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Original Article

ANX A1 PROMOTES THE ODONTOGENIC DIFFERENTIATION OF DENTAL PULP STEM CELLS

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Abstract

Background: Annexin A1 (*ANX A1*) plays a vital role in cell proliferation and differentiation, but its function in dental pulp stem cell (DPSC) odontogenic differentiation remains unclear. This study aims to investigate whether *ANX A1* can boost the odontogenic differentiation of DPSCs and determine the molecular mechanisms involved. **Methods**: Rat DPSCs were cultured *in vitro*, and a stable knockdown cells for *ANX A1* (Sh-*ANX A1*) and *ANX A1* overexpression (Ad-*ANX A1*) cell line was established using short hairpin RNA (shRNA) and overexpression. Cell proliferation, migration, and alkaline phosphatase activity were detected using Cell Counting Kit-8 (CCK-8), cell migration assay, and alkaline phosphatase activity assay. Alizarin red S staining, reverse-transcription quantitative polymerase chain reaction (RT-qPCR), Western blot, and immunofluorescence cell staining were used to assess the capacity for mineral synthesis, expression of odontogenic-related genes, and phosphorylation of P38 MAPK and extracellular signal-regulated kinase 1/2 (ERK1/2). **Results**: Third-generation DPSCs have the potential for adipogenic and osteogenic differentiation. The proliferation level, migration capacity, alkaline phosphatase activity, mineral synthesis capacity and expression of DPSC odontogenic-related genes were significantly suppressed by *ANX A1* knockdown and boosted by *ANX A1* overexpression ($p < 0.05$). Moreover, the phosphorylation of ERK1/2 and osteopontin, runt-related transcription factor 2, and alkaline phosphatase during odontogenic differentiation were significantly promoted by *ANX A1* overexpression and inhibited by *ANX A1* knockdown ($p < 0.05$). **Conclusions**: *ANX A1* promotes the odontogenic differentiation of DPSCs by activating the ERK/mitogen-activated protein kinase (MAPK) signaling pathway. The results offer new insights for dental regenerative medicine.

Keywords: Annexin A1, DPSCs, odontogenic differentiation, extracellular signal-regulated kinase 1/2.

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Introduction

Dental pulp stem cells (DPSCs) are adult stem cells isolated from dental pulp tissue and have self-renewal and multipotent differentiation capabilities [1]. DPSCs are abundant, easy to obtain, have good tissue compatibility, and are easy to preserve. These advantages make them superior to other adult stem cells in periodontal tissue regeneration and functional restoration [2,3]. Calcium deposits and elevated levels of mineralization and alkaline phosphatase (*ALP*) activity have been found in DPSCs [4]. The odontogenic differentiation of DPSCs is a complex process regulated by transcription factors and modulators, such as runt-related transcription factor 2 (*RUNX2*) [4] and glycogen synthase kinase (GSK) 3β [5]. However, the molecular mechanisms that control the odontogenic differentiation of DPSCs are not well understood.

Annexin A1 (*ANX A1*), a 37 kDa protein that binds phospholipids and belongs to the annexin superfamily, plays an important in cell differentiation, apoptosis, and proliferation [6]. *ANX A1* serves as a substrate for the receptor tyrosine kinase associated with epidermal growth factor and is involved in the glucocorticoid signaling pathway of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), thereby contributing significantly to the signaling functions that govern cell proliferation and differentiation [7]. It also plays an important role in various types of diseases and protects periodontal membrane cells from inflammatory responses and cellular senescence [8]. Wei *et al.* [9] found knockdown of annexin-A1 inhibits growth, migration and invasion of glioma cells. Yang *et al.* [10] demonstrated that overexpression of *ANXA1* significantly enhances the migration of PCa cells *in vitro*. In addition, *ANX A1* inhibits the dif-

ferentiation of osteoclasts through suppressing nuclear factor κB (NF-κB) signaling and promoting the peroxisome proliferator-activated receptor gamma (PPAR-γ) pathway [11]. Mechanical stress can target annexins A1 to promote bone cell differentiation [12]. Nevertheless, the effect of *ANX A1* on the odontogenic differentiation of DPSCs remains unknown.

ERK is the most extensively studied member of the MAPK family, and the ERK pathway is a hallmark within the MAPK pathways involved in the regulation of cell differentiation [13]. PD98059 functions as an inhibitor of MAPK kinase (MEK), effectively blocking the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) induced by the extracellular matrix and the osteogenic differentiation of human mesenchymal stromal cells (MSCs) [14]. *ANX A1* regulates tumor necrosis factor (TNF)-induced lung fibroblast proliferation and inflammatory responses through the ERK/MAPK pathway [15]. Therefore, the irregular activation of the ERK pathway may influence the odontogenic DPSCs by modifying the expression levels of *ANX A1*.

Whether *ANX A1* regulates the odontogenic differentiation of DPSCs remains unknown. Therefore, this study investigated the effects and mechanisms of *ANX A1* knockdown and overexpression on the odontogenic differentiation of DPSCs.

Materials and Methods

Isolation of DPSCs

Cervical dislocation: cervical dislocation is performed manually and euthanasia is achieved in approximately 10–15 seconds. Under sterile conditions, five Sprague-Dawley rats were euthanized, and their teeth were extracted from their mandibles. The teeth were soaked for 30 min in Dulbecco's modified Eagle's medium (DMEM) containing 1 $\times$ 10^4 U/L penicillin and 50 mg/L streptomycin, followed by rinsing with phosphate-buffered saline (PBS). The teeth were split longitudinally with a bone chisel, and the apical third of the pulp tissue was collected, rinsed repeatedly with PBS, and digested in a solution of 3 mg/mL collagenase type I (C0130-1g, Sigma-Aldrich, Shanghai, China) for 40 min at 37 °C in a humidified environment with 5 % CO_2. Following digestion, the resulting cells were cultured in DMEM enriched with 10 % fetal bovine serum (FBS) at 37 °C in the presence of 5 % CO_2. No mycoplasma contamination was found during mycoplasma testing.

Multipotential Differentiation

Third-generation DPSCs were plated into a six-well dish and cultured in two different media: one with adipogenic induction liquid (PD-010, Wuhan Pricella Biotechnology Co., Ltd., Wuhan, China) and the other with osteogenic medium for differentiation induction. On day 14, oil red O and alizarin red staining were carried out. The cells were regularly examined and imaged using a phase-contrast inverted microscope (DM6000 M, Leica, Wetzlar, Germany).

