Epithelium-derived SCUBE3 promotes polarized-odontoblast differentiation of dental mesenchymal stem cells and vascularized pulp regeneration

Zijie Wang
  Southern Medical University Nanfang Hospital

Chuying Chen
  Southern Medical University Nanfang Hospital

Jiayi Zhang
  Southern Medical University Nanfang Hospital

Jiangdie He
  Southern Medical University Nanfang Hospital

Lin Zhang
  Southern Medical University School of Basic Medical Sciences

Jiayuan Wu
  Zunyi Medical University

Zhihui Tian (✉ Tianzh@i.smu.edu.cn)
  Southern Medical University Nanfang Hospital   https://orcid.org/0000-0002-6423-2113

Research Article

Abstract

Background

Signal peptide-CUB-EGF domain-containing protein 3 (SCUBE3), a secreted multifunctional glycoprotein, whose transcript expression is restricted to the tooth germ epithelium (Epi) during the development of embryonic mouse teeth, has been demonstrated to play a crucial role in the regulation of tooth development. Based on this, we hypothesized that epithelium-derived SCUBE3 contributed to bio-function in dental mesenchymal cells (Mes) via epithelium-mesenchyme interactions.

Methods

Immunohistochemical staining and a co-culture system were performed to reveal the temporospatial expression of SCUBE3 protein during mouse tooth germ development. In addition, human dental pulp stem cells (hDPSC) were used as a Mes model to study the proliferation, migration, polarized-odontoblast capacity and mechanism of rhSCUBE3. Novel pulp-dentin-like organoid models were constructed to further confirm the polarized odontoblast-induction function of SCUBE3. Finally, semi-orthotopic animal experiments were performed to explore the clinical application of rhSCUBE3.

Results

The epithelium-derived SCUBE3 translocated to the mesenchyme via paracrine during mouse embryonic development, and the differentiating odontoblasts in postnatal tooth germ could subsequently secrete SCUBE3 protein via autocrine. In hDPSCs, exogenous SCUBE3 promoted cell proliferation and migration via TGF-β signalling and accelerated odontoblastic differentiation via BMP2 signalling. In the semi-orthotopic animal experiments, we found that SCUBE3-pretreatment induced polarized odontoblast-like cells attached to the dental walls and better angiogenesis performance.

Conclusion

SCUBE3 protein expression is transferred from the epithelium to mesenchyme during embryonic development and elaborated the function of epithelium-derived SCUBE3 in Mes, including proliferation, migration, and polarized-odontoblastic differentiation, and their mechanisms. These findings shed light on exogenous SCUBE3 application in clinic dental-pulp regeneration.

Introduction

Stem cell-based pulp-dentin regeneration may replace routine root canal treatment and has recently emerged as a promising therapeutic strategy [1, 2]. HDPSCs are an easily accessible population with multipotency and self-renewal ability, properties that have led to their recognition as ideal seed cells for

pulp-dentin regeneration [3]. However, previous studies have suggested that implanting decellularized scaffolds loaded with only hDPSCs is not efficient enough for real dentin-pulp complex regeneration [4, 5]. The odontoblastic differentiation potential of dental stem cells is vital for pulp regeneration and a prerequisite for dentin-pulp complex formation [6, 7]. Thus, it is imperative to optimise the stem cell delivery strategies to enhance the odontoblastic differentiation of hDPSCs.

Many cytokines crucial for tooth development are expressed in a specific temporospatial pattern via epithelial-mesenchymal interactions [8]. Therefore, a further understanding of tooth development could contribute to strategies for pulp-dentin regeneration [9, 10]. SCUBE3, a secreted multifunctional cell plasma membrane-anchored glycoprotein, is a member of the SCUBE family sharing a distinct domain organization of at least five recognizable motifs [11]. Previous studies have reported that *SCUBE1* and *SCUBE3* have dynamic reciprocal expressional patterns within the mesenchyme and epithelium during murine odontogenesis [12]. In brief, *SCUBE3* transcripts are only detected in the epithelial tissue and follow a specific and dynamic transcriptional pattern at the early stage of tooth formation [12, 13].

In a recent report, 18 individuals with biallelic inactivating variants in *SCUBE3* showed a consistent phenotype characterised by abnormal skeletal features, distinctive craniofacial appearance, and dental anomalies. Based on this finding, a novel disease caused by the defective function of *SCUBE3* and linked to processes that control abnormal tooth development was defined [14]. Besides, other studies have reported that the specific knockout of *SCUBE3* leads to a decrease in the height, increase in width of ameloblasts, and loss of representative Tomes processes [15],that changes in ameloblast morphology are related to enamel hypoplasia [16]. Considering the induction of ameloblasts is an indispensable factor for the differentiation of odontoblasts [17], and that SCUBE3 is a secreted glycoprotein [11], we hypothesized that epithelium-derived SCUBE3 was involved in inducing odontoblastic differentiation of Mes during tooth development.

To the best of our knowledge, this study is the first to elaborate on the temporospatial expression of SCUBE3 in mouse embryonic and postnatal tooth germs and the function of epithelium-derived SCUBE3 in dental Mes. Our results demonstrate that epithelium-derived SCUBE3 translocates to Mes via the paracrine pathway and accelerates proliferation, migration, and polarized-odontoblastic differentiation of Mes, and promotes vascularised pulp regeneration.

Materials And Methods

Cell isolation and culture

This study was approved by the institutional review board of the Stomatological Hospital, Southern Medical University (201806). Healthy premolars and third molars were collected from patients aged 18–22 years for orthodontic reasons. Written informed consent was obtained from all participants. After extraction, the teeth were immediately placed in culture medium and kept at a low temperature for delivery to the laboratory for further processing. hDPSCs were isolated as previously reported [18]. Briefly, pulp tissue was extracted from the teeth, minced, and digested with collagenase type I (3 mg/mL;

Invitrogen, Paisley, UK) and dispase (4 mg/mL; Invitrogen) for 1 h at 37 °C. After filtration with 70-mm strainers and centrifugation at 1000 rpm for 5 min, cells were resuspended and cultured in low-glucose Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% foetal bovine serum (Gibco-BRL), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). The medium was refreshed every 2–3 days. Cells were passaged when they reached 70–80% confluency, and all experiments were performed at passages 3–5.

Tooth germs of mandibular first molars from prenatal and new-born C57 BL/6 mice were dissected surgically under a stereomicroscope, followed by isolation of mouse tooth germ epithelial cells (mEpi) and mouse tooth germ mesenchymal cells (mMes) following the method described in our previous study [19]. Briefly, the tooth germs were washed with phosphate-buffered saline (PBS), and the enzymatic reaction was conducted with dispase for 10.5 min at 37 °C. Subsequently, the reaction was stopped, and the tooth germ epithelial and mesenchymal tissues were separated with a 25 gauge needle under a microscope. After filtration and centrifugation, some of the tooth germ Epi and Mes were collected for RNA extraction, and the rest were resuspended and separately cultured in medium. When tooth germ epithelial cells reached 70–80% confluency, the medium was replaced with dermal cell basal medium (ATCC; Manassas, VA, USA) for purification, while tooth germ Mes were cultured in normal medium.

The ameloblast-derived cell line LS-8 was purchased from the State Key Laboratory of Military Stomatology, Department of Dental Technical Laboratory, School of Stomatology, Fourth Military Medical University (Xi'an, China). LS-8 cells were initially cultured in normal medium to induce differentiation, and when the tooth germ epithelial cells reached 70–80% confluency, the medium was replaced with induction medium, which contained 50 mg/ml ascorbic acid and 10 mM β-glycerophosphate, and the cells were cultured for other 2 weeks.

Co-culture system

Transwell permeable systems (Corning, New York, NY, USA) were used for the co-culture of Epi and Mes. LS-8 cells were seeded in the upper compartment, while mMes and hDPSCs were seeded separately in the lower compartment of the dish. After 4 days of co-culture, cells in the lower compartments were collected for subsequent experiments.

shRNA lentivirus infection

The shRNA-targeting *SCUBE3* lentivirus and shRNA negative control (NC) lentivirus were synthesised by Ribo Company (Guangzhou, China). For cell transfection, hDPSCs were seeded into six-well plates and transfected with lentiviral particles, including shSCUBE3#1-#3, and shNC. Untransfected cells were used as the blank control. After 72 h of transfection, the cells were collected to assess the efficiency of SCUBE3 silencing using RT-qPCR and western blotting. The shRNA sequences used in the present study are listed in Table S1.

Migration assays

After starvation, 2×10^5 hDPSCs in medium with 1% FBS were plated in Transwell inserts (Corning), whereas the 10% FBS medium with rhSCUBE3 or SB431542 were added in lower chambers. After 24 h of incubation, the migrated hDPSCs were fixed and stained by 0.5% crystal violet.

HDPSCs were seeded in a medium with rhSCUBE3 or SB431542. When reaching 90% confluency, hDPSCs were scratched by 200 µl pipette tips and photographed after 12 h.

