Loss of Expression of Growth Differentiation Factor-9 (GDF9) in Human Kidney Cancer and Regulation of Growth and Migration of Kidney Cancer Cells by GDF9

PENG DU[1,2,3], LIN YE[1,2], HAN LI[4], FIONA RUGE[1,2], YONG YANG[3] and WEN G. JIANG[1,2]

[1]*Cardiff University-Peking University School of Oncology Joint Institute,*
[2]*Metastasis and Angiogenesis Research Group, Cardiff University School of Medicine, Cardiff, U.K.;*
[3]*Key laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Urology, Peking University Cancer Hospital, Haidian District, Beijing, P.R. China;*
[4]*Department of Urology, Chaoyang Hospital, Capital Medical University, Beijing, P.R. China*

Abstract. *Background: Growth differentiation factor-9 (GDF9), a member of the bone morphogenetic protein (BMP) family and the transforming growth factor (TGF)-beta superfamily, has recently been implicated in the biological control of cancer cell behaviour. It has also been implicated in the development and spread of solid cancer. However, the role of GDF9 in kidney cancer remains to be investigated. In the present study, the expression of GDF9 in normal and malignant human kidney tissues and its molecular and cellular impact on human kidney cancer cells were investigated. Materials and Methods: The expression of GDF9 in human kidney tissues and kidney cancer cell lines (UMRC-2 and CAKI-2) was assessed at both the mRNA and protein levels using reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry, respectively. GDF9 overexpression was induced by a mammalian GDF9 expression construct. The effect of GDF9 expression on cellular functions was examined in kidney cancer cells overexpressing GDF9 using a variety of in vitro assays. Results: In normal kidney tissues, stronger staining of GDF9 was seen in renal tubular epithelial cells, both in the cytoplasm and in the nucleus. In contrast, the staining of GDF9 was notably weak or absent in cells of tumour tissues. Human kidney cancer cell lines UMRC-2 and CAKI-2 had lost their GDG-9 expression. Overexpression of GDF9 reduced in vitro invasion and cellular growth and migration of kidney cell lines in vitro. Using the electric cell-substrate sensing (ECIS) method, it was further revealed that overexpression of GDF9 in these cells markedly reduced cellular migration and adhesion. Conclusion: Human kidney tumours have a reduced or loss of expression of GDF9. In vitro, GDF9 overexpression suppresses the invasiveness, growth and migration of kidney cancer cells. This suggests that GDF9 is a potential tumour suppressor and may have prognostic and therapeutic implications in human kidney cancer.*

Correspondence to: Professor Yong Yang, Department of Urology, Peking University Cancer Hospital, Fucheng Road, Haidian District, Beijing, China. E-mail: yoya_urology@sina.com; or Professor Wen G. Jiang, Metastasis & Angiogenesis Research Group, Cardiff University School of Medicine, Cardiff, CF14 4XN, U.K. Tel: +44 2920742895, Fax: +44 2920742896, e-mail: jiangw@cf.ac.uk

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Growth and differentiation factor 9, GDF9 was initially discovered from the oocytes of mouse ovaries, neonatal and adults alike (1). The human homolog of GDF9 was subsequently identified and found to share 90% identity in its amino acid sequence with the mouse GDF9 (2, 3). GDF9 is a member of the transforming growth factor-beta (TGF-β) superfamily (4). For the past decade, TGF-β has been shown to be a growth-inhibitory cytokine in different cell types, including kidney cancer cells (5, 6). Bone morphogenetic proteins (BMPs) are a subfamily of the TGF-β superfamily and have been shown to be particularly important in the bone formation process, while several members have been implicated in the pathogenesis of different kinds of cancers (7-9). The past decade has witnessed a significant progress in identifying BMPs as regulators of a large variety of important processes, including cell growth, apoptosis, differentiation and invasion, in a range of cell types including cancer cells (8-14).

Although GDF9 was initially thought to be restricted to oocytes of the ovaries and is essential in folliculogenesis (2, 3, 10), recent studies have shown that the factor is distributed more widely in the body. For example, GDF9 has been

reported to be expressed in testis, pituitary gland, adrenal gland and adrenocortical cancer in mouse (11) and in human brain, liver, kidney, prostate, bladder, skin cancer, breast cells and tissues (8, 9, 11-13). The function of GDF9 has been reported, mostly in oocyctes; it promotes the transition of granulosa cells from G_0/G_1 to G_2/M phases and suppresses follistatin (FST) and follistatin-like 3 (FSTL3) production in granulosa cells. In cancer cells, the effects of GDF9 are less well-characterised. In breast cancer, highly aggressive breast cancer cells did not express GDF9. On forced expression of GDF9, breast cancer cells became less invasive (14). GDF9 up-regulation was reported in an aggressive oral carcinoma cell line (13). Recent research has also shown that GDF9 can promote the growth rate of both PC-3 and DU-145 prostate cancer cells by protecting the cells from caspase-3-mediated apoptosis, and suggests that GDF9 may aid in the progression of prostate cancer by acting as a survival factor (15). It has also been recently reported that GDF9 was able to induce epithelial-to-mesenchymal transition (EMT) in prostate cancer cells (16) *via* an activin receptor-like kinase 5 (ALK5)-dependent pathway, an essential receptor signalling pathway for the BMP family (17). Mutations appear to be less common in clinical conditions (18). These studies suggest that GDF9 plays contrasting roles in different malignancies.

