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Role of Wnt/β-Catenin Signaling in Epithelial Differentiation of Lung Resident Mesenchymal Stem Cells

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ABSTRACT

Accumulating evidence has demonstrated that stem cells have the ability to repair the lung tissue injuries following either injection of cultured cells or bone marrow transplantation. As a result, increasing attention has focused on the lung resident mesenchymal stem cells (LR-MSCs) for repairing damaged lung tissues. Meanwhile, some studies have revealed that Wnt/β-catenin signaling plays an important role in the epithelial differentiation of mesenchymal stem cells (MSCs). In the current study, our aim was to explore the roles of Wnt/β-catenin signaling on cell proliferation and epithelial differentiation of LR-MSCs. We have successfully isolated the stem cell antigen (Sca)-$1^{+}CD45^{-}CD31^{-}$ cells which were proposed to be LR-MSCs by magnetic-activated cell sorting (MACS). Furthermore, we demonstrated the expression of epithelial markers on LR-MSCs following indirect co-culture of these cells with alveolar epithelial type II (ATII) cells, confirming the epithelial phenotype of LR-MSCs following co-culture. In order to clarify the regulatory mechanisms of Wnt/β-catenin signaling in epithelial differentiation of LR-MSCs, we measured the protein levels of several important members involved in Wnt/β-catenin signaling in the presence or absence of some canonical activators and inhibitors of the β-catenin pathways. In conclusion, our study demonstrated that Wnt/β-catenin signaling may be an essential mechanism underlying the regulation of epithelial differentiation of LR-MSCs. J. Cell. Biochem. 116: 1532–1539, 2015.

KEY WORDS: LUNG RESIDENT MESENCHYMAL STEM CELLS (LR-MSCs); DIFFERENTIATION; ALVEOLAR EPITHELIAL TYPE II CELLS (ATII CELLS); WNT/β-CATENIN SIGNALING

It is generally believed that tissue-specific stem cells exist in most mammalian tissues. The function of these cells is to maintain tissue homeostasis by supplying new tissue-specific cells either during normal tissue cycling or when existing tissue cells are lost as a result of pathological development [da Silva Meirelles et al., 2006; Liu and Rando, 2011]. The pulmonary system contains a variety of cell populations that reside in distinct anatomical locations. Based on lung injury models it is suggested that the lung contains anatomically and functionally distinct stem cell populations, which may both participate in lung repair and contribute to pulmonary diseases. Furthermore, these studies have logically led to an increasing number of assumptions that LR-MSCs may be more efficient than bone marrow mesenchymal stem cells (BM-MSCs) from a therapeutic perspective [Gill et al., 2004].

Reparative processes in solid organs require mobilization of tissue-resident stem cells and migration of cells within the organs, which plays an essential role in both organized and disorganized repair. Substantial attention has recently focused on the use of lung resident

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mesenchymal stem cells (LR-MSCs) for regenerating damaged organs. A previous study reported the isolation of bronchoalveolar stem cells (BASCs) and suggested that BASCs are responsible for maintaining the homeostasis of bronchiolar and alveolar epithelial cells [Kim et al., 2005]. BASCs may be purified based on positive selection for stem cell antigen (Sca)-1 and CD34 and the absence of CD45 and CD31, the hematopoietic and endothelial cell markers [Kim et al., 2005]. BASCs can be isolated from mouse lung side population cells [Summer et al., 2007], and they express Sca-1, CD44 and CD106, and are negative for CD45 and CD31 [Summer et al., 2007]. These data suggest that the Sca-$1^{+}CD45^{-}CD31^{-}$ cells from adult mouse lungs are the LR-MSCs [Gong et al., 2014], and the LR-MSC population harbors one or more endogenous tissue stem cell populations with defined identity and characteristics of stem cells. Under in vitro co-culture conditions, rat airway epithelial cells can trigger the expression of several epithelial markers on BM-MSCs at an early stage [Wang et al., 2009].

The human lung MSCs (hLMSCs) have the ability to generate non-hematopoietic cells with characteristics of neuroectodermal and mesodermal lineages including neural cells, osteoblasts and sperm-like cells [Hegab et al., 2010]. The potential lung stem cells can differentiate into smooth muscle, bone, fat and cartilage [Summer et al., 2007] with characteristics of "stemness" including high telomerase activity [Summer et al., 2007; Martin et al., 2008]. Recent studies have demonstrated that LR-MSCs can differentiate into alveolar epithelial type II (ATII) cells [Gong et al., 2014]. The functional roles of the ATII cells have been expanded over the years from a surfactant factory to immunomodulation of the alveolus [Martin and Frevert, 2005]. ATII cells express Toll-like receptors, which play an important role in innate host defense of the lung [Armstrong et al., 2004]. In addition, ATII cells are the putative resident alveolar progenitors that can replace damaged alveolar epithelial Type II (ATII) cells after injury [Adamson and Bowden, 1974]. Lung-resident MSCs differ from bone marrow (BM)-derived MSCs with respect to cytokine and gene expression profiles, suggesting that these cells are distinct from those derived from the bone marrow [Badri et al., 2011]. LR-MSCs, thus, represent a reservoir of endogenous organ-specific adult progenitor cells with a potential role in local tissue homeostasis and repair [Jones and McGonagle, 2008]. However, little is known about their contributions because the majority of work studying MSCs in solid organs has previously focused on BM-MSCs. The ability of lung resident MSCs to interact with and modulate the local microenvironment remains to be investigated.

