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Original article

Leptin induces cell invasion and the upregulation of matrilysin in human colon cancer cells

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ABSTRACT

Objective: To investigate the effects of leptin on matrix metalloproteinase-7 (MMP-7) expression and cellular invasion of human colon cancer cells.
Methods: Human colon cancer cells were stimulated with leptin at various concentrations (0 nM, 1.2 nM, 6 nM, 25 nM, and 100 nM), and the stimulatory effects of leptin on the invasion of human colon cancer cells were tested by the transwell invasion assay. The effects of leptin on signaling cascade in colon cancer cells were examined by Western blot assay and zymogram analysis.
Results: This study demonstrated that leptin dose-dependently induced MMP-7 expression and cellular invasion of human colon cancer HT-29 cells. Treatment of MMP-7 small interfering RNA (siRNA) significantly blocked leptin-mediated cellular invasion. Wortmannin [a phosphoinositide-3-kinase (PI3K) specific inhibitor], PD098059 (a Mitogen-activated protein kinases (MAPK)/ extracellular signal-regulated kinase (ERK) kinase (MEK) specific inhibitor), or GM-6001 (a specific inhibitor of MMPs) could suppress leptin-mediated cellular invasion markedly. These aver leptin inducing MMP-7 expression and/or cellular invasion, partly by modulation of phosphoinositide-3-kinase (PI3K)/ AKT and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway in human colon cancer HT-29 cells.
Conclusion: Results suggest a novel mechanism of leptin involved in MMP-7 expression and cellular invasion in human colon cancer HT-29 cells.
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1. Introduction

Colorectal cancer is one of the leading causes of cancer death in many countries, including North America. In the United States alone, thousands of deaths are attributed to this cancer annually [1]. Epidemiological studies identify obesity as a risk factor in certain types of cancer, including colorectal [2–7]. Prior studies link increased blood leptin with malignant progression of colon cancer, suggesting that a greater secretion of adipocyte-derived growth factors and hormones contribute to

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cellular transformation and tumorigenesis [8]. Leptin, a biologically active polypeptide secreted from mature adipocytes and other epithelial cells, circulates as a 16 kDa protein partially bound to plasma proteins and exerts influence via its specific receptors by endocrine, paracrine, or autocrine processes [9,10]. In a physiological status, leptin controls the balance of normal body weight by the regulation of food intake and other biological activities [11]. It has been identified as a mitogen associated with gastrointestinal malignancy [12–14]. In a pathophysiological status, leptin induces tumor angiogenesis and malignant progression of several cancers [12,15–17]. Overexpression of leptin and its receptor (ObR) has been elucidated in human colon cancer cells [18,19]. With blood levels of leptin elevated in obese patients and excess body weight strongly correlated with a risk of colon cancer, studies address the key role of leptin in its etiology [20,21].

Some *in vitro* studies demonstrated that leptin stimulates proliferation and migration of human colon cancer cells [12,14], and activates phosphoinositide-3-kinase (PI3K) and Src kinase pathways in metastatic colon cancer LS174T and HM7 cells [22]. Invasion of such cells into surrounding stroma occurs via the activation of matrix metalloproteinases (MMPs) that degrade extracellular matrix and create a microenvironment for tumor angiogenesis, growth, and metastasis [23–26]. Increased expression of MMPs is associated with tumor invasion and metastasis [28,29]. The MMP gene family divides into subgroups: collagenase, stromelysin, gelatinase, membrane-type MMPs, and other MMPs. Unlike MMP-2, MMP-3, MMP-9, and MMP-11 (produced by stromal cells) [30,31], MMP-7 is expressed in colorectal carcinoma [32–34]. Expression of MMP-2 and MMP-9 is frequently observed in malignant tumors [35], and overexpression of MMP-7 is proven for colorectal tumorigenicity [36]. High MMP-7 expression correlates with a low survival rate of colon cancer cases [37,38]; clinical studies cite MMP-7 (matrilysin) as a key malignant biomarker in human colon cancer. Promoter regions of inducible MMP-2, MMP-7, MMP-9, etc., contain regulatory elements and are tightly modulated in mammalian cells by nuclear transcription factors. Gene expression is regulated by extracellular growth factor, proinflammatory cytokines, etc. [39]. Extracellular stimuli activating Wnt pathways and downstream signal molecules induce nuclear accumulation of β-catenin transcription factor and regulate expression of MMP-7 [40]. Prior studies show a strong linkage between β-catenin and MMP-7 overexpression in several types of cancer [41]. Although earlier studies examined roles of leptin in cell proliferation and migration, the major MMP required to degrade surrounding stroma and cellular invasion in human colon cancer has not been demonstrated yet. We assessed the effects of leptin on MMP-7 expression and cellular invasion of human colon cancer HT-29 cells.

