SLIT3: a Novel Regulator of Odontogenic Differentiation through Akt/Wnt/β-catenin Signaling Pathway

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Article

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Abstract

The odontogenic differentiation of Stem cells from apical papilla (SCAP) is regulated by many extracellular matrix proteins, which plays a crucial role in dentin formation and regeneration. Extracellular matrix protein SLIT3, a classical axon guidance molecule, can link bone resorption to formation as clastokine. However, there is little information about SLIT3 in odontogenesis. Therefore, our study is aimed to explore the effects and possible mechanism of SLIT3 on the proliferation and differentiation of SCAP. Through Immunohistochemical staining and re-analysis of single-cell RNA sequencing and microarray datasets, we found that SLIT3 was expressed in the dental papilla and odontoblast layer of the developing molar tooth of mice. Real time polymerase chain reaction (RT-PCR) and Western blot assays also revealed an increased expression of SLIT3 during the odontogenic differentiation of SCAP. Afterwards, SLIT3 siRNA was used to knockdown SLIT3 and recombinant human SLIT3 (rhSLIT3) protein was used to treat SCAP. Cell Counting Kit-8 assays (CCK8) assays showed SLIT3 promoted proliferation of SCAP. Alkaline phosphatase (ALP) staining and Alizarin red staining were decreased/increased accordingly. Odontogenic markers DMP-1 and DSPP were also down-regulated/up-regulated. In addition, p-Akt and p-GSK3β levels were increased in rhSLIT3-treated SCAP and the movement into cell nucleus of β-catenin was promoted. The effect of SLIT3 was canceled after treatment with the inhibitor of Akt/Wnt/β-catenin signaling pathway. Taken together, our data show that SLIT3 could promote the proliferation and odontogenic differentiation of SCAP by activating Akt/Wnt/β-catenin signaling pathway.

Introduction

Pulp infection and necrosis or periapical inflammation due to trauma or caries often occur in immature permanent teeth, resulting in stagnation of root development. The root length of immature permanent teeth is short, the dentin wall is thin, and there is no apical stop, so root fracture is easy to happen[1–3]. Therefore, the continued development of the root of immature permanent teeth with pulp necrosis is an ideal treatment direction. It is necessary to explore and improve the signal molecules and mechanisms that regulate the process of tooth root development.

Root development occurs after crown formation, which is initiated by the Hertwigs' epithelial root sheath[4,5]. Mesenchymal stem cells in tooth germ receive root development signals from the Hertwigs' epithelial root sheath. They have multi-directional differentiation potential and can differentiate into odontoblast, dental pulp cell, cementoblast and periodontal ligament cell, which play important roles in root development[6,7].

Stem cells from apical papilla (SCAP) are important mesenchymal stem cells derived from apical papilla tissue of immature permanent teeth. They were initially isolated and cultured by Sonanyama, et al[8]. SCAP is widely regarded as the precursor cells of odontoblast, which elongates and polarizes into odontoblast under the induction of epithelial root sheath, and then secretes and mineralizes dentin[9]. Because of its good proliferation ability and multi-directional differentiation potential, SCAP is often used in laboratory studies as an ideal cell model for the study of tooth development and odontoblast differentiation[10,11].

As structural proteins, extracellular matrix proteins can interact with receptors and integrins in cytomembrane, regulate the activities of growth factors and proteases, which plays an important role in dentin formation and mineralization[12,13]. In recent years, SLIT3, a member of the extracellular matrix protein SLIT family, has been found to be involved in many life activities, including angiogenesis, tumorigenesis, inflammation and bone metabolic balance[14–16]. SLIT3 is a new bone coupling factor, which plays a regulatory role in promoting bone formation and inhibiting bone resorption in bone metabolic balance[17]. Although bone and dentin are different mineralized tissues, they have many similarities in the process of mineralization. However, there are few studies on SLIT3 in the field of tooth development until now.

Akt is a very important signal molecule in the cellular kinase signaling network, which is involved in regulating a variety of cell life activities, such as glucose metabolism, cell proliferation, cell differentiation, cell migration and so on[18,19]. GSK3β is not only one of the downstream effector molecules of Akt, but also the key regulator of Wnt/β-catenin pathway, which mediates the crosstalk between P13K/Akt and Wnt/β-catenin pathway[20]. After GSK3β is phosphorylated, the degradation of β-catenin is inhibited so that β-catenin accumulates gradually in the cytoplasm and then is transferred into nuclear[21]. Wnt/β-catenin signaling pathway has been proved to play an important role in tooth development. A number of studies have shown that Wnt/β-catenin signaling pathway has a positive stimulating effect on the proliferation and differentiation of SCAP, which is the key regulatory signal of odontoblast differentiation[22,23].

The purpose of this study was to investigate the spatio-temporal expression of SLIT3 during tooth development *in vivo* and *in vitr*o, and its effect on the proliferation and odontogenic differentiation of SCAP. The role of Akt/Wnt/β-catenin signaling pathway in the effect of SLIT3 on odontogenic differentiation of SCAP was also evaluated.

Results

1. Defining Dental Mesenchyme Subgroups at Different Developmental Stages in the Integrated Single-Cell Transcriptomic Atlas

Data cleaning and clustering analyses were conducted on GSE189381 in a manner similar to that described in a previously published study on cranial neural crest cells[24]. The single-cell RNA sequencing data underwent preprocessing steps, including quality control, integration, principal component analysis, and dimensionality reduction. UMAP plots (Figure 1A) were used to visually represent the single-cell data from E13.5 to PN7.5 in the tooth and surrounding tissue. Based on further subcluster analyses and known markers for dental mesenchyme, the dental mesenchyme subcluster was identified in each UMAP plot, denoted by a dotted frame (Figure 1B).

At E13.5, the early stage of tooth development, the major clusters were classified into two categories: dental mesenchyme and epithelial cell clusters. By E14.5, two distinct clusters representing dental follicle

and dental papilla cells were observed, suggesting lineage segregation at this stage. At E16.5, the dental follicle differentiated into lateral and apical follicles, while the dental papilla formed separate coronal and apical papillae. When reaching PN3.5, nearing the onset of root development, the follicle differentiation pattern displayed similarities to that seen at E16.5, whereas the papilla subclusters included coronal papilla, middle papilla, apical papilla, and odontoblast clusters. Finally, at PN7.5, the cell population resembled that at PN3.5, indicating the likely completion of tooth root development. Notably, Slit3 exhibited constant expression in the dental mesenchyme subclusters, while showing minimal to no expression in the epithelial cell subcluster.

2. SLIT3 is continuously expressed in odontoblasts of developing mandibular first molar in mice.

In immunohistochemical staining of paraffin sections of the first mandibular molar tissues of PN1 mice, SLIT3 was expressed in odontoblasts, ameloblasts, and the middle layer (Figure 1C). On PN7, SLIT3 was expressed in the dental papilla, odontoblasts, ameloblasts and the middle layer (Figure 1D). In PN14 mice, SLIT3 was expressed in the odontoblast layer (Figure 1E), but not in other sites. In the immunohistochemical staining results of tissue sections of PN21 mice, SLIT3 was expressed in both crown and root odontoblasts, and the expression of SLIT3 was stronger in root odontoblasts (Figure 1E and F). In conclusion, SLIT3 is not only positively expressed in the tooth embryo, but also continuously expressed in odontoblasts during the development of mouse mandibular molars, suggesting that SLIT3 plays an important role in the differentiation and maturation of odontoblasts and the formation of hard tissues.