Proliferation assays

1×10^3 hDPSCs were plated into the 96-well plates with rhSCUBE3 or SB431542 and measured by Cell Counting Kit-8 (Sigma-Aldrich) at 450 nm absorbance on the indicated days. HDPSCs were treated with rhSCUBE3 or SB431542. When cells reached 60% confluency, 5-ethynyl-2'-deoxyuridine (EdU) kit was applied per the manufacturer's instruction.

Alkaline phosphatase (ALP) staining

Normal medium was replaced with osteogenic inductive medium (OIM, Cyagen Biosciences, Guangzhou, China) in all groups when hDPSCs reached 70–80% confluency. After 2 weeks of osteogenic induction, the cells were fixed with 4% polyoxymethylene for 15 min, washed with PBS three times for 5 min, and stained with BCIP/NBT alkaline phosphatase substrate (Beyotime Biotechnology, Guangzhou, China).

Alizarin Red (ARS) staining

ARS staining was conducted to analyse the mineralised nodules of the cells in the different treatment groups. After 2 weeks of culturing in OIM, hDPSCs were fixed, washed, and stained with 0.5% ARS solution (Sigma-Aldrich, St Louis, MO, USA) to visualize calcium deposition.

Culture of dentin-pulp-like organoids

Dentin pulp-like organoids were cultured as per a previous method [20]. With manufacturer's instructions, hDPSCs at a density of 5×10^6 cells/mL were mixed with Matrigel (Nitta Gelatin, Osaka, Japan) at a ratio of 1:1, placed onto a sheet of Parafilm (Bemis, Oshkosh, WI, USA), and incubated with for 40 min until polymerization of matrices. The constructs were then transferred and cultured in ultra-low adhesion petri dishes (Corning) for 10 days. The medium was then changed to OIM for the different treatment groups. After 21 days of culture, all organoids were harvested for further experiments.

Immunofluorescent staining

For immunofluorescence staining, cultured cells and organoids were fixed for 20 min, permeabilised with 0.1% Triton X-100 for 15 min, washed with PBS three times for 5 min, and blocked with 10% bovine serum albumin for 1 h. Cells were incubated with the primary antibody anti-SCUBE3 (ab189955, Abcam, Cambridge, MA, USA), and the organoids were incubated with anti-DSPP (sc-73632, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight. Sections of tooth root fragments were stained with DSPP

(sc-73632; Santa Cruz), and α-Tubulin (sc-8035; Santa Cruz). After washing with PBS three times for 5 min, cells and organoids were, respectively, incubated with Dylight 594 (35560, Thermo Scientific, Waltham, MA, USA) or CoraLite 488-conjugated (CL488-66122, ProteinTech Group, Chicago, IL, USA) secondary antibodies for 1 h in the dark. DAPI (Sigma-Aldrich) was used to stain the nuclei. Finally, the cells were observed under a Leica fluorescence microscope (Leica Imaging Systems, Cambridge, UK). Organoids were transferred to observation dishes specified for confocal laser microscopy and examined with a confocal microscope (Carl Zeiss, Göttingen, Germany).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total cellular RNA was extracted from cells and tooth germ tissues using TRIzol reagent (Invitrogen), as per the manufacturer's instructions. Reverse transcription was performed with 1 mg of RNA using the PrimeScript RT reagent kit (Takara, Dalian, China). Quantitative PCR was conducted using the SYBR Premix Ex Taq II kit (Takara) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The expression levels of the genes of interest were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in the same samples. The reactions were performed in triplicate, and three independent experiments were performed. The primer sequences used are listed in Table S2.

Western blot

Total cell proteins were lysed using radioimmunoprecipitation assay (Beyotime) according to the manufacturer's protocol. Proteins in the conditioned medium were extracted using a liquid sample total protein extraction kit (Solarbio). Western blotting was performed as previously described. The primary antibodies used were anti-SCUBE3 (ab189955, Abcam), anti-AMBN (orb155652, Biorbyt, Cambridge, UK), TGFβR1 (ab31013; Abcam), anti-DSPP (sc-73632, Santa Cruz), anti-DMP1 (ab103203, Abcam), anti-OPN (ab8448, Abcam), anti-OSX (ab13418, Abcam), anti-BMP2 (ab14933, Abcam), anti-BMPR1A (ab264043, Abcam), anti-*p*-SMAD1/5 (9516, Cell Signalling Technology, Boston, MA, USA), and anti-SMAD1 (D59D7, Cell Signalling Technology). Anti-GAPDH (Rayantibody, Beijing, China) was used as an internal control. Antibody binding was detected using an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA). The intensity of the bands was quantified using Image J software.

Preparation of root fragments of human teeth

Decellularized tooth root fragments were prepared according to a previous report[20]. In brief, the tooth root fragments were sectioned into 5-mm-thick slices using a high-speed dental handpiece. The canals were enlarged to 3 mm in diameter using ProTaper files (Dentsply-Maillefer, Ballaigues, Switzerland) to remove pulp tissue and partial dentin, soaked with 5% EDTA for 5 min and ultrasonicated for 10 min, followed by sealing one of the open endings with mineral trioxide aggregate (Dentsply Sirona, Yorktown, VA, USA). Tooth root fragments were kept in PBS containing 2% penicillin/streptomycin (Invitrogen) at 4 °C and disinfected using UV sterilisation before use. According to the manufacturer's instructions, hDPSCs at a density of 5×10^5 cells/mL were mixed with Matrigel (Nitta Gelatin) at a ratio of 1:1, injected into the canal cavity, and incubated at 37 °C for 30 min for the subsequent experiments.

Subcutaneous implantation into nude mice

Surgical procedures were operated as formerly reported [10]. In short, 6-week-old immunocompromised nude mice (n = 12) were induced by general anaesthesia with 1.2 μl of 1% pentobarbital sodium (Merck, Darmstadt, Germany) by intraperitoneal injection. A single hydrogel-hDPSC-filled fragment was transplanted subcutaneously into the dorsal side of each mouse. All nude mice were euthanised 4 weeks post-surgery, and all fragments were harvested.

Histological assessment

Tooth root fragments were fixed at 4 °C for 48 h and decalcified for 12 weeks using a 10% EDTA−2Na solution (pH 7.4) on an orbital shaker at room temperature. E12.5, E14.5, E16.5, E18.5, postnatal day 3 (P3), P7, and P14 mice were sacrificed to collect mandible samples as reported previously [21]. Tooth root fragments and mandible samples were embedded in paraffin, sectioned at a thickness of 5-μm, and subjected to haematoxylin and eosin and Masson's trichrome staining (Solarbio). Histomorphology was observed using the Scanscope CS and Image Scope software (Aperio, Sausalito, CA, USA). ImageJ software was used for Quantitative analysis.

Immunohistochemical staining

Immunohistochemical analysis was conducted according to a standard protocol. Briefly, samples were incubated with anti-SCUBE3 (ab189955, Abcam) and anti-mitochondria (ab92824, Abcam) as primary antibodies, followed by washing and incubation with horseradish peroxidase-conjugated secondary antibodies. Histometric observations were performed as described for the histological assessment.

Statistical analysis

Data were statistically analysed using SPSS software (version 20.0; IBM, Armonk, NY, USA) or GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). All data are presented as mean ± standard deviation (SD) from at least three independent experiments. Differences between groups were compared using one-way analysis of variance (ANOVA) with t-tests. Statistical significance was set at $P < 0.05$.

Results

Epithelium-derived SCUBE3 translocated to dental mesenchymal cells via paracrine pathway

Here, we analysed spatiotemporal expression pattern of SCUBE3 in the developing tooth germ using immunohistochemistry. Scube3 expression was initially present at in the tooth germ epithelium at the bud stage (E12.5) and continuously increased after (Fig. 1A1). During the cap stage at E14.5, Scube3 was strongly expressed in the whole epithelial tissue, including epithelial cells and the extracellular matrix (ECM); where it was slightly expressed in the mesenchymal tissue, mainly in the ECM (Fig. 1A2). At the early bell stage (E16.5), Scube3 was gradually downregulated in external enamel epithelium and stratum intermedium while it was still strongly expressed in the internal enamel epithelium. Additionally, Scube3

was more highly expressed in mesenchymal tissue than in the cap stage, and was present in a small number of Mes (Fig. 1A3). During the late bell stage (E18.5), Scube3 expression in the epithelium was gradually attenuated, and it localised to the internal enamel epithelium, while increased in the mesenchyme (Fig. 1A4). With further development until P3 (Fig. 1A5, 6) and P7 (Fig. 1A7, 8), stronger expression was observed in mature odontoblasts, and the expression was significantly weaker in mature ameloblasts than in internal enamel cells at the early differentiation stage. Our results confirmed that Scube3 expression level in the epithelium was reduced during the differentiation of internal enamel cells into ameloblasts consistent with SCUBE3 transcripts as a previous report [12]. However, it was gradually upregulated in Mes, accompanied by odontoblast differentiation. This temporospatial pattern of Scube3 was also observed in the cervical loop of the incisor and its vicinity (Fig. 1B).