2. Materials and methods

2.1. Reagents and antibodies

Anti-MMP-7 antibodies were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA); McCoy's medium and Calcein AM from Invitrogen Inc. (Carlsbad, CA, USA); anti-β-actin antibody, AG 490 [a janus kinase (JAK) inhibitor], SP600125 [a c-Jun N-terminal kinase (JNK) inhibitor], PD098059 (a MEK inhibitor), wortmannin (a PI3K inhibitor), SB 203580 (a p38 inhibitor), and Ipegal CA-630 from Sigma–Aldrich (St. Louis, MO, USA). GM-6001 (a broad-spectrum inhibitor of MMPs) was purchased from Calbiochem Merck Millipore (Concord, MA, USA); human leptin recombinant protein from R&D Systems, Inc. (Minneapolis, MN, USA); human colon cancer HT-29 cell line from American Type Culture Collection (Walkersville, MD, USA); and an NE-PER Nuclear and Cytoplasmic Extract reagent Kit from Pierce Biotechnology (Lackford, IL, USA).

2.2. Cell culture

Briefly, human colon cancer HT-29 cells were cultured in a 37 °C humidified incubator with 5% CO_2 and grown to confluency with fetal bovine serum (FBS) supplemented McCoy's medium. McCoy's medium was supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and 1.5 g/L sodium bicarbonate. Cells used in different experiments have similar passage numbers.

2.3. Preparation of cell lysates

Human colon cancer HT-29 cells were cultured in McCoy's medium in the presence or absence of leptin at diverse concentrations. After the experiment, cells were washed with phosphate buffered saline (PBS) twice and lysed in ice-cold lysis buffer. Lysis buffer contains 1X PBS, 1% Ipegal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) with 100 μM of phenylmethylsulfonyl fluoride (PMSF), aprotinin and specific phosphatase inhibitor, sodium orthovanadate. Lysates were sonicated and centrifuged at 10,000 × g for 15 minutes at 4 °C to remove cell debris, supernatants labeled whole cell lysates. Nuclear and cytoplasm fractions were prepared with an NE-PER Nuclear and Cytoplasmic Extract reagent Kit (Pierce Biotechnology) containing protease inhibitors and phosphatase inhibitors. Cross contamination between nuclear and cytoplasm fractions were barely found (data not shown).

2.4. Western blot analysis

Cellular proteins were fractionated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and then blotted with anti-MMP-7 monoclonal antibody, as per the manufacturer's instructions. Blots were stripped and reprobed with β-actin antibody as a loading control.

2.5. Assessment of cellular invasion

Invasion of tumor cells was analyzed in transwell Boyden chambers with a polyvinylpyrrolidone filter of 8-μm pore size. Each filter was coated with 50 μL of a 1:5 diluted matrigel in cold McCoy's 5A medium to form a thin continuous film on the top of the filter. HT-29 cells pre-stained with Calcein AM (Invitrogen Inc.) and stimulated with leptin were added to each triplicate well in McCoy's 5A medium (5000 cells/well). Following 18 hours of incubation, cells in 10 randomly selective fields were counted by an Olympus IX-71 Inverted Fluorescence Microscope (Olympus, Tokyo, Japan); those invading the lower side of the filter were measured as the invasion index.

2.6. Small interfering RNAs

HT-29 cells (6×10^4 cells in 12-well plates or 1.5×10^5 cells in 6-well plates) were transfected with a small interfering RNA (siRNA) oligonucleotide (Dharmacon Inc., Lafayette, CO, USA) designed to interrupt expression of the human matrilysin gene. Scrambled siGENOME non-targeting siRNA served as the negative control.

