Interactions between Shh, Sostdc1 and Wnt signaling and a new feedback loop for spatial patterning of the teeth

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SUMMARY
Each vertebrate species displays specific tooth patterns in each quadrant of the jaw: the mouse has one incisor and three molars, which develop at precise locations and at different times. The reason why multiple teeth form in the jaw of vertebrates and the way in which they develop separately from each other have been extensively studied, but the genetic mechanism governing the spatial patterning of teeth still remains to be elucidated. Sonic hedgehog (Shh) is one of the key signaling molecules involved in the spatial patterning of teeth and other ectodermal organs such as hair, vibrissae and feathers. Sostdc1, a secreted inhibitor of the Wnt and Bmp pathways, also regulates the spatial patterning of teeth and hair. Here, by utilizing maternal transfer of 5E1 (an anti-Shh antibody) to mouse embryos through the placenta, we show that Sostdc1 is downstream of Shh signaling and suggest a Wnt-Shh-Sostdc1 negative feedback loop as a pivotal mechanism controlling the spatial patterning of teeth.

Furthermore, we propose a new reaction-diffusion model in which Wnt, Shh and Sostdc1 act as the activator, mediator and inhibitor, respectively, and confirm that such interactions can generate the tooth pattern of a wild-type mouse and can explain the various tooth patterns produced experimentally.

KEY WORDS: Shh, Sostdc1, Wnt, Feedback loop, Tooth patterning, Mouse

INTRODUCTION
Ectodermal organs such as teeth, hair, vibrissae and feathers share common morphological features and spatial patterning mechanisms, in which Shh, Wnts and Sostdc1 are key signaling molecules (St-Jacques et al., 1998; Laurikkala et al., 2003; Närhi et al., 2008) but the relationships between these signals are not fully understood. Conditional Shh- and Smo-deficient mice such as K14-Cre;Smoflox/flox and K14-Cre;Shhflox/flox exhibit the same morphological aberrations in tooth patterns: the first (M1) and second (M2) molars are fused and the dental lamina is absent (Dassule et al., 2000; Grilli-Linde et al., 2002). Interestingly, M1-M2 fusion has also been observed in both Sostdc1–/– and Lrp4–/– mice, as have a few supernumerary molars and incisors (Kassai et al., 2005; Ohazama et al., 2008). It has been suggested that Sostdc1 is upstream of Shh and that the molars fusion in K14-Cre;Shhflox/flox, K14-Cre;Sostdc1–/– and Lrp4–/– mice results from reduction of Shh signals (Ohazama et al., 2008). However, a significant increase in Shh signaling was shown in tooth germs of Sostdc1–/– mice, compared with Sostdc1+/– mice (Ahn et al., 2010).

Sostdc1 (also known as USAG-1, ectodin and Wise) is an established secreted inhibitor of the Wnt and Bmp pathways (Laurikkala et al., 2003; Yanagita et al., 2004; Kassai et al., 2005; Ohazama et al., 2008; Munne et al., 2009), and Lrp4 is a negative Wnt co-receptor antagonizing the Lrp5- and Lrp6-mediated activation of Wnt signaling (Johnson et al., 2005). The fact that binding of Sostdc1 to Lrp4 inhibits the Wnt pathway could explain the identical tooth phenotype in Sostdc1+/– and Lrp4–/– mice (Ohazama et al., 2008).

Multiple supernumerary teeth develop in K14-Cre;Ctnnb1(Ex3)fl/+, and K14-Cre;APC(Ex5)fl/kl mice, which show sustained activity of β-catenin in the Wnt pathway in the epithelium (Kuraguchi et al., 2006; Järvinen et al., 2006; Liu et al., 2008). Furthermore, sustained epithelial Wnt/β-catenin signaling in the hair and teeth of K14-Cre;Ctnnb1(Ex3)fl/+, mice upregulates Shh, Dkk1 and Sostdc1, which suggests that Wnt/β-catenin signals are upstream of Shh (Närhi et al., 2008; Liu et al., 2008). Furthermore, deletion of Lef1 or Wnt10b severely diminishes the size and the number of fungiform papillae, and decreases expression of Shh in tongue fungiform papillae (Iwatsuki et al., 2007). It has also been suggested that epithelial Fgf4, dependent on Wnt signaling, targets Fgf3 in dental mesenchyme, which, in turn, induces epithelial Shh expression together with other mesenchymal signals (Kratochwil et al., 2002). By contrast, it has been shown that Shh suppresses Wnt10b in early developing mandibles (Dassule and McMahon, 1998), and that inhibition of Shh signaling by 5E1 (an IgG1 monoclonal antibody against Shh protein) increases the expression of Wnt/β-catenin signaling in fungiform papillae (Iwatsuki et al., 2007). Furthermore, Wnt signaling in tooth germ of Sostdc1+/–;Shh+/– mice is significantly elevated compared with that in Sostdc1+/–; mice (Ahn et al., 2010). All these data taken together suggest that a Wnt-Shh feedback loop involving Sostdc1 and/or other signaling molecules might be involved in patterning the developing teeth.
Previously, it was reported that treatment of pregnant tabby mice with an antibody-like recombinant form of EDA1 permanently rescues the Tabby phenotype in offspring (Gaide and Schneider, 2003). Here, by utilizing maternal transfer of 5E1 through the placenta to block Shh signaling (Wang, L. C. et al., 2000), we investigated the changes in tooth patterning and in gene expression to explore whether a Wnt-Shh negative feedback loop mediates tooth patterning and how Sostdc1 is involved.

