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PTEN/Akt-p27^{kip1} Signaling Promote the BM-MSCs Senescence and Apoptosis in SLE Patients

Wei Tan,[1,2] Zhifeng Gu,[1*] Biyu Shen,[1] Jinxia Jiang,[1] Yan Meng,[1] Zhanyun Da,[1] Hong Liu,[3] Tao Tao,[3] and Chun Cheng[3,4**]

[1]*Department of Rheumatology, Affiliated Hospital of Nantong University, Nantong 226001, China*
[2]*Department of Emergency, The Yangzhou First People's Hospital, Yangzhou 225001, China*
[3]*Department of Immunology, Medical College, Nantong University, Nantong 226001, China*
[4]*Jiangsu Province Key Laboratory for Inflammation and Molecular Drug Target, Medical College, Nantong University, Nantong 226001, China*

ABSTRACT

Recent studies showed that allogeneic bone marrow (BM)-mesenchymal stem cells transplantation (MSCT) was effective in systemic lupus erythematosus (SLE) patients and lupus-prone mice. However, syngeneic BM-MSCT was ineffective. Previous studies, including ours, revealed that BM-MSCs from SLE patients exhibited early signs of senescence and apoptosis such as slow proliferation, increasing senescence-associated β-galactosidase (SA-β-gal)-positive cells and Annexin V-positive cells, and caspase cascade activation. The abnormalities of BM-MSCs might be associated with the pathogenesis of SLE. In this study, we aimed to determine the molecular mechanism of senescent BM-MSCs from SLE patients. We found that the expression of protein 27 kinase inhibition protein 1(p27^{kip1}) increased significantly, which was regulated by phosphatase and tensin homology deleted on chromosome 10 (PTEN)/protein kinase B (Akt) signaling in SLE BM-MSCs. Knockdown of PTEN or p27^{kip1} could reverse the senescent features of BM-MSCs via down-regulating p27^{kip1} expression. When purified CD4$^+$ T cells were incubated with PTEN or p27^{kip1}-silenced SLE BM-MSCs, the ratio of regulatory T (Treg)/T helper type 17 (Th17) cells increased significantly by enhancing regulatory cytokines (IL-10 and TGF-β) and reducing pro-inflammatory cytokines (IL-17 and IL-6). Taken together, we demonstrated that PTEN/Akt signaling played an essential role in the senescent and apoptotic BM-MSCs from SLE patients by up-regulating p27^{kip1} expression. J. Cell. Biochem. 116: 1583–1594, 2015.

KEY WORDS: BONE MARROW-MESENCHYMAL STEM CELLS; SYSTEMIC LUPUS ERYTHEMATOSUS; SENESCENCE; APOPTOSIS; PTEN/AKT SIGNALING; P27^{kip1}; IMMUNOREGULATION

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease characterized by multi-organ involvement and a remarkable variability in clinical presentations, especially common in young female [Julkunen, 2012]. Bone marrow mesenchymal stem cells (BM-MSCs) are widely regarded as a promising cell source because of their multipotent differentiation, self-renewal immunoregulation and hematopoietic regulation through the direct contact with hematopoietic cells, secretion of extracellular matrix and a variety of cytokines [Charbord, 2010]. Recent studies have showed that allogeneic MSCs transplantation (MSCT) is a feasible and secure therapy strategy in lupus-prone mice and SLE patients [Liang et al., 2010; Zhang et al., 2010]. However, syngeneic BM-MSCT is ineffective [Carrion et al., 2010; Gu et al., 2012]. Our and other studies have also revealed that SLE BM-MSCs grow more slowly with early signs of senescence and apoptosis, and exhibit some abnormalities of the cytoskeleton and ultrastructure [Sun et al.,

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*Correspondence to: Zhifeng Gu, Department of Rheumatology, Affiliated Hospital of Nantong University, Nantong, China. E-mail: guzhifeng@126.com
**Correspondence to: Chun Cheng, Department of Immunology, Medical College, Nantong University, Nantong, China. E-mail: chengc@ntu.edu.cn

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2007; Gu et al., 2012; Li et al., 2012]. Thus, we consider that the abnormalities of BM-MSCs are involved in the pathogenesis of SLE. So it is important to search the molecular mechanisms of senescent and apoptotic BM-MSCs from SLE patients.

The golden marker of cellular senescence is the irreversible loss of proliferative potential [Shay and Roninson, 2004]. Proteins regulating cell cycles were reported to be involved in the senescence process of stem cells [Wilson et al., 2010; Attema et al., 2009], among which protein 27 kinase inhibition protein 1 ($p27^{kip1}$) was relatively less studied. $p27^{kip1}$ is taken as a causative factor for familial melanomas and also as one of the important cyclin-dependent kinases (CDK) inhibitors, inhibiting G1 cyclin/CDK complexes and blocking the G1-S transition in the cell cycle [Kudo et al., 2005]. The $p27^{kip1}$ level can be regulated by various signaling pathways [Short et al., 2008; Burgstaller et al., 2009; Kim et al., 2012]. A role for phosphatase and tensin homology deleted on chromosome ten (PTEN)/protein kinase B (Akt) signaling in regulating $p27^{kip1}$ expression was first suggested by Li and Sun [1998], who observed that PTEN-induced cell-cycle arrest was associated with altered $p27^{kip1}$ expression.

PTEN is a potent tumor suppressor gene and a negative regulator of the phosphoinositide 3-kinase (PI3K)/Akt pathway which antagonizes PI3K by dephosphorylating the phospholipid PtdIns (3, 4, 5) P3 (PIP3) at position D3 of the inositol ring [Carnero et al., 2008; Manna and Jain, 2011]. PIP3 is essential for the phosphorylation and activation of the serine/threonine kinase Akt, a key media tor of the PI3K/Akt-signaling pathway [Blanco-Aparicio et al., 2007]. Although some studies show that PTEN/Akt signaling affects cell proliferation via regulating $p27^{kip1}$ expression in cancer cells [van Duijn and Trapman, 2006; Lee et al., 2009; OuYang et al., 2013], its possible connection with the abnormalities of SLE BM-MSCs has not been explored.

The importance of the complex process of cell senescence and apoptosis prompts us to study the roles of PTEN/Akt signaling in the regulation of $p27^{kip1}$ in BM-MSCs from SLE patients. We observe that BM-MSCs from SLE patients exhibit prominent features of senescence and apoptosis, which reduce the ability of up-regulating the ratio of regulatory T (Treg)/T helper type 17 (Th17) cells by enhancing interleukin (IL)-10 and transforming growth factor (TGF)-β and reducing IL-17 and IL-6. Meanwhile, the expression of PTEN and $p27^{kip1}$ increased significantly with the decrease of phosphorylated Akt in SLE BM-MSCs. Furthermore, knockdown of PTEN or $p27^{kip1}$ can reverse the abnormal situation incompletely and improve the immunoregulation ability of BM-MSCs from SLE patients by inhibiting $p27^{kip1}$ expression.

MATERIALS AND METHODS

PATIENTS

Twelve SLE patients aged 15–41 (mean 27.08 ± 8.02 years) were enrolled in the study (Table I). The SLE diagnosis was made based on the criteria proposed by American College of Rheumatology. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was used to measure disease activity. All patients were categorized as active using a cutoff SLEDAI score of 8. Twelve healthy subjects were used as control group. All subjects were female, whose age distribution was similar (mean 26.82 ± 7.41 years). All subjects gave consent to the study, which was approved by the Ethics Committee of the Affiliated Hospital of Nantong University.