2.7. Casein zymography and gelatin zymography

Casein zymography was performed on both the control and treatment groups to measure MMP-7 activation, and gelatin zymography was performed on both groups to rate MMP-2 and MMP-9 activation. Gel was co-polymerized with either 0.1% casein or 0.1% gelatin. For each sample, an equal amount of protein was loaded. Electrophoresis used mini gel slab apparatus Mini Protean 2 (Bio-Rad, Hercules, CA, USA) at a constant 150 V, until the dye reached the bottom of the gel. After electrophoresis, the gel was washed in buffer [2.5% Triton X-100 in 50 mM Tris-HCl (pH 7.5)] for 1 hour in an orbital shaker and zymograms were incubated for 24 hours at 37 °C in buffer [0.15 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 in 50 mM Tris-HCl (pH 7.5)], stained with Coomassie blue, then destained with 7% methanol and 5% acetic acid. Areas of enzymatic activity appeared as clear bands over the dark background.

2.8. Statistical analysis

Biostatistic methodology differentiated cellular invasion among colon cancer cells. In brief, statistical analysis of the invasive capacity among triplicate sets of experimental conditions used SPSS (SPSS Inc., Chicago, IL, USA). Confirmation of statistically significant different invasion indices requires rejection of the null hypothesis: i.e., no difference between mean invasion indices obtained from replicate sets at the $p = 0.05$ level with the one-way analysis of variance model. *Post hoc* tests ascertained differences among groups.

3. Results

3.1. Dose-dependently induced invasion by HT-29 cells

Leptin has emerged as a chief risk factor for many types of cancer. We examined whether leptin could induce cellular invasion. Fig. 1A depicts leptin significantly inducing cellular invasion of human colon cancer HT-29 cells in a dose-dependent manner. In comparison to unstimulated cells, leptin (at concentrations of 1.2 nM, 6 nM, 25 nM, and 100 nM) significantly induced cellular invasion up to 4.2 fold (Fig. 1B). This suggests that leptin plays a prominent role in cellular invasion of human colon cancer HT-29 cells.

3.2. Dose-dependently induced MMP-7 expression in HT-29 cells

To probe the molecular actions of leptin, we measured protein expression levels of MMPs in human colon cancer HT-29 cells. Fig. 2A shows zymogram analysis of leptin inducing

