



Mesenchymal stromal cells can repair radiation-induced pulmonary fibrosis via a DKK-1-mediated Wnt/ β -catenin pathway

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Abstract

Pulmonary injury occurring after thoracic radiotherapy is a main factor limiting the curative effect of radiotherapy. Robust activation of the Wnt signalling pathway induced by ionizing radiation stress plays a critical role in epithelial-mesenchymal transition (EMT) in irradiated type II alveolar epithelial cells and in the proliferation of pulmonary fibroblasts, which contributes to the formation of fibrotic lesions in irradiated lungs. The pathogenesis of radiation-induced pulmonary fibrosis could be restricted by systemic delivery of human adipose-derived mesenchymal stromal cells (Ad-MSCs), as evidenced by the inhibitory effects of Ad-MSCs on EMT in irradiated type II alveolar epithelial cells. The purpose of this study is to observe the effects of mesenchymal stromal cells (MSCs) on repairing fibrosis caused by radiation. We used western blotting and real-time PCR to observe the expression of DKK-1 in MSCs of different origins and passages. After the successful establishment of a radiation-induced lung injury model, we investigated the potency of the supernatant from stromal cells to reduce pro-fibrotic events, including EMT and fibroblast activation. To study the mechanism, we evaluated the levels of active β -catenin, TCF4 and the target genes *Snail*, *Twist* and *c-Myc*. After the injection of Ad-MSCs into mice via the tail vein, proteins related to EMT, fibroblasts and Wnt/ β -catenin signalling were investigated. The TGF- β and IL-10 protein concentrations in peripheral blood were measured by ELISA. Ad-MSC-derived supernatant effectively reversed the decrease in E-cadherin expression and inhibited the increase in vimentin expression induced by ionizing radiation in epithelial cells and suppressed the expression of α -SMA, a mediator of fibroblast proliferation. The canonical Wnt pathway may be activated by irradiation but the nuclear localization of active β -catenin was reduced in the presence of the supernatant from Ad-MSCs. In addition, the expression of target genes involved in EMT was downregulated. Additionally, when DKK-1 in the supernatant was neutralized, all these effects were reversed. Changes in the levels of proteins related to EMT and fibroblast activation, as well as those of active β -catenin and TCF4, were similar in vivo and in vitro. The serum level of the immunosuppressive factor IL-10 was increased after radiation and was further enhanced after Ad-MSC interference for one month. In conclusion, Ad-MSCs medium can contain DKK-1 and inhibit the induction of EMT via Wnt/ β -catenin signalling in vitro and in vivo.

Keywords Radiotherapy · Mesenchymal stromal cells · Epithelial-mesenchymal transition · radiation-induced pulmonary fibrosis

Introduction

Radiotherapy is one of the mainstays of lung cancer treatment. However, pulmonary injury occurring during postthoracic radiotherapy remains unavoidable because epithelial cells and microvascular endothelial cells within alveolar structures are sensitive to ionizing radiation. The pathogenesis of radiation-induced lung injury (RILI) is composed of multiple steps (Huang et al. 2017). Typical lesions include pulmonary edema, interstitial pneumonia and pulmonary fibrosis, which develop in steps. In clinical settings, although doctors try to reduce the pulmonary volume receiving 20-Gy radiation, as V_{20} , RILI

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occurring in some patients is still symptomatic, refractory, or even life-threatening (Wynn et al. 2011). Currently, the standard of care for RILI is alleviation of the symptoms but do not limit the pathogenesis. Several lines of evidence have confirmed the therapeutic potential of mesenchymal stromal cells (MSCs) for RILI such as reducing pneumonitis and inhibiting fibrotic lesion development (Xu et al. 2018). By using the rat model of RILI, we and our colleagues previously reported that endogenous hepatocyte growth factor (HGF) and prostaglandin E2 (PGE2) potentially limited RILI pathogenesis under interference with human adipose-derived MSCs (Ad-MSCs) (Dong et al. 2015). Moreover, in contrast to controls, RILI rats receiving allogenic adipose-derived MSCs exhibited low levels of IL-1, IL-6 and TGF- β in lesioned lungs. However, MSCs infrequently differentiated into functional pulmonary cells because they were rapidly cleared from the lungs within 14 days after infusion. Nevertheless, MSCs can alleviate the progression of later fibrosis by reducing the early inflammatory response.

Recently, the engagement of canonical Wnt signalling in TGF- β -mediated fibrosis was explored. Herein, the stimulation of canonical Wnt by TGF- β was p38-dependent, which enabled fibroblasts to decrease their expression of Dickkopf-1 (DKK-1) if undergoing TGF- β -mediated activation. DKK-1 is a potent antagonist of the canonical Wnt pathway. Functionally, DKK-1 is capable of enhancing the internalization of low-density lipoprotein receptor-related protein 6 (LRP6) on the cell membrane, a mechanism by which LRP6 escapes from activation by Wnt3a-mediated phosphorylation in its intracellular portion, thus inactivating Wnt (Qi et al. 2012; Stewart 2014).

Starting with previous valid evidence, we intend to determine whether Ad-MSCs protect lungs against radiation-induced injury by secreting DKK-1. The results showed that Ad-MSCs were able to produce DKK-1. The supernatant from stromal cells effectively reversed the decrease in E-cadherin expression and inhibited the increase in vimentin expression induced by ionizing radiation in epithelial cells and suppressed the expression of α -SMA, which mediates the activation of fibroblast proliferation. Ad-MSC-CM inhibited the induction of EMT via Wnt/ β -catenin signalling in A549 cells.

Materials and methods

Cell culture and medium collection

BM-MSCs and UC-MSCs (Procell Life Science & Technology Co., Ltd., Wuhan, China) were cultured in complete medium (MSCM, ScienCell, CA, USA). Cells of P5 generation were collected for subsequent detection.

hAd-MSCs (Procell Life Science & Technology Co., Ltd., Wuhan, China) were grown to 80–90% confluence in a T75 culture flask (4×10^6 cells). After the MSCM was removed, the cells were washed three times with phosphate-buffered saline (PBS) to exclude the interference of serum and serum-free DMEM (10 mL) was refilled. The culture was continued for 48 h, because the cells would begin to die by apoptosis if it was too long. The collected medium, termed Ad-MSC-CM, was centrifuged for 10 min at 3000 rpm to remove cell debris and stored at -80°C until use.

