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Microbiota Regulates Dentine Mineralisation and Differentiation of Dental Pulp Stem Cells

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Objective: *To investigate the role of microbiota in dentine formation and the characteristics of dental pulp stem cells (DPSCs) in mouse incisors.*

Methods: *The influence of microbiota on dentine was detected via microcomputed tomography (microCT), microhardness testing and haematoxylin-eosin (HE) staining in incisors from germ-free (GF), specific pathogen–free (SPF) and conventionalised (ConvD) mice. Cell Counting Kit-8 (CCK-8) assay, alizarin red staining and expression of dentine sialophosphoprotein (DSPP), alkaline phosphatase (ALP) and bone sialoprotein (BSP) via real-time polymerase chain reaction (PCR) were used to evaluate the biological characteristics of DPSCs derived from mice of different microbiota status.*

Results: *MicroCT showed that the incisors in the GF and ConvD groups had comparable dentine thickness to those in the SPF group. Microhardness testing showed a lower dentine hardness value in GF incisors compared to SPF, while HE staining showed that GF incisors exhibited thicker predentine than SPF incisors. There was no difference between the ConvD and SPF groups. DPSCs from GF mice showed no significant difference in proliferation rate to SPF and ConvD DPSCs. DPSCs from GF mice formed less mineral deposition and expressed lower levels of osteo-/odontogenic differentiation–related genes including ALP, BSP and DSPP than SPF and ConvD DPSCs. The absence of microbiota in GF mice resulted in a lower dentine hardness value, thicker predentine and impaired osteo-/odontogenic differentiation capacity.*

Conclusion: *The absence of microbiota impaired the dentine mineralisation and osteo-/odontogenic differentiation abilities of DPSCs.*

Key words: *dental pulp stem cells, dentinogenesis, germ-free, microbiota, mineralisation*
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In recent years, the microbiota has been widely discussed, especially the gut microbiota, as it not only affects the gastrointestinal tract but also functions as a hidden organ, altering host metabolism and shaping the host systemic immune function[1,2], and consequently plays an important role in distal target organs such as the brain, lungs, liver, cardiovascular system homeostasis and so on[3-6]. Dysbiosis of the microbiota caused by metabolic disorders such as diabetes mellitus or antibiotic treatment also leads to organ or tissue dysfunction[7,8].

As well as the soft tissue mentioned above, previous studies have also shown the impact of the microbiota on hard tissue such as bone. The absence of gut microbiota in germ-free mice led to increased bone mass[9], which was duplicated in antibiotic-treated mice[8,10]. Bone marrow mesenchymal stem cells (BMMSCs)

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were shown to play an important role in bone homeostasis via osteogenic and immunomodulatory function. Germ-free (GF) mice–derived BMMSCs showed a higher colony-forming ability, proliferation rate and osteogenic capacity, and microbiota replantation normalised the proliferation and differentiation abilities of BMMSCs[11].

The characteristics of rodent incisors including murine incisors are similar to those of bone, which is characterised by continuous growth throughout the lifetime to compensate for the wearing of the tooth at the cut end. During this process, the cells at the proximal end of the incisor continuously proliferate and differentiate into various tooth-forming cell types[12]. In other words, the continuous growth of rodent incisors is enabled by epithelial stem cells (ESCs) and mesenchymal stem cells (MSCs) which unceasingly replenish enamel and dentine, respectively[13,14]. It has been suggested that the odontoblasts arise from MSCs like populations residing in the incisor dental pulp[15], which are thought to be dental pulp stem cells (DPSCs). DPSCs continuously contribute to odontoblast lineage and tooth growth[14]. In vivo, BMMSCs contribute to bone regeneration and reparation, and DPSCs play an important role in the regeneration of the dentine-pulp-like complex[16]. In vitro, DPSCs have similar characteristics to BMMSCs, including proliferation and multi-differentiation abilities[17,18].

We therefore hypothesised that the microbiota maintained the function of DPSCs and dentine formation of incisors, while the absence of microbiota in GF mice disturbed homeostasis.

