GDNF increases cell motility in human colon cancer through VEGF–VEGFR1 interaction

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Abstract

Glial cell line-derived neurotrophic factor (GDNF), a potent neurotrophic factor, has been shown to affect cancer cell metastasis and invasion. However, the molecular mechanisms underlying GDNF-induced colon cancer cell migration remain unclear. GDNF is found to be positively correlated with malignancy in human colon cancer patients. The migratory activities of two human colon cancer cell lines, HCT116 and SW480, were found to be enhanced in the presence of human GDNF. The expression of vascular endothelial growth factor (VEGF) was also increased in response to GDNF stimulation, along with VEGF mRNA expression and transcriptional activity. The enhancement of GDNF-induced cancer cell migration was antagonized by a VEGF-neutralizing antibody. Our results also showed that the expression of VEGF receptor 1 (VEGFR1) was increased in response to GDNF stimulation, whereas GDNF-induced cancer cell migration was reduced by a VEGFR inhibitor. The GDNF-induced VEGF expression was regulated by the p38 and PI3K/Akt signaling pathways. Treatment with GDNF increased nuclear hypoxia-inducible factor 1α (HIF1α) accumulation and its transcriptional activity in a time-dependent manner. Moreover, GDNF increased hypoxia responsive element (HRE)-containing VEGF promoter transcriptional activity but not that of the HRE-deletion VEGF promoter construct. Inhibition of HIF1α by a pharmacological inhibitor or dominant-negative mutant reduced the GDNF-induced migratory activity in human colon cancer cells. These results indicate that GDNF enhances the migration of colon cancer cells by increasing VEGF–VEGFR interaction, which is mainly regulated by the p38, PI3K/Akt, and HIF1α signaling pathways.

Key Words

- GDNF
- cell motility
- VEGF
- colon cancer
- HIF1
Introduction

Vascular and neural neoplastic invasions have been found in patients with colon cancer (Bellis et al. 1993). As vascular and neural infiltrations are known to be ominous prognostic factors, their identification has great clinical relevance. Increasing evidence shows that perineural invasion or the neural cell adhesion molecule might be a prognostic factor for various cancers, such as bile duct cancer (Seki et al. 1993, Kayahara et al. 1994), gallbladder cancer (Seki et al. 1995), breast cancer (Lo et al. 1997), esophageal cancer (Tanaka et al. 1998), pancreatic cancer (Pour et al. 2003, Levy et al. 2006, Kayahara et al. 2007), prostate cancer (Li et al. 2003), and colorectal cancer (Gavert et al. 2005, Zhou et al. 2009, Li et al. 2011). Importantly, a recent report has shown that the severity of the neural invasion in rectal adenocarcinoma is an important factor in a scoring system to assess the prognostic value, showing high correlation with localization and severity (Ceyhan et al. 2010). Patients with a surgically resected pancreatic cancer showed a significantly higher frequency of positive glial cell line-derived neurotrophic factor (GDNF) immunostaining, and the intrapancreatic neural invasion by cancer cells was significantly related to the overexpression of GDNF (Zeng et al. 2008). Importantly, a strongly positive expression of GDNF was significantly more frequent than lesser grades of GDNF expression in patients with pancreatic cancer (Zeng et al. 2008).