Wnt/β-catenin signaling has been suggested to be crucial in regulating embryonic development, cell proliferation and motility, cell fate determination, and the generation of cell polarity [Pinto and Clevers, 2005; Ling et al., 2009]. When extracellular Wnts upon secretion interact with the receptor protein Frizzled (Fzd) and LDL-receptor-related protein (LRP5/6), Wnts suppress the phosphorylation activity of glycogen synthase kinase (GSK)-3β, resulting in dissociation of β-catenin from the APC-Axin-GSK-3β complex. And β-catenin is accumulated in the nucleus where it associates with T cell factor/Lymphoid enhancer factor (TCF/Lef) transcription factors and induces transcription of target genes [Gordon and Nusse, 2006]. Accumulating evidence has supported a vital role that the Wnt/β-catenin pathway plays in the fate determination and differentiation of MSCs, including the differentiation of osteogenesis [Boland et al., 2004], chondrogenesis [Hartmann and Tabin, 2000], adipogenesis [Ross et al., 2000] and myogenesis [Shang et al., 2007]. The importance of Wnt/β-catenin pathway for epithelial stem cell self-renewal and differentiation has been explicitly demonstrated [De Langhe et al., 2005]. In the current study, our aim was to explore the roles of Wnt/β-catenin signaling on the proliferation and differentiation of LR-MSCs into ATII cells.

In this study, we isolated the LR-MSC (Sca-$1^{+}CD45^{-}CD31^{-}$) cells which were next co-cultured with ATII cells to induce differentiation of LR-MSCs into alveolar epithelial cells. To ascertain whether the Wnt/β-catenin pathway was involved in regulating epithelial differentiation of LR-MSCs, we investigated the protein expression levels of the key components of Wnt/β-catenin under co-culture conditions. The roles of β-catenin pathway in the epithelial differentiation of LR-MSCs were also examined.

MATERIALS AND METHODS

ATII CELL LINE CULTURE

The ATII cells were obtained from Yili Bio-technology Co. Ltd. (Shanghai, China). The cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and maintained in a humidified atmosphere of 5% CO_2/95% air at 37 °C. All of the cells were passaged 1:2 or 1:3 using 0.25% trypsin when they reached 70–90% confluence.

ISOLATION OF LUNG STEM CELLS

Lung single-cell suspensions were prepared from the lungs of at least 5 C57BL/6 mice (4–6 week old) as described [Hegab et al., 2010]. In brief, mice were sacrificed by cervical dislocation. The lung parenchyma from mice was digested by fine mincing with a razor blade, followed by incubation in an enzyme mixture containing 0.2% collagenase I (Sigma, St. Louis, MO), 2.4 U/ml dispase (Sigma, St. Louis, MO) and 0.001% DNAse (Sigma, St. Louis, MO) for 1 h at 37 °C with shaking. This suspension was filtered through 100-μm and 40-μm filters, centrifuged, and depleted of red blood cells by RBC lysis buffer. Cells were resuspended in PBS at 1×10^7 cells/ml and stained for Sca-1, CD45 and CD31 followed by sorting using the AutoMACS cell separator system (Miltenyi Biotec, Bergisch Gladbach, Germany).

Freshly isolated LR-MSCs were cultured at a concentration higher than 10^5/ml with DMEM containing 10% fetal bovine serum, 4% L-glutamine, 1% nonessential amino acids, and 1% penicillin and streptomycin, and maintained in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. The culture medium was changed every 48 h, and cells were passaged 1:2 using 0.25% trypsin when they reached 70–90% confluence.

FLOW CYTOMETRIC ANALYSIS

To sort the stem cells and determine the expression of various surface markers, LR-MSCs following the first passage were incubated with fluorescent antibodies at 37 °C for 40 min in the dark followed by two washes with PBS. Flow cytometry was performed on a FACS CaliburTM flow cytometer and the data were analyzed using the

Paint-A-Gate software (Becton Dickinson). The antibodies used were: FITC-conjugated anti-Ly-6A/E (Sca-1), PE-conjugated anti-CD45, PE-conjugated anti-CD90, PE-conjugated anti-CD44 and FITC-conjugated anti-CD106 (eBioscience, San Diego, CA); APC-conjugated anti-CD31 and PE-conjugated anti-CD29 (Bio-legend, San Diego, CA).