Materials and methods

Animals and experimental design

The animal experiment protocol was reviewed and approved by the Ethics Committee of the Peking University Health Science Centre (No. LA2018184). Healthy 6- to 8-week-old female C57B/L6 mice were divided into 3 groups. SPF mice were housed under a 12-hour light-dark cycle and under controlled temperature (22 ± 1°C) and had free access to food and water. GF mice were housed in a germ-free environment and the absence of microbiota was checked. Conventionalised (ConvD) mice were GF mice exposed to a conventional environment by cohousing with SPF mice for 2 weeks.

Microcomputed tomography (microCT) examination

The mouse maxillae were dissected and a stereo microscope (SWZ1000, Nikon, Tokyo, Japan) was used to record the buccal surface of the maxillae. Then, microcomputed tomography (microCT) images were taken of the maxillae (SkyScan 1174, Bruker microCT, Bruker, Kontich, Belgium). The scanning parameters were rotation angle 360 degrees and x-ray exposure time 4750 ms. The scaled image pixels were constructed and analysed using the computer software CTVox (Bruker, Billerica, MA, USA). 2D images were obtained and analysed using the computer software CTAn (Bruker).

Microhardness testing

Three hemimandibles from each group were embedded in methylmethacrylate and metallographically polished to a fine degree. A series of abrasive papers down to 4000 grit were used to polish the teeth until the alveolar crest was reached. Final polishing was then performed using alumina powder slurry pastes of 1 µm, 0.3 µm and 0.05 µm. Each sample was ultrasonically cleaned in distilled water at the end of each polishing step to remove any contaminating materials. The samples were then affixed to a glass backing plate with cyanoacrylate cement and air dried for 24 h prior to instrumentation.

A Leitz Miniload hardness tester (Wild Leitz, Wetzlar, Germany) was used for microhardness testing. Six microindentations were placed in the dentine (25-pound load) of each tooth. The dentine microindentations were placed approximately at the outer one-third point, midpoint and inner one-third point between the chamber and dentinoenamel junction (DEJ) (two in each position). Each microindentation point was orientated with its long axis perpendicular to the cross-section. The length of each indentation point was measured using an image analysis system (Bioquant, R&M Biometrics, Nashville, TN, USA) under 1000× magnification, and a mean value for all microindentations at each site was determined. Microhardness was calculated from the load, indentation length and assumed tip geometry.

Histological analysis

To investigate the histological characteristics of the different groups after clipping the incisors, the mice were treated as mentioned above, then sacrificed with neck-breaking at 12, 24 and 72 h. The hemimandibles were dissected, fixed with 10% formalin for 24h, decalcified in 20% ethylenediaminetetraacetic acid (EDTA) and processed for paraffin embedding. 4-micrometre

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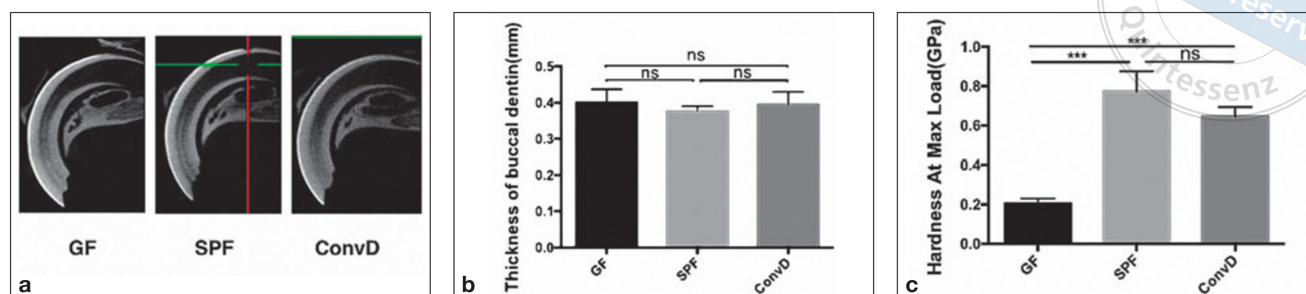

Fig 1 GF mice showed comparable thickness but reduced hardness of dentine compared to SPF mice. **(a)** Representative sagittal plane of mouse maxillary incisors. **(b)** Histogram of statistical analysis of the thickness of dentine. **(c)** Histogram of statistical analysis of the hardness of dentine.

sections were obtained using a Leica rotary microtome (Leica RM2255, Leica Biosystems, Nussloch, Germany), processed for haematoxylin-eosin (HE) staining and examined using a BX51 fluorescence microscope (Olympus, Tokyo, Japan).

