

The Reduction of JunB Inhibits the Odontogenic Differentiation Potential of the DPSCs by Targeting PIN1 in Pulpitis

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Research

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Abstract

Pulpitis is an inflammatory condition that can lead to the loss of tooth vitality. The odontogenic differentiation potential of the dental pulp stem cells (DPSCs) is significantly decreased in the pulpitis microenvironment. The aim of this study was to determine the mechanism of the impaired odontogenic differentiation of the DPSCs in an inflammatory microenvironment. Overexpression and inhibition of the JunB were performed to evaluate its effect on the odontogenic differentiation potential of the DPSCs. The results showed that the JunB was positively correlated with the odontogenic differentiation ability of the DPSCs. The co-immunoprecipitation assay coupled with the mass spectrometry showed that peptidyl-prolyl cis-trans Isomerase NIMA-Interacting 1 (PIN1) was a downstream target of the JunB. Moreover, the expression of PIN1 was negatively correlated with the odontogenic differentiation potential of the DPSCs. Taken together, the decreased expression level of the JunB in an inflammatory state can impair the odontogenic differentiation ability of the DPSCs by upregulating the expression of the PIN1, a negative regulator of the odontogenic differentiation of the DPSCs.

Introduction

Pulpitis is an inflammation of the dental pulp tissue primarily caused by dental caries [1, 2]. During the progression of pulpitis, the gram-negative bacteria interacts with the dental pulp through the dentinal tubules, attracting multiple immune cells to the dentin-pulp interface and triggering various inflammatory cytokines and chemokines involved in this process [3–6]. The dental pulp tissue that contains dental pulp stem cells (DPSCs), is frequently invaded by the gram-negative bacteria, which results in pulpitis as a consequence [7]. Lipopolysaccharide (LPS), a major component of the outer membrane of the gram-negative bacteria, has been widely used to investigate the inflammatory response of the cells or tissues and is a potent inducer of pulpitis [8, 9]. Nowadays, the most common treatment method for pulpitis is root canal therapy. The root canal of the affected tooth is cleaned and shaped and finally obturated with the root canal filling materials and sealers [10]. However, the limitation of this method is that it will irreversibly reduce the tooth substance and decrease the fracture resistance. An endodontically treated tooth is devitalized and presents a high risk of biomechanical failure [11, 12].

DPSCs are adult mesenchymal stem cells (MSCs) with high potential of self-renewal and multi-lineage differentiation (including chondrocytes, adipocytes, neural cells, and osteoblasts), which make them an attractive choice for tissue engineering [13, 14]. During the past decades, numerous in-vitro and in-vivo experiments have confirmed that the DPSCs can differentiate into different tissues (including the dentin) under different favorable microenvironmental conditions [15, 16]. Under physiological conditions, the DPSCs can differentiate into odontoblasts and express the dentin-specific proteins DSPP and DMP-1 (Dentin Matrix Acidic Phosphoprotein 1) [17, 18]. Our previous study demonstrated that the TNF- α triggered osteogenic differentiation of the DPSCs via activating the NF- κ B pathway [19]. However, many other studies have shown that the odontogenic differentiating ability of the DPSCs decreased in the inflammatory microenvironment, thus affecting the restoration and regeneration of tooth tissue [20]. Therefore, there is an urgent need to explore the underlying mechanism of the impaired odontogenic

differentiation capacity of the DPSCs in an inflammatory state, and this knowledge may contribute to the development of more effective clinical treatment strategies.

JunB is a member of the activated protein-1 (AP-1) family, which forms dimeric protein complexes with several protein families, including Jun, Fos, and ATF [21]. It plays a critical role as an activator of transcription. The JunB is involved in the regulation of several basic cellular processes, including cell survival, cell proliferation, senescence, and programmed cell death [22, 23]. Recently, a subset of the MSC transcription factors, including JunB, have been reported to promote osteogenesis and inhibit adipogenesis [24]. Furthermore, the changes in the gene expression profile during the DPSCs differentiation into osteoblastic cells were studied by the microarray technology, and the IGFBP-5, JunB, and NURR1 genes were found to be significantly up-regulated [25]. Another report revealed that the Notch signaling significantly enhanced the cell proliferation but inhibited MSCs osteogenic differentiation induced by the bone morphogenetic protein 9 (BMP 9) via the JunB protein suppression [26]. The JunB severely deficient mice had serious bone problems such as osteopenia and defective endochondral ossification due to the cell-autonomous osteoblast and osteoclast defects [27, 28]. These studies showed that the JunB is widely involved in the osteogenic differentiation of stem cells and the mineralization of bone tissue.

PIN1 is a peptidyl-prolyl cis-trans isomerase that specifically recognizes the phosphorylated Ser/Thr-Pro motifs and isomerizes only the phosphorylated Ser/Thr-Pro (pS/T-P) peptide bonds [29–32]. The PIN1 regulates diverse cellular processes, including growth-signal responses, cell cycle progression, cellular stress responses, neuronal function and immune responses [33, 34]. The PIN1 has been shown to interact with a number of cancer-related phosphoproteins, which suggested that the PIN1 might link to the signal transduction of the pathogenesis of cancer [31, 35, 36]. Moreover, the PIN1 overexpression is associated with the poor differentiation and survival of oral squamous cell carcinoma [37]. Recently, PIN1 has been reported as a negative regulator of odontogenic differentiation of the DPSCs [38]. However, whether the PIN1 is involved in the regulation of odontogenic differentiation of DPSCs in pulpitis remains elusive.

In this study, we proved that the JunB functioned as a positive regulator of odontogenic differentiation in the DPSCs. The inflammatory state reduced the expression of the JunB, which compromised the odontogenic differentiation ability of the DPSCs. The PIN1 was a negative regulator of odontogenic differentiation of the DPSCs in an inflammatory microenvironment. Our study proved that the JunB could participate in the odontogenic differentiation of the DPSCs through interaction with PIN1 indicating that the JunB and its substrate PIN1 may serve as potential diagnostic markers and/or drug targets for the clinical treatment of pulpitis.

Material And Methods

Cell culture

According to the approval guide of Ethics Committee of Affiliated Hospital of Nantong University, clinically healthy and fresh third molars were collected from Oral Comprehensive Department of Affiliated Hospital of Nantong University with patients' informed consent. All subjects (20–25 years old) were free of carious lesions and oral infection. The tooth crown was split and pulp tissues were minced and digested in 3 mg/mL type I collagen (Worthington Biochem, Freehold, NJ) for 30 min at 37°C. To obtain a single cell suspension, the digested tissues were passed through a 70- μ m cell filter (Falcon, BD Labware, Franklin Lakes, NJ). The cell suspension was then seeded into 25-mm² diameter plates and cultured in complete culture medium containing high-glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin. Cells were expanded at 37°C and 5% CO₂, and the culture medium changed every 3 d. Cells were passaged at the ratio of 1:3 when they reached 85–90% confluence. The subsequent experiments were performed with the third generation of DPSCs.

