Effects of lentivirus-mediated silencing of Periostin on tumor microenvironment and bone metastasis via the integrin-signaling pathway in lung cancer

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A B S T R A C T

The study aims to investigate the effects of Periostin gene silencing on tumor microenvironment and bone metastasis via the integrin-signaling pathway in lung cancer (LC). LC patients were divided into bone metastasis and non-bone metastasis groups; Healthy volunteers were selected as normal group. ELISA was performed to detect serum Periostin levels and plasma calcium ion concentration. SBC-5 cells were assigned into blank group (without transfection), negative control (NC) group (transfected with empty plasmid), si-Periostin group (transfected with si-Periostin plasmid), si-Integrin-cyβ3 group (transfected with Integrin-cyβ3 siRNA plasmid) and si-Periostin + si-Integrin-cyβ3 group (transfected with si-Periostin and si-Integrin-cyβ3 plasmid). qRT-PCR and Western blotting were performed to determine mRNA and protein expression of Periostin, metastasis-associated factors of tumor microenvironment and integrin signaling pathway-related proteins. CCK-8, scratch test and transwell assay were applied to detect cell proliferation, migration and invasion respectively. Nude mouse models of LC bone metastasis were established. TRAP Staining was employed to measure the number of osteoclasts. Bone metastasis group exhibited higher levels of Periostin compared to normal and non-bone metastasis groups. Si-Periostin, si-Integrin-cyβ3 and si-Periostin + si-Integrin-cyβ3 groups showed decreased Periostin expression, proliferation rate, migration distance, invasive cells, and expressions of metastasis-associated factors of tumor microenvironment and integrin signaling pathway-related proteins compared to blank and NC groups. Similarly, number of osteoclasts and expression of integrin signaling pathway-related proteins were decreased, and bone injury and calcium ion concentration were reduced. The study demonstrated that down-regulation of Periostin expression modulated tumor microenvironment and inhibited bone metastasis by blocking integrin-signaling pathway in LC.

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1. Introduction

In the past few years, the field of lung cancer (LC) research has advanced remarkably, however LC remains to be the leading cause of cancer-related deaths all over the world [1]. Unfortunately, the majority of LC cases are diagnosed at an advanced stage once the cancer has metastasized, thus limiting the possibility of finding a cure [2]. Brain metastasis, for patients suffering from LC, is a widespread secondary location, which is present in approximately 30–40% of LC patients in the trajectory of the disease [3]. The big challenge of early diagnosis of LC remains to be a difficulty leading to a poor prognosis with short survival time [4]. Periostin, an extracellular matrix protein serves as a key factor in both cell motility and adhesion across the tumor microenvironment [5]. It regulates development of several types of human cancers by means of interactions with Rb/E2F1/p14ARF/Mdm2 pathways or PI3K/Akt/survivin pathways, and is related to tumor growth, invasion, as well as metastasis [6–8].

Lately, Periostin over-expression has been shown to result in poor prognosis as well as an increased risk of metastasis in many malignancies, such as lung cancer, gastric cancer, colon cancer, breast cancer, and head and neck cancer [6,9–12]. Periostin is abnormally up-regulated in LC and furthermore shows close associations with epithelial-mesenchyme transition, metastasis, invasion and angiogenesis [13]. High expression of Periostin plays an important physiological role during...
cell growth at dental, bone, and cardiac tissues levels [14]. Periostin can act as a critical regulator of cell survival, angiogenesis and hypoxia-induced cell apoptosis through activation of intracellular singling pathways and binding to the integrins αvβ3, αvβ5 and α6β4, [15]. Sun et al. found that integrins affect cell growth in LC, functioning as an important factor in the development of more efficient cancer treatment strategies [16]. Interestingly, tumor cells interplay with their environment with the help of several transmembrane proteins, including integrins and some intercellular adhesion molecules that not only mediate tumor metastasis and progression, but also support cell-cell interactions [17]. Moreover, Periostin promotes cell adhesion and motility by activation of integrin-mediated signaling, and also enhances osteoblast-osteocyte differentiation [18]. Periostin acting as a tumor-invasive indicator enhances invasion in esophageal cancer tumor microenvironment [19]. However, the role of Periostin in the tumor microenvironment and bone metastasis remains unknown. Thus, we foremost propose a hypothesis that shows down-regulation of Periostin may alter tumor microenvironment and affects development and progression of bone metastasis in LC through its role in the integrin-signaling pathway. In the present study, we also showed the effects of down-regulation of Periostin on proliferation, migration, invasion of human small cell lung cancer (SBC-5) cells with high bone-metastatic potential in vitro and in vivo.

2. Materials and methods

2.1. Ethics statement

The study was approved by The Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, and written informed consents were obtained from all subjects.