Although GDF9 has been implicated in the progression of certain types of tumours, its role in kidney cancer remains unknown. In the present study, we examined the expression of GDF9 in normal and malignant human kidney tissues, and the effect of GDF9 on invasion, growth, adhesion and migration of kidney cancer cells.

Materials and Methods

Materials, cell lines and tissue samples. All cell lines used in this study were obtained from the European Collection of Animal Cell Culture (ECACC, Porton Down, Salisbury, UK). Cells were routinely maintained in DMEM-F12 medium supplemented with 10% foetal bovine serum and antibiotics. Polyclonal goat anti-GDF9 and monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other reagents or kits were obtained from Sigma-Aldrich, Poole, UK, unless otherwise stated. Twenty-one kidney samples were collected from patients with kidney cancer, immediately after surgery at the Department of Urology, Chaoyang Hospital, Capital Medical University, Beijing, China, including twelve kidney tumour tissues and nine normal-background kidney tissues. These tissues were collected immediate after radical nephrectomy of renal tumour. All protocols were reviewed and approved by the Ethical Committee and all patients gave their written informed consent.

Immunohistochemical staining procedure for kidney tissues. Paraffin-embedded human kidney tissues were sectioned to 6-μm thickness. The slides were first de-waxed by gradient treatment of slides using xylene, 100-50% gradient of ethanol. Antigen retrieval was conducted by microwaving the sections for 20 min in an EDTA buffer. After washing with a Tris balanced buffer solution, the endogenous peroxidase was blocked using H_2O_2 buffer. The sections were then placed in Optimax washing buffer for 5-10 min to rehydrate and incubated for 30 min in horse serum-containing blocking solution and probed with the primary antibody (1:150 dilution). Following extensive washing, sections were incubated for 30 min with a biotinylated secondary antibody (Multilink swine anti-goat/mouse/rabbit immuno-globulin; Dako Inc. Carpinteria, CA, USA). Following washing, an avidin-biotin complex (Vector Laboratories) was applied to the sections followed by extensive washing. Diaminobenzidine chromogen (Vector Laboratories, Petersborough, England, UK) was then added to the sections, which were incubated in the dark for 5 min. Sections were then counterstained in Gill's haematoxylin and dehydrated in ascending grades of methanol before clearing in xylene and mounting under a coverslip.

Construction of GDF9 expression vectors and transfection. The mammalian expression constructs were the same as recently reported (17). Purified GDF9 transgenes and control plasmid vectors were then transfected into UMRC-2 and CAKI-2 cells using an Easyjet Plus electroporator (EquiBio Ltd, Kent, UK). After up to three weeks of selection with blasticidin, the transfectants were verified for their expression of GDF9 and successful clones were used in subsequent studies.

RNA isolation and reverse transcription-polymerase chain reaction (PCR). RNA was isolated using a total RNA isolation kit, Triagent (Sigma). Reverse transcription was performed using the DuraScript™ RT-PCR kit, followed by PCR using a REDTaq™ ReadyMix PCR reaction mix (primer sequences are shown in Table I). Cycling conditions were 94˚C for 5 min, followed by 30 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 40 s. This was followed by a final 10-min extension period at 72˚C. The products were visualized on 1.5% agarose gel stained with ethidium bromide.

Western blot analysis of GDF9 expression. The protein concentraation in cell lysates was determined using the DC Protein Assay kit (BIO-RAD, Hemel Heamstead, England, UK) and an ELx800 spectrophotometer (BIO-TEKTM, Wolf Laboratories, York, England, UK). Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto nitrocellulose sheets. Proteins were then respectively probed with the anti-GDF9 antibody (1:400) and peroxidase-conjugated secondary antibody, with stringent washings between each step. Protein bands were visualized using the Supersignal™ West Dura system (Pierce Biotechnology, Inc., Rockford, IL, USA), and photographed using an UVITech imager (UVITech, Inc., Cambridge, UK).

In vitro cell growth, invasion and matrix adhesion assays. Cell growth was assessed using a method previously reported by our laboratories using a crystal violet method (19, 20). *In vitro* invasion assay was performed according to a standard procedure previously established (21-23), by using 8-μm poresized transwell inserts coated with 50 μg Matrigel (BD Matrigel™ Basement Membrane Matrix, Becton Dickson, Franklin Lakes, NJ, USA). *In vitro* cell matrix adhesion assay was based on a previously described method (22, 23). A total of 30,000 cells were added to each well of a 96-well plate, previously coated with Matrigel (5 μg/well). After 40 min of incubation, non adherent cells were washed-off using balanced saline solution (BSS).

Table I. *Sequences of all the primers used in this study.*

Primer	Sense (5'-3')	Antisense (5'-3')
GDF9 screening	ATGGCACGTCCCAACAAAT	ATTTGACAGCAGAGGAAAAA
GDF9 expression	GCGCTTTTCAAAGTTCTATC	GGTCACATCAATCTGAATCC
GAPDH	ATGATATCGCCGCGCTCGTC	GCTCGGTGAGGATCTTCA

GDF9: Growth and differentiation factor 9; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

The remaining adherent cells were then fixed and stained with crystal violet. The number of adherent cells in random fields were observed and counted under a microscope.

