Circadian clock gene Per2 downregulation in non-small cell lung cancer is associated with tumour progression and metastasis

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Abstract. Period2 (Per2) is a key circadian clock gene, and its deregulation contributes to tumour development, including breast cancer. However, the biological function and clinico-pathological significance of Per2 in non-small cell lung cancer (NSCLC) remain unclear. The present study aimed to explore the role of Per2 and its relative clinical significance in NSCLC. To analyse Per2 expression in NSCLC specimens, reverse transcription-quantitative polymerase chain reaction was performed, and the results indicated that Per2 expression was markedly downregulated in 83.87% (26/31) of NSCLC samples compared with their adjacent matched tissues. Increased Per2 expression was associated with increased differentiation (P<0.01) and reduced lymph node metastasis (P<0.0001). Functional studies identified that enhancing Per2 expression in A549 cells by lentivirus transduction not only significantly suppressed cell growth, migration and invasion (P<0.05) but also inhibited NSCLC growth and metastasis *in vivo*. Animal studies and histopathological analysis identified that Per2 expression in A549 cells not only markedly increased expression of tumour anti-oncogenes Bax, P53 and P21 but also inhibited expression of pro-oncogenes vascular endothelial growth factor, CD44 and c-Myc. These results indicate that the loss of Per2 is one of the factors underlying tumourigenesis in NSCLC, and it may function as a novel molecular target for NSCLC.

Introduction

Lung cancer is the most common cancer type, which is responsible for around 1.4 million deaths worldwide each year (1). NSCLC represents more than 80% of lung cancer cases (2). Although there have been advances in surgical techniques, chemotherapy and radiotherapy for treating lung cancer, the overall survival of patients remains low (3,4). Therefore, it is important to investigate a more complete system for prognostic evaluation. It is necessary to identify a more useful prognostic and predictive markers for early molecular diagnosis or potential targets for more precise risk analysis and development of new therapies.

The daily cycle of light and dark is a driving force for various living organisms. Therefore, circadian rhythms are basic regulators of an organism's biological activities. Circadian rhythms are daily oscillations that are regulated by endogenous clock genes. They make it easier for organisms to adapt to daily environmental changes, including temperature, pressure and light. They are also able to synchronize multiple molecular, biochemical, physiological and behavioural processes (5,6). Common core clock genes include CLOCK, BMAL1, Period1 (Per1), Period2 (Per2), Period3 (Per3), cryptochrome1 (Cry1), cryptochrome2, casein kinase1 epsilon and timeless (7). A number of epidemiological studies have suggested that disruption of the normal circadian rhythm may increase the risk of developing various types of cancer, including breast (7), prostate (8), colorectal, liver and endometrial cancers (9-11). Among all known clock genes, only the members of the Per

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Abbreviations: NSCLC, non-small-cell lung cancer; Per1, Period1; Per2, Period2; Per3, Period3; Cry1, cryptochrome1; VEGF, vascular endothelial growth factor; NC, negative control

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subfamily function as tumour suppressors in mice (8). The neoplastic growth of cancer cells may be restrained by overexpression of Per1 or Per2, and their apoptotic rate may also be increased (8). Importantly, the involvement of Per1 and Per2 in ataxia telangiectasia mutated checkpoint kinase DNA damage response pathways implicates the circadian system in tumour suppression (9).

It has been reported that Per1 is downregulated in NSCLC cell lines and inhibits cancer cell proliferation (7). In addition, Couto et al (10) reported that Per3 was downregulated in NSCLC, and polymorphisms in Per3 genes may be a factor underlying NSCLC in Brazilian patients. According to these reports, the members of the Per subfamily appear to be responsible for tumour progression in NSCLC. However, the biological function and clinicopathological significance of Per2 in NSCLC remains unclear. In the present study, the role of Per2 and its relative clinical significance in NSCLC were explored. It was identified that loss of Per2 was associated with NSCLC progression, and recovery of Per2 expression could suppress A549 cell growth and migration *in vitro* and *in vivo*, suggesting that Per2 may serve as a potential molecular target for NSCLC.

Materials and methods

Clinical samples. A total of 31 pairs of NSCLC and matched para-carcinoma tissues were collected from the Department of Thoracic Surgery, Sichuan Cancer Hospital and Institute (Sichuan, China) between March 2015 and May 2016. All patients were between 30 and 78 years old and among them 20 were male and 31 were female. All patients provided written informed consent, and the study was approved by the Ethical Committee of Sichuan Cancer Hospital & Institute. Lung cancer tissues and matched para-carcinoma tissues were immediately frozen in liquid nitrogen following surgical resection and then stored at -80°C until use. The differentiation and metastasis states were identified by experienced pathologists.

