Growth differentiation factor-15 upregulates interleukin-6 to promote tumorigenesis of prostate carcinoma PC-3 cells

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Results from studies on GDF15 expression in prostate cancer are somewhat controversial. GDF15 was found to be overexpressed in prostate intraepithelial neoplasia; however, decreased expression was observed in higher grade cancer (Iczkowski & Pantazis 2003). Karan et al. (2003) indicated stronger immunohistochemical staining in the areas of adenocarcinoma than in the benign glandular areas of prostate tissues. Another study showed that levels of GDF15 expression were higher in prostate cancer than in normal prostate tissues and were also significantly higher in well-differentiated prostate cancer than in moderately and poorly differentiated adenocarcinomas (Kawahara et al. 2010). Brown et al. (2009) found that increased serum GDF15 concentrations are strongly associated with advancement and progression of prostate cancer. However, relevant in vitro studies have made discrepant findings. Early study showed that upregulation of GDF15 gene expression by vitamin D3 suppresses cell growth of prostate carcinoma LNCaP cells (Lambert et al. 2006). Other studies indicated that GDF15

Abstract

Growth differentiation factor-15 (GDF15), a member of the transforming growth factor-β superfamily, is associated with human cancer progress. We evaluated the role GDF15 plays in tumorigenesis of prostate carcinoma PC-3 cells. Results from real-time RT-PCR and ELISA revealed that expression of GDF15 was approximately threefold higher in LNCaP cells than in PC-3 cells. Other prostate cell lines (PZ-HPV-7, CA-HPV-10, and DU145 cells) expressed extremely low levels of GDF15. Stable overexpression of GDF15 in PC-3 cells enhanced the degree of cell proliferation and invasion as shown in the 3H-thymidine incorporation assay and in the Matrigel invasion assay respectively. Soft agar assays and xenograft animal studies indicated that overexpression of GDF15 in PC-3 cells increased tumorigenesis in vitro and in vivo. Results from RT-PCR, immunoblot, and reporter assays revealed that overexpression of GDF15 resulted in decreased expression of maspin and upregulation of interleukin-6 (IL6), matriptase, and N-myc downstream-regulated gene 1 (NDRG1) expression. Further studies revealed that overexpression of IL6 enhanced GDF15 expression in LNCaP cells while knockdown of IL6 blocked the expression of GDF15 in PC-3 cells, suggesting that expression of GDF15 is upregulated by IL6. This study demonstrated that expression of GDF15 induces cell proliferation, invasion, and tumorigenesis of prostate carcinoma PC-3 cells. The enhancement of tumorigenesis and invasiveness of prostate carcinoma cells that stably overexpress GDF15 may be caused by the dysregulation of maspin, matriptase, and IL6 gene expression. The expression of GDF15 and IL6 is controlled via a positive feedback loop in PC-3 cells.

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Introduction


The objectives of this study were to determine the expression of GDF15 in human prostate carcinoma cells, to determine the tumorigenesis and invasiveness of PC-3 cells engineered to overexpress GDF15, and to evaluate the mechanisms by which GDF15 induces tumorigenesis in human prostate carcinoma cells.

Materials and methods

Cell culture and chemicals

PZ-HPV-7, CA-HPV-10, LNCaP, PC-3, and DU145 cell lines were obtained from the Bioresource Collection and Research Center (BCRC, Taiwan) and were maintained as described previously (Tsui et al. 2012b). FCS was purchased from HyClone (Logan, UT, USA), RPMI 1640 media from Life Technologies, recombinant human GDF15 from PeproTech (Rocky Hill, NJ, USA), doxorubicin from Sigma, and Matrigel from Becton Dickinson Biosciences (Bedford, MA, USA).