Spatial patterning of teeth, characterized by the size and number both of teeth and of their cusps, has been described using a reaction-diffusion mechanism (Jernvall and Thesleff, 2000; Cai et al., 2007), which is based on two main principles (Turing, 1952; Gierer and Meinhardt, 1972; Crampin et al., 2002): (1) the activator promotes its own production and that of an inhibitor, which in turn inhibits activator production; and (2) the inhibitor diffuses faster than the activator. Successful computer models of tooth development already exist (Salazar-Ciudad and Jernvall, 2002; Järvinen et al., 2006; Salazar-Ciudad and Jernvall, 2010). In the most recent mathematical model for tooth patterning, Wnt family genes were suggested as candidates for the activator, and Shh and Sostdc1 as candidates for inhibitors (Salazar-Ciudad and Jernvall, 2010). However, owing to the high spatial resolution for single teeth produced by these models, they are computationally unable to accommodate splitting and development of multiple structures (Järvinen et al., 2006). Here, we produced a new reaction-diffusion mechanism for spatial patterning of the teeth with the aim of hypothesizing how the sequence of tooth primordia positions could be set by a system of chemicals.

MATERIALS AND METHODS

Drug delivery
A monoclonal antibody (mAb) 5E1 (an IgG1 monoclonal antibody against Shh protein) and a control mAb 40-1a (an IgG1 monoclonal antibody against β-galactosidase) were obtained from hybridoma cells at the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA, USA). Cyclopamine was purchased from Toronto Research Chemicals (North York, ON, Canada). A single injection of 5E1 (10 mg/kg body weight), 40-1a (10 mg/kg body weight), cyclopamine (10 mg/kg body weight) or PBS (1 ml) was administered intraperitoneally to pregnant ICR mice at embryonic day (E)10, E12, E14 or E16. A double injection of 5E1 or PBS was administered intraperitoneally to pregnant ICR mice at E14 and E17. All newborn mice were allowed to survive for four weeks, after which they were killed for the analysis of tooth and cusp patterns. Alternatively, cultured embryonic tooth germ explants were treated with mAb 5E1 (130 μg/ml), mAb 40-1a (130 μg/ml), cyclopamine (10 nM) or PBS (100 μl/ml) in solution with Dulbecco’s Modified Eagle’s Medium (DMEM) including 10% foetal bovine serum (FBS). Tooth explants were cultured in vitro for one, two or three days.

Transplantation into kidney
Tooth germs at E12-16 were cultured in medium containing either 5E1 or PBS for two days in vitro and then transplanted into the subcapsular layer of nude mouse kidneys for tooth calcification. All surgical procedures were performed under intraperitoneally administered anaesthesia. No immunosuppressive medication was used. After five weeks the host mice were killed and the kidneys were dissected to obtain the calcified teeth.

Three-dimensional reconstructions
For three-dimensional reconstructions, images of frontal serial sections of the developing molars stained with Haematoxylin and Eosin were imported into the ‘Reconstruct’ software developed by J. C. Fiala and K. M. Harris at Boston University (MA, USA). Images were aligned manually and the shape of the epithelium was traced manually along the basement membrane. Every third image was employed in the reconstruction and the actual reconstructed thickness was 21 μm. Three-dimensional reconstructed computed tomography images were obtained by scanning the calcified teeth using micro-computed tomography (Micro-CT, Skyscan 1076, Skyscan, Antwerp, Belgium). The data were then digitalized using a frame grabber and the resulting images were transmitted to a computer with topographic reconstruction software.

Microarray analysis
Gene-chip expression analysis was performed with RNA from mandibular tooth germs from embryos of pregnant mice at one day after injection (PBS, n=2; 40-1a, n=2; cyclopamine, n=2; 5E1, n=2), using a mouse gene microarray (GeneChip Mouse Genome 430 2.0, Affymetrix, Santa Clara, CA, USA). A gene-chip scanner (GeneChip Scanner 3000, Affymetrix) was used to measure the intensity of the fluorescence emitted by the labeled target. Raw image data were converted to cell-intensity (CEL) files using the Affymetrix GeneChip Operating System, and these CEL files were normalized using the MARS 5.0 algorithm. Following statistical analysis, differentially expressed genes were selected using GenePlex software version 3.0 (ISTECH, Seoul, Korea). Differentially expressed genes with changes of at least 1.5-fold in the 5E1-treated group compared with the control group were selected, and then analyzed statistically using Student’s t-test with the level of statistical significance set at P<0.01. Microarray data have been deposited in GEO with accession number GSE27429.

Quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) analysis
RNA was extracted from mandibular tooth germs from embryos at one day after injection (PBS, n=2; 40-1a, n=2; cyclopamine, n=2; 5E1, n=2). RT-qPCR was performed using a Thermal Cycler Dice Real-Time System and SYBR Premix EX Taq (Takara, Japan) according to the manufacturer’s instructions. For RT-qPCR, the reaction mixture was initially incubated for one minute at 95°C. The amplification program comprised 40 cycles of denaturation at 95°C for 5 seconds, annealing at 55-60°C for 10 seconds, and extension at 72°C for 10 seconds. The RT-qPCR for each sample was performed in triplicate and the amount of each of the RT-qPCR products was normalized using β-2-microglobulin as an internal control. The data were analyzed with the Thermal Cycler Dice Real-Time System analysis software and the 2–ΔΔCt method. The statistical calculations were performed using t-test of variables to determine significant changes at the 95% confidence level (P<0.05).

Protein-bead implantation
Affi-Gel blue beads (Bio-Rad Laboratories, Hercules, CA, USA) were soaked with the Shh recombinant protein (1 μg/μl; mouse Shh-N, R&D Systems, Minneapolis, MN, USA). Control beads were prepared similarly by soaking them in PBS at room temperature for at least 1 hour. The mandibular M1 tooth germs of wild-type mice at E14 were dissected and incubated in Dispase II (Roche, Mannheim, Germany) at 1.2 U/ml in PBS for 20 minutes, and the dental epithelium and mesenchyme were separated. Beads were placed on dental epithelium, mesenchyme or the lingual side of intact tooth germs, which was then cultured for 1 day in DME/M including 10% FBS. Whole-mount in-situ hybridization was then carried out.

In situ hybridization
Tissues were fixed overnight in 4% paraformaldehyde. Hybridizations were performed on these tooth gmerge with digoxigenin-labeled cRNA probes in hybridization buffer for 18 hours at 72°C. Hybridization signals were detected by alkaline-phosphatase-conjugated anti-digoxigenin antibodies plus nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt substrate (Roche, Mannheim, Germany).

Mathematical simulation
In order to model spatial pattern formation for teeth in wild-type mice, a system of reaction-diffusion equations, modified from the Gierer-Meinhardt system (Gierer and Meinhardt 1972) to allow the inclusion of a mediator species, was simulated. The equations simulated are

\[
\frac{\partial A}{\partial t} = D_A \nabla^2 A + \frac{A}{1+M} - \beta A + \alpha,
\]

\[
\frac{\partial M}{\partial t} = D_M \nabla^2 M + \mu \left( A^2 - M \right),
\]

\[
\frac{\partial I}{\partial t} = D_I \nabla^2 I + D \left( M - A I \right).
\]
RESULTS

Molar fusion and supernumerary tooth formation are induced by blocking Shh activity in vivo

Mice delivered from pregnant mice injected with 5E1 between E10 and E18 exhibited normal or abnormal spatial patterns of molars, and reduced body and skull size (Fig. 1; Table 1), whereas all mice delivered from pregnant mice injected with cyclopamine (a specific Smo antagonist), phosphate-buffered saline (PBS) or a control antibody of 40-1a (an IgG1 monoclonal antibody against β-galactosidase), were normal in molar patterning and body size. No changes were detected in the injected pregnant mice. The molar patterns observed in both the maxilla and the mandible following embryonic exposure to 5E1 can be divided into the following five types: type-I, wild-type-like (having three molars); type-II, M1-M2 fusion; type-III, M1-M2 fusion with extra lingual cusps; type-IV, M2-third molar (M3) fusion; type-V, M1-M2-M3 fusion (type-I-V in Fig. 1A-N).

