Wnt/β-catenin signaling mediates the senescence of bone marrow-mesenchymal stem cells from systemic lupus erythematosus patients through the p53/p21 pathway

Zhifeng Gu · Wei Tan · Guijuan Feng · Yan Meng · Biyu Shen · Hong Liu · Chun Cheng

Abstract Recent studies have shown that allogeneic bone marrow (BM)-mesenchymal stem cell transplantation (MSCT) appears to be effective in systemic lupus erythematosus (SLE) patients and lupus-prone mice, contrary to studies in syngeneic BM-MSCT. These studies indicated that the abnormalities of BM-MSCs may be involved in the pathogenesis of SLE. Our studies and other previous studies have revealed that BM-MSCs from SLE patients exhibited early signs of senescence, such as flattened morphology, slow proliferation, increased senescence-associated β-galactosidase (SA-β-gal) activity, and so on. However, the mechanisms by which these cells senescences were still unclear. Previous studies have demonstrated that Wnt/β-catenin signaling plays an important role in stem cell senescence. In the current study, we investigated whether Wnt/β-catenin signaling mediates the senescence of BM-MSCs from SLE patients. We have found that Wnt/β-catenin signaling and the p53/p21 pathway were significantly hyperactivated in senescent SLE BM-MSCs. Treatment with 100 ng/mL Dickkopf-1 (DKK1), a Wnt/β-catenin signaling inhibitor or β-catenin siRNA for 48 h could reverse the senescent features of SLE BM-MSCs. Additionally, the expression levels of p53 and p21 were reduced in treated-SLE BM-MSCs compared with the untreated group. In summary, our study indicated that Wnt/β-catenin signaling may play a critical role in the senescence of SLE BM-MSCs through the p53/p21 pathway.

Keywords Bone marrow-mesenchymal stem cells (BM-MSCs) · Systemic lupus erythematosus (SLE) · Senescence · Wnt/β-Catenin signaling · p53/p21 pathway

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Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease characterized by multi-organ involvement and a remarkable variability in clinical presentations [1]. Deregulated activation of both B and T cells and the aberrant production of pro-inflammatory cytokines are critically involved in the initiation and progression of tissue pathology and organ damage in SLE [2]. Mesenchymal stem cells (MSCs) are multipotent stem cells with the capacity for self-renewal and the potential to differentiate into a variety of cell types, including osteoblasts, chondrocytes, adipocytes, and myoblasts [3]. It has become evident that MSCs can exert immune regulatory functions, both in vivo and in vitro, on a wide range of

immunocompetent cells, such as T cells and B cells [4, 5]. The functional features of MSCs make them attractive therapeutic targets for SLE. Recently, our studies and others have revealed that allogenic MSC transplantation (MSCT) appears to be a feasible and safe therapeutic strategy in lupus-prone mice and SLE patients [6, 7]. However, syngeneic BM-MSCT was ineffective [8, 9]. When the biological characteristics of these cells were studied, our laboratory and others revealed that BM-MSCs from SLE patients showed prominent features of senescence. Sun and colleagues first reported that MSCs from SLE patients grew more slowly than those from normal controls [10]. Then, Nie et al. [11] reported that MSCs from SLE patients appeared larger and flatter in appearance after three passages and grew progressively slower compared with those from normal controls. Li et al. [12] found that there were increased frequencies of apoptosis and aging in SLE BM-MSCs in comparison with those from normal controls. Notably, Gene Ontology analysis showed that the majority of these genes were related to cell cycle and protein binding. Pathway analysis showed that the differentially regulated signal pathways were involved in the actin cytoskeleton, focal adhesion, tight junctions, and the transforming growth factor (TGF)-β pathway [13]. Recently, we reported that MSCs from both untreated and treated SLE patients showed characteristics of senescence [14]. These studies revealed that senescent BM-MSCs may be associated with the pathogenesis of SLE, but the molecular mechanisms were not very clear. The identification of the molecular mechanisms by which senescent BM-MSCs lead to SLE has important significance.

The signs of cell senescence include a diminishing ability to undergo cell division, increased cell size, actin stress fibers, senescence-associated β-galactosidase (SA-β-gal) activity and so on. Cyclin-dependent kinase inhibitors (CDKIs) were reported to be involved in the cell senescence process, among which the p53/p21 pathway has been studied extensively in recent years [15, 16]. Earlier studies had confirmed that senescent cells exhibited extremely low protein levels of cyclin-dependent kinases (CDKs) and cyclins and an increased level of the p53-dependent p21 protein, which inhibits the kinase activity of cyclin/CDK complexes. These characteristic molecular factors and mechanisms result in an irreversible G1-arrest, leading to cell senescence [17]. Furthermore, our recent study demonstrated that the expression levels of p53 and p21 were significantly increased in SLE BM-MSCs, and knockdown of these proteins could reverse the senescence and allow the cells to resume proliferation [18].

Many upstream signaling pathways regulate the expression of p53 and p21 [19–21]. Some studies reported that the p53/p21 pathway may be the main mediator of BM-MSC senescence induced by excessive Wnt/β-catenin

signaling [22–24], which is an evolutionarily conserved intracellular signaling cascade with demonstrated roles in cell proliferation, cell fate determination, and axis polarity induction [25]. Wnt/β-catenin signaling is activated by the binding of Wnt ligands to the frizzled family of receptors. In the absence of Wnt ligands, β-catenin is phosphorylated by glycogen synthase kinase-3β (GSK-3β) and then degraded by the ubiquitin–proteasome system. However, when Wnt ligands bind to frizzled receptors, GSK-3β activity is inhibited, and unphosphorylated β-catenin accumulates in the cytoplasm and translocates into the nucleus, where it promotes the transcription of a variety of target genes [26]. Recent studies have shown that constitutive activation of Wnt/β-catenin signaling can lead to the senescence or dysfunction of certain cells, such as fibroblasts [27], thymocytes [28], and endothelial cells [29]. These data have revealed a new biological role of Wnt/β-catenin signaling in cell senescence. However, whether Wnt/β-catenin signaling plays an important role in the senescence of BM-MSCs from SLE patients through the p53/p21 pathway remained unclear. In the present study, we investigated the effects of Wnt/β-catenin signaling on senescent SLE BM-MSCs and the relationship between Wnt/β-catenin signaling and the p53/p21 pathway.

