ABBS

## Original Article

# Inhibition of lung cancer cell proliferation mediated by human mesenchymal stem cells

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**Human mesenchymal stem cells (hMSCs) are mostly studied for their potential clinical use. Recently, much attention in the field of cancer research has been paid to hMSCs. In this study, we investigated the influence of hMSCs on the proliferation of lung cancer cell lines SK-MES-1 and A549 *in vitro* and *in vivo* by using a co-culture system and the hMSCs-conditioned medium. Our results demonstrated that hMSCs could inhibit the proliferation of SK-MES-1 and A549 cells, and induce the apoptosis of tumor cells *in vitro* via some soluble factors. Animal study showed that these soluble factors from hMSCs could suppress tumorigenesis and tumor angiogenesis by treating preliminarily tumor cells with the hMSCs-conditioned medium. The downregulated expression of vascular endothelial growth factor in tumor cells might be the mechanism of interference in tumor angiogenesis, which was verified by western blot analysis and immunohistochemistry assay. Taken together, our results suggested that the hMSCs could inhibit tumor cell growth by secreting some soluble factors.**

*Keywords*    mesenchymal stem cell; lung cancer; proliferation; vascular endothelial growth factor

## Introduction

Mesenchymal stem cells (MSCs), a rare non-hematopoietic population in the adult bone marrow (BM), could self-renew and differentiate into mesodermal cell lineages, and support hematopoiesis [1,2]. In addition, there was some evidence to show that MSCs have a potent immunosuppressive ability by inhibiting T-lymphocyte proliferation and can suppress graft-versus-host disease [3,4]. Due to these properties, human MSC (hMSC) has been considered as an attractive target as a cell therapeutic agent for endogenous organ and tissue repair [5,6].

Recently, more and more attention has been paid to the relationship between hMSCs and tumor. But there were many inconsistent results about the effects of hMSCs on tumors. For example, some studies showed that hMSCs exerted an antitumorigenic effect in the Kaposis sarcoma and hepatoma animal models [7,8]. However, other studies indicated that hMSCs could promote tumor growth in the tumor microenvironment [9]. Although these results were different, there was evidence to show that hMSCs-induced immunosuppressive and antitumorigenic effects were mediated by the soluble factors released from hMSCs [8,10,11].

To further investigate the interaction of hMSCs with tumors and the related molecular mechanism, we examined the proliferative ability of lung cancer cell lines SK-MES-1 and A549 *in vitro* and *in vivo* by using a co-culture system and the hMSCs-conditioned medium.

## Materials and Methods

### Cells and cell culture

hMSCs (Cyagen, Chicago, USA) were derived from BM of healthy adults and cultured in human mesenchymal stem cell growth medium (hMSC-GM) supplemented with 10% fetal calf serum (Cyagen). hMSCs before the sixth subculture were used in the experiments. Human lung cancer cell lines SK-MES-1 (squamous carcinoma cells) and A549 (adenocarcinoma cells), from Institute of Biochemistry and Cell Biology (Shanghai, China), were cultured in RPMI 1640 supplemented 10% fetal bovine serum (FBS; GIBCO, Gaithersburg, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. All cells were incubated in an incubator at 37°C with 5% $CO_2$.

### Preparation of hMSCs-conditioned medium

When hMSCs were 80–90% confluent in the culture flask, the medium was collected, filtered through a 0.2 µm filter, and stored at −80°C as hMSCs-conditioned medium. hMSC-GM (without FBS) was used as a control.

### Co-cultures of hMSCs and tumor cells

Co-culture systems were established by using transwell using 6-well plates (0.4 µm pore, polycarbonate

membrane; Costar, Cambridge, USA). SK-MES-1 or A549 cell suspensions (1 ml, $5 \times 10^5$ cells) were loaded in the upper inserts, and hMSCs cell suspensions (2 ml, $5 \times 10^5$ cells) were put into the lower compartment of the culture well. hMSC-GM (2 ml) without FBS was used as a control in the lower compartment of well. Each group had three wells. The number of tumor cells in the inserts of the co-culture systems was counted under a microscope after being incubated for 48 h. The cell number in every insert was counted for three times, and the results were expressed as the mean value.

