Activation of Wnt/β-Catenin-p130/E2F4 promotes the differentiation of bone marrow-derived mesenchymal stem cells into type II alveolar epithelial cells through cell cycle arrest

XIWEN ZHANG, MING XUE, AIRAN LIU, HAIBO QIU and FENGMEI GUO

Jiangsu Provincial Key Laboratory of Critical Care Medicine, Department of Critical Care Medicine, Zhongda Hospital, School of Medicine, Southeast University, Nanjing, Jiangsu 210009, P.R. China

Received December 12, 2022; Accepted April 18, 2023

DOI: 10.3892/etm.2023.12029

Abstract. The results of our previous study demonstrated that activation of the Wnt/β-catenin pathway increased the differentiation of mesenchymal stem cells (MSCs) into type II alveolar epithelial (AT II) cells; however, the specific mechanisms remain unclear. The present study aimed to evaluate the role of Wnt/β-catenin-p130/E2F transcription factor 4 (E2F4) in regulating the differentiation of mouse MSCs (mMSCs) into AT II cells, and to determine the specific mechanisms. mMSCs with p130 or E2F4 overexpression were constructed using lentiviral vectors. Differentiation of mMSCs into AT II cells was promoted using a modified coculture system with murine lung epithelial-12 cells incubated in small airway growth medium for 7-14 days. The differentiation efficiency was detected using immunofluorescence, western blot analysis and transmission electron microscopy. To detect the association between the canonical Wnt pathway and p130/E2F4, 4 mmol/l lithium chloride (LiCl) or 200 ng/ml Dickkopf-related protein 1 (DKK-1) was also added to the coculture system. Following differentiation, the cell cycle of mMSCs was evaluated using flow cytometry. The results of the present study demonstrated that surfactant protein C (SP-C) protein expression was higher in the p130 overexpression (MSC-p130) and E2F4 overexpression (MSC-E2F4) groups compared with the normal control mMSCs group following differentiation into AT II cells. Similar results for SP-C protein expression and lamellar body-like structures were also observed using immunofluorescence analysis and electron microscopy. Following the addition of LiCl into the coculture system for activation of the Wnt/β-catenin signaling pathway, phosphorylated (p)-p130/p130 was slightly decreased at 7 days and E2F4 was increased both at 7 and 14 days in mMSCs. Furthermore, the p-p130/p130 ratio was significantly increased at 14 days and E2F4 was decreased both at 7 and 14 days following DKK-1-mediated inhibition of the Wnt pathway. The results of the present study demonstrated that the numbers of cells in G_1 and S phases were increased following activation of the Wnt pathway and decreased following Wnt pathway inhibition. However, the number of cells in G_1 phase was increased following the differentiation of mMSCs overexpressing p130 or E2F4. Therefore, the results of the present study revealed that the canonical Wnt signaling pathway may affect the differentiation of MSCs into AT II cells via regulation of downstream p130/E2F4. The specific mechanisms may be associated with G_1 phase extension in the cell cycle of MSCs.

Correspondence to: Professor Fengmei Guo, Jiangsu Provincial Key Laboratory of Critical Care Medicine, Department of Critical Care Medicine, Zhongda Hospital, School of Medicine, Southeast University, 87 Dingjiaqiao Road, Gulou, Nanjing, Jiangsu 210009, P.R. China
E-mail: fmguo2003@139.com

Abbreviations: ARDS, acute respiratory distress syndrome; AT II cells, type II alveolar epithelial cells; BSA, bovine serum albumin; DAPI, 4,6-diamidino-2-phenylindole; DKK-1, Dickkopf-related protein 1; DMEM/F12, Dulbecco's modified Eagle's media/nutrient mixture F-12; FBS, fetal bovine serum; GFP, green fluorescent protein; GSK 3β, glycogen synthase kinase 3β; LiCl, Lithium chloride; MLE-12, murine lung epithelial-12; mMSCs, mouse mesenchymal stem cells; MSCs, mesenchymal stem cells; MSC-E2F4, mesenchymal stem cells overexpressing E2F transcription factor 4; MSC-NC, mesenchymal stem cells carrying green fluorescent protein (normal control); MSC-p130, mesenchymal stem cells overexpressing p130; PBS, phosphate-buffered saline; pRb, retinoblastoma gene product; Rb, retinoblastoma; RIPA, radioimmunoprecipitation assay; SAGM, small airway growth medium; SP-C, surfactant protein C

Key words: MSCs, p130/E2F transcription factor 4, canonical Wnt pathway, differentiation, AT II cells, cell cycle

Introduction

Mesenchymal stem cells (MSCs), exhibit a high degree of self-renewal and multipotential differentiation, and are widely used in regenerative medicine (1,2). Further investigations into the molecular mechanisms underlying the therapeutic effects of MSCs may lead to increased clinical application of cell-based therapy, and may aid in uncovering the MSC directional differentiation at the molecular level (3). These investigations require precise modulation of the signaling

pathways involved in MSC differentiation, in which the Wnt signaling pathway is one of the key players (4).

The canonical Wnt signaling pathway depends on the accumulation of β-catenin, and is one of the fundamental pathways in cell proliferation and migration, cell fate determination, polarization during embryonic development and tissue homeostasis (5). The canonical Wnt signaling pathway also exerts critical effects on the self-renewal and differentiation of MSCs (6). Results of a previous study demonstrated that lithium chloride (LiCl), an antagonist of glycogen synthase kinase 3β (GSK 3β), reduces the degradation of β-catenin through phosphorylation of GSK 3β at Ser9, subsequently activating the canonical Wnt-β-catenin pathway (7). In addition, Dickkopf-related protein 1 (DKK-1), a member of the Dickkopf family, secretes proteins with two cysteine-rich domains separated by a linker region. Thus, DKK-1 antagonizes canonical Wnt signaling through inhibiting the interaction of low-density lipoprotein receptor-related proteins 5/6 with Wnt (8).

Results of a previous study demonstrated that bone marrow-derived MSCs develop the immunophenotypic and ultrastructural characteristics of type II alveolar epithelial (AT II) cells when cocultured with lung tissue (9). Moreover, MSCs protect against hyperoxic lung injury through increasing the number of distal lung epithelial cells and alveolar differentiation, and decreasing self-renewal (10).

Results of our previous study demonstrated that exogenous addition of Wnt3a or LiCl activated the canonical Wnt/β-catenin pathway, and differentiation of MSCs into AT II cells was promoted using a modified coculture system with murine lung epithelial-12 (MLE-12) cells incubated in small airway growth medium (SAGM) *in vitro*. However, DKK-1-mediated inhibition of the canonical Wnt pathway reduced the expression of AT II-associated markers (11). Moreover, the downstream molecular mechanism by which the canonical Wnt signaling pathway regulates the differentiation of MSCs into AT II cells during mitosis remains unclear.

In the majority of cell lines, checkpoints that regulate cell cycle progression and cell differentiation occur in the G_1/G_0 phase. p130, as a member of the retinoblastoma gene product (pRb) family, forms a repressor complex with the transcription repressor E2F4, initiating the G_1 to S phase transformation in the cell cycle, or maintaining cell cycle arrest in G_0/G_1 phase (12). Activation of the Wnt/β-catenin pathway in mouse MSCs (mMSCs) was associated with the accumulation of β-catenin and pRb family members during MSC cycle progression (13). In addition, results of our previous study revealed that p130 or E2F4 overexpression in mMSCs improved osteogenesis, and inhibited adipogenesis and chondrogenesis through regulating the G_1 phase (14). However, whether the directional differentiation of MSCs and activation of Wnt/β-catenin resulted from the p130/E2F4 pathway remains to be fully elucidated.

Thus, we hypothesized that activation of the canonical Wnt/β-catenin signaling pathway in MSCs may affect the p130/E2F4 pathway through regulating the cell cycle and participating in the differentiation into AT II cells.