Cell culture and lentivirus transduction. A549 cells were purchased from the Institute of Biochemistry and Cell Biology of Chinese Academy of Science (Shanghai, China) and cultured in RPMI-1640 (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) with 15% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified incubator containing 5% CO_2. Overexpression lentivirus of Per2 was purchased from Genechem (Shanghai GeneChem Co., Ltd., Shanghai, China). A549 cells were infected with virus at a multiplicity of infection of 60, and the expression rate of green fluorescence protein was observed with a fluorescence microscope (IX70; Olympus Corporation, Tokyo, Japan) on day 3. Finally, the infected efficiency of A549 following 72 h of lentiviral transduction was confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

Wound healing assay. A549 cells at a density of $3x10^5$ cells/well were seeded in 6-well culture plates and cultured overnight at 37°C. Then, a sterile, plastic 200-μl micropipette tip was used to make a straight scratch on the confluent cell monolayer. The well was washed with medium to remove detached cells, then cells were cultured for a further 48 h at 37°C. Finally, images were obtained under a light microscope and the width of the wound was measured with the microscope and ImageJ software v1.37 (National Institutes of Health, Bethesda, MD USA).

Cell proliferation and colony formation assays. A549 cells with or without lentiviral infection were seeded at a density of $1.5x10^3$ cells/per well into 96-well plates. The cell growth of A549 cell lines was determined by Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) once a day for 1 week. Assays of each group were performed in quintuplicate wells.

For colony formation assays, A549 cells with or without lentiviral transduction were seeded into 6-well plates at a low density (200 cells/per well) and cultured for 2 weeks. Then, cells were fixed with 4% paraformaldehyde at 37°C for 15 min and stained with 1% crystal violet for 30 min at room temperature. Finally, the number of colonies of substantial size (>50 cells per colony) were imaged by light microscope, with three fields of view per well. The experiments were repeated in triplicate.

Cell cycle analysis by DNA content. Briefly, A549 cells with or without lentiviral transduction for 48 h were harvested and washed by centrifugation at 200 x g for 5 min at 25°C in 1X PBS buffer. Next, cells were fixed in 70% ethanol at 4°C overnight and washed twice with cold 1X PBS buffer. Then, cells were resuspended in 100 μl RNase A (10 μg/ml), incubated at 37°C for 30 min and immediately stained with 400 μl propidium iodide (500 μg/ml; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) at 4°C for 30 min in the dark. Finally, data were acquired on a flow cytometer in triplicate (Coulter Epics XL; Beckman Coulter, Inc., Brea, CA, USA) and analysed by Flow Jo7.6 software (FlowJo LLC, Ashland, OR, USA).

In vitro migration and invasion assays. Migration assays and invasion assays were performed using 24-well Transwell filters with 8 μm pore size polycarbonate membrane (Corning, Inc., Corning, NY, USA) and an invasion assay kit that included Matrigel (BD Biosciences, San Jose, CA, USA). Briefly, 48 h following transfection, $2x10^4$ (for the migration assay) or $1x10^5$ cells (for the invasion assay) were resuspended in 200 μl serum-free RPMI-1640 and plated into the upper chamber. The bottom of the lower chambers in the 24-well plates was filled with 0.6 ml medium containing 10% FBS, which was used as a chemoattractant. Following incubation at 37°C for 48 h, the Transwell inserts were removed from the plate, and a cotton-tipped applicator was used to carefully remove the media and remaining cells that had not migrated or invaded from the top of the membrane without damaging it. Then, cells were fixed with 600 μl of 4% paraformaldehyde, stained with 0.5% crystal violet and incubated at room temperature for 5 min at 37°C. Then, the Transwell membranes were allowed to dry. The lower chamber was photographed by a light microscope and cells were counted in at least five different fields of view.

Hematoxylin and eosin (H&E) staining. Xenograft tissues were immersed in 4% paraformaldehyde at 37°C for 4 h, then

transferred to 70% ethanol. Individual lobes of xenograft tissue biopsy material were placed in processing cassettes, dehydrated through a serial alcohol gradient, and embedded in paraffin wax blocks. Prior to staining, 5-μm-thick lung tissue sections were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in PBS. Tissues were then stained at room temperature with H&E. Then, sections were dehydrated through increasing concentrations of ethanol and xylene. A light microscope was used to examine the tissues.

Immunohistochemistry (IHC) analysis. The tumor xenograft were immersed in 4% paraformaldehyde at 37°C for 4 h, and 5 μm thick slices were used for IHC staining by the streptavidin-biotin-peroxidase complex method. The antigen retrieval procedure was performed by heating the samples in antigen retrieval solution containing 10 mM sodium citrate buffer with a pressure cooker at 100°C for 150 sec. When the slices were cooled, normal goat serum blocking solution (Beyotime Institute of Biotechnology, Shanghai, China) was used for blocking at 37°C for 2 h. Rabbit anti human Per2 (1:200; ab179813; Abcam, Cambridge, MA, USA), Ki-67 (1:500; ab8191; Abcam), E-Cadherin (1:100; ab133597; Abcam) and NM23 (1:100; ab154529; Abcam) were incubated at 4°C overnight. Then, the Dako REAL™ EnVision™ kit (K500711; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) was used to incubate the slices at 37°C for 30 min. IHC staining intensity was independently scored under a light microscope by two anatomical pathologists. The staining intensity (negative=0, weak=1, moderate=2 or strong=3) and the proportion of positively stained cells (<25%=1, 25-50%=2, >50 and <75%=3, ≥75%=4) were scored. The immunostaining was semi-quantitatively categorized by multiplying the intensity and the quantity scores to yield a staining index (values from 0 to 12). A staining index of 3-12 was regarded as high expression, while a staining index of 0-2 was regarded as low expression.

