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Effects of ADAM28 shRNA interference vector on biologic functions of human periodontal ligament stem cells

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Abstract

Background/Objective: This study was to investigate the effects of a disintegrin and metalloproteinase 28 (ADAM28) shRNA interference vector on proliferation, differentiation, and apoptosis of human periodontal ligament stem cells (HPDLSCs) and action mechanism.

Methods: After PGPU6/GFP/Neo ADAM28 shRNA1-4 were constructed and identified, and transfected into HPDLSCs for 48h, transfection efficiency and inhibition efficiency of ADAM28 shRNA1-4 were detected by QRT-PCR and Western blot. A group of ADAM28 interference vector with the highest inhibitory efficiency was selected to detect proliferation, differentiation and apoptosis of HPDLSCs. Methabenzthiazuron (MTT) and cell-cycle assay by flow cytometry (FCM) were used to test the HPDLSCs proliferation activity. Alkaline phosphatase (ALP) analysis by enzyme kinetics were performed to measure cytodifferentiation standard. Western blot was carried out to determine the influence of ADAM28-shRNA1 on

HPDLSCs expressing CAP and Pax9. Annexin V fluorescein isothiocyanate (FITC)/propidium iodide (PI) analysis by FCM was performed to measure apoptosis rate and Western blot was used to detect the expression of Bax and Bcl-2 proteins.

Results: PGPU6/GFP/Neo-ADAM28-shRNA1-4 interference vector were successfully constructed and efficiently transfected into HPDLSCs. PGPU6/GFP/Neo-ADAM28-shRNA1-4 all had significant inhibition efficiency, and ADAM28-shRNA1 displayed the highest level. Furthermore, ADAM28-shRNA1 could significantly suppress proliferation and differentiation, and induce apoptosis of HPDLSCs. ADAM28-shRNA1 was able to inhibit the expression levels of CAP and Pax9. Whereas, apoptosis rate of ADAM28-shRNA1 group presented the highest level and ADAM28-shRNA1 could significantly enhance expression levels of Bcl-2 and Bax proteins.

Conclusion: ADAM28 may participate in root development as a major regulator of proliferation, differentiation and apoptosis of HPDLSCs.

KEYWORDS: A disintegrin and metalloprotease 28 (ADAM28); ShRNA interference vector; HPDLSCs; Proliferation; Differentiation; Apoptosis.

Introduction

Previous studies confirmed that periodontal ligament stem cells (PDLSCs) displayed a high proliferative capacity and the ability to differentiate into different cell types in vitro and in vivo to a range of cellular phenotypes, including adipocytes, cementoblasts, chondrocytes and osteoblasts [1]. In addition, PDLSCs have been shown to play a critical role in maintaining periodontal ligament stability, repair of cementum tissue injury and enhancing cell renewal and functionality [2, 3]. Because of broad proliferative and differentiative capability, PDLSCs have tremendous potential to be used in dental regenerative medicine. Therefore, it has been reasonably proposed that PDLSCs play essential roles in the developing process and regenerative therapy of the periodontium and tooth root [4].

A disintegrin and metalloproteinase 28 (ADAM28) gene was shown to possess proteolytic, adhesive properties and autocatalytic activity [5], and was screened from the differential expression genes of patients with congenital hypoplasia of tooth root (CHTR) by Zhao et al [6]. Studies indicated that CHTR is a disease of a physiologic development disorder of the tooth

root that results from ectodermal dysplasia, and the patients usually have tooth mobility, atonia masticatoria, and early slough [7, 8].

Nowadays, no effective therapy of CHTR has been discovered in the world and potential mechanisms of the disease are not clear. Thus, ADAM28 has been regarded as one of the possible virulence genes for CHTR.

Previous studies by Zhao et al.[9-13] concerning the expressions of human ADAM28 in tooth germ suggested that ADAM28 took part in the tooth morphogenesis process, and might actively modulate the proliferation, differentiation and apoptosis of human dental papilla cells (HDPCs), human dental follicle cells (HDFCs) and human gingival fibroblasts (HGFs).

The current study aims to investigate ADAM28 expression features in HPDLSCs and the effects of ADAM28 shRNA interference vector on the biological features of HPDLSCs and action mechanisms.

MATERIALS AND METHODS

The nucleotide sequence data about human ADAM28 in this study appear in the GenBank Nucleotide Sequence Databases under accession number NM-014265. All procedures were approved by the Ethics Committee of Qingdao Stomatological Hospital Affiliated to Qingdao University, Qingdao, China. The study was conducted from August 2024 to September 2025.

Samples and cell culture

The third generation HPDLSCs and pGPU6/GFP/Neo fluorescence vector were obtained from the central laboratory of Qingdao Stomatological Hospital Affiliated to Qingdao University. The HPDLSCs were cultured in 75-cm² cell culture flasks containing Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY), 50 µg/mL ascorbic acid and antibiotics (100 µg/mL streptomycin and 100 U/mL penicillin G, Sigma Chemical Co., St. Louis, MO, USA). The HPDLSCs were subcultured using 0.25% (weight/volume [w/v]) trypsin and 0.05% (w/v) EDTA (Invitrogen, Carlsbad, CA) after reaching 90% confluency. The HPDLSCs between the third and fifth passages were used for the experiments.

Construction and identification of pGPU6/GFP/Neo-ADAM28-shRNA interference vector

Design and synthesis of specific shRNA

The full mRNA sequence of human ADAM28 gene (NM_014265) was found in GenBank.

After the specificity was confirmed by BLAST homology analysis, the secondary structure of the target mRNA sequence was evaluated using RNA structure 4.4 software. Finally, four 21nt-specific shRNA target sequences were selected, and one negative control sequence siRNA-NC was designed.