TABLE I. Details of 12 SLE Patients

Patient	Age	Disease duration	Current treated	SLEDAI
1	15	1 year	Pred 15 mg/day HCQ 0.2/day	11
2	20	8 months	Pred 20 mg/day HCQ 0.2/day LEF 0.2/day	14
3	21	2 years	Pred 15 mg/day HCQ 0.2/day LEF 0.2/day	9
4	26	1 year	Pred 10 mg/day HCQ 0.2/day	13
5	28	16 months	Pred 12.5 mg/day HCQ 0.2/day CTX 0.4/2 weeks	16
6	32	2 years	Pred 12.5 mg/day HCQ 0.2/day	12
7	37	3 years	Pred 12.5 mg/day HCQ 0.2/day	11
8	41	5 years	Pred 7.5 mg/day HCQ 0.2/day	8
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, prednisolone; HCQ, hydroxychloroquine; LEF, leflunomide; CTX, cyclophosphamide.

ISOLATION, CELL CUITURE AND IDENTIFICATION OF BM-MSCS FROM SLE AND CONTROL SUBJECTS

MSCs were isolated and expanded from iliac crest BM of all the SLE patients and control 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*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, and the medium was replaced and non-adherent cells were removed after 5 days and every three days thereafter. When the BM-MSCs became nearly confluent, the adherent cells were released from the dishes with 0.25% trypsin-EDTA (Gibco, NY), and then replanted at a density of 1×10^6 cells per 25 cm^2 dish. Similar to our previous research, the cells were positive for CD29, CD44, CD105, and CD166, but negative for CD14, CD34, CD38, CD45, and HLA-DR [Sun et al., 2007]. After passages 4 (P4), cells were used for the following studies.

SENESCENCE-ASSOCIATED β-GALACTOSIDASE (SA-β-GAL) ASSAY

The SA-β-gal staining is widely used to assess cell senescence, with the positive green or blue-colored staining of β-gal at pH 6.0 being remarkably increased in senescent cells. The SA-β-gal activity was determined using the in situ β-galactosidase staining kit from the Beyotime Institute of Biotechnology following the manufacturer's

instructions. BM-MSCs were passaged into the 6-well culture plates at a density of 5×10^4 cells per well for 72 h. Then cells were washed twice with PBS and fixed with the 4% paraformaldehyde for 15 min. After incubation with staining SA-β-gal detection solution at 37°C without CO_2 overnight, the slips were washed and analyzed under the microscope. We counted at a minimum of 500 cells to determine the percentage of SA-β-gal-positive cells.

CELL PROLIFERATION ASSAY

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8) assay following the manufacturer's instructions. Briefly, cells were plated at a density of 0.3×10^4 cells/well in 96-well plates (Corning, Inc., Corning, NY) in a volume of 100 μl and incubated overnight to allow cell 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, then the absorbance at 490 nm wavelength was quantified on an automated plate reader.

DETERMINATION OF CELL NUMBER

BM-MSCs were seeded at 2×10^4 cells/well into 6-well plates in triplicate for each experimental condition. MSCs were collected from 1 to 11 days after plating and dissociating. The total cell numbers were counted by MTT assay at 405 nm.

THE DETECTION OF APOPTOTIC CELLS

For terminal deoxynucle-otidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay, 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 Hoechst (1:800, Santa Cruz) in the solution for 2 h at room temperature. The Apoptosis Detection System kit (Roche Molecular Biochemicals, Germany) was used for TUNEL assay according to the manufacturer's instructions. For Annexin V analysis, the Annexin V-FITC/PI Apoptosis Detection Kit (Becton Dickinson, Franklin Lakes, NJ) was employed to evaluate the apoptosis of P4 BM-MSCs from SLE and control groups by using FCM analysis. After being washed with iced cold PBS, 1×10^5 cells were resuspended in 100 μl of binding buffer. Five microliter Annexin V and 5 μl PI were added to the cells. After incubation for 15 min (25°C) in the dark, 400 μl of 1X binding buffer was added to each tube and FCM analysis was performed immediately. Data acquisition and analysis were assayed by BD LSRII analyzer (Becton Dickinson, Franklin Lakes, NJ).

$CD4^+$ T CELL ISOLATION, TRANSWELL CULTURE WITH BM-MSCS

The single-cell suspensions of spleens collected from the BALB/c mice were prepared by mechanical disruption in PBS. $CD4^+$ T-cells were isolated by magnetic sorting with dynabead-bound mouse $CD4^+$ cells according to the manufacturer's directions (Dynal Biotech, NY). Positively selected cells were contained an average of 99% $CD4^+$ T-cells as assessed by flow cytometric analysis with CD4 monoclonal antibody. The cell cultures were performed in RPMI-1640 medium supplemented with 10% FCS, 1X nonessential amino acids and 1 mM sodium pyruvate. The purified $CD4^+$ T-cells (1×10^5) were obtained and cultured in the lower chamber of the 24-well diameter transwell plate with a 0.3-μm pore size membrane (Corning, NY). MSCs (1×10^6) were seeded onto the transwell membrane of the inner chamber for 72 h of transwell culture.

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, St. Louis, MO) for 30 min, cells were stained with 50 μg/ml PI solution (Sigma, USA) for 30 min and analyzed by the flow cytometry machine (FACS Calibur, BD Biosciences, Salt Lake). The fraction of cells in the G0/G1, S, and G2/M phases were quantified with the ModFit LT system. There separate experiments were performed.

For Treg and Th17 cells analysis, the ratio of Treg/Th17 among the $CD4^+$ T-cells was analyzed using Treg and Th17 assay kits. After 72 h of transwell culture, $CD4^+$ cells were harvested and washed with PBS, resuspended in 100 μl staining buffer and divided into two aliquots (one for detection and the other for hemotype control). The $CD4^+$ T-cells were stained respectively with anti-CD25-APC and anti-Foxp3-PE or anti-IL-17-PE mAbs for test of Treg and Th17 cells. IgG2a-PE rat isotype was used as a control, and analyzed with a FACS Calibur flow cytometer according to the manufacturer's protocol.

CYTOKINE DETERMINATION BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The concentrations of TGF-β, IL-10, IL-17 and IL-6 cytokine released in the transwell culture supernatants were measured by ELISA using commercially available kits (R&D Systems, Abingdon, Oxon, UK), according to the manufacturer's instructions. Briefly, cells in 100 μl of medium were seeded onto 96-well plates. After 24 h, 100 μl of supernatants were harvested for ELISA assay.

WESTERN BLOTTING

Cells were washed twice with cold-buffered PBS and then lysed in RIPA buffer consisting of 50 mM TRIS, 150 mM NaCl, 2% sodium dodecyl sulfate (SDS) and a protease inhibitor mixture (Roche Molecular Biochemicals, Germany). Equal amounts of protein were separated by 10% SDS polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to polyvinylidene difluoride (PDVF) membranes. Membranes were blocked with 5% nonfat milk and incubated with primary antibodies (1:500) at 4°C overnight and horseradish peroxidase conjugated with mouse anti-rabbit secondary antibody for 2 h at room temperature. The blots were developed using an enhanced chemiluminescence kit. The following primary antibodies were used: PTEN (anti-rabbit, Sigma), p-Akt (anti-rabbit, Sigma), Akt (anti-rabbit, Santa Cruz), $p27^{kip1}$ (anti-rabbit, Santa Cruz), and GAPDH (anti-rabbit, Santa Cruz), which was run as a reference protein.

