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LGR4 is required for sequential molar development

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ABSTRACT

Tooth development requires proliferation, differentiation, and specific migration of dental epithelial cells, through well-organized signaling interactions with mesenchymal cells. Recently, it has been reported that leucine-rich repeat-containing G protein coupled receptor 4 (LGR4), the receptor of R-spondins, is expressed in many epithelial cells in various organs and tissues and is essential for organ development and stem cell maintenance. Here, we report that LGR4 contributes to the sequential development of molars in mice. LGR4 expression in dental epithelium was detected in $SOX2^+$ cells in the posterior end of the second molar (M2) and the early tooth germ of the third molar (M3). In keratinocyte-specific *Lgr4*-deficient mice ($Lgr4^{K5\ KO}$), the developmental defect became obvious by postnatal day 14 (P14) in M3. $Lgr4^{K5\ KO}$ adult mice showed complete absence or the dwarfed form of M3. In M3 development in $Lgr4^{K5\ KO}$ mice, at Wnt/β-catenin signal activity was down-regulated in the dental epithelium at P3, as indicated by lymphoid enhancer-binding factor-1 (LEF1) expression. We also confirmed the decrease, in dental epithelium of $Lgr4^{K5\ KO}$ mice, of the number of $SOX2^+$ cells and the arrest of cell proliferation at P7, and observed abnormal differentiation at P14. Our data demonstrated that LGR4 controls the sequential development of molars by maintaining $SOX2^+$ cells in the dental epithelium, which have the ability to form normal molars.

1. Introduction

Teeth are essential organs required to masticate food for efficient digestion. Mammals, including humans and mice, have three molars at the back of the jaws. These three molars are known to be formed by sequential developmental process. Tooth germs of the second (M2) and third molars (M3) are formed by invagination of the tooth epithelia of the first molar (M1) [1,2]. Recently, it has been reported that dental epithelial stem cells (DESCs) transiently reside in the cervical loop (CL) of M1 [3]. Additionally, SOX2 expression, which is a marker of DESCs in M1 [3], is associated with sequential development directed to the posterior position [1]. Marcia Gaete et al. reported that epithelium of the anterior-molar tail could grow by the posterior movement of epithelial cells, followed by infolding and stratification involving a population of $SOX2^+/SOX9^+$ cells [4].

DESCs in incisors are regulated by various networks including bone morphogenetic protein (BMP), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), Notch, and Wnt [5,6]. Wnt/β-catenin signaling is activated in dental mesenchyme, but not in the epithelium of incisor at developmental stage, and indirectly regulates $Lgr5^+$ DESC in dental epithelium [7,8]. Excess activation of Wnt/β-catenin signaling in the dental epithelium of incisor caused increased differentiation of DESCs [6]. In incisor, the activation of Wnt/β-catenin signaling in the dental mesenchyme and suppression in the dental epithelium is important for maintenance of DESC. However, the mechanism by which DESCs are regulated during the sequential development of molars and their effect on the development of posterior molars are unclear.

In mice, tooth development begins at around embryonic day 11.5 (E11.5) with thickening of the oral epithelium. The epithelium invaginates into the mesenchyme to form a bud by E13.5, and then forms a cap. By E16.5, the tooth germ forms a bell. During the bell stage, the epithelium adjacent to the mesenchyme differentiates into ameloblasts to produce enamel, and the mesenchyme differentiates into odontoblasts to produce dentin. This is the mechanism by which tooth germs develop through epithelial-mesenchymal interactions [9]. Similar interactions between the epithelium and mesenchyme also regulate development of the lungs, kidneys, hair follicles, and mammary glands [10–13]. In addition, it is important to maintain the undifferentiated state of the epithelial cells for the development of the kidneys and mammary glands [14,15].

In these organs, leucine-rich repeat-containing G protein

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coupled receptor 4 (LGR4) regulates the undifferentiated state of the epithelial cells [16,17], and contributes to organ development. LGR4 interacts with the cell-surface transmembrane E3 ubiquitin ligase, zinc and ring finger 3 (ZNRF3), through binding to R-spondins, which are the ligands of LGR4, and clears ZNRF3 from the cell surface. This results in the inhibition of the degradation of Frizzled, a Wnt receptor, and subsequently, Wnt/β-catenin signaling is enhanced [18–20]. LGR4 is widely expressed in stem cells and proliferative components of many epithelial tissues: hair follicle, kidney, and intestine [21,22]. When cultured ex vivo, LGR4-deficient crypts or progenitors, show marked down-regulation of stem-cell markers and Wnt target genes and die rapidly [23]. In the kidney development, LGR4 functions in maintaining the ureteric bud in an undifferentiated state [16]. Thus, LGR4 plays an important role in maintaining stem cells or undifferentiated cells in epithelial tissues. In this study, using $Lgr4^{K5\ KO}$ mice, we demonstrated that LGR4 contributes to sequential molar development.

2. Material and methods

2.1. Animals

$Lgr4^{EGFP\text{-}IRES\text{-}CreERT2/+}$ mice were kindly provided by H.C. [24]. The generation of $Lgr4^{K5\ Ctrl}$ and $Lgr4^{K5\ KO}$ was described previously [25]. We used mice for the analysis at the neonatal stage without distinction of sex. The care and use of mice in this study was approved by the Institutional Animal Care and Use Committee of Tohoku University.

2.2. Histological and immunohistochemical analyses

For molar histology, mice heads were fixed in 4% paraformaldehyde (PFA), and decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 1–2 weeks. After dehydration, the samples were embedded in paraffin, and the paraffin blocks were sectioned at 5 μm thickness and stained with hematoxylin-eosin (H&E). For the frozen sections, the samples were embedded in Optimal Cutting Temperature compound, and the frozen blocks were sectioned at 5 μm thickness. The immunological staining protocol used the Alexa 488-labeled goat anti-rabbit IgG antibody (Invitrogen, A-11034, 1:200), the Alexa 488-labeled goat anti-mouse IgG antibody (Invitrogen, A-11001, 1:200), the Alexa 594-labeled goat anti-mouse IgG antibody (Invitrogen, A-11005, 1:200), the peroxidase-labeled goat anti-rabbit IgG antibody (Vector Laboratories, PI-1000, 1:200), and the avidin-biotinylated enzyme complex. The paraffin-embedded sections and the frozen sections of the heads were analyzed with the following antibodies: rabbit polyclonal antibodies against green fluorescent protein (GFP; MBL, 598, 1:200), mouse monoclonal antibodies against E-cadherin (BD Biosciences, 610182, 1:500), mouse monoclonal antibodies against SOX2 (Abcam, ab79351, 1:200), rabbit polyclonal antibodies against LEF1 (Cell Signaling Technologies, #2230S, 1:100), rabbit monoclonal antibodies against SOX2 (Abcam, ab92494, 1:200), rabbit polyclonal antibodies against Ki67 (Novus Biologicals, NB110-89717, 1:200), mouse monoclonal antibodies against bromodeoxyuridine (BrdU; Roche, 11,170,376,001, 1:200), and rabbit polyclonal antibodies against Amelogenin (Santa Cruz, sc-32892, 1:100). These antibodies were diluted with 5% normal goat serum or the Mouse on Mouse immunodetection kit (Vector Laboratories) in Tris-buffered saline (TBS) or phosphate buffer (PB) and applied to the sections, which were then incubated overnight at 4 °C.

