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Nucleotide-binding domain of phosphoglycerate kinase 1 reduces tumor growth by suppressing COX-2 expression

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Phosphoglycerate kinase 1 (PGK-1) is a multifunctional protein that is involved in the glycolytic pathway and the generation of the angiogenesis inhibitor angiostatin. In a previous study, we showed that the overexpression of full-length PGK-1 in Lewis lung carcinoma (LLC-1) can reduce tumor growth *in vivo* by downregulation of COX-2 expression. Phosphoglycerate kinase 1 has two functional domains: a catalytic domain (CD); and a nucleotide-binding domain (NBD). To identify the functional domain of PGK-1 responsible for its antitumor effects, we evaluated the tumorigenicity of LLC-1 cells overexpressing full-length PGK-1 (LLC-1/PGK), CD (LLC-1/CD), and NBD (LLC-1/NBD). Although no difference in tumor cell growth was observed *in vitro*, the tumor invasiveness was reduced in the LLC-1/PGK, LLC-1/CD, and LLC-1/NBD cells compared to parental LLC-1 cells *in vivo*. In addition, *in vivo* tumor growth retardation by LLC-1/CD and LLC-1/NBD cells was observed, similar to that by LLC-1/PGK cells. However, the reduced stability of COX-2 mRNA and downregulation of the COX-2 protein and its metabolite, prostaglandin E2, was only found in LLC-1/PGK and LLC-1/NBD cells. Low levels of COX-2 were also observed in the tumor mass formed by the modified cells when injected into mice. The results indicate that COX-2 suppression by PGK-1 is independent of its catalytic activity. COX-2 targeting by PGK-1 can be attributed to its NBD and is probably a result of the destabilization of COX-2 gene transcripts brought about by the mRNA-binding property of PGK-1. (*Cancer Sci* 2010; 101: 2411–2416)

Lung cancer is the leading cause of carcinoma-related deaths worldwide, and non-small cell lung carcinoma (NSCLC) constitutes 85% of all lung cancers. Despite advances in lung cancer treatment, poor survival rates are commonly seen in patients with late-stage disease.[1] Therefore, early diagnosis and novel therapeutic approaches are urgently required. Previous studies indicated that the blocking of oncogenic signaling cascades that targeted the epidermal growth factor receptor- and COX-2-regulated pathways yielded promising results in specific subsets of lung cancers.[1,2]

Phosphoglycerate kinase 1 (PGK-1), an enzyme comprising a common nucleotide-binding domain (NBD) and two unique catalytic domains (CDI and CDII), is an interesting multifunctional protein.[3,4,5–9] In addition to being an essential enzyme in the glycolytic pathway,[3] PGK-1 is well known to play an inhibitory role in tumor angiogenesis by facilitating the formation of angiostatin from plasmin.[8] Moreover, PGK-1 can influence DNA replication and repair in the mammalian nucleus and stimulate viral mRNA synthesis in the cytosol.[5,6] The enzyme also serves as an mRNA-binding protein and is implicated in the negative regulation of the stability of urokinase-type plasminogen activator receptor (uPAR) mRNA.[7] The reduction in uPAR expression and cellular motility by overexpression of PGK-1 has been shown in human lung cancer cells.[7] Peptides of PGK-1 isolated from human colon cancer can stimulate γ-interferon secretion from infiltrating T lymphocytes, resulting in enhanced T-cell cytotoxicity against tumor cells.[9]

The COX family comprises key prostaglandin biosynthetic enzymes with clinical significance. Cyclooxygenase-2, the inducible isoform of COX, is overexpressed in early and advanced lung cancer and is associated with a poor prognosis.[2,10–13] Elevated levels of tumor COX-2 and its metabolite prostaglandin E2 (PGE2) contribute to increasing angiogenesis,[11,14] augmentation of tumor invasiveness,[11,15] resistance to apoptosis,[11,16] and suppression of the immune response.[17–19] It has been shown that inhibition of COX-2 in lung cancers can reduce tumorigenicity.[14,19] Thus, COX-2 and PGE2 are considered to play pivotal roles in lung tumorigenesis.

In our previous study, we showed that the COX concentration in PGK-1-overexpressing Lewis lung carcinoma cells (LLC-1) is decreased and the generation of Th1 immunity is enhanced, whereby tumor growth is eventually retarded *in vivo*.[20] In the current study, we further determine the importance of the different PGK-1 domains, that is, CDI and NBD, in the reduction of COX-2 levels and regulation of tumor progression. We established LLC-1 cell lines overexpressing CDI and NBD using the inducible Tet-Off system (BD Clontech, Palo Alto, CA, USA). The expression levels of the CDI and NBD genes in LLC-1 could be negatively modulated by the addition of doxycycline (Dox), which in turns refines the effects of CDI and NBD on tumor cells. Similar to full-length PGK-1, both the CDI and NBD domains could downregulate the invasive ability of the tumor and reduce *in vivo* lung cancer cell growth. Moreover, further analyses showed that tumor growth reduced under conditions of NBD overexpression because of the decrease in the levels of COX-2 and PGE2. The NBD of PGK-1 lowers the stability of COX-2 mRNA, whereby it suppresses COX-2 in lung cancer cells.

