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Accelerated dentinogenesis by inhibiting the mitochondrial fission factor, dynamin related protein 1

Yumiko I. Matsuishi [a, 1], Hiroki Kato [a, 1], Keiji Masuda [a, *], Haruyoshi Yamaza [a], Yuta Hirofuji [a], Hiroshi Sato [a], Hiroko Wada [b], Tamotsu Kiyoshima [b], Kazuaki Nonaka [a]

[a] Section of Oral Medicine for Children, Division of Oral Health, Growth and Development, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan
[b] Laboratory of Oral Pathology, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan

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ABSTRACT

Undifferentiated odontogenic epithelium and dental papilla cells differentiate into ameloblasts and odontoblasts, respectively, both of which are essential for tooth development. These differentiation processes involve dramatic functional and morphological changes of the cells. For these changes to occur, activation of mitochondrial functions, including ATP production, is extremely important. In addition, these changes are closely related to mitochondrial fission and fusion, known as mitochondrial dynamics. However, few studies have focused on the role of mitochondrial dynamics in tooth development. The purpose of this study was to clarify this role. We used mouse tooth germ organ cultures and a mouse dental papilla cell line with the ability to differentiate into odontoblasts, in combination with knockdown of the mitochondrial fission factor, dynamin related protein (DRP)1. In organ cultures of the mouse first molar, tooth germ developed to the early bell stage. The amount of dentin formed under DRP1 inhibition was significantly larger than that of the control. In experiments using a mouse dental papilla cell line, differentiation into odontoblasts was enhanced by inhibiting DRP1. This was associated with increased mitochondrial elongation and ATP production compared to the control. These results suggest that DRP1 inhibition accelerates dentin formation through mitochondrial elongation and activation. This raises the possibility that DRP1 might be a therapeutic target for developmental disorders of teeth.

1. Introduction

Mitochondria have various roles including ATP production, calcium metabolism, apoptosis regulation, and redox homeostasis. Mitochondria continue to divide and fuse throughout the life of a cell. The balance between division and fusion, known as mitochondrial dynamics, is closely linked to various cellular functions [1]. In mammals, dynamin related protein (DRP)1 is a major mitochondrial division factor, and MFN-1/2, and OPA1 are known as major fusion factors [2].

Because mitochondrial dynamics is involved in mitochondrial functions, its dysregulation is associated with various diseases [3]. Mitochondrial dynamics-related diseases are caused by both excessive fusion and excessive division of mitochondria. We have shown that neuronal development and synapse formation are severely impaired due to excessive fusion and elongation of mitochondria in brain-specific DRP1-deficient mice [4]. Other groups have shown that mutations of genes encoding OPA1 and MFN2 are responsible for dominant optic atrophy and Charcot-Marie-Tooth neuropathy type 2A, respectively [5,6].

During tooth formation, ameloblasts differentiate from the odontogenic epithelium and form enamel [7]. Odontoblasts differentiate from undifferentiated dental papilla cells to form dentin [8]. Genes encoding signal transduction factors are involved in these processes, and mutations of these genes cause many kinds of tooth malformations [9–11]. In addition, mitochondria play an important role in functional differentiation of ameloblasts and odontoblasts [7,8]. However, the contribution of mitochondrial dynamics to odontogenesis has not been defined.

The purpose of this study was to clarify how mitochondrial dynamics is involved in odontogenesis by focusing on DRP1. It was difficult to analyze odontogenesis in DRP1 null mice because the

* Corresponding author.
E-mail address: kemasuda@dent.kyushu-u.ac.jp (K. Masuda).
[1] These authors contributed equally to this study.

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embryos die by E10.5 due to severe developmental defects [4]. In this study, we used an organ culture system of mouse tooth germ and a mouse dental papilla cell line (mDP^{E6}) that can differentiate into odontoblasts, in combination with DRP1 gene knockdown technology. We found that the inhibition of DRP1 accelerated odontoblast differentiation and dentin formation.

2. Materials and methods

2.1. Organ culture and tissue preparation

Embryos were collected from C57BL/6JJcl female mice (CLEA Japan, Tokyo, Japan) at 18.5 days of gestation. Tooth germs of the lower first molars were dissected from mandibles of E18.5 embryos. Organ culture was performed according to the protocol of a previous study [12]. Briefly, dissected mandibles and tooth germs were cultured for eight days on nitrocellulose membranes (0.8 μm pore size, EMD Millipore, Hayward, CA, USA) in 24-well plates in BGJb medium (Thermo Fisher, Rockford, IL, USA) supplemented with 10 μg/mL ascorbic acid (Nakarai Tesque, Kyoto, Japan), 5% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 units/mL penicillin (Thermo Fisher), and 100 μg/mL streptomycin (Thermo Fisher) in a humidified atmosphere of 5% CO_2 at 37 °C. All animal experiments were reviewed and approved by the animal ethics committee of Kyushu University and were conducted in accordance with Kyushu University guidelines.