2.3. 5-Bromo-2′-deoxyuridine (BrdU) administration

BrdU (Roche) was dissolved in phosphate buffered saline (PBS) at 5 mg/ml and injected intraperitoneally (50 μg/g body weight).

2.4. Statistics

The results of the experiments are expressed as the means ± SEM. ANOVA and Student's *t*-test were used for statistical analysis of the results, and a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Lgr4 is expressed in the bud epithelium of M1 at the embryonic stage, and its expression shifts to the posterior molar at the postnatal stage

During M1 development, *Lgr4* expression in the dental epithelium from E10.5 to E13.5, and in the collar of the tooth and the outer enamel epithelium at E14.5 has been reported [26]. However, the *Lgr4* expression pattern during M2 or M3 development is not known.

In this study, we analyzed the expression pattern of LGR4 during molar development using $Lgr4^{EGFP\text{-}IRES\text{-}CreERT2/+}$ mice, which express EGFP (and Cre ERT2) inserted in the *Lgr4* locus. Using immunostaining, we detected EGFP colocalized with E-cadherin, an epithelial marker. Strong expression of EGFP was detected in the dental epithelium of M1 at E14.5, indicating that LGR4 was expressed there (Fig. 1A). This result is consistent with those of previous reports [26]. At postnatal day 0 (P0), the EGFP signal was not detectable in the M1 tooth epithelium region (Fig. 1B), but was observed in the collar of M2, and in the bud of M3, which formed from the posterior part of M2 (Fig. 1C, C′). At P3, EGFP was only expressed in the epithelium of M3 (Fig. 1D, D′). No expression was observed in the dental epithelium at P7, by which time M3 develops to the bell stage (Fig. 1E). We found that LGR4 expression shifted to the posterior epithelium, accompanied by the sequential formation of molars from M1 to M3. Next, we performed double immunostaining for EGFP and SOX2, a DESC marker in incisors [27]. The nuclear signal of SOX2 is only detected in the posterior end and in the outer enamel epithelium of M2 and in M3 bud. The expression of EGFP was closely associated with SOX2 expression in the dental epithelium of M2 and M3 at P0 (Fig. 1F-F″). At P3, EGFP was expressed in a part of $SOX2^+$ epithelial cells (Fig. 1G, G′). Taken together, these results suggest that LGR4 is expressed in the $SOX2^+$ epithelial cells during sequential molar development.

3.2. Keratin5-Cre Tg specific deletion of Lgr4 affects the development of M3

To study the role of LGR4 in molar development, we first analyzed the mouse model with targeted deletion of *Lgr4* gene. We histologically analyzed *Lgr4* null mice ($Lgr4^{-/-}$). These mice exhibited neonatal lethality due to renal hypoplasia and impaired erythropoiesis [28,29]. However, they did not show any aberration in the tooth germ of M1 or M2 at P0 (Fig. S1A). To analyze the role of LGR4 in molar development, we generated conditional knockout mice crossed with *Keratin5-Cre* (*K5-Cre*) transgenic (Tg) mice. One of the proteins of the cytokeratin family, Keratin5, is expressed in various epithelial cells including the oral epithelium [30]. *Keratin5* promoter has been used in some mouse models for over expression or gene deletion in tooth germ epithelial cells [31]. *Lgr4* conditional knock-out mice with *K5-Cre* Tg mice ($Lgr4^{K5\ KO}$) circumvented neonatal lethality and kidney abnormalities [25].

