

Glucagon-like Peptide-2 Does Not Modify the Growth or Survival of Murine or Human Intestinal Tumor Cells

Jacqueline A. Koehler, Will Harper, Maja Barnard, Bernardo Yusta, and Daniel J. Drucker

Department of Medicine, and the Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, University of Toronto, Toronto, Ontario, Canada

Abstract

Glucagon-like peptide-2 (GLP-2) secreted from enteroendocrine cells exerts proabsorptive, regenerative, and cytoprotective actions in the normal and injured gut epithelium. Hence, sustained GLP-2 receptor (GLP-2R) activation represents a strategy under investigation for the prevention and treatment of chemotherapy-induced mucositis. Nevertheless, the consequences of increased GLP-2R signaling for the growth and survival of intestinal tumor cells remain poorly understood. We studied the proliferative and cytoprotective actions of GLP-2 in human colon cancer cells stably transfected with the GLP-2R and in nude mice harboring GLP-2R⁺ human colon cancer cells. The importance of the GLP-2R for tumor growth was also examined in *Apc*^{Min/+} mice chronically treated with exogenous GLP-2 and in *Apc*^{Min/+};*Glp2r*^{-/-} mice. GLP-2 increased cyclic AMP accumulation and produced cell-specific activation of growth and survival pathways in DLD-1, SW480, and HT29 cells. However, GLP-2 did not stimulate cell growth or attenuate cycloheximide-, LY294002-, indomethacin-, or chemotherapy-induced cytotoxicity *in vitro*. Moreover, chronic GLP-2 administration had no effect on the growth of human colon cancer cell xenografts in nude mice *in vivo*. Daily GLP-2 treatment for 7 weeks increased growth of normal gut mucosa but did not increase the number or size of polyps in *Apc*^{Min/+} mice, and genetic disruption of the *Glp2r* gene in *Apc*^{Min/+} mice did not modify polyp size or number. Taken together, although GLP-2R activation engages signaling pathways promoting cell proliferation and cytoprotection in the normal gut epithelium, sustained direct or indirect modulation of GLP-2R signaling does not modify intestinal tumor cell growth or survival. [Cancer Res 2008;68(19):7897-904]

Introduction

The gastrointestinal epithelium is composed of distinct specialized cell types important for regulation of nutrient absorption, barrier function, mucosal defense, and regulation of gut motility and energy homeostasis. Enteroendocrine cells encompass distinct subpopulations of cells distinguished by their unique ability to synthesize and secrete peptide hormones that convey signals modulating hunger and satiety, pancreatic and gastric secretion, gall bladder contraction and bile flow, and the control of macro-nutrient absorption and disposal.

Enteroendocrine L cells express proglucagon, which gives rise to a diverse set of proglucagon-derived peptides, including glicentin,

oxyntomodulin, and two glucagon-like peptides, GLP-1 and GLP-2. Glicentin inhibits gastric acid secretion and gut motility and exerts growth-promoting actions in the small bowel; however, the mechanisms through which glicentin exerts these effects remain poorly understood. Oxyntomodulin inhibits food intake in both rodents and humans, actions that require a functional GLP-1 receptor (1, 2). The actions of GLP-1 include the control of satiety, gastrointestinal motility, islet hormone secretion, and the regulation of β -cell proliferation and survival (3). As many of these actions of GLP-1 seem conserved in human subjects, there has been considerable interest in developing drugs for the treatment of type 2 diabetes based on the potentiation of GLP-1 action (4).

GLP-2 is cosecreted with GLP-1 from the gut following meal ingestion (5, 6). Exogenous GLP-2 administration enhances nutrient absorption, stimulates intestinal blood flow, increases barrier function, and inhibits gastric acid secretion and gastric emptying. Moreover, repeated administration of GLP-2 promotes expansion of the intestinal mucosa via stimulation of crypt cell growth and reduction of crypt and enterocyte apoptosis (ref. 7, reviewed in ref. 8).

Although the majority of GLP-2 actions are mediated through a single G protein-coupled receptor (9), the mechanisms through which GLP-2 transduces its multiple actions remain poorly understood. As the GLP-2 receptor (GLP-2R) is localized to subpopulations of enteroendocrine cells (10–12), enteric neurons (12, 13), and subepithelial myofibroblasts (14), the available evidence implicates multiple downstream mediators released from GLP-2R⁺ target cells that are important for GLP-2 action in distinct gut compartments.

The proabsorptive, regenerative, and reparative effects of GLP-2 on the injured mucosal epithelium have fostered efforts directed at elucidating whether GLP-2 administration may be useful for enhancing energy absorption in human patients with short bowel syndrome (15). Nevertheless, the observations that GLP-2 promotes cell proliferation through growth factor-dependent pathways (14, 16) have raised concern about the long-term safety of GLP-2 administration. Here, we examined the actions of GLP-2 on the proliferation and survival of colon cancer cell lines stably expressing the GLP-2R. Due to the phenotypic and genetic similarities between tumors arising in *Apc*^{Min/+} mice and human intestinal cancer, we also assessed the effects of exogenous GLP-2 administration on tumorigenesis in *Apc*^{Min/+} mice. Moreover, we examined the importance of endogenous GLP-2R signaling for intestinal tumor growth in *Apc*^{Min/+};*Glp2r*^{-/-} mice. The results of these studies extend our understanding of the role of GLP-2R signaling in the control of intestinal tumor growth, cell proliferation, and cell survival.

Materials and Methods

Cell lines. Human colon cancer cell lines from the American Type Culture Collection were maintained in DMEM supplemented with 5% fetal

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Daniel J. Drucker, Mt. Sinai Hospital, SLRI 60 Murray Street, Toronto, Ontario, Canada M5T 3L9. Phone: 416-361-2661; Fax: 416-361-2669; E-mail: d.drucker@utoronto.ca.

©2008 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-08-0029

bovine serum (FBS) and 0.5 mg/mL G418. cDNA encoding the human GLP-2R in the pcDNA3.1 eukaryotic expression vector was used to make stable cell lines. Colon cancer cell lines stably expressing the human GLP-2R were made by transiently transfecting cells using Lipofectamine 2000 (Invitrogen). Clones were screened for GLP-2R expression by reverse transcription-PCR (RT-PCR) and Southern blot analysis as previously described (17). Two distinct clones from each cell line (DLD-1 and SW480) that expressed the highest levels of the GLP-2R were then used for further analysis with similar results. Only one clone was found to stably express the human GLP-2R in HT29 cells. Cell growth and survival assays were performed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay (Promega), and cyclic AMP (cAMP) and Western blot analysis was performed as described (18). Antibodies to Akt phosphorylated on Ser⁴⁷³, glycogen synthase kinase 3 α / β (GSK3 α / β) phosphorylated on Ser^{9/21}, and p44/42 mitogen-activated protein kinase (MAPK) phosphorylated on Thr²⁰² and Tyr²⁰⁴ were from Cell Signaling Technology and were used at a 1:1,000 dilution. The KDEL antibody against Grp78/94 (BiP) used at 1:2,000 dilution (Stressgen Bioreagents Corp.) was used as a loading control.

Animals. Animal studies were approved by the Animal Care Committee of the University Health Network and Mt. Sinai Hospital. Mice were housed in plastic-bottomed wired-lid cages and maintained in temperature- and humidity-controlled rooms with a 12-h light/dark cycle and given water and chow *ad libitum* throughout the experiments. C57BL/6J-*Apc*^{Min/+} mice were from The Jackson Laboratory. *Apc*^{Min/+}:*Glp2r* mice were generated by crossing *Apc*^{Min/+} mice with C57BL/6J-*Glp2r*^{-/-} mice recently generated in our laboratory¹ and maintained by crossing male *Apc*^{Min/+}:*Glp2r*^{+/-} with female *Apc*^{+/+}:*Glp2r*^{+/-} mice.

Genotyping. Genomic DNA was prepared from tail biopsies and offspring were genotyped for the *Apc*^{Min/+} allele by PCR. *Glp2r* genotyping was performed using the primers 5'-AGGGGGCATCTCTATTCTC-3', 5'-CTTAGCAGGGCATGGTACCAC-3', and 5'-TGCGAGGCCAGAGGC-CACTTGTGTAGC-3'. Amplification was for 40 cycles at an annealing temperature of 60°C, resulting in the generation of a 400-bp (*Glp2r*^{+/-}) or 500-bp (*Glp2r*^{-/-}) product.

Tumorigenicity assay in nude mice. Tumor implantation experiments used 10-wk-old male BALB/c *nu/nu* athymic (nude) mice (Harlan Labs). DLD-1:hGLP-2R and SW480:hGLP-2R cells ($1 \times 10^6/200 \mu\text{L}$ PBS) were implanted by s.c. injection into opposite flanks of nude mice. Six days later, mice were injected i.p. twice daily for 6 wk with either 100 μL saline ($n = 9$ mice) or 5 μg GLP-2 ($n = 10$ mice; Bachem/Peninsula Laboratories). Mice were injected with 100 mg/kg 5-bromo-2'-deoxyuridine (BrdUrd) in PBS 1 h before euthanasia. Tumors were removed, dissected from connective tissue, and weighed. Small portions were frozen on dry ice and stored at -80°C for RNA or fixed in 10% formalin and paraffin embedded. A 2-cm piece of proximal jejunum was also dissected for subsequent morphometric analysis.

