Journal of Dental Sciences 17 (2022) 145–154

Available online at www.sciencedirect.com

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Original Article

Inactivation of PI3K/Akt promotes the odontoblastic differentiation and suppresses the stemness with autophagic flux in dental pulp cells

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Received 30 March 2021; Final revision received 6 May 2021
Available online 16 June 2021

KEYWORDS
Akt;
Autophagy;
Dental pulp cells;
Odontogenic differentiation;
Stemness

Abstract *Background/purpose:* Autophagy is involved in controlling differentiation of various cell types. The present study aimed to investigate the mechanism related to autophagy in regulating odontogenic differentiation of dental pulp cells.
Materials and methods: Human dental pulp cells (HDPCs) were cultured in differentiation inductive medium (DM) and odontoblastic differentiation and mineralization were evaluated by alkaline phosphatase (ALP) staining and Alizarin red S staining, respectively. Tooth cavity preparation was made on the mesial surface of lower first molars in rat. The expression of autophagy-related signal molecules was detected using Western blot analysis and Immunohistochemistry.
Results: HDPCs cultured in DM showed increased autophagic flux and declined phosphorylation of phosphoinositide 3-kinases (PI3K), protein kinase B (Akt), and mTOR. Dentin matrix protein-1 (DMP-1) and dentin sialoprotein (DSP), markers of odontoblastic differentiation, were upregulated and autophagic activation showing increased LC3-II and decreased p62 levels was observed during

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https://doi.org/10.1016/j.jds.2021.05.013

odontogenic differentiation of HDPCs. However, PI3K blocker 3-methyladenine (3MA), lentiviral shLC3 and Akt activator SC79 attenuated the expression of LC3II as well as DMP-1, ALP activity and mineralization enhanced in HDPCs under DM condition. In addition, 3MA, shLC3 and SC79 recovered the expression of pluripotency factor CD146, Oct4 and Nanog downregulated in DM condition. In rat tooth cavity preparation model, the expression of LC3B and DMP-1 was elevated near odontoblast-dentin layer during reparative dentin formation, whereas 3MA significantly reduced the expression of LC3B and DMP-1.

Conclusion: These findings indicated autophagy promotes the odontogenic differentiation of dental pulp cells modulating stemness via PI3K/Akt inactivation and the repair of pulp.

Introduction

During tooth formation, dental papilla as part of the tooth germ contains mesenchymal cells with potential to differentiate into odontoblast. After tooth eruption, under the condition of mechanical erosion or other stimulation into dentin, some types of cells such as dental pulp cells (DPCs) that reside in dental pulp tissue are known to proliferate and differentiate into odontoblast and form reparative dentin.[1] Healing and repair of human dental pulp (HDP) are results of successive and interrelated processes, including proliferation, chemotaxis, and differentiation of dental pulp cells into odontoblasts, leading to reparative dentin formation.[2] When dental pulp contribute to dentinal regeneration process, odontoblasts secrete several collagenous and non-collagenous proteins, including alkaline phosphatase (ALP), dentin matrix protein-1 (DMP-1), and dentin sialoprotein (DSP).[3–5] These proteins have been used as mineralization markers for osteoblast-/odontoblast-like differentiation of human dental pulp cells (HDPCs).[1]

Autophagy is a general term for conserved and self-degradative process that is crucial in balancing sources of energy during development.[6] The regions of the cytoplasm become sequestered in double-membrane vesicles known as autophagosomes, which undergoes a stepwise maturation process that includes fusion with acidified endosomal and/or lysosomal vesicles, resulting in delivery of cytoplasmic contents to lysosomal compartment and then degradation of contents in autophagosomes. Resulting degradation products are either reused to maintain basal macromolecular synthesis or oxidized by the mitochondria to maintain bioenergetics.[7,8] When cells are exposed to unfavorable stimuli such as nutrient deprivation, radiation, and hypoxia, autophagy is rapidly activated as an adaptive response.[9] In addition to its established roles in homeostasis maintenance and stress adaptation, mounting evidence has revealed that autophagy plays conserved roles in development and controls the differentiation process of various cell types, including lymphocyte, adipocyte, and chondrocyte.[10–14] Several studies using embryonic stem cells, adult cardiac and hematopoietic stem cells have reported that autophagy is required for self-renewal and differentiation.[15–17]

Autophagy and mitochondria biogenesis activated by schisandrin C promotes odontoblastic differentiation of HDPCs.[18] Also, it was reported can shikonin-stimulated odontoblastic differentiation from dental pulp stem cells is involved in the AKT signaling pathway.[19] Although Akt signaling has the potential role in regulating odontoblastic differentiation of dental pulp cells, the relationship between odontoblastic differentiation and stemness modulating by the PI3K/Akt pathway has not been elucidated yet. Therefore, the aim of this study was to investigate the regulation of odontoblastic differentiation and stemness via PI3K/Akt signaling in dental pulp cells cultured under odontogenic induction condition. Also, it was to evaluate whether autophagy-related events are activated to promote odontoblastic differentiation from dental pulp cells for the formation of reparative dentin in the pulp tissue of teeth using cavity preparation model *in vivo*.

Materials and methods

Cell culture

Immortalized HDPCs transfected with human telomerase catalytic component (hTERT) were kindly provided by Professor Takashi Takata (Hiroshima University, Japan).[20] Cells were cultured in growth media (GM) with α-minimum essential medium (Gibco BRL, Grand Island, NY, USA) containing 10% FBS (HyClone, Victoria, Australia), 100 U/mL penicillin (Thermo Fisher Scientific, Waltham, MA, USA), and 100 U/mL streptomycin (Thermo Fisher Scientific). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For differentiation and mineralization experiments, cells were cultured in differentiation inductive media (DM) containing 50 μM/L ascorbic acid (Sigma—Aldrich, St. Louis, MO, USA) and 5 mM/L β-glycerophosphate (Santa Cruz Biotechnology, Dallas, TX, USA).