3. Increased gene and protein expression of SLIT3 during odontogenic differentiation of SCAP.

In this study, SCAP was isolated from an undeveloped caries-free third molar. In the process of mineralization medium induced odontogenic differentiation of SCAP, RT-PCR results indicated that the expression of SLIT3 RNA in the experimental group was significantly increased ($P < 0.05$), which was statistically different from that in the control group, and the difference became more obvious as time went on ($P < 0.0001$) (Figure 1G). Western blot results also indicated that the protein expression level of SLIT3 in the experimental group was also higher than that in the control group during the odontogenic differentiation of SCAP (Figure 1H). In the microarray dataset, Slit3 was found to be highly expressed in both the buccal dental follicle group and the lingual dental papilla group, while its expression was lower in both the buccal and lingual dental epithelial group (Figure 1I).

4. SLIT3 can promote the proliferation of SCAP.

SCAP with low/high expression of SLIT3 were simulated *in vitro*. The results of RT-PCR indicated that SLIT3 expression in SLIT3 siRNA group was significantly lower than NC group, while the expression of SLIT3 in rhSLIT3 treated group was significantly higher than control group (Figure 2A and Figure 2B). It is credible that the SCAP with low/high expression of SLIT3 can be used for the detection of subsequent experiments.

Then, we used CCK8 kit to detect the effect of SLIT3 expression on the proliferation of SCAP (Figure 2C). The results suggested that proliferation of SCAP was enhanced when the expression of SLIT3 was increased, but was weakened in cells with low SLIT3 expression, which proved that SLIT3 could promote the proliferation of SCAP.

5. SLIT3 could promote the mineralization of SCAP and up-regulate the expression of DMP-1 and DSPP.

In the SCAP with low expression of SLIT3, after mineralization induction for 7 days, the ALP staining results showed that the staining degree of SLIT3 siRNA group was lighter than negative control group (Figure 3A). After mineralization induction for 14 days, the ARS staining results also revealed that the mineralized nodules of SLIT3 siRNA group was less than negative control group (Figure 3B), indicating that the odontogenic differentiation ability of SCAP was inhibited followed the decrease of SLIT3 expression.

In the SCAP with rhSLIT3 treated, after mineralization induction for 7 days, the ALP staining results revealed that the degree of mineralization of rhSLIT3 group was higher than the control group (Figure 3H). After mineralization induction for 14 days, the ARS staining results also revealed that the degree of mineralization of rhSLIT3 group was higher than the control group (Figure 3I), indicating that the odontogenic differentiation ability of SCAP was promoted after the increase of SLIT3 expression.

In order to further verify that whether SLIT3 can promote the odontogenic differentiation of SCAP, RT-PCR and Western blot were used to detect the effect of SLIT3 on the expression of dentin formation markers. The RNA and protein samples of SCAP with low/high expression of SLIT3 were collected on the 7th, 10th and 14th day after mineralization induction. The results of RT-PCR demonstrated that the expression of DMP-1 and DSPP decreased in siRNA group, while the expression level of DMP-1 and DSPP was elevated after the increase of SLIT3 in rhSLIT3 group (Figure 3C, D, J and K). The results of Western blot also demonstrated that after 7, 10 and 14 days of mineralization induction, the protein expression of DMP-1 and DSPP decreased in siRNA group, while the protein expression of DMP-1 and DSPP increased in rhSLIT3 group (Figure 3E, F, G, L, M and N).

6. SLIT3 can activate Akt/Wnt/β-catenin signaling pathway leading to the promotion of odontogenic differentiation of SCAP.

In our study, we found that SLIT3 could activate Akt/Wnt/β-catenin signaling pathway. SLIT3 could regulate the activity of pivotal signaling molecule, like Akt, GSK3β and β-catenin, of Akt/Wnt/β-catenin pathway, and ultimately lead to the activation of target genes in the nucleus (Figure 6D), thus exerting its ability to promote the odontogenic differentiation of SCAP. When Akt/Wnt/β-catenin signaling pathway was blocked, the effect of SLIT3 on odontogenic differentiation of SCAP was cancelled.

a) SLIT3 can promote the phosphorylation of Akt and GSK-3β in SCAP.

After the addition of recombinant human SLIT3 protein, the results of Western blot suggested that the levels of p-Akt and p-GSK3β in SCAP were significantly increased after 30- and 60-minutes stimulation

(Figure 4A, B and C) respectively, indicating that SLIT3 can activate and phosphorylate Akt after binding to the receptors on the cell membrane, and then promote the phosphorylation of GSK3β, resulting in the inactivation of GSK3β.

b) SLIT3 can reduce the degradation of β-catenin and promote its nuclear translocation.

After the stimulation of rhSLIT3 protein for 60 mins or later, the results of Western blot revealed that the level of β-catenin in the nucleus of SCAP significantly increased, especially at 90min and 120min (Figure 5A and B). It is suggested that SLIT3 can lead to the accumulation of β-catenin in the nucleus of SCAP.

In order to further clarify the changes of β-catenin in SCAP after the stimulation of rhSLIT3 protein, we localized the distribution of β-catenin in SCAP by cellular immunofluorescence staining (Figure 4C). The results suggested that β-catenin accumulated gradually in SCAP after stimulation for 60 minutes, and a small part of β-catenin transferred into the nucleus. After stimulation for 90 minutes, β-catenin was still accumulating and the level of β-catenin in nucleus increased gradually. After stimulation for 120 minutes, β-catenin continued to accumulate in the SCAP and more β-catenin transferred into the nucleus. These results indicated that SLIT3 can inhibit the degradation of β-catenin in SCAP, making it accumulate and transfer into the nucleus to play a regulatory role.

c) The promotion of SLIT3 on odontogenic differentiation of SCAP was cancelled after inhibition of Akt/Wnt/β-catenin signaling pathway.

After blocking Akt/Wnt/β-catenin signaling pathway with inhibitor Resibufogenin, we explored whether the effect of SLIT3 on odontogenic differentiation of SCAP was changed. The results of RT-PCR and Western blot demonstrated that the expression of dentin formation markers, DMP-1 and DSPP, was decreased after adding inhibitor Resibufogenin, which suggested that the odontogenic differentiation ability of SCAP was inhibited. When Resibufogenin and rhSLIT3 protein were added at the same time, the expression of DMP-1 and DSPP was not significantly up-regulated compared with only adding Resibufogenin, indicating that the effect of SLIT3 on promoting odontogenic differentiation of SCAP was cancelled (Figure 6A, B and C). The above results proved that SLIT3 could promote the odontogenic differentiation of SCAP through Akt/Wnt/β-catenin signaling pathway (Figure 6D).

Discussion

As structural proteins, extracellular matrix proteins can regulate the activities of growth factors and proteases by interacting with receptors and integrins on the cell membrane[25], which plays an important role in dentin formation and mineralization. Extracellular matrix protein SLIT3 initially entered the vision of researchers as guidance for axonal development[26]. With the gradual in-depth and clarified study of SLIT3 in the field of neurodevelopment, many researchers began to explore the role of SLIT3 in other life activities. So far, SLIT3 has been found to be involved in angiogenesis, tumorigenesis, inflammation, regulation of bone metabolism and so on[27,28]. Some studies have found that SLIT3 is a new coupling factor of bone metabolism, which can inhibit the migration and fusion of pre-osteoclasts so that

inhibiting bone resorption; it can also promote the proliferation and migration of osteoblasts, and even indirectly promote bone formation by promoting angiogenic factors[17,29].