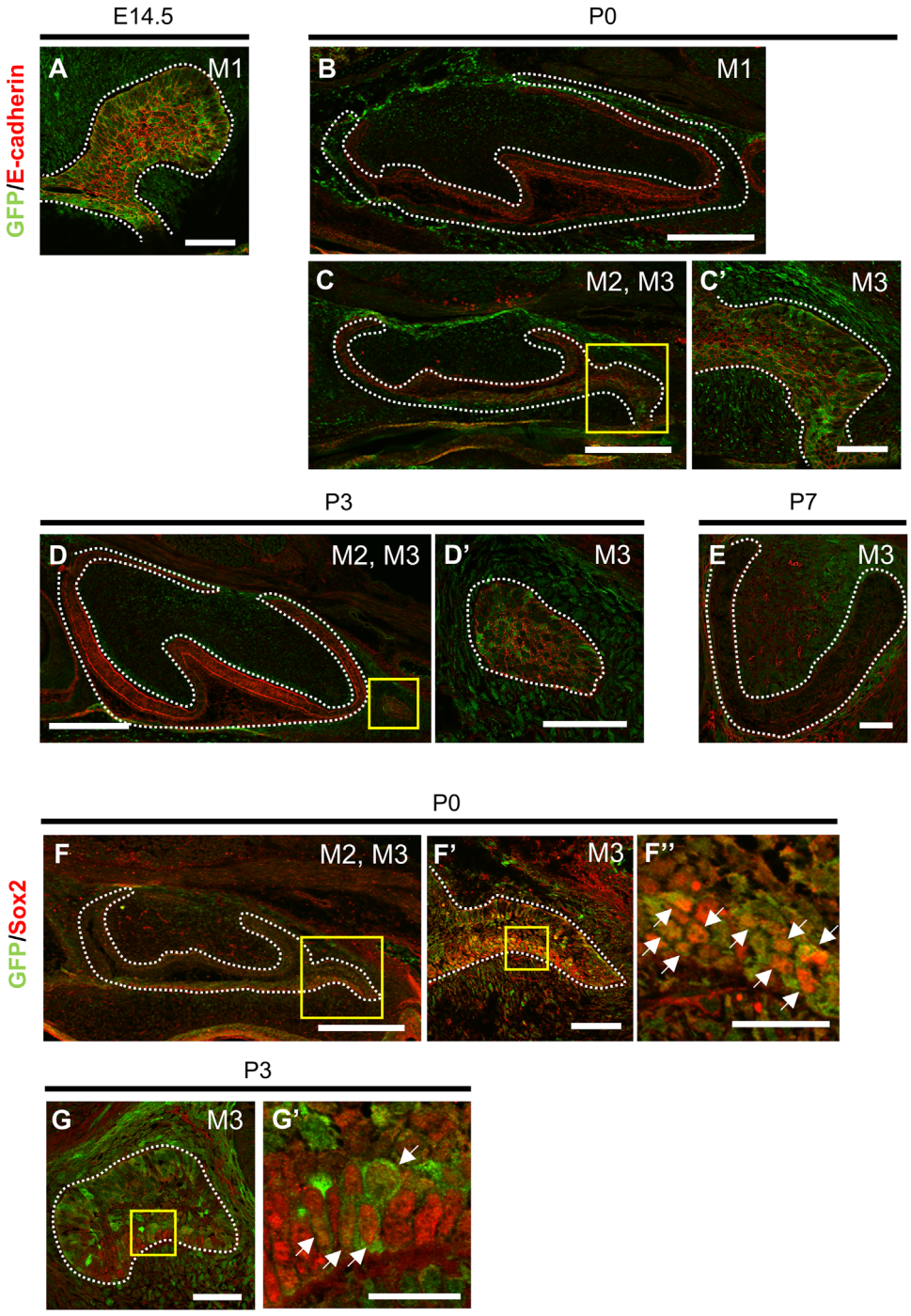

Fig. 1. *Lgr4* is expressed in the bud epithelium of M1 at the embryonic stage, and its expression shifts to the posterior molar at the postnatal stage. (A-E) Immunofluorescence analysis for GFP (green) and E-cadherin (red) of tooth germ in $Lgr4^{EGFP\text{-}IRES\text{-}CreERT2/+}$ mice in coronal (A) and sagittal sections (B-E). (F and G) Immunofluorescence analysis for GFP (green) and SOX2 (red) in $Lgr4^{EGFP\text{-}IRES\text{-}CreERT2/+}$ mice in sagittal sections. Arrows indicate $GFP^+/SOX2^+$ cells. Boxed areas in (C), (D), (F), (F′), and (G) are shown magnified in (C′), (D′), (F′), (F″), and (G′) respectively. Dot-lines indicate dental epithelium. The sample size (n) for each experimental group/condition was 3. Three different individuals were used in the experiment. All experiments were performed in duplicate to confirm the repeatability. Scale bars represent 50 μm (A, C′, D′, E, F′, and G), 200 μm (B-D, and F), and 20 μm (F″ and G′).

The molars in $Lgr4^{K5\ KO}$ mice morphologically developed to their normal structure, similar to in $Lgr4^{K5\ Ctrl}$ mice, until P3 when M3 developed to the bud stage (Fig. 2A-D, A′-D′). At P7, all $Lgr4^{K5\ Ctrl}$ and most $Lgr4^{K5\ KO}$ mice showed development to the cap stage in M3 (Fig. 2E, F, E′, F′), but M3 in only one of the $Lgr4^{K5\ KO}$ mice formed a bud (Fig. 2G, G′). In addition, M3 in $Lgr4^{K5\ Ctrl}$ formed "the late bell" structure and developed the layer of enamel or dentin at P14 (Fig. 2H, H′); however, M3 in $Lgr4^{K5\ KO}$ formed "the early bell" structure and no layer of hard tissue was observed at P14 (Fig. 2I, I′). Additionally, at P14, there was individual with no M3 germ although we observed sequential sections (data not shown). Thus, we confirmed the abnormal development of M3 in $Lgr4^{K5\ KO}$ mice during the period from P7 to P14.

Next, we analyzed molars in male $Lgr4^{K5\ Ctrl}$ or $Lgr4^{K5\ KO}$ mice at postnatal 10 weeks. Some of the $Lgr4^{K5\ KO}$ mice showed the dwarfed form of upper M3 (9/15; 60%, Fig. 2K) and the others showed its complete absence (5/15; 33%, Fig. 2L). Only in one out of the 15 $Lgr4^{K5\ KO}$ mice analyzed, M3 developed to its normal