Materials and methods

Mesenchymal stem cell transduction and culture. Bone marrow-derived mMSCs of C57BL/6 mice were purchased from Cyagen Biosciences, Inc. These MSCs were isolated from the bone marrow of the femurs of C57BL/6 mice. They were uniformly positive for CD29, CD44, and Sca-1 antigens and negative for CD117 and CD31 antigens, and possessed the potential to differentiate into osteocytes, adipocytes, and chondrocytes, as demonstrated in the instructions provided by the supplier. The transduction of mMSCs using lentiviral vectors has been described in our previous study (14). Following transduction, mMSCs tagged with green fluorescent protein (GFP), p130 or E2F4 with GFP; namely, normal control mMSCs (MSC-NC), MSCs overexpressing p130 (MSC-p130) or MSCs overexpressing E2F3 (MSC-E2F4), were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12; Wisent, Inc.) containing 10% foetal bovine serum (FBS; Wisent, Inc.) and 1% antibiotic-antimycotic solution (streptomycin, penicillin and amphotericin B; Wisent, Inc.). Following incubation at 37˚C in a humidified atmosphere with 5% CO_2, cells at passages 6-10 were used for *in vitro* experiments. MLE-12 cells purchased from Xiangfbio Ltd. were cultured in DMEM/F12 with 2% FBS and 1% antibiotic-antimycotic solution (Wisent, Inc.) in a humidified 5% CO_2 incubator at 37˚C.

Differentiation of mMSCs into AT II cells. The coculture system for induced differentiation of mMSCs into AT II cells was described in our previous study (11). Briefly, mMSCs were indirectly cocultured with MLE-12 cells in SAGM (Lonza Group, Ltd.). SAGM was supplemented with 0.5 mg/ml bovine serum albumin (BSA), 30 mg/ml bovine pituitary extract, 0.5 mg/ml hydrocortisone, 0.5 ng/ml epithelial growth factor, 0.5 mg/ml epinephrine, 5 mg/ml insulin, 6.5 ng/ml triiodothyronine, 10 mg/ml transferrin and 0.1 ng/ml retinoic acid. A total of $1x10^4$ mMSCs in 1.5 ml DMEM/F12 supplemented with 10% FBS were seeded in each well of six-well plates. Subsequently, Transwell inserts (0.4-mm pore size, 4.5 cm^2; Corning, Inc.) loaded with $1x10^4$ MLE-12 cells in 1 ml DMEM/F12 supplemented with 10% FBS were added to establish the coculture system. When mMSCs reached 80% confluence after 3 days incubation, the culture medium was replaced with SAGM. Following a further 7 or 14 days, the inserts were removed, and the mMSCs were harvested for subsequent analysis. All of the incubation steps above were at 37˚C in a humidified atmosphere with 5% CO_2. To investigate the role of canonical Wnt signaling in differentiation, 4 mmol/l LiCl (Sigma-Aldrich; Merck KGaA) or 200 ng/ml DKK-1 (PeproTech, Inc.) was used to activate or inhibit the canonical Wnt pathway, respectively. LiCl or DKK-1 were added in the cocultured conditions prior to the addition of SAGM, according to a previous study (11).

Immunofluorescent staining. To analyse the differentiation of mMSCs into AT II cells *in vitro*, immunofluorescent staining was performed. Briefly, following induced differentiation, mMSCs were fixed in 4% paraformaldehyde at 37˚C for 30 min. Following lysis with 0.5% Triton X-100 and blocking with 3% BSA (Roche Diagnostics) for 30 min at room temperature, cells were stained with surfactant protein C (SP-C) primary antibodies (1:100, Santa Cruz Biotechnology, Inc. cat. no. sc-13979) at 4˚C overnight. Cells were subsequently incubated with goat anti-rabbit Alexa Fluor® 647 secondary antibodies (1:250; Abcam; cat. no. ab150079) in 2% BSA for

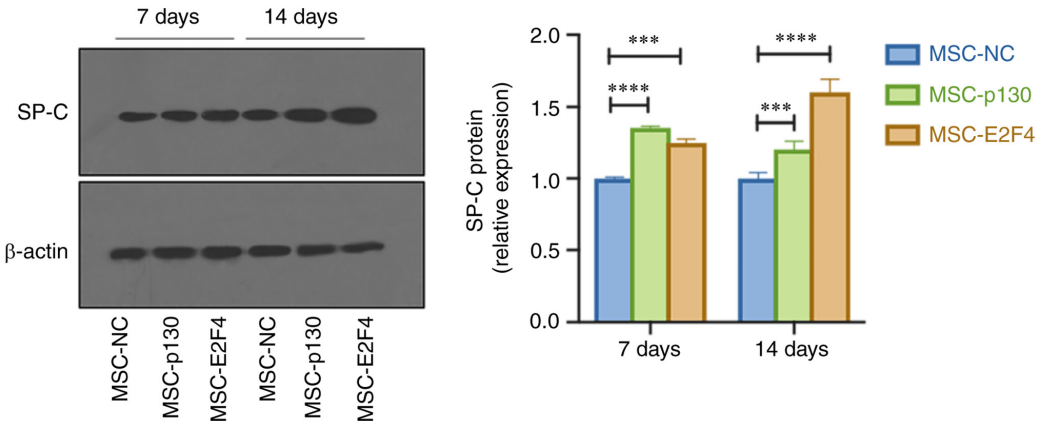


Figure 1. SP-C expression following induced differentiation of MSC-p130 and MSC-E2F4 into type II alveolar epithelial cells *in vitro* (n=3). ***P<0.001, ****P<0.0001 vs. MSC-NC group. MSCs, mouse mesenchymal stem cells; MSC-p130, MSCs overexpressing p130; MSC-E2F4, MSCs overexpressing E2F transcription factor 4; NC, normal control; SP-C, surfactant protein C.

30 min at room temperature in the dark. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Beyotime Institute of Biotechnology) at room temperature for 5-10 min. Images were captured using fluorescence microscopy (Olympus Corporation).

Transmission electron microscopy assay. Trypsin-digested cells were rinsed in phosphate-buffered saline (PBS) and pelleted using centrifugation. Cells were subsequently fixed in 1 ml PBS containing 2.5% glutaraldehyde and 1% osmium tetroxide at 4˚C overnight. Samples were dehydrated in gradient ethanol and embedded in an Araldite resin block at 37, 42, and 60˚C for 24 h each. Ultrathin sections of 80 nm excised from the block were stained with uranyl acetate at room temperature for 3 min prior to imaging with a transmission electron microscope (Hitachi H-7650).

Protein expression of SP-C and p130/E2F4 in MSCs. To analyse the expression of SP-C, phosphorylated (p)-p130, p130 and E2F4 in cells following differentiation, RIPA lysis buffer (Beyotime Institute of Biotechnology) was used to extract total protein lysates, followed by protein determination using a BCA protein assay kit (Beyotime Institute of Biotechnology). Proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beyotime Institute of Biotechnology) with the mass of protein loaded per lane of 100 μg and transferred onto PVDF membranes (MilliporeSigma). Following blocking in 5% BSA for 1 h at room temperature, membranes were incubated at 4˚C overnight with primary antibodies against SP-C (1:1,500; Abcam; cat. no. ab90716), p-p130 (1:1,000; Abcam; cat. no. ab76255), p130 (1:2,000; Abcam; cat. no. ab76234), E2F4 (1:1,500; Proteintech; cat. no. 10923-1-AP) or β-actin (1:4,000; Hangzhou HuaAn Biotechnology Co., Ltd. cat. no. M1210-2). Following primary incubation, membranes were incubated with goat anti-rabbit or goat anti-mouse IgG conjugated with horseradish peroxidase (1:5,000; Zoonbio Biotechnology) for 1 h at room temperature. Protein bands were visualized using Pierce ECL western blotting substrate (Thermo Fisher Scientific, Inc.) and a chemiluminescence imaging system (Bioshine ChemiQ 4800 mini; Ouxiang). Protein expression was quantified using ImageJ software (National Institutes of Health, version 1.52a).

