Glucagon-Like Peptide-1 Receptor Activation Inhibits Growth and Augments Apoptosis in Murine CT26 Colon Cancer Cells

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Obesity, accompanying or independent of type 2 diabetes mellitus (T2DM), is associated with higher rates of malignancy. Hence, there is considerable interest in understanding whether therapies used to treat obese patients with T2DM impact cancer cell growth. Glucagon-like peptide-1 (GLP-1) is produced in enteroendocrine cells and secreted after meal ingestion. GLP-1 regulates blood glucose through multiple mechanisms, principally inhibition of glucagon and stimulation of insulin secretion. GLP-1 also exerts independent effects promoting cell growth and survival, and sustained activation of GLP-1 receptor (GLP-1R) signaling in rodent thyroid glands leads to C-cell hyperplasia and medullary thyroid cancer. Hence, whether therapies based on GLP-1R activation modify growth or survival of cancer cells is of ongoing interest. We studied the biological actions of GLP-1 in mouse CT26 colon cancer cells that express a functional GLP-1R. The GLP-1R agonist exendin (Ex)-4 (exenatide) increased intracellular cAMP levels and inhibited the activity of signaling kinases glycogen synthase kinase 3 and ERK1/2 in CT26 cells. The Ex-4-induced inactivation of glycogen synthase kinase 3, but not ERK1/2, was dependent on protein kinase A and blocked by the GLP-1R antagonist Ex(9–39). Furthermore, Ex-4 altered cell morphology, induced apoptosis, and inhibited proliferation of CT26 cells *in vitro*. Moreover Ex-4 decreased CT26 colony formation in soft agar and augmented apoptosis induced by irinotecan. Twice-daily treatment of CT26 tumor-bearing BALB/c mice with Ex-4 for 2 wk increased tumor apoptosis. Hence, GLP-1R activation reduces growth and survival in CT26 colon cancer cells that express the endogenous classical GLP-1R. (*Endocrinology* 152: 0000–0000, 2011)

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by deficient insulin secretion and resistance to insulin action. The rising incidence of obesity is coincident with a rapidly increasing prevalence of T2DM, with both diagnoses increasingly common in younger individuals in the third or fourth decade of life. Subjects with obesity and T2DM often exhibit hyperinsulinemia and are at risk for development of premature vascular disease, myocardial infarction, and strokes. Moreover, both obesity and T2DM increase incidence rates of multiple cancers, including neoplasms of the breast, pancreas, and colon (1). Hence, there is considerable interest in the mechanisms linking hyperglycemia, insulin resistance, and cancer.

More recent evidence has attributed differential risks for development of certain malignancies to specific antidiabetic therapies. For example, the biguanide metformin is associated with reduced risk of developing some but not all malignancies; however, the unique molecular mechanisms linking metformin action to reduced cell growth

and/or transformation remain incompletely understood (2). Insulin has been used as an essential therapy for type 1 diabetes for almost nine decades and is widely used in T2DM. Activation of the insulin receptor (IR) induces rapid metabolic actions coupled to enhanced glucose uptake and suppression of glucose production in insulin-sensitive tissues. IR signaling also promotes cell proliferation and inhibits apoptosis, providing a molecular mechanism linking IR activation to neoplastic growth (3, 4). Increased levels of insulin may also potentiate signaling through the IGF-I receptor, via regulation of IGF-I-binding proteins and via signaling through hybrid receptors (4). These findings, coupled with reports linking insulin therapy to increased rates of specific cancers (1, 3), have fostered renewed interest in the cellular and molecular actions of different antidiabetic therapies on cancer cells.

Two new classes of antidiabetic therapies, glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) agonists and dipeptidyl peptidase-4 inhibitors, exert their actions through potentiation of GLP-1R signaling (5). GLP-1 controls glucose homeostasis in part via stimulation of insulin secretion; hence, sustained GLP-1R activation may, in some patients, be associated with hyperinsulinemia, a potential mechanism for indirectly promoting colon cancer growth (4). Moreover, GLP-1R activation directly promotes cell proliferation and enhances cell survival (6) independent of insulin in multiple cell types including β-cells, neurons, fibroblasts, and cardiomyocytes, providing additional mechanisms for GLP-1R agonists to modify the development and/or growth of cancer cells. More recent studies have demonstrated that GLP-1R agonists such as exendin (Ex)-4 modify the growth and survival of breast cancer cells, a cell type not previously known to be a direct target of GLP-1R action, through mechanisms involving a nonclassical GLP-1R signaling system (7).

Because T2DM is associated with an increased risk of colon cancer (8, 9), and GLP-1 is produced in the gut and exerts growth factor-like activity in the gastrointestinal tract (10), we examined whether GLP-1R activation regulates the growth or apoptosis of colon cancer cells. We detected expression of the endogenous *Glp1r* in several human cancer expressed sequence tags (EST), including colon cancer. A screen for *Glp1r* expression identified that CT26 murine colon cancer cells express an endogenous functional GLP-1R. Analysis of known signal transduction pathways previously demonstrated to be regulated by GLP-1 (11–13) revealed inhibition of the activity of the growth- and survival-regulating kinases glycogen synthase kinase-3 (GSK3) and extracellular signal-related kinase (ERK1/2). GLP-1R activation robustly stimulated cAMP formation, inhibited cell growth, and promoted apoptosis in CT26 cells. Hence, these results provide the first delineation of the con-

sequences of GLP-1R activation in colon cancer cells that express an endogenous functional GLP-1R.

Materials and Methods

Materials

Tissue culture medium and serum were from Invitrogen Life Technologies (Carlsbad, CA). Forskolin (Fsk), indomethacin, irinotecan, protease inhibitor mixture (P-2714), 3-isobutyl-1-methylxanthine, and phosphatase inhibitor cocktail I were from Sigma Chemical Co. (St. Louis, MO). H-89 and epidermal growth factor (EGF) were from Calbiochem (San Diego, CA). Ex-4 and Ex(9–39), were from Chi Scientific (Maynard, MA). 8-Bromoadenosine- 3′, 5′- cyclic monophosphate (8-Br-cAMP) and 8- (4-Chlorophenylthio)- 2′- O- methyladenosine- 3′, 5′- cyclic monophosphate (pCPT-2′-O-Me-cAMP) were from Biolog Life Science Institute (Bremen, Germany).

Cell lines

Human colon cancer and IEC cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM with 5% fetal bovine serum (FBS). CT26 cells (14) were from American Type Culture Collection, (Rockville, MD) and maintained in RPMI medium containing 10% FBS and penicillin-streptomycin. All experiments were performed using early passage cells (up to passage 8).