All tooth germs are at the primary epithelial band stage at E10. Whereas M1 is at the dental lamina stage, cap stage and bell stage at E12, E14 and E16, respectively, M2 is at the dental lamina stage, cap stage and bell stage at E14, E16 and E18, respectively. A number of mice that were exposed to 5E1 at E10 (E10-5E1) or E12 (E12-5E1) exhibited M1-M2 fusion in the maxillary and/or mandibular quadrants, but M1-M2 fusion was most frequent in 5E1-exposed mice at E14 (E14-5E1) (48/78 in the maxilla and 53/82 in the mandible; Table 1, Fig. 1C-F,K-N; Fig. 2H-K). Mice exposed to 5E1 at E16 (E16-5E1) exhibited no M1-M2 fusion but did exhibit M2-M3 fusion, which was evident in both the maxilla and mandible (Fig. 1G,H). Mice exposed to 5E1 at E18 (E18-5E1) exhibited no fused molars. These findings demonstrate that the pivotal developmental times to enhance M1-M2 fusion and M2-M3 fusion are from E14 to E15 and from E16 to E17, respectively. The finding that 5E1 injection could not induce the M1-M2 fusion at E16 and the M2-M3 fusion at E18 indicates that molars at the bell stage have been already separated from other tooth germs. When 5E1 was injected twice into pregnant mice so that embryos would be exposed at stage E14 and then at stage E17, M1-M2-M3 fusion occurred in both the maxilla and mandible (Fig. 1I,J). In addition, extra incisors or molars, which have been reported in Sostdc1−/− and Lrp4−/− mice, were found in E12-5E1, E14-5E1 and E16-5E1 mice, but not in the PBS-treated E14 mice (E14-PBS) (Fig. 2A-E).
In order to investigate how these tooth fusions arise following 5E1 treatment, sections were taken through the developing teeth of embryos at two days after 5E1 injection at E14. Frontal sections showed that the lingual epithelial bud, which is known to be a rudiment of a secondary tooth (Khajoejbut et al., 1991), and dental lamina were present in E14-PBS (Fig. 1P) but absent in E14-5E1 after two days (Fig. 1S). This result is consistent with findings in K14-Cre;Shhfllox/lox and K14-Cre;Smo+/- mice. Secondly, M1-M2 fusion was evident in sagittal sections of the mandible in E14-5E1 at two days after injection, whereas M1 was separated from M2 in E14-PBS (Fig. 1Q,T). In E14-PBS, after two days M2 could be seen to be clearly separated from M1 in three-dimensional reconstructed images of dental epithelium (red arrowheads in Fig. 1U), whereas the boundary between M1 and M2 was not clear in E14-5E1 (Fig. 1V). Moreover, the buccolingual diameter of M2 was larger in E14-5E1 than in E14-PBS after one day, which suggests that M2 development was accelerated by 5E1 treatment (Fig. 1U,V).

It should be noted that transparent enamel can be seen to cover the dentine in sagittal sections of fused molars in E14-5E1 mice (Fig. 2F,G), in contrast to the enamel defects reported in K14-Cre;Shhfllox/lox and K14-Cre;Smo+/- mice. Enamel formation occurring postnatally might not be affected by a single 5E1 injection at E14.

**Molar fusion is also induced by blocking of Shh activity in vitro**

A parallel series of experiments was carried out in which tooth germs at E14 were cultured for two days in a medium containing 5E1, cyclopamine, 40-1a and PBS, and then grafted under kidney capsules for five weeks to undergo calcification. In contrast to the in vivo results, M1-M2 fusion was induced by cyclopamine as well as 5E1 after two days of culture in vitro (see Fig. S1 in the supplementary material). This discrepancy is attributable to the fact that cyclopamine is no longer active in vivo a few hours after intraperitoneal injection (Lipinski et al., 2008) but is active in culture medium for two days. Consistent with in vivo 5E1 results, M1-M2 fusion was evident in the E14-5E1 calcified teeth developing in the kidney for five weeks, and M2-M3 fusion was evident in the E16-5E1 teeth (see Fig. S1 in the supplementary material), demonstrating again that the pivotal stages for producing M1-M2 fusion and M2-M3 fusion are E14 and E16, respectively.

**Shh activity is effectively blocked by maternal transfer of 5E1**

Many signaling pathways (e.g. those involving Bmp, Fgf, Wnt, Tnf and Shh) are involved in tooth development (Jernvall and Thesleff, 2000), and the enamel knot, a signaling center in dental epithelium, expresses many signaling molecules such as Bmp2, Bmp4, Bmp7, Fgf3, Fgf4, Fgf9, Fgf20, Wnt10a, Wnt10b and Shh (Thesleff, 2003). In order to ascertain whether Shh signaling in developing teeth is blocked by maternal transfer of 5E1, we investigated the transcriptional profiles of tooth germs 24 hours after injection at E14 focusing on genes known to be regulated by Shh signaling. In the microarray analysis, Gli1 and Ptch1, direct targets of hedgehog signaling (Wang, B. et al., 2000), were downregulated in E14-5E1 tooth germs more than fourfold compared with E14-40-1a, E14-PBS and E14-cyclopamine (Table 2), and Ptch1 and Gli1 were downregulated in E14-5E1 tooth germs compared with E14-PBS in the RT-qPCR analysis (Fig. 3). Furthermore, Hhip, another target of hedgehog, was also downregulated in E14-5E1 more than twofold compared with E14-40-1a and E14-PBS in microarray (see Table S1 in the supplementary material), and RT-qPCR confirmed this downregulation of Hhip in E14-5E1 (Fig. 3). Conversely, expression of Smo, Gli2 and Gli3, which are not activated in response to Shh signaling but are involved in Shh signal transcription (Wang, B. et al., 2000), was not changed by 5E1 in the microarray and RT-qPCR analyses (see Table S1 and Fig. S2 in the supplementary material). Shh expression level in the microarrays was not significantly changed but appeared to be increased at least 1.4-fold after 5E1 treatment (see Table S1 in the supplementary material).