IMMUNOFLUORESCENCE

BM-MSCs were fixed with 4% PFA for 1 hour, 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 PTEN (anti-rabbit, Sigma, 1:100), p-Akt (anti-rabbit, Sigma, 1:100), $p27^{kip1}$ (anti-rabbit, Santa Cruz, 1:100), p16 (anti-rabbit, Santa Cruz, 1:100), p53 (anti-rabbit, Sigma, 1:100), p21 (anti-rabbit, Sigma, 1:100) in the same solution overnight at 4°C, washed and incubated in secondary

antibodies for 2 h at room temperature. Nuclei were stained with Hoechst (1:800, Santa Cruz). The cells were examined with a Leica fluorescence microscope (Germany).

siRNA AND TRANSFECTION

The siRNA oligonucleotides were synthesized by Genepharma Co., Ltd. (Shanghai, China). The effective sequence used for the specific silencing of PTEN (siPTEN) and $p27^{kip1}$ ($sip27^{kip1}$) were 5′-AGGCACAAGAGGCCCUAGA-3′ and 5′-GCACGCAGAGUACGA-CUAC-3′. The non-silencing siRNA (NS-siRNA) was an irrelevant siRNA with random nucleotides 5′-UGCUCGACUAGUCACGUTT-3′ and 5′-ACGUTGUCTUGCACUAGUC-3′, and were not homologous to any sequences found in the gene bank. Transfection was carried out by the attractene transfection reagent according to the manufacturer′s protocol (Qiagen, Inc., Valencia, CA). The reaction procedure was as follows: 94°C for 3 min, 94°C for 30 s, 59°C for 30 s, 72°C for 1 min and 72°C for 5 min, for 30 cycles. Following electrophoresis, the images were scanned and analyzed by Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) to determine the integrated optical density (OD) and analyzed using gray values to calculate interference efficiency following 1% agarose gel electrophoresis.

STATISTICAL ANALYSIS

All data were shown as the mean ± standard deviation (SD) from at least three independent experiments. All statistical analyses were performed using SPSS 11.0 software, and were analyzed by Student′s *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

THE BM-MSCS FROM SLE PATIENTS SHOWED SENESCENT AND APOPTOTIC FEATURES

The primary culture of BM-MSCs was successful in 12 cases of SLE patients (Table I) and 12 cases of healthy controls. Our previous study indicated that the BM-MSCs from SLE patients were senescent [Gu et al., 2012], so we didn′t group again in this study. SA-β-gal was regarded as a universal marker of senescence. We found that the BM-MSCs from SLE patients were larger than the normal BM-MSCs, and more and longer podia were found. The number of SA-β-gal-positive cells significantly increased in BM-MSCs from SLE patients in comparison with the BM-MSCs from control group which people unaffected by SLE (Fig. 1A). The proliferation of BM-MSCs was measured with CCK8 assay. The result indicated that BM-MSCs from SLE patients grew more slowly than those from the control group (Fig. 1B). Quantitative analysis showed that the number of SLE BM-MSCs decreased compared to control group from the third day and reached the lowest level at days 9. Then the cell growth was in plateau phase (Fig. 1C). Flow cytometry showed that there were more BM-MSCs restricted in the G1 phase harvested from the treated and untreated SLE patients (73.58 ± 5.1%) than in the BM-MSCs from control group (56.22 ± 4.5%) (Fig. 1D). Moreover, we sought to assess apoptotic cells using TUNEL staining and FCM analysis by Annexin V-FITC/PI staining at the fourth passage. We observed significantly higher TUNEL staining in the BM-MSCs from SLE patients compared with the control group (Fig. 1E). Meanwhile, in Fig. 1F Annexin V-positive cells in the BM-MSCs of SLE patients (22.1 ± 13.4%) showed a significant increase compared to the control group (7.2 ± 4.1%). In summary, we confirmed that the BM-MSCs from SLE patients were senescent and apoptotic cells.

THE IMMUNOREGULATION OF BM-MSCS FROM SLE PATIENTS WAS ABNORMAL

The production of Treg and Th17 cells would be affected by the state of BM-MSCs [Park et al., 2011; Carrión et al., 2011]. After transwell culture of BM-MSCs with $CD4^+$ T cells for 72 h, the number of $CD25^+Foxp3^+$ Treg cells harvested from the $CD4^+$ T-cells decreased in SLE BM-MSCs (2.47 ± 1.1%) compared to the control group (7.84 ± 3.3%). However, the number of $CD4^+IL\text{-}17^+$ Th17 cells increased in SLE group (1.96 ± 0.8%) compared to control group (0.88 ± 0.4%). Therefore, the ratio of Treg/Th17 was significantly down-regulated in SLE BM-MSCs compared with those co-cultured with normal BM-MSCs (Fig. 2A). Moreover, the secretion of regulatory cytokines TGF-β and IL-10 decreased, and pro-inflammatory cytokines IL-17 and IL-6 increased in BM-MSCs from SLE patients, while opposite results were found in normal BM-MSCs (Fig. 2B). From the above data, we found that the abnormities of SLE BM-MSCs could lead to the decline of Treg/Th17 by regulating the secretion of cytokines.

$p27^{kip1}$ EXPRESSION WAS UP-REGULATED RELATING TO PTEN/AKT SIGNALING IN BM-MSCS FROM SLE PATIENTS

There were studies showing that PTEN-induced cell-cycle arrest was associated with altered $p27^{kip1}$ expression [Lee et al., 2009; OuYang et al., Park et al., 2011; Zhang et al., 2008]. In the BM-MSCs from SLE patients, we found that the expression of PTEN was up-regulated, and the phosphorylation of Akt was reduced (Fig. 3A). Meanwhile, the higher expression of PTEN and the lower expression of p-Akt in SLE BM-MSCs were confirmed by immunofluorescence (Fig. 3B). The expression of cell-cycle regulator $p27^{kip1}$ was determined. The results showed that $p27^{kip1}$ increased markedly in BM-MSCs from SLE patients (Fig. 3C) and nuclear fluorescence intensity was enhanced (Fig. 3D). These results suggested that by up-regulating $p27^{kip1}$ expression, the PTEN/Akt signaling might be associated with the senescence of SLE BM-MSCs.

