarding the role of elevated plasma leptin in colon cancer risk. Having established that leptin induced proliferation in a murine model of preneoplastic (Apc$^{Min/+}$; IMCE) colon epithelial cells but not normal (Apc$^{+/+}$; YAMC) cells , we hypothesized that the leptin-associated IMCE cell proliferation was a result of autocrine interleukin-6 (IL-6) production and ensuing IL-6 receptor (IL-6R) signaling. Here we show, for the first time, that leptin induces elevated IL-6 production in IMCE cells but not in YAMC cells. IL-6 treatment induced cell proliferation in IMCE cells, but not in YAMC cells, in a concentration-dependent manner from 0.1 to 100 ng/ml ($P < 0.05$). Interleukin-6-induced IMCE cell proliferation was blocked by the addition of a neutralizing anti-IL-6R antibody. In addition, leptin-induced IMCE cell proliferation was blocked by the addition of an anti-IL-6R neutralizing antibody. Further, we elucidate a novel mechanism by which leptin activates TACE/ADAM17-associated IL-6R shedding and trans-IL-6 signaling in IMCE by induction of IL-6 production. IL-6 treatment of IMCE cells was associated with STAT3, ERK, p38, MEK and JAK2 activation and associated STAT3 nuclear activation and translocation. These data implicate leptin-induced IL-6 production, signaling and subsequent STAT3 activation as early events promoting the survival/proliferation of colon epithelial preneoplastic cells. The elucidation of the leptin-initiated mechanism of preneoplastic cell proliferation establishes a biologically plausible link between the adipoctye-specific cytokine leptin and obesity-associated colon cancer.

Introduction

Obesity is directly associated with an increased risk of cancer at several organ sites, including colon, breast (in postmenopausal women), endometrium, esophagus and kidney (1,2). Adipose tissue secretes hormones and cytokines that may play a role in the development of the systemic inflammatory state that is associated with obesity and subsequent cancer risk. The adipocyte-derived hormone leptin, which plays a crucial role in regulating energy balance, is elevated in obese individuals (3,4). A wide variety of immune, epithelial and normal and tumor cell types, including colon epithelial cells, express the leptin receptor, which mediates many of the biological effects of leptin (5). Homeostatic leptin may act locally within the gastrointestinal tract to influence intestinal functions, such as nutrient absorption. However, it is unclear whether elevated leptin levels have pathophysiological implications in colorectal cancer. In tumor cell lines, leptin treatment induces cell proliferation (6–8). Yet, the mechanism by which leptin causes proliferation has not been elucidated.

Obesity greatly increases the risk of a constellation of disease risk factors now called metabolic syndrome. The clinical presentation includes increased abdominal fat, elevated levels of triglycerides and low-density lipoproteins, low levels of high-density lipoprotein cholesterol, elevated blood pressure, insulin resistance, and prothrombotic and proinflammatory states (9). As such, risk of obesity-associated cancers may be causally related to the proinflammatory state. Colon cancer has been clearly linked to other inflammatory conditions, such as inflammatory bowel disease (IBD), so it is certainly plausible that the increased inflammation that occurs with obesity underlies the obesity–colon cancer association (10).

A growing body of evidence associates inflammation with epithelial cell transformation and the process of carcinogenesis (11,12). Truncating mutations in the adenomatous polyposis coli (*APC*) gene are initiating events in colorectal carcinogenesis; a majority of adenomas in inherited and sporadic forms of colorectal cancers have mutations in *APC* (13). The normal cellular functions of *APC*, including proliferation, migration, differentiation and apoptosis, are disrupted by these truncating mutations (14). A central mechanism by which *APC* participates in these processes is the regulation of Wnt signaling in association with axin and glycogen synthase 3β to target the adhesion protein and transcription factor β-catenin for ubiquitin-mediated proteosomal degradation. Excess cytosolic β-catenin resulting from dysregulated degradation results in nuclear translocation, dimerization with Tcf/LEF (T cell factor/lymphoid enhancer factor) and transcriptional activation of target genes, such as *cyclin D1*, which enhances survival and proliferation of initiated cells (13). Indeed, quantitative measures of gene expression show that constitutional 50% decreases in expression of one *APC* tumor suppressor gene allele can lead to the development of familial adenomatous polyposis (15). In inflammation-associated colorectal cancer, such as cases associated with IBD, non-genetic stimuli also encourage the survival and proliferation of initiated cells (12).

Interleukin-6 (IL-6) is a cytokine secreted by diverse cell types under homeostatic and inflammatory conditions (16).

Abbreviations: *APC,* adenomatous polyposis coli; IBD, inflammatory bowel disease; IL-6, Interleukin-6; sIL-6R, soluble form of the IL-6 receptor; sgp130R, soluble gp130 receptor; STAT3, signal transducer and activator of transcription 3; TACE, tumor necrosis factor-converting enzyme.

Secretion of IL-6 is strongly associated with the pathogenesis of IBD (17,18), and overproduction of IL-6 by intestinal epithelial cells is thought to play a part in the pathogenesis of IBD (19). Although IL-6 is homeostatic at low levels in intestinal epithelial cells, overproduction leads to degradation of the epithelial barrier. In addition, IL-6 stimulates cell proliferation in dysplastic and neoplastic cell lines (20–23).

Interestingly, leptin induces IL-6 transcription and secretion in some cell types (24,25). The only epithelial cells shown to secrete IL-6 in response to leptin are endometrial epithelial cells (26). We hypothesized that the leptin-associated colon epithelial cell proliferation we observed (27) is the consequence of autocrine IL-6 production and resulting autocrine/paracrine trans-IL-6 receptor signaling. We utilized a unique model system of conditionally immortalized colon epithelial cell lines to dissect these early events. These cell lines, YAMC ($Apc^{+/+}$) cells and IMCE ($Apc^{Min/+}$) cells, respectively, display phenotypes consistent with normal and preneoplastic colon epithelial cells observed in human colon epithelial carcinogenesis. In these two cell lines we observed previously that leptin induced cell proliferation in the IMCE ($Apc^{Min/+}$) cells but actually induced low-level apoptosis in the YAMC ($Apc^{+/+}$) cells (27). We demonstrate here, for the first time, that leptin induces IL-6 production, tumor necrosis factor-converting enzyme (TACE)-associated IL-6R signaling, shedding, and trans-IL-6 signaling in $Apc^{Min/+}$ colon epithelial cells. These data implicate leptin-induced IL-6 production and signaling as an early event promoting the survival/proliferation of preneoplastic cells.