Enumeration of intestinal adenomas. Six-week-old littermate control [wild-type (WT)] and *Apc*^{Min/+} mice were injected s.c. once daily for 7 wk with either 200 μL saline solution (six *Apc*^{+/+} and *Apc*^{Min/+} males and five *Apc*^{+/+} and *Apc*^{Min/+} females) or 5 μg GLP-2 (six *Apc*^{+/+} and *Apc*^{Min/+} males and five *Apc*^{+/+} and *Apc*^{Min/+} females). GLP-2 (1-34) was from Bachem/Peninsula Laboratories. To assess whether *Glp2r* genotype modified tumorigenicity, *Glp2r*^{+/+}:*Apc*^{Min/+}, *Glp2r*^{+/-}:*Apc*^{Min/+}, and *Glp2r*^{-/-}:*Apc*^{Min/+} littermate mice were analyzed at 14 to 15 wk of age. In all experiments, mice were fasted overnight and euthanized by anesthesia the following morning. The entire small and large intestine was immediately removed, weighed, and then flushed with saline to remove any remaining fecal debris, and the small intestine was divided into three equal segments (proximal, middle, and distal). Following removal of a 2-cm piece of proximal jejunum for morphometric analysis, the intestines were opened longitudinally, laid flat on Whatman paper (no. 1), and fixed in 10% neutral-buffered formalin for 24 h. Fixed intestines were stained with methylene blue and examined

for polyps by gross inspection with the use of a Leica dissection microscope, and the diameter of the polyps was measured with the aid of an eyepiece micrometer.

Histology, morphometry, and immunocytochemistry. Histologic sections of human tumors obtained from the Department of Surgical Pathology, Mount Sinai Hospital were immunostained using GLP-2R antisera as described (11). A 2-cm piece of proximal jejunum was fixed in 10% formalin, cut into four pieces, embedded in paraffin, and transversely cross-sectioned. Digital images were captured with a camera connected to a Leica DMR microscope and analyzed using Leica QWin software. Crypt+villus height was measured as crypt base to villus tip of an average of 10 to 20 longitudinally, well-orientated crypt+villus units (to make $n = 1$) on H&E-stained sections. A portion of the tumor xenografts were fixed overnight in 10% formalin and embedded in paraffin and immunostained for BrdUrd as previously described (18). Apoptotic cells were scored using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.

Statistical analysis. Statistical significance was assessed by one-way or two-way ANOVA using Bonferroni's multiple comparison post-test and, where appropriate, by unpaired Student's *t* test using GraphPad Prism 4 (GraphPad Software). A *P* value of <0.05 was considered to be statistically significant.

Results

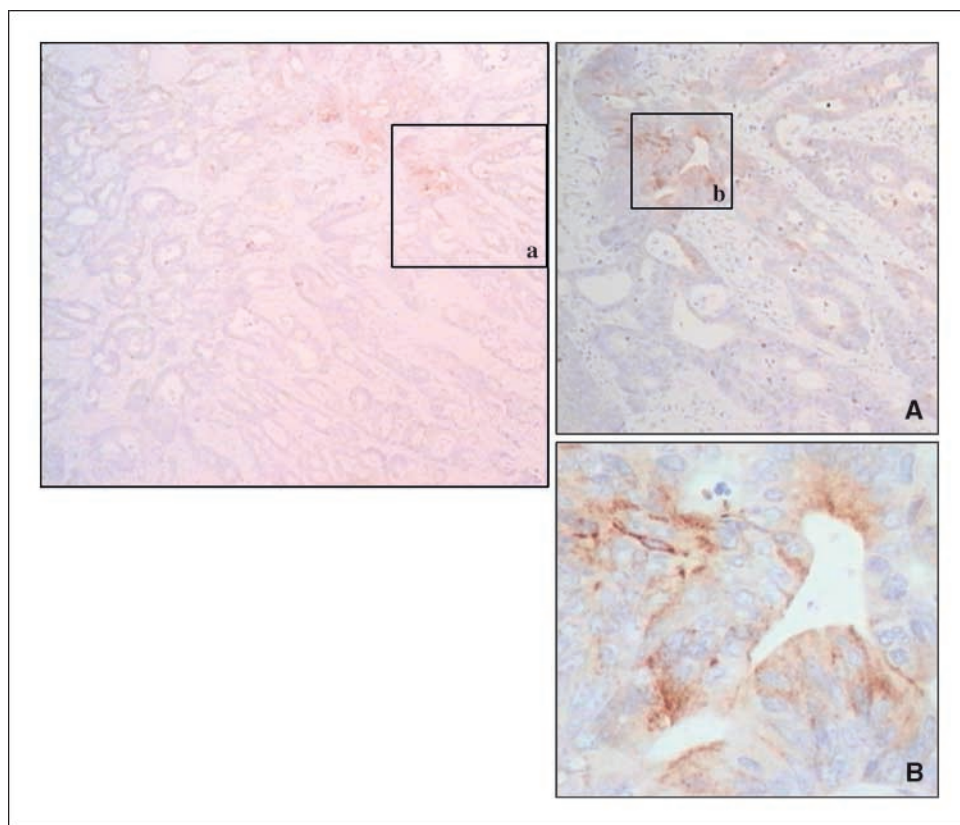
The GLP-2R is expressed in human tumors. To identify human cancers that expressed the GLP-2R, we initially searched the expressed sequence tag (EST) and serial analysis of gene expression (SAGE) tag databases at the National Center for Biotechnology Information using the human GLP-2R nucleotide sequence (UniGene accession number Hs.248202) and found several ESTs or SAGE tags from human cancers, including brain, lung, and cervical cancer, which corresponded to the *GLP-2R* gene (Supplementary Table S1). Remarkably, however, we did not detect GLP-2R ESTs in human colon cancer databases. We next examined a series of human cancers for GLP-2R expression using immunohistochemistry. Although the majority of tumors did not contain any GLP-2R⁺ cells, some tumors contained rare cells exhibiting focal GLP-2R immunopositivity (Fig. 1; Supplementary Fig. S1; Supplementary Table S2).

GLP-2R-mediated effects on downstream signaling pathways in colon cancer cell lines are cell type specific. We and others have not been able to detect endogenous GLP-2R mRNA transcripts by RT-PCR in a variety of colon cancer cell lines, including the adenocarcinoma cell lines DLD-1, SW480, SW48, SW620, SW116, Caco-2, HT29, Colo201, Colo205, and Colo320 as well as the carcinoma cell line T84 (11, 19, 20). To examine whether GLP-2R activation modulates colon cancer growth or survival, we transfected human colon cancer cell lines (including DLD-1, SW480, and HT29 cells) with the human GLP-2R cDNA.

We first assessed whether GLP-2 induces cAMP accumulation in colon cancer cells (9, 21, 22). As shown in Fig. 2A, GLP-2 produced a dose-dependent increase in levels of intracellular cAMP in all three GLP-2R⁺ stable cell lines but not in control cell lines transfected with the expression vector alone. As GLP-2 activates growth and survival pathways, we assessed the effects of exogenous GLP-2 on the phosphorylation levels of p44/p42 MAPK [extracellular signal-regulated kinase (ERK1/2)], GSK3, and Akt [a downstream effector of phosphatidylinositol 3-kinase (PI3K)]. Both GLP-2 and forskolin phosphorylated and thereby inhibited GSK3 in DLD-1:hGLP-2R and SW480:hGLP-2R cells (Fig. 2B). In contrast, GLP-2 increased ERK1/2 phosphorylation in DLD-1:hGLP-2R cells, whereas ERK1/2 phosphorylation decreased following GLP-2

¹ In preparation.

Figure 1. Immunohistochemical detection of GLP-2R expression in human colon neoplasms. Focal GLP-2R immunopositivity was observed in several colon cancer tumors using an antibody raised against the GLP-2R (11) as shown in a human colon adenocarcinoma (magnification, $\times 100$). A, $\times 200$ magnification of inset a. B, $\times 600$ magnification of inset b.



stimulation in SW480:hGLP-2R cells. Similar findings were observed following treatment of the cells with forskolin. Furthermore, EGF, but not GLP-2 or forskolin, induced a robust increase in Akt phosphorylation (Fig. 2B), suggesting that GLP-2 does not activate the PI3K pathway in DLD-1 or SW480 cells. Phosphorylation of these signaling molecules was not observed in stable pcDNA3-transfected control cell lines following GLP-2 stimulation (data not shown). In contrast to findings obtained in DLD-1 and SW480 cells, neither GLP-2 nor forskolin induced the phosphorylation of ERK1/2 or GSK3, and EGF had no effect on levels of phosphorylated Akt in HT29:hGLP-2R cells (Fig. 2B). These findings suggest that GSK3 is constitutively inactivated, and ERK1/2 is constitutively activated, in HT29 cells. Hence, although GLP-2 induces cAMP generation in all three cell lines, the pattern of downstream kinase activation/inactivation induced by GLP-2 is clearly cell type specific.

GLP-2 does not modulate the growth rate of colon cancer cell lines. Previous studies using RT-PCR analyses failed to detect GLP-2R mRNA transcripts in a variety of intestinal epithelial cell lines, including Caco-2, T84, HT29, and SW480 cells (11, 19). Nevertheless, GLP-2 decreased the doubling time of both HT29 and SW480 cell lines (19) and produced a dose-dependent increase in [3 H]thymidine incorporation in both Caco-2 and T84 cell lines (20). Accordingly, we monitored the growth rate of colon cancer cells maintained in serum-free medium (Fig. 3) or in medium containing 5% FBS (Supplementary Fig. S2) with or without GLP-2, forskolin, or FBS for up to 4 days. The doubling time of DLD-1:hGLP-2R cells was comparable in cells maintained in either serum-free medium or medium containing 5% FBS, whereas the growth rate of both SW480:hGLP-2R and HT29:hGLP-2R cells increased when cultured in the presence of serum (Fig. 3). Neither GLP-2 nor forskolin

affected the doubling time of any of these cell lines under serum-free (Fig. 3) or serum-supplemented conditions (Supplementary Fig. S2).