Four interference sequences and one control sequence were named shRNA1, shRNA2, shRNA3, shRNA4, shRNA-NC, respectively. Specific sequences were displayed in Table 1. Then, according to the sequence of siRNA, two DNA template single strands of shRNA were designed and synthesized. The synthesis and total thiomodification of shRNA oligonucleotide chains were completed by Shanghai Shengong Bioengineering Co., LTD.

The loop structure of ShRNA template was TTCAAGAGA to avoid the formation of termination signals, and the transcription termination sequence of shRNA was T6 structure. CACC was added to the 5' end of the sense chain template to complement the sticky end formed by BbsI digestion. GATC was added to the 5' end of the antisense chain template to complement the sticky end formed by BamHI digestion. If the first base of the siRNA is not G, a G is added up after the CACC (Table 2).

Table 1 Four specific siRNA target sequences of ADAM28 gene

Interference sequence number	Target sequence of 21nt siRNA	target site
shRNA1	GCCAGAGCAACAGGAACAATT	248-268
shRNA2	GGAAAGGAGATCACCACAAGC	384-404
shRNA3	GCGTGCCTCTTCAAGTATAA	566-586
shRNA4	GCCACAACCTTTGGAATGTTTC	1135-1155

Table 2 The Sequences of shRNA oligonucleotides targeting ADAM28 gene

Interference sequence number	primer	shRNA oligonucleotide sequence (5'→3')
shRNA1	sense	CACCGCCAGAGCAACAGGAACAATTCAAGAGAATTGTCCTGTGCTCTGGCTTTTTTG
	antisense	GATCCAAAAAAGCCAGAGCAACAGGAACAATTCTCTGAAATTGTCCTGTGCTCTGGC
shRNA2	sense	CACCGGAAAGGAGATCACCACAAGCTTCAAGAGAGCTTGTGGTGATCTCCTTTCCTTTTTTG
	antisense	GATCCAAAAAAGGAAAGGAGATCACCACAAGCTCTCTTGAAGCTTGTGGTGATCTCCTTTCC
shRNA3	sense	CACCGCATGCACTCTTCAAGTATAATTCAAGAGATTATACTTGAAGAGTGCATGCTTTTTTG
	antisense	GATCCAAAAAAGCATGCACTCTTCAAGTATAATCTCTTGAATTATACTTGAAGAGTGCATGC
shRNA4	sense	CACCGCCACAACCTTTGGAATGTTCTTCAAGAGAGAAACATTCCAAAGTTGTGGCTTTTTTG
	antisense	GATCCAAAAAAGCCACAACCTTTGGAATGTTCTCTCTTGAAGAAACATTCCAAAGTTGTGGC
shRNA-NC	sense	CACCGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCCGGAGAATTTTTG
	antisense	GATCCAAAAAATTCTCCGAACGTGTCACGTTCTTGAACGTGACACGTTCCGGAGAAC

The ShDNA template is annealed to generate a transcript

DNA oligo was dissolved with TE (pH8.0) and the concentration was 100 μ mol/L. The corresponding sense and antisense chain oligo solutions were taken, and the annealing reaction system was configured according to the following system: 10 $\times$ shDNA annealing buffer 5 μ L, sense chain (100 μ mol /L) 5 μ L, antisense chain (100 μ mol /L) 5 μ L, distilled water 35 μ L, a total of 50 μ L. Annealing is performed on the PCR machine according to the following procedure: 95 °C 5 min, 85 °C 5 min, 75 °C 5 min, 70 °C 5 min and saved at 4 °C after annealing treatment, two DNA templates expressing shRNA at a concentration of 10 μ mol/L were obtained, and the template solution was diluted 50 times. The final concentration is 200 nmol/L and the transcript is about 60 bp for the ligation reaction.

The linearization of pGPU6/GFP/Neo interference vector

A pGPU6/GFP/Neo vector of 10 μ g was obtained and subjected to enzyme digestion according to the following system: 10 $\times$ Buffer G 15 μ L, BpiI 3 μ L, BamHI 3 μ L, pGPU6/GFP/Neo 10 μ g, and distilled water was added to 150 μ L, a total of 150 μ L. After digestion at 37°C for 1 h, 1% agarose gel electrophoresis was performed and visualised with ethidium bromide staining, the 5117bp pGPU6/GFP/Neo linearized vector was purified and recovered by NucleoTrap Gel Extraction Kit Ver2.0 (Clontech, USA) (**Fig.1A**). Electrophoretic detection was performed to estimate the concentration, and the dilution concentration was reduced to 50 ng/ μ L.

Construction, screening and identification of pGPU6/GFP/Neo-ADAM28-shRNA1-4 recombinant plasmid

The annealed double-stranded Oligo DNA fragments in each group were connected with linearized pGPU6/GFP/Neo interference vectors, and the connection system was as follows: 10 $\times$ T4 connection buffer 2 μ L, pGPU6/GFP/Neo (BpiI+BamHI) 1 μ L, shDNA template (100 n mol/L) 1 μ L, T4 DNA ligase (5 weissU/ μ L) 1 μ L, distilled water 15 μ L, a total of 20 μ L. At 22 °C for 1h, the connection product is transformed into Top10 receptor cells. Transformation and plasmid extraction by alkaline lysis were carried out as described previously [6].

The recombinant plasmid was identified by enzyme digestion of BamH I and Pst I respectively (Fig. 1B). The positive recombinant vector should be able to be incised by BamH I, but not by Pst I. The recombinant plasmid liquid which was confirmed to be positive clones was selected and sent to Shanghai Bioengineering Company (Shanghai,China) for DNA sequencing identification. The positive clones with the correct sequence were expanded and cultured, extracted with a high-purity plasmid medium volume extraction kit (Clontech, USA), and the

concentration and purity were determined by ultraviolet spectrophotometer (BD Biosciences, San Jose, CA, USA). The clones were stored at -20°C for subsequent transfection experiments.

