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Follistatin regulates tooth root furcation morphogenesis

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ABSTRACT Follistatin (Fst), an extracellular inhibitor of the TGF-β signaling pathway, plays a critical role in tooth development, particularly in crown morphogenesis and enamel patterning. While molar root number is conserved within species and between upper and lower jaws, the molecular mechanisms governing root patterning remain largely unknown. To investigate the role of Fst in root formation, we analyzed *K14-Cre*-mediated *Fst* knockout mice. During embryonic and early postnatal stages, *Fst* was dynamically expressed in the dental epithelium including the lingual epithelium, and primary and secondary enamel knots, as well as in the dental mesenchyme at the base of the dental papilla. However, during root formation, *Fst* expression became restricted to the outer layer of Hertwig's epithelial root sheath (HERS). Micro-CT analysis revealed that mutant mice exhibited molar root abnormalities, including enlarged pulp chambers and partial furcation defects on the lingual side of the mandibular molars. Histological analysis showed that, while HERS initially exhibited bucco-lingual asymmetry —with the buccal-side HERS extending more prominently in both control and mutant mice— control mice eventually established bucco-lingual symmetry in HERS extension, ensuring proper root furcation formation. In contrast, mutant mice displayed delayed lingual-side HERS extension, resulting in defective root furcation. Molecular analysis revealed that *Gli1* expression was upregulated, whereas *Cdkn1a* and Wnt signaling molecules (*Wnt4*, *Lhx6*, and *Notum*) were significantly downregulated in the delayed lingual HERS of mutant mice. These findings suggest that Fst regulates tooth root morphogenesis by modulating Shh and Wnt signaling to control lingual epithelial proliferation and HERS extension. This study offers novel insights into the molecular mechanisms governing molar root number and morphology determination.

KEYWORDS: Follistatin, furcation, tooth roots, asymmetry, root patterning.

LABURPENA Folistatina (Fst) TGF-β seinalizazio-bidearen zelulaz kanpoko inhibitzaile bat da, eta funtsezko eginkizuna du hortz-garapenean, bereziki koroaren morfogenesian eta esmaltearen eraketan. Espezie bakoitzaren barruan eta goiko eta beheko barailen artean hagin-sustraien kopurua konstante mantentzen bada ere, oraindik ezezagunak zaizkigu, neurri handi batean, sustraien eraketa-patroia gidatzen duten mekanismo molekularrak. Fst proteinak sustraien eraketan duen papera ikertzeko, *K14-Cre* bidez *Fst* genea desaktibatua zuten *Fst* knockout saguak aztertu genituen. Enbrioi-etapan eta jaio ondoko etapa goiztiarretan, *Fst* genea modu dinamikoan adierazi zen hortz-epitelioan —mihi-epitelioan barne—, esmalte-nodulu primarioan eta sekundarioan, eta hortz-papilaren oinarriko hortz-mesenkiman. Hala ere, sustraiaren eraketan, *Fst* genea Hertwig-en epitelio-zorro erradikularraren (HERS) kanpoko geruzan soilik adierazi zen. Ordenagailu bidezko mikrotomografia bidez egindako analisiak erakutsi zuen sagu mutanteek anomaliak zituztela hagin-sustraietan, besteak beste, pulpa-ganbera handituak eta furkazio-akats partzialak masailezurreko

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haginetan (mihiaren aldean). Azterketa histologikoek erakutsi zutenez, fase goiztiarretan, HERSaren aho aldeko eta mihi aldeko hedapenak asimetrikoak izan arren —ahoaren aldean, HERSa gehiago hedatzen zela ikusi zen, bai kontrol-saguetan, bai sagu mutanteetan—, gerora HERSa simetrikoki hedatzen zen kontrol-saguetan, eta horrek sustraiaren furkazioa egokiro eratzea bermatzen zuen. Sagu mutanteen kasuan, aldiz, HERSaren hedapena atzeratu egiten zen mihiaren aldean, eta horrek sustraiaren furkazioa akastuna izatea eragiten zuen. Ikerketa molekularrek erakusten zutenez, arratoi mutanteen HERS atzeratuan (mihiaren aldekoan), *Gli1* genea gainadierazita zegoen, eta *Cdkn1a* eta Wnt seinalizazio-bideko geneen (*Wnt4*, *Lhx6* eta *Notum*) adierazpena, berriz, nabarmen murriztuta zegoen. Emaitza horiek iradokitzen dute Fst-k hortz-sustraiaren morfogenesia erregulatzen duela, eta horretarako, Shh eta Wnt seinalizazio-bideak modulatzen dituela, mihiko epitelioaren proliferazioa eta HERSaren hedapena kontrolatze aldera. Azterlan honek hortz-sustraien kopurua eta morfologia erregulatzen duten mekanismo molekularrei buruzko ezagutza berriak ematen dizkigu.

HITZ-GAKOAK: Follistatina, amorrua, hortz-sustraiak, asimetria, sustraiak nahastea.

Introduction

Tooth morphogenesis is orchestrated by reciprocal interactions between the epithelium and mesenchyme (Thesleff 2003). Various growth factors and transcription factors contribute to the determination of tooth size and shape (Tummers and Thesleff 2009); however, the mechanisms underlying the regulation of tooth root shape and size remain poorly understood.

Root development begins after crown morphogenesis and is marked by the formation of Hertwig's epithelial root sheath (HERS). As HERS elongates, it induces the proliferation and differentiation of adjacent mesenchymal cells within the dental papilla into odontoblasts, which are essential for secreting dentin that forms the tooth root (Thomas 1995). Thus, HERS plays a critical role in root development (Huang *et al.,* 2009a). Several signaling pathways have been implicated in tooth root development. Epithelial sonic hedgehog (Shh), TGF-β, and Wnt/β-catenin signaling are essential for HERS proliferation and maintenance of its cellular integrity (Gritli-Linde *et al.,* 2002; Huang *et al.,* 2009b; Yang *et al.,* 2021). In contrast, root elongation in the dental mesenchyme is regulated by multiple signaling related genes, including *Ptc1, Smad4, Wls,* and *Sox9*. Ablation of each of these genes in the dental mesenchyme leads to an immediate reduction in mesenchymal cell proliferation, eventually resulting in molars with shortened roots (Nakatomi *et al.,* 2006; Gao *et al.,* 2009; Bae *et al.,* 2015; Lav *et al.,* 2023). These findings suggest that epithelial proliferation is crucial for HERS formation at the onset of root development, whereas mesenchymal proliferation and differentiation play pivotal roles in root elongation.

