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Original Article

Effect of mesenchymal stem cell derived exosomes carrying PDGFD on lung cancer

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Abstract: Mesenchymal stem cells (MSCs) are capable of promoting lung tumor progression, but the underlying mechanism remains unclear. In this study, we investigated the effect of bone marrow MSC-derived exosomes (MSC-exosomes) carrying PDGFD on *in vitro* and *in vivo* lung tumor growth and its underlying mechanism. MSC-exosomes carrying PDGFD promoted lung cancer cell migration, EMT, proliferation and PI3K signaling pathway. Recombinant PDGFD could mimic the effect of MSC-exosomes carrying PDGFD on lung cancer cells. These effects were abolished by a neutralizing antibody against PDGFD *in vitro* and *in vivo*. We found that MSC-exosomes were capable of regulating lung cancer cell progression through PDGFD MSC-exosomes carry. This study may pave the way for novel therapeutic strategy targeting the PDGFD pathway regulating MSC-exosome-mediated cell-cell interactions.

Keywords: MSC, exosome, PDGFD, lung cancer cells

Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality worldwide. Due to rapid disease development, patients with NSCLC have poor prognoses [1]. However, the molecular indicators for NSCLC and its underlying mechanisms are still unclear. Recent studies have revealed that cancer associated fibroblasts (CAF) play important roles in cancer microenvironment and malignant transformation, and are closely related to prognosis [2-4].

Bone marrow derived mesenchymal stem cells (BM-MSCs) are multipotent progenitor cells characterized by their capacity to self-renew and differentiate into various cell types such as chondrocytes, osteocytes, adipocytes or fibroblasts [5, 6]. MSCs are important sources of carcinoma-associated fibroblasts, and can be recruited to tumor microenvironment, thereby differentiate into tumor-associated fibroblasts and constitute tumor stroma [7-9]. It has been established that tumor progression partly depends on the interaction between cancer cells and MSCs [10, 11]. Studies have shown that paracrine signals from MSCs significantly influence tumor cell behavior [12, 13]; however, the mechanism underlying tumor progression mediated by MSCs is still not fully understood.

Platelet-derived growth factors (PDGFs) are activated by two structurally related cell surface receptors tyrosine kinases α-PDGF receptor (PDGFR) and β-PDGFR, which regulate a variety of cellular processes, including cell invasion, proliferation, migration and transformation during pathogenesis [14]. PDGF-D is a newly identified isoform of PDGF and primarily interacts with β-PDGFR. It is up-regulated in various cancers, and plays an important role in tumor metastasis, angiogenesis and growth [15, 16].

Exosomes are small, lipid bilayer membrane vesicles of endocytic origin. They are 50-100 nm in diameter and play an important role in cell to cell communication. Exosomes constitute an interaction network to synergistically promote cancer development [17, 18]. Exosomes from BM-MSCs contain a number of bio-

active substances such as proteins, lipids, mRNAs, and microRNAs (miRNA). It has been found that they are major players in intercellular communication. However, the mechanisms involved in the promotional roles of exosomes in tumor have not yet been fully elucidated. Therefore, the purpose of this study was to determine whether exosomes carrying PDGFD mediated the malignant transformation of BM-MSC to lung cancer.

Materials and methods

Isolation and culture of hBM-MSC

Human MSCs were isolated from the bone marrow of normal donors with informed consent in accordance with the institutional guidelines under the approved protocol. hBM-MSCs were obtained and the characteristics of the isolated hBM-MSCs were investigated as previously described [18, 19]. Osteogenic and adipogenic differentiation assays were also performed on the MSCs, respectively. hBM-MSCs at passage 3 were used for the experiments.

Lung cancer cell lines culture

Lung cancer cells A549 were obtained from cell banks of Shanghai Institutes of Biological Sciences, and maintained in Dulbecco's modified Eagle's medium with low glucose (LG-DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), at 37°C in a humidified atmosphere of 5% CO_2. The cells were passaged every 2-3 days to maintain exponential growth.