Materials and methods

Chemicals

All chemicals were purchased from Sigma (St Louis, MO) unless otherwise noted. Growth media, insulin/transferrin/selenium, murine interferon (IFN-γ) and type IV collagen were purchased from Life Technologies (Rockville, MD). Neonatal calf serum was purchased from Gemini Bio-Products (Woodland, CA). Recombinant murine leptin, IL-6 and murine IL-6R antibody were purchased from R&D Systems (Minneapolis, MN).

Cells and cell culture conditions

YAMC ($Apc^{+/+}$) cells were developed from the transgenic SV40 large T antigen mouse (28). IMCE ($Apc^{Min/+}$) cells were derived from an F1 hybrid between the SV40 large T antigen transgenic mouse and the $Apc^{Min/+}$ mouse (29). Both these cell lines are non-tumorigenic in nude mice, do not grow in soft agar and survive in culture only on extracellular matrix proteins such as collagen I (29). Both YAMC ($Apc^{+/+}$) and IMCE ($Apc^{Min/+}$) cells express the heat-labile SV40 large T antigen under the control of an IFN-γ-inducible promoter. At 33°C the temperature-sensitive SV40 large T antigen is active and drives cell proliferation. At 39°C the temperature-sensitive mutation yields an inactive protein, and cells behave as non-proliferating, differentiated colon epithelial cells (30). Cells were cultured as described previously (31). Briefly, cells were cultured at 33°C on collagen I until reaching ~70% confluence. Once 70% confluent, cells were transferred to 39°C in serum-free and IFN-γ-free medium for 24 h before each experiment. This period allows for cessation of SV40 large T antigen-driven cell proliferation, depletion of residual growth factors (via serum) and a brief stabilization period. Moving the cells to 39°C at 70% confluence prevents contact inhibition of proliferation by allowing enough room for a low level of proliferation while still permitting gap junction formation. These cell lines then behave like normal cells in that they are contact inhibited and undergo apoptosis if they achieve maximal confluence. Therefore, culture conditions are optimized for cells to proliferate slowly for 24 h at 39°C and then undergo cell death over 5–8 days, similar to the lifecycle of a normal colonic epithelial cell.

Leptin/IL-6 proliferation assay

IMCE ($Apc^{Min/+}$) cells were grown in 96-well plates as described above. Briefly, ~1500 cells/well were seeded in 96-well plates coated with collagen I (BD Biosciences; San Jose, CA) as described above. Cells were left at 33°C overnight to adhere and reach 70% confluence. Plates were then moved to 39°C for 24 h in serum-free and IFN-γ-free medium to allow for cessation of SV40 large T antigen-driven cell proliferation and to achieve stability. After the 24 h stabilization period at 39°C the cells were treated (eight wells per treatment) with leptin (1 or 50 ng/ml), IL-6 (0.1, 1, 10 50, 100 ng/ml) or co-treatment combinations. The antibody neutralization experiments were carried out using a monoclonal anti-murine IL-6R antibody (R&D Systems; Minneapolis, MN) or isotype control at 1 μg/ml with a 30 min pretreatment with antibody followed by co-treatment for 48 h.

Cell proliferation was measured after 48 h of treatment as previously described (27). Briefly, cell proliferation was measured using a commercially available compound, calcein AM (Molecular Probes, Eugene, OR), that is colorless, non-fluorescent and cell membrane permeable. The compound fluoresces when cleaved by non-specific esterases in actively proliferating cells. After 48 hours of treatment, the cells were treated with 100 μl of 1 μM calcein AM in phosphate-buffered saline (PBS) for 30 min, and fluorescence was read at an excitation wavelength of 485 nm and emission wavelength of 530 nm in a Cytofluor® fluorescent plate reader (Millipore, Bedford MA). We previously confirmed that this technique measures cell proliferation via flow cytometric analysis in a previous article (27).

IL-6 enzyme-linked immunosorbent assay (ELISA)

The release of IL-6 into the culture medium was quantified by sandwich ELISA according to the manufacturer's instructions (R&D Systems). Briefly, 50 μl of undiluted culture medium was added to each well and incubated according to instructions. Upon completion of the assay procedure, the plate was read at 450 nm wavelength using a Synergy HT plate reader (Bio-Tek; Winooski, VT).

Western blotting

Cells were grown in collagen I coated T-75 flasks and treated as described above prior to collection. Briefly, cells were washed twice with cold PBS and total cell lysate was harvested by scraping cells into 1 ml of cold lysis buffer [30 mM Tris (pH 7.2), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanidate, 1% NP-40 and 10% glycerol] per flask. The cell suspension was then sonicated to insure cell lysis and centrifuged at 4°C for 15 min at 14 000 r.p.m. Nuclear and cytoplasmic fractions were collected using the NE-PER® kit according to the manufacturer's instructions (Pierce Biotechnology; Rockford, IL). For soluble receptor identification, conditioned medium was collected 48 h post-treatment and concentrated 10-fold using Centricon concentrators (5000 MW cutoff) (Millipore, Bedford, MA).

Protein content of the samples was determined by BCA assay (Bio-Rad Laboratories, Hercules, CA), and samples were loaded on an equal protein basis of ~10 μg/lane. Samples were subjected to SDS–polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Bio-Rad Laboratories). Membranes were probed with primary antibodies against IL-6R, gp130, STAT3 and pSTAT3 (Santa Cruz Biotechnology, Santa Cruz; CA) or phospho-specific pairs p38, ERK, MEK, JAK1 and JAK2 (Cell Signaling Technology; Beverly, MA) with shaking overnight at 4°C. Incubation with the primary antibody was followed by the appropriate secondary antibody conjugated to horseradish peroxidase (included with the femtoLucent kit, Geno Technology, St Louis, MO) and detected by chemiluminescence with the femtoLucent kit according to the manufacturer's instructions.

Samples, for both cell types, for either the receptor or signaling experiments were loaded on the same gel. They were processed as a whole for all subsequent steps for optimal comparison. In addition, the same blots were used for each phospo-specific antibody pairs. Each blot was stripped and rerun with other antibodies. Further, the experiments were repeated three times in the same manner. Densitometric analysis represents the actin corrected signal mean ± SE for the three repetitions. Blots shown are from one experiment representative of the three.