Electric cell-substrate impedance sensing (ECIS)-based attachment and migration assay. The ECIS Z-Theta instrument and 96W1E arrays (Applied Biophysics, Inc., NJ, USA) were used in the study, following a method recently reported (24, 25). Briefly, the same number of test cells (60,000 per well) were added to each well of the ECIS arrays. Impedance and resistance of the cell layer were immediately recorded for a period of up to 20 h. When confluence was reached, the monolayer in each well was electrically wounded at 1,400 μA and 6,000 Hz for 30 s to create a 250 μm wound per well. Impedance and resistance of the wounded cells as they migrated into the wound was then recorded for a period of up to 20 h. Data were analysed using the ECIS software, supplied by the manufacturer and are presented here as 3-D models, 2-D migration traces and calculated cell migration.

Statistical analysis. All statistical analysis was performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Non-normally distributed data were assessed using the Mann-Whitney test, while the two sample *t*-test was used for normally-distributed data. Differences were considered to be statistically significant at $p<0.05$.

Results

Expression of GDF9 in human kidney tissues and cell lines. In order to estimate the protein levels of GDF9 in human kidney tissues, we conducted immunohistochemical analyses using archival normal and malignant kidney tissues. As shown in Figure 1, renal tubular epithelial cells of normal tissues stained highly-positive for GDF9 (left column, indicated by arrows). The staining was largely confined to the cytoplasmic region of the cells. It is interesting to note that the kidney glomerulus appear to be completely negative for GDF9 (left column, arrows). Staining for GDF9 was absent in cancer cells of tumour tissues (right two columns). In cases where residual normal tissues were left, a clear contrast between tumour cells and normal tubular epithelial cells was seen (far right column).

The expression of GDF9, at mRNA and protein levels, was also examined in two kidney cancer cell lines , UMRC-2 and CAKI-2. These cells exhibited a complete lack of GDF9 expression (Figure 2).

Overexpression of GDF9 in kidney cancer cells. To investigate the impact of GDF9 on functions of kidney cancer cells, the constructed GDF9 expression vectors were utilised to overexpress GDF9 in kidney cancer cells. After selection using blasticidin, the expression of GDF9 in the transfected cells was verified using both RT-PCR and western blotting (Figure 2). Increased GDF9 expression of both mRNA (Figure 2A) and protein (Figure 2B) was seen in the GDF9-overexpressing UMRC-$2^{GDF9exp}$ cells, in comparison with the controls, UMRC-2^{WT} and UMRC-2^{pEF}. Similarly, overexpression of GDF9 was confirmed in CAKI-$2^{GDF9exp}$ cells, in comparison with CAKI-2^{WT} and CAKI-2^{pEF} control cells.

GDF9 is associated with the growth rate and invasion of kidney cancer cells. UMRC-2 and CAKI-2 cells with forced overexpression of GDF9 displayed a slower growth rate compared with the controls (Figure 3). Using the same cells, the invasiveness was further examined in the genetically modified cells. Interestingly, overexpression of GDF9 in the kidney cancer cell lines, UMRC-2 and CAKI-2, made the cells less invasive compared with the control cells (Figure 4).

Effect of GDF9 overexpression cell matrix adhesion in kidney cancer cells. We first examined the effect of GDF9 on the cell matrix adhesion of kidney cancer cell lines. Overexpression of GDF9 exhibited a significant inhibitory effect on cell matrix adhesion of the UMRC-2 cells ($p<0.01$ *vs.* both controls) (Figure 5A). Similarly, compared with CAKI-2^{WT} and CAKI-2^{pEF}, the number of adherent cells for CAKI-$2^{GDF9exp}$ was significantly reduced ($p<0.01$ *vs.* both controls) (Figure-5B).

The ECIS system was used to further investigate the effect of enhanced expression of GDF9 on UMRC-2 and CAKI-2 cell adhesion. The attachment capacity was markedly reduced in UMRC-$2^{GDF9exp}$ cells compared with UMRC-2^{WT} and UMRC-2^{pEF} cells (Figure 5C and 5D). Similarly, the attachment capacity was markedly reduced in CAKI-$2^{GDF9exp}$ cells compared with CAKI-2^{WT} and CAKI-2^{pEF} cells (Figure 5E and 5F).

Effect of GDF9 on migration of kidney cancer cells. The effect of GDF9 on UMRC-2 and CAKI-2 cellular motility was assessed using the ECIS system. The migration capacity was markedly