## MTT assay

SK-MES-1 and A549 cells were inoculated in the tumor cell culture medium supplemented with 50% hMSCs-conditioned medium using the 96-well plates at a density of $5 \times 10^3$ cells per well, and the control cells were grown in the tumor cell culture medium supplemented with 50% hMSC-GM without FBS. After 24, 48, 72, 96, and 120 h of incubation, 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma, St. Louis, USA) was added (50 μg/well) and incubated for 4 h. Formazan products were solubilized with DMSO, and the optical density was measured at 490 nm to draw cell growth curves.

## Flow cytometry analysis

Lung cancer cells were cultured in the mixture of hMSCs-conditioned medium and RPMI 1640 medium (1:1) supplemented with 2% FBS, which favored apoptosis, and the control cells were cultured in the mixture of RPMI 1640 medium supplemented with 2% FBS and FBS-free hMSC-GM (1:1). Tumor cells were cultured for 48 h and then collected by digesting with 0.25% trypsin without ethylene diamine tetraacetic acid (EDTA) for apoptosis analysis using annexin V-FITC cell apoptosis detection kit (KeyGEN, Nanjing, China). Cell apoptosis was analyzed by flow cytometry (FACSCalibur; BD Pharmingen, Franklin Lakes, USA). Data were analyzed by the software WinMDI2.9 (Phoenix, USA).

## Assay of caspase-3 activity

Cellular caspase-3 activity was determined by using colorimetric assay kit (Beyotime Institute of Biotechnology, Haimen, China). First, SK-MES-1 or A549 cells were cultured in the mixture of RPMI 1640 medium supplemented with 2% FBS and hMSCs-conditioned medium (1:1); and cells cultured in the mixture of RPMI 1640 medium supplemented with 2% FBS and hMSC-GM without FBS (1:1) were used as controls. After incubation for 48 h, cells were treated according to the manufacturer's instructions. Cell lysates were prepared and assays were performed in 96-well plates by incubating 10 μl of cell lysate per sample in 80 μl reaction buffer (1% NP-40, 20 mM Tris–HCl,

137 mM NAD and 10% glycerol) containing 10 μl of caspase-3 substrate (Ac-DEVD-pNA). Lysates were incubated at 37°C for 4 h. An absorbance at 405 nm was read with the Microplate Reader (Rayto, USA). Caspase-3 activities were expressed as the percentage of enzyme activity compared with the control. All experiments were carried out in triplicate.

## Animal experiments

Six-week-old female BALB/c nude mice were obtained from the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). All procedures were carried out in accordance with the advice and permission of the Institutional Ethical Committee of Shandong University. Lung cancer cell lines SK-MES-1 and A549 were firstly treated with the hMSCs-conditioned medium before injection into the mice. After being cultured in the mixture of RPMI 1640 medium supplemented with 10% FBS and hMSCs-conditioned medium (1:1) for 24 h, tumor cells were collected and prepared as single-cell type suspensions ($5 \times 10^6$ cells in 0.1 ml PBS). In the control groups, hMSC-GM replaced the hMSCs-conditioned medium. Cells were injected subcutaneously at the same site of the back of nude mice. Mice were examined once every 5 days and tumor growth was evaluated by measuring the length and width of tumor. Finally, mice were killed and tumor masses were removed and fixed in 10% neutral buffered formalin solution for histological preparations.

## Immunohistochemistry

All tumor masses fixed in 10% neutral formalin were embedded in paraffin at 55°C, and cut into parallel consecutive 4 μm thick sections for the subsequent immunohistochemical study. Briefly, the endogenous peroxidase activity was blocked by using 0.1% of $H_2O_2$ in methanol for 20 min. The sections were permeabilized with EDTA buffer solution (pH 9.0) for 15 min with microwave. Then, the sheep serum was incubated for 30 min to prevent unspecific adherence followed by treatment with primary antibody [mouse anti-vascular endothelial growth factor (VEGF) diluted for 1:100; Zhongshan Biotechnology Co., Beijing, China] at 37°C for 2 h. After being washed by PBS, sections were then incubated with biotin labeled secondary antibody (HRP-conjugated goat antimouse IgG; Zhongshan Biotechnology Co.) diluted for 1:100 at 37°C for 30 min. Then the sections were washed with PBS again. Peroxidase-conjugated streptavidin was added for 20 min and then washed with PBS. Finally, sections were developed with 3,3-diaminobenzidine and hydrogen peroxide and counterstained with hematoxylin. For the negative control, PBS was used instead of primary antibody. The sections were analyzed by light microscopy.