In mammals, the number and morphology of molar roots are highly conserved within species and between upper and lower jaws. This structural consistency is regulated by the epithelial diaphragm, which consists of tongue-like extension of HERS that project inward and fuse between developing roots (Seo *et al.,* 2017). Despite this conservation, the molecular mechanisms governing molar root patterning remain largely unknown. Previous studies have identified mesenchymal proliferation as a key contributor to root morphogenesis (Shon *et al.,* 2014). The formation of a cylindrical root structure requires spatially regulated mesenchymal proliferation, with relatively higher proliferation occurring in the root elongation region compared to the furcation region. To date, several signaling-related genes, including *Nfic, Eda, Lhx6, Wnt10a,* and *Notum,* have been reported to be associated with tooth root patterning (Kim *et al.,* 2015; Fons Romero *et al.,* 2017; Yu *et al.,* 2020, He *et al.,* 2021; Adasooriya *et al.,* 2024). However, the precise interplay between epithelial and mesenchymal signaling that governs the spatial organization and development of molar roots remains largely unexplored and requires further investigation.

Follistatin (Fst) is an extracellular inhibitor of the TGF-β signaling pathway, primarily regulating its activity by binding to ligands such as activin and Bmps (Matzuk *et al.,* 1995). In tooth development, Fst plays a crucial role in cusp patterning and enamel formation and is also implicated in determination of tooth number (Wang *et al.,* 2004a, b; Zhu *et al.,* 2024). Previous studies have demonstrated its essential role in cusp patterning of mouse molars and enamel patterning of mouse incisors (Wang *et al.,* 2004a, b). Overexpression of *Fst* in the dental epithelium inhibits ameloblast differentiation on the labial aspect of the continuously growing mouse incisor, which is considered the crown domain, whereas *Fst* knockout results in ectopic ameloblast differentiation on the lingual enamel-free surface, which is considered the root domain (Wang *et al.,* 2004b; Juuri *et al.,* 2013). In molars of *Fst* knockout mice, aberrant folding of the dental epithelium and shallow cusp formation, accompanied by reduced cell proliferation, have been observed (Wang *et al.,* 2004a). These findings strongly suggest that Fst plays a crucial role in the crown-to-root transition and molar root morphogenesis. However, its precise function in root development remains unclear, as *Fst* knockout mice die shortly after birth due to severe defects in muscle, skeleton, whiskers, and the hard palate (Matzuk *et al.,* 1995). To overcome this limitation, we investigated the role of Fst in tooth root development by employing *K14-Cre*-mediated tissue-specific inactivation of *Fst* in the dental epithelium.

Results

Temporospatial expression of Fst during tooth development

Fst exhibits dynamic spatiotemporal expression in tooth germs during embryonic development. At the bud stage, its expression is observed in the lingual epithelium (Fig. 1A). During

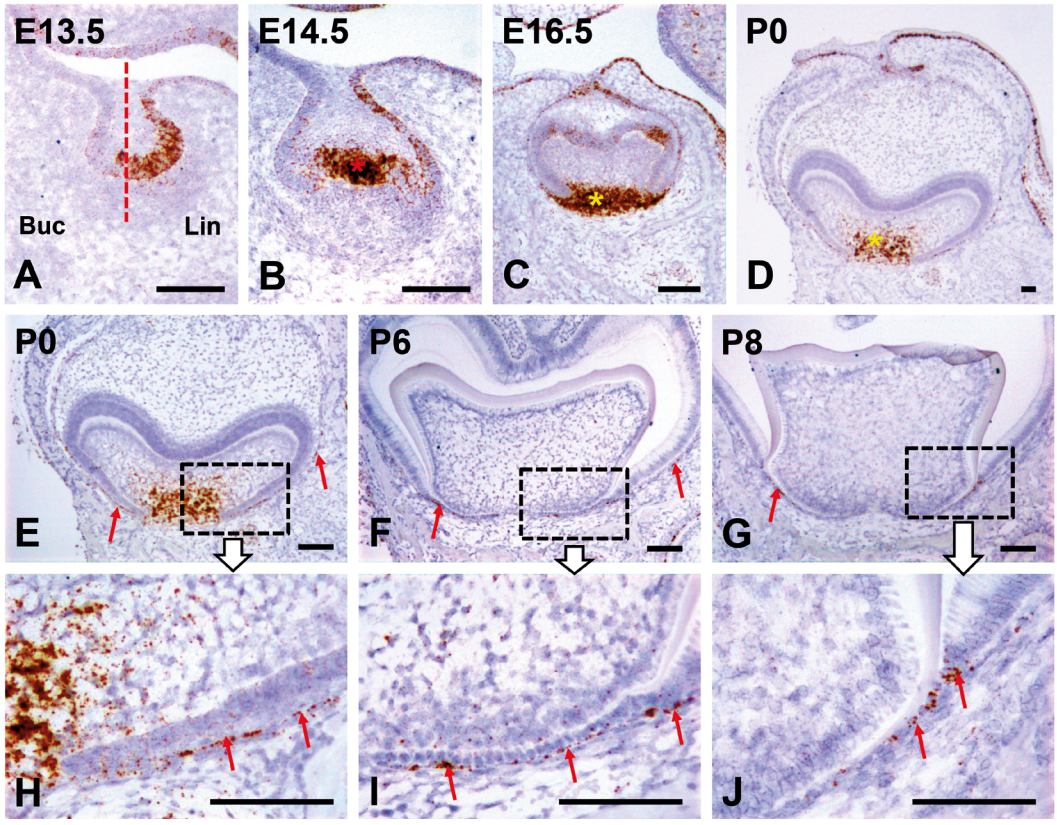


Fig. 1. Spatiotemporal expression of *Fst* during mouse tooth development. (A-J) *In situ* hybridization of *Fst* with coronal sections of mouse mandibular molars at Embryonic day (E) 13.5 (E13.5), E14.5, E16.5, postnatal day (P) 0 (P0), P6, and P8. Dotted boxes in panels E, F, and G are magnified in panels H, I, and J, respectively. A red dotted line marks the central line for cleavage along the bucco-lingual axis of the tooth germ. The red asterisk indicates primary enamel knot with strong expression of *Fst*. Yellow asterisks indicate apical papilla with strong expression of *Fst*. *Fst* expression is detected in the apical papilla, outer enamel epithelium, and the outer layer of HERS in developing mandibular molars at P0. H-J represent higher magnification insets of the boxed areas in E-G respectively. Red arrows indicate *Fst* expression in the outer enamel epithelium (E, H) and the outer layer of HERS (F, G, I, J). Buc, buccal; Lin, lingual. Scale bar: 100 μm (A-J).

the cap stage, *Fst* expression is predominantly detected in the inner enamel epithelium (IEE) on the lingual side and the primary enamel knot (Fig. 1B). By the early bell stage, its expression is clearly observed in the lingual IEE, the secondary enamel knot, and specific regions of the outer enamel epithelium (OEE), as well as in the dental mesenchyme at the base of the dental papilla (Fig. 1C). In the early postnatal period, *Fst* expression persists in the dental mesenchyme between the cervical loops and the outer enamel epithelium (Fig. 1D, E, H). Notably, during root formation, its expression becomes restricted to the outer layer of HERS (Fig. 1F, G, I, J).

