ARTICLE
Journal of Cellular Biochemistry 105:313–320 (2008)

Journal of Cellular Biochemistry

Placenta Growth Factor, PLGF, Influences the Motility of Lung Cancer Cells, the Role of Rho Associated Kinase, Rock1

Jinfeng Chen,[1] Lin Ye,[2] Lijian Zhang,[1**] and Wen G. Jiang[2*]

[1]*Department of Surgery, Peking University School of Oncology, Beijing Cancer Hospital, Beijing 100036, China*

[2]*Metastasis & Angiogenesis Research Group, Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, UK*

ABSTRACT

Placenta growth factor (PlGF) is a member of the VEGF family and has been implicated in the aggressive capacity of solid tumours, partly via its impact on angiogenesis. The present study determined the direct biological function of endogenous PlGF in lung cancer cells. From the human non-small cell lung cancer cell line A549 which expressed good level of PlGF, we created sublines within which PlGF expression was knockdown by way of anti-PlGF ribozyme transgenes. Remarkable reductions of both PlGF mRNA and protein by the ribozyme transgenes were revealed in A549 transfectants ($A549^{\Delta PlGF}$) using RT-PCR and Western blotting respectively. $A549^{\Delta PlGF}$ cells exhibited significantly reduced migration and adhesion compared with the wild-type ($A549^{WT}$) and the empty plasmid control ($A549^{pEF/His}$) cells. Immunocytochemistry and Western blotting further revealed that the expression of ROCK1, Rho associated kinase, was also reduced in $A549^{\Delta PlGF}$ cells, in comparison with wild-type and control cells. In addition, $A549^{\Delta PlGF}$ cells lost its response to a ROCK inhibitor, which otherwise strongly inhibited the motility of $A549^{WT}$ and $A549^{pEF/His}$ cells. These data indicate that PlGF directly regulates the motility of human lung cancer cells and that this regulation critically dependent on ROCK-1. The study further indicates that PlGF is a potential therapeutic target in lung cancer. J. Cell. Biochem. 105: 313–320, 2008.

KEY WORDS: PLACENTAL GROWTH FACTOR; MOTILITY; LUNG CANCER; ROCK

Lung cancer is one of the most prevalent cancers globally. It is the top cancer killer in both men and women [Parkin et al., 2001]. Over the last 30 years lung cancer has become one of the leading cause of death in China and indeed a number of other countries, a subject area currently under intense investigation scientifically and clinically [Parkin et al., 1999]. Most of the patients are already at an advanced stage of the disease at the time of diagnosis, an important contributing factor to the very poor prognosis for these patients [Ettinger and Kris, 2001]. Up to 40% of the patients will have detectable distant disease (metastasis) at presentation [Parker et al., 1997]. Metastasis, the leading cause of death for patients with solid tumours is the outcome of a complex biological phenomenon. Cancer cells would have already escaped from the normal growth control, departed from the primary site, degraded and invaded the basement matrix and extracellular matrix and travelled to the destination organ, before a metastatic tumour is seen. Cellular motility and invasiveness of cancer cells as well as angiogenesis are amongst critical factors in the metastatic process.

Placental growth factor (PlGF), one member of the VEGF family, was first cloned from placenta [Maglione et al., 1991] and has been shown to cooperate with VEGF in the formation of vasculature [Khaliq et al., 1996; Ahmed et al., 2000]. PlGF may activate the VEGF receptor, Flt1, resulting the transphosphorylation of Flk1 and amplifying VEGF derived angiogenesis, in which VEGF/PGF heterodimer can activate VEGF receptors, forming Flk1/Flt1 heterodimers and angiogenesis process [Autiero et al., 2003]. PlGF has been implicated in the progression of a number of solid tumours. This involvement was initially believed to be the results of induction of angiogenesis by PlGF. However, in recent years, evidence began to indicate that PlGF may directly act on cancer cells. PlGF protein level is significantly higher in many tumours compared with non-tumour foci, such as breast cancer, lung cancer, colorectal cancer

Grant sponsor: UICC (International Union Against Cancer) International Fellowship; Grant number: ICR/06/160; Grant sponsor: European Union Framework 6 (LSHB-CT-2006-037386), the Albert Hung Foundation; Grant sponsor: Cancer Research Wales.

*Correspondence to: Prof. Wen G. Jiang, MB, MD, Metastasis & Angiogenesis Research Group, Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, UK. E-mail: jiangw@cf.ac.uk

**Correspondence to: Prof. Lijian Zhang, Department of Thoracic Surgery, Peking University School of Oncology and Beijing Cancer Hospital, Fuchenglu, Haidian, Beijing 100036, China. E-mail: lijzhang@yeah.net

Received 27 November 2007; Accepted 1 May 2008 • DOI 10.1002/jcb.21831 • 2008 Wiley-Liss, Inc.

Published online 9 July 2008 in Wiley InterScience (www.interscience.wiley.com).

and gastric cancer and is correlated with patient's survival [Chen et al., 2004; Parr et al., 2005; Wei et al., 2005; Zhang et al., 2005]. In patients with renal cancer, the plasma PlGF levels were significantly higher than non-cancer people, and plasma PlGF was correlate with patients' survival [Matsumoto et al., 2003]. While the correlation between PlGF and disease progression was thought to be the result of PlGF induced angiogenesis in cancer, recent in vitro and in vivo studies have shown that exogenous PlGF have a direct stimulatory effects on cancer cells, such as the motility and invasion of breast cancer cell [Taylor and Goldenberg, 2007]. The in vitro study has shown over-expression of PlGF-2 in cancer cell lines, such as lung cancer cell A549, colon cancer cell HCT116 and glioblastoma U87-MG.Overexpression of PlGF in in vivo models can inhibit tumour growth by depleting VEGF monodimers [Xu et al., 2006]. The later has provided tentative evidence that PlGF may have a dual action in cancer, direct effect on endothelial cells (induction of angiogenesis) and direct effect on cancer cells (inducing motility and invasion). However, the spectrum that PlGF has a direct impact on cancer and the mechanism(s) by which PlGF acts on cancer is yet to be investigated. We have recently reported that, in clinical lung cancer, PlGF was positively stained in lung cancer cells using immunohistochemistry. We further demonstrated that PlGF mRNA was significantly higher in advanced stage lung tumours and was correlated with poor prognosis in the patients [Zhang et al., 2005]. In the present study, we went on to investigate if PlGF had a direct effect on human lung cancer cells and the possible mechanism by which PlGF may influence the biological behaviours of lung cancer cells.