A549, BEAS-2B and HLF-1 (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) cells were used to study the effects of radiation and Ad-MSCs on the proliferation and activation of fibroblasts or EMT of epithelial cells. Approximately 400 pg/mL of recombinant human TGF- β (Andarawewa et al. 2007) (PeproTech, USA) was added after cells were irradiated with 6 Gy (1.0 Gy/min, 300 kV, 7.86 mA) to establish the fibrotic cell models. Ad-MSC-CMs were immediately used to replace the complete medium after cells were irradiated to study the effects of Ad-MSCs.

Animal model of RILI

Six-week-old male BALB/c mice weighing 20 ± 1 g were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were randomly divided into four groups ($n = 6$ in each group) of two time points each: PBS transplanted with the sham group, CM transplanted with the sham group, PBS transplanted with the irradiation group and CM transplanted with the irradiation group. The mice were intraperitoneally anaesthetized with 100 μL of 0.3% chloral hydrate. A single fraction of 20 Gy (1.0 Gy/min, 300 kV, 7.86 mA) was administered to the chest by using an X-RAD 320 Biological Irradiator (Stone Mountain, GA, USA). The mice were injected with Ad-MSC-CM or PBS at 1-month postirradiation via the tail vein and were sacrificed after another 1 or 6 months. All animal experiments were performed in accordance with the institutional guidelines of Jilin University (Changchun, China) for the Care and Use of Laboratory Animals and this study was approved by the Ethics Committee of First Hospital of Jilin University.

PCR

Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and reverse transcribed to generate cDNA by PrimeScript RT-PCR kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. The thermocycling conditions were as follows: 95°C for 5 min, 40 cycles of 95°C for 10 s followed by 56°C for 30 s. GAPDH were used as

internal controls. All samples were normalized to the internal controls and fold changes were calculated through relative quantification ($2^{-\Delta\Delta C_q}$).

ELISA

The levels of cytokines, including IL-10 and TGF- β 1, were measured using a Mouse ELISA Kit from R&D Systems (Quantikine ELISA Kit, MN, USA). According to the manufacturer's instructions, 20- μ L serum samples were freshly isolated from the peripheral blood of mice and diluted with 80 μ L of 1 \times assay buffer. After incubation in the antibody solution for 1.5 h with rotation, 100 μ L of HRP-conjugated antibody was added to each well and incubated for 30 min. After washing to remove the secondary antibody, 100 μ L of TMR chromogenic solution was added to each well and incubated for 15–20 min. Finally, samples were assessed on an ELISA reader (BioTek Instruments, Inc. Winooski, VT, USA) at wavelengths of 450 nm and 630 nm (wavelength correction).

Western blot analysis

Cells and tissues were harvested and lysed at designed time points postradiation exposure. Proteins were electrophoresed and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). Membranes were incubated with the designated primary antibodies (anti-DKK-1 1:1000, anti- α -SMA 1:5000, anti-vimentin 1:2000, and anti-E-cadherin 1:1000; Abcam, Cambridge, MA, USA; anti- β -actin, anti-TCF4, anti- β -catenin (total), and anti-active β -catenin (phospho): all the dilutions were 1:1000; Cell Signaling Technology, Beverly, MA, USA) overnight at 4 °C after blocking in 5% BSA for 2 h and were then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse diluted at 1:10000, Cell Signaling Technology, Beverly, MA, USA) for 2 h at room temperature. Densitometry was performed using Image J and β -actin was used as internal reference.

Immunofluorescence staining and immunohistochemistry

Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.3% Triton X-100 for 10 min (Solarbio, Beijing, China) after incubation with Ad-MSC-CM or serum-free DMEM postirradiation. Cell slides were then blocked with 5% BSA (BD, NJ, USA) at room temperature and incubated with anti-E-cadherin/vimentin, anti- α -SMA/PCNA, or anti-active β -catenin antibodies at 4 °C overnight. After washing three times with PBS, the corresponding fluorescent secondary antibody (Abcam, Cambridge, MA, USA) was added and incubated for 2 h. After nuclear

counterstaining with DAPI (Beyotime, China), images were examined under a fluorescence microscope (BX53, Olympus, Tokyo, Japan).

The expression of α -SMA and TGF- β in mouse lung tissues was examined by immunohistochemistry. Samples were dissected and fixed in 4% paraformaldehyde, embedded in paraffin and cut into sections 4 mm thick. Sections were then dewaxed in xylene and rehydrated in an ethanol gradient. After blocking with 3% hydrogen peroxide, antigen retrieval was performed using a microwave followed by cooling to room temperature. Sections were blocked in 5% BSA and were then incubated overnight at 4 °C with the corresponding primary antibody (CST, Beverly, MA, USA). Subsequently, a horseradish peroxidase-conjugated secondary antibody was used. The sections were developed with diaminobenzidine tetrahydrochloride and counterstained with haematoxylin. Images were examined with an Olympus microscope and analysed with Image Pro Plus.

Statistical analysis

Statistical analysis was performed using SPSS 21.0 (SPSS Company, Chicago, IL, USA). The results from three independent experiments are presented as the means \pm standard deviations (SDs). Unpaired *t* tests were used to determine the significance of differences between two groups and one-way analysis of variance (ANOVA) was used to determine the significance of differences among three or more groups. $P \leq 0.05$ was considered statistically significant.

Results

Establishment of the in vitro model

Fibrosis is the ultimate lesion occurring in irradiated lungs. EMT of epithelial cells and activation of residual fibroblasts are critical events in radiation-induced pulmonary fibrosis. To mimic the pathogenesis of this condition, human A549 (adenocarcinomic type II alveolar epithelial cells), BEAS-2B (human bronchial epithelial cell line) and HLF-1 (human lung-derived fibroblasts) cells were used. As reported by Andarawewa et al. (2007), IR (2 Gy) plus TGF- β (400 pg/mL) was able to sensitize HMECs to undergo EMT. On this basis, we performed an experiment to determine the optimal IR dose that induced EMT in A549 and BEAS-2B cells and activated HLF-1 cells in vitro. Although biological differences existed among the cell lines, vimentin and α -SMA expression peaked in A549, BEAS-2B and HLF-1 cells irradiated with 6 Gy at an incubation time of 72 h postirradiation (Fig. 1a–c'''). In addition, E-cadherin exhibited the lowest levels in A549 and BEAS-2B cells under the same conditions (Fig. 1a, b). When 400 pg/mL