Cell cycle analysis. Cells were centrifuged at 1,500 rpm and washed twice with ice-cold PBS. Cells were fixed with 70% ethanol and incubated at 4˚C for 30 min. Following fixation, cells were incubated with RNase A (20 μg/ml) for 30 min at 37˚C, and stained with propidium iodide (50 μg/ml; BD Biosciences) for 30 min at room temperature in the dark. Cell cycle analysis was conducted using a BD FACS flow cytometer (BD Biosciences) (14).

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc.). If the data were normally distributed, comparisons among multiple groups were performed using one-way ANOVA followed by Bonferroni's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Overexpression of p130 or E2F4 promotes the differentiation of mMSCs into AT II cells. SP-C protein was analysed to detect the differentiation of mMSCs into AT II cells after 7 and 14 days. Results of the present study demonstrated that SP-C protein expression in the MSC-p130 and MSC-E2F4 groups was significantly higher than in the MSC-NC group (Fig. 1).

Immunofluorescence staining was also performed at 7 and 14 days following the differentiation of mMSCs. Red fluorescence was indicative of SP-C-positive cells, and these were present in both MSC-p130 and MSC-E2F4 groups. The numbers of SP-C-positive cells and DAPI-SP-C double-positive cells (blue and red fusion) in the MSC-p130 and MSC-E2F4 groups were higher than that in the MSC-NC group (Fig. 2A).

In addition, at 7 and 14 days following differentiation, the morphology of numerous mMSCs diversified from fusiform fibroblasts to paving-like epithelial structures. The ultrastructure of the cells was further observed using electron microscopy. Increased levels of vacuolization were observed in the cytoplasm and the vicinity of the membrane in the

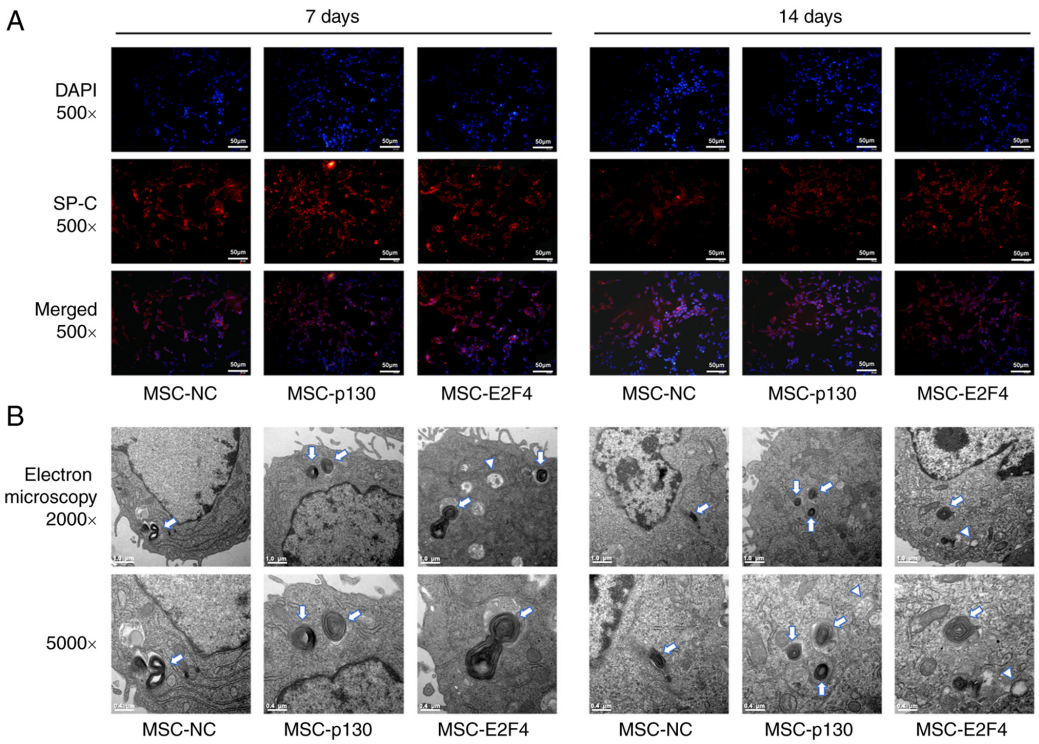


Figure 2. Effects of p130 or E2F4 overexpression on the differentiation of mMSCs into AT II cells *in vitro*. (A) Immunofluorescence staining was used to detect the differentiation of mMSCs. SP-C-positive cells are red. The nuclei stained with DAPI are blue. Representative images at 7 and 14 days after the induced differentiation of mMSCs (magnification, x500; scale bar, 50 μm). (B) Morphological changes in mMSCs following differentiation into AT II cells under a transmission electron microscope. Typical ultrastructure images of mMSCs after 7 and 14 days of differentiation driven by coculture with murine lung epithelial-12 cells and small airway growth medium were observed as lamellar body-like structures and vacuoles [magnification, x2,000 (top row) or x5,000 (bottom row); scale bar, 1.0 μm (top row) or 0.4 μm (bottom row); arrows, lamellar body-like structures; triangles, vacuoles]. AT II cells, type II alveolar epithelial cells; DAPI, 4,6-diamidino-2-phenylindole; E2F4, E2F transcription factor 4; mMSCs, mouse mesenchymal stem cells; MSC-p130, MSCs overexpressing p130; MSC-E2F4, MSCs overexpressing E2F4; NC, normal control; SP-C, surfactant protein C.

MSC-p130 and MSC-E2F4 groups. A small number of characteristic organelles of AT II-lamellar bodies were observed, while there were fewer vacuoles or lamellar structures present in the control group (Fig. 2B).

Activation of the canonical Wnt signaling pathway promotes the differentiation of mMSCs into AT II cells. In our previous study (11), dose-dependent assays using LiCl activation (0.4, 1 or 4 mmol/l) and DKK-1 inhibition were used to modulate the canonical Wnt pathway in mMSCs. Based on the aforementioned findings, 4 mmol/l LiCl, which activated β-catenin to the maximum extent, and 200 ng/ml DKK-1, which effectively inhibited the Wnt signaling pathway, were used in the differentiation experiments of the present study. Compared with the control group, LiCl-mediated activation of the canonical Wnt pathway significantly promoted the expression of SP-C in mMSCs, while the expression of SP-C was significantly decreased following treatment with DKK-1 (Fig. 3A and B) at 7 and 14 days following differentiation.

Activation of the canonical Wnt signaling pathway increases p130/E2F4 expression during mMSC differentiation into AT II cells. To verify whether the differentiation of MSC-p130 and MSC-E2F4 groups into AT II cells was associated with activation of the canonical Wnt signaling pathway, the expression levels of p130 and E2F4 were detected. Results of the present study demonstrated that the phosphorylation level of p130, which was shown by p-p130/p130, was slightly decreased following LiCl activation at 7 days (Fig. 3A) while significantly increased following treatment with DKK-1 at 14 days after differentiation of mMSCs (Fig. 3B). Moreover, the expression level of E2F4 was increased following LiCl activation, compared with the control group but was inhibited following treatment with DKK-1 at 7 days after differentiation (Fig. 3A). Comparable results of E2F4 were also found at 14 days after differentiation of mMSCs in each group (Fig. 3B).

To further elucidate the effects of the canonical Wnt signaling pathway on the differentiation of MSC-p130 and MSC-E2F4 groups into AT II cells, SP-C expression was examined during differentiation using western blotting. The expression levels of SP-C in the DKK-1 + MSC-p130 group were significantly higher than that in the DKK-1 + MSC-NC group at 7 days following differentiation. Moreover, there was no significant change in SP-C expression in the DKK-1 + MSC-E2F4 group (Fig. S1). Comparable results were observed among the three groups at 14 days after differentiation (Fig. S1). These results suggested that overexpression of p130, but not E2F4, may improve the differentiation of mMSCs into AT II cells, while the canonical Wnt signaling pathway was inhibited.