MATERIALS AND METHODS

CELL LINES AND MATERIALS

Human lung cancer cell line A549 (American Type Culture Collection, Manassas, VA), were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. Goat anti-human PlGF, mouse anti-human ROCK1, mouse anti-human GAPDH antibodies and the ROCK inhibitor (Y-27632) were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Matrigel (reconstituted basement membrane) was purchased from Collaborative Research Product (Bedford, MA). Other kits and reagents were obtained from Sigma–Aldrich, Poole, UK, unless otherwise stated.

CONSTRUCTION OF RIBOZYME TRANSGENE TARGETING HUMAN PLGF

Anti-human PlGF hammerhead ribozymes were designed based on the secondary structure of the gene generated using the Zuker RNA mFold program [Zuker, 2003]. Hammerhead ribozymes that specifically target a GTC and a UUC site of human PlGF (GenBank NM_173074) were generated using touch-down PCR (primers as listed in Table I). The ribozymes were then cloned into the pEF6/v5-His vector (Invitrogen), using ampicillin and blasticidin as selection markers for prokaryotic and mammalian cells, respectively. After verification, purified ribozyme transgenes and empty plasmids were transfected into A549 cells individually using an Easyject Plus electroporator (EquiBio, Kent, UK). After up to 2 weeks of selection with Blasticidin (5 μg/ml), stable transfectants were verified for the success in knocking down the PlGF transcript. Successful strains were grown to sufficient number and used in current study. The above procedure generated the following transfected cells from A549: empty vector control: $A549^{pEF/His}$, PlGF knockdown cells, $A549^{\Delta PlGF}$ and the wild-type: $A549^{WT}$.

TABLE I. PCR Primer Sequences

PlGF	Forward	CCCTTGGGTCTCCTCCTTTC
	Reverse	TGCGGCGATGAGAATCTGC
Ribozyme for hPlGF	Forward	CTGCAGCAAGGGAACAGCCTCAT CTGATGAGTCCGTGAGGA
	Reverse	ACTAGTCTGAGAAGATGCCGGTT TCGTCCTCACGGACT
β-actin	Forward	ATGATATCGCCGCGCTCG
	Reverse	CGCTCGGTGAGGATCTTCA

EXTRACTION RNA AND RT-PCR

RNA was obtained using Total RNA Isolation Reagent (ABgene™). 0.25 μg RNA was used to produce the first strand cDNA using a reverse transcription kit (Sigma, Poole, Dorset, UK). PCR was undertaken using a REDTaq™ ReadyMix PCR reaction mix (Sigma–Aldrich, Inc.). Cycling conditions were 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 40 s. This was followed by a final extension of 10 min at 72°C. The quality of cDNA was verified by 580 bp β-actin PCR with the anneal temperature of 55°C. The annealing temperature of PlGF was 58°C. Products were separated by 2% agarose gel and visualised under UV light after staining with ethidium bromide.

IMMUNOPRECIPATAION, SDS–PAGE AND WESTERN BLOTTING

Cells were pelleted and extracted in HCMF buffer (160 mM NaCl, 0.6 mM Na_2HPO_4, 0.1% w/v glucose and 0.01 M Hepes, pH 7.4) containing 0.5% Triton X-100, 2 mM $CaCl_2$, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 1 μg/ml aprotinin for 30 min. Protein concentrations were measured using fluorescamine and quantified by using a multifluoroscanner (Denly, Sussex, UK). Equal amounts of protein from each cell sample were separated on a 12% (for PlGF) or 8% (for ROCK1) polyacrylamide gel. For immunoprecipitation experiments, both cell lysate and condition media were collected. Equal amount of cell lysate and condition medium were incubated with anti-PlGF IgG (4 μg/ml) to precipitate PlGF. The immune complexes subsequently formed were then precipitated by the incubation with protein A/G-agarose conjugate for 1 h and subsequent centrifugation at 13,000*g* for 15 min. After washing the immunoprecipitates with the lysis buffer three times, the samples were boiled in SDS–PAGE sample buffer for 5 min prior to separation using SDS–PAGE. The secondary peroxidase-conjugated secondary antibodies were used at a final dilution 1:1,000. Protein band signal was visualized with the Supersignal™ West Dura system (Pierce Biotechnology, Inc., Rockford, IL), and images obtained using an UVITech imager (UVITech, Inc., Cambridge, England, UK).

IN VITRO CELL GROWTH ASSAY

This was based on a previously reported method [Jiang et al., 2005]. Cells were plated into 96-well plated at 2,000 cells/well followed by a period of incubation. Respective plates were fixed in 10% formaldehyde on the day of plating and daily for the subsequent 5 days. 0.5% crystal violet (w/v) was used to stain cells. Following washing, the stained crystal violet was dissolved with 10% (v/v) acetic acid and the absorbance was determined at a wavelength of 540 nm using an ELx800 spectrophotometer. Absorbance represents the cell number.