2.2. Study subjects

The study population consists of a total of 213 LC patients from the Department of Oncology in Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology from January 2014 and January 2016. All patients were assigned into a bone metastasis group (n = 101) and non-bone metastasis group (n = 112). Inclusion criteria was as follows: (1) complete data were obtained from patients who were pathologically diagnosed as suffering from LC [20]; (2) LC patients undergone bone metastasis were confirmed by bone scans. X-ray, computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET). The calculated mean age of patients in the non-bone metastasis group was 56.43 ± 8.75 years (range 37–73 years), including 65 males and 47 females. The aforementioned patients were established devoid of any tumor tissues in other parts of body. The calculated mean age of patients in the bone metastasis group was 57.50 ± 8.65 years (range 35–73 years), including 59 males and 42 females. Additionally, a total of 73 healthy volunteers aged 23 to 69 years (52 males and 21 females, calculated mean age: 50.37 ± 9.34 years) undergoing medical examination at the same period were selected as the normal group.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Empty stomach venous blood samples (5 ml) were obtained from each subject. 30 min prior to extraction, the blood was centrifuged at rate of 3000 r/min for 15 min to separate the serum from the blood which was subsequently refrigerated at −80 °C. Serum Periostin was employed with ELISA Kit (No. PG48T; Shanghai Yan Ji Bio Technology Co., Ltd. Shanghai, China). Serum samples were diluted at proper proportions, and standard (50 μl) and diluted samples (50 μl) were added into the ELISA plate covered with membrane. The contents of the ELISA plate were discarded after the reaction, and 50 μl of ELISA reagent was added into each well for incubation at 37 °C for 30 min. 50 μl of Chromogenic agent A and 50 μl of chromogenic agent B were mixed together in each well after being washed with phosphate buffered saline (PBS) three times, and at that juncture chromogenic reaction was carried out at 37 °C in the dark for 15 min, followed by the addition of 50 μl stop buffer to halt the reaction. Optical density (OD) value was measured at 450 nm wavelength in a microplate reader. Calculation of the final density of the samples was carried out using the corresponding concentration on the microplate reader, which was multiplied by dilution.

2.4. Construction of Periostin siRNA lentiviral vector

Periostin mRNA (Genbank: NM_002473.4) was employed as the template strand, and the sequences of Periostin siRNA were as follows: sense strand, 5′-GCAACGUGAAUGUGAUAUTT-3′; antisense strand, 5′-AAUUGCAAUUCCAGUUGC-3′. Human Integrin-αv and Integrin-β3 mRNA sequences were employed as the template strand, and the sequence of Integrin-αv siRNA was as follows: 5′-GUAGCA AUUCUAUCAGAdTdTTdTCUAGAGUAUAUCU-3′, and the sequence of Integrin-β3 siRNA was as follows: 5′-GUGCAGUUCUGU ACGUAAadTdTTdTCAGUAAGAACUAUUU-3′. Simultaneously, empty plasmids were selected as the negative control group, and lentivirus vectors were constructed by the Shanghai Genechem Co. Ltd. (Shanghai, China). Single-stranded DNA oligosaccharide fragments were synthesized, and subsequently double-stranded DNA of oligosaccharide was obtained post-primer annealing. Vector linearization was achieved after utilizing restriction enzyme HpaI and XhoI, and followed by lentiviral vector carrying Periostin siRNA sequences construction by connecting to Periostin siRNA sequences through DNA. The recombinant vector was transferred into competent DH5a cells, and positive DH5a cells among the cell culture underwent selection and cloning. Quantitative real-time polymerase chain reaction (qRT-PCR) was employed in order to check for lentiviral vector establishment. Positive clones were amplified post-sequencing, and plasmid isolation kit (No. D0018, Beyotime Institute of Biotechnology, Beijing, China) was used for Periostin siRNA plasmid (si-Periostin) extraction.

2.5. Cell culture and transfection

Human SBC-5 cell line with high bone-metastatic potential was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI-1640 medium (CAS: 16000-044; Gibco, Grand Island, NY, USA) was used in order to culture SBC-5 cells supplemented with 10% fetal bovine serum (FBS) in a 5% CO2 incubator at 37 °C. Upon reaching approximately 80–90% confluence, SBC-5 cells were sub-cultured every 2–3 days. The cells meeting the criterion of being in the logarithmic phase of growth were seeded into 6-well plates (3 × 104 cells/well). SBC-5 cells were divided into the following groups: negative control (NC) group (cells transfected with empty plasmids), si-Periostin group (cells transfected with si-Periostin plasmids), si-Integrin-αvβ3 group (cells transfected with si-Integrin-αvβ3 plasmids), si-Periostin + si-Integrin-αvβ3 group (cells transfected with si-Periostin and si-Integrin-αvβ3 plasmids) and blank group (cells without transfection). Cell transfection was achieved using Lipofectamine 3000 (CAS: L3000-015, Invitrogen, California, USA) following the manufacturer’s instruction. A day prior to transfection, cells were sub-cultured and seeded into 6-well plates. Upon reaching approximately 90–95% confluence, diluted plasmids were mixed with diluted Lipofectamine 3000 in a serum-free medium. After resting at room temperature for 20 min, the mixture was seeded into each well, and further incubated for 4 h at 37 °C in a 5% CO2 incubator, and consequently the medium was changed. Further experiments were carried out 48 h after cell transfection.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA isolation from lung tissues of all subjects and SBC-5 after transfection was carried out using RNA extraction kit (CAS: 74104; Qiagen, Beijing, China). Single-stranded DNA oligosaccharide fragments were synthesized, and subsequently double-stranded DNA of oligosaccharide was obtained post-primer annealing. Vector linearization was achieved after utilizing restriction enzyme HpaI and XhoI, and followed by lentiviral vector carrying Periostin siRNA sequences construction by connecting to Periostin siRNA sequences through DNA. The recombinant vector was transferred into competent DH5a cells, and positive DH5a cells among the cell culture underwent selection and cloning. Quantitative real-time polymerase chain reaction (qRT-PCR) was employed in order to check for lentiviral vector establishment. Positive clones were amplified post-sequencing, and plasmid isolation kit (No. D0018, Beyotime Institute of Biotechnology, Beijing, China) was used for Periostin siRNA plasmid (si-Periostin) extraction.
Valencia, CA, USA) and preserved at −80 °C for further use. Measurement of OD260/280 value was achieved by employing ultraviolet spectroscopy, followed by calculation of RNA concentration. Reverse transcription was performed according to the instructions of reverse transcription kit (CAS: 205311; Qiagen, Valencia, CA, USA) to synthesize cDNA. Primer design was obtained using published data from the Genbank database. Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) synthesized the sequences of primers. Table 1 presents the Primer sequences. PCR reaction system (20 μl in total) was as follows: 10 μl of SYBR PremixExTaq, 0.8 μl of Forward Primer, 0.8 μl of Reverse Primer, 0.4 μl of ROX Reference Dye II, 2 μl of DNA template and 6.0 μl of dH2O. Reaction condition included pre-denaturation at 95 °C for 30 s, and 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The reliability of PCR was evaluated by melt curve analysis with β-actin acting as the internal control. After obtaining the Ct value, and 2^−ΔΔCT method was used to calculate relative expressions of target genes [13].