Transfection efficiency detection of HPDLSCs with recombinant plasmid

The 3rd generation HPDLSCs that had been successfully cultured and identified in the central laboratory of our hospital were subcultured and amplified and then inoculated into 6-well plates and 25 -cm² cell culture flasks with cell density of 3×10⁶ cells /cm³ by using 0.25% (weight/volume [w/v]) trypsin and 0.05% (w/v) EDTA digestion (Invitrogen, Carlsbad, CA) after reaching 90% confluency.

Experimental HPDLSCs were divided into three groups according to pGPU6/GFP/Neo-ADAM28-shRNA1, 2, 3, 4 group, pGPU6/GFP/Neo-shRNA-NC group with 50 μ mol/L transfection concentration and untransfected group. Transient transfection was detected using the Lipofectamine 2000 system (Invitrogen, Carlsbad, CA) and transfection efficiency after being transfected for 48 h was checked under a laser confocal microscope as recorded previously [6].

Blocking efficiency detection of HPDLSCs with recombinant plasmid

The detection of ADAM28 gene expression by real-time fluorescence quantitative reverse transcription polymerase chain reaction (QRT-PCR)

After being transfected for 48 h, the HPDLSCs of each group with 6-well plates were digested by 0.25% trypsin and 0.05% EDTA and collected, and total RNA of each group was extracted with RNeasy Mini Kit (Clontech, USA), and mRNA was reverse-transcribed into cDNA with Prime Scrip RT Reagent Kit (Clontech, USA), and 2μL cDNA of each group was taken as template, ADAM28 gene expression of each group was detected by SYBR Prime Script RT-PCR Kit (Clontech, USA).

The expression difference of ADAM28 protein analyzed by Western blot

After being transfected for 48 h, the HPDLSCs of each group in 25 cm² culture flasks were digested by 0.25% trypsin and 0.05% EDTA and collected, and Western blot analysis was carried out as described previously [11]. Finally, a group of interfering plasmids with the highest inhibitory efficiency was selected as the recombinant plasmid group, and the HPDLSCs of each group were collected for the detections of cell proliferation, differentiation and apoptosis.

Effects of PGPU6/GFP/Neo-ADAM28-ShRNA1 on the biological features of HPDLSCs

After 48h of transfection, 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide

(MTT), cell-cycle by flow cytometry (FCM), alkaline phosphatase (ALP) activity assays and apoptosis analysis by annexin V–FITC/PI were performed, and the HPDLSCs density was retained at $5 \times 10^6/\text{mL}$.

Grouping: 1. Interference vector group: PGPU6/GFP/Neo-ADAM28-ShRNA1,

2. Negative control: pGPU6 / GFP / Neo-shRNA-NC, 3. Untransfected Group

Cell proliferation assay by MTT

The cell proliferation assay was performed in all three groups of HPDLSCs using the MTT method as described previously [9]. Cell proliferation was detected quantitatively using a microplateenzyme-linked immunosorbentassay reader with an absorbance value of 490 nm. The assay was repeated for five times, and the data were presented as the mean $\pm$ standard deviation.

Cell-cycle detection by FCM

To detect the proliferative activity of HPDLSCs, the cellular DNA content from each stage of three groups was determined by FCM as described previously [13]. We analyzed a total of 3×10^5 cells for each sample and determined the percentages of cells in G0/G1, S, and G2+M phases using standard ModiFit and Cell Quest software (Becton Dickinson Biosciences, San Diego, CA).

Alkaline phosphatase (ALP)—activity detection

After 48 hours of transfection, the HPDLSCs of all three groups were washed with 0.01 mol/L PBS and 50 mL of cold 10 mmol/L tris-HCl buffer (pH = 7.4) containing 0.1% TritonX-100 was added before incubation at 4°C overnight as described previously [13]. Alkaline phosphatase (ALP) substrate solution (100 mL) containing 2 mmol/L MgCl_2 and 16 mmol/L p-nitrophenyl phosphate was then mixed with each sample. After incubation for 30 minutes at 37°C, the reaction was stopped by adding 50 mL of 0.2 mol/L NaOH, and the liberated p-nitrophenol was measured spectrophotometrically at 410 nm. Each experiment was repeated five times, and the data were presented as the mean $\pm$ standard deviation.

Influence of PGPU6/GFP/Neo-ADAM28-ShRNA1 on HPDLSCs Expressing cementum attachment protein (CAP) and paired box 9 (Pax9)

After 48 hours of transfection, the HPDLSCs of all three groups were calculated the protein levels by protein quantitative kit (BCA, Clontech, USA) . Western blot and image analysis were performed as described previously [13]. Glyceraldehyde-3-phosphate dehydrogenase

(GAPDH, 900 bp, 1:1000) was used as a control. Polyclonal antibody against CAP (56 kDa) and Pax9 was diluted at 1:500. Control experiments were carried out by replacing the primary antibody with non-immune rabbit serum. The average grayscale (means $\pm$ SDs) of each group were subjected to statistical analyses.

Apoptosis analysis by annexin V–FITC/PI assay

After 48 hours of transfection, the HPDLSCs were harvested, washed with cold PBS, and stained with PI and FITC–conjugated annexin V (annexin V–FITC) using an annexin V–FITC apoptosis detection kit (Apoptosis Detection Kit I, Becton Dickinson, Biosciences) as previously described [12].

The stained cells were kept on ice and subjected to fluorescence-activated cell sorter analysis using a flow cytometer (FACS Calibur flow cytometer, Becton Dickinson, Biosciences) with software (Cell Quest, Becton Dickinson, Biosciences). We measured the FITC fluorescence at 525 nm and the PI fluorescence at 575 nm. Cell Quest software was used to calculate apoptosis rate and the percentage of surviving cells, early apoptotic cells, late apoptotic cells and necrotic cells in each group.