CELL–MATRIX ADHESION ASSAY

This was based on a method we previously reported [Jiang et al., 1995a,b]. Ninety six-well plate were coated with 5 μg of matrigel and allowed to dry. Following rehydration, 40,000 cells were added to each well. After 40 min of incubation non-adherent cells were washed off using BSS buffer. The remaining cells were fixed with 4% formalin and stained with 0.5% crystal violet. The number of adherent cells was then counted under microscopy.

IN VITRO MOTILITY ASSAY USING CYTODEX-2 BEADS (PHARMACIA, PISCATAWAY, NJ)

We followed the protocol described by Rosen et al. [1990]. Cells (1×10^6) were incubated with 100 μl Cytodex-2 beads in 10 ml DMEM medium overnight to coat the cytodex-2 beads. After the medium was aspirated and the beads were washed with DMEM, they were aliquoted into wells of 24-well plate (100 μl/well). After 4 h of incubation, the beads were washed off. The cells that migrated onto the bottom of each well were fixed with 4% formalin for 5 min and were stained with 0.5% crystal violet. The cells were counted under a microscope. The experiment was performed three times.

ELECTRIC CELL-SUBSTRATE IMPEDANCE SENSING (ECIS) BASED MOTILITY ASSAY

The ECIS 1600R model instrument and 8W10 arrays (Applied Biophysics, Inc., NJ) were used in the study [Giaever and Keese, 1991; Keese et al., 2004]. Following treating the array surface with a Cysteine solution, the arrays were incubated with complete medium for 1 h. The same number of lung cancer cells, $A549^{pEF/His}$, $A549^{\Delta PlGF}$, or $A549^{wt}$ (300,000 per well) were added to each wells. After reaching confluence, medium or ROCK inhibitor (10 μM) was added to the respective well. The cells were then immediately subject to wounding using the integrated elevated field module in the instrument in the 1600R model (5v, 30 s for each well). The changes of cellular impedance were immediately recorded after wounding (4,000 Hz). The data was analysed using the ECIS RbA modelling software, supplied by the manufacturer.

IMMUNOCYTOCHEMICAL STAINING FOR ROCK1 PROTEIN

Cells were fixed with 4% formaldehyde and then permeabilized with 0.1% Triton X-100 for 5 min in Tris buffer saline. After blocking with horse serum (10%) for 60 min, the cells were probed with anti-ROCK1 antibody for 1 h, followed by extensive washing. Horseradish peroxidase-conjugated anti-mouse antibody was then added for 1 h and visualized using the Vectastain® ABC system. Slides were mounted with Sterilyte.

STATISTICAL ANALYSIS

Minitab®, version 14 was used for analysis. Normally and Non-normally distributed data were assessed using the two sample *t*-test and Mann–Whitney test respectively.

RESULTS

KNOCKDOWN OF PLGF EXPRESSION BY RIBOZYME TRANSGENES

Following selection, we obtained strains of transfected cells. The expression of PlGF was determined in these transfected cells. As shown by RT-PCR analysis, PlGF mRNA expression was almost completely eliminated from A549 cells by anti-PlGF ribozyme transgene ($A549^{\Delta PlGF}$), a clear contrast to the level of expression in wild-type ($A549^{WT}$) cells and in A549 empty plasmid ($A549^{pEF/His}$) cells (Fig. 1). Knock-down of PlGF mRNA was also reflected by a reduction in its protein level: a decrease in the PlGF protein level was seen in both condition medium and cell lysate from A549 ribozyme transgene ($A549^{\Delta PlGF}$) cells (Fig. 2).

LOSS OF ENDOGENOUS PLGF RESULTED IN A REDUCTION OF MOTILITY AND CELL–MATRIX ADHESION IN A549 CELLS

We first tested the effect of loss of PlGF ($A549^{\Delta PlGF}$) on cell–matrix adhesion of the lung cancer cells. $A549^{\Delta PlGF}$ cells exhibited a significant reduction of cell–matrix adhesion: the number of adherent cells for the $A549^{\Delta PlGF}$ cells being 13.3 ± 3.95, $P < 0.05$ compared with both 549^{wt} (25.6 ± 7.90) and $A549^{pEF/His}$ (28.1 ± 6.90) (Fig. 3B).

In the cell carrier based cell motility assay, we found that the motility reduced significantly in A549 cell with the loss of PlGF. The number of migrating $A549^{\Delta PlGF}$ cells was 23 ± 8 compared with 57.5 ± 13.70 for $A549^{wt}$ cells and 52.12 ± 15.0 for $A549^{\Delta control}$ cells ($P < 0.05$) (Fig. 3A).

To further investigate the effect of knock-down PlGF on cell motility, ECIS system was utilised to determine the migration of

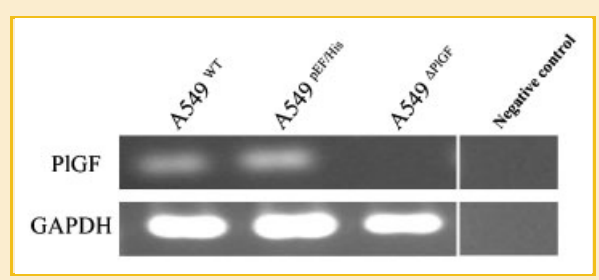

Fig. 1. The effects of ribozyme transgenes on PlGF expression using RT-PCR. mRNA of PlGF was eliminated in A549 $^{\Delta PlGF}$ cells compared with the wild-type ($A549^{WT}$) and empty plasmid control ($A549^{pEF/His}$) cells.