Osteogenic and adipogenic differentiation of hBM-MSCs in vitro

hBM-MSCs in passage 3 were cultured in a medium that contained osteogenic (0.1 mM dexamethasone, 10 mM b-glycerophosphate, and 50 mM ascorbate-phosphate) or adipogenic (0.5 mM isobutylmethylxanthine, 1 mM dexamethasone, 10 mM insulin, and 200 mM indomethacin) reagents, respectively. The reagents were from Sigma-Aldrich. Two weeks later, osteogenic differentiation was assessed by the examination of neutrophil alkaline phosphatase (NAP) with the NAP staining kit (Sun Bio, China) and adipogenic differentiation was examined via intracellular lipid accumulation, which was visualized using Oil-Red-O staining by an inverted microscope (Olympus, Japan).

Isolation and identification of hBMMSC-Exosomes

When the MSCs reached 80%-90% confluence, they were cultured in conditioned medium for 48 h, and then the supernatants containing exosomes were harvested. The exosomes were isolated following the procedure described by Qu et al. with added modifications [20]. Briefly, the conditioned medium was collected and centrifuged at 1,000 g for 20 min to remove cell debris, followed by centrifugation at 2,000 g for 20 min and 10,000 g for 20 min. The supernatant was collected and concentrated using 100 KDa molecular weight cut off (MWCO) (Millipore) at 1,000 g for 30 min. The concentrated supernatant was loaded upon 5 mL of 30% sucrose/D2O cushions, and then ultracentrifuged at 100,000 g for 60 min (optimal-90K; Beckman Coulter). The exosome enriched fraction was diluted with PBS, and then centrifuged thrice at 1,000 g for 30 min using 100 KDa MWCO. Finally, the purified exosomes were subjected to filtration on a 0.22 μm pore filter (Millipore) and stored at -80°C. The protein content of the concentrated exosomes was determined using a BCA protein assay kit (Pierce). hBMMSCs-Ex was identified by transmission electron microscopy (FEI Tecnai 12; Philips), and CD9 (Bioworld Technology, Inc. Louis Park, MN) and CD81 (Epitomics, Burlingame, CA) were measured by western blotting.

Western blot analysis

The cells were collected and lysed in RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 mg/ml aprotinin and 1 g/ml leupeptin) on ice. Aliquots containing identical amounts of protein were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto methanol pre-activated polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked by 5% w/v non-fat dry milk. Following sequential incubation with the primary and secondary antibody (Santa Cruz Biotechnology, Inc., USA), the signal was visualized using HRP substrate (Millipore) and analyzed using MD ImageQuant Software. GAPDH (Kangcheng, China) was used as the loading control. The sources of the primary antibodies were as follows: anti-FAP, anti-

