Abstract

Background: Mesenchymal stem cells (MSCs) are bone marrow stem cells which play an important role in tissue repair. The treatment with MSCs will be likely to aggravate the degree of fibrosis. The Wnt/β-catenin signaling pathway is involved in developmental and physiological processes, such as fibrosis. Dickkopfs (DKKs) are considered as an antagonist to block Wnt/β-catenin signaling pathway by binding the receptor of receptor-related protein (LRP5/6). DKK1 was chosen in attempt to inhibit fibrosis of MSCs by lowering activity of Wnt/β-catenin signaling pathway.

Methods: Stable MSCs were randomly divided into four groups: MSCs control, MSCs + transforming growth factor-β (TGF-β), MSCs + DKK1, and MSCs + TGF-β + DKK1. Flow cytometry was used to identify MSCs. Cell viability was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide test. Immunofluorescence was used to detect protein expression in the Wnt/β-catenin signaling pathways. Western blotting analysis was employed to test expression of fibroblast surface markers and, finally, real-time reverse transcription polymerase chain reaction was employed to test mRNA expression of fibroblast surface markers and Wnt/β-catenin signaling proteins.

Results: Cultivated MSCs were found to conform to the characteristics of standard MSCs: expression of cluster of differentiation (CD) 73, 90, and 105, not expression of 34, 45, and 79. We found that DKK1 could maintain the normal cell morphology of MSCs. Western blotting analysis showed that fibroblast surface markers were expressed in high quantities in the group MSCs + TGF-β. However, the expression was lower in the MSCs + TGF-β + DKK1 group. Immunofluorescence showed high expression of all Wnt/β-catenin molecules in the MSCs + TGF-β group but expressed in lower quantities in MSCs + TGF-β + DKK1 group. Finally, mRNA expression of fibroblast markers vimentin, α-smooth muscle actin and Wnt/β-catenin signaling proteins β-catenin, T-cell factor, and glycogen synthase kinase-3β was significantly increased in MSCs + TGF-β group compared to control (P < 0.05). Expression of the same fibroblast markers and Wnt/β-catenin was decreased to regular quantities in the MSCs + TGF-β + DKK1 group.

Conclusions: DKK1, Wnt/β-catenin inhibitors, blocks the Wnt/β-catenin signaling pathway to inhibit the process of MSCs fibrosis. It might provide some new ways for clinical treatment of certain diseases.

Key words: Dickkopfs-1; Fibrosis; Mesenchymal Stem Cells; Wnt/β-catenin Signaling Pathway

Introduction

Mesenchymal stem cells (MSCs) are bone marrow stem cells which play an important role in cell repair by differentiating into a variety of cell types including adipocytes, osteoblasts, and chondrocytes. Compared with other stem cell types, such as embryonic stem cells and neural stem cells, MSCs have several advantages. First, there are no ethical concerns concerning the use of MSCs, they can be easily separated, proliferate extensively, can self-renew, and have a low risk of tumorigenicity. Furthermore, they can be autologously used. MSCs are immunoprivileged because they express low levels of major histocompatibility complex class I (MHC-I) molecules, but neither MHC-II nor costimulatory molecules cluster of differentiation 80 (CD80), CD86, and CD40. MSCs have low immunogenicity and exhibit unique immune response,

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regulation by which can escape from immune recognition and can inhibit the immune response. In addition, they have great potential for multidirectional differentiation and reproductive activity. Under different inducing factors, MSCs can be developed in different directions. For instance, oxymatrine was able to cause the inhibition of carbon tetrachloride-induced MSC fibrosis, and overexpression of vascular endothelial growth factor (VEGF) inhibited MSC differentiation and osteogenesis in vitro. According to Stockman’s study, using stem cells to treat kidney injury might increase the risk of local tissue fibrosis. Preclinical studies demonstrate promising results, such as using MSCs for diverse lung disorders, including emphysema, bronchopulmonary dysplasia, fibrosis, and acute respiratory distress syndrome.