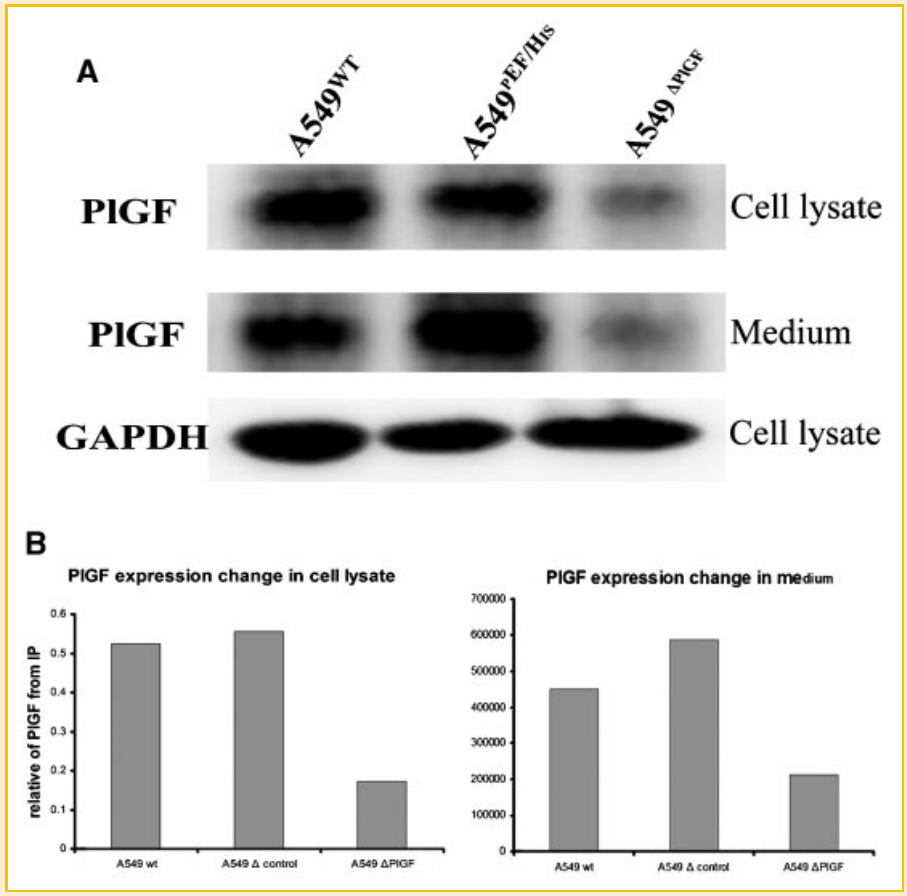

Fig. 2. Knockdown of PlGF by the ribozyme transgene at protein level using immunoprecipitation and western blot analysis. A: PlGF proteins in cell lysates and conditioned medium as shown by immunoprecipitation and Western blotting. Both the cytosolic and secreted PlGF were reduced by the ribozyme transgene. GAPDH was also determined from the original cell lysate and used as an internal reference. B: The bands of PlGF were quantified and the volume of bands were normalised against corresponding GAPDH bands.

A549 cells. The migrating capacity was remarkably reduced in $A549^{\Delta PlGF}$ cells after loss of PlGF, compared with $A549^{WT}$ and $A549^{pEF/His}$ cells (Fig. 5B).

The knockdown of PlGF expression had no effect on the growth of lung cancer cells as shown in Figure 3C.

INFLUENCE ON ROCK1 EXPRESSION IN A549 CELLS BY KNOCK-DOWN OF PLGF

Rho kinase (ROCK) belongs to a family of serine/threonine kinases, and is the Rho GTPase effector. ROCK is involved in regulation of cell adhesion and migration. It has been demonstrated that VEGF/PlGF can induce cell migration resulting Rho GTPase activation in leukaemia [Casalou et al., 2007]. In order to examine the relationship between endogenous PlGF and ROCK family, the expression of ROCK1 was examined in A549 cells with and without the transgenes. Surprisingly, we observed a decrease in the level of ROCK1 protein in $A549^{\Delta PlGF}$ cells. This was reproduced in both Western blot (Fig. 4A,B) and immunocytochemistry (Fig. 4C) analyses.

KNOCK-DOWN OF PLGF IN LUNG CANCER CELLS RENDERED CELLS LESS RESPONSIVE TO ROCK INHIBITOR

In order to test the role of ROCK1 in PlGF induced motility change in A549 cell, we used a small molecule ROCK inhibitor, Y27632. Both cytodex-2 beads motility assay and ECIS assay were employed. The motility was inhibited by exposure to ROCK inhibitor in both $A549^{WT}$ and $A549^{pEF/His}$ cells, the number of migrating cells being 43.75 ± 12.08 without ROCK inhibitor and 28.33 ± 4.830 with the inhibitor for $A549^{WT}$ cells, and 41.92 ± 10.040 and 27.5 ± 5.37 respectively for $A549^{pEF/His}$ cells (Fig. 5A). Interestingly, $A549^{\Delta PlGF}$ lost its response to the ROCK inhibitor in that the motility indices in both ROCK inhibitor treated and non-treated were virtually the same: 17.42 ± 4.89 versus 17.75 ± 6.34.

In ECIS based analysis, ROCK inhibitor was able to inhibit the migration of $A549^{WT}$ and $A549^{pEF/His}$ cells in comparison with the respective controls, but to less extent compared to the $A549^{\Delta PlGF}$ cells (Fig. 5B). Thus similar to the observations seen in the carrier bead motility assay, $A549^{\Delta PlGF}$ cells failed to respond to the ROCK inhibitor.

