Exosomes derived from $PM_{2.5}$-treated lung cancer cells promote the growth of lung cancer via the Wnt3a/β-catenin pathway

HUI XU[1,2*], XINGAI JIAO[1*], YILEI WU[3*], SHUO LI[1], LILI CAO[1] and LIANG DONG[1]

[1]Department of Respiratory Medicine, Qilu Hospital of Shandong University, Jinan, Shandong 250012; [2]Department of Respiratory Medicine, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325000; [3]Department of General Surgery, Ruian People's Hospital, Wenzhou, Zhejiang 325200, P.R. China

Received June 21, 2018; Accepted October 24, 2018

DOI: 10.3892/or.2018.6862

Abstract. Fine particulate matter ($PM_{2.5}$) is associated with an increased lung cancer risk. However, the effect of $PM_{2.5}$ exposure on lung cancer cells is still largely unknown. The present study revealed that A549 lung cancer cells secreted exosomes containing high levels of Wnt3a after treatment with $PM_{2.5}$. These exosomes activated β-catenin signalling in A549 cells. These exosomes exhibited no effects on migration and invasion, but promoted proliferation of A549 cells via the Wnt3a/β-catenin pathway *in vitro*. These exosomes promoted A549 tumour progression in a Wnt3a-dependent fashion *in vivo*. These results demonstrated that $PM_{2.5}$ has a direct effect on promoting lung tumour development. Inhibition of exosome production by tumour cells or blockade of the Wnt3a/β-catenin pathway represents a promising strategy to impede $PM_{2.5}$-mediated lung tumour progression.

Introduction

Fine particulate matter ($PM_{2.5}$) indicates atmospheric aerodynamic equivalent diameter less than or equal to 2.5 micron particles (1,2). Long-term and short-term exposure to $PM_{2.5}$ directly threatens public health (3,4). $PM_{2.5}$ has been related to respiratory disease and cardiovascular disease (5-7). Exposure to $PM_{2.5}$ increases the risk of asthma and exacerbates established asthma (5,6). $PM_{2.5}$ induces inflammation and mucus hyperproduction in the airway epithelium (7). $PM_{2.5}$ is necessary for the migration of human bronchial smooth muscle cells, defining a novel role for PM2.5 in airway remodelling in chronic obstructive pulmonary disease (8). Moreover, $PM_{2.5}$ is associated with the development of atherosclerosis in *ApoE*$^{-/-}$ mice (9).

Many publications have demonstrated that $PM_{2.5}$ is associated with cancer incidence. Suggestive evidence has shown an association between ambient air pollution and the incidence of postmenopausal breast cancer in European women (10). Ambient $PM_{2.5}$ exposure may be a risk factor for hepatocellular carcinoma in the United States (11). $PM_{2.5}$ was also involved in lung cancer burden (12). Enhanced ability of motility and proliferation were observed after $PM_{2.5}$ exposure of non-small cell lung cancer cells (13). $PM_{2.5}$ also induced epithelial-mesenchymal transition of human lung cancer cells (14). In addition, lung cancer stem cell properties were induced by $PM_{2.5}$ (15). However, the definitive relationship between $PM_{2.5}$ exposure and lung cancer has yet to be explored.

Exosomes are vesicles smaller than 150 nm in diameter that are enriched in endosome-derived components. Exosomes have a bilayer lipid structure containing transmembrane proteins, and they enclose soluble proteins, RNA and DNA (16,17). Accumulating evidence has well recognized the important role of exosomes as couriers to mediate communication between different cells (18). Tumour-derived exosomes (TEXs) are closely related to tumour development. TEXs educated dendritic cells to promote tumour metastasis via the HSP72/HSP105-TLR2/TLR4 pathway (19). Lnc-Sox2ot of TEXs promoted EMT and stemness by acting as a ceRNA in pancreatic ductal adenocarcinoma (20). Tumour exosomal RNAs promoted lung pre-metastatic niche formation by activating alveolar epithelial TLR3 to recruit neutrophils (21). Wnt10b in cancer-associated fibroblasts has been shown to promote breast cancer cell metastasis (22). In addition, activation of the Wnt signalling pathway was detected in $PM_{2.5}$-induced pulmonary arterial hypertension of rats (23). Therefore, exosomes from $PM_{2.5}$-treated lung cancer cells may affect tumour progression through activation of Wnt signalling.

The present study demonstrated that Wnt3a was enriched in exosomes from $PM_{2.5}$-treated A549 ($EXO_{PM2.5}$) cells

Correspondence to: Dr Liang Dong, Department of Respiratory Medicine, Qilu Hospital of Shandong University, Jinan, Shandong 250012, P.R. China
E-mail: dl5506@126.com

*Contributed equally

Abbreviations: $PM_{2.5}$, fine particulate matter; TEXs, tumour-derived exosomes; $EXO_{PM2.5}$, exosomes from $PM_{2.5}$-treated A549; EXO_{Ctrl}, exosomes from A549 with mock treatment; EMT, epithelial-mesenchymal transition; ceRNA, competing endogenous RNAs; OD, optical density

Key words: $PM_{2.5}$, exosomes, lung cancer, Wnt3a, β-catenin

(human epithelial cancer cells, which activated Wnt/β-catenin signalling in A549 cells). $EXO_{PM2.5}$ significantly promoted A549 cell proliferation in a Wnt3a-dependent fashion *in vitro*. Furthermore, intratumoural injection of $EXO_{PM2.5}$ accelerated tumour growth and decreased survival rate of mice via Wnt3a. Therefore, these results extend the knowledge of $PM_{2.5}$ exposure and lung cancer progression.

Materials and methods

Reagents. $PM_{2.5}$ was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). Human *Wnt3a* siRNA, negative control (NC) siRNA and antibodies against GRP94 (cat. no. sc-393402), CD63 (cat. no. sc-59284), Tsg101 (cat. no. sc-136111), Alix (cat. no. sc-53540), HSP70 (cat. no. sc-59570), Wnt3a (cat. no. sc-136163) and β-Actin (cat. no. sc-517582) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Human *Wnt1, Wnt3a, Wnt4, Wnt7a, Wnt9a* and *Wnt10b* primers were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). An antibody against β-catenin (cat. no. ab6302) was purchased from Abcam (Cambridge, MA, USA). LF3, a specific inhibitor of Wnt/β-catenin signalling (24), was purchased from Selleckchem (Houston, TX, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Tokyo, Japan). Matrigel matrix basement membrane was purchased from BD Biosciences (San Diego, CA, USA).

Mice and cell line. Female athymic nude mice (aged 6-8 weeks) were purchased from Joint Ventures Sipper BK Experimental Animal Co., Ltd. (Shanghai, China). The mice were maintained in specific pathogen-free facilities with temperature ranging from 22 to 24˚C, humidity ranging from 50 to 60% and 12 h of light/dark cycle at Wenzhou Medical University (Wenzhou, China). Mice had free access to food and water, and all experiments using mice were approved by and performed according to the guidelines of the Animal Ethics Committee of Wenzhou Medical University.

The A549 cell line, a human lung adenocarcinoma cell line, was purchased from the American Type Culture Collection (ATTCC; Manassas, VA, USA), and cultured in RPMI-1640 media supplemented with 10% (v/v) fetal calf serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a 5% CO_2 incubator.