## Western blot analysis

After being cultured in RPMI 1640 medium alone or in the mixture containing hMSCs-conditioned medium (1:1) for 24 h, SK-MES-1 and A549 cells were lysed in lysis buffer at 4°C for 30 min. The lysates were centrifuged at 14,000 $g$ at 4°C for 20 min. The supernatants were collected and stored at −80°C. Protein concentrations were determined by the BCA method. Total protein (50 μg) was separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, USA). After being blocked with 4% skim milk in Tris-buffered saline, the membrane was incubated overnight at 4°C with primary antibody against VEGF or β-actin at a dilution of 1:200 (Santa Cruz, Santa Cruz, USA) for 2 h at room temperature. Then the membrane was incubated with a peroxidase-conjugated secondary antibody for 1 h at room temperature. Signals were detected with ECL. Optical band density was quantified (Imager of Alpha Corporation, San Leandro, USA) and the VEGF values were normalized to β-actin.

## Statistical analysis

SPSS 15.0 software was used for all statistical analysis. Statistical significance was assessed by comparing mean values (means ± SD). The significance level was set at $P < 0.05$.

# Results

## Lung cancer cell proliferation is inhibited by the soluble factors from hMSCs *in vitro*

In the co-culture systems, the numbers of SK-MES-1 and A549 cells co-cultured with hMSCs for 48 h were $(8.28 \pm 0.33) \times 10^5$ and $(9.05 \pm 0.29) \times 10^5$, respectively. However, the numbers of SK-MES-1 and A549 cells of control groups were $(12.20 \pm 0.32) \times 10^5$ and $(12.90 \pm 0.41) \times 10^5$, respectively. Compared with control groups, the hMSCs-induced inhibitory effects on tumor cell proliferation were conspicuous [$P < 0.05$; **Fig. 1(A)**]. Besides, MTT assay also showed the obvious inhibiting effects of SK-MES-1 and A549 cell proliferation treated with the hMSCs-conditioned medium [**Fig. 1(B)**]. Taking together, these results suggested that hMSCs could inhibit the tumor cell proliferation *in vitro* via secreting some soluble factors, which had an inhibitory effect on tumor cells growth.

## hMSCs-conditioned medium promotes the lung cancer cell apoptosis *in vitro*

To investigate the effect of the soluble factors in the hMSCs-conditioned medium on the apoptosis of lung cancer cells, we tested the apoptotic ratios of SK-MES-1 and A549 cells treated with the hMSCs-conditioned medium by flow cytometric analysis. Our results showed
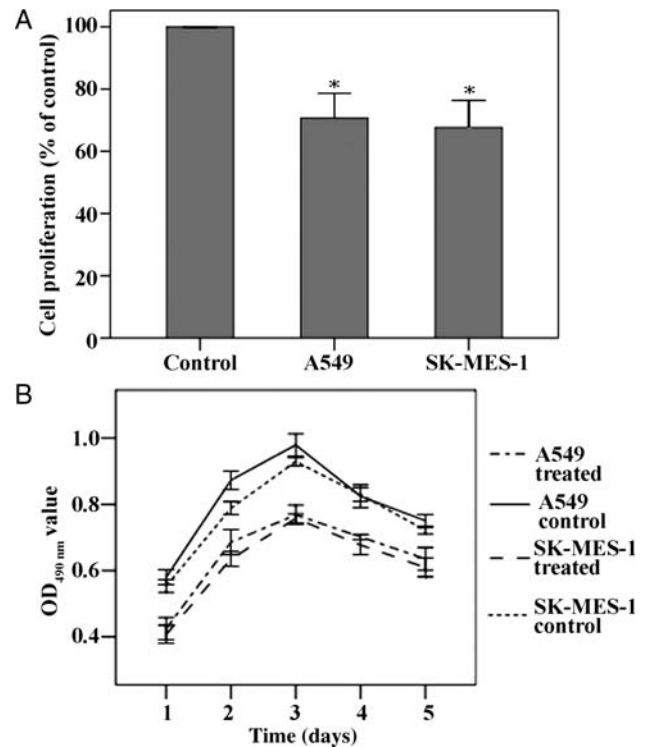


