Int J Clin Exp Med 2017;10(2):2336-2344
www.ijcem.com /ISSN:1940-5901/IJCEM0035892

Original Article

miR-599 and miR-185 down-regulate periostin expression in human lung cancer cells

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Received July 15, 2016; Accepted October 28, 2016; Epub February 15, 2017; Published February 28, 2017

Abstract: Background: Prognosis of lung cancer remains poor. The over-expression of periostin, osteoblast-specific factor, was reported in cancers such as ovarian and breast cancers. However, it is unclear about effects of periostin on the invasion of human lung cancer, and whether the mRNA and protein expression of periostin was regulated by miRNA needs to be explored. Therefore, we explored effects of periostin on the invasion of human lung cancer cells, and how periostin expression was regulated by miRNA. Methods: Human lung cancer cell line A549 cells were treated with different concentrations of periostin protein, and tumor invasion was detected by Transwell assay. A549 and another lung cancer cell line BEAS-2B cells were transfected with mixture of Dicer-siRNA-152 and Drosha-siRNA-1200, or negative control (NC) siRNA. mRNA expression of periostin were detected by qPCR. Protein expression of periostin were examined by Western Blot. HEK293A cells were transfected with vectors (pMIR/report-3'POSTN and pRL-TK), and miRNA (miR-543, miR-296-3P, miR-599, miR-185, miR-202-3P, and NC miRNA, respectively). In addition, HEK293A cells were transfected with periostin gene mutated at binding sites of miR-599 or miR-185. Expression of periostin was determined by measuring relative light unit in dual-luciferase reporter assay. Results: Periostin protein altered invasion of A549 cells in a dose-dependent manner. mRNA expression of periostin in A549 and BEAS-2B cells increased significantly after miRNA was interrupted. Protein expression of periostin in BEAS-2B cells increased markedly after miRNA was interrupted. Expression of periostin was lowest when HEK293A cells were transfected with miR-599 and miR-185. Expression of periostin was not altered by miR-599 or miR-185 mimics when HEK293A cells were transfected with periostin gene mutated at binding sites of miR-599 or miR-185. Conclusions: Periostin protein alters invasion of human lung cancer cells. miR-599 and miR-185 down-regulate periostin expression in lung cancer cells.

Keywords: Periostin, lung cancer, miR-599, miR-185

Introduction

Human lung cancer is the most common cause of cancer-related death in men and second most common in women after breast cancer worldwide [1]. Common treatments for lung cancer include palliative care, surgery, radiation therapy and chemotherapy [2-4]. Nevertheless, prognosis of lung cancer remains poor, with five-year survival rate of less than 20% [5].

Periostin, also known as osteoblast-specific factor, is encoded by the POSTN gene in humans [6]. It functions as a ligand for integrins, and mediates adhesion and migration of epithelial cells [7]. As a secreted extracellular matrix protein, periostin was originally identified in cells from the mesenchymal lineage, including osteoblasts, osteoblast-derived cells, periodontal ligament, and periosteum. It was revealed to be associated with the differentiation of mesenchyme in developing heart, and facilitate the epithelial-mesenchymal transition in endometrial epithelial cells via through ILK-Akt signaling pathway [8, 9]. Periostin over-expression was reported in several types of cancer, such as ovarian and breast cancers [7, 10]. However, it is unclear about effects of different concentrations of periostin on the invasion of human lung cancer.

MicroRNA (miRNA), a small non-coding RNA molecule containing about 22 nucleotides, plays an important role in RNA silencing and

post-transcriptional regulation of gene expression [11, 12]. miRNAs function through base-pairing with complementary sequences within mRNA molecules [13]. The mRNA molecules are subsequently cleaved into two pieces, and destabilized due to shortening of poly (A) tail. As a result, the translation of mRNA into proteins becomes less efficient [14]. Dysregulation of miRNA has been associated with diseases such as hereditary progressive hearing loss, heart diseases, mental disorders, and cancer [15-18]. miRNA deregulation was shown to involve in chronic lymphocytic leukemia [19]. Low miR-324a levels were revealed to be an indicator of poor survival in non-small cell lung cancer [20]. In addition, miR-543 was revealed to promote proliferation of gastric cancer cells by targeting SIRT1 [21]. Dysregulation of miR-296/S100A4 axis promoted tumor invasion by inducing epithelial-mesenchymal transition in human ovarian cancer [22]. Hsa-miR-599 was shown to act as a tumor suppressor and it inhibited cells proliferation, migration and invasion in hepatocellular carcinoma [23]. High miR-185 correlated with metastasis and poor survival in colorectal cancer [24], and miR-202-3p was reported to be a tumor suppressor in gastric cancer [25]. However, whether the mRNA and protein expression of periostin was regulated by miRNA, and if so which particular types of miRNA are involved need to be explored.

