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Therapeutic Potential of Umbilical Cord Mesenchymal Stem Cells for Inhibiting Myofibroblastic Differentiation of Irradiated Human Lung Fibroblasts

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Radiation-induced lung injury (RILI) limits the benefits of radiotherapy in patients with lung cancer. Radiation-induced differentiation of lung fibroblasts to myofibroblasts plays a key role in RILI. Recent studies have shown that mesenchymal stem cells (MSCs) can protect against lung fibrosis and that Wnt/*β*-catenin signaling is involved in fibrotic processes. In the present study, we explored the therapeutic potential of human umbilical cord MSCs (HUMSCs) for preventing radiation-induced differentiation of human lung fibroblasts (HLFs) to myofibroblasts. There are two advantages in the use of HUMSCs; namely, they are easily obtained and have low immunogenicity. Irradiated HLFs were co-cultured with HUMSCs. Expression of *α*-smooth muscle actin (*α*-SMA), a myofibroblast marker, was measured by Western blot analysis and immunohistochemistry. Irradiation (X-rays, 5 Gy) induced the differentiation of HLFs into myofibroblasts, which was inhibited by co-culture with HUMSCs. Irradiation also caused activation of the canonical Wnt/*β*-catenin signaling in HLFs, as judged by increased phosphorylation of glycogen synthase kinase 3*β*, nuclear accumulation of *β*-catenin, and elevated levels of Wnt-inducible signaling protein-1 (WISP-1) in the conditioned medium. However, co-culture with HUMSCs attenuated the radiation-induced activation of the Wnt/*β*-catenin signaling. We also measured the expression of FRAT1 that can enhance the Wnt/*β*-catenin signaling by stabilizing *β*-catenin. Co-culture with HUMSCs decreased FRAT1 protein levels in irradiated nHLFs. Thus, co-culture with HUMSCs attenuated the radiation-induced activation of Wnt/*β*-catenin signaling in HLFs, thereby inhibiting myofibroblastic differentiation of HLFs. Wnt/*β*-catenin signaling is a potential therapeutic target for limiting RILI in patients receiving radiotherapy for lung cancer.

Keywords: lung fibroblasts; radiation-induced lung injury; transwell co-culture; umbilical cord mesenchymal stem cells; Wnt/*β*-catenin signaling
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Introduction

Radiotherapy has a well-established and indispensable role in the treatment of small cell lung cancer (Zhu et al. 2011). However, radiation-induced lung injury (RILI) is a major adverse effect associated with radiotherapy for lung cancer, limiting the therapeutic dose of radiation that can be administered. The clinical manifestations of RILI include acute radiation pneumonitis and radiation-induced fibrosis, but the underlying mechanisms are still not fully understood.

RILI is a complex pathologic process involving several cell types within the lung; among these, fibroblasts are considered to play a key role (Rubin et al. 1995). Radiation can stimulate lung fibroblasts to secrete cytokines, undergo hyperplasia, and differentiate into myofibroblasts (Martin et al. 1993, 2000; Rodemann and Bamberg 1995). Myofibroblasts, which show up-regulated expression of *α*-smooth muscle actin (*α*-SMA), promote the synthesis of additional collagens; this leads to excessive deposition and abnormal remodeling of the extracellular matrix, a hallmark of RILI (Rodemann and Bamberg 1995; Martin et al. 2000; Hu et al. 2006).

The Wnt/*β*-catenin signaling pathway plays important roles in cellular proliferation and differentiation (Morrisey 2003), and has been implicated in the pathogenesis of vari-

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ous fibrotic diseases, including lung fibrosis (Guo et al. 2012). Wnt ligands are extracellular proteins that activate cell surface receptors, which in turn recruit β-catenin in the signal transduction pathway. In the absence of active Wnt ligands, β-catenin is phosphorylated by glycogen synthase kinase-3β (GSK-3β) and subsequently degraded. GSK-3β resides in the cytoplasm as part of a complex with adenomatosis polyposis coli (APC) and axin. In contrast, when Wnt ligands are present, they activate cell surface receptors: frizzled (FZD), and low-density lipoprotein receptor-related proteins (Lrp)-5 and -6. Active signaling results in phosphorylation of GSK-3β and suppression of its activity, allowing β-catenin to accumulate. β-Catenin then translocates to the nucleus where it binds to the T-cell factor/lymphoid enhancer factor (Tcf/Lef) family of transcription factors to induce the expression of target genes.

Certain components of the canonical Wnt/β-catenin signaling pathway have been reported to play a role in lung fibrosis, including Wnt-inducible signaling protein-1 (WISP-1) and frequently rearranged in advanced T cell lymphomas-1 (FRAT1, an inhibitor of GSK-3β-mediated phosphorylation of β-catenin) (Pardo et al. 2005; Königshoff et al. 2009). In addition, Gurung et al. (2009) have shown that irradiation of human lung fibroblasts (HLFs) results in activation of Wnt/β-catenin signaling. Taken together, these findings suggest that selective inhibition of Wnt/β-catenin signaling may have therapeutic potential for the treatment of pulmonary fibrosis.