GDNF, being a neurotrophic factor, promotes the survival and development of various neural cells in the CNS and peripheral nervous system. Previous reports have shown that GDNF also affects the survival of cancer cells and their proliferation in gliomas (Wiesenerhofer et al. 2000b) and enhances the metastasis (Funahashi et al. 2005) and invasion potential (Kikuchi 2004, Ito et al. 2005, Qiao et al. 2009, Lu et al. 2010) of pancreatic cancer. GDNF and its receptor are expressed in human cancers such as gliomas (Wiesenerhofer et al. 2000a). It has also been reported that GDNF is overexpressed at the transcript level in non-small-cell lung carcinomas (Garnis et al. 2005). Increased levels of GDNF in the urine could serve as a basis for the adjunct diagnosis, monitoring, and treatment of interstitial cystitis in patients with bladder cancer (Okragly et al. 1999). Recently, we reported that GDNF increases cell migration and metastasis in gliomas, chondrosarcomas, and oral squamous cell carcinomas (Su et al. 2009, Lu et al. 2010, Chuang et al. 2013). Moreover, it has also been reported that the GDNF expression in colon adenocarcinoma cells may play an important role in the pathogenesis of intestinal ganglioneuromatosis (Qiao et al. 2009), where an increased expression of GDNF is associated with diffuse ganglioneuromatosis (Furuta et al. 2007). Moreover, the enhancement of integrin expression by GDNF strongly influences its invasion of extracellular matrix proteins in colorectal cancer (Furuta et al. 2007). However, little is known about the detailed mechanism by which GDNF mediates colon cancer cell migration.

Vascular endothelial growth factor A (VEGF-A), also simply referred to as VEGF, is divided into different isoforms based upon their sequences of amino acids (Zachary & Gliki 2001). VEGF is a well-known angiogenic factor and is known to play an essential role in neovascularization (Ferrara et al. 1993). The VEGF variant is the predominant and the most critical regulator of the development of the vascular system (Zachary & Gliki 2001). The affinity of VEGF to its receptor 1 (VEGFR1) is higher than to VEGFR2 (Kaplan et al. 2005). Recent studies have shown that VEGFR1 is a critical mediator of physiological and developmental cell migration, as well as tumor-mediated metastasis (Ferrara 2004, Hicklin & Ellis 2005, Kaplan et al. 2005). However, the tyrosine kinase activity of VEGFR1 is much weaker than that of VEGFR2, and thus VEGFR2 has been considered to play the key role in angiogenesis (Ferrara 2004). Generally, increases in VEGF levels have been found to correlate with increases in the incidence of metastasis, decreases in overall survival, and poor prognosis (Takahashi et al. 1995, 1998). Importantly, VEGF is expressed in both primary and metastatic human colon cancers, and VEGF expression is higher in the metastatic cells (Hicklin & Ellis 2005). Moreover, serum levels of VEGF could be useful for screening and detecting the early stages of colon cancer (Ferrara et al. 1993). It has been demonstrated that VEGF is associated with the progression, invasion, and metastasis of colorectal cancer, and the overexpression of VEGF mRNA in the primary tumor is assumed to be closely correlated with a poor prognosis in patients with colorectal cancer (Ishigami et al. 1998). It has also been reported that treatment with monoclonal anti-VEGF antibody reduces metastases to the liver in patients with colorectal cancer (Zachary & Gliki 2001, Ferrara 2004). This study therefore sought to determine whether GDNF affects the expression of VEGF in human colon cancer. We also investigated the intracellular signaling pathways involved in the GDNF-induced upregulation of VEGF expression.
Subjects and methods

Materials

GDNF was purchased from PeproTech (Rocky Hill, NJ, USA). Fetal bovine serum (FBS), DMEM, OPTI-MEM, and Lipofectamine 2000 (LF2000) were purchased from Gibco BRL (Invitrogen Life Technologies). Primary antibodies against β-actin, ERK2, JNK, Akt, p-Akt, and p38 were purchased from Santa Cruz Biotechnology. The MAB specific for GDNF was purchased from R&D Systems (Minneapolis, MN, USA). Primary antibodies against p-p38 and p-JNK were purchased from Cell Signaling and Neuroscience (Danvers, MA, USA). The primary antibody against hypoxia-inducible factor 1α (HIF1α) was purchased from Novus Biologicals (Littleton, CO, USA). The luciferase assay kit was purchased from Promega. The dominant-negative (DN) mutant of HIF1α, HIF1α luciferase, and VEGF-luciferase 1.5 k and 1.2 k (deletion of hypoxia responsive element (HRE)) plasmids were gifts from Dr W-M Fu (National Taiwan University, Taipei, Taiwan). The DN mutant of p38 (DN-p38) was a gift from Dr C-H Tang (China Medical University, Taichung, Taiwan). The HIF1α inhibitor 3-((2-(4-adamantan-1-yl-phenoxy)-acetylamino)-4-hydroxybenzoic acid methyl ester and the VEGF receptor inhibitor N-(4-chlorophenyl)-2-[(pyridin-4-ylmethyl) amino]benzamide were purchased from Merck Co.

