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RESEARCH PAPER

Knockdown of microRNA-584 promotes dental pulp stem cells proliferation by targeting TAZ

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ABSTRACT

Proliferation of dental pulp stem cells (DPSCs) is crucial in tooth development and damage repairing, also includes its therapy application for tissue engineering. MicroRNAs (miRNAs) are key players in biological processes of DPSCs, and transcriptional co-activator with PDZ-binding motif (TAZ) also plays important roles in cell proliferation and differentiation, however, the roles of miR-584 and TAZ in DPSCs are not known. We found up-regulated miR-584 expression and down-regulated TAZ expression levels in aging dental pulp tissue compare to those in young dental pulp tissue. In proliferating DPSCs we demonstrated the decreased miR-584 expression and increased TAZ expression. miR-584 mimics suppressed DPSCs proliferation and migration, and significantly reduced TAZ production, whereas miR-584 inhibition exerted the converse effects. Knocking down of the TAZ in DPSCs had a similar effect as overexpression of miR-584. Furthermore, luciferase reporter assay demonstrated that miR-584 could directly bind to the TAZ mRNA 3′UTR to repress its translation. Overexpression of TAZ can partly rescue miR-584 mimic-mediated the inhibition of proliferation. Additionally, miR-584 inhibited cell proliferation and downregulated expression of cell cycle proteins by AKT signaling pathway. Together, we identified that miR-584 may be a key regulator in the proliferation of DPSCs by regulating TAZ expression via AKT signaling pathway. It would be a promising biomarker and therapeutic target for pulp disease.

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miRNA-584; TAZ; DPSCs; proliferation; cell cycle

1. Introduction

Mesenchymal stem cells (MSCs) are capable of self-renewal and multiple differentiation, usually used as seed cells sources in tissue regeneration and tissue engineering [1,2]. On the other hand, there is a certain limitation on the application of MSCs for their difficult to acquire[3]. Dental pulp stem cells (DPSCs) are obtained from dental pulp tissue. It is a kind of MSCs, has excellent stem cell-like properties, such as high proliferation, self-renewal, and multidirectional differentiation capacity [4,5]. DPSCs can differentiate into odontoblast, osteoblasts, adipocytes, chondrocytes, neuronal cells, cardiomyocytes and so on[6]. When applied in regenerative medicine DPSCs would be a promising cell source [7,8], and could proliferate and regenerate tertiary dentin to repair damage tissue under appropriate conditions in the traumatized dental pulp [9,10]. However, varieties of factors and their roles in regulating the proliferation of DPSCs are rather intricate and still unknown, elucidating the molecular mechanisms is of great significance to developing effective clinical application.

miRNAs belong to non-coding, small (about 19–22 nt), endogenously expressed RNAs. miRNAs play vital roles in mediating various developmental, physiological, and pathologic cellular processes by negatively regulating targets gene expression[10]. miRNAs can directly bind to the 3-untranslated region (3′UTR) of the target mRNAs by base pairing, to evoke the degradation of target mRNA or translational repression [2,11]. There were numerous evidence that miRNAs displayed critical regulatory effects on cell proliferation, differentiation, and tumor initiation [12–14]. Furthermore, miRNA can regulate a variety of physiological function and pathologic processes of DPSCs [15,16]. Previous studies have

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documented miR-584 acted as a potent tumor suppressor in some cancers, involved in stemness in medulloblastoma, microtubule dynamics, and repairing of DNA damage [17–19]. Some reports provided evidence that the expression level of miR-584 was significantly upregulated when the dental pulp was infected[20]. However, there is no report on the physiological function of miR-584 in dental pulp tissue and DPSCs.

Transcriptional co-activator with PDZ-binding domain (TAZ) is an important regulation factor of Hippo pathway, playing an important role in organ development, tissue regeneration, mediating tissue physiological functions and homeostasis [21–23]. In a sense, TAZ acts as "stemness factors" in some stem cells[24]. TAZ is phosphorylated by upstream gene LATS1 and LATS2, and TAZ activity is inhibited through cytoplasmic retention and protein degradation. On the contrary, TAZ interacts with TEA domain family members (TEAD) in the nucleus, and promotes the transcription of downstream gene by interacting with transcription factor [21,25]. According to our previous study, TAZ could promote the proliferation and migration of DPSCs [26]; however, the mechanism of regulating TAZ expression remains largely unknown.

Herein, we confirmed that miR-584 was upregulated in aging dental pulp tissue, and downregulated during the proliferation of DPSCs. Moreover, bioinformatics prediction indicates that miR-584 can target TAZ. Therefore, in this study, we aimed to explore the potential effects of miR-584 on DPSCs and detect the internal molecular mechanism. Further understanding of the potential of miR-584 maybe has great value in the field of dental pulp regenerative treatment.

2. Material and methods

The Ethical Committee of the Second Hospital of Hebei Medical University approved the study. Prior to enrollment in this study, the patients were explained with the protocol. Before specimen collection, the patients had written informed consent.

2.1. Dental pulp tissue samples

A total of 28 healthy teeth were prepared to extract dental pulp tissues. The teeth included removal of third molars or orthodontic extraction teeth. According to age participants were recruited into two groups: orthodontic teeth extracted from young group (12–16 years, n = 14) and health third molars extracted from aging group (50–65 years, n = 14). Pulp tissue was carefully extirpated, and frozen at once into liquid nitrogen and then stored for next protein and mRNA detecting at −80°C.

2.2. DPSCs isolation and culture

Healthy human premolars or impacted third molars (n = 12) were collected from healthy subjects (10 donors; male; aged 16–24 years). Dental pulps were cut into small pieces (1 mm^3) and digested with type I collagenase (3.0 mg/ml; Sigma-Aldrich; Merck KGaA) and dispase (4.0 mg/ml; Sigma-Aldrich) at room temperature for 45 min. The digested mixtures were filtered with a cell strainer (70 μm). Cells were cultured at a density of $1x10^5$/ml in 35 mm culture dishes with Dulbecco's modified Eagle's medium (DMEM; Gibco; shanghai, China), in which 10% fetal bovine serum and 2.0 mM/l glutamine (Invitrogen, Carlsbad, CA, USA) were supplemented (FBS; Bioind, BI, Isreal), and added with 100 units/ml penicillin, 100 μg/ml streptomycin in 5% CO_2 at 37°C. When they reached 70% confluence, adherent DPSCs were digested by 0.2% trypsin and 0.02% EDTA and passaged. The culture medium was changed every 3 days. DPSCs between 3 and 5 passages were selected for future experiments.