Cell Counting Kit-8 Assay

The effect of LPS on cell growth was determined by the CCK8 assay. The LPS (I6143; Sigma-Aldrich Inc., St. Louis, MO, USA) from the intestinal serotype of *Salmonella enterica* was dissolved in water. The DPSCs were seeded on 96-well plates at a cell density of 1 \times 10³ cells/well for 24 h. Next, the cells were treated with different concentrations of the LPS (0, 0.1, 1, and 10 μ g/mL) in a serum-free medium for 6, 12, 24, and 48 h. Then, a 10 μ L CCK-8 solution (Beyotime, Shanghai, China) in 100 μ L fresh media was added to each well, and the cells were further incubated at 37 °C for 2 h. The optical density (OD) was measured at 450 nm using a microplate reader (Thermo Scientific, Eugene, OR, USA).

Odontogenic differentiation

Third generation DPSCs were seeded on 6-well plates at a density of 4000/cm² and cultured with odontogenic differentiation medium (DMEM containing 15% FBS, 10 mmol/L β -glycerophosphate, 50 mg/mL α -ascorbic acid, 10 nmol/L dexamethasone (Sigma-Aldrich), 0.292 mg/mL glutamine, 100 μ g/mL streptomycin, and 100 U/mL penicillin for 3, 7, 14, or 21 d. The medium was changed every 3 d.

ALP staining and Alizarin red staining

The cells were co-cultured in the odontogenic medium for 14 d or 21 d. Then the mineralization potential of the DPSCs was respectively detected by the ALP staining and Alizarin red staining. Briefly, the cells were fixed in 4% paraformaldehyde for 30 min. After washing with PBS, the cells were then incubated with Alkaline Phosphatase (Beyotime) for 12 h for the Alkaline Phosphatase staining or with 2% Alizarin Red S solution (Solarbio) for 15 min for the Alizarin Red staining. The cells were viewed under a microscope.

Western blot analysis

The cells were lysed in a buffer consisting of 50 mM TRIS, 150 mM NaCl, 2 % sodium dodecyl sulfate (SDS), and a protease inhibitor mixture. After centrifugation at 15,000 rpm for 15 min, the protein concentration in the supernatants was quantified using a protein quantification kit. The resulting

supernatant (50 µg protein) was subjected to the SDS polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred onto the polyvinylidene difluoride membranes at 200 mA for 1.5 h in a blotting apparatus (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% nonfat milk for 2 h at room temperature. The primary antibody was incubated at 4°C overnight and the secondary antibody was incubated at room temperature for 2 h. The following primary antibodies were used: rabbit anti-JunB (1:1000, Abcam), rabbit anti-PIN1 (1:1000, Proteintech), and rabbit anti-GAPDH (1:1000, Abcam). The secondary antibody was a goat polyclonal secondary antibody purchased from Abcam (Cambridge, MA, USA).

Real-time polymerase chain reaction analysis

According to the manufacturer's instructions (Invitrogen, Waltham, MA, USA), the total RNA was extracted from cells by Trizol reagent. Complementary DNA (cDNA) for miRNA transcripts were amplified by Maxima™ H Minus cDNA Synthesis Master Mix, with dsDNase (Thermo Fisher Scientific). The polymerase chain reaction (PCR) mixture was prepared using LightCycler 480 SYBR Green (Roche Applied Science, Penzberg, Germany). For GAPDH, DSPP, and DMP-1 detection, cDNA was synthesized by RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific). The AceQ qPCR SYBR Green Master Mix (without ROX) (Vazyme Biotech, Nanjing, China) was then used for quantitative PCR of these genes. The GAPDH was used for normalization. We used a Light Cycler 480 Real-Time PCR System (Roche Diagnostic, Mannheim, Germany) to amplify these genes. The primer sequences used in the experiment were as follows:

GAPDH: 5'-GAAGGTGAAGGTCGGAGTC-3', 5'-GAAGATGGTGATGGGATTTTC-3';

DMP-1: 5'-CGCCCATGGCAAATAGTGAC-3', 5'-CTCCTTATCGGCGTCCATCC-3';

DSPP: 5'-GGAGACAAGACCTCCAAGAGTA-3', 5'-TGCTGGGACCCTTGATTTCTA-3';

JunB: 5'-ACAACTCCTGAAACCGAGCC-3', 5'-CGAGCCCTGACCAGAAAAGTA-3';

PIN1: 5'-ATGGCGGACGAGGAGAAGCTGCC-3', 5'-GTTGCGTTACACCCTTTCTTG-3'

Immunoprecipitation and MS/MS Spectrometry

Pierce Classic Magnetic IP/Co-IP Kit (Thermo Fisher Scientific) was used for the AKAP1 immunoprecipitation (IP). The inflamed pulp tissues preserved in liquid nitrogen were ground and then lysed with the IP lysis buffer (Roche). The protein sample (500 µg) was immunoprecipitated by the 10-µg rabbit anti-JunB (Abcam) at 4 °C overnight. Subsequently, 20–40 µL of fully suspended protein A/G magnetic beads were added and incubated at room temperature for 1 h. The complexes bound to the protein A/G conjugate were washed and resolved in the SDS–PAGE loading buffer and subjected to the MS/MS analysis. The resulting samples were subjected to the LC-MS/MS spectrometry analysis (Shanghai Applied Protein Technology Co., Ltd., Shanghai, China). The MS/MS spectra were searched using MASCOT engine version 2.4 (Matrix Science, London, UK) against the UniProtKB human (188433 total entries, downloaded on 2020-02-17).

Co-Immunoprecipitation

The co-immunoprecipitation protocol was performed using a Pierce co-IP kit (88804; Thermo Scientific™ Pierce™ Classical magnetic bead immunoprecipitation/immunocoprecipitation Kit). The cell lysate was incubated with the rabbit anti-JunB (Abcam) at 4 °C overnight. The antigen/antibody complex was bound to the protein A/G magnetic beads at room temperature for 1 h. The beads were washed twice with the immunoprecipitation lysis/rinse buffer, followed by one wash with pure water. The antigen/antibody complexes were eluted. Finally, immunoblotting experiments for the immune complexes were performed.

Cell transfection

Knockdown of JunB and PIN1 was accomplished using siRNA (synthesized by Genepharma, Shanghai, China). JunB siRNA target sequence was: 5'-CAGCUCAAACAGAAGGUCAdTdT-3' and PIN1 siRNA target sequence was: 5'-CCACCGTCACACAGTATTTAT-3', $2-3 \times 10^5$ cells/well were seeded in 6-well plates one day before siRNA transfection. Transfection was performed according to the manufacturer's instructions using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA) and 80 nM siRNA. The overexpression constructor of the PIN1 and JunB were purchased from GenePharma. Transfection was achieved as described above. Three days later, the transfection efficiency was detected by RT-PCR and Western blot.