Immunohistochemistry

The protocol of immunohistochemistry was described in our previous reports (Lin et al. 2013, Lu et al. 2013). The human colon cancer tissue array was purchased from Biomax (Rockville, MD, USA) in the form of 5 μm sections of paraffin-embedded tissue on glass slides. Tissue specimens were rehydrated and quenched for endogenous peroxidases by soaking in 3% hydrogen peroxide in PBS. After trypsinization, sections were blocked by incubation with 5% BSA. The anti-GDNF primary antibody (1:50) was applied to the tissue specimens at 4°C overnight. Then, after several washes with PBS, the samples were incubated with a biotin-conjugated secondary antibody (1:50). Bound antibodies were detected with an ABC reaction kit (Vector Laboratories, Burlington, CA, USA). The tissue specimens were developed with diaminobenzidine and counterstained with hematoxylin.

Cell cultures

The human colon carcinoma cell lines HCT116 and SW480 were obtained from the American Type Culture Collection (Manassas, VA, USA). The cell culture was performed according to a protocol described previously (Huang et al. 2011). Briefly, cells were grown in plastic cell culture dishes in DMEM, supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin, at 37 °C in a humidified incubator under an atmosphere of 5% CO2 and 95% air.

Migration assay

In vitro migration assay was performed using Costar Transwell inserts (Costar, New York, NY, USA; pore size, 8 μm) and as described in our previous reports (Chen et al. 2011, Lu et al. 2012). Before performing the migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, or transfected with various DN mutants for 24 h. According to a cell viability assay, the various concentrations of inhibitors used did not affect colon cancer cell death (data not shown). Approximately 1 × 10^4 cells in 100 μl of serum-free medium were placed in the upper chamber, and 400 μl of the same medium containing GDNF was placed in the lower chamber. The plates were incubated for 24 h at 37 °C in 5% CO2, and then the cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper sides of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the undersides of the filters were examined and counted under a microscope.

Transfection

HCT116 cells were transiently transfected with 100 nM growth factor receptor z1 (GFR-z1), VEGF-A, or control siRNA by LF2000 for 24 h. Plasmid DNA and LF2000 were premixed in OPTI medium for 20 min and then applied to the cells. An equal volume of medium containing 20% FBS was added 5 h later. After transfection for 24 h, the cell cultures were washed, replenished with fresh serum-free medium, and treated with GDNF for another 24 h.

Reverse transcription PCR

Total RNA was extracted from cells using a TRIzol kit (MDBio, Inc., Taipei, Taiwan). The RT reaction was performed using 2 μg total RNA that was reverse transcribed into cDNA using the oligo(dT) primer and then amplified using the following oligonucleotide primers: VEGF, 5'-CTA-CCTCCACATGCAACGT-3' and 5'-GCAGTAGCTGCGGTGATA-3'; GAPDH, 5'-ACCACAGTCCATGCCATC-3'.
and 5’-TCCACCACCCTGTGCTGTA-3’ Each PCR cycle was carried out for 30 s at 95 °C, 30 s at 55 °C, and 1 min at 68 °C. The PCR products were then separated electrophoretically on a 2% agarose gel and stained with ethidium bromide.

**Western blot analysis**

Cells were treated with GDNF for various times and then lysed briefly in homogenizing buffer on ice. The supernatants were collected by centrifugation. Equal amounts of the samples were separated by SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with non-fat milk in PBS and then probed with primary antibodies. After undergoing three PBS washes, the membranes were incubated with secondary antibodies. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak).