2.3. Transient transfection of miRNA mimics and inhibitors

The negative control (NC), miR-584 mimics and miR-584 inhibitor, or pcDNA3.1-TAZ-3′UTR-MUT and pcDNA3.1-TAZ-3′UTR-WT or pcDNA3.1-TAZ and pcDNA3.1-NC or si-TAZ and si-NC were synthesized by Suzhou GenePharma (Suzhou, China).

At the time of cell transfection, DPSCs were cultured into 96 or 24-well plates for 24 h. mimics-NC, miR-584 mimics, or miR584-inhibitor-NC, miR584-inhibitor or si-NC, si-TAZ or pcDNA3.1-NC, pcDNA3.1-TAZ were transfected, respectively, by lipofectamine 2000 (50 nM; Invitrogen). After transfected for 48 h, total RNA and protein of DPSCs were extracted for the next experiments.

2.4. Quantitative RT-PCR analysis of TAZ and miR-584

A total RNA of DPSCs was extracted with Trizol (Invitrogen). Then, cDNA was reverse-transcribed, respectively, from RNA with PrimeScript RT Reagent kit (Takara, Bio, Tokyo, Japan). qRT-PCR assess was detected by SYBR Ex Taq II Kit (Takara) with the 7500 PCR System. Primers for miR-584, TAZ were synthesized commercially (Suzhou GenePharma). Expression of GAPDH or the endogenous U6 snRNA was acted as internal controls to normalize each mRNA or miRNA expression level, respectively. The expression levels of miR-584 and TAZ were analyzed with the $2^{-\Delta\Delta CT}$ method relative to internal controls. All qRT–PCRs were performed 3 times. The primers sequence as follows: miR-584 forward sequence, 5′-TGC AAT GTG TGT GTT AGC CA-3′ and reverse sequence, 5′-ATC ATT GCT CCT TGG ATG GT-3′; U6 forward sequence, 5′-CTC GCT TCG GCA GCA CAT ATA CT-3′ and reverse sequence, 5′-ACG CTT CAC GAA TTT GCG TGT C-3′; TAZ forward sequence, 5′-ATC CCA GCC AAA TCT CGT GA-3′ and reverse sequence, 5′-GCC CTG CGG GTG GGT-3′; GAPDH forward sequence, 5′- AGA AGG CTG GGG CTC ATT TG −3′ and reverse sequence, 5′-AGG GGC CAT CCA CAG TCT TC −3′.

2.5. Immunoblotting

Total protein of samples was extracted from cells or tissues using RIPA buffer, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed. Polyvinylidene difluoride membranes (PVDF; Millipore, Beijing, China) were used to transfer the protein bands, and blocked for 1 h using nonfat powdered milk. Membranes were treated with diluted monoclonal antibodies at 4°C for one night, primary antibodies as follows: TAZ (1:1000; mAb #83669; Cell Signaling Technology, shanghai, China), CTGF (1:400, sc-101586; Santa Cruz Biotechnology, Suzhou, China), CDK4 (1:1000; mAb #12,790; Cell Signaling Technology), phosphorylated-AKT (p-AKT; 1:2000; mAb #4060; Cell Signaling Technology), GAPDH (1:400; sc-47724; Santa Cruz Biotechnology), AKT (1:1000; mAb #4685, Cell Signaling Technology), cyclin D1 (1:1,000; #2922; Cell Signaling Technology), and β-actin (1:1000 ; mAb #3700 ; Cell Signaling Technology). Subsequently, membranes were treated by secondary antibodies for 1 h at 37°C. Images were captured by chemiluminescence reagents (ECL; Thermo Fisher Scientific, shanghai, China). GAPDH served as an internal control for protein loading. Band intensities were measured and normalized to GAPDH, n = 3 for each experiment.

2.6. Immunofluorescence assay of DPSCs

Cells were inoculated onto coverslips in 24-well plates (2×10^4/well). After cultured for 24 h, DPSCs were immersed in 4% paraformaldehyde at 37°C for 20 min and reacted in 0.3% Triton (Sigma-Aldrich) for 15 min, and then, 10% normal goat serum blocked DPSCs for 2 h. Then, cells incubated with rabbit anti-TAZ polyclonal antibody (1:200; mAb #83669; Cell Signaling Technology) at 4°C overnight. Subsequently, DPSCs were reacted with goat anti-rabbit IgG for 2 h. The nuclei were stained with hoechst 33342 (5 μg/ml) for 15 min. The images were observed and captured by a fluorescence microscope (Olympus Corporation, Tokyo, Japan; ×200 magnification).

2.7. Luciferase assays

Luciferase reporter plasmid with a wild-type 3′UTR of TAZ (pmirGLO-TAZ-3′UTR-WT) or a mutant 3′UTR of TAZ (pmirGLO-TAZ −3′UTR-MUT) were synthesized and purified by Promega (Madison, WI, USA). First, 293 T cells were cultured into a 24-well plate at a density of 1×10^5/well. When the cell fused to 70%, luciferase reporter plasmid (50 ng per well) and miR-584 mimics or miR-NC (50 nM) were added into 293 T cells by lipofectamine 2000 (Invitrogen). After transfection for 48 h at 37°C, luciferase activity of the cells was analyzed by using the Dual-Luciferase reporter assay system (Promega). Renilla activity served as the internal control. All experiments were performed three times.

2.8. Cell proliferation assay

Cells proliferation was evaluated using the CCK-8 assay. DPSCs (1×10^3 cells/well) were seeded onto

96-well plates, and cultured for 24 h. At 3, 5, 7 days post-transfection, CCK-8 reagent 10 μl (Dojindo, Kumamoto, Japan) was added to culture solution. After 2 h reaction at 37°C, the absorbance values (optical density, OD) of the samples were measured at 450 nm.