Statistical analysis

The experimental data were analyzed using the GraphPad Prism software. All experiments were repeated at least three times, with three replicate wells per design and the data are shown as mean \pm standard deviation. The statistical significance was assessed by one-way ANOVA analysis and the minimum significant difference test (Fisher's least significant difference [LSD]). The $P < 0.05$ was considered statistically significant.

Results

The LPS-treatment decreased the odontogenic differentiation ability of the DPSCs

The CCK-8 was used to measure the growth of the DPSCs stimulated by different concentrations of the LPS. Compared with the negative control group, when the LPS concentration was 1 $\mu\text{g}/\text{mL}$, the cell proliferation increased slightly (Fig. 1A). The possibility that the odontogenic differentiation ability of DPSCs was damaged due to the decrease of DPSCs after LPS stimulation was excluded. Therefore, we stimulated the DPSCs with 1 $\mu\text{g}/\text{mL}$ LPS concentration to imitate the inflammatory microenvironment. We added 1 $\mu\text{g}/\text{mL}$ LPS into the odontogenic induction medium and co-cultured with the DPSCs as the inflammatory stimulation group (L-DPSCs) and compared the odontogenic induction ability with the untreated DPSCs group (NC). Then we analyzed the mineralization ability by Alizarin red staining and ALP staining. The results showed that after 14 d, ALP activity of the L-DPSCs group decreased compared with the NC group. And after 21 days of co-culture, Alizarin red staining results showed that the number of mineralized nodules in the L-DPSCs group decreased gradually (Fig. 1B). The DSPP and DMP-1 are

both transcription factors of odontogenic differentiation. The RT-PCR results showed that the expression of the DSPP and DMP-1 in the DPSCs stimulated by the inflammatory microenvironment decreased significantly (Fig. 1C).

Overexpression of the JunB reversed the decreased odontogenic differentiation in the LPS-treated DPSCs

As shown in Fig. 2A, treatment with 1 µg/mL LPS for 72h significantly downregulated the expression level of the JunB in the DPSCs. Next, we overexpressed the JunB in the group L-DPSCs as the group L-DPSCs/JunB. The RT-PCR and western blot were used to verify the overexpression efficiency (Fig. 2B). Compared with the negative control group, the ALP activity significantly increased in the L-DPSCs/JunB group after 14 d of odontogenic induction. Similarly, there were increased numbers of ARS-stained mineralized nodules observed in the L-DPSCs/JunB group after 21 d of induction (Fig. 2C). The mRNA expression level of odontogenic markers DSPP and DMP-1 was also up-regulated with the overexpression of the JunB (Fig. 2D).

Silencing the JunB inhibited the odontogenic differentiation in the DPSCs

We then knocked down the expression of the JunB with siRNA in the DPSCs. The knockdown efficiency was evaluated by RT-PCR and western blot (Fig. 3A.). A reduction of the ALP-stained cells and ARS-stained mineralized modules was observed as compared with the control vector group (Fig. 3B). Silencing the JunB led to the decreased expression level of the DSPP and DMP-1 (Fig. 3C).

The PIN1 was identified as the downstream target of the JunB in the LPS-treated DPSCs

To explore the potential mechanism of the JunB mediating reduction of the odontogenic differentiation of DPSCs in an inflammatory state, we analyzed 20 groups of inflammatory pulp tissues by mass spectrometry to identify the downstream target of the JunB. The mass spectrometry data showed that a total of 1247 proteins could bind to JunB protein in the immunoprecipitation complexes obtained by the anti-JunB (antibody) (Supplementary Figure). Among these proteins, the PIN1 was known to play a critical role in the DPSCs under both physiological and pathological conditions [39, 40, 41]. The co-IP assay further confirmed that the JunB can bind to the PIN1 in the DPSCs in the inflammatory microenvironment (Fig. 4A). Next, we transfected the JunB siRNA and detected the expression changes of the JunB and PIN1 proteins by Western blot. The results showed that the expression of the PIN1 increased significantly after the knockdown of the JunB (Fig. 4B). To explore whether the PIN1 is involved in regulating the JunB expression, JunB protein was measured by Western blot after the cells were transfected with the PIN1 siRNA. The results showed that knocking down of PIN1 could not affect the expression of the JunB (Fig. 4C). Therefore, we believed that the PIN1 plays a role as a downstream regulator of the JunB in the LPS-treated DPSCs.

Silencing the PIN1 promoted the odontogenic differentiation in the LPS-treated DPSCs

We compared the expression level of the PIN1 in the DPSCs with or without 1 µg/mL LPS by Western blot. As shown in Fig. 5A, the LPS treatment increased the expression of the PIN1. We next knocked down the

expression of the PIN1 with siRNA in the L-DPSCs. The knockdown efficiency was evaluated (Fig. 5B). After 14 d of odontogenic induction, the images of ALP staining showed silencing PIN1 significantly increased the ALP activity in the L-DPSCs. In addition, after 21 days of odontogenic induction, the L-DPSCs showed increased numbers of ARS-stained mineralized nodules by the PIN1 inhibition (Fig. 5C). The mRNA expression level of the DSPP and DMP-1 was also found to be up-regulated by silencing PIN1 (Fig. 5D).

Overexpression of the PIN1 inhibited the odontogenic differentiation in the DPSCs

Overexpression of the PIN1 in the DPSCs was verified by the RT-PCR and Western blot (Fig. 6A). After 14 d of odontogenic induction, a reduction in the number of the ALP-stained cells was observed with the overexpression of the PIN1. In addition, after 21 days of odontogenic induction, the DPSCs showed reduced numbers of the ARS-stained mineralized nodules with the overexpression of the PIN1 (Fig. 6B). The RT-PCR results showed that the expression of the DSPP and DMP-1 in the DPSCs decreased significantly with the PIN1 overexpression (Fig. 6C).

Discussion

The development of pulpitis is a sophisticated process regulated by the dentin-pulp complex. The primary goal of endodontic treatment is to retain the natural dentition. Regeneration of a functional pulp-dentin complex is promising for the overall prognosis of the *tooth* [42]. To regenerate the pulp-dentin complex, an appropriate microenvironment is of great importance for stimulating the differentiation of DPSCs. In this regard, the use of bioactive molecules, such as TGF- β , was shown to enhance the regenerative capacity of stem cells and subsequent dentine regeneration [43]. Other approaches that utilized Bioactive compounds known to induce osteogenic effects, demonstrated successful odontogenic differentiation of DPSCs [44]. In addition, our previous research revealed that tumour necrosis factor α (TNF- α) promotes osteogenic differentiation of human DPSCs by activating the NF- κ B signalling pathway [23]. In this study, we tried to clarify the molecular mechanism of impaired odontogenic differentiation of DPSCs under inflammatory state, and then identified that JunB could be a potential target that involved in regulation. It provides a new train of thought for the treatment of pulpitis.