SI-PTEN REVERSED THE SENESCENT AND APOPTOTIC FEATURES, IMPROVED IMMUNOREGULATION OF BM-MSCS FROM SLE PATIENTS

To further assess whether PTEN-$p27^{kip1}$ signaling mediated BM-MSCs senescence and apoptosis, the BM-MSCs were transfected with PTEN siRNA. RT-PCR analysis showed cells transfected with the siRNA decreased the PTEN expression over 80% (Fig. 4A). There were less SA-β-gal-positive cells when PTEN expression was knocked down in BM-MSCs from SLE patients, but it has less effect in the control group′s BM-MSCs (Fig. 4B). And the proliferation ratio of si-PTEN BM-MSCs from SLE patients was partially restored compared with that of the PTEN un-knockdown BM-MSCs, but in the control group′ s BM-MSCs, although PTEN was knockdown, but it has less effect in cell proliferation (Fig. 4C). The further quantitative analysis

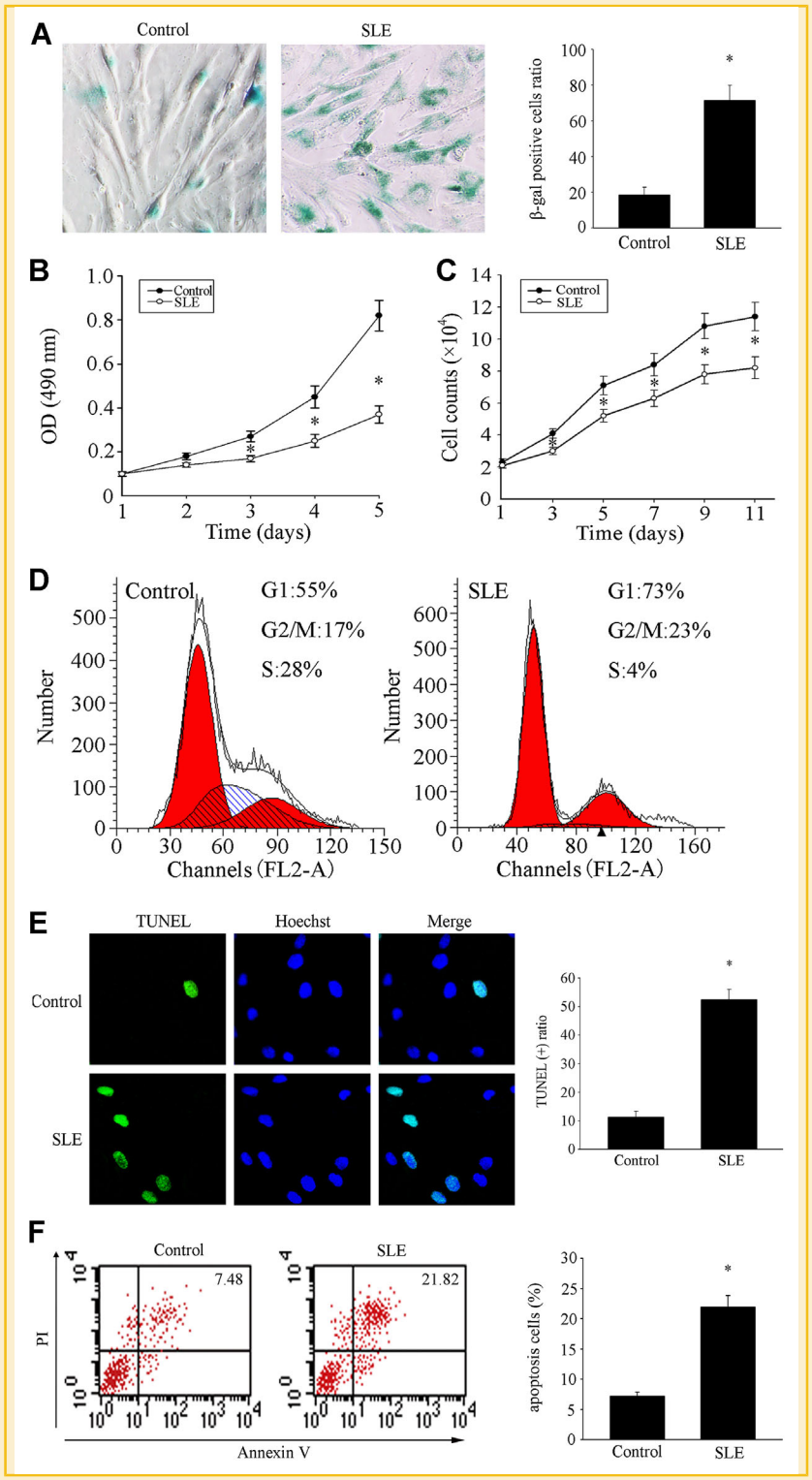

Fig. 1. BM-MSCs from SLE patients were senescent and apoptotic cells. (A) SA-β-gal was used to examine BM-MSCs senescence. The number of SA-β-gal-positive cells obviously increased in SLE BM-MSCs compared to the control group. (B) The P4 BM-MSCs were plated on 96-well plates. After 1–5 days, the cell proliferation ratio was detected using CCK8 assay. The absorbance was shown as the proliferation rate. BM-MSCs from SLE patients grew more slowly than those from the control group. (C) The cell number was determined by counting after 1–11 days. The number of SLE BM-MSCs decreased compared to control group from the third day. (D) The flow cytometry results showed that the ratio of G0 phase increased more in SLE BM-MSCs than control group. (E) TUNEL staining was detected in BM-MSCs from SLE and control groups. The merged images, resulting from the overlap of TUNEL-positive (green) and Hoechst labeled (blue) areas, were observed in MSCs (scale bar = 50 μm). We observed significantly higher TUNEL staining in the BM-MSCs from SLE patients than control group. (F) P4 BM-MSCs from SLE and control groups were analyzed by Annexin V-FITC/PI staining. The results showed a significant increase in Annexin V-positive cells among SLE BM-MSCs. All data were expressed as the mean ± SD. $^{*}P < 0.05$ compared with control group.