Mesenchymal stem cells (MSCs) have been shown to improve bleomycin-induced lung injury and fibrosis in animal models (Ortiz et al. 2003; Rojas et al. 2005; Cui et al. 2007; Zhao et al. 2008; Moodley et al. 2009). Thus, transplanting MSCs may represent a promising strategy for limiting the progression of fibrosis, although more research is needed to understand the underlying mechanisms. Several studies have implicated the canonical Wnt/β-catenin signaling in fibroblast differentiation (Salazar et al. 2009), raising the possibility that this pathway may be involved in the anti-fibrotic actions of MSCs. In the present study, we test this possibility by examining the effects of human umbilical cord MSCs (HUMSCs) on radiation-induced Wnt/β-catenin signaling in HLFs, and on the differentiation of HLFs into myofibroblasts. HUMSCs were used because these cells are easily obtained and have low immunogenicity.

Materials and Methods

HUMSC isolation

All experiments included in this study were approved by the Navy General Hospital Ethical Review Board. The isolation, culture and passaging of HUMSCs were performed as described previously (Gao et al. 2013). Fresh human umbilical cords were obtained from full-term births by cesarean section, following provision of consent by the parents. Cords were stored aseptically in sterile saline immediately after delivery. The umbilical cords were excised, and the Wharton's jelly and umbilical cord blood vessels isolated. Samples were rinsed in sterile phosphate-buffered saline (PBS: 140 mM NaCl; 2 mM KCl; 1.5 mM KH_2PO_4; 15 mM Na_2HPO_4) at room temperature. The Wharton's jelly was cut in cross-section, and 4-5 explants of the matrix, ranging in size from 1-2 mm, were placed in T75 culture flasks with 2-3 ml of culture medium; all procedures were performed under sterile conditions. The isolation and subculture of cells were performed using low-glucose Dulbecco's modified Eagle medium (DMEM; GIBCO, USA) supplemented with 10% fetal bovine serum (GIBCO, USA), 2 mM L-glutamine (Hyclone, USA), 100 IU/ml penicillin (Hyclone, USA) and 100 μg/ml streptomycin (Hyclone, USA). The conditioned medium (CM) was changed every second day. Cultured Wharton's jelly fragments showed characteristic mesenchymal migratory capability. After 15 days of culture, the remnants of the Wharton's jelly fragments were removed from the flask, and the cells attached to the plastic surface were cultured until they reached confluence. During the isolation process, proteases were not used to detach cells from the embedding matrix.

HUMSCs were routinely maintained in DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Hyclone, USA) at 37°C with 5% CO_2 in the atmosphere.

Cell culturing and passaging

Upon reaching confluence, primary cells were routinely subcultured in culture medium. Cells were detached from flasks using accutase (Invitrogen, USA) rather than standard trypsin solution. Primary HUMSCs were cultivated for up to 3-6 passages. Cells at passage 3 were used in the present study.

Immunophenotyping of HUMSCs

Immunophenotyping of the expanded HUMSCs used in this study has been described previously (Gao et al. 2013). Adherent cells were washed with PBS and detached by incubation with trypsin-EDTA for 3 min at 37°C. The harvested cells were washed with staining buffer containing 4% fetal bovine serum (FBS) and 0.1% azide in PBS. A total of 0.1×10^6 cells per tube were used for cell surface antigen expression studies. The cells were stained with phycoerythrin (PE)/fluorescein (FITC)-labeled monoclonal antibodies against human CD44, CD90, CD105, CD73, CD45, CD34, CD3, CD4, CD8, CD80, CD86, HLA-ABC and HLA-DR (Beijing Biosynthesis Biotechnology, China), applied for 45 min at room temperature. The stained cells were examined using a flow cytometer (FACS Calibur, Becton-Dickinson, USA) equipped with a 488-nm argon laser, to confirm the purity of the HUMSCs. Flow cytometric analyses showed that HUMSCs were positive for CD90, CD44, CD105, CD73, HLA-A, -B and -C, but negative for CD31, CD34, CD45, HLA-DR, CD3, CD4, CD8, CD80 and CD86 (Gao et al. 2013).

Differentiation studies

The differentiation potential of the expanded HUMSCs used in this study has been described previously (Gao et al. 2013). HUMSCs showed the capacity to differentiate into osteoblasts, chondrocytes and adipocytes (Gao et al. 2013).

Transwell co-culture of nHLFs with HUMSCs

Normal HLFs (nHLFs) were provided by Dr. Chen Jie (Medical School of Chinese PLA, Beijing, China) (Gorshkova et al. 2012). To investigate the effects of HUMSC-secreted soluble factors on irradiated nHLFs, the nHLFs were co-cultured with HUMSCs in transwell

chambers (0.4-μm pores/6 wells; collagen coated; Corning Costar, Corning, USA). HUMSCs (1.5×10^5 cells/well) were seeded onto the polycarbonate membrane of the upper chamber, and nHLFs (2×10^5 cells/well) grown in the lower chamber. Before exposure to radiation, the nHLFs were cultured alone for 12 h in DMEM/F12 supplemented with 10% FBS (GIBCO) and penicillin-streptomycin (100 U/ml). The CM was then replaced with DMEM/F12 serum-free medium. Co-culture with HUMSCs was initiated immediately after irradiation of the nHLFs. The entire culture system was maintained in 2 ml of serum-free CM at 37°C in an incubator containing 5% CO_2. As a control, nHLFs were cultured in 6-well plates in the absence of co-culture conditions. Three experimental groups were included in this study: the Co-culture group consisted of nHLFs exposed to radiation and co-cultured in the presence of HUMSCs; the Radiation group consisted of nHLFs exposed to radiation but not co-cultured with HUMSCs; and the Normal group (controls) consisted of nHLFs that were neither irradiated nor co-cultured with HUMSCs.