Animal studies. This experiment was conducted in accordance with the Care and Use of Laboratory Animals (12) and was approved by the Ethical Committee of Sichuan Cancer Hospital & Institute. Sixteen 4-week-old male BALB/c-nude mice weighing 12-15 g were purchased from the Laboratory Animal Centre of Sichuan University (Chengdu, China) and reared in specific-pathogen-free conditions. The mice were randomly and equally divided into experimental and control groups, in which the experimental group mice underwent tumour cell transplantation with Per2 overexpression and the control group mice underwent tumour cell transplantation without Per2 overexpression. For the subcutaneous transplantation tumour model, $2x10^6$ A549 cells that did or did not overexpress Per2 were resuspended in 0.1 ml PBS and then injected subcutaneously into the backs of the mice. Tumour growth was measured with Vernier calipers over the course of 30 days following transplantation of tumour cells, and tumour volume was calculated according to the formula: Tumour volume = 0.5x length x width^2. For the lung metastasis model, $1x10^5$ A549 cells diluted in 100 μl PBS was intravenously injected via the mouse tail vein. The metastasis score was determined by the pathological condition of the metastatic mice following treatment for 4 weeks.

RNA extraction and RT-qPCR. Total RNA was extracted from cells using a Total RNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's protocol. Reverse transcription to cDNA was perfroemd using Prime Script™ RT Master mix (Takara Biotechnology Co., Ltd., Dalian, China). The cDNA was used for qPCR using the CFX96 Real-Time Quantitative PCR system (Bio-Rad Laboratories, Inc.) with SYBR® Premix ExTaq™ (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. Relative mRNA expression levels were determined by the cycle threshold (Ct) normalized against GAPDH using the $2^{-\Delta\Delta Ct}$ formula (13). Experiments were performed in triplicate. The primers used in this study were as follows (5'-3'): Per2-forward (F), CAGGTGAAAGCCAATGAAGAG; Per2-reverse (R), GGGAGGTGAAACTGTGGAAC; Bax-F, CCCGAGAGG TCTTTTTCCGAG; Bax-R, CCAGCCCATGATGGTTCT GAT; P53-F, ACTTGTCGCTCTTGAAGCTAC; P53-R, GAT GCGGAGAATCTTTGGAACA; P21-F, CCAACAAACTTA ACGTGCCAC; P21-R, AGGCTCAACAGTAACTGCATC; Nm23-F, CATTGCGATCAAACCAGATG; Nm23-R: CAG AAGTCTCCACGGATGGT; E-Cadherin-F, CGAGAGCTA CACGTTCACGG; E-Cadherin-R, GGGTGTCGAGGGAAA AATAGG; vascular endothelial growth factor (VEGF)-F, GGAGGAGGAAGAAGAGAAGGAA; VEGF-R, GTGGAG GTAGAGCAGCAAGG; CD44-F, CAGCACCATTTCAAC CACAC; CD44-R, GCCAAACCACTGTTCCTTCT; cMyc-F, CCGAGGAGAATGTCAAGAGG; cMyc-R, ACGCACAAG AGTTCCGTAGC; GAPDH-F, GAAGGTGAAGGTCGG AGTC; and GAPDH-R, GAAGATGGTGATGGGATTTC.

Western blot analysis. Antibodies for Per2 (ab179813), p53 (#48818), BAX (#2774), P21 (#2947), E-Cadherin (#14472), NM23 (#3338), VEGF (#2463), CD44 (#3570), c-Myc (#5605) and β-actin (#3700) were purchased from Abcam or Cell Signaling Technology, Inc. (Danvers, MA, USA) and all the antibodies were rabbit anti-human. Cells were harvested, lysed with RIPA buffer (Sigma-Aldrich; Merck KgaA, Darmstadt, Germany) supplemented with 1 mmol/l PMSF (Boster Biological Technology, Pleasonton, CA, USA) and then centrifuged at 14,000 x g at 4°C for 10 min. Protein concentrations of the extracts were measured using a bicinchoninic acid protein assay kit (Nanjing KeyGen Biotech Co., Ltd.). Proteins (20 μg) were separated through 10% SDS-PAGE gels, and then transferred to polyvinylidene difluoride membranes. Following blocking in Tris-buffered saline with Tween-20 containing 5% non-fat milk for 60 min, the membranes were incubated with primary antibodies (P53, Bax, c-Myc, Per2, NM23, CD44, E-cadherin and VEGF primary antibody: 1:1,000 dilution; P21 primary antibody: 1:500 dilution; β-actin as a loading control: 1:2,500 dilution) overnight at 4°C. The membranes were then incubated with the secondary antibodies (mouse anti-rabbit and horseradish peroxidase-linked antibody, 1:5,000 dilution; Cell Signaling Technology, Inc.). Following incubation in enhanced chemiluminescence solution, the proteins on the membranes were detected using Bio-Rad Universal Hood III and analysed using Image Lab™ software 2.0 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are presented as the mean ± standard deviation, with the exception of tissue expression data, which are presented as the median ± range. The differences
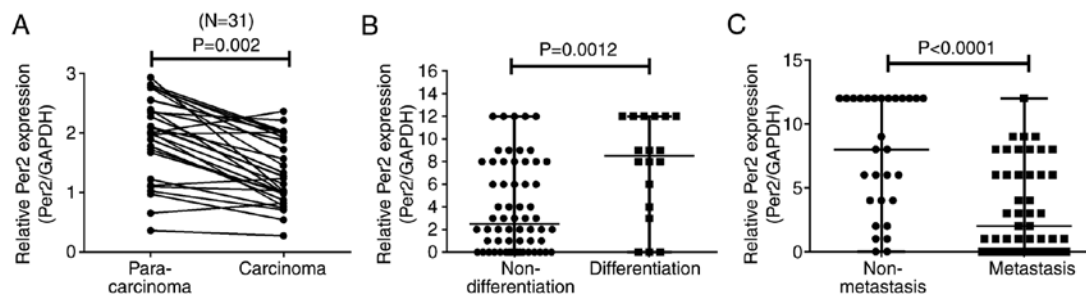
Figure 1. Per2 expression is decreased in NSCLC tissues and associated with clinicopathological characteristics. (A) Per2 expression levels in 31 paired NSCLC tissues and their matched controls were analysed by reverse transcription-quantitative polymerase chain reaction. Evaluation of clinicopathological characteristics identified that low expression of Per2 was significantly associated with (B) pathological differentiation and (C) lymph node metastasis. Data are presented as the median ± range. Per2, Period2; NSCLC, non-small-cell lung cancer.