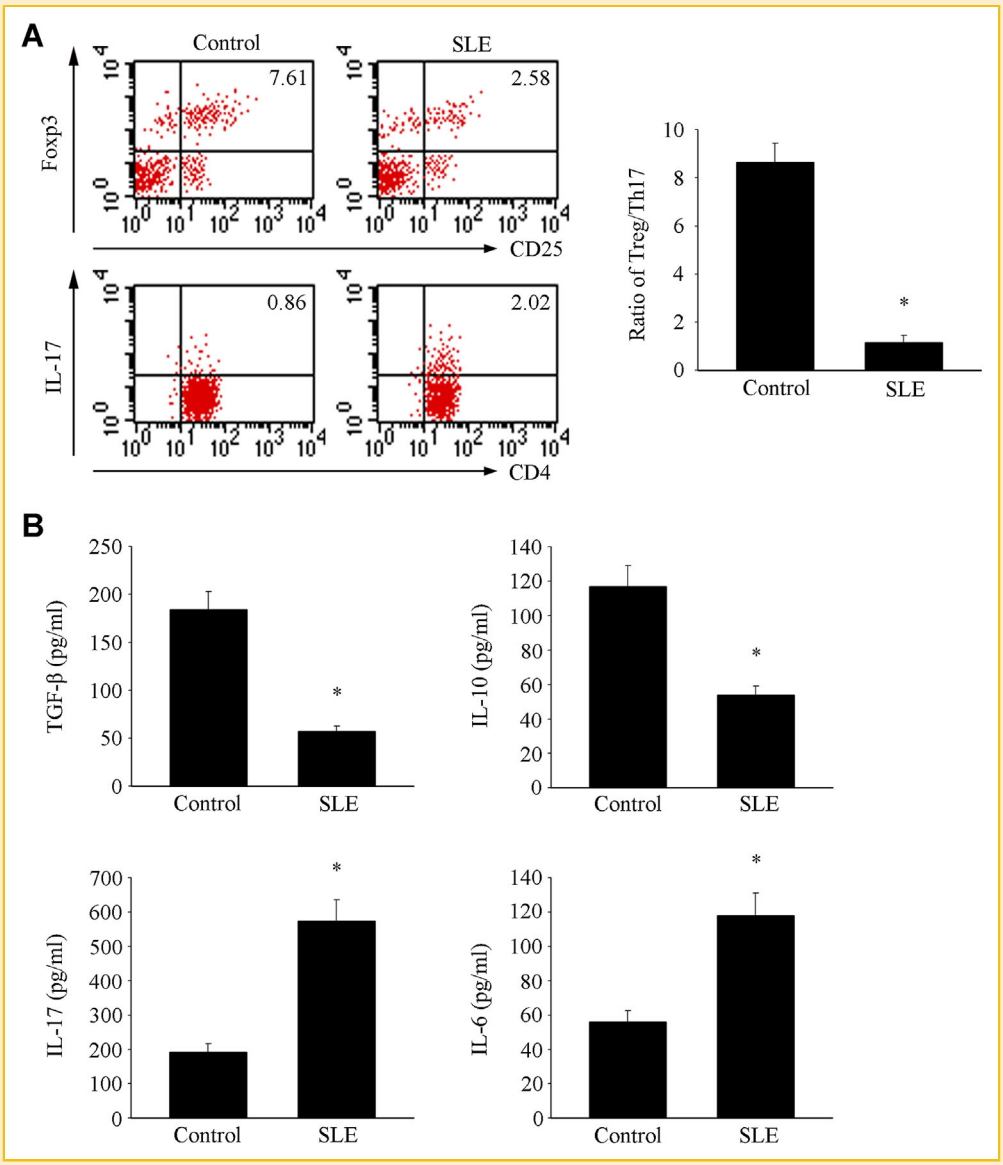

Fig. 2. The immunoregulation of BM-MSCs from SLE patients was abnormal. (A) P4 SLE BM-MSCs transwell cultured with $CD4^+$T-cells for 72 h. The count of $CD4^+CD25^+Foxp3^+$ Treg cells decreased and $CD4^+IL^-17^+$ Th17 cells increased in SLE BM-MSCs compared to the control group by flow cytometry analysis. (B) The supernatants of BM-MSCs were collected. The secretion of IL-10 and TGF-β were reduced but IL-17 and IL-6 increased by ELISA. All data were expressed as the mean ± SD. $^*P < 0.05$ compared with control group.

indicated the number of SLE patients' BM-MSCs increased in si-PTEN-transfected group compared to the untreated group from the third day (Fig. 4D). Cell-cycle analysis revealed that G1 phase arrest was observably reversed in si-PTEN-transfected SLE BM-MSCs (Fig. 4E). In the aspect of cell apoptosis, Figure 4F showed that the TUNEL-positive cells were reduced in SLE BM-MSCs after the treatment of si-PTEN, but it has no differences in the control group's BM-MSCs although PTEN was knockdown. In addition, the Annexin V-positive cells decreased in si-PTEN treated SLE BM-MSCs compared with untreated SLE group (Fig. 4F). After co-culturing BM-MSCs with $CD4^+$ T cells for 72 hours, the ratio of Treg/Th17 increased in si-PTEN-transfected SLE BM-MSCs (Fig. 4G). Additionally, increased secretion of TGF-β and IL-10 and reduced secretion of IL-17 and IL-6 were detected after PTEN knockdown in SLE BM-MSCs (Fig. 4H). These results implied that the PTEN signaling pathway played an essential role in the senescence and apoptosis of SLE BM-MSCs.

$p27^{kip1}$ IS THE DOWN EFFECTOR IN PTEN REGULATED CELL SENESCENCE, APOPTOTIC, AND IMMUNOREGULATION IN BM-MSCS FROM SLE PATIENTS

To further assess whether PTEN/Akt signaling mediated BM-MSCs senescence and apoptosis by regulating $p27^{kip1}$ expression, the expression of $p27^{kip1}$ was detected when PTEN was knockdown. The expression of $p27^{kip1}$ decreased significantly in SLE BM-MSCs transfected with si-PTEN (Fig. 5A), but the expression levels of p16,