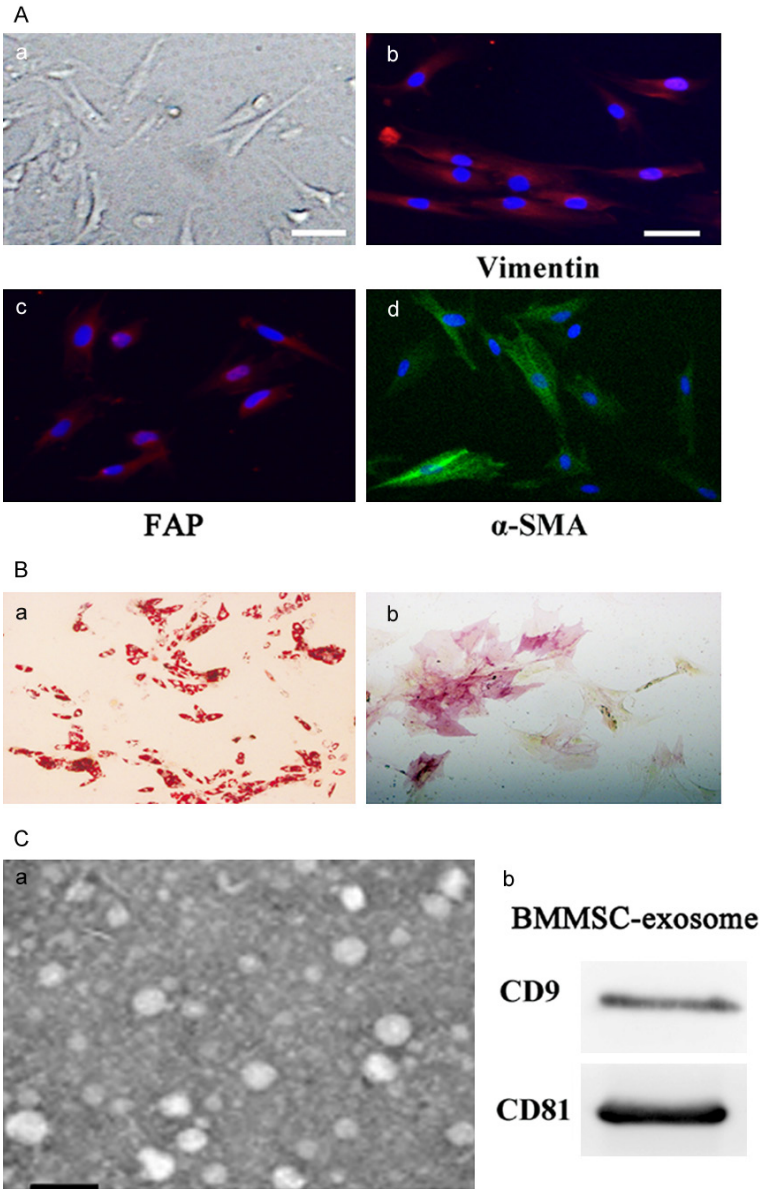

Figure 1. Isolation and identification of MSCs and MSC-exosomes. A. Human BM-MSCs (a) were spindle-shaped and plastic adherent when maintained in Dulbecco's modified Eagle's medium with low glucose supplemented with 15% fetal bovine serum (magnification 200 ×; *scale bar* = 50 μm); hBM-MSCs (b, c, d) express high levels of fibroblast proteins such as vimentin (b), FAP (c) and α-SMA (d) (Magnification 200 ×; *scale bar* = 50 μm); B. Adipogenic and osteogenic differentiation of hBM-MSCs. Oil-Red-O staining (a) of lipids in hucMSCs; Neutrophil alkaline phosphatase (NAP) staining (b) of hucMSCs. Cells were cultured for 14 days before staining (magnification 200 ×); C. Identification of hBMMSCs-exosome. Transmission electronic micrograph (a) of hBM-MSC-Ex, scale bar = 100 nm; Detection of hBMMSC-exosome CD9 and CD81 expression by western blot analysis (b).

vimentin, anti-α-SMA (Abcam, USA); anti-N-cadherin, anti-E-cadherin, anti-PDGFD (Santa Cruz Biotechnology, USA); PI3K, p-PI3K (phosphorylation of PI3K), proliferating cell nuclear antigen (PCNA) (Signalway Antibody Co., Ltd.).

Enzyme-linked immunosorbent assay

PDGF-D in the Dulbecco's modified Eagle's medium with low glucose (LG-DMEM) group, MSC-Ex group were measured using ELISA kit (R&D Systems, USA) in accordance with the manufacturer's instruction. Assays were performed in duplicate, and readings were compared with standard curves obtained with standard proteins provided with the kits.

Transwell migration assay

Following A549 cells treated with MSC-exosome for 72 h, A549 (1×10^5/well) were put into the upper chamber (8 mm) (Corning, NY, USA) in serum-free medium. The complete medium was placed into the lower chamber. Following incubation at 37°C in 5% CO_2 for 8 h, cells remaining at the bottom of the polycarbonate membrane were wiped off with cotton swabs. The cells migrating to the lower surface of the membrane were then fixed with methanol for 30 min. The migrated cells were stained with crystal violet and counted in six random fields under the microscope (Olympus, Japan) for each assay.