Acridine orange staining

HDPCs were treated with 0.5 μg/ml acridine orange (Sigma—Aldrich) in serum-free medium for 15 min at 37 °C. Subsequently, cells were washed with PBS (Sigma—Aldrich) three times and examined under a Confocal Laser Scanning Microscope (Carl Zeiss, Oberkochen, Germany). Depending on acidity, autophagic lysosomes appeared as yellow-orange to bright-red fluorescent cytoplasmic vesicles whereas nuclei were stained green.

Transmission electron microscopy (TEM)

Cells were first fixed with Karnovsky's fixative at room temperature for 4 h, washed with phosphate buffer (pH 7.4, Sigma—Aldrich), and post-fixed in 1% osmium tetroxide (Sigma—Aldrich) in phosphate buffer for 2 h. Specimens were dehydrated through a graded ethanol series (50%—100%, Merck, Darmstadt, Germany) and embedded in a mixture of Epon 812 (Sigma—Aldrich) and Araldite (Sigma—Aldrich). Ultrathin sections (70 nm in thickness) were cut using a Em UC6 Ultramicrotome (Leica, Wetzlar, Germany). A ribbon of serial ultrathin sections from each sample was collected on Ni grids (Sigma—Aldrich) and stained with uranyl acetate (Sigma—Aldrich) and lead citrate (Sigma—Aldrich). Serial fields were photographed with magnification using a FEL Tecnai G2 electron microscope (FEI, Waltham, MA, USA) operated at 120 kV.

Western blot analysis

Cells were washed twice with PBS (Sigma—Aldrich) and proteins were solubilized in a lysis buffer (1% NP-40, 500 mM Tris—HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Benzamid, 1 μl/ml Trypsin inhibitor) containing Xpert Protease Inhibitor Cocktail Solution and Xpert Phosphatase Inhibitor Cocktail Solution (GenDEPOT, Barker, TX, USA). Four tooth crown-pulp pools were added into lysis buffer and crushed using an autoclaved mortar and pestle. They were then homogenized on ice using a homogenizer. Proteins were electro-blotted onto nitrocellulose membranes. The primary antibodies included the following: DMP-1, DSP, LC3, p62, AMPK, p-AMPK, PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR, CD146, Oct4, and Nanog (Cell Signaling Technology, Danvers, MA, USA). Bound antibodies were visualized using an Immobilon Western Chemiluminescent HRP Substrate (Millipore, Burlington, MA, USA). Blots were photographed using a Fusion FX (Vilber Lourmat, Collégien, France). Results are presented as means of densitometry analysis (Image J, Bethesda, MD, USA).

Alkaline phosphatase activity measurement and alizarin red S staining

Cellular alkaline phosphatase (Sigma—Aldrich) activity or Alizarin red S (Sigma—Aldrich) staining as a marker of differentiation were determined at 3, 7, or 14 days in the absence or presence of 3MA (Sigma—Aldrich) for 14 days after differentiation induction. Cells were rinsed with phosphate buffered saline (PBS, Sigma—Aldrich) and fixed in ice-cold 70% ethanol for 1 h at room temperature. Subsequently, cells were stained with BCIP®/NBT Liquid Substrate System (Sigma—Aldrich) for 1 h or 40 mM Alizarin red S (pH 4.2) for 15 min at room temperature with gentle agitation. Non-specific stain was removed by washing with water while remained stain was photographed with a HP officejet Pro L7580 scanner (Palo Alto, CA, USA). For quantitative analysis, the stain was extracted with 10% (w/v) cetylpyridinium chloride (Sigma—Aldrich) for 15 min at room temperature. Intensity was quantified by measuring the absorbance at 570 nm on an Absorbance Microplate Reader (Bio-Tek, Winooski, VT, USA).

shRNA lentiviral particles transduction

HDPCs were seeded into 10-cm dishes (Corning, Glendale, AZ, USA) at density of 4×10^6 cells/dish (50% confluency). After culturing for 24 h, control shRNA lentiviral particles (shNTC, Santa Cruz Biotechnology) and LC3 shRNA lentiviral particles (shLC3, Santa Cruz Biotechnology) were supplemented with 5 μg/ml polybrene (Santa Cruz Biotechnology). Transduced cells were grown in medium containing 5 μg/ml puromycin (Santa Cruz Biotechnology).

Animals

Male Sprague—Dawley rats at 8 weeks old were purchased from Daehan Biolink (Eumseong, South Korea). These animals were kept under standard conditions (temperature of 22 °C, humidity of 55%, 12 h light/12 h dark cycle). They were provided free access to food and water. Rats were divided into the following three groups: non-treated group (control), PBS intraperitoneal (IP) injection group, and 3MA (Sigma—Aldrich) diluted PBS (Sigma—Aldrich) IP injection group (5 rats/group). PBS and 3MA (10 mg/kg weight) were injected to rats every other day. For tooth cavity preparation experiments, rats were subjected to light anesthesia using diethyl ether (Junsei Chemical, Tokyo, Japan) for 1 min followed by immediate IP injection of a solution (1.5 ml/kg weight) of Zoletil 50 (Virbac, Carros, France), Rompun (Bayer, Leverkusen, Germany), and PBS at a ratio of 9:5:8. All experiments were approved by the Institutional Review Board of Chonnam National University Dental Hospital, Gwangju, Korea (CNUDH-2013-002).