Cell cultures

The pulp of intact incisors was obtained from mouse incisors and DPSCs were isolated. The tissue was minced into 0.5-mm pieces and transferred into a T25 culture flask (Corning, Corning, NY, USA), incubated with α-modified minimum essential medium (α-MEM, GIBCO, Thermo Fisher Scientific, Sunnyvale, CA, USA) with 20% foetal bovine serum (GIBCO, Thermo Fisher Scientific) containing ascorbic acid (10 mM, GIBCO, Thermo Fisher Scientific) and glutamate (2 mM, GIBCO, Thermo Fisher Scientific) at 37°C with 5% CO_2. Osteo-/dentinogenic differentiation medium consisted of basal medium plus 10 nm dexamethasone, 50 µg/ml L-Ascorbic acid 2-phosphate and 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA).

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from DPSCs using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Complementary DNA (cDNA) was prepared using the GoScript Reverse Transcription System (Promega, Madison, WI, USA). Real-time polymerase chain reaction (PCR) was performed with an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). β-Actin was used to normalise gene expression, and the relative mRNA expression levels were calculated. The primers used in this study are shown in Table 1.

Statistical analysis of data

Statistical analysis was performed with GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA) using one-way analysis of variance (ANOVA) and statistical significance was determined at $P \leq 0.05$.

Results

GF mice showed comparable dentine thickness but weakened dentine compared to SPF mice

The structure of dentine was analysed by microCT 3D imaging. The representatives of the sagittal planes of maxillary incisors were shown (Fig 1a). At the cross-section located 500 µm apically from the alveolar crest, dentine was thicker in GF incisors compared to SPF and ConvD incisors. However, the difference showed no statistical significance (Fig 1b).

Next, the influence of the microbiota on dentine was investigated by microhardness testing. The results showed that at the level of the alveolar crest, the hardness of dentine at the labial midpoint in GF incisors was lower than that in SPF, while the ConvD incisors showed similar hardness to SPF (Fig 1c).

Table 1 Primers used for real-time PCR.

Target gene	Sequence
β-Actin	Forward: GTGACGTTGACATCCGTAAAGA
	Reverse: GCCGGACTCATCGTACTCC
DSPP	Forward: AACTCTGTGGCTGTGCCTCT
	Reverse: TATTGACTCGGAGCCATTCC
ALP	Forward: CTATCCTGGCTCCGTGCTC
	Reverse: GCTGGCAGTGGTCAGATGTT
BSP	Forward: AAAGTGAAGGAAAGCGACGA
	Reverse: GTTCCTTCTGCACCTGCTTC

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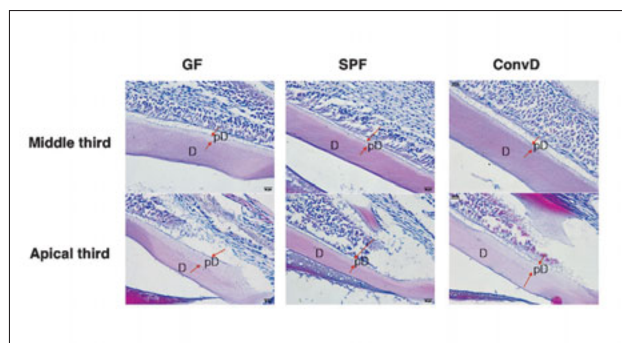

Fig 2 Histological assessment of predentine by HE staining. The incisors of GF mice showed a thicker predentine layer compared to SPF and ConvD mice, while there was no significant difference between SPF and ConvD mice. D, dentin; pD, predentine.

GF mice showed thicker predentine

To clarify the structural alteration of dentine, we observed the apical and middle third of the incisors histologically. HE staining showed that the incisors of GF mice exhibited a thicker predentine layer at both sites (Fig 2).