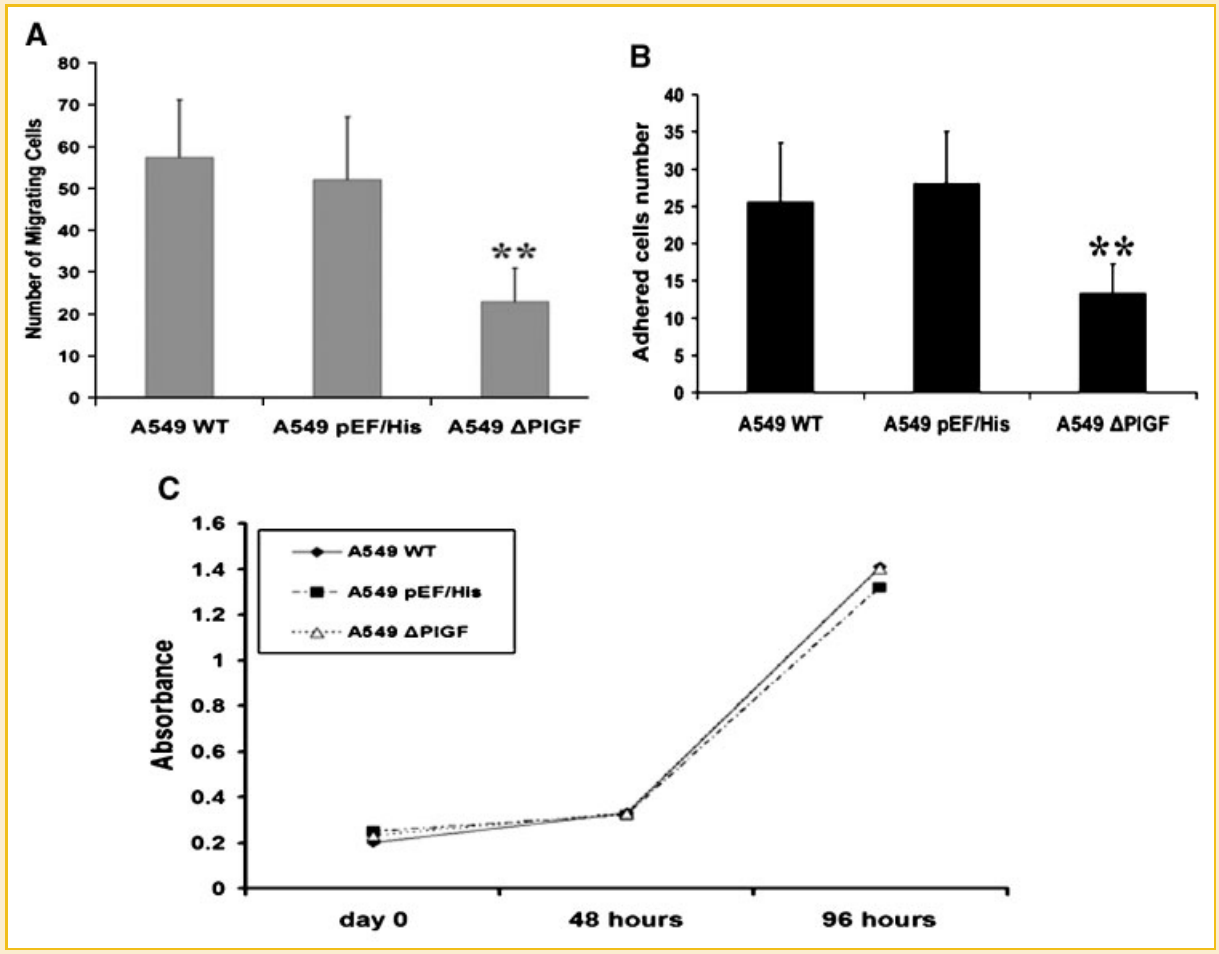

Fig. 3. The effect of ribozyme transgene targeting PlGF on the biological functions of A549 cells. A: motility of $549^{\Delta PIGF}$ cells was reduced remarkably compared with A549 wild-type cells. Assays were performed in triplicate in three preparations each. Asterisk indicates $P < 0.001$ versus $A549^{WT}$ and $A549^{pEF/His}$ cells. B: A decrease of cell–matrix adhesion was seen in $549^{\Delta PIGF}$ cells. Cell matrix adhesion assay was done in quadriplicate per cell line per experiments, $n = 3$. Asterisk indicates $P < 0.001$ versus $A549^{WT}$ and $A549^{pEF/His}$ cells. C: There was no significant change in the rate of cell growth of $549^{\Delta PIGF}$ cells compared with $A549^{WT}$ and $A549^{pEF/His}$ cells. Growth assay was done in six wells per cell line. Error bars represent SD.

DISCUSSION

The present study provides the first line evidence that reduction of PlGF directly affects the motility of lung cancer cell, thus indicating that PlGF has a biological effect on lung cancer cells and that this effect is likely to be via a ROCK dependent pathway. The study has provided further evidence to indicate a therapeutic implication of PlGF in lung cancer.

Levels of PlGF have a demonstrable correlation with tumour progression and poor prognosis in human solid tumours [Wei et al., 2005; Zhang et al., 2005], a connection thought to be linked to PlGF induced angiogenesis [Dull et al., 2001; Castellon et al., 2002; Khurana et al., 2005]. The function of PlGF in tumour cells is poorly understood, although a direct effect on cancer cells has been suggested in recent years. The current study assessed the changes of cellular behaviour with the loss of PlGF expression in A549 cell, a non small cell line that endogenously expresses PlGF. The decrease in endogenous PlGF in A549 cell by way of ribozyme transgenes resulted in reduction of cellular motility and adhesion. This indicates that endogenous PlGF plays an important role in controlling the functions that are critical to the aggressiveness of the lung cancer cell. In the last few years, there has been limited number of reports which demonstrate that PlGF acts on non-endothelial cells.