Materials and Methods

Cells, mice, and reagents. Lewis lung carcinoma (H-2^b; ATCC CRL-1642) cells were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Male C57BL/6 (H-2^b) mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan), and the regulations of the Animal Care Committee of National Yang-Ming University (Taipei, Taiwan) were followed. Recombinant transforming

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growth factor-β1 (TGF-β1) was obtained from eBioscience (San Diego, CA, USA). Recombinant PGE2 and yeast (*Saccharomyces cerevisiae*) PGK proteins, anti-β-actin (AC-15) antibody, and actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Nucleotide-binding domain and CDI constructs. Full-length human PGK-1 cDNA was amplified and cloned into the pRev-TRE vector (Clontech Laboratories, Mountain View, CA, USA) as described previously.[20] The CDI and NBD domains of PGK-1 were amplified from pRevTRE-PGK using specific primers (Table 1). The insert sequences in pRevTRE-PGK, pRev-TRE-CDI, and pRevTRE-NBD were confirmed by automated DNA sequencing.

Preparation of LLC-1 transfectants. Stable clones were established for LLC-1/PGK (clone 30), LLC-1/CDI (clone 14; C14), and LLC-1/NBD (clone 3; N3) (Fig. 1) as described previously.[20] To analyze tumor growth *in vitro*, the transfectants (LLC-1/PGK, LLC-1/CDI, and LLC-1/NBD, 5×10^4/mL) were cultured in 10% FBS-DMEM for 7 days. The cell number was measured daily using the CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA).

***In vivo* tumor model.** Determination of the *in vivo* tumor growth was carried out as described previously.[20]

Examination of invasive ability. The invasive ability of the tumor cells was assessed as described previously.[20]

Detection of gene expression. Total RNA preparation and RT-PCR were carried out as described previously.[20] RT-PCR images were quantified by ImageQuant 5.2 software (Amersham Bioscience, Piscataway, NJ, USA). The levels of COX-2 mRNA in the tumor cells were further quantified using RT–quantitative (q)PCR and SYBR Green I (Bio-Rad, Hercules, CA, USA) with an ABI7700 System (Applied Biosystems, Foster City, CA, USA). All qPCR values were normalized against β-2 microglobulin mRNA. The forward and reverse primers were synthesized by Mission Biotech (Taipei, Taiwan) (Table 1).

Table 1. Sequences of primer pairs used in RT-PCR or RT–quantitative (q)PCR

	Forward primer/reverse primer
PGK-1	5′-CGCGGATCCATGTCGCTTTCTAACAAGCTG-3′
	5′-CATCTGATGGTTCTCTAGAAACTG-3′
PGK-1/CDI	5′-CGGGATCCATGTCGCTTTCTA-ACAAGCTGACG-3′
	5′-CATCTGATGGTTCTCTAGAAACTG-3′
PGK-1/NBD	5′-CGGGATCCATGTTGATGAAGAA-GGAGCTGAAC-3′
	5′-CATCTGATGGTTCTCTAGAAACTG-3′
COX-2	5′-TACCAGTCTCTCAATGAGTACC-3′
	5′-TGGTAGGCTGTGGATCTTGCACATTG-3′
VEGF	5′-GTACCTCCACCATGCCAAGT-3′
	5′-TCACATCTGCAAGTACGTTCG-3′
TGF-β1	5′-CCTGTCCAAACTAAGGC-3′
	5′-GGTTTTCTCATAGATGGCG-3′
HIF-1α	5′-GGAATGGCCCAGTGAGAAAA-3′
	5′-CCAGCAGAGTGAGAGCATCA-3′
ZEB-1	5′-GTTCGTGATTGTTTGCG-3′
	5′-CCAATAGCGTATCCATGC-3′
G3PDH	5′-ACCACAGTCCATGCCATCAC-3′
	5′-TCCACCACCCTGTTGCTGTA-3′
COX-2	5′-GCCTACTACAAGTGTTTCTTTTTGCA -3′
(RT-qPCR)	5′-CATTTTGTTTGATTGTTCACACCAT -3′
Beta-2 microglobulin	5′-CCGGAGAATGGGAAGC -3′
(RT-qPCR)	5′-GTAGACGGTCTTGGGC -3′

CDI, catalytic domain; HIF-1α, hypoxia-inducible factor-1α; NBD, nucleotide-binding domain; PGK-1, phosphoglycerate kinase 1; TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor; ZEB1, zinc finger E box-binding homeobox1.