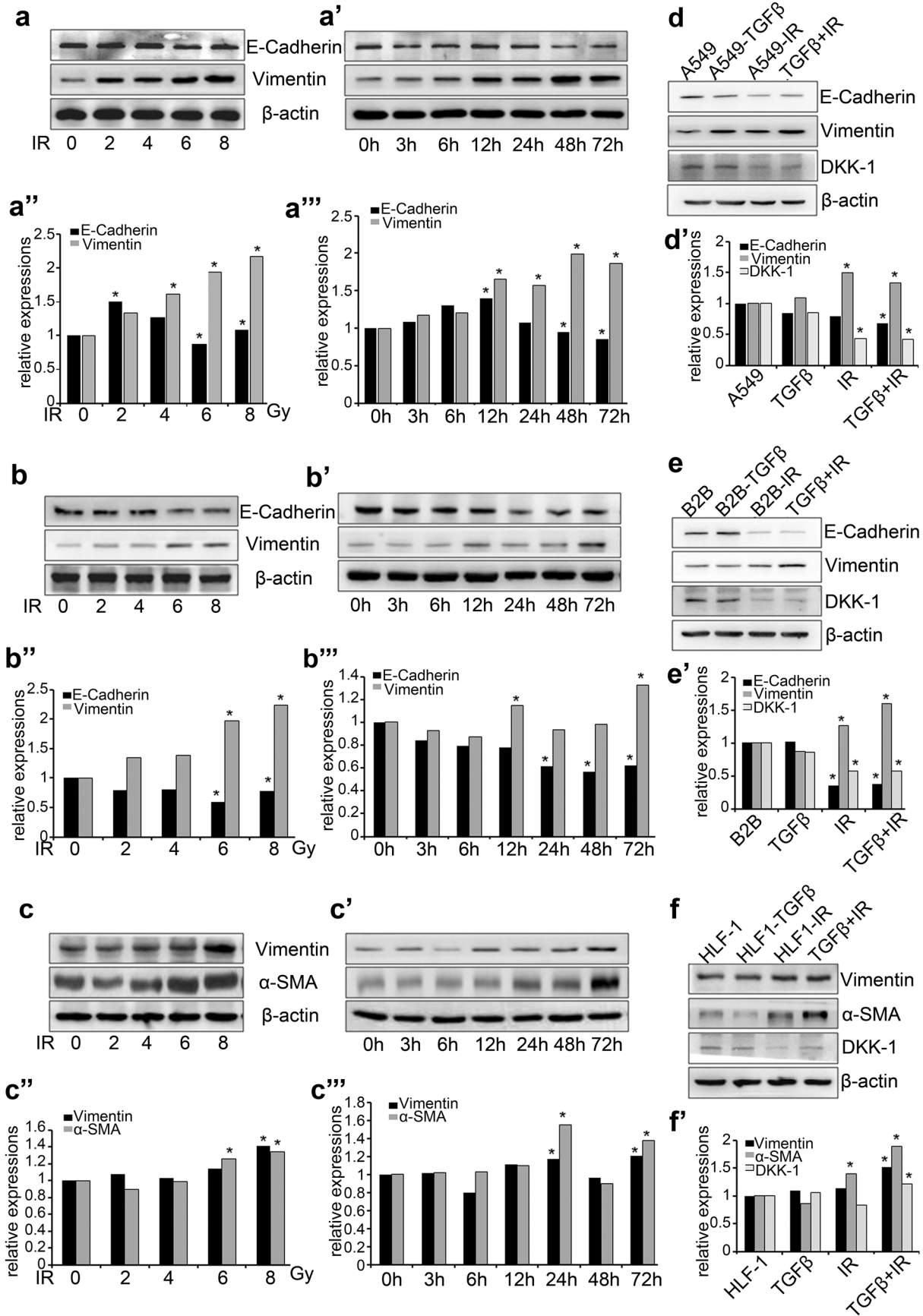


Fig. 1 Establishment of the pulmonary fibrosis model. The protein expression levels of E-cadherin and vimentin in A549 (a, a') and BEAS-2B (b, b') cells and of vimentin and α -SMA in HLF-1 (c, c') cells at different doses or different times were determined by WB assay. Vimentin and α -SMA expression peaked in A549, BEAS-2B and HLF-1 cells irradiated with 6 Gy at an incubation time of 72 h postirradiation. The protein expression levels of E-cadherin, vimentin and DKK-1 after irradiation and/or TGF- β (400 pg/mL) are shown in d, d' for A549 and e, e' for BEAS-2B cells. The expression levels of α -SMA, vimentin and DKK-1 in HLF-1 cells are shown in f, f'

TGF- β was added to the medium, the levels of hallmark proteins associated with EMT or fibroblast activation were further altered (Fig. 1d–e'). Moreover, this treatment also significantly reduced DKK-1 levels in A549, BEAS-2B, or HLF-1 cells (Fig. 1d–f'), suggesting that 6-Gy radiation plus TGF- β could downregulate DKK-1 expression in these cells. Thus, we further measured the expression of DKK-1 in MSCs from different origins to provide a rationale for repairing radiation-induced pulmonary fibrosis via MSCs.

DKK-1 is expressed by MSCs from different tissue origins

To detect the expressions of DKK1, we first compared the protein levels of DKK-1 among MSCs of Ad, BM and UC origin. As shown in Fig. 2(a, b), Ad-MSCs exhibited the highest levels of DKK-1. Then, we compared DKK-1 levels among different passages of Ad-MSCs from the same donor and between different donor-derived Ad-MSCs. Relevant results showed no significant differences in protein levels (Fig. 2a, a'). Moreover, the *DKK-1* mRNA levels exhibited similar alterations (Fig. 2b). Next, to validate the specific role of DKK-1 in antagonizing β -catenin activation, A549 cells were conditioned by using 6-Gy radiation plus TGF- β (400 pg/mL). In this experiment, when DKK-1 was added at a concentration of 100 ng/mL (Liu et al. 2017), the levels of the p- β -catenin were obviously decreased after incubation for 72 h. In addition, EMT was prohibited (Fig. 2c, c'). Collectively, these results showed that DKK-1 can antagonize EMT via β -catenin activation.