GLP-2 does not promote the survival of GLP-2R-transfected colon cancer cells. In contrast to the prosurvival effects of GLP-2R signaling shown in BHK and HeLa cells (17, 21, 23), GLP-2R activation did not attenuate cytotoxicity induced by cycloheximide (a protein synthesis inhibitor), LY294002 (an inhibitor of PI3K), indomethacin (an inhibitor of cyclooxygenase-1 and cyclooxygenase-2), or two chemotherapeutic agents commonly used to treat colon cancer, oxaliplatin and irinotecan, in GLP-2R-transfected colon cancer cell lines (Fig. 4; Supplementary Fig. S3). In contrast, forskolin attenuated oxaliplatin-induced cell death in DLD-1:hGLP-2R (Fig. 4) and HT29:hGLP-2R cells (Supplementary Fig. S3). Similarly, FBS attenuated cycloheximide-induced cell death in DLD-1:hGLP-2R cells and indomethacin- and LY294002-induced cell death in HT29:hGLP-2R cells and increased the number of viable DLD-1:hGLP-2R and SW480:hGLP-2R cells following exposure to irinotecan (Fig. 4; Supplementary Fig. S3). Furthermore, pretreatment with 20 nmol/L GLP-2 or forskolin for 16 h did not increase cell survival following exposure of all three cell lines to cycloheximide, indomethacin, LY294002, oxaliplatin, or irinotecan (data not shown). Taken together, these results show that GLP-2 does not prevent or ameliorate cytotoxic injury in colon cancer cells.

Chronic GLP-2 treatment does not promote the growth of colon cancer xenografts in nude mice. To ascertain whether chronic GLP-2R activation modulates colon cancer cell growth *in vivo*, we injected DLD-1:hGLP-2R and SW480:hGLP-2R cells s.c. into separate flanks of nude mice followed by twice daily injections of saline or 5 μ g GLP-2 for 6 weeks. DLD-1 and SW480 cells were

chosen for this experiment as these cells display an increase in intracellular cAMP levels, but contrasting effects on ERK1/2 activation in response to GLP-2 treatment (Fig. 2). Although chronic treatment with GLP-2 significantly increased crypt+villus height in the jejunum of nude mice (Supplementary Fig. S4), GLP-2 did not augment tumor weight (Fig. 5A). Unexpectedly, SW480:hGLP-2R tumor weights were significantly smaller in GLP-2-treated mice (Fig. 5A, right) and fewer GLP-2-treated mice developed SW480:hGLP-2R tumors (7 of 10 GLP-2-treated mice versus 8 of 9 PBS-treated mice). Similarly, DLD-1:hGLP-2R tumors were also smaller in GLP-2-treated mice, although this trend was not statistically significant (Fig. 5A, left). RT-PCR analysis verified that the level of GLP-2R expression in the tumors at the end of the 6-week treatment period was comparable with levels in the original cell lines used for inoculation (Fig. 5B). Consistent with the much larger size of DLD-1:hGLP-2R relative to SW480:hGLP-2R tumors, the baseline number of BrdUrd⁺ cells was higher in DLD-1 tumors (~20% in DLD-1 compared with ~12% in SW480 cells; Fig. 5D, compare top left with top right). However, GLP-2 had no

effect on the number of BrdUrd⁺ cells in either DLD-1:hGLP-2R or SW480:hGLP-2R tumors (Fig. 5D, top). Furthermore, GLP-2 treatment did not significantly modify the number of TUNEL⁺ cells in either DLD-1:hGLP-2R or SW480:hGLP-2R tumors (Fig. 5D, bottom). Taken together, these results show that chronic treatment with GLP-2 does not promote the growth of DLD-1 or SW480 colon cancer cell xenografts *in vivo*.

Chronic GLP-2 administration does not augment intestinal tumor number or growth in *Apc*^{Min/+} mice. We next examined whether chronic GLP-2 administration affects tumor formation in *Apc*^{Min/+} mice (24). Littermate male and female *Apc*^{Min/+} and WT mice were injected once daily with either saline or 5 μg GLP-2 for 7 weeks. As previously observed in *Apc*^{Min/+} mice, the majority of adenomas developed in the distal jejunum and proximal ileum with very few in the proximal duodenum, distal ileum, or colon (24). No differences were observed in the number of adenomas in the small intestine of mice treated with saline versus GLP-2 (Fig. 6A). Furthermore, we did not observe adenomas in *Apc*^{+/+} mice treated with GLP-2 for 7 weeks (data not shown), and no significant

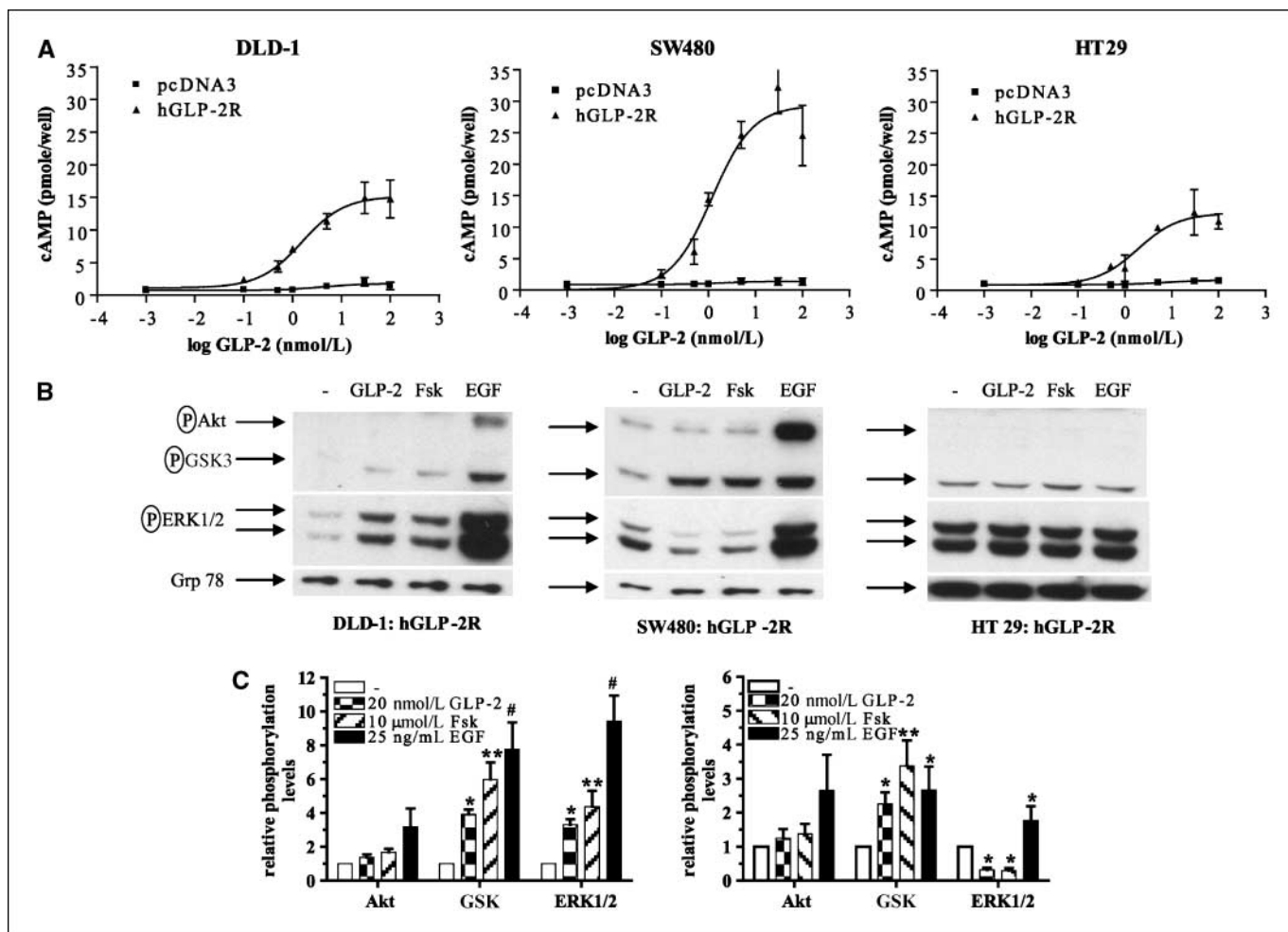


Figure 2. Downstream signaling events activated by GLP-2 in colon cancer cell lines stably expressing the GLP-2R. *A*, DLD-1, SW480, and HT29 human colon cancer cell lines stably expressing either the human GLP-2R or empty vector (pcDNA3) were treated with the indicated concentrations of GLP-2 for 10 min, and cAMP content was measured by a RIA. *Points*, mean of three independent experiments each performed in triplicate; *bars*, SE. *B*, the indicated human colon cancer cell lines stably expressing the human GLP-2R were serum starved overnight and stimulated for 5 min with vehicle (-), 20 nmol/L GLP-2, 10 μmol/L forskolin (*Fsk*), or 25 ng/mL EGF as indicated. Equal amounts of whole-cell lysate were immunoblotted using antisera against phosphorylated ERK1/2 (Thr²⁰²/Tyr²⁰⁴), phosphorylated Akt (Ser⁴⁷³), phosphorylated GSK3α/β (Ser^{9/21}), or Grp78 as a loading control. Shown are representative blots from five independent experiments with similar results. *C*, the signal from the positively phosphorylated kinases was quantified by densitometry, corrected for the intensity of the Grp78 signal, and normalized to the signal from vehicle-treated (-) cells. *Columns*, mean; *bars*, SE. *, *P* < 0.05; **, *P* < 0.01; #, *P* < 0.001 (vehicle versus treatment).