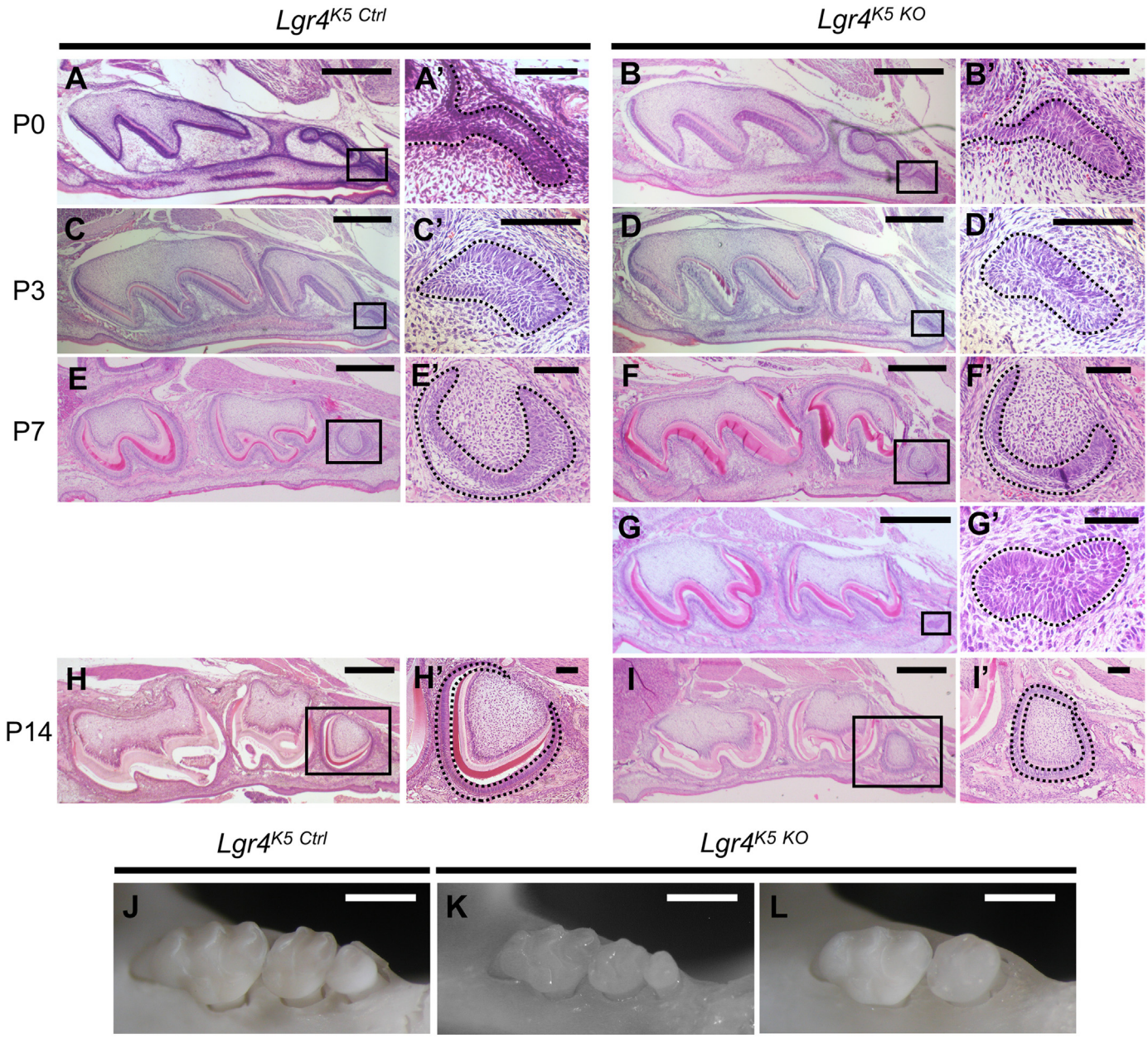

Fig. 2. *Keratin5-Cre* Tg specific deletion of *Lgr4* affects the development of M3. (A-I) H&E staining of $Lgr4^{K5\ Ctrl}$ and $Lgr4^{K5\ KO}$ mice in sagittal sections. Boxed areas in (A)-(I) are shown magnified in (A′)-(I′), respectively. Dot-lines indicate dental epithelium of M3. (J-L) Macroscopic views of maxillary molars in male $Lgr4^{K5\ Ctrl}$ and $Lgr4^{K5\ KO}$ mice at 10 weeks age. The sample size (n) for the experimental groups/conditions is 3 (A-I), and 15 (J-L). Different individuals were used in the experiment. All experiments were performed in duplicate to confirm the repeatability. Scale bars represent 500 μm (A-I), 50 μm (A′-I′), and 1 mm (J-L).

structure (1/15, 7%) similar to that of $Lgr4^{K5\ Ctrl}$ (Fig. 2J). This abnormality was also observed in the lower M3 (Fig. S1B). The size of M1 and M2 in $Lgr4^{K5\ KO}$ mice was slightly, but significantly, decreased compared to that of normal M1 and M2. However, the severest abnormality was observed in M3 (Fig. S1C-F). We found that in $Lgr4^{K5\ KO}$ mice, the development of M3 was abnormal at the early stage, and it was completely absent or dwarfed at adulthood.

3.3. *Lgr4 deletion disrupts the differentiation of dental epithelium into ameloblasts*

In the tooth development, dental epithelium differentiates into ameloblasts to secrete enamel after the bell stage. Amelogenesis is the process of differentiation of dental epithelial cells into ameloblasts, which secrete enamel. To secrete enamel, mature ameloblast forms columnar structure with the localization of nuclei near the stellate reticulum side [32]. Fig. 2 showed that *Lgr4* deletion caused enamel defect in M3 at P14. Therefore, we examined the differentiation of M3 epithelium in $Lgr4^{K5\ KO}$ mice. These mice showed abnormal cell polarity and decreased expression of Amelogenin, the enamel matrix protein, in M3 ameloblasts at P14 (Fig. 3A-A‴, B-B‴). This result indicates that LGR4 was involved in the differentiation of dental epithelial cells into ameloblasts in M3.

3.4. *LGR4 promotes Wnt/β-catenin signaling in M3 development*

LGR4 enhances Wnt/β-catenin signaling by binding its ligand, R-spondin [19,20]. Wnt/β-catenin signaling plays an essential role in the early stage of molar development. It was reported that β-catenin or LEF1 knock-out mice showed developmental arrest of M1 at bud stage [33–35]. Therefore, we evaluated Wnt/β-catenin signaling activity in M3 development by immunostaining for LEF1,

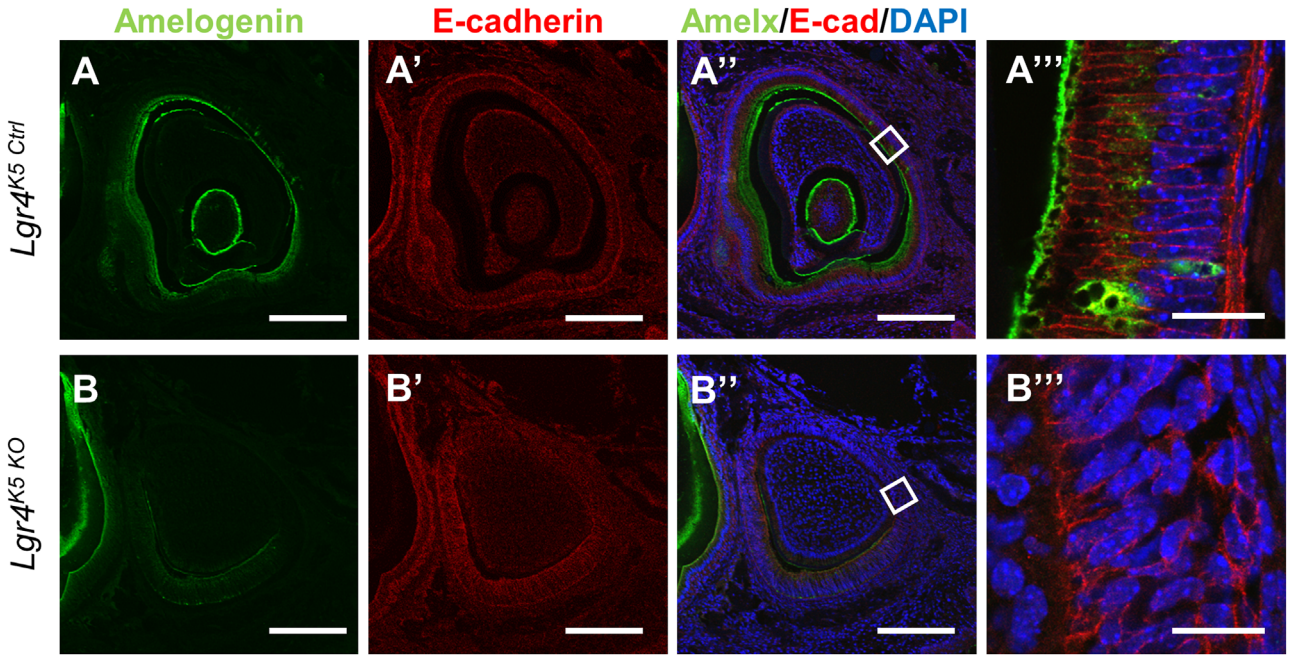

Fig. 3. *Lgr4* deletion disrupts the differentiation of dental epithelium into ameloblasts (A and B) Immunofluorescence analysis for Amelogenin (green) and E-cadherin (red) of M3 tooth germ in $Lgr4^{K5\ Ctrl}$ and $Lgr4^{K5\ KO}$ mice in sagittal sections. Counterstain is DAPI. Boxed areas in (A″) and (B″) are shown magnified in (A‴) and (B‴), respectively. Amelx - Amelogenin; E-cad - E-cadherin The sample size (n) for each experimental group/condition is 3. Three different individuals were used in the experiment. All experiments were performed in duplicate to confirm the repeatability. Scale bars represent 200 μm (A-A″ and B-B″) and 20 μm (A‴ and B‴).

a downstream target of Wnt signaling. $Lgr4^{K5\ Ctrl}$ mice showed strong expression of LEF1 in M3 dental epithelium and mesenchyme at P3 (Fig. 4A, A′). This expression pattern was similar to that of M1 bud stage [33]. However, LEF1 was hardly detected in M3 dental epithelium of $Lgr4^{K5\ KO}$ (Fig. 4B, B′), and the percentage of $LEF1^+$ epithelial cells was significantly decreased (Fig. 4C). These results indicate that LGR4 enhances Wnt/β-catenin signaling in M3 dental epithelium.