RT-PCR and Southern blot analysis

Total RNA was isolated using TRIzol reagent (Life Technologies, Burlington, Ontario, Canada) or the guanidinium thiocyanate method (15). Five micrograms total RNA was reversed transcribed at 50 C for 60 min using Superscript III reverse transcriptase and random hexamer primers (Invitrogen Life Technologies). The mouse GLP-1R was amplified by PCR using primer pairs 5′-GTA CCA CGG TGT CCC TCT CA-3′ and 5′-CCT GTG TCC TTC ACC TTC CCT A-3′. Amplification of mouse GLP-1R cDNA resulted in generation of an approximately 1.4-kb product spanning bases 79–1486 of the GLP-1R open reading frame (16). The human GLP-1R was amplified using the primer pairs 5′-TTC TGC AAC CGG ACC TTC GA-3′ and 5′-ATG AGT GTC AGC GTG GAC TTG-3′ resulting in an 890-bp product spanning bases 181-1070 (amino acids 60–357) of the GLP-1R open reading frame (11). Glyceraldehyde-3-phosphate-dehydrogenase cDNA amplification was performed as described (17).

After gel electrophoresis and transfer to membranes, blots were hybridized with a ^{32}P-labeled mouse (5′-GCTGTATCT-GAGCATAGGCT-3′) or human (5′ TGTATAGCACAGC-CGCCCAG 3′) GLP-1R oligonucleotide probe overnight. After washing, blots were exposed to a phosphorimaging cassette and visualized using Storm 860 Phosphor Screen and Image Quant (version 5.0) software (Molecular Dynamics, Sunnyvale, CA).

cAMP assays

Cells were grown in 24-well plates to approximately 70% confluence and serum starved for 3 h and then reincubated with DMEM lacking serum and treated with the indicated agents supplemented with 100 μM 3-isobutyl-1-methylxanthine for 10 min. cAMP was measured from dried aliquots of ethanol extracts using a RIA kit (Biomedical Technologies, Stoughton, MA).
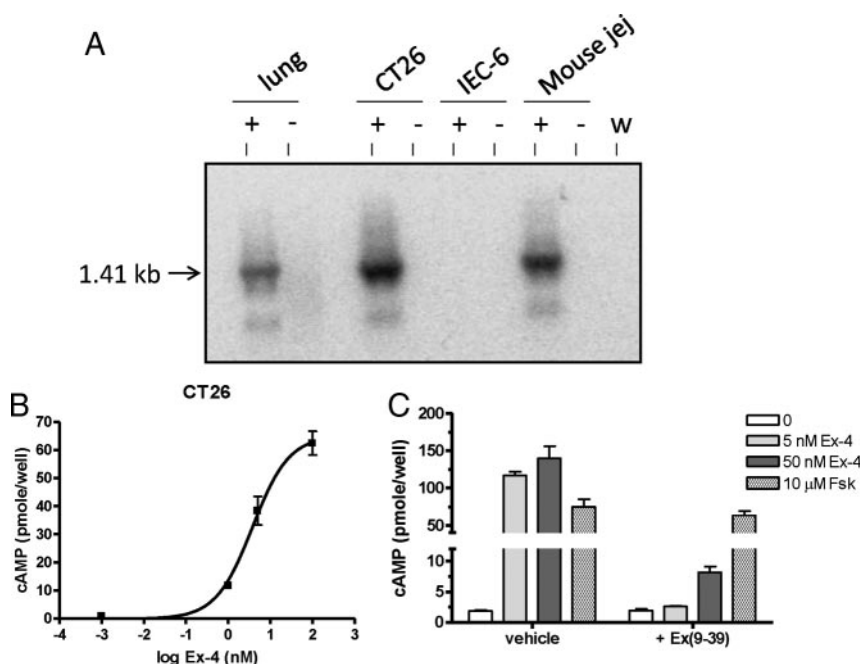
FIG. 1. The GLP-1R is expressed and functional in the CT26 mouse colon cancer cell line. A, RT-PCR using 5 μg total RNA from CT26 cells, the rat small intestinal cell line IEC-6, mouse lung, and jejunum (jej). Control reactions contained identical samples in which the reverse transcriptase (RT −) or RNA (w) was omitted. B, CT26 cells were serum starved for 3 h and incubated with Ex-4 for 10 min before analysis of cAMP content. C, CT26 cells were treated as in B with or without the GLP-1R antagonist Ex(9–39) 1 μM for 30 min and then treated with Ex-4 or 10 μM Fsk for 10 min. Data for B and C represent mean ± SE of three independent experiments performed in triplicate.

Proliferation and cell survival assays

Cells (20–30% confluent) were cultured in 24-well plates for 16 h in serum-free medium or medium containing 10% FBS. Fresh medium was added with or without 5 or 50 nM Ex-4, 10 μM Fsk, or 10% FBS and incubated at 37 C. Medium and treatments were replenished every 24 h. Viable cells were quantified by measuring the bioreduction of a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; tetrazolium salt at 490 nm using the CellTiter 96 AQ$_{ueous}$ cell proliferation assay kit (Promega, Madison, WI). Cell death/survival was assessed in cells maintained for 16 h in medium lacking serum before treatment with indomethacin (600 μM final) or irinotecan (60 μM) in the presence or absence of 5 or 50 nM Ex-4 or 10 μM Fsk for 48 h.

SDS-PAGE and Western blot analysis

Cell lysates were prepared and Western blotting carried out as described (17, 18). Antibodies to Akt phosphorylated on Ser 473, GSK3α/β phosphorylated on Ser 9/21, p44/42 MAPK phosphorylated on Thr 202 and Tyr 204, and caspase 3 were from Cell Signaling Technology (Beverly, MA) and used at 1:1000 dilutions. Poly(ADP-ribose) polymerase (PARP) was from BD Pharmingen (San Diego, CA) and used at a 1:2500 dilution. A primary antibody against heat-shock protein 90 (Transduction Laboratories, San Diego, CA) was used at a 1:2000 dilution. Densitometry was performed on blots exposed to Biomax MR film (Eastman Kodak, Rochester, NY) using a Hewlett-Packard ScanJet 3p scanner (Palo Alto, CA) and Scion Image software.

Soft agar assay

Agarose was suspended at 1.8% in water and autoclaved. A dilution was made with 2× RPMI culture medium and FBS (final concentration, 0.6% agarose, 10% FBS), and 2 ml was added to each well of a six-well plate. Approximately 2×10^4 cells/ml were mixed with 2× RPMI medium, FBS, and 1.8% agarose solution, and 1.5 ml was poured immediately into a six-well plate containing hardened bottom agar (final concentration, 0.3% agarose, 10% FBS, $\sim 1.5 \times 10^4$ cells) and incubated at 37 C and 5% CO_2 atmosphere. The following day, cells were fed with fresh growth medium (RPMI plus 10% FBS) containing 5 or 50 nM Ex-4, and medium was replaced every 2–3 d. Colonies were quantified after 21 d.

Animals and tumor implantation experiments

Experiments were carried out according to protocols approved by the Animal Care Committees of Mount Sinai Hospital. Male BALB/c mice (10–11 wk old) were from the Toronto Centre for Phenogenomics (Toronto, Ontario, Canada).