Overexpression of p130 or E2F4 prolongs the G_1 phase of mMSCs during differentiation into AT II cells. To determine whether the differentiation of MSC-p130 and MSC-E2F4

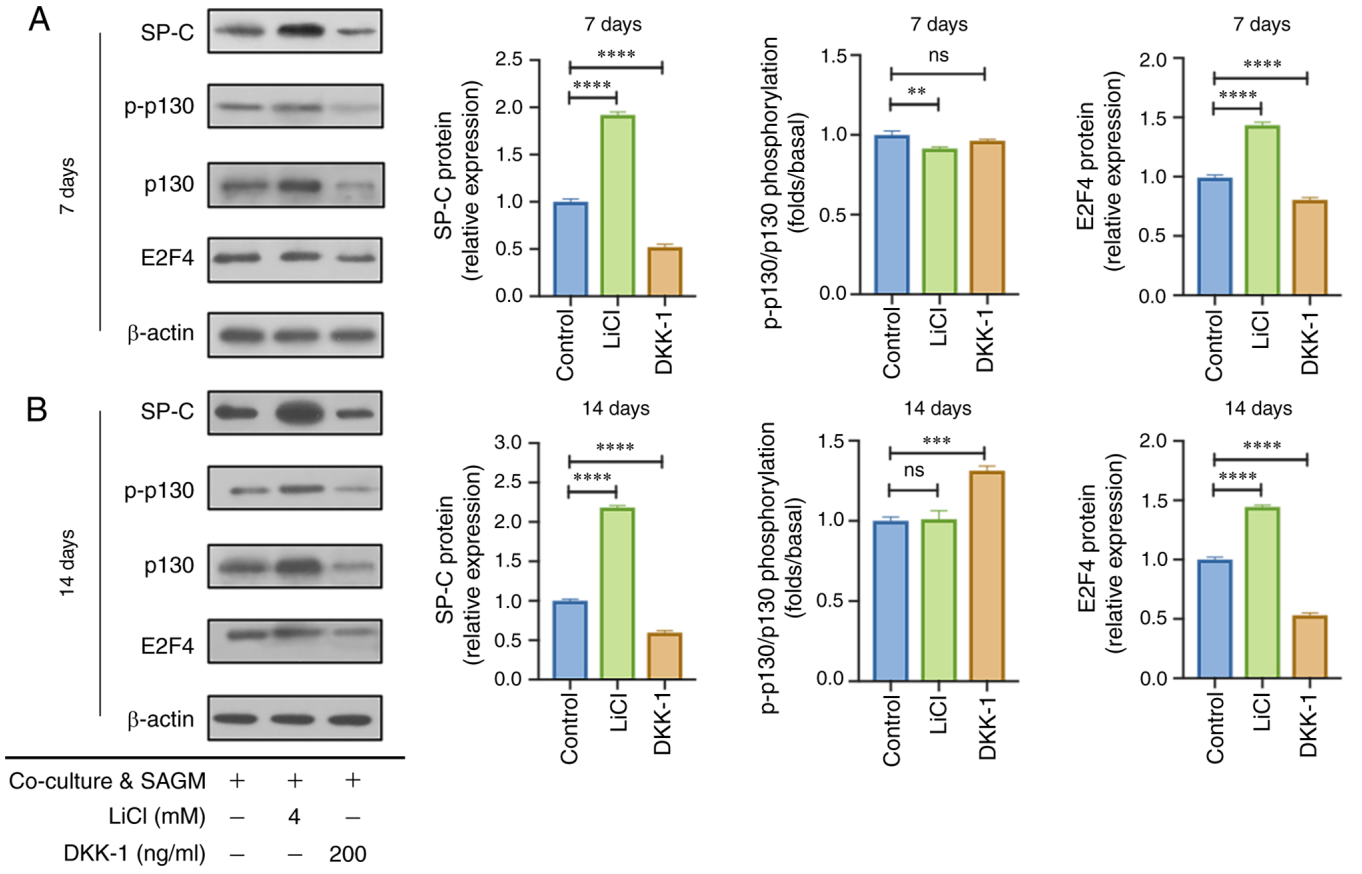


Figure 3. Effects of the canonical Wnt signaling pathway on the expression levels of p130/E2F4 in mMSCs during differentiation into type II alveolar epithelial cells *in vitro*. (A) SP-C, p130 and E2F4 expression was evaluated in mMSCs at 7 days after coculturing with MLE-12 cells and SAGM supplemented with 4 mM LiCl or 200 ng/ml DKK-1 (n=3). (B) SP-C, p130 and E2F4 expression was evaluated in mMSCs at 14 days after coculturing with MLE-12 cells and SAGM supplemented with 4 mM LiCl or 200 ng/ml DKK-1 (n=3). **P<0.01 vs. Control group (mMSCs co-cultured with MLE-12 cells and SAGM), ***P<0.001 vs. Control group, ****P<0.0001 vs. Control group. DKK-1, Dickkopf-related protein 1; E2F4, E2F transcription factor 4; LiCl, lithium chloride; mMSCs, mouse mesenchymal stem cells; p-, phosphorylated; SAGM, small airway growth medium; SP-C, surfactant protein C; ns, not significant.

groups was associated with regulation of the cell cycle, cell cycle stages were detected using flow cytometry. The proportion of G_1-phase cells in the MSC-p130 group was 30.54±0.02% at 7 days following differentiation, which was significantly higher than that in the MSC-NC group. Moreover, the proportion of S-phase cells significantly decreased, and the proportion of G_2/M-phase cells significantly increased in the MSC-p130 group, compared with the MSC-NC group (Fig. 4A). In addition, compared with those in the MSC-NC group, the G_1 and G_2/M phases in the MSC-E2F4 group were also significantly delayed, and the proportion of cells in S phase was significantly reduced (Fig. 4A). Comparable trends were also observed at 14 days following differentiation of mMSCs in each group. Compared with the control group, the proportion of cells in the G_1 and G_2/M phases in the MSC-p130 group was significantly increased, and the proportion of cells in S phase was significantly decreased (Fig. 4B). Comparable trends were observed in the MSC-E2F4 group (Fig. 4B).

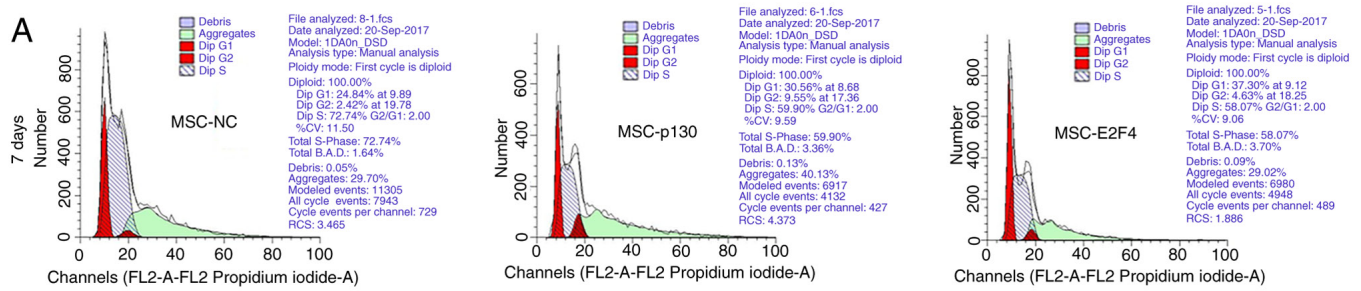
Activation of the canonical Wnt signaling pathway prolongs the G_1 + S phase of mMSCs during differentiation into AT II cells. To further evaluate whether the cell cycle was associated with the canonical Wnt signaling pathway-mediated differentiation of mMSCs into AT II cells, changes in the cell cycle in mMSCs were detected using flow cytometry. The proportion of cells in the G_2/M phase in the LiCl group was 0.29±0.04% at 7 days following differentiation of mMSCs, which was significantly lower than that in the control group (2.61±0.28%). In addition, DKK-1 significantly increased the proportion of cells in the G_2/M phase in mMSCs at 7 days following differentiation (Fig. 5A). Comparable results were observed at 14 days following the differentiation of mMSCs. Compared with the control group, the proportion of cells in the G_2/M phase was significantly decreased in the LiCl group, and significantly increased in the DKK-1 group (Fig. 5B).