MTT assay of nHLFs following irradiation

To select an appropriate dose of radiation in our study, nHLFs were seeded in 96-well plates (3×10^4 cells per well) and treated with 0, 5 or 8 Gy of radiation. Following irradiation, the cells in each well were incubated, at 37°C for 4 h, with 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Subsequently, the CM and MTT were removed, and 200 μl of dimethyl sulfoxide (DMSO) added to each well. The samples were then shaken, and the optical density (OD) values at 570 nm measured using an ELISA meter (3001-122J, Thermo, USA). Assays were performed on days 1, 2, 3, 4, 5 and 6 after irradiation.

In vitro cell irradiation

Once cells reached > 80% confluence, DMEM was replaced with serum-free medium prior to irradiation. Cells were irradiated (at room temperature) with a single dose of 5 Gy X-rays at a dose rate of 3.64 Gy/min, using a medical linear accelerator (Oncor Impression Plus, Siemens Medical Systems, USA).

Western blot analysis

Cells were lysed in RIPA buffer and stored as aliquots at −80°C until assayed. Lysates were mixed with an equal volume of sample buffer, denatured by boiling, and then separated on a 10-15% polyacrylamide minigel. Proteins were transferred to nitrocellulose membranes, blocked with 5% milk, and incubated overnight with rabbit monoclonal anti-α-SMA antibody (1:500, Abcam, USA), rabbit monoclonal IgG anti-GSK3β antibody (1:500, Cell Signaling, USA), rabbit monoclonal anti-p-GSK3β (Ser9) antibody (1:500, Cell Signaling, USA), rabbit monoclonal anti-FRAT1 antibody (1:500, Abcam, USA), or rabbit monoclonal anti-β-catenin antibody (1:500, Epitomics, USA). The next day, the membranes were washed five times with washing buffer and incubated for 30 min with secondary antibody (1:1,000 in blocking buffer). The membranes were then rinsed with washing buffer and incubated with enhanced chemiluminescence (ECL) working solution for 5 min. The signal was detected using Gel software, and the relative intensities of the detected bands compared between groups.

Immunofluorescence

nHLFs were grown on glass coverslips in the lower chamber of a transwell system and cultured for 12 h in DMEM/F12 supplemented with 10% FBS. The CM was then replaced with DMEM/F12 serum-free medium. In the Co-culture group, the nHLFs were irradiated and then immediately co-cultured with HUMSCs that were seeded into the upper chamber of the transwell. Irradiated (Radiation group) and non-irradiated (Normal group) nHLFs cultured in the absence of HUMSCs were used for comparison. After 36 h of culture, the cells were fixed in paraformaldehyde (6 h) and then incubated with mouse anti-α-SMA antibody (1:200, Boster, China). The expression of α-SMA was detected using the SABC (mouse IgG)-Cy3 kit (1:100, Boster, China) and fluorescence microscopy.

Enzyme-linked immunosorbent assay for WISP-1 in the conditioned medium

CM was collected 12, 24 and 36 h after irradiation, and stored at −80°C until required. These time points matched those used for the co-culture experiments. The concentration of WISP-1 in the CM was measured using the WISP1 sandwich ELISA kit (BP-E11700, Shanghai Lengton Biological Technology Co. Ltd, China).

Statistical analysis

Each in vitro experiment was performed at least four times. Data are presented as the mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by the least significant difference (LSD) post-hoc test and Student's t-test were used to compare results between samples. P values < 0.05 were considered statistically significant.

Results

Morphological characteristics of HUMSCs and nHLFs cultured in vitro

Under normal conditions, both adherent nHLFs and passage-3 HUMSCs appeared spindle-shaped under light microscopy (Fig. 1A-D). However, after irradiation, nHLFs became rounded and more irregular in shape (Fig. 1E-H). Interestingly, co-culture with HUMSCs seemed to suppress these radiation-induced changes, with nHLFs retaining their spindle shape and forming contacts with other cells in the culture well (Fig. 1I, J).

Proliferation of nHLFs exposed to different doses of radiation

The MTT assay was used to examine the effects of increasing doses of radiation on the proliferation of nHLFs. The nHLFs were irradiated with 0, 5 or 8 Gy X-rays, and observations made 1, 2, 3, 4, 5 and 6 d after irradiation. Measurement of OD values revealed that the proliferation of non-irradiated nHLFs (0 Gy) increased from day 1 to day 5; this reflects the normal proliferative capacity of these cells (Fig. 2). In contrast to non-irradiated cells, nHLFs treated with 8 Gy of radiation showed significantly inhibited proliferative capacity. Interestingly, cells treated with 5 Gy of radiation displayed increased proliferation compared with non-irradiated cells (Fig. 2). Since the fibrosis that occurs in RILI is associated with increased proliferation of nHLFs (Rodemann and Bamberg 1995; Martin et al. 2000; Hu et al. 2006), we selected 5 Gy as the radiation dose for all subsequent experiments performed in this study.