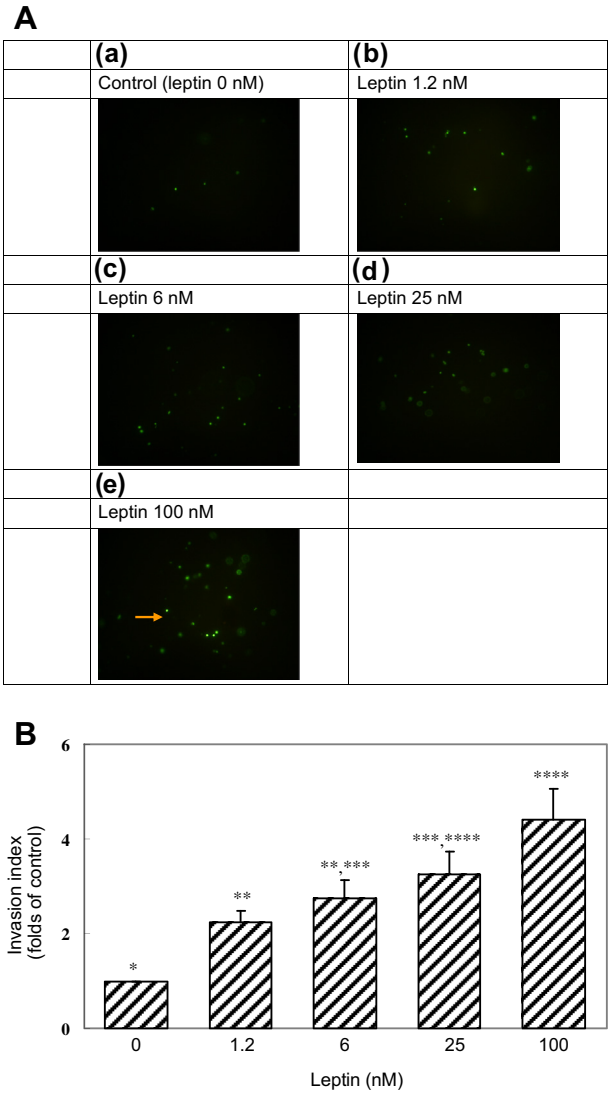

Fig. 1 – Leptin dose-dependently induced cellular invasion of human colon cancer HT-29 cells. Invasion of tumor cells was analyzed in transwell Boyden chambers with a polyvinylpyrrolidone filter of 8-μm pore size. Each filter was coated with 50 μL of 1:5 diluted matrigel in cold McCoy's medium, to form a thin continuous layer on top of the filter. Confluent human colon cancer cells were cultured in McCoy's medium with 10% fetal bovine serum at 37°C. After washing out the media, colon cancer cells were trypsinized, prestained with Calcein AM, and transferred to matrigel coated transwell Boyden chambers. Human colon cancer cells (5000 cells/well) were added to each triplicate well in McCoy's medium containing variant concentrations of leptin (0 nM, 1.2 nM, 6 nM, 25 nM, and 100 nM). After incubation for 18 hours, cells were counted as described above; the number of cells invading the lower side of the filter was measured as the invasive activity. (A) represents the microphotograph of invasive colon cancer cells; (B) represents the level of invasion index. Asterisks represent statistically significant differences, $p < 0.05$. Data shown represent three independent experiments.

expression of MMP-7 strongly, expression of MMP-2 slightly, but expression of MMP-9 barely (data not shown). Western blot also demonstrated leptin inducing expression of MMP-7 in a dose-dependent manner (Fig. 2B). Results suggest that leptin definitely induces MMP-7 expression and activation in human colon cancer HT-29 cells.

3.3. *Treatment of MMP-7 siRNA inhibited leptin-mediated cellular invasion of human colon cancer HT-29 cells*

Because leptin induces cellular invasion and MMP-7 expression in human colon cancer HT-29 cells, it is plausible that MMP-7 plays a vital role in leptin-mediated cellular invasion of such cells. To evaluate the role of MMP-7 in leptin-mediated invasion of human colon cancer HT-29 cells, we used a specific siRNA oligonucleotide designed to interrupt MMP-7 expression. Fig. 3 plots the treatment of specific siRNA against the MMP-7 gene significantly suppressing cellular invasion, even in the presence of leptin. This suggests inducible expression of the MMP-7 molecule modulating cellular invasion and hence acting as a chief therapeutic target in human colon cancer.

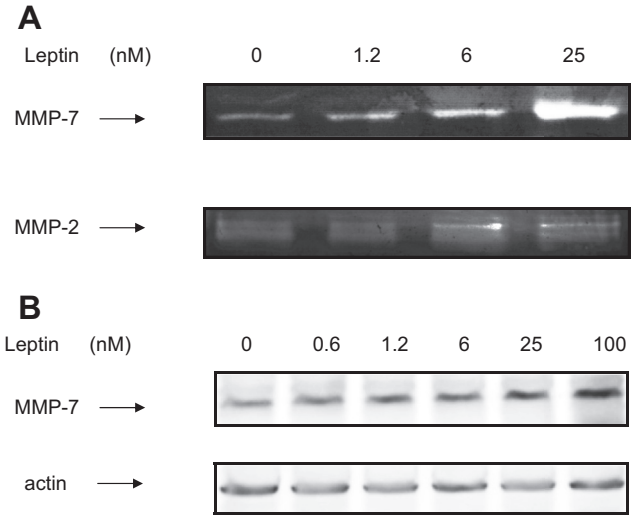

Fig. 2 – Leptin dose-dependently induced expression of matrix metalloproteinase-7 (MMP-7) in human colon cancer HT-29 cells. Post-confluent colon cancer cells cultured on 24-well plates were incubated in McCoy's medium with 10% fetal bovine serum (FBS) at 37°C. After washing out the media, cells were incubated in serum-free (conditioned medium) McCoy's medium in the presence of different leptin concentrations (0 nM, 1.2 nM, 6 nM, and 25 nM) at 37°C for 24 hours. Conditioned medium was collected and loaded into casein- or gelatin-containing zymogram gel stained with Coomassie blue stains (see Materials and methods). The levels of detection represent zymogram expression of different MMPs in human colon cancer cells; MMP-7 and MMP-2 noted with arrow (A). Human colon cancer cells, cultured in McCoy's medium with 10% FBS in a tissue culture dish, were lifted off by trypsinization, pelleted by centrifuge, and resuspended in the same medium. After washing out the media, cells were incubated in McCoy's medium with 10% FBS in a tissue culture dish with variant concentrations of leptin (0 nM, 0.6 nM, 1.2 nM, 6 nM, 25 nM, and 100 nM) for 24 hours. Total cell lysates were blotted with anti-MMP-7 antibody (see Materials and methods). Levels of detection in cell lysates represent the amount of MMP-7 in human colon cancer cells. Blots were stripped and reprobed with anti-actin antibody as the loading control; immunoreactive bands noted with an arrow (B).