Therefore, we explored effects of periostin on the invasion of human lung cancer cells, and how periostin expression was regulated by miRNA in current study.

Methods

Cells and reagents

Cells: A549 (human lung adenocarcinoma cell line; Novobio Inc., Shanghai, China), BEAS-2B (immortalized human bronchial epithelial cells; Novobio Inc., Shanghai, China), and HEK293A cells (human embryonic kidney cells; Novobio Inc., Shanghai, China) were cultured inRPMI 1640 medium which was supplemented with 100 mL/L fetal bovine serum (FBS), 100 kU/L penicillin and 100 mg/L chloramphenicolin a cell incubator with 5% CO_2 at 37°C. Cells were subcultured after being digested by 0.25% trypsin. A549 and BEAS-2B cells that were transfected with the mixture of Dicer-siRNA-152 (Sense: 5'-UGC UUG AAG CAG CUC UGG ATT-3'; Anti-sense: 5'-UCC AGA GCU GCU UCA AGC ATT-3') and Drosha-siRNA-1200 (Sense: 5'-AAC GAG UAG GCU UCG UGA CUU TT-3'; Anti-sense: 5'-AAG UCA CGA AGC CUA CUC GUU TT-3'), or negative control siRNA were purchased from Novobio Inc. (Shanghai, China). The cells were named A549-152/1200, BEAS-2B-152/1200, A549-NC, and BEAS-2B-NC, respectively.

Main reagents: fetal bovine serum (FBS; Gibco Inc., Grand Island, NY, USA); typsin (Gibco Inc., Grand Island, NY, USA); RPMI1640 (GibcoInc., Grand Island, NY, USA); primers and probes (Invitrogen Inc., Grand Island, NY, USA); miRNAeasy Mini Kit (QIAGEN Inc., Valencia, CA, USA); Trizol reagent (Invitrogen Inc., Grand Island, NY, USA); cDNA synthesis kit (Tiangene Biotech Inc., Beijing, China); chloroform (Sinopharm Inc., Shanghai, China); isopropanol (Sinopharm Inc., Shanghai, China); 75% ethanol (Sinopharm Inc., Shanghai, China); DEPC H_2O (Invitrogen Inc., Grand Island, NY, USA); SuperScriptIII reverse transcriptase (Invitrogen Inc., Grand Island, NY, USA); SYBR Green I (Invitrogen Inc., Grand Island, NY, USA); Rnase Inhibitor (Fermentas Inc., Hanover, MD, USA); Platinum Taq DNA Polymerase (Invitrogen Inc., Grand Island, NY, USA); 100 mM dNTPs (Invitrogen Inc., Grand Island, NY, USA); RIPA buffer (Beyotime Inc., Shanghai, China); BCA kit (Beyotime Inc., Shanghai, China); X-ray films (Kodac Inc., Dayton, OH, USA); polyvinylidene fluoride (PVDF) membrane (EMD Millipore Inc., Darmstadt, Germany); enhanced chemiluminescence (ECL) solution (Fermentas Inc., Hanover, MD, USA); anti-periostinprimary antibody (Abcam Inc., Canbridge, MA, USA); anti-actin primary antibody (Novobio Inc., Shanghai, China); secondary goat anti-rabbit antibody (Novobio Inc., Shanghai, China); Dual-Luciferase® Reporter Assay System (Promega Inc., Fitchburg, USA); transwell palte (Sigma-Aldrich Inc., St. Louis, MI); negative controlmiRNA, miR-543, miR-296-3P, miR-599, miR-185, and miR-202-3P (GenePharm Inc., Shanghai, China); vectors (pMIR/report-3'POSTN; pRL-TK; pMIR/report-miR-599-mut; and pMIR/report-miR-185-mut; Novobio Inc., Shanghai, China).

Main equipments: light and fluorescence microscopes (Olympus Inc., Tokyo, Japan); table-type refrigerated centrifuge (Zhengzhou Nanbei Instrument Equipment Inc., Hefei, China); CFX-

96 Touch™ Real-Time PCR Detection System (Bio-Rad Inc., Hercules, CA, USA); cell incubator (Thermo Scientific Inc., Waltham, MA); electrophoresis and transfer system (Tanon Inc., Fullerton CA, USA); Gel-Pro analyzer (Meyer Instruments Inc., Houston, TX, USA); Turner BioSystems GloRunner (BioSurplus Inc., San Diego, CA, USA).

