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Tinkering with the inductive mesenchyme: Sostdc1 uncovers the role of dental mesenchyme in limiting tooth induction

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Like epithelial organs in general, tooth development involves inductive crosstalk between the epithelium and the mesenchyme. Classically, the inductive potential for tooth formation is considered to reside in the mesenchyme during the visible morphogenesis of teeth, and dental mesenchyme can induce tooth formation even when combined with non-dental epithelium. Here, we have investigated induction of mouse incisors using *Sostdc1* (ectodin), a putative antagonist of BMP signaling in the mesenchymal induction of teeth. Deletion of *Sostdc1* leads to the full development of single extra incisors adjacent to the main incisors. We show that initially, *Sostdc1* expression is limited to the mesenchyme, suggesting that dental mesenchyme may limit supernumerary tooth induction. We test this in wild-type incisors by minimizing the amount of mesenchymal tissue surrounding the incisor tooth germs prior to culture in vitro. The cultured teeth phenocopy the extra incisors phenotype of the *Sostdc1*-deficient mice. Furthermore, we show that minimizing the amount of dental mesenchyme in cultured *Sostdc1*-deficient incisors causes the formation of additional de novo incisors that resemble the successional incisor development that results from activated Wnt signaling. Finally, Noggin and Dkk1 prevent individually the formation of extra incisors, and we therefore suggest that inhibition of both BMP and Wnt signaling contributes to the inhibitory role of the dental mesenchyme. Considering the role of mesenchyme in tooth induction and the design of tissue engineering protocols, our work may have uncovered how delicate control of tissue quantities alone influences the outcome between induction and inhibition.

KEY WORDS: Wise, *Usag1*, Vestigial organs, Mouse, Sostdc1 (ectodin)

INTRODUCTION

According to Dollo's Law, evolution is not substantially reversible and a structure lost during evolution is unlikely to reappear. Whereas evolutionary reappearance of recently lost structures can still be assumed to be relatively common (Marshall et al., 1994), long-lost structures are exceedingly rare to reappear. Even long-lost features, however, may still be retained as vestigial structures before disappearing altogether. For example, the developing mouse dentition contains potential to make more teeth than are present in the adult dental pattern, which is often attributed to be due to the presence of vestigial teeth (Peterkova et al., 2002; Keränen et al., 1999). Vestigial tooth germs have been reported in the mouse toothless diastema region between the incisors and the first molar (Peterkova et al., 2002), and also in the mouse incisor region (Peterkova et al., 2002). These tooth rudiments, which would have been retained for over 45 million years, do not develop beyond early invagination of the epithelium, or the early bud stage, and they degenerate apoptotically during later development (Keränen et al., 1999; Peterkova et al., 2002). In addition to these missing tooth loci, rodents have lost the ability for tooth replacement from deciduous to permanent incisors. The loss of successional replacement of molars is evolutionarily basal for all living mammals.

Developmentally, vestigial mouse tooth rudiments disappear after the inductive potential of tooth formation has shifted from the epithelium to the mesenchyme (Vainio et al., 1993; Mina and Kollar, 1987; Kollar and Baird, 1969). The neural crest-derived dental mesenchyme can induce tooth formation when combined with non-dental epithelium, raising the question of what mechanism prevents the full development of rudimentary teeth? Several mutant and transgenic mouse strains have supernumerary molars, predominantly anterior to the first molar, where previous reports have identified vestigial tooth buds (Mustonen et al., 2003; Klein et al., 2006; Kassai et al., 2005). The analysis of the developmental origins of these supernumerary molars has indicated that they develop from the vestigial buds, and thus appear to 'rescue' the teeth lost during evolution (Mustonen et al., 2003; Klein et al., 2006; Kassai et al., 2005). At the molecular level, these works suggest a role for multiple feedback loops activating and inhibiting tooth development and number, but it remains unknown how these interactions may modulate the inductive potential of the dental mesenchyme.

A signaling pathway affecting both incisor and diastema vestigial teeth in mice is the bone morphogenetic protein (BMP) pathway. BMP4 is required for the shift of the inductive potential from the presumptive dental epithelium to the dental mesenchyme after embryonic day 12.5 (E12.5) (Vainio et al., 1993; Chen et al., 1996). Epithelial BMP4 induces the expression of the homeobox gene *Msx1*, which is required for advancing tooth development and for the shift of *Bmp4* expression to mesenchyme (Vainio et al., 1993; Chen et al., 1996). In *Msx1* mutants, development of all teeth is arrested at the early bud stage, and mesenchymal *Bmp4* expression is lacking (Chen et al., 1996). This phenotype can be rescued by forced *Bmp4* expression in the mesenchyme (Zhao et al., 2000; Bei et al., 2000). Tooth development also ceases at the bud stage in mice that lack the functional BMP receptor type 1A in the epithelium (Andl et al., 2004).

Whereas partial downregulation of BMP signaling decreases tooth number (Plikus et al., 2005), deletion of the BMP antagonist *Sostdc1* rescues the vestigial molar tooth buds (Kassai et al., 2005). *Sostdc1*, which was discovered three times independently and has also been called ectodin, *Usag1* and *Wise* (Laurikkala et al., 2003; Yanagita et al., 2004; Itasaki et al., 2003), is a secreted molecule identified as a BMP inhibitor binding to BMPs with high affinity (Laurikkala et al., 2003; Yanagita et al., 2004). BMPs themselves induce the expression

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of *Sostdc1* (Laurikkala et al., 2003). Sostdc1 has also been shown to regulate the Wnt pathway in a context-dependent manner. It is able to compete with Wnts for binding to the Wnt co-receptor Lrp6 (Itasaki et al., 2003).

We have previously shown that Sostdc1 affects the morphology of mouse molars by restricting the size and the placement of epithelial signaling centers: the enamel knots (Kassai et al., 2005). In addition, in *Sostdc1* knockout mice, an extra tooth develops in the location of the vestigial premolar primordium (Kassai et al., 2005) and in the incisor region (Murashima-Suginami et al., 2007) (Fig. 1). In this study, we used *Sostdc1* null mutant mice (*Mus musculus* Linnaeus) to explore the role of the mesenchyme in modulating tooth number. We focused on the incisor because previous reports have suggested the presence of multiple vestigial incisor buds in mice (Peterkova et al., 2002). Compared with living rodents, ancestors of rodents had both higher number of incisors and replacement of incisors from deciduous to permanent teeth. This raises the issue of whether the extra incisors in *Sostdc1*-deficient mice represent rescued teeth or rescued tooth replacement. Our results show that the extra incisors in *Sostdc1*-deficient mice are likely to be the latter: successional incisors. Furthermore, we show the amount of mesenchymal tissue alone may influence the outcome between induction and inhibition of extra teeth and that *Sostdc1* is a central modulator of the inductive potential of dental mesenchyme.