These results suggest that 5E1 blocked Shh activity in developing teeth significantly and selectively, by binding not with Shh mRNA but with Shh protein. In addition, Ptch1 expression disappeared in the dental epithelium and mesenchyme following treatment with 5E1 (compare Fig. 4F,H with 4E,G; see Fig. S3 in the supplementary material). In contrast to the Ptch1 expression, the Shh expression pattern was the same in E14-PBS and E14-5E1 mice in vivo at one day after 5E1 injection (Fig. 4AB; see Fig. S3 in the supplementary material).
Sostdc1 is regulated by Shh during tooth development

Microarray and RT-qPCR analyses showed that blocking Shh signaling via 5E1 treatment downregulated Sostdc1 expression levels more than twofold (Fig. 3, Table 2). E14-PBS mice exhibited Sostdc1 expression in the dental epithelium and mesenchyme in vivo and in vitro (Fig. 4I,K; see Fig. S3 in the supplementary material), but Sostdc1 was weakly expressed in dental epithelium and absent in dental mesenchyme of E14-5E1 (Fig. 4J,L; see Fig. S3 in the supplementary material). Furthermore, exogenous Shh protein induced Sostdc1 expression in dental mesenchyme of wild-type mice at E14 (Fig. 5H) but not in dental epithelium (Fig. 5F). By contrast, ectopic Ptch1 expression was clearly induced around the Shh bead in both the epithelium and mesenchyme, indicating good efficiency of exogenously applied Shh protein (Fig. 5B,D). Interestingly, Ptch1 was widely expressed both in the PBS- and Shh-treated dental epithelium after one day in culture (Fig. 5A,B). Sostdc1 was not induced around either PBS or Shh beads which were placed on the wild-type tooth germs at E14 without separation of epithelium and mesenchyme (Fig. 5I-L).

Wnt and Fgf signaling in epithelium are upregulated after blocking of Shh activity

Expression levels of β-catenin and Lef1 were not changed after blocking Shh activity by 5E1 treatment at E14 according to the microarray analysis, and Lef1 expression pattern was not detectably

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**Table 1. Various spatial patterns of molars in mice after being exposed to 5E1**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Injection</th>
<th>Total number</th>
<th>Type-I (%)</th>
<th>Type-II (%)</th>
<th>Type-III (%)</th>
<th>Type-IV (%)</th>
<th>Type-V (%)</th>
<th>Total number</th>
<th>Type-I (%)</th>
<th>Type-II (%)</th>
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**Table 2. Genes with at least twofold difference in expression as determined by microarray analysis after 5E1 injection compared with after 40-1a, PBS or cyclopamine injection**

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<th>Gene name</th>
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<th>Fold change 5E1 versus cyclopamine</th>
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*Student’s t-test was employed for statistical analysis with level of statistical significance set at P<0.01.
changed as judged by in situ hybridization after in vitro culture (Fig. 4S,T). Nevertheless, Wnt signaling seems to be upregulated as judged by expression of Sp5, which was increased in the microarray and RT-qPCR analyses (Fig. 3; see Table S1 in the supplementary material). Sp5 is a direct transcriptional target of the Wnt/β-catenin pathway, and the promoter region of Sp5 contains multiple binding sites for Lef1 and Tcf family members (Weidinger et al., 2005; Zhang et al., 2008). In addition, Kr73, which is regulated by Sp5 in hair (Zhang et al., 2008), was increased in the microarray and RT-qPCR analyses following 5E1 treatment (Fig. 3, Table 2) and also seen to be expressed more widely by in situ hybridization after culture in vitro (Fig. 4U,V). Sp5 is expressed in dental epithelium of developing M1 at E12 and E14, and its expression is observed mainly in the epithelium of M2 at E16 (see Fig. S4 in the supplementary material). In our experiment, Sp5-expressing areas in M1 and M2 were separated in E14-PBS, but connected in E14-5E1 (Fig. 4M-P).

Although mesenchymal Fgf genes such as Fgf3 and Fgf10 did not display any significant change in gene expression levels, epithelial Fgf genes exhibited significant changes after 5E1 treatment. Fgf9 and Fgf20, expressed in enamel knot, showed significantly increased expression levels in E14-5E1 (Table 2; see Table S1 in the supplementary material) in the microarray analysis, and Fgf4 expression was increased in the RT-qPCR analysis (Fig. 3). An Fgf4-expressing enamel knot appeared in M2 after one day in vitro in E14-5E1 tooth germs but not in E14-PBS (Fig. 4W,X), which indicates accelerated M2 development.