Immunofluorescence Cell Staining

Third-generation DPSCs were cultivated in a 24-well dish under standard conditions. Once the cells reached approximately 80 % confluence, the culture medium was removed and the cells were washed before being fixed at room temperature using 4 % paraformaldehyde (YB36314ES60, Sigma-Aldrich, Shanghai, China). The primary antibodies used on the cells were as follows: anti-STRO-1 (1:400; ab214086, Abcam, Cambridge, UK), anti-vimentin (1:400; ab92547, Abcam, Cambridge, UK), anti-nestin (1:400; ab105389, Abcam, Cambridge, UK), anti-C-Kit (1:300; ab32363, Abcam, Cambridge, UK), and anti-CK14 (1:400; ab119695, Abcam, Cambridge, UK). The nuclei were stained with 4,6-dianmidino-2-phenylindole (DAPI) after washing (C1005, Beyotime, Shanghai, China). The cells were examined using a confocal microscope (FV3000, OLYMPUS, Tokyo, Japan), and the mean fluorescence intensity was determined with ZEN 2.4 software (Zeiss, Oberkochen, Germany).

Flow Cytometry

Third-generation DPSCs in good growth condition were gently washed twice with assay buffer (R1018, Solarbio, Beijing, China) and then digested with 0.25 % trypsin without ethylenediaminetetraacetic acid (EDTA). The digestion was terminated by adding α-MEM containing 10 % FBS. The cells were collected and counted. Each flow cytometry tube was loaded with 5 $\times$ 10^5cells resuspended in 100 μL of assay buffer and then added with 5 μL of directly conjugated antibodies as follows: CD3 (1:200, ab16669, Abcam, Cambridge, UK), CD73 (1:200, ab257311, Abcam, Cambridge, UK), CD34 (1:200, ab81289, Abcam, Cambridge, UK), CD90 (1:200, ab225, Abcam, Cambridge, UK), and CD31 (1:200, ab7388, Abcam, Cambridge, UK). The mixtures were incubated at 4 °C in darkness for 30 min. Afterward, 1 mL of assay buffer was added, and the cells were washed twice and resuspended to a final volume of 500 μL with assay buffer. Flow cytometry (NovoCyte 3000RYB, Agilent, Santa Clara, CA, USA) was then used to analyze the surface markers of the cells.

Lentiviral Transduction

Lentiviral particles obtained from Sigma-Aldrich (TRCN109725, Shanghai, China) were utilized to create stable knockdown cells for *ANX A1* (Sh-*ANX A1*) and cells with *ANX A1* overexpression (Ad-*ANX A1*). As a negative control, cells were transduced with lentiviral particles carrying scrambled sequences (Ad-NC or Sh-NC). Once the cells reached 75 % confluence, normal cells were used as controls, and the transduced cells were selected using 1 μg/mL puromycin.

Table 1. Sequence of RT-qPCR primers.

Primer name	Forward (5′–3′)	Reverse (5′–3′)
ALP	CGGATCCTGACCAAAAACC	TCATGATGTCCGTGGTCAAT
DSPP	ATGACAGTGATAGCACATCAGA	ATTGTTACCATTGCCATTACT
DMP-1	CAGTGAGGAAGATGGCCA	CTTGGCAGTCATTGTCATCTT
RUNX2	GAACCAAGAAGGCACAGAC	AATGCGCCCTAAATCACTG
OPN	CTACAGGGAGAACACCCGGA	ACACTAGAAGACAGCGGGTCC
GAPDH	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTT

RT-qPCR, reverse-transcription quantitative polymerase chain reaction; *ALP*, alkaline phosphatase; *RUNX2*, runt-related transcription factor 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *DSPP*, dentin sialo-phosphoprotein; *DMP-1*, dentin matrix acidic phosphoprotein 1; *OPN*, osteopontin.

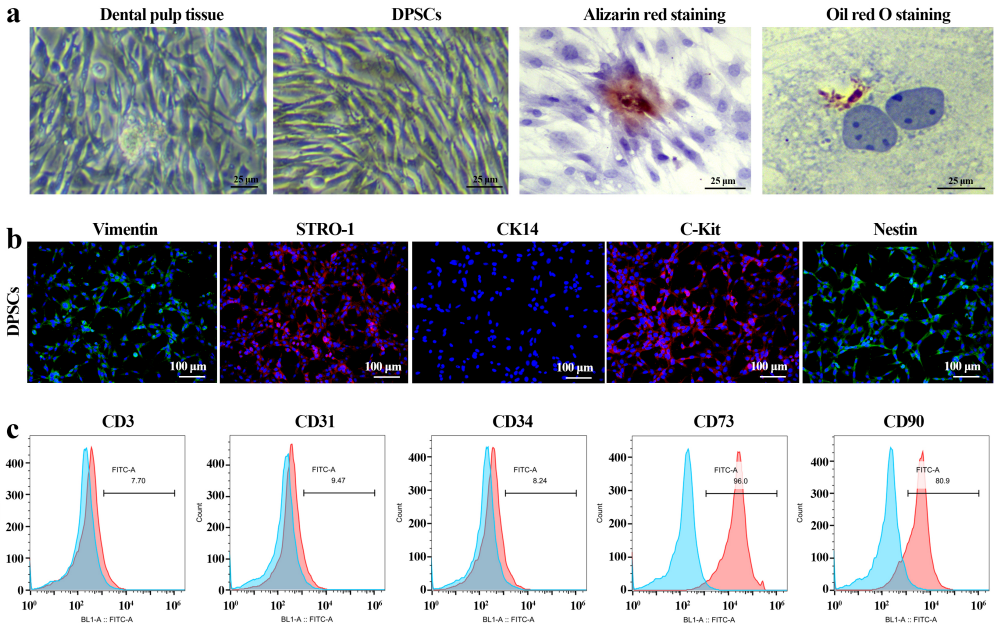

Fig. 1. Characterization of stem cells from the dental pulp. (**a**) The cells of the P3 generation were spindle shaped. Osteogenic or adipogenic medium was used to culture DPSCs. Scale bar = 25 μm. (**b**) DPSC were negative for CK14 and positive for nestin, STRO-1, vimentin, and C-Kit. Scale bar = 100 μm. (**c**) Surface antigens CD3, CD31, CD34, CD73, and CD90 were detected by flow cytometry. DPSC, dental pulp stem cell.

Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

DPSCs from Sprague-Dawley rats, induced to differentiate for 14 days, were collected. Total RNA was isolated using the Trizol method and assessed for quality. Complementary DNA (cDNA) was synthesized through reverse transcription using the Titanium One-Step RT-PCR Kit. RT-qPCR was performed using the SYBR Premix ExTaq^{TM} II kit and primers. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. The primer sequences are shown in Table 1. The messenger RNA (mRNA) expression levels of each gene were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

DPSCs from Sprague-Dawley rats, induced to differentiate for 14 days, were collected. The cells from each group were lysed at 4 °C using whole cell protein extraction reagent. Protein concentration was measured using a Coomassie brilliant blue protein assay kit (ZY1063, ZYbscience, Shanghai, China). Proteins were boiled for denaturation, and 35 μg of each sample was electrophoresed on a 10 % Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel. The proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (1620177, Biorad, Hercules, CA, USA), which was then blocked with 3 % bovine serum albumin for 1.5 hour at room temperature. The following primary antibodies were used for incubation overnight at 4 °C: anti-dentin sialophosphoprotein (*DSPP*) (1:500, ab272929, Abcam, Cambridge, UK), dentin matrix acidic phosphoprotein 1 (*DMP-*

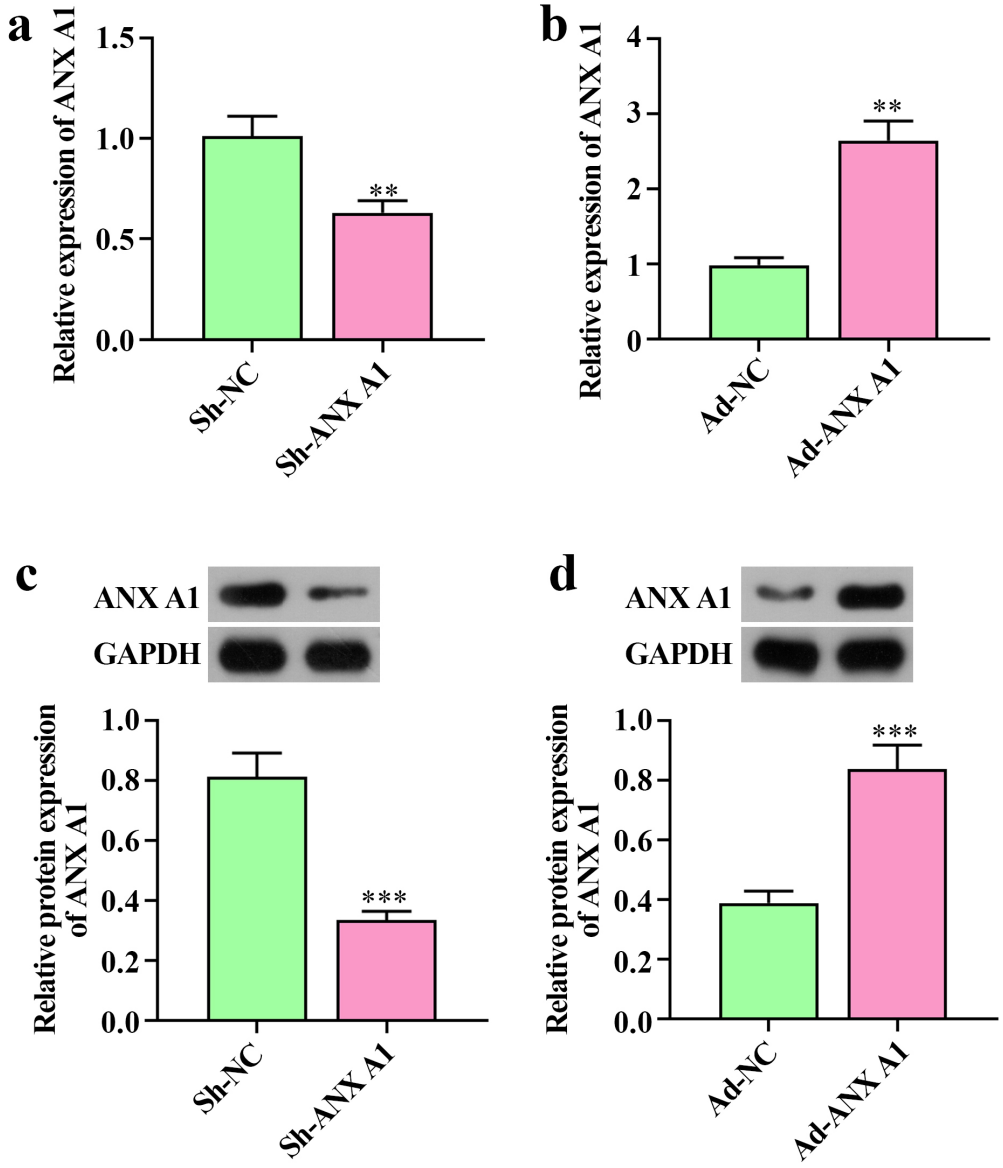

Fig. 2. Expression of *ANX A1* knockdown and overexpression in DPSCs. (**a,b**) RT-qPCR verified the effect of *ANX A1* knockdown or overexpression on the relative expression of *ANX A1* in DPSCs. (**c,d**) Western blot detected the effect of *ANX A1* knockdown or overexpression on the protein expression of *ANX A1* in DPSCs. Data are expressed as the mean ± SD from three independent experiments performed in triplicate. $^{**}p < 0.01$, $^{***}p < 0.001$. *ANX A1*, annexin A1; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SD, standard deviation.

1) (1:1000, ab177246, Abcam, Cambridge, UK), *RUNX2* (1:500, ab76956, Abcam, Cambridge, UK), osteopontin (*OPN*) (1:500, ab216406, Abcam, Cambridge, UK), *ALP* (1:500, ab229126, Abcam, Cambridge, UK) monoclonal antibodies, and rabbit anti-*GAPDH* polyclonal antibody (1:5000, ab8245, Abcam, Cambridge, UK). The following day, the samples were incubated with secondary antibody immunoglobulin G (IgG) (1:2000) for 1.5 hours at room temperature. ECL chemiluminescence reagent kit (HYK2006, MCE, Monmouth Junction, NJ, USA) mixture of solutions A and B was applied to the membrane after washing, and the signals were developed in a gel imaging system. The band intensities were measured using Quantity One software (version 4, Biorad, Hercules, CA, USA). The ratio of the target band intensity to that of the internal control *GAPDH* was used for statistical analysis to semi-quantitatively compare the differences in protein expression levels between groups.