DPSCs from GF mice exhibited impaired mineralised potential

To examine whether the biological characteristics of DPSCs were regulated by the microbiota, DPSCs were isolated from GF, SPF and ConvD mice. Flow cytometry analysis showed that DPSCs from incisors expressed the mesenchymal cell surface marker CD73, but were negative for haematopoietic cell surface markers CD34 and CD45 (Fig 3a). A Cell Counting Kit-8 (CCK-8) assay showed that DPSCs from GF mice displayed no significant difference in proliferation rate when compared to those from SPF and ConvD mice (Fig 3b). Osteogenic study showed that DPSCs from GF mice formed less mineralised deposits than those from SPF and ConvD mice; this was detected by alizarin red staining (Fig 3c). Furthermore, the result of real-time PCR showed the mRNA expression levels of DSPP, ALP and BSP mRNA were decreased in DPSCs from GF mice (Fig 3d). These data demonstrate that the microbiota significantly alters the mineralisation differentiation ability of DPSCs in vitro.

Discussion

In the present study, we proposed the effect of the gut microbiota on the maintenance of dentine formation and odontogenic differentiation potential of DPSCs in mice incisors for the first time. The results demonstrated that the absence of gut microbiota led to reduced dentine mineralisation in rodent incisors and impaired osteo-/dentinogenic function of DPSCs.

Considering that the absence of microbiota would result in increased bone mass, we first examined the formed dentine. MicroCT is a widely used method to evaluate the structure of hard tissues such as tooth and bone. From the microCT imaging, we measured the length of incisors, thickness of enamel and thickness of dentine and found no significant difference among the three groups (the results were not shown). Based on a previous study[19], we chose the cross-section located 500 μm apically from the alveolar crest of the maxillary incisors to measure the thickness of dentine. The results showed that GF mice exhibited a thicker dentine layer in this plane, but the difference between the GF and SPF groups was still not statistically significant.

Dentine microhardness is associated with tubular density, the amount of intertubular dentine and an increase in individual tubular diameter[20]. The tubular density decreased from the pulp chamber to the DEJ, and the hardness value of dentine increased from the inner region to the outer region[21]. Based on a previous study[22], we chose the cross-section at the same level as the alveolar crest of incisors to test the microhardness of dentine. At the midpoint of labial dentine, GF mice showed a lower hardness value compared to SPF and ConvD mice, suggesting there was higher tubular density and less intertubular dentine in GF incisors. As one of the methods used to evaluate the degree of dentine mineralisation, the dentine microhardness suggested compromised dentine mineralisation in GF mice[22].

The HE staining results showed there was thicker predentine in GF incisors, also suggesting impaired dentine mineralisation[23]. After the replantation of microbiota in ConvD mice, the dentine microhardness and predentine thickness recovered; these results indicated that the absence of microbiota leads to impaired dentine mineralisation.

In mouse incisors, it has been suggested that the odontoblasts from DPSCs participate in continuous dentine formation[15]. During the process of continuous growth, stem cells at the proximal end of the incisor give rise to a spatially distinct transit-amplifying cell population of rapidly proliferating cells that differenti-