MATERIALS AND METHODS

Animals

The *Sostdc1*-deficient mouse line used in this study has been described previously (Kassai et al., 2005) and the heterozygous littermates were used as controls. Wild-type embryos were from the NMRI strain and from heterozygous *Shh*-GFP mice, which express a reporter gene [green fluorescence protein (GFP)] under a *Shh*-promoter (Harfe et al., 2004). *Sostdc1*-deficient mice were crossed with the Shh-GFP reporter line. The *Catenb*$^{\Delta ex3k14/+}$ mice have been described previously (Järvinen et al., 2006). Two different reporter mouse lines were crossed with *Sostdc1*-deficient mice to study the localization of Wnt activity: TOPgal (Jackson laboratory) and BATgal (Maretto et al., 2003). The reporter constructs consist of multiple TCF/Lef-binding sites and a WNT target gene minimal promoter driving the expression of *lacZ* reporter gene (DasGupta and Fuchs, 1999; Maretto et al., 2003). The age of the embryos was estimated from the appearance of the vaginal plug (E0) and from their exterior features.

Organ culture

The teeth with some surrounding tissue were dissected from the lower jaw of E12 to E14 embryos and cultured using a Trowell type organ culture system (Sahlberg et al., 2002). The medium was changed every second day and contained DMEM and F12 (Ham's Nutrient Mix: Life Technologies) (1:1) supplemented with 10% fetal calf serum (PAA laboratories, Pasching, Austria), 150 mg/ml ascorbic acid, glutamine and penicillin-streptomycin. The explants were photographed every day using light (Olympus SZX9) or fluorescent (Leica MZFLIII) microscopy.

Recombinant BMP4 (0.5 ng/μl R&D), Noggin (1 ng/μl R&D), Dkk1 (1 ng/μl R&D) and Sostdc1 (1 ng/μl, a kind gift from Dr N. Itoh, Kyoto, Japan) proteins were added into the medium. Canonical Wnt signaling was activated using a specific Gsk3beta inhibitor BIO (2 mM Calbiochem). In bead experiments, Affi-gel agarose beads (Biorad) were soaked in recombinant BMP4 (100 ng/μl R&D) or BSA, and they were placed on top of the tooth explants.

Processing of tissues for histology and in situ hybridization

Heads from wild-type and *Sostdc1* knockout mouse embryos (E12-E17) were dissected and fixed with 4% paraformaldehyde (PFA) at +4°C overnight. They were dehydrated paraffin-embedded and serially sectioned at 7 μm. Sections were counterstained with Hematoxylin and Eosin. Jaws for whole-mount in situ hybridization were fixed similarly. The tissue culture samples were treated with 100% ice-cold methanol for 5 minutes before fixing with 4% PFA. Sample preparation was same for the TUNEL staining.

Three dimensional (3D) reconstructions

Digital pictures were taken of frontal serial sections of the incisor. For the shape 3D reconstruction, the pictures were imported into a stack with Scion Image software (version 4) and the individual slices were aligned with the register function using the midline of the jaw as reference. The epithelial shape was manually traced in Adobe Photoshop CS2 (PS) with the brush function. The distance between the sections (7 μm) was translated to a corresponding pixel distance based on a reference scale bar. The stack with the modified slices was re-sliced at a perpendicular angle in order to create a new stack with only one pixel distance between individual slices. The jagged epithelial shape in the resulting slices were smoothened in PS with a batch command: select white, expand five pixels, smooth 15 pixels, contract four pixels, fill white (shape), invert selection, fill black (background). The E16 *Sostdc1* –/– stack was smoothened entirely manually using the brush function in PS. A projection was made of the stack and the sagittal view selected for presentation.

For the 3D reconstruction of expression patterns, first the shape 3D reconstruction was created according to the previous protocol. Subsequently, the blue X-Gal staining was isolated by applying the high contrast red filter with the 'black&white' command in PS, the levels adjusted and inverted. The epithelial expression of interest was isolated and treated in the same sequence of steps as the shape and projected on the corresponding slice of the shape projection.

In situ hybridization, TUNEL labeling and detection of *lacZ*

Whole-mount in situ hybridization was performed by using the InSituPro robot (Intavis AG, Germany) as described earlier (Laurikkala et al., 2003). BM Purple AP Substrate Precipitating Solution (Boehringer Mannheim Gmbh, Germany) was used to visualize the digoxigenin-labeled probes. Radioactive in situ hybridization for paraffin sections was carried out according to standard protocols using ^{35}S-UTP labeling (Amersham). The following probes were used: murine *Shh* (Vaahtokari et al., 1996), *p21* (Jernvall et al., 1998), *Msx2* (Jowett et al., 1993), *Sostdc1* (Laurikkala et al., 2003; Kassai et al., 2005) and *Bmp4* (Vainio et al., 1993).

Apoptotic cells were visualized by detecting DNA fragmentation in whole-mount samples and sections with the ApoTag Apoptosis Detection Kit.

Localization of Wnt activity through β-galactosidase in the embryonic tissue was revealed using X-Gal staining. E13-14 lower jaws were collected in Dulbecco's 1×PBS and fixed in 2% PFA, 0.2% glutaraldehyde for 30 minutes at 4°C. Tissues were incubated for 3×10 minutes in X-gal washing buffer (2 mM $MgCl_2$, 0.02% NP-40 in PBS) and stained with X-gal staining solution (1 mg/ml X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in X-gal washing buffer) in room temperature. Serial sections were counterstained with Nuclear Fast Red.

DiI-labeling

Microinjections were performed by injecting fluorescent DiI [1,1′-di-octadecyl-6,6-di(4-sulfophenyl)-3,3,3′,3′-tetramethylindocarbocyanine] (Invitrogen) to the in vitro cultured incisor explants. The color (diluted in 2 μg/ml DMSO) was injected to the cells that will later form the extra incisor.

RESULTS

Initial mesenchymally restricted *Sostdc1* expression and enlargement of epithelial gene expression patterns in *Sostdc1*-deficient mice

We first examined the expression pattern of *Sostdc1* during the early stages of wild-type mouse incisor development (Fig. 1A-E). Whole-mount in situ analysis show that *Sostdc1* expression surrounds the incisor placodes at E12 stage (Fig. 1B). At E13, *Sostdc1* is expressed inside the incisor (and molar) tooth germs and around them, but now a *Sostdc1*-negative region has formed immediately next to the tooth germs (Fig. 1D). Histological

sections show that at E12 and E13, *Sostdc1* is expressed in the mesenchyme (Fig. 1A), and is upregulated in the tooth forming area around E13 (Fig. 1C). In the dental epithelium, *Sostdc1* is upregulated at E14 when the incisor has reached the cap stage (Fig. 1E). In the E14 mesenchyme *Sostdc1* expression was localized in the lingual side of incisors (Fig. 1E). In the epithelium, expression was absent from the enamel knot and its underlying mesenchyme (Fig. 1E), agreeing with the earlier reports of developing molar teeth (Laurikkala et al., 2003; Kassai et al., 2005). As in molars, the incisor *Sostdc1* expression correlates with *Bmp4* expression (Fig. 1F), except that in incisors, these genes are strongly expressed in the lingual side (Fig. 1E,F), whereas in the molars they are expressed in the buccal side (Laurikkala et al., 2003; Kassai et al., 2005). Furthermore, compared with *Sostdc1*, *Bmp4* expression is located in closer proximity to the dental epithelium, including in the enamel knot cells themselves and throughout the underlying mesenchyme.