INDIRECT CO-CULTURE EXPERIMENTS

Indirect co-culture was established using cell culture inserts (0.4 μm PET, 4.5 cm^2, Millipore). LR-MSCs and ATII cells were each plated at 10^5/ml. LR-MSCs were plated in the lower chamber and ATII cells were plated in the upper chamber. On days 3, 7, and 14, inserts were removed and LR-MSCs were harvested for Western blotting, electron microscopy and immunocytochemistry analysis. Furthermore, for assessing the regulation of Wnt/β-catenin signaling, 10 ng/ml Wnt3a and 20 ng/ml Dickkopf -1 (DKK-1) (PeproTech, Inc., Rocky Hill, NJ) were added to the lower chamber of the co-culture [Glinka et al., 1998; Bafico et al., 2001]. Wnt3a belongs to the Wnt family proteins which can induce intracellular accumulation of β-catenin and increase translocation of β-catenin into the nucleus to promote gene expression [Gordon and Nusse, 2006]. DKK-1 can bind to LRP5/6, co-receptors for Wnt ligands, and thereby inhibits Wnt/β-catenin. LR-MSCs were cultured in four different regimens: (1) LR-MSCs and ATII cells; (2) LR-MSCs and ATII cells with Wnt3a; (3) LR-MSCs and ATII cells with DKK-1; (4) LR-MSCs alone as control.

IMMUNOFLUORESCENT STAINING AND WESTERN BLOT ASSAY

The immunofluorescence analysis was performed as described (Han, Gong, 2014). The following primary antibodies were employed: Rabbit anti-collagen I, rabbit anti-cytokeratin 18 (CK18), rabbit anti-cytokeratin 19 (CK19), rabbit anti-occludin and rabbit anti-SP-C (all from Abcam Inc. Cambridge, MA). Alexa Fluor 488- or 594-conjugated goat anti-rabbit (Invitrogen, Carlsbad, CA) was used as the secondary antibody. Nuclei were stained with 1 μg/ml DAPI (Sigma, St. Louis, MO). The images were captured using a confocal fluorescence microscope (Olympus, Tokyo, Japan). For Western blotting analysis, whole cell lysates were separated using SDS/12% PAGE and electrophoretically transferred to a PVDF membrane (Roche, Indianapolis, IN) by standard procedures. After the membranes had been blocked, primary antibodies were added to the membranes followed by incubation at 4 °C for 12 h. After three washes in PBST, the membranes were incubated with the secondary antibody at 37 °C for 1 h. The primary antibodies employed were: Rabbit anti-β-Catenin, rabbit anti-Phospho-β-Catenin (Cell Signaling Technology), rabbit anti-Phospho-GSK-3β (Cell Signaling Technology), rabbit anti-GSK-3β, rabbit anti-cytokeratin 18 (CK18), rabbit anti-cytokeratin 19 (CK19), rabbit anti-occludin, and mouse anti-β-actin (Abcam Inc., Cambridge, MA). Horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (Boster, Wuhan, China) was used as the secondary antibody. Immunoreactive protein bands were detected using an Odyssey Scanning System (LI-COR, Inc., Lincoln, NE).

IDENTIFICATION OF AUTOPHAGY BY TRANSMISSION ELECTRON MICROSCOPY

After co-culture for 3, 7 and 14 days, LR-MSCs were harvested by centrifugation, and cell aggregates were fixed with glutaraldehyde, dissected into sections and fixed overnight. Following dehydration with cold alcohol, sections were rinsed in propylene oxide, placed in embedding molds, and incubated in an oven at 64 °C overnight. 70-nm thin sections were prepared and examined with transmission electron microscopy (JEM-1200EX, Japan). The lamellar bodies were identified as a characteristic structure of autophagy.

STATISTICAL ANALYSIS

The data were shown as means ± SD. Statistical analyses were carried out using the SPSS software for windows version 11.0 (SPSS Inc, Chicago, IL). Values at $P < 0.05$ were considered as statistically significant.

RESULTS

ISOLATION AND EXPANSION OF THE LR-MSCS

We have previously demonstrated that the Sca-$1^+$$CD45^-$$CD31^-$ cells were LR-MSCs [Gong et al., 2014]. Therefore, the Sca-$1^+$$CD45^-$$CD31^-$ cells were sorted from the lung cells using MACS.

After culture in DMEM for 7 days, LR-MSCs were found to adhere to the culture surfaces with a long, thin and stellate morphology resembling BM-MSCs. As shown in Figure 1A–D, the LR-MSCs showed a homogeneous fibroblast-like and spindle-shaped morphology after several passages.

LR-MSC CULTURE AND PHENOTYPE IDENTIFICATION

To further evaluate whether the Sca-$1^+$$CD45^-$$CD31^-$ cells were LR-MSCs, the expression by the sorted cells of markers that characterize LR-MSCs was examined by flow cytometry. Indeed, the sorted cells expressed CD44, CD90, CD106, CD29 and Sca-1, but not CD45 or CD31 (Fig. 1E), confirming that these cells were LR-MSCs.