Considering that Scube3 is a secreted protein, to investigate the origin of Scube3 in Mes, we quantified the *Scube3* transcript expression level in E16.5, P1, P5, and P10 tooth germ epithelial and mesenchymal tissue. *Scube3* was hardly expressed in the prenatal mesenchyme, suggesting that the Scube3 protein observed in the immunohistochemical sections of Mes was translocated from the epithelium. Remarkably, *Scube3* expression levels were strongly presented but gradually reduced in the epithelial tissue of the postnatal tooth germ (Fig. 1C). This expression trend is consistent with SCUBE3 protein level during LS-8 (used as a model of tooth germ epithelial cells) ameloblast differentiation (Fig. 1F). Secreted SCUBE3 was detected in the conditioned medium from day 0, increased on day 4, and decreased thereafter (Fig. 1G), suggesting that mEpi produced and released SCUBE3 during ameloblast differentiation. Remarkably, SCUBE3 was hardly expressed in the prenatal mMes but increased in the differentiating mMes of postnatal tooth germ (Fig. 1D). Immunofluorescent staining showed strong Scube3 expression in primary tooth germ epithelial cells (Fig. 1E1-3) and LS-8 (Fig. 1E4-6) but weak expression in tooth germ Mes (Fig. 1E7-9) and hDPSCs (Fig. 1E10-12). Interestingly, after their co-culture, clear Scube3 expression was observed in tooth germ Mes (Fig. 1E13-15) and hDPSCs (Fig. 1E16-18). Western blot also showed significantly higher SCUBE3 expression in co-cultured mMes than the mMes cultured alone (Fig. 1H). However, there is no significant difference in SCUBE3 transcript levels between them (Fig. 1I), demonstrating the increased SCUBE3 protein in co-cultured mMes was translocated from mEpi via paracrine signalling.

Exogenous SCUBE3 accelerated proliferation and migration in dental mesenchymal cells via TGFβ/Smad pathway

To explore the bio-function of epithelium-derived SCUBE3 in Mes, we used hDPSC as a Mes model and replaced epithelium-derived SCUBE3 with rhSCUBE3. CCK-8 (Appendix Fig. 1A) and EdU assay (Appendix Fig. 1B, C) implied increased survival and proliferation ability in rhSCUBE3-treated hDPSCs. In addition, exogenous SCUBE3 induced more migrated cells (Appendix Fig. 1D-G). Of note, we observed TGFβ1, TGFβR1, TGFβR2, Smad4, and p-Smad2/3 expressions significantly upregulated (Fig. 2A, B), suggesting the activation of TGFβ/Smad signaling. To provide further evidence for the mechanism, we exposed hDPSCs with TGFβ pathway inhibitor SB431542 and found that SB431542 repressed the cell growth (Fig. 2C-E) and migration ability (Fig. 2F-I) upregulated by rhSCUBE3. These data verified that SCUBE3

promoted proliferation and migration in hDPSCs via TGFβ/Smad signaling, revealing its capacity for cell self-renewal, recruitment of cells, and even the potential for pulp/dentin regeneration.

ExogenousSCUBE3 was involved in BMP2/Smad pathway-induced odontoblastic differentiation in Mes

In response to epithelium-mesenchyme interactions, *SCUBE3* expression gradually increased in odontoblast-differentiating Mes of the postnatal tooth germ. Therefore, we further explored the temporal relationship between *SCUBE3* and odontoblastic differentiation. Both the mRNA and protein of SCUBE3 in hDPSCs treated with OIM positively correlated with that of DSPP (Appendix Fig. 2A, B), revealing that hDPSCs produced endogenous SCUBE3 during odontoblastic differentiation. We then constructed mesenchymal SCUBE3 knockdown by shRNA (Appendix Fig. 3A, B). shSCUBE3#2-transfected hDPSCs significantly reduced odontoblastic differentiation-related markers expression (Appendix Fig. 3C, D), suggesting *the SCUBE3* gene was crucial for odontoblastic differentiation. Meanwhile, exogenous SCUBE3 positively modulated odontoblastic differentiation in a concentration-dependent manner, with 0.5 µg/ml being the optimum concentration (Appendix Fig. 4). rhSCUBE3-treated cells were robustly Alizarin Red-S (Appendix Fig. 3E) and ALP activity positive (Appendix Fig. 3F). Interestingly, we pre-treated hDPSCs with rhSCUBE3 for 1-6 d and found that exogenous SCUBE3 induced endogenous SCUBE3 expression (Appendix Fig. 5). These data indicated a key role of rhSCUBE3 in modulating odontoblastic differentiation of hDPSCs. We further explored the molecular mechanism and found that BMP2, BMP4, BMPR1A, and BMPR1B significantly upregulated with rhSCUBE3 treatment. Especially, BMP2 and BMPR1A showed the most significant difference (Fig. 3A). Treatment with LDN-193189, an BMP signaling inhibitor, counteracted the upregulation of BMP2, BMPR1A, and *p*-Smad1/5 triggered by rhSCUBE3 (Fig. 3B, Appendix Fig. 6). After inhibiting BMP/Smad signaling, the expressions of differentiation-related markers were downregulated (Fig. 3C, D). And Alizarin Red-S (Fig. 3E) and ALP (Fig. 3F) staining showed less biomineralization in hDPSCs exposed to LDN-193189. These data indicated that SCUBE3 was involved in the BMP2/Smad pathway-induced odontoblastic differentiation of hDPSCs. Interestingly, we observed that BMP2 was increasingly presented (Appendix Fig. 7A) and released into the conditioned medium during differentiation, similar to the expression and secretory pattern of SCUBE3 (Appendix Fig. 7B).

Exogenous SCUBE3 Facilitated Odontoblastic Differentiation in Dentin-Pulp Organoids

Organoids represent a crucial bridge between 2D cell culture and in vivo animal models[26]. To further verify the effect of SCUBE3 on odontoblast differentiation, we constructed dentin-pulp organoids with hDPSCs and observed their development using a light microscope. On the first day of culture, hDPSCs from all groups were dispersed (Fig. 4A-1, 6, 11) and started to aggregate gradually on day 6 (Fig. 4A-2, 7, 12). On day 11, organoids with no difference in each group were exposed to rhSCUBE3 with or without LDN-193189 (Fig. 4A-3, 8, 13). HDPSCs began to form condensed spheroids on day 16 (Fig. 4A-4, 9, 14). On day 21, faster cell aggregation and more condensed organoid formation were observed in rhSCUBE3-treated organoids than in the other groups (Fig. 4A-5, 10, 15). SCUBE3-treated organoids had significantly smaller areas, suggesting that SCUBE3 promotes the development of organoids (Fig. 4B). Additionally,

the organoid development was repressed in the rhSCUBE3/LDN-193189 group. In the SCUBE3 group, the mRNA expression levels of DSPP, DMP1, OPN, and OSX increased (Fig. 4C-F), while that of CD90 significantly decreased (Fig. 4G). These results were confirmed using confocal immunostaining for DSPP (Fig. 4H) and integral optical density (Fig. 4I). Collectively, these findings demonstrate that SCUBE3 promotes odontoblast differentiation of dentin-pulp organoids by activating BMP/Smad signalling.

Exogenous SCUBE3 promoted dentin-pulp regeneration in vivo

A semi-orthotropic animal experiment was conducted to further prove the role of SCUBE3 in promoting vascularised dental pulp regeneration in vivo [22, 23]. After treating hDPSCs with rhSCUBE3 for 1–6 days, the mRNA levels of SCUBE3 and DSPP in hDPSCs were evaluated using RT-qPCR. The hDPSCs pre-treated with rhSCUBE3 for 4 days demonstrated the highest SCUBE3 and DSPP expression levels compared to the other groups (Appendix Fig 5A, B). Subsequently, the decellularized tooth scaffolds were transplanted into the subcutaneous tissue of nude mice under three different conditions: hDPSC-only, hDPSCs pre-treated with rhSCUBE3 for 4 days, and hDPSCs pre-treated with SCUBE3 and LDN-193189. The harvested root canals in the control group were filled with tissue devoid of enough cells but rich in collagen fibres (Fig. 5A1-2, B1-2). The canals of the SCUBE3 group were almost filled with compacted and well-structured connective tissue, along with abundant ECM, collagen fibres, ample cells, and sufficient blood vessels relative to the controls (Figure 5 A3-4, B3-4). There was a significant upregulation of the pulp-like tissue filling rate in the SCUBE3 group compared to that in the controls (Fig. 5C), more attached cell layers (Fig. 5D), and more blood vessels (Fig. 5E) were observed in the SCUBE3 group. Surprisingly, DSPP and a-Tubilin double stain positively cell processes extended in the dentin tubules were observed in the SCUBE3 group (Fig. 5F, H). According to immunohistochemistry results, the cell layer presented human mitochondria (Fig. 5G1-3), revealing that these attached cells were odontoblast-like cells differentiated from hDPSCs. The number of mitochondria-positive cells in the SCUBE3 group was significantly higher than that in the other two groups (Fig. 5I). All these effects of SCUBE3 were reversed by LDN-193189 (Fig. 5A5-6, B5-6, F3, G3). Our results indicated that SCUBE3 promoted the survival of transplanted stem cells, the polarization of odontoblast-like cells, and angiogenesis in vivo.