2.9. Flow cytometry analysis of cell cycle

Post-transfected DPSCs were digested with trypsin. Cells were centrifuged and washed twice, then treated with 70% ethanol for 24 h at −20°C. Cells were incubated with 10 mM RNAse and stained with 10 mM propidium iodide staining (500 μl) at 37°C for 15 min. Transfected DPSCs were analyzed using FACScanto II cytometers (BD, Franklin Lakes, NJ, USA), and data were quantified by FlowJo 10.2 software.

2.10. Cell migration assay

For the transwell migration assay, 5×10^4 of DPSCs transfected with different plasmids were reseeded into the upper chamber with 200 μl of serum-free medium. The chamber was then inserted into the lower chambers containing DMEM (500 μl) supplemented with 10% FBS. After incubation at room temperature for 24 h, the cells remained on the upper surface was scraped using a cotton swab, and the migrated cells through the membrane were treated with 4% paraformaldehyde for 10 min, and then stained with 1% crystal violet (Sigma, Saint Louis, MO, USA) and observed under a microscope (Olympus, Tokyo, Japan). Cells from five random fields were counted to assess the average number. The experiment was repeated 3 times and the mean of three experiments were calculated.

2.11. Statistical analysis

All data were treated as the mean ± standard deviation (SD), and data analysis was selected with SPSS 19.0. Statistical analysis between two groups was performed using Two-tailed Student's t-test. Statistical analysis of multiple groups was performed with one-way ANOVA. $p < 0.05$ was considered statistically significant.

3. Results

3.1. miR-584 expression levels were up-regulated in aging dental pulp tissue

To identify whether miR-584 is involved in young and aging dental pulps tissues, we collected 14 young dental pulp tissue specimens and 14 aging dental pulp tissue specimens to perform miRNA qRT-PCR. The PCR results showed that the expression of miR-584 in the aging dental pulp tissues was significantly up-regulated than those in the young groups (Figure 1(a)). It implied miR-584 may be associated with dental pulp tissue growth potential.

3.2. miR-584 expression is down-regulated in proliferating of DPSCs

To evaluate proliferation capacity of DPSCs, we cultured DPSCs for 3, 5, 7 days and performed CCK-8 assay. The results showed that DPSCs displayed significantly high proliferative potential during DPSC culture over time (Figure 1(b)). To investigate whether proliferation of DPSCs is related to expression level of miR-584, we performed miRNA RT-PCR. We found that miR-584 expression was stepwise downregulated, which was reverse correlation with the proliferation of DPSCs (Figure 1(c)).

3.3. miR-584 represses proliferation and migration of DPSCs

To examine the effect of miR-584 in proliferation of DPSCs, we transfected DPSCs with miR-584 mimics or miR-584 inhibitor. qRT-PCR was performed to detect the transfection efficiency and the results displayed that miR-584 mimics remarkably upregulated miR-584 expression and miR-584 inhibitor downregulated dramatically miR-584 expression in DPSCs (Figure 2(a)). After transfected with miR-584 CCK-8 assay was performed to evaluate DPSCs proliferation. The results revealed that miR-584 mimics significantly inhibited DPSCs proliferation. Whereas miR-584 inhibitor enhanced cell growth compared with the control group and non-transfected group (NT) (Figure 2(b)). DPSCs migration plays vital roles in repairing and regeneration of dental pulp tissue. We performed transwell assay to determine the role of miR-584 in DPSCs migration, miR-584

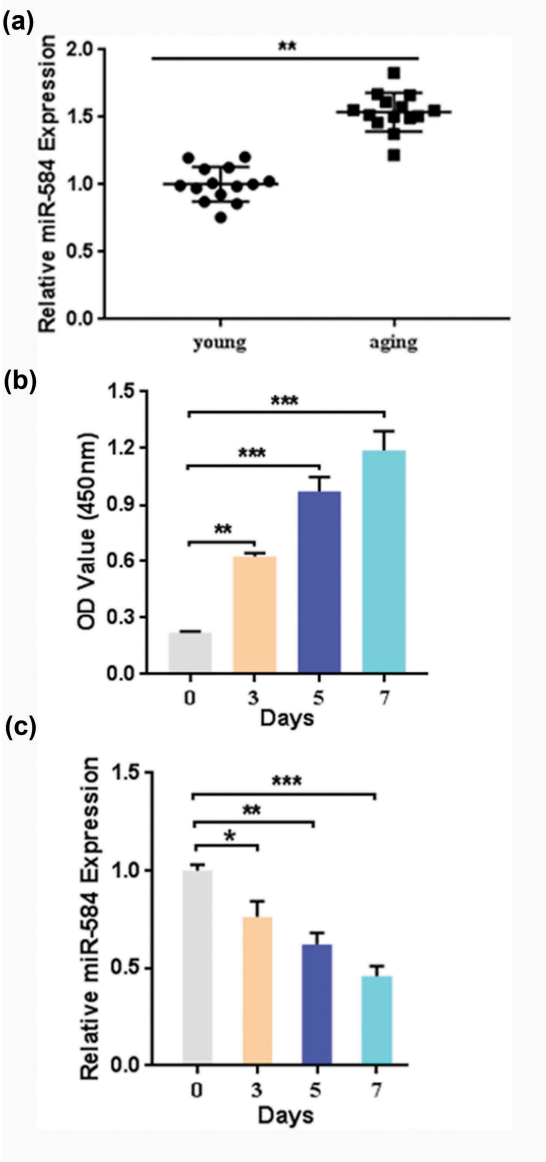

Figure 1. miR-584 expresses in dental pulp tissues and is down-regulated during the proliferation of DPSCs. (a) The expression levels of miR-584 in young and aging human dental pulp tissues were explored using miRNA qRT-PCR. (b) The growth rate of DPSCs during the proliferation at different time points. (c) qRT-PCR was conducted to determine the expression of miR-584 after DPSCs were cultured for 3, 5, and 7 days. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

mimics showed a suppression in migration and miR-584 inhibitor displayed a promotion compared with the control group and non-transfected group (NT) (Figure 2(c,d)). We used Flow cytometry analysis to detect DPSCs cell cycle, and found that miR-584 mimics increased the number of DPSCs in the G0/G1 phase but decreased that in the S phase (Figure 2(e,f)), miR-584 inhibitor decreased the number of cells in the G0/G1 phase, implying that miR-584 may suppress G1-to-S phase transition of DPSCs. These results revealed that miR-584 might inhibit the proliferation and migration of DPSCs.