Transwell invasion assay

A549 cells were digested bytrypsin, and diluted to make concentration of 5×10^5/mL. The membrane of the upper compartment was coated with 50 μL of matrigel (1 g/L), and incubated at 37°C for 1 h in order to reconstruct structure of basal membrane. Eight hundred μL of 1640 medium with 20% fetal bovine serum (FBS), and periostin protein were added into lower compartment. The concentrations of periostin were 200, 20, 2, 0.2, 0.02, and 0 ng/mL during primary screening, and were 0, 0.015, 0.03, 0.06, 0.12, 0.25, 0.5, 1, and 2 ng/mL respectively during concentration gradient study. Two hundred μL of A549 cell suspension were incubated in upper compartment of Transwell, and cells were cultured at a humid incubator with 5% CO_2 at 37°C for 24 h. Transwell was then taken out, and cells on the upper surface of Transwell was erased with cotton swab. Cells on the microporous membrane at lower surface of Transwell was fixed with 4% polyformaldehyde for 30 min, and stained with 1% crystal violet for 10 min. The microporous membrane was washed with phosphate-buffered solution (PBS) for 3 times, and cells that penetrated the membrane were observed under microscope.

Quantitativepolymerase chain reaction (qPCR)

Total RNA of A549-152/1200, A549-NC, A549, BEAS-2B-152/1200, BEAS-2B-NC, and BEAS-2B cells was extracted and purified byTrizolfollowing manufacturer's instructions, respectively. A universal cDNA synthesis kit was utilized for reverse transcription. Each reaction contained 0.5 μL of random primers (0.2 μg/μL) and 1 μLof SuperScrip III reverse transcriptase (200 U/μL). The specific primer for detection of periostin gene was F: GCTGCCATCACATCGGACAT; R: CCTCCCATAATAGACTCAGAACACT. The primer for detecting β-actin gene was F: AGAAAATCTGGCACCACACC; R: AGAGGGTACAGGGATAGCA. qPCR was performed by utilizing MiRcute miRNA qPCR Detection kit. PCR conditions were as follows: pre-denaturing at 95°C for 2 min; denaturing at 95°C for 10 s; and annealing and polymerization at 60°C for 30 s, and 70°C for 45 s. There were 40PCR cycles. PCR was performed in CFX96 Touch™ Real-Time PCR Detection System. The expression of periostin was determined as the ratio of relative optical density of target gene to β-actin.

Western blot

Proteinswere extracted from A549-152/1200, A549-NC, A549, BEAS-2B-152/1200, BEAS-2B-NC, and BEAS-2B cells, respectively. Then they were separated in 10% SDS-polyacrylamide separating gel by eletrophoresis at 120 V, and signal was transferred to PVDF membranes at 100 V for 120 min. Membranes were blocked with 5% non-fat milk for 1 h, and incubated with anti-periostin antibody (1:2000) at 4°C overnight. The membranes were then washed with tris-buffered saline and tween 20 (TBST) for 3 times, and every time lasted for 10 min. Membranes were then incubated with goat anti-rabbit secondary antibody labeled with horseradish peroxidase (HRP) (1/5000) at room temperature for 1 h. Membranes were washed and incubated shortly with ECL solution. Films were exposed in a dark room. Experiments were repeated for 3 times.

Dual-luciferase reporter assay

The sequences for double-stranded miRNA were as follows: negative control (NC) (UUCUCCGAACGUGUCACGUTT); miR-543 (AAACAUUCGCGGUGCACUUCUU); miR-296-3P (GAGGGUUGGGUGGAGGCUCUCC); miR-599 (GUUGUGUCAGUUUAUCAAAC); miR-185 (UGGAGAGAAAGGCAGUUCCUGA); and miR-202-3P (AGAGGUAUAGGGCAUGGGAA). HEK293A cells were cultured in 6-well plates, transfected overnight with vectors (pMIR/report-3'POSTN and pRL-TK) and abovementioned miRNA respectively using lipofectamine 2000. In a parallel experiment, HEK293A cells were divided into 6 groups: (1) miR-599-mut group: cells were transfected with pMIR/report-miR-599-mut (miR-599-mut: periostin gene that has mutation at miR-599 binding site), and pRL-TK; (2) miR-599-mut NC group: cells were transfected with pMIR/report-miR-599-mut, pRL-TK, and NC; (3) miR-599-mut mimics group: cells were transfected with pMIR/report-miR-599-mut, pRL-TK and miR-599 mimic; (4) miR-185-mut group:

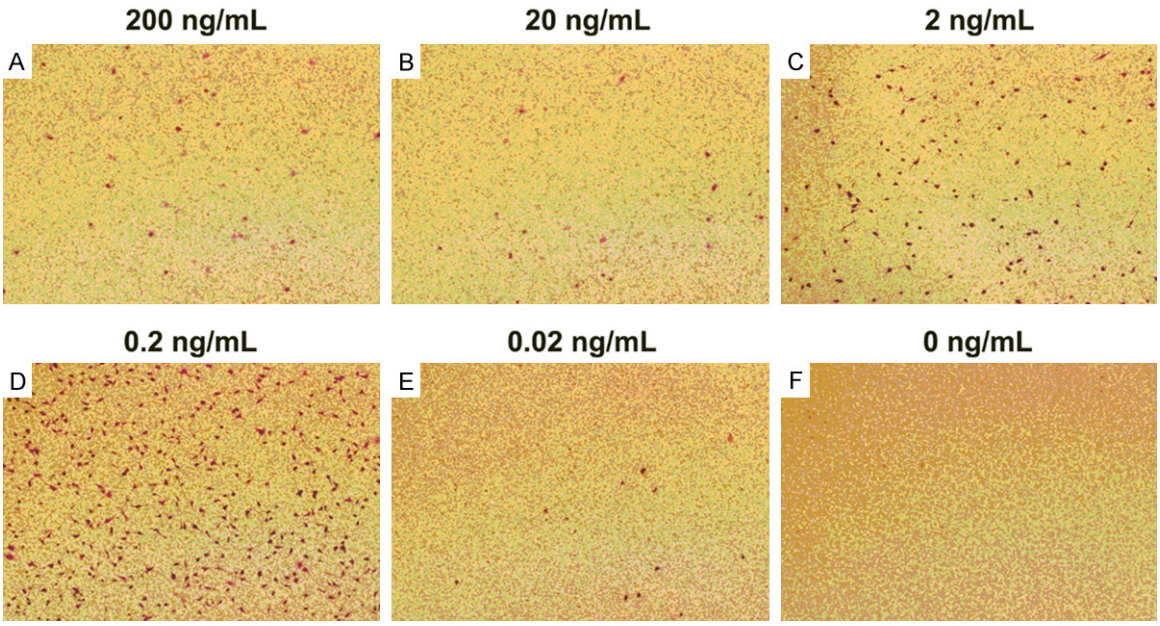

Figure 1. The invasion of A549 cells was highest when concentration of periostin protein was 2 ng/mL during primary screening. Invasion of A549 cells affected by periostin protein was detected by Transwell invasion assay. The concentrations of periostin utilized were 200, 20, 2, 0.2, 0.02, and 0 ng/mL during primary screening.

cells were transfected with pMIR/report-miR-185-mut (miR-185-mut: periostin gene that has mutation at miR-185 binding site), and pRL-TK; (5) miR-185-mut NC group: cells were transfected with pMIR/report-miR-185-mut, pRL-TK, and NC; and (6) miR-185-mut mimics group: cells were transfected with pMIR/report-miR-185-mut, pRL-TK and miR-185 mimic. Cell medium was changed and cells were then cultured for another 36 h. Cells were then lysed with 300 μL of lysis buffer on ice for 10 min, and centrifuged at 13000 rpm for 5 min. One hundred μL of supernatant from each group was mixed with 100 μL of firefly detection reagent, and relative light unit (RLU) was measured. One hundred μL of renilla detection reagent was then added, and a second RLU was measured. The ratio of firefly RLU and renilla RLU was calculated.

Statistical analysis

Results were shown as mean ± SEM. One-way analysis of variance (ANOVA) was used to compare differences among 3 or more groups, followed by Bonferroni post hoc testing for multiple comparisons. *P* values of 0.05 or less were regarded significant. Figures and statistical analysis were made using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA).