**Figure 1 The inhibitory effects of hMSCs on the lung cancer cell proliferation *in vitro*** (A) hMSCs inhibited lung cancer cell proliferation detected by transwell co-culture systems. SK-MES-1 and A549 cells were co-cultured with hMSCs for 48 h, and then cells were harvested for counting. hMSC-GM (without hMSCs) in the co-culture systems was used as a control. The number of cells is represented as the mean ± SD of three independent experiments. Results are shown as percentage of cell number compared with the number of control cells (*$P < 0.05$). (B) The suppressive effect of the hMSCs-conditioned medium on proliferation of SK-MES-1 and A549 cells by MTT assay. The two lower growth curves represented the proliferating activity of SK-MES-1 and A549 cells, respectively, treated with hMSCs-conditioned medium; the superior two were the growth curves of control groups. The absorbance was measured everyday in a period of 5 days.

that the apoptotic ratios significantly increased from $(6.64 \pm 0.75\%)$ and $(5.32 \pm 0.96\%)$ to $(17.79 \pm 0.36\%)$ and $(15.71 \pm 0.82\%)$, respectively, when tumor cells were cultured in the mixture of RPMI 1640 medium and hMSCs-conditioned medium [$P < 0.01$; **Fig. 2(A)**]. Since the activation of caspase-3 proteases was crucial in apoptotic cell death [12], we also detected the caspase-3 activity in tumor cells treated with the hMSCs-conditioned medium. Results showed that there was a more than 2-fold increase in caspase-3 activity in the treated cells compared with controls [$P < 0.01$; **Fig. 2(B)**].

## Soluble factors from hMSCs suppress the lung cancer cell growth *in vivo*

To investigate the effect of soluble factors from hMSCs on lung cancer cell growth *in vivo*, we assessed the growth of SK-MES-1 and A549 cells treated with the
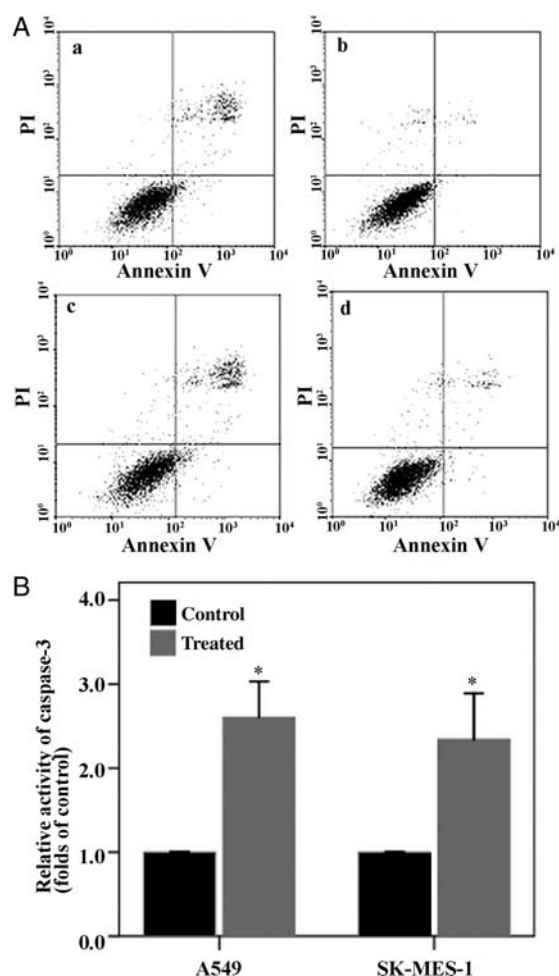
**Figure 2 hMSCs promote the lung cancer cell apoptosis *in vitro*** (A) Flow cytometric analysis showed the apoptosis of A549 (a and b) and SK-MES-1 (c and d) cells cultured in the presence (a and c) or absence (b and d) of hMSCs-conditioned medium. The figures are representative of three experiments. (B) The relative caspase-3 activity in A549 and SK-MES-1 cells treated with the hMSCs-conditioned medium for 48 h compared with untreated controls. The results were expressed as the mean $\pm$ SD of three experiments (*$P < 0.01$).

hMSCs-conditioned medium in BALB/c nude mice. Results showed that the mice ($n = 10$) injected with tumor cells treated with the hMSCs-conditioned medium had a lower tumor incidence than that of control groups ($n = 10$) [**Fig. 3(A)**]. On the 30th day after injection, the mean volume of tumors of the mice injected with tumor cells treated with the hMSCs-conditioned medium was dramatically lower than that of control groups [$P < 0.01$; **Fig. 3(B)**].