In summary, the roles of Wnt/β-Catenin signaling on the senescence of SLE BM-MSCs were investigated. We found that the expression levels of β-catenin and GSK-3β were higher in SLE BM-MSCs than in the normal group. In addition, to evaluate the effects of Wnt/β-catenin signaling on the senescence of SLE BM-MSCs, we used 100 ng/mL Dickkopf-1 (DKK1) and β-catenin siRNA to reverse the senescent features of SLE BM-MSCs. Finally, to determine the mechanisms of Wnt/β-catenin signaling in BM-MSCs with regards to aging, the expression levels of p53 and p21 were examined. We confirmed that Wnt/β-catenin signaling mediated the senescence of SLE BM-MSCs through the p53/p21 pathway.

Materials and methods

Patients

Twelve SLE patients between 15 and 41 years of age (mean 27.08 ± 8.02 years) were enrolled in this study (Table 1). Our previous study had indicated that the BM-MSCs from treated- and untreated-SLE patients were senescent [14], so we didn't group in this study. The SLE diagnosis was made based on the criteria proposed by the American College of Rheumatology. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was used to measure the disease activity. All patients were categorized as active using a cutoff SLEDAI score of eight. Twelve healthy subjects were

Table 1 Details of 12 SLE patients

Patient	Age	Disease duration	Current treated	SLEDAI
1	15	1 year	pred 15 mg/day	11
			HCQ 0.2/day	
2	20	8 months	pred 20 mg/day	14
			HCQ 0.2/day	
			LEF 0.2/day	
3	21	2 years	pred 15 mg/day	9
			HCQ 0.2/day	
			LEF 0.2/day	
4	26	1 year	pred 10 mg/day	13
			HCQ 0.2/day	
5	28	16 months	pred 12.5 mg/day	16
			HCQ 0.2/day	
			CTX 0.4/2 weeks	
6	32	2 years	pred 12.5 mg/day	12
			HCQ 0.2/day	
7	37	3 years	pred 10 mg/day	11
			HCQ 0.2/day	
8	41	5 years	pred 7.5 mg/day	8
			HCQ 0.2/day	
9	18	3 days	None	9
10	24	2 days	None	16
11	28	2 days	None	14
12	35	4 days	None	21

Pred prednisone, *HCQ* hydroxychlorquine, *LEF* leflunomide, *CTX* cyclophophamide

used as the normal group. All research subjects were females with a similar age distributions (mean 26.82 ± 7.41 years). All patients gave consent to the study, which was approved by the Ethics Committee of the Affiliated Hospital of Nantong University.

Isolation, cell culture, and identification of BM-MSCs from SLE and normal subjects

MSCs were isolated and expanded from iliac crest BM of all the SLE patients and normal subjects. Five milliliters of heparinized BM were mixed with an equal volume of phosphate-buffered saline (PBS). Then, the resuspended cells were layered over Ficoll solution (1.077 g/mL) and centrifuged at $2,000 \times g$ for 25 min at room temperature. The mononuclear cells were collected at the interface and resuspended in low-glucose Dulbecco-Modified Eagle Medium (L-DMEM) supplemented with 10 % heat inactivated fetal bovine serum (FBS). Then, the cells were plated at a density of 2×10^7 cells per 25 cm^2 dish and cultured at 37 °C in a 5 % CO$_2$ incubator. The medium was replaced, and non-adherent cells were removed after 5 days and every 3 days thereafter. When the BM-MSCs became nearly confluent, the adherent cells were released from the dishes with 0.25 % trypsin–EDTA (Gibco, USA) and were then replated at a density of 1×10^6 cells per 25 cm^2 dish. After four passages (p4), the cells were used for the following studies.

siRNA and transfection

siRNA oligonucleotides were synthesized by Genepharma Co., Ltd. (Shanghai, China). The effective sequence used for the specific silencing of β-catenin was 5′-CACCTCCCAA GTCCTTTAT-3′. The siRNA sequence was named by si-β-catenin. The non-silencing control siRNA was an irrelevant siRNA with random nucleotides (5′-TTCTCCGAACGTGT-CACGT-3′) and was not homologous to any sequence found in the gene bank. Transfection was carried out according to the manufacturer's protocol (Qiagen Inc., Valencia, CA).

Treatment methods of BM-MSCs

There were four groups in the present study. In the normal or SLE groups, BM-MSCs were cultured for 48 h. In the DKK1 groups, 100 ng/mL DKK1 (R&D Systems, USA) was directly added to the cells and incubated for 48 h. In the si-β-catenin group, the cells were transfected with si-β-catenin for 48 h.

SA-β-gal assay

The SA-β-gal activity was determined using a kit from the Chemical Company following the manufacturer's instructions. BM-MSCs were plated into 6-well culture plates at a density of 5×10^4 cells per well and incubated for 48 h. Then, the cells were washed twice with PBS and fixed with fixing solution for 15 min. After incubation with the staining SA-β-gal detection solution at 37 °C without CO$_2$ overnight, the slips were washed and analyzed under the microscope.

Immunofluorescence assay of the cytoskeleton of BM-MSCs

BM-MSCs were washed twice with PBS and fixed in 4 % paraformaldehyde (PFA) for 1 h. After permeabilization and blocking, they were incubated with fluorescein isothiocyanate (FITC)-conjugated phalloidin. The stained cells were then examined using a Zeiss confocal laser scanning microscope.

Cell proliferation assay

Cell proliferation was measured using the cell counting Kit-8 (CCK-8) assay following the manufacturer's instructions. Briefly, the cells were plated at a density of

0.3×10^4 cells per well in 96-well plates (Corning Inc., Corning, NY) in a volume of 100 μl, and the cells were incubated overnight to allow for adherence. At 1–5 days, CCK-8 (Dojindo, Kumamoto, Japan) reagents were added to a subset of wells under different treatments and incubated for 2 h at 37 °C, we quantified the absorbance using an automated plate reader.