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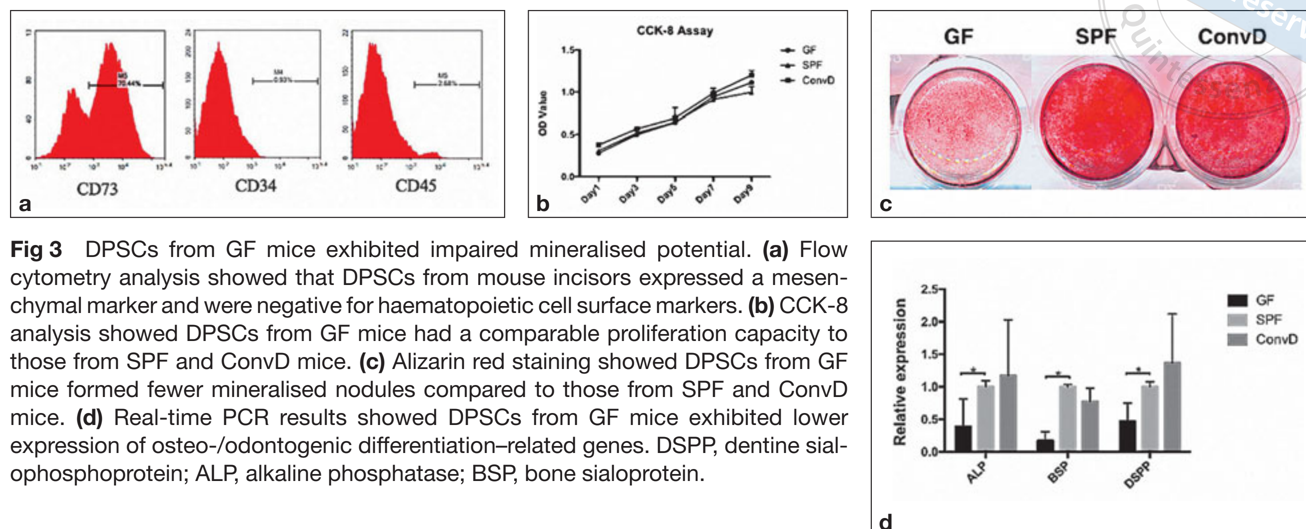

Fig 3 DPSCs from GF mice exhibited impaired mineralised potential. **(a)** Flow cytometry analysis showed that DPSCs from mouse incisors expressed a mesenchymal marker and were negative for haematopoietic cell surface markers. **(b)** CCK-8 analysis showed DPSCs from GF mice had a comparable proliferation capacity to those from SPF and ConvD mice. **(c)** Alizarin red staining showed DPSCs from GF mice formed fewer mineralised nodules compared to those from SPF and ConvD mice. **(d)** Real-time PCR results showed DPSCs from GF mice exhibited lower expression of osteo-/odontogenic differentiation–related genes. DSPP, dentine sialophosphoprotein; ALP, alkaline phosphatase; BSP, bone sialoprotein.

ate into the main specialised tooth-specific cell type, odontoblasts. The fibroblastic pulp cells[12] continuously produce dentine in the apical area, and the newly formed dentine moves apically with the odontoblasts to compensate for the wearing at the cut end[24]. Thus, to illustrate the role of cells in the apical area in incisor growth, we isolated and cultured DPSCs. The proliferative capacity and differentiation potential of DPSCs from different groups were examined in vitro. DPSCs from GF mice exhibited no difference in proliferative capacity compared to those from SPF mice. However, we found that the mineralisation capacity of DPSCs reduced after the depletion of the gut microbiome. Thus, the decline in dentine mineralisation might result from the impaired osteo-/dentinogenic capacity of DPSCs from GF mice. Compared to previous studies on bone marrow, the influence of microbiota on DPSCs was different to that on BMMSCs. The influence of microbiota on DPSCs led to compromised dentine mineralisation, while its influence on BMMSCs led to increased bone density. A previous study suggested that the gut microbiota regulates bone mass in mice with the mechanism involving altered immune status in bone and thereby affected osteoclast-mediated bone resorption[9]. The mechanisms of microbiota that alter dentinogenesis and the biological characteristics of DPSCs remain unclear and need further exploration.

Conclusion

Using a well-established GF mouse model, we found that the microbiota significantly alters dentine mineralisation in mouse incisors and the osteo-/odontogenic differentiation capacity of DPSCs in comparison to DPSCs from SPF mice. Colonisation of GF mice with SPF microbiota (ConvD) normalises the dentine mineralisation and osteo-/odontogenic differentiation capacities of DPSCs.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Dr Shen Ping SU contributed to the collection and assembly of data, data analysis and interpretation and manuscript drafting; Dr Yi REN contributed to the collection and interpretation of data; Dr E Xiao, Prof Yu Ming ZHAO and Prof Yi ZHANG contributed to the overall design of the study and critical editing of the manuscript and provided financial support. All authors read and approved the final manuscript.

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