in Per2 expression between NSCLC tissues and matched para-carcinoma adjacent normal lung tissues were compared by a two-tailed independent samples Student's t-test. All analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Per2 is downregulated in NSCLC tissues and associated with clinicopathological characteristics. Per2 gene exhibits low expression in certain cancer tissues. In the present study, the expression of Per2 in lung cancer was evaluated by performing RT-qPCR in different histological subtypes of NSCLC tissue. As presented in Fig. 1A, compared with matched controls, the Per2 mRNA level was strongly downregulated in 83.87% (26 of 31) of NSCLC samples (P<0.01 vs. control). As indicated in Fig. 1B and C, Per2 expression was significantly associated with pathological differentiation (P<0.01) and lymph node metastasis (P<0.0001). Increased Per2 expression indicated increased differentiation and reduced lymph node metastasis. These findings suggested that Per2 may be involved in NSCLC development.

Overexpression of Per2 decreases A549 cell growth by blocking the cell cycle at G0/G1 phase. Based on the finding that Per2 expression was decreased in NSCLC samples, a Per2-expressing lentivirus was constructed and used to infect A549 cells, in order to determine the potential role of Per2 in NSCLC. As presented in Fig. 2A, the mRNA expression of Per2 increased 11-fold and Per2 protein expression also increased in A549 cells following infection with Per2-expressing lentivirus. Next, a CCK-8 assay, colony formation assay and cell cycle analysis were performed to detect the effect of overexpression of Per2 on A549 cell growth. The results of the CCK-8 and colony formation assays demonstrated that overexpression of Per2 suppressed A549 cell proliferation and colony formation compared with the control group (P<0.05; Fig. 2B and C). Furthermore, flow cytometry was performed to analyse the influence of Per2 on the cell cycle and it was identified that the percentage of A549 cells that remained in G1 phase was significantly increased in the Per2 overexpression group compared with the control group (76.67±10.22 vs. 66.80±11.38%; P<0.01), while fewer cells stayed in S phase

compared with the control group (6.01±2.74 vs. 10.03±3.01%) after 48 h of culture (Fig. 2D). These results indicated that increased Per2 expression in A459 cells inhibits tumour cell proliferation by inducing cell cycle arrest at G0/G1 phase.

Overexpression of Per2 suppresses A549 cell migration and invasion ability. Metastasis begins when the tumour invades into the vascular stroma and migrates to the blood (14). Therefore, the influence of Per2 on tumour cell migration and invasion ability was evaluated with *in vitro* Transwell assays. Overexpression of Per2 significantly inhibited the number of migrating and invading A549 cells compared with the negative control group (migration: 130.80±9.987 vs. 20.20±4.164; invasion: 134.20±7.165 vs. 56.80±6.763; both P<0.0001; Fig. 3A). Similarly, results of the wound healing assay revealed that wound gap closure and migration was significantly decreased for A549 cells overexpressing Per2 (P<0.01; Fig. 3B). These results demonstrated that Per2 may act as an anti-oncogene in suppressing A549 cell growth and metastasis.

Overexpression of Per2 decreases apoptosis- and metastasis-associated gene and protein expression in A549 cells. To further elucidate the molecular mechanisms underlying Per2 effects on cell growth and metastasis, apoptosis-associated genes Bax, P53, P21 and NM23, metastasis-associated genes E-Cadherin, VEGF and CD44, and c-Myc oncogene were evaluated. RT-qPCR and western blotting results are presented in Fig. 4. Overexpression of Per2 markedly increased the expression of Bax, P53, P21 and NM23 and suppressed VEGF, CD44 and c-Myc expression. These findings indicated that the antitumour effect of Per2 is associated with these apoptosis- and metastasis-associated genes.