Bone and teeth are important hard tissue organs, and they have many similarities in the process of formation. For example, bone morphogenetic protein (BMP), osterix (OSX) and bone sialoprotein (BSP) which play important roles in bone metabolism have also been proved to play essential roles in tooth development[30–32]. However, there are few reports on the role of bone metabolic coupling factor SLIT3 in tooth development. At present, it has only been reported that the expression of RNA of Slit family (Slit1-Slit3) during the development of tooth *in vivo*[33]. RNA of Slit3 is mainly expressed in the mesenchymal components of the tooth germ, including dental papilla and dental follicle, and lasts from the bud stage to the bell stage.

The previous landmark study in single-cell transcriptomics primarily aimed to identify cellular differences and transcriptional heterogeneity during mouse root development. In our study, we conducted a comprehensive re-analysis of a previously published dataset, specifically investigating the cellular-specific and temporal expression patterns of SLIT3. Interestingly, although SLIT3 exhibited lower expression levels in epithelial cells, it consistently displayed expression in mesenchymal cell subpopulations. These findings were consistently replicated through the re-analysis of our published microarray datasets. Subsequently, our research explored the spatio-temporal expression of SLIT3 in the root development of postnatal mouse first molar, marking the first investigation of its kind. We discovered that SLIT3 is involved in odontoblast differentiation, maturation, and dentin formation. Furthermore, SLIT3 was also present in the interlayer cells and ameloblasts. This suggests a potential role in enamel genesis and formation since the expression of SLIT3 in these cells gradually diminished and eventually disappeared as enamel development reached its advanced stages.

Stem cells from apical papilla come from the undeveloped dental papilla tissue in the apical region, and have the potential of multi-directional differentiation, such as osteogenesis, odontogenesis, angiogenesis and adipogenesis[34]. Compared with dental pulp stem cells, SCAP have better proliferation efficiency, migration ability and differentiation versatility, so it is an ideal cell model for studying tooth development[35]. We used SCAP as cell model *in vitro* and first found that the expression level of both RNA and protein of SLIT3 increased during the odontogenic differentiation of SCAP. The results are consistent with the results of experiments *in vivo*, which further confirms that SLIT3 plays an important role in tooth development and dentin formation.

However, the regulatory role of SLIT3 on odontogenic differentiation of SCAP and its mechanism are still unclear. Therefore, in our study, we induced SCAP with low/high expression of SLIT3 to odontoblasts *in vitro* and found that the proliferation and odontogenic differentiation ability of SCAP were positively correlated with the expression of SLIT3.

In our study, we qualitatively detected the mineralization ability of SCAP after the change of SLIT3 by alkaline phosphatase staining and alizarin red staining. However, due to the rich and diverse

differentiation potential of SCAP, it is also possible to differentiate into osteoblasts under the condition of mineralization induction. ALP staining and ARS staining cannot accurately indicate the enhancement of osteogenic differentiation or odontogenic differentiation of SCAP. DMP-1 and DSPP are highly phosphorylated proteins belonging to the small integrin-binding ligand N-linked glycoproteins (SIBLINGs) family, which are essential for the development of teeth. DMP-1 and DSPP are common markers of odontogenic differentiation. Therefore, we further selected DMP-1 and DSPP as markers of odontogenic differentiation of SCAP and found that SLIT3 could indeed promote the expression of DMP-1 and DSPP in SCAP, which was consistent with previous results.

SLIT3 is a secretory protein about 200kDa. After SLIT3 is synthesized in the cell, it is transported to the endoplasmic reticulum and Golgi apparatus for further modification, and then secreted into the extracellular matrix[36]. SLIT3, as an extracellular matrix protein, transduces extracellular signals to intracellular by binding to ROBO family receptors on the cell membrane, changing the level of downstream signal molecules, thus regulating cell life activities[37]. The downstream signal molecules of SLIT/ROBO are mainly cytoplasmic protein kinases and cytoskeleton-related regulatory molecules, including Hakai, Myo9b, Abl, small molecule GTPase of Rho family and so on[16]. Because SLIT family proteins are involved in many life activities, there are many kinds of related downstream signal molecules, for example, SLIT2 can regulate the activity of intracellular Akt[28].

Akt is a kind of serine/threonine kinases of the AGC family. It is a key molecule in the signal network of cell kinases and regulates a variety of cell life activities, such as glucose metabolism, apoptosis, cell proliferation and differentiation, cell migration and so on[18,19,38]. Akt is the central node of many signal pathways, which transmits the signals of upstream regulatory proteins to downstream effector molecules including mTOR, FOXO1, GSK3β and so on[39–41]. However, it has not been reported whether SLIT3 can regulate the activity of Akt in SCAP. In our study, we found that exogenous SLIT3 stimulation increased the phosphorylation level of Akt in SCAP, indicating that SLIT3 could lead to the activation of Akt in SCAP.

The classical Wnt/β-catenin signaling pathway is a highly conserved signal pathway in evolution, which plays an important role in embryonic development and postnatal growth[42]. There are many studies about the role of Wnt/β-catenin in tooth morphogenesis and dental hard tissue formation[43]. Wnt/β-catenin signaling pathway has a positive stimulating effect on the proliferation and differentiation of SCAP and is a key regulator of odontoblast differentiation[22,23].

GSK3β is an essential intermediate regulatory molecule in Wnt/β-catenin signaling pathway, which is also downstream signal molecule of Akt. In normal state, GSK3β combines with CK1, APC and Axin to form a protein complex, which can phosphorylate, modify and degrade β-catenin downstream. GSK3β can be phosphorylated by phosphorylated-Akt, resulting in protein complex depolymerization, weakening the degradation of β-catenin. Therefore, β-catenin accumulated in cells, and then transferred into nucleus to affect the downstream biological molecules[21].

In our study, we first proved that SLIT3 could activate Akt, and then phosphorylated and inactivated GSK3β in SCAP. After that, the degradation of β-catenin was inhibited and the level of intracellular β-catenin was increased. When β-catenin accumulated in SCAP, nuclear translocation occurred subsequently and finally the expression of downstream target genes were activated. After inhibiting the phosphorylation of AKT and GSK3β and the accumulation of β-catenin with inhibitor Resibufogenin, SLIT3 could not promote the odontogenic differentiation of SCAP as before, which supported the conclusion that SLIT3 promotes the odontogenic differentiation of SCAP by activating Akt/Wnt β-catenin pathway.

After transfer into the nucleus, β-catenin can interact with TCF/LEF and transcriptional coactivators that regulate the expression of some target genes to form a complex to activate the transcription of downstream target genes[44]. The biological behavior of β-catenin after nuclear translocation in SCAP and the effect changes at the end of the signal pathway are still lack of exploration and verification of related experiments in this study, which will be a direction of follow-up research. In addition, SLIT3/ROBO can promote the proliferation and migration of osteoblasts by regulating the activity of cytoplasmic kinase Abl and then regulating the activity of downstream β-catenin[45]. So, is it possible for SLIT3 to mediate the change of β-catenin through Abl in SCAP, and then regulate the odontogenic differentiation? This is also a problem worthy of future exploration.