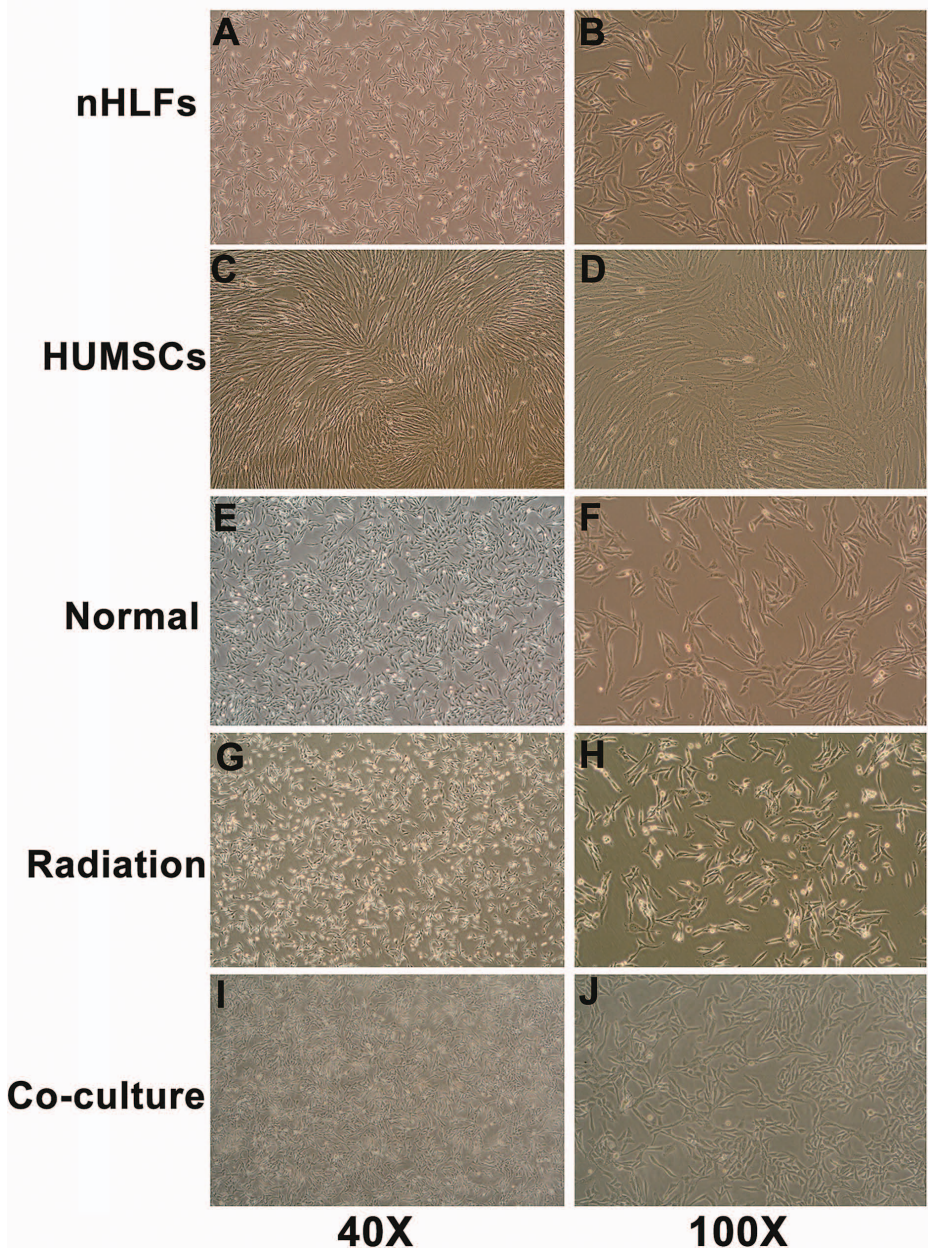

Fig. 1. Morphology of nHLFs and HUMSCs. The images show: nHLFs at 40× (A) and 100× (B) magnification; HUMSCs at 40× (C) and 100× (D) magnification; untreated nHLFs (Normal) at 40× (E) and 100× (F) magnification; nHLFs after radiation (Radiation) at 40× (G) and 100× (H) magnification; and nHLFs after co-culture with HUMSCs (Co-culture) at 40× (I) and 100× (J) magnification. Irradiation caused nHLFs to lose their normal spindle shape (E, F) and become rounded and more irregular in shape (G, H). Co-culture with HUMCs inhibited these effects of irradiation on nHLFs (I, J).