Next, we examined incisor formation in the *Sostdc1*-deficient mice. All the studied (n>25) *Sostdc1*-deficient embryos had extra incisors, which are also well visible after birth. With age, however, adult mice tend to lose the extra incisors, which, when retained, are substantially smaller than the main incisors and lack the yellow-brown pigmentation, typical for the rodent incisors, at their labial surface (Fig. 1G,H).

From E12, the expression of *Shh*, an epithelial placode and enamel knot marker in *Sostdc1*-deficient jaws is stronger and broader in incisors in comparison with the controls (Fig. 1I-L; see Fig. S1 in the supplementary material). The expanded expression of *Shh* in the epithelium correlates with the expanded and stronger expression of *p21* expression (see Fig. S1 in the supplementary material), a differentiation marker of the enamel knots (Jernvall et al., 1998). Moreover, the expanded *Shh* expression in the *Sostdc1*-deficient epithelium corresponds to stages when *Sostdc1* expression is limited to the mesenchyme in the wild-type incisors (Fig. 1C).

Specific lack of epithelial apoptosis in *Sostdc1*-deficient mice

Because rudimentary tooth germs and enamel knots are known to disappear through apoptosis (Keränen et al., 1999; Peterkova et al., 2002), we used TUNEL-staining to study effects of *Sostdc1* on apoptosis (Fig. 2A,B). Almost no apoptosis was detected in the incisor buds at E12 and E13, and no difference was seen between wild-type and mutant incisors (not shown). However, at E14, the cap stage *Sostdc1*-deficient incisor epithelium shows a marked lack of apoptosis (n=6/6) in the enamel knot, and especially in the area closer to the oral epithelium (Fig. 2A,B). There was only limited apoptosis in the mesenchyme and it appeared similar in wild type and the *Sostdc1*-deficient mice.

In order to examine whether the area of apoptosis in the epithelium close to oral surface of E14 wild-type incisor is associated with the removal of a vestigial incisor rudiment, we localized enamel knot markers at an earlier stage – E13-E13.5 (Fig. 2C,D). Because the Wnt pathway is another potential Sostdc1 target, and enamel knots are sites of high Wnt signaling activity (Järvinen et al., 2006), we examined activity of Wnt pathway using TOPgal and BATgal reporter mice crossed with the *Sostdc1*-deficient mice. Epithelial Wnt activity was detected in the incisor enamel knot both in *Sostdc1*-deficient mice and controls at E13-E13.5 (Fig. 2C,D; data not shown). In addition, we detected a small domain of Wnt activity in the area of intense apoptosis in the E14 wild-type incisors (Fig. 2A).

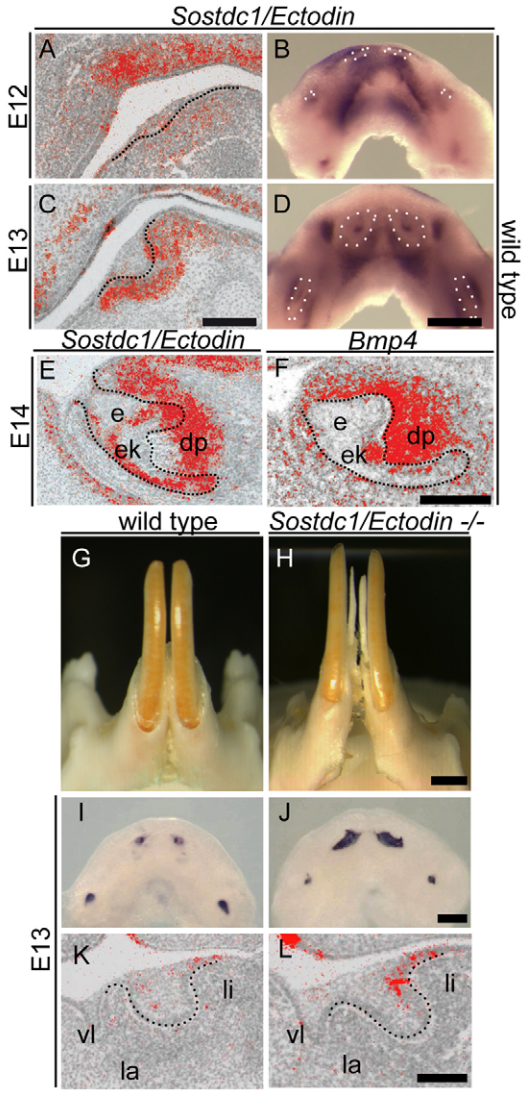

Fig. 1. *Sostdc1* expression is mostly confined to the mesenchyme, and *Sostdc1*-deficient mice have extra incisors and expanded expression patterns of *Shh*. (**A-D**) Radioactive and whole-mount in situ hybridization show *Sostdc1* expression becoming localized to the underlying mesenchyme of lower incisor region between E12 and E13 (C,D). The incisor and molar placodes are outlined in B and D. (**E**) At E14 cap stage, *Sostdc1* expression is intense in the mesenchyme at the lingual side of the incisor and in dental papilla, and it can be detected in the incisor epithelium, whereas it is absent from the mesenchyme under the enamel knot. (**F**) *Bmp4* colocalizes with *Sostdc1* but shows additional expression in the enamel knot and dental papilla at E14. (**G**,**H**) The extra incisors are in the *Sostdc1*-deficient lower jaw, mesiolingual to the main incisors. (**I**,**J**) During development, *Sostdc1*-deficient jaws have enlarged expression of *Shh* in the epithelium. (**K**,**L**) The epithelially restricted *Shh* expression is visible in histological sections. e, dental epithelium; ek, enamel knot; dp, dental papilla; vl, vestibular lamina; la, labial; li, lingual. Scale bars: 0.2 mm in A-F,I-L; 0.5 mm in G,H.