Western blotting for COX-2. An immunoblot assay was carried out as described previously,[20] and we used anti-COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin (Sigma) primary antibodies.

Enzyme-linked immunosorbent assay for PGE2 and TGF-β1. The levels of PGE2 and TGF-β1 in the culture-conditioned medium were measured using commercially available PGE2 enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI, USA) and TGF-β1 ELISA kits (BioSource, Camarillo, CA, USA).

Statistical analysis. Data were expressed as the mean ± SD, and statistical significance was assessed by Student's *t*-test.

Results

Overexpression of PGK-1, CDI, and NBD in lung cancer cells inhibited tumor growth *in vivo* but not *in vitro*. A previous study showed that PGK-1 overexpression in LLC-1 inhibited tumor growth *in vivo*.[20] To determine whether the overexpression of a functional domain of PGK-1 alone (CDI or NBD) can reduce tumor growth, as full-length PGK-1 can, the tumor size was determined at different time points in mice s.c. injected with LLC-1 and LLC-1 transfectants overexpressing full-length PGK-1 (LLC-1/PGK), CDI (LLC-1/CDI), and NBD (LLC-1/NBD) as well as a vector control (LLC-1/EV). The LLC-1/PGK-, LLC-1/CDI-, and LLC-1/NBD-injected mice (0.1 cm^3/21 days) all showed significantly smaller tumor volumes than the control mice (injected with parental LLC-1 or LLC-1/EV; 1.25 cm^3/21 days) (Fig. 2a). In addition, the tumor growth rates of the

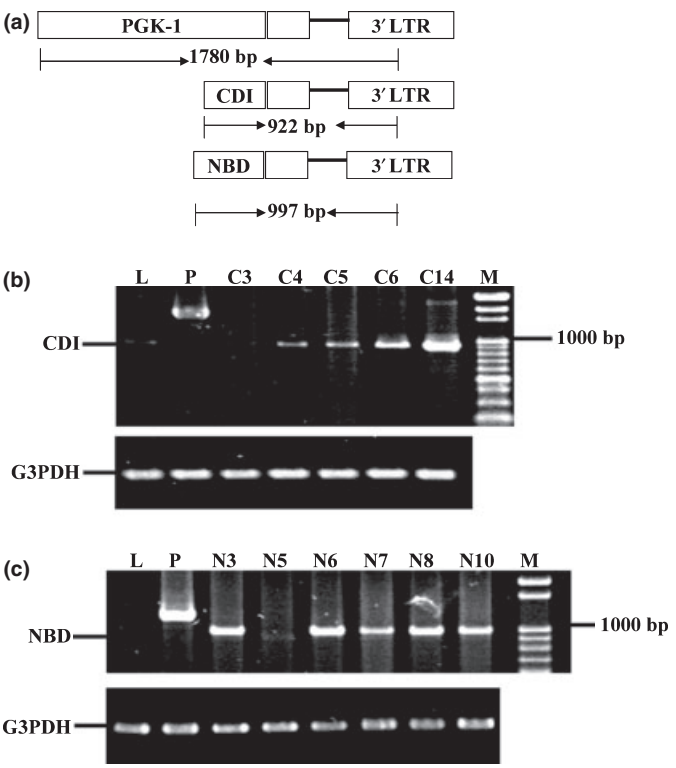

Fig. 1. Expression of catalytic domain (CDI) and nucleotide-binding domain (NBD) of phosphoglycerate kinase 1 (PGK-1) in transfected lung cancer cells. (a) Schematic diagram of PGK-1, CDI, and NBD RT-PCR products. (b,c) Expression of PGK-1, CDI, NBD, and G3PDH were detected in different clones of Lewis lung carcinoma (LLC-1) transfectants. C, LLC-1/CDI clones; L, LLC-1; M, marker; N, LLC-1/NBD clones; P, LLC-1/PGK.

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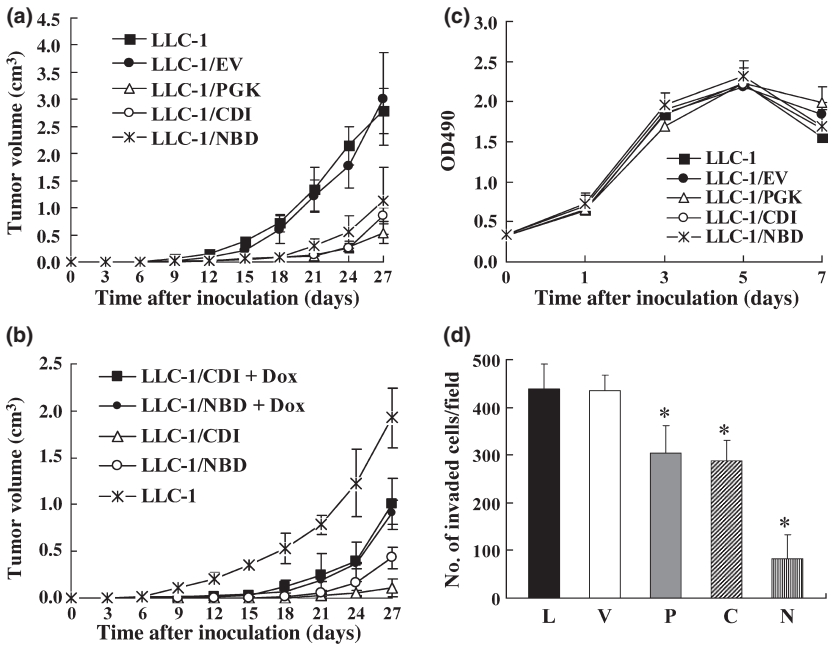