Ablation of *Fst* in the dental epithelium disturbs tooth root morphogenesis

Micro-CT analysis revealed that *K14-Cre;Fst* cKO mutant mice exhibited molar root abnormalities, including enlarged pulp chambers and a partial absence of furcation on the lingual side of the mandibular molar roots (Fig. 2A). To further delineate the tissue-specific roles of *Fst*, we also generated a dental mesenchyme-specific *Fst* knockout using the *Wnt1-Cre* system. However, in contrast to the epithelial deletion, no apparent tooth abnormalities, including defects in root morphology, were observed in *Wnt1-Cre;Fst* cKO mice (Appendix Figure).

To determine the developmental onset of these defects, we focused our histological analysis on the early postnatal stages, particularly postnatal day 6 (P6) and P8. Because the crown-to-root transition in the furcation region exhibits a distinct temporal offset compared to the elongation region, P6 represents the peak dynamic stage when HERS actively undergoes horizontal inward extension to establish the epithelial diaphragm (He *et al.,* 2021; Jeong *et al.,* 2025). Histological analysis showed that in the elongation region, HERS extended asymmetrically, initiating on the buccal side first, followed by the lingual side in both control and mutant mice (Fig. 2B, C, F, G, J, K). However, in control mice, HERS extended symmetrically from both the buccal and lingual sides, successfully forming the root furcation (Fig. 1D, H, L). In contrast, in mutant mice, HERS formation was delayed on the lingual side of the furcation region, ultimately leading to failed root furcation formation (Fig. 2E, I, M).

During root formation, HERS extends symmetrically in the furcation region of control molars but asymmetrically in the elongation region (Fig. 3A, I, C, K). In contrast, mutant molars ex-

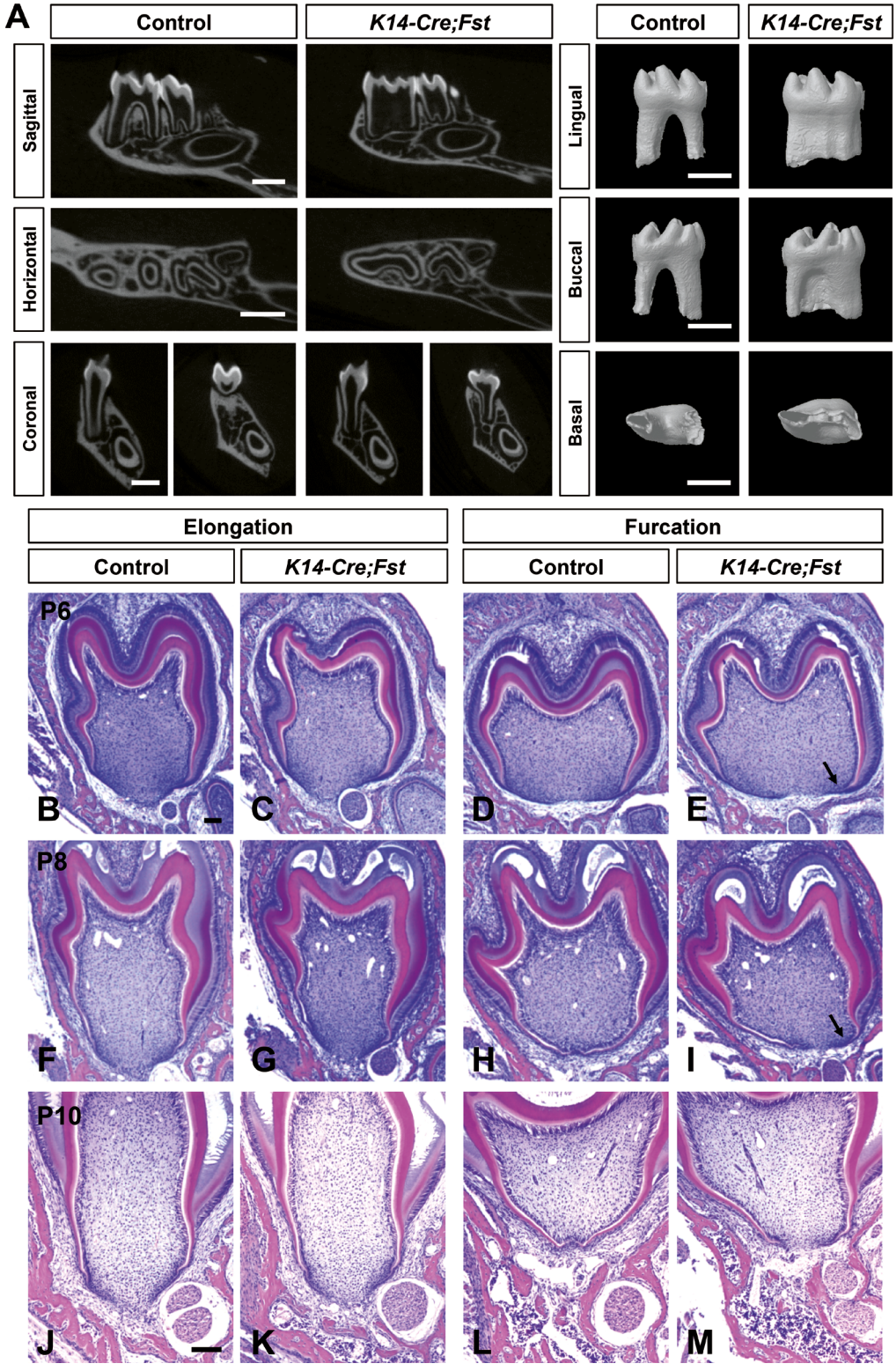


Fig. 2. Molar root abnormalities in *K14-Cre;Fst* mice. (A) Micro-CT images of mandibular molars from control and mutant mice at P28. The left images show segmented molars in sagittal, horizontal, and coronal sections. The right images show 3D reconstructed molars in lingual, buccal, and basal views. (B-M) H&E-stained histological sections of developing molars from control and mutant mice at P6, P8, and P10. Sections are presented to compare the furcation region with elongation region of the developing molar roots. The elongation region exhibits HERS asymmetry both in control and mutant mice (B, C, F, G, J, K). Control molars show symmetric HERS extension during furcation formation, while mutant molars exhibit delayed extension on the lingual side (arrows), resulting in impaired furcation at both P6 (E), P8 (I), and P10 (M). Scale bar: 1 mm (A) and 100 μm (B-M).

hibit asymmetric HERS extension in both the elongation and furcation regions (Fig. 3B, J, D, L). During asymmetric HERS extension in the elongation region of both control and mutant molars, adjacent mesenchymal cells proliferate and differentiate, contributing to root elongation (Fig. 3A-H, Q). In control molars, both buccal and lingual HERS extend, converges, and facilitate mesenchymal cell proliferation and differentiation, leading to furcation formation (Fig. 3I, K, M, O). In contrast, in mutant molars, while the buccal HERS extends similarly to controls, the lingual HERS extends more slowly and remains shorter in the furcation region (Fig. 3J, L, N, P, R, S). Consequently, furcation formation is impaired, resulting in a structure resembling the elongation region rather than a distinct furcation.