Flow cytometry

For cell cycle analysis, BM-MSCs were collected and fixed with 70 % ethanol at 4 °C for 24 h. After being washed with PBS and then treated with 100 μg/ml RNase (Sigma, USA) for 30 min, the cells were stained with 50 μg/ml propidium iodide (PI) solution (Sigma, USA) for 30 min and analyzed using a flow cytometer (FACS Calibur, BD Biosciences, USA). The fraction of cells in the G0/G1, S, and G2/M phases were quantified with the ModFit LT system. Three separate experiments were performed.

Western blotting

To assay the β-catenin protein, cytoplasmic and nuclear proteins from cultured cells were prepared using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Company, Rockford, IL, USA), respectively. β-actin and β-tubulin were used as the internal controls for the cytoplasmic and nuclear proteins, respectively. The
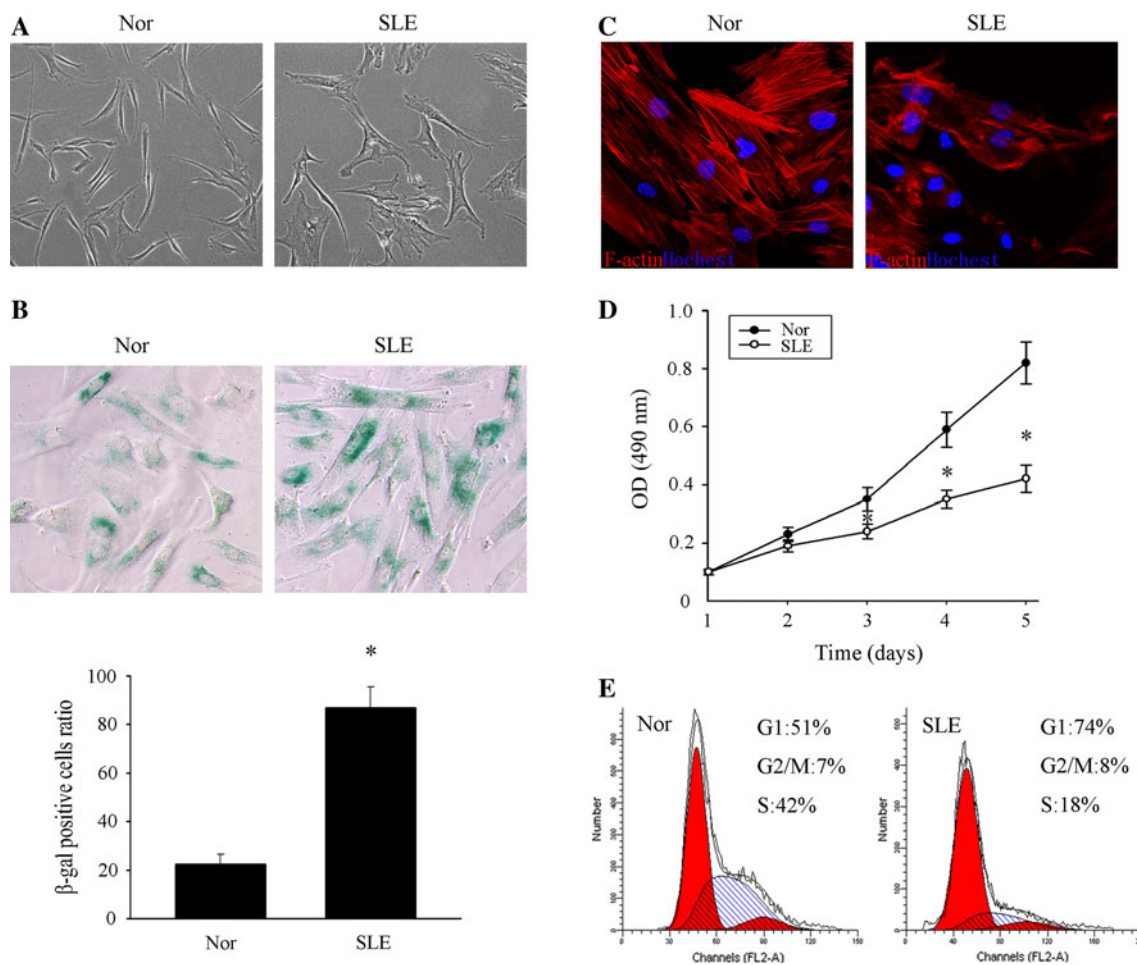