Overexpression of Per2 reduces tumour formation of A549 cells in a nude mouse xenograft model. To evaluate whether Per2 exhibited antitumour effects *in vivo*, A549 cells that were or were not transduced with Per2-overexpressing lentivirus were subcutaneously implanted into the backs of BALB/c-nude mice. Consistent with the current *in vitro* results, Per2-lentivirus A549 NSCLC cells exhibited delayed tumour formation compared with negative controls (P<0.05; Fig. 5A). At 30 days after implanting tumour cells, the tumour weight of the Per2-overexpressing group was 0.328±0.171 g, while that of the negative control group was 0.785±0.249 g; therefore,
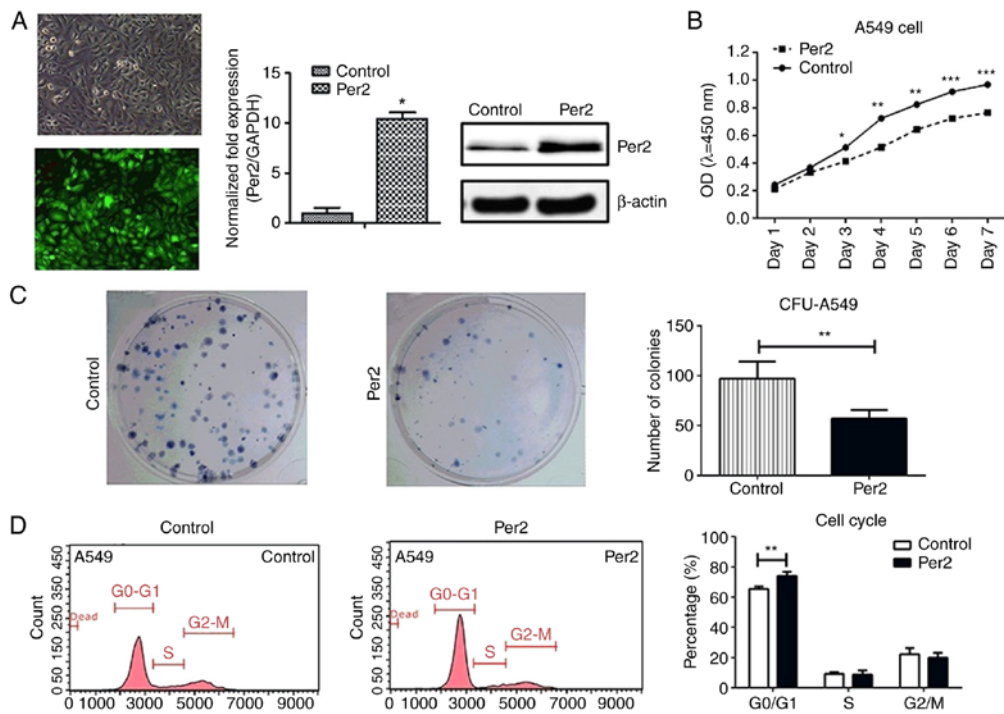
Figure 2. Overexpression of Per2 in A549 cells decreases tumour cell growth by blocking the cell cycle at G0/G1 phase. (A) Overexpression of Per2 in A549 cells was performed by lentivirus infection. Magnification, x40. Per2 overexpression in A549 cells was verified by reverse transcription-quantitative polymerase chain reaction and western blotting. (B) The effect of Per2 overexpression on A549 cell proliferation was evaluated using Cell Counting Kit-8 assay at the indicated time points. (C) The effect of Per2 on colony formation of A549 cells was evaluated at 2 weeks. (D) The cell cycle distribution of A549 cells transduced with Per2-overexpressing lentivirus or negative control for 48 h was analysed by flow cytometry. Similar results were obtained in three experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Per2, cells transfected with lentiviral vector overexpressing Period2.
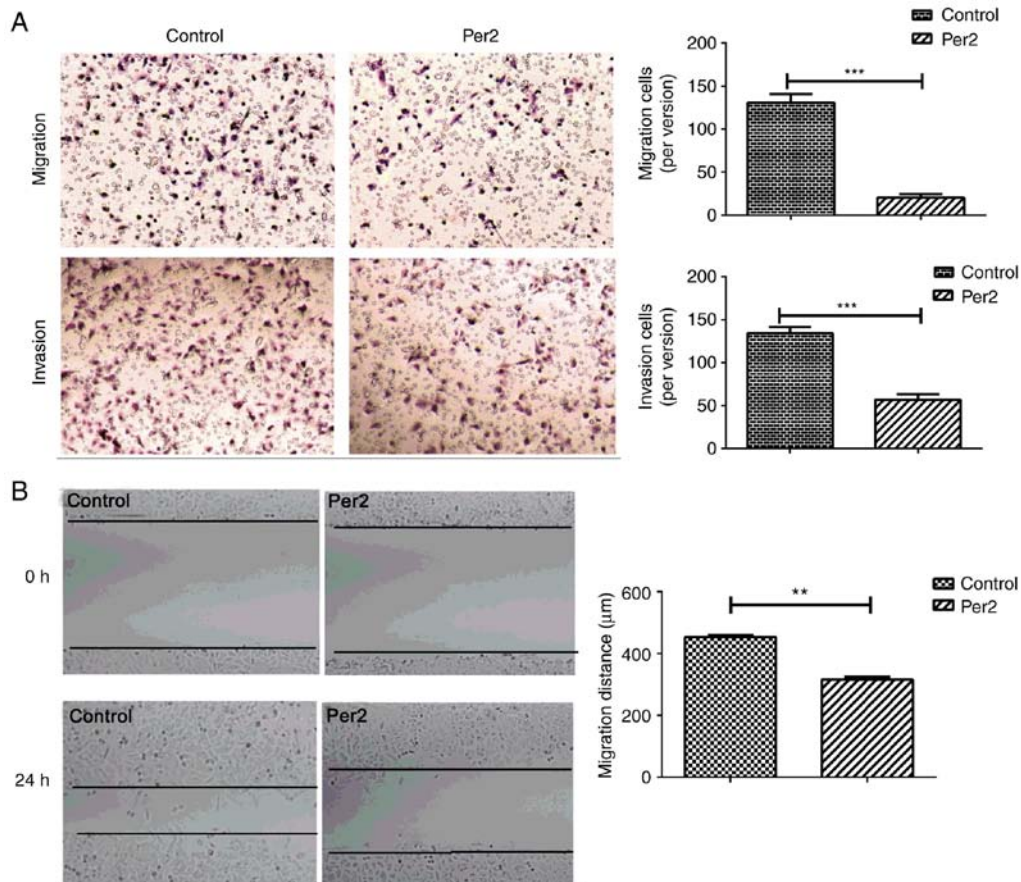