Treatment with MSCs will be likely to aggravate the degree of fibrosis and impact the treatment effect. Therefore, it is necessary to lead MSCs to favorable differentiation. Some studies show that using MSCs in mice might cause the remarkable increase of VEGF and fibroblast growth factor. This is the early performance of fibrosis. Hence, there are key points in MSCs treatment, such as regulating the differentiation of MSCs, exerting its immune regulating function, avoiding the occurrence of fibrosis and promoting tissue repair.

The use of MSCs in drug discovery, supported by mature technologies and established regulatory paths, is expected to receive more feedback. A number of signaling pathways have been identified in various diseases, including cancer, fibrosis, and degenerative diseases. Among them, Wnt/β-catenin is one of the most important signaling pathways which become the research hot spots now. Recent studies have demonstrated that aberrant canonical Wnt/β-catenin signaling pathway facilitates the development of organ fibrosis, which might be a novel therapeutic target in fibrotic disorders. The Wnt/β-catenin signaling pathway is involved in a wide range of developmental and physiological processes, such as cell fate specification, homeostasis, and tissue morphogenesis. The β-catenin complex is composed mainly of adenomatous polyposis coli (APC), axin, glycogen synthase kinase-3β (GSK-3β), and casein kinase 1.

When the Wnt/β-catenin signaling pathway is activated, β-catenin cannot be phosphorylated by GSK-3β and then steadily accumulates in the cytoplasm to activate target gene transcription by T-cell factor (TCF), lymphoid enhancer factor (LEF). As a result, Dickkopfs-1 (DKK1), one member of DKK, is considered as antagonist blocks Wnt/β-catenin signaling by binding the receptor of receptor-related protein (LRP5/6). Inhibiting Wnt/β-catenin pathway will control directional differentiation of MSCs. DKK1 inhibits activation of Wnt/β-catenin signaling pathway, thus inhibiting MSC differentiation of fibroblasts will be important for clinical usage of MSCs for pulmonary diseases theory basis.

This article will discuss the dosage effects of DKK1 on MSC antigen and whether DKK1 can inhibit fibrosis to allow MSCs to be used for better treatment.

**Methods**

**Isolation and culture of mesenchymal stem cell**

SPF C57BL/6 female mice (20–30 g) were chosen from Shanghai Slack Laboratory Animal Co., Ltd. (SCXK [HU] 2007-0003). We provided mouse chow and water on time and put them in 12-h light and 12-h darkness cycle.

Bone marrows from femurs and tibias of 3–4 weeks female mice were separated by density gradient centrifugation (1000 ×g, Eppendorf, Centrifuge 5415R, Hamburg, Germany). The cells from the interface layer were resuspended in low glucose Dulbecco’s modified Eagles medium (DMEM, Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS), 5% of 50 µg/ml penicillin, and streptomycin. A final concentration of 1 × 10⁶ cells was seeded in 25 cm² tissue culture flasks, and grown at 37°C in a humidified atmosphere of 5% CO₂ (Heraeus, RBT, Germany).

Medium was changed 2 days after seeding, and then 3 times/week until the cells reached around 80% confluence. The cells were passaged with 0.25% pancreatic enzyme-ethylenediaminetetraacetic acid (EDTA, Invitrogen Life Science, NY, USA) and grown in MSC growth medium (DMEM with 10% FBS, 50 µg/ml penicillin, and streptomycin). Passages 3–5 were used for all experiments.

**Flow cytometry analysis**

Flow cytometric analysis was used to identify the molecular phenotype of rat-MSCs. In addition, MSCs surface markers’ expression of CD105, CD73, CD90, CD45, CD79, and CD34 were detected using flow cytometry (FACSCalibur, BD, NY, USA). Total 1 × 10⁶ MSCs were diluted by no fluorescence labeling primary antibodies (rabbit anti-mouse CD73, CD105, CD45, CD90, and CD79) with 2 h incubation. Second antibody (goat anti-rabbit Alexa Fluor 488 [Invitrogen Life Science, NY, USA]) was incubated for 1 h in dark place. MSCs were then washed 3 times with PBS and were detected using flow cytometry (BD, NJ, USA). Data were analyzed using FlowJo software (TreeStar Inc. San Carlos, CA).