**Preparation of nuclear extracts**

Nuclear extracts was prepared as described previously (Lin et al. 2011, Chen et al. 2012). Cells were rinsed with PBS and suspended in hypotonic buffer A for 10 min on ice and vortexed for 10 s. The lysates were separated by centrifugation at 12 000 g for 10 min, after which the supernatants containing cytosolic proteins were collected. The pellets containing the nuclear fractions were resuspended in buffer C for 30 min on ice. The suspensions were then centrifuged at 13 000 g for 20 min, whereupon the supernatants containing nuclear proteins were collected and stored at −80 °C.

**Measurement of cell viability**

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoli um bromide (MTT) assay. Cells cultured in 24-well plates were treated with various concentrations of GDNF for 24 h. After incubation, MTT (0.5 mg/ml) was added for 60 min, after which the culture medium was removed and the cells were dissolved in dimethyl sulfoxide and shaken for 10 min. The absorbency (OD) values at 550 nm were immediately measured in a microplate reader. The absorbency values indicate the enzymatic activity of mitochondria and provide information on cell viability.

**Statistical analysis**

Statistical analysis was performed using the software GraphPad Prism 4.01 (GraphPad Software, Inc., San Diego, CA, USA). Values are the mean ± S.E.M. Statistical analysis of the difference between two samples was performed using the Student’s t-test. Statistical comparisons involving more than two groups were performed using one-way ANOVA with Dunnett’s post-hoc test. In all cases, a value of P < 0.05 was considered to be significant.

**Results**

**GDNF increases the metastasis and migration of colon cancer cells**

Higher GDNF expression was found to be associated with higher clinical pathological stages in tissue specimens of human colon cancer (Fig. 1A). The quantification results for GDNF intensity are shown in Fig. 1B. To elucidate a link between the GDNF expression and migration of colon cancer, we examined the migratory activity of human colon cancers by using transwell assays. In our results,
GDNF directed the migration of both human colon cancer HCT116 cells (Fig. 2A) and SW480 cells (Fig. 2B) in a concentration-dependent manner. The pictures of the migrated cells are shown in Fig. 2D. Our results also showed that the GDNF-directed enhancement of colon cancer cell migration was abolished by treatment with a GDNF-neutralizing antibody (Fig. 2C). The receptor for GDNF is thought to be a complex of GFR-α1, which acts as a ligand-binding domain (Jing et al. 1996, Treanor et al. 1996). In order to investigate the role of the GDNF receptor in GDNF-mediated colon cancer cell migration, we transfected GFR-α1 siRNAs into HCT116 human colon cancer cells. The GDNF-induced colon cancer migration was antagonized by transfection with GFR-α1 siRNA in a concentration-dependent manner (Fig. 3A). GDNF has been identified as a ligand for Ret, which acts as the signal-transducing domain (Durbec et al. 1996, Trupp et al. 1996) and increases GDNF-induced pancreatic cancer cell invasion (Sawai et al. 2005). We therefore examined whether any interaction between GDNF and its receptor is involved in the signal transduction pathways leading to GDNF-induced colon cancer cell migration. The level of phosphorylation of the Ret receptor, after GDNF stimulation of HCT116 cells, was assessed by western blot analysis. As shown in Fig. 3B, GDNF induced Ret phosphorylation in a time-dependent manner. These data indicate that the GDNF-induced cancer cell migration.

**Figure 2**

GDNF induces the migratory activity of human colon cancer cells. HCT116 (A) and SW480 (B) human colon cancer cells were treated with various concentrations of GDNF (3, 10, or 30 ng/ml). The in vitro migration activities were measured with the transwell assay after GDNF treatment for 24 h. Results are expressed as the mean ± S.E.M. of three independent experiments. The migrated cells were visualized by phase-contrast imaging (D).

(C) HCT116 cells were incubated with control IgG or GDNF-neutralizing antibody (5 µg), followed by stimulation with 30 ng/ml GDNF. The in vitro migratory activity was measured with the transwell assay after GDNF treatment for 24 h. Results are expressed as the mean ± S.E.M. of at least three independent experiments. *P<0.05 compared with the control IgG group; *P<0.05 compared with the GDNF treatment group.

