Midkine promotes odontoblast-like differentiation and tertiary dentin formation

Yeoung-Hyun Park[1] , Yoon Seon Lee[1] , You-Mi Seo[1] , Hyejin Seo[1], Jung-Su Park[2] , Hyun Sook Bae[3] and Joo-Cheol Park[1]

Keywords : Midkine, Autophagy, Odontoblast, Dentinogenesis, Development, Repair, Biomaterial(s)

[1]Laboratory for the Study of Regenerative Dental Medicine, Department of Oral Histology-Developmental Biology, School of Dentistry and Dental Research Institute, Seoul National University, 1 Gwanakro, Gwanak-gu, Seoul 08826, Republic of Korea

[2]Department of Dental Hygiene, Seoyeong University, Gwang-ju, Republic of Korea

[3]Department of Oral Hygiene, Namseoul University, Cheonan, Republic of Korea

Corresponding Author:

J.C. Park, Department of Oral Histology/Developmental Biology, School of Dentistry and Dental Research Institute, Seoul National University, 1 Gwanakro, Gwanak-gu, Seoul 08826, Republic of Korea

Email: jcapark@snu.ac.kr

Appendix

Materials and Methods

Cell Culture

MDPC-23 cells (passage 17) were cultured in Dulbecco's modified Eagle medium (DMEM ; Gibco BRL, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS ; Gibco BRL) and antibiotic-antimycotic (Gibco BRL) at 37℃ in an atmosphere of 5% CO_2. To induce MDPC-23 cell differentiation, 80-90% confluent cells were cultured with 5% FBS, ascorbic acid (50㎍/㎖), and β-glycerophosphate (10mM) for up to 10 days. Recombinant Midkine (rMK) (TP303995) and Midkine shRNA lentiviral particle (TL501322V) were purchased from Origene (Rockville, MD, USA).

Western Blot Analysis

Cellular proteins (30㎍) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked for 1h with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and incubated overnight at 4℃ with the primary antibody diluted in TBS-T (1:2000). After washing, membranes were incubated for 1h with the appropriate secondary antibodies. Labeled protein bands were detected under an enhanced chemiluminescence system (Amersham Biosciences/GE Healthcare). All reactions were performed in triplicate. Semi-quantitative analyses were performed using Image J software (National Institute of Health, MD, USA).

Real-Time Polymerase Chain Reaction Analysis

Total RNA was extracted from the cells with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (3 μg) was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and oligo (dT) primers (New England Biolabs). The product (1 μL) was amplified by PCR using the following primer pairs: *mMK*, forward 5′-TGGAGCCGACTGCAAATACAA-3′ and reverse 5′-GGCTTAGTCACGCGGATG G -3′; *mGapdh*, forward 5′-AGGTCGGTGTGAACGGATTTG-3′ and reverse 5′-

TGTAGACCATGTAGTGAGGTCA-3′; *mDspp,* forward 5′-CAACCATAGAGAAAGCAAACGCG-3′ and reverse 5′-TTTCTGTTGCCACTGCTGGGAC-3′; *mDmp1,* forward 5′-ACAGGCAAATGAAGACCC-3′ and reverse 5′-TTCACTGGCTTGTATGG-3′; *mNestin,* forward 5′-AGCCCTGACCACTCCAGTTTAG-3′ and reverse 5′-CCCTCTATGGCTGTTTCTTTCTCT-3′. Real-time PCR was performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, USA) using SYBR Green PCR Master Mix (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. PCR conditions were 40 cycles of 95 °C for 1 min, 94 °C for 15 s, and 60 °C for 34 s. All reactions were performed in triplicate, and PCR product levels were normalized to that of the housekeeping gene *Gapdh*. Relative changes in gene expression were calculated using the comparative threshold cycle (Ct) method.