3.5. *Lgr4* deletion decreases the SOX2 positive and proliferative cells in the dental epithelium during molar development

LGR4 activates SOX2 expression and regulates the development and stem cell functions in mammary gland via Wnt/β-catenin/LEF1 signaling [17]. Wnt signal regulates proliferation and differentiation of $SOX2^+$ cells during cochlea development [36]. In the molar development, $SOX2^+$ cells contribute to sequential molar development [1,4]. In the present study, we examined the expression of SOX2, using $Lgr4^{K5\ KO}$ mice.

We confirmed the expression of SOX2 in the dental epithelium of M3, dental cord, and dental lamina connecting M1 to M2, in both $Lgr4^{K5\ Ctrl}$ and $Lgr4^{K5\ KO}$ mice until P3 (Fig. 5A-D, A′-D′). At P7, SOX2 was localized in the CL of M3 in $Lgr4^{K5\ Ctrl}$ (Fig. 5E-E″). In contrast, no expression of SOX2 was observed in the M3 dental epithelium of $Lgr4^{K5\ KO}$ (Fig. 5F-F″). At P14, SOX2 was expressed in the M3 dental cord of $Lgr4^{K5\ Ctrl}$, but not in the CL (Fig. 5G, G′). No expression of SOX2 was observed in M3 dental epithelium of $Lgr4^{K5\ KO}$ (Fig. 5H, H′). In addition, the percentage of $SOX2^+$ cells in M3 epithelium of $Lgr4^{K5\ KO}$ was comparable with that of $Lgr4^{K5\ Ctrl}$ at P3, but significantly decreased at P7 (Fig. 5I, J). Our results strongly demonstrate that the loss of *Lgr4* causes a decrease in the number of $SOX2^+$ cells in the M3 dental epithelium during early developmental stage.

$SOX2^+$ cells in the dental epithelium have high proliferative activity in the M1 development [3]. Therefore, we expected that abnormal cell proliferation occurred in $Lgr4^{K5\ KO}$ mice at P7. We performed immunostaining for antigen Ki67, a typical cell proliferation marker. At P3, there was no difference in M3 between $Lgr4^{K5\ Ctrl}$ (Fig. 6A, A′) and $Lgr4^{K5\ KO}$ mice (Fig. 6B, B′) in the expression of Ki67. However, the expression of Ki67 in the dental epithelium of M3 of $Lgr4^{K5\ KO}$ was significantly decreased at P7 (Fig. 6D, D′) compared to that in $Lgr4^{K5\ Ctrl}$ (Fig. 6C, C′). Therefore, we hypothesized that epithelial cell proliferation was inhibited in M3 of $Lgr4^{K5\ KO}$ from P3 to P7. To determine the proliferative rate of these cells, we conducted pulse chase analysis with BrdU. BrdU, an analog of thymidine, is incorporated into the nuclei of cells during DNA synthesis, and the cells labeled with BrdU can be detected by the *anti*-BrdU antibody. BrdU density in DNA finally decreases to below the detectable level following repeated cell division. However, the cells with slow cell cycle can be detected even after the lapse of a long duration after BrdU administration [37–40].

We administrated BrdU intraperitoneally to $Lgr4^{K5\ KO}$ mice from P0 to P2, dissected these mice at P3 or P7, and detected cells with BrdU by immunostaining (Fig. 6E). At P3, BrdU was detected in the dental epithelium of M3 of $Lgr4^{K5\ Ctrl}$ and $Lgr4^{K5\ KO}$ (Fig. 6F, G). At P7, $BrdU^+$ cells were detected in M3 of $Lgr4^{K5\ KO}$ (Fig. 6I, I′), but no signal was detected in $Lgr4^{K5\ Ctrl}$ (Fig. 6H, H′). In $Lgr4^{K5\ Ctrl}$ mice, BrdU density in DNA may have been below the detectable level because the epithelial cells proliferated actively. In contrast, BrdU was detected at P7 in $Lgr4^{K5\ KO}$ mice suggesting that cell proliferation is inhibited in the dental epithelium of M3 in $Lgr4^{K5\ KO}$ in the period from P3 to P7. Our data demonstrates that *Lgr4* deletion diminishes the cells that actively proliferate in the dental epithelium.

4. Discussion

Three molars develop sequentially from the first tooth germ through invagination of the dental epithelium to form the posterior tooth germ of the next molar. In this report, we clarified the role of LGR4 in sequential molar development using $Lgr4^{K5\ KO}$ mice, which showed an abnormal morphology of the tooth germ