Fig. 2. Overexpression of catalytic domain (CDI), nucleotide-binding domain (NBD), and phosphoglycerate kinase 1 (PGK-1) in Lewis lung carcinoma (LLC-1) cells inhibited *in vivo* tumor growth and invasive ability. (a,b) C57BL/6 mice ($n = 10$) were pretreated with or without doxycycline (Dox; 20 μg/mL) for 3 days, and 5×10^5 cells were then s.c. injected into them. (c) The number of tumor cells was measured on different culture days. (d) The invasive ability of the tumor cells (5×10^4) was determined. Ten fields were counted per filter in each group. Each experiment was repeated in triplicate. C, LLC-1/CDI; EV, LLC-1/EV (vector control); L, parental LLC-1; P, LLC-1/PGK (full-length PGK-1); N, LLC-1/NBD; V, vector. *$P < 0.05$.

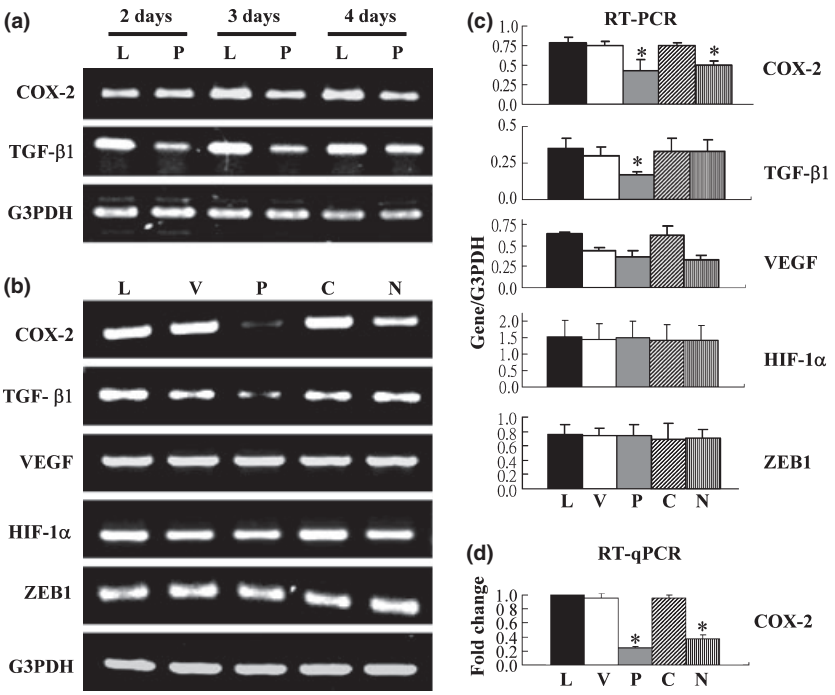

Fig. 3. Expression of COX-2 was downregulated in Lewis lung carcinoma (LLC-1) cells overexpressing phosphoglycerate kinase 1 (PGK) and nucleotide-binding domain (NBD). (a) Cells (8×10^4/mL) were cultured for 2, 3, and 4 days. Gene expression was detected by RT-PCR. (b) Gene expression profiles were surveyed in the 3-day culture of tumor cells by RT-PCR. (c) RT-PCR images were quantified using ImageQuant software and normalized against those of G3PDH. (d) All values from the RT-qPCR analysis of COX-2 were normalized, and the gene expression was plotted as fold change relative to the level in parental LLC-1 cells. L, parental LLC-1; V, LLC-1/EV (vector control); P, LLC-1/PGK; C, LLC-1/CDI; N, LLC-1/NBD; TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible factor-1α; ZEB1, zinc finger E box-binding homeobox1. The means of three independent experiments were calculated. *$P < 0.05$.

