c-Casitas b-Lineage Lymphoma Downregulation Improves the Ability of Long-term Cultured Mesenchymal Stem Cells for Promoting Angiogenesis and Diabetic Wound Healing

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Abstract

The chronic wound induced by diabetes has poor efficacy and could lead to amputation. The repair function of mesenchymal stem cells (MSCs) impaired after long-term culture *in vitro*. Studies have shown that the proto-oncogene c-Casitas b-lineage lymphoma (c-Cbl) can regulate receptor- and non-receptor tyrosine kinase, which was also involved in the angiogenesis process. This study aimed to explore the regulative effect of c-Cbl on the proangiogenic functions of long-term cultured MSCs and evaluate its pro-healing effect on diabetic wounds. In this study, the c-Cbl level was downregulated by locked nucleic acid–modified antisense oligonucleotide gapmers (LNA Gapmers). We detected the effect of c-Cbl downregulation on long-term cultured MSCs in terms of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signal, cellular proliferation, senescence, migration, and angiogenic factors paracrine activity *in vitro*. *In vivo*, we observed the pro-healing effect of long-term cultured MSCs, with or without c-Cbl downregulation, on the diabetic wound. We found that the phosphorylation level of c-Cbl increased and that of Akt decreased in passage 10 (P10) MSCs compared with passage 3 (P3) MSCs ($P < 0.05$). Additionally, the proliferation, paracrine, and migration capacity of P10 MSCs decreased significantly, accompanied by the increase of cellular senescence ($P < 0.05$). However, these functions, including PI3K/Akt activity of P10 MSCs, have been improved by c-Cbl downregulation ($P < 0.05$). Compared with P10 MSCs treatment, treatment with c-Cbl downregulated P10 MSCs accelerated diabetic wound healing, as defined by a more rapid wound closure ($P < 0.05$), more neovascularization ($P < 0.05$), and higher scores of wound histological assessment ($P < 0.05$) in a diabetic rat model. Our findings suggested that c-Cbl downregulation could attenuate the impairment of proangiogenic functions in MSCs induced by long-term culture *in vitro* and improve the effect of long-term cultured MSCs in promoting diabetic wound healing.

Keywords

chronic wounds, mesenchymal stem cells, angiogenesis, c-casitas B-lineage lymphoma (c-Cbl), diabetes

Introduction

Patient with diabetes mellitus (DM) has poor healing of the normal wound and could lead to the diabetic foot ulcer, which is a severe complication. The prevalence of diabetic foot ulcers in DM patients ranges from 15% to 20%, of which 20% need amputating treatment[1]. It brings not only great suffering to patients but also an enormous economic burden on family and society. A variety of complex mechanisms are involved in the delayed wound healing of diabetic ulcers, of which impaired local angiogenesis and decreased

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blood supply are essential pathological changes[2,3]. Therefore, strategies to enhance the angiogenic response in wound sites may effectively promote diabetic wound healing.

Bone marrow mesenchymal stem cells (BM-MSCs) are pluripotent cells that can be mobilized and recruited into the wound site to participate in tissue repair employing specific cell differentiation, paracrine effect, and immune regulation[4]. Recent studies have reported that the diabetic environment could negatively impact the repair function of endogenous mesenchymal stem cells (MSCs) by downregulating the survival factors and attenuating its migration ability[5–7], which might affect wound healing. Recently, stem cell therapy for diabetic wounds is receiving increasing attention. Several studies have confirmed that intravenous or local injection of BM-MSCs can significantly promote acute and chronic wound healing, collagen synthesis, and revascularization[8,9]. Before stem cell therapy, BM-MSCs must be expanded *in vitro* for a long time under specific culture conditions. However, it alters the characteristics of MSCs. Studies have shown that the repair function of MSCs has been significantly reduced after *in vitro* expansion[10]. Although it has a role in promoting wound healing to a certain extent, the effect is still less than expected[11]. Therefore, it will be exciting and essential to find a way of enhancing the repair function of long-term cultured BM-MSCs *in vitro* and further examine its pro-healing effect on diabetic wounds *in vivo*.

The proto-oncogene c-Casitas b-lineage lymphoma (c-Cbl) is an E3 ubiquitin ligase containing the RING Finger (RNF) domain, which can either upregulate or downregulate signal transduction[12,13]. E3 ubiquitin ligase, which presents with the greatest significant tissue and substrate specificity, acts on the last step of the formation of ubiquitin-protein conjugates[14,15]. The protein first labeled by ubiquitin (polypeptide) and then recognized and degraded by the 26 S proteasome complex[16]. c-Cbl can associate with receptor- and non-receptor tyrosine kinase via its tyrosine-binding domain and downregulate these receptors by promoting ubiquitination and lysosomal/proteasome degradation[17]. Additionally, tyrosine phosphorylation of c-Cbl plays a vital role in its function, among which Y700, Y731, and Y774 are the main phosphorylation sites[18]. The phosphorylation of c-Cbl allows its interaction with various signal transducers such as the p85 subunit of phosphatidylinositol 3-kinase (PI3 K), the Crk adaptor proteins, and Src family tyrosine kinases, indicating that c-Cbl plays a vital role in integrating signaling pathways[17,19]. Furthermore, c-Cbl is mainly expressed as a cytoplasmic protein in virtually all cell types and is involved in the angiogenesis process[20]. However, a regulation effect of c-Cbl in BM-MSCs, especially in the function of long-term cultured BM-MSCs, remains largely obscure. In this study, we explored the role of c-Cbl in the impaired function of long-term cultured MSCs *in vitro*. Further, we verified its regulating effects on promoting the healing of diabetic wounds by local injection of modified MSCs *in vivo*.

Materials and Methods

Cell Culture

BM-MSCs of Sprague-Dawley (SD) rat were purchased from Cyagen Biosciences (Guangzhou, China) and human umbilical vein endothelial cells (HUVECs) were purchased from Cobioer Biotechnology (Nanjing, China). BM-MSCs were cultured in MSCs complete medium (Cyagen Biosciences) and HUVECs were cultured in endothelial cell (EC) complete medium (Cyagen Biosciences). All cells were maintained at 37°C in a 5% CO_2 incubator.