Fst regulates asymmetric HERS extension and epithelial proliferation

To investigate the mechanism underlying the shift from symmetric to asymmetric HERS extension in the furcation region, we analyzed *Notch1* expression, a marker for the stratum intermedium (Harada *et al.,* 2006; Mitsiadis *et al.,* 2023) and proliferating dental epithelial cells, in P4 tooth germs, corresponding to the crown-to-root transition (Fig. 4A-D). In the elongation region, both control and mutant mice exhibited asymmetric *Notch1* expression: termination occurred midway in the buccal epithelium but extended to the cervical loop on the lingual side. In the furcation region, control mice displayed symmetric *Notch1* termination in the middle of the dental epithelium, whereas in mutants, *Notch1* termination on the lingual side shifted to the cervical loop (Fig. 4E-H). *Notch1* expression correlated with epithelial proliferation; in the elongation region, proliferation was high in the terminal lingual epithelium and low on the buccal side in both control and mutant mice. However, in the furcation region, control mice exhibited low proliferation bilaterally, while mutant mice displayed high proliferation on the lingual side but low on the buccal side (Fig. 4I-L). These findings suggest that Fst regulates the asymmetric crown-to-root transition by modulating epithelial proliferation, particularly on the lingual side during furcation formation.

Fst regulates molar root morphogenesis via Shh and Wnt signaling modulation

Next, we analyzed molecular changes in the developing furcation region to assess the impact of *Fst* deletion on furcation development at P6. In our *K14-Cre;Fst* cKO model, this stage precisely captures the onset of morphological divergence, specifically, the delayed lingual HERS extension, making it the optimal temporal window to investigate the primary molecular misregulations. In control mice, *Shh* was expressed in cervical ameloblasts and HERS on both the lingual and buccal sides. However, in mutant mice, *Shh* expression was upregulated in cervical ameloblasts and the lingual side of HERS (Fig. 5A, B). Notably, *Gli1* expression was upregulated in the lingual side of HERS in mutant mice (Fig. 5C, D). In contrast, *Gli3* expression was slightly downregulated in the dental epithelium, including HERS, in mutants (Fig. 5E, F). In control mice, *Cdkn1a* was highly expressed in cervical ameloblasts and weakly in HERS, whereas in mutant mice, its expression was strongly downregulated on the lingual side of HERS (Fig. 5G, H). In control mice, *Wnt4* was highly expressed in HERS on both the lingual and buccal sides. However, in mutant mice, *Wnt4* expression was downregulated on both sides, with a more pronounced reduction on the lingual side of HERS (Fig. 5I, J). *Lhx6*, a gene associated with furcation formation, was expressed in the dental mesenchyme along HERS in control mice but was significantly reduced on the lingual side of HERS in mutants (Fig. 5K, L). Additionally, *Notum*, a Wnt inhibitor, was strongly expressed around HERS on both sides in control mice, whereas its expression was markedly restricted in mutant mice (Fig. 5M, N). In control mice, *Smoc2*, a stem cell marker, was absent from the furcation region. However, in mutant mice, *Smoc2* expression emerged in the dental mesenchyme near the tip of the lingual side of HERS (Fig. 5O, P). Taken together, these findings indicate that Fst asymmetrically regulates HERS cell proliferation by modulating Shh and Wnt signaling during furcation formation.

Ablation of *Fst* induces bucco-lingual asymmetry during early crown morphogenesis

Previous studies have demonstrated a correlation between root patterning and crown morphogenesis (Seo *et al.,* 2017; Adasooriya *et al.,* 2024). To further investigate the relationship between root abnormalities and crown development, we analyzed cell proliferation and molecular changes in the developing crown of neonatal mouse molars. In control mice, Ki67-positive proliferating cells were primarily localized in the inner enamel epithelium, with minimal presence in the dental papilla. However, in mutant mice, Ki67-positive cells were more densely distributed in both the inner enamel epithelium and stratum intermedium. Notably, proliferating cells were also observed in the dental papilla beneath the lingual cusp in mutants (Fig. 6A, B). *Fst* expression was detected in the dental mesenchyme between the cervical loops and in the outer dental epithelium of control mice. In contrast, mutant mice exhibited weak *Fst* expression, which was restricted to the dental mesenchyme between the cervical loops (Fig. 6C, D). To evaluate whether *Fst* deletion altered its related signaling pathways, we examined *Inhba* (inhibin/activin bA) expression, but no signal was observed in the tooth germ of either control or mutant mice (Fig. 6E, F). Next, to clearly visualize the morphological shape of the developing cusps, we utilized *Shh* and *Notch1* as structural markers for the dental epithelium and stratum intermedium, respectively. Using these markers, we observed that in both control and mutant mice, *Shh* was strongly expressed in the dental epithelium, except at the tip of the cervical loops. However, in control mice, two distinct epithelial foldings corresponding to the lingual and buccal cusps were observed, whereas mutant mice exhibited an additional epithelial folding. Furthermore, while the buccal and lingual cusps were similar in height in control mice, the lingual cusp was slightly shorter in mutant mice, resulting in bucco-lingual asymmetry in cusp patterning (Fig. 6G, H). This morphological asymmetry was also evident in *Notch1* expression within the stratum intermedium (Fig. 6I, J). Additionally, *Cdkn1a* expression was inversely correlated with the distribution of Ki67-positive cells in both control and mutant mice (Fig. 6K, L). Furthermore, key odontoblast differentiation markers, including *Sp7*, *Dspp*, and *Dmp1*, exhibited symmetrical expression patterns in control mice but showed asymmetrical expression in mutant mice (Fig. 6M-R). Taken together, these findings suggest a potential link between asymmet-