3.4. *PI3K/Akt and MAPK/ERK signaling pathway played key roles in leptin-mediated invasion of human colon cancer HT-29 cells*

To pinpoint the molecular actions of leptin, we treated human colon cancer HT-29 cells with specific inhibitors under stimulation by leptin. Fig. 4 illustrates that wortmannin (a specific

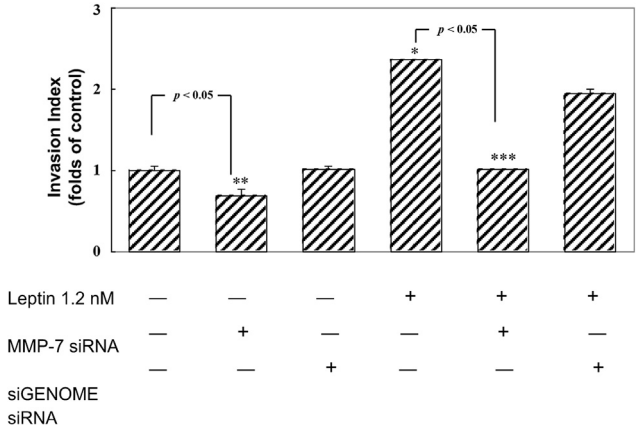

Fig. 3 – Treatment of matrix metalloproteinase-7 (MMP-7) small interfering RNA (siRNA) inhibited leptin-mediated invasion of human colon cancer HT-29 cells. Post-confluent colon cancer cells cultured on 12-well or 6-well plates were cultured in McCoy's medium with 10% fetal bovine serum (FBS) at 37°C. After washing out the media, HT-29 cells (6×10^4 cells in 12-well plates) were transfected with as siRNA oligonucleotide designed to interrupt the expression of the human matrilysin gene. Cells were incubated in serum-free (conditioned medium) McCoy's medium for 24 hours; scrambled siGENOME non-targeting siRNA served as the negative control. Invasion of tumor cells was analyzed in transwell Boyden chambers with polyvinylpyrrolidone filter of 8-μm pore size. Each filter was coated with 50 μL of a 1:5 diluted matrigel in cold McCoy's medium to form a thin continuous layer on top of the filter. Human colon cancer cells transfected with siRNA were cultured in McCoy's medium at 37°C. After washing out the media, cells were trypsinized, prestained with Calcein AM, then transferred to matrigel coated transwell Boyden chambers. Human colon cancer cells (5000 cells/well) were added to each of triplicate wells in serum-free McCoy's medium in the presence or absence of 1.2 nM leptin for 18 hours. * represents statistical difference compared to controls without treatment in the inter-group (leptin vs. untreated control), $p < 0.05$. ** or * represent the statistical difference compared to the control set in the intra-group (MMP-7 siRNA vs. respective control), $p < 0.05$.**

inhibitor of PI3K), PD098059 (a specific inhibitor of MEK), or GM-6001 (a specific inhibitor of MMPs) significantly suppresses leptin-mediated cellular invasion in human colon cancer HT-29 cells. This suggests that the PI3K/Akt and MAPK/ERK signaling pathways take pivotal roles in leptin-mediated MMP-7 expression and cellular invasion in human colon cancer HT-29 cells.

4. Discussion

Colorectal cancer is one of the leading causes of cancer death in many countries. Previous studies suggest that adipocyte-derived growth factors, such as leptin, could contribute to cellular transformation and tumorigenesis [8]. High circulating leptin levels could be associated with malignancy of gastrointestinal cancer [13]. Overexpression of MMP-7 has likewise been demonstrated for tumorigenicity of colon cancer and biomarkers of colorectal carcinoma. Higher circulating levels of MMP-7 are associated with a lower survival rate of colon cancer patients. However, the effects of leptin on MMP-7 expression and the molecular mechanism of cellular invasion in human colon cancer cells have not been demonstrated. This study investigated the molecular actions of leptin on cellular invasion of human colon cancer HT-29 cells *in vitro*.