CT26 cells (1×10^6 cells/200 μl PBS) were injected sc into opposite flanks of BALB/c mice. Three days later, twice-daily ip injections with either 100 μl PBS solution (n = 5 mice) or 10 nmol/kg Ex-4 (n = 5 mice) were commenced for 2 wk. Mice were injected with 100 mg/kg 5-bromo-2′-deoxyuridine (BrdU) in PBS and euthanized by CO_2 inhalation 1 h later. Tissues were removed for RNA, protein, and histological analysis.

Histology and immunocytochemistry

Histology and immunohistochemistry was carried out as described (11, 19, 20). Immunostaining for BrdU employed mouse anti-BrdU (1:1000 dilution; Caltag Laboratories, Burlingame, CA) and terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) staining was performed using ApopTag peroxidase *in situ* apoptosis detection kit (S7100) [Chemicon (Millipore), Billerica, MA], according to the manufacturer's instructions. Slides were examined with a Leica microscope, scanned using an Aperio ScanScope instrument, and analyzed in ImageScope viewing software using the positive pixel algorithms.

Statistical analysis

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

Results

The CT26 colon cancer cell line expresses a functional GLP-1R

To ascertain whether human cancers express the GLP-1R, we searched the EST and serial analysis of gene ex-
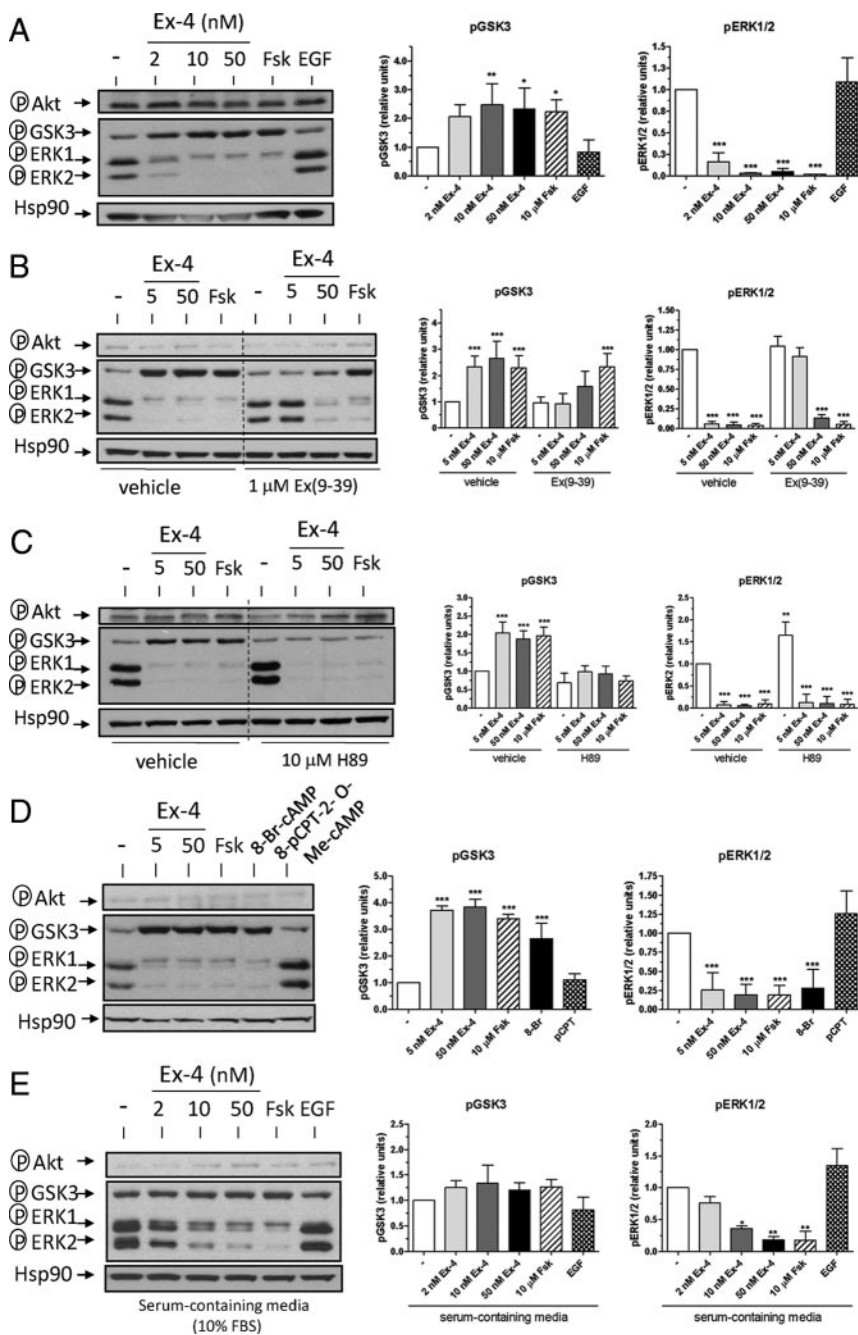
FIG. 2. Ex-4 inhibits GSK3 and ERK1/2 in CT26 cells. A–D, CT26 cells were serum starved overnight (−) and incubated for 15 min with Ex-4 (2–50 nM), 10 μM Fsk, or 25 ng/ml EGF (A); 5 or 50 nM Ex-4 or 10 μM Fsk with or without a 30-min pretreatment with 1 μM Ex(9–39) (B) or 10 μM H89 (C); or 5 or 50 nM Ex-4, 10 μM Fsk, 50 μM 8-Br-cAMP, or 50 μM 8-pCPT-2-O-Me-cAMP (D), as indicated. E, CT26 cells were maintained in serum-containing medium overnight (−) and incubated for 15 min with the indicated concentrations of Ex-4, 10 μM Fsk, or 25 ng/ml EGF. Equal amounts of whole-cell lysates were immunoblotted using antisera against phosphorylated ERK1/2 (Thr202/Tyr204), phosphorylated Akt (Ser 473), phosphorylated GSK3α/β (Ser 9/21), or heat-shock protein 90 (Hsp90). A–E, Representative blots from three independent experiments with similar results; *right panels*, quantification of blots are shown relative to control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (control *vs.* treatment). Data shown are the means ± SD.

colon cancer (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). RT-PCR was employed to screen RNA from human and mouse colon cancer cell lines. *Glp1r* mRNA transcripts were not detected in the adenocarcinoma cell lines DLD-1, SW480, CaCo2, or HT29, the carcinoma cell line T84, or the normal colon cell line 18Co (data not shown); however, CT26 mouse colon carcinoma cells did express the *Glp1r* (Fig. 1A).

The GLP-1R is a member of the class B family of G protein-coupled receptors (GPCR), which signal through G_s leading to activation of adenylate cyclase (21). To determine whether the GLP-1R expressed in CT26 cells was functional, we assessed cAMP production after stimulation with the GLP-1R agonist Ex-4. As shown in Fig. 1B, Ex-4 produced a dose-dependent increase in cAMP levels in CT26 cells (Fig. 1B). Furthermore, the GLP-1R antagonist Ex(9–39) inhibited cAMP production induced by Ex-4, but not Fsk, demonstrating that the actions of Ex-4 in CT26 cells were mediated through the canonical GLP-1R (Fig. 1C).