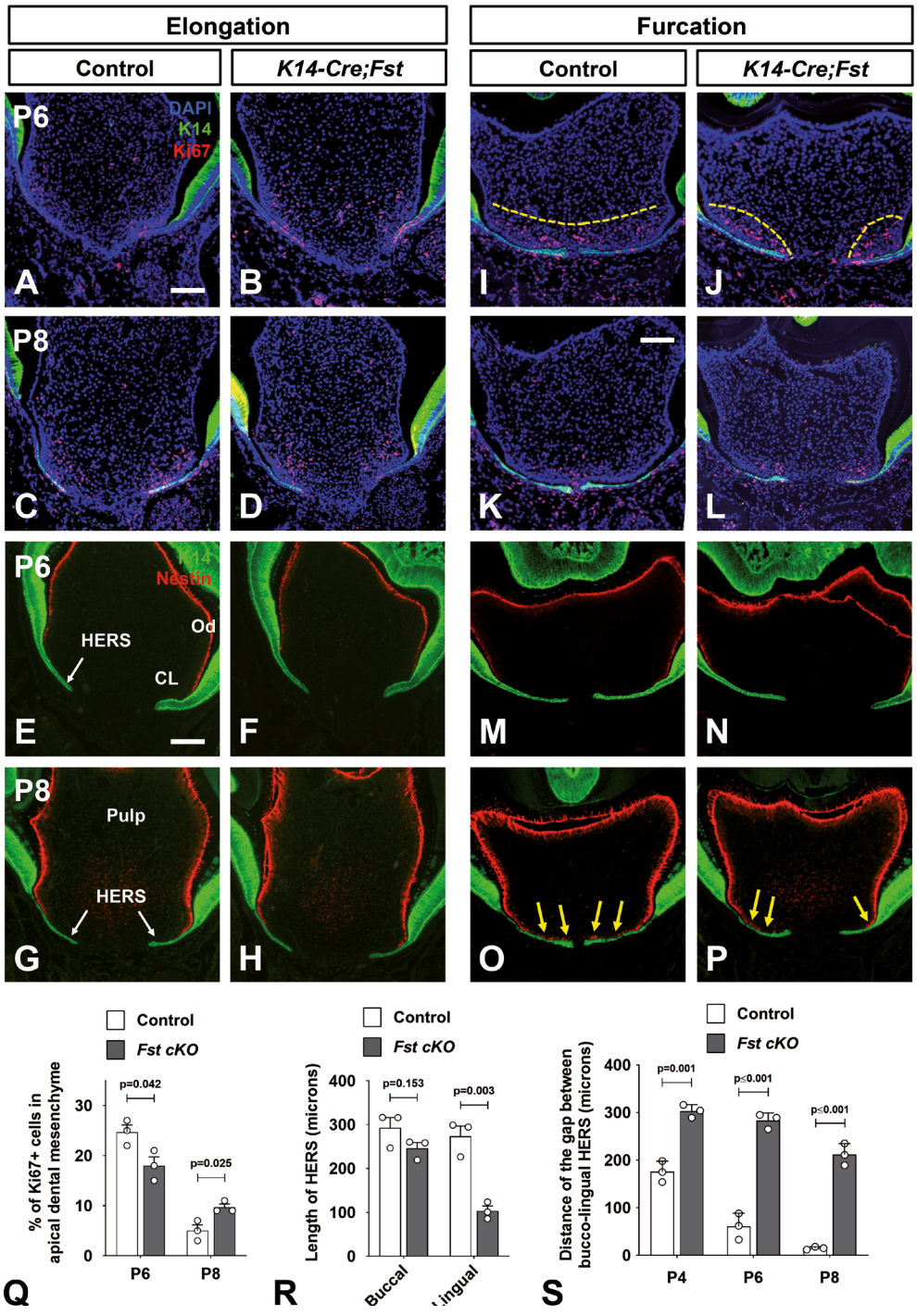


Fig. 3. Ablation of *Fst* in the dental epithelium disrupts symmetrical HERS extension and furcation formation. Sections are presented to compare the furcation region with elongation region of the developing molar roots. (A-L) Representative immunofluorescence images of developing first molars from control and mutant mice at P6 and P8, labeled with antibodies against cytokeratin 14 (K14, green) and Ki67 (red). (E-P) Representative immunofluorescence images of developing molars from control and mutant mice at P6 and P8, labeled with antibodies against K14 (green) and nestin (red). During root development, HERS extends asymmetrically in the elongation region of both control and mutant mice, promoting mesenchymal cell proliferation and differentiation. In the furcation region, HERS exhibits symmetric extension in control mice but remains asymmetric in mutants at P8. White and yellow arrows indicate HERS and differentiated odontoblasts along the extended HERS, respectively. CL, cervical loop; HERS, Hertwig's epithelial root sheath; Od, odontoblasts. (Q) Quantification of the percentage of Ki67-positive cells in the apical dental mesenchyme adjacent to HERS in the furcation region of control and mutant mice (n = 3). (R) Quantification of HERS length in the furcation region at P8 in the control and mutant mice (n = 3). (S) Comparison of the gap distance between bucco-lingual HERS in the furcation region of control and mutant mice (n = 3). Significance for the P-values was assigned as indicated. Scale bar: 100 µm (A-P).

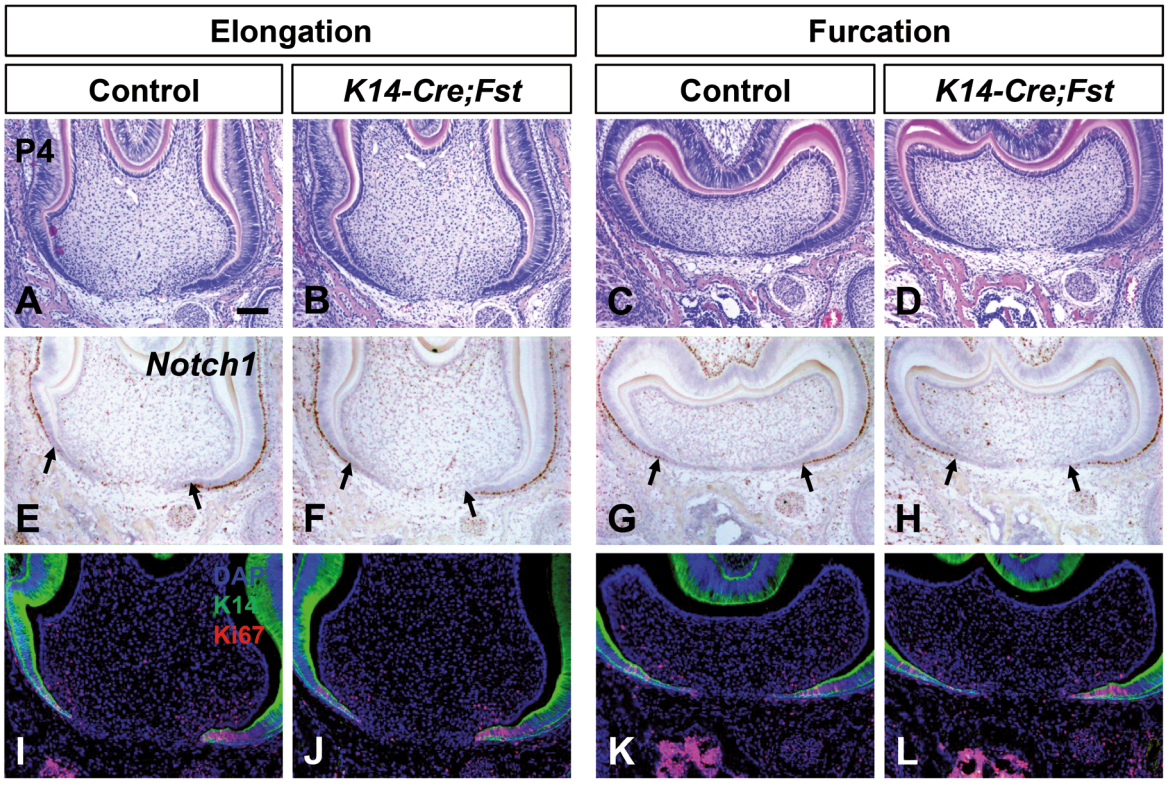


Fig. 4. Regulation of lingual HERS proliferation by Fst during root furcation formation. Sections are presented to compare the furcation region with the elongation region of developing molar roots at P4. (A-D) H&E-stained histological sections of developing molars from control and mutant mice. (E-H) *In situ* hybridization of *Notch1*, a marker for the stratum intermedium, in tissue sections of molars. Black arrows indicate the termination of *Notch1* expression at the tips of developing dental epithelium. (I-L) Immunofluorescence images of developing molars from control and mutant mice, labeled with antibodies against K14 (green) and Ki67 (red). Scale bar: 100 μm.