Pulp Exposure Protocol and Tissue Preparation

The heads of mice are fixed in 4% paraformaldehyde (PFA) in PBS for 24 h at 4 °C. After fixation, the heads of mice were decalcified in 10% ethylenediaminetetraacetic acid (EDTA, pH 7.4), embedded in paraffin, and processed for histological analysis. 4 μm serial sections were mounted on silanized slides and stored in air-tight cases at 4 °C. All tissues were sectioned on frontal plane. Twelve mice (aged 1m) were used for this experiment. Mice were anesthetized with a solution of 2.5% Avertin (Sigma, St. Louis, MO, USA) diluted with RNase-free water (T&I, chuncheon, Korea). The oral cavity was opened with a mouth retractor to expose the molars. Maxillary first molars were cleaned with 0.5% chlorhexidine. Then, the center of the first molar was drilled using a carbide bur (FG1/4) until the pulp was visible, and a 31-mm K-file (M-Access) was used to expose the pulp chamber. The cavities were divided into 4 groups for the experiment. Defect areas were covered with glass ionomer (GI) cement after topical treatment with (1) a PBS-soaked collagen sponge (control(PBS)+GI), (2) an rMK-soaked collagen sponge (rMK+GI), (3) a 3MA-soaked collagen sponge (3MA+GI), or (4) a 3MA and rMK-soaked collagen sponge (3MA+rMK+GI). Each group comprised 2 first molars per animal, totaling 24 first molars. The animals were sacrificed after 4 weeks post-operation and were histologically analyzed (n=3).

Immunocytochemistry and Immunofluorscence

MDPC-23 cells and tissue sections were treated with phosphate-buffered saline (PBS) with 0.5% Triton X-100 for permeabilization. After washing and blocking, cells were incubated for 1h with LC3 antibody (1:100) or DSP

antibody (1:100) in blocking buffer (PBS and 2% bovine serum albumin). Subsequently, anti-fluoresceinisothyocyanate (FITC) anti-rabbit secondary antibody (1:200) or Cy3-conjugated anti-rabbit (1:200) was applied. Cells were visualized using fluorescence microscopy (AX70, Olympus, Tokyo, Japan). The chromosomal DNA in the nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI). Deparaffinized sections were incubated with rabbit polyclonal MK antibody (1:200; Genetex; GTX31198) and LC3 antibody (1:200; Cell Signaling; #3868) overnight at 4°C. After washing, sections were exposed to biotin-labeled goat anti-rabbit IgG (1:200) as the secondary antibody. Each expression was detected using an ABC kit (Vector Labs, Burlingame, CA, USA).

Immunohistochemistry

Briefly, sections were incubated overnight at 4°C with rabbit polyclonal MK antibody (1:200; Genetex; GTX31198), LC3 antibody (1:200; Cell Signaling; #3868), and DSP antibody (1:200; immunization of rabbit with the synthetic peptides NH_2- GNKSIITKESGKLSGS- COOH (amino acid residues 372~387 of DSP)) in 2% bovine serum albumin (BSA)/phosphatebuffered saline (PBS), pH 7.4. Negative control sections were incubated 2% BSA/PBS. Sections were then incubated with a biotin-labeled goat anti-rabbit immunoglobulin G (IgG) (1:200; Vector Labs) as the secondary antibody and then washed and incubated with avidin-biotin-peroxidase complex (Vector Laboratories Inc., Burlingame, USA). Peroxidase was revealed by incubation with methanol containing 3% H2O2. Signals were converted using a diaminobenzidine kit (Vector Laboratories). Nuclei were stained with methylgreen.

Luciferase assay

MDPC-23 cells were seeded on 24-well plates at a density of 5×10^4 cells/well. The cells were transiently transfected using Metafectene Pro reagent (Biontex, Martinsried/Planegg, Germany). Construct pGL3-Dspp was cotransfected into MDPC-23 cells with the rMK. Following the addition of 50 ml luciferin to 50 ml of the cell lysate, luciferase activity was determined using an analytical luminescence luminometer (Promega, Madison, WI, USA) according to the manufacturer's instructions. The analysis was performed in three independent experiments.

Alizarin red S staining

MDPC-23 cells were seeded on 35-mm dishes at a density of 1×10^5 cells/well and were cultured for 7 d with or without rMK groups, 3-methyladenine (3MA, Sigma), or 3MA with rMK groups. A mineralization assay was

performed by staining with alizarin red S (Sigma) solution in 0.1% NH_4OH at pH 4.2 for 20 min at room temperature.

Masson's Trichrome staining

Sections of mouse teeth were deparaffinized in xylene and rehydrated through ethanol. Sections were stained with Weigert's Haematoxylin for 10 minutes, followed by 1% Biebrich-Scarlet-Acid Fuchsin solution for 20 minutes, and 5% phosphotungstic acid for 10 minutes and stained with 2.5% Aniline Blue solution for 20 minutes. Following staining, sections were differentiated in 1% acetic acid for 1minute, dehydrated through 90% and 100% ethanol, cleared in xylene and permanently mounted.