Figure 3. Overexpression of Per2 suppresses A549 cell migration and invasion. (A) Transwell assays were used to analyse the effect of Per2 on A549 cell migration and invasion ability (magnification, x40). (B) Wound healing assay was performed to assess the effect of Per2 on A549 cell migration capacity. Data are presented as the mean ± standard deviation from three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$. Per2, cells transfected with lentiviral vector overexpressing Period2.
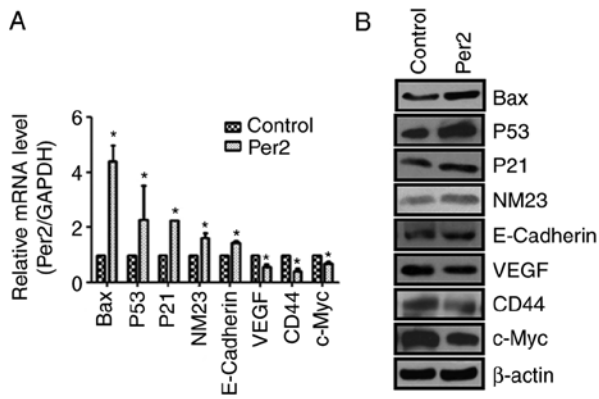
Figure 4. Overexpression of Per2 decrease apoptosis- and metastasis-associated gene and protein expression in A549 cells. Changes in (A) gene and (B) protein expression of Bax, P53, P21, NM23, E-Cadherin, VEGF, CD44 and c-Myc in A549 cells following transduction of Per2-overexpressing lentivirus or negative control for 48 h were detected by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. Per2, cells transfected with lentiviral vector overexpressing Period2; VEGF, vascular endothelial growth factor. *P<0.05.

the tumour growth inhibition rate of the Per2-overexpressing group was 63.56% (P<0.05; Fig. 5B). Furthermore, the number of lung metastasis tumours in the Per2-overexpressing group was significantly decreased when compared with those of the control group, and the H&E staining identified less metastasis in the Per2-overexpressing group (Fig. 5C and D). In addition, IHC analysis was performed on animal tumour samples. As indicated in Fig. 5E, the score of Per2 expression in the Per2-overexpression group (6.38±2.504) was increased compared with the control group (3.12±2.232). For E-cadherin, NM23 and Ki-67 protein expression, IHC indicated that over-expression of Per2 could increase expression of the tumour growth suppressor E-cadherin and tumour metastasis inhibitor NM23, and lower the expression of Ki-67. Collectively, these results suggested that overexpression of Per2 reduces the capacity of A549 cells to form tumours *in vivo*.

Discussion

Core circadian genes play an important role in tissue homeostasis and tumourigenesis. Multiple cancer types are commonly caused by the disruption of circadian rhythms (15). Thus, improved understanding of the underlying biological role of circadian rhythms in NSCLC would help to elucidate the occurrence and development of lung cancer and bring about new ideas for NSCLC diagnosis and therapy. In the current study, it was identified that compared with adjacent non-cancerous tissues, Per2 expression was strongly down-regulated in NSCLC samples and reduced expression was associated with pathological non-differentiation and lymph node metastasis. Functional studies identified that enhanced Per2 expression in A549 non-small cell lung cancer cells significantly suppressed cell proliferation, migration and inva-sion,7 and inhibited NSCLC growth and metastasis *in vivo*. Therefore, Per2 may be a novel molecular target for NSCLC.