**Effect on mesenchymal stem cell differentiation by Wnt/β-catenin signaling pathway**

**Mesenchymal stem cell grouping**

The MSCs were randomly divided into four groups by different mediums. Control: normal MSCs; MSCs + transforming growth factor-β (TGF-β): added 0.5 ng/ml TGF-β (Sigma-Aldrich, Missouri, USA) into medium; MSCs + DKK1: added 20 ng/ml DKK1 (PeproTech Inc., New Jersey, USA) into medium; MSCs + TGF-β + DKK1: added 0.5 ng/ml TGF-β and 20 ng/ml DKK1 simultaneously into medium.

To induce mesenchymal stem cells to be fibroblast under combination of Mesenchymal stem cell + transforming growth factor-β

MSCs were induced to fibroblast differentiation by TGF-β. The 2nd, 3rd, 4th, and 5th generation MSCs were chosen to
subculture with 0.25% pancreatic enzyme-EDTA. DMEM with 10% FBS and 0.5 ng/ml TGF-β was used to resuspend MSCs. The MSCs, which were inoculated in the culture dish with 1 × 10^6/ml density, grown at 37°C in a humidified atmosphere of 5% CO₂.

To inhibit Wnt/β-catenin signaling pathways under combination of mesenchymal stem cell + Dickkopfs-1
Wnt/β-catenin signal pathway was inhibited by DKK1. The 2nd, 3rd, 4th, and 5th generation MSCs were chosen to subculture with pancreatic enzyme-EDTA. DMEM with 10% FBS and 20 ng/ml DKK1 was used to resuspend MSCs. Also, the MSCs were cultivated at 37°C in a humidified atmosphere of 5% CO₂.

To induce mesenchymal stem cells to be fibroblast and inhibition of Wnt/β-catenin signaling pathway under combination of mesenchymal stem cell + transforming growth factor-β + Dickkopfs-1
MSCs were induced into fibroblast by TGF-β. Meanwhile, DKK1 was used to inhibit Wnt/β-catenin signal pathway. The 2nd, 3rd, 4th, and 5th generation MSCs were chosen to subculture with pancreatic enzyme-EDTA. DMEM with 0.5 ng/ml TGF-β, 20 ng/ml DKK1, and 10% FBS was used to cultivate MSCs with 1 × 10^6/ml density.

**Microscopic observation**
Each group was observed by phase contrast ECLIPSE 50i microscope (Nikon, Tochigi, Japan). The number of the survival cells and survival time were recorded under the reverse phase microscope every day.

**Cell viability assay**
Cell viability was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) test according to the protocol (Beyotime, China). After treated, 2 × 10^6 cells of each group were plated in each well of 96-well plates and were incubated with 100 μl culture medium. After incubation, 25 μl MTT solution (5 mg/ml) was added to each well and all the wells underwent further incubation for 4 h. Then, the supernatants were removed and 100 μl dimethyl sulfoxide was added to the wells to dissolve the formazan crystal at 37°C. Thirty minutes later, the absorbance was measured on an automated microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm.

**Mesenchymal stem cell immunofluorescence: To detect the protein expression in the Wnt/β-catenin signaling pathways**
Cell immunofluorescence was used to detect the protein expression of β-catenin, TCF, GSK-3β, Axin1, and c-Jun N-terminal kinase (JNK) of Wnt/β-catenin signaling pathways in each group. We cultivated MSCs for two more generations after grouping. MSCs were fixed in the precool methanol for 15 min. Sealed them in 1% bovine serum albumin (BSA) for 1 h. They were incubated overnight by diluting primary antibodies (rabbit anti-mouse β-catenin, TCF, GSK-3β, Axin1, APC, JNK [Abcam Inc., Cambridge, MA, USA]) in 1% BSA and then incubated 1 h with fluorescein isothiocyanate second antibody (fluorescein labeled antibody to rabbit IgG [H+L]; 1:100). Cell nucleus was incubated with diamidino-phenyl-indole for 5 min at the normal temperature. The cells were cleaned 3 times by PBS between each step. Inverted fluorescence microscope was used to observe.