ric cusp patterns and subsequent asymmetric root furcation defects in *K14-Cre;Fst* cKO mice.

Discussion

Fst is a key regulator of cusp and enamel patterning, yet its role in molar root development remains poorly understood. In this study, we demonstrate the functional significance of *Fst* in molar root formation. Conditional ablation of *Fst* in the dental epithelium resulted in incomplete root furcation formation, characterized by an asymmetric crown-to-root transition and impaired HERS extension in the furcation region. These defects were associated with the regulation of Shh and Wnt signaling pathways, suggesting that Fst is essential for orchestrating molecular signals that drive proper root morphogenesis.

Fst regulates enamel patterning in mouse incisors by asymmetrically inhibiting bone morphogenetic protein signaling and ameloblast differentiation (Wang *et al.,* 2004b). In contrast, molar development follows distinct regulatory mechanisms governing crown morphogenesis, as demonstrated in *Fst* null mice (Wang *et al.,* 2004a). Our spatiotemporal analysis of *Fst* expression reveals both consistencies and notable differences compared to previous reports. Consistent with Wang *et al.* (2004a), we observed strong *Fst* expression in the primary and secondary enamel knots during the cap and bell stages. However, while they reported an absence of epithelial *Fst* expression at the newborn stage, our data demonstrate that *Fst* expression persists in the outer enamel epithelium during the early postnatal period. Furthermore, we precisely localized *Fst* in the lingual inner enamel epithelium during early embryonic stages. These discrepancies are likely attributable to the higher sensitivity of our detection method compared to previous studies, allowing us to capture low-abundance transcripts that might have been missed previously.

To determine whether Fst-mediated regulation similarly influences molar development, we generated a dental epithelium-specific deletion of *Fst* using the *K14-Cre* system, thereby circumventing the perinatal lethality associated with conventional *Fst* knockout mice. Interestingly, conditional inactivation of *Fst* in the dental epithelium led to significant abnormalities in molar root morphogenesis. Indeed, assessing the contribution of mesenchymal *Fst* using the *Wnt1-Cre* system revealed no apparent tooth abnormalities, including defects in root morphology, highlighting that the critical function of Fst in root furcation is epithelium-dependent.

Crown and root development exhibit distinct structural and regulatory characteristics, particularly within the dental epithelium (Ten Cate 1996). The crown epithelium comprises four

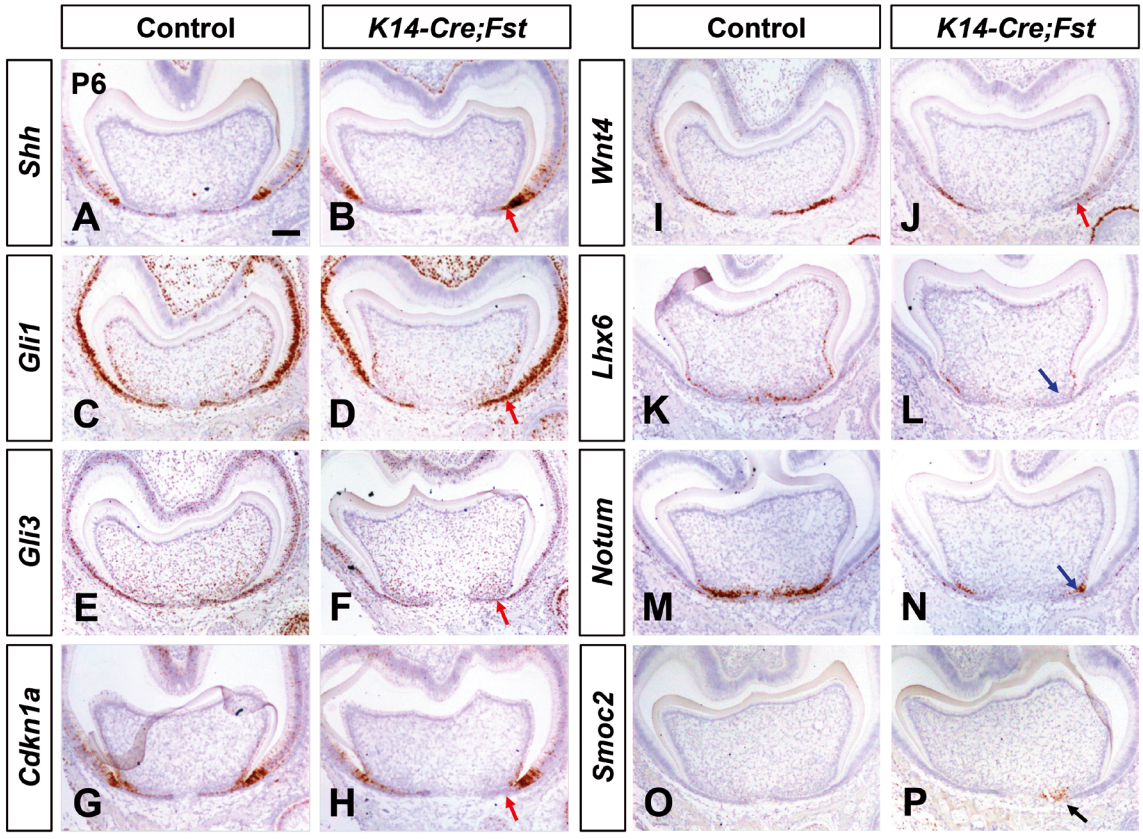


Fig. 5. Regulation of gene expression in lingual HERS by Fst during root furcation formation. (A-P) *In situ* hybridization was performed to compare gene expression at the furcation region of developing molar roots at P6. Red and blue arrows indicate altered expression of genes in the lingual dental epithelium (B, D, F, H, J) and mesenchyme (L, N), respectively. A black arrow indicates upregulated expression of *Smoc2* in the dental mesenchyme near the tip of the lingual side of HERS (P). Scale bar: 100 μm.

well-defined layers, whereas the root epithelium is composed of only two, reflecting fundamental differences in their developmental programs. Several animal models demonstrate root-specific anomalies without affecting crown morphology, suggesting that root development is more sensitive to genetic perturbations and governed by complex regulatory mechanisms (Kim *et al.,* 2013; Kim *et al.,* 2015; Yu *et al.,* 2020). These findings underscore the importance of studying root morphogenesis independently to better understand tissue-specific roles of key developmental regulators.