Cell colony formation assay

Following A549 cells treated with MSC-exosome for 72 h, the A549 cells were trypsinized and resuspended to a concentration of 1,000 cells/2 ml HG-DMEM with 10% FBS and were then incubated for two weeks. At the end of the growth period, cells were fixed with methanol for 30 min and stained with crystal violet for 15 min. The cell colonies were photo-

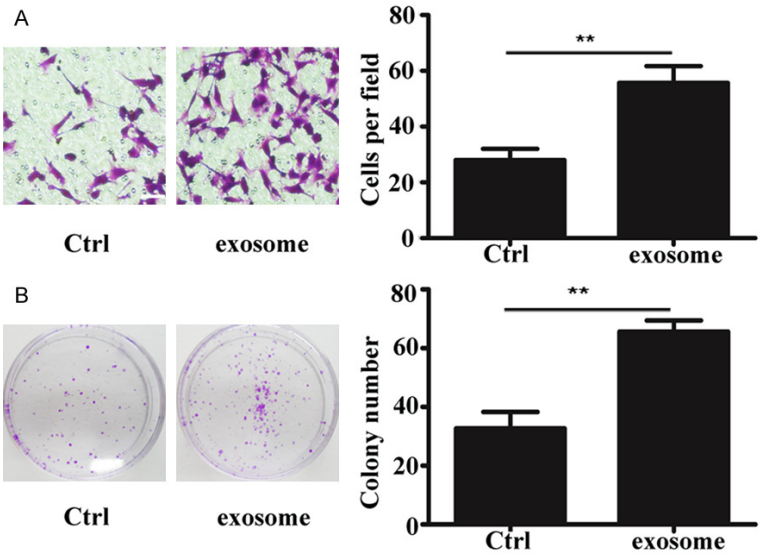

Figure 2. MSC-exosome promoted lung tumor migration and growth. A. Representative images showing that MSC-exosomes improved A549 cell penetration through membrane (magnification 200 ×; *scale bar* = 50 μm); B. Cell colony formation assay showing that MSC-exosome improved the ability of A549 cells to form colonies (***P* < 0.01).

graphed and the number of colonies was counted.

Immunofluorescence staining

Following hBM-MSCs at 3 passage cultured for 48 h, the hBM-MSCs were washed 3 times with cold PBS, fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 5 min, blocked with 5% BSA (Boster Bioengineering, China) and incubated with anti-vimentin, anti-FAP, anti-α-SMA primary antibody (Abcam, USA) at 4°C overnight and followed by Cy3-conjugated anti-rabbit secondary antibody (Sigma, USA). The cells were then stained with DAPI for nuclear staining, and images were acquired using a Nikon Eclipse Ti-S microscope.

Immunohistochemistry

Formalin-fixed paraffin-embedded mouse tumor tissue sections were first deparaffinized in xylene, rehydrated through graded ethanol. The sections were boiled for 10 min in citrate buffer (pH 6.0, 10 mM) for antigen retrieval. The endogenous peroxidase activity was inhibited with exposure to 3% hydrogen peroxide for 10 min. Then the sections were blocked with 5% BSA and incubated with proper diluted PCNA primary antibody (1:200) at 37°C for 1 h. After washed with PBS, the sections were then incubated with diluted secondary antibody for 20 min. Finally, sections were visualized with 3, 3'-diaminobenzidine (DAB) and then counterstained with hematoxylin for examination under a light microscope (Olympus, Japan).

Animal model

Four to five-week-old BALB/c nude mice were purchased from Slac Laboratory Animal Center (Shanghai, China). Animals were maintained in accordance with institutional policies, and all studies were performed with approval of the University Committee on Use and Care of Animals of Jiangsu University. Animals were randomly divided into 4 groups. The animals were injected subcutaneously with untreated A549 cells alone, A549 cells pretreated with MSC-exosome, A549 cells pretreated with MSC-exosome/NA-PDGFD and A549 cells pretreated with MSC-exosome/CtrlA-PDGFD into the backside of mice respectively. Tumor growth was evaluated by measuring the length and width of the tumor mass with calipers every 4 days. Tumor volumes were calculated by the modified ellipsoidal formula: (length × width^2)/2. Tumors were surgically removed, and photographed 40 days after injection.