Radiation-mediated up-regulation of α-SMA expression in nHLFs is attenuated with HUMSCs

α-SMA is a marker of myofibroblasts that play an important role in lung fibrosis. To investigate whether radiation-induced differentiation of nHLFs into myofibroblasts was influenced by the co-culture of HUMSCs, we examined the expression of α-SMA. Western blot analysis showed that nHLFs exhibited increased α-SMA expression 24 h and 36 h after irradiation with 5 Gy of X-rays, as compared to non-irradiated controls (Fig. 3A). Immunofluorescence studies confirmed these findings (Fig. 3B). Importantly, co-culture with HUMSCs attenuated the degree of the radiation-induced up-regulation of α-SMA in nHLFs (Fig. 3).

Activation of the canonical Wnt/β-catenin pathway in irradiated nHLFs is attenuated with HUMSCs

An enhanced level of phosphorylated GSK-3β (p-GSK-3β) is widely used as a marker for activation of the Wnt/β-catenin signaling pathway. Western blot analysis showed that irradiation of nHLFs resulted in a progressive

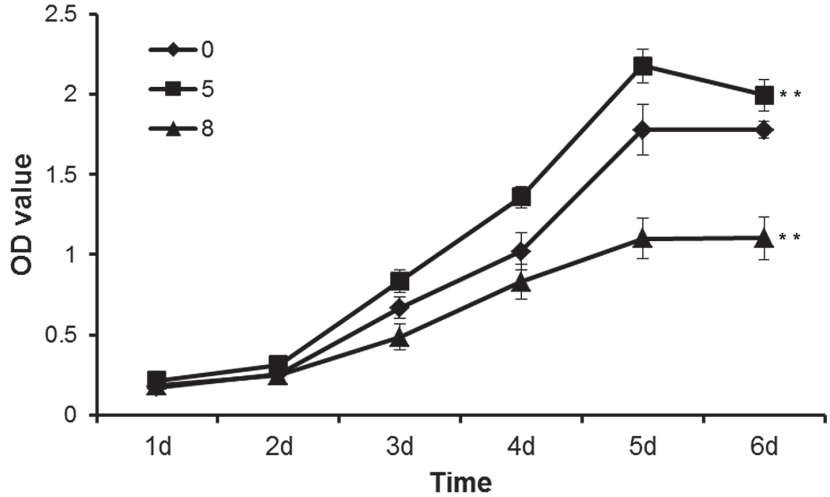

Fig. 2. Effects of increasing doses of radiation on the proliferation of nHLFs, measured using the MTT assay. Optical density (OD) values, determined over a 6-day period following irradiation of the nHLFs, are plotted. The progressive increase in the OD value for non-irradiated nHLFs reflects the normal proliferative capacity of these cells. Compared with no irradiation (0 Gy), 8 Gy of radiation inhibited the proliferation of nHLFs, while 5 Gy of radiation increased the proliferation of nHLFs. Data are presented as the mean ± SD (n = 6). $^{**}P < 0.01$ vs. 0 Gy of radiation.

increase in the level of p-GSK-3β in these cells (Fig. 4A), despite that total levels of GSK-3β were unaffected by irradiation. Co-culture of nHLFs with HUMSCs attenuated the radiation-induced elevation of p-GSK-3β levels (Fig. 4) in nHLFs. We also measured the expression level of FRAT1 that can enhance the Wnt/β-catenin signaling by antagonizing GSK-3β and stabilizing β-catenin. The expression of FRAT1 showed a transient decrease, with a reduction 12 h after irradiation, followed by a return to control levels at 24 h and 36 h (Fig. 4). In addition, co-culture with HUMSCs decreased FRAT1 protein levels in nHLFs.

Accumulation of β-catenin in the nuclei of nHLFs was observed 24 h and 36 h after irradiation, but not 12 h after irradiation (Fig. 5). Co-culture of nHLFs with HUMSCs attenuated the nuclear accumulation of β-catenin in nHLFs.

The gene for WISP-1, a secreted protein, is a downstream target of the canonical Wnt/β-catenin pathway. Therefore, we measured WISP-1 protein levels in the CM of nHLFs as an additional indicator of Wnt/β-catenin signaling. Irradiation of nHLFs increased the WISP-1 levels in the CM, relative to non-irradiated controls (Fig. 6). The radiation-induced increase in WISP-1 protein in the CM was attenuated in nHLFs by co-culture with HUMSCs. Taken together, our data show that irradiation of nHLFs leads to activation of the canonical Wnt/β-catenin signaling pathway.

Discussion

RILI is a major clinical problem for patients undergoing radiotherapy. Radiation induces changes in both epithelial cells and fibroblasts, and promotes the differentiation of fibroblasts into myofibroblasts. The accompanying secretion of cytokines can alter the extracellular matrix, cause inflammation, enhance tissue remodeling and promote fibrosis. Consistent with previous observations, the present study found that irradiated nHLFs showed increased expression of α-SMA, a marker of myofibroblasts that have an important role in lung fibrosis (Hu et al. 2006).