Our study discovered and explored the role of SLIT3 in tooth development and dentin formation for the first time, further improved the signal regulation network of dentin formation, helped us to have a more comprehensive understanding of the process of dentin formation and its influencing factors, and provided a new theoretical basis and treatment ideas for dentin defect repair and regeneration.

Materials and Methods

Re-analysis of single-cell RNA sequencing and microarray datasets

We obtained single-cell RNA sequencing data from the Gene Expression Omnibus (GEO) dataset GSE189381, which pertains to the digestion of teeth and surrounding tissue at five distinct developmental stages (E13.5, E14.5, E16.5, PN3.5, and PN7.5). To analyze the raw read counts at each stage, we utilized the R package Seurat (Ver. 4.0.5) for downstream single-cell level analyses. Cells expressing genes in fewer than 3 cells, mitochondrial genes constituting more than 50% of the expression, and cells with fewer than 200 genes were filtered out. Cell cycle information was obtained for each cell using the 'CellCycleScoring' method. To increase interpretability, the gene expression matrixes were transformed using the 'SCTransform' function in Seurat, with consideration of 4000 highly variable genes. We applied regressions to address the impact of mitochondrial genes and cell cycles, followed by batch effect correction and dataset integration using the “FindIntegrationAnchors” and 'IntegrateData' functions. Subsequently, the “FindNeighbors” and “FindClusters” functions were employed, leveraging unsupervised

clustering based on the first 50 principal components with a resolution of 1. Uniform manifold approximation and projection (UMAP) plots were generated for non-linear dimensional reduction visualization. Differentially expressed genes (DEGs) were determined using the 'FindAllMarkers' function with the 'MAST' package (Ver. 1.20.0). The dental mesenchymal cell subclusters and the epithelial cell subcluster were annotated at different developmental stages based on the DEGs and previously reported markers for the dental mesenchyme.

We conducted a reanalysis of a microarray dataset on mouse dental epithelial, dental papilla, and dental follicle cells groups using the same workflow outlined in our prior study. Our investigation specifically emphasized and visualized the expression level of SLIT3 in different groups.

Animal Models and Immunohistochemical analysis

Mice were obtained from the Dashuo Experimental Animals Co., Ltd., Chengdu, China. The Ethics Committee of West China Hospital of Stomatology approved all experimental procedures. Wild mice (C57/BL6) were harvested at post-natal (PN) 1, 7, 14 and 21. Mandibles were carefully dissected and fixed in freshly prepared 4% paraformaldehyde overnight at 4°C. Then, tissues (except mandible from PN1 mice) were decalcified in 12.5% DEPC-treated EDTA (pH 7.4) for 1–4 weeks depending on the age of the sample. Decalcified tissues were dehydrated and paraffin embedded, and 5-μm thick sections prepared. Immunohistochemical examination using standard procedures were performed to examine the molecular expression. For immunohistochemical examination, polyclonal rabbit anti-SLIT3 (1:100, ab198726, Abcam, Cambridge, UK) were used as primary antibodies and negative control group used phosphate-buffered saline (PBS). Finally, immune complexes were visualized using a diaminobenzidine (DAB) kit (Zhongshan Golden Bridge Bio-technology, Beijing, China).

Cell Isolation and Culture

This research was approved by the Ethics Committee of West China Hospital of Stomatology, and informed consent was obtained from all patients. Human third molars with immature roots were collected from healthy patients 16–20 years old at the oral surgery department of West China Hospital of Stomatology, Sichuan University, Chengdu, China. Briefly, the apical papilla was separated from the immature roots and minced. Then, the tissues were digested in a mixed solution containing 3 mg/mL collagenase type I (Sigma-Aldrich, St Louis, MO) and 4 mg/mL dispase II (Sigma-Aldrich) for 30 minutes at 37℃. Cells were cultured in growth medium including α-modification of Eagle medium (α-MEM; Gibco, Gaithersburg, MD) supplemented with 20% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco) at 37℃ in 5% CO_2. We used the third passage of cells in the following experiments.

Odontogenic Differentiation Induction

For odontogenic differentiation, SCAP were seeded in 6-well plates at a density of 1×10^5/well. When reaching 70–80% confluence, SCAP were cultured in odontogenic differentiation induction medium containing α-MEM, 10% FBS, 50 mg/ml ascorbic acid, 10nM dexamethasone and 10 mM β-

glycerophosphate(Sigma-Aldrich). The mineralization ability of SCAP was evaluated after 1–2 weeks induction.

Down-regulation and Up-regulation of SLIT3

For down-regulation of SLIT3 in SCAP, the ordinary medium was replaced with a serum-free medium to make the cells hungry before transfection. Then, SLIT3 siRNA was transfected into SCAP via Lipo2000 (Thermo Fisher Scientific, MA, USA), while negative control siRNA was transfected into the control group. After incubation for 6–8 hours, the transfection reagent was replaced with the ordinary medium. For up-regulation of SLIT3 in SCAP, recombinant human SLIT3 protein was added into the medium at concentration of 0.5μg/ml. The medium was renewed every two days. The change of expression of SLIT3 was detected by qRT-PCR.

Cell Counting Kit-8 Assay

The proliferation of SCAP with low/high expression of SLIT3 was assessed using a Cell Counting Kit-8 (CCK8; Dojindo, Kumamoto, Japan). In the CCK-8 assay, 5×10^3 SCAP per well were seeded in 96-well plates. CCK-8(10μl) solution was added to each well of the plate at different time points (days 1, 3, 5, 7, 10 and 14). According to the manufacture's instructions, cell viability was determined by measuring the absorbance at 450nm, in which the cell viability of control group was always set to 1.

Alkaline Phosphatase (ALP) Staining and Alizarin Red S (ARS) Staining

SCAP were cultured in 24-well plates supplied with odontogenic differentiation induction medium. The medium was renewed every two days. For ALP staining, after seven days of odontogenic induction, SCAP from each group were fixed with 4% Paraformaldehyde. Then, cells were stained with CIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Shanghai, China). For ARS staining, after 14 days of odontogenic induction, SCAP from each group were fixed with 4% Paraformaldehyde. Then, cells were stained with 1% Alizarin Red S (Solarbio, Beijing, China).

Quantitative Real-time Polymerase Chain Reaction

Total RNA of the SCAP was extracted by TRIzol reagent (TAKARA, Dalian, China) after 7, 10 or 14 days of odontogenic induction. RNA concentrations were measured using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, MA, USA). Complementary DNA was synthesized from 1000 ng RNA using a PrimeScript RT kit (TAKARA, Dalian, China). Real-time quantitative polymerase chain reaction was performed in a Roche 480 Light Cycler with SYBR Green Premix (Yeason, Shanghai, China). The program was set as follows: 40 cycles each involving 5 seconds of denaturation at 95℃ and 30 seconds of amplification at 60℃. The mRNA expression levels of SLIT3, dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP-1) were normalized to GAPDH using the $2^{-\Delta\Delta CT}$ method. The primer sequences for qRT-PCR are listed in Table 1.