Fig. 1 BM-MSCs from SLE patients were senescent cells. **a** The sizes of the SLE BM-MSCs were larger than those from the normal group. **b** SA-β-gal was used to examine BM-MSC senescence. The number of SA-β-gal-positive cells was obviously increased among the SLE BM-MSCs compared to those from a normal person. **c** BM-MSCs were stained with FITC-Phalloidin. Immunofluorescence showed that the F-actin distribution was abnormal in the BM-MSCs from SLE patients. **d** The p4 BM-MSCs were plated in 96-well plates. After 1–5 days, the cell proliferation ratio was detected using a CCK8 assay. The absorbance was shown as the proliferation rate. BM-MSCs from SLE patients grow more slowly than those from the normal group. **e** The flow cytometry results showed that the ratio of cells in G_0 phase increased in the SLE BM-MSCs compared to the normal group. All data were expressed as the mean ± SD (*$P < 0.05$ compared with the normal group)
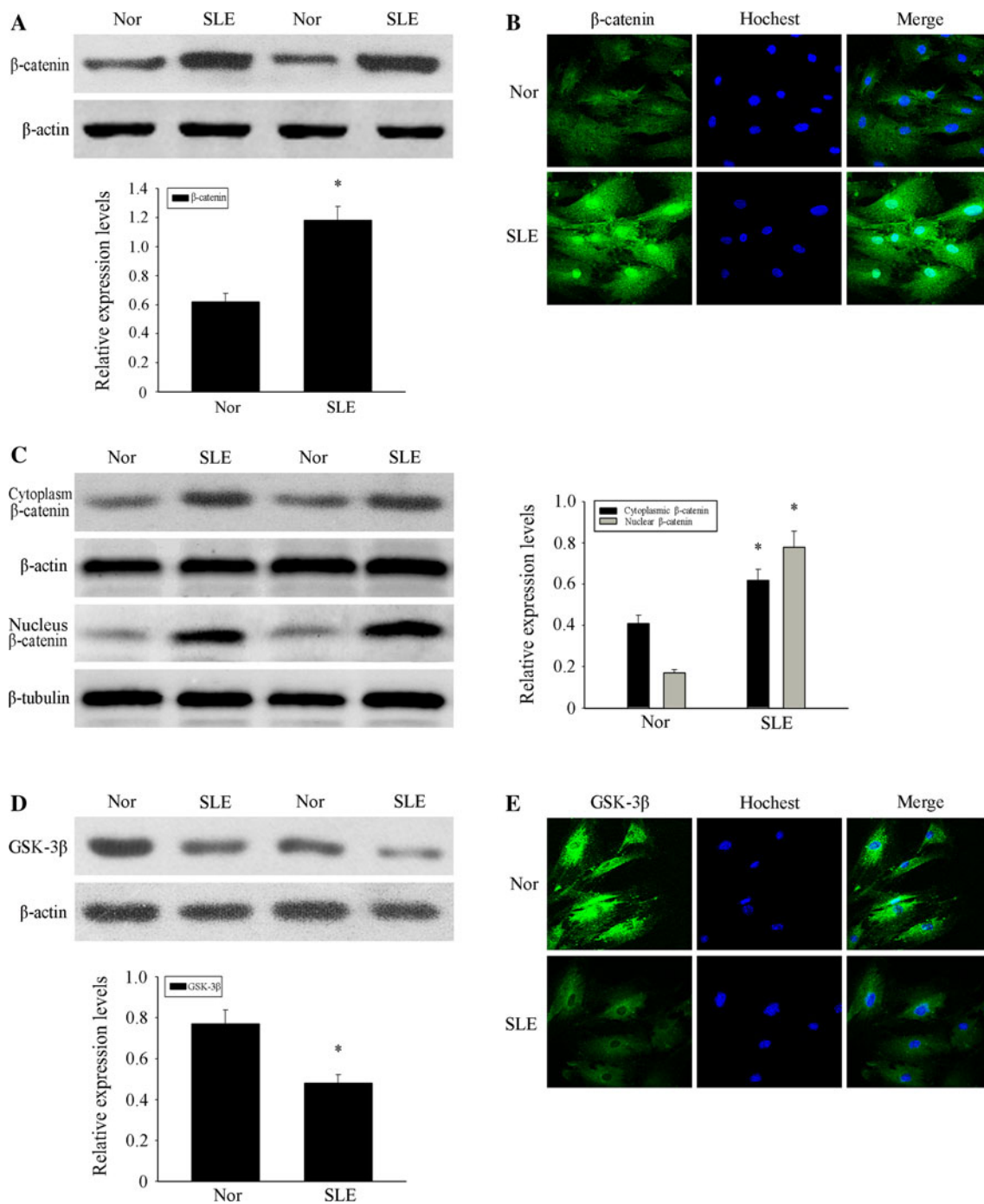
Fig. 2 The over-activation of Wnt/β-catenin signaling in BM-MSCs from SLE patients. **a** β-catenin expression was high in BM-MSCs from SLE patients compared with the normal group, as determined by western blot analysis. β-actin was used as an internal control. **b** P4 BM-MSCs from SLE patients and normal patients were cultured in 24-well plates. Immunofluorescence staining of β-catenin verified the high levels of expression in the SLE group. Counterstaining with Hoechst displayed the localization of the nucleus (*Scale bar* 50 μm). **c** The expression of cytoplasmic and nuclear β-catenin increased in the SLE group compared to the normal group, and the increase in nuclear β-catenin expression was more pronounced in the SLE group. β-actin and β-tubulin were used as the internal controls for the cytoplasm and nucleus, respectively. **d** The expression of GSK-3β was decreased in BM-MSCs from SLE patients compared with the normal group by western blot analysis. β-actin was used as an internal control. **e** Immunofluorescence staining of GSK-3β confirmed the low expression in the SLE BM-MSCs. Counterstaining with Hoechst displayed the localization of the nucleus (*scale bar* 50 μm). All data were expressed as the mean ± SD (*$P < 0.05$ compared with the normal group)
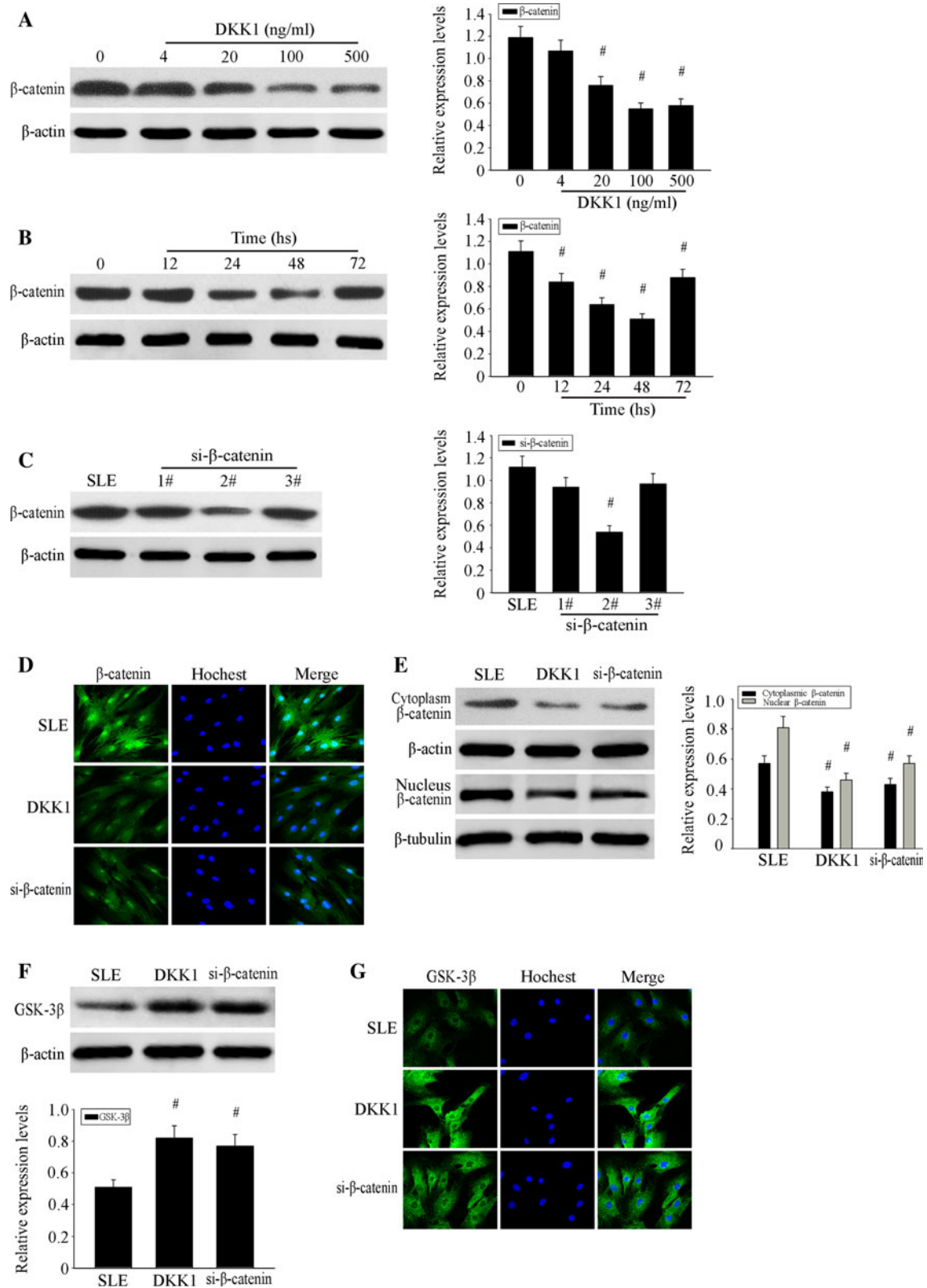
total cellular protein was extracted using the following method. The different BM-MSCs treatment groups were washed in cold-buffered PBS and were then lysed in radio immunoprecipitation assay buffer (150 mM NaCl, 1 % Triton X-1 00, 0.5 % NaDOD, 0.1 % sodium dodecyl sulfate, and 50 mM Tris, pH 8.0). After centrifugation at