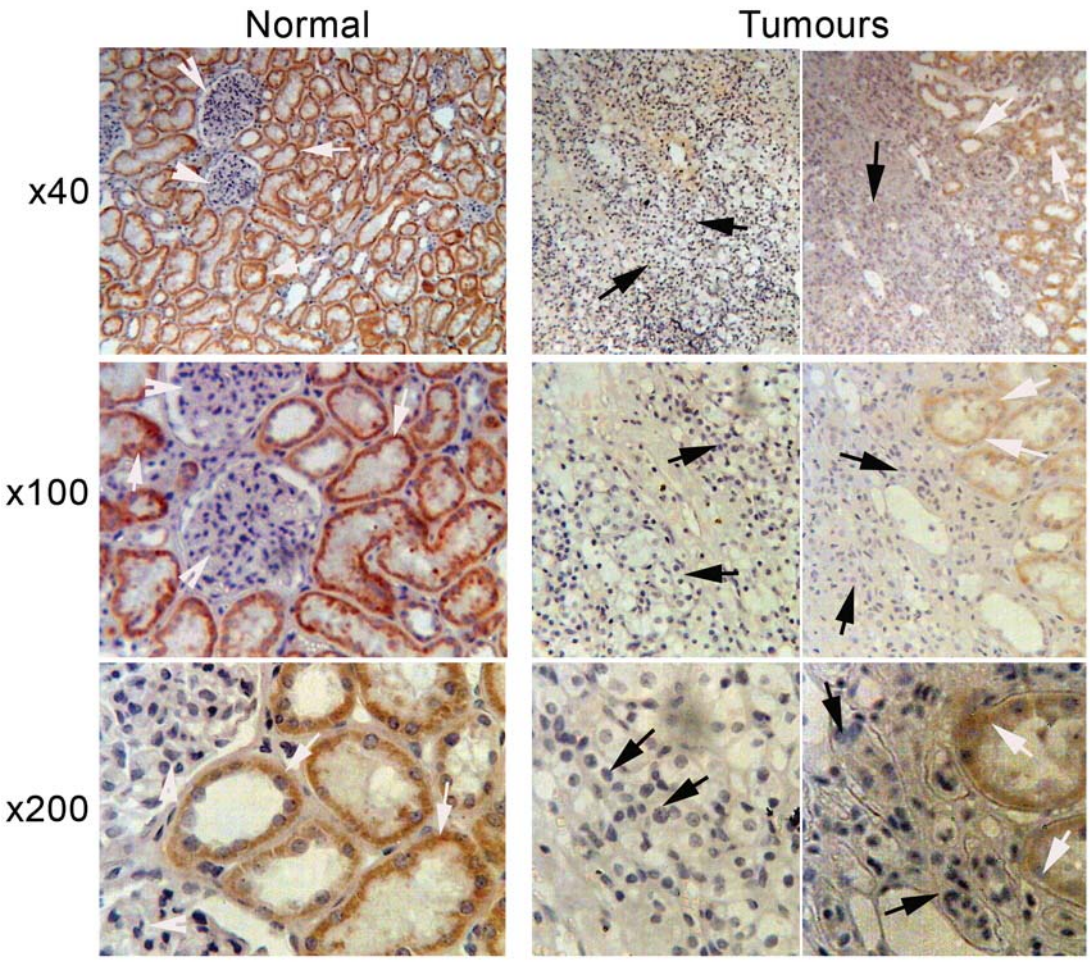

Figure 1. *Staining of GDF9 in human kidney tissues. Left: Normal human kidney tissues. The renal tubular epithelial cells were highly-positively stained for GDF9 (arrows). Interestingly, the glomeruli were completely negative for GDF9 (arrow heads). Stromal cells were also negative for GDF9 staining. Middle and Right: Kidney cancer tissues. Kidney cancer cells were negative for GDF9 (arrows). The right column shows tumour tissues with residual normal tissues. While tumour cells were negative for GDF9 (black arrow), the residual normal tubular epithelium (white arrows) remains positively-stained for GDF9.*

reduced in UMRC-$2^{GDF9exp}$ cells compared with UMRC-2^{WT} and UMRC-2^{pEF} cells (Figure 6A-C). Similarly, the migration capacity was markedly reduced in CAKI-$2^{GDF9exp}$ cells compared with CAKI-2^{WT} and CAKI-2^{pEF} cells (Figure 6D-F).

Discussion

Kidney cancer accounts for approximately 2% of all cancer cases worldwide. Globally, the incidence and mortality rates are increasing by 2-3% per decade. Kidney cancer is relatively uncommon in Asia compared with the West, but its incidence is increasing in more developed Asian nations (26, 27). Accordingly, a common complication in advanced kidney cancer patients is morbidity from bone metastasis. Skeletal metastases occur in around one third of patients with advanced or metastatic kidney cancer (28).

GDF9 is a member of the BMP family also known as a well-established follicular growth factor that has been shown to be vital during early follicular development (3). GDF9 also shares with some of the BMP receptors during its signalling in the cells (29-31). Despite the importance of BMPs in cancer however, the role of GDF9 in cancer remains elusive. The role of GDF9 in tumour progression remains unclear and it is somewhat controversial whether or not GDF9 serves as a metastasis suppressor. In breast cancer, highly aggressive breast cancer cells did not express GDF9. On forced expression of GDF9, breast cancer cells became less invasive (14). Furthermore, another group demonstrated up-regulated levels of GDF9 in an aggressive oral carcinoma cell line (15). Recent research showed that GDF9 can promote the growth rate of both PC-3 and DU-145 prostate cancer cells by protecting the cells from caspase-3-mediated