Results

Periostin protein altered invasion of A549 cells in a dose-dependent manner

Invasion of A549 cells affected by periostin protein was detected by Transwell invasion assay. The concentrations of periostin utilized were 200, 20, 2, 0.2, 0.02, and 0 ng/mL during primary screening, and were 0, 0.015, 0.03, 0.06, 0.12, 0.25, 0.5, 1, and 2 ng/mL respectively during concentration gradient study. During primary screening, the invasion of A549 cells was highest when concentration of periostin protein was 2 ng/mL (**Figure 1**). In addition, the invasion of A549 cells had positive correlation with concentrations of periostin protein between 0 and 0.5 ng/mL, whereas it had negative correlation with concentrations of periostin protein between 0.5 and 2 ng/mL (**Figure 2**).

mRNA and protein expression of periostin in human lung cancer cell line A549 and BEAS-2B increased significantly after miRNA was interrupted

A549 and BEAS-2B cells that were transfected with the mixture of Dicer-siRNA-152 and Drosha-siRNA-1200, or negative control siRNA were cultured. mRNA and protein expression of periostin were detected by qPCR and Western

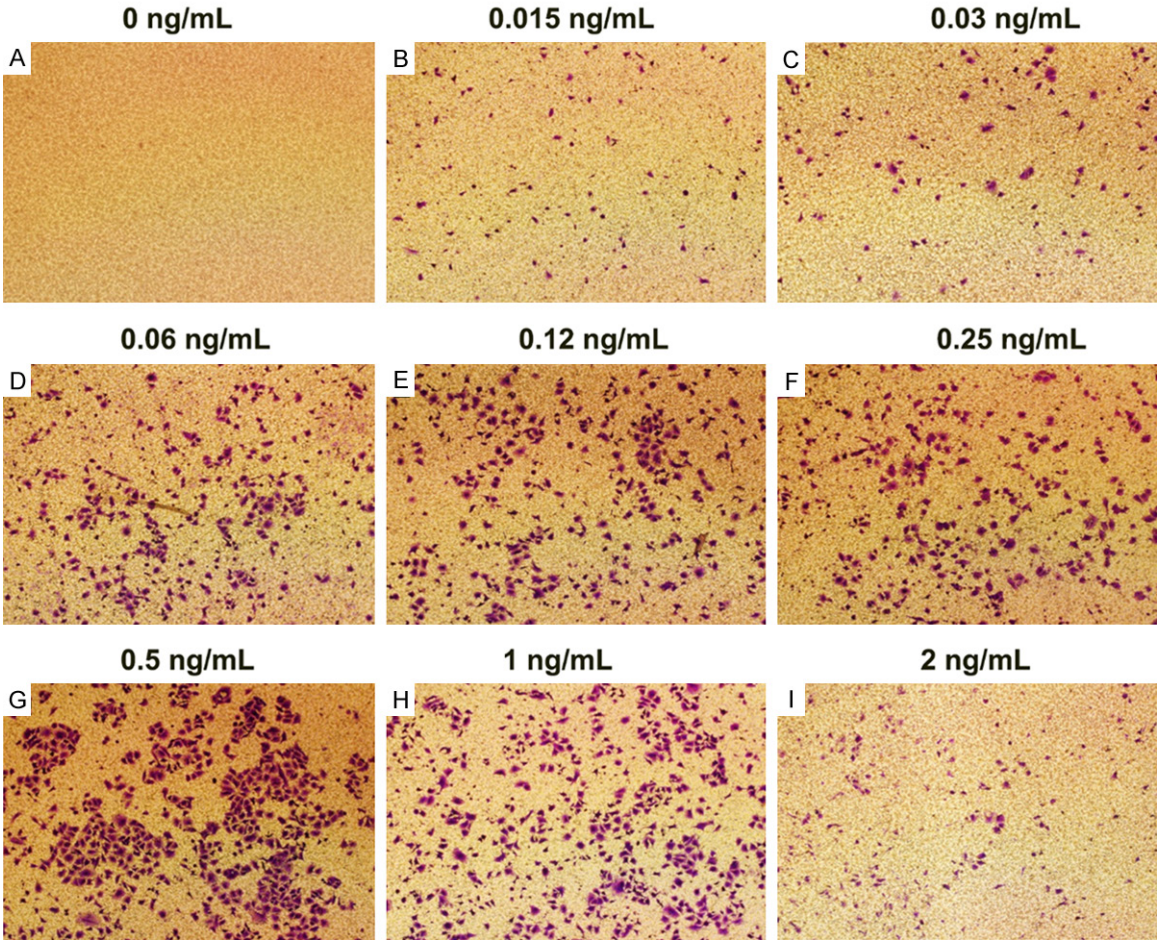

Figure 2. Periostin protein altered invasion of A549 cells in a dose-dependent manner. Invasion of A549 cells affected by periostin protein was detected by Transwell invasion assay. The concentrations of periostin utilized were 0, 0.015, 0.03, 0.06, 0.12, 0.25, 0.5, 1, and 2 ng/mL respectively during concentration gradient study. The invasion of A549 cells had positive correlation with concentrations of periostin protein between 0 and 0.5 ng/mL, whereas it had negative correlation with concentrations of periostin protein between 0.5 and 2 ng/mL.