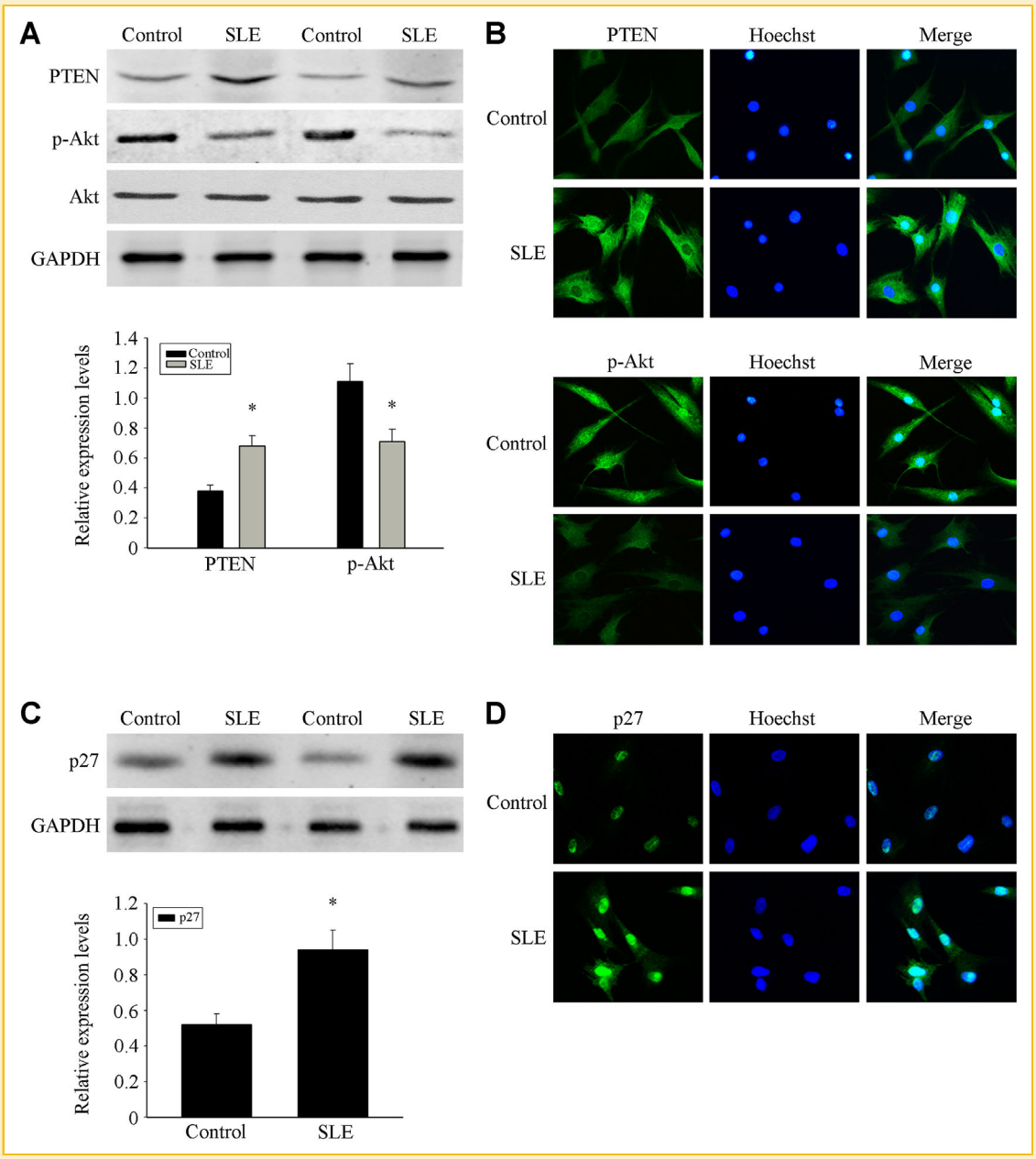

Fig. 3. The expressions of PTEN/Akt signaling and $p27^{kip1}$ in BM-MSCs from SLE patients. (A) The expression of PTEN up-regulated and reduced the phosphorylation level of Akt in BM-MSCs from SLE compared with control group determined by western blot analysis. GAPDH was used as the internal control. (B) P4 BM-MSCs from SLE patients and control group were cultured in 24-well plates. Immunofluorescence staining of PTEN and p-Akt verified their changes in SLE BM-MSCs. Counterstaining with Hoechst displayed the localization of the nucleus (scale bar = 50 μm). (C) The expression of $p27^{kip1}$ was up-regulated in BM-MSCs from SLE compared with control group determined by western blot analysis. GAPDH was used as the internal control. (D) Immunofluorescence staining of $p27^{kip1}$ confirmed its over-activation in 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 control group.

p53, and p21 has not been inhibited by si-PTEN treatment (data not shown). And then, we have also detected the BM-MSCs characterize when $p27^{kip1}$ was knockdown (Fig. 5B). The SA-β-gal-positive cell number was increased when PTEN expression was knocked down in BM-MSCs from SLE patients, but it has less effect in the control group's BM-MSCs (Fig. 5C). And the proliferation ratio of BM-MSCs from SLE patients was restored partially when $p27^{kip1}$ was knockdown, but in the control group's BM-MSCs, although $p27^{kip1}$ was knockdown, but it has less effect (Fig. 5D). In further, the quantitative analysis indicated the number of SLE patients' BM-MSCs increased in si-$p27^{kip1}$ transfected group compared to untransfected group from the third day (Fig. 5E). Cell-cycle analysis also revealed

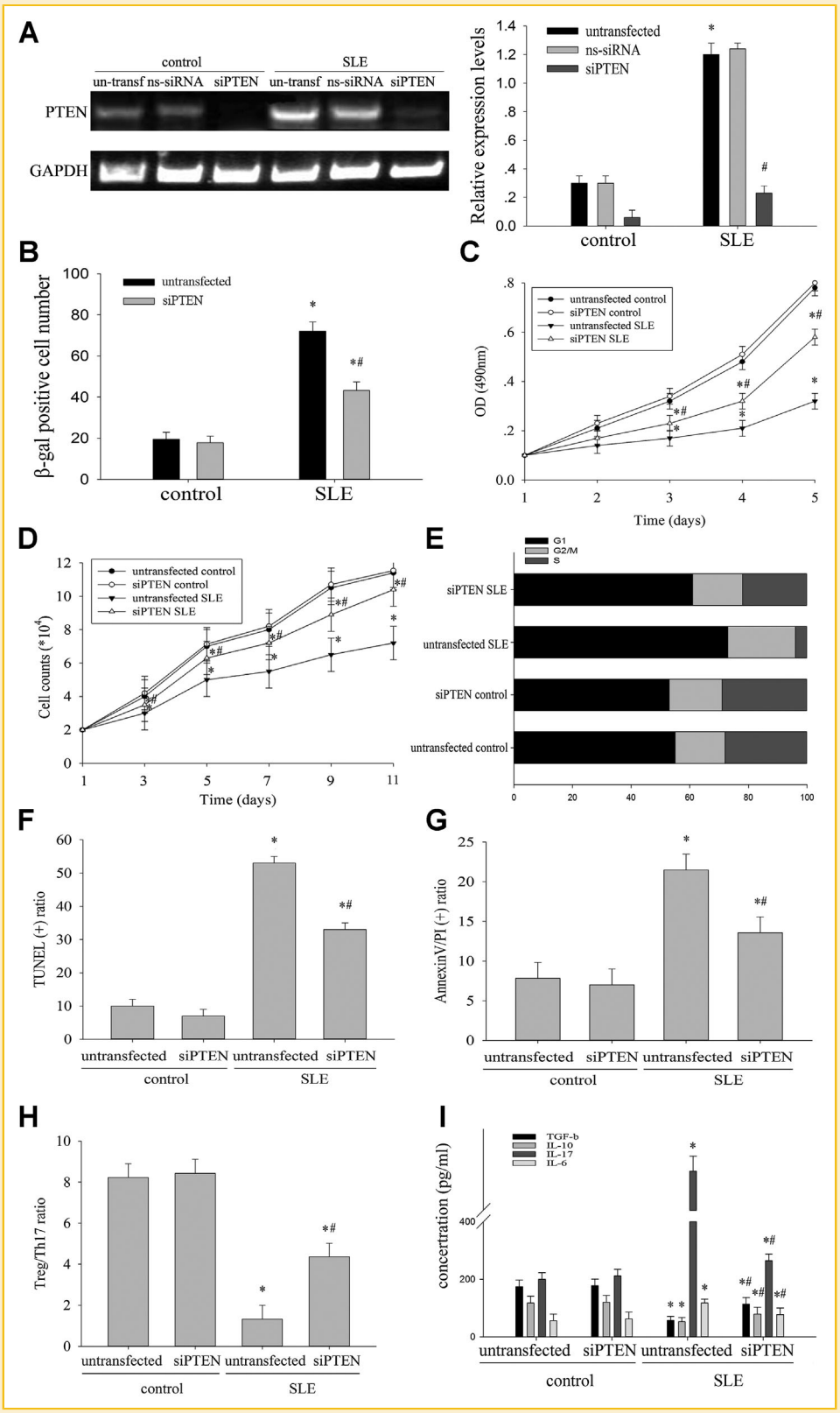

Fig. 4. (*Continued*)

that G1 phase arrest was reversed observably in si-p27^{kip1} transfected SLE BM-MSCs (Fig. 5F). In the aspect of cell apoptosis, Figure 5F showed that the TUNEL -positive cells were reduced in SLE BM-MSCs under the treatment of si-p27^{kip1}, but it has no differences in the normal person′s BM-MSCs although PTEN was knockdown. In addition, the Annexin V-positive cells were also decreasing among si-p27^{kip1} BM-MSCs from SLE patients in comparison with untreated SLE group (Fig. 5G). After co-culturing BM-MSCs with CD4^{+} T cells for 72 hours, the ratio of Treg/Th17 increased in si-p27^{kip1}-transfected SLE BM-MSCs (Fig. 5H). Additionally, we detected increased TGF-β and IL-10 secretion and reduced IL-17 and IL-6 secretion upon p27^{kip1} knockdown in BM-MSCs from SLE patients (Fig. 5I). These results implied that the PTEN-p27^{kip1} signaling pathway played an essential role in the senescence and apoptosis of SLE BM-MSCs.