STAT3 activation

STAT3 (signal transducer and activator of transcription 3) activation was measured using the TransAM STAT3 Transcription Factor Assay Kit according to the manufacturer's instructions (Active Motif; Carlsbad, CA). Briefly, the TransAM STAT3 kit contains a 96-well plate on which has been immobilized oligonucleotide containing the STAT consensus binding site (5′-TTCCCGGAA-3′). The active form of STAT contained in nuclear extract specifically binds to this oligonucleotide. The primary antibody used to detect STAT3 recognizes an epitope on STAT3 that is accessible only when STAT is activated and bound to its target DNA. Substrate-activated HRP-conjugated secondary antibody provides a sensitive colorimetric readout that is quantified by spectrophotometry at 450 nm.

Statistical analysis

Cell proliferation and cell proliferation inhibition data were assessed statistically by comparing treated cell proliferation to control cell proliferation within

each cell type. The experiments were repeated three times and data shown are from one of the experiments representative of all three. The data shown is the mean ± error within one representative experiment. Differences in proliferation were compared using ANOVA in combination with Tukey's multiple comparisons test. IL-6 production data were assessed statistically by comparing supernants isolated from control cells to supernants from leptin-treated cells. Differences in densitometric analysis were compared using ANOVA in combination with Tukey's multiple comparisons test. Pair-wise differences were compared using ANOVA in combination with Tukey's multiple comparisons test. The Prism software package (Graph Pad; San Diego, CA) was utilized for this analysis.

Results

Leptin-induced cell proliferation

YAMC ($Apc^{+/+}$) and IMCE ($Apc^{Min/+}$) cells were treated with concentrations of leptin ranging from 0.01 to 50 ng/ml. These concentrations were chosen to represent a similar physiological range of low to high circulating concentrations of leptin. As observed previously, in YAMC ($Apc^{+/+}$) cells leptin significantly decreased cell proliferation. In contrast, an increase in proliferation was observed with leptin treatment of IMCE ($Apc^{Min/+}$) cells (Figure 1A).

Leptin-induced IL-6 production

IMCE ($Apc^{Min/+}$) cells made significantly more IL-6 than YAMC ($Apc^{+/+}$) cells (78 ± 2 versus 42 ± 4 ng/ml; $P < 0.01$). Leptin treatment induced an increase in IL-6 production in a dose-dependent fashion in IMCE ($Apc^{Min/+}$) but not in YAMC ($Apc^{+/+}$) cells (Figure 1B). Maximal leptin-induced IMCE ($Apc^{Min/+}$) IL-6 production was observed at 1.0–50 ng/ml IL-6.

IL-6-induced cell proliferation

Cell proliferation was determined as described previously (25). YAMC ($Apc^{+/+}$) and IMCE ($Apc^{Min/+}$) cells were treated with concentrations of IL-6 ranging from 0.1 to 100 ng/ml. These concentrations were chosen to represent the physiological range of low to high circulating concentrations of IL-6. In YAMC ($Apc^{+/+}$) cells, IL-6 did not induce proliferation (Figure 2). In contrast, IL-6 induced cell proliferation in IMCE ($Apc^{Min/+}$) cells in a concentration-dependent manner from 0.1 to 100 ng/ml ($P < 0.01$), with a plateau at 10 ng/ml ($P < 0.001$; Figure 2).

IMCE ($Apc^{Min/+}$) cell proliferation induced by 50 ng/ml of IL-6 was completely blocked by the addition of an anti-IL-6 receptor (IL-6R) neutralizing antibody (Figure 3A). In addition, leptin-induced IMCE ($Apc^{Min/+}$) cell proliferation at 1 and 50 ng/ml was blocked by the addition of IL-6R neutralizing antibody (Figure 3B). The addition of a non-specific isotype control antibody did not inhibit IL-6 induced cell proliferation (data not shown).

Leptin induced IL-6 signaling receptors

Western blotting was used to verify the presence of the IL-6R and the cognate gp130 membrane receptor protein in these cell lines. Both cell lines were positive for the IL-6R and gp130 receptor. Leptin treatment caused an increase in IL-6R protein abundance in IMCE ($Apc^{Min/+}$) cells and a decrease in YAMC ($Apc^{+/+}$) cells. In addition, the soluble form of the IL-6 receptor (sIL-6R) was identified in conditioned medium from IMCE ($Apc^{Min/+}$) cells but not in that from YAMC ($Apc^{Min/+}$) cells (Figure 4A). The gp130 membrane receptor protein was detected in both cell types, and membrane gp130 was slightly more abundant in IMCE ($Apc^{Min/+}$) cells than in YAMC