To sum up, a graphical representation to illustrate the molecular mechanism of MSCs differentiation into AT II cells was shown in Fig. 6.

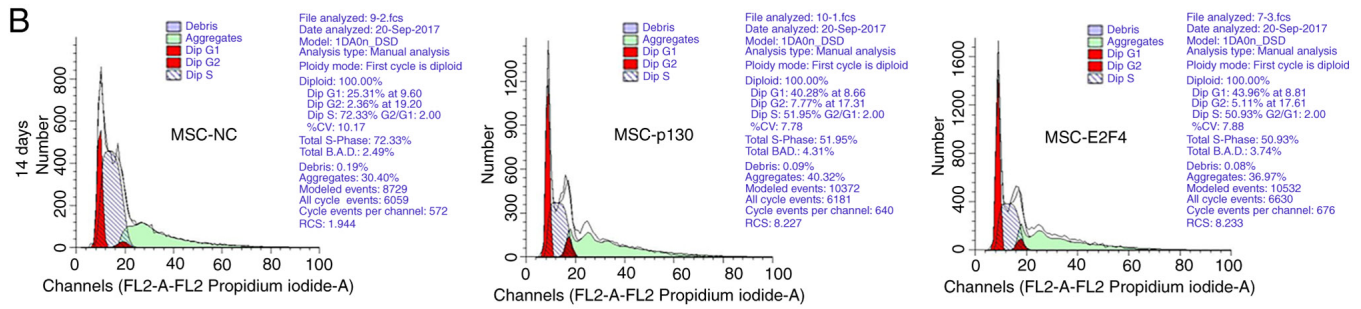
Discussion

MSCs exhibit potential in tissue repair, and in the treatment of cardiovascular, pulmonary and renal diseases. However, the clinical application of MSCs remains limited to phase 2a clinical trials for acute respiratory distress syndrome (ARDS) (15). Large-scale clinical research demonstrating the therapeutic value of MSCs is lacking, due to complexities in the directional differentiation of MSCs (16-18).

In the present study, a coculture system of mMSCs and MLE-12 cells combined with SAGM was established *in vitro,*



Group	G_1 phase (%)	S phase (%)	G_2/M phase (%)
MSC-NC	24.83±0.03	72.79±0.10	2.38±0.07
MSC-p130	30.54±0.02****	60.01±0.10****	9.46±0.08****
MSC-E2F4	37.24±0.05****	58.20±0.12****	4.56±0.08****



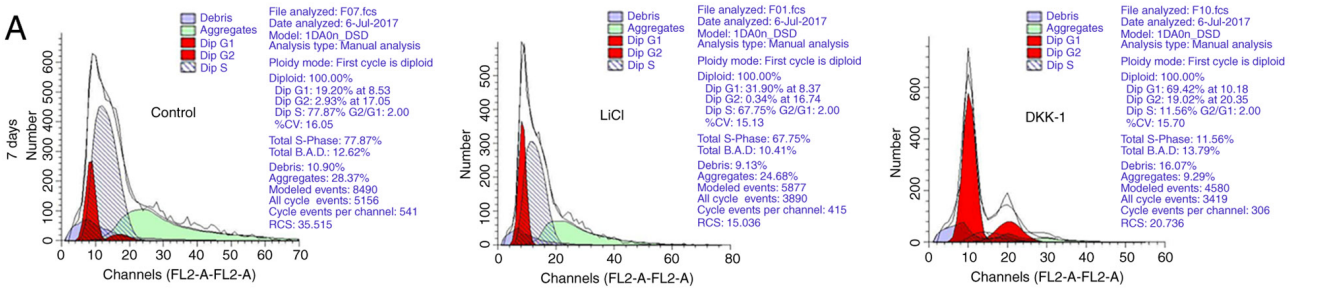
Group	G_1 phase (%)	S phase (%)	G_2/M phase (%)
MSC-NC	25.32±0.01	72.34±0.04	2.34±0.03
MSC-p130	40.30±0.02****	52.19±0.23****	7.51±0.25****
MSC-E2F4	43.87±0.09****	50.52±0.38****	5.62±0.47****

Figure 4. Effects of p130 or E2F4 overexpression on the cell cycle of mMSCs during differentiation into AT II cells *in vitro*. (A) Changes in the cell cycle at 7 days following differentiation of MSC-p130 or MSC-E2F4 into AT II cells (n=3). ****P<0.0001 vs. MSC-NC group. (B) Changes in the cell cycle at 14 days following differentiation of MSC-p130 or MSC-E2F4 into AT II cells (n=3). ****P<0.0001 vs. MSC-NC group. AT II cells, type II alveolar epithelial cells; E2F4, E2F transcription factor 4; mMSCs, mouse mesenchymal stem cells; MSC-p130, MSCs overexpressing p130; MSC-E2F4, MSCs overexpressing E2F4; NC, normal control.

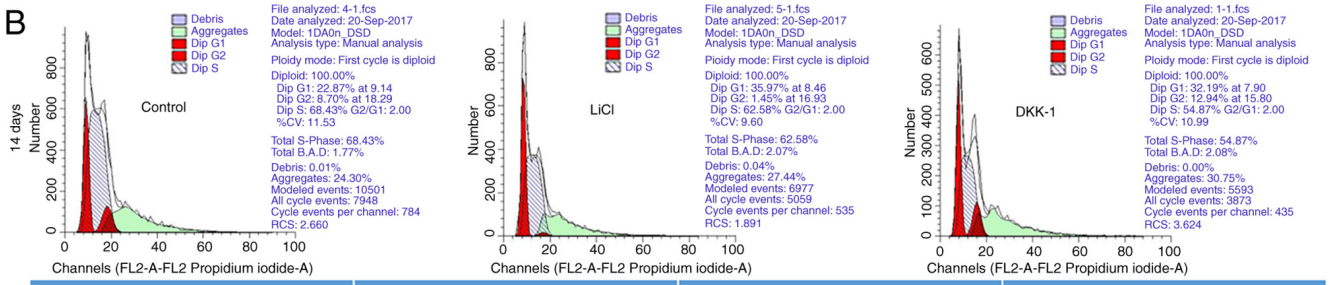
and the Wnt signaling pathway in mMSCs was specifically activated or inhibited. Subsequently, the directed differentiation of mMSCs was observed, and the role of the canonical Wnt signaling pathway in the cell cycle of mMSCs during differentiation into AT II cells was detected. Moreover, the association between the canonical Wnt signaling pathway and p130/E2F4 during the differentiation of mMSCs was also identified, to establish a downstream molecular mechanism during the directed differentiation into AT II cells. To the best of our knowledge, the present study was the first to assess the directional differentiation of mMSCs into AT II cells via regulation of p130/E2F4 and the canonical Wnt pathway.