◀**Fig. 3** The Wnt/β-catenin signaling in BM-MSCs from SLE patients was inhibited by DKK1 and si-β-catenin. **a** Cells were cultured in different concentrations of DKK1 for 48 h. By determining the inhibition of β-catenin, we observed that DKK1 achieved its maximal effects at a dose of 100 ng/ml. **b** P4 BM-MSCs from SLE patients were cultured with 100 ng/ml DKK1 for different times. DKK1 achieved its maximal effects at approximately 48 h, as determined by β-catenin inhibition. **c** Cells were transfected with si-β-catenin for 48 h. Western blot analyses showed that β-catenin expression was significantly decreased in the SLE BM-MSCs treated with the NO.2 siRNA. **d** Immunofluorescence confirmed that the β-catenin expression was obviously reduced in the SLE BM-MSCs treated with DKK1 or si-β-catenin. Counterstaining with Hoechst displayed the localization of the nucleus (Scale bar = 50 μm). **e** After treatment with DKK1 or si-β-catenin, nuclear β-catenin d markedly decreased in SLE BM-MSCs. β-actin and β-tubulin were used as the internal controls for the cytoplasm and nucleus, respectively. **f** The expression of GSK-3β was obviously increased in the DKK1 and si-β-catenin-treated group compared with the normal group by western blot analysis. β-actin was used as an internal control. **g** Immunofluorescence confirmed the increase in GSK-3β expression in SLE BM-MSCs treated with DKK1 and si-β-catenin. Counterstaining with Hoechst displayed the localization of the nucleus (Scale bar 50 μm). All data were expressed as the mean ± SD (#P<0.05 compared with the SLE group)

12,000 rpm for 5 min at 4 °C, the protein supernatant was transferred into new tubes. The protein concentrations of the samples were determined using the bicinchoninic acid protein assay (Pierce Chemical Company, Rockford, IL, USA). A 40 μg sample of the total protein was resolved using 12.5 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) membranes. The membranes were blocked with 5 % nonfat milk at room temperature for 1 h in Tris-buffered saline containing Tween 20. Primary antibodies to detect β-catenin (rabbit anti-human, 1:500; Cell Signaling, Danvers, MA, USA), GSK-3β (rabbit anti-human, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (rabbit anti-human, 1:500; Cell Signaling, Danvers, MA, USA), β-tubulin (rabbit anti-human, 1:500; Cell Signaling, Danvers, MA, USA), p53 (rabbit anti-human, 1:500; Cell Signaling, Danvers, MA, USA), or p21 (rabbit anti-human, 1:500; Cell Signaling, Danvers, MA, USA) were incubated overnight with the membranes at 4 °C. Lastly, the membrane was incubated with a secondary mouse anti-goat or goat anti-rabbit antibody conjugated to horseradish peroxidase (1:2,000; Southern Biotech, Birmingham, AL, USA) for 2 h and were visualized using an enhanced chemiluminescence system (Pierce Chemical Company, Rockford, IL, USA).

Immunofluorescence

BM-MSCs were fixed with 4 % PFA for 1 h, washed with PBS containing 0.1 % Triton X-100 (PBST), and blocked for 30 min in PBST supplemented with 10 % FBS. Cells were then incubated with β-catenin (rabbit anti-mouse, 1:100; Cell Signaling, Danvers, MA, USA), GSK-3β (rabbit anti-mouse, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p53 (rabbit anti-mouse, 1:100; Cell Signaling, Danvers, MA, USA), or p21 (rabbit anti-mouse, 1:100; Cell Signaling, Danvers, MA, USA) antibody in the same solution overnight at 4 °C. The cells were then washed and incubated with secondary antibodies for 2 h at room temperature. Nuclei were stained with Hoechst (1:800, Santa Cruz). The cells were examined using a Leica fluorescence microscope (Germany).

Statistical analysis

All data are presented as the mean ± standard deviation (SD) from at least three independent experiments. All statistical analyses were performed using the SPSS 11.0 software, and the data were analyzed using Student's *t* test with $P < 0.05$ considered statistically significant.