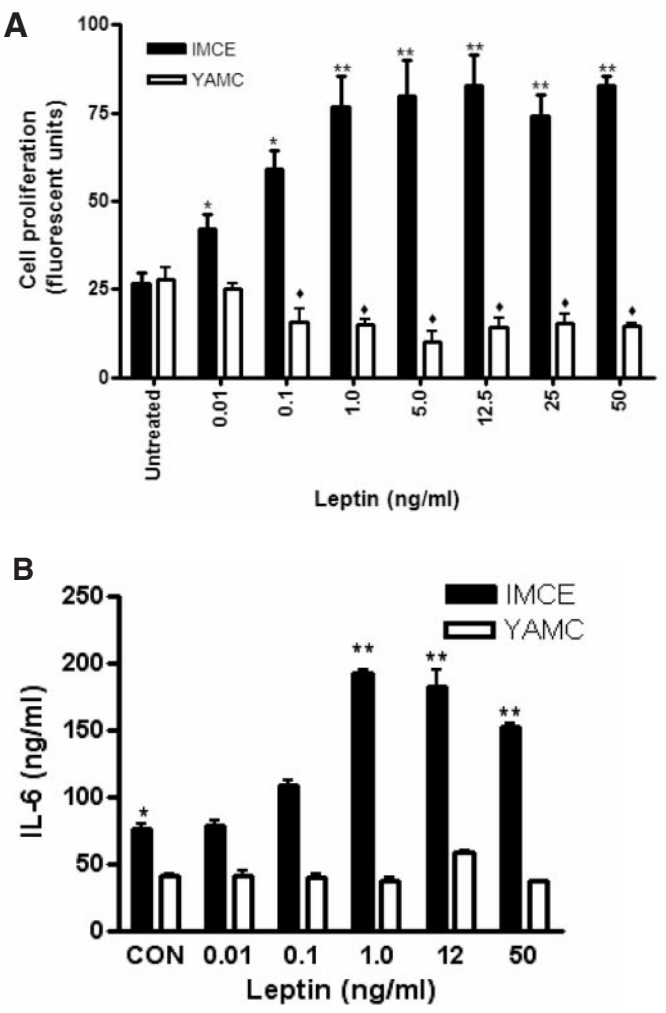

Fig. 1. (**A**) The effect of leptin on the proliferation of IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$) colon epithelial cells. Cells were treated with leptin from 0.01 to 50 ng/ml for 48 h. Con, control. Results are representative of three separate experiments. Closed diamond, $P < 0.01$ (compared with untreated YAMC control); Asterisk, $P < 0.001$ (compared with untreated IMCE control); double asterisk, $P < 0.0001$ (compared with untreated IMCE control). (**B**) The effect of leptin on IL-6 secretion by IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$) colonic epithelial cells. Cells were treated with leptin from 0.01 to 50 ng/ml of leptin for 48 h. Con, control. Results are representative of three separate experiments. Asterisk, $P < 0.01$ (compared with untreated YAMC control); double asterisk, $P < 0.001$ (compared with untreated IMCE control).

($Apc^{+/+}$) cells. The soluble gp130 receptor (sgp130R) was only detectable in medium from IMCE ($Apc^{Min/+}$) cells and not in that from YAMC cells (Figure 4B). TACE protein (also known as ADAM17) was more abundant in IMCE ($Apc^{Min/+}$) cells than in YAMC ($Apc^{+/+}$) cells and its abundance appeared to increase with leptin treatment in both cell types (Figure 4C).

IL-6 induced cell signaling activation

In order to establish the IL-6-dependent signaling pathways associated with the observed proliferative phenotype we surveyed cell signaling pathways consistent with IL-6 signal transduction in IMCE ($Apc^{Min/+}$) total cell lysate by using phospho-specific antibody pairs. IL-6 treatment (50 ng/ml) of IMCE ($Apc^{Min/+}$) cells induced the time-dependent

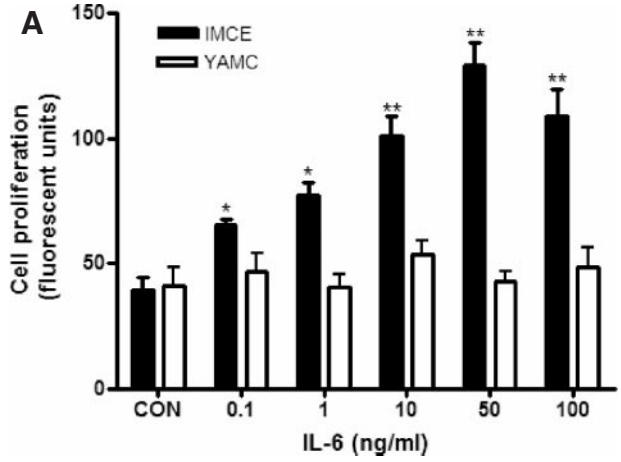

Fig. 2. The effect of IL-6 treatment on the proliferation of IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$) colon epithelial cells. Cells were treated with IL-6 from 0.01 to 100 ng/ml recombinant murine IL-6 for 48 h. Con, control. Asterisk, $P < 0.01$ (compared with IMCE control); double asterisk, $P < 0.001$ (compared with IMCE control). Results are representative of three separate experiments.

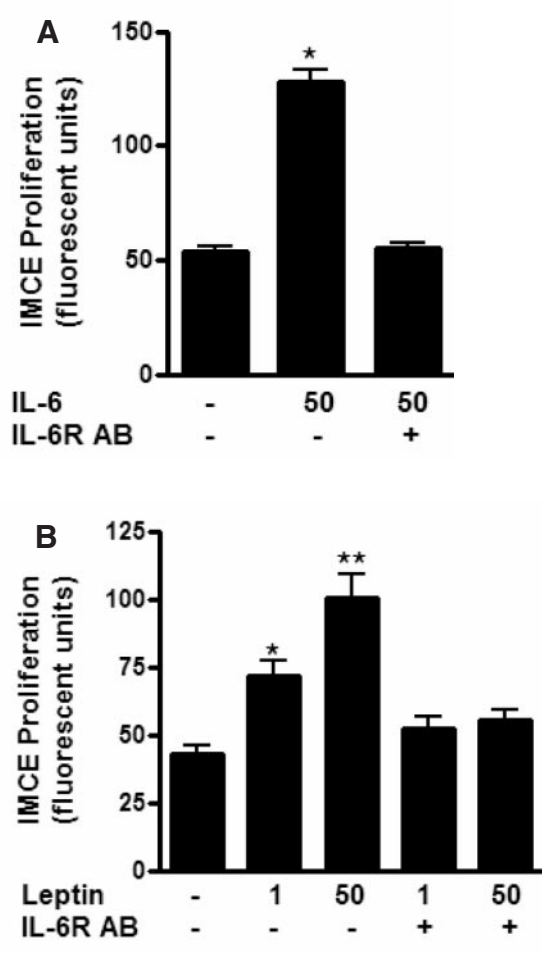

Fig. 3. (**A**) The effect of IL-6 (50 ng/ml) or (**B**) leptin (1 or 50 ng/ml) and co-treatment with anti-IL-6 receptor antibody (IL-6R AB, 1 μg/ml) on proliferation of IMCE ($Apc^{Min/+}$) colon epithelial cells. Cells were treated overnight in serum-free media prior to exposure to treatments. Co-treatments were pretreated for 30 min with anti-IL-6R antibody and then treated with IL-6 (50 ng/ml). Asterisk, $P < 0.01$ (compared with untreated control); double asterisk, $P < 0.001$ (compared with untreated control). Results are representative of three separate experiments.

phosphorylation of ERK, p38, MEK1/2, JAK2, and STAT3 (Figure 5A), but not JAK1 (data not shown). There was no difference in total protein control for pair matched ERK, MEK 1/2, p38, JAK2 and STAT3 (Figure 5C). To show specificity of the response to IL-6, we co-treated IMCE ($Apc^{Min/+}$) cells with a murine anti-IL-6R neutralizing monoclonal antibody. IMCE ($Apc^{Min/+}$) cells treated with antibody alone served as a control. Co-treatment of IMCE ($Apc^{Min/+}$) cells with IL-6 (50 ng/ml) and anti-IL-6R antibody (1 μg/ml) attenuated the time-dependent phosphorylation of ERK and p38, and blocked the phosphorylation of MEK1/2 and STAT3 (Figure 5B). In contrast, JAK2 phosphorylation was activated in the presence of anti-IL-6R antibody treatment alone, as indicated by constitutive phosphorylation of JAK2 in control as well as antibody-treated cells.