As an important protein-coding gene, JunB is best known as an inhibitor of cell division, an inducer of senescence, and a suppressor of tumors, at least in the myeloid lineage. Accumulating evidence supports that the JunB plays a pivotal role in cell differentiation [45, 46, 47, 48]. Recently, scholars have comprehensively characterized the transcriptional and epigenomic changes associated with the osteoblast and adipocyte differentiation of the human MSCs. By using machine learning algorithms for the in-silico modeling of transcriptional regulation, they identified a subset of MSCs transcription factors including JunB that drive the osteogenesis and suppress the adipogenesis [24]. However, deeper validation analysis was not performed. In this study, we compared the expression of the JunB in the normal and LPS-treated DPSCs. We found that the expression of the JunB decreased significantly in the inflammatory state. Furthermore, we found that the odontogenic differentiation ability of the DPSCs

decreased significantly in the inflammatory microenvironment. Overexpression of the JunB in the LPS-treated DPSCs can significantly improve the odontogenic differentiation ability of the DPSCs. Silencing the JunB expression in the DPSCs inhibited the odontogenic differentiation. Therefore, we believed that the JunB functioned as a positive regulator of the odontogenic differentiation of the DPSCs, and the inflammatory conditions could damage the odontogenic differentiation ability of the DPSCs via down-regulation of the JunB expression.

Recent studies have shown that JunB uses Dentin Matrix Protein 1 (DMP1) and Bone Morphogenetic Protein-2 (BMP-2) as two downstream targets to execute its regulation of osteoblast and myogenic differentiation [49, 50]. In this study, we performed co-immunoprecipitation coupled with mass spectrometry and found PIN1 to be a downstream target of JunB in inflammatory pulp tissues. According to the previous reports, undifferentiated mesenchymal stem cells are one of the most important components in dental pulp tissues [51, 52]. Therefore, the co-immunoprecipitation was used to testify whether the PIN1 is the downstream target of the JunB in the L-DPSCs. Our study showed that the PIN1, a member of the parvulin subfamily of peptidyl-prolyl cis/trans isomerase, was a downstream target of the JunB and involved in the regulation the odontogenic differentiation of the DPSCs under the inflammatory state. The increased expression of Pin1 inhibited the odontogenic differentiation of DPSCs, whereas knockdown of PIN1 resulted in the opposite outcome. Our results suggested that unlike JunB, PIN1 could be a negative regulator of the odontogenic differentiation in the DPSCs. Similar to our results, previous reports have pointed out that Pin1 inhibition can improve the condition of periodontitis by inhibiting osteoclast differentiation and inflammation [53].

Functional investigations of JunB extended our knowledge of the mechanism of impaired odontogenic differentiation of DPSCs under inflammatory state in vitro. JunB may function as a positive regulator of odontogenic differentiation of DPSCs and the decline of JunB in inflammatory state can inhibit odontogenic differentiation of DPSCs by targeting Pin1. Our results indicated that JunB may be a potential therapeutic target for inflammation and regeneration in pulpitis. However, further in vivo experiments are warranted to confirm the role of JunB and the mechanism involved.

Conclusion

In summary, our data demonstrated that the decreased expression level of the JunB in an inflammatory state could impair the odontogenic differentiation ability of the DPSCs by upregulating the expression level of the PIN1, a negative regulator of the odontogenic differentiation of the DPSCs. These findings not only contribute to a more comprehensive understanding of the mechanism of stem cells differentiation in an inflammatory microenvironment but also provide a new thought for the dentin-pulp regeneration and treatment of pulpitis.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

SJ and XF conceived and designed the experiments. LL, DJ, YG, RJ, ZX, and QZ, LB, YF performed the experiments. LL and XF analyzed the data. LL and CJ wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Figure

Supplementary Figure is not available with this version.