Tooth cavity preparation of rat molars

Under deep anesthesia, limbs of rats were fixed while mouth was opened with a mouth holder. For tooth cavity preparation, autoclaved low-speed handpiece with medium grit diamond bur (Dentsply, York, PA, USA) was used under water spray cooling. Tooth cavity was made on the mesial surface of tooth crown of lower left first molars. They were drilled only into dentin layer. Lower right first molars were preserved and used as controls for each day. Rats were sacrificed at 10 days after tooth cavity preparation.

Fluorescence immunohistochemistry (FIHC)

Rats were perfused transcardially with 0.1 M PBS (Sigma—Aldrich) and then with fresh cold 4% paraformaldehyde (PFA, Hanlab, Chungju, South Korea) in 0.1 M PBS. Rat mandible and molars were extracted together and fixed in 4% PFA in 0.1 M PBS at 4 °C overnight. They were then decalcified at 4 °C for 3—4 months in RapidCal Immuno (BBC Biochemical, Mount Vernon, WA, USA) with one-week of exchange period. After decalcification, tissues were fixed with 4% PFA in 0.1 M PBS at 4 °C overnight and washed with 0.1 M PBS two times for 3 h each. They were then stored in 0.1 M PBS at 4 °C overnight. On the next day, tissues were dehydrated gradually using 50, 60, 70, 80, 90, and 100% ethyl alcohol (Duksan, Incheon, South Korea) for 1 h each. They were then stored in ACS grade ethyl alcohol (Burdick & Jackson, Muskegon, MI, USA) at 4 °C overnight. Before

paraffin embedding, tissues were immersed in xylene (Duksan) twice for 2 h each at room temperature. Tissues were then embedded in 50, 75, and 100% solution of paraffin wax (CellPath, Powys, UK) in xylene for 1 h each in a vacuum dry oven (65 °C). They were then embedded in new paraffin wax overnight. Paraffin embedded mandible and molars were cut sagittally at thickness of 4 μm using a microtome (Leica Microsystems). Sections were mounted on charged slide glasses for FIHC. Images were acquired using a Zeiss LSM 5 PASCAL laser scanning confocal microscope (Carl Zeiss).

Statistical analysis

Each experiment was repeated at least three times unless stated otherwise. All data are expressed as means ± standard deviation (SD). Statistical significance of difference between treatments was assessed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Student—Newman—Keuls test. Results were considered statistically significant when p value was less than 0.05 using Prism Software (GraphPad Software).

Results

Autophagy is induced during odontogenic differentiation of HDPCs

Autophagy is morphologically characterized by an accumulation of autophagosomes (also called autophagic vacuoles) that can subsequently fuse with lysosomes to form autophagolysosomes.[21] Acridine orange staining performed to detect formation of autophagolysosome displayed considerable red fluorescent dots indicating the formation of acidic autophagolysosome in cytoplasm of HDPCs cultured in DM containing 50 μM/L ascorbic acid and 5 mM/L β-glycerophosphate for 3 and 7 days compared with control cultured in GM, whereas displayed green fluorescence in control cells (Fig. 1A). Furthermore, ultra-structural analysis by TEM revealed that the double-membraned autophagosome was markedly observed at 7 days after differentiation induction. However, treatment of 3MA, a specific inhibitor of autophagic sequestration, decreased autophagic vacuole formation (Fig. 1B). These results demonstrate that autophagy is induced during undergo odontoblast differentiation in HDPCs, which is impaired by inhibition of autophagy, confirming autophagy activation is important in odontogenic differentiation process of HDPCs.

Autophagy activation contributes to odontogenic differentiation process of HDPCs

To observe the change of autophagy activation during odontoblastic differentiation process of HDPCs, protein levels of LC3-II and p62 as classic indicator of autophagy activation were determined by Western blot analysis in cells cultured in DM for 14 days.[22] As shown in Fig. 2A, a time-dependent conversion of LC3-I into LC3-II and down-regulation of p62 were observed when expression of DMP-1 and DSP were gradually increased in cells cultured in DM condition at 7 and 14 days, indicating that autophagy activation was induced during odontoblastic differentiation of HDPCs. To further confirm whether autophagy influences odontogenic differentiation of HDPCs, protein lysates were prepared from HDPCs treated with autophagy inhibiter 3MA (1 mM), a specific blocker of autophagic sequestration under DM condition. Western blot analysis showed that DMP-1 and DSP levels were significantly downregulated by 3MA, which reduced LC3-II expression, but increased p62 level (Fig. 2A). In addition, 3MA (1 mM) induced significant reduction of ALP activity by ALP staining and significant secretion of mineralized matrix measured by Alizarin red S staining compared to cells cultured in DM condition (Fig. 2B).

HDPCs were transduced with lentivirus containing LC3-specific shRNA (shLC3) and then subjected to odontogenic induction for 7 days. The shLC3-expressing cells also showed decreased DMP-1 level, ALP activity and mineralization as well as reduced LC3-II compared with cells infected with non-target control shRNA (shNTC) (Fig. 2C and D). These results demonstrate that the ability of HDPCs to undergo odontoblast differentiation is impaired when autophagy is inhibited, confirming autophagy activation is important in odontogenic differentiation process of HDPCs.

Odontogenic differentiation of HDPCs is regulated via Akt/mTOR pathway

It has been reported that modulation of PI3K/Akt/mTOR and autophagy can control osteogenic differentiation of human mesenchymal stem cells.[23] To examine whether phosphoinositide 3-kinases (PI3K), Akt, and mTOR signal molecules are activated during differentiation of HDPCs, Western blot analysis was performed in HDPCs incubated in DM for 7 days. As shown in Fig. 3A, p-PI3K, p-Akt, and p-mTOR levels were decreased, but p-AMPK level was increased.