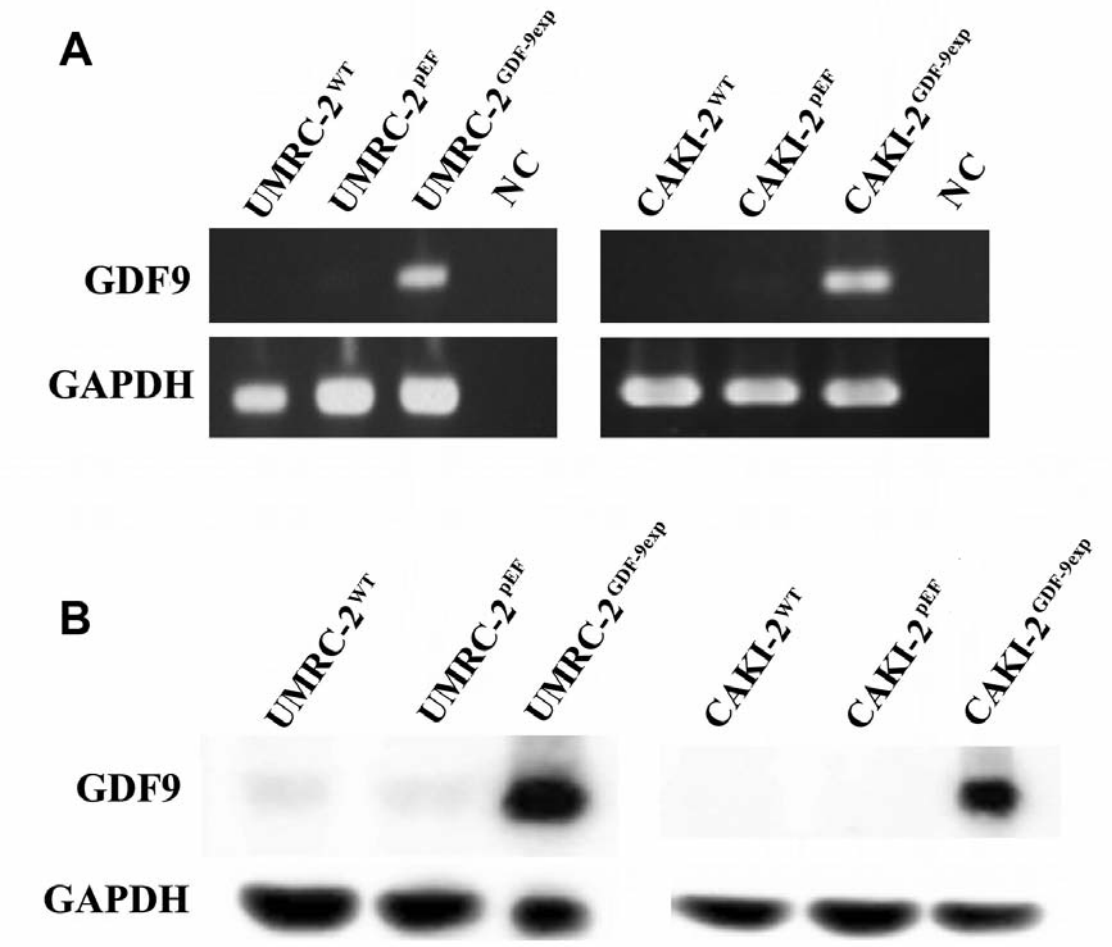

Figure 2. *Overexpression of GDF9 in kidney cancer cells. A: RT-PCR showed increased number of GDF9 transcripts in UMRC-2 and CAKI-2 cells transfected for GDF9 overexpression. GAPDH was examined as a house keeping gene. NC is the negative control. B: Changes of GDF9 protein expression in GDF9-overexpressing kidney cancer cells were verified using western blot analysis. GAPDH was used as the housekeeping control.*

apoptosis, and suggests that GDF9 may aid in the progression of prostate cancer by acting as a survival factor (18). Thus, the role of GDF9 in cancer and cancer metastasis remains open. To the best of our knowledge, the current study is the first report to examine the staining patterns of GDF9 in human kidney tissues and to test the impact on invasion, growth, adhesion and migration of kidney cancer cells by genetically manipulating the expression of GDF9.

Similarly to human breast cancer (14), GDF9 expression was seen at a lower level or was absent from kidney tumour tissue cells, compared with normal renal cells, tubular epithelial cells. Similarly, in kidney cancer cell lines, UMRC-2 and CAKI-2, expressed no GDF9. Moreover, overexpression of GDF9 reduced the invasion, growth, adhesion and migration of kidney cell lines *in vitro*. This is in contrast to what was observed in prostate cancer cells (16, 17). This indicates that in some types of human tumours, GDF9 is expressed at lower levels, although the opposite may be also true. It is interesting to note that both normal renal tubular epithelial cells and kidney cancer cells had little GDF9 in the nucleus. The nuclear existence of GDF9 is particularly interesting as it has been suggested that the cytoplasmic *versus* nuclear distribution pattern of the GDF9 protein may be a key feature in cancer and an important determinant of the contrasting role of GDF9 in different cancer types. The current study further indicates the possibility of a nuclear connection in the function of GDF9. Thus, changes in the overall level of staining of GDF9 in kidney cancer cells and in intracellular distribution appear to be a feature of human kidney tumour tissues. Any conclusions of clinical significance drawn here should be read with caution as the size of the clinical cohort is relative small in the present study. However, a larger cohort size will be very useful in further exploring the clinical and prognostic link.