MORPHOLOGICAL CHANGES OF DIFFERENTIATED LR-MSCS

Co-culture of LR-MSCs with ATII cells for 3 days did not induce obvious morphological changes of LR-MSCs (Fig. 2A). After 7 days of co-culture, about 10% of LR-MSCs showed remarkable morphological changes, as evidenced by changing from a typical fibroblast-like spindle appearance to a round, polygonal morphology. When cultured with ATII cells for 14 days, more than 75% of the LR-MSCs presented an epithelia-like cuboidal cell shape more resembling a monolayer culture of epithelial cells, while the number of cells with the LR-MSC morphology was decreased at the same time. The lamellar bodies, a characteristic structure found only in ATII cells, were visualized by transmission electron microscopy (Fig. 2C).

EPITHELIAL MARKER EXPRESSION IN DIFFERENTIATED LR-MSCS

The epithelial marker expression by LR-MSCs following co-culture with ATII cells for 3, 7 and 14 days was determined by immunofluorescence. As shown in Figure 2B, co-culture of LR-MSCs with ATII cells for 7 days induced the appearance of SP-C, CK18 and occludin in some LR-MSCs. Extended culture for 14 days resulted in a more pronounced increase in the protein expression levels of these epithelial markers in LR-MSCs.

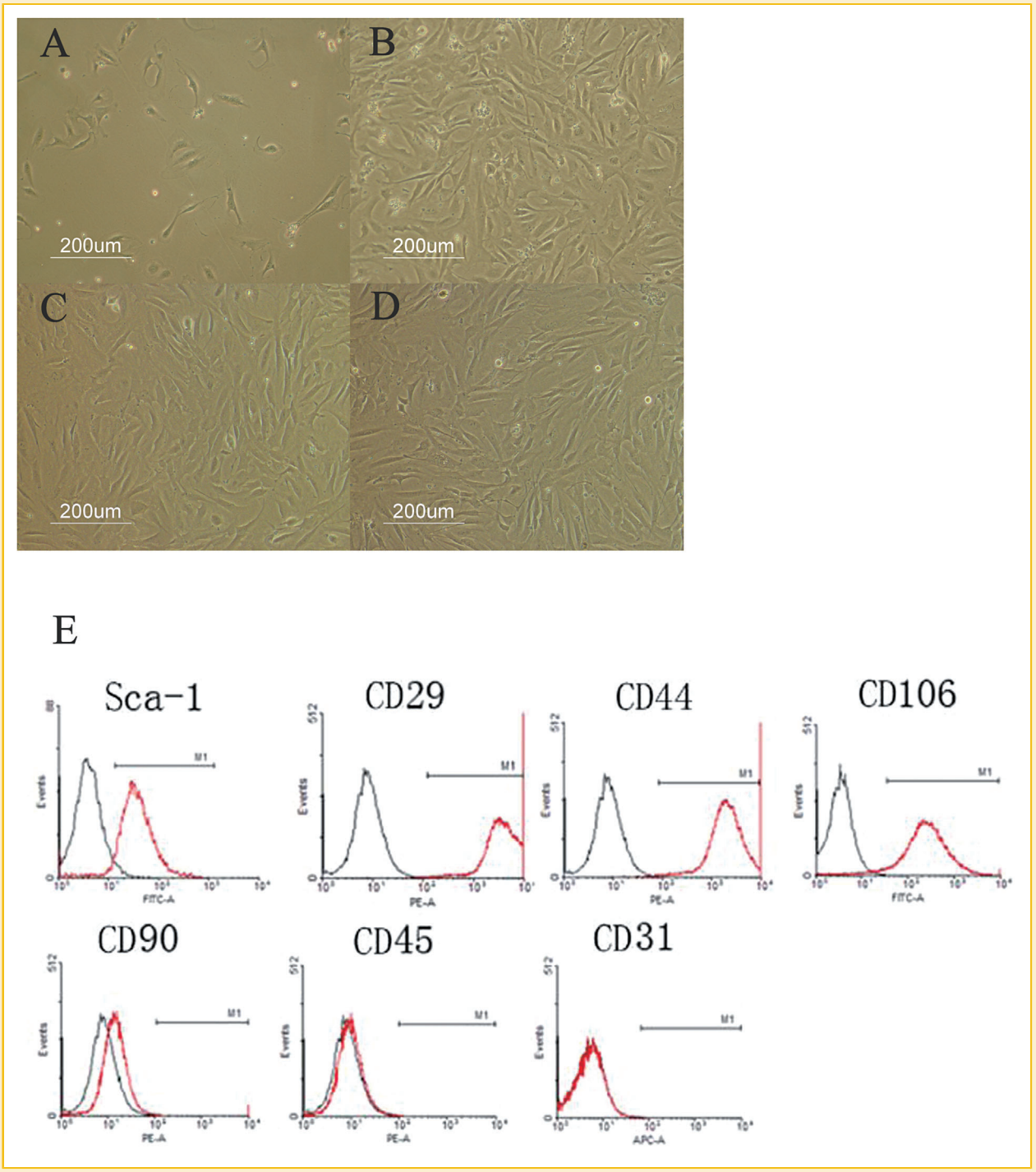

Fig. 1. Morphology and flow cytometric analysis of lung resident mesenchymal stem cells (LR-MSCs). (A, B) Morphology of LR-MSCs cultured in low-glucose DMEM for 3 days (A) and 7 days (B) following isolation by MACS from single-cell suspensions derived from lung tissues was shown. (C, D) Morphology of LR-MSCs in the 3rd (C) and 5th (D) passage was shown by phase-contrast microscopy. (E) Flow cytometric analysis of LR-MSCs in the first passage confirmed the expression of Sca-1, CD29, CD44, CD106 and CD90, but not CD31 or CD45, on LR-MSCs. Black line, isotype control staining; red line, specific antibody staining.