Preparation of $PM_{2.5}$. $PM_{2.5}$ (10 mg) was suspended in 1 ml of normal saline, sonicated at 20% power for 3 pulses of 10 sec each (waiting 5 sec between pulses), and then 4 ml of normal saline was added to a final concentration of 2 mg/ml. The $PM_{2.5}$ solution was aliquoted and stored at 4˚C. Before use, the $PM_{2.5}$ solution was sonicated at 20% power for 3 pulses of 10 sec each (waiting 5 sec between pulses).

$PM_{2.5}$ treatment and exosome isolation. A549 cells ($2.5x10^5$/ml) were seeded into 6-well plates in total volume of 2 ml/well. After 12 h, the supernatant was discarded, and 2 ml of fresh RPMI-1640 media was added with 100 μg/ml $PM_{2.5}$. After 24 h, the supernatant collected from all the wells (240 ml in total) was differentially centrifuged at 300 x g for 10 min, 1,200 x g for 20 min, and 10,000 x g for 30 min at 4˚C. The supernatants from the final centrifugation were ultracentrifuged at 100,000 x g for 1 h at 4˚C. After removing the supernatants, the exosomal pellets were washed in a large volume of ice-cold phosphate-buffered saline (PBS) and centrifuged at 100,000 x g for an additional 1 h at 4˚C. The final pellets were resuspended in PBS. The amount of exosomal proteins was assessed by a BCA assay (Thermo Fisher Scientific, Inc.).

Nanoparticle tracking analysis and electronic microscopy. Nanoparticle tracking analysis of exosomes was assessed by NanoSight NS300 Particle Size Analyzer (Malvern Panalytical Ltd., Malvern, UK). To detect morphology of exosomes, exosomes were isolated and diluted in 100 μl of PBS, and 20 μl of the suspension was placed onto Formvar carbon-coated copper grids (Beijing XXBR Technology Co., Ltd., Beijing, China) at room temperature for 1 min. The excess suspension was removed using filter paper. Exosomes were stained with 2% phosphotungstic acid at room temperature for 5 min. The grids were then fixed with 2.5% glutaraldehyde at room temperature for 5 min followed by rinsing with PBS three times. Images were observed with a Philips Tecnai-10 transmission electron microscope operating at 80 kV (Phillips Electronic Instruments, Inc., Mahway, NJ, USA).

Real-time PCR. Total RNA was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The following PCR conditions were used: 1 cycle at 95˚C for 30 sec; and 40 cycles of 5 sec at 95 and 60˚C at 34 sec. Real-time PCR was performed using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The following primers were used: *Wnt1* forward, 5'-ctcttcggcaagatcgtcaacc-3' and reverse, 5'-cgatggaaccttctgagcagga-3'; *Wnt3a* forward, 5'-atg aaccgccacaacaacgagg-3' and reverse, 5'-gtccttgagg aagtcaccgat g-3'; *Wnt4* forward, 5'-gctggagaagtgcggctgtga-3' and reverse, 5'-ccacaaacgactgtgagaaggc-3'; *Wnt7a* forward, 5'-aggagaagg ctcacaaatgggc-3' and reverse, 5'-cggcaatgatggcgtaggtgaa-3'; *Wnt9b* forward, 5'-cctgcttgagtgccagtttcag-3' and reverse, 5'-aca ccgcgtacaggaaagctgt-3'; *Wnt10b* forward, 5'-ctcgggatttcttggatt ccagg-3' and reverse, 5'-gccatgacacttgcatttccgc-3'.

Immunofluorescence staining. For detection of β-catenin nuclear translocation, A549 cells were treated with 10 μg/ml exosomes for 0, 30 and 60 min. The cells were then fixed, permeabilized and incubated with rabbit polyclonal antibodies against β-catenin (cat. no. ab6302; Abcam) using a dilution of 1:1,000 at 4˚C overnight. Subsequently, the cells were incubated with Alexa Fluor® 647 conjugated goat anti-rabbit antibodies (cat. no. ab150079; Abcam) using a dilution of 1:200 for 1 h. Cells were counterstained with DAPI to indicate DNA. Stained cells were viewed using a confocal microscope (SP2; Leica, Solms, Germany).

Migration assay. A549 cells ($1x10^6$/ml and $2.5x10^5$/ml) were treated with 10 μg/ml exosomes from A549 with mock treatment (EXO_{Ctrl}) or $EXO_{PM2.5}$ for 24 h at 37˚C. Then, $2x10^4$ cells in 100 μl of serum-free media were seeded into the top chamber. The bottom chamber was filled with 800 μl of medium containing 20% serum. After being cultured for

18 h at 37˚C, the cells were fixed with methanol for 20 min and washed with PBS three times. The fixed cells were stained with 10 μg/ml DAPI for 30 min and washed with PBS. The stained cells were examined using a fluorescence microscope.

Invasion assay. After rehydration using a 6-fold volume of serum-free media, 50 μl of Matrigel was added on an 8-μm polycarbonate membrane in 24-well Transwell plates, and the Matrigel was solidified at 37˚C. Then, 1x10^6 A549 tumour cells were incubated with 10 μg/ml EXO_{Ctrl} or $EXO_{PM2.5}$ for 24 h at 37˚C. Subsequently, 5x10^4 A549 cells in 100 μl of serum-free media were seeded into the top chamber. The bottom chamber was filled with 800 μl of medium containing 20% FBS. After being cultured for 48 h at 37˚C, the cells were fixed with methanol for 20 min and washed with PBS three times. The fixed cells were stained with 10 μg/ml DAPI for 30 min and washed with PBS. The stained cells were examined using a fluorescence microscope.

In vitro proliferation assay. A549 cells (2x10^4) were seeded into 96-well plates at 200 μl/well, and 2 μg of exosomes was then added for 24 h. To some of the wells, 10 μM LF3 was added. Four hours before the end of culture, 20 μl of CCK-8 was added. The optical density (OD) of each well was read at 450 nm using an automated microplate reader (Sunrise; Tecan Group, Ltd., Mannedorf, Switzerland).

Western blot analysis. Exosomes or crude proteins were extracted from cell lysates by RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) and then qualified by BCA Protein Assay kit (Beyotime Institute of Biotechnology). A total of 20 μg of proteins was separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% BSA in TBST, and then incubated with corresponding primary antibodies overnight at 4˚C. After incubating with HRP-coupled secondary antibodies for 1 h at room temperature, the membranes were scanned using a Tanon 4500 Gel Imaging System (Tanon Science and Technology Co., Ltd., Shanghai, China). The following primary antibodies and secondary antibodies were used: Rabbit monoclonal antibodies against GRP94 (dilution 1:1,000; cat. no. ab108606), rabbit monoclonal antibodies against Tsg101 (dilution 1:1,000; cat. no. ab125011), mouse monoclonal antibodies against Alix (dilution 1:500; cat. no. ab117600), mouse monoclonal antibodies against HSP70 (dilution 1:500; cat. no. ab47455), mouse monoclonal antibodies against Wnt3a (dilution 1:1,000; cat. no. ab81614), mouse monoclonal antibodies against CD63 (dilution 1:500; cat. no. ab193349), rabbit monoclonal antibodies against β-catenin (dilution 1:2,000; cat. no. ab32572), mouse monoclonal antibodies against β-actin (dilution 1:500; cat. no. ab8226;), HRP-coupled rabbit polyclonal antibodies against mouse (dilution 1:2,000; cat. no. ab6728) and HRP-coupled goat antibodies against rabbit (dilution 1:2,000; cat. no. ab6721; all were from Abcam).