Cell cytotoxicity assay

MDPC-23 cells were seeded in 96-well plates at 3×10^3 cells/well and incubated for 24 h using culture medium (DMEM). The medium was replaced with medium only (control group), 3MA 1mM (3MA+medium), 3MA 2.5mM (3MA+medium), or 3MA 5mM (3MA+medium) (experimental groups) for 5 days. To determine the cellular metabolic activity, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Medifab) was added to the controls and the experimental groups after 4 h. Cell cytotoxicity was measured using a luminometer (FLUOStar OPTIMA; BMC Laboratory), and experiments were performed in triplicate.

TEM analysis

Cells were harvested with trypsin EDTA and fixed in 2% paraformaldehyde (PFA) and 2.5% glutaraldehyde (GA) in 0.1 M sodium cacodylate buffer (pH 7.4). They then were post fixed in buffered 1% osmium tetroxide, embedded and fixed a second time in 2.5% GA, cut into 1 mm sections, and dehydrated through a graded ethanol series and propylene oxide prior to microwave infiltration of 1:1 Spurr/Epon resin. Polymerized blocks were sectioned on a ultramicrotome (Leica, Wetzlar, Germany), and 70-nm sections were re-mounted on 100 mesh grids and stained with uranyl acetate and Reynolds lead citrate. Images were acquired with a JEOL JEM-1400 Flash (Tokyo, Japan).

Cell migration assay

MDPC-23 cells were seeded on 35-mm culture dishes (1×10^5 cells) and grown to confluence for an *in vitro*

wound-healing assay. A scratch was made using a sterile pipette tip in the middle of each dish. Cells were then washed 3 times with PBS and incubated in culture media with or without rMK. Triplicate samples were analyzed from 3 independent experiments.

Tooth Germ Culture

Tooth germs were collected from twelve mouse embryos at 12 days (E12). The mandible was removed, and the mandibular first molars were dissected from surrounding tissues in sterilized PBS. All steps were performed on ice and under sterile conditions. A trowel-type organ culture system was used, in which the explants were grown at the medium-gas interface. The metal grid was placed in a 35-mm culture dish. The samples were transferred onto the filter, which was placed on *the* holds of the grid. Tooth germs were cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 50 U/ml penicillin/streptomycin, and 100 μg/ml ascorbic acid and incubated at 37℃ and 5% CO_2. we conducted *ex vivo* organ culture of mouse molar tooth germs under four different conditions: culture media only (control), rMK-treated, shMK-transfected, or 3MA-treated After 5 days of cultivation (n=3), the tooth germs were fixed in 4%PFA, embedded in paraffin, and serially sectioned at 2㎛.