Locked Nucleic Acid–Modified Antisense Oligonucleotide Gapmers Transfection

The locked nucleic acid–modified antisense oligonucleotide Gapmers (LNA Gapmers) for c-Cbl (5′-3′ sequence: CAGG-CAGCAGGTCTAA) and LNA Gapmers control (5′-3′ sequence: GCTCCCTTCAATCCAA) were purchased from Qiagen Co., Ltd, Hilden, Germany, and the transfection was performed according to the manufacturer's instructions. BM-MSCs of passage 9 (P9) was digested and seeded into six-well plates with 2.5×10^5 per well. These wells were randomly divided into c-Cbl LNA Gapmers transfected and control LNA Gapmers transfected group. Dilute 5 μl (20 μM) LNA Gapmers storage solution with Opti-MEM medium (Invitrogen Corporation, CA, USA), the total volume was 125 μl and then incubated at room temperature for 5 min. The diluted LNA Gapmers was transfected into the MSCs using Lipofectamine 3000 transfection reagent (Invitrogen Corporation, CA, USA), making the final concentration of LNA Gapmers was 50 nM. Culture conditions were set at 37°C and 5% CO_2. The c-Cbl silencing was verified by western blot assay 72 h after transfection.

Animals

The Animal Study Committee of Army Medical University approved all experimental protocols. All operations and procedures conducted on animals were under the guidelines of the United States NIH and ARRIVE. Male SD rats (200–250 g) were used to build the experimental DM rat model. Animals were given by intraperitoneal injection of streptozotocin (STZ, 65 mg/kg; Solarbio Science & Technology Co., Ltd, Beijing, China) in sodium citrate buffer (0.1 M, pH 4.5). Blood glucose levels from tail veins were assessed 3 days after injection. If the blood glucose level is lower than 250 mg/dl, STZ (15 mg/kg) was injected intraperitoneally again. Rats were considered diabetes in the study if glucose concentrations were >250 mg/dl on the third day following the last injection of STZ.

Excisional Wound Model and Treatment

The excisional wound operation was performed on diabetic rats after 2 weeks of observation. Animals were anesthetized by

intraperitoneal injection of 2% pentobarbital sodium solution (Sigma-Aldrich, St. Louis, MO, USA) at a dosage of 0.7 ml/kg before the operation. Two full-thickness dorsal skin wounds (diameter = 1.8 cm) were generated with a sterile punch.

Thirty-six diabetic wound model rats were randomly divided into three groups as follows: (1) Control group (*n* = 12), (2) Passage 10 (P10) + Scramble LNA Gapmers (SCR) group (*n* = 12), and (3) P10 + c-Cbl Knockdown (KD) group (*n* = 12). Cells from different groups were injected intradermally around the wound at four injection sites 24 h after the operation. Every site was injected with 2.5×10^5 cells, which were suspended in 0.3 ml phosphate buffered saline (PBS). The control group was injected with the same volume of PBS. At 0, 3, 7, and 14 days postoperation, the wounds were photographed and analyzed with ImageJ software by calculating the wound size reduction (%). Skin specimens were harvested after rats were sacrificed by CO_2 asphyxiation, fixed in 4% paraformaldehyde, and embedded in paraffin for subsequent experiments.

Western Blotting

According to the manufacturer's instructions, the proteins were extracted from the cells in different groups using radio-immunoprecipitation assay buffer (Cell Signaling Technology, MA, USA). Subsequently, the whole lysates were heated at 95°C with protein loading buffer (5×; Beyotime Biotechnology, Shanghai, China) together in 1:4 ratios for 5 min. Protein extracts were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% bovine serum albumin (BSA) in tris-buffered saline Tween-20 (TBST) at room temperature for 2 h. After that, the membranes were incubated with primary antibody at 4°C overnight, followed by three washes with TBST. Primary antibodies were used and diluted as follows: anti-Y^{731}c-Cbl (1:2,000; Abcam, Shanghai, China), anti-c-Cbl (1:1,000; Cell Signaling Technology, MA, USA), anti-Akt (1:1,000; Cell Signaling Technology, MA, USA), anti-phospho-Akt (1:2,000; Cell Signaling Technology, MA, USA), and anti-beta actin (1:2,000; Abcam, Shanghai, China). The horseradish peroxidase (HRP)-conjugated secondary antibodies (1:50,000; Jackson ImmunoResearch Laboratories, PA, USA) were added to the membrane and incubated for 1 h at room temperature. After incubating and washing, the membranes were added with enhanced chemiluminescence reagents (Bgbiotech, Chongqing, China) and visualized by the imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Finally, the results were analyzed by Image Lab software.

Tube Formation Assay

Passage 3 (P3) MSCs, P10 MSCs, and P10 MSCs (knockdown by c-Cbl LNA Gapmers) were cultured in the EC basal medium containing 0.75% fetal bovine serum (FBS) without growth factors for 24 h. The conditioned culture medium was collected and stored at 4°C for subsequent experiments.

Ninety-six-well plates were coated with 50 µl growth factor reduced Matrigel (Biocoat Incorporation, NY, USA) per well. HUVECs were randomly divided into five groups, which were suspended in different culture medium at the density of 1×10^5/ml, including the positive control group (suspended in EC complete medium), negative control (NC) group (suspended in EC basal medium supplemented with 0.75% FBS alone), P3 group (suspended in P3 conditioned culture medium), P10 group (suspended in P10 conditioned culture medium), P10 + SCR group (suspended in P10 + scramble LNA Gapmers conditioned culture medium), and P10 + c-Cbl KD group (suspended in P10 + c-Cbl LNA Gapmers conditioned culture medium). Each group was added with 100 µl cells per well and incubated at 37°C with 5% CO_2. After incubation for 8 h, tube formation was captured with phase-contrast microscopy (Olympus, Tokyo, Japan) and three fields of each picture were randomly selected for further analysis with ImageJ software.

Transwell Migration Assay

Twenty-four-well transwell plates with 5 µm polycarbonate filters were used to detect the migration ability of BM-MSCs. The BM-MSCs derived from different groups were digested and suspended in low glucose (5.5 mmol/l) Dulbecco's modified Eagle medium (L-DMEM) containing 0.1% BSA, and 200 µl cell suspension was loaded into each of the upper chambers. The lower chambers were added with 500 µl L-DMEM supplemented with 1% FBS and 50 ng stromal cell-derived factor 1 (SDF-1) protein (Novus Biologicals, CO, USA). The transwell plates were then incubated at 37°C and 5% CO_2 for 12 h. The upper chamber cells were wiped, and the migrated cells in the bottom surface were stained and then counted in three separate random fields of each picture by Image-pro Plus 6 software.

Enzyme Linked Immunosorbent Assay (ELISA) Analysis

The different groups of BM-MSCs 24 h conditioned culture medium was collected and then centrifuged at 1,000 rpm/min for 5 min. The supernatant was absorbed and the amount of angiogenic factors was analyzed by vascular endothelial growth factor A (VEGFA) ELISA kit (BOSTER Biological Technology, Wuhan, China), basic fibroblast growth factor (bFGF) ELISA kit (Cusabio Biotech, Wuhan, China), and hepatocyte growth factor (HGF) ELISA kit (BOSTER Biological Technology, Wuhan, China) according to the manufacturer's instructions.