3D analysis reveals the origin of extra incisor in *Sostdc1*-deficient mice

We used 3D analysis of serial sections to determine the relationship between enamel knot dynamics and extra incisor formation. The results show that the small domain of Wnt activity located orally to

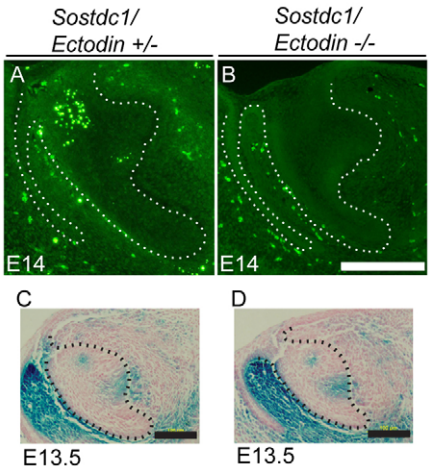

Fig. 2. Apoptosis, seen in wild-type odontogenic epithelium at E14 stage, is absent from *Sostdc1*-deficient jaws and the site correlates with the epithelial Wnt activity seen at E13.5 stage. (**A**,**B**) Sagittal sections show apoptotic cells (detected using TUNEL staining) in regions of the epithelium of control teeth, the enamel knot and a site near the oral surface (A), whereas apoptosis is absent in the *Sostdc1*-deficient teeth (B). (**C**,**D**) Sagittal sections from control (C) and *Sostdc1*-deficient (D) incisor region in the TOPgal-Wnt reporter mouse (at E13.5) show epithelial Wnt activity in the forming enamel knot and at the site that correlates with the extensive epithelial apoptosis in the E14 control teeth (A). Comparable, albeit weaker, Wnt activity was detected in BATgal mice (not shown). Scale bars: 0.2 mm in A,B; 0.1 mm in C,D.

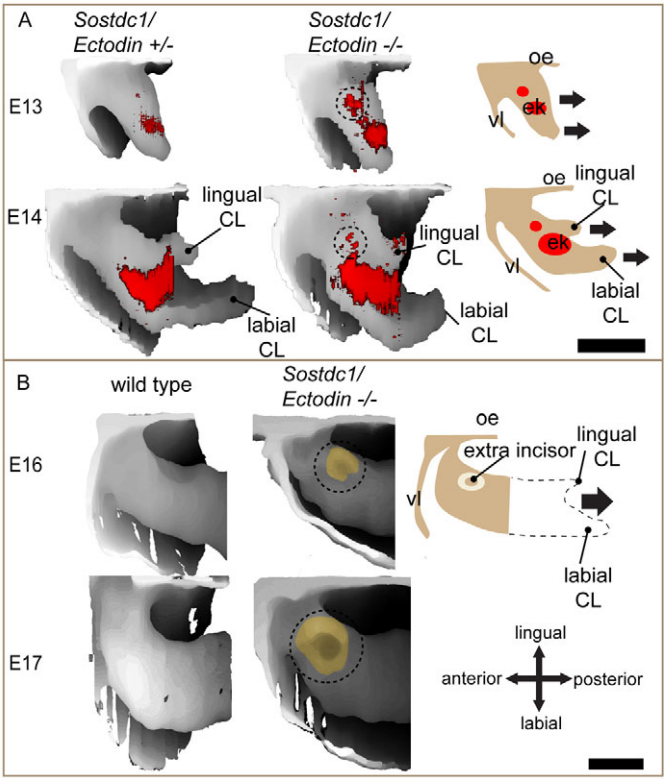

Fig. 3. 3D-reconstruction reveals the origins of the extra incisor in relation to the main incisor. (**A**) *Sostdc1*-deficient and heterozygous controls possessing the TOPgal-Wnt reporter gene show differential epithelial Wnt activity in a sagittal projection of the 3D reconstruction. In addition to the forming enamel knot, an additional epithelial Wnt activity site was observed inside the *Sostdc1*-deficient incisor (circle). (**B**) 3D reconstructions of E16 and E17 *Sostdc1*-deficient and wild-type jaws show the site of extra incisor formation in relation to the main incisor. This location is at the mesiolingual aspect of the main incisor and corresponds to the site of ectopic Wnt activity seen at E13 and E14 (A). oe, oral epithelium; vl, vestibular lamina; CL, cervical loop; ek, enamel knot. Scale bars: 0.2 mm.

the incisor enamel knot is larger and longer in duration in the *Sostdc1*-deficient incisors compared with the wild-type incisors (Fig. 3A). Furthermore, 3D analysis of later developmental stages (Fig. 3B) show that the extra incisor develops at the location previously showing Wnt activity and intense apoptosis in the wild-type incisors. The illustrated 3D renderings are projections from the medial side, and thus the location of the extra incisor closely matches the final position of this tooth in the jaw (Fig. 1H). Taken together, the extra incisor appears not to originate from a separate bud along the dental lamina, but rather to be developmentally part of the main incisor, much like replacement teeth in mammals with more complete tooth replacements.

Extra teeth develop in cultured wild-type incisors in vitro after reducing the amount of tooth-surrounding mesenchyme

Because we observed early expansion of epithelial enamel knot markers at the time when *Sostdc1* expression is limited to the mesenchyme (E12-13), we tested whether we could phenocopy the effect of *Sostdc1* deficiency by minimizing the amount of dental mesenchyme (Fig. 4). We dissected most of the mesenchyme surrounding the incisor tooth germs and cultured the explants in vitro. Removal of mesenchyme resulted in a reduction of *Sostdc1* expression around the dental epithelium, as confirmed by whole-mount in situ hybridization (Fig. 4A,B). Compared with the untrimmed incisors (n=0/>25) (Fig. 4D), the trimmed explants showed development of an extra incisor (n=40/55) (Fig. 4C). The bud of the developing extra incisor was typically seen after 2 days culture at the lingual aspect of the explanted E13 incisor, and during the next 4 days it grew in size and acquired the characteristic shape of an incisor. The extra incisors also developed in these wild-type explants when the incisors were dissected at E12 (n=49/59). When the mesenchyme was trimmed in E14 tooth germs, no extra incisors developed during in vitro culture (not shown).

Additionally, we tested whether the removal of the surrounding mesenchyme from wild-type molar buds would also rescue the development of the vestigial bud anterior to the first molar, which develops to an extra tooth in *Sostdc1* null mutants (Kassai et al., 2005). We dissected the mandibular molar tooth germs at E12 stage and removed most of the surrounding mesenchyme. In a small number of cases (n=4/20) supernumerary teeth formed anterior to the first molars and their development resembled the phenotype of *Sostdc1*-deficient mice (see Fig. S2 in the supplementary material).