**Western blotting analysis of mesenchymal stem cell: To test expression of fibroblast surface markers**
Western blotting was used to test fibroblast surface markers expression of vimentin and α-smooth muscle actin (α-SMA) proteins in four groups. We cultivated MSCs for two more generations after grouping. Then, we used a scraper to scrap cells into centrifuge tubes. The MSCs were cracked in ice for 30 min. Bradford method was used to determine protein concentration. Western Blotting steps included making 5% concentrated gum, adding the samples, running electrophoresis, transferring membrane, closing nonspecific proteins, incubating antibodies (rabbit anti-mouse vimentin and α-SMA [Abcam, Cambridge, UK]; horseradish peroxidase [HRP] labeled second antibody to rabbit IgG), and applying HRP-enhanced chemiluminescence.

**Real-time reverse transcription polymerase chain reaction reaction: To test mRNA expression level of fibroblast markers**
Real-time reverse transcription polymerase chain reaction (RT-PCR) (Takara, DaLiang, China) was used to test mRNA expression level of fibroblast surface markers vimentin, α-SMA and Wnt/β-catenin signaling proteins β-catenin, TCF, and GSK-3β. Primers are shown in Table 1.

### Table 1: PCR primers and probes to test mRNA expression level of fibroblast markers and Wnt/β-catenin signaling molecules in the course of DKK1 administration

| Gene name     | Probe and primer sequence (5′-3′) |
|---------------|-----------------------------------|
| β-catenin     | F:GCCCTGTGAGGACACATCA             |
|               | R:TGCCTGACACCCATGGA               |
|               | P:ACACCCCCGGCG                    |
| TCF           | F:CGGCTAAGGTCGAGA                 |
|               | R:GACCATACGCGCCAACTG             |
|               | P:CGGCACCGATTGAGAACCAC           |
| GSK-3β        | F:CCCCCTGCGACATCT                 |
|               | R:CCCTCTGGCGACATCT               |
| Vimentin      | F:GAGAGAAGAGGCGAAAGCA            |
|               | R:GGCGAGAAGAGCTTACGTC            |
| α-SMA         | F:CGTGAGGCTGAATCAGT              |
|               | R:TTTTCCATGTGCTCCCCAGTTG         |
| GAPDH         | F:CACGCCACTCCATC                 |
|               | R:CGCGACGTGACATCA                |
|               | P:CCCCAAATGGTGCCC                |

PCR: Polymerase chain reaction; DKK1: Dickkopfs-1; TCF: T-cell factor; GSK-3β: Glycogen synthase kinase-3β; α-SMA: α-smooth muscle actin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; F: Forward; R: Reverse; P: Primer.
Statistical analysis
Data are expressed as mean ± standard deviation (SD). All calculations and statistical analyses were performed using SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Dunnett’s t-test was used to analyze differences between groups. A value of \( P < 0.05 \) was considered statistically significant.

RESULTS

Primary culture and identification of mesenchymal stem cells
Some MSCs adhered on the bottom of the flasks which were long spindles in 3 days after first changing the medium. A week later, colonies of MSCs grew uniformly into long shuttle type or spiral shape. After 2 weeks, the cells were mixed 80% fusion. After 3 or 4 generations, MSCs grew fast into a unified form [Figure 1].

Flow cytometry showed that the expression of CD105, CD73, and CD90 on MSCs, but not expression of CD45, CD79, and CD34. This confirmed the high purity of the cultivated MSCs and also demonstrated that the cultivated MSCs conformed with the characteristics of MSCs [Figure 2].