Cell Counting Kit-8 Assay

The Cell Counting Kit-8 (CCK-8) kit (Beyotime Biotechnology, Shanghai, China) was used to detect the cellular

proliferation of BM-MSCs from different groups according to the manufacturer's protocols. Firstly, BM-MSCs were digested and seeded at a density of 3,000 cells per well in 96-well plates. Then, 20 μl of CCK-8 solution was added 2 h before the end of 24, 48, 72, and 96 h incubation, respectively. Finally, the absorbance of each well was determined at a 450 nm wavelength by a spectrophotometer (Thermo Varioskan Flash).

Senescence Associated β-Galactosidase Staining

The senescence of BM-MSCs was determined by using an senescence associated β-galactosidase (SA-β-Gal) staining kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, BM-MSCs that were cultured in a six-well plate were washed with PBS and then fixed in fixative solution for 15 min at room temperature. After that, each well was added with 1 ml staining working solution and maintained at $37^{\circ}C$ without CO_2 for 12 h. The SA-β-gal-positive senescent MSCs were photographed with phase-contrast microscopy (Olympus) and the percentage was calculated from five randomly chosen fields of each well.

Histological Assessment and Immunohistochemistry Analysis

The paraffin-embedded wound specimens were cut into 10 μm thick sections and dewaxed with xylene followed by dehydrated with a series of graded ethanol. For histological assessment, hematoxylin and eosin and Masson's trichrome were applied for wound sections. The staining intensity for Masson was determined using ImageJ software and wound healing was scored by two independent pathologists based on the wound-healing histologic scoring system as described previously[21]. The scoring system including five items—re-epithelialization, neovascularization, granulation tissue amount, chronic inflammation, and collagen deposition—was quantified by wound healing scores. Each item was scored according to the following standard: 0 = None, 1 = Scant or 5 vessels per HPF, 2 = moderate or 6 to 10 vessels per HPF, and 3 = Abundant or more than 10 vessels per HPF. The total wound healing score was calculated by adding up each score.

For immunohistochemistry (IHC) staining, the sections were blocked with 5% goat serum (Beyotime Biotechnology, Shanghai, China) after rehydrated and retrieved for espousing antigen, and then incubated with anti-VEGFA antibody (1:200; Abcam, Shanghai, China) or anti-CD31 antibody (1:200; Novus Biologicals, CO, USA) at $4^{\circ}C$ overnight. Samples were then incubated with biotinylated secondary antibodies (1:250; Beyotime Biotechnology, Shanghai, China) and avidin-HRP solution. Finally, the sections were stained with 3,3-diaminobenzidine and then counterstained with hematoxylin. The images were obtained under the light microscope (Olympus) and analyzed with the Image-Pro Plus 6 software.

Statistical Analysis

The data are presented as mean $\pm$ standard error. All statistical data were calculated by SPSS 13.0 software. Analysis of variance followed by post hoc least significant difference (LSD) comparison was used to analyze data for multiple comparisons between groups. Student's *t*-test was used to analyze the data of two independent groups. Statistical significance was considered if *P*-value < 0.05.

Results

The Phosphorylation Level of Protein Kinase B (Akt) Was Inhibited in Long-term Cultured MSCs and Was Associated with c-Cbl Activation

To evaluate the effect of long-term culture on c-Cbl activation and PI3K/Akt pathway in BM-MSCs, we determined the protein levels of Y^{731}c-Cbl/c-Cbl and S^{473}-Akt/Akt in P3, P6, and P10 MSCs by using western blotting. The result showed that the expression of Y^{731}c-Cbl was significantly increased, and S^{473}-Akt was decreased in P10 MSCs compared with that in P3 MSCs (Fig. 1A, *P* < 0.05). However, there was no significant difference between P3 and P6 MSCs. To further assess the relationship between c-Cbl phosphorylation and activation of the PI3K/Akt signaling pathway, the protein level of c-Cbl in P10 MSCs was knocked down by transfecting with c-Cbl LNA Gapmers and then examined the PI3K/Akt signal alteration. We found that the scramble (SCR) control LNA Gapmers transfection has no effect on the cell protein level, and the expression level of S^{473}-Akt was significantly increased in P10 MSCs after c-Cbl downregulation (Fig. 1B, *P* < 0.05). The above results demonstrated that, to some extent, c-Cbl mediated long-term culture-induced PI3K/Akt signal inhibition in BM-MSCs.

The Angiogenic Factors Paracrine and Migration Ability of BM-MSCs Were Suppressed as Long-term Cultured In Vitro and Were Improved by c-Cbl Knockdown

The results showed that the total length, nodes, and meshes formed by HUVECs was increased when cells were treated with P3 MSCs conditioned culture medium compared to the NC group (Fig. 2A, *P* < 0.05). However, this effect was significantly attenuated by treating with conditioned culture medium from P10 MSCs (Fig. 2A, *P* < 0.05). We also found that c-Cbl knockdown could improve the tube formation of HUVECs induced by P10 MSCs conditioned culture medium (Fig. 2A, *P* < 0.05). Consistently, the ELISA results also showed that the amount of VEGFA, HGF, and bFGF secreted by P3 MSCs was significantly more than those by P10 MSCs. The function of P10 MSCs secreting angiogenic factors was enhanced by c-Cbl downregulation (Fig. 2B, *P* < 0.05).