Removal of surrounding mesenchyme from *Sostdc1*-deficient incisors initiates the development of de novo incisors from the lingual dental epithelium

When the surrounding mesenchyme was trimmed from the *Sostdc1*-deficient incisor explants, the extra incisor that forms in vivo developed also in vitro (Fig. 5). However, the formation of

extra incisors appears to continue, and additional de novo incisors appeared after a few days of culture from the lingual cervical loop area (Fig. 5A-D) (n=48/109). Because the de novo incisors develop in immediate proximity to the other incisors, we examined the origins of these teeth by microinjecting DiI-label in *Sostdc1*-deficient and control explants expressing GFP in the *Shh* locus (Fig. 6A-C). DiI was injected after 4 days of culture to the lingual epithelium of E13 incisor at the site where the 'normal' extra incisor form. The DiI-label spreads during subsequent culture and labels the extra incisor as it grows in both *Sostdc1*-deficient and control explants (Fig. 6A-C; see Fig. S3 in the supplementary material). However, the de novo incisor in the trimmed *Sostdc1*-deficient explant appears as a new DiI-negative outgrowth from, or close to, the cervical loop of the main incisor (Fig. 6A-C).

Activation of BMP signaling accelerates the development of the extra incisors and de novo incisors in the *Sostdc1*-deficient mice

Next, we tested, using the in vitro culture system, the role of BMP signaling in the development of the extra and de novo incisors in *Sostdc1*-deficient mice (Fig. 7). In tooth explants from *Shh*-GFP expressing *Sostdc1*-deficient mice, we observed changes in the enamel knot size after treatment with recombinant BMP4 protein. The results show that overnight treatment with BMP4 causes increased *Shh* expression in *Sostdc1*-deficient incisors, as well as in wild-type incisors where the mesenchyme has been trimmed (Fig. 7A,B).

We introduced exogenous BMP4 to dissected E13 control and *Sostdc1*-deficient incisor explants at the site where *Bmp4* is normally co-expressed with *Sostdc1* in the mesenchyme. BMP4-soaked beads were placed on the lingual side of incisor buds (owing to the flattening of in vitro cultured teeth, lingual position is an approximation) and the day when extra incisors appeared was tabulated (Fig. 7C). In control samples, extra incisors did not develop either in BMP4-treated or in the control explants (Fig. 7F). By contrast, in homozygous *Sostdc1*-deficient explants, BMP4 beads accelerated the formation of extra teeth (Fig. 7F). They had already started to form at the lingual aspect of the incisors during the second culture day, whereas in the absence of BMP bead, the formation of extra incisors was first seen after 4 days of culture (Fig. 7C).

When the timing of the initiation of extra incisor development was compared between the different explants, the *Sostdc1*-deficient teeth cultured with BMP4 beads showed the fastest initiation of extra incisors (Fig. 7C). However, the wild-type incisors with trimmed mesenchyme showed faster initiation of extra incisors than did *Sostdc1*-deficient incisors cultured with mesenchyme, suggesting the presence of other mesenchymal inhibitory factors in addition to *Sostdc1*.

Inhibition of either BMP or Wnt signal activity prevents the formation of the extra incisor in *Sostdc1*-deficient embryos

We tested whether known inhibitors of BMP and Wnt signaling could mimic the function of Sostdc1 and prevent the formation of extra incisors in *Sostdc1*-deficient embryos. Dissected E13 incisor explants were cultured in the presence of Noggin and Dkk1, antagonists of BMP and Wnt signaling, respectively. In addition, recombinant Sostdc1 was used as a control to rescue *Sostdc1* deficiency. All three inhibitors prevented the formation of the extra incisors in *Sostdc1*-deficient and control explants (see Fig. S4 in the supplementary material).

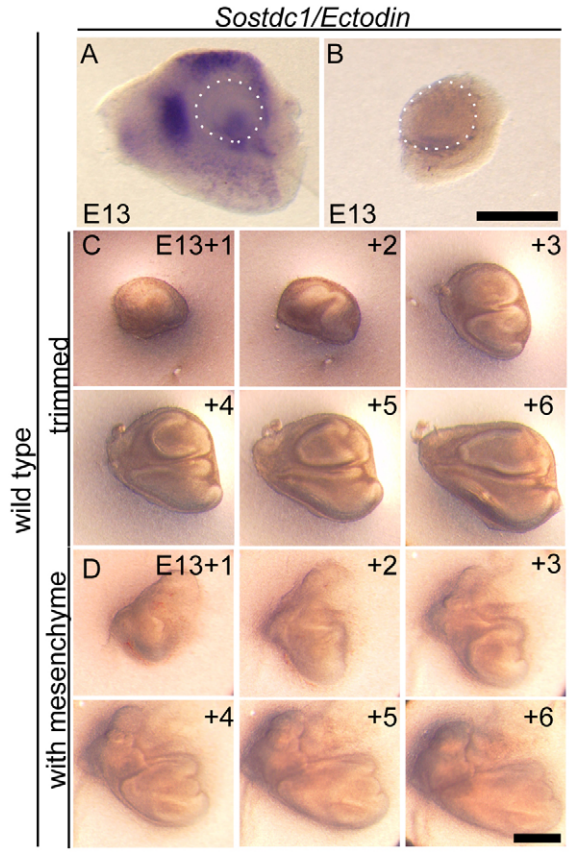

Fig. 4. Extra incisors develop in cultured wild-type incisors after minimizing the amount of tooth-surrounding mesenchyme. (**A**,**B**) A dissected E13 incisor with a large amount of tooth-surrounding mesenchyme (A) exhibits more *Sostdc1* expression than an incisor from which most surrounding mesenchymal tissue has been removed (B). The broken lines show the approximate border between epithelium and mesenchyme. (**C**,**D**) In vitro culture experiments show that extra incisors develop in wild-type explants when most of the surrounding mesenchyme is removed before culture (C). When the surrounding mesenchyme is present in wild-type incisor explants, no extra incisors develop (D). Scale bars: 0.5 mm in A,B; 1 mm in C,D.

Activation of epithelial Wnt signaling mimics the effect of *Sostdc1* deficiency in inducing de novo incisor formation from the lingual epithelium

Stimulation of Wnt signaling in dental epithelium by forced activation of β-catenin causes dramatic stimulation of both molars and incisors in mice (Järvinen et al., 2006). Although the formation of extra molars in these mice was analyzed in detail and it was demonstrated to occur continuously from previously formed teeth, the details of the generation of the extra incisors were not studied. In order to examine whether there were similarities with the formation of the extra teeth in *Sostdc1* knockout mice, we observed the development of $Catenb^{\Delta ex3k14/+}$ incisors in organ culture. E13-E15 incisor tooth germs were dissected from the lower jaws of $Catenb^{\Delta ex3k14/+}$ embryos and cultured for 1 week (see Fig. S5 in the supplementary material). The formation of the supernumerary teeth was progressive, and they formed successively, especially from the lingual incisor epithelium of previously formed incisors (see Fig. S5A in the supplementary material). These supernumerary incisors started to form earlier in vitro than in vivo, which was similar to our