Numerous studies have confirmed that circadian rhythm dysfunction is associated with the pathogenesis of cancer. Disruption of circadian rhythms may promote tumour

progression, and restored circadian rhythms may poten-tially have a positive effect on prognosis (16). For instance, compared with normal breast tissues, the expression levels of Per1 and Per2 genes may decrease in sporadic and familiar breast tumours (17). When compared with sporadic forms, the Per1 gene has lower expression levels, and its form is similar to that of breast cancer. This means that a potential deregula-tion of the circadian clock may result in the inherited form of the disease (16). The breast cancer cells may survive due to the methylation of Per1 and Cry1 gene promoters, inacti-vating expression of these genes and disrupting the circadian cell rhythm (18). Furthermore, factors including patient age, tumour histological grade, invasion depth, lymph node metas-tasis and TNM staging are also associated with Per2 protein expression in patients with breast cancer (19). Although the clinical significance and certain roles of Per2 in the malignant biological behaviour of NSCLC have been reported, including chemoresistance and radioresistance, researchers have not explored the influence of Per2 on NSCLC metastasis *in vitro* and *in vivo* (20). In the current study, the roles of Per2 were explored *in vitro* and a metastasis model was also established *in vivo* by tail vein injection. It was identified that overexpres-sion of Per2 inhibited metastasis of NSCLC. Consistent with previous studies, the current results indicated that Per2 expres-sion was markedly downregulated in ~83.87% (26 of 31) of the NSCLC samples compared with their adjacent control tissues. Furthermore, it was identified that decreased tumour differen-tiation and increased lymph node metastasis were associated with decreased Per2 expression, indicating that NSCLC is closely associated with the circadian gene. In addition, these results suggest Per2 deletion or mutation may result in tumour progression and metastasis.

Numerous studies have reported that Per2 plays an important role in the generation and maintenance of circadian rhythms. It controls 2-10% of all mammalian genes (21). For rodents, ~7% of clock-controlled genes regulate cell proliferation or apoptosis (22,23). In addition, it has been reported that circadian rhythms change with human and other mammalian tumour formation (24,25). Furthermore, studies have suggested that decreased Per2 may disrupt normal circadian rhythms of behaviour and physiology, and could significantly increase the chance of tumour and proliferative phenotypes (26-28). These findings demonstrate that Per2 is of great importance for carcinogenesis. In the current study, Per2 expression was increased in A549 cells by transducing them with a Per2-overexpressing lentivirus and then the role of Per2 in cell proliferation, migration and invasion capacity was evaluated. It was identified that enhanced Per2 expression strongly suppressed A549 cell growth in the form of blocking the cell cycle in G1 phase and restricting cell migration and invasion. The study of NSCLC xenografts in nude mice also confirmed that tumour growth rates and volumes were mark-edly reduced in Per2 lentivirus-transduced A549 NSCLC cells compared with the PBS control group. These findings are consistent with previous studies and suggest that Per2 may act as an anti-oncogene in tumour progression.

The antitumour molecular mechanism of Per2 in NSCLC remains unclear. A previous study reported that the antitu-mour effect of the clock gene Per2 is associated with DNA damage (29). It is now well established that DNA damage
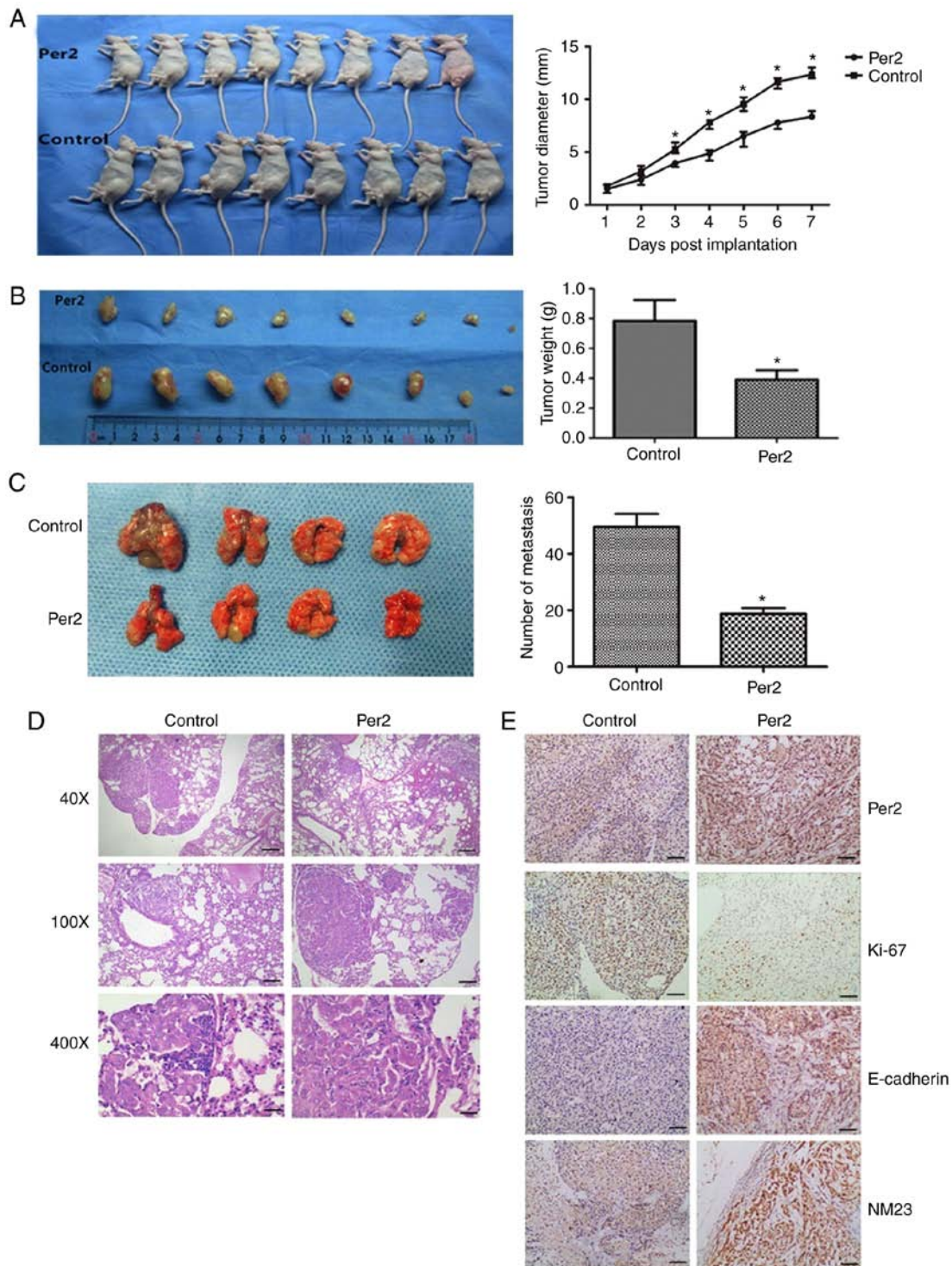
Figure 5. Overexpression of Per2 reduces tumour formation of A549 cells in a nude mouse xenograft model. The effect of Per2 on tumourigenicity in the nude mouse xenograft model was analysed in terms of (A) tumour volume and (B) tumour weight. (C and D) Lung histology in the lung metastasis mouse animal model was analysed by hematoxylin and eosin staining (magnification, x40). (E) Immunohistochemistry was performed to analyse the expression of Per2, Ki-67, E-cadherin and NM23 in mouse tumour samples (magnification, x40). $^*P<0.05$ vs. control. Per2, Period2.