As for migration ability assessment, the transwell assay results showed that P3 MSCs possessed stronger migration
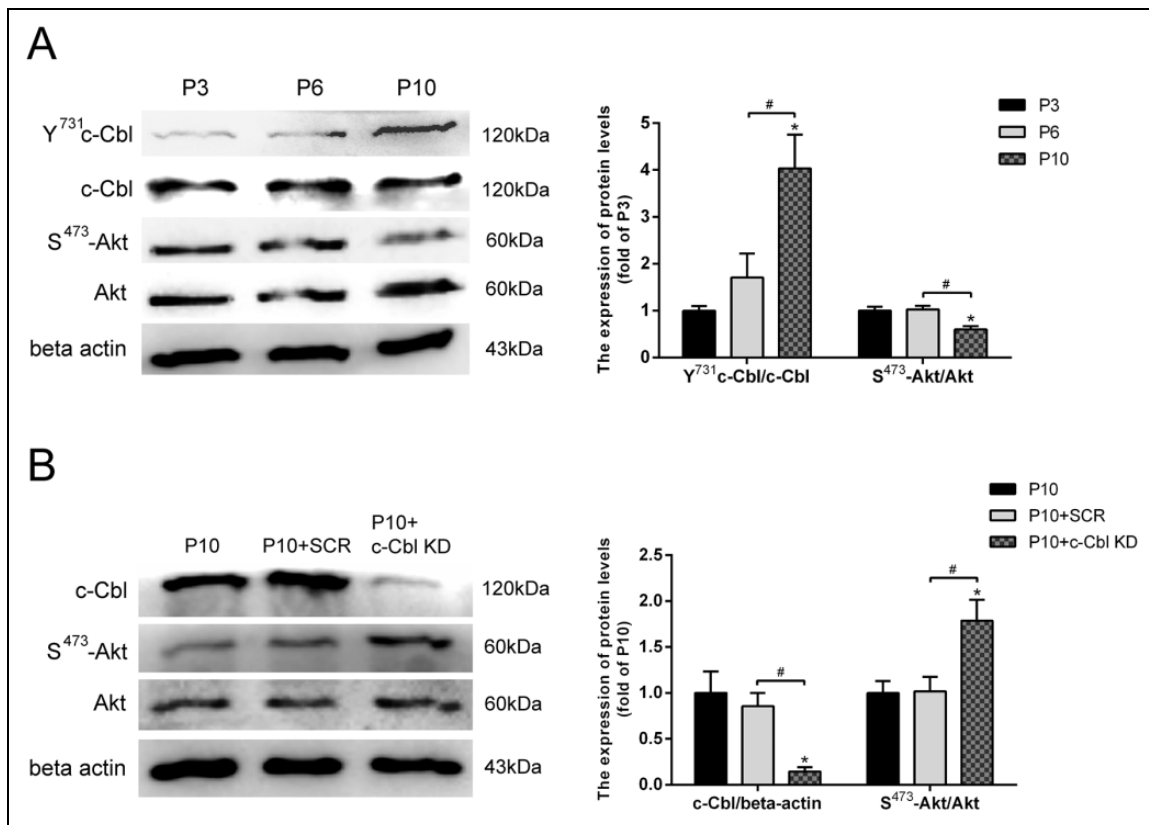
Fig. 1. The phosphorylation level of Akt was decreased in long-term cultured MSCs and increased by treating with c-Cbl LNA Gapmers. (A) Representative immunoblots and histogram analysis of Y731c-Cbl/c-Cbl and S473-Akt/Akt in P3, P6, and P10 MSCs ($n = 3$). Error bars represented the mean $\pm$ SE *$P < 0.05$ compared with the P3 group and #$P < 0.05$ versus between the indicated groups. (B) Representative immunoblots and histogram analysis of c-Cbl and S473-Akt/Akt in P10, P10 + SCR, and P10 + c-Cbl KD group cells ($n = 3$). Error bars represented the mean $\pm$ SE. *$P < 0.05$ compared with the P10 group and #$P < 0.05$ versus between the indicated groups. c-Cbl: c-Casitas b-lineage lymphoma; LNA Gapmers: locked nucleic acid–modified antisense oligonucleotide gapmers; MSC: mesenchymal stem cell; SE: standard error.

than P10 MSCs. Moreover, the number of migrated P10 MSCs was significantly increased after c-Cbl downregulation (Fig. 2C, $P < 0.05$). Altogether, these results proved that c-Cbl played an essential role in suppressing MSCs proangiogenic function induced by long-term culture *in vitro*. The downregulation of c-Cbl may improve the pro-healing efficiency of stem cells on the diabetic wound to a certain extent.

c-Cbl Downregulation Improved Proliferation Capacity and Decreased the Senescence Ratio of Long-term Cultured MSCs

To investigate the effects of c-Cbl downregulation on the proliferation and senescence of long-term cultured MSCs, we performed CCK-8 and SA-β-gal staining assay. The results showed that the ratio of cell senescence was significantly increased in P10 MSCs compared with that in P3 MSCs and c-Cbl downregulation could improve the cellular senescence of MSCs induced by long-term culture *in vitro* (Fig. 3A, B, $P < 0.05$). As expected, compared with P3 MSCs, P10 MSCs showed a repressed capacity for

proliferation and this inhibitory effect mainly occurred in the late stage of cell proliferation. Moreover, the proliferation suppression of P10 MSCs induced by long-term culture could also be improved by c-Cbl knockdown. Still, this improvement did not make P10 MSCs achieve or even exceed the proliferative activity of P3 MSCs (Fig. 3C, $P < 0.05$). These results demonstrated that c-Cbl downregulation could improve the proliferation and senescence status of long-term cultured MSCs to some extent but did not over-activate the proliferative activity of MSCs.

c-Cbl Downregulation Improved the Effect of Long-term Cultured MSCs Treatment on Promoting Angiogenesis in Diabetic Wound