Table 1
Primer Sequences for Quantitative Real-time Polymerase Chain Reaction

Gene	Forward primer sequence	Reverse primer sequence
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
SLIT3	CGGCATCACCGATGTGAAGAA	AGGCGCAGAGTTCGGATCT
DMP-1	CACTCAAGATTCAGGTGGCAG	TCTGAGATGCGAGACTTCCTAAA
DSPP	ATATTGAGGGCTGGAATGGGGA	TTTGTGGCTCCAGCATTGTCA

Western Blot Analysis

Total proteins of the SCAP were extracted by Total Protein Extraction Kit (SAB, Maryland, USA) after 7, 10 or 14 days of odontogenic induction. The protein concentrations were determined by BCA protein assay reagent (Beyotime, Shanghai, China). Equal amount of protein samples were loaded and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane. After blocking with 5% BSA at room temperature for 1 h, the membranes were incubated at 4°C overnight with primary antibodies against SLIT3 (1:500; R&D Systems, Minnesota, USA), DSPP (1:1000; Novus Biologicals, Colorado, USA), DMP-1(1:500; Novus Biologicals, Colorado, USA) and GAPDH (1:5000; Abcam, Cambridge, UK). The membranes were washed in TBST for three times, then they were incubated with the appropriate horseradish peroxidase conjugated secondary antibodies (1:10000; Boster, Wuhan, China) at room temperature for 1h. Finally, the bands were reacted with an enhanced chemiluminescence kit (Epizyme, Shanghai, China) and exposed to a Western blot analysis imaging system. Grayscale analysis was performed with Image J software.

Akt/Wnt/β-catenin Signaling Pathway Validation

To make sure whether Akt/Wnt/β-catenin Signaling Pathway were involved in the process, the total protein and nucleoprotein of SCAP treated with 0.5μg/ml recombinant human SLIT3 protein was extracted at 0, 15, 30, 60, 90 and 120 min respectively. The expression levels of p-Akt/Akt, p-GSK-3β/GSK-3β (1:1000, all from Cell Signaling Technology) and β-catenin (1:5000, Abcam, Cambridge, UK) in nucleus were detected by Western blot assay described previously. Thereafter, the Akt/Wnt/β-catenin inhibitor Resibufogenin (inhibitor of Akt, GSK-3β and β-catenin, 4μM) (Selleck, Houston, TX) was added to the culture medium. The mRNA and protein expression of DSPP and DMP-1 of rhSLIT3-treated SCAP were examined to evaluate the odontogenic differentiation capacity.

Immunofluorescence Staining

For immunofluorescence analysis, cells were seeded on slides. After washing and fixation in 4% paraformaldehyde, the slides were incubated in β-catenin (1:250, Abcam, Cambridge, UK) antibodies at 4℃ overnight. After one hour of incubation with Goat anti-Rabbit secondary antibody (1:1000; Beyotime, Shanghai, China) at 37℃ in the dark, 4,6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus.

Finally, the slides were observed under a fluorescence microscope (Leica, Wetzlar, Germany) and captured.

Statistical Analysis

All experiments were independently performed at least three times. Data are presented as the mean ± standard deviation. Differences between groups were evaluated by Student's t-test or one-way analysis of variance test using SPSS 24.0 software (SPSS Inc., Chicago, USA). P <.05 was considered statistically significant.

Declarations

Conflict of Interests

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

Author Contributions

Lingyu Jiang contributed to conception, design, data acquisition and analysis, drafted and critically revised the manuscript; Liu Liu contributed to data acquisition and analysis, critically revised the manuscript; Fan Yang contributed to design, data analysis; Yujia Cui contributed to conception and design; Jing Xie, Dongzhe Song and Dingming Huang contributed to design, critically revised the manuscript; Jianxun Sun contributed to conception, design and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Figures

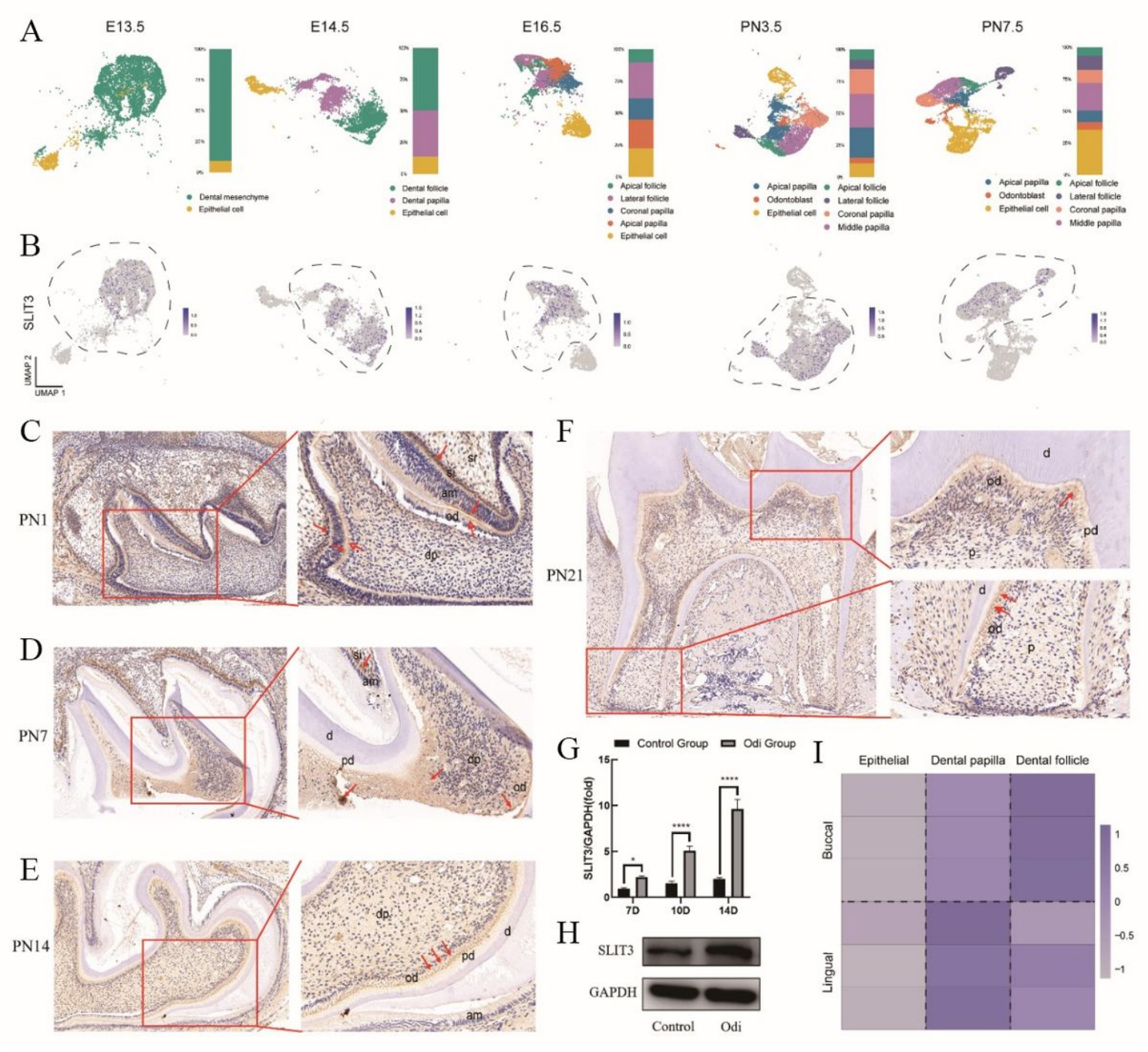

Figure 1

SLIT3 is positively expressed during the differentiation of odontoblast in vivo and vitro. (A) UMAP plots illustrating single-cell data from embryos to postnatal stages in the tooth and surrounding tissues, (B) Expression of SLIT3 at different embryonic and postnatal stages, with dotted lines indicating mesenchymal cell subpopulations, (C) Immunohistochemical staining results of mandibular first molar sections from PN1 mouse (20X), and magnified immunohistochemical staining results of mandibular first molar sections from PN1 mouse (40X), (D) Immunohistochemical staining results of mandibular first molar sections from PN7 mouse (20X), and magnified immunohistochemical staining results of mandibular first molar sections from PN7 mouse (40X) , (E) Immunohistochemical staining results of mandibular first molar sections from PN14 mouse(20X), and magnified immunohistochemical staining