Ad-MSCs-derived supernatant inhibited EMT and fibroblast activation in vitro

Because DKK-1 could be expressed by Ad-MSCs, we investigated whether the supernatant from Ad-MSCs was potent in inhibiting pro-fibrotic events, including EMT and fibroblast activation. As shown in Fig. 3(a, a'), the supernatant from Ad-MSCs obviously reduced vimentin expression by irradiated A549 cells in the presence of TGF- β . In this context, the E-cadherin level was maintained to that in A549 cells without IR plus TGF- β intervention. To confirm this result, immunofluorescence staining for

E-cadherin and vimentin was performed. Under treatment with the supernatant from Ad-MSCs, the morphology of irradiated Ad-MSCs was not obviously changed, even in the presence of TGF- β (Fig. 3b–d'). Consistent with this finding, the supernatant from Ad-MSCs prevented BEAS-2B cells from undergoing EMT in the presence of IR plus TGF- β (Fig. 3e–h'). Regarding fibroblast activation, the supernatant from Ad-MSCs significantly reduced the α -SMA protein level in HLF-1 cells in the presence of IR plus TGF- β (Fig. 3i, i'). The IF staining for α -SMA also confirmed this result (Fig. 3j–l'). Collectively, these results suggested that the supernatant from Ad-MSCs prevents EMT and fibroblast activation in the context of IR plus TGF- β .

Ad-MSCs reduced EMT via DKK-1-primed WNT/ β -catenin inhibition

As mentioned above, the Wnt signalling pathway regulates EMT and DKK-1 was able to inhibit Wnt/ β -catenin activation. To determine whether Ad-MSCs reduced EMT in A549 cells by inhibiting the Wnt signalling pathway, we next treated IR plus TGF- β -conditioned A549 cells with the supernatant from Ad-MSCs. As shown in Fig. 4(a, a'), in the presence of the supernatant from Ad-MSCs, the protein levels of active β -catenin and TCF4 were significantly reduced in A549 cells compared with the IR+TGF- β group. IF staining for active β -catenin showed that this protein was obviously localized in the nucleus in A549 cells conditioned with IR plus TGF- β alone. By contrast, the nuclear localization of active β -catenin was reduced in the presence of supernatant from Ad-MSCs (Fig. 4b–d'). In addition, the expression of the target genes involved in EMT, including *Snail*, *Twist* and *c-Myc*, were downregulated in cells treated with the supernatant from Ad-MSCs (Fig. 4e), probably due to the undegraded β -catenin. These results preliminarily suggested that the supernatant from Ad-MSCs reduced EMT by inhibiting Wnt/ β -catenin activation. On this basis, we added anti-DKK-1 antibody to determine whether Ad-MSC-primed Wnt inhibition was dependent on DKK-1. The relevant data showed that the level of active β -catenin was increased upon neutralization of DKK-1 in the supernatant of Ad-MSCs (Fig. 4f, f'). Moreover, EMT was induced again in the context of DKK-1 neutralization. This result suggested that Ad-MSCs reduced EMT via DKK-1-primed WNT/ β -catenin inhibition.

Ad-MSCs reduced radiation-induced pulmonary fibrosis in vivo

To evaluate the effect of Ad-MSCs on fibrosis in vivo, we subjected C57/B6 mice to 20-Gy whole-thoracic X-ray irradiation (Bickelhaupt et al. 2017). One month later, 1×10^6 Ad-MSC cells were transplanted into the mice via

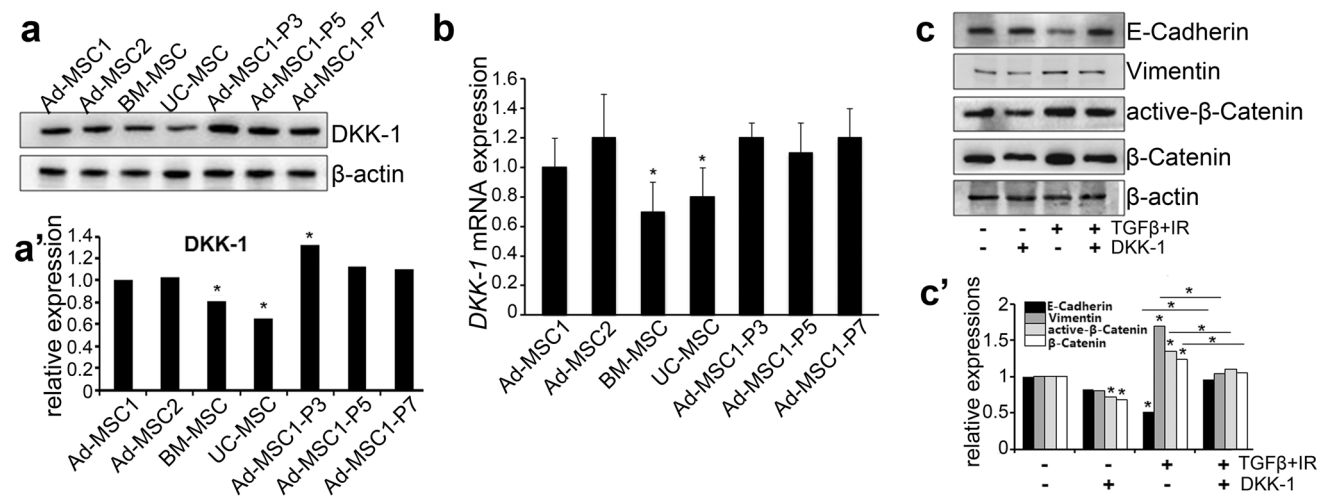


Fig. 2 DKK-1-regulated radiation-induced pulmonary fibrosis (**a**, **a'**) WB was performed to study the expression of DKK-1 in MSCs of different origins and different generations. The DKK-1 exhibited the highest levels in Ad-MSCs and no difference in each generation. (**b**) *DKK-1* mRNA levels in MSCs of different origins and different gen-

erations. * $P < 0.05$ vs Ad-MSC1. The results were consistent with the protein results. (**c**, **c'**) WB was performed to study the expressions of E-cadherin, vimentin, total β -catenin and p- β -catenin in A549 cells after treatment with or without DKK-1. The results showed that DKK-1 can antagonize the changes induced by radiation

the tail vein. After another 1 or 6 months, the mice were sacrificed and assessed. Regardless of Ad-MSC injection status, the color of the exposed area became lighter, even white of the irradiated mice. In the simple irradiation group, inflammatory cells infiltrated the lungs, the alveolae collapsed and the alveolar septum thickened; these changes were more obvious at 6 months. The results shown in Fig. 5(a–b''') indicate that the lung tissue structure of mice treated with Ad-MSCs was more similar to that of mice in the normal group than to that of mice in the simple irradiation group. In addition, the deposition of collagen was reduced in the treatment group.