To further determine the effect of MSCs from different groups on promoting angiogenesis of diabetic wounds, we transplanted the MSCs into the diabetic wounds of SD rats and harvested the wound tissue for subsequent experiments (Fig. 3). We performed IHC staining for CD31 and VEGFA to reveal the number of blood vessels and angiogenesis factors in the dermal defect on the 7th and 14th day after
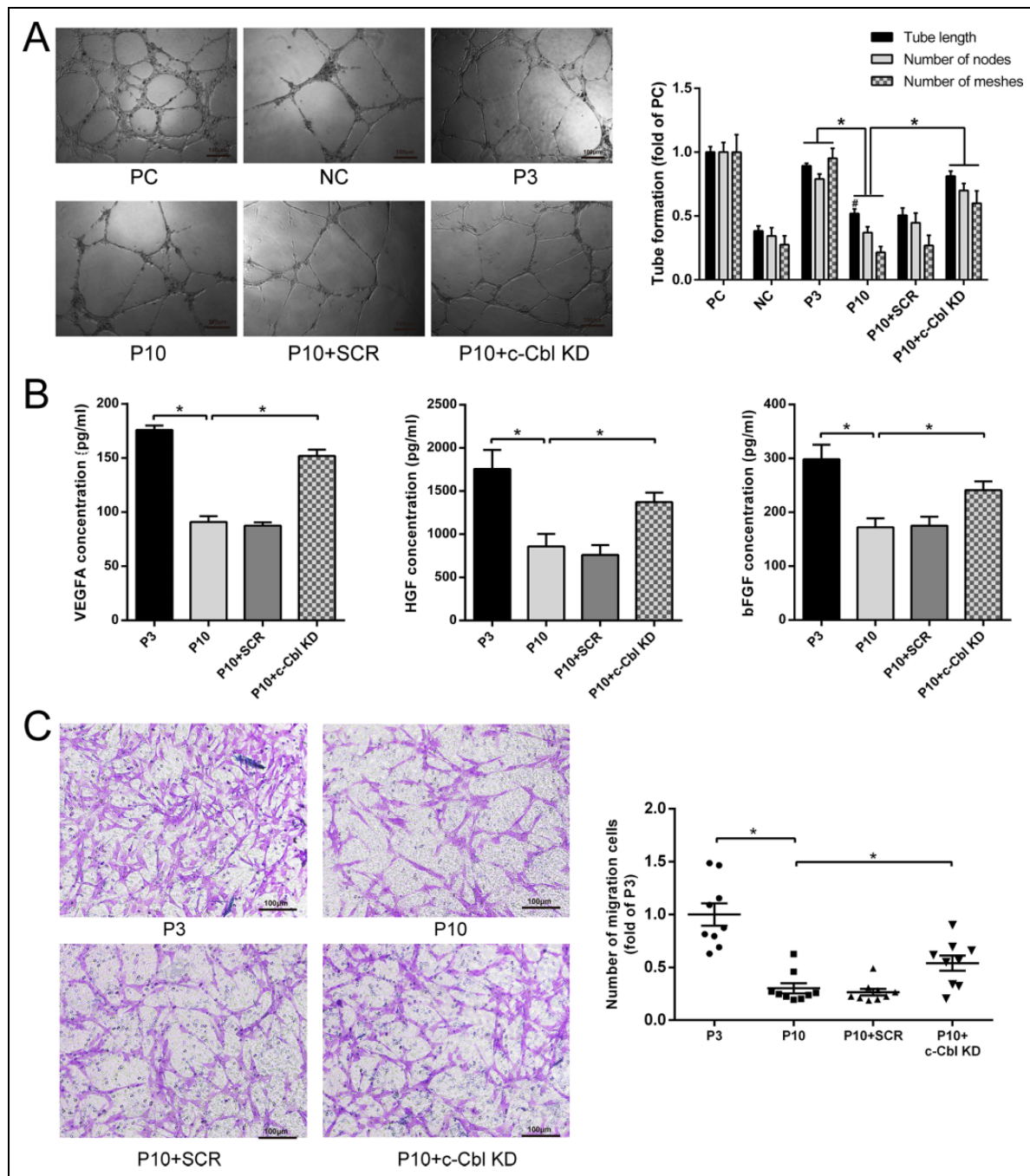
Fig. 2. The migration and angiogenic factors paracrine activity were decreased in long-term cultured MSCs and increased by treating with c-Cbl LNA Gapmers. (A) HUVECs were suspended in EC complete medium (PC) or vehicle (NC)-, P3 MSCs-, P10 MSCs-P10 + scramble LNA Gapmers (P10 + SCR) MSCs-, P10 + c-Cbl LNA Gapmers (P10 + c-Cbl KD) MSCs-conditioned EC basal medium supplemented with 0.75% FBS, and incubated for 8 h (original magnification, 10×) (n = 3). The tube length, nodes, and meshes in different groups counted in three fields per well were statistically analyzed by histogram analysis. (B) The VEGFA, HGF, and bFGF level in the conditioned media were assessed by ELISA assay (n = 3). (C) The representative picture and histogram analysis of the stained MSCs migrated to the bottom surface of the upper chamber with 10× magnification in each group (n = 3). Error bars represent the mean ± SE. #P < 0.05 compared with the NC group and *P < 0.05 versus between the indicated groups. bFGF: basic fibroblast growth factor; c-Cbl: c-Casitas b-lineage lymphoma; EC: endothelial cell; HGF: hepatocyte growth factor; HUVEC: human umbilical vein endothelial cell; LNA Gapmers: locked nucleic acid–modified antisense oligonucleotide gapmers; MSC: mesenchymal stem cell; NC: negative control; PC: positive control; SE: standard error; VEGFA: vascular endothelial growth factor A.
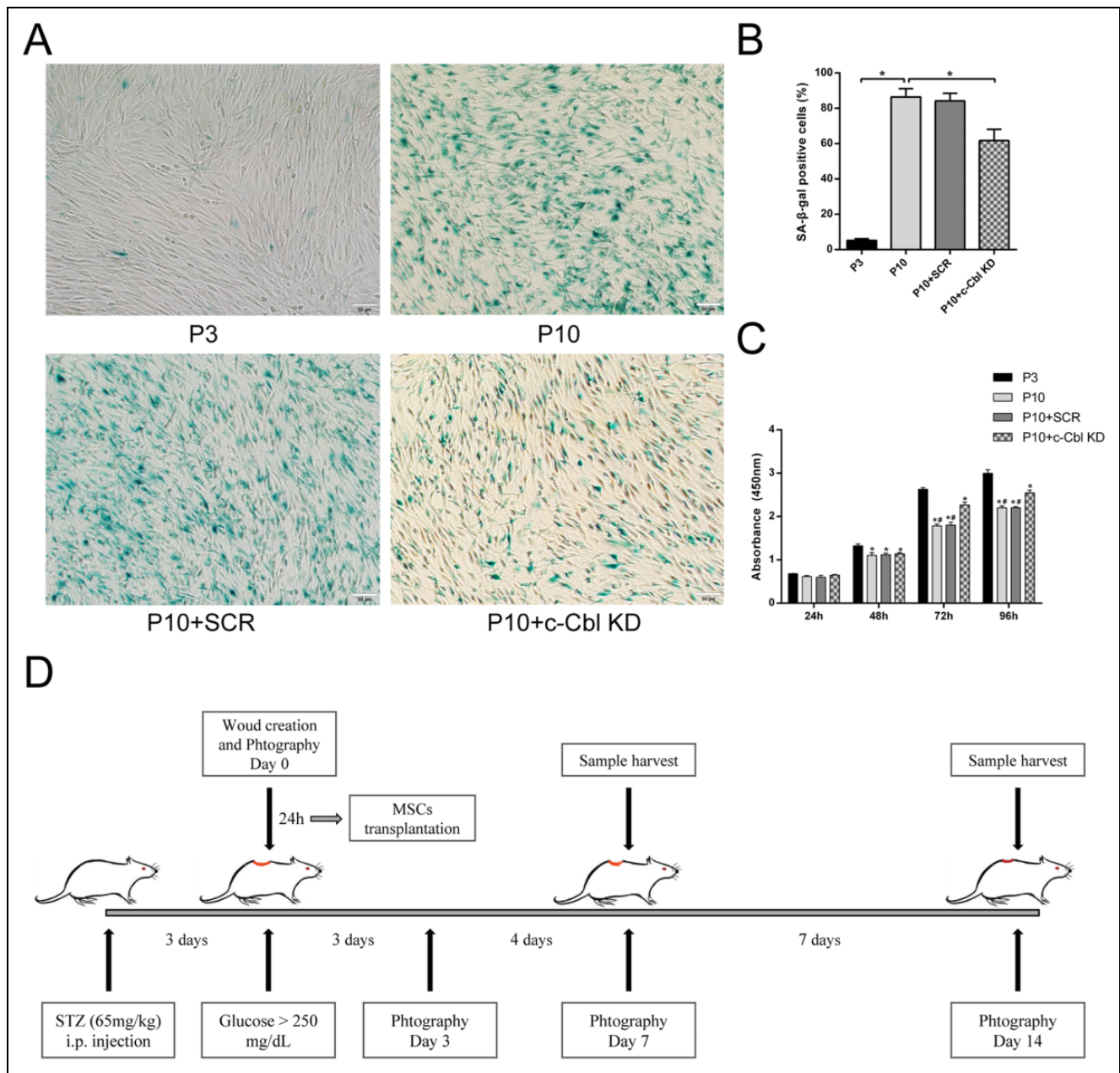
Fig. 3. The effect of c-Cbl downregulation on cellular proliferation and senescence of long-term cultured MSCs and the timeline for diabetic wound observation and sampling. (A) The representative picture of SA-β-gal stained BM-MSCs in different groups. (B) The histogram analysis of SA-β-gal-positive senescent MSCs. Results are expressed as means $\pm$ SE. *P < 0.05 versus between the indicated groups. (C) The cellular proliferation activity was assessed by CCK-8 assay. All data are represented as means $\pm$ SE. #P < 0.05 compared with the P10 + c-Cbl KD group and *P < 0.05 compared with P3 group. (D) After wound induction and injection of MSCs, wounds were photographed at the indicated time points. On days 7 and 14, the wound tissue was harvested and fixed with 10% formalin. c-Cbl: c-Casitas b-lineage lymphoma; CCK-8: Cell Counting Kit-8; LNA Gapmers: locked nucleic acid–modified antisense oligonucleotide gapmers; MSC: mesenchymal stem cell; SA-β-Gal: senescence associated β-galactosidase; SE: standard error.