Results

The BM-MSCs from SLE patients showed senescence characteristics

The primary culture of BM-MSCs was successful in 12 cases of SLE patients and 12 cases of healthy donors. As we have shown previously, BM-MSCs from SLE patients exhibited senescent behavior [14]. Cellular hypertrophy is a marker of cell senescence [30]. From the cell morphology, we found that BM-MSCs from SLE patients were larger than those from the normal group and exhibited more numerous and longer podia (Fig. 1a). SA-β-gal has been proposed to be a universal marker of senescence. We found that the number of SA-β-gal-positive cells was significantly increased in the BM-MSCs from SLE patients in comparison with the BM-MSCs from a normal person (Fig. 1b). The F-actin distribution was disordered and assembled around the nuclear region in BM-MSCs from SLE patients (Fig. 1c). The proliferation of BM-MSCs was measured with a CCK8 assay. The results indicated that BM-MSCs from SLE patients grow more slowly than those from the normal group (Fig. 1d). Flow cytometry showed that there were more BM-MSCs restricted in the G1 phase harvested from the treated- and untreated-SLE patients (73.62 ± 6.4 %) than in the BM-MSCs from the normal patients (52.37 ± 5.1 %) (Fig. 1e). In summary, we confirmed that the BM-MSCs from SLE patients were senescent cells.

Wnt/β-catenin signaling was over-activated in BM-MSCs from SLE patients

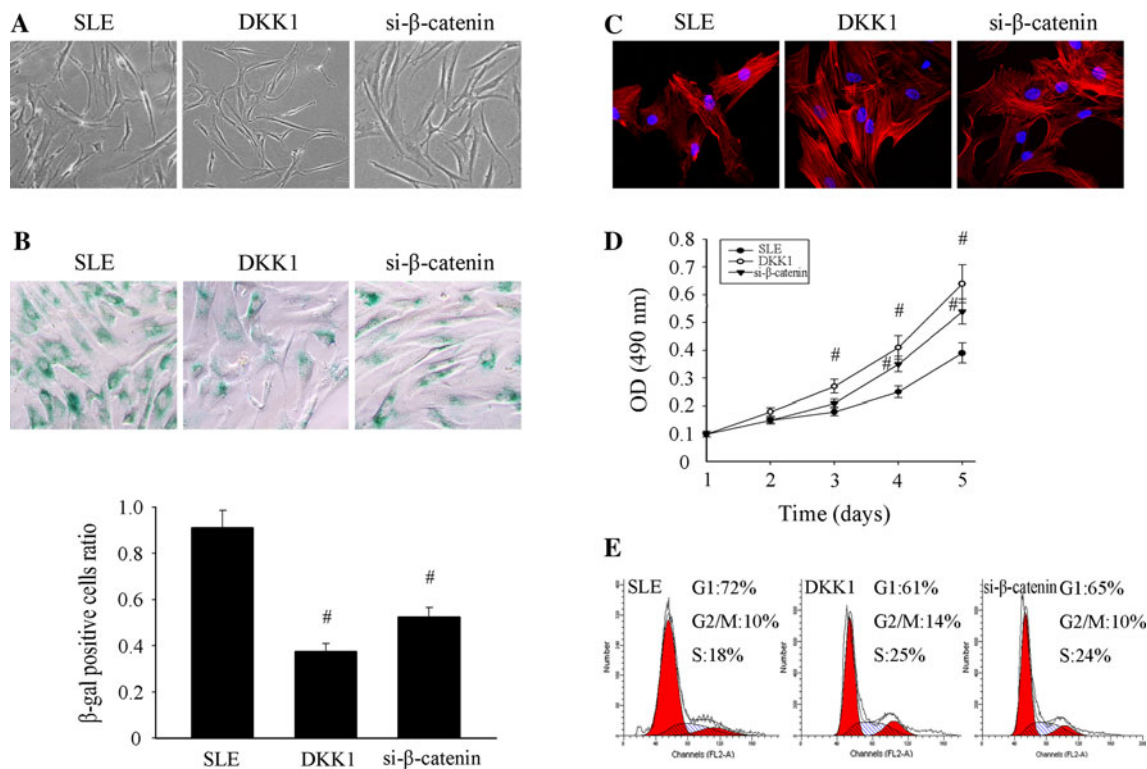To identify the activity of the Wnt/β-catenin signaling pathway in BM-MSCs of SLE patients, the β-catenin

Fig. 4 DKK1 and si-β-catenin decelerated the senescence of BM-MSCs from SLE patients. BM-MSCs from SLE patients were treated with 100 ng/mL DKK1 or si-β-catenin for 48 h. (**a**) The sizes of the SLE BM-MSCs inhibited by DKK1 and si-β-catenin became thinner than the SLE group. (**b**) BM-MSCs were fixed and stained for β-gal. The number of SA-β-gal-positive cells decreased in the DKK1 or si-β-catenin-treated SLE BM-MSCs in comparison with the SLE group. (**c**) Immunofluorescence showed that the abnormal distribution of

F-actin in the BM-MSCs from SLE patients was reversed after β-catenin knockdown through DKK1 treatment or si-β-catenin. (**d**) The p4 BM-MSCs were plated in 96-well plates. After 1–5 days, a CCK8 assay showed that the cell proliferation ratio increased in SLE BM-MSCs treated with DKK1 and si-β-catenin. (**e**) The flow cytometry results showed that the ratio of cells in G_0 phase was decreased in the DKK1 and si-β-catenin-treated group. All data were expressed as the mean ± SD ([#]$P < 0.05$ compared with the SLE group)

expression in the BM-MSCs of the normal and SLE groups was examined by western blot and immunofluorescence analyses. The results showed that β-catenin expression was clearly increased in the senescent SLE BM-MSCs compared to the levels in a normal person (Fig. 2A-B). However, there was no correlation between the expression levels of β-catenin and SLE disease activity. After further study, we found that both cytoplasmic and nuclear β-catenin expression increased in the SLE group compared to the normal group, and the increase in nuclear β-catenin expression was more pronounced in the SLE group (Fig. 2c). GSK-3β is a key enzyme that negatively regulates Wnt/β-catenin signaling, so the Wnt/β-catenin signaling was further studied by determining the levels of GSK-3β expression. Compared with the normal group, GSK-3β expression was notably decreased in the SLE group (Fig. 2d), and the cytoplasmic fluorescence intensity was subdued (Fig. 2e). These results suggested that overactive Wnt/β-catenin signaling may be associated with the senescence of SLE BM-MSCs.