The results for proteins of lung tissues related to EMT and fibroblast activation showed that the expression levels of vimentin, α -SMA and TGF- β in the stromal cell treatment group were significantly lower than those in the simple irradiation group, while opposite results were seen for E-cadherin expression (Fig. 5g, g'). The immunohistochemical results for α -SMA and TGF- β at 6 months were the same as the western blot results (Fig. 5c–d''). To investigate the effect of DKK-1, we assessed the expression of active β -catenin and TCF4 and the results were similar to the in vitro results. Irradiation upregulated these proteins and Ad-MSC cells downregulated them (Fig. 5g, g'). We found that tissue changes at 9 and 12 months were similar to those at 6 months, so we indicated that the changes at 6 months represented the occurrence of radioactive lung injury.

The TGF- β and IL-10 protein concentrations in peripheral blood were measured by ELISA. The serum TGF- β 1 level was significantly increased after irradiation but reduced after hAd-MSCs interference at 1 month and 6 months compared

with that in the non-treated group (Fig. 5e). The serum level of the immunosuppressive factor IL-10 was increased after radiation and was further increased after Ad-MSCs interference for 1 month (Fig. 5f). These results showed the effectiveness of adipose-derived stromal cells in the treatment of radiation-induced lung injury.

Discussion

Pulmonary injury occurring after thoracic radiotherapy is a common complication of radiotherapy. Upon irradiation, alveolar edema is first induced. Next, the expression of several chemotactic ligands is upregulated. On this basis, immune cells will be recruited to injured sites, thus leading to pro-inflammatory responses. In this case, residual pulmonary fibroblasts will be converted to myofibroblasts by several cytokines, such as interleukin-1 beta (IL-1 β), tumor necrosis factor-alpha (TNF- α), transforming growth factor-beta (TGF- β) and connective tissue growth factor (CTGF), which are released by infiltrated immune cells such as macrophages. Myofibroblasts are able to produce collagens, which enable scar tissue formation, thus causing a reduction in respiratory function. More importantly, many scholars have observed that similar lesions can even be found within the outer contour of irradiated, demonstrating that the abscopal effect exists during the pathogenesis of RILI.

Fibrosis is now recognized as a prominent sequel to radiotherapy. The main causes of radiation-induced pulmonary fibrosis include EMT and fibroblast proliferation. The major biomarkers of EMT include the suppression of E-cadherin

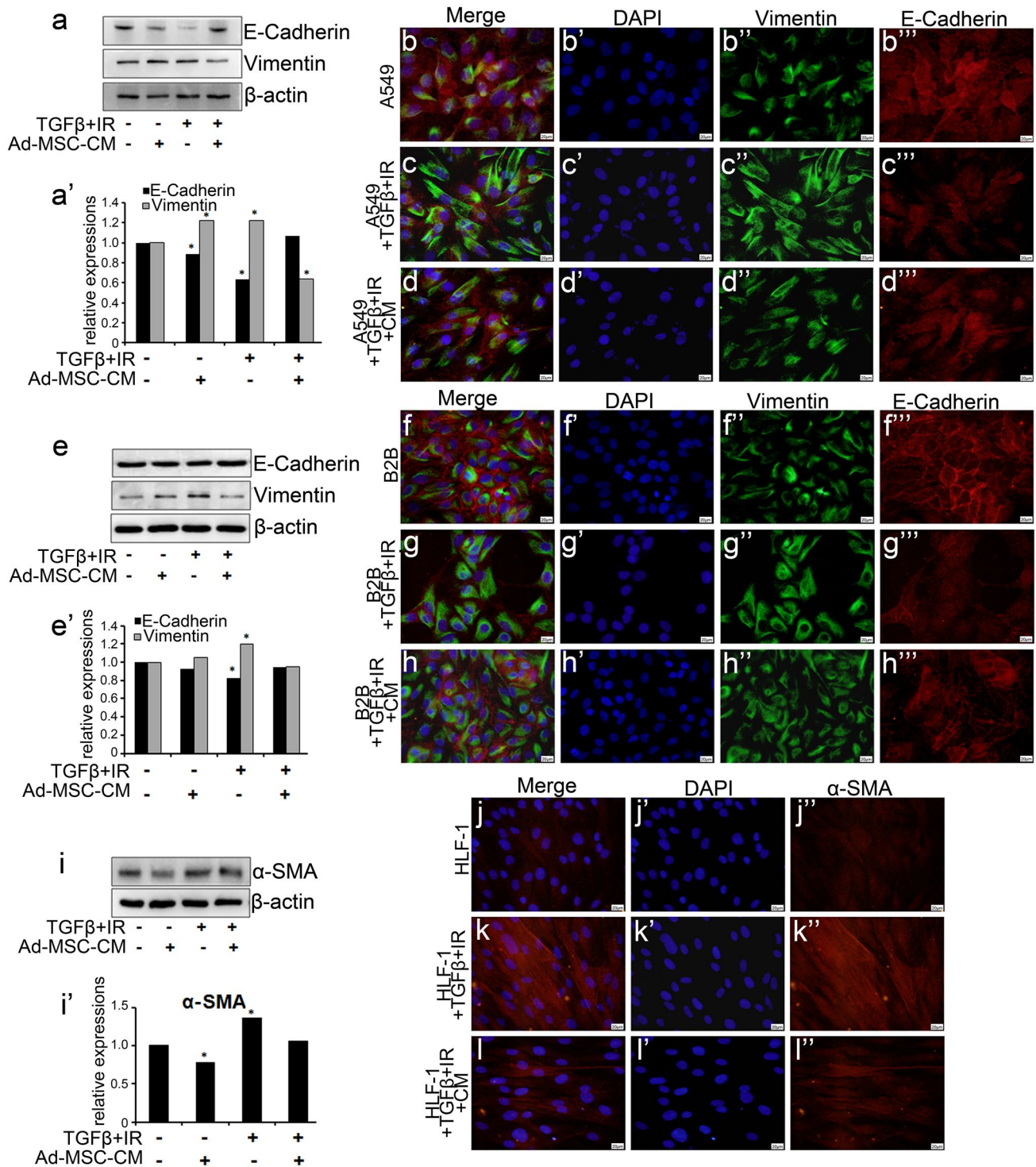


Fig. 3 Ad-MSC-CM inhibited radiation-induced pulmonary fibrosis in vitro. After cells were irradiated for 72 h with the supernatant from Ad-MSCs, we measured the levels of E-cadherin and Vimentin in A549 (a, a') and BEAS-2B (B2B) cells (e, e') and the level of α-SMA in HLF-1 cells (i, i') to study the effect of Ad-MSC-CM on

radiation. Immunofluorescence staining was used to visualize the expression of E-cadherin and vimentin in A549 (b–d''') and B2B (f–h''') cells and the expression of α-SMA in HLF-1 cells (j–l''') under the same conditions. The results all showed that Ad-MSC-CM can mitigate the changes caused by irradiation