Ex-4 inhibits GSK3 and ERK1/2 in CT26 colon cancer cells in a GLP-1R-dependent manner

Consistent with CT26 cells carrying an activating K-ras mutation at codon 12 (G to A transition) (22), ERK1/2 phosphorylation/activation was detectable under basal (serum-starved) conditions (Fig. 2, A–D). Ex-4 treatment reduced ERK1/2 phosphorylation in a dose-dependent manner (Fig. 2A). Furthermore, Ex-4 administration resulted in phosphorylation and thereby inhibition of GSK3 in CT26 cells (Fig. 2A), with inhibition of both GSK3 and ERK1/2 persisting for over 90 min (data not shown). The ERK1/2 and GSK3 inactivation was mimicked by Fsk (an activator of adenylate cyclase) (Fig. 2, A–D). Furthermore, Ex(9–39) markedly diminished the effects of Ex-4 on ERK1/2 and
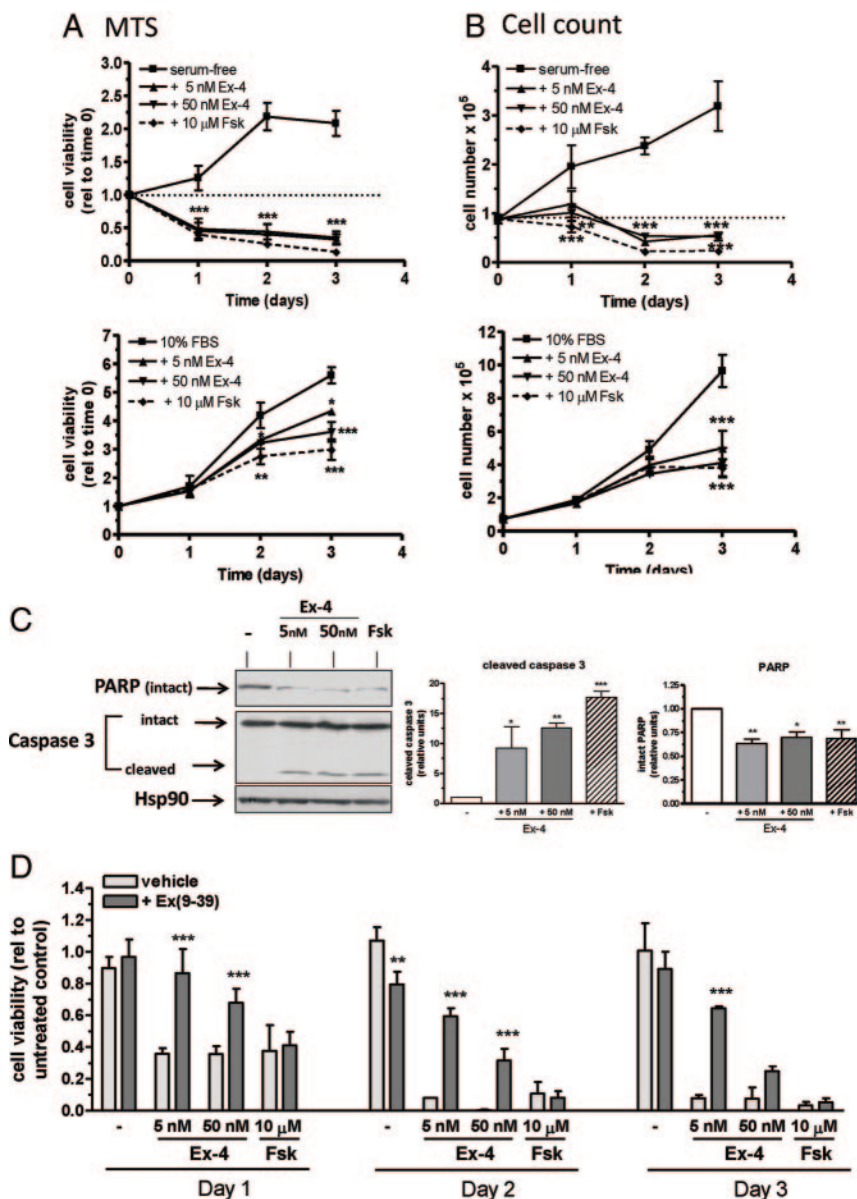
pression (SAGE) tag databases at NCBI and Cancer genome Anatomy project using the human *Glp1r* nucleotide sequence (Unigene access no. Hs.389103); several EST or SAGE tags were detected including brain, prostate, and

FIG. 3. Ex-4 decreases CT26 cell viability and inhibits cell proliferation. A and B, Cells maintained in serum-free medium (*upper panels*) or 10% FBS (*lower panels*) were treated with 5 or 50 nM Ex-4, or 10 μM Fsk. Cell viability was measured using a tetrazolium salt bioreduction assay (MTS assay) (A) or viable cells were counted by staining with trypan blue and counting using a hemocytometer (B). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (control *vs.* treatment). Data shown are the means ± SE from four (A) or three (B) independent experiments. Each MTS assay was performed in triplicate. C, Immunoblot analysis of cell lysates after a 24-h incubation period under serum-free conditions in the presence or absence of Ex-4 or 10 μM Fsk. Twenty-four hours later, floating and attached cells were lysed and Western blot analysis was carried out using antisera against caspase 3, PARP, or heat-shock protein 90 (Hsp90). Shown are representative blots from two independent experiments. *Right panels*, Quantification of blots relative to control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ [control (−) *vs.* treatment]. Data shown are the means ± SD. D, CT26 cells were grown for 3 d in serum-free medium (−) with or without Ex-4 or Fsk, in the presence or absence of 1 μM Ex(9–39). Media and treatments were changed daily, and cell viability was measured using an MTS assay. **, $P < 0.01$; ***, $P < 0.001$ [vehicle *vs.* Ex(9–39)]. Data shown are the means ± SE from three independent experiments, each performed in triplicate.

GSK3 phosphorylation (Fig. 2B). In contrast, neither Ex-4 nor Fsk activated Akt (Fig. 2, A–D), suggesting that Ex-4 does not activate the phosphatidylinositol 3-kinase pathway in CT26 cells. Ex-4 also inhibited ERK1/2 phosphorylation in the presence of serum (Fig. 2E). In contrast, neither Ex-4 nor Fsk modified GSK3 phosphorylation in the presence of serum (Fig. 2E).

Ex-4 inhibits GSK3 in a protein kinase A (PKA)-dependent manner, whereas ERK1/2 inhibition is independent of both PKA and Epac (exchange protein directly activated by cAMP)

We next explored mechanisms linking GLP-1R activation to inhibition of ERK1/2 and GSK3. The PKA inhibitor H89 prevented GSK3 inactivation by Ex-4 but had no effect on Ex-4-dependent reduction of ERK1/2 phosphorylation (Fig. 2C). Because cAMP activates both PKA and Epac (23), and activation of the Epac/ras-related protein 1 pathway can lead to the inactivation of ERK1/2 downstream of Ras (24), we assessed whether activation of Epac induced ERK1/2 inactivation. The cAMP analog (8-Br-cAMP) that activates both Epac and PKA inhibited both GSK3 and ERK1/2; however 8-*p*CPT-2-*O*-Me-cAMP, which selectively activates Epac, did not inactivate GSK3 or ERK1/2 (Fig. 2D), suggesting that control of GSK3 and ERK1/2 phosphorylation by Ex-4 does not involve Epac.