2.7. Western blotting

SBC-5 cells present in all three groups and lung tissues of all subjects were collected separately. In addition, tumor tissues were obtained from mice with LC bone metastasis that were executed 8 weeks post-inoculation with SBC-5 cells in the right hind limb. The total protein extracted with the addition of protein lyses. Measurement of protein concentrations was performed using the Bicinchoninic acid (BCA) Protein Assay Kit (No. P0010; Beyotime Institute of Biotechnology, Beijing, China). Separation of protein samples (30 μg in each well) was achieved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at a voltage from 80 V–120 V, followed by transfer onto a polyvinylidene fluoride (PVDF) membrane at a voltage of 100 mV for 45–70 min, and the process was halted by the use of 5% bovine serum albumin (BSA) at 37 °C for 1 h. Protein samples were incubated overnight with mice anti-human antibodies including Periostin, Integrin-α1, Integrin-αvβ3, FAK (focal adhesion kinase), MCP-1 (monocyte chemo-attractant protein-1), HIF-1α (hypoxygen-inducible factor-1α), VEGF (vascular endothelial growth factor), MMP-9 (matrix metalloproteinase-9), and β-actin monoclonal antibody (Santa Cruz, CA, USA) with a membrane temperature of 4 °C. Once the membranes were washed three times by Tris-buffered saline with Tween 20 (TBST) (5 min per wash), they were subsequently incubated with secondary goat anti-mouse antibody labeled by horse-radish peroxidase at 1 h at room temperature, followed by TBST washing (5 min × 3 times). The PVDF membranes were exposed to electrochemiluminescence (ECL) solution for 3–5 min time periods, and developed in the dark once the ECL solution was filtered out. Calculation of the gray scale of protein bands was carried out by the Image J software, and the relative expression of proteins was represented as the ratio of gray scale between the targeted protein band and β-actin protein band.

2.8. Cell counting kit (CCK-8) assay

SBC-5 cells in the logarithmic growth phase were digested by 0.25% trypsin and consequently the cell concentration was adjusted to 1 × 10^5 cells/ml. A sum total of 200 μl cells were seeded into 96-well plates. Each culture was treated in six reduplicate wells for each group with the addition of sterile PBS in the marginal wells. After 24 h, 48 h and 72 h time periods of cell culture, 10 μl of CCK-8 Kit (No. CK04; Dojindo Laboratories, Tokyo, Japan) was added into each well, followed by incubation of the plate for 2 h. Detection of OD values of each well at 450 nm was carried out by a microplate reader (Varioskan Flash; Thermo Fisher Scientific, Waltham, MA, USA). The proliferation rates of cells in the three groups at different time periods were calculated following zero adjustment of the OD value once the blank well was only filled with the culture medium.

2.9. Scratch test

SBC-5 cells in each group were seeded into 6-well plates (5 × 10^4 cells/well). Upon reaching approximately 80% confluence, a sterile pipette (1 ml) was used to draw a line across each well, and followed by PBS washing to remove the cast-off cells, and a 48-h culture. The remaining cells were observed under a microscope, and subsequently photographed. Scratch widths at 0 h and 48 h time periods were calculated using the Image-Pro Plus 6.0 software. Cell migration distance equates to scratch width at 0 h–scratch width at 48 h.

2.10. Transwell assay

The prepared Matrigel (No. 356234; BD, New Jersey, USA) was melted at a temperature of 4 °C, followed by dilution using a serum-free DMEM at the ratio of 1: 3, embedded to the upper surface of transwell chamber, and finally dried off at room temperature. Posttrypsin-digestion, cells were subsequently suspended in the serum-free DMEM to prepare a cell suspension (1 × 10^5 cells/well), which was added into the upper chamber (200 μl in each chamber). Next, 500 μl of DMEM supplemented with 10% FBS was added into the 24-well plates, avoiding occurrence of bubbles. The transwell chamber was taken out once each well was cultured for 48 h. Subsequently, the cells on the upper chamber surface were removed, and the cells on the lower chamber surface were stained crystal violet after fixation with 95% alcohol for 15 min. The cells penetrating through the base membrane were observed under an inverted microscope, and a calculated average number was recorded.