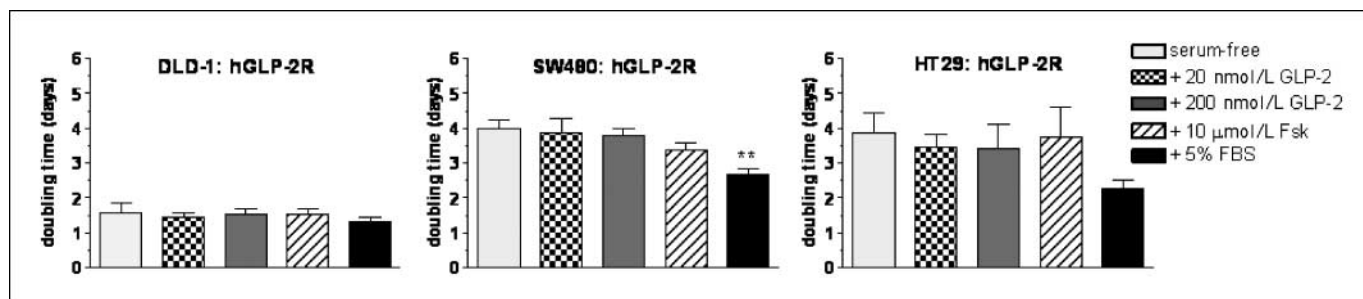


Figure 3. GLP-2 does not modulate the growth rate of colon cancer cell lines stably expressing the GLP-2R. Cells were maintained in serum-free medium for 16 h and then treated with vehicle, 20 nmol/L GLP-2, 200 nmol/L GLP-2, 10 μmol/L forskolin, or 5% FBS. Fresh medium and treatments were replaced every 24 h. At 24-, 48-, 72-, and 96-h time points, cell number was assessed using a tetrazolium salt bioreduction assay (MTS assay). Shown is the doubling time for each cell line under the indicated growth conditions. Columns, mean of four independent experiments each performed in triplicate; bars, SE. **, $P < 0.01$ (serum-free conditions versus treatment).

difference in tumor size was observed between the two treatment groups in male or female $Apc^{Min/+}$ mice (Fig. 6B). In contrast to the lack of effect of GLP-2 on tumor number or size, crypt+villus height was significantly increased in the jejunum of both $Apc^{+/+}$ and $Apc^{Min/+}$ GLP-2-treated mice (Supplementary Fig. S5).

Polyp burden in $Apc^{Min/+}$ mice is not affected by $Glp2r$ genotype. To determine whether $Glp2r$ genotype and levels of endogenous GLP-2R signaling affect tumor development in $Apc^{Min/+}$ mice, adenomas were quantified and measured in

$Apc^{Min/+};Glp2r^{+/+}$, $Apc^{Min/+};Glp2r^{+/-}$, and $Apc^{Min/+};Glp2r^{-/-}$ littermate mice once they reached 14 to 15 weeks of age. No significant differences in polyp number (Fig. 6C) or size (Fig. 6D) were observed between the $Glp2r$ genotypes in either the small or large intestine. In addition, no significant differences in crypt+villus height were observed in the jejunum between the $Glp2r$ genotypes (Supplementary Fig. S6). Thus, endogenous GLP-2 signaling does not influence intestinal adenoma formation or jejunal crypt+villus height in $Apc^{Min/+}$ mice.

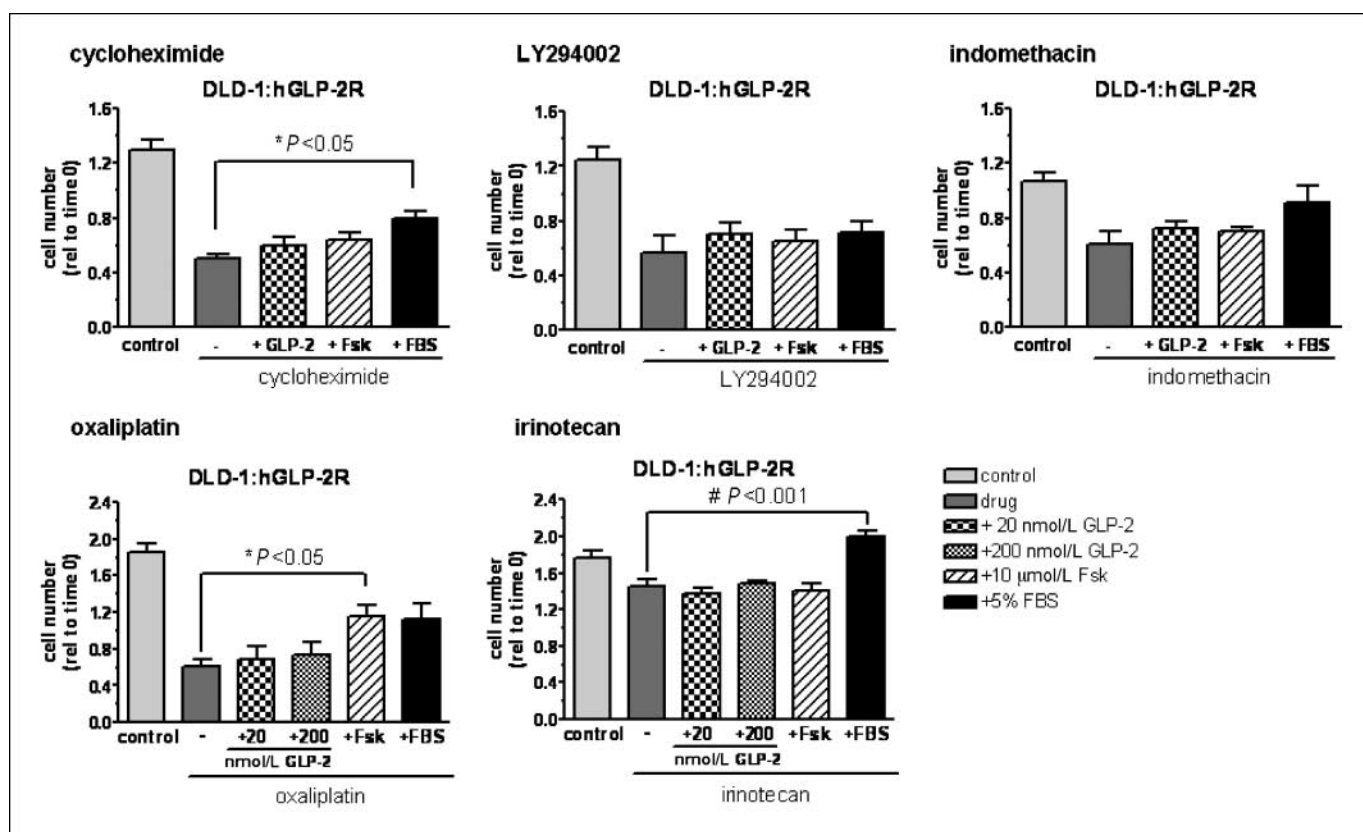


Figure 4. GLP-2 does not protect DLD-1 cells stably expressing the GLP-2R from chemically induced cytotoxicity. DLD-1:hGLP-2R cells were maintained for 16 h in medium lacking serum before treatment with cycloheximide (80 μmol/L final), LY294002 (50 μmol/L final), or indomethacin (400 μmol/L final) in the same medium or were treated with oxaliplatin (100 μmol/L final) or irinotecan (50 μmol/L final) in serum-free medium (without prior serum starvation) in the presence or absence of 20 nmol/L GLP-2, 200 nmol/L GLP-2, 10 μmol/L forskolin, or 5% FBS for 48 h. Cell number was then measured using a tetrazolium salt bioreduction assay (MTS assay), and the values were normalized to the absorbance reading (490 nm) of cells before treatment (time 0). Columns, mean of at least three independent experiments each performed in triplicate; bars, SE. *, $P < 0.05$; #, $P < 0.001$ (treatment alone versus treatment + either GLP-2, forskolin, or FBS).

Discussion

The observation that GLP-2 is a potent intestinotrophic and cytoprotective factor has led to evaluation of GLP-2, or longer-acting GLP-2 analogues, for the treatment of human intestinal

disorders, including short bowel syndrome and inflammatory bowel disease (15). However, the proliferative and antiapoptotic actions of GLP-2 have raised concerns about the tumorigenic potential that may be associated with the long-term use of GLP-2