MSCs have shown therapeutic potential in various models of lung disease, including RILI (Villanueva et al. 1991; Xue et al. 2013). In one animal model of RILI, the beneficial effects of transplanted MSCs involved the regulation of inflammatory cytokines, inhibition of fibrosis, reduced epithelial cell apoptosis, and enhanced differentiation of alveolar epithelium (Xue et al. 2013; Wang et al. 2013). Various in vitro and in vivo studies have reported that MSCs secrete soluble factors that protect host cells against a variety of injuries (Rodman 2008; Brody et al. 2010). This implicates paracrine signaling as an important component of the beneficial effects of MSCs. For this reason, co-culture systems have been used widely to investigate the effects of MSCs on other cell types. Although there is evidence that co-culture with MSCs promotes fibroblast proliferation (Kim et al. 2007; Salazar et al. 2009), information is lacking concerning the effects of MSCs on irradiated lung fibroblasts. Here, we found that co-culture with HUMSCs attenuated the radiation-induced up-regulation of α-SMA in nHLFs. This suggests that HUMSCs protect against RILI via this mechanism.

Aberrant activation of Wnt/β-catenin signaling has been identified in the lungs of patients with idiopathic pulmonary fibrosis (IPF): specifically, β-catenin was detected in the nuclei of myofibroblasts in lung specimens from patients with IPF (Chilosi et al. 2003; Königshoff et al. 2008). Furthermore, inhibition of Wnt signaling (using β-catenin siRNA) can attenuate bleomycin-induced pulmonary fibrosis in mice (Kim et al. 2011). These observations, suggesting an important role for Wnt signaling in fibro-

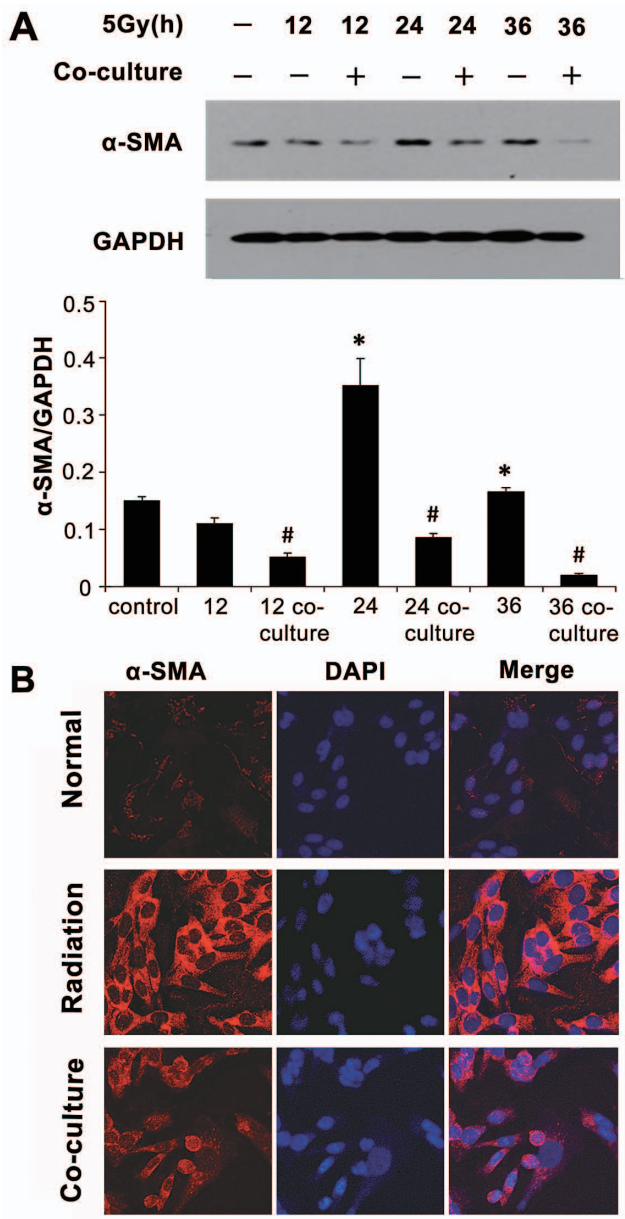

Fig. 3. Expression of α-SMA in untreated nHLFs (Normal group), irradiated nHLFs (Radiation group), and irradiated nHLFs co-cultured with HUMSCs (Co-culture group). nHLFs were harvested 12, 24 and 36 h after irradiation with 5 Gy of X-rays. (A) Representative Western blots and densitometric quantifications of α-SMA protein levels. The expression of GAPDH was used as an internal control. nHLF expression of α-SMA was increased 24 h and 36 h after irradiation, but this up-regulation was attenuated by co-culture with HUMSCs. Data are presented as the mean ± SD (n = 4). *P < 0.05 vs. the Normal group; #P < 0.05 vs. the Radiation group. (B) Immunofluorescence staining of α-SMA (red) and DAPI (blue; used as a counterstain for the nucleus) in the Normal group (top), Radiation group (middle) and Co-culture group (bottom) at 36 h after irradiation (magnification, 200×). Immunostaining of α-SMA was enhanced in the Radiation group compared with the Normal group, but was reduced in the Co-culture group compared with the Radiation group.