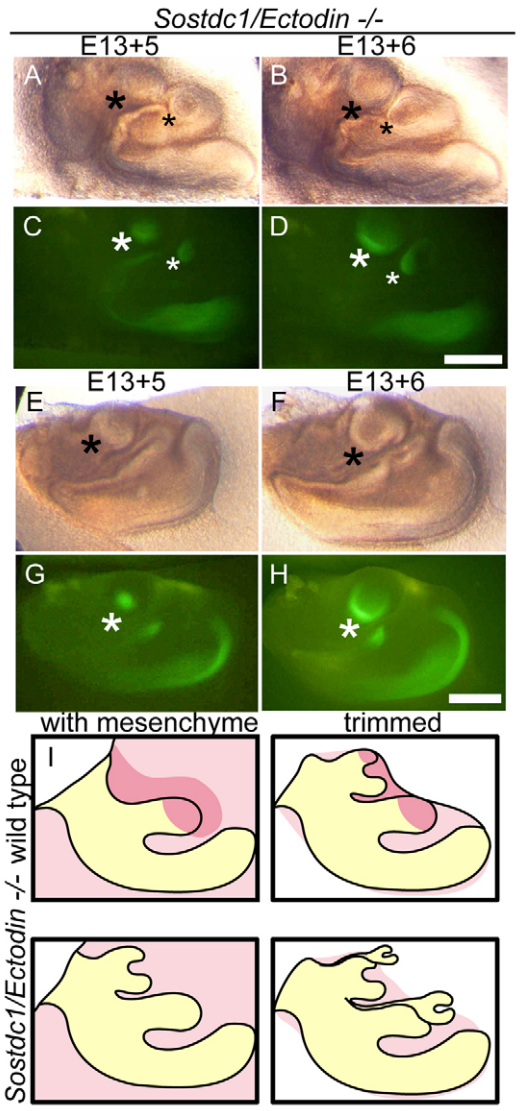

Fig. 5. Additional de novo incisors develop in cultured *Sostdc1*-deficient incisors after minimizing the amount of tooth-surrounding mesenchyme. (**A-D**) De novo incisors form from the lingual epithelium of the *Sostdc1*-deficient incisors after the removal of most of the incisor-surrounding mesenchyme. The larger white asterisks show the extra incisor that appears first and the smaller asterisks indicate de novo incisors (A-D). (**E-H**) *Sostdc1*-deficient incisor explants with the surrounding mesenchyme intact show only formation of one extra incisor (asterisks) (**I**) Schematic drawings summarize the possible ways to produce extra incisors in in vitro cultures. Pink indicates *Sostdc1* expression in the surrounding mesenchyme and in the lingual cervical loop epithelium. Scale bars: 1 mm in A-H.

observations in *Sostdc1*-deficient explants. The de novo formation of incisors was morphologically very similar in the two mouse mutants and indicates that the lingual dental epithelium has the capacity for tooth formation. Additionally, we administered BIO, a specific inhibitor of GSK3b, into the culture medium, thus activating the canonical Wnt signaling pathways throughout the explants. No extra incisor formed in these explants (not shown), but as BIO also had inhibitory effects on the main incisor of the mutant, as well as on control teeth, this may indicate inhibitory effects of enhanced mesenchymal Wnt signaling on tooth development.

DISCUSSION

Classically, the inductive potential of tooth formation is considered to shift from the epithelium to the mesenchyme after E11 (Mina and Kollar, 1987). After this, the neural crest-derived dental mesenchyme can induce tooth formation when combined with non-dental epithelium. Furthermore, several mutants are known that interfere with the mesenchymal induction of the epithelial signaling centers, enamel knots, causing the tooth development to arrest at E13 bud stage (Kratochwil et al., 1996; Bei and Maas, 1998; Peters and Balling, 1999; Åberg et al., 2004). Whereas the inductive role of mesenchyme in tooth development is well established (Vainio et al., 1993; Mina and Kollar, 1987), our results show that induction of additional teeth can be achieved by reducing the amount of dental mesenchyme, perhaps uncovering a role for the mesenchyme in limiting excess tooth induction.

Mesenchymal *Sostdc1* expression inhibits development of extra teeth

In the *Sostdc1*-deficient embryos, markers of tooth formation have broader expression domains, encompassing the areas giving rise to the extra teeth (Fig. 1). In themselves, these kind of broader expression domains are to be expected because similar changes have been reported in the molar region of genetically altered mouse strains with extra molars (Kangas et al., 2004; Mustonen et al., 2003; Klein et al., 2006; Zhang et al., 2003). Our results also agree with previous reports on enlarged expression domains of enamel knot markers, such as *p21*, in *Sostdc1*-deficient molars and incisors (Kassai et al., 2005; Murashima-Suginami et al., 2007; Murashima-Suginami et al., 2008). These patterns are intriguing because, until E14, *Sostdc1* expression is predominantly limited to the mesenchyme (Fig. 1), and the E13 broadened expression patterns in the epithelium are thus suggestive of a role for *Sostdc1* in interfering with epithelio-mesenchymal induction.

The in vitro culture system allowed us to test how reducing the amount of surrounding mesenchyme, and consequently decreasing *Sostdc1* expression, would affect the incisor formation in the wild-type teeth (Fig. 4). Our result, i.e. the in vitro formation of the extra incisor, essentially phenocopies the *Sostdc1*-deficient phenotype. The peak effect of mesenchymal trimming was reached at E13, well within the stage of development when the inductive potential for tooth formation resides in the mesenchyme (Mina and Kollar, 1987). Our work thus appears to have uncovered a complex role for the mesenchyme in the regulation of tooth induction. Initially, the mesenchyme is obviously required for normal tooth morphogenesis and the induction of epithelial enamel knots (Kollar and Baird, 1970a; Kollar and Baird, 1970b; Jernvall et al., 1998), but it might acquire inhibitory roles as the development proceeds and the successional incisor starts to develop. One possibility for the observed activation of the successional tooth after mesenchymal tissue reduction could be slightly different expression domains of genes that induce and inhibit tooth formation. For example, *Sostdc1* appears to have broad expression domains that expand laterally around developing teeth (Fig. 1). Furthermore, peak *Sostdc1* expression intensity in E14 cap stage is further away from the center of the tooth than *Bmp4* expression (Fig. 1), which has been implicated in the induction of enamel knots and the regulation of *Sostdc1* itself (Laurikkala et al., 2003; Kassai et al., 2005; Bei et al., 2000). Interestingly, the bioengineering of tooth germs from dissociated dental cells in vitro has been shown to lead to the formation of multiple incisors (Nakao et al., 2007); it is plausible that these in vitro procedures disrupt the inhibitory mechanisms of in vivo mesenchyme.