results of mandibular first molar sections from PN14 mouse (40X) , (F) Immunohistochemical staining results of mandibular first molar sections from PN21 mouse(20X), and magnified immunohistochemical staining results of crown of mandibular first molar sections from PN21 mouse (40X) (right upper part), and Magnified immunohistochemical staining results of root of mandibular first molar sections from PN21 mouse(40X) (right lower part) , (G) Result of RT-PCR showed that the mRNA expression of SLIT3 was increased in the SCAP differentiated into odontoblasts, (H) Result of Western blot showed that the protein level of SLIT3 was increased in the SCAP differentiated into odontoblasts at 10 days, (I) The expression of SLIT3 in reanalysis of the microarray dataset. E: embryo, PN: postnatal, Sr: star reticular layer, Si: middle layer, Am: ameloblast, Od: odontoblast, Dp: dental papilla, D: Dentin, Pd: pre-dentine, P: Pulp. Figure G magnification: 10X, Figure A, C and E magnification: 20X, Figure B, D, F, H, I magnification: 40X. *P 0.05, ****P 0.0001.

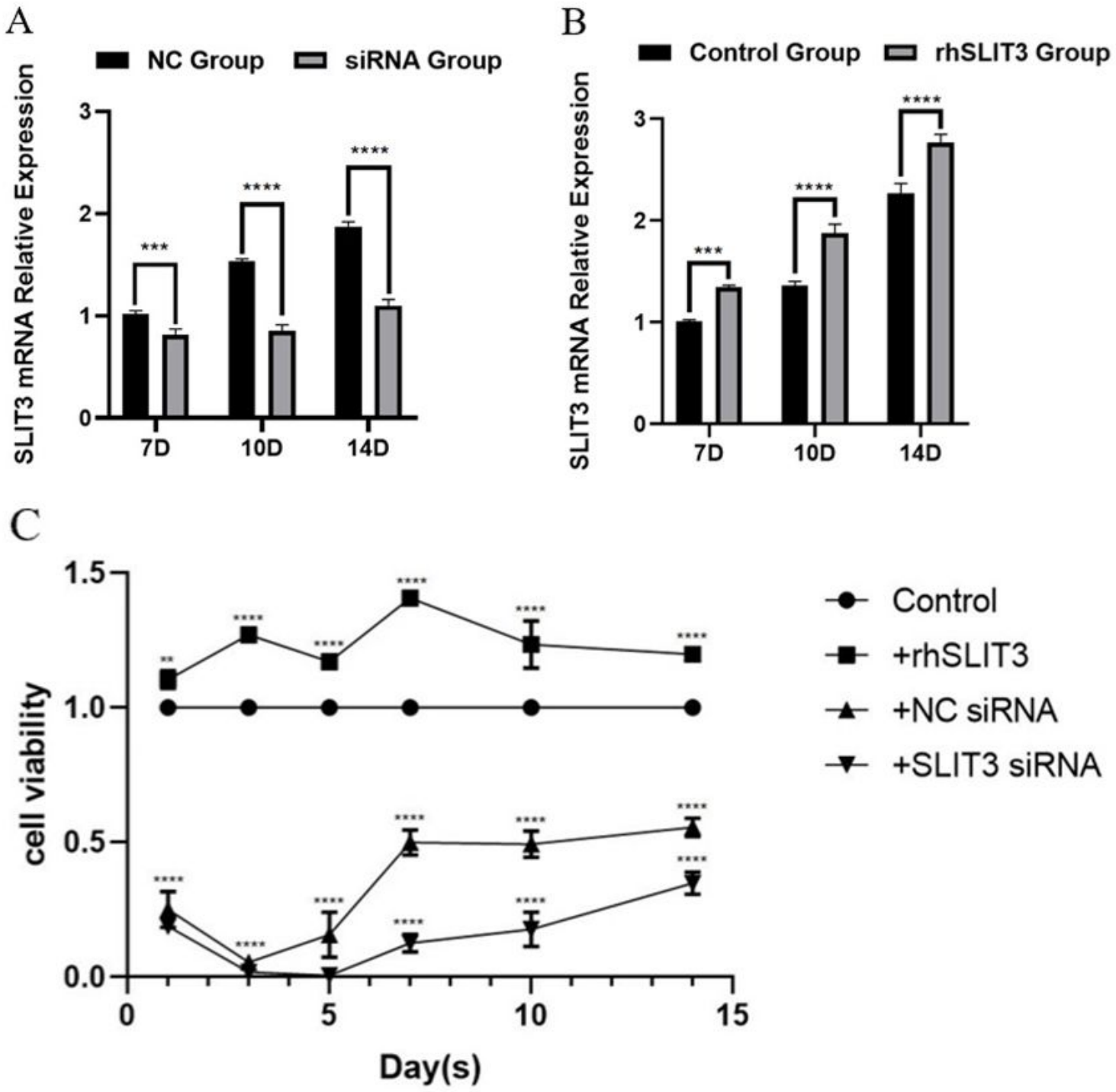

Figure 2

The expression of SLIT3 is positively correlated with the proliferation of SCAP. (A) Result of RT-PCR showed that the expression of SLIT3 in the SLIT3 siRNA group was lower than that in the NC group (B) Result of RT-PCR showed that the expression of SLIT3 in the rhSLIT3 group was higher than that in the control group (C CCK8 assay showed that SCAP of rhSLIT3 group had the strongest proliferation ability, while SCAP of SLIT3 siRNA group had the weakest proliferation ability. At each time point, the cell

viability of control group was set to 1, and the cell viability of the other groups was normalized according to the control group. **P 0.01. ***P 0.001.****P 0.0001.