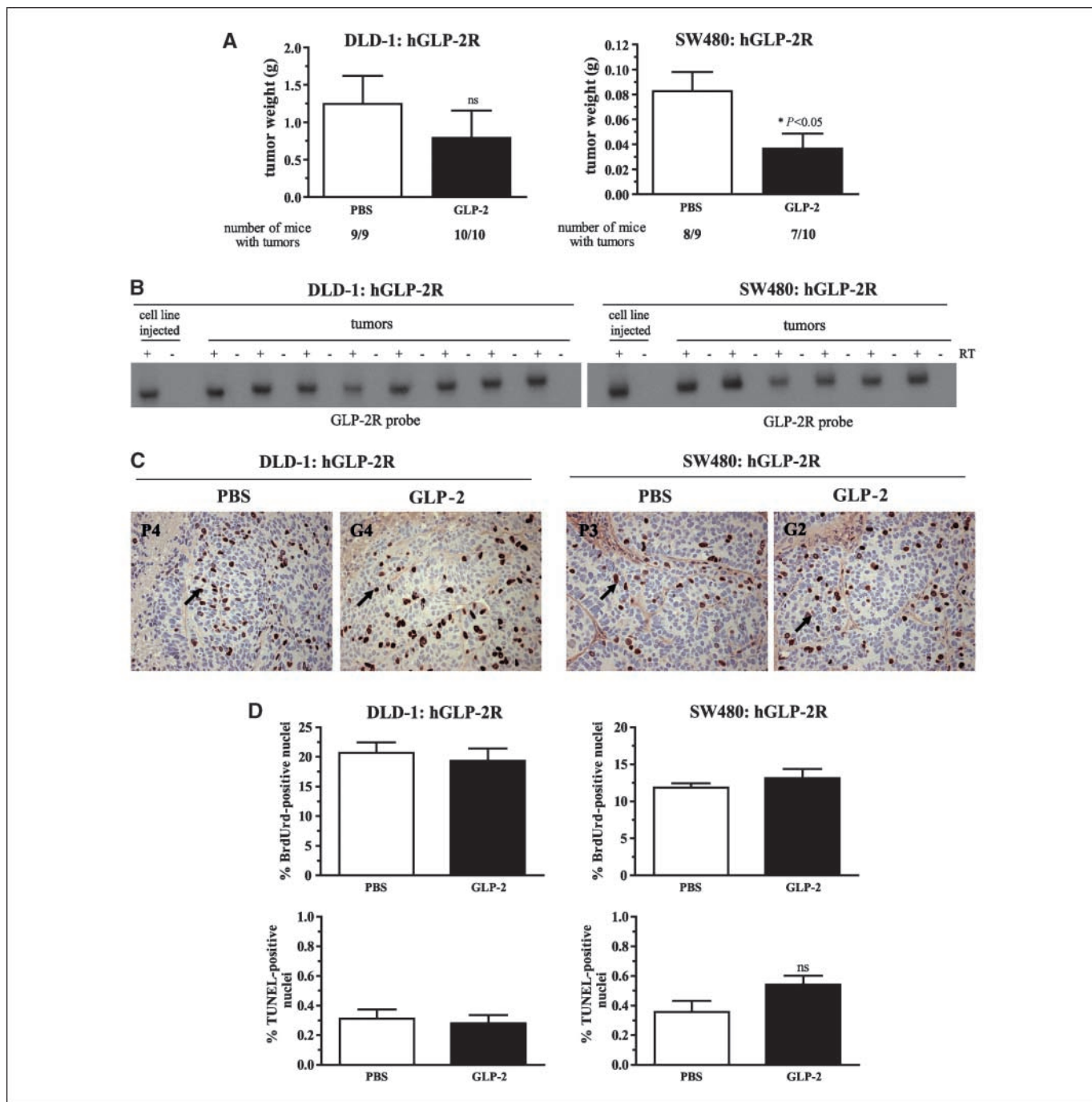
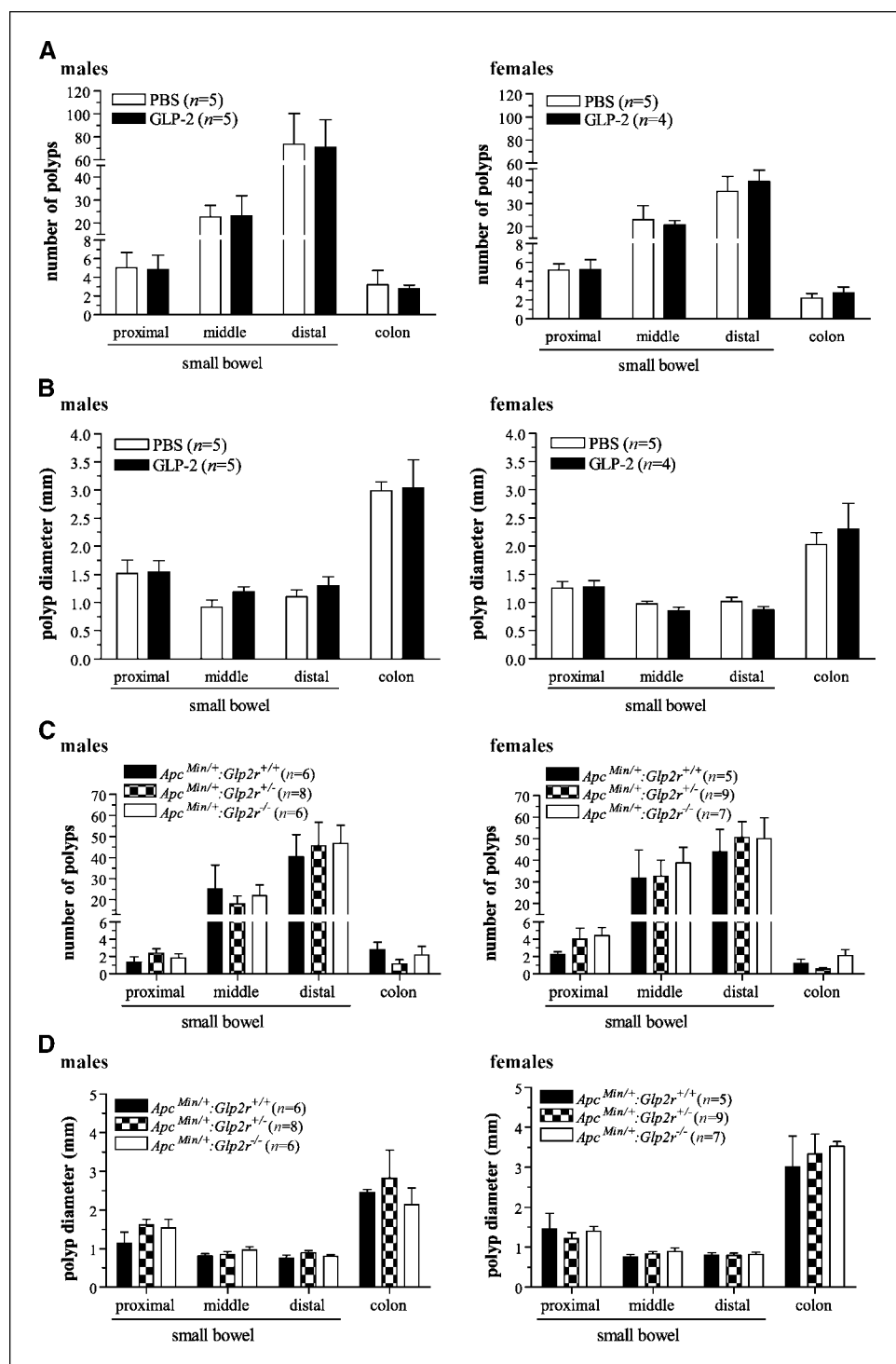


Figure 5. Chronic GLP-2 treatment does not stimulate the growth of GLP-2R-expressing human colon cancer cell xenografts in nude mice. **A**, tumor weights of implanted DLD-1:hGLP-2R (*left*) or SW480:hGLP-2R cells (*right*) at the end of a 6-wk treatment period with PBS or 5 μ g GLP-2 twice daily, as indicated. *Columns*, mean; *bars*, SE. The number of mice that developed tumors is indicated underneath the graph. *, $P < 0.05$ (PBS versus GLP-2 treatment). **B**, GLP-2R expression was assessed by RT-PCR using RNA isolated from an aliquot of the original cell suspension used for xenografts (cell line injected) and from several tumors that developed following implantation of DLD-1:hGLP-2R cells (*left*) or SW480:hGLP-2R cells (*right*). PCR products were analyzed by agarose gel electrophoresis followed by Southern blot analysis using an internal GLP-2R-specific oligonucleotide probe. The specificity of each reaction (RT+) was monitored by control reactions using samples in which the reverse transcriptase (RT) was omitted from the reaction mixture (RT-). **C**, representative photomicrographs of tumors from DLD-1:hGLP-2R (*left*) and SW480:hGLP-2R (*right*) cells following immunostaining with an anti-BrdUrd antibody. Representative BrdUrd⁺ cells are indicated by an arrow. Magnification, $\times 400$. **D**, the percentage of BrdUrd⁺ nuclei (*top*) or TUNEL-positive nuclei (*bottom*) in each tumor is shown for each treatment group, as indicated. For each tumor, at least 1,200 nuclei were scored. A total of six (DLD-1:hGLP-2R) or seven (SW480:hGLP-2R) tumors were assessed for each treatment. *Columns*, mean; *bars*, SE.

Figure 6. Polyp burden in *Apc*^{Min/+} mice is not affected by chronic GLP-2 treatment or *Glp2r* genotype. *A* and *B*, intestinal polyp number, diameter, and distribution along the gastrointestinal tract were assessed in *Apc*^{Min/+} mice treated once daily with PBS or 5 μg GLP-2 for 7 wk. The small intestine was divided into three equal segments (proximal, middle, and distal). *Columns*, mean; *bars*, SE. Data are the total number (*A*) or the average diameter (*B*) of polyps in each section of the small intestine and colon. Although the mean number of polyps in the colon was comparable between male *Apc*^{Min/+} mice in the two treatment groups, two PBS-treated males did not develop any adenomas in the colon, whereas all GLP-2-treated males developed at least two polyps. In contrast, all females, irrespective of treatment, developed at least one polyp in the colon. *C* and *D*, genetic disruption of GLP-2R expression does not alter polyp burden in *Apc*^{Min/+} mice. Intestinal polyp number, diameter, and distribution along the gastrointestinal tract were assessed in *Apc*^{Min/+};*Glp2r*^{+/+}, *Apc*^{Min/+};*Glp2r*^{+/-}, and *Apc*^{Min/+};*Glp2r*^{-/-} mice. The small intestine was divided into three equal segments (proximal, middle, and distal). *Columns*, mean; *bars*, SE. Data are the total number (*C*) or the average diameter (*D*) of polyps in each section of the small intestine and colon. The number of mice in each group is indicated in the legend.



(19, 25). Our data show that activation of GLP-2R signaling does not directly promote the proliferation or enhance survival of GLP-2R-expressing colon cancer cell lines *in vitro*. Moreover, chronic treatment with native GLP-2 did not promote the growth of GLP-2R⁺ colon cancer xenografts in nude mice nor increase adenoma formation or size in *Apc*^{Min/+} mice.