LLC-1/PGK, LLC-1/CDI, and LLC-1/NBD cells were restored when the mice were treated with Dox[20] (Fig. 2b) to further prove the tumor-suppressive effects of PGK, CDI, and NBD. The survival rate of mice injected with LLC-1/PGK, LLC-1/CDI, and LLC-1/NBD cells remained at 100% on day 27, but none of the mice injected with LLC-1 and LLC-1/EV cells survived up to day 27 (data not shown). To rule out the possible cytotoxic effects of overexpressed PGK-1, CDI, and NBD on lung cancer cells, the proliferation rates of the transduced LLC-1 cells and control cells were compared *in vitro*; the growth rates of the transduced cell types were found to be similar (Fig. 2c) and comparable with those of the control cells (LLC-1 and LLC-1/EV). Thus, the *in vivo* antitumor responses are a result of PGK-1, CDI, and NBD expression by LLC-1 and not the toxic effects of the overexpressed proteins.

Overexpression of PGK, CDI, and NBD reduced the invasive ability of the tumor cells. The influence of PGK-1, CDI, and NBD on the invasive ability of the tumor cells was also studied. Compared to the number of LLC-1 and LLC-1/EV control cells, the number of LLC-1/PGK, LLC-1/CDI and LLC-1/NBD cells that invaded the Matrigel-coated filter (Fig. 2d) was significantly lower. A 41%, 40%, and 77% reduction in invasiveness was observed for the LLC-1/PGK, LLC-1/CDI, and LLC-1/NBD cells, respectively, compared to the invasiveness of the controls (Fig. 2d). In addition, overexpression of PGK-1, CDI, or NBD reduced the invasive ability of human A549 lung cancer cells (data not shown). Thus, the overexpression of PGK-1, CDI, or NBD can curb primary tumor growth as well as tumor cell invasion.

Cyclooxygenase-2 transcription was downregulated in LLC-1/PGK and LLC-1/NBD cells. To investigate the mechanisms

by which cellular invasion and tumor growth were inhibited *in vivo* by PGK-1, CDI, and NBD, the transcript levels of COX-2, TGF-β1, angiogenic factor (vascular endothelial growth factor [VEGF]), hypoxia-inducible factor-1α, and transcriptional repressor of E-cadherin (zinc finger E box-binding homeobox1) in the controls and LLC-1 transfectants were analyzed (Fig. 3). The results showed that the COX-2 and TGF-β1 transcripts were downregulated in LLC-1/PGK cells as compared with the control LLC-1 and LLC-1/EV cells (Fig. 3a). In addition, the mRNA level of COX-2, but not that of TGF-β1, significantly decreased in the LLC-1/NBD cells (Fig. 3b,c). However, the overexpression of PGK-1 and NBD in the LLC-1 cells did not significantly affect the levels of VEGF, hypoxia-inducible factor-1α, or zinc finger E box-binding homeobox1. Furthermore, LLC-1/CDI cells displayed the same levels of all the tested genes as the LLC-1 and LLC-1/EV control cells (Fig. 3b,c).

Levels of COX-2 and PGE2 were reduced in the LLC-1/NBD and LLC-1/PGK cells. The mRNA and protein levels of COX-2 in the control cells and transfectants were determined by using RT-qPCR (Fig. 3d) and Western blot analysis (Fig. 4a). Compared with the control cells, the LLC-1/PGK and LLC-1/NBD cells, but not the LLC-1/CDI cells, contained lower amounts of COX-2 mRNA and protein. The levels of COX-2 were also reduced in the A549 cells transfected with PGK-1 or NBD (data not shown). In addition, Dox-treated LLC-1/PGK cells had repressed expression of the PGK transgene, whereby COX-2 protein expression was recovered (Fig. 4b), further confirming the effects of PGK-1 on tumor cells.

We further analyzed the levels of PGE2, a downstream product of COX-2 that has been known to upregulate cellular migration and invasion as well as angiogenesis.[2,10,11] The level of PGE2 was significantly reduced in the conditioned medium in which the LLC-1/PGK (3900 pg/mL) and LLC-1/NBD (4800 pg/mL) cells were cultured in comparison with the levels in the conditioned medium in which the control cells were cultured (6000 pg/mL) (Fig. 4c). Additionally, mice injected with LLC-1/PGK and LLC-1/NBD cells showed reduced COX-2 protein expression in the primary lung tumor mass (Fig. 4d). These results indicated that the levels of COX-2 and PGE2 were decreased by the overexpression of PGK-1 or NBD in LLC-1 cells.

Prostaglandin E2 promoted invasion of LLC-1 cells. The variations in the TGF-β1 mRNA levels (Fig. 3) in the LLC-1 transfectants were confirmed by ELISA. In accordance with the RT-PCR results, only the LLC-1/PGK cells, and not the LLC-1/CDI and LLC-1/NBD cells, displayed a decrease in the amount of secreted TGF-β1 (500 pg/mL) compared to the control LLC-1/EV cells (1100 pg/mL) (Fig. 5a). The role of TGF-β1 and PGE2 in the invasion of lung cancer cells was examined by treating LLC-1 cells with TGF-β1 and PGE2. When the LLC-1 cells were stimulated with PGE2, their invasive ability increased, whereas LLC-1 stimulation with TGF-β1 did not enhance invasiveness (Fig. 5b). These data indicate that the decrease in the level of PGE2 induced by PGK-1 and NBD may reduce the invasive ability of LLC-1 cells.