Expression vector constructs and stable transfection

Full-length human GDF15 cDNA (MCG: 4145) and interleukin-6 (IL6) cDNA (MCG: 9215) vectors were purchased from Invitrogen. The human GDF15 expression vector (pcDNA3-GDF15) was constructed by cloning GDF15 cDNA vector after digestion with EcoRI and NotI into the pcDNA3 expression vector (Invitrogen). The GDF15 expression vector was introduced into the PC-3 cells by electroporation as described previously (Tsui et al. 2011a). Cells were maintained in RPMI medium with 10% FCS and 418 (PAA Laboratories, Linz, Austria) at a final concentration of 800 μg/ml. The two selected resistant colonies were examined to evaluate GDF15 expression by RT-PCR, immunoblot assay, and ELISA as described below and designated as PCGDF15-1 and PCGDF15-2 cells. The mock-transfected PC-3 cells (PCDNA) were transfected with controlled pcDNA3 expression vector and were clonally selected in the same manner as the PCGDF15 cells. The human IL6 expression vector was constructed, introduced into LNCaP cells by electroporation, and maintained in RPMI medium with 10% FCS and Zeocin (Invitrogen) at a final concentration of 100 μg/ml as described previously (Tsui et al. 2011b). The colonies were designated as LN-IL6. The mock-transfected LNCaP cells (LN-DNA) were transfected with the control pcDNA3.1/Zeo expression vector and were selected in the same manner as the LN-IL6 cells. The pSMe2 retroviral vectors containing IL6 short hairpin RNA (shRNA; clone ID: V2HS-111640) and EGFP shRNA (clone ID: RHS1764-9394112) were purchased from Open Biosystems (Huntsville, AL, USA). The IL6 and EGFP knockdown vectors were introduced into PC-3 cells respectively by electroporation and selected by 2 μg/ml puromycin dihydrochloride. The IL6 knockdown PC-3 cells were termed PC-IL6si cells and EGFP knockdown PC-3 cells were termed PC-EGFPsi cells. Cells that expressed IL6 in resistant colonies were evaluated by RT-PCR and ELISA as described below.

siRNA transfection

GDF15-specific siRNA (sc-39798, Sigma) or nonsense siRNA (sc-37007, Sigma) was transiently transfected into PC-3 cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol. To determine the effectiveness of siRNA-mediated knockdown, immunoblot assay and ELISA, as described below, were used to measure the abundance of GDF15.

Semiquantitative and real-time RT-PCR

Total RNA was isolated with Trizol reagent and cDNA was synthesized using the superscript III pre-amplification system as described previously (Pang et al. 2009). The GDF15 primers (5'GGCCAAACCAGAGCTGG-GAAG-3' and 5'GCCCCGAGAGTACCCAGGT G3') were used to amplify sequences specific to human GDF15 mRNA. The sequence of primers used to amplify sequences specific to human maspin, N-myc downstream-regulated gene 1 (NDRG1), matriptase, IL6, and β-actin were previously described (Tsui et al. 2008a, 2011b, 2012a). After separation by 2% agarose gel electrophoresis, the PCR products were visualized by ethidium bromide staining. Real-time PCRs (qPCR) were performed using an ABI StepOne Plus Real-Time PCR system (Applied Biosystems) as described previously (Chung et al. 2012). FAM dye-labeled TaqMan MGB probes and PCR primers for human GDF15 (Hs00171132_m1) were purchased from Applied Biosystems. For the internal positive controls, 18S rRNA (18S; Hs03003631_g1) was used with an FAM reporter dye-labeled TaqMan MGB probe.
Immunoblot assay

Equal quantities of cell extract were loaded onto a 10% SDS–polyacrylamide gel and analyzed using the Western lightning plus-ECL detection system (PerkinElmer, Inc., Waltham, MA, USA) as described previously (Tsui et al. 2012a). The blotting membranes were probed with 1:5000 diluted polyclonal matriptase antiserum (IM1014; Calbiochem, Darmstadt, Germany), 1:1000 diluted maspin antiserum (554292; BD Biosciences, Bedford, MA, USA), 1:5000 diluted NDRG1 antiserum (N-19; Santa Cruz Biotechnology), 1:500 diluted GDF15 antiserum (07-217; Upstate Biotechnology, Charlottesville, VA, USA), or 1:1000 diluted anti-β-actin antiserum (C11, Santa Cruz Biotechnology). The intensity of immunoblotting assay was recorded by the Chemi Genius 2 BioImaging system of (Syngene, Cambridge, UK).