Figures

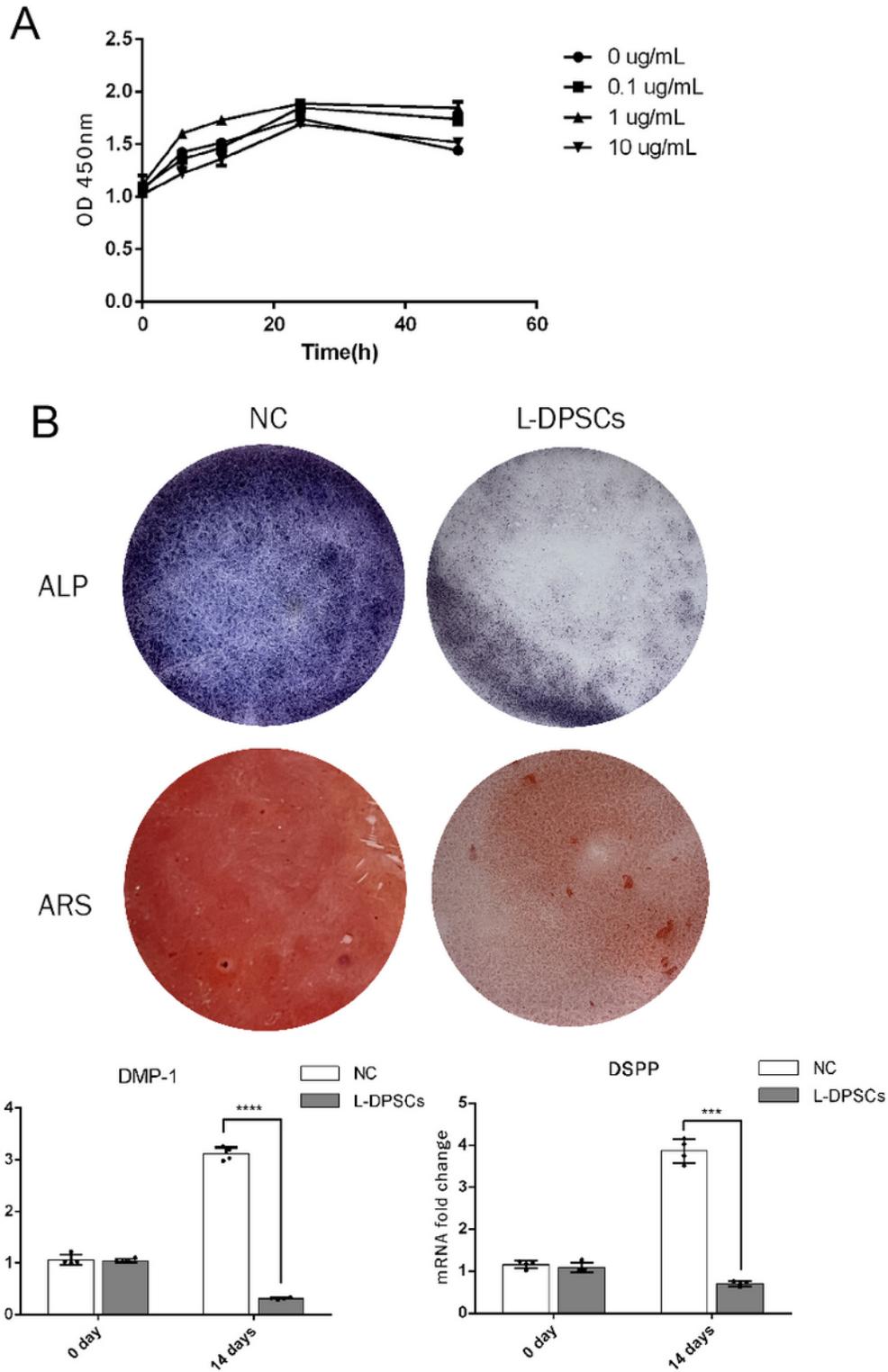


Figure 1

The LPS treatment damaged the odontogenic differentiation ability of the DPSCs. A. The DPSCs were stimulated with 0, 0.1, 1, and 10 $\mu\text{g}/\text{mL}$ LPS for 6, 12, 24, and 48 h, and the cell viability was measured by CCK-8 assay. B. The ALP and Alizarin red staining of the DPSCs with (L-DPSCs) or without (NC) LPS-

stimulation (1 $\mu\text{g}/\text{mL}$) after the odontogenic induction. C. Relative expression of the DSPP and DMP-1 in the NC and L-DPSCs group. **** $P < 0.0001$; *** $P < 0.001$.

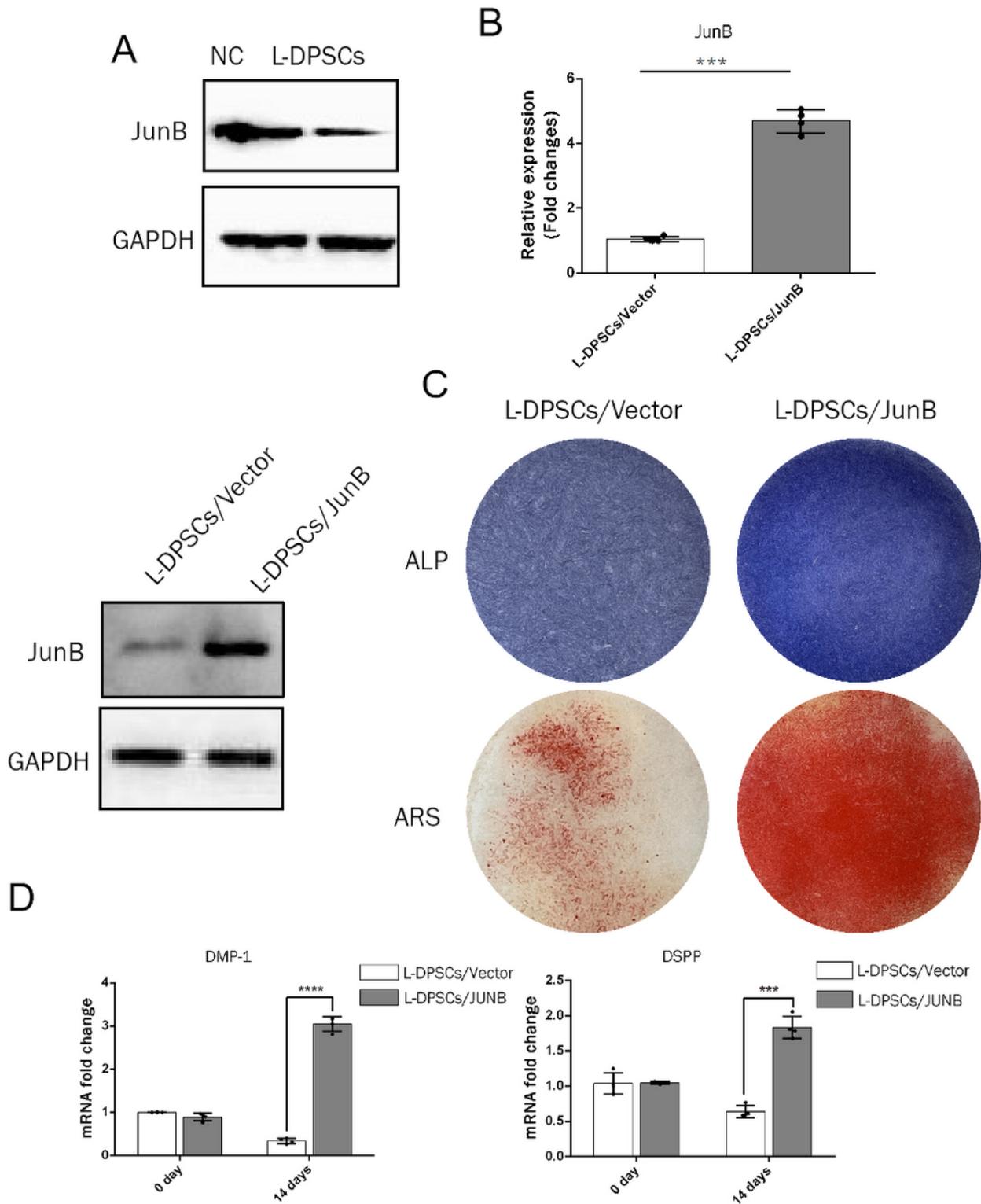


Figure 2

Overexpression of the JunB in the LPS-treated DPSCs promoted odontogenic differentiation. A. The DPSCs treated with 1 $\mu\text{g}/\text{mL}$ LPS (L-DPSCs) exhibited a decreased protein expression level of the JunB. B. The transfection efficiency of the JunB overexpression in the L-DPSCs was testified by RT-PCR and

Western blot assay. C. The ARS and ALP staining after overexpression of the JunB. D. The relative mRNA expression levels of the DMP-1 and DSPP were increased significantly after the JunB overexpression in the L-DPSCs. ****P<0.001; ***P<0.001.