Reduction in COX-2 level due to overexpression of PGK-1 and NBD was through reduction in Cox-2 mRNA stability. In addition to being an intracellular protein, PGK-1 is also secreted into medium by cells, in which the catalytic formation of angiostatin is produced.[8] We investigated whether extracellular PGK downregulates expression of COX-2 in LLC-1 cells. Two and three days after adding the recombinant PGK-1 protein, the PGK-treated cells were tested for protein expression levels of COX-2. We found that there was no difference in COX-2 levels in the PGK-treated LLC-1 and non-treated LLC-1 cells (Fig. 5c). The data suggest that regulation of COX-2 can be attributed to the intracellular

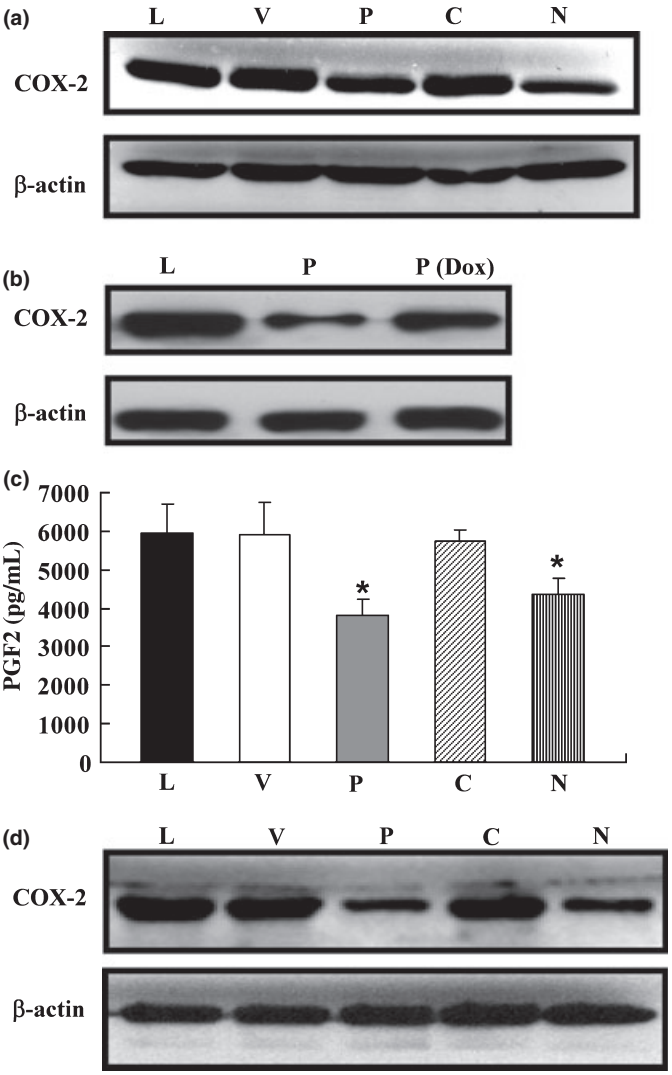

Fig. 4. Expression levels of COX-2 and prostaglandin E2 (PGE2) in Lewis lung carcinoma (LLC-1) cells overexpressing nucleotide-binding domain (NBD) and phosphoglycerate kinase 1 (PGK) were markedly downregulated *in vitro* and *in vivo*. (a,b) Both COX-2 and β-actin proteins were detected. (c) Tumor cells (8×10^4/mL) were cultured for 2 days. The PGE2 concentrations in the culture-conditioned media were measured. (d) C57BL/6 mice were s.c. injected with 5×10^5 tumor cells. After 36 days, COX-2 protein expression in the tumors was detected. L, parental LLC-1; V, LLC-1/EV (vector control); P, LLC-1/PGK; C, LLC-1/CDI; N, LLC-1/NBD; Dox, doxycycline. Each experiment was repeated in triplicate. *$P < 0.05$.

effects of PGK-1. Previous studies have shown that PGK-1 is an mRNA-binding protein and that it downregulated uPAR, interleukin-6 (IL-6), IL-8, and VEGF in cancer cells overexpressing it.[7,21] We inferred that PGK-1 may be directly involved in the post-transcriptional regulation of COX-2 mRNA in LLC-1/PGK cells. Therefore, we measured the post-transcriptional level of COX-2 expression in the PGK- and NBD-overexpressing LLC-1 cells. Transfectants were treated with actinomycin D to inhibit transcription and analyzed for the COX-2 mRNA level by RT-qPCR. The results indicated that the level of COX-2 mRNA was significantly reduced in the LLC-1/PGK and LLC-1/NBD cells (Fig. 5d), but not the LLC-1/CDI cells, compared to the control cells. Therefore, it is likely that the overexpression of PGK-1 or NBD led to the destabilization of COX-2 mRNA in the LLC-1 cells.