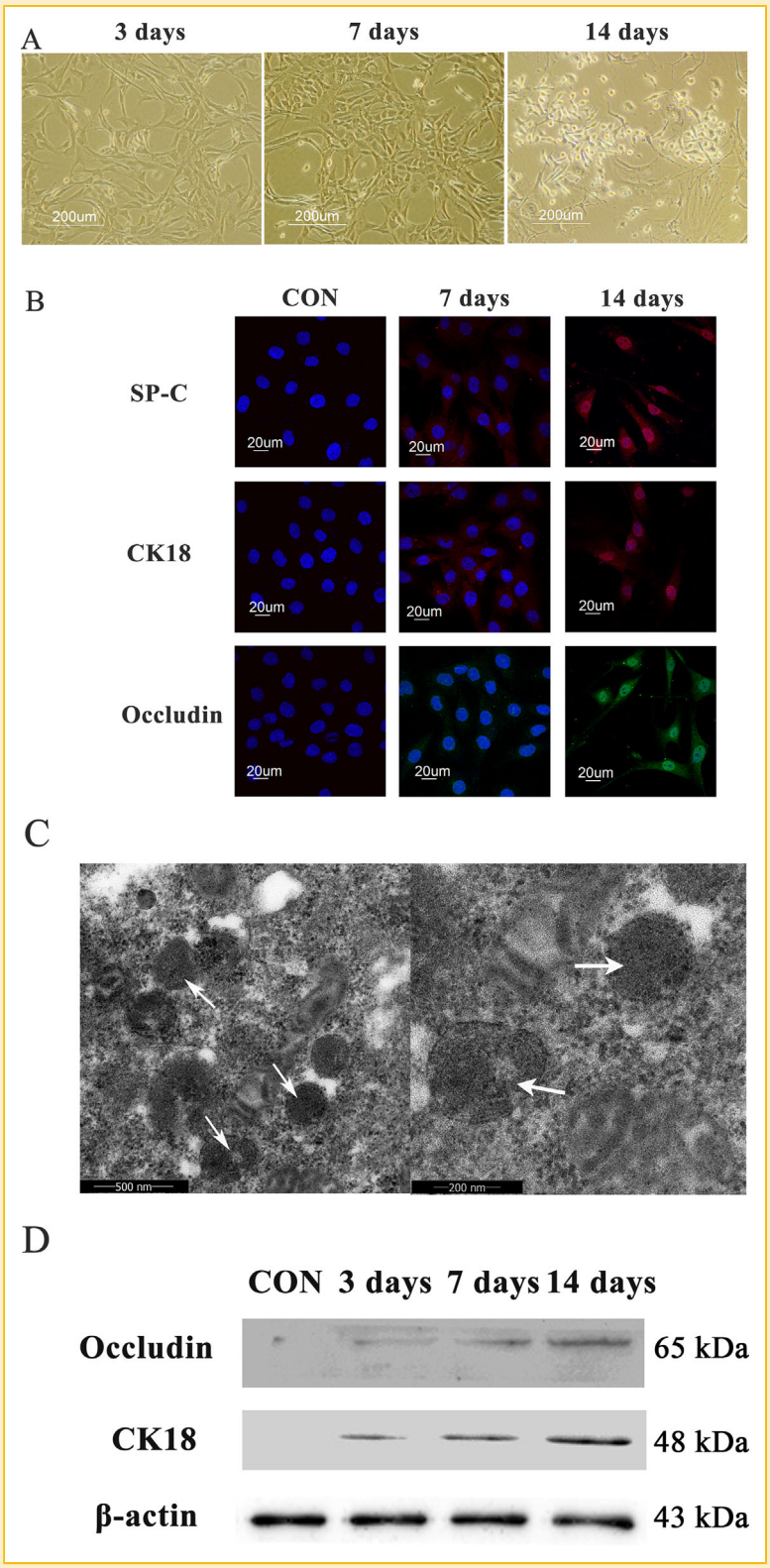

Fig. 2. Morphological changes and expression of markers of epithelial differentiation on lung resident mesenchymal stem cells (LR-MSCs) co-cultured with alveolar epithelial type II cells (ATII cells). (A) Phase-contrast images of the epithelial differentiation of LR-MSCs for 3, 7 and 14 days after co-culture were shown. (B) SP-C, cytokeratin 18 and occludin expression in LR-MSCs co-cultured with ATII cells for 0 (Con), 7 and 14 days was analyzed by immunofluorescent staining. SP-C and cytokeratin 18 were revealed with secondary Alex594-labeled antibodies; occludin with secondary Alex Fluor 488-labeled antibodies; nuclear staining with DAPI. (C) Ultrastuctural (lamellar bodies shown by electron microscopy) features of ATII cells were shown (white arrow). (D) Detection of cytokeratin 18 and occludin expression in LR-MSCs co-cultured with ATII cells for 0 (Con), 7 and 14 days were carried out by Western blotting. β-actin was used as an internal control.

The epithelial marker expression in LR-MSCs following induction for 3, 7 and 14 days was also quantified by Western blotting (Fig. 2D), which supported the immunofluorescence data, confirming that long-term co-culture of LR-MSCs with ATII cells could induce the expression of multiple epithelial markers.

EXPRESSION OF WNT/β-CATENIN SIGNALING COMPONENTS DURING EPITHELIAL DIFFERENTIATION OF LR-MSCS

The expression of Wnt/β-catenin signaling components during epithelial differentiation of LR-MSCs was analyzed by Western blotting. Up-regulation of β-catenin and down-regulation of GSK-3β was observed in LR-MSCs with extended co-culture from 3 to 7 days (Fig. 3). However, further prolongation of the co-culture