Sostdc1 has also been implicated as a Bmp antagonist. Bmp4 expression was downregulated >1.5-fold after 5E1 treatment in the microarray and RT-qPCR analyses (see Table S1 and Fig. S2 in the supplementary material), but other signals in the Bmp pathway such as Bmp2, Bmp5, Bmp7, Bmp1a, Msx1 and Msx2 exhibited minor changes in expression level after 5E1 treatment in the microarray analysis (see Table S1 in the supplementary material). Expression levels of other known Bmp antagonists such as Grem1 and Nog were not changed in either the microarray or RT-qPCR analyses (see Table S1 and Fig. S2 in the supplementary material).

M2 development is accelerated by blocking of Shh activity

The buccolingual diameter of M2 was larger in E14-5E1 than in E14-PBS after one day in vivo (Fig. 1U,V), and Shh, Sp5 and Fgf4 expression in M2 appeared in E14-5E1 but not in E14-PBS after...
one day culture in vitro (Fig. 4C,D,O,P,W,X). Furthermore, Lef1 expression in M2 (red arrow) is larger in E14-5E1 than in E14-PBS after one day in vitro (Fig. 4S,T). These results indicate that M2 expression in M2 (red arrow) is larger in E14-5E1 than in E14-PBS outside the Sostdc1 expression (Laurikkala et al., 2003), whereas Shh expression is not expressed around the control PBS beads (E,G). Sostdc1 is induced by exogenous Shh protein in mesenchyme (arrow in H), but not in epithelium (F). (H-L) Sostdc1 is expressed in dental epithelium and mesenchyme around wild-type tooth germ at E14 but is not induced in either epithelium or mesenchyme around beads soaked in Shh protein. Occlusal views are shown in I and K. Frontal section is shown in J and L at the level of the dashed lines in I and K, respectively. Yellow dashed lines indicate the boundary of the dental epithelium and mesenchyme. Scale bars: 200 μm.

**Fig. 5. Effects of Shh on Sostdc1 expression in mouse.** (A-D) Apart from endogenous Ptch1 (red arrows in A and B), exogenous Ptch1 is expressed around the Shh protein bead in both dental epithelium and mesenchyme (black arrows in B,D), but not around PBS beads (A,C). (E-H) Sostdc1 is not expressed around the control PBS beads (E,G). Sostdc1 is induced by exogenous Shh protein in mesenchyme (arrow in H), but not in epithelium (F). (I-L) Sostdc1 is expressed in dental epithelium and mesenchyme around wild-type tooth germ at E14 but is not induced in either epithelium or mesenchyme around beads soaked in Shh protein. Occlusal views are shown in I and K. Frontal section is shown in J and L at the level of the dashed lines in I and K, respectively. Yellow dashed lines indicate the boundary of the dental epithelium and mesenchyme. Scale bars: 200 μm.

DISCUSSION

**Tooth patterns of 5E1-treated mice are similar to those seen in transgenic mice with Shh signaling defects**

Here, we have used a simple system in which 5E1, a Shh antibody, is injected into pregnant mice at precise times to investigate Shh signaling in patterning of the teeth in embryos. Analysis of expression of Ptch1 and Gli1, known gene targets of Shh signaling, indicated that 5E1 injection blocked Shh activity in developing teeth. The binding efficiency of 5E1 to Shh, Ihh and Dhh proteins has been evaluated in a previous study and it was found that 5E1 (10 μg/ml)-induced differentiation of C3H10T1/2 cells (Wang, L. C. et al., 2000). Ihh and Dhh are not expressed in developing teeth so in our experiments only Shh activity will be blocked. We found, using our treatment regime, that we could replicate various tooth patterns previously observed in K14-Cre;Shhfllox/flox and K14-Cre;SmoI12/12 mice, whereas molar fusion in K14-Cre;APCfl/1 mice (Kuraguchi et al., 2006; Järvinen et al., 2006) as a result of multiple activation and inhibition zones originating from multiple Wnt/β-catenin signals, which act as the inhibitor in the Wnt-Shh-Sostdc1 loop. Furthermore, we verified, by using the new reaction-diffusion model consisting of activator, mediator and inhibitor, that such interactions do indeed lead to tooth patterning consistent with that of wild-type mice (Fig. 6F; see Movie 1 in the supplementary material).
(Dassule et al., 2000; Gritli-Linde et al., 2002) suggests that an early effect of lack of Shh signaling is failure of formation of the dental lamina.