Blot, respectively. Relative mRNA expression of periostin in A549 ($P < 0.05$) and BEAS-2B cells ($P < 0.01$; **Figure 3**) increased significantly after miRNA was interrupted by Dicer- and Drosha-siRNA, as compared to cells treated with negative control siRNA or cells that received no treatment. Similarly, protein expression of periostin in BEAS-2B cells increased markedly after miRNA was interrupted by Dicer- and Drosha-siRNA when compared to negative control and blank groups ($P < 0.01$; **Figure 4**). Protein expression of periostin in A549 cells was too low to be detected by Western Blot.

Expression of periostin was lowest when HEK293A cells were transfected with miR-599 and miR-185

HEK293A cells were transfected with vectors (pMIR/report-3'POSTN and pRL-TK), and miRNA (miR-543, miR-296-3P, miR-599, miR-185, miR-202-3P, and negative control miRNA, respectively). Expression of periostin was determined by measuring relative light unit in dual-luciferase reporter assay. Compared to other miRNA, transfection of miR-599 and miR-185 decreased expression of periostin dramatically ($P < 0.001$, **Figure 5**).

Expression of periostin was not altered by miR-599 or miR-185 mimics when HEK293A cells were transfected with periostin gene mutated at binding sites of miR-599 or miR-185

HEK293A cells were divided into 6 groups as described in Methods: (1) miR-599-mut group; (2) miR-599-mut NC group; (3) miR-599-mut mimics group; (4) miR-185-mut group; (5) miR-185-mut NC group; and (6) miR-185-mut mim-

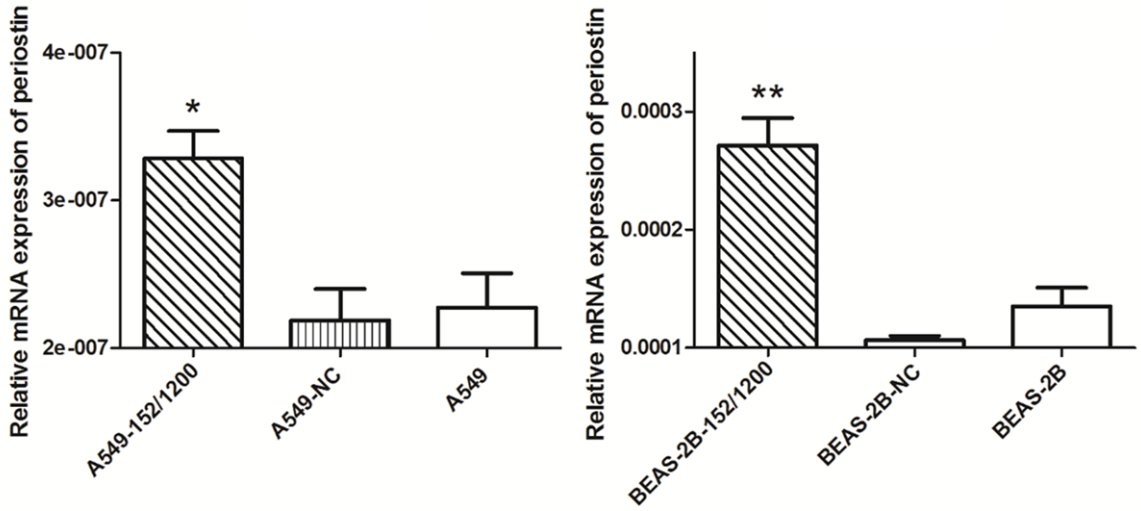

Figure 3. mRNA expression of periostin in human lung cancer cell line A549 and BEAS-2B increased significantly after miRNA was interrupted. A549 and BEAS-2B cells that were transfected with the mixture of Dicer-siRNA-152 and Drosha-siRNA-1200, or negative control siRNA were cultured. mRNA expression of periostin were detected by qPCR. Relative mRNA expression of periostin in A549 and BEAS-2B cells increased significantly after miRNA was interrupted by Dicer- and Drosha-siRNA, as compared to cells treated with negative control siRNA or cells that received no treatment (mean ± SEM, n = 3/group). *indicates that $P < 0.05$ as compared to A549-NC or A549 groups. **indicates that $P < 0.01$ when compared to BEAS-2B-NC and BEAS-2B groups. NC: negative control. 152: Dicer-siRNA-152. 1200: Drosha-siRNA-1200.