Results of the present study demonstrated that overexpression of p130 or E2F4 promoted the differentiation of mMSCs into AT II cells, and Wnt signaling pathway-mediated differentiation may be associated with changes in the G_1 + S phase. Moreover, results of the present study demonstrated that regulation of the canonical Wnt signaling pathway specifically regulated the expression of p130 and E2F4 in mMSCs during differentiation, and the canonical Wnt signaling pathway promoted the differentiation of mMSCs into AT II cells via regulating p130/E2F4. The specific underlying mechanisms may be associated with a prolonged G_1 phase in mMSCs (Fig. 6).

The Wnt signaling pathway is a fundamental regulatory pathway in the development, differentiation and other physiological functions of cells and organisms (5). According to different transduction signals, the Wnt pathway is classified into canonical and noncanonical pathways (19). The canonical Wnt pathway, which is also referred to as the Wnt/β-catenin pathway, plays important regulatory roles in numerous biological processes, such as cell proliferation, differentiation and embryonic development. It is highly conserved in the whole evolutionary process of cells (19). Previous studies demonstrated that both canonical and noncanonical Wnt signaling pathways are involved in the regulation of mMSC differentiation (11,20,21). Results of our previous study demonstrated that activation of the canonical or noncanonical Wnt signaling pathway improved the differentiation of MSCs into AT II cells in lipopolysaccharide-induced



Group	G_1 phase (%)	S phase (%)	G_2/M phase (%)
Control	19.31±0.10	78.08±0.19	2.61±0.28
LiCl	31.87±0.03****	67.83±0.07****	0.29±0.04****
DKK-1	69.79±0.39****	11.29±0.35****	18.92±0.10****



Group	G_1 phase (%)	S phase (%)	G_2/M phase (%)
Control	22.92±0.05	68.33±0.11	8.75±0.06
LiCl	35.99±0.02****	62.16±0.39****	1.85±0.37****
DKK-1	32.24±0.06****	54.71±0.18****	13.05±0.12****

Figure 5. Effects of the canonical Wnt signaling pathway on the cell cycle of mMSCs during differentiation into AT II cells *in vitro*. (A) Changes in the cell cycle at 7 days of mMSCs differentiating into AT II cells in groups treated with 4 mM LiCl or 200 ng/ml DKK-1, respectively (n=3). (B) Changes in the cell cycle at 14 days of mMSCs differentiating into AT II cells in groups treated with 4 mM LiCl or 200 ng/ml DKK-1, respectively (n=3). ****P<0.0001 vs. control group (mMSCs co-cultured with MLE-12 cells and small airway growth medium). AT II cells, type II alveolar epithelial cells; DKK-1, Dickkopf-related protein 1; E2F4, E2F transcription factor 4; LiCl, lithium chloride; mMSCs, mouse mesenchymal stem cells.

ARDS mice (22). Results of the present study also confirmed that regulation of the canonical Wnt signaling pathway specifically regulated the differentiation of MSCs into AT II cells. Cell cycle changes detected using flow cytometry also revealed that the differentiation of mMSCs into AT II cells regulated by the canonical Wnt signaling pathway may be associated with the delay of the G_1 + S phase. Notably, associated literature is lacking at present, and further investigations are required.

p130 plays a role in regulating mitosis, and is an important nuclear transcription factor that initiates the cell cycle and promotes cell differentiation. E2F4, which interacts with p130, is involved in regulation of the cell cycle, from G_1 to S phase transformation (12). Capasso *et al* (23) revealed that the inactivation of retinoblastoma proteins (Rb1 or Rb2/p130) delayed the onset of the last cell division and impaired the terminal adipocyte differentiation of MSCs. Petrov *et al* (13) confirmed that the Wnt/β-catenin and pRb signaling pathways interact and form a common p130/Gsk3β/β-catenin complex during MSC cycle progression. Moreover, results of a previous study demonstrated that levels of p130 and E2F4 in MSCs treated with LiCl were also increased. The p130/E2F4 complex in MSCs was associated with β-catenin, which was co-precipitated from the extracts of asynchronously growing or synchronized at G_0/G_1 and S-phase cells. These results are consistent with those of the present study. However, the effects of the canonical Wnt and p130/E2F4 pathways on the differentiation of MSCs into AT II cells remain unclear. Results of the present study demonstrated that the canonical Wnt signaling pathway impacted the differentiation of mMSCs into AT II cells through regulating p130/E2F4. The specific underlying mechanism may be associated with a prolonged G_1 phase in mMSCs. In addition, results of a previous study demonstrated that mMSCs overexpressing p130 or E2F4 promoted osteogenesis, and inhibited adipogenesis and chondrogenesis. The specific underlying mechanism was associated with regulation of the G_1 phase (14). These results are comparable with those of the present study.

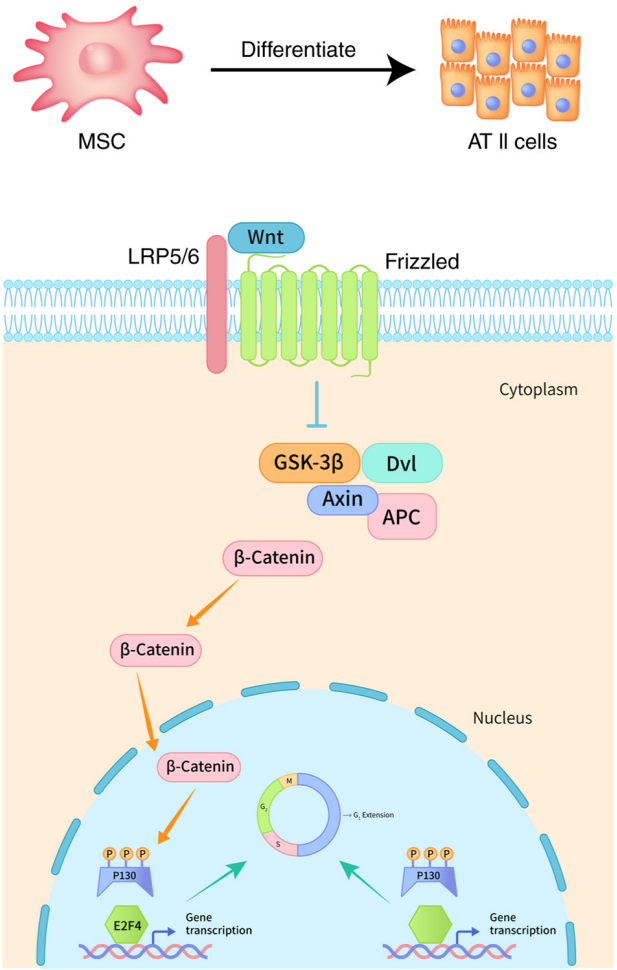


Figure 6. Graphical representation of the mechanisms underlying MSC differentiation into AT II cells. APC, adenomatous polyposis coli; AT II cells, type II alveolar epithelial cells; Dvl, disheveled; E2F4, E2F transcription factor 4; GSK 3β, glycogen synthase kinase 3β; LRP5/6, lipoprotein receptor-related proteins 5/6; MSCs, mesenchymal stem cells; P, phosphorylation.

AT II cells synthesize and secrete alveolar surfactant, and differentiate into type I alveolar epithelial cells. They act as progenitor cells for re-epithelialization of injured alveoli (24). The repair and regeneration of injured AT II cells are therefore critical for patients with acute and chronic pulmonary diseases (25,26). MSCs exhibit potential as therapeutic sources for tissue repair. Results of previous studies demonstrated that MSCs differentiate into AT II cells *in vivo* and *in vitro* (27,28). Moreover, murine bone marrow-derived MSCs and human bone marrow-derived MSCs, with common cell surface markers $CD105^+$ and $CD45^-$ (29), possess similar characteristics, including differentiation into alveolar epithelial cells, alleviation of inflammation, and improvement of pathological impairment both *in vivo* and *in vitro* (30-33). Results of a previous study demonstrated that regulation of the canonical and noncanonical Wnt signaling pathways promoted MSC-mediated protection against epithelial impairment in ARDS mice (22). Moreover, results of the present study revealed that changes in the cell cycle may play a role in the therapeutic effects of MSC differentiation, specifically in repairing lung injuries. Regulating cell cycle changes in MSCs may contribute to further development of their clinical application for the treatment of pulmonary diseases.