IL-6 induced nuclear translocation and activation

IL-6 treatment induced a time-dependent increase in nuclear accumulation of STAT3 in IMCE ($Apc^{Min/+}$) cells, with maximal accumulation at 15 min (Figure 6A). Co-treatment of IMCE ($Apc^{Min/+}$) cells with IL-6 (50 ng/ml) and anti-IL-6R antibody (1 μg/ml) attenuated the time-dependent nuclear accumulation of STAT3 (Figure 6A) with ~3-fold less nuclear STAT3 present at 15 min compared with IL-6 treatment alone (Figure 6B). In contrast, IL-6 treatment did not induce nuclear translocation of NF-kappa B (data not shown).

IL-6 treatment induced a time-dependent increase in nuclear translocation and DNA binding of STAT3 in IMCE ($Apc^{Min/+}$) cells, with maximal activation at 15 min (Figure 6C). Co-treatment of IMCE ($Apc^{Min/+}$) cells with IL-6 (50 ng/ml) and anti-IL-6R antibody (1 μg/ml) inhibited activation of nuclear STAT3 (Figure 6C).

Discussion

The decades long transition from a normal colon stem cell or epithelial progenitor cell, with a typical lifespan of 5 days, to an immortal cell requires initiation by mutational inactivation of one *Apc* allele that promotes cell survival (32). Successive loss of heterozygosity in *Apc* yields a progressive propensity towards the accumulation of mutations in other growth regulatory genes, including *DCC*, *p53*, etc. (33). YAMC ($Apc^{+/+}$) and IMCE ($Apc^{Min/+}$) colon epithelial cell lines are excellent models for studying early events in colon tumorigenesis (34). IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$) cells are non-tumorigenic yet differ in several phenotypes consistent with a transition from a normal to a preneoplastic cell that, *in vivo*, requires decades. There are numerous phenotypic changes in the IMCE ($Apc^{Min/+}$) cells that are consistent with the hypothesis that decreased wild-type *Apc* expression is associated with colorectal carcinogenesis including reduced growth-factor-induced migration, reduced connexin-mediated cell–cell communication and increased inducible nitric oxide synthase/cyclooxygenase-2 (iNOS/COX-2) expression (29,31–35). These preneoplastic phenotypes are consistent with known early phenotypes in human colorectal cancer. In $Apc^{Min/+}$ mice, a heterozygous mutation in *Apc* has been demonstrated to be sufficient to reduce microtubule polymerization and Cx43 expression in intestinal epithelial cell lines (36), These data suggest that the progressive loss of wild-type *Apc* in colorectal carcinogenesis is associated