2.11. A nude mouse model of LC bone metastasis

Thirty BALB/c-nu/nu male nude mice (6-week old, weighing 18 ± 2 g) purchased from the Shanghai Laboratory Animal Center (Shanghai, China) were included in the study. All animals were fed in a specific pathogen free (SPF) animal laboratory under constant humidity and temperature conditions for 1 h. A nude mouse model of LC bone metastasis was established. All nude mice were randomly divided into three groups (10 mice in each group) as follows: blank group (mice injected with SBC-5 cells without transfection), negative control (NC) group (mice injected with SBC-5 cells transfected with empty plasmid), si-Periostin group (mice injected with SBC-5 cells transfected with si-Periostin plasmid), si-Integrin-αvβ3 group (mice injected with SBC-5 cells transfected with si-Integrin-αvβ3 plasmid and si-Periostin + si-Integrin-αvβ3 group (mice injected with SBC-5 cells transfected with si-Periostin and si-Integrin-αvβ3 plasmids). SBC-5 cells in logarithmic phase of growth were obtained, and subsequently the concentration was adjusted to 1 × 10^5 cells/ml. All nude mice were anesthetized with diethyl ether, followed by femoral and tibial skin disinfection. In addition, muscles present in the proximity of tibial joints in the right hind leg of the nude mice in three groups were pierced by a 51/2 needle and 100 μl of cell suspension was injected into the bone, and at that juncture the condition of tumor growth was observed.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Upstream sequence 5′-3′</th>
<th>Downstream sequence 5′-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periostin</td>
<td>GTGACAGAAGTGATCCACGGAGCTCTTGATCGCCTTCTAGACCC</td>
<td>ATCTTGATCGCCTTCTAGACCC</td>
</tr>
<tr>
<td>Integrin-α1</td>
<td>ACTTGATCCCTAAGTCAGCAGTAGATCAGCAGTAATGCAAGGCC</td>
<td>ATCAGCAGTAATGCAAGGCC</td>
</tr>
<tr>
<td>Integrin-αvβ3</td>
<td>CAGACGAGGAGGACACACCAACCACTCGAGAGAGGACACACCCA</td>
<td>TCACAGCAGGACACCAAGCC</td>
</tr>
<tr>
<td>FAK</td>
<td>TCTGAGAAGAACCTGCACTAATG</td>
<td>TCAGCAGGACACCAAGCC</td>
</tr>
<tr>
<td>MMP-9</td>
<td>AACAGCCGCTGCTTCTTGCAGG</td>
<td>CCTGAGTGGACAGCATTCG</td>
</tr>
<tr>
<td>β-actin</td>
<td>TCTCTCTTGCGAACAGACTA</td>
<td>TCAGCAGGACACCAAGCC</td>
</tr>
</tbody>
</table>

Note: MMP-9, matrix metalloproteinase 9; FAK, focal adhesion kinase.
2.12. X-ray film and hematoxylin and eosin (HE) staining

Bone metastasis observations were as follows: 35 days after injections, the nude mice were anesthetized by an intraperitoneal pentobarbital injection. An X-ray film (100 mA; 42 kV; 0.04 s; target distance = 78 cm) was obtained to observe bone metastasis after fixation. The nude mice were executed 8 weeks after inoculation, and their right hind limbs were removed and fixed in 10% formalin made by 10 mmol/l PBS (pH 7.2) for a duration of 4 h. Next, the right hind limbs were rinsed with distilled water, followed by decalcification. After being embedded in paraffin, the limb was sliced into sections and stained with hematoxylin. Once the sections were differentiated in hydrochloric acid alcohol and washed under running tap-water for 10 min, the sections were soaked in ammonia for 5 s, followed by eosin staining. After tap-water washing, the sections were subsequently dehydrated with gradient ethanol, permeabilized with xylene, sealed with neutral gum, and finally bone injuries were analyzed by routine pathological examinations.

2.13. Tartrate resistant acid phosphatase (TRAP) staining

The tissues were decalcified after the removal of soft tissues surrounding the right hind limbs of the nude mice. The Paraffin-embedded tissue sections were subsequently de-waxed. A staining solution was prepared in accordance with the instructions of TRAP Kit (387-1KT, Sigma-Aldrich Chemical Company, St Louis, MO, USA). Six-membered cyclic azo fuchsin, naphthol AS-BI phosphate dissolved in N, N-dimethylformamide and acetate buffer were evenly mixed in proper proportions. After pH adjustment (pH = 5.0), potassium sodium tartrate tetrahydrate was incorporated to prepare the staining solution. The samples were stained with the aforementioned staining solution and incubated for 60 min at 37 °C. Next, the samples were washed using distilled water and stained with hematoxylin, followed by sealing with neutral gum, and observations under a light microscope. Observed red multinucleated cells were regarded as differentiated osteoclasts.
2.14. Measurement of calcium ion concentration

Fasting blood samples were collected from nude mice in each group for calcium determination, and stored in vacuum blood collection tubes (containing heparin acting as an anticoagulant). Calcium Colorimetric Assay Kit (MAK022; Sigma-Aldrich Chemical Company, St Louis, MO, USA) was used to analyze plasma calcium ion concentration.

2.15. Statistical analysis

All statistical analyses were performed using the SPSS 21.0 statistical software (SPSS, Inc., Chicago, IL, USA). Measurement data were expressed as mean ± standard deviation (SD). A t-test was employed when two groups of normally distributed measurement data were compared, and the Mann-Whitey U test was applied when the compared measurement data were not normally distributed. Comparison among multiple groups was analyzed by one-way analysis of variance (ANOVA). \( P < 0.05 \) was considered as statistically significant.

