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研究題目：Investigation of Tooth Organoids from mouse iPSCs

目 的：

The formation of tooth germs involves complex interactions between epithelial cells and mesenchymal cells, which pose challenges in securing both cell types and facilitating their interaction *in vitro*. Additionally, developing an innovative technique capable of producing uniform and abundant quantities of artificial tooth germs in a laboratory setting remains elusive. Induced pluripotent stem cells (iPSCs) emerge as a promising cell source in regenerative medicine due to their high pluripotency and limitless self-renewal capacity (Egusa et al., 2012). iPSCs find applications across various studies, spanning from basic research to tissue regeneration. Recently, numerous techniques have emerged to induce ameloblast differentiation of iPSCs and develop scaffold-free, three-dimensional (3D) structures (organoids) in vitro for diverse organ regeneration (Hermans et al., 2023). Moreover, methods for overexpressing specific genes or proteins have been developed to explore biological mechanisms or devise innovative treatment strategies. Notably, *AmeloD,* a newly identified transcriptional factor highly expressed in inner enamel epithelial cells (IEE), shows promise in promoting ameloblast differentiation (Jia et al., 2022). To overcome these challenges, we propose to establish doxycycline (Dox)-inducible *AmeloD*-expressing mouse iPSCs (*AmeloD*-iPSCs) and investigate organoid culture methods on *AmeloD*-iPSCs to induce epithelial-mesenchymal interactions and generate regenerated tooth germ organoids.

対象および方法：

1) Generation of Doxycycline-inducible *AmeloD* overexpression (*AmeloD*-iPSCs)

AmeloD-iPSCs were generated, using *piggyBac* transposon vector and Neon transfection system (Invitrogen). The pluripotency of transfected iPSCs was confirmed by alkaline phosphatase (ALP) staining, immunofluorescence analysis. An optimal doxycycline concentration (0.02- 2.0 μg/mL) was evaluated after 24 hours by examining GFP expression and *AmeloD* mRNA expression.

2) Fabrication of tooth germ organoids using iPSCs-derived epithelial and mesenchymal cells

AmeloD-iPSCs-derived epithelial and iPSCs-derived mesenchymal cells were generated as previous described (Miao et al., 2021 and Okawa et al., 2016). Subsequently, both iPSCs-derived epithelial and mesenchymal cells were combined by using the shaking culture method for 12 days in DEC and SFD medium to generate tooth germ-like structures. The medium

was changed every 2 days, and addition of doxycycline was performed in an experimental group (Figure 1).

To evaluate tooth germ-like structures, real-time RT-PCR and immunohistochemical analysis (IHC) were conducted to observe the expression of ameloblast or odontoblast related genes and proteins in the constructs.

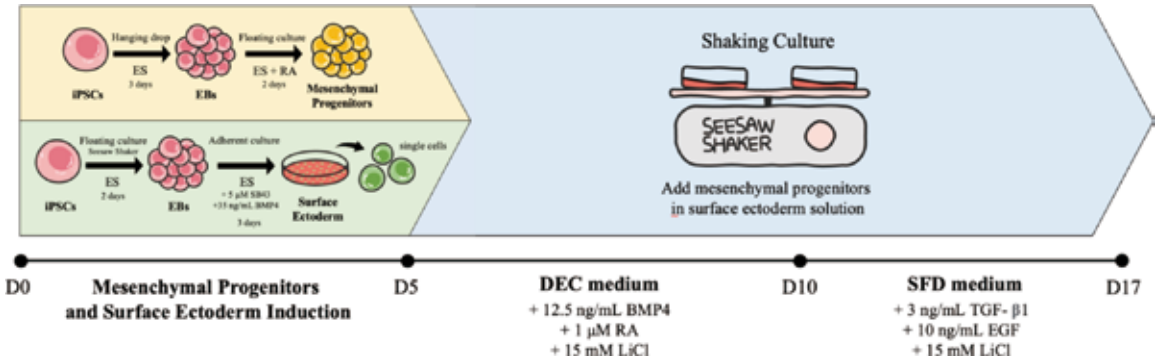

Figure 1. Schematic diagram of tooth germ organoids fabrication

結果および考察：

The Dox-inducible *AmeloD*-expressing *piggyBac* vector with a GFP reporter gene is depicted in Figure 2A. Enhancement of *AmeloD* overexpression using various doxycycline concentrations showed the highest level of *AmeloD* gene expression at both 1 and 2 μg/mL, confirmed through quantitative RT-PCR results, with no significant difference between these two concentrations, consistent with the results of GFP expression. Therefore, the optimal concentration of doxycycline for *AmeloD* overexpression was determined to be 1 μg/mL (Figure 2B and 2C). The pluripotency of *AmeloD*-iPSCs was maintained, as demonstrated by the presence of endogenous stem cell markers (*Sox2, Oct3/4*, and *Nanog*) in semi-quantitative RT-PCR (Figure 2D) and positive staining for ALP and Nanog, consistent with normal iPSC characteristics (Figure 2E).