To examine whether Akt/mTOR pathway is involved in odontoblastic differentiation of HDPCs, Akt activator SC79 (10 μg/ml) was treated in HDPCs under DM condition for 7 days and changes in the expression of autophagy marker and odontoblastic marker were detected using Western blot analysis. The conversion of LC3-I into LC3-II as well as expression of DMP-1 were significantly reduced by SC79, which ameliorated phosphorylation of Akt and mTOR compared with those in the control (Fig. 3B and C). In addition, SC79 significantly diminished ALP activity and mineralization compared with untreated control (Fig. 3D). These results indicate that odontogenic differentiation of HDPCs is mediated through Akt/mTOR pathway.

Activation of Akt/mTOR enhances stemness maintenance during odontoblastic differentiation process of HDPCs

To clarify the role of autophagy in the regulation of stemness during odontoblastic differentiation of HDPCs, cells were grown without or with 1 mM 3MA, shLC3, or 10 μg/ml SC79 for 3, 7, or 14 days under DM condition. Western blot analysis was performed to evaluate expression levels of CD146, Oct4, and Nanog as characteristic markers of primitive stem cells. Cells displayed time-dependently downregulation of CD146, Oct4, and Nanog. However, autophagy inhibition

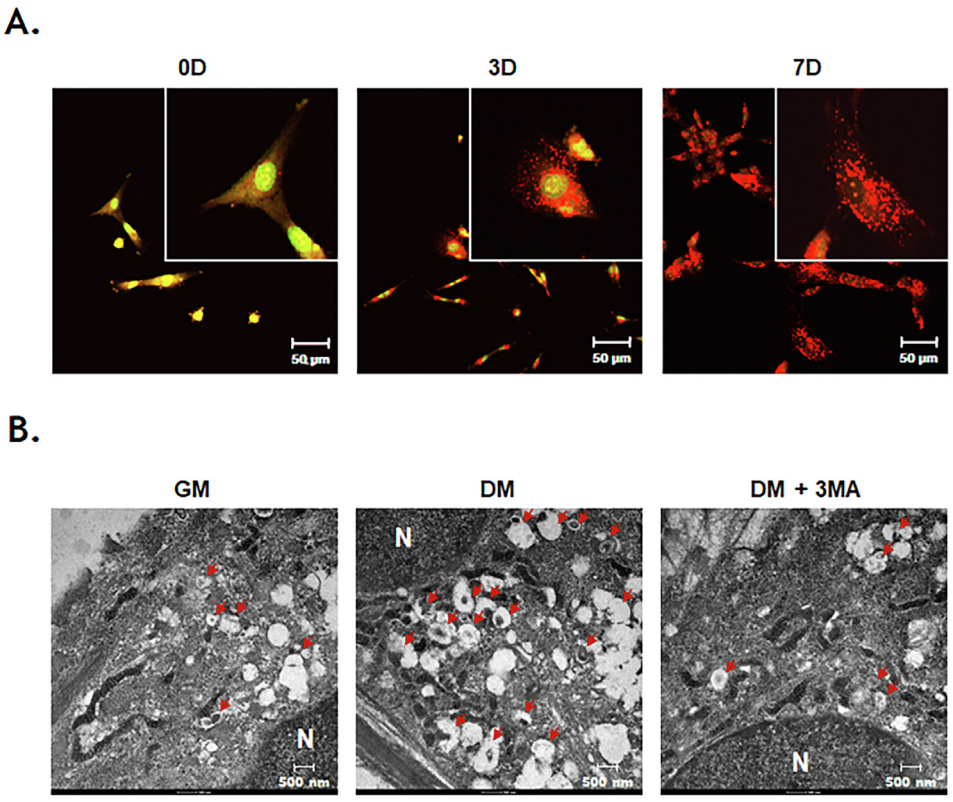

Figure 1 Induction of autophagy during odontogenic differentiation of human dental pulp cells (HDPCs). (A) Cells were cultured in differentiation media (DM) for 0, 3 or 7 days. Cells were stained with Acridine orange (1 μg/ml) and then visualized by a confocal microscope. Scale bars, 50 μm. (B) Cells cultured in growth media (GM) or DM without or with 1 mM 3MA for 7 days were analyzed by transmission electron microscopy and representative images of autophagic vacuoles (red arrows) were shown. Scale bar, 500 nm. N: nucleus.

using 3MA or shLC3 obviously caused upregulation of CD146, Oct4, and Nanog expression for 3 or 7 days compared to the control, respectively (Fig. 4A and B). In addition, Akt activation by SC79 upregulated CD146, Oct4, and Nanog expression for 3 or 7 days (Fig. 4C). These findings demonstrated that autophagy negatively regulates stemness in HDPCs by suppressing Akt activation.

Autophagy is associated with the odontogenic differentiation during reparative dentin formation of rat DPCs

To observe odontogenic differentiation and reparative dentin formation of DPCs, tooth cavity preparation model was set on lower first molars of male Sprague—Dawley rat. Mesial surface of rat molars was drilled into dentin layer without perforation into dental pulp. Fluorescence IHC was performed to detect specific cells with activated autophagy and expression level of odontoblast differentiation-related protein. As shown in Fig. 5A and B, expression of LC3B exhibited high intensity in odontoblast layer of drill teeth, while weak intensity in the control group. In addition, expression of DMP-1 in drilled teeth was highly localized in the odontoblast-dentin layer at day 10 after cavity preparation, compared with the control. These results demonstrate that autophagy activation is accompanied by odontogenic differentiation process during reparative dentin formation after drilling into dentin. However, lower first molars of 3MA injection after tooth cavity preparation maintained low intensity of LC3B and DMP-1 compared with the control (Fig. 5A and B). The protein level of odontogenic markers DMP-1 and DSP in teeth under cavity preparation were increased than those in control group. In addition, LC3-II expression was augmented in a time course, but p62 level was decreased at day 10 after tooth cavity preparation, showing autophagy activation is induced (Fig. 5C). However, 3MA injection with tooth cavity preparation downregulated DMP-1 and DSP levels as well as LC3-II and p62 protein expression compared with preparation group (Fig. 5C). Collectively, these results suggest that autophagy promotes the odontogenic differentiation from dental pulp cells during reparative dentin formation.