3. Results

3.1. Comparison of Periostin level in tissues and serum of subjects among the bone metastasis, non-bone metastasis and normal groups

qRT-PCR was performed in order to determine the expression levels of Periostin mRNA in lung tissues among the bone metastasis, non-bone metastasis and normal groups. The results are shown in Fig. 1A. The Periostin mRNA expression in LC patients in the bone metastasis and non-bone metastasis groups was significantly higher compared to the patients in the healthy control in normal group (both \( P < 0.05 \)). Besides, the bone metastasis group displayed a higher Periostin mRNA expression compared to the non-bone metastasis group (\( P < 0.05 \)).
Additionally, Western blotting was employed in order to measure Periostin protein expression as seen in Fig. 1B & C, and the results indicated that LC patients in the bone metastasis and non-bone metastasis groups exhibited an increased Periostin mRNA expression compared to the patients in the healthy control in normal group (P < 0.05). In addition, Periostin mRNA expression in the bone metastasis group was elevated compared to the non-bone metastasis group (P < 0.05). ELISA tests were performed in order to measure serum Periostin levels in the bone metastasis, non-bone metastasis and normal groups. The results revealed serum Periostin concentrations in normal, non-bone metastasis and bone metastasis groups were 21.91 ± 5.60 ng/ml, 28.46 ± 7.32 ng/ml and 38.28 ± 8.59 ng/ml, respectively. Compared to the normal group, serum Periostin concentrations in LC patients in the bone metastasis and non-bone metastasis groups were evidently increased, and furthermore, serum Periostin concentration in LC patients in the bone metastasis group was significantly increased compared to the non-bone metastasis group (all P < 0.05) (Fig. 1D). These findings indicate that high concentration of serum Periostin was associated with bone metastasis of LC.

3.2. Serum expression levels of tumor microenvironment factor and integrin signaling pathway-related proteins in patients among the bone metastasis, non-bone metastasis and normal groups

ELISA was employed to detect the serum expression levels of MMP-9, Integrin-β1, Integrin-αvβ3 and FAK in patients in bone metastasis, non-bone metastasis and normal groups in order to evaluate relationships among tumor microenvironment factor MMP-9, integrin signaling pathway-related proteins (Integrin-αvβ3 and FAK), LC and bone metastasis of LC. The findings revealed serum MMP-9, Integrin-β1, Integrin-αvβ3 and FAK expression levels were significantly increased in bone metastasis and non-bone metastasis groups compared to the normal group (all P < 0.05), and serum MMP-9, Integrin-β1, Integrin-αvβ3 and FAK expression levels were significantly increased in the bone metastasis group compared to the non-bone metastasis group (P < 0.05) (Fig. 2). These findings indicate that tumor microenvironment factor MMP-9 expression was increased and the integrin signaling pathway was activated in patients with bone metastasis of LC.

3.3. The expression of Periostin in the SBC-5 cells among the blank, NC, si-Periostin, si-Integrin-αvβ3, si-Periostin + si-Integrin-αvβ3 groups

qRT-PCR and Western blotting were employed to measure expression levels of Periostin mRNA and protein in SBC-5 cells among the blank, NC, and si-Periostin, si-Integrin-αvβ3, si-Periostin + si-Integrin-αvβ3 groups in order to assess the mechanism underlying Periostin mediating integrin signaling pathway. As shown in Fig. 3, was no significant Periostin expression differences were detected among blank and NC groups (P > 0.05). Si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups showed a significant decrease in both mRNA and protein expression of Periostin compared to the blank and NC groups, (all P < 0.05), suggesting cell models were successfully established. There were no significant Periostin expression differences among si-Periostin and si-Integrin-αvβ3 groups (P > 0.05). Si-Periostin + si-Integrin-αvβ3 group showed decreased mRNA and protein Periostin expression compared to si-Periostin and si-Integrin-αvβ3 groups (all P < 0.05). The results found that silencing Periostin or blocking integrin signaling pathway decreased Periostin expression, suggesting that Periostin was positively associated with the integrin signaling pathway and silencing Periostin blocked the integrin signaling pathway.

3.4. Downregulation of Periostin inhibited the proliferation, migration and invasion of SBC-5 cells

CCK-8 assay, scratch test and Transwell assays were performed in order to investigate the effects of silencing Periostin on the proliferation, migration and invasion of SBC-5 cells. After Periostin silencing, the cell proliferation rates were detected by CCK-8 assay at 24 h, 48 h and 72 h time periods, respectively. The results showed there was no significant difference in the cell proliferation rate among blank and NC groups at 24 h, 48 h and 72 h time periods, respectively (all P > 0.05). However, the proliferation rates in si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups at 24 h, 48 h and 72 h time periods were significantly decreased compared to blank and NC groups (all P < 0.05). Additionally, the proliferation rates in si-Periostin group and si-Integrin-αvβ3 group at 24 h, 48 h and 72 h time periods did not show any significant differences (P > 0.05), and proliferation
rate in si-Periostin + si-Integrin-αvβ3 group at 24 h, 48 h and 72 h time periods was significantly compared to si-Periostin and si-Integrin-αvβ3 groups ($P < 0.05$) (Fig. 4A).