Influence of PGPU6/GFP/Neo-ADAM28-ShRNA1 on HPDLSCs Expressing Bcl-2 and Bax proteins by Western blot

After 48 hours of transfection, Western blot and image analysis were performed as described previously [13]. GAPDH (1:1000) was used as a control. Polyclonal antibody against Bcl-2 (26 kDa) and Bax (21 kDa) was diluted at 1:500. Control experiments and average grayscale (means $\pm$ SDs) were carried out as mentioned above.

Statistical analyses

Results were analyzed and expressed as the means $\pm$ standard deviations (SDs). Statistical significance was assessed by Student–Newman–Keuls (SNK) test from SPSS-Windows version 26.0 program (SPSS Inc., Chicago, IL, USA). Data were considered to be statistically significant when $p < 0.05$.

RESULTS

Restriction endonuclease digestion identification of pGPU6/GFP/Neo-ADAM28-shRNA 1-4

The results were shown in Fig. 1B. PGPU6/GFP/Neo vector was 5117 bp and ADAM28-shRNA 1-4 oligonucleotide annealed product was 60 bp.

On the upper left side, 1-5 were the digestion results of pGPU6/GFP/Neo-ADAM28-shRNA1 by Pst I and 6-10 are the digestion results of pGPU6/GFP/Neo-ADAM28-shRNA2 by PstI.

On the upper right side, 1-5 were the digestion results of pGPU6/GFP/Neo-ADAM28-shRNA1 by BamH I, and 6-10 were the digestion results of pGPU6/GFP/Neo-ADAM28-shRNA2 by BamH I.

On the lower left side, 1-5 were the digestion results of pGPU6/GFP/Neo-ADAM28-shRNA3 by Pst I and 6-10 were the digestion results of pGPU6/GFP/Neo-ADAM28-shRNA4 by PstI.

On the lower right side, 1-5 were the digestion results of pGPU6/GFP/Neo-ADAM28-shRNA3 by BamH I and 6-10 were the digestion results of pGPU6/GFP/Neo-ADAM28-shRNA4 by BamH I.

These results showed that the digestion identification of recombinant plasmid were correct.

Sequencing identification of pGPU6/GFP/Neo-ADAM28-shRNA 1-4

The positive clones of 4 groups of recombinant vectors correctly identified by enzyme digestion were sent to Shanghai Shenggong Company for DNA sequencing. DNA homology search was performed on the National Center for Biotechnology Information (NCBI) server with Blast 2.0 program, and it was confirmed that 60 bp ADAM28 double-stranded shRNA had been successfully cloned into pGPU6/GFP/Neo vector without mutation, its open reading frame was complete and the recombinant vector was successfully constructed (Fig. 1C).

DNA sequencing result of pGPU6/GFP/Neo-ADAM28-shRNA1 was as follows:

CGGCAGGAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGG
CTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAA
ATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTT
TTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTTCGATTTCTTGGCTTT
ATATATCTTGTGGAAAGGACGAAACACCGCCAGAGCAACAGGAACAATTTCAAGAGA
ATTGTTCTGTTGCTCTGGCTTTTTTGGATCCACTAGTTCTAGAGCGGCCGCCACCGC
GGTGGAGCTCCAGCTTTTGTTCCTTTAGTGAGGGTTAATTGCGCGTTAAGATACAT
TGATGAGTTTGGACAAACCACAACCTAGAAATGCAGTGAAAAAATGCTTTATTTGTG
AAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAACAAGTTAA
CAACAACAATTGCATTCATTTTATGTTTCAGGTTTCAGGGGGAGGTGTGGGAGGTTT
TTTAAAGCAAGTAAAACCTCTACAAATGTGGTATGGCTGATTATGATCAGTTATCTA
GATCCGGTGGATCTGAGTCCGGACTTGTACAGCTCGTCCATGCCGAGAGTGATCCC

GGCGGCGGTACGAACTCCAGCAGGACCATGTGATCGCGCTTCTCGTTGGGGTCT
 TTGCTCAGGGCGGACTGGGTGCTCAGGTAGTGGTTGTCTGGGCAGCAGCACGGGGC
 CGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTC
 CTCGATGTTGTGGCGGATCTTGAAGTTCACCTTGATGCCGTTCTTCTGCTTGTCGGC
 CATGATATAGACGTTGTGGCTGTTGTAGTTGTACTCCAGCTTGTGCCCCAGGATGTT
 GCCGTCCTCCTTGAAGT

Transfection efficiency and blocking effect of pGPU6/GFP/Neo-ADAM28-shRNA1-4 on HPDLSCs

After 48 h of transfection, the morphology and growth characteristics of HPDLSCs in five groups were not significantly different from those before transfection, and the number of adherent cells remained at $3 \times 10^5/\text{cm}^3$. Under Laser confocal microscope, strongly green fluorescence was found in cytoplasm of the five groups (**Fig. 2A**), and the transfection efficiency of the five groups was 92%, 90%, 94%, 91% and 95%, respectively, with no significant difference among five groups.

QRT-PCR detection indicated that there were significant differences between ADAM28-shRNA1-4 group and the other two groups respectively ($P < 0.05$), ADAM28 gene expression in ADAM28-shRNA1-4 groups was significantly inhibited. Moreover, significant differences were detected between ADAM28-shRNA1 group and shRNA2, 3 and 4 groups, respectively ($P < 0.05$), among which shRNA1 had the highest inhibitory efficiency. However, there was no significant difference between shRNA2, 3 and 4 groups ($P > 0.05$). The untransfected group and the negative control shRNA-NC had little effect on ADAM28 expression (Fig. 2B). These results disclosed that pGPU6/GFP/Neo-ADAM28-shRNA1-4 can specifically inhibit the mRNA expression of ADAM28 gene in HPDLSCs.