Discussion

The most notable characteristic of DPCs is that they have the potency to induce into odontogenic differentiation, which can regenerate dentin-pulp-like complexes. Autophagy activation has found in development and the differentiation process of various cell types and their crucial roles have studied.[15,16,23—25] In this study, we investigated that odontoblastic differentiation of dental pulp cells and

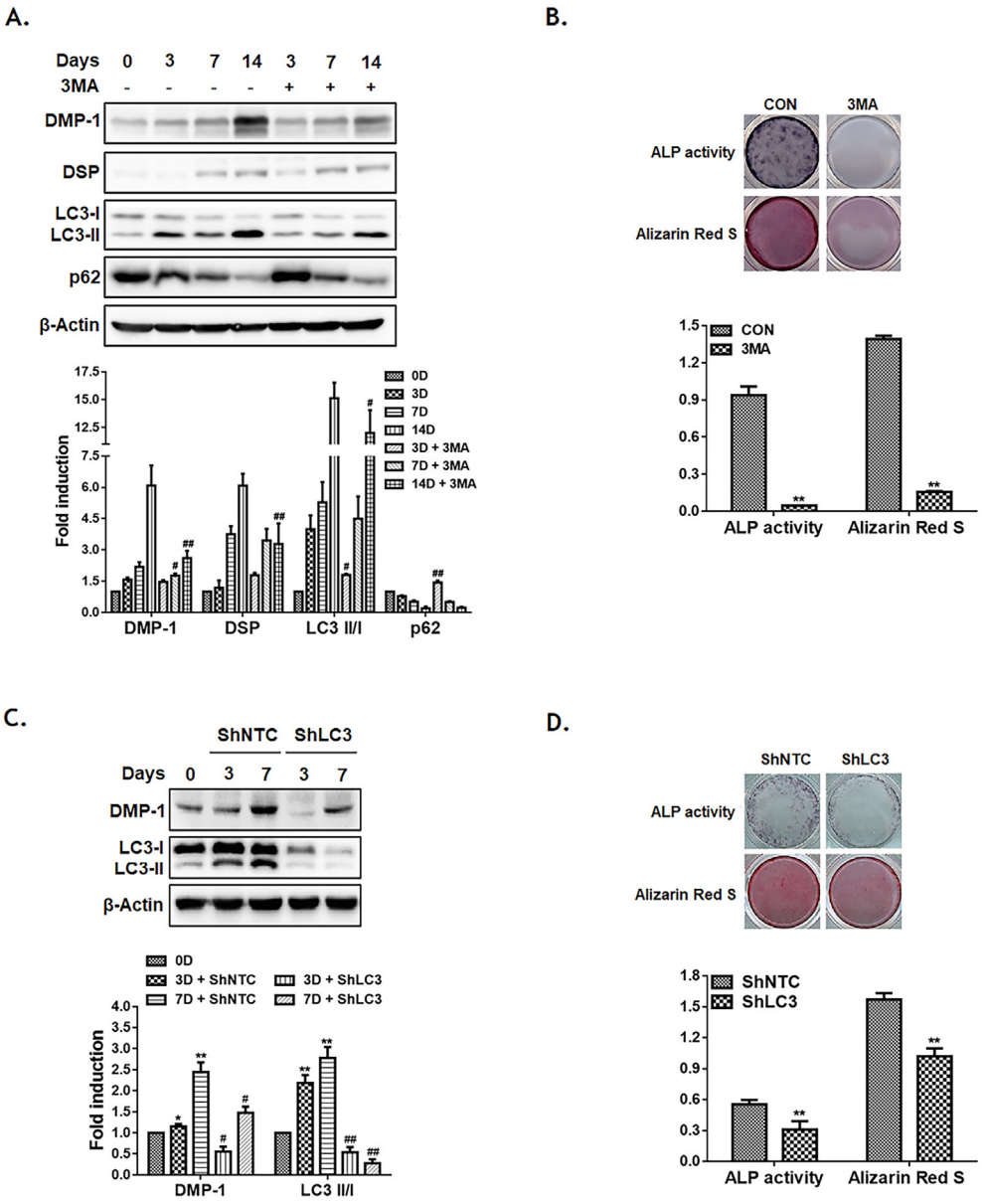

Figure 2 Effect of autophagy inhibition on odontogenic differentiation and mineralization in human dental pulp cells (HDPCs). (A) Cells were cultured in differentiation media (DM) without or with 1 mM 3-methyladenine (3MA) for 3, 7, or 14 days. Cell lysates were subjected to Western blot analysis using anti-dentin matrix protein-1 (DMP-1), dentin sialoprotein (DSP), LC3 and p62 antibodies. (B) Cells cultured in DM with 1 mM 3MA for 14 days were stained with BCIP®/NBT Liquid Substrate System (ALP staining) and with Alizarin red S. Stained cells were dissolved with 10% (w/v) cetylpyridinium chloride and quantified using an absorbance microplate reader at 570 nm. (C) Cells were transduced with lentivirus containing either control shRNA lentiviral particles (shNTC) or LC3 shRNA lentiviral particles (shLC3) and then subjected to odontogenic induction for 3 or 7 days. Cell lysates were subjected to Western blot analysis to determine protein levels of DMP-1 and LC3. (D) Cells were transduced with lentivirus containing either shNTC or shLC3. The degree of differentiation was determined based on ALP activity and Alizarin red S staining at 7 days. Data are presented as mean ± SD from triplicate independent experiments. $^{**}p < 0.01$ vs. control cells or shNTC group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. 3MA-untreated cells or shNTC group.

reparative dentin formation are related to autophagy activation, which regulates maintenance of stemness via suppressing PI3K/Akt/mTOR pathway.