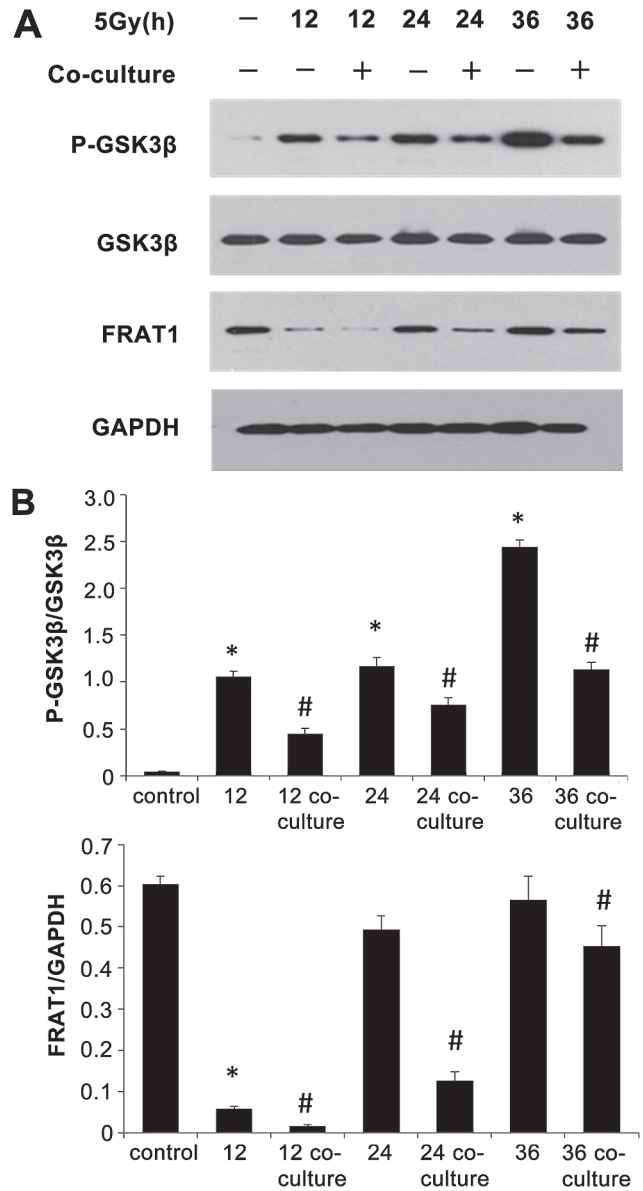

Fig. 4. Changes in p-GSK-3β and FRAT1 levels in non-irradiated and irradiated nHLFs. nHLFs were irradiated with 5 Gy of X-rays, and protein samples collected at 12, 24 and 36 h after irradiation. (A) Representative Western blots for FRAT1, p-GSK-3β and GSK-3β. The expression of GAPDH was used as an internal control. (B) Densitometric quantification of FRAT1, p-GSK-3β and GSK-3β. Irradiated nHLFs showed a progressive increase in p-GSK-3β levels, indicating activation of Wnt/β-catenin signaling. Co-culture with HUMSCs attenuated this radiation-mediated enhancement of p-GSK-3β levels in nHLFs. FRAT1 expression in nHLFs showed only a transient increase in response to irradiation. However, co-culture with HUMSCs reduced the protein levels of FRAT1 in nHLFs. Data are presented as the mean ± SD (n = 4). *P < 0.05 vs. the Normal group; #P < 0.05 vs. the Radiation group.

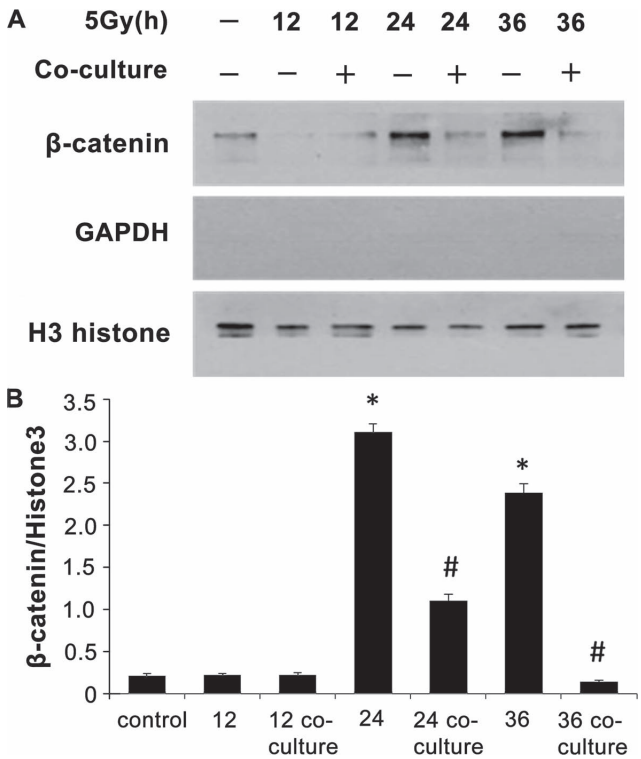

Fig. 5. Changes in nuclear β-catenin levels in non-irradiated and irradiated nHLFs. nHLFs were irradiated with 5 Gy of X-rays, and nuclear protein samples collected at 12, 24 and 36 h after irradiation. (A) Representative Western blots for β-catenin. Histone H3, which is normally expressed in the nucleus, served as the internal control. GAPDH is not expressed in the nucleus; the absence of its expression here was taken to indicate no contamination of the nuclear material with cytoplasm. (B) Densitometric quantification of β-catenin protein levels in the nuclei of nHLFs. Nuclear levels of β-catenin were increased 24 h and 36 h after irradiation, indicating activation of Wnt/β-catenin signaling. Co-culture with HUMSCs attenuated the radiation-mediated increase in nuclear β-catenin in nHLFs. Data are presented as the mean ± SD (n = 4). *P < 0.05 vs. the Normal group; #P < 0.05 vs. the Radiation group.

blasts, have been supported by other studies (Henderson et al. 2010).