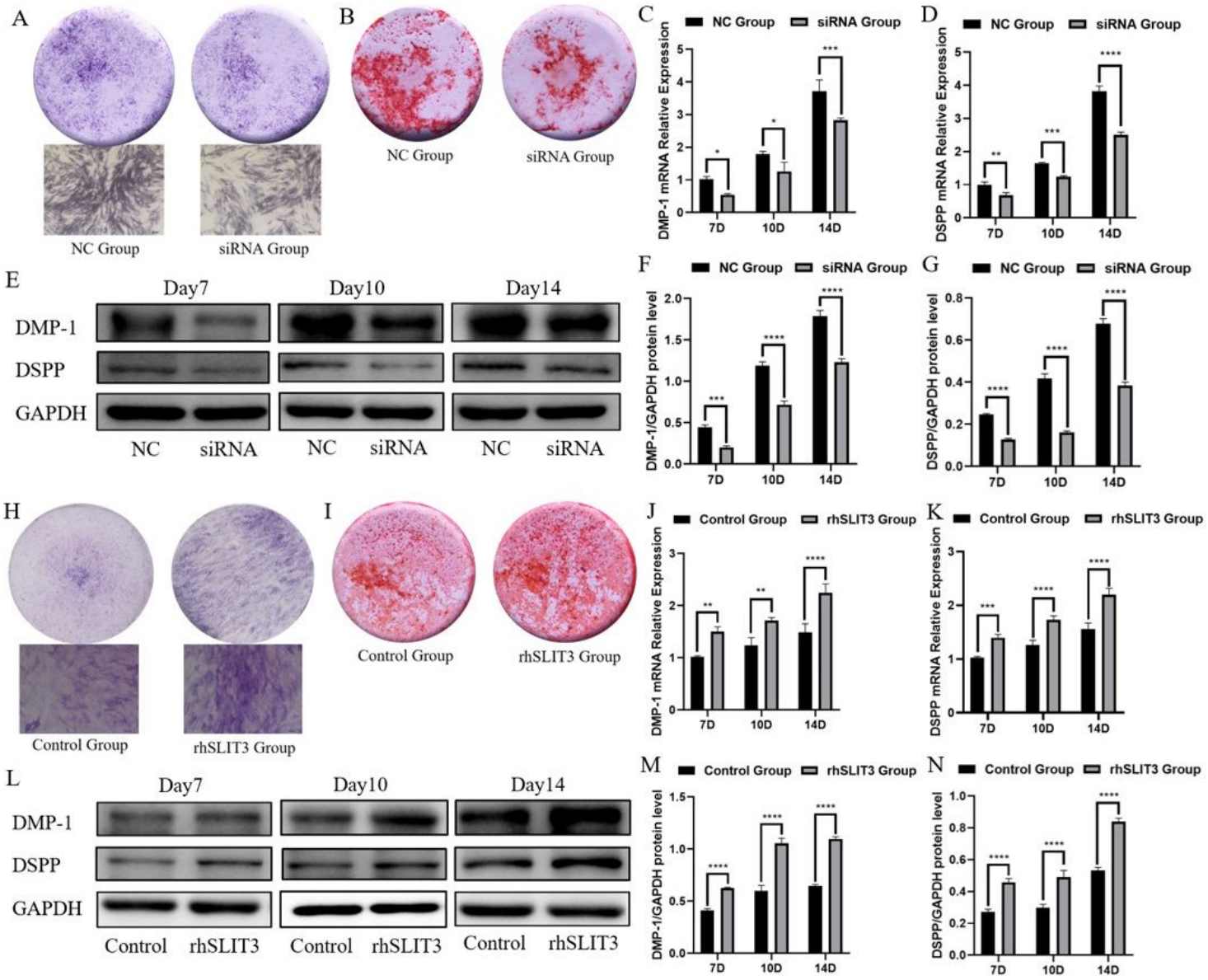

Figure 3

The expression of SLIT3 is positively correlated with the odontogenic differentiation of SCAP. (A) Representative images of alkaline phosphatase (ALP) staining in negative control group and SLIT3 siRNA group of SCAP after 7 days of odontogenic induction. Scale bars: 1000μm, (B) Representative images of alizarin red S (ARS) staining in negative control group and SLIT3 siRNA group of SCAP after 14 days of odontogenic induction. (C and D) RT-PCR analysis of DMP-1 and DSPP mRNA expression in NC group and SLIT3 siRNA group after 7, 10 and 14 days of odontogenic induction. GAPDH was used as an internal control, (E) Western blot analysis of DMP-1 and DSPP expression in NC group and SLIT3 siRNA group after 7, 10 and 14 days of odontogenic induction. GAPDH normalized relative protein levels, (F and G) Quantitative analysis of relative protein levels of DMP-1 and DSPP in NC group and SLIT3 siRNA

group, (H) Representative images of ALP staining in control group and recombination human SLIT3 (rhSLIT3) group of SCAP after 7 days of odontogenic induction. Scale bars: 1000μm, (I) Representative images of ARS staining in control group and rhSLIT3 group of SCAP after 14 days of odontogenic induction, (J and K) RT-PCR analysis of DMP-1 and DSPP mRNA expression in control group and rhSLIT3 group after 7, 10 and 14 days of odontogenic induction. GAPDH was used as an internal control, (L)Western blot analysis of DMP-1 and DSPP expression in control group and rhSLIT3 group after 7, 10 and 14 days of odontogenic induction. GAPDH normalized relative protein levels, (M and N) Quantitative analysis of relative protein levels of DMP-1 and DSPP in control group and rhSLIT3 group.

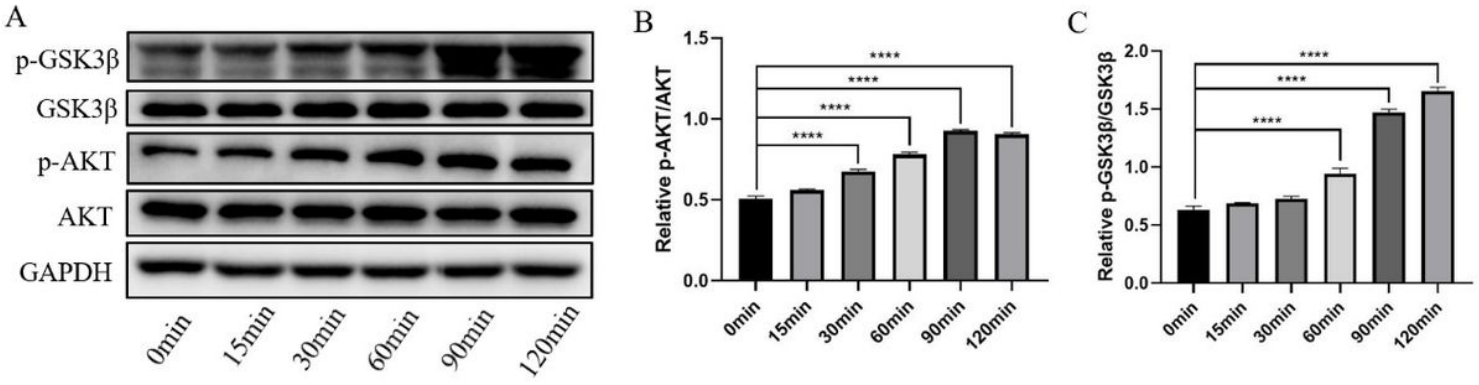

Figure 4

SLIT3 can promote the phosphorylation of Akt and GSK-3β in SCAP. (A) Western blot analysis of phosphorylation levels of Akt and GSK3β in SCAP after the addition of rhSLIT3. GAPDH normalized relative protein levels. (B and C) Quantitative analysis of p-Akt/Akt and p-GSK3β/GSK3β ratio in SCAP after the addition of rhSLIT3.