Statistical analyses

All experiments were conducted at least in triplicate. All data are expressed as the means ± SD. SPSS 11.0 software was used to analyze the data. The means of different treatment groups were compared by two-way ANOVA or the Student's t-test. A *P*-value < 0.05 was considered to indicate a statistically significant difference.

Results

Isolation and identification of MSCs and MSC-exosomes

Human bone marrow MSCs were isolated and confirmed positive for CD29, CD44, CD105,

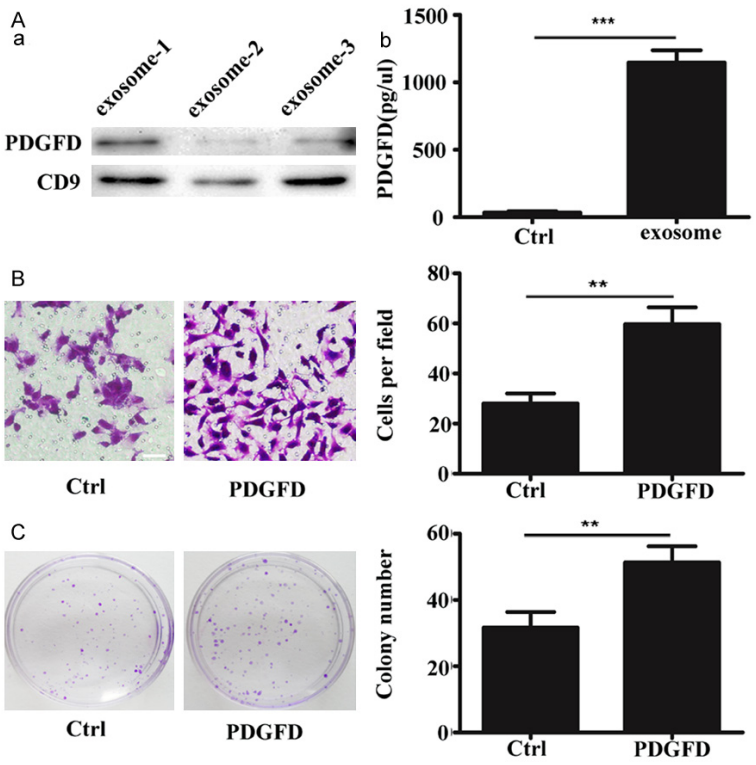

Figure 3. MSC-exosomes carrying PDGFD and the increased growth and migration of PDGFD treated lung tumor cells. A. Western blot analysis (a) showing the expression of PDGFD in MSC-exosome (a). ELISA analysis showing (b) PDGF-DD level was much higher in MSC-exosomes than in the control culture medium. B. Representative images showing that recombinant PDGF-DD (100 ng/ml) improved SGC-7901 cell penetration through membrane (magnification, 200 ×; *scale bar* = 50 μm). C. Cell colony formation assay showing that PDGF-DD could improve the cell colony forming ability of SGC-7901 (***P* < 0.01, ****P* < 0.001).

Influence of MSC-exosome on lung tumor migration and growth

The function of paracrine interactions between BM-MSCs and A549 was evaluated *in vitro* by treating A549 cells with MSC-exosomes for 14 days. The A549 cell migration ability was evaluated by the transwell migration array. After A549 cells were cultured in the presence of MSC-exosomes for 2 weeks, their migration ability was significantly enhanced compared with the control group (**Figure 2A**). Moreover, the proliferation capacity of A549 cells was evaluated by the cell colony formation assay. Similarly, the results showed that cell proliferation was also significantly strengthened compared with the control group after A549 cells were treated with MSC-exosome for 2 weeks (**Figure 2B**).