### VEGF expression is downregulated by the hMSCs-conditioned medium

We found that there was less vessel distribution on the surface of tumor mass of the mice injected with treated tumor cells than that of controls on the 30th day after injection [**Fig. 4(A)**]. In order to explore the molecular mechanism underlying the remodeling of tumor vasculature, we examined the expression of VEGF in the tumor tissue by
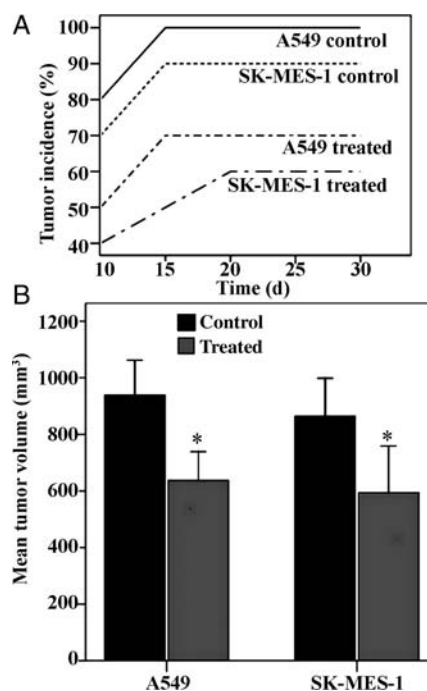
**Figure 3 Soluble factors from hMSCs suppress tumor formation and growth in lung cancer models** (A) SK-MES-1 and A549 cells treated or untreated with the hMSCs-conditioned medium were subcutaneously injected into the mice ($n = 10$). Tumor incidence of mice injected with treated tumor cells was lower than untreated controls. (B) The mean ($\pm$ SD) tumor volume of mice was calculated on the 30th day after injection. Tumor volume (mm$^3$) = $1/2 \times$ length $\times$ width$^2$. Results showed that tumor volume of mice injected with treated tumor cells was smaller than that of control groups (*$P < 0.01$).

immunohistochemistry. Our results showed that VEGF expression in the tumor of the mice injected with treated tumor cells decreased obviously compared with that of controls [**Fig. 4(B)**].

In addition, we investigated the effect of soluble factors from hMSCs on VEGF expression in tumor cells *in vitro* by western blot analysis. Results showed that VEGF expression in lung cancer cells treated with the hMSCs-conditioned medium also decreased dramatically compared with control groups [$P < 0.01$; **Fig.4(C,D)**].