RNA interference assay. For transient silencing of the *Wnt3a* gene, 40 nM siRNA was transfected into cells (2x10^5/well) using 3 μl of INTERFERin siRNA transfection reagent (Polyplus-Transfection, New York, NY, USA) per well in a 24-well plate. The knockdown efficiency of Wnt3a was confirmed by western blotting.

In vivo animal studies. To establish the tumour model, A549 cells (5x10^6) were subcutaneously injected into nude mice. On day 10, the mice were randomly divided into three groups (each group with 5 mice/total 120 mice) and received intratumoural injections of 5 μg of exosomes every other day (total 14 injections). For the survival study, when the largest tumour volume reached 4,000 mm^3 (60 mice), the mice were no longer free to move around. For humanitarian reasons, the mice were sacrificed by cervical dislocation after being intraperitoneally injected with 50 mg/kg pentobarbital sodium approved by Animal Ethics Committee of Wenzhou Medical University. The other 60 mice were also sacrificed by this way on day 36. The length and width of tumours were assessed every four days by vernier caliper and the tumour was calculated according to the following formula: Volume = (length x width^2)/2.

Statistical analysis. Results were expressed as the mean ± SEM. Statistics were analysed by one-way or two-way analysis of variance (ANOVA) with Newman-Keuls post hoc test. The survival curves were calculated using the Kaplan-Meier method and the log-rank test was used for survival analysis. All statistics were analysed by GraphPad Prism 5.0 software (Graphpad Software, Inc., La Jolla, CA, USA). A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

$PM_{2.5}$ exposure increases Wnt3a protein level in $EXO_{PM2.5}$. To assess if $PM_{2.5}$ treatment upregulated expression of Wnt family members, the mRNA expression level of *Wnt* family members in $PM_{2.5}$-treated A549 cells was assessed. The *Wnt3a* mRNA level was greatly increased compared to control cells (Fig. 1A). To investigate if $PM_{2.5}$ exposure affected Wnt3a protein level in $EXO_{PM2.5}$, $EXO_{PM2.5}$ and exosomes from A549 cells with mock treatment (EXO_{Ctrl}) were isolated. Visualization using electron microscopy indicated that EXO_{Ctrl} and $EXO_{PM2.5}$ had similar morphology, and both had diameters ranging from 50 to 150 nm (Fig. 1B), indicating that $PM_{2.5}$ exposure did not affect exosomal morphology. Nanoparticle tracking analysis revealed that the size distribution of EXO_{Ctrl} and $EXO_{PM2.5}$ was 119±41.7 and 119±41.3 nm (mean ± SD), respectively (Fig. 1C). The protein components of EXO_{Ctrl} and $EXO_{PM2.5}$ were examined. Both exosomes were negative for the endoplasmic reticulum-residing protein, GRP94, and positive for HSP70 as well as the multivesicular body-related proteins, CD63, Tsg101 and Alix (Fig. 1D). According to a previous publication, CD63 was used as a loading control (25). Notably, Wnt3a was only detected in $EXO_{PM2.5}$ (Fig. 1D). These results indicated that $EXO_{PM2.5}$ has higher Wnt3a protein levels than EXO_{Ctrl}.

$EXO_{PM2.5}$ induces activation of the Wnt/β-catenin pathway. β-catenin is a key downstream effector in the Wnt signalling pathway (26). In the off-state, β-catenin is phosphorylated by CK1 and subsequently phosphorylated by GSK-3β (27,28), resulting in destabilization of β-catenin (29). In the on-state,

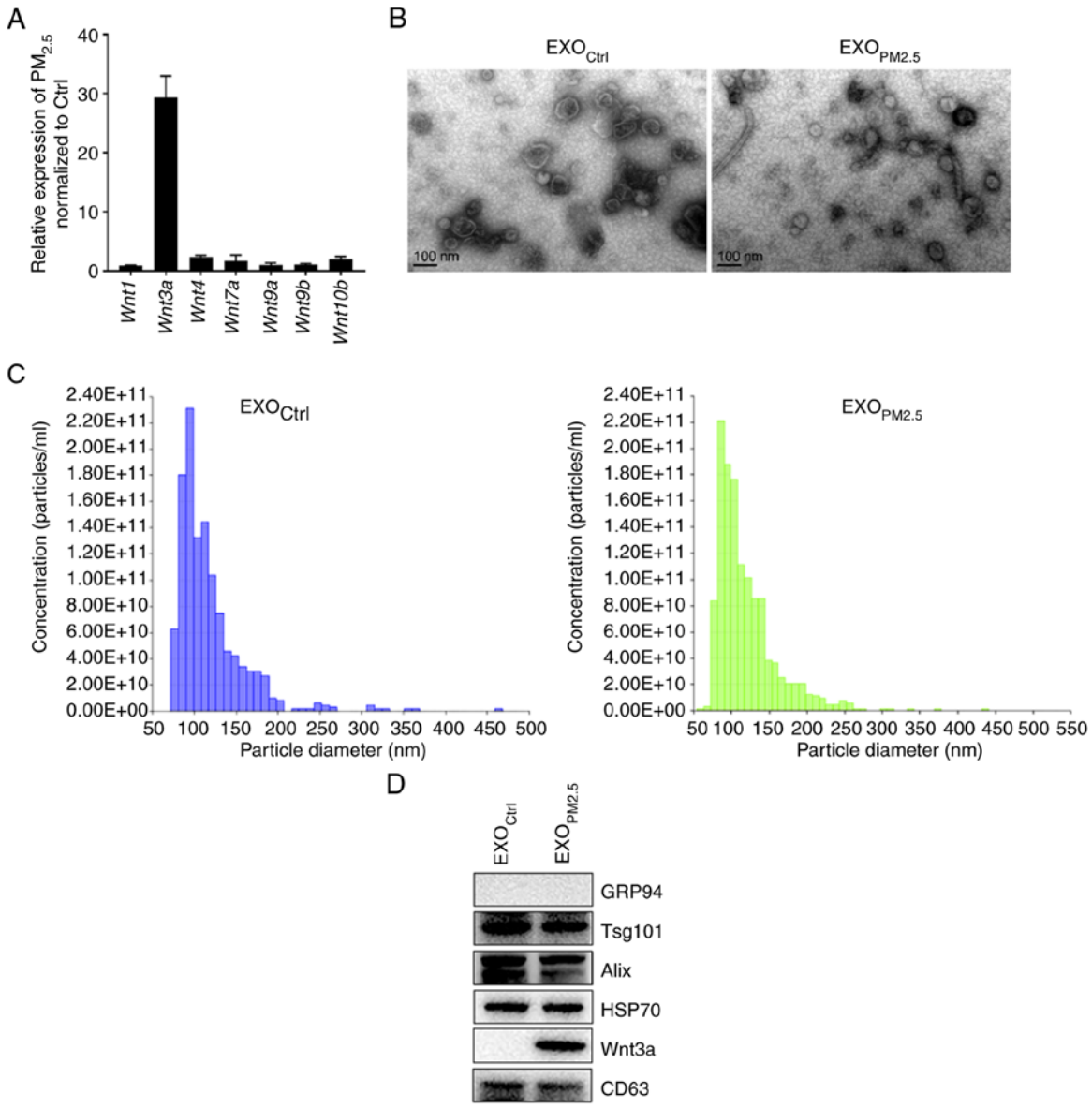

Figure 1. $PM_{2.5}$ exposure increases Wnt3a protein level in $EXO_{PM2.5}$. (A-D) A549 cells ($2.5x10^5$/ml in density) were treated with 100 μg/ml $PM_{2.5}$ for 24 h. (A) The mRNA levels of indicated *Wnt* family members were assessed by real-time PCR. (B) Exosomes in the supernatants were isolated. The morphology of exosomes was detected by electron microscopy. (C) Size distribution of exosomes was analysed by a nanoparticle tracking instrument. (D) Proteins in exosomes were detected by western blotting with CD63 as a loading control. Ctrl and $PM_{2.5}$ in A indicate A549 cells with mock or $PM_{2.5}$ treatment, respectively. Data are representative of three independent experiments.