ELISA

Cells were incubated with 1 ml RPMI 1640 medium with 10% FCS. Following incubation, the conditioned medium from each well was collected for GDF15 or IL6 assay. The IL6 levels in the conditioned media were measured by a IL6 ELISA as described by the manufacturer (Catalog #:2107; Bio Scientific Corporation, Austin, TX, USA). The GDF15 levels in the conditioned media or serum samples collected from animal studies were measured using a GDF15 ELISA according to the manufacturer’s instructions (Catalog #: DY957; R&D Systems, Inc., Minneapolis, MN, USA). The GDF15 and IL6 levels in each sample were adjusted according to the concentrations of protein in the whole cell extract that had been measured using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

Cell proliferation assay

Proliferation of PCDNA and PCGDF15 cells was measured by a 3H-thymidine incorporation assay as described previously (Chung et al. 2011). In this assay, 5×10^5 cells were cultured in each well of a six-well plate in RPMI 1640 medium with 10% FCS. After a required incubation period, 1 μCi/ml 3H-thymidine (PerkinElmer, Boston, MA, USA) was added to each well of a six-well plate. The plate was then incubated at 37°C in a humidified 5% CO2 atmosphere for 4 h. Cells were washed twice with cold PBS and then with cold 5% TCA. Cells were solubilized by adding 0.5 ml 0.5 M NaOH. Then, 400 μl of the solubilized cell solution was mixed with 4 ml scintillation cocktail and counted with a Liquid Scintillation Analyzer (Packard BioScience, Downers Grove, IL, USA).

Matrigel invasion assay

The Matrigel invasion assay was performed as described previously (Tsui et al. 2012b). Briefly, 500 μl RPMI 1640 media with 10% FCS were added to the lower chamber of the 24-well plate. Culture was performed with 200 μl cells in serum-free RPMI 1640 medium in the upper well at a density of 1×10^5 cells/ml. The plates were placed in an incubator with 5% CO2 at 37°C for 24 h. Cells that migrated to the Matrigel-coated transmembrane were fixed in 4% paraformaldehyde and then stained with 0-1% crystal violet solution for 30 min. The results were recorded by a digital camera connected to an inverted microscope (IX71, Olympus, Tokyo, Japan). The membrane was soaked in 10% acetate acid and agitated at 37°C for 1 h. The acetate acid solution was then read by a spectrophotometer at 635 nm (DU640; Beckman, Fullerton, CA, USA).

Soft agar cloning assay

Approximately 10 000 PCDNA and PCGDF15-2 cells were suspended in 2 ml of 0-4% agar (Difco Agar Noble; BD Biosciences) in RPMI 1640 medium and plated on top of 1 ml of 0-8% agar in RPMI 1640 medium in a six-well plate. Plates were incubated for 3 weeks at 37°C and each well was fed with two to three drops of complete growth medium every 2–3 days. Cell colonies were visualized by staining with 0-5 ml p-iodonitrotetrazolium violet (1 mg/ml; Sigma).

Xenograft animal study

The studies were performed in accordance with the Guide for Laboratory Animal Facilities and Care (Council of Agriculture Executive, Taiwan) and approved by the Chang Gung University Animal Research Committee (IACUC approval NO: CGU084074). Male nude mice (BALB/cAnN-Foxn1, 4 weeks old) were purchased from the animal center of the National Science Council in Taiwan. The mice were anesthetized i.p. with 50 μg/kg of body weight of ketamine (100 μg/ml)–xylazine (10 μg/ml). Cells (1×10^6/100 μl) were injected subcutaneously on one side of the lateral back wall close to the shoulder of each mouse. Growth of the xenograft was measured by vernier calipers every 5 days. Tumor volume was calculated with the following formula: Volume = π/6×larger diameter×(smaller diameter)^2 (Tsui et al. 2012b).