Discussion

Members of the SCUBE family, share a distinct domain organisation of at least five recognisable motifs. Previous studies have reported that, during murine odontogenesis, SCUBE1 and SCUBE3 have dynamic reciprocal expressional patterns within the mesenchyme and epithelium, respectively [21]. In contrast, SCUBE2 expression in developing mouse tooth is negligible [24]. In the late bell stage, the dental epithelium can promote odontoblast differentiation of undifferentiated ectomesenchymal cells, which entails interaction of epithelium and mesenchyme via paracrine signalling [25]. The dental epithelium and mesenchyme have been demonstrated to secrete factors that are preferred by reciprocal cells rather than being taken up by themselves and that could evoke differentiation and matrix synthesis [26]. Moreover, epithelium-derived bioactive factors are capable of enhancing odontoblast differentiation in hDPSCs [27, 28]. It was reported that the SCUBE3 transcript was strictly localized to the fetal murine tooth germ

epithelial tissue [12], and its translation product was a secreted glycoprotein. Therefore, we analyzed whether SCUBE3 was involved in epithelium-mesenchyme interactions to affect mesenchymal cells biologically.

Several studies using whole-mount transcript identification and section in situ hybridisation have confirmed that *Scube3* transcripts strictly localise to the foetal murine tooth germ epithelial tissue [12]. However, the spatiotemporal expression of SCUBE3 protein during tooth germ development is still unclear. In the present study, our results showed that, in the Epi of prenatal mice, the expression domains and dynamic changes in SCUBE3 protein were consistent with those of *SCUBE3* mRNA. In postnatal mice, the expression of SCUBE3 in Epi decreased significantly. In tooth germ Mes, a small amount of SCUBE3 protein was found in the ECM of mesenchymal tissue at E14.5 by immunohistochemistry staining. At E16.5, the expression of SCUBE3 started to be detectable in the Mes and increased gradually in the differentiating odontoblasts of postnatal tooth germ. Obviously, SCUBE3 protein can be detected earlier than *SCUBE3* transcript in Mes during tooth development, suggesting that the SCUBE3 protein in the Mes of prenatal tooth germ may derive from the Epi. Notably, a co-culture system of tooth germ Epi and Mes was constructed and convincingly proved that the epithelium-derived SCUBE3 can translocate to Mes via epithelium-mesenchyme interactions.

Afterward, we tried to explore the bio-function of epithelium-derived SCUBE3 on mesenchymal cells via epithelial-mesenchymal interactions. HDPSCs were used as the model of dental mesenchymal cells and rhSCUBE3 was replaced epithelium-derived SCUBE3 protein. CCK-8 and EdU assay showed that rhSCUBE3 could promote the proliferation of hDPSCs. Transwell and wound healing assay both verified the capacity of rhSCUBE3 to accelerate the migration rate of hDPSCs. Though former researches showed that knockdown of *SCUBE3* could suppress lung cancer invasion and metastasis [29], and also suppress breast cancer cells growth, invasion, and migration [30]. Our finding showed that exogenous SCUBE3 also could enhance cell proliferation and migration of dental Mes firstly.

Meanwhile, rhSCUBE3 presented its upregulation of DSPP and DMP1 expression in hDPSCs, demonstrating the application of exogenous SCUBE3 can help promote odontoblastic differentiation of dental Mes. Interestingly, even without the stimulation of rhSCUBE3, both the changes in SCUBE3 mRNA and protein levels were consistent with the increase in DSPP expression during the differentiation of hDPSCs, revealing that odontoblasts themselves can generate SCUBE3 protein via autocrine during odontoblastic differentiation. We used shRNA to knockdown the endogenous *SCUBE3* expression, both DSPP and DMP1were downregulated afterwards. These results thus demonstrated that *SCUBE3* is a vital gene to modulate the odontoblastic differentiation of dental Mes. Notably, exogenous rhSCUBE3 can partly rescue the suppressed odontoblastic differentiation in the cells transfected with shRNA, suggesting that exogenous SCUBE3 could also enhance endogenous SCUBE3 expression and trigger SCUBE3 autocrine/paracrine secretion in differentiating odontoblasts, similar to BMP2 [30]. Since the presence of SCUBE3 protein is earlier than *SCUBE3* transcript in Mes, we speculated that the translocation of epithelium-derived SCUBE3 to the mesenchyme may be involved in the induction of odontoblast

differentiation. Of course, this assumption requires to be further evaluated by more evidences, such as epithelial cell-specific knockout mouse models.

The specific CUB-like structure of SCUBE3 can bind to various signalling receptors, including BMPR, TGF-βR, and VEGF, acting as a ligand or co-receptor to trigger a series of biological effects [31–33]. For an instance, both the SCUBE3 protein and the C-terminal CUB domain fragment can bound to TGF-β type II receptor through the C-terminal CUB domain, activated TGF-β signalling and trigger the epithelial-mesenchymal transition (EMT), deposition of the extracellular matrix, invasion into adjacent tissues and angiogenesis[34]. The TGF-β, BMP, canonical WNT/β-catenin, and MAPK signalling pathways play critical roles in the odontoblastic differentiation of dental Mes [35, 36]. Based on the suggested dual role of BMP/TGF-β as a downstream activator of SCUBE3 and as an essential pathway regulating odontoblastic differentiation [34, 37], we hypothesized that SCUBE3 probably exerted effects on the BMP/TGF-β signalling pathway as an auxiliary modulator. Treating hDPSCs with exogenous SCUBE3 led to the activation of the TGF-β and BMP pathways. According to our results, after the TGF-β signalling was suppressed by SB431542, the proliferation and migration potential were downregulated, while there was no significantly change of differentiation ability. These findings suggested that TGF-β signalling mainly modulated proliferation and migration in the dental Mes treated with rhSCUBE3. However, treatment with the BMP signalling inhibitor LDN-193189 not only downregulated the expression of BMP signalling pathway downstream effectors, BMPR1A and p-Smad1/5, but also inhibited the expression of odontoblast markers, DSPP and DMP1, and osteoblast markers, OPN and OSX, demonstrating that this pathway is essential for SCUBE3-induced odontoblastic differentiation. These results were further verified using dentin-pulp-like organoids. It has been reported that SCUBE3 acted as a BMP2/BMP4 auxiliary receptor and positively modulated signalling by enhancing the specific interactions between BMPs and BMP receptors [14]. Our results demonstrated that SCUBE3 was involved in the BMP-dependent odontoblastic differentiation process of hDPSCs. Furthermore, exogenous SCUBE3 not only promoted the upregulation of odontoblast differentiation-related markers, but also induced the production and secretion of SCUBE3 and BMP2. These suggest that exogenous SCUBE3 could trigger autocrine/paracrine signalling of SCUBE3 during differentiation in hDPSCs, constantly promoting odontoblastic differentiation in the developing tooth germ via epithelium-mesenchyme interactions.

Semi-orthotopic models using subcutaneous tooth fragment implantation have been widely used to investigate pulp-dentin regeneration [19, 22]. We performed the animal model to study the clinical application of SCUBE3. In the SCUBE3 pre-treatment group, we observed well-organized pulp-like tissues and more human mitochondrial-positive cells, along with many DSPP- and α-Tubulin- positive odontoblast-like cells adhered to the dentin walls. The cell layers presented odontoblastic processes extended in the dentin tubules. These results suggested that SCUBE3 pre-treatment could promote transplanted hDPSCs odontoblastic differentiation with polarization of odontoblast-like cells. Nevertheless, pre-dentin wasn't found in any of the groups, which may be ascribed to the relatively short observation time. Surprisingly, although rich new-born blood vessels were observed in the rhSCUBE3 pre-treatment group, both the vascular endothelial cells and perivascular mural cells showed negative staining for human mitochondria, indicating that the new-born vasculature was formed by the host cells.

Moreover, hDPSCs have little capacity to switch to a vascular endothelial phenotype when transplanted into murine hosts [38, 39]. The abundant regenerated vasculature suggests that SCUBE3 pre-treatment might promote angiogenesis by regulating the migration of vascular endothelial cells from the host.