Root formation begins following the completion of crown morphogenesis. During early root development, the cervical loop epithelium extends apically to form HERS. Mesenchymal cells of the dental papilla then differentiate into pre-odontoblasts within HERS and subsequently into odontoblasts to produce root dentin (Thomas 1995). Molar root development is particularly complex, involving both root elongation and furcation to generate multiple roots. Sohn *et al.* (2014) reported that mesenchymal proliferation plays a critical role in determining both root elongation and furcation. In our study, epithelial-specific deletion of *Fst* resulted in sustained proliferation in the cervical loop and/or HERS, accompanied by upregulation of Shh signaling and downregulation of Wnt signaling (Fig. 6S). These findings suggest that the precise crown-to-root transition, mediated by the cervical loop and HERS, is essential for establishing proper root patterning.

Distinct genetic models have demonstrated specific defects in root development. For example, *β-catenin* deficiency results in rootless molars, indicating its essential role in root formation (Kim *et al.,* 2013). Conversely, mutations in genes associated with taurodontism-such as *Eda*, *Lhx6*, and *Wnt10a*-lead to reduced numbers of roots, implicating these genes in furcation development (Fons Romero *et al.,* 2017; Yu *et al.,* 2020; He *et al.,* 2021). Symmetric epithelial diaphragm formation is essential for proper furcation morphogenesis. To date, there have been few reports on asymmetric regulation during tooth development, aside from the well-known labial-lingual asymmetry in mouse incisors, where Fst regulates enamel patterning (Wang *et al.,* 2004b). Here, we introduce a novel developmental concept regarding bucco-lingual asymmetry in molar root patterning. As the inward-bending HERS apices must extend apically without converging, bucco-lingual asymmetry is necessary for proper root elongation. Conversely, successful furcation formation strictly requires symmetric HERS extension, enabling the apices to converge centrally. In this context, Fst plays a critical role in maintaining this required epithelial symmetry during furcation formation. In *K14-*

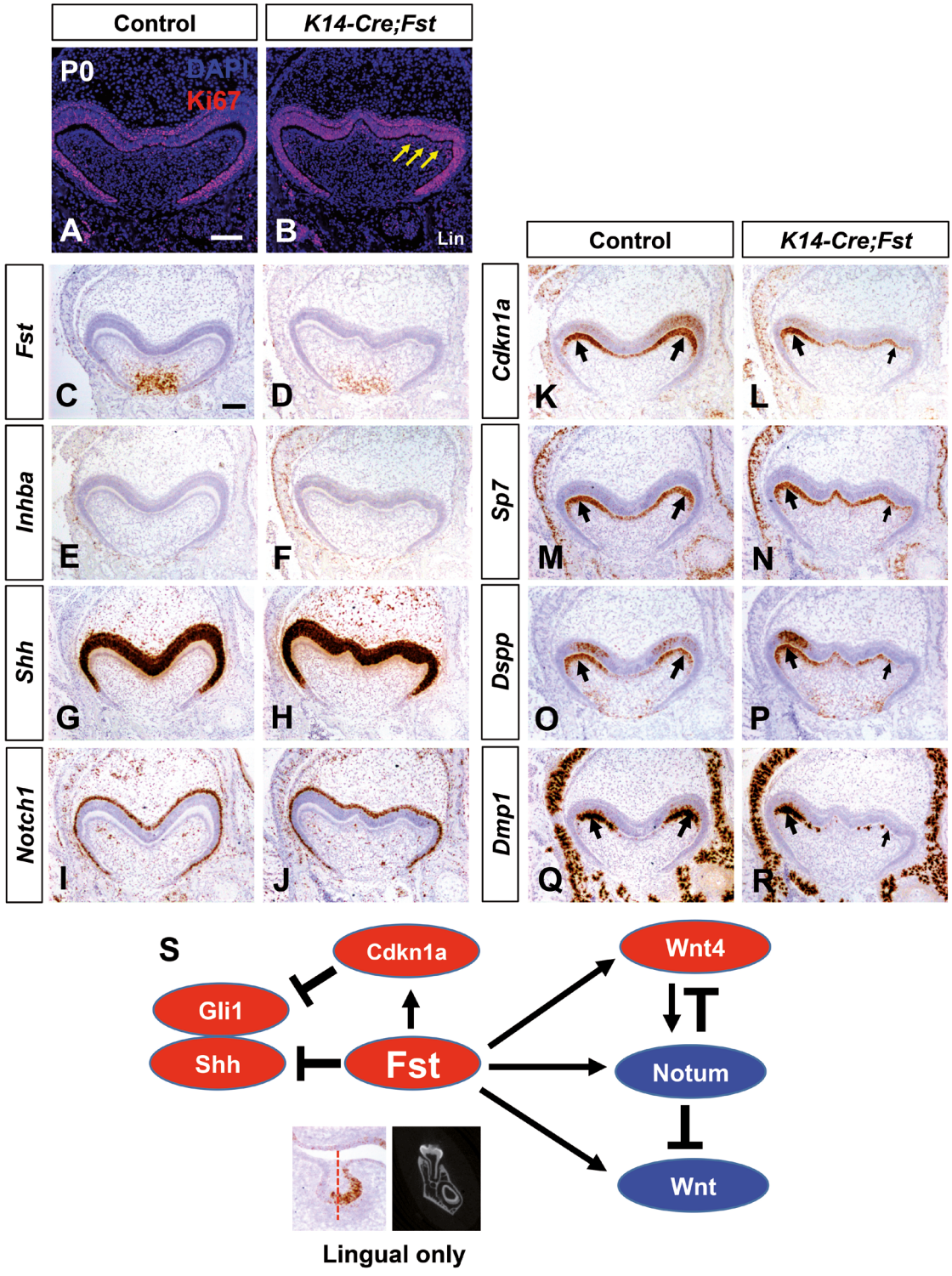


Fig. 6. Potential association between asymmetric crown morphogenesis and root furcation defects in *K14-Cre;Fst* mice. (A, B) Representative immunofluorescence images of Ki67 (red) for evaluating cell proliferation in developing molars at P0. Yellow arrows indicate increased proliferation in the dental papilla. (C-R) *In situ* hybridization of genes in tissue sections of mandibular molars at P0. Thick and thin black arrows represent high and low expression levels of each gene, respectively. (S) Schematic illustration of the molecular regulation of Fst in molar root morphogenesis via Shh and Wnt signaling pathways. Fst modulates key signaling proteins, including Shh, Gli1, and Cdkn1a, and regulates the expression of Wnt4 and Notum. These interactions coordinate the symmetric and asymmetric extension of HERS, essential for proper furcation formation. Loss of *Fst* disrupts epithelial proliferation and signaling, particularly impairing root furcation on the lingual side, underscoring the critical role of Fst in root patterning. Red and blue circles indicate the expression of each molecule in the epithelium and mesenchyme, respectively. Scale bar: 100 μm.