Cell Counting Kit-8 (CCK-8) Assay

DPSCs from Sprague-Dawley rats, induced to differentiate for 14 days, were seeded in disposable 96-well culture plates at a density of 5×10^3 cells per well, with 100 μL of cell suspension per well. After 12 hours of incuba-

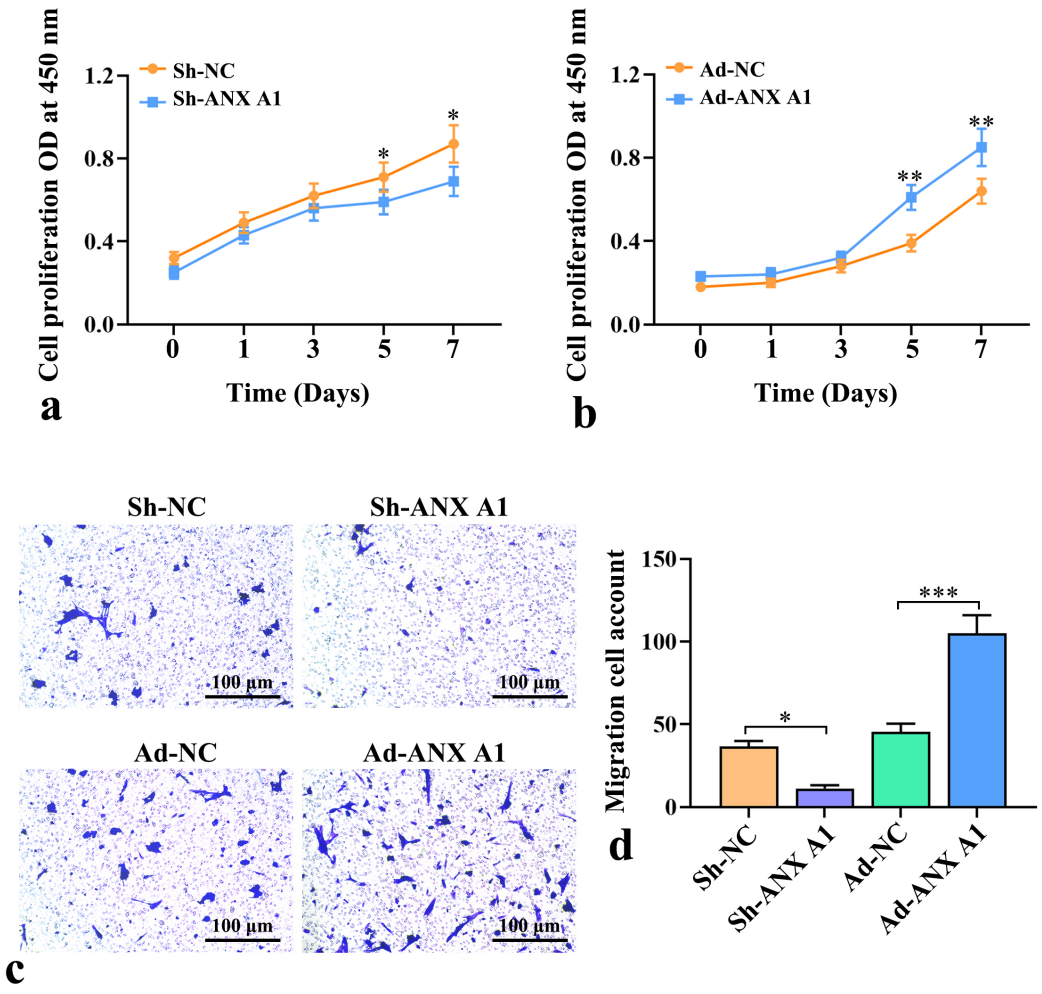

Fig. 3. Effect of *ANX A1* knockdown and overexpression on the proliferation and migration of DPSCs during odontogenic differentiation. (**a,b**) CCK-8 assay detected the proliferation levels of DPSCs. (**c,d**) Transwell analysis evaluated the changes in the migration ability of DPSCs. Scale bar = 100 μm. Data are expressed as the mean ± SD from three independent experiments performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. CCK-8, Cell Counting Kit-8; OD, optical density.

tion, 10 μL of CCK-8 working solution was incorporated to each well and mixed thoroughly, followed by incubation at 37 °C for 2.5 hours. The absorbance of each well was determined at 450 nm using a microplate reader.

Cell Migration Assay

The upper chambers of the Transwell plates were seeded with third-generation DPSCs. The lower chambers were filled with one of the following treatments: Sh-NC, Sh-*ANX A1*, Ad-NC, Ad-*ANX A1*. The cells were cultured at 37 °C for 24 hours and then rinsed with PBS. After fixation with 4 % paraformaldehyde and staining with 0.1 % violet crystals, the DPSCs in each filter were counted in six fields of view under a high magnification microscope (× 200). ImageJ (National Institutes of Health, Bethesda, MD, USA) and GraphPad software (Dotmatics, San Diego, CA, USA) were used to process and analyze the data.

Alkaline Phosphatase Activity Assay

The alkaline phosphatase activity of each group of cells was detected using an alkaline phosphatase activity assay kit. DPSCs from Sprague-Dawley rats, induced to differentiate for 14 days, were lysed with radio immunoprecipitation assay (RIPA) lysis buffer at 4 °C for 20 min. The lysates were spun at 1200 rpm for 12 min at 4 °C, and the supernatant was collected. Buffer working solution and chromogenic solution were added sequentially, and the mixture was incubated at 37 °C for 10 min. The absorbance at 520 nm was determined using a microplate reader. High absorbance values indicate high alkaline phosphatase activity.