**Sostdc1 is a target of Shh signaling in tooth development**

M1-M2 fusions, like those seen in K14-Cre;Shh\textsuperscript{flx/flx} and K14-Cre;Smo\textsuperscript{flx/flx} transgenic mice and produced in our study by injecting 5E1, have also been observed in Sostdc1\textsuperscript{−/−} and Lrp4\textsuperscript{−/−} mice, as have supernumerary molars and incisors (Kassai et al., 2005; Ohazama et al., 2008). We found that Sostdc1 expression was downregulated both in microarray and in RT-qPCR analyses in intact tooth germs compared with dental mesenchyme on its own (Ahn et al., 2010). However, in previously published work on tooth germs from mice, as have supernumerary molars and incisors (Kassai et al., 2005; Munne et al., 2009) and recently, by utilizing Wnt reporter mice, elevated Wnt signaling was reported in Sostdc1\textsuperscript{−/−} and Sostdc1\textsuperscript{+/−} and 5E1-transferred mice enhances molar fusion. Sustained Wnt/β-catenin signals in K14-Cre;Ctnnb1(Ex3)fl/+, 5E1, K14-Cre;Smoflox/flox, Sostdc1\textsuperscript{−/−}, Lrp4\textsuperscript{−/−} and 5E1-transferred mice enhances molar fusion. Sustained Wnt/β-catenin signals in K14-Cre;Ctnnb1(Ex3)fl/+ mice leads to increased Wnt/β-catenin signaling. However, expression levels and patterns of Wnt/β-catenin in dental epithelium. Finally, although blockade of Shh activity by 5E1, would be predicted to lead to increase of Wnt/β-catenin signaling. However, expression levels and patterns of β-catenin and Lef1 were not changed by 5E1 treatment. This result is consistent with previous findings that Lef1 expression showed minor alterations in Sostdc1\textsuperscript{+/−} mice, compared with Sostdc1\textsuperscript{−/−} mice, even though Wnt signaling activity was elevated (Ahn et al., 2010). However, we found that expression levels of Sp5 were increased in microarray and RT-qPCR analyses and ectopic Sp5 expression was also observed. As Sp5, which is expressed only in dental epithelium, at least from E12 to E16, is a direct transcriptional target of the Wnt/β-catenin pathway, and the promoter region of Sp5 contains multiple binding sites for Lef1 and Tcf family members (Weidinger et al., 2005; Zhang et al., 2008), Sp5 can be regarded as a good marker for Wnt/β-catenin activity in dental epithelium. Therefore, we suggest that this upregulation of Sp5 expression being a downstream target of Shh signaling.

It was striking that Sostdc1 was not induced in dental epithelium by applying Shh protein. If one takes Ptc1 expression as an indication that cells have received an Shh signal, then endogenous expression of Ptc1 in both the PBS- and Shh-treated dental epithelium of wild-type mice suggests that both tissues had received the Shh signal. Furthermore, in the same series of experiments, exogenous Shh can induce Ptc1 expression in dental epithelium. Finally, although blockade of Shh activity by 5E1 abolished Ptc1 expression, Sostdc1 was still expressed in dental epithelium. These results suggest that epithelial Sostdc1 is not regulated by Shh and, indeed, it has been reported that epithelial Sostdc1 can be induced by Bmp4 protein (Kassai et al., 2005).

**Sostdc1 and Wnt signaling in spatial patterning of teeth**

Sostdc1 is known to be a secreted inhibitor of the Wnt and Bmp pathways (Laurikkala et al., 2003; Yanagita et al., 2004; Ohazama et al., 2008; Munne et al., 2009) and recently, by utilizing Wnt reporter mice, elevated Wnt signaling was reported in K14-Cre;Sostdc1\textsuperscript{−/−} mice, which show ectopic expression of Sostdc1 in epithelium (Ahn et al., 2010). In our study, decreased expression of Sostdc1, following the blocking of Shh activity by 5E1, would be predicted to lead to increase of Wnt/β-catenin signaling. However, expression levels and patterns of β-catenin and Lef1 were not changed by 5E1 treatment. This result is consistent with previous findings that Lef1 expression showed minor alterations in Sostdc1\textsuperscript{+/−} mice, compared with Sostdc1\textsuperscript{−/−} mice, even though Wnt signaling activity was elevated (Ahn et al., 2010). However, we found that expression levels of Sp5 were increased in microarray and RT-qPCR analyses and ectopic Sp5 expression was also observed. As Sp5, which is expressed only in dental epithelium, at least from E12 to E16, is a direct transcriptional target of the Wnt/β-catenin pathway, and the promoter region of Sp5 contains multiple binding sites for Lef1 and Tcf family members (Weidinger et al., 2005; Zhang et al., 2008), Sp5 can be regarded as a good marker for Wnt/β-catenin activity in dental epithelium. Therefore, we suggest that this upregulation...
of Sp5 indicates increased Wnt/β-catenin signaling following blockade of Shh activity by 5E1. In addition, we found that Sp5-expressing areas in M1 and M2 were connected after blocking of Shh activity by 5E1 treatment, which might be the cause of the M1-M2 fusion.