Conclusions

Our results confirmed that SCUBE3 protein expression showed a dynamic temporospatial distribution pattern during dentin genesis in the developing tooth germ, which was inconsistent with transcripts expression. SCUBE3 translocated from the tooth germ epithelium to the mesenchyme via epithelium-mesenchyme interactions. Additionally, epithelium-derived SCUBE3 promoted cell proliferation, migration, and odontoblastic differentiation of Mes. It was proved that SCUBE3 accelerated proliferation and migration of hDPSCs via TGFβ/Smad pathway. Furthermore, in in-vitro and organoid models, SCUBE3 demonstrated strong potential for inducing odontoblastic differentiation of hDPSCs via the BMP2/Smad pathway. Its capacity to promote vascularised pulp regeneration, including transplanted cell survival, odontoblastic differentiation, cell polarization, and new-born vessels in vivo, were verified using a semi-orthotopic model. These results prove that finding cues from tooth development is a sound strategy for developing novel strategies for pulp-dentin regeneration. To the best of our knowledge, the present study is the first to establish that epithelium-derived SCUBE3 could function as a potential growth factor for dental-pulp regeneration and angiogenesis, and the first to verify that *SCUBE3* is the vital gene to modulate odontoblastic differentiation of mesenchymal cells. Our findings also provide a crucial theoretical framework for the use of SCUBE3 to promote dentin-pulp regeneration.

Abbreviations

SCUBE3
Signal peptide-CUB-EGF domain-containing protein 3
Epi
tooth germ epithelial cells
Mes
tooth germ mesenchymal cells
hDPSC
human dental pulp stem cells
OIM
osteogenic inductive medium
ECM
extracellular matrix
EMT
epithelial-mesenchymal transition.

Declarations

Authors' contributions

Zhihui Tian, Lin Zhang and Jiayuan Wu participated in study concept and design; Zhihui Tian conceived the study, contributed to analysis of data, edited the manuscript; Lin Zhang contributed to analysis of SCUBE3 expression in the developing tooth germ. Zijie Wang performed the technical design and conducted pivotal experiments, and collected and analysed data for figures, and drafted the manuscript. Chuying Chen conducted part of the experiments and edited the manuscript. Jiayi Zhang contributed for Figs 6. Zhihui Tian, Zijie Wang, Chuying Chen, Jiayi Zhang, and Jiangdie He participated in the animal study surgery; All authors read and approved the final paper.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Ethics approval and consent to participate

This study was approved by the institutional review board of the Stomatological Hospital, Southern Medical University (201806).

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Schmalz, G., M. Widbiller and K.M. Galler, Clinical Perspectives of Pulp Regeneration. J Endod, 2020. 46(9S): p. S161-S174.
2. Sui, B., et al., Pulp Stem Cell-Mediated Functional Pulp Regeneration. J Dent Res, 2019. 98(1): p. 27-35.

3. Nuti, N., et al., Multipotent Differentiation of Human Dental Pulp Stem Cells: a Literature Review. Stem Cell Rev Rep, 2016. 12(5): p. 511-523.

4. Xie, Z., et al., Functional Dental Pulp Regeneration: Basic Research and Clinical Translation. Int J Mol Sci, 2021. 22(16).

5. Huang, G.T., et al., Pulp/Dentin Regeneration: It Should Be Complicated. J Endod, 2020. 46(9S): p. S128-S134.

6. Ajay, S.L., A. Sharma and G.J. Dias, Advances in regeneration of dental pulp--a literature review. J Investig Clin Dent, 2015. 6(2): p. 85-98.

7. Huang, X., et al., Microenvironment Influences Odontogenic Mesenchymal Stem Cells Mediated Dental Pulp Regeneration. Front Physiol, 2021. 12: p. 656588.

8. Santosh, A.B. and T.J. Jones, The epithelial-mesenchymal interactions: insights into physiological and pathological aspects of oral tissues. Oncol Rev, 2014. 8(1): p. 239.

9. Huang, D., et al., Tooth Regeneration: Insights from Tooth Development and Spatial-Temporal Control of Bioactive Drug Release. Stem Cell Rev Rep, 2020. 16(1): p. 41-55.

10. Zhou, T., et al., Conditioned medium derived from 3D tooth germs: A novel cocktail for stem cell priming and early in vivo pulp regeneration. Cell Prolif, 2021. 54(11): p. e13129.

11. Wu, B.T., et al., A novel secreted, cell-surface glycoprotein containing multiple epidermal growth factor-like repeats and one CUB domain is highly expressed in primary osteoblasts and bones. J Biol Chem, 2004. 279(36): p. 37485-90.

12. Haworth, K., et al., Expression of the Scube3 epidermal growth factor-related gene during early embryonic development in the mouse. Gene Expr Patterns, 2007. 7(5): p. 630-4.

13. Xavier, G.M., et al., Characterization of a mouse Scube3 reporter line. Genesis, 2010. 48(12): p. 684-92.

14. Lin, Y.C., et al., SCUBE3 loss-of-function causes a recognizable recessive developmental disorder due to defective bone morphogenetic protein signaling. Am J Hum Genet, 2021. 108(1): p. 115-133.

15. Xavier, G.M., L. Panousopoulos and M.T. Cobourne, Scube3 is expressed in multiple tissues during development but is dispensable for embryonic survival in the mouse. PLoS One, 2013. 8(1): p. e55274.

16. Tian, Z., et al., Deletion of epithelial cell-specific Cdc42 leads to enamel hypermaturation in a conditional knockout mouse model. Biochim Biophys Acta Mol Basis Dis, 2018. 1864(8): p. 2623-2632.

17. Arana-Chavez, V.E. and L.F. Massa, Odontoblasts: the cells forming and maintaining dentine. Int J Biochem Cell Biol, 2004. 36(8): p. 1367-73.

18. Zhang, C., et al., Therapeutic effect of dental pulp stem cell transplantation on a rat model of radioactivity-induced esophageal injury. Cell Death Dis, 2018. 9(7): p. 738.

19. He, L., et al., Parenchymal and stromal tissue regeneration of tooth organ by pivotal signals reinstated in decellularized matrix. Nat Mater, 2019. 18(6): p. 627-637.

20. Jeong, S.Y., et al., Fabrication of Dentin-Pulp-Like Organoids Using Dental-Pulp Stem Cells. Cells, 2020. 9(3).

21. Xavier, G.M., P.T. Sharpe and M.T. Cobourne, Scube1 is expressed during facial development in the mouse. J Exp Zool B Mol Dev Evol, 2009. 312B(5): p. 518-24.

22. Zhu, X., et al., A Miniature Swine Model for Stem Cell-Based De Novo Regeneration of Dental Pulp and Dentin-Like Tissue. Tissue Eng Part C Methods, 2018. 24(2): p. 108-120.

23. Huang, G.T., et al., Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model. Tissue Eng Part A, 2010. 16(2): p. 605-15.

24. Xavier, G.M., L. Panousopoulos and M.T. Cobourne, Scube3 is expressed in multiple tissues during development but is dispensable for embryonic survival in the mouse. PLoS One, 2013. 8(1): p. e55274.

25. Thesleff, I., Epithelial-mesenchymal signalling regulating tooth morphogenesis. J Cell Sci, 2003. 116(Pt 9): p. 1647-8.

26. Jiang, N., et al., Exosomes Mediate Epithelium-Mesenchyme Crosstalk in Organ Development. ACS Nano, 2017. 11(8): p. 7736-7746.

27. Oh, H.J., et al., CPNE7, a preameloblast-derived factor, regulates odontoblastic differentiation of mesenchymal stem cells. Biomaterials, 2015. 37: p. 208-17.

28. Lee, J.H., et al., Odontogenic differentiation of human dental pulp stem cells induced by preameloblast-derived factors. Biomaterials, 2011. 32(36): p. 9696-706.

29. Zhao, C., et al., SCUBE3 overexpression predicts poor prognosis in non-small cell lung cancer. Biosci Trends, 2013. 7(6): p. 264-9.

30. O'Connell, D.J., et al., A Wnt-bmp feedback circuit controls intertissue signaling dynamics in tooth organogenesis. Sci Signal, 2012. 5(206): p. ra4.

31. Pawlak, J.B. and G.C. Blobe, TGF-beta superfamily co-receptors in cancer. Dev Dyn, 2022. 251(1): p. 137-163.

32. Yang, X., et al., Activation of TGF-beta1 Pathway by SCUBE3 Regulates TWIST1 Expression and Promotes Breast Cancer Progression. Cancer Biother Radiopharm, 2020. 35(2): p. 120-128.

33. Tu, C.F., et al., SCUBE3 (signal peptide-CUB-EGF domain-containing protein 3) modulates fibroblast growth factor signaling during fast muscle development. J Biol Chem, 2014. 289(27): p. 18928-42.

34. Wu, Y.Y., et al., SCUBE3 is an endogenous TGF-beta receptor ligand and regulates the epithelial-mesenchymal transition in lung cancer. Oncogene, 2011. 30(34): p. 3682-93.

35. Jernvall, J. and I. Thesleff, Reiterative signaling and patterning during mammalian tooth morphogenesis. Mech Dev, 2000. 92(1): p. 19-29.

36. Liang, C., L. Liao and W. Tian, Stem Cell-based Dental Pulp Regeneration: Insights From Signaling Pathways. Stem Cell Rev Rep, 2021. 17(4): p. 1251-1263.