Reporter vector constructs and transient gene expression assay

The human IL6 reporter vector, maspin reporter vector, and NDRG1 reporter vector were constructed as
previously described (Tsui et al. 2008b, 2012a). A 6·5-kbp DNA fragment was subtracted from a BAC clone (RP11-679I18; Invitrogen) and cloned into the pGEM-3 vector (Promega BioScience) with the BamH1 cutting site. The DNA fragment containing the promoter of the 5′-flanking region of the matriptase gene (−3468 to −9) was synthesized with matriptase-promoterR (5′-CCATGGCTCACACTCCGGCCCGGCCGCCCCCA-3′) and T7 primers (Promega BioScience) by PCR and cloned into the pGL3-Basic vector at the KpnI and NcoI cutting sites. Proper ligation and orientation of reporter vectors were confirmed by extensive restriction mapping and sequencing. Cells were plated onto 24-well plates at 1×10⁴ cells/well 1 day before transfection. They were then transiently transfected using TransFast transfection reagent (0·6 µg/well; Promega Biosciences) with 1 µg/well of reporter vector and 0·5 µg/well of pCMVSPORTβGal (Invitrogen) as described previously (Chung et al. 2011). Luciferase activity was determined in relative light units using a LumiCount Luminometer (Packard Bioscience, Meriden, CT, USA) and adjusted according to the β-GAL enzymatic activity.

**Statistical analysis**

Results are expressed as mean ± S.E.M. of at least three independent replications of each experiment. Statistical significance was determined using the one-way ANOVA and Student’s t-test and using the SigmaStat program for Windows, version 2.03 (SPSS, Inc.).

**Results**

GDF15 expression in prostate carcinoma cells was assessed using RT-PCR (Fig. 1A). LNCaP cells had the highest expression of GDF15 of any cells used in this study. Results of RT-qPCR (Fig. 1B) indicated that the quantitative level of GDF15 mRNA was threefold higher in LNCaP cells than in PC-3 cells. Other cell lines used in this study, namely HPV-7-PZ, HPV-10-CA, and DU145, expressed extremely low levels of GDF15. Similar results were obtained by ELISA (Fig. 1C).

The human GDF15 cDNA was stably transfected in the prostate carcinoma PC-3 cells to evaluate the function of native GDF15 on cell proliferation and invasion. The results of immunoblot assay of GDF15 for the whole-cell extract revealed a major band at 35–37 kDa, which represented the pre-cleavage form of GDF15. Results of immunoblot assay (Fig. 2A), RT-qPCR (Fig. 2B), and ELISA (Fig. 2C) confirmed that GDF15 was overexpressed in two PC-3 cell lines that stably overexpressed GDF15 (PCGDF15-1 and PCGDF15-2). Results from the ³H-thymidine incorporation assay revealed that GDF15-transfected cells (PCGDF15-1 and PCGDF15-2) increased 4·75- and 5·79-fold respectively in cell numbers, but mock-transfected PC-3 cells (PCDNA) only increased 3·94-fold in cell numbers after 5-day incubation, indicating that stable overexpression of GDF15 in PC-3 cells enhanced cell proliferation (Fig. 2D).

Results of the in vitro Matrigel invasion assay indicated that the invasive ability of PC-3 cells that overexpressed GDF15 was approximately twofold higher than that of mock-transfected PC-3 cells (Fig. 3A). PC-3 cells that overexpressed GDF15 (PCGDF15-2) increased approximately threefold the ability to grow in soft agar in comparison with mock-transfected PC-3 (PCDNA) cells (Fig. 3B).
GDF15 on the growth of tumors in vivo was evaluated using xenografts in nude mice. PCGDF15-2 and PCDNA cells were injected subcutaneously onto the back of the shoulder of athymic nude mice. The mock-transfected PC-3 cell (PCDNA)-generated tumors grew slower than PC-3 cells that overexpressed GDF15 (PCGDF15-2). After 57 days of growth, tumors derived from PCDNA cells were only ~50% of the size of tumor derived from PCGDF15-2 cells (Fig. 3C). These results were similar to those obtained from in vitro soft agar assays, suggesting that GDF15 enhances tumorigenic activity in prostate carcinoma cells in vivo. ELISA assay of serum samples, which were collected from experimental animals by cardiocentesis, revealed that levels of GDF15 in the circulation were threefold higher in animals that have been injected with PCGDF15-2 cell than in animals that have been injected with PCDNA cells (Fig. 3D).