Influence of Dickkopf-1 on mesenchymal stem cells differentiated into fibroblast
It was found that using the inverted microscope, TGF-β could significantly promote the proliferation of MSCs with the random arrangement in group MSCs + TGF-β. However, in MSCs + DKK1 group, proliferation activity of MSCs was retarded, with maintaining uniform, long spindle and swirling cell morphology, which also proved by MTT test [Figure 3].

Expression of fibroblast surface marker proteins on mesenchymal stem cells
Western blotting analysis was used to test expression of fibroblast surface markers, vimentin, and α-SMA proteins.

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Figure 1: The original generation and subculture mouse MSCs. After 3rd generation, MSCs grew fast into a unified form. (a) MSCs original generation and cultured 7 days, (b) MSCs original generation and cultured 14 days, (c) 1st generation of MSCs, and (d) 3rd generation of MSCs. MSCs: Mesenchymal stem cells.

Figure 2: Flow cytometry identified the surface markers of MSCs. MSCs did not express CD34, CD45, and CD79 (a-c); but expressed CD73, CD90, and CD105 (d-f). X-axis means fluorescence intensity, Y-axis means cell counts. MSCs: Mesenchymal stem cells; CD: Cluster of differentiation.
Expression of cell surface markers vimentin, α-SMA, and TE-7 increased significantly at 3rd generations later in MSCs + TGF-β group. Expression of these two proteins markers in group MSCs + TGF-β + DKK1 was reduced in comparison to MSCs + TGF-β group [Figure 4].

Expression of fibroblast surface marker mRNA on mesenchymal stem cells

RT-PCR was used to test the mRNA expression level of fibroblast markers. The results are shown in Figure 5. Vimentin, α-SMA expression was significantly increased and continued to have high expression in MSCs + TGF-β group. Expression of these fibroblast markers decreased to regular levels in MSCs + TGF-β + DKK1 group.

Wnt/β-catenin signaling molecules expression level in the course of Dickkopf-1 administration

Proteins expression of Wnt/β-catenin signaling pathway

Cell immunofluorescence method was employed to detect the protein expression of β-catenin, TCF, GSK-3β, Axin1, and JNK in the Wnt/β-catenin signaling pathway of MSCs. In MSCs + TGF-β group, β-catenin, TCF, GSK-3β, Axin1, and JNK protein expression increased significantly 3 days later. Expression of these proteins in MSCs + TGF-β + DKK1 group was reduced in comparison with MSCs + TGF-β [Figure 6].

mRNA expression of Wnt/β-catenin signaling proteins

RT-PCR was used to test mRNA expression level of Wnt/β-catenin signaling molecules, including β-catenin, TCF, and GSK-3β. In MSCs + TGF-β group, the Wnt/β-catenin signaling pathway was highly activated. TGF-β had significantly higher mRNA expression level of Wnt/β-catenin signaling molecules β-catenin, TCF, and GSK-3β. The addition of DKK1 showed a low mRNA expression level in Wnt/β-catenin signaling molecules. β-catenin, TCF, and GSK-3β were nearly normal in group MSCs + TGF-β + DKK1 [Figure 7].

Discussion

The characteristic of MSCs, engrafting in organs, suggests that exogenously administered MSCs contribute to repairing the injured tissue. This finding can potentially benefit regeneration of injured lung tissue. However, no matter what organ transplantation or differentiation requires and regulates the differentiation of MSCs, which types of cells differentiated from MSCs is a key question remaining to be clarified. It is known that Wnt/β-catenin signaling pathway also plays a vital role in fate determination and differentiation of MSCs. [20,21]
The fibrotic disease is an increase in the production and accumulation of extracellular matrix (ECM) components. Several molecules are related to the establishment of the fibrotic condition, including TGF-β, angiotensin II, and the connective tissue growth factor (CTGF). The hypothesis is that fibrosis is the transformation resulting from wound healing from defects in the intricate cell-cell signaling events.[22] Meanwhile, researchers have focused on classical fibrogenic mediators, including TGF-β, vimentin, α-SMA proteins, and other signal molecules.[23,24] Recent studies found that Wnt/β-catenin signaling pathways were involved in the development of fibrosis.[21,25,26] DKK-1 could block many of the changes in pericytes required for myofibroblast transition and attenuate established myofibroblast proliferation/activation by mechanisms dependent on LRP-6 and WNT ligands.[27] MSCs from mice and humans produce Wnt/β-catenin proteins which could stimulate matrix components for fibrosis.[28]