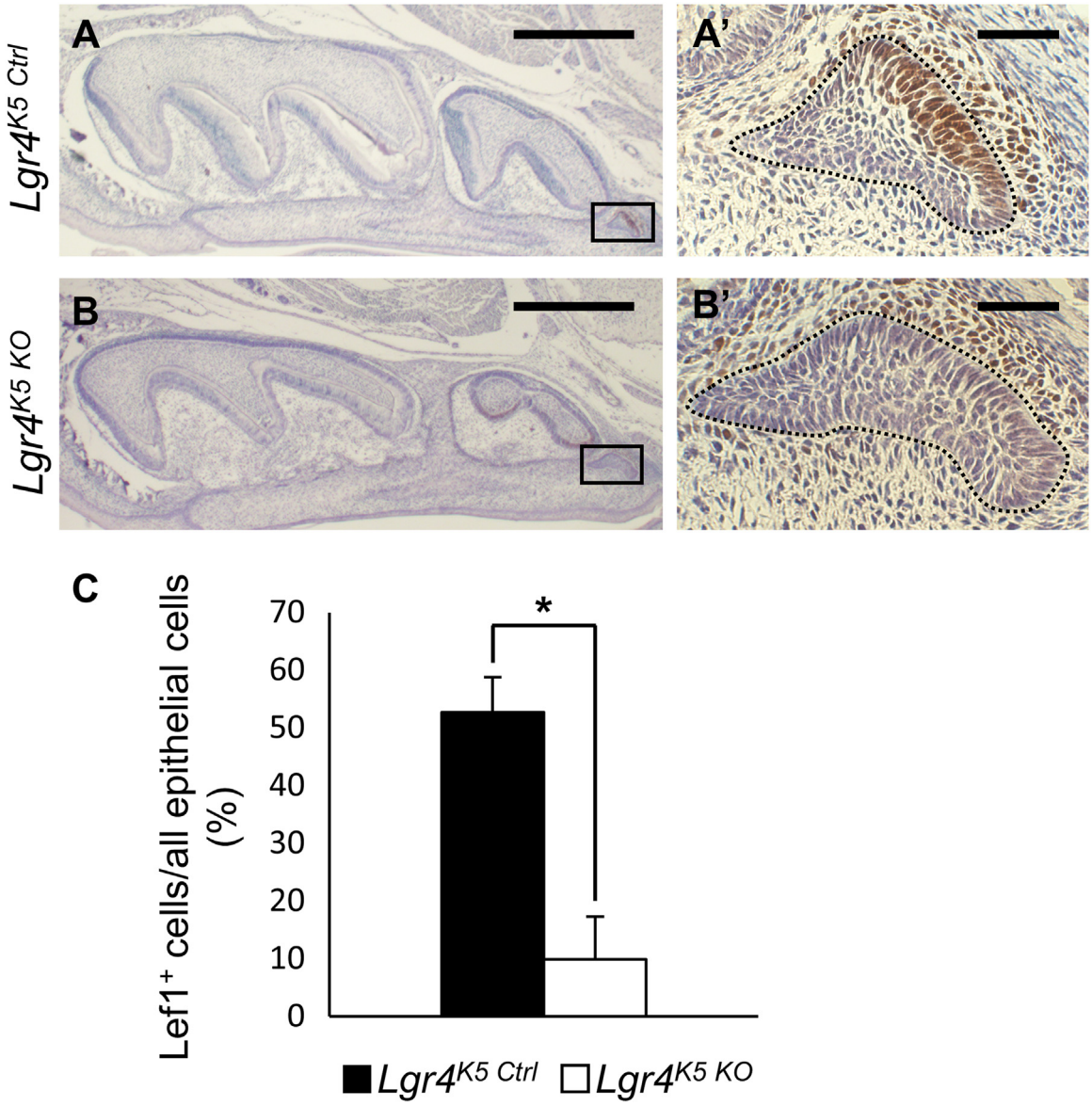

Fig. 4. *Lgr4* promotes Wnt/β-catenin signaling in M3 development (A and B) Immunohistochemistry of LEF1 in $Lgr4^{K5\ Ctrl}$ and $Lgr4^{K5\ KO}$ mice in sagittal sections at P3. Counterstain is hematoxylin. Dot-lines indicate dental epithelium of M3. Boxed areas in (A) and (B) are shown magnified in (A′) and (B′), respectively. Scale bars represent 500 μm (A and B) and 50 μm (A′ and B′). (C) The percentage of $LEF1^+$ cells in M3 dental epithelium of $Lgr4^{K5\ Ctrl}$ and $Lgr4^{K5\ KO}$ mice. N = 3. *$p < 0.05$. Error bars, SEM. ANOVA and Student's *t*-test were used for the statistical analysis. The sample size (n) for each experimental group/condition is 3. Three different individuals were used in the experiment. All experiments were performed in duplicate to confirm repeatability.

and altered gene expression in M3 during the neonatal stage and loss of M3 loss or dwarfed M3 in adults (Table 1).

In the early fetal stage, $SOX2^+$ cells exist in the dental epithelium of M1 [1]. SOX2 expression is translocated to the posterior epithelium of M1 concomitantly with the development and contributes to epithelial cell lineage in M2 or M3 [1]. Using $Lgr4^{EGFP\text{-}IRES\text{-}CreERT2/+}$ mice, we found that the expression of LGR4 was observed in some of the $SOX2^+$ cells and translocated to the posterior epithelium as molars sequentially developed from M1 to M3. This expression pattern and its transition appear to be similar to that observed in $SOX2^+$ cells. These results suggest that $SOX2^+$ cells as well as other undifferentiated epithelial cells express LGR4. LGR4 maintains undifferentiated cells in the kidneys, mammary glands, and intestines and contributes to normal development and homeostasis [16,17].

To study the role of LGR4 in molar development, we used the loss of function approach with gene-deleted mouse models. Interestingly, $Ki67^+$ proliferative cells were not decreased at P3, but clearly decreased at P7 in $Lgr4^{K5\ KO}$ mice. In addition, we administrated BrdU to newborn pups of $Lgr4^{K5\ Ctrl}$ during the period from P0 to P2, and BrdU was undetectable at P7; however it persisted in M3 of $Lgr4^{K5\ KO}$ mice at P7 (Fig. 6). These data demonstrate that the epithelial cells of $Lgr4^{K5\ KO}$ gradually lose their division potential during the M3 tooth germ development phase. Moreover, at P7, $SOX2^+$ cells in CL were not retained in $Lgr4^{K5\ KO}$ mice. These data suggest that LGR4 regulates the sequential development of molars by maintaining $SOX2^+$ cells, which have a higher potential to proliferate.

The role of Wnt/β-catenin signaling in the regulation of sequential development in molars has been unclear. We showed that *Lgr4* ablation caused the suppression of Wnt signal activity in M3, using the experimental model of $Lgr4^{K5\ KO}$ mice. Our data indicate that LGR4 is involved in the activation of Wnt signaling during posterior molar development. However, Wnt signal is downregulated during maintenance of $SOX2^+$ DESC in incisors. The activation of Wnt signal by a drug (BIO) decreases SOX2 expression