Many reports demonstrate the potential role of autophagy in the differentiation of MSCs, macrophage and myocardial differentiation.[17,23,26] Moreover, it was reported that autophagy fragmentarily appears during the development of mouse first molar and odontoblast differentiation in inflammatory environments.[27,28] In the present study, expression pattern of LC3-II and p62 as autophagy flux indicator were significantly increased with formation of acidic autophagolysosomal vacuoles with upregulation of odontogenic differentiation marker DMP-1 and DSP, ALP activity and mineralization of HDPCs cultured in DM

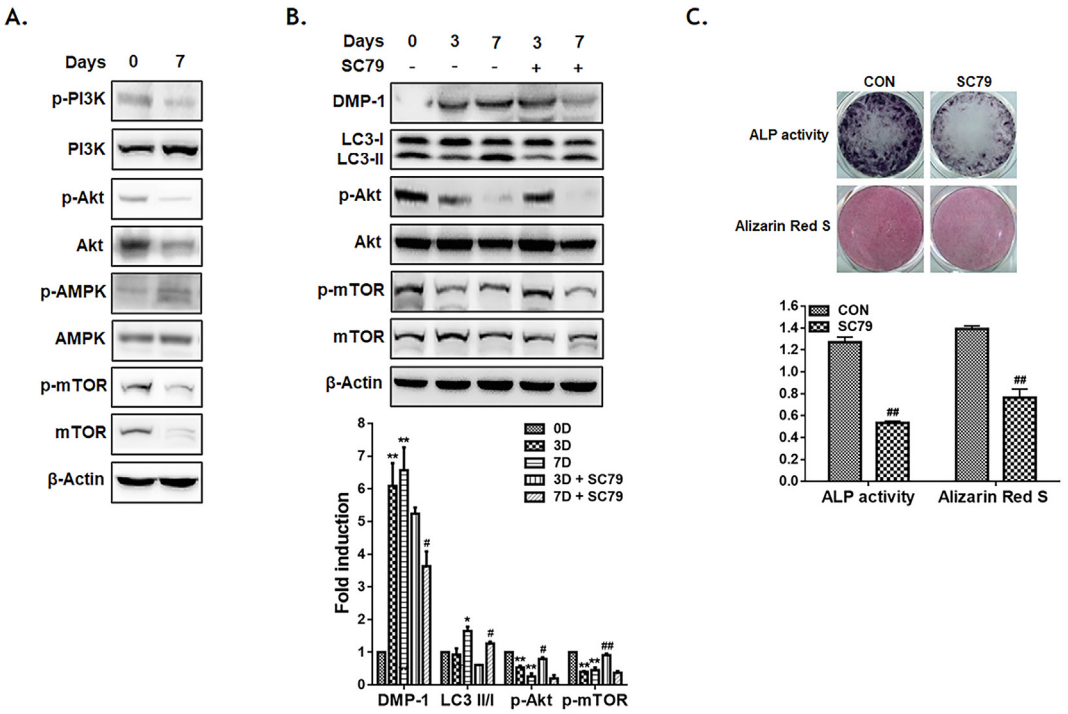

Figure 3 Involvement of Akt/mTOR pathway in odontogenic differentiation of human dental pulp cells (HDPCs). (A) Cells were incubated in differentiation media (DM) for 7 days and total cell lysates were probed with p-PI3K, p-Akt, p-AMPK and p-mTOR antibodies. (B) Cells cultured in DM without or with Akt activator SC79 (10 μg/ml) for 3 or 7 days were harvested at indicated time points and cell lysates were subjected to Western blot analysis. Densitometry analysis results are presented as relative ratio compared with β-Actin. (C) Alkaline phosphatase (ALP) activity analysis and Alizarin red S staining were performed at 7 days under DM condition. ALP activity or matrix mineralization level was normalized with that of untreated cells. Data are presented as mean ± SD from triplicate independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. 0 day or control cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. SC79-untreated cells.

condition. The procedure of cavity preparation induces destructive changes in odontoblasts and an acute inflammatory reaction.[29] At affected site of tooth, the odontoblasts are capable of depositing reactionary dentin or pulpal mesenchymal stem cells take the place of the degenerated odontoblasts to differentiate into odontoblast-like cells, resulting in the formation of reparative dentin.[30] In tooth under cavity preparation, strengthened LC3-II protein level and decreased p62 level were presented at day 10, indicating autophagy activation. Particularly, fluorescence IHC showed high expression of DMP-1 as well as LC3B in the odontoblast layer of drilled teeth. These results demonstrated that autophagy is enhanced with upregulation of odontogenic phenotypic markers in pulp-dentin regeneration process. Overall, it is supposed that autophagy may play a crucial role in the odontogenic differentiation toward odontoblasts from dental pulp cells and the regeneration of odontoblasts during the reparative dentin formation after tooth injury. These results are similar with other studies reported that autophagy is a participant process in the osteogenic differentiation of mesenchymal stem cells and that regulates odontoblast differentiation in an inflammatory environment.[23,27]

It is known that 3MA inhibits autophagy by blocking autophagosome formation via inhibition of PI3K.[31] Expression of odontoblastic differentiation markers in HDPCs was suppressed through the inhibition of autophagic process by 3MA, with evidence of decreased LC3-II and DMP-1 expression and reduced ALP activity and mineralization. Also, inhibition of autophagy process using shLC3 showed same effects with 3MA in HDPCs. It appeared similar results in rat tooth cavity preparation model that 3MA injection attenuated LC3-II and DMP-1 expression compared with the preparation group, demonstrating that autophagy modulates differentiation of subodontoblast layer cells into odontoblast like cells during process of reactionary dentin production. Our *in vivo* data gave strong support to the conjecture that significant role of activated autophagy is to promote odontoblastic differentiation from dental pulp cells for the reparative dentin formation.