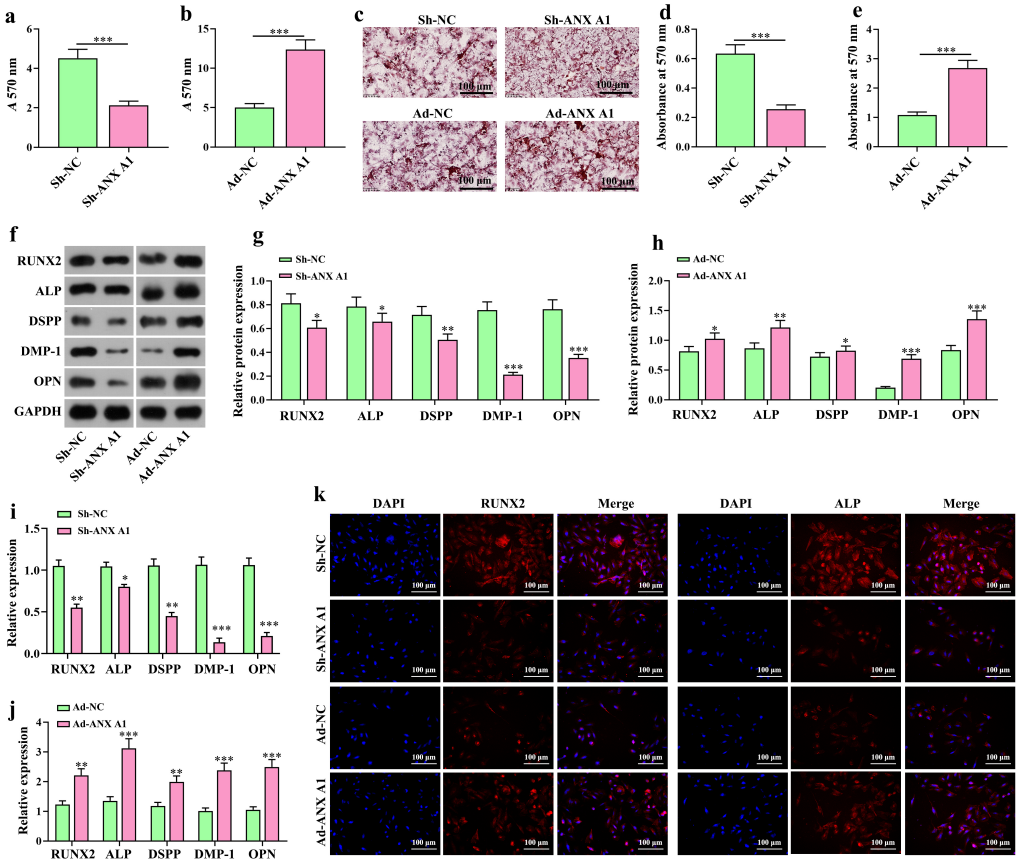

Fig. 4. Effect of *ANX A1* knockdown and overexpression on the odontogenic differentiation of DPSCs. (**a,b**) Comparison of the alkaline phosphatase activity of DPSCs from each group. (**c–e**) Alizarin red S staining analyzed the number of calcium nodules and quantification of matrix mineralization in DPSCs (× 100). Scale bar = 100 μm. (**f–h**) Expression of genes related to dentin differentiation assessed by Western blot. (**i–k**) Immunofluorescence detected the expression of dentin gene proteins. Scale bar = 100 μm. Data are expressed as the mean ± SD from three independent experiments performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *OPN*, osteopontin; *RUNX2*, runt-related transcription factor 2; *ALP*, alkaline phosphatase; *DSPP*, dentin sialo-phosphoprotein; *DMP-1*, dentin matrix acidic phosphoprotein 1; DAPI, 4,6-dianmidino-2-phenylindole.

Alizarin Red S Staining

DPSCs from Sprague-Dawley rats, induced to differentiate for 14 days, were seeded in disposable six-well culture plates with coverslips placed at the bottom. After the cells had grown to cover the coverslips, the coverslips were removed and fixed with pre-cooled acetone for 15 min. The coverslips were rinsed three times with PBS, stained with alizarin red S staining solution for 2 min, dehydrated, made transparent, sealed, and then observed under a light microscope.

Statistical Analysis

Each experiment was repeated three times, and the results were expressed as mean ± standard deviation (SD). Paired *t*-test analysis of variance was used to analyze differences between groups. For all analyses, $p < 0.05$ was considered significant. GraphPad Prism 8 (Dotmatics, San Diego, CA, USA) statistical software was used to analyze relevant data.

Results

Culture and Identification of DPSCs

After approximately 3 days of dental pulp tissue culture, cells began to proliferate from the tissue blocks, displaying a fibroblast-like appearance typical of primary DPSCs. After 7 days, the DPSCs fully spread in the culture flask and maintained their mesenchymal morphology during the passaging. After 14 days in osteogenic/adipogenic medium, mineralized nodules were observed in DPSCs after alizarin red staining and lipid droplets were detected with oil red O staining, suggesting that the DPSCs have the capability for osteogenic and lipogenic differentiation (Fig. 1**a**). In addition, the isolated DPSCs tested positive for nestin, c-Kit, STRO-1, vimentin, CD73 (99.4 %), and CD90 (95.3 %) but negative for CD34 (0.53 %), CD31 (0.43 %), epithelial marker T cell marker CD3 (0.41 %), and CK14 (Fig. 1**b,c**). In summary, these findings indicated that the isolated DPSCs are mesenchymal cells with multipotent differentiation capabilities.