3.4. miR-584 targets TAZ in 3′UTR and negatively regulates its expression

In a previous study, we found that TAZ promotes the proliferation and migration of DPSCs. To reveal the molecular mechanisms of miR-584 inhibiting proliferation and cell cycle of DPSCs, we selected the possible target genes of miR-584 with online prediction bioinformatic analyses (TargetScan, miRanda and PicTar). Among the candidates, we selected TAZ for a potential target gene of miR-584 (Figure 3(a)). To validate whether miR-584 directly binds TAZ, we conducted luciferase activity assay. The results showed luciferase activity was significantly suppressed in co-transfection of miR-584 mimics and the wild-type TAZ 3′UTR compared with those transfected with miR-584 mimics and the mutant TAZ 3′UTR (Figure 3(b)). To further confirm the regulation of miR-584 on TAZ. We transfected DPSCs with miR-584 mimics or miR-584 inhibitor, the results showed miR-584 mimics remarkably reduced TAZ protein expression, and miR-584 inhibitor markedly promoted TAZ expression compared to the control group (Figure 3(c)). Furthermore, western blot results revealed that TAZ expression was similar in DPSCs transfected with pcDNA3.1-TAZ-wt-UTR and pcDNA3.1-TAZ-mut-UTR, however, TAZ expression was significantly reduced in DPSCs treated with miR-584 and the pcDNA3.1-TAZ-wt-UTR, but no difference was observed in cells transfected with miR-584 and the pcDNA3.1-TAZ-mut-UTR. Moreover, miR-584 restoration could partially abrogate the TAZ-wt-UTR-induced acceleration of CTGF (downstream target protein of TAZ) (Figure 3(d)). According to the results, we determined TAZ and miR-584 have a significant inverse correlation, and TAZ could directly bind to miR-584.

3.5. TAZ promotes proliferation and migration of DPSCs

To investigate TAZ expression of dental pulp tissues, we detected TAZ expression by western blot assay. The result showed young dental pulp tissues displayed higher protein levels of TAZ than those in aging tissues (Figure 4(a)). To observe the TAZ expression in DPSCs, we evaluated TAZ expression in DPSCs by immunofluorescence. As showed

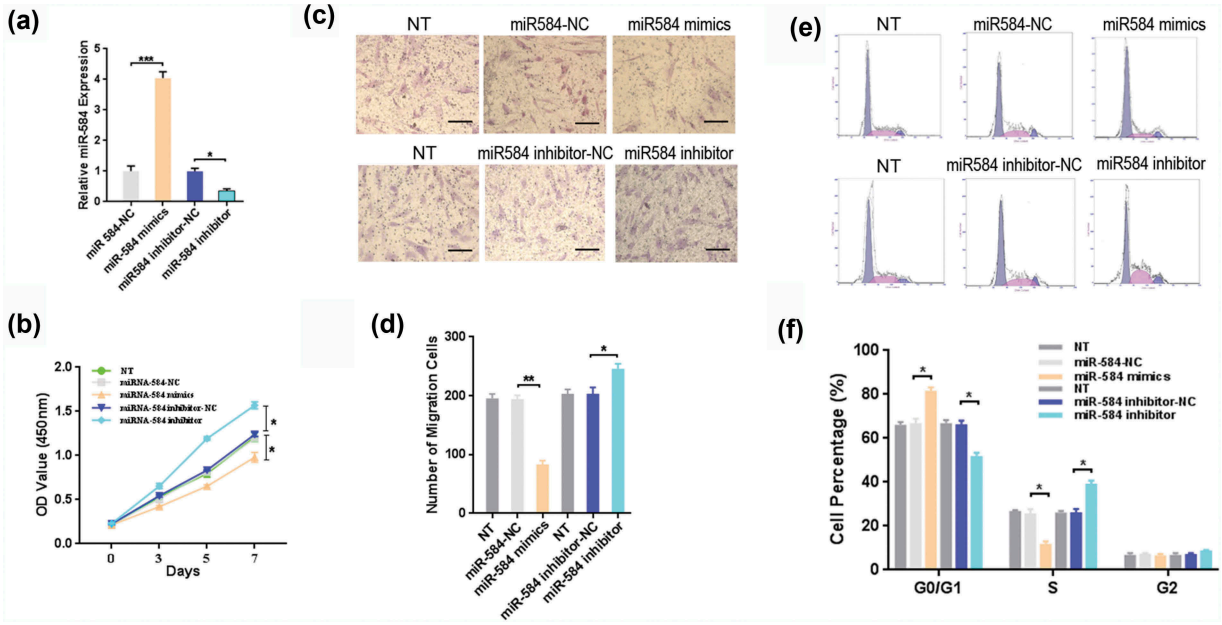

Figure 2. miR-584 inhibits proliferation and migration of DPSCs. (a) Relative mRNA expression of miR-584 was detected in DPSCs transfected with miR-584 mimics, miR-NC or miR-584-inhibitor, miR-584-inhibitor-NC, respectively. (b) CCK-8 assay was used to determine the proliferation of DPSCs transfected with miR-584-mimics or miR-584-inhibitor and corresponding control group and non-transfected group (NT) at 3, 5 and 7 d post-transfection. (c, d) Transwell assay was evaluated cell migration abilities of DPSCs transfected with miR-584 mimics, miR-NC or miR-584-inhibitor, miR-584-inhibitor-NC and non-transfected group (NT). Scale bar = 100 μm. (e, f) Effects of miR-584 on cell cycle alteration of DPSCs were detected by flow cytometry (FCM). *$p < 0.05$, **$p < 0.01$.

in Figure 4(b), TAZ expression was both observed in the nucleus and cytoplasm of DPSCs. We measured TAZ expression during the proliferation of DPSCs by western blot (Figure 4(c)) and qPCR (Figure 4(d)), the results showed TAZ expression was increased gradually after cultured for 0, 3, 5 and 7 days in a time-dependent manner. To determine whether TAZ mediated the proliferation and migration of DPSCs, we transfected DPSCs with TAZ overexpression vector (pcDNA3.1-TAZ) or TAZ silence RNA (siRNA). The results showed that TAZ protein (Figure 4(e)) and mRNA (Figure 4(f)) levels were significantly upregulated by overexpression of TAZ, and suppressed by si-TAZ. We also observed that overexpression of TAZ enhanced proliferation (Figure 4(g)) and migration (Figure 4(h,i)) of DPSCs, and si-TAZ decreased proliferation and migration of DPSCs.