Hollborn et al. [2006] demonstrated that high concentration of exogenous PlGF (at 100 ng/ml) decreased the cell proliferation of retinal pigment epithelial cells. When pre-mixed with soluble HSPGs (Heparin Sulphate Proteoglycans), PlGF-1 can induce concentration-dependent proliferation stimulation of extravillous trophoblasts [Athanassiades and Lala, 1998]. To date, the effect on the growth of cancer cells and indeed tumours in vivo, remains controversial. Xu et al. [2006] showed that over-expression of PlGF can decrease the amount of homodimeric VEGF and thus contribute to the inhibition of tumour growth in vivo. In contrast, Marcellini et al. [2006] reported that melanoma cells over-expressing PlGF grew at a higher rate in transgenic mice compared with the wide type

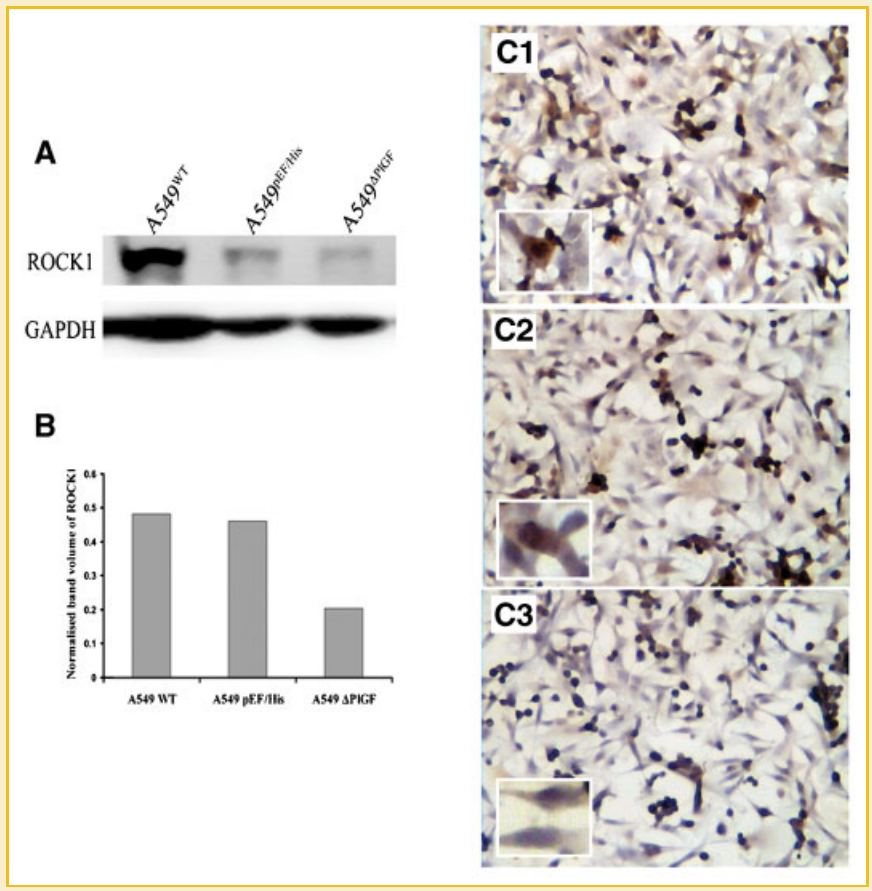

Fig. 4. Knock-down of PlGF in A549 cells resulted in reduction of ROCK1 expression. A: The expression of ROCK1protein was decreased after loss of PlGF in A549$^{\Delta PlGF}$ cells, as shown by Western blot analysis. B: Quantified changes of ROCK1 protein bands. The bands of PlGF were quantified and the volume of bands was normalised against the corresponding GAPDH bands. C: Immunocytochemical staining also demonstrated that ROCK 1 protein level was reduced by knock-down of PlGF. C1: Immunostaining of ROCK1 in A549^{WT}. C2: ROCK1 staining in A549$^{pEF/His}$ control cells. C3: ROCK1 staining in 549$^{\Delta PlGF}$ cells. C1/C2/C3: magnifications were 100×; Insets 400×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

melanoma cells. The present study has clearly added weight to the reports that PlGF does indeed have a direct effect on non-small lung cancer cells in vitro. This, together with previous studies has clearly shown that PlGF acts via both autocrine and paracrine routes in cancer: directly on cancer cells themselves or on the tumour related endothelial cells and, in doing so, PlGF acts as a powerful protein factor in driving the spread of lung cancer.

Rho-associated kinase (ROCK) is a serine/threonine kinase that mediates some of the downstream signalling of RhoA [Riento and Ridley, 2003]. It has two isoforms, namely ROCK1 and ROCKII. The serine/threonine kinase activities of ROCKs have an important role in cell migration, cell death and survival [Shi and Wei, 2007]. ROCKs are known to regulate the metastatic process in cancer such as pancreatic cancer and small cell lung cancer [Kaneko et al., 2002; Li et al., 2006]. Our study clearly demonstrated that there was a decreased ROCK1 expression in protein level with the loss of PlGF expression in A549 cell. ROCK inhibitor could reduce the motility of A549^{WT} and A549$^{pEF/His}$ cells. The effect of the ROCK inhibitor was significantly weaker with the A549$^{\Delta PlGF}$ cells which have lost their endogenous PlGF as the result of ribozyme transgene. This has provided strong evidence that ROCK1 at least partly contributes to the increase of motility in lung cancer with highly expressed PlGF. It is further extrapolated from this result that ROCK1 has a potential therapeutic value in lung cancer.