MSCs have also been implicated in Wnt signaling. Salazar et al. (2009) observed that the CM of MSCs contained Wnt proteins that were capable of stimulating lung fibroblast proliferation through activation of the canonical Wnt/β-catenin signaling pathway. Another group have reported that canonical Wnt signaling is activated in nHLFs following irradiation, and that β-catenin mediates the effects of ionizing radiation on fibroblasts, acting to decrease the severity of radiation-induced soft tissue complications (Gurung et al. 2009).

Importantly, aberrant Wnt/β-catenin signaling contributes to RILI (Gurung et al. 2009). The associations between the Wnt pathway and MSCs (Salazar et al. 2009) were further examined in the present study. We found that irradiation of nHLFs enhanced Wnt signaling, consistent with earlier reports (Gurung et al. 2009). Importantly, co-culture of nHLFs with HUMSCs attenuated the effects of irradiation.

GSK-3β is a negative regulator of the canonical Wnt signaling pathway, and phosphorylation of GSK-3β inhibits its activity. Phosphorylated GSK-3β is increased in IPF (Königshoff et al. 2008) and in irradiated fibroblasts deficient in the ataxia telangiectasia mutated (ATM) protein kinase (Kwon et al. 2008). Consistent with these data, we observed that p-GSK-3β levels were increased in irradiated nHLFs, and this would be expected to result in β-catenin accumulation. Interestingly, studies of lung injury and fibrosis have found that β-catenin and other components of the pathway are up-regulated in samples of diseased lung (Liu et al. 2009). An important observation in our study was that the radiation-induced increases in p-GSK-3β levels were attenuated by co-culture with HUMSCs, suggesting that this effect may contribute to the protective effects of HUMSCs against radiation-mediated nHLF injury.

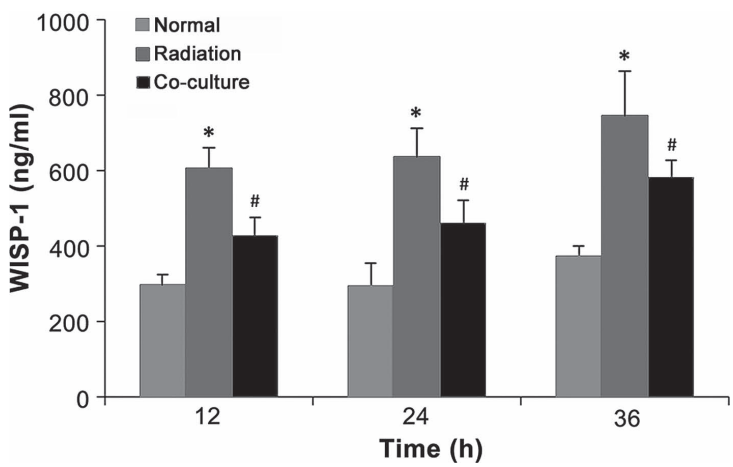

Fig. 6. Analysis of WISP-1 levels by enzyme-linked immunosorbent assay (ELISA). WISP-1 levels were increased in the CM of irradiated nHLFs (Radiation group), compared with non-irradiated controls (Normal group). Co-culture with HUMSCs attenuated this effect (Co-culture group). Data are presented as the mean ± SD (n = 5). *P < 0.05 vs. the Normal group; #P < 0.05 vs. the Radiation group.

FRAT activates canonical Wnt signal transduction by antagonizing GSK-3β and stabilizing β-catenin (van Amerongen and Berns 2005). In the present study, we did not find substantial differences in FRAT1 expression between irradiated and non-irradiated nHLFs. However, the decrease in FRAT1 expression was apparent when irradiated nHLFs were co-cultured with HUMSCs. Additional work is required to elucidate the underlying mechanisms.

WISP-1 is a Wnt target gene that is overexpressed in lung fibrosis (Pardo et al. 2005; Königshoff et al. 2009). Furthermore, inhibition of WISP-1 attenuates the progression of fibrosis in vivo (Königshoff et al. 2009). Here, we observed that WISP-1 levels were increased in the CM of irradiated nHLFs, and that this effect of radiation was inhibited by co-culture with HUMSCs. These findings suggest that enhanced WISP-1 expression contributes to radiation-induced lung injury, and that attenuation of this mechanism contributes to the protective effects of HUMSCs.

In conclusion, the present study has established that the presence of HUMSCs attenuates the Wnt signaling in irradiated nHLFs, thereby suppressing the differentiation of irradiated nHLFs into myofibroblasts. Thus, therapeutic application of HUMSCs may be helpful for attenuating the development and progression of fibrosis. In future, therapeutic targeting of Wnt signaling may prove a useful approach to limiting RILI in patients undergoing radiotherapy.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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