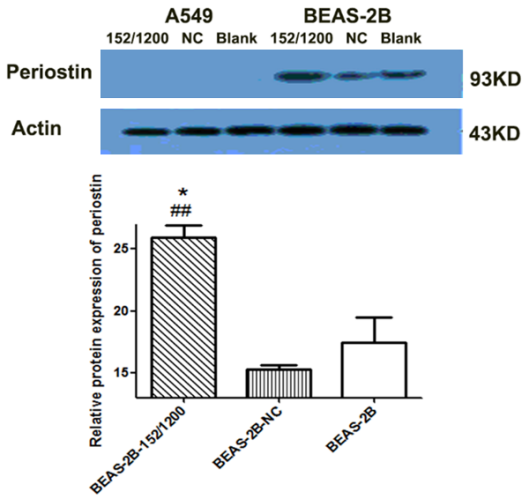

Figure 4. Protein expression of periostin in human lung cancer cell line BEAS-2B increased markedly after miRNA was interrupted. A549 and BEAS-2B cells that were transfected with the mixture of Dicer-siRNA-152 and Drosha-siRNA-1200, or negative control siRNA were cultured. Protein expression of periostin were detected by Western Blot. Protein expression of periostin in BEAS-2B cells increased markedly after miRNA was interrupted by Dicer- and Drosha-siRNA when compared to negative control and blank groups. Protein expression of periostin in A549 cells was too low to be detected by Western Blot (mean ± SEM, n = 3/group). *indicates that $P < 0.05$ as compared to BEAS-2B group. ##indicates that $P < 0.01$ as compared to BEAS-2B-NC group. NC: negative control. 152: Dicer-siRNA-152. 1200: Drosha-siRNA-1200.

ics group. Expression of periostin was determined by measuring relative light unit in dual-luciferase reporter assay. Expression of periostin was not altered by miR-599 or miR-185 mimics when HEK293A cells were transfected with periostin gene mutated at binding sites of miR-599 or miR-185 ($P > 0.05$, **Figure 6**).

Discussion

We have demonstrated that periostin protein alters invasion of human lung cancer cells, and miR-599 and miR-185 down-regulate periostin expression in lung cancer cells.

Periostin was shown to bind to integrins on many cancer cells, and activate Akt/PKB- and FAK-mediated signaling pathways, which resulted in increased cell survival, angiogenesis, metastasis, and the epithelial-mesenchymal transition [26]. In addition, periostin was revealed to be highly upregulated in glioblastomas. The expression of periostinin gliomas correlated directly with tumor grade and recurrence, and inversely with survival [27]. Moreover, glioma stem cells in glioblastomas were reported to secrete periostin, which recruited M2 tumor-associated macrophages from peripheral blood to tumor environment through integrin signaling. M2 tumor-associated macro-

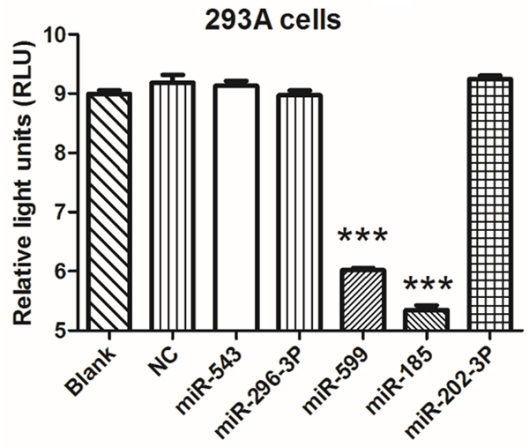

Figure 5. Expression of periostin was lowest when HEK293A cells were transfected with miR-599 and miR-185. HEK293A cells were transfected with vectors (pMIR/report-3'POSTN and pRL-TK), and miRNA (miR-543, miR-296-3P, miR-599, miR-185, miR-202-3P, and negative control miRNA, respectively). Expression of periostin was determined by measuring relative light unit in dual-luciferase reporter assay. Compared to other miRNA, transfection of miR-599 and miR-185 decreased expression of periostin dramatically (mean ± SEM, n = 3/group). ***suggests that P < 0.001 when compared to blank, NC, miR-543, miR-296-3P, and miR-202-3P groups. NC: negative control.