37. Lim, H.C., et al., Delivery of dexamethasone from bioactive nanofiber matrices stimulates odontogenesis of human dental pulp cells through integrin/BMP/mTOR signaling pathways. Int J

Nanomedicine, 2016. 11: p. 2557-67.

38. Baru, O., et al., Angiogenesis in Regenerative Dentistry: Are We Far Enough for Therapy? Int J Mol Sci, 2021. 22(2).

39. Saghiri, M.A., et al., Role of angiogenesis in endodontics: contributions of stem cells and proangiogenic and antiangiogenic factors to dental pulp regeneration. J Endod, 2015. 41(6): p. 797-803.
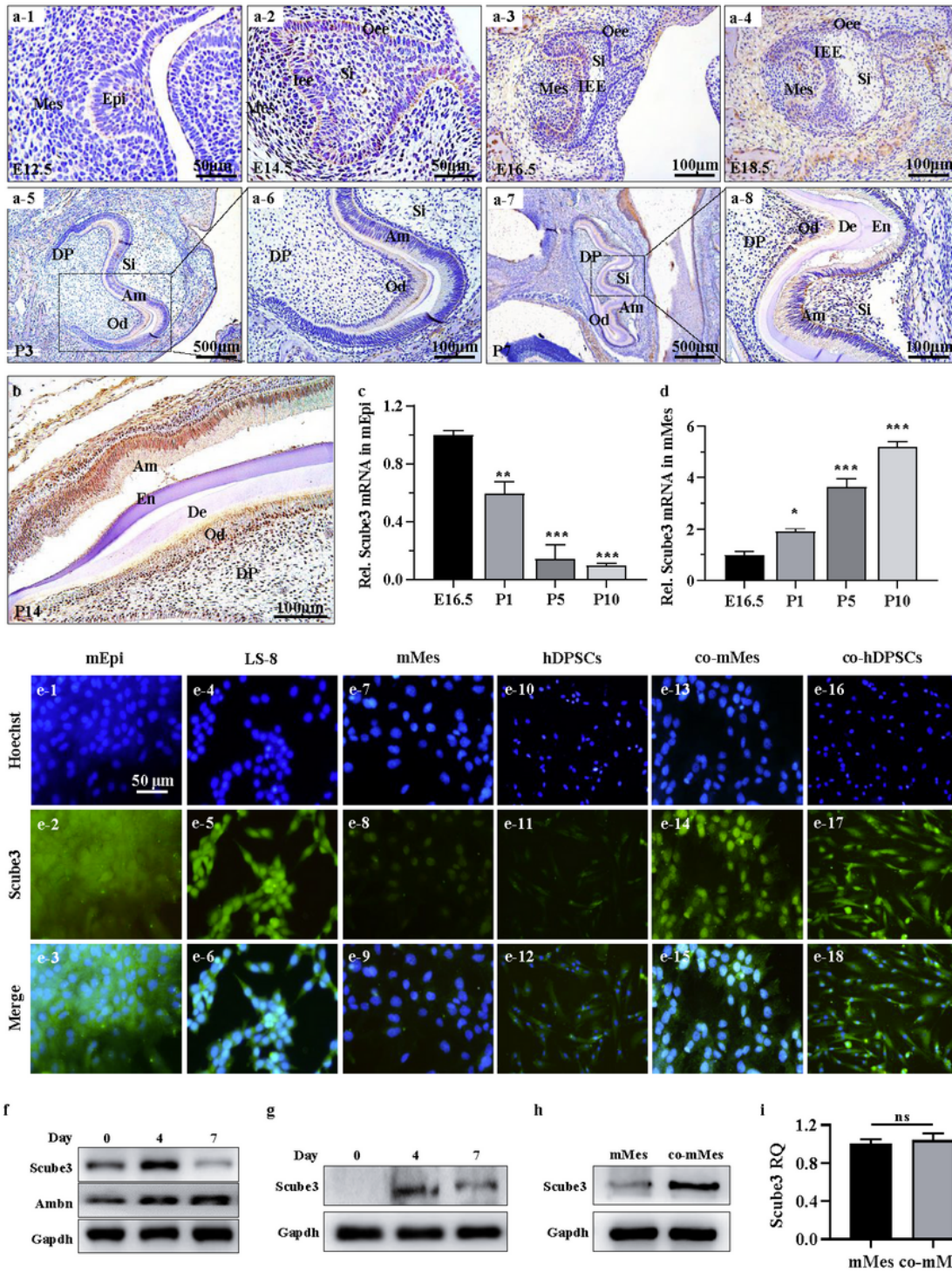
Figures

Figure 1

SCUBE3 was expressed during tooth development and translocated from Epi to Mes via paracrine pathway. (A) SCUBE3 expression in mouse mandibular first molar tooth germ was detected using immunohistochemistry during tooth development on embryonic day 12.5 (E12.5), 14.5 (E14.5), 16.5 (E16.5), 18.5 (E18.5), and postnatal day 3 (P3) and 7 (P7). Boxed areas in (A-6, 8) are shown at higher magnification in (A-5, 7). (B) SCUBE3 immunohistochemistry of mouse incisors at P14. (C, D) SCUBE3

transcript level in epithelial and mesenchymal tissues of the tooth germ on E16.5, P1, P5, and P10 were evaluated using RT-qPCR. (E) Immunofluorescence staining of dental Epi and Mes from mouse mandibular first molar tooth germ, LS-8 cells, co-cultured dental Mes and co-cultured hDPSCs. (F) SCUBE3 in whole-cell lysates of LS-8 cells on the indicated days of ameloblast differentiation were evaluated using western blot. (G) Western blot of secretory SCUBE3 in the conditioned medium of LS-8 during ameloblast differentiation. (H) Expression of SCUBE3 protein in the untreated mMes and co-cultured mMes was evaluated using western blot. (I) Expression of SCUBE3 mRNA in the untreated mMes and co-cultured mMes was evaluated using RT-qPCR. n = 3 independent biological samples. *P < 0.05, **P < 0.01, ***P < 0.001. Am, ameloblasts. Dp, dental papilla. De, dentin. En, enamel. Epi, epithelial. Iee, inner enamel epithelium. Mes, mesenchymal. Si, stratum intermedium. Od, odontoblasts. Oee, outer enamel epithelium. hDPSC, human dental pulp stem cell. Mes, mesenchymal cells. Epi, epithelial cells. ECM, extracellular matrix.
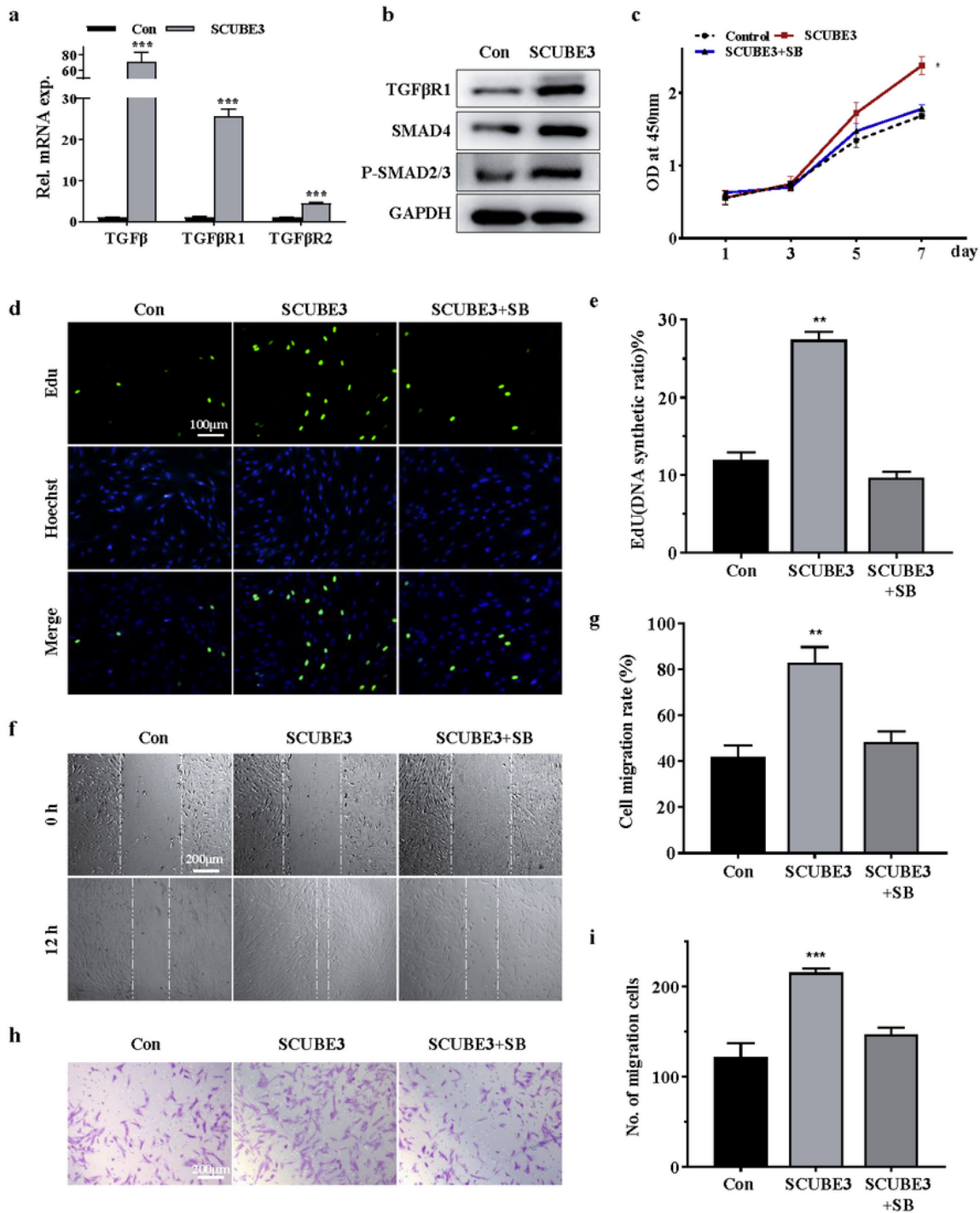
Figure 2