3.6. TAZ overexpression reverses the inhibition of miR-584 mimics on DPSCs proliferation and migration

To determine the relation of miR-584, TAZ and DPSCs proliferation, migration. We performed rescue experiments by overexpression of miR-584 and then overexpression of TAZ in DPSCs. Western blotting analysis confirmed that overexpression of TAZ rescued the reduced protein expression level of TAZ induced by overexpression of miR-584 (Figure 5(a)). The results of CCK-8 (Figure 5(b)) and transwell (Figure 5(c,d)) assay showed the inhibition of miR-584 on DPSCS proliferation and migration was partly restored by overexpression of TAZ. Flow cytometry results revealed that miR-584 mimics increased the number of DPSCs in the G0/G1 phase, pcDNA3.1-TAZ partly abrogated the miR-584-induced inhibition of cell cycle transition (Figure 5 (e,f)). The protein expression levels of cyclin D, CDK4 (the cell cycle promoters) and CTGF (downstream target protein of TAZ) were decreased by miR-584 mimics, and were restored when DPSCs were co-transfected with pcDNA3.1-TAZ (Figure 5(g)). These findings demonstrate that miR-584 inhibits DPSCs proliferation and migration by functionally targeting TAZ.

3.7. miR-584 mimics inhibits the proliferation of DPSCs via PI3K/AKT pathway

PI3K/AKT signaling pathway is an important pathway involving in the proliferation of cells.

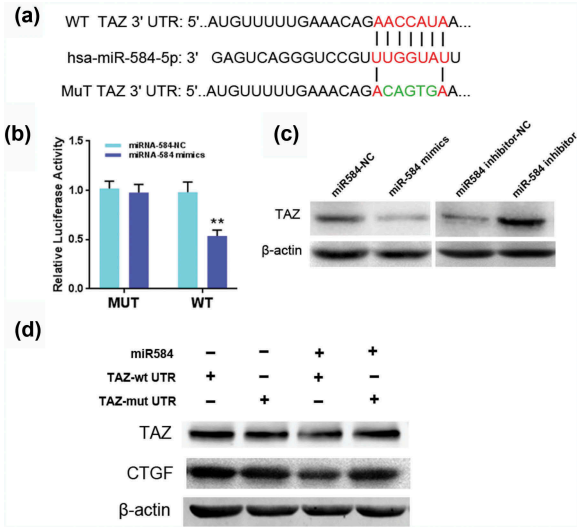

Figure 3. miR-584 directly targets TAZ in 3′UTR and negatively regulates TAZ expression. (a) Schematic description of wild-type or mutated TAZ 3′UTR containing the predicted miR-584 binding sites. (b) Luciferase activity was detected to verify that miR-584 directly bound to the 3′-UTR of TAZ after transfected with miR-584 mimics or miR-NC and pmirGLO-TAZ-3′-UTR WT or pmirGLO-TAZ-3′-UTR Mut. (c) The protein expressions of TAZ were detected by western blot in DPSCs transfected with miR-584 mimics, miR-584 inhibitor, or matched controls. (d) DPSCs were transfected with TAZ-expressing vectors, along with miR-584, and TAZ, CTGF expression were detected by immunoblot. **p < 0.01.

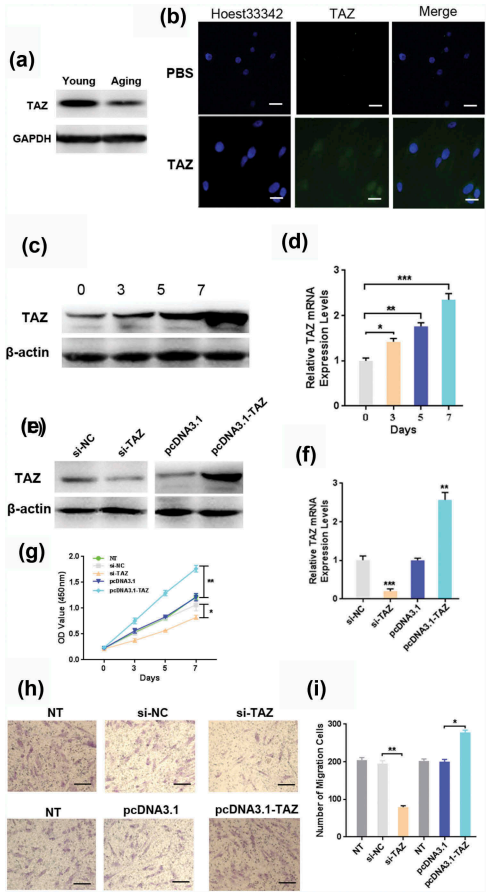

Figure 4. TAZ expresses in dental pulp tissue and promotes the proliferation and migration of DPSCs. (a) TAZ protein level was determined using western blot in young and aging dental pulp tissues. (b) Representative image of TAZ expressed in DPSCs was detected by immunofluorescence staining. Cell nuclei were visualized using Hoechst 33342. (×200) Scale bar = 50 μm. (c) Western blot and (d) qRT-PCR were carried out to evaluate the protein and mRNA expressions of TAZ in DPSCs after incubated for 3, 5, and 7 day(s). (e) The protein and (f) mRNA expressions of TAZ were estimated by western blot and qRT-PCR in DPSCs transfected with si-con, si-TAZ, pcDNA3.1 vector or pcDNA3.1-TAZ vector, respectively. (g) Cell proliferation was assessed by CCK-8, and (H I) migration ability was examined by transwell assays in DPSCs transfected with si-con, si-TAZ, pcDNA3.1 vector or pcDNA3.1-TAZ vector and non-transfected groups (NT), respectively. Scale bar = 100 μm. *p < 0.05, **p < 0.01, ***p < 0.001.