MSC-exosomes carrying PDGFD and increased growth and migration of PDGFD-treated lung tumor cell

and HLA-I surface antigen expressions, and negative for CD34, CD38 and HLADR surface antigen expressions. BMMSCs presented a homogeneous population of spindle fibroblast-like cells. They possessed spindle shape and plastic adherent characteristics (**Figure 1A**). They also expressed high levels of fibroblast proteins, such as vimentin, fibroblast activation protein, FAP and α-SMA (**Figure 1B-D**). Von Kossa staining and Oil Red O staining were used to evaluate their osteogenic differentiation and adipogenic differentiation potential, respectively. They showed spontaneous adipocyte or osteoblast formation (**Figure 2B**). MSC-exomes were isolated and purified using differential centrifugation on a sucrose cushion. By transmission electronic microscopy, it was found that MSC-exosomes were 30-100-nm microvesicles (MVs) (**Figure 3A**). MSC-exosomes showed high levels of CD9 and CD81 expressions (**Figure 3B**).

To examine the molecular mechanism underlying MSC-exosome mediated promotion of tumor migration and proliferation, we investigated the expression of PDGFD in three different batches of MSC-exosome sources. Western blot and ELISA analyses revealed that PDGFD was highly expressed in exosome (**Figure 3A**). Because of the high expression of PDGFD in MSC-exosome, we investigated whether PDGFD was important for exosome in promoting the malignant transformation of lung cancer. To evaluate the effect of PDGFD on lung tumor cells, we performed migration and proliferation experiments after A549 cells were treated with recombinant PDGFD for one week. The results showed that the migration of cells was significantly increased compared with the control group (**Figure 3B**). We also found that the cell cloning ability was significantly enhanced. The untreated A549 cells were served as control in these experiments.

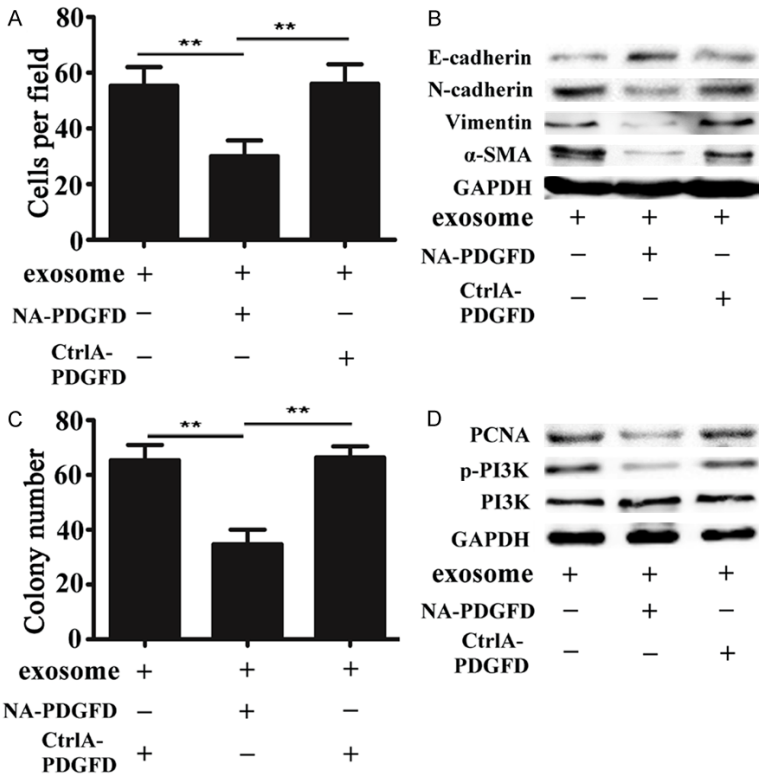

Figure 4. Neutralizing antibody against PDGFD greatly alleviated MSC-exsome-induced lung tumor progression *in vitro*. A. Transwell migration assay histogram of migration cell number in the exosome group, the NA-PDGFD (neutralizing antibody against PDGFD)/exosome group, and the CtrlA-PDGFD (isotype-matched normal antidody) group. B. Western blot analysis showing the expressions of E-cadherin, N-cadherin, α-SMA, and Vimentin in A549 cells in different groups. C. Cell colony formation assay histogram of A549 cells in the exosome group, the NA-PDGFD/exosome group, and the CtrlA-PDGFD group. D. Western blot analysis showing the expressions of PI3k, p-PI3k and PCNA in A549 cells in different groups (**$P < 0.01$).