a Wnt ligand binds to a Frizzled receptor and then prevents β-catenin phosphorylation by GSK-3β, leading to accumulation and increased nuclear import of β-catenin (30). Since Wnt3a was enriched in $EXO_{PM2.5}$, we investigated whether $EXO_{PM2.5}$ activated Wnt/β-catenin signalling. Treatment with $EXO_{PM2.5}$ markedly increased total β-catenin protein in A549 cells (Fig. 2A). In addition, enhanced nuclear translocation of β-catenin was observed in $EXO_{PM2.5}$-treated A549 cells (Fig. 2B). These results indicated that $EXO_{PM2.5}$ activated the Wnt/β-catenin pathway.

$EXO_{PM2.5}$ does not affect A549 cell migration and invasion. Since the Wnt/β-catenin pathway has been implicated in tumour migration and invasion (31,32), we investigated whether $EXO_{PM2.5}$ promoted A549 cell migration. As revealed in Fig. 3A, $EXO_{PM2.5}$ treatment had no effect on A549 cell migration (Fig. 3A and B). The role of $EXO_{PM2.5}$ on invasion of A549 cells was next examined. No difference of invasive ability was observed in A549 cells with or without $EXO_{PM2.5}$ treatment (Fig. 3C and D). These results indicated that $EXO_{PM2.5}$ does not alter the migration and invasion abilities of A549 cells.

$EXO_{PM2.5}$ promotes A549 cell proliferation in a Wnt3a/β-catenin-dependent manner. β-catenin is implicated in tumourigenesis (33), and Wnt3a/β-catenin signalling has also been demonstrated to induce tumour cell proliferation (34). Thus, the effect of $EXO_{PM2.5}$ on A549 cell proliferation was investigated. $EXO_{PM2.5}$, but not EXO_{Ctrl}, significantly promoted A549 cell proliferation (Fig. 4A). To elucidate the role of Wnt3a in this effect, Wnt3a was knocked down in $PM_{2.5}$-treated A549 cells by *Wnt3a* siRNA. Exosomes with low amounts of Wnt3a

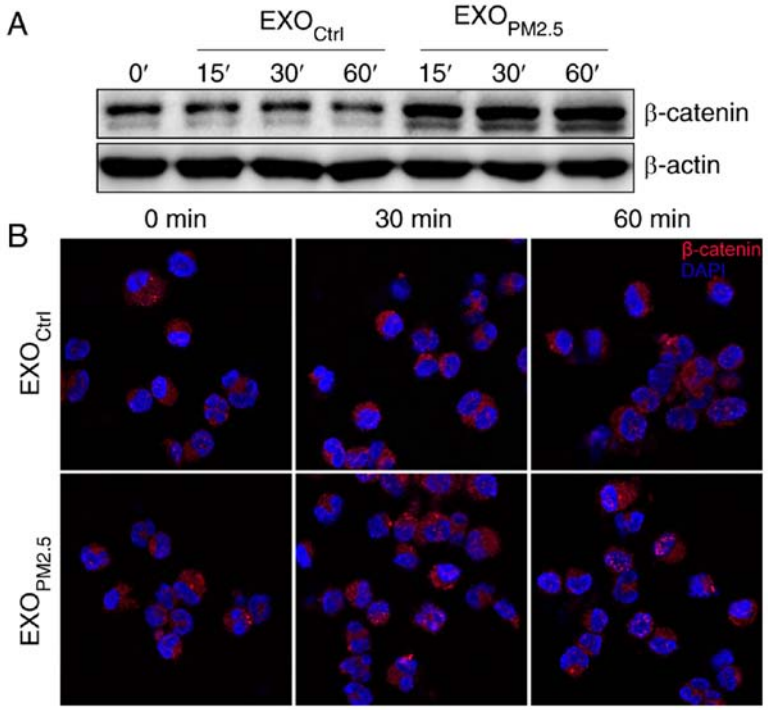

Figure 2. $EXO_{PM2.5}$ induces activation of the Wnt/β-catenin pathway. (A and B) A549 cells ($2.5x10^5$/ml in density) were treated with 10 μg/ml EXO_{Ctrl} or $EXO_{PM2.5}$ for the indicated time-points. (A) The protein level of β-catenin was detected by western blotting. (B) Fluorescence staining of β-catenin and the nucleus. After binding to anti-β-catenin primary antibodies, β-catenin was stained by PE-conjugated secondary antibodies. Nuclear staining was performed by DAPI. Magnification, x400. Data are representative of three independent experiments. $EXO_{PM2.5}$, exosomes from $PM_{2.5}$-treated A549 cells; EXO_{Ctrl}, exosomes from A549 cells with mock treatment.