DISCUSSION

Cell senescence is widely recognized as an irreversible loss of proliferative potential [Shay and Roninson, 2004; Demidenko et al., 2009]. These cells feature irreversible G1 growth arrest involving the up-regulation of cell cycle inhibitors [Wang et al., 2006; Muteliefu et al., 2012]. Moreover, senescent cells display a characteristically enlarged and flattened morphology and an increased SA-β-gal activity [Liu et al., 2011]. Many studies have described the biological changes in senescent BM-MSCs. For example, the growth rate of senescent BM-MSCs decreased and more SA-β-gal-positive cells were found, as well as the morphology and secretion of cytokines were abnormal [Park et al., 2005; Heo et al., 2009]. In our study, we found that the BM-MSCs from both treated and untreated SLE patients displayed prominent senescent characteristics, such as increased SA-β-gal-positive cells, disordered cytoskeleton and slow growth rate compared with BM-MSCs from healthy controls [Gu et al., 2012]. In addition, the SLE BM-MSCs also showed apoptotic features, such as the increased Annexin V-positive cells and caspase cascade activation [Li et al., 2012]. Just as we known, the caspase-cascade activation is the main mechanism in cell apoptosis, and we have also detected in the previous study. In our study, choosing the Annexin V staining in the detection of cell apoptosis was only aimed to shown the early stage of cell apoptosis, which indicated the apoptosis more clearly. These Annexin V assay and TUNEL assay data revealed that BM-MSCs from SLE patients displayed prominent features of senescence and apoptosis. Thus, the abnormal phenomenon of BM-MSCs might be a contributing factor to SLE pathogenesis.

The defects in Treg/Th17 development, maintenance or function have been found to be associated with several human autoimmune diseases [Afzali et al., 2007; Zhang et al., 2011; Kong et al., 2012]. It has been reported that SLE flare which was considered when SLEDAI increased >3 might be linked to the expansion of the Th17 cells population and the depletion of natural Treg cells subpopulations [Yang et al., 2009; Cai et al., 2012]. Treg cells played an essential role in maintaining immune homeostasis and preventing autoimmunity, in contrast to the role of Th17. Our previous study confirmed that BM-MSCs from SLE patients had defective immunoregulatory function compared to healthy controls [Gu et al., 2012]. In this study, we found that after transwell culture of BM-MSCs with CD4^{+} T-cells for 72 h, the ratio of Treg/Th17 in SLE BM-MSCs was reduced compared to those from control group. TGF-β and IL-10 are critical differentiation factor for the generation of Treg cells [O′Hehir et al., 2009], and IL-6 and IL-17 have been taken as the main factors reciprocally regulating the development of pro-inflammatory Th17 cells [Guo et al., 2011]. Our results showed that BM-MSCs from SLE patients down-regulated the secretion of IL-10 and TGF-β, and induced pro-inflammatory cytokines IL-17 and IL-6 after transwell culture with CD4^{+} T cells. Altogether, these date confirmed that there were abnormalities in the immunoregulation of senescent SLE BM-MSCs.

p27^{kip1} is an important negative regulator of cell cycle progression. Its cellular level and localization can be regulated by many different mechanisms [Alkarain and Slingerland, 2004]. One of the signaling pathways that can affect p27^{kip1} is PI3K/Akt signaling [van Duijn and Trapman, 2006; Park et al., 2008]. The cell cycle suppressor PTEN, which is frequently activated in many senescent cells, is a key factor in this signaling route by counteracting the function of PI3K via dephosphorylation of PIP3 [Carnero et al., 2008; Zhang and Yu, 2010; Manna and Jain, 2011]. In this study, we compared the involvement of the PTEN/Akt pathway in the regulation of p27^{kip1} in BM-MSCs from SLE patients with healthy controls. Our results showed that the expression of PTEN was obviously activated in SLE BM-MSCs, which resulted in the reduced phosphorylation level of Akt. Meanwhile, the p27^{kip1} expression was also up-regulated. To further analyze the relationship between PTEN/Akt signaling and p27^{kip1} in SLE BM-MSCs, we detected the influences of si-PTEN on p27^{kip1} expression and senescent SLE BM-MSCs. We found that the expression of p27^{kip1} decreased significantly in si-PTEN-transfected SLE BM-MSCs in comparison with the untreated group, but si-PTEN had no significant

Fig. 4. Si-PTEN reversed the senescence and apoptosis of BM-MSCs from SLE patients. Cells were transfected with the three groups of PTEN siRNA for 48 h. (A) RT-PCR analyses showed that PTEN expression significantly decreased in BM-MSCs from both SLE and normal persons. (B) BM-MSCs were fixed and stained for β-gal. The number of SA-β-gal-positive cells decreased in si-PTEN-transfected SLE BM-MSCs in comparison with untreated group, but it has less effect in control group. (C) The P4 BM-MSCs were plated on 96-well plates. After 1–5 days, CCK8 assay showed that the cell proliferation ratio increased in BM-MSCs when transfected with si-PTEN. (D) The cell number was determined by counting after 1–11 days. The number of BM-MSCs increased in si-PTEN-transfected group compared to untransfected group from the third day. (E) The flow cytometry results showed that the ratio of G0 phase decreased in si-PTEN-transfected group. (F) TUNEL staining was detected in BM-MSCs from si-PTEN-transfected group and untransfected group. (G) P4 BM-MSCs from treated and untreated SLE groups were analyzed by Annexin V-FITC/PI staining. The results showed the Annexin V-positive cells decreased among si-PTEN-transfected group. (H) P4 BM-MSCs transwell were cultured with CD4^{+} T-cells for 72 h. The count of CD4^{+}CD25^{+}Foxp3^{+} Treg cells increased and CD4^{+}IL^{-}17^{+} Th17 cells decreased in si-PTEN-transfected SLE BM-MSCs compared to untreated group by flow cytometry analysis. (I) The supernatants of BM-MSCs were collected. The si-PTEN-transfected group induced the secretion of IL-10 and TGF-β but reduced IL-17 and IL-6 by ELISA. All data were expressed as the mean ± SD. $^{*}P < 0.05$ compared with the untransfected control group, $^{*}P < 0.05$ compared with the untransfected SLE group.