**Figure 3**

Involvement of Ret and GFR-α1 in GDNF-induced migration of human HCT116 colon cancer cells. (A) Cells were transfected with siRNA against control (200 µM) or GFR-α1 (100 or 200 µM) for 24 h, followed by stimulation with GDNF. The in vitro migratory activity was measured with the transwell assay after GDNF (30 ng/ml) treatment for another 24 h. Results are expressed as the mean ± S.E.M. of at least three independent experiments. *P<0.05 compared with the control group; #P<0.05 compared with the GDNF treatment group. (B) Cells were treated with GDNF (30 ng/ml) for the indicated times (5, 10, 30, 60, or 120 min) and cell lysates were extracted. The phosphorylation of Ret was determined by western blot analysis. Results are representative values from three independent experiments.
migration may occur via activated Ret and GFR-α1 receptors in human colon cancers.

**GDNF-directed colon cancer cell migration involves VEGF expression**

Because angiogenesis has been found to be associated with increases in the growth and migratory potential of tumor cells, we studied the GDNF-induced expression of the angiogenic factor VEGF in HCT116 cells. As shown in Fig. 4A and B, GDNF increased the VEGF protein expression in concentration- and time-dependent manners. In addition, GDNF increased the VEGF mRNA expression in HCT116 cells (Fig. 4C). The VEGF-luciferase reporter gene assay was further used to examine whether the GDNF upregulated VEGF through a transcriptional pathway. As shown in Fig. 4D, GDNF also increased the VEGF transcriptional activity in HCT116 cells. Furthermore, the incubation of colon cancer cells with a VEGF-neutralizing antibody markedly inhibited the GDNF-induced cancer cell migration (Fig. 4E). VEGF exhibits high binding affinity to VEGFR1 (Kaplan et al. 2005). We therefore examined whether VEGFR1 is involved in the GDNF-induced migration of human colon cancer cells. After GDNF stimulation of the HCT116 cells, the expression of VEGFR1 was assessed by western blot analysis. As shown in Fig. 5A and B, GDNF induced VEGFR1 upregulation in concentration- and time-dependent manners. Moreover, pretreatment with a VEGFR pharmacological inhibitor, N-(4-chlorophenyl)-2-[(pyridin-4-ylmethyl)amino]benzamide, effectively reduced the enhancement of GDNF-induced colon cancer migration (Fig. 5C). These data indicate that GDNF-induced colon cancer migration may occur through the interaction of VEGF and VEGFR1.

**MAP kinase signaling pathways are involved in GDNF-mediated VEGF upregulation and colon cancer cell migration**

Previous studies and our recent report demonstrated that GDNF activates MAP kinase, and that Akt exerts biological and pathophysiological functions (Tremblay et al. 2006, Lai et al. 2010, Gu et al. 2011, Tomas et al. 2012). Thus, we examined whether MAP kinase and the Akt signaling pathway are involved in GDNF-induced VEGF expression and colon cancer migration. Our results showed that the stimulation of cells with GDNF increased the phosphorylation of ERK, p38, and JNK (Fig. 6A, B, and C respectively). On the other hand, GDNF also increased Akt

**Figure 4**

GDNF-directed migratory activity involves upregulation of VEGF in HCT116 cells. Cells were incubated with various concentrations of GDNF (3, 10, or 30 ng/ml) for 24 h (A) or 8 h (C), and the VEGF protein and mRNA levels were determined using western blot and RT-PCR analyses respectively. (B) Cells were treated with GDNF (30 ng/ml) for the indicated times (4, 8, 16, and 24 h), and VEGF protein expression was analyzed by western blot analysis. Results are representative values for three independent experiments. (D) Cells were transfected with VEGF-promoter luciferase plasmid for 24 h, followed by stimulation with 30 ng/ml GDNF for another 24 h. VEGF transcriptional activity was determined by the luciferase assay. (E) Cells were incubated with control IgG or VEGF antibody (5 μg), followed by stimulation with 30 ng/ml GDNF. The in vitro migratory activity was measured with the transwell assay after GDNF (30 ng/ml) treatment for another 24 h. Results are expressed as the mean ± s.e.m. of three independent experiments. *P < 0.05 compared with the control group; #P < 0.05 compared with the GDNF treatment group.
phosphorylation in a time-dependent manner (Fig. 6D). Moreover, pretreatment with SB203580, LY294002, or Akt inhibitors, but not PD98059 or SP600125, reduced the GDNF-induced VEGF transcriptional activity (Fig. 6E) and VEGF expression (Fig. 6F). Transfection with p38-DN, p85-DN, and Akt-DN reduced the GDNF-enhanced migration of HCT116 cells. These results indicate that the GDNF-mediated VEGF upregulation and cell migration occur via the p38 and PI3K/Akt signaling pathways.