In order to evaluate the mechanisms involved in the effect of GDF15 on cell proliferation and metastasis, we measured differences in the gene expression of matriptase, maspin, NDRG1, and IL6 between PCDNA and PCGDF15-2 cells. Results of RT-PCR (Fig. 4A) and immunoblot assays (Fig. 4B) revealed that stable overexpression of GDF15 in PC-3 cells enhanced matriptase, NDRG1, and IL6 gene expression but blocked maspin gene expression. Results of the transient gene expression assays also indicated that overexpression of GDF15 enhanced matriptase (Fig. 4C) and NDRG1 (Fig. 4D) promoter activity and attenuated the promoter activity of the maspin gene (Fig. 4E). The ELISA assays revealed that levels of IL6 secretion of PC-3 cells were about twofold higher in PC-3 cells (PCGDF15-2) that stably overexpressed GDF15 than in mock-transfected (PCDNA) cells (Fig. 4F).

To further investigate the function of GDF15 on cell proliferation and invasion, GDF15 was transiently knocked down using siRNA in PC-3 cells. Both the expression levels of GDF15 in GDF15 knockdown PC-3 (PC-GDF15si) cells and mock knockdown (PC-COLsi) cells were determined by immunoblot assay and ELISA. Results of immunoblot assay (Fig. 5A) and ELISA (Fig. 5B) indicated that expression of GDF15 was blocked in GDF15 knockdown PC-3 (PC-GDF15si) cells. Results of immunoblot assays also found that transient knockdown of GDF15 in PC-3 cells augmented maspin protein expression but blocked matriptase and NDRG1 protein expression (Fig. 5A). Moreover, 38-6% of IL6 secretion was blocked as GDF15 was transiently knocked down in PC-3 cells (Fig. 5B).

Results from 3H-thymidine incorporation assays revealed that GDF15 knockdown PC-3 cells increased
only 2.06-fold in cell numbers but the mock-transfected PC-3 cells (PC-COLsi) increased 3.61-fold in cell numbers after 5 days of incubation, suggesting that transient knockdown GDF15 in PC-3 cells attenuated cell proliferation (Fig. 5C). Results of the in vitro Matrigel invasion assays indicated that the invasive ability of mock-transfected PC-3 cells was ~1.4-fold higher than that of GDF15 knocked down cells (Fig. 5D).

In order to further determine the correlation between the expressions of IL6 and GDF15, we cloned two subculture cell lines, namely LNCaP cells that overexpressed IL6 (LN-IL6) and IL6-knockdown PC-3 cells (PC-IL6si), as described in Materials and methods section. The expression levels of IL6 in the LN-IL6 cells (Fig. 6A), in the PC-IL6si cells (Fig. 6B), and in the mock-transfected cells (LN-DNA and PC-COLsi) were determined by RT-PCR and ELISA. Immunoblot assays indicated that levels of GDF15 in LN-IL6 cells were 1.8-fold higher than that of GDF15 in LN-DNA cells (Fig. 6C) and that levels of GDF15 in PC-IL6si cells were 31% lower than that of GDF15 in the PC-COLsi cells (Fig. 6D). The ELISA results demonstrated similar findings, namely that overexpression of IL6 led to an increase in GDF15 expression in LNCaP cells (Fig. 6E) and that expression of GDF15 protein was blocked in PC-3 cells when IL6 was knocked down (Fig. 6F). Interestingly, immunoblot assays revealed that knockdown IL6 in PC-3 cells blocked matriletase but enhanced maspin and MDRG1 gene expression (Fig. 6G).

We also evaluated the effect of recombinant human GDF15 on the cell proliferation of PC-3 and LNCaP cells. The 3H-thymidine incorporation assays indicated that GDF15 treatment enhanced the proliferation of PC-3 cells; however, GDF15 decreased the proliferation of LNCaP cells after 48 h of incubation (Fig. 7A). The ELISA results also indicated that GDF15 treatment for 24 h induced IL6 secretion in a dose-dependent manner (Fig. 7B). Results of immunoblot assays revealed that GDF15 treatment enhanced NDRG1 and