reversed the trend. The expression levels of β-catenin decreased following co-culture for 14 days compared to the levels after 7 days. Again, GSK-3β expression followed an opposite trend compared with the regulation of β-catenin levels (Fig. 3). Furthermore, the phosphorylation levels of both β-catenin and GSK-3β in stimulated LR-MSCs showed opposite trends compared with their respective protein modulation (Fig. 3).

INHIBITION OF WNT/β-CATENIN SIGNALING PROMOTES EPITHELIAL DIFFERENTIATION OF LR-MSCS

To reveal the mechanisms by which Wnt/β-catenin signaling regulates epithelial differentiation of LR-MSCs, Wnt3a or DKK-1 were added to the co-culture of LR-MSCs with ATII cells.

The addition of Wnt3a increased the expression levels of β-catenin following incubation for 3, 7 and 14 days. Conversely, DKK-1 decreased the expression levels of β-catenin in these conditions. The expression level of GSK-3β increased. Meanwhile, the results indicated that the phosphorylation expression of β-catenin and GSK-3β presented a reverse trend compared to their respective protein expression (Fig. 4A–C).

Next, we investigated the morphologic effects of Wnt3a and DKK-1 on the epithelial differentiation of LR-MSCs following co-culture

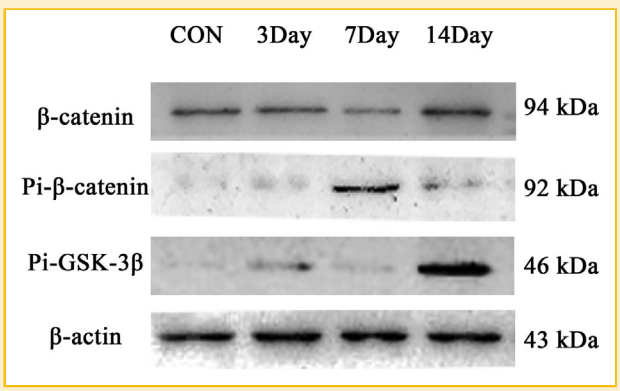

Fig. 3. Co-culture with alveolar epithelial type II (ATII) cells induced the regulation of proteins at several important nodes of Wnt/β-catenin signaling pathways in lung resident mesenchymal stem cells (LR-MSCs). Both the protein expression and phosphorylation levels of GSK-3β and β-catenin in LR-MSCs co-cultured with ATII cells for 0 (Con), 3, 7 and 14 days were determined by Western blotting. β-actin was used as an internal control.