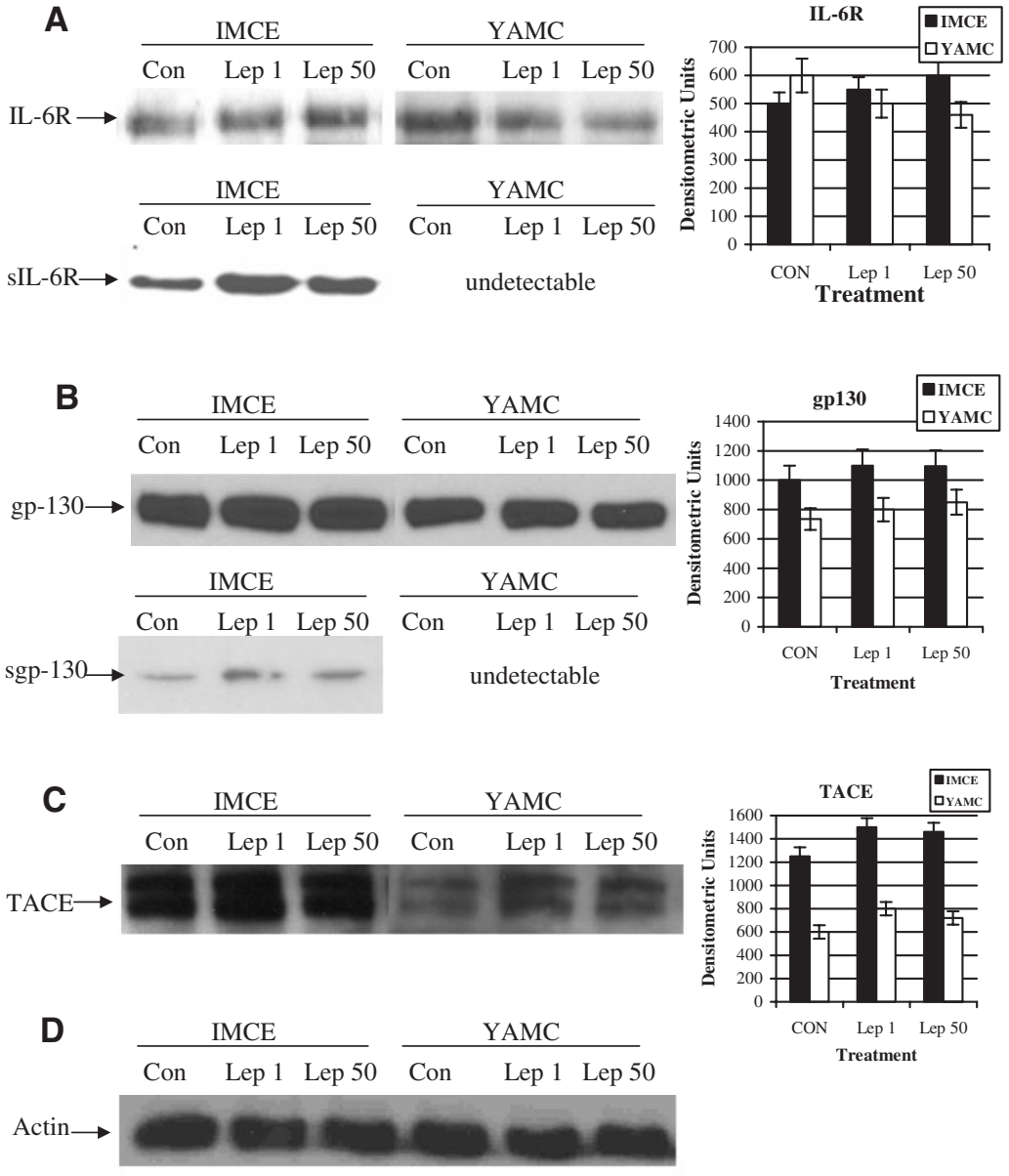

Fig. 4. The effect of 24 h leptin treatment (1 or 50 ng/ml) on membrane or soluble IL-6R (**A**), or gp130 membrane and soluble receptor (**B**) or TACE (**C**) abundance as measured by western blot and corresponding densitometric analysis in IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$) colon epithelial cells. (**D**) Actin, protein loading control for membrane receptors. Densitometric values represent the actin corrected means ± SE of three blots.

with the acquisition of growth promoting and apoptosis inhibitory capabilities that promote neoplasia.

We recently established that leptin causes a striking increase in IMCE ($Apc^{Min/+}$) cell proliferation but not in YAMC ($Apc^{+/+}$) cells as observed in Figure 1A (25).

We were motivated to identify a leptin-inducible factor responsible for this observed differential proliferative effect by the kinetics or temporality of this response to leptin in the IMCE ($Apc^{Min/+}$) cells (27). Because the proliferative effects were observed over a 48 h leptin treatment regimen, we surmised that a secreted autocrine growth factor might be responsible for this effect. We established a time-dependent and concentration-dependent increase in IL-6 secretion after leptin treatment using an IL-6 ELISA. IL-6, as a prospective autocrine factor candidate, was concordant with observations that intestinal epithelial cells secrete IL-6 in response to inflammatory signals, such as those occurring in IBD (17–18), and that IL-6 is involved in intestinal inflammation (37).

To date, IL-6 secretion has been observed in response to leptin in disparate cell types, including endometrial epithelial cells and adipocytes (24–26). However, the observation that leptin can induce IL-6 production in preneoplastic colon epithelial cells is novel (Figure 1B). Here we show that IL-6 treatment, in a concentration-dependent fashion, induces IMCE ($Apc^{Min/+}$) but not YAMC ($Apc^{+/+}$) cell proliferation (Figure 2). The IL-6-induced proliferation was blocked by treatment with IL-6R antibody, indicating specificity of the effect of IL-6 (Figure 3A). In addition, leptin-induced cell proliferation was also blocked by anti-IL-6R co-treatment (Figure 3B). These data demonstrate that IL-6, either added exogenously or induced by leptin, can stimulate IMCE ($Apc^{Min/+}$) cell proliferation. This observation is consistent