Ex-4 inhibits proliferation and induces apoptosis in CT26 cells

To determine whether GLP-1R activation modulates CT26 cell proliferation or survival, we quantified cell viability. CT26 cell numbers increased in the absence or presence of serum (Fig. 3, A and B), whereas Ex-4 and Fsk significantly reduced the number of CT26 cells (Fig. 3, A and B), suggesting that increased levels of cAMP reduce CT26 proliferation and/or survival. Moreover, both Ex-4 and Fsk increased levels of cleaved (active) caspase-3 and decreased levels of intact PARP, a downstream caspase-3 substrate (Fig. 3C). Furthermore, the reduction in cell viability induced by Ex-4, but not Fsk, was reversed or significantly attenuated by Ex(9–39) (Fig. 3D). Taken together, these results suggest that GLP-1R activation inhibits growth and survival pathways in CT26 cells.
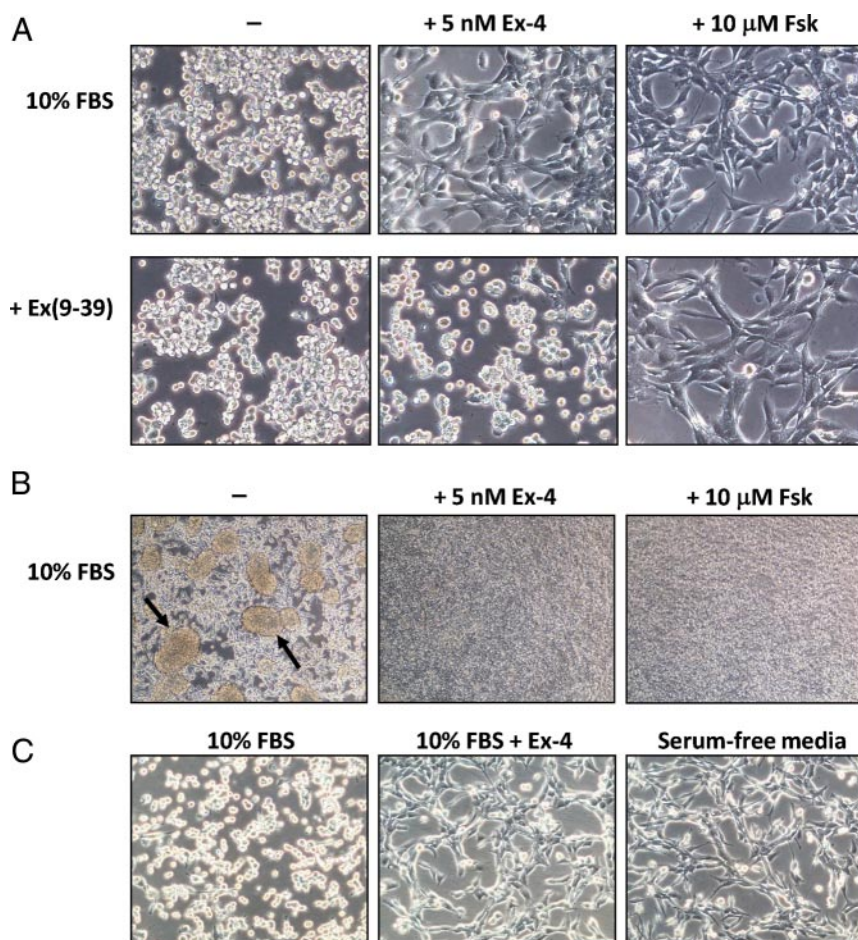
FIG. 4. The effect of Ex-4 on CT26 cell morphology is blocked by the GLP-1R antagonist Ex(9–39). A, CT26 cells were grown in serum-containing medium to 20–30% confluence and then pretreated with or without Ex(9–39) for 30 min before treatment with 5 nM Ex-4 or 10 μM Fsk for 48 h in the presence of serum. Magnification, $\times$200. B, Cells were grown in serum-containing medium in the presence or absence of 5 nM Ex-4 or 10 μM Fsk for 96 h. Magnification, $\times$200. C, CT26 cells grown in serum-free medium or medium containing 10% FBS plus Ex-4 exhibit similar morphology. CT26 cells were maintained in serum-free medium or medium plus 10% FBS in the presence or absence of 50 nM Ex-4 for 48 h. Magnification, $\times$200.

Ex-4 induces morphological changes in CT26 colon cancer cells

CT26 cells grown in the presence of serum grew in clusters of small rounded cells (Fig. 4A, *upper left panel*). However, in the presence of Ex-4 or Fsk, the cells became less round, flattened, and occupied more surface area (Fig. 4A, *top panels*), similar to the morphology observed when cells were cultured under serum-free conditions (Fig. 4C). Furthermore, CT26 cells formed rounded spheres and foci when cultured past confluence in serum-containing medium (Fig. 4B, *left panel*), whereas in the presence of serum and Ex-4 or Fsk, these cells remained as a monolayer rather than forming foci (Fig. 4B, *middle and right panels*). Furthermore, the GLP-1R antagonist Ex(9–39) reversed the morphological effects of Ex-4 but not Fsk (Fig. 4A, *lower panels*). These results suggest that GLP-1R activation restores contact growth inhibition of CT26 cells.

Ex-4 inhibits colony formation in soft agar

We next examined whether Ex-4 modified the ability of CT26 cells to form colonies in soft agar, a measurement of anchorage-independent growth. Approximately 350 of 1.5×10^4 cells initially embedded in agar formed colonies. However, colonies formed in the presence of Ex-4 appeared smaller and significantly less numerous than those cultured with serum alone (Fig. 5, A and B), suggesting that GLP-1R activation impedes anchorage-independent growth of CT26 cells.

Ex-4 enhances irinotecan-induced apoptosis in CT26 cells

To determine whether GLP-1R activation modulates drug-induced cell death, CT26 cells were treated with or without indomethacin (a nonsteroidal antiinflammatory drug that inhibits cyclooxygenase-1 and -2) or irinotecan (a topoisomerase I inhibitor used to treat colon cancer) in the presence or absence of Ex-4. In contrast to prosurvival effects of GLP-1R signaling in rat and human β-cells and cell lines (25, 26), Ex-4 did not attenuate cytotoxicity induced by indomethacin (Fig. 6A). Furthermore, Ex-4 or Fsk alone decreased cell viability under serum-free conditions (Fig. 6A, control), and both agents augmented the cytotoxic effects of irinotecan (Fig. 6A). Consistent with these findings, both Ex-4 and Fsk increased the levels of cleaved (active) caspase-3, robustly decreased the abundance of intact PARP, and increased levels of cleaved PARP relative to irinotecan alone (Fig. 6B).