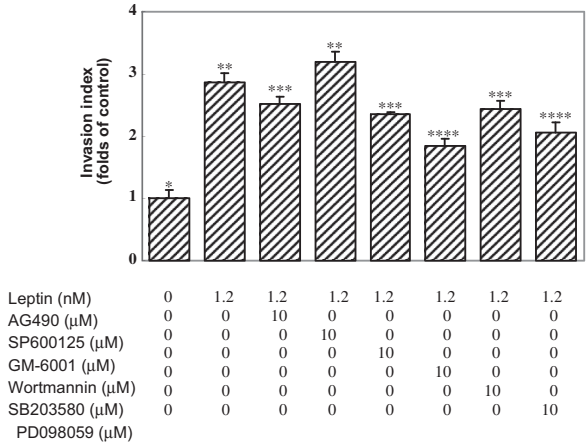

Fig. 4 – PI3K/Akt and MAPK/ERK signaling pathways played key roles in leptin-mediated invasion of human colon cancer HT-29 cells. Invasion of tumor cells was analyzed in transwell Boyden chambers with a polyvinylpyrrolidone filter of 8-μm pore size. Each filter was coated with 50 μL of a 1:5 diluted matrigel in cold McCoy's medium to form a thin continuous layer on top of the filter. Confluent human colon cancer cells were cultured in McCoy's medium with 10% fetal bovine serum at 37°C. After washing out the media, colon cancer cells were trypsinized, prestained with Calcein AM, then transferred to matrigel coated transwell Boyden chambers. Human colon cancer cells (5000 cells/well) were treated with different inhibitors and stimulated with leptin (1.2 nM) in serum-free McCoy's medium. After incubation for 18 hours, cells were counted as described above; the number of cells invading the lower side of the filter was gauged as invasive activity. Asterisks represent statistical significance, $p < 0.05$. Data shown represent three independent experiments.

Our results demonstrate that leptin dose-dependently induces cellular invasion and expression of MMP-7 molecules in human colon cancer HT-29 cells. However, leptin barely influenced the expression of other types: e.g., MMP-2 and MMP9. It is plausible that leptin induces MMP-7 expression and cellular invasion in human colon cancer HT-29 cells. To investigate whether the inducible form of MMP-7 is responsible for leptin-mediated cellular invasion, we used specific siRNA designed to interfere with MMP-7 gene expression. Fig. 3 proves that treatment of siRNA can significantly inhibit cellular invasion, suggesting the MMP-7 molecule as the major molecule involved in leptin-mediated cellular invasion in human colon cancer cells. This suggests that MMP-7 is an inducible molecule which plays a critical role in cellular invasion under leptin stimulation. Prior studies indicated strong immunohistochemical staining of MMP-7 in various types of colonic adenomas [36]. Our results concur with earlier findings and provide a novel mechanism of leptin in tumorigenesis of human colon cancer. Studies also indicate that expression of MMP-7 is significantly correlated with the presence of nodal or distant metastases [42,43].

To examine the molecular mechanism of leptin in cellular invasion, we investigated major signaling pathways responsible for cellular invasion of human colon cancer cells. Using variant specific inhibitors against signaling pathways, we observed that treatment with wortmannin (a specific inhibitor of PI3K), PD098059 (a specific inhibitor of MEK), and GM-6001 (a specific inhibitor of MMPs) significantly blocked cellular invasion. The results hint that PI3K/Akt and MAPK/ERK signaling pathways play key roles in leptin-mediated cellular invasion in human colon cancer HT-29 cells.

In summary, a mechanism by which leptin may exert malignant effects is partly through modulation of MMP-7 expression, as needed for cellular invasion during tumor development. Findings yield novel mechanistic insights into tumorigenic effects of leptin on the malignant progression of human colorectal cancer. We only tested the effects of leptin on human colon cancer HT-29 cell lines, yet our *in vitro* results are consistent with other findings, that high circulating levels of leptin might be a risk factor associated with tumor malignancy, as evident from population studies and animal experiments showing enhanced tumor progression and cancer development. Our results also suggest a novel hypothesis that MMP-7 might act as a malignant biomarker in an autocrine regulation manner in human colon cancer cells.

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