Cre;Fst cKO mice, the loss of epithelial *Fst* results in altered inner enamel epithelium folding, particularly affecting the lingual side of the tooth germ, and leads to asymmetric furcation formation. Consequently, the furcation region inappropriately retains the bucco-lingual asymmetry characteristic of the elongation region, leading to an abnormal and incomplete furcation formation. *Fst* is predominantly expressed in the lingual epithelium of the developing tooth. Its absence disrupts the balance of epithelial growth, contributing to morphological asymmetry during root development. These findings suggest that lingual *Fst* expression is crucial for establishing epithelial symmetry, which in turn ensures proper furcation formation. As previously established, Fst regulates cusp patterning during crown morphogenesis (Wang *et al.,* 2004a). Consistent with previous studies demonstrating that lateral growth and cusp patterning directly dictate root furcation patterns (Seo *et al.,* 2017), tooth root development is a sequential process highly dependent on the morphological and molecular state of the preceding crown stage. Collectively, these findings suggest that asymmetric crown morphogenesis may influence asymmetric root development, particularly in the furcation region rather than during root elongation. Further investigation is required to elucidate the molecular mechanisms underlying asymmetric crown morphogenesis in *K14-Cre;Fst* cKO mice.

Taken together, our results indicate that Fst regulates tooth root morphogenesis by controlling epithelial proliferation and HERS extension on the lingual side during furcation formation. We acknowledge that delayed lingual HERS extension may not be the sole cause of defective root furcation, given that other mesenchymal factors or complex morphogenetic events are likely involved. Nevertheless, our transgenic model demonstrates that it represents at least one highly possible and critical contributing mechanism. These findings highlight a critical role for Fst in both root furcation and overall root patterning and provide new insights into the molecular mechanisms governing the shape and number of molar roots.

Materials and Methods

Mouse strains and X-gal staining

All experimental procedures were approved by the Animal Welfare Committee of Jeonbuk National University (NON2019-112). This study is compliant with the ARRIVE guidelines (Animal Research Reporting of In Vivo Experiments). The *Fst* floxed allele (Fst^{fl}) and *K14-Cre* mice have been previously described (Dassule *et al.,* 2000; Jorgez *et al.,* 2004). *K14-Cre* and *R26R* reporter mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). To generate *K14-Cre;*$Fst^{fl/fl}$ (hereafter referred to as mutant) mice, *K14-Cre;*$Fst^{fl/+}$(hereafter referred to as control) mice were crossed with $Fst^{fl/fl}$ mice. Mouse offspring were genotyped by polymerase chain reaction analysis using previously described primers (Soriano 1999; Dassule *et al.,* 2000; Jorgez *et al.,* 2004). To analyze the level of Cre activity, *K14-Cre* mice were crossed with *R26R* reporter mice and the mandibles of the double transgenic mice were processed for *X-gal* staining as described previously (Kim *et al.,* 2013).

Micro-computed tomography

Mandibles were dissected from 4-wk-old control or mutant mice, bisected at the symphysis, and fixed in 4% PFA. The mandibles were scanned by means of a desktop scanner (1076 Skyscan Micro-CT, Skyscan, Kontich, Belgium). They were subsequently reconstructed and analyzed with CTAn software (Skyscan).

Tissue preparation and histology

For histological analysis, mice were sacrificed and the mandibles were carefully dissected. The tissues were fixed in 4% paraformaldehyde (PFA) and decalcified in 10% EDTA solution for 2 to 4 wk at 4 °C. The decalcified tissues were dehydrated through a graded ethanol series, embedded in paraffin, and sectioned at 5-μm thickness. Slides were stained with hematoxylin and eosin (H&E).

In situ hybridization

In situ hybridization was performed with the RNAscope® 2.5 High-Definition Assay-Brown (Advanced Cell Diagnostics, Newark, CA, USA) according to the user manual 322452 (FFPE sample preparation and pretreatment) and 322310 (RNAscope® 2.5 High-Definition Detection Reagent-Brown user manual) provided by the manufacturer.-The following RNA probes were used for *In situ* hybridization: Mm-Fst (454331, targeting NM_001301373.1, nucleotide 497-1469), Mm-Dspp (448301, targeting NM_010080.2, nucleotide 355-1420), Mm-Ambn (508241, targeting NM_001303431.1, nucleotide 186-1086), Mm-Shh (314361, targeting NM_009170.3, nucleotide 307-1197), Mm-Cdkn1a (408551, targeting NM_007669.4, nucleotide 199-1240), Mm-Wnt4 (401101, targeting NM_009523.2, nucleotide 2147-3150), Mm-Notum (428981, targeting NM_175263.4, nucleotide 406-1623), Mm-Lhx6 (422791, targeting NM_001083127.1, nucleotide 753-1991), Mm-Sfrp2 (400381, targeting NM_009144.2, nucleotide 709-1703), Mm-Dmp1 (441171, targeting NM_016779.2, nucleotide 689-1543), Mm-Sp7 (403401, targeting NM_130458.3, nucleotide 837-2230),Mm-Smoc2 (318541, targeting NM_022315.2, nucleotide 292-1231), Mm-Gli1 (311001, targeting NM_010296.2, nucleotide 25-1205), Mm-Gli3 (445511, targeting NM_008130.2, nucleotide 716-1711), Mm-Inhba (455871, targeting NM_008380.1, nucleotide 445-1390), Mm-Notch1 (404641, targeting NM_008714.3, nucleotide 1153-1960).

Measurement of cell proliferation, HERS length, bucco-lingual distance, and statistical analysis

To assess the proliferation of apical dental mesenchyme, the percentage of Ki67-positive cells adjacent to HERS at the furcation region was quantified in control and mutant mice (n = 3). Quantification was performed within the apical one-third of the dental pulp using immunofluorescence staining for Ki67. For morphological analysis of HERS, measurements were conducted on H&E-stained coronal sections. At P8, the length of HERS at the furcation region was defined as the linear distance from the crown-root transition point to the apical terminus, measured separately on both buccal and lingual sides in control and mutant mice (n = 3). In addition, the distance between the buccal and lingual HERS extensions was measured in coronal sections of the molars to assess the buc-

co-lingual gap at the furcation region in control and mutant mice (n = 3).

All data are presented as means ± SEM. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Statistical differences were determined by Student's t test, and values $P < 0.05$ were considered statistically significant.

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Conflict of interest

The authors declare no conflicts of interest.

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