Thus, western blotting analysis was performed to examine the expression levels of AKT, p-AKT, Cyclin D and CTGF in DPSCs after transfection with miR-NC or miR-584-mimics. We found that miR-584 overexpression suppressed the expression of AKT, p-AKT, Cyclin D, CTGF (Figure 6(a,b)). These results implied that miR-584 inhibits PI3K/AKT signaling pathway in DPSCs.

4. Discussion

In the present study, we identify that miR-584 inhibits the proliferation and migration of DPSCs by suppressing its downstream target TAZ expression.

microRNAs have shown to regulate many cell biological functions, such as cell cycle, migration, proliferation, apoptosis and tissue development [27–29]. miRNAs provide new insights into additional molecular mechanisms of post-transcriptional regulation beyond that of transcription factors[30]. Some studies have indicated miR-584 could inhibit tumor growth in some human cancers, including lung cancer[31], thyroid cancer[32], glioma, and gastric cancer[19]. Over-expression of miR-584 suppressed cell proliferation, migration, invasion and increased apoptosis by repressing different mRNA translations in various tumor cells [31,32]. Previous reports confirmed that miRNAs play crucial roles in mediating odontogenic differentiation of DPSCs[14], immune response, and inflammation of dental pulp [4,20]. It is reported that miR-584 was positively expressed in DPSCs, and was remarkable upregulated in inflamed pulps compared

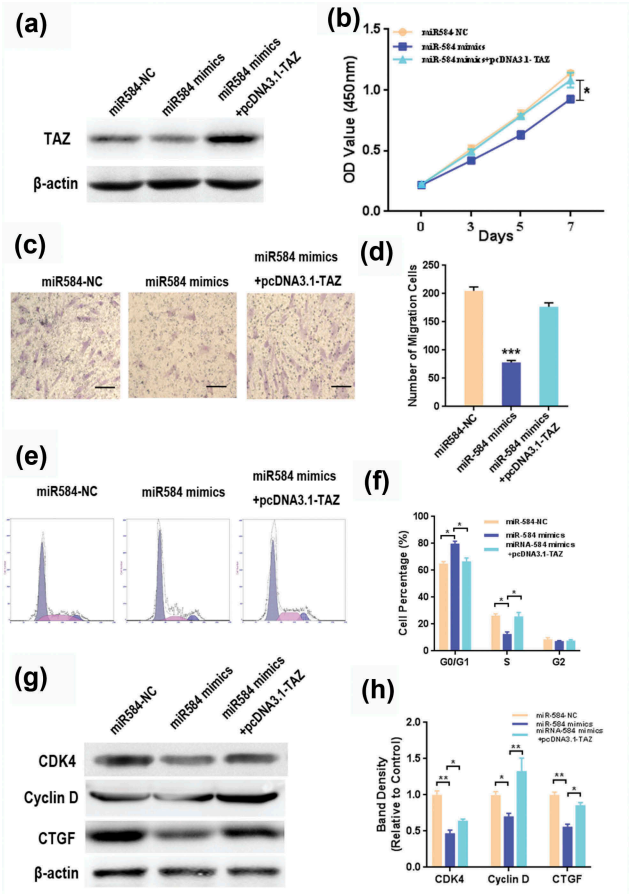

Figure 5. TAZ overexpression partially reverses the inhibition effects of miR584-mimics on DPSCs proliferation and migration. (a) Western blot analysis of TAZ protein expression levels in DPSCs transfected with miR-584 NC, miR-584 mimics or miR-584 mimics + pcDNA3.1-TAZ. (b) Cell proliferation and (c d) migration ability were assessed by CCK-8 and transwell assays transfected with miR-584 NC, miR-584 mimics or miR-584 mimics + pcDNA3.1-TAZ. Scale bar = 100 μm. (e f) Cell cycle distribution was detected by FCM in DPSCs transfected with miR-584 NC, miR-584 mimics or miR-584 mimics + pcDNA3.1-TAZ. (g) The protein expression levels and relative quantity results (h) of CDK4, Cyclin D, CTGF were detected by western blot in DPSCs transfected with miR-584 NC, miR-584 mimics or miR-584 mimics + pcDNA3.1-TAZ. **P < 0.01, *p < 0.05, **p < 0.01, ***p < 0.001.

to normal pulps [15,20], and involved in various biological processes [33]. In our study, miRNA-584 expression was upregulated in aging dental pulp tissue compared to young dental pulp tissue. Aging is a complicated process in which dental pulp tissues undergo age-related senescence, and the proliferation, differentiation and regenerative capacity of DPSCs progressively declines [34,35]. Previous studies indicated that the decline of proliferation potency is a hallmark of aging stem cells and DPSCs isolated from the young sample has higher proliferation abilities than DPSCs from an aging sample[36]. Next, we found the expression level of miR-584 was stepwise down-regulated followed the duration of proliferation in DPSCs. It suggested miR-584 may regulate DPSCs proliferation. Here in, we surveyed functional and mechanism researches of miR-584. The results revealed that miR-584 could suppress DPSCs proliferation and migration. The cell cycle results showed miR-584 may regulate cell-cycle arrest in G0/G1 phase, thus cell proliferation was inhibited. The current study suggests that miR-584 could be used as a negative regulatory factor in DPSCs growth.

There are various target genes of miR-584 which had been verified, such as WWP1 in gastric cancer [19], MTDH in NSCLC[31], ROCK-1 in thyroid cancer[32], PTTG1IP in glioma. To clarify the underlying mechanisms of miR-584 mediating proliferation and migration in DPSCs, based on bioinformatic analyses, we predicted TAZ as the candidate target gene of miR-584. TAZ is the major downstream effector of the Hippo pathway. TAZ plays important roles in regulating tissue homeostasis, organ development, regeneration, and tumorigenesis [37,38] by regulating cell proliferation, apoptosis, migration[39]. A recent study also indicates that TAZ promotes osteogenic differentiation of DPSCs, and inhibits adipogenic differentiation [40]. Our study provided evidence that TAZ expression was down-regulated in aging dental pulp tissues, and stepwise increased during DPSCs proliferation, which indicated an inverse correlation with the expression of miR-584. Functional study showed that TAZ promoted DPSCs proliferation and migration. Luciferase reporter results demonstrated that miR-584 directly bound TAZ to its 3′ UTR, and inhibited TAZ protein expression level by suppressing the translation of TAZ mRNA. Here, we also demonstrated that miR-584 negatively regulated TAZ expression in DPSCs. Knocking down of TAZ had the same effects as over-expression of miR-584. Furthermore, restoration of TAZ can rescue the inhibition of miR-584 mimics in DPSCs. All in all, the results implied that miR-584 may be a key factor involved in regulating DPSCs proliferation and migration, and TAZ was a direct downstream target gene of miR-584.