**Figure 3 Stable overexpression of GDF15 enhances cell invasiveness and tumorigenesis of PC-3 cells.** (A) The invasive activity of cells was determined by the in vitro Matrigel invasion assay. Data are presented as the mean percentage (± S.E.M.) of the OD in relation to that of the PCDNA cell group. (B) In vitro tumorigenesis of PCDNA and PCGDF15-2 cells was determined by soft agar assays. Cells were grown in 0.4% soft agar for 3 weeks, stained with p-iodonitrotetrazolium violet solution, and then photographed. The cells were counted and the results are represented graphically. (C) Nude mice were inoculated subcutaneously with PCDNA (white circle) or PCGDF15-2 (black circle) cells. During the indicated days, tumor size was measured with vernier calipers. Data are presented as tumor size in mm3 (± S.E.M.; n=6) of inoculated cells measured at indicated time intervals. Blood samples were collected from experimental animals by cardiocentesis immediately after killing and were assayed for GDF15 (D) levels by ELISA. Data are presented as mean (± S.E.M.; n=6) of the GDF15 levels (∗P<0.01).
matriptase protein expression but blocked maspin protein expression (Fig. 7C). The immunoblot assays indicated that doxorubicin treatment not only induced p53 expression but also enhanced GDF15 gene expression in LNCaP cells (Fig. 7D, top). The ELISA results also showed that doxorubicin treatment for 24 h induced GDF15 secretion in a dose-dependent manner (Fig. 7D, bottom).

**Discussion**

GDF15 has divergent effects and pleiotropic functions in cancer and might contribute to the proliferation, migration, invasion, metastasis, and treatment resistance of cancer cells as well as tumor-induced anorexia and weight loss in the late stages of cancer (Mimeault & Batra 2010). In vitro and in vivo studies have shown that the involvement of GDF15 in cell growth inhibition, apoptosis induction, and enhancement of cancer invasiveness is dependent on cancer cell types (Liu et al. 2003, Lambert et al. 2006, Gokar et al. 2007).

The effect of GDF15 on cell proliferation in prostate carcinoma cells was inconclusive in previous studies. Studies of upregulation of GDF15 by vitamin D3 or isochaihulactone suggested cell growth suppression in prostate carcinoma LNCaP cells (Lambert et al. 2006, Chiu et al. 2011). However, a recent study indicated that a low dose of GDF15 (≤0·1 µg/ml) did not affect cell proliferation but attenuated cell invasion of PC-3 cells (Cheng et al. 2011). For comparison, we treated PC-3 cells with a higher concentration (0·2–0·8 µg/ml) of GDF15 in this study, which showed that GDF15 did enhance cell proliferation. Moreover, our results also demonstrated that exogenous GDF15 divergently interfered with the cell proliferation of p53 WT LNCaP cells and p53-null PC-3 cells, suggesting that...
were determined by immunoblotting assays. Controls were treated with recombinant human GDF15 as indicated for 24 h. Cell extracts were used in the immunoblot assays for determination of GDF15 and p53 expressions (top) and the relative density of the target gene/β-actin ratio (bottom). Data are presented as mean ± S.E.M. of the IL6 levels to that of the control group. (B) The PC-3 cells were cultured with various concentrations of doxorubicin as indicated for 24 h. Cell extracts were treated with various concentrations of recombinant human GDF15 as indicated for 24 h. Cell extracts were used in the immunoblot assays for determination of NDRG1, matriptase, and maspin expressions. (C) The PC-3 cells were cultured with various concentrations of recombinant human GDF15 as indicated for 48 h. Cell extracts were used in the immunoblot assays for determination of NDRG1, matriptase, and maspin expressions. (D) The LNCaP cells were cultured with various concentrations of doxorubicin as indicated for 24 h. Cell extracts were used in the immunoblot assays for determination of NDRG1, matriptase, and maspin expressions. The intensities of the different bands were analyzed using the ChemiGenius-associated GeneTools program (Syngene). The quantitative analysis was done by determining the intensity of target genes and β-actin in each band from three independent experiments. Data are presented as the fold-induction of the relative density of the target gene/β-actin (± S.E.M.) in relation to the mock-transfected controls. ELISA was used to determine the expression of GDF15 levels in LNCaP (E), PC-IL6si (F), and in the mock-transfected controls (LN-DNA and PC-COLsi). Data are presented as mean ± S.E.M. (n=6) of the GDF15 levels in LN-IL6 (E), PC-IL6si (F), and in the mock-transfected controls (LN-DNA and PC-COLsi). Data are presented as mean ± S.E.M. (n=6) of the IL6 levels. Expression levels of the GDF15 gene in LN-IL6 cells (G) and PC-IL6si cells (D) in relation to their mock-transfected controls (LN-DNA and PC-COLsi) were determined by immunoblot assays. The intensities of the different bands were analyzed using the ChemiGenius-associated GeneTools program (Syngene). The quantitative analysis was done by determining the intensity of target genes and β-actin in each band from three independent experiments. Data are presented as the fold-induction of the relative density of the target gene/β-actin (± S.E.M.) in relation to the mock-transfected controls. ELISA was used to determine the expression of GDF15 levels in LN-IL6 (E), PC-IL6si (F), and in the mock-transfected controls (LN-DNA and PC-COLsi). Data are presented as mean ± S.E.M. (n=6) of the GDF15 levels in LN-IL6 (E), PC-IL6si (F), and in the mock-transfected controls (LN-DNA and PC-COLsi). Data are presented as mean ± S.E.M. (n=6) of the IL6 levels. Expression levels of the GDF15 gene in LN-IL6 cells (G) and PC-IL6si cells (D) in relation to their mock-transfected controls (LN-DNA and PC-COLsi) were determined by immunoblotting assay.