GAPDH was used as a control to detect the relative expression of ADAM28 protein. Western blot analysis revealed that there were significant differences between ADAM28-shRNA1-4 group and the other two groups respectively ($P < 0.01$). The results indicated that pGPU6/GFP/Neo-ADAM28-shRNA1-4 carrying ADAM28 gene interference fragment could significantly inhibit the expression of ADAM28 protein, and distinct differences were found between shRNA1 and shRNA2, 3 and 4 groups, respectively ($P < 0.05$), shRNA1 had the best inhibitory effect. Meanwhile there was no significant difference between shRNA2, 3 and 4 groups ($P > 0.05$). The protein expression of ADAM28 was not decreased in the untransfected group and the negative control (Fig. 2C), indicating that the ADAM28 interference vector could

specifically inhibit the expression level of ADAM28 protein in HPDLSCs. **pGPU6/GFP/Neo-ADAM28-shRNA1** was selected as interference vector group in the subsequent experiment. Quantitative data are expressed as means $\pm$ SDs.

Effects of pGPU6/GFP/Neo-ADAM28-shRNA1 on biological functions of HPDLSCs

The MTT assay showed that in the **pGPU6/GFP/Neo-ADAM28-shRNA1** group, cell proliferative activity was markedly inhibited which exhibited a lower absorbance value than the other groups at 48 h (**Fig. 3A**), There was a prominent difference between pGPU6/GFP/Neo-ADAM28-shRNA1 and the other two groups ($P<0.01$) while the P values between the groups were 0.012 and 0.015, respectively. There was no significant difference between the negative control and untransfected group, that is, pGPU6/GFP/Neo-ADAM28-shRNA1 could significantly inhibit the proliferation of HPDLSCs.

After 48 h of transfection, cell-cycle distributions were analyzed by FCM indicated that transfection of pGPU6/GFP/Neo-ADAM28-shRNA1 resulted in a notably lower percentage of HPDLSCs in S/G2 + M phases and PI (S+G2M) compared to the other groups (**Fig. 3B**, $P<0.05$), also demonstrating that the blocking of ADAM28 could significantly inhibit the proliferation of HPDLSCs. There was no significant difference between negative control and untransfected group.

Similar to the cell-proliferation assay, analysis of the ALP activity displayed that the pGPU6/GFP/Neo-ADAM28-shRNA1 group kept a lower level of ALP absorbance than the other groups after 48 h of transfection (**Fig. 3C**, $P<0.01$) while the P values between the groups were 0.025 and 0.031, respectively. The results revealed that blocking of ADAM28 could significantly restrain the differentiation of HPDLSCs.

Western blot and image analysis indicated that the expression levels of CAP and Pax9, and relative grayscale of pGPU6/GFP/Neo-ADAM28-shRNA1 group were notably lower than those of the other groups, and a distinct difference was inspected between pGPU6/GFP/Neo-ADAM28-shRNA1 and the other groups (**Fig. 3D**, $P<0.01$),

while the P values between the groups were 0.035 and 0.041, respectively. These results demonstrated that blocking of ADAM28 could significantly suppress the expression levels of CAP and Pax9 protein, namely, ADAM28 had positive correlation with CAP and Pax9. There was no significant difference between negative control and untransfected group.

Cell-apoptosis assay suggested that there were many more apoptotic cells in the pGPU6/GFP/Neo-ADAM28-shRNA1 group than in the other groups, and a significant

difference was found between the pGPU6/GFP/Neo-ADAM28-shRNA1 and the other groups, demonstrating that transfection of pGPU6/GFP/Neo-ADAM28-shRNA1 could induce apoptosis of HPDLSCs (**Fig. 3E**, $P < 0.05$), while the P values between the groups were 0.028 and 0.023, respectively.

Western blot and image analysis indicated that the relative grayscale of Bcl-2 and Bax expressions in pGPU6/GFP/Neo-ADAM28-shRNA1 group were notably higher than those of the other groups, and a significant difference was detected between pGPU6/GFP/Neo-ADAM28-shRNA1 and the other groups (**Fig. 3F**, $P < 0.01$), while the P values between the groups were 0.052 and 0.061, respectively. These results showed that transfection of pGPU6/GFP/Neo-ADAM28-shRNA1 could significantly increase the expression levels of Bcl-2 and Bax proteins in HPDLSCs.