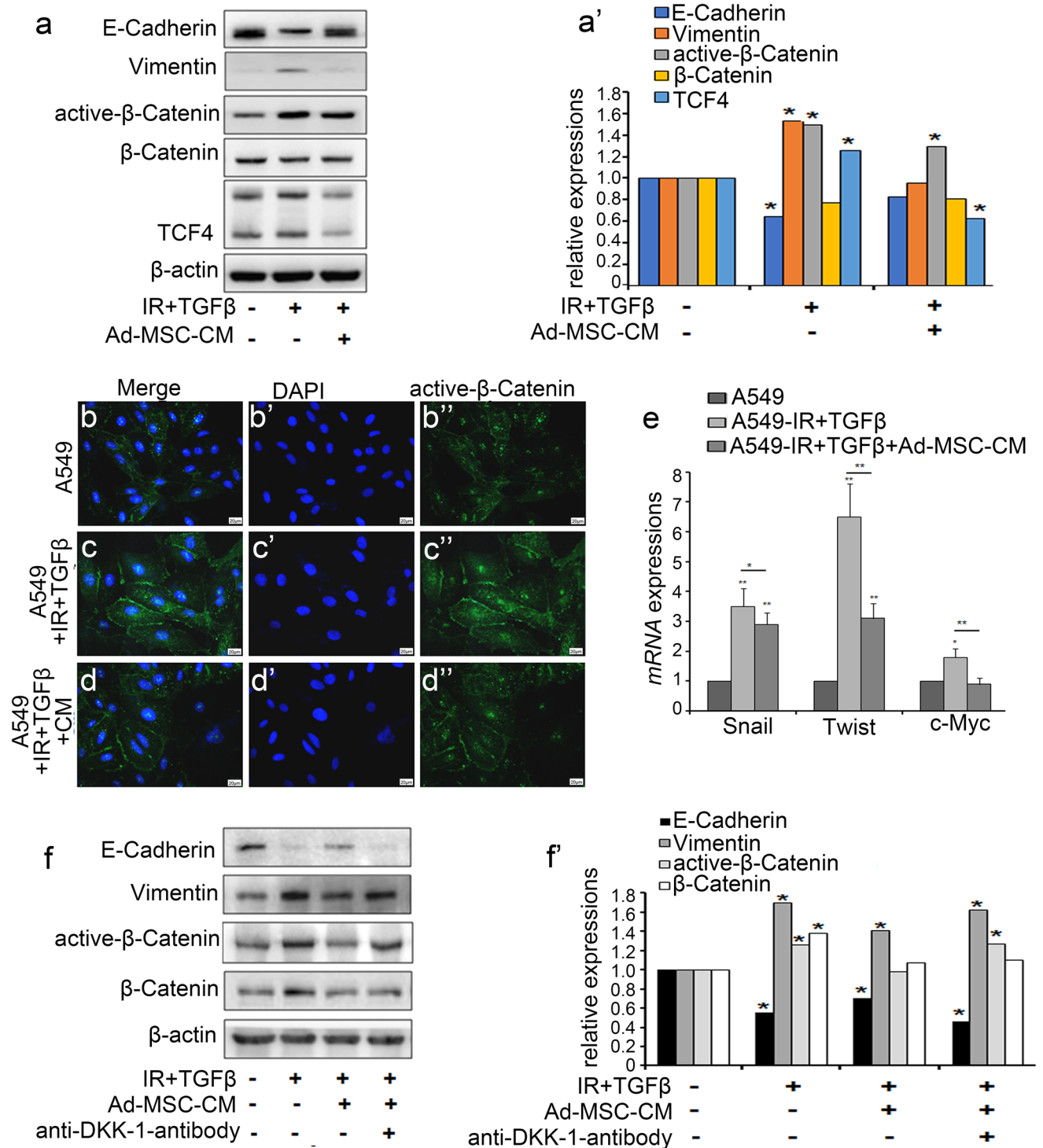


Fig. 4 DKK-1 secreted in Ad-MSC-CM regulated radiation-induced pulmonary fibrosis via the WNT/β-catenin pathway in A549 cells. (**a**, **a'**) Wnt/β-catenin pathway proteins in A549 cells were detected by WB after the cells were irradiated and treated by Ad-MSC-CM. The expression of nuclear-localized p-β-catenin (**b–d''**) in A549 cells were showed by IF. (**e**) *Snail*, *Twist1* and *c-Myc* mRNA levels

were measured by Q-PCR after the cells were irradiated and treated by Ad-MSC-CM. * $P < 0.05$ vs control, ** $P < 0.01$ vs control. (**f**, **f'**) E-cadherin, vimentin and β-catenin were detected by WB in A549 cells after neutralization with anti-DKK-1 antibody. The remission of EMT induced by radiation from Ad-MSC-CM occurred again