Results of this study indicate that stable overexpression of GDF15 in PC-3 cells affects cell proliferation and colony formation in soft agar, suggesting that GDF15 induces tumorigenic activity in prostate carcinoma cells. Our results indicated that GDF15 overexpression enhanced the invasive and metastatic properties of prostate carcinoma PC-3 cells while knocking down GDF15 attenuated cell motility, which is consistent with findings reported by Senapati et al. (2010). However, in a study by Cheng et al. (2011), it is shown that cell motility did not significantly enhance when GDF15 was transiently knocked down in PC-3 cells, although exogenous GDF15 treatments (0.02–0.1 μg/ml) did significantly block the invasive ability of PC-3 cells.

Studies have shown that GDF15 gene expression is controlled by multiple factors, such as p53, vitamin D3, TNF, Sp1, and Egr-1 (Shim & Eling 2005, Lambert et al. 2006, Zimmers et al. 2006). We used doxorubicin-treated LNCaP cells, which expressed wild-type p53 and the effects of GDF15 on prostate carcinoma cells may depend on both the GDF15 levels and the cell types.

Several clinical studies have also demonstrated that enhancement of GDF15 expression is positively correlated with poor prognosis and patient survival rates.
(Tsui et al. 2004), indicating that p53 upregulates GDF15 expression in LNCaP cells. Similar results have been found in a previous study, which indicated that ectopic expression p53 induced GDF15 expression in PC3 and ALVA-3 PCa cells (Lambert et al. 2006, Cheng et al. 2011).

The mechanisms governing the effect of GDF15 on the expression of other genes are still not well known. Iczkowski & Pantazis (2003) suggested that TGF-βRII is likely to form part of the complex where GDF15 exerts its effect on body weight through the regulation of the neuropeptide Y (NPY) gene and the pro-opiomelanocortin (POMC) gene in the mouse hypothalamus. An in vitro study found that GDF15 induces apoptosis by blocking expression of metallothionein 1E, RhoE, and catenin δ1 genes (Liu et al. 2003), and the xenograft animal study indicated that GDF15 inhibits tumorigenesis by suppressing insulin-like growth factor-1 and cyclin D1 of DU145 cells (Wang et al. 2012). Chiu et al. (2011) suggested that isochaihulactone induces the expression of GDF15 via JNK activation in LNCaP cells. A recent study showed that overexpression of GDF15 induces metastasis of human prostate cancer cells through a FAK-RhoA signaling pathway (Senapati et al. 2010). This study is the first to report the dysregulation of IL6, matriptase, maspin, and NDRG1 expression by GDF15 in PC-3 cells.