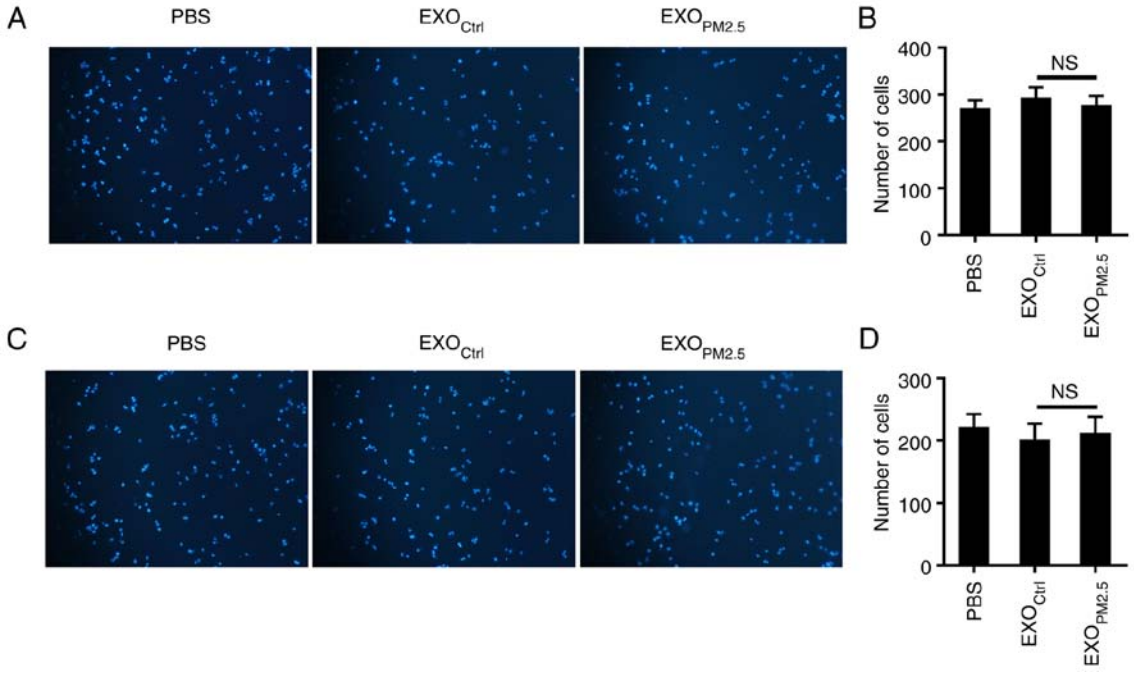

Figure 3. $EXO_{PM2.5}$ does not affect A549 cell migration and invasion. (A) A549 tumour cells were incubated with 10 μg/ml EXO_{Ctrl} or $EXO_{PM2.5}$ for 24 h, and the cells were then plated in the top chamber of a Transwell plate. After 18 h, the cells on the bottom of the Transwell filter were imaged and quantified. Magnification, x100. (B) The results of A were statistically analysed (n=5). (C) A549 tumour cells were incubated with 10 μg/ml EXO_{Ctrl} or $EXO_{PM2.5}$ for 24 h, and cells were then plated in the top chamber, which was precoated with 50 μl of Matrigel. After 48 h, the cells on the bottom of the Transwell filter were imaged and quantified. Magnification, x100. (D) The results of C were statistically analysed (n=5). Data are representative of three independent experiments. NS, not significant; $EXO_{PM2.5}$, exosomes from $PM_{2.5}$-treated A549 cells; EXO_{Ctrl}, exosomes from A549 cells with mock treatment.

protein were obtained from $PM_{2.5}$-treated A549 cells (Fig. 4B). Exosomes from $PM_{2.5}$-treated A549 cells transfected with NC siRNA ($EXO_{PM2.5}$/NC siRNA) promoted A549 cell proliferation (Fig. 4C). However, exosomes from $PM_{2.5}$-treated A549

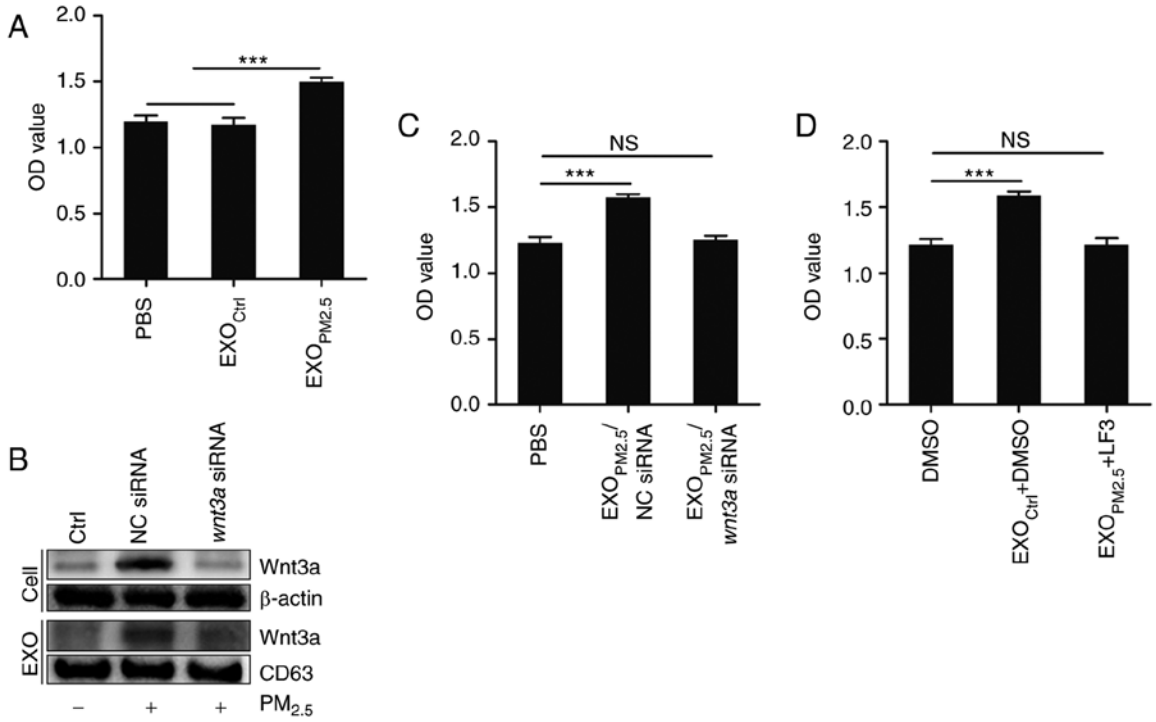

Figure 4. $EXO_{PM2.5}$ promotes A549 cell proliferation in a Wnt3a/β-catenin-dependent manner. (A) A549 cells ($2.5x10^5$/ml in density) were treated with 10 μg/ml EXO_{Ctrl} or $EXO_{PM2.5}$ for 24 h, and A549 cell proliferation was then assessed using a CCK-8 assay. (B) A549 cells ($2.5x10^5$/ml in density) were treated with 100 μg/ml $PM_{2.5}$. Concurrently, cells were transfected with NC or *Wnt3a* siRNA. After 24 h, Wnt3a protein in A549 cells or cell-derived exosomes was detected by western blotting. (C and D) A549 cells ($2.5x10^5$/ml in density) were treated with (C) 10 μg/ml $EXO_{PM2.5}$/NC siRNA or $EXO_{PM2.5}$/*Wnt3a* siRNA, or (D) cells were treated with 10 μg/ml $EXO_{PM2.5}$ in the presence of 10 μM LF3. After 24 h, A549 cell proliferation was assessed using a CCK-8 assay. Data are representative of three independent experiments. ***P<0.001. NS, not significant; $EXO_{PM2.5}$, exosomes from $PM_{2.5}$-treated A549 cells; EXO_{Ctrl}, exosomes from A549 cells with mock treatment.

cells transfected with *Wnt3a* siRNA ($EXO_{PM2.5}$/*Wnt3a* siRNA) had no effect on A549 cell proliferation (Fig. 4C). In the presence of LF3, a specific inhibitor of Wnt/β-catenin signalling, $EXO_{PM2.5}$ did not promote A549 cell proliferation (Fig. 4D). These results demonstrated that the effect of $EXO_{PM2.5}$ on the enhanced A549 cell proliferation was dependent on Wnt3a/β-catenin signalling.