In our research, TGF-β plays a positive role in fibrogenesis. TGF-β is a key mediator in fibrosis, inducing epithelial to mesenchymal transition, fibroblast-to-myofibroblast activation, and ECM deposition.[29] The cooperation of TGF-β and Wnt/β-catenin signaling has been demonstrated in the process of fibrosis in recent years.[30] Under the condition of TGF-β in our study, expression of fibroblast surface marker proteins increased. TGF-β also increased β-catenin proteins levels and enhanced β-catenin/TCF/LEF transcription activities in MSCs. This result is in accordance with mouse and human findings.
with Zhou’s research in 2011.[31] TGF-β activates β-catenin signaling pathway via TGF-β type I receptor kinase activin receptor-like kinase 5, Smad3, protein kinase-A, and phosphatidylinositol 3-kinase pathways; the interaction between TGF-β and β-catenin signaling supports the view that β-catenin signaling is a mediator of TGF-β’s effects on differentiation of human MSCs.

Under resting condition, cytoplasmic β-catenin is phosphorylated by a destruction complex which is composed of Axin, APC, and GSK-3β. Once activated by TGF-β or other Wnt/β-catenin ligands, Wnt/β-catenin proteins will then bind to their receptors, which leads to the disassembly of the destruction complex and results in dephosphorylation of β-catenin. The quantity of expression from Wnt/β-catenin signaling molecules indeed increases with MSCs in our study.

The secreted DKK family (DKK1-4), exemplified by DKK1, could block the Wnt/β-catenin signaling pathway by combining LRP5/6 receptor.[17,18] It is known that regulating Wnt/β-catenin signaling is via interaction with Kremen receptors. The result tells us Wnt/β-catenin signaling pathways is in the highly activated status when MSCs are differentiating into fibroblast. After getting Dkk1, the expression of MSCs in β-catenin, TCF, GSK-3β, Axin1, and JNK, which are molecules in Wnt/β-catenin pathway, decreases more than that in control group. After getting Dkk1, less cells transform into fibrosis than that in control group as shown by immunofluorescence studies.

Pfaff et al.[32] found that in idiopathic fibrosis, DKK proteins affected epithelial cell proliferation. Wnt-induced epithelial cell proliferation is regulated by DKK1 in a dose-dependent fashion through functional studies. It is consistent with our study results to prevent fibrosis by preventing Wnt/β-catenin pathways. DKK proteins might, therefore, be suitable therapeutic targets for MSC transplantation. In conclusion, inhibiting the Wnt/β-catenin pathway could control directional differentiation of MSCs.

Activating the Wnt/β-catenin signaling pathway will accelerate the process of MSC fibrosis. It has been proved DKK-1 blocks activation and proliferation of established myofibroblasts in vitro and blocks pericyte proliferation, pericyte migration, gene activation, and cytoskeletal reorganization to TGF-β or CTGF. Dkk1 could block Wnt/β-catenin signaling pathway to stop the process of MSCs fibrosis.[27] In light of those possible effects, DKK-1 can have on MSCs, can be direct MSC clinical treatment and the related research.

**Study limitation**

This study confirmed Dkk1 could stop the process of MSCs fibrosis in vitro while its function in vivo and its mechanism still need further discussed.

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Conflicts of interest
There are no conflicts of interest.

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