Matriptase is an epithelium-derived type II transmembrane serine protease that has been implicated in activating substrates such as pro-hepatic growth factor and pro-uromodulin plasminogen activator (Lin et al. 1999, Wu et al. 2010). Inhibition of endogenous matriptase synthesis or treatment of cells with matriptase inhibitors has been shown to reduce invasion of prostate carcinoma cells, suggesting that matriptase plays a role in the properties of the invasive phenotype (Forbs et al. 2005, Sanders et al. 2006, Tsui et al. 2008a). In this study, we found that overexpression of GDF15 resulted in increased matriptase expression in vitro, which may account for the increasing invasiveness of PC-3 cells with forced overexpression of GDF15.

Maspin, a member of the serine protease inhibitor family, has been demonstrated to induce a more prominent actin cytoskeleton, to reduce invasion capacity, to increase the rate of spontaneous apoptosis, and to alter proteasome function in maspin-expressing cells (Li et al. 2007). In prostate cancer, maspin inhibits tumor growth, reduces bone metastasis, and decreases angiogenesis in vitro and in vivo (Chen et al. 2003, Watanabe et al. 2005, Hall et al. 2008). We found that PC-3 cells engineered to overexpress GDF15 blocked maspin gene expression. This finding might explain, in part, how overexpression of GDF15 leads to an increased proliferation and invasiveness of prostate carcinoma cells by GDF15.

NDRG1 is known to play important roles in both androgen-induced cell differentiation and inhibition of prostate metastasis (Mostaghel et al. 2007, Tu et al. 2007, Chung et al. 2012). The expression of NDRG1 was shown to be inversely correlated with Gleason score and overall survival rate in patients with prostate cancer (Bandyopadhay et al. 2003, Song et al. 2010). Interestingly, results of this study indicated that GDF15 upregulates NDRG1 gene expression, while knockdown of GDF15 downregulates NDRG1 protein expression in PC-3 cells. The mechanisms and physiological function governing the upregulation of NDRG1 by GDF15 in PC-3 cells are still unknown. As results of this study indicated that GDF15 treatment enhanced proliferation of PC-3 cells but attenuated proliferation of LNCaP cells, therefore, the upregulation of NDRG1 by GDF15 may explain the divergent effects of GDF15-related proliferation and invasion of different types of cancer cells (Mimeault & Batra 2010).

IL6 is a multifunctional cytokine, and an activator of androgen receptor in prostate cancer may yield either tumor cell proliferation or differentiation. Prolonged treatment with IL6 results in generations of sublines that express a more malignant prostate phenotype (Culig 2011). Previous studies have shown that plasma IL6 levels have prognostic significance in patients with metastatic hormone-refractory prostate cancer (George et al. 2005). An increase in serum cytokines including IL6 was found in cachectic patients with prostate carcinoma, and immunostaining levels of IL6 and its receptor increased with Gleason score (Pfitzenmaier et al. 2003, Royuela et al. 2004). In vitro studies have also indicated that IL6 enhanced the expression of prostate-specific antigen, a tumor marker of prostate cancer, in LNCaP cells (Tsui et al. 2011b). Results from in vitro studies have shown that both IL6 and IL6 receptors are expressed and that IL6 should be considered a positive growth factor in PC-3 cells (Selander et al. 2006, Tsui et al. 2008b). In this study, we found that IL6 was upregulated in PC-3 cells that have been engineered to overexpress GDF15; moreover, IL6 secretion was blocked when GDF15 was knocked down in PC-3 cells. It seems that the expression of GDF15 and that of IL6 are regulated by a positive feedback loop in prostate carcinoma cells. Our results indicated that IL6 and GDF15 have similar effects on the gene expression of matriptase and maspin. However, our results also showed that the expression of NDRG1 protein was enhanced in PC-3 cells while IL6 was knocked down, suggesting that GDF15 and IL6 may also regulate target genes in a divergent way. Further studies are warranted to investigate the mechanisms governing the effects of IL6 on GDF15 expression.

In conclusion, we have demonstrated that overexpression of GDF15 increases cell proliferation, tumorigenesis, and invasiveness of PC-3 cells. The
tumor-inductive effects of GDF15 on prostate carcinoma cells may be caused by the dysregulation of gene expression of maspin, matriptase, and IL6. The regulatory mechanisms governing the expression of GDF15 and IL6 are regulated by a positive feedback loop in prostate carcinoma cells.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of the research reported.

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