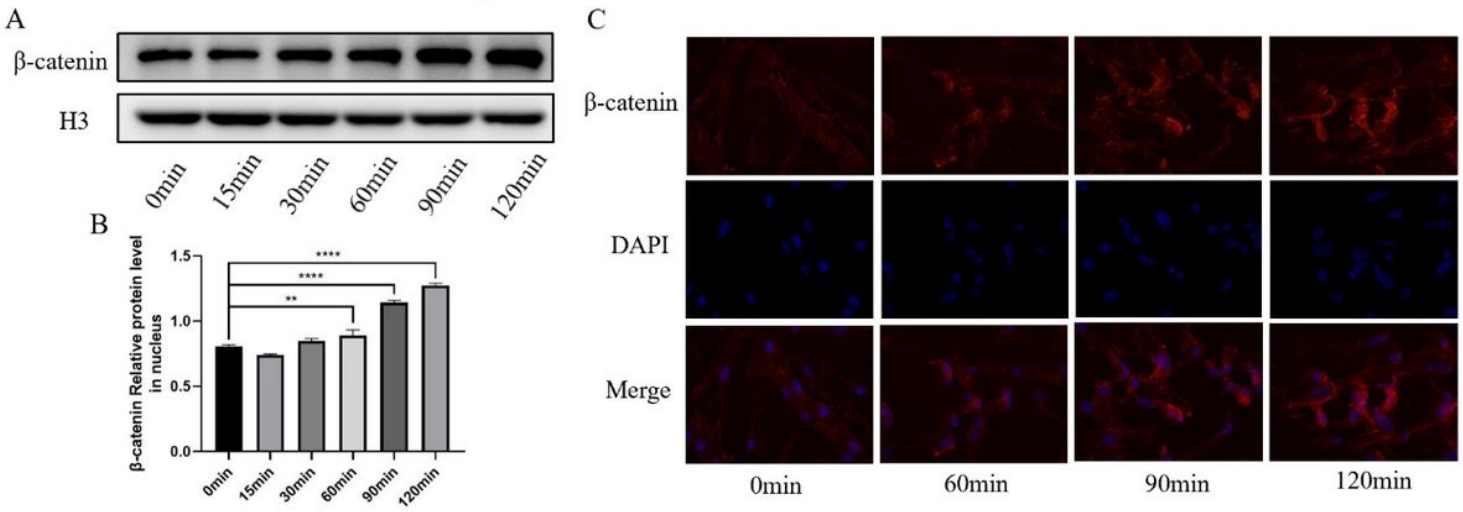

Figure 5

SLIT3 can reduce the degradation of β-catenin and promote its nuclear translocation. (A) Western blot analysis of β-catenin in nucleus of SCAP after the addition of rhSLIT3, H3 normalized relative protein

level, (B) Quantitative analysis of relative protein level of β-catenin in nucleus of SCAP after the addition of rhSLIT3, (C) Representative images of cyto-immunofluorescence staining of β-catenin in SCAP after the addition of rhSLIT3 for 60, 90 and 120mins.

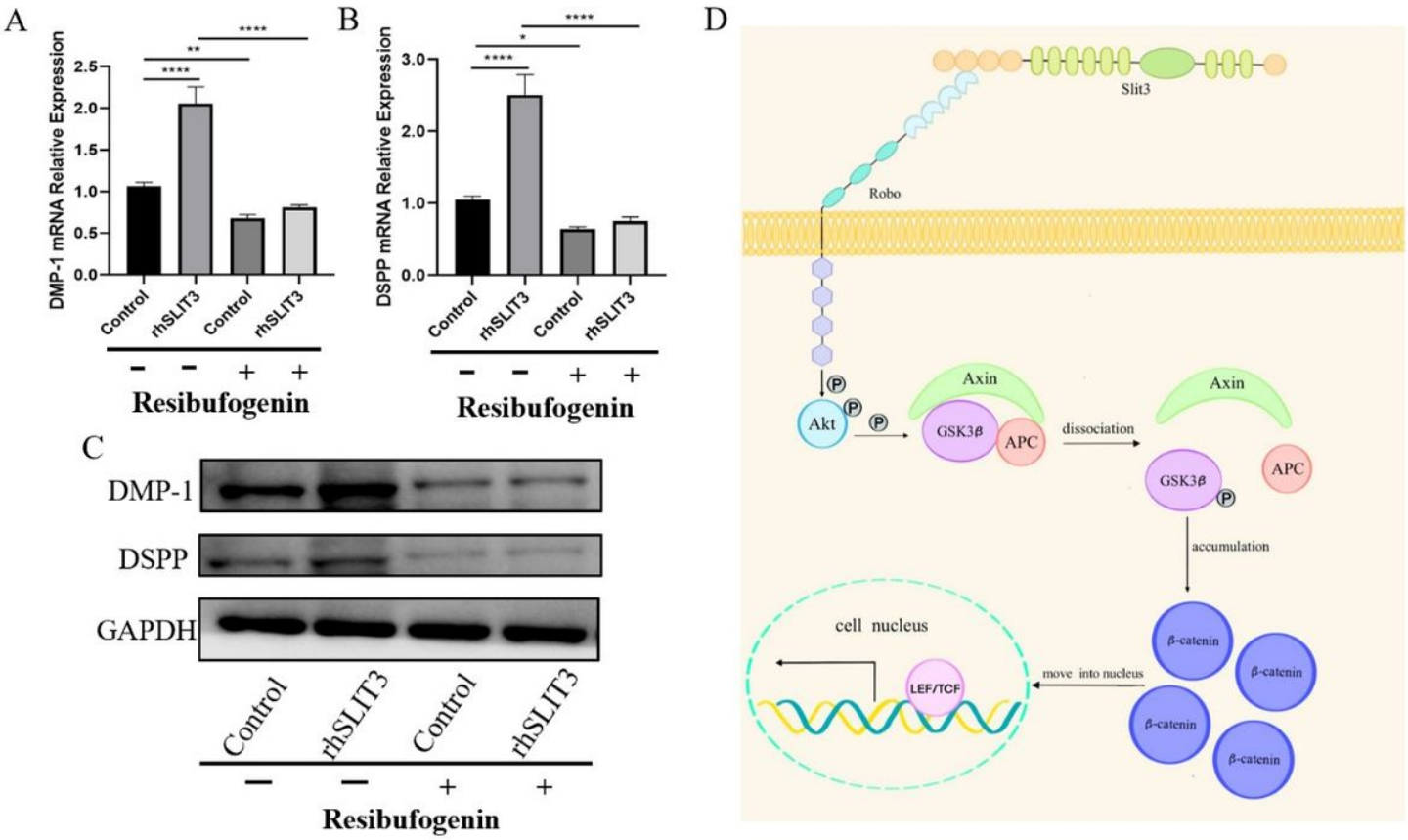

Figure 6

The promotion of SLIT3 on odontogenic differentiation of SCAP was cancelled after inhibition of Akt/Wnt/β-catenin signaling pathway. (A&B) RT-PCR analysis of DMP-1 and DSPP mRNA expression in SCAP after blocking Akt/Wnt/β-catenin signaling pathway and/or adding rhSLIT3, (C) Western blot analysis of DMP-1 and DSPP in SCAP after blocking Akt/Wnt/β-catenin signaling pathway and/or adding rhSLIT3, (D) Schematic diagram of SLIT3 regulating odontogenic differentiation of SCAP via the Akt/Wnt/β-catenin pathway.