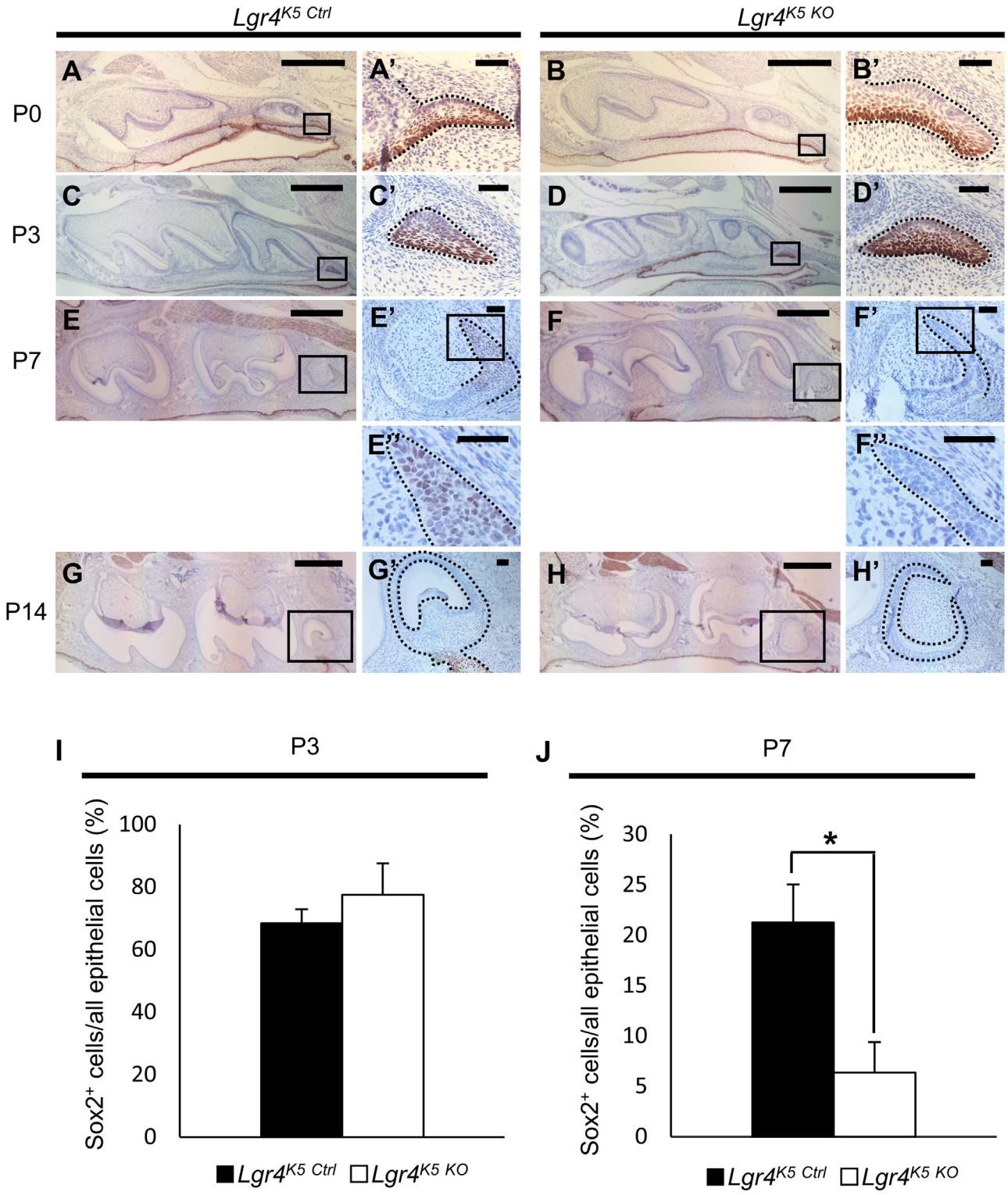

Fig. 5. *Lgr4* deletion decreased the SOX2$^+$ cells in the dental epithelium during molar development. (A-H) Immunohistochemistry of SOX2 in *Lgr4*$^{K5\ Ctrl}$ and *Lgr4*$^{K5\ KO}$ mice in sagittal sections at P0, P3, P7, and P14. Counterstain is hematoxylin. Dot-lines indicate dental epithelium of M3. Boxed areas in (A)-(H), (E′), and (F′) are shown magnified in (A′)-(H′), (E″), and (F″), respectively. Scale bars represent 500 μm (A-H) and 50 μm (A′-H′, E″, and F″). (I and J) The percentage of SOX2$^+$ cells in M3 dental epithelium of *Lgr4*$^{K5\ Ctrl}$ and *Lgr4*$^{K5\ KO}$ mice at P3 and P7. N=3. $^*p<0.05$. Error bars, SEM. ANOVA and Student's *t*-test were used for the statistical analysis. The sample size (n) for each experimental group/condition is 3. All experiments were performed in duplicate to confirm the repeatability.

and inhibits cell proliferation in CL of incisor [41]. In addition, SOX2$^+$ DESCs transiently express the Wnt inhibitor Sfrp5 and contribute to all epithelial cell lineages of the tooth [27]. These observations suggest that Wnt signal plays different roles in DESC regulation: the maintenance of SOX2$^+$ cells in the molar and the promotion of differentiation in incisor. Additionally, decreased LEF1 expression in *Lgr4*$^{K5\ KO}$ mice was observed at P3, whereas the number of SOX2$^+$ cells and the proliferative ability were decreased after P7 (Table 1). This temporal difference in the timing of the alteration between SOX2 and LEF1 expression might reflects