In summary, the present study shows that ribozyme designed to target human PlGF is an effective approach to inhibit the action of PlGF in lung cancer cells. It can specifically reduce the expression of PlGF at both mRNA and protein level in the cancer cells. The lung cancer cells carrying the ribozyme transgene exhibit low level of migration and adhesion ability, providing the evidence that PlGF is an important molecule in the control of dissemination of lung cancer cells by directly promoting the aggressiveness of cancer cells. These results indicate that PlGF is an important biological regulator of lung cancer cells and may be a valuable target in the therapeutic development in lung cancer.

ACKNOWLEDGMENTS

Dr. Chen is supported by an UICC International Fellowship. WGJ would like to thank the European Union (the Angiostop

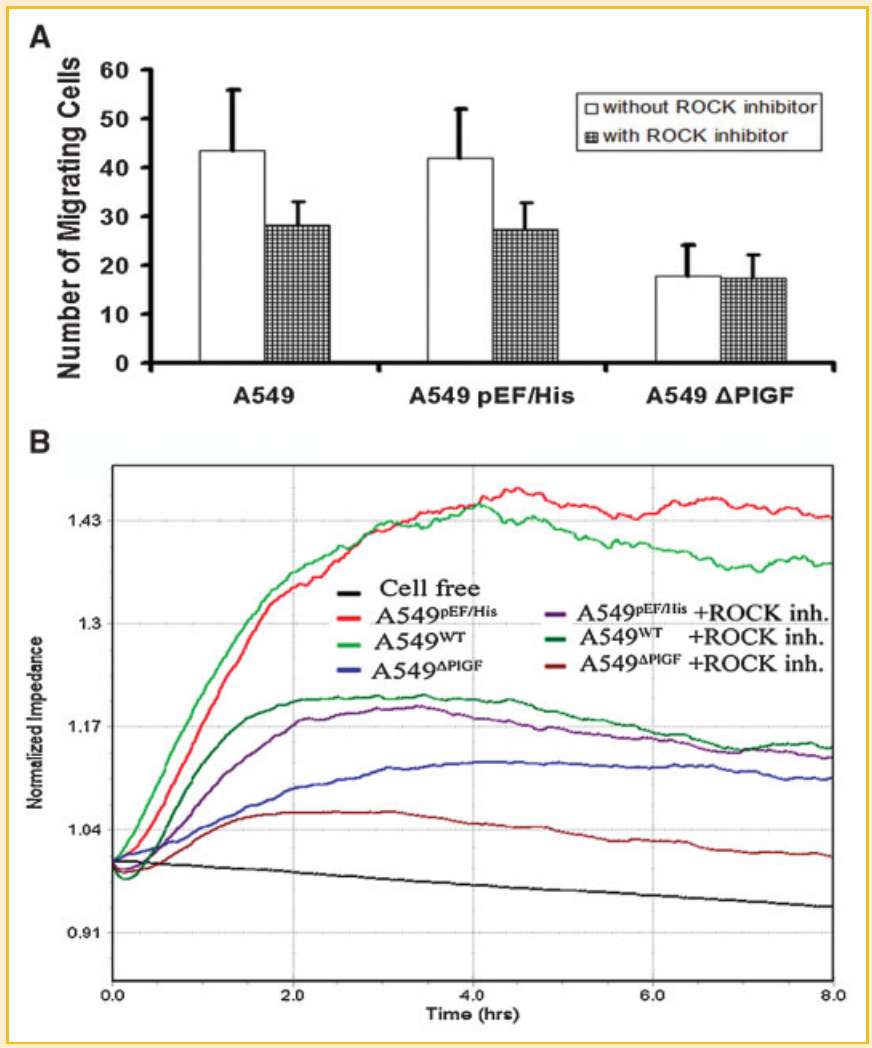

Fig. 5. The effects of ROCK inhibitor and the ribozyme transgene targeting on cell motility. A: Cytodex carrier assay. ROCK inhibitor (10 μM) significantly reduced motility of $A549^{WT}$ and $A549^{pEF/His}$ cells, but only had an marginal effect on $549^{\Delta PIGF}$ cells. B: ECIS-based wounding motility assay. $A549^{WT}$ and $A549^{pEF/His}$ cells had significantly reduced migration in response to the ROCK inhibitor. The $549^{\Delta PIGF}$ cells failed to show a significant change. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Programme-FP6), Cancer Research Wales and the Albert Hung Foundation for supporting.

REFERENCES

Ahmed A, Dunk C, Ahmad S, Khaliq A. 2000. Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF) and soluble Flt-1 by oxygen—A review. Placenta 21(Suppl A): S16–S24.

Athanassiades A, Lala PK. 1998. Role of placenta growth factor (PIGF) in human extravillous trophoblast proliferation, migration and invasiveness. Placenta 19:465–473.

Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dombrowski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin DJ, Persico G, Herbert JM, Communi D, Shibuya M, Collen D, Conway EM, Carmeliet P. 2003. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nat Med 9:936–943.

Casalou C, Fragoso R, Nunes JF, Dias S. 2007. VEGF/PLGF induces leukemia cell migration via P38/ERK1/2 kinase pathway, resulting in Rho GTPases activation and caveolae formation. Leukemia 21:1590–1594.

Castellon R, Hamdi HK, Sacerio I, Aoki AM, Kenney MC, Ljubimov AV. 2002. Effects of angiogenic growth factor combinations on retinal endothelial cells. Exp Eye Res 74:523–535.

Chen CN, Hsieh FJ, Cheng YM, Cheng WF, Su YN, Chang KJ, Lee PH. 2004. The significance of placenta growth factor in angiogenesis and clinical outcome of human gastric cancer. Cancer Lett 213:73–82.

Dull RO, Yuan J, Chang YS, Tarbell J, Jain RK, Munn LL. 2001. Kinetics of placenta growth factor/vascular endothelial growth factor synergy in endothelial hydraulic conductivity and proliferation. Microvasc Res 61: 203–210.