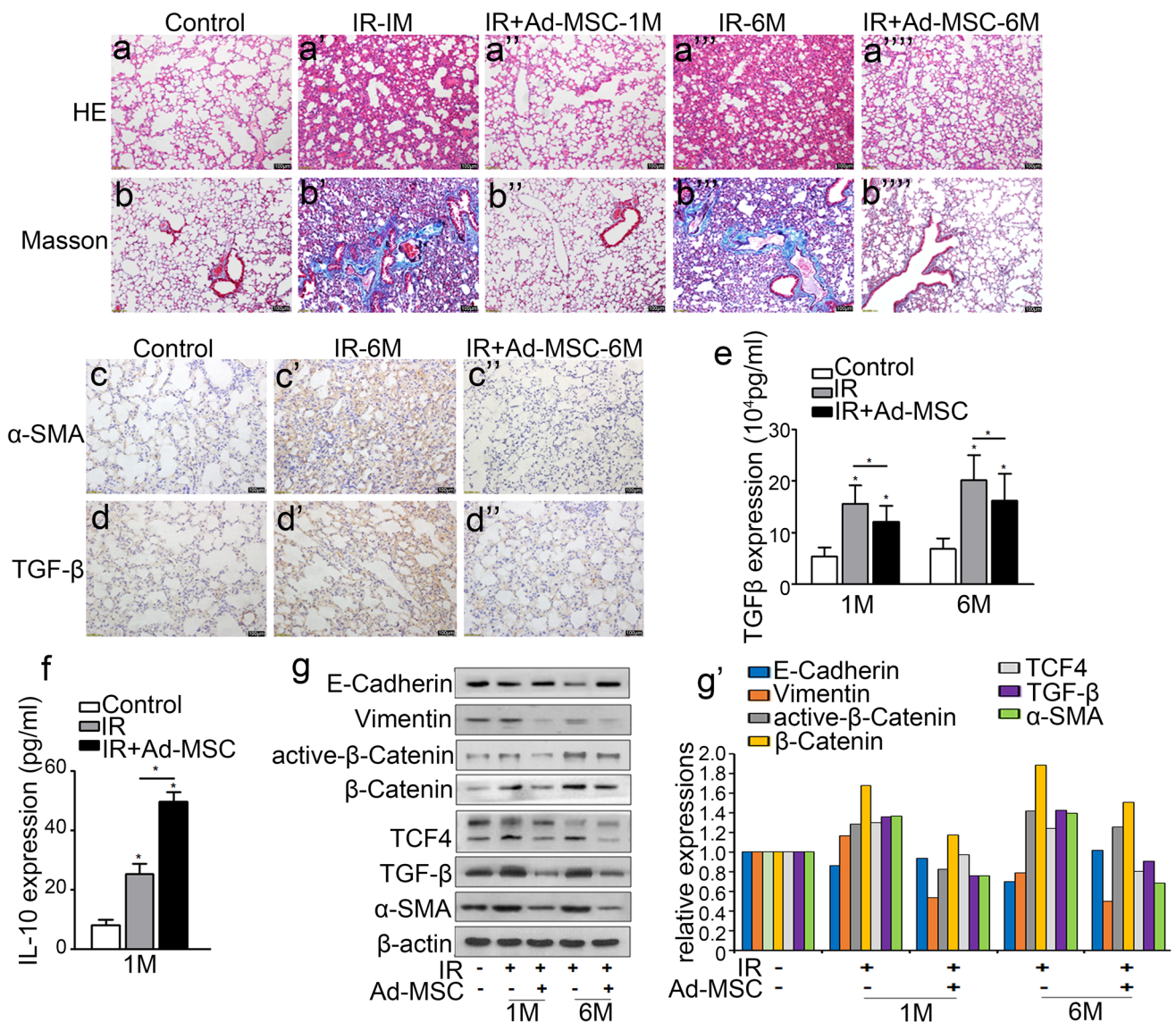


Fig. 5 Ad-MSCs inhibited radiation-induced pulmonary fibrosis in vivo. (a–b'') Histo-morphological analysis of the lungs from irradiated mice were detected at 1 or 6 months (H&E and Masson staining) with or without the treatment of Ad-MSCs. (c–d'') Immunohistochemistry was conducted to assess the expression of

α-SMA and TGF-β in lung tissues. (e) ELISA was used to measure the serum TGF-β levels in mice after exposure 1 or 6 months later. (f) Under similar conditions, an ELISA was performed to study the levels of IL-10 in the serum of each group. (g, g') Proteins were tested by western blot analysis from the mice of different groups

expression and the acquisition of N-cadherin and vimentin expression (Nagaraja and Nagarajan, 2018). The major biomarker of fibroblast proliferation is α-SMA. (Andarawewa et al. 2007 and Park et al. 2003), found that a single exposure to 2-Gy IR sensitizes HMECs to undergo TGF-β-mediated EMT and to exhibit all the classic hallmarks of EMT. It is widely known that TGF-β is an EMT inducer. However, we found that 6-Gy radiation may induce EMT in epithelial cells and the activation of fibroblasts with 400 pg/mL TGF-β.

We found that radiation can not only induce fibrosis but also decrease the basal expression of DKK-1 in epithelial cells and fibroblasts. DKK1 can block the canonical Wnt

pathway, which is an important pathway leading to fibrosis, through interaction with LRP5/6 and Kremen receptors. The decrease in DKK1 expression may be related to EMT, so we considered whether it is necessary to introduce DKK1 from external sources. We first measured the DKK-1 level in stromal cells of different sources or different generations and found significantly higher levels in Ad-MSCs than in BM-MSCs and UC-MSCs but slightly different levels in different passages of Ad-MSCs. Our previous research measured the cytokine of DKK-1 and suggested that the concentration had a high expression (Chang et al. 2018); thus, we selected Ad-MSCs as the source.

Radiation-induced EMT and fibrosis can be influenced by numerous signalling pathways such as the NF- κ B, MAPK and Wnt pathways; mi-RNAs; and histone modifications (Nagaraja et al. 2018). Irradiation can inhibit β -catenin transfer to the nucleus, which leads to an increase in active β -catenin and TCF4 levels. We evaluated the expression of Wnt/ β -catenin-related proteins in epithelial cells after treatment with medium to verify whether Wnt/ β -catenin can be impacted by Ad-MSCs and found that the induction of pulmonary fibrosis markers by radiation can be obviously remitted by Ad-MSC-CM. The level of non-phospho- β -catenin was increased, especially in the nucleus; this molecule binds to the sequence-specific transcription factors LEF/TCF and functions as a transcriptional coactivator to promote the expression of Wnt target genes (Mao et al. 2013). The levels of Snail, Twist and c-Myc, which can bind to the promoter of E-cadherin and further inhibit its expression, were elevated by irradiation. In association with the phosphorylation and degradation of β -catenin, DKK-1 can also increase the E-cadherin level and decrease the vimentin level (Mikheev et al. 2008). To confirm the role of DKK-1 in Ad-MSC-CM, we added an antibody to neutralize DKK-1. When the content of DKK-1 in the culture solution was reduced, the expression of β -catenin decreased further but remained higher than that in the single irradiation group, demonstrating the role of DKK-1 in Ad-MSC-CM.

Although some studies have regarded MSC as a factor that can promote EMT (Feng et al. 2018; Iser et al. 2016; Xu et al. 2018), numerous studies have shown that MSCs can repair fibrosis caused by radiation (Su et al. 2019; Böhrnsen et al. 2015). We believe that one of the main reasons for the differing results is that we used the supernatant, whereas others used cocultured stem cells. The cytokines released by cells in different environments vary greatly and different stimuli change the levels of factors in the microenvironment. We used the supernatant containing substances naturally secreted by stromal cells, which was fairly stable and contained high levels of DKK-1. Ad-MSCs, as a kind of stromal cell derived from low-radiosensitivity adipocytes, are also less affected by irradiation than BM-MSCs and UC-MSCs.