**HIF1α is involved in GDNF-induced migration of human HCT116 colon cancer cells**

As mentioned previously, intratumoral hypoxia correlates with poor prognosis and enhanced metastases formation. HIF1α is a key molecule in the hypoxic response and plays critical roles in cell detachment, invasion, energy metabolism, and induction of angiogenesis (Gort et al. 2008).

Moreover, HIF1α also plays a role in this relationship by promoting expression by tumor cells of growth factors, such as VEGF (Ikeda et al. 1995, Forsythe et al. 1996). We sought to determine whether HIF1α activation is involved in GDNF-induced VEGF transcriptional activity. Our results showed that GDNF increased HIF1α accumulation in the nucleus (Fig. 7A). HIF1 binds to the HRE, which regulates the expression of a variety of genes that mediate the cellular response to hypoxic stress (Harris 2002). HCT116 cells were transfected with a VEGF promoter containing the HRE construct (1.5 k) for 24 h. As shown in Fig. 7B, the GDNF-stimulated VEGF transcriptional activity increased. However, in cells that were transfected with an HRE-deletion VEGF promoter construct (1.2 k), GDNF could not increase the VEGF transcriptional activity (Fig. 7B). Furthermore, pretreatment with an HIF1 inhibitor for 30 min or transfection with HIF1α-DN for 24 h antagonized the GDNF-enhanced migration of the HCT116 human colon cancer cells (Fig. 7C). These results indicate that the activation of HIF1α plays an important role in GDNF-induced VEGF expression and migration of human colon cancer cells.

**Discussion**

Colon cancer ranks among the most frequent malignancies in Taiwan and is one of the leading causes of cancer-related deaths worldwide. The metastatic dissemination of primary tumors is linked directly to patient survival and accounts for about 90% of all colon cancer deaths. Current clinical predictions on whether colon cancer will metastasize are defined mainly by histopathological staging, describing the tumor spread within a surgical specimen. It should therefore be obvious that tools and methodologies that allow early cancer detection directly will affect the patient survival time. The molecular mechanisms controlling the invasion, dissemination to blood or lymphatic systems, and spread of tumor cells to distant organs are still poorly understood. Beyond a potential application to new therapeutics, the underlying novel hypotheses and mechanistic studies will have substantial potential to reveal the interlocking nature of growth factor pathways and the way they modulate pathophysiological states. Thus, perineural invasion and hypoxia would be related to GDNF and VEGF expression in colon cancer cells, and subsequently, the induction of cancer angiogenesis and cellular migration. Our study also elucidated that downstream signaling of GDNF-receptor, together with HIF1α activation and VEGF expression, is responsible for cancer cell migration. The successful implementation of these
screening procedures will contribute to a reduction in the disease-associated mortality of colon carcinoma.

Although beneficial for patients with stage III colon cancer, the role of adjuvant chemotherapy is not yet established for stage II disease. Currently, patients with stage II colonic cancer are treated mostly by surgery alone. However, patients with high-risk stage II disease, with a prognosis similar to stage III patients, should be offered chemotherapy under the current guidelines (O’Connor et al. 2011). In spite of the potentially curative resection, about 15–30% of patients with stage II disease will eventually develop recurrent locoregional disease or distant metastases within 5 years (Koebrugge et al. 2011).