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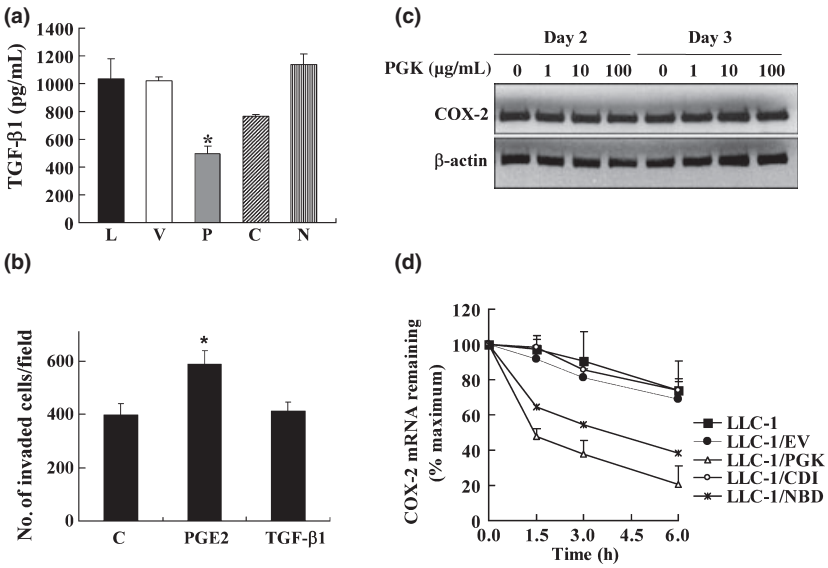

Fig. 5. Expression of COX-2 in Lewis lung carcinoma (LLC-1) cells overexpressing nucleotide-binding domain (NBD) and phosphoglycerate kinase 1 (PGK) cells was reduced because of decreased COX-2 mRNA stability. (a) Transfected tumor cells (8×10^4/mL) were cultured for 4 days. The transforming growth factor-β1 (TGF-β1) concentrations in the culture supernatants were measured. (b) LLC-1 cells (2×10^5/mL) were stimulated with prostaglandin E2 (PGE2; 10 μg/mL) or TGF-β1 (6 ng/mL) for 2 days. The invasive ability of the treated cells was assessed. (c) LLC-1 cells were treated with yeast PGK at different concentrations for 2 or 3 days. The level of COX-2 protein in the treated cells was detected. (d) LLC-1 and LLC-1 transfectants were treated with actinomycin D (5 μg/mL) for different time periods. The COX-2 mRNA levels were determined by RT–quantitative PCR. C, LLC-1/catalytic domain (CDI); EV, LLC-1/EV (vector control); L, parental LLC-1; V, LLC-1/EV (vector control); P, LLC-1/PGK; C, LLC-1/CDI; N, LLC-1/NBD; EV, vector control. The means of three independent experiments were calculated. *$P < 0.05$.

Discussion

Despite recent improvements in chemotherapy and radiotherapy for lung cancer management, novel multimodality treatments are still required to further benefit patients.[1,2] The inhibition of COX-2 represents one such treatment method.[2] In this study, we showed that the inhibition of COX-2 by overexpression of the NBD of PGK-1 in lung cancer cells could retard tumor growth *in vivo*. In addition to reducing COX-2 expression, PGK-1 can further limit tumorigenicity by downregulating uPAR,[7] generating angiostatin,[8] and promoting immune responses against cancer cells.[9] The tumor vascularity and growth rate were found to be reduced in fibrosarcoma- and pancreatic tumor-bearing mice injected with recombinant PGK-1 protein.[8] In prostate cancer, enhanced expression of PGK-1 was found to lead to the arrest of angiogenesis as a result of angiostatin generation and reduced secretion of pro-angiogenic factors (VEGF, IL-6, and IL-8). However, it has been shown that when prostate cancers metastasize to tissues with high chemokine (C-X-C motif) ligand (CXCL)-12 production, the expression of PGK-1 is inhibited by CXCL-12 signaling through chemokine[C-X-C motif] receptor 4 (CXCR4), which results in angiogenic switch and metastatic growth.[21] In NSCLC, PGK-1 overexpression not only activated peripheral immune cells to trigger specific antitumor immunity, but also inhibited the expression of oncogenic factors (COX-2, PGE2, and TGF-β1), tumor invasion, and angiogenesis, resulting in the retardation of tumor growth in both immunocompetent and immunodeficient mice.[20] In the current study, we first showed that the overexpression of the functional domains CDI and NBD of PGK-1 could also lead to reduced tumor invasive ability (Fig. 2d) and *in vivo* lung cancer growth (Fig. 2a). Thus, the CDI and NBD domains of PGK-1 play an important role in the regulation of tumor development.