## Discussion

Tumor growth is dependent on tumor angiogenesis that results from remodeling of preexisting vasculature in host tissues surrounding the growing tumor mass, and tumor cells play an important role in the molecular mechanisms underlying the remodeling of host vasculature [13–15]. For example, tumor-derived VEGF has been thought as the most important molecular mechanism to regulate tumor vessel formation [16,17]. And researches have provided many new targets for lung cancer therapy [18]. Recently, more attention has been paid to the interaction between mesenchymal stem cells and tumor cells. Some studies
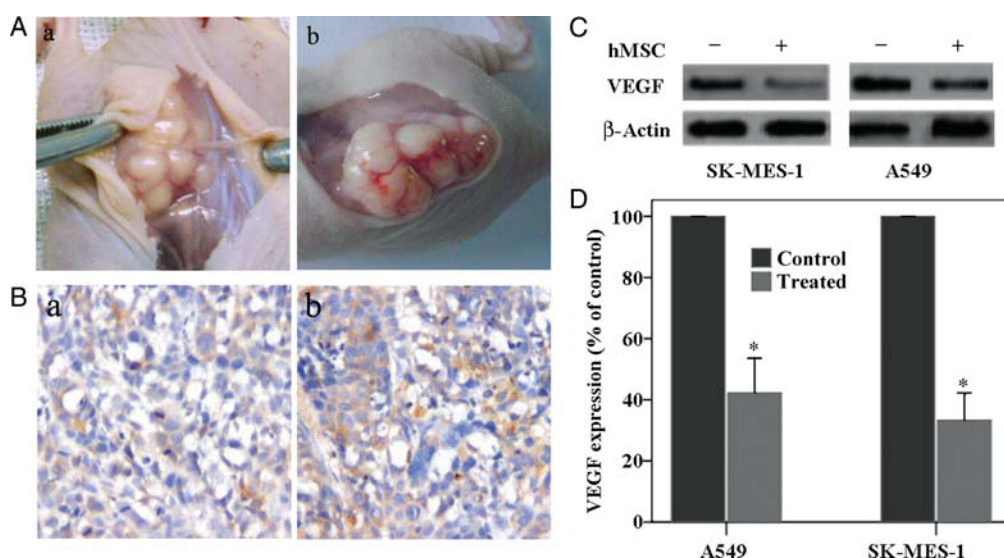
**Figure 4 The decrease of tumor vessel formation and downregulation of VEGF expression mediated by the soluble factors from hMSCs** (A) Vessel distribution in differently treated mice. There is less on the surface of tumor mass of the mice injected with tumor cells treated (a) than that of controls (b). (B) Immunohistochemical staining of VEGF in tumor tissue. These figures were representative ones. Cells treated (a) or untreated (b) with the hMSCs-conditioned medium were shown. Strongly yellow brown stained tumor cells were considered as the positivity of VEGF expression (magnification, ×200). (C) Western blot analysis of VEGF expression. After being treated with hMSCs-conditioned medium for 24 h, SK-MES-1 or A549 cells were lysed and proteins were subjected to SDS-PAGE analysis, transferred to nitrocellulose membranes and incubated with primary antibodies against VEGF or β-actin. β-Actin was used as internal control. (D) Relative expression of VEGF. Results were expressed as the mean ± SD of three independent experiments (*$P < 0.01$).

demonstrated that hMSCs had an inhibitory effect on tumorigenesis [7,8,19]. But others observed that hMSCs favored tumorigenesis and tumor growth through several ways: such as differentiating into tumor stromal fibroblasts, promoting tumor vessel formation and constructing cancer stem cell niches [10,20–23]. All animals were co-injected with tumor cells and hMSCs in the animal experiments of these studies. To prevent hMSCs from differentiating into tumor stromal fibroblasts to favor tumorigenesis *in vivo*, and moreover to investigate the molecular mechanisms underlying the remodeling of host vasculature in tumor cells, we used the hMSCs-conditioned medium as the regulator of tumor cells to investigate the effect of the soluble factors from hMSCs on tumorigenesis *in vivo*.

Our findings demonstrated that the soluble factors from hMSCs were able to suppress tumorigenesis and tumor vessel formation via treating preliminarily tumor cells with the hMSCs-conditioned medium. We observed that BALB/ c mice subcutaneously injected with lung cancer cells treated with the hMSCs-conditioned medium had a much lower tumor incidence, smaller tumor volume and less vessel distribution than control groups. *In vitro*, our results showed the proliferation of lung cancer cells treated with hMSCs-conditioned medium was obviously inhibited compared with the controls. Flow cytometric analysis revealed that lung cancer cells treated with hMSCs-conditioned medium had a much higher apoptosis ratio than the controls. In addition, the expression levels of VEGF in lung cancer cells and in the tumor tissue were downregulated

when these tumor cells were treated with the hMSCs-conditioned medium. These data suggested that hMSCs could really inhibit the lung cancer cell proliferation by the secretion of the soluble factors, which could also interfere in tumor angiogenesis via the downregulation of VEGF expression in tumor cells. Ramasamy *et al*. showed that hMSCs could arrest tumor cells in the G1 phase by downregulating the expression of positive cell cycle regulators, such as cyclin D2, *in vitro* [10]. Qiao *et al*. [8] showed that the soluble factors released from Z3 hMSCs could inhibit tumor cell proliferation via the Wnt signaling pathway. But, the precise factors from hMSCs with these anti-tumor functions remain fairly elusive so far and need to be investigated further.

Lung cancer has been known as the leading worldwide cause of cancer death. The cure rate remains less than 15% despite of improvements in surgery, radiotherapy and chemotherapy [24]. Therefore, new effective and better-tolerated treatment strategies are needed. Our studies suggested that hMSCs could inhibit lung cancer cell growth via secreting the soluble factors, which hint possibly a new therapeutic strategy for lung cancer.

## Funding

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