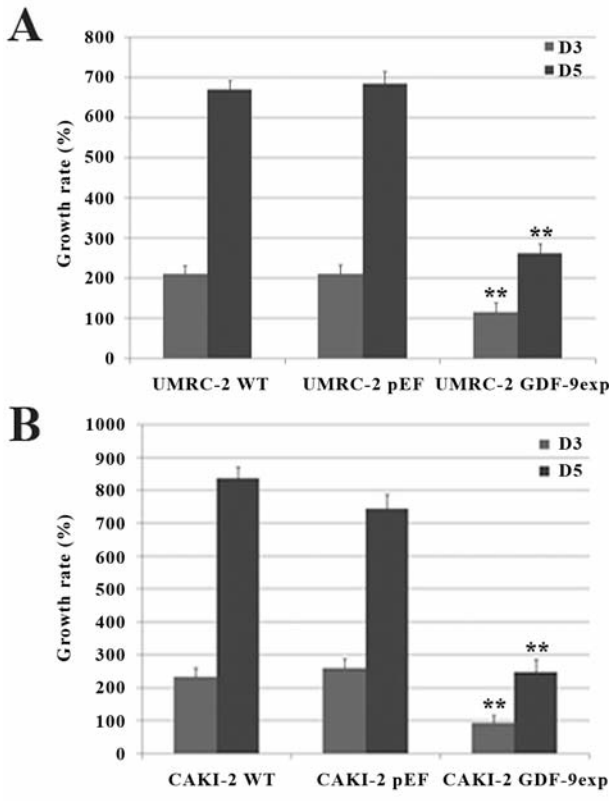

Figure 3. *Effects of GDF9 on the in vitro growth of kidney cancer cells. A: The cell growth (third day and fifth day, both being normalized by that of the first day) of UMRC-2^{GDF9exp} cells was significantly reduced in comparison to that of the two controls, UMRC-2^{WT} and UMRC-2^{pEF}. B: The cell growth of CAKI-2^{GDF9exp} cells was significantly reduced in comparison to that of the two controls, CAKI-2^{WT} and CAKI-2^{pEF}. **p<0.01 vs. control. Representative data from three independent experiments are shown.*

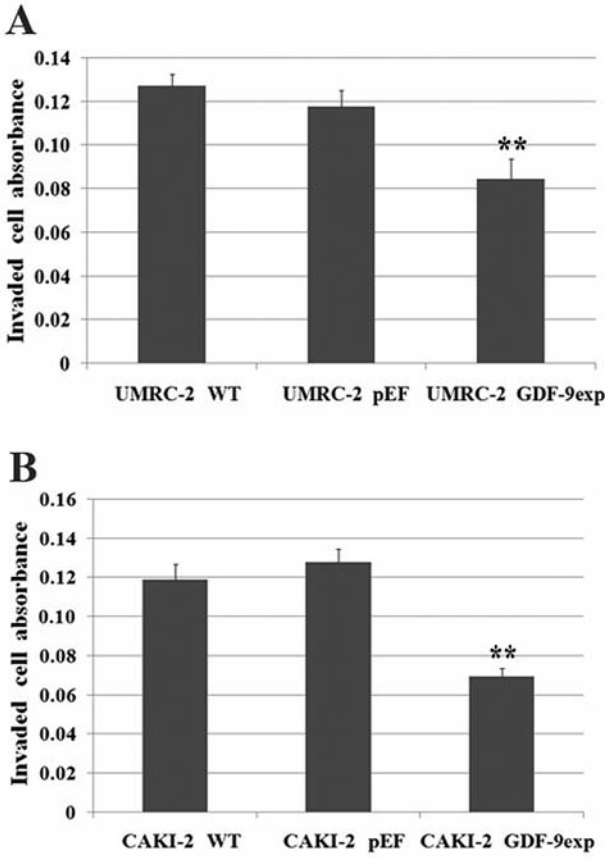

Figure 4. *Effects of GDF9 overexpression on the invasion of kidney cancer cells. A: UMRC-2 cells; B: CAKI-2 cells. **p<0.01 versus the controls. Representative data from three independent experiments are shown.*

In the present study, we employed methods to genetically alter the expression of GDF9 in kidney cancer cells, namely the overexpression approach. In clear contrast to normal expression, forced overexpression of GDF9 in two kidney cancer cell lines, UMRC-2 and CAKI-2, resulted in a reduction of invasion, adhesion and migration. This indicates that GDF9 plays a key role in the control of the aggressiveness of kidney cancer cells. The current study has also demonstrated that over-expression of GDF9 also resulted in reduced cell growth *in vitro*. Primary breast cancer samples from patients with good predicted prognosis, had significantly higher levels of GDF9 compared to patients with poor prognosis including those who exhibited metastasis, local recurrence, or had died from breast cancer (16). This evidence strongly suggests that GDF9 is a potential tumour suppressor in these tumours. This suggestion is further supported by our *in vitro* results, in which GDF9 expression exhibited an inhibitory effect on the growth of kidney cancer cells.