Cell migration and invasion were evaluated using scratch test and Transwell assay post-Periostin silencing. Fig. 4B showed that slower a scratch wound healing rates as well as significantly lower migration distance in si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups compared to blank and NC groups ($P < 0.05$). There was no significant difference among blank and NC groups ($P > 0.05$). There was no significant migration distance difference among si-Periostin group and si-Integrin-αvβ3 group ($P > 0.05$). Si-Periostin + si-Integrin-αvβ3 group showed significantly reduced migration distance compared to si-Periostin and si-Integrin-αvβ3 groups ($P < 0.05$). Moreover, Transwell assay results showed occurrence of massive

Fig. 4. Proliferation rates, migration and invasion of SBC-5 cells in blank, NC, si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups. Note: A, proliferation rates of SBC-5 cells in blank, NC, si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups detected by CCK-8 assay at 0 h, 24 h, 48 h and 72 h time periods, respectively; B, images of scratch wound healing and comparison of migration distance of SBC-5 cells among blank, NC, si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups detected by scratch test; C, images and the comparison of invasive SBC-5 cells among blank, NC, si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups detected by Transwell assay; CCK-8, cell counting kit-8; NC, negative control. *, $P < 0.05$ compared to the blank group; #, $P < 0.05$ compared to the NC group; $\dagger$, $P < 0.05$ compared to the si-Periostin and si-Integrin-αvβ3 groups.
invasive cells in both blank and NC groups, whereas the number of invasive cells in si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups was still relatively low. It was confirmed via transwell assay results that the number of invasive cells in the si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups was significantly decreased compared to blank and NC groups (all \( P < 0.05 \)). There was no significant difference among blank and NC groups (\( P > 0.05 \)). There was no significant difference in the number of invasive cells among si-Periostin and si-Integrin-αvβ3 groups (\( P > 0.05 \)). Si-Periostin + si-Integrin-αvβ3 group showed a decrease in the number of invasive cells compared to si-Periostin and si-Integrin-αvβ3 groups (\( P < 0.05 \)) (Fig. 4C). The results indicate that silencing Periostin or blocking the integrin signaling pathway reduced proliferation, migration and invasion abilities of cells, suggesting that silencing Periostin might block the integrin signaling pathway in order to inhibit LC cell metastasis.

### 3.5. Downregulation of Periostin decreased the expressions of integrin signaling pathway-related proteins

qRT-PCR and Western blotting tests were conducted to detect the expression of MMP-9, Integrin-α1, Integrin-αvβ3 and FAK mRNA and protein in order to further assess the underlying mechanism Periostin mediating integrin signaling pathway and the effect of silencing Periostin on tumor microenvironment factor MMP-9, the. As shown in Fig. 5, there was no significant difference in the mRNA and protein expression levels of MMP-9, Integrin-α1, Integrin-αvβ3 and FAK among blank and NC groups (all \( P > 0.05 \)). Si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups showed significantly decreased levels of mRNA and protein expressions of MMP-9, Integrin-α1, Integrin-αvβ3 and FAK compared to blank and NC groups (all \( P < 0.05 \)). There was no significant difference in the mRNA and protein expressions of proteins among si-Periostin and si-Integrin-αvβ3 groups (\( P > 0.05 \)). Furthermore, si-Periostin + si-Integrin-αvβ3 group showed significantly decreased levels of mRNA and protein expression of MMP-9, Integrin-α1, Integrin-αvβ3 and FAK compared to si-Periostin and si-Integrin-αvβ3 groups (all \( P < 0.05 \)). These findings indicate that silencing Periostin resulted in blocked integrin signaling pathway and inhibited the expression of tumor microenvironment factor MMP-9.

### 3.6. Downregulation of Periostin prevented bone injury in the nude mice with LC bone metastasis

X-ray and HE staining were used to show the degree of bone injury. TRAP staining was used to detect osteoclasts, and the ELISA was used to determine the plasma calcium concentration in order to investigate the effect of silencing Periostin on bone metastasis of LC in vivo. X-ray films indicated severe damage to right hind tibia of nude mice in both blank and NC groups, in addition multiple bone metastatic foci occurred at the inoculation site. However, si-Periostin and si-Integrin-αvβ3 groups showed relatively mild tibial damage to the right hind limbs of the nude mice, and occurrence of a few bone metastatic foci occurred at the inoculation site in the si-Periostin + si-Integrin-αvβ3 group (Fig. 6A). HE staining method was additionally employed to observe bone injury in nude mice in the three groups. In the blank and NC groups, structure disturbance of bone tissues was observed to induce bone destruction, original bone tissue was vanished, and tumor development was out of control. Whereas, occurrence of relatively mild bone damage was observed, and in addition bone tissues relatively retained intact structure with local reactive bone, and a few tumor cells presented in bone marrow in si-Periostin and si-Integrin-αvβ3 groups, and correspondingly no bone damage was present, bone tissues retained intact structure, and no tumor cells presented in bone marrow in si-Periostin + si-Integrin-αvβ3 group (Fig. 6B).

TRAP is a histochemical marker of osteoclasts. Osteoclasts appear as big, red and multinucleated cells after staining. TRAP staining was used in order to observe the number of osteoclasts in nude mice bone tissues. Si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups showed a significant decline in the number of osteoclasts with relatively mild osteolytic bone destruction compared to blank and NC groups (\( P < 0.05 \)). There was no significant difference in the number of osteoclast among blank and the NC groups (\( P > 0.05 \)). There was no significant difference in the number of osteoclasts among si-Periostin and si-Integrin-αvβ3 groups (\( P > 0.05 \)). Si-Periostin + si-Integrin-αvβ3 group showed a significant decline in the number of osteoclasts compared to si-Periostin and si-Integrin-αvβ3 groups (Fig. 6C & D).