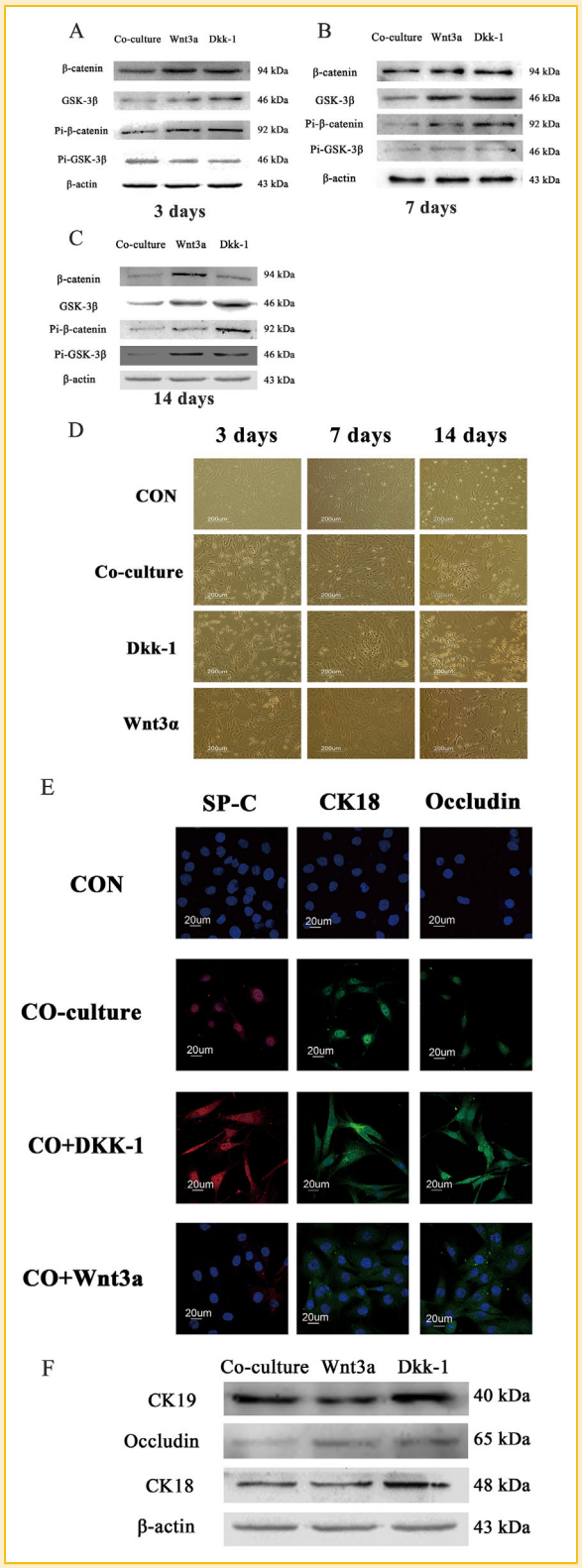

Fig. 4. The effect of Wnt3a (10 ng/ml) and DKK-1 (20 ng/ml) on epithelial differentiation of lung resident mesenchymal stem cells (LR-MSCs). (A–C) Both the protein and phosphorylation levels of β-catenin and GSK-3β in LR-MSCs treated with Wnt3a and DKK-1 for 3, 7 and 14 days following co-culture with alveolar epithelial type II(ATII) cells were determined by Western blotting. (D) Wnt3a and DKK-1 induced morphological changes of LR-MSCs following co-

for 7 days. As demonstrated in Figure 4D, Wnt3a induced remarkable morphologic changes in LR-MSCs without exhibiting epithelial-like changes. The cell morphology was significantly different from that of the control in the absence of co-culture. DKK-1 was able to stimulate epithelial differentiation of LR-MSCs by blocking the Wnt/β-catenin signaling.

We also measured the expression levels of epithelial markersSP-C, CK18 and occluding which demonstrated similar regulation patterns. Wnt3a caused a distinct decrease in the expression levels of SP-C, CK18 and occludin. In contrast, DKK-1 was capable of up-regulating these three markers on LR-MSCs (Fig. 4E). The findings on the regulation of CK18, CK19 and occludin using immunofluorescence staining were confirmed by Western blotting (Fig. 4F). In conclusion, the activation of Wnt/β-catenin signaling induced by Wnt3a prevented epithelial differentiation in LR-MSCs. Conversely, the inhibition of the Wnt/β-catenin pathway by DKK-1 promoted epithelial differentiation of LR-MSCs.

In contrast, LR-MSCs upon treatment with Wnt3a without co-culture up-regulated β-catenin, and the treatment promoted the differentiation of LR-MSCs to a myofibroblastic but not an epithelial morphology (data not shown). This finding is consistent with other reports[Akhmetshina et al., 2012]. Furthermore, DKK had no significant effects on LR-MSCs in terms of either morphological changes or the regulation of the relevant proteins (data not shown).