Constitutive COX-2 overexpression in tumors is responsible for the dysregulation of post-transcriptional and translational mechanisms, whereby tumorigenesis is stimulated.[22] AU-rich sequence element (ARE)-binding proteins bind to the ARE of COX-2 3′-untranslated region and influence the fate of COX-2 mRNA by regulating mRNA degradation, stabilization, or translation.[22,23] In serous cancer, the enhanced binding of HuR, an RNA-binding protein, to COX-2 ARE was found to contribute to an increase in COX-2 mRNA stability.[22,24,25] An mRNA-binding protein, PGK-1 has been shown to interact directly with the mRNA coding region sequence of uPAR, resulting in reduced uPAR mRNA stability in H157 human lung carcinoma cells.[7] Despite lacking enzyme activity, mutant PGK-1 overexpressed in lung carcinoma cells can downregulate uPAR expression and the invasive ability of the tumor cells.[7] We also showed that the overexpression of wild-type PGK-1 accelerated COX-2 mRNA degradation (Fig. 5d), leading to reduced levels of COX-2 protein and tumorigenicity attenuation (Figs 2a,4a). Furthermore, even though it lacks catalytic activity, the NBD of PGK-1 destabilized COX-2 mRNA (Fig. 5d), which in turn contributed to hampered tumor cell invasive ability and tumor growth (Fig. 2a,d). As PGK-1 is an mRNA binding protein, the NBD domain of PGK-1 may interact directly with the mRNA coding region sequence of COX-2, resulting in reduced COX-2 mRNA stability. Therefore, the NBD is essential for the PGK-1-mediated reduction in COX-2 mRNA expression at the post-transcriptional level, and the downregulation of COX-2 mRNA is independent of the catalytic activity of PGK-1. The catalytic domain of PGK-1 (CDI) contains many cysteine residues that may be involved in thiol reductase activity of PGK-1 on plasminogen for the generation of angiostatin, leading to reduction of tumor vascularity *in vivo*.[8] Therefore, the CDI domain may be important for PGK-1-mediated tumor suppression (Fig. 2a) due to the generation of angiostatin.

In the early phases of tumorigenesis, TGF-β1 inhibits the proliferation of epithelial cells. Owing to changes in the differentiation of tumors and their sensitivity to TGF-β1, an increase in tumor TGF-β1 production enhances cancer progression in the

late phase.[26] Elevated TGF-β1 secretion by many types of tumors is positively related to poor clinical outcome.[27] Overproduction of TGF-β1 influences proliferation, differentiation, migration, invasion, angiogenesis, and immune surveillance in tumor cells.[28] We showed that the overexpression of full-length PGK-1 in lung carcinoma cells reduced the level of TGF-β1, but the overexpression of NBD or CDI did not (Figs 3c,5a). The regulation of TGF-β1 may require coordination between NBD and CDI domains of PGK-1. However, TGF-β1 treatment did not enhance the migratory or invasive abilities of LLC-1 cells (Fig. 5b). Our previous result found that overexpression of full-length PGK-1 in LLC-1 may trigger an effective antitumor immune response *in vivo*.[20] In the LLC study, Young *et al.* found that LLC cells can induce immune suppressive activity of immature myeloid cells through production of TGF-β1.[28,29] When anti-TGF-β antibodies were added to the TGF-β1-containing supernatants, the suppressive activity of bone myeloid cells was diminished. This effect promoted CD4 T cell proliferation in an LLC tumor cell–dentritic cell coculture system.[30] Hence tumor-derived TGF-β1 is able to regulate differentiation of myeloid dendritic cells and function of T cells, which can provide a tumor-supporting microenvironment to accelerate tumor growth. Based on previous studies and our experimental data, the reduction in the levels of tumor-derived TGF-β1 by wild-type PGK-1 may be involved in the activation of antitumor immunity.

In addition to the downregulation of uPAR and the generation of angiostatin,[7,8] PGK-1 expressed in lung cancer cells also limits the tumor levels of COX-2 and TGF-β1, the tumor invasive ability, angiogenesis as well as tumor immunosurveillance, leading to suppression of tumor growth.[20] Interestingly, both CDI and NBD of PGK-1 possess the ability to inhibit tumor cell invasion and delay tumor growth. The antitumor effects of NBD are evident by decreasing COX-2 and PGE2 expression. The targeting of COX-2 by PGK-1 is independent of its catalytic activity. Taken together, our findings show the detailed mechanism of action of PGK-1, which may be an effective target for pharmacological treatment or gene therapy intervention for NSCLC.

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