Generally, blood calcium is mainly found in the plasma, further classified into free calcium ions and bound calcium. The plasma calcium ion concentration directly affects physiological changes. In addition, the
plasma calcium ion concentration was notably decreased in the si-Periostin and si-Periostin + si-Integrin-αvβ3 groups compared to blank and NC groups (both \( P < 0.05 \)). Fig. 6E shows that there was no significant difference observed in the plasma calcium ion concentration among blank and NC groups (\( P > 0.05 \)). Moreover, no significant difference was observed in the plasma calcium ion concentration among si-Periostin and si-Integrin-αvβ3 groups (\( P > 0.05 \)). Si-Periostin + si-Integrin-αvβ3 group showed a significant decrease in plasma calcium ion concentration compared to si-Periostin and si-Integrin-αvβ3 groups (\( P < 0.05 \)). The results suggest that silencing Periostin or blocking integrin signaling pathway protects against bone injury in rats with bone metastasis of LC, which further indicates the inhibitory effect of silencing Periostin on bone metastasis of LC by blocking the integrin signaling pathway.

3.7. Serum expression levels of Periostin, MMP-9, Integrin-β1, Integrin-αvβ3 and FAK in the nude mice among the blank, NC, si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups

ELISA tests were conducted to determine serum expression levels of Periostin, MMP-9, Integrin-β1, Integrin-αvβ3 and FAK in rats in each group in order to assess the mechanism underlying Periostin mediating integrin signaling pathway and the effect of silencing Periostin on in vivo tumor microenvironment factor MMP-9. The findings reveal that

![Fig. 6. Bone injury of nude mice and plasma calcium ion concentration in blank, NC, si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups detected by X-ray, HE staining and TRAP staining. Note: A, X-ray films of bone injury of nude mice in blank, NC, si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups (white arrows referring to the location of bone injury); B, images of injured bone tissues of nude mice in blank, NC, si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups detected by HE staining (×200); C, images of osteoclast in bone tissues of nude mice in blank, NC, si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups detected by TRAP staining; D, comparison of the number of osteoclast in bone tissues of nude mice among blank, NC, si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups detected by TRAP staining; E, comparison of the plasma calcium ion concentration among blank, NC, si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups; NC, negative control; HE, hematoxylin and eosin; TRAP, tartrate resistant acid phosphatase.]

there were no significant protein expression differences among blank and NC groups (P > 0.05). si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups showed significantly decreased serum levels of Periostin, MMP-9, Integrin-α1, Integrin-αvβ3 and FAK compared to blank and NC groups (all P < 0.05). There were no significant serum expression level differences of Periostin, MMP-9, Integrin-α1, Integrin-αvβ3 and FAK among si-Periostin and si-Integrin-αvβ3 groups (P > 0.05). Si-Periostin + si-Integrin-αvβ3 group showed significantly decreased serum levels of Periostin, MMP-9, Integrin-α1, Integrin-αvβ3 and FAK compared to si-Periostin and si-Integrin-αvβ3 groups (all P < 0.05) (Fig. 7). These findings indicate that silencing Periostin might block the integrin signaling pathway and inhibit in vivo expression of tumor microenvironment factor MMP-9.

3.8. Downregulation of Periostin suppressed the expression of integrin signaling pathway-related proteins in the nude mice with LC bone metastasis

Western blotting techniques were used to detect the expression levels of Periostin, MMP-9, Integrin-α1, Integrin-αvβ3 and FAK in tumor tissues of nude mice in order to further assess the mechanism underlying Periostin mediating integrin signaling pathway and the effect of silencing Periostin on in vivo tumor microenvironment factor MMP-9. There were no significant protein expression differences among blank and NC groups (P > 0.05). Si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups showed significantly decreased expression of Periostin, MMP-9, Integrin-β1, Integrin-αvβ3 and FAK compared to blank and NC groups (all P < 0.05). There were no significant Periostin, MMP-9, Integrin-β1, Integrin-αvβ3 and FAK expression differences among si-Periostin and si-Integrin-αvβ3 groups (P > 0.05). Si-Periostin + si-Integrin-αvβ3 group showed significantly decreased levels of MMP-9, Integrin-β1, Integrin-αvβ3 and FAK expression compared to si-Periostin and si-Integrin-αvβ3 groups (Fig. 7). These aforementioned findings indicate that silencing Periostin might block the integrin signaling pathway and inhibit in vivo expression of tumor microenvironment factor MMP-9.

4. Discussion

Recent studies have indicated that Periostin is over-expressed in various human cancers further promoting tumor metastasis and growth [21,22]. Sung et al. illustrated that high Periostin expression in tumor microenvironment is associated with advanced and recurrent tumors in patients suffering from epithelial ovarian cancer [23]. In addition, our study demonstrates Periostin down-regulation could modulate tumor microenvironment and inhibits bone metastasis in LC by means of blocking the integrin signaling pathway.

We found increased serum levels of Periostin in bone metastasis group, and down-regulation of Periostin inhibited the proliferation, migration and invasion of the LC cells with high metastatic potential, suggesting that Periostin participates in Lung cancer progression, as well as
tumor metastasis. Additionally, Periostin-induced signaling pathways facilitate cell growth along with angiogenesis, and inhibit hypoxia-induced cell death [24]. Moreover, Periostin expression is elevated in response to hypoxia stress in human A549 non-small cell lung cancer (NSCLC) cell line through Akt/PKB signaling pathways [25]. Li et al. reported Periostin over-expression in colorectal cancer was positively-correlated with lymph node metastasis [26]. Liu et al. also revealed Periostin expression in primary tumor tissues and metastatic tissues stoma can promote cancer development and progression by the means of isoprenaline stimulation [27]. Wang et al. demonstrated Periostin as a potential target for prevention and treatment of breast tumor metastasis [28]. Wu et al. also reported Periostin silencing reduced cell proliferation and invasion in patients suffering from LC [13]. Consequently, Periostin silencing restrained LC progression and metastasis.