Neutralizing antibody against PDGFD greatly alleviated MSC-exosome-induced lung tumor progression in vitro

In order to study whether PDGFD was a key molecule in exosome mediated lung cancer development, we added either a neutralizing antibody against PDGFD (NA-PDGFD) or an isotype-matched normal antibody (CtrlA-PDGFD) in the exosome group. Notably, this migration effect was abrogated by a PDGFD-specific blocking antibody, but not by a control antibody (**Figure 4A**). The results of western blot analysis also showed that the expression of E-cadherin, an important feature of epithelial cell, was increased after A549 cells was treated with exosome in the presence of neutralizing antibody against PDGFD. The increased expression of E-cadherin was accompanied by decreased expressions of the important mesenchymal markers N-cadherin, Vimentin and α-SMA (**Figure 4B**). Similarly, compared with the control group, the proliferation ability of cells was significantly decreased in the PDGFD blocking antibody group (**Figure 4C**). In order to study the signal proteins associated with cell proliferation, we tested p-PI3K, PI3K and PCNA. Western blot analysis showed that the expressions of p-PI3K and PCNA were weakened in the PDGFD blocking antibody group (**Figure 4D**).

Neutralizing antibody against PDGFD abolished MSC-exosome growth-promoting effect on lung cancer cells tumor xenografts in vivo

To evaluate the effect of PDGFD-carrying exosomes on lung tumor growth, we performed an animal model experiment using a neutralizing antibody against PDGFD. A549 cells were treated with exosome in the presence of either a neutralizing antibody against PDGFD or a control normal antibody for one week before injection. Five weeks later, the xenograft tumors were removed, photographed and weighed. Our data revealed that the size of the tumor from the neutralizing antibody group was significantly smaller than those of the exosome group and the control antibody group; however, it was approximately equal to that in the control group (**Figure 5A** and **5B**). In addition, we performed PCNA immunohistochemical staining on tissue sections from tumor tissue in each group. As shown by the increased number and intensity of PCNA-positive cells, the results revealed that the percentage of PCNA-positive cells in the neutralizing antibody group was markedly lower than those in the exosome group and the control antibody group (**Figure 5C**).

Discussion

Although MSCs is believed to be critically involved in tumor development and progression