MSCs have potential to differentiate into alveolar epithelial cells *in vitro* and *in vivo*. Different media containing epithelial differentiation inducers or matrix were tested *in vitro* to prove MSCs differentiation into specific epithelial tissue-related properties (34,35). Recently, Zeng *et al* (36) proved that overexpression of Fork head box protein M1 facilitates MSCs differentiation into AT II cells *in vitro* using the co-culture system combined with SAGM. Besides, Perng *et al* (37) demonstrated that the addition of transforming growth factor-β1 and extracellular matrix collagen are required to facilitate MSCs differentiation into lung epithelial-like cells *in vitro*. And Chanda *et al* (38) established an organoid model in Matrigel mixed with MTEC/plus media to study stem cell behavior and intercellular communication between lung resident MSCs and AT II with age-related phenotypes, revealing that young MSCs formed normal 3D structures with both young and aged AT II cells, but aged L-MSCs developed abnormal, loose structures with AT II cells. In addition, different models were also used to prove the therapeutic effects of MSCs by differentiation into AT II cells *in vivo*. Recently, Shao *et al* (39) found that overexpression of CXCR7 promoted differentiation of MSCs into AT II cells and enhanced the ability of MSCs to modulate the inflammatory response in phosgene-induced ALI. And Huang *et al* (40) demonstrated that MSCs possess the differentiation potential into AT II cells, as a primary mechanism through which MSCs exert a therapeutic effect, specifically within a microenvironment of injury of pulmonary fibrosis.

Surfactant protein is a specific protein in the lung, which is divided into four subtypes; namely, SP-A, -B, -C and -D. SP-A and SP-D directly interact with pathogens and allergens, stimulate immune cells, and influence cytokine and chemokine profiles during the host defense response. In addition, SP-B and SP-C play critical roles in reducing the surface tension of the lung, and in the regulation of intracellular processes required for the production of surfactant and for the maintenance of AT II cell function (41). SP-C mRNA is limited to lung tissue and is detectable in isolated AT II cells. Compared with SP-C mRNA, SP-A, SP-B and SP-D are mainly present in type II cells and Clara cells (42).

Notably, the present study exhibits numerous limitations. Results of the present study demonstrated that changes in the cell cycle were not synchronized between canonical Wnt pathway activation and p130/E2F4 overexpression during the differentiation of mMSCs into AT II cells. This may be due to the complex crosstalk that occurs during cell signaling, making it difficult to target specific factors. Moreover, further investigations are required to determine the interaction between β-catenin and p130 during the differentiation of MSCs into AT II cells, including specific cell cycle changes and interaction domains. In addition, p130 and E2F4 on Day 7 and Day 14 in the MSC-NC, MSC-p130 and MSC-E2F4 groups after induced differentiation is required to further determine the effects of overexpression of p130 or E2F4. However, results of our previous study demonstrated that lentivirus-mediated p130 or E2F4 transduction is stable and efficient, even after 20 passages of mMSCs (14). A further limitation of the present study included the low magnification used during fluorescence microscopy, which may lead to bias during fluorescent counting. However, the protein expression levels of SP-C verified the increased levels of differentiation.

In conclusion, results of the present study demonstrated that the canonical Wnt signaling pathway may impact the differentiation of MSCs into AT II cells through regulating downstream p130/E2F4. The specific underlying mechanism may be associated with regulation of the cell cycle. Results of the present study may provide a novel theoretical basis for promoting the differentiation of MSCs into AT II cells for the treatment of pulmonary diseases.

Acknowledgements

The authors would like to thank Dr Shixia Cai (Department of Critical Care Medicine, The Affiliated Hospital of Qingdao University, Qingdao, China) and Dr Jianxiao Chen (Department of Critical Care Medicine, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China) for their advice and guidance on the design of the experiments.

Funding

The study was supported by the National Natural Science Foundation of China (grant nos. 81871602 and 81930058), the Jiangsu Provincial Special Program of Medical Science (grant no. BE2019749), National Science and Technology Major Project for Control and Prevention of Major Infectious Diseases of China (grant no. 2017ZX10103004), Natural Science Foundation of Jiangsu Province (grant no. BK20200356), and the Fundamental Research Funds for the Central Universities of China (grant no. ZDYYZXKT2020027).

Availability of data and materials

All data generated and/or analyzed during this study are included in this published article.

Authors' contributions

XZ participated in the study design, performed laboratory work and statistical analysis, prepared the drafts of the manuscript, and revised the manuscript according to advice from the other authors. MX participated in the laboratory work, performed statistical analysis and drafted the manuscript. AL participated in the study design and helped revise the manuscript. HQ and FG confirm the authenticity of all the raw data and were responsible for the study design and revised the manuscript for important intellectual content. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Galipeau J and Sensébé L: Mesenchymal stromal cells: Clinical challenges and therapeutic opportunities. Cell Stem Cell 22: 824-833, 2018.
2. Huppert LA, Liu KD and Matthay MA: Therapeutic potential of mesenchymal stromal cells in the treatment of ARDS. Transfusion 59: 869-875, 2019.
3. Schwede M, Wilfong EM, Zemans RL, Lee PJ, Dos Santos C, Fang X and Matthay MA: Effects of bone marrow-derived mesenchymal stromal cells on gene expression in human alveolar type II cells exposed to TNF-α, IL-1β, and IFN-γ. Physiol Rep 6: e13831, 2018.
4. Visweswaran M, Pohl S, Arfuso F, Newsholme P, Dilley R, Pervaiz S and Dharmarajan A: Multi-lineage differentiation of mesenchymal stem cells-to Wnt, or not Wnt. Int J Biochem Cell Biol 68: 139-147, 2015.
5. Logan CY and Nusse R: The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20: 781-810, 2004.
6. Etheridge SL, Spencer GJ, Heath DJ and Genever PG: Expression profiling and functional analysis of wnt signaling mechanisms in mesenchymal stem cells. Stem Cells 22: 849-860, 2004.
7. Sasaki T, Han F, Shioda N, Moriguchi S, Kasahara J, Ishiguro K and Fukunaga K: Lithium-induced activation of Akt and CaM kinase II contributes to its neuroprotective action in a rat microsphere embolism model. Brain Res 1108: 98-106, 2006.
8. Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, Delius H, Hoppe D, Stannek P, Walter C, *et al*: Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signaling. Nature 417: 664-667, 2002.
9. van Haaften T, Byrne R, Bonnet S, Rochefort GY, Akabutu J, Bouchentouf M, Rey-Parra GJ, Galipeau J, Haromy A, Eaton F, *et al*: Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats. Am J Respir Crit Care Med 180: 1131-1142, 2009.
10. Leeman KT, Pessina P, Lee JH and Kim CF: Mesenchymal stem cells increase alveolar differentiation in lung progenitor organoid cultures. Sci Rep 9: 6479, 2019.
11. Liu AR, Liu L, Chen S, Yang Y, Zhao HJ, Liu L, Guo FM, Lu XM and Qiu HB: Activation of canonical wnt pathway promotes differentiation of mouse bone marrow-derived MSCs into type II alveolar epithelial cells, confers resistance to oxidative stress, and promotes their migration to injured lung tissue in vitro. J Cell Physiol 228: 1270-1283, 2013.
12. Beshiri ML, Holmes KB, Richter WF, Hess S, Islam AB, Yan Q, Plante L, Litovchick L, Gévry N, Lopez-Bigas N, *et al*: Coordinated repression of cell cycle genes by KDM5A and E2F4 during differentiation. Proc Natl Acad Sci USA 109: 18499-18504, 2012.
13. Petrov N, Zhidkova O, Serikov V, Zenin V and Popov B: Induction of Wnt/β-catenin signaling in mouse mesenchymal stem cells is associated with activation of the p130 and E2f4 and formation of the p130/Gsk3β/β-catenin complex. Stem Cells Dev 21: 589-597, 2012.
14. Zhang X, Chen J, Liu A, Xu X, Xue M, Xu J, Yang Y, Qiu H and Guo F: Stable overexpression of p130/E2F4 affects the multi-potential abilities of bone-marrow-derived mesenchymal stem cells. J Cell Physiol 233: 9739-9749, 218.
15. Matthay MA, Calfee CS, Zhuo H, Thompson BT, Wilson JG, Levitt JE, Rogers AJ, Gotts JE, Wiener-Kronish JP, Bajwa EK, *et al*: Treatment with allogeneic mesenchymal stromal cells for moderate to severe acute respiratory distress syndrome (START study): A randomised phase 2a safety trial. Lancet Respir Med 7: 154-162, 2019.
16. Laffey JG and Matthay MA: Fifty years of research in ARDS. Cell-based therapy for acute respiratory distress syndrome. biology and potential therapeutic value. Am J Respir Crit Care Med 196: 266-273, 2017.
17. Wilson JG, Liu KD, Zhuo H, Caballero L, McMillan M, Fang X, Cosgrove K, Vojnik R, Calfee CS, Lee JW, *et al*: Mesenchymal stem (stromal) cells for treatment of ARDS: A phase 1 clinical trial. Lancet Respir Med 3: 24-32, 2015.
18. Walter J, Ware LB and Matthay MA: Mesenchymal stem cells: Mechanisms of potential therapeutic benefit in ARDS and sepsis. Lancet Respir Med 2: 1016-1026, 2014.
19. Ling L, Nurcombe V and Cool SM: Wnt signaling controls the fate of mesenchymal stem cells. Gene 433: 1-7, 2009.