Our study findings indicate that mRNA and protein expression of MCP-1, HIF-1α, VEGF and MMP-9 acting as metastasis-associated factors of tumor microenvironment were inhibited in the si-Periostin group, thus we conclude that silencing of Periostin might modulate tumor microenvironment. HIF-1α enhances the proliferative and angiogenic potential of small cell lung cancer (SCLC) cells by regulating functional genes such as VEGF-A and interleukin-6 [29]. MCP-1, a chemokine secreted by breast tumor cells, recruits inflammatory monocytes that produce VEGF that in turn promotes lung metastasis extravasation, and furthermore enhances prostate tumor growth and angiogenesis [30]. MK et al.’s findings reveal notable activity of MMP-9 in lung cancer progression and metalloproteinase associations with pathologic type and clinical stages of cancer, indicating that MMP-9 could serve as a therapeutic target for LC [31]. Similarly, Matsuza et al. found increased Periostin expression may indirectly promote angiogenesis by increasing in vitro VEGF expression through the Integrin-αvβ3 integrin/FAK/P3K/Akt signaling pathways [32]. Interestingly, MCP-1, HIF-1α, VEGF and MMP-9 have been considered as key factors in tumor microenvironment modulation affecting cell differentiation, invasion and metastasis [33-36]. Consistent with these findings, our results further conclude that expressed Periostin suppressed the aforementioned four factors to inhibit metastasis within tumor microenvironment.

In addition, our study also observes the severity of bone injury, and Periostin could mediate the number of osteoclasts, thus silencing of Periostin will be beneficial for LC treatment. Daubine et al. observed bone metastasis, osteolysis, ectopic ossification and bone deformation in rat models of prostate carcinoma, which is consistent with the results of HE staining in our study [37]. Yang et al. demonstrated activation of bone osteoclasts results in tumor growth and metastasis induction [38]. We observed a decrease in the number of osteoclasts in the si-Periostin group; confirming down-regulation of Periostin may suppress bone metastasis in LC. Besides, presented results similarly showed that plasma calcium ion concentration was notably decreased after silencing of Periostin. Calcium ion channels such as TRPV6, SK3 and P2X-receptor, are capable of activating several signaling pathways in order to induce bone metastasis where the tumor microenvironment serves as an essential factor [39]. Furthermore, high concentration of extracellular calcium functions with extracellular calcium-sensing receptor (CaSR) as a tumor promoter leading to bone metastasis in some cancers [40,41]. In the present paper, Periostin down-regulation decreased calcium concentration, thereby inhibit the formation of bone metastasis.

Notably, mRNA and protein expression of integrin-αvβ1, integrin-αvβ3 and FAK were reduced in the si-Periostin group, indicating silencing of Periostin in the integrin-signaling pathway during the course of LC. Periostin is capable of binding to integrins-αvβ3,-αvβ5, and -αvβ4, thereby promoting activation of FAK-mediated signaling pathways [15]. It was found that FAK is often linked to a poor prognosis in breast cancer, and it interacts with β4 integrin in tumor microenvironment [42]; down-regulated FAK expression via the inhibition of ERK/MAPK signaling pathway results in suppressed proliferation, migration and invasion of NSCLC cells [43]. Furthermore, evidence indicates Periostin loss decreased MMP-2 and VEGF expression and induced ERK phosphorylation; Periostin-induced ERK and MMP-2 can be reduced by the means of integrin-αvβ3 blocking antibody, further revealing positive correlation between Periostin and integrins [44]. Zhong et al. also implied suppression of integrin expression inhibits metastasis.

Fig. 8. The mRNA and protein expression of integrin signaling pathway-related proteins in the nude mice with LC bone metastasis among blank, NC and si-Periostin groups. Note: A, bands of Periostin, MMP-9, Integrin-αvβ3, Integrin-αvβ3 and FAK proteins in blank, NC, si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups detected by Western blotting; B, comparisons of relative expressions of Periostin, MMP-9, Integrin-αvβ3 and FAK proteins in blank, NC, si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups; LC, lung cancer; NC, negative control; MMP-9, matrix metalloproteinase 9; FAK, focal adhesion kinase; *, P < 0.05 compared to the blank group; #, P < 0.05 compared to the si-Periostin and si-Integrin-αvβ3 groups.
of LC cells [45]. Consequently, we may assume that Periostin silencing may inhibit LC progression and metastasis by blocking the integrin-signaling pathway. Additionally, integrin-β1, integrin-αvβ3, FAK expression declined in LC bone metastasis rat models in the si-Periostin group, which further confirmed Periostin may affect bone metastasis of LC via the integrin-signaling pathway.

In conclusion, our study provides evidence for the hypothesis that down-regulated Periostin might control tumor microenvironment and suppress LC bone metastasis via blocking the integrin-signaling pathway. Meanwhile, knockdown of Periostin mediated by siRNA contributed to suppressing proliferation, migration and invasion of SBC-5 with high metastatic potential. The findings may provide a novel therapeutic target for the treatment of LC patients with bone metastasis. However, treatment protocols such as the selection of Periostin inhibitors or silencing and most effective levels of Periostin require further investigations.

Disclosure statement

None.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 81202095). We would also like to thank all participants enrolled in the present study.

References