Ex-4 does not modulate the propagation of CT26 cells *in vivo*

To ascertain whether GLP-1R activation regulates growth of CT26 cells *in vivo*, we implanted CT26 cells sc into both flanks of syngeneic BALB/c mice followed by twice-daily injections with either PBS or a bioactive dose of Ex-4 previously shown not to cause sustained weight loss in mice (11, 19). Unexpectedly, tumor-bearing BALB/c mice treated with Ex-4, but not PBS, started to lose weight and became lethargic after 10 d of treatment and shortly thereafter exhibited signs of dehydration, soft
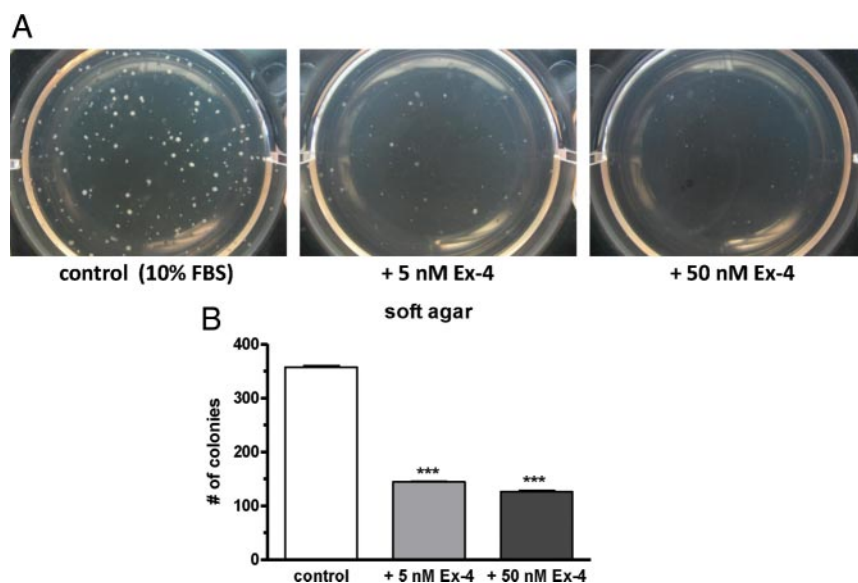
FIG. 5. Ex-4 decreases CT26 colony formation in soft agar. CT26 cells ($\sim 1.5 \times 10^4$) were suspended in agar and cultured in growth medium (10% FBS) with or without Ex-4 for 3 wk. Media and treatments were changed every 2–3 d. A, Representative pictures of soft agar plates demonstrating reduction in CT26 colony formation in the presence of Ex-4; B, quantification of CT26 colonies formed in the presence or absence of Ex-4. ***, $P < 0.001$ (control vs. treatment). Data shown are the means ± SE from two independent experiments, each performed in duplicate.

stools, and further weight loss resulting in termination of the experiment (Fig. 7A). No significant difference in tumor weight was observed after 2 wk of Ex-4 vs. PBS treatment (Fig. 7B). However, extensive areas of apoptosis were observed in tumors from Ex-4-treated mice, exemplified by positive TUNEL staining, chromatin condensation (including crescent or ring-like structures), and nuclear fragmentation (Fig. 7, C and D, and Supplemental Fig. 1), along with a corresponding decrease in the proliferative area of tumors from Ex-4-treated mice (Fig. 7E). Thus, although the tumor weights were similar, Ex-4 treatment affected the survival/maintenance of cells within these tumors.

Discussion

The use of GLP-1R agonists such as exenatide and liraglutide as antidiabetic agents has fostered considerable interest in the long-term safety of these peptides (27). Sustained GLP-1R activation expands β-cell mass in normal and diabetic rodents via stimulation of cell proliferation and inhibition of apoptosis (28); however, insulinomas have not been reported in the context of long-term GLP-1R activation. The GLP-1R is also expressed on rodent thyroid C cells, and genetic disruption of the *Glp1r* leads to reduced calcitonin expression and osteoporosis in mice (29). Conversely, acute GLP-1R activation rapidly increases calcitonin gene expression and calcitonin secre-

tion in rodents (30), and continuous GLP-1R stimulation leads to C cell hyperplasia and, in some instances, medullary thyroid cancer to a greater extent in rats compared with mice (30). However, monkey thyroid C cells do not exhibit a proliferative response to GLP-1R activation (30), and calcitonin levels do not rise after therapy with GLP-1R agonists in human subjects with T2DM (30, 31).

Although data demonstrating GLP-1 promotes cell proliferation in the gastrointestinal tract are limited, continuous infusion of GLP-1 produces hyperplasia in the rat small bowel (32). Moreover, Ex-4 promotes robust small bowel growth in rats; however, no proliferative effects were noted in the rat colon (10). Although the structurally related proglucagon-derived peptide GLP-2 also promotes cell proliferation in the bowel, GLP-2 exerts its actions through a structurally different receptor, and GLP-1R agonists such as Ex-4 exhibit very weak affinity for the GLP-2 receptor (10, 33). Furthermore, the *Glp1r* is expressed in the gastrointestinal tract (34). Hence, the available evidence supports a distinct role for GLP-1 in the promotion of intestinal growth through the known GLP-1R.