$EXO_{PM2.5}$ promotes A549 cell growth in vivo via Wnt3a. Finally, the effect of $EXO_{PM2.5}$ on A549 cell progression *in vivo* was assessed. An A549 tumour model in nude mice was established, and intratumoural injection of $EXO_{PM2.5}$ was performed every other day. $EXO_{PM2.5}$ increased A549 cell growth (Fig. 5A) and reduced the survival rate of tumour-bearing mice (Fig. 5B). Immunohistochemical staining of Ki-67 revealed that $EXO_{PM2.5}$ significantly promoted tumour cell proliferation (Fig. 5C and D). To dissect the role of Wnt3a in $EXO_{PM2.5}$ in this process, intratumoural injection of $EXO_{PM2.5}$/NC siRNA or $EXO_{PM2.5}$/*Wnt3a* siRNA was performed every other day. $EXO_{PM2.5}$/NC siRNA, but not $EXO_{PM2.5}$/*Wnt3a* siRNA, increased tumour growth and reduced the survival rate of tumour-bearing mice (Fig. 5E and F). Immunohistochemical results also revealed that $EXO_{PM2.5}$/*Wnt3a* siRNA did not promote tumour cell proliferation (Fig. 5G and H). In the survival study, when the largest tumour volume reached 4,000 mm^3, for humanitarian reasons, the mice were sacrificed. However, in Fig. 5B, compared with EXO_{Ctrl} or PBS-treated mice, the tumour volume of $EXO_{PM2.5}$-treated mice reached 4,000 mm^3 much earlier. Similarly, in Fig. 5F, compared with $EXO_{PM2.5}$/*Wnt3a* siRNA or PBS-treated mice, the tumour volume of $EXO_{PM2.5}$/NC siRNA-treated mice reached 4,000 mm^3 much earlier. Therefore, these results reflected the real tendency of survival time of tumour mice with different treatments. Altogether, these results indicated that $EXO_{PM2.5}$-induced tumour growth *in vivo* was dependent on Wnt3a.

Discussion

Since humans are required to breathe in air, the $PM_{2.5}$ pollution in atmosphere directly affects the physiological environment of the respiratory tract, especially the lungs. $PM_{2.5}$ is implicated in increased risk of lung cancer (35). However, there is no direct evidence of the effect of $PM_{2.5}$ exposure on lung cancer cells. The present study revealed that $PM_{2.5}$-treated A549 lung cancer cells produced exosomes containing high levels of Wnt3a, which promoted A549 cell proliferation by activating Wnt/β-catenin signalling. We also detected Wnt3a by flow cytometry after adsorbing exosomes onto latex, but we did not detect Wnt3a this way (data not shown) which suggests that Wnt3a does not exist in exosomes. IL-10 and TGF-β1 are in exosomes, where they can exert immunosuppressive functions via the expression of their receptors on the cell membrane (36,37). Therefore, even if Wnt3a is in exosomes, it still can activate β-catenin signalling. In tumour patients who inhale $PM_{2.5}$, $PM_{2.5}$ stimulates lung epithelial cells. $PM_{2.5}$

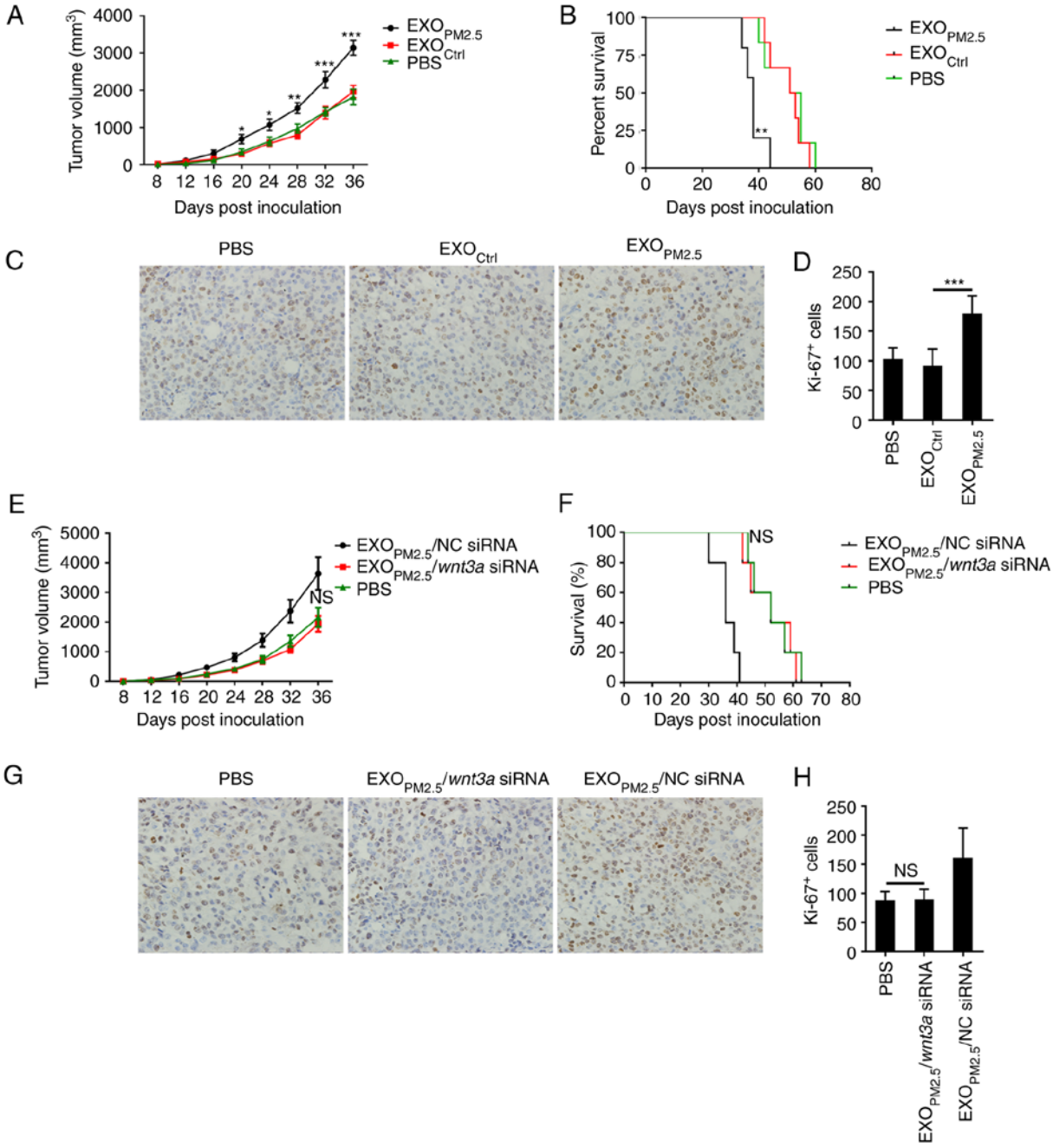

Figure 5. $EXO_{PM2.5}$ promotes A549 cell growth *in vivo* via Wnt3a. (A-H) A549 cells ($5x10^6$) were subcutaneously injected into nude mice on day 0. On day 10, mice were randomly divided into three groups (n=5) and (A and B) received an intratumoural injection of 5 μg of EXO_{Ctrl} or $EXO_{PM2.5}$ or (E and F) received an intratumoural injection of 5 μg $EXO_{PM2.5}$/NC siRNA or $EXO_{PM2.5}$/*Wnt3a* siRNA every other day. (A and E) The tumour size was measured every four days and (B and F) the survival rate of mice was analysed. (C, D, G and H) On day 30, the mice were sacrificed, and the tumour tissues were subjected to immunohistochemical staining using Ki-67. (C and G) Representative images are shown. Magnification, x200. (D and H) Ki-67$^+$ cells were statistically analysed. Data are representative of two independent experiments. *P<0.05; **P<0.01; ***P<0.001 vs. EXO_{Ctrl} in A and B; NS vs. $EXO_{PM2.5}$/*Wnt3a* siRNA in E and F. NS, Not significant; $EXO_{PM2.5}$, exosomes from $PM_{2.5}$-treated A549 cells; EXO_{Ctrl}, exosomes from A549 cells with mock treatment.

may also induce lung epithelial cells to secrete exosomes containing Wnt3a, which activates Wnt/β-catenin signalling in tumour cells, leading to tumour progression.