DKK1 and si-β-catenin decreased the expression of Wnt/β-catenin signaling in BM-MSCs from SLE patients

To further assess the role of Wnt/β-catenin signaling in the progression of BM-MSC senescence, we used different concentrations of DKK1, a Wnt/β-catenin signaling inhibitor, and si-β-catenin to interfere with the expression of the Wnt/β-catenin signaling pathway. First, we determined the cell's dose response to DKK1 by measuring the expression of β-catenin. The results showed that DKK1 inhibited β-catenin expression at concentrations of 20 ng/ml or higher and achieved maximal effects at doses of 100–500 ng/ml (Fig. 3a). Additionally, we found that 100 ng/ml DKK1 rapidly inhibited β-catenin expression and achieved its maximal effects at 48 h (Fig. 3b). Thus, we performed these experiments using DKK1 at a concentration of 100 ng/ml for 48 h. To further assess the role of Wnt/β-catenin signaling in BM-MSC senescence, we used BM-MSCs transfected with si-β-catenin or a non-specific siRNA. Over 50 %
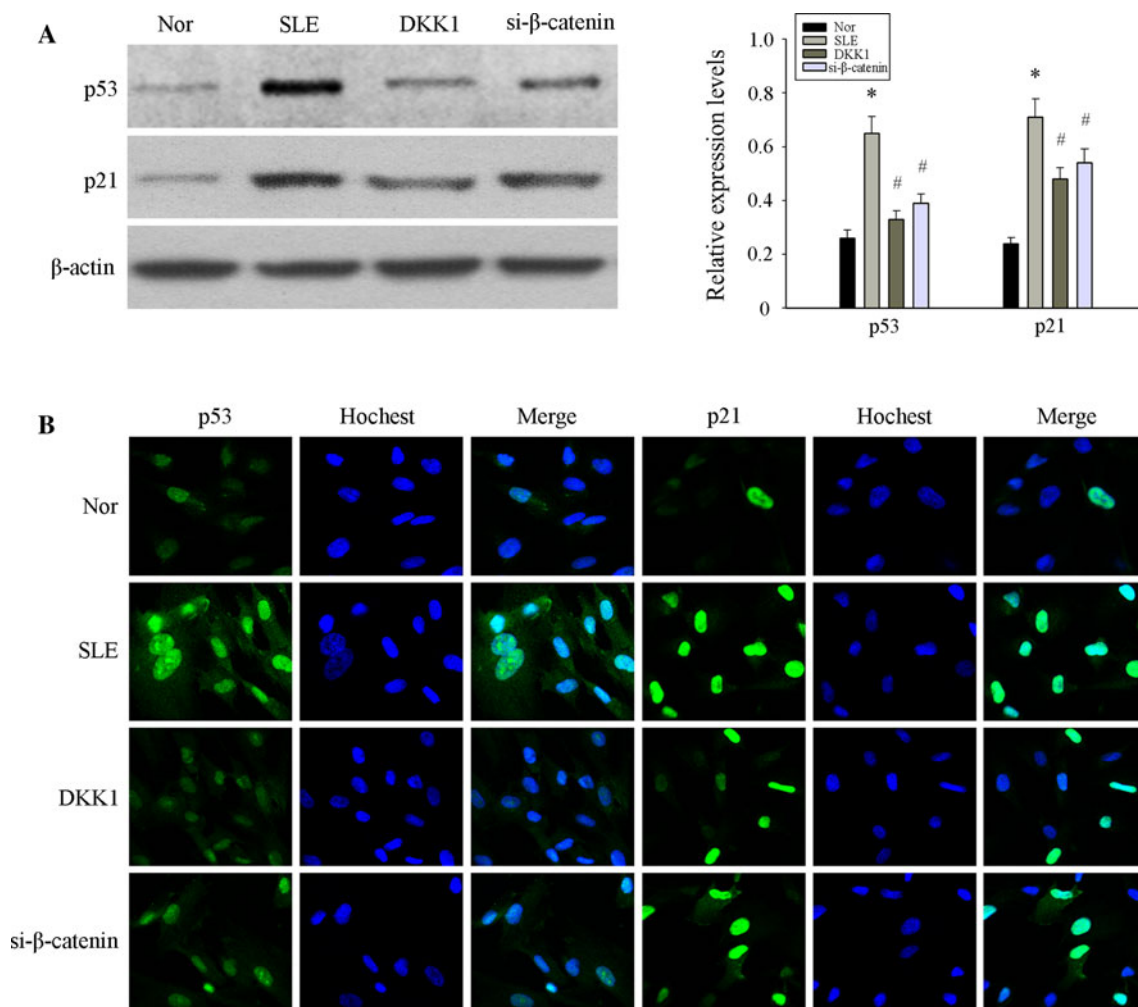
Fig. 5 The connection of Wnt/β-catenin signaling and the p53/p21 pathway in BM-MSCs from SLE patients. (**a**) The expression levels of p53 and p21 were high in BM-MSCs from SLE patients compared with the normal group, as determined by western blot analysis; the expression of these proteins could be inhibited by DKK1 or si-β-catenin treatment. β-actin was used as an internal control. (**b**) P4 BM-MSCs from the SLE group and the normal group were cultured in 24-well plates. Half of the SLE group was treated with DKK1 or si-β-catenin for 48 h. Immunofluorescence staining of p53 and p21 verified their high expression levels in the nuclei of SLE BM-MSCs, and this expression was inhibited by DKK1 or si-β-catenin treatment. Counterstaining with Hoechst displayed the localization of the nucleus (*Scale bar* 50 μm). All data were expressed as the mean ± SD (*$P<0.05$ compared with the normal group; #$P<0.05$ compared with the SLE group)

of the cells were transfected with the siRNA constructs only in the second group, which was chosen to add in the medium of SLE BM-MSCs. We found that β-catenin expression was considerably decreased in the si-β-catenin-transfected BM-MSCs (Fig. 3c). However, cell viability was not affected in the β-catenin knockdown BM-MSCs. After treatment with 100 ng/mL DKK1 or si-β-catenin, we observed, by immunofluorescence analysis, that the high expression of β-catenin in the SLE BM-MSCs was reversed, especially in the nucleus (Fig. 3d). Next, we confirmed that nuclear β-catenin expression markedly decreased in the treated groups by isolating the nuclear and cytoplasmic fractions of the BM-MSCs (Fig. 3e). Meanwhile, GSK-3β expression

significantly increased in SLE BM-MSCs treated with DKK1 or si-β-catenin (Fig. 3f–g).