20. Cai SX, Liu AR, He HL, Chen QH, Yang Y, Guo FM, Huang YZ, Liu L and Qiu HB: Stable genetic alterations of β-catenin and ROR2 regulate the Wnt pathway, affect the fate of MSCs. J Cell Physiol 229: 791-800, 2014.
21. Liu A, Chen S, Cai S, Dong L, Liu L, Yang Y, Guo F, Lu X, He H, Chen Q, *et al*: Wnt5a through noncanonical Wnt/JNK or Wnt/PKC signaling contributes to the differentiation of mesenchymal stem cells into type II alveolar epithelial cells in vitro. PLoS One 9: e90229, 2014.
22. Cai SX, Liu AR, Chen S, He HL, Chen QH, Xu JY, Pan C, Yang Y, Guo FM, Huang YZ, *et al*: The orphan receptor tyrosine kinase ROR2 facilitates MSCs to repair lung injury in ARDS animal model. Cell Transplant 25: 1561-1574, 2016.
23. Capasso S, Alessio N, Di Bernardo G, Cipollaro M, Melone MA, Peluso G, Giordano A and Galderisi U: Silencing of RB1 and RB2/P130 during adipogenesis of bone marrow stromal cells results in dysregulated differentiation. Cell Cycle 13: 482-490, 2014.
24. Matthay MA, Robriquet L and Fang X: Alveolar epithelium: Role in lung fluid balance and acute lung injury. Proc Am Thorac Soc 2: 206-213, 2005.
25. Mason RJ: Thoughts on the alveolar phase of COVID-19. Am J Physiol Lung Cell Mol Physiol 319: L115-L120, 2020.
26. Ruaro B, Salton F, Braga L, Wade B, Confalonieri P, Volpe MC, Baratella E, Maiocchi S and Confalonieri M: The history and mystery of alveolar epithelial type II cells: Focus on their physiologic and pathologic role in lung. Int J Mol Sci 22: 2566, 2021.
27. Rojas M, Xu J, Woods CR, Mora AL, Spears W, Roman J and Brigham KL: Bone marrow-derived mesenchymal stem cells in repair of the injured lung. Am J Respir Cell Mol Biol 33: 145-152, 2005.
28. Ma N, Gai H, Mei J, Ding FB, Bao CR, Nguyen DM and Zhong H: Bone marrow mesenchymal stem cells can differentiate into type II alveolar epithelial cells in vitro. Cell Biol Int 35: 1261-1266, 2011.
29. Li H, Ghazanfari R, Zacharaki D, Lim HC and Scheding S: Isolation and characterization of primary bone marrow mesenchymal stromal cells. Ann N Y Acad Sci 1370: 109-118, 2016.
30. Ortiz LA, Gambelli F, McBride C, Gaupp D, Baddoo M, Kaminski N and Phinney DG: Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. Proc Natl Acad Sci USA 100: 8407-8411, 2003.
31. Yamada M, Kubo H, Kobayashi S, Ishizawa K, Numasaki M, Ueda S, Suzuki T and Sasaki H: Bone marrow-derived progenitor cells are important for lung repair after lipopolysaccharide-induced lung injury. J Immunol 172: 1266-1272, 2004.
32. Kotton DN, Ma BY, Cardoso WV, Sanderson EA, Summer RS, Williams MC and Fine A: Bone marrow-derived cells as progenitors of lung alveolar epithelium. Development 128: 5181-5188, 2001.
33. Chen QH, Liu AR, Qiu HB and Yang Y: Interaction between mesenchymal stem cells and endothelial cells restores endothelial permeability via paracrine hepatocyte growth factor in vitro. Stem Cell Res Ther 6: 44, 2015.
34. Shannon JM, Mason RJ and Jennings SD: Functional differentiation of alveolar type II epithelial cells in vitro: Effects of cell shape, cell-matrix interactions and cell-cell interactions. Biochim Biophys Acta 931: 143-156, 1987.
35. Olsen CO, Isakson BE, Seedorf GJ, Lubman RL and Boitano S: Extracellular matrix-driven alveolar epithelial cell differentiation in vitro. Exp Lung Res 31: 461-482, 2005.
36. Zeng M, Chen Q, Ge S, He W, Zhang L, Yi H and Lin S: Overexpression of FoxM1 promotes differentiation of bone marrow mesenchymal stem cells into alveolar type II cells through activating Wnt/β-catenin signaling. Biochem Biophys Res Commun 528: 311-317, 2020.
37. Perng DW, Yang DM, Hsiao YH, Lo T, Lee OK, Wu MT, Wu YC and Lee YC: miRNA-146a expression positively regulates tumor necrosis factor-α-induced interleukin-8 production in mesenchymal stem cells and differentiated lung epithelial-like cells. Tissue Eng Part A 18: 2259-2267, 2012.
38. Chanda D, Rehan M, Smith SR, Dsouza KG, Wang Y, Bernard K, Kurundkar D, Memula V, Kojima K, Mobley JA, *et al*: Mesenchymal stromal cell aging impairs the self-organizing capacity of lung alveolar epithelial stem cells. Elife 10: e68049, 2021.
39. Shao Y, Zhou F, He D, Zhang L and Shen J: Overexpression of CXCR7 promotes mesenchymal stem cells to repair phosgene-induced acute lung injury in rats. Biomed Pharmacother 109: 1233-1239, 2019.
40. Huang K, Kang X, Wang X, Wu S, Xiao J, Li Z, Wu X and Zhang W: Conversion of bone marrow mesenchymal stem cells into type II alveolar epithelial cells reduces pulmonary fibrosis by decreasing oxidative stress in rats. Mol Med Rep 11: 1685-1692, 2015.
41. Mulugeta S and Beers MF: Surfactant protein C: Its unique properties and emerging immunomodulatory role in the lung. Microbes Infect 8: 2317-2323, 2006.
42. Beers MF and Fisher AB: Surfactant protein C: A review of its unique properties and metabolism. Am J Physiol 263: L151-L160, 1992.