DISCUSSION

In this study, LR-MSCs ($Sca\text{-}1^{+}CD45^{-}CD31^{-}$) were isolated efficiently from mouse lungs by MACS. It has been previously demonstrated that LR-MSCs undergo extensive self-renewal and can differentiate into endothelial, Clara, ATI and ATII cells, as well as cells of mesenchymal origin in vitro [Hegab et al., 2010]. The roles and fate of LR-MSCs during development in both physiological and pathological conditions in the lung have attracted substantial attention. Progress in stem cell biology suggests that the distal lung harbors various other cells with progenitor properties, including endothelial progenitor cells, side-population cells and bronchoalveolar stem cells [Borthwick et al., 2001; Engelhardt, 2001; Otto, 2002; Summer et al., 2007]. During embryogenesis, lung-specific mesenchyme is crucial for epithelial differentiation and morphogenesis [Maeda et al., 2007], and in vitro co-culture of embryonic stem cells with lung mesenchyme promotes their differentiation to alveolar epithelium [Vranken et al., 2005]. A recent study demonstrated that lung MSCs are multipotent vascular precursors, a population that has the capacity to participate in vascular remodeling and the function of these MSCs is likely subject to regulation in part by the Wnt/β-catenin signaling pathway [Chow et al., 2013]. Human lung-resident MSCs have been recently demonstrated to secrete KGF, an important epithelial growth factor, suggesting that human lung-resident MSCs can possibly modulate type II alveolar epithelial cells (AECs) in a paracrine fashion. Furthermore, it is also demonstrated that human lung-resident MSCs may establish gap junctional communication with epithelial cells, thereby permitting direct intercellular transfer of molecules [Badri et al., 2011]. In the present work, we demonstrated for the first time that co-culture of LR-MSCs with ATII cells could promote the differentiation of LR-MSCs into alveolar epithelial type II cells by impacting the signaling pathways.

LR-MSCs cultured alone did not express cytokeratins or other markers of epithelial differentiation. Furthermore, treatment with Wnt3a in the absence of co-culture promoted the differentiation of LR-MSCs to a myofibroblastic but not an epithelial morphology. DKK had no significant effects on LR-MSCs without co-culture. Only under co-culture conditions, did the morphology of LR-MSCs gradually become cubical or polygonal, resembling ATII cells. These observations are consistent with the prediction that appropriate local environmental factors are required for the differentiation of stem cells. ATII cells may be able to provide essential stimuli for regulating epithelial differentiation of LR-MSCs.

Considerable evidence suggests a critical role of Wnt/β-catenin signaling in cell fate decision of mesenchymal stem cells [Ishii et al., 2008]. A recent study demonstrated that airway epithelial cells can trigger epithelial differentiation of BM-MSCs, which can be promoted by blocking Wnt/β-catenin signaling [Wang et al., 2009]. In the current study, we demonstrated that β-catenin was up-regulated in LR-MSCs during initial co-culture, and that Wnt/β-catenin signaling was involved in the regulation of epithelial differentiation of LR-MSCs under co-culture conditions.

The canonical concept supports that Wnt/β-catenin signaling keeps stem cells in a self-renewal and undifferentiated state [Boland et al., 2004; Cho et al., 2006]. Exogenous application of Wnt3a to cell cultures expands the multipotential population of MSCs as well as human adipose-derived stem cells due to increased self-renewal and decreased apoptosis [Boland et al., 2004]. The proliferative effect of Wnt3a on MSCs is presumably achieved through up-regulation of *cyclin D1* and *c-Myc*, both of which drive cell cycle progression and promote cell growth [Baek et al., 2003]. In the present study, we focused on the roles of Wnt/β-catenin signaling in the epithelial differentiation capacity of LR-MSCs. Under co-culture conditions, DKK-1 was able to reduce the intracellular expression of β-catenin in LR-MSCs. Thus, it is possible to prevent LR-MSCs from re-entering cell cycle and subsequent proliferation to promote the epithelial differentiation of LR-MSCs through antagonizing the canonical Wnt/β-catenin pathway. Furthermore, GSK-3β plays a key role in the down-regulation of Wnt/β-catenin signaling by phosphorylation and inactivation of β-catenin. The GSK-3β activity is mediated through a phosphorylation-dependent mechanism, with the phosphorylation occurring at a serine residue in the N-terminus [Wang et al., 2009]. These findings suggest intense communication between Wnt/β-catenin signaling and the epithelial differentiation of LR-MSCs. However, LR-MSCs can also differentiate into myofibroblasts in response to profibrotic factors such as transforming growth factor (TGF)-β and an altered profibrotic phenotype is noted in LR-MSCs derived from lungs in diseases such as bronchiolitis obliterans [Walker et al., 2011]. Above all, these results indicate a complex mechanism by which canonical Wnt signaling regulates the differentiation of LR-MSCs into epithelial cells and further studies are needed to clarify the detailed mechanisms.

In summary, under in vitro co-culture conditions, ATII cells can induce the expression of several epithelial markers on LR-MSCs. Wnt/β-catenin signaling is involved in the differentiation of LR-

MSCs into the epithelial lineage. The blocking of Wnt/β-catenin pathway may promote the differentiation of LR-MSCs towards alveolar epithelial cells.

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