Cell cycle comprises complex pathways at different biological proccess[41]. PI3K/AKT signaling pathway plays important roles in the mediation of

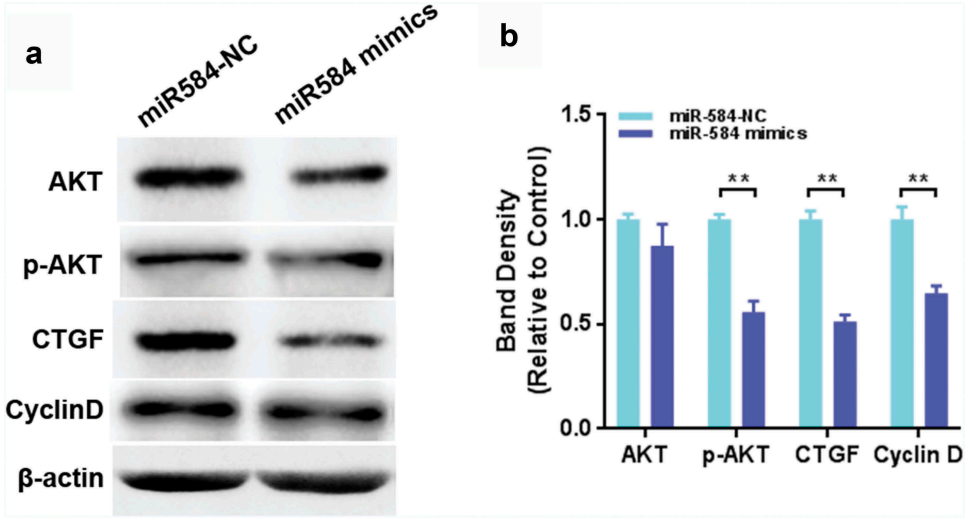

Figure 6. miR-584-mimics suppress proliferation of DPSCs via PI3K/AKT pathway. (a) Western blot analysis of AKT, p-AKT, CTGF, cyclin D protein expression in DPSCs transfected with miR-584 NC or miR-584 mimics. (b) The protein bands of AKT, p-AKT, CTGF, cyclin D were quantified by Image J software and normalized to GAPDH. **P < 0.01.

cell cycle. AKT can cause a series of positively cascade reactions in G1/S cell cycle progression[42]. The previous study has reported that TAZ could regulate the proliferation of retinoblastoma cells by AKT signaling pathway[38]. In our study, we confirmed that overexpression of miR-584 inhibited the protein expression levels of AKT, p-AKT, CTGF. Taken together, our findings suggested that the regulation of cell cycle caused by miR-584 is associated with the AKT pathway in DPSCs.

In summary, our results elucidated that miR-584 regulates the development and progression of DPSCs by directly binding to TAZ via the AKT pathway. Therefore, miR-584 could be acted as a promising molecular target in pulp disease treatment.

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Disclosure statement

The authors have no potential conflict of interest.

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References

[1] Chamberlain G, Fox J, Ashton B, et al. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells. 2007;25:2739–2749.

[2] Li T, Guo H, Li H, et al. MicroRNA-92a-1-5p increases CDX2 by targeting FOXD1 in bile acids-induced gastric intestinal metaplasia. Gut. 2019;68(10):1751–1763.

[3] Gu Y, Bai Y, Zhang D. Osteogenic stimulation of human dental pulp stem cells with a novel gelatin-hydroxyapatite-tricalcium phosphate scaffold. J Biomed Mater Res A. 2018;106:1851–1861.

[4] Liu W, Gong Q, Ling J, et al. Role of MiR-424 on angiogenic potential in human dental pulp cells. J Endod. 2014;40:76–82.

[5] Zhao X, Gong P, Lin Y, et al. Characterization of alpha-smooth muscle actin positive cells during multilineage differentiation of dental pulp stem cells. Cell Prolif. 2012;45:259–265.

[6] Sonoda S, Mei YF, Atsuta I, et al. Exogenous nitric oxide stimulates the odontogenic differentiation of rat dental pulp stem cells. Sci Rep. 2018;8:3419.

[7] Gu S, Ran S, Liu B, et al. miR-152 induces human dental pulp stem cell senescence by inhibiting SIRT7 expression. FEBS Lett. 2016;590:1123–1131.

[8] Arthur A, Rychkov G, Shi S, et al. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem Cells. 2008;26:1787–1795.

[9] Zhang P, Yang W, Wang G, et al. miR-143 suppresses the osteogenic differentiation of dental pulp stem cells by inactivation of NF-kappaB signaling