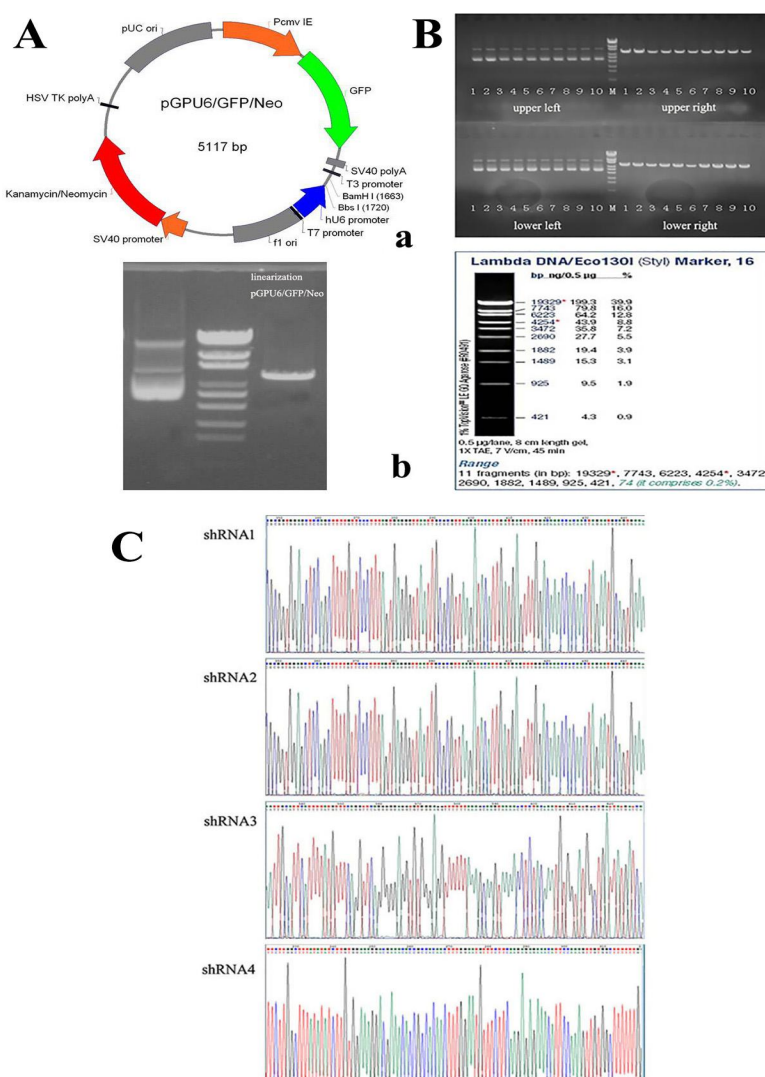


Fig. 1 Construction and identification of pGPU6/GFP/Neo-ADAM28-shRNA1-4 recombinant plasmid

A. PGPU6/GFP/Neo vector decomposition diagram (a) and electrophoresis diagram of the linearized vector after enzyme digestion (b).

B. Restriction endonuclease digestion identification of pGPU6/GFP/Neo-ADAM28-shRNA 1-4 showed that PGPU6/GFP/Neo vector was 5117 bp and ADAM28-shRNA 1-4 product was 60 bp, recombinant plasmids were correctly constructed.

C. DNA sequencing peak figures of pGPU6/GFP/Neo-ADAM28-shRNA1-4 indicated that its open reading frame was complete without mutation.

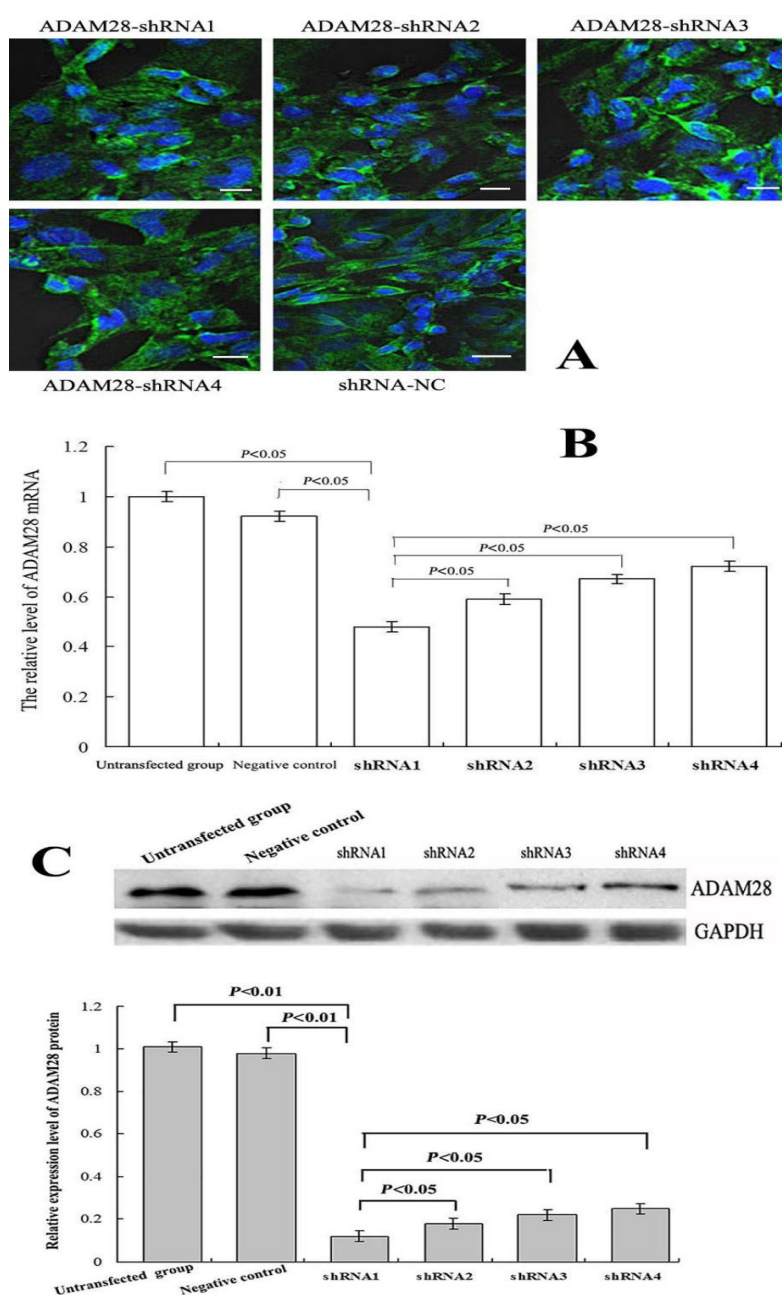


Fig. 2 Transfection efficiency and blocking effect of pGPU6/GFP/Neo-ADAM28-shRNA1-4 on HPDLSCs

A. Detection of transfection efficiency revealed a strong green fluorescence was displayed in cytoplasm of the five groups at almost the same level, FITC / DAPI fluorescent labelling, laser confocal microscopy, Scale bar = 20 μ m.

B. RT-QPCR detection disclosed that there were significant differences between ADAM28 shRNA1-4 group and the other two groups respectively ($P < 0.05$), and shRNA1 had the highest inhibitory efficiency. The quantitative analysis of the relative grayscale value was indicated by ADAM28 shRNA1-4/GAPDH. Quantitative data are expressed as means $\pm$ SDs.

C. Western blot analysis of ADAM28 protein expression indicated that the relative grayscale value of the pGPU6/GFP/Neo-ADAM28-shRNA1-4 group was notably lower than those of the other two groups, as shown by ADAM28 shRNA1-4/GAPDH. There were significant differences between ADAM28-shRNA1-4 group and the other two groups respectively ($P < 0.01$). Moreover, distinct differences were found between shRNA1 and shRNA2, 3 and 4 groups, respectively ($P < 0.05$), shRNA1 had the best inhibitory effect. Quantitative data are expressed as means $\pm$ SDs.