Possibly, other high-risk factors can influence or predict the outcome in patients with stage II colon cancer, and further research seems warranted (Iddings et al. 2006, van Schaik et al. 2009, Bilchik et al. 2010). The elucidation of the molecular biology of cancer cells is currently being exploited to develop potential therapeutic targets. In this study, we found that GDNF enhances the migratory ability of human colon cancer cells. Interestingly, it has been reported that the exogenous administration of GDNF could increase VEGF expression in spinal cord injury (Kao et al. 2008). It has also been reported that GDNF induces the migration of lung (Garnis et al. 2005) and pancreatic cancer cells (Okada et al. 2003, Funahashi et al. 2005, Gil et al. 2010).

The combination of radiotherapy and surgery has significantly improved local control and overall survival in patients with colon cancer, but cell migration and

Figure 6
GDNF induces MAP kinase and Akt signaling activation in HCT116 colon cancer. Cells were incubated with GDNF for the indicated times. The phosphorylation of ERK (A), p38 (B), JNK (C), and Akt (D) was determined by western blot analysis. Results are representative values for three independent experiments. (E) Cells were transfected with the VEGF luciferase plasmid for 24 h and then treated with PD98059, SB203580, SP600125, LY294002, or Akt inhibitor for 30 min, before incubation with GDNF for another 24 h. VEGF luciferase activities were measured and the results were normalized to the β-galactosidase activity. The results are expressed as the mean ± S.E.M. from three independent experiments. *P < 0.05 compared with the control group; #P < 0.05 compared with the GDNF treatment group. (F) Cells were transfected with DN-p38, DN-85, DN-Akt, or control vector for 24 h, followed by stimulation with GDNF for another 24 h. The in vitro migratory activity was measured using the transwell assay. The results are expressed as the mean ± S.E.M. of three independent experiments. *P < 0.05 compared with the control group; #P < 0.05 compared with the GDNF treatment group.
GDNF induces VEGF-A upregulation through HIF1α activation in HCT116 colon cancer cells. (A) Cells were treated with GDNF for the indicated times, and the levels of nuclear HIF1α were determined by immunoblotting with HIF1α-specific antibody. The results are representative values for three independent experiments. (B) Cells were transfected with the VEGF-A-luciferase expression plasmid (1.5 k) or HRE deletion VEGF-A-luciferase (1.2 k) for 24 h. The VEGF-A-luciferase activity was assayed after GDNF stimulation for another 8 or 24 h. Results are expressed as the mean ± S.E.M. of three independent experiments. (C) Cells were pretreated with the HIF1α inhibitor for 30 min, or transfected with HIF1α-DN for 24 h, followed by stimulation with GDNF for another 24 h. The in vitro migratory activity was measured using the transwell assay. The results are expressed as the mean ± S.E.M. of three independent experiments. *P < 0.05 compared with the control group; #P < 0.05 compared with the GDNF treatment group.

Metastasis formation still remain a significant problem (S.R.C. 1997). Metastasis formation is a major factor in cancer progression and in the majority of mortalities. Recent observations have indicated that the metastatic phenotype may already be present during the angiogenic switch of tumors. In order for solid tumors to grow, an increase in oxygen delivery to cells via angiogenesis and glycolysis activation have to occur (Seagroves et al. 2001). Intratumoral hypoxia correlates with poor prognosis and enhanced metastases formation, where HIFs play critical roles during tumor cell expansion. Increasing evidence implicates HIF function in metastatic cell characteristics such as cell detachment, invasion, energy metabolism, and induction of angiogenesis (Gort et al. 2008). HIF1α is a heterodimeric transcription factor that is activated by low oxygen tension and it subsequently binds to HRE, which regulates the expression of a variety of genes that mediate the cellular response to hypoxic stress (Harris 2002). HIF1α plays a role in this relationship by promoting tumor cells' expression of growth factors, such as VEGF (Ikeda et al. 1995, Forsythe et al. 1996). It has been shown that blocking VEGF results in an increase in the radiosensitivity of the tumor vasculature and increases the overall tumor radiation response (Geng et al. 2001, Töiyama et al. 2010). Importantly, xenograft studies have revealed that HIF1α-deficient human colon cancer cells displayed lower rates of proliferation and migration, effectively inhibiting tumor growth (Imamura et al. 2009).