surgery. We found that the skin wound treated with c-Cbl downregulated P10 MSCs showed a significantly higher blood vessel density than the control and P10 + SCR group both on the 7th day and 14th day after the operation (Fig. 4A, B, P < 0.05). Additionally, the CD31-stained blood vessels in P10 + SCR group were also higher than those in the control group on the 14th day after surgery. Meanwhile, VEGFA level in wound tissue was significantly increased on the 7th day after operation in P10 + c-Cbl KD group compared with control and P10 + SCR group; however, this difference was not detected at postoperative day 14 between three groups (Fig. 4A, C, P < 0.05). Furthermore, the
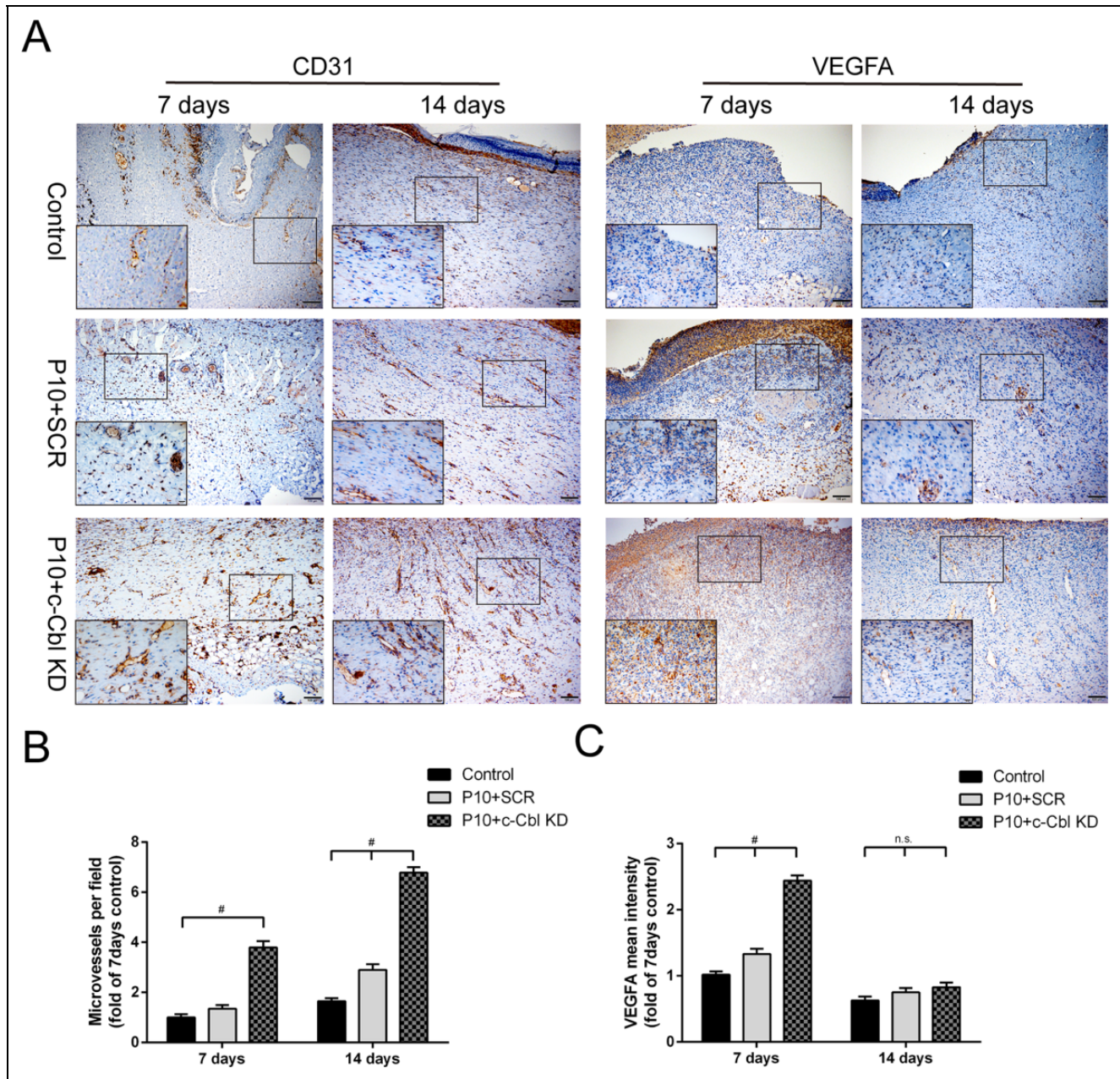
Fig. 4. c-Cbl downregulation increased the effect of long-term cultured MSCs on promoting VEGFA expression and diabetic rat wound angiogenesis. (A) The staining of CD31 and VEGFA in diabetic wounds was detected by immunohistochemistry on the 7th and 14th day after surgery (original magnification, 10× and 40×) (*n* = 4). (B) Microvessels were showed as positive CD31 staining and typical round or oval structure. (C) Fluorescent intensity of VEGFA was quantified by densitometry and normalized to the control group. The values are presented as means ± SE. #*P* < 0.05 versus between the indicated groups. c-Cbl: c-Casitas b-lineage lymphoma; MSC: mesenchymal stem cell; SE: standard error; VEGFA: vascular endothelial growth factor A.