Ettinger DS, Kris MG. 2001. NCCN: Non-small cell lung cancer. Cancer Control 8:22–31.

Giaever I, Keese CR. 1991. Micromotion of mammalian cells measured electrically. Proc Natl Acad Sci USA 88:7896–7900.

Hollborn M, Tenckhoff S, Seifert M, Kohler S, Wiedemann P, Bringmann A, Kohen L. 2006. Human retinal epithelium produces and responds to placenta growth factor. Graefes Arch Clin Exp Ophthalmol 244:732–741.

Jiang WG, Hiscox S, Hallett MB, Horrobin DF, Mansel RE, Puntis MC. 1995a. Regulation of the expression of E-cadherin on human cancer cells by gamma-linolenic acid (GLA). Cancer Res 55:5043–5048.

Jiang WG, Hiscox S, Hallett MB, Scott C, Horrobin DF, Puntis MC. 1995b. Inhibition of hepatocyte growth factor-induced motility and in vitro invasion of human colon cancer cells by gamma-linolenic acid. Br J Cancer 71:744–752.

Jiang WG, Davies G, Martin TA, Parr C, Watkins G, Mason MD, Mokbel K, Mansel RE. 2005. Targeting matrilysin and its impact on tumor growth in vivo: The potential implications in breast cancer therapy. Clin Cancer Res 11:6012–6019.

Kaneko K, Satoh K, Masamune A, Satoh A, Shimosegawa T. 2002. Expression of ROCK-1 in human pancreatic cancer: Its down-regulation by morpholino oligo antisense can reduce the migration of pancreatic cancer cells in vitro. Pancreas 24:251–257.

Keese CR, Wegener J, Walker SR, Giaever I. 2004. Electrical wound-healing assay for cells in vitro. Proc Natl Acad Sci USA 101:1554–1559.

Khaliq A, Li XF, Shams M, Sisi P, Acevedo CA, Whittle MJ, Weich H, Ahmed A. 1996. Localisation of placenta growth factor (PIGF) in human term placenta. Growth Factors 13:243–250, color plates I–II, pre bk cov.

Khurana R, Moons L, Shafı S, Luttun A, Collen D, Martin JF, Carmeliet P, Zachary IC. 2005. Placental growth factor promotes atherosclerotic intimal thickening and macrophage accumulation. Circulation 111:2828–2836.

Li B, Zhao WD, Tan ZM, Fang WG, Zhu L, Chen YH. 2006. Involvement of Rho/ROCK signalling in small cell lung cancer migration through human brain microvascular endothelial cells. FEBS Lett 580:4252–4260.

Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG. 1991. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. Proc Natl Acad Sci USA 88:9267–9271.

Marcellini M, De Luca N, Riccioni T, Ciucci A, Orecchia A, Lacal PM, Ruffini F, Pesce M, Cianfarani F, Zambruno G, Orlandi A, Failla CM. 2006. Increased melanoma growth and metastasis spreading in mice overexpressing placenta growth factor. Am J Pathol 169:643–654.

Matsumoto K, Suzuki K, Koike H, Okamura K, Tsuchiya K, Uchida T, Takezawa Y, Kobayashi M, Yamanaka H. 2003. Prognostic significance of plasma placental growth factor levels in renal cell cancer: An association with clinical characteristics and vascular endothelial growth factor levels. Anticancer Res 23:4953–4958.

Parker SL, Tong T, Bolden S, Wingo PA. 1997. Cancer statistics. CA Cancer J Clin 47:5–27.

Parkin DM, Pisani P, Ferlay J. 1999. Estimates of the worldwide incidence of 25 major cancers in1990. Int J Cancer 80:827–841.

Parkin DM, Bray F, Ferlay J, Pisani P. 2001. Estimating the world cancer burden: Globocan2000. Int J Cancer 94:153–156.

Parr C, Watkins G, Boulton M, Cai J, Jiang WG. 2005. Placenta growth factor is over-expressed and has prognostic value in human breast cancer. Eur J Cancer 41:2819–2827.

Riento K, Ridley AJ. 2003. Rocks: Multifunctional kinases in cell behaviour. Nat Rev Mol Cell Biol 4:446–456.

Rosen EM, Meromsky L, Setter E, Vinter DW, Goldberg ID. 1990. Smooth muscle-derived factor stimulates mobility of human tumor cells. Invasion Metastasis 10:49–64.

Shi J, Wei L. 2007. Rho kinase in the regulation of cell death and survival. Arch Immunol Ther Exp (Warsz) 55:61–75.

Taylor AP, Goldenberg DM. 2007. Role of placenta growth factor in malignancy and evidence that an antagonistic PlGF/Flt-1 peptide inhibits the growth and metastasis of human breast cancer xenografts. Mol Cancer Ther 6:524–531.

Wei SC, Tsao PN, Yu SC, Shun CT, Tsai-Wu JJ, Wu CH, Su YN, Hsieh FJ, Wong JM. 2005. Placenta growth factor expression is correlated with survival of patients with colorectal cancer. Gut 54:666–672.

Xu L, Cochran DM, Tong RT, Winkler F, Kashiwagi S, Jain RK, Fukumura D. 2006. Placenta growth factor overexpression inhibits tumor growth. angiogenesis, and metastasis by depleting vascular endothelial growth factor homodimers in orthotopic mouse models. Cancer Res 66:3971–3977.

Zhang L, Chen J, Ke Y, Mansel RE, Jiang WG. 2005. Expression of Placenta growth factor (PlGF) in non-small cell lung cancer (NSCLC) and the clinical and prognostic significance. World J Surg Oncol 3:68.

Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31:3406–3415.