Tooth germ organoids were formed using the shaking culture method, combining *AmeloD*-iPSCs-derived epithelial cells and iPSCs-derived mesenchymal spheroids. Immunohistochemical staining of the tooth germ organoids revealed expression of all ameloblast- and odontoblast-related proteins. However, there was significantly higher expression of AMGN, DMP-1, and DSPP in the *AmeloD* overexpression group compared to the control. Morphologically, some combinations in the *AmeloD* overexpression group exhibited cuboidal cells surrounded by multilayered AMGN-positive epithelial-like cells (Figure 3).

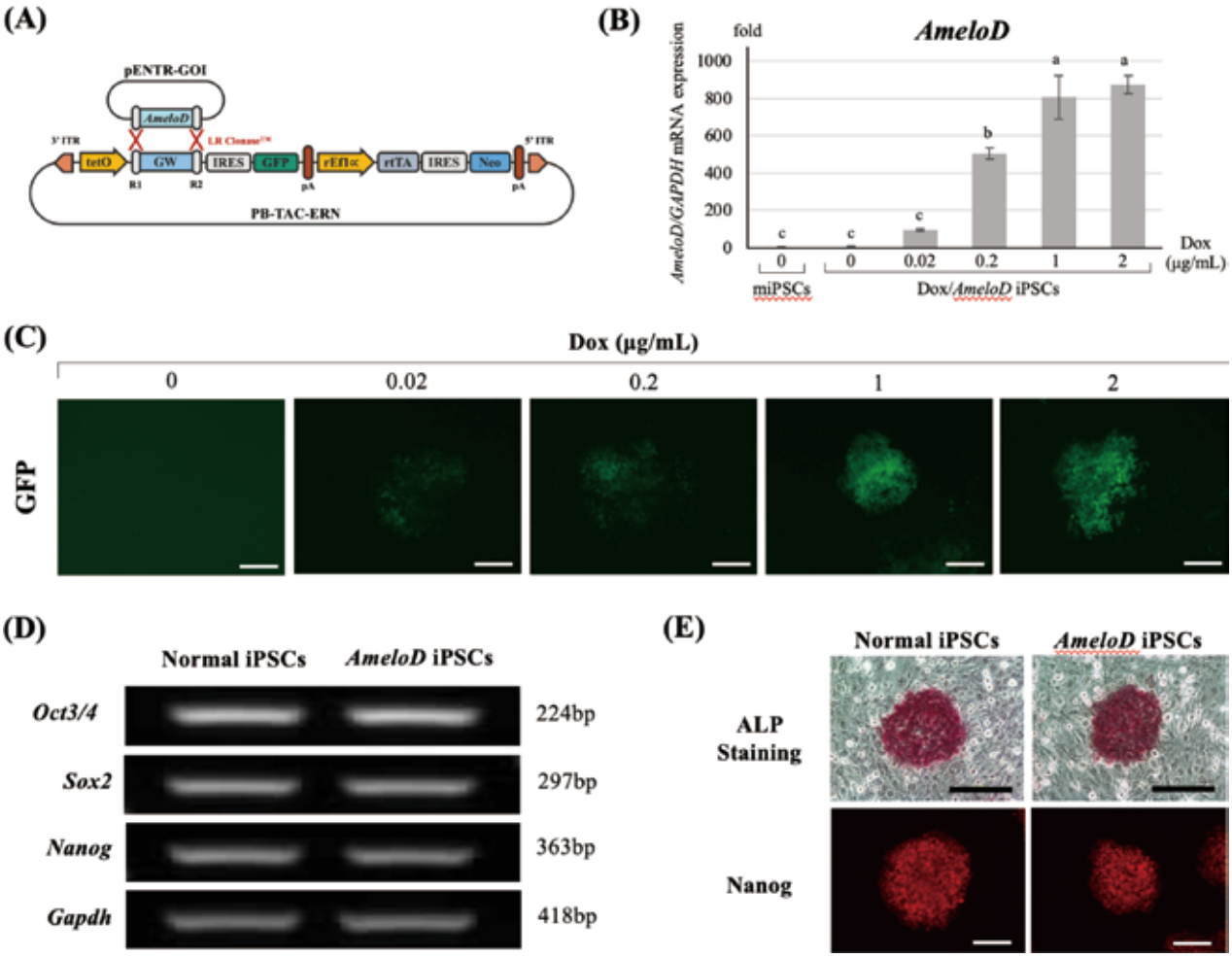

Figure 2. Establishment of doxycycline (Dox)-inducible *AmeloD* overexpression (*AmeloD* iPSCs)
(A) Dox-inducible *AmeloD*-expressing piggy*Bac* transposon vector, (B, C) Optimization of doxycycline concentration (0.02- 2.0 μg/mL), evaluated by quantitative RT-PCR (B) and GFP expression (C), (D, E) Maintenance of pluripotency of *AmeloD*-iPSCs, indicated by pluripotent marker gene expression (quantitative RT-PCR) (D), ALP staining, and immunofluorescence for Nanog (E), Scale bars: 200 μm.