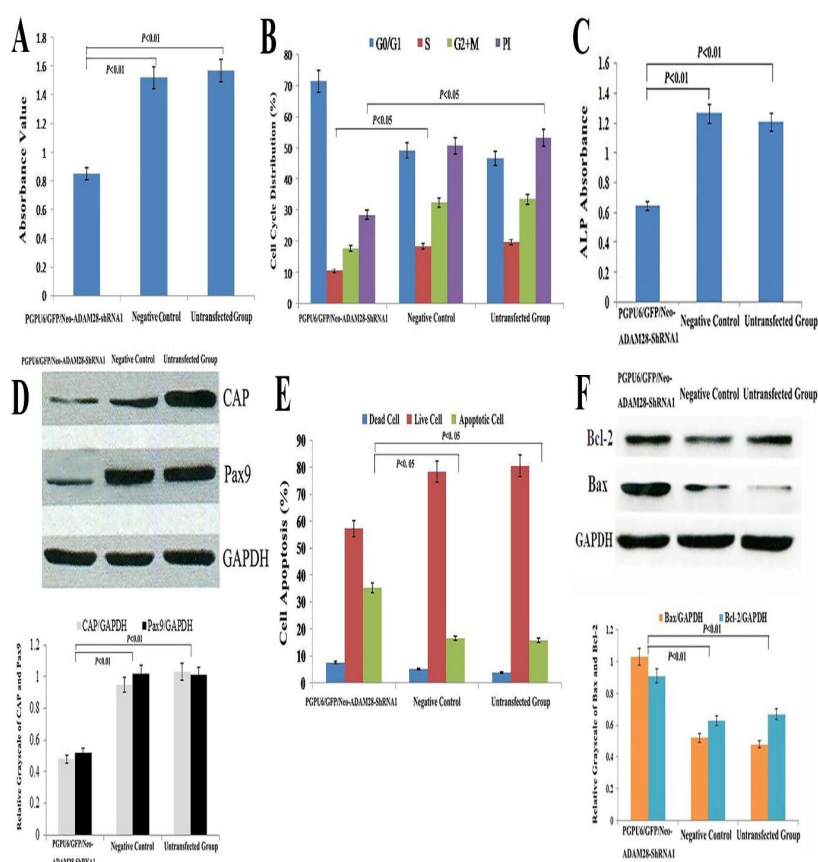


Fig. 3 Influence of pGPU6/GFP/Neo-ADAM28-shRNA1 on the proliferation, differentiation and apoptosis of HPDLSCs

A. The MTT assay indicated that the HPDLSCs of the pGPU6/GFP/Neo-ADAM28-shRNA1 group had a markedly lower absorbance value than the values of the other groups at 48h. Cell proliferation was notably inhibited at a statistically significant level. $P < 0.01$ versus the other groups. Data are presented as the means $\pm$ SDs from all groups.

B. Cell-cycle distributions that were analyzed by flow cytometry showed that ADAM28-shRNA1-treated HPDLSCs displayed a markedly lower percentage of cells in the S/G2 + M phases and PI (S+G2M), compared to the other groups ($P < 0.05$).

C. The ALP-activity assay disclosed that the pGPU6/GFP/Neo-ADAM28-shRNA1 group remained a lower level of ALP absorbance than the other groups after 48 h of transfection. $P < 0.01$ versus the other groups.

D. Western blot and image analysis indicated that the expression levels of CAP and Pax9, and relative grayscale of pGPU6/GFP/Neo-ADAM28-shRNA1 group were distinctly lower than those of the other groups, and a significant difference was detected between pGPU6/GFP/Neo-ADAM28-shRNA1 and the other groups.

E. Cell apoptosis assay indicated that there were more apoptotic cells in the pGPU6/GFP/Neo-ADAM28-shRNA1 group than in the other groups, and a significant difference was found between the pGPU6/GFP/Neo-ADAM28-shRNA1 and the other groups. $P < 0.05$ versus the other groups.

F. Western blot and image analysis showed that the relative grayscale of Bcl-2 and Bax expressions in pGPU6/GFP/Neo-ADAM28-shRNA1 group were notably higher than those of the other groups, and a significant difference was detected between pGPU6/GFP/Neo-ADAM28-shRNA1 and the other groups.

DISCUSSION

To our knowledge, this is the first assay of effects of ADAM28 shRNA interference vector on proliferation, differentiation and apoptosis of HPDLSCs in vitro. The defining feature of ADAM28 is a conserved modular-domain organization consisting of an N-terminal signal sequence and a prodomain, metalloprotease domain, disintegrin-like domain, cysteine-rich domain, epidermal growth factor (EGF)-like domain, a transmembrane domain, and cytoplasmic domain [14, 15].

According to the clinical situation, CHTR is separated into dentin developmental anomaly of tooth root, cementum developmental defect of tooth root, root paramorphia (absence, short cone

shape), and root adhesive organ dysplasia [8]. CHTR has been proposed to relate with the proliferation and differentiation disorder of odontogenic mesenchymal stem cells. In this study we provide evidence to show that ADAM28 appears to play some pathogenic role in the process of CHTR. Therefore, ADAM28 shRNA interference vector was constructed, and its influences on the biological features of HPDLSCs were determined.