Primary cell culture and *ex vivo* transplantation

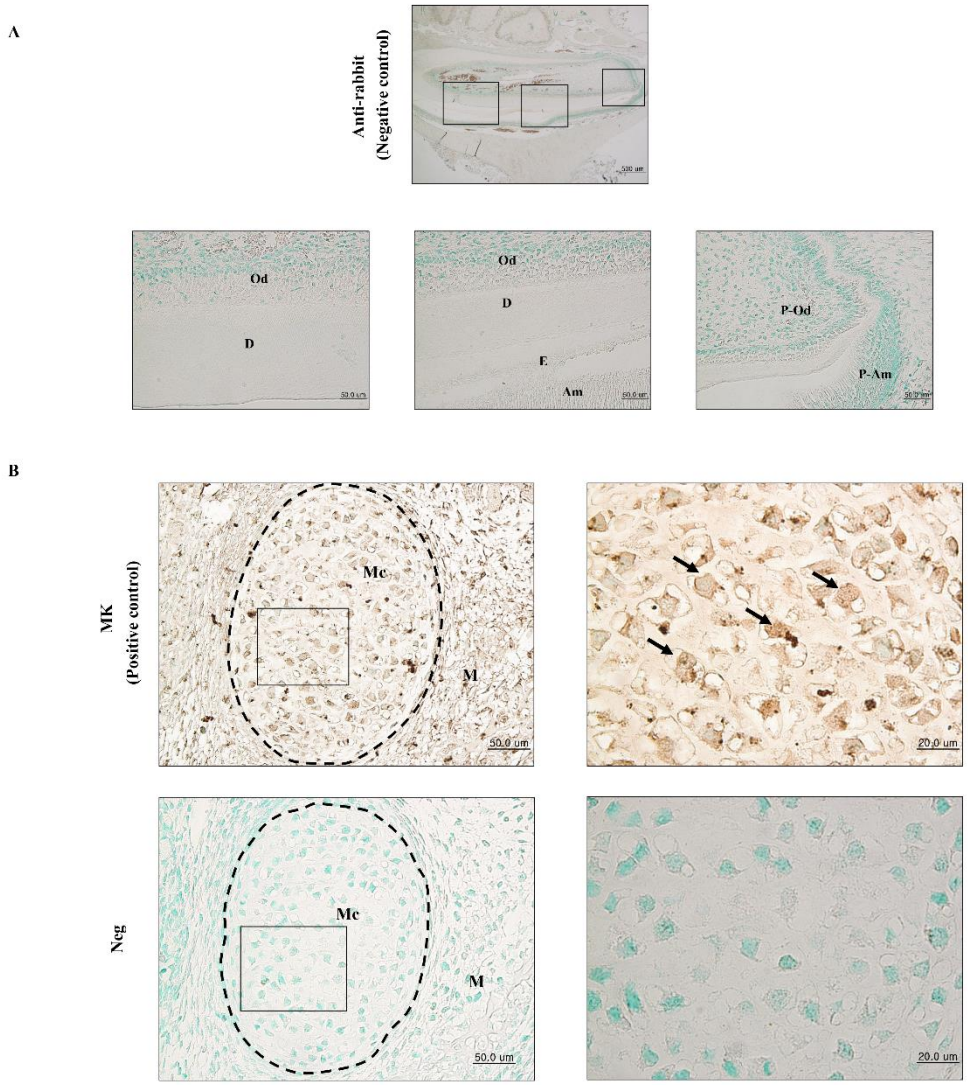
The hDPCs were obtained from Seoul National University Dental Hospital (Seoul, Republic of Korea), and the experimental protocol was accepted by the Institutional Review Board of Seoul National University (S-D20140006). Informed consent was obtained from patients. We collected normal human impacted third molars (at the root developing stage) from 15 adults (15–18 years of age) at the Seoul National University Dental Hospital (Seoul, Korea). The experimental protocol was approved by the hospital's Institutional Review Board. The experiments were performed with the understanding and written consent of each participating subject according to the Declaration of Helsinki. We isolated hDPCs for use in vitro as previously described. The hDPCs (2×10^6) were mixed with 100 mg hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer) alone, with rMK (2 ug), with 3MA (40mM), or with rMK and 3MA in a 0.5% fibrin gel. We then transplanted them subcutaneously into immunocompromised mice (NIHbg-nu-xid; Harlan Laboratories, Indianapolis, IN, USA) for 6 weeks (n=3).

Masson's trichrome staining

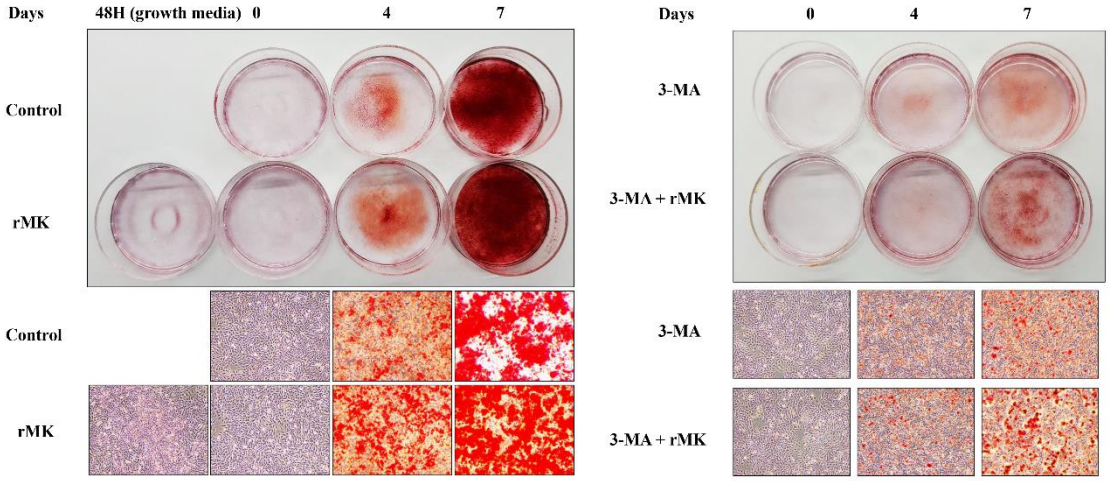
Sections of mouse teeth were deparaffinized in xylene and rehydrated using ethanol. Sections were stained with

Weigert's Haematoxylin for 10 minutes, followed by a 1% Biebrich-Scarlet-Acid Fuchsin solution for 20 minutes, and 5% phosphotungstic acid for 10 minutes. They were then stained with 2.5% Aniline Blue solution for 20 minutes. Following staining, sections were differentiated in 1% acetic acid for 1 minute, dehydrated through 90% and 100% ethanol, cleared in xylene, and permanently mounted.