Although GLP-2 stimulated cAMP accumulation in GLP-2R-transfected colon cancer cell lines studied here, we observed cell-specific differences in GLP-2R-dependent effects on ERK1/2

and GSK3 phosphorylation. These differential effects may reflect the extent of cAMP accumulation induced by GLP-2 and/or differences in mutations of specific signaling molecules inherent to these colon cancer cells. All three cell lines contain mutations in the *Apc* and *p53* tumor suppressor genes. Furthermore, both epidermal growth factor receptor and HER2 are overexpressed and constitutively active in SW480 and HT29 cells, whereas all but HT29 cells contain oncogenic *K-ras* mutations. Thus, it seems plausible that the multiple genetic alterations inherent to specific

cancer cells contribute to cell-specific differences in signaling events following GLP-2R activation, and may also underlie the lack of an effect of GLP-2 on the proliferation or survival of these colon cancer cell lines.

Previous studies have shown that GLP-2 increased the proliferation and/or migration of several colon cancer cell lines *in vitro*, including Caco-2, T84, HT29, and SW480 (19, 20). However, expression of the known GLP-2R was not detected in these cells, suggesting that the GLP-2-mediated effects on cell proliferation may be through activation of a different receptor. In contrast, we expressed the cloned human GLP-2R in colon cancer cell lines to specifically examine GLP-2R-dependent effects on colon cancer proliferation and survival. We did not detect a GLP-2-dependent increase in proliferation of the colon cancer cell lines examined here, in contrast with previous results (19). Moreover, our studies show that GLP-2 did not promote the growth of GLP-2R⁺ DLD-1 or SW480 xenografts in nude mice. Thus, our results do not support a direct or indirect role for GLP-2 in the growth of GLP-2R-expressing colon cancer cells.

As GLP-2 and the GLP-2R agonist h[Gly2]GLP-2 accelerated the growth of chemically induced colonic neoplasms in mice (25), we explored the tumorigenic potential of chronic GLP-2R activation using *Apc*^{Min/+} mice as an alternative genetic model of intestinal tumorigenesis. No increase in either the number or size of adenomas that developed in *Apc*^{Min/+} mice was observed following chronic GLP-2 administration. Hence, administration of GLP-2 at levels sufficient to induce growth of the intestinal mucosa does not promote adenoma formation or accelerate the growth of existing tumors in *Apc*^{Min/+} mice. Moreover, disruption of the *Glp2r* gene

did not alter polyp formation in *Apc*^{Min/+};*Glp2r*^{-/-} mice, suggesting that endogenous GLP-2R signaling does not influence tumor initiation or growth in *Apc*^{Min/+} mice. Hence, although GLP-2 acutely activates the β -catenin pathway in mouse intestinal crypt cells (26), this does not seem to play an important role in tumorigenesis in *Apc*^{Min/+} mice, where β -catenin transit into the nucleus is an early consequence of *Apc*⁺ loss observed in *Apc*^{Min/+} tumors (27).

In conclusion, our results do not reveal a role for GLP-2 in promoting the growth or survival of intestinal tumor cells. Nevertheless, there are several limitations to our studies, which include the dose and duration of exposure to GLP-2, the use of native GLP-2 and not more potent GLP-2 analogues, and the specific cell and murine models used for these experiments. As longer-acting GLP-2 analogues such as teduglutide are currently being studied in patients with intestinal dysfunction (15), it seems prudent to understand the consequences of sustained GLP-2R activation in a diverse number of additional experimental contexts.

Disclosure of Potential Conflicts of Interest

DJ. Drucker is a party to a GLP-2 licensing agreement between NPS Pharmaceuticals, Inc., the University of Toronto, and the University Health network. The other authors disclosed no potential conflicts of interest.

Acknowledgments

Received 1/9/2008; revised 6/17/2008; accepted 7/10/2008.

Grant support: NCIC grant 017428 and CIHR grant MOP-14799.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

- Dakin CL, Small CJ, Batterham RL, et al. Peripheral oxyntomodulin reduces food intake and body weight gain in rats. *Endocrinology* 2004;145:2687-95.
- Baggio LL, Huang Q, Brown TJ, Drucker DJ. Oxyntomodulin and glucagon-like peptide-1 differentially regulate murine food intake and energy expenditure. *Gastroenterology* 2004;127:546-58.
- Drucker DJ. The biology of incretin hormones. *Cell Metab* 2006;3:153-65.
- Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* 2006;368:1696-705.
- Orskov C, Holst JJ. Radio-immunoassays for glucagon-like peptides 1 and 2 (GLP-1 and GLP-2). *Scand J Clin Lab Invest* 1987;47:165-74.
- Xiao Q, Boushey RP, Drucker DJ, Brubaker PL. Secretion of the intestinotropic hormone glucagon-like peptide 2 is differentially regulated by nutrients in humans. *Gastroenterology* 1999;117:99-105.
- Drucker DJ, Ehrlich P, Asa SL, Brubaker PL. Induction of intestinal epithelial proliferation by glucagon-like peptide 2. *Proc Natl Acad Sci U S A* 1996;93:7911-6.
- Estall JL, Drucker DJ. Glucagon-like peptide-2. *Annu Rev Nutr* 2006;26:391-411.
- Munroe DG, Gupta AK, Kooshesh F, et al. Prototypic G protein-coupled receptor for the intestinotrophic factor glucagon-like peptide 2. *Proc Natl Acad Sci U S A* 1999;96:1569-73.
- Nelson DW, Sharp JW, Brownfield MS, Raybould HE, Ney DM. Localization and activation of glucagon-like peptide-2 receptors on vagal afferents in the rat. *Endocrinology* 2007;148:1954-62.
- Yusta B, Huang L, Munroe D, et al. Enteroendocrine localization of GLP-2 receptor expression. *Gastroenterology* 2000;119:744-55.
- Guan X, Karpen HE, Stephens J, et al. GLP-2 receptor localizes to enteric neurons and endocrine cells expressing vasoactive peptides and mediates increased blood flow. *Gastroenterology* 2006;130:150-64.
- Bjerknes M, Cheng H. Modulation of specific intestinal epithelial progenitors by enteric neurons. *Proc Natl Acad Sci U S A* 2001;98:12497-502.
- Orskov C, Hartmann B, Poulsen SS, Thulesen J, Hare KJ, Holst JJ. GLP-2 stimulates colonic growth via KGF, released by subepithelial myofibroblasts with GLP-2 receptors. *Regul Pept* 2005;124:105-12.
- Jeppesen PB, Sanguinetti EL, Buchman A, et al. Teduglutide (ALX-0600), a dipeptidyl peptidase IV resistant glucagon-like peptide 2 analogue, improves intestinal function in short bowel syndrome patients. *Gut* 2005;54:1224-31.
- Dube PE, Forse CL, Bahrami J, Brubaker PL. The essential role of insulin-like growth factor-1 in the intestinal tropic effects of glucagon-like peptide-2 in mice. *Gastroenterology* 2006;131:589-605.
- Koehler JA, Yusta B, Drucker DJ. The HeLa cell glucagon-like peptide-2 receptor is coupled to regulation of apoptosis and ERK1/2 activation through divergent signaling pathways. *Mol Endocrinol* 2005;19:459-73.
- Koehler JA, Drucker DJ. Activation of GLP-1 receptor signaling does not modify the growth or apoptosis of human pancreatic cancer cells. *Diabetes* 2006;55:1369-79.
- Masur K, Schwartz F, Entschladen F, Niggemann B, Zaenker KS. DPPIV inhibitors extend GLP-2 mediated tumour promoting effects on intestinal cancer cells. *Regul Pept* 2006;137:147-55.
- Dasle J, Ashley SW, Shimoda N, Zinner MJ, Whang EE. Glucagon-like peptide 2 stimulates intestinal epithelial proliferation *in vitro*. *Dig Dis Sci* 2002;47:1135-40.
- Yusta B, Boushey RP, Drucker DJ. The glucagon-like peptide-2 receptor mediates direct inhibition of cellular apoptosis via a cAMP-dependent protein kinase-independent pathway. *J Biol Chem* 2000;275:35345-52.
- Yusta B, Estall J, Drucker DJ. GLP-2 receptor activation engages Bad and glycogen synthase kinase 3 in a protein kinase A-dependent manner and prevents apoptosis following inhibition of phosphatidylinositol 3-kinase. *J Biol Chem* 2002;277:24896-06.
- Yusta B, Somwar R, Wang F, et al. Identification of glucagon-like peptide-2 (GLP-2)-activated signaling pathways in baby hamster kidney fibroblasts expressing the rat GLP-2 receptor. *J Biol Chem* 1999;274:30459-67.
- Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 1990;247:322-4.
- Thulesen J, Hartmann B, Hare KJ, et al. Glucagon-like peptide 2 (GLP-2) accelerates the growth of colonic neoplasms in mice. *Gut* 2004;53:1145-50.
- Dube PE, Rowland KJ, Brubaker PL. Glucagon-like peptide-2 activates β -catenin signaling in the mouse intestinal crypt: role of insulin-like growth factor-1. *Endocrinology* 2008;149:291-301.
- Sheng H, Shao J, Williams CS, et al. Nuclear translocation of β -catenin in hereditary and carcinogen-induced intestinal adenomas. *Carcinogenesis* 1998;19:543-9.