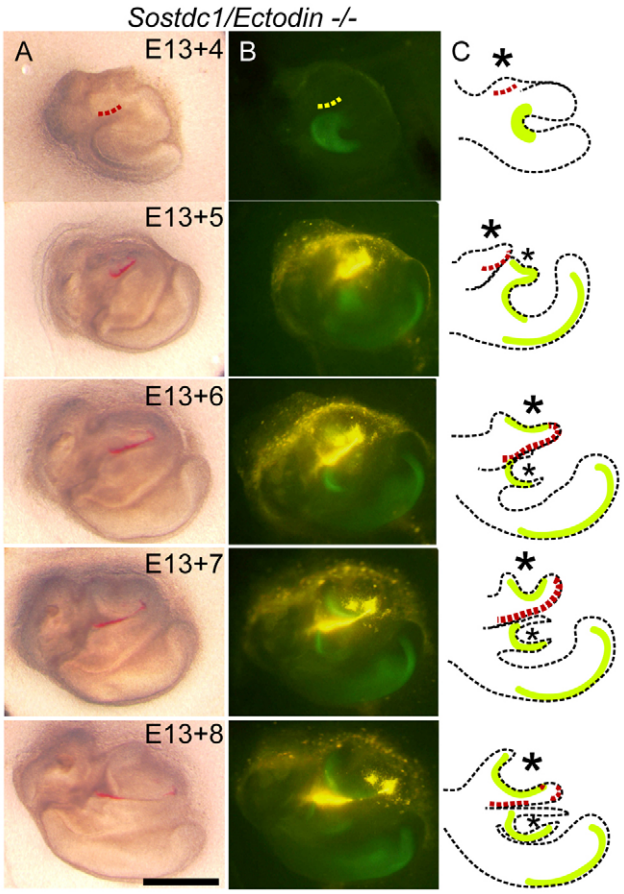

Fig. 6. Fluorescent DiI label reveals the different origins of extra and de novo incisors in *Sostdc1*-deficient mouse. (**A**,**B**) DiI was injected in the in vitro cultured E13 *Sostdc1*-deficient (A) and wild-type incisor explants (see Fig. S3 in the supplementary material) that expressed GFP under the *Shh* promoter. The dye was injected at the epithelial site where the extra incisor starts to form. The dye became incorporated into the forming extra incisor (large asterisks), but not into the main incisor. The de novo incisor (small asterisks) forms from or close to the lingual cervical loop epithelium of the main incisor and lacks DiI label (B). (**C**) Schematic drawings summarize the bright-field (A) and fluorescent (B) pictures; green is the GFP expression and red is the injected DiI. Asterisk indicates extra incisors. Scale bar: 0.5 mm.

Both *Bmp4* and *Sostdc1* are intensely expressed in the mesenchyme at the lingual side of incisor, corresponding to the location of extra incisors in the *Sostdc1*-deficient mouse. This contrasts with the buccal expression bias of *Sostdc1* and *Bmp4* in molars (Jernvall et al., 1998; Laurikkala et al., 2003; Kassai et al., 2005). However, in molars of *Sostdc1*-deficient mice, the cuspal morphology is substantially altered in the buccal side. We interpret these results as further supporting the role of *Sostdc1* as a feedback inhibitor of BMP4 in tooth development, and the role of BMP4 in regulating the lateral bias in tooth patterning (Kassai et al., 2005).

In the toothless mouse diastema, both the epithelium (Cobourne et al., 2004) and the mesenchyme (Yamamoto et al., 2005; Yuan et al., 2008) have been proposed to inhibit diastema tooth development. Recently Yuan et al. (Yuan et al., 2008) have shown that, whereas post E12 diastema mesenchyme retains the capacity to form teeth, it cannot initiate tooth formation by inducing epithelium. Because *Sostdc1* expression domains partially cover the diastemal regions, we propose that *Sostdc1* may be one of the genes suppressing diastemal tooth formation.

Normal removal of incipient extra incisors involves epithelial apoptosis

In our in vitro cultures of wild-type teeth, the experimental phenocopying of the *Sostdc1*-deficient extra incisors was possible until the incisors reached E14 cap-stage. By this developmental stage, *Sostdc1* expression is upregulated also in the epithelium (Fig. 1). This alone suggests that trimming the E14 dental mesenchyme in vitro may not reduce *Sostdc1* expression enough to allow the development of extra incisors. Another possibility for the decreased tooth making potential of E14 explants may be apoptotic removal of the epithelial area that has potential to form extra incisors. Increased apoptosis has been reported in multiple rudimentary teeth (Tureckova et al., 1996; Peterkova et al., 2002; Keränen et al., 1999) and we detected a distinct population of apoptotic cells in the dental epithelium of the wild-type incisors at the site where the extra incisors of *Sostdc1* mutants develop. There was a dramatic lack of apoptotic bodies in the dental epithelium of *Sostdc1*-deficient teeth at E14, both in the enamel knot and especially in the region of the epithelium giving rise to the extra incisors (Fig. 2). Unlike Murashima-Suginami (Murashima-Suginami, 2007; Murashima-Suginami, 2008), we did not detect marked changes in mesenchymal apoptosis in the *Sostdc1*-deficient teeth. Taken together, because earlier removal of mesenchyme expressing *Sostdc1* causes the development of the extra incisors in the wild-type explants and because this ability is lost with the upregulation of *Sostdc1* expression and apoptosis in the epithelium, we suggest that apoptosis may be a relatively downstream event in the in vivo suppression of extra tooth formation.

Sostdc1 may not be the only mesenchymal factor inhibiting development of extra teeth

Because the trimming of the mesenchyme in the wild-type explants consistently produced the extra incisor found in *Sostdc1*-deficent mice in vivo and in vitro, we tested whether the trimming of mesenchyme in the mutants had any effect. The results showed that, in addition to the one extra incisor, additional teeth were initiated later from the lingual cervical loop epithelium of the previously initiated incisors (Figs 5 and 6). This de novo incisor formation in the trimmed *Sostdc1* null explants suggests that there are additional genes expressed in the mesenchyme that suppress extra teeth. The phenotype of these de novo incisors resembles the supernumerary teeth that form when epithelial β-catenin is constitutively stabilized in transgenic mice ($Catenb^{\Delta ex3k14/+}$) (see Fig. S5 in the supplementary material). Normally, the stabilization of epithelial β-catenin requires canonical Wnt signaling and the additional mesenchymal factors may thus involve modulators of Wnt signaling pathway. As *Sostdc1* has been implicated in the inhibition of both BMP and Wnt signaling (Laurikkala et al., 2003; Itasaki et al., 2003; Yanagita et al., 2004), there may be partly redundant signaling pathways that restrict extra tooth formation.