In conclusion, the current study shows that GDF9 expression in kidney cancer is reduced. GDF9 overexpression can suppress the aggressiveness of kidney cancer cells through inhibiting cell invasion, growth, adhesion and migration. The current study, thus,] suggests that GDF9 may be putative tumour suppressor in kidney cancer.

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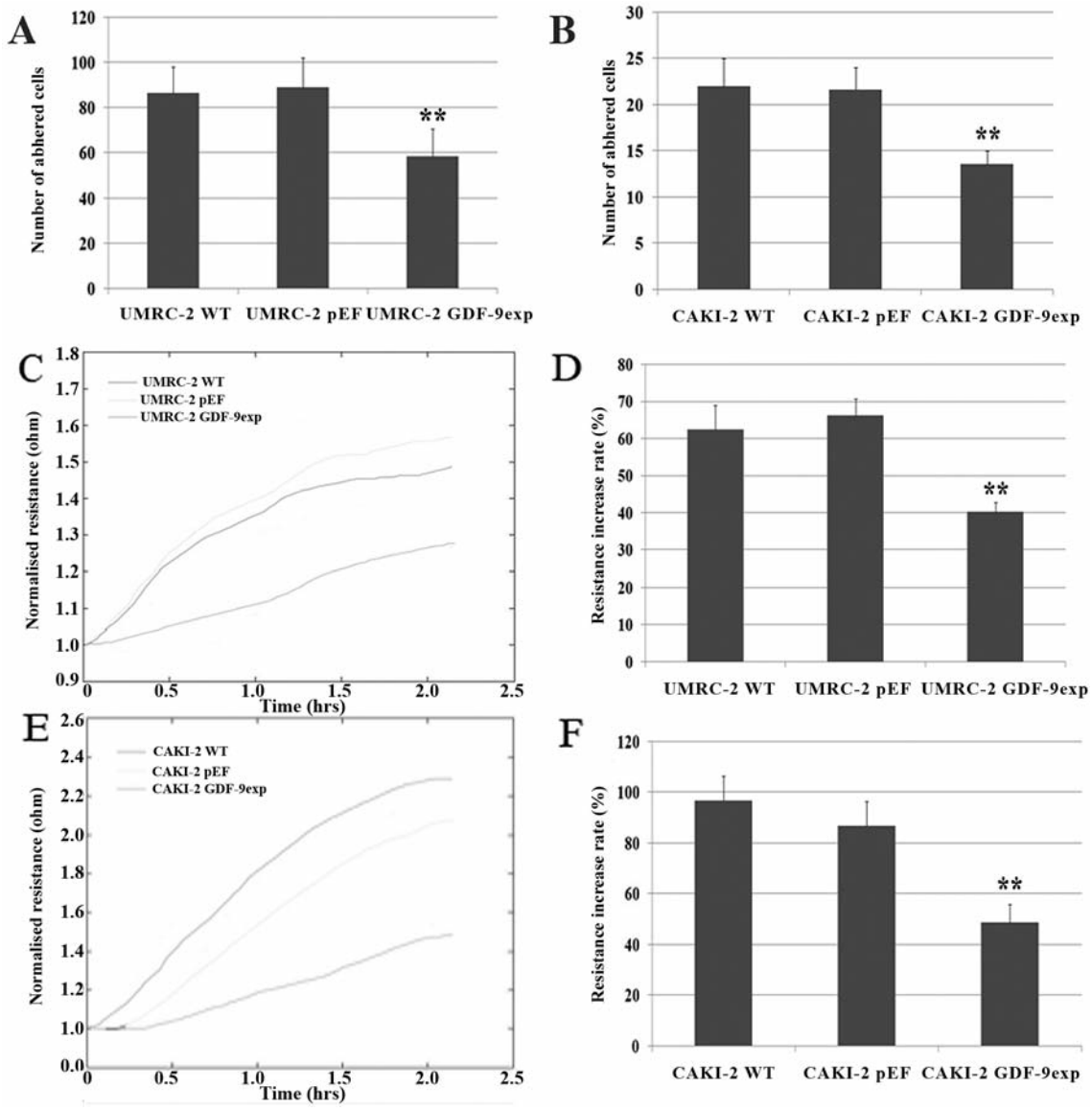

Figure 5. *Effects of GDF9 on adhesion of kidney cancer cells in vitro. A: Overexpression of GDF9 reduced the number of adherent UMRC-2^{GDF9exp} cells. **p<0.01 versus UMRC-2^{WT} and UMRC-2^{pEF} cells. B: Overexpression of GDF9 also reduced the number of adherent CAKI-2^{GDF9exp} cells. **p<0.01 versus CAKI-2^{WT} and CAKI-2^{pEF} cells. C and D: The UMRC-2^{GDF9exp} cells over-expressing GDF9 showed a markedly reduced attachment using an ECIS model. E and F: CAKI-2^{GDF9exp} cells over-expressing GDF9 also exhibited a markedly reduced attachment. ECIS RbA modelling of cell attachment indicated a significant reduction of attachment in GDF9 transfected cells. **p<0.01 versus wild-type and control plasmid control cells. All experiments were repeated three times.*