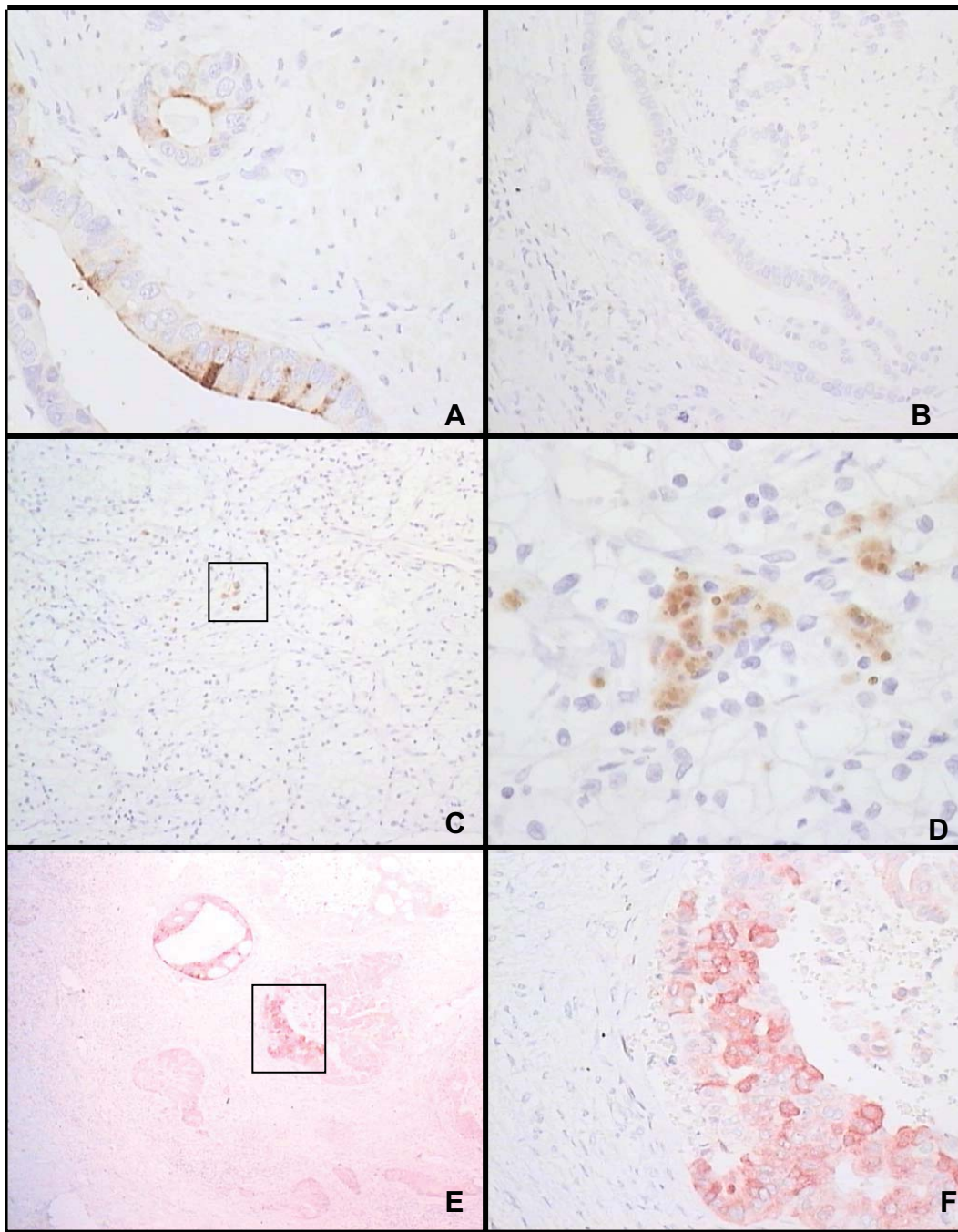
Supplementary Table 1:

Cancer library	Unigene	Cancer Genome Anatomy Project	
	EST	EST	SAGE tags
lung/respiratory tract	8/103 986	8/172 645	0/159 059
cervix	1/ 34 156	3/44 142	-
Non-glioma/brain	1/131 223	0/201 640	3/ 4 997 811
peripheral nervous system	-	1/662	-
gastrointestinal tract	0/119 520	0/12 682	-
colon	0/113 595	0/153 290	0/701 542

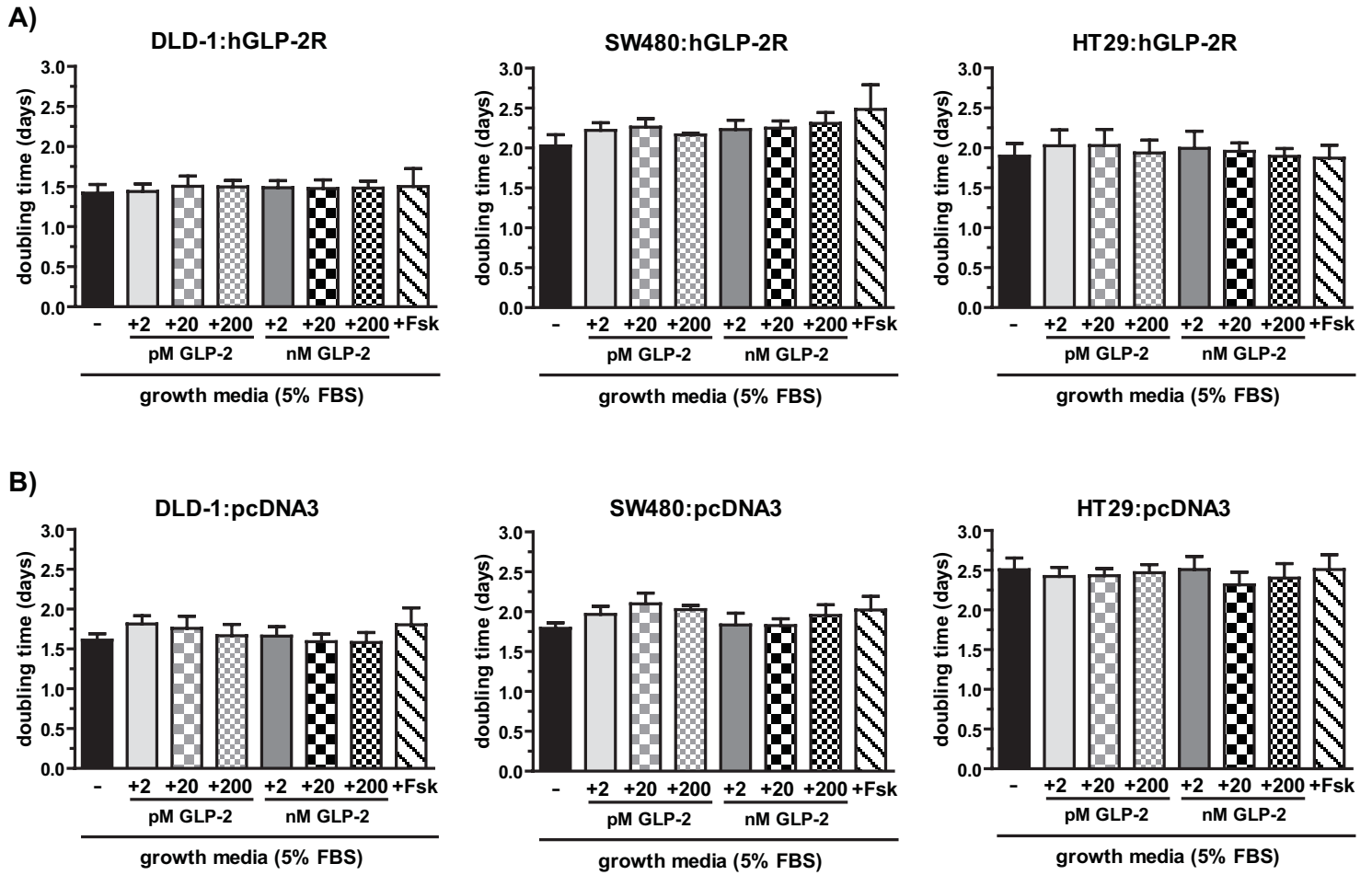
Supplementary Table 2:

Tumor Type	N	Focal GLP-2R positivity
Colon adenocarcinoma	45	14
Papillary Thyroid Cancer	6	1
Medullary Thyroid Cancer	1	1
Melanoma	3	-
Small cell cancer (H&N)	3	-
Schwannoma	1	-
Buccal carcinosarcoma	1	1
Prostate adenocarcinoma	19	1
Lung carcinoid	1	1
Lung Adenocarcinoma	3	1
Lung bronchoalveolar cancer	3	2
Uterine endometrial cancer	5	2
Ovary adenocarcinoma	9	2
Cervix adenocarcinoma	3	1

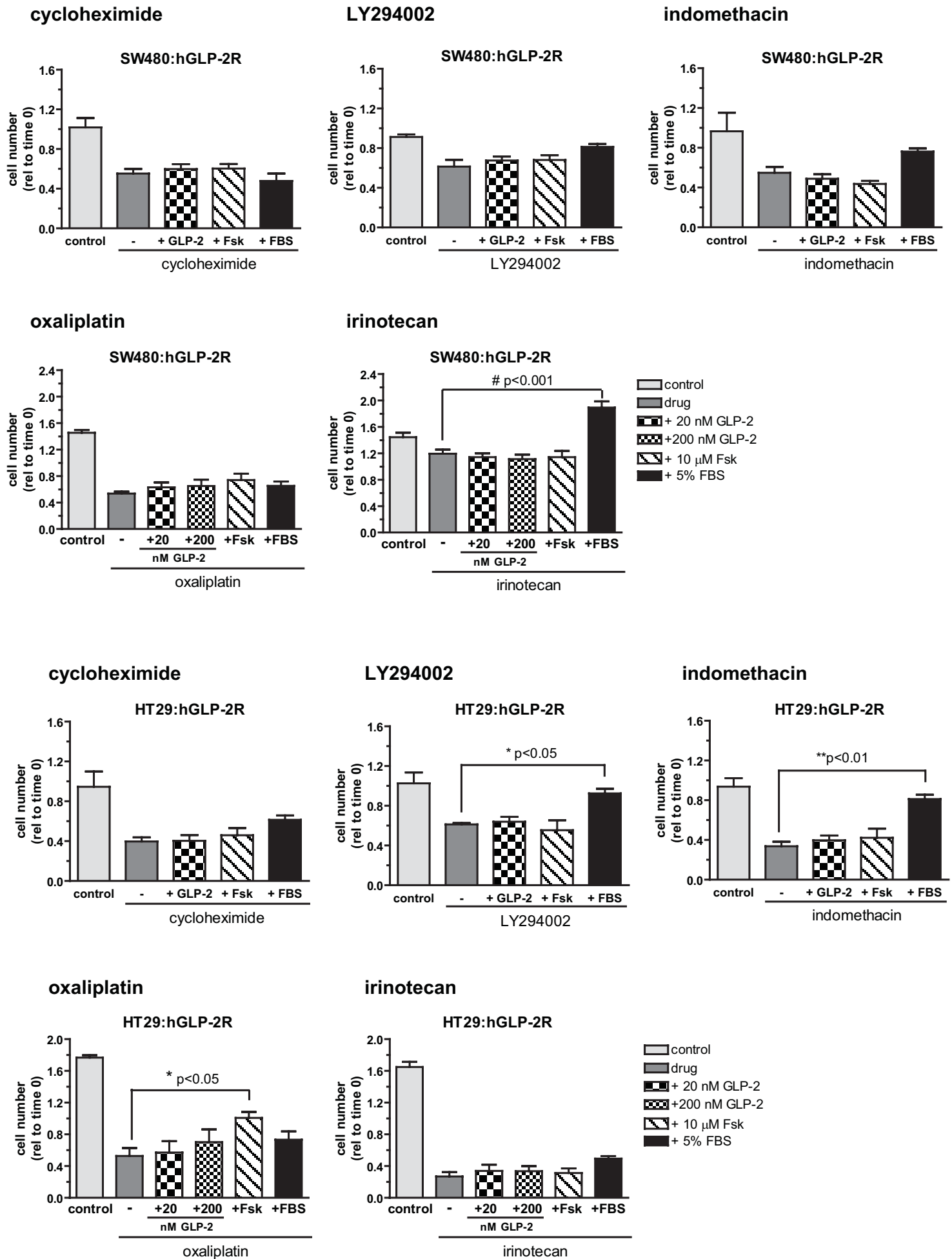
Supplementary Figure 1:



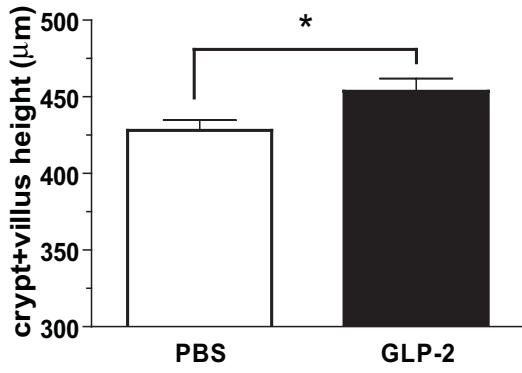
Supplementary Figure 2:



Supplementary Figure 3:

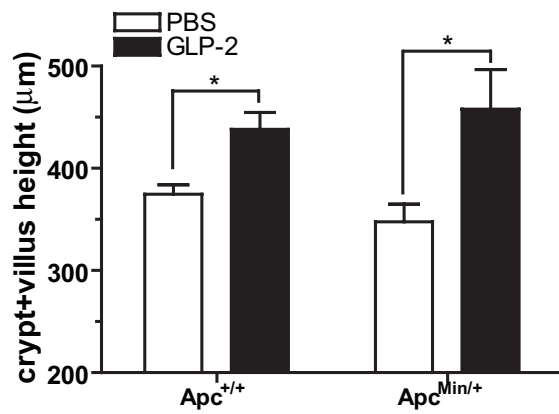


Supplementary Figure 4:

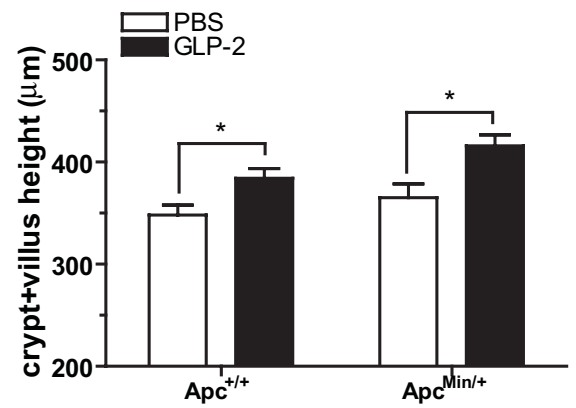


Supplementary Figure 5:

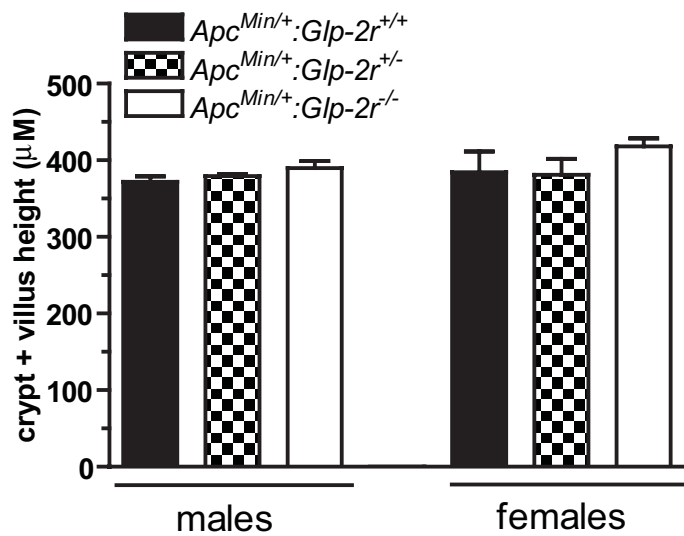
males



females



Supplementary Figure 6:



Supplementary Table 1: GLP-2R gene expression in human cancers. The human GLP-2R nucleotide sequence (Unigene access # Hs.248202) was used to query EST and SAGE databases using Unigene's EST ProfileViewer (www.ncbi.nlm.nih.gov/Unigene) or the Cancer Genome Anatomy Project (cgap.nci.nih.gov) virtual Northern. Shown are the number of ESTs or SAGE tags corresponding to the GLP-2R / total number of ESTs or SAGE tags in the cancer library for the given tissue.

Supplementary Table 2

Analysis of GLP-2R immunopositivity by immunocytochemistry in human tumors. Focal immunopositivity was observed in less than 1% of all tumor cells examined for GLP-2R immunopositivity.

Supplementary Figure 1: Immunohistochemical analysis of human tumours

expressing the GLP-2 receptor. Focal immunopositivity was observed in a human colon cancer tumour (A) that was eliminated following preabsorption of antiserum with recombinant GLP-2R (B) Mag x 200, and in Renal Cell Adenocarcinoma (C; Mag x 100, D; Mag x 600) and in Buccal Carcinosarcoma (E; Mag x 100, F; Mag x 600) using an antibody raised against the GLP-2 receptor (11).

Supplementary Figure 2: GLP-2 does not modulate the growth rate of colon cancer

cell lines stably expressing the GLP-2 receptor. Colon cancer cells lines stably transfected with the GLP-2R (A) or pcDNA3 control (B) were maintained in growth media (5% FBS) and treated with 2 pM - 200 nM GLP-2 or 10 μ M forskolin. Fresh

medium and treatments were replaced every 24 h. At 24, 48, 72 and 96 h time points, cell number was assessed using a tetrazolium salt bio-reduction assay (MTS assay). Shown is the doubling time for each cell line under the indicated growth conditions. Data shown are means \pm SE from three independent experiments, each performed in triplicate.

Supplementary Figure 3: GLP-2 does not protect GLP-2R-transfected colon cancer cell lines from chemically-induced cytotoxicity. SW480 and HT29 human colon cancer cell lines stably transfected with the human GLP-2R were maintained for 16 h in media lacking serum prior to treatment with cycloheximide (80 μ M final), LY294002 (50 μ M final), or indomethacin (400 μ M final) in the same media or were treated with oxaliplatin (100 μ M final) or irinotecan (50 μ M final) in serum-free media (without prior serum starvation) in the presence or absence of 20 nM GLP-2, 200 nM GLP-2, 10 μ M forskolin or 5% FBS for 48 h (HT29 cells were treated with indomethacin for 24 h). Cell number was then measured using a tetrazolium salt bio-reduction assay (MTS assay), and the values normalized to the absorbance reading (490 nm) of cells prior to treatment (time 0). Data shown are the means \pm SE from at least three independent experiments, each performed in triplicate. * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$ (treatment alone *versus* treatment + either GLP-2, forskolin or FBS).

Supplementary Figure 4: Chronic GLP-2 treatment increases small intestinal villus height in nude mice. Crypt+villus heights of intestinal segments from the proximal jejunum of nude mice (carrying xenografts) injected intraperitoneally with PBS (n=9) or

5 μg GLP-2 (n=10) twice a day for 6 weeks. Shown are means \pm SE. *p<0.05 (PBS *versus* GLP-2 treatment).

Supplementary Figure 5: Chronic GLP-2 treatment increases small intestinal villus height in $Apc^{Min/+}$ mice. Crypt+villus heights of intestinal segments from the proximal jejunum of age and sex matched $Apc^{+/+}$ and $Apc^{Min/+}$ littermates (n=12 $Apc^{+/+}$, 10 $Apc^{Min/+}$ males, 10 $Apc^{+/+}$, 9 $Apc^{Min/+}$ females) injected subcutaneously with PBS or 5 μg GLP-2 once a day for 7 weeks. Data shown are means \pm SE *p<0.05 (PBS *versus* GLP-2 treatment).

Supplementary Figure 6: Genetic disruption of GLP-2R expression does not alter crypt + villus height in $Apc^{Min/+}$ mice. Crypt+villus heights of intestinal segments from the proximal jejunum was assessed in age- and sex-matched $Apc^{Min/+}:Glp-2r^{+/+}$, $Apc^{Min/+}:Glp-2r^{+/-}$, and $Apc^{Min/+}:Glp-2r^{-/-}$ mice (n = 4-5 mice in each group). Data shown are means \pm SE.