Although GLP-1 clearly promotes cell proliferation and inhibits cell death in nonneoplastic tissues (5, 26, 28, 35), there is little information as to whether and how GLP-1R activation regulates cell growth and survival pathways in cancer cells. We previously studied the consequences of activated GLP-1R signaling in human pancreatic cancer cells that expressed the endogenous GLP-1R. Although GLP-1R activation increased levels of cAMP and inhibited ERK1/2 phosphorylation in some cell types, no effect of Ex-4 on cell proliferation or cell survival was noted (11). There is even less information about the role of GSK3 as a downstream target for GLP-1 action. Here we show that the GLP-1R agonist Ex-4 inhibits both ERK1/2 and GSK3 in the CT26 colon cancer cells. Surprisingly, unlike findings observed in β-cells, cardiomyocytes, and neurons (26, 36, 37), where GLP-1, acting through cAMP, enhances cell proliferation and/or survival, Ex-4 or Fsk induced apoptosis under serum-free conditions in CT26 cells and inhibited cell proliferation in the presence of serum. Moreover, Ex-4 decreased CT26 colony formation in soft agar, augmented apoptosis induced by the chemotherapeutic agent irinotecan, and induced apoptosis within tumors *in vivo*.
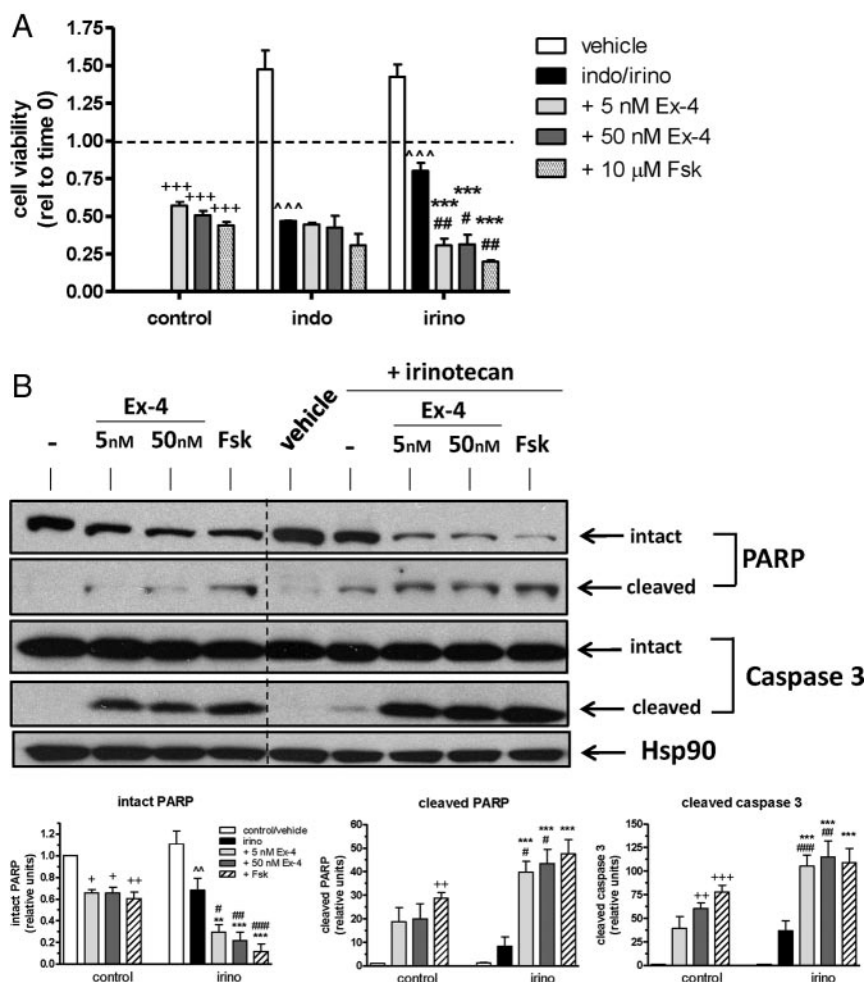
FIG. 6. Ex-4 enhances irinotecan-induced apoptosis in CT26 cells. A, Cells were maintained for 16 h in medium lacking serum before treatment with indomethacin (indo) (600 μM final) or irinotecan (irino) (60 μM) in the presence or absence of 5 or 50 nM Ex-4 or 10 μM Fsk for 24 h; cell viability was measured using a tetrazolium salt bioreduction assay (MTS assay) and is shown relative to cell viability before treatment (time 0). ^^^, $P < 0.001$ (vehicle (DMSO) *vs.* drug); ***, $P < 0.001$ (irinotecan alone *vs.* irinotecan plus Ex-4 or Fsk); #, $P < 0.05$; ##, $P < 0.01$ (Ex-4 or Fsk *vs.* irinotecan plus Ex-4 or Fsk); +++, $P < 0.001$ (control *vs.* Ex-4 or Fsk). Data shown are the means ± SE from three independent experiments, each performed in triplicate. B, Immunoblot analysis of cell lysates after a 24-h incubation period under serum-free conditions in the presence or absence of irinotecan and/or Ex-4 or 10 μM Fsk. Twenty-four hours after the initial treatment, cell lysates were immunoblotted against caspase 3, PARP, or heat-shock protein 90 (Hsp90) as a loading control. Shown are representative blots from two independent experiments. *Lower panels*, Quantification of blots are shown relative to control. ^^, $P < 0.01$ (vehicle *vs.* drug); **, $P < 0.01$; ***, $P < 0.001$ (irinotecan alone *vs.* irinotecan plus Ex-4 or Fsk); #, $P < 0.05$; ##, $P < 0.01$; ##, $P < 0.001$ (Ex-4 or Fsk *vs.* irinotecan plus Ex-4 or Fsk); +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$ (control *vs.* Ex-4 or Fsk). Data shown are the means ± SD.

Because degradation of native GLP-1 yields one or more bioactive metabolites that may also regulate cell survival (38), we purposefully employed the degradation-resistant GLP-1R agonist Ex-4 in our studies. Importantly, the actions of Ex-4 to stimulate cAMP formation, inhibit GSK3 and ERK1/2, and reduce cell viability were all attenuated by the GLP-1R antagonist Ex(9–39). Hence, taken together with the demonstration that CT26 cells express the endogenous GLP-1R, the available evidence strongly suggests that Ex-4 reduces cell growth and in-

creases apoptosis in CT26 cells through the canonical GLP-1R.

Enteroendocrine-derived peptide hormones such as neurotensin acting through GPCRs have previously been shown to stimulate colon cancer growth or survival (39). Administration of human [Gly^2] GLP-2 to mice administered azoxymethane or dimethylhydrazine results in more intestinal polyps (40), increased aberrant crypt foci, and development of colonic adenocarcinomas (41). Similarly, progastrin promotes colon cancer cell growth via the *CCK2R*, and reducing *CCK2R* expression attenuates the development of experimental colon cancer in mice (42). The GPCR ligand prostaglandin E2 also increases colon cancer cell growth in part through a β-catenin-dependent pathway that requires $G_{\alpha s}$ but not PKA activation (31). Hence, the current findings that Ex-4 reduced colon cancer cell growth and induced apoptosis were unexpected because the majority of gut peptides analyzed in similar models act as promoters of cancer cell growth.

The precise mechanisms through which GLP-1R activation alters the morphology, growth and survival characteristics of CT26 cells remain unclear but may involve down-regulation of ERK1/2 activity. The ERK1/2 MAPK signaling pathway is frequently activated in human colon cancer cells and represents a therapeutic target for inhibition of cell growth (43). Moreover, constitutive expression of either mitogen-activated protein kinase kinase 1 or mitogen-activated protein kinase kinase 2 in intestinal epithelial cells leading to ERK1/2 activation produces profound morphological alterations, enhances anchorage-independent cell growth, and leads to neoplastic transformation (44). Conversely, inhibition of ERK1/2 often promotes apoptosis through dysregulation of cell death-regulating proteins that may sustain cancer cell survival (45).