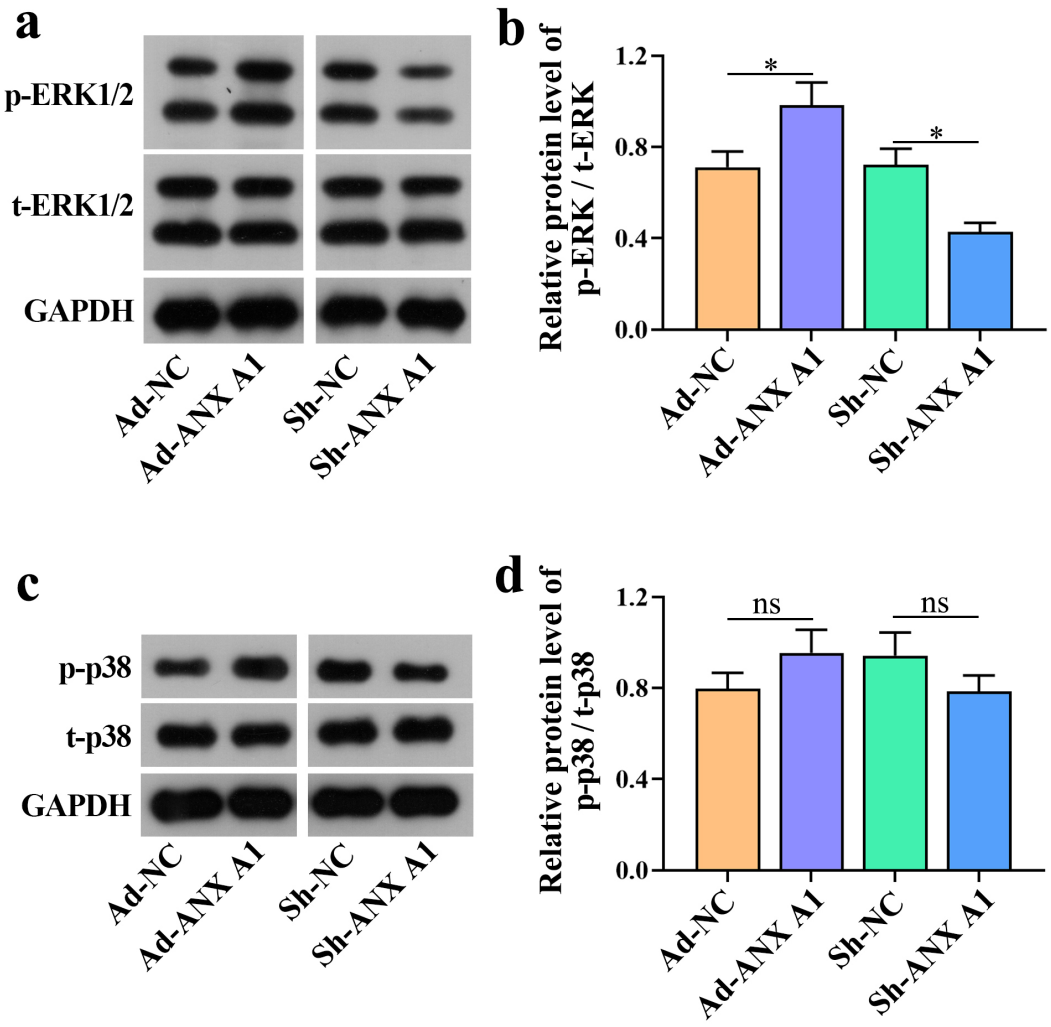

Fig. 5. *ANX A1* effect on ERK1/2 and p38 MAPK phosphorylation during odontogenic differentiation. (**a,b**) Expression levels of p-ERK and t-ERK1/2 proteins assessed by Western blot. (**c,d**) Expression levels of p-p38 and t-p38 proteins assessed by Western blot. Data are expressed as the mean ± SD from three independent experiments performed in triplicate. $^{*}p < 0.05$, ns, no significant difference. ERK1/2, extracellular signal-regulated kinase 1/2; MAPK, mitogen-activated protein kinase.

Expression of ANX A1 in DPSCs

To clarify the effect of *ANX A1* on DPSCs, we constructed *ANX A1* knockdown and overexpression vectors and transfected them into DPSCs cells. Sh-NC and Ad-NC served as control groups, providing strong support for validating the transfection efficiency and expression of *ANX A1* overexpression and knockdown. RT-qPCR analysis showed that the mRNA expression in the Sh-*ANX A1* group was significantly reduced compared with that in the Sh-NC group. Meanwhile, the mRNA expression in the Ad-*ANX A1* group was significantly higher than that in the Ad-NC group (Fig. 2**a,b**). Western blot analysis showed that the protein expression was markedly reduced in the Sh-*ANX A1* group compared with that in the Sh-NC group. Meanwhile, the protein expression in the Ad-*ANX A1* group was significantly higher than that in the Ad-NC group (Fig. 2**c,d**).

Effect of ANX A1 on the Proliferation and Migration of DPSCs

To further explore the effect of *ANX A1* on the proliferation and migration of DPSCs during odontogenic differentiation, we used a CCK-8 assay to detect the proliferation levels of DPSCs after *ANX A1* knockdown or overexpression. Compared with those in the Sh-NC group, the cell proliferation rate decreased and the proliferation speed significantly slowed down in the Sh-*ANX A1* group. Compared with those in the Ad-NC group, the cell proliferation rate increased and the proliferation speed significantly accelerated in the Ad-*ANX A1* group (Fig. 3**a,b**). The migration ability of DPSCs after *ANX A1* knockdown or overexpression were detected by transwell assay. The results showed that the cell migration rate was increased by *ANX A1* overexpression but reduced by *ANX A1* knockdown (Fig. 3**c,d**).