Tissue preparation was according to the protocol of a previous study [12]. Briefly, cultured organs were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) for 12 h at 4 °C, followed by embedding in paraffin wax. Serial sections were cut at 5 μm thick in the anterior-posterior direction to obtain the largest part of the tooth germ, and were stained with hematoxylin and eosin as described previously [12].

2.2. Culture of a mouse dental papilla cell line

The mDP^{E6} mouse dentate papilla cell line was used for analysis of differentiation into odontoblasts as described previously [13]. Briefly, mDP^{E6} cells were cultured in alpha minimum essential medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 units/mL penicillin (Thermo Fisher), and 100 μg/mL streptomycin (Thermo Fisher) in a humidified atmosphere of 5% CO_2 at 37 °C. Differentiation into odontoblasts was induced by culturing for five days in the presence of 10 nM dexamethasone (Sigma-Aldrich), 1.8 mM potassium hydrogen phosphate (Nakarai Tesque), 100 μM ascorbic acid, and 10 ng/mL basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA).

2.3. Inhibition of DRP1

In organ culture, we used an antisense oligodeoxynucleotide (5′-GCGCCTCCATGACCCCCGAA-3′) against mouse DRP1 mRNA, and a corresponding sense oligodeoxynucleotide (5′-TTCGGGGGTCATGGAGGCGC-3′) as the control. These oligodeoxynucleotides were transfected using an HVJ Envelope Transfection kit (GenomeONE™-Neo, Ishihara Sangyo, Osaka, Japan) according to the manufacturer's protocol. The culture medium containing oligodeoxynucleotides was changed every 24 h as described previously [12].

In cell culture, mDP^{E6} cells were seeded in 6-well plates at 9.6×10^3 cells per well. After 24 h, ON-TARGETplus Mouse Dnm11 siRNA (No. 74006; GE Healthcare, Buckinghamshire, UK) against DRP1 mRNA, and ON-TARGETplus non-targeting siRNA (#1; GE Healthcare) as control siRNA were transfected using Lipofectamine RNAi Max (Thermo Fisher). After 48 h, siRNA against DRP1 mRNA and control siRNA were transfected and cultured for a further 72 h.

2.4. Analysis of predentin formation by tooth germ in organ culture

Two different 50 μm wide areas including predentin were selected around the starting point of dentin formation in each tooth germ. This was followed by measuring the areas of predentin using Image J software (National Institutes of Health, Bethesda, MD, USA). The average values of four different areas were calculated in the antisense DRP1 and control groups.

2.5. Immunohistochemical staining of DRP1 in tooth germs in organ culture

For immunohistochemical analysis of DRP1 expression in cultured tooth germs, we used monoclonal mouse anti-DRP1 (clone: 8/DLP1, BD Biosciences, NJ, USA) antibody according to the manufacturer's protocol (EnVision + HRP kit, Agilent, Santa Clara, CA, USA). After blocking peroxidases, the primary antibody were added for 60 min at room temperature followed by reaction with the horseradish peroxidase (HRP)-labeled secondary antibodies for 30 min. 3,3′-Diaminobenzidine was applied to detect a positive signal and samples were counterstained with hematoxylin.

2.6. Western blot analysis

mDP^{E6} cells were washed three times with phosphate-buffered saline, suspended in Laemmli sample buffer (62.5 mM Tris HCl, pH 6.8, containing 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 10% glycerol) and incubated at 95 °C for 10 min for whole cell lysate preparation. Protein concentrations were quantified using the BradfordUltra assay (Expedeon, Cambridge, UK). Whole cell lysates containing 10 μg protein were separated by SDS polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (EMD Millipore). For detecting DRP1, monoclonal mouse anti-DRP1 (clone: 8/DLP1, BD Biosciences) and HRP-labeled goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as primary and secondary antibodies, respectively. For control, primary rabbit anti-Hsp90 and secondary HRP-labeled goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology) were used. The primary and secondary antibodies were added for 60 min at room temperature, followed by membrane development with the ECL Prime kit (GE Healthcare). Immunoreactive bands were analyzed using LAS-1000 Pro (Fujifilm, Tokyo, Japan). The relative expression level of DRP1 was calculated using the expression level of Hsp90 as an internal control.

2.7. Analysis of mitochondrial morphology

After induction of odontoblast differentiation, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by permeabilization for 5 min with 0.1% Triton-X 100 (Nacarai Tesque). A rabbit anti-Tom20 antibody (Santa Cruz Biotechnology) was used as the primary antibody at room temperature for 90 min, and an Alexa Fluor 594-labeled goat anti-rabbit IgG antibody (Thermo Fisher) was reacted as the secondary antibody at room temperature for 60 min. Fluorescent images were captured with an Axio Imager M2 using Apotome 2 (Zeiss, Oberkochen, Germany).

2.8. RNA extraction and mRNA expression analysis

Total RNA was recovered using the RNeasy Mini kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized using the

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