Firstly, pGPU6/GFP/Neo-ADAM28-shRNA1-4 interference vector were successfully constructed and efficiently transfected into HPDLSCs. ADAM28-shRNA1-4 all had significant inhibition efficiency, and ADAM28-shRNA1 had the highest efficiency, which disclosed that ADAM28-shRNA1-4 may specifically affect the function of promoters and enhancers, and finally down-regulate the expression levels of ADAM28 mRNA and protein in HPDLSCs. Subsequently, MTT and cell-cycle detection showed that the blocking of ADAM28 could significantly inhibit the proliferation of HPDLSCs, which demonstrated that ADAM28 had an upright-regulatory effect on the proliferation of HPDLSCs. The critical step required for the proliferation of cells is their transition from cell-cycle arrest to entry into the active phase of the cell cycle through the G1/S restriction point; the fast transition could enhance higher rates of cell proliferation [16]. These results suggested that ADAM28 could enhance the HPDLSCs proliferation by promoting the transition process from the G1 to S phases, further accelerating the DNA reproduction and protein synthesis of HPDLSCs. It is speculated that ADAM28 contains an EGF-like domain, which may possess some functions of EGF, such as the stimulation or preservation of undifferentiated cell proliferation [17, 18]. Therefore, our results confirmed the positive correlation between ADAM28 and HPDLSCs proliferation.

To date, the only cementum-specific protein is the cementum attachment protein (CAP), which plays a critical role in cementogenesis during regeneration of periodontal tissues and wound healing [19, 20, 21]. Moreover CAP promotes cell attachment of fibroblasts and other periodontal cells, and its activity is mediated by $\alpha 5 \beta 1$ integrin receptors on cell surfaces [22]. PAX9 gene is strongly expressed in the oral mesenchyme and required for the mesenchymal expression of BMP4, MSX1, and LEF1 during tooth development [23]. Moreover, PAX9 is involved in the formation of the posterior teeth and mandibular incisors [24, 25]. Therefore, PAX9 is essential for the development of a variety of organs and skeletal elements. Mutations in PAX9 are the most common genetic cause of tooth agenesis [26].

The present experiments showed that blocking of ADAM28 could significantly suppress the differentiation of HPDLSCs, and expression levels of CAP and Pax9 protein, namely, ADAM28 has positive regulation on CAP and Pax9 expressions, which was in accordance with above

proliferation detection. Therefore, it is reasonably supposed that some elements of ADAM28 could act as promoters or depressors for the proliferation and differentiation of HPDLSCs.

Apoptosis is considered to be a vital component of various processes including normal cell turnover, proper growth and functioning of the immune system, embryonic development and chemical-induced cell death [27]. Functionally, the Bcl-2 family of proteins play an important role in the control of apoptosis and Bcl-2 gene codes for a 25-26 kDa protein. It has been demonstrated that insertion into membranes is closely associated with the ability of Bcl-2 to regulate apoptosis [28, 29, 30]. Previous experiments have established that Bcl-2 is capable of blocking a wide range of apoptotic stimuli in a variety of different cell types [31]. Bax was isolated as a Bcl-2 binding protein in immunoprecipitation experiments and Bax is a 21 kDa protein that is known to play an essential role in apoptosis [32, 33]. In overexpression experiments, Bax committed the cell to apoptosis and could antagonise the protective effect of Bcl-2. Taken together, with the demonstration of the two proteins as binding partners in cells, this lead to the hypothesis that regulation of the formation of bax/bcl-2 heterodimers might be a key point of control in apoptosis [31].

In this study, the transfection of ADAM28-shRNA1 could significantly promote apoptosis of HPDLSCs and expression levels of Bcl-2 and Bax proteins in HPDLSCs, which strongly demonstrated that ADAM28-shRNA1 had positive regulatory effect on apoptosis of HPDLSCs and exerted opposite effects on the proliferation and differentiation of HPDLSCs.

Data on teeth development have disclosed that proper expression of Notch signalling is critical for differentiation of ameloblasts, odontoblasts and osteoblasts, secretion of enamel and dentine matrix, proliferation and apoptosis of dental stem cells, generation of tooth roots and further functions [34, 35, 36, 37, 38]. The Notch signalling pathway is involved in cell differentiation, apoptosis and proliferation, thus controlling organ formation and morphogenesis [34, 35, 36, 37, 38]. Furthermore, the Notch gene encodes a transmembrane receptor with an extracellular domain carrying multiple EGF-like repeats and a cytoplasmic domain required for signal transduction [39].

As mentioned above, ADAM28 protein also contains a transmembrane domain, a cytoplasmic domain and an EGF-like domain, which include some components of the extracellular matrix and may play a major role in signalling transduction, intracellular protein maturation [40]. Therefore, Notch and ADAM28 are highly similar in molecular structure, and have analogous biological features [41]. It is postulated that some components of ADAM28 molecular structure

can not only act as promoters of proliferation and differentiation of HPDLSC, but also as inhibitors of apoptosis.

Based on above findings, we propose the action mechanism might be that ADAM28-shRNA1 inhibit the catalytic cracking of metalloproteinase and disintegrin-like domain, and suppress the promoting cell proliferation of epidermal growth factor (EGF)-like domain, block Notch signal transmission and participate in the negative regulation of apoptosis.

CONCLUSIONS

In summary, this study testifies convincingly the successful construction and transfection of pGPU6/GFP/Neo-ADAM28-shRNA1-4 into HPDLSCs and confirms that ADAM28-shRNA1 could effectively manipulate the proliferation, differentiation, and apoptosis of HPDLSCs by interacting with Notch signal pathway. Evidence provides that ADAM28 inhibitor could be a potential therapeutic target against CHTR.

Declaration of conflicting interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Author contribution statement

All authors had full access to the data, contributed to the study, approved the final version for publication, and take responsibility for its accuracy and integrity. All authors have made substantial contributions to the study.

Zheng Zhao has been involved in conception and design of the work, acquisition of data, analysis or interpretation of data, specific experimental operations, drafting the manuscript and revising it critically, and has given final approval of the version to be published.

Jie Li has been involved in data collection and statistical analyses.

Xia Zhao and **Xin Tian** has been involved in data interpretation, image acquisition and analysis.

All authors declare adherence to Quality improvement studies: SQUIRE depending on the study design.

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