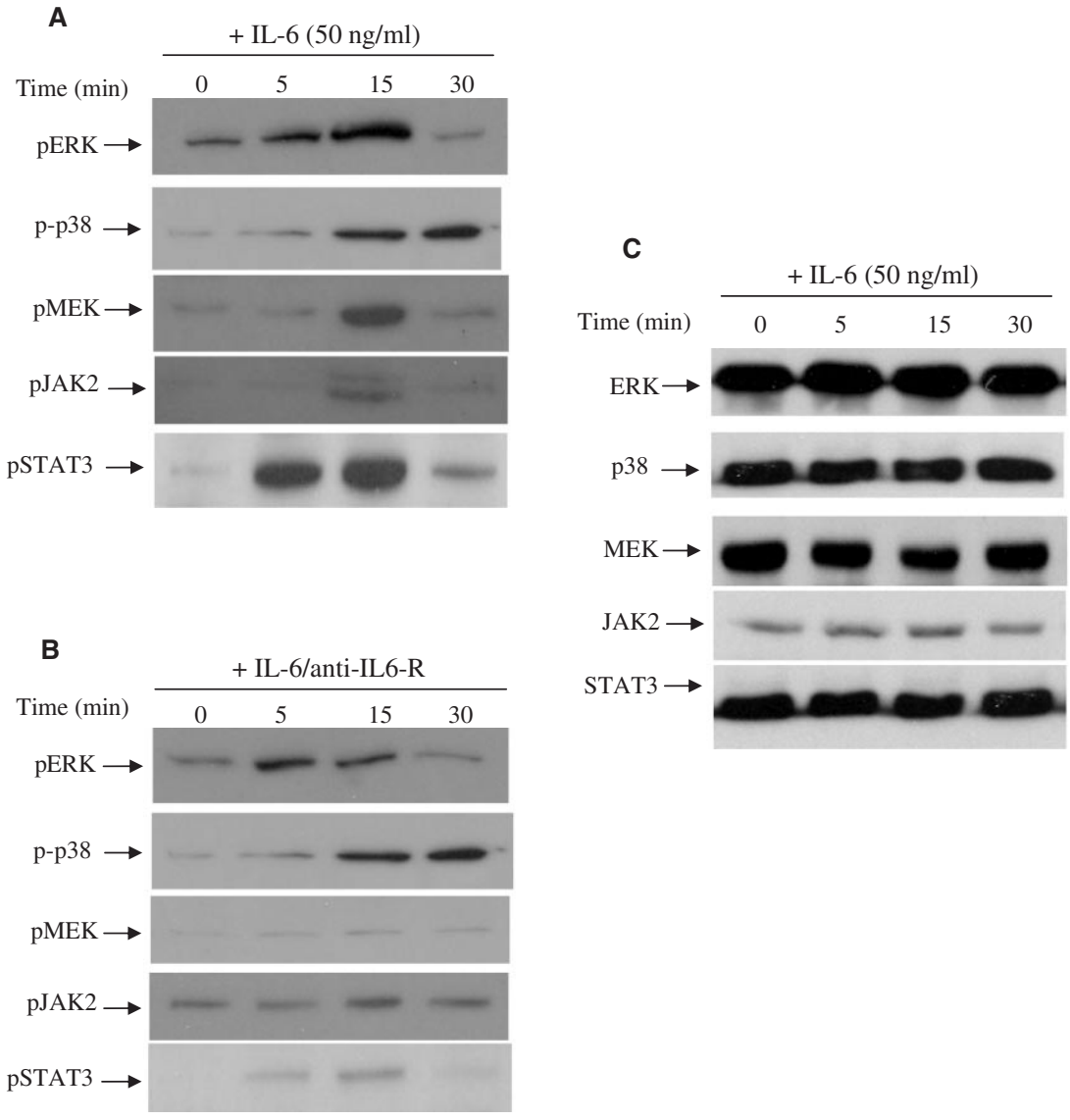

Fig. 5. (**A**) IL-6 treatment or (**B**) co-treatment with anti-IL-6R of IMCE ($Apc^{Min/+}$) cells and phosphorylation (activation) of ERK, MEK 1/2, p38 JAK2 and STAT3. (**C**) Total protein control for pair matched ERK, MEK 1/2, JAK2, p38 and STAT3. Cells were incubated overnight in serum-free medium prior to exposure to IL-6 (50 ng/ml). Co-treatment experiments were performed by pretreating cells for 30 min with anti-IL-6R antibody and then treating with IL-6 (50 ng/ml). At the indicated times, cellular extracts were prepared and western-blot analysis was performed. Blots shown are one representative experiment of two.

with the proliferative phenotype induced by leptin in tumor cell types (38).

However, our data are not consistent with the observation that leptin administration to $Apc^{Min/+}$ mice did not enhance polyp formation (39). We propose at least two important differences, aside from the *in vivo* versus *in vitro* experimental systems including dose of leptin and cell type, which may explain the apparent contradictory findings. First, the serum levels of leptin achieved by administration to $Apc^{Min/+}$ mice (7.4 versus control 2.3 ng/ml) did not achieve concentrations consistent with $Apc^{Min/+}$ mice with high-fat-diet-induced obesity (14.39 vs. control 5.93 ng/ml) (40). The concentrations of leptin used in the present *in vitro* study are more reflective of leptin levels in C57BL/6J mice with diet-induced obesity (30 ng/ml) (41). As such, the concentrations of leptin used in this study are relevant to obese mice but not in murine leptin administration studies, which achieve only modest increases in serum leptin concentrations. Finally, the cell lines used in this study are derived from the colon of the min mice while most polyps form in the small intestines of $Apc^{Min/+}$ mice. It may be that small intestinal epithelial cells respond differently to leptin than colon epithelial cells.

In order to understand the proliferative response in IMCE ($Apc^{Min/+}$) cells, we investigated the mechanistic basis of IL-6 receptor signaling in these cells. IL-6 binds to a receptor complex comprises membrane-associated IL-6R and gp130. The binding between IL-6 and the membrane-bound receptors causes signal transduction and activation of intracellular signaling pathways. Membrane-bound IL-6R and gp130 may produce, by proteolytic shedding, two soluble forms of these membrane-bound receptors. sIL-6R is an agonistic circulating receptor of IL-6; sIL-6 can bind IL-6R and this binary complex (IL-6/sIL-6R) can bind membrane-bound gp130 and, thereby, activate target cells, even in those that do not express