depends on the activation of p53 and p53-associated transcriptional activation, including p21 (30-32). Furthermore, hypoxia of tumour cells usually results in VEGF agonist activity, which may be restrained by Per2; therefore, tumour angiogenesis may be inhibited (33). It was investigated in the current study whether the antitumour mechanism of Per2 in NSCLC was associated the p53-p21-induced DNA damage pathway.

In vitro and *in vivo* observations indicated that increased Per2 expression in A549 cells not only significantly increased expression of the tumour anti-oncogenes Bax, P53, and P21, but also inhibited expression of the pro-oncogenes VEGF, CD44 and c-Myc (34). These results indicate that loss of Per2 inhibits tumour cell growth and metastasis by activating the P53/P21 DNA damage-induced apoptosis pathway in NSCLC

cells. Therefore, it was speculated that reducing Per2 may promote the proliferation and metastasis of NSCLC. This area will be investigated further in future studies.

The Per family may function as a cancer suppressor in the pathogenesis of NSCLC. However, the detailed mechanisms of the downregulation of Per still require elucidation. CpG methylation of promoter sequences may inactivate promoter functions, particularly in tumour suppressor or related genes, and may also lead to silencing of these genes. A key factor in silencing cancer suppressor genes is commonly considered to be methylation (32). Therefore, it is speculated that methylation of the promoters of Per genes may result in the inhibition of Per. Chen *et al* (35) noted that the abnormal expression of Period in breast cancer is not caused by genetic mutations but by the methylation of the Per1 or Per2 promoter. However, Nagel *et al* (36) identified that the microRNA-192/194 cluster is an effective inhibitor of the entire Per gene family using a forward genetic screen. This introduced a new mechanism to control the downregulation of Per at the post-transcriptional level. Based on the aforementioned reported, the reason for the inhibition of Per genes in cancer is complex, and not solely due to methylation. There were also certain limitations to the current study; only A549 cells were available in our laboratory at that time, therefore Per2 overexpression in A549 was studied according to the laboratory conditions. Further investigation is required to clarify the effect of Per on NSCLC development and its underlying mechanisms. In the future, further experimental studies of Per2 in NSCLC will be performed, and several cell lines will be selected to conduct a comprehensive study.

In conclusion, the present study identified that loss of Per2 may be closely associated with the development of NSCLC. Recovery of Per2 expression could suppress A549 cell growth and migration *in vitro* and *in vivo* by increasing apoptosis-associated gene expression and decreasing metastasis-associated gene expression. These findings suggest that Per2 may serve as a novel molecular target for NSCLC.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RX and YC performed the experiments; JL, TX and YW provided technical support, critical comments and suggestions. RX, TX and XY edited the paper; RX, YC and ZW analyzed the data; XY, JL and QL designed the experiments, analyzed the data and wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided written informed consent, and the study was approved by the Ethical Committee of Sichuan Cancer Hospital & Institute (Sichuan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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