A Wnt-Shh-Sostdc1 negative feedback loop in tooth patterning

In tooth development, it has been reported that Wnt/β-catenin signals are upstream of Shh (Kratovich et al., 2002; Jarvinen et al., 2006; Liu et al., 2008; Närvä et al., 2008) and Sostdc1 (Jarvinen et al., 2006; Närvä et al., 2008; Liu et al., 2008). By contrast, it was also reported that Shh suppresses Wnt/β-catenin signaling in early developing mandible (Dassule and McMahon, 1998). Furthermore, a Wnt-Shh negative feedback loop in tooth development was recently suggested by the significant elevation of Wnt signaling in Sostdc1+/−;Shh−/− mice, compared with that in Sostdc1+/+ mice (Ahn et al., 2010). In our study, the induction of Sostdc1 by Shh protein in dental mesenchyme and the elevation of Wnt/β-catenin signaling after blocking Shh signaling activity suggest a Wnt-Shh-Sostdc1 negative feedback loop, in which Wnt signaling induces Shh and suppresses the Wnt/β-catenin pathway indirectly via Sostdc1, as a candidate mechanism for tooth patterning.

Fgf4 and Fgf3 have been reported as downstream genes of Wnt/β-catenin pathway (Chamorro et al., 2005; Hendrix et al., 2006; Kratovich et al., 2002), and the Fgf pathway was suggested as one of major downstream targets of Wnt signaling regulated by Sostdc1 (Ahn et al., 2010). Therefore, the overactivation of Wnt/β-catenin signaling via downregulation of Sostdc1 after 5E1 injection might be the cause of the increase of Fgf gene expression. In our study, mesenchymal Fgf genes, such as Fgfβ3 and Fgfl0, did not display any significant change in gene expression levels, whereas epithelial Fgf genes, such as Fgf4, Fgf9 and Fgf20, exhibited significant changes following 5E1 treatment, which might be attributable to the elevation of Sp5 expression in enamel knot.

Another pathway regulated by Sostdc1 downstream of Wnt signaling appears to be the Shh pathway itself. Shh expression was also found to be elevated in Sostdc1−/− mice (Ahn et al., 2010) and in our study we found 1.4-fold increases in Shh expression after 5E1 treatment and premature Shh expression in M2 development. An increase in Shh expression would be consistent with a Wnt-Shh-Sostdc1 negative feedback loop.

Sostdc1 is a Bmp antagonist as well as an antagonist of the Wnt/β-catenin pathway. Recent work comparing tooth germs of E13.5 Sostdc1+/+;Shh−/− mice with those of Sostdc1−/− mice, showed major changes in components of the Wnt, Fgf and Shh pathways but minor alterations in Bmp and/or Tgfβ or other pathways (Ahn et al., 2010). In addition, our results showing minor changes in expression levels in the Bmp pathway after 5E1 treatment highlight the role of Sostdc1 as a Wnt antagonist in tooth development. Nevertheless, Sostdc1 might have a close relationship with Bmp signals in teeth. For example, the development of extra molars and incisors in Sostdc1−/− mice was accelerated by exogenous Bmp4 protein compared with wild-type mice (Kassai et al., 2005; Munne et al., 2009). However, the role of Bmp signals in the spatial patterning of teeth still remains to be elucidated, because conditional Bmpr1a-deficient mice such as K14-Cre43;Bmpr1aloxp/lox mice exhibit failure of tooth development after the bud stage (Andl et al., 2004), and Sostdc1−/−;Bmpr1aloxp/lox mice, in which levels of Bmp signaling were reduced through removal of a copy of the Bmpr1a type I receptor gene, do not show tooth phenotypes different from those of Sostdc1−/− mice (Ahn et al., 2010).

Our results show that injection of an antibody against a specific signaling molecule into pregnant mice at chosen time points can be used to explore the relationships between signaling pathways in developing organs at various embryonic stages. By utilizing this method, we found evidence to suggest a Wnt-Shh-Sostdc1 negative feedback loop governing the spatial patterning of teeth. The loop described here might be a general reaction-diffusion mechanism for achieving the spatial pattern of other organs in vertebrates from fish to human. In our new model for spatial patterning of the teeth, Wnt, Shh and Sostdc1 act as activator, mediator and inhibitor, respectively (Fig. 6E). In this ‘proof of concept’ model of the spatial patterning of teeth, we have simply chosen the boundary conditions and form of growth to allow the system to recapitulate experimental observations. We need the Dirichlet (fixed) boundary conditions to stop pattern forming on the sides of the domain and we need the Neumann (zero flux) boundary conditions to stop diffusion of the mediator out of the domain. These boundary conditions are possible from a biological viewpoint, as they represent sinks and impermeability, respectively. They should be seen as predictions of the model. Our model is in contrast with a recent Salazar-Ciudad and Jernvall model (Salazar-Ciudad and Jernvall, 2010), which suggests that both Shh and Sostdc1 act as inhibitors. These two models are complementary in that we are trying to understand position, whereas they are trying to understand shape.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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