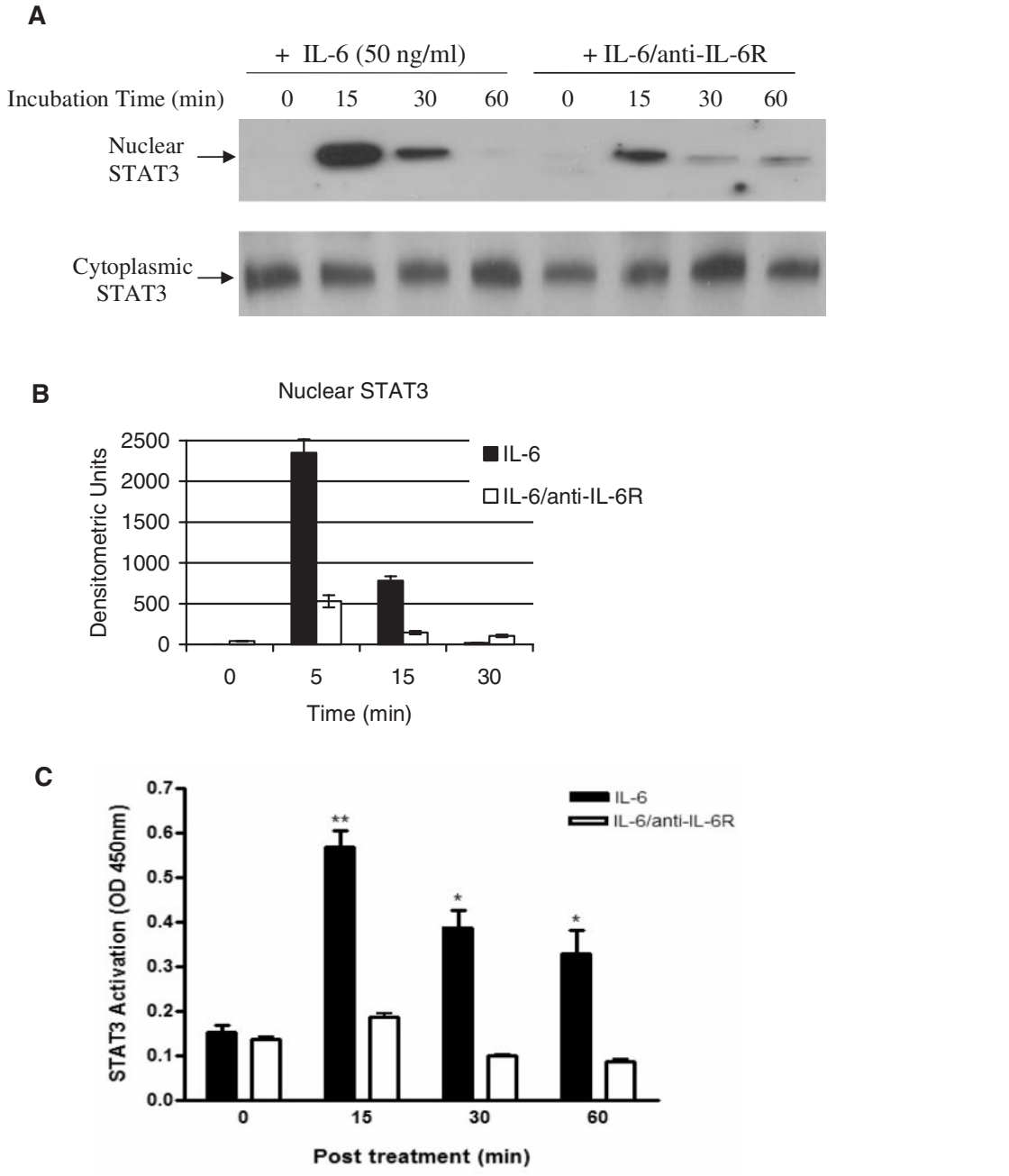

Fig. 6. (**A**) IL-6 (50 ng/ml) treatment or co-treatment with anti-IL-6R of IMCE ($Apc^{Min/+}$) cells and nuclear translocation of STAT3 in IMCE ($Apc^{Min/+}$) cells. (**B**) Densitometric values of the western data. Cells were treated overnight in serum-free medium prior to exposure to IL-6. Co-treatments were pre-treated for 30 min with anti-IL-6R antibody and then treated with IL-6. At the indicated times after IL-6 addition, cellular extracts were prepared and western-blot analysis was performed. Densitometric values represent the actin corrected means ± SE of the three blots. (**C**) IL-6 (50 ng/ml) treatment or co-treatment with anti-IL-6R of IMCE ($Apc^{Min/+}$) cells and activation of nuclear STAT3 in IMCE ($Apc^{Min/+}$) cells using the TransAM(tm) STAT3 transcription factor assay (Active Motif).

IL-6R on their surface (42,43). Conversely, sgp130 can inhibit IL-6 signal transduction (44).

Upon further investigation, we found that there were dramatic differences in IL-6 receptor complex regulation between IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$) cells. Untreated YAMC ($Apc^{+/+}$) cells had more membrane bound IL-6R than IMCE ($Apc^{Min/+}$) cells. However, in response to leptin treatment IMCE ($Apc^{Min/+}$) cells shed soluble IL-6R into conditioned media, while the YAMC ($Apc^{+/+}$) cells did not (Figure 4A). The gp130 membrane receptor was present on the membranes of both cell types. We observed some shedding of gp130 only in IMCE ($Apc^{Min/+}$) cells in response to leptin treatment (Figure 4B).

Consistent with IL-6 signaling, IL-6 treatment resulted in a time-dependent phosphorylation of ERK, MEK 1/2, p38, JAK2 and STAT3. These phosphorylation events were attenuated (p38 and ERK) or blocked (MEK 1/2) by anti-IL-6R antibody co-treatment. The activation of these signaling proteins led to the phosphorylation of STAT3 (Figure 5A) and translocation of activated and functional STAT3

(Figure 6) into the nucleus of IMCE ($Apc^{Min/+}$) cells consistent with IL-6 signaling in intestinal epithelial cells (45,46). As such, our data imply that IL-6 production can regulate the fine balance of intestinal epithelial cell proliferation in a homeostatic fashion through a balanced activation of different signaling cascades, thereby, resulting in negative and positive signals during biological responses to cytokines.

These findings are consistent with IL-6 signaling homeostatically via the membrane-bound receptor and with deregulated IL-6 signaling via the leptin-induced generation of the soluble IL-6 receptor complex (47). It is plausible that the shedding of soluble gp130 in response to leptin was an attempt by the cell to block the sIL-6R signaling, but clearly it was not shed in a quantity capable of acting in an inhibitory manner (44). These data illuminate our previous observation that leptin-induced IMCE ($Apc^{Min/+}$) cell proliferation is probably the result of autocrine IL-6 production. We extend this observation here by demonstrating that leptin-induced IL-6 signaling via shedding of the sIL-6R is primarily responsible for this effect. A soluble form of the IL-6R can be generated by proteolytic cleavage by a sheddase, by membrane protein-solubilizing proteases (MPSP) that may be TACE/ADAM17 or by alternative mRNA splicing of the IL6R mRNA (48). Indeed, the TACE/ADAM17 protein was more abundant in the IMCE ($Apc^{Min/+}$) cells consistent with the elevated levels of sIL-6R observed (Figure 4C). Current investigations are underway in our laboratory to elucidate this potential mechanism regulating the mechanism underlying the differential effects of leptin on IL-6 production, differential shedding of the IL-6R and resultant IMCE ($Apc^{Min/+}$) cell proliferation.

The ascription of cause-and-effect using data drawn from IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$) cells, or from any cells grown in culture, is problematic. As with all cells grown in culture, genetic drift may occur during multiple passages, which may introduce genotypic changes that may not be accounted for in the experimental design. As such, the ascription of differences in response to leptin reported here to differences in *Apc* genotype may be at least partially affected by unmeasured changes in the structure or expression of genes other than *Apc*. Our attempts to express full-length *Apc* in IMCE cells to restore the phenotype observed in cells expressing wild-type *Apc* have not been successful. Overexpression of *Apc* in these cells induces apoptotic cell death within 24 h, consistent with the association of wild-type *Apc* with induction of apoptosis using an inducible expression system (49). Even as we acknowledge the limitations for drawing inferences from these data, our observations are consistent with the hypothesis that the observed differences in leptin-induced cell growth are associated with *Apc* genotype.

We demonstrate here, for the first time, that leptin induces IL-6 production, IL-6R membrane shedding and trans-IL-6 signaling in IMCE ($Apc^{Min/+}$) colon epithelial cells. These differences in IL-6 signaling in IMCE ($Apc^{Min/+}$) cells compared with that in YAMC ($Apc^{+/+}$) cells indicate that this, like defective cell migration, increased NOS2 and COX-2 expression and decreased cell–cell communication, may be a very early event in colon epithelial cell tumorigenesis. Our findings illustrate the utility of these cell lines as models of normal and preneoplastic colon epithelial cells, respectively. We implicate leptin-induced IL-6 signaling as an early event promoting the survival/proliferation of colon epithelial preneoplastic cells. The elucidation of this leptin-initiated mechanism of preneoplastic cell proliferation establishes a biologically plausible link between this adipokine and obesity-associated cancer risk. These data illustrate the utility of cancer stage-specific and context-specific models for cancer prevention research and highlight potential biomarkers of risk for obesity-related cancer prevention.

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Conflict of Interest statement. None declared.

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