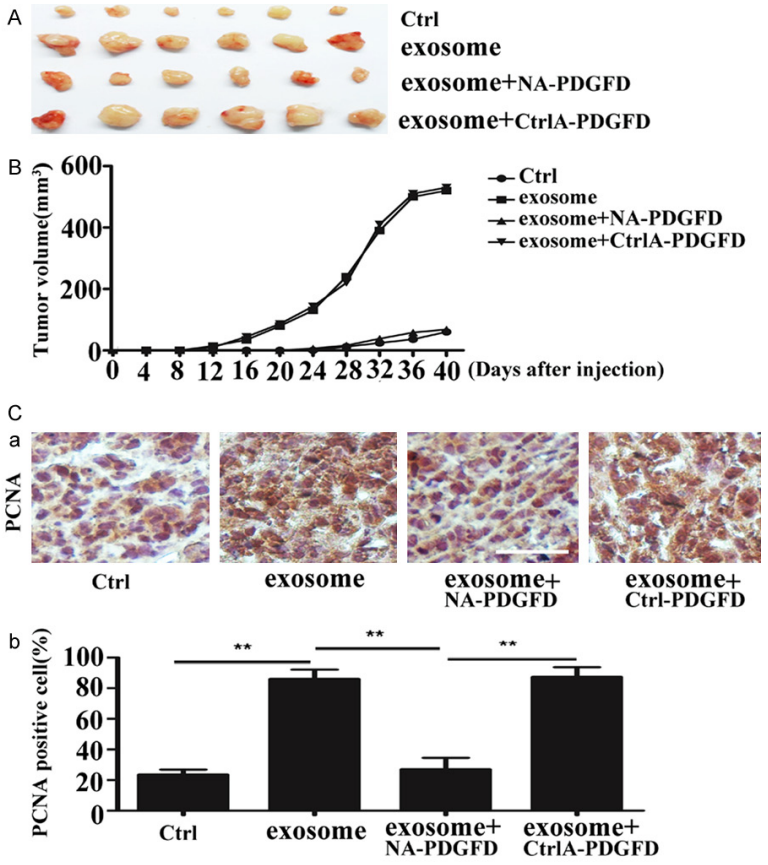

Figure 5. Neutralizing antibody against PDGFD abolished the growth promoting effect of MSC-exosome growth-promoting on lung cancer cell tumor xenografts *in vivo*. A. Representative photographs of tumor tissues in different groups. B. The tumor volume of mice in different groups. C. Immunohistochemical analysis of PCNA expression in tumor tissues in different groups. (a) Representative photomicrograph; (b) percentage of proliferating cell nuclear antigen (PCNA)-positive cells (magnification, 200 ×; scale bar = 200 μm) (***P* < 0.01).

[21, 22], the exact mechanism is still not very clear and poorly documented. The paracrine interactions that involve tumor growth between MSCs and cancer cells may be related to those responses. In addition to soluble factors, exosomes have also been deemed as an important mechanism in the paracrine action of MSCs. Recent studies have found that exosomes contain functional nucleic acids and proteins when transferred into recipient cells, which indicated signal transmission for tumor progression [23, 24]. Here, we report an important finding that MSC-exosomes carrying PDGFD play a significant role in lung tumor progression.

In this study, we cultured BM-MSCs from human bone specimens. The successful isolation and purification of exosomes from bone marrow MSCs was by confirming their diameters (50-100 nm) and the protein expressions of CD9 and CD81. Carrying proteins, DNAs, mRNAs, and non-coding RNAs, exosomes play critical roles in tumorigenesis [25], growth [26], angiogenesis [27], metastasis [28] and drug resistance [29]. At present, there is few research on the effect of MSC-exsomes on lung cancer. Therefore, we investigated whether MSC-exosomes promoted lung tumor migration and growth *in vitro*. In order to study the mechanism, we evaluated the expression of PDGFD in MSC-exsome. PDGFD is an isoform of the PDGF family, and may play a key role in the development and progression of human cancers [30]. The results of western blot and ELISA analyses showed high expression of PDGFD in MSC-exosome.

To address the question of whether MSC-exosome is mediated by PDGFD to promote the development of lung cancer, firstly, we treated lung tumor cells A549 with recombinant PDGFD for one week. The results showed that the effect of recombinant PDGFD on lung tumor progression was similar to that of MSC-exosome. Afterwards, we treated A549 cells with an antibody against PDGFD in the MSC-exsome group. The results demonstrated that the effect of MSC-exosome on migration and proliferation of tumor was greatly weakened compared to that that in the non-treated/control-antibody group *in vitro*. It has been suggested that epithelial-mesenchymal transition (EMT) and migration are closely related [31]. Besides, PI3k signaling pathway is important for cell proliferation [32]. Western blot analysis demonstrated that EMT and PI3K signaling is significantly attenuated in the antibody against PDGFD group. Consistent with the *in vitro* results, our *in vivo* data showed that tumors

grew slower in the control and the antibody against PDGFD group than in the other two groups. Moreover, immunohistochemical analysis of PCNA expression in tumor tissues indicated that percentage of PCNA-positive cells was lower in the control and the antibody against PDGFD group than in the other two groups. These results suggest that PDGFD might play a key role in MSC-exosome mediated lung tumor progression. Further studies are needed to investigate whether there are other mechanisms involved.

In summary, it was revealed in this study that MSC-exosomes promote lung tumor progression through exosome carrying PDGFD. Novel features of the complex dialogue between MSC-exosome and tumor cells were discovered in this study. These finding establishes an unprecedented link between both. This could be a lung tumor diagnostic biomarker and a novel selective therapeutic strategy targeting PDGFD exosomes carrying.

Acknowledgements

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Disclosure of conflict of interest

None.

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