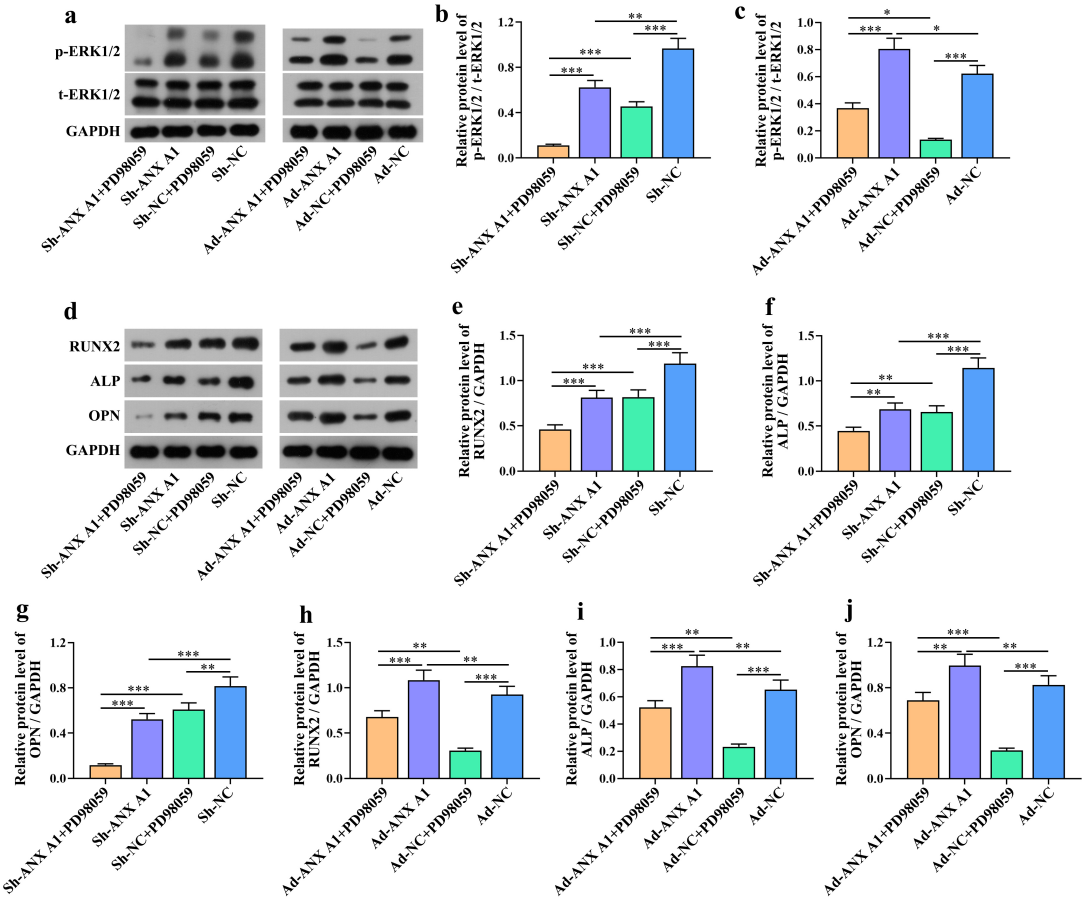

Fig. 6. Effect of PD98059 on the odontogenic differentiation ability of DPSCs. (**a–c**) Treatment of DPSCs with PD98059 markedly suppressed the phosphorylation of ERK1/2. (**d–j**) Western blot analysis of the protein expression of odontogenic differentiation-related genes *RUNX2*, *ALP*, and osteopontin (*OPN*) after treatment with PD98059. Data are expressed as the mean ± SD from three independent experiments performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Effect of ANX A1 on the Odontogenic Differentiation of DPSCs

To analyze the effect of *ANX A1* knockdown and overexpression on odontogenic differentiation of DPSCs, we compared the alkaline phosphatase activity of DPSCs in each group. The results showed that the alkaline phosphatase activity was significantly inhibited by *ANX A1* knockdown but significantly promoted by *ANX A1* overexpression (Fig. 4**a,b**). Weak alizarin red S staining and reduced number of calcium nodules were found in the sh-*ANX A1* group, and the opposite was observed in the Ad-*ANX A1* group (Fig. 4**c–e**). Western blot results showed *ANX A1* knockdown markedly suppressed the expression of *RUNX2*, *ALP*, *DSPP*, *DMP-1*, and *OPN* genes and proteins, and its overexpression significantly promoted their expression (Fig. 4**f–h**). These results are in agreement with those for immunofluorescence (Fig. 4**i–k**).

ANX A1 Enhanced the Phosphorylation of ERK1/2 during Odontogenic Differentiation

To determine whether *ANX A1* promotes the odontogenic differentiation of DPSCs by activating ERK MAPK and p38 MAPK, we analyzed the phosphorylation status of ERK and p38 during the odontogenic differentiation of DPSCs. Compared with that in the Sh-NC group, the phosphorylation of ERK1/2 was decreased in the Sh-*ANX A1* group. Meanwhile, the phosphorylation of ERK1/2 was higher in the Ad-*ANX A1* group than in the Ad-NC group (Fig. 5**a,b**). The phosphorylation status of p38 was not different between *ANX A1* overexpression and knockdown (Fig. 5**c,d**). Therefore, we conducted further research on ERK1/2.

ANX A1 Promoted the Odontogenic Differentiation of DPSCs by Activating ERK1/2

ANX A1 can regulate the ERK signaling pathway at proximal sites [16], and Sh-*ANX A1* can inhibit ERK1/2 activity [17]. In this study, the MEK/ERK inhibitor PD98059 was used to simulate the inhibitory or promotive effects of Sh-*ANX A1* and overexpression on ERK1/2 activity. A

marked suppression of ERK1/2 activity was observed when the Sh-NC cells were treated with PD98059. Furthermore, Sh-*ANX A1* cells and Ad-*ANX A1* cells exposed to PD98059 exhibited a pronounced decrease in ERK1/2 activity (Fig. 6**a–c**). PD98059 was also evaluated for its effect on the expression of odontogenic differentiation genes. In agreement with the data derived from the ERK1/2 activity, the protein expression levels of *RUNX2*, *OPN* and *ALP* were significantly reduced in the PD98059-treated Sh-*ANX A1* group compared with those in the Sh-*ANX A1* without treatment group. (Fig. 6**d–j**). Furthermore, the treatment of 20 μm PD98059 to Ad-*ANX A1* cells resulted in a marked reduction in the expression levels of proteins *RUNX2*, *OPN*, and *ALP* compared with those in Ad-*ANX A1* cells without treatment (Fig. 6**d–j**). The above results proved that the activation of ERK1/2 is essential for odontogenic differentiation, and PD98059 attenuates the promotive effect of *ANX A1* overexpression on odontogenic differentiation.

Discussion

Annexins are a family of proteins with the common property of binding calcium and phospholipids and have been implicated in cellular processes, such as cell growth [7]. Accumulating evidence indicates that *ANX A1* has a role in cell differentiation [6,18]. This study aimed to explore the effects and mechanisms of *ANX A1* knockdown and overexpression on the odontogenic differentiation of DPSCs. Results showed that transfecting Ad-*ANX A1* in DPSCs resulted in increased cell migration, proliferation, and odontogenic differentiation ability. *ANX A1* overexpression increased the phosphorylation of ERK1/2 and expression of odontogenic-related genes during odontogenic differentiation.

Owing to the presence of nerves, blood vessels, and connective tissue in the pulp tissue, the conventional method for identifying DPSCs is to distinguish them from endothelial cells, fibroblasts, and hematopoietic stem cells according to their surface markers [19,20]. In this study, the DPSC surface markers CD90 and CD73 were positively expressed, and CD3, CD31, and CD34 were negatively expressed. This finding indicated that the extracted cells possess the biological characteristics of DPSCs and can be used for subsequent experimental investigations.