VEGFA expression of P10 + SCR group was also higher than that of the control group 7 days after surgery. These data suggested that, compared with the control, local injection of long-term cultured MSCs with downregulated c-Cbl could significantly promote the early expression of VEGFA in wound tissue and finally enhance angiogenesis in the diabetic wound.

c-Cbl Downregulation Improved the Effect of Long-term Cultured MSCs Treatment on Promoting Diabetic Wound Healing

The progress of wound closure was photographed and analyzed at different time points. During the progress, no negative effects were observed in wound sites (Fig. 5A). The
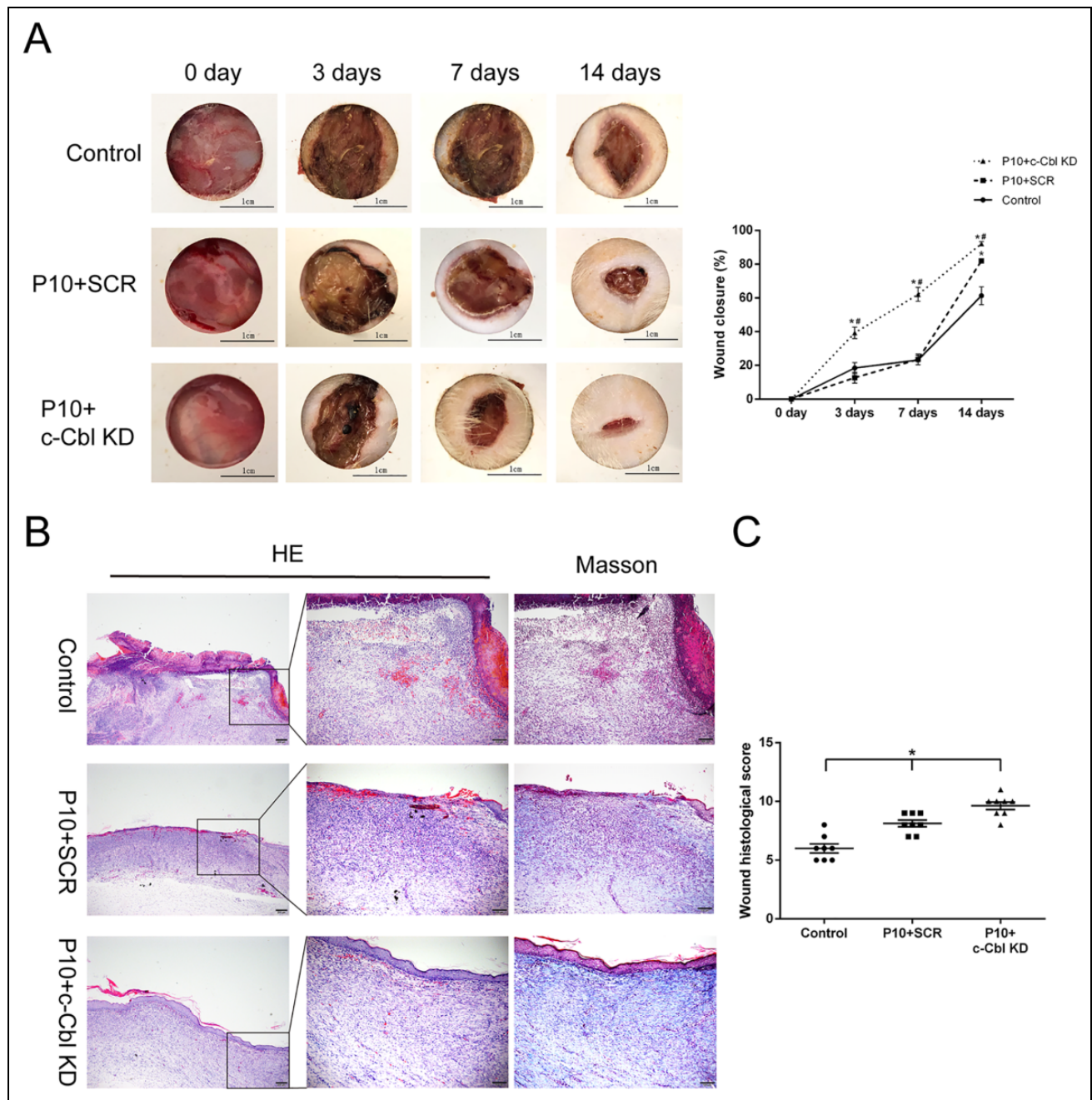
Fig. 5. c-Cbl downregulation increased the effect of long-term cultured MSCs on wound healing in diabetic rats. (A) General view of wound healing in the diabetic rat model treated with PBS (control), P10 MSCs + scramble LNA Gapmers (P10 + SCR), and P10 MSCs + c-Cbl LNA Gapmers (P10 + c-Cbl KD) at 0, 3, 7, and 14 days postoperation. At the indicated times, the wound closure rate of all treatment groups was analyzed and represented in the linear diagram ($n = 8$). The values are presented as means $\pm$ SE. *$P < 0.05$ compared with control and #$P < 0.05$ compared with P10 + SCR group. (B) Representative micrographs of H&E and Masson's staining of skin wound tissues at 14 days after the operation (original magnification, 4× and 10×) ($n = 4$). (C) The histogram analysis of wound healing scores in three groups ($n = 8$). The values are presented as means $\pm$ SE. *$P < 0.05$ versus between the indicated groups. c-Cbl: c-Casitas b-lineage lymphoma; H&E: hematoxylin and eosin; LNA Gapmers: locked nucleic acid–modified antisense oligonucleotide gapmers; MSC: mesenchymal stem cell; PBS: phosphate buffered saline; SE: standard error.

results showed that the P10 + c-Cbl KD group's wound closure rate was higher than that of the control and P10 + SCR group at 3, 7, and 14 days after the operation (Fig. 5A, $P < 0.05$). What's more, the wound treated with P10 MSCs + scramble LNA Gapmers also resulted in a higher wound closure rate at 14 days postoperation compared with the

control group (Fig. 5A, $P < 0.05$). The wound healing scores quantified the histological alterations, including re-epithelialization, neovascularization, granulation tissue amount, chronic inflammation, and collagen deposition. The results showed that the score of wounds treated with the P10 MSCs, downregulated by c-Cbl LNA Gapmers, was higher than that of the control and P10 + SCR group on day 14. Besides, the P10 + SCR group's wound healing score was also higher than that of the control group (Fig. 5B, C, $P < 0.05$). These results collectively confirmed the critical role of c-Cbl downregulation in improving the efficiency of long-term cultured MSCs on promoting diabetic wound healing, including its histological presentation.