Since pneumonia is the basis for pulmonary fibrosis, we measured the levels of inflammation in mice at 1 month and the degree of fibrosis at 6 months after exposure. In addition, the western blot results revealed that the results for fibrosis and Wnt/ β -catenin protein expression were consistent with the in vitro results and that Ad-MSCs can upregulate endogenous IL-10 expression at 1 month. RILI can lead to the increased expression of inflammatory factors. Meanwhile, the expressions of anti-inflammatory factors were also increased for the purpose of protection. After the injection of Ad-MSCs, to reduce the inflammation in the body, the expression of IL-10 was also increased. Coincidentally, this

anti-inflammatory effect has also been shown in radiation-induced intestinal injury (Chang et al. 2018).

Conclusion

Unexpectedly, our study showed that the canonical Wnt pathway may be activated by irradiation and that Ad-MSCs can reverse the induction of these effects in A549 cells, indicating that fibrosis promoted by ionizing radiation can be suppressed by Ad-MSCs through the Wnt/ β -catenin signalling pathway in vitro and in vivo.

Author contributions LS was responsible for writing the first draft of the manuscript. YZ contributed to the linguistic polish of the article. LS and WS were responsible for all parts of the experiments. LM was responsible for a critical review of the manuscript. PC and LD were responsible for the concept of the review. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All animal studies were performed in compliance with the regulations and guidelines of the Medical ethics committee of the First Hospital of Jilin University, Changchun, Jilin Province, China. The document ID is 2017-169.

References

- Andarawewa KL, Erickson AC, Chou WS, Costes SV, Gascard P, Mott JD, Bissell MJ, Barcellos-Hoff MH (2007) Ionizing radiation predisposes nonmalignant human mammary epithelial cells to undergo transforming growth factor beta induced epithelial to mesenchymal transition. *Cancer Res.* 67(18):8662–70
- Bickelhaupt S, Erbel C, Timke C, Wirkner U, Dadrich M, Flechsig P, Tietz A, Pföhler J, Gross W, Peschke P, Hoeltgen L, Katus HA, Gröne HJ, Nicolay NH, Saffrich R, Debus J, Sternlicht MD, Seeley TW, Lipson KE, Huber PE (2017) Effects of CTGF blockade on attenuation and reversal of radiation-induced pulmonary fibrosis. *J Natl Cancer Inst.* 109(8)
- Böhrnsen F, Fricke M, Sander C, Leha A, Schliephake H, Kramer FJ (2015) Interactions of human MSC with head and neck squamous cell carcinoma cell line PCI-13 reduce markers of epithelial-mesenchymal transition. *Clin Oral Investig.* 19(5):1121–8
- Chang P, Zhang B, Shao L, Song W, Shi W, Wang L, Xu T, Li D, Gao X, Qu Y, Dong L, Wang J (2018) Mesenchymal stem cells

- over-expressing cxcl12 enhance the radioresistance of the small intestine. *Cell Death Dis.* 9(2):154
- Dong LH, Jiang YY, Liu YJ, Cui S, Xia CC, Qu C, Jiang X, Qu YQ, Chang PY, Liu F (2015) The anti-fibrotic effects of mesenchymal stem cells on irradiated lungs via stimulating endogenous secretion of HGF and PGE2. *Sci Rep.* 5:8713
- Feng H, Zhao JK, Schiergens TS, Wang PX, Ou BC, Al-Sayegh R, Li ML, Lu AG, Yin S (2018) Thasler WE Bone marrow-derived mesenchymal stromal cells promote colorectal cancer cell death under low-dose irradiation. *Br J Cancer.* 118(3):353–365
- Huang Y, Zhang W, Yu F, Gao F (2017) The cellular and molecular mechanism of radiation-induced lung injury. *Med Sci Monit.* 23:3446–3450
- Iser IC, Ceschini SM, Onzi GR, Bertoni AP, Lenz G, Wink MR (2016) Conditioned medium from adipose-derived stem cells (ADSCs) promotes epithelial-to-mesenchymal-like transition (EMT-Like) in glioma cells in vitro. *Mol Neurobiol.* 53(10):7184–7199
- Liu T, Zhang L, Wang Y, Zhang H, Li L, Bao X (2017) Dickkopf-1 inhibits Wnt3a-induced migration and epithelial-mesenchymal transition of human lens epithelial cells. *Exp Eye Res.* 161:43–51
- Mao Y, Xu J, Li Z, Zhang N, Yin H, Liu Z (2013) The role of nuclear beta-catenin accumulation in the Twist2-induced ovarian cancer EMT. *PLoS One.* 8(11):e78200
- Mikheev AM, Mikheeva SA, Maxwell JP, Rivo JV, Rostomily R, Swisshelm K, Zarbl H (2008) Dickkopf-1 mediated tumor suppression in human breast carcinoma cells. *Breast Cancer Res Treat.* 112(2):263–73
- Nagaraja SS, Nagarajan D (2018) Radiation-induced pulmonary epithelial-mesenchymal transition: a review on targeting molecular pathways and mediators. *Curr Drug Targets.* 19(10):1191–1204
- Park CC, Henshall-Powell RL, Erickson AC, Talhouk R, Parvin B, Bissell MJ, Barcellos-Hoff MH (2003) Ionizing radiation induces heritable disruption of epithelial cell interactions. *Proc Natl Acad Sci U S A.* 100(19):10728–33
- Qi L, Sun B, Liu Z, Li H, Gao J, Leng X (2012) Dickkopf-1 inhibits epithelial-mesenchymal transition of colon cancer cells and contributes to colon cancer suppression. *Cancer Sci.* 103(4):828–35
- Stewart DJ (2014) Wnt signaling pathway in non-small cell lung cancer. *J Natl Cancer Inst.* 106(1): djt356
- Su F, Ahn S, Saha A, DiGiovanni J, Kolonin MG (2019) Adipose stromal cell targeting suppresses prostate cancer epithelial-mesenchymal transition and chemoresistance. *38(11):1979–1988*
- Wynn TA (2011) Integrating mechanisms of pulmonary fibrosis. *J Exp Med.* 208(7):1339–50
- Xu T, Yuyu Z, Pengyu C, Shouliang G, Lihong S, Lihua D (2018) Mesenchymal stem cell-based therapy for r radiation-induced lung injury. *Stem cell and therapy.* 9:18

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