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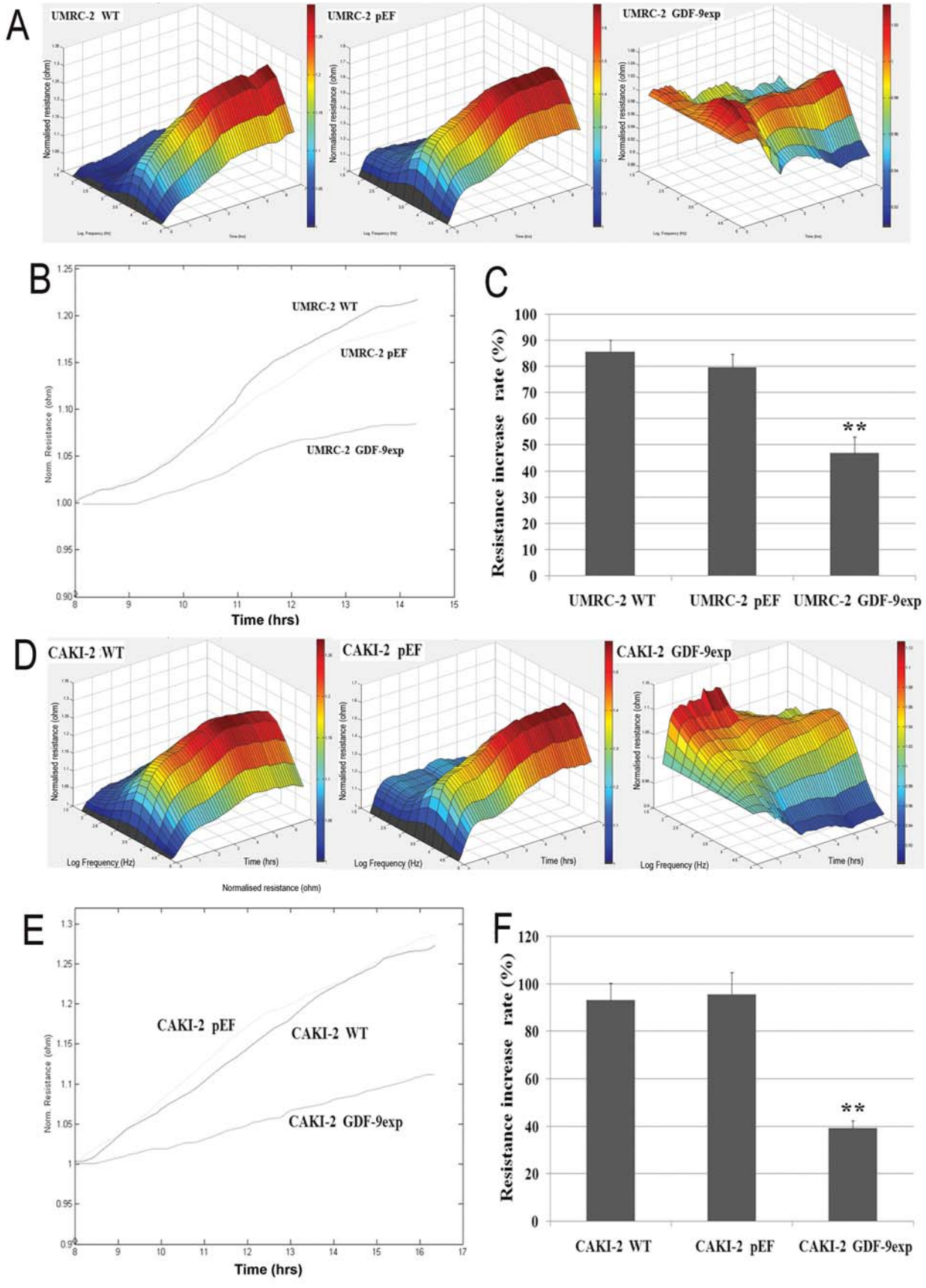
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UMRC-2 WT
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UMRC-2 WT
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Figure 6. *Effect of GDF9 on migration of kidney cancer cells as analysed by ECIS (wounding assays). The impedance changes during the migration process are shown. A-C: UMRC-2 cells. A: Cell migration after electric wounding demonstrated as a 3-D model. Wild-type (left) and control transfected (middle) cells exhibited a time-dependent migration into the wounds with the peak changes seen at 4,000 Hz. Over-expression of GDF9 (UMRC-2^{GDF9exp}, right) resulted in a marked reduction of migration, although the peak remained at 4,000 Hz. B: The UMRC-2^{GDF9exp} cells, over-expressing GDF9 exhibited markedly reduced migration, as shown by 2-D traces. C: CAKI-2^{GDF9exp} cells over-expressing GDF9 showed a markedly reduced migration. D-F: CAKI-2 cells. D: 3-D modelling of cell migration. E: ECIS RbA modelling of cell attachment indicated a significant reduction of attachment of GDF9-transfected UMRC-2^{GDF9exp} cells. **p<0.01 versus UMRC-2^{WT} and UMRC-2^{pEF} cells. F: ECIS RbA modelling of cell attachment indicated a significant reduction of attachment of GDF9-transfected CAKI-2^{GDF9exp} cells. **p<0.01 versus CAKI-2^{WT} and CAKI-2^{pEF} cells. All experiments were repeated three times.*

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