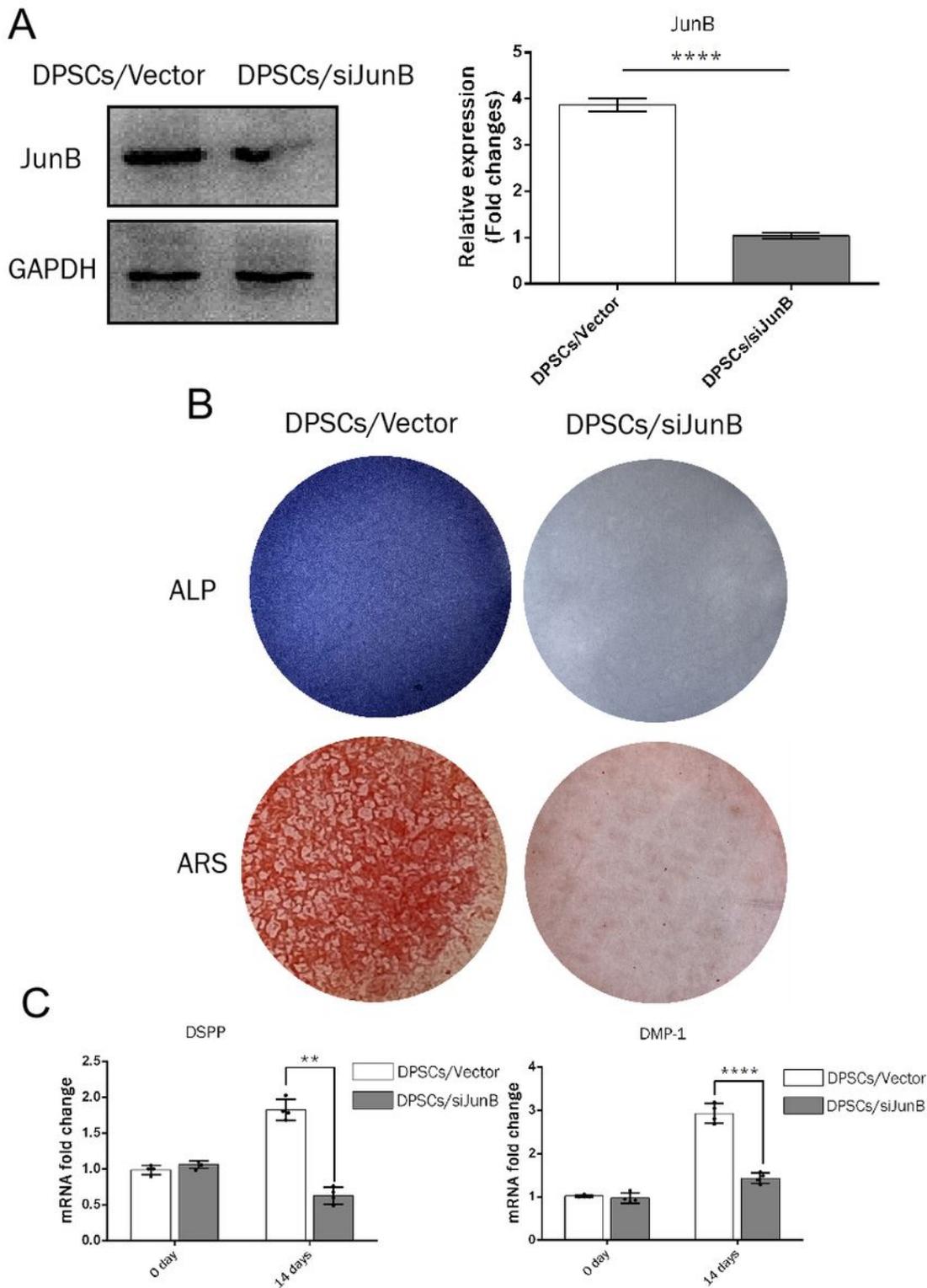


Figure 3

Silencing the JunB expression inhibited odontogenic differentiation in the DPSCs. A. The transfection efficiency of the JunB siRNA in the DPSCs was testified by RT-PCR and Western blot. B. The ALP staining

and ARS staining of the DPSCs after knockdown of the JunB expression. C. The relative mRNA expression of DMP-1 and DSPP in DPSCs decreased significantly after silencing the JunB. ** $P < 0.01$; **** $P < 0.0001$.

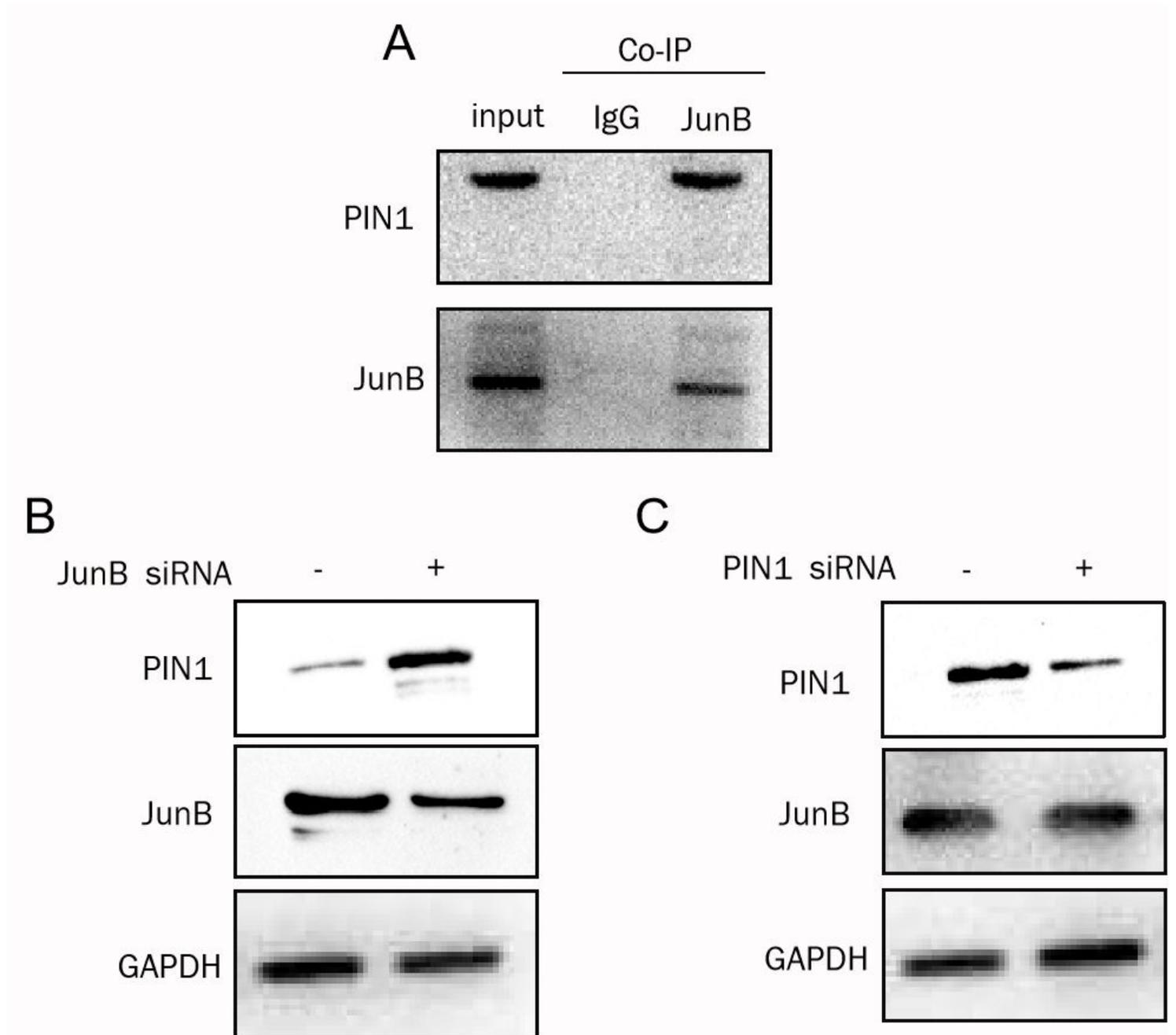


Figure 4

The PIN1 was identified as the downstream target of the JunB. A. The co-IP of the JunB and PIN1. B. Changes of the JunB and PIN1 protein levels in the LPS-treated DPSCs after the JunB siRNA transfection. C. Changes of the JunB and PIN1 protein levels in the LPS-treated DPSCs after the PIN1 siRNA transfection.