Although our findings demonstrate that GLP-1 inhibits growth and induces apoptosis in a murine tumor that expresses the GLP-1R, we cannot be certain whether these find-
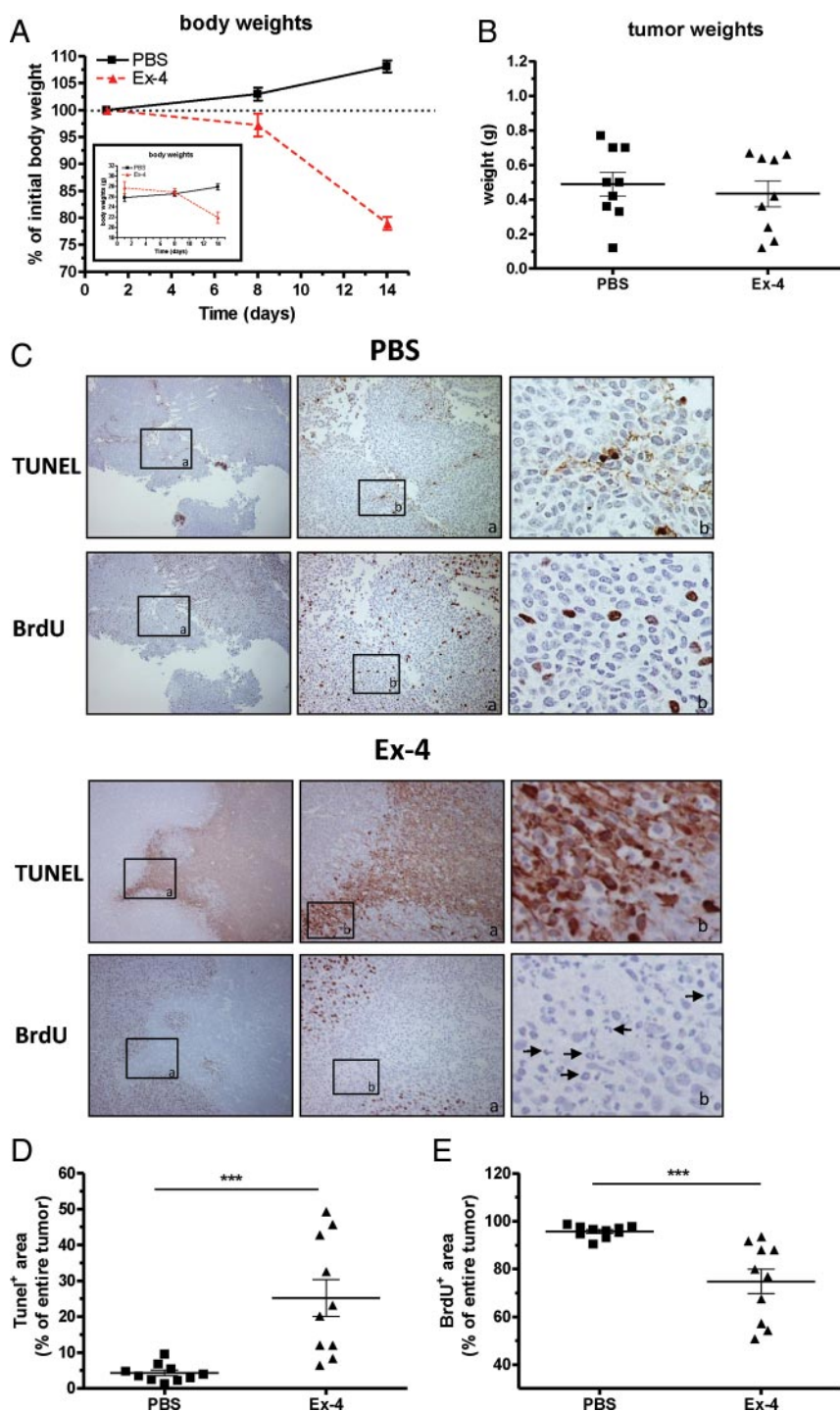
FIG. 7. Ex-4 induces apoptosis in CT26 tumors implanted into BALB/c mice. A, Body weight of BALB/c mice treated with PBS or Ex-4; tumor implantation was carried out at d −3. Data shown are the means ± SD from PBS- or Ex-4-treated mice (n = 5 mice per group). B, Tumor weights at the end of the 2-wk treatment period with PBS or Ex-4, as indicated. Data shown are the weights of each individual tumor, and the mean tumor weight is indicated with a *horizontal line* ± SE. All mice developed tumors in both flanks. C, TUNEL (*upper panels*) and BrdU (*lower panels*) staining of tumors from BALB/c mice treated as indicated. Magnification, ×50 (*left panel*), ×200 (*middle panel*, *inset a*), or ×5 (*right panel*, *inset b*). Chromatin condensation (including crescent or ring-like structures) and nuclear fragmentation are indicated by *arrowheads*. D and E, Quantification of TUNEL-positive (D) and BrdU-positive (E) area of tumors from BALB/c mice treated with PBS or Ex-4 (10 nmol/kg twice daily) as indicated. Data shown are TUNEL-positive or BrdU-positive area (as a percentage of the entire tumor area) of each individual tumor with the mean indicated by a *horizontal line* ± SE, for tumors from PBS- or Ex-4-treated mice (n = 10 per group). ***, $P <$ 0.001 PBS *vs.* Ex-4.

ings will apply to other cancers or cell lines that express a functional GLP-1R. CT26 cells contain an activating Ras mutation and secrete TGFβ, which could contribute to how these cells respond to GLP-1R activation (22). Glucose-dependent insulinotropic peptide, a peptide hormone structurally and functionally related to GLP-1, also increases intracellular cAMP yet stimulated proliferation of the mouse MC-26 colorectal cancer cell line (46). By contrast, intracellular cAMP levels had no effect on the proliferation or survival of colon cancer cell lines stably expressing the GLP-2R (20), whereas GLP-1R agonists reduce the growth and survival of breast cancer cells via a cAMP-dependent mechanism that does not appear to require the known classical GLP-1R (7). Thus, the effects of GPCR and specifically GLP-1R activation on the growth and survival of malignant cells likely depends on the cell type, existing perturbations in signal transduction pathways and secreted factors, and cellular context.

Our data demonstrating reduction of growth and promotion of CT26 cell apoptosis *in vitro*, together with enhanced apoptosis in implanted tumor cells exposed to Ex-4 *in vivo*, clearly demonstrate a cytostatic proapoptotic antitumor effect pursuant to activation of the known GLP-1R. However, these findings were obtained using a single murine colon cancer cell line and do not provide information on whether the use of GLP-1R agonists for the therapy of diabetes might influence the incidence or behavior of human colon cancer in subjects treated with GLP-1R agonists. However, because we detected *Glp1r* mRNA transcripts (EST) in several human cancers, including colon cancer (Supplemental Table 1), it seems prudent to understand the direct and indirect GLP-1R-dependent mechanisms that regulate cancer cell growth in diverse models. Because colorectal cancer is more common in diabetic subjects, and the use of exenatide and liraglutide is slowly gaining popularity, future mechanistic and epidemiological investiga-

tions assessing whether specific antidiabetic therapies differentially impact the biological behavior or risk of colon cancer development are clearly of interest.

Acknowledgments

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Supplemental Table 1:

Cancer library	Unigene EST	Cancer Genome Anatomy Project EST	SAGE tags
brain	-	0/201860	3/4 997 811
cerebellum	-	0/2	1/1 495 938
skin	0/125579	0/125591	4/34522313
prostate	0/103891	0/107302	1/491 794
gastrointestinal tract	0/119 870	0/12 814	-
colon	0/114 992	0/153 788	1/643586

Supplemental Figure 1:

PBS	Ex-4