Discussion

Skin wound under diabetes environment is challenging to heal. Numerous studies have been carried out on autologous BM-MSCS therapy for diabetic wounds, and their results also show part efficiency[8,22]. Unlike animal experiments, the long-term expansion of BM-MSCs *in vitro* is inevitable before clinical application. It is reported that stem cells' repair function significantly decreased after being cultured for a long time[10]. In this study, we found PI3K/Akt signal was inhibited after BM-MSCs culture *in vitro* for a long time, which was related to the c-Cbl activation. Consistently, the proangiogenic functions, including migration and paracrine ability of P10 MSCs, were also improved by c-Cbl knockdown. *In vivo*, we found that c-Cbl downregulated P10 MSCs improved its effect on promoting angiogenesis and wound healing in STZ-induced diabetic rats. Our results suggested that c-Cbl downregulation may enhance the proangiogenic function of MSCs after long-term expansion *in vitro* by activating PI3K/Akt signals, thereby accelerating revascularization and cutaneous wound healing of diabetic rats with P10 MSCs treatment.

Patients with diabetic ulcers were more prone to delayed wound healing, which involved reduced blood supply and insufficient local angiogenesis[2,23,24]. VEGF expression at the wound site was decreased since the delayed delivery of immune cells or MSCs to dermal defects induced by reduced angiogenesis[23]. Meanwhile, the impairment of the SDF-1/C-X-C chemokine receptor type 4 (CXCR4) signal axis under the diabetic environment was also attributed to the decreased numbers of migrating MSCs around the dermal defect, which further impaired angiogenesis and diabetic wound healing[25–27]. Besides the reduced numbers, the angiogenic properties of MSCs also attenuated significantly after exposure to the diabetic serum *in vitro* by inducing autophagy signaling[28]. Similarly, the diabetic condition could also affect the angiogenic/cardiogenic capacity of cardiac c-kit+ cells *in vitro* and vivo[29]. Therefore, improving the angiogenic and migration function of long-term expanded MSCs *in vitro* is of great significance before the cell therapy of diabetic wounds. Moreover, studies have reported that the proangiogenic functions, including proliferation, migration,

and paracrine activity, could be regulated by the PI3K/Akt signal pathway in MSCs[30]. Recently, researchers found that c-Cbl knockout promoted angiogenesis by activating Akt phosphorylation and vascular repair in myocardial ischemia mouse models[31]. Based on these pieces of evidence, we chose to detect changes in the phosphorylation levels of Akt and c-Cbl during the MSCs expansion *in vitro*, for related reports showed that they were upregulated in angiogenesis. Similarly, our results showed that c-Cbl downregulation could increase the phosphorylation level of Akt, decrease the ratio of cell senescence and improve the cellular proliferation, angiogenic factors paracrine, and migration ability of long-term cultured MSCs. All these data suggested the fact that c-Cbl downregulation played an essential role in promoting the proangiogenic function of long-term cultured MSCs, and this effect was achieved, to some extent, by activating the PI3K/Akt signaling.

As an essential cytokine, VEGF promotes angiogenesis and regulates other cellular biological behaviors related to wound healing. Chronic inflammation, a hallmark of diabetic wounds, is correlated with impaired macrophage transition from proinflammatory M1 to anti-inflammatory M2 phenotype[32,33]. Furthermore, the decreased expression of local VEGF participated in regulating phenotypic transformation of macrophages and led to an increase in the proportion of M1 cells, thereby promoting the sustained inflammatory response of diabetic wound tissue[34,35]. Studies also found that local VEGF could increase endothelial progenitor cells recruitment, granulation tissue formation, collagen deposition, and re-epithelialization, thus promoting wound healing[36,37]. Consistent with the above findings, our study demonstrated that the c-Cbl downregulated P10 MSCs could induce a significant improvement in terms of VEGFA protein level, neo-formed vessel count, diabetic wound healing, and histological scores compared with control P10 MSCs treatment group. Our results suggested that c-Cbl was a critical regulator in improving the efficiency of long-term cultured MSCs on promoting angiogenesis and wound healing in the diabetic rat model.

To our knowledge, the present study firstly explored the regulatory relationship between c-Cbl activity and PI3 K/Akt signal after a long-term expansion of MSCs *in vitro*. It verified the effects of c-Cbl downregulation on proangiogenic functions of long-term cultured MSCs and the improvement of the curative effect on chronic wounds in diabetic rats. However, our research had some limitations: firstly, our experiment was carried out only on the STZ-induced type I diabetic rat model, but the effect of c-Cbl downregulated MSCs might also be studied in the type II diabetic rat model. Secondly, we did not provide the specific downstream molecular mechanism to demonstrate how the c-Cbl/Akt signal pathway regulated the proangiogenic functions of long-term cultured MSCs. Previous studies suggested the migration and paracrine activity of MSCs could be regulated by SDF-1/CXCR4 signaling[38]. Moreover, the up-expression of SDF-1/CXCR4 signaling was associated with activated

PI3K/Akt pathway[30], which provided clues for further study of the mechanism. Even though the complete mechanism needs to be further explored, this study's primary purpose is preliminary validation.

In conclusion, our results showed that c-Cbl downregulation effectively augmented the proangiogenic functions of long-term cultured MSCs and improved its pro-healing effect for the chronic wound of diabetic rats, which was manifested as faster wound closure, activated neovascularization, and higher histological scores. In this process, the regulatory effect of c-Cbl may be due to its influence on the PI3K/Akt signal pathway. Our findings also suggested that the functional impairment of MSCs induced by long-term culture *in vitro* could be improved by c-Cbl downregulation, which may provide a better basis for the clinical application of MSCs on the diabetic wound.

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