pathway via targeting TNF-alpha. Arch Oral Biol. 2018;87:172–179.
[10] Wang BL, Wang Z, Nan X, et al. Downregulation of microRNA-143-5p is required for the promotion of odontoblasts differentiation of human dental pulp stem cells through the activation of the mitogen-activated protein kinases 14-dependent p38 mitogen-activated protein kinases signaling pathway. J Cell Physiol. 2019;234:4840–4850.
[11] Higashi T, Hayashi H, Ishimoto T, et al. miR-9-3p plays a tumour-suppressor role by targeting TAZ (WWTR1) in hepatocellular carcinoma cells. Br J Cancer. 2015;113:252–258.
[12] Lujambio A, Lowe SW. The microcosmos of cancer. Nature. 2012;482:347–355.
[13] Luo H, Gao H, Liu F, et al. Regulation of Runx2 by microRNA-9 and microRNA-10 modulates the osteogenic differentiation of mesenchymal stem cells. Int J Mol Med. 2017;39:1046–1052.
[14] Gong Q, Wang R, Jiang H, et al. Alteration of microRNA expression of human dental pulp cells during odontogenic differentiation. J Endod. 2012;38:1348–1354.
[15] Sehic A, Tulek A, Khuu C, et al. Regulatory roles of microRNAs in human dental tissues. Gene. 2017;596:9–18.
[16] Zhan FL, Liu XY, Wang XB. The role of MicroRNA-143-5p in the differentiation of dental pulp stem cells into odontoblasts by targeting Runx2 via the OPG/RANKL signaling pathway. J Cell Biochem. 2018;119:536–546.
[17] Ueno K, Hirata H, Shahryari V, et al. Tumour suppressor microRNA-584 directly targets oncogene Rock-1 and decreases invasion ability in human clear cell renal cell carcinoma. Br J Cancer. 2011;104:308–315.
[18] Abdelfattah N, Rajamanickam S, Panneerdoss S, et al. MiR-584-5p potentiates vincristine and radiation response by inducing spindle defects and DNA damage in medulloblastoma. Nat Commun. 2018;9:4541.
[19] Li Q, Li Z, Wei S, et al. Overexpression of miR-584-5p inhibits proliferation and induces apoptosis by targeting WW domain-containing E3 ubiquitin protein ligase 1 in gastric cancer. J Exp Clin Cancer Res. 2017;36:59.
[20] Zhong S, Zhang S, Bair E, et al. Differential expression of microRNAs in normal and inflamed human pulps. J Endod. 2012;38:746–752.
[21] Debaugnies M, Sanchez-Danes A, Rorive S, et al. YAP and TAZ are essential for basal and squamous cell carcinoma initiation. EMBO Rep. 2018;19. DOI:10.15252/embr.201845809
[22] Plouffe SW, Lin KC, Moore JL 3rd, et al. The Hippo pathway effector proteins YAP and TAZ have both distinct and overlapping functions in the cell. J Biol Chem. 2018;293:11230–11240.
[23] Wu X, Zhang Y, Xing Y, et al. High-fat and high-glucose microenvironment decreases Runx2 and TAZ expression and inhibits bone regeneration in the mouse. J Orthop Surg Res. 2019;14:55.
[24] Zheng L, Xiang C, Li X, et al. STARD13-correlated ceRNA network-directed inhibition on YAP/TAZ activity suppresses stemness of breast cancer via co-regulating Hippo and Rho-GTPase/F-actin signaling. J Hematol Oncol. 2018;11:72.
[25] Kim AR, Park JI, Oh HT, et al. TAZ stimulates liver regeneration through interleukin-6-induced hepatocyte proliferation and inhibition of cell death after liver injury. Faseb J. 2019;33(5):5914–5923.
[26] Tian S, Tian X, Liu Y, et al. Effects of TAZ on human dental pulp stem cell proliferation and migration. Mol Med Rep. 2017;15:4326–4332.
[27] Tome M, Lopez-Romero P, Albo C, et al. miR-335 orchestrates cell proliferation, migration and differentiation in human mesenchymal stem cells. Cell Death Differ. 2011;18:985–995.
[28] Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136:215–233.
[29] Fan J, An X, Yang Y, et al. MiR-1292 targets FZD4 to regulate senescence and osteogenic differentiation of stem cells in TE/SJ/Mesenchymal tissue system via the Wnt/beta-catenin pathway. Aging Dis. 2018;9:1103–1121.
[30] Han TS, Voon DC, Oshima H, et al. Interleukin 1 up-regulates MicroRNA 135b to promote inflammation-associated gastric carcinogenesis in mice. Gastroenterology. 2019;156:1140–55 e4.
[31] Zhang Y, Wang Y, Wang J. MicroRNA-584 inhibits cell proliferation and invasion in non-small cell lung cancer by directly targeting MTDH. Exp Ther Med. 2018;15:2203–2211.
[32] Xiang J, Wu Y, Li DS, et al. miR-584 suppresses invasion and cell migration of thyroid carcinoma by regulating the target oncogene ROCK1. Oncol Res Treat. 2015;38:436–440.
[33] Vasanthan P, Govindasamy V, Gnanasegaran N, et al. Differential expression of basal microRNAs' patterns in human dental pulp stem cells. J Cell Mol Med. 2015;19:566–580.
[34] Alt EU, Senst C, Murthy SN, et al. Aging alters tissue resident mesenchymal stem cell properties. Stem Cell Res. 2012;8:215–225.
[35] Iohara K, Murakami M, Nakata K, et al. Age-dependent decline in dental pulp regeneration after pulpectomy in dogs. Exp Gerontol. 2014;52:39–45.
[36] Ning T, Shao J, Zhang X, et al. Ageing affects the proliferation and mineralization of rat dental pulp stem cells under inflammatory conditions. Int Endod J. 2020;53:72–83.
[37] Zhan T, Huang X, Tian X, et al. Downregulation of MicroRNA-455-3p links to proliferation and drug

resistance of pancreatic cancer cells via targeting TAZ. Mol Ther Nucleic Acids. 2018;10:215–226.

[38] Zhang Y, Xue C, Zhu X, et al. Suppression of microRNA-125a-5p upregulates the TAZ-EGFR signaling pathway and promotes retinoblastoma proliferation. Cell Signal. 2016;28:850–860.

[39] Zuo QF, Zhang R, Li BS, et al. MicroRNA-141 inhibits tumor growth and metastasis in gastric cancer by directly targeting transcriptional co-activator with PDZ-binding motif, TAZ. Cell Death Dis. 2015;6:e1623.

[40] Suh JS, Kim KS, Lee JY, et al. A cell-permeable fusion protein for the mineralization of human dental pulp stem cells. J Dent Res. 2012;91:90–96.

[41] Nussinov R, Tsai CJ, Jang H, et al. Oncogenic KRAS signaling and YAP1/beta-catenin: similar cell cycle control in tumor initiation. Semin Cell Dev Biol. 2016;58:79–85.

[42] Liang J, Slingerland JM. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. Cell Cycle. 2003;2:339–345.