In developed countries, the concentration of $PM_{2.5}$ in the atmosphere is generally less than 10 $\mu g/m^3$, and in developing countries it is generally above 35 $\mu g/m^3$, and the highest is likely to reach 200-300 $\mu g/m^3$. An adult breathes more than 20,000 times a day, inhaling ~20 m^3 of air. Therefore, an adult inhales at least 200 μg/day of $PM_{2.5}$. The concentration we used was 100 μg/ml in 2 ml and the quality of $PM_{2.5}$ was 200 μg. Thus, in humans, it is likely to be exposed to such a concentration. Notably, at a concentration of 100 μg/ml, we did not observe increased apoptosis of A549 cells (data not shown). On the contrary, $PM_{2.5}$ promoted lung tumor cell proliferation by inducing the cells to secrete exosomes with high levels of Wnt3a. With $PM_{2.5}$ treatment, A549 cells notably upregulated Wnt3a expression. The mechanism of the effect of $PM_{2.5}$ was not investigated in this study. Environmental ultrafine particulate matter has been reported to activate NF-κB and AP-1 (7). Wnt10a and Wnt10b are the target genes

of NF-κB (38). Bioinformatics analysis predicted binding sites of NF-κB and AP-1 in the *Wnt3a* promoter, indicating that $PM_{2.5}$ may promote *Wnt3a* transcription by activating NF-κB and AP-1. If the mechanism is unveiled in the future, it will be beneficial to identify the specific target to prevent lung cancer progression caused by $PM_{2.5}$ exposure. Wnt signalling through its receptors (Frizzled) activate β-catenin signalling, which is often called the canonical pathway (39). As a ligand of the canonical pathway, the downstream effector of Wnt3a in $EXO_{PM2.5}$ is β-catenin, which was supported by the increased protein level and nuclear translocation of β-catenin in $EXO_{PM2.5}$-treated A549 cells. Use of the LF3 inhibitor confirmed that Wnt3a contained in $EXO_{PM2.5}$ promoted A549 cell proliferation through activation of β-catenin signalling *in vitro*. However, the role of β-catenin in $EXO_{PM2.5}$-mediated tumour inhibition was not ascertained *in vivo*, but the findings did indicate that all of the effects were Wnt3a-dependent.

The present study demonstrated that $EXO_{PM2.5}$ significantly promoted A549 cell proliferation *in vitro*. Exosomes isolated from Wnt3a knockdown $EXO_{PM2.5}$-treated A549 cells had extremely low levels of Wnt3a and did not induce A549 cell proliferation *in vitro*. These results indicated that Wnt3a was responsible for $EXO_{PM2.5}$-mediated A549 cell proliferation *in vitro*. Inhibition of β-catenin signalling in A549 cells prevented $EXO_{PM2.5}$-induced A549 cell proliferation *in vitro*, indicating that Wnt3a contained in $EXO_{PM2.5}$ activated β-catenin signalling in A549 cells. The mouse tumour model revealed that $EXO_{PM2.5}$ promoted A549 cell growth and decreased the survival rate of tumour-bearing mice. In the mouse tumour model, $EXO_{PM2.5}$ did promote A549 cell proliferation. The Wnt/β-catenin pathway was also involved in tumour cell migration and invasion by mediating epithelial-mesenchymal transition of tumour cells (40). However, the migration and invasion promoting effect of $EXO_{PM2.5}$ on A459 cells *in vitro* could not be observed.

In summary, $PM_{2.5}$ exposure induced high expression of Wnt3a in A549 lung cancer cells. Isolated exosomes with a high level of Wnt3a activated β-catenin signalling in A549 cells and promoted their proliferation *in vitro*. Furthermore, these exosomes also promoted tumour progression *in vivo*. Therefore, these results indicated that inhibition of the Wnt/β-catenin pathway or exosome secretion may prevent $PM_{2.5}$-mediated lung cancer progression.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Zhejiang Medicines Health Science and Technology Program (no. 2016KYB193), the Key Research Project of Shandong Province (nos. 2016 GSF201028 and 2017GSF218056) and the National Natural Science Foundation of China (no. 81770029).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HX, XJ and YW performed the real-time PCR, the immunofluorescent staining, migration, invasion, cell proliferation and the animal experiments. SL and LC performed the cell culture, the exosome isolation and the western blotting experiments. LD conceived and designed the study. HX wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experiments using mice were approved by and performed according to the guidelines of the Animal Ethics Committee of Wenzhou Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Huang L, Pu Z, Li M and Sundell J: Characterizing the indoor-outdoor relationship of fine particulate matter in non-heating season for urban residences in Beijing. PLoS One 10: e0138559, 2015.
2. Ho SM: Environmental epigenetics of asthma: An update. J Allergy Clin Immunol 126: 453-465, 2010.
3. Honda T, Pun VC, Manjourides J and Suh H: Anemia prevalence and hemoglobin levels are associated with long-term exposure to air pollution in an older population. Environ Int 101: 125-132, 2017.
4. Zanobetti A and Schwartz J: The effect of fine and coarse particulate air pollution on mortality: A national analysis. Environ Health Perspect 117: 898-903, 2009.
5. Mirabelli MC, Vaidyanathan A, Flanders WD, Qin X and Garbe P: Outdoor PM2.5, ambient air temperature, and asthma symptoms in the past 14 days among adults with active asthma. Environ Health Perspect 124: 1882-1890, 2016.
6. Song J, Kang J, Lin B, Li J, Zhu Y, Du J, Yang X, Xi Z and Li R: Mediating role of TRPV1 Ion channels in the co-exposure to PM2.5 and formaldehyde of Balb/c mice asthma model. Sci Rep 7: 11926, 2017.
7. Chen ZH, Wu YF, Wang PL, Wu YP, Li ZY, Zhao Y, Zhou JS, Zhu C, Cao C, Mao YY, *et al*: Autophagy is essential for ultrafine particle-induced inflammation and mucus hyperproduction in airway epithelium. Autophagy 12: 297-311, 2016.
8. Ye X, Hong W, Hao B, Peng G, Huang L, Zhao Z, Zhou Y, Zheng M, Li C, Liang C, *et al*: PM2.5 promotes human bronchial smooth muscle cell migration via the sonic hedgehog signaling pathway. Resp Res 19: 37, 2018.
9. Du X, Jiang S, Zeng X, Zhang J, Pan K, Zhou J, Xie Y, Kan H, Song W, Sun Q and Zhao J: Air pollution is associated with the development of atherosclerosis via the cooperation of CD36 and NLRP3 inflammasome in $ApoE^{-/-}$ mice. Toxicol Lett 290: 123-132, 2018.
10. Andersen ZJ, Stafoggia M, Weinmayr G, Pedersen M, Galassi C, Jørgensen JT, Oudin A, Forsberg B, Olsson D, Oftedal B, *et al*: Long-term exposure to ambient air pollution and incidence of postmenopausal breast cancer in 15 European cohorts within the ESCAPE project. Environ Health Perspect 125: 107005, 2017.
11. VoPham T, Bertrand KA, Tamimi RM, Laden F and Hart JE: Ambient $PM_{2.5}$ air pollution exposure and hepatocellular carcinoma incidence in the United States. Cancer Causes Control 29: 563-572, 2018.