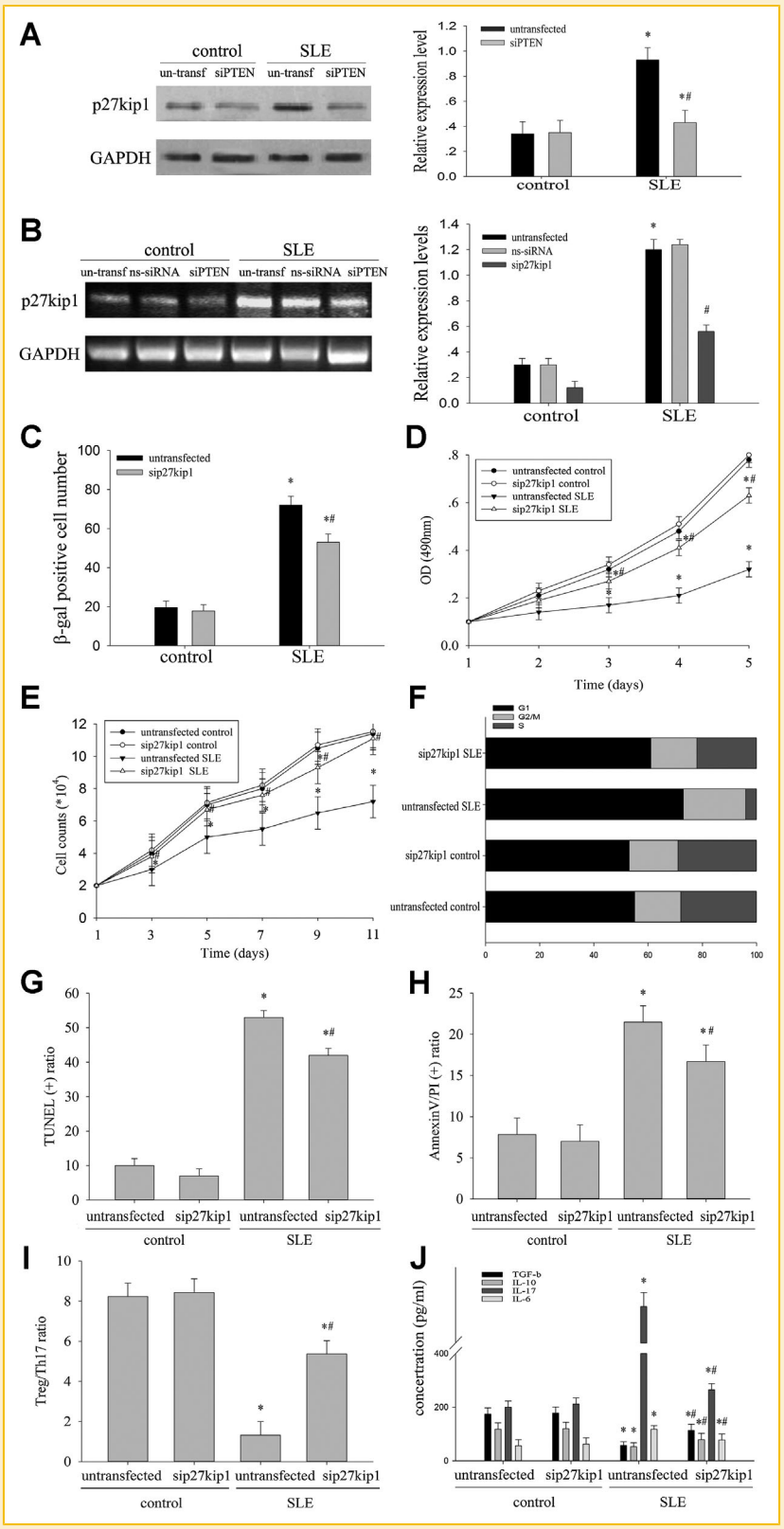

Fig. 5. (*Continued*)

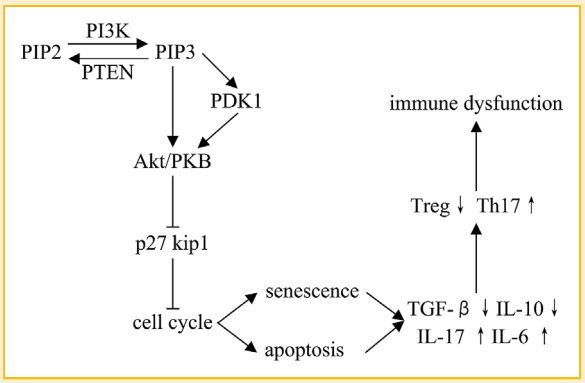

Fig. 6. PTEN-p27^{kip1} signaling mediated the cell senescence and apoptosis of SLE BM-MSCs. PTEN signaling was avtivation in the SLE BM-MSCs, which can up-regulated p27^{kip1} expression. And the increased p27^{kip1} expression caused cell cycle arrest, and then induced cell senescence and apoptosis. After that the SLE BM-MSCs induced the abnormal immunomodulatory phenomena by cytokines secretion.

influence on the expressions of p16, p53 and p21. Besides, knockdown of PTEN could also partially reverse the senescent and apoptotic features of SLE BM-MSCs and prompt immunoregulation of BM-MSCs from SLE patients by enhancing the ratio of Treg/Th17 cells and affecting related cytokines secretion. Our previous study showed that the upregulation of p16^{INK4A} promoted cellular senescence of BM-MSCs from SLE patients [Gu et al., 2012]. And in the study, we have found knockdown PTEN expression only affected p27^{kip1} expression, but it has no effected in p16^{INK4A}, p53 and p21 expression. These data indicated in the SLE patients' BM-MSCs senescence and apoptosis, many mechanism may involve in the process, and the changes of cell characterize may controlled by several ways.

In summary, we characterized the prominent senescent and apoptotic features and abnormal immunoregulation in BM-MSCs from SLE patients. Our results indicated that PTEN/Akt signaling played an essential role in the senescence and apoptosis of SLE BM-MSCs by up-regulating p27^{kip1} expression. The senescent and apoptotic SLE BM-MSCs were closely related to their abnormal immunomodulatory phenomena (Fig. 6). This study will further our understanding in cellular and molecular mechanisms underlying the senescent BM-MSCs from SLE patients.

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Fig. 5. Si-p27^{kip1} reversed the senescence and apoptosis of BM-MSCs from SLE patients. (A) Cells were transfected with the three groups of PTEN siRNA for 48 h, Western blot analysis showed that the expression of p27^{kip1} obviously decreased in si-PTEN-transfected SLE BM-MSCs compared with untransfected group. GAPDH was used as the internal control. Cells were transfected with the three groups of p27^{Kip1} siRNA for 48 h. (B) RT-PCR analyses showed that p27^{kip1} expression significantly decreased in BM-MSCs from both SLE and normal persons. (C) BM-MSCs were fixed and stained for β-gal. The number of SA-β-gal-positive cells decreased in si-p27^{kip1}-transfected SLE BM-MSCs in comparison with untreated group, but it has less effect in control group. (D) The P4 BM-MSCs were plated on 96-well plates. After 1–5 days, CCK8 assay showed that the cell proliferation ratio increased in BM-MSCs when transfected with si-p27^{kip1}. (E) The cell number was determined by counting after 1–11 days. The number of BM-MSCs increased in si- p27^{kip1}-transfected group compared to untransfected group from the third day. (F) The flow cytometry results showed that the ratio of G0 phase decreased in si- p27^{kip1}-transfected group. (G) TUNEL staining was detected in BM-MSCs from si- p27^{kip1}-transfected group and untransfected group. (H) P4 BM-MSCs from treated and untreated SLE groups were analyzed by Annexin V-FITC/PI staining. The results showed the Annexin V-positive cells decreased among si-p27^{kip1}-transfected group. (I) P4 BM-MSCs transwell were cultured with CD4$^+$ T-cells for 72 h. The count of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells increased and CD4$^+$IL$^-$17$^+$ Th17 cells decreased in si-PTEN-transfected SLE BM-MSCs compared to untreated group by flow cytometry analysis. (J) The supernatants of BM-MSCs were collected. The si-p27^{kip1}-transfected group induced the secretion of IL-10 and TGF-β but reduced IL-17 and IL-6 by ELISA. All data were expressed as the mean ± SD. $^*P < 0.05$ compared with the untransfected control group, $^*P < 0.05$ compared with the untransfected SLE group.

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