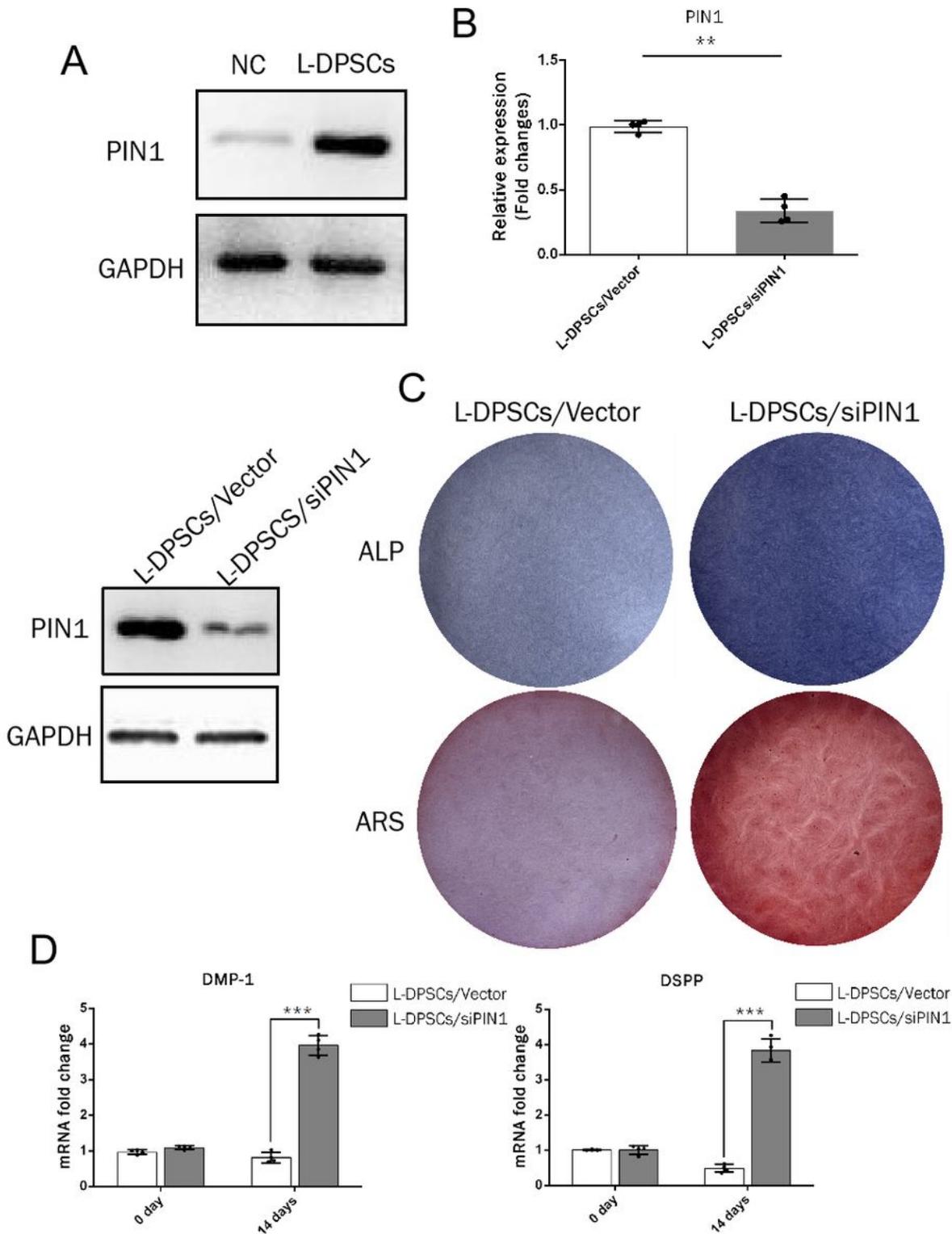


Figure 5

Silencing PIN1 promoted odontogenic differentiation in the LPS-treated DPSCs. A. Treatment with 1 $\mu\text{g}/\text{mL}$ LPS (L-DPSCs) up-regulated the protein expression of the PIN1 in the DPSCs. B. The transfection efficiency of the PIN1 siRNA in the L-DPSCs was testified by RT-PCR and Western blot assay. C. The ALP staining and ARS staining of the L-DPSCs after silencing PIN1. D. Relative mRNA expression of DMP-1 and DSPP after silencing the PIN1. *** $P < 0.001$.