Cell proliferation and migration ability of hDPSCs treated with exogenous rhSCUBE3. (A) The expression of TGFβ pathway downstream effectors, TGFβ1, TGFβR1, and TGFβR2 was assessed using RT-qPCR. (B) The expression of TGFβ pathway downstream effectors was assessed using Western blot analysis. (C) CCK-8 assay. (D, E) EdU assay and quantification. (F, G) Wound healing assay and quantification. (H, I)

Transwell migration assay and quantification. n = 3 independent biological samples. *P < 0.05, **P < 0.01, ***P < 0.001. SB, SB431542. hDPSC, human dental pulp stem cell.
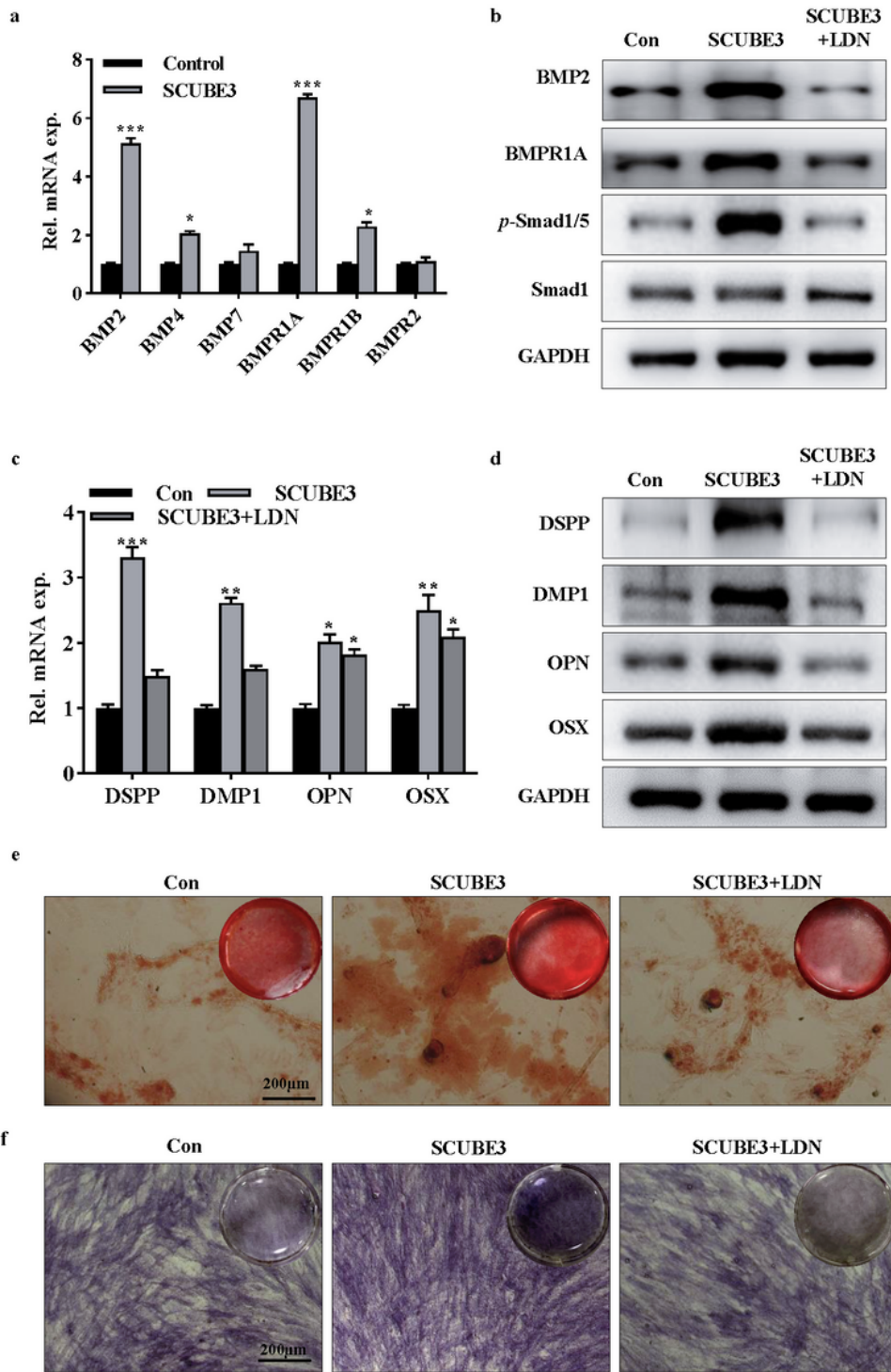

Figure 3

Exogenous SCUBE3 promoted odontoblastic differentiation of hDPSCs via BMP2/Smad pathway. The cells were exposed to exogenous rhSCUBE3 to explore the differentiation and mechanism of exogenous SCUBE3 in hDPSCs. (A) RT-qPCR was performed to verify the activation of BMP signaling pathway downstream effectors BMP2, BMP4, BMPP1A, and BMPR1B by SCUBE3. BMP2 and BMPR1A presented the most significant difference relative to the control. (B) HDPSCs were further treated with exogenous rhSCUBE3, or rhSCUBE3 with BMP2 pathway inhibitor LDN-193189. Western blot analysis was use to detected the BMP signaling pathway downstream effectors BMP2, BMPR1A, Smad1, and p-Smad1/5 expression. (C) The expression of the odontoblastic differentiation markers DSPP, DMP1, OPN, and OSX in hDPSCs treated with rSCUBE3 or rhSCUBE3 with LDN-193189 was assessed using RT-qPCR. (D)The expression of the odontoblastic differentiation markers in hDPSCs treated with rhSCUBE3 or rhSCUBE3 with LDN-193189 was assessed using Western blot. (E) Alizarin R staining. (F) Alkaline phosphatase activity staining. n = 3 independent biological samples. *P < 0.05, **P < 0.01, ***P < 0.001. LDN, LDN-193189. hDPSCs, human dental pulp stem cells.
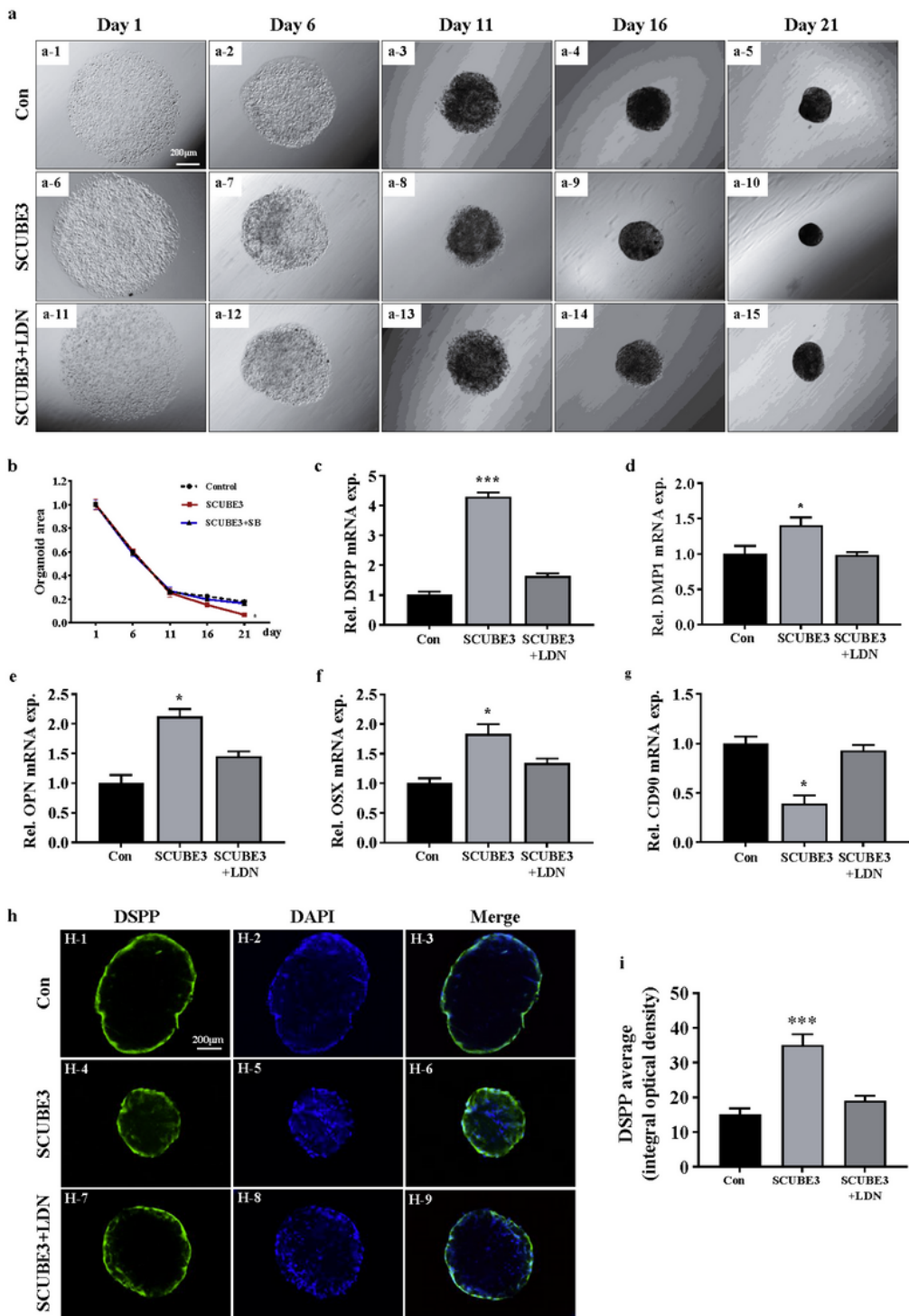
Figure 4