Up-regulated *ANX A1* promotes differentiation of mouse c2c12 myoblasts into myoblasts [21]. Pan *et al.* [22] found that Sh-*ANX A1* inhibits the osteogenic differentiation of rat BM-MSC. Although *ANX A1* expression is linked to the differentiation of several cell types [23,24], data suggesting that *ANX A1* is involved in the odontogenic differentiation of DPSCs are limited. To verify the role of *ANX A1* in odontogenic differentiation, we transfected DPSCs with *ANX A1* knockdown and overexpression constructs. The results showed that Ad-*ANX A1* significantly boosted the proliferation and migration of DPSCs and significantly increased the alkaline phosphatase activity, number of calcium nodules, and expression of odontogenic-related genes. The results for Sh-*ANX A1* were opposite, indicating that *ANX A1* promotes the odontogenic differentiation of DPSCs.

The odontogenic differentiation of DPSCs is a complex process involving many signaling pathways, such as the Wnt signaling pathways [25], NF-κB signaling pathways [26], and MAPK signaling pathways [27]. Despite studies on the role of the MAPK signaling pathway in DPSCs, whether *ANX A1* promotes the odontogenic differentiation of DPSCs by activating the MAPK signaling pathway remains unknown. The cell signaling pathways p38, ERK, and c-Jun N-terminal kinase (JNK) MAPKs play crucial roles in cell differentiation. The function of p38 MAPK in osteogenic differentiation remains ambiguous, and previous research results are controversial. Some studies showed that the activation of ERK1/2 (but not JNK or p38 MAPK) can promote osteogenic differentiation [22], and others indicated that p38 MAPK can promote osteogenic differentiation [28]. The ERK signaling pathway influences osteogenesis through various mechanisms throughout the differentiation [29]. *ANX A1* plays a specific regulatory role in the ERK signaling cascade at upstream locations. An elevation in *ANX A1* expression results in the persistent activation of ERK1/2 kinases [14]. A recent study found that silencing *ANX A1* enhances the sensitivity of cancer cells to low concentrations of arsenic trioxide by inhibiting the ERK MAPK activity [17]. The p38 MAPK signaling pathway plays an important role in transducing extracellular signaling pathways and can be activated in response to inflammatory stimuli and hormones, thereby regulating the differentiation of DPSCs [30]. Vandomme *et al.* [31] found that p38-MAPK and the downstream gene *STAT3* are involved in the differentiation of DPSCs into odontoblasts and mediate intracellular signaling in DPSCs. Bai *et al.* [32] demonstrated that transforming growth factor β1 (TGF-β1) promotes the early differentiation of DPSCs into odontoblasts by activating the p38 MAPK signaling pathway. The current study suggested that only the regulation of ERK1/2 phosphorylation by *ANX A1*, not the p38 pathway, could be involved in odontogenic differentiation. This result is consistent with previous research [22]. Based on the findings of the present study, we propose the hypothesis that the p38 pathway does not play a role in the odontogenic differentiation of DPSCs as influenced by *ANX A1*.

To further validate whether the ERK1/2 pathway is involved in the osteogenic differentiation of DPSCs, this study analyzed the effect of PD98059 on the odontogenic differentiation ability of DPSCs. The findings showed that the expression of odontogenic genes (*RUNX2*, *ALP*, and *OPN*) was reduced in DPSCs when ERK1/2 phosphorylation was blocked. This result is in line with the study of Wang *et al.* [33]. Although transfection with si-*ANX A1* led to decreased ERK1/2 phosphorylation and the overexpression of *ANX A1* led to increased ERK1/2 phosphoryla-

tion, whether PD98059 would attenuate the promotive effect of *ANX A1* overexpression on odontogenic differentiation and the inhibitory effect of Sh-*ANX A1* on odontogenic differentiation remains uncertain. Therefore, Ad-*ANX A1* cells were treated with PD98059. As expected, the reduction in odontogenic gene expression became pronounced in Ad-*ANX A1* cells after PD98059 intervention. These results further demonstrated that the odontogenic differentiation of DPSCs requires the activation of ERK1/2, and the promotive effect of *ANX A1* overexpression on the odontogenic differentiation of DPSCs involves ERK1/2 activation.

Conclusions

The results of this study regarding the effect of *ANX A1* on DPSCs will enhance its role in odontogenic differentiation and tooth regeneration. In particular, *ANX A1* has a positive effect on odontogenic differentiation. In DPSCs, *ANX A1* promotes odontogenic differentiation by enhancing proliferation levels, migration capacity, alkaline phosphatase activity, mineral synthesis capacity, and increasing the expression of odontogenic-related genes. Subsequently, we preliminarily explored the effect of *ANX A1* on the ERK/MAPK pathway, suggesting that *ANX A1* may influence the odontogenic differentiation of DPSCs through the ERK/MAPK pathway. This study offers new insights for dental regenerative medicine.

List of Abbreviations

DPSCs, dental pulp stem cells; *ANX A1*, annexin A1; CCK-8, Cell Counting Kit-8; RT-qPCR, reverse transcription quantitative polymerase chain reaction; ERK1/2, extracellular signal-regulated kinase 1/2; *OPN*, osteopontin; *RUNX2*, runt-related transcription factor 2; *ALP*, alkaline phosphatase; DMEM, Dulbecco's modified Eagle's medium; cDNA, complementary DNA; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *DSPP*, dentin sialo-phosphoprotein; *DMP-1*, dentin matrix acidic phosphoprotein 1; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; Sh-*ANX A1*, knockdown cells for *ANX A1*; Ad-*ANX A1*, *ANX A1* overexpression; GSK, glycogen synthase kinase; NF-κB, nuclear factor κB; MEK, MAPK kinase; TNF, tumor necrosis factor; SD, standard deviation; DAPI, 4,6-dianmidino-2-phenylindole; mRNA, messenger RNA.

Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

Author Contributions

LZ contributed to the design of this work. YZH and JW contributed to the interpretation of data. JW and YZH analyzed the data. YZH and JW drafted the work. LZ revised critically for important intellectual content. All authors read and approved the final manuscript. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Ethics Approval and Consent to Participate

The study was reviewed and approved by Animal Experimental Ethics Committee of Xi'an Jiaotong University Medical Science Center (Ethical approval number: XJTUAE2024-2171).

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Conflict of Interest

The authors declare no conflict of interest.

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