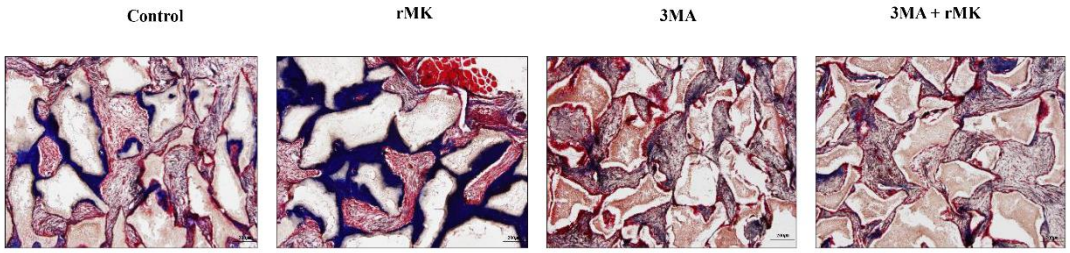
Appendix Figures



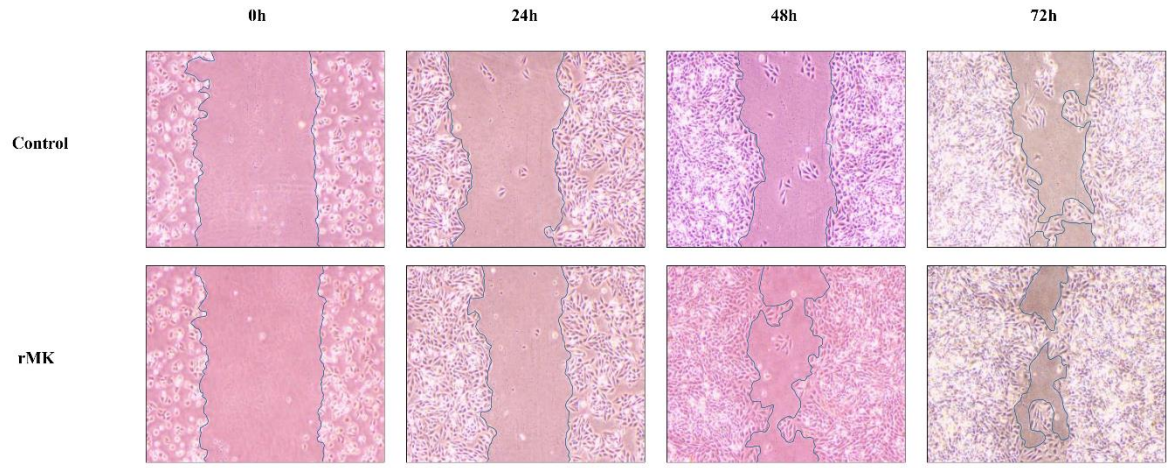
Appendix Figure 1. Expression of MK in mouse tissue. (A) Negative control group was detected by immunohistochemistry on mouse postnatal day 14 (P14) incisor, scale bars 500㎛ and 50㎛. Boxed areas were shown at higher magnification. Am, ameloblast; E, enamel; D, dentin; Od. odontoblast; P-am, pre-ameloblast; P-od, pre-odontoblast; P, dental pulp. (B) MK expression (arrows) and negative control group were detected by immunohistochemistry on mouse embryo day 4 (E4) meckel's cartilage, 50㎛ and 20㎛. Boxed areas were shown at higher magnification. Mc, meckel's cartilage; M, mesenchyme.



Appendix Figure 2. Effect of MK on the mineralized nodule formation *in vitro*. Effects of rMK, 3MA, and 3MA+rMK on mineralized nodule formation of MDPC-23 cells *in vitro* analyzed by alizarin red S staining during odontoblast differentiation.



Appendix Figure 3. Effect of MK in *ex vivo* mineralization. The hDPCs were mixed with 100 mg HA/TCP particles alone (control), with rMK (rMK), with 3MA (3MA), or with 3MA+rMK (3MA+rMK) in a 0.5% fibrin gel and transplanted subcutaneously into immunocompromised mice for 6 weeks (n=3). Sectioned- samples were stained with Masson's trichrome staining (MT stain). Scale bars 200㎛.



Appendix Figure 4. Effect of MK on migration of MDPC-23 cells. Effect of rMK on migration of MDPC-23 cells is determined using a wound-healing assay for up to 72 hours.