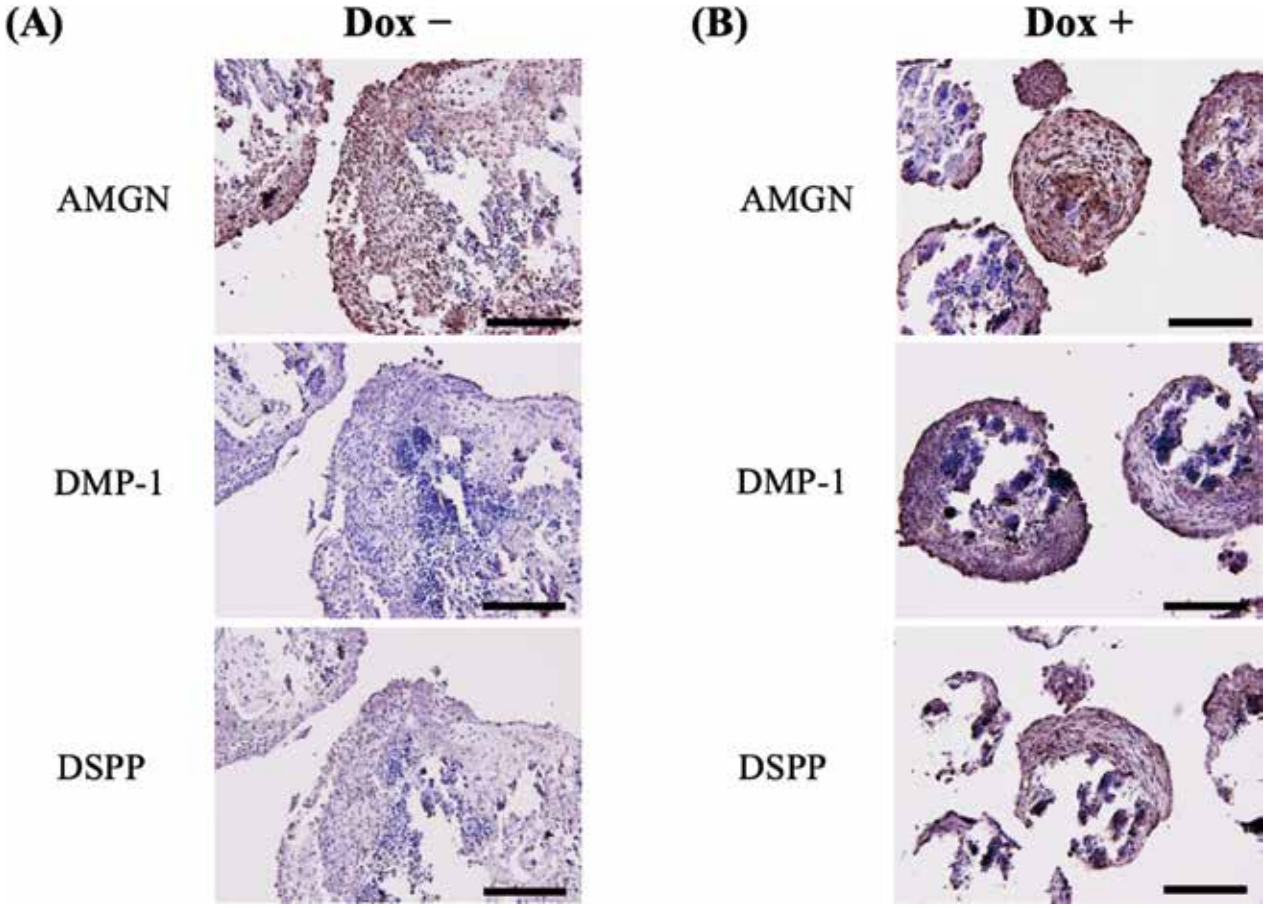

Figure 3. Immunohistochemical staining images of generated tooth germ organoids; (A) Control group, and (B) *AmeloD* overexpression group, Scale bars: 100 μm.

Although these preliminary results demonstrated ameloblast- and odontoblast-positive constructs, further experiments are required to enhance the established protocol for tooth germ organoid regeneration. Self-organization and cell polarization of ameloblast-like cells are necessary to reproduce a mimic tooth-like structure.

成果発表：（予定を含めて口頭発表、学術雑誌など）

・Naruephorn Vinaikosol, Hiroko Okawa, Hiroshi Egusa, Effects of Controlled Overexpression of *AmeloD* on Ameloblast Differentiation of Induced Pluripotent Stem Cells, The 133rd Annual Meeting of the Japan Prosthodontic Society/The 14th Biennial Congress of the Asian Academy of Prosthodontics (AAP), Chiba, July 6-7, 2024.