Inhibition of both BMP and Wnt signaling contribute to the inhibitory role of the dental mesenchyme

We have previously linked *Sostdc1* to BMP signaling in tooth development (Laurikkala et al., 2003; Kassai et al., 2005). BMP4 can induce the expression of *Sostdc1*, which in turn antagonizes the

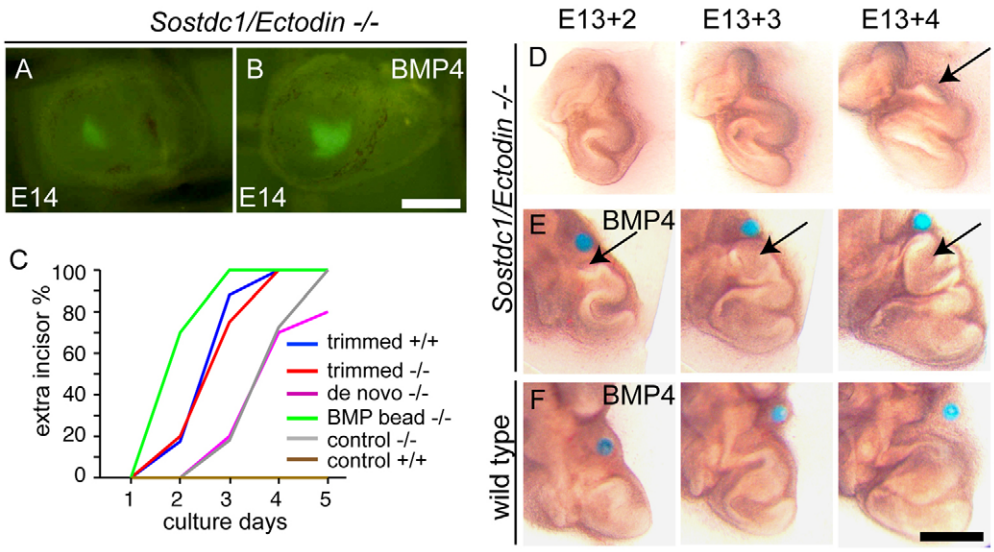

Fig. 7. Acceleration of extra incisor formation by BMP4. (**A**,**B**) Incisors from *Sostdc1*-deficient mice, that express GFP under the *Shh* promoter, have wider enamel knot area after BMP4 treatment. (**C**) Cumulative percentage curves show the appearance of extra incisor. *Sostdc1*-deficient explants (*n*=18/18), *Sostdc1*-deficient explants with BMP4-soaked bead (*n*=16/16), trimmed *Sostdc1*-deficient explants (*n*=24/24) (of which 80% formed de novo incisors), trimmed wild-type explants *n*=17/17. (**D-F**) In vitro culture experiments show that *Sostdc1*-deficient tissue has increased sensitivity to BMP4; a BMP4-soaked bead (blue) placed on top of the lingual incisor surrounding mesenchyme accelerates the development of extra incisors in the *Sostdc1*-deficient incisors (E) but not in the control (F). Arrow indicates the extra incisor. Scale bars: 0.25 mm in A,B; 1 mm in D-F.

induction of enamel knot marker *p21* by BMPs in tissue cultures (Laurikkala et al., 2003; Kassai et al., 2005). The role of BMP4 as mesenchymally expressed enamel knot inducer was strengthened in the absence of Sostdc1, as shown by our experiments where we placed BMP4-releasing beads on the lingual side of the in vitro mutant explants (Fig. 7). BMP4 accelerated extra incisor formation even further than did the trimming of the mesenchyme. However, we observed acceleration of extra incisor formation in only the *Sostdc1*-deficient explants, perhaps further supporting the role of *Sostdc1* as a feedback inhibitor of BMP signaling (Kassai et al., 2005). Additionally, we have previously shown a relatively subtle acceleration of normal molar initiation by BMP4 (Kavanagh et al., 2007), and it is conceivable that the 'rescue' of extra teeth may require additional factors.

In addition to BMP signaling, *Sostdc1* has been linked to Wnt signaling (Itasaki et al., 2003). This link is indirectly supported by the resemblance of the de novo supernumerary tooth formation in *Sostdc1*-deficient mice with the phenotype of *Catenb*$^{\Delta ex3k14/+}$ mice (see Fig. S5 in the supplementary material). In addition, Dkk1, a potent Wnt signal inhibitor inhibited extra incisor formation in vitro. The BMP antagonist Noggin had a similar inhibitory effect, as also shown recently by Murashima-Suginami et al. (Murashima-Suginami et al., 2008) (see Fig. S4 in the supplementary material). These inhibitory effects agree with previous works implicating both Wnt and BMP as activators of tooth initiation (Järvinen et al., 2006; Bei et al., 2000; Wise and Stock, 2006). It is noteworthy that the de novo incisors formed only in the absence of epithelial *Sostdc1* expression. Whereas mesenchymal *Sostdc1* expression may predominantly antagonize mesenchymally expressed *Bmp4*, the epithelially expressed *Sostdc1* may also interfere with epithelial Wnt signaling crucial for tooth induction, a possibility supported by the increased Wnt activity in the *Sostdc1*-deficient incisors (Fig. 3). In developing hair placode epithelia, *Bmp* expression is upregulated as a result of β-catenin stabilization (Närhi et al., 2008), and it is conceivable that Wnt and BMP signaling may function both up- and downstream from each other at different stages of development.

Conclusions and a hypothesis on the identity of the extra incisors

Our results are indicative of a role for mesenchyme, through *Sostdc1* and at least one additional factor, in inhibiting extra tooth formation. The exact developmental origin of the extra incisor (Fig. 3) is reminiscent of the location from which normal replacement teeth are initiated in many mammals (e.g. Luckett, 1985; Järvinen et al., 2009). Therefore, we propose that the most parsimonious interpretation of the extra incisor in *Sostdc1*-deficient mice is to consider it as a replacement tooth. This fits with the views that, evolutionarily, rodent incisors are interpreted to be second, and sometimes first, deciduous incisors (Luckett 1985; Meng et al., 2003; Asher et al., 2005). The permanent replacement incisor was lost early in the evolutionary history of rodents, together with the reduction of the number of incisors to the one pair found in all rodents. This scenario for the role of *Sostdc1* may be further supported by the de novo supernumerary incisors formed in the *Sostdc1* mutants with trimmed mesenchyme. These teeth resemble the continuous tooth budding found in the *Catenb*$^{\Delta ex3k14/+}$ mice. Hence, a central role of *Sostdc1* in normal tooth development may be to modulate BMP and Wnt signaling in limiting tooth replacement. In addition to tooth replacement, the Sostdc1-Wnt-BMP signaling may be part of the developmental program limiting the induction of teeth spatially. Interestingly, the region-specific absence of dentition in teleost fish seems to correlate with the lack of *Bmp* expression (Wise and Stock, 2006), and it remains to be tested whether *Sostdc1*-like inhibition may also be involved in spatial delineation of tooth-forming areas in other vertebrates.

Considering the role of mesenchyme in tooth induction and the design of tissue engineering protocols, our work may have uncovered how delicate control of tissue quantities alone may influence the outcome between induction and inhibition.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/3/393/DC1

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