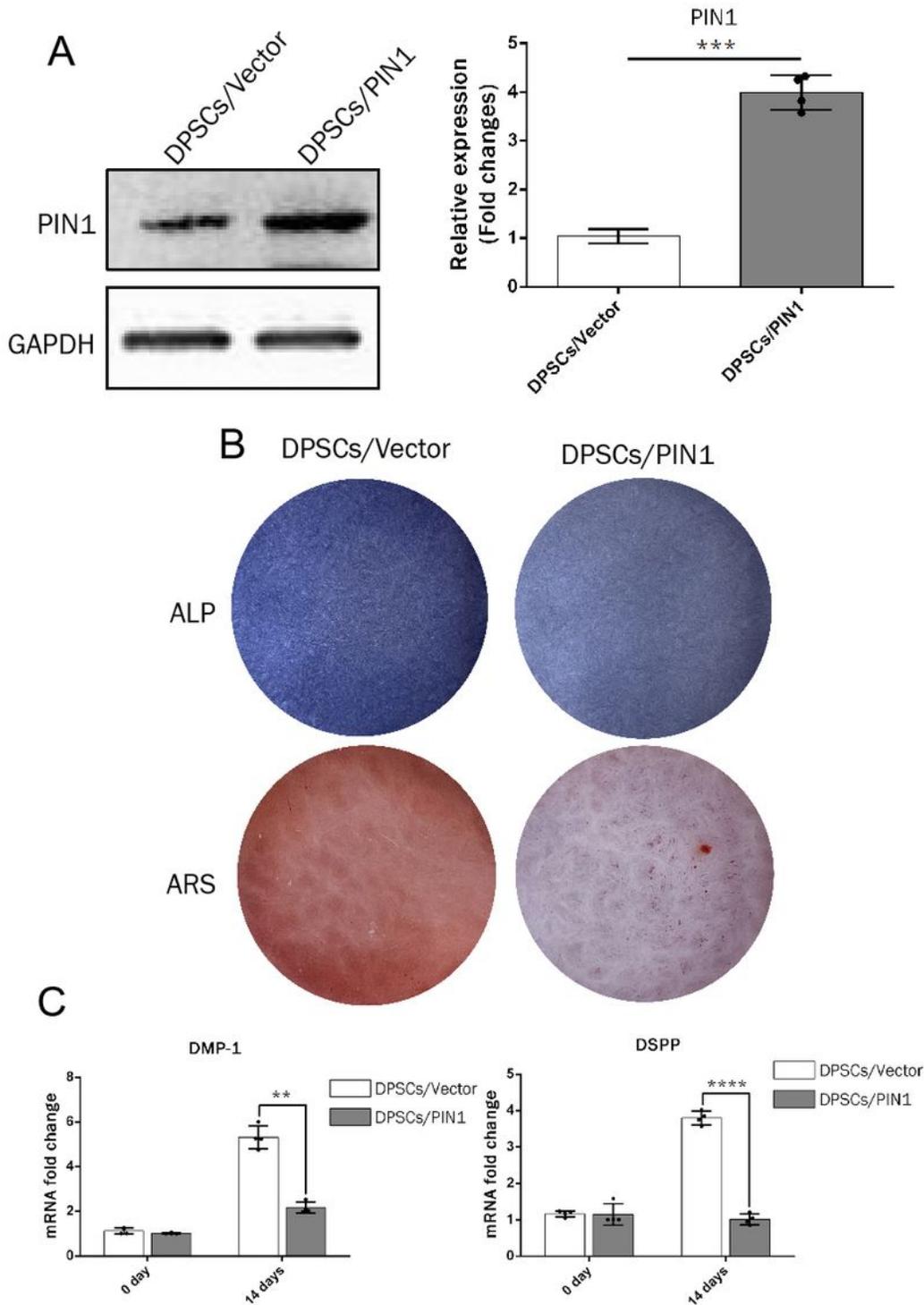


Figure 6

Overexpression of the PIN1 inhibited odontogenic differentiation in the DPSCs. A. The transfection efficiency of the PIN1 overexpression in the DPSCs was testified by RT-PCR and Western blot. B. The ARS and ALP staining of the DPSCs after overexpression of the PIN1. C. Relative mRNA expression of DSPP and DMP-1 in the DPSCs after overexpression of the PIN1. ** $P < 0.01$; **** $P < 0.0001$.

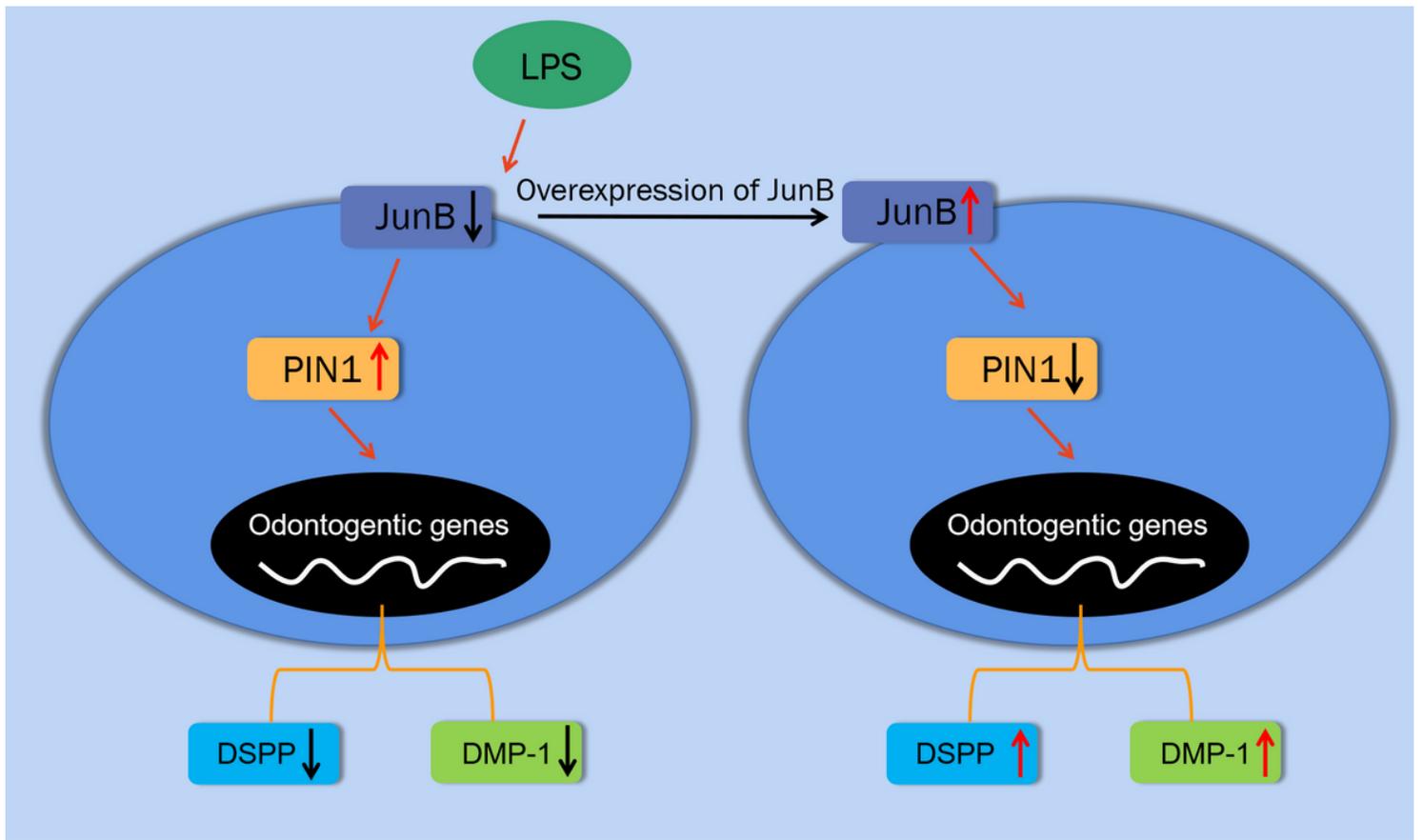


Figure 7

A schematic illustration of the function of JunB and PIN1 in the odontogenic differentiation of LPS-treated DPSCs.