DKK1 and si-β-catenin slowed cell senescence in BM-MSCs from SLE patients

We compared the effects of DKK1 and si-β-catenin on the cell morphology of BM-MSCs from SLE patients, and we found that the treatment decelerated cell hypertrophy (Fig. 4a). There were less SA-β-gal-positive cells in the BM-MSCs from SLE patients when treated with DKK1 or si-β-catenin (Fig. 4b). Furthermore, the disordered distribution of F-actin in BM-MSCs from SLE patients was

effectively reversed after treatment with DKK1 or si-β-catenin (Fig. 4c). The CCK8 assay showed that the proliferation rate of the si-β-catenin BM-MSCs from SLE patients was restored to that of normal BM-MSCs (Fig. 4d). Cell-cycle analysis revealed that the G1 phase arrest was reversed in SLE BM-MSCs treated with DKK1 or si-β-catenin (61.85 ± 4.3 % and 64.71 ± 4.8 %) (Fig. 4e). These results implied that the Wnt/β-catenin pathway played an essential role in the senescence of SLE BM-MSCs.

Activated Wnt/β-catenin signaling promoted the expression of p53 and p21 in BM-MSCs from SLE patients

To investigate the effects of Wnt/β-catenin signaling on the p53/p21 pathway, we first examined p53 and p21 expression using western blot and immunofluorescence analyses. The results show that p53 and p21 expression obviously increased in the SLE group compared to the normal group. However, after treating the SLE BM-MSCs with DKK1 or si-β-catenin, p53 and p21 levels significantly decreased (Fig. 5a). Furthermore, in the normal group, p53 and p21 expression was very weak, whereas the SLE group showed a clear increase in p53 and p21 expression. Compared with the SLE group, p53 and p21 expression was substantially inhibited in the DKK1 or si-β-catenin groups (Fig. 5b). Therefore, we considered that the target genes of p53 and p21 were closely related to the cell senescence induced by excessive activation of Wnt/β-catenin signaling.

Discussion

Recently, some researchers have postulated that SLE is a stem cell disorder disease. A better understanding of the dysfunction of the BM-MSCs in SLE patients could have major clinical implications in SLE treatment. In our study, we confirmed that the BM-MSCs from SLE patients displayed prominent senescent characteristics, such as increased SA-β-gal staining, disordered cytoskeletons and slow growth rates, which were similar to our previous studies [14].

Wnt/β-catenin signaling is an evolutionarily conserved intracellular signaling cascade with demonstrated roles in cell proliferation and cell fate determination [25]. Besides, Wnt signaling pathways also control lineage specification in vertebrate embryos and maintain pluripotency in embryonic stem cells [31]. In recent years, several lines of evidence suggested that Wnt/β-catenin signaling plays pivotal roles in cell or tissue senescence. For instance, Brack et al. [32] confirmed that the Wnt/β-catenin signaling pathway was elevated in tissue-specific stem cell aging and increased in tissue fibrosis with age. Liu H et al. [33] also found that both

in vitro and in vivo, continuous Wnt exposure triggered accelerated cellular senescence in a mammalian mouse model of accelerated senescence. In relation to BM-MSCs, Da-yong Zhang et al. [22, 24] indicated that Wnt/β-catenin signaling played a critical role in the BM-MSC senescence induced by the serum of aged animals. In our study, we show that β-catenin expression and the nuclear accumulation of β-catenin increased in BM-MSCs from SLE patients, and this accumulation could be inhibited by treatment with DKK1 or si-β-catenin. These results indicated that Wnt/β-catenin signaling in SLE BM-MSCs may mediate their senescence. To further explore the relationship between SLE BM-MSC senescence and the activity of Wnt/β-catenin signaling, we examined the changes in the SLE BM-MSCs after modulating Wnt/β-catenin signaling. In the SLE group, after treatment with 100 ng/ml DKK1 or si-β-catenin for 48 h, the number of SA-β-gal-positive cells significantly decreased, and the proliferation was obviously enhanced. Taken together, we concluded that Wnt/β-catenin signaling was an important mediator of senescence in BM-MSCs from SLE patients.

Previous studies have found that the p53/p21 pathway may be the main mediator of BM-MSC senescence induced by excessive Wnt/β-catenin signaling [22–24]. A recent study reported that the expression levels of p53 and p21 were increased in BM-MSCs from SLE patients [18]. In our current study, we examined whether Wnt/β-catenin signaling mediated the senescence of BM-MSCs from SLE patients through the p53/p21 pathway. Our results showed a clear increase in p53 and p21 expression in the SLE group compared with their expression in the normal group. After treatment with 100 ng/ml DKK1 or si-β-catenin, the expression of p53 and p21 substantially decreased. These results implied that the activation of Wnt/β-catenin signaling induced SLE BM-MSC senescence by activating the p53/p21 pathway. We confirmed that the p53/p21 pathway could mediate the cell senescence induced by excessive Wnt/β-catenin signaling. Therefore, there is a correlation between Wnt/β-catenin signaling and the p53/p21 pathway, and both pathways could be potential molecular targets to delay the onset of senescence of BM-MSCs in SLE patients.

In conclusion, Wnt/β-catenin signaling played a critical role in the senescence of BM-MSCs from SLE patients. The p53/p21 pathway may be the main mediator of the BM-MSC senescence induced by excessive Wnt/β-catenin signaling. Deciphering the mechanisms of Wnt/β-catenin signaling involved in the senescence of SLE BM-MSCs will help improve transplantation efficacy of BM-MSCs in SLE patients.

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