The Akt, mTOR, and AMPK signaling pathway are known as principal mediators involved in autophagic process and participant regulators in the differentiation of various cell types.[10,11] Akt and mTOR are involved in differentiation of bone marrow MSCs and osteogenic cell lines to osteoblasts.[23,32] In the present study, the role of PI3K, Akt, mTOR, and their interplays in odontogenic differentiation process of HDPCs were investigated. In the present study, the phosphorylation of PI3K/Akt/mTOR was diminished during odontogenic differentiation of HDPCs in DM condition, whereas Akt activator SC79 enhanced phosphorylation of mTOR and decreased LC3-II levels, autophagy marker. In addition, Akt activation by SC79 led to reduction of DMP-1 levels, ALP activity and mineralization, indicating that

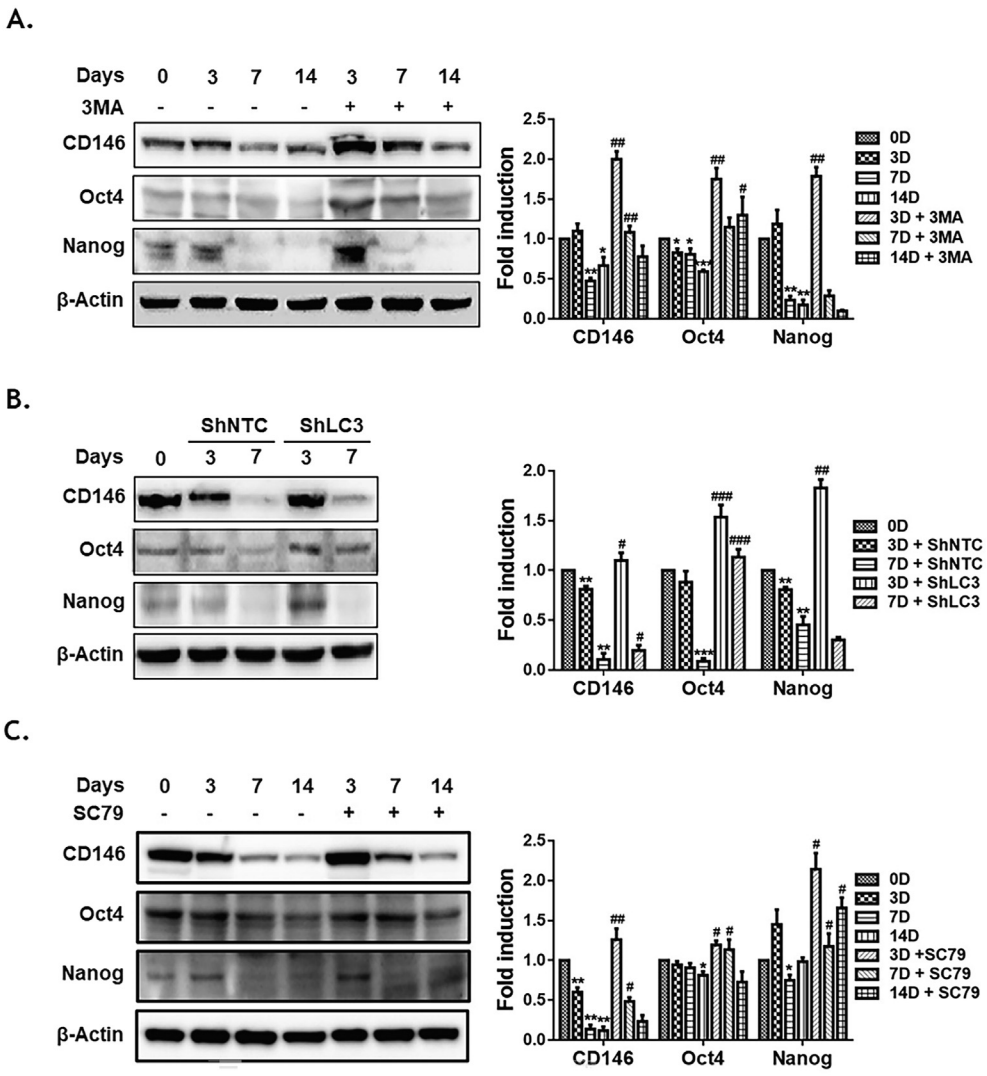

Figure 4 Effect of autophagy inhibition on expression of stem cell multi/pluri-potency markers during odontogenic differentiation in human dental pulp cells (HDPCs). (A—C) Cells were grown in differentiation media (DM) without or with 3-methyladenine (3MA) (1 mM), LC3 shRNA lentiviral particles (shLC3) and Akt activator SC79 (10 μg/ml) for 3, 7, or 14 days and Western blot analysis was performed with CD146, Oct4, and Nanog antibodies. Densitometry analysis is presented as relative ratio compared with β-Actin. Data are presented as mean ± SD from triplicate independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. 0 day; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. 3MA or SC79-untreated cells.