SCUBE3 accelerated formation and odontoblastic differentiation of dentin-pulp-like organoids. (A) hDPSCs were treated with or without rhSCUBE3 and LDN-193189. The organoids were observed and photographed under a light microscope on day 1, 6, 11, 16, and 21. (B) Quantification of organoid area. (C-F) Expression levels of the odontoblastic/osteoblastic markers DSPP, DMP1, OPN, and OSX mRNA in dentin-pulp-like organoids were evaluated by RT-qPCR. (G) Levels of the undifferentiated cell marker CD90

mRNA in dentin-pulp-like organoids were evaluated using RT-qPCR. (H, I) Photomicrographs of dentin-pulp-like organoids under confocal immunofluorescence microscopy and quantification with integral optical density (IOD). n = 3 independent biological samples. *P < 0.05, **P < 0.01, ***P < 0.001. hDPSC, human dental pulp stem cell. LDN, LDN-193189.
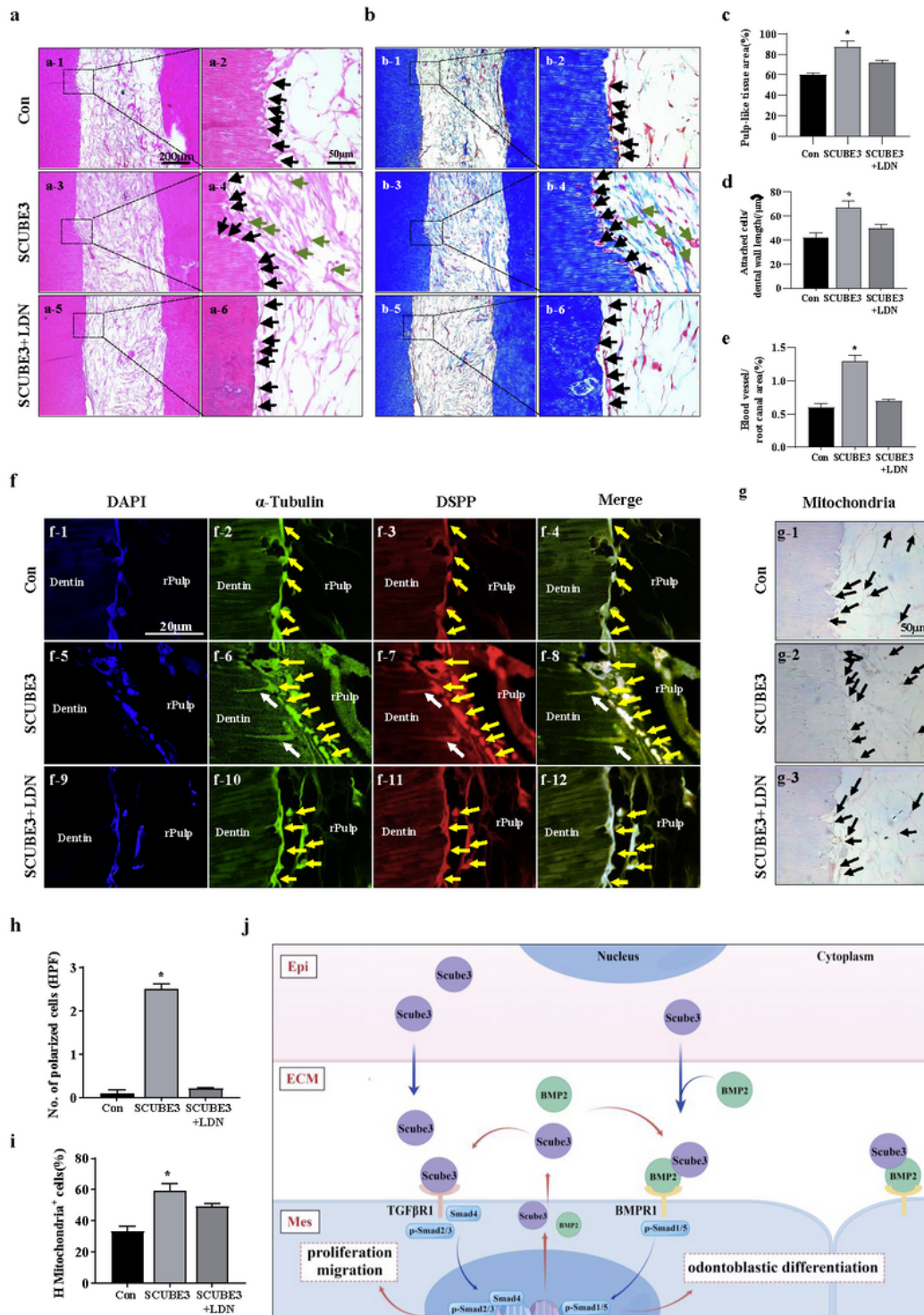
Figure 5

SCUBE3 pre-treatment enhanced cell survival, odontoblastic differentiation, cell polarization, and pulp-like tissue formation of transplanted stem cells in vivo, while LDN-193189 inhibited the effects. (A) Hematoxylin and eosin images of the control, SCUBE3 group, SCUBE3+LDN-193189 group (A-1, 3, 5) and their higher magnifications (A-2, 4, 6). (B) Masson staining images of control, SCUBE3 group, SCUBE3+LDN-193189 group (B-1, 3, 5) and their higher magnifications (B-2, 4, 6.) Black arrowheads show the cells attached to dentin wall. Green arrowheads show the new-born blood vessels. (C, D, E) Quantification of new-born pulp-like tissue, attached cell layer, new-born blood vessels. (F) Confocal immunofluorescence of α-Tubulin and DSPP. Odontoblastic processes with a length of about 20μm extended in the dentin tubules were observed in the SCUBE3 pre-treatment group (Fig. 5F). (G) Human mitochondria immunohistochemistry. Black arrowheads show the human mitochondria+ cells. (H) Quantification of polarized cells observed in (F). (I) Quantification of cells positively stained of human mitochondria. (J) Overall schematic diagram of SCUBE3 in the regulation of odontoblastic differentiation. SCUBE3 is produced and secreted into the ECM by Epi via paracrine. As a co-receptor of BMP2, secreted SCUBE3 can bind to BMP2 to form a complex. The SCUBE3-BMP2 complex then recruits BMPR1A into raft microdomains, augments the specific interactions between BMP2 and BMPR1A, and activates BMP2/Smad-mediated odontoblastic differentiation in Mes. Also, as a ligand of TGFβR1, SCUBE3 can promote cell proliferation and migration via TGF signaling. The SCUBE3 also improves endogenous SCUBE3 expression and triggers the autocrine/paracrine secretion of SCUBE3 and BMP2 during differentiation, which can positively promote odontoblastic differentiation of the developing tooth germ in response to epithelium-mesenchyme interactions. n = 3 independent biological samples. *P < 0.05, **P < 0.01, ***P < 0.001. LDN: LDN-193189. rPulp, regenerative pulp. Epi, tooth germ epithelial cells. Mes, tooth germ mesenchymal cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile.doc