12. Liao Y, Xu L, Lin X and Hao YT: Temporal trend in lung cancer burden attributed to qmbient fine particulate matter in Guangzhou, China. Biomed Environ Sci 30: 708-717, 2017.
13. Yang B, Chen DM, Zhao H and Xiao CL: The effects for PM2.5 exposure on non-small-cell lung cancer induced motility and proliferation. Springerplus 5: 2059, 2016.
14. Yang D, Ma MY, Zhou WC, Yang BA and Xiao CL: Inhibition of miR-32 activity promoted EMT induced by PM2.5 exposure through the modulation of the Smad1-mediated signaling pathways in lung cancer cells. Chemosphere 184: 289-298, 2017.
15. Wei H, Liang F, Cheng W, Zhou R, Wu X, Feng Y and Wang Y: The mechanisms for lung cancer risk of PM2.5: Induction of epithelial-mesenchymal transition and cancer stem cell properties in human non-small cell lung cancer cells. Environ Toxicol 32: 2341-2351, 2017.
16. Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, Dingli F, Loew D, Tkach M and Théry C: Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. Proc Natl Acad Sci USA 113: E968-E977, 2016.
17. Sansone P, Savini C, Kurelac I, Chang Q, Amato LB, Strillacci A, Stepanova A, Iommarini L, Mastroleo C, Daly L, *et al*: Packaging and transfer of mitochondrial DNA via exosomes regulate escape from dormancy in hormonal therapy-resistant breast cancer. Proc Natl Acad Sci USA 114: E9066-E9075, 2017.
18. Tkach M and Théry C: Communication by extracellular vesicles: Where we are and where we need to go. Cell 164: 1226-1232, 2016.
19. Shen Y, Guo D, Weng L, Wang S, Ma Z, Yang Y, Wang P, Wang J and Cai Z: Tumor-derived exosomes educate dendritic cells to promote tumor metastasis via HSP72/HSP105-TLR2/TLR4 pathway. Oncoimmunology 6: e1362527, 2017.
20. Li Z, Jiang P, Li J, Peng M, Zhao X, Zhang X, Chen K, Zhang Y, Liu H, Gan L, *et al*: Tumor-derived exosomal lnc-Sox2ot promotes EMT and stemness by acting as a ceRNA in pancreatic ductal adenocarcinoma. Oncogene 37: 3822-3838, 2018.
21. Liu Y, Gu Y, Han Y, Zhang Q, Jiang Z, Zhang X, Huang B, Xu X, Zheng J and Cao X: Tumor exosomal RNAs promote lung pre-metastatic niche formation by activating alveolar epithelial TLR3 to recruit neutrophils. Cancer Cell 30: 243-256, 2016.
22. Chen Y, Zeng C, Zhan Y, Wang H, Jiang X and Li W: Aberrant low expression of p85α in stromal fibroblasts promotes breast cancer cell metastasis through exosome-mediated paracrine Wnt10b. Oncogene 36: 4692-4705, 2017.
23. Cong LH, Du SY, Wu YN, Liu Y, Li T, Wang H, Li G and Duan J: Upregulation of Klotho potentially inhibits pulmonary vascular remodeling by blocking the activation of the Wnt signaling pathway in rats with PM2.5-induced pulmonary arterial hypertension. J Cell Biochem 119: 5581-5597, 2018.
24. Fang L, Zhu Q, Neuenschwander M, Specker E, Wulf-Goldenberg A, Weis WI, von Kries JP and Birchmeier W: A small-molecule antagonist of the β-catenin/TCF4 interaction blocks the self-renewal of cancer stem cells and suppresses tumorigenesis. Cancer Res 76: 891-901, 2016.
25. Wang GJ, Liu Y, Qin A, Shah SV, Deng ZB, Xiang X, Cheng Z, Liu C, Wang J, Zhang L, *et al*: Thymus exosomes-like particles induce regulatory T cells. J Immunol 181: 5242-5248, 2008.
26. Cadigan KM and Nusse R: Wnt signaling: A common theme in animal development. Genes Dev 11: 3286-3305, 1997.
27. Amit S, Hatzubai A, Birman Y, Andersen JS, Ben-Shushan E, Mann M, Ben-Neriah Y and Alkalay I: Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: A molecular switch for the Wnt pathway. Genes Dev 16: 1066-1076, 2002.
28. Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X and He X: Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 108: 837-847, 2002.
29. Yost C, Torres M, Miller JR, Huang E, Kimelman D and Moon RT: The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. Genes Dev 10: 1443-1454, 1996.
30. MacDonald BT, Tamai K and He X: Wnt/beta-catenin signaling: Components, mechanisms, and diseases. Dev Cell 17: 9-26, 2009.
31. Sha J, Han Q, Chi C, Zhu Y, Pan J, Dong B, Huang Y, Xia W and Xue W: PRKAR2B promotes prostate cancer metastasis by activating Wnt/β-catenin and inducing epithelial-mesenchymal transition. J Cell Biochem 119: 7319-7327, 2018.
32. Zhang T, Ma Z, Liu L, Sun J, Tang H, Zhang B, Zou Y and Li H: DDX39 promotes hepatocellular carcinoma growth and metastasis through activating Wnt/beta-catenin pathway. Cell Death Dis 9: 675, 2018.
33. Polakis P: The oncogenic activation of beta-catenin. Curr Opin Genet Dev 9: 15-21, 1999.
34. Sun GL, Li Z, Wang WZ, Chen Z, Zhang L, Li Q, Wei S, Li BW, Xu JH, Chen L, *et al*: miR-324-3p promotes gastric cancer development by activating Smad4-mediated Wnt/beta-catenin signaling pathway. J Gastroenterol 53: 725-739, 2017.
35. Yang H, Li S, Sun L, Zhang X, Hou J and Wang Y: Effects of the ambient fine particulate matter on public awareness of lung cancer risk in China: Evidence from the internet-based big data platform. JMIR Public Health Surveill 3: e64, 2017.
36. Kim SH, Lechman ER, Bianco N, Menon R, Keravala A, Nash J, Mi Z, Watkins SC, Gambotto A and Robbins PD: Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis. J Immunol 174: 6440-6448, 2005.
37. Yu L, Yang F, Jiang L, Chen Y, Wang K, Xu F, Wei Y, Cao X, Wang J and Cai Z: Exosomes with membrane-associated TGF-β1 from gene-modified dendritic cells inhibit murine EAE independently of MHC restriction. Eur J Immunol 43: 2461-2472, 2013.
38. Voutilainen M, Lindfors PH, Lefebvre S, Ahtiainen L, Fliniaux I, Rysti E, Murtoniemi M, Schneider P, Schmidt-Ullrich R and Mikkola ML: Ectodysplasin regulates hormone-independent mammary ductal morphogenesis via NF-κB. Proc Natl Acad Sci USA 109: 5744-5749, 2012.
39. Logan CY and Nusse R: The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20: 781-810, 2004.
40. Gu J, Cui CF, Yang L, Wang L and Jiang XH: Emodin inhibits colon cancer cell invasion and migration by suppressing epithelialmesenchymal transition via the Wnt/β-catenin pathway. Oncol Res: Jan 4, 2018 (Epub ahead of print). doi: 10.3727/096504018 X15150662230295.