mechanism related to Akt signaling negatively regulates the odontoblast differentiation in HDPCs. The present study provided possible evidence that autophagy activation via Akt/mTOR signaling pathway is involved in the modulation of odontogenic differentiation of HDPCs. It is consistent to previous report that suppression of AKT-mTOR signal pathway stimulates osteogenic/dentinogenic capacity of stem cells from apical papilla.[33]

Autophagy regulates the stemness maintenance in MSC with decreased reactive oxygen species generation.[34] Also, it was found that increased autophagy in hepatocellular carcinoma promotes cell survival and stem cell-like property.[35] Until recently, the implication of autophagy in the properties of stemness in stem cells have been extensively studied in several cells.[25,26] In the present study, to investigate whether autophagy involves stemness maintenance in HDPCs during differentiation process, the expression of CD146, Oct4, and Nanog as key multi/pluri-potency factors were examined.[36,37] CD146, Oct4, and Nanog levels were time-dependently downregulated in differentiation condition of HDPCs, whereas were significantly increased by autophagy inhibition with 3MA or shLC3. These results strongly demonstrated that autophagy downregulates expression of stemness-related markers during odontogenic differentiation of HDPCs. However, the present results were disagreed with the several studies showed that autophagy participates in maintaining or promoting the stemness phenotype in some cancer stem cells.[35,38,39] Furthermore, the present study showed that Akt activator SC79 restored the expression of stemness markers CD146, Oct4, and Nanog downregulated during odontogenic differentiation of HDPCs, suggesting that Akt signaling regulates stemness maintenance during

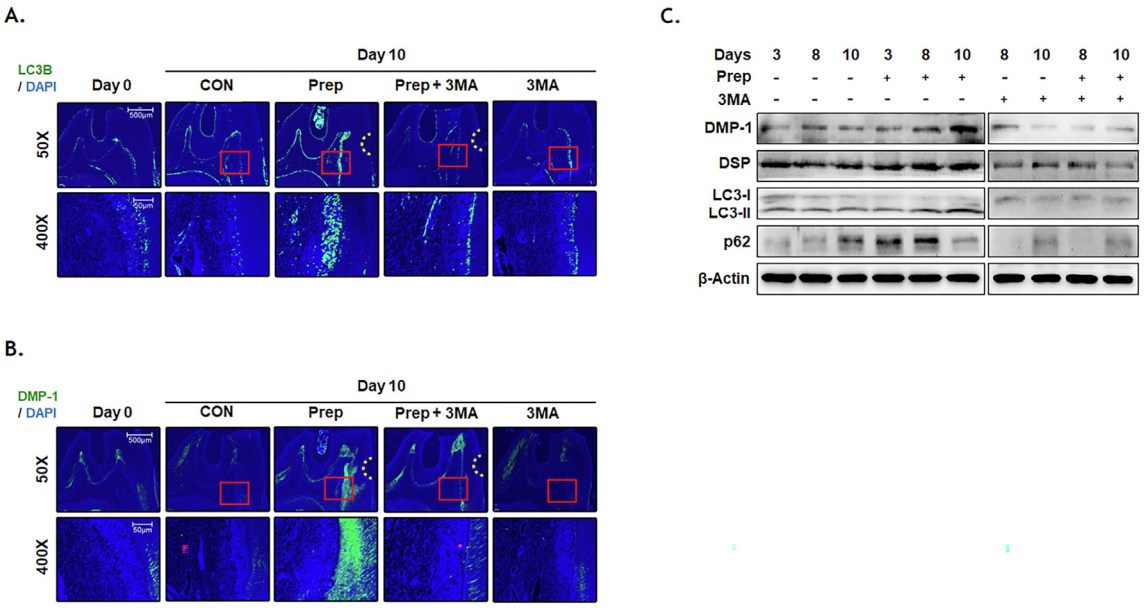

Figure 5 Effect of autophagy inhibition on odontogenic differentiation in rat tooth crown-pulp lysates after tooth cavity preparation. (A, B) Localization of proteins was visualized after fluorescence immunohistochemistry staining in sagittal tooth sections from rats without or with 3-methyladenine (3MA) intraperitoneal (IP) injection (10 mg/kg weight) at 10 days after tooth cavity preparation (n = 4). Green fluorescence indicates LC3B-positive and blue fluorescence indicates DMP-1-positive cells. Yellow dotted curves indicate tooth cavity preparation sites. Scale bars, 500 μm for 50× magnification and 50 μm for 400× magnification. (C) Pooled samples of tooth crown-pulp lysates isolated from rats with or without 3MA IP injection were analyzed by Western blot analysis (n = 4). β-Actin was used as an internal control.

odontoblastic differentiation of HDPCs. It was different with previous study reported that undifferentiated mouse embryonic palatal mesenchyme (MEPM) cells exhibited low p-PTEN, p-Akt and p-mTOR levels, indicating that MEPM cells maintain their stemness through PTEN/Akt/mTOR signaling.[40] The results of present study on the relationship between autophagy and stemness in HDPCs will enhance understanding of the mechanisms through which autophagy contributes to modulate self-renewal and odontoblastic differentiation in HDPCs, but it needs further study on the association of autophagy and stemness-related transcription factor participated in this process.

Taken together, our results revealed that autophagy plays a significant role in induction of odontogenic differentiation and regulation of stemness of HDPCs via suppressing PI3K/Akt/mTOR signal pathway. A better understanding of the mechanism facilitating the crucial role of autophagy in odontogenesis of DPCs may offer therapeutic benefit for dental pulp regeneration.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2019R1A5A2027521). It was also supported by Chonnam National University (No. 2018-3404).

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