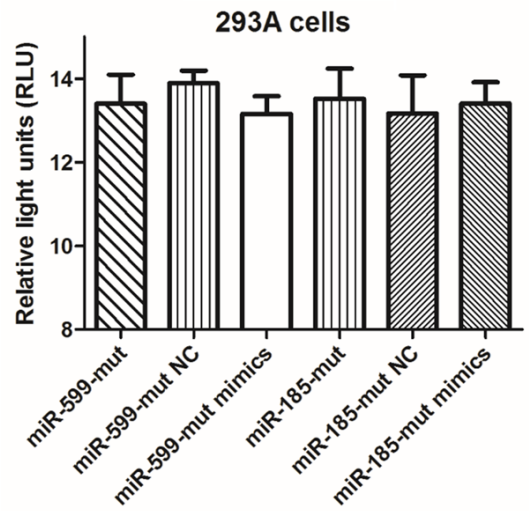

Figure 6. Expression of periostin was not altered by miR-599 or miR-185 mimics when HEK293A cells were transfected with periostin gene mutated at binding sites of miR-599 or miR-185. HEK293A cells were divided into 6 groups: (1) miR-599-mut group: transfection with pMIR/report-miR-599-mut (miR-599-mut: periostin gene mutated at miR-599 binding site), and pRL-TK; (2) miR-599-mut NC group: transfection with pMIR/report-miR-599-mut, pRL-TK, and NC; (3) miR-599-mut mimics group: transfection with pMIR/report-miR-599-mut, pRL-TK and miR-599 mimic; (4) miR-185-mut group: transfection with pMIR/report-miR-185-mut (miR-185-mut: periostin genemutated at miR-185 binding site), and pRL-TK; (5) miR-185-mut NC group: transfection with pMIR/report-miR-185-mut, pRL-TK, and NC; and (6) miR-185-mut mimics group: transfection with pMIR/report-miR-185-mut, pRL-TK and miR-185 mimic. Expression of periostin was determined by measuring relative light unit in dual-luciferase reporter assay. Expression of periostin was not altered by miR-599 or miR-185 mimics when HEK293A cells were transfected with periostin gene mutated at binding sites of miR-599 or miR-185 (mean ± SEM, n = 3/group). ***suggests that *P* < 0.001 when compared to blank, NC, miR-543, miR-296-3P, and miR-202-3P groups. NC: negative control.

phages are tumor-supportive and immunosuppressive. Therefore, through possible recruitment mechanism, periostin supported tumor progressionin gliomas [28].

The incidence and mortality of lung cancer are the highest among all cancer types in men worldwide. It has the third highest incidence, and is second after breast cancer in mortality among women [1]. We have unveiled that periostin protein altered invasion of human lung cancer cells in current study. The invasion of A549 cells had positive correlation with concentrations of periostin protein between 0 and 0.5 ng/mL, whereas it had negative correlation with concentrations of periostin protein between 0.5 and 2 ng/mL. Future investigations are needed to elucidate dosage responses of perostin protein in animal models. Since we investigated human lung cancer cells alone in current study, chemoattraction of M2 tumor-associated macrophages might not be able to explain the altered tumor invasion. It is possible other molecular signaling pathways in lung cancer cells are involved, which require further research efforts.

Expression levels of miRNA was linked to prognosis of various cancers. Either high miR-185 or low miR-133b levels was reported to correlate with metastasis and poor survival in colorectal cancer [24]. Tumor cell proliferation in hepatocellular carcinoma resulted from the interaction of miR-21 with MAP2K3, a tumor repressor gene [29]. Plasma miR-21, miR-494, and miR-1973 were revealed to be promising biomarkers for Hodgkin lymphoma [30]. In addition, miR-205 was shown to inhibit the metastatic nature of breast cancer [31]. MiRNA-200 family, including miR-200a, miR-200b,

miR-200c, miR-141 and miR-429, were down-regulated in tumor progression of breast cancer [32]. We revealed in current study that miR-599 and miR-185 down-regulated periostin expression in lung cancer cells, whereas the inhibition was abolished after binding sites of miR-599 or miR-185 were muted. This provides novel approaches of targeting periostin in lung cancer therapy.

In conclusion, we have demonstrated novel data suggesting that periostin protein alters invasion of human lung cancer cells. In addition, miR-599 and miR-185 down-regulate periostin expression in lung cancer cells. Although future research is needed to reveal how to regulate perostin precisely in animal models, peritostin may serve as a promising therapeutic target for human lung cancer.

Acknowledgements

The research was supported by "1255 Plan for Discipline Construction" funds at Changhai Hospital, Second Military Medical University (CH125521106).

Disclosure of conflict of interest

None.

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