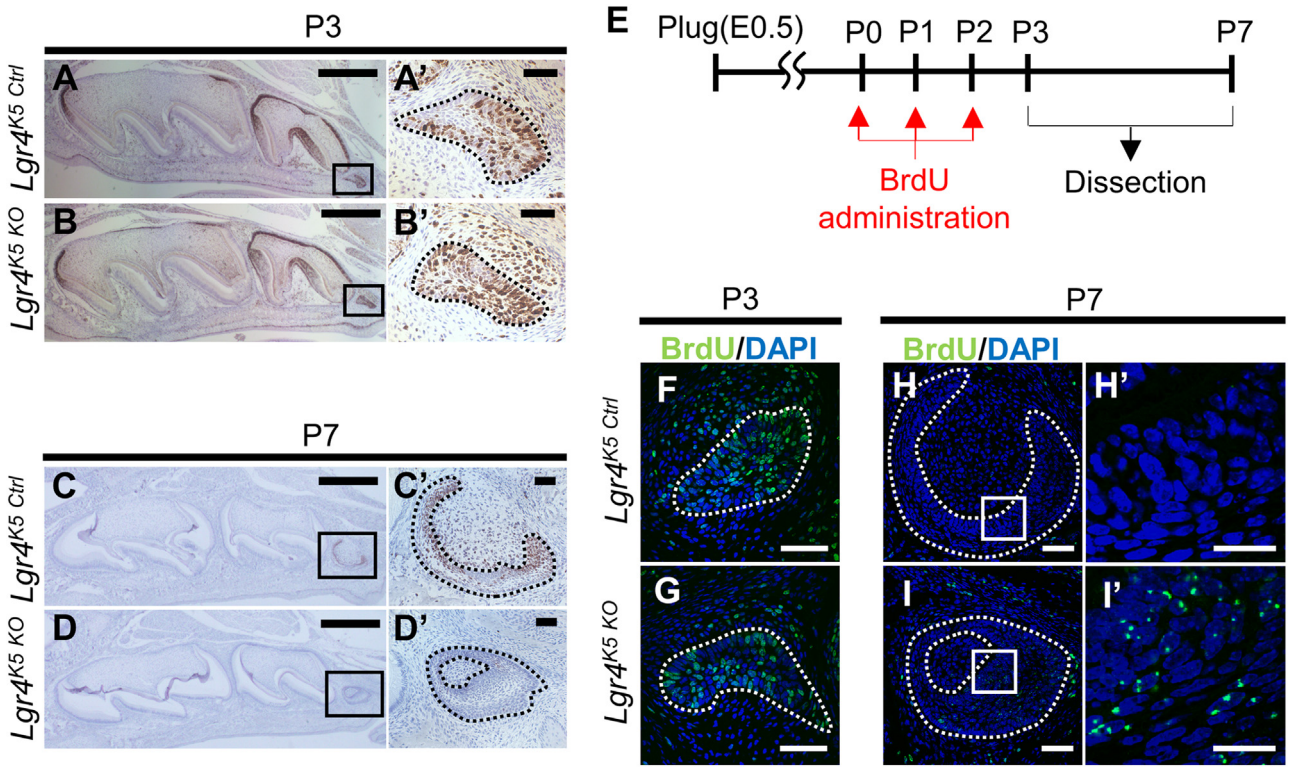

Fig. 6. LGR4 regulated the cell proliferation of the dental epithelium in M3. (A-D) Immunohistochemistry of Ki67 in *Lgr4*$^{K5\ Ctrl}$ and *Lgr4*$^{K5\ KO}$ mice in sagittal sections at P3 and P7. Counterstain is hematoxylin. (E) Timing of BrdU administration and sample harvest in *Lgr4*$^{K5\ Ctrl}$ and *Lgr4*$^{K5\ KO}$ mice. (F-I) Immunofluorescence analysis for BrdU in *Lgr4*$^{K5\ Ctrl}$ and *Lgr4*$^{K5\ KO}$ mice in sagittal sections at P3 and P7. Counterstain is DAPI. Dot-lines indicate dental epithelium of M3. Boxed areas in (A)-(D), (H), and (I) are shown magnified in (A′)-(D′), (H′), and (I′), respectively. The sample size (n) for each experimental group/condition is 3. Three different individuals were used in the experiment. All experiments were performed in duplicate to confirm the repeatability. Scale bars represent 500 μm (A-D), 50 μm (A′-D′, F-I), and 20 μm (H′ and I′).

Table 1
The summary of time points and differences between *Lgr4*$^{K5\ Ctrl}$ and *Lgr4*$^{K5\ KO}$.

	Lgr4$^{K5\ Ctrl}$	*Lgr4*$^{K5\ KO}$
P0	M3 is formed by invagination of M2 epithelium.	
P3	– M1 and M2 form a bell and M3 forms a bud. – *Lgr4* is expressed in a part of M3.	– The morphology of the molars is normal. – LEF1 expression is decreased in M3.
P7	– M3 develops to the cap stage. – SOX2^{+} cells remained in the CL of M3.	– M3 develops to the cap stage or stops at bud stage. – SOX2 and Ki67 expression is decreased.
P14	– M3 develops to the late bell stage. – The enamel layers are formed in M3.	– M3 develops to the early bell stage or tooth germ is completely lost. – Ameloblast differentiation is disrupted.
Adult	Molar development is completed.	A dwarfed form or the complete absence of M3

the indirect relationship between Wnt signaling and SOX2 expression. However, it still remains unclear whether Wnt signaling regulates SOX2 expression per this report. Further experiments will be required to investigate the involvement between Wnt signaling and SOX2^{+} epithelial cells.

In the M3 ameloblast of *Lgr4*$^{K5\ KO}$ mice, the cell polarity was disrupted with abnormal localization and the enamel secretion was inhibited at P14 (Fig. 3). This result indicates that *Lgr4*$^{K5\ KO}$ mice lack mature ameloblasts in the tooth germ of M3. However, *Lgr4* was only expressed during the early developmental stage of M3, but not expressed after P7 at which time ameloblast differentiation and maturation occurs. Therefore, LGR4 does not directly regulate ameloblast differentiation. These data suggested that LGR4 maintains the germ cells of ameloblast in the dental epithelium during early developmental stage. The maintenance of dental epithelium during developmental phase by *Lgr4* probably influences the characteristics of M3, such as structure, existence, and stability to jaws in adult mice.

There was a variation in the phenotypes of M3 within the experimental group of *Lgr4*$^{K5\ KO}$ mice: small structure, dislocation from jaws, and complete loss. These variations were observed during the neonatal stage, where some *Lgr4*$^{K5\ KO}$ mice showed an abnormal M3 germ that failed to form a cap at P7 (Fig. 2G). At P14, in addition to individuals with defective enamel, the M3 germ was completely lost in some *Lgr4*$^{K5\ KO}$ mice (data not shown). These results suggest the possibility that the progenitor is disrupted in *Lgr4*$^{K5\ KO}$ mice with severe phenotypes at the adult stage.

The full length of M1 and M2 was slightly decreased in *Lgr4*$^{K5\ KO}$ mice at the adult stage (Fig. S1C-F). In contrast, the tooth germ of M1 and M2 was normally developed in *Lgr4*$^{K5\ KO}$ mice or in *Lgr4*$^{-/-}$ mice at P0, and no critical differences were observed by morphological analysis (Fig. 2A, B and Fig. S1A, B). We presume that the subtle abnormalities in the dental epithelial progenitor at the early developmental stage, which we were unable to determine in this study, are caused by *Lgr4* deletion, and they finally caused little abnormality in M1 and M2 at the adult stage. These subtle abnormalities, for example, the decrease of division frequency or the reduction of the period for maintaining the undifferentiated state, might affect the size of the tooth germ or the differentiation potential to ameloblasts in M1 and M2

development.

In this study, we used both male and female *Lgr4*$^{K5\ KO}$ mice in the analysis at neonatal stage. At adult, male *Lgr4*$^{K5\ KO}$ mice showed the dwarfed form or complete absence in M3 (Fig. 2J-L). We also performed the morphological analysis of female *Lgr4*$^{K5\ KO}$ mice at adult. As a result, female showed similar phenotype as seen in male. No gender-related differences were observed in adult mice, which probably true in neonatal mice as well.

In mice, molars have no potential to grow continuously or to be replaced, and this feature is similar to that of molars in humans. Additionally, the mechanism underlying the sequential development of molars is probably similar in mice and humans. However, this mechanism has been mostly unclear. In this study, using *Lgr4* deficient mice, we demonstrated for the first time that LGR4 is required for the maintenance of SOX2$^+$ cells and their proliferation during the sequential development of molars from the anterior to posterior epithelia of the tooth germs. This study has afforded a better understanding of the maintenance mechanism of DESCs during molar development, which should accelerate the development of new approaches for studying the mechanisms underlying tooth regeneration.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.08.018.

Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.08.018.

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