Numerous studies have indicated that VEGF and VEGFR1 are implicated in colon cancer progression. Evidence has shown that expression of VEGF and VEGFR1 correlates with vascularity, metastasis, and proliferation in human colon cancer specimens (Takahashi et al. 1995, Ellis et al. 2000). Recent studies have also demonstrated that VEGF and VEGFR1 are expressed in human colon cancer tissue specimens, and that VEGF generated by colon cancer cells stimulates their growth directly through an autocrine mechanism that is independent of its primary function of angiogenesis induction (Ahluwalia et al. 2013). Moreover, in clinical studies that compared the efficacy of standard metastatic colorectal chemotherapy (irinotecan, 5-fluorouracil, and leucovorin) with and without bevacizumab, an anti-VEGF antibody, increased median survival with bevacizumab indicates a relationship between VEGF and metastatic colon cancer (Ferrara et al. 2004, Roskoski 2007). Several lines of evidence indicate that VEGFR2 mediates the stimulatory effects of VEGF on angiogenesis, whereas VEGFR1 actually attenuates the actions of VEGF by binding VEGF without activating angiogenesis (Zachary & Gliki 2001). Importantly, a previous study reported that colon cancer cells express VEGFR1, but not VEGFR2 (Fan et al. 2005). In addition, VEGFR1 has been reported to stimulate endothelial cell migration in response to VEGF-A (Kanno et al. 2000). A recent report has also shown that prostaglandin E2 regulates cell migration via the induction of VEGFR1 in HCA7 human colon cancer cells (Fujino et al. 2011). Similarly, in this study, GDNF induced VEGFR1 upregulation in concentration- and time-dependent manners. Moreover, pretreatment with a
VEGFR pharmacological inhibitor effectively reduced the enhancement of GDNF-induced colon cancer migration. Conclusively, therefore, GDNF directs cell migration by the interaction of VEGF and VEGFR1.

Signaling molecules, including ERK, p38, JNK, and Akt, have been demonstrated to play important roles in colon cancer cell invasion and migration (Tremblay et al. 2006, Lai et al. 2010, Gu et al. 2011, Tomas et al. 2012). The activation of Ret by GDNF results in the activation of the MAP kinase pathways (van Weering & Bos 1997). Specifically, Ret activates the ERK and JNK signaling pathways (Marek et al. 2004, Asai et al. 2006). Our results showed that GDNF induced ERK, p38, JNK, and Akt phosphorylation in human colon cancer cells. Furthermore, SB203580 and Ly294002, as well as an Akt inhibitor, effectively antagonized the GDNF-induced VEGF transcriptional activity and protein expression. Knockdown of p38 and Akt also reduced the GDNF-induced cell migration in human colon cancer. These results indicate that GDNF directs the migration of colon cancer cells through the activation of the p38 and Akt signaling pathways via Ret activation. Our results indicate that GDNF directs the migration of colon cancer through the activation of Ret and GFR-z1, as well as via p38 and Akt activation. Moreover, GDNF increased VEGF expression via HIF-1α activation, thereby contributing to tumor migration. Our findings, which reveal the molecular mechanisms by which GDNF promotes colon cancer cell migration, may lead to a better understanding of the malignant progression of human colon cancers.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
S-M Huang, T-S Chen, C-M Chiu, and L-K Chang performed experiments, acquisition and analysis of data, and drafted the manuscript. K-F Liao, H-M Tan and W-L Yeh helped to perform experiments and prepare the manuscript. S-M Huang, M-Y Wang and D-Y Lu conceived the study and participated in its design and coordination, S-M Huang, M-Y Wang and D-Y Lu were involved in drafting the manuscript and revising it for intellectual content. All authors read and approved the final manuscript.

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